Methods in Molecular Biology 1827

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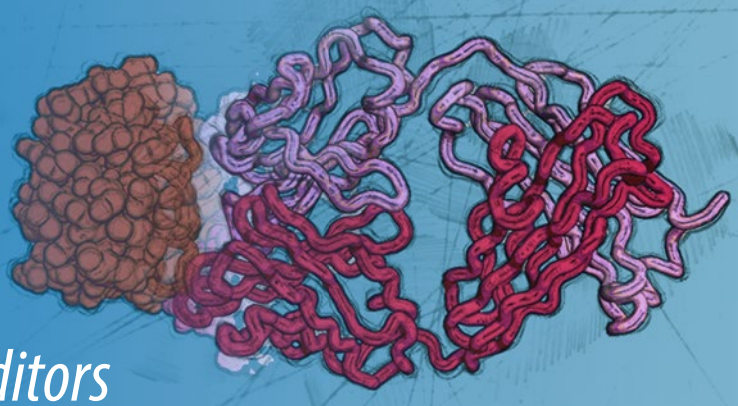

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Patrick Chames *Editors*

Antibody Engineering

Methods and Protocols

Third Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Third Edition

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-8647-7 ISBN 978-1-4939-8648-4 (eBook)
https://doi.org/10.1007/978-1-4939-8648-4

Library of Congress Control Number: 2018950851

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Printed on acid-free paper

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The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

The use of antibody-based therapeutics has grown exponentially in the past few decades, now representing a large component of therapeutic drugs that was dominated by small organic molecules up until the late 1990s. Antibodies have proven versatile in treating a variety of diseases including cancer, autoimmunity, infectious diseases, or even neurodegenerative disorders. As of 2017, 70 therapeutic antibodies have been approved by the FDA, and more than 550 promising candidates are in different phases of clinical trials. They currently represent 20% of the top 100 selling drugs, up from just 1% in 2007. However, major improvements and breakthroughs have been necessary to achieve these impressive results.

The 1970s were the start of great revolutions in the field: Gerald Edelman and Rodney Porter were awarded the Nobel Prize for their work on the molecular structure of antibodies, the first atomic resolution structure of an antibody fragment was published, followed by the groundbreaking development of hybridoma technology by Georges J. F. Köhler and César Milstein. This technology allowed antibodies to be produced and characterized as monoclonals, starting the modern era of antibody engineering.

Despite this revolution, the success of antibodies as therapeutic molecules was not immediate, and most clinical studies led to disappointments. First murine antibodies used as treatment had many limitations, such as a short in vivo half-life, limited tumor penetration, inefficient recruitment of host effector functions, and most of all, immune response from the patient against the injected antibody, also called "HAMA" response, referring to the production of neutralizing human anti-mouse antibodies.

For many years, researchers developed strategies to abrogate this problem; the journey toward antibody humanization began. Fully murine antibodies first progressed to chimeras, where variable regions from murine origin were assembled onto human constant domains, then to humanized antibodies by insertion of only the relevant CDRs onto human antibody scaffolds. Finally, fully human antibodies were generated, directly in genetically modified mice, selected from human synthetic antibody libraries or by sequencing of human plasma cells.

However, immunogenicity was not the only factor holding up the development of antibodies. Indeed, the classical architecture of immunoglobulin molecules bears some inherent limitations. Many innovative formats have been explored to overcome these major hurdles, such as reducing the antibody to its minimal functional size, modulating the valency, the (multi) specificity, increasing the half-life, and enhancing the recruitment of immune effector cells.

As the demand for monoclonal antibodies in research and clinical applications continues to increase, the necessity to develop even more efficient molecules is crucial. Antibody engineering has become a key discipline for generation of innovative antibodies-based molecules used in research, diagnostics, and therapy.

This third edition of *Antibody Engineering: Methods and Protocols* remains in the lineage of its predecessors and gives the readers complete and easy access to a variety of antibody engineering techniques. From the generation of native, synthetic, or immune antibody libraries, the selection of lead candidates thanks to different powerful and innovating display technologies, to their production, characterization, and optimization, this handbook provides the reader with an extensive toolbox to create the powerful molecules of tomorrow.

Darlinghurst, NSW, Australia ***Damien Nevoltris***
Marseille, France ***Patrick Chames***

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Part VI Characterization, Optimization, and Innovative Formats

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Part I

In Silico Methods

Chapter 1

Antibody Design and Humanization via In Silico Modeling

Vinodh B. Kurella and Reddy Gali

Abstract

Antibody humanization process converts any nonhuman antibody sequence into humanized antibodies. This can be achieved using different methods of antibody design and engineering. This chapter will primarily focus on antibody design using a homology model followed by framework shuffling of murine to human germline sequence for humanization. Historically, mouse antibodies have been humanized using sequence-based approaches, in which all the murine frameworks are replaced with most homologous human germline sequence or related scaffold. Most often this humanized antibody design, when tested, has a significantly reduced binding or no binding to the cognate antigen. This is due to noncompatibility of mouse CDRs being supported by non-native human framework scaffold. This mismatch between mouse, human structural fold, and elimination of key conformational residues often leads to antibody humanization failures. Recently, there has been advent of homology modelor structure-guided antibody humanization. Instead of humanization based on linear sequence, this approach takes into account the tertiary structure and fold of the mouse antibody. A mouse homology model of the fragment variable is created, and based on sequence alignment with human germline, residues that are different in mouse are replaced with humanized sequence in the model. Energy minimization is applied to this humanized model that also delineates residues which might have steric clashes due to change in the overall tertiary conformation of the humanized antibody. Therefore, a homology model-guided with rational mutations, and reintroduction of key conformational residues from mouse antibody not only eliminates steric clashes but might also restore function in relation to binding affinity to its antigen.

Key words Antibody design, Humanization, De-immunization, Antibody homology model, PIGS, Rosetta, Antibody model, Prediction of Immunoglobulin Structures, Mouse antibody humanization, Homology model-guided humanization, Structure-based antibody engineering

1 Introduction

Historically, antibodies have been generated mainly using mouse models. These antibodies worked best for research and diagnostic applications, but did not fare well in human therapeutic use. In the early days of therapeutic antibody development and use, mouse monoclonal antibody against a specific cancer target was administered directly to human patients. This led to generation of human anti-mouse antibody response (HAMA), which not only neutralized this therapeutic antibody but also led to severe allergic

Damien Nevoltris and Patrick Chames (eds.), *Antibody Engineering: Methods and Protocols*, Methods in Molecular Biology, vol. 1827, https://doi.org/10.1007/978-1-4939-8648-4_1, © Springer Science+Business Media, LLC, part of Springer Nature 2018

reaction in humans. Researchers then replaced mouse Fc (fragment crystalline) and part of Fab (fragment antigen binding) with human antibody sequences, a chimeric antibody. This chimera, based on the sequence, did reduce overall immunogenicity of the mouse mAb but still did contain substantial mouse residues. Greg Winter at Medical Research Council (MRC, UK) came up with a novel idea of taking the mouse CDRs of both heavy and light chains and transplanting them directly onto a fully human antibody scaffold. In this method, CDRs are the only sequences that are mouse, and the rest of the antibody contains human sequence. This technique is popularly known as CDR replacement [1]. It was a groundbreaking technique at that time and is still considered a gold standard in antibody humanization.

Over the years, there have been numerous methods developed for undertaking antibody humanization or antibody de-immunization. Although CDR replacement dramatically reduces the mouse residues in the humanized antibody, however, in most cases it results in a significant drop in affinity toward the antigen. This is due to sequence and structural differences between mouse CDRs being supported by non-native human frameworks. Therefore, humanization becomes an iterative process in which numerous designs have to be made, tested, and ranked based on functional readout (e.g., affinity measurement). Other humanization technique adopts sequence-based conversion of a mouse mAb to a humanized antibody. It involves replacement of only those residues to human germline sequences, which are not conserved between mouse and human. Another technique involves a more conservative approach, in which replacement is done only for one framework at a time instead of all the four frameworks [2]. In this approach, designs are tested, and sequential framework replacement of mouse to human is accomplished, driven by affinity measurements and functional data. Framework shuffling is a method to create humanized antibody variants based on sequence conservation and randomization of heavy and light chains from different variable chain germline sequences [3]. Super humanization is another technique in which mouse CDRs are also humanized along with the frameworks [4]. Given HAMA responses against a mouse mAb are antibody mediated, researchers have mapped out the surface-exposed residues in the mouse mAb and replaced only those with corresponding human sequences, also known as antibody resurfacing [5]. This is achieved by delineating surface-exposed residues using either X-ray/NMR structures or homology model of the mouse mAb fragment variable region (Fv). Recently, this approach is further optimized, in which the mouse mAb Fv model is first generated, and then non-conserved residues in mouse are mutated to reflect human germline sequences. This humanized homology model has mouse CDRs and human frameworks, which is energy minimized using one of these force fields (GROMOS,

CHARMM). If there are steric clashes between frameworks and CDRs/frameworks regions, these can be visualized in the chimeric homology model. Sequence or structure-based back or novel mutations can be introduced to avoid these steric clashes. This approach is well adopted by the scientific community as a homology model-guided antibody humanization [6–9].

1.1 Antibody Structures

Antibodies can be classified into canonical and noncanonical classes. Antibody heavy chain can be further divided into four frameworks, which encompass CDRs (H1, H2, H3) and light chain containing CDRs (L1, L2, L3). All these frameworks have a very high degree of similar structural fold. CDRs L1–L3 and H1 and H2 can be assigned to canonical classes. Based on the length and sequence, these loops can be modeled based on the structural templates available from these canonical classes in protein data bank. Given the diversity and uniqueness of CDR H3, it does not belong to any canonical family. In the recent past, there has been plethora of antibody structures determined via NMR, X-ray crystallography, as well as cryo-EM techniques. Most of these antibodies have sequence and structural similarity between them that has led to creation and development of different algorithms for antibody homology model prediction, as opposed to resource-intensive experimental structure determination.

These algorithms primarily use antibody sequence information to extract structural templates from protein data bank (PDB), which results in creation of homology model of an antibody. Given tremendous growth in biologics in the recent past over small molecules, different researchers, as well as commercial companies, have developed antibody model prediction algorithms. Publically available antibody modeling algorithms are Rosetta antibody modeling, Web antibody modeling (WAM), structure-based antibody prediction server (SAbPred), Prediction of ImmunoGlobulin Structures (PIGS), and Kotai (antibody builder). Commercially, there are a number of antibody modeling products, such as Biovia's Discovery Studio, Schrodinger's BioLuminate, and Chemical Computing Group's MOE and Macromoltek. A brief description of some of these prediction algorithms is described.

1.2 Web Antibody Modeling (WAM)

Antibody modeling algorithm follows a series of steps to obtain a homology model from the sequences of variable heavy and light chains. It consists of sequence-based search for framework and canonical loop regions to find the most homologous structure from protein data bank. Noncanonical antibody regions are built either using knowledge-based search from databases or ab initio model building, using CONGEN conformational search [10]. Final conformational homology model is selected from the five lowest energy models, ranked based on torsion angles closest to the original PDB template.

1.3 Rosetta Antibody Modeling

In this algorithm, antibody variable heavy and light chain is split into frameworks and CDR sequences. These sequences then become input for template search in the curated protein data bank for the most similar sequences, and use the most homologous structure as a template to build a homology model. As framework regions are mostly conserved across different antibodies, a framework 3D model is built. CDR-H3 structure is the most variable and challenging to predict; hence its structural fold is created by de novo model building. Once VH–VL orientation is chosen via sequence blast of the whole Fv (fragment variable—VH + VL) against the PDB, the modeled CDR loops for VH and VL are grafted onto this framework template for further refinement and model building. Engrafted model is energy minimized, and an ensemble of high-resolution models <3000 are ranked based on Rosetta score that reflects probable entropies in each model, and the lowest energy is ranked as the top model [11].

1.4 Prediction of Immunoglobulin Structures (PIGS)

In general, this antibody variable homology modeling server algorithm is similar to those mentioned above. However, there are some differences in the workflow and strategies implemented in PIGS. A sequence-based analysis is performed to obtain homologous framework sequences of known antibody structures from protein data bank. Antibody CDRs with canonical loops are modeled based on known antibody structures and grafted upon the framework model. Both heavy and light chain models (if the parental templates are different) then are packed together based on conserved interface residues at the heavy and light chains from known antibody structures. Finally, energy minimization of the side chains is carried out via SCWRL4.0 (side chain with backbone-dependent rotamer library), results in the final antibody homology model generation [12].

2 Materials

Antibody humanization can be undertaken either using commercially available tools (Schrodinger's BioLuminate, Biovia's Discovery Studio, Chemical Computing Group's MOE and Macromoltek) or publically available web servers (Prediction of Immunoglobulin Structures- PIGS; Rosetta antibody modeling; structure-based antibody prediction server- SAbPred; and Kotai -antibody builder). To describe and delineate differences and limitations between these algorithms will be beyond the scope of this chapter. Given, publically available resources and servers can be accessed by everyone with a computer and internet access. This chapter will primarily focus on utilizing public servers for antibody homology model building and humanization.

1. To delineate boundaries of mouse heavy and light chain, user can input the whole heavy chain and light chain from the mouse IgG sequence separately into IMGT (the international ImMunoGeneTics information system®) DomainGapAlign alignment tool using default settings (http://imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi) [13]. As an example, a mouse antibody from protein data bank code (3MBX) with the variable heavy (VH) and light (VL) sequence (*see* **Notes 1** and **2**).

VH—
EVTLKESGPGILQPSQTLSLTCSFSGFSLSTYGMGVGWIRQPSGKGLEWLA
HIWWDDVKRYNPALKSRLTISKDTSGSQV
FLKIASVDTSDTATYYCARMGSDYDVWFDYWGQGTLVTVSA

VL—
DIVMSQSPSSLAVSVGEKVTMSCKSSQSLLYNNNQKNYLAWYQQKPGQS
PKLLIYWASTRESGVPDRFTGSGSGTDFTLT
ISSVKAEDLAVYYCQQYYSYPFTFGSGTKLEIK

2. Once the variable heavy (VH) and variable light (VL) chains are defined as above, these VH and VL sequences will become the starting material to be used as input for antibody homology model building. Model can be built by accessing any of the antibody modeling servers:

   PIGS—http://circe.med.uniroma1.it/pigs/index.php
   Rosetta—http://rosie.rosettacommons.org/antibody
   SAbPred—http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/WelcomeSAbPred.php
   Kotai—https://sysimm.ifrec.osaka-u.ac.jp/kotaiab/

3. Mouse antibody homology model is built by one of the above servers; the model can be visualized via molecular visualization software programs, such as PyMOL, DeepView-Swiss-PDBViewer, or UCSF Chimera. These programs can be downloaded as given below:

   PyMOL—https://www.pymol.org/
   DeepView-Swiss-PDBViewer—http://spdbv.vital-it.ch/disclaim.html
   UCSF Chimera—http://www.cgl.ucsf.edu/chimera/

4. Energy minimization of the humanized antibody model can be undertaken by DeepView-Swiss-PDBViewer. This software can be downloaded from this website.
   http://spdbv.vital-it.ch/disclaim.html

3 Methods

1. The first step is to obtain the mouse antibody variable and heavy and light sequences and create a homology model via any of the antibody homology modeling software. Input mouse antibody sequences (VH and VL) into either antibody homology modeling software (BioLuminate, Discovery studio, or MOE) or any of these public web servers (e.g., PIGS, Rosetta, SAbPred, or Kotai). (For an example, refer to Subheading 2, **step 1**, for a mouse antibody sequence.) Use PIGS web server as a starting tool for this exercise (Fig. 1a, b).

   PIGS—http://circe.med.uniroma1.it/pigs/index.php
   Rosetta—http://rosie.rosettacommons.org/antibody
   SAbPred—http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/WelcomeSAbPred.php
   Kotai—https://sysimm.ifrec.osaka-u.ac.jp/kotaiab/

2. Human framework selection can be primarily accomplished from two sources:

   (a) IMGT database can be used to obtain most identical (percentage identity) human germline repertoire for both VH chain and VL chains. IMGT's DomainGapAlign tool can be used to obtain the most similar corresponding human germline sequences for heavy and light chain separately (http://imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi) (*see* Fig. 2). Use default settings; under species drop-down menu, choose Homo sapiens (Humans), and then click "Align and IMGT-gap my sequence for VH and VL separately.

   (b) Blast antibody sequences VH and VL separately to find the most identical human framework in PDB. Igblast tool can be utilized for this analysis (https://www.ncbi.nlm.nih.gov/igblast/).
   Human framework selection criteria can be made based on percentage identity to individual VH and VL chains or human frameworks with highest overall identity for both chains (VH and VL).

3. Mutate and replace mouse residues in the homology model created in **step 1** to human residues, either found in the alignment using IMGT's DomainGapAlign alignment (**step 2a**) or human antibody structure obtained from PDB (**step 2b**). Replacement of mouse residues to human residues can be performed using PyMOL>wizard> mutagenesis tool https://www.pymol.org/ (*see* **Note 3**). In addition, user can subject the resulting chimeric model to Ramachandran plot validation using PDBsum Generate option (this step is optional) (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html).

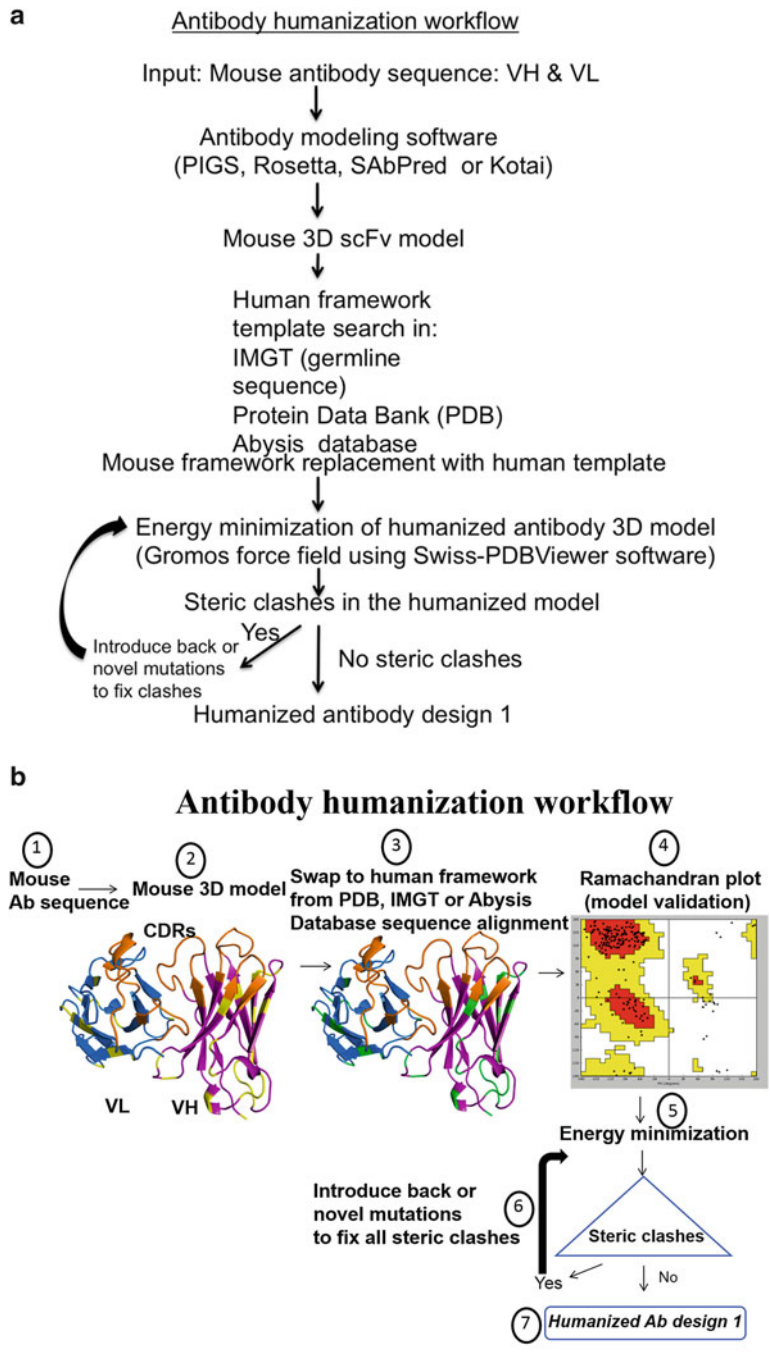

Fig. 1 (**a**) Antibody humanization steps are described in this workflow. As described in this flow chart, there are numerous steps in this process that need usage of different antibody modeling servers, sequence alignment tools, and protein visualization software. (**b**) Visual representation of antibody humanization design process. Different steps involved in antibody humanization are annotated based on each stage of the design development. A mouse antibody

4. This humanized homology model has human frameworks and mouse CDRs (chimeric), which is subjected to energy minimizations using Swiss-PDBViewer (spdbv) software. Upload this humanized model in the spdbv software, and select all, and then under tools, select "compute energy (Force Field)." The output "energy report" can be copied into a word document for review. Residues with high total energies need to be examined further. This can be carried out using PyMOL software (*see* **Note 4**).
5. Energy-minimized humanized model is then examined both visually and energetically for steric clashes. Based on the degree of entropies score, as well as extent of steric clashes, some residues may be replaced either to novel mutations or back mutation (parental mouse) residues. For example (Figs. 3 and 4), if there is a steric clash between two residues in the framework regions, then the residue that is not conserved across different germlines is mutated to fix the steric clash (*see* **Note 5**).
6. Once residue replacement is completed, humanized homology model is again subjected to energy minimization (**step 4**) to examine amelioration of these steric clashes (Fig. 3b). If no further steric clashes are found, then this humanized model is designated as design one.
7. To design additional humanized variants, one can choose the second highest identical human framework from **step 2** and carry out the engineering until **step 6** to obtain design two. Repeat **steps** from **2** to **6** to obtain a minimal set of 20 different humanized variants for experimental testing (*see* Fig. 1a, b).

4 Notes

1. To explore some additional examples of mouse antibodies for humanization, refer to [14], as it lists 17 different mouse antibodies for humanization using the same methods as mentioned in this chapter.

Fig. 1 (continued) sequence was utilized to create a 3D homology model, CDRs in orange, light chain in blue, and heavy chain in purple, and sequences that are different in mouse are highlighted in yellow. Once human germline sequence is obtained from either IMGT or Abysis database and aligned with mouse antibody sequence, those residues that were different are changed to reflect human sequences (green—step 3). This chimeric humanized model can also be validated via Ramachandran plot using PDBsum Generate option (step 4—optional). The humanized model (human framework and mouse CDRs) was then subjected to energy minimization using Swiss-PDBViewer (spdbv) software (step 5). If no steric clashes are found, this will result in creation of the first humanized antibody design (step 7)

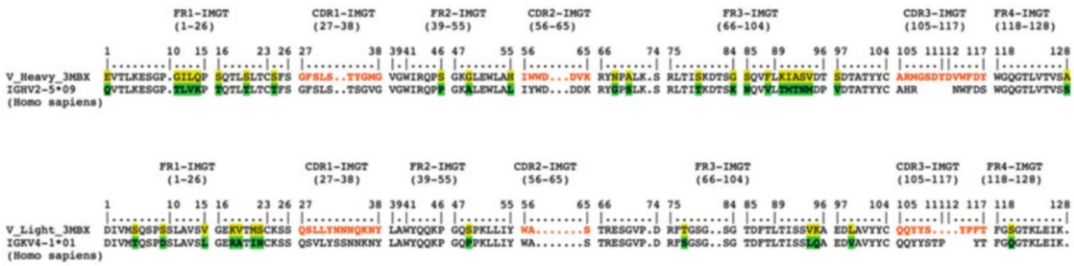

Fig. 2 Mouse antibody and corresponding human germline sequence alignment using IMGT DomainGapAlign tool. Mouse antibody sequence (PDB ID—3MBX) is aligned with the most identical human germline sequence. Mouse residues in both heavy and light chain (yellow) were mutated to human germline residues (green), whereas the mouse CDRs (orange) were unchanged. Mouse variable heavy chain is 71.4% (identical) to human germline IGHV2-5*09; a total of 24 amino acids needed to be replaced. Whereas mouse variable light chain is 82.3% (identical) to human germline IGKV4-1*01, as such, only 12 amino acids needed to be replaced

2. If using PIGS for antibody model building with VH and VL as input sequence from PDB ID—3MBX antibody, there will be two warnings after the submission for template selection. One being "No IG satisfying search criteria among first 20, and other is H3 canonical structure not defined." To circumvent first warning, under results, select threshold 40 results, instead of 20 (default).
3. Mutagenesis tool in PyMOL is recommended for mutating and replacing mouse antibody residues in the heavy chain and light chain to corresponding residues in the human germline gene (Fig. 2). In PyMOL, select wizard and mutagenesis, it will prompt to "pick a residue" of the mouse amino acid, and mutate to corresponding human amino acid from Fig. 2. (If using 3MBX as an example, in the heavy chain (VH) position 1, mouse glutamate (E) needs to be mutated to glutamine (Q) for humanization.) (*see* Fig. 2).
4. Energy minimization of the humanized chimeric model using Swiss-PDBViewer (spdbv). Once the hybrid-humanized model is opened using Swiss-PDBViewer, it might give an error of "unrealistic B factor." Please ignore and close this generic error. Compute energy (force field), under tools, is first selected. Make sure the pop-up window has all the boxes checked, including "show energy report." The energy report is usually saved in "temp" folder, which can be found inside the original folder, where Swiss-PDBViewer is installed (SPDBV_4.1.0_OSX "folder name." It is recommended to use desktop folder as a destination for software installation). In 3MBX, there are many residues with high total energies. As an example, only a couple of them are described here. Residues in the heavy chain position 24 (PHE) and in the light chain position 46 (LEU) have very high electrostatic or total score.