Hybridoma Technology for the Win

A Direct Comparison Between Common Antibody Generation Platforms

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 explore some of the benefits of using the hybridoma technique, and consider some alternative approaches that currently exist for mAb generation. We also address some perceived misconceptions relating to hybridoma technology.

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 purification of monoclonal antibodies for use in downstream applications.

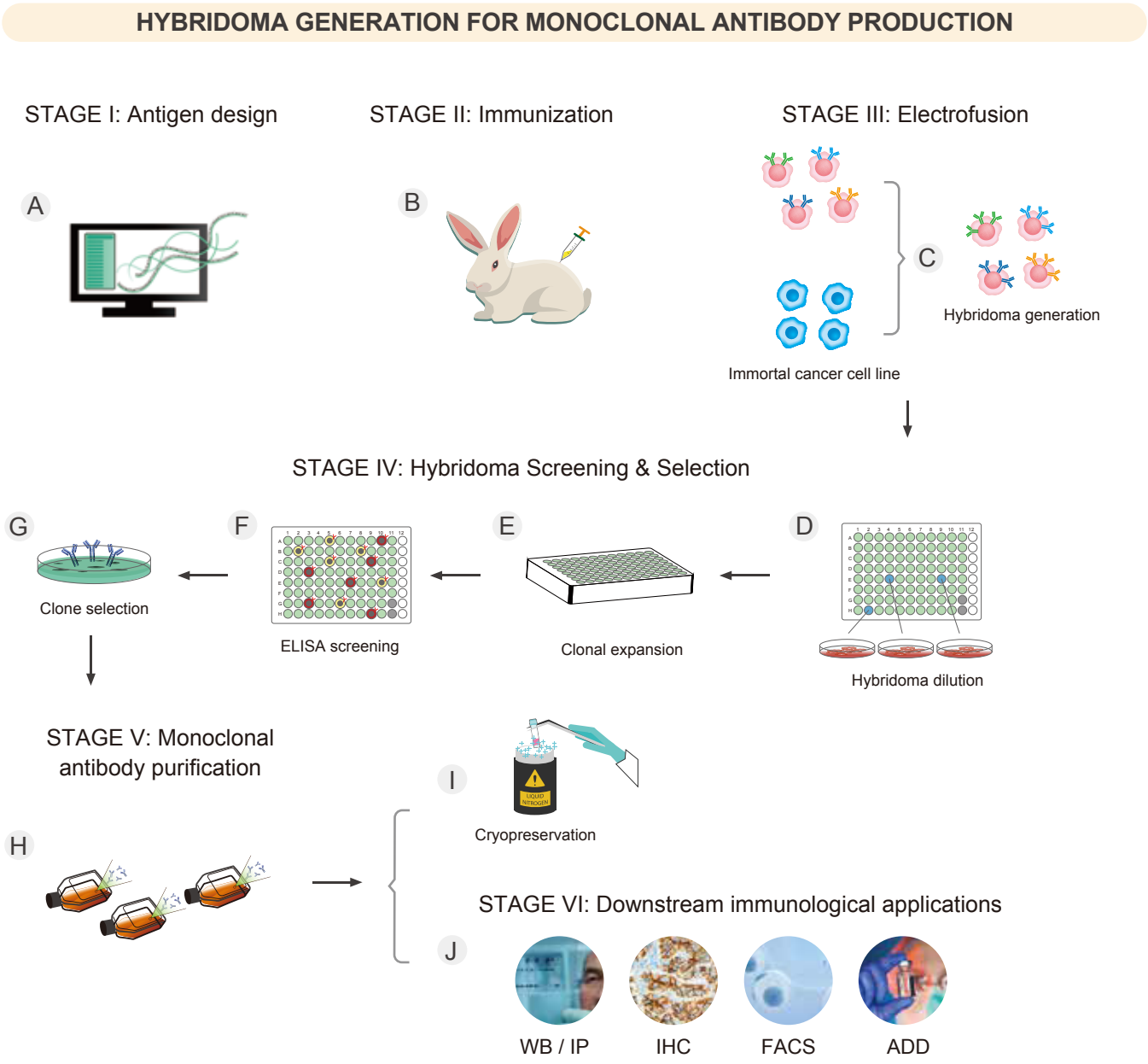

Figure 1: The different stages of hybridoma-based monoclonal antibody generation.
(A) Stage 1: The specific antigen is designed and generated. (B) Stage 2: A host animal is immunized with the antigen. (C) Stage 3: B cells are isolated from the host animal's spleen and fused with myeloma cells to form 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) Purified monoclonal antibodies can be used in a wide variety of downstream immunological applications.

The Benefits of Monoclonal Antibody Production using Hybridoma Technology

While newer technologies for mAb generation have been developed, as a mature and optimized technique, hybridoma technology is still the leading method for mAb discovery.

High specificity

Monoclonal antibodies are prized for their high specificity since they are directed against just one epitope. This increases the accuracy that the antibody has of detecting the protein of interest while decreasing the likelihood of cross-reactivity with other Ags, minimizing the incidence of non-specific background signals. However, the type of mAb generation technique can significantly affect an antibody's specificity towards its Ag.

Since hybridoma technology relies on mature B cells taken from secondary lymphatic organs, such as the spleen, any purified antibodies derived from these cells will have already gone through multiple stages of stringent genetic modifications within the host animal even before a researcher begins their own screening. During the 6- to 8-week immunization period, researchers can simultaneously screen the host animal's sera for antibody specificity, titer, and affinity as the animal optimizes its own response to the immunogen. Therefore, by the time B cells are isolated from the host animal's spleen, the animal would have already been screened multiple times to ensure that its immune response was as strong as it could be. It is only after the host animal's immune system undergoes a significant amount of antibody modification that its B cells are isolated and fused to form hybridomas for further antibody screening and characterization. By continually conducting specificity screening, from initial immunization through to hybridoma subcloning, hybridoma technology is able to naturally generate the highest specificity antibodies available.

Naturally high affinity through host animal affinity maturation

Natural affinity maturation is an extremely complex antibody refinement process that occurs within the GCs of the host animal. During affinity maturation, DNA encoding the variable regions of antibody chains are diversified through a combination of DNA rearrangements and hypermutations, mentioned previously as somatic hypermutation (SHM)[6,7]. Through SHM, mutations accumulate in the complementary determining regions (CDRs) of the variable genes, and the tightest binders to the Ag are actively selected for, resulting in the production of antibodies with high Ag affinity. In addition to SHM, several mammalian species (rabbits) and birds also go through gene conversion (GCV)[8]. GCV takes place when a dsDNA break occurs within the already rearranged V(D)J segments of an antibody's variable region. This dsDNA break is then repaired via a homologous recombination-like mechanism, introducing affinity increasing mutations in the process. SHM and GCV improve antibody affinity within the variable region, however the constant domain of the antibody also has to go through its own maturation process.

Newly generated antibodies will generally be of the IgM isotype, with a short half-life and limited functional capacity. In order to change the constant region, antibodies will go through class switch recombination (CSR), a process which facilitates isotype switching[9]. IgG is most common isotype used in biological research and drug development, however, CSR can generate antibodies from all five isotypes in order ensure that the antibody is equipped with the necessary effector functions and different biodistribution.

In vivo affinity maturation is critically important to natural antibody development. It is a dynamic, self-refining

process that always results in high affinity, functional antibodies of native conformation. Rabbit mAbs developed through natural affinity maturation can achieve Kd values in the 10^{-12} range. *In vitro* methods, such as phage display (discussed later), need to artificially engineer this affinity maturation step through the development of DNA mutant libraries and rigorous screening methods. Antibodies developed *in vitro* usually achieve Kd values in the 10^{-9} to 10^{-12} range.

Batch-to-batch consistency

Since hybridoma technology fuses antibody producing B cells with an immortal cancer cell line, the resulting hybridomas are able to constitutively produce a large population of mAbs, even after going through multiple rounds of cryopreservation, reconstitution, and subcloning. Rather than relying on antibody sequencing and recombinant expression, maintaining stable hybridoma cultures via cryopreservation is the preferred method for retaining batch-to-batch consistency, as many researchers believe natural antibody production results in a lower mutation rate and higher stability. Identical, functional antibodies are important in all aspects of experimental research where reliability and reproducibility of results is key. While less straightforward than cryopreservation, hybridomas can also be sequenced and have their DNA sequence form the basis for recombinant antibody (rAb) expression, though the parameters for rAb purification will likely need to be optimized.

Efficient production method

Hybridoma generation has prevailed as the top choice for mAb production for the past 5 decades. During this time, hybridoma technology has gone through multiple rounds of optimization in order to increase Ag immunogenicity, clonal numbers, and application-tailored specificity. Because of this, hybridoma technology is one of the most efficient methods currently available to generate high quality mAbs in a timely and cost effective manner. Specifically, mAb generation via hybridoma takes on average about 4 months and costs just a few thousand dollars. Newer mAb generation techniques, while each having their own advantages, can be more time-consuming, costly, and less optimized for generating mAbs of high specificity and affinity. Also, since most antibody maturation takes place within the host animal during the immunization process, generating mAbs from hybridomas is also significantly less labor intensive. Furthermore, since hybridomas are cell lines, they can be easily scaled up for high efficiency large scale mAb production through in vitro or in vivo methods, depending on the researchers' preferences.

Some Alternatives to Hybridoma Technology

In addition to hybridoma technologies, display libraries and single B cell sorting are two other popular methods that can be used for monoclonal antibody discovery. Each technology has its own strengths and weaknesses, but the decision for which method to use ultimately depends on the goal of each individual research project (Tables 1 and 2).

Combinatorial display technologies

Combinatorial display technologies, such as phage, ribosome, and yeast display, are all powerful ways to develop antibodies *in vitro*[10–12]. Established over 30 years ago, advances in these technologies have recently

gained momentum as a result of progress made in the fields of DNA/genome sequencing and high throughput biology.

In antibody phage display, large, randomized DNA libraries are constructed based on variable sequences from naïve, immunized, or synthetic B cell repertoires. Variable region containing antibody fragments, such as Single Chain Variable Fragments (ScFv) or Antigen-binding Fragments (Fab), are expressed on the surface of bacteriophages through E. coli-based transfection[10,13,14]. Once the display libraries are constructed, the Ag is washed over the antibody fragments and analyzed for high specificity and high affinity binders[15]. After the binders are identified, the variable domain sequences are cloned into an expression cassette and used for recombinant antibody production[10]. After the recombinant antibodies are purified they can be further refined by an unnatural form of affinity maturation, in which the positive binders are slightly mutated, screened, and analyzed again for specificity and affinity.

Advantages: Display technologies are a high throughput, animal-free method where a lot of control is given to the user to produce antibodies of great diversity and high affinity (Kd ~10^{-12} range). If DNA libraries are available, the same library can be used to generate new mAbs in a fast and efficient manner. Combinatorial display methods are also an alternative strategy for developing antibodies that are otherwise difficult to generate in animals. This difficulty might stem from Ags that have low immunogenicity in animals, those that contain subdominant epitopes, are toxic, or show self-recognition[10–12].

Disadvantages: A strong disadvantage of combinatorial display methods is that they can be expensive to execute if the library needs to be generated from scratch using host animal immunization. Generating a variable chain library can take an extended length of time as it is very important that the DNA library is large and possesses high sequence diversity in order to increase the chances of identifying strong Ag binders. Furthermore, even though display methods are high throughput, *in vitro* strategies to increase antibody affinity are costly, time consuming, and not always successful. In fact, unnatural affinity maturation and isotype switching in vitro can actually result in antibodies with weaker affinities, especially when antibody fragments are converted into full length immunoglobulins[16]. In contrast, natural affinity maturation and CSR *in vivo* actively selects for high affinity antibodies, eliminating the possibility of accidently reducing antibody affinity. Additionally as a method heavily dependent on molecular cloning techniques, combinatorial display libraries are highly susceptible to technical errors associated with plasmid generation and protein expression.

On top of the disadvantages of combinatorial libraries as a whole, each type of library comes with its own negatives. For example, phage display libraries are commonly prone to selection bias, where some antibody fragments are unable to bind to an Ag because they contain unpaired cysteine residues which form disulfide bonds and negatively affect antibody fragment display[17]. Another display format, synthetic libraries, can generate strong binders that may turn out to be immunogenic if used *in vivo*. This is because the sequences used in synthetic libraries are often highly mutated and not naturally present in the human or rodent Ab repertoire.

Single B cell antibody technologies

A newer strategy to develop mAbs involves directly screening and selecting for individual IgG-secreting B cells from the natural B cell antibody repertoire of humans and animals. This technique, known as B cell sorting, relies on the intact, in vivo immune system rather than on hybridoma cell lines or combinatorial display library generation[18–20]. Coupled with advanced, high throughput DNA sequencing technologies, such as next generation sequencing, it is possible to obtain natively paired antibodies with high *in vivo* specificity and affinity.

Depending on the antibody of interest, B-cell isolation can be done in a random fashion using FACS (most common), laser capture microdissection, immunospot assays on a chip, microengraving, or droplet microfluidics[18]. B cells can also be isolated in an Ag-selective manner using a variety of methods such as Ag-coated magnetic beads or fluorochrome-labeled Ags via multi-parameter FACS[18]. Once the target B cells are isolated, each individual cell goes through mRNA purification and complementary DNA (cDNA) is subsequently generated for sequence analysis and recombinant mAb expression.

Advantages: Single B cell technologies allow for the direct selection and isolation of functional mAbs from human and animal B cell populations that are otherwise difficult to produce using *in vitro* techniques. In particular, the strength of this technique is that it does not require a lot of cells, and B cells can be sorted according to their developmental stage based on their cell surface markers. As such, mature B cells, like plasma and memory B cells, can be specifically isolated using this method. Having already gone through the *in vivo* processes of affinity maturation and CSR, the antibodies produced by these cells already bear high affinities towards the Ag of interest and also retain their natural, cognate heavy and light antibody chain pairings. As a result, recombinant expression of antibodies using this method are likely to be stable and possess high Ag specificity and affinity.

Disadvantages: Single B cell technologies are most commonly used for therapeutic human antibody drug discovery because mAb development using this technique is extremely expensive, at times costing upwards of $100,000. In contrast, mAb generation from hybridomas can cost under $5,000. Also, since Ag-specific B cells are present at low frequencies in peripheral blood, it may be difficult to establish an initial population of leads for mAb development. Lastly, while it may be an efficient method, B cell sorting is a relatively new technology that is actively being optimized. Thus, the duration of mAb screening to recombinant expression using this method does not differ much from other currently available mAb development technologies.

Table 1: Advantages & Disadvantages between Different mAb Generation Methods

Method for mAb Development	Advantages	Disadvantages
Hybridoma Technology	-Mature, reliable, optimized technology -Uses natural *in vivo* affinity maturation to generate high affinity mAbs -Cost-effective and efficient (high yields, fast turnaround time, unlimited mAb supply) -Multiple opportunities to screen antibody pools during development process -Humanized transgenic animals available for therapeutic mAb development -Straightforward, mostly hands-off technique -Flexible: Direct or recombinant mAb purification	-Limited to immunogenic Ags -Requires animal handling -Inherent immunogenicity when derived from non-humanized transgenic hosts
Combinatorial Display Libraries	-Established technology where user has full control -Animal-free *in vitro* method -High throughput -Efficient & cost-effective if using pre-established phage libraries & screening methods -mAbs can be developed against non-immunogenic and toxic Ags -Excellent for optimizing antibody leads	-Expensive if phage libraries must be established -Dependent on DNA library quality, size & sequence diversity -mAb can only be characterized after recombinant expression -*In vitro* affinity maturation can result in loss of cognate chain pairing -Binding affinity loss possible during isotype switching -Selection process susceptible to affinity bias -mAbs may possess unnatural immunogenic sequences -Susceptible to technical errors during multiple rounds of screening, molecular cloning and recombinant antibody purification
Single B Cell Technologies	-mAbs are fully human -High throughput -Can specifically select for mature antibody-secreting plasma cells and memory B cells -Good for identifying rare antibodies	-Expensive, relatively new technology -Needs specialized equipment -Limited to certain types of Ags -Lack of Ag-specific B cells in peripheral blood -Requires recombinant protein expression

Table 2: Best Use Applications of mAbs based on Generation Method

Method for mAb Development	Best Use Application
Hybridoma Technology	-Research reagents (WB/IP, IHC, FACS) -mAb lead generation -Therapeutic antibody drug discovery (humanized transgenics)
Combinatorial Display Libraries	-mAb lead generation -mAb lead optimization -Therapeutic antibody drug discovery
Single B cell technologies	-Therapeutic antibody drug discovery

Common misconceptions about hybridoma technology

With all these different technologies for mAb generation available, it can sometimes be difficult to decide which is best suited for your research purposes. As a mature technology, it may appear that using hybridomas to generate mAbs is less desirable and will eventually be superseded by newer technologies. At this time we would like to address some of the misconceptions that can lead to reservations when considering the use of hybridoma technology.

Hybridomas take a long time to generate

In general, the entire process of hybridoma development, from Ag design, to immunization and mAb production usually takes between 4 to 6 months. However, depending on the Ag and immunization protocol, it is possible to generate hybridomas in as little as 8 weeks, with many options for screening and evaluating antibody quality over the course of the antibody generation process. *In vitro* technologies, like phage display, can be more efficient if high quality libraries and optimized screening protocols are already in place. However, the process of establishing combinatorial display libraries from scratch, to mAb screening and selection, through to recombinant expression, can sometimes take up to 8 months. Furthermore, with display technologies, it is only after mAbs are recombinantly expressed that the quality of an antibody can be evaluated. Therefore, there is a significant chance that display libraries can take longer than hybridoma-based mAb generation, without the tremendous benefit of a natural affinity maturation process.

In vitro approaches do not require the use of animals

Animal handling is an indispensable part of the hybridoma generation process. Housing animals in a research facility can be costly, as well as a large time and administrative commitment. Outsourcing mAb production is a cost effective and convenient option that eliminates the need for researchers to have access to an in-house vivarium, yet still be able to generate the desired mAbs through an *in vivo* approach. There are many ways to outsource the animal housing and immunization steps at dedicated research facilities, while still reaping the benefits of harnessing an animal's natural immune system to generate specific, high affinity, functional mAbs. For example, GenScript's animal research facility has Office of Laboratory Animal Welfare (OLAW) Assurance, an Institutional Animal Care and Use Committee (IACUC), as well as AAALAC accreditation.

In vitro approaches are more diverse and allow for screening of unnatural sequences

It is true that *in vitro* approaches harnessing diverse libraries can select for unnatural antibody fragments that possess a high affinity towards an Ag. However, this method is not without its drawbacks, as these unnatural sequences can lead to a loss in natural cognate antibody chain pairing and disrupt antibody function. *In vitro* display methods are also prone to affinity bias due to the presence of unpaired cysteine residues, which disproportionately form disulfide bonds and interfere with the antibody screening and selection process. Additionally, if the antibody is destined for an *in vivo* use or application, there is a chance that the unnatural antibody sequences may be immunogenic and therefore unsuitable for use.

It isn't possible to generate fully human antibodies in animals

Hybridoma development, despite being a mature technology, still holds great potential for the optimization and development of therapeutic mAbs. Increasingly, transgenic animals expressing human immunoglobulins are being developed and utilized to generate mAbs for clinical use. The therapeutic mAbs generated by these 'humanized' animals have reduced immunogenicity, compared to chimeric or murine antibodies, and are more likely to lead to FDA approval success, further emphasizing the ongoing relevance and importance of the hybridoma platform. While single B cell technologies allow for the isolation and development of fully human antibodies, they are dependent on human peripheral blood donors and limited to the types Ags that the donors have been exposed to.

Summary

In this white paper, we covered the basic principles and benefits of using hybridoma technology to generate mAbs. We also compared the hybridoma technique to two other popular mAb generating technologies, combinatorial display libraries and single B cell sorting, in order to shed some light on the strengths and weaknesses of each technology for various applications. Finally, we cleared up some misconceptions relating to the use of hybridomas to generate 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 that 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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