

Production of Human Monoclonal Antibodies by the Epstein–Barr Virus Method

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Abstract

Epstein–Barr virus (EBV) is a herpes virus which in vitro efficiently immortalizes nearly all human B lymphocytes. The lymphoblastoid diploid cell lines (LCL's) thus generated preserve the characteristics of the cells initially infected by the virus: the cells produce and secrete immunoglobulins and also express these molecules on their surface. A selection of specific antibody-producing cells (i.e., antigen-committed cells) before EBV-infection or when LCL's have already been established, enables isolation of monoclonal cell lines that secrete specific antibodies. If selection of antigen-committed cells is not feasible, secretion of specific antibodies by cloned LCL's in limiting dilution cultures enables isolation of the desired cell lines. The method allows the production of human IgM, IgG, IgA, and IgE monoclonal antibodies from any individual. Monoclonal antibodies produced by the EBV method resemble the antibody repertoire of the donor of the lymphocytes. Human monoclonal antibodies are promising reagents for passive immunization.

Key words Epstein–Barr virus, Immortalization, Diploid, Lymphoblastoid cell line, Passive immunization, Antibody repertoire/

1 Introduction

In the early 1970s, when monoclonal antibodies were still an unknown entity, I started my post doctoral studies in George Klein's laboratory in Stockholm, Sweden, a worldwide known Epstein–Barr virus (EBV) research center. The exciting finding that EBV efficiently immortalizes human B-cells [1] led George Klein to suggest that the virus might be used to establish cell lines which in vitro produce human-specific antibodies. In a series of studies we generated for the first time human monoclonal antibodies, with prospects for future clinical applications [2].

EBV, a member of the herpes virus family is one of the most common viruses found in humans [3]. Most of the Western population is infected with the virus, each individual maintaining a

life-long active immune response against the virus and against the cells infected by it.

In vivo, EBV infects B cells expressing on their surface the CD21 EBV receptor [4]. Under some conditions even other cells, devoid of the receptor, are infected by the virus. The initial lytic virus infection of B cells is normally followed by an EBV latency that persists within CD27 positive memory B lymphocytes for life [5].

In vitro, resting human B lymphocytes infected with EBV show typical viral latency, reflected by the expression of a limited number of viral proteins and two types of non-translated RNA molecules. The virus-induced-molecules transform the B cells into immortalized diploid cells that usually do not release virus particles. EBV efficiently infects the majority of human B lymphocytes, including IgM, IgG, IgA, and IgE cells [6–10]. The immortalized cells, which grow rapidly as a suspension in standard cell culture media supplemented with fetal calf serum or in serum-free medium, usually reach a density of about 10^6 cells/ml. They maintain the characteristics of the initially infected B lymphocytes, including expression and secretion of immunoglobulins. The cells can be cloned with the support of a feeder layer. Depending on each specific cell line, the tissue culture medium derived from cells at saturation contains 0.5–10 μ g immunoglobulin/ml. Because of their human origin, these cells cannot be grown as peritoneal ascites in normal mice.

EBV can be used to make specific antibody-producing cell lines, provided that the virus-targeted lymphocytes are derived from a donor who has been exposed to the corresponding antigen. This limitation might be overcome if antigen-specific sensitization in vitro systems were developed (*see* Chapter 15 in this volume by Tomimatsu and Shirahata). Somatic mutations and isotype switching take place during in vitro growth of the LCL's [11–13]. The enzymatic activity of activation-induced cytidine deaminase (AID) plays a major role in inducing these changes in the immunoglobulin genes [14]. In contrast to the corresponding process in vivo, the ongoing somatic mutations during in vitro growth do not lead to improved affinity of the antibody but may lower antibody activity.

Because of the very low frequency of cells that produce any specific antibody even in an immunized individual, there is an obvious need to apply a highly sensitive selection method to enrich for the specific cells. Selection takes advantage of the cell-surface expression of the specific antibody on B cells. Alternatively, individual cells (or clones) can be selected simply by assessing their capacity to secrete the specific antibody.

As a rule, peripheral blood lymphocytes are used as a source for establishing antibody-producing cell lines, but even spleen and lymph node-derived cells can serve this purpose.

Numerous laboratories have successfully applied the EBV method to generate IgM, IgG, and IgA-specific antibodies against proteins, carbohydrates and peptides. The monoclonal antibodies

produced by the EBV method resemble antibodies that have emerged in vivo against a specific pathogen. These antibodies may be relevant to future clinical applications such as anti-pathogen passive immunization. Moreover, monoclonal antibodies that are produced by the EBV method from patients with immune disorders and which resemble pathogenic antibodies are valuable reagents for the study of these abnormalities.

The monoclonal antibodies secreted by EBV-immortalized cell lines are purified from the cell culture medium. Alternatively, the cDNA of the immunoglobulin genes can be readily isolated from the antibody-secreting LCL's and used for transfection of cells and bacteria in order to produce large amounts of the antibody.

2 Materials

2.1 Isolation of Lymphocytes

1. A freshly drawn blood sample (10 ml or more) supplemented with any type of anticoagulant or a buffy coat derived from a blood center, is a suitable source of lymphocytes. The blood samples and buffy coats are diluted with phosphate-buffered-saline (PBS).
2. PBS: 0.14 M NaCl (8.0 g), 2.7 mM KCl (0.2 g), 8.0 mM Na₂HPO₄ (1.15 g), 1.4 mM KH₂PO₄ (0.2 g), in double distilled water (1 l), adjusted to pH 7.5. An autoclaved stock solution of 10× concentrated PBS can be prepared in advance.
3. Sterile Ficoll Isopaque (1.077 g/ml) is commercially available.
4. A concentrated (×100) stock solution of 100 µg/ml sterile cyclosporin A (Sandoz, Holzkirchen, Germany) in PBS can be prepared in advance, divided into small aliquots and frozen at -20 °C until use.

2.2 Infection of Cells with EBV and Outgrowth of Lymphoblastoid Cell Lines

1. The EBV reagent used for immortalization is the cell supernatant harvested from the B95-8 marmoset cell line [15] (*see Note 1*).
2. CpG ODN 2006 (5-tcgtcgttttgcgttttgcgtt-3), a commercially available 24-mer oligodeoxy nucleotide, is used to activate in vitro the isolated blood lymphocytes. The bases are phosphorothioated to render the compound nuclease-resistant. CpG ODN 2006 is dissolved in RPMI-1640, 10 % FCS at a concentration of 25 µg/ml, sterile-filtered and, kept at -20 °C. It is added to cell cultures at a final concentration of 2.5 µg/ml.
3. RPMI-1640 supplemented with penicillin and streptomycin 10 % FCS is used to grow EBV-immortalized lymphoblastoid cell lines. For some purposes this medium is supplemented with 20 % FCS.

2.3 Selection of Lymphocytes

2.3.1 Selection Based on Cell Surface Expression of Immunoglobulins

Selection of antigen-committed B cells is carried out with antigen-coated magnetic beads (*see Note 2*). The kind of magnetic device required for the isolation of the bead-attached cells depends on the type of magnetic beads used (*see Note 3*).

1. Selection of isotype-specific B lymphocytes.

Commercially available magnetic beads coated with anti-immunoglobulin heavy chain-specific antibodies are used to remove or enrich blood derived specific Ig surface-positive lymphocytes. Both microscopic and submicroscopic magnetic beads are suitable for selection.

2. Selection of antibody-specific B lymphocytes.

Activated magnetic beads (i.e., tosyl activated beads, Dynal, Invitrogen, USA) are coated under sterile conditions with the molecule in question, e.g., proteins, peptides, carbohydrates, or nucleic acids. If the peptides are very short they have to be linked to a large polymer before being coated onto the magnetic beads. It is recommended to prepare the specific peptides with an additional cysteine to enable conjugation to a carrier protein (such as albumin). A variety of chemical reagents for this kind of conjugation are commercially available (Pierce, USA).

2.3.2 Selection Based on Secretion of a Specific Antibody

Flat bottom 96-well microplates are used for oligoclonal growing cells.

2.4 Cloning of Cells

1. Flat bottom 96-well microplates are used to clone cells.
2. 2000 R irradiated blood-derived lymphocytes from any blood donor are used as feeder layer.

2.5 ELISA

Standard ELISA microplates are used for the detection of specific antibodies.

1. Saturation buffers (any one of the following solutions can be used): 50 % FCS in PBS.
3 % bovine serum albumin (BSA) in PBS.
Low-fat milk.
0.5 % gelatin in PBS.
2. Wash buffer.
1/10 diluted PBS with 0.05 % Tween 20.
3. Enzyme-conjugated anti-human immunoglobulin antibodies. These reagents should be diluted according to the manufacturer's instructions.
4. The ELISA substrate should be chosen in accordance with the type of conjugated enzyme reagent used in the test.

3 Methods

There are several alternative strategies for establishing EBV-immortalized lymphoblastoid cell lines that secrete human monoclonal antibodies. Figure 1 is a schematic of these options.

3.1 Isolation of Lymphocytes (See Note 4)

1. Carry out the entire procedure under sterile conditions.
2. Dilute the blood sample with one volume of PBS. If a buffy coat is used, dilute it with 100 ml PBS.
3. Separate the lymphocytes on a discontinuous Ficoll Isopaque gradient. Layer carefully two volumes of cell suspension onto one volume of Ficoll Isopaque in a 15 or 50 ml test tube, avoiding mixing of the two layers.

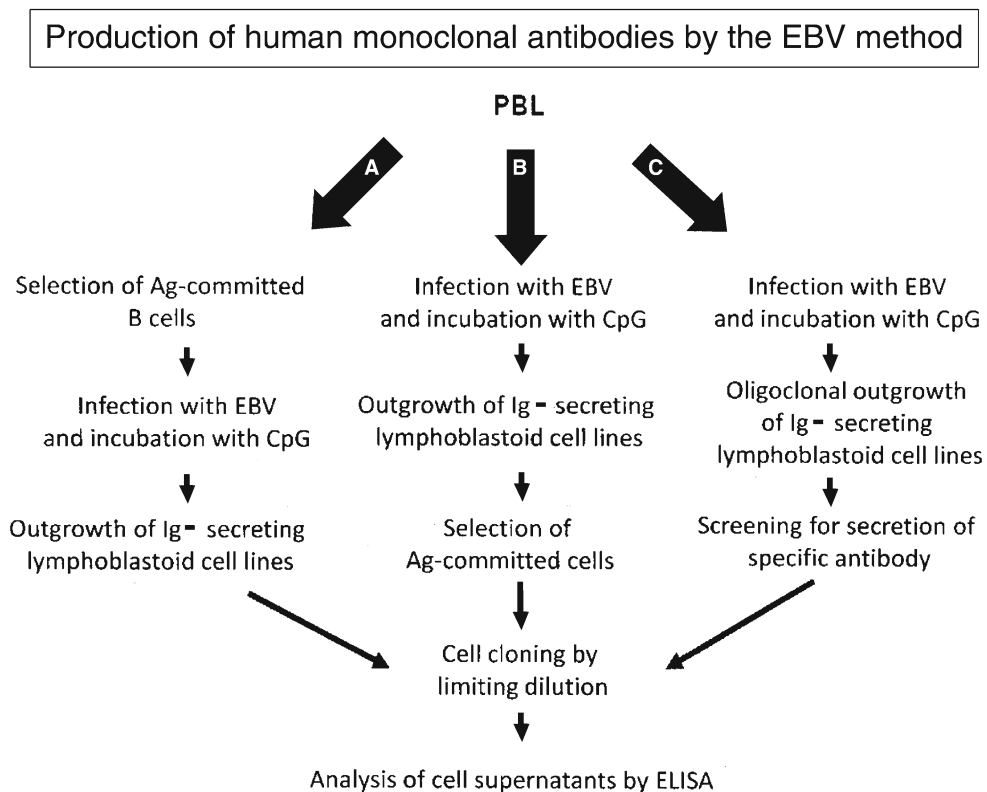


Fig. 1 Schematic showing the strategies involved in producing human monoclonal antibodies from peripheral blood lymphocytes, applying the EBV method. **(A)** Antigen-committed B lymphocytes are selected before EBV infection. **(B)** B lymphocytes, which first are infected with EBV, are allowed to proliferate in vitro and then, upon reaching around 10^7 cells, antigen-committed cells are selected. **(C)** Lymphocytes, which first are infected with EBV, are allowed to grow as oligoclonal in micro-wells and their supernatants are tested for the presence of specific antibodies. In all three schemes the cells are cloned by limiting dilution and finally the cell supernatants are tested, usually by ELISA, for the presence of the specific antibody

4. Centrifuge the test tubes for 20 min at 2,200 rpm (about $800\times g$), with no brake. The erythrocytes form a pellet at the bottom of the test tube; the lymphocytes (and platelets too) are located in the interphase above the Ficoll Isopaque layer.
5. Use a pipette to discard about 4/5 of the upper layer. Then collect into a test tube the interphase, which contains mainly lymphocytes and platelets. The interphase may be contaminated with some erythrocytes (often due to the fact that the blood sample is not fresh), which do not interfere with the process. Obviously, the recovered cell fraction will also contain some Ficoll Isopaque. Dilute the cell fraction with one volume of PBS, 1 % FCS and centrifuge for 10 min, 2,200 rpm (about $800\times g$) (*see Note 5*).

Discard the supernatant (*see Note 6*).

6. Suspend the cells in PBS, 1 % FCS and centrifuge the test tube at 1,100 rpm (about $300\times g$), 5 min. If the supernatant is turbid (reflecting the presence of platelets), washes should be repeated as above (i.e., centrifugation at 1,100 rpm, 5 min) until the supernatant is clear (*see Note 7*).

Suspend the cells in RPMI-1640, 10 % FCS and count. Usually, 10^6 lymphocytes are recovered from 1 ml blood and about 6×10^8 cells are recovered from the entire buffy coat.

3.2 Infection with EBV

1. Spin down the lymphocytes, gently resuspend, and add B95-8 virus-containing reagent (1 ml per 10^7 lymphocytes). Incubate the cells for 1 h at 37 °C. Then spin down the cells at 1,000 rpm for 5 min and discard the supernatant. Resuspend the cells at 10^6 cells/ml in RPMI-1640, 20 % FCS, 1 $\mu\text{g}/\text{ml}$ cyclosporin A, 2.5 $\mu\text{g}/\text{ml}$ CpG ODN (*see Notes 8 and 9*).

The direct result of the viral infection is that the B cells start to proliferate, with an average doubling time of 24 h. In bulk cultures of EBV-infected non-selected blood-lymphocytes (*see Note 10*) the T cells die out within 1–2 weeks. Cyclosporin A, added to the medium at 1 $\mu\text{g}/\text{ml}$, prevents the cytotoxic activity of the T cells against the EBV-immortalized cells. The lymphoblastoid cells, which grow as single cell suspension, forming typical clumps, usually reach a concentration of about 1.0×10^6 cells/ml.

3.3 Selection of Lymphocytes and Establishment of Antibody-Secreting Cell Lines

Cells should be selected before EBV infection (Fig. 1, option A) or when they have already been immortalized by the virus (Fig. 1, option B). When the aim is to establish cell lines that secrete IgG antibodies, it is recommended first to enrich for lymphocytes expressing IgG or to deplete IgM-positive cells before infection with EBV.

Selection of lymphocytes (and of cells derived from EBV-immortalized lines) takes advantage of the fact that the target cells of EBV (and also the lymphoblastoid cells) are

immunoglobulin surface-positive. Usually selection is performed with the aid of ligand-coated magnetic beads and an appropriate magnetic device (*see Note 3*).

1. On the day the lymphocytes are isolated and infected with the virus, resuspend them at a concentration of up to 50×10^6 cells/ml in RPMI-1640, 10 % FCS. It is important to have a single cell suspension and to avoid clumps. The mixture of cells and magnetic beads is now placed in a 1–2 or 5 ml capped test tube, depending on the total number of cells and the type of magnetic device used for selection. The entire procedure should be carried out at 4 °C. Centrifuge the suspension 15 min, 800 rpm (about $250 \times g$) and then keep on ice for 1 h.
2. Gently suspend the pellet, using a pipette with a relatively wide opening. The magnetic device is then used to isolate the beads with their attached cells. Keep the test tube adjoined to the magnet for 2 min, then open the tube and, while keeping it attached to the magnet, decant the fluid, leaving behind the beads and the beads specifically-bound B cells, still attached to the wall of the test tube. Close the tube, remove the magnet and add fresh cold medium as before. Gently resuspend the pellet and repeat the selection procedure five times.
3. Thoroughly resuspend the pellet and add 2×10^7 irradiated feeder cells in 20 ml RPMI-1640, 20 % FCS, 2,5 µg/ml CpG ODN. Seed 200 µl/well into a flat bottom 96-well microplate. Allow the cells to grow for about 4 weeks. There is no need to feed the cells during this period. The magnetic beads do not interfere with the growth of the cells.
4. Collect 100 µl supernatant samples from wells in which there is substantial cell growth and check for the presence of specific antibodies by ELISA or any other rapid assay. Count the cells in wells in which antibodies were detected and clone in flat bottom microplates at 0.5 cells/well with $1-2 \times 10^5$ irradiated feeder cells/well in RPMI-1640, 20 % FCS.
5. Transfer the antibody-producing clones to large tissue culture flasks and freeze in liquid nitrogen, $2-10^6$ cells per ampoule.

3.4 Establishment of Cell Lines that Secrete a Specific Antibody Without Selection of Antigen-Committed Cells

Sometimes, the number of available PBL is small and, in addition, selection of antigen-committed cells is not feasible: the antigen may not be available as a pure reagent or it is not possible to coat the beads with the antigen used for selection. In such cases, a different strategy is called for (*see Fig. 1*, option C). A small number of virus-immortalized antigen-non-selected cells are allowed to grow as oligoclones in flat-bottom microwells together with irradiated allogeneic lymphocytes as feeder layer. When cell saturation is reached, the supernatants should be tested (usually by ELISA) for the presence of a specific antibody.

1. Dilute EBV-infected cells to a final concentration of 0.5×10^3 to 0.5×10^4 cells/ml in RPMI-1640, 20 % FCS, 1 $\mu\text{g}/\text{ml}$ cyclosporin A, 2.5 $\mu\text{g}/\text{ml}$ CpG ODN, and mix with 10^6 irradiated lymphocytes/ml. Then seed 200 μl in wells of 96-well flat-bottom microplates. Thus, each well will contain only 100–1,000 virus-infected B cells (*see Note 11*).

Incubate the microplates at 37 °C for about 4 weeks without refeeding (*see Notes 12 and 13*).

2. When the cells reach a concentration of about 2×10^5 cells/well, use a multichannel micropipette to collect and transfer a 100 μl supernatant sample from each well into a “master plate.” Then test the supernatants for the presence of specific antibodies (usually by a rapid ELISA).
3. Clone cells from wells that were positive in the ELISA.

3.5 Cloning of EBV-Immortalized Cells

EBV-immortalized cells cannot develop from a single cell unless supported by a feeder cell layer. Moreover, technically it is very difficult to get the cells to grow as monoclonal colonies in soft agar. The cells are therefore cloned by limiting dilution in microplate wells in the presence of 2000 R irradiated allogeneic blood-derived lymphocytes. These cells can be prepared in advance and kept frozen until used as feeder.

Mix one volume of irradiated lymphocytes derived from any blood donor, suspended in medium ($2\text{--}4 \times 10^6$ cells/ml) with one volume of the cell suspension (10 cells/ml) to be cloned. The cell mixture (200 μl) is now seeded in flat-bottom wells in a 96-well microplate, using a multichannel micropipette. Theoretically, under these conditions each well should contain one cell that is capable of proliferating. In practice, we suggest that cloning be performed in 5–10 microplates at a time, 2–3 of them seeded with 1–0.3 cells/well (*see Note 14*).

Wrap the microplates with aluminum foil and keep them in the incubator for 4 weeks without refeeding (*see Note 12*). Eventually the outgrowth of the LCL is clearly observed under an inverted microscope (*see Note 15*).

Transfer the cells collected from the wells to round bottom tissue culture test tubes containing 0.5 ml RPMI-1640, 20 % FCS. One week later transfer the cells to a tissue culture flask with 5 ml medium, as above (*see Note 16*).

3.6 ELISA

The supernatants derived from hundreds of microwells must be rapidly checked for the presence of specific antibodies. A standard ELISA is often the test of choice. Because of the small volume of available supernatant, ELISA should be performed using a 45 μl sample volume. For each supernatant a specific ELISA and a control ELISA are performed simultaneously. The control ELISA is performed identically with the exception that the wells are not coated with the specific antigen.

The ELISA wells should be coated with a relatively pure antigen solution (45 µl/well) in PBS buffer. Saturate the wells with 250 µl of a non-reactive protein solution (*see Note 17*). Then take two samples from every cell supernatant, each consisting of 45 µl, add one to an antigen-coated well, the other to its control well and leave for 1 h at RT (room temperature). Wash the plate as above and add enzyme-conjugated antibody against human immunoglobulins and leave for 1 h at RT. A color reaction develops upon the addition of a specific substrate (*see Note 18*).

4 Notes

1. The cells of this line grow partly as a suspension and are partly surface-attached in RPMI-1640, 10 % fetal calf serum (FCS). To prepare a culture supernatant with a high virus titer, the B95-8 cells are allowed to grow for 4–5 weeks in tissue culture flasks without refeeding. Virus-containing supernatants can be stored at 4 °C or frozen at –20 °C until use. The B95-8 cells are kept frozen in liquid nitrogen, with 10 % dimethyl sulfoxide (DMSO), 90 % FCS. In our experience not all the cells survive the freezing and thawing. However, the cells have an extremely high cloning efficiency and when cultured after being thawed, the cell line is rescued even if very few cells are alive.
2. Blood-derived B lymphocytes express IgM, IgG, IgA, and IgE isotypes, but it should be emphasized that the majority of the cells are IgM-positive. Therefore, if the aim is to establish cell lines that secrete IgG antibodies, it is recommended first to enrich for lymphocytes which express IgG or, alternatively, to deplete IgM-positive cells before infection with EBV.
As the target cells of EBV are immunoglobulin surface-positive B cells, their selection is straightforward. Usually selection is achieved with the aid of ligand-coated magnetic beads.
3. In the past when magnetic beads were not available, specific ligand-coated erythrocytes were used for this purpose [16]. At 4 °C the ligand-coated erythrocytes form rosettes with B cells which express on their surface the corresponding antibody. The rosettes are then easily and very efficiently separated from non-rosetting cells on a discontinuous Ficoll Isopaque gradient, similarly to the separation of blood lymphocytes described elsewhere (*see Subheading 3.1*).
4. The end product of this procedure should be a single cell suspension of lymphocytes with no cell clumps or platelets. Platelets tend to aggregate and also to cause aggregation of lymphocytes. Therefore it is essential to repeat the washes described in Subheading 3.1 until no more platelets remain.

The presence of clumped cells will dramatically worsen selection, as performed in the following steps.

5. In the first wash, the cells are centrifuged at high speed because of the presence of some Ficoll Isopaque in the sample. Under these conditions both cells and platelets are pelleted. However, in the subsequent washes, the test tubes are spun at 1,100 rpm, 5 min, so that the lymphocytes are pelleted, whereas the platelets remain in the supernatant.
6. After discarding the supernatant and prior to any addition of wash solution the cell pellet should be gently resuspended by thoroughly stirring the test tube. This is crucial to avoid formation of cell clumps.
7. Usually three washes are sufficient to remove the platelets but sometimes more washes are required.
8. We usually infect the cells immediately after the lymphocytes have been isolated. However, lymphocytes can be kept overnight on ice in RPMI-1640, 10 % FCS before being infected with the virus. Leaving the cells in the incubator at 37 °C may decrease cell viability and cause non-desired activation of the B cells.
9. CpG ODN was shown to increase dramatically the efficiency of EBV-induced immortalization of B cells [17].
10. The initially isolated cells are a mixture of cells containing only about 10 % B cells.
11. When EBV-immortalized cells are seeded as described, only about 5 % will proliferate into cell lines. Therefore, only 15–150 different B cell clones will develop in each well.
12. The irradiated cells of the feeder layer disintegrate within a short time, whereas the number of the lymphoblastoid cells gradually increases. If the cells are widely dispersed, outgrowth will be poor. It is therefore recommended that about 8 days after seeding, the microplate should be tilted so that all the cells are allowed to slide down to the lower “corner” of the wells. An object, such as an ordinary pencil, is positioned under the microplate at its edge to keep the plate permanently tilted. The fact that the cells are now closely positioned to each other facilitates their outgrowth.
13. There is no need under the conditions described above to refeed the wells during the 4 weeks of culture. It is, however, mandatory to cover each microplate with aluminum foil to decrease evaporation.
14. When cloning the cells it is preferable to seed an average 0.3 cells/well to increase the chances of outgrowth monoclonality.
15. EBV-immortalized cells (either freshly infected or cells derived from established cell lines) have a low efficiency of cloning, even when grown together with a feeder layer. We have cloned

LCL's numerous times using the limiting dilution method, with allogeneic irradiated blood-derived lymphocytes as feeder. The average cloning efficiency is 15 %. This means that when seeding in a microplate at 0.3 cell/well, there should be cell line outgrowth in about three to four wells per 96-well microplate.

16. It is recommended to place the flask upright, slightly tilted, so that the cells will slide down to one corner of the vessel. Lymphoblastoid cells in close proximity tend to grow out better, particularly when starting as a sparse culture.
17. Usually, 50 % FCS in PBS is the saturation buffer of choice. However, if the background absorption values in the control wells are high (because of the interaction of ELISA reagents with FCS) PBS with BSA, low-fat milk or gelatin is used for saturation (*see* Subheading 2.5).
18. Supernatants derived from five microplates (i.e., 480 wells) can easily be tested simultaneously by ELISA during one day and by one person. The day before the assay, add to each well 45 μ l, antigen, incubate for 1 h at room temperature and then leave overnight at 4 °C. The concentration of antigen used to coat ELISA microwell varies, depending on the type of molecule used. If the antigen is a protein, then usually 1–2 μ g/ml is optimal. High concentrations of antigen might cause interference whereas low concentrations will result in a weak optical signal. The following day knock out the plates to empty the wells, add 250 μ l of a saturation buffer to block the remaining free surface of the wells. Similarly saturate five control plates (devoid of antigen) with saturation solution. On the day before the assay, use a multichannel micropipette to collect supernatants (100 μ l/well) from the five tissue culture microplates in which the immortalized cells grew. Add the supernatants to five similarly marked 96-well round bottom “master microplates” at the locations corresponding to those of the original tissue culture microplates.

On the day the assay is performed, wash the ELISA plates four times by dipping each plate in a dish with the wash buffer and knock out the contents. It should take about 5 min to wash ten plates (i.e., five test microplates and the corresponding five control microplates), four washes each. After the last wash, leave the wash solution in the microplates. Empty one antigen-coated microplate and the corresponding control plate by knocking out the fluids. Use a multichannel micropipette to transfer the supernatants' samples (each 45 μ l) from the “master microplate” to the corresponding wells in the antigen-coated ELISA microplate and to the corresponding control microplate. Repeat the procedure for the remaining microplates. Handle only two plates at a time (as described above) to avoid drying out the ELISA microplates.

If the antigen is a costly reagent then it is recommended to collect the antigen solution from the wells before adding the saturation buffer and save it for future use in ELISA. Obviously, the concentration of rescued antigen will be somewhat lower compared with the original antigen solution.

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References

1. Nilsson K, Ponten J (1975) Classification and biological nature of established human hematopoietic cell lines. *Int J Cancer* 15:321–341
2. Steinitz M, Klein G, Koskimies S, Mäkelä O (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* 269:420–422
3. Kuppers R (2003) B cells under influence: transformation of B cells by Epstein-Barr virus. *Nat Rev Immunol* 3:801–812
4. Nemerow GR, Wolfert R, McNaughton ME, Cooper NR (1985) Identification and characterization of the Epstein-Barr virus receptor on human lymphocytes-B and its relationship to the C3d complement receptor (Cr-2). *J Virol* 55:347–351
5. Heath E, Begue-Pastor N, Chaganti S, Croom-Carter D, Shannon-Lowe C, Kube D, Feederle R, Delecluse HJ, Rickinson AB, Bell AI (2012) Epstein-Barr virus infection of naive B cells in vitro frequently selects clones with mutated immunoglobulin genotypes: implications for virus biology. *PLoS Pathog* 8(5):e1002697
6. Ehlin-Henriksson B, Gordon J, Klein G (2003) B-lymphocyte subpopulations are equally susceptible to Epstein-Barr virus infection, irrespective of immunoglobulin isotype expression. *Immunology* 108:427–430
7. Brown NA, Miller G (1982) Immunoglobulin expression by human lymphocytes-B clonally transformed by Epstein-Barr virus. *J Immunol* 128:24–29
8. Miyawaki T, Kubagawa H, Butler JL, Cooper MD (1988) Ig isotypes produced by Ebv-transformed B-cells as a function of age and tissue distribution. *J Immunol* 140:3887–3892
9. Stein LD, Chan MA, Hibi T, Dosch HM (1986) Epstein-Barr virus-induced IgE production in limiting dilution cultures of normal human B-cells. *Eur J Immunol* 16:1167–1170
10. Steinitz M, Klein G (1980) Ebv-transformation of surface IgA-positive human-lymphocytes. *J Immunol* 125:194–196
11. Shannon-Lowe C, Baldwin G, Feederle R, Bell A, Rickinson A, Delecluse HJ (2005) Epstein-Barr virus-induced B-cell transformation: quantitating events from virus binding to cell outgrowth. *J Gen Virol* 86:3009–3019
12. Gil Y, Levy-Nabot S, Steinitz M, Laskov R (2007) Somatic mutations and activation-induced cytidine deaminase (AID) expression in established rheumatoid factor-producing lymphoblastoid cell line. *Mol Immunol* 44:494–505
13. Chezar I, Lobel-Lavi L, Steinitz M, Laskov R (2008) Ongoing somatic hypermutation of the rearranged VH but not of the V-lambda gene in EBV-transformed rheumatoid factor-producing lymphoblastoid cell line. *Mol Immunol* 46:80–90
14. Laskov R, Yahud V, Hamo R, Steinitz M (2011) Preferential targeting of somatic hypermutation to hotspot motifs and hypermutable sites and generation of mutational clusters in the IgVH alleles of a rheumatoid factor producing lymphoblastoid cell line. *Mol Immunol* 48:733–745
15. Miller G, Lipman M (1973) Comparison of yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by Epstein-Barr-virus. *J Exp Med* 138:1398–1412
16. Steinitz M, Tamir S, Frodin JE, Lefvert AK, Mellstedt H (1988) Human monoclonal anti-idiotypic antibodies. I. Establishment of immortalized cell-lines from a tumor patient treated with mouse monoclonal-antibodies. *J Immunol* 141:3516–3522
17. Traggiai E, Becker S, Subbarao K, Kolesnikova L, Uematsu Y, Gismondo MR, Murphy BR, Rappuoli R, Lanzavecchia A (2004) An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 10:871–875