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(57) Abrégé/Abstract:
The invention relates to the field of structural biology. More specifically, the invention relates to methods for the identification and characterization of biomolecular tools allowing the selective recognition and/or stabilization of distinct conformational states of protein complexes, including transient protein-protein interactions and protein-nucleic acid complexes. Such tools can then be used for purification purposes, crystallization and structure determination of these stabilized protein complexes, for drug discovery, as research tools, as well as for diagnosis and treatment of diseases.

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(54) **Title**: METHODS TO SELECT FOR AGENTS THAT STABILIZE PROTEIN COMPLEXES

(57) **Abstract**: The invention relates to the field of structural biology. More specifically, the invention relates to methods for the identification and characterization of biomolecular tools allowing the selective recognition and/or stabilization of distinct conformational states of protein complexes, including transient protein-protein interactions and protein-nucleic acid complexes. Such tools can then be used for purification purposes, crystallization and structure determination of these stabilized protein complexes, for drug discovery, as research tools, as well as for diagnosis and treatment of diseases.

METHODS TO SELECT FOR AGENTS THAT STABILIZE PROTEIN COMPLEXES

FIELD OF THE INVENTION

The invention relates to the field of structural biology. More specifically, the invention relates to methods for the identification and characterization of biomolecular tools allowing the selective recognition and/or stabilization of distinct conformational states of protein complexes, including transient protein-protein interactions and protein-nucleic acid complexes. Such tools can then be used for purification purposes, crystallization and structure determination of these stabilized protein complexes, for drug discovery, as research tools, as well as for diagnosis and treatment of diseases.

BACKGROUND

Transient molecular interactions between macromolecules provide a powerful mechanism in biology to regulate function and cell processes. A crucial step towards the full understanding of cellular systems consists of mapping the networks of physical DNA-, RNA- and protein-protein interactions of an organism of interest as completely and accurately as possible, the “interactome network”. Recently a large number of biological pathway and network databases have been developed to capture the expanding knowledge of molecular interactions. However, the complete understanding of molecular interactions requires high resolution 3D structures as they provide key atomic details about binding interfaces and information about structural changes that accompany molecular interactions.

The interactions of two or more dissimilar proteins, the so called protein–protein interactions (PPIs), are central to most biological processes. Critical cellular functions, including cell growth, DNA replication, transcription activation, translation and transmembrane signal transduction, are all regulated by multiprotein complexes and therefore quaternary protein structures represent a large and attractive emerging class of targets for human therapeutics[1-4]. It is now well established that human diseases can be caused by aberrant PPIs, either through the loss of an essential interaction or through the formation and/or stabilization of a protein-protein interaction at an inappropriate time or location. Proteins themselves are dynamic and can exist in multiple conformations, often induced by interaction with another protein in a (transient) protein-protein interaction. These conformational changes are often functionally important and reflect allosteric regulation that activate or inactivate specific protein functions. The diversity and complexity of these highly dynamic PPIs present many opportunities and challenges for the identification of drug-like molecules with the ability to modulate the PPI with the necessary selectivity and potency.

PPIs can occur between identical or non-identical chains (homo- or hetero-oligomers). Besides composition, non-obligate and obligate complexes can be distinguished. In an obligate PPI, the protomers are not found as stable structures on their own *in vivo*. The components of non-obligate complexes are independently stable. In contrast to a permanent interaction that only exists in its complexed form, a transient interaction associates and dissociates *in vivo*. We distinguish weak transient interactions that feature a dynamic oligomeric equilibrium in solution, where the interaction is broken and formed continuously and strong transient associations that require a molecular trigger to shift the oligomeric equilibrium. Many PPIs do not fall into distinct types. Rather, there is a continuum between obligate and non-obligate interactions, and the stability of transient complexes varies much depending on physiological conditions and environment[5].

Yeast two-hybrid screens have been used extensively to map binary transient interactions and tandem affinity purification run in conjunction with mass spectroscopy and chemical crosslinking has been developed to detect transiently formed complexes. A large number of biological network databases have been developed to capture the expanding knowledge of protein-protein interactions but rigorous assessment of high-throughput as well as literature-curated PPI data has shown that experimental data can be prone to error and are not completely comprehensive[5]. Therefore, computational methods can be applied to increase confidence and predict interactions currently hidden from the experimental techniques[6].

Ultimately, the complete understanding of molecular interactions requires high resolution 3D structures as they provide key atomic details about binding interfaces and information about structural changes that accompany protein-protein interactions. The structural details of these interactions, often necessary to understand their function, are only available for a tiny fraction and this gap is growing[7]. Modern overexpression and purification procedures can usually supply sufficient material for structural studies on a single protein, but obtaining sufficient material can be an enormous problem for large multiprotein complexes. But even if expression and purification problems can be overcome, we are still confronted with the intrinsic property that these complexes are transient, complicating their structural characterization X-ray crystallography, Nuclear Magnetic Resonance (NMR), Small Angle X-ray Scattering (SAXS) or Electron microscopy (EM). The transient nature of these PPIs also makes them difficult targets for drug discovery.

The structural characterization of multiprotein complexes is currently limited to permanent protein-protein interactions or to transient interactions that can be stabilized by i) binding of small molecule effectors (such as nucleotides, substrates, ions, or analogs thereof), ii) naturally occurring ligands or iii) introduction of stabilizing mutations to the interacting protomers of the PPI. Antibodies and fragments

derived thereof have been identified that are able to bind quaternary protein structures[8]. However, none of these antibodies (or fragments) selectively stabilize the PPI, i.e. preferentially interact with the protein complex versus one of the interacting protomers. Camelid single domain antibody fragments (VHHs or Nanobodies) have been identified that bind conformational epitopes of (membrane) proteins and complexes thereof (Pardon et al. 2014). For example, Nanobodies that stabilize a complex composed of agonist-occupied monomeric β2-adrenergic receptor and nucleotide-free Gs heterotrimer were identified by (i) immunizing llamas with the complex after chemical cross-linking of the associating proteins to mature Nanobodies that bind allosteric epitopes on the transient complex, and (ii) two different panning strategies. These Nanobodies were able to protect the complex from dissociation by GTPγS and provide stabilization to the G protein subunits, which was essential for determining its crystal structure. Conformational antibodies that stabilize particular conformers of single proteins and methods to identify these have also been described (Rasmussen et al., 2011; Kruse et al., 2013). However, generic methods to identify allosteric modulators that bind at a site orthogonal to the protein-protein interface, inducing conformational changes that affect the protomers' propensity to form a complex are lacking.

There is thus a need for straightforward methods for the selection of novel tools that selectively stabilize transient protein complexes, making them amenable for structural investigation and drug discovery.

SUMMARY OF THE INVENTION

Many protein-protein interactions (PPIs) are regulated by allosteric modulators that bind at a site orthogonal to the protein-protein interface, inducing/stabilizing conformational changes that affect the protomers' propensity to form a complex. Here provided is a generic method for the generation and selection of allosteric binding agents that stabilize transient protein complexes. Such binding agents are instrumental to purify, crystallize and solve the structures of transient complexes that have been resistant to structural investigation by conventional methods. These binding agents are also useful for drug discovery against a target protein complex, as research tools, as well as for diagnosis and treatment of diseases that are associated with a particular protein complex conformation.

Owing to the cooperative nature of structural transitions in proteins, the molecular mechanisms behind protein association are poorly understood. However, thermodynamic cycles are a well-established approach for analyzing the energetics of interactions within or between macromolecules. The thermodynamic cycle presented in Fig. 1 is useful for quantifying the contribution of a binding agent (a Nanobody is taken as the example) to the stability of a transient complex.

The association of two proteins (A and B in Figure 1) generates new conformational epitopes in the transient complex A-B. On the one hand, new epitopes are formed by the A-B interface. On the other hand, A and B may undergo significant conformational changes upon association, generating new conformational epitopes that are not represented in the protomers. Protein-protein interaction (PPI) stabilizing tools (for example, a Nanobody in Figure 1) selectively bind epitopes that are unique to the transient complex.

Thermodynamics imply that any binding agent that preferentially binds an allosteric structural feature, unique to the transient complex ($K_{binding\ agent}$ / $K'_{binding\ agent}$ > 1), will stabilize this complex proportionally:

$$K_{binding\ agent} \times K'_{d} = K'_{binding\ agent} \times K_{d} \quad \text{or} \quad K_{binding\ agent} / K'_{binding\ agent} = K_{d} / K'_{d}$$

This means that, for example, an allosteric antibody raised against a (conformational) epitope, unique to the transient complex, will stabilize this complex by the principle of mass action. This thermodynamic concept can be expanded beyond binary interactions.

Thus, here provided is a selection method for binding agents that specifically bind to a protein complex and not to the constituting members of the protein complex. It should be clear that the transient protein complex within the context of the present invention can be formed from the association of two or more constituting members (as defined further herein). Binding agents that are selected by the method of the invention are able to selectively stabilize distinct conformations of a protein complex.

Preferably, these binding agents can not only be used to stabilize the protein complex itself but will also stabilize one of the constituting members in the protein complex conformation, in absence of its interacting partner(s).

Notably, the selection method that is provided herein can simultaneously be used to

(i) select binding agents that selectively bind to one of the constituting members of the protein complex (and not to the protein complex itself nor the other constituting member(s) of the protein complex), and/or

(ii) select binding agents that selectively bind to one of the individual members of a protein complex and to the protein complex (and not to the other individual member(s) of the complex).

Thus, according to a first aspect, the invention relates to a method to select conformation-selective binding agents of a protein complex, the method comprising the steps of:

a) Displaying a collection of binding agents at the extracellular surface of a population of cells

b) Using cell sorting to separate, from said population of cells of b), cells displaying binding agents that

i. specifically bind to a protein complex conformation and not to the individual members of the complex, and/or

ii. specifically bind to one of the individual members of a protein complex and not to the protein complex conformation nor to the other individual member(s) of the complex, and/or

iii. specifically bind to one of the individual members of a protein complex and to a protein complex conformation and not to the other individual member(s) of the complex.

In a particular embodiment of the above method, the individual members of the protein complex are distinguishably tagged and step b) comprises the steps of:

a) Incubating a mixture of distinguishably tagged individual members of a protein complex with the population of cells under suitable conditions to allow binding to the cells

b) Using cell sorting to select, from said population of cells, cells displaying binding agents that

i. specifically bind to a protein complex conformation and not to the individual members of the complex, and/or

ii. specifically bind to one of the individual members of a protein complex and not to the protein complex conformation nor to the other individual member(s) of the complex, and/or

iii. specifically bind to one of the individual members of a protein complex and to a protein complex conformation and not to the other individual member(s) of the complex.

In a preferred embodiment of the above method, at least one of the individual members of the protein complex is labelled with a fluorescent label. More preferably, the individual members of the protein complex are each labelled with a distinguishable fluorescent label.

According to a specific embodiment of the above method, the cell sorting in step b) is done using FACS.

Preferably, the protein complex in any of the above methods is a protein-protein complex that is constituted of at least two interacting monomeric proteins. Alternatively, the protein complex is a

protein-nucleic acid complex that is constituted of at least one monomeric protein interacting with at least one nucleic molecule.

The method may further comprise the step of isolating the binding agent from the cell sorted in step b). The method may also comprise the step of measuring the binding specificity and/or affinity of the binding agent for the protein complex conformation as compared to the individual members of the protein complex by any suitable technique, such as biophysical method or FACS.

In a preferred embodiment of any of the above methods, the plurality of binding agents is a library of antibodies or antibody fragments. More preferably, said antibodies fragments are immunoglobulin single domain antibodies, in particular nanobodies. Said antibodies or antibody fragments are preferably obtained from an animal that has been immunized with the protein complex in a cross-linked form.

In one specific embodiment, the population of cells as used in any of the above methods is a population of cells is a population of yeast cells.

According to another aspect, the invention also encompasses a composition comprising a binding agent obtained by any of the above methods.

In still another aspect, the invention relates to a composition comprising a protein complex and a binding agent obtained by any of the above methods, whereby the protein complex is stabilized by the binding agent.

Further embodiments will be found in the detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Allosteric ternary binding model to analyze the effect of conformational nanobodies on the oligomeric state of a transient PPI. Equivalent cycles can be drawn for Nbs that bind changing conformational epitopes on A or for antibodies that bind changing conformational epitopes consisting of elements of A and B.

Figure 2. FACS analysis of a library of yeast cells displaying nanobodies that recognize different conformational features of a transient complex composed of proteins A and B. A and B are separately labeled with different fluorescent dyes.

Figure 3. SDS-PAGE of the cross-linked RIC8A●Gαi1 transient complex. Lane 1 Molecular weight marker (Bio-Rad). Lanes 2 and 3 are duplicates of samples cross-linked for 5 minutes with K100 (Covalx) and BS3 (Thermo Scientific). Lanes 4 and 5 are duplicates of samples cross-linked for 30 minutes, respectively.

Figure 4. Stepwise co-selection by FACS of Nanobodies that bind to RIC8A•Gαi but not to RIC8A alone or Gαi alone. RIC8A was separately labeled with the fluorescent dye Dylight405 (Life Technologies). Gαi1 was separately labelled with the fluorescent dye Dylight488 (Life Technologies). For each round of selection, a three-color FACS sort was performed on R-Phycoerythrin fluorescence (confirming the expression of the displayed Nb, not shown), Dylight405 fluorescence (measuring the binding of RIC8A to the yeast cells), and Dylight488 (measuring the binding of Gαi1 to the yeast cells).

Figure 5. AA sequence alignment (CLC viewer) of Nanobodies resulting from the selection for binders to the RIC8A•Gαi complex.

Figure 6. Stepwise co-selection by FACS of Nanobodies that bind to RIC8A alone but not the RIC8A•Gαi complex or Gαi alone. For each round of selection round, a three-color FACS sort was performed on R-Phycoerythrin fluorescence (not shown), Dylight405 fluorescence (measuring the binding of RIC8A to the yeast cells), and Dylight488 (measuring the binding of Gαi1 to the yeast cells).

Figure 7. AA sequence alignment (CLC viewer) of Nanobodies resulting from the selection for RIC8A binders.

Figure 8. Summary of the different interaction profiles of Nanobodies in a FACS screening experiment. Target molecules for nanobody binding: RIC8A-Dylight405, Gai1-Dylight488, RIC8A-Dylight405 / Gai1-Dylight488 complex, Results are shown as dot blots or histograms where the signal of a specific Nanobody (black) is compared to the FACS signal of an irrelevant Nanobody (gray). For each clone, a three-color FACS analysis was performed on R-Phycoerythrin fluorescence (not shown), Dylight405 fluorescence (measuring the binding of RIC8A to the yeast cells), and Dylight488 (measuring the binding of Gαi1 to the yeast cells). Following representative clones were chosen to illustrate the binding profiles: CA8316 (type 1), CA8322 (type 2) and CA8417 (type 3).

Figure 9. Octet Red96 Sensorgram of binding of CA8332 to RIC8A, Gαi1 and RIC8A•Gαi1 complex. A. Chart showing association (0-600 sec) and dissociation (600-1500 sec) of CA8332 to RIC8A (alone) in grey, to Gαi1 (alone) in light gray and to the RIC8A•Gαi1 complex in black.

B. Dose responds curves showing association (0-900 sec) and dissociation (900-2000 sec) using 125, 12.5, 1.25, 0.125, 0.0125, 0uM of the purified RIC8A•Gai1 complex.

Figure 10. SDS-PAGE of the E. coli DNA gyrase. Lane 1 Molecular weight marker (Fermentas). Lane 2 DNA gyrase. Lane 3 ternary covalent complex (DNA gyrase•DNA•Ciprofloxacin).

Figure 11. Stepwise selection by FACS of Nanobodies that bind to DNA•gyrase•CFX but not to gyrase alone. DNA was separately labeled with the fluorescent dye Alexa488 (Life Technologies). Gyrase was unlabeled, since this interferes with complex formation. For each round of selection, a two-color FACS sort was performed on R-Phycoerythrin fluorescence (confirming the expression of the displayed Nb) and Alexa488 (measuring the binding of DNA, or more generally DNA•gyrase•CFX to the yeast cells). There is a destinction made in FACS sort round 3 and 4 between condition a) DNA•gyrase•CFX and condition b) DNA•gyrase.

Figure 12. AA sequence alignment (CLC viewer) of Nanobodies resulting from the selection for DNA•gyrase•CFX and DNA•gyrase binders.

Figure 13. Stepwise selection by FACS of Nanobodies that bind to gyrase alone. Gyrase was labeled with Dylight 405. For each round of selection, a two-color FACS sort was performed on R-Phycoerythrin fluorescence (confirming the expression of the displayed Nb) and Dylight405 (measuring the binding of gyrase to the yeast cells).

Figure 14. AA sequence alignment (CLC viewer) of Nanobodies resulting from the selection for gyrase binders.

Figure 15. Summary of the different interaction profiles of Nanobodies in a FACS screening experiment. Target molecules for nanobody binding (from left to right): DNA-Alexa647•gyrase•CFX, DNA-Alexa647•gyrase, gyrase-Dylight405. Results are shown as dot blots or histograms where the signal of a specific Nanobody (black) is compared to the FACS signal of an irrelevant Nanobody (gray). For each clone, a two-color FACS analysis was performed on R-Phycoerythrin fluorescence (not shown), Alexa647 fluorescence (measuring the binding of DNA or to the yeast cells) or Dylight405 (measuring the binding of gyrase to the yeast cells). Following representative clones were chosen to illustrate the binding profiles: CA9302 (type 1), CA9306 (type 2).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and

are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

Unless otherwise defined herein, scientific and technical terms and phrases used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclatures used in connection with, and techniques of molecular and cellular biology, structural biology, biophysics, pharmacology, genetics and protein and nucleic acid chemistry described herein are those well-known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, for example, Sambrook et al. Molecular Cloning: A Laboratory Manual, 3th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992, and Supplements to 2002); Rupp, Biomolecular crystallography: principles, Practice and Applications to Structural Biology, 1st edition, Garland Science, Taylor & Francis Group, LLC, an informa Business, N.Y. (2009); Limbird, Cell Surface Receptors, 3d ed., Springer (2004); Flow Cytometry Protocols, 2nd ed. Humana Press (2004); Antibody engineering, 2nd ed. Springer (2010).

As used herein, the terms "polypeptide", "protein", “peptide” are used interchangeably herein, and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. Throughout the application, the standard one letter notation of amino acids will be used. Typically, the term “amino acid” will refer to “proteinogenic amino acid”, i.e. those amino acids that are naturally present in proteins. Most particularly, the amino acids are in the L isomeric form, but D amino acids are also envisaged.

As used herein, the terms "nucleic acid molecule", "polynucleotide", “polynucleic acid”, “nucleic acid” are used interchangeably and refer to a polymeric form of nucleotides of any length, either

deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, control regions, isolated RNA of any sequence, nucleic acid probes, and primers. The nucleic acid molecule may be linear or circular.

The term “binding agent”, as used herein, means the whole or part of a proteinaceous (protein, protein-like or protein containing) molecule that is capable of binding using specific intermolecular interactions to a target protein. In particular, the term “binding agent” is not meant to include a naturally-occurring binding partner of the protein. More specifically, the term “binding agent” refers to a polypeptide, more particularly a protein domain. A suitable protein domain is an element of overall protein structure that is self-stabilizing and that fold independently of the rest of the protein chain and is often referred to as “binding domain”. Such binding agents vary in length from between about 25 amino acids up to 500 amino acids and more. Many binding domains can be classified into folds and are recognizable, identifiable, 3-D structures. Some folds are so common in many different proteins that they are given special names. Non-limiting examples are binding agents selected from a 3- or 4-helix bundle, an armadillo repeat domain, a leucine-rich repeat domain, a PDZ domain, a SUMO or SUMO-like domain, a cadherin domain, an immunoglobulin-like domain, phosphotyrosine-binding domain, pleckstrin homology domain, src homology 2 domain, amongst others. A binding agent can thus be derived from a naturally occurring molecule, e.g. from components of the innate or adaptive immune system, or it can be entirely artificially designed. A binding agent can thus be immunoglobulin-based or it can be based on domains present in proteins, including but limited to microbial proteins, protease inhibitors, toxins, fibronectin, lipocalins, single chain antiparallel coiled coil proteins or repeat motif proteins. Particular examples of binding agents which are known in the art include, but are not limited to: antibodies, heavy chain antibodies (hcAb), single domain antibodies (sdAb), minibodies, the variable domain derived from camelid heavy chain antibodies (VHH or nanobodies), the variable domain of the new antigen receptors derived from shark antibodies (VNAR), alphabodies, protein A, protein G, designed ankyrin-repeat domains (DARPins), fibronectin type III repeats, anticalins, knottins, engineered CH2 domains (nanoantibodies), engineered SH3 domains, affibodies, peptides and proteins, lipopeptides (e.g. pepducins) (see, e.g., Gebauer & Skerra, (2009) Current opinion in chemical biology 13, 245-255; Skerra, J. Molecular Recognition, 13:167-187 (2000); Starovasnik et al., Proc. Natl. Acad. Sd. USA, 94: 10080- 10085 (1997); Binz et al, Nature Biotech., 22: 575-582 (2004); Koide et al, J. Mol Biol, 284: 1141-1151 (1998); Dimitrov, MAbs. 2009 Jan-Feb;1(1):26-8; Nygren, P-A. (2008), FEBS J. 275, 2668-2676.; WO2010066740). Frequently, when generating a particular type of binding agent

using selection methods, combinatorial libraries comprising a consensus or framework sequence containing randomized potential interaction residues are used to screen for binding to a molecule of interest, such as a protein.

As used herein, the term “protein complex” refers to a group of two or more associated macromolecules, whereby at least one of the macromolecules is a protein. A protein complex, as used herein, typically refers to associations of macromolecules that can be formed under physiological conditions. Individual members of a protein complex are linked by non-covalent interactions. Within the scope of the invention, a protein complex can be a non-covalent interaction of only proteins, and is then referred to as a protein-protein complex (as defined hereafter); for instance, a non-covalent interaction of two proteins, of three proteins, of four proteins, etc. As used herein, a protein complex can also be a non-covalent interaction of at least one protein and at least other macromolecule, such as a nucleic acid, and is then referred to as a protein-nucleic acid complex (as defined hereafter); for instance a non-covalent interaction of one protein and one nucleic acid, two proteins and one nucleic acid, two proteins and two nucleic acids, etc. It will be understood that a protein complex can be multimeric. Each interacting macromolecule of a protein complex is herein referred to as an “individual member” or “member” of the protein complex. Accordingly, an individual member of a protein complex can be a monomeric protein, a nucleic acid, or another macromolecule. Protein complex assembly can result in the formation of homo-multimeric or hetero-multimeric complexes. Moreover, interactions can be stable or transient. More details are provided further in the Description.

In general, the term “naturally-occurring” in reference to a member protein of a protein complex means a protein that is naturally produced (e.g., by a wild-type mammal such as a human). Such proteins are found in nature. The term “non-naturally occurring” means a protein that is not naturally-occurring. Naturally-occurring proteins that have been mutated by an amino acid substitution, deletion, and/or insertion, and variants of naturally-occurring proteins, e.g., epitope-tagged proteins or proteins lacking their native N and/or C-terminus, are examples of non-naturally occurring proteins. Non-limiting examples of either naturally-occurring or non-naturally occurring proteins within the context of the present invention are provided further herein.

An “epitope”, as used herein, refers to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spatial conformation, which is unique to the epitope. Generally an epitope consists of at least 4, 5, 6, 7 such amino acids, and more usually, consists of at least 8, 9, 10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and multi-dimensional nuclear magnetic resonance.

A “conformational epitope”, as used herein, refers to an epitope comprising amino acids in a spatial conformation that is unique to a folded 3-dimensional conformation of a polypeptide. Generally, a conformational epitope consists of amino acids that are discontinuous in the linear sequence but that come together in the folded structure of the protein. However, a conformational epitope may also consist of a linear sequence of amino acids that adopts a conformation that is unique to a folded 3-dimensional conformation of the polypeptide (and not present in a denatured state). In protein complexes, conformational epitopes consist of amino acids that are discontinuous in the linear sequences of one or more polypeptides that come together upon folding of the different folded polypeptides and their association in a unique quaternary structure. Similarly, conformational epitopes may here also consist of a linear sequence of amino acids of one or more polypeptides that come together and adopt a conformation that is unique to the quaternary structure.

The term "conformation" or "conformational state" of a protein refers generally to the range of structures that a protein may adopt at any time. One of skill in the art will recognize that determinants of conformation or conformational state include a protein's primary structure as reflected in a protein's amino acid sequence (including modified amino acids) and the environment surrounding the protein. The conformation or conformational state of a protein also relates to structural features such as protein secondary structures (e.g., α-helix, β-sheet, among others), tertiary structure (e.g., the three dimensional folding of a polypeptide chain), and quaternary structure (e.g., interactions of a polypeptide chain with other protein subunits). Post-translational and other modifications to a polypeptide chain such as ligand binding, phosphorylation, sulfation, glycosylation, or attachments of hydrophobic groups, among others, can influence the conformation of a protein. Furthermore, environmental factors, such as pH, salt concentration, ionic strength, and osmolality of the surrounding solution, and interaction with other proteins and co-factors, among others, can affect protein conformation. The conformational state of a protein may be determined by either functional assay for activity or binding to another molecule or by means of physical methods such as X-ray crystallography, NMR, or spin labeling, among other methods. For a general discussion of protein conformation and conformational states, one is referred to Cantor and Schimmel, Biophysical Chemistry, Part I: The Conformation of Biological. Macromolecules,.W.H. Freeman and Company, 1980, and Creighton, Proteins: Structures and Molecular Properties, W.H. Freeman and Company, 1993.

The term “stabilizing” or “stabilized”, as used herein, refers to the capability of a binding agent to selectively bind a specific conformation of a protein complex and to maintain the protein complex in this specific conformation. Within this context, a binding agent that selectively binds to a specific conformation of a protein complex refers to a binding agent that binds with a higher affinity to the

protein complex than to the individual interacting members. One of skill in the art will recognize that binding agents that specifically or selectively bind to a specific conformation of a protein complex will stabilize this specific conformation.

The term "affinity", as used herein, refers to the degree to which a ligand binds to an antigen on a target protein so as to shift the equilibrium of protein and ligand toward the presence of a complex formed by their binding. Thus, for example, where an antigenic target and antibody (fragment) are combined in relatively equal concentration, an antibody (fragment) of high affinity will bind to the available antigen so as to shift the equilibrium toward high concentration of the resulting complex. The dissociation constant is commonly used to describe the affinity between the ligand and the antigenic target. Considering a simple bi-molecular association (L+A↔LA, L: ligand, A: protein A), the equilibrium dissociation constant (Kd in M) is defined as the product of the concentration of the unbound species A and L divided by the concentration of the complex AL: $K_d = \frac{[A][L]}{[AL]}$. In a similar way, the equilibrium dissociation constant can also be defined for ternary complexes. For example, $K_d = \frac{[A][B][L]}{[ABL]}$ (L forms a ternary complex with proteins A and B) which would have units in M^2. To calculate how tightly the Ligand (L) binds to the existing complex AB, a Kd for that reaction can easily be defined as follows: $K_d = \frac{[AB][L]}{[ABL]}$. This is effectively a bimolecular reaction in which the complex AB is treated as if it were a single molecule. However, it should be noticed that the concentration of the binary complex AB is itself a function of [A] and [B]. Typically, the dissociation constant is lower than 10-5 M. Preferably, the dissociation constant is lower than 10-6 M, more preferably, lower than 10-7 M. Most preferably, the dissociation constant is lower than 10-8 M. Other ways of describing the affinity between a ligand and its target protein are the association constant (Ka), the inhibition constant (Ki), or indirectly by evaluating the potency of ligands by measuring the half maximal inhibitory concentration (IC50) or half maximal effective concentration (EC50). For example, within the scope of the invention, the ligand may be a binding agent, preferably an immunoglobulin, such as an antibody, or an immunoglobulin fragment, such as a VHH or Nanobody, that binds a conformational epitope on a protein complex, that is not represented on the individual members of the complex. Alternatively, the ligand may be a binding agent, preferably an immunoglobulin, such as an antibody, or an immunoglobulin fragment, such as a VHH or Nanobody, that binds a conformational epitope on one of the individual members of a protein complex, that is not represented on the protein complex.

The term "specificity", as used herein, refers to the ability of a binding agent, in particular an immunoglobulin or an immunoglobulin fragment, such as a VHH or Nanobody, to bind preferentially to one antigen, versus a different antigen, and does not necessarily imply high affinity.

The terms "specifically bind" and "specific binding", as used herein, generally refers to the ability of a binding agent, in particular an immunoglobulin, such as an antibody, or an immunoglobulin fragment, such as a VHH or Nanobody, to preferentially bind to a particular antigen that is present in a homogeneous mixture of different antigens. In certain embodiments, a specific binding interaction will discriminate between desirable and undesirable antigens in a sample, in some embodiments more than about 10 to 100-fold or more (e.g., more than about 1000- or 10,000-fold). Within the context of the invention, the terms particularly refer to the ability of a binding agent to preferentially recognize and/or bind to a protein complex and not to the individual interacting members.

As used herein, the term “conformation-selective binding agent” refers to a binding agent that binds to a target protein in a conformation-selective manner. A binding agent that selectively binds to a particular conformation of a protein refers to a binding agent that binds with a higher affinity to a protein in a subset of conformations than to other conformations that the protein may assume. One of skill in the art will recognize that binding agents that selectively bind to a specific conformation will stabilize or retain the protein in this particular conformation. For example, a conformation-selective binding agent for a protein complex will preferentially bind to the protein complex and will not or to a lesser degree bind to any of the individual members of the complex, and will thus have a higher affinity for said protein complex conformation; or vice versa. The terms “specifically bind”, “selectively bind”, ”preferentially bind”, and grammatical equivalents thereof, are used interchangeably herein. The terms “conformational specific” or “conformational selective” are also used interchangeably herein.

As used herein, the term "ligand" means a molecule that specifically binds to a protein or a protein complex. A ligand may be, without the purpose of being limitative, a polypeptide, a lipid, a small molecule, an antibody, an antibody fragment, a nucleic acid, a carbohydrate. A ligand may be synthetic or naturally occurring. A ligand also includes a "native ligand" which is a ligand that is an endogenous, natural ligand for a native protein complex. A ligand may be an agonist, a partial agonist, an inverse agonist, an antagonist, an allosteric modulator, and may bind at either the orthosteric site or at an allosteric site. In particular embodiments, a ligand may be a “conformation-selective ligand” or “conformation-specific ligand”, meaning that such a ligand binds the protein complex in a conformation-selective manner. A conformation-selective ligand binds with a higher affinity to a particular conformation of the protein complex than to the individual members of the complex.

The term “antibody” is intended to mean an immunoglobulin or any fragment thereof that is capable of antigen binding. The term “antibody” also refers to single chain antibodies and antibodies with only one binding domain.

As used herein, the terms "complementarity determining region" or "CDR" within the context of antibodies refer to variable regions of either H (heavy) or L (light) chains (also abbreviated as VH and VL, respectively) and contains the amino acid sequences capable of specifically binding to antigenic targets. These CDR regions account for the basic specificity of the antibody for a particular antigenic determinant structure. Such regions are also referred to as "hypervariable regions." The CDRs represent non-contiguous stretches of amino acids within the variable regions but, regardless of species, the positional locations of these critical amino acid sequences within the variable heavy and light chain regions have been found to have similar locations within the amino acid sequences of the variable chains. The variable heavy and light chains of all canonical antibodies each have 3 CDR regions, each non- contiguous with the others (termed L1, L2, L3, H1, H2, H3) for the respective light (L) and heavy (H) chains. Immunoglobulin single variable domains, in particular Nanobodies, generally comprise a single amino acid chain that can be considered to comprise 4 “framework sequences or regions” or FRs and 3 “complementary determining regions” or CDRs. The nanobodies have 3 CDR regions, each non-contiguous with the others (termed CDR1, CDR2, CDR3). The delineation of the FR and CDR sequences can, for example, be based on the IMGT unique numbering system for V-domains and V-like domains (Lefranc et al. 2003, Developmental and Comparative Immunology 27:55).

As used herein, the terms “determining”, ”measuring”, ”assessing”, “monitoring” and “assaying” are used interchangeably and include both quantitative and qualitative determinations.

As used herein, the term “cell sorting” refers to a technique by which individual cells of a sample are separated according to their properties, including intracellular or extracellular properties. Currently there are several methods for cell sorting. The three major types of cell sorting are fluorescent activated cell sorting (FACS), magnetic cell selection, including magnetic activated cell sorting (MACS), and single cell sorting.

Other definitions may be found in the detailed description.

DETAILED DESCRIPTION

In various embodiments, the invention provides a method to select conformation-selective binding agents that are specific for a protein complex. These binding agents specifically bind conformational epitopes that are prevalent in a protein complex structure and not represented on the individual members of the complex. These binding agents have lower affinity for the structures of the individual members of the complex. These binding agents can be used to stabilize a protein complex in a conformation that is otherwise only transiently formed, whereby the stabilization occurs via a

monovalent interaction with the complex of interest (or in other words, a multispecific or multivalent type of interaction is not required for such stabilization of a protein complex of interest). For instance, and without the purpose of being limitative, a conformation-selective binding agent can be used to stabilize and purify large quantities of transient protein complexes in a particular conformational state for research purposes, including studies such as X-crystallography. In particular, the conformation-selective binding agent identified by the method of the invention can be used to isolate active or inactive conformations of transient protein complexes, thus eliminating the need for ligands, co-factors, or other molecules. In addition, the conformation-selective binding agents can also be used to isolate active or inactive conformations of one of the constituting members in its complex selective conformation, in absence of its interacting partner(s).

In general terms, the method to select conformation-selective binding agents of a protein complex involves displaying a collection of binding agents at the extracellular surface of a population of cells and using cell sorting to select, from said population of cells, cells displaying binding agents that specifically bind to a protein complex and not to the individual members of the complex.

The herein described selection method may be performed on any type of macromolecular complex, including protein complexes (as defined herein), such as a protein-protein complex or a protein-nucleic acid complex. For example, a protein complex may be an interaction of one or more membrane receptors with one or more intracellular proteins (e.g. a GPCR with a G protein and/or β-arrestin[10]), an interaction of two or more membrane receptors (e.g. a dimeric/multimeric complex of receptor tyrosine kinases[11]), an interaction of two or more intracellular proteins, an interaction of a DNA binding protein and a DNA molecule, etc.

In a preferred embodiment, the selection method is performed on a protein-protein complex that is constituted of at least two interacting monomeric proteins. The terms “protein-protein complex” and “protein-protein interaction” (PPI), which are used interchangeably herein, refer to a non-covalent interaction that can be formed between two or more monomeric proteins under physiological conditions. Herein, said two or more monomeric proteins are also referred to as the “individual members” or “members” of the protein-protein interaction. It should be clear that multimeric protein-protein complexes are also envisaged here, comprising more than two interacting monomeric proteins.

For the sake of clarity, a protein-protein interaction can be an interaction between identical or non-identical member proteins (homo-multimeric vs. hetero-multimeric protein-protein complex, respectively).

Even more preferably, the invention envisages a method to select for conformation-selective binding agents that specifically bind to a transient protein-protein complex. The term “transient protein-

protein interaction" or "transient protein-protein complex", as used herein, refers to a protein-protein interaction that, under physiological conditions, can either associate into a complex hereby adopting a particular conformation or dissociate into a free form of two or more individual members that can stably exist on their own. Thus, in a transient interaction, a protein may interact briefly and in a reversible manner with other proteins in certain cellular contexts – cell type, cell cycle stage, external factors, presence of other binding proteins, etc. – as it happens with most of the proteins involved in biochemical cascades. For example, G protein-coupled receptors only transiently bind to G proteins when they are activated by extracellular ligands. Transient protein-protein interactions can be either weak or strong, depending on subcellular localization, physiological conditions, environment etc. Typically, weak transient protein-protein interactions refer to transient complexes that under physiological conditions show a dynamic mixture of different oligomeric states. Strong transient protein-protein interactions refer to transient complexes that change their quaternary structure only when triggered for example by ligand binding. For the sake of clarity, a transient protein-protein interaction is different from a "permanent protein-protein interaction" which is a protein-protein interaction that only exists in its complex form. Permanent or stable interactions involve proteins that interact for a long time, taking part of permanent complexes as subunits, in order to carry out structural or functional roles. These are usually the case of homo-oligomers (e.g. cytochrome c), and some hetero-oligomeric proteins, as the subunits of ATPase.

A member protein forming part of a (transient) protein-protein interaction that can be used in the herein described selection method, can be an intracellular protein, such as a small GTPase, a kinase, a phosphatase, etc., or a (trans)membrane protein, such as a receptor protein, e.g. a GPCR, an ion channel, transport proteins etc., or a secreted protein such as a hormone, etc. The nature of the member protein that forms part of a (transient) protein-protein interaction is not critical to the invention and can be from any organism including a fungus (including yeast), nematode, virus, insect, plant, bird (e.g. chicken, turkey), reptile or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, camelid, or human). Preferably, the member protein is of mammalian origin, even more preferably of human origin. Also, a member protein forming part of a (transient) protein-protein interaction may be naturally occurring or non-naturally occurring (i.e., altered or designed by man), as long as protein complex formation can still occur. Mutants or variants of naturally-occurring proteins are examples of non-naturally occurring proteins. Non-naturally occurring proteins may have an amino acid sequence that is at least 70% identical to, at least 80% identical to, at least 90% identical to, at least 95% identical to or at least 99% identical to, a naturally-occurring protein. In one specific embodiment, a member protein may have a deletion (e.g. N- and/or C-terminal deletion, loop deletion, etc.), or a substitution, or an insertion or addition in relation to its

amino acid or nucleotide sequence, or any combination thereof. In addition, the term “non-naturally occurring” is intended to encompass wild-type polymorphic variants, interspecies homologues, and alleles of member proteins forming part of a (transient) protein-protein interaction according to the invention.

The current method can be applied on any protein complex that is described in the art[5]. Molecular interactions can occur between molecules belonging to different biochemical families (proteins, nucleic acids, lipids, carbohydrates, etc.) and also within a given family. Whenever such molecules are connected by physical interactions, they form molecular interaction networks that are generally classified by the nature of the compounds involved. In general, “interactome” refers to an interaction network, such as “protein–protein interaction networks” (for example the human interactome[12]) or subsets thereof. Another extensively studied type of interactome is the protein–DNA interactome, also called a ”gene-regulatory network”, a network formed by transcription factors, chromatin regulatory proteins, and their target genes. Notably, there are a multitude of methods to detect currently unknown interactions. For example, methods to detect protein-protein interactions which are known by the person skilled in the art and reviewed in e.g. V. Srinivasa Rao et al. (2014), Protein-Protein Interaction Detection: Methods and Analysis. Int J Proteomics 2014: 147648, and incorporated herein by reference. Protein-protein interaction detection methods are categorically classified into three types, namely, *in vitro*, *in vivo*, and *in silico* methods. In *in vitro* techniques, a given procedure is performed in a controlled environment outside a living organism. The *in vitro* methods in PPI detection are tandem affinity purification, affinity chromatography, coimmunoprecipitation, protein arrays, protein fragment complementation, phage display, X-ray crystallography, NMR spectroscopy, and proximity based methods like Fluorescence Resonance Energy Transfer (FRET), Bioluminescence Resonance Energy Transfer (BRET), Amplified Luminescent Proximity Homogeneous Assay Screen (ALPHA Screen). In *in vivo* techniques, a given procedure is performed on the whole living organism itself. The *in vivo* methods in PPI detection are yeast two-hybrid (Y2H, Y3H) and synthetic lethality. *In silico* techniques are performed on a computer or via computer simulation. The *in silico* methods in PPI detection are sequence-based approaches, structure-based approaches, chromosome proximity, gene fusion, *in silico* 2 hybrid, mirror tree, phylogenetic tree, and gene expression-based approaches. The most conventional and widely used high-throughput methods are yeast two-hybrid screening and affinity purification coupled to mass spectrometry. It will be understood that the herein described method also encompasses the use of such novel identified protein complex targets.

Alternatively, the method may also be performed on a protein-nucleic acid complex, more specifically a protein-DNA complex or a protein-RNA complex. The term “protein-nucleic acid complex” refers to a

non-covalent interaction that can be formed between at least one monomeric protein and at least one nucleic acid, such as a DNA or an RNA molecule, under physiological conditions. Herein, said at least one monomeric protein and at least one nucleic acid are also referred to as the “individual members” or “members” of the protein-nucleic acid interaction. It should be clear that multimeric protein-nucleic acid complexes are also envisaged here.

Although in principle any collection or library of binding agents may contain conformation-selective binding agents against protein complexes, a preferred method is to generate an immune library of binding agents, in particular an immune library of antibodies or antibody fragments, by immunizing an animal with an (optionally cross-linked) protein complex to expose the immune system of the animal with the conformational epitopes that are unique to the complex. In a preferred embodiment of the method, an immune library of immunoglobuling single variable domains (as defined hereafter) is generated.

In principle, animals could be immunized with mixtures of the interacting members of a protein complex. However, considering the short half-life of many protein complexes, which is particularly the case for transient PPIs (0,1 to 1s), it is preferred to stabilize transient complexes by chemical cross-linking of the individual interacting members of the complex. In this way, animals can be immunized with antigens that are in a covalent association that is very similar to the transient complex to trigger and mature immunoglobulins that bind conformational epitopes of this transient complex. Methods for cross-linking members of protein-protein complexes or protein-nucleic acid complexes may be performed in accordance with any of the techniques known to those skilled in the art. Obviously, regardless of the reagent used, it is required that the reaction proceeds under conditions that preserve the native state of the protein complex. Reagents and protocols are reviewed in e.g. Leitner, A. et al. Probing Native Protein Structures by Chemical Cross-linking, Mass Spectrometry, and Bioinformatics. Molecular & Cellular Proteomics 9, 1634-1649 (2010); Bich, C. et al. Reactivity and applications of new amine reactive cross-linkers for mass spectrometric detection of protein-protein complexes. Anal Chem 82, 172-9 (2010), all of which are incorporated herein by reference.

Chemical cross-linking reagents known in the art may be classified in several categories according to their reactivity (*e.g.* amine- or thiol-reactive and homo- and heterobifunctional) or the incorporation of additional functional groups (*e.g.* cleavable sites and affinity tags). Conventional chemical cross-linking reagents consists of two reactive sites connected through a spacer or linker region, typically an alkyl chain. Most commonly, the reactive groups of cross-linkers target the primary amino group of lysine (and the protein N termini). For this purpose, *N*-hydroxysuccinimidyl or sulfosuccinimidyl esters are almost exclusively used. Common succinimide-type linkers are disuccinimidyl suberate (DSS; six-carbon

linker) and disuccinimidyl glutarate (DSG; three-carbon linker) as well as their sulfo analogs bis(sulfosuccinimidyl) suberate (BS^3) and bis(sulfosuccinimidyl) glutarate. Lysine cross-linking has several advantages, including the high prevalence of Lys residues (about 6%) and relatively high reaction specificity. Similar specific cross-linking reactions can be carried out when targeting cysteine residues, *e.g.* by maleimides, but the low abundance of Cys (<2%) makes this less attractive. Other cross-linking chemistries include arginine-specific cross-linking or acidic cross-linking (Zhang et al. 2008, Nested Arg-specific bifunctional crosslinkers for MS-based structural analysis of proteins and protein assemblies. Anal. Chim. Acta 627, 117–128; Novak P., and Kruppa G. H., 2008, Intra-molecular cross-linking of acidic residues for protein structure studies. Eur. J. Mass Spectrom. 14, 355–365). Glutaraldehyde is also frequently used in biochemistry applications as an amine-reactive homobifunctional crosslinker. In addition to homobifunctional cross-linkers, several heterobifunctional linkers have been described. These may incorporate two different reactive groups, *e.g.* Lys- and Cys-reactive, or may combine different cross-linking concepts, *e.g.* chemical and photoinduced cross-linking. Also encompassed as a suitable cross-linking reagent is formaldehyde, which only contains a single aldehyde group but is able to connect two amino acid side chains via a two-step reaction. Formaldehyde is a less specific reagent, although lysine and tryptophan residues are primarily targeted (Sutherland B. W., et al. 2008, Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. J. Mass Spectrom. 43, 699–715; Toews J., et al. 2008, Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. Anal. Chim. Acta 618, 168–183).

Functionalized cross-linking reagents include linkers carrying stable isotope labels, affinity tags, or moieties that give characteristic fragmentation patterns in tandem mass spectrometry experiments. For example, different stable isotope-labeled cross-linking reagents such as DSS or BS^3 are commercially available from suppliers such as Creative Molecules and the Pierce division of Thermo Scientific, and more complex reagents have also been prepared in labeled form. Amongst the affinity-tagged cross-linking reagents, biotin is most frequently used as the affinity group, allowing the isolation of modified peptides by avidin affinity chromatography (Trester-Zedlitz et al. (2003) A Modular Cross-Linking Approach for Exploring Protein Interactions. J. Am. Chem. Soc. 125, 2416–2425; Kang et al. et al. 2009, Synthesis of biotin-tagged chemical cross-linkers and their applications for mass spectrometry. Rapid Commun. Mass Spectrom. 23, 1719–1726). Other examples include an azide-containing cross-linking reagent (Nessen M. A., et al. 2009, Selective enrichment of azide-containing peptides from complex mixtures. J. Proteome Res. 8, 3702–3711). Another variety of functionalized reagents uses linkers with specially designed fragmentation properties. Most frequently, these linkers contain labile bonds that are easily cleaved during collision-induced dissociation (Tang X. et al. 2005,

Mass spectrometry identifiable cross-linking strategy for studying protein-protein interactions. Anal. Chem. 77, 311–318; Chowdhury et al. 2009, Identification of cross-linked peptides after click-based enrichment using sequential collision-induced dissociation and electron transfer dissociation tandem mass spectrometry. Anal. Chem. 81, 5524–5532; Zhang H., et al. 2009, Identification of protein-protein interactions and topologies in living cells with chemical cross-linking and mass spectrometry. Mol. Cell. Proteomics 8, 409–420).

Methods of immunization are well-known in the art. For the immunization of an animal with a (cross-linked) protein complex, the complex may be produced and purified using conventional methods that may employ expressing a recombinant form of the protein complex in a host cell, and purifying the protein complex using affinity chromatography and/or antibody-based methods. In particular embodiments, the baculovirus/Sf-9 system may be employed for expression, although other expression systems (e.g., bacterial, yeast or mammalian cell systems) may also be used. Methods for purifying protein complexes comprising membrane proteins are described in, for example, Kobilka, B. K., Anal Biochem 231, 269-271 (1995), Eroglu et al EMBO 2002 3: 491^96, Chelikani et al Protein Sci. 2006 15:1433-40 and the book "Identification and Expression of G Protein-Coupled Receptors" (Kevin R. Lynch (Ed.), 1998), among many others. Such membrane protein complexes may be reconstituted in phospholipid vesicles. Likewise, methods for reconstituting membrane proteins in phospholipid vesicles are known, and are described in: Luca et al Proc. Natl. Acad. Sci. 2003 100 :10706-l 1, Mansoor et al Proc. Natl. Acad. Sci. 2006 103: 3060-3065, Niu et al, Biophys J. 2005 89: 1833-1840, Shimada et al J. Biol. Chem. 2002 277:31774-80, and Eroglu et al Proc. Natl. Acad. Sci. 2003 100: 10219-10224, among others. Methods for recombinant expression[13] and purifying protein complexes[14] of soluble proteins are well-known in the art. Other immunization methods include, without limitation, the use of complete cells expressing a protein complex or fractions thereof, immunization with viruses or virus like particles expressing a protein complex of interest, amongst others (e.g. as described in WO2010070145, WO2011083141). Any suitable animal, in particular a mammal such as a rabbit, mouse, rat, camel, sheep, cow, shark, pig, amongst others, or a bird such as a chicken or turkey, may be immunized using any of the techniques well known in the art suitable for generating an immune response.

In one embodiment, the method as described herein involves displaying a collection of binding agents, preferably an immune library, at the extracellular surface of a population of cells. Surface display methods are reviewed in [15] and include bacterial display, yeast display, mammalian display.

Preferably, the population of cells are yeast cells. Any means to display a protein on the surface of yeast is encompassed by the present disclosure. The different yeast surface display methods all provide

a means of tightly linking each binding agent encoded by the library to the extracellular surface of the yeast cell which carries the plasmid encoding that protein. Most yeast display methods described to date use the yeast *Saccharomyces cerevisiae*, but other yeast species, for example, *Pichia pastoris*, could also be used. More specifically, in some embodiments, the yeast strain is from a genus selected from the group consisting of *Saccharomyces, Pichia, Hansenula, Schizosaccharomyces, Kluyveromyces, Yarrowia*, and *Candida*. In some embodiments, the yeast species is selected from the group consisting of *S. cerevisiae, P. pastoris, H. polymorpha, S. pombe, K. lactis, Y. lipolytica,* and *C. albicans*.

Most yeast expression fusion proteins are based on GPI (Glycosyl-Phosphatidyl-Inositol) anchor proteins which play important roles in the surface expression of cell-surface proteins and are essential for the viability of the yeast. One such protein, alpha-agglutinin consists of a core subunit encoded by AGA1 and is linked through disulfide bridges to a small binding subunit encoded by AGA2. Proteins encoded by the nucleic acid library can be introduced on the N-terminal region of AGA1 or on the C-terminal or N-terminal region of AGA2. Both fusion patterns will result in the display of the polypeptide on the yeast cell surface.

In some embodiments, fusion proteins for yeast display include an engineered protein fused to the N-terminal or C-terminal part of a protein capable of anchoring in a eukaryotic cell wall (e.g., α-agglutinin, AGA1, Flo1 or major cell wall protein of lower eukaryotes, see U.S. Pat. Nos. 6,027,910 and 6,114,147 which are hereby incorporated by reference), for example, proteins fused with the GPI fragment of Flo1 or to the Flo1 functional domain (Kondo et al., Appl. MicroBiol. Biotechn., 2004, 64: 28-40).

In certain embodiments, a method that relies on in vivo biotinylation of the protein to be displayed, followed by its capture on the yeast cell surface is used. For example, the protein to be displayed is genetically fused to a yeast secretory protein of choice and to a biotin-acceptor peptide (BAP). An epitope tag, such HA or FLAG®, is also engineered immediately downstream from the sequence encoding the protein variant. Common secretory proteins include yeast alpha mating factor prepro 1 (WTαMFpp), the invertase leader, synthetic leaders (Clements et al., *Gene*, 106:267-271 (1991)), and the engineered alpha mating factor prepro αMFpp8 (Rakestraw et al., *Biotechnol. Bioeng.*, 103:1192-1201 (2009)). The gene encoding the fusion may be controlled by an inducible promoter, such as, for example, the galactose-inducible promoter, GAL1-10. Before inducing expression of the protein to be displayed, the outside surface of the yeast cell is chemically conjugated to avidin. Upon induction, the biotin-acceptor peptide in the fusion protein is biotinylated inside the cell by a co-expressed biotin ligase. The biotinylated fusion protein is then secreted from the cell and captured on the cell-surface avidin due to the extremely high-affinity interaction between avidin and biotin.

In certain embodiments, the protein variants to be displayed are genetically fused to a GPI (Glycosyl-Phosphatidyl-Inositol) anchor protein, such as the mating type protein agglutinin-a-1 (Aga1), flocculin proteins (e.g., Flo1), as well as Sed1, Cwp1, Cwp2, Tip1and Tir1/Srp1. In certain embodiments, the anchor protein is selected from the group consisting of a GP1 anchor, a modified GP1 anchor, a major cell wall protein, CCW14, CIS3, CWP1, PIR1, and PIR3.

It is also contemplated that the methods disclosed herein are carried out using mammalian host cells. Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, Chinese hamster ovary cells/−DHFR(CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216 (1980)), and CHO cells engineered to produce controlled fucosylation (MAbs. 1(3):230-36 (2009)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham et al., J. Gen Virol. 36: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, (Biol. Reprod. 23: 243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., Annals N.Y. Acad. Sci. 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

When the host cell is a mammalian cell, examples of portions of cell surface proteins that retain the ability to display proteins on the cell surface include suitable transmembrane domain of any known cell membrane proteins, or a polypeptide with a GPI anchor sequence, or a non-cleavable type II signal anchor sequence. Examples of membrane anchor sequences used for cell display in mammalian cells include PDGFR transmembrane domain (Chesnut et al., J Immunol Methods 193(1): 17-27, (1996); Ho et al., Proc Natl Acad Sci USA 103(25): 9637-42, (2006); incorporated by reference in their entirety), GPI anchor from human decay-accelerating factor (Akamatsu et al., J Immunol Methods, 327(1-2): 40-52 (2007); incorporated by reference in its entirety) and T-cell receptor (TCR) chain (Alonso-Camino et al., PLoS One 4(9): e7174 (2009); incorporated by reference in its entirety). Another example is the use of type II signal anchor sequences (U.S. Pat. No. 7,125,973; incorporated by reference in its entirety). Alternatively, a capture molecule such as an antibody or protein can be fused to a membrane anchor sequence, and displayed on the cell surface in order to capture the protein of interest (U.S. Pat. No. 6,919,183; incorporated by reference in its entirety). In certain embodiments, an artificial cell surface anchor sequence is assembled into, or attached to, the cell membrane of mammalian cells.

The methods disclosed herein may also be carried out using prokaryotic host cells. Thus, in some or any embodiments, the host cell is a prokaryotic cell. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis (e.g., B. licheniformnis 41 P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as P. aeruginosa, and Streptomyces. One preferred E. coli cloning host is E. coli 294 (ATCC 31,446), although other strains such as E. coli B, E. coli X1776 (ATCC 31,537), and E. coli W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

When the host cell is a prokaryotic cell, examples of suitable cell surface proteins include suitable bacterial outer membrane proteins. Such outer membrane proteins include pili and flagella, lipoproteins, ice nucleation proteins, and autotransporters. Exemplary bacterial proteins used for heterologous protein display include LamB (Charbit et al., EMBO J, 5(11): 3029-37 (1986); incorporated by reference in its entirety), OmpA (Freudl, Gene, 82(2): 229-36 (1989); incorporated by reference in its entirety) and intimin (Wentzel et al., J Biol Chem, 274(30): 21037-43, (1999); incorporated by reference in its entirety). Additional exemplary outer membrane proteins include, but are not limited to, FliC, pullulunase, OprF, OprI, PhoE, MisL, and cytolysin. An extensive list of bacterial membrane proteins that have been used for surface display and are contemplated for use in the present invention are detailed in Lee et al., Trends Biotechnol, 21(1): 45-52 (2003), Jose, Appl Microbiol Biotechnol, 69(6): 607-14 (2006), and Daugherty, Curr Opin Struct Biol, 17(4): 474-80 (2007), all incorporated by reference in their entirety. In certain embodiments, the anchor protein is an artificial sequence that is assembled into, or attaches to the outer surface of the bacterial cell.

In a preferred embodiment of the herein described selection method, at least one of the individual members of the protein complex is distinguishably labeled, so to allow detection and separation via cell sorting of cells displaying binding agents that

i. specifically bind to a protein complex and not to the individual members of the complex, and/or

ii. specifically bind to one of the individual members of a protein complex and not to the protein complex nor to the other individual member(s) of the complex, and/or

iii. specifically bind to one of the individual members of a protein complex and to a protein complex and not to the other individual member(s) of the complex.

Libraries of cells displaying binding agents on the surface are screened for antigen binding using cell sorting, e.g by either magnetic activated cell sorting (MACS) or fluorescent activated cell sorting (FACS).

The current method thus involves a step of incubating a mixture of distinguishably tagged individual members of a protein complex with the population of cells under suitable conditions to allow binding to the cells.

In a preferred embodiment, cell sorting will be performed by FACS. In this scenario, it is preferred that at least one of the individual members of the protein complex is labeled with a fluorophore that can be detected in FACS with any technique known to the person skilled in the art. For example, this can be achieved by recombinant expression of a member protein with a fluorescent tag (GFP, YFP, etc.). Proteins can also be indirectly labeled by incubating with a fluorescent Ab conjugate / cascade directed against a tag (e.g. FLAG, polyhistidine, etc.) or the protein itself. In a preferred embodiment, the individual member is directly labeled via random covalent coupling of fluorescent dyes (e.g. Alexa, Dylight) to amine or cysteine reactive groups of the protein. In an alternative embodiment, the cell sorting is performed with MACS. In such a scenario, it is preferred that at least one of the individual members of the protein complex is labeled with a magnetic label, for example via streptavidin or Ab conjugated magnetic beads.

FACS or MACS can be used simultaneously (1) to analyse the properties of each binding agent that is displayed multivalently on an individual cell and (2) to selectively recover cells displaying binding agents with a particular property (e.g. Figure 2). Cells displaying binding agents that selectively bind a first member (A in Figure 2) but not a second member (e.g. B in Figure 2) or the protein complex (AB in Figure 2) can be enriched if cells of the top-left quadrant are recovered by FACS. Cells displaying binding agents that selectively bind the second member (B in Figure 2) but not the first member (e.g. A in Figure 2) or the protein complex (AB in Figure 2) can be enriched if cells of the bottom-right quadrant are recovered by FACS. Cells displaying binding agents that selectively bind the protein complex (AB in Figure 2) but not to the first (A in Figure 2) alone or the second member (B in Figure 2) alone can be enriched if cells of the top-right quadrant are recovered by FACS. These processes can be repeated in several rounds to enrich for cells displaying binding agents with the desired characteristics. The selection of appropriate conditions for cell sorting by FACS or MACS is well within the skill in the art and illustrated, in a non-limiting way, in the Example section.

In additional embodiments, the current selection method may involve additional steps to further characterize the binding properties of the binding agent, for example and without limitation, a step of affinity maturation, a step of expressing the desired amino acid sequence, a step of screening for binding and/or for activity against the desired antigen (in this case, the protein complex and/or the individual members of the complex), a step of determining the desired amino acid sequence or

nucleotide sequence, a step of introducing one or more humanizing substitutions, a step of formatting in a suitable multivalent and/or multispecific format, a step of screening for the desired biological and/or physiological properties (i.e. using a suitable assay known in the art), and/or any combination of one or more of such steps, in any suitable order.

Various methods may be used to determine specific binding (as defined hereinbefore) between the binding agent and a target protein complex, including for example, enzyme linked immunosorbent assays (ELISA), flow cytometry, radioligand binding assays, surface plasmon resonance assays, phage display, and the like, which are common practice in the art, for example, in discussed in Sambrook et al. (2001), Molecular Cloning, A Laboratory Manual. Third Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and are further illustrated in the Example section. It will be appreciated that for this purpose often a unique label or tag will be used, such as a peptide label, a nucleic acid label, a chemical label, a fluorescent label, or a radio frequency tag, as described hereafter.

The conformation-selective binding agents in the above described method may also be modified and/or may comprise (or can be fused to) other moieties. Examples of modifications, as well as examples of amino acid residues within the binding agent of the invention that can be modified (i.e. either on the protein backbone but preferably on a side chain), methods and techniques that can be used to introduce such modifications and the potential uses and advantages of such modifications will be clear to the skilled person. For example, such a modification may involve the introduction (e.g. by covalent linking or in another suitable manner) of one or more functional groups, residues or moieties into or onto the binding agent. Examples of such functional groups and of techniques for introducing them will be clear to the skilled person, and can generally comprise all functional groups and techniques mentioned in the art as well as the functional groups and techniques known per se for the modification of pharmaceutical proteins, and in particular for the modification of antibodies or antibody fragments (including ScFv's and single domain antibodies), for which reference is for example made to Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980). Such functional groups may for example be linked directly (for example covalently) to the binding agent, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

In the case a binding agent is of potential therapeutic value, one of the most widely used techniques for increasing the half-life and/or reducing immunogenicity of pharmaceutical proteins comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv's); reference is made to for example

Chapman, Nat. Biotechnol., 54, 531-545 (2002); by Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003), by Harris and Chess, Nat. Rev. Drug. Discov., 2, (2003) and in WO04060965. Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA. Preferably, site-directed pegylation is used, in particular via a cysteine-residue (see for example Yang et al., Protein Engineering, 16, 10, 761-770 (2003). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in an binding agent, or the binding agent may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus of an binding agent, all using techniques of protein engineering known per se to the skilled person. Preferably, for the binding agents of the invention, a PEG is used with a molecular weight of more than 5000, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the range of 20,000-80,000. Another, usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the immunoglobulin single variable domain or polypeptide of the invention. Another technique for increasing the half-life of a binding agent may comprise the engineering into bifunctional constructs (for example, one Nanobody against the target opioid receptor and one against a serum protein such as albumin) or into fusions of binding agents with peptides (for example, a peptide against a serum protein such as albumin).

A usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the selected binding agents.

Yet another modification may comprise the introduction of one or more detectable labels or other signal-generating groups or moieties, depending on the intended use of the labeled binding agent. Suitable labels and techniques for attaching, using and detecting them will be clear to the skilled person, and for example include, but are not limited to, fluorescent labels, (such as IRDye800, VivoTag800, fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine and fluorescent metals such as Eu or others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes, metals, metals chelates or metallic cations or other metals or metallic cations that are particularly suited for use in in vivo, in vitro or in situ diagnosis and imaging, as well as chromophores and enzymes (such as malate dehydrogenase, staphylococcal nuclease, delta- V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase,

asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels will be clear to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy. Such labeled binding agents of the invention may for example be used for *in vitro*, *in vivo* or *in situ* assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays", etc.) as well as *in vivo* diagnostic and imaging purposes, depending on the choice of the specific label. As will be clear to the skilled person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable chelating groups for example include, without limitation, 2,2',2''-(10-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DOTA), 2,2'-(7-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (NOTA), diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the binding agent to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a binding agent of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated binding agent may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may for example also be used to bind the binding agent of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example is the liposomal formulations described by Cao and Suresh, Journal of Drug Targetting, 8, 4, 257 (2000). Such binding pairs may also be used to link a therapeutically active agent to the binding agent of the invention.

In case binding agents are modified by linking particular functional groups, residues or moieties (as described hereinabove) to the binding agent, then often linker molecules will be used. Preferred “linker molecules” or “linkers” are peptides of 1 to 200 amino acids length, and are typically, but not necessarily, chosen or designed to be unstructured and flexible. For instance, one can choose amino acids that form no particular secondary structure. Or, amino acids can be chosen so that they do not form a stable tertiary structure. Or, the amino acid linkers may form a random coil. Such linkers include, but are not limited to, synthetic peptides rich in Gly, Ser, Thr, Gln, Glu or further amino acids that are frequently associated with unstructured regions in natural proteins (Dosztányi, Z., Csizmok, V., Tompa, P., & Simon, I. (2005). IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics (Oxford, England), 21(16), 3433-

4.). Non-limiting examples of suitable linker sequences include (GS)5 (GSGSGSGSGS), (GS)10 (GSGSGSGSGSGSGSGSGSGS), (G4S)3 (GGGGSGGGGSGGGGS), llama IgG2 hinge (AHHSEDPSSKAPKAPMA) or human IgA hinge (SPSTPPTPSPSTPPAS) linkers. For certain applications, it may be advantageous that the linker molecule comprises or consists of one or more particular sequence motifs. For example, a proteolytic cleavage site can be introduced into the linker molecule such that detectable label or moiety can be released. Useful cleavage sites are known in the art, and include a protease cleavage site such as Factor Xa cleavage site having the sequence IEGR, the thrombin cleavage site having the sequence LVPR, the enterokinase cleaving site having the sequence DDDDK, or the PreScission -or 3C- cleavage site LEVLFQGP.

Alternatively, in case the binding agent is linked to a detectable label or moiety using chemoenzymatic methods for protein modification, the linker moiety may exist of different chemical entities, depending on the enzymes or the synthetic chemistry that is used to produce the covalently coupled molecule in vivo or in vitro (reviewed in: Rabuka 2010, Curr Opin Chem Biol 14: 790-796).

According to a preferred embodiment, it is particularly envisaged that the binding agents in the above method are derived from an innate or adaptive immune system. Preferably, said binding agent is derived from an immunoglobulin. Preferably, the binding agent according to the invention is derived from an antibody or an antibody fragment. The term "antibody" (Ab) refers generally to a polypeptide encoded by an immunoglobulin gene, or a functional fragment thereof, that specifically binds and recognizes an antigen, and is known to the person skilled in the art. An antibody is meant to include a conventional four-chain immunoglobulin, comprising two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50 kDa). Typically, in conventional immunoglobulins, a heavy chain variable domain (VH) and a light chain variable domain (VL) interact to form an antigen binding site. The term “antibody” is meant to include whole antibodies, including single-chain whole antibodies, and antigen-binding fragments. In some embodiments, antigen-binding fragments may be antigen-binding antibody fragments that include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (dsFv) and fragments comprising or consisting of either a VL or VH domain, and any combination of those or any other functional portion of an immunoglobulin peptide capable of binding to the target antigen. The term “antibodies” is also meant to include heavy chain antibodies, or fragments thereof, including immunoglobulin single variable domains, as defined further herein.

The term “immunoglobulin single variable domain” defines molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain (which is different from conventional immunoglobulins or their fragments, wherein typically two immunoglobulin variable domains interact

to form an antigen binding site). It should however be clear that the term "immunoglobulin single variable domain" does comprise fragments of conventional immunoglobulins wherein the antigen binding site is formed by a single variable domain. Preferably, the binding agent is an immunoglobulin single variable domain.

Generally, an immunoglobulin single variable domain will be an amino acid sequence comprising 4 framework regions (FR1 to FR4) and 3 complementary determining regions (CDR1 to CDR3), preferably according to the following formula (1):

FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 (1)

or any suitable fragment thereof (which will then usually contain at least some of the amino acid residues that form at least one of the complementarity determining regions). Immunoglobulin single variable domains comprising 4 FRs and 3 CDRs are known to the person skilled in the art and have been described, as a non-limiting example, in Wesolowski, et al. (2009), Med. Microbiol. Immunol. 198, 157-174. Typical, but non-limiting, examples of immunoglobulin single variable domains include light chain variable domain sequences (e.g. a VL domain sequence) or a suitable fragment thereof, or heavy chain variable domain sequences (e.g. a VH domain sequence or VHH domain sequence) or a suitable fragment thereof, as long as it is capable of forming a single antigen binding unit. Thus, according to a preferred embodiment, the binding agent is an immunoglobulin single variable domain that is a light chain variable domain sequence (e.g. a VL domain sequence) or a heavy chain variable domain sequence (e.g. a VH domain sequence); more specifically, the immunoglobulin single variable domain is a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or a heavy chain variable domain sequence that is derived from a heavy chain antibody. The immunoglobulin single variable domain may be a domain antibody, or a single domain antibody, or a "dAB" or dAb, or a Nanobody (as defined herein), or another immunoglobulin single variable domain, or any suitable fragment of any one thereof. For a general description of single domain antibodies, reference is made to the following book: "Single domain antibodies", Methods in Molecular Biology, Eds. Saerens and Muyldermans, 2012, Vol 911. The immunoglobulin single variable domains, generally comprise a single amino acid chain that can be considered to comprise 4 "framework sequences" or FR's and 3 "complementary determining regions" or CDR's (as defined hereinbefore). It should be clear that framework regions of immunoglobulin single variable domains may also contribute to the binding of their antigens (Desmyter et al, J Biol Chem. 2002 Jun 28;277(26):23645-50; Korotkov et al, Structure. 2009 Feb 13;17(2):255-65). The delineation of the CDR sequences (and thus also the FR sequences) can be based on the IMGT unique numbering system for V-domains and V-like domains (Lefranc et al.

2003, Developmental and Comparative Immunology 27:55). Alternatively, the delineation of the FR and CDR sequences can be done by using the Kabat numbering system as applied to VHH domains from Camelids in the article of Riechmann and Muyldermans (J. Immunol. Methods 2000; 240: 185-195).

It should be noted that the immunoglobulin single variable domains as binding agent in their broadest sense are not limited to a specific biological source or to a specific method of preparation. The term "immunoglobulin single variable domain" encompasses variable domains of different origin, comprising mouse, rat, rabbit, donkey, human, shark, camelid variable domains. According to specific embodiments, the immunoglobulin single variable domains are derived from shark antibodies (the so-called immunoglobulin new antigen receptors or IgNARs), more specifically from naturally occurring heavy chain shark antibodies, devoid of light chains, and are known as VNAR domain sequences. Preferably, the immunoglobulin single variable domains are derived from camelid antibodies. More preferably, the immunoglobulin single variable domains are derived from naturally occurring heavy chain camelid antibodies, devoid of light chains, and are known as VHH domain sequences or Nanobodies.

According to a particularly preferred embodiment, the binding agent in the above method is an immunoglobulin single variable domain that is a Nanobody (as defined further herein, and including but not limited to a VHH). The term "Nanobody" (Nb), as used herein, is a single domain antigen binding fragment. It particularly refers to a single variable domain derived from naturally occurring heavy chain antibodies and is known to the person skilled in the art. Nanobodies are usually derived from heavy chain only antibodies (devoid of light chains) seen in camelids (Hamers-Casterman et al, Nature 363, 446-448 (1993); Desmyter et al. Nat Struct Biol. 1996 Sep;3(9):803-11) and consequently are often referred to as VHH antibody or VHH sequence. Camelids comprise old world camelids (*Camelus bactrianus* and *Camelus dromedarius*) and new world camelids (for example *Lama paccos*, *Lama glama, Lama guanicoe* and *Lama vicugna*). Nanobody® and Nanobodies® are registered trademarks of Ablynx NV (Belgium). For a further description of VHH's or Nanobodies, reference is made to the book "Single domain antibodies", Methods in Molecular Biology, Eds. Saerens and Muyldermans, 2012, Vol 911, in particular to the Chapter by Vincke and Muyldermans (2012), as well as to a non-limiting list of patent applications, which are mentioned as general background art, and include: WO 94/04678, WO 95/04079, WO 96/34103 of the Vrije Universiteit Brussel; WO 94/25591, WO 99/37681, WO 00/40968, WO 00/43507, WO 00/65057, WO 01/40310, WO 01/44301, EP 1 134 231 and WO 02/48193 of Unilever; WO 97/49805, WO 01/21817, WO 03/035694, WO 03/054016 and WO 03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO 04/041867, WO 04/041862, WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858, WO 06/40153, WO 06/079372, WO

06/122786, WO 06/122787 and WO 06/122825, by Ablynx N.V. and the further published patent applications by Ablynx N .V. As will be known by the person skilled in the art, the Nanobodies are particularly characterized by the presence of one or more Camelidae “hallmark residues” in one or more of the framework sequences (according to Kabat numbering), as described for example in WO 08/020079, on page 75, Table A-3, incorporated herein by reference). It should be noted that the Nanobodies, of the invention in their broadest sense are not limited to a specific biological source or to a specific method of preparation. For example, Nanobodies, can generally be obtained: (1) by isolating the VHH domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring VHH domain; (3) by "humanization" of a naturally occurring VHH domain or by expression of a nucleic acid encoding a such humanized VHH domain; (4) by "camelization" of a naturally occurring VH domain from any animal species, and in particular from a mammalian species, such as from a human being, or by expression of a nucleic acid encoding such a camelized VH domain; (5) by "camelisation" of a "domain antibody" or "Dab" as described in the art, or by expression of a nucleic acid encoding such a camelized VH domain; (6) by using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences known per se; (7) by preparing a nucleic acid encoding a Nanobody using techniques for nucleic acid synthesis known per se, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of one or more of the foregoing. A further description of Nanobodies, including humanization and/or camelization of Nanobodies, can be found e.g. in WO08/101985 and WO08/142164, as well as further herein.

The term “immunoglobulin single variable domain” also encompasses variable domains that are “humanized” or “camelized”, in particular Nanobodies that are “humanized” or “camelized”. For example both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes a naturally occurring VHH domain or VH domain, respectively, and then changing, in a manner known per se, one or more codons in said nucleotide sequence in such a way that the new nucleotide sequence encodes a "humanized" or "camelized" immunoglobulin single variable domains of the invention, respectively. This nucleic acid can then be expressed in a manner known per se, so as to provide the desired immunoglobulin single variable domains of the invention. Alternatively, based on the amino acid sequence of a naturally occurring VHH domain or VH domain, respectively, the amino acid sequence of the desired humanized or camelized immunoglobulin single variable domains of the invention, respectively, can be designed and then synthesized *de novo* using techniques for peptide synthesis known per se. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring VHH domain or VH domain, respectively, a nucleotide sequence encoding the desired humanized or camelized immunoglobulin single variable domains of the

invention, respectively, can be designed and then synthesized *de novo* using techniques for nucleic acid synthesis known per se, after which the nucleic acid thus obtained can be expressed in a manner known per se, so as to provide the desired immunoglobulin single variable domains of the invention. Other suitable methods and techniques for obtaining the immunoglobulin single variable domains of the invention and/or nucleic acids encoding the same, starting from naturally occurring VH sequences or preferably VHH sequences, will be clear from the skilled person, and may for example comprise combining one or more parts of one or more naturally occurring VH sequences (such as one or more FR sequences and/or CDR sequences), one or more parts of one or more naturally occurring VHH sequences (such as one or more FR sequences or CDR sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a Nanobody of the invention or a nucleotide sequence or nucleic acid encoding the same.

In a preferred embodiment, the binding agent as selected in the above method stabilizes a specific conformational state of a protein complex upon binding. The term “stabilizes” or “stabilizing”, as used herein, is to be understood within the context of thermodynamic reaction equilibria and implies a higher binding affinity (or a preferred binding) of the binding agent for the protein complex versus a lower binding affinity (or less preferred binding, including no binding) for its individual members. In particular, the binding agent may specifically bind to a newly formed conformational epitope that is induced or becomes more accessible upon association of said protein complex, and that is not formed by or less accessible in each of the individual members alone of said protein complex. Consequently, the binding agent will also increase the binding affinity between the individual members of said protein complex upon binding. It should be clear that the conformational epitopes selectively recognized by the binding agent as described herein can be either member protein specific epitopes, which for example become more accessible upon interaction with the one or more other member proteins, or otherwise protein complex specific epitopes, which are only formed by combining amino acid residues of the two or more constituting member proteins.

In another embodiment, the binding agent selected in the above method is further characterized in that it also stabilizes upon binding the protein complex-selective conformation of one of the constituting members in the absence of the other interacting member(s).

In specific embodiments, the selective conformation of a protein complex as described herein may be either an inactive conformation or an active conformation (or any other thermodynamically intermediary state) and depends on the type of proteins/nucleic acids involved in the interaction. For example, the conformation of a PPI is "active" when said conformation increases, opens, activates, facilitates, enhances activation, enhances binding, or upregulates a PPI’s activity by at least 10% over

another conformation of the PPI or over a conformation of each of the individual members of the PPI. The conformation of a PPI is "inactive" when said conformation decreases, closes, deactivates, hinders, diminishes activation, or diminishes binding, or down regulates the PPI's activity by at least 10% over another conformation of the PPI or over a conformation of each of the individual members of the PPI.

One aspect of the invention also relates to a composition comprising a binding agent obtained by the current method. In one embodiment, the invention encompasses a composition comprising a protein complex (as defined hereinbefore) and a binding agent (as described above) obtained by the current method, whereby the protein complex is stabilized in a complex conformation by the binding agent.

The binding agents identified by the methods described above have a number of applications.

For example, the binding agents identified by the methods described above may be used to purify a protein complex of interest. Suitable purification methods include, without limitation, affinity-based methods such as affinity chromatography, affinity purification, immunoprecipitation, protein detection, immunochemistry, surface-display, size exclusion chromatography, ion exchange chromatography, amongst others, and are all well-known in the art.

Also, binding agents that are specific for a protein complex conformation can be used as co-crystallization aid, or in other words, can be used to facilitate crystallogenesis of a transient protein complex, using any of a variety of crystallization methods. For example, in case a membrane protein forms part of a protein complex, crystallization methods as reviewed in Caffrey (2003), J Struct. Biol. 2003 142:108-32 and Caffrey et al, Nat Protoc 4, 706-731, (2009) may be preferred. In general terms, the methods are lipid-based methods that include adding lipid to the complex prior to crystallization. Many of these methods, including the lipidic cubic phase crystallization method and the bicelle crystallization method, exploit the spontaneous self-assembling properties of lipids and detergent as vesicles (vesicle-fusion method), discoidal micelles (bicelle method), and liquid crystals or mesophases (in meso or cubic-phase method). Lipidic cubic phases crystallization methods are described in, for example: Landau et al, Proc. Natl. Acad. Sci. 1996 93:14532-5; Gouaux, Structure. 1998 6:5-10; Rummel et al, J. Struct. Biol. 1998 121 :82-91; Nollert et al, Methods. 2004 34:348-53; Rasmussen et al. (2011) Nature 469, 175-180, which publications are incorporated by reference for disclosure of those methods. Bicelle crystallization methods are described in, for example: Faham et al., Protein Sci. 2005 14:836-40. 2005; Faham et al, J Mol Biol. 2002 Feb 8;316(1): 1-6, which publications are incorporated by reference for disclosure of those methods. Crystallization methods for soluble proteins are known in the art, and are described for example in [17].

Further, the binding agents identified by the methods described above may also be used in compound screening methods, for example to identify compounds that specifically bind a protein complex in a particular conformation and/or that induce or enhance the association of a protein complex in a particular conformation upon binding, or alternatively to identify compounds that disrupt a protein complex in a conformation-selective manner. Such compounds can have a therapeutic benefit, for instance, if the compound is able to activate or increase protein complex-mediated signaling or prolong the duration of the protein complex-mediated signaling or alternatively decrease or inhibit protein complex-mediated signaling, depending on the disease indication.

Further, the binding agents identified by the methods described above may also be used as a diagnostic tool to detect and/or capture a protein complex that is characteristic of a given disease in a patient sample.

Further, the binding agents identified by the methods described above may also be used as research tool, for example to detect or visualize a protein complex in a sample. In particular, it will be appreciated that the binding agents may be particularly useful tools for the development or improvement of cell-based assays. Cell-based assays are critical for assessing the mechanism of action of new biological targets and biological activity of chemical compounds, for example, cell-based assays for measurement of protein complex pathway (in)activation; measurement of protein trafficking by tagging one or more member(s) of a protein complex with a fluorescent protein; direct measures of interactions between proteins using Fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) or yeast two-hybrid approaches. Also relevant to the invention, binding agents may be key building blocks of (intracellular) conformational biosensors for proteins or protein complexes inside a cell[18].

The following examples are intended to promote a further understanding of the invention. While the invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the invention is limited only by the claims attached herein.

EXAMPLES

PART 1. Binding agents for the stabilization of transient protein-protein interactions.

GENERAL DESCRIPTION OF THE METHOD

It was shown that crosslinking a transient protein complex, followed by immunization of llama's with this complex, followed by display of the in vivo matured antibody repertoire, and followed by co-selection, delivers Nanobodies with the unique property to bind conformational epitopes on transient protein complexes that are not represented on the constituting monomers.

Step 1: Cross-link association proteins and immunize llamas for the generation of Nbs that bind conformational features of the complex

a) Chemical cross-linking of the interacting monomers. In principle, animals could be immunized with mixtures of the interacting monomers. However, considering the short half-life of transient PPIs (0,1 to 1s), we envisage to stabilize these transient complexes by chemical cross-linking. In this way, animals can be immunized with antigens that are in a covalent association that is very similar to the native transient complex to trigger and mature immunoglobulins that bind allosteric conformational epitopes of the transient complex.

Method: Over the last decades, various cross-linking strategies have been used to preserve labile protein-protein interactions in stable macromolecular assemblies[10]. As an example, we use a cocktail of highly efficient bifunctional reagents[11] containing diverse reactive groups that can react with amines or carboxylic acids (www.covalx.com). We found that these reagents are effective in cross-linking a wide range of interacting proteins in native-like covalent complexes under physiological conditions.

b) Generation of in vivo matured conformational Nanobodies by immunization. Although naive or synthetic libraries of antibodies may contain conformational binders against protein-protein complexes, a preferred method is to immunize llamas with the (optionally cross-linked) protein-protein complex to expose their immune system with the conformational epitopes that are unique to the (transient) complex. The Nanobody platform has the competitive advantage over other recombinant scaffold libraries in that large numbers (10^9) of fragments harboring the full antigen-binding capacity of genuine *in vivo* matured antibodies can be screened for high affinity binders in a couple of days, allowing one to fully exploit the humoral response of large mammals against native antigens [12,13].

Method: A llama is immunized with a cross-linked complex of interacting proteins), prepared as described above (of note, also free partners can be present in the sample). The antigen is diluted in an antigen compatible buffer (ag-buffer) and equal volumes of the cross-linked antigen and Gerbu adjuvant are mixed via up-and-down pipetting. Once a stable emulsion is obtained, this preparation can be injected into Llama's following a standard immunisation scheme as described in Table 1[14].

Table 1: Immunization scheme with cross-linked antigens

Day	Llama injection	Tissue collection
Day 0	200µg antigen + 0µl Ag buffer + 600µl Gerbu	10ml pre-immune blood
Day 14	100µg antigen + 100µl Ag buffer + 400µl Gerbu	
Day 21	100µg antigen + 100µl Ag buffer + 400µl Gerbu	
Day 28	100µg antigen + 100µl Ag buffer + 400µl Gerbu	10ml immune blood
Day 35	100µg antigen + 100µl Ag buffer + 400µl Gerbu	
Day 42	100µg antigen + 100µl Ag buffer + 400µl Gerbu	
Day 45		100ml immune blood

Blood is collected from the immunized animal at days 28 and 42 and the humoral immune response is evaluated by ELISA[14]. 100µg of a 1µg/ml solution of the crosslinked complex was coated on a Maxisorp plate. Serial dilutions of the pre-immune (day 0) and immune (day 28) sera are added to the coated wells. Bound llama immunoglobulins are detected using anti-llama IgG antibodies conjugated horse radish peroxidase conjugate (Imtec) using a colorimetric assay based on the ABTS substrate (Sigma).

Step 2: Display and co-selection method for Nbs that selectively bind the transient complexes or Nbs that selectively bind the separate interacting monomers

Immunizations of llamas with cross-linked complexes (step 1) inevitably generate different classes of Nanobodies: Nbs that bind to protomers only, binders that recognize a protomer and the complex with similar affinity and antibodies that selectively bind to the complex. The second technological challenge is to combine this first step with highly efficient selection methods that can discriminate Nbs that exclusively bind the transient (non-cross-linked) complex from Nbs that bind to the dissociated monomers.

a) Display immune library on yeast cells.

As an example, we exploited the multivalent display of Nanobodies on yeast cells in combination with the resolving power of fluorescent-activated cell sorting (FACS). In yeast display each Nb is displayed as a fusion to the Aga2p protein at about 50.000 copies on the surface of an individual yeast cell[15]. For co-selection by FACS, we label the protomers that associate in a transient PPI separately with different fluorescent dyes. Next, the Nb-displaying yeast library can be stained with a mixture of these

fluorescent protomers. FACS can then be used to analyse the properties of each Nb that is displayed on a particular yeast cell and to recover particular populations as illustrated in Fig 2. When 2 proteins A and B are seperately fluorenscently labelled with a different dye, a two-color FACS analysis will discriminate populations of (1) yeast cells displaying aspecific binders, (2) yeast cells displaying Nbs that only bind A, (3) yeast cells displaying Nbs that only bind B and (4) yeast cells displaying Nbs that specifically bind the complex AB (Figure 2).

Method: A blood sample of 100ml of an immunized llama contains sufficient expressing B-cells to clone the full diversity of the in-vivo matured Nanobodies with high specificity for their cognate antigen. Therefore 100ml of fresh anti-coagulated blood is collected from the immunized animal (day 45) and peripheral blood lymphocytes are purified as described[14].

Total RNA is extracted from the purified peripheral blood lymphocytes following the instructions provided with the RNeasy extraction kit (Qiagen). Next, double stranded cDNA is synthesised by reverse transcription of 50µg of total RNA using the SyperScriptIII First –Strand cDNA kit (Invitrogen) and random hexanucleotides.

To amplify all immunoglobulin heavy chains from the cDNA, forward primer CALL001 and reverse primer CALL002 are used in a first PCR reaction[14]. The Nanobody encoding DNA fragments are separated from the variable domain of the heavy chain of the conventional antibody repertoire via gel electrophoresis. The appropriate DNA-band is purified from the gel using a Qiaquick gel extraction kit and further amplified using a second nested PCR. This second PCR with nested primers GAPVHH5_fw and GAPVHH3_rv annealing at framework 1 and framework 4 respectively amplifies Nanobody encoding genes as NheI-BamHI fragments. Primers GAPVHH5_fw and GAPVHH3_rv create two 30bp overlaps with the pCTCON2 yeast display vector[15] to allow GAP repair cloning via homologues recombination in yeast.

CALL001	5’ GTC CTG GCT GCT CTT CTA CAA GG 3’
CALL002	5’ GGT ACG TGC TGT TGA ACT GTT CC 3’
GAPVHH5_fw	5’ CGG TAG CGG AGG CGG AGG GTC GGC TAG CCA GGT GCA GCT GGT GGA GTC TGG GG 3’
GAPVHH3_rv	5’ GGG ACC CAG GTC ACC GTC TCC AGC GGA TCC GAA CAA AAG CTT ATT TCT GAA G- 3’

The display vector pCTCON2 allows the expression of the Nanobody as a fusion protein at the C-terminus of the Aga2p on the cell surface of yeast. The expression of this construct is under control of a galactose-induced promotor and results in 50.000 copies per yeast cell. For GAP repair cloning, 10µg of the amplified Nanobody repertoire is mixed with 10µg of NheI-BamHI (Fermentas) digested pCTCON2 and co-transformed in electrocompetent EBY100 yeast cells. Homologues recombination in EBY100 yeast cells results in the insertion of the full Nanobody repertoire into the linearized pCTCON2 vector with a diversity of 1xE6 to 1xE7 transformants and an insert rate up to 100%. The presence of an N-terminal HA-tag and a C-terminal myc-tag on the displayed Nanobody allows to monitor Nanobody expression on each individual yeast cell in FACS by use of fluorescently labeled antibodies that bind HA or myc.

b) Fluorescent labelling of individual protein partners

Essential to this invention is the distinctive labelling of at least one of the partners, for example with specific fluorescent dyes that can be detected in FACS. This can be achieved by recombinant expression of the protein with a fluorescent tag (GFP, YFP). Proteins can also be indirectly labeled by incubating with a fluorescent Ab conjugate / cascade directed against a tag or the protein itself. The method applied in this example is random covalent coupling of fluorescent dyes (Alexa, Dylight) to amine or cysteine reactive groups of the protein. If possible, the effect of any type of labeling on the protein-protein interaction should be checked in advance, since labeling could be interfering with complex formation. If this is case, an alternative labeling method would be advisable.

c) Yeast display and co-selection by FACS

FACS can be used simultaniously (1) to analyse the properties of each Nanobody that is displayed multivalently on an individual yeast cell and (2) to selectively recover yeast cells displaying Nbs with a particular property (Figure 2). Yeast cells displaying Nanobodies that selectively bind A but not B or AB can be enriched if cells of the top-left quadrant are recovered by FACS. Yeast cells displaying Nanobodies that selectively bind B but not A or AB can be enriched if cells of the bottom-right quadrant are recovered by FACS. Yeast cells displaying Nanobodies that selectively bind the AB complex but not to A alone or B alone can be enriched if cells of the top-right quadrant are recovered by FACS. These processes can be repeated in several rounds to enrich for yeast cells displaying nanobodies with the desired characteristics.

Method: For a first selection round by yeast display, a sufficiently large aliquot of the library is thawed (number of yeast cells > 10x the library diversity) and inoculated in fresh SDCAA 1:100 pen/strep medium according to Chao et al[15]. Yeast cells are induced with galactose for Nb expression in SGCAA

medium for minimum 16h and 1x10^8 cells are harvested by centrifugation. Induced yeast cells are washed 3 times by centrifugation with Ag-buffer supplemented with 2% BSA to block aspecific binding sites on the yeast surface. From this point on samples are kept at 4°C during further manipulations to minimize the dissociation of the detection antibodies and labeled proteins from the yeast displayed Nanobodies. To monitor the expression level of the displayed Nb on each cell during FACS, the yeast cells were subsequently incubated with 1ml Ag- buffer supplemented with 1:100 anti c-myc mouse monoclonal antibody (Roche) for 30min, washed three times by centrifugation, and subsequently labelled with 1ml 1:100 R-Phycoerythrin goat anti-mouse antibody (Lucron) for 30min. In parallel with these labelling steps, the separately labelled proteins are mixed at such a concentration (1 – 100µM) that at least a small fraction of transient complex will be formed after 1h of incubation. Next, the labelled yeast cells are washed and resuspended in 1ml of this mixture of labelled proteins. During 1h incubation, a fraction of the displayed Nanobodies will specifically bind the double-labelled transient complex or the constituting single-labelled proteins. To remove excess amounts of unbound labelled proteins, yeast cells are washed three times by centrifugation with Ag-buffer supplemented with 0.2% BSA.

All selection rounds are performed using 3-color Fluorescence Activated Cell Sorting (FACS) on a FACS AriaIII (BD Biosciences). Figure 4 provides an illustrative example of the stepwise enrichment for yeast cells that express Nanobodies that selectively bind to complex AB, but not to A or B separately. Typically 0,1% to 10% of the yeast cells contained in the top-right quadrant, corresponding to yeast cells that bind significant amounts of both of the separately labelled proteins constituting the (transient) complex are sorted and collected by FACS in 500µl 2xSDCAA 1:100 pen/strep medium. Sorted yeast cells are plated out for single colonies on SDCAA 1:100 pen/strep agar plates and incubated 3 days at 30°C. This sub-library is scraped, cultured in liquid SDCAA 1:100 pen/strep and induced with SGCAA 1:100 pen/strep for the next selection round.

After a few rounds of selection, single yeast colonies are picked from the enriched FACS outputs. These clones are cultured in 1ml SDCAA 1:100 pen/strep medium and a 96-well masterplate for long term storage is produced by adding 30% glycerol. From the same culture, VHH genes are amplified via PCR and the sequence of these fragments is determined using pCTCON_fw and pCTCON2_rv as the sequencing primers[15]

pCTCON2_fw	5’ GTTCCAGACTACGCTCTGCAGG 3’
pCTCON2_rv	5’ GATTTTGTTACATCTACACTGTTG 3’

Step 3: Analysis of the binding properties of single Nanobodies by FACS

Yeast cells deriving from a single colony all express the same Nanobody and FACS on a culture of these cells can easily be used to analyze the binding properties of the Nanobody that is expressed according to the same principles that are illustrated in Figure 2.

Based on sequence analysis families of all clones contained in the master plate, Nanobody sequences can be grouped in sequence families of related Nanobodies[14]. Based on such analysis individual clones were selected for individual analysis by FACS.

Method: Each selected clone is grown ON at 30°C and 200rpm in 96 deepwell plates containing 1ml SDCAA 1:100 pen/strep. Nanobody expression is induced with SGCAA 1:100 pen/strep medium for 16h at 30°C and 200rpm.

In total 2x10^6 cells are harvested and washed by centrifugation with Ag buffer complemented with 2% BSA. To monitor the expression level of the displayed Nb on each cell during FACS, the yeast cells are subsequently incubated with 1ml of Ag-buffer complemented with 1:100 anti c-myc mouse monoclonal antibody (Roche) for 30min, washed three times by centrifugation, and subsequently labelled with 1ml 1:100 R-Phycoerythrin goat anti-mouse antibody (Lucron) for 30min. Yeast cells are then incubated for 1h with mixtures of separately labelled proteins constituting the (transient) complex. To remove excess amounts of the labelled proteins, yeast cells were washed three times with Ag buffer supplemented with 0.2% BSA. Stained cells are subsequently analysed by FACS according to Figure 2 to assess if the Nanobody under investigation is (1) aspecific for the antigen, (2) binds selectively to A but not to B or AB, (3) binds selectively to B but not to A or AB and (4) binds selectively to AB but not to A alone or B alone. It should be noted that not all Nanobodies fall into these distinct types. Rather, there is a continuum between Nanobodies that exclusively bind to AB, to A or to B and Nanobodies that bind stronger to AB compared to A or B, and Nanobodies that bind stronger to A or B compared to AB.

Step 4: Expression of selected Nanobodies in *E. coli* and purification by IMAC

For periplasmic expression in *E. coli*, individual Nanobody sequences were subcloned in a pMESy4 vector[14] containing a pelB signal for periplasmic expression and an ampicillin resistance gene. The C-terminal HA and N-terminal $(His)_6$EPEA tag allows efficient purification via affinity chromatography and detection in ELISA with appropriate antibodies [24].

Method: The yeast plasmid is recovered from an ON culture using the Zymoprep kit. Nanobody genes are amplified by PCR as PstI-BSTEII fragments using VHH_for and VHH_back as primers. The VHH

fragments are digested with PstI and BstEII restriction enzymes (Fermentas) and ligated into the linearized pMESy4 vector with complementary ends. Ligation products are transformed in electrocompetent WK6 cells and single colonies are picked.

VHH_for	5' GAT GTG CAG CTG CAG GAG TCT GGR GGA GG 3'
VHH_back	5' GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT 3'

Nanobodies are purified from the periplasm of *E. coli* by IMAC according to Pardon et al [14].

EXAMPLE 1

In order to identify Nanobodies that stabilize and selectively bind to a transient protein complex, the RIC8A/Gαi1 complex was chosen as a model system.

Heterotrimeric G protein alpha subunits (Gα) are activated upon exchange of GDP for GTP at the nucleotide binding site of Gα, catalyzed by guanine nucleotide exchange factors (GEFs). In addition to transmembrane G protein-coupled receptors (GPCRs), which act on G protein heterotrimers, members of the family cytosolic proteins typified by mammalian RIC8A (resistance to inhibitors of cholinesterase 8A) are GEFs for Gi/q/12/13-class Gα subunits. RIC8A binds to Gα-GDP, resulting in the release of GDP. The RIC8A complex with nucleotide-free Gαi1 is stable, but dissociates upon binding of GTP to Gαi1.

As members of the Ras superfamily of regulatory GTP binding proteins, heterotrimeric G protein alpha subunits (Gα) undergo cycles of activation and deactivation driven by binding and hydrolysis of GTP[16]. Conversion to the basal, inactive state results from the intrinsic GTP hydrolyase activity of the G protein. Reactivation is achieved by replacement of GDP by GTP at the nucleotide-binding site, catalyzed by guanine nucleotide exchange factors (GEFs). Although the structural events that accompany GEF catalyzed nucleotide exchange on Ras-like G proteins are relatively well understood[17], the mechanism of heterotrimeric G protein activation remains enigmatic. Agonist-activated, transmembrane G protein-coupled receptors (GPCRs)[18] are the best characterized heterotrimeric G protein GEFs. GPCRs act on plasma membrane-localized G protein heterotrimers that consist of GDP-bound Gα tightly associated with heterodimers of Gβ and Gγ subunits. Members of a family of predominantly cytosolic proteins, typified by mammalian RIC8A, identified as non-receptor GEFs, catalyze nucleotide exchange directly on Gα subunits of the Gi/o/q/12/13 families[19]. Across phylogeny, RIC8A paralogs act in GPCR-independent pathways to orient mitotic spindles in asymmetric cell division, as demonstrated in C. elegans[20,21], Drosophila[22], and mammalian cells[24].

a) Preparation of a protein complex as immunogen for camelid immunization

Recombinant rat RIC8A (uniprot:Q80ZG1) and Gαi1 (uniprot:P10824) were produced and purified as described previously[24]. The (non-covalent) complex was purified by a size exclusion chromatography in an amine-free buffer (20mM PBS buffer pH 6,8 without DTT). The chemical cross-linking reaction was performed using K100 reagent purchased from Covalx (http://www.covalx.com/)[11]. 10uM of the complex in 20mM PBS buffer pH 6,8 was incubated with 10ul of 2mg/ml of K100 cross-linker for 5 and 30minutes at room temperature. Removal of the unreacted cross-linker species was done using a 1ml HiTrap desalting matrix (GE Healthcare). The presence of the cross-linked complex (100kDa) was monitored by SDS/PAGE (Figure 3). In fact this was still a non-homogeneous mixture containing free RIC8a (50kDa) and Gαi1 (37kDa).

b) Induction of an antibody mediated immune response in llama against the cross-linked RIC8A/Gαi1 complex.

This preparation of the cross-linked (30 minutes) RIC8A-Gαi1 complex was diluted into 20mM PBS pH7,4 with 1mM DTT and injected into a llama according to step 1 of the general method. A clear antigen-specific signal was detected, as the ELISA signals of the immune serum (day 28) were minimally 2-fold higher than those obtained with the pre-immune serum.

c) Fluorescent labeling of RIC8A and Gαi1

Purified recombinant RIC8A and Gαi1 were dialyzed extensively against 20mM PBS pH 7,4 with 1mM DTT prior to the separate labeling reactions. RIC8A was incubated with a 5fold excess of Dylight 405 NHS Ester (Pierce,Thermo Scientific) according to the manufacturer's instructions. Gαi1 was incubated with a 5fold excess of Dylight 488 NHS Ester (Pierce,Thermo Scientific).

The labeling reaction was performed at room temperature in the dark for 30minutes. Unreacted dye was removed by size exclusion using a NAP5 column (GE Healthcare). Labeling was checked by SDS-PAGE and visualization of the fluorescent protein was performed using a Biorad gel imager.

d) Yeast display and Co-selection of Nanobodies that selectively bind RIC8A●Gαi1 complex but not to RIC8A or Gαi1 alone.

Aiming at stabilizing the RIC8A●Gαi1 complex for further biophysical/biochemical investigation of this complex, we selected Nanobodies that selectively bind the RIC8A●Gαi1 complex but not to RIC8A or Gαi1 by FACS according to the principle that is illustrated in Figure 2. For the first selection round the yeast display library was diluted in 1ml 20mM PBS pH 7.4, 150mM NaCl, 2mM DTT supplemented with

10µM of separately labeled RIC8A Dylight 405 and 10µM of separately labeled Gαi1 Dylight 488. Before adding the yeast cells, the complex was allowed to form by mixing equimolar amounts of RIC8A Dylight 405 and Gαi1 Dylight 488 and incubating 1h at RT. At 10µM there will be a mixture of free RIC88A Dylight 405, free Gαi1 Dylight 488 and RIC8A Dylight 405 / Gαi1 Dylight 488 complex, since the Kd of this PPI is around 12µM in the presence of GDP [24]. To enrich yeast cells displaying nanobodies that selectively bind the RIC8A●Gαi1 complex but not to RIC8A or Gαi1 alone, the following sorting gates were applied in a three-color FACS experiment (R-Phycoerythrin, Dylight 405 and Dylight 488). High R-Phycoerythrin fluorescence indicates that the yeast cell expresses a Nanobody on its surface. High Dylight 405 fluorescence indicates that the displayed Nanobody traps RIC8A and high Dylight 488 fluorescence indicates that the Nanobody traps. Yeast cells scoring on three colors express a Nanobody that binds the RIC8A●Gαi1 complex but not to RIC8A or Gαi1 alone. In a first round of selection (See figure 4), about 0,1 – 1% of triple positive cells (binding RIC8A●Gαi1, Q2) were sorted and yeast clones expressing aspecific Nanobodies (Q4) or Nanobodies that bind RIC8A only (Q1) or Nanobodies that bind Gαi1 only (Q3) were discarded.

To obtain Nanobodies that bind the RIC8A●Gαi1 complex only, two more rounds of co-selection by FACS were performed to stepwise enrich the top 0,1% - 0,5% fraction of yeast cells with the highest fluorescence of Dylight 405 and Dylight 488. In the second and third FACS selection, the number of stained cells to be sorted by FACS was lowered to 1x10^7 and 5x10^6 cells, respectively and the staining volume was lowered accordingly to 500µl and 200µl. The concentration of separately labelled RIC8A Dylight 405 and Gαi1 Dylight 488 complex was kept constant at 10µM. In round 2, about 0.3% of the best scoring cells were collected (P3 in Figure 4). In the 3rd round, the best scoring yeast cells were collected in two pools (Figure 4). P3 contains all yeast cells with a high fluorescence in the Dylight 405 channel and the Gαi1 Dylight channel, P4 contains the 0.5 best scoring fraction of these cells.

Results

Figure 4 illustrates that three rounds of co-selection by FACS enriches for yeast cells that display nanobodies (positive signal in the R-Phycoerythrin channel) and show increasing fluorescence of Dylight 405 (coupled to RIC8) and Dylight 488 (coupled to Gαi1). Remarkably, yeast cells with a high signal in the Dylight 405 channel only or the Dylight 488 channel only did not enrich after 3 rounds of selection indicating that co-selection by FACS is a powerful method to select Nanobodies that bind the RIC8A●Gαi1 complex but not to RIC8A or Gαi1 alone. .

To further characterize the Nanobodies that were obtained by co-selection by FACS the sequence was determined for 100 clones recovered from round 2 (P3 in Figure 4) and 100 clones recovered from round 3 (P3 and P4).

Sequence alignment of all these clones shows that the selected Nanobodies belong to 21 different sequence families only (characterized by differences in CDR3 loop[14]). Representative sequences of the 15 largest sequence families are aligned in Figure 5. The large occurrence (Table 2) of some families provides confidence that enriched binders that are binding to the complex rather than just aspecific binders.

Table 2

CA pCTCON2 *S. cerevisiae*	CA pMESy4 *E.coli*	Family	Occurrence	CDR3 Sequence
CA8312	CA8328	1	21	CAGDRQPYVYDLPTAQYQYDY
CA8411	CA8435	3	40	CASSSIEFGPLEDTYDY
CA8314	CA8330	4	2	CAAEAREFSVGSYYATEYDY
CA8315	CA8331	5	14	CAADRKPYSYYPSDFGSW
CA8316	CA8332	7	11	CAATPADSAFMRNLRVYDY
CA8318	CA8334	8	8	CVARVGSPSSSDRAYQY
CA8319	CA8335	10	34	CAATRRDFYIIRNSRPQFDY
CA8322	CA8338	16	4	CARCPAGAACKVEYDY
CA8402	CA8431	19	6	CAATPADLTVVAGPPRIEMWY
CA8403	CA8432	20	3	CAATAADYVLRSRPSVYSY
CA8405	CA8433	21	3	CAAAIRDGHNYYASDMRRYDY
CA8418		23	6	ASDRRPYRYNIGTAEGEYNY
CA8419		24	4	GKGWFLNRRDES
CA8421		26	2	AADRVPYRFGVPSINEYDY
CA8424	CA8444	29	2	NFNVRYYGEY

e) Yeast display and co-selection of Nanobodies that selectively bind to RIC8A or Gαi1 alone but not to the RIC8A●Gαi1 complex.

So far, we only focussed on binders that selectively bind to (transient) protein complexes but not to the constituting monomers because such binders will stabilize the complex. Nanobodies that only bind to one of the constituting monomers but not to the protein complex are equally important in other biochemical, biophysical or therapeutic applications because such Nanobodies will disrupt a (transient) protein-protein complex.

Selection of binders that recognize only one partner instead of the complex but do not bind the complex were selected in a similar manner as described in the previous section with the following

modifications. In the first round (Figure 6) Nanobody expressing yeast cells were sorted that exclusively bind to RIC8A Dylight 405 (Q1). Cells binding the complex (Q2), the other partner Gαi1 Dylight 488 (Q3) and cells that display aspecific binders (Q4) were discharged. In the following round 2 the induced yeast sub-library was incubated with 100µl of 1µM free Ric-8A Dylight 405.

Results

Figure 6 nicely illustrates that two rounds of co-selection by FACS enrich for yeast cells with a high signal in the Dylight 405 channel only. From the selection output of round 3 (P3 and P4), 100 clones were sequenced and aligned (Figure 7). All clones belong to 4 sequence families only of which two with very high occurrence (Table 3). These Nanobodies have completely different sequences in the CDR3 loop compared to the ones found that bind the RIC8A•Gαi complex (Table 2).

Table 3

CA pCTCON2 *S. cerevisiae*	CA pMESy4 *E.coli*	Family	Occurrence	CDR3 Sequence
CA8408	CA8109	6	3	ARTSRASVTTRVADFGY
CA8406		11	13	NLQNRYDAMDY
CA8417	CA8440	13	30	NTYPVNSY
CA8407		22	3	NAEPRYYGAAYL

f) Characterization of Nanobodies by FACS screening

To identify if we indeed selected for Ric-8A●Gαi1 specific complex binders in paragraph d and for RIC8A only binders in paragraph e, a FACS screening assay was performed on single yeast clones. Screening for Nanobody binding was performed in 20mM PBS pH7.4, 150mM NaCl, 2mM DTT, 0.2% BSA buffer on following target proteins: 1µM RIC8A Dylight 405, 1µM Gαi1 Dylight 488, 1µM complex RIC8A Dylight 405●Gαi1 Dylight 488.

Results

This screening method allowed us to characterize the Nanobodies and to distinguish between three interaction profiles **(Figure 8):**

- Type 1: Nanobodies that specifically bind the Ric-8A●Gαi1 complex and not the individual partners. At this concentration (1µM of individual partners) the complex is not fully formed, so the fact that double stained yeast cells could be detected indicates that these Nanobodies act as complex

stabilizers. Also this Nanobody specifically recognizes an epitope that is only present in the RIC8A●Gαi1 complex. This can be explained by the availability of new epitopes formed by the binding interface or due to conformational changes in the interaction partners upon binding.

- Type 2: Nanobodies that interact with the complex if the sample is incubated with a RIC8A Dylight 405●Gαi1 Dylight 488 mixture. In addition this Nanobody binds as well with one of the free partners if incubated with a single Ag. This means that the Nanobody can bind an epitope that is present in the free partner and in the complex.
- Type 3: Nanobodies that show a preferable binding to the individual partner RIC8A even if the RIC8A Dylight 405●Gαi1 Dylight 488 complex is presented. They also show a high binding towards the single partner in solution. This implies that upon Nanobody binding the complex cannot be formed.

Results from this screening are summarized **(Table 4)** and show that clones coming from the sort for RIC8●Gαi1 complex binders result in enrichment for type 1 and type 2 binders. This is in contrast with the output clones from the RIC8A selection method where only type 3 binders were selected.

Table 4

CA pCTCON2 S. cerevisiae	CA pMESy4 E.coli	Ric8A	Gαi1	Ric8A - Gαi1	Type
CA8312	CA8328	no	no	yes	1
CA8411	CA8437	yes	no	yes	2
CA8314	CA8330	no	no	yes	1
CA8315	CA8331	no	no	yes	1
CA8316	CA8332	no	no	yes	1
CA8318	CA8334	no	no	yes	1
CA8319	CA8336	no	no	yes	1
CA8322	CA8338	yes	no	yes	2
CA8402	CA8431	no	no	yes	1
CA8403	CA8432	no	no	yes	1
CA8405	CA8433	no	no	yes	1
CA8418		yes	no	yes	2
CA8419		yes	no	yes	2
CA8421		yes	no	yes	2
CA8424	CA8444	yes	no	yes	2
CA8408	CA8109	yes	no	free Ric8A	3
CA8406		yes	no	free Ric8A	3
CA8417	CA8440	yes	no	free Ric8A	3
CA8407		yes	no	free Ric8A	3

g) Biophysical characterization of Nanobodies that bind to RIC8A●Gαi1 but not to RIC8A alone or Gαi1 alone.

A representative Nb that binds to RIC8A●Gαi1 complex but not to RIC8A alone or Gαi1 alone (CA8332) was further characterized by biolayer interferometry (BLI) using an Octet Red96 (FortéBio, Menlo Park, Calif., USA). 40ug/ml of the purified Nanobody was immobilized through the C-terminal Histag on a NiNTA-coated biosensor (part N°:18-5102). Immobilization was performed in the binding buffer (10mM Tris pH8.5, 150mM, 1mM DTT, 0,2mg/ml BSA) for 900sec shaken at 1KRPM. This step is enough to saturate the biosensor with the Nanobody. Then the tips are washed for 600sec at 1.2KRPM in the binding buffer. Next, a baseline is measured for 600sec at 1KRPM in a well containing the binding buffer. The association kinetics of the different analytes with the immobilized Nanobody can then be measured by transferring the biosensor to a well containing RIC8A●Gαi1 complex, RIC8A alone or Gαi1 alone for 600sec at 1KRPM. Next, dissociation can be followed by transferring the biosensor to buffer only for 900sec.

Using this technique, we first confirmed that CA8332 binds to RIC8A●Gαi1 complex but not to RIC8A alone or Gαi1 alone. For this purpose, the different analytes were dispensed in three different wells containing RIC8A (5uM), Gαi1 (5uM), or the RIC8A●Gαi1 complex (5uM). The binding isotherms in Figure9A illustrate that CA8332 binds to RIC8A●Gαi1 complex but not to RIC8A alone or Gαi1 alone. After 600 seconds of association, the biosensors were transferred to buffer allowing dissociation to be measured for 900sec at 1KRPM. These dissociation isotherms indicate that the ternary CA8332●RIC8A●Gαi1 complex is tight and that CA8332 dissociates slowly from RIC8A●Gαi1.

In order to accurately measure the affinity of CA8332 for the RIC8A●Gai1 complex we immobilized CA8332 on the biosensor (as described previously) and measured its binding kinetics at different concentration of the purified RIC8A●Gαi1 complex (using 125, 12.5, 1.25, 0.125, 0.0125, 0uM), the association was measured for 900 sec and the dissociation for 1100sec (Figure9B). The raw data acquired for the interaction between the Nanobody (CA8332) and the RIC8A●Gαi1 complex were processed and fit to a curve in order to extract values of *k*on, *k*off and *K*d. Processing began with reference correction to compensate for signal drift of the immobilized biosensor with the binding buffer. Using a reference biosensor, the signal generated by a biosensor with only the Nanobody that probed the binding buffer was subtracted from both the association and dissociation steps for the interaction between the Nanobody and the complex. The binding to the complex showed a dose dependent signal, the curve fitting suggests that the binding occurs in a 1:1 model with an estimated *K*d value of 0.2nM.

PART 2. Binding agents for the stabilization of protein-DNA interactions.

EXAMPLE 1

a) Preparation of a protein complex as immunogen for camelid immunization.

In order to identify Nanobodies that stabilize and selectively bind to a transient protein/DNA complex, the *Escherichia coli* DNA gyrase in complex with a DNA fragment was chosen as a model.

Bacterial topoisomerases IIa (DNA gyrase and Topoisomerase IV) introduce negative DNA supercoils [35]and remove positive supercoils, functions essential for bacterial DNA transcription and replication[36]. DNA gyrase is composed of two heterodimeric subunits, GyrA comprising the DNA ligation activity, and GyrB comprising the ATPase activity. The DNA gyrase supercoiling reaction is a complex process that is incompletely understood. However, the catalytic cycle gives ample opportunity for disruption by inhibitors, which can for example, interfere with DNA binding, DNA cleavage, DNA strand passage and ATP hydrolysis[37]. A variety of natural products (simocyclinones)[38], synthetic small molecules (quinolones)[35] and protein-based entities (CcdB)[39], have been identified as potent inhibitors. Interestingly, all these topoisomerase inhibitors block the enzyme at different stages of the catalytic cycle. Hence, the different inhibitor/protein complexes must have different conformations. We used Ciprofloxacin (CFX) a second-generation fluoroquinolone which mechanism of action is to block DNA gyrase in the religation stage[40], producing a stable covalent ternary complex DNA/Gyrase/CFX[35]. The *E.coli* DNA gyrase construct used is a fusion of GyrB (388-804) (NP_418154) and GyrA (1-525) (NP_416734) as described previously[41, 42]. And a 34bp DNA fragment containing a DNA gyrase hotspot in the center was used to prepare the complex.

Gyr_fw	5'-ACCAAGGTCATGAATGACTATGCACGTAAAACAG-3'
Gyr_rv	5'-CTGTTTTACGTGCATAGTCATTCATGACCTTGGT-3'

5uM of DNA gyrase was incubated with a 10-fold excess of DNA (reaction buffer: 100mM Tris, 100mM KCl, 12mM MgCl2, 10mM DTT and 20% glycerol). 1mM of Cirprofloxacin (17850-25G-F, Fluka) was added to the complex and further incubated for 16h at 37°C. The covalent complex was detected on SDS/PAGE gel (Figure 10).

b) Induction of an antibody mediated immune response in llama against the cross-linked DNA●gyrase●CFX complex.

One llama was immunized with the DNA/gyrase/CFX complex as immunogen, prepared as described above (point a). The immunization scheme is identical as described in Step 1b of the General Method.

c) Cloning of the llama Nanobody immune repertoires in yeast display vector.

Identical to Step1c of the General Method.

d) Fluorescent labeling of DNA●gyrase complex

Random labeling on amines inhibits the binding of DNA gyrase to the DNA. Hence we used a labeled DNA fragment containing an Alexa488 dye on the 5' end.

Gyr_fwAlexa488	A488-5'-ACCAAGGTCATGAATGACTATGCACGTAAAACAG-3'

e) Selection of Nanobodies binding to the DNA●gyrase●CFX complex.

For the first selection round, induced yeast cells were incubated with 1ml DNA-Alexa488●gyrase●CFX complex diluted in PBS buffer. The DNA-Alexa488●gyrase●CFX complex was formed in 100mM Tris, 100mM KCl, 12mM MgCl2, 10mM DTT and 20% glycerol buffer as described in point a. To select for Nanobodies that specifically stabilize DNA●gyrase●CFX complex, but not gyrase alone, a different FACS sorting strategy was used as illustrated in Figure 2. Since labeling of both partners interfered with complex formation (point d), it was irrelevant to look for such kind of double positive events in FACS. Therefore we chose to label only the DNA molecule, especially because we did not expect the presence of DNA-only binding Nanobodies in this immune library. This means that following sorting gates (Figure 11) could be applied to select for complex binders in a two-color FACS experiment (R-Phycoerythrin, Alexa488). High R-Phycoerythrin fluorescence indicates that the yeast cell expresses a Nanobody on its surface. High Alexa488 fluorescence indicates that the displayed Nanobody binds DNA, and thus in this case DNA-Alexa488●gyrase●CFX complex.

Yeast cells scoring on two colors express a Nanobody that binds the DNA●gyrase●CFX complex but not to unlabeled gyrase alone. In a first round of selection (See figure 10), about 10% of double positive cells (binding DNA●gyrase●CFX, P3) were sorted and yeast clones expressing aspecific Nanobodies or Nanobodies that bind gyrase only were discarded.

In the subsequent FACS rounds, the amount of stained yeast was lowered to 1x10^7 in round 2 and 3 and 5x10^6 cells in round 4. The staining volume was lowered accordingly to 500µl and 200µl. Until no significant shift in Alexa488 fluorescence was seen (round 2 and 3), a very mild sorting strategy was used, where 10% of the total yeast cells were collected (P3 in Figure 11). Also, from round 3 on, a more stringent selection was performed for complex stabilizing Nanobodies. Therefore we included a

condition where labeled DNA and unlabeled gyrase were mixed in the absence of the stabilizing antibiotic CFX (Round3b and 4b in Figure 11). To obtain Nanobodies that bind DNA●gyrase●CFX (condition a in Figure 11) or DNA●gyrase complex (condition b in Figure 11) only the top 2% of PE and Alexa-488 positive cells where sorted in round 4 (P4 in Figure 11).

Results

As illustrated in Figure 11, an enrichment for the selected population (P4) is seen after four rounds of selection by FACS. The population of yeast cells that display Nanobodies (positive signal in the R-Phycoerythrin channel) showed increased binding to DNA●gyrase●CFX complex (positive signal in the Alexa488 channel). As expected, this enrichment is smaller when no CFX is present, a smaller fraction of Nanobodies are able to bind the DNA●gyrase in the absence of CFX.

To further characterize the Nanobodies that were obtained by co-selection by FACS the sequence was determined for 100 clones recovered from round 2 (P3 in Figure 11), 100 clones recovered from round 3a and round 3b (P3) and 100 clones recovered from round 4a and 4b (P4).

Sequence alignment of all these clones shows that the selected Nanobodies belong to 22 different sequence families only (characterized by differences in CDR3 loop[14]). Representative sequences of the 6 largest sequence families are aligned in Figure 12 and shown in Table 5.

Table 5

CA pCTCON2 *S. cerevisiae*	CA pMESy4 *E.coli*	Family	Occurrence	CDR3 Sequence
CA9302	CA9317	1	11	AAALRPNSVQYKY
CA9303	CA9318	3	8	AATPGYTSASKVPSDYAY
CA9304	CA9319	5	4	GADSAGWFRIRQVPADYDY
CA9305	CA9320	7	3	ARGAFSFATTVQSDYNY
CA9307	CA9322	10	2	TADHALRLSSRLTDYDY
CA9309	CA9324	12	4	AADPSRWYFCSSDSNPNTFDS

f) Selection of gyrase only binding nanobodies

Selection of binders that recognize only one partner instead of the complex but do not bind the complex were selected in a similar manner as described in the previous section with the following modifications. Since we were not looking for complex binding Nbs, gyrase could be fluorescently labeled as described in paragraph c of Example1. In two selection rounds (Figure 13) Nanobody expressing yeast cells were sorted that exclusively bind to gyrase Dylight 405 (P3 and P4).

Results

Even though there was no clear enrichment seen (Figure 13), we were confident that we enriched for yeast cells expressing a Nanobody (high PE fluorescence) that binds to gyrase (high Dylight 405 fluorescence). From the selection output of round 2 (P4), 100 clones were sequenced and aligned (Figure 14). All clones belong to 9 sequence families (Table 6) and some of them were identical to the ones selected on the DNA●gyrase●CFX complex in the previous paragraph (Table 5).

Table 6

CA pCTCON2 *S. cerevisiae*	CA pMESy4 *E.coli*	Family	Occurrence	CDR3 Sequence
CA9302	CA9317	1	11	AAALRPNSVQYKY
CA9303	CA9318	3	8	AATPGYTSASKVPSDYAY
CA9304	CA9319	5	4	GADSAGWFRIRQVPADYDY
CA9305	CA9320	7	3	ARGAFSFATTVQSDYNY
CA9306	CA9321	9	2	ALQYGWRWSWDDGSARDMRY
CA9308	CA9323	11	2	ATKTRGGDWRSGKNWNY
CA9309	CA9324	12	4	AADPSRWYFCSSDSNPNTFDS
CA9310	CA9325	15	2	AASTGYGTNSRYDYDY

g) Nanobody characterization by FACS screening

In order to characterize the selected Nanobody families, a FACS screening was performed on single yeast cells expressing a particular Nanobody. Induced yeast cells were incubated with DNA-Alexa647●gyrase●CFX, DNA-Alexa647●gyrase●CFX and 1µM gyrase-Dylight405.

Results

With this screening method, we could characterize all Nanobody families and classify them into two interaction profiles (Figure 15):

- Type 1: Nanobodies that show binding to DNA●gyrase●CFX complex and to free gyrase. In contrast, type 1 binders do not show binding to a DNA●gyrase and thus are not able to stabilize the complex in absence of CFX. However, this does not mean that these Nanobodies are not binding a complex specific conformation of gyrase, they might just not be as efficient as CFX to keep DNA in complex with the gyrase.
- Type 2: Nanobodies that only show binding for free gyrase and do not bind DNA●gyrase●CFX or DNA●gyrase complexes. They are not able to recognize the complex conformation of gyrase, hence they inhibit DNA binding.

Results from this screening are summarized (Table 8) and show that both described selection methods (paragraph e and f) results both in type 1 and type 2 binders.

Table 8

CA pCTCON2 *S. cerevisiae*	CA pMESy4 *E.coli*	DNA-gyrase-CFX	DNA-gyrase	gyrase	Type
CA9302	CA9317	yes	no	yes	1
CA9303	CA9318	yes	no	yes	1
CA9304	CA9319	yes	no	yes	1
CA9305	CA9320	yes	no	yes	1
CA9306	CA9321	no	no	yes	2
CA9307	CA9322	no	no	yes	2
CA9308	CA9323	yes	no	yes	1
CA9309	CA9324	yes	no	yes	1
CA9310	CA9325	no	no	yes	2

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CLAIMS

1. A method to select conformation-selective binding agents of a protein complex, the method comprising the steps of:
    a) Displaying a collection of binding agents at the extracellular surface of a population of cells
    b) Using cell sorting to separate, from said population of cells of b), cells displaying binding agents that
        i. specifically bind to a protein complex conformation and not to the individual members of the complex, and/or
        ii. specifically bind to one of the individual members of a protein complex and not to the protein complex conformation nor to the other individual member(s) of the complex, and/or
        iii. specifically bind to one of the individual members of a protein complex and to a protein complex conformation and not to the other individual member(s) of the complex.
2. The method of claim 1, wherein the individual members of the protein complex are distinguishably tagged and step b) comprises the steps of:
    a) Incubating a mixture of distinguishably tagged individual members of a protein complex with the population of cells under suitable conditions to allow binding to the cells
    b) Using cell sorting to select, from said population of cells, cells displaying binding agents that
        i. specifically bind to a protein complex conformation and not to the individual members of the complex, and/or
        ii. specifically bind to one of the individual members of a protein complex and not to the protein complex conformation nor to the other individual member(s) of the complex, and/or
        iii. specifically bind to one of the individual members of a protein complex and to a protein complex conformation and not to the other individual member(s) of the complex.
3. The method of claims 1-2, wherein at least one of the individual members of the protein complex is labeled with a fluorescent label.
4. The method of claims 1-3, wherein the individual members of the protein complex are each labeled with a distinguishable fluorescent label.

5. The method of claims 1-4, wherein the cell sorting in step b) is done using FACS.
6. The method of claim 1-5, wherein said protein complex is a protein-protein complex that is constituted of at least two interacting monomeric proteins.
7. The method of claim 1-5, wherein said protein complex is a protein-nucleic acid complex that is constituted of at least one monomeric protein interacting with at least one nucleic molecule.
8. The method of claim 1-6, wherein said method comprises isolating the binding agent from the cell sorted in step b).
9. The method of claim 8, further comprising measuring the binding specificity and/or affinity of the binding agent for the protein complex conformation as compared to the individual members of the protein complex by any suitable technique, such as biophysical method or FACS.
10. The method of claims 1-9, wherein said plurality of binding agents is a library of antibodies or antibody fragments.
11. The method of claim 10, wherein said antibodies fragments are immunoglobulin single domain antibodies, in particular nanobodies.
12. The method of claim 10, wherein said antibodies or antibody fragments are obtained from an animal that has been immunized with the protein complex in a cross-linked form.
13. The method of claim 12, wherein said animal is a camelid.
14. The method of claims 1-13, wherein said population of cells is a population of yeast cells.
15. A composition comprising a binding agent obtained by any of the above methods.
16. A composition comprising a protein complex and a binding agent obtained by any of the above methods, whereby the protein complex is stabilized by the binding agent.

Figure 1

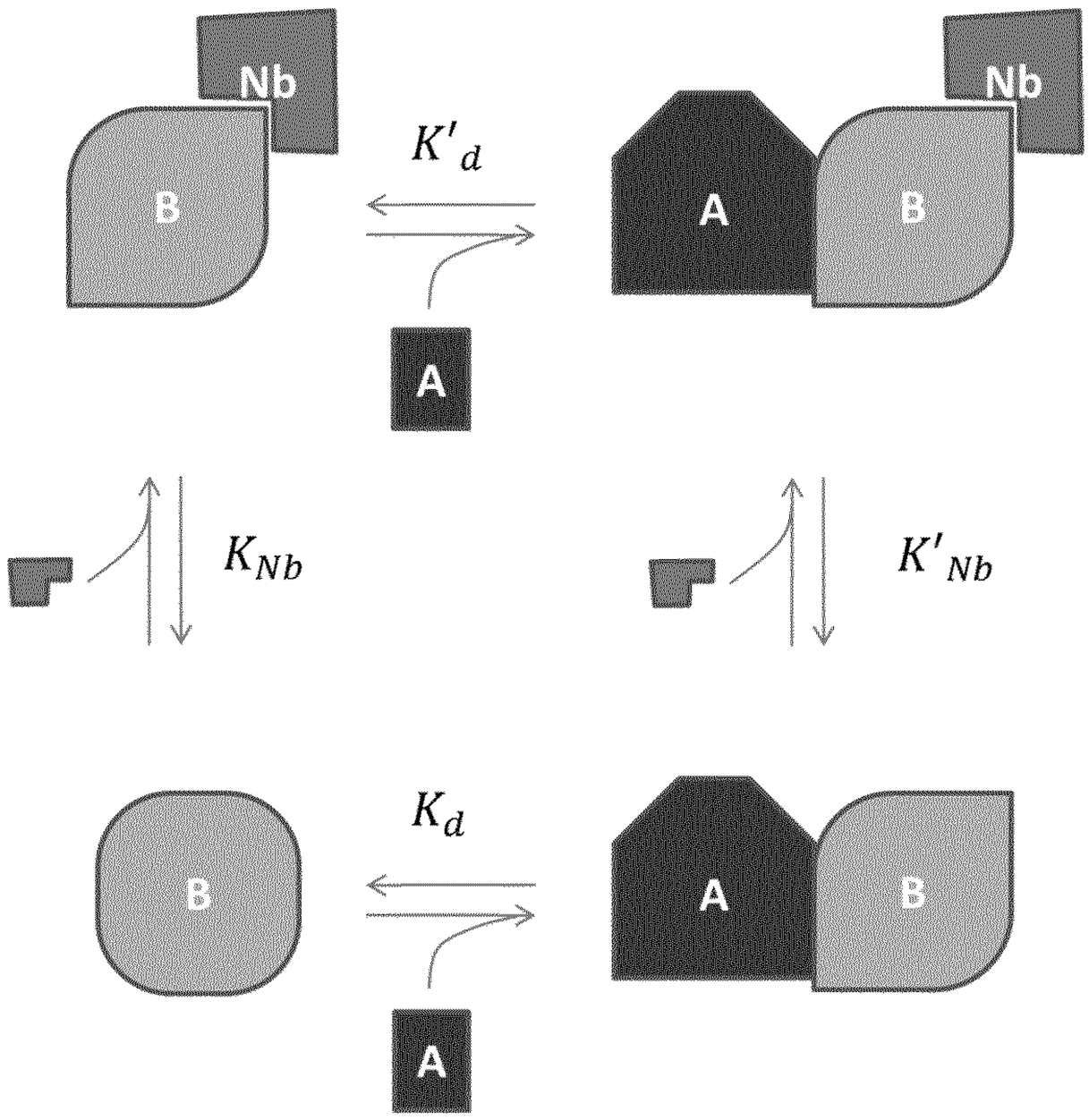


Figure 2

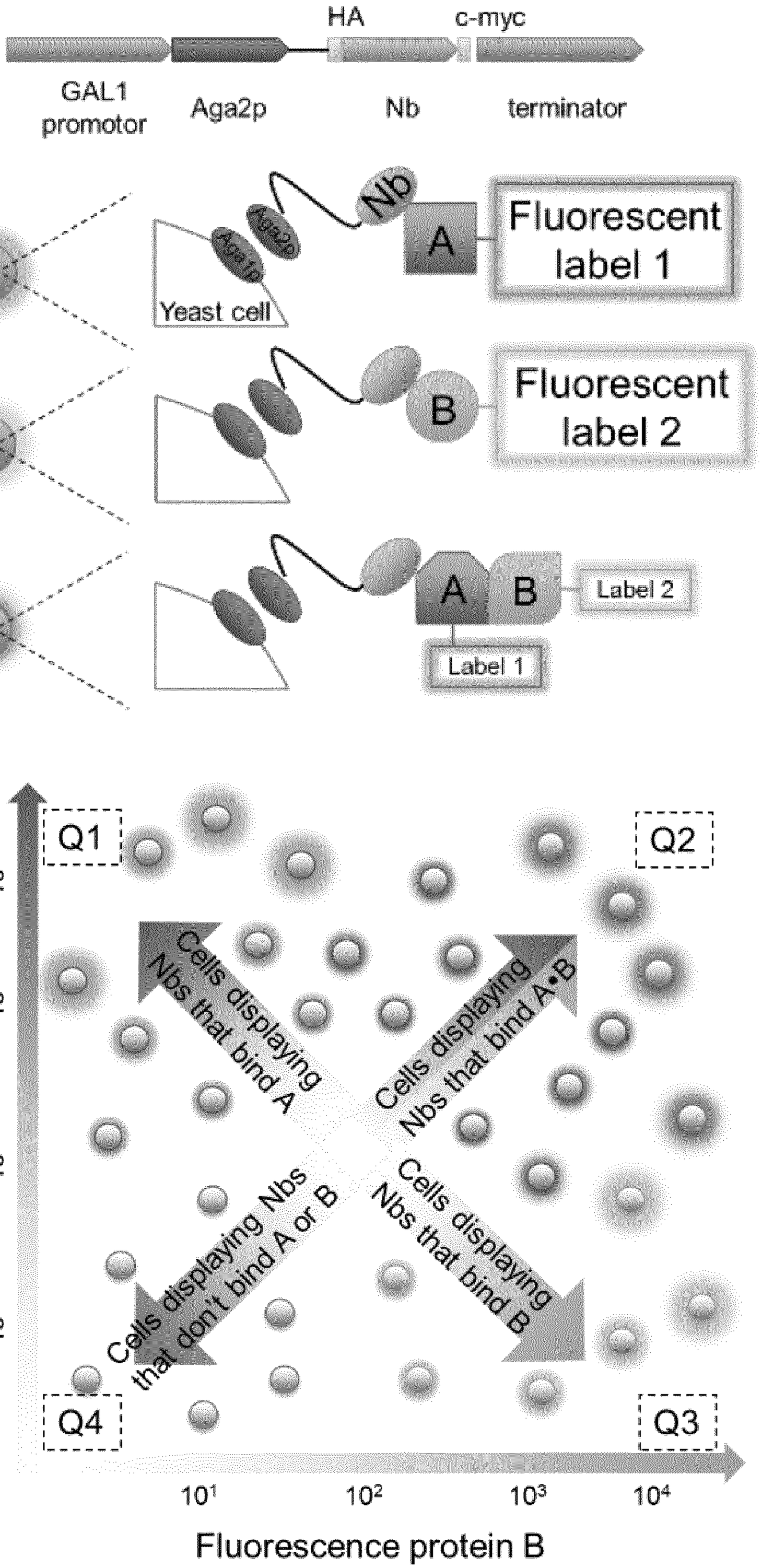

HA
c-myc
GAL1 promotor
Aga2p
Nb
terminator
Aga1p
Aga2p
Nb
A
Fluorescent label 1
Yeast cell
B
Fluorescent label 2
A
B
Label 2
Label 1
Q1
Q2
Q3
Q4
Cells displaying Nbs that bind A
Cells displaying Nbs that bind A•B
Cells displaying Nbs that don't bind A or B
Cells displaying Nbs that bind B
Fluorescence protein A
Fluorescence protein B
10^1
10^2
10^3
10^4

Figure 3

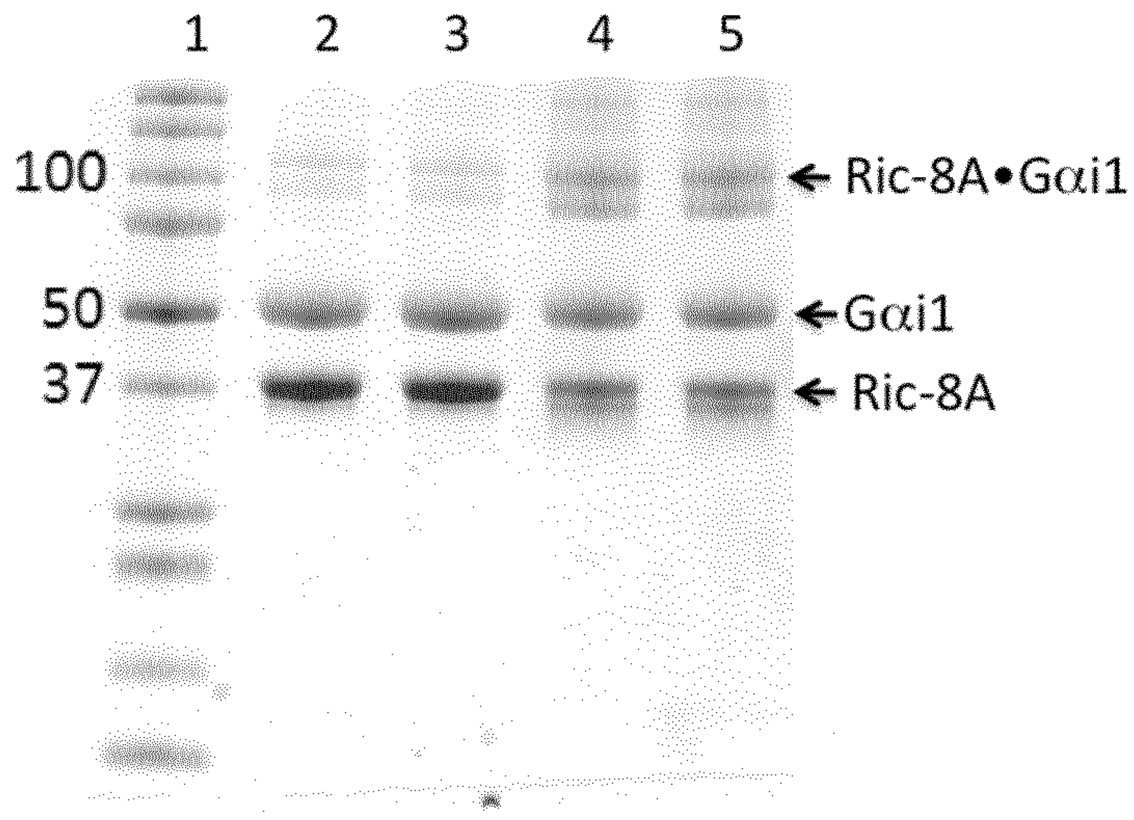
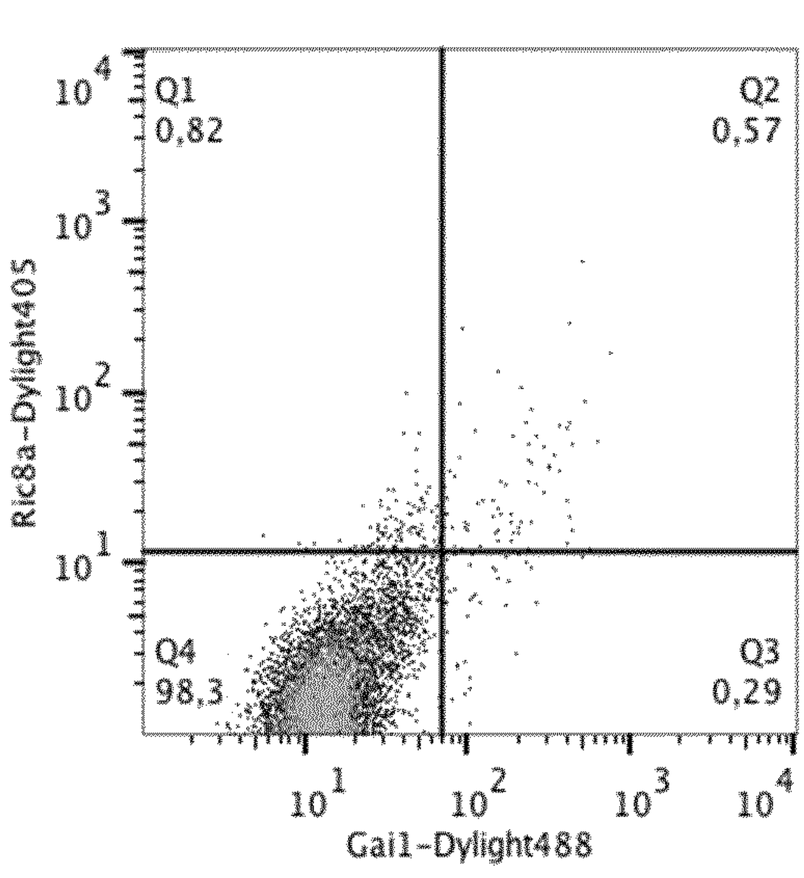
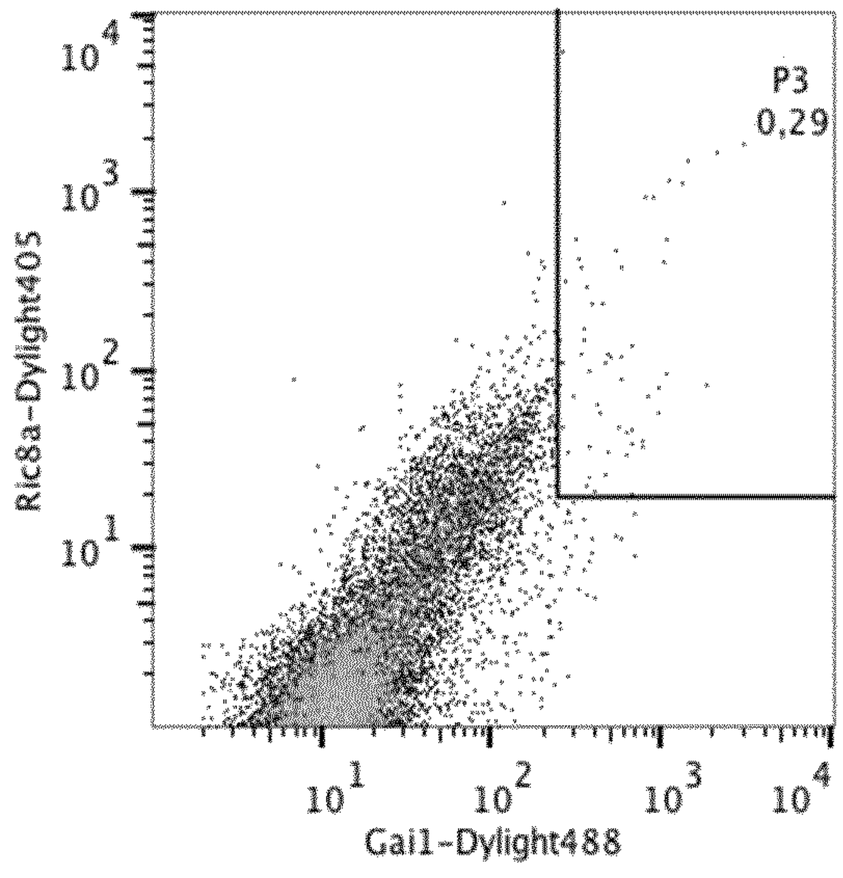


Figure 4

Round 1



Round 2



Round 3

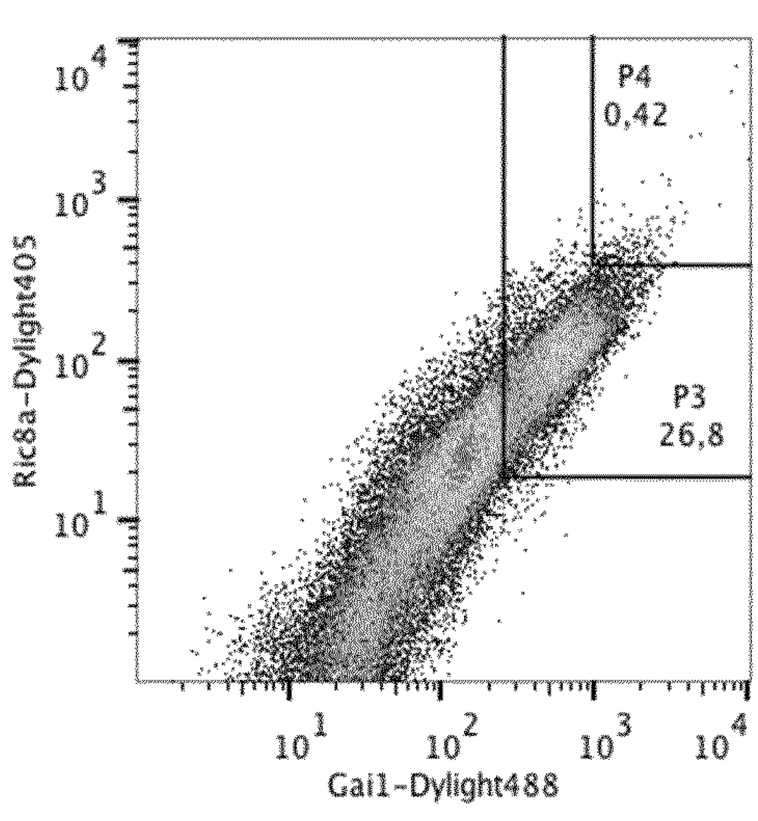
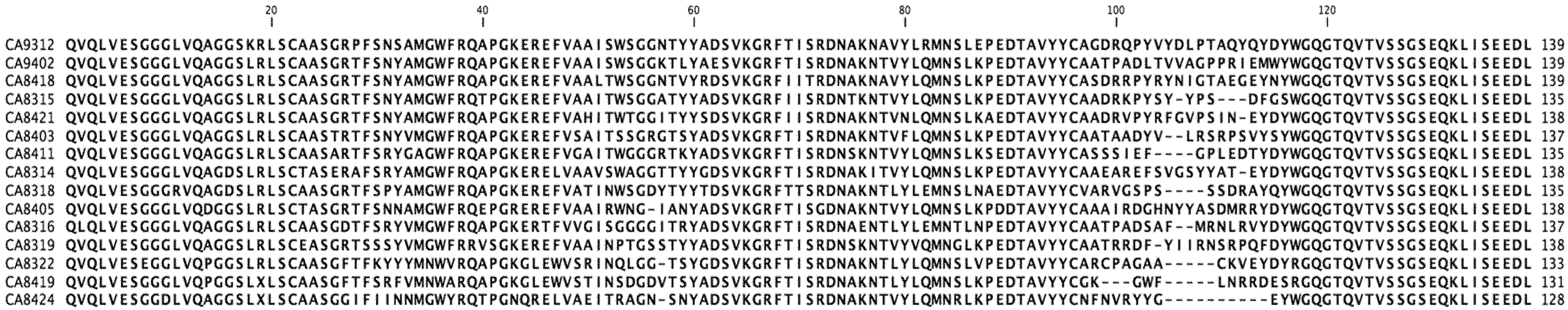


Figure 5


20
40
60
80
100
120
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CA9402 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFVAAISWSGGKTLYAESVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAATPADLTVVAGPPRIEMWYWGQGTQVTVSSGSEQKLISEEDL 139
CA8418 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFVAALTWSGGNTVYRDSVKGRFIITRDNAKNAVYLQMNSLKPEDTAVYYCASDRRPYRYNIGTAEGEYNYWGQGTQVTVSSGSEQKLISEEDL 139
CA8315 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQTPGKEREFVAAITWSGGATYYADSVKGRFIISRDNTKNTVYLQMNSLKPEDTAVYYCAADRKPYSY-YPS---DFGSWGQGTQVTVSSGSEQKLISEEDL 135
CA8421 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFVAHITWTGGITYYSDSVKGRFIISRDNAKNTVNLQMNSLKAEDTAVYYCAADRVPYRFGVPSIN-EYDYWGQGTQVTVSSGSEQKLISEEDL 138
CA8403 QVQLVESGGGLVQAGGSLRLSCAASTRTFSNYVMGWFRQAPGKEREFVSAITSSGRGTSYADSVKGRFTISRDNAKNTVFLQMNSLKPEDTAVYYCAATAADYV--LRSRPSVYSYWGQGTQVTVSSGSEQKLISEEDL 137
CA8411 QVQLVESGGGLVQAGGSLRLSCAASARTFSRYGAGWFRQAPGKEREFVGAITWGGGRTKYADSVKGRFTISRDNSKNTVYLQMNSLKSEDTAVYYCASSSIEF----GPLEDTYDYWGQGTQVTVSSGSEQKLISEEDL 135
CA8314 QVQLVESGGGLVQAGDSLRLSCTASERAFSRYAMGWFRQAPGKERELVAAVSWAGGTTYYGDSVKGRFTISRDNAKITVYLQMNSLKPEDTAVYYCAAEAREFSVGSYYAT-EYDYWGQGTQVTVSSGSEQKLISEEDL 138
CA8318 QVQLVESGGGRVQAGDSLRLSCAASGRTFSPYAMGWFRQAPGKEREFVATINWSGDYTYYTDSVKGRFTTSRDNAKNTLYLEMNSLNAEDTAVYYCVARVGSPS----SSDRAYQYWGQGTQVTVSSGSEQKLISEEDL 135
CA8405 QVQLVESGGGLVQDGGSLRLSCTASGRTFSNNAMGWFRQEPGREREFVAAIRWNG-IANYADSVKGRFTISGDNAKNTVYLQMNSLKPDDTAVYYCAAAIRDGHNYYASDMRRYDYWGQGTQVTVSSGSEQKLISEEDL 138
CA8316 QLQLVESGGGLVQAGGSLRLSCAASGDTFSRYVMGWFRQAPGKERTFVVGISGGGGITRYADSVKGRFTISRDNAENTLYLEMNTLNPEDTAVYYCAATPADSAF--MRNLRVYDYWGQGTQVTVSSGSEQKLISEEDL 137
CA8319 QVQLVESGGGLVQAGGSLRLSCEASGRTSSSYVMGWFRRVSGKEREFVAAINPTGSSTYYADSVKGRFTISRDNSKNTVYVQMNGLKPEDTAVYYCAATRRDF-YIIRNSRPQFDYWGQGTQVTVSSGSEQKLISEEDL 138
CA8322 QVQLVESEGGLVQPGGSLRLSCAASGFTFKYYYMNWVRQAPGKGLEWVSRINQLGG-TSYGDSVKGRFTISRDNAKNTLYLQMNSLVPEDTAVYYCARCPAGAA-----CKVEYDYRGQGTQVTVSSGSEQKLISEEDL 133
CA8419 QVQLVESGGGLVQPGGSLXLSCAASGFTFSRFVMNWARQAPGKGLEWVSTINSDGDVTSYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCGK---GWF-----LNRRDESRGQGTQVTVSSGSEQKLISEEDL 131
CA8424 QVQLVESGGDLVQAGGSLXLSCAASGGIFIINNMGWYRQTPGNQRELVAEITRAGN-SNYADSVKGRFTISRDNAKNTVYLQMNRLKPEDTAVYYCNFNVRYYG----------EYWGQGTQVTVSSGSEQKLISEEDL 128

Figure 6

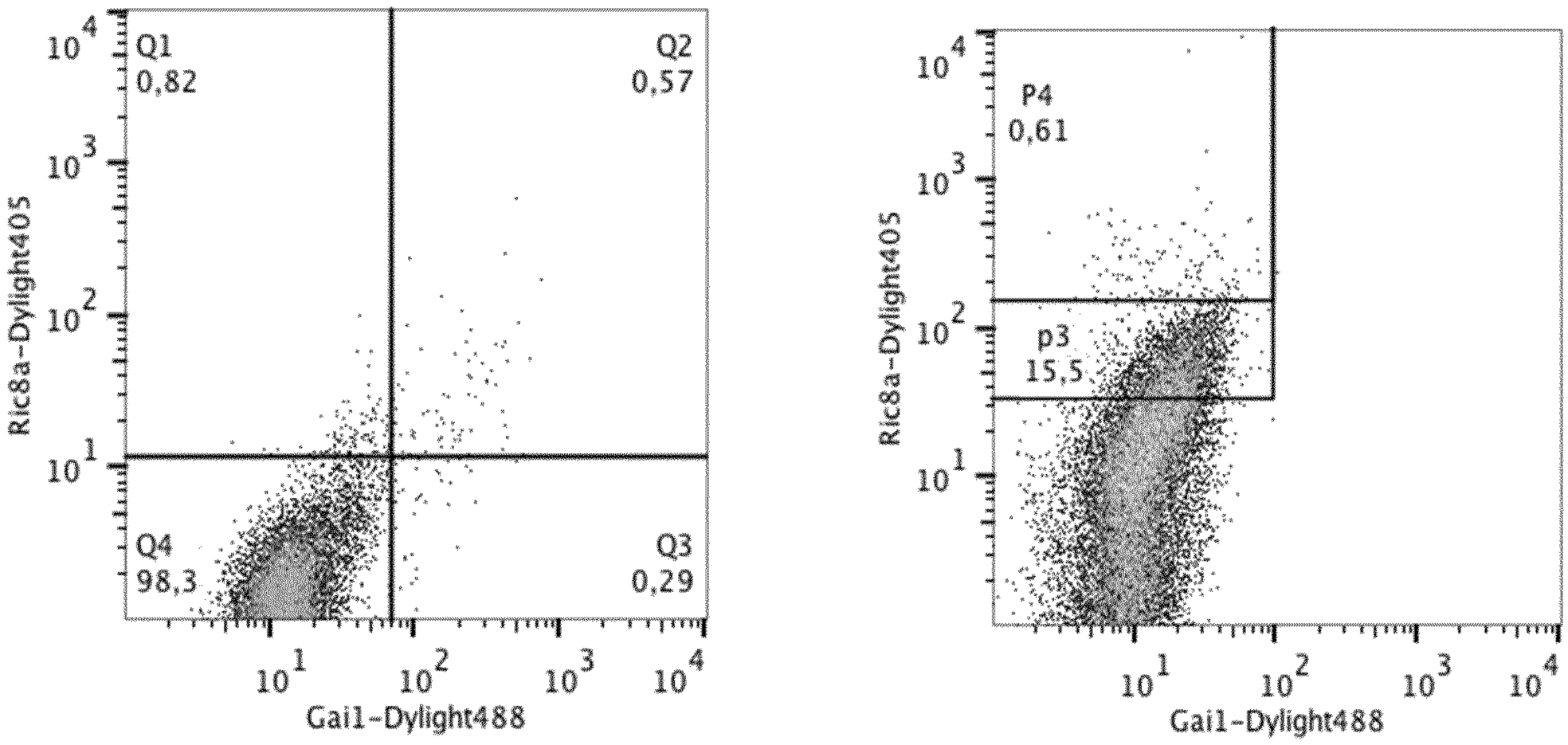
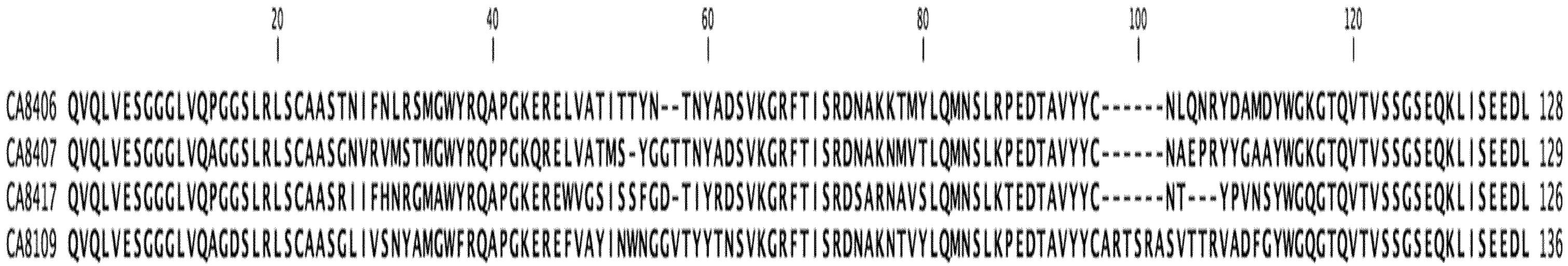

Round 1
Round 2
Q1
0,82
Q2
0,57
Q4
98,3
Q3
0,29
P4
0,61
p3
15,5
Ric8a-Dylight405
Gai1-Dylight488
10^1
10^2
10^3
10^4

Figure 7


20
40
60
80
100
120
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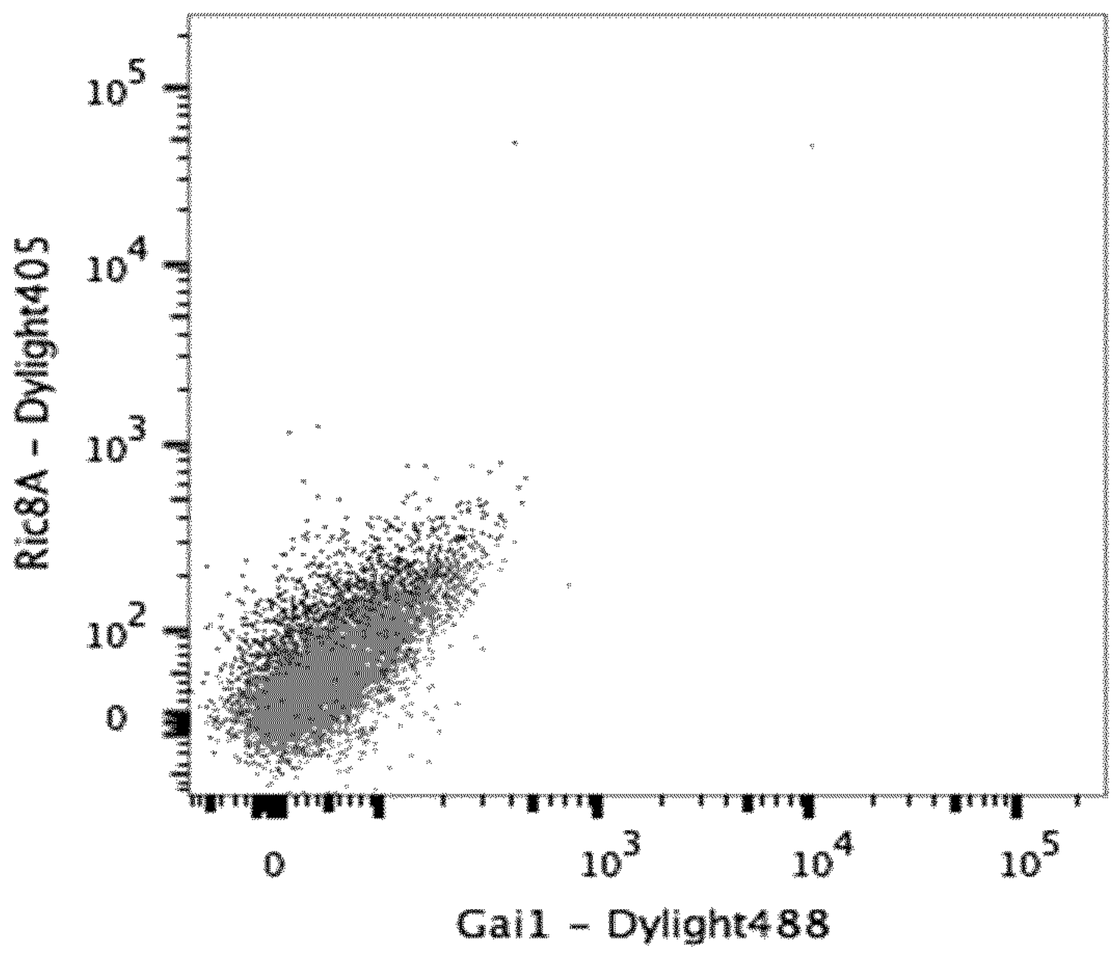
CA8407 QVQLVESGGGLVQAGGSLRLSCAASGNVRVMSTMGWYRQPPGKQRELVATMS-YGGTTNYADSVKGRFTISRDNAKNMVTLQMNSLKPEDTAVYYC------NAEPRYYGAAYWGKGTQVTVSSGSEQKLISEEDL 129
CA8417 QVQLVESGGGLVQPGGSLRLSCAASRIIFHNRGMAWYRQAPGKEREWVGSISSFGD-TIYRDSVKGRFTISRDSARNAVSLQMNSLKTEDTAVYYC------NT---YPVNSYWGQGTQVTVSSGSEQKLISEEDL 126
CA8109 QVQLVESGGGLVQAGDSLRLSCAASGLIVSNYAMGWFRQAPGKEREFVAYINWNGGVTYYTNSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCARTSRASVTTRVADFGYWGQGTQVTVSSGSEQKLISEEDL 136

Figure 8

Ric8A/ Type 1



Gαi1 (GDP)/ Type 1

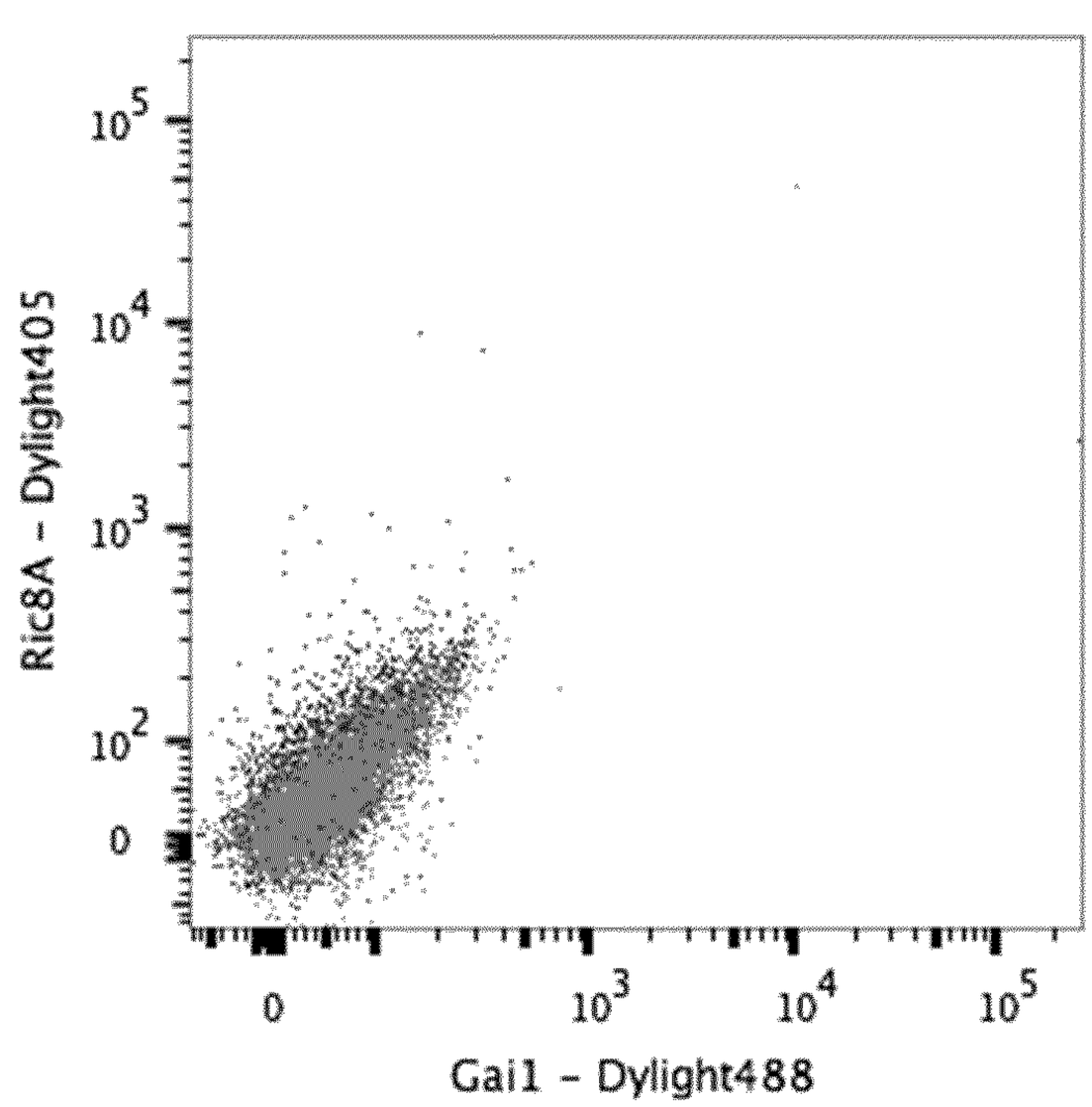
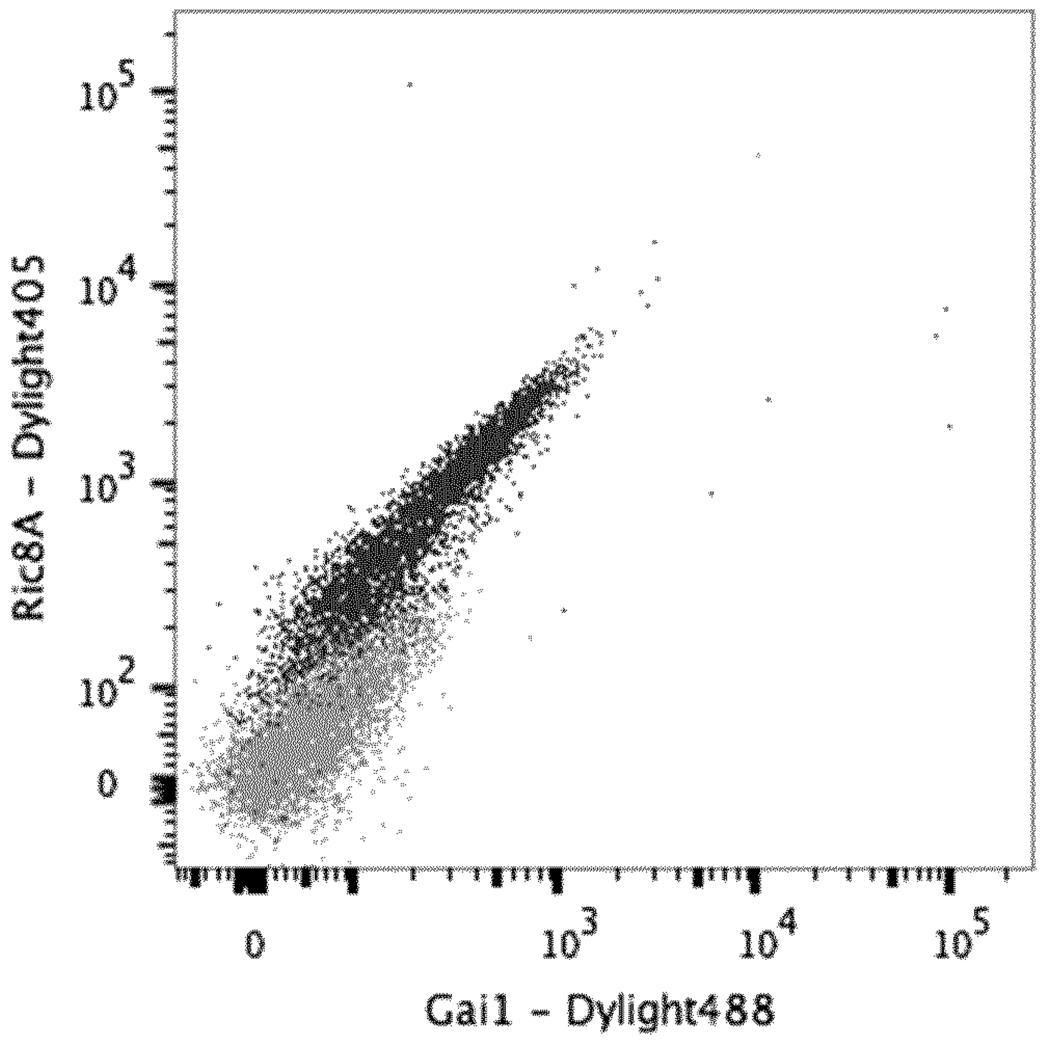


Figure 8 continued

Ric8A/ Gαi1/ Type 1



Ric8A /Type 2

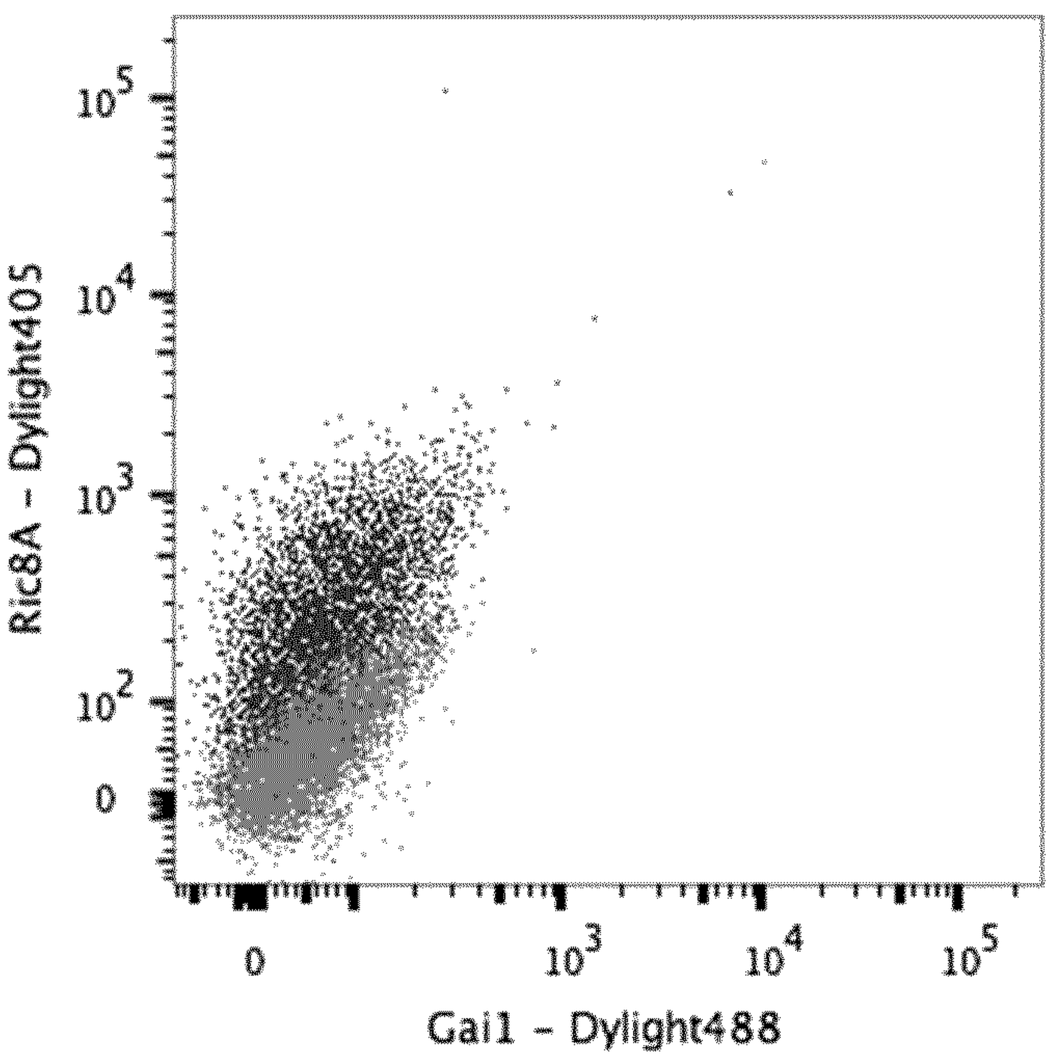
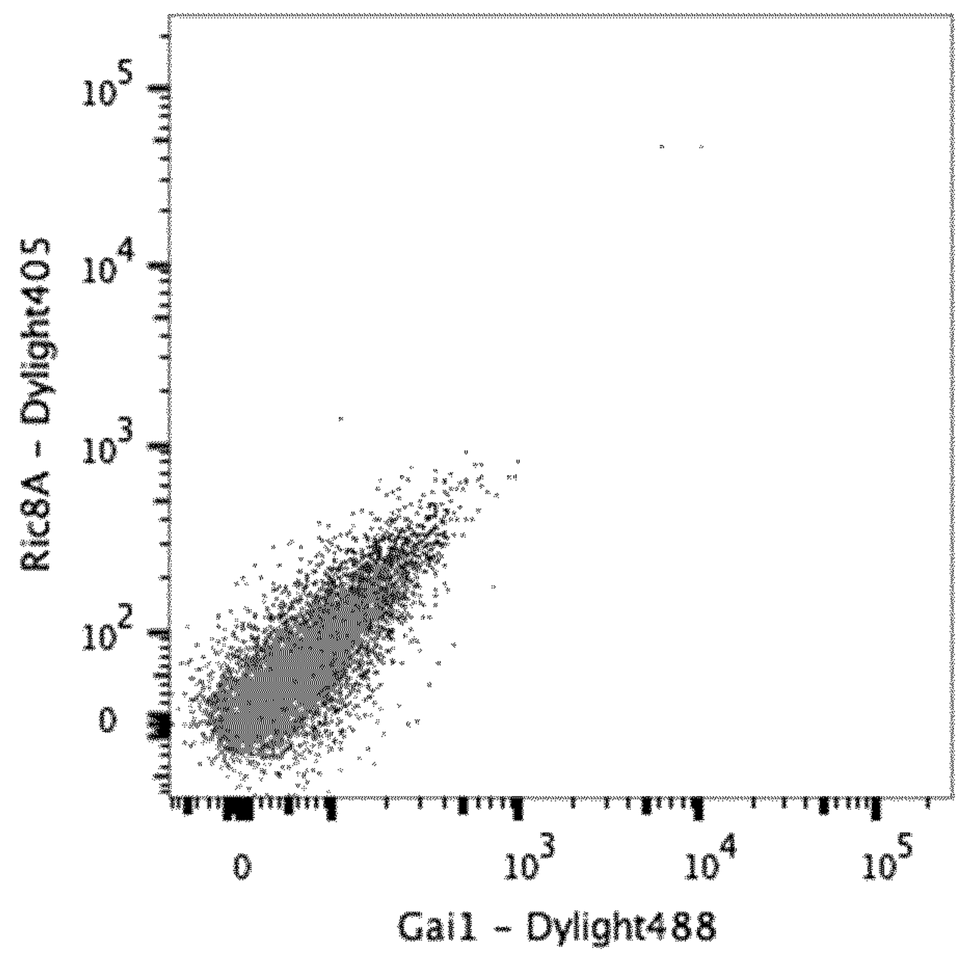


Figure 8 continued

Gαi1 (GDP)/ Type 2



Ric8A/ Gαi1/ Type 2

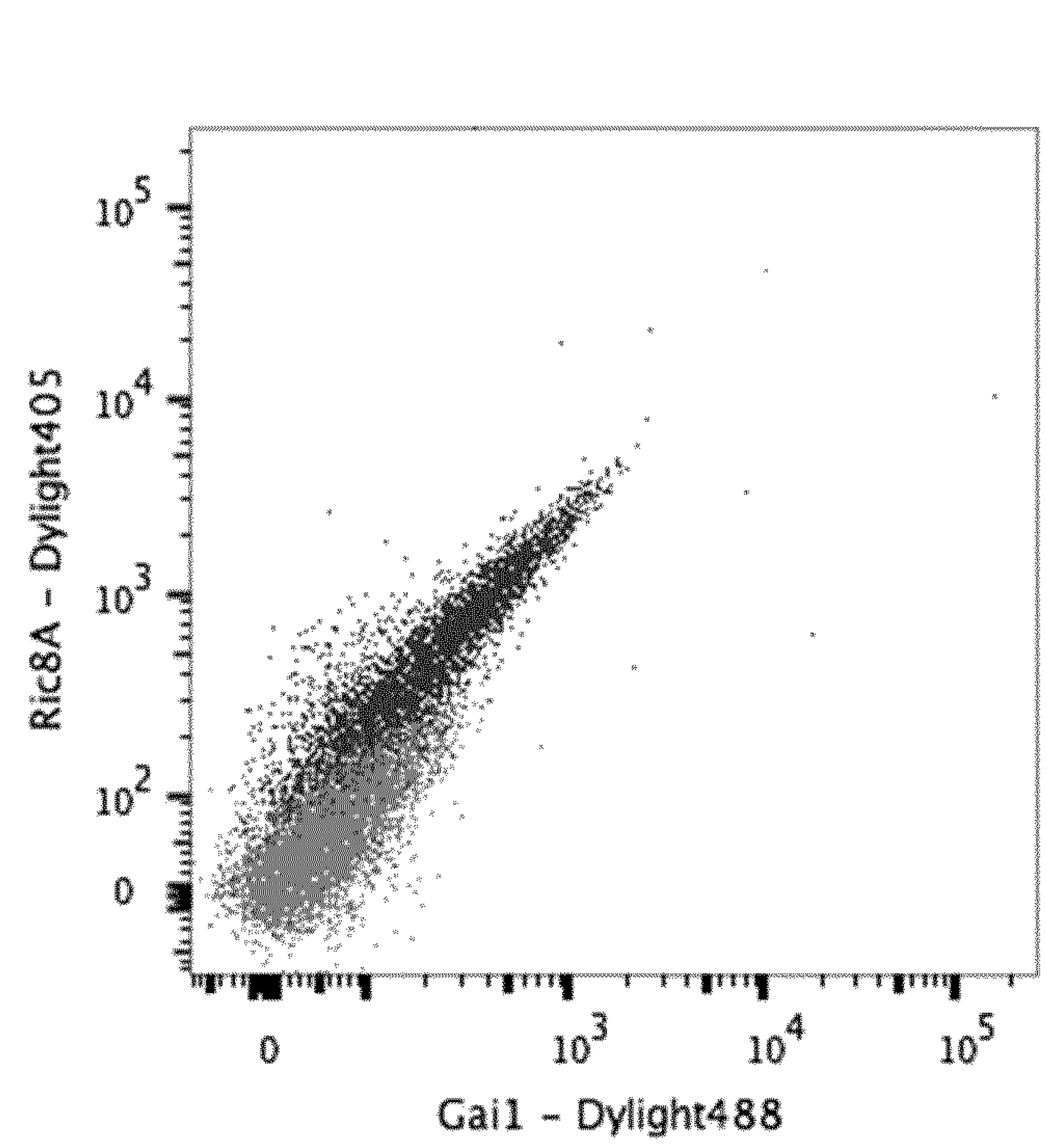
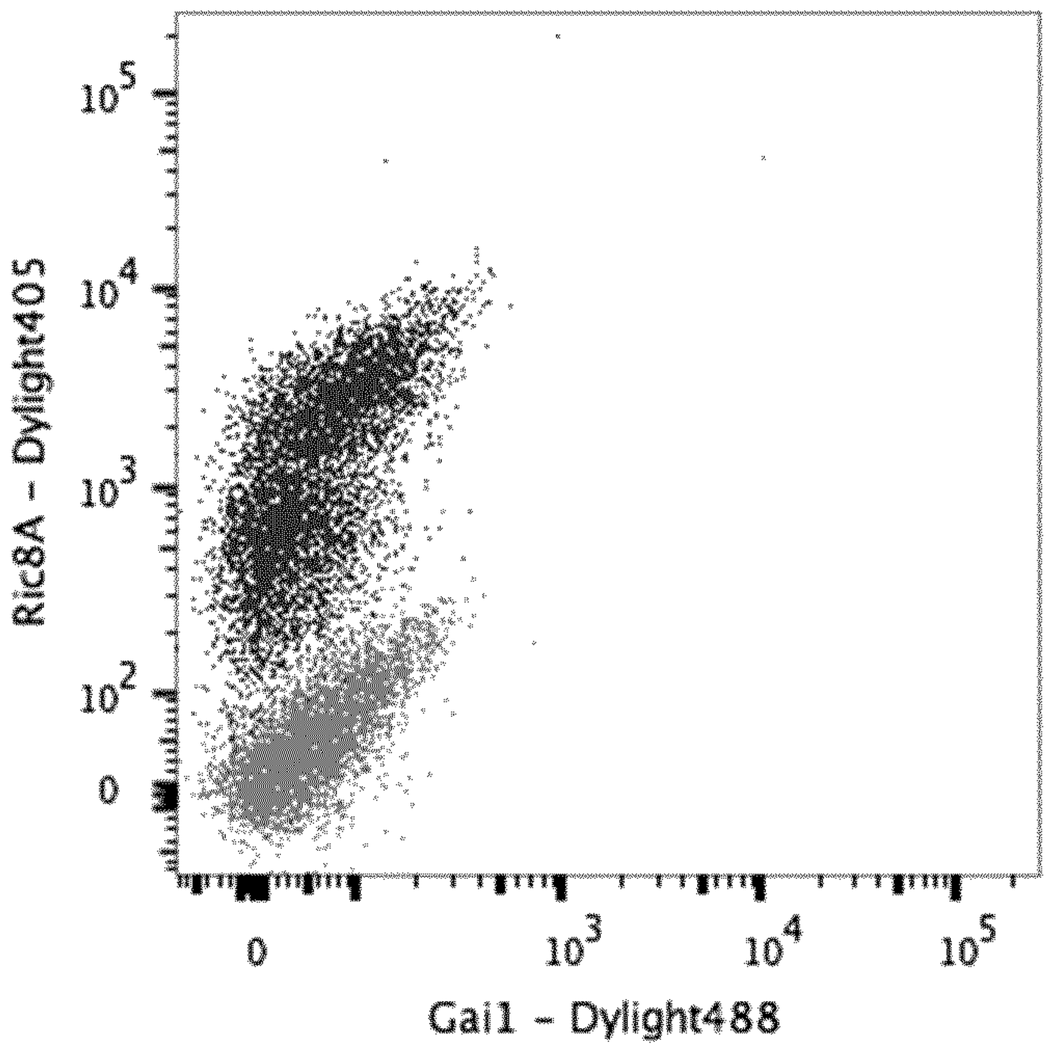


Figure 8 continued

Ric8A/ Type 3



Gαi1 (GDP)/ Type 3

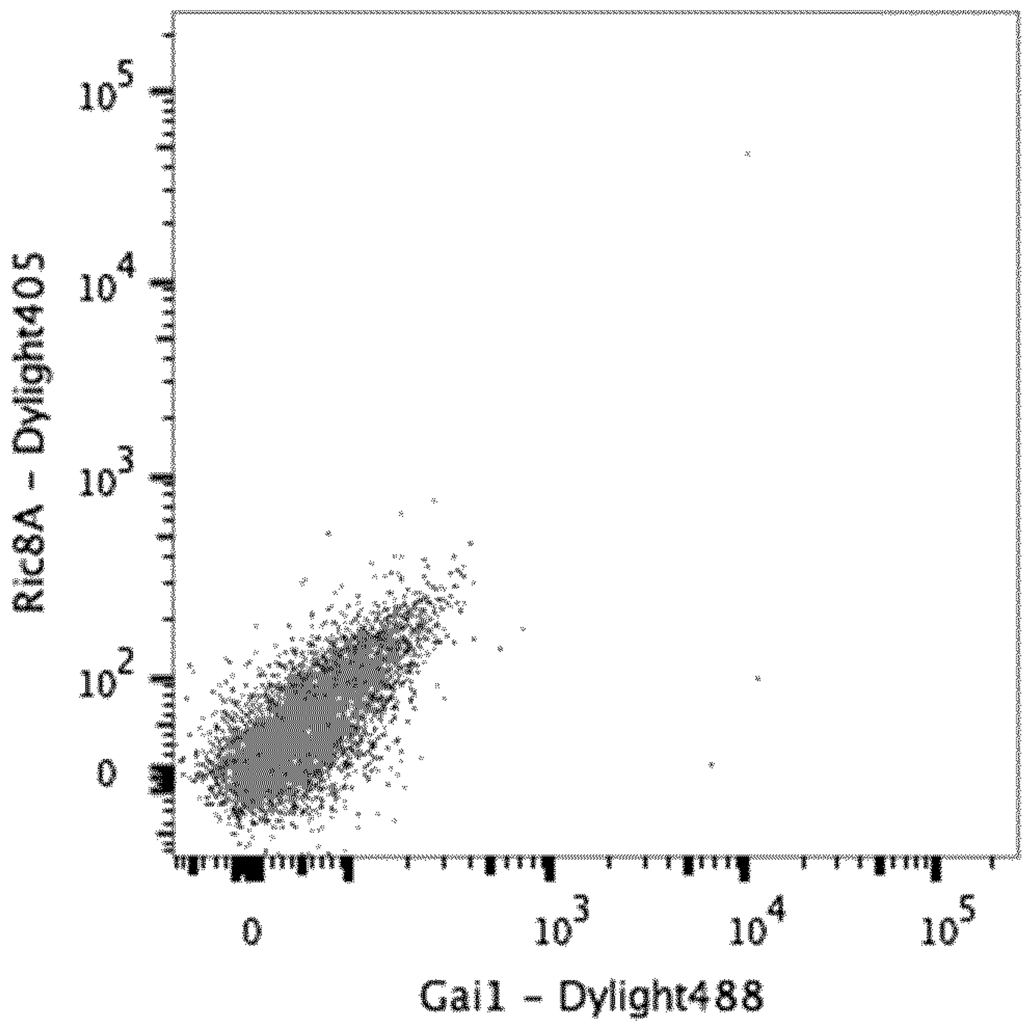


Figure 8 continued

Ric8A/ Gαi1/ Type 3

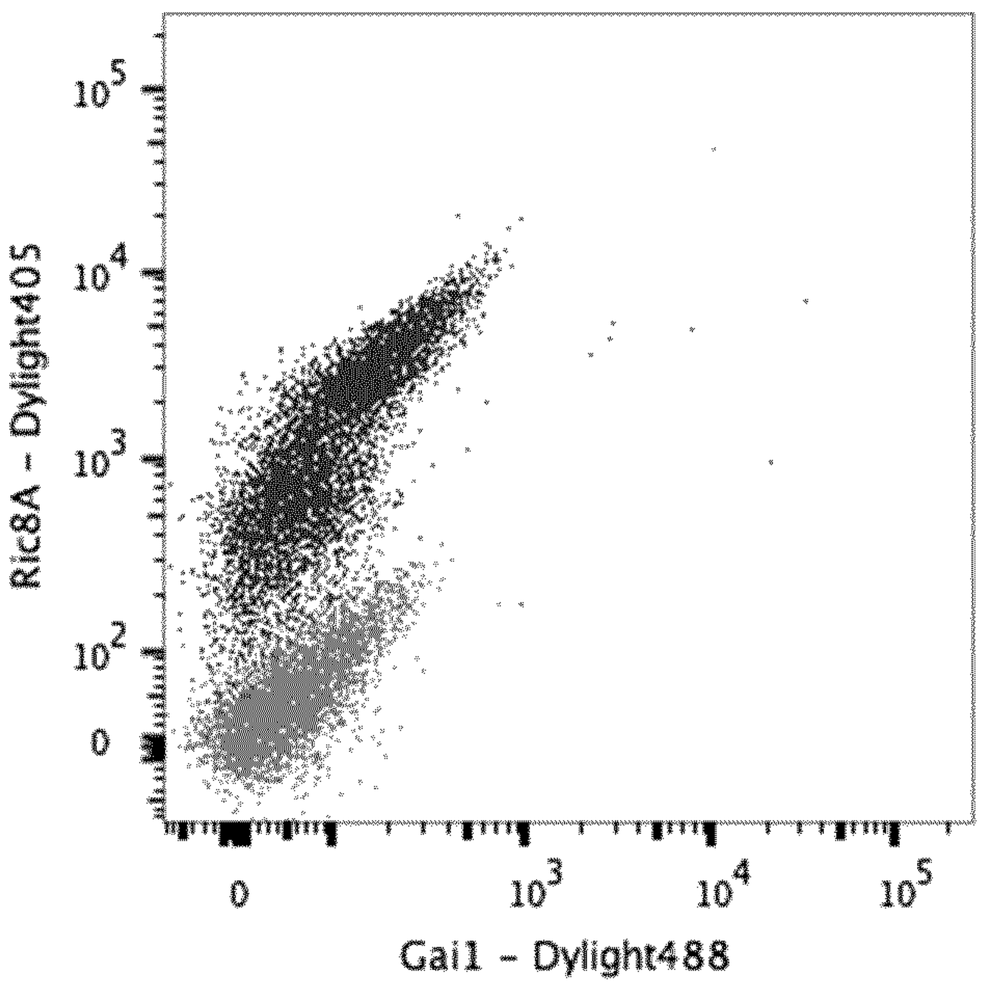


Figure 9

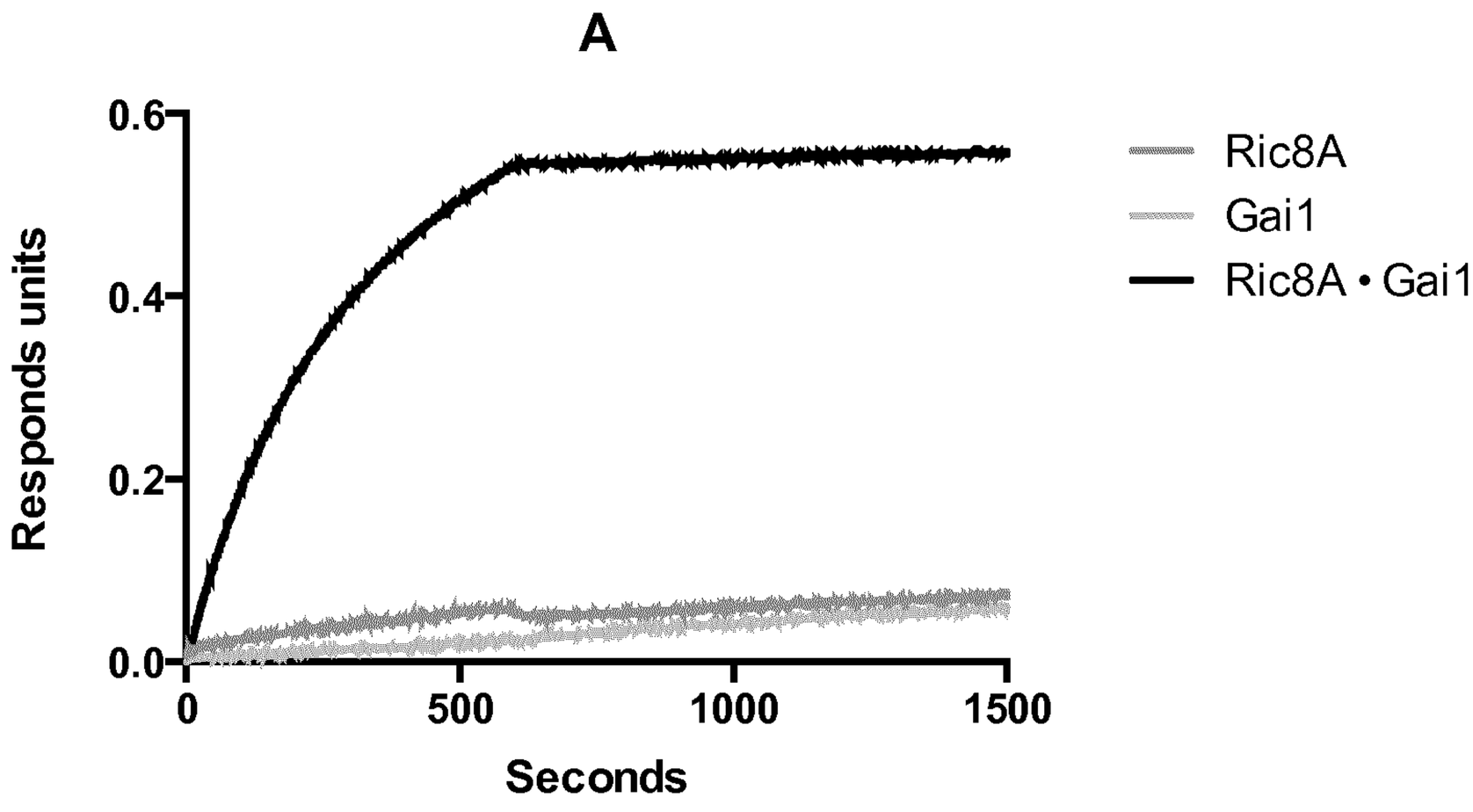


Figure 9 continued

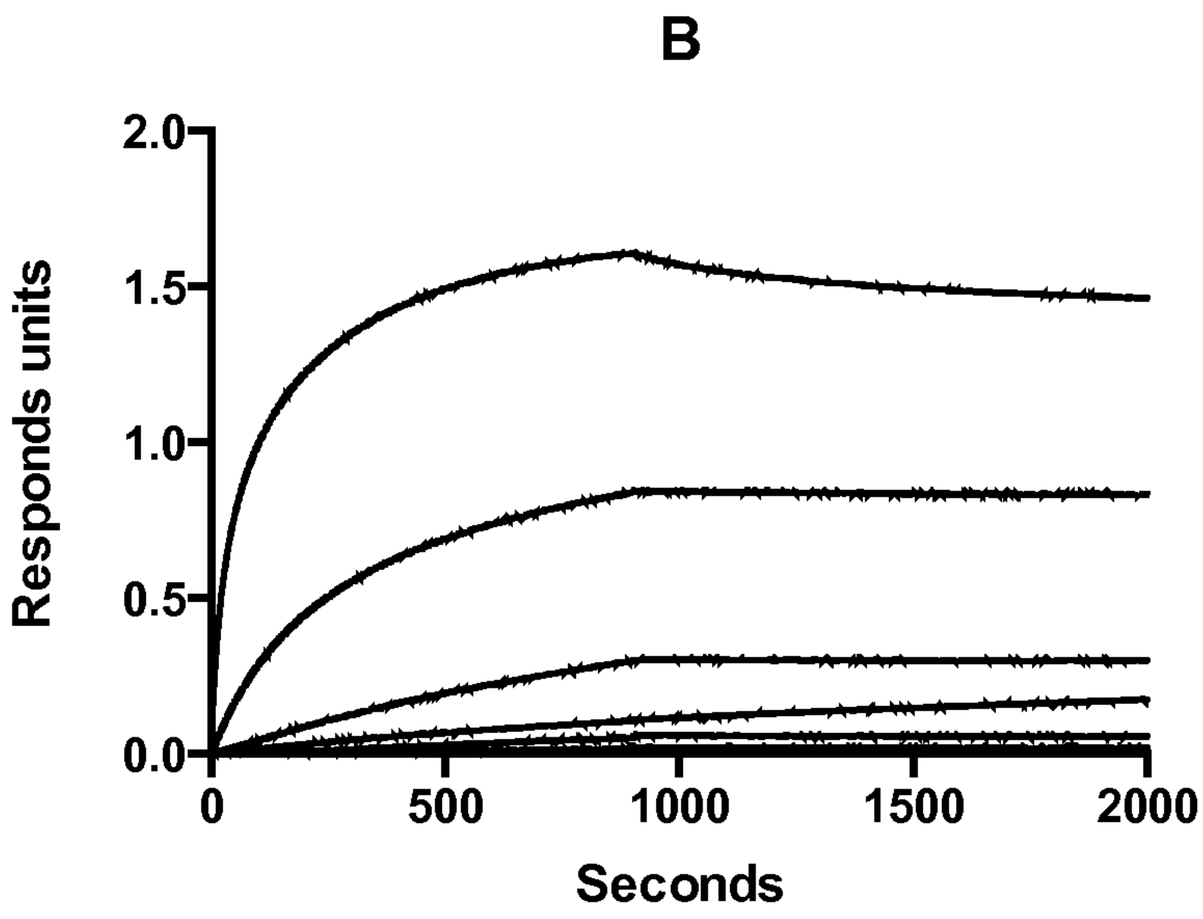


Figure 10

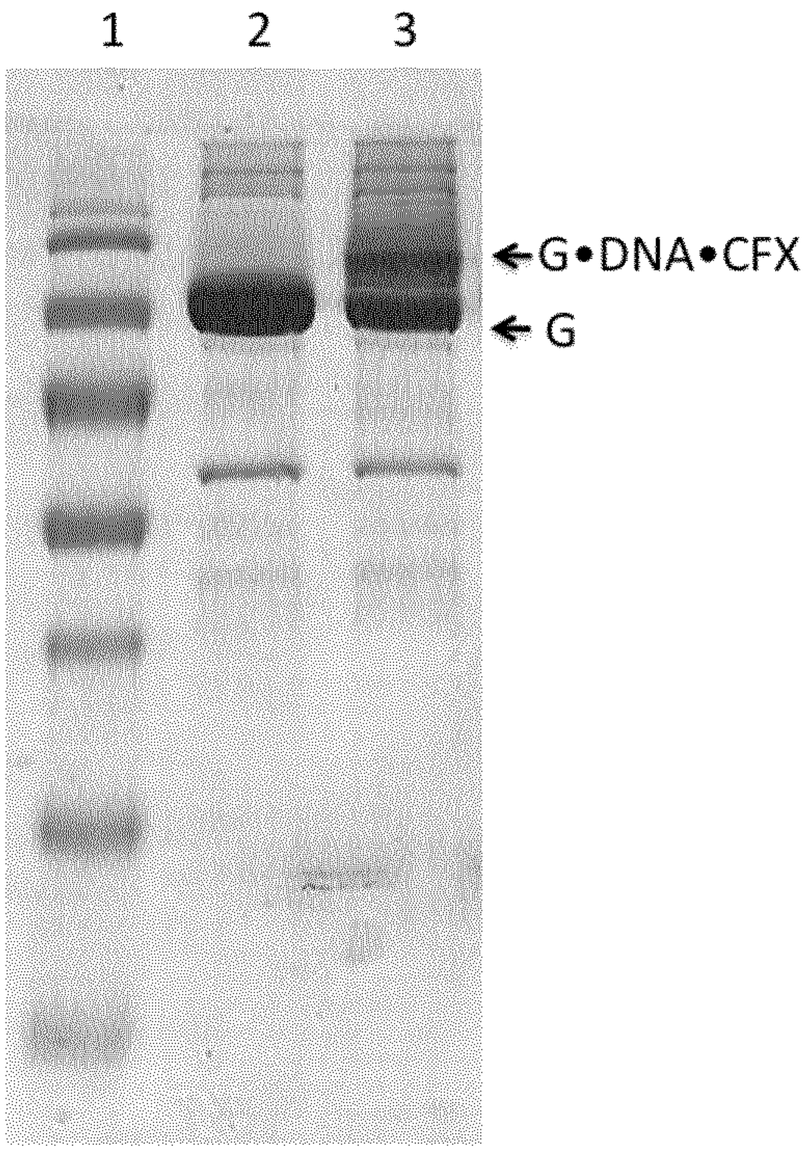
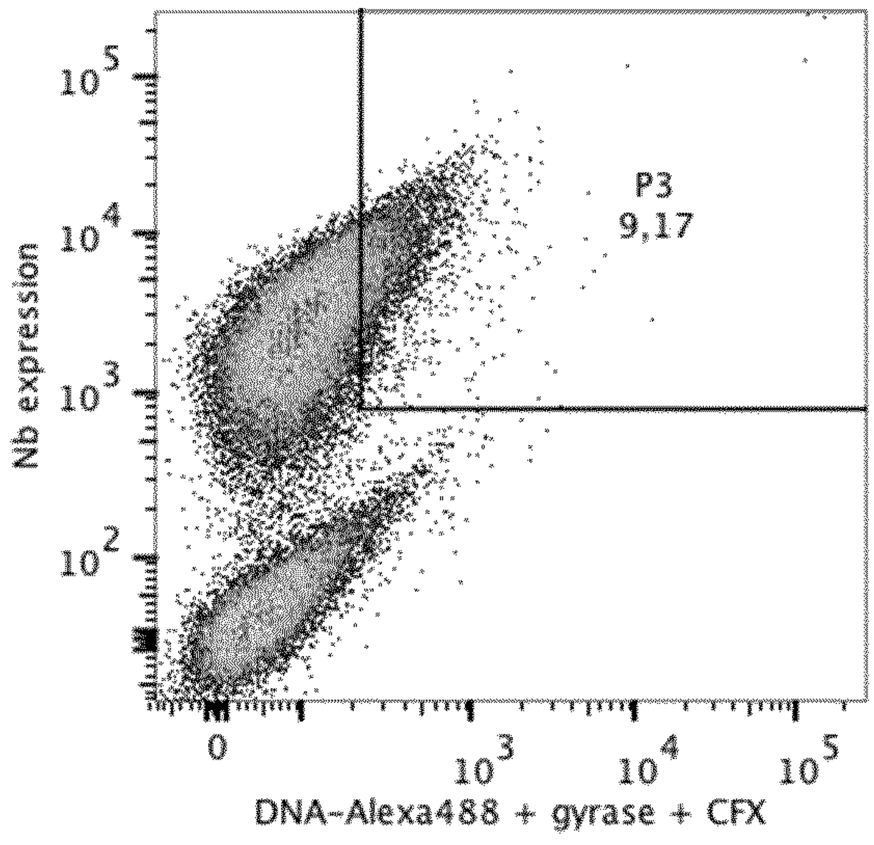
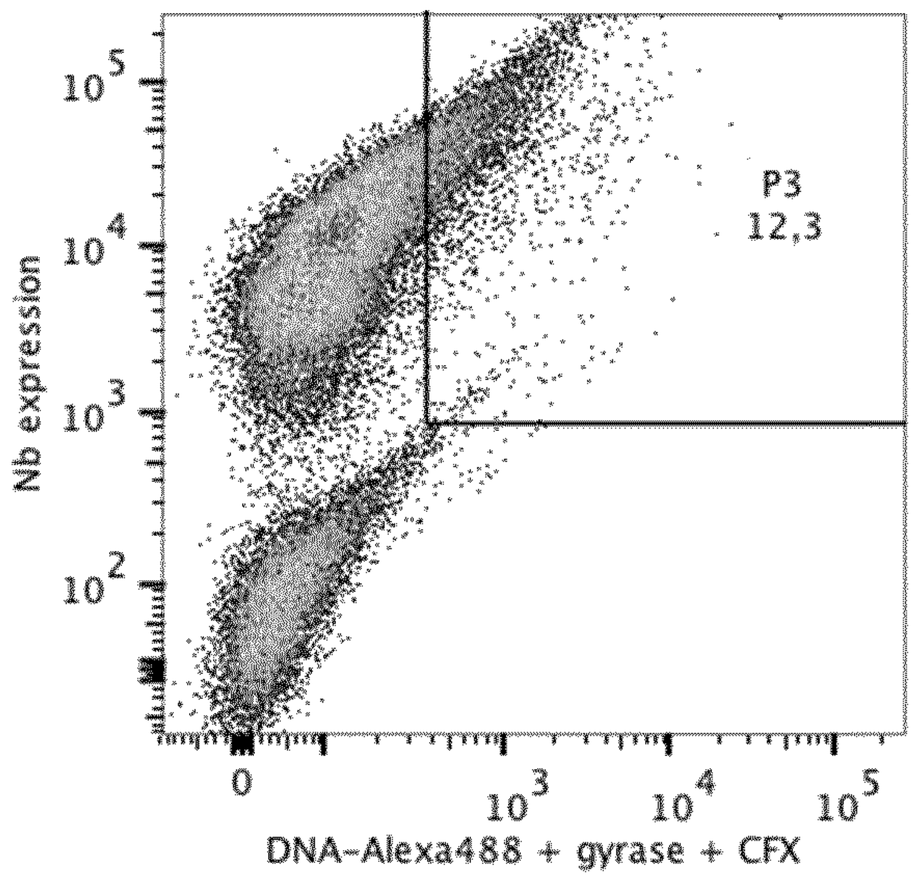
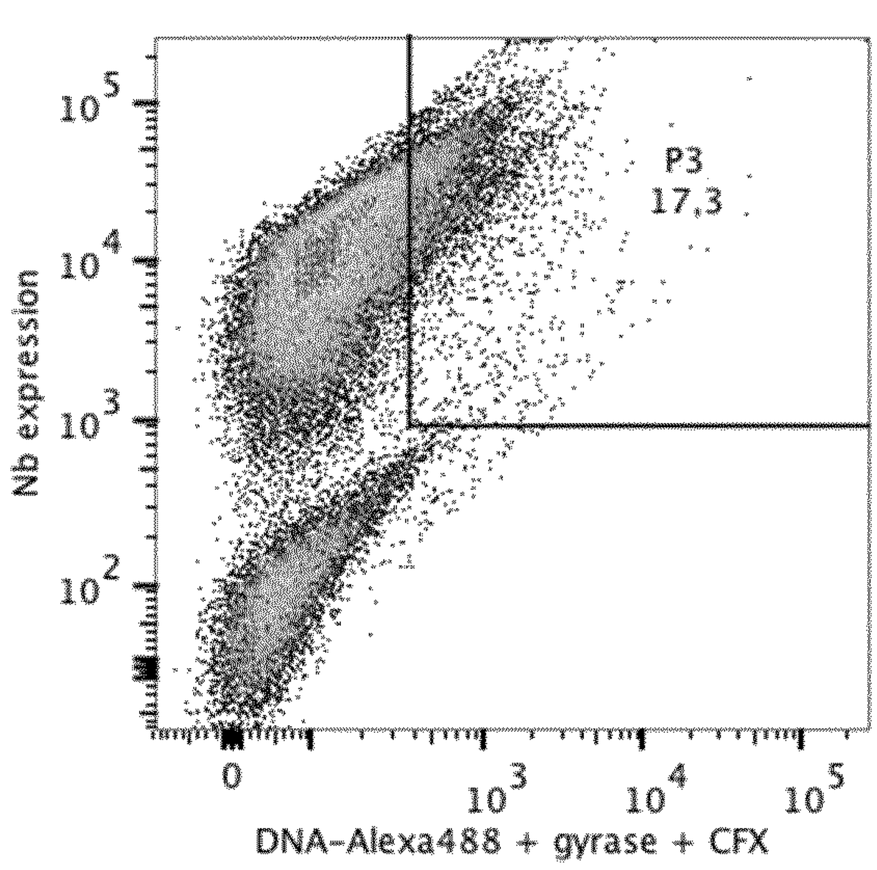


Figure 11


Round 1
P3
9,17
Nb expression
DNA-Alexa488 + gyrase + CFX


Round 2
P3
12,3
Nb expression
DNA-Alexa488 + gyrase + CFX


Round 3a
P3
17,3
Nb expression
DNA-Alexa488 + gyrase + CFX

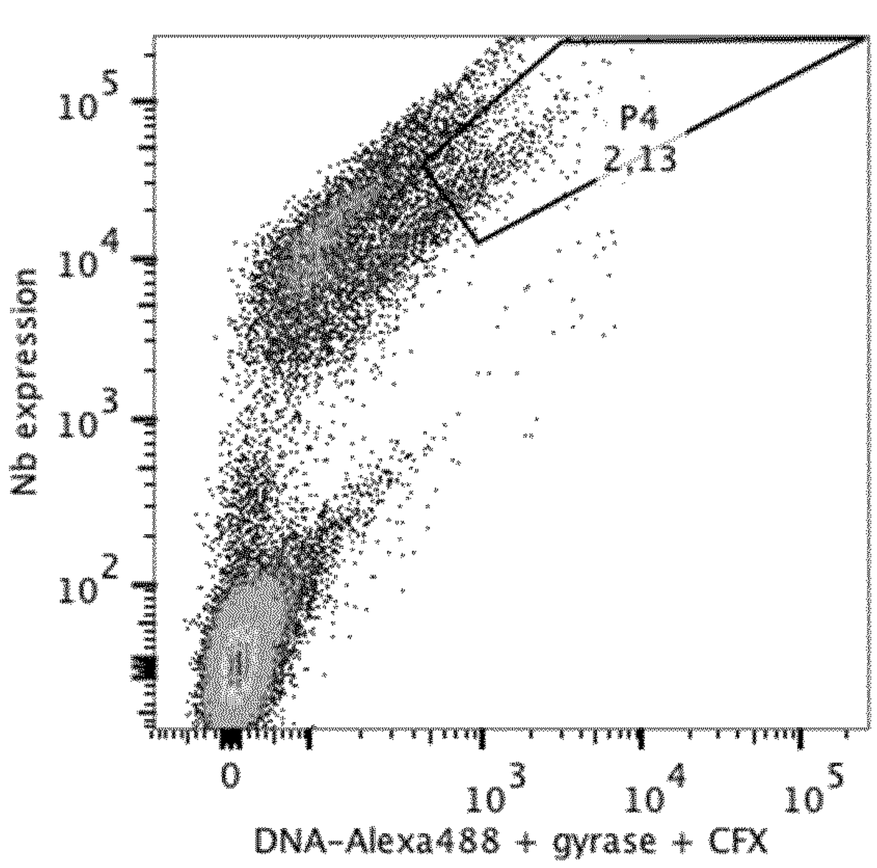
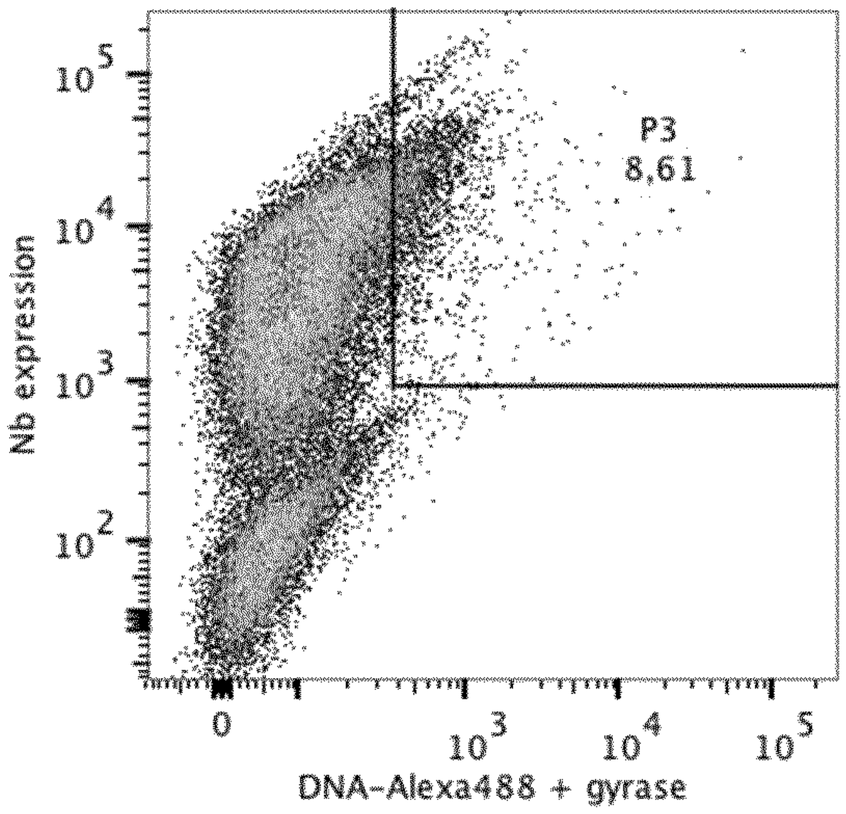

Round 4a
P4
2,13
Nb expression
DNA-Alexa488 + gyrase + CFX

Figure 11 continued

Round 3b



Round 4b

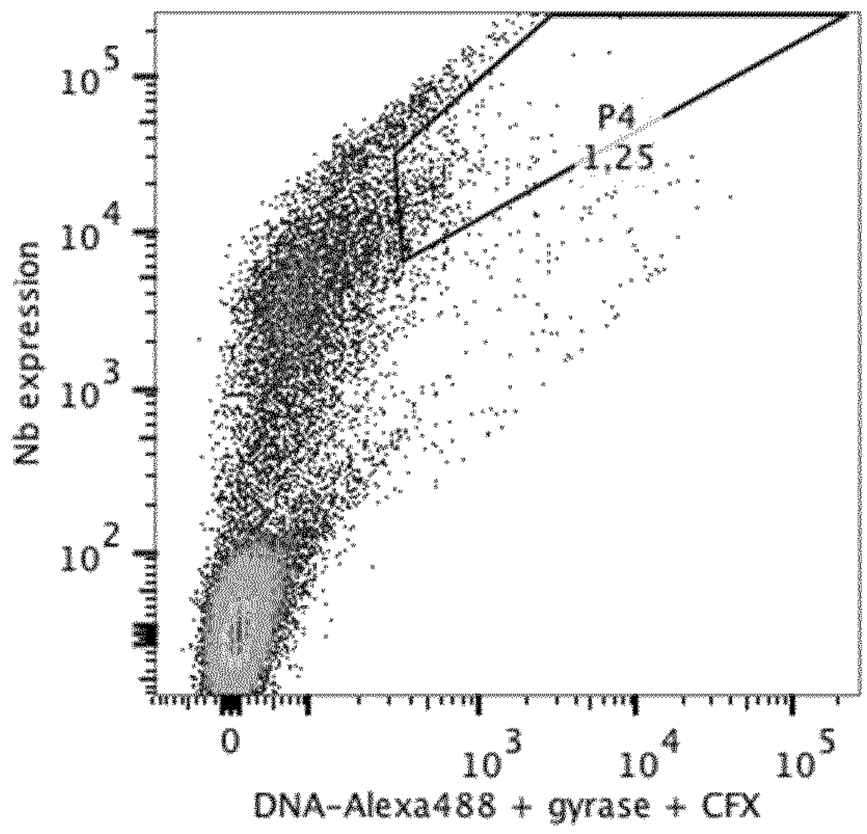


Figure 12

```
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                    |         |         |         |         |         |         |
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CA9305 QVQLVESGGGLVQAGGSLRLSXAASGRSFDIHAMGWFRQAPGKEREFVAAISWNSGNTEYADVVKGRFTISRDNAKDTVYLQMNGLKPEDTAVYYCARGAFSF----ATTVQSDYNYWGQGTQVTVSSGSEQKLISEEDL 136
CA9302 QVQLVESGGGLVQPGGSLRLSCTASEGTFSVYNLAWFRQAPGKERELVASINWSGSVTYYTDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCAA-ALRPN--SVQ-----YKYWGQGTQVTVSSGSEQKLISEEDL 132
CA9303 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYRMGWFRQPPGKEREFVARISPNGGSTYYTDSMRGRFIISRDNVKNTVYLQMNSLKHEDTAVYYCAA-TPGYT--SASKVPSDYAYWGQGTQVTVSSGSEQKLISEEDL 137
CA9307 QVQLVESGGGLVQAGGSLRLSCAASGRTFSSDYMAWIRQPPGKESEFVAVIARSNAGTFYADSVKGRFTISRDNAKNTVYLQMNSLNREDTAVYYCTADHA--L--RLSSRLTDYDYWGQGTQVTVSSGSEQKLISEEDL 136
CA9309 QVQLVESGGGVVQAGGSLRLSCAASGRTLGSYDVGWFRQAPGKEREFLSISRQSGASIYCADSVKGRFTVSRDNAKNLVYLQMNSLKPEDTAVYYCAADPSRWYFCSSDSNPNTFDSWGQGTQVTVSSGSEQKLISEEDL 140
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Figure 13

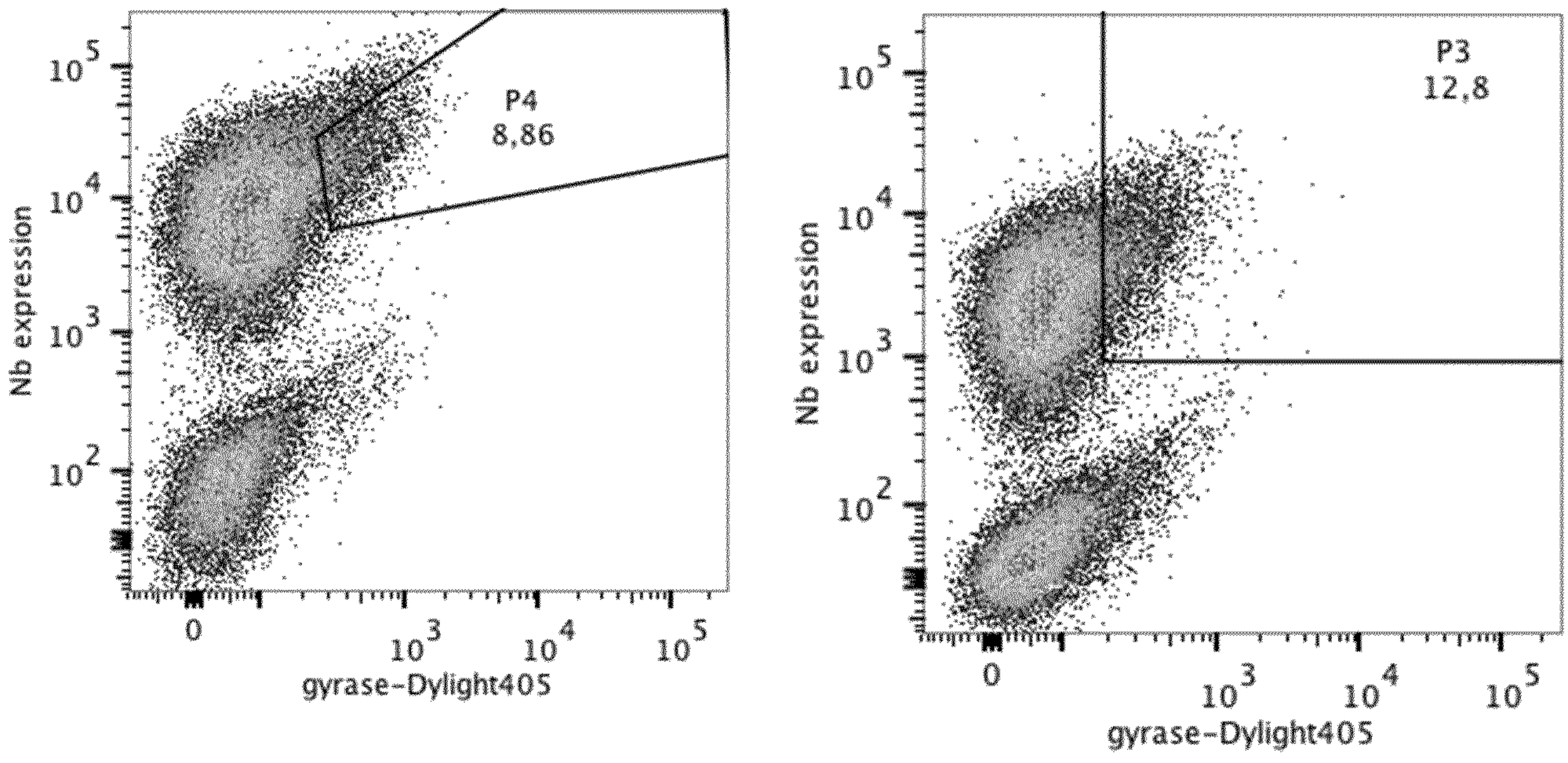
Round 1
Round 2
P4
8,86
P3
12,8
Nb expression
gyrase-Dylight405
0
10^2
10^3
10^4
10^5

Figure 14

```
                    20        40        60        80        100       120       140
                    |         |         |         |         |         |         |
CA9304 QVQLVESGGGLVQAGGSLRLSCVASGLTLTNYVMGWFRQAPGKEREFVAAISRSGSGTDYVDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCGADSAGWFRIRQVPA--DYDYWGQGTQVTVSSGSEQKLISEEDL 138
CA9305 QVQLVESGGGLVQAGGSLRLSXAASGRSFDIHAMGWFRQAPGKEREFVAAISWNSGNTEYADVVKGRFTISRDNAKDTVYLQMNGLKPEDTAVYYCARGAFSF--ATTVQS--DYNYWGQGTQVTVSSGSEQKLISEEDL 136
CA9308 QVQLVESGGGLAQAGASLRLSCVASGRTFESYPMGWFRQAPGKEREFVGAISWNGGVIDYADSVKGRFTISRDNAKNTVYLQMNNLKPEDTAIYYCATKTRG--GDWRSGK--NWNYWGQGTQVTVSSGSEQKLISEEDL 136
CA9306 QVQLVESGGGLVQAGGSLRLSCAASGHASSTYTMGWFRQAPGKEREFVSGINWSDDSTSYAESVKGRFTISRDGAKNTVYLQMNSLKPEDTAVYYCALQ-YGWRWSWDDGSARDMRYWGKGTQVTVSSGSEQKLISEEDL 139
CA9302 QVQLVESGGGLVQPGGSLRLSCTASEGTFSVYNLAWFRQAPGKERELVASINWSGSVTYYTDSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCAA-ALRPNSVQ-------YKYWGQGTQVTVSSGSEQKLISEEDL 132
CA9303 QVQLVESGGGLVQAGGSLRLSCAASGRTFSNYRMGWFRQPPGKEREFVARISPNGGSTYYTDSMRGRFIISRDNVKNTVYLQMNSLKHEDTAVYYCAA-TPGYTSASKVPS--DYAYWGQGTQVTVSSGSEQKLISEEDL 137
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CA9309 QVQLVESGGGVVQAGGSLRLSCAASGRTLGSYDVGWFRQAPGKEREFLSISRQSGASIYCADSVKGRFTVSRDNAKNLVYLQMNSLKPEDTAVYYCAADPSRWYFCSSDSNPNTFDSWGQGTQVTVSSGSEQKLISEEDL 140
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Figure 15

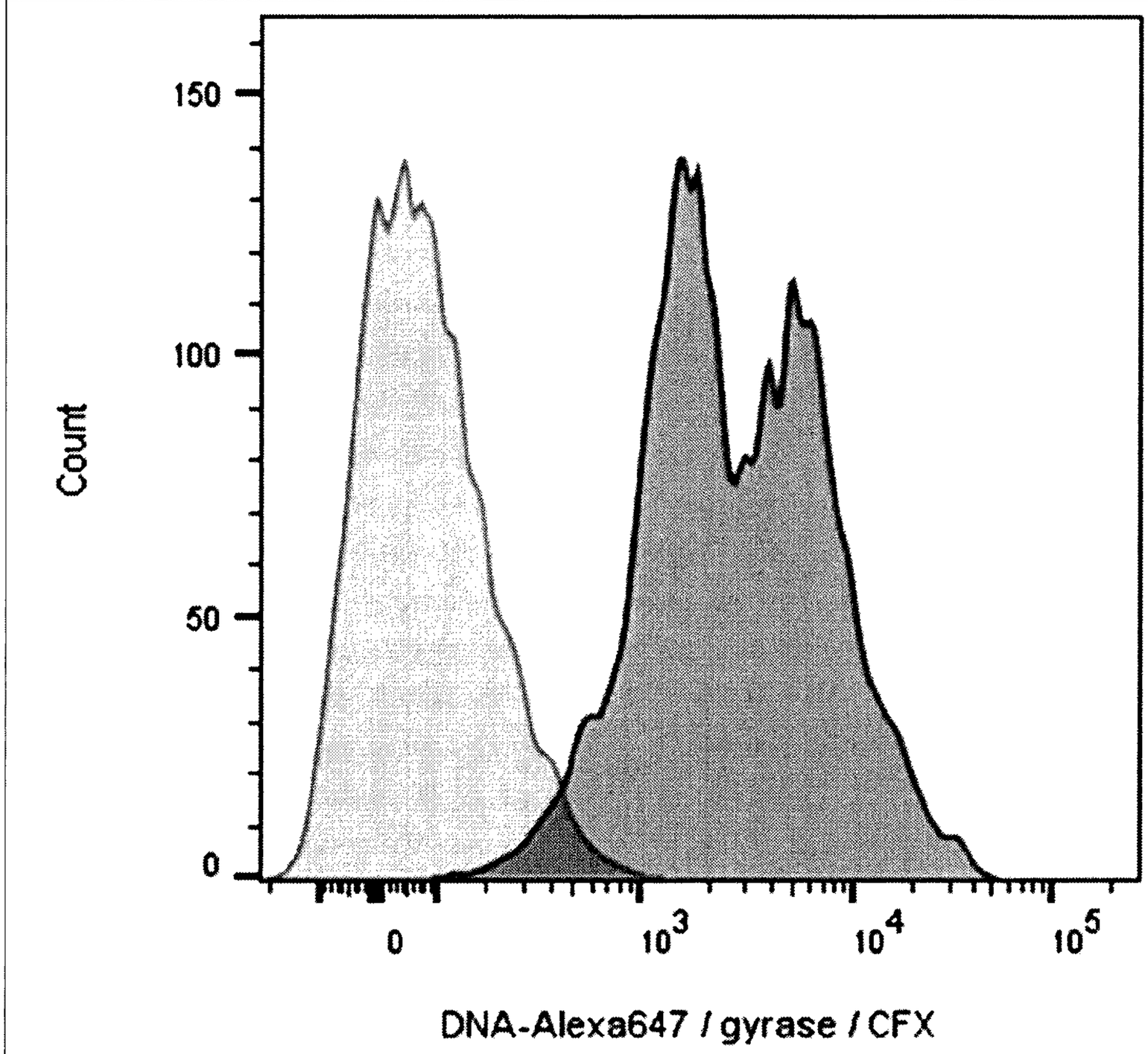
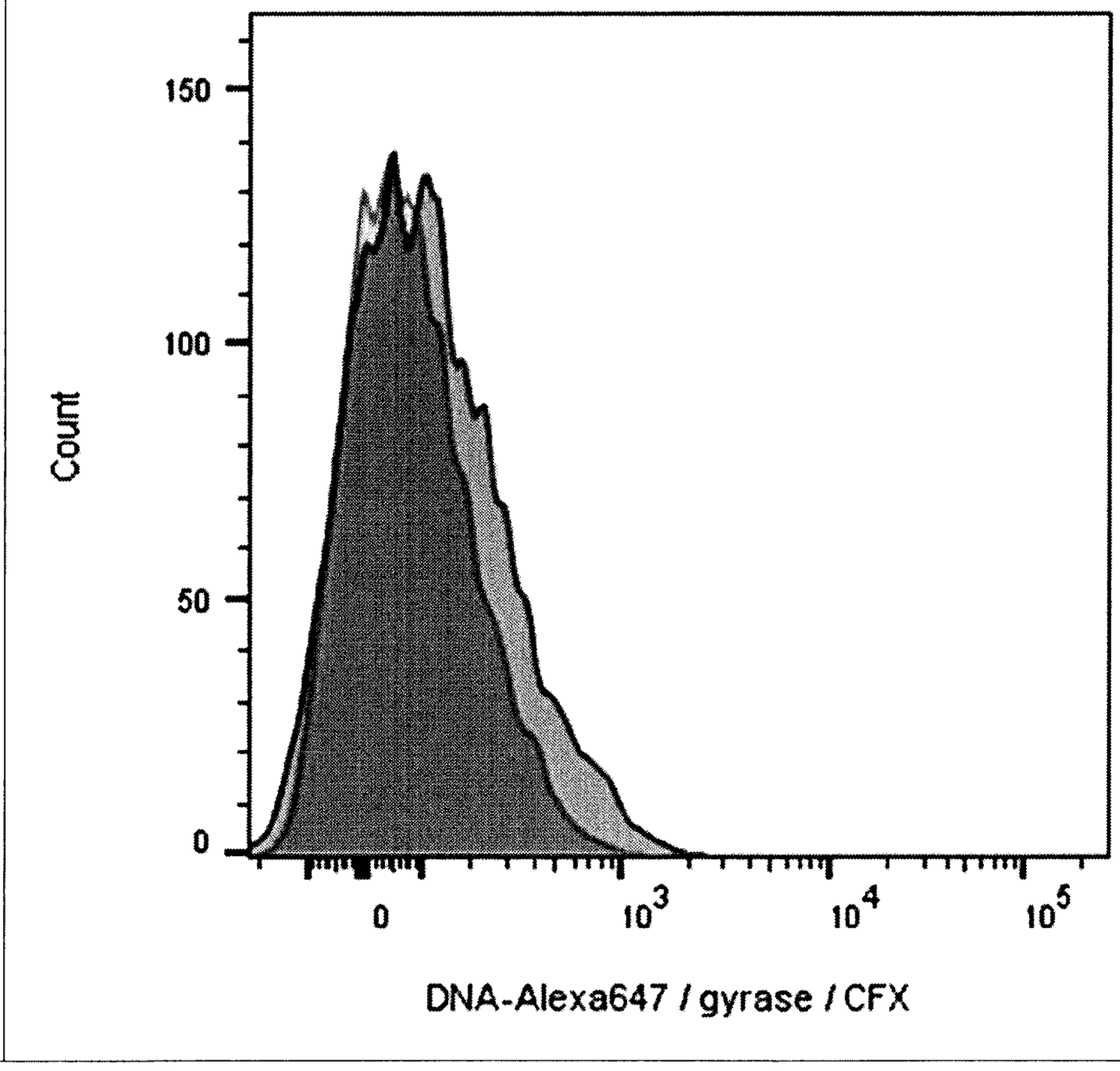
Type	DNA / gyrase /CFX
1	
2	

Figure 15 continued

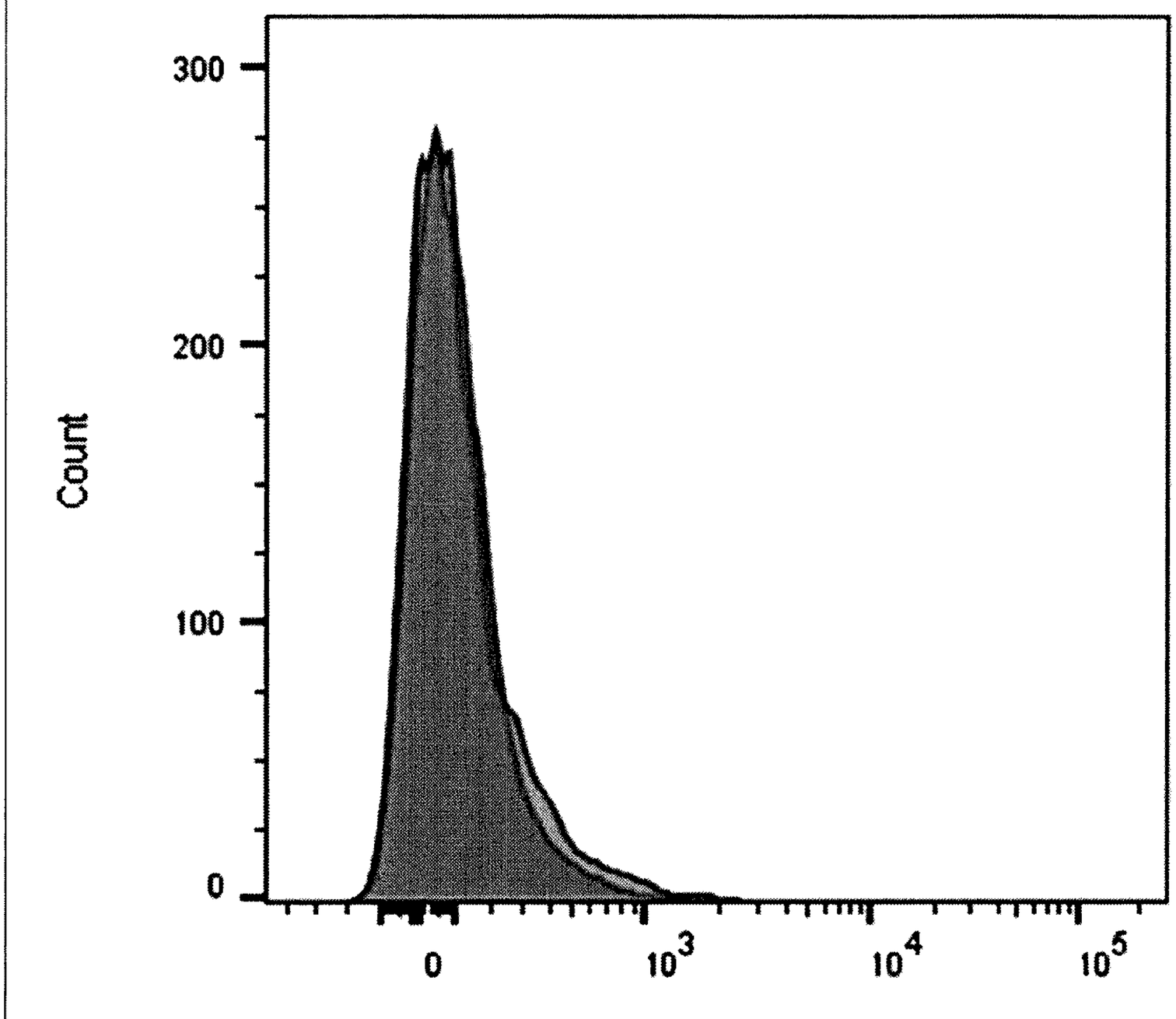
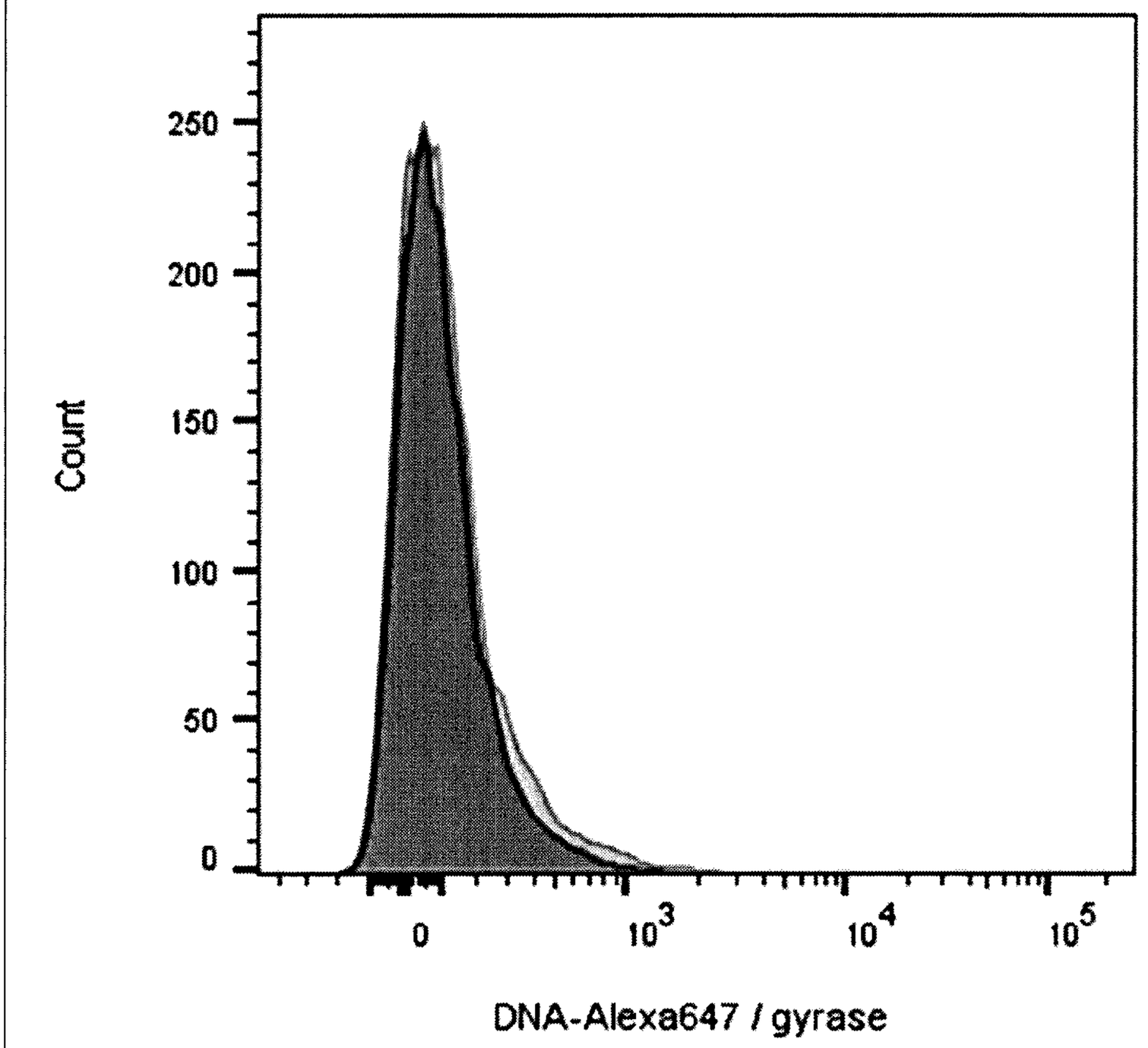
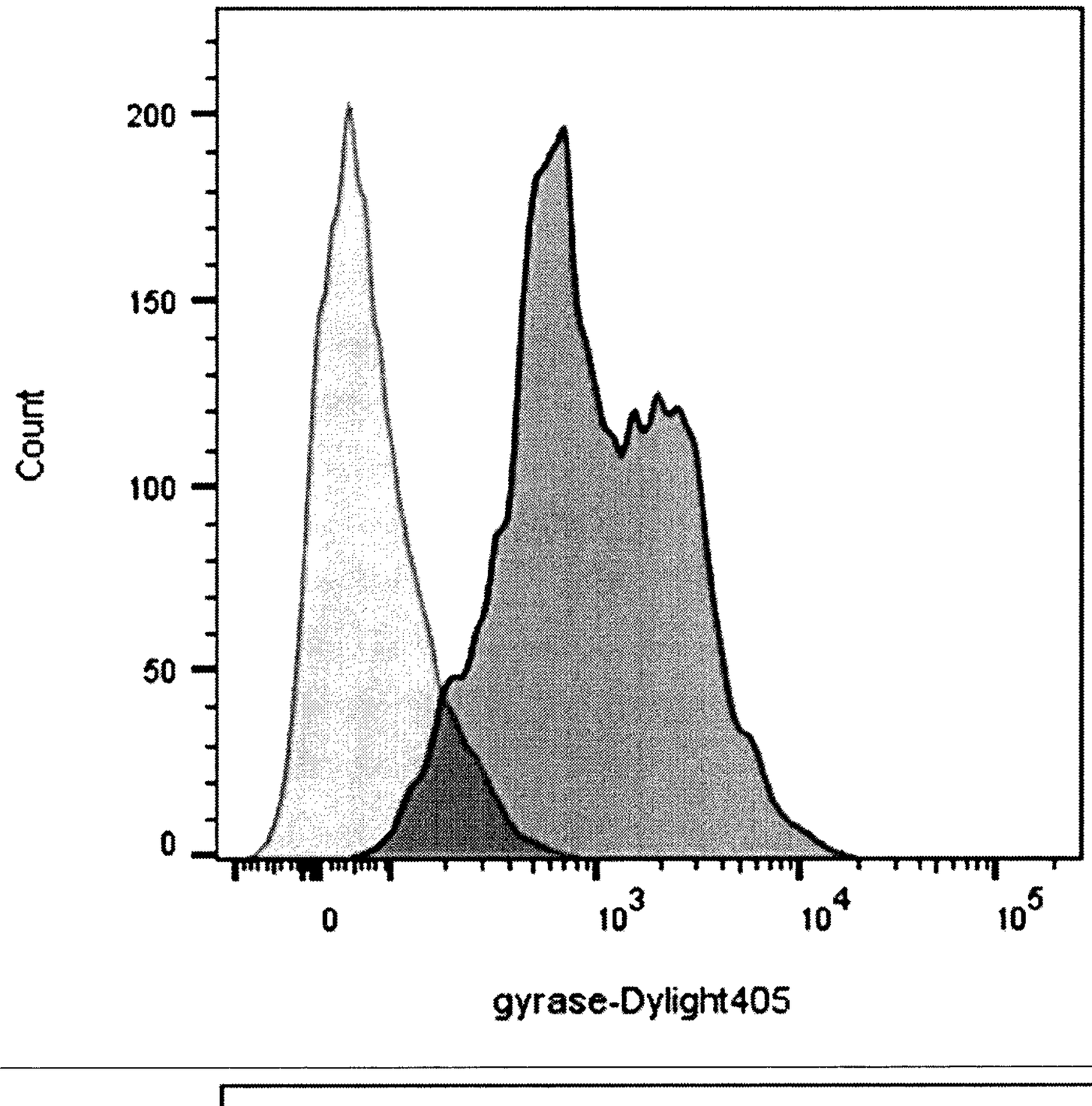
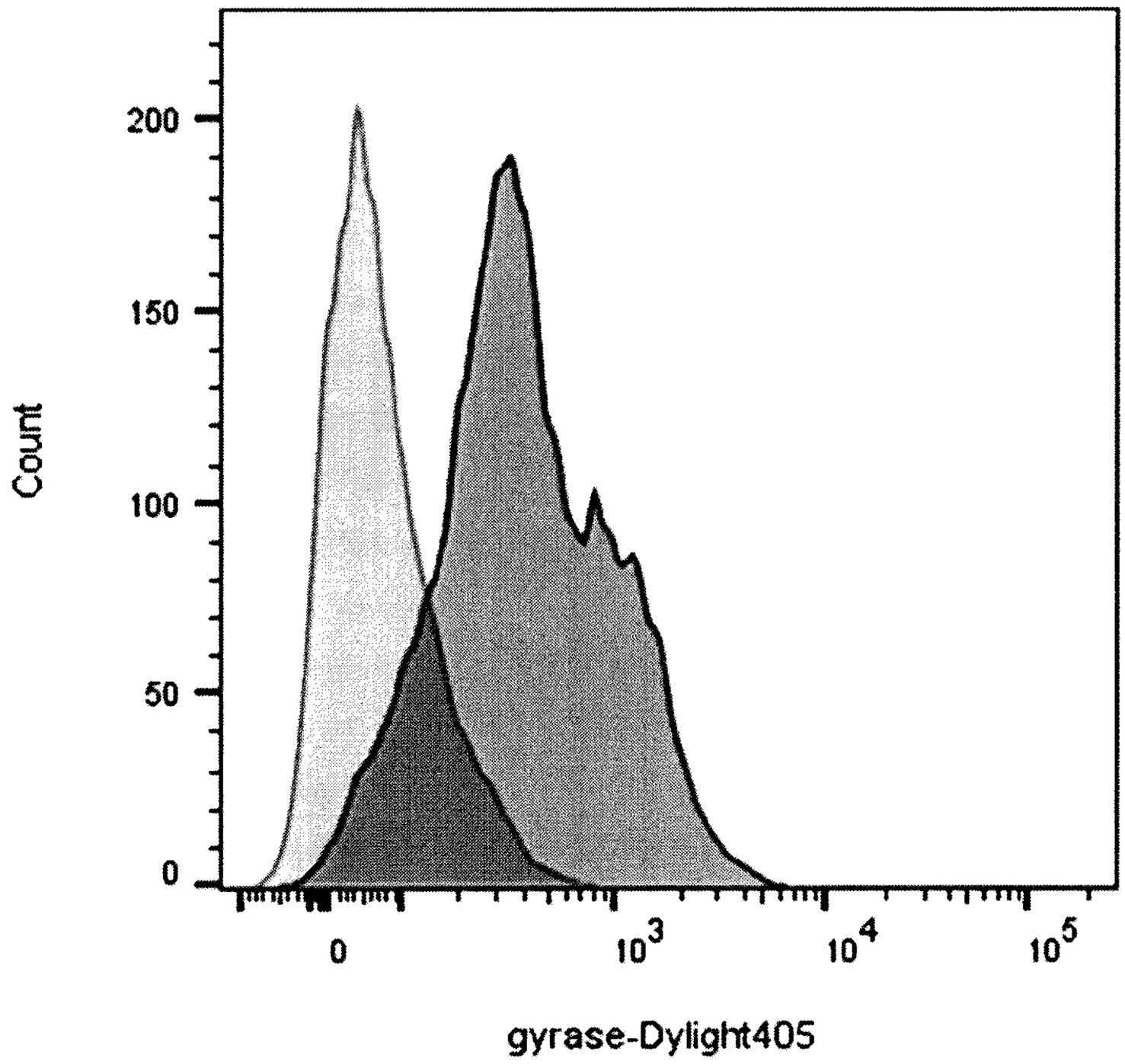
Type	DNA / gyrase
1	
2	

Figure 15 continued

Type	gyrase
1	
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