Generation of Antibodies to Difficult Membrane Protein Targets

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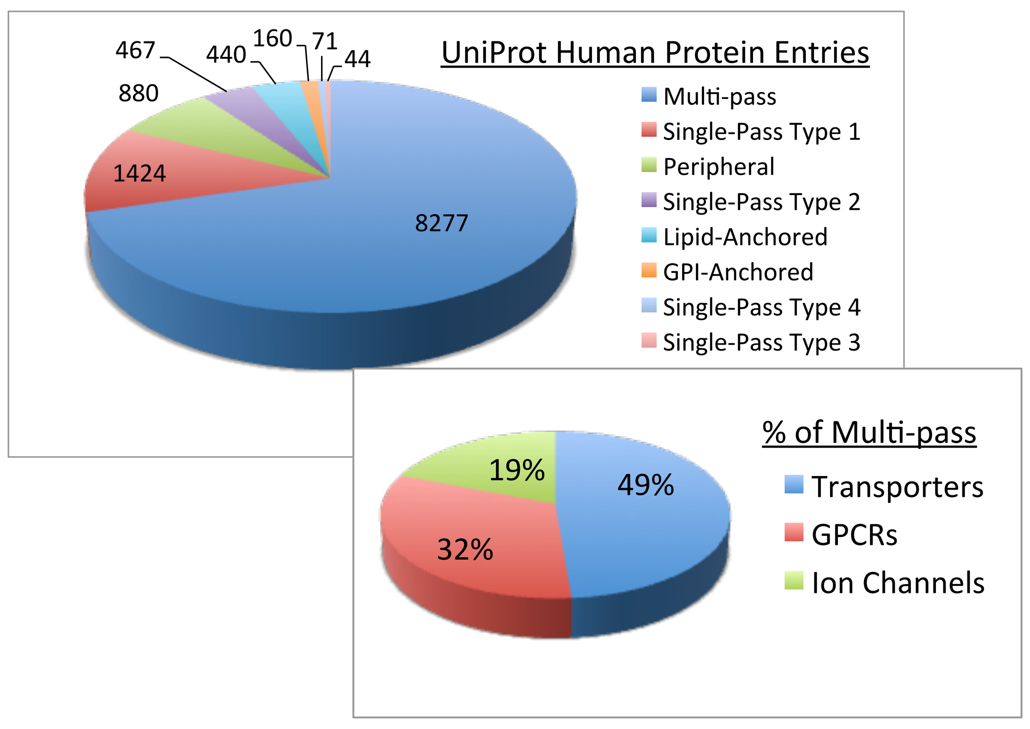
Introduction

Multipass transmembrane and multimeric membrane proteins are targets for the development of therapeutic monoclonal antibodies, but they present challenges for antibody generation. Membrane proteins represent ~25% of the entire genome and the majority of those are multi-pass, complex proteins that are challenging to express in a native, bio-active state with appropriate quaternary structure.



Potential Immunogens for Difficult Membrane Proteins

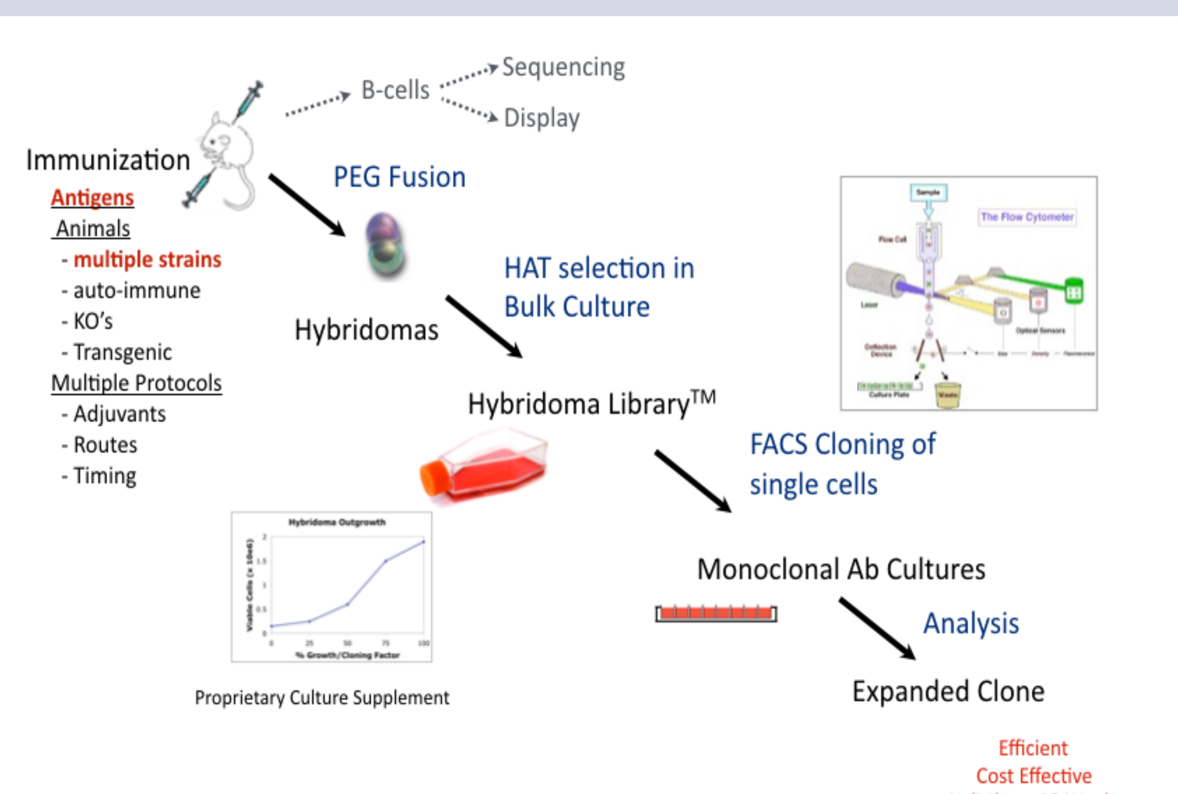
Immunized animals continue to be the most robust source for the discovery of therapeutic antibodies, but immunogens for multi-pass and complex membrane proteins are difficult to generate. Various surrogate platforms exist to allow the expression of properly folded protein, including antigen-expressing cells, viral lipoparticles, and nanodiscs.

Immunogen	Advantages	Disadvantages
Peptide Conjugates	Lowest Cost	Non-native structure
Extracellular Domains	Native Structure	Multipass or multi-meric problematic
Human Cells	Native Structure	Low Expression / High Background
DNA	Natural expression/Low cost	Boosting problematic
Syngeneic Transfectants	Native structure / High Expression /Low Background	Must have cells compatible with host
Viral Lipoparticles / VLPs	Native structure	Low Ag purity, Cost
Nanodiscs	Native structure, High Ag Purity prevalence	Both extra- and intra cellular loops, Cost

DNA-based immunization strategies have been shown by us and others to generate antibodies against native conformational epitopes of membrane proteins. Generally, target-specific hybridomas are best obtained when DNA-immunization is combined with injections of target protein or target-expressing cells. To this end, Antibody Solutions has developed a hybrid immunization strategy that combines the use of DNA immunization with other immunogens to prime and final boost animals for the generation of activated B-cells for hybridoma fusion.

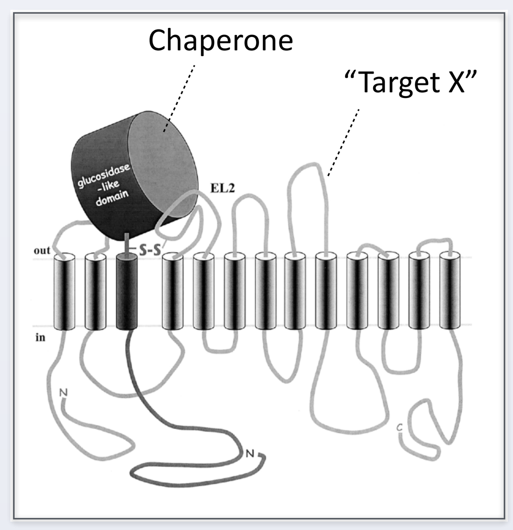
Antibody Discovery Platform

Antibody Solutions has established a robust platform for capturing the antibody response of immunized mice and rats principally through our Hybridoma Library™ approach, which integrates effective immunization protocols, optimized fusion technology, FACS cloning of hybridomas to ensure clonality, and high throughput screening on target protein expressing cells. Both conventional and human Ab-producing rodents can be used.



Case Study: "Target X" - Heteromeric Multipass Protein Target

"Target X" is a multipass heteromeric protein involved in amino acid transport across the cell membrane. In addition to the challenging structure that includes 12 trans-membrane domains and 6 extracellular loops, the target has high homology to mouse and rat (~90%) protein and is co-expressed with a large chaperone protein.



Properties of Target X

Heteromeric Amino Acid Transporter
12 Transmembrane Domains
6 Extracellular Loops
~90% Murine Homology
Heavy Chain Chaperone Protein (>400aa)
Chaperone is potentially most Immunogenic but has 70% AA homology with the murine homolog.

Target X Immunogens

These Immunogens and a proprietary immunization protocol were used to obtain Target X antibodies.

Immunogen	Source	Use
DNA	Antibody Solutions	Most Booster Injections
Syngeneic Transfectants	Antibody Solutions	Initial & intermediate injections, final boost
Viral Lipoparticles / VLPs	Integral Molecular	Initial & intermediate injections, final boost
Nanodiscs	Cube Biotech	Initial & intermediate injections, final boost

Target X Cell Line Development

Syngeneic rodent cells expressing the target are critical reagents for immunization and antibody screening.

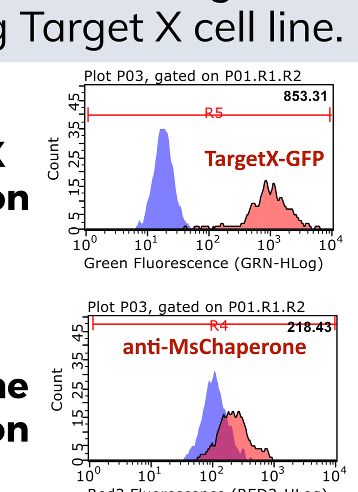
Creation of stable clones expressing Target X

Design and Synthesis of Plasmid (IRES + GFP)
↓
Transfection of syngeneic cells NIH-3T3 and P3X63Ag8.653
↓
Antibiotic-selection
↓
Cell surface Ag detection using GFP
↓
Flow sorting high Ag expressors
↓
Isolation of Stable clones
↓
Production of Cell Banks for immunization or screening

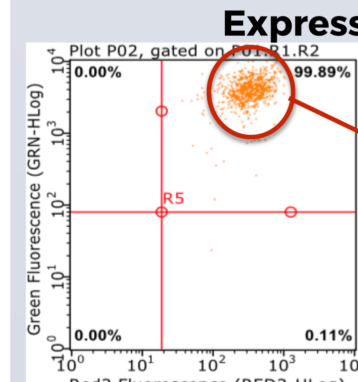
We used a mouse cell line known to express the chaperone protein required for Target X expression to create a stable, high-expressing Target X cell line.

Target X Expression

Chaperone Expression



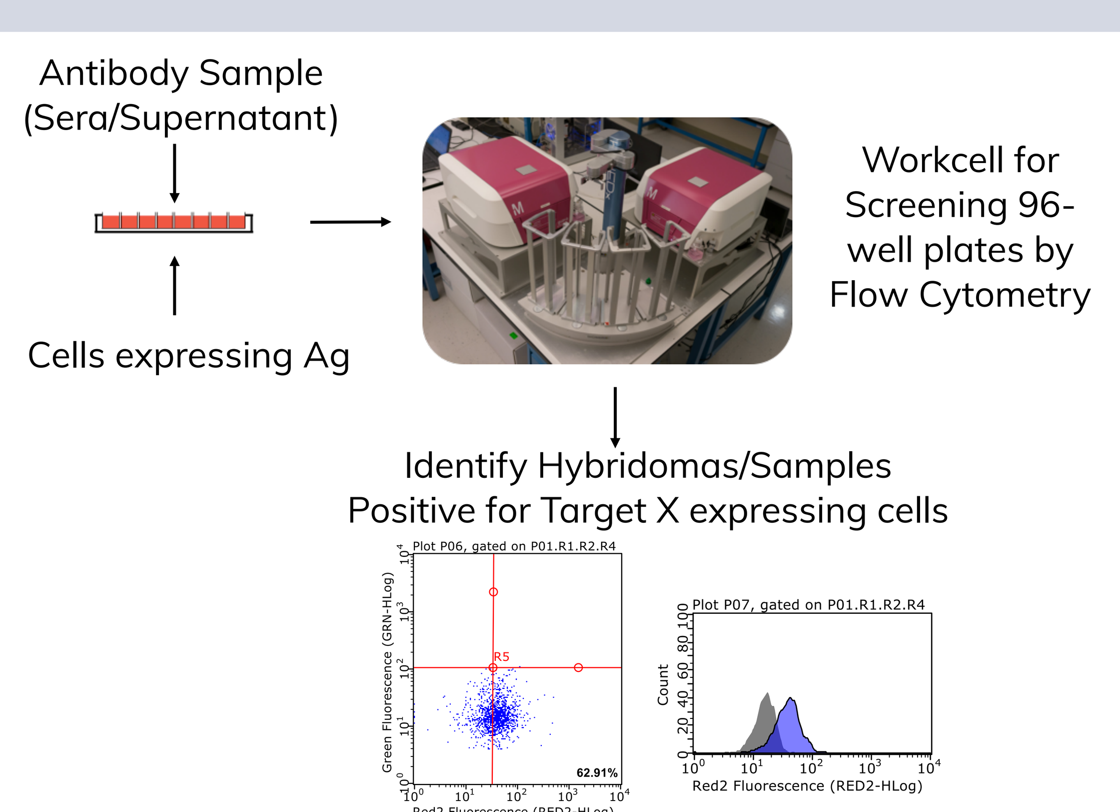
Target X + Chaperone Expression



Note the uniform, high expressing population of Target X + Chaperone expressing cells.

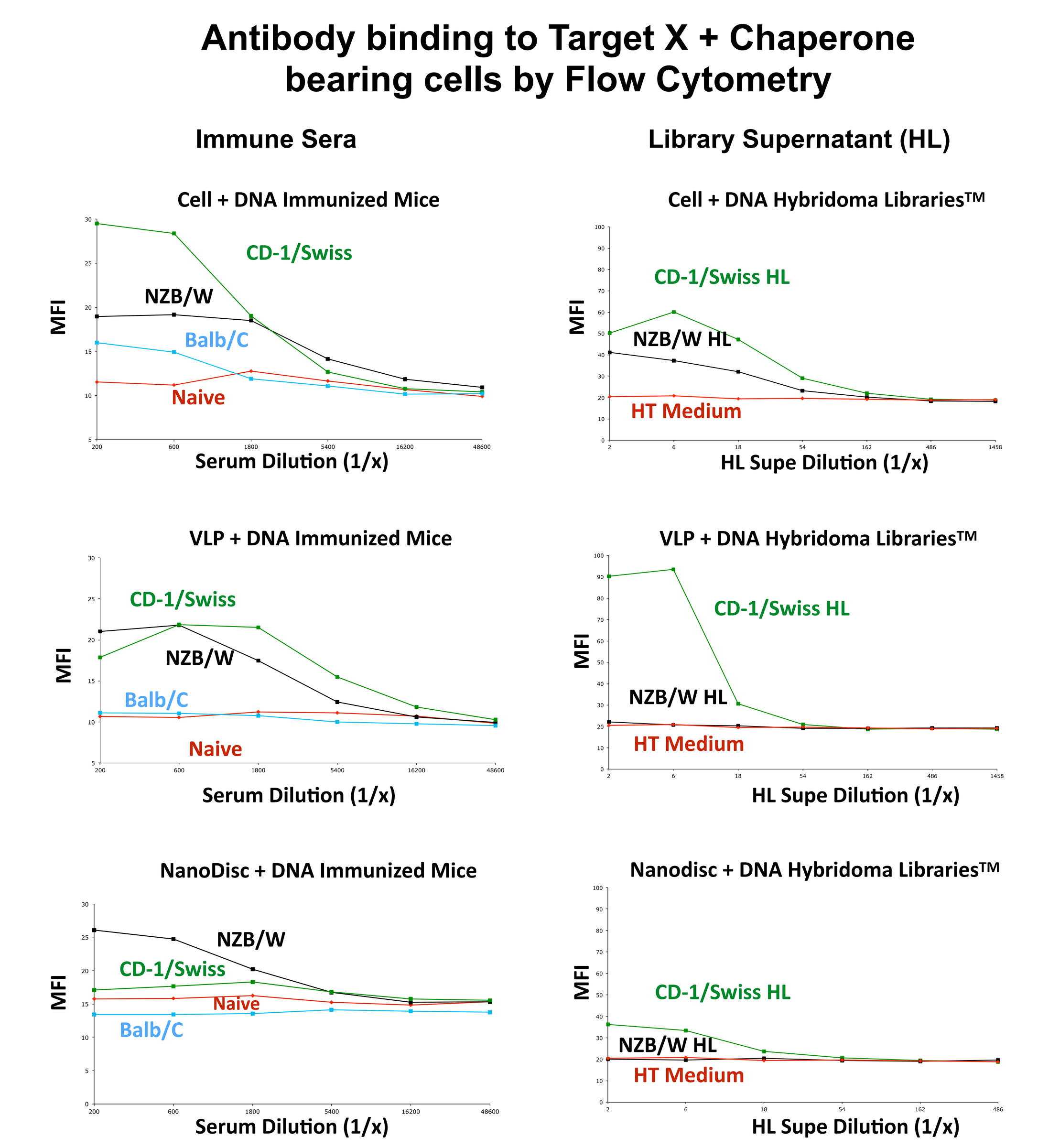
Primary Screening by Flow Cytometry

Primary screening of antibody binding by flow cytometry using Ag-expressing cells is critical for finding relevant antibodies. Cell screening ensures that antibodies recognized the native Target X expressed on the cell surface. We used a work cell designed for high-throughput screening by flow cytometry.



Serum and Hybridoma Library™ Results

In combination with DNA immunization, syngeneic Target X-cells, -VLP's and -Nanodiscs were used in 3 conventional mouse strains: CD-1/Swiss, NZB/W, and Balb/c. All three immunogen/immunization strategies raised Target X antibodies in the sera of mice. Hybridoma Libraries™ made from CD-1/Swiss mice showed the highest binding activity to Target X cells.



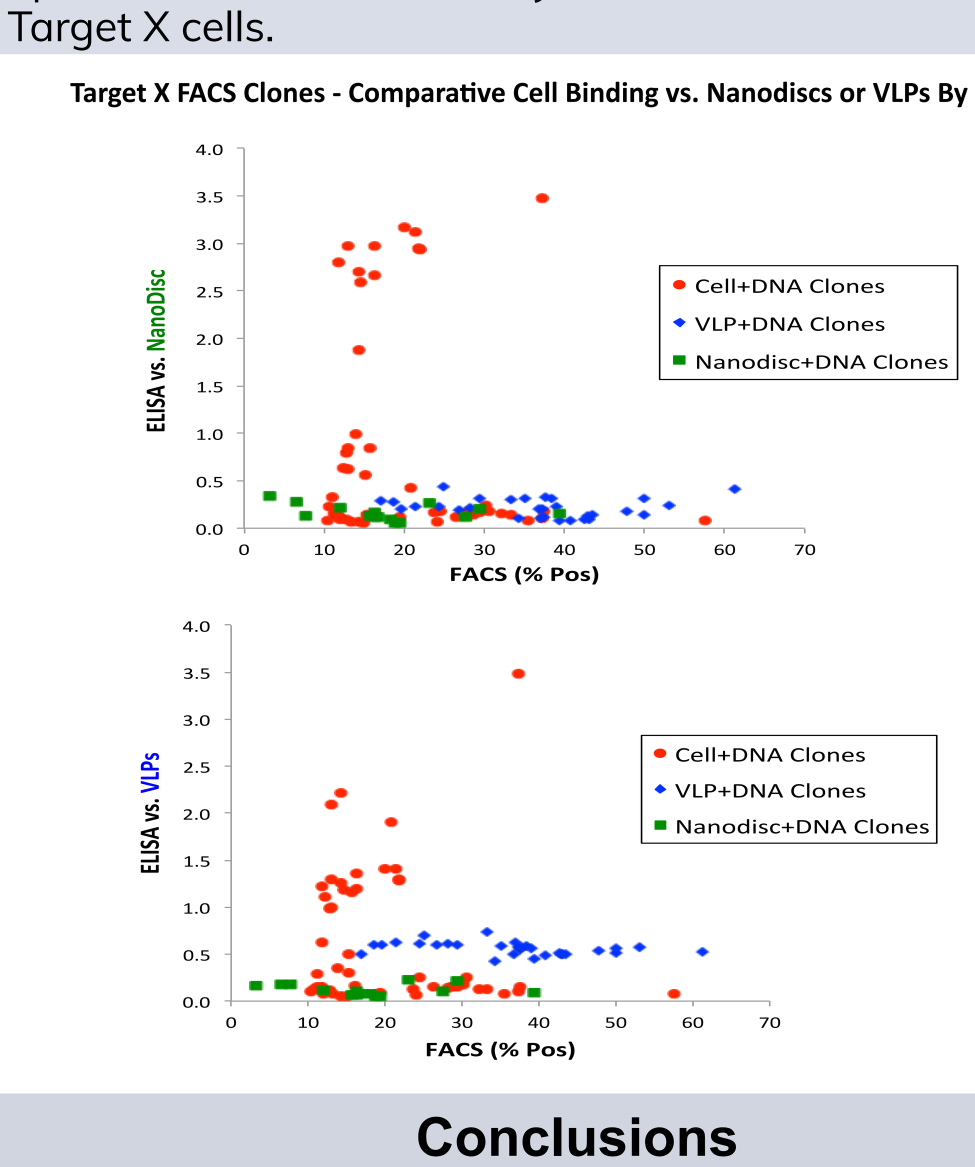
Target X Hybridoma Clones

Clones were prepared from the 3 CD-1/Swiss Hybridoma Libraries™ raised using the 3 immunogen/immunization strategies. Clones were identified in all three Hybridoma Libraries™ that had antibody binding activity to Target X - expressing cells. While Cells + DNA gave the highest number of positive clones, other strategies gave similar numbers of positive clones per plates prepared.

Immunogen	Library	No. 96-Well Plates	# Cell Binding Clones
Cells + DNA	CD-1/Swiss	30	50
VLPs + DNA	CD-1/Swiss	30	35
Nanodiscs + DNA	CD-1/Swiss	10	15

FACS Positive Clones and Nanodisc or VLP Binding

Target X antibody binding clones were further analyzed for antibody binding to Nanodiscs or VLPs. Generally there was a poor correlation between the FACS binding activity to cells and their ELISA binding activity to Nanodiscs or VLPs. Immunization with Cells and DNA appeared to give the most diverse set of clones based upon differential reactivity to Nanodiscs, VLPs and Target X cells.



Conclusions

- All three immunogen/immunization approaches raised Target X Abs.
- There was a mouse strain bias (e.g., CD-1/Swiss) in obtaining positive Hybridoma Libraries™.
- Antibody diversity, based upon screening results, was highest with the Cell + DNA immunization approach.