integral molecular

Shotgun Mutagenesis

Epitope Mapping
Protein Engineering
Antibody Optimization

Sample Final Report

This is a Sample Final Report from a project and contains genuine data. All antibody and target names have been coded.

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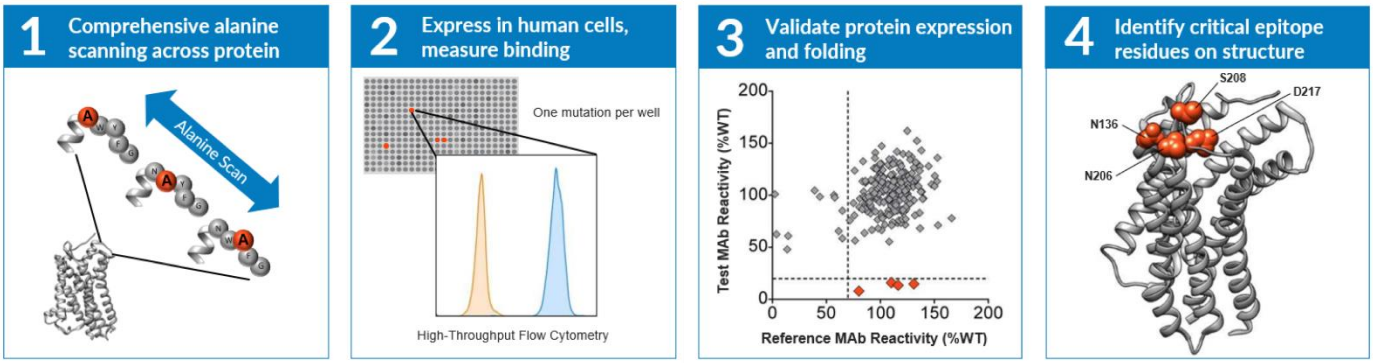
Technology Overview



Shotgun Mutagenesis is Integral Molecular's proprietary epitope mapping and protein engineering platform. This platform allows hundreds of specified mutations to be introduced into target proteins and individually tested in human cells by high-throughput flow cytometry. The combination of large-scale mutagenesis and rapid cellular testing of natively folded proteins enables us to rapidly identify and engineer critical domains in structurally complex proteins.

Using these comprehensive mutation libraries, the binding site of an antibody, drug, or other protein can be mapped, even on conformationally-complex proteins such as GPCRs, ion channels, transporters and viral envelope proteins. Custom epitope mapping services include automated creation of a mutation library (e.g. Ala scan) for a customer-specified target sequence and testing of antibody binding to determine contact residues. Pre-validated Shotgun Mutagenesis libraries are also available for epitope mapping projects, allowing for expedited project turnaround time.

Project Summary

Protein (Target):	<Target>
Library Size:	<LibSize>
Antibodies:	<Abs>
Mutation Strategy:	Alanine scan mutagenesis
Cell type:	<Cells>
Assay For Mapping	Flow cytometry
Epitope Tag:	None

1 Assay Setup

Evaluate and identify optimal conditions for binding and screening of three anti-Target1 MAbs and Fabs, with wild-type protein in the Shotgun Mutagenesis screening assay setup.

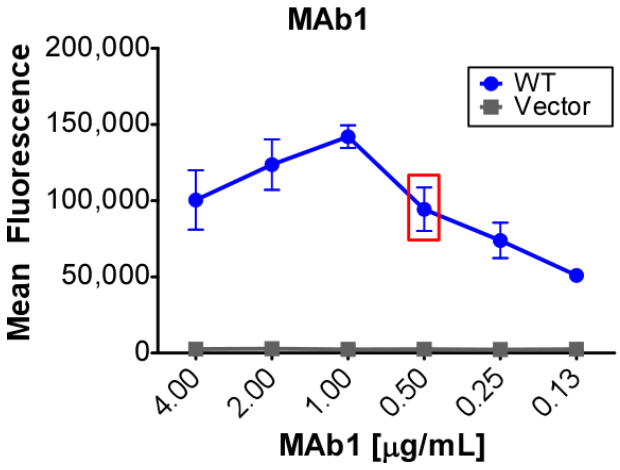
2 Epitope Mapping

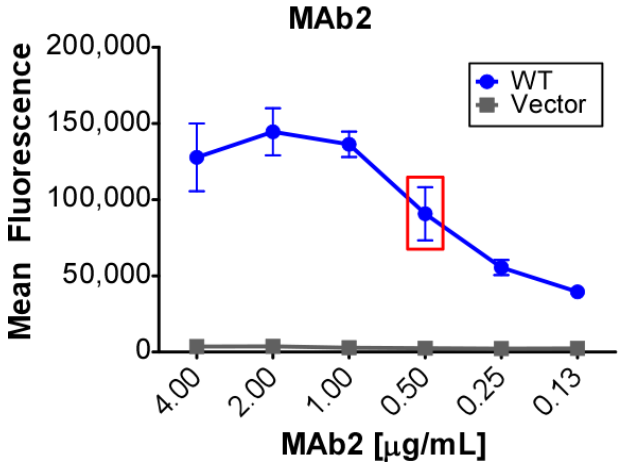
Prepare a Target1 Ala-scan library and map the epitopes of 3 anti-Target1 antibodies at a single amino acid resolution using Shotgun Mutagenesis technology.

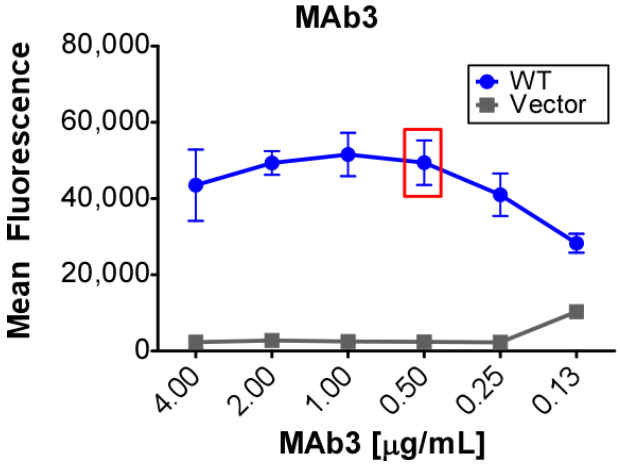
1 Assay Setup

MAbs

Figure 1. Optimization of anti-Target1 MAbs in HEK-293T cells. To optimize detection of binding by anti-Target1 MAbs, HEK-293T cells were transfected with a wild-type (WT) construct or vector alone in 384-well format, followed by detection using a high throughput flow cytometry assay. Serial dilutions of each test MAb (beginning with 4 µg/ml) were tested for immunoreactivity against cells expressing WT or vector alone. Each point represents the average of four replicates. The recommended working concentration for screening each MAb is highlighted (red box or blue highlight) based on signal to background (S/B) calculations and raw signal values.







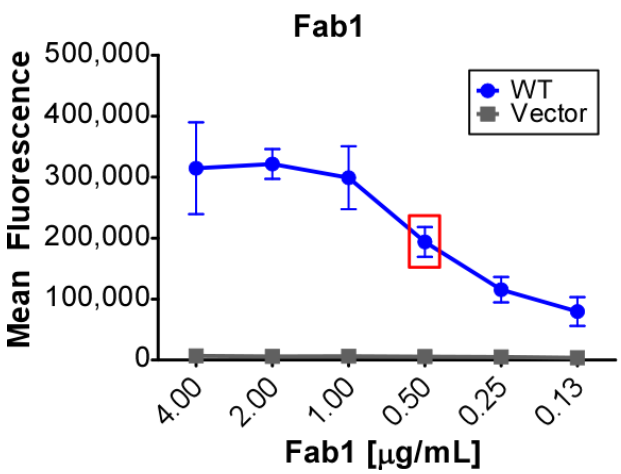
Binding Protein	MAb1	MAb2	MAb3
µg/mL	S/B	S/B	S/B
4.00	38	36	19
2.00	43	39	18
1.00	58	48	21
0.50	38	37	20
0.25	32	24	18
0.13	21	16	3

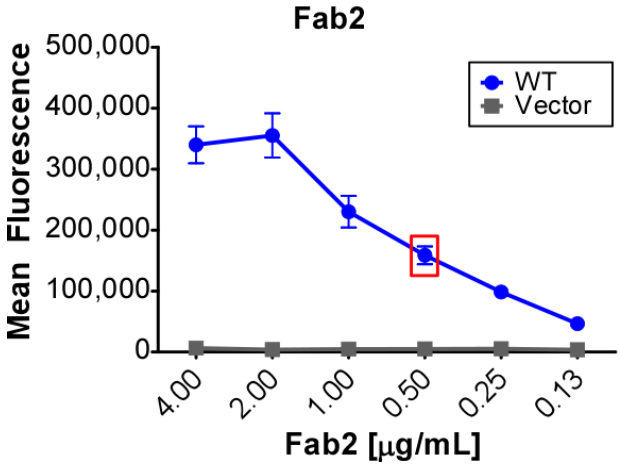
Table 1. Experimental parameters optimized for high-throughput flow cytometry of MAbs.

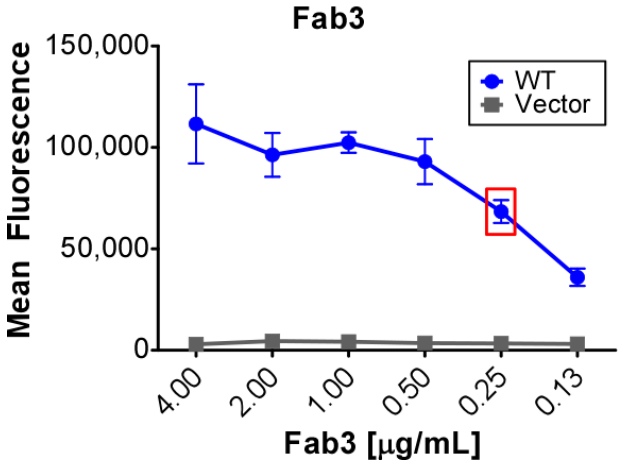
Experimental Parameter	Test MAb	Test MAb	Test MAb
Cells	HEK293T	HEK293T	HEK293T
Fixative	none	none	none
Blocking Buffer	10% Goat Serum	10% Goat Serum	10% Goat Serum
1° Ab			
Ab name	MAb1	MAb2	MAb3
Target	Target1	Target1	Target1
Optimal Conc.	0.5 µg/ml	0.5 µg/ml	0.5 µg/ml
Incubation (RT)	60 min	60 min	60 min
2° Ab			
Target	Mouse IgG	Mouse IgG	Mouse IgG
Optimal Conc.	1:400 (3.75 µg/ml)	1:400 (3.75 µg/ml)	1:400 (3.75 µg/ml)
Incubation (RT)	30 min	30 min	30 min
Manufacturer	Jackson ImmunoResearch	Jackson ImmunoResearch	Jackson ImmunoResearch
Cat #	115-545-003	115-545-003	115-545-003
Antibody ID	AlexaFluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)	AlexaFluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)	AlexaFluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L)
Washes	PBS (Ca^{2+}, Mg^{2+} free)	PBS (Ca^{2+}, Mg^{2+} free)	PBS (Ca^{2+}, Mg^{2+} free)
Signal:Background	38:1	37:1	20:1

Fabs

Figure 2. Optimization of anti-Target1 Fabs in HEK-293T cells. To optimize detection of binding by anti-Target1 Fabs, HEK-293T cells were transfected with a wild-type (WT) construct or vector alone in 384-well format, followed by detection using a high throughput flow cytometry assay. Serial dilutions of each test Fab (beginning with 4 µg/ml) were tested for immunoreactivity against cells expressing WT or vector alone. Each point represents the average of four replicates. The recommended working concentration for screening each Fab is highlighted (red box or blue highlight) based on signal to background (S/B) calculations and raw signal values.







Binding Protein	Fab1	Fab2	Fab3
µg/mL	S/B	S/B	S/B
4.00	45	52	38
2.00	54	87	21
1.00	49	47	25
0.50	34	31	27
0.25	23	19	20
0.13	21	12	12

Experimental Parameter	Test Fab	Test Fab	Test Fab
Cells Fixative Blocking Buffer	HEK293T none 10% Goat Serum	HEK293T none 10% Goat Serum	HEK293T none 10% Goat Serum
1º Ab			
Ab name Target Optimal Conc. Incubation (RT)	Fab1 Target1 0.5 µg/ml 60 min	Fab2 Target1 0.5 µg/ml 60 min	Fab3 Target1 0.25 µg/ml 60 min
2º Ab			
Target Optimal Conc. Incubation (RT) Manufacturer Cat # Antibody ID	Mouse F(ab')2 1:200 (7.50 µg/ml) 30 min Jackson ImmunoResearch 115-546-006 Alexa Fluor® 488-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, F(ab')2	Mouse F(ab')2 1:200 (7.50 µg/ml) 30 min Jackson ImmunoResearch 115-546-006 Alexa Fluor® 488-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, F(ab')2	Mouse F(ab')2 1:200 (7.50 µg/ml) 30 min Jackson ImmunoResearch 115-546-006 Alexa Fluor® 488-AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, F(ab')2
Washes Signal:Background	PBS (Ca^{2+}, Mg^{2+} free) 34:1	PBS (Ca^{2+}, Mg^{2+} free) 31:1	PBS (Ca^{2+}, Mg^{2+} free) 20:1

Table 2. Experimental parameters optimized for high-throughput flow cytometry of Fabs.

Recommendations for Screening

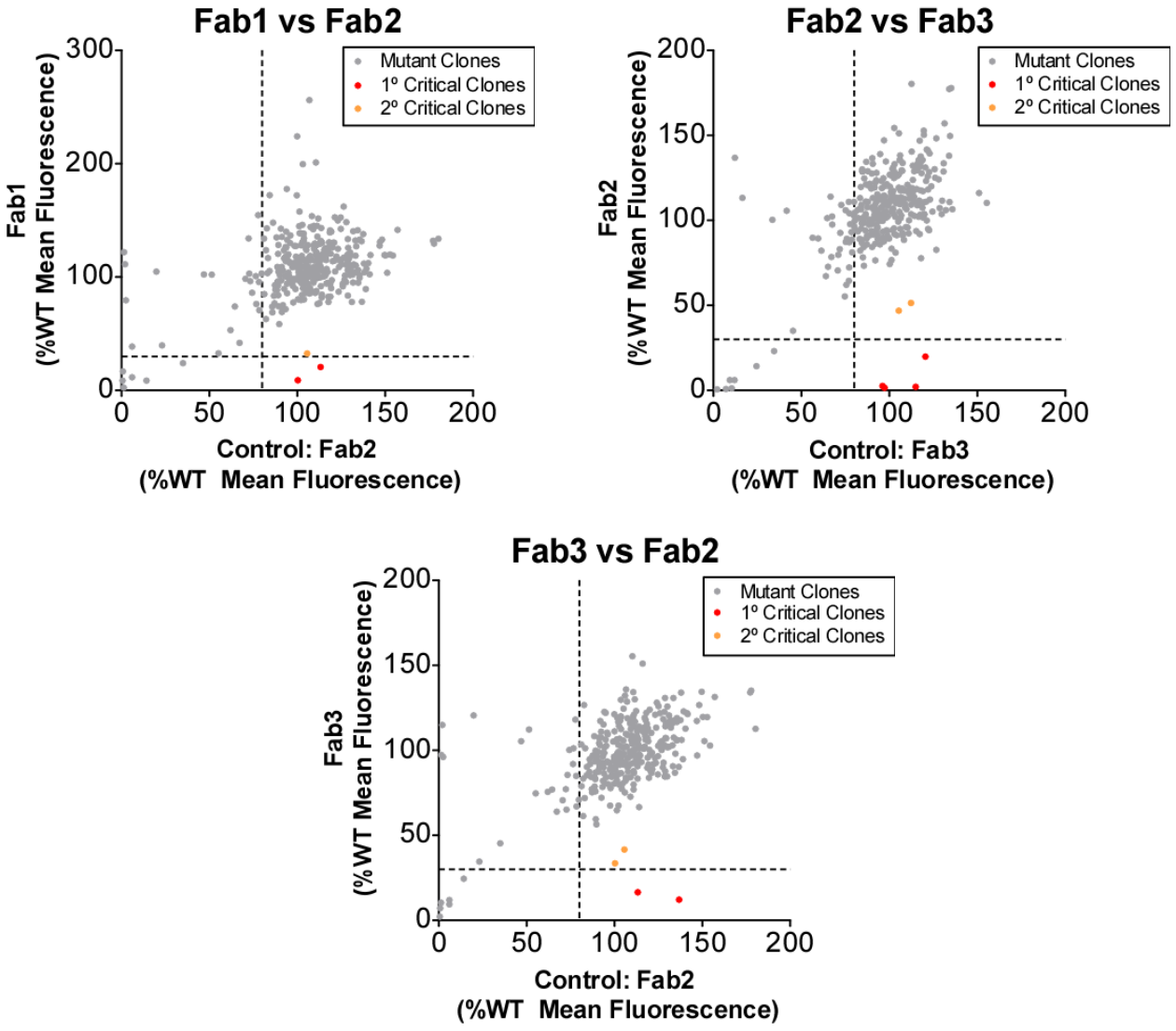
Optimal screening conditions were determined for detection of anti-Target1 MAbs and Fabs binding to WT Target1. Using these conditions, each antibody demonstrated a robust signal, high signal-to-background values, and low variability between replicates. These data indicate that the selected conditions are suitable for high-throughput epitope mapping. Recommended screening conditions for the Target1 mutation library are: 0.5 µg/mL for MAb1, MAb2, MAb3, Fab1, and Fab2, and 0.25 µg/mL for Fab3 and the Control MAb. The recommended concentrations give an optimal separation between Target1-expressing and vector-transfected cells, to allow the detection of critical clones displaying decreased Ab binding. Secondary antibodies from Jackson ImmunoResearch will be used at 1:400 for MAb detection and 1:200 for Fab detection.

High Stringency Screening Conditions

Due to the high affinity nature of anti-Target1 MAbs, screening began with a Fab version. Library screens of high-affinity MAbs often fail to yield critical residues for Ab binding. Conversion of a high affinity MAb to Fab usually weakens binding sufficiently to allow us to identify critical residues for Fab binding.

2 Epitope Mapping

Figure 3. Identification of critical clones for anti-Target1 Fabs. The Target1 mutation library was assayed in a flow cytometry assay, in duplicate, for binding by Fab1, Fab2, and Fab3. Each raw data point was background-subtracted and normalized to the value for the Fab reactivity with Target1 wild-type (WT). To identify clones that had high Target1 expression but gave low binding with Target1 Abs, each test Fab was compared to a control Fab. For each clone, the mean binding value is plotted as a function of the clone's mean Target1 expression value (gray circles), as reported by reactivity with the control Ab. To identify preliminary critical clones, thresholds (dashed lines) of >70% WT binding to control Ab and <30% WT reactivity to experimental Fabs were applied. Preliminary critical clones identified using these thresholds are shown as red circles. Also identified are secondary clones (orange circles) with mutated residues which do not meet the criteria for being critical, but whose decreased binding activity and proximity to critical residues suggest that they may be part of the epitope.

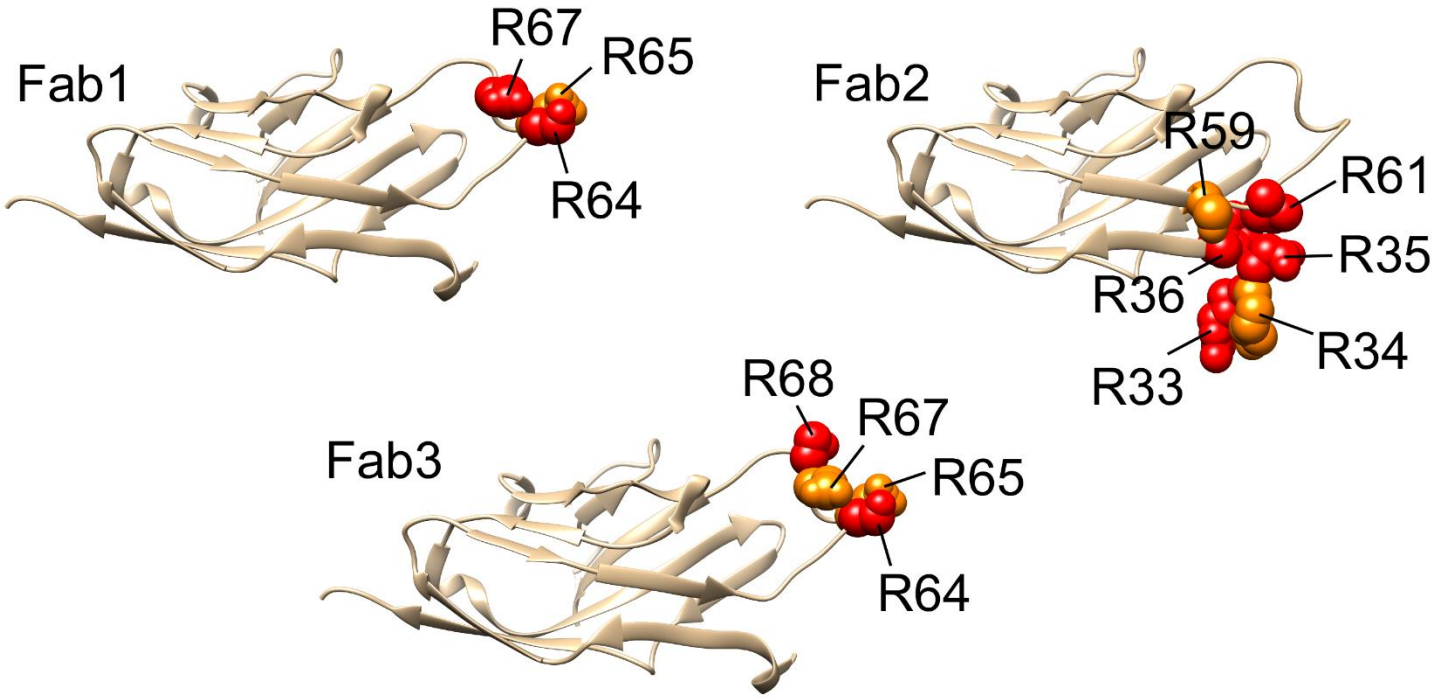


Critical Residues

Table 3. Identification of critical residues for anti-Target1 Fabs. The mean binding reactivities (and ranges) are listed for all critical residues identified in our screen. Critical residues (outlined in red) were identified as those that were negative for Fab binding (<30% of binding to WT) but positive for control antibody binding (>70%). Secondary residues (outlined in orange) did not meet the threshold guidelines but their decreased binding activity and proximity to critical residues suggest that they may be part of the epitope.

Binding Reactivity (% WT)			
Residue	**Fab1**	**Fab2**	**Fab3**
R33	79.5 (1)	2.6 (5)	96.1 (24)
R34	102.4 (52)	46.9 (22)	105.3 (3)
R35	111.4 (23)	2.1 (1)	115.0 (17)
R36	122.0 (37)	1.5 (0)	97.2 (32)
R59	102.1 (11)	51.4 (3)	112.3 (4)
R61	104.8 (8)	19.9 (4)	120.5 (17)
R64	20.6 (2)	113.3 (4)	16.5 (24)
R65	32.7 (0)	105.7 (13)	41.6 (29)
R67	8.9 (2)	100.4 (24)	33.6 (3)
R68	78.2 (44)	136.8 (11)	12.2 (3)

Figure 4. Visualization of critical residues. The primary and secondary critical residues (red and orange spheres, respectively) were identified on a 3D structure of Target1.



Conclusions

The screening of the Target1 mutation library with anti-Target1 Fabs has been completed successfully. The following residues were identified as critical for binding, along with secondary residues that may contribute to antibody epitopes but are of lesser importance for binding:

	Critical Residues	**Secondary Residues**
Fab1:	R64, **R67**	R65
Fab2:	**R33**, **R35**, **R36**, R61	R34, R59
Fab3:	**R64**, **R68**	R65, R67

Validated critical residues represent amino acids whose side chains make the highest energetic contributions to the MAb-epitope interaction (Bogan and Thorn, 1998; Lo Conte et al., 1999). Critical residues that gave the lowest reactivities with specific antibodies (bolded and underlined above) are the major energetic contributors to binding by these antibodies, with lesser contributions by the other critical residues, and by the secondary residues.

Publication Support and Acknowledgement

Upon request, Integral Molecular will be pleased to assist customers in preparing publication-quality figures for presenting Shotgun Mutagenesis epitope mapping data in patents, publications, posters, and scientific presentations. Integral Molecular requests to have Shotgun Mutagenesis and Integral Molecular acknowledged and referenced (citing Davidson and Doranz, 2014) in the Materials and Methods section of publications.

References

Bogan, A.A. and Thorn, K.S. (1998). Anatomy of hot spots in protein interfaces. J. Mol. Biol. *280*, 1-9.

Davidson, E. and Doranz, B.J. (2014). A high-throughput shotgun mutagenesis approach to mapping B-cell antibody epitope. Immunology *143*, 13-20.

Lo Conte, L., Chothia, C., and Janin, J. (1999). The atomic structure of protein-protein recognition sites. J. Mol. Biol. *285*, 2177-2198.

Additional resources at Integral Molecular

Integral Molecular brings to this project over 15 years of experience focusing on membrane proteins and antibodies. Our technologies and applications have been published in over 50 papers and patents, including manuscripts published in *Cell*, *Science*, and *Nature*. The leadership of Integral Molecular, including its three co-founders, Ben Doranz, Ph.D, MBA, Sharon Willis, Ph.D., and Joe Rucker, Ph.D., is involved in each project to ensure its success. In addition to the membrane Proteome Array (MPA), Integral Molecular offers a suite of services to enable membrane protein antibody discovery and characterization:

- **MPS Antibody Discovery Engine.** Utilizes Integral Molecular's suite of proprietary technologies to deliver high quality antibody leads against intractable membrane protein targets.
- **Lipoparticle Technology.** High-concentration membrane proteins on virus-like particles retain the native structure of the membrane protein for antibody discovery or biosensor analyses.
- **Membrane Proteome Array.** A platform to profile antibody and ligand binding against 5,300 membrane proteins for de-orphaning and specificity profiling.