(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 November 2024 (07.11.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/229309 A2

(51) International Patent Classification:
G01N 33/58 (2006.01) *G01N 33/68* (2006.01)

(21) International Application Number:
PCT/US2024/027569

(22) International Filing Date:
02 May 2024 (02.05.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

63/463,844	03 May 2023 (03.05.2023)	US
63/598,007	10 November 2023 (10.11.2023)	US
63/633,381	12 April 2024 (12.04.2024)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: METHODSAND COMPOSITIONS FOR HIGH-THROUGHPUT PROTEIN DELIVERY, SCREENING, AND DETECTION

(57) Abstract: Methods and systems for delivery using one or more delivery particles (*e.g.*, virions (*e.g.*, AAV), lipid-based delivery particles, etc.) of one or more cargos (*e.g.*, cargo polypeptides) and subsequent quantification of an abundance of one or more cargos (*e.g.*, proteins) in a mixture (*e.g.*, a complex mixture (*e.g.*, in vivo)) using barcodes (*e.g*, peptide barcodes), binders (*e.g.*, polypeptide binders), and binding agents (*e.g*, phage) are provided herein.

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METHODS AND COMPOSITIONS FOR HIGH-THROUGHPUT PROTEIN DELIVERY, SCREENING, AND DETECTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 63/463,844 filed May 3, 2023, U.S. Provisional Application No. 63/598,007 filed November 10, 2023, and U.S. Provisional Application No. 63/633,381 filed April 12, 2024, the contents of all of which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

[0002] The present specification makes reference to a Sequence Listing (submitted electronically as a .xml file named "2013703-0026_ST26.xml" on May 2, 2024). The .xml file was generated on October 18, 2022, and is 12,323,567 bytes in size. The entire contents of the Sequence Listing are herein incorporated by reference.

BACKGROUND

[0003] Assessments of agents (*e.g.*, cargo components encoding cargo polypeptides, delivery particles, etc.) are central to much of molecular and pharmaceutical biology.

SUMMARY

[0004] The present disclosure provides insights and technologies that achieve improved or otherwise desirable assessment of agents (*e.g.,* nucleic acids comprising and/or encoding cargo agents (*e.g.*, one or more nucleic acid sequence components, *e.g.*, cargo polypeptide agents), therapeutic agents, and/or in some embodiments delivery particles comprising such nucleic acids comprising and/or encoding cargo agents and/or therapeutic agents).

[0005] Among other things, the present disclosure appreciates that many current technologies for assessing, and in particular for determining presence and/or abundance of, one or more agents of interest, may typically rely on mass spectroscopy and/or affinity (*e.g.,*

immuno-) detection. The present disclosure appreciates that many available affinity detection technologies are slow and/or costly to perform or implement; many such technologies must be performed one at a time and many are constrained, for example, by availability of fluorogenic substrates (*e.g.,* that may be assessed by relevant technologies – *e.g.,* light microscopy).

[0006] The present disclosure further appreciates that certain other technologies, such as DNA barcoding technologies, that are sometimes utilized to assess agents of interest, can also suffer disadvantages. DNA barcodes, for example, can lack stability and/or display undesirable immunogenicities, *e.g.,* when utilized *in vivo.* The present disclosure appreciates that such technologies therefore can encounter problems, particularly for assessing agents (*e.g.,* cargo agents) in complex environments (*e.g., in vivo*)

[0007] Among other things, the present disclosure encompasses the recognition of the source of certain problems with available technologies typically utilized to assess agents of interest, and in particular to assess cargo agents and/or delivery particles of interest. In particular, the present disclosure identifies the source of certain problems encountered by such technologies for assessment (*e.g.,* detection and/or measurement of quantity, such as concentration; *e.g.*, inabilities to detect and/or quantify functional information) of multiple agents, and in particular when such agents are present in a complex system (*e.g.,* in a complex solution and/or *in vivo*).

[0008] Furthermore, the present disclosure provides certain technologies that achieve such assessments, in some embodiments with surprisingly high accuracy. Those skilled in the art will appreciate that a number of contexts exist in which detection and/or measurement (*e.g.,* of a precise amount), of a plurality of agents within a complex system is desirable; moreover, those skilled in the art will appreciate the benefit of high accuracy in many such contexts.

[0009] Among other things, the present disclosure provides technologies that achieve detection and/or measurement (*e.g.,* highly accurate and/or otherwise precise measurement) of one or more, and in some embodiments of a plurality of agents (*e.g.,* nucleic acids comprising and/or encoding cargo agents, *e.g.*, delivery particles comprising nucleic acids comprising and/or encoding cargo agents), including in complex systems (*e.g., in vivo*). In some embodiments, detected agent(s) may be or comprise cargo agents and/or forms thereof (*e.g.,* aggregated;

complexed; covalently modified such as by disulfide bond formation, glycosylation, pegylation, phosphorylation; truncated such as by proteolytic cleavage, *etc.*).

[0010] In some embodiments, detected agent(s) may be delivered via delivery particles (*e.g.,* viral particles, virus-like particles, lipid-based particles, polymer-based particles, bead-based, metal-based, or polysaccharide-based particles of interest; *e.g.*, of same and/or different types) in varying conditions (*e.g.,* physiological conditions, *e.g.*, target tissues of interest). In some embodiments, nucleic acids are disposed within delivery particles. In some embodiments, a nucleic acid sequence comprising (a) a cargo component that encodes a cargo polypeptide; (b) a barcode component wherein the cargo component is operably linked to the barcode component. In some embodiments, cargo components include other types of cargo as described herein. In some embodiments, detection of a cargo component associated with a barcode component can be used in turn to assess and/or quantify phenotypes of a delivery particle of interest (*e.g.*, tropism, etc.).

[0011] In some embodiments, provided technologies are particularly useful or effective for assessment of therapeutic agents. For example, in some embodiments, provided technologies may be particularly useful for the assessment of one or more features (*e.g.,* properties (*e.g.,* concentration, localization, persistence, affinity, *etc*)) of agent(s) of interest; in some such embodiments, relevant agent(s) may be characterized by one or more attributes appropriate or desirable for therapeutic use. For example, in some embodiments, provided technologies may be used to screen potential therapeutic agents (*e.g.,* polypeptide entities) for one or more features (*e.g.,* properties, attributes) suitable for therapeutic use. In some embodiments, features of potential therapeutic agent(s) may be measured one at a time. In some embodiments, two or more features of potential therapeutic agent(s) may be measured simultaneously. For example, in some embodiments, one or more therapeutic agents may be screened for an affinity to a target agent - yet other desirable properties for example molecular stability in a physiologically relevant environment are not yet known. In some embodiments, for example, one or more therapeutic agents may be screened for an affinity to a target agent, along with other desirable properties, for example molecular stability in a physiologically relevant environment.

[0012] The present disclosure appreciates that many current methods of polypeptide measurement rely on determining the abundance of light of a certain wavelength, or overall

luminescence, such as western blot or ELISA (Towbin 1979, Engvall 1972). Due to constraints of visible light wavelength, these methods allow for only a small number of different polypeptides, often fewer than 4, to be measured at a single time within a single reaction (Elshal, 2006). The present disclosure appreciates that many applications, including drug discovery applications, would benefit from (and, in some cases, require) dramatically higher throughput.

[0013] The present disclosure further appreciates that nucleic acid sequencing technologies (*e.g.*, DNA sequencing technologies) have been developed that can analyze billions of individual DNA molecules in a single experiment (Shendure, 2005). Various strategies have been developed to try to apply this massive throughput achievable with nucleic acid sequencing techniques to protein detection and measurement, in particular by tagging proteins with an attached piece of DNA (typically referred to as a "DNA barcode"), which may then be sequenced to indirectly detect the protein (Trads, 2017) or one or more features of the protein.

[0014] The present disclosure appreciates the power of applying high-throughput nucleic acid sequencing technologies to assessment of other agents, and in particular of cargo agents, but also identifies the source of certain problems associated with many approaches utilized to study polypeptides by attachment of DNA barcodes. For example, the present disclosure appreciates that modification of a protein by attachment of a DNA barcode can often alter its functionality (Trads, 2017), which can defeat the purpose of using the DNA barcode to assess a polypeptide.

[0015] Known techniques to quantify or screen a plurality of therapeutic moieties is described by WO2020097254 (Gordian Biotechnology). The present disclosure identifies the source of a problem with such approaches, however, and moreover provides certain advantages relative to them, including the ability to assess and/or quantify cargo polypeptides directly. Approaches such as those described by Gordian Biotechnology fail to describe such a feature and rely on cell-based analyses. Furthermore, as may be appreciated by a person of ordinary skill in the art, reading the present disclosure, cell-based analyses are limited by experimental complexity, number of outputs, require additional enrichment steps, and cost. In comparison, the present disclosure is not limited by such disadvantages since nucleic acid sequences that encode one or more polypeptide binders that are associated with one or more peptide barcodes can be sequenced and measured to quantify the cargo component without the need of additional analyses and/or enrichment steps.

[0016] Known techniques to quantify barcoded cargo polypeptides include those as presented in Egloff et al. (2019), that use mass spectrometry to determine the presence or absence of a protein sequence in a mixture (Egloff 2019). The present disclosure identifies the source of a problem with such approaches, however, and moreover provides certain advantages relative to them, including, for example, by using nucleic acids (*e.g.,* DNA) for amplification of the original signal; approaches such as those described in Egloff et al. fail to include (or to benefit from) such a feature. Furthermore, as may be appreciated by a person of ordinary skill in the art, reading the present disclosure, mass spectrometry only reads out the mass-to-charge ratio of an associated sequence; thus methods using mass spectrometry are limited in their total throughput, since different sequences can have the same mass-to-charge ratio. By comparison, the present invention is not limited by such disadvantages since the nucleic acid sequence associated with one or more binding agents in turn associated with each barcode is sequenced and measured to determine and quantify the barcoded cargo polypeptide.

[0017] Other techniques available in the art use antibodies displayed on phage (Fab-phage) to determine presence of endogenous proteins expressed on cell surfaces (Pollock, 2018). In such methods, one Fab-phage is generated per endogenous protein (*i.e.,* target protein to be assessed) and no barcodes are utilized. In contrast, the present technology envisions the use of engineered barcode sequences that are generalizable, such that they can be used to mark any protein, whether endogenous or exogenous to the context in which it is applied, and subsequently measured using one or more binding agents to which each barcode, and therefore each barcoded cargo polypeptide, is uniquely associated with (*i.e.,* a "barcode fingerprint" as described elsewhere in this disclosure). Such complex association of one or more binding agents with a barcode is then measured and precise quantification of the associated protein is achieved, *e.g.,* using a complex algorithm (*i.e.,* 'decoding' as described elsewhere in this disclosure).

[0018] The present disclosure recognizes the ability of antigens displayed on phages to determine epitopes of antibodies within the blood to which the phages are able to bind (Mohan, 2018). However, this method is not able to determine the sequence of the antibody to which the antigen binds, and thus only provides limited information on any antibodies that specifically bind to the antigens displayed on phage. However, the present disclosure provides systems, compositions, and methods that provide the advantage of using generic barcodes with known

affinities to one or more binders or binding agents, that can be used to tag any target(s) of interest in a complex mixture, including but not limited to blood, to determine and quantify the target(s).

[0019] The present disclosure, among other things, provides technologies that can achieve assessment (*e.g.*, detection and/or quantification) of multiple agents (*e.g.*, multiple nucleic acids comprising and/or encoding cargo agents, multiple delivery particles comprising nucleic acids encoding cargo agents, and/or a combination thereof) within a pool of such agents, using DNA sequencing without requiring (direct or indirect) covalent association of the DNA with the assessed agent, or otherwise constraining the assessed agent.

[0020] Described herein are peptide barcodes (also known as "barcodes") and technologies to make and/or utilize them. In some embodiments, barcodes are utilized to mark cargos. Among other things, such an approach can achieve pooled measurement of cargos without amending non-polypeptide identifiers. In some embodiments, a peptide barcode is an amino acid polypeptide sequence. In some embodiments, a peptide barcode is contained within a cargo (*e.g.*, a polypeptide (*e.g.*, an antibody, *e.g.*, a cell-surface antigen) to be measured; *e.g.*, is endogenous to a cargo to be measured). In some embodiments, a peptide barcode is not contained with a cargo polypeptide (*e.g.*, an antibody, *e.g.*, a cell-surface antigen) to be measured; *e.g.*, is exogenous to a cargo polypeptide to be measured). In some embodiments, a barcode, for example, is a sequence (*e.g.*, a designed sequence) contained within a cargo (*e.g.*, a cargo to be measured). In some embodiments, a cargo comprises a cargo polypeptide. In some embodiments, a barcode is associated (*e.g.*, bound (*e.g.*, covalently)) to the N terminus of a cargo polypeptide (*e.g.*, a cargo polypeptide to be measured). In some embodiments, a barcode is associated (*e.g.*, bound (*e.g.*, covalently)) to the C terminus of a cargo polypeptide (*e.g.*, a cargo polypeptide to be measured). In some embodiments, a barcode is associated (*e.g.*, bound (*e.g.*, covalently)) proximal to the N terminus (*e.g.*, internal to a cargo polypeptide (*e.g.*, a loop region that is proximal to the N terminus)) of a cargo polypeptide (*e.g.*, a cargo polypeptide to be measured). In some embodiments, a barcode is associated (*e.g.*, bound (*e.g.*, covalently)) proximal to the C terminus *e.g.*, internal to a cargo polypeptide (*e.g.*, a loop region that is proximal to the C terminus)) of a cargo polypeptide (*e.g.*, a cargo polypeptide to be measured).

[0021] The methods disclosed herein may use peptide barcodes that are designed to have varying lengths. In some embodiments, a peptide barcode may have a length ranging between 1-

100, 5-50, 8-25, 9-25, or 9-15 amino acids. In some embodiments, a peptide barcode may have a length of at least 25 amino acids. In some embodiments, a peptide barcode may have a length of at most 8 amino acids. In some embodiments, a peptide barcode may have a length of 10 amino acids.

[0022] Barcode sequences as described herein may be reused, so as to be able to quantify different agents (*e.g.,* nucleic acids comprising and/or encoding cargos of interest, *e.g.*, delivery particles of interest, each comprising a nucleic acid encoding a cargo of interest) or mixture of agents (mixture of cargos of interest to be measured and/or mixture of delivery particles to be measured, *e.g.*, via detection of a barcoded cargo). In some embodiments, a barcode is generated such that it can be easily reused between several different agents (*e.g.,* nucleic acids comprising and/or encoding cargos of interest, *e.g.*, delivery particles of interest, each comprising a nucleic acid encoding a cargo of interest) across different experiments.

[0023] Among other things, barcodes described herein are designed to be distinct from each other (*e.g.*, unique). In some embodiments, a barcode is designed to have a distinct sequence (*e.g.,* distinct from another barcode). For example, each barcode is designed to be distinct (*e.g.,* unique) from every other barcode used in an experiment, such that each agent (*e.g.,* nucleic acids comprising cargos to be measured, *e.g.*, delivery particles comprising a nucleic acid comprising a cargo to be measured) is associated (*e.g.*, operably linked) with at least one barcode, and each barcode (*e.g.,* barcode with a specific sequence) is only associated with one cargo. As may be understood by a person of ordinary skill in the art, the diversity of barcodes contained within a pool is limited only by the possible diversity of amino acid sequences for a given barcode length. For example, for a barcode length 'N', there exists 20^N distinct amino acid barcode sequences of length N.

[0024] Methods described herein relate to the detection of one or more barcodes using a binding agent. In some embodiments, a barcode is contacted with a binding agent that is associated with or comprises a detectable nucleic acid. For example, in some embodiments, a binding agent may be or comprises a phage, a ribosome, mRNA, DNA, etc. In some embodiments, a binding agent is a phage with a binding motif on its surface (*e.g.,* a polypeptide binder as described herein). In some embodiments, a binding agent comprises a detectable nucleic acid. In some embodiments, a binding agent expresses a detectable nucleic acid. In some

embodiments, a binding agent expresses a detectable nucleic acid on (*e.g.,* on a surface of) the binding agent (*e.g.,* a binder). In some embodiments, a binder is a polypeptide. In some embodiments, a binder associates with a barcode (*e.g.,* with known specificity and affinity). In some embodiments a binder associates with one or more barcodes (*e.g.,* with different known specificities and affinities). In some embodiments, a binder is an antibody (*e.g.,* expressed on a surface of a binding agent). In some embodiments, for example, to detect the presence of a specific (*e.g.,* distinct) barcode, the present disclosure envisions the association of a distinct detectable nucleic acid (*e.g.,* a DNA sequence, an RNA sequence, etc.) to a specific barcode. This is achieved through the contact of a binder, which may be expressed on (*e.g.,* on a surface of) a binding agent that comprises the distinct detectable nucleic acid.

[0025] Described herein are binders. In some embodiments, a binder is a polypeptide. In some embodiments, for example, a binder is generated to have known specificity and affinity for a given barcode. In some embodiments, a binder is generated to have known specificity and affinity for one barcode. In some embodiments, a binder is generated to have known specificity and affinity for multiple (*e.g.,* two or more, three or more, etc.) barcodes. In some embodiments, a binder is generated to have known specificity and affinity for at least one barcode. In some embodiments, a binder, for example, is expressed on the surface of a binding agent (*e.g.,* a phage, a ribosome, etc.) using methods known to those skilled in the art.

[0026] Among other things, systems and methods described, for example, as described herein, identify the advantages of nucleic acid sequencing techniques and apply them effectively to protein detection and measurement methods. For example, methods described herein may use several binders, with known specificities and affinities to different barcodes, which can be expressed on binding agents and mixed together in a single pool. Upon mixing with a pool of barcoded cargo (*i.e.,* cargo polypeptides, each associated with a barcode as described herein), a binder expressed on a binding agent binds to any given barcode in the pool with known but varying affinities. Such a spectrum of affinities of a binder to various barcodes is termed herein as a 'Binder Fingerprint'. Conversely, a barcode may bind to any given binder in a pool of binders with known but varying affinities. Such a spectrum of affinities of a barcode to various binders is termed herein as a 'Barcode Fingerprint'. Thus, the presence of specific barcoded cargos can be detected, for example, in a complex solution, by extracting and sequencing the

associated nucleic acid (*e.g.*, detectable nucleic acid (*e.g.*, DNA sequence, RNA sequence, etc.)) of the population of binding agents (*e.g.*, phage) bound to barcodes associated with cargos.

[0027] Other methods to use binders to identify polypeptide sequences have been developed. However, these methods encounter a number of challenges, including difficulty in generating and characterizing binders, and effectively decoding their binding to specifically identify polypeptides. Another limitation with previously developed binders is their non-specific binding that results in poor signal-to-noise ratios, thereby negatively affecting the accuracy of detection. In contrast, the present technology generates many binders rapidly (*e.g.*, in about a week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, or about 1 year). In some embodiments, for example, between about 100 to about 1000 binders may be generated rapidly. In some embodiments, between about 10 to about 1000 binders may be generated rapidly. In some embodiments, between about 10 to about 10,000 binders may be generated rapidly. In some embodiments, at least about 10,000 binders may be generated rapidly.

[0028] Binders as described herein are robust. Binders can bind to barcodes (*e.g.*, with robust affinities to one or more barcodes) as described herein in a variety of conditions and/or environments. For example, binders as described herein can bind to barcodes (*e.g.*, with robust affinities to one or more barcodes) in various complex environments (*e.g.*, in blood, tissue, serum, plasma, etc.). Thus, binders of the present disclosure may be used to detect targets (*e.g.*, a nucleic acid encoding a cargo of interest) in varying conditions (*e.g.*, physiological conditions, *e.g.*, target tissues of interest). Moreover, binders of the present disclosure may also be used to detect delivery particles (*e.g.*, viral particles, virus-like particles, lipid-based particles, polymer-based particles, bead-based, or polysaccharide-based particles of interest) in varying conditions (*e.g.*, physiological conditions, *e.g.*, target tissues of interest).

[0029] Analogously, barcodes, as described herein, may be generated in a rapid and robust manner. In some embodiments, barcodes as described herein are specific to binders as described herein. In some embodiments, for example, between about 100 to about 2000 barcodes may be generated rapidly (*e.g.*, in about a week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, or about 1 year). In some embodiments, between about 10 to about 1000 barcodes may

be generated rapidly. In some embodiments, between about 10 to about 10,000 barcodes may be generated rapidly. In some embodiments, at least about 10,000 barcodes may be generated rapidly.

[0030] Barcodes as described herein are robust. Barcodes can bind to binders (*e.g.,* with robust affinities to one or more binders) as described herein in a variety of conditions and/or environments. For example, barcodes as described herein can bind to binders (*e.g.,* with robust affinities to one or more binders) in various complex environments (*e.g.,* in blood, tissue, serum, plasma, etc.). Thus, barcodes of the present disclosure may be used to detect targets (*e.g.,* agents of interest) in varying conditions (*e.g.,* physiological conditions). The present disclosure, therefore corrects for the disadvantages and defects of existing methods (*e.g.,* non-specific binding, variable binding in different environments, etc.) by generating large numbers of robust binders and barcodes rapidly, which may be used in combination with computational methods (*e.g.,* deconvolution methods) described herein, to allow for specific, well-characterized binder-barcode binding/association and accurate detection methods.

[0031] The present disclosure also envisions an ability to modify sequence(s) of one or more peptide barcode sequences such that they are readily distinguishable from each other, and/or from potential background protein sequence. Analogously, the present disclosure also envisions the ability to modify the sequence(s) of one or more polypeptide binder sequences such that they are readily distinguishable from each other, and/or from potential background protein sequence.

[0032] Among other things, the present invention as described herein provides methods of testing 'n' distinct protein candidates where $n \geq 1$, in a single assay or animal model. In some embodiments, a protein candidate is a therapeutic protein candidate. In some embodiments, multiple protein candidates are designed and each distinct protein candidate is associated with its own unique peptide barcode as described herein. Such barcoding has many advantages, including but not limited to injecting all protein candidates in a single injection into an assay and/or an animal in a cost- and time-efficient manner. Subsequently, a sample (*e.g.,* tissue sample, serum sample, blood sample, extracellular sample, single cell sample etc.) from an injected animal may be obtained and barcodes extracted. In some embodiments, such extracted barcodes provide a measure of the relative abundance of protein candidates originally injected. For example, one or

more extracted barcodes may be identified by contacting them with a pool of binders (*e.g.,* expressed on a binding agent) known to bind to the barcodes originally bound to the protein candidates. Following binding of barcodes and binders, bound binding agents (*e.g.,* phage) are selected and their detectable nucleic acid (*e.g.,* DNA sequence, RNA sequence, etc.) extracted. In some embodiments, extracted nucleic acids are subjected to sequencing (*e.g.,* next generation sequencing). The sequenced nucleic acid may then be used to identify the one or more barcodes they were designed to bind to, which along with the previously established information on binding affinities between various binder-barcode pairs may be used to identify and determine the relative abundance of each protein originally injected.

[0033] Also described herein, are methods used to translate nucleic acid counts, for example from a sequencing experiment, to relative or absolute protein quantifications. In some embodiments, nucleic acid sequences are counted and *in silico* translated into protein sequences. As is described herein, a nucleic acid sequence corresponds to a binder sequence, with established and characterized affinity for every barcode given in a pool. In some embodiments, binder counts are compared to a database of known propensities for binding to a single barcode. In some embodiments, binder counts are compared to a database of known propensities for binding to multiple barcodes (*e.g.,* two or more, three or more, etc.). In some embodiments, for example in a sequencing experiment, relative proportions of binder counts are compared directly in order to determine relative proportions of barcodes and/or proteins associated with barcodes. In some embodiments, as may be known to a person of ordinary skill in the art, sequences (*e.g.,* control sequences or accessory sequences) of known abundance (*e.g.,* count, quantification, concentration, etc.) are utilized (*e.g.,* added to the sequencing experiment) to determine an absolute abundance (*e.g.,* count, quantification, concentration, etc.) for a given binder or binders, which may be used to estimate an absolute abundance (*e.g.,* count, quantification, concentration, etc.) for a barcode or barcodes, and/or protein(s) associated with barcode(s) using either direct counts or a linear model as described herein.

[0034] In some embodiments, a nucleic acid comprises a cargo component which encodes a cargo polypeptide. In some embodiments, a cargo polypeptide is or comprises a therapeutic polypeptide. In some embodiments, a cargo component further comprise one or more sequence elements. In some embodiments, a cargo component is associated with (*e.g.,* operably linked to) nucleotide sequences encoding barcodes as described herein.

[0035] Among other things, the present disclosure provides a method of assessing barcodes, binders (*e.g.,* binding agents (*e.g.,* with binders expressed on a surface)), cargos, (*e.g.,* barcoded cargos (*e.g.,* barcoded cargo polypeptides)) as described herein. In some embodiments, a method comprises subjecting a population of barcoded cargos (*e.g.,* barcoded cargo polypeptides) to an assessment; separating those members of a population that satisfy an assessment from those that do not, so that either a positive population or a negative population, or both is identified; contacting a positive population, or a negative population, or each population separately from the other, with a set of binders which includes at least one particular binder specific for each barcode in a population; and determining which binders bind to separated members, thereby determining which barcoded cargos (*e.g.,* barcoded cargo polypeptides) are present in a contacted population(s).

[0036] The present disclosure provides a method comprising contacting a set of binders either with a first population, with a second population, or separately with each of a first and second populations, of barcoded cargos (*e.g.,* barcoded cargo polypeptide); and determining which binders of a set bind to a member of a first population, a second population, or both, thereby determining which barcoded cargos (*e.g.,* barcoded cargo polypeptide) are present in contacted population(s). In some embodiments, each binder binds specifically (*e.g.,* with known affinities) to one or more barcodes. In some embodiments, a set of binders, collectively, includes at least one binder specific for each of the barcodes in the first and second populations. In some embodiments, a first and second populations have been separated from one another based on performance in an assessment.

[0037] In some embodiments, a method further comprises determining differences between a first and second population, to determine a functional effect of a performance assessment. In some embodiments, a method comprises separating binders that bind to at least one cargo (*e.g.,* barcoded cargo (*e.g.,* barcoded cargo polypeptide)).

[0038] In some embodiments, a step of determining comprises quantifying a number of binders that bind to a barcoded cargo (*e.g.,* barcoded cargo polypeptide). In some embodiments, quantifying may be performed by decoding a nucleotide sequence of each binder that binds to a barcoded cargo (*e.g.,* barcoded cargo polypeptide). In some embodiments, quantifying a number

of binders that bind to a cargo (*e.g.,* barcoded cargo polypeptide) provides measure of a cargo (*e.g.,* protein) in a population.

[0039] In some embodiments, a step of determining comprises amplifying nucleic acids of bound phage particles. In some embodiments, a step of determining comprises determining nucleotide sequences of amplified nucleic acids. In some embodiments, one or more of determined nucleotide sequences corresponds to a coding sequence of a binder. In some embodiments, a step of determining comprises detecting one or more cargos (*e.g.,* proteins) from a population of barcoded cargos (*e.g.,* barcoded cargo polypeptides) using determined sequence(s) of a coding sequence of a binder. In some embodiments, a step of determining comprises identifying one or more barcoded cargos (*e.g.,* barcoded cargo polypeptides) as a therapeutic or a target to treat a disease, disorder, or condition.

[0040] In some embodiments, a step of determining comprises performing one or more of amplification, propagation, and sequencing (*e.g.,* nucleic acid (*e.g.,* DNA, RNA) amplification, propagation, and/or sequencing). In some embodiments, amplification may be performed using one or more of Polymerase Chain Reaction (PCR), Loop-mediated Isothermal Amplification (LAMP), Rolling Circle Amplification (RCA), or a similar known technique. In some embodiments, sequencing may be performed using one or more of Illumina, Next Generation Sequencing (NGS), nanopore sequencing, Pac Bio long-read sequencing, or a similar known technique.

[0041] In some embodiments, a step of separating comprises purifying one or more barcoded cargos (*e.g.,* barcoded cargo polypeptides) from a sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are purified from a complex sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are purified from a complex mixture. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are purified using affinity purification methods (*e.g.,* FLAG IP, protein G/A) or protein precipitation methods.

[0042] In some embodiments, a method further comprises injecting a population of barcoded cargos into an animal. In some embodiments, a method further comprises injecting a population of barcoded cargos (*e.g.,* barcoded cargo polypeptides) into an animal. In some embodiments, each barcode is bound to a specific binder expressed on a phage. In some

embodiments, a method further comprises obtaining a sample from an animal to subject to an assessment.

[0043] In some embodiments, a method as described herein comprises determining relative amounts of each binder present in a sample, thereby identifying a subset of an injected population of barcoded cargos (*e.g.,* barcoded cargo polypeptides) present in a sample. In some embodiments, a method as described herein comprises comparing relative amounts to a standard of known concentration to determine an absolute quantity of each binder present in a sample.

[0044] In some embodiments, a method as described herein comprises optionally, repeating steps one or more of method steps described herein using an identified subset of cargos (*e.g.,* proteins).

[0045] In some embodiments, a method as described herein comprises identifying one or more cargos (*e.g.,* cargo polypeptides) as a therapeutic or a target to treat a disease, disorder, or condition.

[0046] In some embodiments, a method as described herein comprises identifying one or more delivery particles as a therapeutic or a target to treat a disease, disorder, or condition.

[0047] In some embodiments, a method as described herein comprises removing any unassociated (*e.g.,* unbound) binders. In some embodiments, removing may be performed by washing.

[0048] In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a complex sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a complex mixture. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a purified sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a mammal.

[0049] In some embodiments, delivery particles (*e.g.,* comprising a nucleic acid described herein) are in a sample. In some embodiments, delivery particles (*e.g.,* comprising a nucleic acid described herein) are in a complex sample. In some embodiments, delivery particles (*e.g.,* comprising a nucleic acid described herein) are in a complex mixture. In some

embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a purified sample. In some embodiments, barcoded cargos (*e.g.,* barcoded cargo polypeptides) are in a mammal.

[0050] In some embodiments, a sample is or comprises one or more of serum, blood, tissue, or a tumor. In some embodiments, a sample is a control (*e.g.,* positive control or negative control). In some embodiments, a sample is or comprises a cell or a population of cells.

[0051] In some embodiments, a sample is a complex sample. In some embodiments, a complex sample is or comprises a tissue. In some embodiments, a complex sample is or comprises blood. In some embodiments, a complex sample is a complex mixture. In some embodiments, a complex sample is or comprises one or more of serum, blood, or tissue.

[0052] In some embodiments, a barcode is or comprises one or more amino acids. In some embodiments, a barcode is comprised in a Complementarity-Determining Regions (CDR) of a cargo (*e.g.,* a protein). In some embodiments, a barcode is synthetic. In some embodiments, a barcode is 1-100, 5-50, 8-25, 9-25, or 9-15 amino acids in length. In some embodiments, a barcode is 10 amino acids in length. In some embodiments, a barcode has relatively little or no effect on cargo (*e.g.,* a protein) function. In some embodiments, a barcode does not elicit an immune response. In some embodiments, barcodes are orthogonal to each other. In some embodiments, at least one barcode is linked with a polypeptide (*e.g.,* a polypeptide binder, a cargo) of interest.

[0053] In some embodiments, a barcode is attached to a cargo (*e.g.,* a cargo polypeptide). In some embodiments, a barcode is attached to a suitable position on a cargo (*e.g.,* a cargo polypeptide). In some embodiments, a suitable position is an N-terminus or a C-terminus.

[0054] In some embodiments, a binder is or comprises a binding moiety displayed on a phage. In some embodiments, each binder of a set of binders is expressed on a phage. In some embodiments, a binder is expressed on a surface of a phage particle.

[0055] In some embodiments, a phage is selected from a group consisting of M13, T4, T7, Lambda, and filamentous phage. In some embodiments, a phage is M13.

[0056] The present disclosure provides, among other things, a nucleic acid whose nucleotide sequence is or comprises a sequence encoding a peptide barcode. In some embodiments, a peptide barcode has a length within a range of 1 to 100, 5 to 50, 8 to 25, 9 to 25,

or 9 to 15 amino acids. In some embodiments, a peptide barcode has a length of 8 to 25 amino acids. In some embodiments, a peptide barcode has a length of 10 amino acids. In some embodiments, a peptide barcode has been determined to bind specifically to a particular group of polypeptide binders within a set of binders.

[0057] In some embodiments, a peptide barcode has an amino acid sequence selected from a group consisting of SEQ ID NOs: 5347-8398. In some embodiments, an encoding sequence is selected from a group consisting of SEQ ID NOs: 1148-4199.

[0058] The present disclosure provides a library comprising a plurality of nucleic acids. In some embodiments, a plurality of nucleic acids together encodes, among other things, a collection of peptide barcodes. In some embodiments, each nucleic acid comprises, in order from 5' to 3' or 3' to 5', one or more of: a) a first invariant sequence (*e.g.,* a linker sequence or a cargo sequence); b) a variant sequence that is at least 9 nucleotides long; and c) a second invariant sequence (*e.g.,* a linker sequence, a stop codon, or a cargo sequence).

[0059] In some embodiments, a variant sequence is at least 15, 24, 27, 45, 150, or 300 nucleotides long.

[0060] In some embodiments, a library further comprises one or more of: d) sequence encoding one or more short helical motifs; e) sequence encoding one or more disordered motifs; f) an invariant sequence linking a sequence (*e.g.*, a barcode component) to a cargo (*e.g.*, a cargo component).

[0061] In some embodiments, each peptide barcode of a collection binds specifically to a particular group of polypeptide binders within a set of binders. In some embodiments, each peptide barcode of a collection binds specifically to one or more polypeptide binders within a set of binders.

[0062] The present disclosure provides a nucleic acid whose nucleotide sequence is or comprises a sequence encoding a polypeptide binder moiety. In some embodiments, a polypeptide binder moiety has a length within a range of 10 to 400 amino acids. In some embodiments, a polypeptide binder moiety has been determined to bind specifically to a particular group of peptide barcodes within a collection of barcodes.

[0063] In some embodiments, a polypeptide binder moiety has an amino acid sequence selected from a group consisting of SEQ ID NOs: 4200- 5346. In some embodiments, an encoding sequence is selected from a group consisting of SEQ ID NOs: 1-1147.

[0064] The present disclosure provides a library comprising a plurality of nucleic acids. In some embodiments, a plurality together encodes a set of polypeptide binder moieties. In some embodiments, each nucleic acid comprises, in order from 5' to 3' or 3' to 5': a) a first invariant sequence (*e.g.,* an antibody germline sequence (*e.g.,* IGHV/IGKV)); b) a first variant sequence that is at least 10 nucleotides long (*e.g.,* a CDR (*e.g.,* CDR3) sequence); and c) a second invariant sequence (*e.g.,* an antibody germline sequence (*e.g.,* IDHJ/IGKJ)).

[0065] In some embodiments, each nucleic acid further comprises one or more of: d) a stop codon (*e.g.,* after a second invariant sequence); e) a linker sequence; f) a third invariant sequence (*e.g.,* an antibody germline sequence (*e.g.,* IGHV/IGKV)); g) a second variant sequence that is at least 10 nucleotides long (*e.g.,* a CDR (*e.g.,* CDR3) sequence); and h) a fourth invariant sequence (*e.g.,* an antibody germline sequence (*e.g.,* IDHJ/IGKJ)).

[0066] The present disclosure provides, among other things, a library of phage particles, each phage particle comprising one or more nucleic acids as described herein.

[0067] In some embodiments, a phage is selected from a group consisting of M13, T4, T7, Lambda, and filamentous phage. In some embodiments, a phage is M13.

[0068] The present disclosure provides a set of barcode and binders. In some embodiments, each barcode is a peptide between 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids in length that binds specifically to a particular group of binders among binders in a set. In some embodiments, each binder is a polypeptide that binds specifically to at least one barcode among barcodes in a set.

[0069] In some embodiments, specific binding is observed when binders are expressed on phage that are contacted with barcodes. In some embodiments, each binder is expressed on a phage.

[0070] The present disclosure provides a kit comprising a set of binders, each of which is a polypeptide that binds specifically to at least a particular peptide barcode in a collection barcodes. In some embodiments, each binder is provided as a polypeptide, a nucleic acid

encoding a polypeptide, or both. In some embodiments, one or more of binders is provided as a phage particle, or collection thereof, engineered to express a binder. In some embodiments, one or more of binders is provided as a nucleic acid in a phagemid vector, or as an insert suitable for cloning into a phage vector.

[0071] In some embodiments, a kit further comprises information designating peptide barcodes for each binder. In some embodiments, each binder has been determined to bind to at least a particular peptide barcode within a collection of barcodes that each bind specifically to at least one binder in a set.

[0072] In some embodiments, a kit further comprises a set of instructions to perform sequencing of one or more phage particles bound to one or more barcodes. In some embodiments, a kit further comprises a computer readable program for decoding sequencing data. In some embodiments, a kit further comprises reagents to express a binder on a phage particle.

[0073] In some embodiments, a kit comprises nucleic acids that encode one or more barcodes. In some embodiments, a kit comprises nucleic acids that encode one or more binders.

[0074] The present disclosure provides a method of pharmacokinetic screening. In some embodiments, a method comprises injecting a set of barcoded therapeutic candidate cargo polypeptides into an animal. In some embodiments, each barcoded therapeutic candidate protein comprises a specific peptide barcode. In some embodiments, a method comprises obtaining a sample from an animal; purifying one or more barcoded therapeutic candidate cargo polypeptide from a sample; contacting a sample with a set of binders (*e.g.,* binding agents with binders expressed on them) which includes at least one particular binder specific for each barcode in a sample; and determining relative amounts of each binder present in a sample to determine each barcoded therapeutic candidate proteins' pharmacokinetic properties or biodistribution.

[0075] In some embodiments, purified proteins may be a subset of barcoded therapeutic candidate cargo polypeptide which are administered to an animal.

[0076] In some embodiments, multiple samples may be obtained from an animal.

[0077] In some embodiments, an animal is a mammal. In some embodiments, an animal is a human. In some embodiments, an animal is genetically modified to express barcoded cargos (*e.g.,* barcoded cargo polypeptides).

[0078] In some embodiments, an animal is a model for a disease, disorder, or condition. In some embodiments, a disease, disorder, or condition is cancer, autoimmune, neurodegenerative, or a pathogenic (*e.g.,* viral/bacterial) disease, disorder, or condition.

[0079] In some embodiments, a step of determining comprises (i) sequencing nucleic acid from binding agents expressing a binder; (ii) decoding relative amounts of each barcode present thereby determining relative amounts of each therapeutic candidate protein; and/or (iii) performing one or more of FACS, or MACS (magnetic activated cell sorting), affinity-based purification.

[0080] In some embodiments, a step of determining comprises quantifying number of binders that bind to a barcoded cargo (*e.g.,* barcoded cargo polypeptide (*e.g.,* barcoded therapeutic cargo polypeptide)). In some embodiments, quantifying is performed by decoding a nucleotide sequence of each binder that binds to a barcoded cargo (*e.g.,* barcoded cargo polypeptide). In some embodiments, a step of determining comprises identifying one or more delivery particles, *e.g.*, via a barcoded cargo polypeptide.

[0081] In some embodiments, a number of nucleotide sequences provides a measure of cargo (*e.g.,* target protein) in a population of barcoded cargos (*e.g.,* barcoded cargo polypeptides).

[0082] In some embodiments, a step of administering comprises administering barcoded cargos (*e.g.,* barcoded cargo polypeptides, barcoded therapeutic candidate proteins, nucleic acids encoding barcoded cargo polypeptides, nucleic acids encoding therapeutic candidate proteins, etc.) disposed within a delivery particle. In some embodiments, a step of administering comprises administering barcoded cargos (*e.g.,* nucleic acids encoding barcoded cargo polypeptides, nucleic acids encoding therapeutic candidate proteins, etc.) decorating a surface of a delivery particle.

[0083] The present disclosure provides a method of characterizing a collection of peptide barcodes comprising: providing: (i) a library of phage particles, wherein each phage particle is

designed to express a polypeptide binder, and wherein each binder binds to one or more peptide barcodes; (ii) a collection of peptide barcodes; contacting each phage particle with each barcode to form bound phage-barcode particles; determining an amount of binding between each phage particle and barcode; and identifying phage-barcode pairs that bind specifically to each other among barcodes in a collection and phages in a library.

[0084] The present disclosure provides a method of characterizing a collection of peptide barcodes comprising: providing (i) a set of binders, wherein each binder is a polypeptide that binds to one or more peptide barcodes, and (ii) a collection of peptide barcodes; contacting each binder with each barcode to form bound binder-barcode particles; determining a relative amount of binding between each polypeptide binder and peptide barcode; and identifying binder-barcode pairs that bind specifically to each other among barcodes in a collection and binders in a set.

[0085] The present disclosure provides a database of amino acid or encoding nucleic acid sequences for a collection of peptide barcodes, which database is embodied in a computer readable format. In some embodiments, each barcode sequence has a length within a range of 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids. In some embodiments, each barcode sequence has been determined to bind specifically to one or more polypeptide binders within a set of binders that each bind specifically to one or more of barcodes in a collection.

[0086] In some embodiments, a binding pattern of one or more polypeptide binders to a barcode is used to identify a peptide barcode.

[0087] The present disclosure provides a database of amino acid or encoding nucleic acid sequences for a set of polypeptide binders. In some embodiments, a database is embodied in a computer readable format. In some embodiments, each binder sequence has a length within a range of 10 to 400 amino acids. In some embodiments, each binder sequence has been determined to bind specifically to one or more peptide barcodes within a collection of barcodes that each bind specifically to one or more of binders in a set.

[0088] The present disclosure provides, among other things, a database of amino acid or encoding nucleic acid sequences for a set of barcode-binder associations, embodied in a computer readable format. In some embodiments, each barcode is a peptide between 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids in length. In some embodiments, each binder is a polypeptide that binds specifically to one or more barcodes among barcodes in a set.

[0089] The present disclosure provides a set of barcode-binder association designations, embodied in a computer readable format. In some embodiments, each barcode is a peptide between 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids in length. In some embodiments, each binder is a polypeptide that binds specifically to one or more barcodes among barcodes in a set.

[0090] In some embodiments, specific binding is observed when binders are expressed on a phage particle that are then contacted with barcodes.

[0091] The present disclosure provides a method of treatment using technologies described herein. In some embodiments, a method comprises administering a therapeutic cargo polypeptides, or a characteristic portion thereof, that has been determined to satisfy an assessment. In some embodiments, satisfying an assessment may be by a process comprising steps of: a) subjecting a population of barcoded cargo polypeptides to an assessment; b) separating those members of a population that satisfy an assessment from those that do not, so that either a positive population or a negative population, or both, is identified; c) contacting a positive population, or a negative population, or each population separately from the other, with a set of binders which includes at least one particular binder specific for each barcode in a population; d) determining which binders bind to separated members, thereby determining which barcoded cargo polypeptides are present in a contacted population(s); and e) identifying a therapeutic cargo polypeptides from barcoded cargo polypeptides determined to be present in a contacted population(s).

[0092] The present disclosure provides a method of treatment comprising administering a therapeutic cargo polypeptides, or a characteristic portion thereof, that has been determined to satisfy an assessment by a process comprising steps of: a) contacting a set of binders either with a first population, with a second population, or separately with each of a first and second populations, of barcoded cargo polypeptides; b) determining which binders of a set bind to a member of a first population, a second population, or both, thereby determining which barcoded cargo polypeptides are present in a contacted population(s); and c) identifying a therapeutic cargo polypeptides from barcoded cargo polypeptides determined to be present in a contacted population(s). In some embodiments, each binder binds specifically to one or more barcodes relative to other barcodes. In some embodiments, a set of binders, collectively, includes a binder

specific for each barcode in a first and second populations. In some embodiments, a first and second populations have been separated from one another based on performance in an assessment.

[0093] Among other things, the present disclosure is directed to a cell comprising a nucleic acid as described herein, a library of nucleic acids as described herein, a plurality of delivery particles as described herein, or a delivery particle as described herein.

[0094] Among other things, the present disclosure is directed to a population of cells comprising a nucleic acid as described herein, a library of nucleic acids as described herein, a plurality of delivery particles as described herein, or a delivery particle as described herein.

[0095] Among other things, the present disclosure is directed to a composition (*e.g.*, pharmaceutical composition) comprising a nucleic acid as described herein, a library of nucleic acids as described herein, a plurality of delivery particles as described herein, or a delivery particle as described herein.

[0096] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof, wherein the therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0097] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more barcoded cargo polypeptides, or characteristic portion thereof, wherein the barcoded cargo polypeptides are generated by a method as described herein.

[0098] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof, wherein the one or more therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0099] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more barcoded cargo polypeptides, or characteristic portion thereof, wherein the one or more barcoded cargo polypeptides are generated by a method as described herein.

[0100] Among other things, the present disclosure provides for a method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof, wherein the one or more therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0101] Among other things, the present disclosure provides for a method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof, wherein the therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0102] Among other things, the present disclosure provides nucleic acids. In some embodiments, a nucleic acid comprising (a) a cargo component whose nucleotide sequence is or comprises a sequence encoding a cargo polypeptide, (b) a barcode component whose nucleotide sequence is or comprises a sequence encoding a peptide barcode. In some embodiments, a barcode component may be characterized in that: (i) a peptide barcode has a length within a range of 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids; and (ii) has been determined to bind specifically to a particular group of polypeptide binders within a set of binders. In some embodiments, a cargo component is operably linked to a barcode component.

[0103] In some embodiments, a cargo component further comprises one or more sequence elements, or a complement thereof. In some embodiments, a cargo component further comprises one or more sequence elements, or a complement thereof selected from a group consisting of: a promoter, an enhancer, a silencer, an insulator, a transcriptional regulatory element, a translational regulatory element, a splice donor, a splice acceptor, a transcriptional terminator, a translational start site, a translational stop site, a packaging signal, an integration signal, and any combination thereof. In some embodiments, a cargo component further comprises one or more of a capping moiety, a 5' untranslated region (UTR), 3' UTR, a polyadenylation (polyA) tail, or a complement thereof, or any combination thereof. In some embodiments, a cargo component comprises an internal ribosome entry site (IRES). In some embodiments, a cargo component further encodes a cleavable moiety (*e.g.*, a self-cleaving

peptide (*e.g.*, a 2A peptide)). In some embodiments, a cargo component, or a portion thereof, is codon-optimized.

[0104] In some embodiments, a cargo polypeptide further comprises a localizing moiety. In some embodiments, a localizing moiety is selected from a group consisting of: a secretory signal and an intracellular localization moiety. In some embodiments, a cargo polypeptide further comprises an intermediate or a pro component. In some embodiments, a cargo polypeptide further comprises a tag moiety. In some embodiments, a cargo polypeptide further comprises a targeting moiety (e.g., a shuttle moiety). In some embodiments, a cargo polypeptide further comprises a liganding moiety (e.g., a shuttle moiety). In some embodiments, a cargo polypeptide further comprises a stability modifying moiety. In some embodiments, a cargo polypeptide further comprises a masking moiety. In some embodiments, a cargo polypeptide further comprises an allosteric modulation moiety. In some embodiments, a localizing moiety, a tag moiety, a targeting moiety, a liganding moiety, a stability modifying moiety, a masking moiety, or an allosteric modulation moiety is cleavable.

[0105] In some embodiments, a cargo polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide. In some embodiments, a cargo polypeptide is or comprises a variant polypeptide (*e.g.*, a variant cargo polypeptide). In some embodiments, a variant polypeptide is a variant of a reference polypeptide, which reference polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide. In some embodiments, a variant polypeptide is or comprises at least one mutation relative to a reference polypeptide (*e.g.*, a wild-type polypeptide).

[0106] In some embodiments, a variant cargo polypeptide is associated with (*e.g.*, operably linked to) a barcode, as described herein (*i.e.*, a barcoded variant cargo polypeptide). In some embodiments, a variant cargo polypeptide possesses improved functionality (*e.g.*, reduced toxicity, improved pharmacokinetic measures (*e.g.*, dissociation constant (Kd), improved biophysical properties, improved developability, improved expression, etc.) relative to a reference polypeptide (*e.g.*, a wild-type polypeptide).

[0107] In some embodiments, a cargo nucleic acid (*e.g.*, a cargo component) is or comprises a wild-type (*e.g.*, naturally occurring) nucleic acid. In some embodiments, a cargo nucleic acid (*e.g.*, a cargo component) is or comprises a variant nucleic acid (*e.g.*, a variant cargo nucleic

acid). In some embodiments, a variant nucleic acid is a variant of a reference nucleic acid, which reference nucleic acid is or comprises a wild-type (*e.g.*, naturally occurring) nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide). In some embodiments, a variant nucleic acid is or comprises at least one mutation relative to a reference nucleic acid (*e.g.*, a wild-type nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide)).

[0108] In some embodiments, a variant cargo nucleic acid (*e.g.*, a variant cargo component) is associated with (*e.g.*, operably linked to) a barcode, as described herein (*i.e.*, a barcoded variant cargo nucleic acid). In some embodiments, a variant cargo nucleic acid possesses improved functionality (*e.g.*, reduced toxicity, improved pharmacokinetic measures (*e.g.*, dissociation constant (Kd), improved biophysical properties, improved developability, improved expression, etc.) relative to a reference nucleic acid (*e.g.*, a wild-type nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide)).

[0109] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more variant polypeptides, or characteristic portion thereof, wherein the one or more variant polypeptides are identified from a population of barcoded variant polypeptides by a method as described herein.

[0110] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more variant nucleic acids, encoding one or more variant polypeptides, or characteristic portion thereof, wherein the one or more variant nucleic acids are identified from a population of barcoded variant nucleic acids by a method as described herein.

[0111] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more variant nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof, wherein the therapeutic polypeptides are identified from a population of barcoded variant cargo polypeptides by a method as described herein.

[0112] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more variant nucleic acids encoding one or more barcoded variant cargo polypeptides, or characteristic portion thereof, wherein the barcoded variant cargo polypeptides are generated by a method as described herein.

[0113] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof, wherein the one or more therapeutic polypeptides are identified from a population of barcoded variant cargo polypeptides by a method as described herein.

[0114] Among other things, the present disclosure provides for a composition (*e.g.*, pharmaceutical composition) comprising one or more barcoded variant cargo polypeptides, or characteristic portion thereof, wherein the one or more barcoded variant cargo polypeptides are generated by a method as described herein.

[0115] In some embodiments, an encoded peptide barcode has an amino acid sequence selected from a group consisting of SEQ ID NOs: 5347-8398. In some embodiments, an encoded peptide barcode is encoded by a nucleic acid sequence selected from a group consisting of SEQ ID NOs: 1148-4199. In some embodiments, an encoded peptide barcode has a length of 8 to 25 amino acids. In some embodiments, an encoded peptide barcode has a length of 10 amino acids.

[0116] In some embodiments, a nucleotide sequence of a barcode component comprises, in order from 5' to 3' or 3' to 5', one or more of: (a) a first invariant sequence (*e.g.,* a linker sequence or a payload sequence); (b) a variant sequence that is at least 9 nucleotides long; and (c) a second invariant sequence (*e.g.,* a linker sequence, a stop codon, or a payload sequence). In some embodiments, a nucleotide sequence of a barcode component further comprises one or more of: (d) a sequence encoding a short helical motif; (e) a sequence encoding a disordered motif; (f) an invariant sequence linking a barcode component to a cargo component.

[0117] In some embodiments, a variant sequence is at least 15, 24, 27, 45, 150, or 300, nucleotides long.

[0118] In some embodiments, each polypeptide binder of a group of polypeptide binders has an amino acid sequence selected from a group consisting of SEQ ID NOs: 4200- 5346. In some embodiments, each polypeptide binder of a group of polypeptide binders is encoded by a nucleic acid sequence selected from a group consisting of SEQ ID NOs: 1-1147. In some embodiments, each polypeptide binder is expressed on a phage. In some embodiments, a phage is selected from a group consisting of M13, T4, T7, Lambda, and filamentous phage. In some embodiments, a phage is M13.

[0119] In some embodiments, a nucleic acid encodes a barcoded cargo polypeptide. In some embodiments, a barcoded cargo polypeptide, or a characteristic portion thereof, is expressed on a surface of a delivery particle (*e.g.*, a viral particle, a lipid-based particle [*e.g.*, cell-produced or not cell-produced, a lipid nanoparticle (LNP), a liposome, a micelle, an extracellular vesicle (*e.g.*, exosomes, microparticles, etc.)], a polymer-based particle (*e.g.*, PGLA), a polysaccharide-based particle, etc.).

[0120] In some embodiments, a nucleic acid is or comprises DNA. In some embodiments, a nucleic acid is or comprises RNA.

[0121] In some embodiments, a nucleic acid is disposed within a delivery particle. In some embodiments, a nucleic acid is disposed on a surface of a delivery particle.

[0122] The present disclosure provides a library comprising a plurality of nucleic acids. In some embodiments, each nucleic acid is a nucleic acid of as described herein.

[0123] The present disclosure provides a plurality of delivery particles. In some embodiments, one or more of delivery particles in a plurality comprises a nucleic acid as described herein. In some embodiments, a nucleic acid in each delivery particle in a plurality is same. In some embodiments, delivery particles comprise at least two different nucleic acids. In some embodiments, delivery particles that comprise at least two different nucleic acids comprise different cargo components. In some embodiments, delivery particles comprise cargo components encoding at least two different cargo polypeptides. In some embodiments, cargo polypeptides are variants of a reference polypeptide, which reference polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide. In some embodiments, variants comprise amino acid sequences. In some embodiments, variants comprise amino acid sequences that are at least 70% identical to each other (*e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to each other).

[0124] In some embodiments, delivery particles comprise one or more associated (*e.g.*, covalently or non-covalently) targeting moieties. In some embodiments, one or more targeting moieties are of the same type. In some embodiments, one or more targeting moieties are of different types.

[0125] In some embodiments, a plurality of delivery particles are substantially a same type of delivery particle. In some embodiments, a plurality of delivery particles comprises two or more types of delivery particles. In some embodiments, a plurality of delivery particles is or comprises a viral particle, a lipid-based particle [*e.g.*, cell-produced or not cell-produced, a lipid nanoparticle (LNP), a liposome, a micelle, an extracellular vesicle (*e.g.*, exosomes, microparticles, etc.)], a polymer-based particle (*e.g.*, PGLA), a polysaccharide-based particle, or a combination thereof.

[0126] In some embodiments, a plurality of delivery particles are or comprise a viral particle. In some embodiments, a plurality of delivery particles are or comprise two or more types of viral particles. In some embodiments, viral particles are or comprise one or more of AAV delivery particles, lentivirus delivery particles, adenovirus delivery particles, herpesvirus delivery particles, and anellovirus delivery particles. In some embodiments, AAV delivery particles are or comprise two or more serotypes (*e.g.*, AAV2, AAV5, AAV6, AAV8, AAV9, AAV.DJ, AAV.PHP, any variant thereof, or a combination thereof).

[0127] In some embodiments, two or more types of delivery particles are or comprise two or more types of lipid-based particles (*e.g.*, LNPs)(*e.g.*, having different formulations).

[0128] Among other things, the present disclosure provides a delivery particle comprising a nucleic acid as described herein.

[0129] Among other things, the present disclosure provides a population of delivery particles comprising a nucleic acid as described herein.

[0130] Among other things, the present disclosure provides a cell comprising a nucleic acid as described herein, a library as described herein, a plurality of delivery particles as described herein, or a delivery particle as described herein.

[0131] The present disclosure provides a population of cells comprising a nucleic acid of as described herein, a library as described herein, a plurality of delivery particles o as described herein, a delivery particle as described herein, or a population of delivery particles as described herein.

[0132] The present disclosure provides a composition (*e.g.*, pharmaceutical composition). In some embodiments, a composition comprises a nucleic acid as described herein, a library as

described herein, a plurality of delivery particles as described herein, a delivery particle as described herein, or a population of delivery particles as described herein.

[0133] Among other things, the present disclosure provides a kit comprising: (a) a set of nucleic acids; and (b) a set of binders, each of which is a polypeptide, or a nucleic acid encoding a polypeptide, that binds specifically to at least a particular peptide barcode in a collection of barcodes. In some embodiments, each nucleic acid of a set is as described herein. In some embodiments, a kit comprises one or more of binders is provided as a phage particle, or collection thereof, engineered to express a binder. In some embodiments, a kit comprises one or more of binders is provided as a nucleic acid in a phagemid vector, or as an insert suitable for cloning into a phage vector.

[0134] In some embodiments, a kit further comprises information designating peptide barcodes for each binder. In some embodiments, each binder has been determined to bind specifically to at least a particular peptide barcode within a collection of barcodes. In some embodiments, each peptide barcode binds specifically to at least one binder in a set.

[0135] In some embodiments, a kit further comprises a set of instructions to perform sequencing of one or more phage particles bound to one or more barcodes. In some embodiments, a kit further comprises a computer readable program for decoding sequencing data. In some embodiments, a kit further comprises reagents to express a binder on a phage particle.

[0136] In some embodiments, a kit comprises nucleic acids that encode one or more barcodes. In some embodiments, a kit comprises nucleic acids that encode one or more binders.

[0137] Among other things, the present disclosure provides a method for identifying a therapeutic polypeptide or a target polypeptide to treat a disease, disorder, or condition. In some embodiments, a method comprises steps of: a) subjecting a population of barcoded cargo polypeptides to an assessment; b) separating those members of a population that satisfy an assessment from those that do not, so that a positive population or a negative population, or both, is identified; c) contacting a positive population, or a negative population, or each population separately from the other, with a set of binders which includes at least one binder specific for each barcode in a population; and d) determining which binders bind to separated members, thereby determining which barcoded cargo polypeptides are present in a contacted population(s).

In some embodiments, barcoded cargo polypeptides are encoded by nucleic acids as described herein. In some embodiments, a method further comprises: a) administering a population of nucleic acids that encode barcoded cargo polypeptides to an animal; and b) obtaining a sample from an animal to subject to further assessment.

[0138] In some embodiments, a step of separating comprises purifying one or more barcoded cargo polypeptides from a sample. In some embodiments, barcoded cargo polypeptides are purified from a complex sample. In some embodiments, a complex sample is tissue. In some embodiments, a complex sample is blood. In some embodiments, barcoded cargo polypeptides are purified using affinity purification methods (*e.g.,* FLAG IP, protein G/A) or protein precipitation methods.

[0139] In some embodiments, each binder of a set of binders is expressed on a phage.

[0140] In some embodiments, a step of determining comprises: a) amplifying nucleic acids of bound phage particles; b) determining nucleotide sequences of amplified nucleic acids, wherein one or more of determined nucleotide sequences corresponds to a coding sequence of a binder; c) detecting one or more cargo polypeptides from a population of barcoded cargo polypeptides using determined sequence(s) of a coding sequence of a binder; and f) identifying one or more barcoded cargo polypeptides as a therapeutic or a target to treat a disease, disorder, or condition.

[0141] Among other things, the present disclosure provides a method of pharmacokinetic screening. In some embodiments, a method comprises: a) administering a population of nucleic acids that encode a set of barcoded therapeutic candidate polypeptides, or characteristic portion thereof, to an animal; b) obtaining a sample from an animal; c) purifying one or more barcoded therapeutic candidate polypeptides from a sample; d) contacting a sample with a set of binders (*e.g.,* binding agents with binders expressed on them) which includes at least one binder specific for each barcode in a sample; and e) determining (*e.g.*, simultaneously) relative amounts of each binder present in a sample to determine each barcoded therapeutic candidate polypeptides' pharmacokinetic properties, biodistribution, half-life, tissue-mediated drug disposition (TMDD), epitope properties, affinity properties, thermostability properties, pH sensitivity properties, or *in vivo* stability. In some embodiments, each therapeutic candidate polypeptide comprises a specific peptide barcode.

[0142] In some embodiments, multiple samples are obtained from an animal. In some embodiments, an animal is a model for a disease, disorder, or condition.

[0143] In some embodiments, an animal is a mammal. In some embodiments, an animal is a human. In some embodiments, an animal is genetically modified to express barcoded therapeutic candidate polypeptides.

[0144] In some embodiments, a disease, disorder, or condition is cancer, autoimmune, neurodegenerative, or a pathogenic (*e.g.,* viral/bacterial) disease, disorder, or condition.

[0145] In some embodiments, purified therapeutic candidate polypeptides are a subset of barcoded therapeutic candidate polypeptides administered to an animal.

[0146] In some embodiments, a sample is blood, tissue, a tumor. In some embodiments, a sample is a control.

[0147] In some embodiments, a step of determining comprises (i) sequencing nucleic acid from binding agents expressing a binder; (ii) decoding relative amounts of each barcode present thereby determining relative amounts of each therapeutic candidate polypeptide; and/or (iii) performing one or more of FACS, MACS (magnetic activated cell sorting), or affinity-based purification.

[0148] In some embodiments, a method comprises removing any unassociated (*e.g.,* unbound) binders. In some embodiments, removing is performed by washing.

[0149] In some embodiments, a step of determining comprises performing one or more of amplification, propagation, and sequencing (*e.g.,* nucleic acid (*e.g.,* DNA, RNA) amplification, propagation, and/or sequencing). In some embodiments, amplification is performed using PCR, LAMP, or RCA. In some embodiments, sequencing is performed using Illumina, NGS, nanopore sequencing, or Pac Bio long-read sequencing.

[0150] In some embodiments, a step of determining comprises quantifying a number of binders that bind to a barcoded therapeutic candidate polypeptide. In some embodiments, quantifying is performed by decoding a nucleotide sequence of each binder that binds to a barcoded therapeutic candidate polypeptide. In some embodiments, a number of nucleotide sequences provides measure of target polypeptide in a population of barcoded therapeutic candidate polypeptides.

[0151] In some embodiments, a step of administering comprises administering barcoded therapeutic candidate polypeptides orally or intravenously.

[0152] In some embodiments, barcoded therapeutic candidate polypeptides are delivered by a plurality of delivery particles as described herein, a delivery particle as described herein, or a population of delivery particles as described herein.

[0153] Among other things, the present disclosure provides a method of treatment comprising: administering a therapeutic polypeptide or nucleic acid that encodes a therapeutic polypeptide, or characteristic portion thereof, that has been determined to satisfy an assessment by a process comprising steps of: a) subjecting a population of nucleic acids that encode a set of barcoded cargo polypeptides to an assessment; b) separating those members of a population that satisfy an assessment from those that do not, so that a positive population or a negative population, or both, is identified; c) contacting a positive population, or a negative population, or each population separately from the other, with a set of binders which includes at least one binder specific for each barcode in a population; d) determining which binders bind to separated members, thereby determining which barcoded cargo polypeptides are present in a contacted population(s); and e) identifying a therapeutic polypeptide from barcoded cargo polypeptides determined to be present in a contacted population(s).

[0154] Among other things, the present disclosure provides a method of treatment comprising: administering a therapeutic polypeptide or nucleic acid that encodes a therapeutic polypeptide, or characteristic portion thereof, that has been determined to satisfy an assessment by a process comprising steps of: a) contacting a set of binders either with a first population, with a second population, or separately with each of a first and second populations of barcoded cargo polypeptides; b) determining which binders of a set bind to a member of a first population, a second population, or both, thereby determining which barcoded cargo polypeptides are present in a contacted population(s); and c) identifying a therapeutic polypeptide from barcoded cargo polypeptides determined to be present in a contacted population(s). In some embodiments, i) each binder binds specifically to one barcode relative to the other barcodes; and ii) a set of binders, collectively, includes a binder specific for each of barcodes in a first and second populations. In some embodiments, barcoded cargo polypeptides are encoded by nucleic acids as

described herein. In some embodiments, a first and second populations have been separated from one another based on performance in an assessment.

[0155] Among other things, the present disclosure provides a method of treatment comprising: administering a therapeutic polypeptide, or characteristic portion thereof. In some embodiments, a therapeutic polypeptide is identified from a population of barcoded cargo polypeptides by a method as described herein.

[0156] The present disclosure provides a method of treatment comprising: administering a nucleic acid encoding a therapeutic polypeptide, or characteristic portion thereof. In some embodiments, a therapeutic polypeptide is identified from a population of barcoded cargo polypeptides by a method as described herein.

[0157] The present disclosure provides a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof. In some embodiments, one or more therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0158] The present disclosure provides a composition (*e.g.*, pharmaceutical composition) comprising one or more barcoded cargo polypeptides, or characteristic portion thereof. In some embodiments, one or more barcoded cargo polypeptides are generated by a method as described herein.

[0159] The present disclosure provides a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof. In some embodiments, therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0160] The present disclosure provides a method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof. In some embodiments, one or more therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0161] The present disclosure provides a method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof. In some embodiments, therapeutic

polypeptides are identified from a population of barcoded cargo polypeptides by a method as described herein.

[0162] These, and other aspects encompassed by the present disclosure, are described in more detail below and in the claims.

BRIEF DESCRIPTION OF THE DRAWING

[0163] **FIG. 1A** is a schematic of barcoded cargo as described herein, according to an illustrative embodiment. It illustrates a barcoded cargo and the corresponding DNA encoding a barcoded cargo. LN refers to "linker N terminus" and LC refers to "linker C terminus". In some embodiments, LN and LC sequences are constant and encode amino acids that connect the cargo to the barcode. In some embodiments, LN and LC sequences are constant and are nucleic acid sequences used for modular cloning of barcodes with different cargos. In some embodiments, LN and LC sequences are flanked by Type IIS restriction site sequences.

[0164] **FIG. 1B** is a schematic of barcoded cargo as described herein, according to an illustrative embodiment. It illustrates a nucleic acid sequence encoding a barcode and/or a barcoded cargo. LN refers to "linker N terminus" and LC refers to "linker C terminus". In some embodiments, LN and LC sequences are constant and encode amino acids that connect the cargo to the barcode. In some embodiments, LN and LC sequences are constant and are nucleic acid sequences used for modular cloning of barcodes with different cargos. In some embodiments, LN and LC sequences are flanked by Type IIS restriction site sequences.

[0165] **FIG. 2** is a schematic of a method to detect and/or quantify and/or characterize cargos (*e.g.,* cargo polypeptides) in a pool using barcodes and binding agents as described herein, according to an illustrative embodiment. A library of barcoded cargo is contacted with a library of binding agents containing identifying DNA. A wash step is applied that removes binding agents that do not associate (*e.g.,* link (*e.g.,* form strong linkages)) to any of the barcoded cargo, while leaving only binding agents that associate with barcodes. Following the wash, a process of DNA sequencing is applied to associated binding agents. In some embodiments, sequencing may be performed using next-generation sequencing (NGS) (*e.g.,* as operated by an Illumina sequencer). The relative abundances of DNA sequences are reported as a computer file (*e.g.,*

.fastq data). A computer algorithm is applied on the .fastq data combined with prior biophysical characterization of the binding agents to infer the abundance of each of barcoded cargo in a pool.

[0166] **FIG. 3A** is a schematic for capturing a barcode as described herein, so that it may be contacted by a binding agent as described herein, according to an illustrative embodiment. It illustrates a capture scaffold that may have a barcode associated with it (*e.g.,* immobilized on its surface), and a binding agent (*e.g.,* phage with binder expressed on its surface (*e.g.,* with binder DNA in phage)) is contacted to characterize biophysical interaction. In some embodiments, the biophysical characterization is a measure of dissociation constant (Kd) between the binding agent and the peptide barcode.

[0167] **FIG. 3B** is a schematic for capturing a barcode as described herein, so that it may be contacted by a binding agent as described herein, according to an illustrative embodiment. It is a schematic of a barcode-binder platform as described herein, according to an illustrative embodiment. The schematic shows a magnetic bead with a bead binding domain conjugated to a universally tagged (*e.g.,* HALO, Chitin BD, Avitag (Strep), etc.) barcoded cargo. To detect the captured barcoded cargo, a binding agent (*e.g.,* phage expressing a binder on its surface (*e.g.,* phage with binder DNA/lib)) with known affinity to the barcode is bound to the immobilized cargo. The DNA within the phage that encodes for the binder is then amplified and subjected to NGS to detect the cargo.

[0168] **FIG. 3C** is a schematic for capturing a barcode as described herein, so that it may be contacted by a binding agent as described herein, according to an illustrative embodiment. It is a schematic of a barcode-binder platform as described herein, according to an illustrative embodiment. The schematic shows a magnetic bead with an Fc / Protein A conjugated barcoded cargo. To detect the captured barcoded cargo, a binding agent (*e.g.,* phage expressing a binder on its surface (*e.g.,* phage with binder DNA/lib) with known affinity to the barcode is bound to the immobilized cargo. The DNA within the phage that encodes for the binder is then amplified and subjected to NGS to detect the cargo.

[0169] **FIG. 4** is a schematic of a method to learn the barcode fingerprint of a given barcode as described herein, according to an illustrative embodiment. A peptide barcode displayed on a capture scaffold is contacted with a library of binding agents containing identifying DNA. A wash step is applied that removes binding agents that do not associate (*e.g.,*

link (*e.g.*, form strong linkages)) with any of the barcodes, while leaving only binding agents that do associate with barcodes. After the wash, a process of DNA sequencing is applied to associated binding agents. In some embodiments, sequencing may be performed using next-generation sequencing (NGS) (*e.g.*, as operated by an Illumina sequencer). The relative abundances of DNA sequences are reported as a computer file (*e.g.*, in .fastq format). A computer algorithm is applied on the .fastq data to computer a barcode fingerprint. This is a vector of the relative counts of the members of the binding agent library. The method of learning a barcode fingerprint can be repeated for any barcode to identify a unique fingerprint. In some embodiments, steps 1 – 4 of FIG. 4 may be repeated, each time starting with a focused binding agent library in order to improve the fingerprint, for a barcode with an existing fingerprint or a new barcode. In some embodiments, the focused binding agent library is made by oligonucleotide library synthesis.

[0170] **FIG. 5** is a schematic of a method to use a fingerprint matrix of a set of barcodes to determine the relative abundance of a mixture of barcodes, according to an illustrative embodiment. A set of barcodes for which individual fingerprints have been determined are combined in a known ratio and displayed (*e.g.*, on a scaffold) for subsequent contact with a binding agent library. A binding agent library is contacted with the set of barcodes and non-specific binding agents are washed away. The specific binding agents are quantified by NGS and reported as a mixed measurement computer file (*e.g.*, in .fastq format). These data are provided to a computer algorithm that uses the mixed measurement to learn the relative scalings of readouts relative to the original fingerprints, and assembles the scaled fingerprints together into a scaled matrix. This scaled fingerprint matrix can then be used to quantify the relative abundance of barcoded cargos.

[0171] **FIGS. 6A-6C** show results of quantifying a complex mixture of barcodes. Up to 6 barcodes were pooled and then measured using the decoding method described herein. **FIG. 6A** shows the actual relative proportion of a given barcode (left panel) and the measured relative proportion of a given barcode (right panel). Rows are individual experimental conditions, columns are barcodes, color is measurements (100% barcode = white, 0% barcode = black). **FIG. 6B** shows a plot of measured concentration of barcodes against actual concentration of barcodes for all experiments compared across all barcodes. Across all experiments and mixtures, a pearson of 0.95 between measured and actual proportions was calculated. **FIG. 6C** shows a plot of NGS count values, normalized to counts per million, for each single barcode

measurement as well as mixture that were used to predict the relative abundance of each barcode within the mixture. Rows are experiments, thus all values in a row are generated from a single .fastq file and columns are binding agents. **FIG. 6C** discloses SEQ ID NOS. 8400-8413, respectively, in order of appearance.

[0172] **FIGS. 7A-7B** show a schematic of a method and data obtained using the decoding method on cargo polypeptide with barcodes contained within internal regions of the polypeptide sequences (*i.e.,* endogenous barcodes). The schematic shows results from a synthetic pooled barcode measurement assay. **FIG. 7A** shows two barcoded cargos (BC1 and BC2) combined at various known concentrations in different wells of a 96-well plate. Each mixture was subjected to contact with the same pool of binding agents and decoded as described herein. Each mixture was quantified and then compared to the known values of the barcoded cargos. **FIG. 7B** shows relative actual proportions (X axis) of each barcode correlate to relative measured proportions (Y axis) with a pearson of .96

[0173] **FIGS. 8A-8C** show a schematic of a method to detect cargo polypeptides in serum using the barcode-binder platform as described herein, according to an illustrative embodiment. The cargo polypeptides have barcodes contained within internal regions of the polypeptide sequences (*i.e.,* endogenous barcodes). **FIG. 8A** shows the barcoded therapeutic antibody agents of interest (barcoded-mAbs) were mixed at known concentrations and then added to serum. The barcoded cargos were then purified, contacted with binding agents, and subjected to decoding. **FIG. 8B** shows the relative actual barcoded antibody proportion (left) and the relative measured antibody proportion (right) for 3 experimental conditions, with 3 replicates each. Rows correspond to experimental condition, columns to barcodes, and color of heat-map cell is a measure of the proportion of barcoded antibody present. **FIG. 8C** shows a scatterplot of all the data across all experimental conditions for all barcodes with a Spearman correlation of .926 across all experimental measurements.

[0174] **FIG. 9A** shows a schematic of the experiment provided in Examples 1, 2, and 8. Six unique barcodes (BC1, BC2, BC3, BC4, BC5, and BC6) were mixed at known proportions, contacted with binding agents, and subjected to decoding as described herein. Two barcodes were experimentally held out as negative controls, but prediction for these barcodes was allowed, thus allowing determination of background prediction.

[0175] **FIG. 9B** and **FIG. 9C** show data on accuracy of decoding procedure across a 10-fold range of concentrations for the 6 unique barcodes. **FIG. 9B** shows plot of actual data (input) and measured data obtained after decoding for one mixture of known barcode concentrations. Input known concentrations (left bar) are shown next to predictions/measured data (right bar) for each barcode across 3 replicates. **FIG. 9C** shows plots of actual data (input) and measured data obtained after decoding for five different mixtures (*i.e.,* pools 1-5) of known barcode concentrations. Input known concentrations (left bar) are shown next to predictions/measured data (right bar) for each barcode across 3 replicates.

[0176] **FIGS. 10A-10C** show a method and data for determining the absolute concentration of a single test barcode as described herein. **FIG. 10A** shows a schematic of an experiment. A single test barcode was assayed at several concentrations, while a "spike-in" barcode (*i.e.,* a reference barcode) was added to each assay mixture at a known concentration. The various concentrations of the test barcode were contacted with binding agents and decoding was performed as described herein. The prediction of the "spike-in" barcode was used to determine the absolute amount of the test barcode being measured. **FIG. 10B** shows a plot of the measured absolute quantities of the test barcode (right bar) compared to known input concentrations of the test barcode (left bar) for each titration of the test barcode. The Y-axis is the logarithm of the test barcode concentration in nanograms per milliliter (ng/mL). **FIG. 10C** shows the results of determination of absolute concentration for 6 different barcodes. Plots show known input concentrations (left bar) and measured concentration (right bar) for six (6) different barcodes.

[0177] **FIG. 11** shows a method for determining the relative abundance of two polypeptides after injection *in vivo*, using the binder-barcode system described herein according to an illustrative embodiment. The figure shows a graphical depiction of experimental setup. In group 1 (top), mice were injected with 1 barcoded cargo. In group 2 (middle), 2 barcoded cargos were injected. In group 3 (bottom), no barcoded cargos were injected. For each of the three groups, at 24 hours, a serum sample was taken; the barcoded cargo(s) captured using binding agents as described herein, and subjected to decoding. The measured barcoded cargo concentrations (right bar) compared to known input concentrations (left bar) for each group are shown.

[0178] **FIGS. 12A-12E** show determination of twenty-four (24) barcodes contained within a single mixture. **FIG. 12A** shows a graphical depiction of the experiment. Of 24 total barcodes the algorithm can predict, 10 were present within a mixture at equal concentrations. The rest were held out from the pool, but prediction was computationally allowed. Three (3) separate pools, which cover all possible barcodes, were measured in replicate. **FIG. 12B** shows prediction for the first pool. Input concentration (left bar) and measured concentration (right bar) are displayed. **FIG. 12C** shows predictions across all three pools. As in B, input concentration is left bar and measured is right bar. **FIG. 12D** shows the barcode fingerprint for the 24 barcodes used to computationally determine the relative abundance of the barcodes within the 3 pools. Columns represent barcode fingerprints, and rows represent binding agent fingerprints. **FIG. 12D** discloses SEQ ID NOS. 8414, 8415, 8414, 8416, 8414, 8413, 8414, 8417, 8414, 8418, 8414, 8419, 8414, 8420-8425, 8422, 8426, 8427, 8426, 8428-8431, 8430, 8432, 8433, 8432, 8434, 8432, 8435, 8432, 8430, 8432, 8436-8453, 8413, 8453, 8454, 8453, 8455-8475, 8474, 8476-8480, 8479, 8481-8484, 8483, 8484-8493, 8472, 8494, 8472, 8495, 8472, 8496, 8472, 8497, 8472, 8498-8502, 8501, 8503-8505, 8504, 8506, 8504, 8507, 8504, 8508-8516, 8515, 8517, 8518, 8417, 8519, 8520, 8519, 8521-8532, 8403, 8533-8542, 8541, 8543-8545, 8544, 8546, 8544, 8547, 8544, 8548-8552, 8551, 8553, 8551, 8554-8562, respectively, in order of appearance. **FIG. 12E** shows the binding agent counts from the three pools, used to computationally determine the proportion of the pools. Rows are the binding agent counts, columns are the pools, the cell is the binding agent count within a specific pool. **FIG. 12E** discloses SEQ ID NOS. 8414, 8415, 8414, 8416, 8414, 8413, 8414, 8417, 8414, 8418, 8414, 8419, 8414, 8420-8425, 8422, 8426, 8427, 8426, 8428-8431, 8430, 8432, 8433, 8432, 8434, 8432, 8435, 8432, 8430, 8432, 8436-8453, 8413, 8453, 8454, 8453, 8455-8475, 8474, 8476-8480, 8479, 8481-8484, 8483, 8484-8493, 8472, 8494, 8472, 8495, 8472, 8496, 8472, 8497, 8472, 8498-8502, 8501, 8503-8505, 8504, 8506, 8504, 8507, 8504, 8508-8516, 8515, 8517, 8518, 8417, 8519, 8520, 8519, 8521-8532, 8403, 8533-8542, 8541, 8543-8545, 8544, 8546, 8544, 8547, 8544, 8548-8552, 8551, 8553, 8551, 8554-8562, respectively, in order of appearance.

[0179] **FIG. 13A** is a schematic of a method to detect and/or quantify and/or characterize fourteen (14) exemplary cargos (*e.g.,* cargo polypeptides) in a pool using a binder-barcode platform as described herein. A library of barcoded cargo was contacted with a library of binding

agents containing identifying DNA ("binder-barcode particles"). Binder-barcode particles were injected as a pooled library into wild-type (wt) BALB/c mice (n=3 per timepoint) *in vivo*. Blood was collected from individual mice at timepoints 30 min, 6 hours, 24 hours, and 48 hours, (n=3 per timepoint), and serum was extracted. Binder-barcode particles were captured and subjected to a decoding procedure as described herein.

[0180] **FIG. 13B** depicts plots showing clearance of fourteen (14) exemplary binder-barcode particles injected into wild-type (wt) BALB/c mice (n=3 per timepoint) *in vivo*. Data were collected at time points 30 min, 6 hours, 24 hours, and 48 hours as measured by a decoding procedure described herein. Y-axis is normalized to 100% of injection volume for each exemplary binder-barcode particle. Plots shown in **FIG. 13B** were measured simultaneously. Each plot contains exemplary binder-barcode particles that were characterized as having certain measurable phenotypes. The left plot shows clearance (% injection) of clinical controls with known properties. The middle plot shows clearance (% injection) of exemplary binder-barcode particles that were characterized has having slow clearance properties. The right plot shows clearance (% injection) of exemplary binder-barcode particles that were characterized as having fast clearance properties.

[0181] **FIG. 14A** is a schematic of a method to detect and/or quantify and/or characterize thirty-six (36) cargos (*e.g.*, cargo polypeptides) in a pool using a binder-barcode platform as described herein. A library of barcoded cargo was contacted with a library of binding agents containing identifying DNA ("binder-barcode particles"). Binder-barcode particles were injected as a pooled library into tumor bearing NSG mice, which had been previously implanted with two tumor cell lines ("Tumor 1", "Tumor 2"), (n=2-4 per timepoint) *in vivo*. Blood and tumor tissue was collected from individual mice at timepoints 30 min, 6 hours, 24 hours, and 48 hours, (n=3 per timepoint). Tissue was lysed using standard lysis buffer, and serum was separated from blood. Binder-barcode particles were captured and subjected to a decoding procedure as described herein.

[0182] **FIG. 14B** is a heat-map of data collected from thirty-six (36) exemplary binder-barcode particles using a decoding procedure described herein. Rows identify each exemplary binder-barcode particle tested in the present example. Columns indicate data for a mouse across each time point for serum, Tumor 1, or Tumor 2. Color intensity indicates relative units of drug

as measured via a decoding procedure described herein. Color intensity indicates a normalized readout of relative concentration as measured via next generation sequencing (NGS).

[0183] **FIG. 14C** depicts plots of binder-barcode particles described by **FIG. 14B** using a decoding procedure described herein. A diversity of properties was simultaneously measured. For example, binder-barcode particle P14_A5 was rapidly cleared from serum, with minimal accumulation in Tumor 1 or Tumor 2, while binder-barcode particle P17_A10 was more slowly cleared and maintained in tumor 1 over time.

[0184] **FIGS. 15A-15C** depict plots showing ELISA quantitation of two groups of cargos (Group 1: cargo polypeptides with no barcode; Group 2: a pool of eight (8) binder-barcode particles where each particle includes the same cargo polypeptides used in Group 1, and each particle is barcoded with a different barcode) (**FIG. 15A**), quantification of Group 2 using a decoding procedure described herein (**FIG. 15B**), and a comparison of half-life measurements for Group 1 and Group 2 quantified using ELISA and a decoding procedure described herein, respectively (**FIG. 15C**).

[0185] **FIG. 16A** is a schematic of a method to detect and/or quantify and/or characterize thirty-five (35) cargos (*e.g.,* cargo polypeptides) in distinct pools with different number of barcoded cargos at different concentrations using a binder-barcode platform as described herein.

[0186] **FIG. 16B** depicts a plot showing measured barcode level (arbitrary units) versus expected barcoded-cargo level (ng) generated by arraying ninety-six (96) distinct mixtures comprising 10-35 barcoded cargo with each barcoded cargo at a known concentration between 1 pg and 1 µg. Each data point in FIG. 16B represents a comparison between a known concentration of a binder-barcode particle from one of the ninety-six (96) distinct mixtures and a concentration determined by a decoding procedure described herein.

[0187] **FIG. 17** depicts a schematic of an exemplary method that provides for high throughput cargo delivery, production, screening, identification, and/or characterization as described herein. Nucleic acids comprising (1) a cargo component whose nucleotide sequence is or comprises a sequence encoding a cargo polypeptide and (2) a barcode component whose nucleotide sequence is or comprises a sequence encoding a peptide barcode are disposed within one or more delivery particles and are administered to an animal (*e.g.*, a mammal). Functional

cargos are expressed in a tissue of interest. Decoding methods are used to determine cargos and/or delivery particles with desired properties.

[0188] **FIG. 18** depicts a schematic of an exemplary method that provides for tracking and/or assessment and/or quantification of different nucleic acids encoding a cargo component disposed within different types of delivery particles, according to an embodiment of the present disclosure. Two exemplary nucleic acid constructs were designed: (1) a first nucleic acid comprising (a) a cargo component encoding a cargo polypeptide comprising a secretion signal peptide, and (b) a barcode component; and (2) a second nucleic acid comprising (a) a cargo component encoding a cargo polypeptide without a secretion signal peptide, and (b) a barcode component. Each nucleic acid design was disposed within different delivery particles (*e.g.*, AAV delivery particles, *e.g.*, AAV2, AAV9, AAV.PHPB) that exhibit different tissue tropisms. Delivery particles were administered into mice and decoded according to methods described herein.

[0189] **FIGS. 19A-19C** depict bar graphs showing high-throughput screening, identification, and/or quantification of two different cargo polypeptides (with or without a secretion signal peptide) delivered via different delivery particles (AAV2, AAV9, AAV.PHPB) across different tissue types (brain, liver, serum).

[0190] **FIG. 20** depicts a schematic showing that high-throughput screening provides for screening of multiple cargos, formats, targets, and tissues simultaneously in different models.

[0191] **FIG. 21** depicts octet biolayer interferometry (BLI) data that show respective dissociation of cargo polypeptides against the transferrin receptor (TfR).

[0192] **FIG. 22** depicts ELISA data that show respective dissociation of cargo polypeptides against the transferrin receptor (TfR).

[0193] **FIG. 23** depicts a schematic of an exemplary method showing that variant cargos of a previously detected, assessed, and/or characterized cargo (*e.g.*, wild-type cargo) may be generated and subject to further detection, assessment, and/or characterization, for example, using methods as described herein. In some embodiments, such variant cargos may possess improved functionality (*e.g.*, improved developability, improved expression, improved affinity, etc.).

[0194] **FIG. 24** depicts a plot showing a high-throughput *in vivo* screen of brain shuttle candidates using the binder-barcode platform described herein. Panel (a) shows anti-TfR VHHs with unique properties including: epitope, affinity, thermostability, and pH sensitivity, that were nominated for screening *in vivo*. Panel (b) shows 239 anti-TfR VHHs that were simultaneously screened for abundance *in vivo* in sets of 15 to 96, at doses ranging from 0.5 to 1 mg/kg, depending on batch size, in brain, serum, and other tissue using the binder-barcode platform at 24 hours.

[0195] **FIG. 25** depicts a plot showing PK analysis across brain, cell-free fraction (parenchyma), serum, and muscle tissues of select screened TfR1 brain shuttle candidates analyzed in a multiplexed experiment using the binder-barcode platform described herein.

DEFINITIONS

[0196] ***About:*** The term "about", when used herein in reference to a value, refers to a value that is similar, in context to the referenced value. In general, those skilled in the art, familiar with the context, will appreciate the relevant degree of variance encompassed by "about" in that context. For example, in some embodiments, the term "about" may encompass a range of values that within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less of the referred value.

[0197] ***Administer***: The term "administer" or "administering", when used herein typically refers to the administration of a composition to a subject or system to achieve delivery of an agent that is, or is included in, the composition. Those of ordinary skill in the art will be aware of a variety of routes that may, in appropriate circumstances, be utilized for administration to a subject, for example a human. For example, in some embodiments, administration may be ocular, oral, parenteral, topical, etc.. In some particular embodiments, administration may be bronchial (*e.g.*, by bronchial instillation), buccal, dermal (which may be or comprise, for example, one or more of topical to the dermis, intradermal, interdermal, transdermal, etc), enteral, intra-arterial, intradermal, intragastric, intramedullary, intramuscular, intranasal, intraperitoneal, intrathecal, intravenous, intraventricular, within a specific organ (*e.g.*, intrahepatic), mucosal, nasal, oral, rectal, subcutaneous, sublingual, topical, tracheal (*e.g.*, by intratracheal instillation), vaginal, vitreal, etc. In some embodiments, administration may involve

only a single dose. In some embodiments, administration may involve application of a fixed number of doses. In some embodiments, administration may involve dosing that is intermittent (*e.g.*, a plurality of doses separated in time) and/or periodic (*e.g.*, individual doses separated by a common period of time) dosing. In some embodiments, administration may involve continuous dosing (*e.g.*, perfusion) for at least a selected period of time.

[0198] ***Affinity*:** As is known in the art, "affinity" is a measure of the tightness with which two or more binding partners associate with one another. Those skilled in the art are aware of a variety of assays that can be used to assess affinity, and will furthermore be aware of appropriate controls for such assays. In some embodiments, affinity is assessed in a quantitative assay. In some embodiments, affinity is assessed over a plurality of concentrations (*e.g.*, of binding partner at a time). In some embodiments, affinity is assessed in the presence of one or more potential competitor entities (*e.g.*, that might be present in a relevant – *e.g.*, physiological – setting). In some embodiments, affinity is assessed relative to a reference (*e.g.*, that has a known affinity above a particular threshold [a "positive control" reference] or that has a known affinity below a particular threshold [a "negative control" reference"]. In some embodiments, affinity may be assessed relative to a contemporaneous reference; in some embodiments, affinity may be assessed relative to a historical reference. Typically, when affinity is assessed relative to a reference, it is assessed under comparable conditions.

[0199] ***Agent*** : In general, the term "agent", as used herein, is used to refer to an entity (*e.g.*, for example, a lipid, metal, nucleic acid, polypeptide, polysaccharide, small molecule, etc, or complex, combination, mixture or system [*e.g.*, cell, tissue, organism] thereof), or phenomenon (*e.g.*, heat, electric current or field, magnetic force or field, etc.). In appropriate circumstances, as will be clear from context to those skilled in the art, the term may be utilized to refer to an entity that is or comprises a cell or organism, or a fraction, extract, or component thereof. Alternatively or additionally, as context will make clear, the term may be used to refer to a natural product in that it is found in and/or is obtained from nature. In some instances, again as will be clear from context, the term may be used to refer to one or more entities that is man-made in that it is designed, engineered, and/or produced through action of the hand of man and/or is not found in nature. In some embodiments, an agent may be utilized in isolated or pure form; in some embodiments, an agent may be utilized in crude form. In some embodiments, potential agents may be provided as collections or libraries, for example that may be screened to identify

or characterize active agents within them. In some cases, the term "agent" may refer to a compound or entity that is or comprises a polymer; in some cases, the term may refer to a compound or entity that comprises one or more polymeric moieties. In some embodiments, the term "agent" may refer to a compound or entity that is not a polymer and/or is substantially free of any polymer and/or of one or more particular polymeric moieties. In some embodiments, the term may refer to a compound or entity that lacks or is substantially free of any polymeric moiety.

[0200] ***Amino acid:*** in its broadest sense, as used herein, refers to any compound and/or substance that can be incorporated into a polypeptide chain, *e.g.,* through formation of one or more peptide bonds. In some embodiments, an amino acid has the general structure H_2N–C(H)(R)–COOH. In some embodiments, an amino acid is a naturally-occurring amino acid. In some embodiments, an amino acid is a non-natural amino acid; in some embodiments, an amino acid is a D-amino acid; in some embodiments, an amino acid is an L-amino acid. "Standard amino acid" refers to any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid" refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. In some embodiments, an amino acid, including a carboxy- and/or amino-terminal amino acid in a polypeptide, can contain a structural modification as compared with the general structure above. For example, in some embodiments, an amino acid may be modified by methylation, amidation, acetylation, pegylation, glycosylation, phosphorylation, and/or substitution (*e.g.,* of the amino group, the carboxylic acid group, one or more protons, and/or the hydroxyl group) as compared with the general structure. In some embodiments, such modification may, for example, alter the circulating half-life of a polypeptide containing the modified amino acid as compared with one containing an otherwise identical unmodified amino acid. In some embodiments, such modification does not significantly alter a relevant activity of a polypeptide containing the modified amino acid, as compared with one containing an otherwise identical unmodified amino acid. As will be clear from context, in some embodiments, the term "amino acid" may be used to refer to a free amino acid; in some embodiments it may be used to refer to an amino acid residue of a polypeptide.

[0201] ***Animal:*** as used herein refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans, of either sex and at any stage of development. In some

embodiments, "animal" refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.,* a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In some embodiments, an animal may be a transgenic animal, genetically engineered animal, and/or a clone.

[0202] ***Antibody:*** As used herein, the term "antibody" refers to a polypeptide that includes canonical immunoglobulin sequence elements sufficient to confer specific binding to a particular target antigen. As is known in the art, intact antibodies as produced in nature are approximately 150 kD tetrameric agents comprised of two identical heavy chain polypeptides (about 50 kD each) and two identical light chain polypeptides (about 25 kD each) that associate with each other into what is commonly referred to as a "Y-shaped" structure. Each heavy chain is comprised of at least four domains (each about 110 amino acids long)– an amino-terminal variable (VH) domain (located at the tips of the Y structure), followed by three constant domains: CH1, CH2, and the carboxy-terminal CH3 (located at the base of the Y's stem). A short region, known as the "switch", connects the heavy chain variable and constant regions. The "hinge" connects CH2 and CH3 domains to the rest of the antibody. Two disulfide bonds in this hinge region connect the two heavy chain polypeptides to one another in an intact antibody. Each light chain is comprised of two domains – an amino-terminal variable (VL) domain, followed by a carboxy-terminal constant (CL) domain, separated from one another by another "switch". Intact antibody tetramers are comprised of two heavy chain-light chain dimers in which the heavy and light chains are linked to one another by a single disulfide bond; two other disulfide bonds connect the heavy chain hinge regions to one another, so that the dimers are connected to one another and the tetramer is formed. Naturally-produced antibodies are also glycosylated, typically on the CH2 domain. Each domain in a natural antibody has a structure characterized by an "immunoglobulin fold" formed from two beta sheets (*e.g.,* 3-, 4-, or 5-stranded sheets) packed against each other in a compressed antiparallel beta barrel. Each variable domain contains three hypervariable loops known as "complement determining regions" (CDR1, CDR2, and CDR3) and four somewhat invariant "framework" regions (FR1, FR2, FR3, and FR4). When natural antibodies fold, the FR regions form the beta sheets that provide the structural framework for the domains, and the CDR loop regions from both the heavy and light

chains are brought together in three-dimensional space so that they create a single hypervariable antigen binding site located at the tip of the Y structure. The Fc region of naturally-occurring antibodies binds to elements of the complement system, and also to receptors on effector cells, including for example effector cells that mediate cytotoxicity. As is known in the art, affinity and/or other binding attributes of Fc regions for Fc receptors can be modulated through glycosylation or other modification. In some embodiments, antibodies produced and/or utilized in accordance with the present invention include glycosylated Fc domains, including Fc domains with modified or engineered such glycosylation. For purposes of the present invention, in certain embodiments, any polypeptide or complex of polypeptides that includes sufficient immunoglobulin domain sequences as found in natural antibodies can be referred to and/or used as an "antibody", whether such polypeptide is naturally produced (*e.g.,* generated by an organism reacting to an antigen), or produced by recombinant engineering, chemical synthesis, or other artificial system or methodology. In some embodiments, an antibody is polyclonal; in some embodiments, an antibody is monoclonal. In some embodiments, an antibody has constant region sequences that are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, antibody sequence elements are humanized, primatized, chimeric, etc, as is known in the art. Moreover, the term "antibody" as used herein, can refer in appropriate embodiments (unless otherwise stated or clear from context) to any of the art-known or developed constructs or formats for utilizing antibody structural and functional features in alternative presentation. For example, embodiments, an antibody utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE or IgM antibodies; bi- or multi-specific antibodies (*e.g.,* Zybodies®, etc); antibody fragments such as Fab fragments, Fab' fragments, F(ab')2 fragments, Fd' fragments, Fd fragments, and isolated CDRs or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (*e.g.,* shark single domain antibodies such as IgNAR or fragments thereof); cameloid antibodies; masked antibodies (*e.g.,* Probodies®); Small Modular ImmunoPharmaceuticals ("SMIPs™"); single chain or Tandem diabodies (TandAb®); VHHs; Anticalins®; Nanobodies® minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies;, Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®; and KALBITOR®s. In some embodiments, an antibody may lack a covalent modification (*e.g.,* attachment of a glycan) that it would have if produced naturally. In some embodiments, an

antibody may contain a covalent modification (*e.g.,* attachment of a glycan, a cargo [*e.g.,* a detectable moiety, a therapeutic moiety, a catalytic moiety, etc], or other pendant group [*e.g.,* poly-ethylene glycol, etc.]

[0203] ***Antibody agent*:** As used herein, the term "antibody agent" refers to an agent that specifically binds to a particular antigen. In some embodiments, the term encompasses any polypeptide or polypeptide complex that includes immunoglobulin structural elements sufficient to confer specific binding. Exemplary antibody agents include, but are not limited to monoclonal antibodies or polyclonal antibodies. In some embodiments, an antibody agent may include one or more constant region sequences that are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, an antibody agent may include one or more sequence elements are humanized, primatized, chimeric, etc, as is known in the art. In many embodiments, the term "antibody agent" is used to refer to one or more of the art-known or developed constructs or formats for utilizing antibody structural and functional features in alternative presentation. For example, embodiments, an antibody agent utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE or IgM antibodies; bi- or multi-specific antibodies (*e.g.,* Zybodies®, etc); antibody fragments such as Fab fragments, Fab' fragments, F(ab')2 fragments, Fd' fragments, Fd fragments, and isolated CDRs or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (*e.g.,* shark single domain antibodies such as IgNAR or fragments thereof); cameloid antibodies; masked antibodies (*e.g.,* Probodies®); Small Modular ImmunoPharmaceuticals ("SMIPs™"); single chain or Tandem diabodies (TandAb®); VHHs; Anticalins®; Nanobodies® minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies;, Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®; and KALBITOR®s. In some embodiments, an antibody may lack a covalent modification (*e.g.,* attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody may contain a covalent modification (*e.g.,* attachment of a glycan, a cargo [*e.g.,* a detectable moiety, a therapeutic moiety, a catalytic moiety, etc], or other pendant group [*e.g.,* poly-ethylene glycol, etc.]. In many embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes one or more structural elements recognized by those skilled in the art as a complementarity determining region (CDR); in some embodiments an antibody agent is or comprises a polypeptide whose amino acid sequence

includes at least one CDR (*e.g.,* at least one heavy chain CDR and/or at least one light chain CDR) that is substantially identical to one found in a reference antibody. In some embodiments an included CDR is substantially identical to a reference CDR in that it is either identical in sequence or contains between 1-5 amino acid substitutions as compared with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 96%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes structural elements recognized by those skilled in the art as an immunoglobulin variable domain. In some embodiments, an antibody agent is a polypeptide protein having a binding domain which is homologous or largely homologous to an immunoglobulin-binding domain.

[0204] ***Associated:*** Two events or entities are "associated" with one another, as that term is used herein, if the presence, level, degree, type and/or form of one is correlated with that of the other. For example, a particular entity (*e.g.,* polypeptide, genetic signature, metabolite, microbe, etc) is considered to be associated with a particular disease, disorder, or condition, if its presence, level and/or form correlates with incidence of and/or susceptibility to the disease, disorder, or

condition (*e.g.*, across a relevant population). In some embodiments, two or more entities are physically "associated" with one another if they interact, directly or indirectly, so that they are and/or remain in physical proximity with one another. In some embodiments, two or more entities that are physically associated with one another are covalently linked to one another; in some embodiments, two or more entities that are physically associated with one another are not covalently linked to one another but are non-covalently associated, for example by means of hydrogen bonds, van der Waals interaction, hydrophobic interactions, magnetism, and combinations thereof.

[0205] ***Barcode, Barcode component, or Barcode peptide or Peptide barcode:*** As used herein, the term "barcode" refers to a sequence (nucleic acid or amino acid), which associates (*e.g.*, covalently or non-covalently) with a cargo as described herein. In some embodiments, a nucleic acid comprises a "barcode component" encoding a peptide barcode. In some embodiments, a barcode component is operably linked to a cargo component that encodes a cargo polypeptide. In some embodiments, a peptide barcode is linked to cargo polypeptide. As described herein a barcode associates with a binder with known specificity and affinity. In some embodiments, a barcode binds to a specific antibody-agent. In some embodiments, a barcode may be contained within a specific cargo of interest. In some embodiments, a barcode may be terminal to a specific cargo of interest. In some embodiments, a barcode may be synthetic. In some embodiments, a barcode may be designed. For example, a barcode sequence may be ordered as a DNA polynucleotide and cloned into a cargo of interest using methods of molecular cloning known to a person of ordinary skill in the art.

[0206] ***Binder:*** As used herein, the term "binder" or "binder moiety" refers to a polypeptide sequence, which associates with a barcode with known specificity and affinity. In some embodiments, a binder is or comprises an antibody agent. In some embodiments, a binder is expressed on a surface of a binding agent. In some embodiments, a binder may bind to one or more barcodes.

[0207] ***Binding***: It will be understood that the term "binding" or "bind", as used herein, typically refers to a non-covalent association between or among two or more entities. "Direct" binding involves physical contact between entities or moieties; indirect binding involves physical interaction by way of physical contact with one or more intermediate entities. Binding between

two or more entities can typically be assessed in any of a variety of contexts – including where interacting entities or moieties are studied in isolation or in the context of more complex systems (*e.g.,* while covalently or otherwise associated with a carrier entity and/or in a biological system or cell).

[0208] ***Binding agent***: In general, the term "binding agent" is used herein to refer to any entity that binds to a target of interest as described herein (*e.g.,* a barcode, a barcoded target, etc.). In many embodiments, a binding agent of interest is one that binds specifically with its target in that it discriminates its target from other potential binding partners in a particular interaction context. In general, a binding agent may be or comprise an entity of any chemical class (*e.g.,* polymer, non-polymer, small molecule, polypeptide, carbohydrate, lipid, nucleic acid, etc) or biological class (*e.g.,* bacteria, phage, ribosome, mRNA, DNA, etc.). In some embodiments, a binding agent is a single chemical entity. In some embodiments, a binding agent is a complex of two or more discrete chemical entities associated with one another under relevant conditions by non-covalent interactions. For example, those skilled in the art will appreciate that in some embodiments, a binding agent may comprise a "generic" binding moiety (*e.g.,* one of biotin/avidin/streptavidin and/or a class-specific antibody) and a "specific" binding moiety (*e.g.,* an antibody or aptamers with a particular molecular target) that is linked to the partner of the generic biding moiety. In some embodiments, such an approach can permit modular assembly of multiple binding agents through linkage of different specific binding moieties with the same generic binding moiety partner. In some embodiments, binding agents are or comprise phages. In some embodiments, binding agents are or comprise polypeptides (including, *e.g.,* antibodies or antibody fragments). In some embodiments, binding agents are or comprise small molecules. In some embodiments, binding agents are or comprise nucleic acids. In some embodiments, binding agents are or comprise aptamers. In some embodiments, binding agents are polymers; in some embodiments, binding agents are not polymers. In some embodiments, binding agents are non-polymeric in that they lack polymeric moieties. In some embodiments, binding agents are or comprise carbohydrates. In some embodiments, binding agents are or comprise lectins. In some embodiments, binding agents are or comprise peptidomimetics. In some embodiments, binding agents are or comprise scaffold proteins. In some embodiments, binding agents are or comprise mimeotopes. In some embodiments, binding agents are or comprise stapled peptides. In certain embodiments, binding agents are or comprise nucleic acids, such as DNA or RNA.

[0209] ***Biological Sample***: As used herein, the term "biological sample" typically refers to a sample obtained or derived from a biological source (*e.g.,* a tissue or organism or cell culture) of interest, as described herein. In some embodiments, a source of interest comprises an organism, such as an animal or human. In some embodiments, a biological sample is or comprises biological tissue or fluid. In some embodiments, a biological sample may be or comprise bone marrow; blood; blood cells; ascites; tissue or fine needle biopsy samples; cell-containing body fluids; free floating nucleic acids; sputum; saliva; urine; cerebrospinal fluid, peritoneal fluid; pleural fluid; feces; lymph; gynecological fluids; skin swabs; vaginal swabs; oral swabs; nasal swabs; washings or lavages such as a ductal lavages or broncheoalveolar lavages; aspirates; scrapings; bone marrow specimens; tissue biopsy specimens; surgical specimens; feces, other body fluids, secretions, and/or excretions; and/or cells therefrom, etc. In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, obtained cells are or include cells from an individual from whom the sample is obtained. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. For example, in some embodiments, a primary biological sample is obtained by methods selected from the group consisting of biopsy (*e.g.,* fine needle aspiration or tissue biopsy), surgery, collection of body fluid (*e.g.,* blood, lymph, feces *etc.*), etc. In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (*e.g.,* by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to techniques such as amplification or reverse transcription of mRNA, isolation and/or purification of certain components, *etc.*

[0210] ***Cargo, Cargo component, or Cargo polypeptide:*** As used herein, the term "cargo" refers to a payload, which may be associated (*e.g.*, covalently or non-covalently) to a barcode. In some embodiments, a cargo comprises a nucleic acid (referred to herein as a "cargo component") encoding a cargo polypeptide. In some embodiments, a cargo is or comprises a cargo component (*e.g.*, that encodes a cargo polypeptide). In some embodiments, a cargo is or comprises a cargo polypeptide (*e.g.*, encoded by a cargo component). In some embodiments, a cargo component is operably linked to a nucleic acid referred to herein as a "barcode

component". In some embodiments, a barcode component encodes a peptide barcode. In some embodiments, a peptide barcode is linked (*e.g.*, covalently and/or non-covalently) to a cargo polypeptide. In some embodiments, a cargo polypeptide is detected in a pool of polypeptides. In some embodiments, a cargo polypeptide is an unmodified polypeptide that is to be detected in a pool of polypeptides without association of a peptide barcode. In some embodiments, a cargo polypeptide is a modified polypeptide that is to be detected in a pool of polypeptides. In some embodiments, a cargo polypeptide may not be associated with a barcode (*e.g.,* a peptide barcode). In some embodiments, a cargo comprises one or more sequences (nucleic acid sequence or amino acid sequence) that modify expression of a cargo polypeptide. In some embodiments, such one or more sequences are associated (directly or indirectly) with a barcode throughout a period of assessment of cargo polypeptides, as described herein.

[0211] ***CDR:*** As used herein, "CDR" refers to a complementarity determining region within an antibody variable region. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the variable regions. A "*set of CDRs*" or "*CDR set*" refers to a group of three or six CDRs that occur in either a single variable region capable of binding the antigen or the CDRs of cognate heavy and light chain variable regions capable of binding the antigen. Certain systems have been established in the art for defining CDR boundaries (*e.g.,* Kabat, Chothia, etc.); those skilled in the art appreciate the differences between and among these systems and are capable of understanding CDR boundaries to the extent required to understand and to practice the claimed invention.

[0212] ***Characteristic portion***: As used herein, the term "characteristic portion," in the broadest sense, refers to a portion of a substance whose presence (or absence) correlates with presence (or absence) of a particular feature, attribute, or activity of the substance. In some embodiments, a characteristic portion of a substance is a portion that is found in a given substance and in related substances that share a particular feature, attribute or activity, but not in those that do not share the particular feature, attribute or activity. In some embodiments, a characteristic portion shares at least one functional characteristic with the intact substance. For example, in some embodiments, a "characteristic portion" of a protein or polypeptide is one that contains a continuous stretch of amino acids, or a collection of continuous stretches of amino acids, that together are characteristic of a protein or polypeptide. In some embodiments, each such

continuous stretch generally contains at least 2, 5, 10, 15, 20, 50, or more amino acids. In general, a characteristic portion of a substance (*e.g.*, of a protein, antibody, *etc.*) is one that, in addition to a sequence and/or structural identity specified above, shares at least one functional characteristic with the relevant intact substance. In some embodiments, a characteristic portion may be biologically active.

[0213] ***Characteristic sequence***: As used herein, the term "characteristic sequence" is a sequence that is found in all members of a family of polypeptides or nucleic acids, and therefore can be used by those of ordinary skill in the art to define members of the family.

[0214] ***Characteristic sequence element:*** As used herein, the phrase "characteristic sequence element" refers to a sequence element found in a polymer (*e.g.*, in a polypeptide or nucleic acid) that represents a characteristic portion of that polymer. In some embodiments, presence of a characteristic sequence element correlates with presence or level of a particular activity or property of a polymer. In some embodiments, presence (or absence) of a characteristic sequence element defines a particular polymer as a member (or not a member) of a particular family or group of such polymers. A characteristic sequence element typically comprises at least two monomers (*e.g.*, amino acids or nucleotides). In some embodiments, a characteristic sequence element includes at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, or more monomers (*e.g.*, contiguously linked monomers). In some embodiments, a characteristic sequence element includes at least first and second stretches of contiguous monomers spaced apart by one or more spacer regions whose length may or may not vary across polymers that share a sequence element.

[0215] ***Combination therapy***: As used herein, the term "combination therapy" refers to those situations in which a subject is simultaneously exposed to two or more therapeutic regimens (*e.g.*, two or more therapeutic agents). In some embodiments, two or more agents may be administered simultaneously. In some embodiments, two or more agents may be administered sequentially. In some embodiments, two or more agents may be administered in overlapping dosing regimens.

[0216] ***Comparable***: As used herein, the term "comparable" refers to two or more agents, entities, situations, sets of conditions, that may not be identical to one another but that are sufficiently similar to permit comparison there between so that one skilled in the art will appreciate that conclusions may reasonably be drawn based on differences or similarities observed. In some embodiments, comparable sets of conditions, circumstances, individuals, or

populations are characterized by a plurality of substantially identical features and one or a small number of varied features. Those of ordinary skill in the art will understand, in context, what degree of identity is required in any given circumstance for two or more such agents, entities, situations, sets of conditions, to be considered comparable. For example, those of ordinary skill in the art will appreciate that sets of circumstances, individuals, or populations are comparable to one another when characterized by a sufficient number and type of substantially identical features to warrant a reasonable conclusion that differences in results obtained or phenomena observed under or with different sets of circumstances, individuals, or populations are caused by or indicative of the variation in those features that are varied.

[0217] ***Comprising:*** A composition or method described herein as "comprising" one or more named elements or steps is open-ended, meaning that the named elements or steps are essential, but other elements or steps may be added within the scope of the composition or method. To avoid prolixity, it is also understood that any composition or method described as "comprising" (or which "comprises") one or more named elements or steps also describes the corresponding, more limited composition or method "consisting essentially of" (or which "consists essentially of") the same named elements or steps, meaning that the composition or method includes the named essential elements or steps and may also include additional elements or steps that do not materially affect the basic and novel characteristic(s) of the composition or method. It is also understood that any composition or method described herein as "comprising" or "consisting essentially of" one or more named elements or steps also describes the corresponding, more limited, and closed-ended composition or method "consisting of" (or "consists of") the named elements or steps to the exclusion of any other unnamed element or step. In any composition or method disclosed herein, known or disclosed equivalents of any named essential element or step may be substituted for that element or step.

[0218] ***Decoding***: As used herein, the term “decoding”, refers to a laboratory and/or bioinformatics process of identifying and quantifying a unique set of amino acids within a barcode. In some embodiments, such identification and quantification is achieved using nucleic acid (*e.g.,* DNA) counts form a sequencing experiment and measuring an abundance of binder counts. In some embodiments, previously measured fingerprints (*e.g.,* binder fingerprint or barcode fingerprint) are used to determine the relationship between an unknown barcode mixture, which is being decoded, for example, by comparing to a previously known mixture’s

binder counts, across binders with known and varying affinities to several barcodes within the pool.

[0219] ***Designed:*** As used herein, the term "designed" refers to an agent (i) whose structure is or was selected by the hand of man; (ii) that is produced by a process requiring the hand of man; and/or (iii) that is distinct from natural substances and other known agents.

[0220] ***Determine:*** Many methodologies described herein include a step of "determining". Those of ordinary skill in the art, reading the present specification, will appreciate that such "determining" can utilize or be accomplished through use of any of a variety of techniques available to those skilled in the art, including for example specific techniques explicitly referred to herein. In some embodiments, determining involves manipulation of a physical sample. In some embodiments, determining involves consideration and/or manipulation of data or information, for example utilizing a computer or other processing unit adapted to perform a relevant analysis. In some embodiments, determining involves receiving relevant information and/or materials from a source. In some embodiments, determining involves comparing one or more features of a sample or entity to a comparable reference.

[0221] ***Engineered:*** In general, the term "engineered" refers to the aspect of having been manipulated by the hand of man. For example, in some embodiments, a small molecule may be considered to be engineered if its structure and/or production is designed and/or implemented by the hand of man. Analogously, in some embodiments, a polynucleotide may be considered to be "engineered" when two or more sequences, that are not linked together in that order in nature, are manipulated by the hand of man to be directly linked to one another in the engineered polynucleotide. For example, in some embodiments of the present invention, an engineered polynucleotide comprises a regulatory sequence that is found in nature in operative association with a first sequence (*e.g.,* coding sequence) but not in operative association with a second sequence (*e.g.,* coding sequence), is linked by the hand of man so that it is operatively associated with the second sequence. Comparably, a cell or organism is considered to be "engineered" if it has been manipulated so that its genetic information is altered (*e.g.,* new genetic material not previously present has been introduced, for example by transformation, mating, somatic hybridization, transfection, transduction, or other mechanism, or previously present genetic material is altered or removed, for example by substitution or deletion mutation, or by mating

protocols). As is common practice and is understood by those in the art, expression products of an engineered polynucleotide, and/or progeny of an engineered polynucleotide or cell are typically still referred to as "engineered" even though the actual manipulation was performed on a prior entity.

[0222] ***Expression:*** As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.,* by transcription); (2) processing of an RNA transcript (*e.g.,* by splicing, editing, 5' cap formation, and/or 3' end formation); (3) translation of an RNA into a polypeptide or protein; and/or (4) post-translational modification of a polypeptide or protein.

[0223] ***Fingerprint***: As used herein, the term "fingerprint" refers to the counts of one or more unknown agents that a known agent may bind to or be associated with. In some embodiments, a fingerprint may be for a known barcode or barcode mixture. In some embodiments, a fingerprint may be for a known binder or binder mixture. For example, in some embodiments, a fingerprint (*e.g.,* barcode fingerprint) may refer to the counts of one or more binders (*e.g.,* determined through sequencing analysis) to bind specifically to a known barcode or barcode mixture. That is, in some embodiments, a fingerprint for a barcode refers to the counts of one or more binders, some of which may have high affinity for the barcode, and some of which may have low affinity for the barcode. In some embodiments, a fingerprint may be used in the decoding process, which process is used to determine the relative or absolute abundance of a given barcode within a pool of barcodes. As is understood to a person of ordinary skill in the art a fingerprint may be determined for a known barcode or barcode mixture, or for a known binder or binder mixture. For example, in some embodiments, a fingerprint (*e.g.,* binder fingerprint) may refer to the counts of one or more barcodes (*e.g.,* determined through sequencing analysis) that bind specifically to a known binder or binder mixture. That is, in some embodiments, a fingerprint for a binder refers to the counts of one or more barcodes, some of which may have high affinity for the binder, and some of which may have low affinity for the binder. Accordingly, a fingerprint may also be used in the decoding process, in some embodiments, to determine the relative or absolute abundance of a given binder within a pool of binders.

[0224] ***Fragment:*** A "fragment" of a material or entity as described herein has a structure that includes a discrete portion of the whole, but lacks one or more moieties found in

the whole. In some embodiments, a fragment consists of such a discrete portion. In some embodiments, a fragment consists of or comprises a characteristic structural element or moiety found in the whole. In some embodiments, a polymer fragment comprises or consists of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more monomeric units (*e.g.,* residues) as found in the whole polymer. In some embodiments, a polymer fragment comprises or consists of at least about 5%, 10%, 15%, 20%, 25%, 30%, 25%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more of the monomeric units (*e.g.,* residues) found in the whole polymer. The whole material or entity may in some embodiments be referred to as the "parent" of the whole.

[0225] ***Human***: In some embodiments, a human is an embryo, a fetus, an infant, a child, a teenager, an adult, or a senior citizen.

[0226] ***Improve, increase, inhibit*** or ***reduce:*** As used herein, the terms "improve", "increase", "inhibit', "reduce", or grammatical equivalents thereof, indicate values that are relative to a baseline or other reference measurement. In some embodiments, an appropriate reference measurement may be or comprise a measurement in a particular system (*e.g.,* in a single individual) under otherwise comparable conditions absent presence of (*e.g.,* prior to and/or after) a particular agent or treatment, or in presence of an appropriate comparable reference agent. In some embodiments, an appropriate reference measurement may be or comprise a measurement in comparable system known or expected to respond in a particular way, in presence of the relevant agent or treatment. In some embodiments, "improve", "increase", "inhibit", "reduce" may be referred to collectively as "modify".

[0227] ***Invariant sequence***: As used herein, the term "invariant sequence" indicates a sequence that is substantially identical in a library of nucleic acids. In some embodiments, each nucleic acid comprises, among other things, a barcode component. As an example, in some embodiments, a barcode component may further comprise one or more of: (1) a nucleic acid sequence encoding a short helical motif, (2) a nucleic acid encoding a disordered motif, and (3) an invariant sequence linking the barcode component to the cargo component. A nucleic acid sequence encoding a short helical motif and a nucleic acid encoding a disordered motif may each

respectively vary across the library of nucleic acids. In contrast, each invariant sequence in a pool of nucleic acids is substantially identical.

[0228] ***In vitro*:** The term "in vitro" as used herein refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

[0229] ***In vivo:*** as used herein refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

[0230] ***Library:*** The term "library" as used herein refers to a mixture of one or more distinct molecules. In some embodiments, all elements of a library share one or more common components. In some embodiments, all elements of a library share no common components. In some embodiments, one or more elements of a library are distinguished by one or more unique components. In some embodiments, as may be apparent from the context, a library may refer to a mixture of binding agents. In some embodiments, a library may be a phage library. In some embodiments, for example, a phage library may consist of phage with distinct binders displayed on (*e.g.*, on a surface) of the phage and encapsulating DNA encoding for this binder within the phage. In some embodiments, a library may refer to a mixture of barcoded cargo proteins. In some embodiments, a library may refer to a mixture of barcodes (*e.g.*, peptide barcodes).

[0231] ***Linker:*** as used herein, is used to refer to that portion of a multi-element agent that connects different elements to one another. For example, those of ordinary skill in the art appreciate that a polypeptide whose structure includes two or more functional or organizational domains often includes a stretch of amino acids between such domains that links them to one another. In some embodiments, a polypeptide comprising a linker element has an overall structure of the general form S1-L-S2, wherein S1 and S2 may be the same or different and represent two domains associated with one another by the linker. In some embodiments, a polyptide linker is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length. In some embodiments, a linker is characterized in that it tends not to adopt a rigid three-dimensional structure, but rather provides flexibility to the polypeptide. A

variety of different linker elements that can appropriately be used when engineering polypeptides (*e.g.,* fusion polypeptides) known in the art (see *e.g.,* Holliger, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (1994) Structure 2: 1 121-1123).

[0232] ***Nucleic acid***: As used herein, in its broadest sense, refers to any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. As will be clear from context, in some embodiments, "nucleic acid" refers to an individual nucleic acid residue (*e.g.,* a nucleotide and/or nucleoside); in some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising individual nucleic acid residues. In some embodiments, a "nucleic acid" is or comprises RNA; in some embodiments, a "nucleic acid" is or comprises DNA. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, a nucleic acid analog differs from a nucleic acid in that it does not utilize a phosphodiester backbone. For example, in some embodiments, a nucleic acid is, comprises, or consists of one or more "peptide nucleic acids", which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. Alternatively or additionally, in some embodiments, a nucleic acid has one or more phosphorothioate and/or 5'-N-phosphoramidite linkages rather than phosphodiester bonds. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleosides (*e.g.,* adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxy guanosine, and deoxycytidine). In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleoside analogs (*e.g.,* 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3 -methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5 -propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a nucleic acid comprises one or more modified sugars (*e.g.,* 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared with those in natural nucleic acids. In some embodiments, a nucleic acid has a nucleotide sequence that encodes a

functional gene product such as an RNA or protein. In some embodiments, a nucleic acid includes one or more introns. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 1 10, 120, 130, 140, 150, 160, 170, 180, 190, 20, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long. In some embodiments, a nucleic acid is partly or wholly single stranded; in some embodiments, a nucleic acid is partly or wholly double stranded. In some embodiments a nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide. In some embodiments, a nucleic acid has enzymatic activity.

[0233] ***Operably linked***: As used herein, refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control element "operably linked" to a functional element is associated in such a way that expression and/or activity of the functional element is achieved under conditions compatible with the control element. In some embodiments, "operably linked" control elements are contiguous (*e.g.*, covalently linked) with coding elements of interest; in some embodiments, control elements act in trans to or otherwise at a from the functional element of interest. In some embodiments, "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. In some embodiments, for example, a functional linkage may include transcriptional control. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences can be contiguous with each other and, *e.g.*, where necessary to join two protein coding regions, are in the same reading frame. In some embodiments, a cargo component is operably linked to a barcode component.

[0234] ***Peptide:*** The term "peptide" as used herein refers to a polypeptide that is typically relatively short, for example having a length of less than about 100 amino acids, less than about

50 amino acids, less than about 40 amino acids less than about 30 amino acids, less than about 25 amino acids, less than about 20 amino acids, less than about 15 amino acids, or less than 10 amino acids.

[0235] ***Pharmaceutical composition***: As used herein, the term "pharmaceutical composition" refers to a composition in which an active agent is formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, an active agent is present in unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when administered to a relevant population. In some embodiments, a pharmaceutical composition may be specially formulated for administration in solid or liquid form, including those adapted for, *e.g.*, administration, for example, an injectable formulation that is, *e.g.*, an aqueous or non-aqueous solution or suspension or a liquid drop designed to be administered into an ear canal. In some embodiments, a pharmaceutical composition may be formulated for administration via injection either in a particular organ or compartment, *e.g.*, directly into an ear, or systemic, *e.g.*, intravenously. In some embodiments, a formulation may be or comprise drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes, capsules, powders, etc. In some embodiments, an active agent may be or comprise an isolated, purified, or pure compound.

[0236] ***Polypeptide:*** As used herein refers to any polymeric chain of residues (*e.g.*, amino acids) that are typically linked by peptide bonds. In some embodiments, a polypeptide has an amino acid sequence that occurs in nature. In some embodiments, a polypeptide has an amino acid sequence that does not occur in nature. In some embodiments, a polypeptide has an amino acid sequence that is engineered in that it is designed and/or produced through action of the hand of man. In some embodiments, a polypeptide may comprise or consist of natural amino acids, non-natural amino acids, or both. In some embodiments, a polypeptide may comprise or consist of only natural amino acids or only non-natural amino acids. In some embodiments, a polypeptide may comprise D-amino acids, L-amino acids, or both. In some embodiments, a polypeptide may comprise only D-amino acids. In some embodiments, a polypeptide may comprise only L-amino acids. In some embodiments, a polypeptide may include one or more pendant groups or other modifications, *e.g.*, modifying or attached to one or more amino acid side chains, at the polypeptide's N-terminus, at the polypeptide's C-terminus, or any combination thereof. In some embodiments, such pendant groups or modifications may be selected from the

group consisting of acetylation, amidation, lipidation, methylation, pegylation, etc., including combinations thereof. In some embodiments, a polypeptide may be cyclic, and/or may comprise a cyclic portion. In some embodiments, a polypeptide is not cyclic and/or does not comprise any cyclic portion. In some embodiments, a polypeptide is linear. In some embodiments, a polypeptide may be or comprise a stapled polypeptide. In some embodiments, the term "polypeptide" may be appended to a name of a reference polypeptide, activity, or structure; in such instances it is used herein to refer to polypeptides that share the relevant activity or structure and thus can be considered to be members of the same class or family of polypeptides. For each such class, the present specification provides and/or those skilled in the art will be aware of exemplary polypeptides within the class whose amino acid sequences and/or functions are known; in some embodiments, such exemplary polypeptides are reference polypeptides for the polypeptide class or family. In some embodiments, a member of a polypeptide class or family shows significant sequence homology or identity with, shares a common sequence motif (*e.g.,* a characteristic sequence element) with, and/or shares a common activity (in some embodiments at a comparable level or within a designated range) with a reference polypeptide of the class; in some embodiments with all polypeptides within the class). For example, in some embodiments, a member polypeptide shows an overall degree of sequence homology or identity with a reference polypeptide that is at least about 30-40%, and is often greater than about 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more and/or includes at least one region (*e.g.,* a conserved region that may in some embodiments be or comprise a characteristic sequence element) that shows very high sequence identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99%. Such a conserved region usually encompasses at least 3-4 and often up to 20 or more amino acids; in some embodiments, a conserved region encompasses at least one stretch of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more contiguous amino acids. In some embodiments, a useful polypeptide may comprise or consist of a fragment of a parent polypeptide. In some embodiments, a useful polypeptide as may comprise or consist of a plurality of fragments, each of which is found in the same parent polypeptide in a different spatial arrangement relative to one another than is found in the polypeptide of interest (*e.g.,* fragments that are directly linked in the parent may be spatially separated in the polypeptide of interest or vice versa, and/or fragments may be present in a different order in the

polypeptide of interest than in the parent), so that the polypeptide of interest is a derivative of its parent polypeptide. In some embodiments, a polypeptide may be a protein.

[0237] ***Pro Component***: As used herein, the term "pro component" refers to an inactive component. In some embodiments, a pro component once expressed can take an active form, for example, to have an intended effect. In some embodiments, a pro component may be expressed *in vitro*. In some embodiments, a pro component may be expressed *in vivo*. In some embodiments, a pro component may be expressed in a tissue (*e.g.*, of an animal (*e.g.*, a mammal)).

[0238] ***Protein***: As used herein, the term "protein" refers to a polypeptide (*i.e.,* a string of at least two amino acids linked to one another by peptide bonds). Proteins may include moieties other than amino acids (*e.g.,* may be glycoproteins, proteoglycans, *etc.*) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete polypeptide chain as produced by a cell (with or without a signal sequence), or can be a characteristic portion thereof. Those of ordinary skill will appreciate that a protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. Polypeptides may contain l-amino acids, d-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.,* terminal acetylation, amidation, methylation, *etc.* In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

[0239] ***Reference:*** As used herein describes a standard or control relative to which a comparison is performed. For example, in some embodiments, an agent, animal, individual, population, sample, sequence or value of interest is compared with a reference or control agent, animal, individual, population, sample, sequence or value. In some embodiments, a reference or control is tested and/or determined substantially simultaneously with the testing or determination of interest. In some embodiments, a reference or control is a historical reference or control, optionally embodied in a tangible medium. Typically, as would be understood by those skilled in the art, a reference or control is determined or characterized under comparable conditions or

circumstances to those under assessment. Those skilled in the art will appreciate when sufficient similarities are present to justify reliance on and/or comparison to a particular possible reference or control.

[0240] ***Regulatory Element***: As used herein, the term "regulatory element" or "regulatory sequence" refers to non-coding regions of DNA that regulate, in some way, expression of one or more particular genes. In some embodiments, such genes are apposed or "in the neighborhood" of a given regulatory element. In some embodiments, such genes are located quite far from a given regulatory element. In some embodiments, a regulatory element impairs or enhances transcription of one or more genes. In some embodiments, a regulatory element may be located in cis to a gene being regulated. In some embodiments, a regulatory element may be located in trans to a gene being regulated. For example, in some embodiments, a regulatory sequence refers to a nucleic acid sequence which is regulates expression of a gene product operably linked to a regulatory sequence. In some such embodiments, this sequence may be an enhancer sequence and other regulatory elements which regulate expression of a gene product.

[0241] ***Sample:*** As used herein, the term "sample" typically refers to an aliquot of material obtained or derived from a source of interest. In some embodiments, as would be appreciated from the context by a person of ordinary skill in the art, the term "sample" may be used interchangeably with terms like "mixture", or "complex mixture", or "complex sample". In some embodiments, a source of interest is a biological or environmental source. In some embodiments, a source of interest may be or comprise a cell or an organism, such as a microbe, a plant, or an animal (*e.g.,* a human). In some embodiments, a source of interest is or comprises biological tissue or fluid. In some embodiments, a biological tissue or fluid may be or comprise cells, serum, extracellular matrix, CSF, and/or combinations or component(s) thereof. In some embodiments, a biological tissue or fluid may be or comprise amniotic fluid, aqueous humor, ascites, bile, bone marrow, blood, breast milk, cerebrospinal fluid, cerumen, chyle, chime, ejaculate, endolymph, exudate, feces, gastric acid, gastric juice, lymph, mucus, pericardial fluid, perilymph, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum, semen, serum, smegma, sputum, synovial fluid, sweat, tears, urine, vaginal secreations, vitreous humour, vomit, and/or combinations or component(s) thereof. In some embodiments, a biological fluid may be or comprise an intracellular fluid, an extracellular fluid, an intravascular fluid (blood plasma), an interstitial fluid, a lymphatic fluid, and/or a transcellular fluid. In some embodiments, a

biological fluid may be or comprise a plant exudate. In some embodiments, a biological tissue or sample may be obtained, for example, by aspirate, biopsy (*e.g.,* fine needle or tissue biopsy), swab (*e.g.,* oral, nasal, skin, or vaginal swab), scraping, surgery, washing or lavage (*e.g.,* brocheoalvealar, ductal, nasal, ocular, oral, uterine, vaginal, or other washing or lavage). In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (*e.g.,* by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to one or more techniques such as amplification or reverse transcription of nucleic acid, isolation and/or purification of certain components, *etc.*

[0242] ***Specific:*** The term "specific", when used herein with reference to an agent having an activity, is understood by those skilled in the art to mean that the agent discriminates between potential target entities or states. For example, in some embodiments, an agent is said to bind "specifically" to its target if it binds preferentially with that target in the presence of one or more competing alternative targets. In many embodiments, specific interaction is dependent upon the presence of a particular structural feature of the target entity (*e.g.,* an epitope, a cleft, a binding site). It is to be understood that specificity need not be absolute. In some embodiments, specificity may be evaluated relative to that of the binding agent for one or more other potential target entities (*e.g.,* competitors). In some embodiments, specificity is evaluated relative to that of a reference specific binding agent. In some embodiments specificity is evaluated relative to that of a reference non-specific binding agent. In some embodiments, the agent or entity does not detectably bind to the competing alternative target under conditions of binding to its target entity. In some embodiments, binding agent binds with higher on-rate, lower off-rate, increased affinity, decreased dissociation, and/or increased stability to its target entity as compared with the competing alternative target(s).

[0243] ***Subject:*** As used herein, the term "subject" refers to an organism, typically a mammal (*e.g.,* a human, in some embodiments including prenatal human forms). In some embodiments, a subject is suffering from a relevant disease, disorder or condition. In some

embodiments, a subject is susceptible to a disease, disorder, or condition. In some embodiments, a subject displays one or more symptoms or characteristics of a disease, disorder or condition. In some embodiments, a subject does not display any symptom or characteristic of a disease, disorder, or condition. In some embodiments, a subject is someone with one or more features characteristic of susceptibility to or risk of a disease, disorder, or condition. In some embodiments, a subject is a patient. In some embodiments, a subject is an individual to whom diagnosis and/or therapy is and/or has been administered.

[0244] ***Substantially:*** As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0245] ***Therapeutic agent:*** As used herein, the phrase "therapeutic agent" in general refers to any agent that elicits a desired pharmacological effect when administered to an organism. In some embodiments, an agent is considered to be a therapeutic agent if it demonstrates a statistically significant effect across an appropriate population. In some embodiments, the appropriate population may be a population of model organisms. In some embodiments, an appropriate population may be defined by various criteria, such as a certain age group, gender, genetic background, preexisting clinical conditions, etc. In some embodiments, a therapeutic agent is a substance that can be used to alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. In some embodiments, a "therapeutic agent" is an agent that has been or is required to be approved by a government agency before it can be marketed for administration to humans. In some embodiments, a "therapeutic agent" is an agent for which a medical prescription is required for administration to humans. In some embodiments, a therapeutic agent is a therapeutic protein.

[0246] ***Variant:*** As used herein, the term "variant" refers to a version of something, *e.g.*, a gene sequence, that is different, in some way, from another version. To determine if something is a variant, a reference version is typically chosen and a variant is different relative to that

reference version. In some embodiments, a variant can have the same or a different (*e.g.*, increased or decreased) level of activity or functionality than a wild type sequence. For example, in some embodiments, a variant can have improved functionality as compared to a wild-type sequence if it is, *e.g.*, mutated to confer reduced toxicity in a cell. As another example, in some embodiments, a variant can have improved functionality as compared to a wild-type sequence if it is, *e.g.*, mutated to confer improved protein production in a cell. As another example, a "variant polypeptide" as used herein is a variant polypeptide that comprises one or more mutations relative to a reference polypeptide.

DETAILED DESCRIPTION

I. Barcoded Cargos

[0247] Methods and systems to generate and use barcodes and barcoded cargo are described herein. In some embodiments, a cargo polypeptide is encoded by a cargo component. In some embodiments, a peptide barcode is encoded by a barcode component. In some embodiments, cargo components are operably linked to barcode components. Other exemplary cargos are described throughout the present disclosure.

[0248] Among other things, the present disclosure provides for methods used to detect and/or characterize cargos. In some embodiments, methods disclosed herein are used to detect and/or characterize cargo polypeptides (*e.g.*, therapeutic polypeptides) encoded by cargo components. In some embodiments, methods disclosed herein are used to detect and/or characterize therapeutic or non-therapeutic polypeptides. In some embodiments, methods disclosed herein are used to detect and/or characterize cargos by tagging them with barcodes (*e.g.,* barcoded cargo components). In some embodiments, methods disclosed herein are used to detect and/or characterize cargos *in vitro*. In some embodiments, methods disclosed herein are used to detect and/or characterize cargos *in vivo*. In some embodiments, methods disclosed herein are used to detect and/or characterize a cargo. In some embodiments, methods disclosed herein are used to detect and/or characterize multiple (*e.g.,* two or more, three or more, four or more, etc.) cargos.

i. Barcodes

[0249] In some embodiments, a barcode is or comprises an amino acid sequence. In some embodiments, a barcode is or comprises an amino acid sequence that occurs in nature. In some embodiments, a barcode is or comprises an amino acid sequence that does not occur in nature. In some embodiments, a barcode is or comprises an amino acid sequence that is synthetic. In some embodiments, a barcode comprises naturally occurring amino acids. In some embodiments, a barcode comprises non-naturally occurring amino acids (*e.g.,* modified amino acids). In some embodiments, a barcode is or comprises a peptide barcode.

[0250] Barcodes of the present disclosure can be of varying lengths. For example, in some embodiments, a barcode may have a length ranging between 1 and 100 amino acids. In some embodiments, a barcode may have a length ranging between 5 and 50 amino acids. In some embodiments, a barcode may have a length ranging between 8 and 25 amino acids. In some embodiments, a barcode may have a length ranging between 9 and 25 amino acids. In some embodiments, a barcode may have a length ranging between 9 and 15 amino acids. In some embodiments, a barcode may have a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids. In some embodiments, a barcode may have a length of at least 5 amino acids. In some embodiments, a barcode may have a length of at most 100 amino acids.

[0251] Barcodes, as described herein may be available in a library in different formats. For example, in some embodiments a barcode as described herein may be described as a nucleic acid sequence. In other instance, a barcode as described herein may be described as an amino acid sequence. A person of ordinary skill in the art will appreciate that barcodes described in one format may be converted to another format using basic biological principles. Accordingly, barcodes described as nucleic acid sequences may be translated into proteins, which may be used to detect the presence or absence of a cargo (*e.g.,* cargo polypeptide) in a mixture. Such a translated barcode is referred to herein as a peptide barcode.

[0252] Accordingly, barcodes of the present disclosure when described using nucleic acids may have lengths different from amino acid sequence lengths disclosed in the paragraph above. For example, in some embodiments, a barcode may have a length ranging between 3 and 300 nucleotides. In some embodiments, a barcode may have a length ranging between 15 and

150 nucleotides. In some embodiments, a barcode may have a length ranging between 24 and 75 nucleotides. In some embodiments, a barcode may have a length ranging between 27 and 75 nucleotides. In some embodiments, a barcode may have a length ranging between 27 and 45 nucleotides. In some embodiments, a barcode may have a length of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 , 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, or 75 nucleotides. In some embodiments, a barcode may have a length of at least 15 nucleotides. In some embodiments, a barcode may have a length of at most 300 nucleotides.

[0253] Barcodes of the present disclosure may have one or more properties. In some embodiments, a barcode may be naturally occurring. In some embodiments, a barcode may not be naturally occurring (*e.g.,* synthetic). In some embodiments, a barcode may have relatively no effect on cargo function. For example, in some embodiments, tagging a cargo (*e.g.,* a cargo component) with a barcode as described herein does not alter or change relatively the function of the tagged cargo. In some embodiments, a barcode may have an effect (*e.g.,* positive or negative) on cargo function. For example, in some embodiments, tagging a cargo (*e.g.,* a cargo component) with a barcode as described herein may alter or change relatively a function (*e.g.,* half-life (*e.g.,* longer half-life), enhance targeting to specific tissue, etc.) of the tagged cargo. In some embodiments, a barcode may not elicit an immune response (*e.g.,* an IgG response, a complement response, etc.). In some embodiments, barcodes are orthogonal to each other. In some embodiments, barcodes are not orthogonal to each other.

[0254] Barcodes of the present disclosure may be attached to various positions of a cargo. For example, in some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a suitable position on a cargo. In some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a non-suitable position on a cargo. In some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a suitable position on a cargo. For example, in some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to an N-terminus of a cargo polypeptide. In some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a C-terminus of a cargo polypeptide. In some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a non-terminal

position on a cargo polypeptide (*e.g.,* side chain). In some embodiments, a barcode may be associated (*e.g.*, covalently or non-covalently) to a non-suitable position on a cargo polypeptide.

[0255] Among other things, barcodes (*e.g.,* peptide barcodes or barcode components encoding peptide barcodes) of the present disclosure may be flanked by additional sequences (*e.g.,* nucleic acid sequences, amino acid sequences, etc.). In some embodiments, a barcode may be flanked by additional sequences on a barcode's 5' end. In some embodiments, a barcode may be flanked by additional sequences on a barcode's 3' end. In some embodiments, a barcode may be flanked by additional sequences on a barcode's 3' and 5' end. In some embodiments, an additional sequence may be a primer binding site, a restriction endonuclease recognition sequence, a restriction enzyme site (*e.g.,* a cleavage site), a sequence that encodes an amino acid sequence, a sequence that does not encode an amino acid sequence, an amino acid sequence, or a nucleic acid sequence. For example, in some embodiments, a barcode may be flanked by nucleic acid sequences encoding an amino acid sequence. In some embodiments, a barcode may be flanked by nucleic acid sequences that does not encode an amino acid sequence. In some embodiments, a barcode may be flanked by amino acid sequences. In some embodiments, a peptide barcode may be flanked by amino acid sequences (*e.g.,* Glycine - Serine (GS), *e.g.*, other linker amino acid sequences). Analogously, in some embodiments, a barcode component encoding a peptide barcode of the present disclosure may be flanked by additional sequences (*e.g.,* nucleic acid sequences, amino acid sequences, etc.). In some embodiments, a barcode component encoding a peptide barcode may be flanked by nucleic acid sequences on a 5' end. In some embodiments, a barcode component encoding a peptide barcode may be flanked by nucleic acid sequences on a 3' end. In some embodiments, a barcode component encoding a peptide barcode may be flanked by nucleic acid sequences on a 3' and 5' end. In some embodiments, a barcode component encoding a peptide barcode may be flanked by nucleic acid sequences encoding an amino acid sequence comprising a Glycine - Serine (GS). In some embodiments, a barcode component encoding a peptide barcode may be flanked by nucleic acid sequences encoding an amino acid sequence comprising a linker amino acid sequence as described herein.

[0256] In some embodiments, a barcode may be flanked by restriction endonuclease recognition sequences. In some embodiments, a barcode may be flanked by restriction endonuclease recognition sequences on a barcode's 5' end. In some embodiments, a barcode may be flanked by restriction endonuclease recognition sequences on a barcode's 3' end. In some

embodiments, barcode may be flanked by restriction endonuclease recognition sequences on a barcode's 3' and 5' end. In some embodiments, a nucleic acid encoding a peptide barcode may be flanked by restriction endonuclease recognition sequences. In some embodiments, a nucleic acid encoding a peptide barcode may be flanked by restriction endonuclease recognition sequences on a 5' end. In some embodiments, a nucleic acid encoding a peptide barcode may be flanked by restriction endonuclease recognition sequences on a 3' end. In some embodiments, a nucleic acid encoding a peptide barcode may be flanked by restriction endonuclease recognition sequences on a 3' and 5' end. In some embodiments, a restriction endonuclease recognition sequence may be recognized by one or more restriction enzymes (*e.g.,* BsaI, BsmBI, BbsI, SapI, etc.). In some embodiments, restriction endonuclease recognition sequences are Type I, Type II, or Type IIs restriction endonuclease recognition sequences. Such recognition sequences, for example, may be used to produce universal overhangs that may be used in cloning peptide barcodes into different locations of various cargo. Such flexibility allows a barcode to be used to detect different cargo polypeptides in different experiments.

[0257] In some embodiments, a barcode component encoding a barcode (*e.g.,* a peptide barcode) may be associated with (*e.g.,* attached to, linked to) a second cargo component encoding a cargo polypeptide (*e.g.,* a cargo polypeptide of interest). Such nucleic acid sequences, for example, may be translated to form barcoded cargos (*e.g.,* barcoded cargo polypeptides). In some embodiments, a barcode component encoding a barcode (*e.g.,* a peptide barcode) is separate from a cargo component encoding a cargo polypeptide (*e.g.,* a cargo polypeptide of interest). For such nucleic acid sequences, for example, a barcode component encoding a peptide barcode may be translated separately from a cargo component sequence encoding a cargo polypeptide, and subsequently attached using one or more methods known in the art to join distinct amino acid sequences (*e.g.,* using linkers).

[0258] Barcodes of the present disclosure may be associated (*e.g.,* directly or indirectly attached) to cargos so as to form barcoded cargos (or barcoded cargo components as described herein). For example, in some embodiments, each barcode sequence (*e.g.,* peptide barcode sequence) may be associated to only one cargo of interest (*e.g.,* cargo polypeptide of interest) within a mixture. In some embodiments, each barcode sequence may be associated to more than one cargo of interest (*e.g.,* cargos with different sequences) within a mixture. In some embodiments, multiple (*e.g.,* two or more, three or more, four or more, etc.) barcode sequences

may be associated to one cargo of interest within a mixture. For example, in some embodiments, one or more barcode sequences may be associated to various different positions on a given cargo – such a setup may be useful in, for example, in studying and identifying the stability and/or cleavage of such barcoded cargos. In some embodiments, each cargo in a mixture is a unique sequence (*e.g.,* each cargo has a different sequence from every other cargo in the mixture). In some embodiments, each cargo in a mixture is a non-unique sequence.

[0259] Various methods and parameters may be used to select suitable barcodes for a given cargo. For example, stability of a barcoded cargo is key in determining if a cargo may be tagged by said barcode. In some embodiments, a barcode may be tagged to a specific cargo across different experiments. In some embodiments, a barcode may be tagged to different cargos in different experiments. For example, in some embodiments, a barcode may be tagged to two or more, three or more, four or more, ten or more, 100 or more, 1000 or more, or 10,000 or more different cargos across different experiments.

[0260] In some embodiments, a barcode may be associated with only one cargo in a given experiment. In some embodiments, a barcode may be associated with multiple cargos in a given experiment. For example, in some embodiments, one or more barcodes are associated with multiple cargos (*i.e.,* a barcode is tagged to multiple cargos) in a mixture, such that each cargo is associated with a unique set of barcodes within the mixture. That is, each cargo may be associated with a unique "pattern" of barcodes in the mixture. Analogously, in some embodiments, several cargos may be associated with the same barcode.

[0261] Among other things, barcodes described herein are designed to have a distinct (*i.e.,* unique) sequence. In some embodiments, a barcode is designed to have a distinct sequence (*e.g.,* distinct from another barcode). For example, each barcode is designed to be distinct (*e.g.,* unique) from every other barcode used in an experiment, such that each cargo (*e.g.,* protein to be measured) is attached to at least one barcode, and each barcode (*e.g.,* barcode with a specific sequence) is only attached to one cargo. As may be understood by a person of ordinary skill in the art, the diversity of barcodes contained within a pool is limited only by the possible diversity of amino acid sequences for a given barcode length. For example, for a barcode length 'N', there exists 20^N distinct amino acid barcode sequences of length N (if only unmodified/naturally

occurring amino acids are used). That is, for a barcode length of 15, the theoretical limit is 20^{15}, or 3.2768×10^{19}.

[0262] In some embodiments, barcodes, as described herein, can be designed and/or developed through machine-learning methods.

[0263] Example of barcodes according to various embodiments of the present disclosure are listed in sequence listing filed herewith. In some embodiments, a barcode (*e.g.,* peptide barcode) is or comprises an amino acid sequence selected from SEQ ID NOs: 5347-8398. In some embodiments, a barcode (*e.g.,* peptide barcode) is encoded by a sequence that is or comprises a nucleic acid sequence selected from SEQ ID NOs: 1148-4199.

ii. Cargo Polypeptides

[0264] Methods and systems disclosed herein are may be used for detection of one or more cargos as described herein.

[0265] In one aspect, systems and methods disclosed herein may be used for detecting a cargo (*e.g.,* cargo polypeptide) in a mixture. Specifically, barcodes disclosed herein tagged to a cargo (*e.g.,* barcoded cargo component) in a mixture and used to detect said cargo in the mixture. In some embodiments, each cargo is different from every other cargo in a mixture. In some embodiments, each cargo in a mixture is different from every other cargo in a mixture by at least one amino acid. In some embodiments, each cargo in a mixture is different from every other cargo in a mixture by two or more amino acids. In some embodiments, a cargo (*e.g.,* in a mixture) may be tagged with a barcode. In some embodiments, each cargo (*e.g.,* in a mixture) may be tagged with a same barcode. In some embodiments, each cargo (*e.g.,* in a mixture) may be tagged with different barcode. In some embodiments, a cargo (*e.g.,* in a mixture) may be tagged with a barcode that is different from every other barcode (*e.g.,* associated with other cargos) in a mixture by at least one amino acid. In some embodiments, a cargo (*e.g.,* in a mixture) may be tagged with a barcode that is different from every other barcode (*e.g.,* associated with other cargos) in a mixture by two or more amino acids.

[0266] As discussed elsewhere in the specification, a cargo may be tagged with different barcodes (*e.g.,* in different mixtures, different experiments, etc.). For example, as noted above, in

some embodiments, each barcode sequence may be associated (*e.g.*, covalently or non-covalently) with only one cargo of interest within a mixture. In some embodiments, each barcode sequence may be associated (*e.g.*, covalently or non-covalently) with more than one cargo of interest (*e.g.*, cargos with different sequences) within a mixture. In some embodiments, multiple (*e.g.*, two or more, three or more, four or more, etc.) barcode sequences may be attached to one cargo of interest within a mixture. For example, in some embodiments, one or more barcode sequences may be attached to various different positions on a given cargo – such a setup may be useful in, for example, in studying and identifying the stability of such barcoded cargos. In some embodiments, each cargo in a mixture is a unique sequence (*e.g.*, each cargo has a different sequence from every other cargo in the mixture). In some embodiments, each cargo in a mixture is a non-unique sequence.

[0267] In some embodiments, a cargo may be tagged to a specific barcode across different experiments. In some embodiments, a cargo may be tagged to different barcodes in different experiments. For example, in some embodiments, a cargo may be tagged to two or more, three or more, four or more, ten or more, 100 or more, 1000 or more, or 10,000 or more different barcodes across different experiments.

[0268] In some embodiments, a cargo may be associated with only one barcode in a given experiment. In some embodiments, a cargo may be associated with multiple barcodes in a given experiment. For example, in some embodiments, one or more barcodes (*e.g.*, in a mixture) are associated with multiple cargos (*i.e.*, a barcode is tagged to multiple cargos) in a mixture, such that each cargo is associated with a unique set of barcodes within the mixture. That is, each cargo may be associated with a unique "pattern" of barcodes in the mixture. In some embodiments, several cargos may be associated with the same barcode.

[0269] Among other things, the present disclosure provides for nucleic acids comprising, for example, a cargo component encoding a cargo polypeptide of interest. In some embodiments, a cargo polypeptide has a therapeutic function. In some embodiments, a cargo polypeptide does not have a therapeutic function (*e.g.*, may aid another cargo with a therapeutic function). For example, possible cargo polypeptides which one may wish to screen as drugs, such as monoclonal antibodies, single domain antibodies, enzymes, bispecific antibodies, or any other cargo polypeptide which may have therapeutic function.

[0270] In some embodiments, a cargo polypeptide further comprises a targeting moiety. In some embodiments, a targeting moiety targets a cargo polypeptide to a location of interest (*e.g.*, a cell of interest, a tissue of interest, an organ of interest). In some embodiments, a targeting moiety targets a cargo polypeptide to a cell-receptor agent of interest. In some embodiments, a targeting moiety is expressed on a surface of a delivery particle described herein. Targeting moieties are known in the art.

[0271] In some embodiments, a cargo polypeptide further comprises a localizing moiety. In some embodiments, a localizing moiety is a secretion peptide signal. In some embodiments, a localizing moiety is a nuclear localization signal. Other localizing moieties are known in the art.

[0272] In some embodiments, a cargo polypeptide further comprises a pro component. In some embodiments, a pro component, as described herein, refers to an inactive component that, once expressed in a tissue of interest, takes an active form so that it exhibits an intended effect. For example, pro components include moieties such as carboxylic, hydroxyl, amine, or phosphate/phosphonate groups. In some embodiments, pro components may be activated once exposed to environmental conditions such as pH, presence (or absence) of an agent, etc.

[0273] In some embodiments, a cargo polypeptide further comprises a tag moiety. In some embodiments, a tag moiety comprises a detectable moiety. Tag moieties are known in the art.

[0274] In some embodiments, a cargo polypeptide further comprises a liganding moiety. In some embodiments, a liganding moiety targets a cargo polypeptide to a tissue of interest. In some embodiments, a liganding moiety targets a cargo polypeptide to a target agent within a cell, tissue, or organ (*e.g.*, *in vivo*). In some embodiments, a liganding moiety targets a cargo polypeptide to a target agent on a surface of a cell, tissue, or organ (*e.g.*, *in vivo*). In some embodiments, a cargo polypeptide further comprises a stability modifying moiety. In some embodiments, a cargo polypeptide further comprises a masking moiety. In some embodiments, a cargo polypeptide further comprises an allosteric modulation moiety.

[0275] In some embodiments, a targeting moiety may also be referred to as a shuttle moiety (or a "shuttle" as described herein). In some embodiments, a liganding moiety may also be referred to as a shuttle moiety (or a "shuttle" as described herein). In some embodiments, a shuttle moiety is or comprises an antibody. In some embodiments, a shuttle moiety is or

comprises a variant or a fragment of an antibody. In some embodiments, a liganding moiety is or comprises a targeting moiety. In some embodiments, a targeting moiety is or comprises a liganding moiety.

[0276] In some embodiments, a targeting moiety (e.g., a shuttle moiety), as described herein, can be designed and/or developed through machine-learning methods. In some embodiments, a liganding moiety (e.g., a shuttle moiety), as described herein, can be designed and/or developed through machine-learning methods.

[0277] In some embodiments, a cargo is or comprises an antibody. In some embodiments, a cargo is or comprises an antibody associated with a targeting moiety (e.g., a shuttle moiety), as described herein. In some embodiments, a cargo is or comprises an antibody associated with a liganding moiety (e.g., a shuttle moiety), as described herein. In some embodiments, a cargo is or comprises an antibody drug conjugate (ADC). In some embodiments, a cargo is or comprises an ADC associated with a targeting moiety (e.g., a shuttle moiety), as described herein. In some embodiments, a cargo is or comprises an ADC associated with a liganding moiety (e.g., a shuttle moiety), as described herein. In some embodiments, a cargo is or comprises an antibody associated with (e.g., covalently, e.g., non-covalently) an oligonucleotide). In some embodiments, a cargo is or comprises an antibody associated with an oligonucleotide that is associated with a targeting moiety (e.g., a shuttle moiety), as described herein. In some embodiments, a cargo is or comprises an antibody associated with an oligonucleotide that is associated with a liganding moiety (e.g., a shuttle moiety), as described herein.

[0278] In some embodiments, an oligonucleotide comprises DNA. In some embodiments, and oligonucleotide comprises RNA. In some embodiments, an oligonucleotide comprises DNA and RNA. In some embodiments, an oligonucleotide comprises or is an RNA interference (RNAi) molecule. In some embodiments, an oligonucleotide comprises or is an DNA interference (DNAi) molecule. In some embodiments, an oligonucleotide comprises or is an antisense oligonucleotide (ASO). In some embodiments, an oligonucleotide comprises or is an shRNA. In some embodiments, an oligonucleotide comprises or is an miRNA. In some embodiments, an oligonucleotide comprises or is a gRNA. In some embodiments, an oligonucleotide comprises or is an siRNA.

[0279] In some embodiments, a cargo polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide. In some embodiments, a cargo polypeptide is or comprises a variant polypeptide (*e.g.*, a variant cargo polypeptide). In some embodiments, a variant polypeptide is a variant of a reference polypeptide, which reference polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide. In some embodiments, a variant polypeptide is or comprises at least one mutation relative to a reference polypeptide (*e.g.*, a wild-type polypeptide).

[0280] In some embodiments, a variant cargo polypeptide is associated with (*e.g.*, operably linked to) a barcode, as described herein (*i.e.*, a barcoded variant cargo polypeptide). In some embodiments, a variant cargo polypeptide possesses improved functionality (*e.g.*, reduced toxicity, improved pharmacokinetic measures (*e.g.*, dissociation constant (Kd), improved biophysical properties, etc.) relative to a reference polypeptide (*e.g.*, a wild-type polypeptide).

[0281] In some embodiments, cargos, as described herein, can be designed and/or developed through machine-learning methods. In some embodiments, cargo polypeptides, as described herein, can be designed and/or developed through machine-learning methods. For example, in some embodiments, a cargo polypeptide (e.g., comprising a targeting moiety or a liganding moiety as described herein (e.g., a shuttle moiety)) can be designed and/or developed (e.g., may be refined through multiple iterations) through machine-learning methods.

[0282] An assessment of pharmacokinetic (PK) properties is a key criteria in the nomination of therapeutic leads, but typically occurs in the later stages of drug discovery and only for a limited number of candidates. The binder-barcode platform described herein allows to characterize PK of many therapeutic candidates earlier in drug discovery.

[0283] In some embodiments, cargos, designed and/or developed through machine-learning methods possess improved functionality (e.g, reduced toxicity, improved pharmacokinetic (pK) measures (*e.g.*, dissociation constant (Kd), improved biophysical properties, epitope properties, affinity properties, thermostability properties, pH sensitivity properties, etc.) relative to a reference cargo (*e.g.*, a wild-type cargo). In some embodiments, cargo polypeptides, designed and/or developed through machine-learning methods possess improved functionality (e.g, reduced toxicity, improved pharmacokinetic (pK) measures (*e.g.*, dissociation constant (Kd), improved biophysical properties, epitope properties, affinity

properties, thermostability properties, pH sensitivity properties, etc.) relative to a reference cargo polypeptide (*e.g.*, a wild-type cargo polypeptide).

iii. Linkers

[0284] Among other things, systems and methods described herein may use linkers. In some embodiments, a cargo as described herein and a barcode as described herein are separated by a linker. In some embodiments, linkers (L) provide distance between a cargo (P) and a barcode (b). That is, structurally a barcoded cargo, in some embodiments, may have a sequence of P-L-b. This, for example, may contribute to folding characteristics, cargo functionality, and/or cargo stability.

[0285] In some embodiments, linkers may be nucleic acids. In some embodiments, linkers may be amino acids. Linkers as described herein may have varying lengths. For example, in some embodiments, a linker may have a length of at least 3 amino acids. In some embodiments, a linker may have a length of between 1 and 50 amino acids (*e.g.*, between 1 and 30 amino acids). In some embodiments, for example, a linker is or comprises a sequence GGGS.

[0286] In some embodiments, linkers of the present invention may be cleaved upon treatment. For example, in some embodiments, a linker may comprise one or more motifs that may be cleaved upon treatment.

[0287] In some embodiments, linkers of the present invention may be resistant to cleavage. In some embodiments, linkers of the present invention may be resistant to cleavage in assays. In some embodiments, linkers of the present invention may be resistant to cleavage *in vivo*.

[0288] In one aspect, linkers may be used to tag barcodes. In some embodiments, each linker sequence is associated with a distinct barcode sequence. For example, in some embodiments, a linker sequence may be used as a unique tag associated with a distinct barcode sequence (*e.g.*, nucleic acid sequence) in a mixture. That is, in some embodiments, such a linker may be used to amplify an associated barcode sequence. For example, in some embodiments, such a linker may be used as a primer to amplify an associated barcode sequence. Subsequently, in some embodiments, an amplified linker may be used to isolate an associated barcode

sequence, allowing for retrieval of the barcode sequence (*e.g.,* nucleic acid sequence) from a given linker-barcode pair. In some embodiments, a linker-barcode pair may be subject to DNA sequencing for identification of the barcode sequence.

[0289] In some embodiments, a nucleic acid sequence encoding for a linker-barcode pair may be used to associate (*e.g.,* link) the linker-barcode pair to a new cargo.

II. Binders and Binding Agents

i. Binders

[0290] In some embodiments, a binder (*i.e.,* a binder moiety) is or comprises a nucleic acid sequence. In some embodiments, a binder is or comprises a nucleic acid sequence that occurs in nature. In some embodiments, a binder is or comprises a nucleic acid sequence that does not occur in nature. In some embodiments, a binder is or comprises a nucleic acid sequence that is synthetic. In some embodiments, a binder comprises naturally occurring nucleic acids. In some embodiments, a binder comprises non-naturally occurring nucleic acids (*e.g.,* modified nucleic acids).

[0291] In some embodiments, a binder nucleic acid sequence is or comprises a sequence that encodes for a polypeptide sequence. For example, in some embodiments, a binder nucleic acid sequence may contain a region, which encodes for a polypeptide sequence conferring high affinity and/or specificity for a given barcode (*e.g.,* peptide barcode). In some embodiments, a binder nucleic acid sequence is or comprises a sequence that encodes for an antibody. In some embodiments, a binder nucleic acid sequence is or comprises a sequence that encodes for a fragment of an antibody. In some embodiments, a binder nucleic acid sequence is or comprises a sequence that encodes for a single-chain variable Fragment (scFv). As maybe known to those of ordinary skill in the art, a scFv is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins. In some embodiments, a V_H and V_L chain may be connected with a short linker peptide (*e.g.,* linker of about 5-50 amino acids in length, 10-25 amino acids in length, etc.).

[0292] In some embodiments, for example, a binder is generated to have known specificity and affinity for a given barcode. In some embodiments, a binder is generated to have

known specificity and affinity for one barcode. In some embodiments, a binder is generated to have known specificity and affinity for multiple (*e.g.,* two or more, three or more, etc.) barcodes. In some embodiments, a binder is generated to have known specificity and affinity for at least one barcode. In some embodiments, a binder, for example, is expressed on the surface of a binding agent (*e.g.,* a phage, a ribosome, etc.) using methods known to those skilled in the art.

[0293] In some embodiments, a binder associates with a barcode (*e.g.,* with known specificity and affinity).

[0294] In some embodiments, a binder is or comprises a polypeptide sequence that occurs in nature. In some embodiments, a binder is or comprises a polypeptide sequence that does not occur in nature. In some embodiments, a binder is or comprises a polypeptide sequence that is synthetic. In some embodiments, a binder comprises naturally occurring amino acids. In some embodiments, a binder comprises non-naturally occurring amino acids (*e.g.,* modified amino acids).

[0295] Binders of the present invention may be of varying lengths. For example, in some embodiments, a binder may have a length ranging between 5 to 1000 amino acids. In some embodiments, a binder may have a length ranging between 5 to 800 amino acids. In some embodiments, a binder may have a length ranging between 6 to 500 amino acids. In some embodiments, a binder may have a length ranging between 10 to 400 amino acids. In some embodiments, a binder may have a length ranging between 5 to 500 amino acids. In some embodiments, a binder may have a length ranging between 5 to 1000 amino acids. In some embodiments, a binder may have a length of 10 amino acids. In some embodiments, a binder may have a length of at least 5 amino acids. In some embodiments, a binder may have a length of at most 1000 amino acids.

[0296] Binders, as described herein may be available in a library in different formats. For example, in some embodiments a binder as described herein may be described as a nucleic acid sequence. In other instance, a binder as described herein may be described as an amino acid sequence. A person of ordinary skill in the art will appreciate that binders described in one format may be converted to another format using basic biological principles. Accordingly, binders described as nucleic acid sequences may be translated into proteins, which may be used to detect the presence or absence of a cargo (*e.g.,* barcoded cargo (*e.g.,* barcoded cargo

polypeptide)) in a mixture. Such a translated binder is referred to herein as a polypeptide binder or polypeptide binder moiety.

[0297] Accordingly, binders of the present disclosure when described using nucleic acids may have lengths different from amino acid sequence lengths disclosed in the paragraph above. For example, in some embodiments, a binder may have a length ranging between 15 to 3000 nucleotides. In some embodiments, a binder may have a length ranging between 15 to 2400 nucleotides. In some embodiments, a binder may have a length ranging between 24 to 1500 nucleotides. In some embodiments, a binder may have a length ranging between 30 to 1200 nucleotides. In some embodiments, a binder may have a length of 30 nucleotides. In some embodiments, a binder may have a length of at least 15 nucleotides. In some embodiments, a binder may have a length of at most 3000 nucleotides.

[0298] Binders of the present disclosure may have one or more specific properties. In some embodiments, a binder may be naturally occurring. In some embodiments, a binder may not be naturally occurring (*e.g.,* synthetic). In some embodiments, a binder may not elicit an immune response (*e.g.,* an IgG response, a complement response, etc.).

[0299] Among other things, binders (*e.g.,* polypeptide binders, nucleic acids encoding binders) of the present disclosure, like barcodes discussed above, may be flanked by additional sequences (*e.g.,* nucleic acid sequences, amino acid sequences, etc.). In some embodiments, a binder may be flanked by additional sequences on a binder's 5' end. In some embodiments, a binder may be flanked by additional sequences on a binder's 3' end. In some embodiments, a binder may be flanked by additional sequences on a binder's 3' and 5' end. In some embodiments, an additional sequence may be a primer binding site, a restriction endonuclease recognition sequence, a restriction enzyme site (*e.g.,* a cleavage site), a sequence that encodes an amino acid sequence, a sequence that does not encode an amino acid sequence, an amino acid sequence, or a nucleic acid sequence.

[0300] In one aspect of the present invention, a binder nucleic acid sequence may be associated with (*e.g.,* attached to, linked to) another nucleic acid sequence. For example, in some embodiments, a binder nucleic acid sequence may be associated with a nucleic acid sequence encoding one or more genes. In some embodiments, a binder nucleic acid sequence may be associated with a nucleic acid sequence encoding one or more genes of a phage (*e.g.,* m13). In

some embodiments, a binder nucleic acid sequence may be associated with a nucleic acid sequence encoding a polypeptide. In some embodiments, a binder nucleic acid sequence may be associated with a nucleic acid sequence encoding a polypeptide of a phage (*e.g.*, m13 gene3 protein). The binder-gene3 protein fusion can be expressed and incorporated into m13 phage.

[0301] Among other things, binders described herein are designed to have a distinct (*i.e.*, unique) sequence. In some embodiments, a binder is designed to have a distinct sequence (*e.g.*, distinct from another binder). For example, each binder is designed to be distinct from every other binder used in an experiment (*i.e.*, to be unique).

[0302] In some embodiments, binders, as described herein, can be designed and/or developed through machine-learning methods.

[0303] In one aspect of the present invention, binders bind to barcodes or barcoded cargos, as described herein, with high specificity and high affinity. In some embodiments, a barcode or barcoded cargo (*e.g.*, barcoded cargo polypeptide to be measured) binds to one binder, and each binder (*e.g.*, binder with a specific sequence) binds to one barcode or barcoded cargo. In some embodiments, a barcode or barcoded cargo (*e.g.*, barcoded cargo polypeptide to be measured) binds to at least one binder. In some embodiments, each binder (*e.g.*, binder with a specific sequence) binds to at least one barcode or barcoded cargo. In some embodiments, multiple binders (*e.g.*, with different sequences (*e.g.*, polypeptide sequences)) bind to a single barcode. In some embodiments, multiple barcodes (*e.g.*, with different sequences (*e.g.*, peptide sequences)) bind to a single binder.

[0304] Example of binders according to various embodiments of the present disclosure are listed in sequence listing filed herewith. In some embodiments, a binder (*e.g.*, polypeptide binder) is or comprises an amino acid sequence selected from SEQ ID NOs: 4200-5346. In some embodiments, a binder (*e.g.*, polypeptide binder) is encoded by a sequence that is or comprises a nucleic acid sequence selected from SEQ ID NOs: 1-1147.

ii. Binding Agents

[0305] Methods described herein relate to the detection of one or more barcodes using a binding agent. In some embodiments, a binding agent is associated with or comprises a

detectable nucleic acid. In some embodiments, a binding agent expresses a detectable nucleic acid. In some embodiments, a binding agent expresses a detectable nucleic acid on its surface (*e.g.,* a binder). In some embodiments, a binding agent expresses an antibody on its surface.

[0306] In some embodiments, for example, to detect the presence of a specific (*e.g.,* distinct) barcode, the present invention envisions the association of a distinct detectable nucleic acid (*e.g.,* a DNA sequence, an RNA sequence, etc.) to a specific barcode. This is achieved through contacting a barcode with a binding agent. In some embodiments, one or more barcodes may be contacted with a binding agent. In some embodiments, one or more binding agents may be contacted with a barcode.

[0307] In some embodiments, a binding agent may be or comprises a phage, a ribosome, mRNA, DNA etc. In some embodiments, a binding agent is a phage. In some embodiments, a binding agent is may be a M13 phage, T4 phage, T7 phage, Lambda phage, or filamentous phage. In some embodiments, a binding agent is may be a M13 phage.

[0308] Binders as disclosed herein may be expressed on binding agents using methods known in the art. For example, a person of ordinary skill in the art may be able to express a nucleic acid encoding a polypeptide binder on (*e.g.,* on a surface) of a phage using techniques and methods available in the art.

III. Production

i. Production of Barcodes

[0309] Disclosed herein are methods and systems for the production of barcodes for use in systems and methods of the present disclosure. In some embodiments, barcodes, as described herein, may be generated rapidly (*e.g.,* in about a week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, or about 1 year) . In some embodiments, for example, between about 100 to about 1,000 barcodes may be generated rapidly. In some embodiments, between about 10 to about 1000 barcodes may be generated rapidly. In some embodiments, between about 10 to about 10,000 barcodes may be generated rapidly. While large numbers of barcodes, as described herein, may

be generated rapidly, such barcodes are also robust, in that barcodes generated using the methods disclosed herein may bind specifically and with different affinities to a known set of binders.

[0310] In accordance with various embodiments, barcodes as described herein may be synthesized using a nucleic acid (*e.g.,* oligonucleotide) array. In some embodiments, barcodes as described herein may be synthesized using a DNA array. In some embodiments, nucleic acids (*e.g.,* oligonucleotides) of a nucleic acid array are expressed into barcodes. In some embodiments, barcodes as described herein may be synthesized using nucleic acid library. In some embodiments, a nucleic acid library is synthesized using a nucleic acid array. In some embodiments, nucleic acids (*e.g.,* oligonucleotides) of a nucleic acid library are expressed into barcodes.

[0311] In some embodiments, a barcode nucleic acid library comprises about 1 or more, about 2 or more, about 3 or more, about 4 or more, about 5 or more, about 10 or more, about 50 or more, about 100 or more, about 200 or more, about 300 or more, about 400 or more, about 500 or more, about 600 or more, about 700 or more, about 800 or more, about 900 or more, about 1000 or more, about 2000 or more, about 3000 or more, about 4000 or more, or about 5000 or more potential barcodes. In some embodiments, a nucleic acid library comprises one or more potential barcode sequences. Such potential barcode sequences may be screened for functionality as peptide barcodes (*i.e.,* after translation of potential barcode nucleic acid sequences) using one or more methods described herein.

[0312] Barcodes of the present disclosure may be screened for one or more specific properties. In some embodiments, a barcode may be screened for specific binding (*e.g.,* specificity, binding affinity) to a binder. In some embodiments, a barcode may be screened for specific binding to one or more binders. In some embodiments, a barcode may be screened for specific binding to at least a binder. In some embodiments, a barcode may be screened for specific binding to at most a binder. In some embodiments, a barcode may be screened for specific binding to multiple binders.

[0313] As may be understood by a person of ordinary skill in the art, a barcode is designed to be distinct (*i.e.,* unique (*e.g.,* have a unique sequence)) in a pool of barcodes. Such distinction may be achieved, in some embodiments, by changing one or more amino acids in a barcode. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes

by 1 amino acid. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes by at least 1 amino acid. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes by at most 1 amino acid. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes by 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes by at least 2 amino acids. In some embodiments, a barcode is distinct from other barcodes in a pool of barcodes by at most 50 amino acids.

ii. Production of Barcoded Cargos

[0314] Barcoded cargos in accordance with the present invention may be produced in various ways. In some embodiments, cargo-barcode nucleic acid sequence pairs may be inserted into a plasmid to allow for expression in different expression systems (*e.g.,* protein expression systems). In some embodiments, at least one cargo-barcode nucleic acid sequence pair is inserted into a plasmid. In some embodiments, at least two cargo-barcode nucleic acid sequence pairs are inserted into a plasmid. In some embodiments, at least three cargo-barcode nucleic acid sequence pairs are inserted into a plasmid. In some embodiments, one or more cargo-barcode nucleic acid sequence pairs are inserted into a plasmid.

[0315] In some embodiments, a cargo-barcode nucleic acid sequence may comprise additional sequences. In some embodiments, a cargo-barcode nucleic acid sequence may comprise additional nucleic acid sequences. In some embodiments, a cargo-barcode nucleic acid sequence may comprise a universal motif sequence. In some embodiments, a cargo-barcode nucleic acid sequence may comprise at least one universal motif sequence. In some embodiments, a cargo-barcode nucleic acid sequence may comprise at least two universal motif sequences. In some embodiments, a cargo-barcode nucleic acid sequence may comprise two or more universal motif sequences.

[0316] In some embodiments, at least one cargo-barcode nucleic acid sequences in a pool of cargo-barcode nucleic acid sequences may comprise a universal motif sequence. In some embodiments, all cargo-barcode nucleic acid sequences in a pool of cargo-barcode nucleic acid sequences may comprise a universal motif sequence.

[0317] Different plasmids may be used to produce technologies described herein. In some embodiments, a plasmid is a DNA plasmid. In some embodiments, a plasmid is an RNA plasmid. In some embodiments, a plasmid is a fertility F-plasmid. In some embodiments, a plasmid is a resistance plasmid. In some embodiments, a plasmid is a virulence plasmid. In some embodiments, a plasmid is a degradative plasmid. In some embodiments, a plasmid is a Col plasmid.

[0318] Different hosts (*e.g.,* host cell, host cell line, etc.) may be used to produce technologies described herein. In some embodiments, a host is a mammalian host. In some embodiments, a host is a non-mammalian host. In some embodiments, a host is an insect. In some embodiments, a host is a bacteria. In some embodiments, a host is *E. coli*.

[0319] In some embodiments, a cargo-barcode pair is expressed *in vitro*. In some embodiments, a cargo-barcode pair is expressed *in vivo*. In some embodiments, a cargo-barcode pair is expressed from RNA. In some embodiments, a cargo-barcode pair is expressed from transcribed RNA. In some embodiments, a cargo-barcode pair is expressed from DNA. In some embodiments, a cargo-barcode pair is expressed using protein components (*e.g.,* required for protein translation).

[0320] After expression of barcoded cargo constructs, constructs may be purified from the pool. In some embodiments, purification may be performed using a universal motif. In some embodiments, purification may be performed using HIS tag, FLAG tag, HALO tag, SNAP tag, Avitag, Twin strep tag, or any other tag based method of protein purification known in the art.

iii. Production of Binders

[0321] Disclosed herein are methods and systems for the production of binders for use in systems and methods of the present disclosure. In some embodiments, binders, as described herein, may be generated rapidly (*e.g.,* in about a week, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, or about 1 year) . In some embodiments, for example, between about 100 to about 1000 binders may be generated rapidly. In some embodiments, between about 10 to about 1000 binders may be generated rapidly. In some embodiments, between about 10 to about 10,000

binders may be generated rapidly. While large numbers of binders, as described herein, may be generated rapidly, such binders are also robust, in that binders generated using the methods disclosed herein may bind specifically and with different affinities to a known set of barcodes.

[0322] In some embodiments, a binder nucleic acid library comprises about 1 or more, about 2 or more, about 3 or more, about 4 or more, about 5 or more, about 10 or more, about 50 or more, about 100 or more, about 200 or more, about 300 or more, about 400 or more, about 500 or more, about 600 or more, about 700 or more, about 800 or more, about 900 or more, about 1000 or more, about 2000 or more, about 3000 or more, about 4000 or more, or about 5000 or more potential binders. In some embodiments, a nucleic acid library comprises one or more potential binder sequences. Such potential binder sequences may be screened for functionality as polypeptide binders (*i.e.,* after translation of potential nucleic acid binder sequences) using one or more methods described herein.

[0323] Binders in accordance with the present invention may be produced in various ways. In some embodiments, a binder nucleic acid sequence may be inserted into a plasmid to allow for expression in different expression systems. In some embodiments, at least one binder nucleic acid sequence is inserted into a plasmid. In some embodiments, at least two binder nucleic acid sequences are inserted into a plasmid. In some embodiments, at least three binder nucleic acid sequences are inserted into a plasmid. In some embodiments, one or more binder nucleic acid sequences are inserted into a plasmid.

[0324] In some embodiments, a binder nucleic acid sequence is attached to one or more genes. In some embodiments, a binder nucleic acid sequence is attached to one or more genes prior to insertion into a plasmid. In some embodiments, a binder nucleic acid sequence is attached to one or more genes after insertion into a plasmid. In some embodiments, a binder nucleic acid sequence is attached to a bacteriophage gene. In some embodiments, a binder nucleic acid sequence is attached to an m13 bacteriophage gene. In some embodiments, a binder nucleic acid sequence is attached to gene 3 (*i.e.,* that encodes for gene 3 protein) of m13 bacteriophage.

[0325] In some embodiments, plasmids (*e.g.,* containing binder sequences, containing binder and bacteriophage sequences, etc.) may be transformed into a host. In some embodiments,

plasmids may be transformed into a host and expressed. In some embodiments, plasmids are transformed into a bacterium. In some embodiments, plasmids are transformed into *E. coli*.

[0326] In some embodiments, expression of plasmids results in phage production. In some embodiments, expression of plasmids results in display of a binder on a surface of a phage. In some embodiments, expression of plasmids results in display of two binders on a surface of a phage. In some embodiments, expression of plasmids results in display of at least one binder on a surface of a phage. In some embodiments, expression of plasmids results in display of one or more binders on a surface of a phage. In some embodiments, expression of plasmids results in display of one or more binders on one or more surfaces of a phage. In some embodiments, expression of plasmids results in display of at least one binder on one or more surfaces of a phage.

[0327] Following phage production, the resulting pool may be purified to determine the presence of one or more polypeptide binders. In some embodiments, purification may be performed using a universal motif. In some embodiments, purification may be performed using HIS tag, FLAG tag, HALO tag, SNAP tag, Avitag, Twin strep tag, or any other tag based method of protein purification known in the art.

[0328] In some embodiments, a purified binder pool may be highly diverse. In some embodiments, a purified binder pool may not be highly diverse. In some embodiments, a purified binder pool is subjected to screening methods to select binders of interest.

[0329] Binders of the present disclosure may be screened for one or more specific properties. In some embodiments, a binder may be screened for specific binding to a barcode. In some embodiments, a binder may be screened for specific binding to one or more barcodes. In some embodiments, a binder may be screened for specific binding to at least a barcode. In some embodiments, a binder may be screened for specific binding to at most a barcode. In some embodiments, a binder may be screened for specific binding to multiple barcodes.

[0330] As may be understood by a person of ordinary skill in the art, a binder is designed to be distinct (*i.e.,* unique (*e.g.,* have a unique sequence)) in a pool of binders. Such distinction may be achieved, in some embodiments, by changing one or more amino acids in a binder. In some embodiments, a binder is distinct from other binders in a pool of binders by 1 amino acid. In some embodiments, a binder is distinct from other binder in a pool of binders by at least 1

amino acid. In some embodiments, a binder is distinct from other binder in a pool of binders by at most 1 amino acid. In some embodiments, a binder is distinct from other binder in a pool of binders by 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids. In some embodiments, a binder is distinct from other binder in a pool of binders by at least 2 amino acids. In some embodiments, a binder is distinct from other binder in a pool of binders by at most 1000 amino acids.

IV. Characterization

i. Samples

[0331] As described elsewhere in the present disclosure, a sample may be a biological sample. In some embodiments, a sample may contain one or more barcoded cargos. In some embodiments, a sample may contain one or more barcoded cargo polypeptides.

[0332] In some embodiments, a sample is derived from an organism. In some embodiments, a sample is derived from an animal. In some embodiments, a sample is derived from an animal model of disease. In some embodiments, a sample is derived from a non-mammal. In some embodiments, a sample is derived from a mammal (*e.g.,* a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, a sample is derived from a mouse. In some embodiments, a sample is derived from a human. In some embodiments, a sample is derived from cells (*e.g., in vitro*). In some embodiments, a sample is a human cell line.

[0333] In some embodiments, a sample may be purified. In some embodiments, a sample may not be purified.

[0334] In some embodiments, a sample is obtained from cells that was treated with barcoded cargos. In some embodiments, a sample is obtained from cells that was not treated with barcoded cargos. In some embodiments, a sample is obtained from an animal that was treated with barcoded cargos. In some embodiments, a sample is obtained from an animal that was not treated with barcoded cargos. For example, in some embodiments, a sample is obtained from a human that was treated with barcoded cargo polypeptides.

[0335] In some embodiments, a sample is obtained from cells that was genetically modified. In some embodiments, a sample is obtained from cells that was modified by gene therapy. In some embodiments, a sample is obtained from cells that was genetically modified to include one or more barcoded cargos. In some embodiments, a sample is obtained from cells that was genetically modified to express a barcoded cargos. In some embodiments, a sample is obtained from cells that was genetically modified to include one or more barcodes. In some embodiments, a sample is obtained from cells that was genetically modified to express a barcodes. In some embodiments, a sample is obtained from cells that was genetically modified to include one or more binders. In some embodiments, a sample is obtained from cells that was genetically modified to express a binders.

[0336] In some embodiments, a sample is obtained from an animal that was genetically modified. In some embodiments, a sample is obtained from an animal that was modified by gene therapy. In some embodiments, a sample is obtained from an animal that was genetically modified to include one or more barcoded cargos. In some embodiments, a sample is obtained from an animal that was genetically modified to express a barcoded cargos. In some embodiments, a sample is obtained from an animal that was genetically modified to include one or more barcodes. In some embodiments, a sample is obtained from an animal that was genetically modified to express a barcodes. In some embodiments, a sample is obtained from an animal that was genetically modified to include one or more binders. In some embodiments, a sample is obtained from an animal that was genetically modified to express a binders.

ii. Fingerprints

[0337] Among other things, systems and methods described herein identify the advantages of nucleic acid sequencing techniques and apply them effectively to protein detection and measurement methods. For example, methods described herein may use several binders, with known binding specificities and affinities to different barcodes, that can be expressed on binding agents and mixed together in a single pool. Upon mixing with a pool of barcoded cargo polypeptides (*i.e.,* proteins, each associated with a barcode as described herein), each binder expressed on a binding agent binds to a one or more barcodes in the pool with known but varying affinities. Such a spectrum of affinities for a given barcode to one or more binders results in a

distinct distribution of binder counts for a given barcode that can be determined through NGS, and is termed herein a 'Barcode Fingerprint'. In some embodiments, the collective barcode fingerprints for a set of barcodes is termed herein a 'Fingerprint Matrix'. Analogously, a spectrum of affinities of a binder to various (*e.g.,* one or more) barcodes is termed herein as a 'Binder Fingerprint'. In some embodiments, using the provided technologies the presence of a barcoded cargo polypeptide(s) can be detected, for example, in a complex solution, by extracting and sequencing the associated nucleic acid (*e.g.,* detectable nucleic acid (*e.g.,* DNA sequence, RNA sequence, etc.)) of the population of binding agents (*e.g.,* phage) that bind to the barcoded cargo polypeptide(s). That is, for example, in some embodiments, the presence of a protein in a complex solution is determined not through a single binder, but through a specific combination of multiple binders that bind to a barcode associated with said protein in fixed, known proportions.

[0338] Fingerprints, as disclosed herein, have many advantages. In some embodiments, a fingerprint approach of detection allows for reduction of noise. For example, the use of multiple binders to detect a barcode in a complex solution introduces a redundancy into the detection methods that in turn reduces signal noise. Additionally, another advantage of the "fingerprint" approach is that partial non-specificities in the binders (*e.g.,* to barcodes other than the barcode of interest to be detected) can be tolerated and compensated for by the computational prediction methods.

[0339] In some embodiments, binder sequences may be modified in order to change a fingerprint. In some embodiments, binder sequences may be modified in order to improve a fingerprint.

[0340] A barcode fingerprint, as described herein, for a given barcode may include affinity information of a given barcode to one or more binders. In some embodiments, a barcode fingerprint may include affinity information of a given barcode to one binder. In some embodiments, a barcode fingerprint may include affinity information of a given barcode to at least one binder. In some embodiments, a barcode fingerprint may include affinity information of a given barcode to 2, 3, 4, 5, 10, 20, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more binders. In some

embodiments, a barcode fingerprint may include affinity information of a given barcode to at most 10,000 binders.

[0341] A binder fingerprint, as described herein, for a given binder may include affinity information of a given binder to one or more barcodes. In some embodiments, a binder fingerprint may include affinity information of a given binder to one barcode. In some embodiments, a binder fingerprint may include affinity information of a given binder to at least one barcode. In some embodiments, a binder fingerprint may include affinity information of a given binder to 2, 3, 4, 5, 10, 20, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more barcodes. In some embodiments, a binder fingerprint may include affinity information of a given binder to at most 10,000 barcodes.

[0342] As discussed herein, in some embodiments, multiple barcode fingerprints for a set of barcodes may be grouped together and is termed herein a 'Fingerprint Matrix'. In some embodiments, a fingerprint matrix may comprise one barcode fingerprint. In some embodiments, a fingerprint matrix may comprise at least one barcode fingerprint. In some embodiments, a fingerprint matrix may comprise 2, 3, 4, 5, 10, 20, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more barcode fingerprints. In some embodiments, a fingerprint matrix may comprise at most 10,000 barcode fingerprints.

[0343] The technologies described herein allow for the generation and characterization of unique fingerprints for each barcode. This allows, for example, availability of methods of cargo (*i.e.,* target (*e.g.,* protein)) detection that may not require orthogonality between barcode-binder pairs. In some embodiments, barcode-binder pairs may be orthogonal. In some embodiments, barcode-binder pairs may not be orthogonal. As may be evident to a person of ordinary skill in the art, barcode-binder pairs as described herein provide the advantage of being more robust, as the availability of unique fingerprints makes non-specific binding less of a concern, a major advantage in complex environments (*e.g.,* serum, blood, etc.).

iii. Decoding

Analysis

[0344] A key component of the invention is the method used to deduce relative or absolute protein concentrations from the DNA sequencing of binders. In the invention, the DNA sequences are translated in silico into amino acid sequences corresponding to each binder and tabulated to yield a table of binder counts. The binder count table measured for any given barcode in isolation is henceforth known as a "fingerprint" of a barcode. When applying the invention to an unknown mixture of barcoded cargos, the relative or absolute abundance of individual barcodes is determined by comparing the binder count table to the predetermined fingerprints of the individual barcodes and applying a computational prediction method described below. In some embodiments, the binder count table of a mixture of m unknown barcodes is assumed to be a linear combination of their respective fingerprints; the coefficients of the linear combination are inferred through least-squares fitting of the equation $A x = b$, where A is an n-by-m matrix of fingerprints, b is a length-n vector of binder counts, and x is an undetermined length-m vector of the abundances of each of the barcodes. In some embodiments, the abundances of each of the barcodes is inferred using a Bayesian method, whereby a suitable prior probability distribution over the barcode abundances is assumed, a likelihood ratio of the observed count table given barcode abundances is calculated from a model of the uncertainties in the experimental system, and a posterior probability distribution is inferred the product of the prior with the likelihood ratio. In some embodiments, the posterior distribution is estimated using Monte Carlo sampling methods. In some embodiments, the maximum of the posterior distribution is determined with a computational optimization procedure. In some embodiments, the binder count table is assumed to be a non-linear function of the abundances of various barcodes to account for saturation of particular barcode-binder interactions or competition between distinct barcodes or distinct binders.

[0345] In some embodiments, relative proportions of binder counts are compared directly in order to determine relative proportions of barcodes. In some embodiments, sequences of known abundance are mixed into the experiment, and utilized to determine the absolute abundance of a given binder, which is used to estimate an absolute concentration for a barcode.

V. Nucleic Acids

i. Cargo Nucleic Acid

[0346] Among other things, the present disclosure provides nucleic acids, *e.g.*, that can be disposed within a delivery particle as described herein. Nucleic acids according to the present disclosure include all those known in the art, including cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viral constructs (*e.g.*, lentiviral, retroviral, adenoviral, and adeno-associated viral constructs) that incorporate a nucleic acid encoding a cargo polypeptide, or characteristic portion thereof. Those of skill in the art will be capable of selecting suitable constructs, as well as cells, for making any of the polynucleotides described herein. In some embodiments, a nucleic acid is a plasmid (*i.e.,* a circular DNA molecule that can autonomously replicate inside a cell). In some embodiments, a nucleic acid can be a cosmid (*e.g.*, pWE or sCos series).

[0347] In some embodiments, a cargo nucleic acid (*e.g.*, a cargo component) is or comprises a wild-type (*e.g.*, naturally occurring) nucleic acid. In some embodiments, a cargo nucleic acid (*e.g.*, a cargo component) is or comprises a variant nucleic acid (*e.g.*, a variant cargo nucleic acid). In some embodiments, a variant nucleic acid is a variant of a reference nucleic acid, which reference nucleic acid is or comprises a wild-type (*e.g.*, naturally occurring) nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide). In some embodiments, a variant nucleic acid is or comprises at least one mutation relative to a reference nucleic acid (*e.g.*, a wild-type nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide)).

[0348] In some embodiments, a variant cargo nucleic acid (*e.g.*, a variant cargo component) is associated with (*e.g.,* operably linked to) a barcode, as described herein (*i.e.,* a barcoded variant cargo nucleic acid). In some embodiments, a variant cargo nucleic acid possesses improved functionality (*e.g.*, reduced toxicity, improved pharmacokinetic measures (*e.g.,* dissociation constant (Kd), improved biophysical properties, improved developability, improved expression, etc.) relative to a reference nucleic acid (*e.g.*, a wild-type nucleic acid (*e.g.*, a nucleic acid encoding a wild-type polypeptide)).

1. Viral Nucleic Acid

[0349] In some embodiments, a nucleic acid is a viral construct. In some embodiments, a viral construct is a lentivirus, retrovirus, adenovirus, or adeno-associated virus construct. In some embodiments, a nucleic acid is an adeno-associated virus (AAV) construct (see, *e.g.*, Asokan et al., Mol. Ther. 20: 699-7080, 2012, which is incorporated in its entirety herein by reference). In some embodiments, a viral construct is an adenovirus construct. In some embodiments, a viral construct may also be based on or derived from an alphavirus. Alphaviruses include Sindbis (and VEEV) virus, Aura virus, Babanki virus, Barmah Forest virus, Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalitis virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Me Tri virus, Middelburg virus, Mosso das Pedras virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Rio Negro virus, Ross River virus, Salmon pancreas disease virus, Semliki Forest virus, Southern elephant seal virus, Tonate virus, Trocara virus, Una virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus, and Whataroa virus. Generally, the genome of such viruses encode nonstructural (*e.g.*, replicon) and structural proteins (*e.g.*, capsid and envelope) that can be translated in the cytoplasm of the host cell. Ross River virus, Sindbis virus, Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEEV) have all been used to develop viral constructs for coding sequence delivery. Pseudotyped viruses may be formed by combining alphaviral envelope glycoproteins and retroviral capsids. Examples of alphaviral constructs can be found in U.S. Publication Nos. 20150050243, 20090305344, and 20060177819; constructs and methods of their making are incorporated herein by reference to each of the publications in its entirety.

[0350] In some embodiments, a nucleic acid is a viral construct and can have a total number of nucleotides of up to 10 kb. In some embodiments, a viral construct can have a total number of nucleotides in the range of about 1 kb to about 2 kb, 1 kb to about 3 kb, about 1 kb to about 4 kb, about 1 kb to about 5 kb, about 1 kb to about 6 kb, about 1 kb to about 7 kb, about 1 kb to about 8 kb, about 1 kb to about 9 kb, about 1 kb to about 10 kb, about 2 kb to about 3 kb, about 2 kb to about 4 kb, about 2 kb to about 5 kb, about 2 kb to about 6 kb, about 2 kb to about 7 kb, about 2 kb to about 8 kb, about 2 kb to about 9 kb, about 2 kb to about 10 kb, about 3 kb to about 4 kb, about 3 kb to about 5 kb, about 3 kb to about 6 kb, about 3 kb to about 7 kb, about 3 kb to about 8 kb, about 3 kb to about 9 kb, about 3 kb to about 10 kb, about 4 kb to about 5 kb,

about 4 kb to about 6 kb, about 4 kb to about 7 kb, about 4 kb to about 8 kb, about 4 kb to about 9 kb, about 4 kb to about 10 kb, about 5 kb to about 6 kb, about 5 kb to about 7 kb, about 5 kb to about 8 kb, about 5 kb to about 9 kb, about 5 kb to about 10 kb, about 6 kb to about 7 kb, about 6 kb to about 8 kb, about 6 kb to about 9 kb, about 6 kb to about 10 kb, about 7 kb to about 8 kb, about 7 kb to about 9 kb, about 7 kb to about 10 kb, about 8 kb to about 9 kb, about 8 kb to about 10 kb, or about 9 kb to about 10 kb.

[0351] In some embodiments, a nucleic acid is a lentivirus construct and can have a total number of nucleotides of up to 8 kb. In some examples, a lentivirus construct can have a total number of nucleotides of about 1 kb to about 2 kb, about 1 kb to about 3 kb, about 1 kb to about 4 kb, about 1 kb to about 5 kb, about 1 kb to about 6 kb, about 1 kb to about 7 kb, about 1 kb to about 8 kb, about 2 kb to about 3 kb, about 2 kb to about 4 kb, about 2 kb to about 5 kb, about 2 kb to about 6 kb, about 2 kb to about 7 kb, about 2 kb to about 8 kb, about 3 kb to about 4 kb, about 3 kb to about 5 kb, about 3 kb to about 6 kb, about 3 kb to about 7 kb, about 3 kb to about 8 kb, about 4 kb to about 5 kb, about 4 kb to about 6 kb, about 4 kb to about 7 kb, about 4 kb to about 8 kb, about 5 kb to about 6 kb, about 5 kb to about 7 kb, about 5 kb to about 8 kb, about 6 kb to about 8kb, about 6 kb to about 7 kb, or about 7 kb to about 8 kb

[0352] In some embodiments, a nucleic acid is an adenovirus construct and can have a total number of nucleotides of up to 8 kb. In some embodiments, an adenovirus construct can have a total number of nucleotides in the range of about 1 kb to about 2 kb, about 1 kb to about 3 kb, about 1 kb to about 4 kb, about 1 kb to about 5 kb, about 1 kb to about 6 kb, about 1 kb to about 7 kb, about 1 kb to about 8 kb, about 2 kb to about 3 kb, about 2 kb to about 4 kb, about 2 kb to about 5 kb, about 2 kb to about 6 kb, about 2 kb to about 7 kb, about 2 kb to about 8 kb, about 3 kb to about 4 kb, about 3 kb to about 5 kb, about 3 kb to about 6 kb, about 3 kb to about 7 kb, about 3 kb to about 8 kb, about 4 kb to about 5 kb, about 4 kb to about 6 kb, about 4 kb to about 7 kb, about 4 kb to about 8 kb, about 5 kb to about 6 kb, about 5 kb to about 7 kb, about 5 kb to about 8 kb, about 6 kb to about 7 kb, about 6 kb to about 8 kb, or about 7 kb to about 8 kb.

[0353] Any of the nucleic acids described herein can further include a control sequence, *e.g.*, a control sequence selected from the group of a transcription initiation sequence, a transcription termination sequence, a promoter sequence, an enhancer sequence, an RNA splicing sequence, a polyadenylation (poly(A)) sequence, a Kozak consensus sequence, and/or additional

untranslated regions which may house pre- or post-transcriptional regulatory and/or control elements. In some embodiments, a promoter can be a native promoter, a constitutive promoter, an inducible promoter, and/or a tissue-specific promoter. Non-limiting examples of control sequences are described herein.

[0354] In some embodiments, the present disclosure provides for a cargo component that further comprises one or more sequence elements, or the complement thereof, selected from the group consisting of: a promoter, an enhancer, a silencer, an insulator, a transcriptional regulatory element, a translational regulatory element, a splice donor, a splice acceptor, a transcriptional terminator, a translational start site, a translational stop site, a packaging signal, an integration signal, inverted terminal repeats (ITRs), and any combination thereof. Exemplary sequence elements are described herein.

2. Plasmid

[0355] In some embodiments, a nucleic acid (*e.g.*, cargo nucleic acid) is or comprises a plasmid. In some embodiments, a nucleic acid is a DNA plasmid. In some embodiments, a nucleic acid is an RNA plasmid. In some embodiments, a plasmid is able to replicate independently in a cell. In some embodiments, a plasmid comprises an origin of replication sequence. In some embodiments, a plasmid is a nanoplasmid.

[0356] Nucleic acids provided herein can be of different sizes. In some embodiments, a nucleic acid is a plasmid and can include a total length of up to about 1 kb, up to about 2 kb, up to about 3 kb, up to about 4 kb, up to about 5 kb, up to about 6 kb, up to about 7 kb, up to about 8 kb, up to about 9 kb, up to about 10 kb, up to about 11 kb, up to about 12 kb, up to about 13 kb, up to about 14 kb, or up to about 15 kb. In some embodiments, a nucleic acid is a plasmid and can have a total length in a range of about 1 kb to about 2 kb, about 1 kb to about 3 kb, about 1 kb to about 4 kb, about 1 kb to about 5 kb, about 1 kb to about 6 kb, about 1 kb to about 7 kb, about 1 kb to about 8 kb, about 1 kb to about 9 kb, about 1 kb to about 10 kb, about 1 kb to about 11 kb, about 1 kb to about 12 kb, about 1 kb to about 13 kb, about 1 kb to about 14 kb, or about 1 kb to about 15 kb.

[0357] In some embodiments, the present disclosure provides for a plasmid comprising a cargo component that further comprises one or more sequence elements, or the complement thereof, selected from the group consisting of: a promoter, an enhancer, a silencer, an insulator, a transcriptional regulatory element, a translational regulatory element, a splice donor, a splice acceptor, a transcriptional terminator, a translational start site, a translational stop site, a packaging signal, an integration signal, inverted terminal repeats (ITRs), and any combination thereof. Exemplary sequence elements are described herein.

3. RNA

[0358] In certain embodiments, the disclosed compositions comprise nucleic acids. In some embodiments, nucleic acids are RNAs. In some embodiments, nucleic acids comprise modified nucleic acids. In some embodiments, nucleic acids comprise modified RNAs. Among other things, the present disclosure describes that selection and combination of nucleic acids as described herein impacts characteristics of cargo nucleic acid such as stability and ionizability.

A. Modified RNAs

[0359] In certain embodiments, the disclosed compositions and/or nucleic acids comprise modified nucleic acids, including modified RNAs.

[0360] Modified nucleosides or nucleotides can be present in an RNA, for example a mRNA. A mRNA comprising one or more modified nucleosides or nucleotides, for example, is called a "modified" RNA to describe the presence of one or more non-naturally and/or naturally occurring components or configurations that are used instead of or in addition to the canonical A, G, C, and U residues. In some embodiments, a modified RNA is synthesized with a non-canonical nucleoside or nucleotide, here called "modified."

[0361] Modified nucleosides and nucleotides can include one or more of: (i) alteration, *e.g.*, replacement, of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens in the phosphodiester backbone linkage (an exemplary backbone modification); (ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar (an exemplary sugar modification); (iii) wholesale replacement of

the phosphate moiety with "dephospho" linkers (an exemplary backbone modification); (iv) modification or replacement of a naturally occurring nucleobase, including with a non-canonical nucleobase (an exemplary base modification); (v) replacement or modification of the ribose-phosphate backbone (an exemplary backbone modification); (vi) modification of the 3' end or 5' end of the oligonucleotide, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, cap or linker (such 3' or 5' cap modifications may comprise a sugar and/or backbone modification); and (vii) modification or replacement of the sugar (an exemplary sugar modification). Certain embodiments comprise a 5' end modification to an mRNA or nucleic acid. Certain embodiments comprise a 3' end modification to an mRNA or nucleic acid. A modified RNA can contain 5' end and 3' end modifications. A modified RNA can contain one or more modified residues at non-terminal locations. In certain embodiments, an mRNA includes at least one modified residue.

[0362] Unmodified nucleic acids can be prone to degradation by, *e.g.*, intracellular nucleases or those found in serum. For example, nucleases can hydrolyze nucleic acid phosphodiester bonds. Accordingly, in one aspect the RNAs (*e.g.*, mRNAs) described herein can contain one or more modified nucleosides or nucleotides, *e.g.*, to introduce stability toward intracellular or serum-based nucleases. The term "innate immune response" includes a cellular response to exogenous nucleic acids, including single stranded nucleic acids, which involves the induction of cytokine expression and release, particularly the interferons, and cell death.

[0363] Accordingly, in some embodiments, RNA or nucleic acids in the disclosed the disclosed compositions, preparations, nanoparticles, and/or nanomaterials comprise at least one modification which confers increased or enhanced stability to the nucleic acid, including, for example, improved resistance to nuclease digestion *in vivo*. As used herein, the terms "modification" and "modified" as such terms relate to the nucleic acids provided herein, include at least one alteration which preferably enhances stability and renders the RNA or nucleic acid more stable (*e.g.*, resistant to nuclease digestion) than the wild-type or naturally occurring version of the RNA or nucleic acid. As used herein, the terms "stable" and "stability" as such terms relate to the nucleic acids of the present invention, and particularly with respect to the RNA, refer to increased or enhanced resistance to degradation by, for example nucleases (*i.e.*, endonucleases or exonucleases) which are normally capable of degrading such RNA. Increased stability can include, for example, less sensitivity to hydrolysis or other destruction by

endogenous enzymes (*e.g.*, endonucleases or exonucleases) or conditions within the target cell or tissue, thereby increasing or enhancing the residence of such RNA in the target cell, tissue, subject and/or cytoplasm. The stabilized RNA molecules provided herein demonstrate longer half-lives relative to their naturally occurring, unmodified counterparts (*e.g.*, the wild-type version of the mRNA). Also contemplated by the terms "modification" and "modified" as such terms related to the mRNA of the LNP compositions disclosed herein are alterations which improve or enhance translation of mRNA nucleic acids, including for example, the inclusion of sequences which function in the initiation of protein translation (*e.g.*, the Kozac consensus sequence). (Kozak, M., Nucleic Acids Res 15 (20): 8125-48 (1987), the contents of which are hereby incorporated by reference herein in its entirety).

[0364] In some embodiments, an RNA or nucleic acid of the disclosed compositions, preparations, nanoparticles, and/or nanomaterials disclosed herein have undergone a chemical or biological modification to render it more stable. Exemplary modifications to an RNA include the depletion of a base (*e.g.*, by deletion or by the substitution of one nucleotide for another) or modification of a base, for example, the chemical modification of a base. The phrase "chemical modifications" as used herein, includes modifications which introduce chemistries which differ from those seen in naturally occurring RNA, for example, covalent modifications such as the introduction of modified nucleotides, (*e.g.*, nucleotide analogs, or the inclusion of pendant groups which are not naturally found in such RNA molecules).

[0365] In some embodiments of a backbone modification, the phosphate group of a modified residue can be modified by replacing one or more of the oxygens with a different substituent. Further, the modified residue, *e.g.*, modified residue present in a modified nucleic acid, can include the wholesale replacement of an unmodified phosphate moiety with a modified phosphate group as described herein. In some embodiments, the backbone modification of the phosphate backbone can include alterations that result in either an uncharged linker or a charged linker with unsymmetrical charge distribution. Examples of modified phosphate groups include, phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. The phosphorous atom in an unmodified phosphate group is achiral. However, replacement of one of the non-bridging oxygens with one of the above atoms or groups of atoms can render the phosphorous atom chiral. The stereogenic phosphorous atom can possess either the "R"

configuration (herein Rp) or the "S" configuration (herein Sp). The backbone can also be modified by replacement of a bridging oxygen, (*i.e.,* the oxygen that links the phosphate to the nucleoside), with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at either linking oxygen or at both of the linking oxygens. The phosphate group can be replaced by non-phosphorus containing connectors in certain backbone modifications. In some embodiments, the charged phosphate group can be replaced by a neutral moiety. Examples of moieties which can replace the phosphate group can include, without limitation, *e.g.*, methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino.

4. Other

[0366] In certain embodiments, cargo nucleic acids comprise other components. In some embodiments, cargo nucleic acids comprise one or more of components such as promoters, enhancers, untranslated regions (UTRs), internal ribosome entry sites (IRES), splice sites, polyadenylation sequences, sequences comprising destabilization domains, reporter sequences or elements, and/or other additional sequences. Among other things, the present disclosure describes that selection and combination of one or more of the components as described herein impacts characteristics of nucleic acids such as stability, expression, localization and tropism.

A. Promoters

[0367] In some embodiments, a nucleic acid comprises a promoter. The term "promoter" refers to a DNA sequence recognized by enzymes/proteins that can promote and/or initiate transcription of an operably linked gene (*e.g.*, a nucleic acid encoding a cargo polypeptide). For example, a promoter typically refers to, *e.g.*, a nucleotide sequence to which an RNA polymerase and/or any associated factor binds and from which it can initiate transcription. Thus, in some

embodiments, a nucleic acid (*e.g.*, disposed within a delivery particle) comprises a promoter operably linked to one of the non-limiting example promoters described herein.

[0368] In some embodiments, a promoter is an inducible promoter, a constitutive promoter, a mammalian cell promoter, a viral promoter, a chimeric promoter, an engineered promoter, a tissue-specific promoter, or any other type of promoter known in the art. In some embodiments, a promoter is a RNA polymerase II promoter, such as a mammalian RNA polymerase II promoter. In some embodiments, a promoter is a RNA polymerase III promoter, including, but not limited to, a HI promoter, a human U6 promoter, a mouse U6 promoter, or a swine U6 promoter. A promoter will generally be one that is able to promote transcription in a cell, tissue, organ, organoid, or organism of interest. In some embodiments, a promoter is a mammalian cell-specific promoter.

[0369] A variety of promoters are known in the art, which can be used herein. Non-limiting examples of promoters that can be used herein include: human EFlα, human cytomegalovirus (CMV) (US Patent No. 5,168,062, which is incorporated in its entirety herein by reference), human ubiquitin C (UBC), mouse phosphoglycerate kinase 1, polyoma adenovirus, simian virus 40 (SV40), β-globin, β-actin, α-fetoprotein, γ-globin, β-interferon, γ-glutamyl transferase, mouse mammary tumor virus (MMTV), Rous sarcoma virus, rat insulin, glyceraldehyde-3-phosphate dehydrogenase, metallothionein II (MT II), amylase, cathepsin, MI muscarinic receptor, retroviral LTR (*e.g.*, human T-cell leukemia virus HTLV), AAV ITR, interleukin-2, collagenase, platelet-derived growth factor, adenovirus 5 E2, stromelysin, murine MX gene, glucose regulated proteins (GRP78 and GRP94), α-2-macroglobulin, vimentin, MHC class I gene H-2_K b, HSP70, proliferin, tumor necrosis factor, thyroid stimulating hormone a gene, immunoglobulin light chain, T-cell receptor, HLA DQa and DQ , interleukin-2 receptor, MHC class II, MHC class II HLA-DRa, muscle creatine kinase, prealbumin (transthyretin), elastase I, albumin gene, c-fos, c-HA-ras, neural cell adhesion molecule (NCAM), H2B (TH2B) histone, rat growth hormone, human serum amyloid (SAA), troponin I (TN I), duchenne muscular dystrophy, human immunodeficiency virus, and Gibbon Ape Leukemia Virus (GALV) promoters. Additional examples of promoters are known in the art. See, *e.g.*, Lodish, Molecular Cell Biology, Freeman and Company, New York 2007, each of which is incorporated in its entirety herein by reference. In some embodiments, a promoter is the CMV immediate early promoter. In some embodiments, the promoter is a CAG promoter or a CAG/CBA promoter. The term "constitutive" promoter

refers to a nucleotide sequence that, when operably linked with a nucleic acid encoding a cargo polypeptide, causes RNA to be transcribed from the nucleic acid in a cell under most or all physiological conditions.

[0370] Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter, the cytomegalovirus (CMV) promoter (see, *e.g.*, Boshart et al, *Cell* 41:521-530, 1985, which is incorporated in its entirety herein by reference), the SV40 promoter, the dihydrofolate reductase promoter, the beta-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EFl-alpha promoter (Invitrogen).

[0371] Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech, and Ariad. Additional examples of inducible promoters are known in the art.

[0372] Examples of inducible promoters regulated by exogenously supplied compounds include the zinc-inducible sheep metallothionein (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088, which is incorporated in its entirety herein by reference); the ecdysone insect promoter (see, *e.g.*, No et al, Proc. Natl. Acad Sci. US.A. 93:3346-3351, 1996, which is incorporated in its entirety herein by reference), the tetracycline-repressible system (see, *e.g.*, Gossen et al, Proc. Natl. Acad Sci. US.A. 89:5547-5551, 1992, which is incorporated in its entirety herein by reference), the tetracycline-inducible system (see, *e.g.*, Gossen et al, Science 268:1766-1769, 1995; and Harvey et al, Curr. Opin. Chem. Biol. 2:512-518, 1998, each of which is incorporated in their entirety herein by reference), the RU486-inducible system (see, *e.g.*, Wang et al, Nat. Biotech. 15:239- 243, 1997; and Wang et al, Gene Ther. 4:432-441, 1997, each of which is incorporated in their entirety herein by reference), and the rapamycin-inducible system (see, *e.g.*, Magari et al. J Clin. Invest. 100:2865-2872, 1997, which is incorporated in its entirety herein by reference).

[0373] The term "tissue-specific" promoter refers to a promoter that is active only in certain specific cell types and/or tissues (*e.g.*, transcription of a specific gene occurs only within cells

expressing transcription regulatory and/or control proteins that bind to the tissue-specific promoter).

[0374] In some embodiments, regulatory and/or control sequences impart tissue-specific gene expression capabilities. In some cases, tissue-specific regulatory and/or control sequences bind tissue-specific transcription factors that induce transcription in a tissue-specific manner.

[0375] In some embodiments, provided nucleic acids comprise a promoter sequence selected from a CAG, a CBA, a CMV, or a CB7 promoter.

B. Enhancers

[0376] In some instances, a construct can include an enhancer sequence. The term "enhancer" refers to a nucleotide sequence that can increase the level of transcription of a nucleic acid encoding a protein of interest (*e.g.*, a cargo polypeptide). Enhancer sequences (generally 50-1500 bp in length) generally increase the level of transcription by providing additional binding sites for transcription-associated proteins (*e.g.*, transcription factors). In some embodiments, an enhancer sequence is found within an intronic sequence. Unlike promoter sequences, enhancer sequences can act at much larger distance away from the transcription start site (*e.g.*, as compared to a promoter). Non-limiting examples of enhancers include a RSV enhancer, a CMV enhancer, and/or a SV40 enhancer.

C. Flanking Untranslated Regions, 5′ UTRs and 3′ UTRs

[0377] In some embodiments, any of the nucleic acids described herein can include an untranslated region (UTR), such as a 5′ UTR or a 3′ UTR. UTRs of a gene are transcribed but not translated. A 5′ UTR starts at the transcription start site and continues to the start codon but does not include the start codon. A 3′ UTR starts immediately following the stop codon and continues until the transcriptional termination signal. The regulatory and/or control features of a UTR can be incorporated into any of the constructs, compositions, kits, or methods as described herein to enhance or otherwise modulate the expression of a cargo polypeptide.

[0378] Natural 5′ UTRs include a sequence that plays a role in translation initiation. in some embodiments, a 5′ UTR can comprise sequences, like Kozak sequences, which are commonly

known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus sequence CCR(A/G)CCAUGG, where R is a purine (A or G) three bases upstream of the start codon (AUG), and the start codon is followed by another "G". The 5′ UTRs have also been known to form secondary structures that are involved in elongation factor binding.

[0379] In some embodiments, a 5′ UTR is included in any of the constructs described herein. Non-limiting examples of 5′ UTRs, including those from the following genes: albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, and Factor VIII, can be used to enhance expression of a nucleic acid molecule, such as an mRNA.

[0380] 3′ UTRs are known to have stretches of adenosines and uridines (in the RNA form) or thymidines (in the DNA form) embedded in them. These AU-rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU-rich elements (AREs) can be separated into three classes (see, *e.g.*, Chen et al., Mal. Cell. Biol. 15:5777-5788, 1995; Chen et al., Mal. Cell Biol. 15:2010-2018, 1995, each of which is incorporated herein by reference in its entirety): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. For example, c-Myc and MyoD mRNAs contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A) (U/A) nonamers. GM-CSF and TNF-alpha mRNAs are examples that contain class II AREs. Class III AREs are less well defined. These U-rich regions do not contain an AUUUA motif, two well-studied examples of this class are c-Jun and myogenin mRNAs.

[0381] Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message in vivo.

[0382] In some embodiments, the introduction, removal, or modification of 3′ UTR AREs can be used to modulate the stability of an mRNA encoding a cargo polypeptide. In other embodiments, AREs can be removed or mutated to increase the intracellular stability and thus increase translation and production of a cargo polypeptide.

[0383] In other embodiments, non-ARE sequences may be incorporated into the 5′ or 3′ UTRs. In some embodiments, introns or portions of intron sequences may be incorporated into the flanking regions of the polynucleotides in any of the constructs, compositions, kits, and methods provided herein. Incorporation of intronic sequences may increase protein production as well as mRNA levels.

D. Internal Ribosome Entry Sites (IRES)

[0384] In some embodiments, a nucleic acid comprising a cargo component can include an internal ribosome entry site (IRES). An IRES forms a complex secondary structure that allows translation initiation to occur from any position with an mRNA immediately downstream from where the IRES is located (see, *e.g.*, Pelletier and Sonenberg, Mal. Cell. Biol. 8(3):1103-1112, 1988).

[0385] There are several IRES sequences known to those in skilled in the art, including those from, *e.g.*, foot and mouth disease virus (FMDV), encephalomyocarditis virus (EMCV), human rhinovirus (HRV), cricket paralysis virus, human immunodeficiency virus (HIV), hepatitis A virus (HAV), hepatitis C virus (HCV), and poliovirus (PV) (see *e.g.*, Alberts, Molecular Biology of the Cell, Garland Science, 2002; and Hellen et al., Genes Dev. 15(13):1593-612, 2001, each of which is incorporated in its entirety herein by reference).

[0386] In some embodiments, the IRES sequence that is incorporated into a construct that encodes a cargo polypeptide, or a C-terminal portion of a cargo polypeptide is the foot and mouth disease virus (FMDV) 2A sequence. The Foot and Mouth Disease Virus 2A sequence is a small peptide (approximately 18 amino acids in length) that has been shown to mediate the cleavage of polyproteins (see, *e.g.*, Ryan, MD et al., EMBO 4:928-933, 1994; Mattion et al., J Virology 70:8124-8127, 1996; Furler et al., Gene Therapy 8:864-873, 2001; and Halpin et al., Plant Journal 4:453-459, 1999, each of which is incorporated in its entirety herein by reference). The cleavage activity of the 2A sequence has previously been demonstrated in artificial systems including plasmids and gene therapy constructs (AAV and retroviruses) (see, *e.g.*, Ryan et al., EMBO 4:928-933, 1994; Mattion et al., J Virology 70:8124-8127, 1996; Furler et al., Gene Therapy 8:864-873, 2001; and Halpin et al., Plant Journal 4:453-459, 1999; de Felipe et al., Gene Therapy 6:198-208, 1999; de Felipe et al., Human Gene Therapy I I: 1921-1931, 2000; and

Klump et al., Gene Therapy 8:811-817, 2001, each of which is incorporated in its entirety herein by reference).

[0387] An IRES can be utilized in a delivery particle described herein. In some embodiments, a nucleic acid encoding a C-terminal portion of a cargo polypeptide can include a polynucleotide internal ribosome entry site (IRES). In some embodiments, an IRES can be part of a composition comprising more than one nucleic acid. In some embodiments, an IRES is used to produce more than one cargo polypeptide from a single gene transcript.

E. Splice Sites

[0388] In some embodiments, any of the nucleic acids provided herein can include splice donor and/or splice acceptor sequences, which are functional during RNA processing occurring during transcription. In some embodiments, splice sites are involved in trans-splicing.

F. Polyadenylation Sequences

[0389] In some embodiments, a construct provided herein can include a polyadenylation (poly(A)) signal sequence. Most nascent eukaryotic mRNAs possess a poly(A) tail at their 3′ end, which is added during a complex process that includes cleavage of the primary transcript and a coupled polyadenylation reaction driven by the poly(A) signal sequence (see, *e.g.*, Proudfoot et al., Cell 108:501-512, 2002, which is incorporated herein by reference in its entirety). A poly(A) tail confers mRNA stability and transferability (see, *e.g.*, Molecular Biology of the Cell, Third Edition by B. Alberts et al., Garland Publishing, 1994, which is incorporated herein by reference in its entirety). In some embodiments, a poly(A) signal sequence is positioned 3′ to the coding sequence.

[0390] As used herein, "polyadenylation" refers to the covalent linkage of a polyadenylyl moiety, or its modified variant, to a messenger RNA molecule. In eukaryotic organisms, most messenger RNA (mRNA) molecules are polyadenylated at the 3′ end. A 3′ poly(A) tail is a long sequence of adenine nucleotides (*e.g.*, 50, 60, 70, 100, 200, 500, 1000, 2000, 3000, 4000, or 5000) added to the pre-mRNA through the action of an enzyme, polyadenylate polymerase. In some embodiments, a poly(A) tail is added onto transcripts that contain a specific sequence, *e.g.*,

a poly(A) signal. A poly(A) tail and associated proteins aid in protecting mRNA from degradation by exonucleases. Polyadenylation also plays a role in transcription termination, export of the mRNA from the nucleus, and translation. Polyadenylation typically occurs in the nucleus immediately after transcription of DNA into RNA, but also can occur later in the cytoplasm. After transcription has been terminated, an mRNA chain is cleaved through the action of an endonuclease complex associated with RNA polymerase. A cleavage site is usually characterized by the presence of the base sequence AAUAAA near the cleavage site. After the mRNA has been cleaved, adenosine residues are added to the free 3′ end at the cleavage site.

[0391] As used herein, a "poly(A) signal sequence" or "polyadenylation signal sequence" is a sequence that triggers the endonuclease cleavage of an mRNA and the addition of a series of adenosines to the 3′ end of the cleaved mRNA.

[0392] There are several poly(A) signal sequences that can be used, including those derived from bovine growth hormone (bGH) (see, *e.g.*, Woychik et al., Proc. Natl. Acad Sci. US.A. 81(13):3944-3948, 1984; U.S. Patent No. 5,122,458, each of which is incorporated herein by reference in its entirety), mouse-β-globin, mouse-α-globin (see, *e.g.*, Orkin et al., EMBO J 4(2):453-456, 1985; Thein et al., Blood 71(2):313-319, 1988, each of which is incorporated herein by reference in its entirety), human collagen, polyoma virus (see, *e.g.*, Batt et al., Mal. Cell Biol. 15(9):4783-4790, 1995, which is incorporated herein by reference in its entirety), the Herpes simplex virus thymidine kinase gene (HSV TK), IgG heavy-chain gene polyadenylation signal (US 2006/0040354, which is incorporated herein by reference in its entirety), human growth hormone (hGH) (see, *e.g.*, Szymanski et al., Mal. Therapy 15(7):1340-1347, 2007, which is incorporated herein by reference in its entirety), the group consisting of SV40 poly(A) site, such as the SV40 late and early poly(A) site (see, *e.g.*, Schek et al., Mal. Cell Biol. 12(12):5386-5393, 1992, which is incorporated herein by reference in its entirety).

[0393] The poly(A) signal sequence can be AATAAA. The AATAAA sequence may be substituted with other hexanucleotide sequences with homology to AATAAA and that are capable of signaling polyadenylation, including ATTAAA, AGTAAA, CATAAA, TATAAA, GATAAA, ACTAAA, AATATA, AAGAAA, AATAAT, AAAAAA, AATGAA, AATCAA, AACAAA, AATCAA, AATAAC, AATAGA, AATTAA, or AATAAG (see, *e.g.*, WO 06/12414, which is incorporated herein by reference in its entirety).

[0394] In some embodiments, a poly(A) signal sequence can be a synthetic polyadenylation site (see, *e.g.*, the pCl-neo expression construct of Promega that is based on Levitt el al, Genes Dev. 3(7):1019-1025, 1989, which is incorporated herein by reference in its entirety). In some embodiments, a poly(A) signal sequence is the polyadenylation signal of soluble neuropilin-1 (sNRP) (AAATAAAATACGAAATG) (see, *e.g.*, WO 05/073384, which is incorporated herein by reference in its entirety). In some embodiments, a poly(A) signal sequence comprises or consists of the SV40 poly(A) site.

G. Destabilization Domains

[0395] In some embodiments, any of the nucleic acids provided herein can optionally include a sequence encoding a destabilizing domain ("a destabilizing sequence") for temporal control of protein expression. Non-limiting examples of destabilizing sequences include sequences encoding a FK506 sequence, a dihydrofolate reductase (DHFR) sequence, or other exemplary destabilizing sequences.

[0396] In the absence of a stabilizing ligand, a protein sequence operatively linked to a destabilizing sequence is degraded by ubiquitination. In contrast, in the presence of a stabilizing ligand, protein degradation is inhibited, thereby allowing the protein sequence operatively linked to the destabilizing sequence to be actively expressed. As a positive control for stabilization of protein expression, protein expression can be detected by conventional means, including enzymatic, radiographic, colorimetric, fluorescence, or other spectrographic assays; fluorescent activating cell sorting (FACS) assays; immunological assays (*e.g.*, enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and immunohistochemistry).

[0397] Additional examples of destabilizing sequences are known in the art. In some embodiments, the destabilizing sequence is a FK506- and rapamycin-binding protein (FKBP12) sequence, and the stabilizing ligand is Shield-1 (Shld1) (see, *e.g.*, Banaszynski et al. (2012) Cell 126(5): 995-1004, which is incorporated in its entirety herein by reference). In some embodiments, a destabilizing sequence is a DHFR sequence, and a stabilizing ligand is trimethoprim (TMP) (see, *e.g.*, Iwamoto et al. (2010) Chem Biol 17:981-988, which is incorporated in its entirety herein by reference).

[0398] In some embodiments, a destabilizing sequence is a FKBP12 sequence, and a presence of nucleic acids carrying the FKBP12 gene in a subject cell (*e.g.*, a cell of interest (*e.g.*, a glial cell, a liver cell, a tumor cell, etc.)) is detected by Western blotting. In some embodiments, a destabilizing sequence can be used to verify the temporally-specific activity of delivery particles described herein.

H. Reporter Sequences or Elements

[0399] In some embodiments, nucleic acids provided herein can optionally include a sequence encoding a reporter polypeptide and/or protein ("a reporter sequence"). Non-limiting examples of reporter sequences include DNA sequences encoding: a beta-lactamase, a beta-galactosidase (LacZ), an alkaline phosphatase, a thymidine kinase, a green fluorescent protein (GFP), a red fluorescent protein, an mCherry fluorescent protein, a yellow fluorescent protein, a chloramphenicol acetyltransferase (CAT), and a luciferase. Additional examples of reporter sequences are known in the art. When associated with control elements which drive their expression, the reporter sequence can provide signals detectable by conventional means, including enzymatic, radiographic, colorimetric, fluorescence, or other spectrographic assays; fluorescent activating cell sorting (FACS) assays; immunological assays (*e.g.*, enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and immunohistochemistry).

[0400] In some embodiments, a reporter sequence is the LacZ gene, and the presence of a construct carrying the LacZ gene in a mammalian cell (*e.g.*, a cell of interest (*e.g.*, a glial cell, a liver cell, a tumor cell, etc.)) is detected by assays for beta-galactosidase activity. When the reporter is a fluorescent protein (*e.g.*, green fluorescent protein) or luciferase, the presence of a construct carrying the fluorescent protein or luciferase in a mammalian cell (*e.g.*, a cell of interest (*e.g.*, a glial cell, a liver cell, a tumor cell, etc.)) may be measured by fluorescent techniques (*e.g.*, fluorescent microscopy or FACS) or light production in a luminometer (*e.g.*, a spectrophotometer or an IVIS imaging instrument). In some embodiments, a reporter sequence can be used to verify the tissue-specific targeting capabilities and tissue-specific promoter regulatory and/or control activity of any of the constructs described herein.

[0401] In some embodiments, a reporter sequence is a FLAG tag (*e.g.*, a 3xFLAG tag), and the presence of a construct carrying the FLAG tag in a mammalian cell (*e.g.*, a cell of interest

(*e.g.*, a glial cell, a liver cell, a tumor cell, etc.)) is detected by protein binding or detection assays (*e.g.*, Western blots, immunohistochemistry, radioimmunoassay (RIA), mass spectrometry).

I. Additional Sequences

[0402] In some embodiments, nucleic acids of the present disclosure may comprise a T2A element or sequence. In some embodiments, nucleic acids of the present disclosure may include one or more cloning sites. In some such embodiments, cloning sites may not be fully removed prior to manufacturing for administration to a subject. In some embodiments, cloning sites may have functional roles including as linker sequences, or as portions of a Kozak site. As will be appreciated by those skilled in the art, cloning sites may vary significantly in primary sequence while retaining their desired function.

J. Inverted Terminal Repeat Sequences (ITRs)

[0403] In some embodiments, a delivery particle is an AAV delivery particle. AAV derived nucleic sequences of a construct typically comprises the cis-acting 5′ and 3′ ITRs (see, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990), which is incorporated in its entirety herein by reference). Generally, ITRs are able to form a hairpin. The ability to form a hairpin can contribute to an ITRs ability to self-prime, allowing primase-independent synthesis of a second DNA strand. ITRs can also aid in efficient encapsidation of an AAV construct in an AAV delivery particle.

[0404] An rAAV delivery particle (*e.g.*, an AAV2 delivery particle) of the present disclosure can comprise a nucleic acid comprising a cargo component encoding a cargo polypeptide and associated elements flanked by a 5′ and a 3′ AAV ITR sequences. In some embodiments, an ITR is or comprises about 145 nucleic acids. In some embodiments, all or substantially all of a sequence encoding an ITR is used. An AAV ITR sequence may be obtained from any known AAV, including presently identified mammalian AAV types. In some embodiments an ITR is an AAV2 ITR.

[0405] An example of a construct molecule employed in the present disclosure is a "cis-acting" construct containing a transgene, in which the selected transgene sequence and

associated regulatory elements are flanked by 5′ or "left" and 3′ or "right" AAV ITR sequences. 5′ and left designations refer to a position of an ITR sequence relative to an entire construct, read left to right, in a sense direction. For example, in some embodiments, a 5′ or left ITR is an ITR that is closest to a promoter (as opposed to a polyadenylation sequence) for a given construct, when a construct is depicted in a sense orientation, linearly. Concurrently, 3′ and right designations refer to a position of an ITR sequence relative to an entire construct, read left to right, in a sense direction. For example, in some embodiments, a 3′ or right ITR is an ITR that is closest to a polyadenylation sequence (as opposed to a promoter sequence) for a given construct, when a construct is depicted in a sense orientation, linearly. ITRs as provided herein are depicted in 5′ to 3′ order in accordance with a sense strand. Accordingly, one of skill in the art will appreciate that a 5′ or "left" orientation ITR can also be depicted as a 3′ or "right" ITR when converting from sense to antisense direction. Further, it is well within the ability of one of skill in the art to transform a given sense ITR sequence (*e.g.*, a 5′/left AAV ITR) into an antisense sequence (*e.g.*, 3′/right ITR sequence). One of ordinary skill in the art would understand how to modify a given ITR sequence for use as either a 5′/left or 3′/right ITR, or an antisense version thereof.

VI. Delivery Particles

[0406] Among other things, the present disclosure provides delivery particles. In some embodiments, a delivery particle is a viral particle, a lipid-based particle [(*e.g.*, cell-produced or not cell-produced), a lipid nanoparticle (LNP), a liposome, a micelle, an extracellular vesicle (*e.g.*, exosomes, microparticles, etc.)], a polymer-based particle (*e.g.*, PGLA), a polysaccharide-based particle, etc. In some embodiments, delivery particles as described herein comprise nucleic acids In some embodiments, a nucleic acid described herein is disposed within a delivery particle. In some embodiments, a nucleic acid described herein is associated (*e.g.*, covalently or non-covalently) with a surface of delivery particle. In some embodiments, a nucleic acid comprises, among other things, a cargo component encoding a cargo polypeptide, that, when expressed, is expressed on a surface of a delivery particle.

i. Virions:

[0407] Among other things, the present disclosure provides virions that comprise a nucleic acid and a capsid as described herein. In some embodiments, virions are delivery particles that comprise a nucleic acid comprising a cargo component encoding a cargo polypeptide or characteristic portion thereof described herein, and a capsid described herein. An exemplary delivery particle is an AAV delivery particle. An exemplary delivery particle is a lentivirus delivery particle. However, other delivery particles may be used.

[0408] In some embodiments, a delivery particle is an AAV delivery particle. AAV delivery particles that comprise a nucleic acid comprising a cargo component encoding a cargo polypeptide or characteristic portion thereof described herein, and a capsid described herein. In some embodiments, AAV delivery particles can be described as having a serotype, which is a description of the construct strain and the capsid strain. For example, in some embodiments an AAV delivery particle may be described as AAV2, wherein the particle has an AAV2 capsid and a construct that comprises characteristic AAV2 Inverted Terminal Repeats (ITRs). In some embodiments, an AAV delivery particle may be described as a pseudotype, wherein the capsid and construct are derived from different AAV strains, for example, AAV2/9 would refer to an AAV delivery particle that comprises a construct utilizing the AAV2 ITRs and an AAV9 capsid.

1. AAV Construct

[0409] The present disclosure provides nucleic acids that comprise a cargo component encoding a cargo polypeptide or characteristic portion thereof. In some embodiments described herein, a nucleic acid that comprises a cargo component encoding a cargo polypeptide or characteristic portion thereof can be disposed within an AAV delivery particle.

[0410] In some embodiments, a nucleic acid comprises one or more components derived from or modified from a naturally occurring AAV genomic construct. In some embodiments, a sequence derived from an AAV construct is an AAV1 construct, an AAV2 construct, an AAV3 construct, an AAV4 construct, an AAV5 construct, an AAV6 construct, an AAV7 construct, an AAV8 construct, an AAV9 construct, an AAV2.7m8 construct, an AAV8BP2 construct, an AAV293 construct, an AAV.DJ construct, or AAV Anc80 construct. In some embodiments, an

rAAV Anc80 capsid is an rAAV Anc80L65 capsid. Additional exemplary AAV constructs that can be used herein are known in the art (see, *e.g.*, Kanaan et al., Mol. Ther. Nucleic Acids 8:184-197, 2017; Li et al., Mol. Ther. 16(7): 1252-1260, 2008; Adachi et al., Nat. Commun. 5: 3075, 2014; Isgrig et al., Nat. Commun. 10(1): 427, 2019; and Gao et al., J. Virol. 78(12): 6381-6388, 2004; each of which is incorporated in its entirety herein by reference).

[0411] In some embodiments, provided nucleic acids comprise a cargo component, *e.g.*, encoding a cargo polypeptide, one or more regulatory and/or control sequences, and optionally 5′ and 3′ AAV derived inverted terminal repeats (ITRs). In some embodiments wherein a 5′ and 3′ AAV derived ITR is utilized, the polynucleotide construct may be referred to as a recombinant AAV (rAAV) construct. In some embodiments, provided rAAV constructs are packaged into an AAV capsid to form an AAV delivery particle.

[0412] In some embodiments, AAV derived sequences (which are comprised in a polynucleotide construct) typically include the cis-acting 5′ and 3′ ITR sequences (see, *e.g.*, B. J. Carter, in "Handbook of Parvoviruses," ed., P. Tijsser, CRC Press, pp. 155 168, 1990, which is incorporated herein by reference in its entirety). Typical AAV2-derived ITR sequences are about 145 nucleotides in length. In some embodiments, at least 80% of a typical ITR sequence (*e.g.*, at least 85%, at least 90%, or at least 95%) is incorporated into a construct provided herein. The ability to modify these ITR sequences is within the skill of the art. (see, *e.g.*, texts such as Sambrook et al., "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York, 1989; and K. Fisher et al., J Virol. 70:520 532, 1996, each of which is incorporated in its entirety by reference). In some embodiments, any of the coding sequences and/or constructs described herein are flanked by 5′ and 3′ AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including presently identified AAV types.

[0413] In some embodiments, nucleic acids described in accordance with this disclosure and in a pattern known to the art (see, *e.g.*, Asokan et al., Mol. Ther. 20: 699-7080, 2012, which is incorporated herein by reference in its entirety) are typically comprised of, a coding sequence or a portion thereof, at least one and/or control sequence, and optionally 5′ and 3′ AAV inverted terminal repeats (ITRs). In some embodiments, provided constructs can be packaged into a capsid to create an AAV delivery particle. An AAV delivery particle may be delivered to a selected target cell. In some embodiments, provided constructs comprise an additional optional

coding sequence that is a nucleic acid sequence (*e.g.*, inhibitory nucleic acid sequence), heterologous to the nucleic sequences, which encodes a polypeptide, protein, functional RNA molecule (*e.g.*, miRNA, miRNA inhibitor) or other gene product, of interest. In some embodiments, a nucleic acid coding sequence is operatively linked to and/or control components in a manner that permits coding sequence transcription, translation, and/or expression in a cell of a target tissue.

[0414] In some embodiments, a nucleic acid is an rAAV nucleic acid. In some embodiments, an rAAV nucleic acid can include at least 500 bp, at least 1 kb, at least 1.5 kb, at least 2 kb, at least 2.5 kb, at least 3 kb, at least 3.5 kb, at least 4 kb, or at least 4.5 kb. In some embodiments, an AAV construct can include at most 7.5 kb, at most 7 kb, at most 6.5 kb, at most 6 kb, at most 5.5 kb, at most 5 kb, at most 4.5 kb, at most 4 kb, at most 3.5 kb, at most 3 kb, or at most 2.5 kb. In some embodiments, an AAV construct can include about 1 kb to about 2 kb, about 1 kb to about 3 kb, about 1 kb to about 4 kb, about 1 kb to about 5 kb, about 2 kb to about 3 kb, about 2 kb to about 4 kb, about 2 kb to about 5kb, about 3 kb to about 4 kb, about 3 kb to about 5 kb, or about 4 kb to about 5 kb.

[0415] Any of the nucleic acids described herein can further include regulatory and/or control sequences, *e.g.*, a control sequence selected from the group of a transcription initiation sequence, a transcription termination sequence, a promoter sequence, an enhancer sequence, an RNA splicing sequence, a polyadenylation (poly(A)) sequence, a Kozak consensus sequence, and/or any combination thereof. In some embodiments, a promoter can be a native promoter, a constitutive promoter, an inducible promoter, and/or a tissue-specific promoter. Non-limiting examples of control sequences are described herein.

2. AAV Capsids

[0416] The present disclosure provides one or more nucleic acids disposed with an AAV capsid. In some embodiments, an AAV capsid is from or derived from an AAV capsid of an AAV2, 3, 4, 5, 6, 7, 8, 9, 10, DJ, PHP-B, rh8, rh10, rh39, rh43 or Anc80 serotype, or one or more hybrids thereof. In some embodiments, an AAV capsid is from an AAV ancestral serotype

[0417] As provided herein, any combination of AAV capsids and AAV nucleic acids (*e.g.*, comprising AAV ITRs) may be used in recombinant AAV (rAAV) particles of the present disclosure. For example, wild type or variant AAV2 ITRs and Anc80 capsid, wild type or variant AAV2 ITRs and AAV6 capsid, etc. In some embodiments of the present disclosure, an AAV delivery particle is wholly comprised of AAV2 components (*e.g.*, capsid and ITRs are AAV2 serotype). In some embodiments, an AAV delivery particle is an AAV2/6, AAV2/8 or AAV2/9 particle (*e.g.*, an AAV6, AAV8 or AAV9 capsid with an AAV construct having AAV2 ITRs).

ii. Lipid-Based Delivery Particles

[0418] Among other things, the present disclosure provides for compositions, preparations, and/or delivery particles that comprise lipids (*e.g.*, lipid-based delivery particles). In some embodiments, lipid-based delivery particles are produced by a cell. In some embodiments, lipid-based delivery particles are not produced by a cell. The present invention provided for lipid-based delivery particles that may be of various types. In some embodiments, lipid-based delivery particles may be lipid nanoparticles (LNPs). In some embodiments, lipid-based delivery particles may be liposomes. In some embodiments, lipid-based delivery particles may be micelles. In some embodiments, lipid-based delivery particles may be extracellular vesicles (*e.g.*, exosomes).

1. Lipid Nanoparticles (LNPs)

[0419] In some embodiments, the present disclosure provides for compositions, preparations, and/or delivery particles that comprise lipid nanoparticles. In some embodiments, lipid nanoparticles comprise one or more components. In some embodiments, lipid nanoparticles comprise one or more components such as compounds, ionizable lipids, sterols, conjugate-linker lipids, and phospholipids. Among other things, the present disclosure describes that selection and combination of one or more of the components as described herein impacts characteristics of lipid nanoparticles such as diameter, pKa, stabilization, and ionizability.

[0420] Among other things, the present disclosure describes that selection and combination of one or more of the components as described herein impacts functional activity of

lipid nanoparticles such as tropism, stabilization, and drug delivery efficacy. For example, the present disclosure describes that a combination of components may better suit delivery of nucleic acids comprising a cargo (*e.g.*, a nucleic acid encoding a cargo polypeptide) as described herein. In some embodiments, a cargo comprises RNA. In some embodiments, a cargo comprises DNA.

[0421] In some embodiments, lipid nanoparticles comprise one or more compounds as described herein. In some embodiments, lipid nanoparticles comprise one or more ionizable lipids as described herein. In some embodiments, lipid nanoparticles comprise one or more sterols as described herein. In some embodiments, lipid nanoparticles comprise one or more conjugate-linker lipids as described herein. In some embodiments, lipid nanoparticles comprise one or more phospholipids as described herein.

A. Ionizable Lipids

[0422] Among other things, the present disclosure describes compositions, preparations, delivery particles, and/or methods that comprise one or more ionizable lipids as described herein.

[0423] Among other things, the present disclosure describes that different ratios of ionizable lipids influence one or more functional activities such as desired tropisms, stabilization, and drug delivery efficacy of compositions, preparations, nanoparticles, and/or nanomaterials described herein. For example, in some embodiments, compositions, preparations delivery particles, and methods can be used to identify an amount of ionizable lipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein. In some embodiments, compositions, preparations delivery particles, and methods can be used to identify characteristic features of an ionizable lipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein.

B. Sterols

[0424] Among other things, the present disclosure describes compositions, preparations, delivery particles, and/or methods that comprise one or more sterols as described herein.

[0425] In some embodiments, a sterol is a cholesterol, or a variant or derivative thereof. In some embodiments, a cholesterol is modified. In some embodiments, a cholesterol is an oxidized cholesterol. In some embodiments, a cholesterol is esterified cholesterol. Unmodified cholesterol can be acted upon by enzymes to form variants that are side-chain or ring oxidized. In some embodiments, a cholesterol can be oxidized on the beta-ring structure or on the hydrocarbon tail structure. In some embodiments, a sterol is a phytosterol. Exemplary sterols that are considered for use in the disclosed lipid nanoparticles include but are not limited to 25-hydroxycholesterol (25-OH), 20α-hydroxycholesterol (20α-OH), 27-hydroxycholesterol, 6-keto-5α-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 7β-25-dihydroxycholesterol, beta-sitosterol, stigmasterol, brassicasterol, campesterol, or combinations thereof. In some embodiments, a side-chain oxidized cholesterol can enhance cargo delivery relative to other cholesterol variants. In some embodiments, a cholesterol is an unmodified cholesterol. In some embodiments, a cholesterol is campesterol.

[0426] For example, in some embodiments, compositions, preparations delivery particles, and methods can be used to identify an amount of sterol that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein. In some embodiments, compositions, preparations delivery particles, and methods can be used to identify characteristic features of a sterol that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein.

C. Conjugate-Linker Lipids

[0427] Among other things, the present disclosure describes compositions, preparations, delivery particles, and/or methods that comprise one or more conjugate-linker lipids as described herein.

[0428] In some embodiments, a conjugate-linker lipid is or comprises a polyethylene glycol (PEG)-lipid or PEG-modified lipid. In some embodiments, PEG or PEG-modified lipids may be alternately referred to as PEGylated lipids or PEG-lipids. Inclusion of a PEGylating lipid can be used to enhance lipid nanoparticle colloidal stability *in vitro* and circulation time *in vivo*. In some embodiments, the PEGylation is reversible in that the PEG moiety is gradually released

in blood circulation. Exemplary PEG-lipids include but are not limited to PEG conjugated to saturated or unsaturated alkyl chains having a length of C6-C20. PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides (PEG-CER), PEG-modified dialkylamines, PEG-modified diacylglycerols (PEG-DAG), PEG-modified dialkylglycerols, and mixtures thereof. For example, in some embodiments, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPE, PEG-DSG or a PEG-DSPE lipid.

[0429] In some embodiments, a conjugate-linker lipid comprises a polyethylene glycol lipid. In some embodiments, the conjugate-linker lipid comprises DiMystyrlGlycerol (DMG), 1,2-Dipalmitoyl-rac-glycerol, methoxypolyethylene Glycol (DPG-PEG), or 1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene (DSG – PEG). In some embodiments, a conjugate-linker lipid has an average molecular mass from about 500 Da to about 5000 Da. In some embodiments, a conjugate-linker lipid has an average molecular mass of about 2000 Da.

[0430] For example, in some embodiments, compositions, preparations delivery particles, and methods can be used to identify an amount of conjugate-linker lipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein. In some embodiments, compositions, preparations delivery particles, and methods can be used to identify characteristic features of a conjugate-linker lipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein.

D. Phospholipids

[0431] Among other things, the present disclosure describes compositions, preparations, delivery particles, and/or methods that comprise one or more phospholipids as described herein.

[0432] In some embodiments, one or more phospholipids may assemble into one or more lipid bilayers. In some embodiments, one or more phospholipids may include a phospholipid moiety. In some embodiments, one or more phospholipids may include one or more fatty acid moieties. In some embodiments, one or more phospholipids may include a phospholipid moiety and one or more fatty acid moieties. In some embodiments, a phospholipid moiety includes but

is not limited to phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2-lysophosphatidyl choline, and sphingomyelin. In some embodiments, a fatty acid moiety includes but is not limited to lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alphalinolenic acid, erucic acid, phytanic acid, arachidic acid, arachidonic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid. Non-natural species including natural species with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. For example, a phospholipid may be functionalized with or cross-linked to one or more alkynes *(e.g.,* an alkenyl group in which one or more double bonds is replaced with a triple bond). Under appropriate reaction conditions, an alkyne group may undergo a copper-catalyzed cycloaddition upon exposure to an azide. Such reactions may be useful in functionalizing a lipid bilayer of a nanoparticle composition to facilitate membrane permeation or cellular recognition or in conjugating a nanoparticle composition to a useful component such as a targeting or imaging moiety *(e.g.,* a dye).

[0433] Exemplary phospholipids include but are not limited to 1,2-distearoyl-*sn*glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycerophosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-*sn*-glycerophosphocholine (DUPC), l-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-*sn*-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoy l-*sn*-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl snglycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phospho-rac-(1 -glycerol) sodium salt (DOPG), dipalmitoylphosphatidylglycerol (DPPG), palmitoyloleoylphosphatidylethanolamine (POPE), distearoyl-phosphatidyl-ethanolamine (DSPE), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine

(DMPE), 1-stearoyl-2-oleoyl-phosphatidy ethanolamine (SOPE), 1-stearoyl-2 oleoylphosphatidylcholine (SOPC), sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine (LPE), or combinations thereof. In some embodiments, a phospholipid is DSPC. In some embodiments, a phospholipid is DMPC.

[0434] In some embodiments, the phospholipid comprises 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(succinyl) (succinyl PE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(succinyl) (succinyl-DPPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), or a combination thereof.

[0435] For example, in some embodiments, compositions, preparations delivery particles, and methods can be used to identify an amount of phospholipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein. In some embodiments, compositions, preparations delivery particles, and methods can be used to identify characteristic features of a phospholipid that is useful and/or critical to functional activity of lipid nanoparticles such as desired tropisms, stabilization, and drug delivery efficacy as described herein.

E. Diameter

[0436] Among other things, the present disclosure describes compositions and/or delivery particles that have an average hydrodynamic diameter from about 30 to about 220 nm. In some embodiments, compositions, preparations, nanoparticles, and/or nanomaterials described herein have an average hydrodynamic diameter that is about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, 150 nm, 155 nm, 160 nm, 165 nm, 170 nm, 175 nm, 180 nm, 185 nm, 190 nm, 195 nm, 200 nm, 205 nm, 210 nm, 215 nm, 220 nm, or any range having endpoints defined by any two of the aforementioned values. For

example, in some embodiments, compositions, preparations, nanoparticles, and/or nanomaterials described herein have an average hydrodynamic diameter from between 50 nm to 200 nm.

[0437] In some embodiments, lipid nanoparticles described herein have an average hydrodynamic diameter from about 30 to about 220 nm. In some embodiments, lipid nanoparticles described herein have an average hydrodynamic diameter that is about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, 150 nm, 155 nm, 160 nm, 165 nm, 170 nm, 175 nm, 180 nm, 185 nm, 190 nm, 195 nm, 200 nm, 205 nm, 210 nm, 215 nm, 220 nm, or any range having endpoints defined by any two of the aforementioned values. For example, in some embodiments, lipid nanoparticles described herein have an average hydrodynamic diameter from between 50 nm to 200 nm.

F. Methods of Manufacturing LNPs

[0438] Methods of manufacturing lipid nanoparticles are known in the art. In some embodiments, the described compositions, preparations, and/or delivery particles are manufactured using microfluidics. For instance, exemplary methods of using microfluidics to form lipid nanoparticles are described by Leung, A.K.K, et al., *J Phys Chem*, 116:18440-18450 (2012), Chen, D., et al., *J Am Chem Soc*, 134:6947-6951 (2012), and Belliveau, N.M., et al., *Molecular Therapy- Nucleic Acids*, 1: e37 (2012), the disclosures of which are hereby incorporated by reference in their entireties. Additionally, exemplary methods as described in U.S. Patent Nos. 8,569,256, 5,965,542 and U.S. Patent Publication Nos. 2016/0199485, 2016/0009637, 2015/0273068, 2015/0265708, 2015/0203446, 2015/0005363, 2014/0308304, 2014/0200257, 2013/086373, 2013/0338210, 2013/0323269, 2013/0245107, 2013/0195920, 2013/0123338, 2013/0022649, 2013/0017223, 2012/0295832, 2012/0183581, 2012/0172411, 2012/0027803, 2012/0058188, 2011/0311583, 2011/0311582, 2011/0262527, 2011/0216622, 2011/0117125, 2011/0091525, 2011/0076335, 2011/0060032, 2010/0130588, 2007/0042031, 2006/0240093, 2006/0083780, 2006/0008910, 2005/0175682, 2005/017054, 2005/0118253, 2005/0064595, 2004/0142025, 2007/0042031, 1999/009076 and PCT Pub. Nos. WO 99/39741, WO 2018/081480, WO 2017/004143, WO 2017/075531, WO 2015/199952, WO 2014/008334, WO 2013/086373, WO 2013/086322, WO 2013/016058, WO 2013/086373, W02011/141705,

and WO 2001/07548, the full disclosures of each of which are herein incorporated by reference in their entirety for the purposes described herein.

[0439] Briefly, a cargo, such as a cargo described herein, is prepared in a first buffer solution. Other lipid nanoparticle components (such as ionizable lipid, conjugate-linker lipids, cholesterol, and phospholipid) are prepared in a second buffer solution. In some embodiments, a syringe pump introduces the two solutions into a microfluidic device. The two solutions come into contact within the microfluidic device to form lipid nanoparticles encapsulating the cargo.

[0440] For example, in some embodiments, cationic lipids, neutral lipids (*e.g.*, DSPC, and/or cholesterol) and polymer-conjugated lipids can be solubilized in ethanol at a pre-determined molar ratio (*e.g.*, ones described herein). In some embodiments, lipid nanoparticles (lipid nanoparticle) are prepared at a total lipid to polyribonucleotides weight ratio of approximately 10: 1 to 30: 1. In some embodiments, such polyribonucleotides can be diluted to 0.2 mg/mL in acetate buffer.

[0441] In some embodiments, using an ethanol injection technique, a colloidal lipid dispersion comprising polyribonucleotides can be formed as follows: an ethanol solution comprising lipids, such as cationic lipids, neutral lipids, and polymer-conjugated lipids, is injected into an aqueous solution comprising polyribonucleotides (*e.g.*, ones described herein).

[0442] In some embodiments, lipid and polyribonucleotide solutions can be mixed at room temperature by pumping each solution at controlled flow rates into a mixing unit, for example, using piston pumps. In some embodiments, the flow rates of a lipid solution and a RNA solution into a mixing unit are maintained at a ratio of 1:3. Upon mixing, nucleic acid-lipid particles are formed as the ethanolic lipid solution is diluted with aqueous polyribonucleotides. The lipid solubility is decreased, while cationic lipids bearing a positive charge interact with the negatively charged RNA.

[0443] In some embodiments, a solution comprising RNA-encapsulated lipid nanoparticles can be processed by one or more of concentration adjustment, buffer exchange, formulation, and/or filtration.

[0444] In some embodiments, RNA-encapsulated lipid nanoparticles can be processed through filtration.

[0445] In some embodiments, particle size and/or internal structure of lipid nanoparticles (with or without RNAs) may be monitored by appropriate techniques such as, *e.g.*, small-angle X-ray scattering (SAXS) and/or transmission electron cryomicroscopy (CryoTEM).

2. Extracellular Vesicles (EVs)

[0446] As described herein, an extracellular vesicle (EV) is a lipid-bound vesicle-like structure. In some embodiments, EVs have a membrane. In some embodiments, EVs have a membrane that is a double layer membrane (*e.g.*, a lipid bilayer). In some embodiments, EVs have a membrane that originates from a cell. In some embodiments, EVs have a membrane that originates from the plasma membrane of a cell.

[0447] The term extracellular vesicle encompasses exosomes, microvesicles, membrane microparticles, ectosomes, blebs or apoptotic bodies. In some embodiments, an EV is classified as an exosome, microvesicle, membrane microparticle, ectosome, bleb or apoptotic body based on the origin of formation.

[0448] In some embodiments, EVs are substantially transparent. In some embodiments, EVs are substantially spherical.

A. Populations

[0449] In some embodiments, an EV has a diameter within a range of 50 to 1000 nm. In some embodiments, an EV has a diameter within a range of 50 to 750 nm. In some embodiments, an EV has a diameter within a range of 50 to 500 nm. In some embodiments, an EV has a diameter within a range of 50 to 300 nm. In some embodiments, an EV has a diameter within a range of 50 to 200 nm. In some embodiments, an EV has a diameter within a range of 50 to 150 nm. In some embodiments, an EV has a diameter within a range of 100 to 1000 nm. In some embodiments, an EV has a diameter within a range of 100 to 750 nm. In some embodiments, an EV has a diameter within a range of 100 to 500 nm. In some embodiments, an EV has a diameter within a range of 100 to 300 nm. In some embodiments, an EV has a

diameter within a range of 100 to 200 nm. In some embodiments, an EV has a diameter of at least 100 nm. In some embodiments, an EV has a diameter of at most 300 nm.

[0450] A population of EVs (*e.g.*, as present in a composition, pharmaceutical composition, medicament, preparation or otherwise) will comprise EVs with a range of diameters. In some embodiments, the median diameter of EVs within a population is 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, or 300 nm (± 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nm). In some embodiments, the mean diameter of EVs within a population is 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, or 300 nm (± 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nm).

[0451] A population of EVs may comprise at least 10, 100, 1000, 104, 105, 106, 107, 108, 109, 1010, 1011, 1012, 1013 or 1014 EVs. A population of EVs may comprise at least 10, 100, 1000, 104, 105, 106, 107, 108, 109, 1010, 1011, 1012, 1013 or 1014 EVs per mL of carrier.

B. Production

[0452] In some embodiments, an extracellular vesicle is derived from a cell into the extracellular environment. An extracellular vesicle may be derived from various cell types under both pathological and physiological conditions. In some embodiments, EVs have a similar composition to the cell from which it is derived from. In some embodiments, an EV is produced from outward budding and fission of cellular membrane. An EV may be produced via a natural process or a chemically-induced or enhance process.

[0453] In some embodiments, EVs are derived from cells that are contacted with a vesicle-inducing agent. A vesicle-inducing agent may be calcium ionophore, lysophosphatidic acid (LPA), or phorbol-12-myristat-13-acetate (PMA).

[0454] In some embodiments, EVs are derived from human cells, or cells of human origin. In some embodiments, EVs are derived from cells that are not modified (*e.g.*, transduced, transfected, infected, or otherwise modified). In some embodiments, EVs are derived from cells that are *ex vivo*.

[0455] In some embodiments, EVS are derived from hematopoietic cells. In some embodiments, EVs are derived from immune cells. For example, EVs may be derived from red blood cells, white blood cells, cancer cells, stem cells, dendritic cells, macrophages, or other cell types.

3. Liposomes

[0456] In some embodiments, the present disclosure provides for compositions, preparations, and/or delivery particles that comprise liposomes.

[0457] The present disclosure contemplates liposomes capable of attaching and releasing nucleic acids, nucleic acid conjugates, polypeptides, and/or fusion proteins as described herein. Liposomes are microscopic spherical lipid bilayers surrounding an aqueous core that are made from amphiphilic molecules such as phospholipids. For example, a liposome may trap a nucleic acid between the hydrophobic tails of the phospholipid micelle. In some embodiments, water soluble agents can be entrapped in the core and lipid-soluble agents can be dissolved in the shell-like bilayer.

[0458] Those skilled in the art will appreciate that, in some embodiments liposomes allow water soluble and water insoluble chemicals to be used together in a medium without the use of surfactants or other emulsifiers. In some embodiments, liposomes can form spontaneously. For example, liposomes can form by forcefully mixing phospholipids in aqueous media. In some embodiments, liposomes can be formed, for example, when water oluble compounds are dissolved in an aqueous solution capable of hydrating phospholipids. Upon formation of the liposomes, these water soluble compounds are trapped within the aqueous liposomal center. The liposome wall, being a phospholipid membrane, holds fat soluble materials such as oils.

[0459] In some embodiments, liposomes provide controlled release of incorporated compounds. In some embodiments, liposomes do not provide controlled release of incorporated compounds. In addition, in some embodiments, liposomes can be coated with water soluble polymers such as polyethylene glycol to increase the pharmacokinetic half-life. Also contemplated is an ultra high-shear technology to refine liposome production, resulting in stable, unilamellar (single layer) liposomes having specifically designed structural characteristics. These

unique properties of liposomes allow the simultaneous storage of normally immiscible compounds and the capability of their controlled release.

[0460] The present disclosure contemplates cationic and anionic liposomes, as well as liposomes having neutral lipids. Preferably, cationic liposomes comprise negatively-charged materials by mixing the materials and fatty acid liposomal components and allowing them to charge-associate. Clearly, the choice of a cationic or anionic liposome depends upon the desired pH of the final liposome mixture. Examples of cationic liposomes include lipofectin, lipofectamine, and lipofectace.

[0461] Also contemplated is a delivery particle comprising liposomes that provides controlled release of at least one cargo as described herein. Preferably, liposomes that are capable of controlled release: i) are biodegradable and non-toxic; ii) carry both water and oil soluble compounds; iii) solubilize recalcitrant compounds; iv) prevent compound oxidation; v) promote protein stabilization; vi) control hydration; vii) control compound release by variations in bilayer composition such as, but not limited to, fatty acid chain length, fatty acid lipid composition, relative amounts of saturated and unsaturated fatty acids, and physical configuration; viii) have solvent dependency; iv) have pH-dependency and v) have temperature dependency.

[0462] Compositions of liposomes are broadly categorized into two classifications. Conventional liposomes are generally mixtures of stabilized natural lecithin (PC) that may comprise synthetic identical-chain phospholipids that may or may not contain glycolipids. Special liposomes may comprise: i) bipolar fatty acids; ii) the ability to attach antibodies for tissue-targeted therapies; iii) coated with materials such as, but not limited to lipoprotein and carbohydrate; iv) multiple encapsulation and v) emulsion compatibility.

[0463] Liposomes may be easily made in the laboratory by methods such as, but not limited to, sonication and vibration. Alternatively, compound-delivery liposomes are commercially available. For example, Collaborative Laboratories, Inc. are known to manufacture custom designed liposomes for specific delivery requirements.

iii. Carbohydrate-based Delivery Particles

[0464] The present disclosure provides for compositions, preparations, and/or delivery particles that comprise carbohydrate-based delivery particles (*e.g.*, polysaccharide-based delivery particles).

[0465] A variety of carbohydrate-based delivery particles are known in the art. Non-limiting examples of carbohydrate-based delivery particles comprise, but are not limited to, chitosan, hyaluronic acid, dextran, arabinogalactan, starch, cyclodextrin, cycloamylose, pullulan, inulin, cellulose, hemicellulose, alginic acid, chondroitin sulfate, heparin, and gums (*e.g.*, natural polysaccharide gums (*e.g.*, Guar gum, xanthum gum, gum Arabic, carrageenan gum, pectin, etc.)) (see, *e.g.*, Barclay, T. G., et al, Carbohydrate Polymer, 2019 Oct 1; 221;94-112, which is incorporated in its entirety herein by reference).

[0466] As may be appreciated by a skilled person, carbohydrate-based delivery particles are advantageous for cargo delivery as they have relatively high cargo loading. In some embodiments, a cargo may be released from a carbohydrate-based delivery particle by one or more external stimuli. In some embodiments, a cargo may be released from a carbohydrate-based delivery particle without one or more external stimuli.

[0467] In some embodiments, carbohydrate-based delivery particles are microspheres as described herein. In some embodiments, carbohydrate -based delivery particles are microparticles as described herein. In some embodiments, carbohydrate -based delivery particles are nanoparticles as described herein. In some embodiments, carbohydrate-based delivery particles are microcapsules as described herein. For example, microsphere, microparticle, nanoparticle, and microcapsule construction as described for polymer-based delivery particles may also be used to construct carbohydrate-based delivery particles as described herein.

[0468] Carbohydrate-based delivery particles may be constructed in a number of ways. In some embodiments, for example, a carbohydrate-based delivery particle may be constructed using polysaccharide chemistry. For example, a skilled person may appreciate that carbohydrate-based delivery particles as described herein may be constructed by exploiting functional groups in the polysaccharide. In some embodiments, a carbohydrate-based delivery particle as described herein may be constructed by exploiting a hydroxyl. In some embodiments, a carbohydrate-based delivery particle as described herein may be constructed by exploiting a carboxylic acid. In some

embodiments, a carbohydrate-based delivery particle as described herein may be constructed by exploiting an amino group. The chemical nature of these groups makes them useful in the formation of carbohydrate-based delivery particles and binding of cargos (*e.g.*, a cargo polypeptide, a therapeutic polypeptide, nucleic acids encoding a cargo polypeptide, nucleic acids encoding a therapeutic polypeptide, nucleic acids encoding a barcoded cargo polypeptide, nucleic acids encoding a barcode, nucleic acids encoding a binder, etc.). In some embodiments, formation of carbohydrate-based delivery particles and binding of cargos is or comprises non-covalent interactions. In some embodiments, formation of carbohydrate-based delivery particles and binding of cargos is or comprises covalent linking reactions.

[0469] In some embodiments, carbohydrate-based delivery particles may self-assemble. In some embodiments, carbohydrate-based delivery particles may not self-assemble. In some embodiments, carbohydrate-based delivery particles may self-assemble spontaneously. In some embodiments, carbohydrate-based delivery particles may not self-assemble spontaneously. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble to form, for example, hydrogels. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble to not form hydrogels. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble to form semicrystalline microparticles. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble to form crystalline microparticles. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble to form hydrophobically modified polysaccharides. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble by ionic crosslinking. In some embodiments, carbohydrate-based delivery particles as described herein, may self-assemble by ionic assembly.

[0470] In some embodiments, carbohydrate-based delivery particles may be constructed by chemical crosslinking. In some embodiments, carbohydrate-based delivery particles may be constructed using covalent crosslinks. In some embodiments, carbohydrate-based delivery particles may be constructed using non-covalent crosslinks. In some embodiments, carbohydrate-based delivery particles may be constructed using click chemistry crosslinking. In some embodiments, carbohydrate-based delivery particles may be constructed using disulphide bridges. In some embodiments, carbohydrate-based delivery particles may be constructed using

multifunctional reagents. In some embodiments, carbohydrate-based delivery particles may be constructed using alkenyl modification.

[0471] Sizes of carbohydrate-based delivery particles are generally tunable and may be engineered to be of different sizes. In some embodiments, carbohydrate-based delivery particles, as described herein, may be between 10 nm and 1000 nm in diameter. In some embodiments, carbohydrate-based delivery particles, as described herein, may be between 1 μm and 1000 μm.

iv. Polymer-Based Delivery Particles

[0472] The present disclosure provides for compositions, preparations, and/or delivery particles that comprise polymer-based delivery particles.

[0473] For example, in some embodiments, delivery particles described herein comprise poly(lactide-co-glycolide), aliphatic polyesters including, but not limited to, poly-glycolic acid and poly-lactic acid, hyaluronic acid, polyurethanes, polyacrylic acids, pseudo-poly(amino acids), polyhydroxybutyrate-related copolymers, polyanhydrides, polymethylmethacrylate, poly(ethylene oxide), lecithin and phospholipids.

[0474] In some embodiments, polymer-based delivery particles are microspheres as described herein. In some embodiments, polymer-based delivery particles are microparticles as described herein. In some embodiments, polymer-based delivery particles are microcapsules as described herein.

[0475] Microspheres and microcapsules are useful due to their ability to maintain a generally uniform distribution, provide stable controlled compound release and are economical to produce and dispense. Preferably, an associated delivery gel or the compound-impregnated gel is clear or, alternatively, said gel is colored for easy visualization by medical personnel.

[0476] Microspheres are obtainable commercially (ProLease™, Alkermes: Cambridge, Mass.). For example, a freeze-dried medium comprising at least one therapeutic agent is homogenized in a suitable solvent and sprayed to manufacture microspheres in the range of 20 to 90 μm. Techniques are then followed that maintain sustained-release integrity during phases of purification, encapsulation, and storage. Scott et al., Improving Protein Therapeutics With Sustained Release Formulations, Nature Biotechnology, Volume 16:153-157 (1998).

Modification of the microsphere composition by the use of biodegradable polymers can provide an ability to control the rate of nucleic acid release. Miller et al., Degradation Rates of Oral Resorbable Implants, Polylactates and Polyglycolates: Rate Modification and Changes in PLA/PGA Copolymer Ratios, J. Biomed. Mater. Res., Vol. 11:711-719 (1977).

[0477] Alternatively, a sustained- or controlled-release microsphere preparation is prepared using an in-water drying method, where an organic solvent solution of a biodegradable polymer metal salt is first prepared. Subsequently, a dissolved or dispersed medium of nucleic acid(s) is added to the biodegradable polymer metal salt solution. The weight ratio of nucleic acid(s) to the biodegradable polymer metal salt may for example be about 1:100000 to about 1:1, preferably about 1:20000 to about 1:500 and more preferably about 1:10000 to about 1:500. Next, the organic solvent solution containing the biodegradable polymer metal salt and nucleic acid(s) is poured into an aqueous phase to prepare an oil/water emulsion. The solvent in the oil phase is then evaporated off to provide microspheres. Finally, these microspheres are then recovered, washed and lyophilized. Thereafter, the microspheres may be heated under reduced pressure to remove the residual water and organic solvent.

[0478] Other methods useful in producing microspheres that are compatible with a biodegradable polymer metal salt and nucleic acid mixture are: i) phase separation during a gradual addition of a coacervating agent; ii) an in-water drying method or phase separation method, where an antiflocculant is added to prevent particle agglomeration and iii) by a spray-drying method.

[0479] Also contemplated in the disclosure is a medium comprising a microsphere or microcapsule capable of delivering controlled release of a nucleic acid for a duration of approximately between 1 day and 6 months. The microsphere or microparticle may be colored to allow the user the ability to see the medium clearly as it is dispensed. The microsphere or microcapsule may be clear. The microsphere or microparticle may be impregnated with a radio-opaque fluoroscopic dye.

[0480] Controlled-release microcapsules may be produced by using known encapsulation techniques such as centrifugal extrusion, pan coating and air suspension. Such microspheres and/or microcapsules can be engineered to achieve desired release rates. For example, Oliosphere™ (Macromed) is a controlled-release microsphere system. These particular

microspheres are available in uniform sizes ranging between 5-500 μm and composed of biocompatible and biodegradable polymers. Specific polymer compositions of a microsphere can control the nucleic acid release rate such that custom-designed microspheres are possible, including effective management of the burst effect. ProMaxx™ (Epic Therapeutics, Inc.) is a protein-matrix delivery system. The system is aqueous in nature and is adaptable to standard pharmaceutical delivery models. In particular, ProMaxx™ are bioerodible protein microspheres that deliver both small and macromolecular drugs, and may be customized regarding both microsphere size and desired release characteristics.

[0481] A microsphere or microparticle may comprise a pH sensitive encapsulation material that is stable at a pH less than the pH of the internal mesentery. The typical range in the internal mesentery is pH 7.6 to pH 7.2. Consequently, the microcapsules should be maintained at a pH of less than 7. However, if pH variability is expected, the pH sensitive material can be selected based on the different pH criteria needed for the dissolution of the microcapsules. The encapsulated nucleic acid, therefore, will be selected for the pH environment in which dissolution is desired and stored in a pH preselected to maintain stability. Examples of pH sensitive material useful as encapsulants are Eudragit™ L-100 or S-100 (Rohm GMBH), hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate phthalate, and cellulose acetate trimellitate. Lipids may comprise the inner coating of the microcapsules. In these compositions, these lipids may be, but are not limited to, partial esters of fatty acids and hexitiol anhydrides, and edible fats such as triglycerides. Lew C. W., Controlled-Release pH Sensitive Capsule And Adhesive System And Method. U.S. Pat. No. 5,364,634 (herein incorporated by reference).

[0482] The present invention contemplates a microparticle comprising a gelatin, or other polymeric cation having a similar charge density to gelatin (*i.e.*, poly-L-lysine) and is used as a complex to form a primary microparticle. A primary microparticle is produced as a mixture of the following composition: i) Gelatin (60 bloom, type A from porcine skin), ii) chondroitin 4-sulfate (0.005%-0.1%), iii) glutaraldehyde (25%, grade 1), and iv) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC hydrochloride), and ultra-pure sucrose (Sigma Chemical Co., St. Louis, Mo.). The source of gelatin is not thought to be critical; it can be from bovine, porcine, human, or other animal source. Typically, the polymeric cation is

between 19,000-30,000 daltons. Chondroitin sulfate is then added to the complex with sodium sulfate, or ethanol as a coacervation agent.

[0483] Following the formation of a microparticle, a nucleic acid is directly bound to the surface of the microparticle or is indirectly attached via a "bridge" or "spacer". The amino groups of the gelatin lysine groups are easily derivatized to provide sites for direct coupling of a compound. Alternatively, spacers (*i.e.,* linking molecules and derivatizing moieties on targeting ligands) such as avidin-biotin are also useful to indirectly couple targeting ligands to the microparticles. Stability of the microparticle is controlled by the amount of glutaraldehyde-spacer crosslinking induced by the EDC hydrochloride. A controlled-release medium is also empirically determined by the final density of glutaraldehyde-spacer crosslinks.

[0484] The present invention contemplates microparticles formed by spray-drying a composition comprising fibrinogen or thrombin with a nucleic acid. Preferably, these microparticles are soluble and the selected protein (*i.e.,* fibrinogen or thrombin) creates the walls of the microparticles. Consequently, the nucleic acids are incorporated within, and between, the protein walls of the microparticle. Heath et al., Microparticles And Their Use In Wound Therapy. U.S. Pat. No. 6,113,948 (herein incorporated by reference). Following the application of the microparticles to living tissue, the subsequent reaction between the fibrinogen and thrombin creates a tissue sealant, thereby releasing the incorporated compound into the immediate surrounding area.

[0485] One having skill in the art will understand that the shape of the microspheres need not be exactly spherical, only to be small particles capable of being administered by an appropriate route to the site or subject of interest. Microparticles may be comprised of a biocompatible and/or biodegradable material selected from the group consisting of polylactide, polyglycolide and copolymers of lactide/glycolide (PLGA), hyaluronic acid, modified polysaccharides and any other well-known material.

v. Other Delivery Particles

[0486] In some embodiments, delivery particles, as contemplated herein. may be any delivery particle known in the art. For example, in some embodiments, delivery particles may be beads, metal-based (*e.g.*, Au, Si, Zn, Fe) particles, micelles, etc.

vi. Production Nucleic Acids and Cells

[0487] Among other things, the present disclosure provides for production nucleic acids, *e.g.*, from which a viral delivery particle as described herein is produced. Moreover, among other things, the present disclosure provides for production cells that contain and/or produce nucleic acids used to produce a viral delivery particle as described herein.

1. Production Nucleic Acids and Cells for Viral Delivery Particles

[0488] For example, in some embodiments, a viral delivery particle is an AAV delivery particle. AAV delivery particles, and related AAV systems and AAV constructs are generally well known in the art (see, *e.g.*, Kelleher and Vos, Biotechniques, 17(6):1110-17 (1994); Cotten et al., P.N.A.S. U.S.A., 89(13):6094-98 (1992); Curiel, Nat Immun, 13(2-3):141-64 (1994); Muzyczka, Curr Top Microbiol Immunol, 158:97-129 (1992); and Asokan A, et al., Mol. Ther., 20(4):699-708 (2012), each of which is incorporated in its entirety herein by reference). Methods for generating and using AAV constructs are described, for example, in U.S. Pat. Nos. 5,139,941, 4,797,368 and PCT filing application US2019/060328, each of which is incorporated in its entirety herein by reference.

[0489] Methods for obtaining viral constructs are known in the art. For example, to produce AAV constructs, the methods typically involve culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein or fragment thereof; a functional rep gene; a recombinant AAV construct composed of AAV inverted terminal repeats (ITRs) and a coding sequence; and/or sufficient helper functions to permit packaging of the recombinant AAV construct into the AAV capsid proteins.

[0490] In some embodiments, components to be cultured in a host cell to package an AAV construct in an AAV capsid may be provided to the host cell in trans. Alternatively, any one or

more components (*e.g.*, recombinant AAV construct, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell that has been engineered to contain one or more such components using methods known to those of skill in the art. In some embodiments, such a stable host cell contains such component(s) under the control of an inducible promoter. In some embodiments, such component(s) may be under the control of a constitutive promoter. In some embodiments, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated that is derived from HEK293 cells (which contain E1 helper functions under the control of a constitutive promoter), but that contain the rep and/or cap proteins under the control of inducible promoters. Other stable host cells may be generated by one of skill in the art using routine methods.

[0491] Recombinant AAV construct, rep sequences, cap sequences, and helper functions required for producing an AAV of the disclosure may be delivered to a packaging host cell using any appropriate genetic element (*e.g.*, construct). A selected genetic element may be delivered by any suitable method known in the art, *e.g.*, to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques (see, *e.g.*, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., which is incorporated in its entirety herein by reference). Similarly, methods of generating AAV particles are well known and any suitable method can be used with the present disclosure (see, *e.g.*, K. Fisher et al, J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745, which are incorporated in their entirety herein by reference).

[0492] In some embodiments, recombinant AAVs may be produced using a triple transfection method (*e.g.*, as described in U.S. Pat. No. 6,001,650, which is incorporated in its entirety herein by reference). In some embodiments, recombinant AAVs are produced by transfecting a host cell with a recombinant AAV construct (comprising a coding sequence) to be packaged into AAV particles, an AAV helper function construct, and an accessory function construct. An AAV helper function construct encodes "AAV helper function" sequences (*i.e.,* rep and cap), which function in trans for productive AAV replication and encapsidation. In some embodiments, the AAV helper function construct supports efficient AAV construct production without generating any detectable wild type AAV particles (*i.e.,* AAV particles containing functional rep and cap genes). Non-limiting examples of constructs suitable for use with the present disclosure include pHLP19 (see,

e.g., U.S. Pat. No. 6,001,650, which is incorporated in its entirety herein by reference) and pRep6cap6 construct (see, *e.g.*, U.S. Pat. No. 6,156,303, which is incorporated in its entirety herein by reference). An accessory function construct encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). Accessory functions may include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based accessory functions can be derived from any known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus.

[0493] Additional methods for generating and isolating AAV viral constructs suitable for delivery to a subject are described in, *e.g.*, U.S. Pat. No. 7,790,449; U.S. Pat. No. 7,282,199; WO 2003/042397; WO 2005/033321, WO 2006/110689; and U.S. Pat. No. 7,588,772, each of which is incorporated in its entirety herein by reference. In one system, a producer cell line is transiently transfected with a construct that encodes a coding sequence flanked by ITRs and a construct(s) that encodes rep and cap. In another system, a packaging cell line that stably supplies rep and cap is transiently transfected with a construct encoding a coding sequence flanked by ITRs. In each of these systems, AAV particles are produced in response to infection with helper adenovirus or herpesvirus, and AAVs are separated from contaminating virus. Other systems do not require infection with helper virus to recover the AAV--the helper functions (*i.e.*, adenovirus E1, E2a, VA, and E4 or herpesvirus UL5, UL8, UL52, and UL29, and herpesvirus polymerase) are also supplied, in trans, by the system. In such systems, helper functions can be supplied by transient transfection of the cells with constructs that encode the helper functions, or the cells can be engineered to stably contain genes encoding the helper functions, the expression of which can be controlled at the transcriptional or posttranscriptional level.

[0494] Other types of viral delivery particles can be produced in accordance with embodiments described herein.

2. *Production Nucleic Acids and Cells for Extracellular Vesicle Delivery Particles*

[0495] Among other things, the present disclosure provides for production of nucleic acids, *e.g.*, from which an extracellular vesicle delivery particle (EV) as described herein is produced. Moreover, among other things, the present disclosure provides for production cells that contain and/or produce nucleic acids used to produce an extracellular vesicle delivery particle as described herein.

[0496] EVs, *e.g.*, exosomes, of the present disclosure can be produced from a cell grown in vitro or a body fluid of a subject. When exosomes are produced from in vitro cell culture, various producer cells, *e.g.*, HEK293 cells, CHO cells, C2C12, and MSCs, can be used. In some aspects, producer cells can be selected from HEK293 cells, HEK293S cells, HEK293SF cells, Chinese Hamster Ovary (CHO) cells, mesenchymal stem cells (MSCs), BJ human foreskin fibroblast cells, fHDF fibroblast cells, AGE.HN® neuronal precursor cells, CAP® amniocyte cells, adipose mesenchymal stem cells, RPTEC/TERT1 cells, dendritic cells, macrophages, B cells, mast cells, neutrophils, Kupffer-Browicz cells, PER.C6 cells, Induced pluripotent stem cells (iPSCs), or C2C12 cells. In some aspects, the producer cells are stem cells.

[0497] In some embodiments, producer cells can be genetically and/or pharmacologically modified to reduce gene and/or protein function in a cholesterol biosynthetic pathway, as described herein. In some aspects, modified producer cells can be further modified (*e.g.*, genetically) to comprise exogenous sequences encoding a protein to produce EVs described herein. Genetically-modified producer cells can contain the exogenous sequence by transient or stable transformation. Nucleic acids encoding cargo polypeptides can be transformed as a plasmid. Nucleic acids encoding cargo polypeptides can be stably integrated into a genomic sequence of a producer cell, at a targeted site or in a random site. In some embodiments, a stable cell line is generated for production of lumen-engineered exosomes. In some embodiments modified producer cells comprise a recruiting polypeptide. In some embodiments, a recruiting polypeptide comprises a nucleic acid binding moiety. In some embodiments, a recruiting polypeptide comprises a membrane-associating moiety. In some embodiments, a recruiting polypeptide comprises a nucleic acid binding moiety and a membrane-associating moiety.

[0498] Nucleic acids encoding cargo polypeptides can be inserted into a genomic sequence of a producer cell, located within, upstream (5 '-end) or downstream (3 '-end) of an endogenous

sequence encoding an exosome protein. Various methods known in the art can be used for the introduction of nucleic acids encoding cargo polypeptides into a producer cell. For example, cells modified using various gene editing methods (*e.g.*, methods using a homologous recombination, transposon-mediated system, loxP-Cre system, CRISPR/Cas9 or TALEN) are within the scope of the present disclosure.

[0499] In some embodiments, the present disclosure provides a cell composition comprising modified cells that produce EVs, wherein modified cells have a reduced gene and/or protein function in a cholesterol biosynthetic pathway. In some embodiments, reduced gene and/or protein comprises protein in a cholesterol biosynthesis comprises one or more genes selected from 3 -hydroxy-3 -methylglutaryl-coenzyme A reductase (HMGCR), sterol regulatory element-binding protein 2 (SREBF2), Squalene epoxidase (SQLE), or 7-Dehydrocholesterol reductase (DHCR7) or a protein encoded by the gene.

[0500] In some embodiments, the present disclosure provides producer cells for use in the methods described herein. In some aspects, producer cells are prepared according to the methods described herein.

[0501] Also disclosed are methods for delivering a nucleic acid as described herein to a cell (*e.g.*, a target cell). Methods may include contacting a cell with exosomes disclosed herein. Targeted exosomes may comprise a ligand at the N-terminus of a fusion protein that targets the exosomes to target cells. As such, a ligand may be present on the surface of the exosome where the ligand binds specifically to a receptor on the surface of the target cells. Cargo components may include a candidate therapeutic nucleic acid or a therapeutic nucleic acid for treating a disease or disorder when a cargo component is delivered to the target cells (*e.g.*, a hybrid RNA comprising a miRNA, shRNA, mRNA, ncRNA, or any combination of any of these RNAs fused to the RNA-motif that binds to the RNA-binding domain of the fusion protein). Exosomes may be formulated as a pharmaceutical composition for treating the disease or disorder.

VII. Systems

i. Expression Systems

[0502] Numerous expression systems exist that comprise at least a part or all of the compositions discussed herein. Prokaryote- and/or eukaryote-based systems can be employed to produce nucleic acid sequences, or their cognate cargo polypeptides, proteins and peptides. For example, component components, barcode components, and related cargo polypeptides and peptide barcodes may utilize an expression system, such as an inducible or constitutive expression system. Many such systems are commercially and widely available.

[0503] For example, an insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents 5,871,986, 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

[0504] In addition to the disclosed expression systems, other examples of expression systems include STRATAGENE®'s COMPLETE CONTROL Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an E. coli expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

ii. Cells, Tissues, Organs, Organoids, Organisms

[0505] Among other things, the present disclosure provides for expression of cargo polypeptides in cells (*e.g.*, eukaryotic cells, *e.g.*, prokaryotic cells), tissues, organs, organoids, or organisms. In some embodiments, cells, tissues, organs, organoids, or organisms are mammalian

cells, tissues, organs, organoids, or organisms. Nucleic acids, or cargo polypeptides encoded by nucleic acids, described herein may be comprised within a cell, tissue, organ, organoid, or organism. Nucleic acids may be in a eukaryotic cell, such as a mammalian cell or a plant cell. Mammalian cells, tissues, organs, organoids, or organisms may be of human, non-human primate, bovine, porcine, rodent or mouse origin. Cells, tissues, organs, organoids, or organisms may be of non-mammalian origin, such as poultry, fish, or shrimp.

[0506] A mammalian cell, tissue, organ, organoid, or organism may be of human or non-human mammalian origin, *e.g.*, primate, bovine, ovine, porcine, canine, rodent, Leporidae such as monkey, cow, sheep, pig, dog, rabbit, rat or mouse. A cell, tissue, organ, organoid, or organism may be of non-mammalian origin such as poultry bird (*e.g.*, chicken), vertebrate fish (*e.g.*, salmon) or shellfish (*e.g.*, oyster, clam, lobster, shrimp). A cell, tissue, organ, organoid, or organism may be or comprise a tumor cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a benign cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a kidney cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise an adipose cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a brain cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a liver cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be a blood cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a skin cell, tissue, organ, organoid, or organism. A cell, tissue, organ, or organoid may be or comprise a muscle cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a cardiac cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be an ocular cell, tissue, organ, or organoid. A cell, tissue, organ, or organoid may be or comprise a nerve cell, tissue, organ, organoid, or organism. A cell, tissue, organ, or organoid may be a parenchyma cell, tissue, organ, or organoid. Populations of cells may be or comprise one or more cells described herein, or any other cell type present in the tissue, organ, organoid or organism of interest.

[0507] As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of

replicating a vector or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors or viruses. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid, such as a recombinant protein-encoding sequence, is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

[0508] Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above-described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

VII. Use

i. Assessing Cargos

[0509] Technologies described herein may be used to detect, assess, and/or characterize cargos (*e.g.,* cargo polypeptides (*e.g.*, variant cargo polypeptides); cargo nucleic acids (*e.g.*, variant cargo nucleic acids)). In some embodiments, provided technologies may be used, for example, to assay cargos in complex environments (*e.g.,* serum, blood, tissue, etc.). In some embodiments, cargos may be nucleic acids encoding cargo polypeptides. In some embodiments, cargos may be nucleic acids encoding candidate therapeutic polypeptides or therapeutic polypeptides.

[0510] As described herein, a cargo may be associated with a barcode (*i.e.,* a barcoded cargo). In some embodiments, a barcoded cargo may be assayed using binding agents (*e.g.,* phages with binders expressed on them) using methods as described herein. In some embodiments, a barcoded cargo may be captured (*e.g.,* using affinity reagents) on a surface (*e.g.,* beads or plates). In some embodiments, a barcoded cargo may be immobilized for barcode assaying. In some embodiments, a barcoded cargo is contacted with one or more binders and subject to decoding as described herein.

[0511] In some embodiments, cargos may be detected, assessed, and/or characterized *in vitro*. In some embodiments, cargos may be detected, assessed, and/or characterized *in vivo*.

[0512] In some embodiments, cargos that were previously detected, assessed, and/or characterized may be pooled together and subject to further detection, assessment, and/or characterization, for example, using methods as described herein. In some embodiments, such further detection, assessment, and/or characterization may be performed *in vitro*. In some embodiments, such further detection, assessment, and/or characterization may be performed *in vivo*.

[0513] In some embodiments, cargos that were previously detected, assessed, and/or characterized may be subject to mutagenesis (*e.g.*, random mutagenesis, targeted mutagenesis, or using machine learning (*e.g.*, pre-trainer large language model)). In some embodiments, for example, such mutated cargos (*e.g.*, variant cargos (*e.g.*, variant cargo nucleic acids, variant cargo polypeptides) may be pooled together and subject to further detection, assessment, and/or characterization, for example, using methods as described herein. **FIG. 23** depicts a schematic of such an exemplary method. **FIG. 23** shows that variants of a previously detected, assessed, and/or characterized cargo may be generated and subject to further detection, assessment, and/or characterization, for example, using methods as described herein. In some embodiments, such iterative methods, for example, could result in a variant cargo that for example shows improved or enhanced functionality (*e.g.*, improved developability, expression, affinity, etc.). In some embodiments, such further detection, assessment, and/or characterization may be performed *in vitro*. In some embodiments, such further detection, assessment, and/or characterization may be performed *in vivo*.

[0514] In some embodiments, cargos may be disposed within one or more delivery particles as described herein.

[0515] For example, **FIG. 17** depicts a schematic of an exemplary method that provides for high throughput cargo delivery, production, screening, identification, and/or characterization as described herein. In some embodiments, nucleic acids comprising (1) a cargo component whose nucleotide sequence is or comprises a sequence encoding a cargo polypeptide and (2) a barcode component whose nucleotide sequence is or comprises a sequence encoding a peptide barcode are disposed within one or more delivery particles and are administered to an animal

(*e.g.*, a mammal). In some embodiments, functional cargos are expressed in a tissue of interest, and decoding methods are used to determine cargos and/or delivery particles with desired properties.

[0516] In some embodiments, methods described herein determine simultaneous *in vivo* assessment of phenotypes of cargos in multiple tissues. In some embodiments, a phenotype includes biodistribution information related to cargos. In some embodiments, a phenotype includes pharmacokinetic (clearance) information of cargos. In some embodiments, a phenotype includes half-life information of cargos. In some embodiments, a phenotype includes tissue-mediated drug disposition (TMDD) of cargos. In some embodiments, a phenotype includes properties related to *in vivo* stability of cargos. In some embodiments, multiple phenotypes of cargos may be determined simultaneously.

[0517] In some embodiments, methods described herein determine simultaneous *in vivo* tracking and/or assessment and/or quantification of different cargos. In some embodiments, methods described herein determine simultaneous *in vivo* tracking and/or assessment and/or quantification of different types of delivery particles.

[0518] For example, **FIG. 20** depicts a schematic showing that high-throughput screening using the delivery and assessment platform as described herein provides for screening of multiple cargos, formats, targets, and tissues simultaneously in different models (*e.g.*, mouse, NHP, etc.).

ii. Compositions

[0519] Among other things, the present disclosure provides compositions. In some embodiments, a composition comprises a nucleic acid as described herein. In some embodiments, a composition comprises one or more nucleic acids as described herein. In some embodiments, a composition comprises a plurality of nucleic acids as described herein. In some embodiments, when more than one construct is included in the composition, the nucleic acids are each different.

[0520] In some embodiments, a composition comprises a delivery particle as described herein. In some embodiments, a composition comprises one or more delivery particles as

described herein. In some embodiments, a composition comprises a plurality of delivery particles. In some embodiments, when more than one delivery particle is included in the composition, the delivery particles are each different.

[0521] In some embodiments, a composition comprises an AAV delivery particle as described herein. In some embodiments, a composition comprises one or more AAV delivery particles as described herein. In some embodiments, a composition comprises a plurality of AAV delivery particles. In come embodiments, when more than one AAV delivery particle is included in the composition, the AAV delivery particles are each different.

[0522] In some embodiments, a composition comprises a barcode as described herein. In some embodiments, a composition comprises one or more barcodes as described herein. In some embodiments, a composition comprises a plurality of barcodes as described herein. In some embodiments, when more than one barcode is included in the composition, the barcodes are each different.

[0523] In some embodiments, a composition comprises a binder as described herein. In some embodiments, a composition comprises one or more binders as described herein. In some embodiments, a composition comprises a plurality of binders as described herein. In some embodiments, when more than one binder is included in the composition, the binders are each different.

[0524] In some embodiments, a composition comprises a binding agent as described herein. In some embodiments, a composition comprises one or more binding agents as described herein. In some embodiments, a composition comprises a plurality of binding agents as described herein. In some embodiments, when more than one binding agent is included in the composition, the binding agents are each different.

[0525] In some embodiments, a composition comprises a cargo polypeptide as described herein. In some embodiments, a composition comprises one or more cargo polypeptides as described herein. In some embodiments, a composition comprises a plurality of cargo polypeptides as described herein. In some embodiments, when more than one cargo polypeptide is included in the composition, the cargo polypeptides are each different.

[0526] In some embodiments, a composition comprises a candidate therapeutic polypeptide or therapeutic polypeptide as described herein. In some embodiments, a composition comprises one or more candidate therapeutic polypeptides or therapeutic polypeptides as described herein. In some embodiments, a composition comprises a plurality of candidate therapeutic polypeptides or therapeutic polypeptides as described herein. In some embodiments, when more than one candidate therapeutic polypeptides or therapeutic polypeptides is included in the composition, the candidate therapeutic polypeptides or therapeutic polypeptides are each different.

[0527] In some embodiments, a composition is or comprises a pharmaceutical composition.

iii. Kits

[0528] Technologies as described herein may be provided in the form of a composition. For example, in some embodiments, a composition may comprise one or more elements (*e.g.*, nucleic acid, amino acid, etc.) to produce or generate one or more barcodes and/or binders as described herein. In some embodiments, a composition may comprise one or more elements to produce or generate a set of barcodes. In some embodiments, a composition may comprise one or more elements to produce or generate a set of binders. In some embodiments, a composition may comprise one or more elements to produce or generate a pool of barcode-binder pairs. In some embodiments, a composition may comprise one or more elements to produce or generate binding agents (*e.g.*, phage expressing binders). In some embodiments, a composition may be a barcode composition. In some embodiments, a composition may be a binder composition. In some embodiments, a composition may be a barcode-binder composition. In some embodiments, a composition may be a binding agent composition. In some embodiments, a composition may comprise one or more of barcodes, binders, binding agents, and/or components thereof. In some embodiments, a composition may comprise one or more sets/pools of barcodes, binders, binding agents, and/or components thereof.

[0529] Provided herein are compositions comprising nucleic acids, delivery particles, barcodes, binders, binding agents, polypeptides (*e.g.*, cargo polypeptides, therapeutic polypeptides, etc.), or components thereof. In some embodiments, a composition comprises nucleic acids, delivery particles, barcodes, binders, binding agents, polypeptides (*e.g.*, cargo polypeptides, therapeutic polypeptides, etc.), components thereof and/or combinations thereof,

which have been assessed, identified, characterized or assayed using methods as described herein. In some embodiments, a composition provided herein comprises one or more, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more nucleic acids, delivery particles, barcodes, binders, binding agents, polypeptides (*e.g.*, cargo polypeptides, therapeutic polypeptides, etc.), components thereof and/or combinations thereof, which have been assessed, identified, characterized or assayed using methods as described herein.

[0530] In some embodiments, a composition provided herein comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more nucleic acid sequences or amino acid sequences as listed in sequence listing filed herewith.

[0531] A composition as described herein may be formulated in various forms. For example, in some embodiments, a composition as described herein may be formulated in a powder form (*e.g.,* lyophilized). In some embodiments, a composition as described herein may be formulated in a liquid form.

[0532] In some embodiments, compositions for use in accordance with the present disclosure are pharmaceutical compositions, *e.g.,* for administration (*e.g.,* topical, oral, subcutaneous, intravenous, intramuscular, intracerebral, intrathecal, rectal (*e.g.,* rectal intubation), opthalmical, intravitreal, or suprachoroidal administration) to a subject (*e.g.,* a mammal (*e.g.,* a human)). In some embodiments, such compositions are administered to a subject to detect, characterize, and/or assess one or more attributes of one or more cargos administered or to be administered to the subject. Pharmaceutical compositions typically include an agent to be administered (*e.g.,* nucleic acids, delivery particles, polypeptides (*e.g.*, cargo polypeptides, therapeutic polypeptides, etc.), and/or components thereof), and a pharmaceutically acceptable carrier. Certain exemplary pharmaceutically acceptable carriers include, for instance saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Pharmaceutical compositions are typically formulated to be compatible with its intended route of administration. Examples of routes of administration include topical, oral, subcutaneous,

intravenous, intramuscular, intracerebral, intrathecal, rectal, (*e.g.*, rectal intubation), opthalmical, intravitreal, or suprachoroidal administration.

[0533] In some embodiments, pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of a pharmaceutical composition. In some particular embodiments, a pharmaceutical composition can contain, *e.g.*, any one or more of the following inactive ingredients, or compounds of a similar nature: a binder, an excipient, a lubricant, a glidant, or some similar such compound.

[0534] Compositions can be included in a kit, container, pack, or dispenser, together with instructions for administration (*e.g.*, to a subject) or for use in a method described herein. In some embodiments, instructions may include methods to reconstitute a powder form composition to a liquid form composition for further use. In some embodiments, a kit may include instructions that allows a user to generate new set of binders for a new set of barcodes. In some embodiments, a kit comprises a set of instructions to perform sequencing of one or more phage particles bound to one or more barcodes.

[0535] In some embodiments, a kit comprises information designating peptide barcodes for each binder. In some embodiments, a kit comprises a computer readable program for decoding sequencing data.

[0536] In some embodiments, a kit comprises reagents to express a binder on a phage particle. In some embodiments, a kit comprises nucleic acids that encode one or more barcodes. In some embodiments, a kit comprises nucleic acids that encode one or more binders.

[0537] Those skilled in the art, reading the present disclosure, will appreciate that, in some embodiments, a composition (*e.g.*, a nucleic acid composition, a delivery particle composition, a binder composition, a barcode composition, a binding agent composition, a polypeptide composition (*e.g.*, a cargo polypeptide composition, a therapeutic polypeptide composition), etc.) as described herein may be or comprise one or more cells, tissues, or organisms (*e.g.*, plant or microbe cells, tissues, virus, or organisms) that produce (*e.g.*, have produced, and/or are producing) a relevant binder, barcode, binding agent, and/or polypeptide (*e.g.*, a cargo polypeptide, a therapeutic polypeptide) as described herein.

[0538] Those skilled in the art will appreciate that, in some embodiments, technologies for preparing compositions and/or preparations, and/or for preparing (and particularly for preparing pharmaceutical compositions) may include one or more steps of assessing or characterizing a compound, preparation, or composition, *e.g.*, as part of quality control. In some embodiments, if an assayed material does not meet pre-determined specifications for the relevant assessment, it is discarded. In some embodiments, if such assayed material does meet the pre-determined specifications, then it continues to be processed as described herein.

[0539] In some embodiments, a composition is tailored to a specific subject (*e.g.*, a specific mammal, *e.g.*, a patient). In some embodiments, a composition is specific for a cargo to be assessed for an individual subject (*e.g.*, mammal (*e.g.*, human, mouse, etc.)). In some embodiments, a composition is specific for cargos to be assessed for an individual subject (*e.g.*, mammal (*e.g.*, human, mouse)). In some embodiments, a composition is specific for cargos of a population of subjects (*e.g.*, mammals (*e.g.*, humans, mice, etc.)). Populations of subjects can include, but are not limited to: families, subjects in the same regional location (*e.g.*, neighborhood, city, state, or country), subjects with the same disease or condition, subjects of a particular age or age range, subjects that consume a particular diet (*e.g.*, food, food source, or caloric intake).

EXEMPLIFICATION

Example 1: Identification of barcodes, corresponding binding agents, and determining fingerprints

[0540] The present Example demonstrates methods for identifying barcodes, corresponding binding agents (*e.g.*, binders expressed on binding agents), and determining fingerprints (*e.g.*, barcode fingerprints) and using the information to determine the proportion of a barcode in a given mixture. The resulting materials can then be used to measure and quantify different cargos.

Design and synthesis of a barcode library:

[0541] Barcode sequences were designed which contain specific sequence motifs thought to fold into a given helical or loop structure. All sequences from the Protein Data Bank (PDB) were downloaded, along with their corresponding secondary structure predictions. Sequences were selected and subsetted from the full sequence if they met the criteria of: being a contiguous helix or loop sequence for a length of 8-25 amino acids. A random subset of 100,000 of the peptide sequences matching this criteria were then ordered as an oligo pool, containing constant overhangs and type IIS sites for cloning into a vector (see FIG. 1B).

Cloning barcode library into expression plasmid:

[0542] The designed pool of barcodes was cloned into a pET expression vector to yield a barcode attached to a cargo protein. A plasmid was constructed containing 6xHIS-HALO-TEV-LN-IIS-LC, allowing for direct cloning of the oligo pool via golden gate assembly. LN and LC represent the constant overhangs in the oligo pool used for ligation (FIG. 1B). 1 µg of vector was predigested with BsaI at 37° C and purified. Oligo pools were amplified via Polymerase Chain Reaction (PCR). A 1:10 molar ratio of purified vector and insert were added to a golden gate assembly reaction using NEB golden gate assembly mix (Cat: E1601S), and incubated at 37°C for 1 hour, then heat killed at 70°C for 5 min. This material was then purified, drop dialyzed into pure H_20 for 60 min, and electroporated into electrocompetent BL21 bacteria (lucigen). Serial dilution were plated to recover individual colonies. Colonies were then picked, grown in media containing 1% glycerol and 100 µg/ml carbenicillin, and glycerol stocked in 20% glycerol at -80°C. After cloning, a construct is generated containing a barcode attached to a protein using a linker (FIG. 1A).

Expression of individual barcodes:

[0543] Expression was performed either using *in vitro* transcription translation (IVTT) or BL21 induction. For IVTT, PCR was performed directly from glycerol stock by adding primers specific for T7 and T7 terminator sequences in BL21. The resulting amplicon contains T7 and T7 terminator for expression, and makes the protein **8xHIS-HALO-TEV-LN-Barcode-LC**. 1 µL of

PCR product, containing approximately 50 ng of DNA was added to a 10 µL IVTT reaction using NEBPure (cat no: E6800S) assembled according to the manufacturer's instructions. The reaction was then incubated for 4 hours at 37˚C. For *escherichia coli* (*E. coli*) expression, cultures were grown to an OD of 0.5 at 37˚C, then induced using Isopropyl β-d-1-thiogalactopyranoside (IPTG) and grown overnight at 25˚C. Cells were lysed the next day using sonication in the lysis buffer, and the lysed material separated from the inclusion bodies via centrifugation, taking the supernatant containing protein. Supernatant was purified using affinity chromatography Ni-NTA resin and stored for future use.

Capture of individual barcodes on HALO magnetic beads:

[0544] 10 µL of IVTT was diluted to 50 µL in PBS supplemented with BSA at 1mg/ml . To this mixture, 30 µL of Halo tag magnetic beads (cat: G7281) were added, and incubated with shaking at 400 rpm for 2 hours, then 4˚C with shaking overnight. Beads were captured on magnetic stand, and the supernatant removed. The beads were then washed 2x with PBS-T with 0.1% Tween 20 (PBS-T). A schematic of a captured barcode is shown in FIG. 3B.

Construction of a phage display library containing binders with varying affinities to barcodes:

[0545] Binders with strong affinities to at least one barcode were generated via methods known to those skilled in the art (*e.g.,* phage display, hybridoma, etc.). These binders were then displayed on phage as scFv fragments fused to m13 gene 3 protein (g3). Briefly, oligos containing the scFv binding sequences were generated via DNA synthesis. The oligo were cloned into a plasmid containing the constant regions of the scFv connected to G3 via a G4S linker (SEQ ID NO.: 8399) and myc tag. 30 µg of the library was electroporated into TG1 (lucigen) and plated on several 25 mm plates containing carbenecllin at 100 µg/ml and glucose 1%. Dilutions of electroporation were plated for diversity analysis. The Q trays were scraped and glycerol stocked. To produce phage, a 2L culture was inoculated at ~ OD 0.05 and grown to OD 0.5 at 37˚C with 100 µg/ml carbenicillin and 1% glycerol. At OD 0.5, helper phage was added at a 10:1 phage:cell ratio, and incubated with shaking at 250 rpm. After 1 hour, shaking was reduced to 150 rpm and temperature to 30˚C, and incubated overnight. The next day, phage was prepared

via PEG precipitation (Barbas et al. 2001), resuspended in 10 mL, and titered. Phage was stored at 4°C until use.

Assessment of phage-binder-barcode interaction:

[0546] 10 µL of the phage library prepared using methods described above was added to the captured barcode and incubated at room temperature for 2 hours, to allow binding of the phage to the barcode (FIG. 3C). After incubation, the mixture was washed by successive transfers of the magnetic beads to fresh PBS-T 3 times. Phage were eluted from beads by resuspension in PBS containing TEV protease + 0.1% DTT and incubation at 37°C for 30 min. Beads were collected on a magnetic stand, and the supernatant collected. To the supernatant, trypsin was added and further incubated for 30 min. To propagate phage, the supernatant was added to 50 mL of TG1 *E. coli* grown at 37°C to an OD of 0.5 in 2xYT and incubated with shaking at 37°C for 1 hour. Then 100 µg/ml carbenicillin and 1% glucose were added with incubation under the same conditions for 1 hr. Helper phage (cat: PH050L) was added and further incubated for 1 hour at 37°C. The culture was centrifuged and placed into new media containing 100 µg/ml carbenicillin and 50 µg/ml kanamycin, and incubated overnight at 30°C. Following this, centrifugation at 4000g for 20 minutes was performed, and the supernatant containing phage was collected.

Analysis and establishment of a fingerprint for a given barcode:

[0547] After selection was performed from the original phage pool against each barcode individually, phage-scfv (*i.e.*, phage-binder) selectivity was analyzed via NGS. Phage were lysed via heating at 98°C for 10 minutes, and the resulting genomes were PCRed using primers which flank the CDR regions of both the heavy CDR3 (5 prime) and light chain CDR3 (3 prime). A second round of PCR was performed to add required illumina sequences (i5/i7, sequencing primer binding region) for NGS. The result DNA was pooled, quantified and subject to NGS using an Illumina instrument. This process is shown in FIG. 2 and FIGS. 3A-3C.

[0548] NGS reads are demultiplexed using the illumina software bcl-convert, such that each final .fastq contains the DNA sequences from a given phage CDR3 pair which correspond

to the output from a given barcode well. The corresponding CDR3 sequences are then counted using a computer program, revealing the distribution of binders present for a given barcode. The fingerprint of a barcode corresponds to the vector of counts for each scFv binder within the given pool. Each fingerprint is the median of n=3 individual barcode replicates. The process and resulting fingerprint for a single barcode using the pool of phage binders is shown in FIG. 4.

Determination of proportion of barcodes in a given mixture using a fingerprint matrix:

[0549] Once the fingerprints of each barcode of a set of barcodes have been determined, the proportions of barcodes in an unknown sample were measured in the following manner. The binder-barcode interactions were assessed as described above, and the resulting NGS readout was fit to a linear combination of the known fingerprints via least squares. That is, the coefficients of the linear combination are chosen by minimizing the sum of squares of the differences between the measured NGS count and the expected NGS count for all binding species as described in Example 8. The expected NGS count is given by the matrix product of the fingerprint matrix with a set of barcode abundance coefficients. Once the coefficients have been obtained, they are normalized to sum to 1 to obtain proportions.

Decoding of equal proportion barcode mixture to asses fingerprint scaling:

[0550] In order to determine any scaling issues that may arise due to varying affinities between binders and barcodes, a scaling factor is generated via a measurement of barcodes mixed in equal proportions. Briefly, all barcodes validated are mixed at even concentrations after production. Phage binder interactions were assessed as described above, and the resulting phage was subject to NGS. Using the fingerprints determined for individual barcodes above, the proportion of barcodes in the mixture was estimated via least squares regression as described in Example 8. The proportions predicted using the least squares form the basis for scaling factors (sf) where sf = 1/p , where p=proportion predicted. The process is described in FIG. 5.

Assessment of mixtures of barcodes of known proportions:

[0551] Barcodes produced above were mixed at known proportions and subject to assessment to determine their accuracy. Barcodes were mixed at different proportions (FIG. 6A), and then analysis of phage binder (*i.e.,* binding agent) interactions was performed as described above. The NGS counts for each binder within the pool were counted. Least squares was used to determine the proportion of barcodes given the fingerprint constructed above using single barcodes. Predictions from the least squares analysis were then rescaled using the established scaling factor by p' = p*sf, where p' is the new prediction, p is the original prediction and sf is the established scaling factor. The new predictions are then re-normalized to sum to 1.

[0552] FIG. 6B shows the accuracy of proportion measurement for this method. Six (6) different barcodes were measured, normalized via a scaling factor, and relative barcode proportions were estimated with a global pearson correlation of .95 across all measurements made. Measurements were across a 100-fold gradient of barcodes. FIG. 6C shows a plot of NGS count values, normalized to counts per million, for each single barcode measurement as well as mixture that were used to predict the relative abundance of each barcode within the mixture. Rows are experiments, thus all values in a row are generated from a single .fastq file and columns are binding agents.

Example 2: ***In vitro*** **detection of a cargo in a mixture using binder-barcode platform**

[0553] The present example demonstrates a method of measuring the presence or absence of a given cargo in a mixture using a binder-barcode platform as described herein.

[0554] Barcodes generated in Example 1 were transferred onto a novel cargo using DNA cloning. Briefly, barcodes were amplified out of the pET 6xHIS-HALO-TEV-LN-Barcode-LC such that the LN-Barcode-LC part is amplified. The barcode insert was cloned using gibson into a new pET vector containing 6xHIS-Cargo-LN-Barcode-LC, where cargo was a novel protein of interest. Cargo-barcodes were produced in *E. coli* as described above. Barcoded cargo proteins are then purified via affinity chromatography using Ni NTA, washed in 500 mM NaCl, 50 mM Tris-HCl, 50 mM imidazole, and eluted using 500 mM imidazole. Barcoded cargo proteins were then subject to decoding using the phage binder library described in Example 1. FIGS. 6, 9, and 12 show experimental setups to detect cargo using barcodes previously generated in a different

context, and results of such experiments thus showing these barcodes contain generalized detection properties across varying numbers of barcodes within a pool.

[0555] In the experiment described in FIG. 9, six unique barcodes (BC1, BC2, BC3, BC4, BC5, and BC6) were mixed at known proportions, contacted with binding agents, and subjected to decoding as described herein (FIG. 9A). Two barcodes were experimentally held out as negative controls, but prediction for these barcodes was allowed, thus allowing determination of background prediction. FIGS. 9B and 9C show data on accuracy of decoding procedure across a 10-fold range of concentrations for the 6 unique barcodes. FIG. 9B shows plot of actual data (input) and measured data obtained after decoding for one mixture of known barcode concentrations. Input known concentrations (left bar) are shown next to predictions/measured data (right bar) for each barcode across 3 replicates. FIG. 9C shows plots of actual data (input) and measured data obtained after decoding for five different mixtures (*i.e.*, pools 1-5) of known barcode concentrations. Input known concentrations (left bar) are shown next to predictions/measured data (right bar) for each barcode across 3 replicates.

[0556] In the experiment described in FIG. 12, the quantification of twenty-four (24) barcodes contained within a single mixture was determined. FIG. 12A shows a graphical depiction of the experiment. Of 24 total barcodes the algorithm can predict, 10 were present within a mixture at equal concentrations. The rest were held out from the pool, but prediction was computationally allowed. Three (3) separate pools, which cover all possible barcodes were measured in replicate. These pools were mixed, then captured on HALO beads, described in Example 1. The immobilized barcoded cargo was then contacted with a pool of binding agents (*i.e.*, binding agents with binders expressed on them), and decoded to CDR3 sequence counts as described in Example 1. The CDR3 sequence counts, determined via NGS, were then utilized to determine the presence and total concentration of each barcoded cargo in the sample via decoding (see Example 8). FIG. 12B shows prediction for the first pool; the input concentration (left bar) and measured concentration (right bar) are plotted for each barcode in the pool. FIG. 12C plots predictions for each of the three pools. As in FIG. 12B, the bar graphs plot input concentration on the left and measured concentration on the right. FIG. 12D shows the barcode fingerprint for each of the 24 barcodes used to computationally determine the relative abundance of the barcodes within each of the 3 pools. Columns represent barcode fingerprints, and rows represent binding agent fingerprints. FIG. 12E shows the binding agent counts from the three

pools, used to computationally determine the proportion of the pools. Rows are the binding agent counts, columns are the pools, and each cell is the binding agent count within a specific pool.

Example 3: ***In vitro*** **assessment of cargo stability within a pool of cargos using binder-barcode platform**

[0557] The present example demonstrates a method to determine the general aggregative tendencies of several cargos in a pool using barcode decoding.

[0558] A purified pool of barcoded cargos is generated using the method described in Example 2. The purified pool is then subject to size exclusion chromatography using standard methods. Different fractions are collected - corresponding to monomeric vs aggregated cargos. The general presence or absence of a given barcoded cargo within the purified pool is not known. The separated fractions, containing an unknown abundance of each barcoded cargo, are then immobilized on beads or immunosorbent assay plates, contacted with a pool of binders (*i.e.*, binding agents with binders expressed on them), and decoded to CDR3 sequence counts as described in Example 1. The CDR3 sequence counts, determined via NGS, are then utilized to determine the presence and total concentration of barcoded cargos in each fraction. The concentration of barcoded cargos in the different fractions are then compared to determine the percent of each barcoded cargo which is monomeric vs aggregated within the purified pool.

Example 4: ***In vivo*** **assessment of cargo pharmacokinetics within a pool of cargos using binder-barcode platform**

[0559] The present example demonstrates a method to determine the overall residence and clearance time of a given cargo, contained within a pool of cargos, using a mouse model, as demonstrated in FIG. 11.

[0560] Three pools of barcoded cargos were injected into three different groups (pool 1 into group 1; pool 2 into group 2, and pool 3 into group 3) of mice (n=3). Pool one contained a single barcoded antibody at 10mg/kg. Pool two contained two barcoded antibodies pooled at equal concentrations and injected at 20 mg/kg. Pool three contained PBS only. Injection volumes were held constant at 100μL per pool. At 24 hours after injection, blood was collected from each

mouse and serum separated. 10μL of serum was diluted 1:10 in PBS and captured using anti-human IgG magnetic beads (Ray biotech cat# 801-101-1) by incubation overnight at 4°C with mixing at 700 rpm. The immobilized barcoded cargos were washed using PBS-T three (3x) times, to remove all serum proteins not associated with the affinity reagent. The immobilized barcoded cargo was then contacted with a pool of binding agents (*i.e.,* binding agents with binders expressed on them), and decoded to CDR3 sequence counts as described in Example 1. The CDR3 sequence counts, determined via NGS, were then utilized to determine the presence and total concentration of each barcoded cargo in the sample via decoding (see Example 8). The proportion of barcoded cargos measured at 24 hours was compared to the injected concentration to determine the relative rate of clearance for each barcoded cargo from the organism. In each of the groups, only the injected barcoded antibody was detected by decoding as evidenced by the graph plotted in FIG. 11, with high accuracy. In group 2 mice, which were injected with pool 2 that contains both antibodies at equal concentrations, slightly differing amounts are measured via decoding in the serum at 24 hours. It is hypothesized that this difference is due to differences in clearance rates between the two barcoded antibodies. As expected, the control group showed almost no antibody.

Example 5: ***In vivo*** **assessment of cargo biodistribution using binder-barcode platform**

[0561] The present example demonstrates a method to determine the overall distribution of a barcoded cargo across a diverse set of tissues using a mouse model.

[0562] A pool of purified cargos is injected intravenously into a BALB-6 mouse. After at least 24 hours, different tissue samples, such as liver, lung, and brain, are taken from the organism. The tissues are then processed into a single-cell suspension via vigorous shaking with beads. The suspension is then lysed using a lysis buffer to liberate the barcoded cargos (*e.g.,* barcoded cargo polypeptides) contained within the tissues. The lysed suspension is then purified using a universal tag affinity reagent contained within the cargos to separate the barcoded cargos. Purified barcoded cargos are then immobilized, and barcode decoding is performed according to method described in Example 1. The CDR3 sequence counts, determined via NGS, are then utilized to determine the presence and total concentration of each barcoded cargo in each sample. Cargo abundance across different tissue samples is then compared, to determine the

percent of each cargo which is contained within each tissue. This data may then be used to select the best cargo with specific biodistribution properties.

Example 6: ***In vitro*** **demonstration of recovering known mixture of unmodified antibodies**

[0563] The present example demonstrates how a known mixture of antibody proteins with no barcode attached was quantified using the protein quantitation invention described herein.

[0564] Briefly, scFv binders to the antibodies were generated using methods known to those skilled in the art. The binder were then cloned and displayed on phage as described in Example 1. The two antibodies of interest were expressed in CHO cells and purified from the media using Protein A affinity chromatography. Antibodies were mixed together in known proportions (FIG. 7A). Antibodies were then captured using 50 µL anti-Human Fc magnetic beads and incubated in PBS. The antibodies were then subject to phage assessment as described in Example 1, and the relative abundance of each antibody estimated using the algorithm described in Example 8. An accuracy of pearson .96 at determining the relative concentration of these two antibodies in varying proportion mixtures was calculated (FIG. 7B).

Example 7: ***In vitro*** **demonstration of recovering known mixture of antibodies in presence of serum**

[0565] The present example demonstrates how a known mixture of antibody proteins with barcodes contained within internal regions of the protein sequences was quantified in mouse serum using the protein quantitation technology described herein.

[0566] Briefly, antibodies were produced as described in Example 6, and a similar experiment was performed, except after production, the antibodies were mixed with mouse serum, incubated at 37°C for 30 minutes, and then captured using anti-Fc magnetic beads (FIG. 8A). The immobilized barcoded antibodies were then contacted with a pool of binding agents (*i.e.,* binding agents with binders expressed on them), and decoded to CDR3 sequence counts as described in Example 1. The CDR3 sequence counts, determined via NGS, were then utilized to determine the presence and total concentration of each barcoded antibodies in the sample via

decoding (Example 8). The relative concentration of three antibodies was estimated with a spearman of .926 across 3 mixtures of antibody concentration as shown in FIG. 8B and FIG. 8C. These results show the ability using technologies described herein to rank the antibodies present in a sample with high accuracy after incubation with serum *i.e.,* in a complex environment.

Example 8: Detailed description of the decoding algorithm used to infer barcode abundance

Method of inferring barcode amounts:

[0567] Before decoding an unknown sample, a set of barcodes and its interaction with a binder pool (*i.e.,* phage binder pool) must first be characterized. This is done by decoding a set of known samples under known conditions. The binder pool and experimental conditions is held fixed between all samples.

[0568] To characterize a set of barcodes, we measure a set of fingerprints, one for each barcode. A fingerprint represents the ideal readout of an individual barcode. Roughly speaking, it is the spectrum of affinities between a given barcode and all the binder species in the pool. A fingerprint can be estimated by decoding multiple identical samples containing purely one barcode, averaging together the readouts of the replicates, and rescaling accordingly. Alternatively, fingerprints can be learned by decoding samples containing known mixtures of barcodes and deconvoluting appropriately to isolate individual fingerprints. Together, the fingerprints of a set of barcodes is known as a "fingerprint matrix".

[0569] Once the fingerprint matrix of a set of barcodes is determined, it can be used to infer the barcode composition in an unknown sample. The decoding algorithm accomplishes this by fitting the readout of the unknown sample to a linear combination of fingerprints. This is described in further detail in the algorithmic section described herein. A key assumption of the algorithm is that the decoding process is linear: if a sample contains two barcodes mixed in equal proportions, it is assumed that its readout is equal to the sum of the fingerprints of the two barcodes (plus noise). More generally, the readout of a mixture of barcodes is assumed to be the

sum of the fingerprints of each barcode, appropriately weighted by its prevalence in the mixture. This assumption has empirically been found to be true.

[0570] The task of barcode quantification has varying levels of difficulty. From easiest to hardest, these include

- Binary classification: detecting the presence or absence of a barcode in a sample
- Rank-order quantification: ranking barcodes from most to least prevalent in a sample
- Relative quantification: determining the ratios between barcodes in a sample
- Absolute quantification: determining the absolute amount of each barcode in a sample

[0571] In this Example, absolute quantification is discussed in further detail.

Mathematical model of decoding:

[0572] The decoding process can be represented with the following mathematical model, where:

x is a length-n vector representing the input sample and each entry is the amount of a barcode species in units of ng; y is a length-m vector representing the bound binder fraction and each entry is the number of particles of a binder species in units of pfu; z is a length-m vector representing the NGS readout and each entry is the number of counts for a binder species.

[0573] The bound binder fraction is modelled as a linear combination of fingerprints, and the NGS readout is modelled as multiplying the bound binder fraction by a conversion factor:

$$y_j = \sum_{i=1}^{n} A_{ji} x_i + \epsilon_1$$

$$z_j = s_j y_j + \epsilon_2$$

where A_{ji} is the fingerprint matrix, an m by n matrix with units of pfu bound per ng of barcode. The 'ji' entry represents the propensity for barcode i to bind binder j in the binder pool; s_j is the conversion factor between pfu bound and number of NGS reads for binder species j; ε_1 is noise associated with the binding steps; ε_2 is noise associated with the post-binding steps.

[0574] The model assumes that binding between barcodes and binders is linear. In other words, if a sample contains a mixture of barcodes, its readout is assumed to be equal to the sum of the fingerprints of the individual barcodes, weighted by the relative barcode abundances. In Appendix A we provide a detailed biophysical model which justifies the linear assumption under one key condition: the amount of available binder cannot be significantly depleted by binding to the barcodes in the sample. Thus, as in typical immunoassays, the binding agent must be in excess and cannot be the limiting reagent.

Fingerprint matrix A_{ji}:

[0575] Each column of the mn-fingerprint matrix is a fingerprint. Each fingerprint represents the ideal, properly normalized readout of a pure barcode. The entry A_{ji} represents the contribution of the j'th binder to the fingerprint of barcode i.

[0576] The fingerprint of a barcode depends on the binding affinity to all the binders in the pool as well as the relative abundance of each binder species in the pool. Furthermore, the fingerprint is sensitive to the binding, equilibration, and elution steps. In the simplest case, the fingerprint matrix is given by

$$A_{ji} = d_j / K_{ji}$$

where d_j is the concentration of binder j in the binder pool, and K_{ji} is the dissociation constant of the complex between barcode i and binder j (see Appendix A). In more complicated cases, A_{ji} could also include effects of adhesion to surfaces, unbinding during washing steps, etc.

[0577] The matrix product of A with a barcode mixture x gives the composition of the ideal bound binder fraction (*i.e.,* in absence of noise), in units of number of phage particles.

[0578] The fingerprint matrix can be determined from measuring the readout of multiple known samples. Multiple replicates are performed to average over noise. In addition, the fingerprints are properly scaled, either with respect to one another or to an absolute standard (see the Normalization section).

Conversion factors s_j:

[0579] The post-binding steps introduce a conversion factor between the number of bound phage particles to the number of NGS reads. This is represented by s_j. In the simplest case, s_j is identical for all binder species and represents an overall normalization,

$$s = C \,/\, \sum_{j=1}^{m} y_j$$

such that the total number of reads/counts is C. This models the situation where there is some sort of bottleneck in the processing, such as saturation of the propagation culture, such that the end result is a fixed number of reads, irrespective of the amount of bound phage. In a more complicated case, s_j could depend on the binder species, reflecting amplification bias or differential phage fitness in propagation.

[0580] In the case where the conversion factor is identical for all binder species, it is a single number which must be determined on a sample-by-sample basis. This can be done by using a DNA sequence spiked in at some point of the process (as described herein).

Noise ε:

[0581] Noise sources are represented by the terms ε_1 and ε_2. In the absolute simplest case, ε_1 is absent and is ε_2 is Gaussian noise of fixed variance, in which case prediction can be done with ordinary least-squares regression. In reality, the noise arises from multiple, non-Gaussian sources, as detailed in the sections above. These include log-normal noise involved with exponential steps, such as phage propagation and PCR amplification, Poisson noise due to finite sequencing depth (and possibly stochasticity in binding/elution at low concentrations), and Gaussian noise from all sorts of other processes, such as sample degradation, etc.

Conversion from read counts to phage counts:

[0582] One feature of the NGS is that the readout is a relative measurement: it gives the ratio of abundances between different binder species, but not necessarily the absolute

concentrations of binder species. To obtain an absolute readout, the raw readout must be divided by a conversion factor between the number of phage particles bound and the NGS read count.

[0583] Without knowing the conversion factor, it is only possible to determine the relative abundances of barcode in a sample (*i.e.,* proportions). Absolute quantification requires a reference of known concentration (either barcode or binder) to be spiked into the process.

Methods of absolute quantification:

Spiking a phage ladder into eluate:

[0584] One method of normalizing is to add a unique binder species into the eluate at a known concentration, $y_{spike\text{-}in}$. This reference species should be distinct from the existing binders in the pool. In the subsequent steps (where the eluted phage are propagated, DNA extracted, PCR'ed, and sequenced), the reference phage will be amplified by (ideally) the same factor as the other phage in the pool, *i.e.,*

$$z_{spike-in} = s \cdot y_{spike-in}$$

[0585] The conversion factor can be estimated by dividing the number of reads corresponding to the reference phage by the (known) concentration at which it was added, *i.e.,*

$$\hat{s} = y_{spike-in}/z_{spike-in}$$

[0586] A generalization is to spike multiple binder species into the eluate. Each reference species can be spiked in at a different concentration. If the concentrations are evenly spaced, this forms a "phage ladder", in analogy with ladders used in gel electrophoresis. To estimate the conversion factor, the number of reads of each reference sequence can be compared to the (known) concentration at which it was added to the eluate. Averaging across the species then yields a more precise estimate of the conversion factor.

Spiking barcode into sample:

[0587] Alternatively, a reference barcode of known concentration can be added to the sample at the beginning of the decoding process. This reference barcode should be distinct from

the existing barcodes in the sample. The decoding algorithm can use the raw readout to determine the proportions of all barcodes within the sample, including the reference and the sample barcodes. By dividing the reference barcode concentration by its predicted proportion, a barcode conversion factor can be determined. Multiplying all the predicted proportions by this factor then yields the absolute abundances of barcode.

[0588] Note that this method is only applicable for decoding an unknown sample after a set of properly normalized fingerprints has been determined.

Scaling fingerprints:

[0589] An important subtlety is that readouts have to be scaled even in the case of relative quantification. Specifically, the fingerprints of a set of barcodes must be properly scaled with respect to one another. Intuitively, this is because raw, unscaled fingerprints cannot be directly compared between barcodes; the read count of a binder has a different meaning in the context of a different barcode's fingerprint, because each barcode has a different conversion factor between bound binder count and read count. To ensure that fingerprints are measured in the same units, each unscaled fingerprint must each be scaled by (the inverse of) its conversion factor.

[0590] To illustrate this, consider a case with two barcodes. Suppose that, due to differences in binding affinity across the binder pool, the total amount of binder bound to 100ng of barcode *A* is 10 times greater than that bound to 100 ng of barcode *B*. However, due to the nature of the method, the raw readouts end up having the same number of reads. In this example, one NGS read in barcode *A*'s raw fingerprint corresponds to 10 reads in barcode *B*'s raw fingerprint. The conversion factors are different. Now consider a sample containing a 1:1 mixture of the two barcodes. Due to the difference in affinity, the bound binder fraction in this sample is 10:1; consequently, the number of NGS reads corresponding to *A* and *B* would also be in a 10:1 ratio. In other words, the readout is proportional to $10a+b$, where a is the raw readout of *A* and b is the raw readout of *B*. On the basis of this, one would come to the incorrect conclusion that *A* and *B* are in a 10:1 ratio. To correct for this, the raw fingerprint of barcode *A* must be multiplied by a factor of 10, compared to *B*, to obtain a correctly scaled fingerprint, $a'=10a$ and $b'=b$. Now, when the two barcodes are mixed in equal proportions, the correct result is determined: the readout is an equally weighted mixture of the two correctly scaled fingerprints, $a'+b'$.

[0591] This example shows that the relative scaling factor between barcodes' raw fingerprints can be determined by measuring the readout of a known mixture of the barcodes. If the barcodes are mixed in equal proportions, the composition of the mixture readout will be each raw fingerprint, weighted by their scaling relative to each other. (Note that this gives only the relative conversion factor between the barcodes and not an absolute conversion factor to absolute barcode quantities.)

The decoding algorithm:

[0592] There are two phases of the decoding algorithm:

- "Training phase": Learn the fingerprint matrix A_{ji} by measuring the readout of a number of known samples
- "Testing phase": Predict the barcode amounts in an unknown sample by measuring the readout and comparing it to A_{ji}

Training phase: learning the fingerprint matrix

[0593] In the training phase, the fingerprints of a set of barcodes are determined by measuring the readouts of a set of samples with known composition. The fingerprints must be correctly scaled with respect to one another. One method of measuring the fingerprint matrix is outlined below.

[0594] First, a set of samples, each containing purely a single barcode are prepared. Each sample is decoded. The fingerprint of each barcode is estimated by taking multiple replicates of a barcode and averaging together their readouts. The error is reduced if more replicates are averaged. This yields an unscaled fingerprint for each barcode.

[0595] Next, the fingerprints are correctly scaled with respect to each other. This is done by multiplying each of the unscaled fingerprints by a scaling factor. To determine the scaling factor of each barcode, a sample consisting of all barcodes mixed in equal proportion is decoded. Theoretically, this readout of this sample should be the sum of the normalized fingerprints of all the barcodes, weighted equally. However, if we fit this mixture readout to the set of

unnormalized fingerprints determined from the previous step, the weights of the barcodes would not be equal. The coefficients to the linear fit are precisely the factor by which each barcode's fingerprint should be multiplied by to obtain a correctly normalized fingerprint. By averaging together multiple replicates of this, a more precise estimate of the scaling factors can be determined.

[0596] This method is detailed further below. Let $\tilde{A}$ be the unscaled fingerprint matrix obtained from averaging multiple unscaled measurements of single barcodes. Each column should sum to a fixed number of reads, so the units of $\tilde{A}$ are counts per million. The correctly scaled fingerprint matrix A is obtained by multiplying each column of $\tilde{A}$ by a rescaling factor S, such that $A = \tilde{A}S$, where S is a $n \times n$ diagonal "rescaling matrix". Here S_{ii} is the scaling factor for the i'th barcode, with units of pfu eluted per count per million. Let y be the readout of a mixture of known composition x. One way to determine scaling factors is to use the unscaled fingerprint matrix to infer a set of "biased" predictions as $\tilde{x} = \tilde{A}^{+}y = SA^{+}y$, where $\tilde{A}^{+}$is the pseudoinverse of $\tilde{A}$ (see the prediction section below). The ratio of the "biased" prediction to the actual amount is an estimate of the scaling factor: $S_{ii} \approx \tilde{x}_i/x_i$. Multiplying each unscaled fingerprint by this scaling factor gives our best estimate of the properly scaled fingerprint $A = \tilde{A}S$.

[0597] Note that there are other methods for measuring the fingerprint matrix. This present method only uses the single-barcode samples to determine (unnormalized) fingerprints, and the mixture samples to determine the scaling factors. More sophisticated methods may use uneven mixtures to determine scaling factors and/or use the information in these samples to better estimate the fingerprint (beyond just learning its scaling factor).

Testing phase: predicting barcode amounts in unknown samples

[0598] In the testing phase we are supplied with the readout y of an unknown sample x and we aim to infer its composition, $\hat{x}$. This is done by fitting the readout to a linear combination of fingerprints, $y = A\hat{x}$, where A is the (properly scaled) fingerprint matrix learned from the training phase.

[0599] The fitting is done by choosing a set of coefficients x_j, which minimizes a loss function. The loss function measures the deviation between the expected and measured readout. The expected readout is the matrix product A_x, based on the determined fingerprints and proposed mixture coefficients. In the simplest case, the loss function is a sum of squared errors,

$$(\hat{x}) = \sum_{j=1}^{m}(\sum_{i=1}^{n} A_{ji}\hat{x}_i - y_j)^2 = ||A\hat{x} - y||_2{}^2$$

and the inferred mixture composition is the minimizer of this loss, $\hat{x} = argmin\, L(x)$. If the number of binders is greater than the number of barcodes, then A_x=y is an overdetermined system and there is a unique minimizer of the loss. The solution is given by

$$\hat{x} = A^+ y$$

where $A^+ \equiv (A^T A)^{-1} A^T$ is the Moore-Penrose inverse of the fingerprint matrix. If relative abundances (proportions) are desired, the coefficients can be normalized to sum to 1.

[0600] Note that the L2 loss function above is the simplest case. It is (proportional to) the negative log likelihood in the case where ε_1 is absent (no noise in binding) and ε_2 is Gaussian noise of fixed variance. To model more realistic forms of noise, other loss functions can be chosen.

Appendix A: Biophysical model of binding process

[0601] Let n be the number of barcoded cargo polypeptide species in a sample, and let $x_i, i = 1, \ldots, n$ be the concentrations of each barcode species added to the decoding well. Likewise, let m be the number of decoder species in the decoder pool, and let $d_j, j = 1, \ldots, m$ be the concentrations of each decoder species added to the decoding well. Suppose that any decoder can interact with any barcode in a one-to-one stoichiometry to form a bound complex. A total number of nm such complexes can be formed, one for each barcode-decoder pair. Let $c_{ij}, i = 1, \ldots, n, j = 1, \ldots, m$ be the concentration of a complex between barcode i and decoder j, and let K_{ij} be the equilibrium dissociation constant characterizing the affinity of this interaction, with $\Delta G_{binding} = -RT\ ln\, K_{ij}$.

[0602] For any decoder species, we assume that the amount which binds to the sample and subsequently eluted is given by $y_j \equiv \sum_{i=1}^{m} c_{ij}$, the bound complexes of that decoder summed over all possible barcode pairs.

[0603] In the simplest case, we assume that each species is an ideal solute in a dilute solution, and that the binding between barcodes and decoders is allowed to approach thermodynamic equilibrium. At equilibrium, some fraction of the decoders will bind to the barcodes, but there will still be remaining unbound barcodes, with concentrations $\tilde{x}_i, i = 1, \ldots n$, as well as unbound decoders, with concentrations $\tilde{d}_j, j = 1, \ldots m$.

[0604] The equilibrium state is given by minimizing the overall free energy of the system. This is shown to be equivalent to solving the following set of equations:

$$x_i = \tilde{x}_i + \sum_{j=1}^{m} c_{ij} \text{ for } i = 1, \ldots n$$

$$d_j = \tilde{d}_j + \sum_{i=1}^{n} c_{ij} \text{for } j = 1, \ldots m$$

$$K_{ij} = \tilde{x}_i \tilde{d}_j / c_{ij} \text{for } i = 1, \ldots, n, j = 1, \ldots, m$$

[0605] The first equation ensures the conservation of mass: the total amount of a barcode species is the unbound amount plus the amounts bound in complexes with all possible decoder partners. The second equation is an analogous statement for the decoders. The last equation is the definition of an equilibrium constant of a barcode-decoder pair.

[0606] The known values are the equilibrium constants K_{ij} and the x_i and d_j, representing the total concentration of each species of barcode and decoder, respectively, added to the well. The unknown variables are $\tilde{x}_i$, $\tilde{d}_j$, and c_{ij}. The values of the unknown variables are determined by solving the system of equations above.

[0607] In the decoding process, a decoder pool with fixed values of K_{ij} and d_j is added to a sample of unknown barcode composition, x_i. The observable output of the binding process is the amount of each decoder species which binds to the sample, $y_j \equiv \sum_{i=1}^{m} c_{ij}$. A key question of the binding process is how the input, the barcode concentrations x_i, affects the output, the bound decoder amounts y_j.

[0608] In general, the system of equations above is non-linear, but when certain conditions are met, the binding process can be well approximated by a set of linear equations. The decoding process is greatly simplified if the underlying equations are linear. A linear system implies that, at the least:

1) If the concentration of a barcode is doubled, the decoders bound to that barcode are all correspondingly doubled. (no saturation)
2) If two barcodes are mixed together, the decoders bound to the mixture are the sum of the decoders bound to each barcode alone. (no competition)

These two criteria can be thought of as linearity of single and multiple barcode detection, respectively. They are necessary, but not sufficient conditions.

[0609] To see how these criteria play in practice, consider the following binding situations which violate one or more of the criteria. Suppose that the decoder is the limiting reagent in a one-to-one stoichiometry of barcodes and decoders. Above a certain barcode concentration, all the available decoders would become saturated; an increase in x_i would not lead to a proportional increase in y_j. Thus, to avoid saturation, the barcode concentrations should be kept below the Kd of the interaction (or the decoder concentration, whichever is greater -- see below).

[0610] As another example, consider a situation where two barcodes A and B both have affinity for a certain decoder D, but barcode A has a much stronger affinity than barcode B. In the absence of A, the binding of barcode B has a certain binding curve. However, if there is enough A to deplete much of the available D, the binding of barcode B to the remaining D will be significantly altered. This is a situation where A and B compete for available decoder D. Note, however, that if the binding of A did not deplete the amount of D remaining in the pool, then the binding between B and D would not be altered by the presence of A. The competitive behavior occurs only in situations where the available D is significantly consumed by binding to A -- if A is highly abundant and/or the A-D affinity is strong. In both examples of non-linear situations, one or more decoders in the pool is significantly depleted by binding to barcodes.

[0611] These examples indicate that the binding process is linear when a small fraction of the decoding pool is bound to the sample. Indeed, if this condition is met, the equations above

can be simplified into a simple linear system. Under this assumption, the amount of unbound decoder $\tilde{d}_j$ is well approximated by the total amount of decoder, d_j. If we further assume that the bound complexes deplete a small fraction of the available barcodes, then the system of equations simplifies to

$$c_{ij} \simeq x_i d_j / K_{ij}$$

and the output can be represented with a simple matrix multiplication,

$$y_j = \sum_{i=1}^{n} A_{ji} x_i \text{ with } A_{ji} \equiv d_j / K_{ij}$$

Example 9: Assessment of absolute cargo abundance of a single test barcode using a reference barcode (or spike-in barcode)

[0612] The present example demonstrates a method to determine absolute cargo abundance of cargos in a pool using a reference barcode and barcode decoding.

[0613] FIG. 10A shows a schematic of an experiment. A single test barcode attached to cargo was assayed at several concentrations, ranging from 0 ng/mL to 1250ng/mL. In the same sample, a "spike-in" barcode attached to a cargo (*i.e.,* a reference barcode) was added to each assay mixture at 250ng/mL. Various concentrations of a test barcoded cargo were contacted with binding agents (*i.e.,* binding agents with binders expressed on them) and decoding was performed as described herein. Prediction of a reference or "spike-in" barcode was used to determine an absolute amount of a test barcode, and by extension a barcoded cargo, being measured (see Example 8). FIG. 10B shows a plot of measured absolute quantities of the test barcode (right bar) compared to known input concentrations of the test barcode (left bar) for each titration of the test barcode. The Y-axis is a logarithm of the test barcode concentration in nanograms per milliliter (ng/mL). FIG. 10C shows results of determination of absolute concentration for 6 different barcoded cargos. The plots show known input concentrations (left bar) and measured concentration (right bar) for six (6) different barcoded cargos.

Example 10: Simultaneous *in vivo* assessment of phenotypes of cargos using binder-barcode platform

[0614] The present example demonstrates a method to perform simultaneous *in vivo* assessment of phenotypes of cargos using a binder-barcode platform described herein. In some embodiments, a phenotype includes pharmacokinetic (or clearance) data as described herein.

[0615] FIG. 13A is a schematic of a method to detect and/or quantify and/or characterize fourteen (14) exemplary cargos (*e.g.*, proteins) in a pool using a binder-barcode platform described herein. Fourteen (14) exemplary binder molecules were produced with different barcodes as described herein ("binder-barcode particles"). Binder-barcode particles were injected as a pool into wild-type (wt) BALB/c mice. At timepoints 30 min, 6 hours, 24 hours, and 48 hours, blood was collected from individual mice (n=3 per timepoint), and serum was extracted. Binder-barcode particles were captured and subjected to the decoding procedure as described.

[0616] FIG. 13B shows simultaneous *in vivo* assessment of clearance phenotypes of cargos using a binder-barcode platform described herein. In particular, FIG. 13B shows plots of clearance of multiple cargos, measured simultaneously and grouped by rate of clearance phenotype (*e.g.*, slow vs. fast clearance). FIG. 13B (left) depicts controls with known properties that were measured. FIG. 13B (center) depicts cargos identified as having slow clearance properties over time. FIG. 13B (right) depicts cargos identified as having fast clearance properties over time. Data was normalized to 100% of injection volume for each binder-barcode particle.

[0617] Accordingly, the present example confirms that a binder-barcode platform described herein can be used for simultaneous characterization of phenotypes *in vivo*.

Example 11: Simultaneous *in vivo* assessment of phenotypes of cargos in multiple tissues using binder-barcode platform

[0618] Among other things, the present disclosure provides an insight that on-target, off-tumor toxicity is a biodistribution challenge. The present example demonstrates a method for simultaneous *in vivo* assessment of phenotypes of cargos in multiple tissues using a binder-

barcode platform described herein. In some embodiments, a phenotype includes biodistribution data as described herein. In some embodiments, a phenotype includes pharmacokinetic (clearance) data as described herein. In some embodiments, a phenotype includes pharmacokinetic data and biodistribution data as described herein.

[0619] FIG. 14A is a schematic of a method to detect and/or identify and/or quantify and/or characterize thirty-six (36) exemplary cargos (*e.g.*, proteins) in a pool using a binder-barcode platform described herein. Thirty-six (36) binder molecules were produced with different barcodes ("binder-barcode particles"). Binder-barcode particles were injected as a pool into tumor bearing NSG mice, which had been previously implanted with two tumor cell lines (Tumor 1, Tumor 2). At timepoints 30 min, 6 hours, 24 hours, and 48 hours, blood and tumor tissue was collected from individual mice (n=2-4 per timepoint). In some embodiments, other tissue can be harvested, including lung, liver, brain, etc. Tissue was lysed using standard lysis buffer. Serum was separated from blood. Binder-barcode particles were captured binder from each tissue and subjected to a decoding procedure as described herein.

[0620] FIG. 14B is a heat-map of all binder-barcode particle data collected as described herein. Rows indicate different binder constructs identifiers (IDs) that correlate to binder-barcode particles tested by the present example. Columns indicate data for a mouse across each time point for serum, Tumor 1, or Tumor 2. Color intensity indicates relative units of drug as measured via a decoding procedure described herein. Color intensity indicates a normalized readout of relative concentration as measured via next generation sequencing (NGS).

[0621] FIG. 14C depicts plots of binder-barcode particles described by FIG. 14B using a decoding procedure described herein. A diversity of properties was simultaneously measured. For example, binder-barcode particle P14_A5 was rapidly cleared from serum, with minimal accumulation in Tumor 1 or Tumor 2, while binder-barcode particle P17_A10 was more slowly cleared and maintained in tumor 1 over time.

[0622] Accordingly, the present example confirms that a binder-barcode platform described herein can be used for simultaneous characterization of phenotypes of cargos across multiple tissue types *in vivo*.

Example 12: Simultaneous *in vivo* assessment of phenotypes of cargos using binder-barcode platform

[0623] The present example demonstrates a method for simultaneous *in vivo* assessment of phenotypes of cargos (*e.g.*, proteins) using a binder-barcode platform described herein. In some embodiments, a phenotype is a half-life measurement as described herein.

[0624] FIG. 15A depicts a plot showing ELISA quantitation of two groups of cargos (Group 1: cargo polypeptide with no barcode; Group 2: a pool of eight (8) binder-barcode particles where each particle includes the same cargo polypeptide used in Group 1, and each particle is barcoded with a different barcode). Group 1 was injected into a cohort of wild-type (wt) BALB/c mice. Group 2 was also injected into a cohort of wild-type (wt) BALB/c mice. At timepoints 6 hours, 24 hours, and 48 hours, blood was collected from individual mice (n=3 per timepoint), and serum was extracted. ELISA quantitation showed similar measurements between Group 1 and Group 2, confirming that barcode does not affect cargo function. FIG. 15B shows a plot depicting simultaneous and individual assessment of eight (8) distinct entities in Group 2 using a decoding procedure described herein. FIG. 15C shows a comparison of half-life measurements for Group 1 and Group 2. Half-life measurements for Group 1 was quantified using ELISA (dashed line). Half-life measurements for Group 2 was quantified using a decoding procedure described herein (bars). As shown in FIG. 15C, variation in half-life measurements across different binder-barcode particles within a pool can be resolved using a binder-barcode platform described herein. Moreover, ELISA cannot perform simultaneous measurements of phenotypes in one experiment.

[0625] Accordingly, the present example confirms that a binder-barcode platform described herein can be used for simultaneous, accurate characterization of phenotypes *in vivo*. Moreover, the present example confirms that attachment of a barcode to a cargo using a method described herein does not disrupt *in vivo* properties of a cargo.

Example 13: Sensitivity and dynamic range of binder-barcode platform

[0626] The present example demonstrates a method to determine sensitivity and dynamic range of a binder-barcode platform described herein.

[0627] An array of ninety-six (96) mixtures comprising ten (10) to thirty-five (35) barcoded cargos (*e.g.,* proteins), with each barcoded cargo having a known concentration between 1 picogram (pg) and 1 microgram (μg), was designed to determine sensitivity and dynamic range of a binder-barcode platform described herein (FIGS. 16A-16B). Each data point in FIG. 16B represents a comparison between a known concentration of a barcoded cargo particle from one of the ninety-six (96) distinct mixtures and a concentration determined by a decoding procedure described herein. As shown in FIG. 16B, cargos were quantified across a 10,000-fold range of concentrations. FIG. 16B also shows cargos down to 0.1 nanograms (ng) were also quantified.

[0628] Accordingly, the present example confirms that a binder-barcode platform described herein can be used for simultaneous, sensitive characterization of barcoded cargos across a broad dynamic range of concentrations in diverse mixtures.

Example 14: Comparison of *in vitro* and *in vivo* assessment of phenotypes of cargos using binder-barcode platform

[0629] The present example provides an insight that *in vitro* models sometimes fail to accurately model *in vivo* environments. Among other things, the present disclosure provides an insight that certain *in vitro* systems can be limited (*e.g.,* with respect to their modeling of *in vivo* performance) by one or more of the following: two-dimensional monolayer of *in vitro* models versus complex three-dimensional architecture of *in vivo* environments, differences in target post translation modification or accessibility, differences in gene expression, absence of stromal cells and/or extracellular matrix, lack of circulation and diffusion from vasculature, or off-target and antigen sink effects not modeled in *in vitro* models.

[0630] Binder-barcode particles tested in Example 11 were also tested in an *in vitro* T cell activation assay (data not shown). A binder-barcode particle that exhibited highest T cell activation *in vitro* exhibited poor tumor tissue accumulation and rapid clearance *in vivo*. Moreover, a binder-barcode particle that exhibited modest T cell activation *in vitro* exhibited high tumor accumulation and slow clearance *in vivo*. Accordingly, the present example confirms that a binder-barcode platform described herein can be used to identify binders with unexpected *in vivo* properties. Moreover, the present example confirms that a binder-barcode platform

described herein can be used to select therapeutic candidates exhibiting desirable phenotypes *in vivo*.

Example 15: Barcode does not significantly affect cargo properties

[0631] The present example confirms that a barcode has relatively little or no effect on cargo (*e.g.*, protein) properties. In some embodiments, a property includes cargo affinity as described herein. In some embodiments, a property includes cargo production (or yield) as described herein.

[0632] Affinity and production (or yield) of a cargo (*e.g.*, protein) with and without a barcode were assessed using biolayer interferometry (BLI). Ten different barcodes were tested. Affinity of a barcoded cargo exhibited similar affinity of a cargo without a barcode, as measured by BLI (data not shown). Moreover, cargo production (or yield) of a barcoded cargo exhibited similar cargo production (or yield) of a cargo without a barcode, as measured by BLI (data not shown).

[0633] Accordingly, the present example confirms that cargo performance is not significantly impacted by a barcode. Moreover, the present example confirms that cargo production is not significantly impacted by a barcode.

Example 16: Assessment and quantification of expression and/or localization of barcoded cargo polypeptides delivered using multiple serotypes of AAV delivery particles

[0634] The present example demonstrates a multiplexed, high-throughput method of measuring a barcoded cargo polypeptide as described herein. Moreover, the present example demonstrates that a binder-barcode platform described herein can be used to quantify effects (*e.g.*, phenotypes such as localization, expression, etc.) that result from modifying cargo polypeptides as described herein. Further, the present example demonstrates that a binder-barcode platform described herein can be used to quantify effects (*e.g.*, phenotypes such as localization, expression, etc.) that result from a type of delivery particle that is used to deliver a cargo component as described herein. In particular, the present example demonstrates effects of a secretion tag on expression and/or localization of cargo polypeptides using three different

serotypes of AAV delivery particles (AAV2, AAV9, AAV.PHPB) in a pooled mixture. The present example demonstrates that expression and/or tropism of cargo polypeptides can be quantified using a binder-barcode platform as described herein.

[0635] Other types of cargo components and modifications to a cargo polypeptide can be assessed using a binder-barcode platform as described herein. Phenotypes other than expression and localization can also be quantified and/or assessed using a binder-barcode platform as described herein.

Method:

[0636] **FIG. 18** depicts a schematic of an exemplary method described herein that provides for tracking and/or identification and/or assessment and/or quantification of different nucleic acids encoding a cargo component disposed within different types of delivery particles (*e.g.*, AAV delivery particles, *e.g.*, AAV2, AAV9, AAV.PHPB) that exhibit different tissue tropisms.

[0637] Two constructs comprising barcoded cargo components were synthesized. A first construct was formatted as Sec-ALFA-GOI-BC ("Construct 1"), where Sec is a nucleotide sequence encoding a secretory signal peptide, ALFA is a nucleotide sequence encoding a universal capture tag, GOI is a cargo component whose nucleotide sequence is or comprises a sequence encoding a cargo polypeptide of interest, and BC is a barcode component whose nucleotide sequence is or comprises a sequence encoding a peptide barcode. A second construct was formatted as ALFA-GOI-BC ("Construct 2"). A difference between Construct 1 and Construct 2 was that Construct 2 lacked a secretion signal peptide (Sec). For Construct 1, six (6) different versions were synthesized, and for Construct 2 nine (9) different version were synthesized, each associated with a different barcode (see Table 3). Each version of Construct 1 and Construct 2 were cloned into a backbone containing human cytomegalovirus (HCMV) promoter and an inverse terminal repeat (ITR) sequence for packaging in an adeno associate virus (AAV). The resulting plasmids were then used to produce AAV using methods known in the art. Three different AAV serotypes were used to package constructs, as shown in Table 3, namely AAV2, AAV9, and AAV-PHPB. Each version of Construct 1 and Construct 2 were

individually packaged into each serotype of AAV. It is noted that AAV2 was only measured with a secretion tag. Table 3 shows experimental designs for Construct 1 and Construct 2.

[0638] Table 3. Different AAV variants produced and their content

Construct #	Secretion tag	Barcode ID	AAV Serotype
1	Yes	1	AAV-2
1	Yes	2	AAV-2
1	Yes	3	AAV-2
1	Yes	4	AAV-9
1	Yes	5	AAV-9
1	Yes	6	AAV-9
1	Yes	7	AAV-PHPB
1	Yes	8	AAV-PHPB
1	Yes	9	AAV-PHPB
2	No	10	AAV-9
2	No	11	AAV-9
2	No	12	AAV-9
2	No	13	AAV-PHPB
2	No	14	AAV-PHPB
2	No	15	AAV-PHPB

[0639] AAV delivery particles were titered via qPCR to determine genome copies per mL (GC/mL). AAV delivery particles were pooled such that GC/mL was equal across all variants.

[0640] C57 female mice (n=15) were injected via tail vein with $1x10^{11}$ total genome copies of the pooled AAV delivery particles. Three (3) mice were then euthanized at each time point (1 day, 2 days, 7 days, 14 days, and 21 days) and brain, liver and serum tissues were collected. Tissues were lysed using Radioimmunoprecipitation Assay (RIPA) buffer with a HALT protease inhibitor cocktail and two (2) rounds of bead beating for 60 seconds at 25Hz, and barcodes were subject to decoding as previously described herein.

Results:

[0641] A person of ordinary skill in the art would understand tropisms conferred by AAV serotypes, such as AAV2, AAV9, AAV-PHPB. For example, AAV2 generally has poor expression

and primarily infects liver, AAV9 has mostly high liver expression and low levels of expression in the brain, and AAV.PHPB infects and expresses cargo polypeptides in neuron cells (see Weinmann et al, Nature Communications, 11, 5432 (2020)). However, these results are either usually performed as single measurements when looking at protein, or via RNA and/or DNA sequencing of the viral genomes when looking in a pool. Furthermore, effects of incorporating one or more components such as a secretion tag on expression and/or localization of a cargo polypeptide and assessing such effects is unknown. Moreover, other technologies would not be able to assess effects on delivered cargo polypeptides using different types of delivery particles in a multiplexed and high-throughput format.

[0642] **FIGS. 19A**, **19B**, and **19C** show data for three replicate mice at 14 days, averaged across barcode and mouse replicates. It is noted that data from other time points look similar. The Y-axis in each of the graphs of **FIGS. 19A-19C** show a fold-change in decoding measurement for a given barcoded cargo component and serotype pair as compared to a background measurement. The X-axis in each of **FIGS. 19A-19C** shows various tissues analyzed for each of the three AAV serotypes. Green bars correspond to Construct 2 (*i.e.,* constructs without a secretion tag), and orange bars correspond to Construct 1 (*i.e.,* constructs with a secretion tag. AAV2, which was only measured with a secretion tag, had general poor expression across tissues collected compared to other serotypes (see **FIG. 19A**). Strikingly, when AAV9 was used for packaging, a cargo polypeptide was expressed and localized over 100-fold more in serum compared to liver when a secretion tag was associated with a cargo polypeptide (see **FIG. 19B**). Additionally, as shown in **FIG. 19B**, cargo polypeptides were not localized in serum without a secretion tag. Further, AAV9 cargo polypeptides were expressed in brain tissue, which was detected marginally over background for both Construct 1 and Construct 2. AAV.PHPB is known to infect and express exclusively from neurons. However, when a secretion tag was associated with a cargo polypeptide and packaged in AAV.PHPB, almost equal amounts of cargo polypeptide localized in serum and brain tissue (see **FIG. 19**). This finding indicates that a secretion tag is leading to leakage from brain tissue. Conversely, when no secretion tag is added to a cargo polypeptide, no cargo polypeptide was seen in serum and very high levels of cargo polypeptide expression was seen in brain tissue (see **FIG. 19C**).

Discussion:

[0643] As described herein, peptide barcodes were used to assess effects of localization and/or expression of cargo polypeptides delivered by a variety of AAV serotypes in a single mixture. This workflow would have been previously impossible to perform and would not previously be explicitly capable of measuring delivered cargo polypeptides directly. If a skilled person were to look only at DNA and RNA of AAV, a skilled person would obtain results that are not well correlated with actual localization or expression of a cargo polypeptide. Accordingly, the present example demonstrates quantification of expression and/or localization of different types of cargo polypeptides using different types delivery particles in a single experiment using a binder-barcode platform described herein.

[0644] It is an insight of the present disclosure that other forms of delivery vehicles (*e.g.*, viral particles, lipid-based particles ((*e.g.*, cell-produced or not cell-produced), lipid nanoparticles (LNPs), liposomes, micelles, extracellular vesicles (*e.g.*, exosomes, microparticles, etc.)), polymer-based particles (*e.g.*, PGLA-based), polysaccharide-based particles, etc.) and/or nucleic acids (*e.g.*, RNA) may be used in accordance with the Examples described herein.

[0645] Moreover, delivery particles and/or nucleic acids (*e.g.*, RNA) may be designed and/or tested via a binder-barcode platform to assess and/or quantify nucleic acids using a binder-barcode platform described herein to address challenges with correlating an amount of nucleic acid in a tissue with functional performance (*e.g.*, expression). Furthermore, the present example may be used to assess large pools of therapeutic polypeptides delivered simultaneously to cells, tissues, or organs. This workflow reduces a number of burdens of conventional methods, including production, assessment, and ability to capture functional readouts of cargo polypeptides and/or delivery particles in a high-throughput and multiplexed format.

Example 17: *In vivo* discovery and assessment of barcoded cargo polypeptides that cross the Blood-Brain Barrier (BBB) using AAV delivery particles

[0646] The present example demonstrates a multiplexed, high-throughput method of measuring a barcoded cargo polypeptide as described herein. Moreover, the present example demonstrates that a binder-barcode platform described herein can be used to identify and/or

quantify cargo polypeptides for certain phenotypes such as expression and/or localization. For example, in some embodiments, cargo polypeptides can be identified and/or assessed for ability to cross the blood-brain barrier (BBB) as described by this example.

Method:

[0647] A set of cargo polypeptides were measured via octet biolayer interferometry (BLI) to determine their respective dissociation against the transferrin receptor (TfR). Dissociation against TfR for another set of cargo polypeptides were determined via ELISA according to standard methods (see, *e.g.,* Engvall, E., Clinical Chemistry, Volume 56, Issue 2, 2010, Pages 319-320). Results of an ELISA assay are shown in **FIG. 22**. Each cargo component encoding a cargo polypeptide was then associated with a unique barcode component. Each peptide barcode encoded by a barcode component is associated (*e.g.*, covalently or not covalently) with the C-terminus of the cargo polypeptide via a linker. Nucleic acids (or constructs) were synthesized with cargo components including a sequence encoding a cargo polypeptide, promoter elements, terminator elements, and/or ITR elements for AAV packaging according to standard methods. Table 4 below shows experimental designs for constructs tested in this example.

[0648] Table 4. Different AAV delivery particles produced and their content

Construct #	Cargo	Barcode ID	AAV Serotype
1	bMB-TfR-116	bc-28	AAV-DJ
2	bMB-TfR-541	bc-211	AAV-DJ
3	bMB-TfR-305	bc-100	AAV-DJ
4	bMB-TfR-359	bc-170	AAV-DJ
5	bMB-TfR-085	bc-248	AAV-DJ
6	bMB-TfR-002	bc-266	AAV-DJ
7	bMB-TfR-353	bc-189	AAV-DJ
8	bMB-TfR-231	bc-280	AAV-DJ
9	bMB-TfR-267	bc-238	AAV-DJ
10	bMB-TfR-143	bc-8	AAV-DJ
11	bMB-TfR-300	bc-62	AAV-DJ
12	bMB-TfR-299	bc-84	AAV-DJ
13	bMB-TfR-248	bc-20	AAV-DJ

Construct #	Cargo	Barcode ID	AAV Serotype
14	bMB-TfR-106	bc-318	AAV-DJ
15	bMB-TfR-352	bc-161	AAV-DJ
16	bMB-TfR-139	bc-155	AAV-DJ
17	bMB-TfR-152	bc-27	AAV-DJ
18	bMB-TfR-154	bc-141	AAV-DJ
19	bMB-TfR-158	bc-135	AAV-DJ
20	bMB-TfR-160	bc-283	AAV-DJ
21	bMB-TfR-326	bc-43	AAV-DJ
22	bMB-TfR-354	bc-79	AAV-DJ
23	bMB-TfR-144	bc-224	AAV-DJ
24	bMB-TfR-121	bc-187	AAV-DJ
25	bMB-TfR-160	bc-33	AAV-DJ
26	bMB-TfR-117	bc-340	AAV-DJ
27	bMB-TfR-136	bc-276	AAV-DJ
28	bMB-TfR-318	bc-180	AAV-DJ
29	bMB-TfR-122	bc-86	AAV-DJ
30	bMB-TfR-236	bc-228	AAV-DJ
31	bMB-TfR-140	bc-45	AAV-DJ
32	bMB-TfR-343	bc-130	AAV-DJ
33	bMB-TfR-329	bc-4	AAV-DJ
34	bMB-TfR-193	bc-22	AAV-DJ
35	bMB-TfR-559	bc-10	AAV-DJ
36	bMB-TfR-086	bc-167	AAV-DJ
37	bMB-TfR-265	bc-251	AAV-DJ
38	bMB-TfR-324	bc-304	AAV-DJ
39	bMB-TfR-313	bc-292	AAV-DJ
40	bMB-TfR-356	bc-73	AAV-DJ
41	bMB-TfR-357	bc-168	AAV-DJ
42	bMB-TfR-379	bc-162	AAV-DJ
43	bMB-TfR-345	bc-32	AAV-DJ
44	bMB-TfR-016	bc-7	AAV-DJ
45	bMB-TfR-847	bc-278	AAV-DJ
46	bMB-TfR-994	bc-150	AAV-DJ
47	bMB-TfR-525	bc-17	AAV-DJ
48	bMB-TfR-387	bc-195	AAV-DJ
49	bMB-TfR-365	bc-249	AAV-DJ
50	bMB-TfR-334	bc-142	AAV-DJ

Construct #	Cargo	Barcode ID	AAV Serotype
51	bMB-TfR-151	bc-226	AAV-DJ
52	bMB-TfR-136	bc-122	AAV-DJ
53	bMB-TfR-310	bc-196	AAV-DJ
54	bMB-TfR-295	bc-93	AAV-DJ
55	bMB-TfR-302	bc-59	AAV-DJ
56	bMB-TfR-312	bc-25	AAV-DJ
57	bMB-TfR-321	bc-145	AAV-DJ
58	bMB-TfR-142	bc-40	AAV-DJ
59	bMB-TfR-989	bc-41	AAV-DJ
60	fc5_vhh	bc-270	AAV-DJ
61	lag16	bc-53	AAV-DJ
62	fc5_vhh	bc-116	AAV-DJ
63	lag16	bc-52	AAV-DJ
64	fc5_vhh	bc-327	AAV-DJ
65	lag16	bc-97	AAV-DJ
66	bMB-TfR-113	bc-28	AAV-DJ
67	bMB-TfR-896	bc-211	AAV-DJ
68	bMB-TfR-962	bc-100	AAV-DJ
69	bMB-TfR-309	bc-170	AAV-DJ
70	bMB-TfR-876	bc-248	AAV-DJ
71	bMB-TfR-064	bc-266	AAV-DJ
72	bMB-TfR-215	bc-189	AAV-DJ
73	bMB-TfR-141	bc-280	AAV-DJ
74	bMB-TfR-147	bc-238	AAV-DJ
75	bMB-TfR-979	bc-8	AAV-DJ
76	bMB-TfR-341	bc-62	AAV-DJ
77	bMB-TfR-383	bc-84	AAV-DJ
78	bMB-TfR-278	bc-20	AAV-DJ
79	bMB-TfR-445	bc-318	AAV-DJ
80	bMB-TfR-264	bc-161	AAV-DJ
81	bMB-TfR-109	bc-155	AAV-DJ
82	bMB-TfR-157	bc-27	AAV-DJ
83	bMB-TfR-151	bc-141	AAV-DJ
84	bMB-TfR-933	bc-135	AAV-DJ
85	bMB-TfR-157	bc-283	AAV-DJ
86	bMB-TfR-125	bc-43	AAV-DJ
87	bMB-TfR-306	bc-79	AAV-DJ

Construct #	Cargo	Barcode ID	AAV Serotype
88	bMB-TfR-192	bc-224	AAV-DJ
89	bMB-TfR-320	bc-187	AAV-DJ
90	bMB-TfR-126	bc-33	AAV-DJ
91	bMB-TfR-360	bc-340	AAV-DJ
92	bMB-TfR-953	bc-276	AAV-DJ
93	bMB-TfR-363	bc-180	AAV-DJ
94	bMB-TfR-250	bc-86	AAV-DJ
95	bMB-TfR-898	bc-228	AAV-DJ
96	bMB-TfR-888	bc-45	AAV-DJ
97	bMB-TfR-328	bc-130	AAV-DJ
98	bMB-TfR-348	bc-4	AAV-DJ
99	bMB-TfR-119	bc-22	AAV-DJ
100	bMB-TfR-296	bc-10	AAV-DJ
101	bMB-TfR-293	bc-167	AAV-DJ
102	bMB-TfR-350	bc-251	AAV-DJ
103	bMB-TfR-335	bc-304	AAV-DJ
104	bMB-TfR-155	bc-292	AAV-DJ
105	bMB-TfR-315	bc-73	AAV-DJ
106	bMB-TfR-373	bc-168	AAV-DJ
107	bMB-TfR-301	bc-162	AAV-DJ
108	bMB-TfR-872	bc-32	AAV-DJ
109	bMB-TfR-280	bc-7	AAV-DJ
110	bMB-TfR-140	bc-278	AAV-DJ
111	bMB-TfR-149	bc-150	AAV-DJ
112	bMB-TfR-126	bc-17	AAV-DJ
113	bMB-TfR-162	bc-195	AAV-DJ
114	bMB-TfR-143	bc-249	AAV-DJ
115	bMB-TfR-337	bc-142	AAV-DJ
116	bMB-TfR-123	bc-226	AAV-DJ
117	bMB-TfR-112	bc-122	AAV-DJ
118	bMB-TfR-393	bc-196	AAV-DJ
119	bMB-TfR-377	bc-93	AAV-DJ
120	bMB-TfR-107	bc-59	AAV-DJ
121	bMB-TfR-252	bc-25	AAV-DJ
122	bMB-TfR-319	bc-145	AAV-DJ
123	bMB-TfR-137	bc-40	AAV-DJ
124	bMB-TfR-098	bc-41	AAV-DJ

[0649] DNA of the aforementioned nucleic acid constructs were pooled in equimolar ratios to form two non-overlapping pools. These pools were then used to construct AAV delivery particles according to standard methods. Two pools were then titered using a qPCR assay to determine the genome copies present per ml (GC/mL). Titers for the two pools are shown in a table below.

[0650] Table 5. Table showing GC/mL for two pools AAV

Pool #	AAV Library	GC/mL
1	AAV-library-1	$7.48x10^{12}$ GC/mL
2	AAV-library-2	$1.82x10^{13}$ GC/mL

[0651] AAV delivery particles were diluted to $5X10^{11}$ GC /mL and 200 µL of each of the two AAV libraries was injected into transgenic female C57 containing a humanized transferrin receptor allele (Mouse strain C57BL/6-Tfr1tm1TFR1/Bcgen). Each AAV library was injected into 15 individual mice (n=15). Three (3) mice are euthanized at each time point (7 days, 14 days, 21 days, and 28 days). Mice are perfused with 2x blood volumes of Phosphate buffered Saline (PBS). Blood is then collected and serum is separated from blood. After perfusion, brain, liver, muscle, skin, heart, lung, kidney, stomach, intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described herein.

Results:

[0652] The present example demonstrates that a binder-barcode platform as described herein can quantify localization and/or expression of cargo polypeptide in certain tissues, including brain tissue, and can determine if said cargo polypeptide is also localizing and/or being expressed in other tissue (such as serum), including a ratio of amount of cargo polypeptide being expressed across different tissue types. For example, data in **FIG. 21** shows respective

dissociation of cargo polypeptides against the transferrin receptor (TfR1) according to embodiments described herein.

[0653] Moreover, delivery particles, including AAV delivery particles, provide continuous expression of cargo polypeptides, which increases overall sensitivity of a binder-barcode platform as described herein. Ability to quantify potent and functional delivery of cargo polypeptides across different tissues can help identify potent cargo polypeptides that not only cross the BBB but are retained and/or more significantly expressed in brain tissue compared to other tissues.

[0654] It is an insight of the present disclosure that other forms of delivery vehicles (*e.g.*, viral particles, lipid-based particles ((*e.g.*, cell-produced or not cell-produced), lipid nanoparticles (LNPs), liposomes, micelles, extracellular vesicles (*e.g.*, exosomes, microparticles, etc.)), polymer-based particles (*e.g.*, PGLA-based), polysaccharide-based particles, etc.) and/or nucleic acids (*e.g.*, RNA) may be used in accordance with the Examples described herein.

[0655] Accordingly, the present example demonstrates a multiplexed and high-throughput approach that can be used to identify and quantify cargo polypeptides that cross the BBB.

Example 18: *In vivo* discovery and assessment of barcoded cargo polypeptides that target tumors using AAV delivery particles

[0656] The present example demonstrates a multiplexed method of measuring pooled barcoded cargo polypeptides to assess tumor targeting using AAV delivery particles and a binder-barcode platform as described herein.

Method:

[0657] A set of cargo polypeptides is measured via octet biolayer interferometry (BLI) to determine their respective dissociation against a tumor target. Another set of cargo polypeptides is determined via ELISA according to standard methods (see, *e.g.,* Engvall, E., Clinical Chemistry, Volume 56, Issue 2, 2010, Pages 319-320). Each cargo component encoding a cargo polypeptide was then associated with a unique barcode component. Each peptide barcode

encoded by a barcode component is associated (*e.g.*, covalently or not covalently) with the C-terminus of the cargo polypeptide via a linker. Nucleic acids (or constructs) were synthesized with cargo components including a sequence encoding a cargo polypeptide, promoter elements, terminator elements, and/or ITR elements for AAV packaging according to standard methods. Table 6 below shows experimental designs for constructs tested in this example.

[0658] Table 6. Different AAV delivery particles produced and their content

Construct #	Cargo	Barcode ID	AAV Serotype
1	bMB-N4-3	1	AAV-8
2	bMB-N4-4	2	AAV-8
3	bMB-N4-8	3	AAV-8
4	bMB-N4-11	4	AAV-8
5	bMB-N4-13	5	AAV-8
6	bMB-N4-34	6	AAV-8
7	bMB-N4-40	7	AAV-8
8	bMB-N4-52	8	AAV-8
9	bMB-N4-53	9	AAV-8
10	bMB-N4-59	10	AAV-8
11	bMB-N4-69	11	AAV-8
12	bMB-N4-79	12	AAV-8
13	bMB-N4-82	13	AAV-8
14	bMB-N4-86	14	AAV-8
15	bMB-N4-87	15	AAV-8
16	bMB-N4-94	16	AAV-8
17	bMB-N4-98	17	AAV-8
18	bMB-N4-99	18	AAV-8
19	bMB-N4-102	19	AAV-8
20	bMB-N4-103	20	AAV-8
21	bMB-N4-108	21	AAV-8
22	bMB-N4-111	22	AAV-8
23	bMB-N4-113	23	AAV-8
24	bMB-N4-115	24	AAV-8
25	bMB-N4-119	25	AAV-8
26	bMB-N4-122	26	AAV-8
27	bMB-N4-126	27	AAV-8
28	bMB-N4-127	28	AAV-8
29	bMB-N4-131	29	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
30	bMB-N4-135	30	AAV-8
31	bMB-N4-138	31	AAV-8
32	bMB-N4-143	32	AAV-8
33	bMB-N4-146	33	AAV-8
34	bMB-N4-147	34	AAV-8
35	bMB-N4-148	35	AAV-8
36	bMB-N4-154	36	AAV-8
37	bMB-N4-155	37	AAV-8
38	bMB-N4-160	38	AAV-8
39	bMB-N4-162	39	AAV-8
40	bMB-N4-166	40	AAV-8
41	bMB-N4-170	41	AAV-8
42	bMB-N4-179	42	AAV-8
43	bMB-N4-183	43	AAV-8
44	bMB-N4-185	44	AAV-8
45	bMB-N4-190	45	AAV-8
46	bMB-N4-193	46	AAV-8
47	bMB-N4-194	47	AAV-8
48	bMB-N4-198	48	AAV-8
49	bMB-N4-199	49	AAV-8
50	bMB-N4-208	50	AAV-8
51	bMB-N4-209	51	AAV-8
52	bMB-N4-215	52	AAV-8
53	bMB-N4-216	53	AAV-8
54	bMB-N4-219	54	AAV-8
55	bMB-N4-220	55	AAV-8
56	bMB-N4-221	56	AAV-8
57	bMB-N4-222	57	AAV-8
58	bMB-N4-223	58	AAV-8
59	bMB-N4-234	59	AAV-8
60	bMB-N4-236	60	AAV-8
61	bMB-N4-240	61	AAV-8
62	bMB-N4-248	62	AAV-8
63	bMB-N4-251	63	AAV-8
64	bMB-N4-253	64	AAV-8
65	bMB-N4-264	65	AAV-8
66	bMB-N4-295	66	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
67	bMB-N4-297	67	AAV-8
68	bMB-N4-314	68	AAV-8
69	bMB-N4-325	69	AAV-8
70	bMB-N4-327	70	AAV-8
71	bMB-N4-349	71	AAV-8
72	bMB-N4-351	72	AAV-8
73	bMB-N4-356	73	AAV-8
74	bMB-N4-359	74	AAV-8
75	bMB-N4-364	75	AAV-8
76	bMB-N4-377	76	AAV-8
77	bMB-N4-385	77	AAV-8
78	bMB-N4-387	78	AAV-8
79	bMB-N4-388	79	AAV-8
80	bMB-N4-389	80	AAV-8
81	bMB-N4-390	81	AAV-8
82	bMB-N4-394	82	AAV-8
83	bMB-N4-398	83	AAV-8
84	bMB-N4-400	84	AAV-8
85	bMB-N4-401	85	AAV-8
86	bMB-N4-409	86	AAV-8
87	bMB-N4-446	87	AAV-8
88	bMB-N4-448	88	AAV-8
89	bMB-N4-467	89	AAV-8
90	bMB-N4-483	90	AAV-8
91	bMB-N4-559	91	AAV-8
92	bMB-N4-627	92	AAV-8
93	bMB-N4-638	93	AAV-8
94	bMB-N4-768	94	AAV-8
95	bMB-N4-772	95	AAV-8
96	bMB-N4-775	96	AAV-8
97	bMB-N4-785	97	AAV-8
98	bMB-N4-787	98	AAV-8
99	bMB-N4-788	99	AAV-8
100	bMB-N4-789	100	AAV-8
101	bMB-N4-790	101	AAV-8
102	bMB-N4-796	102	AAV-8
103	bMB-N4-797	103	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
104	bMB-N4-810	104	AAV-8
105	bMB-N4-819	105	AAV-8
106	bMB-N4-820	106	AAV-8
107	bMB-N4-822	107	AAV-8
108	bMB-N4-846	108	AAV-8
109	bMB-N4-857	109	AAV-8
110	bMB-N4-860	110	AAV-8
111	bMB-N4-861	111	AAV-8
112	bMB-N4-870	112	AAV-8
113	bMB-N4-876	113	AAV-8
114	bMB-N4-877	114	AAV-8
115	bMB-N4-879	115	AAV-8
116	bMB-N4-881	116	AAV-8
117	bMB-N4-883	117	AAV-8
118	bMB-N4-890	118	AAV-8
119	bMB-N4-922	119	AAV-8
120	bMB-N4-934	120	AAV-8
121	bMB-N4-940	121	AAV-8
122	bMB-N4-942	122	AAV-8
123	bMB-N4-955	123	AAV-8
124	bMB-N4-960	124	AAV-8
125	bMB-N4-975	125	AAV-8
126	bMB-N4-985	126	AAV-8
127	bMB-N4-988	127	AAV-8
128	bMB-N4-997	128	AAV-8
129	bMB-N4-1000	129	AAV-8
130	bMB-N4-1003	130	AAV-8
131	bMB-N4-1004	131	AAV-8
132	bMB-N4-1007	132	AAV-8
133	bMB-N4-1009	133	AAV-8
134	bMB-N4-1011	134	AAV-8
135	bMB-N4-1023	135	AAV-8
136	bMB-N4-1025	136	AAV-8
137	bMB-N4-1031	137	AAV-8
138	bMB-N4-1032	138	AAV-8
139	bMB-N4-1033	139	AAV-8
140	bMB-N4-1037	140	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
141	bMB-N4-1050	141	AAV-8
142	bMB-N4-1052	142	AAV-8
143	bMB-N4-1059	143	AAV-8
144	bMB-N4-1060	144	AAV-8
145	bMB-N4-1063	145	AAV-8
146	bMB-N4-1065	146	AAV-8
147	bMB-N4-1068	147	AAV-8
148	bMB-N4-1069	148	AAV-8
149	bMB-N4-1071	149	AAV-8
150	bMB-N4-1072	150	AAV-8
151	bMB-N4-1093	151	AAV-8
152	bMB-N4-1097	152	AAV-8
153	bMB-N4-1098	153	AAV-8
154	bMB-N4-1103	154	AAV-8
155	bMB-N4-1106	155	AAV-8
156	bMB-N4-1124	156	AAV-8
157	bMB-N4-1131	157	AAV-8
158	bMB-N4-1132	158	AAV-8
159	bMB-N4-1133	159	AAV-8
160	bMB-N4-1135	160	AAV-8
161	bMB-N4-1137	161	AAV-8
162	bMB-N4-1141	162	AAV-8
163	bMB-N4-1143	163	AAV-8
164	bMB-N4-1145	164	AAV-8
165	bMB-N4-1161	165	AAV-8
166	bMB-N4-1162	166	AAV-8
167	bMB-N4-1167	167	AAV-8
168	bMB-N4-1172	168	AAV-8
169	bMB-N4-1177	169	AAV-8
170	bMB-N4-1180	170	AAV-8
171	bMB-N4-1184	171	AAV-8
172	bMB-N4-1193	172	AAV-8
173	bMB-N4-1196	173	AAV-8
174	bMB-N4-1205	174	AAV-8
175	bMB-N4-1206	175	AAV-8
176	bMB-N4-1210	176	AAV-8
177	bMB-N4-1215	177	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
178	bMB-N4-1217	178	AAV-8
179	bMB-N4-1223	179	AAV-8
180	bMB-N4-1230	180	AAV-8
181	bMB-N4-1239	181	AAV-8
182	bMB-N4-1250	182	AAV-8
183	bMB-N4-1251	183	AAV-8
184	bMB-N4-1252	184	AAV-8
185	bMB-N4-1257	185	AAV-8
186	bMB-N4-1259	186	AAV-8
187	bMB-N4-1277	187	AAV-8
188	bMB-N4-1278	188	AAV-8
189	bMB-N4-1279	189	AAV-8
190	bMB-N4-1284	190	AAV-8
191	bMB-N4-1292	191	AAV-8
192	bMB-N4-1293	192	AAV-8
193	bMB-N4-1307	193	AAV-8
194	bMB-N4-1310	194	AAV-8
195	bMB-N4-1312	195	AAV-8
196	bMB-N4-1313	196	AAV-8
197	bMB-N4-1330	197	AAV-8
198	bMB-N4-1338	198	AAV-8
199	bMB-N4-1357	199	AAV-8
200	bMB-N4-1360	200	AAV-8
201	bMB-N4-1361	201	AAV-8
202	bMB-N4-1362	202	AAV-8
203	bMB-N4-1363	203	AAV-8
204	bMB-N4-1367	204	AAV-8
205	bMB-N4-1371	205	AAV-8
206	bMB-N4-1379	206	AAV-8
207	bMB-N4-1381	207	AAV-8
208	bMB-N4-1388	208	AAV-8
209	bMB-N4-1398	209	AAV-8
210	bMB-N4-1407	210	AAV-8
211	bMB-N4-1408	211	AAV-8
212	bMB-N4-1414	212	AAV-8
213	bMB-N4-1416	213	AAV-8
214	bMB-N4-1417	214	AAV-8

Construct #	Cargo	Barcode ID	AAV Serotype
215	bMB-N4-1425	215	AAV-8
216	bMB-N4-1436	216	AAV-8
217	bMB-N4-1443	217	AAV-8
218	bMB-N4-1447	218	AAV-8
219	bMB-N4-1452	219	AAV-8
220	bMB-N4-1467	220	AAV-8

[0659] DNA of the aforementioned nucleic acid constructs are pooled in equimolar ratios to form two non-overlapping pools. These pools are then used to construct AAV delivery particles according to standard methods. Two pools are then titered using a qPCR assay to determine the genome copies present per ml (GC/mL).

[0660] AAV delivery particles are diluted to about $5X10^{11}$GC /mL and 200 µL of each of the two AAV libraries is injected via tail vein into transgenic NSG mice. In parallel, the mice are injected subcutaneously with $5*10^6$ SW780 cells, a cell line exhibiting epithelial morphology that was isolated from the urinary bladder of a human patient with transitional cell carcinoma, in the left flank, and $5*10^6$ HCT116 cells, a cell line that was isolated from the colon of an adult human male with colon cancer, in the right flank in a 200 µL volume composed of 50% Matrigel (Corning® Matrigel® Matrix) and 50% DMEM. When tumors reach 200-250 mm^3 in size, mice are euthanized. Mice are perfused with 2x blood volumes of Phosphate buffered Saline (PBS). Blood is then collected and serum is separated from blood. After perfusion, brain, liver, muscle, skin, heart, lung, kidney, stomach, intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described previously herein.

Discussion:

[0661] The present example demonstrates that a binder-barcode platform as described herein can quantify localization and/or expression of cargo polypeptide in certain tissues, including tumor tissue. Moreover, the present example demonstrates that a binder-barcode platform as described herein can determine if said cargo polypeptide is also localizing and/or

being expressed in other tissue (such as serum), including a ratio of amount of cargo polypeptide being expressed across different tissue types.

[0662] Moreover, delivery particles, including AAV delivery particles, provide continuous expression of cargo polypeptides, which increases overall sensitivity of a binder-barcode platform as described herein. Ability to quantify targeted and functional delivery of cargo polypeptides across different tissues can help determine potent cargo polypeptides that not only specifically target tumor tissues but are retained and/or more significantly expressed in tumor tissue compared to other tissues.

[0663] It is an insight of the present disclosure that other forms of delivery vehicles (*e.g.*, viral particles, lipid-based particles ((*e.g.*, cell-produced or not cell-produced), lipid nanoparticles (LNPs), liposomes, micelles, extracellular vesicles (*e.g.*, exosomes, microparticles, etc.)), polymer-based particles (*e.g.*, PGLA-based), polysaccharide-based particles, etc.) and/or nucleic acids (*e.g.*, RNA) may be used in accordance with the Examples described herein.

[0664] Accordingly, the present example demonstrates a multiplexed and high-throughput approach that can be used to identify and quantify cargo polypeptides that target tumor tissue.

Example 19: *In vivo* discovery and assessment of barcoded cargo polypeptides delivered using Lipid Nanoparticles (LNPs)

[0665] The present example demonstrates a multiplexed method of measuring barcoded polypeptides administered in a pooled mixture using a lipid nanoparticle delivery particles (LNPs) and a binder-barcode platform as described herein.

Method:

[0666] LNPs containing a barcoded cargo component are synthesized according to methods known in the art. 1 mg of LNP for each barcoded cargo component is generated. A table describing different variations of LNPs and/or cargo components is provided below. LNP Formulation "A" refers to an exemplary formulation that may be used in accordance with

embodiments of the present disclosure. In other embodiments, other types of formulations may be used.

[0667] Table 7. Different LNP variants produced and their content

Construct #	Cargo	Barcode ID	LNP Formulation
LNP1	ALFA-Sec-GOI	1	A
LNP2	ALFA-Sec-GOI	2	A
LNP3	ALFA-Sec-GOI	3	A
LNP4	ALFA-GOI	4	A
LNP5	ALFA-GOI	5	A
LNP6	ALFA-GOI	6	A

[0668] LNPs are pooled equally by mass and 200 µg of the LNP pool is injected via tail vein into female C57 mice (n=10). At 24 hour time point, mice are euthanized and brain, liver, muscle, skin, heart, lung, kidney, stomach, intestine tissues are collected. Tissue is then lysed and subjected to decoding as described herein.

Discussion:

[0669] Accordingly, the present example demonstrates that multiple cargo polypeptides can be delivered using pooled LNPs and assessed in parallel. An amount of each barcoded component delivered to each tissue of the mouse is readily assessed using a binder-barcode platform. Moreover, a functional readout, including expression of each cargo polypeptide, is assessed and quantified using a binder-barcode platform described herein.

Example 20: *In vivo* discovery and assessment of tissue-specific barcoded cargo polypeptides using AAV delivery particles

[0670] The present example demonstrates a multiplexed method of measuring a barcoded polypeptide to assess tissue-specific expression of a cargo polypeptide using a binder-barcode platform as described herein. Moreover, the present example describes methods for quantifying and/or assessing a library of cargo components that encode a cargo polypeptide. As described herein, in some embodiments, each cargo component may further comprise a tissue-specific promoter. As described herein, in some embodiments, each cargo component may further

comprise an untranslated region (UTR). As described herein, in some embodiments, each cargo component may further comprise a tissue-specific promoter and a UTR. In such an embodiment, the present example describes demonstrates quantification of effects of promoters on cargo polypeptide expression and/or localization. A binder-barcode platform described herein is used to quantify effects of cargo polypeptide localization and/or expression based on additional variables included in the cargo component As such, a binder-barcode platform as described herein can be used to identify cargo components based on desired phenotypes of cargo polypeptides.

[0671] Other types of cargo components and modifications to a cargo polypeptide can be assessed using a binder-barcode platform as described herein. Phenotypes other than expression and localization can also be quantified and/or assessed using a binder-barcode platform as described herein.

Method:

[0672] Different nucleic acids comprising barcoded cargo components were designed as follows. Cargo components that encode a cargo polypeptide can also include other components such as promoters, UTRs, or combinations thereof. Each cargo component encoding a cargo polypeptide was then associated with a unique barcode component. Each peptide barcode encoded by a barcode component is associated (*e.g.*, covalently or not covalently) with the C-terminus of the cargo polypeptide via a linker. Nucleic acids (or constructs) were synthesized with cargo components including a sequence encoding a cargo polypeptide, promoter elements, terminator elements, and/or ITR elements for packaging into AAV delivery particles. A table describing different variations of AAV delivery particles and/or cargo components is provided below.

[0673] Table 8. Different variants produced and their content

Construct #	Cargo	Barcode ID	AAV Serotype	Promoter	UTR
1	ALFA-Sec-GOI	1	AAV-9	CMV	WPRE
2	ALFA-Sec-GOI	2	AAV-9	CMV+intron	WPRE
3	ALFA-Sec-GOI	3	AAV-9	EF1A	WPRE

Construct #	Cargo	Barcode ID	AAV Serotype	Promoter	UTR
4	ALFA-Sec-GOI	4	AAV-9	EFS	WPRE
5	ALFA-Sec-GOI	5	AAV-9	CAG	WPRE
6	ALFA-Sec-GOI	6	AAV-9	CBh	WPRE
7	ALFA-Sec-GOI	7	AAV-9	CBA	WPRE
8	ALFA-Sec-GOI	8	AAV-9	SFFV	WPRE
9	ALFA-Sec-GOI	9	AAV-9	MSCV	WPRE
10	ALFA-Sec-GOI	10	AAV-9	SV40	WPRE
11	ALFA-Sec-GOI	11	AAV-9	mPGK	WPRE
12	ALFA-Sec-GOI	12	AAV-9	hPGK	WPRE
13	ALFA-Sec-GOI	13	AAV-9	UBC	WPRE
14	ALFA-Sec-GOI	14	AAV-9	Mammalian Tissue-Specific Promoters	WPRE
15	ALFA-Sec-GOI	15	AAV-9	Name	WPRE
16	ALFA-Sec-GOI	16	AAV-9	Nanog	WPRE
17	ALFA-Sec-GOI	17	AAV-9	Nes	WPRE
18	ALFA-Sec-GOI	18	AAV-9	Tuba1a	WPRE
19	ALFA-Sec-GOI	19	AAV-9	Camk2a(long)	WPRE
20	ALFA-Sec-GOI	20	AAV-9	Camk2a(short)	WPRE
21	ALFA-Sec-GOI	21	AAV-9	SYN1	WPRE
22	ALFA-Sec-GOI	22	AAV-9	Hb9	WPRE
23	ALFA-Sec-GOI	23	AAV-9	Th	WPRE
24	ALFA-Sec-GOI	24	AAV-9	Thy1	WPRE
25	ALFA-Sec-GOI	25	AAV-9	NSE	WPRE
26	ALFA-Sec-GOI	26	AAV-9	GFAP(long)	WPRE
27	ALFA-Sec-GOI	27	AAV-9	GFAP(short)	WPRE
28	ALFA-Sec-GOI	28	AAV-9	Iba1	WPRE
29	ALFA-Sec-GOI	29	AAV-9	ProA1	WPRE
30	ALFA-Sec-GOI	30	AAV-9	hRHO	WPRE
31	ALFA-Sec-GOI	31	AAV-9	hBEST1	WPRE
32	ALFA-Sec-GOI	32	AAV-9	Grm6	WPRE
33	ALFA-Sec-GOI	33	AAV-9	Grm6(short)	WPRE
34	ALFA-Sec-GOI	34	AAV-9	Grm6(long)	WPRE
35	ALFA-Sec-GOI	35	AAV-9	Prnp	WPRE
36	ALFA-Sec-GOI	36	AAV-9	Cnp	WPRE
37	ALFA-Sec-GOI	37	AAV-9	K14	WPRE
38	ALFA-Sec-GOI	38	AAV-9	K19	WPRE

Construct #	Cargo	Barcode ID	AAV Serotype	Promoter	UTR
39	ALFA-Sec-GOI	39	AAV-9	BK5	WPRE
40	ALFA-Sec-GOI	40	AAV-9	mTyr	WPRE
41	ALFA-Sec-GOI	41	AAV-9	cTnT	WPRE
42	ALFA-Sec-GOI	42	AAV-9	αMHC(long)	WPRE
43	ALFA-Sec-GOI	43	AAV-9	αMHC(short)	WPRE
44	ALFA-Sec-GOI	44	AAV-9	Myog	WPRE
45	ALFA-Sec-GOI	45	AAV-9	ACTA1	WPRE
46	ALFA-Sec-GOI	46	AAV-9	MHCK7	WPRE
47	ALFA-Sec-GOI	47	AAV-9	SM22a	WPRE
48	ALFA-Sec-GOI	48	AAV-9	EnSM22a	WPRE
49	ALFA-Sec-GOI	49	AAV-9	Runx2	WPRE
50	ALFA-Sec-GOI	50	AAV-9	OC	WPRE
51	ALFA-Sec-GOI	51	AAV-9	Col1a1	WPRE
52	ALFA-Sec-GOI	52	AAV-9	Col2a1	WPRE
53	ALFA-Sec-GOI	53	AAV-9	aP2	WPRE
54	ALFA-Sec-GOI	54	AAV-9	Adipoq	WPRE
55	ALFA-Sec-GOI	55	AAV-9	Tie1	WPRE
56	ALFA-Sec-GOI	56	AAV-9	Cd144	WPRE
57	ALFA-Sec-GOI	57	AAV-9	CD68(short)	WPRE
58	ALFA-Sec-GOI	58	AAV-9	CD68(long)	WPRE
59	ALFA-Sec-GOI	59	AAV-9	CD11b	WPRE
60	ALFA-Sec-GOI	60	AAV-9	Afp	WPRE
61	ALFA-Sec-GOI	61	AAV-9	Alb	WPRE
62	ALFA-Sec-GOI	62	AAV-9	TBG	WPRE
63	ALFA-Sec-GOI	63	AAV-9	TBG+intron	WPRE
64	ALFA-Sec-GOI	64	AAV-9	MMTV	WPRE
65	ALFA-Sec-GOI	65	AAV-9	Wap	WPRE
66	ALFA-Sec-GOI	66	AAV-9	HIP	WPRE
67	ALFA-Sec-GOI	67	AAV-9	Pdx1	WPRE
68	ALFA-Sec-GOI	68	AAV-9	Ins2	WPRE
69	ALFA-Sec-GOI	69	AAV-9	Hcn4	WPRE
70	ALFA-Sec-GOI	70	AAV-9	NPHS2	WPRE
71	ALFA-Sec-GOI	71	AAV-9	SPB	WPRE
72	ALFA-Sec-GOI	72	AAV-9	CD144	WPRE
73	ALFA-Sec-GOI	73	AAV-9	TERT	WPRE
74	ALFA-Sec-GOI	74	AAV-9	CMV	oPRE
75	ALFA-Sec-GOI	75	AAV-9	CMV+intron	oPRE

Construct #	Cargo	Barcode ID	AAV Serotype	Promoter	UTR
76	ALFA-Sec-GOI	76	AAV-9	EF1A	oPRE
77	ALFA-Sec-GOI	77	AAV-9	EFS	oPRE
78	ALFA-Sec-GOI	78	AAV-9	CAG	oPRE
79	ALFA-Sec-GOI	79	AAV-9	CBh	oPRE
80	ALFA-Sec-GOI	80	AAV-9	CBA	oPRE
81	ALFA-Sec-GOI	81	AAV-9	SFFV	oPRE
82	ALFA-Sec-GOI	82	AAV-9	MSCV	oPRE
83	ALFA-Sec-GOI	83	AAV-9	SV40	oPRE
84	ALFA-Sec-GOI	84	AAV-9	mPGK	oPRE
85	ALFA-Sec-GOI	85	AAV-9	hPGK	oPRE
86	ALFA-Sec-GOI	86	AAV-9	UBC	oPRE
87	ALFA-Sec-GOI	87	AAV-9	Mammalian Tissue-Specific Promoters	oPRE
88	ALFA-Sec-GOI	88	AAV-9	Name	oPRE
89	ALFA-Sec-GOI	89	AAV-9	Nanog	oPRE
90	ALFA-Sec-GOI	90	AAV-9	Nes	oPRE
91	ALFA-Sec-GOI	91	AAV-9	Tuba1a	oPRE
92	ALFA-Sec-GOI	92	AAV-9	Camk2a(long)	oPRE
93	ALFA-Sec-GOI	93	AAV-9	Camk2a(short)	oPRE
94	ALFA-Sec-GOI	94	AAV-9	SYN1	oPRE
95	ALFA-Sec-GOI	95	AAV-9	Hb9	oPRE
96	ALFA-Sec-GOI	96	AAV-9	Th	oPRE
97	ALFA-Sec-GOI	97	AAV-9	Thy1	oPRE
98	ALFA-Sec-GOI	98	AAV-9	NSE	oPRE
99	ALFA-Sec-GOI	99	AAV-9	GFAP(long)	oPRE
100	ALFA-Sec-GOI	100	AAV-9	GFAP(short)	oPRE
101	ALFA-Sec-GOI	101	AAV-9	Iba1	oPRE
102	ALFA-Sec-GOI	102	AAV-9	ProA1	oPRE
103	ALFA-Sec-GOI	103	AAV-9	hRHO	oPRE
104	ALFA-Sec-GOI	104	AAV-9	hBEST1	oPRE
105	ALFA-Sec-GOI	105	AAV-9	Grm6	oPRE
106	ALFA-Sec-GOI	106	AAV-9	Grm6(short)	oPRE
107	ALFA-Sec-GOI	107	AAV-9	Grm6(long)	oPRE
108	ALFA-Sec-GOI	108	AAV-9	Prnp	oPRE
109	ALFA-Sec-GOI	109	AAV-9	Cnp	oPRE
110	ALFA-Sec-GOI	110	AAV-9	K14	oPRE

Construct #	Cargo	Barcode ID	AAV Serotype	Promoter	UTR
111	ALFA-Sec-GOI	111	AAV-9	K19	oPRE
112	ALFA-Sec-GOI	112	AAV-9	BK5	oPRE
113	ALFA-Sec-GOI	113	AAV-9	mTyr	oPRE
114	ALFA-Sec-GOI	114	AAV-9	cTnT	oPRE
115	ALFA-Sec-GOI	115	AAV-9	αMHC(long)	oPRE
116	ALFA-Sec-GOI	116	AAV-9	αMHC(short)	oPRE
117	ALFA-Sec-GOI	117	AAV-9	Myog	oPRE
118	ALFA-Sec-GOI	118	AAV-9	ACTA1	oPRE
119	ALFA-Sec-GOI	119	AAV-9	MHCK7	oPRE
120	ALFA-Sec-GOI	120	AAV-9	SM22a	oPRE
121	ALFA-Sec-GOI	121	AAV-9	EnSM22a	oPRE
122	ALFA-Sec-GOI	122	AAV-9	Runx2	oPRE
123	ALFA-Sec-GOI	123	AAV-9	OC	oPRE
124	ALFA-Sec-GOI	124	AAV-9	Col1a1	oPRE
125	ALFA-Sec-GOI	125	AAV-9	Col2a1	oPRE
126	ALFA-Sec-GOI	126	AAV-9	aP2	oPRE
127	ALFA-Sec-GOI	127	AAV-9	Adipoq	oPRE
128	ALFA-Sec-GOI	128	AAV-9	Tie1	oPRE
129	ALFA-Sec-GOI	129	AAV-9	Cd144	oPRE
130	ALFA-Sec-GOI	130	AAV-9	CD68(short)	oPRE
131	ALFA-Sec-GOI	131	AAV-9	CD68(long)	oPRE
132	ALFA-Sec-GOI	132	AAV-9	CD11b	oPRE
133	ALFA-Sec-GOI	133	AAV-9	Afp	oPRE
134	ALFA-Sec-GOI	134	AAV-9	Alb	oPRE
135	ALFA-Sec-GOI	135	AAV-9	TBG	oPRE
136	ALFA-Sec-GOI	136	AAV-9	TBG+intron	oPRE
137	ALFA-Sec-GOI	137	AAV-9	MMTV	oPRE
138	ALFA-Sec-GOI	138	AAV-9	Wap	oPRE
139	ALFA-Sec-GOI	139	AAV-9	HIP	oPRE
140	ALFA-Sec-GOI	140	AAV-9	Pdx1	oPRE
141	ALFA-Sec-GOI	141	AAV-9	Ins2	oPRE
142	ALFA-Sec-GOI	142	AAV-9	Hcn4	oPRE
143	ALFA-Sec-GOI	143	AAV-9	NPHS2	oPRE
144	ALFA-Sec-GOI	144	AAV-9	SPB	oPRE
145	ALFA-Sec-GOI	145	AAV-9	CD144	oPRE
146	ALFA-Sec-GOI	146	AAV-9	TERT	oPRE

[0674] DNA of the aforementioned nucleic acid constructs is pooled in equimolar ratios. This is then used to construct AAV delivery particles according to standard methods. Pools are then titered using a qPCR assay to determine the genome copies present per ml (GC/mL).

[0675] AAV delivery particles are diluted to about $5X10^{11}$GC /mL and 200 µL of the AAV libraries is injected into transgenic mice

[0676] Mice are euthanized at 7 days, 14 days, 21 days, and 28 days. At these endpoints, blood is collected and serum is separated from blood. Afterwards, mice are perfused with 2x blood volumes of Phosphate buffered Saline (PBS) and brain, liver, muscle, skin, heart, lung, kidney, stomach and intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described herein.

[0677] In some embodiments, nucleic acids (constructs) are also delivered to mice via other types of delivery particles, including LNPs (see, *e.g.*, Example 4). Mice are euthanized at 7, 14, 21 and/or 28 days. Blood is collected and serum is separated from blood. Then, mice are perfused with PBS and brain, liver, muscle, skin, heart, lung, kidney, stomach and intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described herein.

Discussion:

[0678] Accordingly, the present example demonstrates the ability to measure localization and/or expression of multiple cargo polypeptides delivered through different delivery particles (*e.g.*, AAV, lipid-based, etc.) in parallel. Accordingly, multiple cargo component designs can be assessed in parallel across multiple tissues of interest as described herein.

Example 21: *In vivo* inducible expression of barcoded cargo polypeptides delivered using AAV delivery particles

[0679] The present example demonstrates a multiplexed method of measuring a barcoded cargo polypeptide to assess inducible expression of the cargo polypeptide delivered using delivery particles as described herein.

[0680] Other types of cargo components and modifications to a cargo polypeptide can be assessed using a binder-barcode platform as described herein. Phenotypes other than expression and localization can also be quantified and/or assessed using a binder-barcode platform as described herein.

Method:

[0681] Different nucleic acids comprising barcoded cargo components were designed as follows. Cargo components that encode a cargo polypeptide, can include other components such as inducible promoters. In some embodiments, inducible promoters require an exogenous signal (*e.g.*, Doxycycline (DOX)) to be activated and drive expression of a cargo polypeptide. Each cargo component encoding a cargo polypeptide was then associated with a unique barcode component. Each peptide barcode encoded by a barcode component is associated (*e.g.*, covalently or not covalently) with the C-terminus of the cargo polypeptide via a linker. Nucleic acids (or constructs) were synthesized with cargo components including a sequence encoding a cargo polypeptide, promoter elements, terminator elements, and/or ITR elements for packing into AAV delivery particles. A table describing different variations of AAV delivery particles and/or cargo components is provided below.

[0682] Table 9. Different variants produced and their content

Construct #	AAV Serotype	Cargo	Barcode ID	Promotor	Exogenous Signal Required
1	AAV-9	ALFA-Sec-GOI	1	Tert	Tetracycline
2	AAV-9	ALFA-Sec-GOI	2	Dox	Doxycycline

[0683] DNA of the aforementioned nucleic acid constructs is pooled in equimolar ratios. This is then used to construct AAV delivery particles according to standard methods. Pools are then titered using a qPCR assay to determine the genome copies present per ml (GC/mL).

[0684] AAV delivery particles are diluted to about $5X10^{11}$GC /mL and 200 µL of the AAV libraries is injected into transgenic mice. Mice are euthanized at 7 days, 14 days, 21 days, and 28 days. At these endpoints, blood is collected and serum is separated from blood.

Afterwards, mice are perfused with 2x blood volumes of Phosphate buffered Saline (PBS) and brain, liver, muscle, skin, heart, lung, kidney, stomach and intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described herein.

[0685] In some embodiments, nucleic acids (constructs) are also delivered to mice via other types of delivery particles, including LNPs (see, *e.g.*, Example 4). Mice are euthanized at 7, 14, 21 and/or 28 days. Blood is collected and serum is separated from blood. Then, mice are perfused with PBS and brain, liver, muscle, skin, heart, lung, kidney, stomach and intestine tissues are all collected. Tissue is then lysed and subjected to decoding as described herein.

Discussion:

[0686] Accordingly, the present example demonstrates the ability to measure localization and/or expression of multiple cargo polypeptides delivered through different delivery particles (*e.g.*, AAV, lipid-based, etc.) in parallel. Accordingly, multiple cargo component designs can be assessed in parallel across multiple tissues of interest as described herein.

Example 22: *In vivo* discovery and assessment of tissue-specific barcoded cargo polypeptides using lentivirus delivery particles

[0687] Example 20 is repeated using lentivirus delivery particles.

Example 23: *In vivo* multiplexed screening of TfR1 liganding moieties that cross the Blood-Brain Barrier (BBB)

[0688] The present example demonstrates a multiplexed, high-throughput method of screening liganding moieties as described herein. The present example also demonstrates that a binder-barcode platform described herein can be used to assess inducible expression of a delivered cargo polypeptide. Moreover, the present example demonstrates that a binder-barcode platform described herein can be used to identify and/or quantify cargo polypeptides and/or components thereof for certain phenotypes such as expression and/or localization. For example, in some embodiments, components of cargo polypeptides (e.g., liganding moieties) can be

identified and/or assessed for ability to cross the blood-brain barrier (BBB) as described by this example. In some embodiments, a liganding moiety is or comprises a shuttle moiety.

[0689] Other types of cargo components can be assessed using a binder-barcode platform as described herein. Phenotypes other than expression and localization can also be quantified and/or assessed using a binder-barcode platform as described herein. Exemplary phenotypes include epitope properties, affinity, thermostability, pH sensitivity, or other phenotypes described herein.

Method:

[0690] Transferrin receptor 1 (TfR1) was selected as an initial brain target to be targeted by a liganding moiety as described herein.

[0691] Following phage-display discovery, 239 unique anti-TfR VHHs with unique properties including: epitope, affinity, thermostability, and pH sensitivity, were nominated for screening *in vivo* (see FIG. 24(a)). Protein language models (not shown in FIG. 24(a)) and phage-based readouts were used to inform this selection.

[0692] The 239 VHHs were simultaneously screened *in vivo* in sets of 15 to 96, at doses ranging from 0.5 to 1 mg/kg, depending on batch size (see FIG. 24(b)). Brain, serum, cell-free fraction (parenchyma), and muscle tissue abundances were quantified using barcodes at 24 hours (1 day). Hits demonstrating favorable brain uptake and stable PK (robust serum levels) were advanced for further screening and validation.

Example 24: Multiplexed *in vivo* assessment of phenotypes of liganding moieties using binder-barcode platform

[0693] The present example demonstrates a method to perform multiplexed *in vivo* assessment of phenotypes of shuttles (e.g., brain liganding moieties) using a binder-barcode platform described herein. In some embodiments, a phenotype includes pharmacokinetic (or clearance) data as described herein.

Method:

[0694] Select candidates from a TfR1 screen performed in Example 23 were analyzed in a multiplexed PK analysis. Results of this experiment are shown in FIG. 25. As shown in FIG. 25, liganding moiety "shuttle" candidates showed a diversity of different PK properties across blood, brain, and peripheral tissues.

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OTHER EMBODIMENTS

[0705] It is to be appreciated by those skilled in the art that various alterations, modifications, and improvements to the present disclosure will readily occur to those skilled in the art. Such alterations, modifications, and improvements are intended to be part of the present disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawing are by way of example only and any invention described in the present disclosure if further described in detail by the claims that follow.

[0706] Those skilled in the art will appreciate typical standards of deviation or error attributable to values obtained in assays or other processes as described herein. The publications, websites and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice, including those listed in the above References section, are hereby incorporated by reference in their entireties.

[0707] It is to be understood that while embodiments of the invention have been described in conjunction with the detailed description thereof, the foregoing description is

intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

EQUIVALENTS

[0708] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the following claims:

CLAIMS

We claim:

1. A nucleic acid comprising:

(a) a cargo component whose nucleotide sequence is or comprises a sequence encoding a cargo polypeptide;

(b) a barcode component whose nucleotide sequence is or comprises a sequence encoding a peptide barcode characterized in that:

(i) the peptide barcode has a length within a range of 1 to 100, 5 to 50, 8 to 25, 9 to 25, or 9 to 15 amino acids; and

(ii) has been determined to bind specifically to a particular group of polypeptide binders within a set of binders,

wherein the cargo component is operably linked to the barcode component.

2. The nucleic acid of claim 1, wherein the cargo component further comprises one or more sequence elements, or the complement thereof, selected from the group consisting of: a promoter, an enhancer, a silencer, an insulator, a transcriptional regulatory element, a translational regulatory element, a splice donor, a splice acceptor, a transcriptional terminator, a translational start site, a translational stop site, a packaging signal, an integration signal, and any combination thereof.

3. The nucleic acid of any of the preceding claims, wherein the cargo component comprises an internal ribosome entry site (IRES).

4. The nucleic acid of any of the preceding claims, wherein the cargo component further encodes a cleavable moiety (*e.g.*, a self-cleaving peptide (*e.g.*, a 2A peptide)).

5. The nucleic acid of any of the preceding claims, wherein the nucleic acid is or comprises DNA.

6. The nucleic acid of any of the preceding claims, wherein the nucleic acid is or comprises RNA.

7. The nucleic acid of claim 6, wherein the cargo component further comprises one or more of a capping moiety, a 5' untranslated region (UTR), 3' UTR, a polyadenylation (polyA) tail, or the complement thereof, or any combination thereof.

8. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises a localizing moiety.

9. The nucleic acid of claim 8, wherein the localizing moiety is selected from the group consisting of: a secretory signal and an intracellular localization moiety.

10. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises an intermediate or a pro component.

11. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises a tag moiety.

12. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises a liganding moiety (e.g., a shuttle moiety).

13. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises a stability modifying moiety.

14. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises a masking moiety.

15. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide further comprises an allosteric modulation moiety.

16. The nucleic acid of any of claims 8 to 15, wherein the localizing moiety, tag moiety, liganding moiety, stability modifying moiety, masking moiety, or a allosteric modulation moiety is cleavable.

17. The nucleic acid of any of the preceding claims, wherein the cargo polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide.

18. The nucleic acid of any of claims 1 to 16, wherein the cargo polypeptide is or comprises a variant polypeptide.

19. The nucleic acid of claim 18, wherein the variant polypeptide is a variant of a reference polypeptide, which reference polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide.

20. The nucleic acid of any of the preceding claims, wherein the nucleic acid is disposed within a delivery particle.

21. The nucleic acid of any of the preceding claims, wherein the nucleic acid is disposed on a surface of a delivery particle.

22. The nucleic acid of any one of the preceding claims, wherein the encoded peptide barcode has an amino acid sequence selected from the group consisting of SEQ ID NOs: 5347-8398.

23. The nucleic acid of any one of the preceding claims, wherein the encoded peptide barcode is encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1148-4199.

24. The nucleic acid of any one of the preceding claims, wherein the encoded peptide barcode has a length of 8 to 25 amino acids.

25. The nucleic acid of any one of the preceding claims, wherein the encoded peptide barcode has a length of 10 amino acids.

26. The nucleic acid of any one of the preceding claims, wherein the nucleotide sequence of the barcode component comprises, in order from 5' to 3' or 3' to 5', one or more of:

(a) a first invariant sequence (*e.g.,* a linker sequence or a payload sequence);

(b) a variant sequence that is at least 9 nucleotides long; and

(c) a second invariant sequence (*e.g.,* a linker sequence, a stop codon, or a payload sequence).

27. The nucleic acid of claim 26, wherein the variant sequence is at least 15, 24, 27, 45, 150, or 300, nucleotides long.

28. The nucleic acid of any one of the preceding claims, wherein the nucleotide sequence of the barcode component further comprises one or more of:

(d) a sequence encoding a short helical motif;

(e) a sequence encoding a disordered motif;

(f) an invariant sequence linking the barcode component to the cargo component.

29. The nucleic acid of any one of the preceding claims, wherein each polypeptide binder of the group of polypeptide binders has an amino acid sequence selected from the group consisting of SEQ ID NOs: 4200- 5346.

30. The nucleic acid of any one of the preceding claims, wherein each polypeptide binder of the group of polypeptide binders is encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-1147.

31. The nucleic acid of any one of the preceding claims, wherein each polypeptide binder is expressed on a phage.

32. The nucleic acid of claim 31, wherein the phage is selected from the group consisting of M13, T4, T7, Lambda, and filamentous phage.

33. The nucleic acid of claim 31, wherein the phage is M13.

34. The nucleic acid of any one of the preceding claims, wherein the nucleic acid encodes a barcoded cargo polypeptide, wherein the barcoded cargo polypeptide, or a characteristic portion thereof, is expressed on the surface of a delivery particle (*e.g.*, a viral particle, a lipid-based particle [*e.g.*, cell-produced or not cell-produced, a lipid nanoparticle (LNP), a liposome, a micelle, an extracellular vesicle (*e.g.*, exosomes, microparticles, etc.)], a polymer-based particle (*e.g.*, PGLA), a polysaccharide-based particle, etc.).

35. The nucleic acid of any one of the preceding claims, wherein the cargo component, or a portion thereof, is codon-optimized.

36. A library comprising a plurality of nucleic acids, wherein each nucleic acid is a nucleic acid of any one of the preceding claims.

37. A plurality of delivery particles, wherein one or more of the delivery particles in the plurality comprises a nucleic acid of any one of claims 1 to 35.

38. The plurality of delivery particles of claim 37, wherein the nucleic acid in each of the delivery particles is the same.

39. The plurality of delivery particles of claim 37, wherein the delivery particles comprise at least two different nucleic acids.

40. The plurality of delivery particles of claim 39, wherein the at least two different nucleic acids comprise different cargo components.

41. The plurality of delivery particles of claim 39 or 40, wherein the delivery particles comprise cargo components encoding at least two different cargo polypeptides.

42. The plurality of delivery particles of any one of claims 39 to 41, wherein the cargo polypeptides are variants of a reference polypeptide, which reference polypeptide is or comprises a wild-type (*e.g.*, naturally occurring) polypeptide.

43. The plurality of delivery particles of claim 42, wherein the variants comprise amino acid sequences that are at least 70% identical to each other (*e.g.*, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to each other).

44. The plurality of delivery particles of any one of claims 37 to 43, wherein the delivery particles comprise one or more associated (*e.g.*, covalently or non-covalently) targeting moieties.

45. The plurality of delivery particles of claim 44, wherein the one or more targeting moieties are of the same type.

46. The plurality of delivery particles of claim 44, wherein the one or more targeting moieties are of different types.

47. The plurality of delivery particles of any one of claims 37 to 46, wherein the plurality of delivery particles are substantially a same type of delivery particle.

48. The plurality of delivery particles of any one of claims 37 to 46, wherein the plurality of delivery particles comprises two or more types of delivery particles.

49. The plurality of delivery particles of any one of claims 37 to 48, wherein the plurality of delivery particles is or comprises a viral particle, a lipid-based particle [*e.g.*, cell-produced or not cell-produced, a lipid nanoparticle (LNP), a liposome, a micelle, an extracellular vesicle (*e.g.*, exosomes, microparticles, etc.)], a polymer-based particle (*e.g.*, PGLA), a polysaccharide-based particle, or a combination thereof.

50. The plurality of delivery particles of any one of claims 37 to 49, wherein the delivery particles are or comprise a viral particle.

51. The plurality of delivery particles of any one of claims 37 to 50, wherein the delivery particles are or comprise two or more types of viral particles.

52. The plurality of delivery particles of any one of claims 49 to 51, wherein the viral particles are or comprise one or more of AAV delivery particles, lentivirus delivery particles, adenovirus delivery particles, herpesvirus delivery particles, and anellovirus delivery particles.

53. The plurality of delivery particles of claim 51, wherein the AAV delivery particles are or comprise two or more serotypes (*e.g.*, AAV2, AAV5, AAV6, AAV8, AAV9, AAV.DJ, AAV.PHP, any variant thereof, or a combination thereof).

54. The plurality of delivery particles of claim 48 or 49, wherein the two or more types of delivery particles are or comprise two or more types of lipid-based particles (*e.g.*, LNPs)(*e.g.*, having different formulations).

55. A delivery particle comprising the nucleic acid of any one of claims 1 to 35.

56. A population of delivery particles comprising the nucleic acid of any one of claims 1 to 35.

57. A cell comprising the nucleic acid of any one of claims 1 to 35, the library of claim 36, the plurality of delivery particles of any one of claims 37 to 54, or the delivery particle of claim 53.

58. A population of cells comprising the nucleic acid of any one of claims 1 to 35, the library of claim 36, the plurality of delivery particles of any one of claims 37 to 54, the delivery particle of claim 55, or the population of delivery particles of claim 56.

59. A composition (*e.g.*, pharmaceutical composition) comprising the nucleic acid of any one of claims 1 to 35, the library of claim 36, the plurality of delivery particles of any one of claims 37 to 54, the delivery particle of claim 55, or the population of delivery particles of claim 56.

60. A kit comprising:

(a) a set of nucleic acids, wherein each nucleic acid of the set is according to any one of claims 1 to 35; and

(b) a set of binders, each of which is a polypeptide, or a nucleic acid encoding a polypeptide, that binds specifically to at least a particular peptide barcode in a collection of barcodes.

61. The kit of claim 60, wherein one or more of the binders is provided as a phage particle, or collection thereof, engineered to express the binder.

62. The kit of claim 60, wherein one or more of the binders is provided as a nucleic acid in a phagemid vector, or as an insert suitable for cloning into a phage vector.

63. The kit of any one of claims 60 to 62, further comprising information designating peptide barcodes for each binder, wherein each binder has been determined to bind specifically to at least a particular peptide barcode within the collection of barcodes, and wherein each peptide barcode binds specifically to at least one of the binders in the set.

64. The kit of any one of claims 60 to 63, further comprising a set of instructions to perform sequencing of one or more phage particles bound to one or more barcodes.

65. The kit of claim 64, further comprising a computer readable program for decoding sequencing data.

66. The kit of any one of claims 60 to 65, further comprising reagents to express a binder on a phage particle.

67. The kit of any one of claims 60 to 66, comprising nucleic acids that encode one or more barcodes.

68. The kit of any one of claims 60 to 67, comprising nucleic acids that encode one or more binders.

69. A method for identifying a therapeutic polypeptide or a target polypeptide to treat a disease, disorder, or condition comprising steps of:

a) subjecting a population of barcoded cargo polypeptides to an assessment, wherein the barcoded cargo polypeptides are encoded by the nucleic acids of any one of claims 1 to 35;

b) separating those members of the population that satisfy the assessment from those that do not, so that a positive population or a negative population, or both, is identified;

c) contacting the positive population, or the negative population, or each population separately from the other, with a set of binders which includes at least one binder specific for each barcode in the population; and

d) determining which binders bind to the separated members, thereby determining which barcoded cargo polypeptides are present in the contacted population(s).

70. The method of claim 69, further comprising:

a) administering the population of nucleic acids that encode the barcoded cargo polypeptides to an animal; and

b) obtaining a sample from the animal to subject to further assessment.

71. The method of claim 70, wherein the step of separating comprises purifying one or more barcoded cargo polypeptides from the sample.

72. The method of claim 71, wherein the barcoded cargo polypeptides are purified from a complex sample.

73. The method of claim 72, wherein the complex sample is tissue.

74. The method of claim 73, wherein the complex sample is blood.

75. The method of any one of claims 71 to 74, wherein the barcoded cargo polypeptides are purified using affinity purification methods (*e.g.,* FLAG IP, protein G/A) or protein precipitation methods.

76. The method of any one of claims 69 to 75, wherein each binder of the set of binders is expressed on a phage.

77. The method of claim 76, wherein the step of determining comprises:

a) amplifying nucleic acids of the bound phage particles;

b) determining nucleotide sequences of the amplified nucleic acids, wherein one or more of the determined nucleotide sequences corresponds to the coding sequence of the binder;

c) detecting one or more cargo polypeptides from the population of barcoded cargo polypeptides using the determined sequence(s) of the coding sequence of the binder; and

f) identifying the one or more barcoded cargo polypeptides as a therapeutic or a target to treat a disease, disorder, or condition.

78. A method of pharmacokinetic screening, the method comprising:

a) administering a population of nucleic acids that encode a set of barcoded therapeutic candidate polypeptides, or characteristic portion thereof, to an animal, wherein each therapeutic candidate polypeptide comprises a specific peptide barcode;

b) obtaining a sample from the animal;

c) purifying one or more barcoded therapeutic candidate polypeptides from the sample;

d) contacting the sample with a set of binders (*e.g.,* binding agents with binders expressed on them) which includes at least one binder specific for each barcode in the sample; and

e) determining (*e.g.*, simultaneously) the relative amounts of each binder present in the sample to determine each barcoded therapeutic candidate polypeptides' pharmacokinetic properties, biodistribution, half-life, tissue-mediated drug disposition (TMDD), epitope properties, affinity properties, thermostability properties, pH sensitivity properties, or *in vivo* stability.

79. The method of claim 78, wherein multiple samples are obtained from the animal.

80. The method of claim 78, wherein the animal is a model for a disease, disorder, or condition.

81. The method of claim 80, wherein the disease, disorder, or condition is cancer, autoimmune, neurodegenerative, or a pathogenic (*e.g.,* viral/bacterial) disease, disorder, or condition.

82. The method of any one of claims 78 to 81, wherein the purified therapeutic candidate polypeptides are a subset of barcoded therapeutic candidate polypeptides administered to the animal.

83. The method of any one of claims 78 to 81, wherein the sample is blood, tissue, a tumor.

84. The method of any one of claims 78 to 83, wherein the sample is a control.

85. The method of any one of claims 78 to 84, wherein the step of determining comprises (i) sequencing nucleic acid from the binding agents expressing the binder; (ii) decoding the relative

amounts of each barcode present thereby determining the relative amounts of each therapeutic candidate polypeptide; and/or (iii) performing one or more of FACS, MACS (magnetic activated cell sorting), or affinity-based purification.

86. The method of any one of claims 78 to 85, comprising removing any unassociated (*e.g.,* unbound) binders.

87. The method of claim 86, wherein the removing is performed by washing.

88. The method of any one of claims 78 to 87, wherein the step of determining comprises performing one or more of amplification, propagation, and sequencing (*e.g.,* nucleic acid (*e.g.,* DNA, RNA) amplification, propagation, and/or sequencing).

89. The method of claim 88, wherein the amplification is performed using PCR, LAMP, or RCA.

90. The method of claim 88, wherein the sequencing is performed using Illumina, NGS, nanopore sequencing, or Pac Bio long-read sequencing.

91. The method of any one of claims 78 to 90, wherein the step of determining comprises quantifying the number of binders that bind to a barcoded therapeutic candidate polypeptide, wherein the quantifying is performed by decoding the nucleotide sequence of each binder that binds to the barcoded therapeutic candidate polypeptide.

92. The method of claim 91, wherein the number of nucleotide sequences provides measure of target polypeptide in the population of barcoded therapeutic candidate polypeptides.

93. The method of any one of claims 78 to 92, wherein the step of administering comprises administering the barcoded therapeutic candidate polypeptides orally or intravenously.

94. The method of any one of claims 78 to 93, wherein the barcoded therapeutic candidate polypeptides are delivered by the plurality of delivery particles of any one of claims 37 to 54, the delivery particle of claim 55, or the population of delivery particles of claim 56.

95. The method of any one of claims 70 to 94, wherein the animal is a mammal.

96. The method of any one of claims 70 to 95, wherein the animal is a human.

97. The method of any one of claims 70 to 96, wherein the animal is genetically modified to express the barcoded therapeutic candidate polypeptides.

98. A method of treatment comprising:

administering a therapeutic polypeptide or nucleic acid that encodes a therapeutic polypeptide, or characteristic portion thereof, that has been determined to satisfy an assessment by a process comprising steps of:

a) subjecting a population of nucleic acids that encode a set of barcoded cargo polypeptides to the assessment;

b) separating those members of the population that satisfy the assessment from those that do not, so that a positive population or a negative population, or both, is identified;

c) contacting the positive population, or the negative population, or each population separately from the other, with a set of binders which includes at least one binder specific for each barcode in the population;

d) determining which binders bind to the separated members, thereby determining which barcoded cargo polypeptides are present in the contacted population(s); and

e) identifying the therapeutic polypeptide from the barcoded cargo polypeptides determined to be present in the contacted population(s).

99. A method of treatment comprising:

administering a therapeutic polypeptide or nucleic acid that encodes a therapeutic polypeptide, or characteristic portion thereof, that has been determined to satisfy an assessment by a process comprising steps of:

a) contacting a set of binders either with a first population, with a second population, or separately with each of the first and second populations of barcoded cargo polypeptides, wherein the barcoded cargo polypeptides are encoded by the nucleic acids of any one of claims 1 to 35, wherein:

i) each binder binds specifically to one barcode relative to the other barcodes; and

ii) the set of binders, collectively, includes a binder specific for each of the barcodes in the first and second populations,

wherein the first and second populations have been separated from one another based on performance in the assessment;

b) determining which binders of the set bind to a member of the first population, the second population, or both, thereby determining which barcoded cargo polypeptides are present in the contacted population(s); and

c) identifying the therapeutic polypeptide from the barcoded cargo polypeptides determined to be present in the contacted population(s).

100. A method of treatment comprising:

administering a therapeutic polypeptide, or characteristic portion thereof, wherein the therapeutic polypeptide is identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

101. A method of treatment comprising:

administering a nucleic acid encoding a therapeutic polypeptide, or characteristic portion thereof, wherein the therapeutic polypeptide is identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

102. A composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof, wherein the one or more therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

103. A composition (*e.g.*, pharmaceutical composition) comprising one or more barcoded cargo polypeptides, or characteristic portion thereof, wherein the one or more barcoded cargo polypeptides are generated by a method of any one of claims 69 to 97.

104. A composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof, wherein the therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

105. A method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more therapeutic polypeptides, or characteristic portion thereof, wherein the one or more

therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

106. A method of manufacturing a composition (*e.g.*, pharmaceutical composition) comprising one or more nucleic acids encoding one or more therapeutic polypeptides, or characteristic portion thereof, wherein the therapeutic polypeptides are identified from a population of barcoded cargo polypeptides by the method of any one of claims 69 to 97.

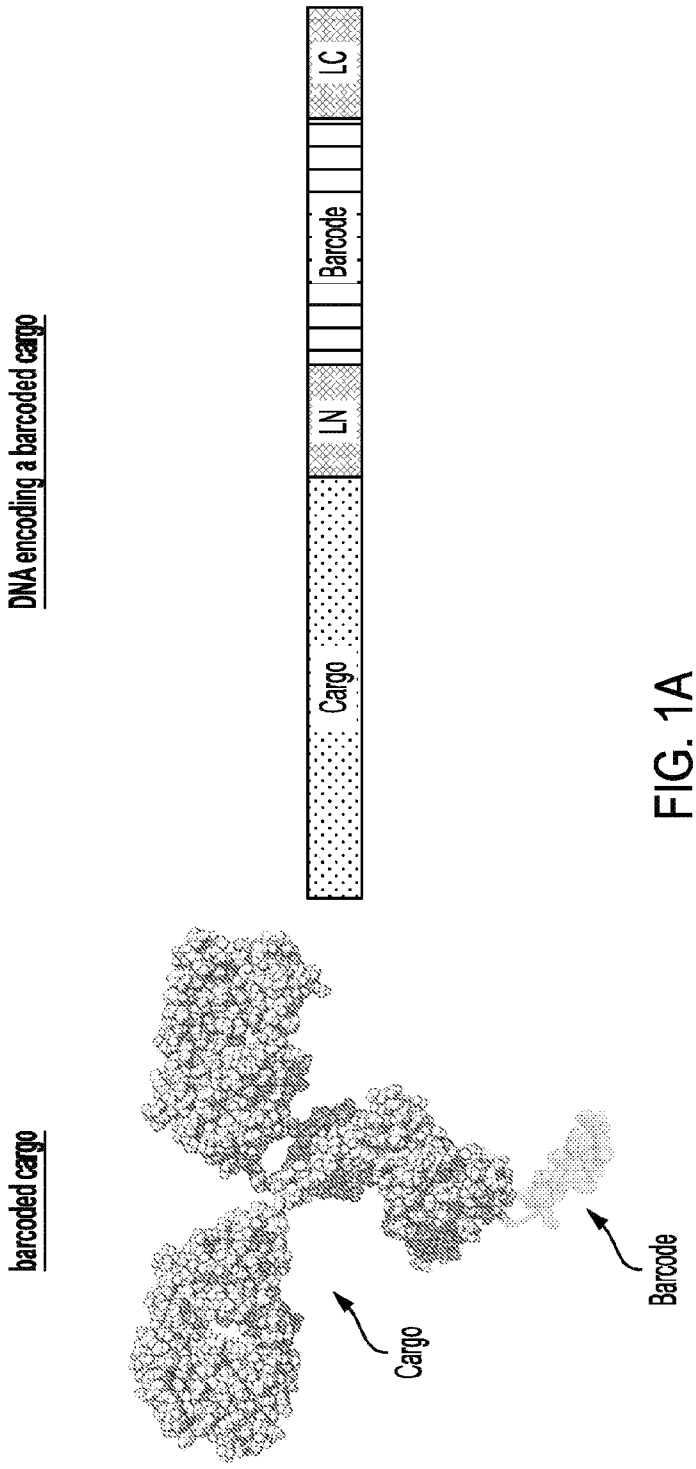
DNA encoding a barcoded cargo
Cargo
LN
Barcode
LC
barcoded cargo
Cargo
Barcode

FIG. 1A

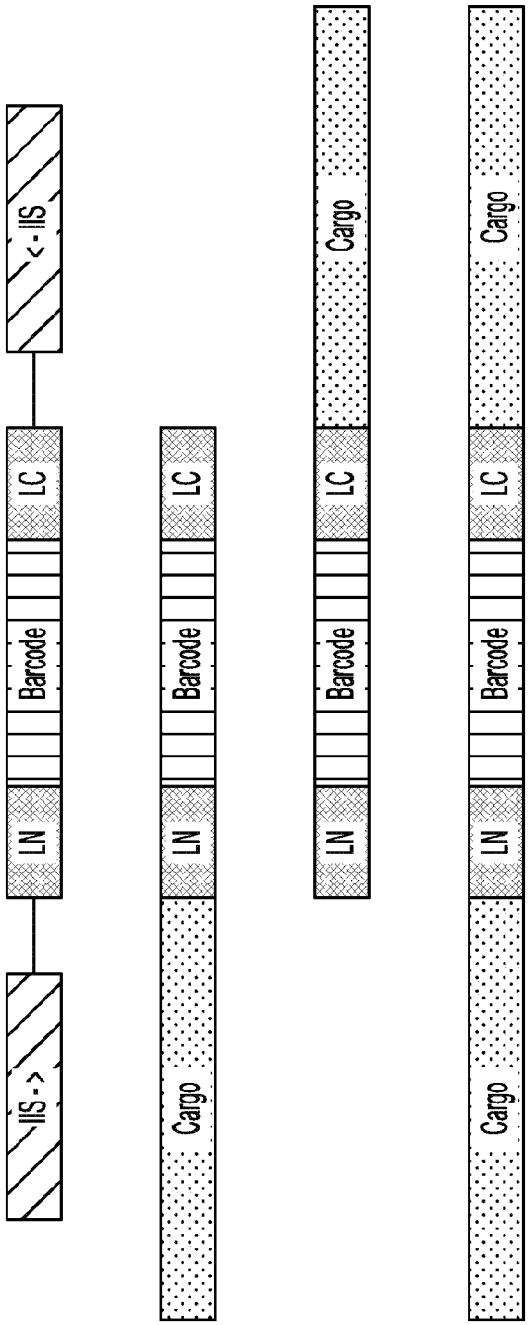
IIS ->
LN
Barcode
LC
< - IIS
Cargo
LN
Barcode
LC
Cargo
LN
Barcode
LC
Cargo

FIG. 1B

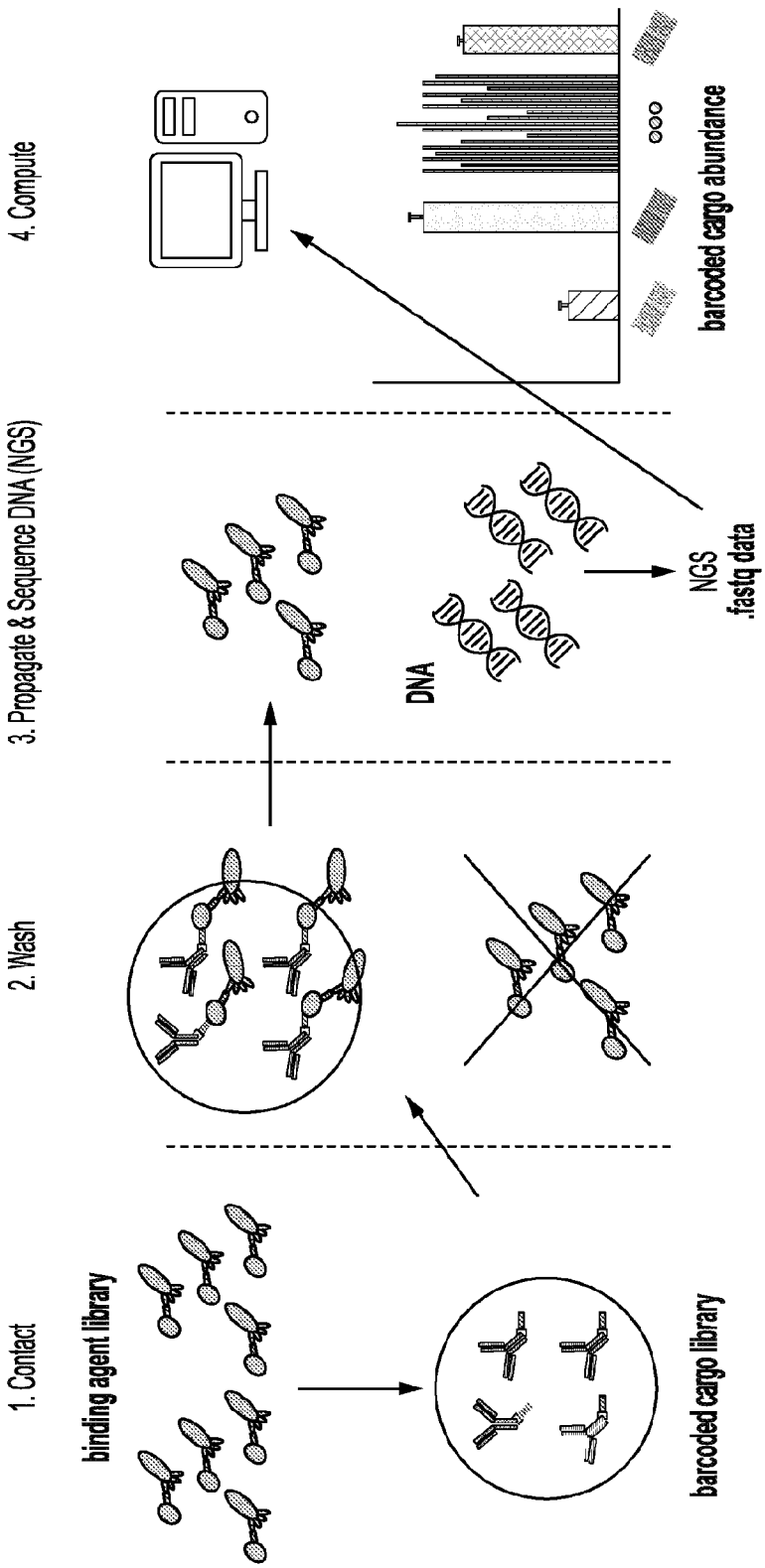
1. Contact
binding agent library
barcoded cargo library
2. Wash
3. Propagate & Sequence DNA (NGS)
DNA
NGS
.fastq data
4. Compute
barcoded cargo abundance

FIG. 2

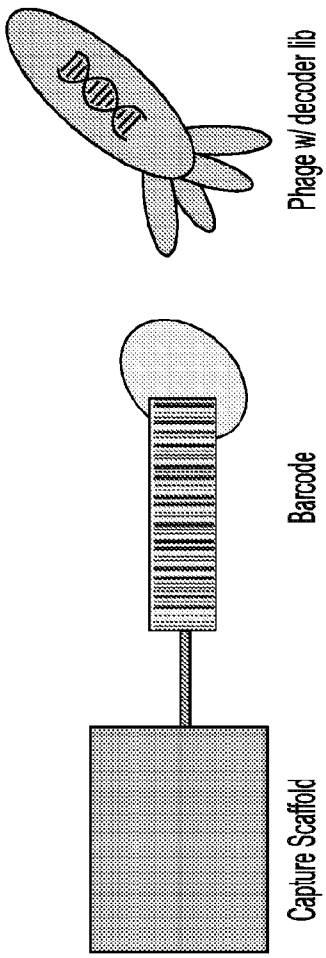

FIG. 3A

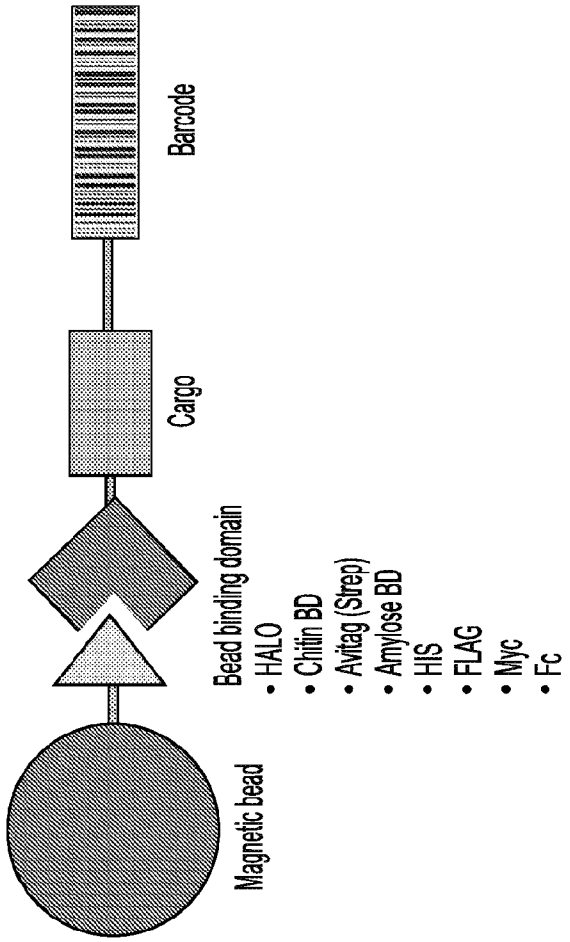
Barcode
Cargo
Bead binding domain
• HALO
• Chitin BD
• Avitag (Strep)
• Amylose BD
• HIS
• FLAG
• Myc
• Fc
Magnetic bead

FIG. 3B

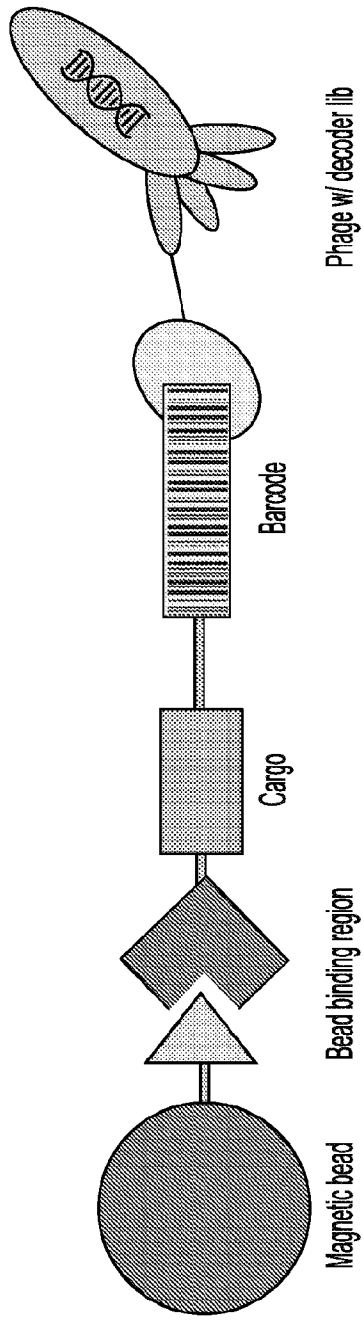
Phage w/ decoder lib
Barcode
Cargo
Bead binding region
Magnetic bead

FIG. 3C

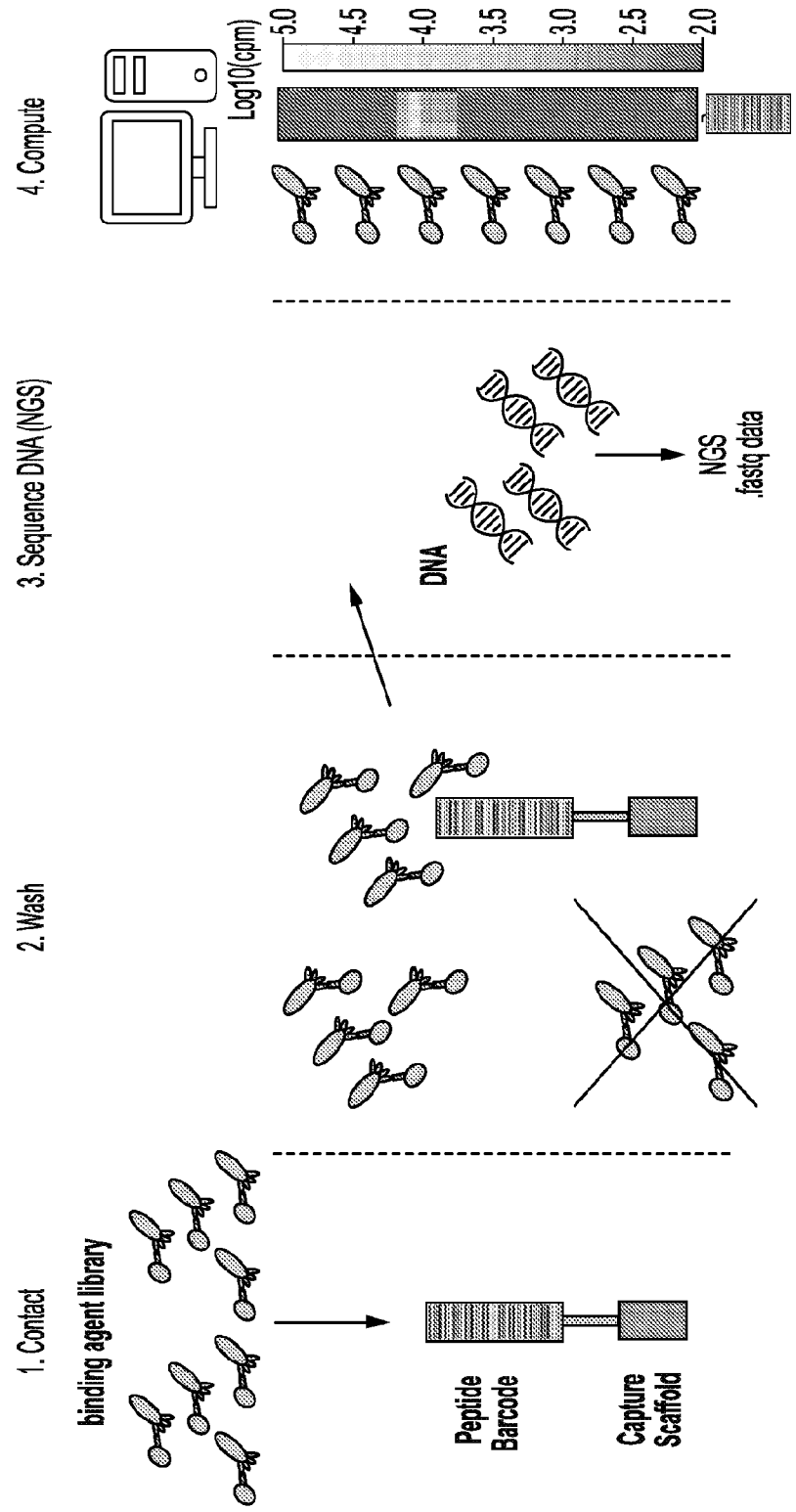
1. Contact
binding agent library
Peptide Barcode
Capture Scaffold
2. Wash
3. Sequence DNA (NGS)
DNA
NGS .fastq data
4. Compute
Log10(cpm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0

FIG. 4

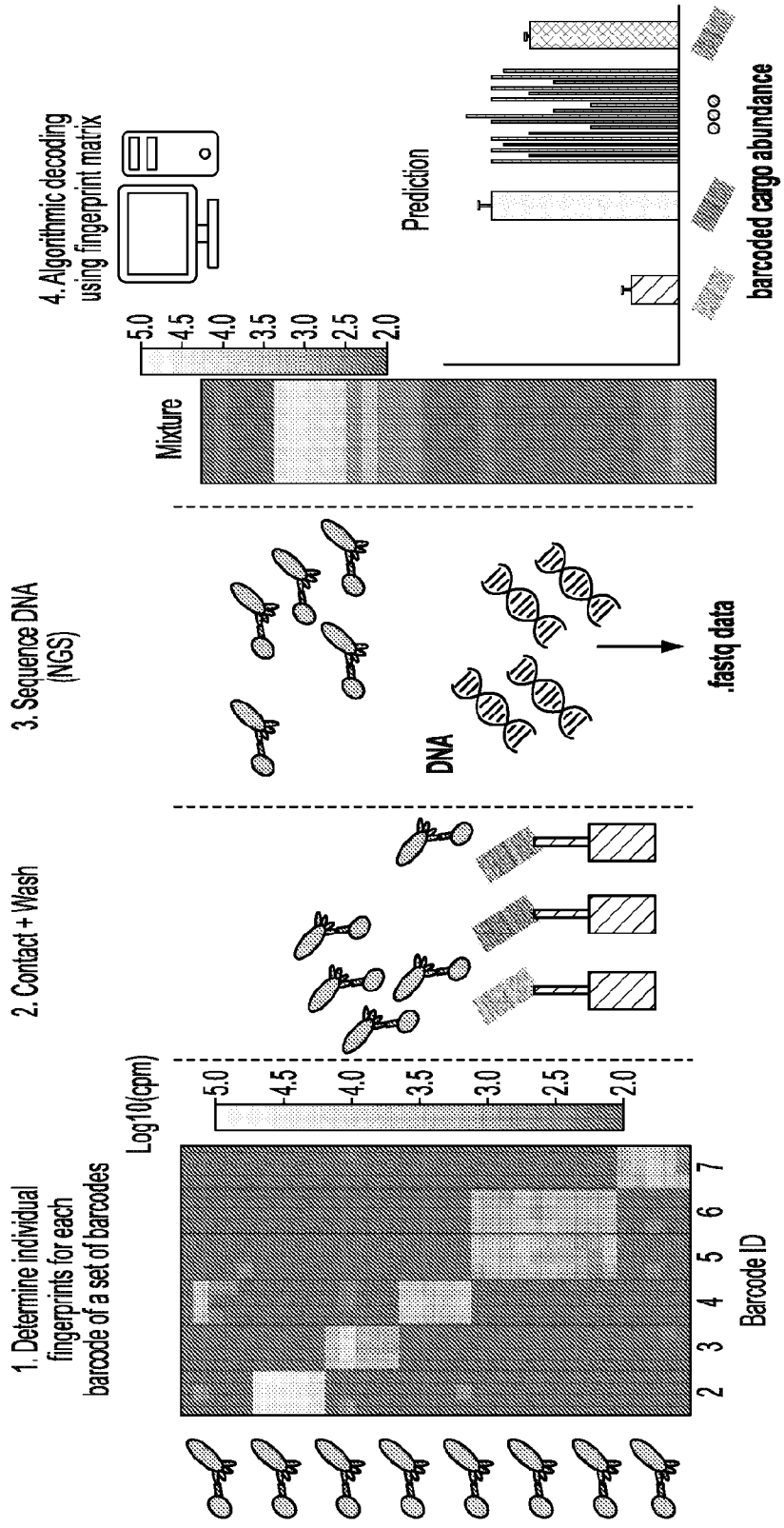
1. Determine individual fingerprints for each barcode of a set of barcodes
Log10(cpm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
2 3 4 5 6 7
Barcode ID
2. Contact + Wash
3. Sequence DNA (NGS)
DNA
.fastq data
4. Algorithmic decoding using fingerprint matrix
Mixture
Prediction
barcoded cargo abundance

FIG. 5

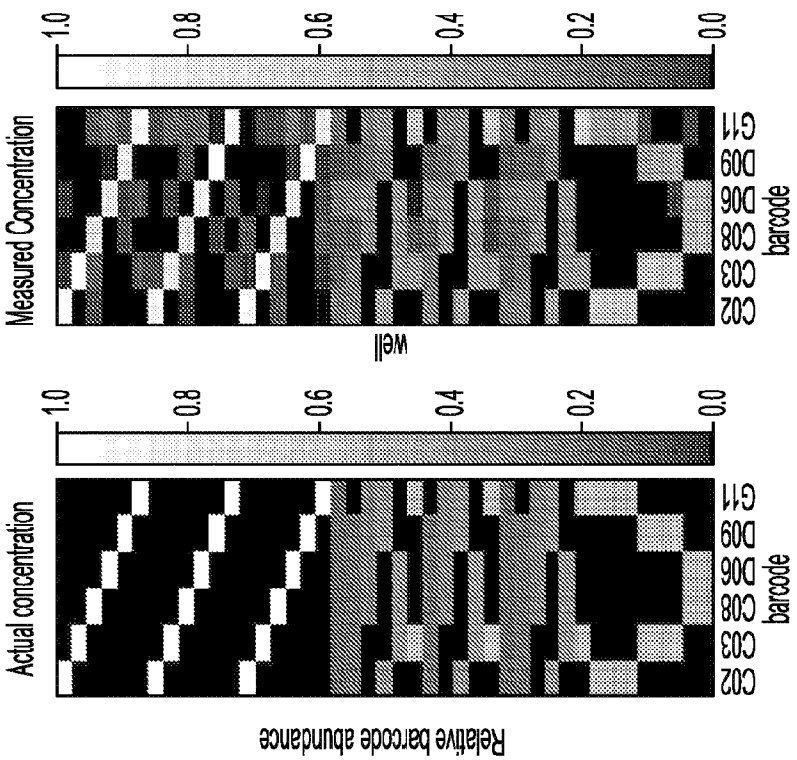
Relative barcode abundance
Actual concentration
barcode
C02
C03
C08
D06
D09
G11
1.0
0.8
0.6
0.4
0.2
0.0
well
Measured Concentration
barcode
C02
C03
C08
D06
D09
G11
1.0
0.8
0.6
0.4
0.2
0.0

FIG. 6A

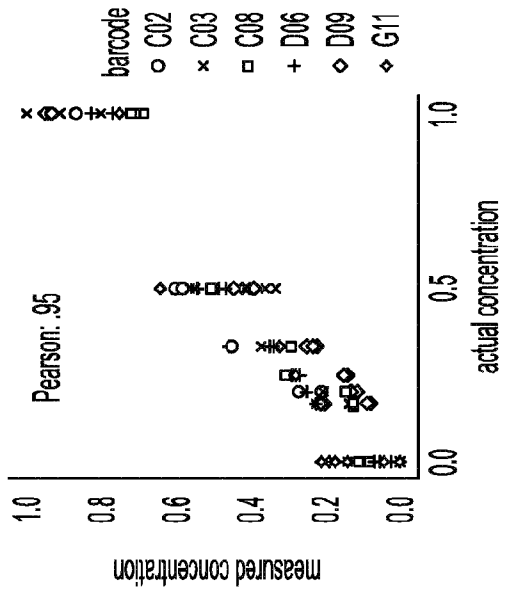
measured concentration
1.0
0.8
0.6
0.4
0.2
0.0
Pearson: .95
0.0
0.5
1.0
actual concentration
barcode
C02
C03
C08
D06
D09
G11

FIG. 6B

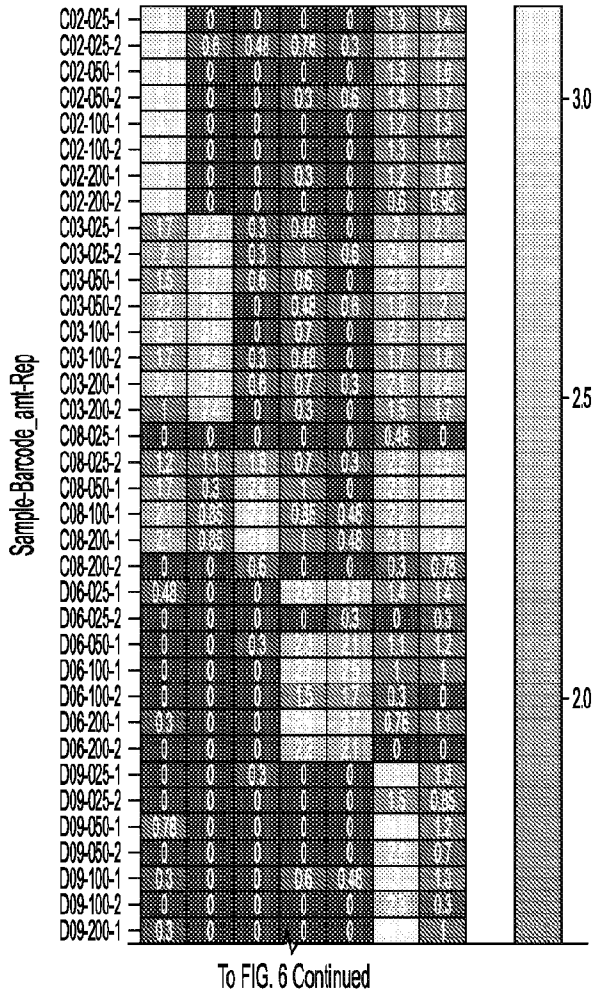
Sample-Barcode_amt-Rep
C02-025-1
C02-025-2
C02-050-1
C02-050-2
C02-100-1
C02-100-2
C02-200-1
C02-200-2
C03-025-1
C03-025-2
C03-050-1
C03-050-2
C03-100-1
C03-100-2
C03-200-1
C03-200-2
C08-025-1
C08-025-2
C08-050-1
C08-100-1
C08-200-1
C08-200-2
D06-025-1
D06-025-2
D06-050-1
D06-100-1
D06-100-2
D06-200-1
D06-200-2
D09-025-1
D09-025-2
D09-050-1
D09-050-2
D09-100-1
D09-100-2
D09-200-1
3.0
2.5
2.0
To FIG. 6 Continued

FIG. 6C

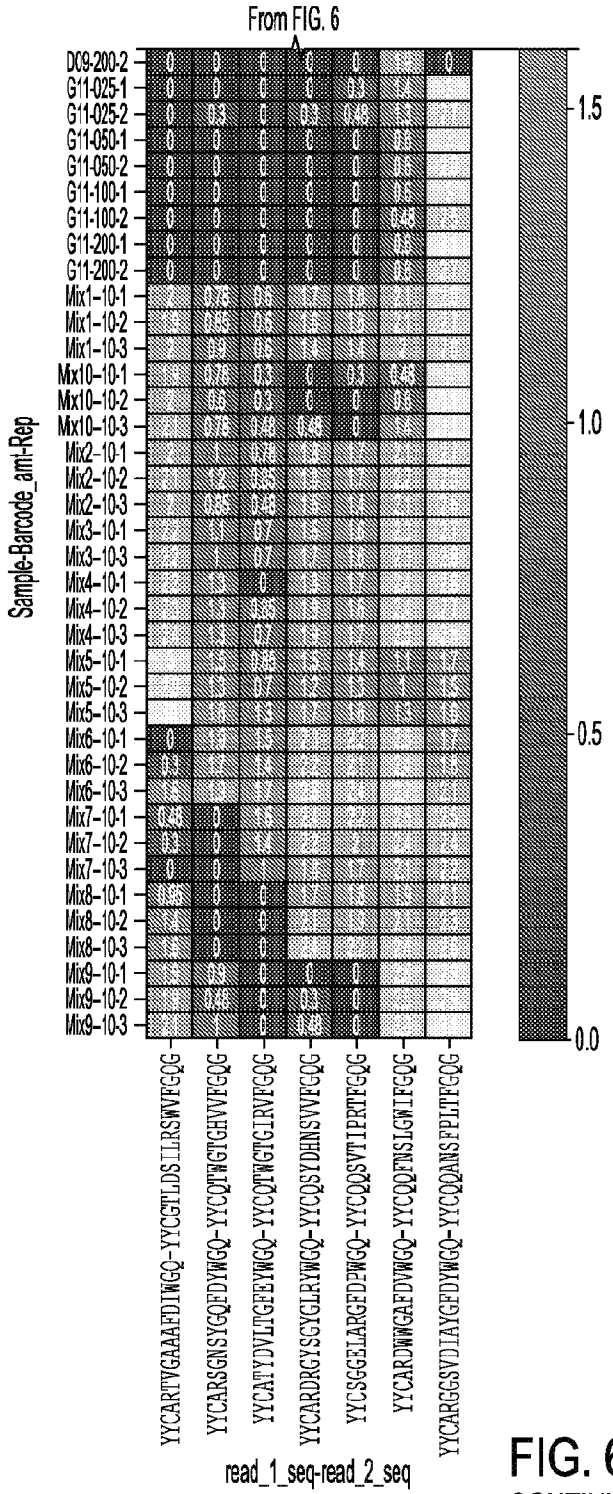

FIG. 6C
CONTINUED

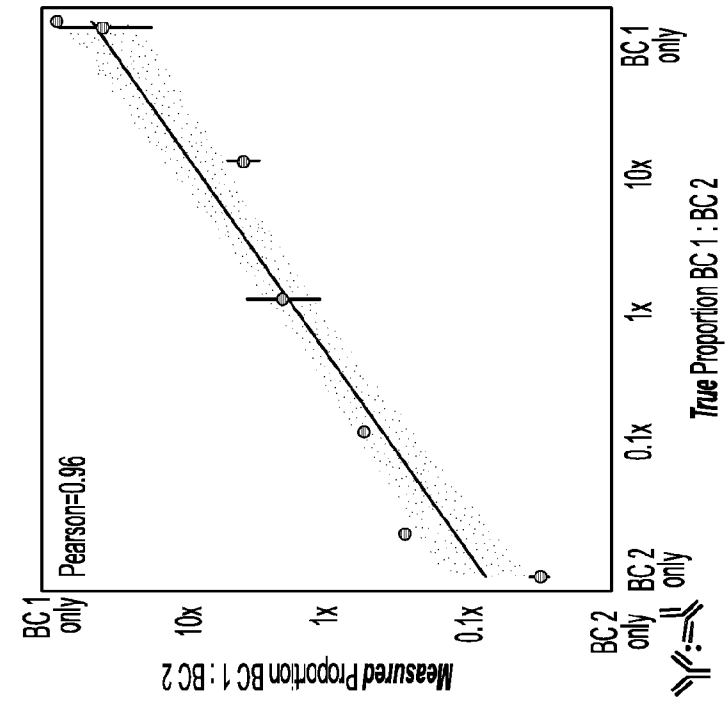
Measured Proportion BC 1 : BC 2
BC 1 only
10x
1x
0.1x
BC 2 only
Pearson=0.96
BC 2 only
0.1x
1x
10x
BC 1 only
True Proportion BC 1 : BC 2

FIG. 7B

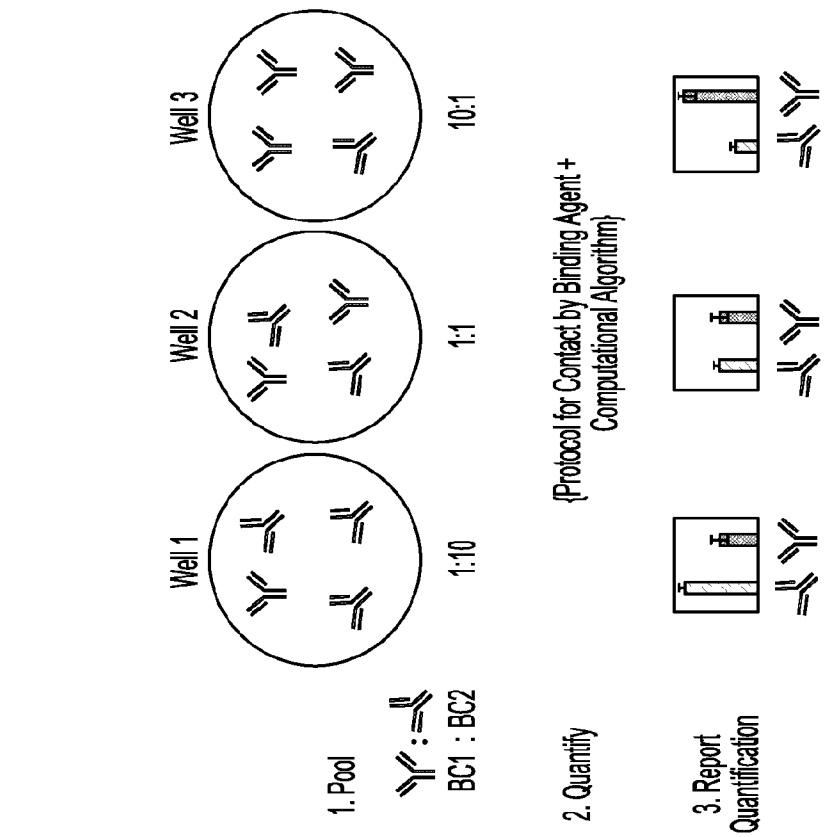
1. Pool
BC1 : BC2
Well 1
1:10
Well 2
1:1
Well 3
10:1
2. Quantify
{Protocol for Contact by Binding Agent + Computational Algorithm}
3. Report Quantification

FIG. 7A

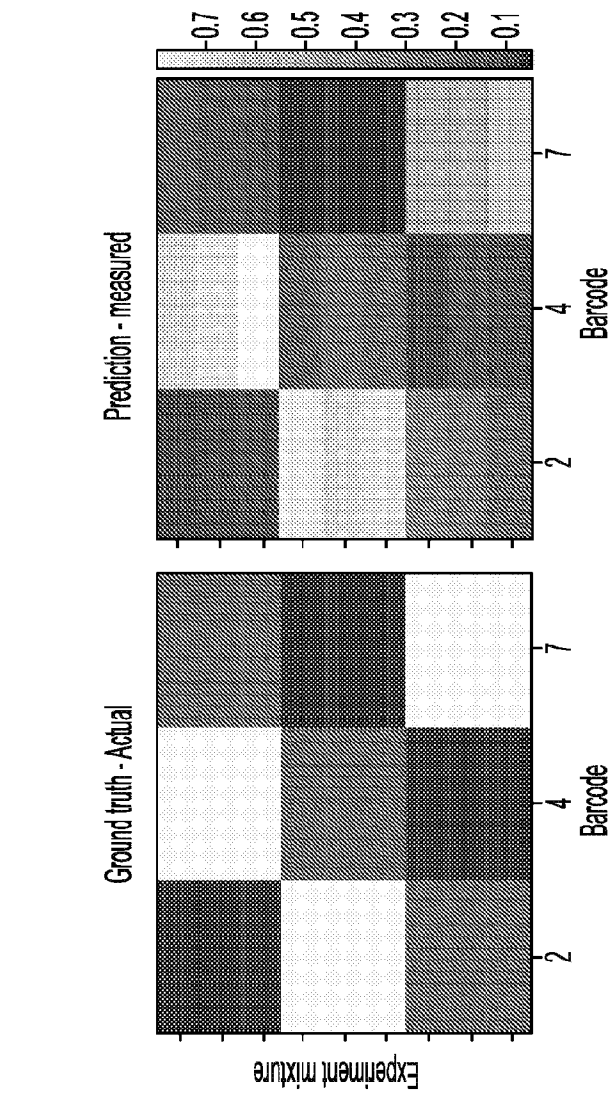
Barcoded-mAbs
Add serum
Capture & Decode
Experiment mixture
Ground truth - Actual
Prediction - measured
Barcode
2
4
7
0.7
0.6
0.5
0.4
0.3
0.2
0.1

FIG. 8A

FIG. 8B

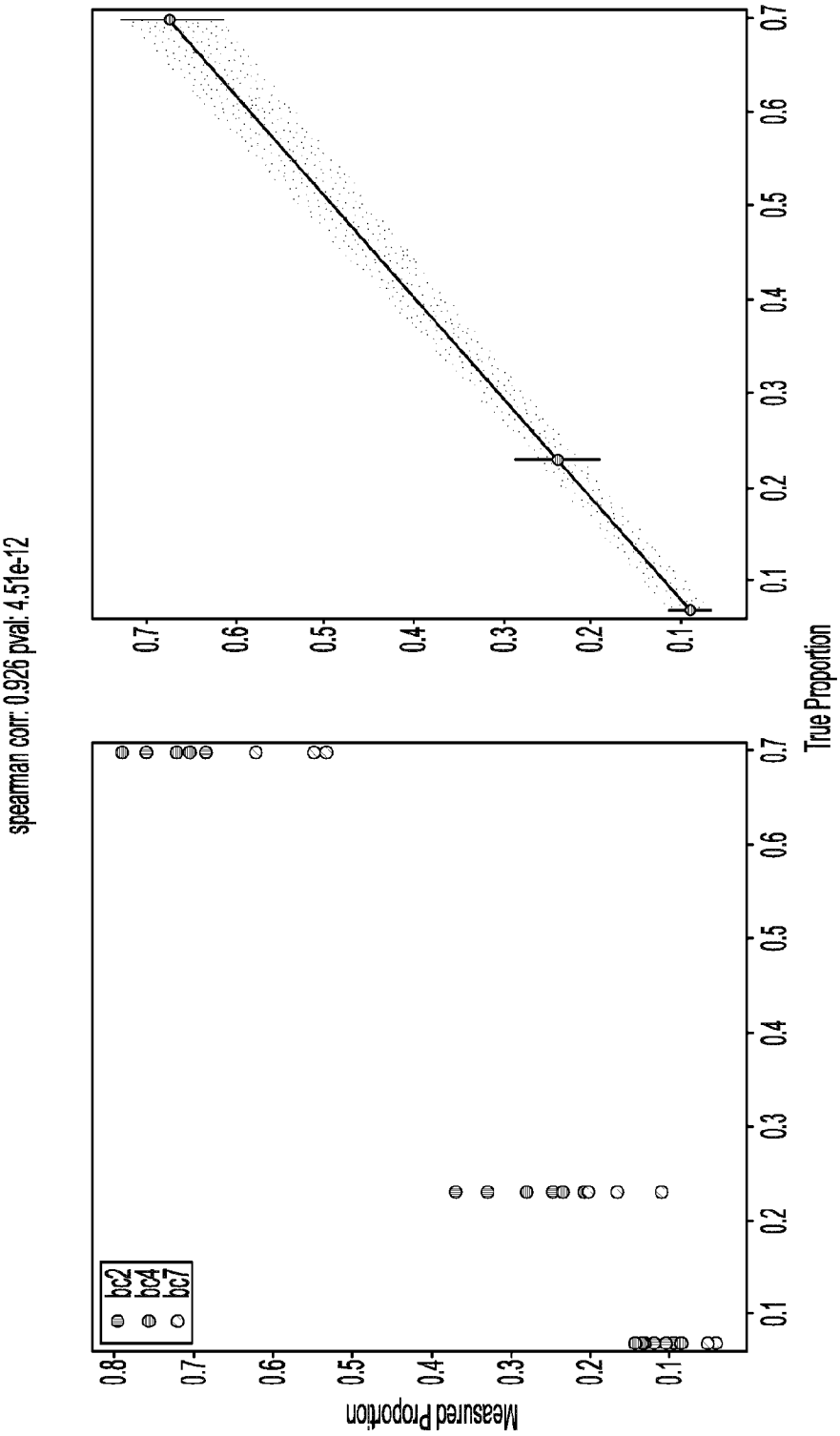
spearman corr: 0.926 pval: 4.51e-12
bc2
bc4
bc7
Measured Proportion
True Proportion
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

FIG. 8C

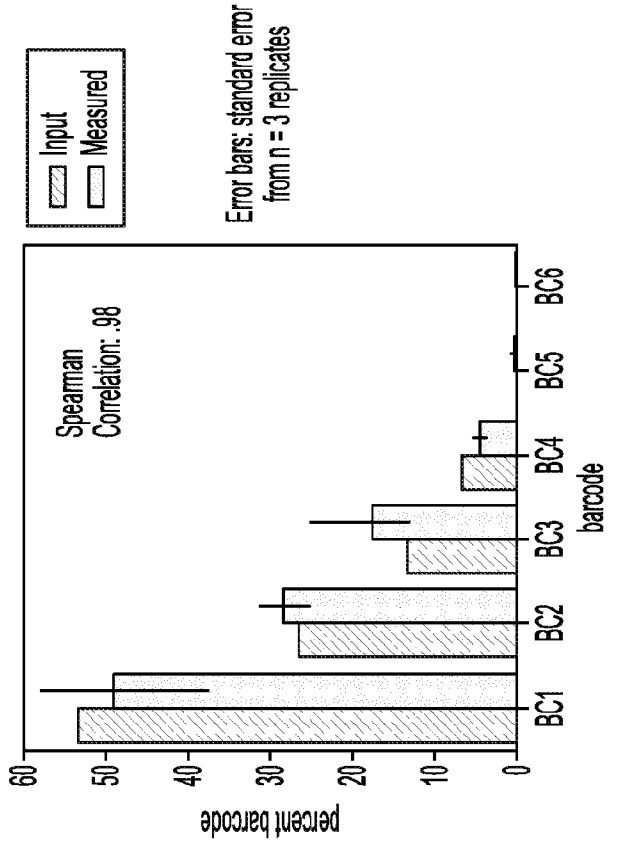
Input
Measured
Error bars: standard error
from n = 3 replicates
Spearman
Correlation: .98
percent barcode
60
50
40
30
20
10
0
BC1
BC2
BC3
BC4
BC5
BC6
barcode

FIG. 9B

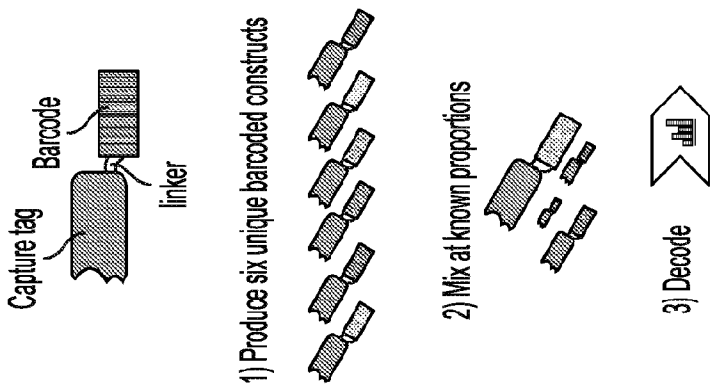
Capture tag
Barcode
linker
1) Produce six unique barcoded constructs
2) Mix at known proportions
3) Decode

FIG. 9A

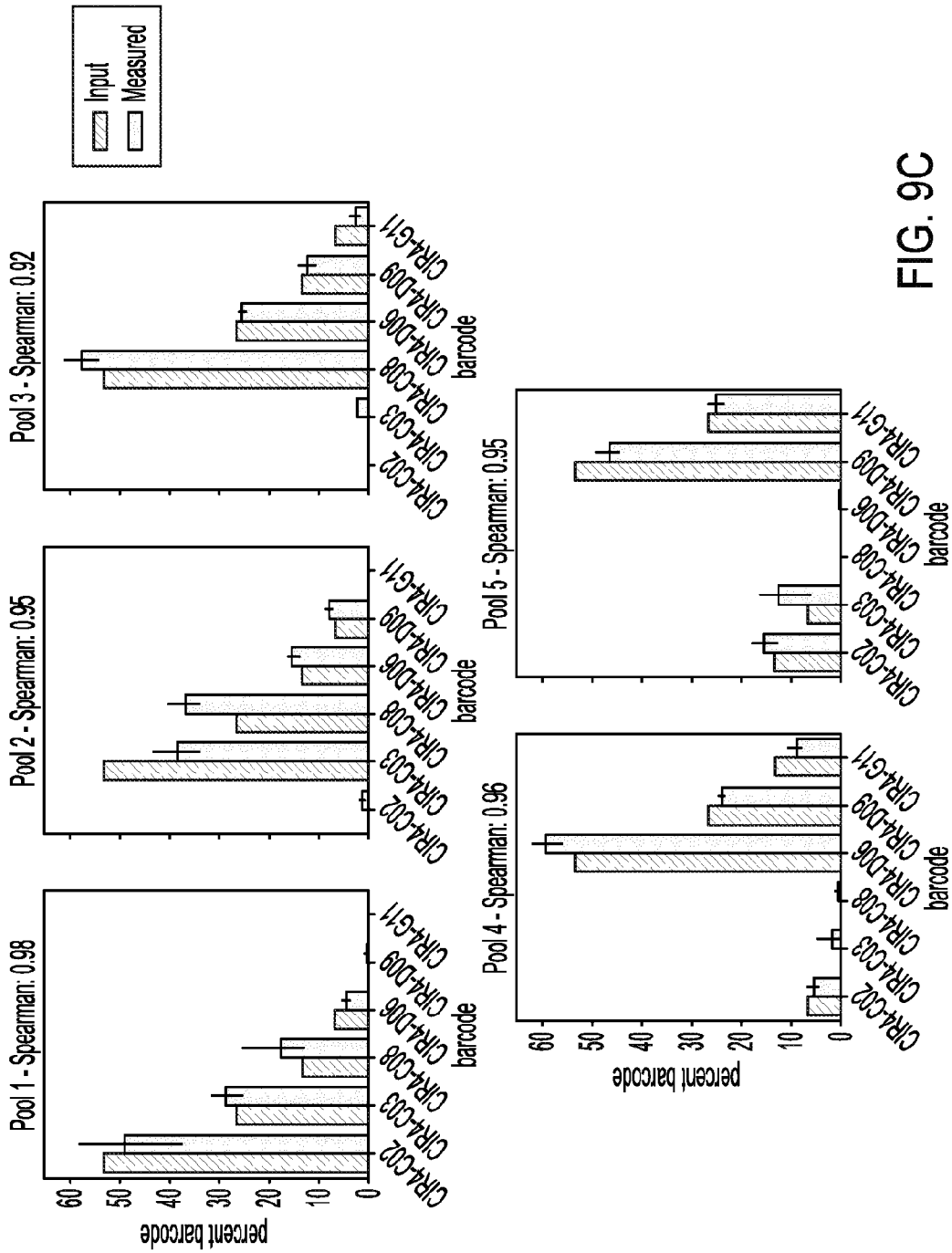
Input
Measured
Pool 1 - Spearman: 0.98
Pool 2 - Spearman: 0.95
Pool 3 - Spearman: 0.92
Pool 4 - Spearman: 0.96
Pool 5 - Spearman: 0.95
percent barcode
barcode
CIR4-C02
CIR4-C03
CIR4-C08
CIR4-D06
CIR4-D09
CIR4-G11

FIG. 9C

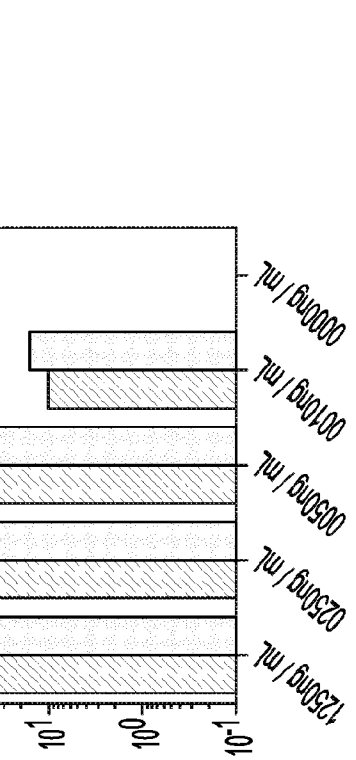

FIG. 10B

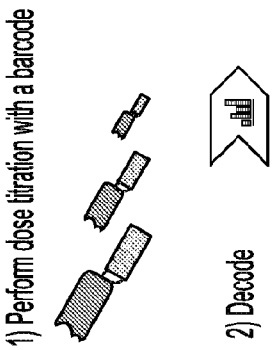

FIG. 10A

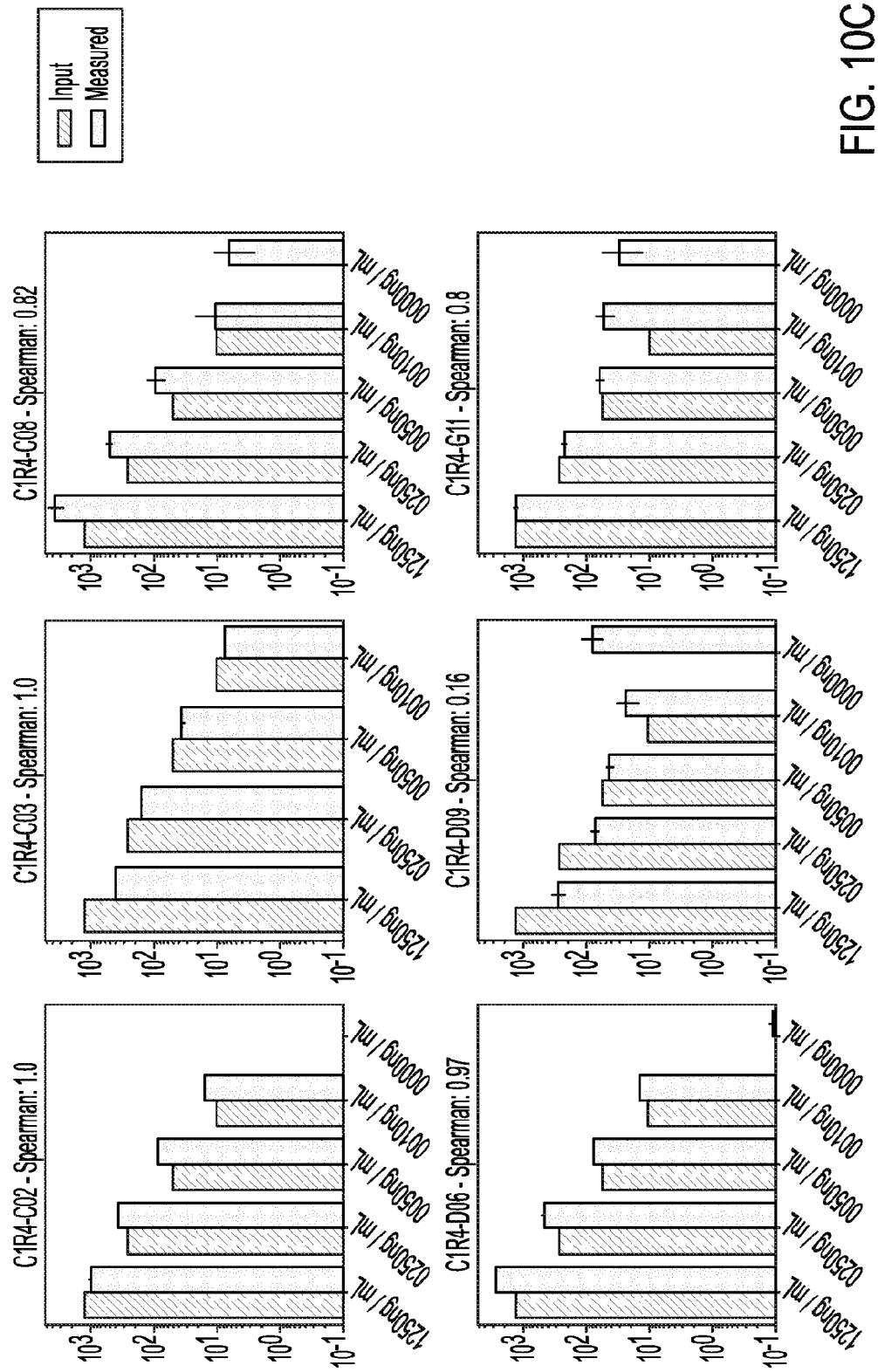
Input
Measured
C1R4-C02 - Spearman: 1.0
C1R4-C03 - Spearman: 1.0
C1R4-C08 - Spearman: 0.82
C1R4-D06 - Spearman: 0.97
C1R4-D09 - Spearman: 0.16
C1R4-G11 - Spearman: 0.8
125ng / mL
025ng / mL
005ng / mL
001ng / mL
000ng / mL
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}

FIG. 10C

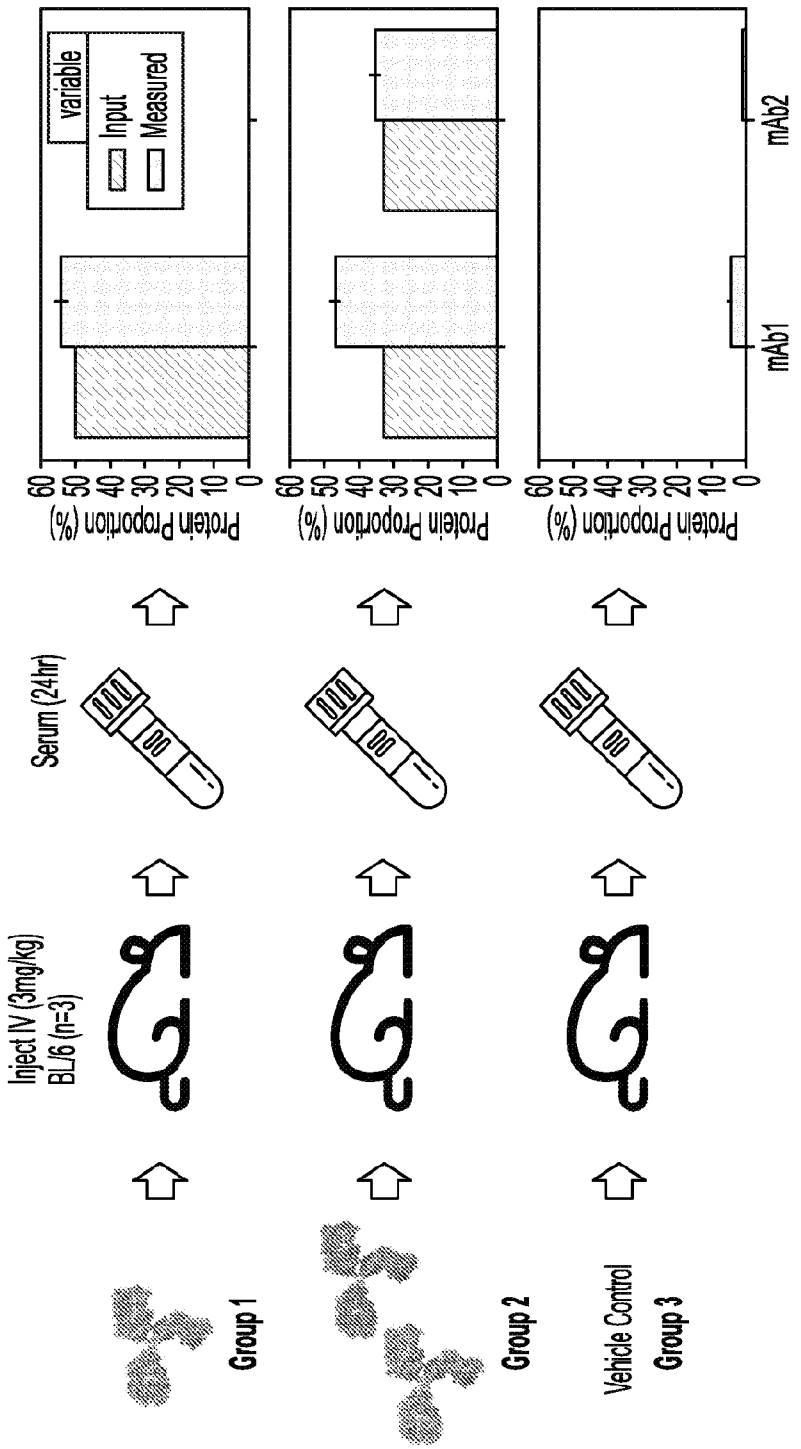
Group 1
Group 2
Vehicle Control
Group 3
Inject IV (3mg/kg)
BL/6 (n=3)
Serum (24hr)
Protein Proportion (%)
variable
Input
Measured
mAb1
mAb2

FIG. 11

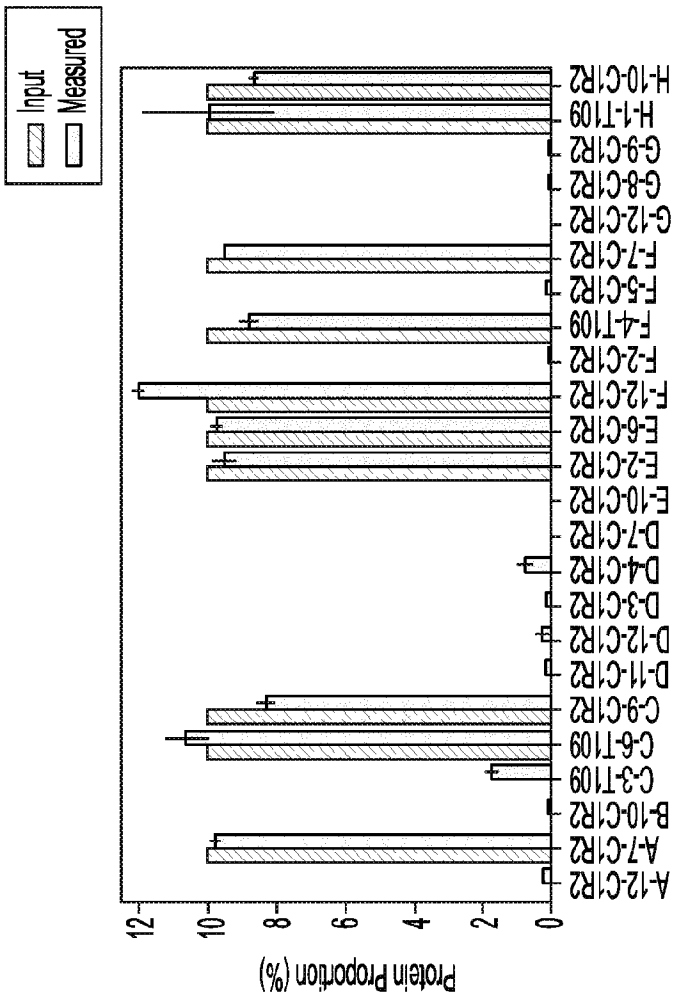

FIG. 12B

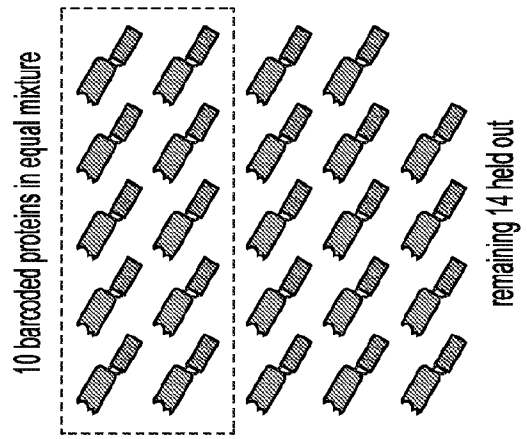

FIG. 12A

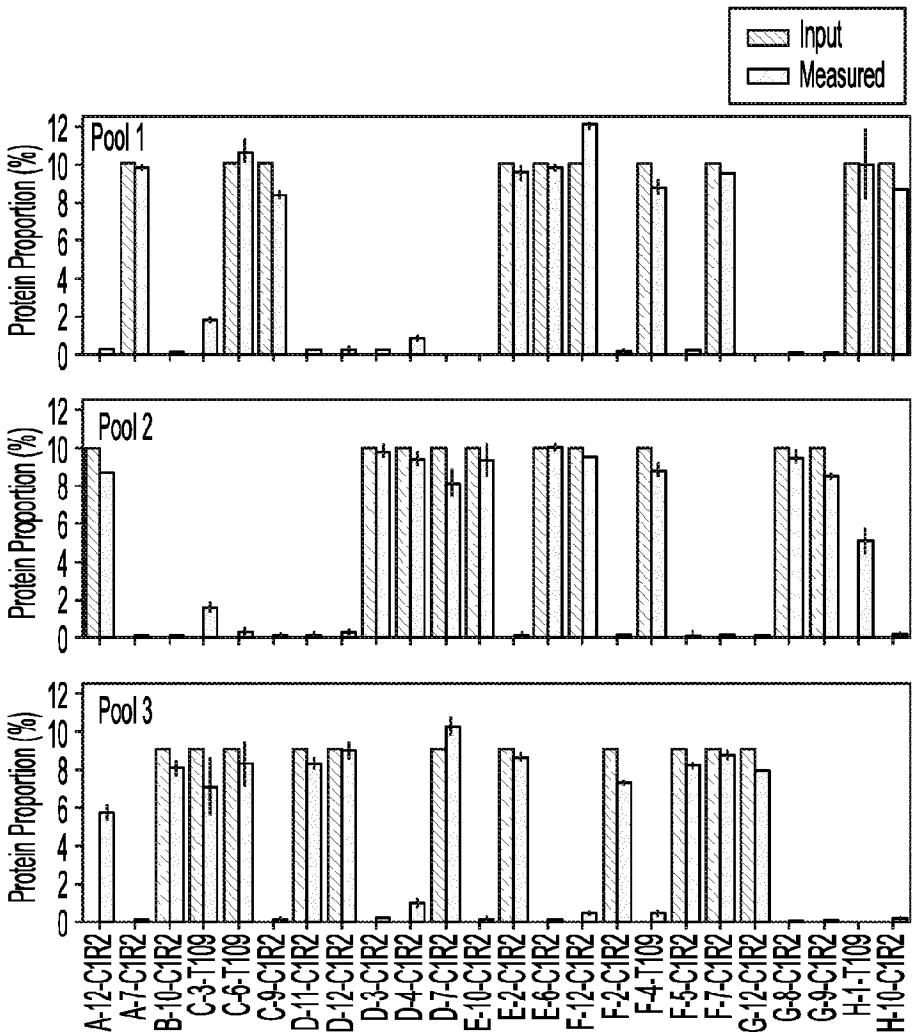
Input
Measured
Pool 1
Pool 2
Pool 3
Protein Proportion (%)
0
2
4
6
8
10
12
A-12-C1R2
A-7-C1R2
B-10-C1R2
C-3-T109
C-6-T109
C-9-C1R2
D-11-C1R2
D-12-C1R2
D-3-C1R2
D-4-C1R2
D-7-C1R2
E-10-C1R2
E-2-C1R2
E-6-C1R2
F-12-C1R2
F-2-C1R2
F-4-T109
F-5-C1R2
F-7-C1R2
G-12-C1R2
G-8-C1R2
G-9-C1R2
H-1-T109
H-10-C1R2

FIG. 12C

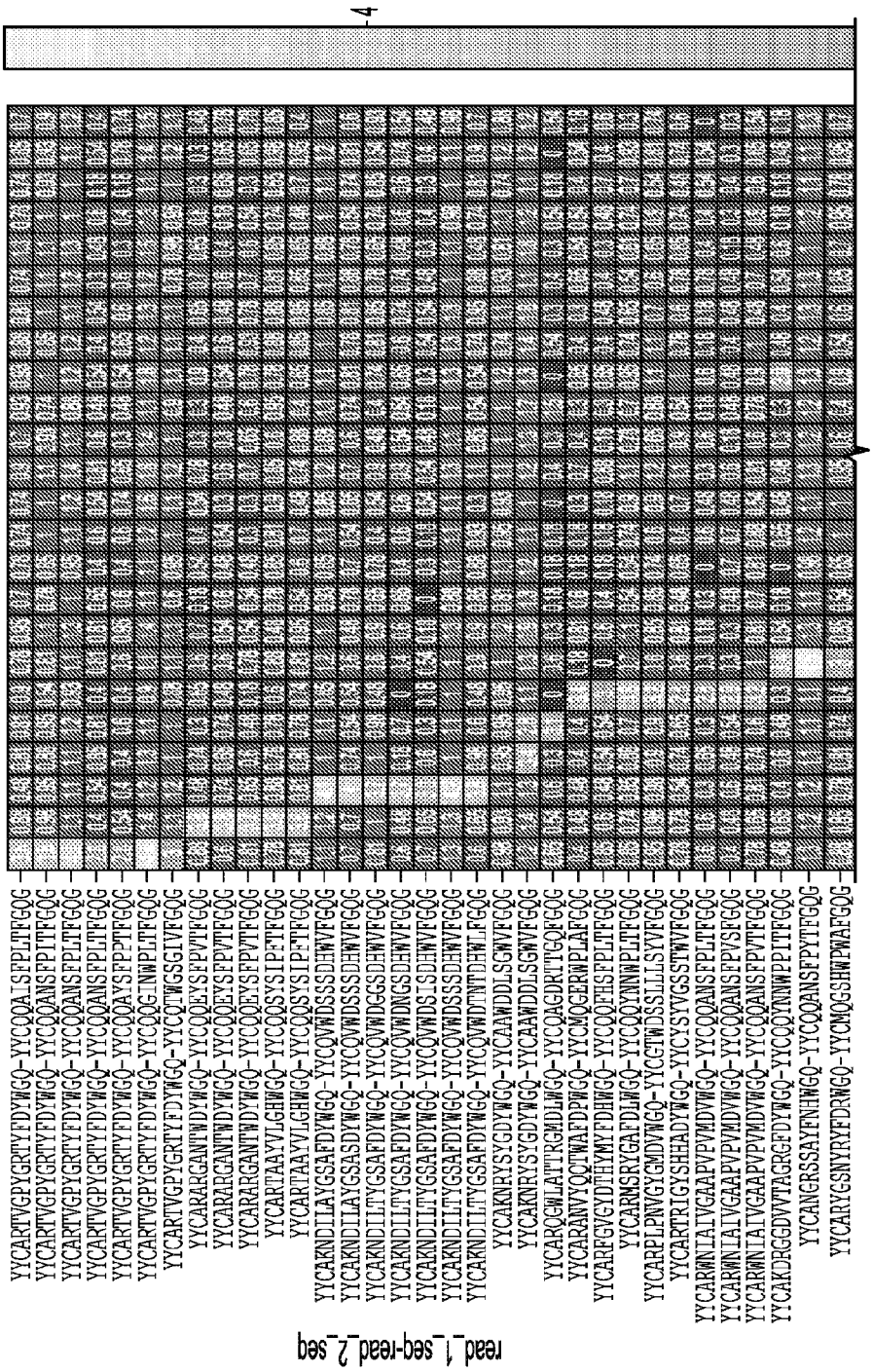

FIG. 12D

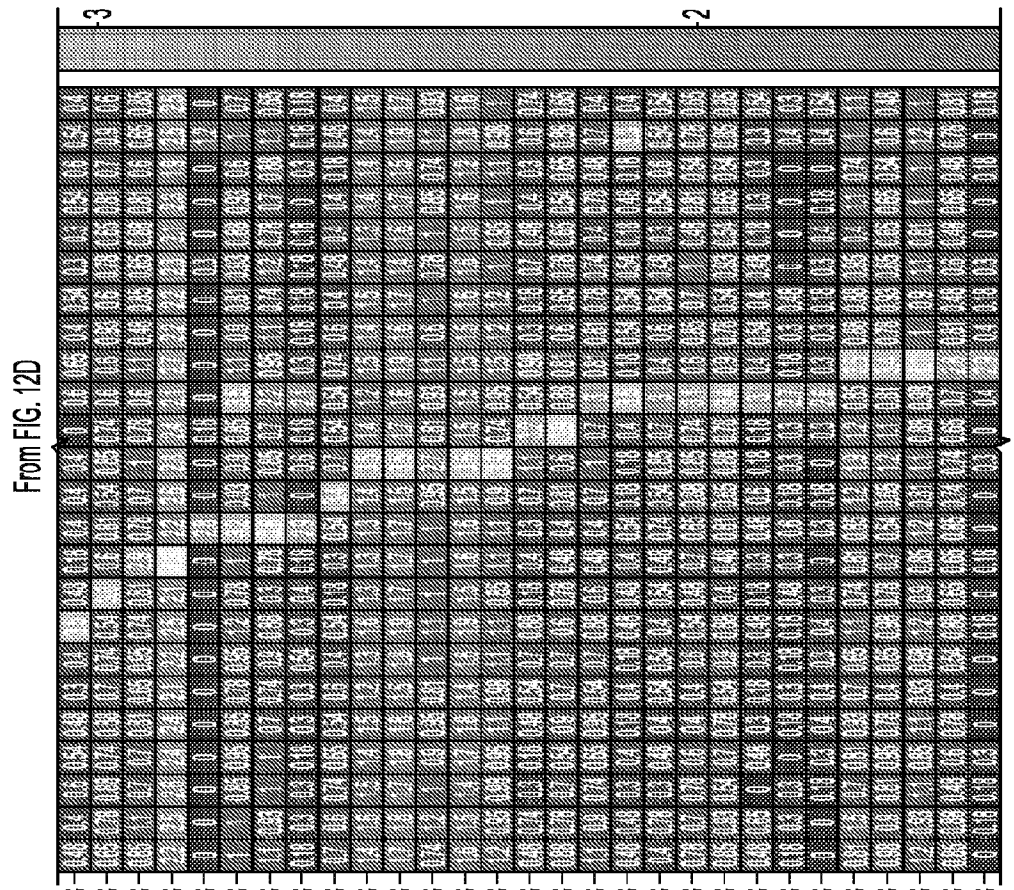

FIG. 12D
CONTINUED

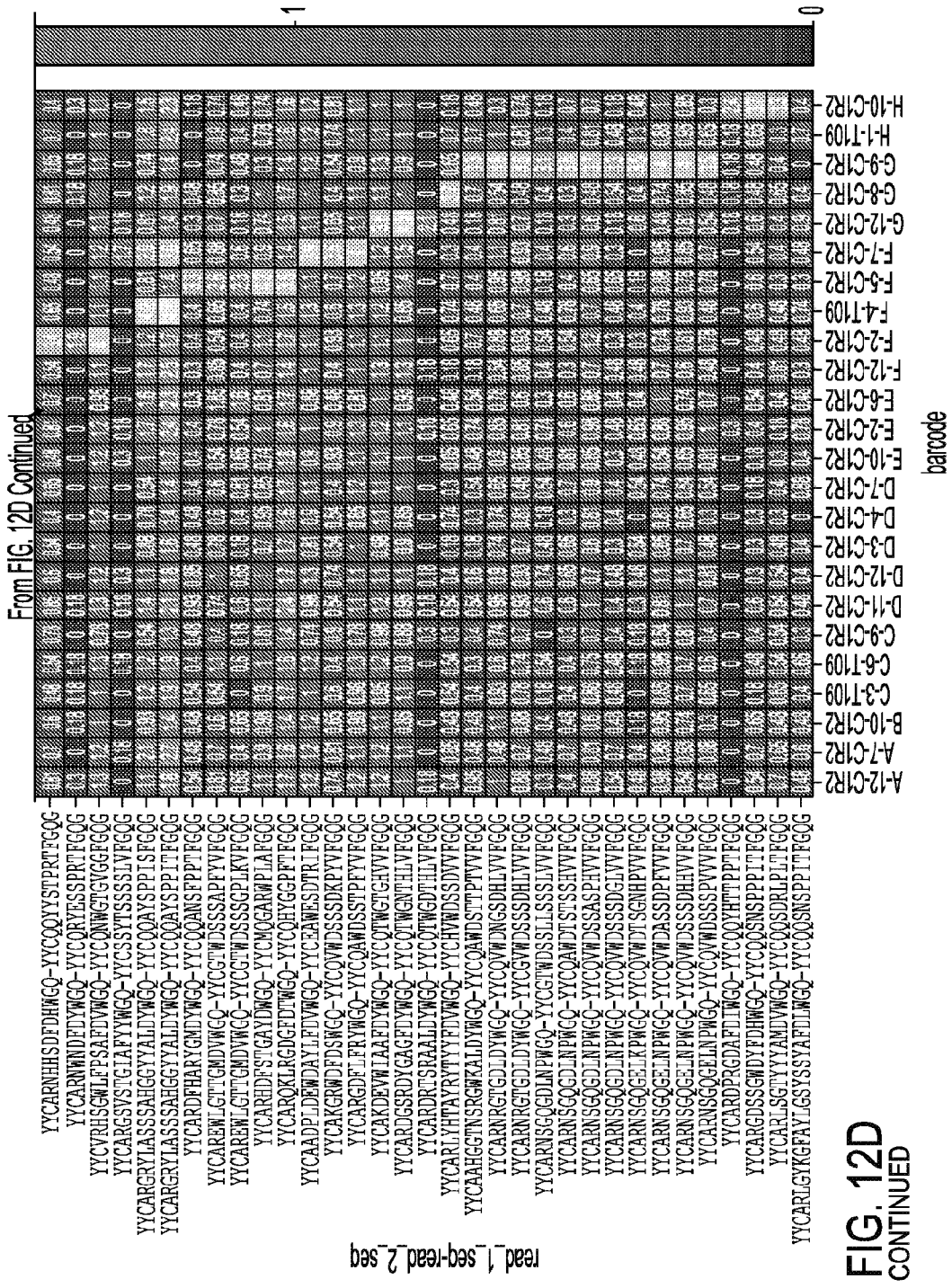

FIG. 12D
CONTINUED

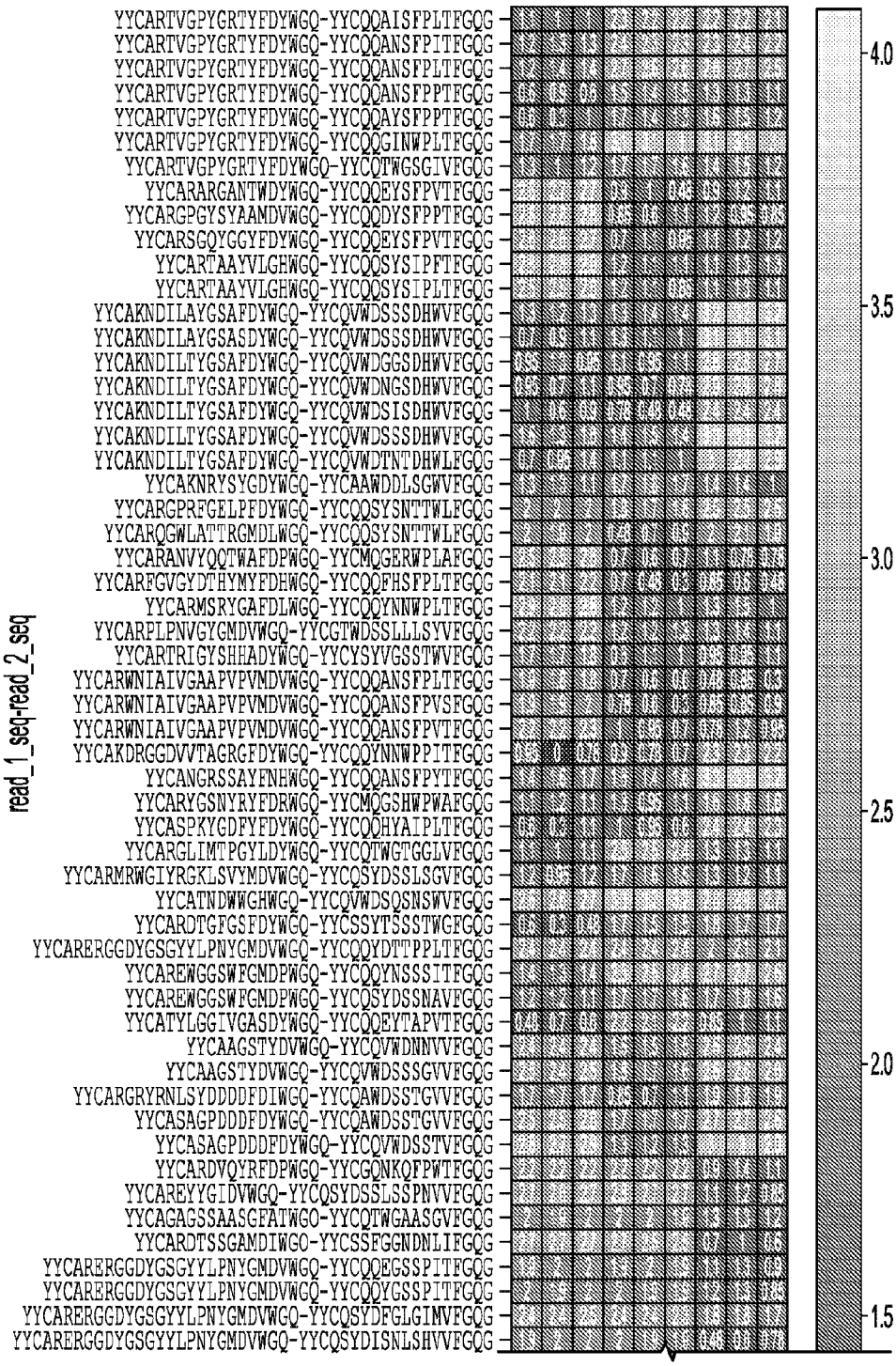

FIG. 12E

To FIG. 12E Continued

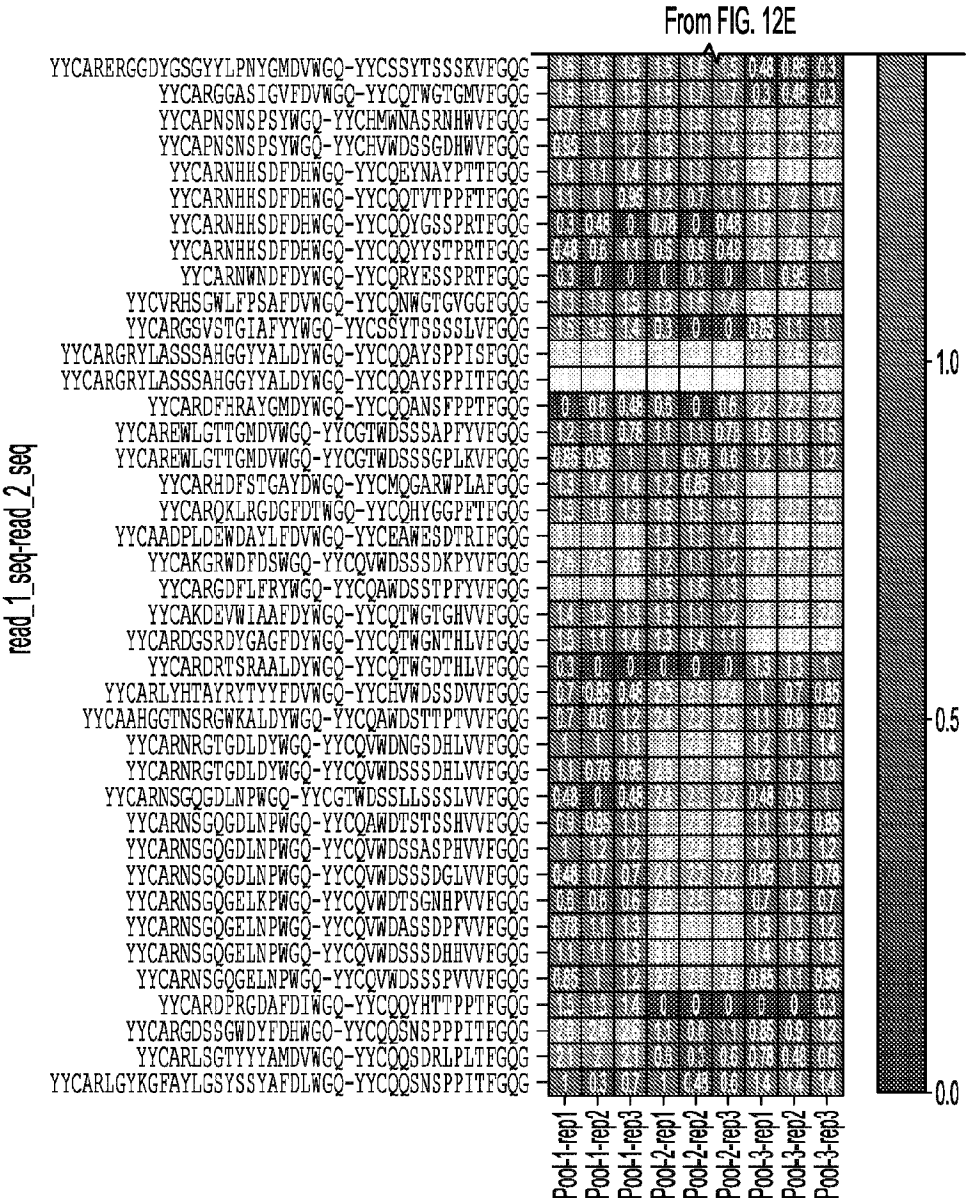

FIG. 12E
CONTINUED

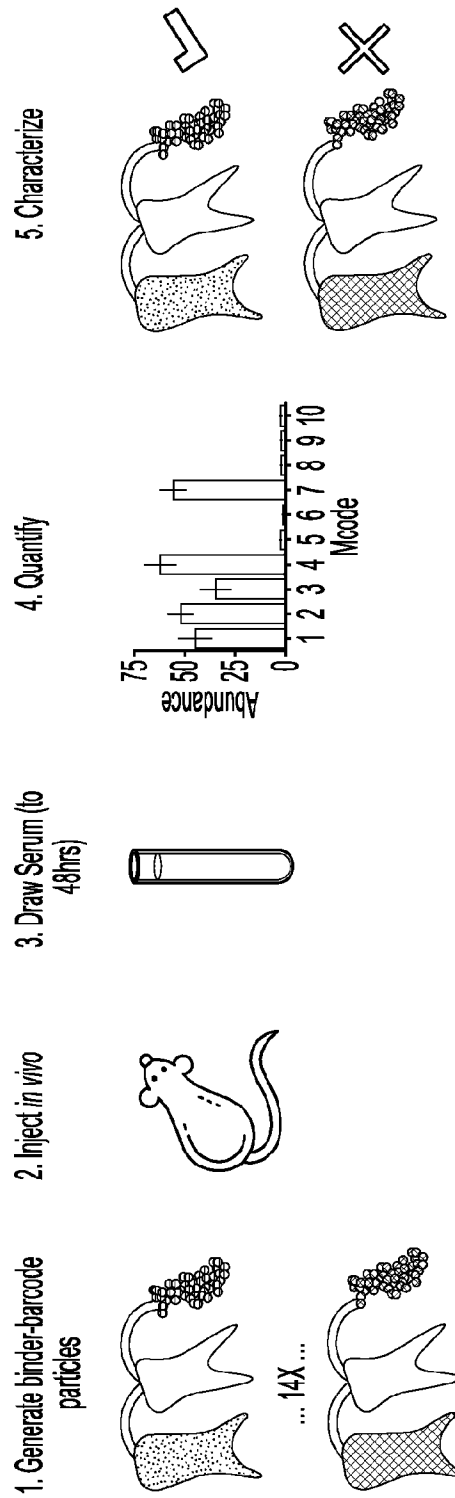
1. Generate binder-barcode particles
... 14X ...
2. Inject in vivo
3. Draw Serum (to 48hrs)
4. Quantify
Abundance
75
50
25
0
1 2 3 4 5 6 7 8 9 10
Mcode
5. Characterize

FIG. 13A

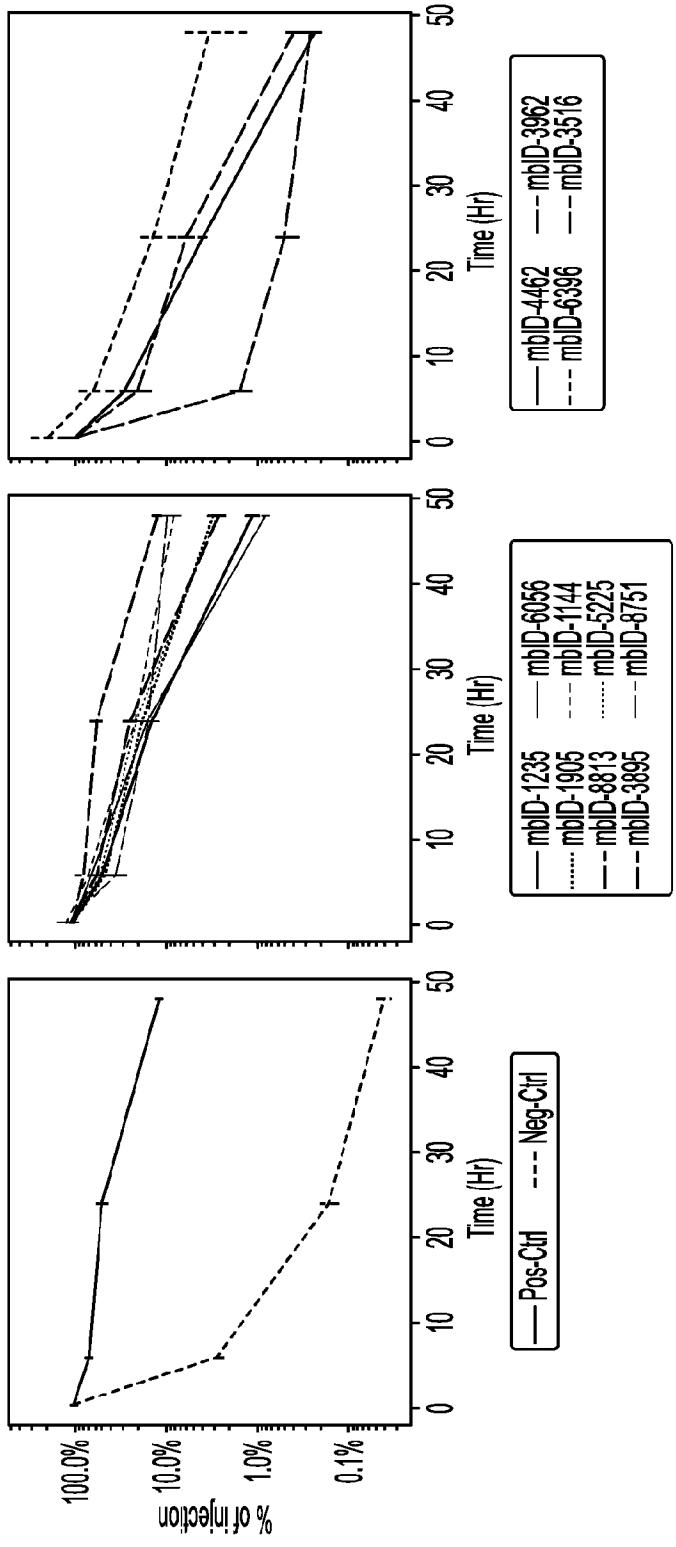
% of injection
100.0%
10.0%
1.0%
0.1%
0
10
20
30
40
50
Time (Hr)
Pos-Ctrl
Neg-Ctrl
mbID-1235
mbID-1905
mbID-8813
mbID-3895
mbID-6056
mbID-1144
mbID-5225
mbID-8751
mbID-4462
mbID-6396
mbID-3962
mbID-3516

FIG. 13B

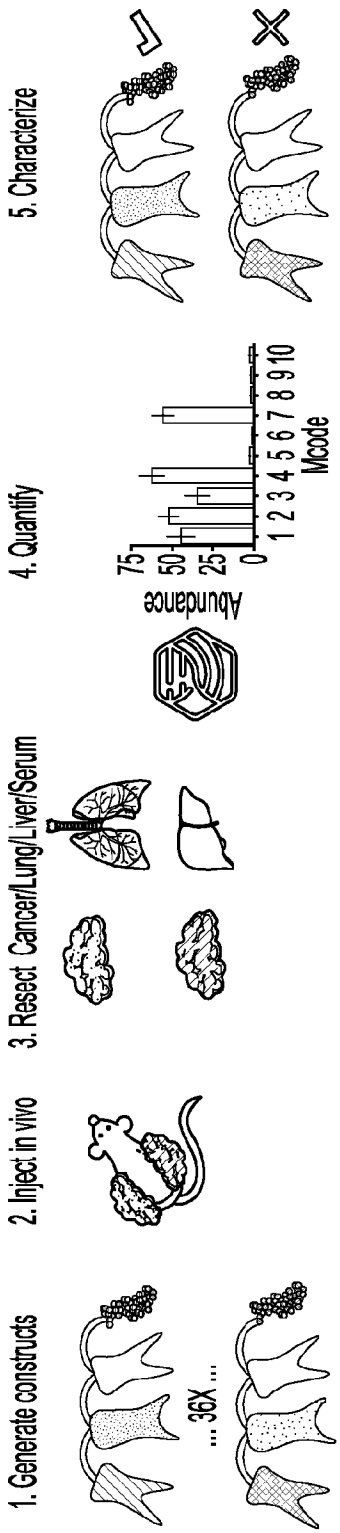
1. Generate constructs
...36X...
2. Inject in vivo
3. Resect Cancer/Lung/Liver/Serum
4. Quantify
Abundance
75
50
25
0
1 2 3 4 5 6 7 8 9 10
Mcode
5. Characterize

FIG. 14A

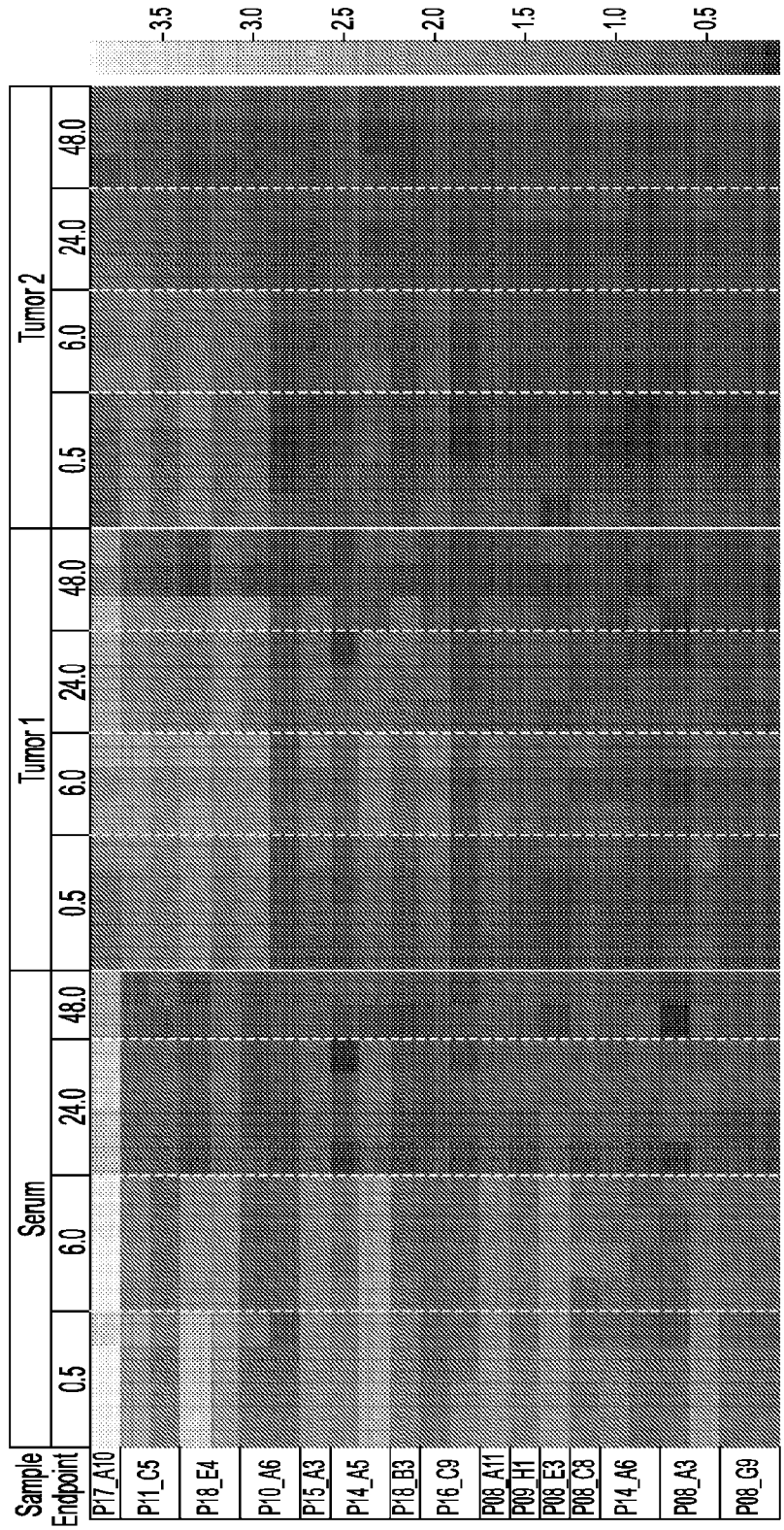
Sample
Endpoint
Serum
Tumor 1
Tumor 2
0.5
6.0
24.0
48.0
0.5
6.0
24.0
48.0
0.5
6.0
24.0
48.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
P17_A10
P11_C5
P18_E4
P10_A6
P15_A3
P14_A5
P18_B3
P16_C9
P08_A11
P09_H1
P08_E3
P08_C8
P14_A6
P08_A3
P08_G9
Construct

FIG. 14B

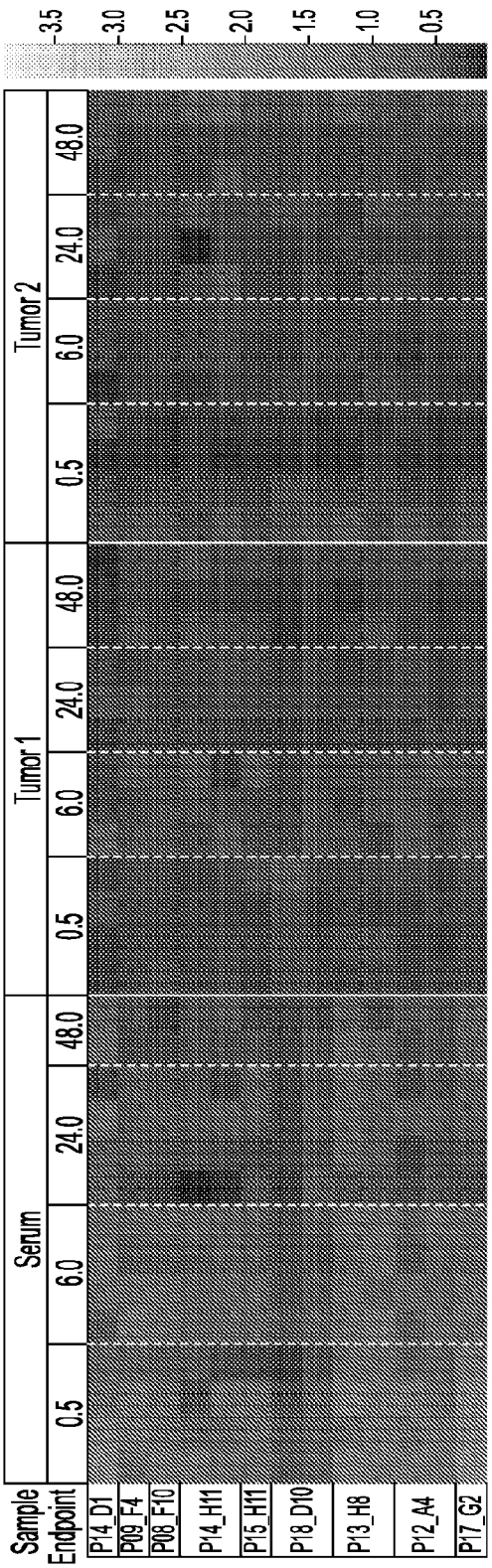

FIG. 14B
CONTINUED

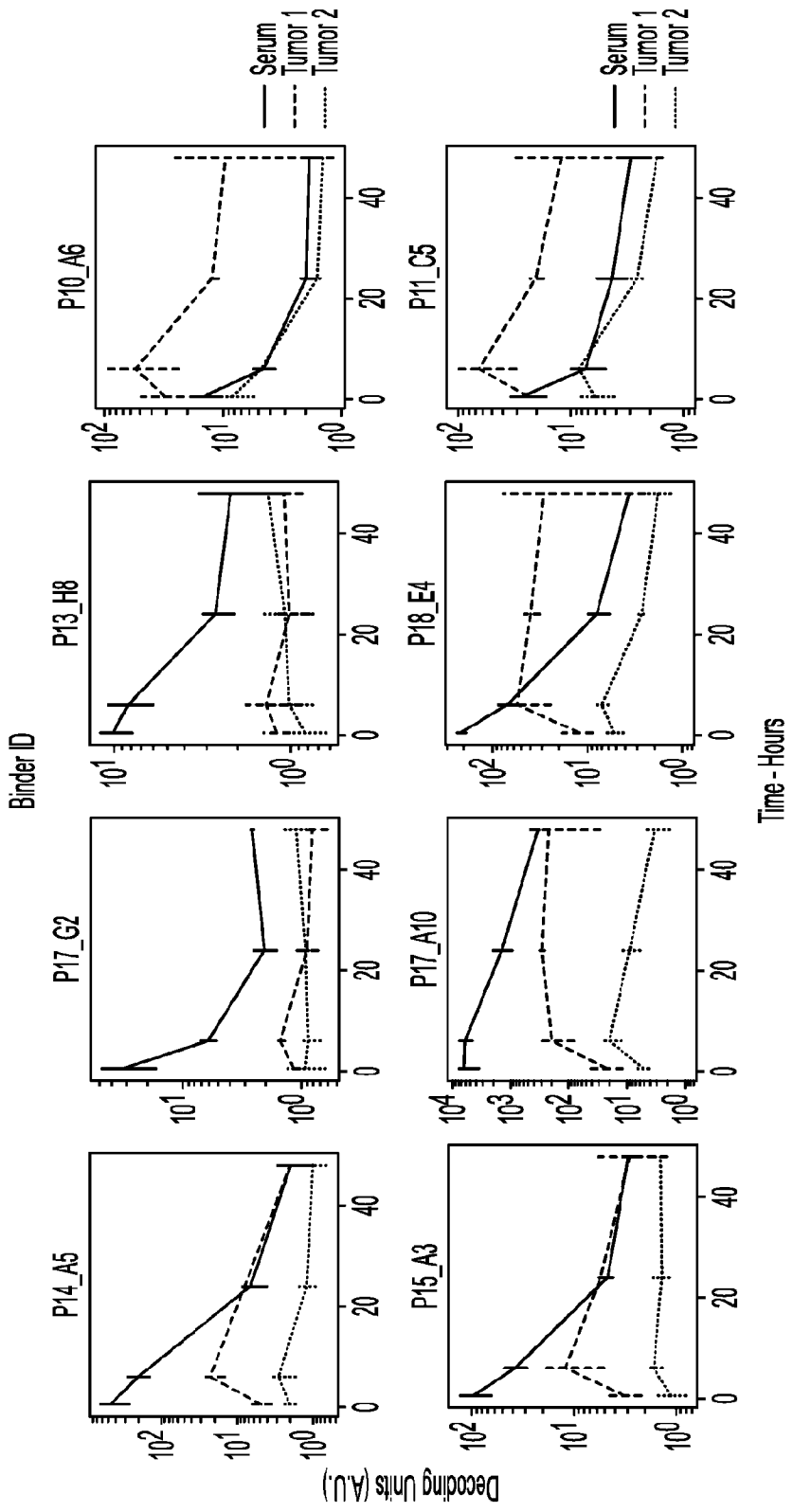
Binder ID
P14_A5
P17_G2
P13_H8
P10_A6
P15_A3
P17_A10
P18_E4
P11_C5
Serum
Tumor 1
Tumor 2
Decoding Units (A.U.)
Time - Hours

FIG. 14C

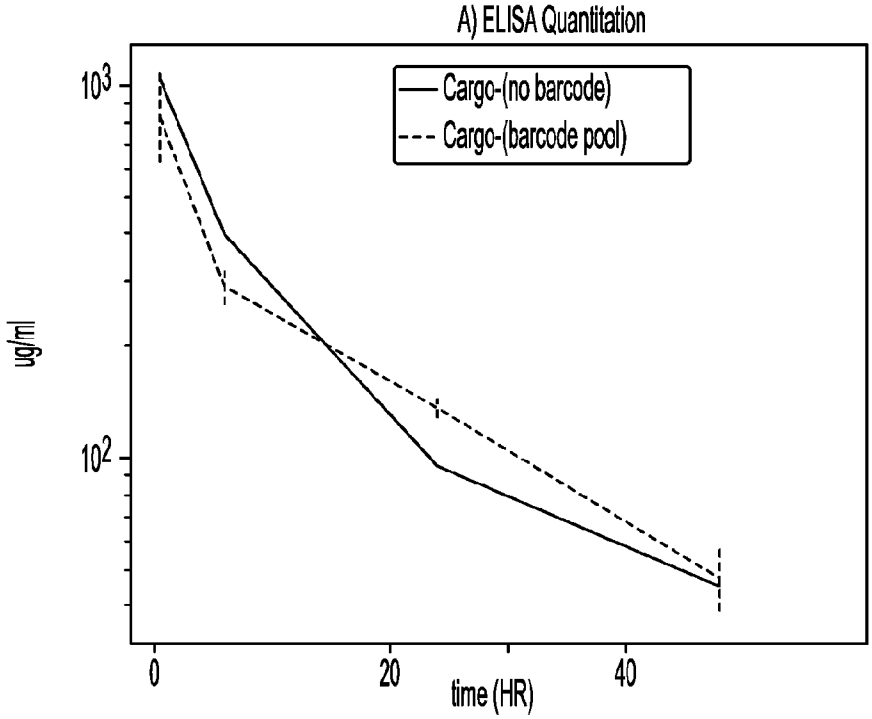

FIG. 15A

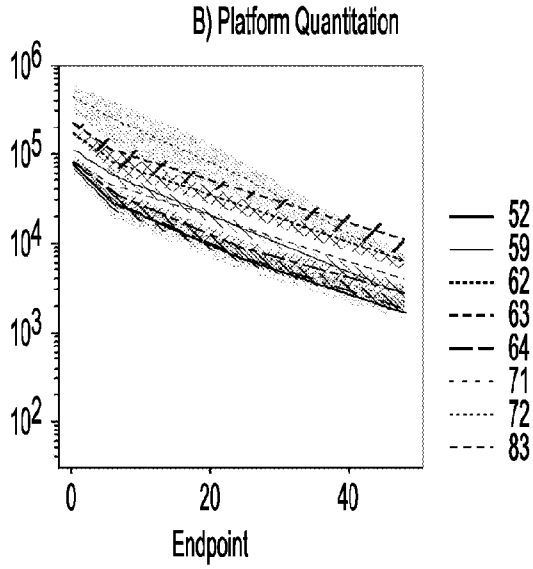

FIG. 15B

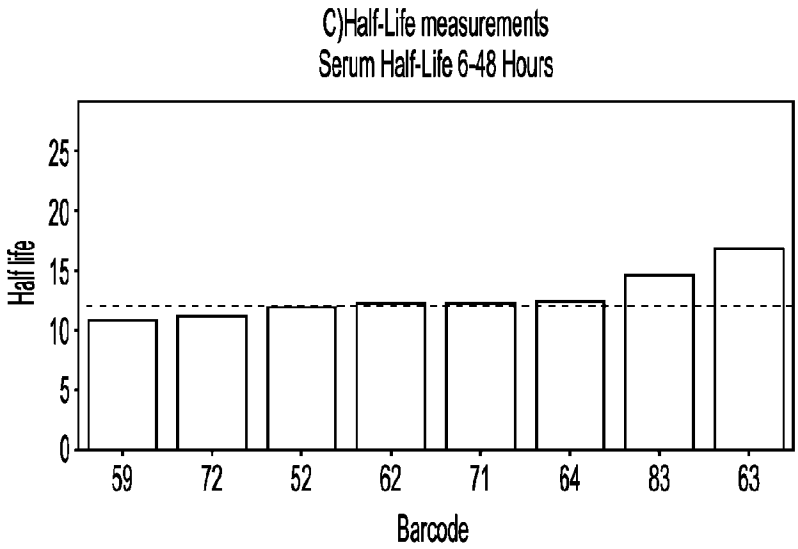
C)Half-Life measurements
Serum Half-Life 6-48 Hours
Half life
25
20
15
10
5
0
59
72
52
62
71
64
83
63
Barcode

FIG. 15C

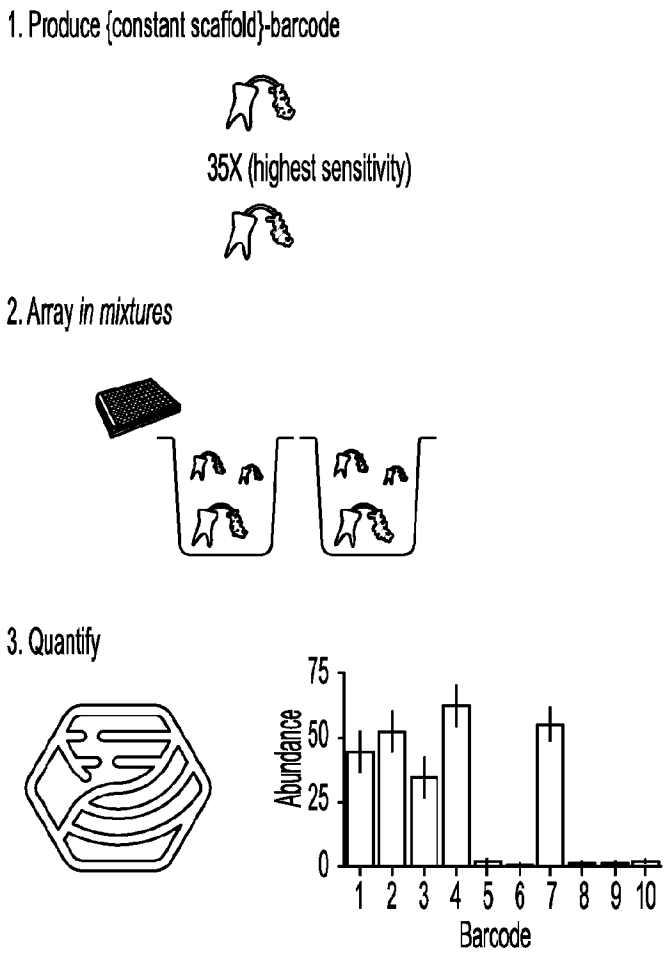
1. Produce {constant scaffold}-barcode
35X (highest sensitivity)
2. Array *in mixtures*
3. Quantify
Abundance
75
50
25
0
1 2 3 4 5 6 7 8 9 10
Barcode

FIG. 16A

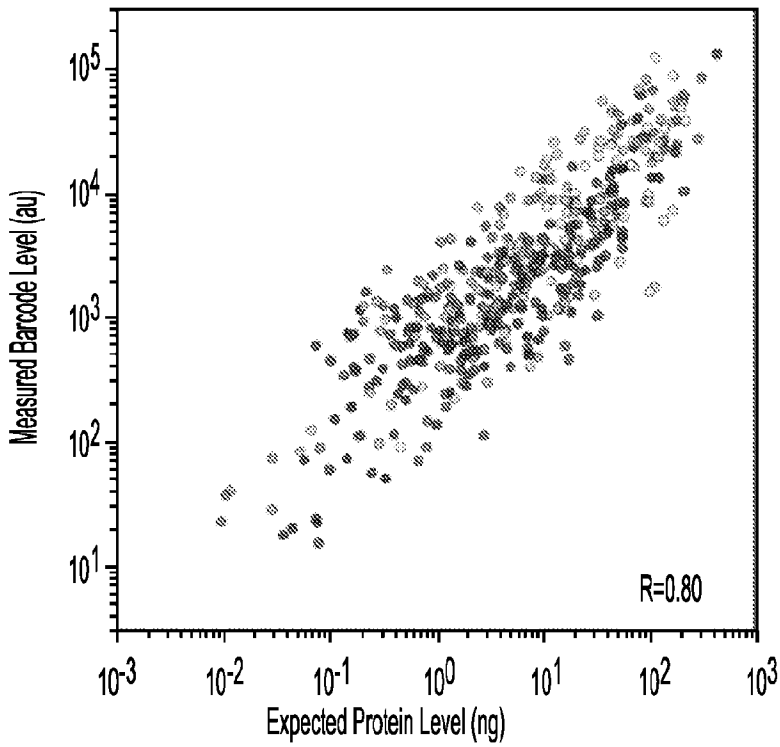
Measured Barcode Level (au)
10^5
10^4
10^3
10^2
10^1
R=0.80
10^{-3}
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Expected Protein Level (ng)

FIG. 16B

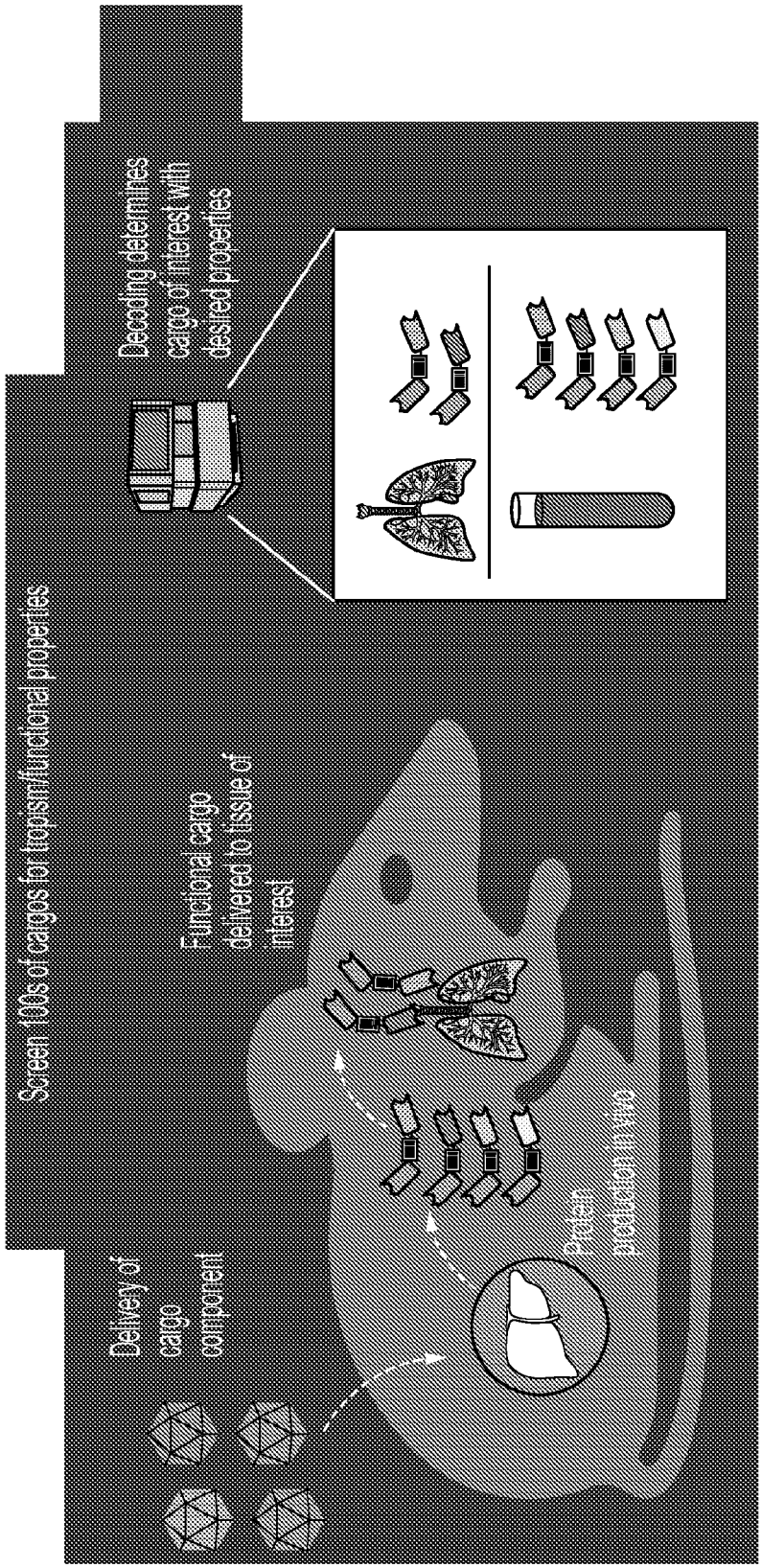
Screen 100s of cargos for tropism/functional properties
Decoding determines cargo of interest with desired properties
Functional cargo delivered to tissue of interest
Delivery of cargo component
Patient population

FIG. 17

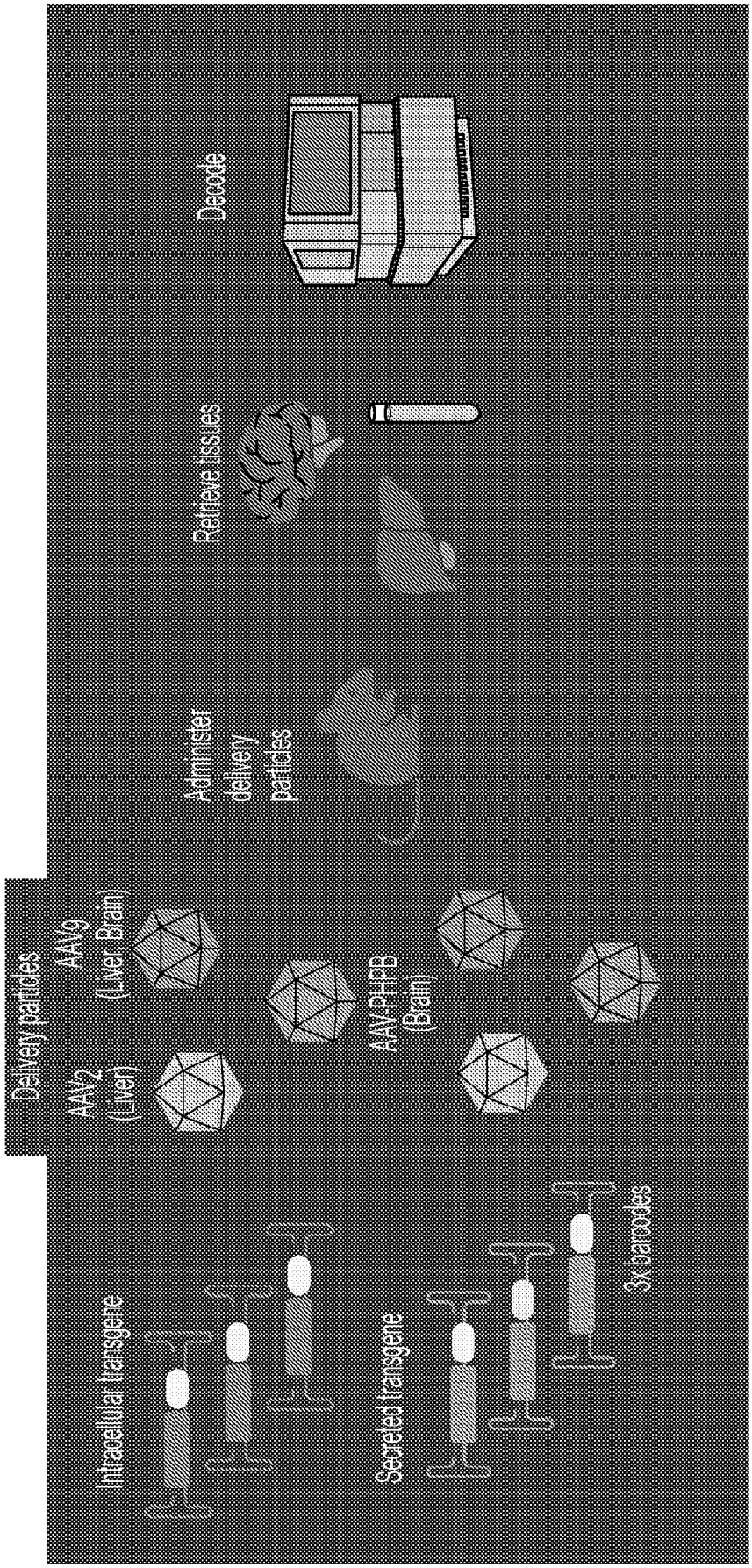
Delivery particles
AAV2 (Liver)
AAV9 (Liver, Brain)
AAV-PHP.B (Brain)
Intracellular transgene
Secreted transgene
3x barcodes
Administer delivery particles
Retrieve tissues
Decode

FIG. 18

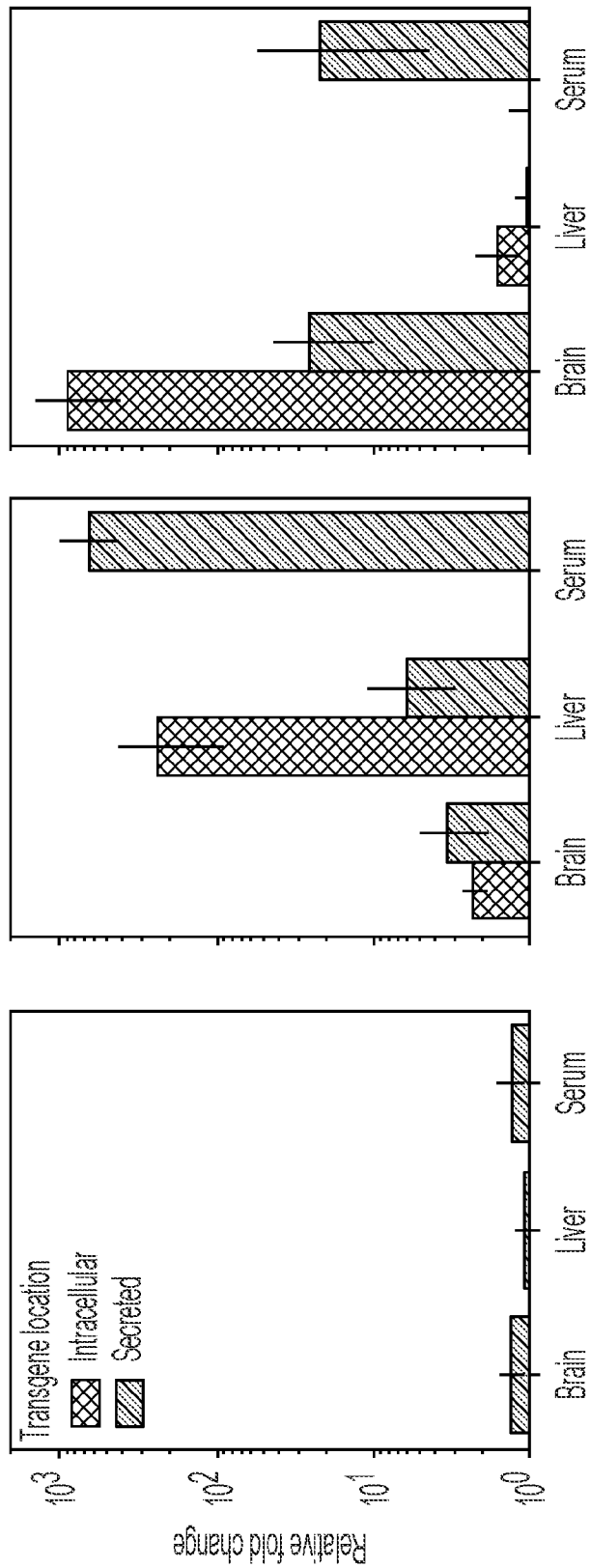

FIG. 19A

FIG. 19B

FIG. 19C

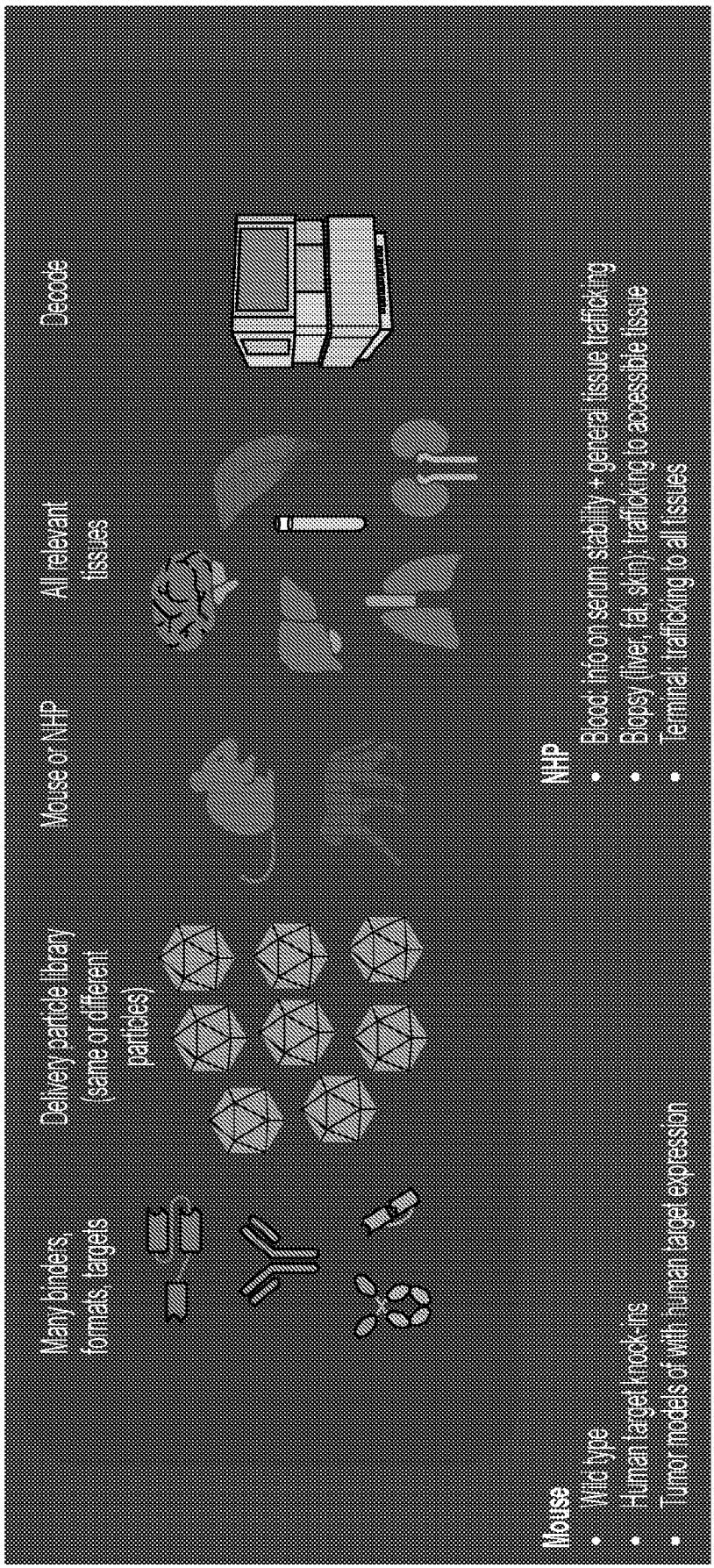
Many binders, formats, targets
Delivery particle library (same or different particles)
Mouse or NHP
All relevant tissues
Decode
Mouse
Wild type
Human target knock-ins
Tumor models of with human target expression
NHP
Blood info on serum stability + general tissue trafficking
Biopsy (liver, fat, skin): trafficking to accessible tissue
Terminal: trafficking to all tissues

FIG. 20

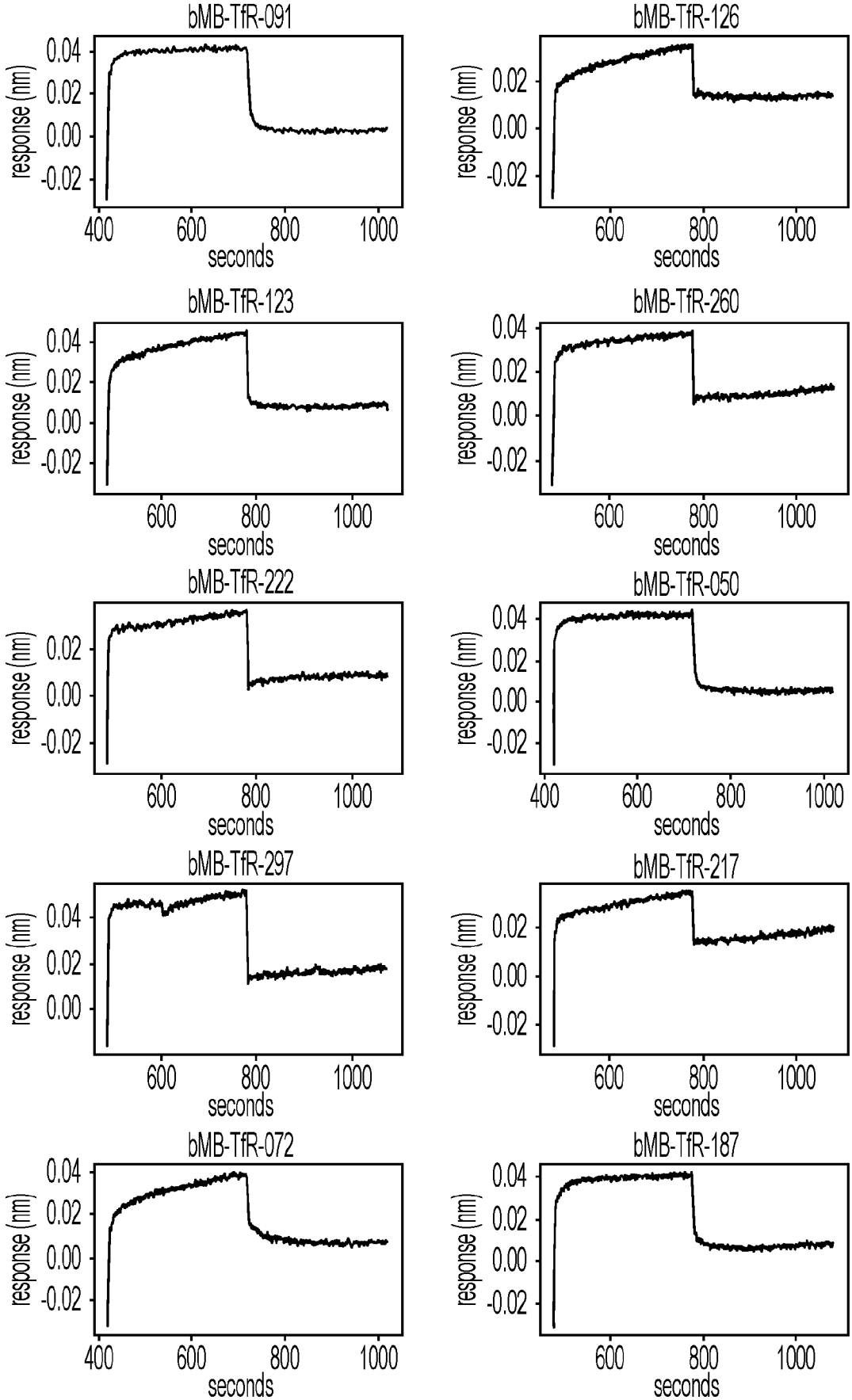
bMB-TfR-091
response (nm)
0.04
0.02
0.00
-0.02
400
600
800
1000
seconds
bMB-TfR-126
response (nm)
0.02
0.00
-0.02
600
800
1000
seconds
bMB-TfR-123
response (nm)
0.04
0.02
0.00
-0.02
600
800
1000
seconds
bMB-TfR-260
response (nm)
0.04
0.02
0.00
-0.02
600
800
1000
seconds
bMB-TfR-222
response (nm)
0.02
0.00
-0.02
600
800
1000
seconds
bMB-TfR-050
response (nm)
0.04
0.02
0.00
-0.02
400
600
800
1000
seconds
bMB-TfR-297
response (nm)
0.04
0.02
0.00
600
800
1000
seconds
bMB-TfR-217
response (nm)
0.02
0.00
-0.02
600
800
1000
seconds
bMB-TfR-072
response (nm)
0.04
0.02
0.00
-0.02
400
600
800
1000
seconds
bMB-TfR-187
response (nm)
0.04
0.02
0.00
-0.02
600
800
1000
seconds

FIG. 21

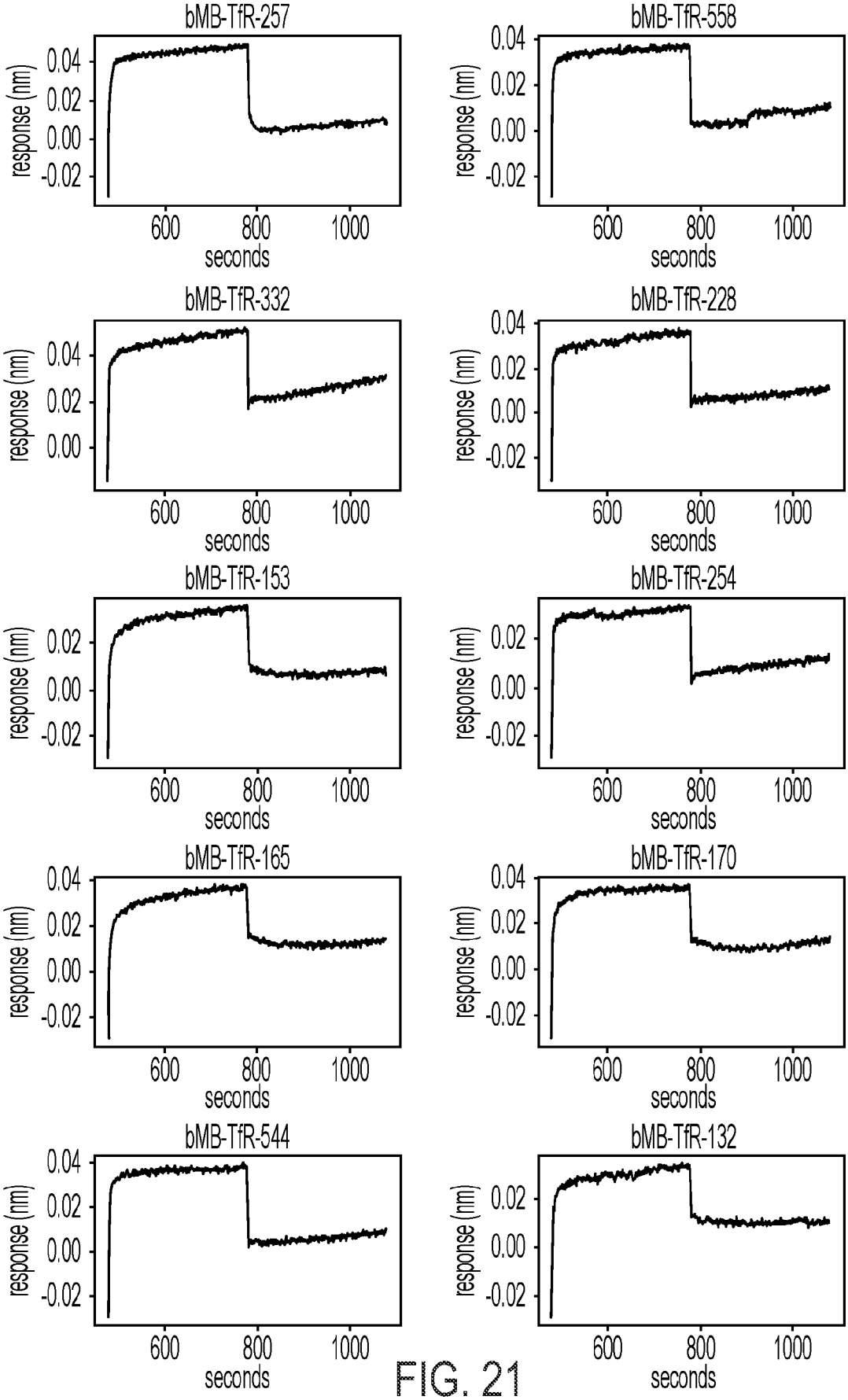

FIG. 21
CONTINUED

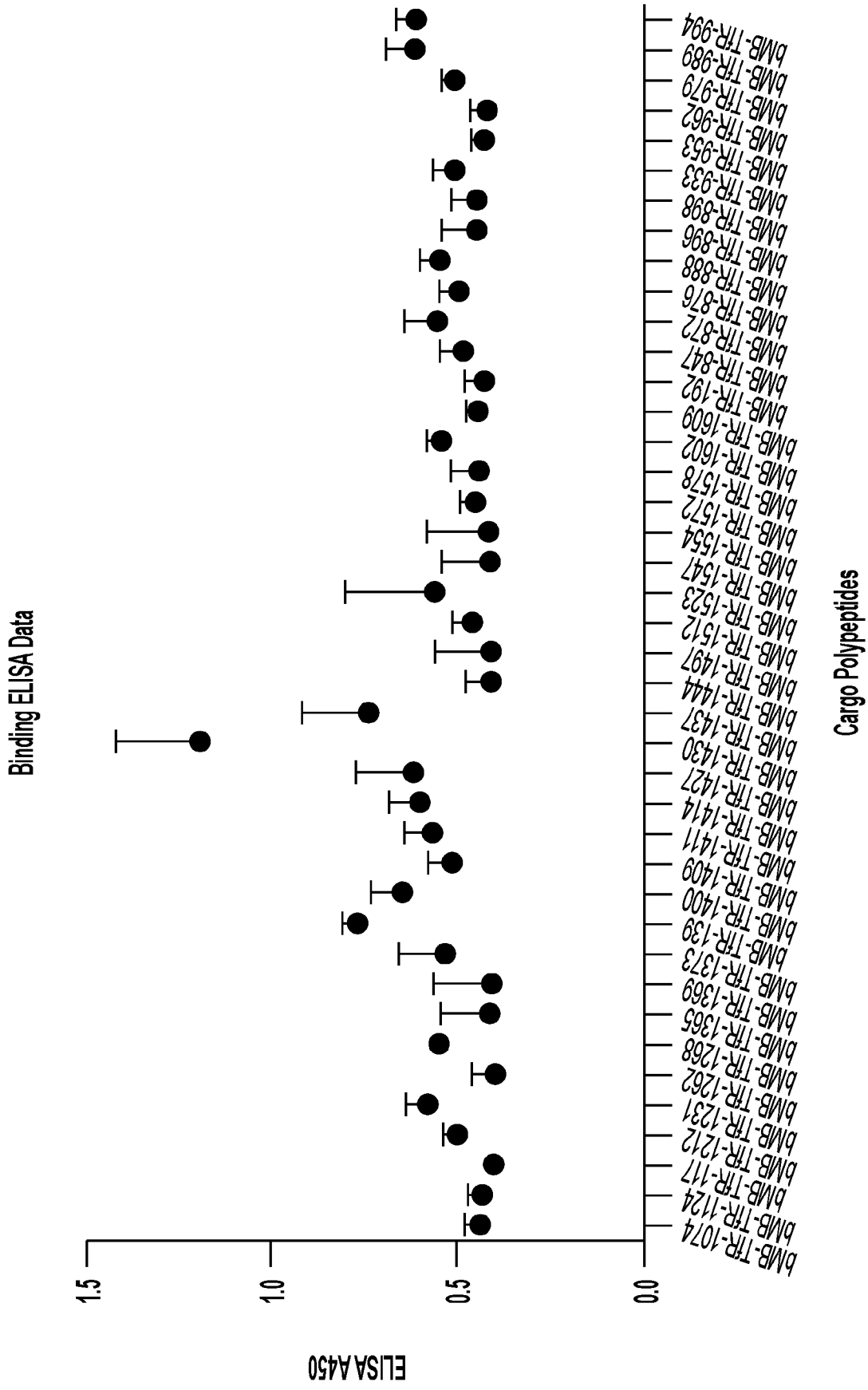
Binding ELISA Data
ELISA A450
1.5
1.0
0.5
0.0
Cargo Polypeptides
bMB-TR-1074
bMB-TR-1124
bMB-TR-117
bMB-TR-1212
bMB-TR-1231
bMB-TR-1262
bMB-TR-1268
bMB-TR-1365
bMB-TR-1369
bMB-TR-1373
bMB-TR-139
bMB-TR-1400
bMB-TR-1409
bMB-TR-1411
bMB-TR-1414
bMB-TR-1427
bMB-TR-1430
bMB-TR-1437
bMB-TR-1444
bMB-TR-1497
bMB-TR-1512
bMB-TR-1523
bMB-TR-1547
bMB-TR-1554
bMB-TR-1572
bMB-TR-1578
bMB-TR-1602
bMB-TR-1609
bMB-TR-192
bMB-TR-847
bMB-TR-872
bMB-TR-876
bMB-TR-888
bMB-TR-896
bMB-TR-898
bMB-TR-933
bMB-TR-953
bMB-TR-962
bMB-TR-979
bMB-TR-989
bMB-TR-994

FIG. 22

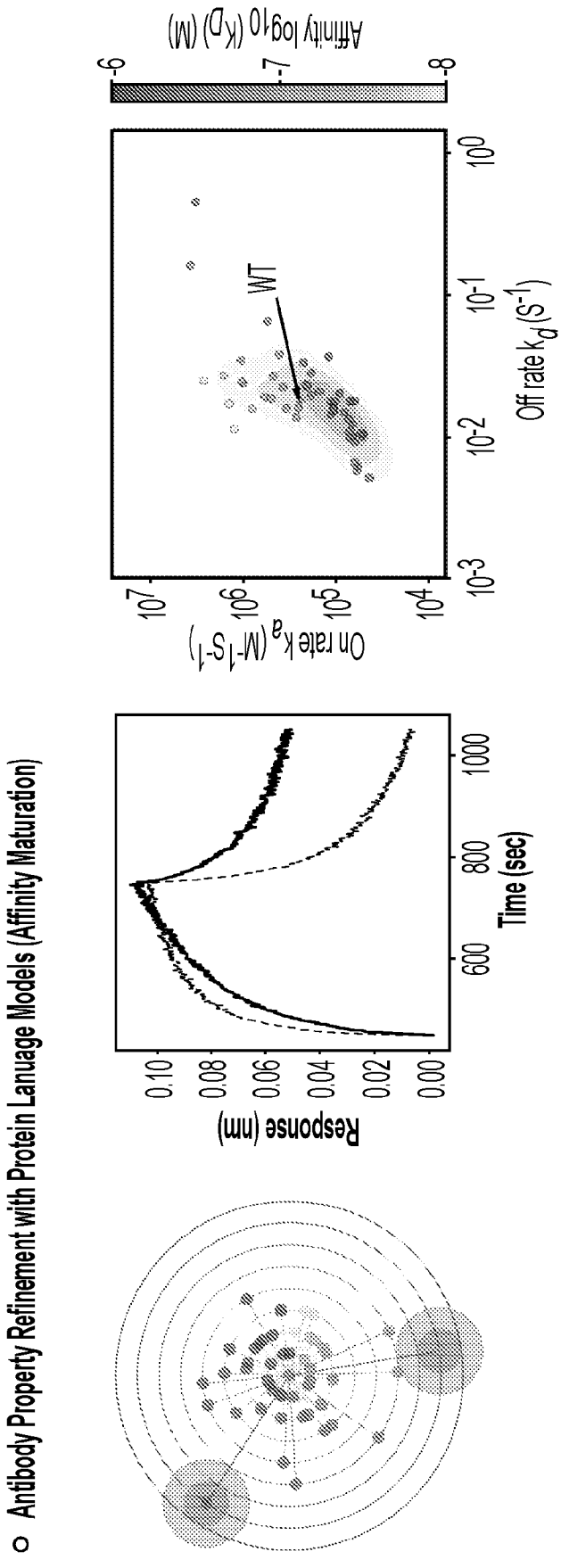
o Antibody Property Refinement with Protein Lanuage Models (Affinity Maturation)
Response (nm)
0.10
0.08
0.06
0.04
0.02
0.00
600
800
1000
Time (sec)
On rate k_a ($M^{-1}S^{-1}$)
10^7
10^6
10^5
10^4
10^{-3}
10^{-2}
10^{-1}
10^0
Off rate k_d (S^{-1})
WT
Affinity $\log_{10}$ (K_D) (M)
-6
-7
-8

FIG. 23

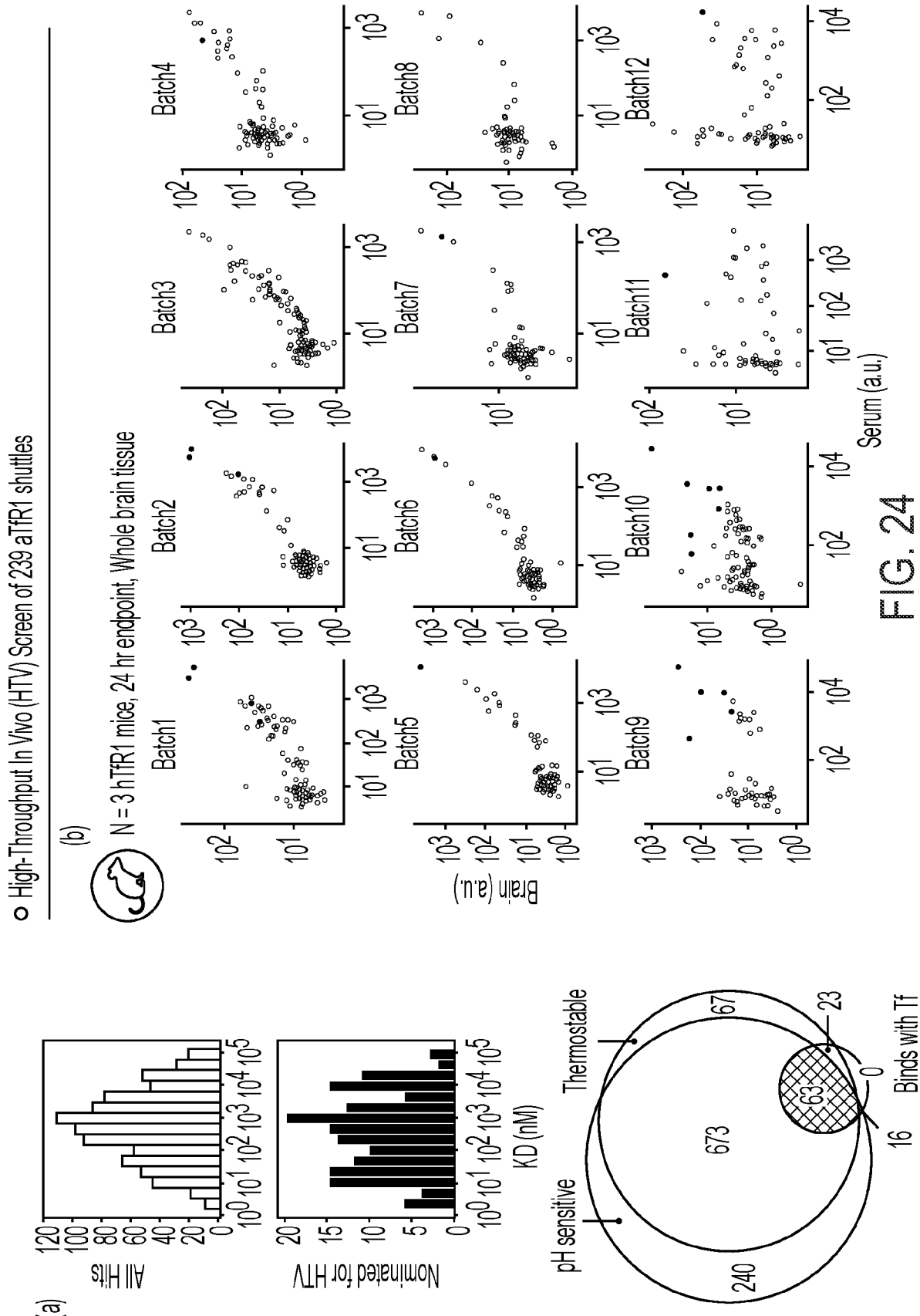
o High-Throughput In Vivo (HTV) Screen of 239 aTfR1 shuttles
(a)
All Hits
Nominated for HTV
KD (nM)
pH sensitive
Thermostable
673
240
67
23
63
0
16
Binds with Tf
(b)
N = 3 hTfR1 mice, 24 hr endpoint, Whole brain tissue
Batch1
Batch2
Batch3
Batch4
Batch5
Batch6
Batch7
Batch8
Batch9
Batch10
Batch11
Batch12
Brain (a.u.)
Serum (a.u.)

FIG. 24

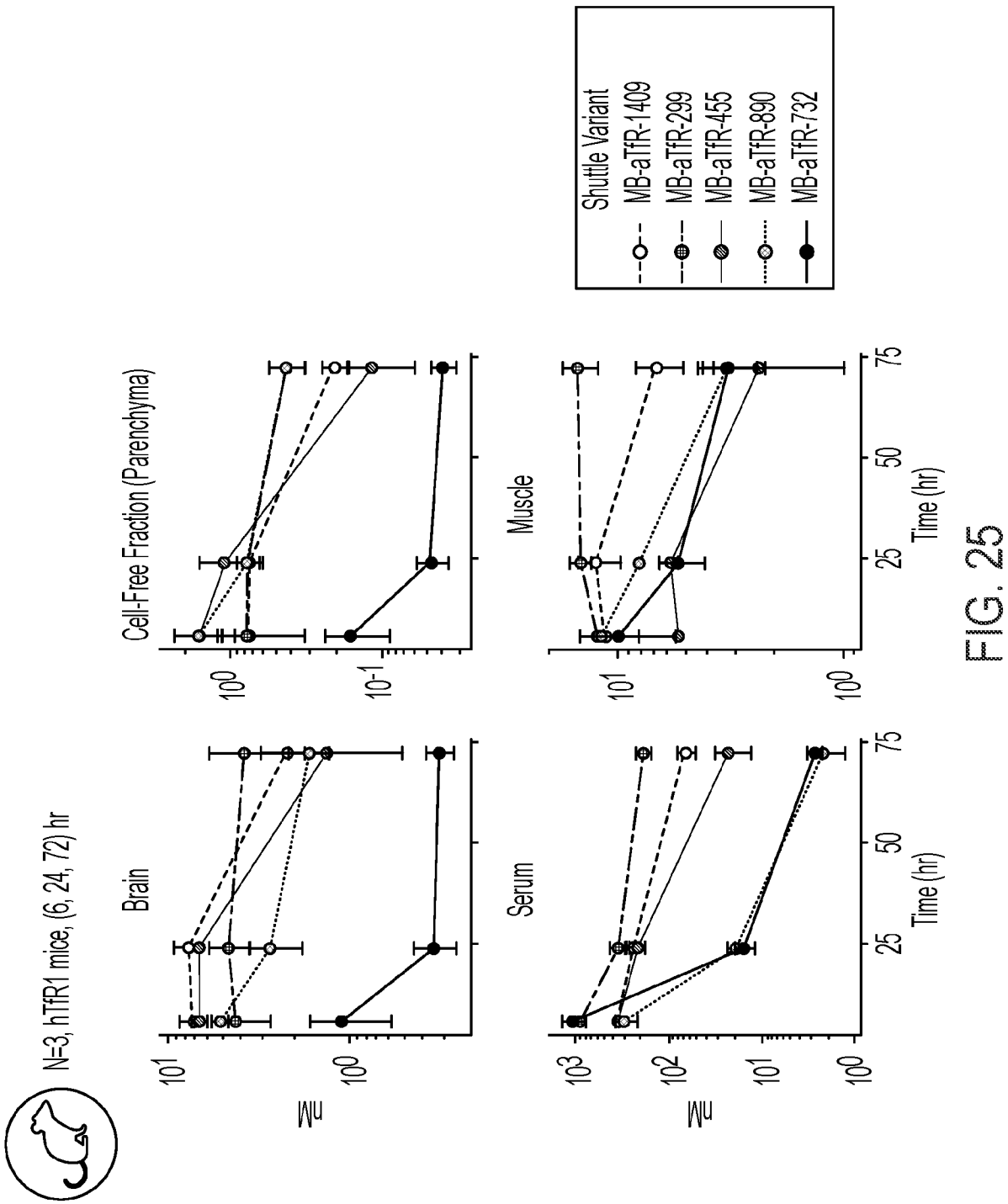
N=3, hTfR1 mice, (6, 24, 72) hr
Brain
Cell-Free Fraction (Parenchyma)
Serum
Muscle
nM
Time (hr)
Shuttle Variant
MB-aTfR-1409
MB-aTfR-299
MB-aTfR-455
MB-aTfR-890
MB-aTfR-732

FIG. 25