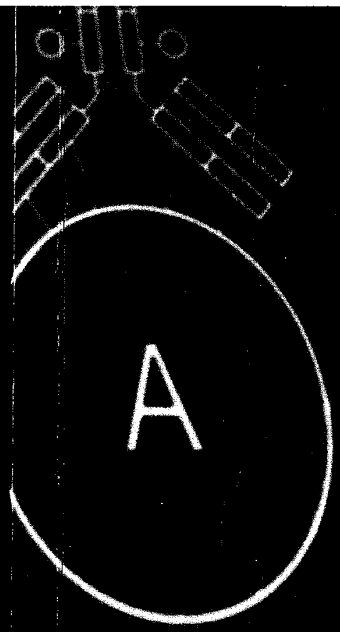

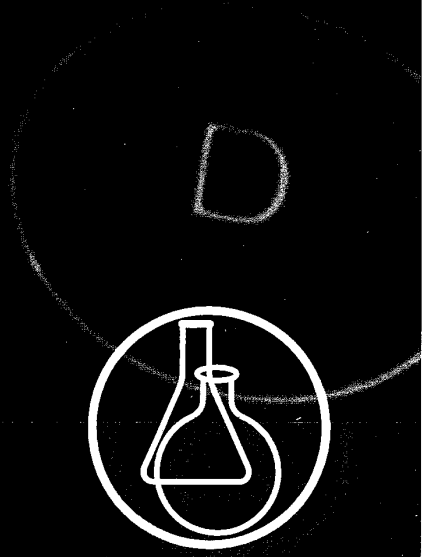

Monoclonal Antibodies

PRACTICAL APPROACH

ALXN2035
IPR2023-01069

ALXN2035
IPR2019-00739

Monoclonal Antibodies

A Practical Approach

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OXFORD
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Great Clarendon Street, Oxford OX2 6DP

Oxford University Press is a department of the University of Oxford.
It furthers the University's objective of excellence in research, scholarship, and education by publishing worldwide in

Oxford New York

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Chennai Dar es Salaam Delhi Florence Hong Kong Istanbul Karachi
Kuala Lumpur Madrid Melbourne Mexico City Mumbai Nairobi Paris
São Paulo Singapore Taipei Tokyo Toronto Warsaw

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Published in the United States by Oxford University Press Inc., New York

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First published 2000

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British Library Cataloguing in Publication Data
Data available

Library of Congress Cataloging in Publication Data

Monoclonal antibodies : a practical approach / edited by Philip S. Shepherd, Christopher J. Dean.
p. ; cm.—(The practical approach series)
Includes bibliographical references and index.
ISBN 0-19-963723-7 (hbk.: alk. paper)—ISBN 0-19-963722-9 (pbk.: alk. paper)
1. Monoclonal antibodies—Laboratory manuals. I. Shepherd, Philip S.
II. Dean, Christopher J. III. Series.
[DNLM: 1. Antibodies, Monoclonal. 2. Immunologic Techniques. QW 575.5.A6 M7455 2000]
QR186.85 M65646 2000 616.07'98—dc21 99-059426
1 3 5 7 9 10 8 6 4 2

ISBN 0-19-963723-7 (Hbk.)
ISBN 0-19-963722-9 (Pbk.)

Typeset in Swift by Footnote Graphics, Warminster, Wilts
Printed in Great Britain on acid-free paper
by The Bath Press (Avon) Ltd

Appendix

1 The design of the humanized antibody

Humanization by CDR grafting (also called reshaping) is now a well-established procedure for reducing the immunogenicity of monoclonal antibodies (mAbs) from xenogeneic sources (commonly rodent) and for improving their activation of the human immune system; in fact there are many humanized mAbs in clinical trials and a few have been given approval to be used as drugs. Although the mechanics of producing the engineered mAb using the techniques of molecular biology are relatively straightforward, simple grafting of the donor rodent complementarity determining regions (CDRs) into human acceptor frameworks does not always reconstitute the binding affinity and specificity of the original mAb. The design of the engineered mAb is now the critical step in reproducing the function of the original molecule. This design includes various choices: the extents of the CDRs, the human frameworks to use, and the substitution of residues from the rodent mAb into the human framework regions (backmutations). The positions of these backmutations have been identified principally by sequence/structure analysis or by analysis of an homology model of the variable regions 3D structure. Recently, phage libraries have been used to vary the amino acids at chosen positions (1, 2). Similarly, many approaches have been used to choose the most appropriate human frameworks in which to graft the rodent CDRs. Early experiments used a limited subset of well-characterized human mAbs (often where the structure was available), irrespective of the sequence identity to the rodent mAb (the so-called 'fixed frameworks' approach). Some groups use variable regions with high amino acid sequence identity to the rodent variable regions ('homology matching' or 'best-fit'); others use consensus or germline sequences, while still others select fragments of the framework sequences within each light or heavy chain variable region from several different human mAbs. There are also approaches to humanization developed which replace the surface rodent residues with the most common residues found in human mAbs ('re-surfacing' or 'veneering') and those which use differing definitions of the extents of the CDRs.

Not surprisingly, some rodent mAbs have proved difficult to humanize using standard protocols (3). The design and engineering of humanized mAbs are still very much areas of research, as much for the light they shed on protein structure and function as well as for the potential therapeutic and diagnostic benefits. For

this reason, an iterative protocol called the 'Humanization design cycle' (see *Protocol 1*) is described in the accompanying sections. These sections additionally supply data and raise issues of concern to the prospective antibody designers to help them through the bewildering variety of choices. The data is also available on the WWW under either of the two following 'Humanization bY Design' urls:
`http://mathbio.nimr.mrc.ac.uk/jsaldan` or
`http://www.cryst.bbk.ac.uk/~ubcg07s`
with hypertext links to the various resources for database search, sequence analysis, and structural modelling. Search forms for Sequence and Text input are also available under these urls to aid navigation through the list of previously humanized antibodies taken from the literature.

Protocol 1

The humanization design cycle

Equipment

- Any computer of reasonable power (for instance Pentium processor, 32 Mb RAM) which is connected to the WWW through a standard browser (e.g. Netscape) is needed to accomplish the design protocol

Method

1. Examine the source donor amino acid sequences for each chain (see Section 1.1.1).
2. Produce a structural model of the Fv regions (see Section 1.1.2).
3. Consider the human acceptor framework sequences for each chain (see Section 1.1.3).
4. Identify putative backmutations in the chosen frameworks (see Section 1.1.4).
5. Reconsider framework choice and design humanized antibody sequence.
6. Construct humanized and chimeric antibody sequences.
7. Test constructs.
8. Success? If 'No' then return to step 3.

1.1 Issues to consider

1.1.1 The source donor amino acid sequences

In the source donor sequence for each chain (heavy and light) it is important to consider:

(a) The complementarity determining regions. These contain the residues most likely to bind antigen and thus they must be retained in the humanized antibody (unless the precise residues involved in binding are known which is unlikely). CDRs are defined by sequence according to the Kabat scheme or by structure according to the Chothia scheme (see *Table 2*). The advantage of using the latter definitions is that the CDRs are shorter, therefore the humanized antibody will have fewer xenogeneic fragments. However, the

experience of other groups (4) has shown that using the Kabat definition generally requires fewer iterations in the humanization design cycle. A third scheme for defining CDR extents (see *Table 2*) called the 'contact definition' from Andrew Martin is based on an analysis of antibody/antigen interactions. It is likely to be useful in humanization but is as yet untested.

(b) Canonical residues. These are key residues which determine to a large extent the structural conformation of the CDR loop. They were originally defined by Chothia, but have now been revised by Andrew Martin. His WWW form at url: `http://www.biochem.ucl.ac.uk/~martin/abs/chothia.html` allows input of the variable region sequences and automatic identification of the canonical structure class and important residues. Canonical residues are almost always retained in the humanized antibody if they are different to those in the human frameworks. Numerous examples of the importance of canonical residues can be found in the literature which can be searched for in the 'Humanization bY Design' web sites.

(c) Interchain packing residues. Defined by Novotny and Haber, and Chothia at the interface between the variable heavy and variable light chain domains (see *Table 3*). Generally, unusual packing residues should be retained in the humanized antibody if they differ from those in the human frameworks. Their importance is illustrated in the humanization of mAb 1B4 (5).

(d) Unusual framework residues. These can be located using Andrew Martin's WWW form at url: `http://www.biochem.ucl.ac.uk/~martin/abs/seqtest.html`. For mouse antibodies, it is better to determine the subgroup using the form on the Kabat Database WWW site at url: `http://immuno.bme.nwu.edu/subgroup.html` and identify the residue positions which differ from the consensus. These donor-specific differences may point to somatic mutations which enhance activity. Unusual residues close to the binding site may possibly contact the antigen. However, if they are not important for binding, then it is desirable to remove them because they may create immunogenic neoepitopes in the humanized antibody. Note that sometimes unusual residues in the donor sequence are actually common residues in the human acceptor sequence. Unusual residues in the acceptor frameworks are not desirable because of the possibility of immunogenicity.

(e) Potential *N*- or *O*-glycosylation sites. It is well known that potential *N*-glycosylation sites are specific to a consensus pattern N-X-S/T. These sites can be located in the donor sequence using the Prosite WWW form at url: `http://www.expasy.ch/sprot/scnpsite.html`. Removal of potential *N*-glycosylation sites has not destroyed the binding avidity of a humanized antibody thus far, and in one case actually increased it (6). *O*-glycosylation sites are usually found in helical segments which means that they are not common in the beta-sheet structure of antibodies. They have no consensus pattern, but differences in *O*-linked glycosylation between chimeric and humanized forms of BrE-3 was hypothesized to cause the increased binding of the humanized mAb (7).

Table 2 CDR loop definitions (numbering according to Kabat *et al.*, ref. 15)

Loop	Kabat	Chothia	Contact
CDR-L1	L24–L34	L26–L32	L30–L36
CDR-L2	L50–L56	L50–L52	L46–L55
CDR-L3	L89–L97	L91–L96	L89–L96
CDR-H1	H31–H35	H26–H32	H30–H35
CDR-H2	H50–H65	H52A–H55	H47–H58
CDR-H3	H95–H102	H95–H101	H93–H101

Table 3 Residues at the VL/VH domain interface[a]

	Kabat number[b]	Mouse[c]	Human[c]
VK	34	H678 N420 A408 Y147 E114	A531 N147 D66
	36	Y1653 F198 L96	Y748 F80
	38	Q1865 H47	Q799 H22
	44(+)	P1767 V132 I40	P839 L5
	46	L1381 R374 P97	L760 V37
	87	Y1457 F448	Y795 F41
	89	Q1170 L206 F144	Q687 M107
	91	W376 S374 G356 Y295 H182	Y404 R115 S105 A84
	96(+)	L537 Y380 W285	L134 Y215 F78 W73 I71
	98(+)	F1724	F654
VH	35	H1001 N636 S402	E184 S527 H340 G167 A143
	37	V2336 I200	V1037 I477 L27
	39	Q2518 K67	Q1539 R16
	45(+)	L2636 P16	L1531 P24
	47	W2518 L64 Y50	W1534 Y21
	91	Y2149 F479	Y1429 F116
	93	A2202 T222 V102	A1346 T90 V71
	95	Y399 G375 S340 D340 R226	D268 G266 R109 E100
	100k(+)	F1285 M450	F540 M109 L33
	103(+)	W1469	W323

[a] The positions of interdomain residues were as defined by Chothia *et al.* (16).

[b] Numbering is according to Kabat *et al.* (15). Residues underlined are in the framework, other residues are in the CDRs. (+) residues are the six that form the core of the VL/VH interface according to Chothia *et al.* (16).

[c] The number following the one-letter amino acid code is the frequency taken from Kabat (November 1997 dataset; see url in the text). The number of sequences examined is identical for each domain and equal to 1997 and 852 for mouse and human sequences, respectively; except for residue 103 in the heavy chain where numbers are 1651 and 369 for mouse and human sequences, respectively.

1.1.2 Modelling the Fv regions

A carefully built model of the Fv regions of the source antibody and in some cases of the designed humanized antibody, is a common denominator in many of the successful humanization experiments. It is possible to build a model completely automatically using commercial packages or academic servers such as Swiss-Model at url: `http://www.expasy.ch/swissmod/SWISS-MODEL.html`. However, the danger of allowing a computer to make all the decisions is highlighted in the humanization of antibody AT13/5 (8) where the interaction between residues at positions 29 and 78 (Kabat numbering) in the heavy chain was not modelled correctly. The experience of an expert in protein sequence analysis and modelling is always worthwhile.

1.1.3 Choice of human acceptor frameworks

Despite this being the most critical part of the humanization design cycle, there are no hard-and-fast rules for choosing the acceptor frameworks into which to graft the donor CDRs. This is because the benefit of the various choices (in terms of immunogenicity in the patient) has not clearly been proved in the clinic. Therefore, there are only a set of approaches which have arisen from the collective experience of previous humanization experiments.

(a) Fixed frameworks or homology matched. Some groups prefer to use fixed frameworks (usually antibody NEW for the heavy chain and REI for the light chain since their structures are solved) for all their humanized antibodies. Other groups try to use the frameworks that are most similar to their donor sequence (homology matching or best-fit). A subtle comparison of the two methods in terms of the ease of producing a functional humanized antibody appears to favour homology matching (9). Note that it is entirely possible to 'mix and match' framework regions (i.e. choose frameworks 1–4 from different human antibodies according to sequence similarity) although this approach hasn't been tested exhaustively.

(b) Light/heavy chain frameworks from the same/different clone. In general, light and heavy chains from the same antibody are more likely to form a functional binding site than light and heavy chains from different antibodies. However, since the interface between variable domains is so well conserved (see *Table 3*), this is not usually a problem. A comparison of the two approaches once again appears to favour the latter (10).

(c) Human subgroup consensus or individual framework sequences. Being limited to frameworks from particular human antibodies runs the risk of the somatic mutations creating immunogenic epitopes, even though the frameworks are human. An alternative approach is to use the frameworks from human consensus sequences where idiosyncratic somatic mutations will have been 'evened out'. The two approaches have been compared; in one case showing no difference in binding avidities (11) and in the other case showing better binding with individual frameworks (12). Consensus sequences for human and mouse subgroups can be found under urls:

http://mathbio.nimr.mrc.ac.uk/jsaldan/cons/cons.html; or
http://www.cryst.bbk.ac.uk/~ubcg07s/cons/cons.html.

(d) Frameworks from germline sequences. Consensus sequences are artificial and although having no idiosyncratic residues, may create unnatural sequence motifs which are immunogenic. An alternative is to use human germline sequences which have been compiled in the database VBase at url: http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html.

Having decided on an approach to take in order to choose the human frameworks; which particular human antibody, consensus, or germline sequence should be used? This is simple in the fixed framework approach since the choice is always NEW for the heavy chain and REI for the light (although since modifications to both frameworks are apparent from the literature (13) even there a choice needs to be made!). The choice is usually made by performing a search for the most homologous sequence over the appropriate database. There are many database search programs available, the two most common being *BLAST* and *FASTA* which can be found at the following two urls respectively: http://www.ncbi.nlm.nih.gov/BLAST/ and http://www2.ebi.ac.uk/fasta3/. *BLAST* is often preferable since it allows searching over the Kabat database. In order to search the consensus or germline sequences, they need to be downloaded onto the local computer. Choice of the particular human frameworks for the light and heavy chain variable regions should be made by trying to match the length of the CDRs, the canonical residues and the interface packing residues (as described in Section 1.1.1), as well as trying to find the highest percentage identity between the donor and acceptor sequences. Unusual residues in the acceptor frameworks are not usually tolerated since they may lead to immunogenic epitopes in the resultant humanized antibody. Try to find human frameworks which are similar (in terms of percentage identity) to the source sequences and also require the least number of backmutations (see next section).

1.1.4 Identify putative backmutations

This is the most difficult and unpredictable procedure in the humanization of monoclonal antibodies. It is also the area which throws much light on protein structure and function. A solid body of data for helping to identify strategic alterations (using the sequence and text search forms) is available on the Humanization bY Design web sites under mirrored urls:
http://mathbio.nimr.mrc.ac.uk/jsaldan/selhum.html;
http://www.cryst.bbk.ac.uk/~ubcg07s/selhum.html.

(a) Analysis of donor and acceptor sequences. Putative point backmutations from the human framework residue back to the original source residue will already have been identified from the analysis of the donor and acceptor sequences for canonical residues, interchain packing residues, unusual residues, and glycosylation sites. Experience has shown that it is especially important to retain the source's canonical and interchain packing residues (though unfortunately not in all cases).

(b) Vernier zone and CDR-H3. Consider residues in the Vernier zone (see *Table 4*) (14) which form a 'platform' on which the CDRs rest and therefore potentially affect their conformation. Due to its extreme variability, special attention should be paid to CDR-H3, analysing the structural model for residues which may potentially affect its conformation.

(c) Proximity to binding site. Using the structural model, analyse residues within 5–6 Å of any CDR residues. These residues are likely to bind to antigen, especially if they are unusual as classified by the analysis of the source sequence.

(d) Glycosylation sites. Consider removal of any potential glycosylation sites, especially if they are on the surface of the structural model, since experience has shown that this can enhance the avidity of the humanized antibody.

Table 4 Residues in the VERNIER zone

Heavy chain	Light chain
2	2
27–30	4
47–49	35–36
67	46–49
69	64
71	66
73	68–69
78	71
93–94	98
103	–

Having decided on the residues to backmutate, return to the question of human acceptor frameworks. It is not unlikely that an overlooked human framework may actually contain the backmutations which are to be retained. If this is the case, then there is no need to introduce residues from the source sequence, thus making the humanized antibody more 'human'.

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