AlivaMab DISCOVERY SERVICES

Generating Diverse Antibodies Against Multi-Spanning Membrane Protein Targets

A Case Study Using AlivaMab Discovery Services' Propriety Technologies to Target G-Protein-Coupled Receptors (GPCRs)

Abstract

Multi-spanning membrane proteins are the target for over half of all FDA-approved drugs. They continue to play an outsized role in drug discovery and represent some of the most important protein families, including G-protein-coupled receptors (GPCRs) and ion channels. Given the size of membrane protein families and the need to target individual multi-spanner proteins with low off-target effects, many companies have sought to pursue selective and potent antibody therapies instead of small molecule drugs. However, it can be difficult to generate sufficient drug-quality antibody hits using traditional immunization and hybridoma workflows and even more difficult using *in vitro* display technologies. To address this need, AlivaMab Discovery Services (ADS) has developed strategies that can generate large, high potency hit panels in less than half the time required by standard immunization and *in vitro* display methods. Below, the AlivaMab Discovery Services approach to antibody discovery is applied to multiple GPCRs, resulting in rapid production of diverse high-affinity antibody hits.

Through a project targeting multi-spanning membrane proteins, AlivaMab Discovery Services delivered:

- Immunization strategies that generated potent antibody responses
- Recovery of large hit panels with high affinity and functional antibodies with fully human variable regions
- Wide epitope diversity resulting in multiple distinct leads
- Faster turnaround compared with *in vitro* display approaches

Antibody Drug Discovery for Multi-spanning Integral Membrane Proteins: A Challenging Yet Critical Therapeutic Target

The antibody therapeutics market is expected to generate a global revenue of $300 billion by 2025.[1] Its continued growth reflects its critical role in treating a number of illnesses, including inflammatory, metabolic, and infectious diseases, and various cancers.[2] A lot of opportunity remains, particularly as new targets and targeting approaches are developed and validated.

One key area at the forefront of antibody drug development is the targeting of membrane proteins, particularly those with multiple transmembrane domains (referred to as complex integral or multi-spanning membrane proteins). Incredibly, membrane proteins make up ~23% of the human proteome, yet represent >60% of current drug targets, largely due to their accessibility and role in modulating cell signaling pathways.[3] Of these, a few multi-spanning membrane proteins families are particularly prominent, namely G-protein-coupled receptors (GPCRs), ion channels, and transporters.[4,5] Therapies targeting GPCRs and ion channels represent one-third and one-fifth of all FDA-approved drugs, respectively.[6,7]

Membrane proteins were historically targeted with small molecule drugs. However, the specificity of antibodies offers a tremendous advantage when targeting specific multi-spanning membrane proteins, especially within large protein families (there are ~800 known GPCRs and ~400 known ion channels).[4] The lack of off-target effects, coupled with low toxicity and long residence times, makes antibody therapies even more appealing.[8]

Generating a diversity of potent antibody drug candidates is a challenge for multi-spanning membrane proteins, due to their commonly low expression, low immunogenicity, and highly conserved sequences.[9] Arguably, the biggest hurdle is generating high quantities of properly folded recombinant multi-spanning membrane proteins to use as antigens during immunization.[4,9] Multi-spanning membrane proteins usually require a lipid membrane environment for proper protein folding and stability. Maintaining the functional native conformations of these proteins when used as antigens is critical to ensure that the antibodies produced can recognize the membrane protein in its true state *in vivo*.

The AlivaMab Discovery Services Approach

AlivaMab Discovery Services has developed two proprietary immunization strategies (**Figure 1**) that circumvent challenges associated with traditional recombinant membrane protein-based immunizations.

Both immunization strategies for multi-spanning proteins make use of the AlivaMab® Mouse (offered by Ablexis, LLC),[10] which is specifically designed to rapidly generate large panels of exceptionally diverse, high-affinity antibodies with human variable regions. These AlivaMab Mouse Prime Diversity (AMMPD) immunization strategies save significant time by eliminating the need to express and purify large amounts of target protein.

For AMMPD-DNA (**Figure 1**, Top), instead of purified recombinant protein, the team genetically introduces the antigen into the AlivaMab Mouse and expresses it using a proprietary vector that sustains high expression *in vivo*. This provides native expression of the correctly folded antigen, enhanced immunogenicity, and prevents the genes of interest from being silenced. In AMMPD-VAC (**Figure 1**, Bottom), a mouse tumor cell line (syngeneic to the AlivaMab Mouse) is transfected with the target antigen gene with a different proprietary vector that elicits high antigen expression and enhances the likelihood of a robust immune response. These antigen-expressing cells are then used to generate a tumor in AlivaMab Mice, which cause the mice to mount an immune response

against the tumor with the only foreign element being the target antigen. The tumor itself acts as a excellent adjuvant, making AMMPD-VAC a particularly powerful method for generating strong and diverse immune responses. Furthermore, tracking tumor regression is a useful mechanism for identifying mice with strong target-specific antibody responses.

To access the collective power of these two multi-spanner immunization strategies, both were used to generate diverse antibody candidate libraries targeting multiple human GPCRs (referred to as GPCR-A, -B, and -D).

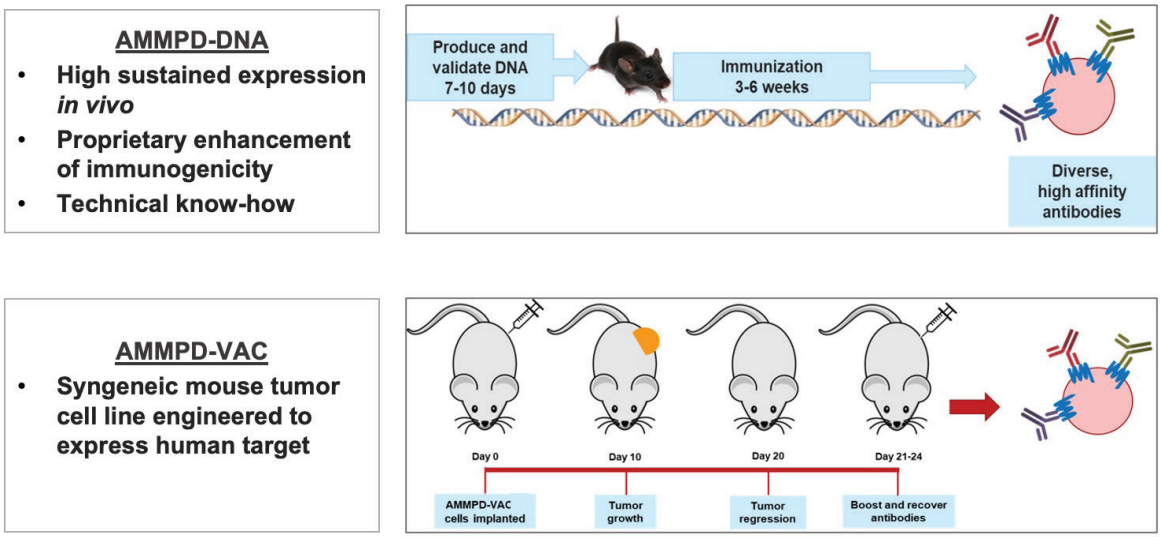

Figure 1: ADS's two proprietary immunization systems (Top: AMMPD-DNA and Bottom: AMMPD-VAC) for generating antibodies against multi-spanning membrane proteins.

High Titer Antibody Generation Against GPCR Targets Using AMMPD

For any antibody generation project, evaluating immunization success requires an understanding of serum antibody titers. Ideally, very dilute serum samples still exhibit a detectable response towards the target antigen, indicating a robust immune response. Both AMMPD-DNA and AMMPD-VAC produced mouse sera with high titer anti-GPCR antibody activity against cells expressing each GPCR (**Figure 2**). Flow cytometry-based serum binding studies indicated that separate AMMPD-DNA immunizations could generate potent antibody titers in response to both GPCR-A and -B antigens (**Figure 2**, GPCR-A & GPCR-B). In both cases, flow histograms collected using dilute serum samples were significantly shifted relative to parental cell controls not expressing the GPCRs. For AMMPD-VAC immunization against GPCR-D, titers were even higher. Impressively, the highest AMMPD-VAC serum dilution (1:43,740) still led to a log shift in the flow histogram compared with the parental cell control at the same dilution (**Figure 2**, GPCR-D). This titer analysis shows the capacity of these two immunization strategies to generate potent immune response against multi-spanning membrane proteins. It also shows that these approaches work on multiple different target proteins.

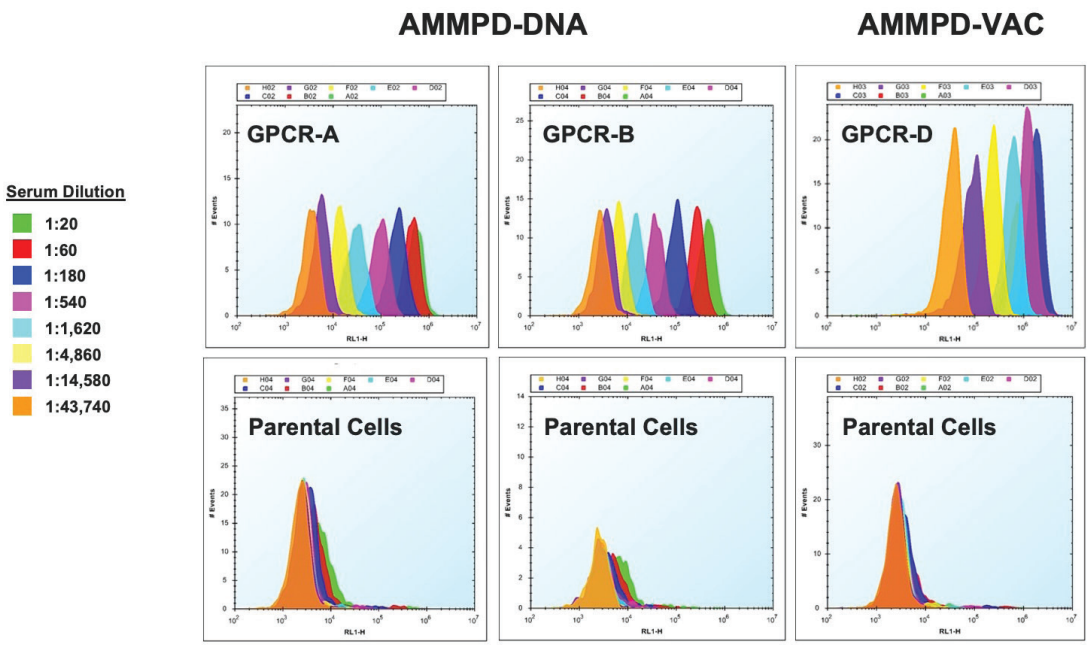

Figure 2: AMMPD Immunizations Quickly Produce Strong Anti-GPCR IgG Titers in Mouse Serum

AMMPD Strategies Produce Large Panels of Antibody Hits with Therapeutic Quality Potency

After measuring serum titers, the next step is to recover antibody-expressing B-cells, use them to form hybridomas, and screen them for binding to the target antigen to identify panels of confirmed hits against each unique antigen. While traditional hybridoma strategies and *in vitro* display systems tend to produce a few dozen hits per iteration, the AMMPD strategies generated hundreds of confirmed hits for each GPCR.

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ADS' immunization workflows are also highly amenable to rapid expansion. Ultimately, this enables even larger antibody panels with thousands of hits – without significantly increasing the project timeline.

While promising, the question remains: Are these hits viable drug candidates? To assess potency for each antibody isolated from the respective serum samples, functional assays were performed to determine both 50% effective concentration (EC_{50}) for binding to cells expressing the target antigen and 50% inhibitory concentration (IC_{50}) for antibody antagonist activity. These assays were performed using comparator antibodies to help contextualize their efficacy in relation to existing commercial and therapeutic antibodies.

The high potencies observed in the sera dilutions are maintained in many of the recovered individual antibody samples (two examples shown in **Figure 3** and **Table 1**). Importantly, in these cases, antibody hits demonstrate strong affinities (EC_{50} in low pM range) and functional activity (IC_{50} = <nM) similar to – or even better than – comparable therapeutic antibodies that binds the same GPCR (**Table 1**). Taken together, this approach results in excellent antibody recovery from immunized AlivaMab Mice, large hit panels, and numerous highly potent candidate antibodies against multi-spanning membrane proteins.

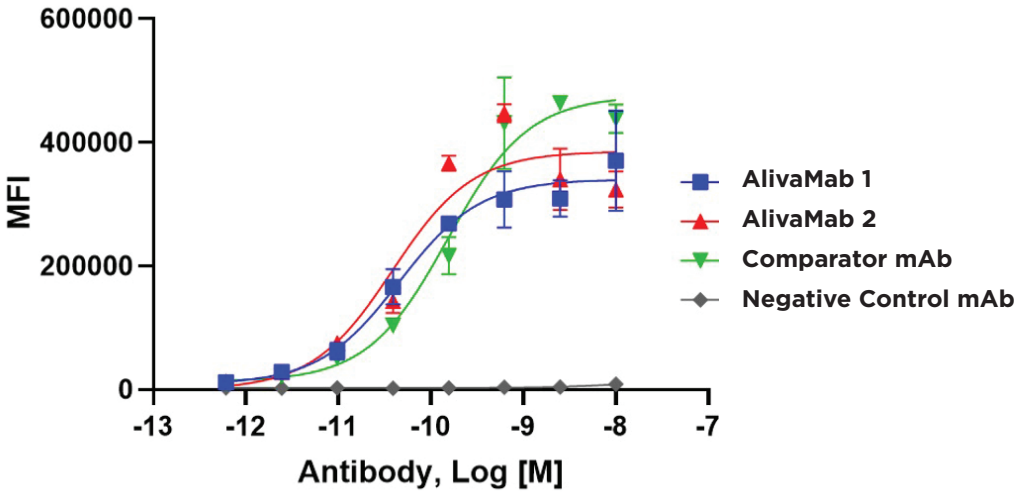

Figure 3: Functional Assay Performance of Two Example Therapeutic-Quality GPCR Antibody Hits.

GPCR mAb	EC_{50} (M)	IC_{50} (M)
ADS 1	5×10^{-11}	5×10^{-10}
ADS 2	1×10^{-10}	7×10^{-10}
Comparator	1×10^{-10}	6×10^{-9}
Neg. Control	N.A.	N.A.

Table 1: EC_{50}'s and IC_{50}'s of Two AlivaMab Anti-GPCR Antibody Hits and an Existing Comparator Therapeutic Antibody

Cell-Based FACS Assay Enables Efficient Epitope Binning and Reveals Antibody Panel Diversity

As noted earlier, the likelihood of success in drug discovery often hinges on the production of diverse antibody leads, since this diversity can increase the likelihood of success during *in vivo* research. One essential determinant of antibody diversity is the epitope they recognize. As a result, it's important to assess whether your hits bind a number of unique epitopes.

ADS has developed a proprietary cell-based process to bin antibody candidates by the unique epitopes they bind immediately after hit identification. Since the assay is performed with fluorescence assisted cell sorting (FACS) using cells, competitive antibody binding is accessed within the complete context of the cell membrane, as required for multi-spanners. In this process, individual hits are pre-bound to cells expressing target multi-spanning proteins on their surface. Then, a second antibody is added to determine if both can bind simultaneously, which indicates unique epitopes for the two hits. This is repeated for the entire panel to determine the total number of epitope bins and organize antibodies that share the same epitopes. Applying this method to a 384-member anti-GPCR-D panel results in 26 epitope bins (**Figure 4**), demonstrating an impressive diversity of antibodies against an example multi-spanning membrane protein. Combining the ADS epitope binning approach and its high diversity immunization strategies provides well-characterized candidate panels that cover a wide range of binding modalities – ideal for drug discovery efforts.

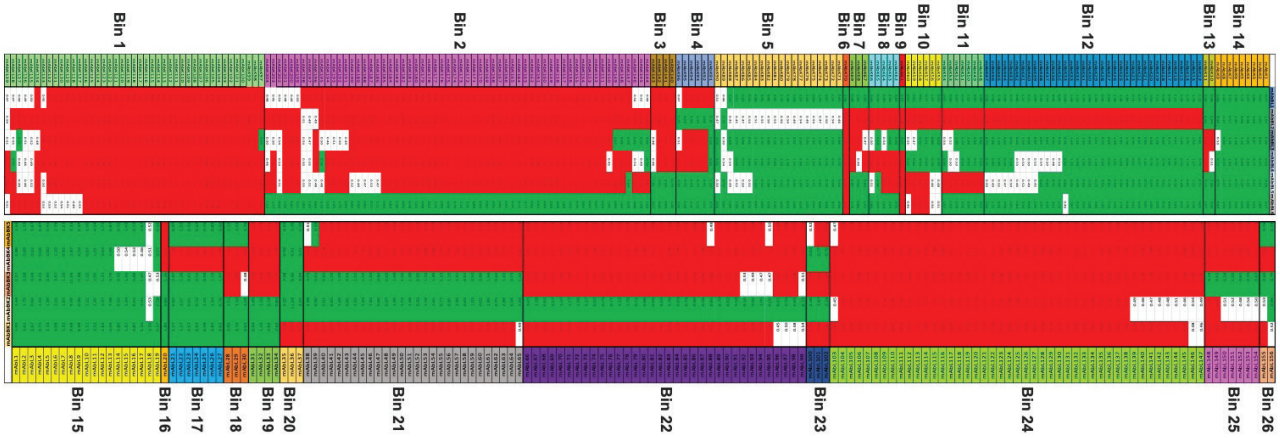

Figure 4: Epitope Binning of an anti-GPCR-D Antibody Panel Identifies 26 Epitope Bins

Faster Antibody Discovery: Delivery of Quality Candidates for Membrane Protein Drug Discovery

While traditional approaches struggle to target multi-spanning integral membrane proteins, ADS's novel and synergistic approach generates potent sera, large hit panels with high-affinity functional candidates, and broad epitope diversity. ADS workflows generate many strong binding, well-characterized candidates, which increase the likelihood of success in pre-clinical research and beyond. Importantly, the workflows can achieve this in just 11 weeks, generating results well before other methods like *in vitro* display, which can take more than twice as long (**Figure 5**).[1] Collectively, drug discovery clients that partner with AlivaMab Discovery Services on their multi-spanning drug targets can accelerate their projects and achieve success in competitive landscapes.

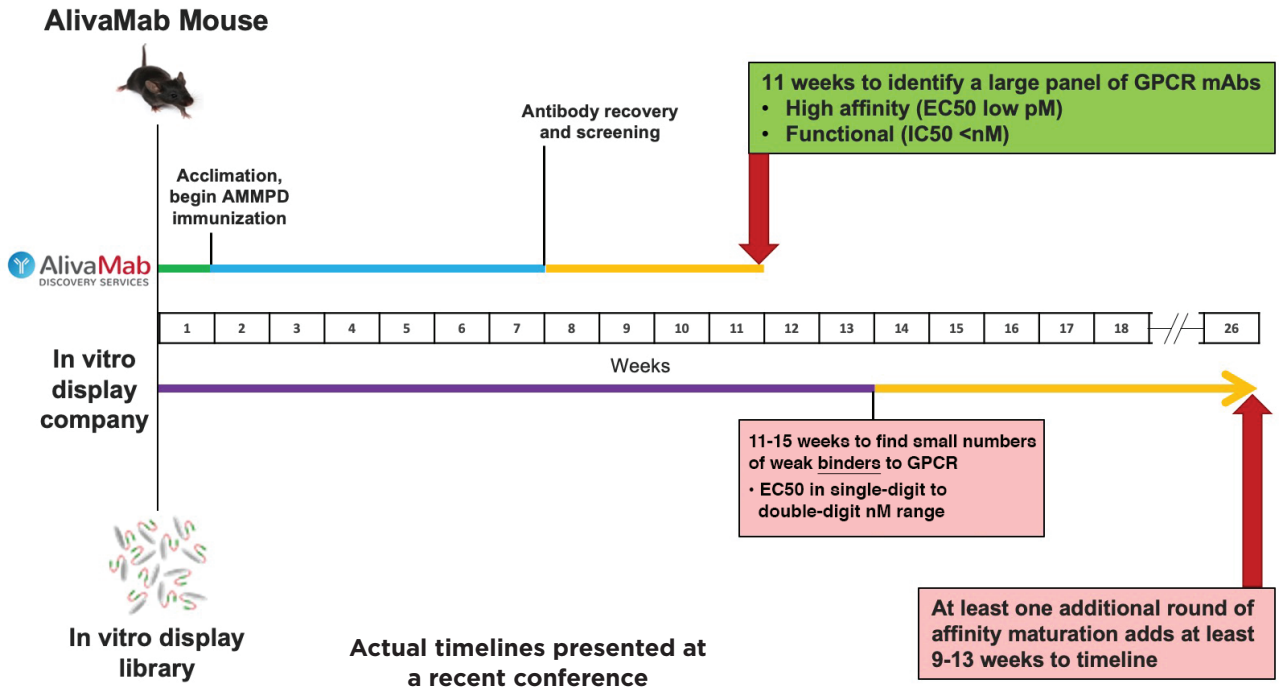

Figure 5: Delivering Quality Candidates for GPCRs Faster Than *in Vitro* Display

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