Monoclonal Antibodies Technology. Protocols

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Immunization.

The first step in preparing useful monoclonal antibodies (MAbs) is to immunize an animal (Balb/c for example) with an appropriate *antigen*.

Methods (only for soluble antigen):

Solubilize selected antigen in Phosphate buffer solution (PBS) at pH 7.2-7.4, ideally at a final concentration per animal between 10 to 50 μg/ml.

It is recommended that the antigen under consideration be incorporated into the emulsion adjuvants in 1:1 volumetric relation. We commonly use Frend's adjuvant (FA) to prepared inmunized solution. The first immunization should be prepared with complete FA, and the another could be prepared with incomplete FA.

It is recommended to inject mice with 0.2 ml intraperitoneal (ip) or subcutaneous (sc). Our experience suggests the sc route is the preferred route. A minimum protocol for immunizing mice to generate cells for preparing hybridomas is s follows: immunize sc on day 0, boost sc on day 21, take a trial bleeding on day 26; if antibody titters are satisfactory, boost ip on day 35 with antigen only, and remove the spleen to obtain cells for fusion on day 38.

Fusion protocol

The myeloma cell line we are using is X63 Ag8.653. At the moment of fusion myeloma cells need a good viability (at least a 95%).

1. Remove the spleen cells from immunized mice using sterile conditions. An immune spleen should yield between 7 a 10 $x10^7$ nucleated cells.
2. Place the spleen in 20 ml of serum-free RPMI 1640 in a Petri dish. Using a needle and syringe, inject the spleen with medium to distend and disrupt the spleen stroma and free the nucleated cells.
3. Flush the cell suspension with a Pasteur pipet to disperse clumps of cells.
4. Centrifuge the spleen cell suspension at 250g for 10 min. Resuspend the pellet in serum-free RPMI 1640. Determine cell concentration using Neuhabuer chamber.
5. Mix the myeloma cells and spleen cells in a conical 50-ml tube in serum-free RPMI 1640, 1 $x10^7$ spleen cells to 1 $x10^6$ myeloma cells (ratio 10:1). Centrifuge the mixture

of cells at 300g for 10 minutes. While the cells are centrifuging, set aside 30 ml of serum-free RPMI 1640 in another 50-ml tube. Prepare the 50% PEG and place the timer in the hood.

6. Remove all the supernatant from the cell pellet. Overlay the pellet of cells with 0.5 ml of 50% PEG with a Pasteur pipet during 1 minute. Then add 5 ml of serum-free RPMI 1640 during 3 minutes. At the end of 3 minutes, add during a minute 15 ml of the same medium.

7. Centrifuge at 250g for 10 minutes. Gently resuspend the fused cells at a concentration of 5 $x10^4$ cells/ml, in a RPMI medium containing serum at 20%, HAT (selective growth) and antibiotics.

8. Distribute the cell suspension into 96-well-flat-bottom-tissue-culture plates add 0.2 ml per well. Incubate the plates at 37°C in 5% CO_2. Three to 4 days in HAT medium is sufficient for arresting the proliferation of unfused myeloma cells.

Screening and establishing a Hybridoma line.

The screening could be perform using different immunoassay alternatives (ELISA, RIA, etc). The principal requisite is have been developed a reproducible method before to make the fusion. These method could be improve using the serum of mice immunized as sample.

Approximately 1 week after the fusion, colonies of hybrid cells will be ready to screen. During the screening, samples of tissue culture media are removed from wells that have growing hybridomas and are tested for the presence of the desired antibodies. Successful fusions will produce between 2 000 and 5 000 hybridomas colonies. Depending on the fusion, individual wells will become ready to screening over 2 to 6 day period. Typically, the first wells could be ready to screen in day 7 or 8, and most of the wells will need to be screened within the next 4 or 5 days.

When you detected a possible positive clone, you could transfer hybrids of interest to 24-plates adding 1 ml of HAT medium (RPMI 1640) supplemented with serum at 20%. Cryopreserve each culture of cells when they have reached at least 3/4 confluency, 2 ampules/cell culture. Save the supernatants for further studies.

Regarding to separate the interested cells of irrelevant cells, you must been do a limited diluted procedures (see Methods in Molecular Biology, vol.46).

Ascitis production.

To produce ascitis the number of cells depend of the every particular clone. Usually we inoculated ip between 1 to 2 x10^6 cells/ml/mice in PBS buffer.

The animals must be inoculated intraperitonealy with Pristan (Sigma) or mineral oil using 0.5 ml per mice. The inoculation could be occurred 7 to 10 days before to inoculated the clone. The weight of the animals must be controlled in order to obtain better yield.

The bioreactors are an alternative method to produce AcMs without it have not used animals models.

IgG purification process on protein A columns.

Mouse antibodies of the IgG_1 subclass do not have a high affinity for protein A. Every subclass could be eluted at different peaks using a gradient pH. Usually we use 3M NaCl/1.5 M Gly pH 8.9 as a coupling buffer. For eluting IgGs we use 0.1M Citric Acid in a gradient pH (6 to IgG_1, 5 to IgG_{2a} or IgG_{2b}, 4 IgG_3).

1. Adjust a Sepharose Protein A (Pharmacia) beads using NaCl/1.5 M Gly pH 8.9 (coupling buffer).
2. Diluted the ascitis 1:10 in coupling buffer using a filter paper for remove the aggregations.
3. Pass the solution trough a Protein A bead column at a standarized flow according your equipment. The capacity of the Protein A beads for IgG1 is approximately 5 mg/ml of wet beads. Ascitis contain between 1-10 mg/ml.
4. Wash the colums with 10 volumes of coupling buffer.
5. Elute the column with 0.1M Citric Acid in a gradient pH starting at 6 to 4. Collect the eluate in appropriate tube, and identify the immunoglobulin-containing fractions by absorbance a 280 nm (1 DO = 0.8 mg/ml).