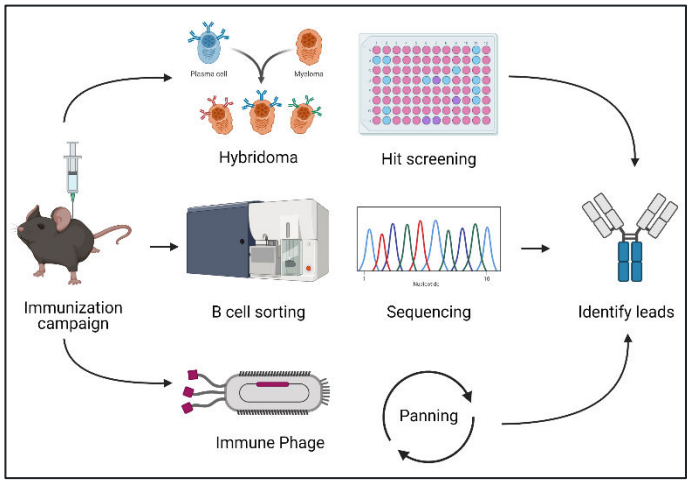
Alloy Therapeutics

Approaches to binder recovery following immunization campaigns in mouse models

When embarking on an antibody discovery campaign, the choice of binder recovery system is central to the proposed workflow. While the end goal of each approach is the same—to recover sequences that encode for correct heavy- and light-chain paired antibodies with binding specificity for your target of interest—each individual path to identifying these lead candidates brings its own unique strengths and weaknesses. The choice of binder recovery approaches for your discovery campaign can be informed by numerous factors, including the nature of the target antigen and/or the technical capabilities available to your research program. Each of the three approaches discussed here—hybridoma generation, single B cell sorting & sequencing, or immune phage libraries—can be utilized to successfully recover high-quality binders for further pre-clinical research and development. By using multiple approaches in parallel, you can potentially increase the sequence diversity recovered from a single *in vivo* immunization campaign, leading to a larger and more diverse panel of potential leads to test for your desired characteristics.

Which approach is best for my program?



During an antibody discovery campaign using *in vivo* immunization methods, once seroconversion to your target antigen has been confirmed in your animal cohort, the next step is to plan for recovery of antibody sequences. This can be accomplished using three general approaches: generation of antibody-secreting *B cell hybridomas*, isolation of target-specific B cells using *fluorescence-activated cell sorting (FACS)* coupled with *single-cell sequencing*, or generation of *immune bacteriophage libraries* from genetic material recovered from lymphoid tissues of immunized animals.

Hybridomas

Process: Spleen and/or lymph node cells are fused with myeloma cells to generate immortalized cells, which are then screened for the production of target-binding antibodies. Hybridoma technology is a time-tested approach to binder recovery from immunization campaigns, and while the recovery

process may be long and labor-intensive, it can reliably generate a large panel of potential leads from any manner of antibody discovery campaign.

Benefits: By coupling hybridoma generation with cell culture methods such as limited dilution cloning or culture in semi-solid media, the hybridoma process can yield monoclonal antibodies with relative ease. Additionally, as long as the cloning procedure was performed correctly, these antibodies will retain their correct heavy- and light-chain pairing, as well as any post-translational modifications that improve stability and limit aggregation.

Potential drawbacks: The production and screening of hybridomas can be a time-intensive process, especially when tasked with screening hundreds of potential clones for target-binding antibody production.

B cell sorting

Process: B cells are isolated from single cell suspensions of lymphoid tissue based on their ability to produce antibodies specific to your target of interest. This is often accomplished using FACS with a fluorescently labelled target antigen as the staining reagent, along with other fluorescent antibodies for identifying particular B cell lineages.

Benefits: Once isolated to high purity, target-specific B cells can be processed directly for antibody sequencing, as the sorting has already served as the target-binding screening step in this binder recovery process. This allows for more rapid progression from cell isolation to binder recovery compared to the extended culture times required for hybridoma screening.

Potential drawbacks: The ability to perform B cell sorting is critically dependent on the nature of your target antigen, requiring extensive validation of the proposed sorting process prior to its use for a specific antibody discovery campaign. For example, if your target is a soluble protein or a single pass transmembrane protein that can be expressed as a soluble construct (e.g. Fc-fusion), then you should be able to generate fluorescently labelled antigen as a FACS reagent. However, if your target is more challenging, such as a multi-pass transmembrane receptor, the ability to generate soluble FACS reagents may be limited. Additionally, B cell sorting methodologies often require antibody display on the surface of cells in the form of B cell receptors, meaning that sorting methodologies often focus on memory B cells, unlike hybridomas that can also encompass antibody-secreting plasma cells. Newer technologies are becoming available that allow for sorting of plasma cells based on the target-binding specificities of their secreted antibodies, but these approaches may not be available to all research programs.

Immune phage library

Process: mRNAs encoding antibodies can be directly recovered from lymphoid organs and used to build a library of variable regions for display on the surface of bacteriophages, usually in either the Fragment antigen-binding (Fab) format or as single-chain fragment variables (scFv). These antibody fragment-displaying phages can then be “panned” against your target of interest to enrich phages that express variable regions with target-binding properties.

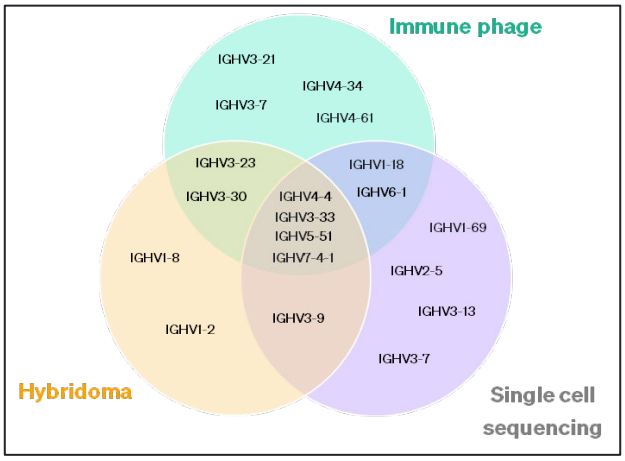
Benefits: Immune phage display allows for enrichment of particular target-binding properties during the panning process—for example, binding to particular epitopes on your target, or cross-reactivity to multiple species. Panning can also be used in a subtractive fashion, such as to enrich for antibodies that bind only one variant of a larger related family of proteins. Phage libraries can also be run at a higher throughput than hybridoma or single B cell, potentially yielding more target binders in a shorter amount of time. Additionally, immune phage libraries can be interrogated multiple times in future panning rounds to recover additional potential binders. Immune phage libraries can also be leveraged for *in vitro* affinity maturation of antibodies, a powerful tool for improving the binding affinities of potential leads.

Potential drawbacks: As immune phage libraries rely on display of Fab or scFv versions of full-length antibodies, there may be unforeseen developability issues with identified leads when they are re-formatted back to full-length antibodies. In addition, as the library generation process randomizes the heavy- and light-chain pairing, there is an increased risk that leads identified through phage panning may lead to less well-behaved full-length antibodies.

Considering that each unique approach has specific strengths and weaknesses, how do you choose the approach that is best for your antibody discovery program? As mentioned above, the nature of your target antigen is often the defining factor. If you have a well-behaved antigen that can function as a soluble construct, single B cell sorting is a powerful approach. However, if your target antigen is more challenging, hybridoma or immune phage may be the optimal approach. Ultimately, it is important to recognize that no single approach is necessarily best for identifying the best diversity of potential leads.

A multiplexed approach to binder recovery can yield the broadest antibody diversity from a single campaign

In the example shown at left, a single antibody discovery campaign performed in-house by Alloy Discovery Services utilized all three distinct approaches discussed above—hybridoma, single B cell sorting and sequencing, and immune phage display. Following the completion of all three binder recovery approaches, the heavy chain germline usage of the best clones was compared, with heavy chain germlines common to multiple approaches identified in this Venn diagram. As you can see, while there were numerous heavy chain germlines that were common to multiple approaches, including



four distinct IGHVs that were represented in all three, many heavy chain germlines were only identified when individual approaches were performed. In this example, four distinct heavy chain germlines were unique to immune phage libraries, another four set of germlines were distinct to single B cell sorting, and two germlines were only recovered via hybridoma generation. Therefore, by utilizing all three distinct approaches to binder recovery in parallel, the greatest diversity of germline gene usage was obtained. These results highlight the importance of taking a multiplexed approach to binder recovery—even by running two of the three discussed recovery approaches, your program can significantly increase the potential for antibody diversity in your panel of potential leads.

ATX-Gx™ Platform

As shown in the above example, the ATX-Gx™ mouse platform is amenable to each of the specific binder recovery methodologies discussed here. Our internal Alloy Discovery Services team has extensive expertise in multiple binder recovery technologies and is working to expand our internal toolkit of state-of-the-art approaches for target-specific B cell recovery and antibody sequencing. When coupled with the diverse suite of transgenic animals available within the ATX-Gx™ mouse platform, including animals with diverse MHC haplotypes and those with human kappa or lambda light chains, the ATX-Gx™ platform can enable your antibody drug discovery program for success.

ATX-GK BL/6		Complete functional human Gamma heavy chain and Kappa light chain on a BL/6 background (MHC Haplotype H-2b); custom KO strains available.
ATX-GK BALB/c		The same ATX-GK antibody diversity on a BALB/c background (MHC Haplotype H-2d).
ATX-GK MIX		Complete functional human Gamma heavy chain and Kappa light chain on a mix BL/6 & BALB/c background (MHC Haplotypes H-2b & H-2d).
ATX-pGK		First half of human Gamma heavy chain with full Kappa light chains on a BL/6 background to limit immunodominance.
ATX-dGK	Q4-21	Second half of human Gamma heavy chain with full human Kappa light chains on a BL/6 background to limit immunodominance.
ATX-GL		Complete functional human Gamma heavy chain and 21/30 Lambda light chain genes on a BL/6 background.
ATX-HYPERIMMUNE	Q4-21	ATX-Gx™ strain genetically engineered to produce a diverse Ig response to high homology targets.

Alloy Therapeutics is a biotechnology ecosystem company empowering the global scientific community to make better medicines together. Through a community of partners, Alloy democratizes access to tools, technologies, services, and company creation capabilities that are foundational for discovering and developing therapeutic biologics. The company facilitates affordable, non-exclusive access to the entire drug discovery community from academic scientists, small and medium biotech, to the largest biopharma. Alloy's lead offering, the ATX-Gx™ platform, is a human therapeutic antibody discovery platform consisting of a growing suite of proprietary transgenic mice strains. Founded in 2017 and privately funded by visionary investors, Alloy is headquartered in Boston, MA with European labs in Cambridge, UK. As a reflection of Alloy's relentless commitment to the scientific community, Alloy reinvests 100% of its revenue in innovation and access to innovation.

Your Lab

- We send the ATX-Gx mice directly to your lab for immunizations; mice ready to ship immediately
- Compatible with various specific-pathogen-free requirements
- 24/7 support from our partner success team to help with experimental design and to troubleshoot any of your problems during discovery

Certified CRO or Partner

- We send the ATX-Gx mice to your Certified CRO of choice
- Certified CROs: Over 18 certified CROs across North America, Europe and Asia, each offering distinct discovery capabilities/advantages
- Alloy can certify a licensee's preferred partner upon request

Three ways to access the Alloy mice

Alloy Discovery Services

- Work directly with Alloy's in-house Discovery Services team in Boston, MA or Cambridge, UK for your immunizations and discovery work
- Alloy Discovery Services is available only to Alloy Platform Licensees
- Our services team is highly aligned with your long-term drug discovery goals

Flexible offerings for all levels of antibody discovery capabilities

Regardless of your choice, you will always have access to Alloy's best practices and our highly dedicated scientific teams in the US and UK

Schedule a 15-minute call to learn more: www.alloytx.com/bd