

CHAPTER 7

Generating Monoclonal Antibodies

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SERUM CONTAINS MANY DIFFERENT TYPES of antibodies (polyclonal) that are specific for many different antigens. Even in hyperimmune animals, seldom are more than one-tenth of the circulating antibodies specific for one antigen. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques. Therefore, the preparation of homogeneous, or monoclonal, antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production.

The first isolation of a homogeneous population of antibodies came from studies of B-cell tumors. Clonal populations of these cells can be propagated as tumors in animals or grown in tissue culture. Because all of the antibodies secreted by a B-cell clone are identical, these tumor cells provide a source of homogeneous antibodies. Unfortunately, B-cell tumors secreting antibodies of a predefined specificity cannot be isolated conveniently.

In the animal, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibodies. Köhler and Milstein (1975) developed a technique that allows the growth of clonal populations of cells secreting homogeneous antibodies with a defined specificity. In this technique, an antibody-secreting cell is isolated from an immunized animal, then fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or hybridomas can be maintained *in vitro* and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as monoclonal antibodies.

CHARACTERISTICS OF MONOCLONAL ANTIBODIES

Monoclonal Antibodies Are Powerful Immunochemical Tools

The usefulness of monoclonal antibodies stems from three characteristics—their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. The production of monoclonal antibodies allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope. Hybridoma cell lines also provide an unlimited supply of antibodies. Even the most farsighted researchers have found that large supplies of valuable antisera eventually run out. Hybridomas overcome these difficulties. In addition, one unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single-cell-cloned before use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens.

Hybridomas secreting monoclonal antibodies specific for a wide range of epitopes have been prepared. Any substance that can elicit a humoral response can be used to prepare monoclonal antibodies. Their specificities range from proteins to carbohydrates to nucleic acids. However, monoclonal antibodies are often more time-consuming and costly to prepare than polyclonal antibodies. They are not necessarily the best choice for certain immunochemical techniques. In theory, either as single antibody preparations or as pools, monoclonal antibodies can be used for all of the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job. Researchers should be certain that they need these types of reagents before they begin constructing hybridoma cell lines. Table 1 describes some of the uses of monoclonal antibodies.

Hybridomas Are Immortal Somatic Cell Hybrids That Secrete Antibodies

In the early 1970s, several research groups worked on different methods to extend the life span of antibody-secreting cells *in vitro*. For murine cells, the practical aspects of this goal were solved by applying techniques used in somatic cell genetics. By fusing two cells, each of which have properties necessary for a successful hybrid cell line, Köhler and Milstein (1975) showed that antibody-secreting cell lines could be established routinely and then maintained *in vitro*. The two cells that are commonly used as partners in these fusions are antibody-secreting cells isolated from immunized animals and myeloma cells. The myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody-secreting cells provide the functional immunoglobulin genes.

Early work solved the three technical problems for achieving a successful fusion: (1) finding appropriate fusion partners, (2) defining conditions for efficient fusion, and (3) choosing an appropriate system to select for hybrid cells against the background of unfused cells. Choosing an appropriate and reliable screening assay can be the difference between a successful fusion and an unsuccessful fusion.

TABLE 1. Immunochemical techniques: Polyclonal versus monoclonal antibodies

Technique	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
Cell staining	Usually good	Antibody dependent	Excellent
Immunoprecipitation	Usually good	Antibody dependent	Excellent
Immunoblots	Usually good	Antibody dependent	Excellent
Immunoaffinity purification	Poor	Antibody dependent	Poor
Immunoassays with labeled antibody	Difficult	Good	Excellent
Immunoassays with labeled antigen	Usually good	Antibody dependent	Excellent

TABLE 2. Myeloma cell lines used as fusion parents

Cell line	Reference(s)	Derived from	Chains expressed	Secreting	Comments
Mouse lines					
P3-X63Ag8	Köhler and Milstein 1975	P3 K	$\gamma 1, \kappa$	IgG1	Not recommended
X63Ag8.653	Kearney et al. 1979	P3-X63Ag8	None	No	Recommended
Sp2/0-Ag14	Köhler and Milstein 1976; Shulman et al. 1978	P3-X63Ag8 × BALB/c	None	No	Recommended
FO	de St. Groth and Scheidegger 1980	Sp2/0-Ag14	None	No	Recommended
NSI/1-Ag4-1	Köhler et al. 1976	P3-X63Ag8	Kappa	No	Recommended
NSO/1	Galfre and Milstein 1981	NSI/1-Ag4-1	None	No	Recommended
FOX-NY	Taggart and Samloff 1983	NSI/1-Ag4-1	Kappa(?)	No	
Rat lines					
Y3-Ag1.2.3	Galfre et al. 1979	Y3	Kappa	No	Not recommended
YB2/0	Kilmartin et al. 1982	YB2/3HL	None	No	Recommended
IR983F	Bazin 1982	LOU/c rats	None	No	Recommended

Myelomas from BALB/c Mice Are Good Cells for Fusion

Myelomas can be induced in a few strains of mice by injecting mineral oil into the peritoneum. Many of the first examples of these myelomas were isolated from BALB/c mice by Potter (1972), and these cells are referred to by the abbreviation MOPC (for mineral oil plasmacytoma). Derivatives of BALB/c myelomas have become the most commonly used partners for fusions. Table 2 lists some of the myeloma cell lines used for hybridoma construction. Myelomas have all the cellular machinery necessary for the secretion of antibodies, and many secrete these proteins. To avoid the production of hybridomas that secrete more than one type of antibody, myelomas that are used for fusions have been selected for the lack of production of functional antibodies. Figure 1 shows the derivation of many of the commonly used myeloma cell lines.

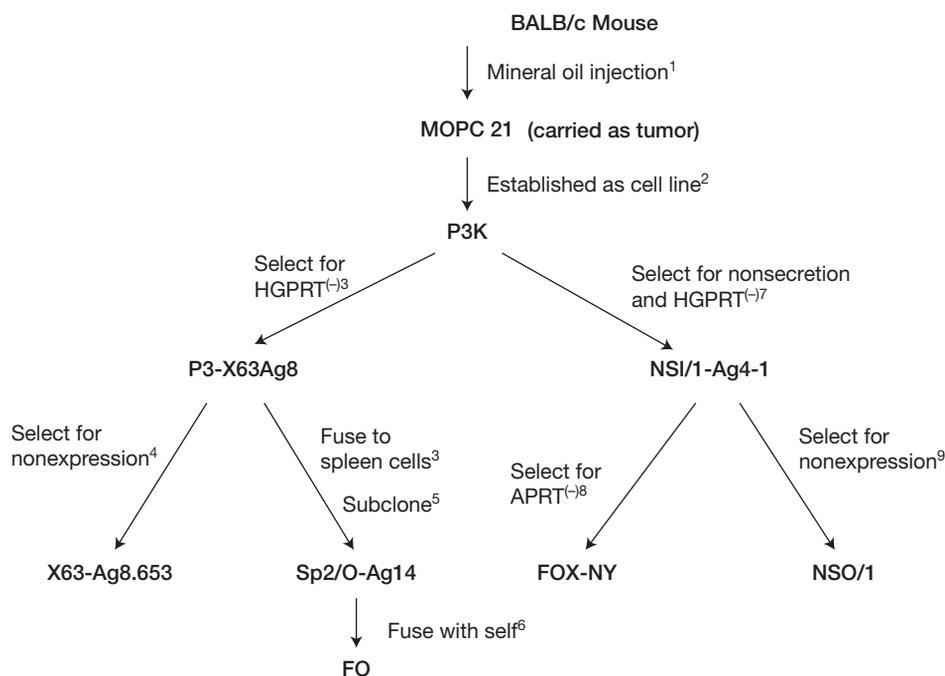


FIGURE 1. Myeloma family tree. ¹Potter (1972); ²Horibata and Harris (1970); ³Köhler and Milstein (1975); ⁴Kearney et al. (1979); ⁵Shulman et al. (1978); ⁶de St. Groth and Scheidegger (1980); ⁷Köhler et al. (1976); ⁸Taggart and Samloff (1983); ⁹Galfre and Milstein (1981).

The other cell for the fusion is isolated from immunized animals. These cells must carry the rearranged immunoglobulin genes that specify the desired antibody. Because of the difficulties in purifying cells that can serve as appropriate partners, fusions are normally performed with a mixed population of cells isolated from a lymphoid organ of the immunized animal. Although a number of studies have helped to characterize the nature of this B-cell-derived partner, the exact state of differentiation of this cell is still unclear.

Hybridomas can be prepared by fusing myelomas and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore, fusions are normally performed with cells from the same species. All commonly used mouse strains can serve as successful fusion partners with BALB/c myelomas; however, immunizations are normally performed in BALB/c mice, because this allows the resulting hybridomas to be grown as tumors in this mouse strain to produce ascites. With the development of in vitro systems and modern bioreactors, the strain of mouse fused with the BALB/c myelomas is less of an issue when producing monoclonal antibodies. Interspecies hybridomas (heterohybridomas) can also be produced in quantity this way.

Polyethylene Glycol Is the Most Commonly Used Agent to Fuse Mammalian Cells

In theory, the fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In practice, hybridoma fusions became routine after the introduction of the use of polyethylene glycol (PEG). The use of PEG as a fusing agent for mammalian cells was first shown by Pontecorvo (1975) and was quickly adopted by somatic cell geneticists. PEG is the method of choice for hybridoma production, allowing the rapid and manageable fusion of mammalian cells.

PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve before mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy- or light-chain gene is lost, production of the antibody is lost. In a culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer in the supernatant. When this occurs, the hybridoma is considered unstable. If the hybridoma loses the chromosome containing the gene used in drug selection (see below), then the growth of the hybridoma will be unstable, and these cells will die during selection. In practice, the selection for the stable segregation of the drug selection marker is so strong that within a short time (3–5 d) the hybridoma is either lost completely or a variant is isolated that stably retains the selectable marker.

Unfused Myeloma Cells Are Eliminated by Drug Selection

Even in the most efficient hybridoma fusions, only ~1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in the culture. The cells from the immunized animal do not continue to grow in tissue culture and thus do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed. Most hybridoma constructions achieve this by drug selection. Commonly, the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (first reported by Littlefield 1964). For example, selection with 8-azaguanine often yields a cell line harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (*HPRT*). The addition of any compound that blocks the de novo nucleotide synthesis pathway will force cells to use the salvage pathway. Cells containing a nonfunctional HGPRT protein will die in these conditions (Fig. 2A). Hybrids between myelomas with a nonfunctional *HPRT* and cells with a functional *HPRT* will be able to grow. Commonly used drugs for hybridoma selection are aminopterin, methotrexate, or azaserine, all of which inhibit the de novo nucleotide synthesis pathway (Fig. 2B).

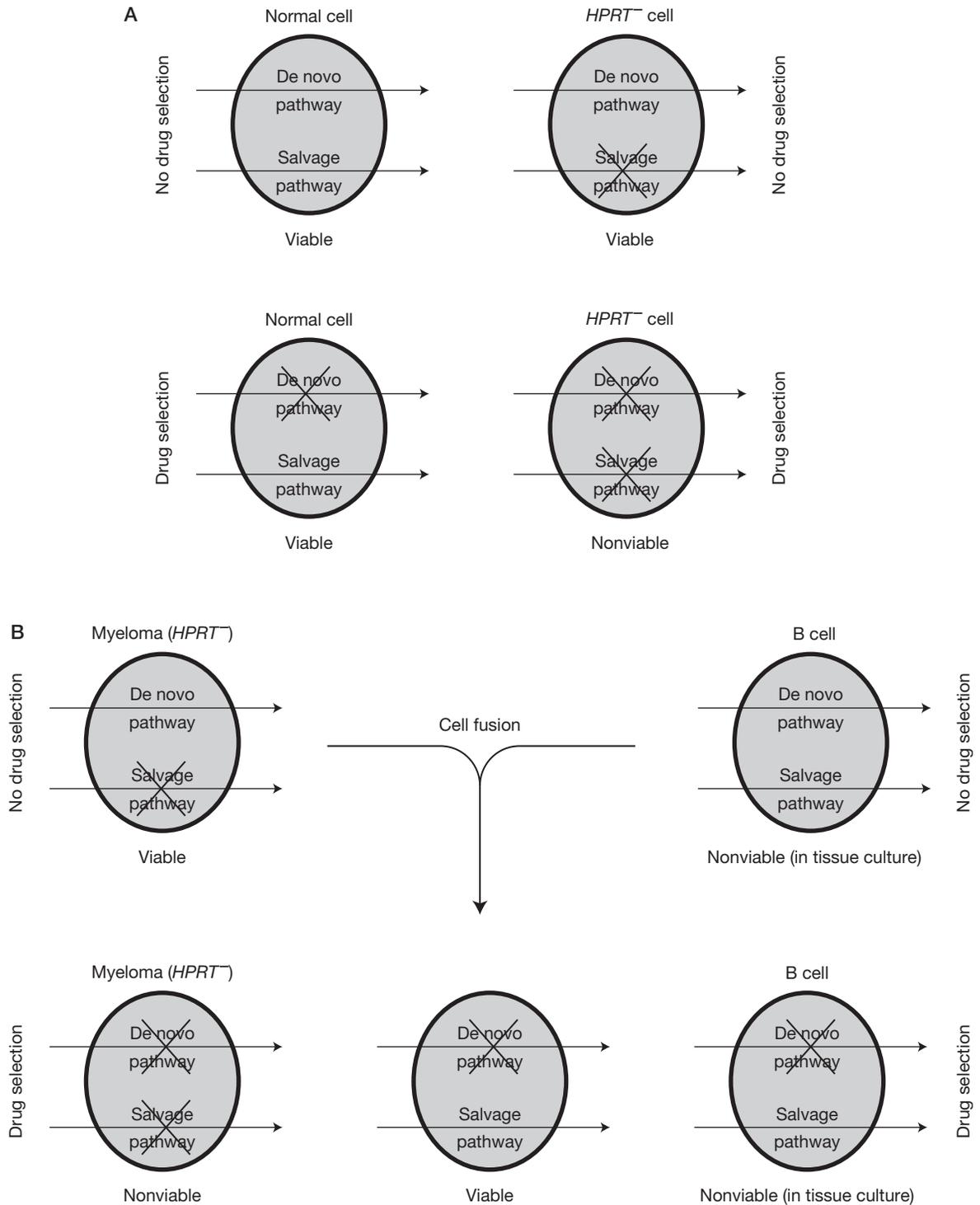


FIGURE 2. Cells obtain nucleotides from two sources, either by de novo synthesis or through a salvage pathway. The hybridoma selection process works because drugs like aminopterin are able to block the de novo nucleotide synthesis pathway, and myeloma partner cells have a defective *HPRT* enzyme, blocking their ability to use the salvage pathway. B cells do not live long in culture without special supplements. The only cells that can survive are the hybridomas. (A) Pathways of nucleotide synthesis. (B) Drug selection for viable hybridomas.

PRODUCTION OF MONOCLONAL ANTIBODIES

Although hybridomas can be prepared from animals other than mice, all of the techniques below use mice as examples. Similar techniques can be used for fusions of rat myelomas and rat antibody-secreting cells, as well as rabbit plasmacytomas and rabbit antibody-secreting cells. More specialized fusions using interspecies crosses or human cells are discussed briefly below in this chapter.

Stages of Hybridoma Production

Figure 3 outlines the steps in the production of monoclonal antibodies. Animals are injected with an antigen preparation. Once a good humoral response (titer) has appeared in the immunized animal and an appropriate screening procedure is developed, a fusion can be considered. The sera from test bleeds are used to develop and validate the screening procedure. After an appropriate screening assay has been established, the actual production of the hybridomas can begin. Several days before the fusion, animals are boosted with a sample of the antigen. For the fusion, antibody-secreting cells are prepared from the immunized animal, mixed with the myeloma cells, and fused. After the fusion, cells are diluted in selective medium and plated in multiwell tissue culture dishes. Hybridomas are ready to test beginning ~1 wk after the fusion. Cells from positive wells are expanded and then single-cell-cloned. Hybridoma production seldom takes <2 mo from start to finish, and it can take well over a year to obtain stable hybridomas. It is convenient to divide the production of monoclonal antibodies into three stages: (1) immunizing mice, (2) developing the screening procedure, and (3) producing hybridomas. Any one of these stages may proceed very quickly, but all have inherent problems that should be considered before the start of the project. Immunizing mice is discussed in Chapter 6. Screening methods and hybridoma generation are discussed in this chapter.

DEVELOPING THE SCREENING METHOD

Because most hybridoma cells grow at approximately the same rate, the tissue culture supernatants from all the fusion wells usually are ready to screen within a few days of one another. This makes screening the most labor-intensive segment of hybridoma production. Care in developing the proper screening assay will help to keep the amount of work needed to identify positive wells to a minimum and is essential in selecting a hybridoma that produces a monoclonal antibody with the desired characteristics.

Approximately 1 wk 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 200 and 20,000 hybridoma colonies, with 500–1000 colonies being the norm. Depending on the fusion, individual wells will become ready to screen over a 5-d to 12-d period. Typically, the first wells would be ready to screen on Day 7 or 8, and most of the wells will need to be screened within the next 4 or 5 d. By Day 14, all initial screening of the fusion plates should be completed and potential positive wells expanded to 24-well plates. Cells will be overgrown in the fusion plates by this time and will eventually die off.

A good screening procedure must (1) reduce the number of cultures that must be maintained to a reasonable level (seldom more than 50 cultures at one time); (2) identify potential positives in 48 h or less (24 h or less is ideal); and (3) be easy enough to perform for all the needed wells (high throughput). Positive wells may be as rare as 1 in 500 or as common as 9 out of 10. It is best to expand any potentially interesting hybridoma population after the initial screen and then rescreen it, rather than risk losing a unique antibody. The expanded hybridoma culture from a positive well will provide a larger volume of supernatant to evaluate the potential antibody in additional assays. Following the initial screen, the effort needs to change to one of elimination, that is, identifying false positives and nonspecific antibodies as soon as possible. Several different screening assays can be combined to identify the hybridomas with desired characteristics, as long as they aim at reducing the tissue

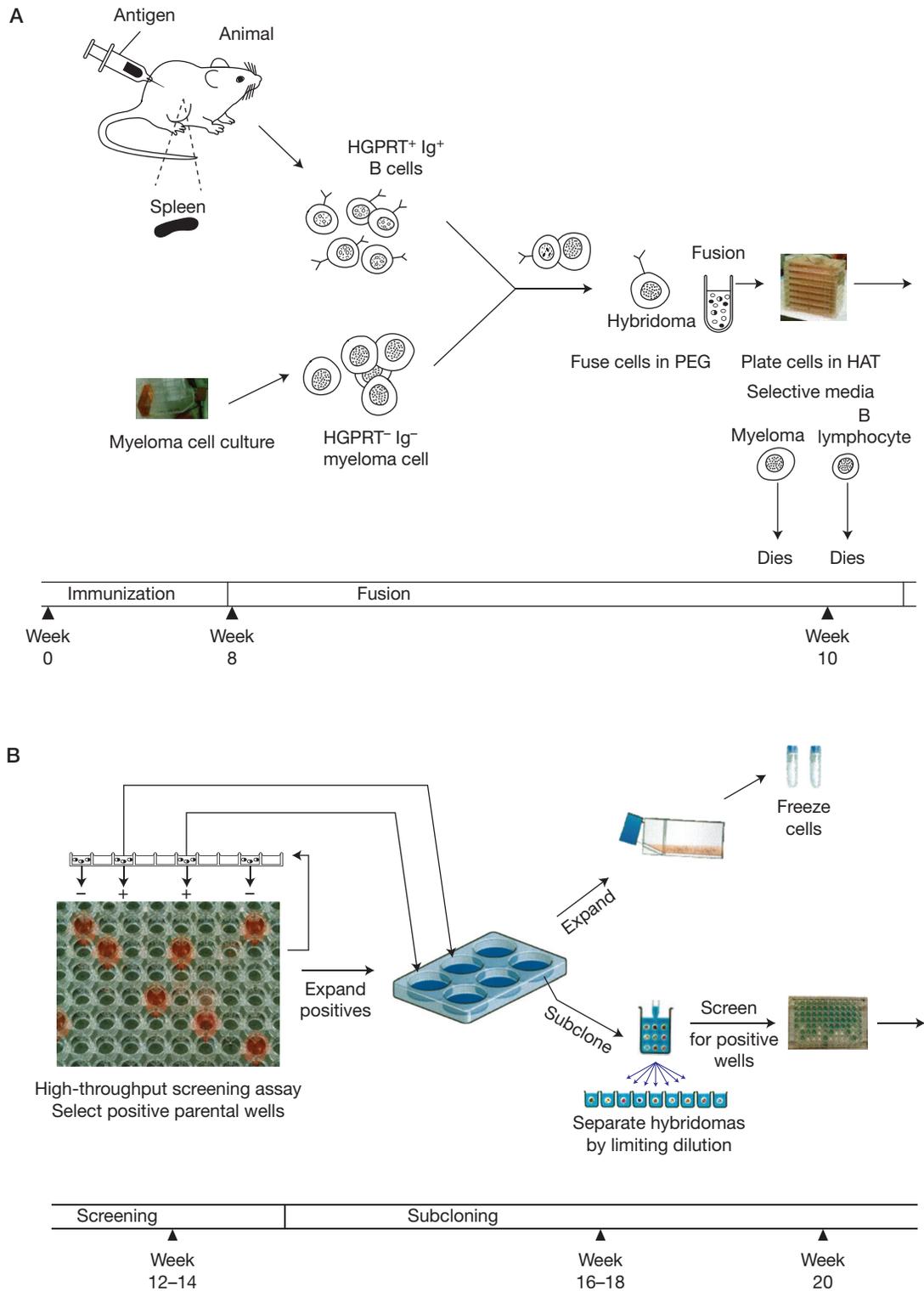


FIGURE 3. Stages of hybridoma production. (Figure continues on facing page.)

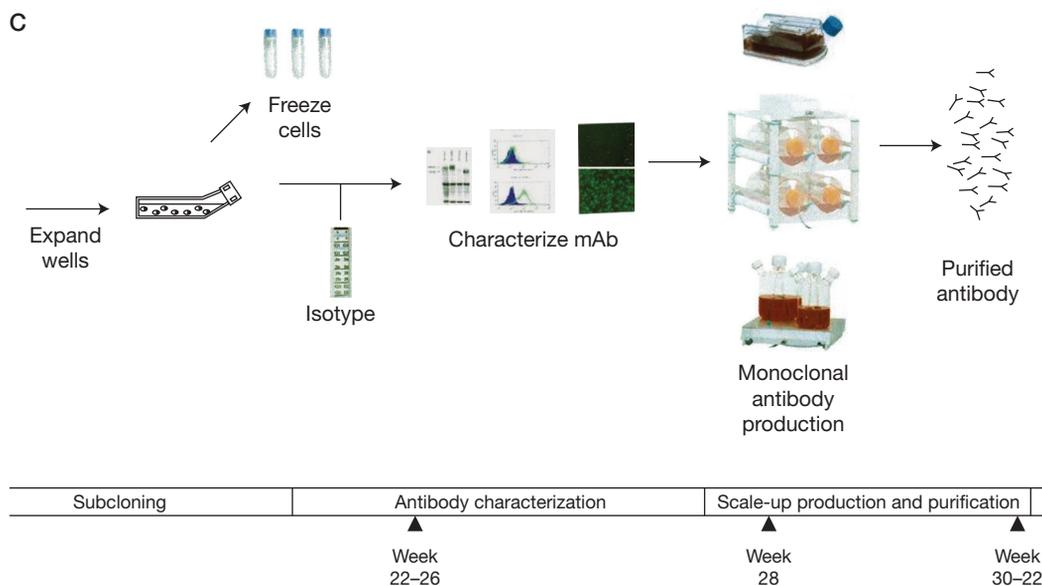


FIGURE 3. (Continued)

culture work to a manageable level. After the first round of screens, handling the tissue culture necessary for 100 wells is difficult for one person, 50 wells is reasonable, and less than 20 is relatively simple.

It must be emphasized that all screening procedures must be tested and validated before the fusion is performed. It is often wise to have a backup assay in place in case there are unforeseen issues found with the original planned assay. After the fusion, there is seldom enough time to try out new ideas or to refine methods. The test bleeds can and should be used to set up and test the screening assay.

Screening Strategies

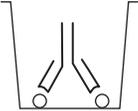
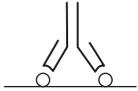
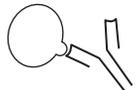
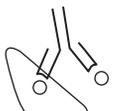
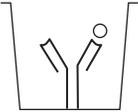
There are three classes of screening methods: (1) antibody capture assays, (2) antigen capture assays, and (3) functional screens. Currently, the most common screens are either antibody or antigen capture, but because functional assays attain higher throughput, more fusions will be screened by these methods. Table 3 depicts several of the more common screening techniques.

In general, the more antigens there are combined in the immunizing injections, the more difficult the screen because there will be a complex immune response and it is unlikely that animals will respond to all antigens equally. Researchers immunizing with pure or partially pure antigens should use methods for antibody capture (e.g., enzyme-linked immunosorbent assay [ELISA] or fluorescence-activated cell sorting [FACS]). If the subcellular location of an antigen is known, positive tissue culture supernatants can be identified by cell staining. If the immunizations used complex antigen solutions, procedures such as immunoprecipitation, western blot, or other antigen capture assays may be the only alternatives. In addition to the tests described below, any of the assays used for analyzing antigens can be adapted for use as a screen (see Chapters 13–16).

Antibody Capture Assays

Antibody capture assays are often the easiest and most convenient of the screening methods. In an antibody capture assay, the following sequence takes place: The antigen is bound to a solid substrate; the antibodies in the hybridoma tissue culture supernatant are allowed to bind to the antigen; the unbound antibodies are removed by washing; and then the bound antibodies are detected by a secondary reagent

TABLE 3. Methods for screening hybridoma fusions

Method	Examples	Advantages	Disadvantages
Antibody capture	PVC wells 	Easy; rapid	Need pure or partially pure antigen; does not discriminate between high- and low-affinity Ab
	Nitrocellulose 		
	Whole cells 		
	Permeabilized cells 		
Antigen capture	Ab/Ag in solution 	Only detect high-affinity Ab	Unless you have pure labeled Ag, assay is tedious and slow
	Ab/Ag on solid phase 	Rapid	Need pure labeled Ag; setting up solid phase is tricky
Functional	Blocking 	Ab immediately useful	False positives; potentially tedious
	Depletion 	Ab immediately useful; only detect high-affinity Ab	Tedious; Ag must be limiting

that specifically recognizes the antibody. In this assay, the detection method identifies the presence of the antibody, thus determining a positive reaction (Figs. 4 and 5).

Most antibody capture assays rely on an indirect method of detecting the antibody. This is commonly performed with a secondary reagent such as rabbit anti-mouse immunoglobulin antibodies (monoclonal or polyclonal). These antibodies are usually purchased from commercial suppliers or can be prepared by injecting purified mouse immunoglobulins into rabbits. The rabbit antibodies can be purified; labeled with an easily detectable tag, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), or biotin (see Chapter 12); and used to determine the presence of mouse, rat, or hamster monoclonal antibodies (see Protocol 1).

Alternatively, positives can be located by other reagents that will bind specifically to antibodies. Two proteins that may be used for these reactions are Protein A and Protein G. Both of these

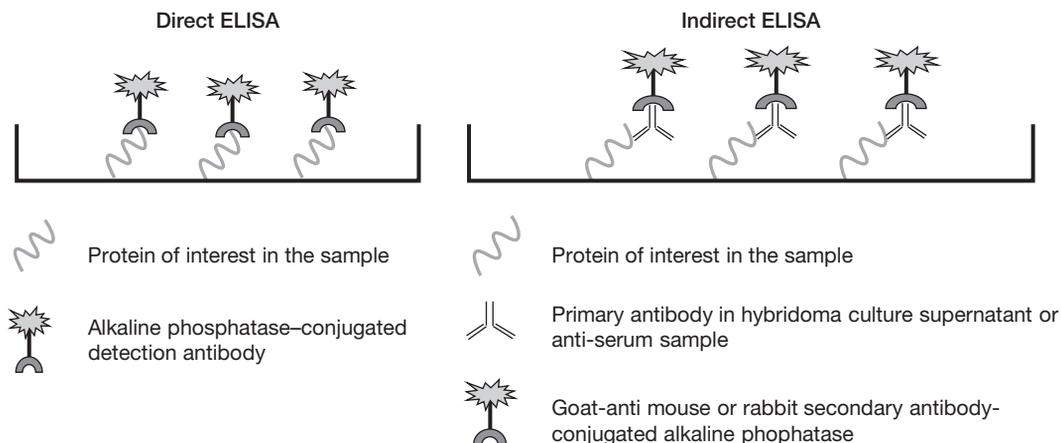


FIGURE 4. Direct and indirect ELISA. In direct ELISA, the target antigen, which is immobilized on a plastic surface, is detected by anti-target antibody conjugated to an enzyme label. In indirect ELISA, the target antigen, which is immobilized on a plastic surface, binds to anti-target antibody, forming an immune complex. This complex is then detected by a secondary antibody conjugated to an enzyme label.

polypeptides are bacterial cell wall proteins that have high affinities for a site in the Fc portion of some antibodies. Protein A and Protein G can be purified and labeled with an appropriate tag. Protein L is another bacterial protein that has an affinity for the antibody light chain (kappa chain only). Although not specific for any given species, they do have varied binding affinities for different subclasses of immunoglobulins and do not bind all species/subclasses of immunoglobulin equally well (Table 4).

Immunoglobulin-fusion proteins may have been used as immunogens or screening proteins. These reagents are made by combining the Fc region of an antibody molecule (often a mouse IgG2a or human IgG1) with the extracellular domain of a cell-surface protein. If these reagents are directly bound to ELISA plates and a secondary HRP-conjugated, general anti-mouse antibody is used to detect the hybridoma antibody, the assay will not be readable. This is because the secondary antibody will bind to both the hybridoma antibody and the immunoglobulin-fusion protein, resulting in a positive reaction in every well. This issue can be avoided by using an Fc region from a species that does not react to the immunoglobulin tail (like human Ig) or by switching the secondary detecting antibody to an anti-light-chain HRP antibody (kappa, lambda, or both). If the subclass of immunoglobulin used in the fusion protein is known, then a cocktail of anti-heavy-chain-specific

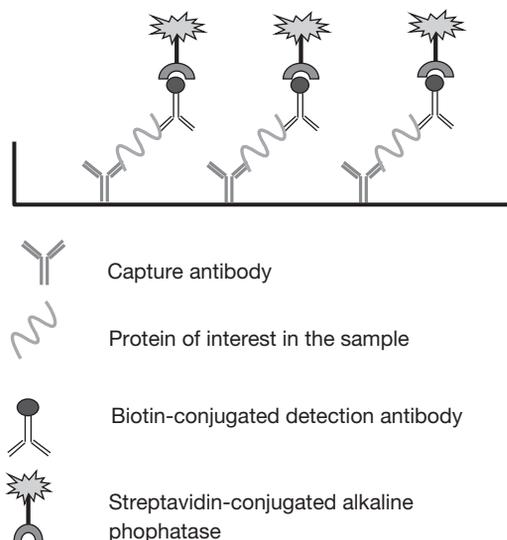


FIGURE 5. Capture or sandwich ELISA. Unlabeled anti-target capture antibody, which is immobilized on a plastic surface, binds to the target in solution (sample of interest). The biotin-labeled anti-target antibody, known as the detection antibody, then binds to the target, forming a sandwich, which is then detected by streptavidin conjugated to an enzyme label. “Sandwich ELISA” and “capture ELISA” are synonymous. The “capture” name comes from using an antibody to capture a protein in a mixture instead of directly binding the protein to the ELISA plate. The “sandwich” name comes from the observation that the antigen (protein) is sandwiched between the capture antibody and the detecting antibody.

TABLE 4. Binding characteristics of antibody-binding proteins—Protein A, Protein G, Protein A/G, and Protein L

Species	Antibody class	Protein A	Protein G	Protein A/G	Protein L ^a
Human	Total IgG	S	S	S	S ^a
	IgG1	S	S	S	S ^a
	IgG2	S	S	S	S ^a
	IgG3	W	S	S	S ^a
	IgG4	S	S	S	S ^a
	IgM	W	NB	W	S ^a
	IgD	NB	NB	NB	S ^a
	IgA	W	NB	W	S ^a
	Fab	W	W	W	S ^a
ScFv	W	NB	W	S ^a	
Mouse	Total IgG	S	S	S	S ^a
	IgM	NB	NB	NB	S ^a
	IgG1	W	M	M	S ^a
	IgG2a	S	S	S	S ^a
	IgG2b	S	S	S	S ^a
Rat	Total IgG	W	M	M	S ^a
	IgG1	W	M	M	S ^a
	IgG2a	NB	S	S	S ^a
	IgG2b	NB	W	W	S ^a
	IgG2c	S	S	S	S ^a
Cow	Total IgG	W	S	S	NB
	IgG1	W	S	S	NB
	IgG2	S	S	S	NB
Goat	Total IgG	W	S	S	NB
	IgG1	W	S	S	NB
	IgG2	S	S	S	NB
Sheep	Total IgG	W	S	S	NB
	IgG1	W	S	S	NB
	IgG2	S	S	S	NB
Horse	Total IgG	W	S	S	?
	IgG(ab)	W	NB	W	?
	IgG(c)	W	NB	W	?
	IgG(T)	NB	S	S	?
Rabbit	Total IgG	S	S	S	W ^a
Guinea pig	Total IgG	S	W	S	?
Pig	Total IgG	S	W	S	S ^a
Dog	Total IgG	S	W	S	?
Cat	Total IgG	S	W	S	?
Chicken	Total IgY	NB	NB	NB	NB

Reprinted, with permission, from Thermo Fisher Scientific Inc., © 2008, <http://www.piercenet.com/files/TR0034-Ab-binding-proteins.pdf>. Proteins A, G, A/G, and L are native and recombinant proteins of microbial origin that bind to mammalian immunoglobulins. The binding specificities and affinities of these proteins differ between source species and antibody subclass. Use the table to select the antibody-binding protein that is best for your application.

(S) Strong binding; (M) medium binding; (W) weak binding; (NB) no binding; (?) information not available.

^aBinding to Protein L will occur only if the immunoglobulin has the appropriate kappa light chains. The stated binding affinity refers only to species and subtypes with appropriate kappa light chains. Lambda light chains and some kappa light chains will not bind.

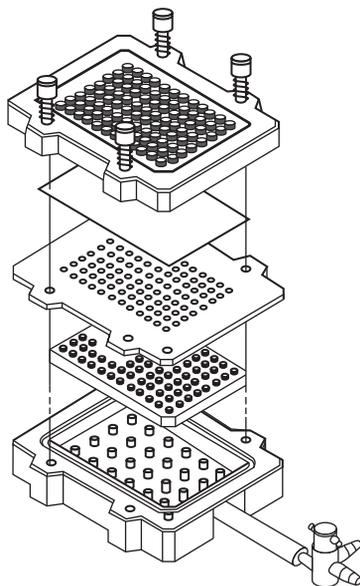


FIGURE 6. Schematic of the dot blot apparatus. (With permission, from http://www.biorad.com/webroot/web/images/lsr/products/electrophoresis/product_overlay_content/global/lsr_bio_dot_assembly.jpg.)

antibodies can be made, excluding the one used in the fusion protein. For instance, if the fusion protein were made using a mouse IgG2a Fc, the detecting cocktail of anti-mouse (IgG1 + IgG2b + IgG3 + kappa light chain) could be used (see Protocol 2).

If the antigen is a protein that is available in large amounts, dot blots are one of the assays of choice. Apparatus for performing dot blots is available commercially in a 96-well format (Fig. 6). The antigen is bound directly to a nitrocellulose sheet, and many assays can be performed on a single sheet; therefore, the manipulations are simple (see Protocols 3 and 4).

Whole cells can also be adapted for use in a screening assay. Both immunofluorescence stainings of cells grown on coverslips or flow cytometry (FACS) have been used successfully to identify hybridomas secreting monoclonal antibodies to cell-surface proteins (see Protocols 5 and 7). Each of these techniques can also be adapted for internal proteins by permeabilizing the cells before applying the hybridoma supernatants (see Protocols 6 and 8). These assays have the advantage of screening the potential antibodies on native, properly folded forms of the antigen with intact post-translational modifications.

To determine the subcellular location of an antigen, hybridoma tissue culture supernatants can be screened using immunohistochemistry (see Protocol 9). One major advantage of using cell staining in hybridoma screens is that the assays give an extra level of information. Unlike other antibody capture assays that rely on the simple detection of antibody, cell staining also determines the localization. This extra information makes cell staining a good assay when using complex antigens.

Both fluorochrome- and enzyme-labeled reagents can be used to detect the presence of antibodies (for more details, see Chapter 16), but if the levels are high enough to be detected using enzyme-labeled reagents, enzyme methods should be used. Enzyme-labeled assays can be scored by using a light microscope. Scoring assays using a fluorescent microscope will give more resolution, but long-term observation under this microscope is disorienting for most people.

Antigen Capture Assays

In an antigen capture assay, the detection method identifies the presence of the antigen. Often this is achieved by labeling the antigen directly. These assays require the monoclonal antibody to have a high affinity for antigen because the labeled antigen is normally added at very low concentration in free solution.

There are two types of antigen capture assays, and these assays differ by the order in which the steps are performed. In one variation, the antibodies in the tissue culture supernatant are bound to a

solid phase first, and then the antigen is allowed to react with the antibody. In the second variation, the antibody–antigen complex is allowed to form before the binding of the antibody to a solid phase. In either case, once the antibody–antigen complexes are bound to the solid support, the unbound antigen is removed by washing, and positives are identified by detecting the antigen.

Detection of the antigen can be performed by several techniques. If the antigen is available in pure form, it can be labeled by fluorescent tagging or enzyme coupling. If the antigen itself is an enzyme, positives may be determined by the presence of the enzymatic activity. Any property that is unique to the antigen can be used to identify positives (Fig. 5).

In a capture or sandwich ELISA assay, the polyvinyl chloride (PVC) wells of a high-binding-capacity ELISA plate are first coated with an anti-immunoglobulin antibody and then incubated with hybridoma tissue culture supernatant. Monoclonal antibodies in the supernatant are thus “captured” on the coated solid support, and they are detected by screening with, for example, biotin- or histidine-tagged antigen (see Protocol 10). The antigen can be labeled to a high specific activity and thus very little antigen is required for this procedure. The reverse dot-blot assay described in Protocol 11 is similar, except that nitrocellulose is used as the solid support. Reverse dot-blot assays are more complicated to use than many of the other screening assays, but they are particularly valuable if pure or partially pure antigen is available, although only in limited quantities. However, the assays are tricky to set up and demand careful use. The handling of individual assays is easier in capture ELISA assays than in reverse dot-blot assays because each well is used for a separate assay; with the nitrocellulose, this is achieved by using a dot-blot apparatus. Another advantage of capture ELISA assays is that individual reactions can be analyzed using a 96-well plate reader and the results reported as absorbance units.

Immunoprecipitation is another antigen capture assay (see Protocol 12), but it is seldom used for screening hybridoma fusions because the assays are tedious and time-consuming. However, because the antigen is normally detected after SDS–polyacrylamide electrophoresis, it is simple to discriminate potential positives from authentic ones. The added information gained about the molecular weight of an antigen makes these assays particularly useful when using complex antigens.

Functional Screens

Screening for Phospho-Specific Antibodies. Phospho-specific antibodies are difficult to raise in rodents. The immune system of the rabbit is much more amenable to recognizing the phospho-specific epitope. The down side to using rabbits is that a stable fusion partner is not currently freely available. Affinity deletion/purification of rabbit sera has produced some very good phospho-specific polyclonal antibodies. The process involves generating the specific response in rabbits using peptides 12–22 amino acids in length (smaller is better) in which the phosphorylated amino acid (tyrosine, serine, or threonine) is in the center of the peptide. This peptide is injected into rabbits and serum is collected. The serum is exhaustively affinity-depleted using a nonphosphorylated peptide, leaving the phospho-specific antibodies in the depleted sera. The sera can then be further purified by Protein A or phospho-peptide affinity purification. This is not practical in mice or rats because the volume of sera (50–100 μ L) obtainable is significantly less than from rabbits (30–50 mL). Commercial companies (e.g., Eptomics and Cell Signaling Technology) have developed stable rabbit plasmacytoma partner cells for performing hybridoma generation. The advantage here is that after a good polyclonal phospho-specific response is verified, the same rabbit can be used for hybridoma generation.

The key to successful isolation of phospho-specific hybridomas is more dependent on a very specific screening assay. A simple ELISA comparing phospho-peptide to non-phospho-peptide and an irrelevant phospho-peptide may work but often is not sufficient. A better screening assay would be to test indirectly for the presence of phospho-specific antibodies by their ability to block the phosphorylation site of interest in a biological activity assay. Even if antibodies to the nonphosphorylated protein were present from other hybridomas, they should not affect the readout of this type of assay. An example of this kind of assay would look at the up-regulation or down-regulation of another protein in the

signaling pathway downstream from the phospho-protein against which the monoclonal antibody has been generated that is dependent on the phosphorylation event. The screening will be more challenging but will pay off in the identification of the desired antibody.

Screening for Blocking/Neutralizing or Activating Antibodies. Blocking or neutralizing antibodies inhibit the interaction of cells (receptor/ligand) or block reactions (chemical or cell signaling events). Antibodies can be blocking either by binding an epitope directly that interferes with these types of interactions or indirectly because antibody molecules are large and can sterically hinder the interaction. Activating antibodies induce a specific activity upon binding, often by cross-linking a receptor. Antibodies that activate cell functions by cross-linking can often be made into a blocking reagent by fragmenting the antibody molecule and using only the monovalent Fab fragment. Not all antibodies are blocking or activating. Some antibodies bind a receptor or enzyme and have no effect on the cell or a signaling pathway. They simply tag the protein.

Once again, success in obtaining blocking or activating antibodies is dependent on a good screening assay and the right immunogen. Information on the target site and structure of the protein is extremely useful for generating hybridomas in these types of projects. If the binding site is known, then a peptide can be synthesized for immunization. This may help direct the immune response to the desired site, but the immune serum should be checked on the native (endogenous) protein for recognition and blocking/activating ability before planning a fusion. Peptides do not have any secondary structure or posttranslational modifications that may be crucial for defining the necessary epitope for blocking/activating antibodies. One good strategy is to use immunoglobulin fusion proteins of the extracellular domains for immunization and screen the serum and fusion on transfected cell lines expressing active forms of the target protein. A mock transfectant should always be made using the same vector and tag for use as a negative control cell line if flow cytometry will be used as the screening assay. If the target protein is internal, then shRNA/RNAi knockdown transfectants (immunoprecipitation [IP] screen) should be used as the negative control. In either case, the peptide, immunoglobulin fusion protein, or recombinant protein can be used for an initial screen or in parallel with the flow cytometry or IP screen. The blocking/activating property of the monoclonal antibody should be the basis of the secondary screen, once the number of samples has been reduced to a more manageable number.

Screening for Internalizing Antibodies. Internalizing antibodies are usually directed at cell-surface receptors, which internalize after they bind their ligand or are cross-linked by the ligand or an antibody molecule. Therefore, all monoclonal antibodies that bind the receptor at the same site as the ligand for that receptor will be internalizing antibodies. Similar strategies for designing the immunogen as described for blocking/activating antibodies can be used. The main difference is in the screening assay. At some point during the screening of antibodies determined to bind the receptor (usually a flow cytometry screen), the ability of the antibody to induce internalization needs to be ascertained. The best way to screen for internalization is by confocal microscopy. Comparing immunofluorescently stained cells at 37°C and 4°C using a confocal microscope will clearly show the internalization process (Fig. 7). Cell membrane fluidity and internalization are arrested at 4°C or in the presence of sodium azide. Staining the cells with the hybridoma supernatants at 4°C then warming them up to 37°C will initially show antibody staining on the cell surface that will move laterally in the cell membrane (a process called capping) to produce a cap of receptors that is internalized as the cells warm up. This process can be recorded. Antibodies that do not induce internalization will only show surface staining at both the colder and warmer temperatures. Screening to identify the internalizing antibodies should be performed as early as possible in the fusion screening process.

Functional Assays

In functional assays, the antibodies in the hybridoma tissue culture supernatants are used to induce a reaction, block a reaction, or as a molecular handle to deplete an essential component of a reaction

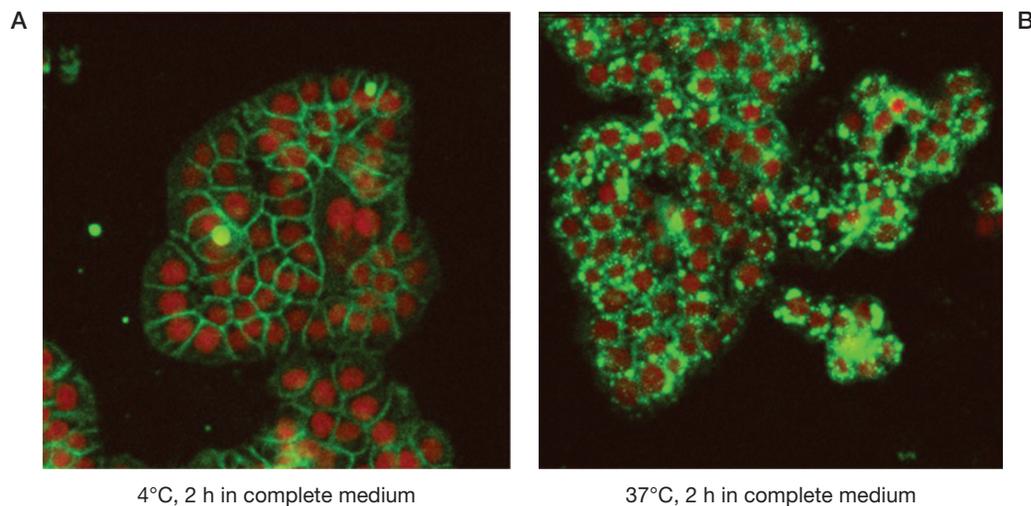


FIGURE 7. Cell-surface receptor internalization. (A) Cell-surface binding. (B) Internalization of surface receptor. The red dye is a nuclear stain (propidium iodide), and the green stain is from a fluorescein-tagged mAb against a cell-surface receptor. At 4°C, no internalization occurs. Once the temperature is increased to 37°C, internalization of the receptor can occur, dragging the mAb inside the cell. Cross-linking the receptor using the mAb triggers the internalization. (With permission, from Millenium Pharmaceuticals, Inc.)

mix. Any antibodies that are identified using these assays form an immediately useful set of reagents. However, the assays are difficult to perform in high throughput and interpret, and therefore are seldom used.

GENERATING HYBRIDOMAS

Although hybridoma production is the most discussed of the stages of monoclonal antibody preparation, most of the steps have been analyzed in enough detail that they are now routine. This stage is dependent on how well the previous stages of immunization and development of the screen have gone. A strong immune response and the use of a good screening method will make the production of the hybridomas an easier task.

Once a good immune response has developed in an animal and an appropriate screening procedure has been developed, the construction of hybridomas is ready to begin. For the actual fusion, antibody-secreting cells are isolated from the appropriate lymphoid tissue (spleen and lymph nodes), mixed with myeloma cells, centrifuged to generate good cell-to-cell contacts, and fused with the aid of a fusing agent like PEG (see Protocol 19), Sendai virus (see Protocol 20), or electric current (electrofusion) (see Protocol 21). The fused cells are then removed from the fusing agent, diluted into selective medium, and plated in multiwell tissue culture dishes. Beginning ~1 wk later, samples of the tissue culture supernatants are removed from wells that contain growing hybridomas and tested for the presence of the appropriate antibodies. Cells from positive wells are grown, single-cell-cloned, and frozen. Finally, the monoclonal antibodies are collected and used.

Hybridoma production demands good tissue culture facilities and a researcher with tissue culture experience. An experienced cell culturist will be able to perform the entire fusion procedure from removal of the lymphoid tissue to the plating of the final fused cells in <2 h. Little work is then required until the screening begins in ~1 wk. Screening is the most labor-intensive part of the entire project. The initial screening must be completed in ~1 wk before the hybridoma colonies become overgrown in the wells and start to die off. If the fusion has been successful, another 2 wk of tissue culture work will be needed until a suitable stage for a break has been reached. Do not begin hybridoma production without a good screening assay in place and the time needed for these operations.

Although resultant hybridomas are relatively easy to grow, in the first stages following the fusion, they may be particularly fragile and need extra care. Because they are the final result of a long series of operations and because they are produced as individual clones with no backup, the cells are quite valuable. Contaminated cultures cannot be recovered at the early stages.

Chapter 8 contains descriptions of the techniques used for growing and maintaining hybridoma and myeloma cell lines as well as lists of appropriate growth media and advice for dealing with low levels of contamination.

PREPARATION FOR FUSIONS

Before the time of fusion, several solutions must be prepared. In addition, unless you have purchased batches of fetal bovine serum (FBS) and PEG that have been prescreened by manufacturers for their use in fusions, these solutions should be qualified ahead of time (see Protocols 13 and 18). Some lots of FBS are better at supporting hybridoma growth than others. The key constituents that distinguish good batches of serum from bad are not known. Order test batches from several suppliers or purchase prescreened serum directly from the distributor.

Low-Density Growth Supplements

Fusion plates are typically seeded at low cell density (one to five cells/well) to allow individual hybridoma cells to be isolated and screened. Cells produce growth factors that help them survive and remain healthy. There are not enough cells present in each well of the fusion plates to provide the necessary environment to support good growth of the newly fused hybridoma cells unless some form of growth supplement is provided. This significantly increases the fusion efficiency and overall success of the fusion. Commonly used methods to enhance low-density hybridoma growth include the use of Hybridoma Cloning Factor, oxaloacetate pyruvate insulin (OPI solution) (see Protocol 16), recombinant murine IL-6, or the use of feeder layer plates.

Using Hybridoma Cloning Supplements

The idea behind the use of hybridoma cloning supplements is to replace feeder layers or other conditioned media and growth factors used in hybridoma generation. This increases the yield of hybridomas surviving hypoxanthine/aminopterin/thymidine (HAT) selection, the cloning efficiency of B-cell hybridomas, and the number of antibody-producing hybridoma colonies. There are many different supplements available commercially (e.g., from PAA Laboratories, Roche, MP Biomedicals, GENTAUR, and Santa Cruz Biotechnology). Each hybridoma supplement is slightly different in composition and should be used according to the manufacturer's instructions. Many of these supplements contain interleukin-6 (IL-6) as the active ingredient along with other growth supplements and nutrients to help hybridoma survival in low-density culture.

Preparing Recombinant Murine IL-6

Recombinant murine IL-6 can be added to the selection medium when plating out a fusion or cloning plate to enhance survival of the newly formed hybridoma cell or single-cell-cloned hybridoma subclones. IL-6 is an interleukin that stimulates myeloma/plasmacytoma growth. It has also been found to increase antibody secretion from some hybridoma cell lines. IL-6 exerts its biological activity in the concentration range of 10–100 units/mL (0.1–1 ng/mL). A concentration of 100 units/mL (1 ng/mL) is recommended for the preparation of B-cell hybridomas. Care should be taken not to use too much IL-6 because it can become toxic to the cells. Here too much of a good thing can be a bad thing!

Preparing Feeder Layer Plates

Cultured cells secrete growth factors that help each other grow and maintain good health. When cells are present in very low densities, these growth factors get diluted to the point at which they may be ineffective until the cells grow up in sufficient numbers to bring up the level of these growth factors. Feeder layer plates are prepared with cells that provide growth factors to support the growth of the hybridoma cells until they can expand in number and provide their own. Peritoneal macrophages, myeloma cells, splenocytes, and MRC-5 cells (a human lung fibroblast line) are the most common feeder layer cells used in hybridoma fusions. Methods for their preparation are found in Protocols 14–17.

Drug Selections

Hybridoma cell lines are selected by the addition of drugs that block the *de novo* synthesis of nucleotides (for details of the theory of drug selection, see Chapter 8). The myeloma cells have a defect in the nucleotide salvage pathway, making them dependent on *de novo* nucleotide synthesis. The most commonly used agents are aminopterin, methotrexate, and azaserine. All are effective agents to select against the growth of the myeloma fusion partner. When using aminopterin or methotrexate, *de novo* purine and pyrimidine synthesis is blocked, whereas azaserine blocks only purine biosynthesis. Consequently, aminopterin and methotrexate are supplemented with hypoxanthine and thymidine. Recipes for HAT and HT selection media are provided in Protocol 19. Azaserine solutions are supplemented with hypoxanthine.

Final Boost

Three to five days before the fusion, the immunized animal (mouse, rat, hamster, or rabbit) is given a final boost. This boost should be performed at least 3 wk after the previous injection. This interval will allow most of the circulating antibodies to be cleared from the bloodstream by the animal. Serum titers in the mouse begin dropping ~14 d after an immunization. Rabbits tend to hold high serum titers for a week or so longer than mice. If the levels of circulating antibodies are high, they will bind to the antigen and lower the effective strength of the boost.

The final boost is used to induce a good, strong response and to synchronize the maturation of the response. If this synchronization occurs, a large number of antigen-specific lymphocytes will be present in the local lymphoid tissue ~3–4 d after the boost. This will allow an increase in the relative concentration of the appropriate B-lymphocyte fusion partners. Consequently, the final boost should be directed to the source of the cell collection. In most cases, the spleen is the best choice for lymphocyte isolation, and, therefore, the final boost should try to localize the response to the spleen. This is best achieved by an intravenous injection or performed concurrently with an intraperitoneal injection. If your source of antigen is limited, a single intravenous injection should be used. Remember that the antigen solution should be compatible with an intravenous boost (no Freund's adjuvant, sodium dodecyl sulfate [SDS] concentration below 0.1%, urea below 1 M, etc.) (see Chapter 6). If the antigen cannot be injected directly into the bloodstream, an intraperitoneal injection should be used, and the fusion should be performed 5 d after the final boost.

In some specialized cases, for example, footpad injections or preparing IgA monoclonal antibodies, a regional lymph node may be the preferred site of lymphocyte collection. In the two examples given, the B-cell partners would be prepared from the inguinal node or from the Peyer's patches, respectively. The final boost should be given as close to the lymphoid organ(s) to be fused as possible.

Preparing the Partner Cells for Fusions

Before the fusion, the myeloma cells that will serve as fusion partners must be removed from frozen stocks and grown. The technique below is designed to place the cells in the best conditions for the fusion, but any tissue culture techniques that keep the cells healthy and rapidly growing in log phase

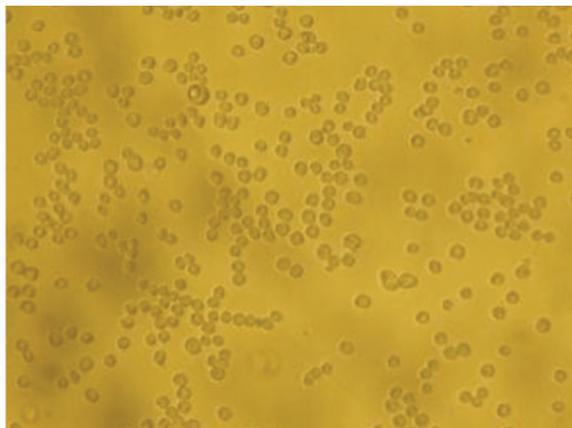


FIGURE 8. Myeloma cells in culture. (Reprinted from [http://en.wikipedia.org/wiki/File:J558L_\(Mouse_B_Myeloma\)_Cell_Line.jpg](http://en.wikipedia.org/wiki/File:J558L_(Mouse_B_Myeloma)_Cell_Line.jpg).)

are suitable. On the day of the fusion, the antibody-secreting cells (splenocytes or lymph node cells) are isolated from the mouse. Both types of cells are mixed together just before the fusion.

Preparing Myeloma Cells for Fusions

Myeloma cells should be thawed from liquid nitrogen stocks at least 6 d before the fusion. Longer times may be necessary if the viability of frozen stocks is poor. The myeloma cells should be checked routinely for mycoplasma contamination, particularly if your laboratory or your tissue culture facility has a history of mycoplasma problems (see Chapter 8). Any cells that test positively for mycoplasma should be replaced. Mycoplasma infections are not visible by light microscopy and require specific testing methods for detection. Fluorescent staining (4',6-diamidino-2-phenylindole [DAPI]), reporter cell kits, and PCR kits are available commercially to test for mycoplasma. Specialty laboratories are also available to screen cells for mycoplasma. The myeloma cells should be growing rapidly and healthy before the fusion (Fig. 8). For further details regarding the collection of myeloma cells for fusions, see Protocol 19.

Preparing Splenocytes for Fusions

For details regarding the collection of splenocytes for fusions, see Protocol 19. Figures 9–13 illustrate various stages of this procedure. If possible, at the time of the fusion, collect the blood from the immunized animal to use as polyclonal sera specific for your immunogen that can subsequently be

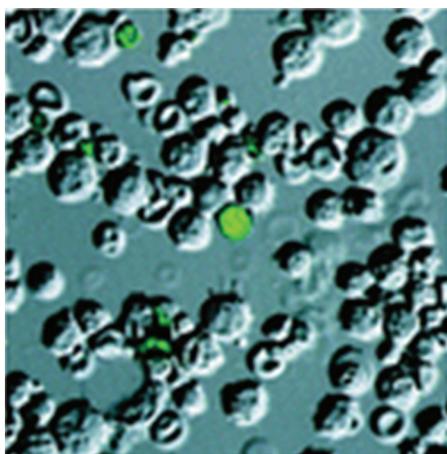


FIGURE 9. Spleen cell suspension. Spleen cell suspension containing B lymphocytes, T lymphocytes, blood cells, and splenic stromal cells. It is not possible to differentiate these cell types under standard light microscopy without staining for specific surface markers. (Reprinted from Arvola et al. 2000.)



FIGURE 10. Harvesting mouse spleen. (A) Mouse spleen in situ (tip of scissor). (B) Dissected mouse spleen. (B, Reprinted, with permission, from Liddell 2005, © Wiley.)

used as a positive control in the fusion screening assay. Collecting sera from laboratory animals is discussed in Chapter 6.

FUSIONS

Over the last 35 years, several variations in fusion techniques have evolved. Most are based on the techniques of Galfre et al. (1977) or Gefter et al. (1977) and Kennett (1978). Normally the fusion technique that is used does not make a major difference in the success of the fusion. Use the method that is easiest and most convenient. Fusion methods using PEG, Sendai virus, and electric current are described in Protocols 19–21.

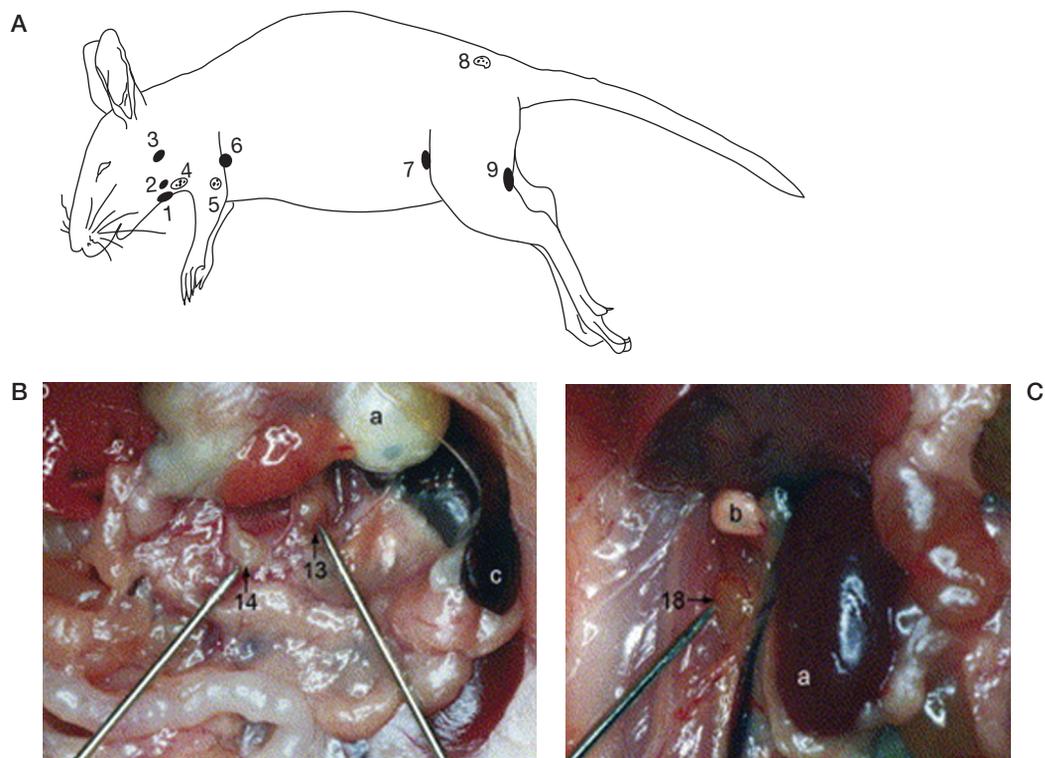


FIGURE 11. Harvesting mouse lymph nodes. (A) Lymph node sites in mouse. (B,C) Intra-abdominal lymph nodes (labeled “14” in B and “b” in C). (Reprinted, with permission, from Van den Broeck et al. 2006, © Elsevier.)

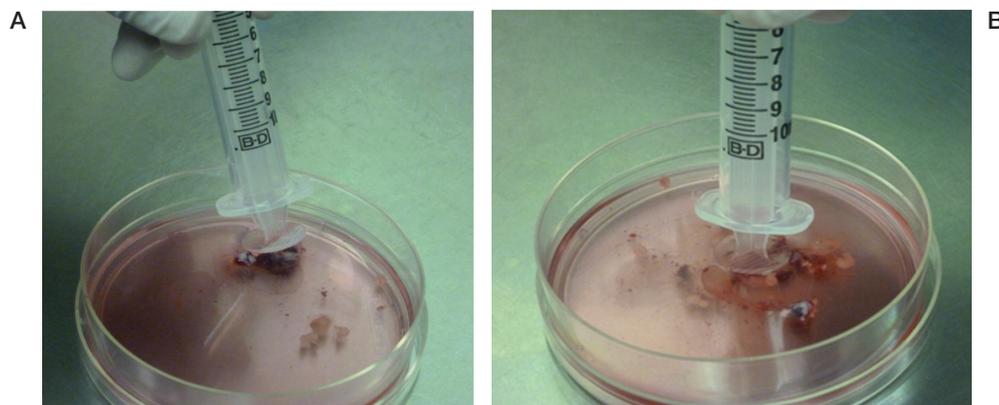


FIGURE 12. Preparing splenocytes for fusion. (A) A mouse spleen that is about to be spread apart using the plunger of a syringe to prepare a spleen cell suspension. (B) The spleen has been mashed up.

Although the original paper published by Köhler and Milstein in 1975 used inactivated Sendai virus as the fusing agent, most fusions are now performed with PEG. PEG is a dehydrating agent that aids somatic cell fusion by an unidentified mechanism. What is known regarding PEG-assisted fusions is that they work best when cell-to-cell contact is maximized and the environment is hydrophobic. PEG preparations often contain impurities that make it toxic to cells and will reduce fusion efficiency. Any PEG being considered for use in hybridoma fusions should be pretested (see Protocol 18). Reliable and inexpensive commercial brands of PEG certified for hybridoma fusion are available.

Sendai virus has been used for somatic cell hybridization since the early 1950s. Sendai is a paramyxovirus, also known as hemagglutinating virus of Japan (HVJ), which aids cellular membrane fusion at neutral pH. This process involves binding of the virus to the plasma membrane of the cell, a step that is mediated by the viral hemagglutinating/neuraminidase protein and the viral fusion protein F. The fusion activity resides at the amino terminus of the F protein in a hydrophobic region. Cell fusion is triggered when the F protein binds to a receptor on the cell membrane, which has a terminal sialic acid molecule, and it then penetrates the cell membrane. A Japanese company, Cosmo Bio Co., Ltd., has developed a kit (GenomONE-CF) in which the HVJ envelope protein is prepared from inactivated HVJ with its membrane-fusing ability intact (see Protocol 20).

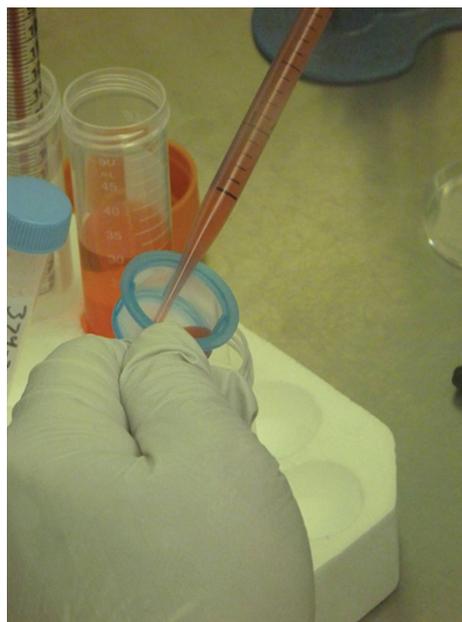


FIGURE 13. Removing clumps of cells. After the spleen cells have been made into a suspension (Fig. 12), they are passed through a cell strainer to remove clumps of cells and debris. B and T lymphocytes, as well as blood cells, pass through the strainer and are collected in a tube for fusion with myeloma cells to generate hybridomas.