Antibody Engineering

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Roland Kontermann • Stefan Dübel
Editors

Antibody Engineering Volume 1

Second Edition

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Foreword

Antibodies, naturally produced for protection by a variety of organisms, are also extremely powerful tools for research, diagnosis, and therapy. Since publication of the first edition of *Antibody Engineering* in 2001, the field of antibody research and development (R&D) has continued to grow at a remarkable pace. The research arena has seen advances in understanding structure-function relationships, antibody engineering techniques, and production of various antibody fragments. Clinical development has expanded, with novel monoclonal antibodies directed toward an array of targets entering the study at a rapid pace and the study of more than 200 monoclonal antibodies as treatments for a wide variety of diseases on-going.

A key feature of the global surge in antibody R&D activity is the need for updated information by both novice and experienced researchers. The publication of this second edition of *Antibody Engineering* is thus timely. In this manual, Roland Kontermann and Stefan Dübel provide comprehensive coverage of both new and well-established techniques. Volume 1 reviews techniques that serve as the foundation for antibody research (e.g. humanization, antibody production in eukaryotic expression systems), key information on the measurement of antibody structure and function, and current thinking on preclinical development practices. Volume 2 focuses on antibody fragment or derivative research. This area has greatly increased in importance, as the limitations of full-size antibodies have become more apparent. Up-to-date information on techniques to generate single-chain variable fragments, bispecific antibodies, and single domain antibodies are included.

The manual provides topic overviews that place information in context, and materials and methods that are described in clear, concise language. Newcomers to the field will benefit from the practical advice included, and experts will appreciate both the wealth of information collected and the extensive reference lists provided for each section. *Antibody Engineering* 2nd edition will, thus, be an invaluable resource to anyone engaged in antibody R&D.

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Preface

More than a century after the first Nobel Prize was awarded for an antibody-based therapy, these molecules continue to fascinate researchers and inspire novel therapeutic approaches. More than ever, antibodies are used for a very broad and still steadily expanding spectrum of applications – from proteomics to cancer therapy, from microarrays to in vivo diagnostics. Responsible for the renaissance of this class of molecules are recombinant approaches that allow the modification and improvement of almost all properties. Today, affinity, valency, specificity, stability, serum half-life, effector functions, and even the species origin and thus the immunogenicity, just to name a few aspects, can be engineered at will. More than 20 antibodies are approved for clinical use, and almost all are genetically engineered, recombinant molecules. The next generations of these antibodies are already in the pipeline, and a plethora of alternative antibody formats are under development for various applications.

We look back on exciting 25 years of development from humble beginnings in the early 1980s, when the mere production of an antibody chain in *Escherichia coli* was a goal hard to achieve, to today's impressive list of protein engineering tools. Among them, in particular, the methods that allow us to make human antibodies outside the human body, such as transgenic human Ig mice and phage display, have shaped and driven the developments during the past decade.

Ten years ago, in the preface of the first edition of *Antibody Engineering* – which was comprehensive at its time with less than half of the pages – we predicted that "...it can be expected that recombinant antibody based therapies will be a widespread and acknowledged tool in the hands of the physicians of the year 2010." This vision has become true within the past decade, and even was exceeded, since we also see that these technologies have broadly entered basic research, allowing us to bring to reality the vision of generating sets of antibodies to entire proteomes – in high throughput robots without a single animal involved.

Antibody Engineering aims to provide the toolbox for many exciting developments, and it will help the reader to stay up-to-date with the newest developments in this still fast moving field. It is designed to lead the beginners in this technology in their first steps by supplying the most detailed and proven protocols, and also by supplying professional antibody engineers with new ideas and approaches.

Stuttgart and Braunschweig — Roland Kontermann and Stefan Dübel

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Nina Strebe, Frank Breitling, Dieter Moosmayer, Bodo Brocks, and Stefan Dübel

1.1 Introduction

Despite a growing number of recombinant antibodies being isolated from phage display libraries, most known antibody specificities are available from hybridoma cell lines. Here, a method is presented to obtain the genetic information for the antigen-binding part of the antibody from hybridoma cells, and to assemble it into a functional bacterially produced fusion protein (scFv fragment). To achieve this, vectors have been constructed, which combine the two variable regions (Vh and Vl) with a peptide linker. The genetic information for Vh and Vl is amplified from hybridoma cells using the polymerase chain reaction (PCR) with antibody-specific primers.

What are the reasons for cloning an scFv from a hybridoma? First, some hybridoma cell lines are very low producers, or antibody production is lost upon prolonged culture. In this case, a recombinant "hybridoma immortalization" can rescue a valuable antibody specificity. Second, the recombinant format can be

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required for the desired application. Examples are complex fusion proteins such as immunotoxins and antibody-enzyme fusions, or bispecific antibodies, which cannot be prepared with defined stoichiometry and coupling points by conventional chemical modification. Another example is the application as an intrabody (Strebe et al. 2009; Breitling and Dübel 1999). It has also been shown that the scFv format itself can be beneficial for the desired function. In one example, a monoclonal antibody against TNFα-Receptor (TNFR1), with limited TNF-agonistic activity was converted into a potent TNFα antagonist by producing it as a monomeric scFv fragment, thus preventing ligand binding and receptor cross linking (Moosmayer et al. 1995). In another example, the scFv fragment, but not the original antibody, was able to act as an enzyme inhibitor, probably because of its smaller size, avoiding sterical hindrance (Liu et al. 1999).

A problem frequently obstructing the functional cloning of V region DNA from hybridoma cell lines is their sequence heterogenicity. Point mutations and insertions as well as entirely different V regions may be found in the PCR products. Even if an antibody sequence has already been determined from the hybridoma, e.g., by PCR sequencing, it is not necessarily the sequence coding for the functional V regions, as shown, e.g., for the Myc1-9E10 (anti c-myc) hybridoma cell line (Fuchs et al. 1997; Schiweck et al. 1997). Various explanations for this sequence heterogenicity can be considered. Mutations can accumulate in the hybridoma cell population upon prolonged culture, which are not evident from functional analysis of the supernatant as long as a sufficient fraction of the cells still produce the correct antibody chains. These types of mutations can be minimized by preparing the cDNA from a freshly produced hybridoma subclone. Entirely different V sequences might derive from traces of expressed mRNA from the myeloma fusion partner, or even from the second allele of the B cell partner, since hybridoma cells are not controlled anymore by the rigid regulation mechanisms of the immune system. Even antibody pseudogene transcripts with stop codons inside the V region have been found in PCR products. Further, point mutations at both ends of the sequence can result from base pair mismatches during PCR priming.

In conclusion, an assay for antigen-binding function should be done as early as possible in the process of cloning. Creating a small phage display library from the PCR products, and screening for function is recommended where possible, e.g., when sufficient amounts of soluble antigen are available to perform a panning (see "troubleshooting"). This process is described in detail in Sect. 1.1.3. In this chapter, we present a direct cloning approach for hybridoma antibodies, recognizing antigens that are not available in significant amounts, such as cell surface antigens.

1.2 Outline

The method comprises hybridoma subcloning, RNA isolation, cDNA synthesis, PCR, stepwise cloning into a bacterial expression vector, and initial characterization steps for structure, production, and function of the antibody. The key to the successful

cloning is the PCR primer set. Two different choices for PCR primers are given. The first is a minimal set, which was empirically tested and evolved over more than 7 years (Dübel et al. 1994). To date, it has allowed successful amplification of V region DNA from over 40 hybridoma lines, including several rat hybridomas, and we have not observed a case so far where no PCR amplification is possible. However, this primer design strategy resulted in quite long oligonucleotides, thus introducing primer mutations at mismatch nucleotides, which may interfere with antigen binding of folding. The second primer set has been designed based on more recent and extended knowledge of antibody sequences and also includes IgM and lambda primers. It has not been tested to a similar extent, but proved to amplify V region DNA from several hybridoma antibodies, and it also has been successfully applied for cloning highly diverse repertoires from immunized mice (Brocks et al. 2001). In general, primers designed for the generation of murine V region libraries (see Sect. 1.1.2) might be used as well for cloning of V regions from hybridoma.

The entire procedure is outlined in Fig. 1.1. Bacterial culture, DNA manipulations, transformation, and gel electrophoresis methods are performed according to standard protocols (Sambrook et al. 1989).

1.3 Materials

1.3.1 Equipment

- ELISA reader
- PCR thermocycler.

1.3.2 Reagents

- RNA extraction Kit (RNeasy mini kit, Qiagen)
- Reverse transcriptase (SuperScript II, Invitrogen)
- Oligonucleotide primers, e.g., as described in Tab.1
- DNA polymerase (CombiZyme polymerase, Invitek)
- PCR-reaction buffer (supplied with the enzyme)
- Nucleotide stock solution containing 10 mM of each dNTP
- Bacterial culture, gel electrophoresis and agarose gel extraction equipment
- *Escherichia coli* K12, JM109, TG1 or XL1-blue competent cells
- Bacterial growth medium (LB) agar plates containing 100 μg/mL glucose and 100 μg/mL ampicillin
- Bacterial growth medium (LB) agar plates containing 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG)
- Media and agar plates are prepared according to standard protocols as previously described (Sambrook et al. 1989)

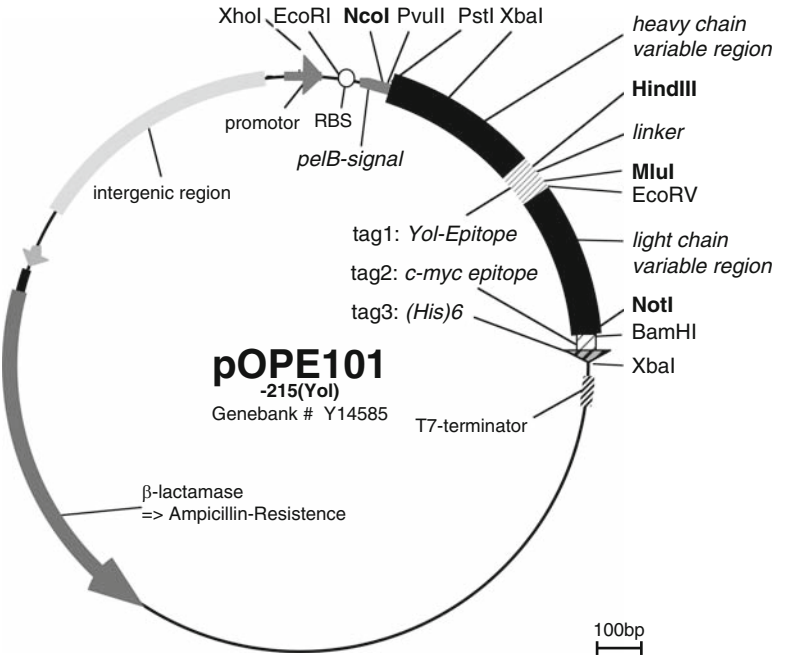

Fig. 1.1 The *E. coli* scFv expression vector pOPE101

- Tris–NaCl–Tween (TNT): 20 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4
- Tris-buffered-saline (TBS): 20 mM Tris–HCl, 0.15 M NaCl, pH 7.4
- Nitrocellulose membrane filters fitting the petri dish used for plating of the transformed bacteria, e.g., BA85 0.45 μM Ø82 mm, Schleicher & Schuell
- Indian Ink (Pelikan, Braunschweig)
- Chloroform
- Sodium azide powder
- 1% skimmed milk powder in TNT
- Antibody recognizing a tag peptide coded by the expected fusion protein
- Enzyme-labeled antiserum recognizing the first antibody
- Precipitating substrate system for the enzyme labeled antiserum
- Autoclaved glycerol.

1.4 Procedure

1.4.1 Isolation of Antibody DNA

1. Prepare a freshly subcloned culture of the hybridoma cells and check the supernatant for antigen binding. Collect at least 10^6 hybridoma cells by centrifugation.

2. Isolate the total RNA using a kit.
3. 9 µL total RNA, 2.5 µL oligo(dT)12–18 primer (10 µM), and 5 µL dNTPs were mixed. They were incubated for 5 min at 70°C and for 5 min on ice.
4. Afterward, 5 µL RT buffer (5×), 2.5 µL DTT (0.1 M) and 1 µL reverse transcriptase were added and incubated for 60 min at 42°C.
5. The reaction was stopped by incubation for 15 min at 70°C. The generated cDNA was stored at −20°C.
6. Mix the first strand cDNA with the PCR constituents on ice. Each 50 µL reaction contains 25 pmol of each primer, polymerase buffer as described by the supplier and 250 µM dNTPs. Use 1 µL of cDNA for each 50 µL PCR reaction.
7. Preheat the thermocycler to 95°. Add 0.5 U per 50 µL reaction volume of DNA polymerase on ice and mix. Avoid warming to room temperature, put the tubes quickly into the preheated thermocycler. Denature for max. 60 s. **Note**: *Taq*-DNA polymerase may be used, but there is a higher risk of introducing mutations during amplification. Some enzyme products consist of a mixture of proofreading/nonproofreading enzymes; they may be used as well. Longer denaturation than 60 s at the start of the program is not necessary, and may even result in loss of yield.
8. Perform 25–30 cycles of 30 s denaturation at 95°C, 1 min hybridization at the appropriate primer hybridization temperature, and 1 min polymerization at 72°C. Use the primers described in Tables 1.1 and 1.2 in individual reactions for each appropriate primer pair. After the end of the cycles, immediately cool down to 4°C. **Note:** A hybridization temperature of 55°C should be tried initially. If no products are amplified, perform a set of 4 PCRs, differing only by their hybridization temperatures of 42, 45, 48, and 51°C. Buffer, nucleotides, and primers may be stored as a premix at −20°C. Overcycling with *vent*-DNA polymerase may lead to product degradation.
9. Purify the PCR products. In case you have used the primer set of Table 1.1, you can directly proceed to Step 10. In case you have used the primer set of Table 1.2, you have to perform a second PCR to introduce the restriction cited necessary for cloning. To do this, use 1 µL of the purified first PCR reaction as a template in a reaction similar to step 7 and 8, but do only nine cycles, employing a hybridization temperature of 57°C.
10. Collect 1/5 volume of the reaction for analysis on a 1.5% agarose gel, containing ethidium bromide. Phenol extract the PCR product or freeze the PCR tubes immediately, until you have time to extract it. **Note**: The remaining activity of DNA polymerase needs to be removed after completion of the PCR reaction by phenol extraction to prevent digestion of 3′ overhanging ends by the intrinsic 3′-5′-exonuclease activity of the Polymerases. This step is essential for efficient cloning after the subsequent restriction digest. Omission of this step may result in drastically reduced ligation efficiency. Be aware that gel purification or spin columns do not remove this polymerase activity completely.

Table 1.1 Minimal Oligonucleotide set for the amplification of mouse and rat immunoglobulin variable region DNA. According to Dübel et al. (1994)

κ chain FR1 region:	(EcoRV)
Bi6	5′-GGTGATATCGTGAT(A/G)AC(C/A)CA(G/A) GATGAACTCTC
Bi7	5′-GGTGATATC(A/T)TG(A/C)TGACCCAA(A/T) CTCCACTCTC
Bi8	5′-GGTGATATCGT(G/T)CTCAC(C/T)CA(A/G) TCTCCAGCAAT
κ chain constant domain:	(BamHI)
Bi5	5′-GGGAAGATGGATCCAGTTGGTGCAGCATCAGC
Heavy chain FR1 region: (PstI, PvuII)	
Bi3	5′-GAGGTGAAGCTGCAGGAGTCAGGACCTAGCCTGGTG
Bi3b	5′-AGGT(C/G)(A/C)AACTGCAG(C/G)AGTC(A/T)GG
Bi3c	5′-AGGT(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG
Bi3d	5′-AGGT(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG
γ chain CH1 domain: (HindIII)	
Bi4	5′-CCAGGGGCCAGTGGATAGACAAGCTTGGGTGTCGTTTT
Reamplification primers for the introduction of other restriction sites	
Heavy chain FR1 region: Bi3f	5′- CAGCCGGCCATGGCGCAGGT(C/G)CAGCTGCAG(C/G) AG NcoI PvuII,PstI
κ chain constant domain: Bi5c	5′- GAAGATGGATCCAGCGGCCGCAGCATCAGC BamHI NotI
κ chain FR1 region: Bi8b	5′- AATTTTCAGAAGCACGCGTAGATATC(G/T)TG(A/C)T(G/C)ACCCAA(T/A)CTCCA MluI EcoRV

As a standard set, the combination Bi3f + Bi4 should be used for Vh and Bi8b + Bi5c for Vl. If no product is found in this first approach, other combinations can be tried. Preferentially, Bi7 should be tried instead of Bi8b, and Bi3b/3c instead of 3f. In addition, in case that restriction sites required for cloning are present internally in the amplification products, they should be reamplified (not more than 5–8 PCR cycles) to introduce alternative cloning sites. In case internal restriction sites of enzymes essential for cloning are present in the amplification products, they can be reamplified (not more than 5–8 PCR cycles) with the primers containing alternative sites. Please note that overcycling with *vent*-DNA polymerase may lead to a degradation of the correct product

11. Double digest the purified PCR product with the appropriate restriction endonucleases. **Note:** Calculate the amount of required enzyme carefully. Over digestion may reduce the ligation efficiency .
12. Purify the digested PCR fragment. We recommend spin column kit systems.

1.4.2 Cloning and Colony Screening

1. Ligate the appropriate dephosphorylated vector fragment (see Fig. 1.2) with the digested PCR product.

Table 1.2 Extended Oligonucleotide set for the two step amplification of mouse and rat immunoglobulin variable region DNA. According to Brocks et al. (2001)

First PCR	
Heavy chain	
Gamma chain CH1 domain:(IgG)	
Bi4	CCA GGG GCC AGT GGA TAG ACA AGC TTG GGT GTC GTT TT
Mu chain CH1 domain:(IgM)	
Bi4m	GGA GAC GAG GGG GAA AAG CTT TGG GAA GGA CTG ACT CTC
Heavy chain FR1:	
MHV.B1	GAT GTG AAG CTT CAG GAG TC
MHV.B2	CAG GTG CAG CTG AAG GAG TC
MHV.B3	CAG GTG CAG CTG AAG CAG TC
MHV.B4	CAG GTT ACT CTG AAA GAG TC
MHV.B5	GAG GTC CAG CTG CAA CAA TCT
MHV.B6	GAG GTC CAG CTG CAG CAG C
MHV.B7	CAG GTC CAA CTG CAG CAG CCT
MHV.B8	GAG GTG AAG CTG GTG GAG TC
MHV.B9	GAG GTG AAG CTG GTG GAA TC
MHV.B10	GAT GTG AAC TTG GAA GTG TC
MHV.B11	GAG GTC CAG CTG CAA CAG TC
MHV.B12	GAG GTG CAG CTG GAG GAG TC
Light chain	
Kappa chains	
kappa chain constant domain	
MKC. F	GGA TAC AGT TGG TGC AGC ATC
Kappa chain FR1	
MKV.B1	GAT GTT TTG ATG ACC CAA ACT
MKV.B1	GAT GTT TTG ATG ACC CAA ACT
MKV.B2	GAT ATT GTG ATG ACG CAG GCT
MKV.B3	GAT ATT GTG ATA ACC CAG
MKV.B4	GAC ATT GTG CTG ACC CAA TCT
MKV.B5	GAC ATT GTG ATG ACC CAG TCT
MKV.B6	GAT ATT GTG CTA ACT CAG TCT
MKV.B7	GAT ATC CAG ATG ACA CAG ACT
MKV.B8	GAC ATC CAG CTG ACT CAG TCT
MKV.B9	CAA ATT GTT CTC ACC CAG TCT
MKV.B10	GAC ATT CTG ATG ACC CAG TCT
Lambda chains	
Lambda chain constant domain	
MLC.F	GGT GAG TGT GGG AGT GGA CTT GGG CTG
Lambda chain FR1 region	
MLV.B	CAG GCT GTT GTG ACT CAG GAA
Second PCR	
Heavy chain	
Gamma chain CH1 domain:(IgG), Hind III site	
Bi4	(identical to 1st PCR)
Mu chain CH1 domain:(IgM), Hind III site	
Bi4m	(identical to 1st PCR)

(*continued*)

Table 1.2 (continued)

Heavy chain FR1 with NcoI site:	
MHV.B1.Nco	GAA TAG GCC ATG GCG GAT GTG AAG CTG CAG GAG TC
MHV.B2.Nco	GAA TAG GCC ATG GCG CAG GTG CAG CTG AAG GAG TC
MHV.B3.Nco	GAA TAG GCC ATG GCG CAG GTG CAG CTG AAG CAG TC
MHV.B4.Nco	GAA TAG GCC ATG GCG CAG GTT ACT CTG AAA GAG TC
MHV.B5.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAA CAA TCT
MHV.B6.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAG CAG TC
MHV.B7.Nco	GAA TAG GCC ATG GCG CAG GTC CAA CTG CAG CAG CCT
MHV.B8.Nco	GAA TAG GCC ATG GCG GAG GTG AAG CTG GTG GAG TC
MHV.B9.Nco	GAA TAG GCC ATG GCG GAG GTG AAG CTG GTG GAA TC
MHV.B10.Nco	GAA TAG GCC ATG GCG GAT GTG AAC TTG GAA GTG TC
MHV.B11.Nco	GAA TAG GCC ATG GCG GAG GTC CAG CTG CAA CAG TC
MHV.B12.Nco	GAA TAG GCC ATG GCG GAG GTG CAG CTG GAG GAG TC
Light chain	
Kappa chains	
Kappa chain constant domain with NotI site	
MKC. F.Not	TGA CAA GCT TGC GGC CGC GGA TAC AGT TGG TGC AGC ATC
Kappa chain FR1 with MluI site	
MKV.B1.Mlu	TA CAG GAT CCA CGC GTA GAT GTT TTG ATG ACC CAA ACT
MKV.B2.Mlu	TA CAG GAT CCA CGC GTA GATATT GTG ATG ACG CAG GCT
MKV.B3.Mlu	TA CAG GAT CCA CGC GTA GAT ATT GTG ATA ACC CAG
MKV.B4.Mlu	TA CAG GAT CCA CGC GTA GAC ATT GTG CTG ACC CAA TCT
MKV.B5.Mlu	TA CAG GAT CCA CGC GTA GAC ATT GTG ATG ACC CAG TCT
MKV.B6.Mlu	TA CAG GAT CCA CGC GTA GAT ATT GTG CTA ACT CAG TCT
MKV.B7.Mlu	TA CAG GAT CCA CGC GTA GAT ATC CAG ATG ACA CAG ACT
MKV.B8.Mlu	TA CAG GAT CCA CGC GTA GAC ATC CAG CTG ACT CAG TCT
MKV.B9.Mlu	TA CAG GAT CCA CGC GTA CAA ATT GTT CTC ACC CAG TCT
MKV.B10.Mlu	TA CAG GAT CCA CGC GTA GAC ATT CTG ATG ACC CAG TCT

(*continued*)

Table 1.2 (continued)

Lambda chains	
lambda chain constant domain with NotI site	
MLC.F.Not	GA CAA GCT TGC GGC CGC GGT GAG TGT GGG AGT GGA CTT GGG CTG
lambda chain FR1 region with MluI site	
MLV.B.Mlu	TA CAG GAT CCA CGC GTA CAG GCT GTT GTG ACT CAG GAA

2. Transform *E.coli* cells and plate on LB agar plates containing 100 μg/mL glucose and 100 μg/mL ampicillin. Incubate overnight at 28–32°C to obtain small colonies. **Note**: The glucose should not be omitted since it is necessary for the tight suppression of the synthetic promoter of pOPE vectors, and thus, for maintaining the stability of the insert.
3. When colonies with a diameter of about 0.5 mm have formed, put a nitrocellulose filter on the plate, wait a few seconds until it is entirely moistured.
4. Label the orientation of the filter on the agar plate by piercing a syringe needle dipped into Indian ink through the filter into the agar.
5. Use a scalpel or razor blade to cut out a section of about one fifth of the filter for the negative control.
6. Carefully remove both pieces of the filter with forceps, put them on new plates with the attached bacteria pointing upward. Put the negative control onto selection medium with glucose, the major section onto selection medium containing 100 μM IPTG. **Note**: With pOPE-vectors in *E.coli* JM109, we achieved optimal protein secretion with 20 μM IPTG at 25°C. This optimal IPTG concentration can vary between different Fv-sequences by a factor of about two. Higher IPTG concentrations lead to higher amounts of total protein, but in this case, most of the scFv fragments still carry the bacterial leader sequence (Dübel et al. 1992) and form aggregates. However, for the immunoblot analysis of total cellular SDS extracts, it is not necessary to discriminate between unprocessed and processed protein. Therefore, a higher IPTG concentration is used to increase the intensity of the protein band on the blot.
7. Incubate for 3 h at 37°C.
8. Expose the filters for 15 min to chloroform vapor (in a glass chamber containing an open chloroform vessel) **Note**: This step increases the staining intensity at the margin of a colony, thus improving the signal. It might be omitted in the case of strong reactions.
9. Wash the filters 2× in an excess of TNT (50 mL for a filter with a diameter of 10 cm) with the colonies pointing downwards. Remove the bacteria by gently rubbing the filters on the bottom of the washing vessel.
10. Wash the filters in TNT containing 0.01% NaN_3 (Caution: sodium azide is very toxic) with the colonies pointing downward, to kill residual bacteria. **Note**: NaN_3 should not be stored as a stock solution since it degrades rapidly in water.
11. Wash the filters 3× in an excess of TNT with the colonies pointing downwards.

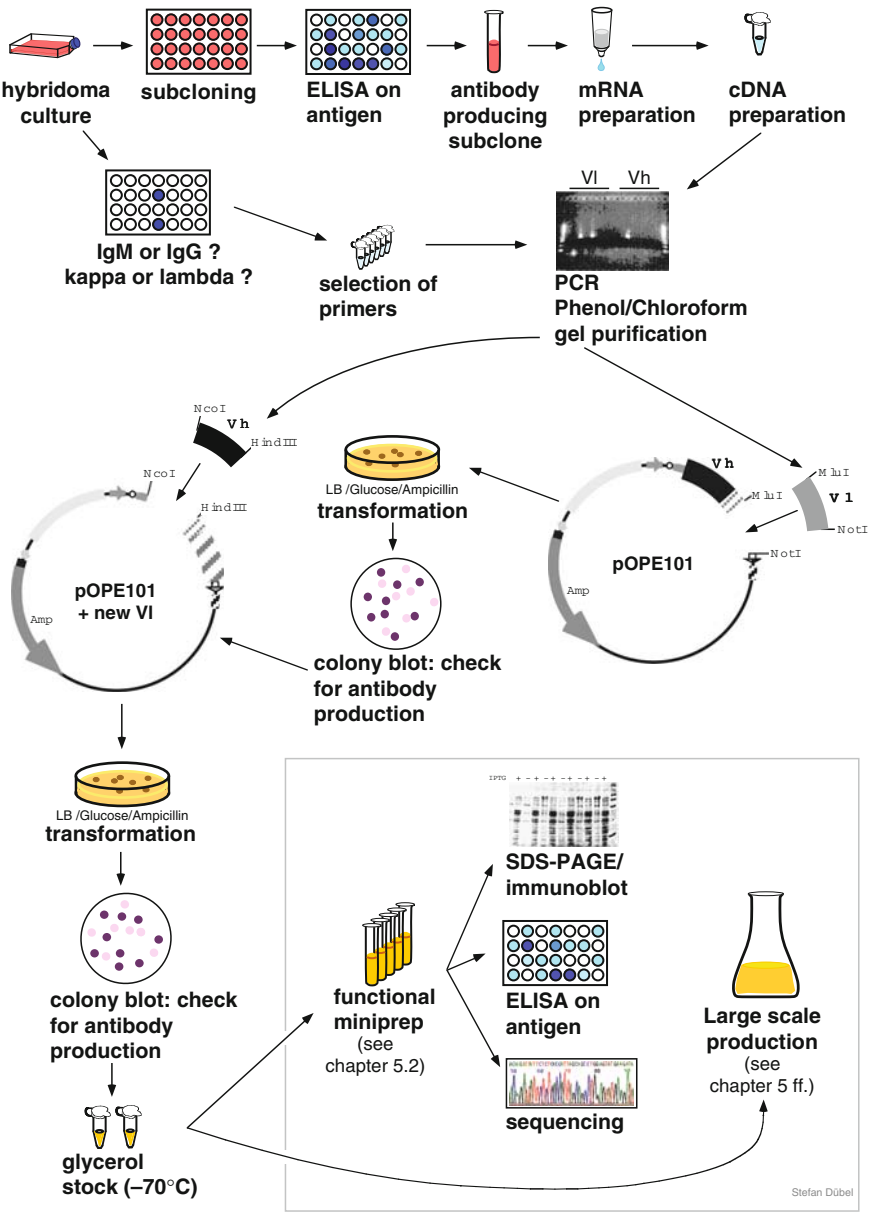

Fig. 1.2 Flow chart of scFv cloning from hybridoma cell lines ("hybridoma immortalization")

12. Block unspecific binding sites by shaking for 30 min in 1% milk powder in TNT. **Note**: Store 1% milk powder in TNT at 4°C for a maximum of 1 day; for longer storage, freeze aliquots. For a filter of 10 cm diameter, 4–5 mL of blocking or antibody solution are usually enough to obtain an even staining.

The best results are obtained with a platform shaker, which has a tilting motion in only one direction. The use of platform shakers with a tumbling movement requires larger incubation volumes to obtain an even staining. Only one filter should be used per incubation vessel.

13. Incubate in 1% milk powder/TNT containing the first antibody. **Note:** For scFv fragments cloned into pOPE51 or pOPE101, the monoclonal antibody Myc1-9E10 that binds to the internal epitope EQKLISEEDLN (Evan et al. 1985, commercially available from Cambridge Research Biochemicals) can be used. The recommended dilution is 1/10,000. The most specific result is obtained after incubation overnight at 4°C. For most applications, however, 1–2 h incubation at room temperature is sufficient. Alternatively, the His-tag can be utilized for detection with Ni-NTA HRP conjugate (Qiagen, Hilden, Germany). In this case, a second antibody is not necessary; proceed directly with Step 17 after incubation.
14. Wash 3× for 5 min in TNT.
15. Incubate in 1% milk powder/TNT containing the second antibody. **Note**: Commercially available antimouse IgG antisera, labeled with horseradish peroxidase (HRP), usually require a dilution of 1:1,000–1:5,000 in a 1–2 h incubation.
16. Wash 3× for 5 min in TNT, and briefly in TBS to remove detergent
17. Place the filters in substrate solution until the desired noise/signal ratio between the induced and the non-induced piece of the filter is reached. **Note**: We use cobalt enhanced diaminobenzidine/H_2O_2 – substrate system (Caution: diaminobenzidine is a cancerogene): Dilute 200 µL of diaminobenzidine stock solution (25 mg/mL in water, stock solution should be stored at −20°C in aliquots for max. 6 months) in 10 mL TBS, containing 0.02% (w/v) $CoCl_2$ and add 1 µL of 30% (v/v) H_2O_2, use immediately. The addition of $CoCl_2$ enhances sensitivity about 30 fold. Commercially available premixed TMB substrate solutions may be used instead.
18. After sufficient substrate reaction (usually not more than 10 min), wash 3× for 5 min in tap water. Air dry for documentation. **Note**: Scan or photograph within 1 day since bleaching may occur.
19. Pick a few positive colonies. Inoculate 1 mL of LB_{GA} (LB medium containing 100 mM glucose and 50 µg/mL ampicillin). Grow overnight at 37°C. Add 250 µL glycerol, mix and freeze at −30°. **Note**: The master plates should not be stored for longer than 1 day at 4°C. Cultures freshly inoculated directly from the frozen glycerol stock should be used for all subsequent experiments. Never thaw the glycerol stock; it is sufficient to scrape a bit of ice from the surface for inoculation.

1.4.3 Troubleshooting

Frequently, more than one primer pair amplifies a PCR product of the correct size. The reasons are discussed in the introduction. In this case, it is recommended to

pick, clone, and sequence 5–10 clones of each product. In case different sequences are found, all have to be tested for function. A set of clones has to be generated containing all possible combinations of Vh and Vl regions. Alternatively, a phage display screening (panning) can be employed after cloning the PCR products directly into a phagemid surface expression vector (e.g., pHAL, see chapter 5) and screened for functional antibody fragments. The latter procedure is always recommended if soluble and purified antigen is available in amounts above a few micrograms. Only if the antigen is not available, e.g., in case of cell surface antigens, the first approach has to be used.

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Chapter 2
Coning Hybridoma cDNA by RACE

Andrew Bradbury

2.1 Introduction

V region primer PCR is usually successful in the amplification of hybridoma V genes, especially if using diverse primer sets (Wang et al. 2000; Krebber et al. 1997; Coloma et al. 1991; Gavilondo-Cowley et al. 1990; Rohatgi et al. 2008). However, there are a number of potential pitfalls in using V region PCR. Mutations within the 5′ or 3′ ends of the V genes may inhibit primer annealing, and so, prevent amplification. In some cases, the use of universal V region primers can introduce mutations that can reduce stability, production yield, and antigen affinity (Honegger and Pluckthun 2001; Jung et al. 2001). Another problem is the presence of other V genes within the hybridoma that are preferentially amplified. These arise for two reasons. The first is nonproductive rearrangments, which, not being mutated, are very good PCR templates (Carroll et al. 1988; Storb et al. 1980), while the second is probably caused by the fusion of more than one spleen cell to the myeloma cell line, resulting in multiple functional (as well as nonfunctional) V genes (Zack et al. 1995). In this situation, an alternative to V gene PCR is to use either traditional cDNA cloning or rapid amplification of cDNA ends (RACE) (Frohman et al. 1988). This technique relies on knowledge of a small part of gene sequence to amplify from that gene sequence to either end of the cDNA. For both cases, an oligo-dT primer containing a specific tag is used to amplify the cDNA end. In the case of the 3′ end, the sequence to which it anneals is the naturally occurring poly-A tail, while in the case of the 5′ end (which is that used when RACE is used to clone hybridoma V genes), a poly-A tail is added using terminal transferase. PCR specificity can be

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subsequently improved by using the specific tag primer and a nested sequence specific primer (Pescatori et al. 1995).

When applied to immunoglobulins (Ruberti et al. 1994; Doenecke et al. 1997), the isotype of the monoclonal to be cloned provides the sequence knowledge, which can be used for the internal primer. This is used to create the cDNA. A poly-A tail is added to the 5′ end of the cDNA using terminal transferase, and the complete V gene, including 5′ untranslated region, leader sequence, and a small part of the constant region, can then be amplified. Once amplified, the V genes can then be cloned into standard cloning vectors such as pUC, from which they may be sequenced or reamplified using V region primers for cloning into specific phage display (McCafferty et al. 1990; Sblattero and Bradbury 2000) or eukaryotic antibody expression vectors (Persic et al. 1997a, b). However, the annealing temperature required may be as low as 37°C, if failure to amplify from total cDNA is due to mutations in the primer annealing sites (Ruberti et al. 1994; Ruberti et al. 1993). Mutations in the V genes can be avoided by using high amounts of cloned V gene (1 μg) and a DNA polymerase with proofreading activity (such as T. litoralis [Vent, New England Biolabs] or P. furiosus [Stratagene]) to reduce errors introduced by PCR.

Table 2.1 gives the sequences of hinge region primers of different heavy chain isotypes, which can be used for cDNA synthesis. Additional restriction sites should be added to the 5′ end with a nucleotide tail to allow efficient digestion. Hinge-specific primers are used, as these tend to be the most isotype-specific part of the constant region genes. Nested primers that can be used are located at the 5′ end of CH1 and these are also shown in Table 2.1. There is no equivalent to hinge region primers for the light chain. As a result, irrelevant light chains will also be amplified, although one can distinguish between mouse and rat light chains, and there are slight differences between the different isotypes. The light chain cDNA synthesis primer is found at the 3′ end of the constant region and the PCR primer overlaps it with a 6 bp extension to preserve specificity.

2.2 Procedure

1. Prepare cytoplasmic mRNA from 5×10^6 hybridoma cells.
2. Synthesize cDNA[a] with the following protocol: denature 1 μg of poly (A) mRNA at 65°C for 5 min in DEPC treated water, put on ice, and then add to a mixture containing 5 μl 5×RT buffer, 10 μl RNasin (Promega), 10 pmol cDNA synthesis-specific primer (see Table 2.1), 250 μM of each of the four deoxynucleotide triphosphates (dNTPs), and 10U of Moloney murine leukemia virus reverse transcriptase in a total volume of 25 μl. The reaction mixture is incubated at 42°C for 60 min and then at 52°C for 30 min. After inactivation at 95°C for 5′ the reverse transcription mixture is diluted with 2 ml of 0.1 TE (1 mM Tris pH7.0, 0.1 mM EDTA).

Table 2.1 Primers for cDNA synthesis of mouse or rat immunoglobulin genes

cDNA primers specific for heavy chain isotypes (all priming in hinge)

RACEMOG1 (mouse IgG1)	TAT GCA AGG CTT ACA ACC ACA
RACEMOG2a (mouse IgG2a)	AGG ACA GGG CTT GAT TGT GGG
RACEMOG2b (mouse IgG2b)	AGG ACA GGG GTT GAT TGT TGA
RACEMOG3 (mouse IgG3)	GGG GGT ACT GGG CTT GGG TAT
RACERAG1 (rat IgG1)	AGG CTT GCA ATC ACC TCC ACA
RACERAG2a (rat IgG2a)	ACA AGG ATT GCA TTC CCT TGG
RACERAG2b (rat IgG2b)	GCA TTT GTG TCC AAT GCC GCC
RACERAG2c (rat IgG2c)	TCT GGG CTT GGG TCT TCT GGG

Light chain primers (all prime at 3′ end of CL)

CKFOR (mouse and rat K)	CTC ATT CCT GTT GAA GCT CTT GAC
MOCKFOR (mouse K)	CTC ATT CCT GTT GAA GCT CTT GAC AAT
RACKFOR (rat K)	CTC ATT CCT GTT GAA GCT CTT GAC GAC

MOCKFOR and RACKFOR are identical to CKFOR except for the last three bases. If a V region from a rat mouse hybrid is to be cloned, and the mouse myeloma partner expresses a light chain V region mRNA, it can be excluded by the use of RACKFOR

CL1FOR (mouse λ1 λ4; rat λ1)	ACA CTC AGC ACG GGA CAA ACT CTT CTC
CL2FOR (mouse λ2, λ3; rat λ2)	ACA CTC TGC AGG AGA CAG ACT CTT TTC

These may be used individually if the lambda isotype is known or as an equimolar mixture to prime all lambda chains.

RACE PCR primers

Heavy chain primers (all prime at 3′ end of CH1)

MOCG12FOR (mouse IgG1, IgG2a; rat IgG1, IgG2a, IgG2b)	CTC AAT TTT CTT GTC CAC CTT GGT GC
MOCG2bFOR (mouse IgG2b)	CTC AAG TTT TTT GTC CAC CGT GGT GC
RACG2cFOR (rat IgG2c)	CTC AAT TCT CTT GAT CAA GTT GCT TT
MOCG3FOR (mouse IgG3)	CTC GAT TCT CTT GAT CAA CTC AGT CT
MOCMFOR (mouse IgM)	TGG AAT GGG CAC ATG CAG ATC TCT

These may be used individually or as an equimolar mixture to prime all heavy chains.

Light chain primers (all prime at 3′ end of CL)

CKRAsp	CTC ATT CCT GTT GAA GCT CTT GAC GAC GGG

(*continued*)

Table 2.1 (continued)

(this is identical to CKFOR, except that at the 3′ end, it has 6 extra bases to increase its specificity for rat)	
CKMOsp	CTC ATT CCT GTT GAA GCT CTT GAC AAT GGG
(this is identical to CKFOR, except that at the 3′ end, it has 6 extra bases to increase its specificity for mouse)	
CL1FOR	ACA CTC AGC ACG GGA CAA ACT CTT CTC
(mouse λ1 λ4; rat λ1)	
CL2FOR	ACA CTC TGC AGG AGA CAG ACT CTT TTC
(mouse λ2, λ3; rat λ2)	
CL1FORsp	ACA CTC AGC ACG GGA CAA ACT CTT CTC CAC AGT
(mouse and rat λ1)	
CL2FORsp	ACA CTC TGC AGG AGA CAG ACT CTT TTC CAC AGT
(mouse λ2, λ3; rat λ2)	
CL4FORsp	ACA CTC AGC ACG GGA CAA ACT CTT CTC CAC ATG
(mouse λ4)	
These may be used individually or in a pooled equimolar mixture. They are identical to the corresponding CLFOR primers, except that at the 3′ end, there are 6 extra bases to increase the specificity for each λ.	
None of the constant region primers described above has restriction sites at the 5′ end included for cloning. This should be inserted according to the vector to be subsequently used.	
RACE PCR primer	
XSCTnTag	GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT
Anneals to the poly A tail are added by terminal transferase, and provide XhoI, SalI, ClaI sites at the 5′ end. This is the original primer described by Frohman et al. However, other restriction sites can also be used.	

3. Remove excess primer using a Centricon 100 spin filter (20 min at 1,000 g, twice). The first retained liquid is collected and diluted to 2 ml before repeating the Centricon concentration. The second is concentrated to 10 µl and used in the following steps.
4. Synthesize a polyA tail at the 5′ end of the cDNA by adding 4 µl 5× Tailing buffer (supplied by Promega with the enzyme), 4 µl dATP 1 mM and 10U of Terminal deoxynucleotidyl transferase (Promega). The mix is incubated for 5 min at 37°C and then 5 min at 65°C. The volume of the cDNA/tailing reaction is adjusted to 500 µl.
5. Amplify 10 µl of reaction with Vent polymerase as follows:
   1 precycle: 5 min 95°C, 5 min 60°C, 40 min 72°C; 40 cycles: 1 min 95°C, 1 min 60°C, 3 min 72°C.
   PCR is performed using the oligonucleotide XSCTnTag (Table 2.1), which hybridizes to the poly(A) tail added to the 5′ end of cDNA and one PCR primer, specific for the light or heavy chain (Table 2.1).
   [a]Although we use this protocol, which is based upon the original published method by Frohman et al. (1988), other protocols, including cDNA synthesis and RACE kits should also be effective.

2.3 Results

After following this procedure, a single band should be obtained. This can be excised and purified from the agarose gel, digested with the appropriate enzymes, and cloned into the vector of choice. Sometimes a smear may be obtained. This can be reduced by changing the PCR conditions, or can be ignored if not too strong.

2.4 Troubleshooting

We have found this method extremely successful in amplifying V regions, which cannot be amplified by V region primers. As in any PCR reaction, varying the annealing temperature, the Mg concentration or the polymerase may improve the quality of the product.

In all cases, the specificity of amplified V genes should be confirmed by functional analysis of expressed antibodies or antibody fragments after expression in either bacteria or mammalian cells. If more than one V gene is obtained, both should be tested, in combination with the other V genes, to determine the correct combination.

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Chapter 3
Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly

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Abbreviations

BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
HRP	Horse radish peroxidase
IPTG	Isopropylthiogalactoside
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
scFv	Single-chain Fv fragment
cfu	Colony forming units
tet	Tetracycline

3.1 Introduction

Today, antibodies can be obtained from naive repertoires (Winter et al. 1994; Vaughan et al. 1996) or libraries of fully synthetic genes (Knappik et al. 2000), and in the last decade, numerous libraries have been described (reviewed in Mondon et al. 2008). Nonetheless, hybridomas have remained the predominant source of antibodies, and a wealth of well characterized and even unique clones exist and are continuing to be generated. There is, thus, great interest in immortalizing these clones, in the extreme case, as a computer file of the sequences, as well as in accessing the antibody in a variety of new formats. To obtain enough material

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