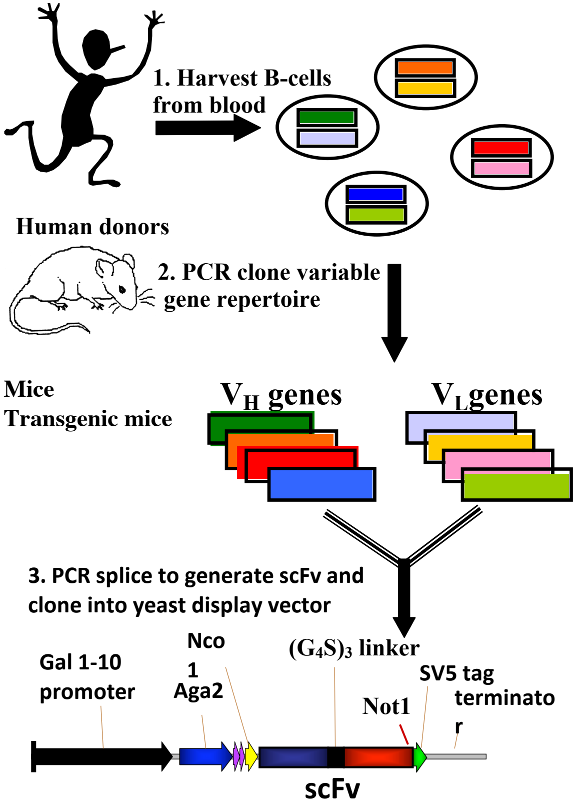
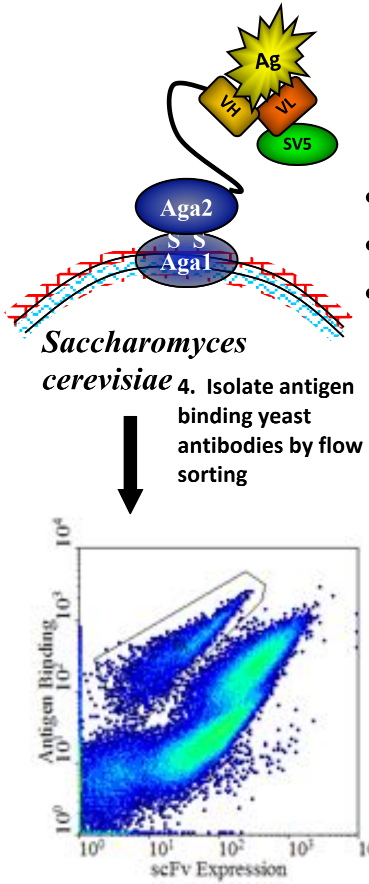
Yeast display and immune libraries

Overview of Yeast display of antibodies





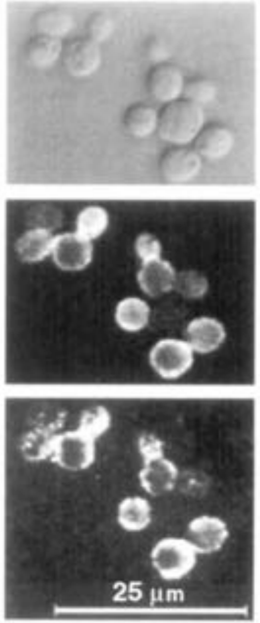
- Isolation of lead mAbs
- Affinity maturation
- Broaden specificity

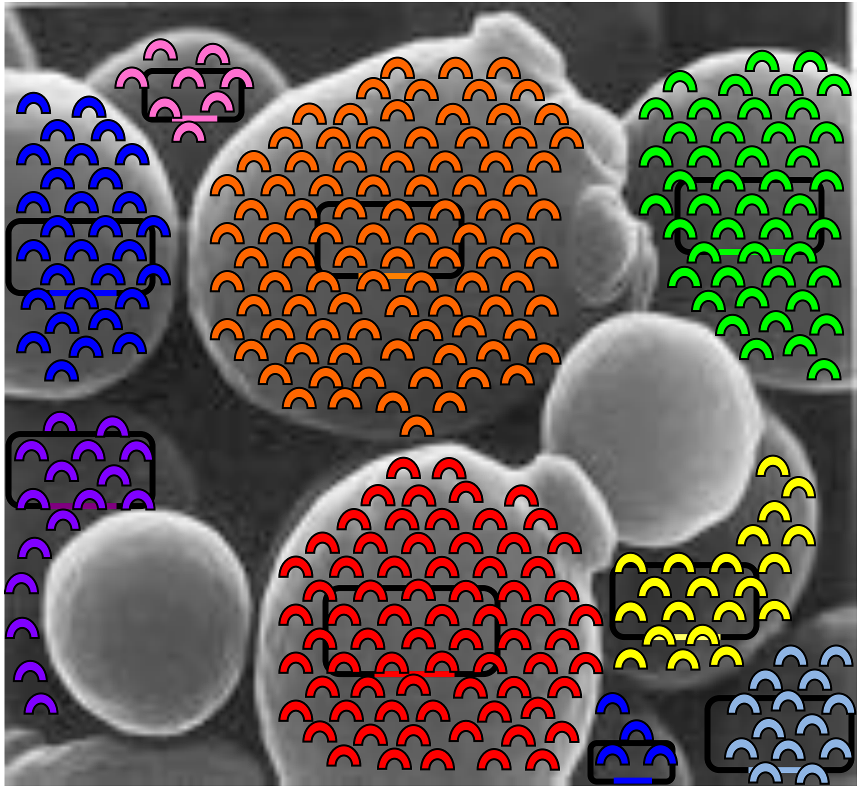
Convert scFv to IgG

Yeast display: each yeast displays hundreds of thousands of copies of a single antibody

scFv

target





Boder & Wittrup, 1997, Nat Biotechnol, 15, 553

Phage vs. Yeast Display

Phage

- Larger primary libraries
- Selection from naïve libraries
- Relatively straightforward
- Soluble scFv or Fab easily made in *E. coli*
- General familiarity with *E. coli*

- Selection is a "black box"
- Antibody must be expressed and purified to measure affinity
- Repertoires incompletely sampled

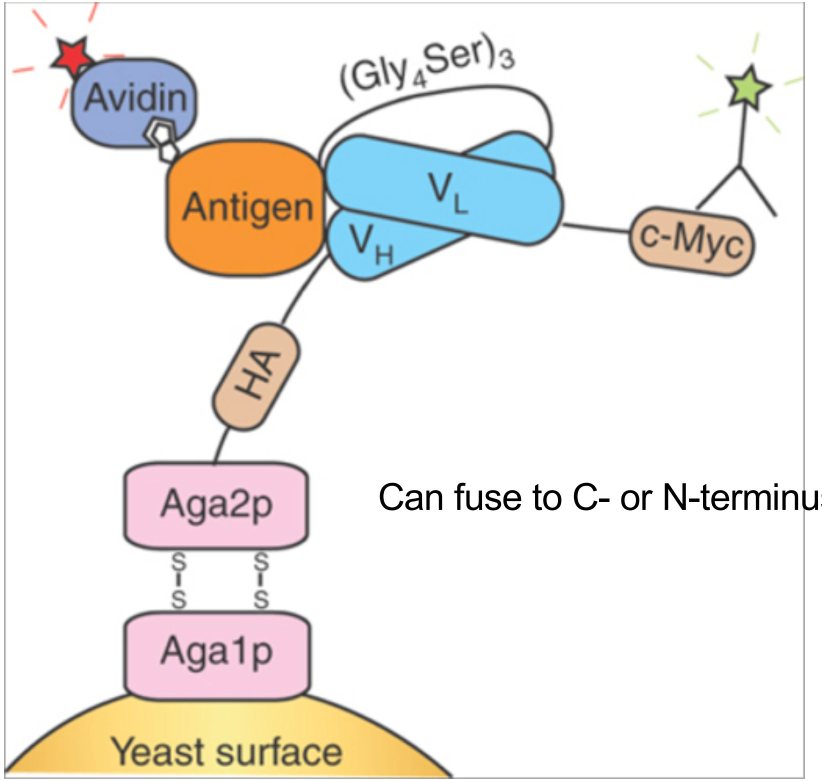
Yeast

- Smaller primary libraries
  - Libraries ≤10^8 with gap repair
  - Immune libraries and affinity maturation
- Naive library selections more challenging
- Requires flow cytometry
- Less general familiarity with yeast
- Need to subclone to make native Ab fragment

- Precise selection calibration
- Direct characterization on yeast without antibody purification:
  - Affinity; epitopes
- Repertoires sampled more completely as greater proportion of antibodies displayed*

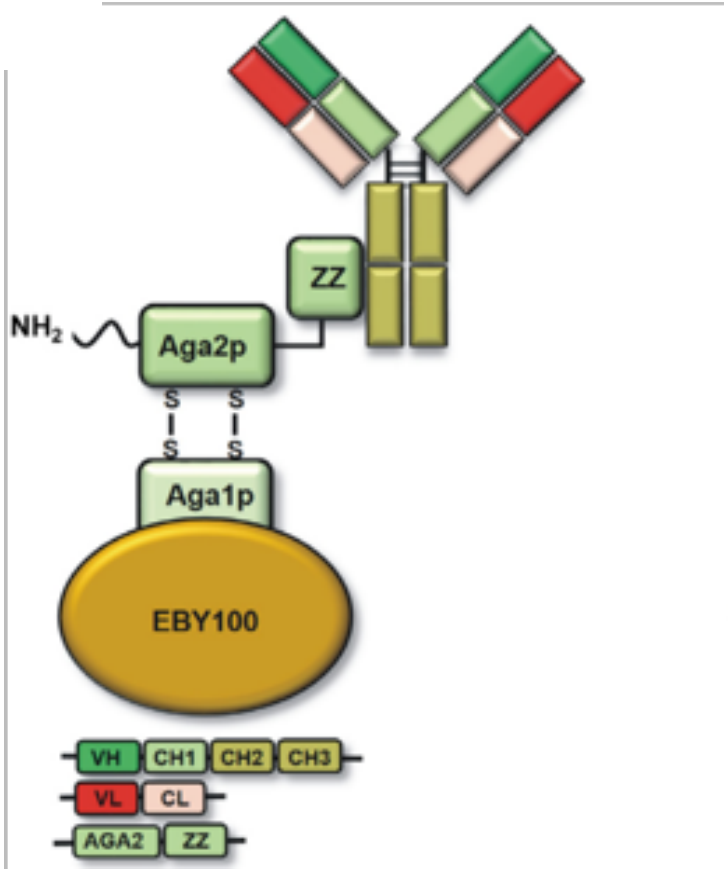
*Bowley et al. (2007) PEDS, **20** 81-90

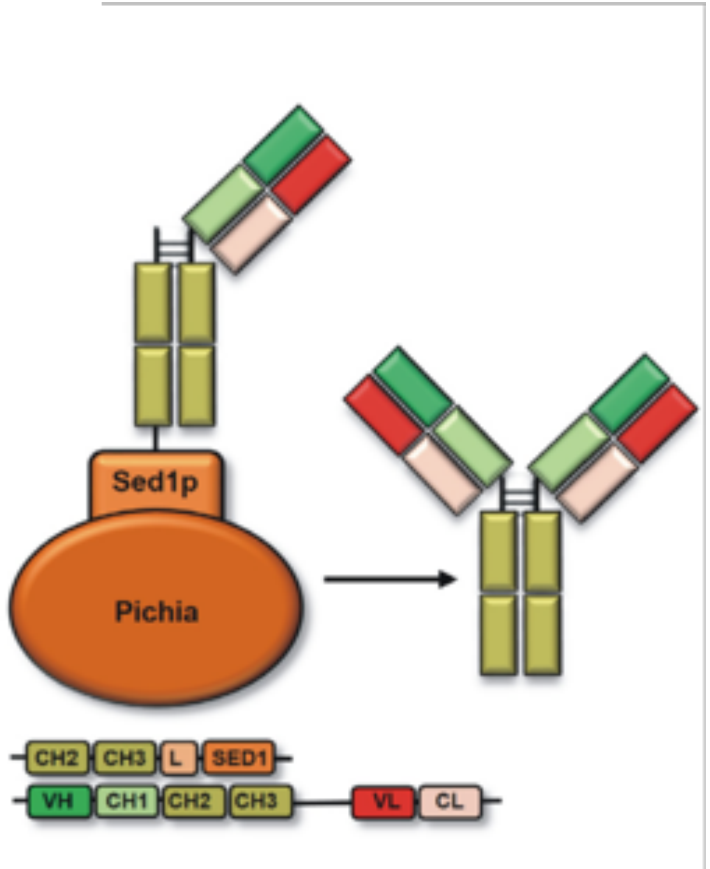
Aga2 for antibody display



Can fuse to C- or N-terminus

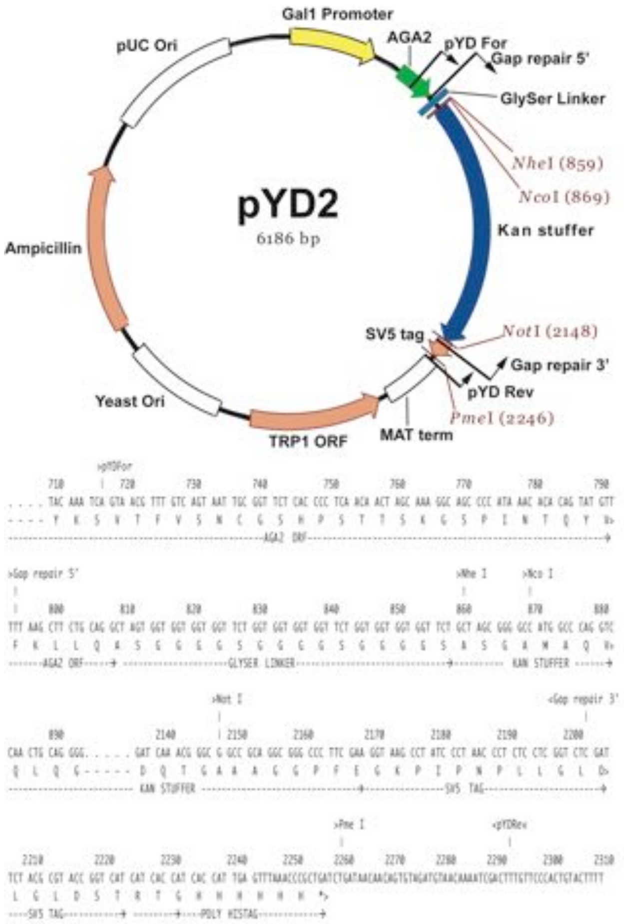
Additional yeast display formats





Cloning yeast display libraries

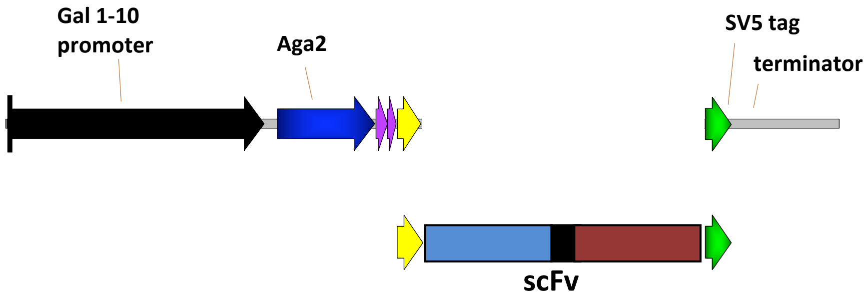
Yeast display vector systems: scFv



Razai et al, 2005,J. Mol. Biol. 351: 158-169

Yeast antibody library construction by gap repair

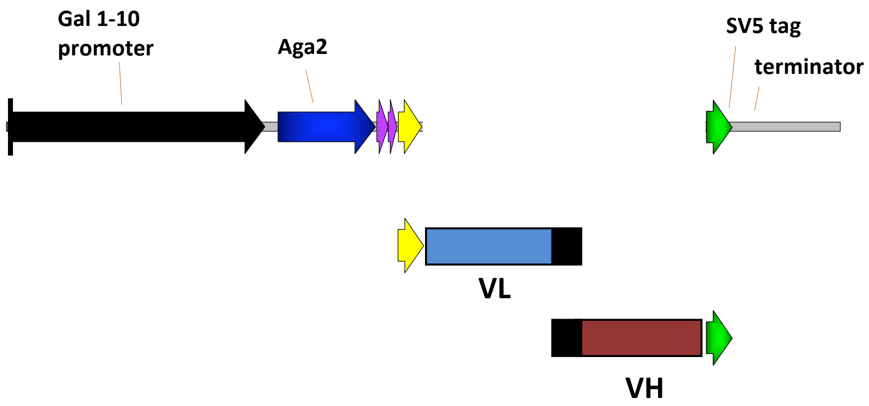
Clone scFv directly into yeast using gap repair:



- Create scFv by cloning or assembly
- Double cut vector
- Generate PCR fragment with > 25 bp overhang
- Mix vector & insert and transfect
- Efficiency ~10^6/ug insert

Yeast antibody library construction by gap repair

Clone VH and VL directly into yeast together using gap repair:



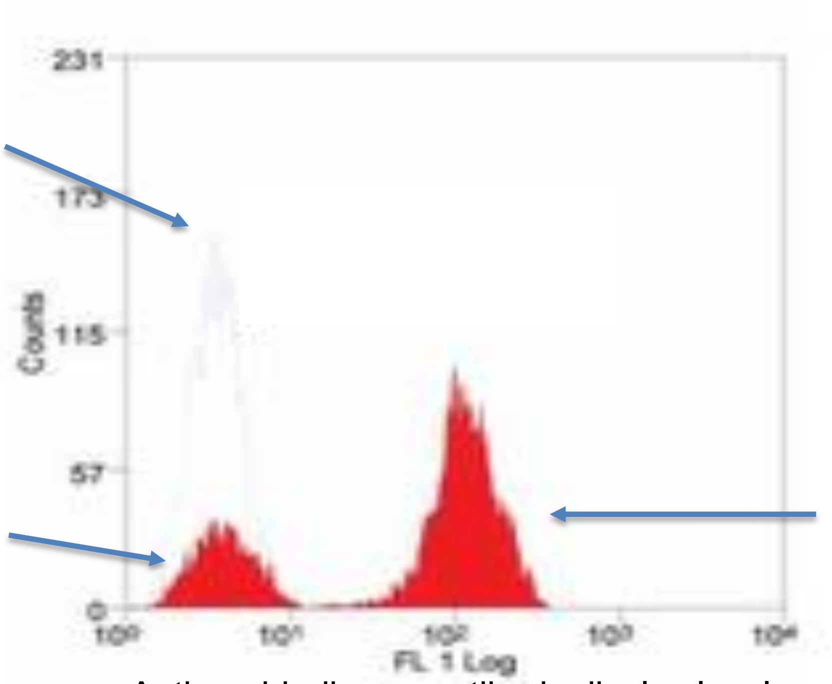
- Double cut vector
- Generate PCR fragments with > 25 bp overhang
- Mix vector & insert and transfect
- Efficiency 1->100E6/ug insert
- Can use 3 or more fragments
- Useful for chain shuffling

Selecting from yeast display libraries

Display level and antigen binding vary per yeast

Negative control

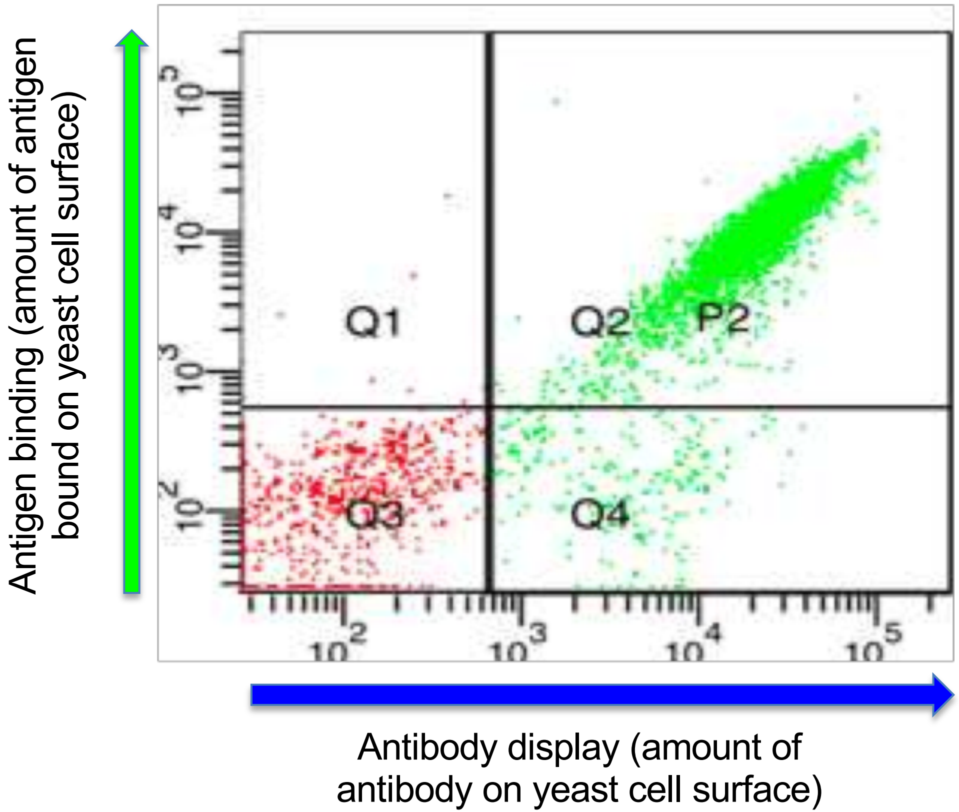
Daughter yeast cells that do not display antibodies

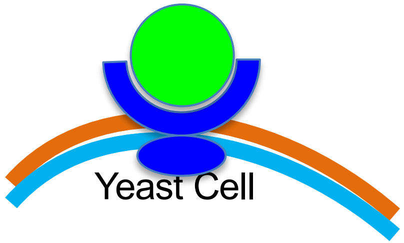


Yeast cells displaying antibodies that bind the target

Antigen binding or antibody display level

Interpreting antibody yeast display flow cytometry plots



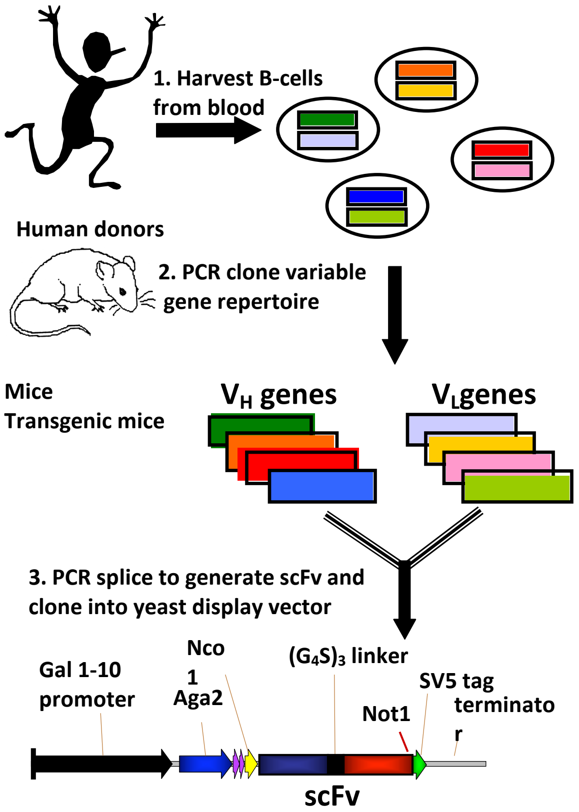


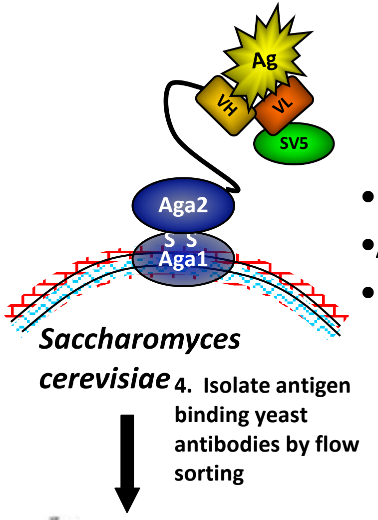
Example of selecting botulinum neurotoxin specific scFv from immune yeast display libraries

Botulinum Toxin: Molecular Structure

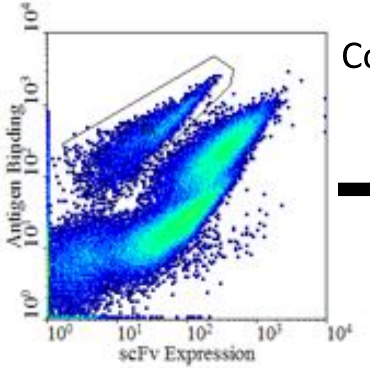
Lacy DB et al. Nat Struc Bio 1998;5(10):898-902.

Studying the human immune response to BoNT by making mAbs and dissecting the structure/function relationship





- Isolation of lead mAbs
- Affinity maturation
- Broaden specificity

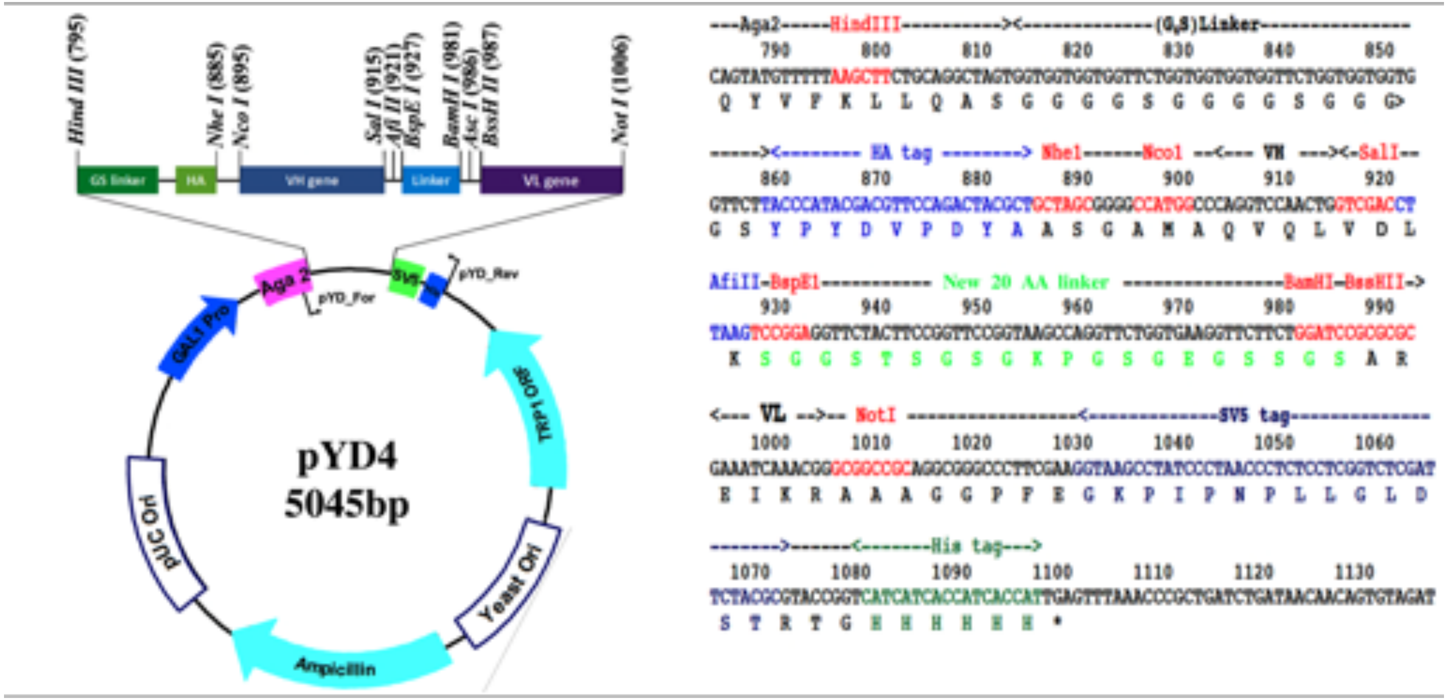


Convert scFv to IgG

- affinity
- epitope
- neutralization

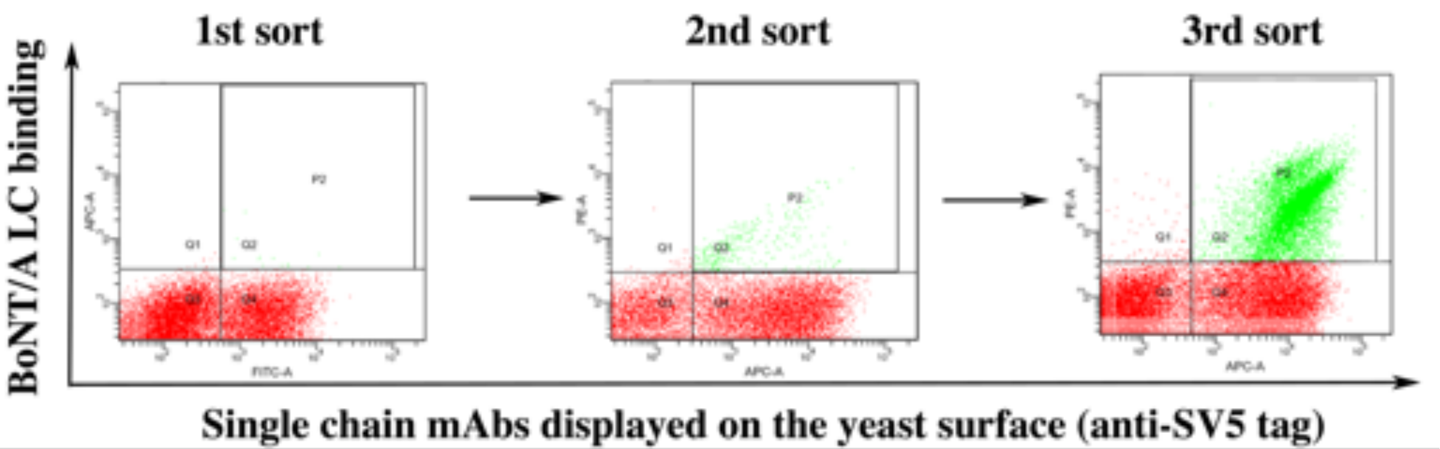
Immune library construction

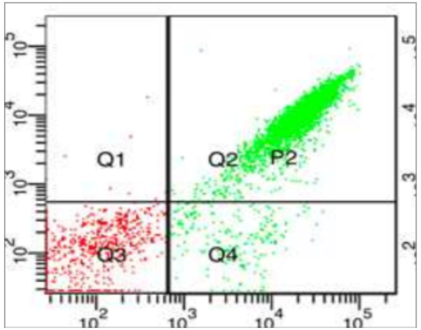
- PCR amplify VH and VK genes using human or murine family based primers



Fan et al, 2015. PLoS One 10: e0135306

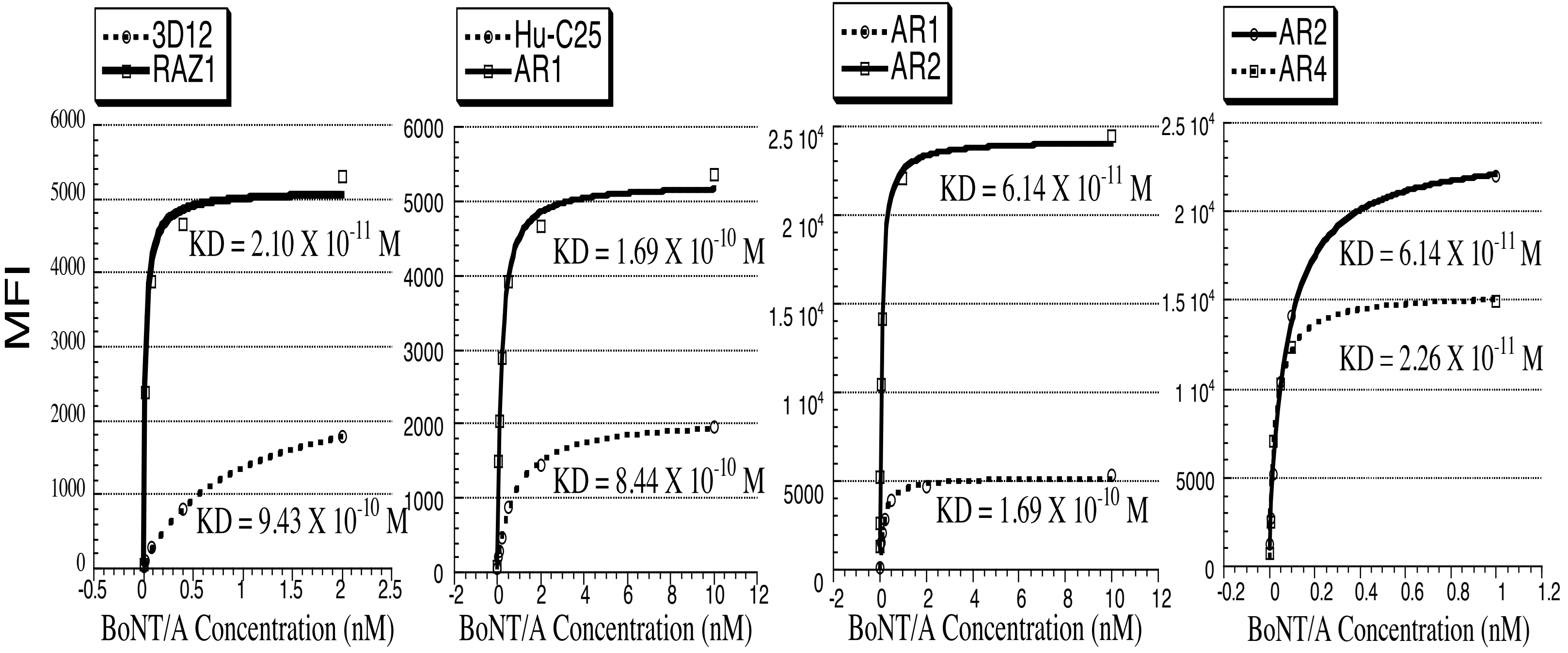
Selecting yeast antibodies by flow cytometry





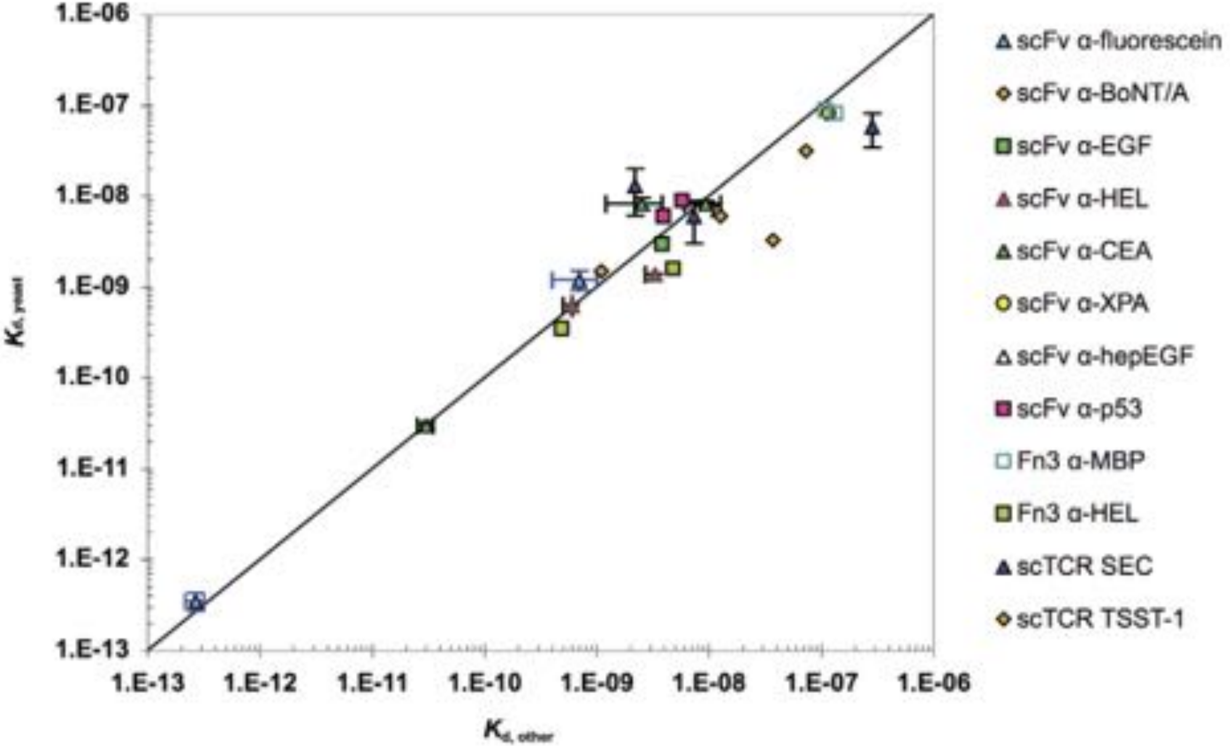
- scFv mAb dot plot
- normal distribution of binding/display
- Need to be mindful of this in setting library sort gates
    - esp. in initial rounds from 1° libraries
    - Need to separate clones for affinity maturation

Measuring KD's of yeast displayed scFv



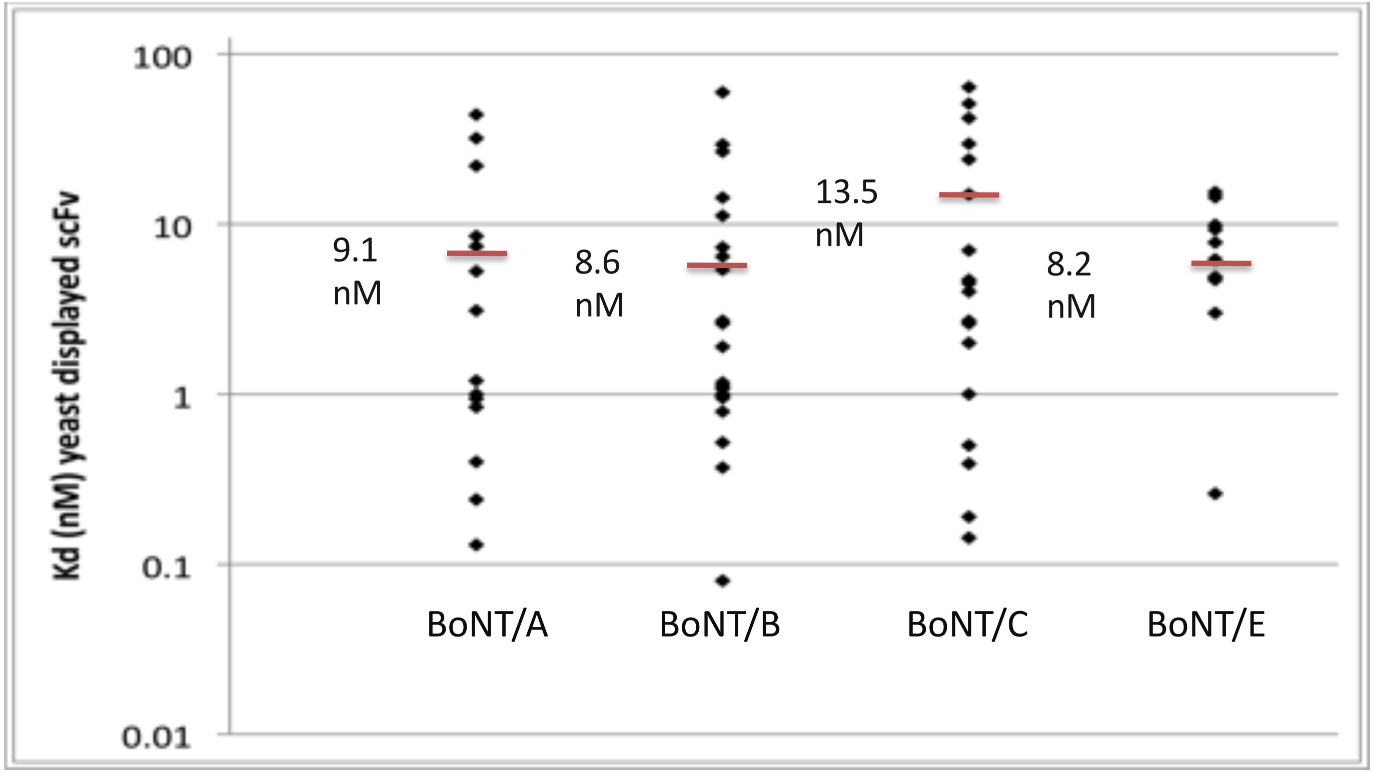
Razai A, et al J. Mol. Biol. 351:158-169, 2005.

Measurement of antibody affinity directly on the yeast surface: correlation with other methods



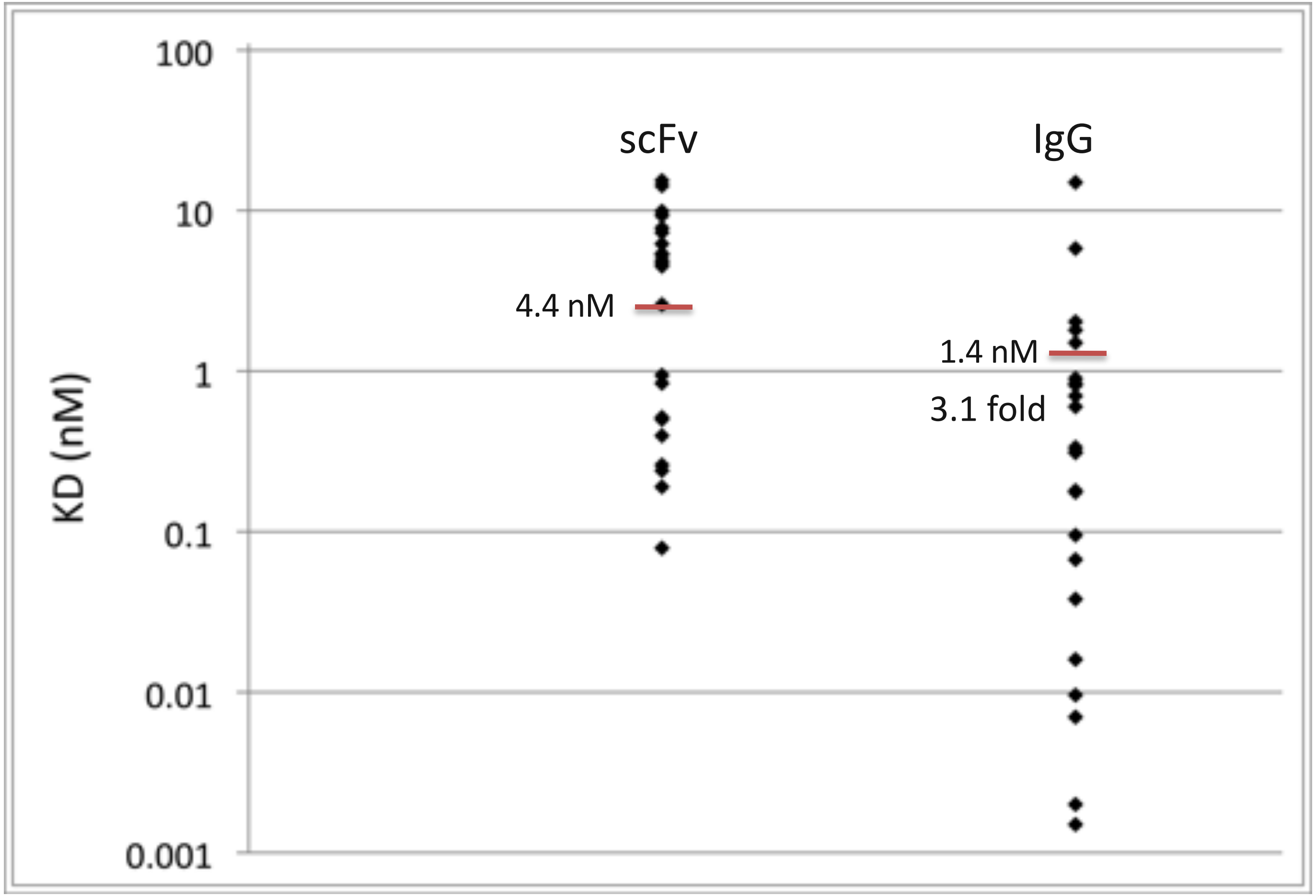
Gai & Wittrup (2007) Current Opinion in Structural Biology, **17** 467–473

Yeast displayed BoNT scFv (n = 66)



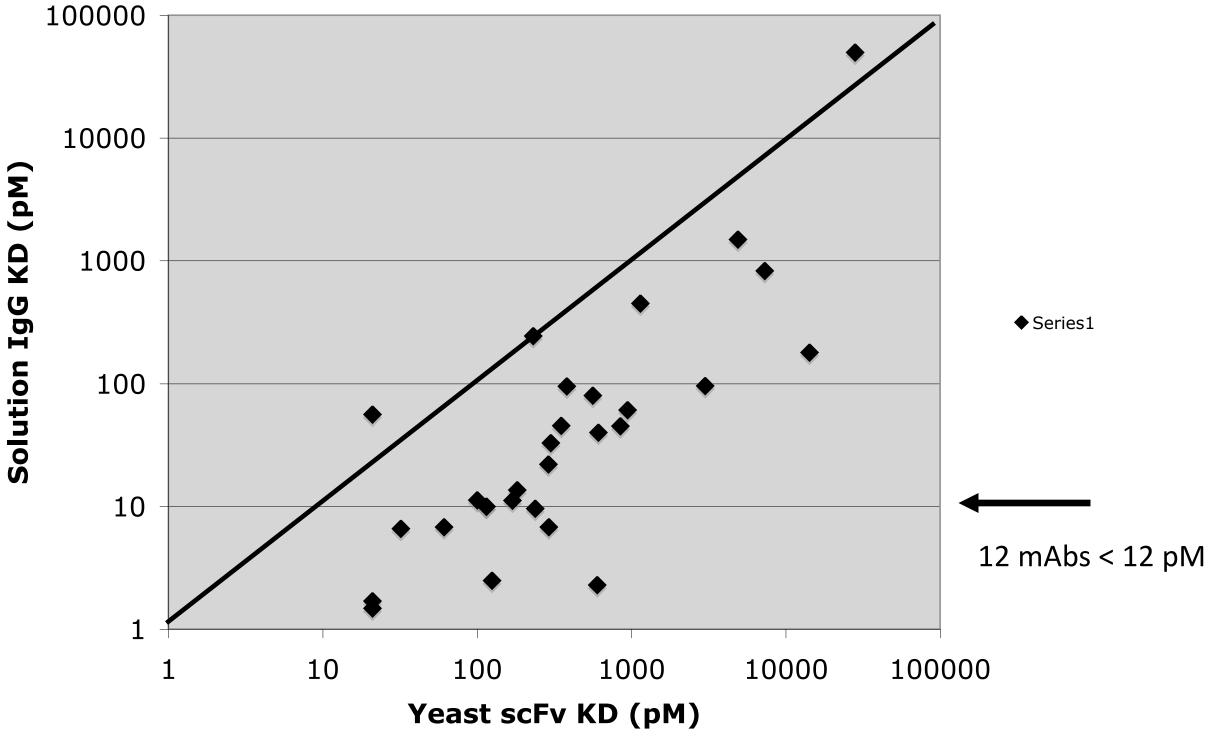
Repertoire usage: VH1: 29%; VH2: 4%; VH3: 50%; VH4: 14%;VH5:3%
Vk1:50%; Vk2: 6%; Vk3: 22%; Vk4: 4%; Vl1: 12%; Vl3: 6%

Affinities of lead BoNT scFv and their IgG (n = 23)

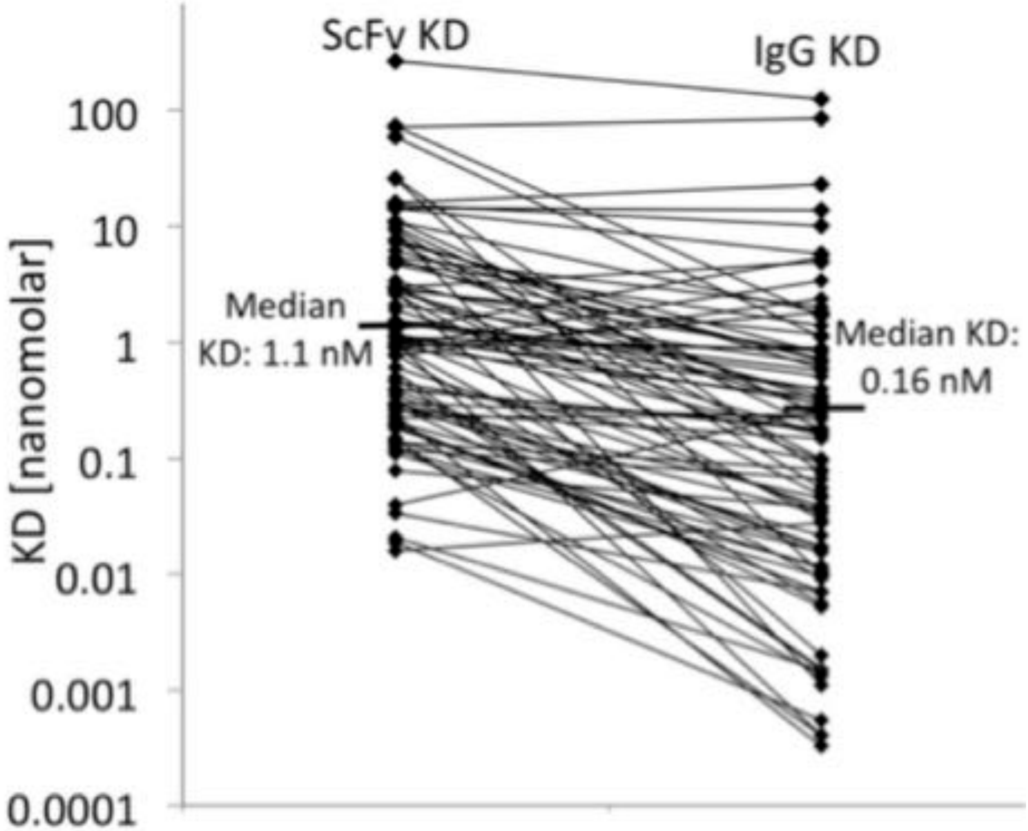


Affinities of BoNT yeast displayed scFv and IgG in solution

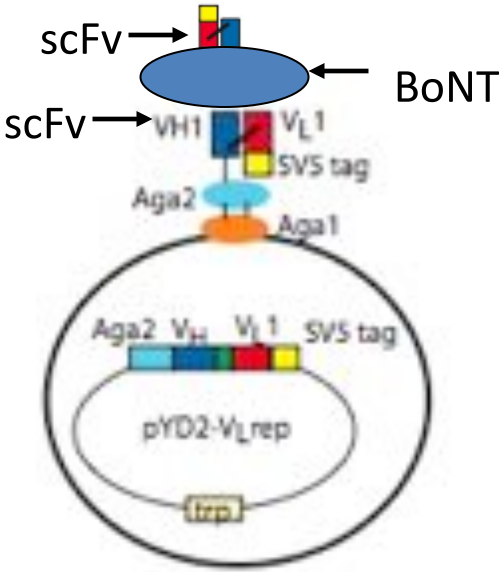
12 primary libraries, 30 secondary libraries: 30 scFv converted to IgG



Affinites of scFv converted to IgG (85 scFv)



Mapping antibody epitopes for overlap



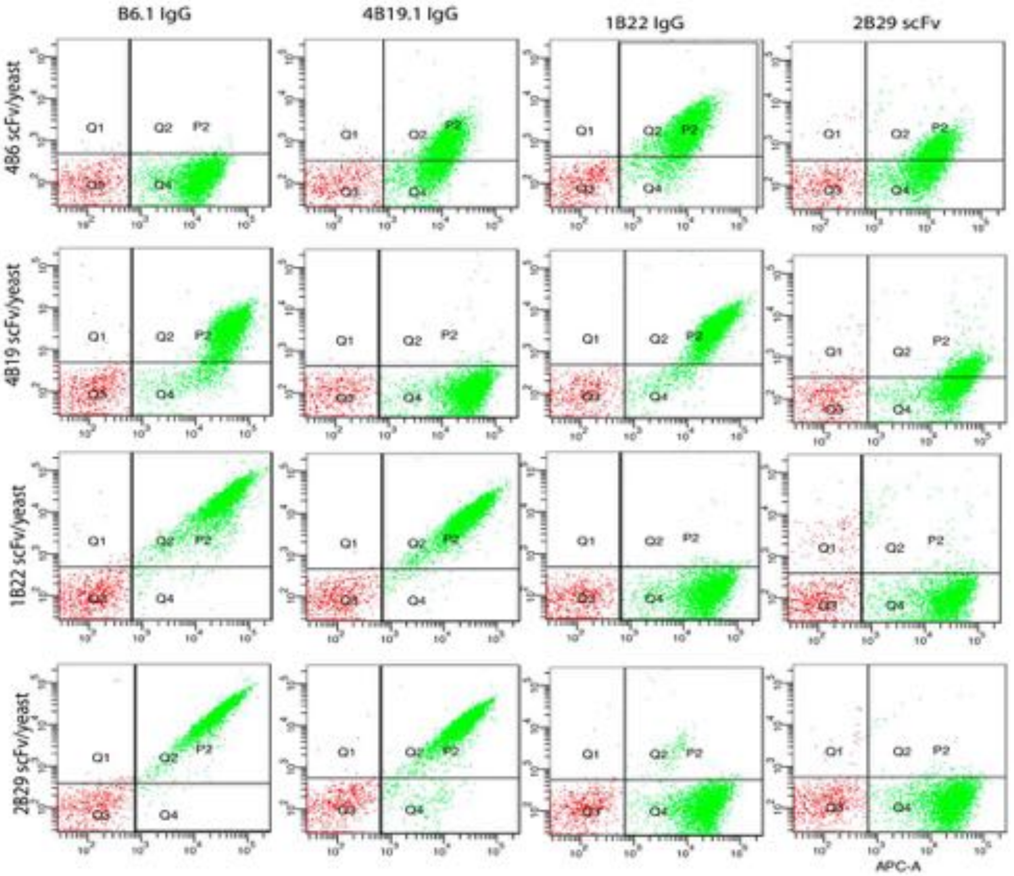
Epitope mapping antibodies by flow cytometry

B6scFv yeast

4B19scFv yeast

1B22scFv yeast

2B29scFv yeast

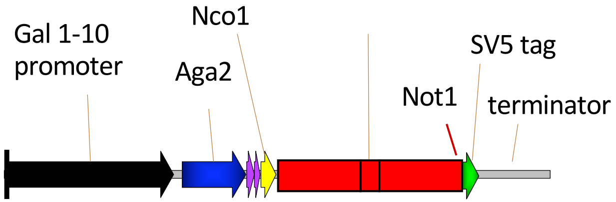


Antigen yeast display

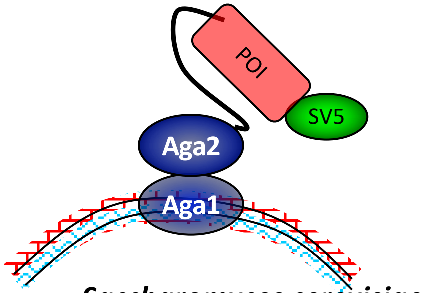
- Use to domain and fine epitope map antibodies
- Use to select phage antibodies

Antigen yeast display

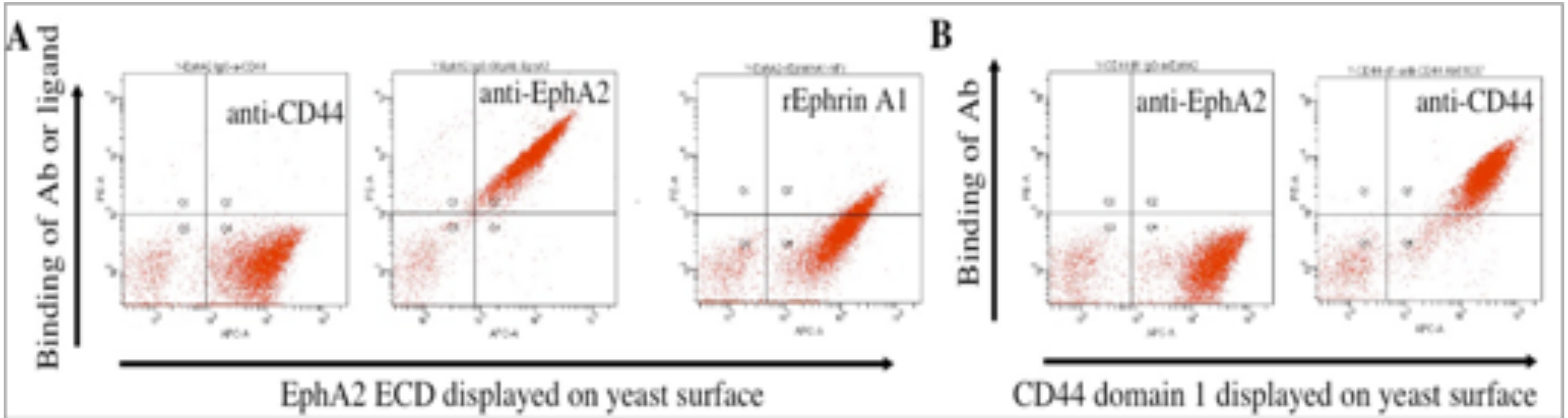
Gene of interest



Protein of interest

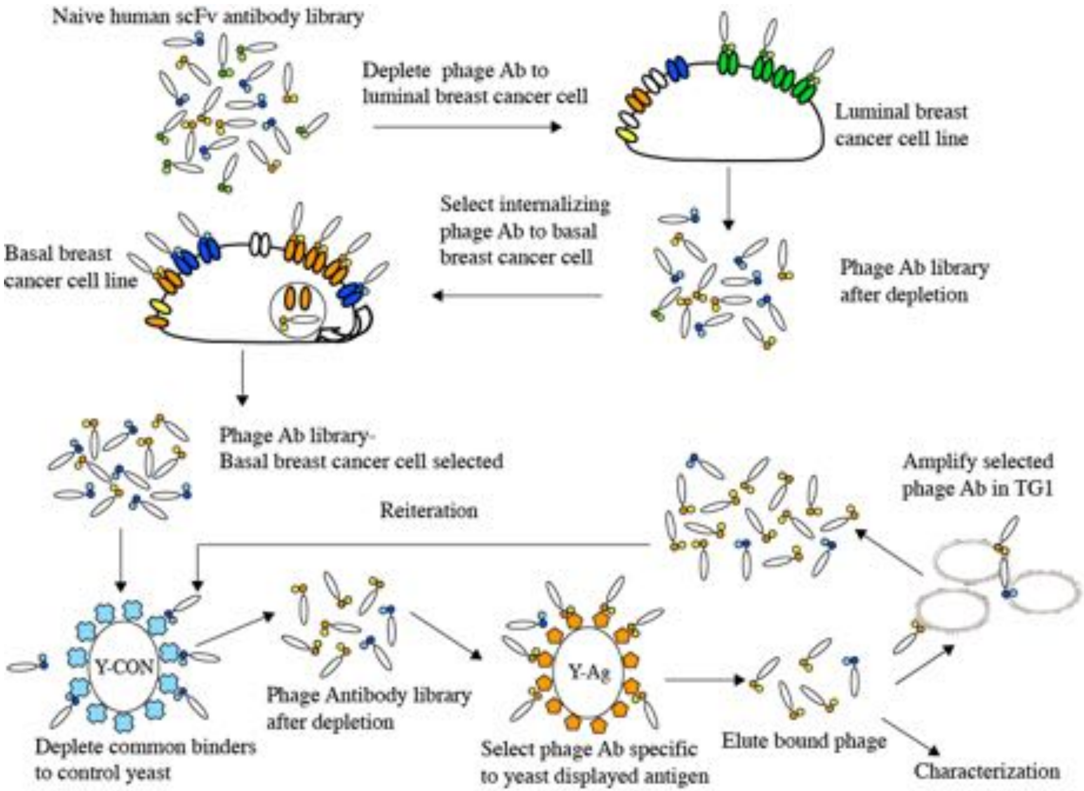


Saccharomyces cerevisiae



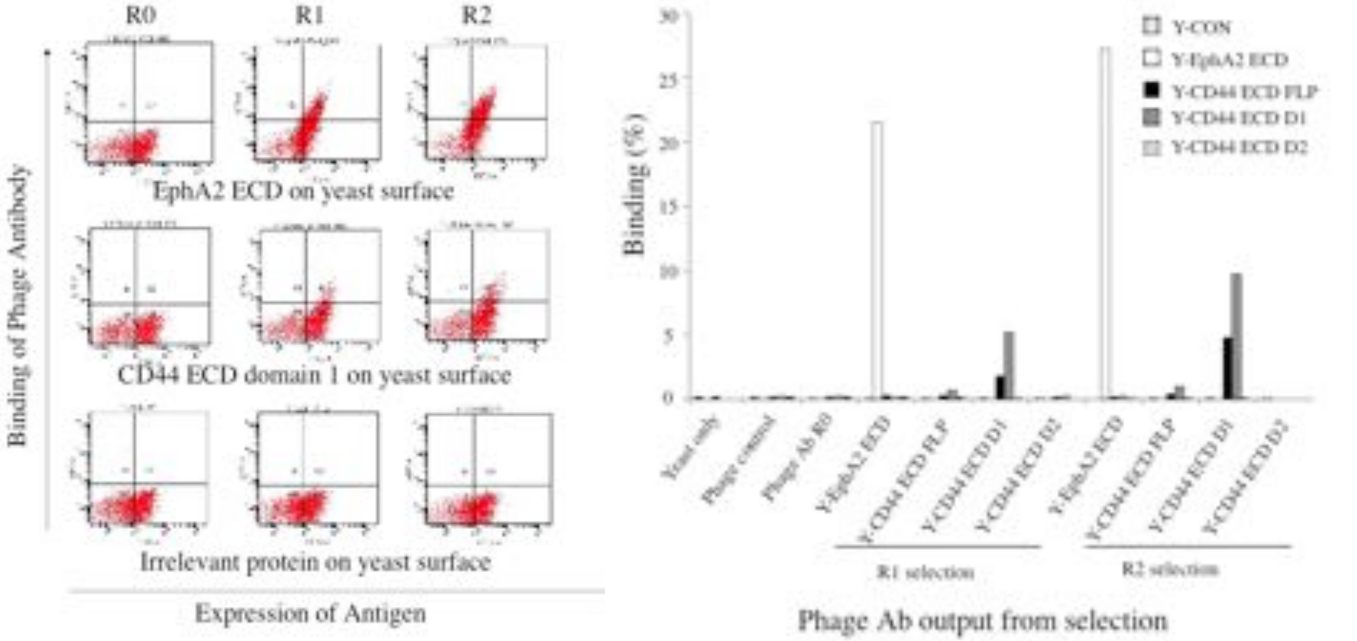
Zhou et. al., JMB, 404: 88-99. 2010

Yeast displayed antigen combined with cell selection



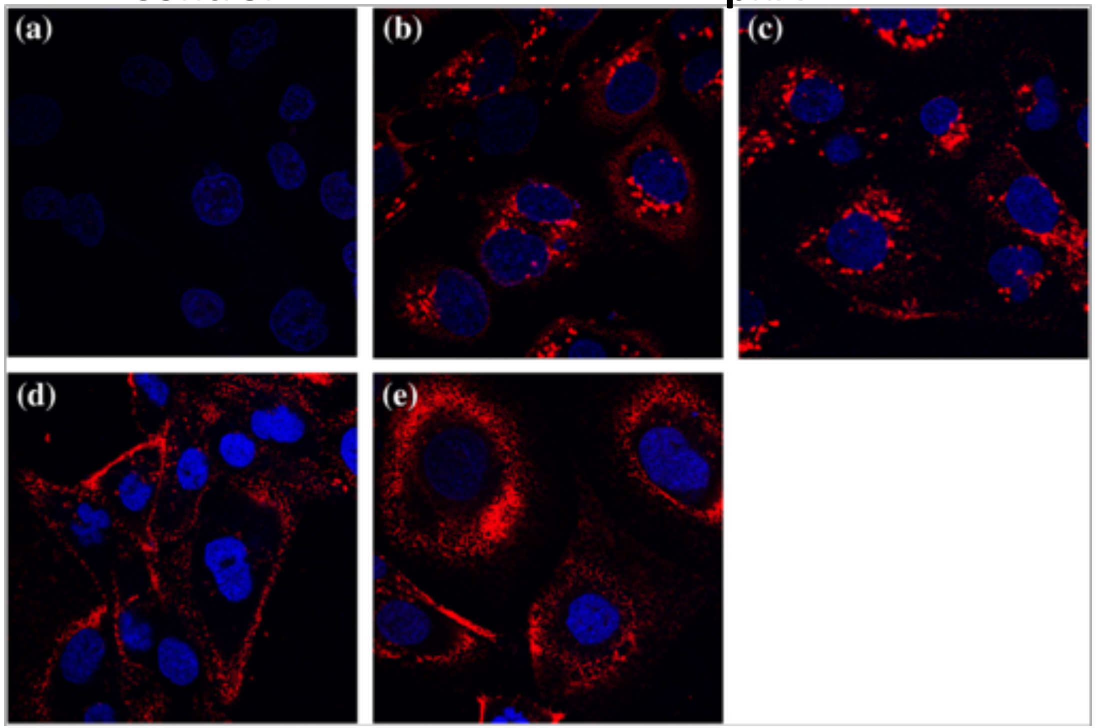
Zhou et. al., JMB, 404: 88-99. 2010

Enriched Phage Abs on Yeast displayed Ags



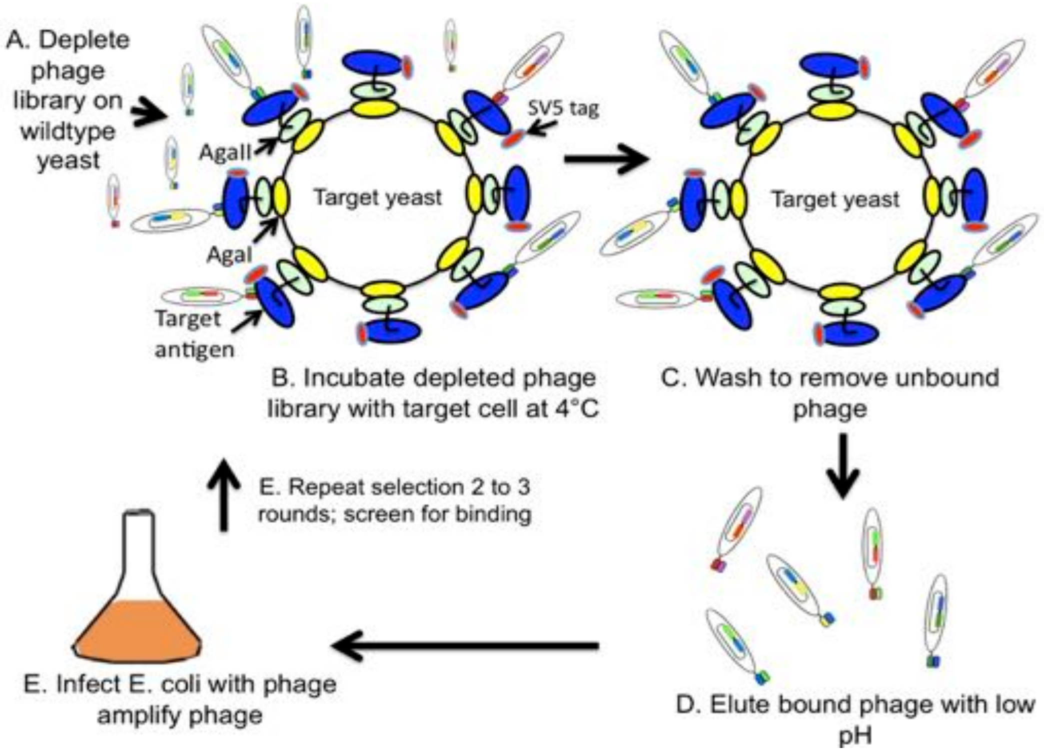
Zhou et. al., JMB, 404: 88-99. 2010

Internalizing mAbs to Basal Breast Cancer Cells



Zhou et. al., JMB, 404: 88-99. 2010

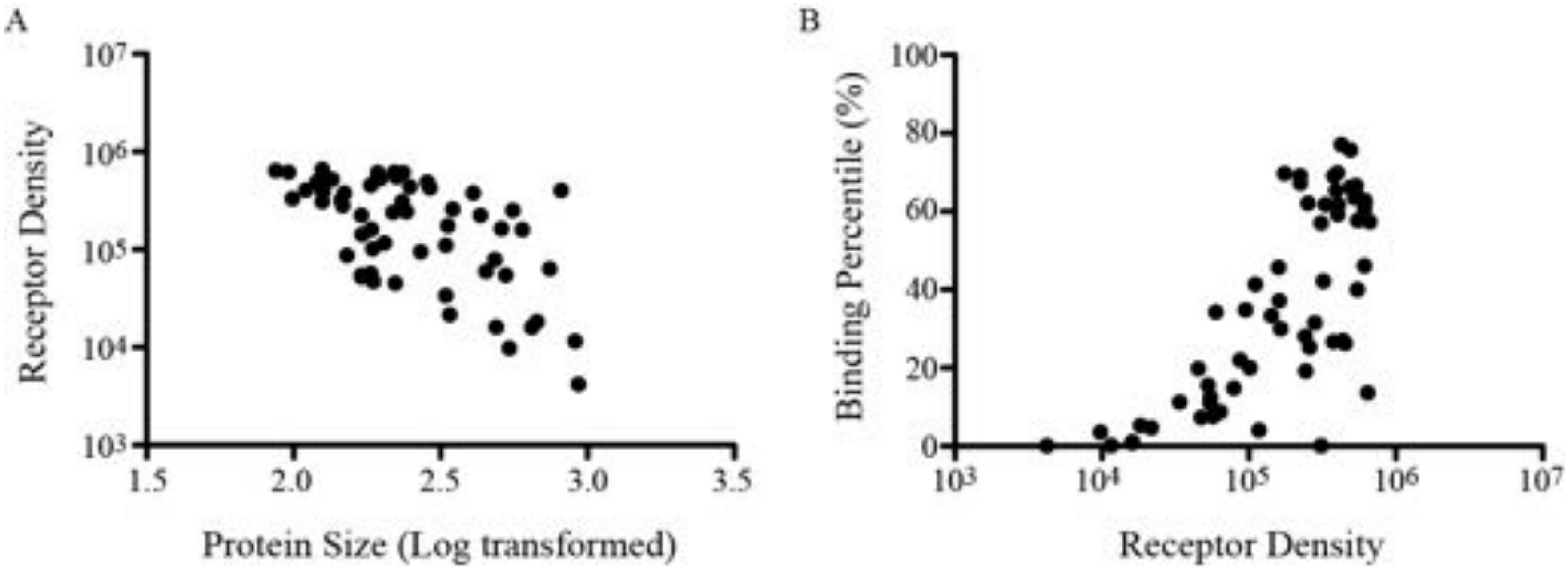
Yeast displayed Ag Used for High Throughput Phage Ab Selection



Selected Secretome for Yeast Ag Display and Phage Ab Selections

Gene Name	Display	Domain Classes	Yeast Display Strategy (# residues displayed)
EGFR	Type 1 TM	Receptor L domain, Furin-like cysteine rich region, Furin-like repeats	4 Individual domains (185, 125, 171, 135)
HER2	Type 1 TM	Receptor L domain, Furin-like cysteine rich region, Furin-like repeats	4 Individual domains (193, 126, 170, 145)
HER3	Type 1 TM	Receptor L domain, Furin-like cysteine rich region, Furin-like repeats	4 Individual domains (187, 124, 170, 147)
HER4	Type 1 TM	Receptor L domain, Furin-like cysteine rich region, Furin-like repeats	4 Individual domains (183, 125, 170, 152)
EPHA2	Type 1 TM	Ligand Binding Domain, SAM (Sterile alpha motif), FN3 domain	Full-length ECD (510)
EPHB3	Type 1 TM	Ligand Binding Domain, SAM, FN3	Full-length ECD (527)
VEGFR2	Type 3 TM	Ig	ECD (744) & Ig2-3 (204)
FGFR1	Type 1 TM	Ig	ECD (348), Ig1 (87), Ig2-3 (218)
c-Met	Type 1 TM	Sema (semaphorin domain), PSI (Plexin repeat), IPT (Ig-like, plexins, transcription factors)	Full-length ECD & individual domain combinations (489, 908, 271, 340)
MST1R	Type 1 TM	Sema (semaphorin domain), PSI, IPT	ECD & 3 individual domain combinations (933, 647, 222, 330)
ICAM1	Type 1 TM	Ig	Ig1-5 (452) & Ig1 (99)
PECAM	Type 1 TM	Ig	Ig1-2 (236)
VCAM	Type 1 TM	Ig	Ig2-7 (673) & Ig2-3 (197)
EpCAM	Type 1 TM	Thyroglobulin type-1	Full-length ECD (242)
E-Cad	Type 1 TM	Cadherin like domain	Cad domains 1-5 (542), 1-2 (220)
CD44	Type 1 TM	Link domain	7 variant domains (149, 409, 558, 249, 291, 335, 433)
CD47	5 TM	Ig-like V-type	N-terminal ECD 1 (123)
CD73	GPI anchor	N-terminal metallophosphatase domain	Full-length ECD (523)
CD168	GPI anchor	Hyaluronan-binding fragment	63kDa isoform (561)
MSLN	Secreted	No domain superfamily	Cleaved form (285)
MMP9	Secreted	Fibronectin type-II, Hemopexin	82 kDa (601) & FNII 1-3 (184)
TIMP1	Secreted	NTR	Full-length protein (183)
TIMP2	Secreted	NTR	Full-length protein (193) & NTR (125)
Robo1	Type 1 TM	Ig-like C2-type, Fibronectin type-III	Full-length ECD & individual domain combinations (96, 110, 330, 484, 814)

Many proteins display on yeast and can be used to select antigen specific mAbs



Zhao *et al. PLoS One* **9**, e111339 (2014).

Mammalian cell display

Advantages of mammalian cell display

- Screen 10’s millions clones
- Gain info on expression level, affinity and specificity
- Appears to provide information on antibody developability
  - More developable antibodies appear to display better
- Ability to work in IgG format
- Potential for screening directly for function in mammalian cells
- Screen directly in production cell type used for IgG production

Challenges in mammalian cell display

- Making large libraries
  - Transformation efficiency much lower than bacteria or yeast
- Genotype phenotype coupling
  - Standard transfection/electroporation integrates antibody genes as a linear array with variable copy number of the transfected transgene
    - Results in multiple different antibody genes into each cell
    - Expression of multiple distinct antibodies per cell
    - Mixing of different heavy and light chain monomers for IgG or Fab formatted libraries
    - Co-isolation of many passenger antibody genes
    - VH and VL genes on separate plasmids
- Slow growth rate
  - 24 hour doubling time

Solutions to the multiple gene problem: Vector mediated display

- Episomally replicating vectors
  - EBV origin based
  - Eventually resolve into a cell population with limited number of antibody genes per cell
  - HEK cells
  - Simultaneous display and secretion
    - Lox mediated removal of tm domains to allow secretion
    - Alternative splicing to remove tm domains
  - Inducible AID allows somatic hypermutation
- Standard expression vectors
  - Transient transfections
    - Plasmids need to be isolated after each selection round
    - HEK cells
      - Separate VH and VK libraries as full length with murine H2K tm region
      - scFvs with PDGFR tm
    - Each round takes ~7 days
  - Stable transfections
    - Suitable for multiple rounds of selection
    - Small libraries
    - Takes longer

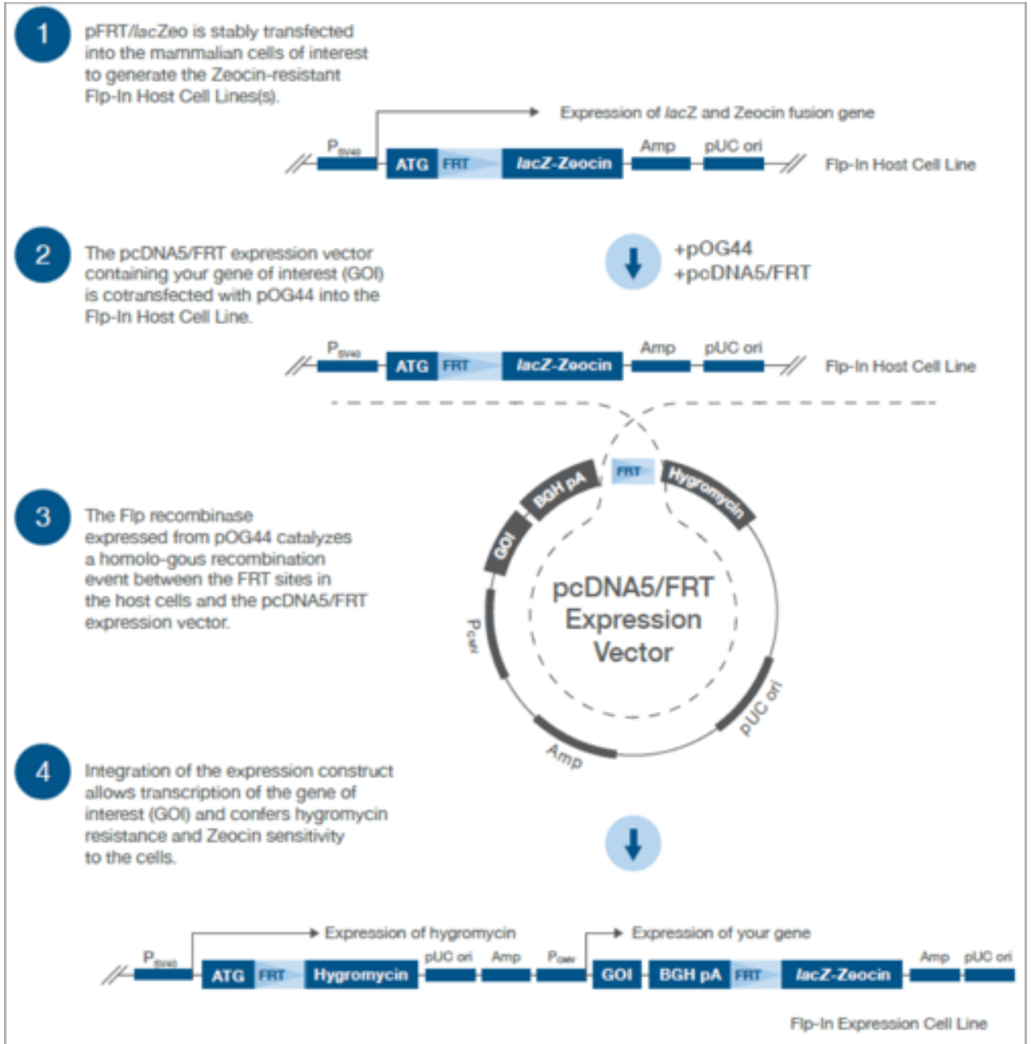
Solutions to the multiple gene problem: viral vectors

- Retrovirus
  - Separate VH and VK libraries in retroviruses
    - Full length IgG
    - IgM tm region
    - VH and VK are not linked, so binding specificity needs to be recreated
- Sindbis virus
  - scFvs fused in frame with PDGFR beta chain tm domain
    - VH and VL recloned as full length IgGs after selection
- Vaccinia
  - Phage display like selection on surface of vaccinia virus
  - After infection into mammalian cells, mammalian display
  - Separate VH and VK libraries
    - Full length IgG
    - IgG tm region
    - VH and VK are not linked, so binding specificity needs to be recreated

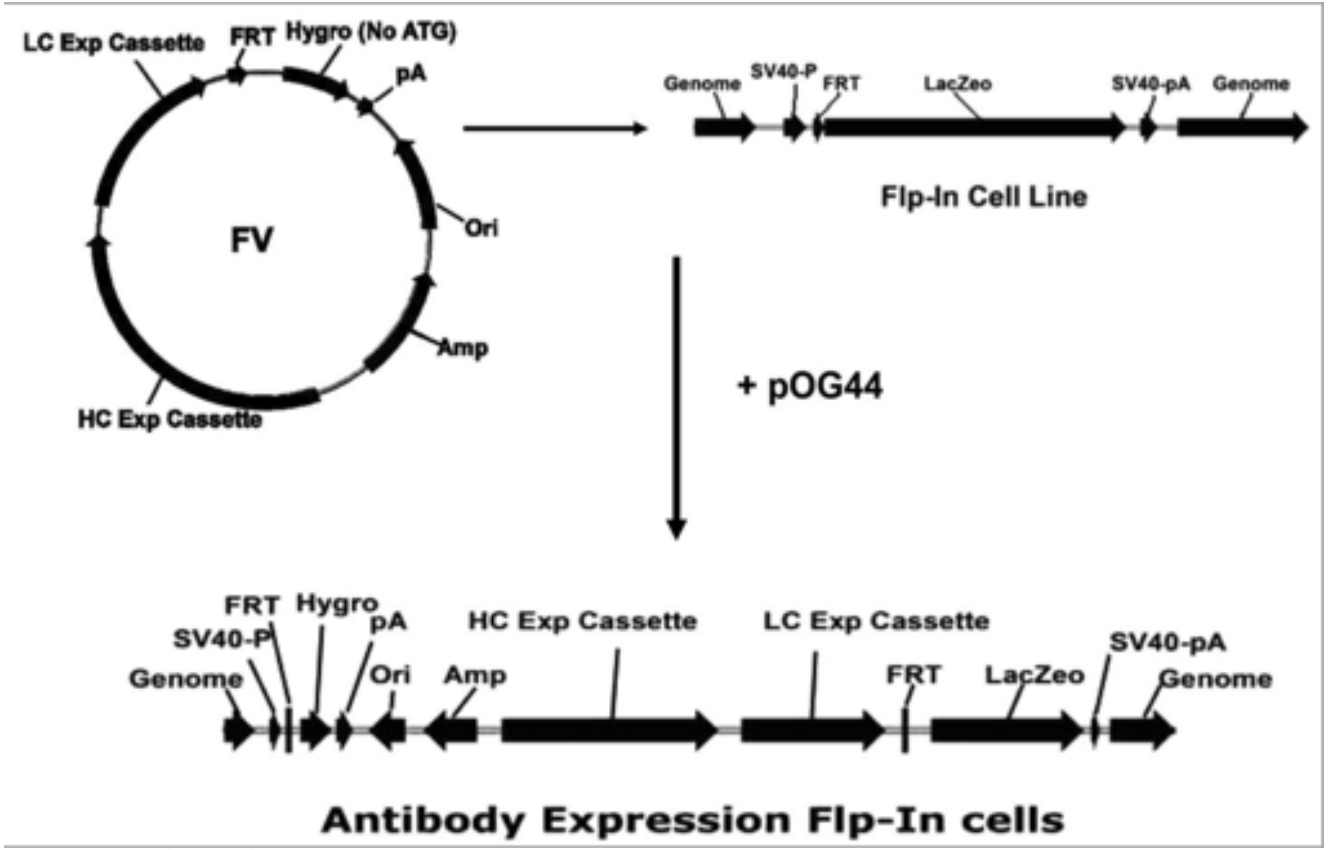
Solutions to the multiple gene problem: genetic approaches

- FLP site specific integration (three different groups)
  - Flp Recombination Target (FRT) inserted into genome
  - Affinity maturation libraries only, not naïve
  - Vector contains two FRT sites allowing site specific genome integration
  - Flp In CHO line (Invitrogen) used
    - Full length IgG
    - VH and VL on same expression vector
    - PDGFR tm domain
    - Promoterless Hygromycin gene
      - Only expressed when integrated in-frame with ATG codon and up-stream promoter of the FRT site within host genome
      - Allows selection of cells expressing antibodies
    - Insertion of part of vector into genome mediated by Flp recombinase
    - Lox sites flanking tm domain allow conversion from display to secretion by cre recombinase expression

Flp-In system



Applied to antibody display



Zhou *et al.* *MAbs* **2**,508-514 (2010).

Solutions to the multiple gene problem: genetic approaches

- Transposase mediated gene insertion
  - Separate vectors for Transposase, VH and VL
    - Co-transform all vectors
      - Hyperactive piggyback transposase transfers inserts VH and VL expression cassettes into genome
      - Full length IgG
      - VH and VL transcriptional units contain downstream IRES and downstream hygromycin B and puromycin
    - Membrane bound and secreted IgG produced by natural alternative splicing between CH3 and membrane domain

Solutions to the multiple gene problem: genetic approaches

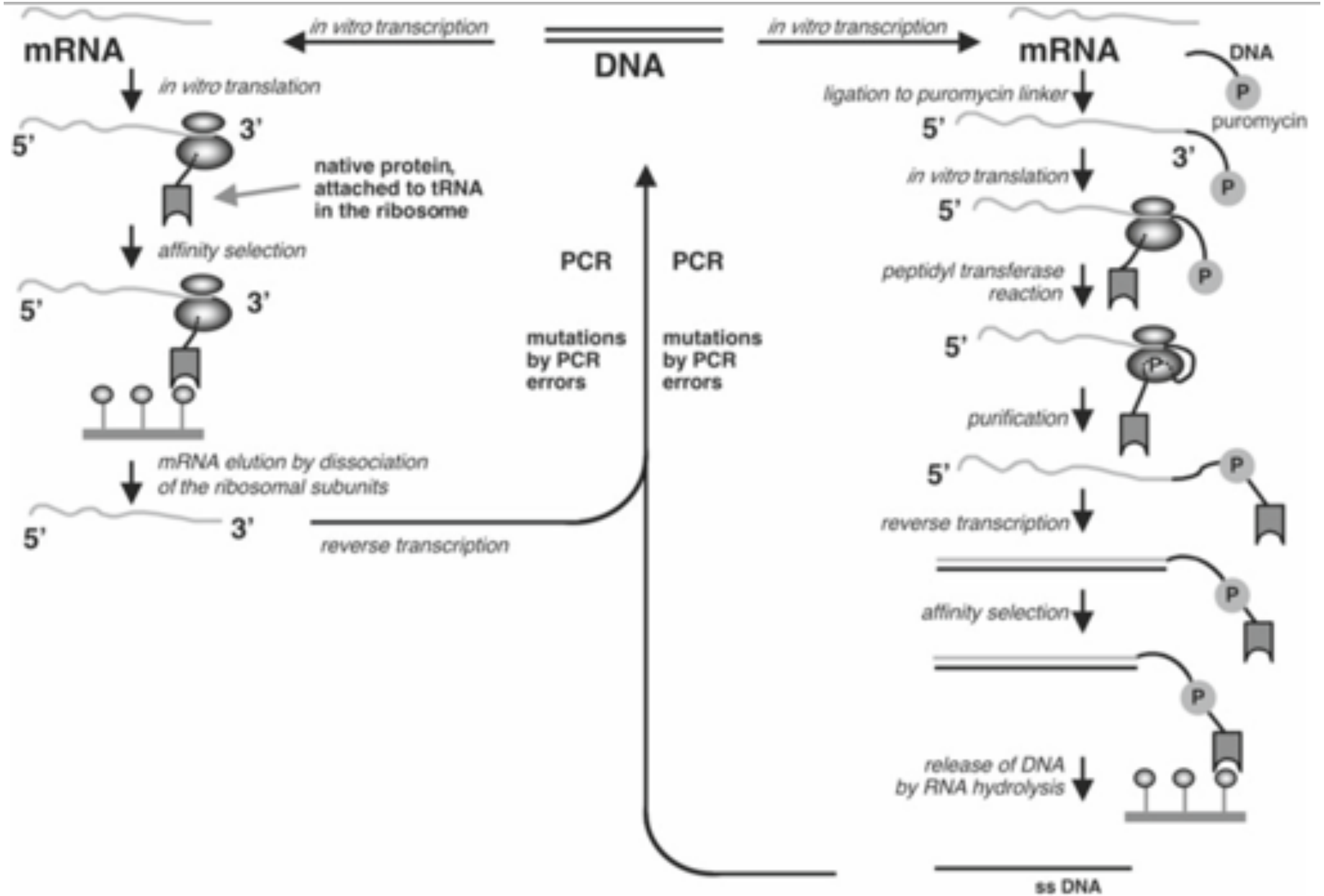
- Talen mediated integration (McCafferty)
  - AAVS locus targeted (McCafferty)
    - Maxcyte based transfection
  - scFv or full length IgG integrated
  - Output from phage selections
  - More recently carried out with CRISPR-Cas9
- CRISPR-Cas9 mediated integration
  - Hybridoma engineered to remove VL locus, and replace VH locus with ruby RFP (Reddy)
  - Reprogrammed to express full length antibodies with CRISPR-Cas9
    - Inserted construct: VK-CK (2A peptide) VH-CH1
    - VH-CH1 upstream of genomic CH2-CH3 genomic construct
    - Natural alternative splicing produces both displayed and secreted IgG

In vitro display methods

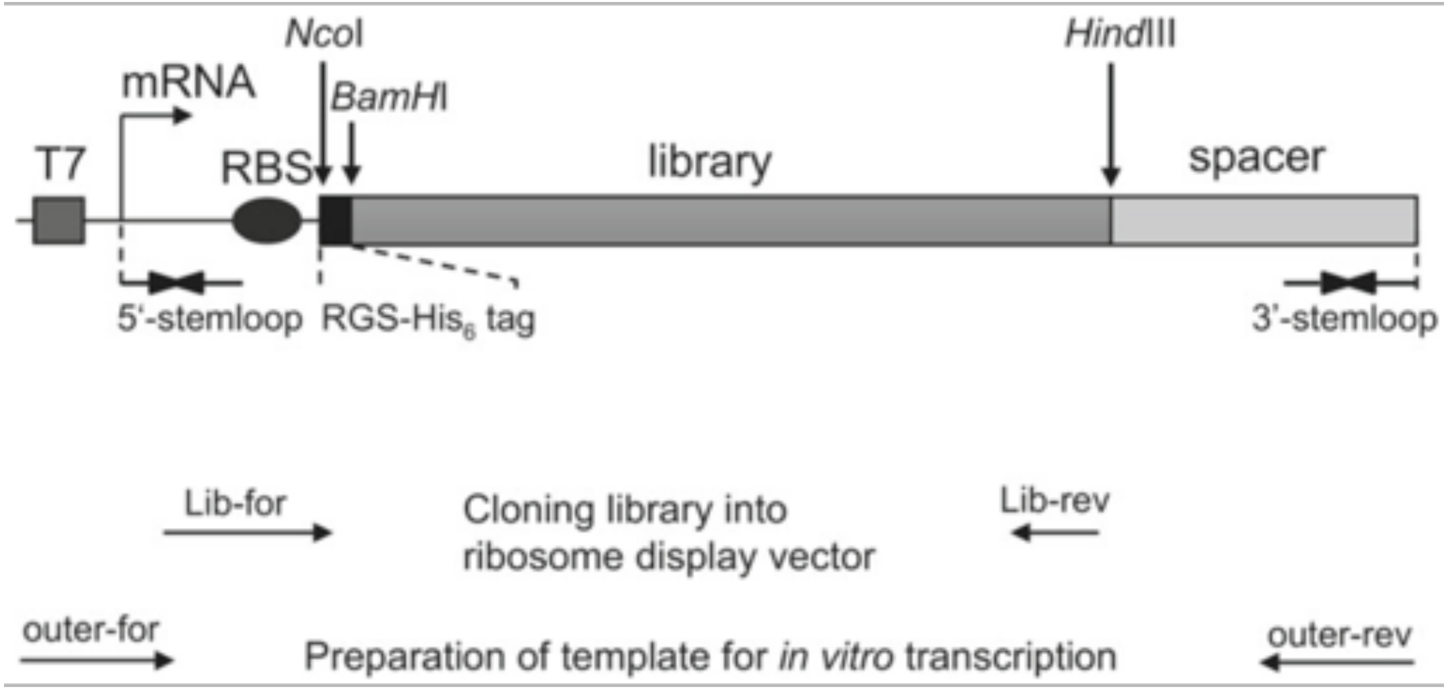
In vitro methods

- For both ribosome and mRNA display
    - Very large libraries – 10^{12-13} molecules
    - Selection acts on single molecules
    - Requires single protein construct
        - scFvs or scFabs, not Fabs or full length IgG
    - PCR step in every cycle introduces in built affinity maturation
        - Selection is stochastic
            - » Repeat selections tend to coalesce around different clonotypes
            - » Each selection gives different solutions
    - Screening requires final output to be cloned into alternative system
        - Expression vector and screening on target
        - Two hybrid system and screening on endogenously expressed protein
- Ribosome display
    - Ribosome acts as link between mRNA and encoded protein
- mRNA display
    - Puromycin acts as link between mRNA and encoded protein

Ribosome display mRNA display



Construct for ribosome display

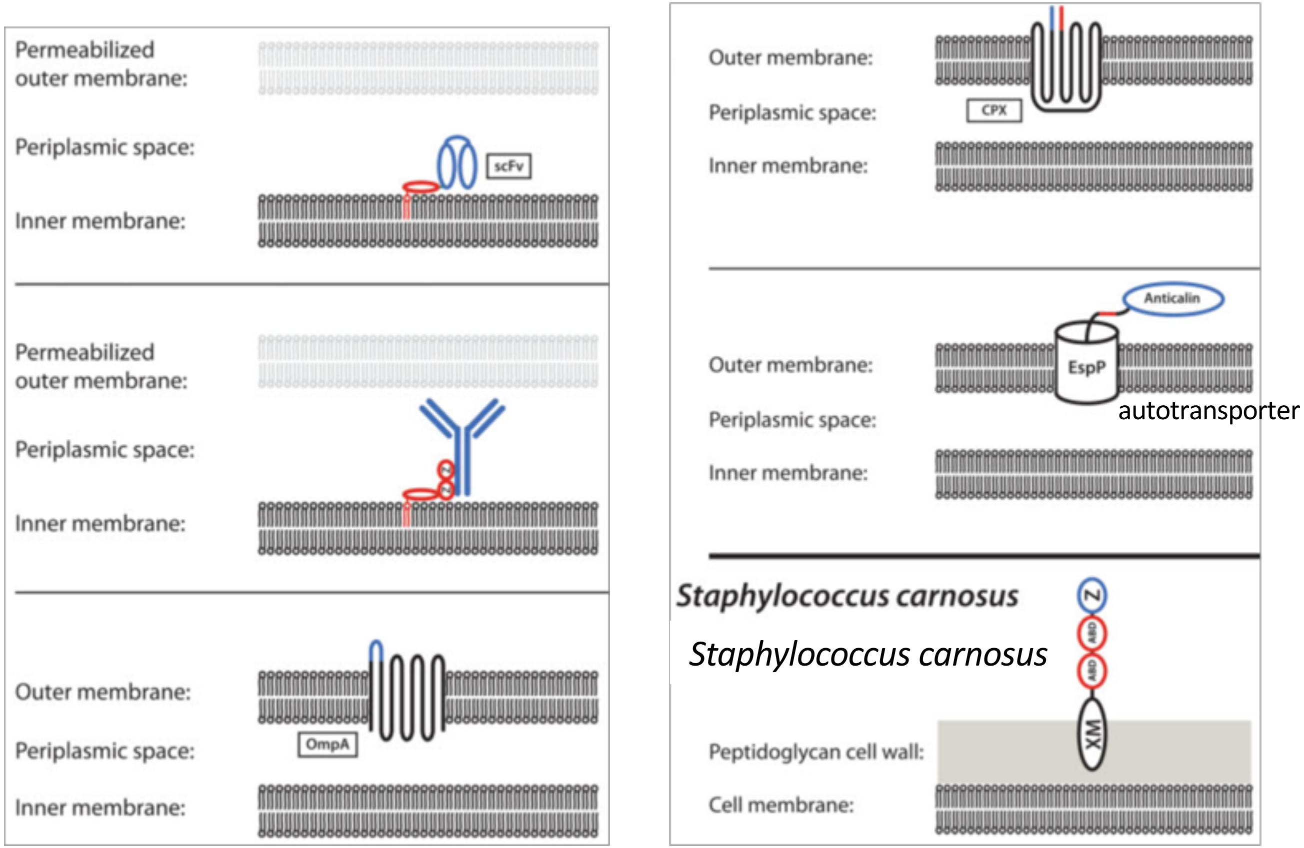


Bacterial display

Bacterial display

- Similar advantages to yeast display
  - FACS based selection
  - Analysis of epitopes on surface
- Gram negative
  - All E. coli based
  - Many different systems
    - Fundamental problem is display beyond outer membrane
      - Permeabilization of outer membrane
        - » Requires PCR to rescue antibodies
      - Difficult to display antibodies in intact E. coli
    - Autotransporters solve the problem by using endogenous mechanisms to display
      - Applied to anticalin libraries and many enzymes but not antibodies
      - Difficult to predict which proteins will display
      - Some proteins can be displayed without being folded
- Gram positive
  - S. carnosus most popular
    - Many publications from limited number of groups
    - Main problem is low level of transformation
      - Difficult to generate libraries >10^6 diversity
      - Good for affinity maturation or selection from small libraries
      - Single domain proteins displayed, no scFv
        - » Nanobodies, peptides, affibodies,

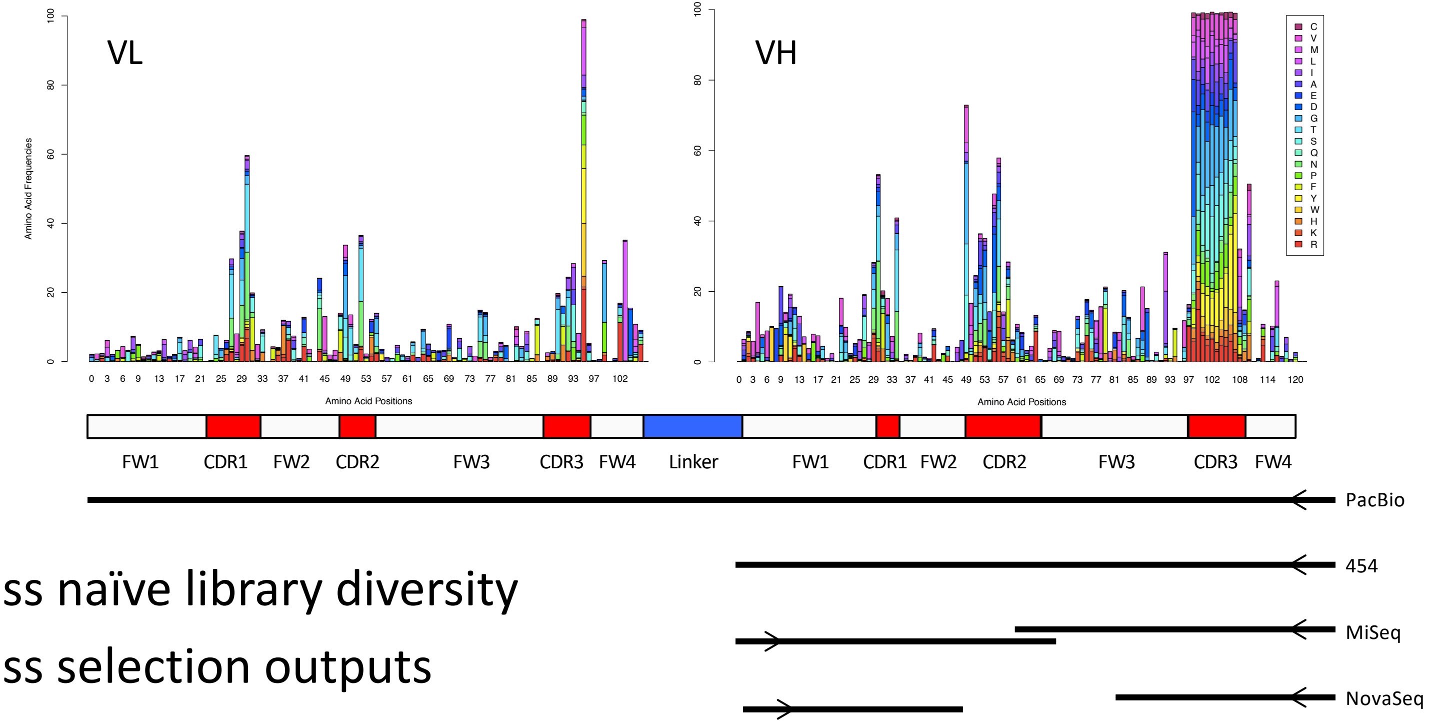
Bacterial display



Lofblom, *Biotechnol J* **6**, 1115-1129, (2011)

Next generation sequencing

NGS platforms and antibodies



- Assess naïve library diversity
- Assess selection outputs

Glanville, J. et al. (2015) Curr Opin Struct Biol **33**, 146

Comparison of Sequencing Platforms

Platform	Type of sequencing	Max read length (bp)	Throughput	Cost (lowest)	Accuracy	Time	Type of error	Cost per 10^6 V domains
NovaSeq (Illumina)	2 x 150	300	S4 $3.3x10^9$/lane	~$24K/lane		40h		$7.27
	2 x 150	300	S2 10^9/lane	~$8K/lane	>75% at 99.9%	40h		$8.00
MiSeq (Illumina) v2/v3	2 x 300	600	$25x10^6$/lane	$3100/lane	>70% reads at 99.9%	55h	Substitution	$124
	2 x 150	300	$16x10^6$/lane	$1100/lane	>80% reads at 99.9%	24h		$78
IonTorrent (LifeTech)-316	1 x 400 1 x 200	400 200	$2x10^6$/chip	$900/chip	> 99%	5 h 3 h	InDel	$450
PacBio-RSII	1 x 8500	8500	47,000	$1050	11-15%	≤4 h	InDel	$22,340
		e.g. 10 passes of 850 bp			99.999% depends on no. passes			
454 (Roche)-GS-FLWX+	1 x 700	700	50,000 in 1/8 plate	$2400/1/8 plate	99.997%	23 h	InDel	
	1 x 450	450		$1900/1/8 plate	99.995%	10 h		$38,000

Some considerations on the use of NGS

- Naïve libraries
  - The diversity of most libraries is measured by counting colonies, with claimed diversities $>10^9$
  - The greatest number of reads possible with NGS is $3x10^9$ (NovaSeq)
  - Even using NovaSeq most naïve libraries will be undersampled, sometimes massively so
  - NovaSeq can provide a minimum measured diversity
- Selections
  - After selection diversity is significantly reduced (1,000 to 10,000 different sequences)
    - NGS can cover the diversity of an antibody selection output
  - Theoretically[1]
    - Libraries of 10^{5-6} should have enough "shape space" to recognize all antigens (affinity threshold)
  - Practically[2,3]
    - Mean (and median) of 4 antibodies per antigen from libraries of $\sim10^7$ antibodies
  - There should be 4,000 - 100,000 different binders from a library of 10^{10} antibodies
    - But usually no more than 30 antibodies in standard selections
    - CAT (now MedImmune) selected >1000 antibodies with >568 different HCDR3s from 10^{11} library against one target (with massive effort)
  - NGS identifies many more antibodies

[1]Perelson & Oster *J Theor Biol* **81**, 645-670, (1979)
[2]Griffiths, *et al. The EMBO journal* **13**, 3245-3260 (1994)
[3]Marks *et al. Journal of molecular biology* **222**, 581-597 (1991).

Understanding Hamming Distances

	Clone Identification at:			
HCDR3	**Hamming 0**	**Hamming ≤1**	**Hamming ≤2**	**Hamming ≤3**
CARLVPETHLRYFDYWG	A	A	A	A
CARLIPETHLRYFDYWG	B	A	A	A
CARLVPDTHLRYFDYWG	C	A	A	A
CARLVPETHIRYFDYWG	D	A	A	A
CARLVPESHLRYFDYWG	E	A	A	A
CARLVPETHLRFFDYWG	F	A	A	A
CARVVPDTHLRYFDYWG	G	B	A	A
CARLVPETHIRYGDYWG	H	C	A	A
CARLVSESHLRYFDYWG	I	D	A	A
CARLVPETRLRFFDYWG	J	E	A	A
CARLIPETRLKYFDYWG	K	F	B	A
CARVVPDTHLRYWDYWG	L	G	C	A
CARLVAETHIRYGDYWG	M	H	D	A
CAKLVSESHLRYFDYWG	N	I	E	A
CARLVPETRLRFFDFWG	O	J	F	A
Total number of clonotypes	**15**	**10**	**6**	**1**

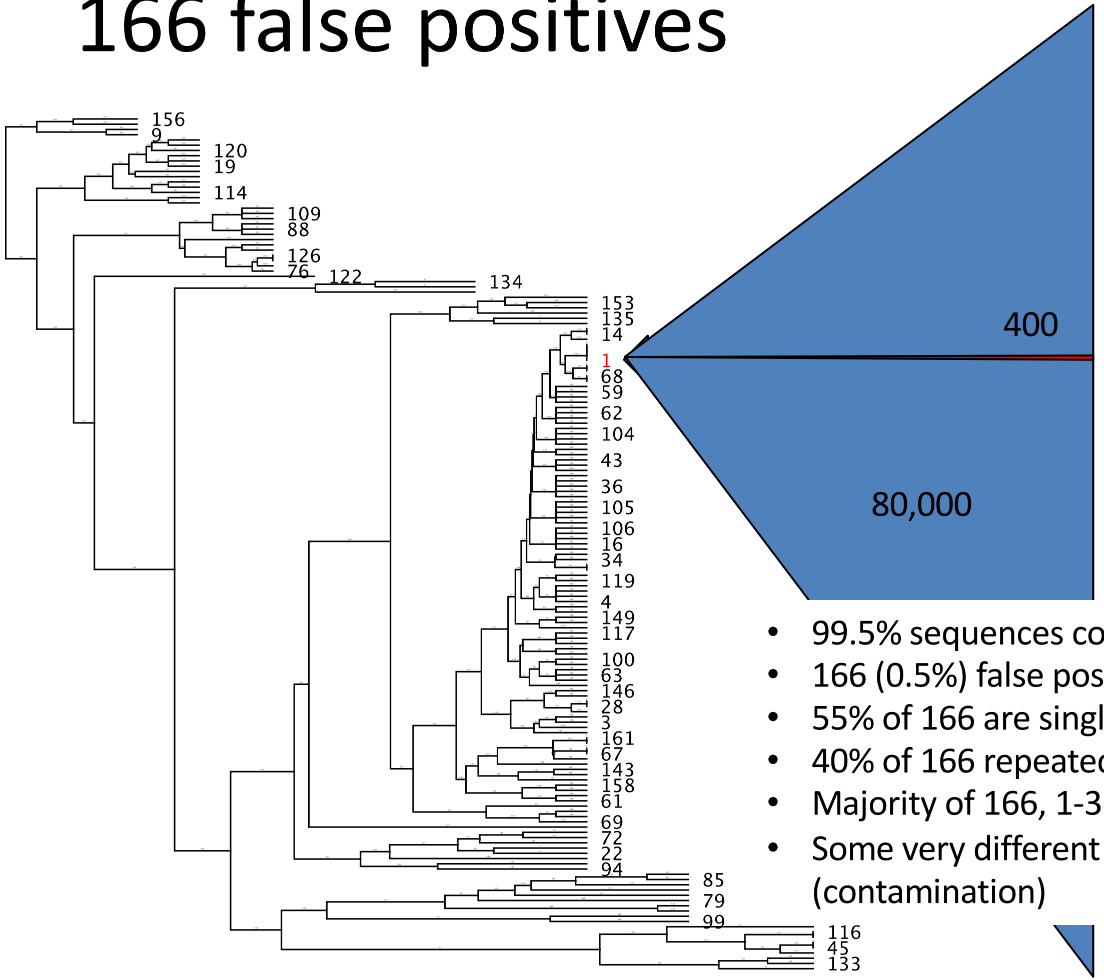
Different identified clonotypes, according to specified Hamming distance

Naïve library analysis with NGS

Challenges in using Next Generation Sequencing to assess library diversity

- Error rates
  - Error rates similar to somatic hypermutation rates
  - Most sequences from a given clone are correct
    - Sequence one HCDR3 80,000 times
      - >99.5% correct
      - <0.5% comprise 166 unique false positive HCDR3s
- What constitutes a different antibody?
  - Hamming distance of ≥2?
    - Pure Hamming is probably insufficient
    - Functional Hamming in which amino acids grouped into functional categories probably more appropriate
    - Most measures concentrate on HCDR3 alone
- Read length
  - NGS Doesn't cover full scFv or Fab length
- Read numbers
  - Except for the smallest libraries, NGS will always undersample diversity
  - More data leads to different conclusions

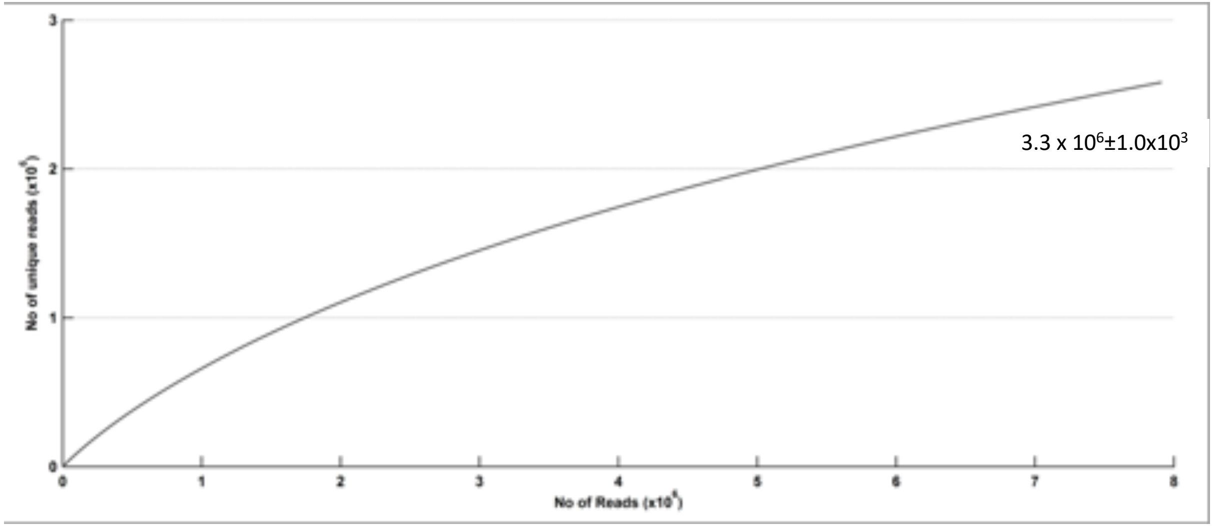
166 false positives



- 99.5% sequences correct
- 166 (0.5%) false positives
- 55% of 166 are singletons
- 40% of 166 repeated false positives
- Majority of 166, 1-3 mutations away
- Some very different HCDR3s (contamination)

Early attempts to assess library diversity with NGS

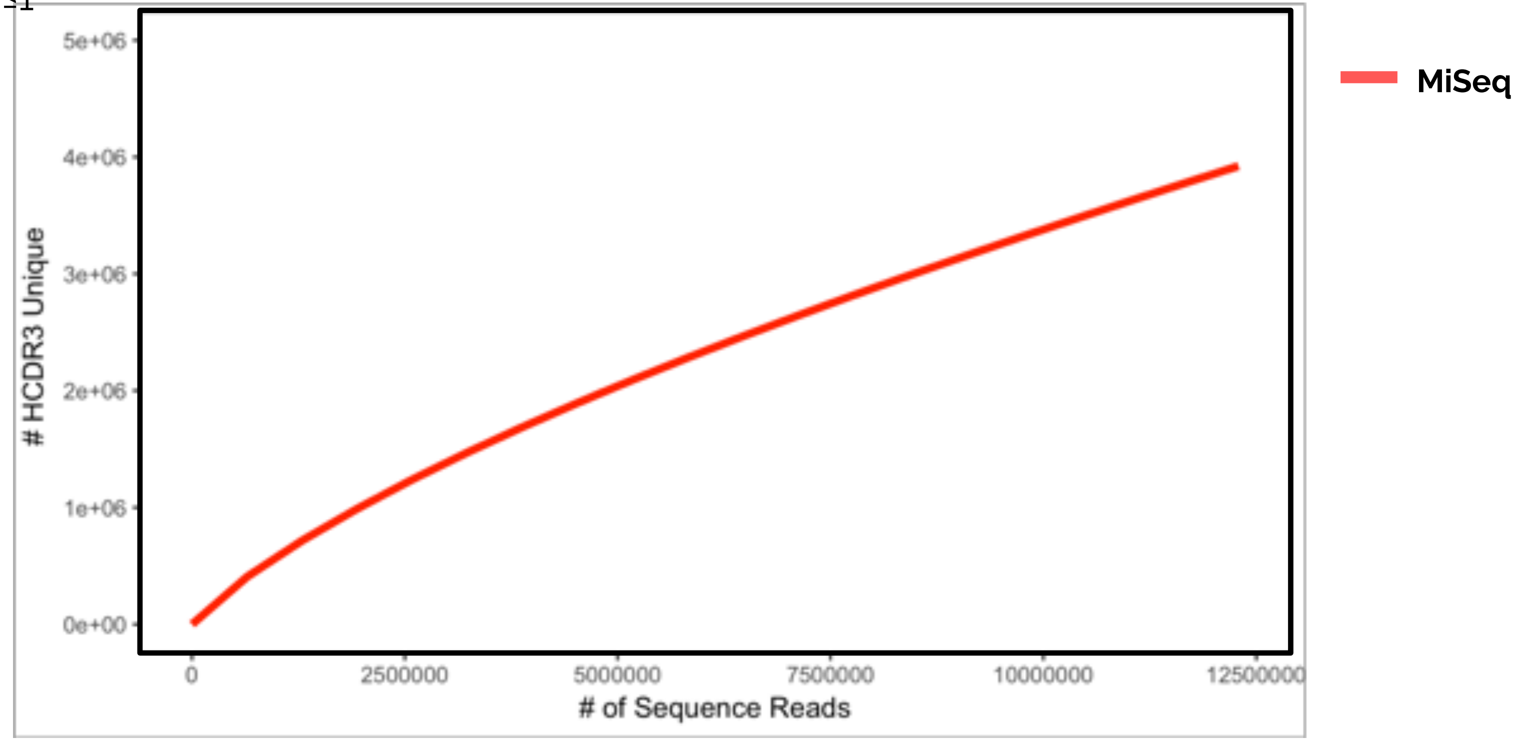
Accumulation plot observed (unique HCDR3 vs tot. # sequences)



- 10^8 transformants, if true VH diversity is ~3x10^6, this is ~30 fold less than expected on basis of number of transformants
  - Additional diversity in HCDR1/2 not accounted for here
- Glanville, 2009: 3x10^{10} transformants (650 donors), estimated VH diversity (non-redundant capture-recapture) 2x10^5
  - ~10^5 less than expected
- The diversity of natural libraries is probably much smaller than that estimated by counting the number of transformants

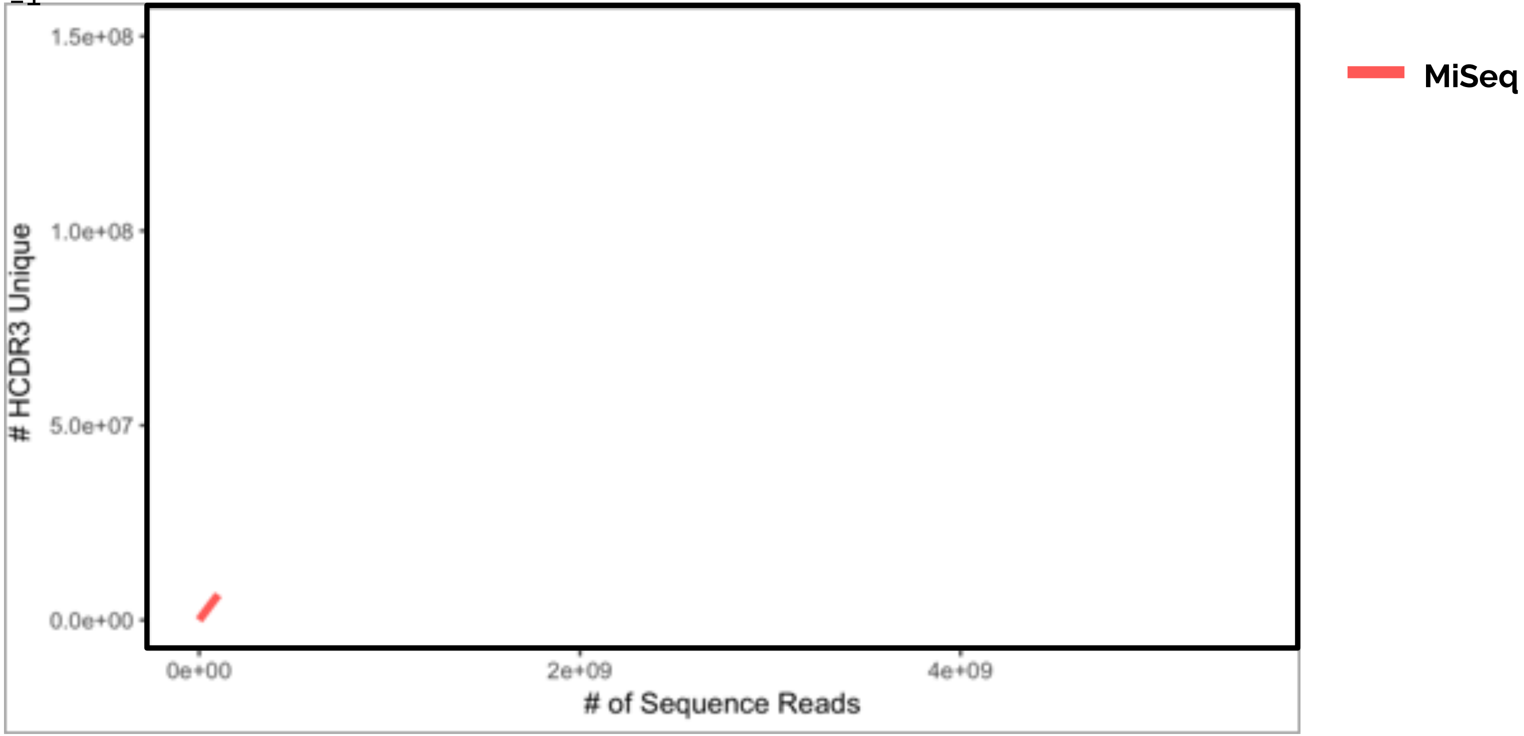
Measuring HCDR3 Diversity of the single donor library by MiSeq…

Hamming ≤1 in HCDR3



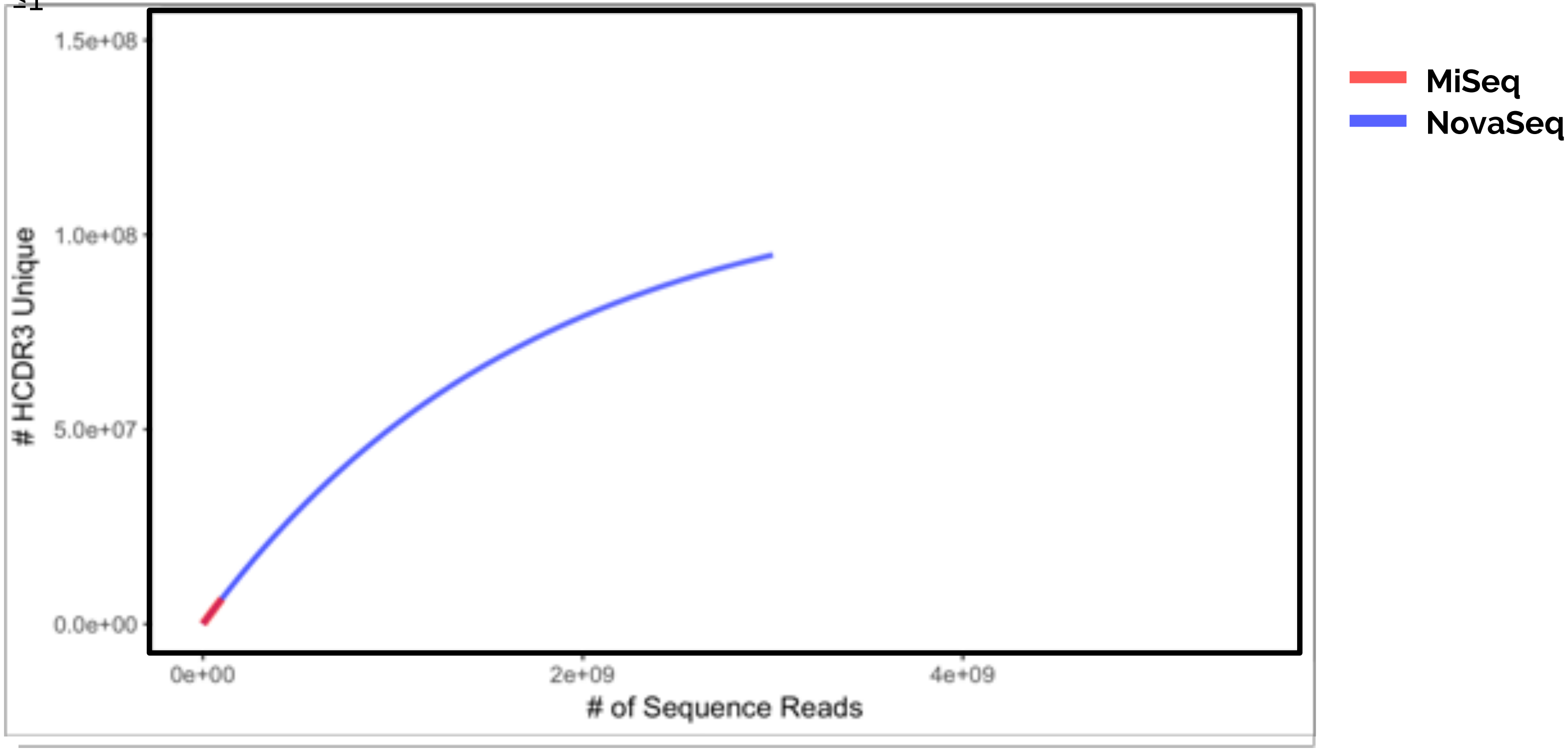
…on a NovaSeq Scale…

Hamming ≤1 in HCDR3

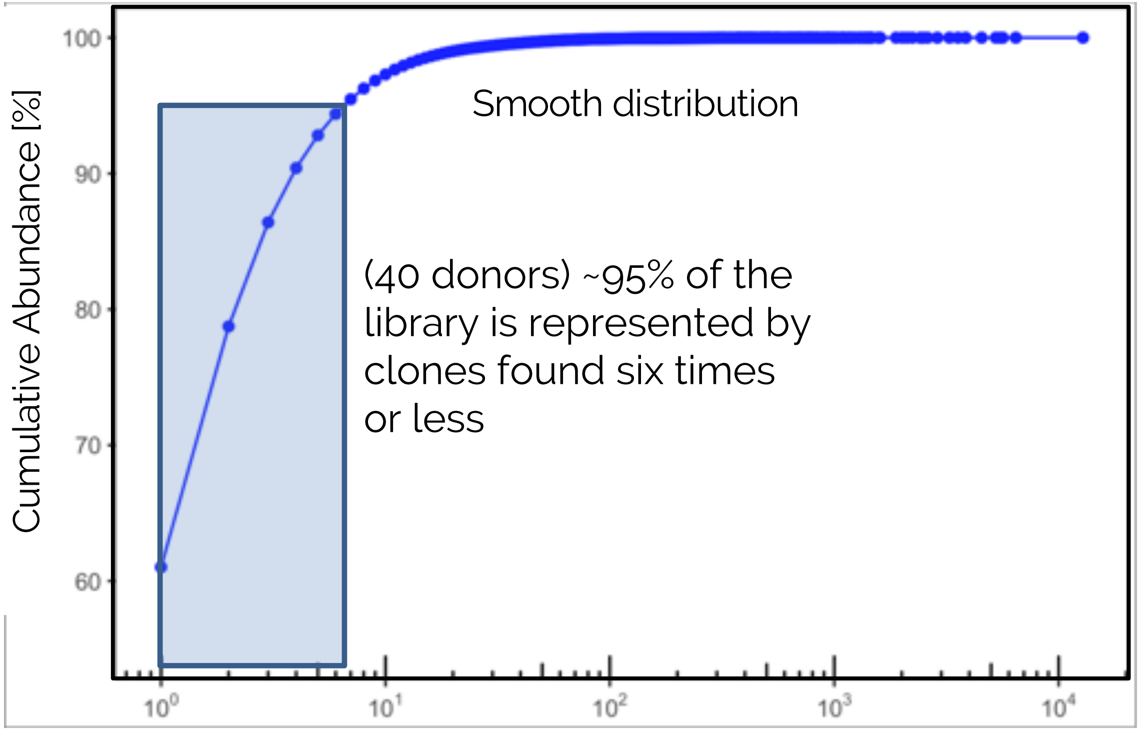


…with NovaSeq Sequence

Hamming ≤1
in HCDR3



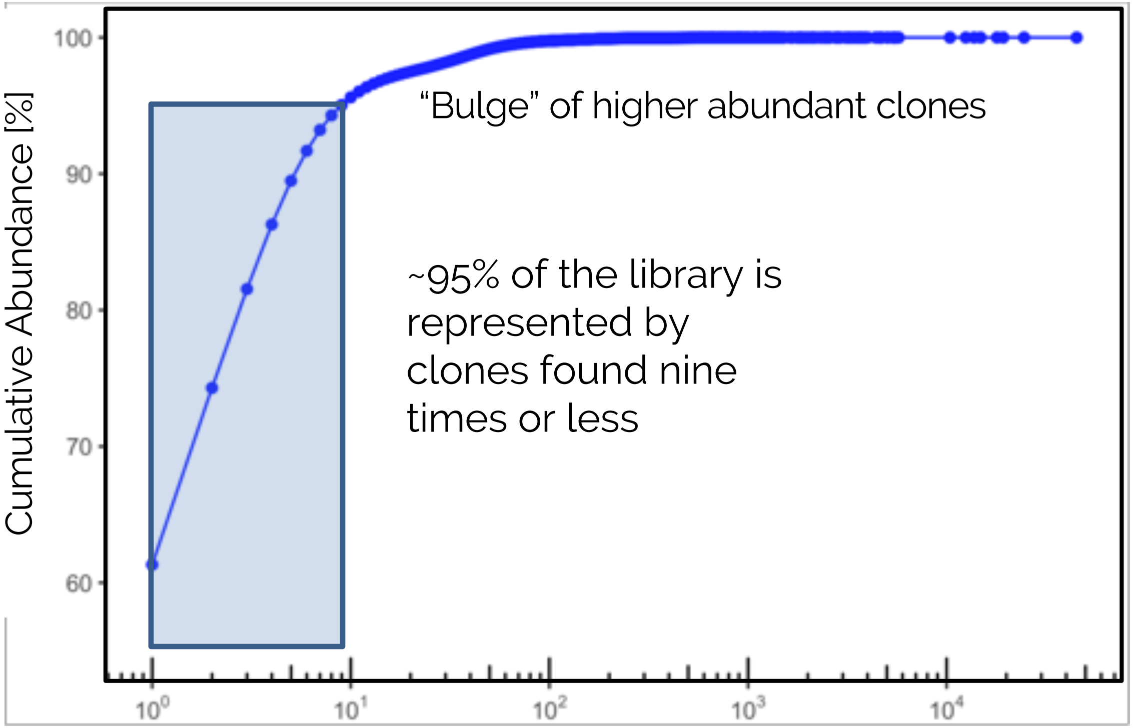
First Generation Multi-Donor Library



Hamming ≤1 amino acid

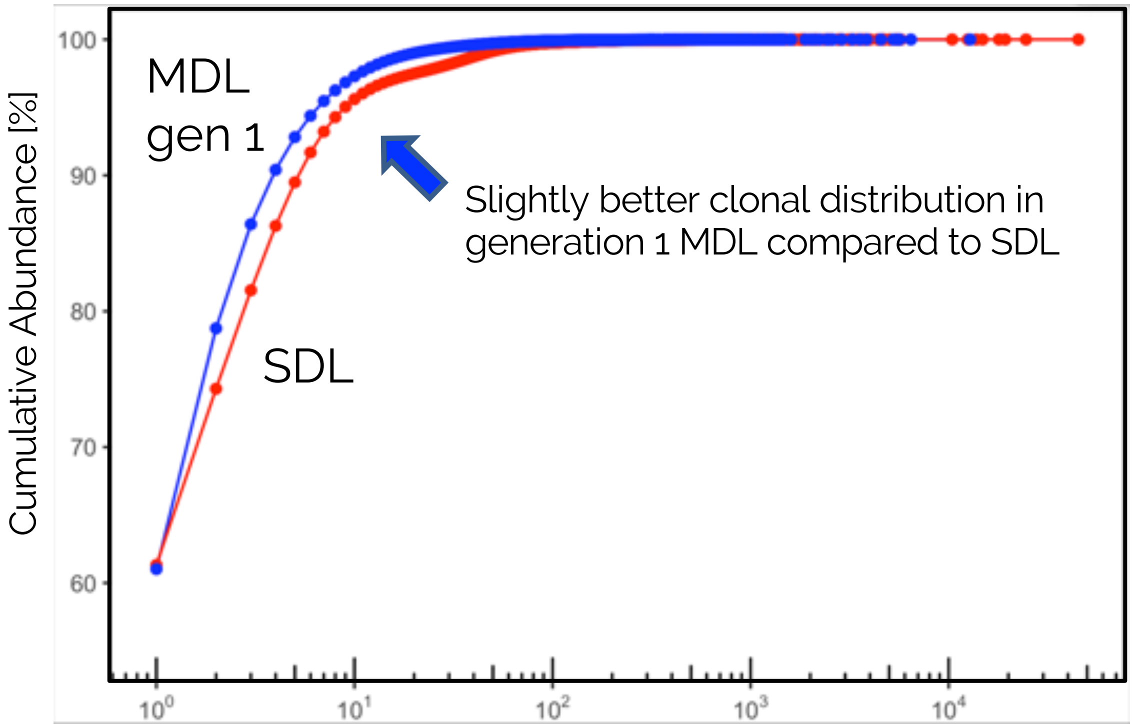
Sblattero, D. and Bradbury, A. (2000) Nat Biotechnology

Single-Donor Library



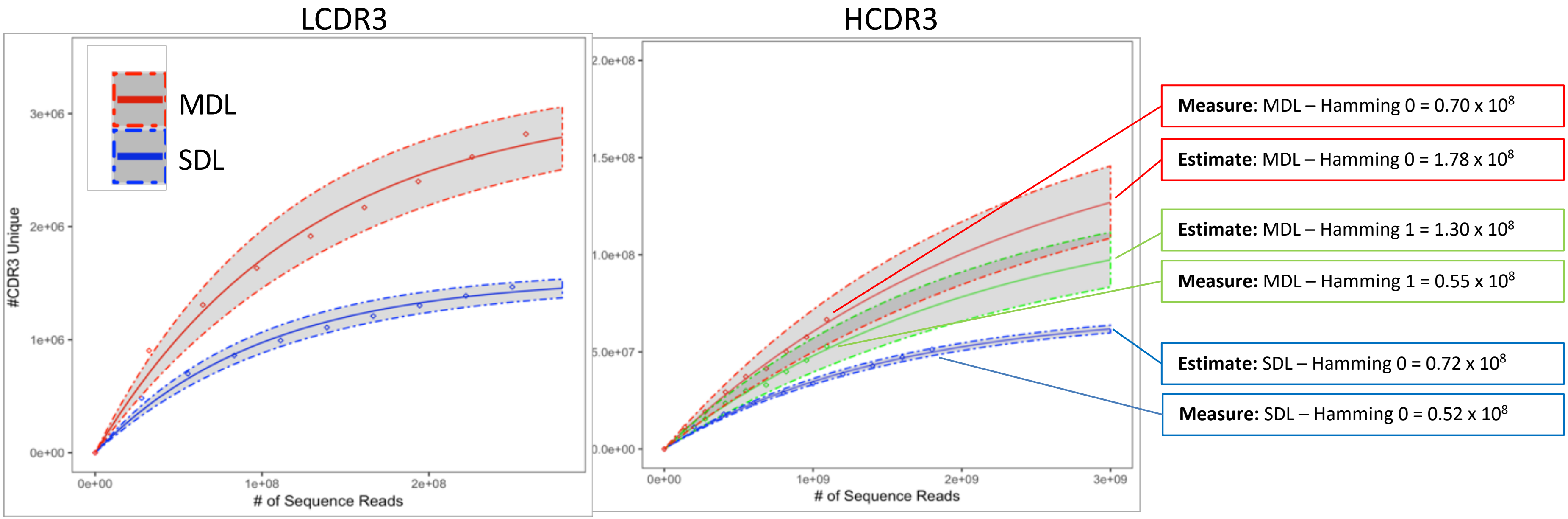
Hamming ≤1 amino acid

Comparison Between First Generation Multi- and Single- Donor Libraries



Hamming ≤1 amino acid

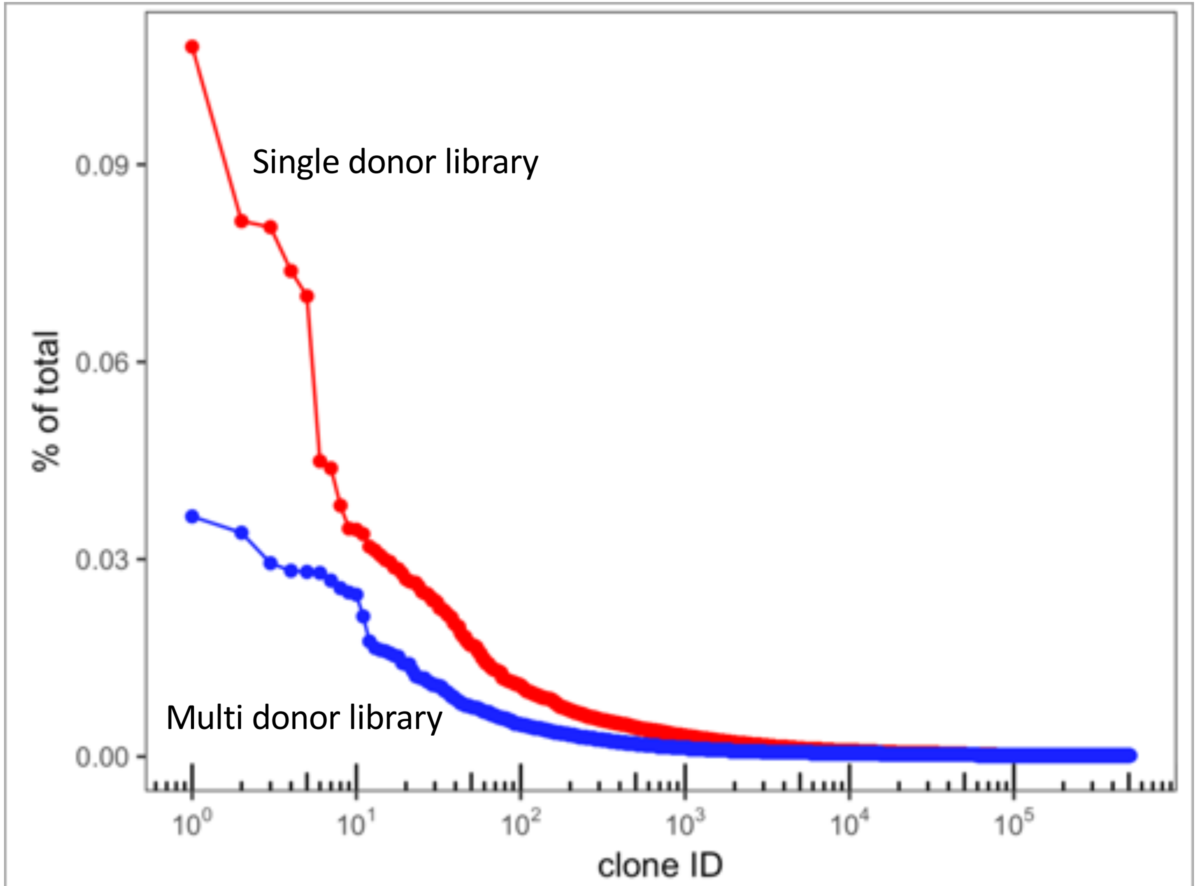
Analysis of Cloned Diversity (NovaSeq): CDR3 Accumulation Plot



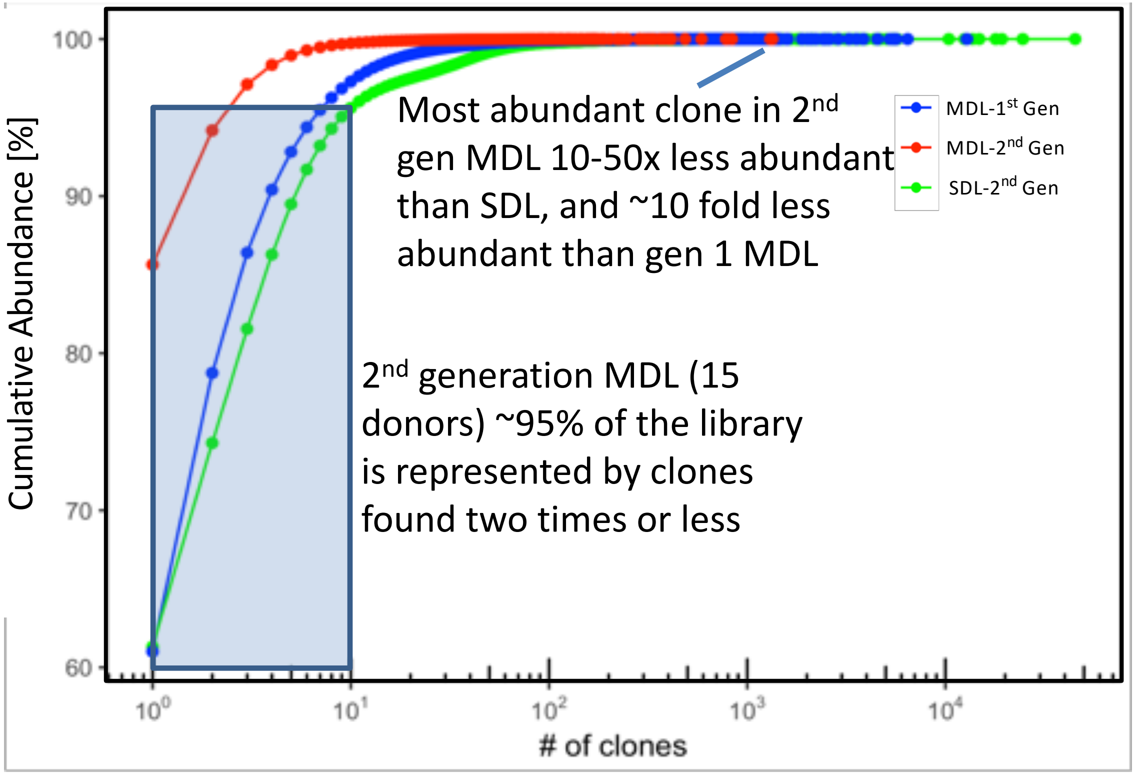
	LCDR3		HCDR3	
Diversity	**SDL**	**MDL**	**SDL**	**MDL**
Estimate	1.55 x 10^6	3.63 x 10^6	0.72 x 10^8	1.78 x 10^8
Measure	1.55 x 10^6	3.63 x 10^6	0.52 x 10^8	0.70 x 10^8

MDL at Hamming 1 shows greater measured and estimated diversity than SDL at Hamming 0 with 7.0x10^8 fewer reads

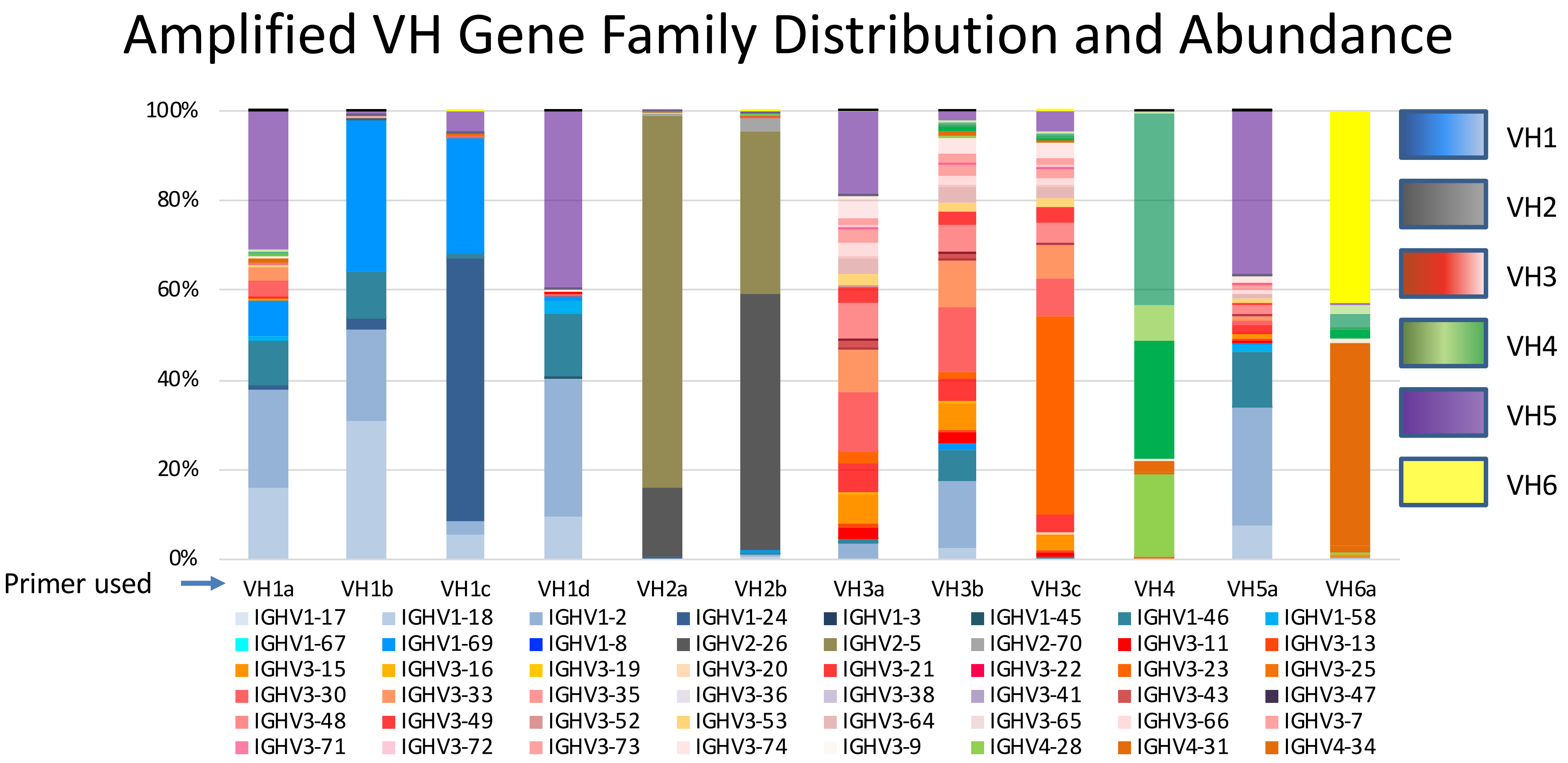
Reduced Clonal Dominance in Multi Donor Library



Normalized comparison of libraries

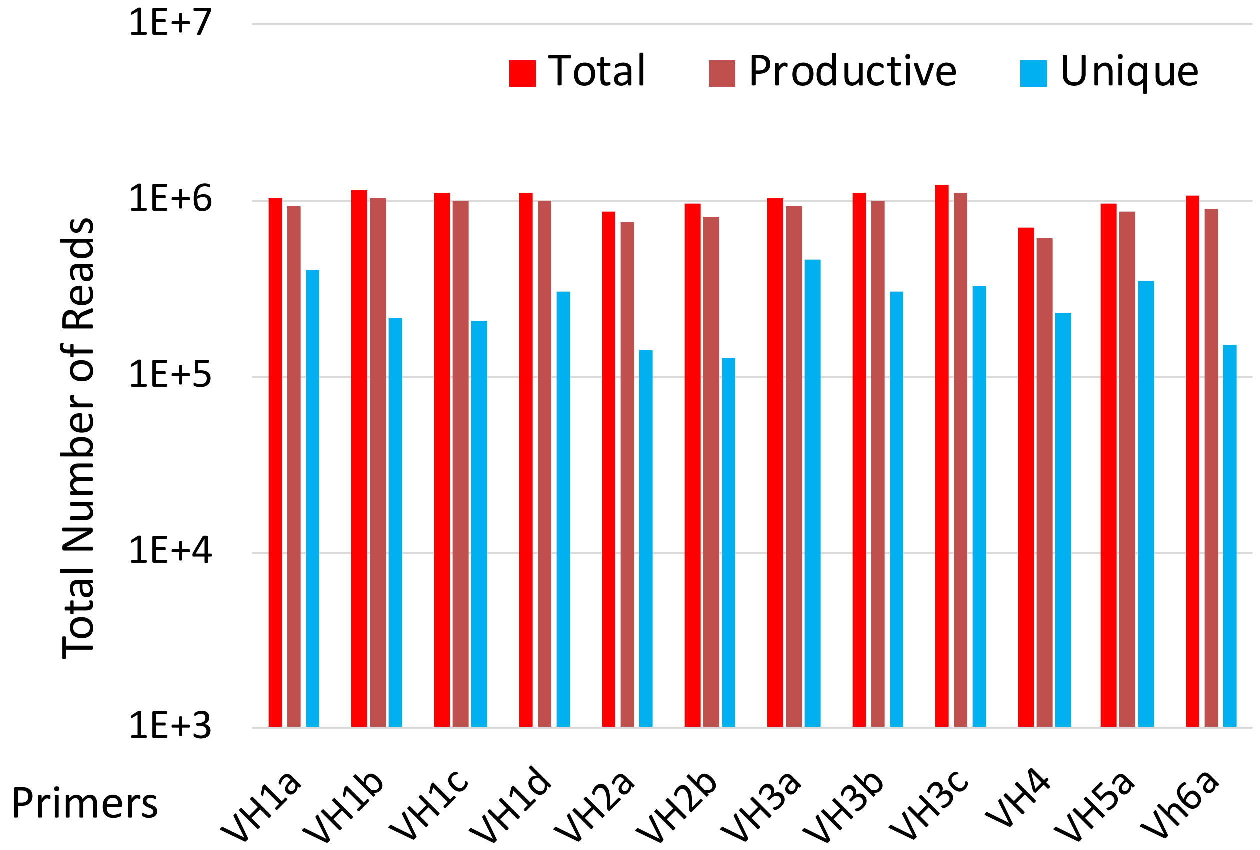


Hamming ≤1 amino acid



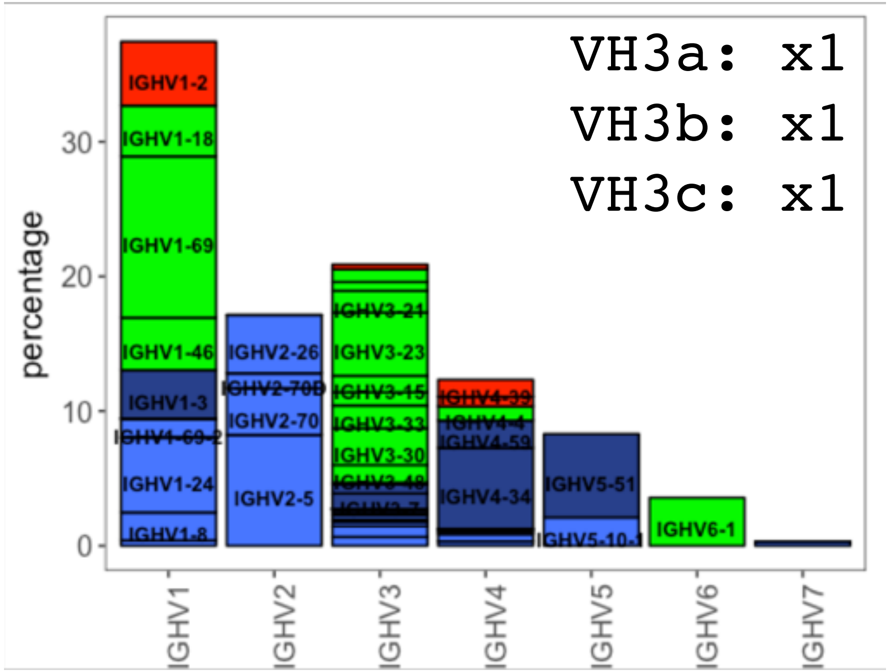
Amplified VH Gene Family Distribution and Abundance
100%
80%
60%
40%
20%
0%
Primer used
VH1a
VH1b
VH1c
VH1d
VH2a
VH2b
VH3a
VH3b
VH3c
VH4
VH5a
VH6a
VH1
VH2
VH3
VH4
VH5
VH6
IGHV1-17
IGHV1-18
IGHV1-2
IGHV1-24
IGHV1-3
IGHV1-45
IGHV1-46
IGHV1-58
IGHV1-67
IGHV1-69
IGHV1-8
IGHV2-26
IGHV2-5
IGHV2-70
IGHV3-11
IGHV3-13
IGHV3-15
IGHV3-16
IGHV3-19
IGHV3-20
IGHV3-21
IGHV3-22
IGHV3-23
IGHV3-25
IGHV3-30
IGHV3-33
IGHV3-35
IGHV3-36
IGHV3-38
IGHV3-41
IGHV3-43
IGHV3-47
IGHV3-48
IGHV3-49
IGHV3-52
IGHV3-53
IGHV3-64
IGHV3-65
IGHV3-66
IGHV3-7
IGHV3-71
IGHV3-72
IGHV3-73
IGHV3-74
IGHV3-9
IGHV4-28
IGHV4-31
IGHV4-34

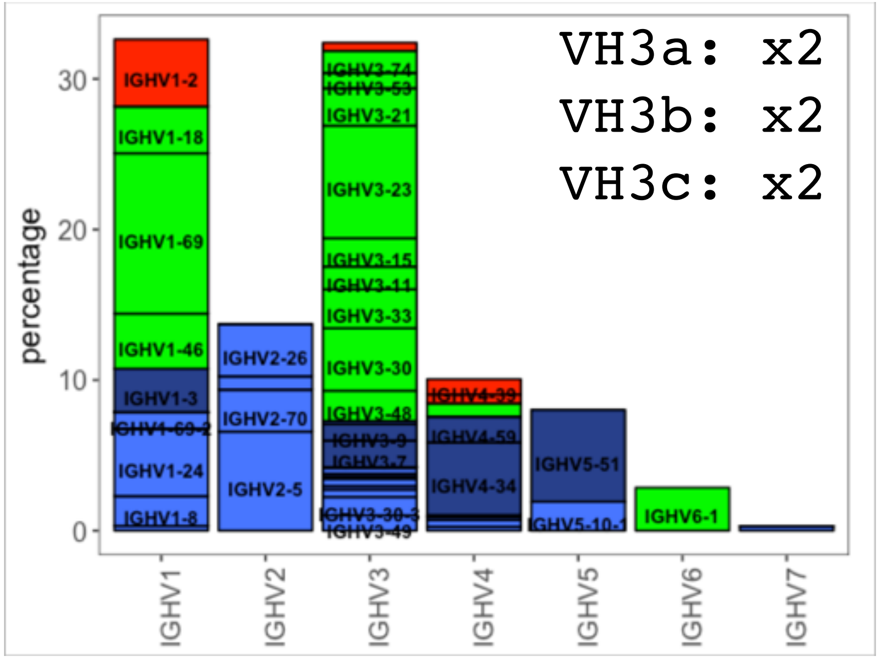
MiSeq NGS analysis of VH Gene PCR Products

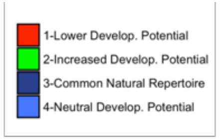


MiSeq Analysis of MDL1 PCR products: Projected VH Family Distribution

original distribution







NGS Analysis for naïve libraries

- NovaSeq provides measure of absolute diversity
  - Greater sequence depth provides better diversity estimates
  - Diversity estimates limited to HCDR3
    - Known to underestimate diversity: HCDR1/2 and VL
- Germline VH and VL gene diversity
  - If carried out at PCR level can adjust final library composition
- Functional diversity (open reading frames)

- VH MDL 2-3 times more diverse than SDL
  - Rate of unique clone accumulation 2-3 times faster
  - Projected final diversity 2-3 times higher
  - Final analysis still to be completed
- MDL shows reduced clonal dominance compared to SDL
  - MDL should perform better if diversity is what counts

Analysis of selections outputs with NGS

Evolution of in vitro antibody discovery in our laboratory

Display platform

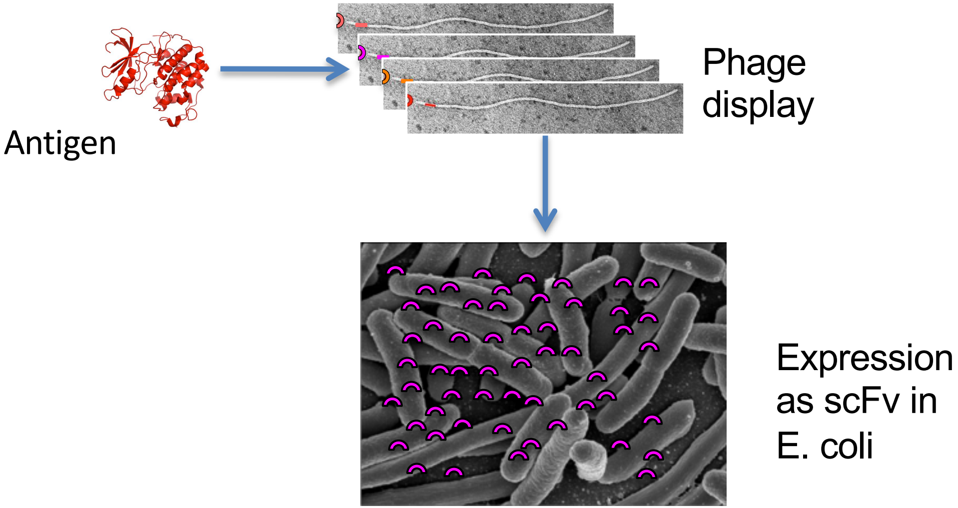
- 1999 Phage display
- 2012 Phage + Yeast
- 2015 Phage + Yeast + NGS

Number of scFvs

- One to tens selected
- Tens to hundreds selected
- Thousands identified

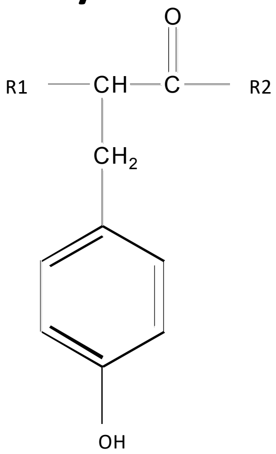
- Selected scFvs are clones available for further study
- Identified scFvs are sequenced identified scFvs that need to be isolated

Selection by phage display

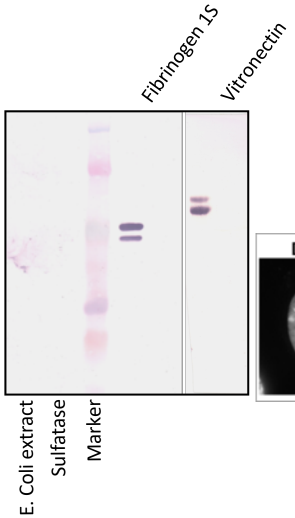


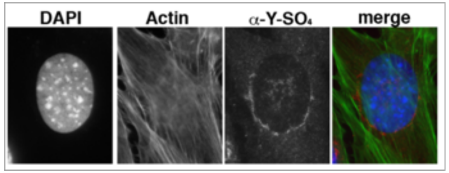
Selecting antibodies against the tyrosine sulfate modification using phage display

- Tyrosine sulfate found on secreted and membrane proteins
  - Computational analysis suggests up to 33% proteins entering secretory pathway are tyrosine sulfated, but less than 70 shown experimentally
- Numerous attempts to derive specific antibodies by immunization have failed
  - "Impossible antigen" due to expression in secretory pathway
  - Tyrosine sulfate, ubiquitous non-immunogenic modification



- 8000 antibodies screened by phage display
  - One scFv found that recognizes the tyrosine sulfate modification
  - Western blotting, immunofluorescence
  - Affinity ~1µM
  - One student, 18 months work





- Reformatted to a mouse IgG
  - Sold by Millipore as a mouse monoclonal!
  - millipore.com/catalogue/item/05-1100x

Kehoe et al., (2006) Mol. Cell. Proteomics, **5**, 2350-2363
Lassen, K. S. et al., (2008) Electrophoresis **29**, 2557
Ronai, Z. et al. (2009) Biochemical Journal **418**, 155

Phage vs. Yeast Display

Phage

- Larger primary libraries
- Selection from naïve libraries
- Relatively straightforward
- Soluble scFv or Fab easily made in *E. coli*
- General familiarity with *E. coli*

- Selection is a "black box"
- Antibody must be expressed and purified to measure affinity
- Repertoires incompletely sampled

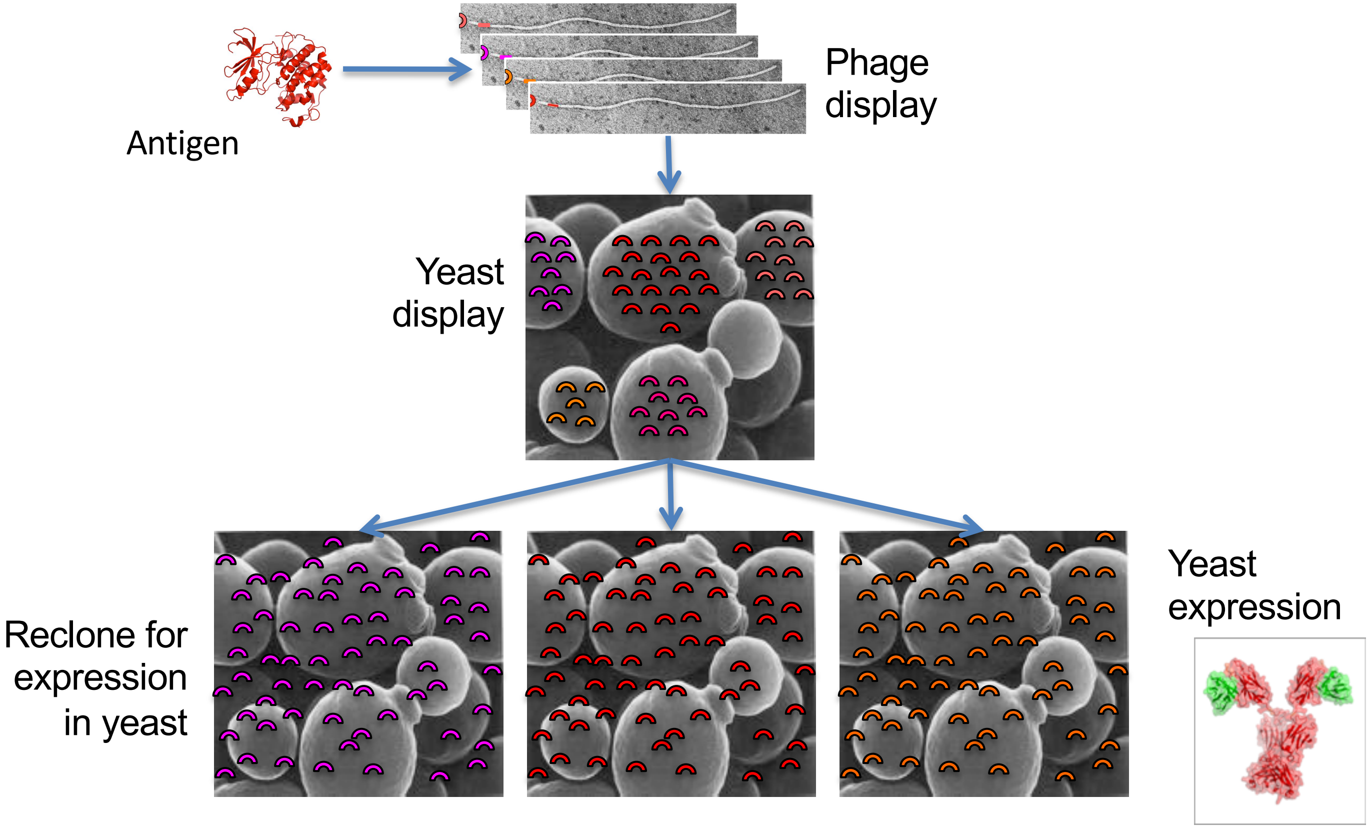
Yeast

- Smaller primary libraries
  - Libraries $\leq 10^8$ with gap repair
  - Immune libraries and affinity maturation
- Naive library selections more challenging
- Requires flow cytometry
- Less general familiarity with yeast
- Need to subclone to make native Ab fragment

- Precise selection calibration
- Direct characterization on yeast without antibody purification:
  - Affinity; epitopes
- Repertoires sampled more completely as greater proportion of antibodies displayed*

*Bowley et al. (2007) PEDS, **20** 81-90

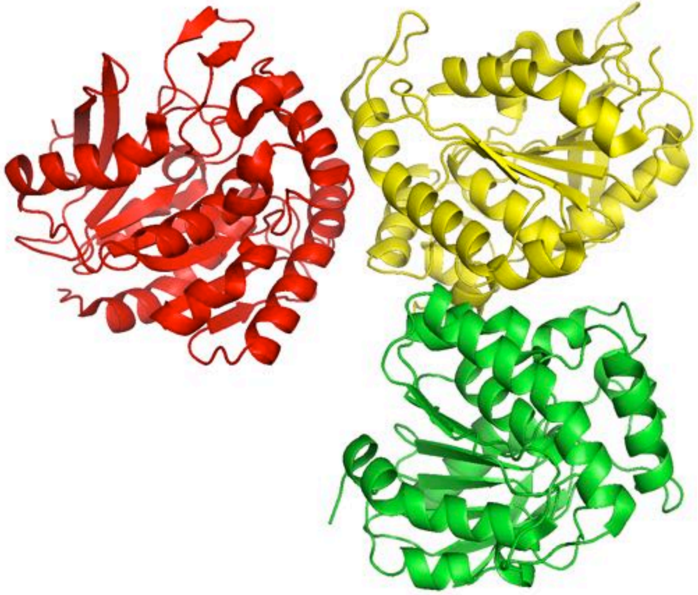
Combining phage and yeast display to select antibodies



Pick 96 clones, test and sequence

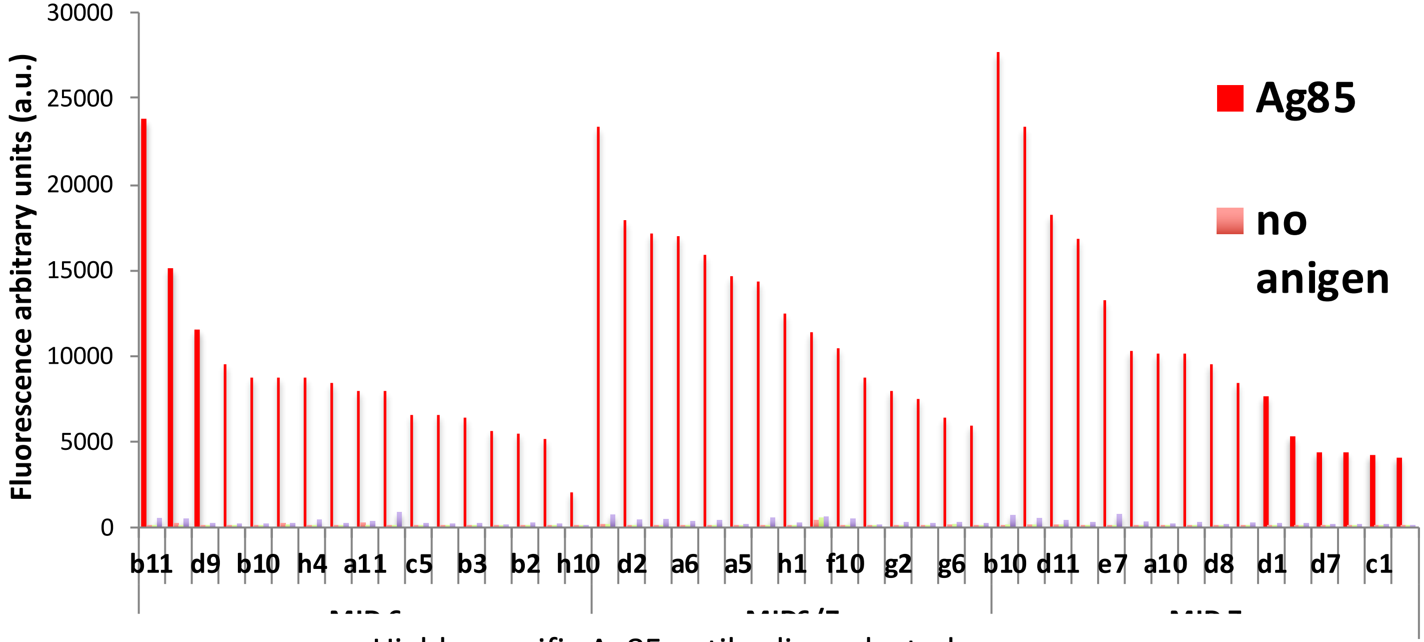
Ag85 as a *M. tuberculosis* biomarker

- Ag85 is a three protein complex, one of the major secreted protein of *M. tuberculosis*
- Mycolyl transferase involved in cell wall synthesis
- Present in the *sputum* of pulmonary patients, also detected in serum and urine
- Ag85 could be used as a “early” reporter for TB infection assay
- No specific, stable antibodies available for Ag85 detection



Selected anti-Ag85 human monoclonals

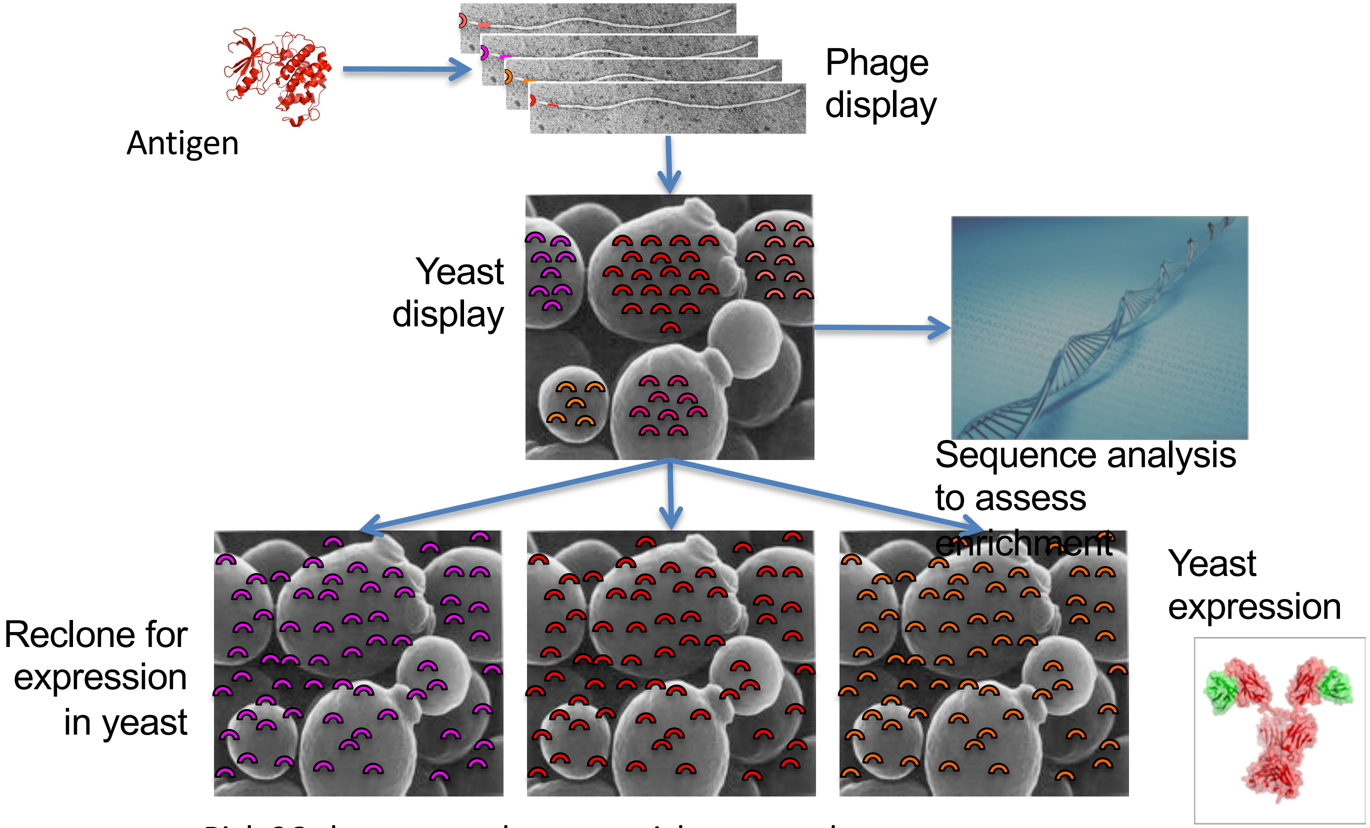
>111 (of 200 tested) different monoclonals selected
Kd 20-430 nM



Highly specific Ag85 antibodies selected

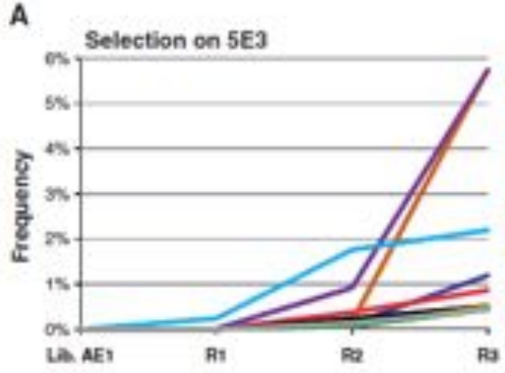
Adding Next Generation Sequencing

Integrating NGS into antibody selection screening



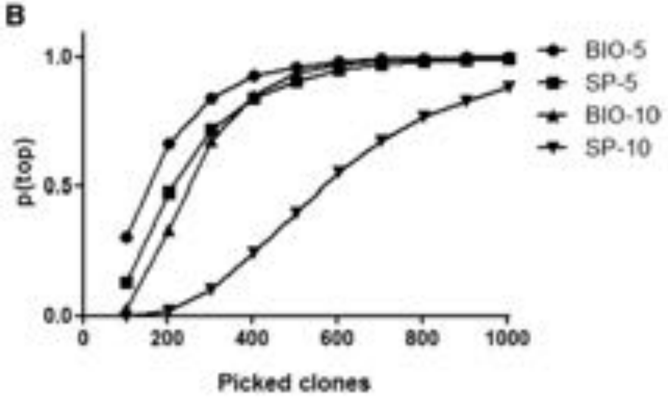
Pick 96 clones, correlate to enrichment rank

Traditional ELISA screening does **not** isolate all top binders



Clone #	VH CDR3 Sequence (IMGT)	Length	Family	Round 3 frequency	Rescued clones	Corresponding screening hit
1	ARGNRRAYMDY	11	VH1	37017	5E3R-1	D11
2	ARGTYANTTSMDY	13	VH5	36901	5E3R-2	
3	ARGDSNTDDADAMDY	15	VH3	14096	5E3R-3	D6
4	ARGRKKEKGMDY	12	VH3	7661	5E3R-4	
5	ARGRPSLFDY	10	VH1	5573	5E3R-5	
6	ARGRRTGGMDY	11	VH5	3290	5E3R-6	D4
7	ARSAGMDY	8	VH3	3159		
8	ARDSRDGTPMDY	12	VH3	2867		
9	ARRKKMDY	8	VH3	2818		
10	ARDAPTGDPFDY	12	VH1	2815		

Ravn *et al*. (2010). Nucleic Acids Res 38(21): e193.



Di Niro *et al*., (2010). Nucleic Acids Res 38(9): e110.

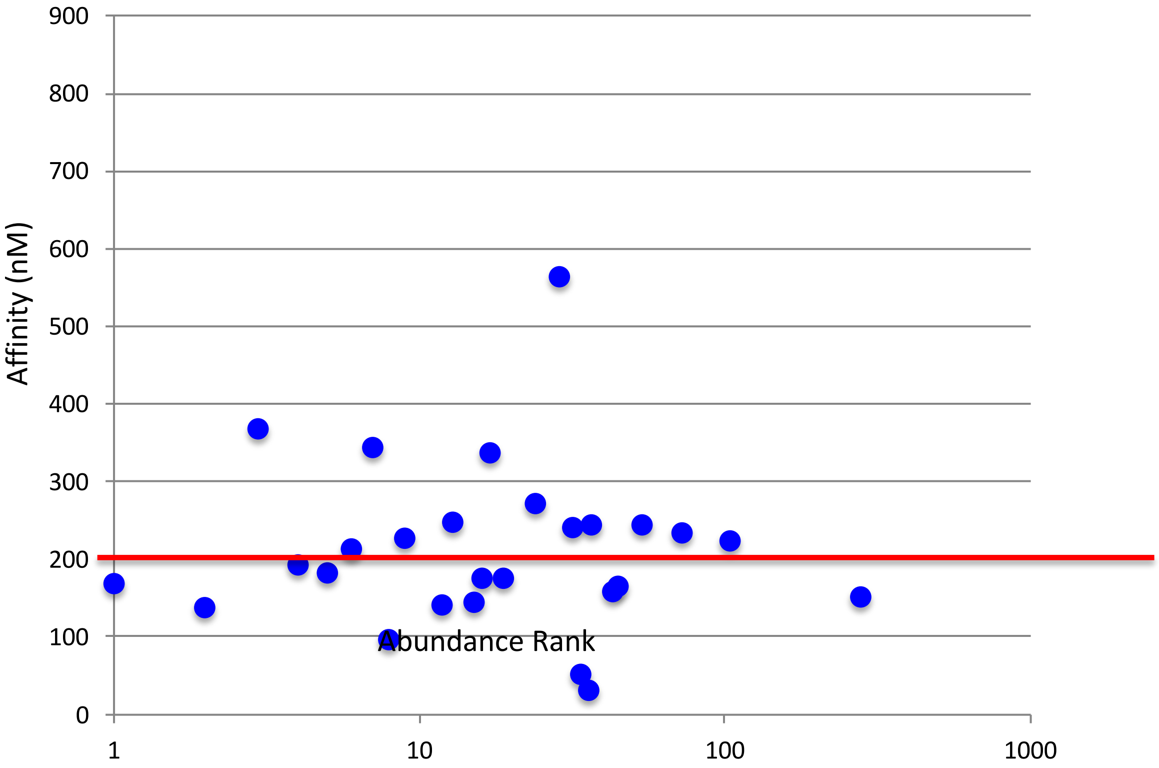
How Diverse? deep sequencing analysis of selection outputs

Antigen	Total # sequences	#clusters to 99%
MAP2K5	25342	**934**
CDK2	32138	**880**
CTBP2	41608	**731**
MAPK8	56525	**198**
PLAA	41996	**289**
SF3A1	4602	**216**
USP11	41924	**1148**
Ubiquitin	33710	**175**

- 200-1100 different HCDR3s found per target
- Diversity ten fold greater due to different VL and HCDR1/HCDR2's
- Estimated 2,000 to 10,000 different antibodies per target

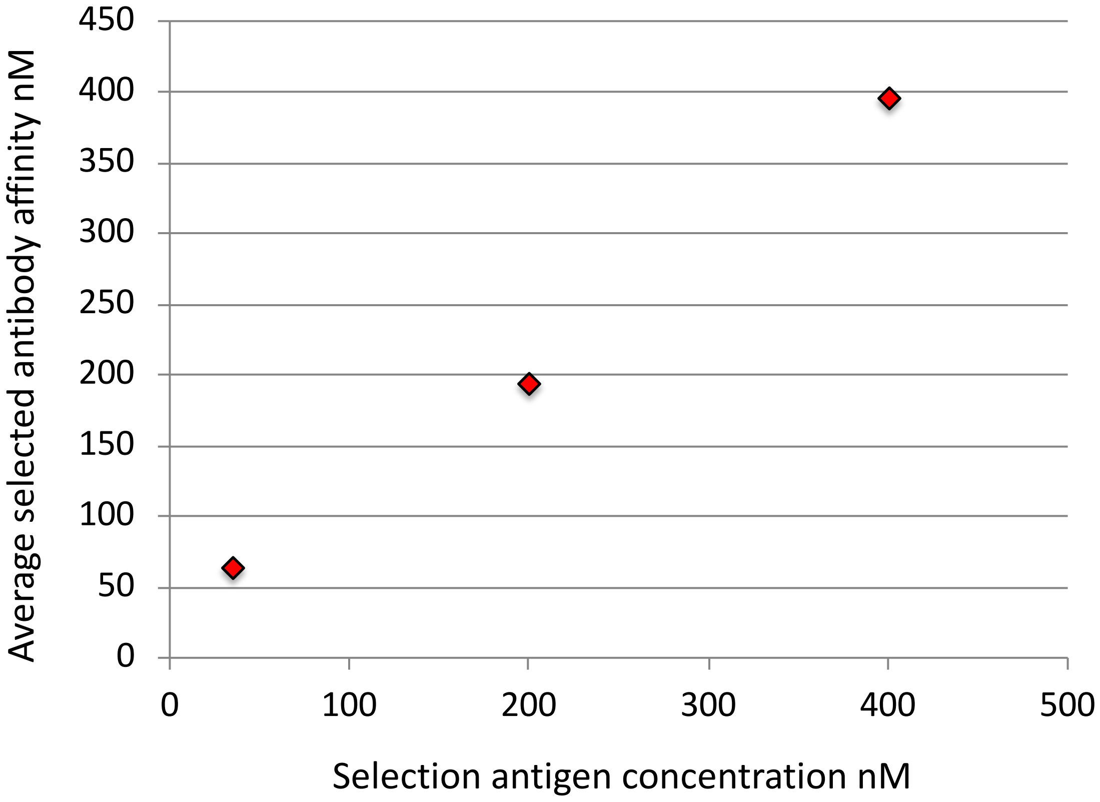
D'Angelo et al., (2014). mAbs **6** 160

Affinity of monoclonals selected against ubiquitin

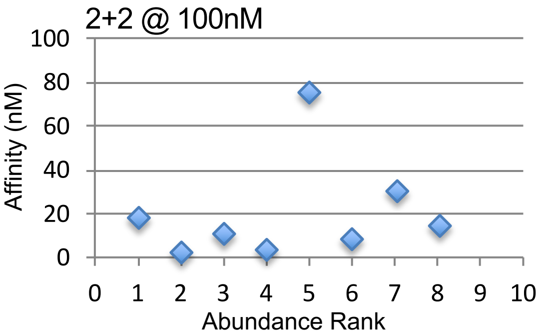


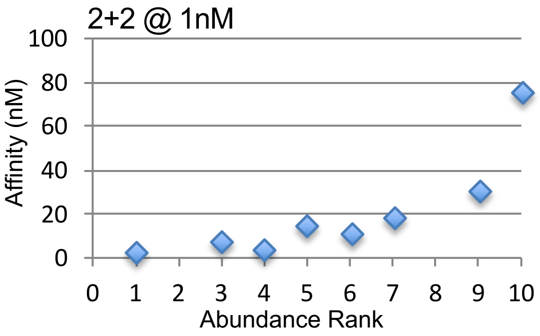
- Little correlation between affinity and abundance rank
- Antibody affinities cluster around selection antigen concentration (200nM)

Improving average polyclonal affinity?



Correlating affinity with abundance





HCDR3 sequences

2+2@100nM	%	affinity
CAKGFRAGDAFDIW	18.7	18nM
CASQGFQGDAFDIW	17.5	2 nM
CASHSGNLGTNGVGDAFDIW	10.7	11 nM
CARPYYGSGDAFDYW	8.5	4 nM
CAHSYGDPFDYW	7.6	75 nM
CARPLSGWYGDAFDIW	5.5	8 nM
CARGSSGSFDIW	4.4	30 nM
CATHSSGWYGDAFDIW	2.6	15 nM
CARVSAFGETFDLW	2.3	
CARADWIDAFDIW	2.2	
	80.2	

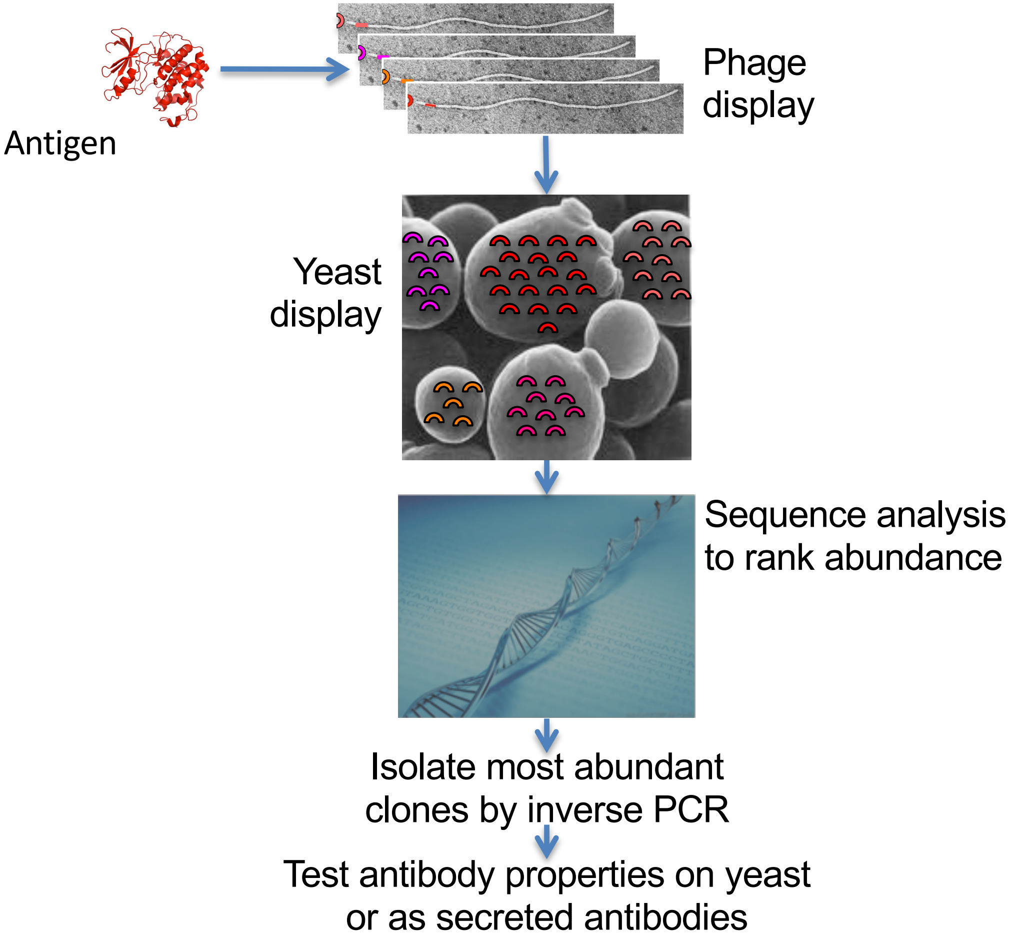
2+2@1nM	%	affinity
CASQGFQGDAFDIW	37.7	2 nM
CARGTEGWFDPW	15.9	
CARPLSGWYGDAFDIW	14.7	8 nM
CARPYYGSGDAFDYW	6.1	4 nM
CATHSSGWYGDAFDIW	2.8	15 nM
CASHSGNLGTNGVGDAFDIW	2.5	11 nM
CAKGFRAGDAFDIW	2.3	18 nM
CARDLGSDYYDSSGYPGGDAFDIW	1.9	
CARGSSGSFDIW	1.4	30 nM
CAHSYGDPFDYW	1.2	75 nM
	86.5	

Are we accessing the full diversity of our libraries?

- Phage selection on CDK2
  - 8 different scFvs identified
  - Affinities, 30-83 nM
- Phage and yeast display
  - 27 different scFvs
  - Affinities 32-565 nM
- Phage, yeast and deep sequencing
  - There may be some mutations and recombination going from phage to yeast
  - 880 different HCDR3s identified
  - All antibodies tested, down to 277th in abundance, bind target
  - Affinities, 2-565 nM
    - Improved affinities with selections on reduced target concentrations

- Full library diversity not accessed by standard selections

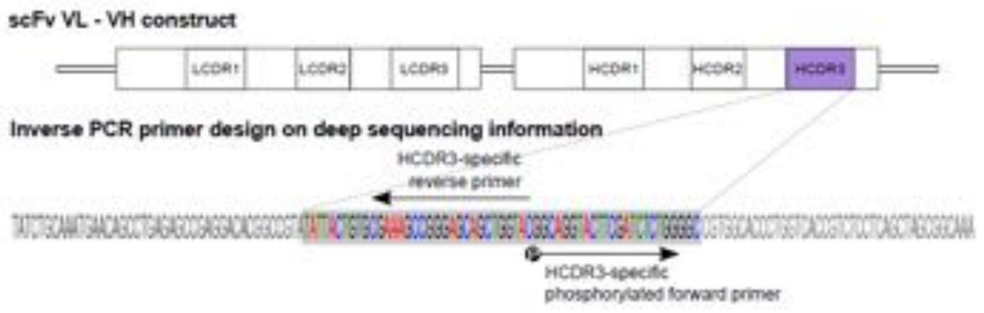
Using NGS to directly isolate most abundant antibodies

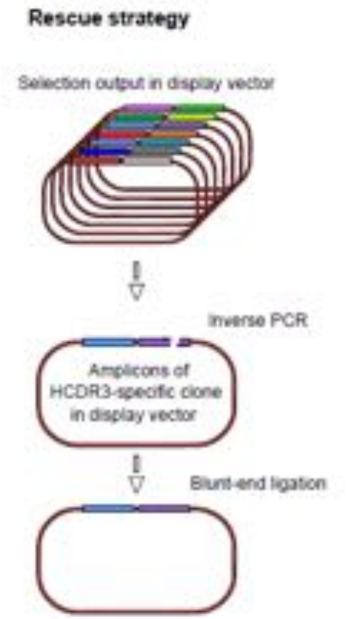


From sequences to clones

- HCDR3 specific primers designed from DNA sequence
- Inverse PCR performed on selected output and ligated
- Single clones with same HCDR3 obtained
- Clones tested for specificity

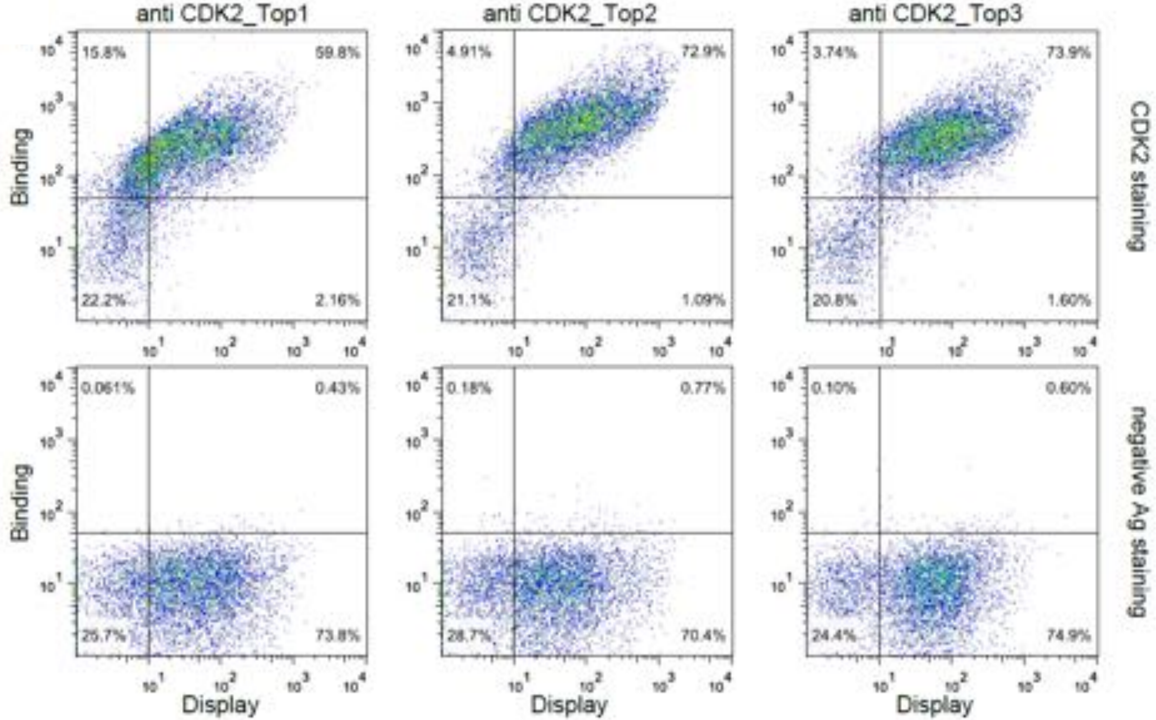
antiCDK2 top ranking	%
CAKGFRAGDAFDIW	18.7
CASQGFQGDAFDIW	17.5
CASHSGNLGTNGVGDAFDIW	10.7
CARPYYGSGDAFDYW	8.5
CAHSYGDPFDYW	7.6
CARPLSGWYGDAFDIW	5.5
CARGSSGSFDIW	4.4
CATHSSGWYGDAFDIW	2.6
CARVSAFGETFDLW	2.3
CARADWIDAFDIW	2.2
	80.2





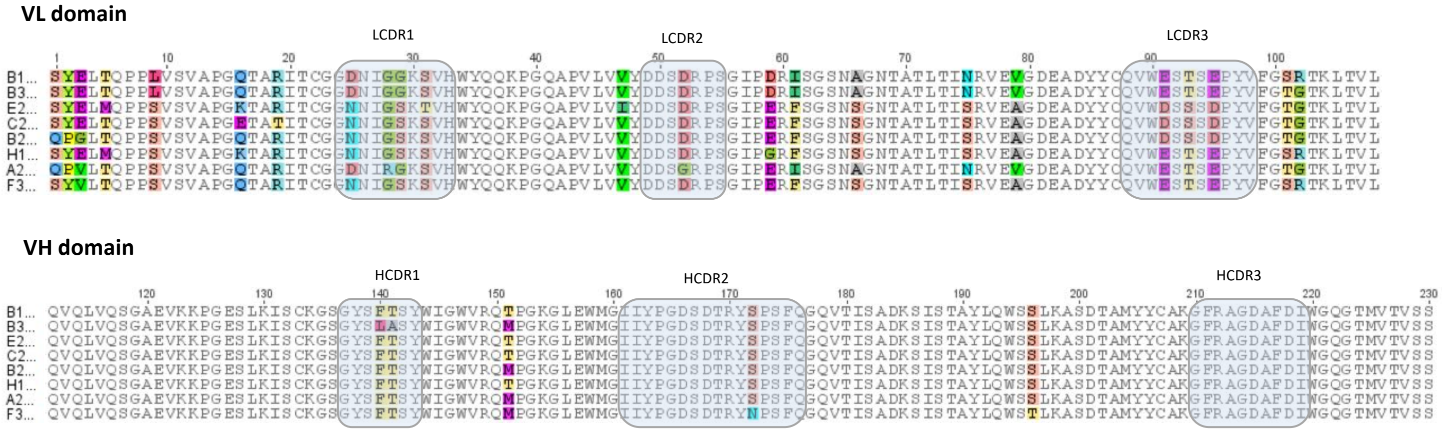
D'Angelo, S. et al. MAbs **6**, 160, (2014).
D'Angelo, S. et al. Protein Eng Des Sel **27**, 301, (2014).

Inverse PCR applied to 3 most abundant clones in anti-CDK2 selections



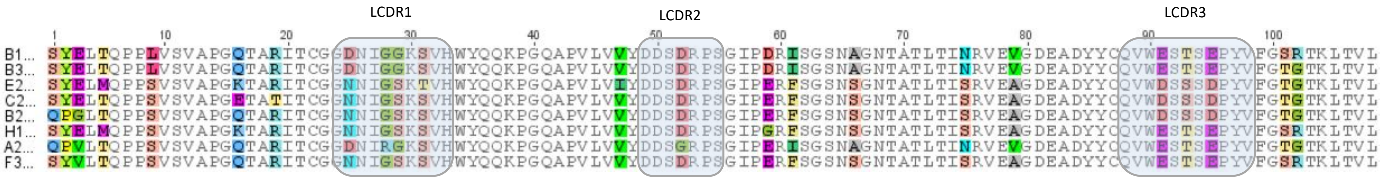
- We can successfully design HCDR3 specific primers to rescue full-length scFvs from selected polyclonal pools
- Top ranking clones are binders

Dissecting the monoclonality

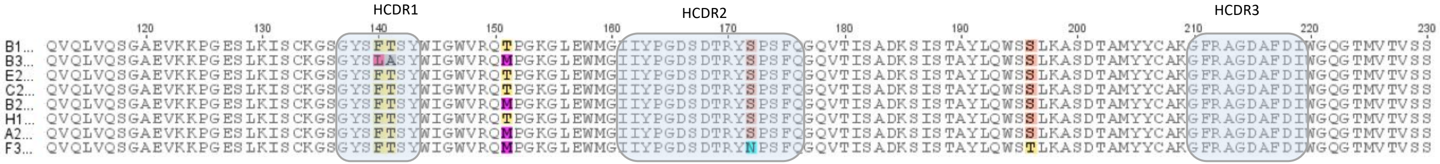


Dissecting the monoclonality

VL domain



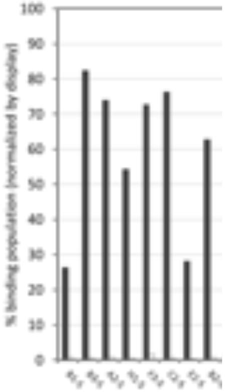
VH domain



	Kd
B1_1	30.1
B3_1	61.6
E2_2	64.5
C2_1	84.4
B2_2	135.8
H1_1	173.6
A2_2	203.9
F3_1	352.5

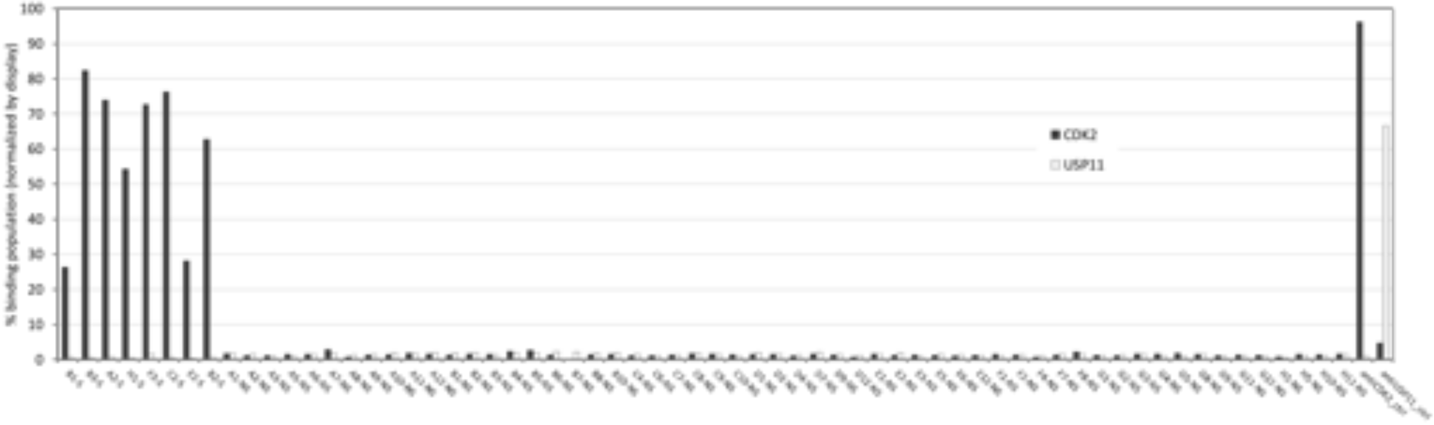
- Antibodies with same HCDR3 show a **30-350 nM** range of affinities
- Polyclonality is extended to the antibodies sharing the same HCDR3
  - Antibodies <98% homology considered different
  - Selected Abs 91.6 – 97.8% homologous
- Pairing with different VL domains and additional VH mutation leads to a suite of antibodies recognizing the same epitope with a wide affinity range

What about in the naïve unselected library?
Binding data for clones with identical HCDR3s



Selected binding clones

What about in the naïve unselected library?
Binding data for clones with identical HCDR3s

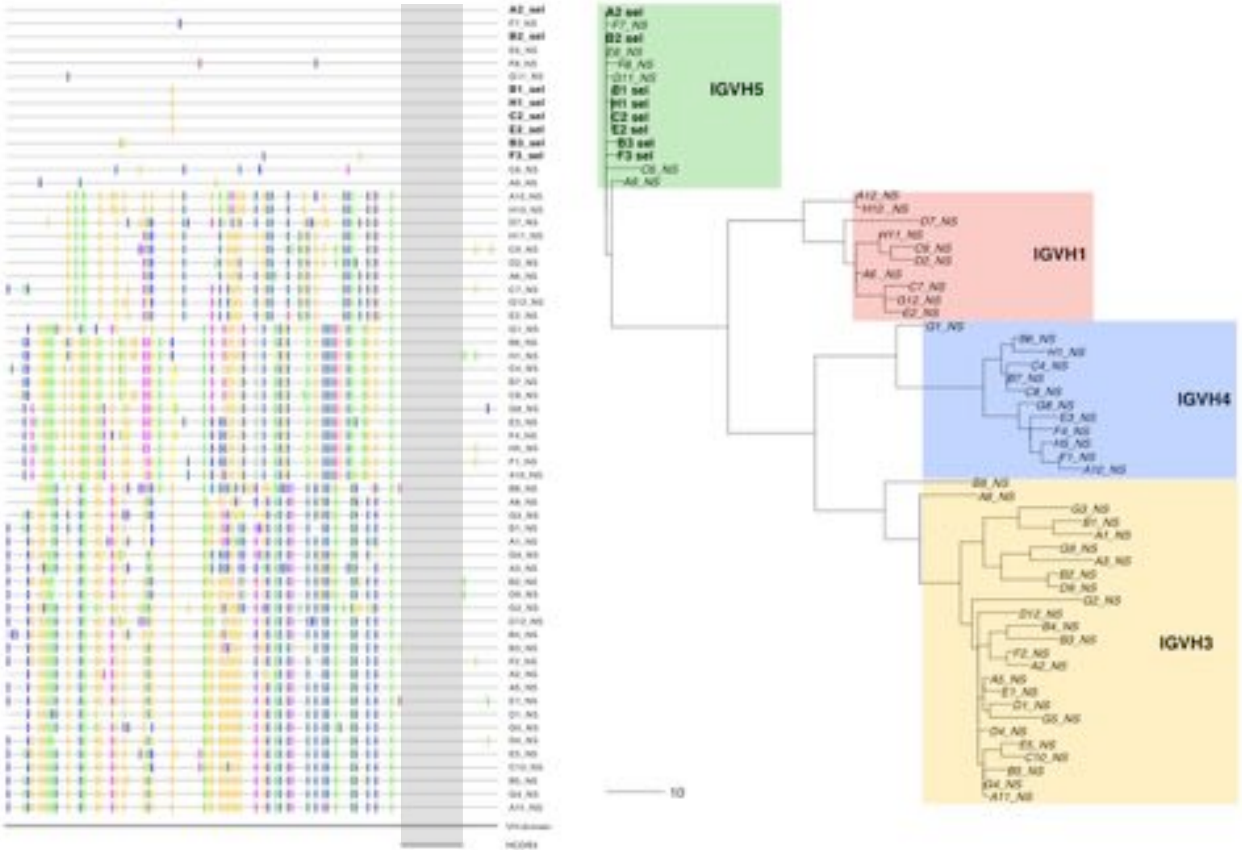


Selected binding clones

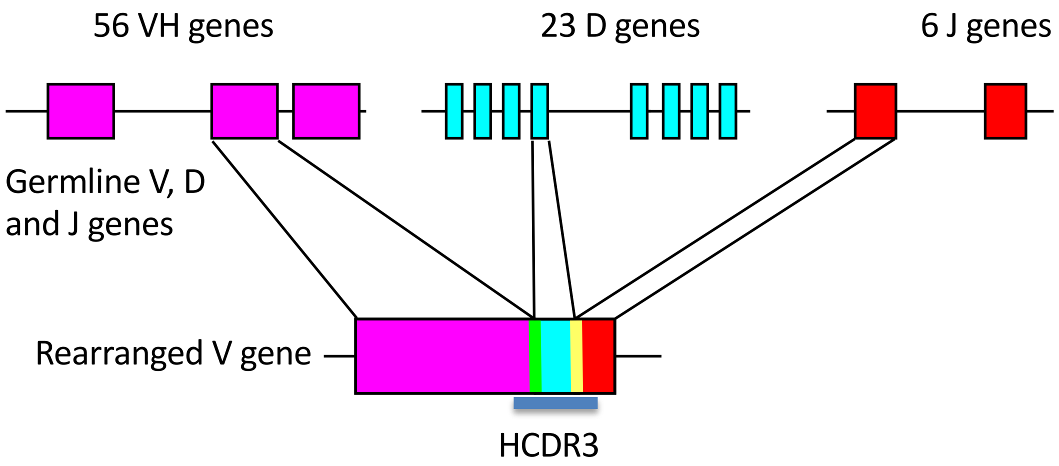
Unselected clones with same HCDR3

- HCDR3 is necessary but not sufficient for binding
- Clones with the same HCDR3 but different VL do not bind to antigen

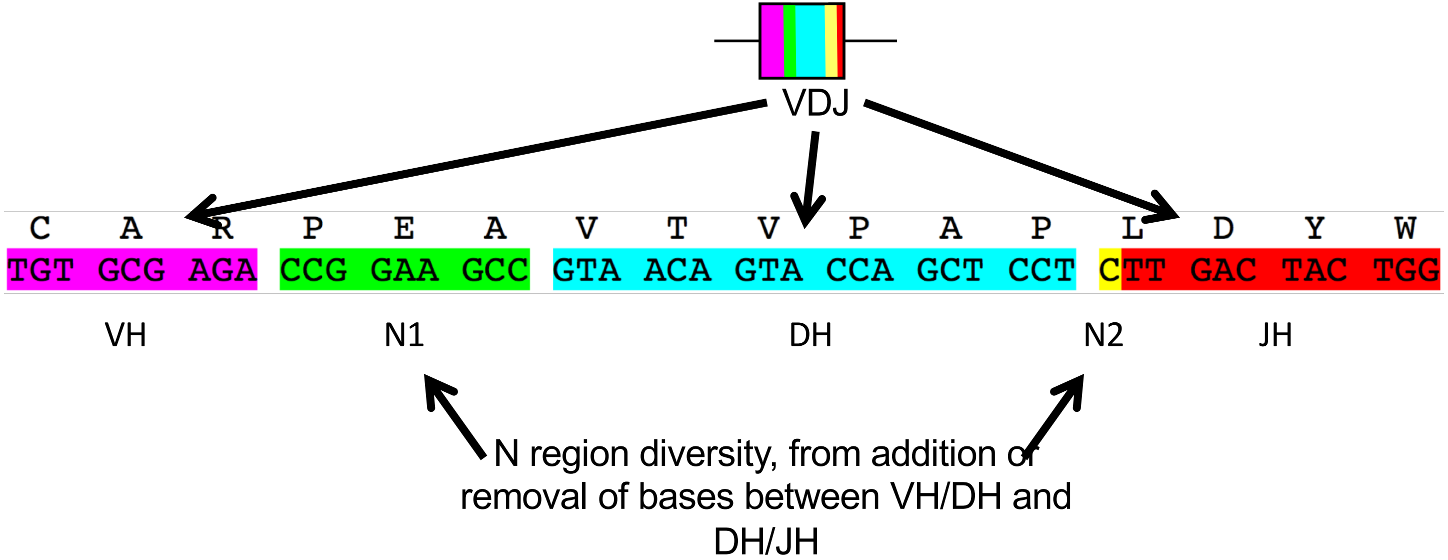
Diversity of VHs with the same HCDR3



How VH genes are made in vivo

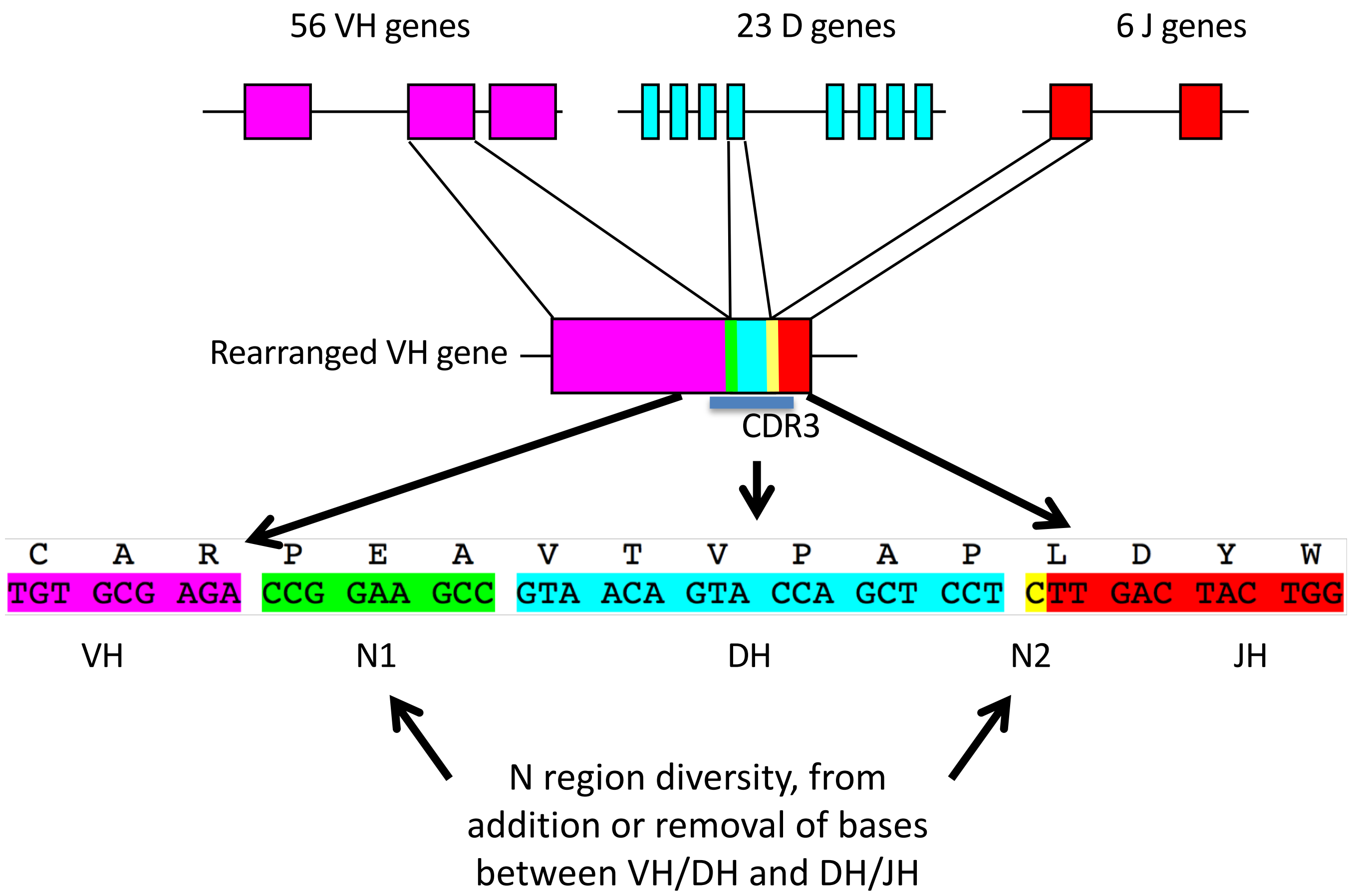


Detailed examination HCDR3 origins



IMGT Junction/Analysis

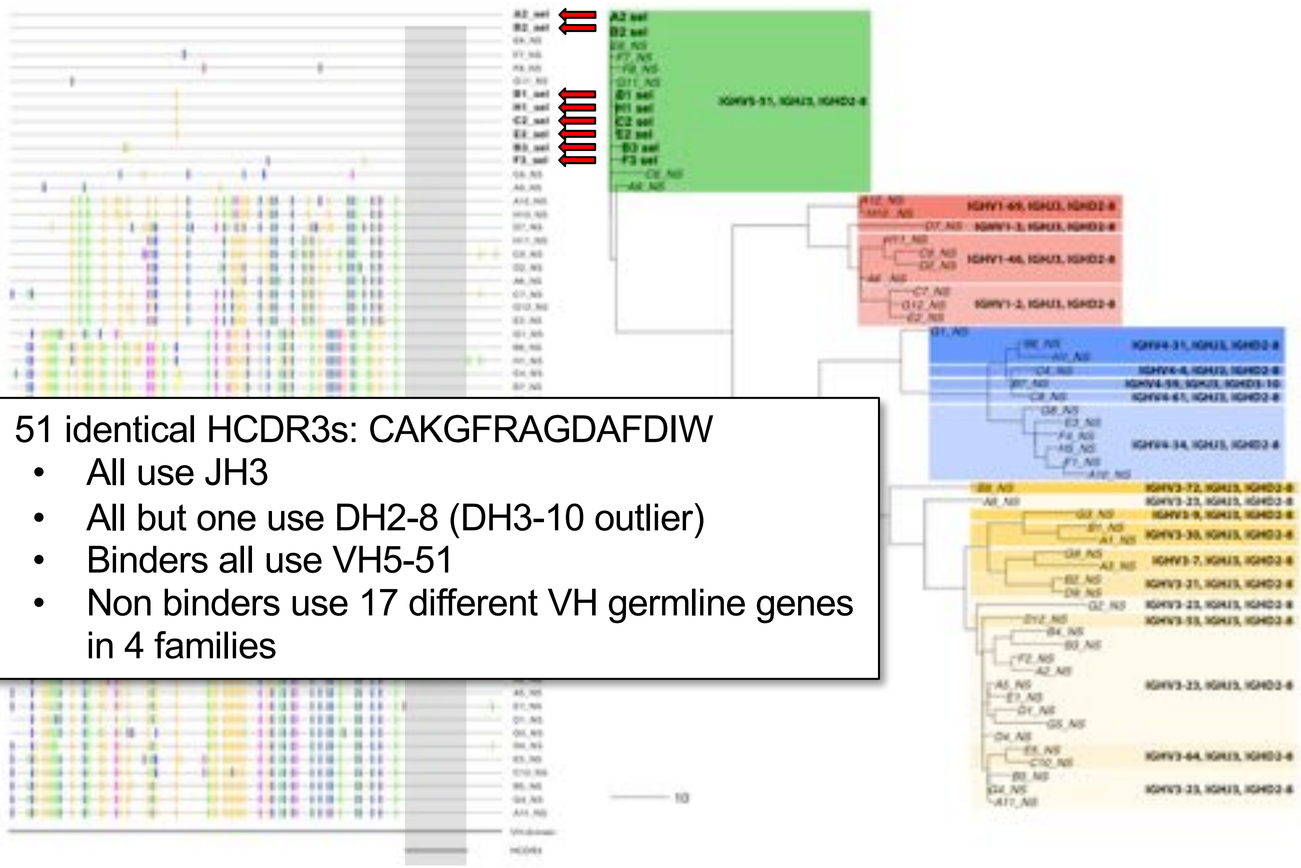
How VH genes are made in vivo



IMGT Junction/Analysis

Germline gene origins

← binders



- 51 identical HCDR3s: CAKGFRAGDAFDIW
  - All use JH3
  - All but one use DH2-8 (DH3-10 outlier)
  - Binders all use VH5-51
  - Non binders use 17 different VH germline genes in 4 families

What happens in vivo?

- Two in vivo generated NGS datasets
  - DeKosky – ~55,000 HCDR3 sequences from naïve B cells of 3 subjects
    - 23 pairs of identical HCDR3s shared between 2 subjects (0.08% all reads)
      - All discordant for VH, 7 discordant for DH, identical JH
    - No identical HCDR3s in all 3 subjects
  - DeWitt – total 37M HCDR3 sequences from naïve B cells of single subject
    - 8,596,145 productive MiSeq reads comprising 7,984,053 unique HCDR3s from naïve B cells of three donors
      - 568 identical HCDR3s (0.007% of the total unique HCDR3s) generated by different VDJ recombinations
        - » Generated with 2-26 different VDJ rearrangements
        - » 176 rearrangements found in all three donors.

DeKosky, B. J. *et al. Proc Natl Acad Sci U S A* **113**, E2636, (2016).
DeWitt, W. S. *et al. PLoS One* **11**, e0160853, (2016).

HCDR3 aa Sequence	# rearrangements	VH family	VH gene	DH gene	JH gene	Donor Representation
CARDRGDYW	26	IGHV01	IGHV01-02	IGHD03-10	IGHJ04-01	donor1
CARDRGDYW	26	IGHV01	IGHV01-02	IGHD05-24	IGHJ04-01	donor3
CARDRGDYW	26	IGHV01	IGHV01-03	IGHD01-26	IGHJ04-01	donor3
CARDRGDYW	26	IGHV01	IGHV01-03	IGHD03-10	IGHJ04-01	donor3
CARDRGDYW	26	IGHV01	IGHV01-03	IGHD06-25	IGHJ04-01	donor1
CARDRGDYW	26	IGHV01	IGHV01-18	IGHD03-10	IGHJ04-01	donor3
CARDRGDYW	26	IGHV01	IGHV01-18	IGHD03-16	IGHJ04-01	donor1, donor3
CARDRGDYW	26	IGHV01	IGHV01-18	IGHD05-24	IGHJ04-01	donor2
CARDRGDYW	26	IGHV01	IGHV01-18	IGHD06-25	IGHJ04-01	donor3
CARDRGDYW	26	IGHV01	IGHV01-46	IGHD03-10	IGHJ04-01	donor1, donor2, donor3
CARDRGDYW	26	IGHV01	IGHV01-46	IGHD03-16	IGHJ04-01	donor2
CARDRGDYW	26	IGHV01	IGHV01-69	IGHD03-10	IGHJ04-01	donor1, donor2, donor3
CARDRGDYW	26	IGHV01	IGHV01-69	IGHD03-16	IGHJ04-01	donor2
CARDRGDYW	26	IGHV03	IGHV03-11	IGHD03-10	IGHJ04-01	donor1
CARDRGDYW	26	IGHV03	IGHV03-11	IGHD03-16	IGHJ04-01	donor1
CARDRGDYW	26	IGHV03	IGHV03-13	IGHD03-10	IGHJ04-01	donor1
CARDRGDYW	26	IGHV03	IGHV03-48	IGHD03-10	IGHJ04-01	donor3
CARDRGDYW	26	IGHV03	IGHV03-53	IGHD03-10	IGHJ04-01	donor1, donor3
CARDRGDYW	26	IGHV03	IGHV03-53	IGHD03-16	IGHJ04-01	donor3
CARDRGDYW	26	IGHV03	IGHV03-53	IGHD05-24	IGHJ04-01	donor1
CARDRGDYW	26	IGHV03	IGHV03-64	IGHD03-10	IGHJ04-01	donor1, donor3
CARDRGDYW	26	IGHV03	IGHV03-66	IGHD03-10	IGHJ04-01	donor2, donor3
CARDRGDYW	26	IGHV04	IGHV04-39	IGHD03-10	IGHJ04-01	donor1, donor3
CARDRGDYW	26	IGHV04	IGHV04-39	IGHD03-16	IGHJ04-01	donor3
CARDRGDYW	26	IGHV05	IGHV05-51	IGHD03-16	IGHJ04-01	donor2
CARDRGDYW	26	IGHV07	IGHV07-04_1	IGHD03-10	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV01	IGHV01-02	IGHD06-19	IGHJ04-01	donor1, donor2, donor3
CARDSSGWYYFDYW	20	IGHV01	IGHV01-03	IGHD06-19	IGHJ04-01	donor1, donor2, donor3
CARDSSGWYYFDYW	20	IGHV01	IGHV01-08	IGHD06-19	IGHJ04-01	donor1
CARDSSGWYYFDYW	20	IGHV01	IGHV01-18	IGHD06-19	IGHJ04-01	donor1, donor2, donor3
CARDSSGWYYFDYW	20	IGHV01	IGHV01-46	IGHD06-19	IGHJ04-01	donor1, donor3
CARDSSGWYYFDYW	20	IGHV01	IGHV01-69	IGHD06-19	IGHJ04-01	donor2, donor3
CARDSSGWYYFDYW	20	IGHV02	IGHV02-70	IGHD06-19	IGHJ04-01	donor1, donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-11	IGHD06-19	IGHJ04-01	donor1
CARDSSGWYYFDYW	20	IGHV03	IGHV03-20	IGHD06-19	IGHJ04-01	donor1
CARDSSGWYYFDYW	20	IGHV03	IGHV03-23	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-48	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-53	IGHD06-19	IGHJ04-01	donor1, donor2, donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-64	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-66	IGHD06-19	IGHJ04-01	donor2, donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-72	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV03	IGHV03-74	IGHD06-19	IGHJ04-01	donor1, donor2
CARDSSGWYYFDYW	20	IGHV04	IGHV04-39	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV05	IGHV05-51	IGHD06-19	IGHJ04-01	donor3
CARDSSGWYYFDYW	20	IGHV06	IGHV06-01	IGHD06-19	IGHJ04-01	donor1, donor2
CARDSSGWYYFDYW	20	IGHV07	IGHV07-04_1	IGHD06-19	IGHJ04-01	donor3

Two examples of HCDR3s derived from multiple rearrangements

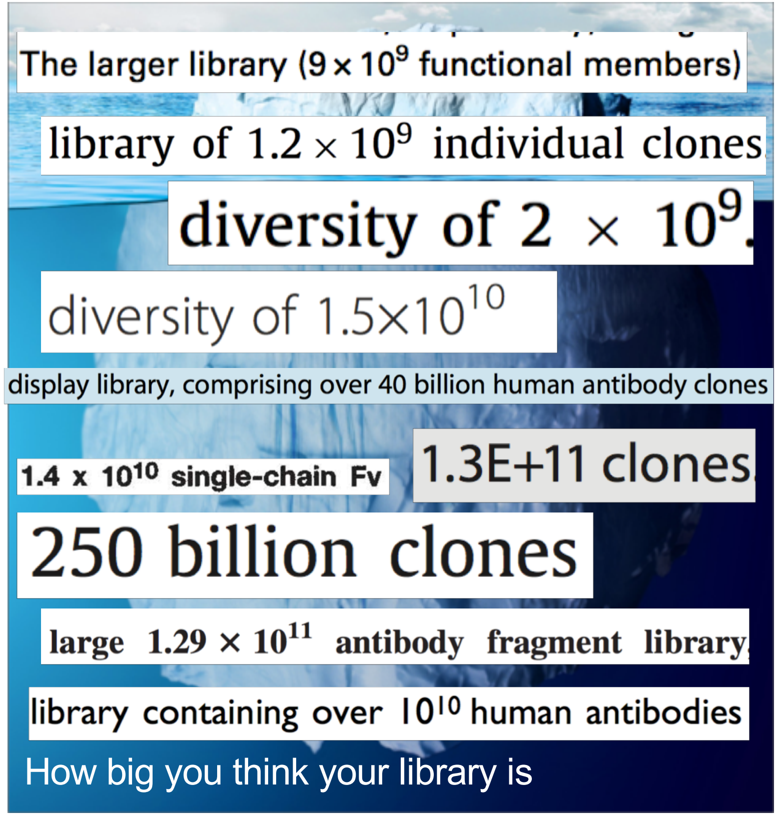
- 14 different VH germlines from 5 different VH families
- 5 DH genes
- 1 JH

- 20 different VH germlines from all VH families
- 1 DH gene
- 1 JH

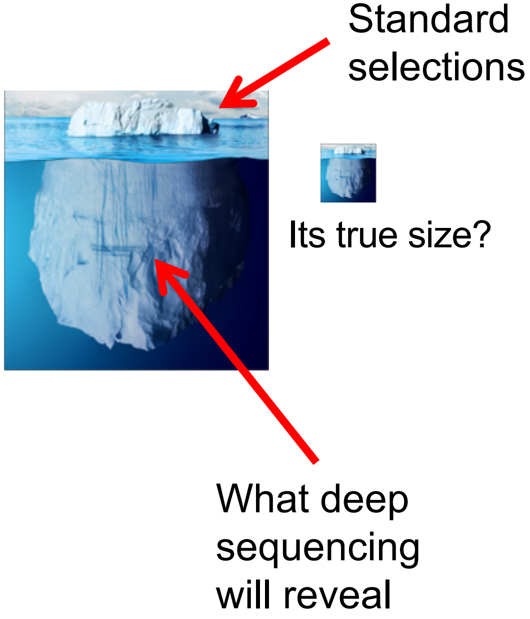
Examples of HCDR3s with identical rearrangements found in 3 donors

HCDR3	VH gene	DH gene	JH gene	number
CARDSSGWYYFDYW	IGHV01-02	IGHD06-19	IGHJ04-01	4
CARDSSGWYYFDYW	IGHV01-03	IGHD06-19	IGHJ04-01	4
CARDSSGWYYFDYW	IGHV01-18	IGHD06-19	IGHJ04-01	4
CARDSSGWYYFDYW	IGHV03-53	IGHD06-19	IGHJ04-01	4
CARGYSSGWYYFDYW	IGHV01-02	IGHD06-19	IGHJ04-01	4
CARGYSSGWYYFDYW	IGHV01-46	IGHD06-19	IGHJ04-01	4
CARGYSSGWYYFDYW	IGHV03-53	IGHD06-19	IGHJ04-01	4
CARGYSSGWYYFDYW	IGHV05-51	IGHD06-19	IGHJ04-01	4
CARGYSSSWYYFDYW	IGHV01-18	IGHD06-13	IGHJ04-01	3
CARGYSSSWYYFDYW	IGHV01-69	IGHD06-13	IGHJ04-01	3
CARGYSSSWYYFDYW	IGHV05-51	IGHD06-13	IGHJ04-01	3
CAKDSGSYYFDYW	IGHV03-23	IGHD01-26	IGHJ04-01	2
CAKDSGSYYFDYW	IGHV03-43	IGHD01-26	IGHJ04-01	2
CARDCSSTSCYDYW	IGHV01-02	IGHD02-02	IGHJ04-01	2
CARDCSSTSCYDYW	IGHV01-18	IGHD02-02	IGHJ04-01	2
CARDRGDYW	IGHV01-46	IGHD03-10	IGHJ04-01	2
CARDRGDYW	IGHV01-69	IGHD03-10	IGHJ04-01	2
CARDRGWFDPW	IGHV01-18	IGHD03-10	IGHJ05-01	2
CARDRGWFDPW	IGHV03-74	IGHD03-10	IGHJ05-01	2
CARDRGYSGYDFDYW	IGHV01-02	IGHD05-12	IGHJ04-01	2
CARDRGYSGYDFDYW	IGHV01-18	IGHD05-12	IGHJ04-01	2

Conclusion



The larger library (9 × 10⁹ functional members)
library of 1.2 × 10⁹ individual clones
diversity of 2 × 10⁹.
diversity of 1.5×10¹⁰
display library, comprising over 40 billion human antibody clones
1.4 x 10¹⁰ single-chain Fv
1.3E+11 clones
250 billion clones
large 1.29 × 10¹¹ antibody fragment library
library containing over 10¹⁰ human antibodies
How big you think your library is



Standard selections
Its true size?
What deep sequencing will reveal

Antibody affinity maturation

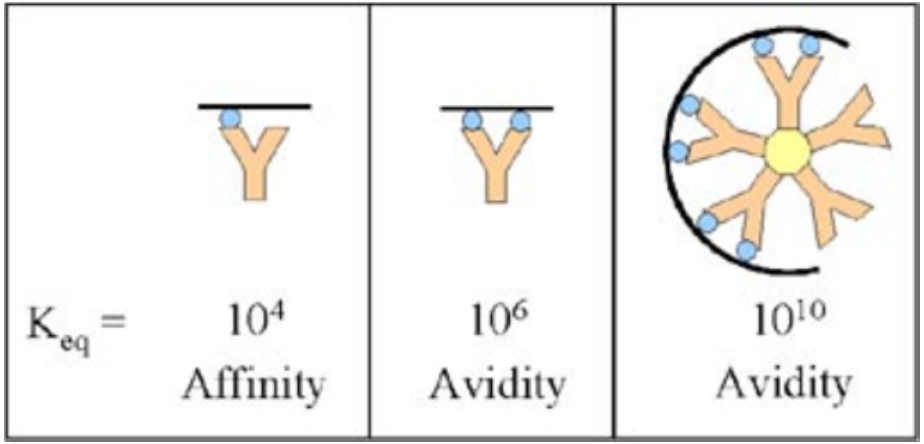
Why increase affinity

- Increase ‘biologic’ activity of the mAb
  - Reduce the concentration of a soluble toxin or ligand
  - Use a lower dose of mAb to achieve the same potency

$$mAb + Ag \leftrightarrow mAb\text{-}Ag$$

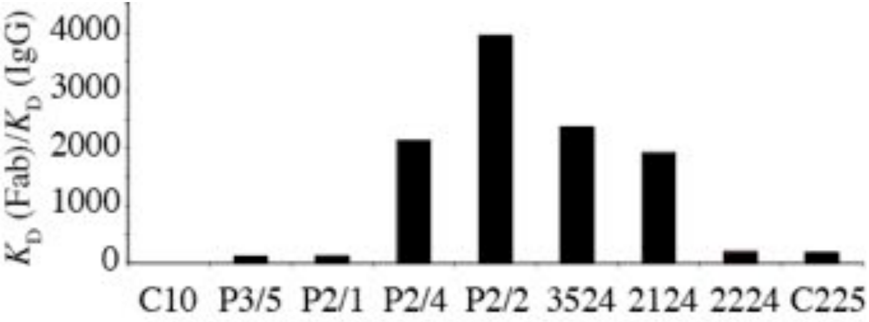
- Importance less clear for multivalent cell surface antigen where only binding is desired (as opposd to blocking ligand
- Not useful for scFvs used in CAR T cells

The difference between affinity and avidity



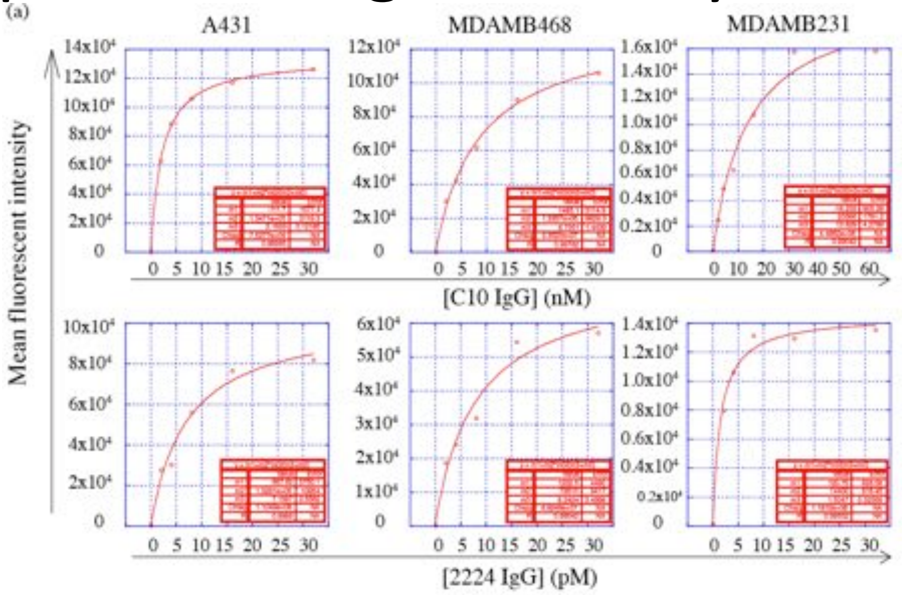
Impact of intrinsic affinity on avidity

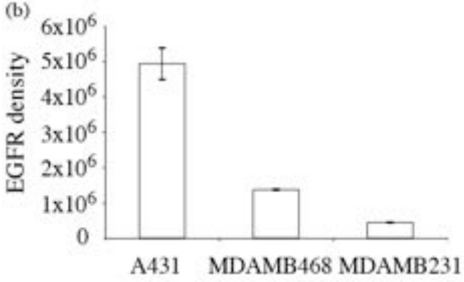
Antibody	scFv K_D (nM)	Fab K_D (nM)	IgG K_D (nM)	K_D (Fab)/ K_D (IgG)
C10	263.67	124.23	1.17	106
P3/5	88.24	58.24	0.5	116
P2/1	14.81	25.4	0.012	2117
P2/4	15.39	25.2	0.0064	3938
P2/2	17.01	18.1	0.0077	2351
3524	7.47	15.4	0.012	1903
2124	9.90	1.31	0.007	187
2224	0.94	1.2	0.007	171
C225	NA	0.013	0.006	2



Zhou et al. 2012, Mol. Cancer Ther. 11:1467-1476

Impact of antigen density on avidity



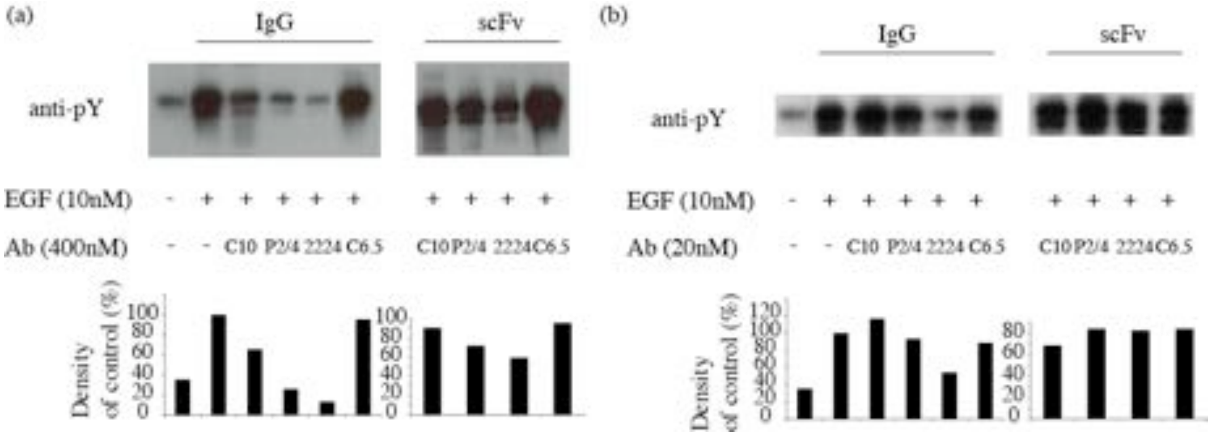


(c)

	Density (10^6)	K_D C10-IgG (10^{-9}M)	K_D 2224-IgG (10^{-12}M)
A431	4.90	2.18	7.16
MDAMB468	1.37	8.74	8.24
MDAMB231	0.44	13.83	1.5

Zhou et al. 2012, Mol. Cancer Ther. 11:1467-1476

Impact of intrinsic affinity of signaling inhibition

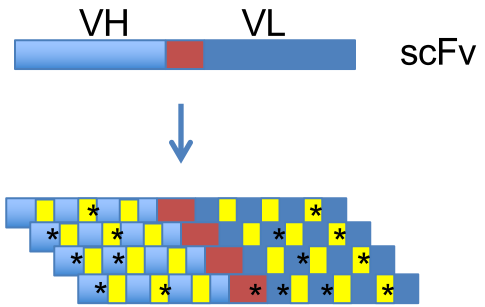


Zhou et al. 2012, Mol. Cancer Ther. 11:1467-1476

Overview of affinity maturation

- How and where to introduce mutations
- Display platform to use
- Selecting rare higher affinity binders from lower affinity binders
- Identifying and characterizing the higher affinity antibodies

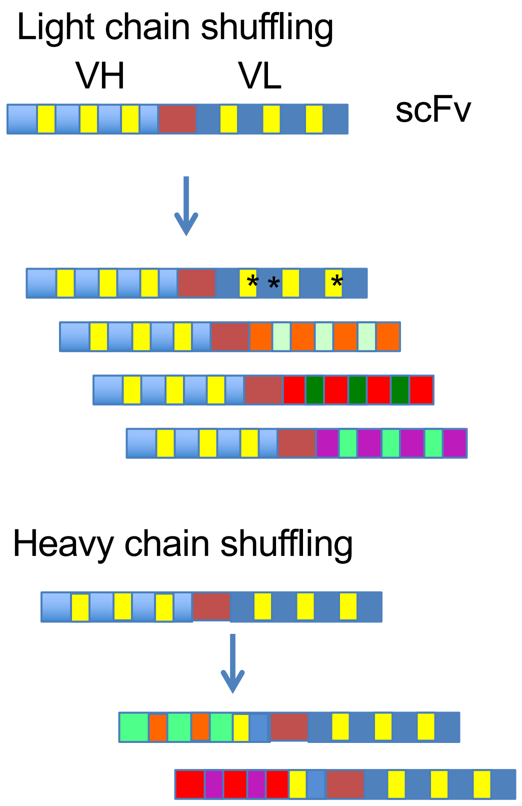
How and where to introduce mutations



- Randomly
  - Error prone PCR
  - Mutator strains of E. coli
  - Intrinsic in ribosome display
  - Advantages
    - Simple, requires no design effort
  - Disadvantages
    - Frequently obtain multiple mutations in both CDRs and frameworks
    - Some mutations in frameworks and affect stability, expression, aggregation
    - Some mutations may have no impact or negative impact on affinity

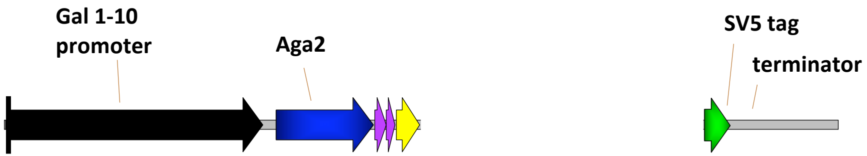
How and where to introduce mutations

- Chain shuffling
  - Esp. for immune libraries
    - Library size results in binding VH sampling only one or a few VL
  - Sample variants of same germline V-gene as well as many other germline genes
  - Tests mutations in 3 CDRs simultaneously
  - Most common is light chain shuffling
  - If VH shuffling, frequently maintain VH CDR3

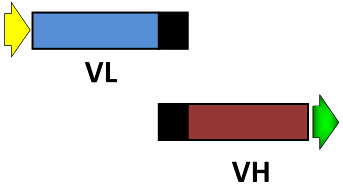


Light chain shuffling by yeast display

Clone VH and VL directly into yeast together using gap repair:



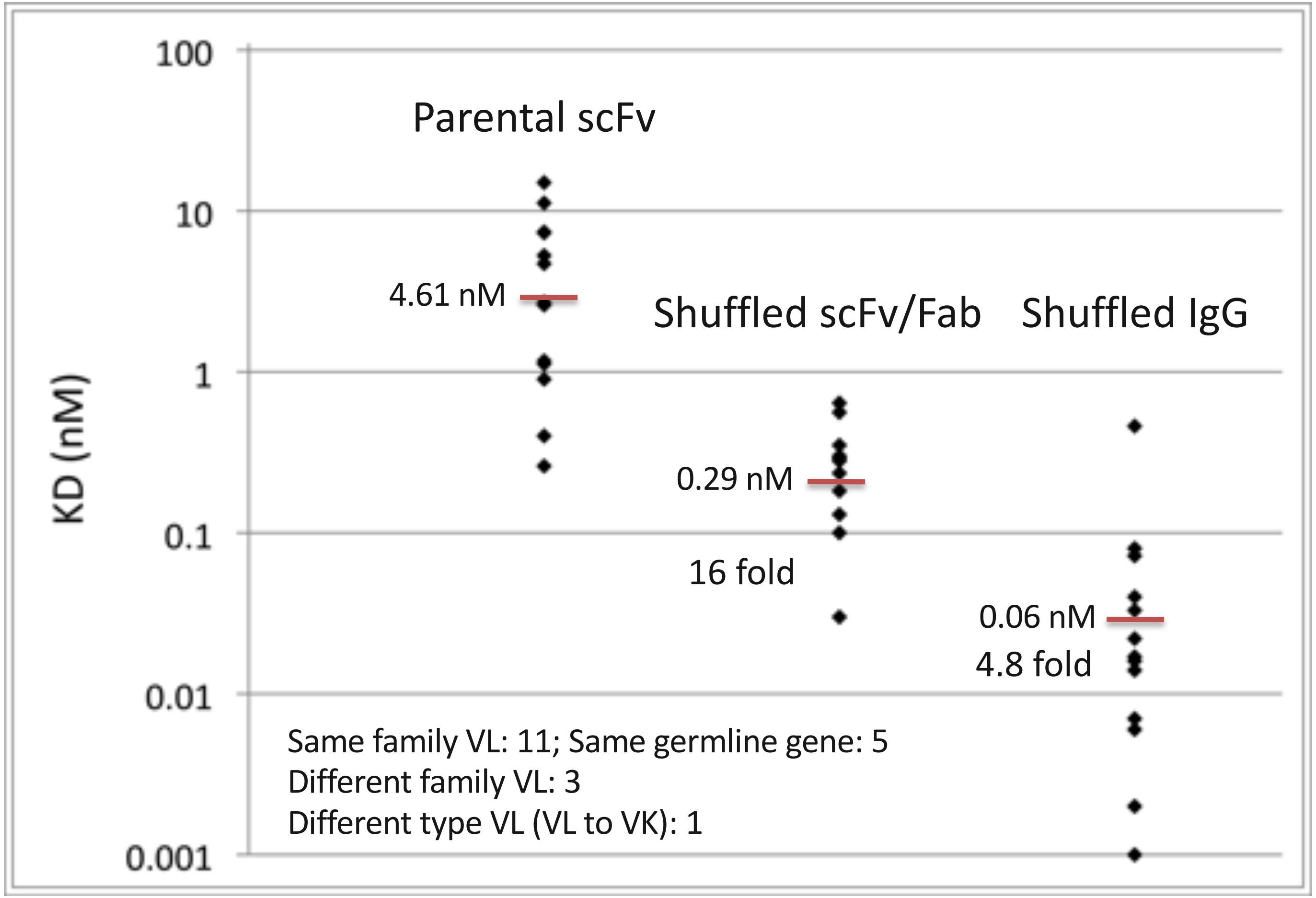
VL library – doesn’t have to be from original immune repertoires



Original VH

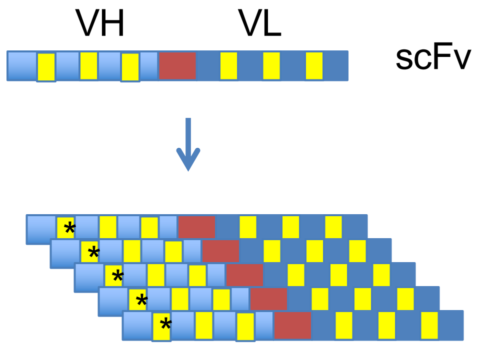
- Double cut vector
- Generate PCR fragments with > 25 bp overhang
- Mix vector & insert and transfect
- Efficiency 1->100E6/ug insert

BoNT mAb affinity maturation by chain shuffling (10 scFv, 3 Fab)



How and where to introduce mutations

- Site directed into the CDRs
  - Only insert mutations into CDRs, not frameworks
  - Most likely to generate new antigen contacts or modify side chain position
  - Allows iterative improvement by moving from CDR to CDR

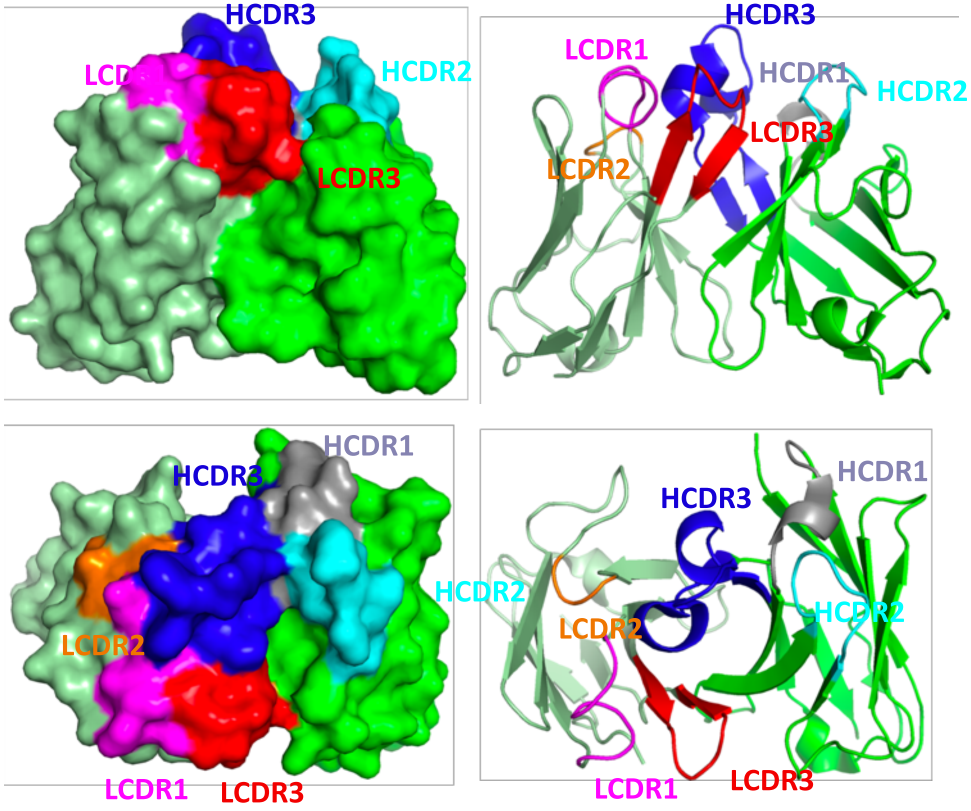


How to introduce CDR mutations

- Random using NNS coding
  - Cheap
  - Requires no thinking
  - Introduces stop codons, cysteines and many non-naturally occurring amino acids at each positions.
  - Most sequences do not encode any of the original amino acids in the CDR
    - Destroys binding
  - Limited sampling of sequence space
    - 32e5 = 3.4e7
- Spiked oligonucleotides
  - Preferred approach
    - 25-50% wild type aa at each position
      - 70:10:10:10 nucleotide mix at positions 1 and 2, G/C at position 3
      - Allows testing of every possible single double and triple mutation at any position randomizing 6 aa
      - Usually enough for any single CDR
      - 4 CDRs maximum for any antibody
      - Sequentially or simultaneously
- Array based oligonucleotides
  - No redundancy
  - More expensive
  - All single and double mutations for one CDR can be exhaustively analyzed

Where to introduce CDR mutations

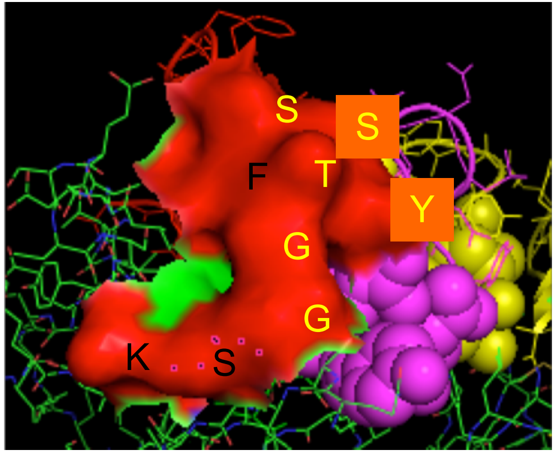
- Greatest germline diversity in center of combing site
  - VH CDR3 >>>>> VL CDR3
  - Best to leave HCDR3 to last because of possible effect on affinity
- Preferred to start with VL shuffling, then, order of mutation:
  - H1, H2, L3, L1, H3
  - Mimics somatic hypermutation
- If no chain shuffling, order of mutation:
  - L3, H1, H2, L1, H3
- Can perform sequentially, or simultaneously and combine mutations



Solvent (antigen) accessible residues are a subset of CDR residues

HV1-69 & KV3-15

SCKASGGTFSSYAISWV

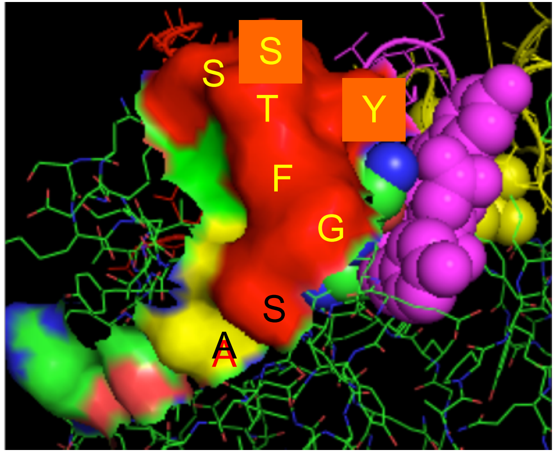


Surface residues red

Mutant oligo = GTFSSY

HV3-23 & KV1-69

SCAASGFTFSSYAMSWV



Surface residues red

Mutant oligo = FTFSSY

KABAT CDR residues

CDR residues common to KABAT and structure (IMGT)

Structural (IMGT) residues

H3 in magenta;
CDRLs in yellow

What display system to use

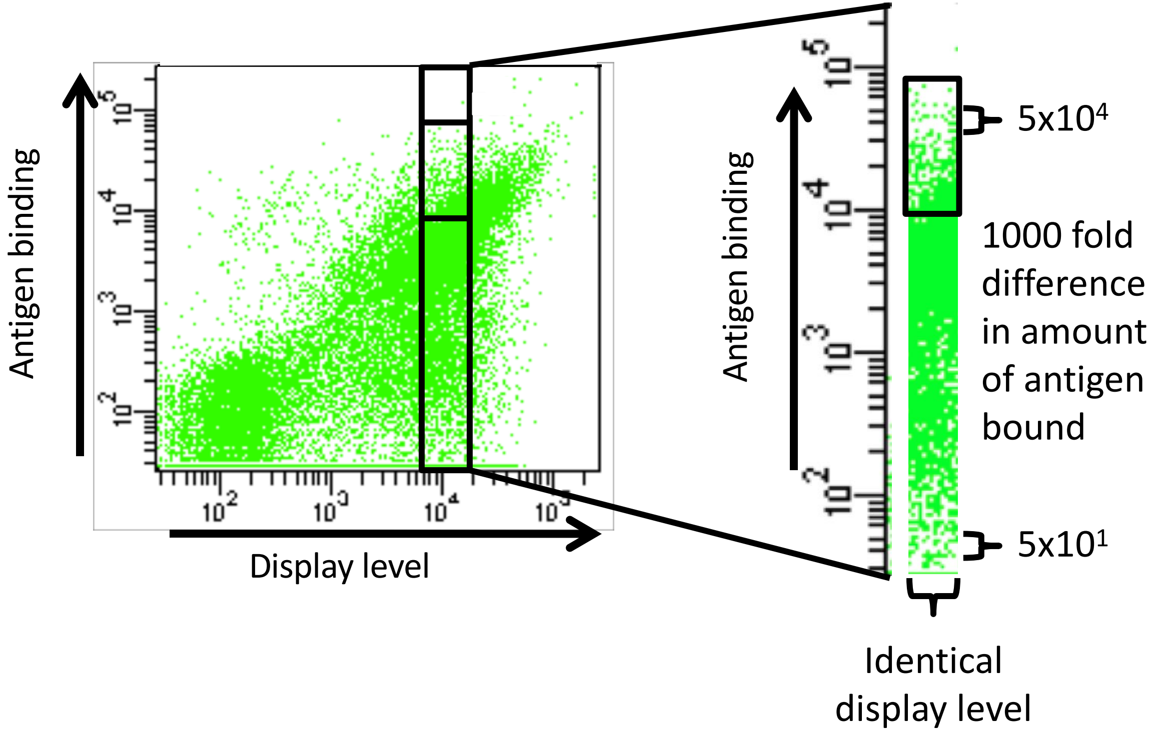
- Phage display
  - Simple
  - Examples of > 1000 fold increases in affinity using sequential spiked oligo libraries
  - Use biotinylated monovalent antigen, decrease concentration each round
  - Need to secrete Ab fragment for affinity measurement
  - Can obtain off rate with unpurified fragment, need purified fragment for KD due to variability in fragment concentration
  - Black box: cannot see what is happening each round of selection

What display system to use

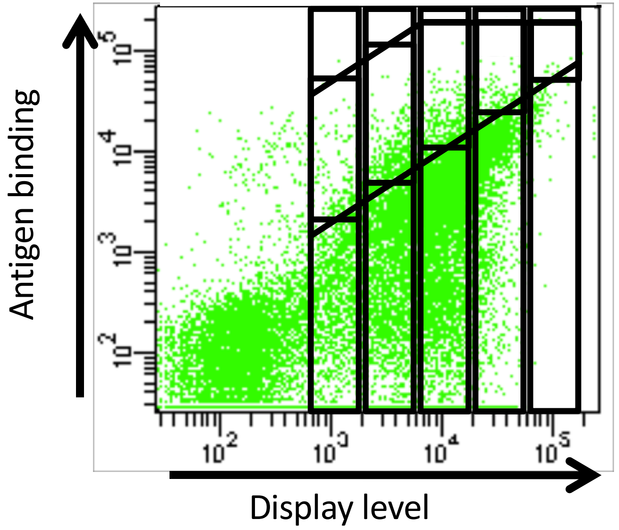
- Yeast display
  - More complex, need to understand flow cytometry
  - Sense that large libraries hard to construct, but straightforward using gap repair
  - Can measure ‘library KD’ at each round of selection to tailor antigen concentration, and can see on cytometer success of staining in separating clones of different affinity
  - Can measure KD on the display platform

Affinity maturation using yeast display

Sorting for higher affinity

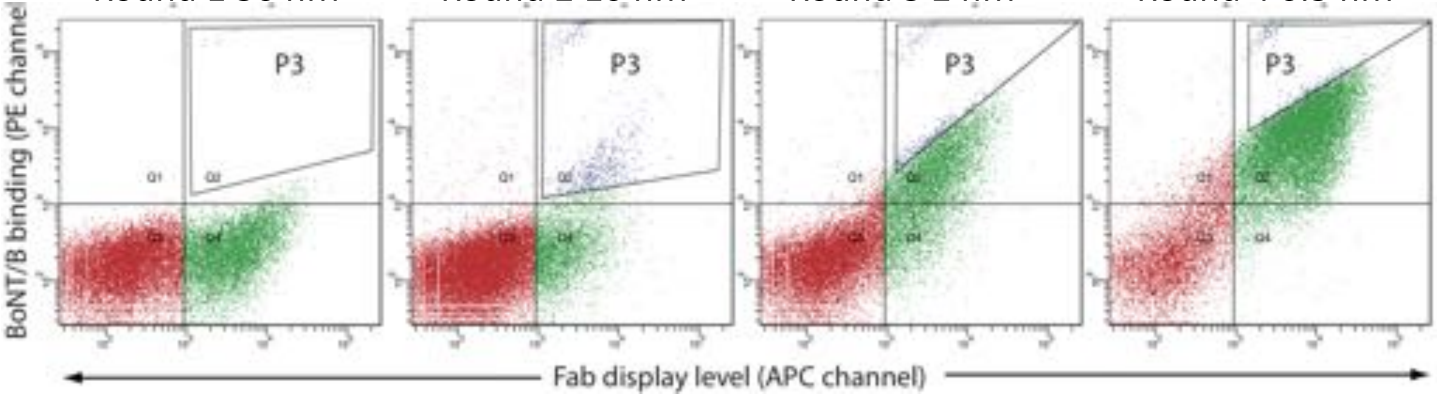


Sorting for higher affinity



Affinity maturation points to consider

Round 1 50 nM | Round 2 10 nM | Round 3 2 nM | Round 4 0.5 nM



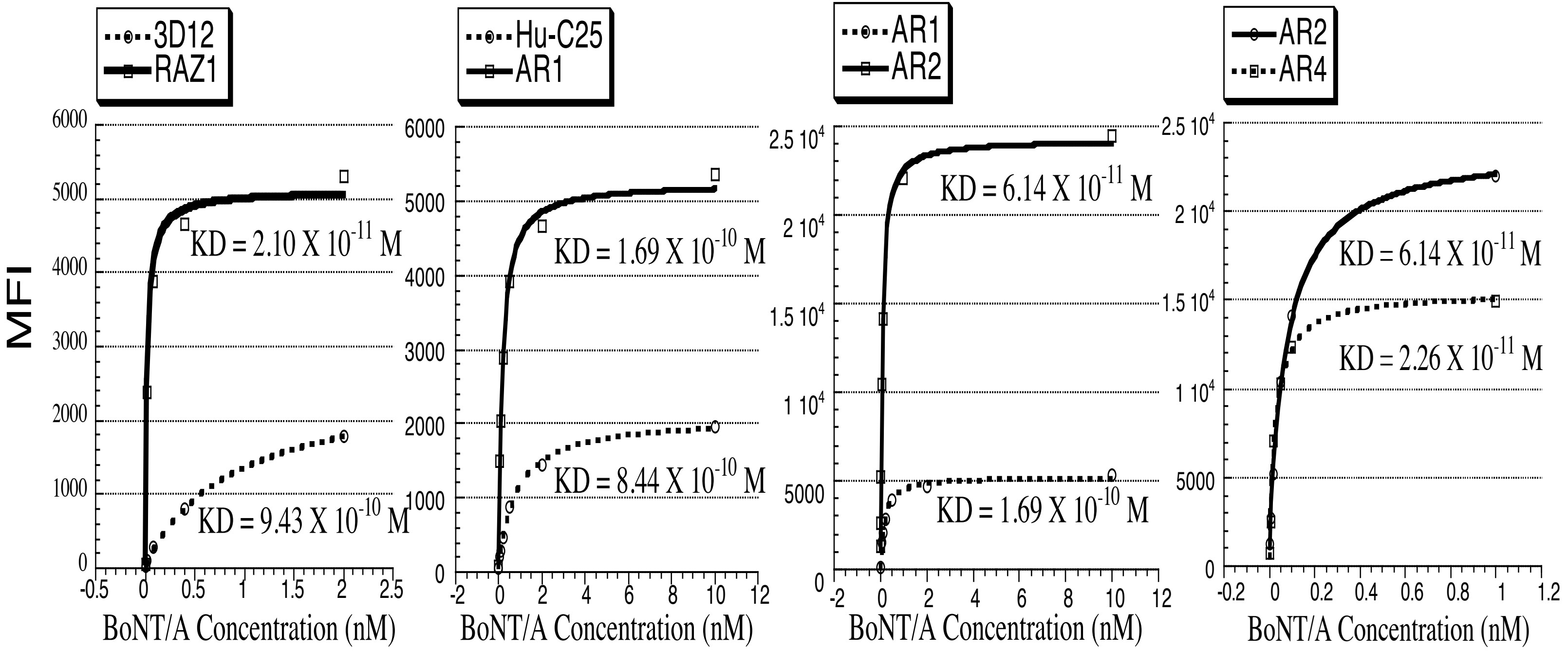
- Generally gate 0.1% of the binding population, except 1st round
- Sort with different antigen concentrations, and use concentration that separates the binding population most, but is above background
- If doing equilibrium sorts, calculate the time to eq. and try to reach this, also ideally in antigen (mole) excess:
- e.g. typically 5e5 scFv/yeast, staining 10e6 yeast
- at 7.5 pM requires 100mL volume to be in 10 fold antigen excess
- Library Kd should improve between rounds
- Below KD of 1 nM, consider on rate/off rate staining
  - Stain for short period of time not achieving equillibrium
  - Wash, incubate in large volume and in presence of unlabelled Ag to prevent rebinding
- Convert multiple Ab fragments to IgG

$$t_{\Theta} = \frac{-\ln(1-\Theta)}{k_a \cdot C + k_d}$$

Individual clone characterization

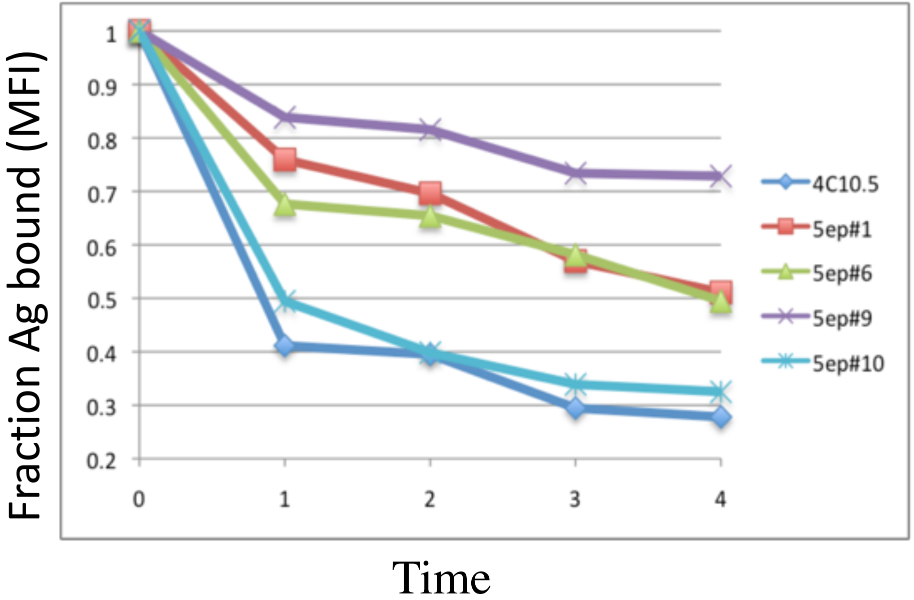
- Between rounds stay in liquid culture
- Plate after last round of sorting
- Pick individual colonies, sequence and induce unique clones
- Measure KD of antibody fragment on yeast surface
- Convert to IgG and measure KD

Measuring KD's of yeast displayed scFv



Razai A, et al J. Mol. Biol. 351:158-169, 2005.

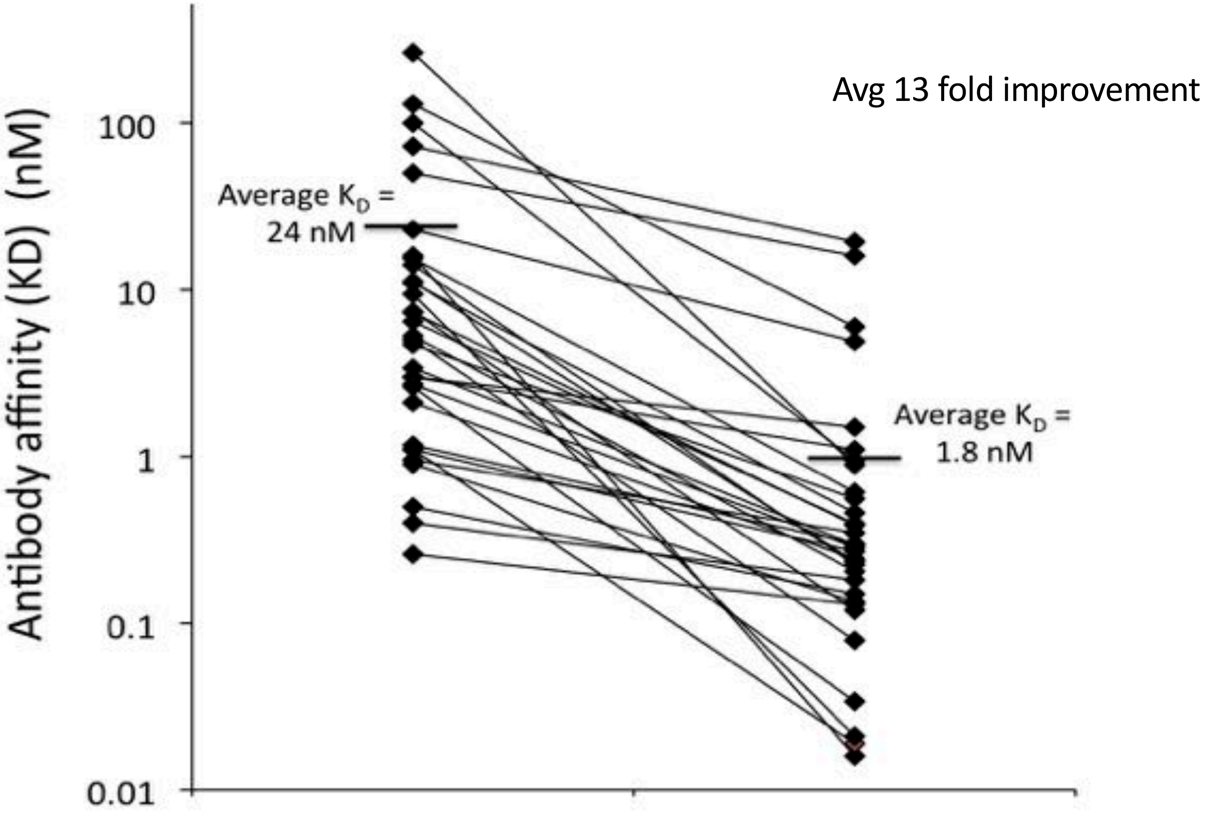
Use of yeast based off-rate screening to identify higher affinity mAbs



All of these have similar Kds

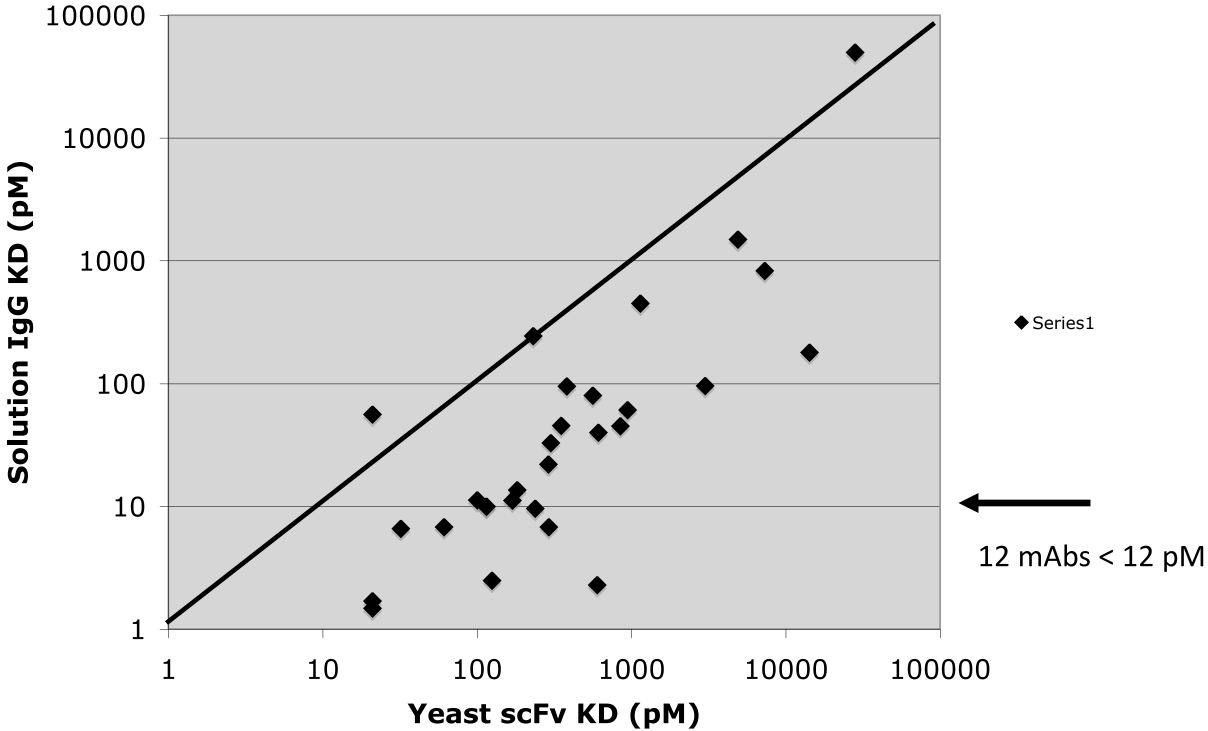
- Incubate yeast with Ag to equilibrium
- Wash, let dissociate and block rebinding
- Measure MFI
- Allows prediction of antibodies that will perform better as IgG

Affinites of lead and affinity matured scFv and Fab
(32 antibodies, 26 scFv, 6 Fab)



Affinities of BoNT yeast displayed scFv and IgG in solution

12 primary libraries, 30 secondary libraries: 30 scFv converted to IgG



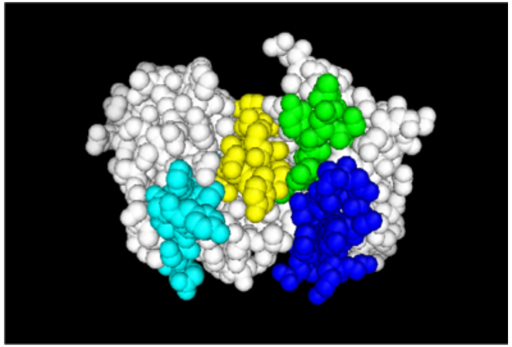
Evolving cross reactive mAbs

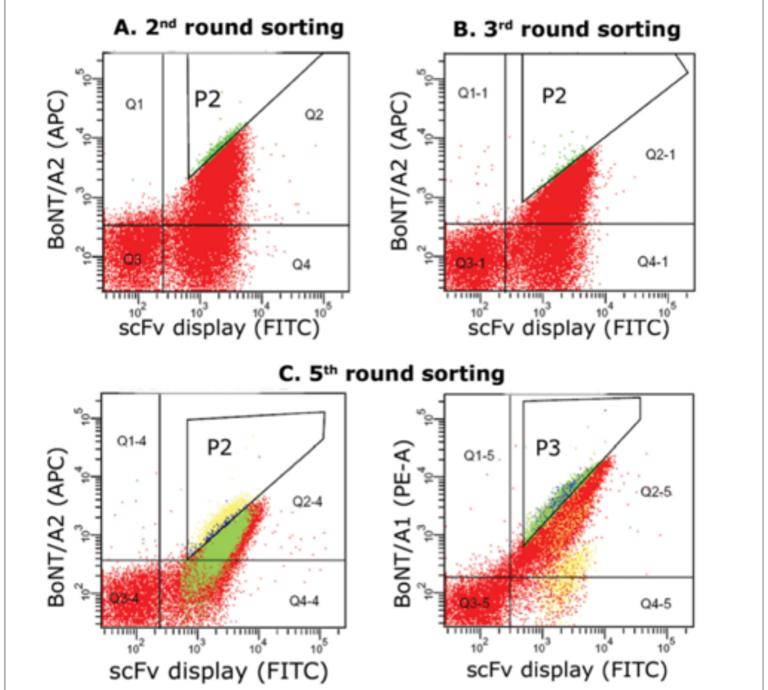
Four separate libraries made (H1, H2, H3 L1) but only HCDR1 library cross-reactive Abs

AR2

KD A1 = 5.0 x10^{-11} M

KD A2 = 2.0 x10^{-7} M





CR2

KD A1 = 6.5 x10^{-11} M

KD A2 = 3.0 x10^{-10} M

Four separate libraries made (H1, H2, H3 L1) but only HCDR1 library cross-reactive Abs

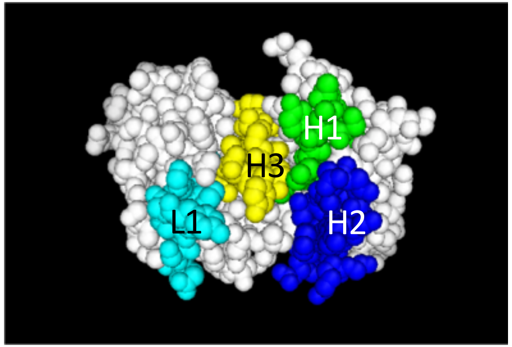
Sort for P2+P3 in 5th round

VH CDR1 GFTFS DHYMY

VH CDR1 GFTFK YDYMY

Garcia C, et al. Nature Biotech. 25:107-116, 2007.

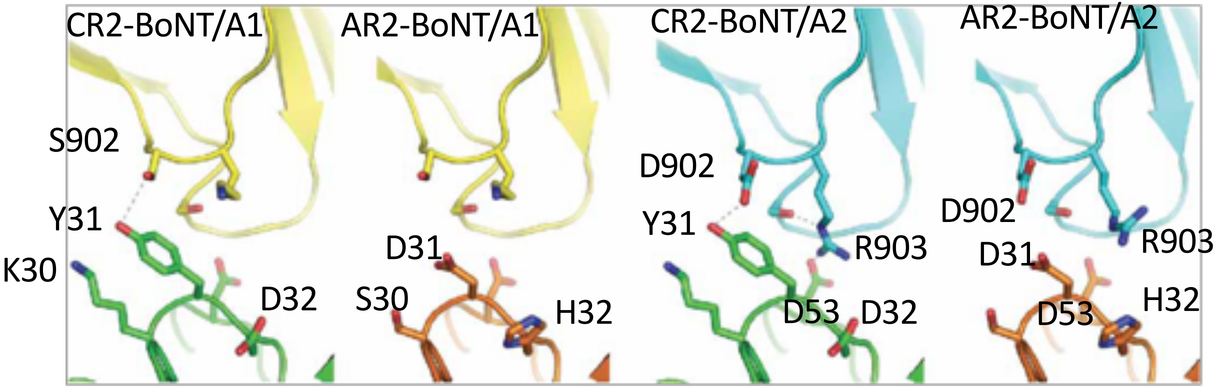
Evolving cross reactive mAbs



AR2
KD A1 = 5.0 x10^{-11} M
KD A2 = 2.0 x10^{-7} M

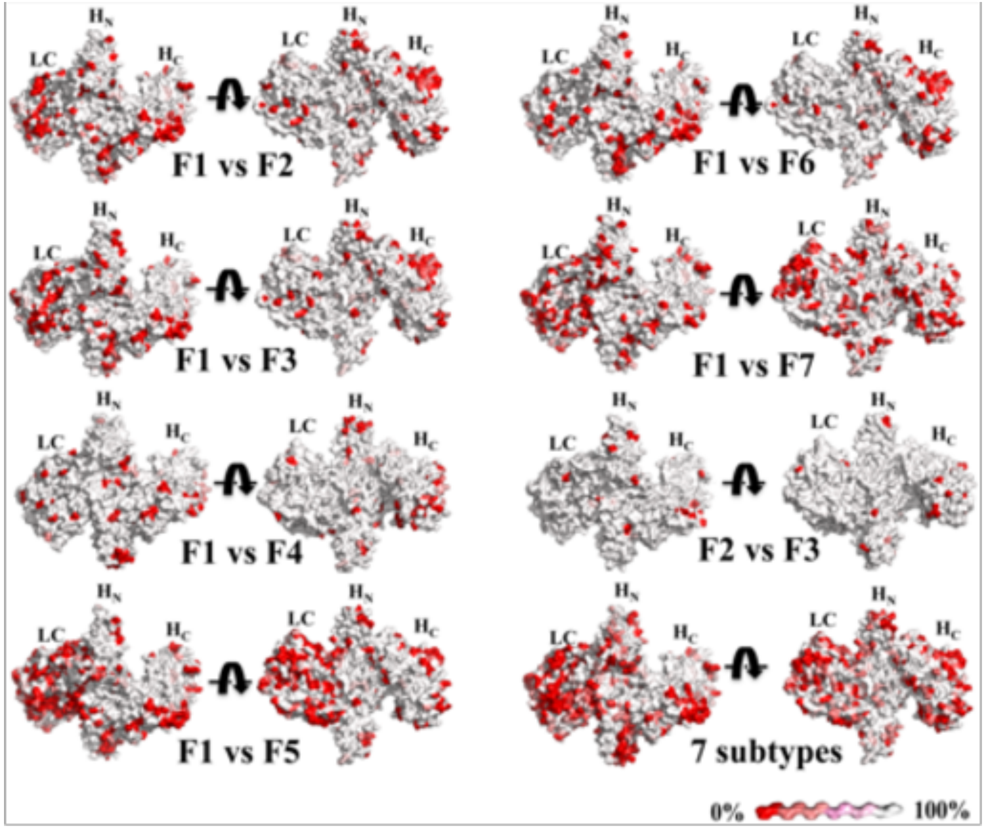
CR2
KD A1 = 6.5 x10^{-11} M
KD A2 = 3.0 x10^{-10} M

31
VH CDR1 GFTFS D<u>H</u>YMY
VH CDR1 GFTF<u>K</u> <u>YD</u>YMY
30 32



Garcia C, et al. Nature Biotech. 25:107-116, 2007

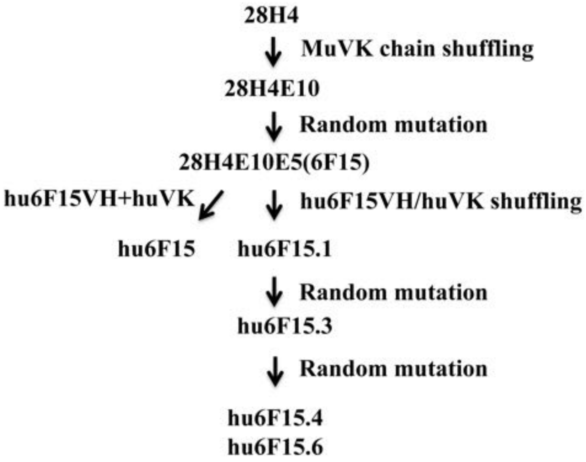
Evolving cross reactive BoNT/F mAbs



BoNT/F subtypes differ by up to 36%

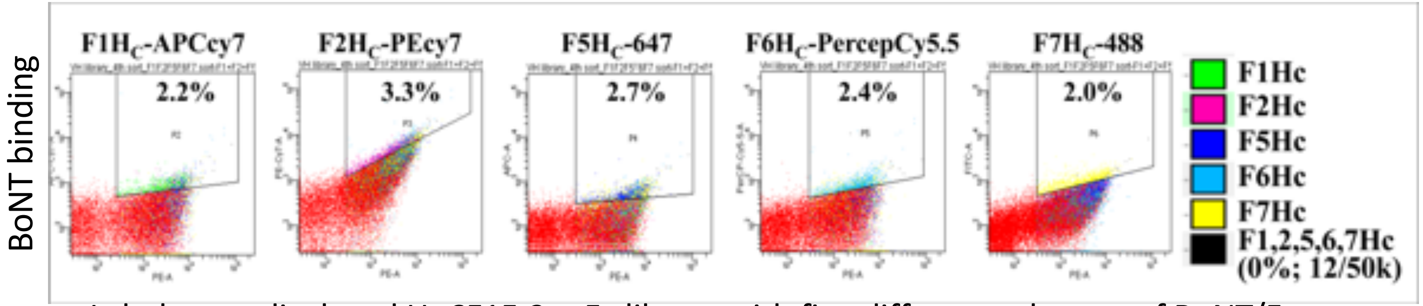
Fan, Y. *et al. PLoS One* **12**, e0174187, (2017).

Engineering cross reactive BoNT/F mAbs



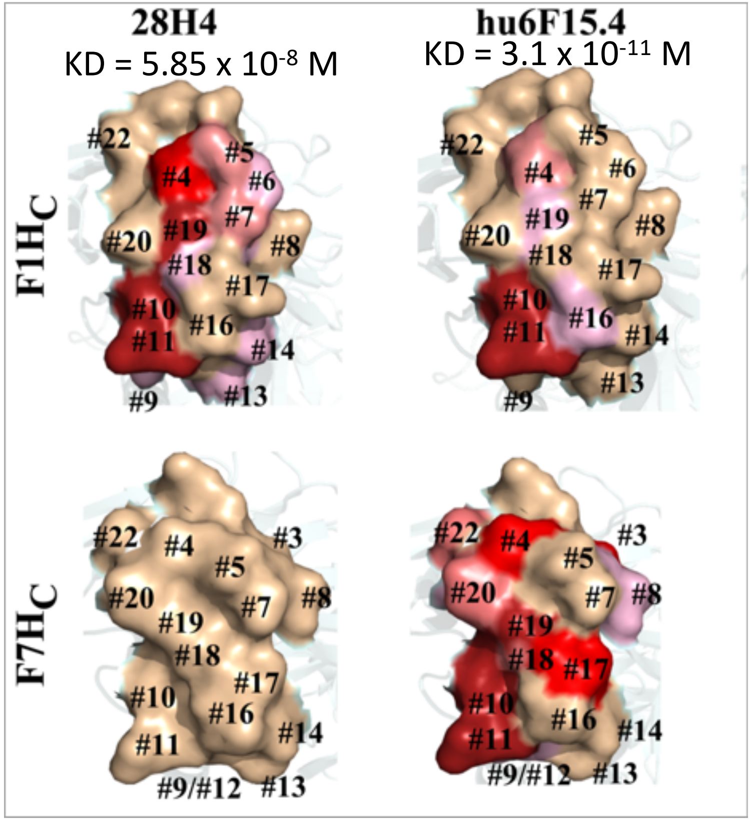
KD = BoNT/F1 = 5.85 x 10^{-8} M

KD BoNT/F7 < 10 uM



Label yeast displayed Hu6F15.3 scFv library with five different subtypes of BoNT/F labeled with different fluorescent dyes

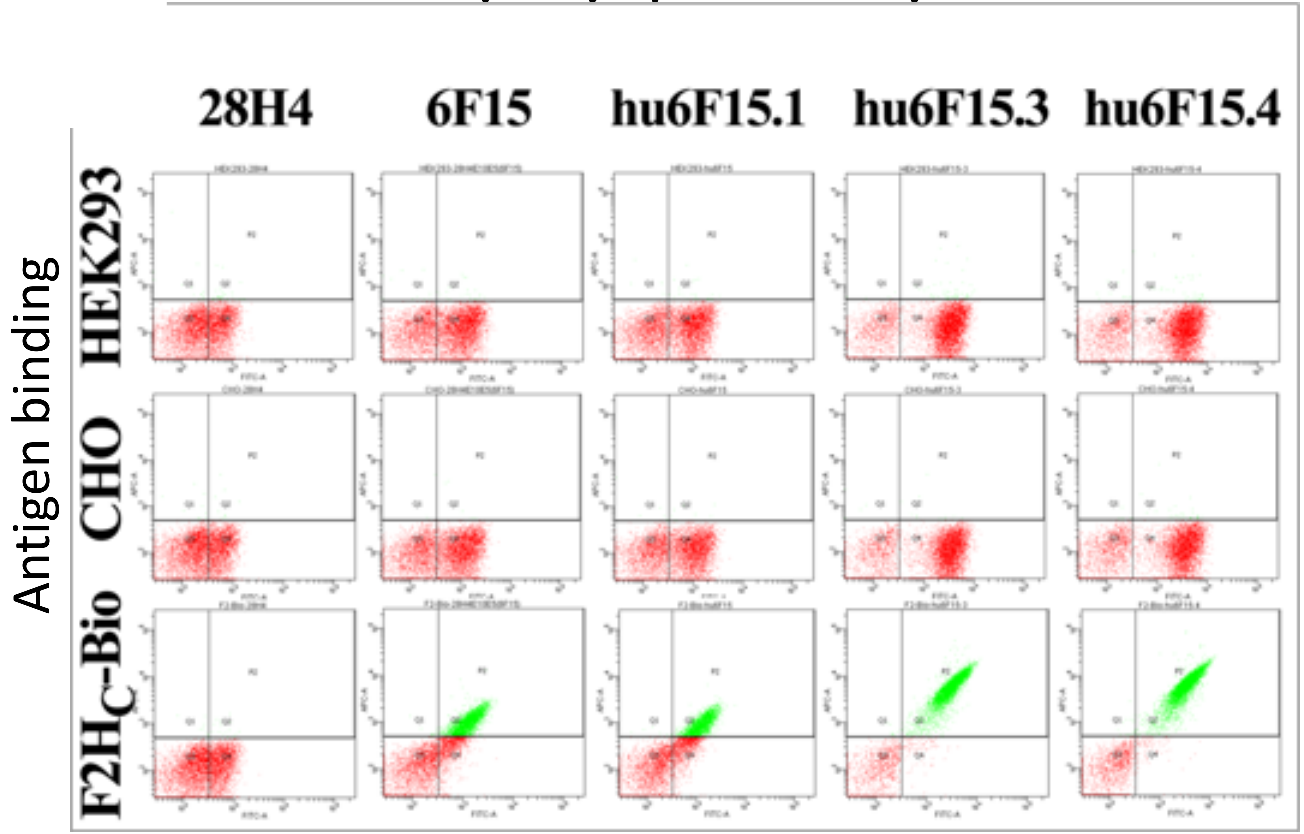
Mechanism of increased cross reactivity



KD < 10 uM   KD = 6.6×10^{-10} M

- Increase affinity for shared contacts between subtypes
- Reduce importance of different contacts for binding to BoNT/F1 subtype
- Increase affinity for different contacts in BoNT/F7

Engineered cross reactivity does not result in polyspecificity



Selecting clones to convert to IgG

- Pick multiple of different sequences and highest KD
  - Some may not be developable
  - At KD < 1 nM, similar yeast KD may give very different IgG KD
    - Measure clones with slowest off rates
    - Pick top 6 clones to make as full length IgGs

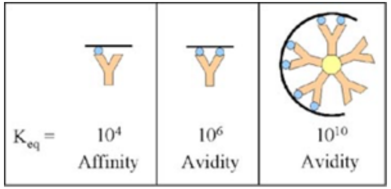
Antibody characterization and developability

Antibody characterization

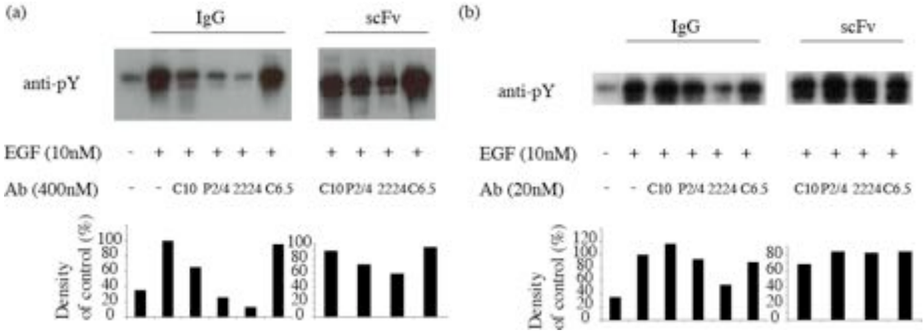
- Affinity
- Biologic activity
- Epitope
- Expression
- Sequence
- Specificity
  - Self-aggregation
  - Solubility
- Stability

Antibody characterization

- Affinity & biologic activity
  - Monovalent intrinsic affinity vs avidity
    - Be sure that measurement not being performed where avidity may confound (e.g. Fc fusion protein)



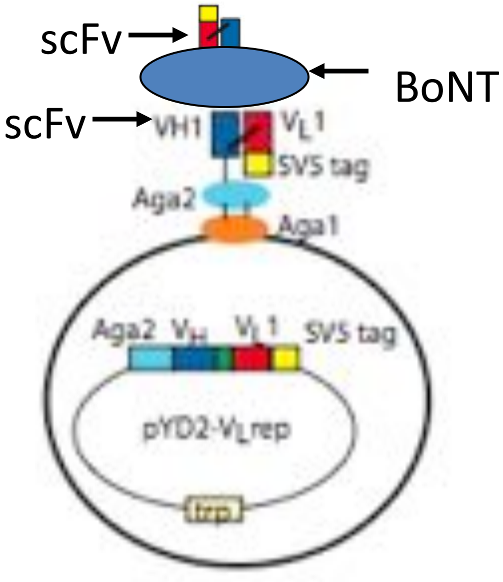
  - Biologic activity
    - In vitro vs in vivo



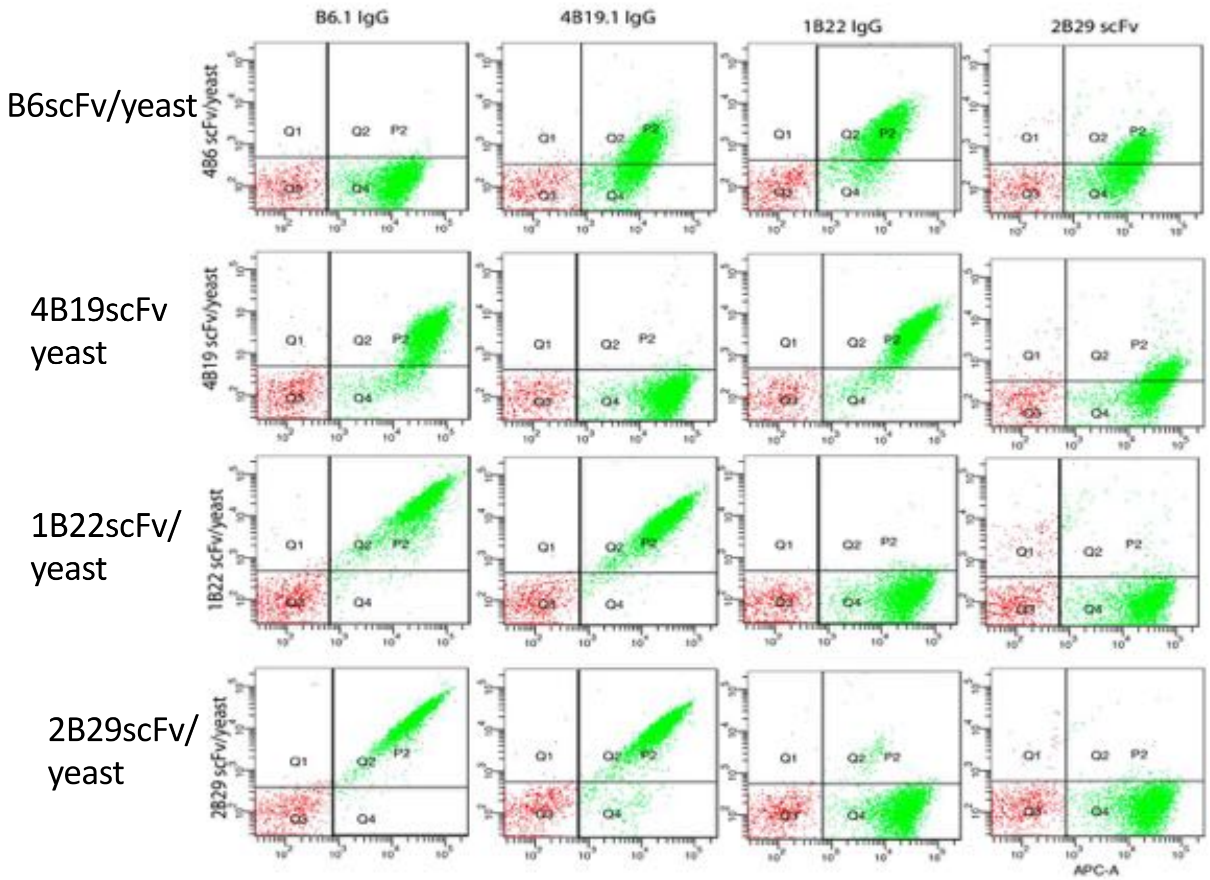
Antibody characterization

- Epitope
  - Important to
    - Understand structure function relationship of mAb-Ag interaction
    - May be necessary if cannot isolate cross reactive mAb to show homologous epitopes for murine and human binding mAbs
  - Binning mAbs by overlap
  - At a domain level
  - Fine epitope

Mapping antibody epitopes for overlap

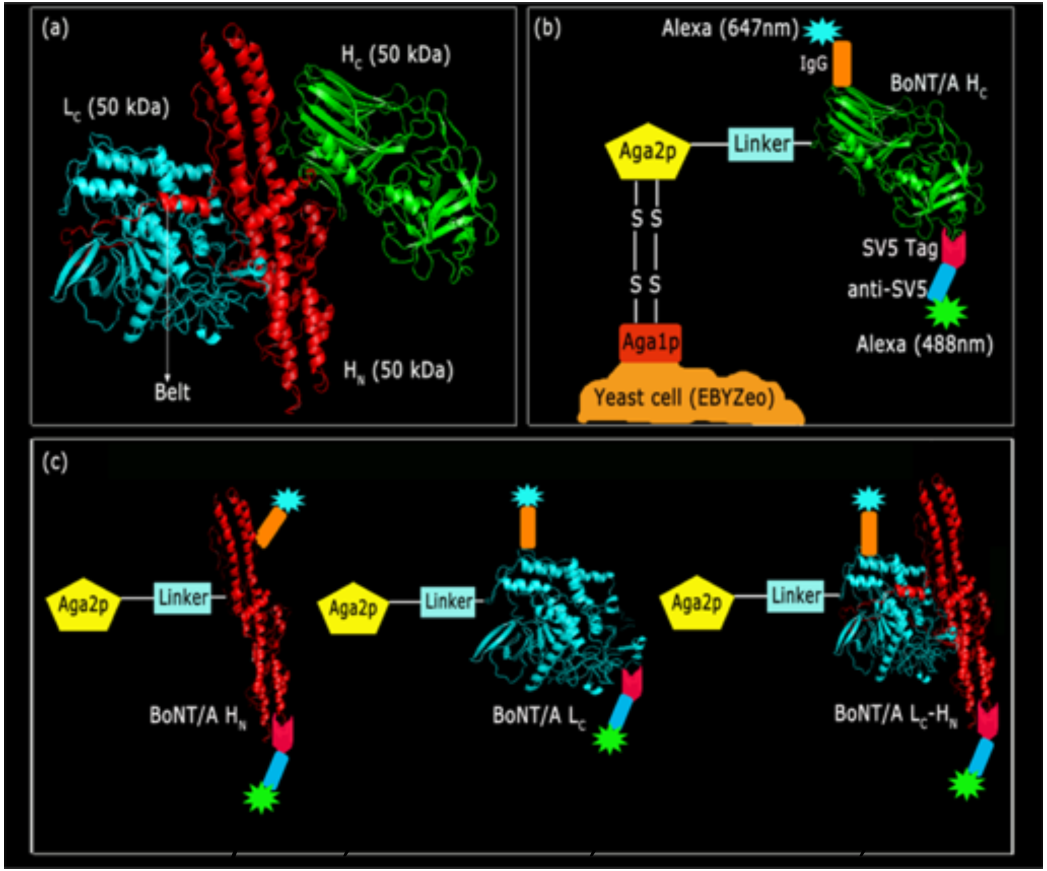


Epitope mapping antibodies by flow cytometry

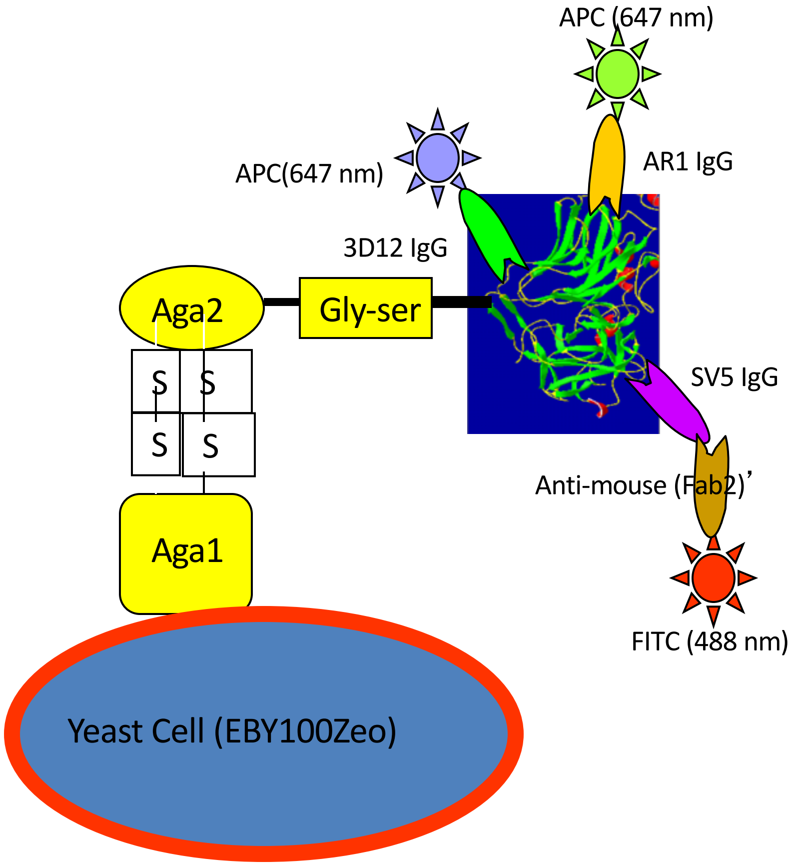


Yeast displayed (BoNT) domains

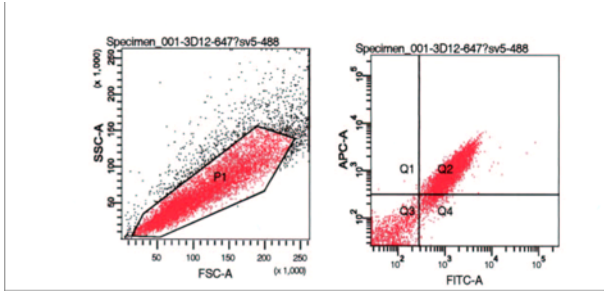
Domain and fine epitope mapping



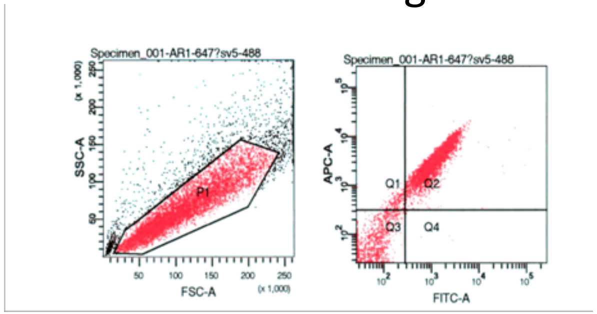
BoNT/HC Yeast display for domain epitope mapping



3D12 binding

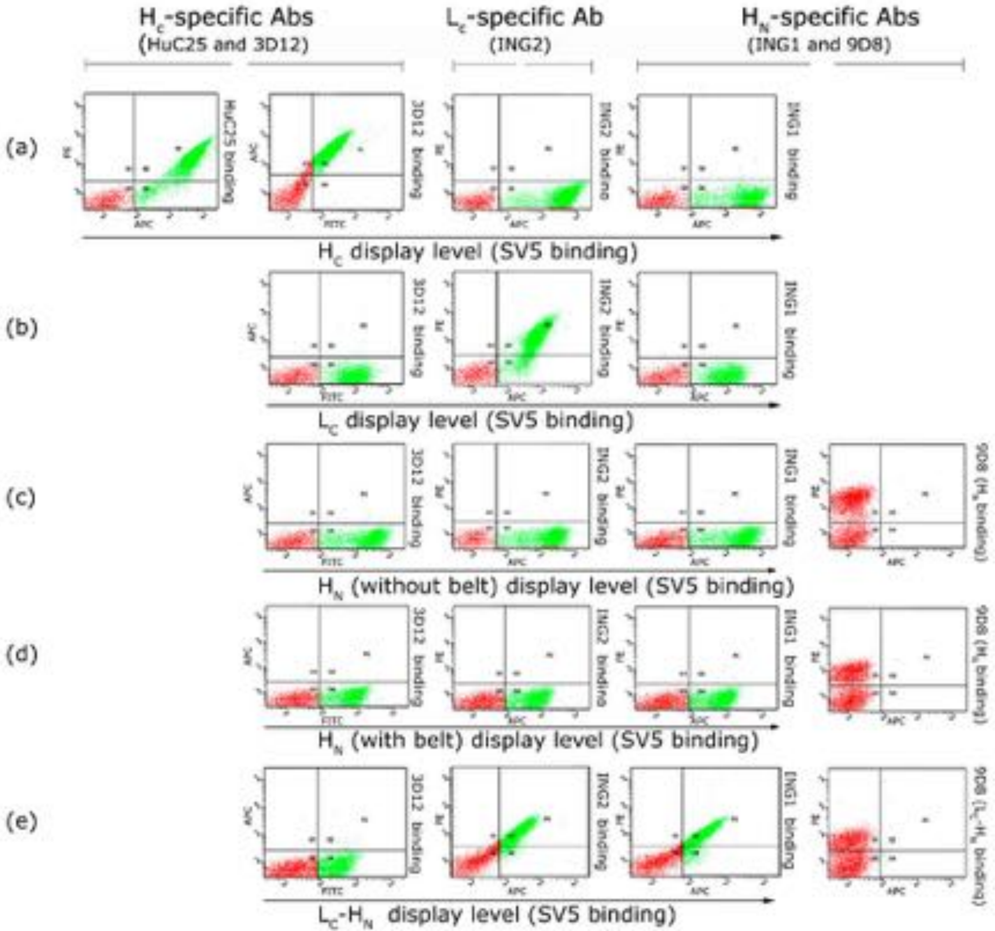


AR1 binding



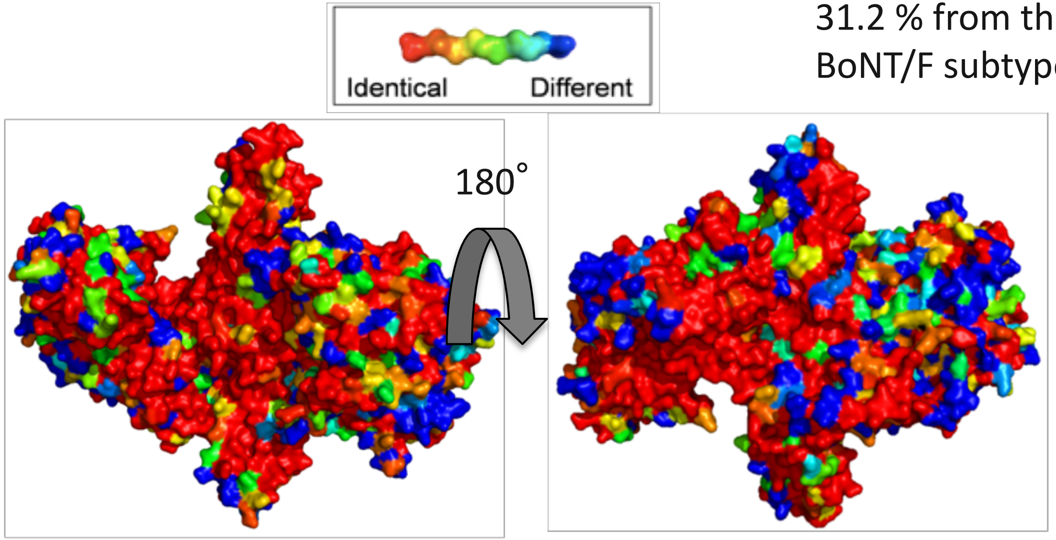
Antibodies binding to yeast displayed Bot domain

BoNT/A domain display



BoNT/F subtype sequence variability
Seven subtypes

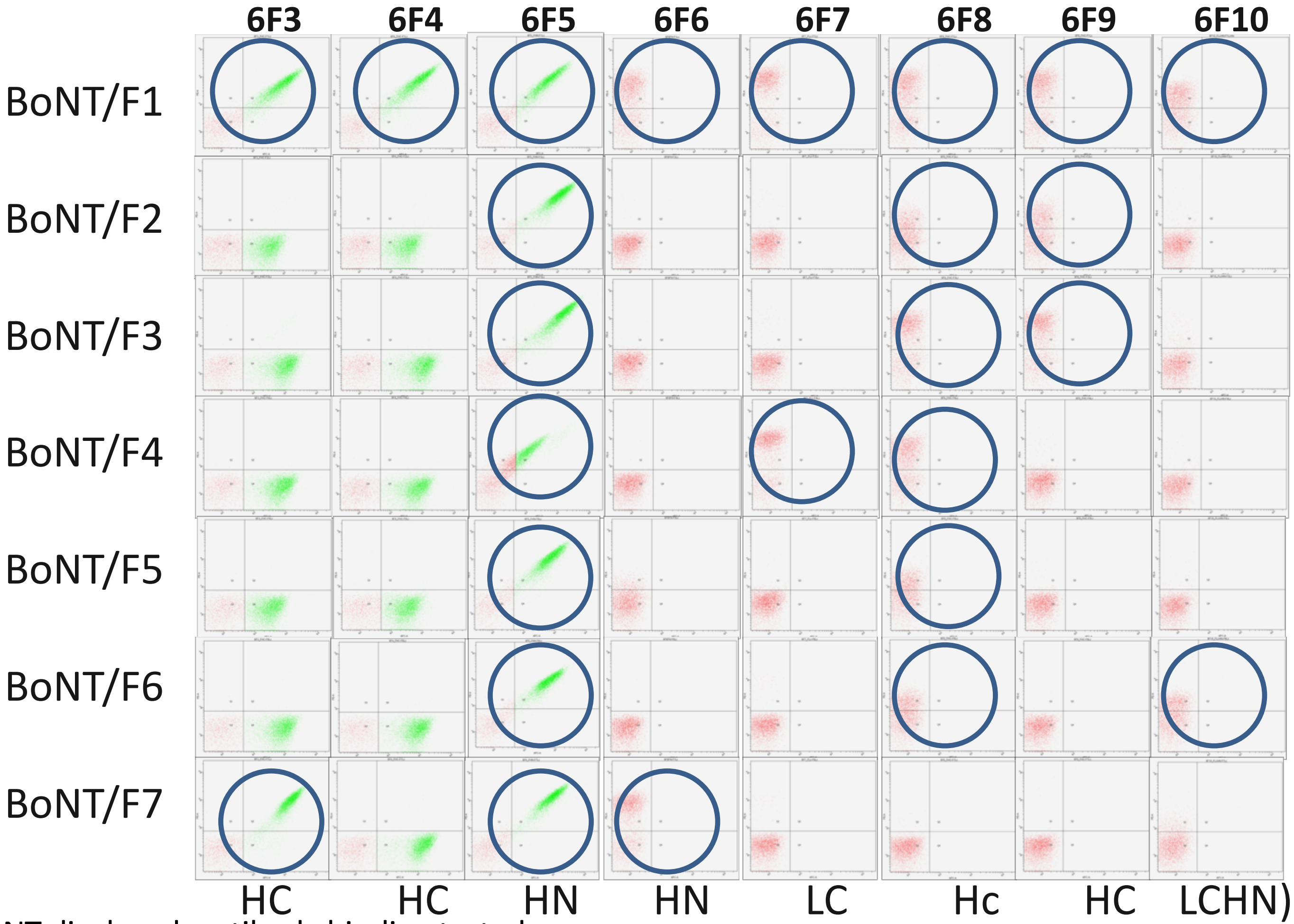
BoNT/F1 differs by 16.6 to 31.2 % from the other BoNT/F subtypes



HC HN LC

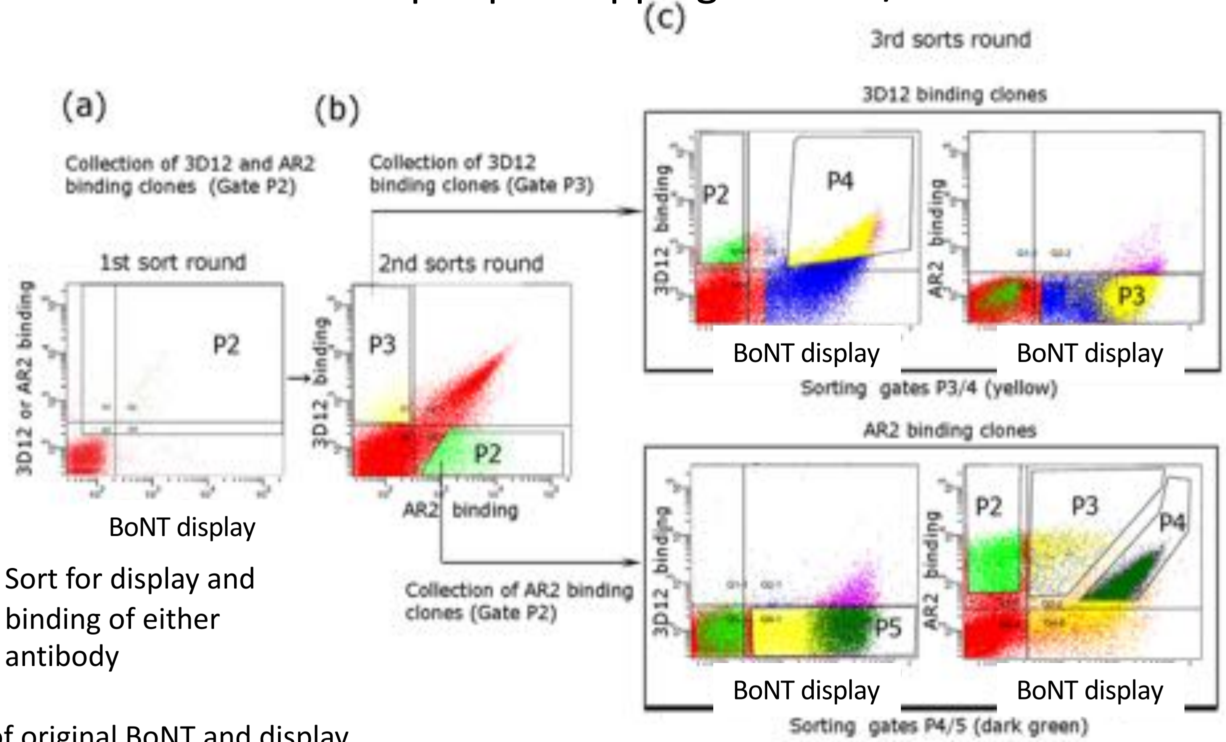
HC HN LC

BoNT/F mAb Cross reactivity



BoNT displayed, antibody binding tested

Sorts for fine epitope mapping of BoNT/A antibodies



Sort for display and binding of either antibody

Error PCR of original BoNT and display

Use 3D12 and AR2 to select for correctly folded BoNT

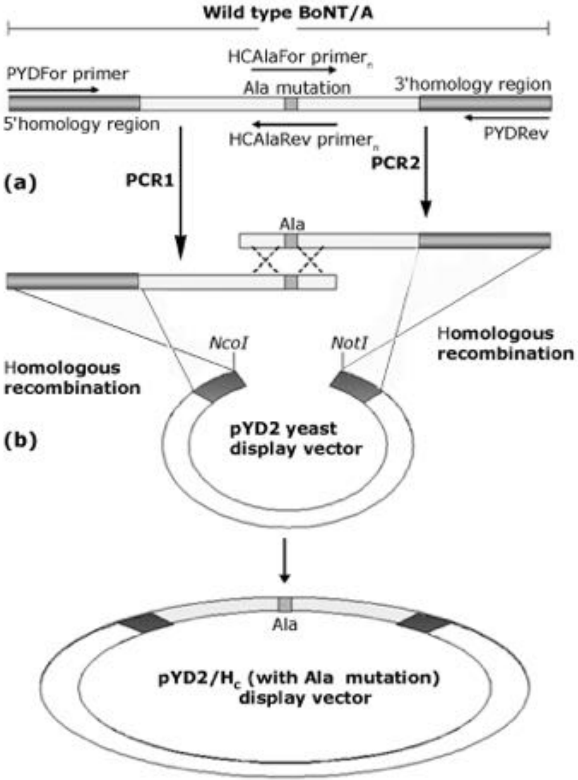
Sort for lack of binding for other antibody

Sequence single clones of desired phenotype

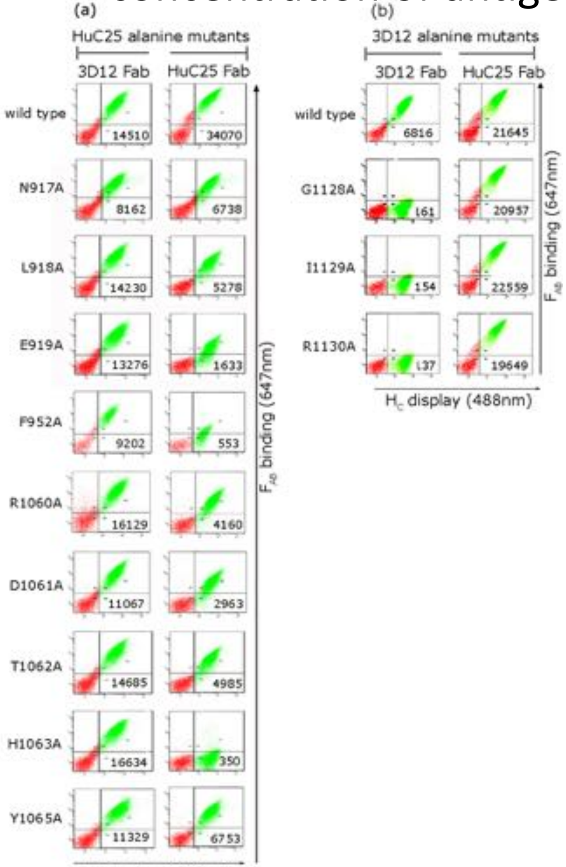
Model location of mutations to identify putative epitope

Levy et al, J. Mol. Biol., 365:196-210, 2007

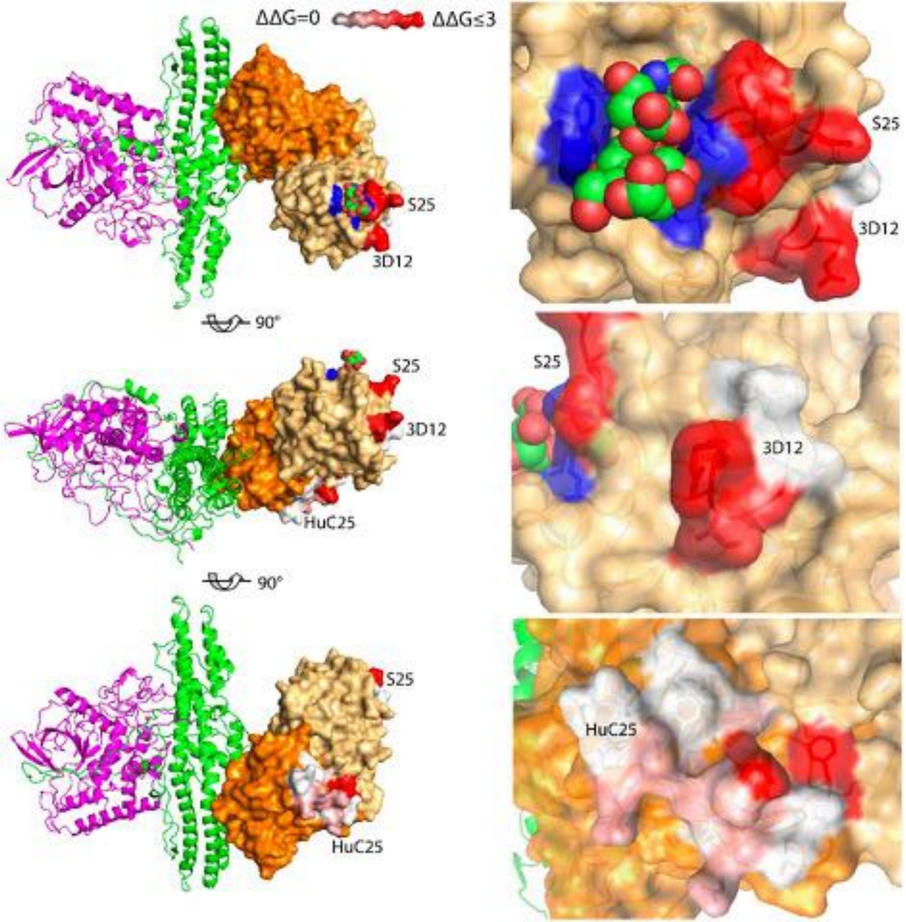
Generate single alanine mutants by homolgous recombination



Identify mutants that affect binding, measure MFI at KD concentration of antigen



BoNT/A domain display



mAb epitopes

S25

3D12/RAZ1

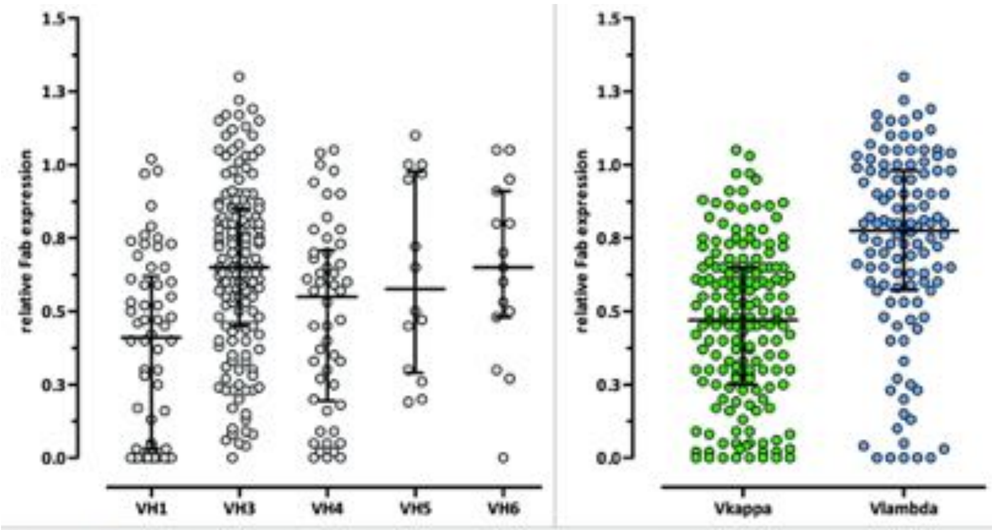
HuC25/CR2

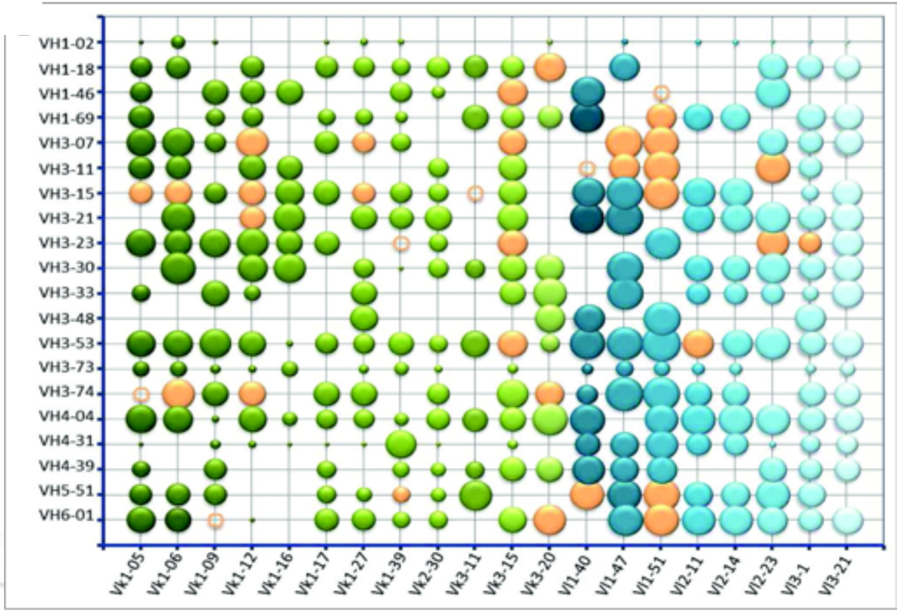
Levy et al, J. Mol. Biol., 365:196-210, 2007

Antibody characterization

- Expression
  - Need to be able to express mAb in desired format at scale
    - For IgG > 1gm/L typically from CHO
    - Transient HEK expression does not always reflect ability to achieve high titer CHO expresion

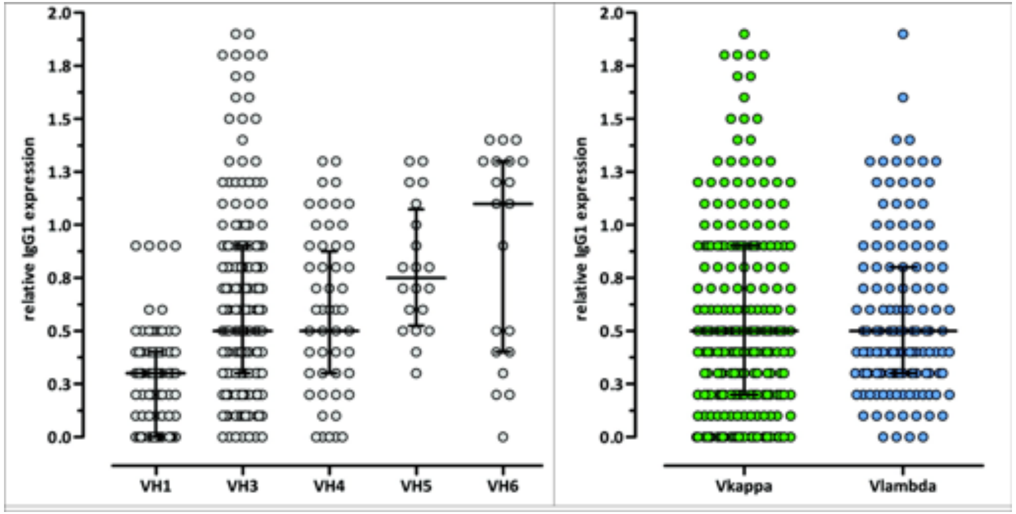
Relative Fab expression differs by V-gene

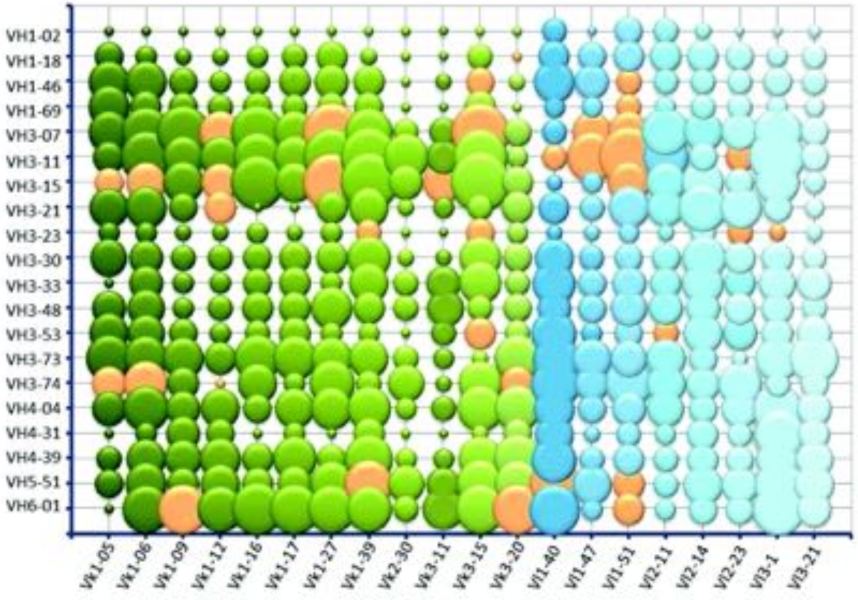




Tiller, T. *et al. MAbs* **5**, 445, (2013).

Relative IgG expression differs by V-gene

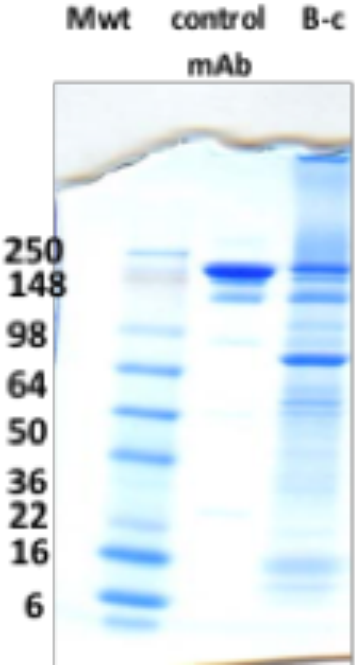




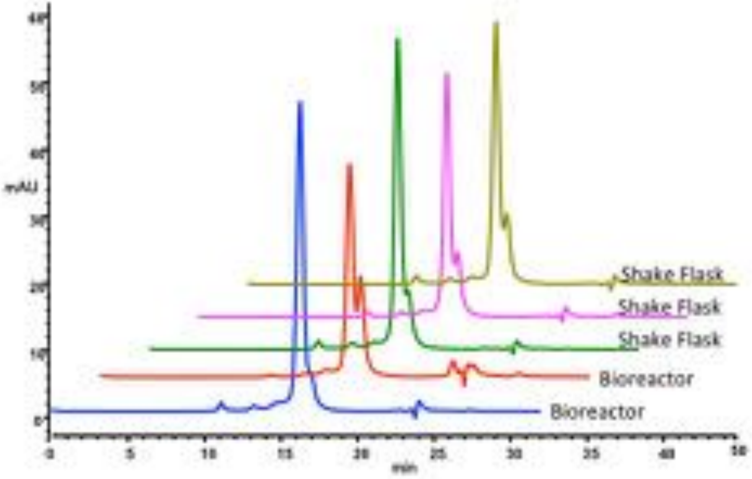
Tiller, T. *et al. MAbs* **5**, 445, (2013).

Improving antibody expression by engineering

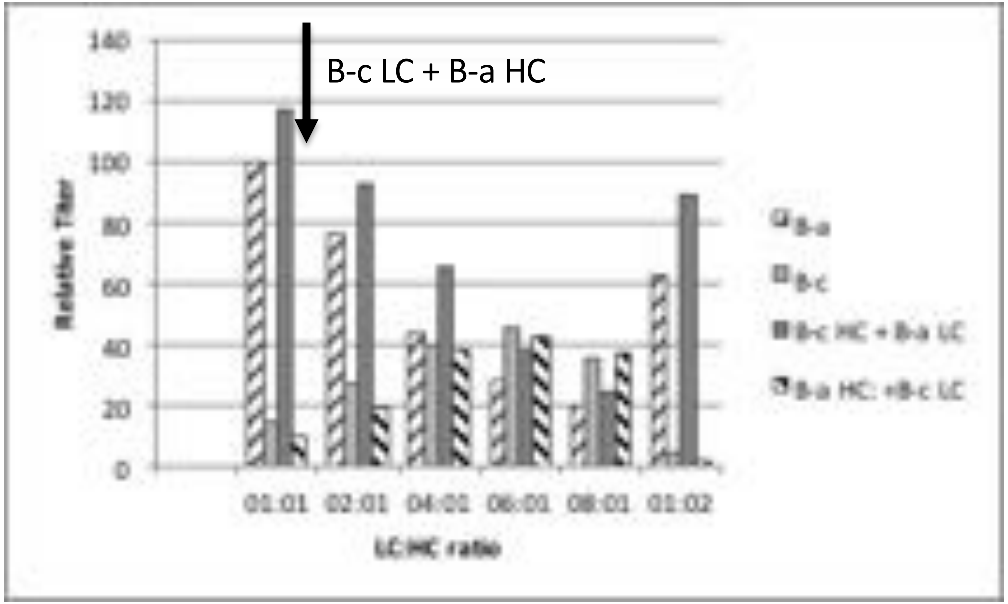
Engineering to improve CHO expression and IgG quality



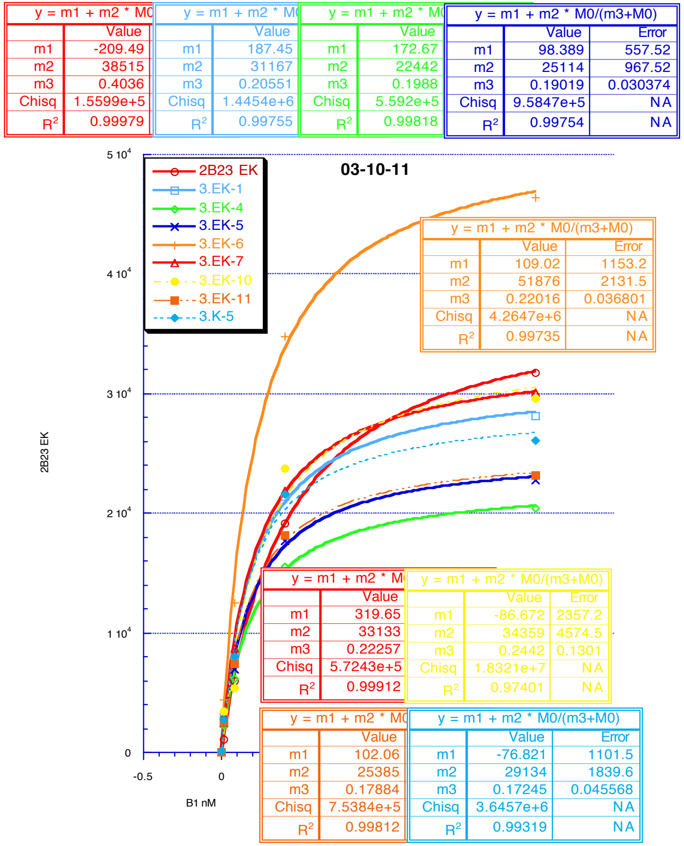
mAb XB23 = Mab B-c



Expression yield due to light chain



Evolution of 2B23 to improve IgG expression in CHO limited by light chain



KD on BoNT/B1 at concentrations = 2.0, 0.4, 0.08, 0.016 and 0.0 nM

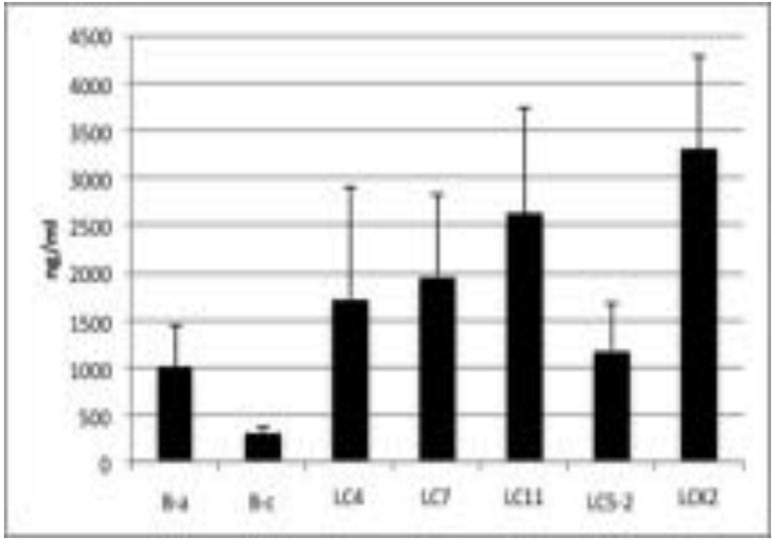
Clone	KD/B1 nM
2B23 EK	0.404
3.2B23EK-1	0.205
3.2B23EK-4	0.199
3.2B23EK-5	0.190
3.2B23EK-6	0.220
3.2B23EK-7	0.223
3.2B23EK-10	0.244
3.2B23EK-11	0.179
3.2B23K-5	0.172
2B23 wt	0.637

IgG affinities of yeast displayed 2B23 mutants

Clone	Yeast KD	Kinexa KD (pM)
Wild type	0.637	37.7
EK4	0.199	0.077
EK7	0.223	2.73
EK11	0.179	13.4
EK5-2	0.172	4.99
K2	0.180	1.89
K11	0.158	2.9

All six much better behaved and with better expression

All six mutants much better behaved and with better expression



Clone	Shake Flask Titer (µg/ml)	CE-SDS (%)		SEC-HPLC(%)		IEX-HPLC (%)		
		IgG	**HH[a]**	**Monomer/ Shoulder**	**HMW[b]**	**Acidic**	**Main**	**Basic**
EK4-43	450	94.31	1.29	96.28/0	3.62	18.7	59.9	21.4
EK4-78	373	94.90	0.89	98.66/0	1.34	22.2	58.4	19.5
EK4-54	331	93.84	1.80	96.36/0	3.51	39.2	47.3	13.5
mAb B-c	50-160	75.20	20.70	71.94/19.45	5.54	ND[c]	ND	ND

Antibody characterization

- Sequence
  - Undesirable sequence features
    - V-region glycosylation (coded NXS/T)
      - Germline encoded in some V-genes; both framework and CDRs
      - May be preferentially selected using yeast display
    - Cysteines
      - Can usually mutate to Ser without affecting binding
    - Solvent accessible methionine
      - Deamidation
    - Solvent accessible aspartate
      - Oxidation

Antibody characterization

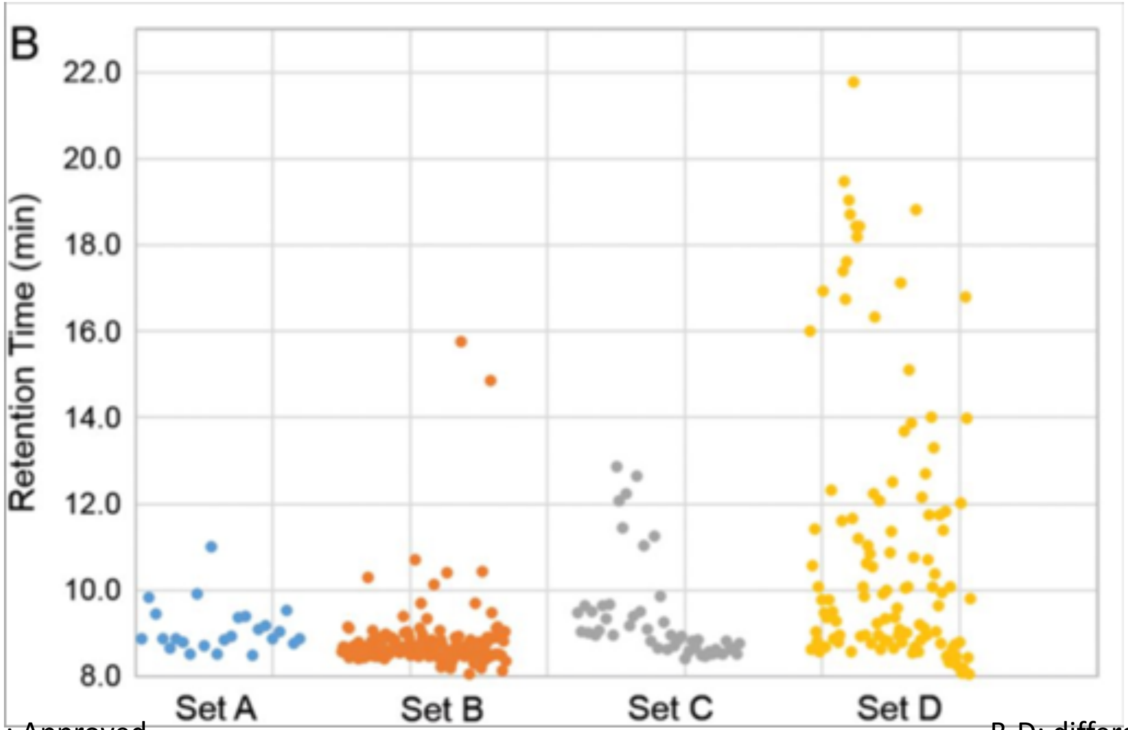
- Specificity
  - Measure binding on panel of relevant and irrelevant antigens
    - For therapeutic mAbs useful for tox. studies to have cross reactive antibody (murine and human)
  - Serum half-life in rodent can reflect cross reactivity (or antigen sink)

		Ag1	Ag2	Ag3	Ag4	Ag5	Ag6	Ag7	Ag8	
hyb 5	200	2,3	25,1	0,9	1,0	3,9	1,1	15,8	44,9	recAb stronger than hyb mAb, but both have strong polyreactivity
	20	0,5	18,5	0,8	0,8	1,1	0,8	1,1	1,4	
recAb 5A	5 to 50	0,6	8,5	2,3	3,3	55,1	9,8	71,9	274,8	
recAb 5B	5 to 50	0,3	0,9	1,3	1,6	1,4	0,6	0,7	3,2	

Polyspecificity

- Can be classified into two types
  - Self specificity: Aggregation, which can be quantified by
    - Cross interaction chromatography (CIC)
      - Polyclonal or monoclonal antibody column
      - Run mAbs under study over column and measure retention times
      - Found to correlate with solubility
    - AC-SINS
      - Gold particles with capture reagent (anti-FC e.g.)
      - Measure plasmon change as particles closer if self aggregation
  - Polyspecificity for other antigens
    - Can be quantitated using ‘polyspecificity reagents
      - Baculovirus lysates
      - Biotinylated CHO or mammalian membrane lysates

CIC of approved drugs and library mAbs

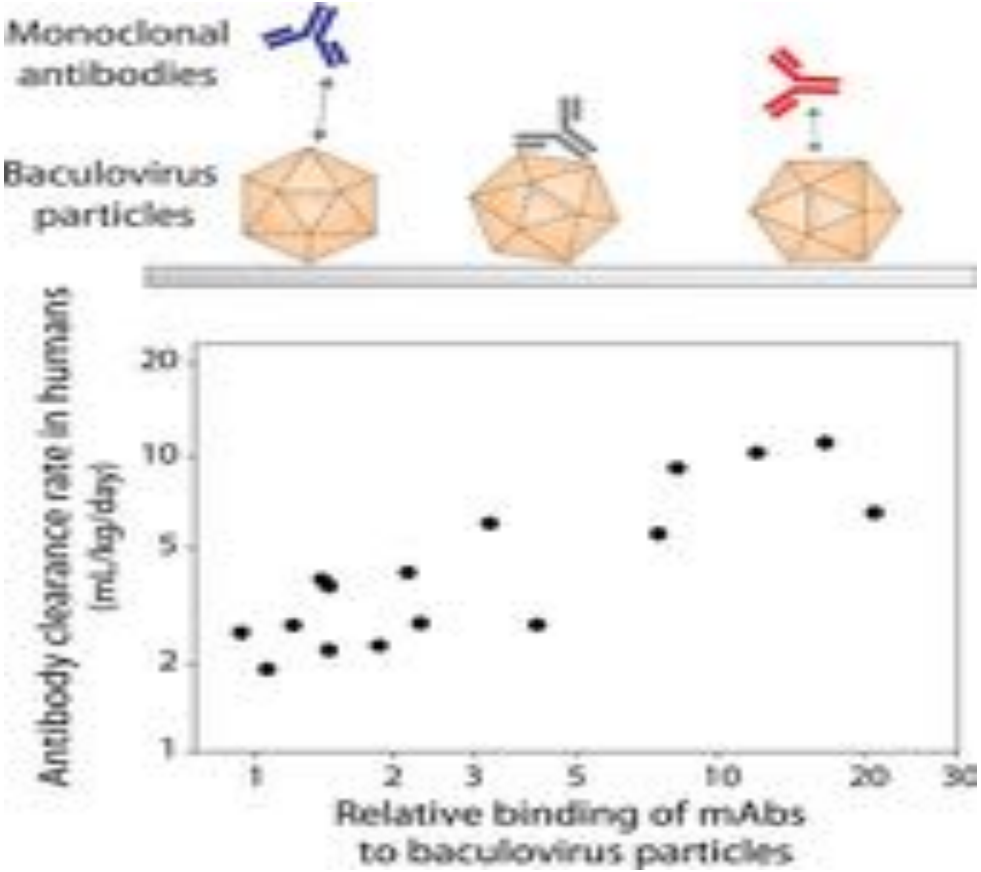


A: Approved therapeutics

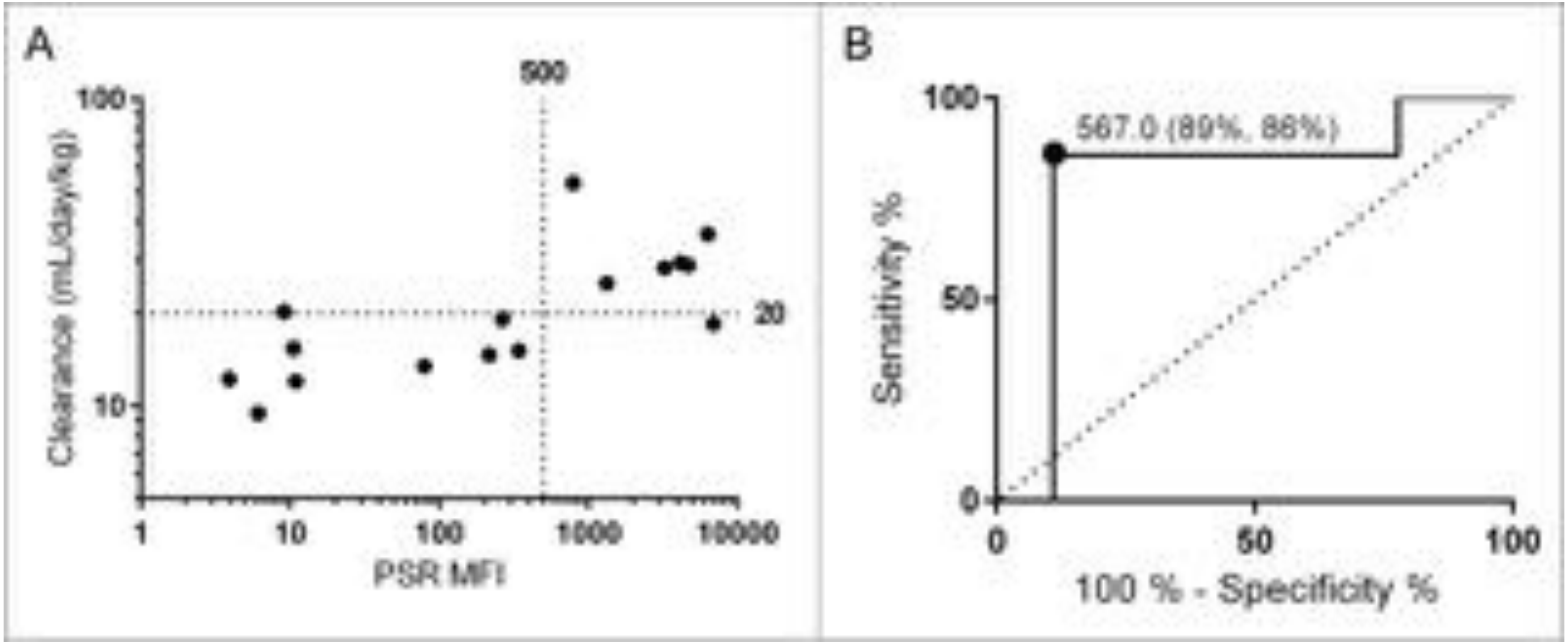
B-D: different Adimab campaigns

Yingda Xu et al. Protein Engineering, Design and Selection 2013;26:663-670

Polyspecificity correlates with reduced serum half life

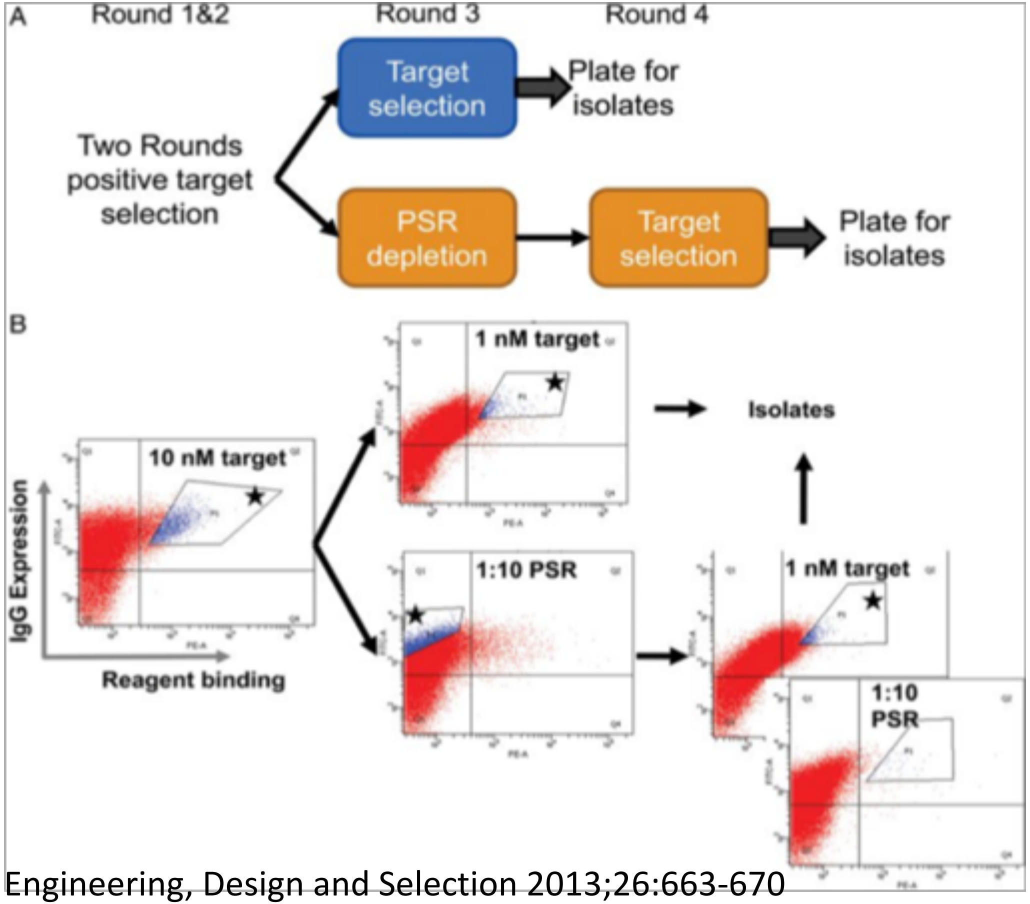


Polyspecificity correlates with reduced serum half life



Yingda Xu et al. Protein Engineering, Design and Selection 2013;26:663-670

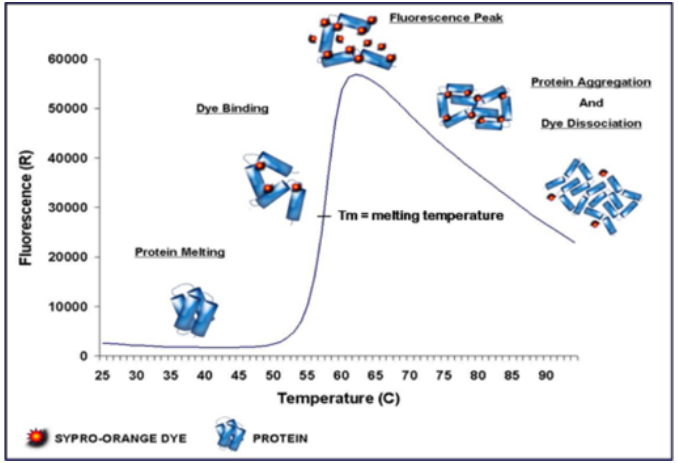
Selecting to remove polyspecificity



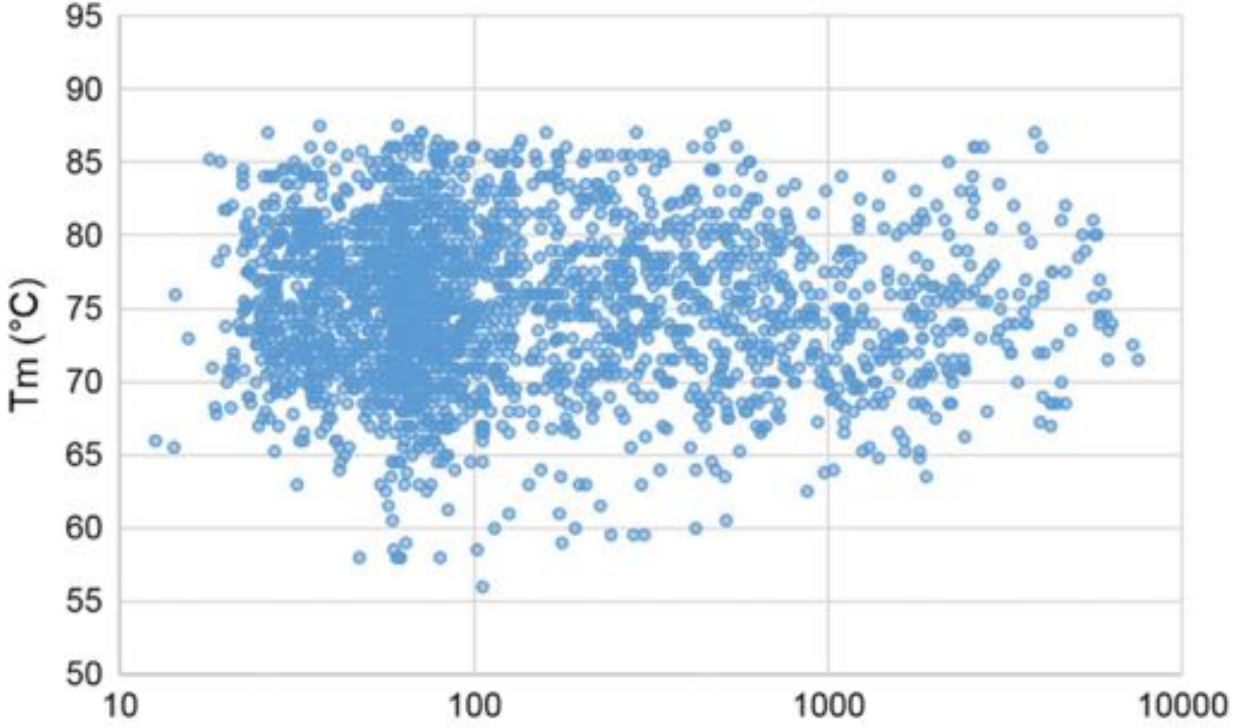
Yingda Xu et al. Protein Engineering, Design and Selection 2013;26:663-670

Antibody characterization

- Stability
  - Reflected in Tm of the variable region (usually Fab)
  - Varies significantly as a function of sequence
  - Can be measured using:
    - DSC
    - SYPRO Orange



Some assays have no cross correlation



Polyspecificity reagent

Yingda Xu et al. Protein Engineering, Design and Selection 2013;26:663-670

Assessment of 137 clinical antibodies

- 48 from approved antibodies
- 42 phase 2/3 or 3
- 47 phase 2
  - 124 Kappa, 13 lambda
  - 58 fully human
  - 67 humanized
  - 12 at least one non-human V region
- All recloned with IgG1 Fc
- Expressed in HEK

Jain, T. *et al PNAS* **114**, 944 (2017)

Tests performed

- Antibody self-interaction
  - AC-SINS: affinity-capture self-interaction nanoparticle spectroscopy
  - CSI-BLI: clone self-interaction by bio-layer interferometry
- Cross interactions
  - PSR: poly-specificity reagent binding (cell membrane)
  - BVP: baculovirus particle
  - CIC: cross-interaction chromatography
  - ELISA with a panel of commonly used antigens
- HEKt: Expression titer in HEK cells
- Tm: Melting temperature of Fab
- HIC: Hydrophobic interaction chromatography
- SGAC-100: (salt-gradient affinity-capture self-interaction nano- particle spectroscopy)
- SMAC: Standup monolayer adsorption chromatography
- Monomeric species assessment
  - AS: Size-exclusion chromatography in the context of accelerated stability

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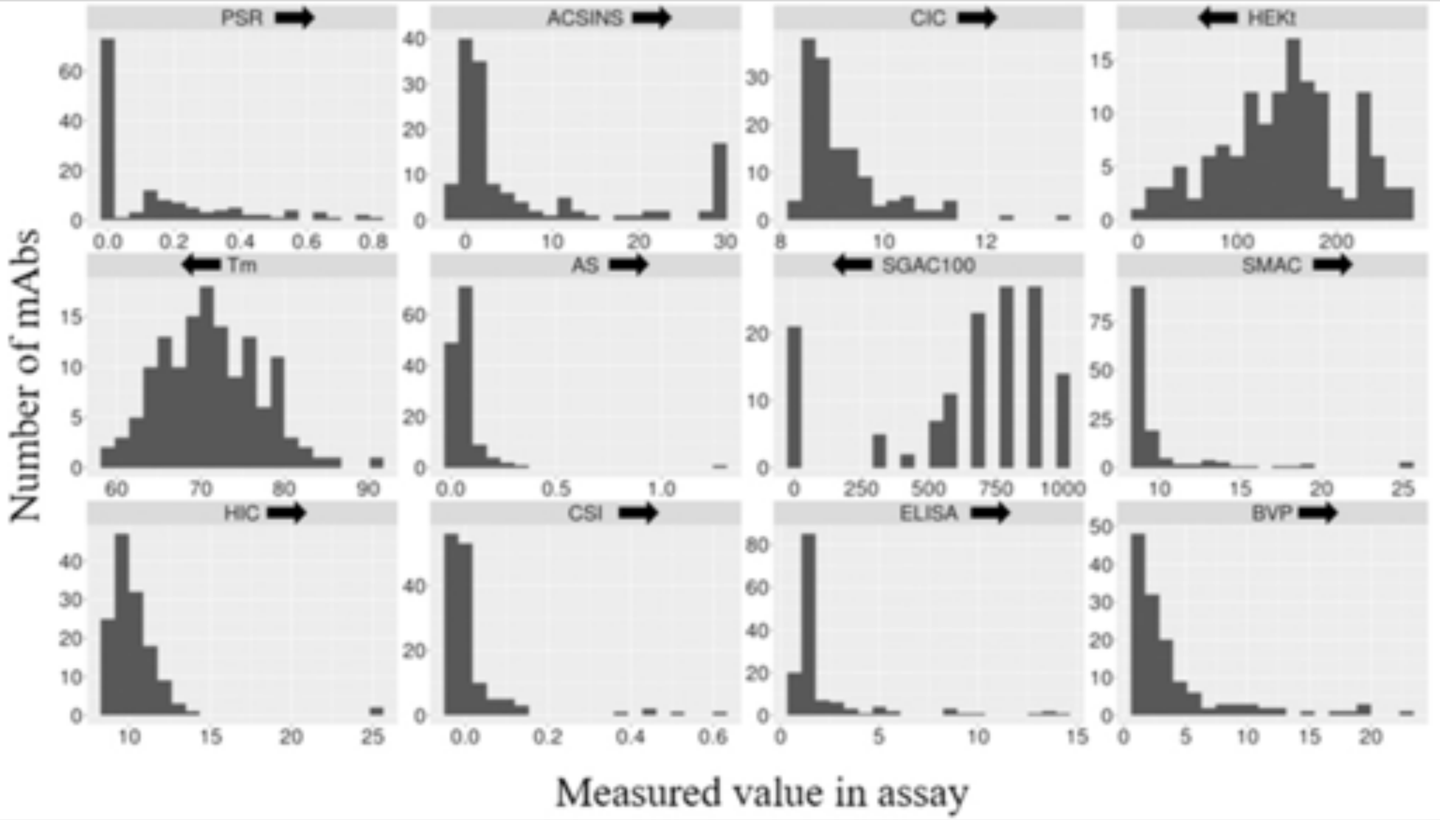
Long tails



Arrows indicates direction of worse developability

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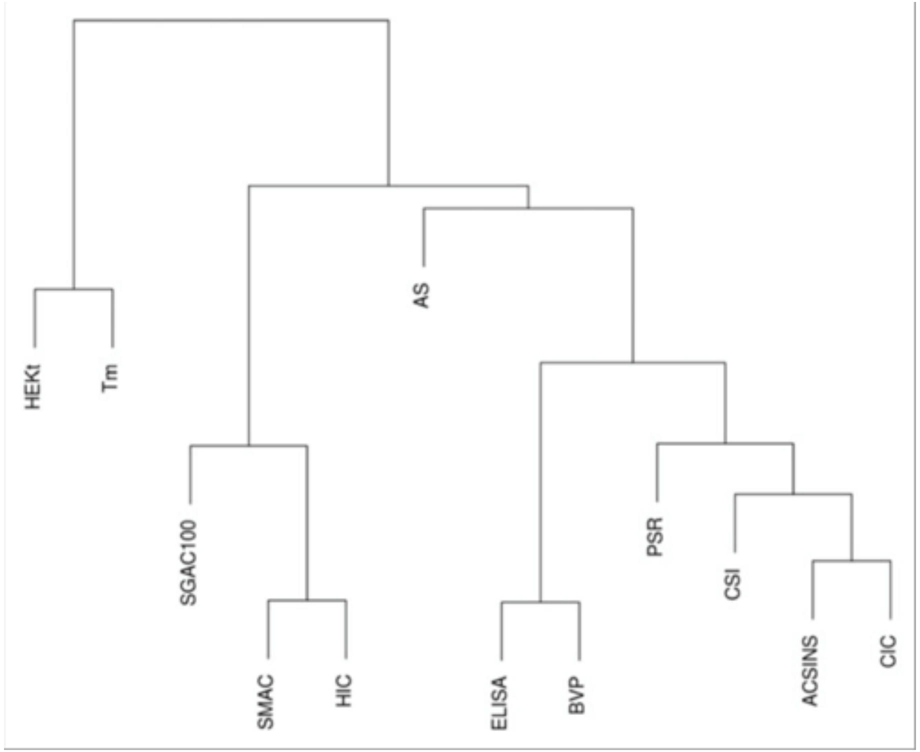
Hierarchical clustering of biophysical properties



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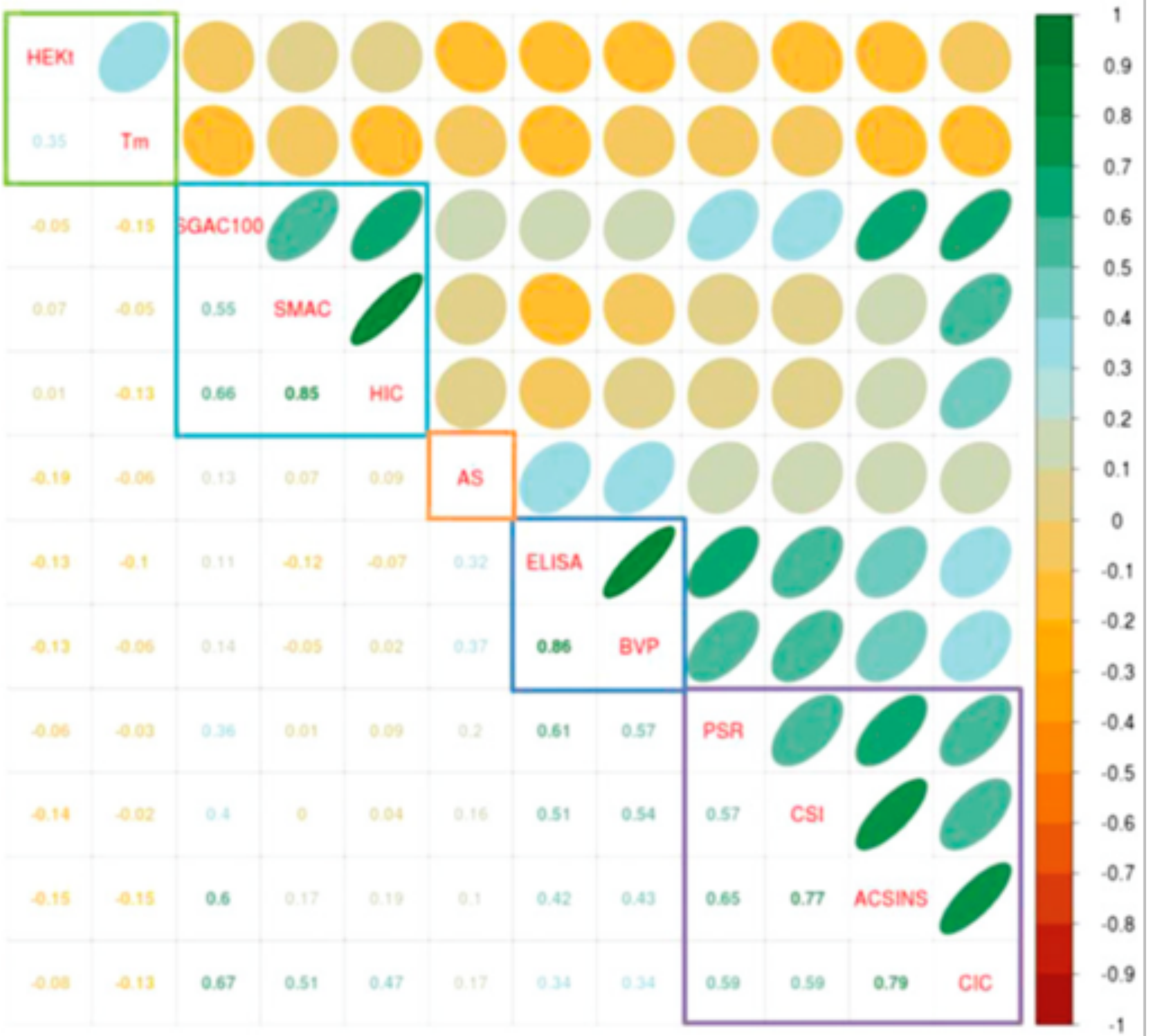

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Concept of red flags

Group	Assay	Worst 10% threshold	Units (flag)
Group 1	PSR	0.27 ± 0.06	None (>)
	ACSINS	11.8 ± 6.2	Nanometers (wavelength change) (>)
	CSI	0.01 ± 0.02	BLI response units (>)
	CIC	10.1 ± 0.5	Retention time (min) (>)
Group 2	HIC	11.7 ± 0.6	Retention time (min) (>)
	SMAC	12.8 ± 1.2	Retention time (min) (>)
	SGAC-SINS	370 ± 133	Salt concentration (mM) (<)
Group 3	BVP	4.3 ± 2.2	Fold-over-background (>)
	ELISA	1.9 ± 1.0	Fold-over-background (>)
Group 4	AS	0.08 ± 0.03	Monomer percentage loss per day (>)

- 48 approved antibodies
  - For each measure a “red flag” corresponds to a value in the worst 10%
- Document the red flags for antibodies at different stages of development

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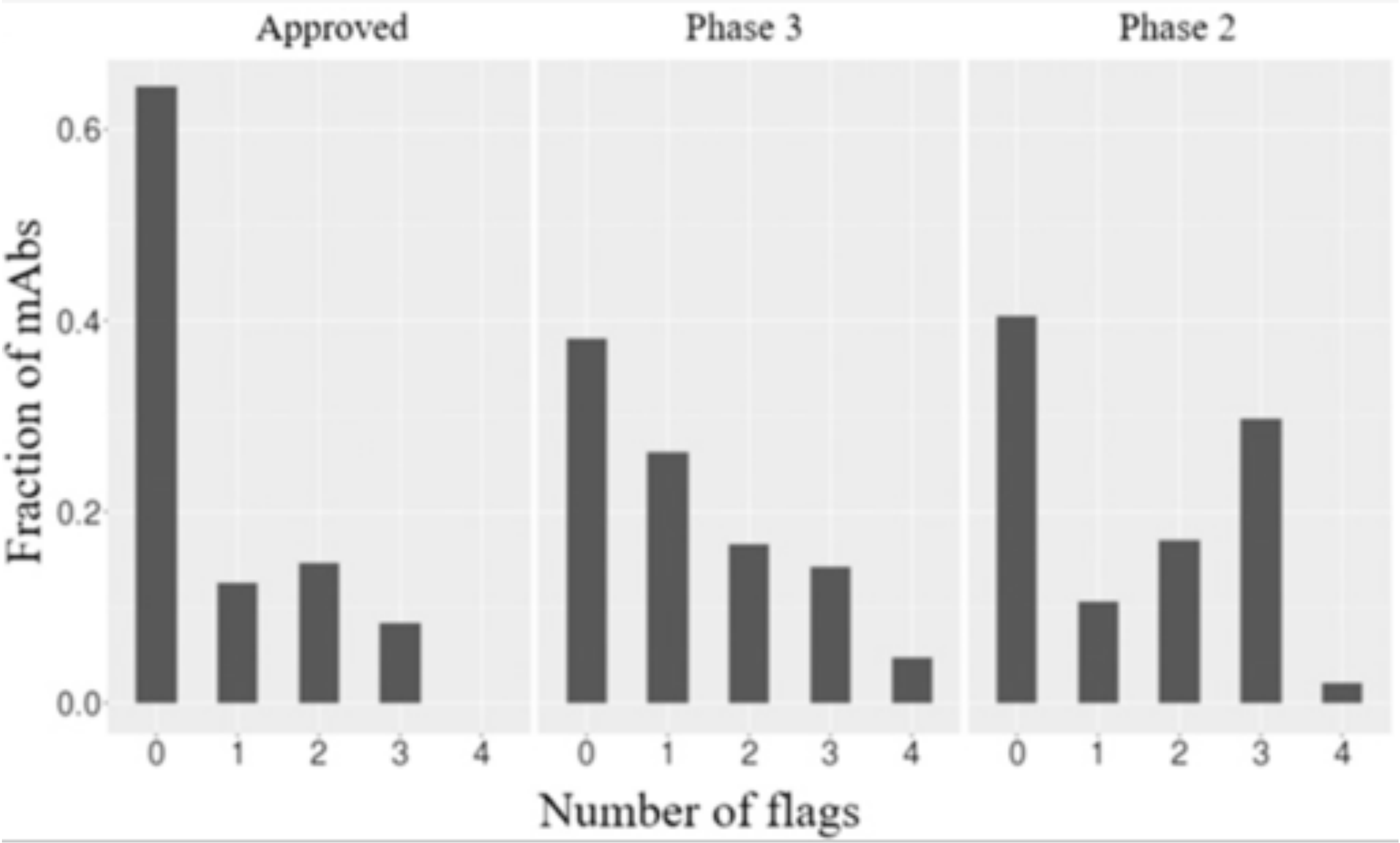
Red flags through development



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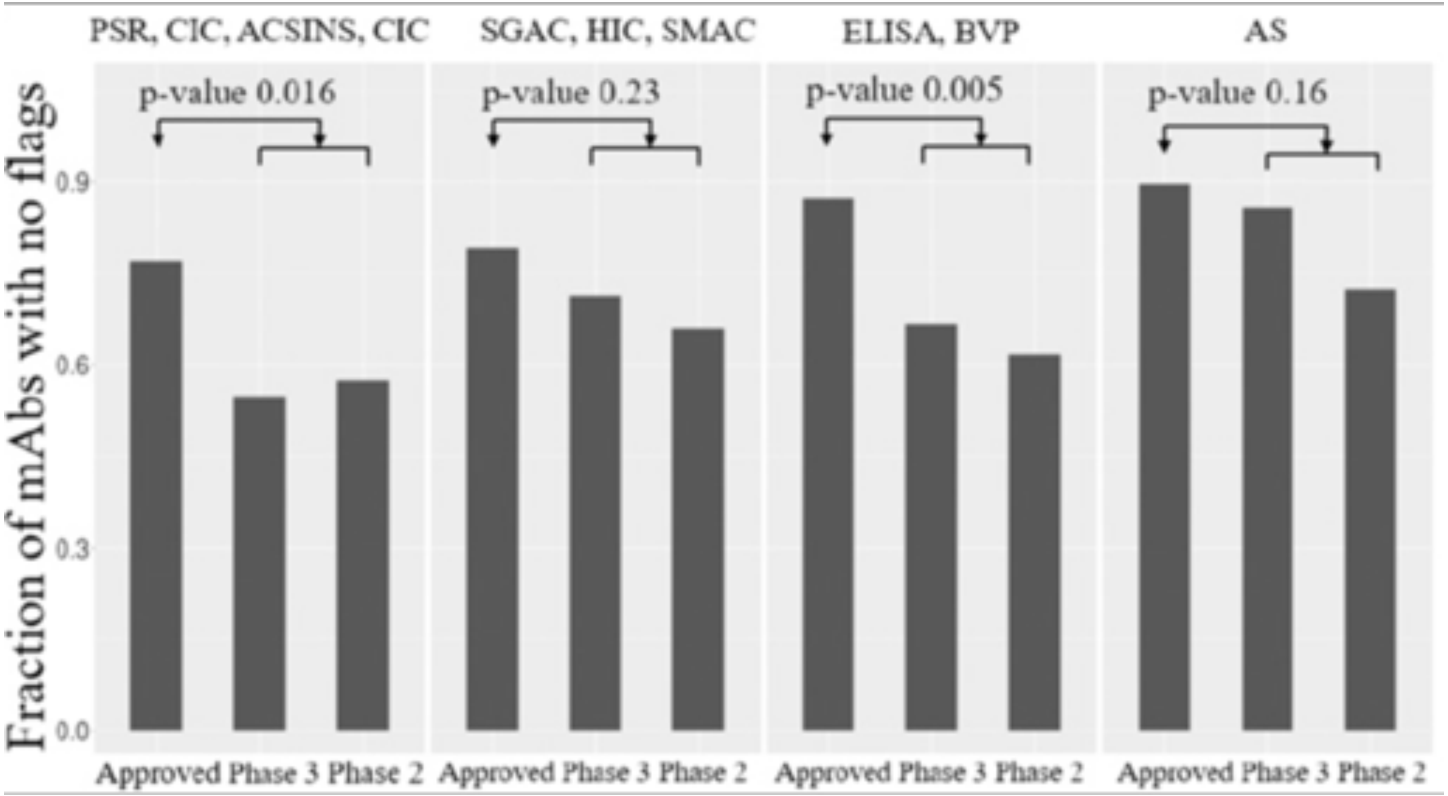
Red flags by assay



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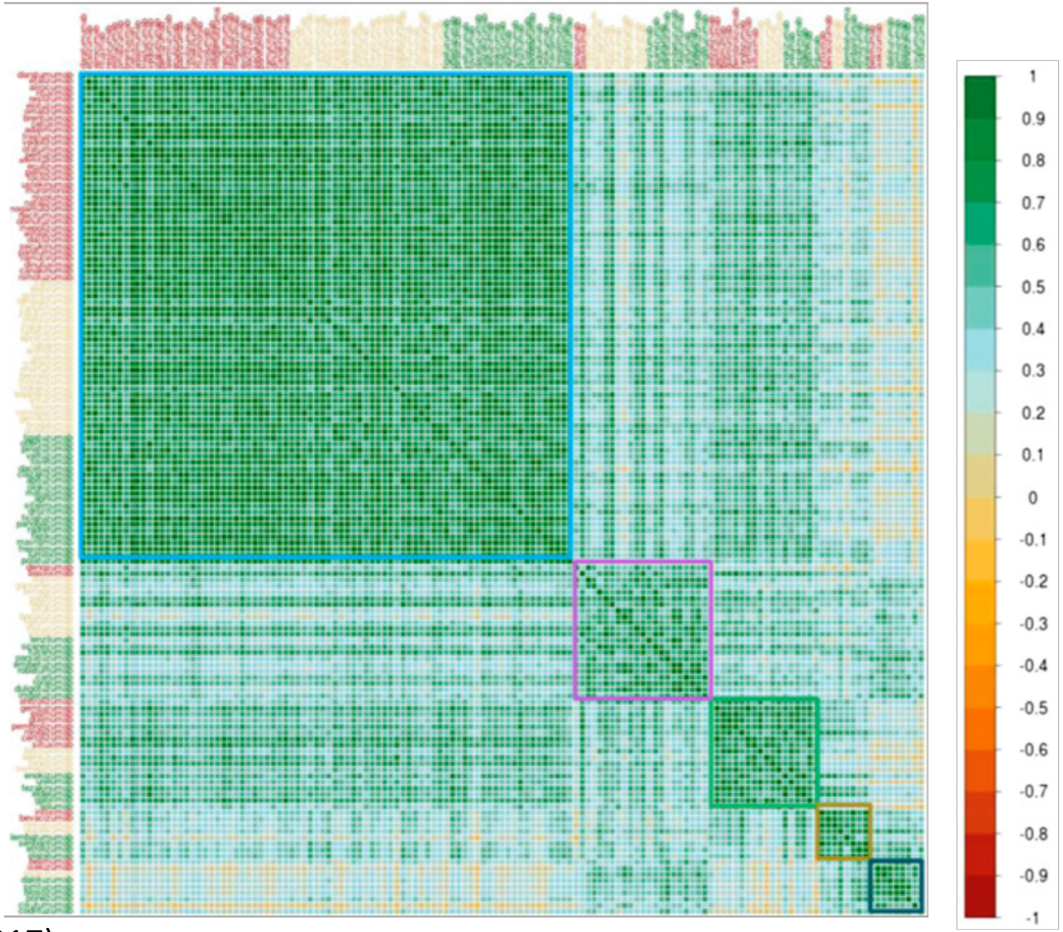
Cross clustering of all 137 antibodies

Approved

Phase 3

Phase 2



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