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(71) Applicant: **PRESIDENT AND FELLOWS OF HARVARD COLLEGE** [US/US]; 17 Quincy Street, Cambridge, Massachusetts 02138 (US).

(72) Inventors: **CHURCH, George M.**; 218 Kent Street, Brookline, Massachusetts 02446 (US). **GU, Liangcai**; 109 Queensberry Street, Apt. 15, Boston, Massachusetts 02215 (US).

(74) Agent: **IWANICKI, John P.**; 28 State Street, Suite 1800, Boston, Massachusetts 02109 (US).

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(54) Title: BARCODED PROTEIN ARRAY FOR MULTIPLEX SINGLE-MOLECULE INTERACTION PROFILING

(57) Abstract: Methods for attaching barcodes to polypeptides are provided. Methods for detecting molecular interactions at the single molecule level are provided.

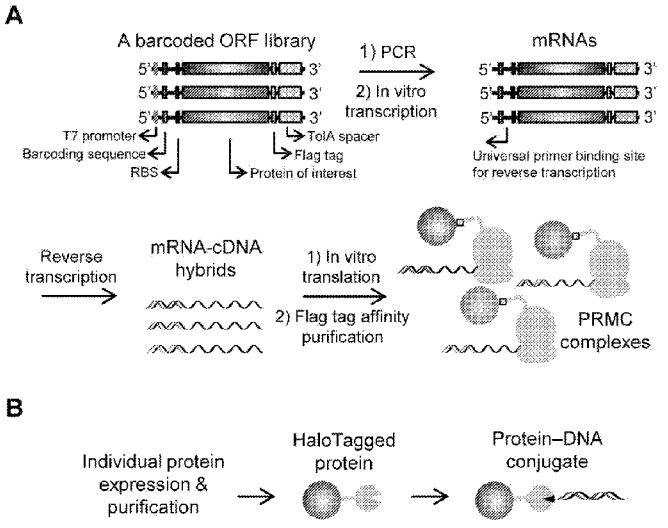

Figure 1

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BARCODED PROTEIN ARRAY FOR MULTIPLEX SINGLE-MOLECULE INTERACTION PROFILING

RELATED APPLICATION DATA

This application claims priority to U.S. Provisional Patent Application No. 61/969,997, filed on March 25, 2014 and is hereby incorporated herein by reference in its entirety for all purposes.

STATEMENT OF GOVERNMENT INTERESTS

This invention was made with government support under DE-FG02-02ER63445 awarded by the U.S. Department of Energy. The government has certain rights in this invention.

FIELD

The present invention relates to methods and compositions for massively parallel quantitative analyses of molecular interactions at a single-molecule level.

BACKGROUND

Compared with recent advances in massively parallel DNA sequencing (Shendure, J. & Ji, H. Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135-1145 (2008)), high-throughput protein analyses, such as yeast-two-hybrid screening (Dreze, M. et al. High-quality binary interactome mapping. Methods Enzymol. 470, 281-315 (2010)), protein microsrray (MacBeath, G. & Schreiber, S. L. Printing proteins as microarrays for high-throughput function determination. Science 289, 1760-1763 (2000)), and affinity purification–mass spectrometry (Gavin, A. C. et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141-147 (2002)), are impeded by ensemble measurements, which necessitate individual analyte separation or enrichment and, therefore, compromise throughput and cost-effectiveness. Single molecule protein detection has been achieved using optical methods (Weiss, S. Fluorescence spectroscopy of single biomolecules. Science 283, 1676-1683 (1999)), but the multiplexity is limited to the number of spectrally non-overlapping chromophores.

SUMMARY

Embodiments of the invention are directed to a DNA barcoded protein array technology for parallel protein interaction profiling on a single molecule basis. DNA barcodes are attached to proteins collectively via ribosome display (Hanes, J. & Pluckthun, A. In vitro selection and

evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. U.S.A. 94, 4937-4942 (1997)) or individually via enzymatic conjugation. Barcoded proteins are assayed en masse in aqueous solution and subsequently immobilized in a polyacrylamide (PAA) thin film to construct a random SM array, where barcoding DNAs are amplified into in situ polymerase colonies (polonies) (Mitra, R. D. & Church, G. M. In situ localized amplification and contact replication of many individual DNA molecules. Nucleic Acids Res. 27, e34 (1999)) and analyzed by DNA sequencing. This method allows precise quantification of various proteins with a throughput of over one billion molecules per array. Novel methods are described herein that measure protein interactions based on the statistical analysis of co-localized polonies arising from barcoding DNAs of interacting proteins. Two highly demanding applications, G-protein coupled receptor (GPCR) and antibody screening and binding profiling, were demonstrated. The methods described herein for the first time enable library vs. library screening in a single assay, which can simultaneously interrogate molecular binding affinity and specificity.

In certain exemplary embodiments, a method for attaching a plurality of barcodes to a plurality of polypeptides. The method includes the steps of attaching a barcode to a plurality of DNA template sequences to produce a plurality of barcoded templates comprising a barcode sequence and a protein coding sequence, performing in vitro transcription to synthesized barcoded mRNA templates and then reverse transcription of the barcoded mRNA templates to produce a plurality of mRNA-cDNA hybrid sequences, and performing in vitro translation of the mRNA-cDNA hybrid sequences to generate a plurality of protein-ribosome-mRNA-cDNA complexes.

In certain aspects, the step of attaching is performed using PCR and in vitro transcription. In other aspects, the plurality of protein-ribosome-mRNA-cDNA complexes are formed by ribosome stalling. In other aspects, the plurality of barcoded sequences are synthesized in parallel on an immobilized support or individually synthesized as a mixture of random sequences on a support. In still other aspects, each of the steps is performed in a single container, and a correlation between a barcoding sequence and a protein sequence is determined using massively parallel DNA sequencing.

In certain aspects, the barcoded templates contain a polymerase (e.g., T7 polymerase) promoter, and mRNAs are synthesized from the barcoded DNA templates by in vitro transcription in a single container.

In other aspects, reverse transcription is performed using universal primers, and the cDNA sequences are complementary upstream to a ribosome binding site of the barcoded template. In certain aspects, ribosomes stall at the 3' end of the mRNA-cDNA hybrid sequences during in vitro translation due to one or both of a lack of stop codons or the presence of ribosome stalling peptide sequences. In other aspects, primers for cDNA synthesis contain one or both of 5' desthiobiotin

modifications and 5' acrydite modifications. In yet other aspects, the protein coding sequence encodes one or more affinity tags (e.g., FLAG tags and the like), e.g., at the C-terminal of a protein of interest. In other aspects, the protein-ribosome-mRNA-cDNA complexes, which contain the full-length protein of interest, are purified using a protein affinity tag and a cDNA desthiobiotin tag

In certain exemplary embodiments, a method for attaching a barcode to a polypeptide is provided, comprising the steps of providing a DNA template comprising an enzyme ligand at its 5' end, providing a fusion protein comprising an enzyme tag specific for the ligand, and allowing the enzyme to covalently bind the ligand to produce a polypeptide comprising a barcode.

In certain aspects, the method is performed using an automated high-throughput platform. In other aspects, at least 1000, 10,000, 1,000,000 or more polypeptides comprising a barcode sequence are prepared in parallel. In certain aspects, an enzyme tag is selected from the group consisting of one or more of HaloTag, CLIP tag, SNAP tag and the like. In other aspects, both the DNA template and the polypeptide comprise an affinity tag (e.g., the DNA template comprises a desthiobiotin tag and the polypeptide comprises a His tag), e.g., performing affinity purification using two steps.

In certain exemplary embodiments, a method of detecting and quantifying a plurality of polypeptides in situ is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides comprising a barcode, immobilizing the plurality of polypeptides on a substrate, performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides, and identifying and quantifying amplified barcode sequences and recording their locations by in situ DNA sequencing.

In certain aspects, the polypeptides comprising barcodes are made according to one of the methods described above. In certain aspects, the plurality of polypeptides are randomly immobilized in a crosslinked polyacrylamide gel layer having a thickness of about a few microns. In other aspects, the nucleic acid sequences have a 5' end modification (e.g., an acrydite modification) and are copolymerized into the gel matrix to avoid template drifting. In yet other aspects, the nucleic acid sequences are amplified into polonies using solid-phase PCR. In still other aspects, the polonies are approximately 1-2 microns in diameter, and/or greater than about 1,000,000 polonies are analyzed on 1 mm2 array area. In certain aspects, the polonies are analyzed using sequencing-by-synthesis or sequencing-by-ligation to identify barcode sequences and location coordinates.

In certain exemplary embodiments, a method of detecting a protein-protein interaction between two or more polypeptides is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides comprising a barcode under defined conditions (e.g., selected from the group consisting of one or any combination of ligands, cofactors, buffers and temperature) to allow formation of protein-protein interactions, stabilizing the protein-protein

interactions by chemical crosslinking, immobilizing the plurality of polypeptides on a substrate, performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides, and detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when a protein-protein interaction has occurred between two or more polypeptides.

In certain aspects, the polypeptides comprising barcodes are made according to one of the methods described above. In certain aspects, co-localized barcodes are deconvoluted by DNA sequencing using different sequencing primers. In other aspects, the degree of co-localization of polonies is quantitatively analyzed by co-localization statistics using polony colocalization ratios and pair cross-correlation function (PCCF). In still other aspects, protein binding affinity can be quantitatively correlated with polony co-localization ratios. In still other aspects, the polypeptide is selected from the group consisting of a natural polypeptide, a recombinant polypeptide, and a de novo synthesized polypeptide. In other aspects, about 1,000,000,000 polypeptides are immobilized on half the area of a standard microscopic slide (e.g., a 25 × 75 mm2 slide). In certain aspects, a first library of at least 100,000 or more different polypeptides can be screened against a second other library of at least 100,000 or more different polypeptides or other barcoded molecules in a single assay. In certain aspects, both molecular binding affinity and specificity can analyzed in a single assay.

In certain exemplary embodiments, a method of detecting an interaction between polypeptides and nucleic acid sequences is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides and nucleic acid sequences comprising a barcode under defined conditions to allow formation of polypeptide-nucleic acid interactions, stabilizing polypeptide-nucleic acid interactions by chemical crosslinking, immobilizing nucleic acid sequences on a substrate, performing in situ amplification of the barcodes bound to the immobilized polypeptide-nucleic acids, and detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when polypeptide-nucleic acid interactions have occurred between polypeptides and nucleic acid sequences.

In certain exemplary embodiments, a method of detecting an interaction between polypeptides and small molecules is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides and small molecules comprising a barcode under defined conditions to allow formation of polypeptide-small molecule interactions, stabilizing polypeptide-small molecules interactions by chemical crosslinking, immobilizing polypeptides and small molecules on a substrate, performing in situ amplification of the barcodes bound to the immobilized polypeptides and small molecules, and detecting amplified barcode sequences, wherein co-

localized amplified barcode sequences are detected when polypeptides and small molecule interactions have occurred between polypeptides and small molecules.

In certain exemplary embodiments, a method of detecting binding affinity of a plurality of polypeptides to an unlabeled ligand in a solution is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides comprising a barcode, providing in the aqueous medium one or more substrates, wherein the substrates exhibits altered binding affinity to the polypeptides when bound by a ligand, introducing a barcode which is associated with a compound assayed in a well, and quantifying the co-localization of barcodes determine protein and ligand interactions.

In certain aspects, the polypeptides comprising a barcode are made according to one of the methods described above. In certain aspects, the substrates are proteins. In certain aspects, the ligand increases or decreases binding affinity of a polypeptide to one or more substrates. In certain aspects, a barcode associated with a compound assayed in a well can be introduced by linking the barcode to the substrate assayed in the well according to one of the methods described above. In certain aspects, a barcode is added to the original barcoding DNAs of the polypeptides using PCR, which is compatible with standard sample barcoding protocols used in next-generation sequencing methods. In other aspects, the ligand is selected from the group consisting of unlabelled small molecules and polypeptide. In yet other aspects, the polypeptide is an antibody or a binder protein, e.g., a nanobody, adnectin, an affibody, DARPin, or the like. In still other aspects, upon polypeptide binding to a ligand, the polypeptide participates in a protein-substrate interaction. In other aspects, a library of unlabelled ligands is assayed with a polypeptide library in multi-well plate for automatic high-through screening, and wherein, in each well, one ligand is profiled using a polypeptide library. In other aspects, both the polypeptide screening and compound profiling are performed at the same time to minimize assay time. In still other aspects, mixed proteins at approximately a zeptomole amount can be analyzed in a picoliter reactor to minimize reagent costs.

In certain exemplary embodiments, a method of detecting binding affinities of polypeptide library to ligand library is provided. The method includes the steps of providing a plurality of polypeptides having a barcode bound thereto, contacting the plurality of polypeptides with one or more test ligands, performing in situ amplification of the barcodes bound to the plurality of polypeptides and the plurality of substrates pooled from multiple wells, and detecting amplified barcode sequences, wherein co-localized amplified barcode sequences of polypeptides and substrates are detected when polypeptides have bound to ligands, and wherein the number of co-localized amplified barcode sequences relative to all amplified barcode sequences correlates with binding affinity to the substrate and thus the ligand efficacy to activate polypeptides.

In certain aspects, the ligand increases or decreases binding affinity of a polypeptide to a substrate. In other aspects, the ligand modulates one or more activities of the polypeptide. In other aspects, the ligand is a small molecule or a polypeptide. In other aspects, upon polypeptide binding to a ligand, the polypeptide participates in a protein-protein interaction.

In certain exemplary embodiments, a method of detecting binding affinity of a polypeptide to a compound is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides having a barcode bound thereto, contacting the medium with one or more test compounds, immobilizing the plurality of polypeptides on a substrate, performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides, and detecting amplified barcodes, wherein co-localized amplified barcodes are detected when a polypeptide has bound to a compound, and wherein the number of co-localized amplified barcodes relative to non-co-localized amplified barcodes correlates with binding affinity to the compound.

In certain exemplary embodiments, a method of screening for a test compound that modulates an activity of a polypeptide is provided. The method includes the steps of providing a plurality of polypeptides having a barcode bound thereto, contacting the plurality of polypeptides with one or more test compounds, wherein polypeptide binding to a test compound alters the ability of the polypeptide to participate in a protein-protein interaction, performing in situ amplification of the barcode sequences bound to the plurality of polypeptides, and detecting amplified barcode sequences, wherein altered co-localization of amplified barcode sequences in the presence of the test compound is observed when the test compound modulates an activity of the polypeptide.

In certain aspects, test compound binding to a polypeptide modulates the ability of the polypeptide to participate in a protein-protein interaction.

In certain exemplary embodiments, a method of screening for a test compound that modulates an activity of a polypeptide is provided. The method includes the steps of providing in an aqueous medium a plurality of polypeptides having a barcode sequence bound thereto, contacting the medium with one or more test compounds, wherein polypeptide binding to a test compound alters the ability of the polypeptide to participate in a protein-protein interaction, immobilizing the plurality of polypeptides on a substrate, performing in situ amplification of the barcode sequences bound to the immobilized plurality of polypeptides, and detecting amplified barcode sequences, wherein altered co-localization of amplified barcode sequences in the presence of the test compound is observed when the test compound modulates an activity of the polypeptide

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains drawings executed in color. Copies of this patent or patent application publication with the color drawings will be provided by the Office upon request and payment of the necessary fee. The foregoing and other features and advantages of the present invention will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings in which:

Figures 1A-1B schematically depict protein barcoding methods according to certain aspects of the invention. (A) Collective barcoding via ribosome display. A short synthetic barcoding sequence can be introduced into DNA templates via PCR. PRMC complexes are formed via ribosome stalling induced by a C-terminal E. coli SecM peptide. Displayed proteins bearing a C-terminal Flag tag are separated from the ribosomes by an E. coli TolA spacer domain. (B) Individual barcoding via a HaloTag-mediated conjugation. A 220-base pair (bp) double-stranded barcoding DNA is modified with a HaloTag ligand (black triangle).

Figures 2A-2C depict amplification and quantification of barcoding DNAs. (A) Schematic of in situ polony amplification and sequencing. Barcoded proteins were immobilized in a PAA gel matrix attached to a Bind-Silane treated glass slide. The slide was assembled into a flow cell, where barcoding DNAs could be amplified into polonies for fluorescence imaging-based sequencing. (B) Representative merged images of polonies hybridized with Cy5 (red), Cy3 (green) and fluorescein (blue)-labelled oligos (20× objective). (C) Polony quantification of mixed protein binders and antigens. Pearson correlation coefficient R was calculated for different coverages grouped by dotted lines.

Figures 3A-3E depict analyses of protein interactions via polony co-localization. (A) Interaction of DsRed subunits resulted in co-localized polonies. DsRed polonies were identified by SBE with Cy5 (red) or Cy3 (green)-labelled ddNTPs. (B) The correlation between the polony co-localization ratios and KDs of Ras–Raf-RBD complexes. Means of measurements at 100 imaging positions ± 95% confidence level (CL; refer to Figure 13). Fitting equation, $R = R_{max} \times P/(K_D + P)$, where R is the predicted Raf-RBD polony co-localization ratio, R_{max} is the maximum polony co-localization ratio when Raf-RBD is saturated by Ras, and P is the Ras concentration. (C) Schematic of multiplex GPCR screening and profiling by the binding assay of mixed barcoded GPCRs to barcoded β-arr2. (D) Comparison of β-arr2 binding to isoproterenol-activated β2-adrenergic receptor with or without GRK2-mediated phosphorylation. β-arr2 titration data were fitted by the one-site-specific model using GraphPad Prism 6. (E) Parallel compound profiling

with three GPCRs. Data represent mean values of 50 measurements; error bars, 95% CL (refer to Figure 14). **P < 0.01, ***P < 0.001, one-tailed paired Student's t test.

Figures 4A-4B depict parallel antibody binding profiling. (A) Heat map of the mean co-localization ratios measured at 100 imaging positions. ScFvs share the same origins were grouped by their numbers (Figure 12). (B) The correlation between the co-localization statistics with the immunoprecipitation results. Selected scFvs were fused to a C-terminal streptavidin binding peptide (SBP) tag and captured by streptavidin-coated magnetic beads to pull down bound human protein fusions with a HaloTag, which could be labelled by Halo-TMR. Proteins were analyzed by fluorescent gel imaging. Error bars, 95% CL. ***P < 0.001, one-tailed paired Student's t test.

Figures 5A-5B depict the improved stability of PRMC complexes generated in a reconstituted E. coli IVT (PURE) system. (A) HaloTag or HaloTagged proteins were applied to measure the PRMC complex stability. The 5'-acrydite modification of cDNAs is required for the array analysis of PRMC complexes, but not relevant to this stability assay. (B) Comparison of the PRMC complex stability in the PURE and an E. coli crude extract (S30) IVT system. Nucleic acid degradation or ribosome dissociation results in the loss of barcoding DNAs. IVT reactions were performed at 37°C for 30 min and PRMC complexes were further incubated at room temperature for indicated periods of time before the stability analysis. Means of three independent experiments ± standard deviations.

Figures 6A-6B depict HaloTag-based protein–DNA conjugation method. (A) A general barcoding protocol adaptable to an automatic platform. Fusion proteins carrying an N or C-terminal HaloTag and an affinity tag were conjugated to a 220 bp barcoding DNA bearing the 5' and 3' modifications. (B) Agarose gel electrophoresis of selected barcoding DNA and protein–DNA conjugate.

Figures 7A-7B depict that covalent immobilization of barcoding DNAs is required for in situ polony amplification. Representative images of polonies amplified from barcoding DNA templates without (A) or with (B) a 5'-acrydite modification (refer to Figures 5A and 6A). Oversized polonies or tiny adjacent polonies depicted in (A) can be resulted from template-drifting-induced multiple seeding events during the amplification.

Figures 8A-8B graphically depict polony quantification of various barcoded proteins. (A) Plot showing the average number of polonies detected at a single imaging position vs. the average number of barcoding DNA templates predicted by real-time PCR quantification. Data represent mean values of 100 measurements; error bars, 95% CL. (B) Log–log plot of total numbers of polonies detected vs. dilution factors. Data represent mean values of two technical replicates.

Figures 9A-9B depict that crosslinking efficiency of DsRed is improved by a lysine-rich TolA domain. (A) SDS-PAGE analysis of purified DsRed (Clontech) and HaloTag–DsRed–TolA proteins before (lanes 1 and 3) and after (lanes 2 and 4) the crosslinking. 10 μM purified proteins were crosslinked by 1 mM BS(PEG)5 in 20 mM HEPES buffer, pH 8.0, 150 mM KOAc at 4°C for 1 h; Proteins were stained with Coomassie blue. Only a minor band of the crosslinked dimer was observed for DsRed (lane 2), and, in contrast, HaloTag–DsRed–TolA was all crosslinked as the tetramer or trimer (lane 4). Co-purified E. coli proteins (protein bands below the major band in the lane 3), which likely interact with the TolA, were efficiently crosslinked to HaloTag–DsRed–TolA. (B) Comparison of different crosslinking conditions. Proteins were labelled by Halo-TMR and analyze by fluorescent gel imaging. Only a minor fraction of HaloTag–TolA, a control to detect non-specific crosslinking, was crosslinked at increased protein concentrations.

Figure 10 schematically depicts Expression vectors described herein. pRD-NHA-SecM, the vector for ribosome display; pEco-CSBPHis, pEco-CHaloFlagHis and pEco-NHalo-CHis, vectors for E. coli in vivo and in vitro protein expression; pBac-NFlagHA, the Baculovirus expression vector for GPCRs; pIRES-CHaloFlagHis and pIRES-CHaloFlagHis-Gateway, vectors for expression of fusion proteins in the human IVT system. Refer to Figure 16 for their DNA sequences.

Figure 11 depicts polony quantification of mixed binder proteins and antigens.

Figure 12 depicts scFvs and human proteins used in the screening methods described further herein.

Figure 13 depicts polony quantification for Ras-Raf-RBD binding assays used in methods described further herein.

Figures 14A-14B depict polony quantification for GPCR-β-arrestin binding assays.

Figure 15 depicts proteins screened using methods described herein. The protein sequences are publically available.

Figure 16 depicts vector and primer sequences according to certain aspects of the invention.

Figure 17 graphically depicts simulation results of the mathematical modeling for a protein library vs. a probe library screening in a single assay. The mathematical modeling description provides additional details.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

Embodiments of the present invention are directed to novel methods for attaching barcodes to polypeptides. Embodiments of the present invention are directed to novel methods for detecting

interactions, e.g., protein-protein interactions, protein-nucleic acid interactions, protein-mall molecules interaction, binding affinities and the like. In certain aspects, the methods described herein are performed using massively parallel techniques.

In various embodiments, the methods disclosed herein comprise amplification of nucleic acids including, for example, polynucleotides, oligonucleotides and/or oligonucleotide fragments. Amplification methods may comprise contacting a nucleic acid sequence with one or more primers (e.g., primers that are complementary to barcode sequences) that specifically hybridize to the nucleic acid under conditions that facilitate hybridization and chain extension. Exemplary methods for amplifying nucleic acids include the polymerase chain reaction (PCR) (see, e.g., Mullis et al. (1986) Cold Spring Harb. Symp. Quant. Biol. 51 Pt 1:263 and Cleary et al. (2004) Nature Methods 1:241; and U.S. Patent Nos. 4,683,195 and 4,683,202), anchor PCR, RACE PCR, ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:360-364), self-sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. U.S.A. 87:1874), transcriptional amplification system (Kwoh et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:1173), Q-Beta Replicase (Lizardi et al. (1988) BioTechnology 6:1197), recursive PCR (Jaffe et al. (2000) J. Biol. Chem. 275:2619; and Williams et al. (2002) J. Biol. Chem. 277:7790), the amplification methods described in U.S. Patent Nos. 6,391,544, 6,365,375, 6,294,323, 6,261,797, 6,124,090 and 5,612,199, isothermal amplification (e.g., isothermal bridge amplification (IBA), rolling circle amplification (RCA), hyperbranched rolling circle amplification (HRCA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), PWGA or any other nucleic acid amplification method using techniques well known to those of skill in the art.

"Polymerase chain reaction," or "PCR," refers to a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer binding sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer binding sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g., exemplified by the references: McPherson et al., editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature greater than 90 °C, primers annealed at a temperature in the range 50-75 °C, and

primers extended at a temperature in the range 72-78 °C. In certain aspects, a double stranded target nucleic acid may be denatured at a temperature greater than 90 °C in a conventional PCR using Taq DNA polymerase, or by adding formamide at 60 °C in isothermal bridge amplification using Bst polymerase.

The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, assembly PCR and the like. Reaction volumes range from a few hundred nanoliters, e.g., 200 nL, to a few hundred microliters, e.g., 200 microliters. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g., Tecott et al., U.S. Patent No. 5,168,038. "Real-time PCR" means a PCR for which the amount of reaction product, i.e., amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g., Gelfand et al., U.S. Patent No. 5,210,015 ("Taqman"); Wittwer et al., U.S. Patent Nos. 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al., U.S. Patent No. 5,925,517 (molecular beacons). Detection chemistries for real-time PCR are reviewed in Mackay et al., Nucleic Acids Research, 30:1292-1305 (2002). "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al. (1999) Anal. Biochem., 273:221-228 (two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified. "Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references: Freeman et al., Biotechniques, 26:112-126 (1999); Becker-Andre et al., Nucleic Acids Research, 17:9437-9447 (1989); Zimmerman et al., Biotechniques, 21:268-279 (1996); Diviacco et al., Gene, 122:3013-3020 (1992); Becker-Andre et al., Nucleic Acids Research, 17:9437-9446 (1989); and the like.

In certain embodiments, methods of determining the sequence of one or more nucleic acid sequences of interest, e.g., polynucleotides, oligonucleotides and/or oligonucleotide fragments, are provided. Determination of the sequence of a nucleic acid sequence of interest can be performed using variety of sequencing methods known in the art including, but not limited to, sequencing by hybridization (SBH), sequencing by ligation (SBL), quantitative incremental fluorescent nucleotide

addition sequencing (QIFNAS), stepwise ligation and cleavage, fluorescence resonance energy transfer (FRET), molecular beacons, TaqMan reporter probe digestion, pyrosequencing, fluorescent in situ sequencing (FISSEQ), FISSEQ beads (U.S. Pat. No. 7,425,431), wobble sequencing (PCT/US05/27695), multiplex sequencing (U.S. Serial No. 12/027,039, filed February 6, 2008; Porreca et al (2007) Nat. Methods 4:931), polymerized colony (POLONY) sequencing (U.S. Patent Nos. 6,432,360, 6,485,944 and 6,511,803, and PCT/US05/06425); nanogrid rolling circle sequencing (ROLONY) (U.S. Serial No. 12/120,541, filed May 14, 2008), allele-specific oligo ligation assays (e.g., oligo ligation assay (OLA), single template molecule OLA using a ligated linear probe and a rolling circle amplification (RCA) readout, ligated padlock probes, and/or single template molecule OLA using a ligated circular padlock probe and a rolling circle amplification (RCA) readout) and the like. High-throughput sequencing methods, e.g., on cyclic array sequencing using platforms such as Roche 454, Illumina Solexa, AB-SOLiD, Helicos, Polonator platforms and the like, can also be utilized. High-throughput sequencing methods are described in U.S. Serial No. 61/162,913, filed March 24, 2009. A variety of light-based sequencing technologies are known in the art (Landegren et al. (1998) Genome Res. 8:769-76; Kwok (2000) Pharmocogenomics 1:95-100; and Shi (2001) Clin. Chem. 47:164-172).

Embodiments of the present invention are directed to polynucleotides, oligonucleotides, small molecules, substrates, test compounds and the like having one or two or more labels (e.g., barcode sequences) attached thereto. As used herein, the term "barcode" refers to a unique oligonucleotide sequence that allows a corresponding nucleic acid sequence (e.g., an oligonucleotide fragment) to be identified, retrieved and/or amplified. In certain embodiments, barcodes can each have a length within a range of from 4 to 36 nucleotides, or from 6 to 30 nucleotides, or from 8 to 20 nucleotides. In certain exemplary embodiments, a barcode has a length of 4 nucleotides. In certain aspects, the melting temperatures of barcodes within a set are within 10 °C of one another, within 5 °C of one another, or within 2 °C of one another. In other aspects, barcodes are members of a minimally cross-hybridizing set. That is, the nucleotide sequence of each member of such a set is sufficiently different from that of every other member of the set that no member can form a stable duplex with the complement of any other member under stringent hybridization conditions. In one aspect, the nucleotide sequence of each member of a minimally cross-hybridizing set differs from those of every other member by at least two nucleotides. Barcode technologies are known in the art and are described in Winzeler et al. (1999) Science 285:901; Brenner (2000) Genome Biol. 1:1 Kumar et al. (2001) Nature Rev. 2:302; Giaever et al. (2004) Proc. Natl. Acad. Sci. USA 101:793; Eason et al. (2004) Proc. Natl. Acad. Sci. USA 101:11046; and Brenner (2004) Genome Biol. 5:240.

In certain embodiments, one or more markers are used to detect and/or retrieve (i.e. purify) polynucleotides, oligonucleotides, small molecules, substrates, test compounds and the like

described herein. Examples of detectable and/or retrievable markers include various radioactive moieties, enzymes, prosthetic groups, fluorescent markers, luminescent markers, bioluminescent markers, metal particles, protein-protein binding pairs, protein-antibody binding pairs and the like. Detectable markers are commercially available from a variety of sources.

In certain aspects of the invention, detectable and/or retrievable proteins and/or protein tags are provided. Examples of detectable fluorescent proteins include, but are not limited to, yellow fluorescent protein (YFP), green fluorescence protein (GFP), cyan fluorescence protein (CFP), umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrin and the like. Examples of detectable bioluminescent proteins include, but are not limited to, luciferase (e.g., bacterial, firefly, click beetle and the like), luciferin, aequorin and the like. Examples of detectable and/or retrievable enzyme systems include, but are not limited to, galactosidases, glucorinidases, phosphatases, peroxidases, cholinesterases and the like.

Biotin, or a derivative thereof, may also be used as a detectable and/or retrievable label, and subsequently bound by a detectably labeled avidin/streptavidin derivative (e.g. phycoerythrin-conjugated streptavidin), or a labeled anti-biotin antibody. Digoxigenin may be expressed subsequently bound by a labeled anti-digoxigenin antibody (e.g. fluoresceinated anti-digoxigenin). In general, any member of a conjugate pair may be incorporated into a detection oligonucleotide provided that a detectably labeled conjugate partner can be bound to permit detection. As used herein, the term antibody refers to an antibody molecule of any class, or any sub-fragment thereof, such as an Fab.

Other suitable labels for detection and/or retrieval include one or more protein tags. As used herein, the term "protein tag" refers to a heterologous polypeptide sequence linked to a polymerase of the invention. Protein tags include, but are not limited to, Avi tag (GLNDIFEAQKIEWHE) (SEQ ID NO:22), calmodulin tag (KRRWKKNFIAVSAANRFKKISSSGAL) (SEQ ID NO:23), FLAG tag (DYKDDDDK) (SEQ ID NO:24), HA tag (YPYDVPDYA) (SEQ ID NO:25), His tag (HHHHHH) (SEQ ID NO:26), Myc tag (EQKLISEEDL) (SEQ ID NO:27), S tag (KETAAAKFERQHMDS) (SEQ ID NO:28, SBP tag (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQ GQREP) (SEQ ID NO:29), Softag 1 (SLAELLNAGLGGS) (SEQ ID NO:30), Softag 3 (TQDPSRVG) (SEQ ID NO:31), V5 tag (GKPIPNPLLGLDST) (SEQ ID NO:32), Xpress tag (DLYDDDDK) (SEQ ID NO:33), Isopeptag (TDKDMTITFTNKKDAE) (SEQ ID NO:34), SpyTag (AHIVMVDAYKPTK) (SEQ ID NO:35), streptactin tag (Strep-tag II: WSHPQFEK) (SEQ ID NO:36) and the like.

Detection and/or retrieval method(s) used will depend on the particular detectable labels used in the microorganism. In certain exemplary embodiments, microorganisms may be selected for,

screened for and/or retrieved using a microscope, a spectrophotometer, a tube luminometer or plate luminometer, x-ray film, magnetic fields, a scintillator, a fluorescence activated cell sorting (FACS) apparatus, a chromatography apparatus, a microfluidics apparatus, a bead-based apparatus or the like.

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g., Komberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See Kanehisa (1984) Nucl. Acids Res. 12:203.

"Complex" refers to an assemblage or aggregate of molecules in direct or indirect contact with one another. In one aspect, "contact," or more particularly, "direct contact," in reference to a complex of molecules or in reference to specificity or specific binding, means two or more molecules are close enough so that attractive noncovalent interactions, such as van der Waal forces, hydrogen bonding, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules. In such an aspect, a complex of molecules is stable in that under assay conditions the complex is thermodynamically more favorable than a non-aggregated, or non-complexed, state of its component molecules. As used herein, "complex" refers to a duplex or triplex of polynucleotides or a stable aggregate of two or more proteins. In regard to the latter, a complex is formed by an antibody specifically binding to its corresponding antigen.

"Duplex" refers to at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. In one aspect, stable duplex means that a duplex structure is not destroyed by a stringent wash, e.g., conditions including temperature of about 5 °C less that the Tm of a strand of the duplex and low monovalent salt concentration, e.g., less than 0.2 M, or less than 0.1 M. "Perfectly matched" in reference to a duplex means that the polynucleotide or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term "duplex" comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, PNAs, and the like, that may be employed. A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," or "locus" refers to a contiguous sub-region or segment of a genome. As used herein, genetic locus, or locus, may refer to the position of a nucleotide, a gene, or a portion of a gene in a genome, including mitochondrial DNA, or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. In one aspect, a genetic locus refers to any portion of genomic sequence, including mitochondrial DNA, from a single nucleotide to a segment of few hundred nucleotides, e.g. 100-300, in length. Usually, a particular genetic locus may be identified by its nucleotide sequence, or the nucleotide sequence, or sequences, of one or both adjacent or flanking regions. In another aspect, a genetic locus refers to the expressed nucleic acid product of a gene, such as an RNA molecule or a cDNA copy thereof.

"Hybridization" refers to the process in which two single-stranded polynucleotides bind non-covalently to form a stable double-stranded polynucleotide. The term "hybridization" may also refer to triple-stranded hybridization. The resulting (usually) double-stranded polynucleotide is a "hybrid" or "duplex." "Hybridization conditions" will typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and even more usually less than about 200 mM. Hybridization temperatures can be as low as 5 °C, but are typically greater than 22 °C, more typically greater than about 30 °C, and often in excess of about 37 °C. Hybridizations are usually performed under stringent conditions, i.e., conditions under which a probe will hybridize to its target subsequence. Stringent conditions are sequence-dependent and are different in different circumstances. Longer fragments may require higher hybridization temperatures for specific hybridization. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of

any one alone. Generally, stringent conditions are selected to be about 5 ºC lower than the Tm for the specific sequence at s defined ionic strength and pH. Exemplary stringent conditions include salt concentration of at least 0.01 M to no more than 1 M Na ion concentration (or other salts) at a pH 7.0 to 8.3 and a temperature of at least 25 ºC. For example, conditions of 5XSSPE (750 mM NaCl, 50 mM Na phosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30 ºC are suitable for allele-specific probe hybridizations. For stringent conditions, see for example, Sambrook, Fritsche and Maniatis, Molecular Cloning A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press (1989) and Anderson Nucleic Acid Hybridization, 1st Ed., BIOS Scientific Publishers Limited (1999). "Hybridizing specifically to" or "specifically hybridizing to" or like expressions refer to the binding, duplexing, or hybridizing of a molecule substantially to or only to a particular nucleotide sequence or sequences under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., primers, enzymes, microarrays, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials for assays of the invention. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains primers.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g., oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references: Whitely et al., U.S. Patent No. 4,883,750; Letsinger et al., U.S. Patent No. 5,476,930; Fung et al., U.S. Patent No. 5,593,826; Kool, U.S. Patent No. 5,426,180; Landegren et al., U.S. Patent No. 5,871,921; Xu and Kool (1999) Nucl. Acids Res. 27:875; Higgins et al., Meth. in Enzymol. (1979) 68:50; Engler et al. (1982) The Enzymes, 15:3 (1982); and Namsaraev, U.S. Patent Pub. 2004/0110213.

"Amplifying" includes the production of copies of a nucleic acid molecule of the array or a nucleic acid molecule bound to a bead via repeated rounds of primed enzymatic synthesis. "In situ" amplification indicated that the amplification takes place with the template nucleic acid molecule

positioned on a support or a bead, rather than in solution. In situ amplification methods are described in U.S. Patent No. 6,432,360.

"Support" can refer to a matrix upon which nucleic acid molecules of a nucleic acid array are placed. The support can be solid or semi-solid or a gel. "Semi-solid" refers to a compressible matrix with both a solid and a liquid component, wherein the liquid occupies pores, spaces or other interstices between the solid matrix elements. Semi-solid supports can be selected from polyacrylamide, cellulose, polyamide (nylon) and crossed linked agarose, dextran and polyethylene glycol.

"Randomly-patterned" or "random" refers to non-ordered, non-Cartesian distribution (in other words, not arranged at pre-determined points along the x- or y- axes of a grid or at defined "clock positions," degrees or radii from the center of a radial pattern) of nucleic acid molecules over a support, that is not achieved through an intentional design (or program by which such design may be achieved) or by placement of individual nucleic acid features. Such a "randomly-patterned" or "random" array of nucleic acids may be achieved by dropping, spraying, plating or spreading a solution, emulsion, aerosol, vapor or dry preparation comprising a pool of nucleic acid molecules onto a support and allowing the nucleic acid molecules to settle onto the support without intervention in any manner to direct them to specific sites thereon. Arrays of the invention can be randomly patterned or random.

"Heterogeneous" refers to a population or collection of nucleic acid molecules that comprises a plurality of different sequences. According to one aspect, a heterogeneous pool of oligonucleotide sequences is provided with an article of manufacture (e.g., a microarray).

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Komberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90:543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al., Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al., Current Opinion in Structural Biology, 5:343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids (LNAs), and like

compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

As used herein, the terms "nucleic acid molecule," "nucleic acid sequence," "nucleic acid fragment," "oligonucleotide," "oligonucleotide fragment" and "polynucleotide" are used interchangeably and are intended to include, but are not limited to, a polymeric form of nucleotides that may have various lengths, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Nucleic acid molecules include single stranded DNA (ssDNA), double stranded DNA (dsDNA), single stranded RNA (ssRNA) and double stranded RNA (dsRNA). Different nucleic acid molecules may have different three-dimensional structures, and may perform various functions, known or unknown. Non-limiting examples of nucleic acid molecules include a gene, a gene fragment, a genomic gap, an exon, an intron, intergenic DNA (including, without limitation, heterochromatic DNA), messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, small interfering RNA (siRNA), miRNA, small nucleolar RNA (snoRNA), cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of a sequence, isolated RNA of a sequence, nucleic acid probes, and primers. Nucleic acid molecules useful in the methods described herein may comprise natural nucleic acid sequences and variants thereof, artificial nucleic acid sequences, or a combination of such sequences.

An oligonucleotide sequence refers to a linear polymer of natural or modified nucleosidic monomers linked by phosphodiester bonds or analogs thereof. The term "oligonucleotide" usually refers to a shorter polymer, e.g., comprising from about 3 to about 100 monomers, and the term "polynucleotide" usually refers to longer polymers, e.g., comprising from about 100 monomers to many thousands of monomers, e.g., 10,000 monomers, or more An "oligonucleotide fragment" refers to an oligonucleotide sequence that has been cleaved into two or more smaller oligonucleotide sequences. Oligonucleotides comprising probes or primers usually have lengths in the range of from 12 to 60 nucleotides, and more usually, from 18 to 40 nucleotides. Oligonucleotides and polynucleotides may be natural or synthetic. Oligonucleotides and polynucleotides include deoxyribonucleosides, ribonucleosides, and non-natural analogs thereof, such as anomeric forms thereof, peptide nucleic acids (PNAs), and the like, provided that they are capable of specifically binding to a target genome by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like.

Usually nucleosidic monomers are linked by phosphodiester bonds. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5' to 3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, "T" denotes deoxythymidine, and "U"

denotes the ribonucleoside, uridine, unless otherwise noted. Usually oligonucleotides comprise the four natural deoxynucleotides; however, they may also comprise ribonucleosides or non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed in methods and processes described herein. For example, where processing by an enzyme is called for, usually oligonucleotides consisting solely of natural nucleotides are required. Likewise, where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g., single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al., Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references. Oligonucleotides and polynucleotides may be single stranded or double stranded.

Nucleic acid molecules may optionally include one or more non-standard nucleotide(s), nucleotide analog(s) and/or modified nucleotides. Examples of modified nucleotides include, but are not limited to diaminopurine, S2T, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-D46-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine and the like. Nucleic acid molecules may also be modified at the base moiety (e.g., at one or more atoms that typically are available to form a hydrogen bond with a complementary nucleotide and/or at one or more atoms that are not typically capable of forming a hydrogen bond with a complementary nucleotide), sugar moiety or phosphate backbone.

In certain exemplary embodiments, large polynucleotides are provided. In certain aspects, isolation techniques that maximize the lengths of polynucleotides (e.g., DNA molecules) obtained are used. For example, in situ lysis or deproteinization (e.g., with EDTA, detergent, protease, any combinations thereof and the like) after agarose embedding (as routinely performed for pulsed field gel electrophoresis) can be used to obtain polynucleotides.

Nucleic acid molecules may be isolated from natural sources or purchased from commercial sources. Oligonucleotide sequences (e.g., barcodes) may also be prepared by any suitable method, e.g., standard phosphoramidite methods such as those described by Beaucage and Carruthers ((1981) Tetrahedron Lett. 22: 1859) or the triester method according to Matteucci et al. (1981) J. Am. Chem. Soc. 103:3185), or by other chemical methods using either a commercial automated oligonucleotide synthesizer or high-throughput, high-density array methods known in the art (see U.S. Patent Nos. 5,602,244, 5,574,146, 5,554,744, 5,428,148, 5,264,566, 5,141,813, 5,959,463, 4,861,571 and 4,659,774, incorporated herein by reference in its entirety for all purposes). Pre-synthesized oligonucleotides may also be obtained commercially from a variety of vendors.

Nucleic acid molecules may be obtained from one or more biological samples. As used herein, a "biological sample" may be a single cell or many cells. A biological sample may comprise a single cell type or a combination of two or more cell types. A biological sample further includes a collection of cells that perform a similar function such as those found, for example, in a tissue. Accordingly, certain aspects of the invention are directed to biological samples containing one or more tissues. As used herein, a tissue includes, but is not limited to, epithelial tissue (e.g., skin, the lining of glands, bowel, skin and organs such as the liver, lung, kidney), endothelium (e.g., the lining of blood and lymphatic vessels), mesothelium (e.g., the lining of pleural, peritoneal and pericardial spaces), mesenchyme (e.g., cells filling the spaces between the organs, including fat, muscle, bone, cartilage and tendon cells), blood cells (e.g., red and white blood cells), neurons, germ cells (e.g., spermatozoa, oocytes), amniotic fluid cells, placenta, stem cells and the like. A tissue sample includes microscopic samples as well as macroscopic samples.

In certain aspects, nucleic acid sequences derived or obtained from one or more organisms are provided. As used herein, the term "organism" includes, but is not limited to, a human, a non-human primate, a cow, a horse, a sheep, a goat, a pig, a dog, a cat, a rabbit, a mouse, a rat, a gerbil, a frog, a toad, a fish (e.g., Danio rerio) a roundworm (e.g., C. elegans) and any transgenic species thereof. The term "organism" further includes, but is not limited to, a yeast (e.g., S. cerevisiae) cell, a yeast tetrad, a yeast colony, a bacterium, a bacterial colony, a virion, virosome, virus-like particle and/or cultures thereof, and the like.

Isolation, extraction or derivation of nucleic acid sequences may be carried out by any suitable method. Isolating nucleic acid sequences from a biological sample generally includes treating a biological sample in such a manner that nucleic acid sequences present in the sample are extracted and made available for analysis. Any isolation method that results in extracted nucleic acid sequences may be used in the practice of the present invention. It will be understood that the particular method used to extract nucleic acid sequences will depend on the nature of the source.

Methods of DNA extraction are well-known in the art. A classical DNA isolation protocol is based on extraction using organic solvents such as a mixture of phenol and chloroform, followed by precipitation with ethanol (J. Sambrook et al., "Molecular Cloning: A Laboratory Manual," 1989, 2nd Ed., Cold Spring Harbour Laboratory Press: New York, N.Y.). Other methods include: salting out DNA extraction (P. Sunnucks et al., Genetics, 1996, 144: 747-756; S. M. Aljanabi and I. Martinez, Nucl. Acids Res. 1997, 25: 4692-4693), trimethylammonium bromide salts DNA extraction (S. Gustincich et al., BioTechniques, 1991, 11: 298-302) and guanidinium thiocyanate DNA extraction (J. B. W. Hammond et al., Biochemistry, 1996, 240: 298-300). A variety of kits are commercially available for extracting DNA from biological samples (e.g., BD Biosciences Clontech (Palo Alto, CA): Epicentre Technologies (Madison, WI); Gentra Systems, Inc. (Minneapolis, MN); MicroProbe Corp. (Bothell, WA); Organon Teknika (Durham, NC); and Qiagen Inc. (Valencia, CA)).

Methods of RNA extraction are also well known in the art (see, for example, J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" 1989, 2nd Ed., Cold Spring Harbour Laboratory Press: New York) and several kits for RNA extraction from bodily fluids are commercially available (e.g., Ambion, Inc. (Austin, TX); Amersham Biosciences (Piscataway, NJ); BD Biosciences Clontech (Palo Alto, CA); BioRad Laboratories (Hercules, CA); Dynal Biotech Inc. (Lake Success, NY); Epicentre Technologies (Madison, WI); Gentra Systems, Inc. (Minneapolis, MN); GIBCO BRL (Gaithersburg, MD); Invitrogen Life Technologies (Carlsbad, CA); MicroProbe Corp. (Bothell, WA); Organon Teknika (Durham, NC); Promega, Inc. (Madison, WI); and Qiagen Inc. (Valencia, CA)).

"Polymorphism" or "genetic variant" means a substitution, inversion, insertion, or deletion of one or more nucleotides at a genetic locus, or a translocation of DNA from one genetic locus to another genetic locus. In one aspect, polymorphism means one of multiple alternative nucleotide sequences that may be present at a genetic locus of an individual and that may comprise a nucleotide substitution, insertion, or deletion with respect to other sequences at the same locus in the same individual, or other individuals within a population. An individual may be homozygous or heterozygous at a genetic locus; that is, an individual may have the same nucleotide sequence in both alleles, or have a different nucleotide sequence in each allele, respectively. In one aspect, insertions or deletions at a genetic locus comprises the addition or the absence of from 1 to 10 nucleotides at such locus, in comparison with the same locus in another individual of a population (or another allele in the same individual). Usually, insertions or deletions are with respect to a major allele at a locus within a population, e.g., an allele present in a population at a frequency of fifty percent or greater.

"Primer" includes an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process are determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers usually have a length in the range of between 3 to 36 nucleotides, also 5 to 24 nucleotides, also from 14 to 36 nucleotides. Primers within the scope of the invention include orthogonal primers, amplification primers, constructions primers and the like. Pairs of primers can flank a sequence of interest or a set of sequences of interest. Primers and probes can be degenerate in sequence. Primers within the scope of the present invention bind adjacent to a target sequence (e.g., an oligonucleotide fragment, a barcode sequence or the like).

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as an amplification or sequencing primer to a barcode sequence, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecules in a reaction or sample, it forms the largest number of the complexes with the second molecule. In certain aspects, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak non-covalent chemical interactions, such as van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

"Spectrally resolvable" in reference to a plurality of fluorescent labels means that the fluorescent emission bands of the labels are sufficiently distinct, i.e., sufficiently non-overlapping, that molecular tags to which the respective labels are attached can be distinguished on the basis of the fluorescent signal generated by the respective labels by standard photodetection systems, e.g., employing a system of band pass filters and photomultiplier tubes, or the like, as exemplified by the systems described in U.S. Patent Nos. 4,230,558; 4,811,218, or the like, or in Wheeless et al., pgs. 21-76, in Flow Cytometry: Instrumentation and Data Analysis (Academic Press, New York, 1985). In one aspect, spectrally resolvable organic dyes, such as fluorescein, rhodamine, and the

like, means that wavelength emission maxima are spaced at least 20 nm apart, and in another aspect, at least 40 nm apart. In another aspect, chelated lanthanide compounds, quantum dots, and the like, spectrally resolvable means that wavelength emission maxima are spaced at least 10 nm apart, and in a further aspect, at least 15 nm apart.

"Tm" is used in reference to "melting temperature." Melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the Tm of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation. Tm =81.5+0.41 (% G+C), when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, "Quantitative Filter Hybridization," in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H. T. & Santa Lucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of Tm.

It is to be understood that the embodiments of the present invention which have been described are merely illustrative of some of the applications of the principles of the present invention. Numerous modifications may be made by those skilled in the art based upon the teachings presented herein without departing from the true spirit and scope of the invention. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference in their entirety for all purposes.

The following examples are set forth as being representative of the present invention. These examples are not to be construed as limiting the scope of the invention as these and other equivalent embodiments will be apparent in view of the present disclosure, figures, tables and accompanying claims.

EXAMPLE I

Barcoded Protein Array Construction

To analyze proteins in a massively parallel SM format proteins that were molecularly coupled to a DNA bearing a barcoding sequence were generated. One barcoding approach was to in vitro translate and display proteins on protein–ribosome–mRNA–cDNA (PRMC) complexes, in which the cDNA contains a synthetic barcode (Figure 1A). Specifically, the ribosome display was performed by using mRNA–cDNA hybrids as templates and an in vitro translation (IVT) system reconstituted with purified components8 that was shown to stabilize PRMC complexes (Figure 5). PRMC complexes bearing full-length proteins of interest were enriched by Flag-tag affinity purification. This approach was applicable to proteins of various sizes and size-related biases during barcode detection can be avoided by using uniformly sized barcoding DNAs. To barcode a

large protein library, millions of chip-synthesized9,10 or random barcoding sequences could be introduced to the 5' end of protein open reading frames (ORFs) by polymerase chain reaction (PCR) and later matched to the ORF sequences by high-throughput DNA sequencing. Alternatively, some proteins that could only be functionally expressed in vivo were required to be individually barcoded. Thus, fusion proteins were constructed with an engineered enzyme tag, HaloTag11, which mediates an efficient covalent conjugation to a HaloTag ligand-modified double stranded DNA (Figure 1B). This method could be readily adapted to a microtiter plate format for automated parallel protein production (Figure 6).

A complex mixture of barcoded proteins could be identified and quantified by in situ sequencing their barcodes (Figure 2A). They were immobilized into an ultrathin-layer crosslinked PAA gel attached to a microscopic slide, and their barcoding DNAs bearing a 5'-acrydite modification (Figures 5A and 6A) were covalently crosslinked to the gel matrix to prevent template drifting (Figure 7). A solid-phase PCR, with two gel-anchored primers, was performed according to an adapted isothermal bridge amplification protocol12 in an assembled flow cell. This amplification showed a high efficiency of ~80% barcode detection (Figure 8A), and resulted in polonies of 1-2 μm diameter (Figure 2B) similar to the clusters generated on an Illumina platform12. Polonies were analyzed by hybridization with fluorescent probes7, single-base extension (SBE)13 or ligation-based sequencing14 in our work. As the polony density could reach over 1 million polonies per square millimeter, about one billion protein molecules can be measured in half the area of a 25 × 75 mm slide.

To test the accuracy of this method, nine immunoglobulin and non-immunoglobulin binding proteins and three antigens (e.g., human, bacterial and viral proteins) of a molecular weight range between 3.4 and 120 kDa (Figure 11) were selected. Mixed PRMC complexes were prepared in six barcoded dilutions, with concentrations spanning six orders of magnitude, pooled together and subjected to the SM quantification. Barcode detection efficiencies of different proteins were found to be almost identical at various concentrations (Figure 8). The quantification showed high reproducibility, e.g., the Pearson correlation coefficient R was above 0.99 when over 1,000 protein polonies were detected (Figure 2C). Without intending to be bound by scientific theory, because the proteins were highly diluted (e.g., at less than picomolar concentrations) prior to array deposition, protein monomers should have been the predominant form.

EXAMPLE II

Detection of Protein-Protein Interactions

To detect protein–protein interactions, it was hypothesized that barcoding DNAs of proteins forming complexes would be amplified into co-localized polonies. To test this, DsRed, which

naturally forms a tetramer15, was generated with monomers each bearing one of two different barcodes. To avoid dissociation of any complexes during the array analysis, they were crosslinked with an amine-reactive crosslinker, bis-N-succinimidyl-(pentaethylene glycol) ester (BS(PEG)5). The crosslinking was shown to be efficient due to the presence of a lysine-rich TolA spacer domain (Figure 1A and Figure 9). It was evident that barcoding DNAs of the co-localized monomers (DsReda and DsRedb) were co-amplified into overlapping polonies (Figure 3A), providing a solid basis for further applications.

Because this approach detected polonies of both unbound and bound proteins in a single solution, it was queried whether it could provide a measure of protein binding affinities. A model system, the GTP-dependent binding of human H-Ras (Ras) to Ras-binding domain of c-Raf-1 (Raf-RBD)16, was chosen. A Raf-RBD polony co-localization ratio—the percentage of Raf-RBD polonies co-localized with Ras polonies—was measured for wild-type (WT) Ras and Raf-RBD and eight Raf-RBD mutants. The Ras protein concentration was titrated over three orders of magnitude (Figure 3B). Although the co-localization ratio was sensitive to experimental variables (e.g., the crosslinking conditions, polony array density, etc.), all the proteins within a single assay were basically under the same reaction conditions. Given a similar proportion of active protein and crosslinking efficiency, polony co-localization ratios could be correlated with ratios of bound proteins at equilibrium and thus their binding affinities. To test this, the co-localization ratios were plotted against previously reported dissociation constants (KDs) ranging from nanomolar to micromolar17,18 (Figure 3B and figure 11), and fitted by using a one-site-specific binding model (dashed curves). The fitted and observed average co-localization ratios show relatively high agreement ($R > 0.96$), except for the A85K mutant of significantly lower experimental values than predicted by the model, likely due to the disruption of Lys85-mediated interactions by the crosslinking18. Therefore, this method could be useful for high-throughput screening of protein binding affinities.

EXAMPLE III

High-Throughput Screening

As a first high-throughput screening application, small molecule-mediated protein–protein interactions were studied. Importantly, a significant advantage of the novel method described herein over traditional solid-phase techniques such as protein microarrays3 is that proteins are both stored and assayed in aqueous solution. To exploit this, G-protein coupled receptors (GPCRs), the largest membrane protein family and premier drug targets19, were used to address challenges in screening. Current GPCR–ligand screening techniques mainly rely on cell-based assays20, which are subject to limitations such as the inhomogeneous nature of the samples, the presence of other cellular components that can cause false positives or negatives, and limited miniaturization and

multiplexing capability (e.g., one receptor per assay). To prepare a homogenous SM GPCR sample compatible the methods described herein, receptors were stabilized in phospholipid bilayer nanodiscs21 by assembling detergent-solubilized GPCRs, phospholipids and a membrane scaffold protein, MSP1E3D1, into GPCR–nanodisc complexes22,23. GPCR activation upon ligand binding could be functionally assessed by β-arrestin binding to the active receptors, which is a G-protein independent assay applicable to almost all GPCRs including orphan receptors24.

A compound library could be screened in multi-well plates, and in each well, one compound was assayed with many barcoded GPCRs and a β-arrestin-2 (β-arr2) protein bearing a well-position-associated barcode (Figure 3C). All the samples were pooled and deposited on one slide, and the interaction of a GPCR with a compound was detected by the increased GPCR polony co-localization with the corresponding β-arr2 polonies. Efforts to obtain functional GPCRs using IVT systems were not successful, so they were instead expressed in baculovirus infected insect cells, purified using nanodiscs and individually barcoded (Figure 1B). To establish assay conditions, β-arr2 binding to an agonist (isoproterenol) saturated β2-adrenergic receptor (ADRB2) was assessed, with and without GPCR kinase 2 (GRK2)-mediated receptor phosphorylation and under varied β-arr2 protein concentrations (Figure 3D). The co-localization ratios were measured at 50 imaging positions on the array for statistical analysis. As expected, coupling the receptor phosphorylation to the assay improves the β-arr2 binding, e.g., a ~3 to 11-fold increase (largest $P = 0.002$) of the average co-localization ratios after the phosphorylation. The fitting of β-arr2 titration data for the phosphorylated receptor yielded an apparent KD of 0.95 nM, which is close to the KD of 0.23 nM obtained from traditional binding assays using radiolabeled β-arr225.

To test the screening performance, three GPCRs, ADRB2, M1 and M2 muscarinic acetylcholine receptors (CHRM1 and CHRM2), were assayed with six compounds including antagonists and full, partial, subtype selective and non-selective agonists (Figure 3E and Figure 12). The co-localization statistical analysis based on measurements of ~13,000-17,000 polonies for each receptor precisely identified the full agonists (isoproterenol and carbachol) from the antagonists and inactive compounds (largest $P < 2.7\times10\text{-}10$). Moreover, different types of agonists could be distinguished by comparing their polony co-localization ratios, e.g., the full and partial agonists for ADRB2 (isoproterenol and pindolol, respectively; $P < 0.004$), and the orthosteric and allosteric agonists for CHRM1 (carbachol and xanomeline, respectively; $P < 3\times10\text{-}6$). Thus, the methods described herein enabled both GPCR screening and compound profiling at the same time. Importantly, this method was capable of investigating a large number of GPCRs at the zeptomole level, which will drastically reduce the average screening time and reagent cost per receptor–ligand test.

EXAMPLE IV

Antibody Library Screening

A most remarkable feature of the methods described herein is their ability to screen two barcoded libraries in a single assay. Available techniques (e.g., yeast two-hybrid system2) for library vs. library screening are cell-based and require matching genes from two libraries in positive clones by performing individual PCR reactions26. To demonstrate this capability, a test of a demanding application, the binding profiling of antibody repertoire, was prototyped. The screening of natural or semisynthetic monoclonal antibody (mAb) libraries using methods known to others at the time of filing typically included binding affinity selection and specificity profiling which have to be conducted separately with current techniques. The traditional specificity profiling was costly, usually requiring at least one protein chip for a single antibody test27, and thus has only been commercially applied to therapeutic antibodies. However, both processes could be integrated using the methods described herein by screening an antibody library with a target protein library.

Specifically, 200 antibodies, generated in single-chain variable fragment (scFv) form, were tested against 55 human cytokines, growth factors and receptors (Figure 12). Twenty scFvs were derived by random mutagenesis from each of ten scFvs, the genes of which were previously synthesized from a programmable DNA microchip10. Barcoded scFv proteins were collectively generated in the reconstituted IVT system supplemented with disulfide bond enhancing factors (New England Biolabs). To set up a binding assay, the human proteins were assigned as probes, the concentrations of which are required to be adjustable to ensure formation of enough complexes with target scFvs. Although a human ORF library was recently synthesized using an Escherichia coli IVT system28, it was determined to be better for this application to individually synthesize the probes in a human IVT system (Thermo Scientific) and stabilize membrane proteins by adding assembled nanodiscs. Approximately 0.64 million polonies were sequenced and the co-localization ratios were measured for 11,000 scFv–probe pairs at 100 imaging positions (Figure 4A and). 148 out of 200 scFvs were found with the highest co-localization ratios, and thus the highest specificity, to their predicted targets. Substantial cross-reactivity could be sensitively detected, e.g., 3474 scFv–probe pairs showed 10-fold higher polony co-localization than random distribution ($P < 0.01$). ScFv mutants of a same scFv, grouped by their numbers, exhibited similar but not identical binding patterns to the probes.

Next, the results of 40 scFv–probe pairs were confirmed by immunoprecipitation. The co-localization statistics were consistent with relative fluorescence intensities of the protein bands (Figure 4B). Moreover, to further assess multiplexing potential, a mathematical model was developed (described further herein) that integrated parameters including KDs of protein–probe complexes to be detected and numbers of proteins and probes that can be assayed simultaneously.

Notably, the model indicated that tens of thousands of proteins and probes can be quantitatively analyzed within a single assay.

EXAMPLE V

Discussion

Taken together, these data provide a next-generation method for protein-protein interaction profiling with fundamentally enhanced sensitivity, throughput and cost-effectiveness. Because the polony amplification and sequencing protocol is adaptable to industrial next-generation sequencing platforms, the methods described herein can be readily translated into many platforms and applications. The methods described herein are not limited to the studies of natural or recombinant proteins, and will be applicable to de novo proteins (e.g., with unnatural amino acids or modifications) which could be synthesized with IVT systems of high manipulability8, nucleic acids and barcoded small molecules29. Finally, the methods described herein, along with our ongoing research on single cell-based transcriptome sequencing30, profoundly extends imaging-based sequencing technology by demonstrating that new information can be derived from analyzing the spatial patterning as well as the sequence content and numbers of arrayed DNAs.

EXAMPLE VI

Mathematical Model

Co-localization Statistics

To compare degrees of co-localization between different protein and probe pairs in an experiment, co-localization ratios, defined as the percentages of protein polonies co-localized with corresponding probe polonies, were measured and Student's t-tests were performed for the measurements at multiple imaging positions. The contribution from random co-localization can be estimated by calculating the mean value of pair cross-correlation function (PCCF) over the distance interval of zero to the co-localization threshold. In addition, the PCCF statistic (1) can be applied to characterize co-localization patterns of two polony species that were overlapped or partially overlapped. Below is how the PCCF values were calculated.

Let i and j be two types of objects for co-localization analysis and A be a sampled array area. A cross-correlation Ripley K-function $\widehat{\boldsymbol{K}}(\boldsymbol{r})$ can be estimated (2) as

$$\widehat{\boldsymbol{K}}_{\boldsymbol{i},\boldsymbol{j}}(\boldsymbol{r}) = \frac{\mathbf{1}}{\boldsymbol{A}\hat{\boldsymbol{\lambda}}_{\boldsymbol{i}}\hat{\boldsymbol{\lambda}}_{\boldsymbol{j}}} \sum_{\boldsymbol{k}} \sum_{\boldsymbol{l}} \boldsymbol{\omega}(\boldsymbol{i}_{\boldsymbol{k}}, \boldsymbol{j}_{\boldsymbol{l}}) \mathbf{I}(\boldsymbol{d}_{\boldsymbol{ik,jl}} < \boldsymbol{r})$$

where $\boldsymbol{d}_{\boldsymbol{ik,jl}}$ is the distance between the centroids of k'th location of type i objects and the l'th location of type j objects, and $\mathbf{I}(\boldsymbol{d}_{\boldsymbol{ik,jl}} < \boldsymbol{r})$ is the indicator function with the value 1 if $\boldsymbol{d}_{\boldsymbol{ik,jl}} < \boldsymbol{r}$ is true and 0 otherwise. The density of type i objectives $\hat{\boldsymbol{\lambda}}$ can be estimated as

$$\hat{\lambda}_i = \frac{N_i}{A}$$

where N_i is the total number of i objects. The weight function, $\omega(i_k, j_l)$ provides an edge correction but was here ignored ($\omega(i_k, j_l) \approx 1$). The function $\hat{K}_{i,j}(r)$ can be interpreted as the ratio of the number of i and j objects localized within radius r of each other, over the number that would be expected by chance. Following (1), a PCCF that considered co-localization was computed within a radial interval $[r, r + \Delta r)$ via

$$\frac{1}{A\hat{\lambda}_i\hat{\lambda}_j(2\pi r\Delta r + \pi\Delta r^2)}\sum_i\sum_j I(r \le d_{ik,jl} < r + \Delta r)$$

where $\sum_i \sum_j I(r \le d_{ik,jl} < r + \Delta r)$and $A\hat{\lambda}_i\hat{\lambda}_j(2\pi r\Delta r + \pi\Delta r^2)$ are, respectively, an actual count of co-localized objects i and an average number of objects i that are co-localized with objects j by chance. The PCCF mean values were calculated over the interval of 0 to the co-localization threshold ($r = 0$ and $\Delta r =$ the co-localization threshold). In computing a PCCF value for an experiment in which Q images were analyzed, co-localization events were aggregated over all images and divided by Q times the expected number of random co-localization per image. By definition, randomly co-localized objects should have PCCF values of 1. However, to assess whether PCCFs derived in actual experiments were statistically significantly different from 1, following (1) 95% confidence intervals of the PCCFs of randomly co-localized objects were estimated using Monte-Carlo simulations. Specifically, each simulation assumed Q images, and within each image, Ni and Nj polony and probe objects, respectively, where Q was the number of images analyzed in the experiment whose PCCF was being evaluated, and Ni and Nj were the mean numbers of polony and probe objects observed in the actual experiment. Coordinates for the protein and probe polonies were randomly picked using uniform locations. All dimensions were scaled to actual image dimensions in pixels. For each simulation, a PCCF was computed in the same manner as in the actual experiment by aggregating co-localization events over Q random images. Finally, means and confidence intervals for these random PCCFs were obtained from 1000 simulations.

Initial Mathematical Model of SM-based Protein Library vs. Probe Library Binding Assay

This describes a mathematical model whose aim is to assist understanding of the sensitivity and specificity of detection of protein–probe interactions in complex mixtures. The following items are assumed:

1) n species of barcoded proteins $P_1, P_2, \ldots, P_n$ are allowed to interact with m species of barcoded probes $R_1, R_2, \ldots, R_m$ in a one-pot assay. It is assumed that each protein is present in the same concentration and that the total protein concentration is $P_\#$. Similarly, it is assumed that

the total concentration of probes is $R_{\#}$ and the concentration of each R_j is $R_{\#}/m$. It is assumed that probe concentrations are titratable and that $R_{\#}/m \gg P_{\#}/n$. For simplicity, it will be assumed here that $m = n$ and that for each protein P_i one probe R_i (denoted with the same index) has been chosen or designed to specifically target the protein.

2) Due to folding and other issues relating to the efficiency of ribosome display, only a fraction α of each protein is in an active form that is capable of binding specifically to their targeting probes. The active and inactive forms of the protein P_i will be denoted P_i^+ and P_i^-, with total concentrations $\frac{\alpha P_{\#}}{n}$ and $\frac{(1-\alpha)P_{\#}}{n}$, respectively. For similar reasons, only a fraction τ of probes are active and can specifically bind to their targeted proteins, and their active and inactive forms will similarly be denoted R_j^+ and R_j^-, with concentrations $\frac{\tau R_{\#}}{n}$ and $\frac{(1-\tau)R_{\#}}{n}$. These fractions are assumed to be stable throughout the assay, and active and inactive forms of the proteins and probes are assumed not to be able to interconvert. The fractions α and τ will be assumed to apply to all proteins and all probes, respectively.

3) For $i = 1, 2, \ldots n$, the active forms of protein P_i and its specifically targeting probe R_i will interact according to the reaction

$$\text{(S1)} \quad P_i^+ + R_i^+ \overset{K_D}{\leftrightarrow} (P_i^+ R_i^+)_S$$

where $(P_i^+ R_i^+)_S$ denotes the complex formed from the specific interaction, and K_D the dissociation constant of this complex, and where K_D applies equally to each such protein–probe pair. All forms of protein P_i will also interact non-specifically with all forms of all probes, including with specific probe R_i. This leads to four reactions between the active or inactive protein P_i and each of the n probes R_j $(j = 1, 2, \ldots n)$, all of which are assumed to be characterized by the same non-specific dissociation constant U:

$$\text{(U1)} \quad P_i^+ + R_j^+ \overset{U}{\leftrightarrow} \left(P_i^+ R_j^+\right)_U \qquad \text{(j=1,..,n)}$$

$$\text{(U2)} \quad P_i^+ + R_j^- \overset{U}{\leftrightarrow} \left(P_i^+ R_j^-\right)_U \qquad \text{(j=1,..,n)}$$

$$\text{(U3)} \quad P_i^- + R_j^+ \overset{U}{\leftrightarrow} \left(P_i^- R_j^+\right)_U \qquad \text{(j=1,..,n)}$$

$$\text{(U4)} \quad P_i^- + R_j^- \overset{U}{\leftrightarrow} \left(P_i^- R_j^-\right)_U \qquad \text{(j=1,..,n)}$$

It will also be assumed that (i) non-specific interactions between probes and proteins are always binary, and we can therefore neglect the possibility of ternary or higher complexes, and (ii) probes only non-specifically interact with proteins, and proteins only with probes, and thus that probes and probes, and proteins and proteins, will not interact.

4) After these reactions reach equilibrium, protein–probe complexes of all of these sorts are irreversibly captured by chemical crosslinking, and free probes are removed from the solution, leaving a residual concentration $\boldsymbol{R^0}$. It is assumed that both free and complexed protein and probe molecules are then deposited on the surface of the array in proportion to their solution concentrations, and then immobilized on the array. Of these, it is assumed that only a fraction β of protein and a fraction γ of probe molecules bear barcoding DNAs that can be successfully amplified into polonies and detected on the array, and that the ability of protein and probe DNAs to be amplified is independent of whether the proteins and probes are free or in complex.

5) The following simplifications will be made regarding computation of PCCF statistics (see above): Instead of computing PCCFs by counting all pairs of $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ polonies within a specified distance threshold, PCCFs will be calculated from the numbers of $\boldsymbol{P_i}$ polonies that are found co-localized with $\boldsymbol{R_i}$ polonies in either of the following ways: (i) specific and non-specifically bound $\boldsymbol{P_i \cdot R_i}$ complexes in which both components form polonies (as per the assumption 4) will be counted as intrinsically co-localized polonies; (ii) $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ polonies that are formed on the array by other means may be found to be randomly co-localized. A central value for random co-localization will be computed as the number of non-$\boldsymbol{P_i \cdot R_i}$-derived $\boldsymbol{P_i}$ polonies that are expected to be found by chance within the distance threshold from non-$\boldsymbol{P_i \cdot R_i}$-derived $\boldsymbol{R_i}$ polonies, given the numbers of these polonies obtained from 4 above. The sum of (i) and (ii) will be used to compute a central PCCF for $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ on the array, and variation from this central value will be estimated by random simulations described below. This calculation of PCCF differs from the formal definition given above and in (1) by being non-symmetrical in $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$. Also, in counting $\boldsymbol{P_i}$ polonies that are near $\boldsymbol{R_i}$ polonies instead of counting all pairs of neighboring $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ polonies, it ignores the extra pairs that would be taken into account in the PCCF as formally defined should a $\boldsymbol{P_i}$ polony be found near multiple $\boldsymbol{R_i}$ polonies, and is thus conservative regarding co-localization counts compared to its formal definition.

The equilibriums of the five reactions in the assumption 3, and the assumption 1 that $\boldsymbol{R_\#/n \gg P_\#}$, yield $\boldsymbol{2n+1}$ equations involving the concentration $[\boldsymbol{P_i^+}]$ of free $\boldsymbol{P_i^+}$ and $\boldsymbol{2n}$ equations involving the concentration $[\boldsymbol{P_i^-}]$ of free $\boldsymbol{P_i^-}$

$$(S1') \quad [\boldsymbol{P_i^+}]\frac{\boldsymbol{\tau R_\#}}{\boldsymbol{nK_D}} = [(\boldsymbol{P_i^+ R_i^+})_S]$$

(U1′) $[P_i^+]\frac{\tau R_\#}{nU} = \left[\left(P_i^+ R_j^+\right)_U\right]$ (j=1,..,n)

(U2′) $[P_i^+]\frac{(1-\tau)R_\#}{nU} = \left[\left(P_i^+ R_j^-\right)_U\right]$ (j=1,..,n)

and

(U3′) $[P_i^-]\frac{\tau R_\#}{nU} = \left[\left(P_i^- R_j^+\right)_U\right]$ (j=1,..,n)

(U4′) $[P_i^-]\frac{(1-\tau)R_\#}{nU} = \left[\left(P_i^- R_j^-\right)_U\right]$ (j=1,..,n)

Note that here there is a single (S1′) equation involving the one specifically targeting probe R_i, but n instances each of (U1′)-(U4′), one for each R_j for j=1,..,n.

From the assumption 2 and the equations (S1′), (U1′) and (U2′), we get

$$[P_i^+] + [(P_i^+ R_i^+)_S] + \sum_{j=1}^{n}\left[\left(P_i^+ R_j^+\right)_U\right] + \sum_{j=1}^{n}\left[\left(P_i^+ R_j^-\right)_U\right] = \frac{\alpha P_\#}{n}$$

or

$$[P_i^+]\left(1 + \frac{\tau R_\#}{nK_D} + \sum_{j=1}^{n}\frac{\tau R_\#}{nU} + \sum_{j=1}^{n}\frac{(1-\tau)R_\#}{nU}\right) = [P_i^+]\left(1 + \frac{\tau R_\#}{nK_D} + \frac{R_\#}{U}\right) = \frac{\alpha P_\#}{n}$$

which leads in turn to

$$[P_i^+] = \frac{\alpha P_\#}{n + R_\#\left(\frac{\tau}{K_D} + \frac{n}{U}\right)} = \frac{\alpha P_\#}{n + \frac{R_\#}{\widetilde{K}_D}} = \frac{\alpha P_\# \widetilde{K}_D}{n\widetilde{K}_D + R_\#}$$

where $\widetilde{K}$ can be interpreted as an adjusted specific dissociation constant

$$\widetilde{K}_D = \frac{1}{\frac{\tau}{K_D} + \frac{n}{U}}$$

Similarly, from the assumption 2 and equations (U3′) and (U4′), one gets

$$[P_i^-] = \frac{(1-\alpha)P_\#}{n + \frac{nR_\#}{U}} = \frac{(1-\alpha)P_\# U}{n(U + R_\#)}$$

Using equations (S1′) and (U1′)-(U4′), the total concentration $[(\boldsymbol{P_i \cdot R_i})]$ of $(\boldsymbol{P_i \cdot R_i})$ complexes between the protein $\boldsymbol{P_i}$ and its specifically targeting probe $\boldsymbol{R_i}$ in any of their active and inactive forms is

$$[(\boldsymbol{P_i \cdot R_i})] = \frac{\boldsymbol{\alpha P_\# \tilde{K}_D}}{\boldsymbol{n\tilde{K}_D + R_\#}}\left(\frac{\boldsymbol{\tau R_\#}}{\boldsymbol{nK_D}} + \frac{\boldsymbol{R_\#}}{\boldsymbol{nU}}\right) + \frac{\boldsymbol{(1-\alpha)P_\# U}}{\boldsymbol{n(U+R_\#)}}\left(\frac{\boldsymbol{R_\#}}{\boldsymbol{nU}}\right)$$

$$= \frac{\boldsymbol{P_\# R_\#}}{n}\left(\frac{\boldsymbol{\alpha\left(1 - \frac{(n-1)\tilde{K}_D}{U}\right)}}{\boldsymbol{n\tilde{K}_D + R_\#}} + \frac{\boldsymbol{(1-\alpha)}}{\boldsymbol{n(U+R_\#)}}\right)$$

Total free protein concentration can also be computed as

$$[\boldsymbol{P_i^+}] + [\boldsymbol{P_i^-}] = \frac{\boldsymbol{\alpha P_\# \tilde{K}_D}}{\boldsymbol{n\tilde{K}_D + R_\#}} + \frac{\boldsymbol{(1-\alpha)P_\# U}}{\boldsymbol{n(U+R_\#)}} = \boldsymbol{P_\#}\left(\frac{\boldsymbol{\alpha\tilde{K}_D}}{\boldsymbol{n\tilde{K}_D + R_\#}} + \frac{\boldsymbol{(1-\alpha)U}}{\boldsymbol{n(U+R_\#)}}\right)$$

There is also a total concentration $\left[\left(\boldsymbol{P_i \cdot R_{j\neq i}}\right)\right]$ of $\left(\boldsymbol{P_i \cdot R_j}\right)$ complexes between $\boldsymbol{P_i}$ and $\boldsymbol{R_j}$ probes ($j \neq i$) that are not targeted to $\boldsymbol{P_i}$, in any of their active and inactive forms. This is simplified as

$$\left[\left(\boldsymbol{P_i \cdot R_{j\neq i}}\right)\right] = ([\boldsymbol{P_i^+}] + [\boldsymbol{P_i^-}])\frac{\boldsymbol{(n-1)R_\#}}{\boldsymbol{nU}}$$

Finally, it must also be considered that probe $\boldsymbol{R_i}$ will be in non-specific complexes with other proteins $\boldsymbol{P_{j\neq i}}$ than its specific target. By our assumptions above, since all proteins $\boldsymbol{P_j}$ $(\boldsymbol{j \neq i})$ behave identically with respect to their targeting and non-targeting probes to $\boldsymbol{P_i}$, we have $\left[\boldsymbol{P_j^+}\right] + \left[\boldsymbol{P_j^-}\right] = [\boldsymbol{P_i^+}] + [\boldsymbol{P_i^-}]$ for all $\boldsymbol{j \neq i}$, and therefore that

$$\left[\left(\boldsymbol{P_{j\neq i} \cdot R_i}\right)\right] = ([\boldsymbol{P_i^+}] + [\boldsymbol{P_i^-}])\frac{\boldsymbol{(n-1)R_\#}}{\boldsymbol{nU}}$$

Arraying, polony formation, and co-localization statistics

It is now assumed that the mixture is arrayed for SM assaying, and that polonies are formed on the array. Following assumption 4, the fractions of polonies relevant to evaluation of $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ co-localization can be computed as follows:

$\boldsymbol{f_{(PR)} = \frac{\beta\gamma[(P_i \cdot R_i)]}{C}}$	Fraction of $(\boldsymbol{P_i \cdot R_i})$ complexes between $\boldsymbol{P_i}$ and its specifically targeting probe $\boldsymbol{R_i}$ that are detectable on the array as intrinsically co-localized polonies

$f_{(PX)} = \frac{\beta\gamma[(P_i \cdot R_{j\neq i})]}{C}$	Fraction of $(P_i \cdot R_j)$ complexes between P_i and other probes R_j (j ≠ i) that are detectable on the array as polonies of P_i that are intrinsically co-localized with those of other probes.
$f_P = \frac{\beta(1-\gamma)([(P_i \cdot R_i)] + [(P_i \cdot R_{j\neq i})]) + \beta([P_i^+] + [P_i^-])}{C}$	Fraction of P_i polonies that do not appear intrinsically co-localized with probe polonies
$f_{(XR)} = \frac{\beta\gamma[(P_{j\neq i} \cdot R_i)]}{C}$	Fraction of $(P_j \cdot R_i)$ complexes between probe R_i and other proteins P_j (j ≠ i) that are detectable on the array as polonies of R_i that are intrinsically co-localized with the other proteins.
$f_R = \frac{(1-\beta)\gamma([(P_i \cdot R_i)] + [(P_{j\neq i} \cdot R_i)]) + \gamma\frac{R^0}{n}}{C}$	Fraction of R_i polonies that do not appear intrinsically co-localized with protein polonies

where

$$C = (1-(1-\beta)(1-\gamma))[(P_i \cdot R_i)] + \beta[(P_i \cdot R_{j\neq i})] + \gamma[(P_{j\neq i} \cdot R_i)] + \beta([P_i^+] + [P_i^-]) + \gamma\frac{R^0}{n}$$

Note that as per the assumption 5, $f_{(PR)}$ determines the number of intrinsically co-localized $\mathbf{P_i}$ and $\mathbf{R_i}$ polonies found on the array. The other fractions will be used in calculation of the number of randomly co-localized polonies below. First the numbers of polonies of the various sorts is computed, and then random co-localization is calculated.

Let it now be assumed that N_i polonies are detected for the protein P_i. These N_i polonies may be apportioned as

$n_{(PR)} = \frac{N_i f_{(PR)}}{f_{(PR)} + f_{(PX)} + f_p}$	Polonies of P_i intrinsically co-localized with polonies of R_i

$n_{(PX)} = \frac{N_i f_{(PX)}}{f_{(PR)} + f_{(PX)} + f_p}$	Polonies of P_i intrinsically co-localized with polonies of other probes R_j $(j \neq i)$
$n_p = \frac{N_i f_P}{f_{(PR)} + f_{(PX)} + f_p}$	Polonies of P_i that are not intrinsically co-localized with probe polonies.

It follows from the frequencies derived above that the following numbers of polonies are detected for the probe R_i apart that are not counted with the N_i P_i protein polonies above (the only R_i polonies considered with the N_i polonies above are the $n_{(PR)}$ instances of R_i polonies co-localized with P_i polonies).

$n_{(XR)} = \frac{N_i f_{(XR)}}{f_{(PR)} + f_{(PX)} + f_p}$	Polonies of R_i intrinsically co-localized with polonies of other proteins P_j $(j \neq i)$
$n_R = \frac{N_i f_R}{f_{(PR)} + f_{(PX)} + f_p}$	Polonies of R_i that are not intrinsically co-localized with protein polonies.

In preparing to compute random co-localization and the final PCCF statistic, a question arises in the context of our highly multiplexed SM assay as to whether P_i polonies from both uncomplexed P_i^+ and P_i^- objects vs. P_i polonies formed from $P_i \cdot R_{j \neq i}$ complexes should be treated equivalently regarding whether they can be randomly co-localized (and similarly for R_i polonies). It could be the case that P_i polonies formed within complexes cannot be co-localized with R_i polonies to the degree that P_i polonies formed from uncomplexed P_i objects can due to steric constraints or other factors. In non-multiplexed assays, such as those considered in (1), this question never arises because the non-targeting partners in $P_i \cdot R_{j \neq i}$ and $P_{j \neq i} \cdot R_i$ complexes would never be surveyed for detection, and the resulting P_i and R_i polonies would all be considered isolated objects that could appear near each other by chance in the same way. A broader issue concerns the fact that the PCCF is specifically a Pair Cross-Correlation Function (1), and the question arises whether for multiplexed assays it might be better to develop and employ a higher-order multi-variate statistic that compares actual vs. expected random co-localization for many kinds of objects at once, somewhat like multi-variate ANOVAs analyze variances of many variables and interactions at once. However, in this initial model, we will in fact treat polonies derived from free probe and protein molecules vs. complexes equivalently in terms of their potential for random co-localization within the constraints indicated in the assumption 5. Notably, even when only considering pairwise co-localization, such as the application of PCCF in (1), where objects are labeled antibodies, the prima facie distinction between objects co-localized by virtue of targeting physical

interactions and isolated objects that appear as random background is an idealization, since the apparently isolated objects are likely interacting non-specifically with many other kinds of unsurveyed molecules and complexes in the cell matrix, and PCCF remains a useful statistic even though these interactions are ignored.

Random Co-localization

As noted in assumption 5 and discussed in the comment above, random co-localization will be considered between $\boldsymbol{P_i}$ and $\boldsymbol{R_i}$ polonies that do not arise from intrinsic co-localization from $\boldsymbol{P_i \cdot R_i}$ complexes. The number of such polonies is known to be $\boldsymbol{n_{(PX)} + n_P}$ for $\boldsymbol{P_i}$, and $\boldsymbol{n_{(XR)} + n_R}$ for $\boldsymbol{R_i}$. Given imaged array area A and polony radius r, the density of these $\boldsymbol{R_i}$ polonies that could appear anywhere on the array by chance can be estimated as

$$\boldsymbol{\rho_R = \frac{n_R + n_{(XR)}}{A}}$$

and the probability of a probe $\boldsymbol{R_i}$ polony appearing in the vicinity of a $\boldsymbol{P_i}$ protein polony by chance would then be

$$\boldsymbol{\pi(2r)^2 \rho_R}$$

Thus, the expected number of the $\boldsymbol{n_{(PX)} + n_P}$ $\boldsymbol{P_i}$ polonies that will have an $\boldsymbol{R_i}$ polony localized nearby by chance will be

$$\boldsymbol{n_{(PR)}^{rand} = \pi(2r)^2 \rho_R \left(n_{(PX)} + n_P\right)}$$

Thus, the total number of $\boldsymbol{P_i}$ polonies co-localized with $\boldsymbol{R_i}$complexes will be

$$\boldsymbol{n_{(PR)}^{tot} = n_{(PR)} + n_{(PR)}^{rand}}$$

PCCF Statistic

To complete the PCCF statistic as specified in assumption 5, $\boldsymbol{n_{(PR)}^{tot}}$ must be divided by the expected number of $\boldsymbol{P_i}$ polonies co-localized with $\boldsymbol{R_i}$ polonies assuming that all of these individual polonies (including the ones in $\boldsymbol{P_i \cdot R_i}$ complexes) could be co-localized by chance. Similar to the logic above, the total density of R_i objects will now be

$$\lambda_R = \frac{n_R + n_{(PR)} + n_{(XR)}}{A}$$

and the probability of a probe R_i polony appearing in the vicinity of a P_i protein polony will then be

$$\pi(2r)^2 \lambda_R N_i$$

and, therefore

$$PCCF = PCCF(P_{\#}, R_{\#}, R^0, n, \alpha, \tau, K_D, U, \beta, \gamma, N_i, A, r) = \frac{n_{(PR)}^{tot}}{\pi(2r)^2 \lambda_R N_i}$$

Random Simulations

To estimate the degree of variation to which the PCCF statistic may be subject under a given set parameters, distribution of PCCF values were computed using the formula above assuming that the six terms n_P, $n_{(PR)}$, $n_{(PX)}$, $n_{(XR)}$, n_R, and $n_{(PR)}^{rand}$ are all randomly drawn from Poisson distributions whose means are the values computed above within the model. Because these simulations do not take into account variation in actual samples or assay conditions, and because Poisson error may itself underrepresent the variability inherent in the underlying system vs. the model, these estimates must be considered lower bounds for the variance that will be encountered in actual assays.

Detection of Specific vs. Non-Specific Binding as a Function of K_D and n

As an application of the model, the PCCF values computed were compared for a mixture of n proteins and targeting probes that specifically interact with dissociation constant K_D, where n is allowed to vary over a large range, with the PCCF for mixtures of the same numbers of proteins and probes, in which all the proteins and probes interact only non-specifically with dissociation constant U. In particular, an array in which 5×108 protein polonies can be detected is assumed, and these are divided equally among the n proteins, where n is allowed to range between 500 and 500,000 (so that the number of detected polonies per protein species N_i correspondingly varies between 1,000,000 and 1,000). Three specific dissociation constants are considered K_D, and non-specific PCCFs are computed by letting $K_D \to \infty$. All parameters other than K_D, n, and N_i are assigned the following fixed values consistent with literature and experimental data.

$P_{\#} = 20\ pmol\ /100\ \mu L$	Approximate values which can be used in the assay
$R_{\#} = 200\ pmol\ /100\ \mu L$	
$R^0 = 100\ nM$	
$A = 75 \times 25\ mm^2$	Standard microscope slide area
$r = 0.7\ \mu m$	Co-localization threshold distance used in our experiments
$\alpha = 0.8$	Approximate values based on the ACP functional assay
$\tau = 0.8$	
$U = 10\ \mu M$	Assumed non-specific protein–probe complex

	dissociation constant
$\beta = 0.75$	Refer to (3)
$\gamma = 0.75$	

Results are summarized in Figure 17. In Figure 17, error bars span the range of the 1st and 99th percentiles of randomly simulated PCCF distributions as described above, with the following exception(s): (i) For large values of n, the 99th percentile of the non-specific PCCF distribution was no more than the central value computed by the model so that the upper error bar could be at or below the central value. In these cases the maximum value observed in the PCCF distribution was used to set the upper error bar instead of the 99th percentile, and the upper end of the error bar was marked with an asterisk (*). This situation arises because the number of P_i and R_i polonies becomes very small so that simulations result in no or very few co-localized polonies except for a small number of outliers. (ii) Because PCCFs are presented below via their log10 values, PCCF values of 0 cannot be portrayed directly. However, in some cases the 1st percentiles of PCCF values were 0, and this is indicated by the use of a downward pointing arrowhead on the lower error bars. Note that markers and error bars are slightly jittered in order to allow overlapping error bars to be seen clearly. For each set of K_D, n, and N_i values, 10,000 random simulations were performed.

A conclusion that may be drawn from these simulations is that order-of-magnitude differences between specific K_Ds can be clearly distinguished from each and from non-specific binding in mixtures of up to ~63,000 distinct protein and probe species under the conditions assumed in the model. Note, however, that while the lack of overlap between error bars that indicate 1st and 99th percentiles implies that the PCCF distributions for these different K_Ds overlap with $P < 0.0001$, these probabilities are not corrected for multiple hypotheses.

References In This Example

(1) Philimonenko et al. (2000) J. Struct. Biol. 132:201

(2) Hanisch and Stoyan (1979) Math. Operationsforsch. Statist., Ser. Statitics 14:559

(3) Mitra and Church (1999) Nucleic Acids Res. 27:e34

Example VII

Methods

DNA Construction

Protein coding sequences were synthesized (Genewiz and IDT), PCR amplified from plasmids or genomic DNA, or transferred from Gateway-adapted human ORF clones31 (refer to Figure 15 for DNA sources, sequences and construction methods), and inserted into expression vectors containing a multiple cloning site or Gateway recombination sites (refer to Figure 10 for plasmid construction and Figure 16 for plasmid and primer sequences) for in vitro or in vivo protein translation.

Ribosome Display-Based Protein Barcoding

For protein libraries of relatively small size (e.g., ≤ 200 in this work), a barcoding sequence can be introduced to DNA templates by performing individual PCR reactions with a barcoded primer. Barcoded linear DNA templates were pooled and in vitro transcribed using a HiScribe T7 kit (NEB). Transcribed mRNAs were treated with a DNA-free kit (Ambion), purified with an RNeasy Mini kit (Qiagen) and quantified by Nanodrop 1000 (Thermo Scientific). To generate mRNA–cDNA hybrids, cDNAs were synthesized by incubating ~0.10 μM mRNA, 1 μM 5'-acrydite and desthiobiotin-modified primer, 0.5 mM each dNTP, 10 U/μL SuperScript III, 2 U/μL RNaseOUT (Invitrogen) and 5 mM dithiothreitol (DTT) in a buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM MgCl2) at 50°C for ~30 min. Resultant mRNA–cDNA hybrids were enriched by isopropanol precipitation and purified with streptavidin-coated magnetic beads (Dynabeads M-270 Streptavidin, Life Technologies). A PURExpress Δ Ribosome kit (NEB) was applied to display proteins on E. coli ribosomes. Typically, a 250 μL IVT reaction with ~0.40 μM mRNA–cDNA hybrids and ~0.30 μM ribosome was incubated at 37°C for 30 min, quenched by addition of 250 μL ice-cold buffer HKM (50 mM HEPES, pH 7.0, 250 mM KOAc, 25 mM Mg(OAc)2, 0.25 U/mL RNasin (Promega), 0.5 mg/mL chloramphenicol, 5 mM 2-mercaptoethanol and 0.1% (v/v) Tween 20) and centrifuged (14,000 g, 4°C) for 10 min to remove insoluble components. PRMC complexes, always kept on ice or in cold room, were subjected to two-step Flag tag and desthiobiotin tag affinity purification to enrich full-length and barcoded target proteins. Thus, proteins were sequentially purified using anti-Flag M2 (Sigma-Aldrich) and the streptavidin magnetic beads, which were blocked with the buffer HKM supplemented with 100 μg/mL yeast tRNA and 10 mg/mL BSA. The bound proteins were eluted with the buffer HKM containing 100 μg/ml Flag peptide or 5 mM biotin, and their barcoding DNAs were quantitated by real-time PCR.

Protein Expression and Purification and HaloTag-Based Barcoding

His-tagged HaloTag–TolA, HaloTag–DsRed–TolA, Ras–TolA–HaloTag and β-arr2–TolA–HaloTag were expressed in E. coli or using an E. coli IVT system for relatively large or small-scale production. Proteins were expressed in an OverExpress C41(DE3) strain (Lucigen) with 1 mM isopropyl- -D-galactopyranoside (IPTG) induction at 30°C for 8-10 h and purified using immobilized metal affinity chromatography (IMAC) at 4°C. In brief, harvested cells were

resuspended in a lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole and 20% glycerol) and disrupted by French press. Supernatants of cell lysates were loaded on a 5 ml HisTrap column (GE Healthcare) and non-specifically bound components were washed off with a buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole and 10% glycerol). His-tagged proteins were eluted with a buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole and 10% glycerol), concentrated with Amicon Ultra-15 centrifugal filter units (Millipore), buffer exchanged to a storage buffer (50 mM HEPES, pH 7.0, 150 mM KOAc and 20% glycerol) using a PD10 desalting column (GE Healthcare), flash frozen in 100-500 L aliquots by liquid N2 and stored at -80°C. Relatively small amounts of proteins were synthesized in an E. coli crude extract (RTS 100 E. coli HY, 5 PRIME) at 30°C for 4 h, and similarly purified with His-tag magnetic beads (Dynabeads His-tag, Life Technologies).

Human ADRB2, CHRM1 and CHRM2 were expressed in baculovirus-infected Sf9 cells (Life Technologies) and solubilized with detergents similarly as previously described32,33. To reduce protein denaturation during purification, a recently developed method was followed in which solubilized GPCRs are immediately assembled into GPCR–nanodisc complexes before affinity purification34. Briefly, synthesized GPCR genes were inserted into a pBac-NFlagHA vector and thus GPCRs were expressed as a fusion protein bearing an N-terminal Flag and a HA tag and a HaloTag. Cells were harvested at two days after transfection, homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl and 1 mM EDTA) supplemented with a protease inhibitor (PI) cocktail (Roche) and centrifuged to collect the membrane fractions. The membranes were solubilized in a solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM CaCl2, 5 mM MgCl2, 2 mM EDTA, 10% glycerol and 1% n-dodecyl-β-d-maltopyranoside (DDM)) supplemented with a PI cocktail (Set III, EMD Biosciences) and centrifuged at 15,000 g for 15 min; the supernatants were subjected to a bicinchoninic acid assay (Thermo Scientific) to determine protein concentration. The nanodiscs were assembled by incubating 90 μM MSP1E3D1 (Sigma-Aldrich), 8 mM POPC, 40 mM DDM and 180 μg total membrane protein in a reconstitution buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM CaCl2, 5 mM MgCl2, 2 mM EDTA and 2% glycerol) on ice for 45 min, followed by removal of the detergent using Bio-Beads SM-2 (Bio-Rad)34. GPCR–nanodisc complexes were bound to anti-Flag M1 agarose resin (Sigma-Aldrich) and eluted with a conjugation buffer C1 (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA and 5% glycerol) in the presence of 0.2 mg/mL Flag peptide. Ligand binding activities of purified GPCRs were measured by a time-resolved fluorescence resonance energy transfer assay (Cisbio Bioassays), in which the HaloTagged GPCRs were labelled with HaloTag–terbium cryptate (Lumi4-Tb) and interacted with fluorescent ligands, L0011GRE (ADRB2 antagonist) and L0040RED (CHRM1 and CHRM2 agonist).

Human protein genes were sub-cloned into pIRES–CHaloFlagHis or pIRES–CHaloFlagHis–Gateway containing an internal ribosome entry site (IRES) for in vitro protein synthesis with a human IVT kit (Thermo Scientific). Proteins were translated at 30°C for 2 h and purified with the anti-Flag M2 or His-tag magnetic beads. Membrane proteins were stabilized by addition of preassembled nanodiscs (2 μL MembraneMax reagent/50 μL reaction, Life Technologies). HaloTag fusion proteins can be semi-quantitatively analyzed by in-gel fluorescence detection. Thus, HaloTag domains were covalently labeled with a fluorescent reporter Halo-TMR (Promega) and subsequently analyzed by SDS-PAGE and fluorescent gel imaging with a Typhoon Trio Imager (GE Healthcare).

220-bp double stranded barcoding DNAs were prepared in parallel by PCR amplification with a universal template (Figure 16) and one primer bearing a barcoding sequence. Modifications of desthiobiotin, acrydite and HaloTag ligand were introduced by a secondary PCR with the modified primers. A HaloTag ligand was conjugated to the primer by incubating the amino modified oligo (100 μM) and 5 mM succinimidyl ester (O4) ligand (Promega) in a conjugation buffer C2 (50 mM Na2HPO4, pH 8.0 and 150 mM NaCl) at room temperature for 1 h; the modified oligo was purified by reverse-phase high-performance liquid chromatography using a Source 15RPC column (GE Healthcare) and an elution gradient of 3-70% CH3CN/H2O (0.1 M triethylamine acetate). To generate protein–DNA conjugates, we typically incubated equal molar amounts (e.g., ~0.5 μM) of modified barcoding DNAs and HaloTagged proteins in the conjugation buffer C1 with gentle shaking at room temperature for 4-6 h; the conjugates were purified with the anti-Flag M2 or His-tag and then the streptavidin magnetic beads. Barcoded proteins can be eluted in corresponding assay buffers (see below) in the presence of 5 mM biotin.

Ras–Raf-RBD Binding Assay

Prior to the barcoding, the E. coli expressed and purified Ras protein was saturated with a non-hydrolyzable GTP analog, Gpp(NH)p, by EDTA-enhanced nucleotide exchange as previously described35. 2 nM mixed Raf-RBD WT and mutants displayed on PRMC complexes were incubated with different concentrations of barcoded Ras in an assay buffer A1 (50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 0.5 mM DTT and 0.1% (v/v) Tween 20) in the presence of 0.5 mM Gpp(NH)p for 1 h. After reaching equilibrium, Ras–Raf-RBD complexes were crosslinked with 0.5 mM BS(PEG)5 at 4°C for 1 h. The reaction was quenched by adding Tris-HCl, pH 8.0 to a final concentration of 50 mM. Free barcoded Ras protein can contribute to random Raf-RBD polony co-localization which affects accuracy of the measurement, and thus was removed by affinity purification to enrich HA-tagged Raf-RBD proteins. Thus, the samples were incubated with anti-HA magnetic beads (Thermo Scientific) at 4°C for ~2 h and eluted with an array deposition buffer (20 mM HEPES, pH 7.0, 50 mM KOAc, 6 mM Mg(OAc)2, 0.5 mg/mL

chloramphenicol, 0.25 U/mL RNasin (Promega) and 0.1% Tween 20) in the presence of 2 mg/mL HA peptide.

GPCR Profiling Assay

Mixed barcoded GPCRs were assayed with 100 μM alprenolol, pindolol, isoproterenol, atropine and carbachol (Sigma-Aldrich) and 100 nM xanomeline (Tocris Bioscience). A GPCR–β-arr2 binding assay was performed by mixing a ligand with ~1 nM GPCR–nanodisc complexes in an assay buffer A2 (20 mM HEPES, pH 7.5, 50 mM KOAc, 2 mM EDTA and 5 mM MgCl2), followed by addition of 10 nM GRK2 (Life Technologies), 0.1 mM ATP, 10 nM G protein β1γ2 subunits (KeraFAST) and 5 nM barcoded β-arr2 in a total volume of 25 μL. Compounds were assayed in parallel using a multi-well plate, and the reactions were incubated at 30°C for 30 min. Similarly as described above, GPCR–β-arr2 complexes were crosslinked and both crosslinked and free GPCRs were purified from the reactions with the anti-HA magnetic beads. Proteins were pooled and analyzed on a single array.

ScFv Binding Profiling and Immunoprecipitation Assay

Error-prone PCR was performed for ten scFv genes previously synthesized10 by using a random mutagenesis kit (Clontech) at the condition of 3.5 mutations per 1,000 bp. Twenty mutants for each scFv were randomly picked and pooled to constructed a scFvs library. Ribosome display of the scFv library was specifically performed with the PURExpress Δ Ribosome kit supplemented with disulfide bond enhancers (NEB, 10 μL of the enhancer 1 and 2 for a 250 μL reaction). The binding assay was performed by incubating scFvs (~5.5 nM) and mixed barcoded human proteins (~1.8 μM) in a buffer A3 (50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl2 and 0.1% (v/v) Tween 20) at 4°C for 4 h. Similarly as above, scFv–human protein complexes were crosslinked and HA-tagged scFvs in free and bound forms were enriched by the anti-HA magnetic beads and eluted in the buffer HKM.

For the immunoprecipitation assay, selected scFv genes were subcloned into pEco-CSBP and the scFv fusions bearing a C-terminal SBP tag were in vitro synthesized using a PURExpress IVT kit (NEB) supplemented with the disulfide bond enhancers. In each binding assay, a 10 μL IVT reaction (typically containing 0.1-0.4 μM translated scFvs) was incubated with 2 μL human protein (4.6-9.5 nM) labelled by Halo-TMR in the buffer A3 at 4°C for 4 h. ScFvs and bound human proteins were pulled down with the streptavidin magnetic beads and then analyzed by SDS-PAGE and fluorescence gel imaging.

Array Deposition

Barcoded proteins were diluted with the deposition buffer to a 10× deposition concentration between 0.1 to 1 nM. Because the presence of oxygen can inhibit the gel polymerization, a gel-

casting solution (6.66% acrylamide/bis-acrylamide (19:1, molecular grade, Ambion) and two 5'-acrydite-modified bridge amplification primers (111 μM each) in the deposition buffer) were degassed with argon and mixed with diluted proteins by a 9:1 volume ratio in an anaerobic chamber (Coy Lab). To form a gel layer of less than 5-μm thickness, ~20 μL of the gel-casting mix, immediately after addition of 0.1% (v/v) TEMED and 0.05% (w/v) ammonium persulfate, was applied to a glass microscope slide surface pretreated with Bind-Silane (GE Healthcare)7,13, and a coverslip was placed on the top of the liquid and tightly pressed to form a liquid layer evenly spread over the glass surface. The gel was polymerized in the anaerobic chamber for 4 h. After removal of the coverslip, the slide was washed with Milli-Q H2O and dried by a quick spin.

Polony Amplification, Linearization and Blocking

The protein-loaded slide was assembled in a FC 81 transmission flow cell containing a 1.85-mm-thick polycarbonate flow channel (BioSurface Technologies), and thus reagents and buffers can be sequentially added in cycles for polony amplification, linearization and blocking. Flow cell components including the channel, a coverslip and tubing were sequentially cleaned by sonication in 5% Contrad 70, 1 M NaOH, 0.1 N HCl and Milli-Q H2O, and air dried in an AirClean PCR hood. Prior to the amplification, mRNAs can be digested with 10 U/mL RNase H (NEB) in a digesting buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2 and 0.1% (v/v) Triton X-100) at 37°C for 20 min. Polony amplification, linearization and blocking were performed similarly as a Illumina cluster generation protocol12. Typically, immobilized barcoding DNAs were subjected to 32-35 cycles of isothermal bridge amplification at 60°C. For each cycle, the flow cell was washed with deionized formamide (Ambion) and an amplification buffer (20 mM Tris-HCl, pH 8.8, 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1% (v/v) Triton X-100, 1.3% (v/v) DMSO and 2M betaine) and incubated with 200 μM dNTPs and 80 U/mL Bst polymerase (NEB) in the amplification buffer for 5 min. Resulted double-stranded polonies were linearized by incubated the flow cell with 10 U/mL USER enzyme (NEB) in a linearization buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium sulfate and 0.1% (v/v) Triton X-100) at 37°C for 1 h; excised strands were eluted with a wash buffer W1 (1×SSC and 70% formamide). Exposed 3'-OH ends of DNAs were blocked by incubating the flow cell for three times with 10 μM ddNTPs and 250 U/mL terminal transferase (NEB) in a blocking buffer (20 mM Tris-acetate, pH 7.9, 50 mM KOAc, 10 mM Mg(OAc)2 and 0.25 mM CoCl2) at 37°C for 10 min.

DNA Sequencing

Linearized and 3'-OH blocked polonies were analyzed by hybridization with fluorescently labeled oligos, SBE or sequencing by ligation similarly as previously described13,14. The assays can be performed within the flow cell or a gasket chamber assembled with the slide taken out of the flow

cell and a microarray gasket slide (Agilent Technologies). Oligos (IDT) and dideoxynucleotides (PerkinElmer) were labelled by fluorescein/FAM, Cy3/Ty563 or Cy5/Ty665 for three-color imaging. Although four-color imaging is typical for DNA sequencing, only three channels were applied in our platform to minimize crosstalk between channels. In brief, polonies were hybridized with oligos (2 μM each) in a hybridization buffer (5×SSC and 0.1% (v/v) Tween 20) at 60°C for 10 min, and the flow cell was cooled to 40°C and washed with a wash buffer W2 (0.3×SSC and 0.1% (v/v) Tween 20). The SBE was performed by incubating primer-bound polonies with fluorescently labeled ddNTPs (1 μM each) and 0.32 U/μl Thermo Sequenase (GE Healthcare) in an extension buffer (26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl2 and 0.05% (v/v) Tween 20) at 60°C for 5 min; excess ddNTPs were removed with the wash buffer W2. For each sequencing-by-ligation cycle, sequencing primer-bound polonies were probed with a query primer set (fluorescent nonamers, 2 μM each subpool) in a ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 1 mM ATP and 5 mM DTT) in the presence of 30 U/μl T4 DNA ligase (Enzymatics). The ligation was incubated at room temperature for 20 min, then increased to 35°C and stayed for 40 min. Before the next cycle, the hybridized primers were stripped with the buffer W1 at 60°C, followed by wash with the buffer W2. To facilitate deconvolution of co-localized polonies from two protein libraries, each library was separately sequenced by using a distinct sequencing primer.

Image Acquisition, Processing and Base Calling

Fluorescence imaging was conducted with a Leica AM TIRF MC system including a DMI6000 B inverted microscope, a motorized scanning stage and a Hamamatsu C9100-02 electron multiplying CCD camera (1000 × 1000 pixels, Hamamatsu Photonics). Polony images were acquired under an epi-illumination mode by using a 20× objective (HCX PL Fluotar L, N.A. 0.40, Leica) or 40× (HCX PL APO, N.A. 0.85, Leica) and from three channels (fluorescein, Cy3 and Cy5) using, respectively, 488, 561 and 635 nm lasers and excitation–emission filter pairs of 490/20–525/50, 552/24–605/65 and 635/10–720/60, respectively. Raw images were exported by LAS AF Lite software (Leica) and processed using ImageJ and MATLAB (R2011a) scripts to remove background fluorescence and exclude small-size impurities and large-scale structures. Image analyses and base calling were conducted similarly as previously described14. In brief, MATLAB scripts were applied to identify polony coordinates by finding local maxima or weighted centroids, construct a reference image containing all detected polonies by super-imposing images taken in the first cycle, and align images from later cycles to the reference image. Thus, a set of fluorescence values for each acquisition cycle as well as the coordinates were obtained for barcode identification and polony co-localization analyses. Given programmable synthetic barcodes, only a few sequencing cycles were required for the protein libraries used in this work (e.g., 5 cycles for the library of 200 scFvs barcoded by a 5-bp sequence composed of A, T and C).

Co-localization Analysis and Statistics

To align reference images of two protein libraries, polonies were hybridized with both sequencing primers labelled by Cy3 or Cy5, and thus their images were super-imposed to serve as a cross-library reference. MATLAB scripts calculated the offset of reference images generated from two sequencing rounds, measured distances between all polony positions identified from the two libraries, and compared them to a defined threshold to determine the co-localization. A polony exclusion effect36,37 was considered usually observed for competitive co-amplification of co-localized templates, and an optimized threshold distance was set to be 0.7 μm. Total and co-localized polony numbers were computed for each paired polony species at each imaging position. Co-localization statistics were calculated using Student's t-tests based on measurements at all imaged positions. In addition, a pair cross-correlation function (PCCF) statistic38 was applied to compare observed with random polony co-localization and study their co-localization patterns.

Example VIII

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What is claimed:

1. A method for attaching a plurality of barcodes to a plurality of polypeptides comprising the steps of:

attaching a barcode to a plurality of DNA template sequences to produce a plurality of barcoded templates comprising a barcode sequence and a protein coding sequence;

performing reverse transcription of the barcoded templates to produce a plurality of mRNA-cDNA hybrid sequences; and

performing in vitro translation of the mRNA-cDNA hybrid sequences to generate a plurality of protein-ribosome-mRNA-cDNA complexes.

2. The method of claim 1, wherein the step of attaching is performed using PCR and in vitro transcription.

3. The method of claim 1, wherein the plurality of protein-ribosome-mRNA-cDNA complexes are formed by in vitro translation and ribosome stalling.

4. The method of claim 1, wherein the plurality of barcoded sequences are synthesized in parallel on an immobilized support or individually synthesized as a mixture of random sequences on a support.

5. The method of claim 1, wherein each of the steps is performed in a single container, and a correlation between a barcoding sequence and a protein sequence is determined using massively parallel DNA sequencing.

7. The method of claim 1, wherein the barcoded templates contain a polymerase promoter, and mRNAs are synthesized from the barcoded DNA templates by in vitro transcription in a single container.

8. The method of claim 7, wherein the polymerase is T7 polymerase.

9. The method of claim 1, wherein the reverse transcription is performed using universal primers, and the cDNA sequences are complementary upstream to a ribosome binding site of the barcoded template.

10. The method of claim 1, wherein ribosomes stall at the 3' end of the mRNA-cDNA hybrid sequences during in vitro translation due to one or both of a lack of stop codons or the presence of ribosome stalling peptide sequences.

11. The method of claim 1, wherein primers for cDNA synthesis contain one or both of 5' desthiobiotin modifications and 5' acrydite modifications.

12. The method of claim 1, wherein the protein coding sequence encodes one or more affinity tags at its C-terminus.

13. The method of claim 12, wherein the affinity tag is a FLAG tag.

14. The method of claim 1, wherein the protein-ribosome-mRNA-cDNA complexes are purified using a protein affinity tag and a cDNA desthiobiotin tag.

15. A method for attaching a barcode to a polypeptide comprising the steps of:
providing a DNA template comprising an enzyme ligand at its 5' end;
providing a protein comprising an enzyme specific for the ligand; and
allowing the enzyme to bind the ligand to produce a polypeptide comprising a barcode sequence.

16. The method of claim 15, performed using an automated high-throughput platform.

17. The method of claim 16, wherein 10,000 or more polypeptides comprising a barcode sequence are prepared in parallel.

18. The method of claim 15, wherein an enzyme ligand is selected from the group consisting of one or more of HaloTag, CLIP tag and SNAP tag.

19. The method of claim 15, wherein both the DNA template and the polypeptide comprise an affinity tag.

20. The method of claim 19, further comprising the step of performing affinity purification using two steps.

21. The method of claim 20, wherein the DNA template comprises a desthiobiotin tag and the polypeptide comprises a His tag.

22. A method of detecting and quantifying a plurality of polypeptides in situ comprising the steps of:

providing in an aqueous medium a plurality of polypeptides comprising a barcode;

immobilizing the plurality of polypeptides on a substrate;

performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides; and

identifying and quantifying amplified barcode sequences and recording their locations by in situ DNA sequencing.

23. The method of claim 22, wherein the polypeptides comprising barcodes are made according to the method of claim 1 or claim 15.

24. The method of claim 22, wherein the plurality of polypeptides are randomly immobilized in a crosslinked polyacrylamide gel layer having a thickness of about a few microns.

25. The method of claim 22, wherein the nucleic acid sequences have a 5' end modification and are copolymerized into the gel matrix to avoid template drifting.

26. The method of claim 25, wherein the 5' end modification is an acrydite modification.

27. The method of claim 22, wherein the nucleic acid sequences are amplified into polonies using solid-phase PCR.

28. The method of claim 27, wherein the polonies are approximately 1-2 microns in diameter.

29. The method of claim 28, wherein greater than about 1,000,000 polonies are analyzed on 1 mm2 array area.

30. The method of claim 28, wherein the polonies are analyzed using sequencing-by-synthesis or sequencing-by-ligation to identify barcode sequences and location coordinates.

31. A method of detecting a protein-protein interaction between two or more polypeptides comprising the steps of:

providing in an aqueous medium a plurality of polypeptides comprising a barcode under defined conditions to allow formation of protein-protein interactions;

stabilizing the protein-protein interactions by chemical crosslinking;

immobilizing the plurality of polypeptides on a substrate;

performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides; and

detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when a protein-protein interaction has occurred between two or more polypeptides.

32. The method of claim 31, wherein the polypeptides comprising barcodes are made according to the method of claim 1 or claim 15.

33. The method of claim 31, wherein defined conditions are selected from the group consisting of one or any combination of ligands, cofactors, buffers and temperature.

34. The method of claim 31, wherein co-localized barcodes are deconvoluted by DNA sequencing using the same or different sequencing primers.

35. The method of claim 31, wherein the degree of co-localization of polonies is quantitatively analyzed by co-localization statistics using polony colocalization ratios and pair cross-correlation function (PCCF).

36. The method of claim 30, wherein protein binding affinity can be quantitatively correlated with polony co-localization ratios.

37. The method of claim 30, wherein the polypeptide is selected from the group consisting of a natural polypeptide, a recombinant polypeptide, and a de novo synthesized polypeptide.

38. The method of claim 30, wherein at least about 1,000,000,000 polypeptides are immobilized on half the area of a standard microscopic slide.

39. The method of claim 38, wherein the microscopic slide is 25 × 75 mm2.

40. The method of claim 30, wherein a first library of at least 100,000 different polypeptides can be screened against a second other library of at least 100,000 different polypeptides or other barcoded molecules in a single assay.

41. The method of claim 30, wherein both molecular binding affinity and specificity can analyzed in a single assay.

42. A method of detecting an interaction between polypeptides and nucleic acid sequences comprising the steps of:

providing in an aqueous medium a plurality of polypeptides and nucleic acid sequences comprising a barcode under defined conditions to allow formation of polypeptides-nucleic acid interactions;

stabilizing polypeptide-nucleic acid sequence interactions by chemical crosslinking;

immobilizing polypeptides and nucleic acid sequences on a substrate;

performing in situ amplification of the barcodes bound to the immobilized polypeptides and nucleic acids; and

detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when polypeptide-nucleic acid sequence interactions have occurred between polypeptides and nucleic acid sequences.

43. A method of detecting an interaction between polypeptides and small molecules comprising the steps of:

providing in an aqueous medium a plurality of polypeptides and small molecules comprising a barcode under defined conditions to allow formation of polypeptide-small molecule interactions;

stabilizing polypeptide-small molecule interactions by chemical crosslinking;

immobilizing polypeptides and small molecules on a substrate;

performing in situ amplification of the barcodes bound to the immobilized polypeptides and small molecules; and

detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when polypeptide-small molecule interactions have occurred between polypeptides and small molecules.

44. A method of detecting binding activity changes of a plurality of polypeptides triggered by binding to an unlabeled ligand in solution comprising the steps of:

providing in an aqueous medium a plurality of polypeptides comprising a barcode;

providing in the aqueous medium a substrate comprising a barcode, wherein the substrate exhibits altered binding affinity to the polypeptides when bound by a ligand; and

quantifying the co-localization of barcodes determine protein and ligand interactions.

45. The method of claim 44, wherein the polypeptides comprising a barcode are made according to the method of claim 1 or claim 15.

46. The method of claim 44, wherein the barcoded substrate is a protein.

47. The method of claim 44, wherein the ligand increases or decreases binding affinity of a polypeptide to a substrate.

48. The method of claim 44, wherein a barcode is associated with a ligand assayed in a well.

49. The method of claim 44, wherein the ligand is an unlabelled small molecule or a protein.

50. The method of claim 49, wherein the protein is selected from the group consisting of an antibody, a nanobody, adnectin, an affibody and DARPin.

51. The method of claim 44, wherein upon polypeptide binding to a ligand, the polypeptide participates in a protein-protein interaction.

52. The method of claim 44, wherein a library of unlabelled ligands are assayed with a polypeptide library in multi-well plate for automatic high-through screening, and wherein, in each well, one ligand is profiled using a polypeptide library.

53. The method of claim 52, wherein both the polypeptide screening and ligand profiling are performed at the same time to minimize assay time.

54. The method of claim 44, wherein mixed proteins at approximately a zeptomole amount are analyzed in a picoliter reactor to minimize reagent costs.

55. A method of detecting binding affinity of a polypeptide to a compound comprising the steps of:

providing a plurality of polypeptides having a barcode bound thereto;

contacting the plurality of polypeptides with one or more test compounds;

performing in situ amplification of the barcodes bound to the plurality of polypeptides; and

detecting amplified barcode sequences, wherein co-localized amplified barcode sequences are detected when a polypeptide has bound to a compound, and wherein the number of co-localized

amplified barcode sequences relative to non-co-localized amplified barcode sequences correlates with binding affinity to the compound.

56. The method of claim 57, wherein the compound increases or decreases binding affinity of a polypeptide to a substrate.

57. The method of claim 57, wherein the compound modulates one or more activities of the polypeptide.

58. The method of claim 57, wherein the compound is a small molecule, an antibody or a polypeptide.

59. The method of claim 58, wherein upon polypeptide binding to a small molecule, the polypeptide participates in a protein-protein interaction.

60. A method of detecting binding affinity of a polypeptide to a compound comprising the steps of:

providing in an aqueous medium a plurality of polypeptides having a barcode bound thereto;

contacting the medium with one or more test compounds;

immobilizing the plurality of polypeptides on a substrate;

performing in situ amplification of the barcodes bound to the immobilized plurality of polypeptides; and

detecting amplified barcodes, wherein co-localized amplified barcodes are detected when a polypeptide has bound to a compound, and wherein the number of co-localized amplified barcodes relative to non-co-localized amplified barcodes correlates with binding affinity to the compound.

61. A method of screening for a test compound that modulates an activity of a polypeptide comprising the steps of:

providing a plurality of polypeptides having a barcode bound thereto;

contacting the plurality of polypeptides with one or more test compounds, wherein polypeptide binding to a test compound alters the ability of the polypeptide to participate in a protein-protein interaction;

performing in situ amplification of the barcode sequences bound to the plurality of polypeptides; and

detecting amplified barcode sequences, wherein altered co-localization of amplified barcode sequences in the presence of the test compound is observed when the test compound modulates an activity of the polypeptide.

62. The method of claim 61, wherein test compound binding to a polypeptide modulates the ability of the polypeptide to participate in a protein-protein interaction.

63. A method of screening for a test compound that modulates an activity of a polypeptide comprising the steps of:

providing in an aqueous medium a plurality of polypeptides having a barcode sequence bound thereto;

contacting the medium with one or more test compounds, wherein polypeptide binding to a test compound alters the ability of the polypeptide to participate in a protein-protein interaction;

immobilizing the plurality of polypeptides on a substrate;

performing in situ amplification of the barcode sequences bound to the immobilized plurality of polypeptides; and

detecting amplified barcode sequences, wherein altered co-localization of amplified barcode sequences in the presence of the test compound is observed when the test compound modulates an activity of the polypeptide.

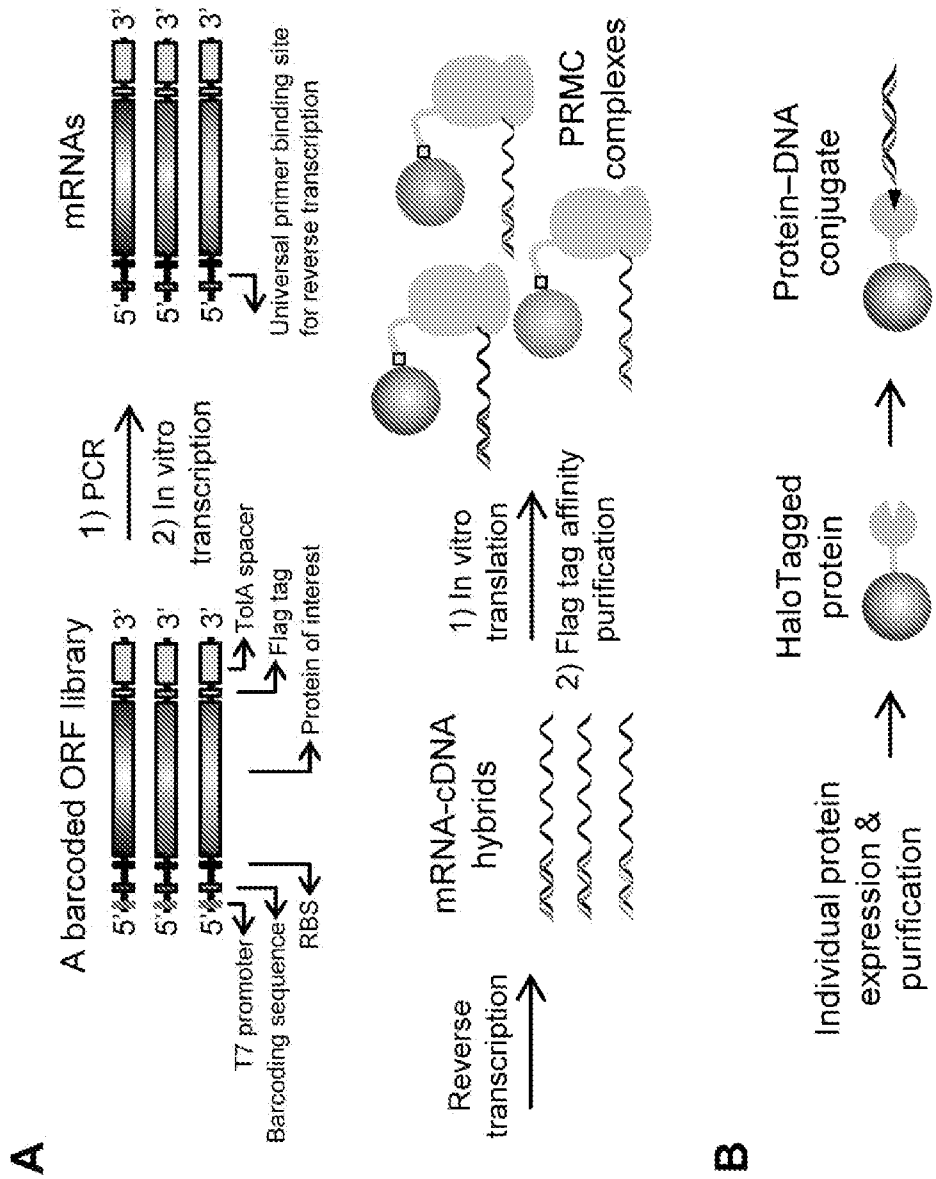

A
A barcoded ORF library
1) PCR
2) In vitro transcription
mRNAs
5'
3'
T7 promoter
Barcoding sequence
RBS
TolA spacer
Flag tag
Protein of interest
Universal primer binding site for reverse transcription
Reverse transcription
mRNA-cDNA hybrids
1) In vitro translation
2) Flag tag affinity purification
PRMC complexes
B
Individual protein expression & purification
HaloTagged protein
Protein–DNA conjugate

Figure 1

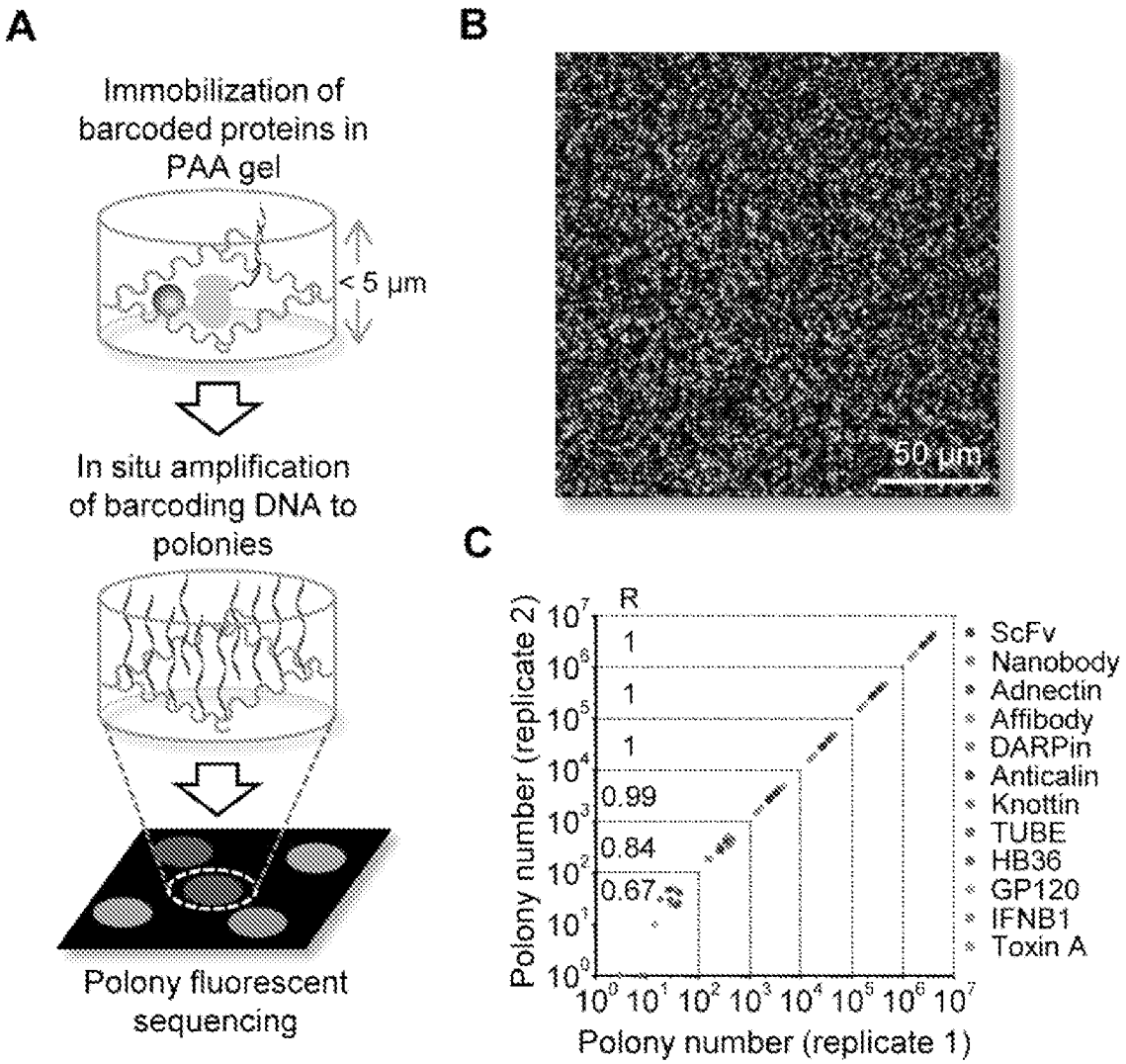

A
Immobilization of barcoded proteins in PAA gel
< 5 μm
In situ amplification of barcoding DNA to polonies
Polony fluorescent sequencing
B
50 μm
C
R
1
1
1
0.99
0.84
0.67
Polony number (replicate 2)
Polony number (replicate 1)
ScFv
Nanobody
Adnectin
Affibody
DARPin
Anticalin
Knottin
TUBE
HB36
GP120
IFNB1
Toxin A

Figure 2

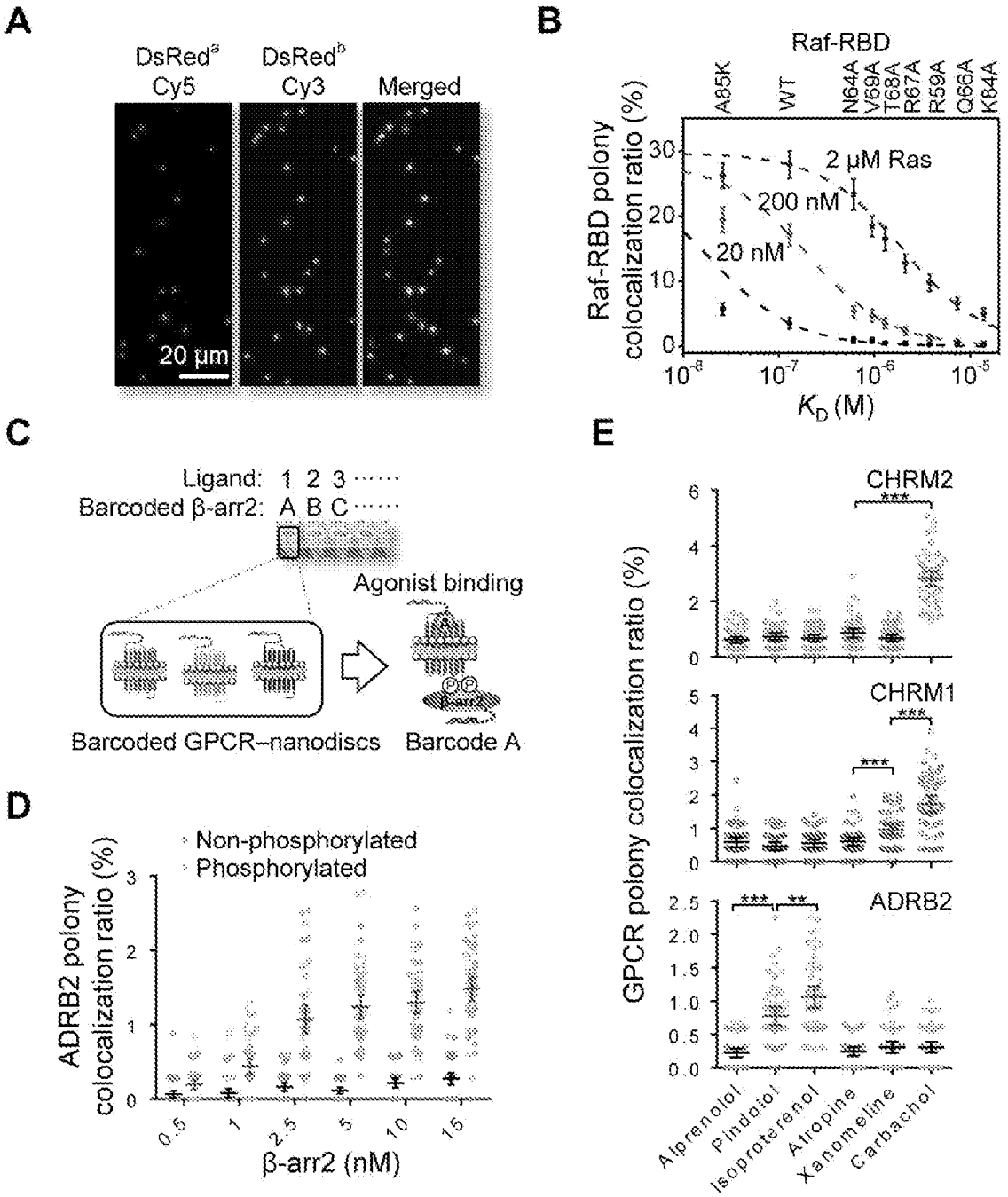

A
DsRed^a Cy5
DsRed^b Cy3
Merged
20 μm
B
Raf-RBD
A85K
WT
N64A
V69A
T68A
R67A
R59A
Q66A
K84A
Raf-RBD polony colocalization ratio (%)
2 μM Ras
200 nM
20 nM
K_D (M)
C
Ligand: 1 2 3 ·····
Barcoded β-arr2: A B C ·····
Agonist binding
Barcoded GPCR–nanodiscs
Barcode A
D
Non-phosphorylated
Phosphorylated
ADRB2 polony colocalization ratio (%)
β-arr2 (nM)
E
CHRM2
CHRM1
ADRB2
GPCR polony colocalization ratio (%)
Alprenolol
Pindolol
Isoproterenol
Atropine
Xanomeline
Carbachol

Figure 3

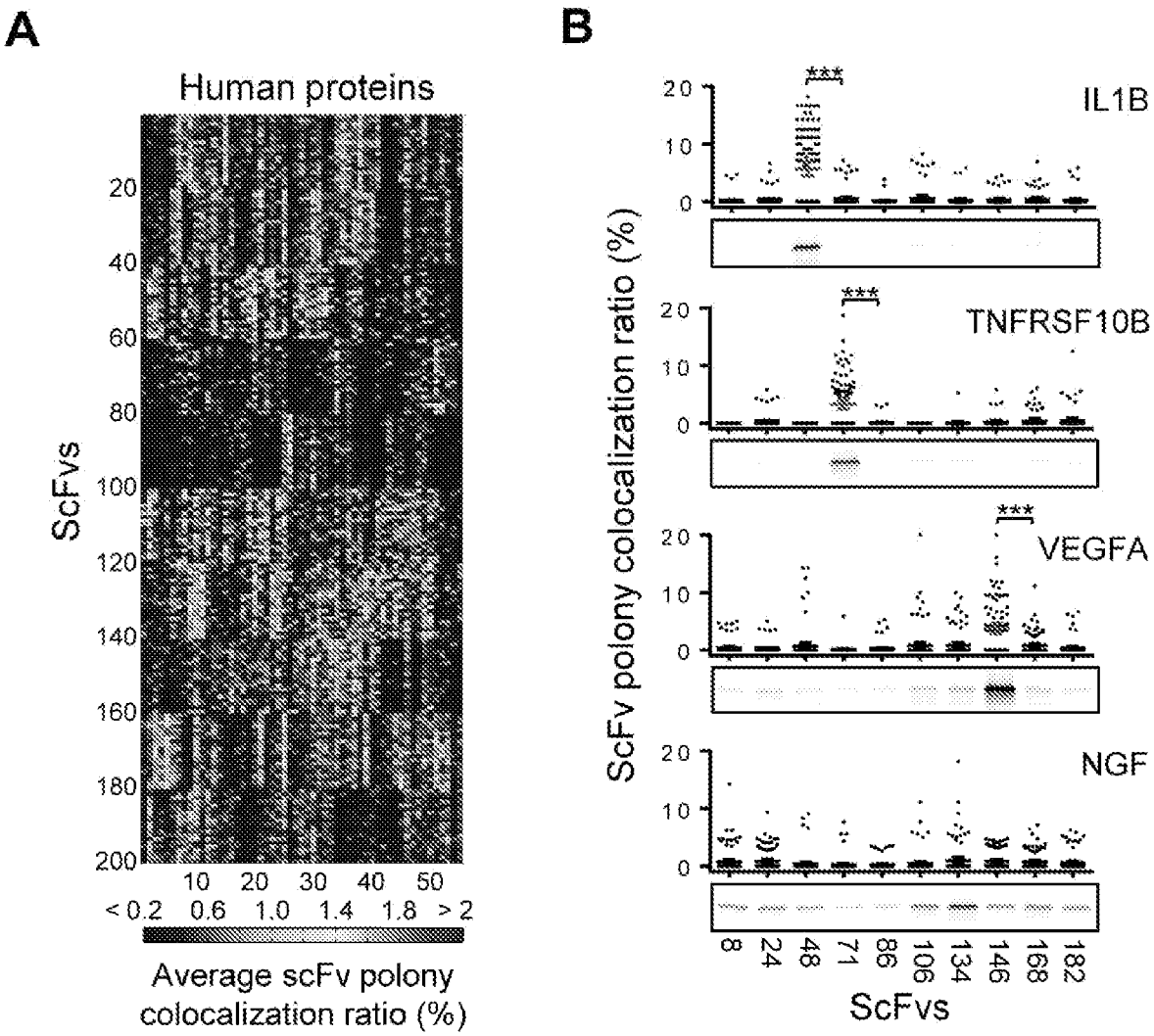
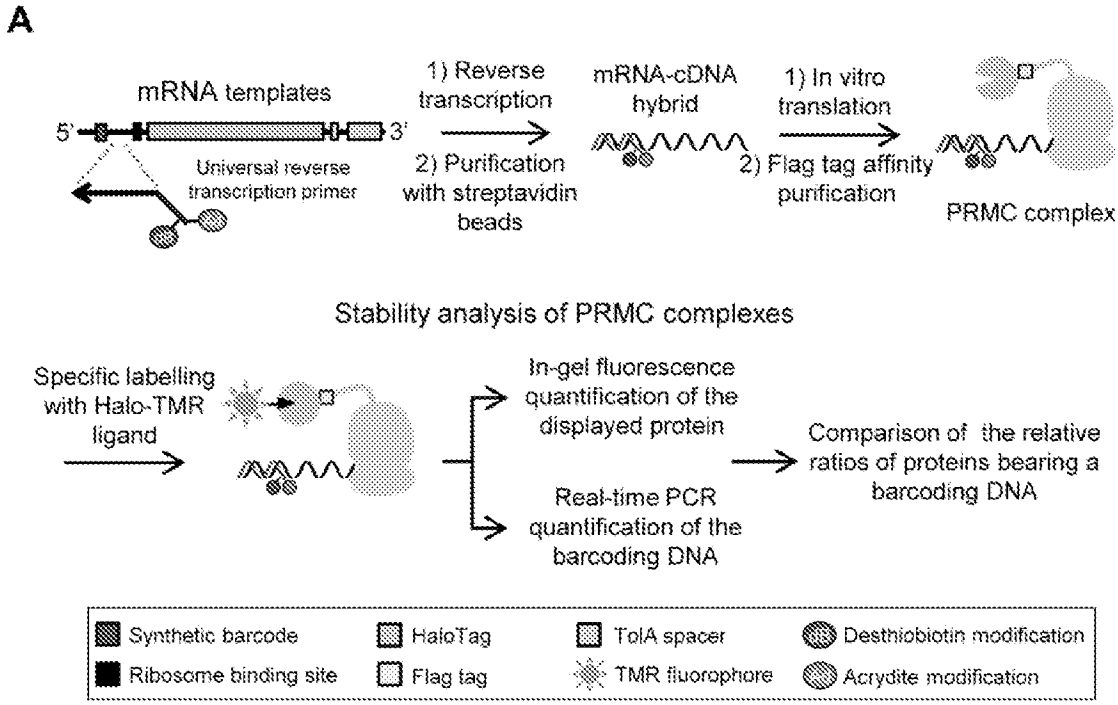

A
Human proteins
ScFvs
20
40
60
80
100
120
140
160
180
200
10 20 30 40 50
< 0.2 0.6 1.0 1.4 1.8 > 2
Average scFv polony colocalization ratio (%)
B
ScFv polony colocalization ratio (%)
IL1B
TNFRSF10B
VEGFA
NGF
***
20
10
0
8 24 48 71 86 106 134 146 168 182
ScFvs

Figure 4


A
mRNA templates
5'
3'
Universal reverse transcription primer
1) Reverse transcription
2) Purification with streptavidin beads
mRNA-cDNA hybrid
1) In vitro translation
2) Flag tag affinity purification
PRMC complex
Stability analysis of PRMC complexes
Specific labelling with Halo-TMR ligand
In-gel fluorescence quantification of the displayed protein
Real-time PCR quantification of the barcoding DNA
Comparison of the relative ratios of proteins bearing a barcoding DNA
Synthetic barcode
Ribosome binding site
HaloTag
Flag tag
TolA spacer
TMR fluorophore
Desthiobiotin modification
Acrydite modification

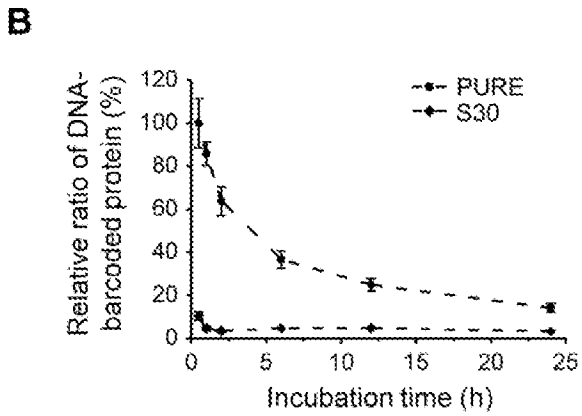

B
Relative ratio of DNA-barcoded protein (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
Incubation time (h)
PURE
S30

Figure 5

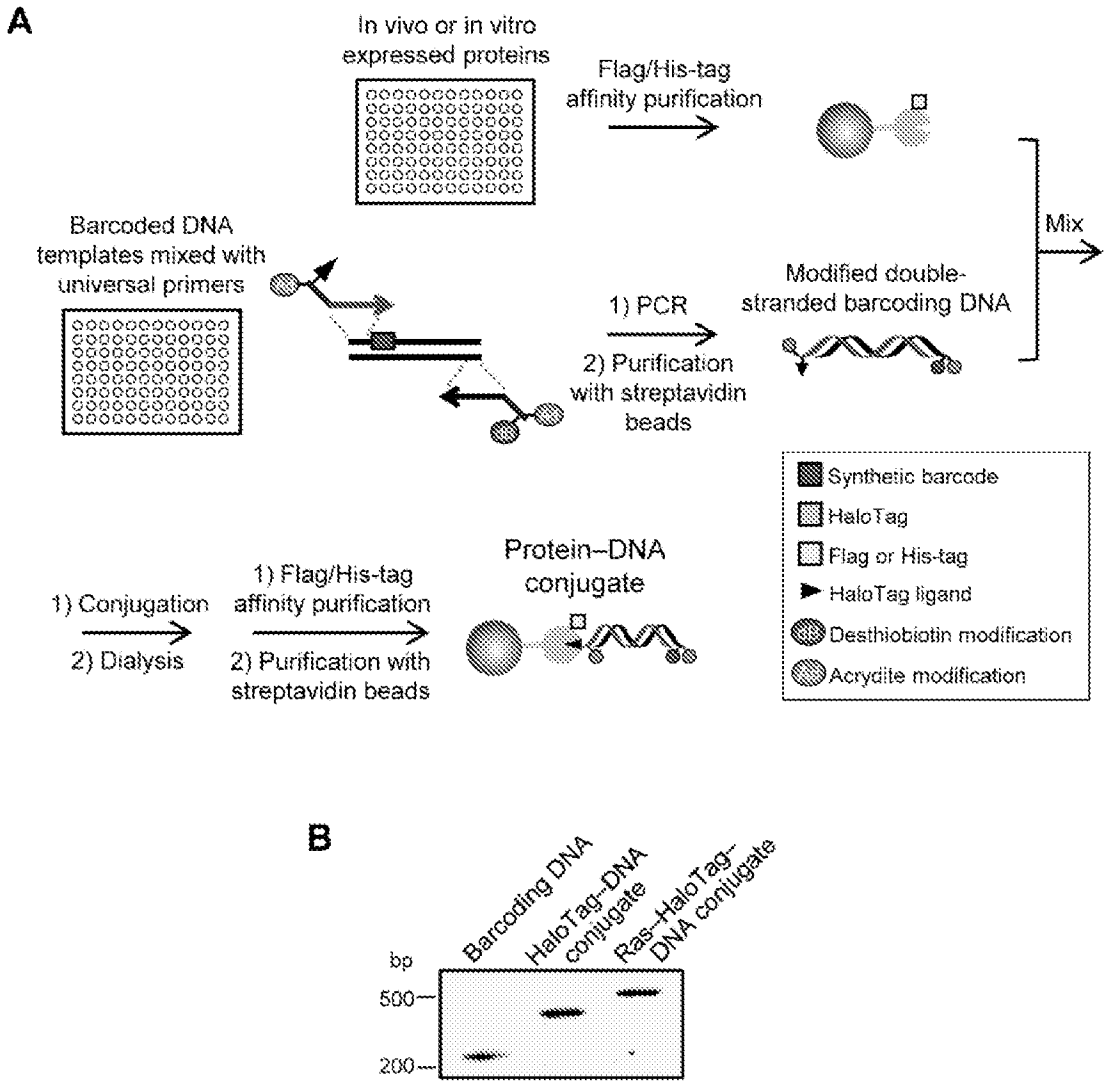

A
In vivo or in vitro expressed proteins
Flag/His-tag affinity purification
Barcoded DNA templates mixed with universal primers
1) PCR
2) Purification with streptavidin beads
Modified double-stranded barcoding DNA
Mix
Synthetic barcode
HaloTag
Flag or His-tag
HaloTag ligand
Desthiobiotin modification
Acrydite modification
1) Conjugation
2) Dialysis
1) Flag/His-tag affinity purification
2) Purification with streptavidin beads
Protein–DNA conjugate
B
Barcoding DNA
HaloTag–DNA conjugate
Ras–HaloTag–DNA conjugate
bp
500
200

Figure 6

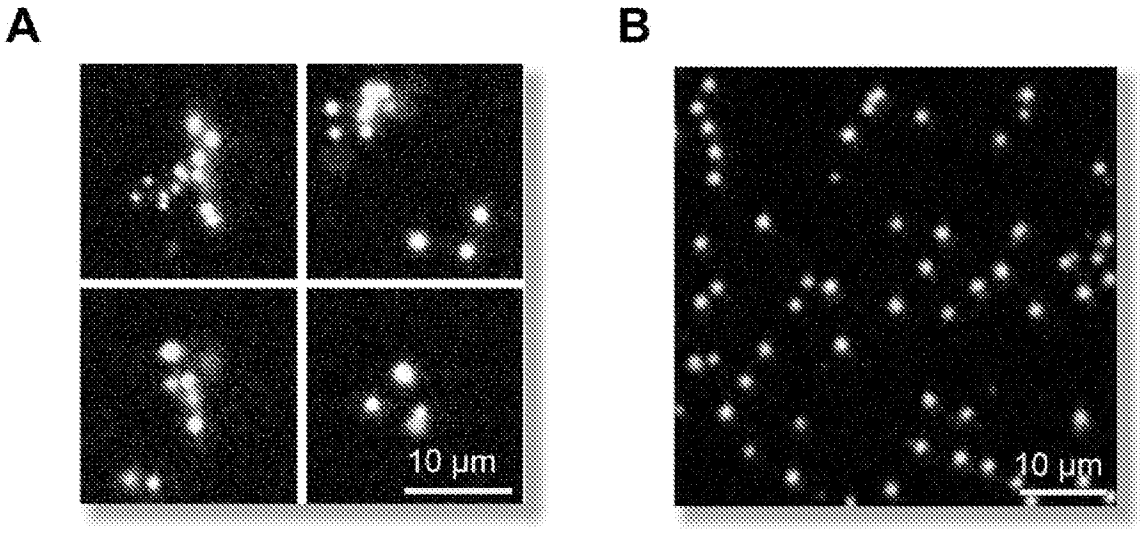


Figure 7

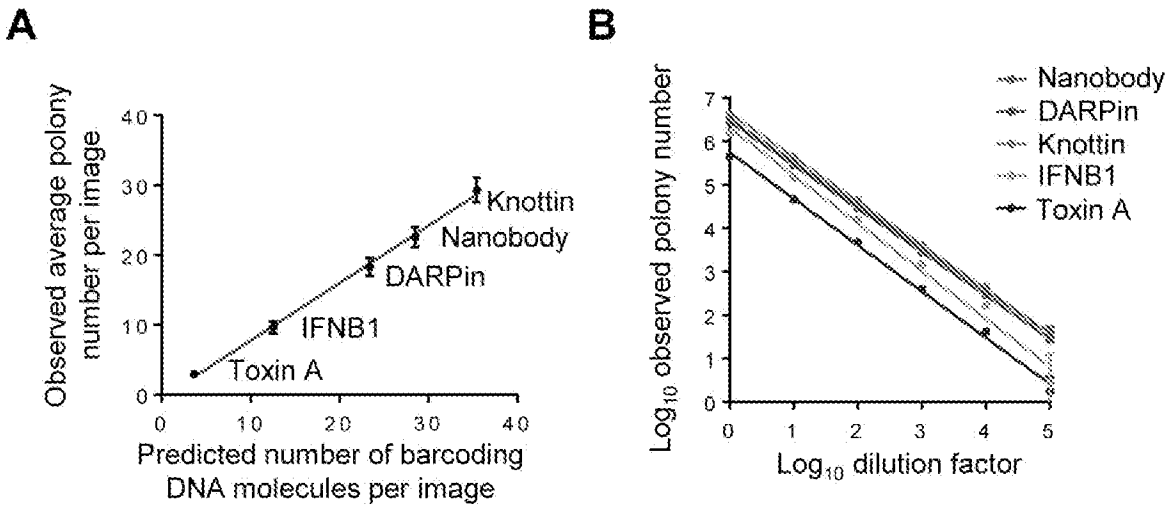

A
Observed average polony number per image
40
30
20
10
0
0
10
20
30
40
Predicted number of barcoding DNA molecules per image
Knottin
Nanobody
DARPin
IFNB1
Toxin A
B
Log10 observed polony number
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Log10 dilution factor
Nanobody
DARPin
Knottin
IFNB1
Toxin A

Figure 8

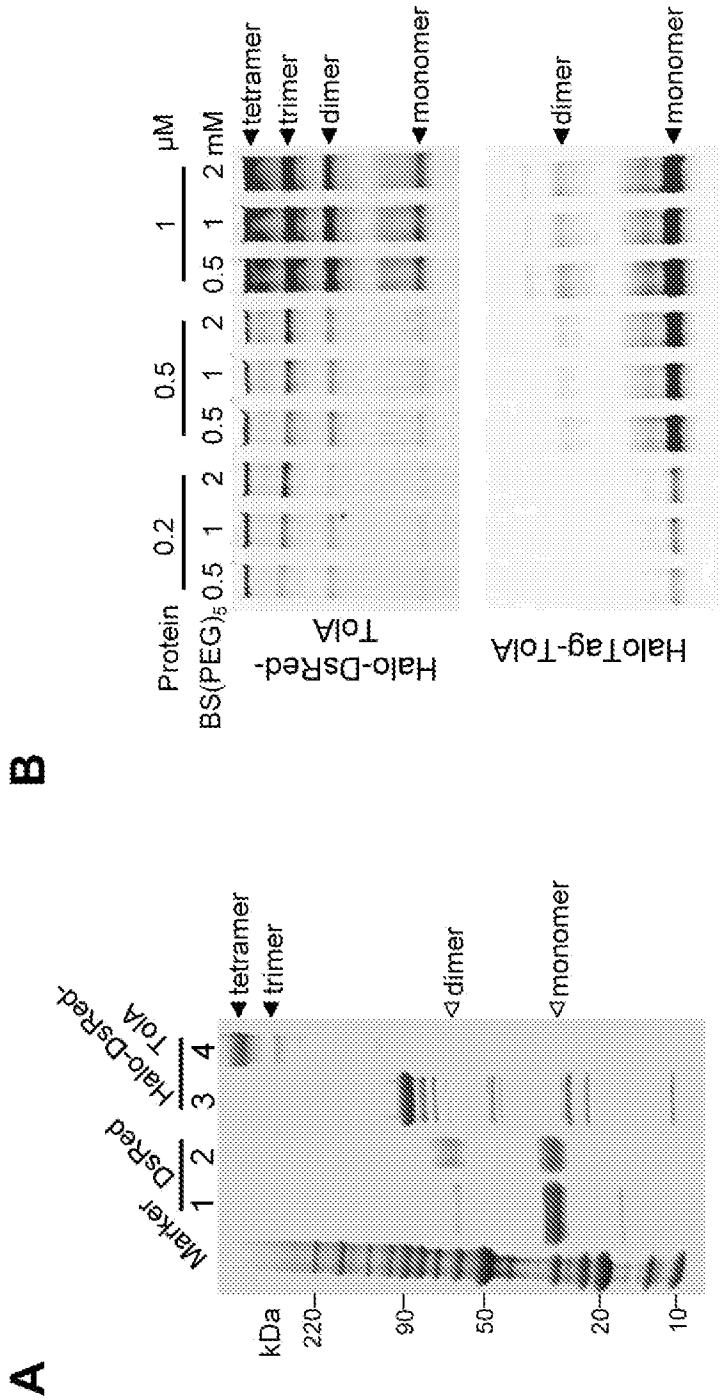

A
Marker
DSRed
Halo-DSRed-TolA
1 2 3 4
kDa
220
90
50
20
10
tetramer
trimer
dimer
monomer
B
Protein
BS(PEG)5
0.2
0.5
1
2
0.5
1
2
μM
mM
Halo-DSRed-TolA
HaloTag-TolA
tetramer
trimer
dimer
monomer
dimer
monomer

Figure 9

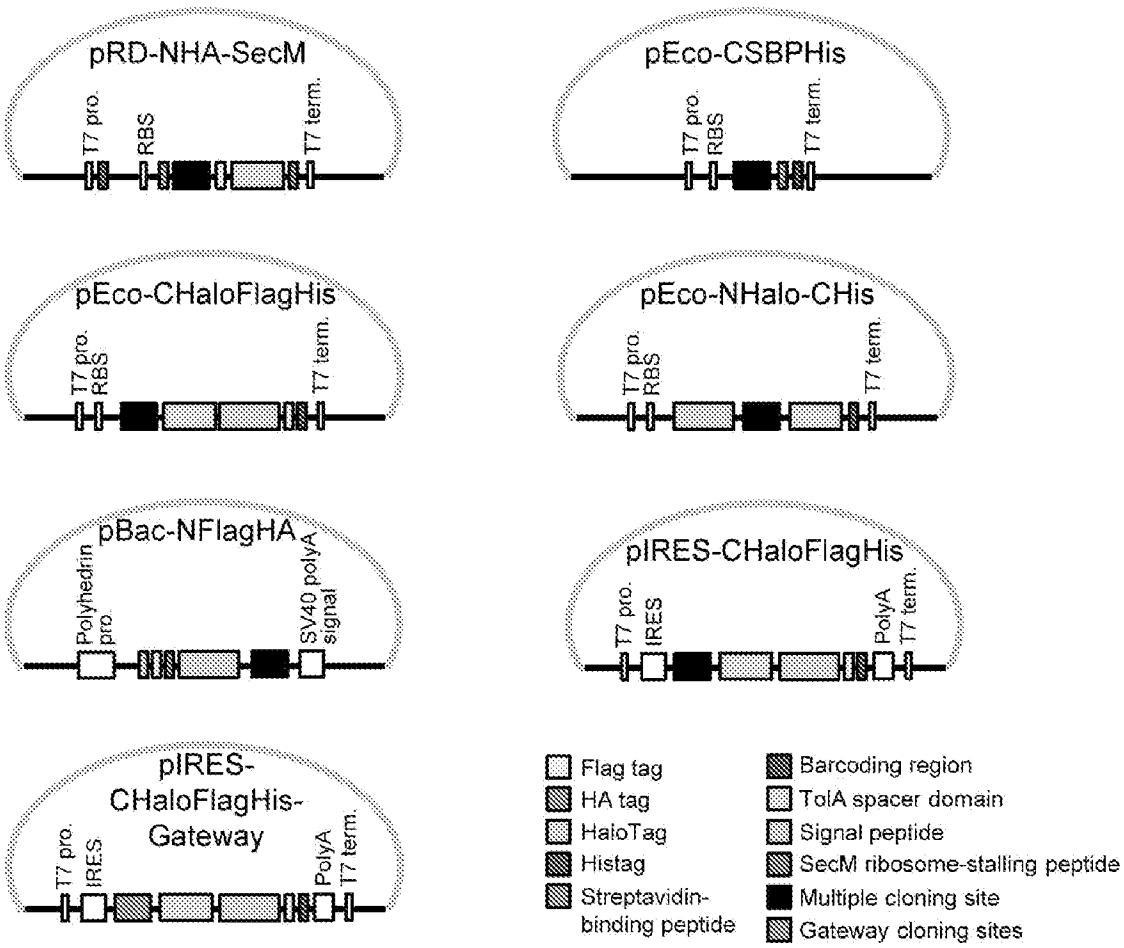

pRD-NHA-SecM
T7 pro.
RBS
T7 term.
pEco-CSBPHis
T7 pro.
RBS
T7 term.
pEco-CHaloFlagHis
T7 pro.
RBS
T7 term.
pEco-NHalo-CHis
T7 pro.
RBS
T7 term.
pBac-NFlagHA
Polyhedrin pro
SV40 polyA signal
pIRES-CHaloFlagHis
T7 pro.
IRES
PolyA
T7 term.
pIRES-CHaloFlagHis-Gateway
T7 pro.
IRES
PolyA
T7 term.
Flag tag
HA tag
HaloTag
Histag
Streptavidin-binding peptide
Barcoding region
TolA spacer domain
Signal peptide
SecM ribosome-stalling peptide
Multiple cloning site
Gateway cloning sites

Figure 10

Name	Details	Protein length (a. a.)	Observed polonies for different dilutions					
			10×	100×	1000×	10,000×	100,000×	1000,000×
Scfv	Single-chain variable fragment (Oportuzumab)	254	3,457,566 3,783,673	354,491 384,958	37,034 40,638	3,343 3,595	342 415	43 41
Nanobody	Single-domain antibody (Caplacizumab)	260	3,139,771 3,419,354	319,974 349,061	33,354 36,785	2,932 3,257	394 345	31 49
Adnectin	Engineered the tenth fibronectin type III domain, CT-322	103	3,386,196 3,722,036	346,682 379,665	36,263 39,761	3,238 3,745	378 391	28 30
Affibody	Engineered Z domain of protein A, ABY-025	59	3,904,925 4,307,520	398,140 440,476	41,749 45,790	3,773 4,335	373 509	41 25
DARPin	Engineered ankyrin repeat proteins, MP0112	136	2,632,662 2,893,576	267,989 295,016	28,038 30,697	2,563 2,990	265 327	30 25
Anticalin	Engineered lipocalin, PRS-050	155	2,579,188 2,830,314	263,339 289,320	28,315 30,149	2,520 2,704	279 269	26 46
Knottin	Engineered cystine-knot miniprotein, 2.5D	34	3,990,730 4,407,769	406,311 448,907	42,426 47,177	3,885 4,174	468 452	41 26
TUBEs	Tandem ubiquitin-binding entities, ubiquilin-1	252	2,311,215 2,525,796	235,068 258,409	24,739 27,117	2,339 2,429	288 286	18 31
HB36	Computationally designed hemagglutinin binding protein	93	2,391,775 2,622,536	242,272 267,086	25,598 28,051	2,322 2,662	255 275	37 23
GP120	The outer domain of HIV envelope glycoprotein GP120	192	1,693,019 1,854,037	172,836 188,939	18,255 19,322	1,671 1,878	169 182	14 10
IFNB1	Human interferon beta 1	166	1,396,290 1,527,030	142,750 155,242	15,082 16,298	1,390 1,492	151 189	8 1
Toxin A	*Clostridium difficile* toxin A receptor-binding domain	1048	441,523 461,514	45,073 47,540	4,717 4,784	439 364	37 46	3 1

Figure 11

ScFv #	Original mAb	Construction method	Binding target
1-20	Anrukinzumab	V_H-linker-V_L	IL13
21-40	Ustekinumab	V_H-linker-V_L	IL12B
41-60	Canakinumab	V_H-linker-V_L	IL1B
61-80	Conatumumab	V_H-linker-V_L	TNFRSF10B
81-100	Ibalizumab	V_H-linker-V_L	CD4
101-120	Oportuzumab	V_H-linker-V_L	CD326
121-140	Otelixizumab	V_H-linker-V_L	CD3E
141-160	Ranibizumab	V_H-linker-V_L	VEGFA
161-180	Siltuximab	V_H-linker-V_L	IL6
181-200	Tanezumab	V_H-linker-V_L	NGF

Human protein #	Entrez Gene symbol	Entrez Gene ID	Protein length (a.a.)
1	IL1B	3553	270
2	IL2	3558	154
3	IL3	3562	153
4	IL4	3565	154
5	IL6	3569	212
6	IL7	3574	178
7	IL8	3576	100
8	IL9	3578	145
9	IL10	3586	179
10	IL12B	3593	328
11	IL13	3596	147
12	IL15	3600	163
13	IL17A	3605	156
14	IL17B	27190	181
15	IL17F	112744	164
16	IL18	3606	194
17	IL20	50604	177
18	IL25	64806	178
19	IL26	55801	172
20	IL28A	282616	201
21	IL29	282618	201

Human protein #	Entrez Gene symbol	Entrez Gene ID	Protein length (a.a.)
22	IL31	386653	165
23	CD1A	909	312
24	CD1E	913	334
25	CD3E	916	208
26	CD4	920	459
27	CD7	924	241
28	CD27	939	261
29	CD38	952	301
30	CD40	958	278
31	CD52	1043	62
32	CD58	965	241
33	CD59	966	129
34	CD79B	974	230
35	CD74	972	233
36	CD80	941	289
37	CD207	50489	329
38	CD247	919	165
39	CD274	29126	291
40	CD302	9936	171
41	CD320	51293	283
42	CD326	4072	314
43	CNTF	1270	201
44	CSF2	1437	145
45	CSF3	1440	201
46	NGF	4803	241
47	NODAL	4838	348
48	OSM	5008	253
49	THPO	7066	350
50	VEGFA	7422	237
51	FASLG	356	282
52	LTA	4049	206
53	TNF	7124	234
54	TNFRSF10B	8795	440
55	TNFRSF13B	23495	248

Figure 12

Raf-RBD	Ras (nM)	Total Raf-RBD polonies	Total Ras polonies	Colocalized Raf-RBD polonies	Mean of colocalization ratio (%)	Lower 95% CI of mean*	Upper 95% CI of mean*
WT	**20**	2489	3276	89	3.532	2.696	4.368
R59A		2859		9	0.292	0.087	0.496
N64A		2277		21	0.920	0.478	1.361
Q66A		2504		7	0.281	0.073	0.489
R67A		2239		10	0.443	0.132	0.754
T68A		1885		10	0.463	0.183	0.744
V69A		2835		23	0.860	0.480	1.240
K84A		2582		7	0.246	0.066	0.427
A85K		2600		148	5.687	4.778	6.596
WT	**200**	2721	4556	465	17.080	15.420	18.750
R59A		2795		39	1.399	0.976	1.821
N64A		2849		150	5.316	4.490	6.141
Q66A		3092		24	0.759	0.411	1.107
R67A		2420		58	2.352	1.704	3.000
T68A		1935		69	3.565	2.743	4.388
V69A		2356		111	4.693	3.782	5.605
K84A		2020		9	0.414	0.091	0.737
A85K		2614		509	19.440	17.530	21.350
WT	**2000**	2211	6408	610	27.900	25.770	30.040
R59A		2536		245	9.732	8.457	11.010
N64A		1915		441	23.310	20.970	25.640
Q66A		2677		176	6.558	5.658	7.458
R67A		2112		274	12.730	11.310	14.150
T68A		2296		377	16.490	14.730	18.250
V69A		2525		475	18.440	16.860	20.030
K84A		2566		127	4.900	3.980	5.819
A85K		2564		673	26.180	24.260	28.100

Note:
* N=100

Figure 13

A

Assay conditions	β-arr2 (nM)	Total β-arr2 polonies	Total ADRB2 polonies	Colocalized ADRB2 polonies	Mean of colocalization ratio (%)	Lower 95% CI of mean*	Upper 95% CI of mean*
GRK2 phosphorylated	**0.5**	390	16841	33	0.195	0.131	0.259
	1	648		73	0.439	0.333	0.545
	2.5	1186		179	1.071	0.884	1.257
	5	1306		207	1.238	1.080	1.396
	10	1425		219	1.298	1.149	1.446
	15	1467		248	1.485	1.320	1.649
Non-phosphorylated	**0.5**	574	17386	11	0.063	0.018	0.108
	1	733		14	0.079	0.023	0.136
	2.5	1259		28	0.160	0.106	0.214
	5	1583		20	0.113	0.07	0.156
	10	1521		37	0.214	0.155	0.274
	15	1446		47	0.273	0.192	0.353

Figure 14

B

Ligands	GPCR	Total β-arr2 polonies	Total GPCR polonies	Colocalized GPCR polonies	Mean of colocalization ratio (%)	Lower 95% CI of mean*	Upper 95% CI of mean*
alprenolol	ADRB2	1356	16783	37	0.221	0.152	0.289
pindolol		1626		131	0.778	0.642	0.914
isoproterenol		1513		178	1.060	0.898	1.222
atropine		1778		41	0.248	0.181	0.315
xanomeline		1787		51	0.307	0.219	0.395
carbachol		1530		51	0.305	0.224	0.385
alprenolol	CHRM1	1356	13077	76	0.590	0.446	0.734
pindolol		1626		61	0.465	0.349	0.581
isoproterenol		1513		72	0.552	0.437	0.667
atropine		1778		79	0.604	0.487	0.722
xanomeline		1787		128	0.974	0.819	1.129
carbachol		1530		225	1.729	1.477	1.981
alprenolol	CHRM2	1356	16291	99	0.614	0.500	0.728
pindolol		1626		117	0.723	0.602	0.844
isoproterenol		1513		108	0.670	0.551	0.790
atropine		1778		137	0.849	0.691	1.008
xanomeline		1787		110	0.675	0.559	0.791
carbachol		1530		455	2.804	2.543	3.065

Note:

* N=50

Figure 14 (Cont.)

Expression vector	Protein name	DNA source	Construction method
pRD-NHA-SecM	Scfv	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Nanobody	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Adnectin	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Affibody	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	DARPin	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Anticalin	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Knottin	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	TUBEs	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	HB36	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	GP120	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	IFNB1	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	Toxin A	Clostridium difficile gDNA (ATCC)	NdeI/HindIII
pRD-NHA-SecM	DsRed	pDsRed2 (Clontech)	NdeI/SacI
pEco-NHalo-Chis	DsRed	pDsRed2 (Clontech)	NdeI/SacI
pEco-CHaloFlagHis	Ras	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_R59A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_N64A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_Q66A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_R67A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_T68A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_V69A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_K84A	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	Raf-RBD_A85K	Gene synthesis	NdeI/SacI
pBac-NFlagHA	ADRB2	Gene synthesis	NcoI/EcoRI
pBac-NFlagHA	CHRM1	Gene synthesis	NcoI/EcoRI
pBac-NFlagHA	CHRM2	Gene synthesis	NcoI/EcoRI
pEco-CHaloFlagHis	ARRB2	Gene synthesis	NdeI/HindIII
pRD-NHA-SecM	ScFv_Anrukinzumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Ustekinumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Canakinumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Conatumumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Ibalizumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Oportuzumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Otelixizumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Ranibizumab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Siltuximab	Gene synthesis	NdeI/SacI
pRD-NHA-SecM	ScFv_Tanezumab	Gene synthesis	NdeI/SacI
pEco-CSBPHis	ScFvs & mutants	Synthetic & random	NdeI/SacI

		mutagenesis	
pIRES-CHaloFlagHis	IL1B	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	IL2	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL3	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL4	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	IL6	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	IL7	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL8	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL9	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL10	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	IL12B	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis	IL13	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	IL15	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL17A	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL17B	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL17F	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL18	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL20	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL25	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	IL26	hORFeome v8.1	Gateway
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pIRES-CHaloFlagHis-Gateway	CD1A	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD1E	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	CD3E	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis	CD4	Gene synthesis	NdeI/XhoI

pIRES-CHaloFlagHis-Gateway	CD7	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD27	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD38	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD40	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD52	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD58	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD59	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD79B	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD74	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD80	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD207	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD247	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD274	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD302	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CD320	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	CD326	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	CNTF	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CSF2	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	CSF3	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	NGF	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	NODAL	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	OSM	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	THPO	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	VEGFA	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	FASLG	hORFeome v8.1	Gateway

pIRES-CHaloFlagHis-Gateway	LTA	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis-Gateway	TNF	hORFeome v8.1	Gateway
pIRES-CHaloFlagHis	TNFRSF10B	Gene synthesis	NdeI/XhoI
pIRES-CHaloFlagHis-Gateway	TNFRSF13B	hORFeome v8.1	Gateway

SEQUENCES OF EXPRESSION VECTORS:

pRD-NHA-SecM:

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AGAGAATGAGGAACCCGGGAACAATGATGGAATCCCTAGGTTGGCTTACCAAAAATGAAGCTGATGAA
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ATAGAATCCTATTTACTAGGAAAGGAAAAGCCTCCTATTTATAAAAATTGGATAGAGCTTTCTCAACAAC
AGTGGAATATCAATGATAGAACAATTGCCGATTTATTAGATGGGGTCTTAATAATACCATCGTATGCCGT
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TCAAGCTTGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGCGGCGATTATAAAGATGATGATGATAA
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GGTCTTGAGGGGTTTTTTGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTGATCCGGCTGCTAAC
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ACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTGCGTTTTC
CCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTAC
GTGTTCCGCTTCCTTTAGCAGCCCTTGCGCCCTGAGTGCTTGCGGCAGCGTGAGCTTCAAAAGAATTGCC
AGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAG
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ATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCA
TCGAGCGAGCACGCACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAG
GGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGTATGCCGGATGGTGAGGATCTCGTCGT
GACTCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGT
GGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCT
TGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGC
CTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGC

Figure 16

CCAACCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAA
AGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTC
CATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACA
GGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGC
TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTAT
CTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC
CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA
ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA
GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
AGATTACGCGCAGAAAAAAAGGATTTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT
GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
ATAGTCCGGAAATACAGGAACGCACGCTGGATGGCCCTTCGCTGGGATGGTGAAACCATGAAAAATGG
CAGCTTCAGTGGATTAAGTGGGGGTAATGTGGCCTGTACCCTCTGGTTGCATAGGTATTCATACGGTTA
AAATTTATCAGGCGCGATTGCGGCAGTTTTTCGGGTGGTTTGTTGCCATTTTTACCTGTCTGCTGCCGTG
ATCGCGCTGAACGCGTTTTAGCGGTGCGTACAATTAAGGGATTATGGTAAATCCACTTACTGTCTGCCCT
CGTAGCCATCGAGATAAACCGCAGTACTCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTTTTC
AGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAG
CGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGG
GGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTG
GGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGAT
TTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCA
AATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTGTTTATTTTTCTAA
ATACATTCAAATATGTATCCGCTCAT (SEQ ID NO:1)

Note: A pFN18K (Promega) derivative containing a kanamycin resistance gene for ribosome display.

pEco-CSBP

AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA
GAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAATTCTTAATTAAGCTGCAGGAGCTCGT
CGACGCGGCCGCACTCGAGAAGCTTGGCGGCAGCGGCATGGACGAGAAGACCACCGGCTGGCGGGGC
GGCCACGTGGTGGAGGGCCTGGCCGGCGAGCTGGAGCAGCTGCGGGCCAGGCTGGAGCACCACCCTC
AGGGCCAGCGGGAGCCCGGCGGCAGCGGCCACCACCACCACCACCACTGAGCTGAGCAATAACTAGCA
TAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTG
GCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGAC
CGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGG
CTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACC
CCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTT
GACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCG
GTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACA
AAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTCAGGTGGCACTTTTCGGGGAAATGTG

Figure 16

CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAATTAATTCTTAGAA
AAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAA
GCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGT
CTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGT
GAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACT
TGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGA
TTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCA
ACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTG
GAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTT
GATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCA
ACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCG
CACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAAT
CGCGGCCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAG
CAGACAGTTTTATTGTTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGT
AGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAA
CCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCT
TCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTC
TGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG
TGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGG
TTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATG
AGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACA
GGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCA
CCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAA
CGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGA
TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCG
CAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCG
GTATTTCACACCGCATATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
ACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGC
CCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGT
GTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGT
GAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGT
CTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAA
GGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTAC
TGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGG
ACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTA
GCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGAC
TTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGT
CGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCG
GGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCT
TCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAAT

Figure 16

ACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAG
CGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCAT
GCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTG
CCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTC
GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTG
GTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCA
GCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATAT
AACATGAGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTC
GGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGC
CCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATC
GGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTT
AATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTA
CCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGA
ACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCA
CTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCG
ACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGT
GCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACG
CGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGC
TGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACG
TTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTT
TTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCC
AGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCA
ACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGC
GAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGA
TGCCGGCCACGATGCGTCCGGCGTAGAGGATCG (SEQ ID NO:2)

Note: A pET24b (Novagen) derivative containing a kanamycin resistance gene for E. coli expression of fusion proteins bearing a C-terminal SBP tag and His-tag.

pEco-CHaloFlagHis

AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA
GAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAATTCTTAATTAAGCTGCAGGAGCTCGT
CGACGCGGCCGCACTCGAGAAGCTTGGCGGCAGCGGCCAGAAGCAAGCTGAAGAGGCGGCAGCGAAA
GCGGCGGCAGATGCTAAAGCGAAGGCCGAAGCAGATGCTAAAGCTGCGGAAGAAGCAGCGAAGAAA
GCGGCTGCCGACGCAAAGAAAAAAGCAGAAGCAGAAGCCGCCAAAGCCGCAGCCGAAGCGCAGAAAA
AAGCCGAGGCAGCCGCTGCGGCACTGAAGAAGAAAGCGGAAGCGGCAGAAGCAGCTGCCGCTGAAGC
AAGAAAGAAAGCGGCAACTGAAGGCGGCAGCGGCATGGCTGAGATCGGCACCGGTTTCCCCTTCGACC
CCCACTACGTGGAGGTGCTGGGTGAGCGTATGCACTACGTGGACGTGGGTCCCCGTGACGGCACCCCC
GTGCTGTTCCTGCACGGTAACCCCACCTCCTCCTACGTGTGGCGTAACATCATCCCCCACGTGGCTCCCAC
CCACCGTTGCATCGCTCCCGACCTGATCGGTATGGGTAAGTCCGACAAGCCCGACCTGGGTTACTTCTTC
GACGACCACGTGCGTTTCATGGACGCTTTCATCGAGGCTCTGGGTCTGGAGGAGGTGGTGCTGGTGATC
CACGACTGGGGTTCCGCTCTGGGTTTCCACTGGGCTAAGCGTAACCCCGAGCGTGTGAAGGGTATCGCT

Figure 16

TTCATGGAGTTCATCCGTCCCATCCCCACCTGGGACGAGTGGCCCGAGTTCGCTCGTGAGACCTTCCAGG
CTTTCCGTACCACCGACGTGGGTCGTAAGCTGATCATCGACCAGAACGTGTTCATCGAGGGCACCCTGC
CTATGGGTGTGGTGCGTCCCCTGACCGAGGTGGAGATGGACCACTACCGTGAGCCCTTCCTGAACCCCG
TGGACCGTGAGCCCCTGTGGCGTTTCCCCAACGAGCTGCCCATCGCTGGTGAGCCCGCTAACATCGTGG
CTCTGGTGGAGGAGTACATGGACTGGCTGCACCAGTCCCCCGTGCCCAAGCTGCTGTTCTGGGGCACCC
CCGGTGTGCTGATCCCCCCCGCTGAGGCTGCTCGTCTGGCTAAGTCCCTGCCCAACTGCAAGGCTGTGG
ACATCGGTCCCGGTCTGAACCTGCTCCAGGAGGACAACCCCGACCTGATCGGTTCCGAGATCGCTCGTT
GGCTGTCCACCCTGGAGATTTCCGGTGGTGGTTCCGGTGATTATAAAGATGATGATGATAAAGGCGGCG
CGGGCCACCACCACCACCACCACTGAGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGG
TCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCG
GCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCG
CCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGG
GGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATG
GTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAA
TAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGA
TTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAA
AATATTAACGTTTACAATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTT
TCTAAATACATTCAAATATGTATCCGCTCATGAATTAATTCTTAGAAAAACTCATCGAGCATCAAATGAAA
CTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAA
AACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACA
TCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGA
CTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACG
CTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAAT
ACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAG
CGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCG
CAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAAT
TCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAG
AAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCG
CGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTT
CCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGAC
CAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCT
TGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTT
GTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTC
GCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAA
GACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTG
GAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGA
AGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCT
TCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTT
TTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTG

Figure 16

GCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACC
GCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGA
AGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGT
GCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGAC
TGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCG
GCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCAC
CGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCC
TGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCA
TGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGT
AATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACT
GGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGGT
CAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAG
ATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAAG
ACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCG
GTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGA
TCATGCGCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGG
GACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATC
GTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTACGAGT
TGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCT
GACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATT
AATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGG
GCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCC
CCAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTATCGT
CGTATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCA
GCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTG
TTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGA
TATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGGCCCGCTAACAGCGCGATT
TGCTGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATA
CTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACA
GCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTG
TGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTT
GATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCA
ACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCC
GCCATCGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAA
CGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCT
GAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGG
ATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAG
CACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTG
CCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGA

Figure 16

TGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCG
TAGAGGATCG (SEQ ID NO:3)

Note: A pET24b derivative containing a kanamycin resistance gene for E. coli expression of fusion proteins bearing a C-terminal TolA spacer, HaloTag, Flag and His-tag.

pEco-NHalo-CHis

GGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCGAATTGTGAGC
GGATAACAATAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATATCGATGGCAGAAATCGGTACTGG
CTTTCCATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTCGATGTTGGTCCGCGC
GATGGCACCCCTGTGCTGTTCCTGCACGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGC
ATGTTGCACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAATCCGACAAACCAGACCT
GGGTTATTTCTTCGACGACCACGTCCGCTTCATGGATGCCTTCATCGAAGCCCTGGGTCTGGAAGAGGTC
GTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTTCCACTGGGCCAAGCGCAATCCAGAGCGCGTC
AAAGGTATTGCATTTATGGAGTTCATCCGCCCTATCCCGACCTGGGACGAATGGCCAGAATTTGCCCGC
GAGACCTTCCAGGCCTTCCGCACCACCGACGTCGGCCGCAAGCTGATCATCGATCAGAACGTTTTTATCG
AGGGTACGCTGCCGATGGGTGTCGTCCGCCCGCTGACTGAAGTCGAGATGGACCATTACCGCGAGCCG
TTCCTGAATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGCCA
GCGAACATCGTCGCGCTGGTCGAAGAATACATGGACTGGCTGCACCAGTCCCCTGTCCCGAAGCTGCTG
TTCTGGGGCACCCCAGGCGTTCTGATCCCACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGCCTAAC
TGCAAGGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGATCGGCAG
CGAGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGCGAGCCAACCACTGAGGATCTGTACTTTCA
GAGCGATAACGCGATCGCCATGGAACATATGGCTAGCGAATTCGAGCTCAAGCTTCAGAAGCAAGCTG
AAGAGGCGGCAGCGAAAGCGGCGGCAGATGCTAAAGCGAAGGCCGAAGCAGATGCTAAAGCTGCGG
AAGAAGCAGCGAAGAAAGCGGCTGCAGACGCAAAGAAAAAAGCAGAAGCAGAAGCCGCCAAAGCCGC
AGCCGAAGCGCAGAAAAAAGCCGAGGCAGCCGCTGCGGCACTGAAGAAGAAAGCGGAAGCGGCAGA
AGCAGCTGCAGCTGAAGCAAGAAAGAAAGCGGCAACTGAAGCTGCTGAAAAAGCCAAAGCAGAAGCT
GAGAAGAAAGCGGCTGCTGAAAAGGCTGCAGCTGATAAGAAAGCGGCAGCAGAGAAAGCTGCAGCCG
ACAAAAAAGCAGCAGAAAAAGCGGCTGCTGAAAAGGCAGCAGCTGATAAGAAAGCAGCGGCAGAAAA
AGCCGCCGCAGACAAAAAAGCGGCAGCGGCAAAAGCTGCAGCTGAAAAAGCCGCTGCAGCAAAAGCG
GCCGCAGAGGCAGATGATATTTTCGGTGAGCTACACCACCACCACCACCACTGAGGATCCTCTAGAGTC
GACCTGCAGGCATGCAAGCTGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCC
ACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAA
GGAGGAACTATATCCGGTTCGCTTGCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCAC
TGCAAGCTACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATC
CGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGTTCCGCTTCCTTTAGCAGCCCTTGCGCCCTG
AGTGCTTGCGGCAGCGTGAGCTTCAAAAGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGC
CCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATC
AAGAGACAGGATGACGGTCGTTTCGCATGCTTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTT
GGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCC
GGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGC
AGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTT

Figure 16

GTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCA
CCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCT
ACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGCACTCGGATGGAAGCCGGTCTT
GTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAA
GGCGCGTATGCCGGATGGTGAGGATCTCGTCGTGACTCATGGCGATGCCTGCTTGCCGAATATCATGGT
GGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACAT
AGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTA
CGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGA
CTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCGGTATCAGCTCACTCAAAGGCGGTAATACG
GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGG
AACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATC
GACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC
TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAG
CGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGC
TGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACC
CGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTA
GGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATC
TGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACC
GCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATTTCAAGAAGAT
CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGA
GATTATCAAAAAGGATCTTCACCTAGATCCTTTTATAGTCCGGAAATACAGGAACGCACGCTGGATGGC
CCTTCGCTGGGATGGTGAAACCATGAAAAATGGCAGCTTCAGTGGATTAAGTGGGGGTAATGTGGCCT
GTACCCTCTGGTTGCATAGGTATTCATACGGTTAAAATTTATCAGGCGCGATTGCGGCAGTTTTTCGGGT
GGTTTGTTGCCATTTTTACCTGTCTGCTGCCGTGATCGCGCTGAACGCGTTTTAGCGGTGCGTACAATTA
AGGGATTATGGTAAATCCACTTACTGTCTGCCCTCGTAGCCATCGAGATAAACCGCAGTACTCCGGCCAC
GATGCGTCCGGCGTAGAGGATCGAGATCTTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGT
CTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAA
GTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATC
AAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCT
CCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGG
GCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTT
GCGTTTCTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT (SEQ ID NO:4)

Note: A pFN18K derivative containing a kanamycin resistance gene for E. coli expression of fusion proteins bearing an N-terminal HaloTag and a C-terminal Histag.

pBac-NFlagHA

GACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACT
TGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCG
TCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAA
CTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGG
AGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCT

Figure 16

TTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAA
CGCGAATTTTAACAAAATATTAACGTTTACAATTTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACC
CCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGC
ATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGT
GCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAA
CGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGC
AAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAA
AGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTG
CGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGG
ATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACA
CCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTC
CCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC
GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACT
GGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG
AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTT
ACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTG
ATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGAT
CAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTAC
CAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGC
GCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCG
CCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCG
GGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCAC
ACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCG
CCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCG
CACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTT
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTT
TTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA
TAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTC
AGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACA
CCGCAGACCAGCCGCGTAACCTGGCAAAATCGGTTACGGTTGAGTAATAAATGGATGCCCTGCGTAAGC
GGGTGTGGGCGGACAATAAAGTCTTAAACTGAACAAAATAGATCTAAACTATGACAATAAAGTCTTAAA
CTAGACAGAATAGTTGTAAACTGAAATCAGTCCAGTTATGCTGTGAAAAAGCATACTGGACTTTTGTTAT
GGCTAAAGCAAACTCTTCATTTTCTGAAGTGCAAATTGCCCGTCGTATTAAAGAGGGGCGTGGCCAAGG
GCATGGTAAAGACTATATTCGCGGCGTTGTGACAATTTACCGAACAACTCCGCGGCCGGGAAGCCGATC
TCGGCTTGAACGAATTGTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTATGC
CCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTAGATCACATAAGCACC
AAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGGTGGCAATGCCCTGCCTCCGGTGCTCGCC
GGAGACTGCGAGATCATAGATATAGATCTCACTACGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCG
CGAGAGCGCCAACAACCGCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAG

Figure 16

TTCCCGAGGTAATCGGAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACGACCGAAA
AGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTACATGTGCGAATGATGCCCATA
CTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTGCGTAACATCG
TTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATA
GACTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCG
GTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTACGAACCGAACAGGCT
TATGTCAACTGGGTTCGTGCCTTCATCCGTTTCCACGGTGTGCGTCACCCGGCAACCTTGGGCAGCAGCG
AAGTCGAGGCATTTCTGTCCTGGCTGGCGAACGAGCGCAAGGTTTCGGTCTCCACGCATCGTCAGGCAT
TGGCGGCCTTGCTGTTCTTCTACGGCAAGGTGCTGTGCACGGATCTGCCCTGGCTTCAGGAGATCGGAA
GACCTCGGCCGTCGCGGCGCTTGCCGGTGGTGCTGACCCCGGATGAAGTGGTTCGCATCCTCGGTTTTC
TGGAAGGCGAGCATCGTTTGTTCGCCCAGGACTCTAGCTATAGTTCTAGTGGTTGGCTACGTATACTCCG
GAATATTAATAGATCATGGAGATAATTAAAATGATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTT
CGTAACAGTTTTGTAATAAAAAAACCTATAAATATTCCGGATTATTCATACCGTCCCACCATCGGGCGCG
GATCCTCGATGAAGACCATCATCGCTCTGTCCTACATCTTCTGCCTGGTGTTCGCTGACTACAAGGACGA
CGACGACGCTTCCTCCTACCCCTACGACGTGCCCGACTACGCTATGGCTGAGATCGGCACCGGTTTCCCC
TTCGACCCCCACTACGTGGAGGTGCTGGGTGAGCGTATGCACTACGTGGACGTGGGTCCCCGTGACGG
CACCCCCGTGCTGTTCCTGCACGGTAACCCCACCTCCTCCTACGTGTGGCGTAACATCATCCCCCACGTG
GCTCCCACCCACCGTTGCATCGCTCCCGACCTGATCGGTATGGGTAAGTCCGACAAGCCCGACCTGGGTT
ACTTCTTCGACGACCACGTGCGTTTCATGGACGCTTTCATCGAGGCTCTGGGTCTGGAGGAGGTGGTGC
TGGTGATCCACGACTGGGGTTCCGCTCTGGGTTTCCACTGGGCTAAGCGTAACCCCGAGCGTGTGAAGG
GTATCGCTTTCATGGAGTTCATCCGTCCCATCCCCACCTGGGACGAGTGGCCCGAGTTCGCTCGTGAGAC
CTTCCAGGCTTTCCGTACCACCGACGTGGGTCGTAAGCTGATCATCGACCAGAACGTGTTCATCGAGGG
CACCCTGCCTATGGGTGTGGTGCGTCCCCTGACCGAGGTGGAGATGGACCACTACCGTGAGCCCTTCCT
GAACCCCGTGGACCGTGAGCCCCTGTGGCGTTTCCCCAACGAGCTGCCCATCGCTGGTGAGCCCGCTAA
CATCGTGGCTCTGGTGGAGGAGTACATGGACTGGCTGCACCAGTCCCCCGTGCCCAAGCTGCTGTTCTG
GGGCACCCCCGGTGTGCTGATCCCCCCCGCTGAGGCTGCTCGTCTGGCTAAGTCCCTGCCCAACTGCAA
GGCTGTGGACATCGGTCCCGGTCTGAACCTGCTCCAGGAGGACAACCCCGACCTGATCGGTTCCGAGAT
CGCTCGTTGGCTGTCCACCCTGGAGATCTCCGGTGGTGGTTCCGGTGCCATGGAACATATGGCTAGCGA
ATTCAAAGGCCTACGTCGACGAGCTCACTAGTCGCGGCCGCTTTCGAATCTAGAGCCTGCAGTCTCGAG
GCATGCGGTACCAAGCTTGTCGAGAAGTACTAGAGGATCATAATCAGCCATACCACATTTGTAGAGGTT
TTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGT
TAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCAT
TTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCTGATCAC
TGCTTGAGCCTAGGAGATCCGAACCAGATAAGTGAAATCTAGTTCCAAACTATTTTGTCATTTTTAATTTT
CGTATTAGCTTACGACGCTACACCCAGTTCCCATCTATTTTGTCACTCTTCCCTAAATAATCCTTAAAAACT
CCATTTCCACCCCTCCCAGTTCCCAACTATTTTGTCCGCCCACAGCGGGGCATTTTTCTTCCTGTTATGTTT
TTAATCAAACATCCTGCCAACTCCATGTGACAAACCGTCATCTTCGGCTACTTTTTCTCTGTCACAGAATG
AAAATTTTTCTGTCATCTCTTCGTTATTAATGTTTGTAATTGACTGAATATCAACGCTTATTTGCAGCCTGA
ATGGCGAATGG (SEQ ID NO:5)

Note: A pFastBac1 derivative containing ampicillin and gentamicin resistance genes for

Figure 16

Baculovirus expression of fusion proteins bearing an N-terminal signal peptide, Flag and HA tags, and HaloTag.
pIRES-CHaloFlagHis

TAATACGACTCACTATAGGGCGAATTAATTCCGGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGA
GGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAAT
GCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGT
AGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGT
ATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAG
TCAAATGGCTCACCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGG
GATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCC
CCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACCACCCATATGGGATC
CGAATTCGATATCTTAATTAAGCTGCAGGAGCTCGTCGACGCGGCCGCACTCGAGCAGAAGCAAGCTGA
AGAGGCGGCAGCGAAAGCGGCGGCAGATGCTAAAGCGAAGGCCGAAGCAGATGCTAAAGCTGCGGA
AGAAGCAGCGAAGAAAGCGGCTGCCGACGCAAAGAAAAAAGCAGAAGCAGAAGCCGCCAAAGCCGCA
GCCGAAGCGCAGAAAAAAGCCGAGGCAGCCGCTGCGGCACTGAAGAAGAAAGCGGAAGCGGCAGAA
GCAGCTGCCGCTGAAGCAAGAAAGAAAGCGGCAACTGAAGGCGGCAGCGGCATGGCTGAGATCGGCA
CCGGTTTCCCCTTCGACCCCCACTACGTGGAGGTGCTGGGTGAGCGTATGCACTACGTGGACGTGGGTC
CCCGTGACGGCACCCCCGTGCTGTTCCTGCACGGTAACCCCACCTCCTCCTACGTGTGGCGTAACATCAT
CCCCCACGTGGCTCCCACCCACCGTTGCATCGCTCCCGACCTGATCGGTATGGGTAAGTCCGACAAGCCC
GACCTGGGTTACTTCTTCGACGACCACGTGCGTTTCATGGACGCTTTCATCGAGGCTCTGGGTCTGGAG
GAGGTGGTGCTGGTGATCCACGACTGGGGTTCCGCTCTGGGTTTCCACTGGGCTAAGCGTAACCCCGAG
CGTGTGAAGGGTATCGCTTTCATGGAGTTCATCCGTCCCATCCCCACCTGGGACGAGTGGCCCGAGTTC
GCTCGTGAGACCTTCCAGGCTTTCCGTACCACCGACGTGGGTCGTAAGCTGATCATCGACCAGAACGTG
TTCATCGAGGGCACCCTGCCTATGGGTGTGGTGCGTCCCCTGACCGAGGTGGAGATGGACCACTACCGT
GAGCCCTTCCTGAACCCCGTGGACCGTGAGCCCCTGTGGCGTTTCCCCAACGAGCTGCCCATCGCTGGT
GAGCCCGCTAACATCGTGGCTCTGGTGGAGGAGTACATGGACTGGCTGCACCAGTCCCCCGTGCCCAAG
CTGCTGTTCTGGGGCACCCCCGGTGTGCTGATCCCCCCCGCTGAGGCTGCTCGTCTGGCTAAGTCCCTGC
CCAACTGCAAGGCTGTGGACATCGGTCCCGGTCTGAACCTGCTCCAGGAGGACAACCCCGACCTGATCG
GTTCCGAGATCGCTCGTTGGCTGTCCACCCTGGAGATTTCCGGTGGTGGTTCCGGTGATTATAAAGATG
ATGATGATAAAGGCGGCGCGGGCCACCACCACCACCACCACTGAGATCTGACTGAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAGTTTAAACACTAGTCCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTA
AACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGGCTTCCTCGCTCACTGACTCGCT
GCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGA
ATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG
GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTC
AGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCT
CTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT
CATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA

Figure 16

GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTT
CTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAA
GGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACT
TGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT
AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGC
AATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGC
CGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA
GTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCT
CGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTT
GTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATC
ACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTG
GTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA
TACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGC
GAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATC
TTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAA
GGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTAT
CAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG
CGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA
ATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGC
AGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCG
TCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGT
GCACCATATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTA
AACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGA
AATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAA
CAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATG
GCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGA
ACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGG
GAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACC
ACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCGCGCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAG
GCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTG
(SEQ ID NO:6)

Note: A pT7CFE1 (Thermo Scientific) derivative containing a multiple cloning site, an ampicillin resistance gene for human IVT expression of fusion proteins bearing a C-terminal TolA spacer, HaloTag, Flag and His-tags.

pIRES-CHaloFlagHis-Gateway

TAATACGACTCACTATAGGGCGAATTAATTCCGGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGA
GGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAAT

Figure 16

GCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGT
AGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGT
ATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAG
TCAAATGGCTCACCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGG
GATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTCTAGGCCCC
CCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACCACCCATATGGGAAC
AAGTTTGTACAAAAAAGCTGAACGAGAAACGTAAAATGATATAAATATCAATATATTAAATTAGATTTTG
CATAAAAAACAGACTACATAATACTGTAAAACACAACATATCCAGTCACTATGGCGGCCGCATTAGGCA
CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGATTTTGAGTTAGGATCCGTCGAGATTT
TCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGG
CATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGG
ATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTT
GCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGAT
AGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCA
CGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTAT
TTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGA
TTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGC
GACAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGA
ATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAAACGCGTGGATCCGGCTT
ACTAAAAGCCAGATAACAGTATGCGTATTTGCGCGCTGATTTTTGCGGTATAAGAATATATACTGATATG
TATACCCGAAGTATGTCAAAAAGAGGTATGCTATGAAGCAGCGTATTACAGTGACAGTTGACAGCGACA
GCTATCAGTTGCTCAAGGCATATATGATGTCAATATCTCCGGTCTGGTAAGCACAACCATGCAGAATGAA
GCCCGTCGTCTGCGTGCCGAACGCTGGAAAGCGGAAAATCAGGAAGGGATGGCTGAGGTCGCCCGGTT
TATTGAAATGAACGGCTCTTTTGCTGACGAGAACAGGGGCTGGTGAAATGCAGTTTAAGGTTTACACCT
ATAAAAGAGAGAGCCGTTATCGTCTGTTTGTGGATGTACAGAGTGATATTATTGACACGCCCGGGCGAC
GGATGGTGATCCCCCTGGCCAGTGCACGTCTGCTGTCAGATAAAGTCTCCCGTGAACTTTACCCGGTGGT
GCATATCGGGGATGAAAGCTGGCGCATGATGACCACCGATATGGCCAGTGTGCCGGTCTCCGTTATCGG
GGAAGAAGTGGCTGATCTCAGCCACCGCGAAAATGACATCAAAAACGCCATTAACCTGATGTTCTGGGG
AATATAAATGTCAGGCTCCCTTATACACAGCCAGTCTGCAGGTCGACCATAGTGACTGGATATGTTGTGT
TTTACAGTATTATGTAGTCTGTTTTTTATGCAAAATCTAATTTAATATATTGATATTTATATCATTTTACGTT
TCTCGTTCAGCTTTCTTGTACAAAGTGGTGCTCGAGCAGAAGCAAGCTGAAGAGGCGGCAGCGAAAGC
GGCGGCAGATGCTAAAGCGAAGGCCGAAGCAGATGCTAAAGCTGCGGAAGAAGCAGCGAAGAAAGC
GGCTGCCGACGCAAAGAAAAAAGCAGAAGCAGAAGCCGCCAAAGCCGCAGCCGAAGCGCAGAAAAAA
GCCGAGGCAGCCGCTGCGGCACTGAAGAAGAAAGCGGAAGCGGCAGAAGCAGCTGCCGCTGAAGCAA
GAAAGAAAGCGGCAACTGAAGGCGGCAGCGGCATGGCTGAGATCGGCACCGGTTTCCCCTTCGACCCC
CACTACGTGGAGGTGCTGGGTGAGCGTATGCACTACGTGGACGTGGGTCCCCGTGACGGCACCCCCGT
GCTGTTCCTGCACGGTAACCCCACCTCCTCCTACGTGTGGCGTAACATCATCCCCCACGTGGCTCCCACCC
ACCGTTGCATCGCTCCCGACCTGATCGGTATGGGTAAGTCCGACAAGCCCGACCTGGGTTACTTCTTCGA
CGACCACGTGCGTTTCATGGACGCTTTCATCGAGGCTCTGGGTCTGGAGGAGGTGGTGCTGGTGATCCA
CGACTGGGGTTCCGCTCTGGGTTTCCACTGGGCTAAGCGTAACCCCGAGCGTGTGAAGGGTATCGCTTT

Figure 16

CATGGAGTTCATCCGTCCCATCCCCACCTGGGACGAGTGGCCCGAGTTCGCTCGTGAGACCTTCCAGGCT
TTCCGTACCACCGACGTGGGTCGTAAGCTGATCATCGACCAGAACGTGTTCATCGAGGGCACCCTGCCT
ATGGGTGTGGTGCGTCCCCTGACCGAGGTGGAGATGGACCACTACCGTGAGCCCTTCCTGAACCCCGTG
GACCGTGAGCCCCTGTGGCGTTTCCCCAACGAGCTGCCCATCGCTGGTGAGCCCGCTAACATCGTGGCT
CTGGTGGAGGAGTACATGGACTGGCTGCACCAGTCCCCCGTGCCCAAGCTGCTGTTCTGGGGCACCCCC
GGTGTGCTGATCCCCCCCGCTGAGGCTGCTCGTCTGGCTAAGTCCCTGCCCAACTGCAAGGCTGTGGAC
ATCGGTCCCGGTCTGAACCTGCTCCAGGAGGACAACCCCGACCTGATCGGTTCCGAGATCGCTCGTTGG
CTGTCCACCCTGGAGATTTCCGGTGGTGGTTCCGGTGATTATAAAGATGATGATGATAAAGGCGGCGCG
GGCCACCACCACCACCACCACTGAGATCTGACTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTTTA
AACACTAGTCCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTT
GCTGAAAGGAGGAACTATATCCGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGG
CGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA
GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC
ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACA
GGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGC
TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTAT
CTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC
CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA
ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA
GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
AGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGT
GGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
AAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGC
TTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGT
GTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACG
CTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTG
CAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA
TAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCA
TTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCT
CCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACT
GCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCAT
TCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCAC
ATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACC
GCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCA
GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAA
ATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCG
GATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCC
ACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTT

Figure 16

CGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCT
TGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTC
GGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATATGCGGTGTGAAAT
ACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTAAACGTTAATATTTTGTTAAAATTC
GCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATC
AAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGT
GGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTA
ATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAG
AGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGC
TAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCT
ACAGGGCGCGTCGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCT
CTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGG
TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTG (SEQ ID NO:7)

Note: A pT7CFE1 derivative containing a Gateway cloning site, an ampicillin resistance gene for human IVT expression of fusion proteins bearing a C-terminal TolA spacer, HaloTag, Flag and His-tags

SEQUENCES OF PRIMERS:

Ribosome display-based barcoding:

Template barcoded (F):
CGAAATTAATACGACTCACTATAGGGAATGATACGGCGACCACCGAGGG*****AGA
GAATGAGGA (Note: Multiple oligos, and asterisks denote a barcoding sequence composed of A/T/C) (SEQ ID NO:8)

Template (R): TGCTAGTTGCTCCCGCAC (SEQ ID NO:9)

RT primer: /5Acryd/T/ideSBioTEG/TTTTTTTTTCAAGCAGAAGACGGCATACGA (SEQ ID NO:10)

HaloTag-based barcoding:

Universal template of barcoding DNA:
ACTTCAGTACGTCACTACCAACAATGATGGAATCCCTAGGTTGGCTTACCAAAAATG
AAGCTGATGAATACTCCCTCAAAGACAATTGTCAACCTTACTTGTCCATTCCTGAAG
AAATATTATATTTATACAACTTACCCATAGAATCCTATCGTATGCCGTCTTCTGCTTG
(SEQ ID NO:11)

Barcoding DNA (F):
AATGATACGGCGACCACCGAGGGG****ACTTCAGTACGTCACTACCAAC (SEQ ID NO:12) (Note: Multiple oligos, and asterisks denote a barcoding sequence composed of A/T/C)

Barcoding DNA (R): CAAGCAGAAGACGGCATACGA (SEQ ID NO:13)

Barcoding DNA modification (F):
/5Acryd/T/iUniAmM/TTTTTTTTTAATGATACGGCGACCACCGA (SEQ ID NO:14)

Barcoding DNA modification (R):
/5Acryd/T/ideSBioTEG/TTTTTTTTTCAAGCAGAAGACGGCATACGA (SEQ ID NO:15)

Figure 16

Polony amplification:
Bridge amplification (F): /5Acryd/TTTTTTTTTTAATGATACGGCGACCACCGA (SEQ ID NO:16)
Bridge amplification (R): /5Acryd/TTTTTTTTTTCAAGCAGAAGACGGCA/ideoxyU/ACGA (SEQ ID NO:17)
Fluorescence hybridization:
Sequencing 1_Cy3: CCCGGGTTCCTCATTCTCT/Cy3/ (SEQ ID NO:18)
Sequencing 2_Cy5: GGTAGTGACGTACTGAAGT/Cy5/ (SEQ ID NO:19)
Sequencing:
Sequencing 1: CCCGGGTTCCTCATTCTCT (SEQ ID NO:20)
Sequencing 2: GGTAGTGACGTACTGAAGT (SEQ ID NO:21)

Figure 16

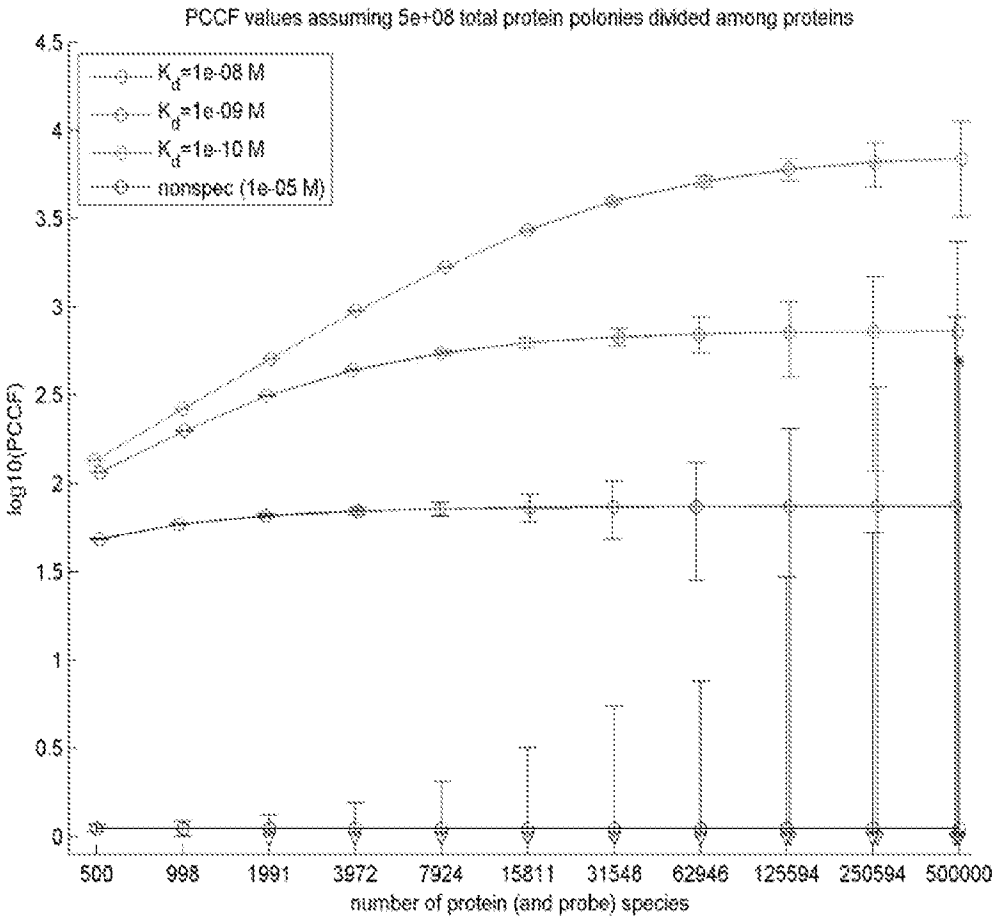
PCCF values assuming 5e+08 total protein colonies divided among proteins
K_d=1e-08 M
K_d=1e-09 M
K_d=1e-10 M
nonspec (1e-05 M)
log10(PCCF)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
500
998
1991
3972
7924
15811
31546
62946
125594
250594
500000
number of protein (and probe) species

Figure 17