

Video Article

Generation of Recombinant Human IgG Monoclonal Antibodies from Immortalized Sorted B Cells

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Abstract

Finding new methods for generating human monoclonal antibodies is an active research field that is important for both basic and applied sciences, including the development of immunotherapeutics. However, the techniques to identify and produce such antibodies tend to be arduous and sometimes the heavy and light chain pair of the antibodies are dissociated. Here, we describe a relatively simple, straightforward protocol to produce human recombinant monoclonal antibodies from human peripheral blood mononuclear cells using immortalization with Epstein-Barr Virus (EBV) and Toll-like receptor 9 activation. With an adequate staining, B cells producing antibodies can be isolated for subsequent immortalization and clonal expansion. The antibody transcripts produced by the immortalized B cell clones can be amplified by PCR, sequenced as corresponding heavy and light chain pairs and cloned into immunoglobulin expression vectors. The antibodies obtained with this technique can be powerful tools to study relevant human immune responses, including autoimmunity, and create the basis for new therapeutics.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52830/>

Introduction

The goal of this article is to describe in detail a methodology to generate and characterize human IgG monoclonal antibodies obtained from human peripheral blood mononuclear cells (PBMCs).

The interest to study human antibodies has grown in many different fields of research. In particular, many research groups are interested in the pathology caused by auto-antibodies¹⁻³. We have cloned and characterized pathogenic auto-antibodies¹. The study of auto-antibodies can help to identify their targets and to develop therapeutic strategies, e.g., using competitor antibodies⁴. Moreover, the study of human antibodies can also be of interest in other fields of research, i.e., to evaluate the immune response after vaccination⁵, to characterize the antibody profile of individuals that were exposed and became resistant to specific pathogens⁶ or to study which antibodies are in the natural repertoire^{7,12}.

Several techniques have been developed to generate recombinant human monoclonal antibodies⁸⁻¹²; most of these use phage display and B-cell immortalization. The use of phage display has been extensively applied for the discovery of new antibodies¹³. However it has a major disadvantage, namely that the heavy and light chain pairs of the human immunoglobulin become dissociated in the process. Production of hybridomas with human B cells or EBV transformation overcomes this drawback.

We use infection of thymic B cells with EBV in combination with polyclonal B cell stimulation via Toll-like receptor 9 (TLR-9)^{6,12}.

In this paper, we describe in detail the technology that we use for the development of IgG human antibodies, with a complete overview of all the steps from PBMC isolation to the *in vitro* antibody generation. This protocol can be used for the analysis of any type of human IgG profile. In our laboratory, B cells producing IgG antibodies have been successfully separated from the rest of PBMCs after sorting. Fifty sorted B cells⁸ can then be plated in multi-well plates and immortalized by EBV and TLR-9 activation, for the clonal expansion of single B cells. As feeder cells, fibroblasts from human embryonic lung tissue have been used, cell line wi38, which facilitates the visualization of the immortalized B cells. From these B cells, the sequences of the heavy and light chains of the immunoglobulin can be obtained by PCR, and the antibodies' genes cloned in immunoglobulin G expression vectors and produced *in vitro*. Using this technique, single antibodies with exactly the same antibody sequence found in the donor can be studied.

Protocol

Informed consent was obtained from the participants of the study. The study was approved by the institutional ethics committee.

1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

1. Centrifuge 25 ml of the participants' heparinized blood at 900 x g for 15 min, as soon as possible after the blood extraction. If there is less blood, scale down the reagents accordingly. Perform all the next steps in a hood.
2. Transfer the serum to a clean tube. It can be used in later steps for freezing PBMCs or in case of autoimmune patients, to test auto-antibodies.
3. Dilute the blood with 10 ml of RPMI 1640 media and resuspend the cells by pipetting up and down.
4. Add 15 ml of a solution containing polysucrose and sodium diatrizoate (1.077 g/ml) to a new 50 ml tube.
5. Gently layer the blood on top of the solution in step 1.4 by bringing the two openings of the tubes close together until the two liquids come very slowly in contact, and then decant the PBMC suspension slowly on top of the 50 ml tube. This step is critical to have a good separation of the PBMCs.
6. Centrifuge the tube obtained in step 1.5 at 400 x g without brake at RT for 20 min.
7. After the centrifugation, recover with the pipet the white ring that appears in the middle part of the tube, which contains the PBMCs.
8. Wash the cells with 25 ml of RPMI 1640 media.
9. Centrifuge 300 x g at RT for 10 min.
10. Discard the supernatant and wash the cells with 10 ml of RPMI 1640. Centrifuge again as in step 1.9.
11. Count the PBMCs with a Neubauer chamber as previously reported¹⁴.
12. Process the PBMCs as in section 2 or store them for later use as follows: 10 million PBMCs for freezing per Cryovial tube diluted in 1 ml of 10% dimethyl sulfoxide (DMSO) and 90% serum obtained in step 1.2. Freeze progressively and for long storage in liquid nitrogen.

2. Staining PBMCs for Sorting CD22+ and IgG+ by Cell Cytometry

1. Plate the PBMCs in a 25 cm² cell culture flask with 6 ml of complete RPMI 1640 medium (supplemented with L-glutamine, 10 mM HEPES buffer, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% of fetal bovine serum) and let them recover O/N in the incubator at 37 °C at 5% CO₂.
2. Block sterile FACS tubes with sterile 4% albumin phosphate buffered saline (PBS) solution O/N. Wash the tubes twice with PBS and fill them with 500 µl of complete RPMI 1640 medium. Use these tubes for recovering the cells after sorting.
3. Collect the PBMCs in a tube and centrifuge at 400 x g at RT for 5 min.
4. Resuspend the cells in labeling buffer (see steps 2.5 and 2.6) and count them¹⁴. The labeling buffer is sterile 2% fetal bovine serum and 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS.
5. Prepare 3 fluorescence-activated cell sorting (FACS) tubes with 10⁵ cells each and resuspend them in 100 µl of labeling buffer. These tubes will serve for defining the gates of the sorting.
 1. In the first tube add 5 µl of anti CD22 PerCP antibody (recommended in manufacturer's datasheet), in the second tube 20 µl of anti IgG PE (recommended in manufacturer's datasheet), and nothing in the third tube. Incubate on ice and in dark during 30 min.
6. Resuspend 5 million cells in 200 µl of labeling buffer in a FACS tube and add 10 µl of anti CD22 PerCP and 40 µl of anti IgG PE. Incubate the cells on ice and dark during 30 min. If the sorting of a higher number of cells is desired, scale up all the reagents.
7. Centrifuge cells at 400 x g with a low brake for 5 min at RT.
8. Decant softly the supernatant.
9. Resuspend the cells in 500 µl of labeling buffer. Centrifuge cells at 400 x g with a low brake for 5 min at RT. Repeat steps 2.7 and 2.8
10. Decant softly the supernatant and resuspend the cells in 300 µl of RPMI complete media.
11. Pass the cells through a 0.45 µm filter. Cells are ready to sort.

3. Sorting of the B Cells CD22+ and IgG+

1. Use 3 control tubes to establish the viability of the cells. In the control with no staining, use the forward and size scatter plot to define the lymphocyte gating. The controls with the CD22 PerCP and anti-IgG PE serve to establish the gates, and the sorting gate. Perform following previously reported data¹⁵.
Note: A gate is a set of value limits (boundaries) that serve to isolate a specific group of cytometric events, in our case cells, from a large set. In the case of a sorting gate the value limits allows the recovery of the cytometric events, cells, which are inside of the boundaries defined: CD22+ and IgG+.
2. As a collection tube in the sorting, use the blocked tubes with 500 µl of complete RPMI 1640 medium, obtained from step 2.2.
3. Proceed to sort double positive¹⁵: CD22+ IgG+. Note the final number of cells in each condition. Plate sorted cells on top of feeder cells as soon as possible.

4. Irradiation of Feeder Cells

Note: Perform the preparation of the feeder cells between 1 - 3 days before sorting. At least 5,000 wi38 cells are needed per well in a 96 round well plate. Perform steps 4.1, 4.2 and 4.4 in a hood.

1. Cultivate the wi38 cells at 37 °C and 5% CO₂ in complete RPMI 1640 medium (same as for PBMCs) until the desired number of cell are reached.

2. Irradiate them at 50 Grays, following a protocol described before¹⁶. Perform irradiation once the wi38 cells have been trypsinized and resuspended in complete RPMI medium, or with wi38 attached to the culture flask and trypsinized after the irradiation. Follow the method for trypsinization indicated by the supplier of wi38 cells. Irradiate the number of cells necessary to cover the wells needed for plating IgG+ B cells (1% of the total PBMC counted in step 1.11).
3. Plate 90 μ l containing 5,000 irradiated wi38 cells in each well of the 96 round well plate. Leave the wells in the outside row of the plate empty (because they evaporate faster), and only use the 60 wells in the middle of the plate for plating cells. Fill the outer wells that are framing the plate with 200 μ l of PBS.
4. Place them in an incubator at 37 °C at 5% CO₂, until needed.

5. Plating Sorted PBMCs, EBV Infection and Growing

1. Dilute the sorted PBMCs, to plate 50 cells in 50 μ l of RPMI 1640 complete medium per well in the 96 round well plate (previously plated with wi38 irradiated cells). Perform steps in a hood equipped for EBV work.
Note: the infection of 50 cells per well is optimized and increases the likelihood to produce a monoclonal antibody⁸, however, it is recommended to verify this by PCR as described before^{1,6}.
2. Add 60 μ l of EBV supernatant (containing 3 - 4 x 10⁸ viral copies/ml) to each well in the 96 round well plate¹⁷. CAUTION should be taken during EBV work.
3. Add 1 μ g/ml of CpG (ODN2006) per well.
4. Leave the cells in an incubator at 37 °C at 5% CO₂.
5. Visually monitor clone growing under the microscope after one week.
Note: Some examples of B cell growth can be seen below in the results section. Notice that growth rates of the clones may vary, and extra time might be necessary to start seeing some cell mass growing in the middle of the well.
6. After the 1st two weeks monitor clone growing under the light microscope and proceed to the first media exchange. Slowly pipet 90 μ l of medium from the top part of the well and collect in a clean 96-well plate. (B cells lie in the bottom of the well, so there is no risk to aspirate them).
 1. Add to each well 100 μ l of complete RPMI 1640 media supplemented with 1 μ g/ml of CpG (ODN2006) and 50 U/ml of IL2. If clones are growing, analyze this first media exchange by ELISA as in step 6.
7. Monitor clone growing and change media after the 3rd and the 4th week of growing again by taking 90 μ l of media and adding 100 μ l of complete RPMI 1640 media supplemented with 1 μ g/ml of CpG (ODN2006) and 50 U/ml of IL2. Use the collected supernatant to monitor IgG production by ELISA as in step 6.
Note: After one month clone growing should be evident by observing aggregates of round cells that usually lie in the middle of the round bottom well.
8. At this step, change media in the same way that the previous weeks but only with complete RPMI 1640 media. A good indicator to know the right moment to change media is the media color, if it is becoming yellowish cells need a media exchange.
9. When 96-well plates are containing big clones (approximately 5 x 10⁵ cells), transfer them to a flat well of a 24-well plate. Add 300 μ l of complete RPMI 1640 media to the new well. Resuspend the 96-well growing clone, by pipetting up and down several times, and transfer cells to the new well, distributing homogeneously. Add new media when needed.
 1. When wells of the 24-well plate are full of cells (approximately 5 x 10⁶ cells), transfer to a flat well of a 6-well plate. Add 2 ml of complete RPMI 1640 media to the new well. Resuspend the cells in the 24-well plate by pipetting up and down several times, and distribute them homogeneously in the new well.
10. Add media when needed. If cells are not to be maintained in culture, pellet the cells at 400 x g for 5 min at RT. Store the supernatants for ELISA testing. Wash the pellet of cells once with 1 x PBS, centrifuge again at 400 x g for 5 min, and stored dry at -80 °C until RNA extraction.
 1. When the cells in the 6-well plate become confluent (approximately 20 x 10⁶ cells), transfer to a 60 mm culture plate. Add 4 ml of complete RPMI 1640 media to the new plate. Resuspend the cells in 6-well plate and transfer them as done in steps 5.9 and 5.10.
11. Add new media when needed. At this step, freeze cells at 10 – 30 million/ml in 90% fetal calf serum and 10% DMSO. If larger amounts of cells are wanted, continue expansion of the clones in bigger surface plates.

6. ELISA for IgG Antibody Detection

1. Dispense 50 μ l/well in a ELISA plate of goat F(ab)₂ anti-human Fc antibody diluted 1:200 in coating buffer. Coating buffer contains 50 mM Na₂CO₃ pH 9.6.
2. Seal the plates with a plastic sticker, and incubate 1 hr at 37 °C. Alternatively, incubate at 4 °C O/N.
3. Wash each well 6 times with 200 μ l of washing buffer. Washing buffer contains 0.05% Tween-20 in 1 x PBS. Do not touch or scratch the well surface where the antibody has bound.
4. Block with blocking buffer, 100 μ l/well. Blocking buffer contains 4% of non-fat dry milk in PBS. Seal the plate and incubate 1 hr at 37 °C.
5. Do not wash. Discard blocking buffer and slap the plate 3 times upside down on a paper towel to remove residual liquid.
6. Incubate clones' supernatants and standards.
 1. For a first test, dilute clone supernatants obtained in step 5.6 or 5.7 1/3 in RPMI. For the standards dilute with RPMI medium human IgG solution to get: 1,000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.6 ng/ml and blank. Use 50 μ l/well and incubate at 37 °C for 60 min. Analyze further dilutions of the clone' supernatant to test the antibody affinity, such as 1/10 or 1/100.
7. Wash as done in step 6.3.
8. Use 50 μ l/well of goat F (ab)₂ anti-human IgG Fc peroxidase conjugated diluted 1:20,000 in incubation buffer. Incubation buffer contains 1% BSA and 0.02% Tween 20 in 1x PBS. Incubate at 37 °C for 60 min.
9. Wash as done in step 6.3.

10. Add 100 µl/well substrate solution containing 0.1% 3,3',5,5'-Tetramethylbenzidine (TMB). Incubate 10 min until a stable blue color forms in the wells. Be careful; do not wait too long!
11. Stop the reaction by adding 50 µl of 2 M H₂SO₄. Measure the absorption at 450 nm, within 30 min after stopping the reaction.
Note: All the clones' supernatants with 3 standard deviations over the blank will be considered positive for IgG human antibodies. For confirmation of the positive clones this ELISA should be repeated at least three times in different supernatants of the same clone. Also to discard the possibility of unspecific cross reactivity is recommended to assure that there is no signal when supernatants are incubated in uncoated but blocked wells. At this step, a detection assay to screen for the antigenic specificity of the antibodies of interest can be performed prior to proceed to section 7.

7. RNA Isolation and First Strand cDNA Synthesis of the Producing IgG Clones

1. As soon as the production of IgG is confirmed by ELISA, extract the RNA of the clone.
2. For extracting RNA from cells growing in steps 5.7 or 5.9, use the superscript III cells direct cDNA synthesis kit, which allows obtaining DNA from 10,000 cells to one cell.
3. For extracting RNA from larger amounts of cells, or from the pellet stored in step 5.11 use the high pure RNA isolation kit. Other systems of RNA extraction can also be suitable at this step. Follow the instructions of the manufacturer.
4. For cell numbers between 1×10^6 and 1×10^4 use the reverse transcription system, following the instruction of the manufacturer. Other systems for first strand cDNA synthesis can also be used.

8. 1st and 2nd PCR for Amplification of the Heavy and Light Chains of the IgG-producing B-cell Clones

1. With the cDNA of the cell clones, obtained in step 5.6 and 5.7 and positive in the IgG ELISA in step 6, perform PCR with the primers listed in **Table 1**.
2. Set up independent PCRs for each IgG heavy, kappa and lambda chain. Make a stock solution with forward and reverse primers in equal concentrations. Add them to the reaction at 0.4 µM final concentration.
3. Use 1 µl of the cDNA synthesis to perform the 1st PCR. Here, perform 20 µl reactions using Takara Taq manufacturer instructions. Alternatively, use other polymerases.
4. Place the 1st PCR under the following cycle conditions: 94 °C for 5 min; (50 cycles of: 94 °C for 30 sec; 58 °C for 30 sec, and 72 °C for 45 sec; 72 °C for 7 min; let it cool down at 4 °C. Include a blank in the reaction, to detect possible contaminations.
5. Prepare 1x TBE (89 mM Tris-borate and 2 mM EDTA). In a volume of 100 ml of 1x TBE add 1 g of agarose (1% agarose gel). Heat the solution in the microwave for approximately 1 min, until the agarose gets dissolved. Then add 4 µl of 10 mg/ml ethidium bromide (or equivalent DNA dye) and mix.
 1. Pour the content in a tray and wait until gets polymerized. Run 5 µl of the PCR mix in the gel to visualize the bands. Heavy chain fragments should be around 400 - 550 bp, while light chain should be around 300 - 400 bp. If the bands are not detected, proceed likewise to 2nd PCR.
6. Use 1 µl of the first PCR mix to perform the 2nd PCR. Use the same reagents that in step 8.2 but using the suitable mix of primers (see **Table 1**). For the 2nd PCR use the following conditions: 94 °C for 5 min; (50 cycles of: 94 °C for 30 sec; 56.5 °C for 30 sec and 72 °C for 55 sec); 72 °C for 7 min; let it cool down at 4 °C. Include a blank in the reaction, to detect possible PCR contaminations.
7. Prepare an agarose gel as described in 8.5. Run the gel to visualize the PCR product as in 8.5.1 Use the amplifications which contain the right size fragments for cloning in step 9 and produce the antibodies in step 10.
8. Sequence the DNA, by using 10 µl reaction containing: 100 ng of the 2nd PCR, 0.2 µM of the primer or primer mix, 1 µl of the terminator and 1 µl of the buffer. Perform the sequencing reaction under these conditions: (25 cycles of: 96 °C for 10 sec; 50 °C for 5 sec; and to 60 °C for 4 min); 60 °C for 7 min; let it cool down at 4 °C.
9. Purify the sequencing reactions with microspin G50 columns following manufacturer's instructions. Evaluate the sequences of the ligation in an automatic sequence analyzer as described before¹⁸. Electropherograms should be clean and correspond to a single immunoglobulin sequence.
Note: If the PCR products show unclear or mixed immunoglobulin sequences, please consider to clone them using the TopoTA system, to obtain isolated sequences and then proceed to step 9.

9. Cloning and Sequencing of the Heavy and Light Chains of the Producing IgG clones

1. Prepare 1 µg of DNA of vectors pFUSE2ss-CLlg-hk, pFUSE2ss-CLlg-hl2 and pFUSEss-CHlg-hG1.
2. Digest these vectors with the appropriate restriction enzymes. Use *EcoRI* and *BsWI* for pFUSE2ss-CLlg-hk, *EcoRI* and *AvrII* for pFUSE2ss-CLlg-hl2, and *EcoRI* and *NheI* for pFUSEss-CHlg-hG1. Use 3 units of each restriction enzyme for 1 µg of DNA of vector.
 1. Digest with one enzyme and purify the digested product with a PCR Purification Kit. Digest with the second enzyme and purify with a Gel Extraction Kit (according to manufacturer's protocol). Cut the fragment of interest from the agarose gel. Prepare agarose gel as in step 8.5.
3. Digest the 2nd PCR products of the producing IgG clone: *EcoRI* and *BsWI* for kappa chain amplification, *EcoRI* and *AvrII* for lambda chain amplification, and *EcoRI* and *NheI* for heavy chain amplification. Digest with one enzyme and purify the digested product with a PCR Purification Kit. Digest with the second enzyme and purify the digested product with a PCR Purification Kit. Use same units of enzymes and DNA amount as in step 9.2.
4. Ligate the PCR fragments inside of the vectors with T4 DNA ligase 16 °C O/N following manufacturer's recommendations. The ratio used should be 1 : 2 (vector : insert).
5. Use 1 µl of the ligation to transform DH5α bacteria. Follow DH5α manufacturer' conditions for transformation. Spread bacteria in blasticidin or zeocin plates, depending on the type of vector used. Incubate the plates O/N at 37 °C.

6. Grow single colonies, and extract DNA with a miniprep kit, following manufacturer' instructions. Use for single colony grow and DNA extraction the manufacturer's, recommendations from the DNA miniprep kit. Analyze them by digestion with the enzymes used for preparing vectors and inserts as in steps 9.2 and 9.3.
7. Sequence the DNA, by using 100 ng of the vector as done in steps 8.8 and 8.9.

10. Production of Antibodies in HEK cells

1. Grow HEK cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 1% L-glucose, 1% penicillin/streptavidin (DMEM+) to a confluency of 75% in a 150 mm cell culture plate. Perform in a hood steps 10.1 to 10.7.
2. Before transfection, exchange the medium to medium as before but without fetal calf serum.
3. For each heavy, light chain transfection, prepare a transfection mix with 2.5 ml of the DMEM (without serum), 9 µg of the vector with the heavy chain, 6 µg of the vector with the light chain, and 100 µl of polyethylenimine (PEI) solution (from a 1 µg/µl stock). Incubate at RT for 15 min.
4. Gently add 2.5 ml of the transfection mix prepared in step 10.3 to the HEK cells in the plate. Rock the plate to distribute homogeneously.
5. Incubate the cells in the incubator at 37 °C with 5% CO₂ for 24 hr.
6. Change the culture medium to medium without fetal calf serum.
7. Collect the medium from the plates 4 days later.

Note: The antibodies in the media can be used to characterize their reactivity *in vitro* and *in vivo*.

Representative Results

The sorting gating after staining CD22 and IgG positive cells is shown in **Figure 1**. In this image the area of the double positive cells – B cells producing IgG antibodies – is selected to sort all these cells in a separate tube. In the analysis, approximately 1% of the total PBMCs correspond to this double positive population. The number of sorted cells obtained will depend on the number of cells obtained in section 1.

The different outcomes after 5 weeks of EBV immortalization and CpG (ODN2006) stimuli are shown in **Figure 2**. The detection of the growing clones is easy; Wi38 feeder cells have a more elongated fibroblast shape, and the B cell clones appear as very small round cells growing clustered in the middle of the round bottom multi-well plate. At this stage, it is evident that some clones start growing. However, the growing speed can be variable and of course some of the wells containing immortalized cells may not have any cells growing at all.

The supernatant of the growing clones is tested in an ELISA for detecting IgG as shown in **Table 2**. In this ELISA a standard curve for IgG is tested, together with the supernatant of the clones and the blanks. A positive clone is considered when the value in the ELISA is 3 standard deviations over the blank value. Negative clones' values are under the 3 standard deviations of the blank. For confirmation, positive clones should be positive in the ELISA at least three times in different supernatants of the same clone and if possible verified by an additional screening method.

The complete sequence of a human IgG antibody obtained applying the steps described in this manuscript is shown in **Figure 3**. This sequence has been obtained after cloning the immunoglobulin heavy and lambda chain pair from a clonally expanded B cell. Variable and constant regions of the heavy and light chain can be characterized with this technique. After obtaining the sequences, antibodies sequences can be cloned and produced *in vitro* in HEK cell cultures.

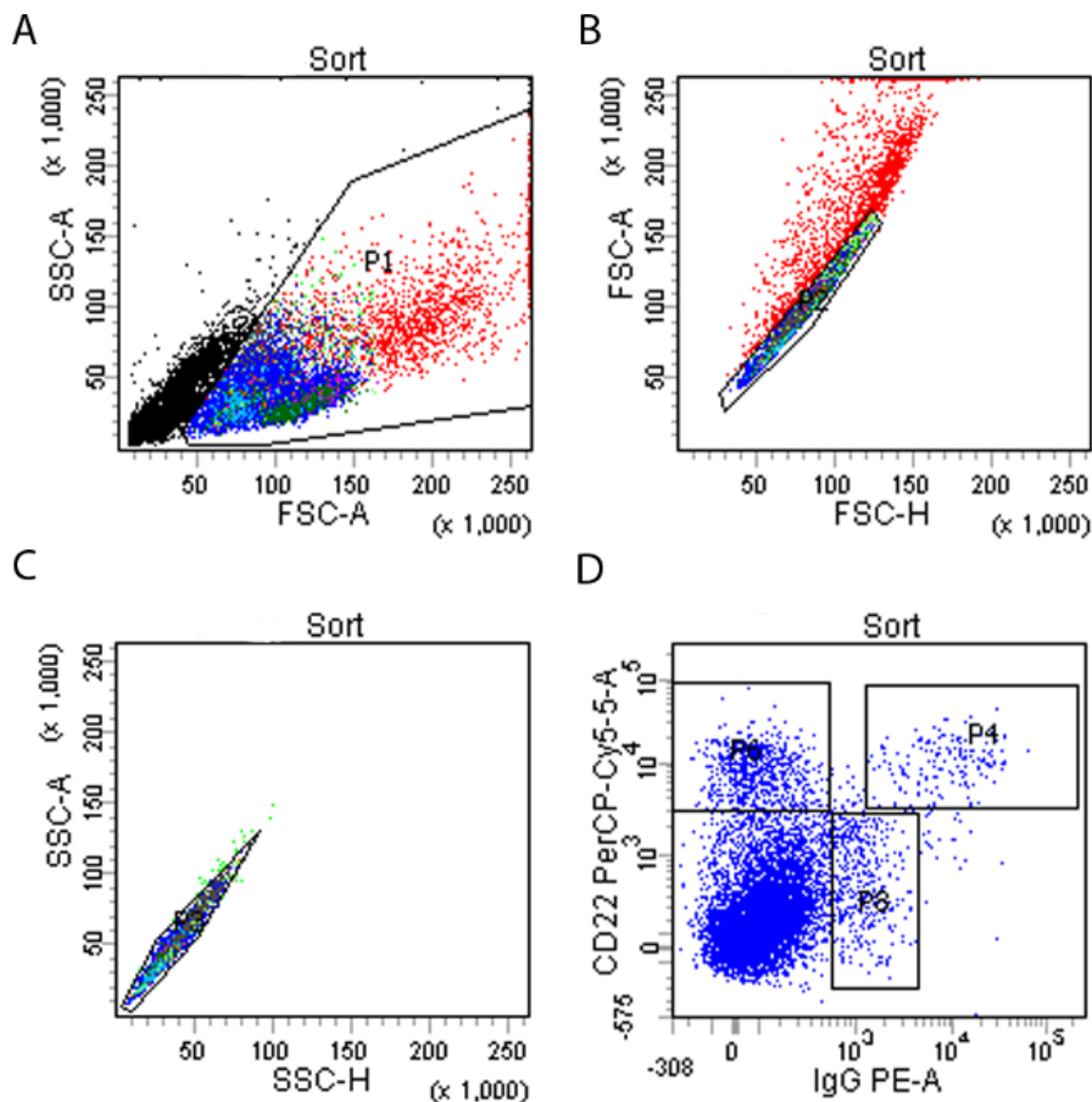


Figure 1. Flow cytometric analysis of CD22+ and IgG+ cells from human peripheral blood mononuclear cells. **(A)** Selection of the population of living cells is shown in P1. **(B)** Forward scatter plot. **(C)** Size scatter plot. **(D)** The Y axis shows cells separated by anti-CD22-PerCP and on the X axis separated by IgG-PE. P4 square indicates the cell fraction that has been sorted (CD22+, IgG+) and recovered for culture. [Please click here to view a larger version of this figure.](#)

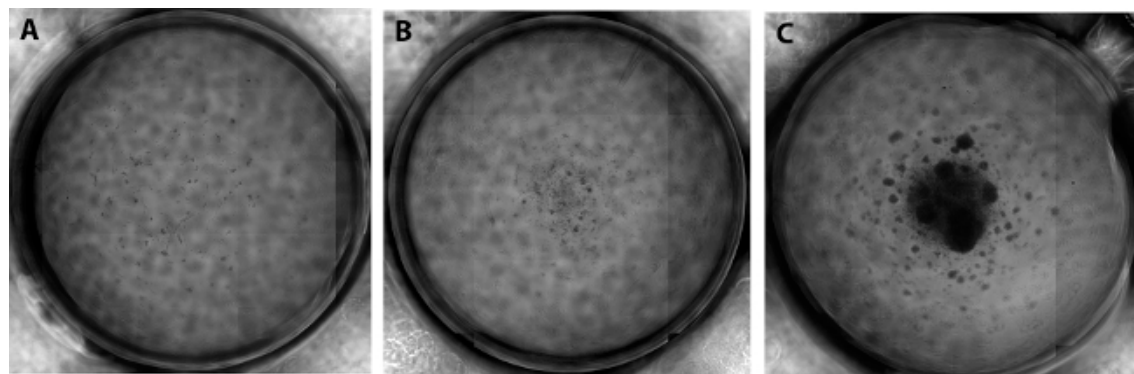


Figure 2. Representative image of B cell immortalized clones in a 96 well-plate after 5 weeks of culture. **(A)** In this well no immortalization was observed after 5 weeks, but wi38 irradiated feeder cells can be observed. **(B)** A slowly growing clone was observed in this well, with tiny round aggregates in the middle. **(C)** Fast growing clone showing round aggregates of immortalized B cells. [Please click here to view a larger version of this figure.](#)

F5.2 VH

CAG GTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCA CAG ACC CTG TCC CTC ACC TGC
Q V Q L Q E S G P G L V K P S Q T L S L T C

← VH-CDR1 →

ACT GTC TCT GGT GCC TCC ATG AGC AGT GGT GCT TAT TAC TGG AGC TGG ATC CGC CAG CAC CCA GGG
T V S G A S M S S G A Y Y W S W I R Q H P G

← VH-CDR2 →

AAG GGC CTG GAG TGG ATT GGG TAC ATC TAT TAC AGT GGG AGC ACC GAC TAC AAC CCG TCC CTC AAG
K G L E W I G Y I Y Y S G S T D Y N P S L K

AGT CTA CTT TTC ATA TCA AAA GAC ACG TCT AAG AAT CAG TTC TCC CTG AAG CTG AGC TCT GTG ACT GCC
S L L F I S K D T S K N Q F S L K L S S V T A

← VH-CDR3 →

GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA GTG ATG TCG TTT TAC TAT AAA TCA GGG AGT TCC CCC CGG
A D T A V Y Y C A R V M S F Y Y K S G S S P R

→

GCT TTT GAT ATC TGG GGC CAA GGG ACA ATG GTC ACC GTC TCT CAG CTA GCT AGC
A F D I W G Q G T M V T V S Q L A S

F5.2 VA

CAG TCT GTG GTG ACT CAG CCT CGC TCA GTG TCC GGG TCT CCT GGA CAG TCA GTC ACC ATC TCC TGC
Q S V V T Q P R S V S G S P G Q S V T I S C

← VA-CDR1 →

ACT GGA ACC AGC AGT GAT GTT GGT GGT TAT AAC TAT GTC TCC TGG TAC CAA CAA CAC CCA GGC AAA
T G T S S D V G G Y N Y V S W Y Q Q H P G K

← VA-CDR2 →

GCC CCCAAA CTC ATG ATT TAT GAT GTC ACT AAG CGG CCCTCA GGG GTC CCT GAT CGC TTC TCT GGC
A P K L M I Y D V T K R P S G V P D R F S G

TCC AAG TCT GGC AAC ACG GCC TCC CTG ACC ATC TCT GGA CTC CAG GCT GAG GAT GAG GCT GAT TAT
S K S G N T A S L T I S G L Q A E D E A D Y

← VA-CDR3 →

TAC TGC TGT TCA TAT GCA GGC AGC TGT TGG GTG TTC GGC GGA GGG ACC AAG GTG ACC GTC CTC CTA
Y C C S Y A G S C W V F G G G T K V T V L L

Figure 3. Human antibody sequences of heavy and light chain pair from a human immortalized B cell clone, F5.2, indicating V CDR1, CDR2 and CDR3 sequences. [Please click here to view a larger version of this figure.](#)

1 st PCR primers		
	Forward (5'-3')	Reverse (3'-5')
IgG	5' L-VH 1 ACAGGTGCCCACTCCAGGTGCAG	3' Cy CH1 GGAAGGTGTGCACGCCGCTGGTC
	5' L-VH 3 AAGGTGTCCAGTGTGARGTGCAG	
	5' L-VH 4/6 CCCAGATGGGTCTGTCCAGGTGCAG	
	5' L-VH 5 CAAGGAGTCTGTTCCGAGGTGCAG	

K	5' L Vκ 1/2 ATGAGGSTCCCYGCTCAGCTGCTGG	3' Cκ 543 GTTCTCGTAGTCTGCTTTGCTCA
	5' L Vκ 3 CTCTTCCTCCTGCTACTCTGGCTCCAG	3' Cκ 494 GTGCTGTCCTTGCTGTCCTGCT
	5' L Vκ 4 ATTTCTCTGTTGCTCTGGATCTCTG	
	5' Pan Vκ ATGACCCAGWCTCCABYCWCCCTG	
λ	5' L Vλ 1 GGTCTGCGGCCAGTCTGTGCTG	3' Cλ CACCAGTGTGGCCTTGTGGCTTG
	5' L Vλ 2 GGTCTGCGGCCAGTCTGCCCTG	
	5' L Vλ 3 GCTCTGTGACCTCCTATGAGCTG	
	5' L Vλ 4/5 GGTCTCTCTCSCAGCYTGTGCTG	
	5' L Vλ 6 GTTCTTGGGCCAATTTATGCTG	
	5' L Vλ 7 GGTCCAATTCYAGGCTGTGGTG	
	5' L Vλ 8 GAGTGGATTCTCAGACTGTGGTG	
2 nd PCR primers		
	Forward (5'-3')	Reverse (3'-5')
IgH	5' EcoRI VH1 CAACCGGAATTCGAGGTGCAGCTGG TGCAG	3' NheI JH 1,2,4,5 CTGCTAGCTAGCTGAGGAGACGGT GACCAG
	5' EcoRI VH1 to 5 CAACCGGAATTCAGAGGTGCAGCTG GTGCAG	3' NheI JH 3 CTGCTAGCTAGCTGAGGAGACGGTGA CCATTG
	5' EcoRI VH3 CAACCGGAATTCAGAGGTGCAGCTG GTGGAG	3' NheI JH 6 CTGCTAGCTAGCTGAGGAGACGGTG ACCGTG
	5' EcoRI VH3 23 CAACCGGAATTCAGAGGTGCAGCT GTTGGAG	
	5' EcoRI VH4 CAACCGGAATTCACAGGTGCAGCT GCAGGAG	
	5' EcoRI VH 4 34 CAACCGGAATTCACAGGTGCAGCTAC AGCAGTG	
	5' EcoRI VH 1 18 CTTCCGGAATTCACAGGTTACAGCT GGTGCAG	
	5' EcoRI VH 1 24 CTTCCGGAATTCACAGGTCCAGCT GGTACAG	
	5' EcoRI VH3 33 CTTCCGGAATTCACAGGTGCAGCT GGTGGAG	
	5' EcoRIVH 3 9 GATCCGGAATTCAGAAGTGCAGCT GGTGGAG	
	5' EcoRI VH4 39 GATCCGGAATTCACAGCTGCAGCT GCAGGAG	
	5' EcoRI VH 6 1 GATCCGGAATTCACAGGTACAGCT GCAGCAG	
K	5' EcoRI Vκ 1 5 CAACCGGAATTCAGACATCCAGATGA CCCAGTC	3' BsiWI Jκ 1 to 4 GCCACCGTACGTTTGATYTCCACCTTGGTC

	5' EcoR1 Vκ 1 9 CTTCCGGAATTCAGACATCCAGTTGAC CCAGTCT	3' BsiWI Jκ 2 GCCACCGTACGTTTGATCTCCAG CTTGGTC
	5' EcoR1 Vκ 1D 43 CTTGGCGAATTCAGCCATCCGGATGA CCCAGTC	3' BsiWI Jκ 3 GCCACCGTACGTTTGATATCCACT TTGGTC
	5' EcoR1 Vκ 2 24 CTTCCGGAATTCAGATATTGTGATGA CCCAGAC	
	5' EcoR1 Vκ 2 28 CTTCCGGAATTCAGATATTGTGATG ACTCAGTC	
	5' EcoR1 Vκ 2 30 CTTCCGGAATTCAGATGTTGTGATGA CTCAGTC	
	5' EcoR1 Vκ 3 11 CTTCCGGAATTCAGAAATTGTGTTG ACACAGTC	
	5' EcoR1 Vκ 3 15 CTTCCGGAATTCAGAAATAGTGATG ACGCAGTC	
	5' EcoR1 Vκ 3 20 CTTCCGGAATTCAGAAATTGTGTTGA CGCAGTCT	
	5' EcoR1 Vκ 4 1 CTTCCGGAATTCAGACATCGTGATG ACCCAGTC	
λ	5' EcoR1 Vλ 1 CTTCCGGAATTCACAGTCTGTGCT GACKCAG	3' AvrII Jλ 1 to 3 CTGGTTACCTAGGAGGACGGTSACCT TGGTCCC
	5' EcoR1 Vλ 2 CTTCCGGAATTCACAGTCTGCCC TGA CT CAG	3' AvrII Jλ 4 CTGGTTACCTAGGAAAATGATCAGC TGGGTTC
	5' EcoR1 Vλ 3 CTTCCGGAATTCATCCTATGAGC TGACWCAG	3' AvrII Jλ 5 CTGGTTACCTAGGAGGACGGTCAGC TCGGTCCC
	5' EcoR1 Vλ 4 to 5 CTTCCGGAATTCACAGCYTG TG CTGACTCA	3' AvrII Jλ 6 CTGGTTACCTAGGAGGACGGTCAGCT GGGTGCC
	5' EcoR1 Vλ 6 CTTCCGGAATTCAAATTTTATGC TGA CT CAG	3' AvrII Jλ 7 CTGGTTACCTAGGAGGACGGTCAC TTGGTCCAT
	5' EcoR1 Vλ 7 to 8 CTTCCGGAATTCACAGRCTGTG GTGACYCAG	

Table 1. Primers used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.076	0.077	2.003	0.080	0.138	0.102	0.188	0.338	0.040	2.041	0.051	0.081
B	2.011	0.085	0.074	0.069	0.081	0.122	0.372	2.133	0.119	0.097	0.072	0.072
C	0.068	0.179	0.091	0.073	0.077	0.097	0.606	1.882	0.081	2.071	0.094	0.075
D	0.063	0.070	0.065	0.071	0.082	2.071	0.339	2.089	0.076	0.086	0.066	0.069
E	1.921	0.077	0.065	0.085	0.095	1.968	1.910	0.122	0.072	0.070	0.065	0.066
F	0.113	0.068	0.066	0.082	0.088	0.090	0.460	0.070	0.079	0.952	0.098	0.065
G	2.041	2.108	1.472	0.665	0.331	0.194	0.123	0.094	0.080	0.072	0.070	0.072
H	2.146	2.132	1.634	0.665	0.341	0.178	0.132	0.094	0.082	0.080	0.074	0.068

Standards ng/μl	1000	500	250	125	62.5	31.25	15.6	7.8	3.9	1.95	0.975	0
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Table 2. Representative results of IgG screening by ELISA. Clone supernatants are in A1-A10, B1-B10, C1-C10, D1-D10, E1-E10, F1-F10. Blanks are in A11, B11, C11, D11, E11, F11, A12, B12, C12, D12, E12, F12. Standard curve is duplicated: G1-G12 and H1-H12. The corresponding concentration of immunoglobulins in each duplicate is shown below. Positive or IgG producing clones are shown in dark grey. Negative or non-IgG producing clones are shown in light grey

Discussion

In this manuscript, all the steps for the generation of IgG antibodies from human PBMCs are presented in detail. This protocol includes some advantages over previously published techniques. One of the advantages is that the antibody produced keeps the heavy and light chains corresponding to the original pair in the B cell clone. The identification of IgG antibodies can be done in any type of human donor, and there is no need for exacerbation of the immune response due to vaccination⁵. The use of the fibroblast cell line wi38 as a feeder cell, allows a more rapid detection of the growing clones, since they are morphologically different and very easy to differentiate, compared to the PBMCs used as feeder cell in previously described works^{1,6-8}. Moreover the use of wi38 as a feeder cell favors the freezing of big amounts of cell aliquots that can be easily thawed and cultured before every experiment.

One of the critical steps in this protocol is the starting material: the PBMCs. If the blood has been waiting too long for centrifugation, or after the extraction of the PBMCs, they are not stored in the appropriate freezing conditions; as such the number of viable cells will be reduced, along with the number of the B cell IgG producing clones obtained. For that reason, an early extraction and a good preservation of the PBMCs are recommended for a successful outcome. The number of IgG producing clones will be limited to the number of PBMCs at the beginning of the experiment. Higher numbers of PBMCs will give higher numbers of IgG producing clones and a diverse antibody production. Another critical step is the cloning of the PCR fragments of the light and heavy chains of the antibody. If the ligation is not successful after several attempts, an extra step of cloning the 2nd PCR product in a TopoTA system is recommended. The digestion of the insert with the appropriate enzymes can be easier in the TopoTA vector, for a subsequent ligation in the pFUSEss expression vectors.

This technique can be transferred to any type of human tissue in which human B cells are enriched³. It can also be applied to the study of other types of immunoglobulins, just by changing the labelled antibodies used for sorting (antibodies against IgM, IgE, IgA or IgD), the primer design for the PCR, and the expressing vectors. The studies of these immunoglobulins can be of interest to understand, initial immune responses, mucosa antibody secretion and allergy profiles, among others.

In conclusion, we have described a technique to produce human recombinant monoclonal IgG antibodies that leaves the heavy and light chains pairs of the human immunoglobulin intact. The technique is useful and easy to perform starting from a blood sample. The monoclonal antibodies obtained through this method are potentially useful in studies on human immune responses. Furthermore, monoclonal antibodies produced with this method might be a good starting point for the development of immuno-therapeutics for different pathological conditions.

Disclosures

The authors declare that they have no competing financial interests.

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