

Isolation of Natural Anti-Fc ϵ RI α Autoantibodies from Healthy Donors

Monique Vogel and Michael P. Horn

Abstract

Natural antibodies are defined as antibodies detected in a healthy individual without active immunization. These antibodies are specific for exoantigens, as well as for autoantigens, mostly without any pathogenic role. Most of the studies conducted with natural (auto-) antibodies have been performed using affinity purified antibodies from individual sera or polyclonal Ig-preparations such as Intravenous Ig (IVIg). For in-depth analysis of such autoantibodies affinity-purified Ig-preparations from healthy individuals are of no use, as they are oligoclonal or polyclonal. Thus, there is a need of human monoclonal autoantibodies. Human monoclonal autoantibodies can be produced from B cells isolated from humans; however, this requires the screening of a large number of antibodies to identify one among them specific to an antigen. Using the phage display technology we generated such autoantibodies against the alpha subunit of the high-affinity IgE receptor (Fc ϵ RI α). Here we describe the step-by-step protocol for the generation of such libraries and the isolation of autoantibodies by affinity panning.

Key words Natural antibodies, Anti-Fc ϵ RI α autoantibodies, IVIg, Phage display technology, Isolation

1 Introduction

The pathogenic role of natural antibodies in autoimmune disease is controversial. These antibodies are often polyreactive with a variety of both self and foreign antigens. However, the presence of such physiological autoantibodies does not generally precipitate autoimmune disease. Our investigations of autoantibodies associated with autoimmune urticaria suggest they are indistinguishable from natural antibodies found in healthy subjects.

Autoantibodies reacting with the α -subunit of the human high-affinity immunoglobulin E (IgE) receptor (Fc ϵ RI α) have been described in autoimmune urticaria [1, 2]. However, we previously reported the presence of anti-Fc ϵ RI α autoantibodies in the serum of healthy donors as well as in multidonor intravenous IgG (IVIg) preparations [3]. Thus, these anti-Fc ϵ RI α autoantibodies may

belong to the natural antibody repertoire reacting with a restricted set of self-antigens [4, 5]. In general, functional studies and in-depth characterizations of natural autoantibodies using serum or IVIg preparations are difficult to interpret due to the polyclonal nature of the respective antibody preparations, which may contain mixtures of different types (specificities) of autoantibodies. Therefore, to characterize these natural anti-Fc ϵ RI α autoantibodies and investigate their functional activity in vitro and in vivo, we generated human recombinant anti-Fc ϵ RI α antibodies by repertoire cloning from a nonimmune IgM library displayed on the filamentous phage M13 [6, 7].

The generation of monoclonal antibodies against an exogenous antigen is a very well-established method in rodents, but also in humans, monoclonal antibodies can be generated upon immunization with the antigen and subsequent isolation of antigen-specific B cells. These cells can further be immortalized by EBV infection or by cell fusion using heterohybridoma cell lines [8]. Using antibody phage display technology, we generated human monoclonal Fab fragments against different antigens, such as Tetanus Toxoid (TTd, *see ref.* [9]), Rhesus D (RhD, *see ref.* [10, 11]), or even IgE [12]. For TTd and RhD, individuals have been actively immunized, and the B cells for generation of the libraries have been isolated thereafter. However, in order to generate monoclonal natural autoantibodies, active immunization is not feasible. Thus, we use as a source of the antibody repertoire IgM-positive B cells isolated from children's tonsils.

There are mainly two different phage display systems for antibody repertoire cloning, single-chain Fv (scFv) and Fab-display. In the scFv system, the variable regions of the heavy and light chains were cloned in one gene segment using a spacer element [13, 14], whereas in the Fab system the light chains and the CH1 domain and the variable region of the heavy chains are cloned individually into the vector. Even though scFv proteins might be easier to handle, Fab libraries have in our view one big advantage. The combination of the heavy and light chains after expressing these proteins is based on their conformation. The heavy chain–light chain combinations as such have a huge impact on the specificity as well as the affinity of the antibody [15], and furthermore, not all random VH/VL pairings are represented in a natural antibody repertoire [16]. Thus, taking these two points into consideration, the Fab display system might represent a more natural antibody repertoire compared to the scFv system.

2 Materials

2.1 Isolation of PBMC or B Cells from Children's Tonsils

1. Fresh tonsils from children under routine tonsillectomy.
2. Nunc™ petri dishes.
3. Hank's balanced salt solution (HBSS).

4. Ficoll-Paque™ PLUS. Store at 4 °C.
5. Trypan Blue stain (0.4%). Store at room temperature (RT).
6. Phosphate-buffered saline (PBS): 5.6 mM Na₂HPO₄, 154 mM NaCl, 1.06 mM KH₂PO₄, pH 7.4. Adjust the pH with HCl. Dilute in sterile water and store at RT.
7. RNase-free water.
8. PBS containing 2% (wt/vol) fetal calf serum (FCS) and 1 mM EDTA.
9. Plastic pipettes (1, 5, 10, and 25 mL).
10. Micropipettes and tips (20, 200, and 1000 μ L).
11. 15- and 50-mL conical tubes.
12. Human B Cell enrichment kit (STEMCELL Technologies).
13. Hemocytometer.
14. RLT (RNeasy lysis buffer; Qiagen).

2.2 RNA Preparation

1. Purified B cells from fresh tonsils.
2. RNase-free 1.5 mL microfuge tubes.
3. RNase-free water.
4. RNase midi kit (Qiagen).
5. Sodium acetate solution (3 M, pH 5.2). Adjust pH with glacial acetic acid. Store at RT.
6. 100% ethanol.

2.3 RT-PCR Amplification of VH and VL cDNA

1. RNase-free 1.5 mL microfuge tubes.
2. 0.5 mL PCR tubes.
3. ThermoScript™ Reverse Transcriptase.
4. Oligo(dT)15 Primer, 500 μ g/mL (Promega).
5. dNTP Mix, PCR-Grade, 100 mM (25 mM each dNTP) (Agilent Technologies).
6. 5 $\times$ cDNA Synthesis Buffer.
7. 0.1 M DTT.
8. RNasin.
9. 5 U/ μ L Herculanase II Fusion DNA Polymerase (Agilent Technologies).
10. 5 $\times$ Herculanase II Reaction Buffer (Agilent Technologies).
11. 100 mM dNTP mix: 25 mM of each dATP, dCTP, dGTP, dTTP diluted in sterile water from 100 mM stock concentration (Agilent Technologies).
12. 96-well thermocycler.
13. MinElute Gel Extraction Kit (Qiagen).

14. 6× Gel loading dye solution (Fermentas). Store at RT.
15. TBE Buffer, 10×. Molecular biology grade (Promega).
16. 100-bp DNA ladder (Fermentas). Store at −20 °C
17. Agarose, LE, analytical grade.
18. red[®]—personal gel imaging system (Bucher Biotec AG).

2.4 Cloning of the Light Chain

1. Phagemid vector pMVS (3.49 kb) which has been reconstructed based on pComb3H-SS (Scripps Research Institute, La Jolla, Cal., USA); an oligonucleotide containing the myc and 6 His tags has been inserted at the XbaI site.
2. 20 U/μL *XbaI*.
3. 20 U/μL *SacI*.
4. 10× CutSmart[®] Buffer (New England Biolabs Inc.).
5. Agarose, LE, analytical grade.
6. TBE Buffer, 10×. Molecular biology grade.
7. 6× gel loading dye solution.
8. 100 bp DNA ladder.
9. QIAquick Gel extraction Kit.
10. 2 U/μL T4 DNA ligase and 10× T4 DNA ligase buffer.
11. Electrocompetent XL1-Blue cells with an efficiency of $>1 \times 10^{10}$ colony forming units (cfu)/μg pUC18/DNA (Agilent Technologies). Store at −80 °C.
12. *E. coli* Pulser[™] Transformation apparatus (Bio-Rad).
13. 0.2 cm Electroporation Cuvettes Plus (BTX, 1 mm)
14. Super Optimal Broth with catabolite repression (SOC) medium for *E. coli* (Cold Spring Harbor Protocols).
15. Super broth (SB) medium: 30 g tryptone, 20 g yeast extract, 10 g 3-(N-morpholino)-propanesulfonic acid (MOPS). Dissolve 30 g tryptone, 20 g yeast extract, 10 g MOPS in water. Bring to 1 L total volume with H₂O, stir until dissolved, and bring to pH 7.0 with 1 N NaOH. Sterilize by autoclaving in 1 L glass bottle. Store at RT.
16. 100 mg/mL carbenicillin. Dissolve 1 g of carbenicillin disodium in 10 mL of water. Sterilize by filtration through 0.22 μm. Store at −20 °C.
17. 10 mg/mL tetracycline. Dissolve 100 mg of tetracycline hydrochloride in 10 mL of ethanol. Store at −20 °C.
18. LB plates with 100 μg/mL ampicillin. Store at 4 °C.
19. Pasteur pipettes.
20. 50 mL Falcon tubes.

2.5 Production of Helper Phage

1. Electrocompetent XL1-Blue cells.
2. SB medium.
3. 10 mg/mL tetracycline.
4. VCSM13 helper phage (Agilent Technologies). Store at -80°C .
5. Dimethyl sulfoxide (DMSO).
6. PEG 8000. Store at RT.
7. PBS.

2.6 Cloning of the Heavy Chain and Phage Library Preparation

1. 20 U/ μL *Xho*I.
2. 10 U/ μL *Spe*I.
3. 10 $\times$ CutSmart[®] Buffer.
4. Agarose.
5. QIAquick Gel extraction Kit.
6. 2 U/ μL T4 DNA Ligase and 10 $\times$ T4 DNA ligase buffer.
7. Electrocompetent XL1-Blue cells with an efficiency of $>1 \times 10^{10}$ colony forming units (cfu)/ μg pUC18/DNA.
8. *E. coli* Pulser[™] Transformation apparatus.
9. 100 mg/mL carbenicillin.
10. 5 mg/mL tetracycline.
11. LB plates with 100 μg /mL ampicillin.
12. 100 mg/mL kanamycin sulfate. Dissolve 100 mg of kanamycin sulfate in 10 mL of water. Store at -20°C .
13. PEG 8000.
14. 12% NaCl

2.7 Library Selection on FcεR1α

1. 5 mL polystyrene immunotubes.
2. PBS.
3. Blocking solution: PBS containing 1.5 mg/mL casein.
4. PBS containing 0.1% Tween 20.
5. 0.1 M glycine-HCl, pH 2.2.
6. 2 M Tris-HCl pH 9.5.
7. SB Medium.
8. 0.05 M bicarbonate buffer pH 9.6. Prepare 0.05 M buffer with a pH of 9.6 by mixing 16 mL of 0.2 M sodium carbonate (21.2 g of Na_2CO_3 in 1 L of H_2O) and 34 mL of 0.2 M sodium bicarbonate (16.8 g of NaHCO_3 in 1 L of H_2O) and dilute to a volume of 200 mL.

2.8 Selection of Specific Anti-FcεR1α Clones

1. Protran[™] BA85 nitrocellulose membranes (Krackeler).
2. 5 mM isopropyl- β -D-thiogalactopyranoside (IPTG, Axon lab).

3. Lysozyme buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 0.4 mg/mL lysozyme, 1 U/mL DNase I recombinant, RNase-free.
4. Blocking solution.
5. 20 µg/mL human serum albumin (HSA)-FcεRIα. A fusion protein of two moieties of the extracellular part of the alpha subunit of the high affinity IgE receptor with one moiety of HSA (Novartis AG).
6. 10 µg/mL HSA.
7. Peroxidase-labeled sheep anti-human Fab antibody (Nordic).
8. 4-chloro-1-α-naphthol.
9. SB Medium.
10. Qiagen plasmid maxi kit (Qiagen).
15. LB agar plates containing 100 µg/mL ampicillin.

2.9 ELISA with Single Phage Clones for Definition of Recognition Pattern

1. Costar assay plates (Costar).
2. 5 mM IPTG.
3. PBS containing 1.5 mg/mL casein.
4. 5 µg/mL HSA-FcεRIα.
5. 5 µg/mL HSA.
6. 50 µg/mL β-casein from bovine milk.
7. PBS containing 0.1% Tween 20.
8. PBS.
9. 3,3',5,5'-tetramethylbenzidine.
10. 1 M H₂SO₄.
11. ELISA microplate reader (Spectra Max).

2.10 Nucleotide Sequencing

1. SB medium.
2. 100 mg/mL carbenicillin.
3. QIAprep Spin Miniprep Kit (Qiagen).
4. Sense and antisense primers (Microsynth AG).
5. V-base sequence directory (<http://www2.mrc-lmb.cam.ac.uk/vbase/vbase>).

2.11 Generation of Full-Length IgG for Functional Studies and Affinity Measurements

1. 25 U/µL *Bss*HII.
2. 10 U/µL *Sal*I.
3. 12.5 U/µL *Apa*LI.
4. 2.5 U/µL *Spe*I.
5. 10× CutSmart[®] Buffer (New England Biolabs).
6. QIAquick Gel extraction Kit.

7. 2 U/ μ L T4 DNA ligase and 10 $\times$ T4 DNA ligase buffer.
8. Human embryonic kidney cells 293 (HEK-293) expressing SV40 large T antigen (HEK-293T)
9. Lipofectamine[®] 2000 Transfection reagent (ThermoFisher Scientific).
10. Dulbecco's Modified Eagle Medium–Nutrient Mixture F-12 (DMEM–F-12) (ThermoFisher Scientific).
11. Fetal calf serum (FCS).
12. HiTrap Protein G Sepharose High Performance column (GE Healthcare Life Sciences).
13. Sodium dodecyl sulfate (SDS)–polyacrylamide gel.
14. Goat anti-human IgG (Fc specific) and peroxidase-labeled-goat anti-human IgG (whole molecule) antibodies.
15. Tissue culture (TC) flasks (Becton Dickinson).
16. 6-well TC plates (Becton Dickinson).

3 Methods

The generation of a human Fab library is summarized in Fig. 1. The single steps are depicted in detail in the following sections. The following section can also be used for the isolation of peripheral blood mononuclear cells (PBMC).

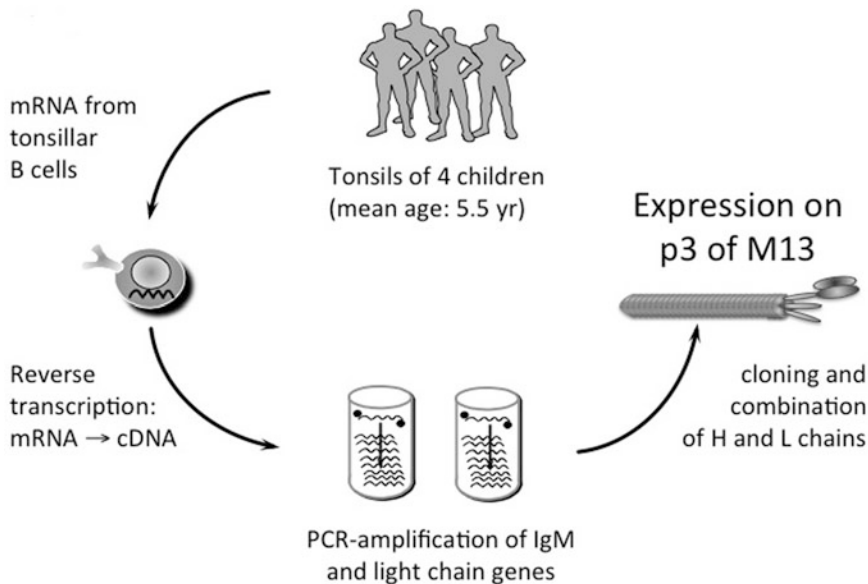


Fig. 1 Schematic illustration of the library construction. Tonsillar B cells are used as the source for the antibody library. Individual clones are amplified from the total mRNA from these cells. Light chain and Fc fragment were cloned into the phagemid vector and expressed on the surface of the bacteriophage M13

3.1 Isolation of Mononuclear Immune Cells (MNC) and B Cells from Children's Tonsils

1. Obtain fresh tonsils from children undergoing routine tonsillectomy.
2. To isolate MNC, place the tonsils in a petri dish, cut them into fine pieces with scissors or razor blades, then push out the cells with pincers into a 50 mL tube, rinse the plate with HBSS and pool with the first supernatant.
3. Let stand for 5–10 min until precipitation of the cell debris.
4. Carefully layer the supernatant over 15 mL Ficoll-Paque in a 50 mL centrifuge tube. Centrifuge at $800 \times g$ for 20 min at RT without brake.
5. Collect the mononuclear cells from interface leaving the cell pellet containing fibroblast and other cell debris.
6. Wash the cells three times with HBSS and resuspend in PBS containing 2% FCS and 1 mM EDTA (*see Note 1*).
7. Determine the number of viable cells by Trypan Blue staining using a hemocytometer. The expected yield is between 5×10^8 and 10^9 cells per tonsil.
8. Purify B cells by positive selection with a human CD19⁺ selection kit. Proceed according to the manufacturer's instruction.
9. After purification, assess the number of viable cells as above.
10. To assess the loss during the procedure determine the number of recovered B cells as a percentage of the input number. Expected percentage number is between 50 and 70% with a viability of >95%.
11. After isolation of B cells, resuspend the cells in RLT Buffer (2 mL up to 5×10^7 cells and 4 mL for more than 5×10^7 up to 10^8) cells and proceed according to Subheading 3.2.

3.2 Total RNA Preparation

1. For total RNA preparation we use the RNeasy Midi kit preparation. After resuspension of the B cells in either 2 or 4 mL of RLT buffer, proceed according to the manufacturer's instruction.
2. Following the last centrifugation step, elute the RNA in 200 μ L of RNase-free water. Immediately remove a 1.5 μ L aliquot and measure the absorbance at 260 nm in a NanoDrop instrument to calculate the total RNA concentration. A ratio of around 2.0 is generally considered as "pure" RNA and a concentration of 40 μ g/mL RNA gives an absorbance of 1.
3. Store remaining total RNA at -80°C for up to a year. For long-term storage precipitate the RNA by adding 0.1 volumes RNase-free 3 M sodium acetate (pH 5.2) and 2.2 volumes of 100% ethanol, vortex, and store at -80°C .

3.3 RT-PCR

Amplification of Fd (See Note 2) and Light Chains cDNA

For this step we use the primers depicted in Table 1.

1. In a RNase-free 1.5 mL Eppendorf tube, premix 2.5 µg total RNA, 1 µL of oligo(dT) (500 µg/mL), 10 mM dNTP mix, and RNase-free water up to 12 µL. Incubate for 5 min at 4 °C store on ice for at least 1 min and then collect by brief centrifugation.
2. Prepare reverse transcriptase mix by combining 4 µL of 10× Thermoscript Buffer, 1 µL of 0.1 M DTT, 1 µL of RNAsin, and 1 µL of Thermoscript™ Reverse Transcriptase, and RNase-free water up to 8 µL.
3. Add the prepared reverse transcriptase reaction mixture to the prepared RNA-Oligo(dT)-dNTP sample (12 µL), incubate at

Table 1
Primer sequences

Name	Sequences
<i>Reverse</i>	
IgM (CM1)	5'-GCTCACACTAGTGGCAATCACTGGAAGAGG-3'
IgG ₁ (CG1d)	5'-GCATGTACTAGTTTTGTCAAGATTGG-3'
Cκ (CLκ)	5'-GCGCCGTCTAGAATTAACTCTCCCCTGTTGAAGCTCTTTGTGA
CL (CLλ)	CGGGCGAACTCAG-3'
	5'-GCGCCGTCTAGAATTATGAACATTCTGTAGG-3'
<i>VH forward</i>	
VH135	5'-AGGTGCAGCTGCTCGAGTCTGG-3'
VH135b	5'-AGGTGCAGCTCGAGCAGTCTGG-3'
VH2	5'-CAGATCACCTTGCTCGAGTCTGG-3'
VH2F	5'-CAGGTGCAGCTACTCGAGTCGGG-3'
VH4F	5'-CAGGTGCAGCTGCTCGAGTCGGG-3'
VH4G	5'-CAGGTGCAGCTACTCGAGTGGGG-3'
VH6A	5'-CAGGTACAGCTCGAGCAGTCAGG-3'
VH6F	5'-CAGGTACAGCTCGAGCAGTCAGG-3'
<i>Vκ forward</i>	
Vκ1	5'-GAGCCGCACGAGCCCCGAGCTCCAGATGACCCAGTCTCC-3'
Vκ2	5'-GAGCCGCACGAGCCCCGAGCTCGTGATGACCCAGTCTCC-3'
Vκ3	5'-GAGCCGCACGAGCCCCGAGCTCGTGATGACCCAGTCTCC-3'
Vκ4	5'-GAGCCGCACGAGCCCCGAGCTCGTGATGACACAGTCTCC-3'
Vκ5	5'-GAGCCGCACGAGCCCCGAGCTCACACTCACGCAGTCTCC-3'
<i>Vλ forward</i>	
Vλ1a	5'-GAGCCGCACGAGCCCCGAGCTCGTGTTGACGCAGCCGCCCTC--3'
Vλ1b	5'-GAGCCGCACGAGCCCCGAGCTCGTGCTGACTCAGCCACCCTC-3'
Vλ2	5'-GAGCCGCACGAGCCCCGAGCTCGCCCTGACTCAGCCTCCCTCCGT-3'
Vλ4	5'-GAGCCGCACGAGCCCCGAGCTCGTGCTGACTCAGCCCCCGTC-3'
Vλ5	5'-GAGCCGCACGAGCCCCGAGCTCGTGCTGACTCAGCCACCTTC-3'
Vλ3-9	5'-GAGCCGCACGAGCCCCGAGCTCGAGCTGACTCAGCCACCCTC-3'
Vλ7-8	5'-GAGCCGCACGAGCCCCGAGCTCGTGGTGACTCAGGAGCCCTC-3'
<i>Sequencing primers</i>	
pCH3H	5'-GGAGGAATTTAAAATGAAATAC-3'
pCL3H	5'-GTGGAATTGTGAGCGGATAAC-3'

50 °C for 60 min, followed by 5 min incubation at 85 °C. First strand cDNA can be stored for weeks at –20 °C.

4. For Fd amplification, use 2 µL of the first strand cDNA with 1.25 µL 10 µM of the eight VH forward primers (*see* Table 1) in 0.5 mL PCR tubes.
5. For light chain amplification, mix 2 µL of the first strand cDNA with 1.25 µL 10 µM of each 5 V κ or V λ forward primers (*see* Table 1) in 0.5 mL PCR tubes.
6. Prepare three PCR master mixes for 8 reactions of Fd, 5 reactions of kappa, and 7 reactions of lambda amplifications.
 - (a) Fd master mix: In 1.5 mL Eppendorf tubes, combine 80 µL 5 $\times$ Herculase Buffer, 6.4 µL of 25 mM dNTP mix, 10 µL of 10 µM of either CG1D or CM1 reverse primers, 281.6 µL of water, and 4 µL 5 U/µL Herculase polymerase.
 - (b) Kappa master mix: In 1.5 mL Eppendorf tubes, combine 50 µL 5 $\times$ Herculase Buffer, 4 µL of 25 mM dNTP mix, 6.25 µL of 10 µM of CL κ reverse primer, 176 µL of water, and 2.5 µL 5 U/µL Herculase polymerase.
 - (c) Lambda master mix: In 1.5 mL Eppendorf tubes, combine 70 µL of 5 $\times$ Herculase Buffer, 5.6 µL of 25 mM dNTP mix, 8.75 µL of 10 µM of CL λ reverse primer, 246.4 µL of water, and 3.5 µL 5 U/µL Herculase polymerase.
7. Add 47.75 µL of the master mix to the corresponding prepared first strand cDNA/forward primer.
8. In a 96-well thermocycler, use the following PCR conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30, 68 °C for 1 min. Followed by 72 °C for 10 min and then by cooling to 4 °C.
9. To each sample add 10 µL of 6 $\times$ gel loading dye solution and separate by gel electrophoresis on a 1% (wt/vol) agarose gel in TBE buffer using a 100 bp DNA ladder as reference. The PCR band should run at 660 bp for both heavy and light chains.
10. Cut the band with a razor blade and elute DNA using Qiagen MinElute Gel extraction kit. Measure the absorbance at 260 nm and calculate the DNA concentration based on the formula that 50 µg/mL DNA gives an absorbance of 1.

3.4 Cloning of the Light Chain

The cloning of both chains is illustrated in Fig. 2. The cloning of the light chain represents the first step of the library construction. Both light chains will be first digested with *SacI* and *XbaI* and then into cloned into vector pMVS.

1. For digestion, combine 1 µg of each light chain kappa and lambda with 10 $\times$ CutSmart Buffer, 3.5 µL of 20 U/µL *XbaI*,

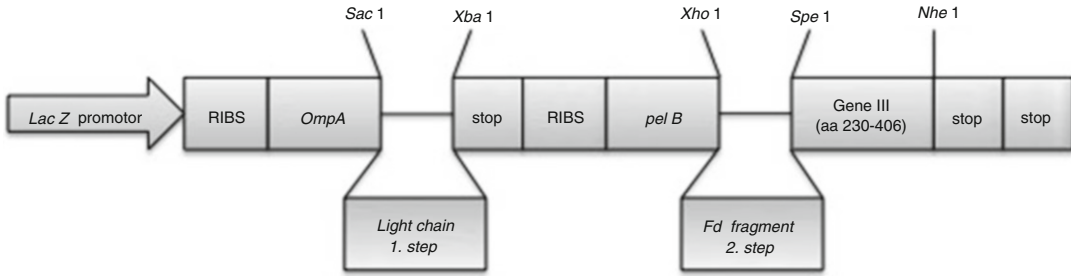


Fig. 2 Cloning site of the phagemid vector for the integration of the Fd fragment and the light chains

and 0.9 μL of 40 U/ μL *SacI* and add water to a final volume of 100 μL . Incubate at 37 °C for 90 min.

2. After inactivation of the enzyme by heating 25 min at 80 °C, purify the DNA with the QIAquick PCR Purification Kit. Resuspend the DNA in 10 μL of water and measure the absorbance at 260 nm as previously. Store at -20 °C.
3. Perform a test ligation by mixing 100 ng of *XbaI/SacI*-digested pMVS vector with 33 ng of *XbaI/SacI*-digested light chain, 1 μL of 10 $\times$ T4 DNA ligase buffer, 1 μL of 1 U/ μL T4 DNA ligase, and water to obtain a final volume of 10 μL . Prepare another test ligation using only *XbaI/SacI*-digested pMVS vector. Incubate for 3 h at RT.
4. For *E. coli* transformation, we set an *E. coli* Pulser apparatus to 2.5 kV. For each ligation use 40 μL of electrocompetent cells and add 1 μL of the ligation mixture. Mix well and let stand for 1 min on ice.
5. Transfer the mixture of cells and DNA to an ice-cold electroporation cuvette and shake the suspension to the bottom. Place the cuvette into the chamber unit and pulse once. Remove the cuvette and immediately add 1 mL of SOC medium, resuspend with a Pasteur pipette and transfer to a 15 mL tube and shake at 37 °C and 250 rpm for 1 h.
6. For estimation of the library size, plate 10 and 1 μL of the culture on Luria-Bertani broth (LB) agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin plates. Incubate overnight at 37 °C. Calculate the number of independent clones that can be expected from the ligation, e.g., 50 colonies from a 1 μL aliquot would give 1×10^7 independent clones (50 colonies $\times$ 1000 (dilution factor) $\times$ 20 (fraction of ligations) $\times$ 10 (adjusted to library scale)). To perform the library ligation of light chain, the number of independent clones should be at least 1×10^7 independent clones with a background less than 10%.
7. For cloning the light chain combine 1 μg of *XbaI/SacI*-digested pMVS vector with 330 ng of *XbaI/SacI*-digested

light chain fragmentation, add 10 μ L of 10 $\times$ T4 DNA ligase buffer, water, and T4 DNA ligase 10 U/mL. Incubate at RT for 3 h.

8. Purify the DNA with the QIAquick PCR Purification Kit. Elute the DNA in 10 μ L of elution buffer and store on ice. As for the test ligation electrocompetent *E. coli* cells are thawed, put on ice and cuvettes required for the ligation are also put on ice.
9. Add to the eluted DNA 100 μ L of thawed *E. coli*, divide the sample into two cuvettes, store 1 min on ice and then electroporate at 2.5 V. Transfer the cells into a 50 mL Falcon tube. Shake at 37 °C and 250 rpm for 1 h.
10. Add 10 mL SB medium containing 20 μ g/mL carbenicillin and 10 μ g/mL tetracycline and shake at 37 °C and 250 rpm for 1 h.
11. To assess the number of independent transformants remove immediately after adding the 10 mL SB medium 20, 1 and 0.1 aliquots and plate on LB medium plus 100 μ g/mL ampicillin. Shake at 37 °C and 250 rpm for 1 h.
12. After 1 h incubation, transfer the 10–100 mL SB medium containing 50 μ g/mL carbenicillin and 10 μ g/mL tetracycline. Shake at 37 °C and 250 rpm overnight.
13. Centrifuge at 3000 $\times g$ for 20 min at 4 °C. Remove the supernatant and prepare from the cell pellet plasmid DNA using Qiagen plasmid maxi kit. Elute DNA in 50 μ L and measure the absorbance at 260 nm as previously. Store at –20 °C.

3.5 Production of Helper Phage

1. Streak out *E. coli* strain XL1-Blue onto an LB tetracycline Plate and incubate overnight at 37 °C.
2. Pick a single colony and inoculate into 10 mL of SB medium with 10 μ g/mL tetracycline and incubate at 37 °C until OD₆₀₀ = 0.3 (approx. 2.0×10^8 cfu/mL).
3. Then two alternatives for proceeding.
Add helper phage at a multiplicity of infection (MOI) of 20:1 phage to bacterial cells(= 4.0×10^{10} pfu/10 mL).Add a single plaque of M13K07 helper phage from a fresh plate to the 10 mL culture.
4. Incubate at 37 °C for 30 min without shaking.
5. Transfer to 100 mL of SB (LB) medium containing 10 μ g/mL tetracycline and 70 μ g/mL kanamycin and incubate at 37 °C with vigorous aeration (225 rpm) for 8 h.
6. Heat the culture to 65 °C for 15 min and then centrifuge at 2794 $\times g$ for 15 min to discard cell debris.
7. Titer the helper phage produced (Titer should be 1×10^{11} – 1×10^{12} pfu/mL).

8. Store at 4 °C (*see* **Note 3**).
9. If the titer is too low, then precipitate the phage by adding 4% of PEG and 3% NaCl. Dissolve by swirling and precipitate the phage on ice for 30 min.
10. Centrifuge at $14,145 \times g$ for 20 min at 4 °C. Resuspend the phage pellet in 2 mL of PBS, centrifuge for 5 min at $16,000 \times g$ and store the supernatant at 4 °C long-term storage.

3.6 Cloning of the Heavy Chain and Phage Library Preparation

The cloning of the heavy chain depicted in Fig. 2 is followed by the phage preparation using the helper phage VCSM13 helper phage.

1. For cloning, the Fd fragment is first digested with *Xho*I and *Spe*I by mixing 1 μ g of Fd DNA with 10 μ L of 10 $\times$ CutSmart Buffer, 3.5 μ L of 20 U/ μ L *Xho*I, and 0.5 μ L of 40 U/ μ L *Spe*I and add water to a final volume of 100 μ L. Incubate at 37 °C for 90 min.
2. After inactivation of the enzyme and purification of the DNA (purification as above for the light chains, *see* Subheading 3.4) clone into the *Spe*I/*Xho*I-digested pMVS containing the light chains. For ligation and transformation proceed as previously for the light chains.
3. After transformation and incubation in 1 mL of SOC for 1 h at 37 °C transfer the culture to 100 mL SB containing 20 μ g/mL carbenicillin and 10 μ g/mL tetracycline. Remove aliquots as before to determine the size of the library.
4. Then shake for 1 h at 250 rpm at 37 °C. After increase the concentration of carbenicillin to 50 μ g/mL by adding 3 μ L of 1 mg/mL carbenicillin. Shake at 37 °C and 250 rpm for an additional 1 h.
5. Add 1 mL of VCSM13 helper phage (10^{12} pfu) and transfer to 100 mL of SB medium containing 50 μ L of 100 mg/mL carbenicillin and 70 μ L of 10 mg/mL tetracycline. Continue shaking for 1 h at 37 °C and at 250 rpm.
6. Add 70 μ L of 100 mg/mL kanamycin and continue shaking at 37 °C, 250 rpm overnight.
7. Centrifuge at $3000 \times g$ for 20 min at 4 °C. Keep supernatant and cell pellet. With the cell pellet carry out phagemid isolation using Qiagen plasmid maxi kit.
8. Elute DNA in 50 μ L and measure the absorbance at 260 nm as previously. Store at -20 °C.
9. For the phage preparation, add to the supernatant 33 mL of 16% PEG 8000/12% NaCl. Mix and let stand 30 min on ice. Centrifuge at $15,000 \times g$ for 30 min at 4 °C.

10. Discard the supernatant and resuspend in 2 mL PBS containing 1.5 mg/mL of casein and 1.5 mg/mL of HSA and store at 4 °C.
11. Transfer to microfuge tube and centrifuge at $16,000 \times g$ for 5 min.
12. Remove the supernatant and store on ice for panning on the same day. For long-term storage, add 1 vol. of glycerol and store at -20 °C.

3.7 Library Selection on FcεRIα

This step is performed on immobilized human recombinant FcεRIα and carried out during six rounds of panning. After each round of panning, specific binders are eluted and amplified by infection of *E. coli*. The procedure is illustrated in Fig. 3.

1. For each round of biopanning, coat one immunotube using 4 mL of 20 µg/mL FcεRIα or 20 µg/mL HSA in 0.05 M bicarbonate buffer. Incubate overnight at 4 °C or 1 h at 37 °C.
2. Remove the coating solution, fill up the tube with blocking solution. Mix on a rotator tube at 37 °C for 1 h.
3. Shake out the blocking solution, add 4 mL of the amplified phage preparation in 1% BSA to the HSA-coated tube and incubate at RT for 2 h.
4. Transfer the phage solution to the FcεRIα-coated tube and incubate at RT for 2 h.

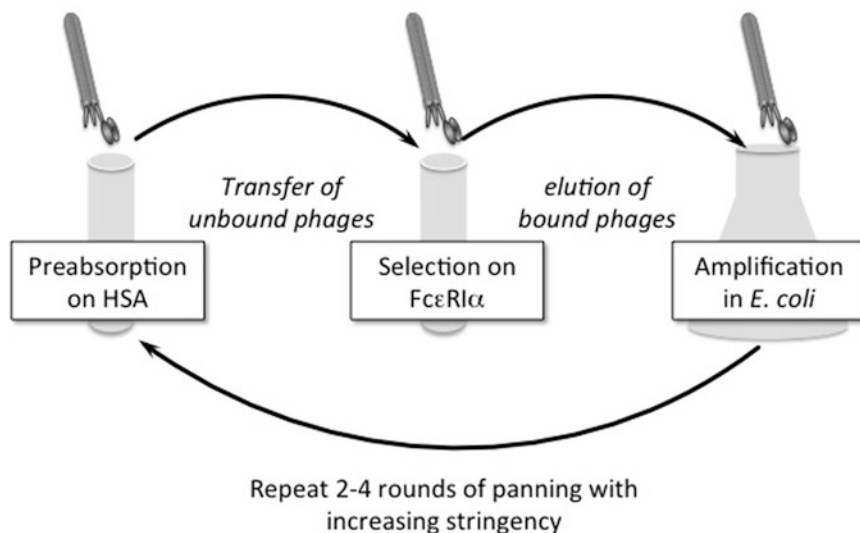


Fig. 3 Schematic illustration of the biopanning procedure for the selection of FcεRIα-specific Fab phages. The total phage population is preabsorbed on HSA, the carrier protein of the used recombinant FcεRIα. Then, the nonbinding phages are transferred to FcεRIα. After washing off the nonspecific phages, the bound phages are eluted by acid treatment and amplified in *E. coli*

5. Remove the phage solution, wash nonbinding phages with 10×5 mL PBS containing 0.1% Tween 20 and 10×5 mL PBS.
6. Wash the tubes with 3×5 mL PBS and elute the remaining phages for 15 min with 500 μ L of 0.1 M glycine-HCl and neutralize with 30 μ L of 2 M Tris pH 9.5.
7. Pool all eluted phages and incubate for 15 min at room temperature with 4 mL of *E. coli* XL-1 cells ($OD_{600} = 1$) for infection. Amplify and precipitate the phages as described above (*see* Subheading 3.6).
8. For output titting remove aliquots of 10, 1, and 0.1 μ L, add to 100 μ L of SB medium and plate on LB plates containing 100 μ g/mL of ampicillin. Incubate at 37 °C overnight.
9. For input titting, prepare 10^{-7} , 10^{-8} , and 10^{-9} NaCl dilutions of phage in SB medium.
Add 5 μ L of each dilution to 50 μ L of XL1-Blue cells ($OD_{600} = 1$). Incubate 15 min at RT and then plate on LB plates containing 100 μ g/mL of ampicillin. Incubate overnight at 37 °C.

3.8 Selection of Specific Anti-FcεRIα Clones

1. If the phage panning reveals an enrichment of phage titer after six rounds of panning, streak clones from 50 colonies on LB agar plates for replica-plating. After 6 h growing at 37 °C, put nitrocellulose filters, soaked in 5 mM IPTG on the plates and bacteria. Incubate overnight at 30 °C.
2. Freeze the filters for 15 min at -80 °C followed by 15 min incubation in chloroform area.
3. To digest bacteria, incubate each filter in 5 mL lysozyme buffer for 2×1 h.
4. Block each filter with 5 mL of blocking solution for 1 h at RT.
5. Incubate overnight each nitrocellulose filter on a tabletop shaker with FcεRIα (10 μ g/mL), 10 μ g/mL HSA or peroxidase-conjugated sheep anti-human Fab antibody (1/1000 in blocking solution). After washing with 3×10 mL PBS-Tween, incubate the filters previously incubated with FcεRIα and HSA, respectively, with peroxidase-conjugated sheep anti-human albumin (1/1000 in blocking solution) for 2 h at room temperature.
6. Develop the filters with 4-chloro-1- α -naphthol. Reclone the positive clones on LB plates containing 100 μ g/mL of ampicillin and isolate DNA from 100 mL cultures using Qiagen plasmid maxi kit according to the manufacturer's instructions.

3.9 ELISA with Single Phage Clones for Definition of Recognition Pattern

If the colony screening reveals positive clones, these should be produced as single phage clones and analyzed by ELISA for recognition pattern.

1. For the production of single Fab phage (Phab) clones, inoculate single colonies in 20 mL of SB medium and incubate overnight at 37 °C.
2. Dilute the cultures 1/100 into 100 mL of fresh SB medium and incubate at 37 °C until OD₆₀₀ = 0.4. Add 1 mL of VCSM13 helper phage in 20 fold excess and precipitate the phages as described above (*see* Subheading 3.6).
3. To determine the recognition pattern of the Fab phage clones, coat RIA/EIA plates with the following antigens in 0.05 M bicarbonate buffer as follows: 5 µg/mL of FcεRIα, 5 µg/mL of HSA, and 50 µg/mL of casein. Incubate overnight at 4 °C followed by incubation with blocking solution for 2 h at 37 °C.
4. After discarding blocking buffer, incubate Fab clones in three-fold dilutions (1/3–1/6561) for 4 h at 37 °C. As negative control, use phagemid phage supernatants containing no Fab fragments.
5. Wash plates with 5× PBS–Tween 20 and 5× PBS and then incubate plates with peroxidase-conjugated rabbit anti-phage antibodies for 2 h at 37 °C.
6. Shake out the detecting antibody solution. Wash the wells as before and add 50 µL of HRP substrate solution (TMB) and incubate for 5–20 min at RT.
7. Stop the reaction with 50 µL/well 1 M H₂SO₄. Determine the absorbance at 450 nm with an ELISA microplate reader.

3.10 Nucleotide Sequencing

If the ELISA revealed specific phage clones, they should be analyzed by DNA sequencing after phagemid preparation.

1. Prepare ten tubes containing 5 mL of SB medium supplemented with 50 µg/mL of carbenicillin.
2. Inoculate each tube with one colony from a master plate containing the bacteria corresponding to the positive clones. Shake at 37 °C at 250 rpm overnight.
3. Centrifuge at 3000 × *g* for 20 min at 4 °C. Discard the supernatant and resuspend the pellet in 250 µL of Qiagen buffer P1 from the QIAprep Spin Miniprep Kit. Continue with the phagemid preparation according to the protocol supplied by the QIAprep Spin Miniprep Kit. Elute DNA in 50 µL of elution and store at –20 °C.
4. For sequencing use sense and antisense primers (*see* Table 1). After sequencing, compare heavy and light chain sequences with the V Base Sequence Directory.

3.11 Generation of Full-Length IgG for Functional Studies and Affinity Measurements

For further characterization of affinities and epitope specificity, specific Fab clones are expressed as full-length IgG using an integrated vector system [kindly provided by Dr. A. Bradbury, Trieste, Italy, for details *see* ref. 6].

1. Amplify the VH regions using for amplification forward and reverse primers hybridizing to the 5' end the VH region: 5'-TCC ACA GGC GCG CAC TCC GAG GTG CAG CTG CTC GAG TCT GG-3' and the 3' end of the FR4: 5'-ACC ACG GTC ACC GTC TCC TCA GGT GAG TCC TGT CGA CGG ATC CA-3'.
2. For cloning of the VH regions the restriction sites of *Bss*HII are used at the 5' end and *Sa*I at the 3' end of the forward and reverse primers, respectively. For the light chains, the sequence of the Cκ constant region in the vector is replaced by the cDNA coding for the whole λ light chains using the following primers: Cλ reverse: 5'-AAA GTA CTA GTC TAC TAT GAA CAT TCT GTA GGG GCC AC-3', Vλ forward: 5'-TAT CCG TGC ACT CCA ATT TTA TGC TGA CTC AGC CCC ACT CTG TG-3'. For the cloning in the light chain, backbone plasmid the *Apa*LI and *Spe*I sites are used, which are located at the 5' end and 3' end of the Vλ forward and Cλ reverse primers, respectively.
3. After cloning, transfect both plasmids (heavy chain–light chain, 7:3, total 10 μg/2 × 10⁶ cells) into HEK-293T cells with Lipofectamine 2000 reagent.
4. Propagate HEK-293T cells in DMEM–F12 medium supplemented with 2% of FCS at 37 °C in 5% CO₂. For transformation, seed 2 × 10⁶ cells/well in 6-well TC plates and grow overnight at 37 °C. Prior to transformation, exchange the medium with 2.5 mL/well of serum-free medium.
5. Mix plasmid DNA (heavy chain–light chain, 7:3, total 10 μg/2 × 10⁶ cells) in 250 μL serum-free medium with 15 μL Lipofectamine 2000 reagent diluted in 250 μL serum-free medium and incubated at room temperature for 20 min. After incubation add the mix to the cells and incubate overnight at 37 °C in 5% CO₂. After overnight incubation replace medium with DMEM–F12 medium supplemented with 2% FCS and transfer the cells to TC-Flasks (Becton Dickinson) and culture for 5 days.
6. Purify the antibodies in the cell supernatant on protein G sepharose columns and check the purity on a 9% SDS–polyacrylamide gel.
7. Determine the concentration of the purified IgG by sandwich ELISA using two goat anti-human IgG Abs.
8. The purified full-length anti-FcεRIα IgG can be further tested for their anti-FcεRIα specificity in an ELISA as described in

Subheading 3.9. The purified human monoclonal autoantibodies can then be used for the in vitro characterization as well as for functional studies [6].

4 Notes

1. The solution should be Ca^{2+} and Mg^{2+} free.
2. Fd fragment: variable and first constant domain of the Ig heavy chain.
3. Add DMSO to 7% for storage at -80°C .

References

1. Hide M, Francis DM, Grattan CE, Hakimi J, Kochan JP, Greaves MW (1993) Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 328(22):1599–1604. doi:10.1056/NEJM199306033282204
2. Fiebiger E, Hammerschmid F, Stingl G, Maurer D (1998) Anti-FcεpsilonRIα autoantibodies in autoimmune-mediated disorders. Identification of a structure-function relationship. *J Clin Invest* 101(1):243–251. doi:10.1172/JCI511
3. Horn MP, Gerster T, Ochensberger B, Derer T, Kricek F, Jouvin MH et al (1999) Human anti-FcεpsilonRIα autoantibodies isolated from healthy donors cross-react with tetanus toxoid. *Eur J Immunol* 29(4):1139–1148
4. Lacroix-Desmazes S, Kaveri SV, Mouthon L, Ayoub A, Malanchere E, Coutinho A et al (1998) Self-reactive antibodies (natural autoantibodies) in healthy individuals. *J Immunol Methods* 216(1–2):117–137
5. Mouthon L, Lacroix-Desmazes S, Nobrega A, Barreau C, Coutinho A, Kazatchkine MD (1996) The self-reactive antibody repertoire of normal human serum IgM is acquired in early childhood and remains conserved throughout life. *Scand J Immunol* 44(3):243–251
6. Horn MP, Pachlopnik JM, Vogel M, Dahinden M, Wurm F, Stadler BM et al (2001) Conditional autoimmunity mediated by human natural anti-Fc(epsilon)RIα autoantibodies? *FASEB J* 15(12):2268–2274
7. Barbas CF 3rd, Kang AS, Lerner RA, Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci U S A* 88(18):7978–7982
8. Horn MP, Zuercher AW, Imboden MA, Rudolf MP, Lazar H, Wu H et al (2010) Pre-clinical in vitro and in vivo characterization of the fully human monoclonal IgM antibody KBPA101 specific for *Pseudomonas aeruginosa* serotype IATs-O11. *Antimicrob Agents Chemother.* doi:10.1128/AAC.01142–09. AAC.01142–09 [pii]
9. Vogel M, Lai L, Rudolf MP, Curcio-Vonlanthen V, Miescher S, Stadler BM (1996) Cross reactive anti-tetanus and anti-melittin Fab fragments by phage display after tetanus toxoid immunisation. *Hum Antibodies Hybriomas* 7(1):11–20
10. Miescher S, Zahn-Zabal M, De Jesus M, Moudry R, Fisch I, Vogel M et al (2000) CHO expression of a novel human recombinant IgG1 anti-RhD antibody isolated by phage display. *Br J Haematol* 111(1):157–166
11. Stadler BM, Rudolf MP, Zurcher AW, Miescher S, Vogel M (1996) Anti-IgE in allergic sensitization. *Immunol Cell Biol* 74(2):195–200. doi:10.1038/icb.1996.27
12. Vogel M, Miescher S, Biaggi C, Stadler BM (1994) Human anti-IgE antibodies by repertoire cloning. *Eur J Immunol* 24(5):1200–1207
13. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NB, Hamid M (2012) scFv antibody: principles and clinical application. *Clin Dev Immunol* 2012:980250. doi:10.1155/2012/980250
14. Lennard S (2002) Standard protocols for the construction of scFv libraries. *Methods Mol Biol* 178:59–71
15. Giles IP, Haley J, Nagl S, Latchman DS, Chen PP, Chuukwuocha RU et al (2003) Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. *Mol Immunol* 40(1):49–60
16. DeKosky BJ, Ippolito GC, Deschner RP, Lavinder JJ, Wine Y, Rawlings BM et al (2013) High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire. *Nat Biotechnol* 31(2):166–169. doi:10.1038/nbt.2492