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Hybridoma Technology for the Win

An In-Depth Introduction to Hybridoma-Based Antibody Development

White Paper

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Introduction

In 1975, Köhler and Milstein developed hybridoma technology in order to produce a never-ending supply of monoclonal antibodies[1]. This discovery enabled researchers all over the world to share their antibodies with one another, unlocking a vast amount of potential for biological research. Monoclonal antibodies (mAbs) are prized for their specificity, because unlike polyclonal antibodies, they only recognize one unique antigenic epitope. This high degree of selectivity favors the use of mAbs in a wide range of research, diagnostic, and therapeutic applications that frequently involve the identification, tracking, quantitation or pharmacological targeting of proteins of interest from complex biological mixtures.

Four decades in, hybridoma technology continues to be a leading and important method for mAb discovery and development. So much so that the majority of the ~80 FDA-approved mAb therapeutics are derived from mouse hybridomas[2]. And now, with the development of human-like transgenic animals, stable rabbit hybridomas, and additional methods for the humanization of mAbs from rare species, this number is only expected to increase for many more years to come[3–5].

In this white paper, we seek to equip readers with some basic knowledge on what hybridomas are, and how they are used for mAb production.

Hybridoma Generation for Monoclonal Antibody Production

A hybridoma is, as its name suggests, a type of hybridized cell that forms when an antibody-producing B-lymphocyte is fused with an immortal myeloma cell to generate an immortal B cell which can constitutively generate mAbs.

Hybridoma generation is a five-step process that takes advantage of a host animal's natural ability to generate functional, highly specific, high-affinity mAbs. In brief, the first stage involves the development and optimization of the antigen that a host animal is immunized with. Next, a host animal is immunized with the immunogen in order to elicit an immune response and initiate the process of B cell maturation. The third stage involves the isolation of these B cells from the spleen of the host animal and fusing them with immortal myeloma cells to generate hybridomas. During the fourth stage, the generated hybridomas are subject to multiple rounds of screening and selection in order to identify the hybridomas which produce the best mAbs for the intended downstream application. The fifth and final stage is the amplification of these specific hybridomas and subsequent antibody purification.

In this section, we further explore each stage of the hybridoma generation process.

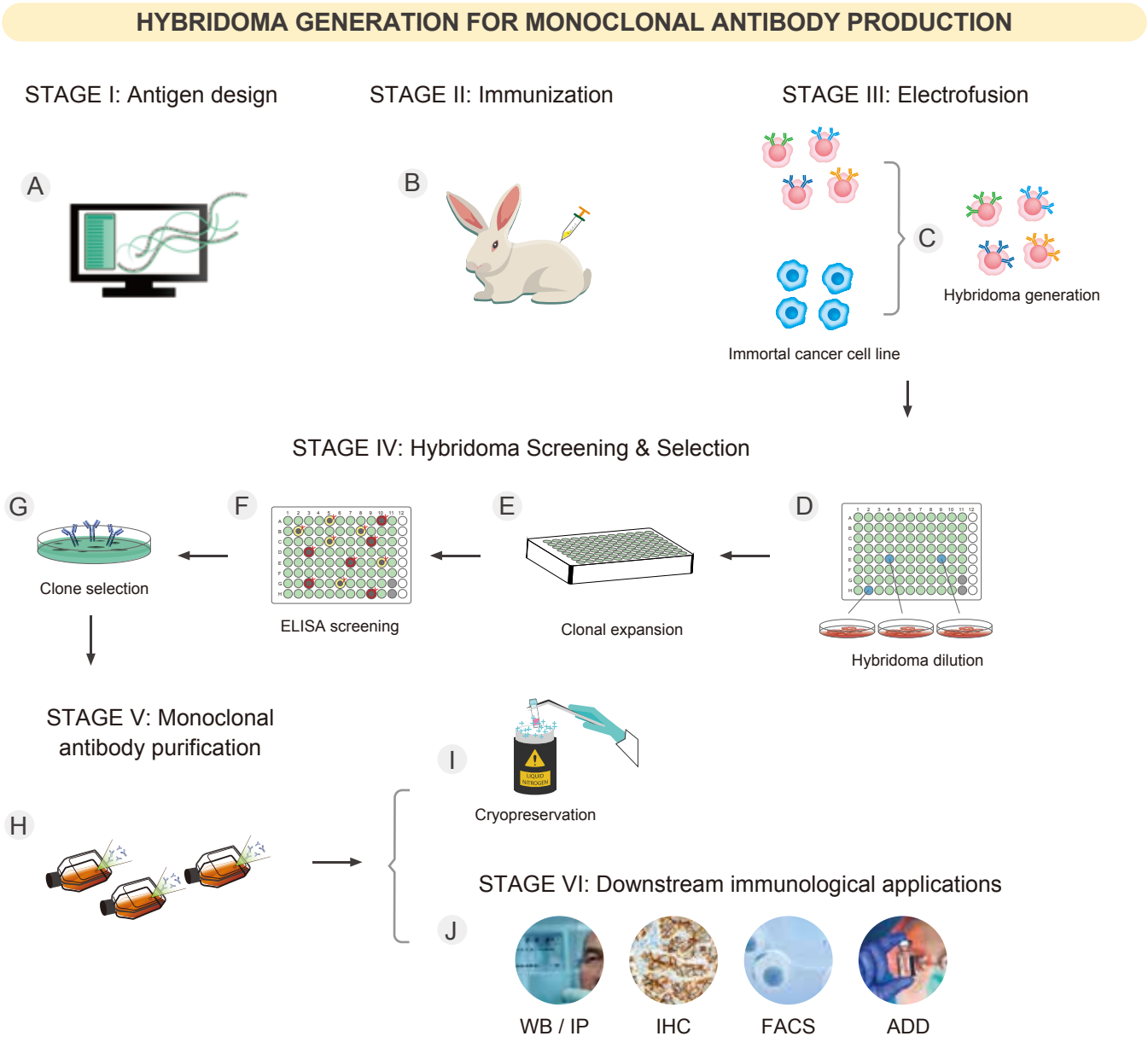

Figure 1: The different stages of hybridoma-based monoclonal antibody generation.
(A) Stage 1: The specific antigen is designed and generated. (B) Stage 2: A host animal is immunized with the antigen. (C) Stage 3: B cells are isolated from the host animal's spleen and fused with myeloma cells to form hybridomas. (D – G) Stage 4: The hybridomas are (D) diluted into selective culture medium and plated in multi-well tissue culture plates (1 clone/well), (E) individual hybridoma clones are allowed to expand, after which (F) tissue culture supernatants that contain monoclonal antibodies are screened via ELISA in order to (G) select hybridoma clones for stage 5. (H, I) Stage 5: Selected hybridoma clones are used for (H) monoclonal antibody purification, and hybridoma cell lines can also be (I) cryopreserved for future antibody production. (J) Stage 6: Purified monoclonal antibodies can be used in a wide variety of downstream immunological applications such as Western blot (WB), immunoprecipitation (IP), immunohistochemistry (IHC), fluorescence activated cell sorting (FACS), and antibody drug discovery (ADD).

Stage One: Antigen design

An antigen (Ag), or immunogen, is a foreign molecule, such as a protein, peptide, polysaccharide, small molecule, overexpression cell line, DNA fragment, or even an antibody drug, that is able to induce a host animal's immune response. The goal of which, is usually to generate antibody-producing B cells against that specific Ag.

A desirable Ag will have high immunogenicity as well as antigenicity. An Ag's immunogenicity, which refers to its ability to induce a host immune response, is closely tied to its antigenicity, which is defined as the ability of an antibody to bind to a specific site on the Ag known as the epitope[6]. An epitope's antigenicity is greatly dependent on characteristics such as structural conformation, amino acid sequence, and accessibility, all of which can significantly affect antibody binding. To make Ag selection more challenging, an Ag with high antigenicity may have poor immunogenicity. An example of this are certain small molecules, such as lipids and nucleic acids, which have a tendency to fail at meeting the size threshold (~10–20 kDa) necessary to activate the immune response. In this situation, these small molecules, also known as haptens, will have to be conjugated to a carrier protein, such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) to be rendered immunogenic[7,8].

Since successful Ag design can be quite a difficult process, many bioinformatic tools have been developed with complex algorithms to assist with generating a well-designed, immunogenic Ag. GenScript's **OptimumAntigen™** Design Program measures each peptide Ag against several protein databases to confirm the desired epitope specificity, and helps users avoid designing Ags against unexposed epitopes. Users can also specify their desired Ag cross-reactivity and explore application-tailored Ag conjugation and presentation options.

Stage Two: Immunization of host animal

Injection: Once an immunogenic Ag is prepared, it is used to immunize the host animal. While there are several different kinds of immunization strategies, in general, mice are intraperitoneally injected with the Ag at approximately 6 weeks of age. This immunization step can be repeated at 14, and 21 days after the first injection to boost the immune response and further encourage the development of mature, antibody-producing B cells. Most immunization methods, such as neonatal tolerization, drug induced subtractive immunization, and masking subtractive immunization, include an extra step that involves the administration of a tolerogen ahead of Ag immunization[9]. Tolerogens are cells, plasma membrane lysates, or proteins that contain multiple immunodominant epitopes. The goal of tolerization, is to allow the host animal's immune system to develop antibodies against the tolerogen in order to eventually recognize them as self-antigens. Then, when the actual experimental Ag is later administered, only mAbs specific to those antigenic epitopes will be developed rather than mAbs belonging to the native host species.

Besides the addition of tolerogens to the immunization protocol, adjuvants can also be co-administered with an Ag to elicit a more vigorous host immune response. There are many different types of adjuvants that can be used to enhance an animal's natural immune response, ranging from organic compounds like mineral oils and squalene to inorganic particulate aluminum salts[10]. Adjuvants exert their effects in several ways. First, they help to encourage the development of high antibody titers by retaining and extending the release of the Ag at the injection site, known as the depot effect, keeping the immune system continually stimulated[10]. Adjuvants can also mimic microbial pathogen-associated molecular patterns which are small structures on microorganisms that activate a host animal's innate immune response[11]. Additionally, particulate adjuvants can stimulate the adaptive immune response by inducing cells to release molecular signals associated with tissue damage, such as nucleic

acids, reactive oxygen species, and cytokines[12]. The initiation of the host animal's innate immune response subsequently activates the animal's adaptive immune response, and allows the immune system to develop a repertoire of Ag-specific antibodies in a faster and stronger manner.

Customizing an animal's immunization protocol is a delicate balance between scientific knowledge, experience, and luck. Many different decisions must be made in just the right combination in order to ensure the animal has a strong immune response to the Ag. Some of these decisions include the age of host animal, the immunization method, tolerization method, Ag concentration, ratio of Ag to adjuvant, immunization/boost schedule, test bleed titer, and much more. For these reasons, it is extremely important to consult with an antibody generation expert to design a suitable immunization protocol, rather than solely relying on the bioinformatics analysis of the Ag.

The immune response: Upon administration of the Ag or Ag/adjuvant combination, the animal's body begins to mount an immune response. This process is initiated when an Ag binds to a naïve B cell receptor, becomes internalized, and degraded[13]. Once degraded, the antigenic fragments will be presented on the outside of the naïve B cell through the major histocompatibility complex (MHC), resulting in the generation of Ag-presenting cells (APC) (APC's also regularly are macrophages, dendritic cells, and activated T cells)[13,14]. The antigenic peptides bound to the MHC will bind to and activate the T-cell receptor (TCR) of a neighboring T Helper cell (TH). Then, the activated TH cells differentiate into TH1 in order to assist macrophages in killing pathogens, or TH2, which secrete chemokines and cytokines to activate the differentiation of B lymphocytes[13,14].

In order to generate highly specific antibodies, TH2-activated B lymphocytes will undergo clonal expansion and differentiate into several different cell types. The first group of differentiated B cells are known as B lymphoblasts, which differentiate into short-lived plasmablasts (immature B cells) through a process known as the extrafollicular response[15]. Plasmablasts generate antibodies that possess only moderate affinity towards the Ag. However, these antibodies provide the host with immediate protection towards the foreign Ag. Plasmablasts can undergo further maturation, in which they migrate to a B cell follicle within a secondary lymphoid organ, and multiply rapidly to form a germinal center (GC)[15,16.] It is within the microenvironment of the GC where plasmablasts differentiate into plasma cells or memory B cells. Plasma cells undergo a complex antibody maturation process in order to secrete large amounts of extremely specific, high affinity antibodies, while memory B cells are responsible for assisting the immune system in generating a much faster immune response if the same Ag was to re-infect the body in the future[15].

High-affinity antibodies are developed within the GC via a process known as affinity maturation in which immature plasma cells modify their existing genomes to generate high affinity antibodies against their activating Ag. Affinity maturation takes place through a phenomenon known as somatic hypermutation (SHM), where a series of random mutations and rearrangements occur within the variable segments of the immunoglobulin genome[17,18]. The purpose of this rearrangement is to produce high-affinity antibodies against the B cell-activating antigenic epitope. In addition to SHM, antibodies also undergo a process known as class-switch recombination (CSR), where the modified antibody 'switches' immunoglobulin classes by swapping the DNA of their heavy chain constant region until the correct class (IgG, IgM, IgE, IgD, or IgA) is expressed[19]. Once the antibody sequence is optimized, immature plasma cells will either differentiate into mature plasma cells which begin secreting large amounts of mature antibodies, or into memory B cells which remain quiescent until Ag re-exposure leads to their activation and subsequent plasma cell differentiation[15].

Since the spleen is the key location within the host animal where B cell maturation and antibody development takes place, it is the organ that is harvested after an animal's immunization regimen for mature plasma cell isolation.

Stage Three: Cell fusion for hybridoma generation

After isolation, the B cells are fused with immortal cancer cells in order to form hybridomas. There are two primary ways to fuse these cells together – polyethylene glycol- (PEG-) mediated, or by electrofusion. The mechanism of action for PEG-mediated cell fusion is not well understood, but it has been posited that PEG, being a hydrophilic molecule, causes cells to aggregate and their cell membranes to become dehydrated[20–22]. In doing so, PEG increases the local concentration of cells, forces contact between their membranes and disrupts the packing between their connected monolayers, eventually leading them to fuse[20]. Electrofusion, on the other hand, is a highly optimized and reliable process that results in a greater number of successful hybridomas formed per unit of spleen cells (at least 4 times the fusion rate of PEG), making it the preferred method over PEG for hybridoma generation[23–27]. During electrofusion, B cells and cancer cells are mixed together and exposed to a pulsed electrical field of different voltages and frequencies. The first electrical pulse is an AC waveform that draws cells into close proximity and aligns them, the second pulse is a DC waveform that fuses the cells, and the third AC pulse maintains and encourages the completion of the cell fusion process[27].

Stage Four: Hybridoma screening & selection

After fusion, successfully formed hybridomas are isolated from their unfused B cell and cancer cell counterparts by growth in HAT medium (hypoxanthine-aminopterin-thymidine medium)[1]. Screening via HAT media works because only successfully fused hybridoma cells are able to survive more than a few days. This is because their unfused cancer cell predecessors have a mutation which leaves them without the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Due to this mutation, unfused cancer cells are unable to synthesize nucleotides through their de novo nucleotide synthesis pathway and their salvage pathway, and quickly die off within days after fusion. On a similar note, unfused B cells will also die off due to their natural inability to survive more than a day or two without the appropriate media and growth factors. Thus, after just a few days, fused hybridomas will be the only cell lines left alive to move forward with additional screening.

The surviving hybridoma cells are then diluted into multi-well tissue culture plates and subjected to a rigorous screening process to select for the highest specificity and affinity binders against the immunogen. There are several different stages throughout the immunization process where screening can be done before the antibodies are finally purified from the hybridomas. The enzyme-linked immunosorbent assay (ELISA) is a popular method used for screening antibody-Ag binding from hybridoma supernatants[28,29]. There are a variety of approaches using ELISA assays (e.g. direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA) to evaluate and select for antibody binding and affinity[30]. High-throughput, automated hybridoma screening methods have also been developed through the use of technologies such as Biacore™ Surface Plasmon Resonance (SPR), fluorescence-activated cell sorting (FACS), and microfluidics[31–34]. These techniques greatly increase the efficiency and accuracy of the screening process to select for antibodies with high Ag-specificity (Table 1)

Table 1: High Throughput Hybridoma Screening Technologies

Technology	Detection method	Protocol	Efficiency
Biacore™	Surface plasmon resonance antibody-screening using biosensors	mAb is immobilized from crude hybridoma supernatant onto flow cells, and antibody-Ag association/dissociation binding rate measured	Can obtain binding kinetics of ~200 antibody supernatants/day
Fluorescence-activated cell sorting (FACS)	Flow cytometer	Hybridoma cells are labeled with fluorescently-tagged Ag and screened (detection of intracellular & cell surface-bound mAbs)	Can screen >300,000 individual hybridoma clones/day
Microfluidic fluorescence-activated droplet sorting (FADS)	Flow cytometer	Hybridoma cells are labeled with fluorescently-tagged Ag encapsulated within droplets and screened (detection of intracellular, cell surface-bound, and secreted mAbs)	Can screen >300,000 individual hybridoma clones/day

After hybridomas are screened for antibody-Ag binding specificity and affinity, many researchers will further characterize their mAbs. The antibody attributes that are most commonly elucidated include antibody concentration, titer, isotype, epitope bin, and variable sequence. It is the combination of these characteristics, together with the results of the affinity and specificity analysis, which will determine the hybridoma clone that will be used for mAb production.

Stage Five: Monoclonal antibody purification

There are several ways to scale up and purify a mAb from a specific hybridoma clone. Some of these purification methods are listed in tables 2 and 3, such as from *in vitro* hybridoma culture supernatants, *in vitro*-based recombinant antibody expression, and *in vivo*-based ascites production[35]. Monoclonal antibodies purified via any of these methods can be used in a wide variety of immunological assays such as Western blotting, immunoprecipitation, ELISA, immunohistochemistry (IHC), flow cytometry, FACS, and much more. However, if the antibody needs to be constitutively generated over a period of time, hybridoma cell lines can be cryopreserved in liquid nitrogen for long term storage and eventual reconstitution.

Table 2: Methods of Antibody Purification

	Production method	Unpurified mAb Yield	Production details
in vitro	Hybridoma culture supernatant	1 – 3 mg/ml	-Hybridoma culture is expanded by incubating in spinner flasks or roller bottles (most common) at 37°C for 2 weeks -mAb-containing cell culture supernatant is isolated -mAbs are concentrated and purified using affinity purification methods (e.g. protein A or G affinity columns)
in vitro	Recombinant antibody expression	Ranges from <1 to >100 mg/ml Concentration varies widely depending on mAb, expression system, and method	-Hybridoma is sequenced to determine DNA sequence of variable antibody regions -DNA constructs are designed, synthesized and cloned into appropriate mAb expression vectors -Recombinant mAb expression vectors are transiently or stably transfected into desired expression system and expressed -mAbs are harvested, concentrated & purified using affinity purification methods
in vivo	Ascites fluid	2.5 – 10 mg/ml	-Hybridoma cells are injected directly into the peritoneal cavity of a mouse or rat -mAbs are produced at high concentrations in ascitic fluids in animal's abdomen -mAb-containing ascitic fluids are withdrawn -mAbs can be concentrated and purified using affinity purification methods

Table 3: Advantages and Disadvantages between Different mAb Production Methods

	Production method	Advantages	Disadvantages
in vitro	Hybridoma culture supernatant	-Straightforward, cost-effective, efficient process (mAbs in ~3 wks) -Minimal hands-on batch production protocol -Hybridoma cell-line can be cryopreserved for future supply of identical mAb clones	-May require processing of large volumes of culture medium to yield sufficiently high mAb concentrations for research applications (0.1 – 10 mg/ml)
in vitro	Recombinant antibody expression	-Generated mAb expression vector can be used for future mAb production -Can be high yielding depending on expression system, growth and purification conditions	-Expensive start up costs and technical experience required -Need to determine correct expression vector & expression system -mAb expression protocol must be established and optimized -Labor intensive and susceptible to technical errors associated with designing and producing recombinant DNA
in vivo	Ascites fluid	-Alternative method to produce mAbs from hybridomas that do not adapt to in vitro growth conditions -Efficient and simple way to generate mAbs at a high concentration in vivo (mAbs in ~3 wks)	-Requires animal handling -mAbs may contain endogenous mouse immunoglobulins and can be contaminated with mouse pathogens

Summary

In this white paper, we have covered the basic principles plasma cell maturation, as well as how hybridoma technology is used to produce mAbs. We here at GenScript believe that depending on your application, hybridoma technology is a highly optimized, valuable and cost effective platform for *in vivo* mAb generation, which we are confident to use when generating custom antibodies for customers just like you. For more information on our services, please visit www.genscript.com/custom-antibody-production-services.html.

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