Generation of functional antibodies for mammalian membrane protein crystallography

Tomoya Hino^{1,2}, So Iwata^{1,3,4} and Takeshi Murata^{1,5,6}

Address

¹Iwata Human Receptor Crystallography Project, ERATO, JST, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

²Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, 4-101, Koyama-Minami, Tottori 680-8552, Japan

³Department of Cell Biology, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

⁴Division of Molecular Bioscience, Membrane Protein Crystallography Group, Imperial College London, Exhibition Road, London SW7 2AZ, UK.

⁵Department of Chemistry, Graduate School of Science, Chiba University, 1-33 Yayoi-cho, Inage, Chiba 263-8522, Japan

⁶JST, PRESTO, 1-33 Yayoi-cho, Inage, Chiba 263-8522, Japan

Corresponding author: Murata, Takeshi (t.murata@faculty.chiba-u.jp) and Iwata, So (s.iwata@mfour.med.kyoto-u.ac.jp)

Membrane proteins act as gateways to cells, and they are responsible for much of the communication between cells and their environments. Crystallography of membrane proteins is often limited by the difficulty of crystallization in detergent micelles. Co-crystallization with antibody fragments has been reported as a method to facilitate the crystallization of membrane proteins; however, it is widely known that the generation of mouse monoclonal antibodies that recognize the conformational epitopes of mammalian integral membrane proteins is typically difficult. Here, we present our protocols to generate functional mouse antibodies for the membrane protein crystallography, which have enabled us to solve crystal structures of mammalian receptors and transporters complexed with antibody fragments.

Introduction

Human cells have a wide variety of membrane proteins, which comprise approximately 30% of proteins encoded by the genome [1]. Many membrane proteins are located at cell surface; therefore, they are readily available to small molecule drugs circulating in the blood. Therefore, membrane proteins such as G-protein-coupled receptors (GPCRs), transporters, and channels are important targets for drug therapy, with approximately 70% of drugs currently available in the market known to target membrane proteins. In order to design new drugs and to understand their molecular mechanisms, it is essential to have information about their 3D structure, and this is usually obtained by X-ray crystallography. However, the number of determined and deposited membrane protein structures constitutes only approximately 0.5% of all deposited protein structures in the Protein Data Bank, owing to difficulties in the structural analyses of membrane proteins, with a major factor being the problems associated with their crystallization.

Membrane proteins are localized within membranes; therefore, for purification purpose, they are solubilized using detergent. In this procedure, the detergent forms micelles covering the hydrophobic surface of the membrane protein, and the purified protein is generally crystallized as a complex with the detergent micelles. Detergent

micelles are not small; micelles consisting of the variable detergent, n-dodecyl β -D-maltoside (frequently used in membrane protein crystallography) have a molecular weight of approximately 65 kDa [2], which is higher than that of many integral membrane proteins. Thus, crystallization of a membrane protein–detergent micelle complex can be complicated by steric hindrance from the micelles, which can impede the protein-protein interactions that are desirable for crystallization. In this review, we describe a method for the crystallization of membrane proteins by using antibodies, and our protocols for the generation of functional antibodies to facilitate crystallization of membrane proteins including mammalian integral membrane proteins such as GPCRs and transporters.

Co-crystallization with antibody fragments

Fab and Fv fragments of mouse monoclonal antibodies have been shown to facilitate crystallization of several membrane proteins by increasing the polar surface area for protein-protein interactions within the crystal lattice [3-6] (Fig. 1). Iwata *et al.* were the first to succeed in solving the co-crystal structure of bacterial cytochrome *c* oxidase with

antibody fragment (Fv) by using this strategy in 1995. Surprisingly, no direct interactions were found to exist between the membrane protein molecules, whereas Fv fragments were found to be involved in all crystal contacts [7]. Subsequently, various research groups successfully used this strategy to obtain structures of several important membrane proteins, including ion channels and membrane transporters (Table 1) [7-19,20^{**},21,22^{**}-24^{**}]. Thus, crystallization using antibodies is a method that can be applied to any type of membrane protein.

However, commercially available antibodies against membrane proteins seldom facilitate crystal formation. Most of these available antibodies are used for western blotting, and they recognize linear epitopes of membrane proteins because most of them have been raised against peptides or denatured protein. In this case, the fluctuations between the membrane protein and the bound antibody via a flexible linear epitope might be not conducive for a stable crystal lattice formation. In contrast, antibodies that bind to membrane proteins by recognizing their specific conformational epitopes are expected to facilitate crystallization with less fluctuation and through a larger hydrophilic region (Fig. 1). In fact, most antibodies known to facilitate the crystallization of membrane proteins

recognize their conformational epitopes rather than a linear epitope. However, it is not simple to generate such an antibody for mammalian integral membrane proteins. Recently, Kobilka *et al.* have succeeded in raising camelid single-chain antibody fragments (nanobodies) that can recognize and stabilize the active state structure of human β 2-adrenergic receptor, and they obtained the first active state crystal structure of the receptor in complex with the nanobody [22^{**}]. They also obtained the crystal structure of the receptor with a Fab fragment from a mouse monoclonal antibody; however, the structure, particularly on the extracellular side, was heavily disordered [5,21]. The problem may have been caused by fluctuations between the GPCR and the Fab fragment because the antibody recognized a flexible loop. We have modified the screening protocols to obtain mouse monoclonal antibodies that recognize the conformational epitopes of mammalian integral membrane proteins, and these methods are summarized below.

Protocols of mouse antibody generation

Liposome immunization---In conventional immunization methods for the preparation

of antibodies against membrane proteins, partial oligopeptides or purified samples solubilized with detergent are used to immunize mice; however, obtaining antibodies that recognize the conformational epitopes is difficult using these methods. Antibodies that bind to a part of the oligopeptides can be obtained using partial oligopeptide immunization, and the majority of antibodies obtained using purified sample immunization recognize denatured structures because blood dilutes the detergent, resulting in immediate denaturation of the purified membrane protein. To avoid this difficulty, we use a reconstituted liposome for immunization ("liposome immunization"); the target membrane protein is reconstructed without denaturation in phospholipid vesicles consisted of hen egg phosphatidyl choline with the adjuvant Lipid A, a lipid component derived from gram-negative bacteria, which acts on Toll-like receptor 4 to enhance innate immune activity as described elsewhere [5] (Fig. 2a). Use of immunodeficient mice (MRL/lpr) allowed efficient preparation of monoclonal antibodies against mammalian membrane proteins, without occurrence of immunotolerance. Adoption of this method led to a greater than 10-fold elevation of antibody titers compared to immunization of normal mice (BALB/c) with a detergent solubilized

receptor.

First screening: liposome ELISA---Besides immunization and screening methods, procedures were conducted according to the method developed by Milstein *et al.* for the preparation of mouse monoclonal antibodies [25]. After the antibody titer increased, the mouse spleen was excised, and myeloma cells were fused with antibody-producing B cells by using polyethylene glycol (PEG) to prepare hybridomas by HAT selection. Enzyme linked immunosorbent assay (ELISA) is frequently used as the first screening assay for antibodies. In general, antigens are fixed on a plastic plate to detect antibodies that recognize them; however, using purified membrane proteins as antigens caused problems with the use of ELISAs, in that either the presence of detergent resulted in few antigens being fixed to the plastic plate, or repeated washings denatured the membrane protein. Thus, we seldom obtained useful antibodies that recognize conformational epitopes by using this method. Therefore, we developed a method, “liposome ELISA” by using purified samples reconstituted into liposomes containing biotinylated lipids (biotinyl phosphatidylethanolamine) to maintain the protein in its native conformation and effectively immobilize liposomes onto streptavidin-coated plates as described in

international patent WO 2010/126115 (Fig. 2b). Although we were concerned that the use of liposomes for immunization may result in antibodies against lipids, in practice only approximately 10%–20% of the antibodies obtained recognized lipids, and this was regarded as an acceptable level.

Second screening: denatured dot blot---The first ELISA screening, described above, selected antibodies recognizing the native structure of the target membrane protein. To eliminate antibodies that recognize a linear epitope, N and C termini or unstructured regions in the native structure, we performed a dot blot (“denatured dot blot”) by using purified samples denatured with 1% sodium dodecyl sulfate (Fig. 2c). Thus, we selected antibodies that did not bind to the denatured protein, suggesting that they recognize the conformational epitopes present only in the native form of the protein. In practice, approximately 10%–20% of the antibodies were positive by the liposome-ELISA assay and negative by the denatured dot blot technique, which were selected.

Third screening: binding affinity selection by Biacore---Although antibodies recognizing conformational epitopes are obtained by the methods described above, if they have weak binding affinities and can dissociate immediately, they are not suitable for

co-crystallization. Thus, it is important to screen for antibodies with high binding affinities. We constructed an evaluation system by using a Biacore T100 analyzer that works on the principle of surface plasmon resonance (Fig. 2d). Monoclonal anti-mouse Fc fragment antibody is immobilized on a sensor chip (CM5) and antibodies in hybridoma culture supernatants are tightly trapped by the Fc antibody fixed on the sensor chip. Purified sample in detergent is then passed over the surface, and the specific binding is monitored to screen for those with dissociation constants (K_D) below 10 nM. In this experiment, almost half of the selected antibodies that passed the first and second screenings also met this criterion. This method allows testing of approximately 100 different culture supernatants in half a day by using the same chip.

The combination of these immunization-screening techniques enabled us to obtain high affinity antibodies that recognize conformational epitopes against various membrane proteins, including GPCRs, membrane transporters, and ion channels from various species—spanning humans to bacteria. Further, we succeeded in obtaining the crystal structure of a human GPCR in complex with the selected antibody fragments as

described below.

Generation of functional antibodies for a human GPCR

Human A_{2A} adenosine receptor (A_{2A}AR) is an important GPCR involved in the control of various physiological activities, including regulation of glutamine and dopamine release in the brain [26]. Specific inhibitors of the receptor are in advanced clinical trials for the treatment of Parkinson's disease [27]. Several crystal structures of stabilized A_{2A}AR mutants (T4 lysozyme [or b₅₆₂RIL]-fusion mutants in intercellular loop 3 [ICL3] and alanine-scanning mutants) have been obtained by breakthroughs in protein engineering and crystallography over the past 5 years [28,29*,30*,31,32]. We purified A_{2A}AR with a complete ICL3 [33] and raised mouse monoclonal antibodies against it by using the conventional mouse hybridoma system combined with improved screening methods as described above. We obtained 24 monoclonal antibodies that bound to native A_{2A}AR with high affinity ($K_D < 10$ nM) but did not recognize the denatured receptor [24**]. Eight of the antibodies variably inhibited agonist binding to the purified receptor, and one (IgG2838) completely inhibited agonist binding but did not affect antagonist

binding. In addition, these functional antibodies also drastically improved the thermostability of A_{2A}AR, indicating that they are likely to be effective for co-crystallization.

Crystal structure of the GPCR-Fab complex

The Fab fragment was generated from the functional antibody (IgG2838, which completely inhibited agonist binding) by papain cleavage. The A_{2A}AR-Fab complex was purified by size-exclusion chromatography and crystallized in detergent. We obtained the crystal structures of the complex with antagonist (ZM241385) at 2.7 Å [24^{**}]. The Fab fragment recognized the intracellular side of the receptor (Fig. 3a), and its complementarity-determining region, CDR-H3, was unusually extended and penetrated into an internal cavity formed by an α -helical bundle of the receptor, which interacted with helices II, III, VI, and VIII by forming 7 hydrogen bonds (Fig. 3b). Furthermore, other CDRs also interacted with helices I, II, III, VI, VIII, and ICL3 through 11 hydrogen bonds. Thus, the Fab fragment recognized the wide intracellular surface of A_{2A}AR with high affinity. Despite the fact that the antibody bound to the intracellular side of the

receptor, it blocked the binding of the agonist on the extracellular side but did not affect the binding of the antagonist, as described above. These findings indicate that the receptor is locked into an inactive conformation by tight binding of the functional antibody and that the conformation of the intracellular side and the ligand binding on the extracellular side are strongly coupled.

Conclusion

We succeeded in obtaining several antibodies to facilitate the crystallization of other mammalian integral membrane proteins such as membrane transporters by using this strategy (unpublished data). In the most trials, we were also able to obtain functional antibodies that modulate the activity of the target. This is probably because the antibody binds to the protein by recognizing several conformational epitopes in the native structure, thus stabilizing a particular conformation and/or preventing the conformational changes of the target protein, as in the case of the A_{2A}AR. Co-crystallization using these functional antibodies might enable us to determine the transitional 3D structures that are usually very difficult to capture. We expect that our technique could be used to determine the

crystal structures of different snapshots of targeted membrane proteins to increase understanding of molecular mechanisms and dynamics, and it will also lead to the development of new drugs based on the structural information obtained.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

Antibodies were prepared in collaboration with the T. Hamakubo group at the University of Tokyo. We deeply appreciate all those who were involved in this study. This work was supported by a grant from the ERATO Human Receptor Crystallography Project from the Japan Science and Technology Agency, by the Targeted Proteins Research Program and Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, *et al.*: **Initial sequencing and analysis of the human genome.** *Nature* 2001, **409**:860-921.
2. Aoudia M, Zana R: **Aggregation Behavior of Sugar Surfactants in Aqueous Solutions: Effects of Temperature and the Addition of Nonionic Polymers.** *J Colloid Interface Sci* 1998, **206**:158-167.
3. Ostermeier C, Iwata S, Ludwig B, Michel H: **Fv fragment-mediated crystallization of the membrane protein bacterial cytochrome c oxidase.** *Nat Struct Biol* 1995, **2**:842-846.
4. Hunte C, Michel H: **Crystallisation of membrane proteins mediated by antibody**

- fragments.** *Curr Opin Struct Biol* 2002, **12**:503-508.
5. Day PW, Rasmussen SGF, Parnot C, Fung JJ, Masood A, Kobilka TS, Yao XJ, Choi H-J, Weis WI, Rohrer DK, *et al.*: **A monoclonal antibody for G protein-coupled receptor crystallography.** *Nat Meth* 2007, **4**:927-929.
 6. Lim HH, Fang Y, Williams C: **High-efficiency screening of monoclonal antibodies for membrane protein crystallography.** *PLoS One* 2011, **6**:e24653.
 7. Iwata S, Ostermeier C, Ludwig B, Michel H: **Structure at 2.8 Å resolution of cytochrome c oxidase from *Paracoccus denitrificans*.** *Nature* 1995, **376**:660-669.
 8. Hunte C, Koepke J, Lange C, Rossmanith T, Michel H: **Structure at 2.3 Å resolution of the cytochrome bc(1) complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment.** *Structure* 2000, **8**:669-684.
 9. Hino T, Matsumoto Y, Nagano S, Sugimoto H, Fukumori Y, Murata T, Iwata S, Shiro Y: **Structural basis of biological N₂O generation by bacterial nitric oxide reductase.** *Science* 2010, **330**:1666-1670.
 10. Inaba K, Murakami S, Nakagawa A, Iida H, Kinjo M, Ito K, Suzuki M: **Dynamic**

- nature of disulphide bond formation catalysts revealed by crystal structures of DsbB.** *EMBO J* 2009, **28**:779-791.
11. Tsukazaki T, Mori H, Fukai S, Ishitani R, Mori T, Dohmae N, Perederina A, Sugita Y, Vassilyev DG, Ito K, *et al.*: **Conformational transition of Sec machinery inferred from bacterial SecYE structures.** *Nature* 2008, **455**:988-991.
12. Zhou Y, Morais-Cabral JH, Kaufman A, MacKinnon R: **Chemistry of ion coordination and hydration revealed by a K⁺ channel-Fab complex at 2.0 Å resolution.** *Nature* 2001, **414**:43-48.
13. Uysal S, Vásquez V, Tereshko V, Esaki K, Fellouse FA, Sidhu SS, Koide S, Perozo E, Kossiakoff A: **Crystal structure of full-length KcsA in its closed conformation.** *Proc Natl Acad Sci U S A* 2009, **106**:6644-6649.
14. Dutzler R, Campbell EB, MacKinnon R: **Gating the selectivity filter in ClC chloride channels.** *Science* 2003, **300**:108-112.
15. Jiang Y, Lee A, Chen J, Ruta V, Cadene M, Chait BT, MacKinnon R: **X-ray structure of a voltage-dependent K⁺ channel.** *Nature* 2003, **423**:33-41.
16. Lee S-Y, Lee A, Chen J, MacKinnon R: **Structure of the KvAP voltage-dependent**

K⁺ channel and its dependence on the lipid membrane. *Proc Natl Acad Sci U S*

A 2005, **102**:15441-15446.

17. Hibbs RE, Gouaux E: **Principles of activation and permeation in an**

anion-selective Cys-loop receptor. *Nature* 2011, **474**:54-60.

18. Fang Y, Jayaram H, Shane T, Kolmakova-Partensky L, Wu F, Williams C, Xiong Y,

Miller C: **Structure of a prokaryotic virtual proton pump at 3.2 Å resolution.**

Nature 2009, **460**:1040-1043.

19. Shaffer PL, Goehring A, Shankaranarayanan A, Gouaux E: **Structure and**

mechanism of a Na⁺-independent amino acid transporter. *Science* 2009,

325:1010-1014.

20. Krishnamurthy H, Gouaux E: **X-ray structures of LeuT in substrate-free**

outward-open and apo inward-open states. *Nature* 2012, , **481**:469-474.

••This paper describes the crystal structures of the bacterial homologue, LeuT, of neurotransmitter sodium symporter in complexes with conformation-specific antibody fragments in the outward-open and inward-open state, establishing a structural framework for the molecular mechanism of the symporters.

21. Rasmussen SGF, Choi H-J, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, *et al.*: **Crystal structure of the human beta2 adrenergic G-protein-coupled receptor.** *Nature* 2007, **450**:383-387.

22. Rasmussen SGF, Choi H-J, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, *et al.*: **Structure of a nanobody-stabilized active state of the β_2 adrenoceptor.** *Nature* 2011, **469**:175-180.

••This paper describes the first active-state structure of a human β_2 adrenergic receptor in complex with a camelid nanobody that acts like as a G-protein, which provides insights into the process of agonist binding and activation.

23. Rasmussen SGF, Devree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, *et al.*: **Crystal structure of the β_2 adrenergic receptor-Gs protein complex.** *Nature* 2011, **477**:549-555.

••This paper describes the first structure of the active state ternary complex composed of an agonist bound human β_2 adrenergic receptor and a camelid nanobody bound Gs

heterotrimer, representing the first high-resolution view of transmembrane signaling by a GPCR.

24. Hino T, Arakawa T, Iwanari H, Yurugi-Kobayashi T, Ikeda-Suno C, Nakada-Nakura Y, Kusano-Arai O, Weyand S, Shimamura T, Nomura N, *et al.*: **G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody.** *Nature* 2012, **482**:237-240.

••This paper describes the crystal structure of a human adenosine A_{2A} receptor in complex with a Fab fragment which prevents agonist but not antagonist binding, suggesting a new strategy to modulate the activity of GPCRs.

25. Köhler G, Milstein C: **Continuous cultures of fused cells secreting antibody of predefined specificity.** *Nature* 1975, **256**:495-497.

26. Fredholm BB, Chen J-F, Masino SA, Vaugeois J-M: **Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs.** *Annu Rev Pharmacol Toxicol* 2005, **45**:385-412.

27. Müller CE, Jacobson KA: **Recent developments in adenosine receptor ligands and their potential as novel drugs.** *Biochim Biophys Acta* 2010, **1808**:1290-1308.

28. Jaakola V-P, Griffith MT, Hanson MA, Cherezov V, Chien EYT, Lane JR, Ijzerman

AP, Stevens RC: **The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist.** *Science* 2008, **322**:1211-1217.

29. Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao Z-G, Cherezov V, Stevens RC:

Structure of an Agonist-Bound Human A_{2A} Adenosine Receptor. *Science* 2011, **332**:322-327.

•This paper describes the crystal structure of a human adenosine A_{2A} receptor-T4 lysozyme fusion protein bound to an agonist UK-432097 which acts as a ‘conformationally selective agonist’ capable of receptor stabilization in a specific active-state configuration.

30. Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AGW, Tate CG:

Agonist-bound adenosine A(2A) receptor structures reveal common features of GPCR activation. *Nature* 2011, **474**:531-525.

•This paper describes crystal structures of the agonist (adenosine and NECA) bound thermostabilized human adenosine A_{2A} receptor mutant, representing an intermediate conformation between the active and inactive states of the receptor.

31. Doré AS, Robertson N, Errey JC, Ng I, Hollenstein K, Tehan B, Hurrell E, Bennett K, Congreve M, Magnani F, *et al.*: **Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine.** *Structure* 2011, **19**:1283-1293.
32. Liu W, Chun E, Thompson AA, Chubukov P, Xu F, Katritch V, Han GW, Roth CB, Heitman LH, Ijzerman AP, *et al.*: **Structural basis for allosteric regulation of GPCRs by sodium ions.** *Science* 2012, **337**:232-236.
33. Yurugi-Kobayashi T, Asada H, Shiroishi M, Shimamura T, Funamoto S, Katsuta N, Ito K, Sugawara T, Tokuda N, Tsujimoto H, *et al.*: **Comparison of functional non-glycosylated GPCRs expression in *Pichia pastoris*.** *Biochem Biochys Res Commun* 2009, **380**:271-276.

Figure legends

Figure 1. Schematic illustration of co-crystallization of integral membrane protein with

antibody fragment. Fab fragment of mouse monoclonal antibody facilitates crystallization of the membrane protein by increasing the polar surface area.

Figure 2. Graphical protocols to obtain mouse monoclonal antibodies that facilitate the crystallization of mammalian integral membrane proteins. See text for details.

Figure 3. Structure of the A_{2A}AR-Fab complex. **a**, Overall structure viewed parallel to the membrane. A_{2A}AR and the Fab light and heavy chains are shown in blue-gray, green and yellow, respectively. The bound antagonist ZM241385 in the ligand-binding pocket is shown as a space-filling model. **b**, Transparent surface representation of the interface between A_{2A}AR (top) and Fab fragment (bottom). Relative to **a**, A_{2A}AR has been rotated 90° around a horizontal axis, whereas Fab fragment is shown in the same orientation. Surface within 4 Å of CDR-H3 are colored red.

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Table 1

Table 1. List of co-crystal structures of membrane protein with antibody fragment.

Membrane Protein	PDB code	ligand format	Resolution	Source	TM helices	Reference
<i>Enzymes</i>						
Cytochrome c oxidase	1QLE	Fv	2.8	<i>P. denitrificans</i>	22	[7]
Cytochrome bc1 complex	1EZV	Fv	2.3	<i>S. cerevisiae</i>	12	[8]
Nitric oxide reductase	3O0R	Fab	2.7	<i>P. aeruginosa</i>	13	[9]
Disulfide bond formation protein B (DsbB)	2ZUQ	Fab	3.3	<i>E. coli</i>	4	[10]
<i>Translocon</i>						
Sec YE	2ZJS	Fab	3.2	<i>T. thermophilus</i>	11	[11]
<i>Ion channel</i>						
KcsA K ⁺ channel, channel domain	1K4D	Fab	2.0	<i>S. lividans</i>	2	[12]
KcsA K ⁺ channel, full length	3EFF	Fab	3.8	<i>S. lividans</i>	2	[13]
CIC H ⁺ /Cl ⁻ antiporter	1OTS	Fab	2.5	<i>E. coli</i>	12	[14]
KvAP voltage-gated K ⁺ channel, full length	1ORQ	Fab	3.2	<i>A. pernix</i>	6	[15]
KvAP voltage-gated K ⁺ channel, full length	2A0L	Fv	3.9	<i>A. pernix</i>	6	[16]
KvAP voltage-gated K ⁺ channel, sensor domain	1ORS	Fab	1.9	<i>A. pernix</i>	4	[15]
GluCl glutamate-gated Cl ⁻ channel	3RHW	Fab	3.26	<i>C. elegans</i>	4	[17]
<i>Transporter</i>						
AdiC Arg/Agmatine antiporter	3NCY	Fab	3.2	<i>S. enterica</i>	12	[18]
ApcT Amino acid transporter	3GI9	Fab	2.5	<i>M. janaschii</i>	12	[19]
LeuT Leucine transporter (outward open)	3TT1	Fab	3.1	<i>A. aeolicus</i>	12	[20 ^{db}]
LeuT Leucine transporter (inward open)	3TT3	Fab	3.22	<i>A. aeolicus</i>	12	[20 ^{db}]
<i>G protein coupled receptor</i>						
β2 adrenergic receptor, antagonist bound	2R4R	Fab	3.4	<i>H. sapiens</i>	7	[21]
β2 adrenergic receptor, agonist bound	3P0G	V _H H	3.5	<i>H. sapiens</i>	7	[22 ^{db}]
β2 adrenergic receptor, Gs complex	3SN6	V _H H	3.2	<i>H. sapiens</i>	7	[23 ^{db}]
A _{2A} adenosine receptor, antagonist bound	3VG9	Fab	2.7	<i>H. sapiens</i>	7	[24 ^{db}]

a. Liposome immunization

b. 1st screening: Liposome ELISA

c. 2nd screening: Denatured dot blot

d. 3rd screening: Binding affinity selection