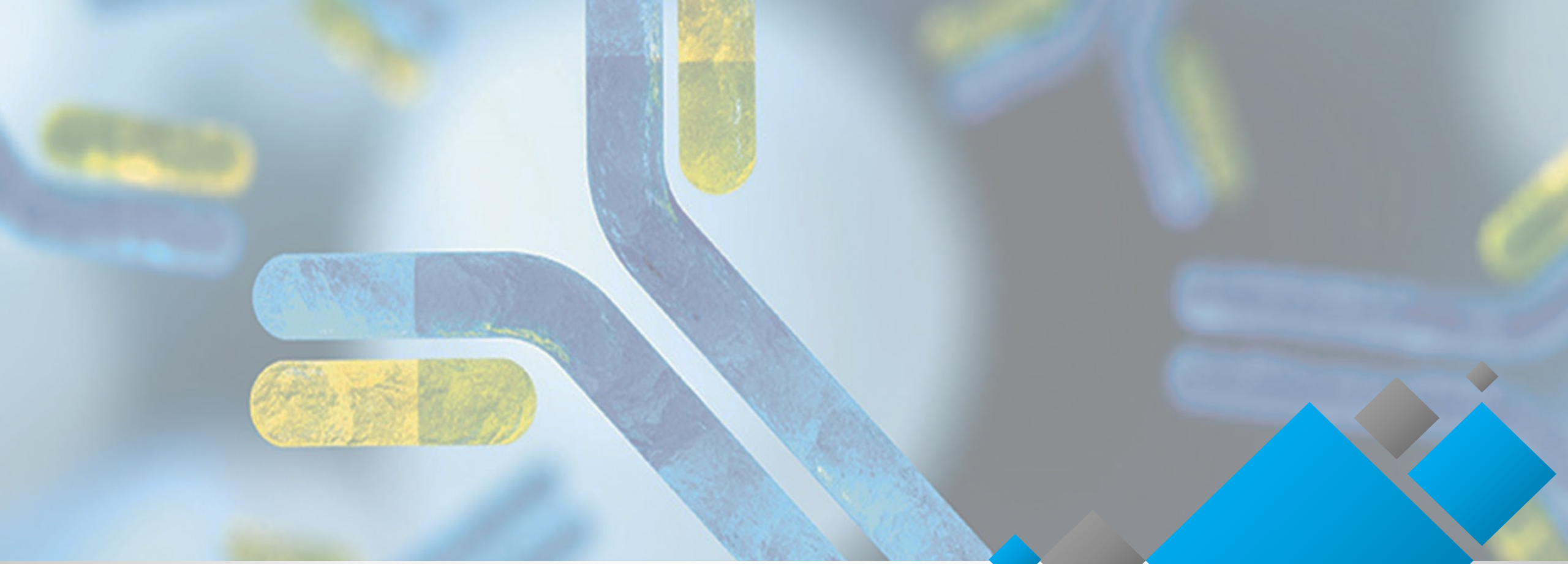
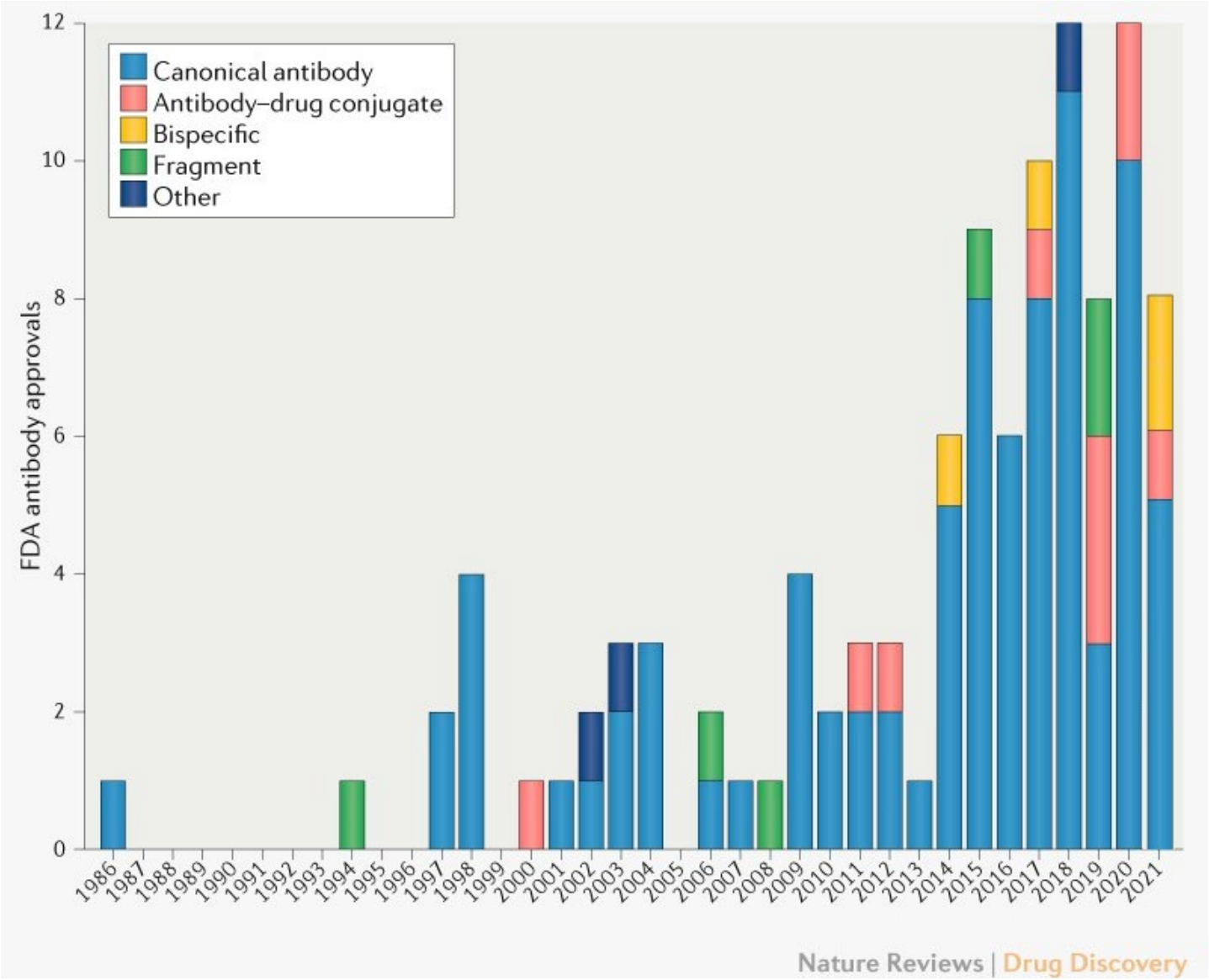


Innovative Techs & Platforms Applied in Antibody Discovery

Dr. Long Xu

百英生物
BIOINTRON

We’ve Entered the Era of “100+ Antibody Drugs”



Adapted from Nature Reviews Drug Discovery 20, 10 (2021)

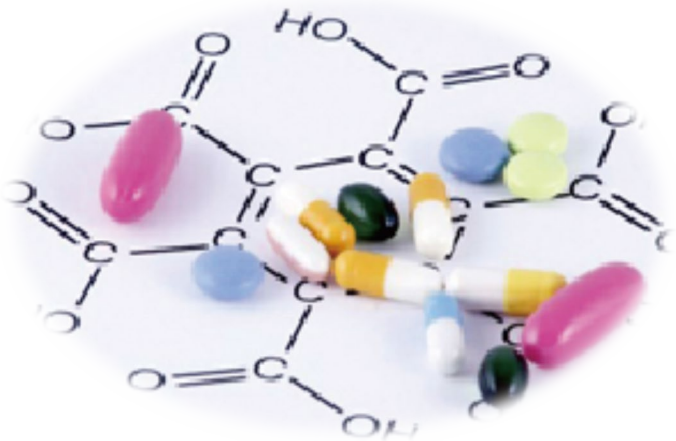
7 of Top 10 Best-Selling Drugs in H1 2023 are Antibodies

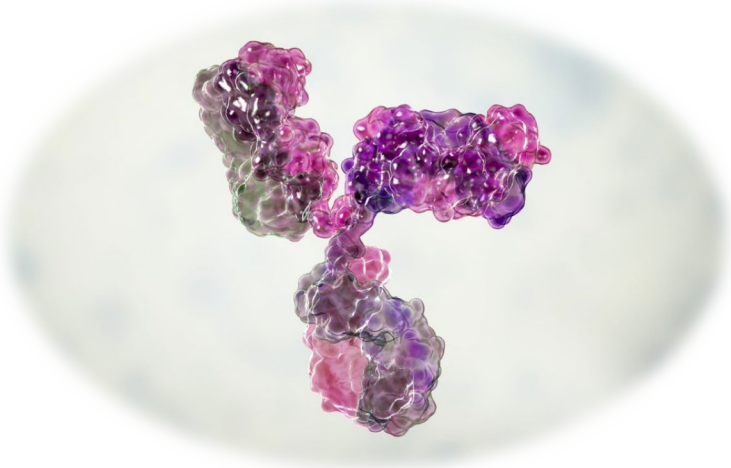
百英生物 BIOINTRON

	Drug	Target	Company	Sales (in billion U.S. dollars)			
				2020	2021	2022	2023
1	Keytruda	PD-1	Merck	14.38	17.19	20.94	12.07
2	Humira	TNFa	AbbVie/Eisai	19.83	20.70	21.24	7.55
3	Eliquis	FXa	BMS/Pfizer	9.17	10.76	11.79	6.63
4	Ozempic	GLP-1R	Novo Nordisk	3.51	5.86	8.47	6.18
5	Biktarvy		Gilead	7.26	8.62	10.39	5.66
6	Dupixent	IL-4R	Sanofi/Regeneron	4.05	6.20	8.68	5.35
7	Stelara	IL-12/IL-23	Janssen/Mitsubishi Tanabe	7.95	9.55	9.72	5.24
8	Opdivo	PD-1	BMS/Ono	7.92	8.48	9.36	4.88
9	Jardiance	SGLT2	ELI LILLY/BI	385.40	5.90	7.30	4.80
10	Darzalex	CD38	JANSSEN	4.19	6.02	7.98	4.70

Data from mp.weixin.qq.com/s/4ZLWCJJulGa0Ejv0HNu9yg

Small Molecule Drugs vs Biologics





	Small Molecule Drugs	Biologics
Size	Small	Large
Structure	Defined structure & physicochemical properties	Structure & physicochemical properties less well defined
Stability	Stable; not heat-labile	Not stable; heat– and shear-labile
Affinity	Low	Better
Specificity	Low	Better specificity for target binding
Selectivity	Low tissue selectivity	Better tissue selectivity

Bottlenecks in Antibody Drug Discovery

百英生物 BIOINTRON

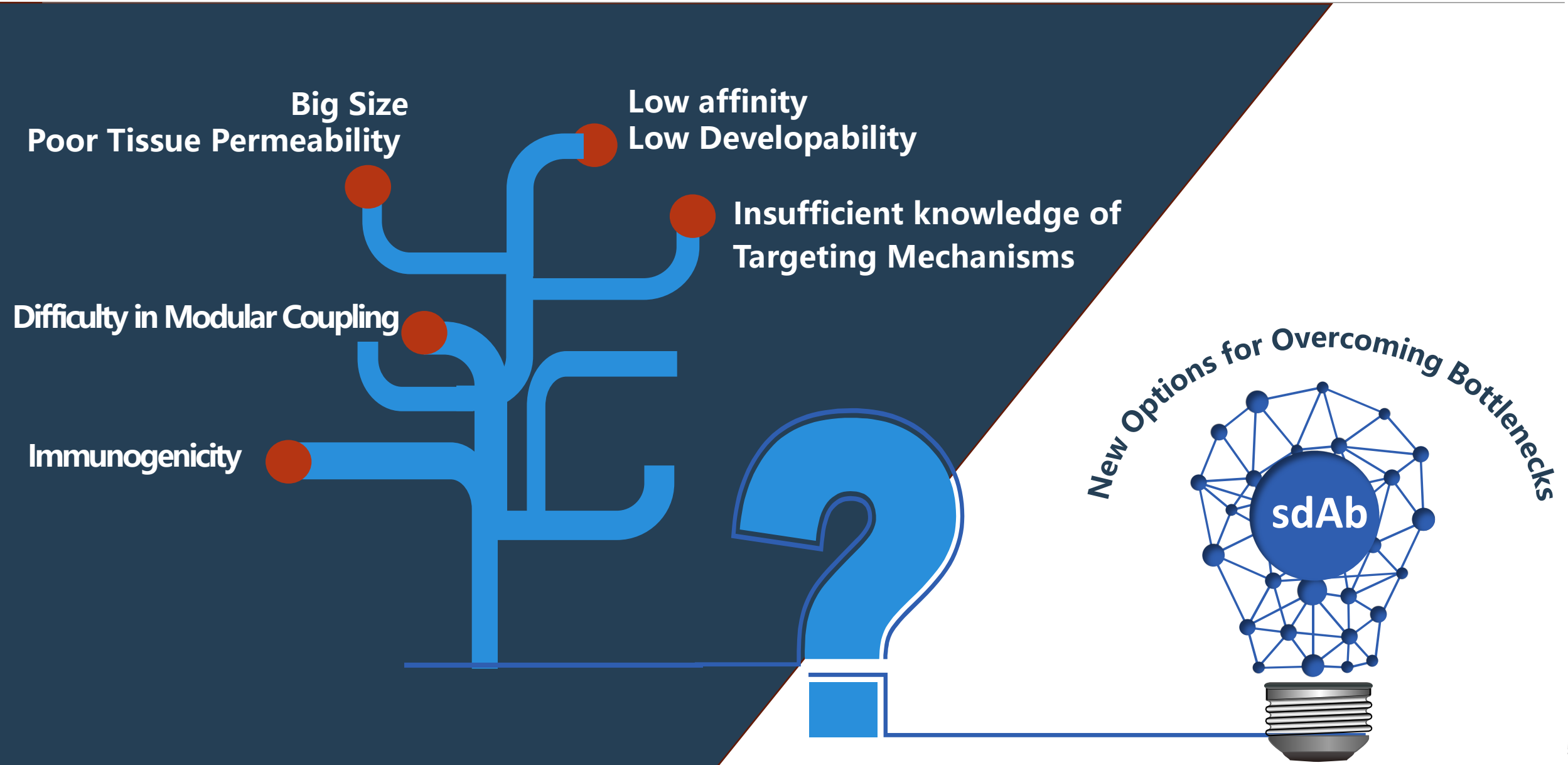
Big Size
Poor Tissue Permeability

Low affinity
Low Developability

Insufficient knowledge of
Targeting Mechanisms

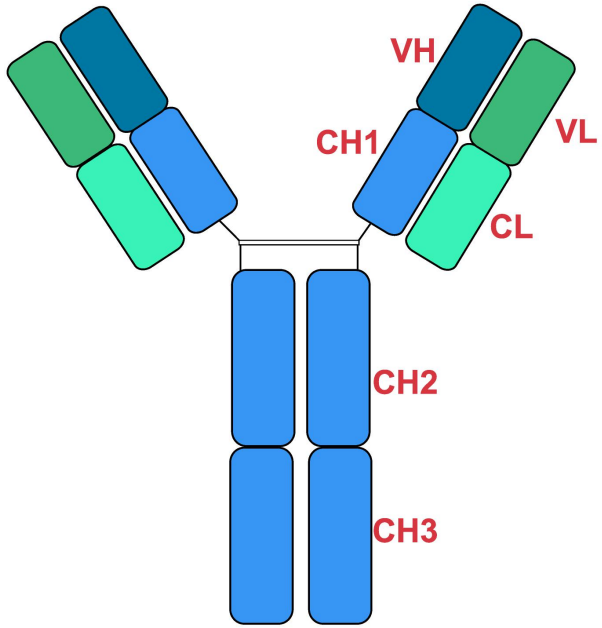
Difficulty in Modular Coupling

Immunogenicity

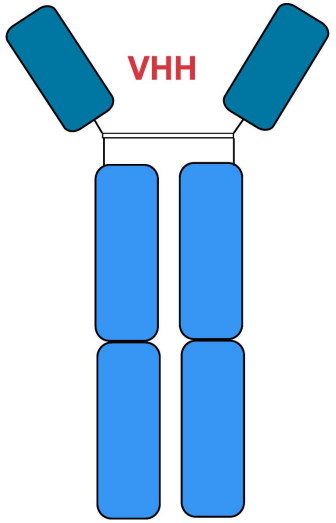


New Options for Overcoming Bottlenecks

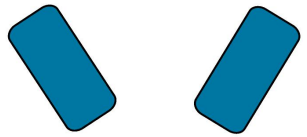
sdAbs Offer New Options for Antibody Drug Discovery



Conventional antibody
150 kDa



Heavy chain antibody
90 kDa



VHH/nanobody
15 kDa

VHH (variable domain of heavy chain of heavy-chain antibody)

Favorable Biophysical-chemical Properties of VHH

01 Small size, better permeability

02 High thermal stability

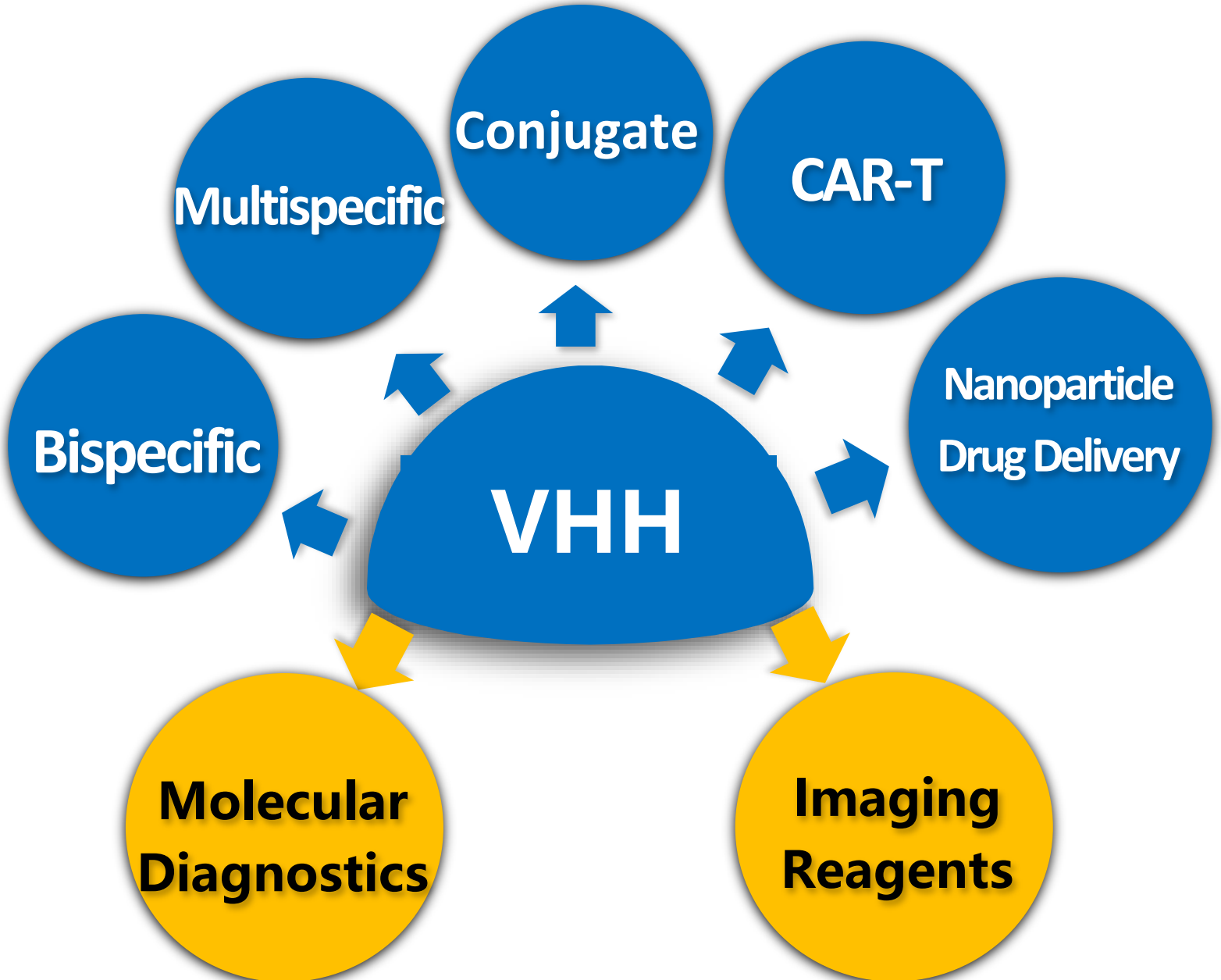
03 Modularity for multispecific applic.

04 Good solubility

05 High homology to hVH

06 Flexible production system

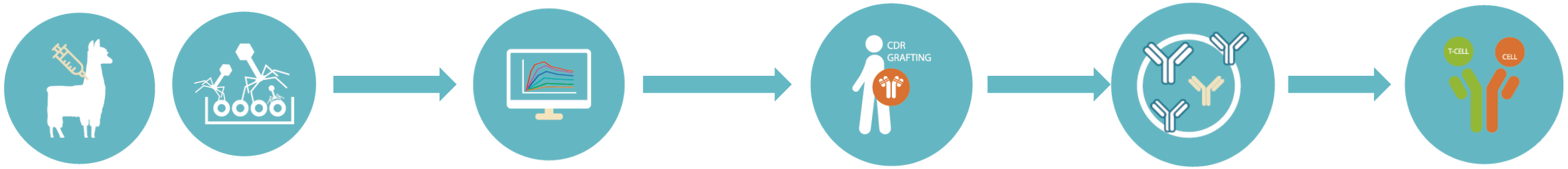
Wide Range of Applications of VHH



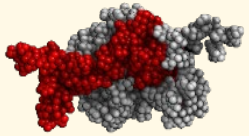
VHH Antibody Discovery

百英生物 BIOINTRON

Hit Generation & Screening → Hit Characterization → Lead Humanization → Lead Affinity Maturation → Applications

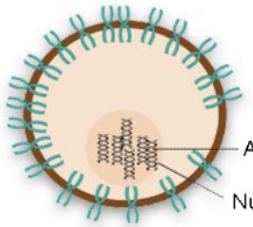


Immunization strategy



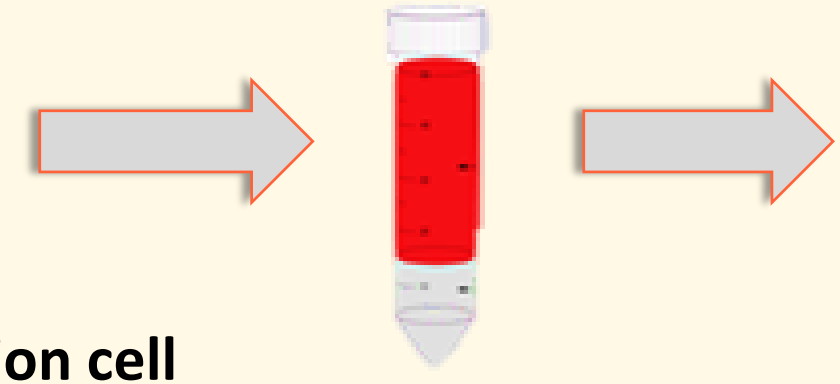
Protein

DNA



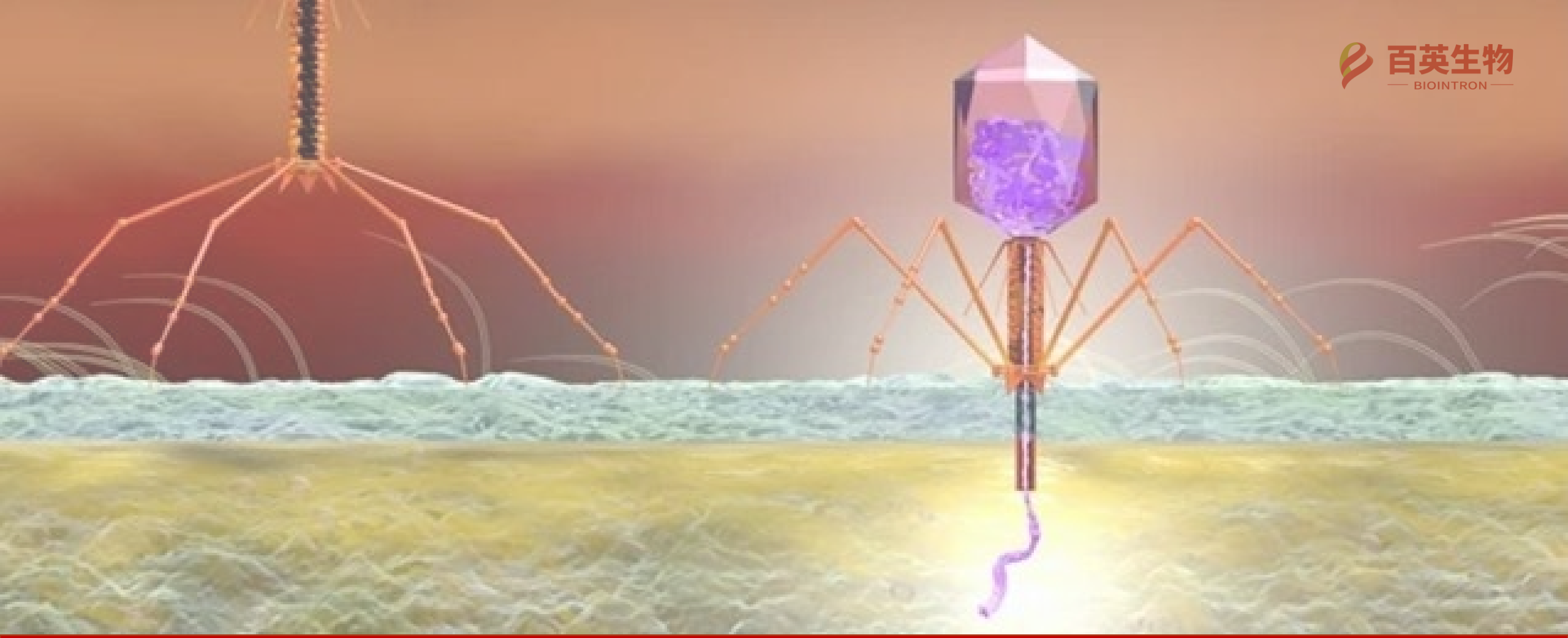
Over-expression cell

PBMC isolation



Phage Display
1.5 month/high-throughput

NGS Sequencing & Data Mining

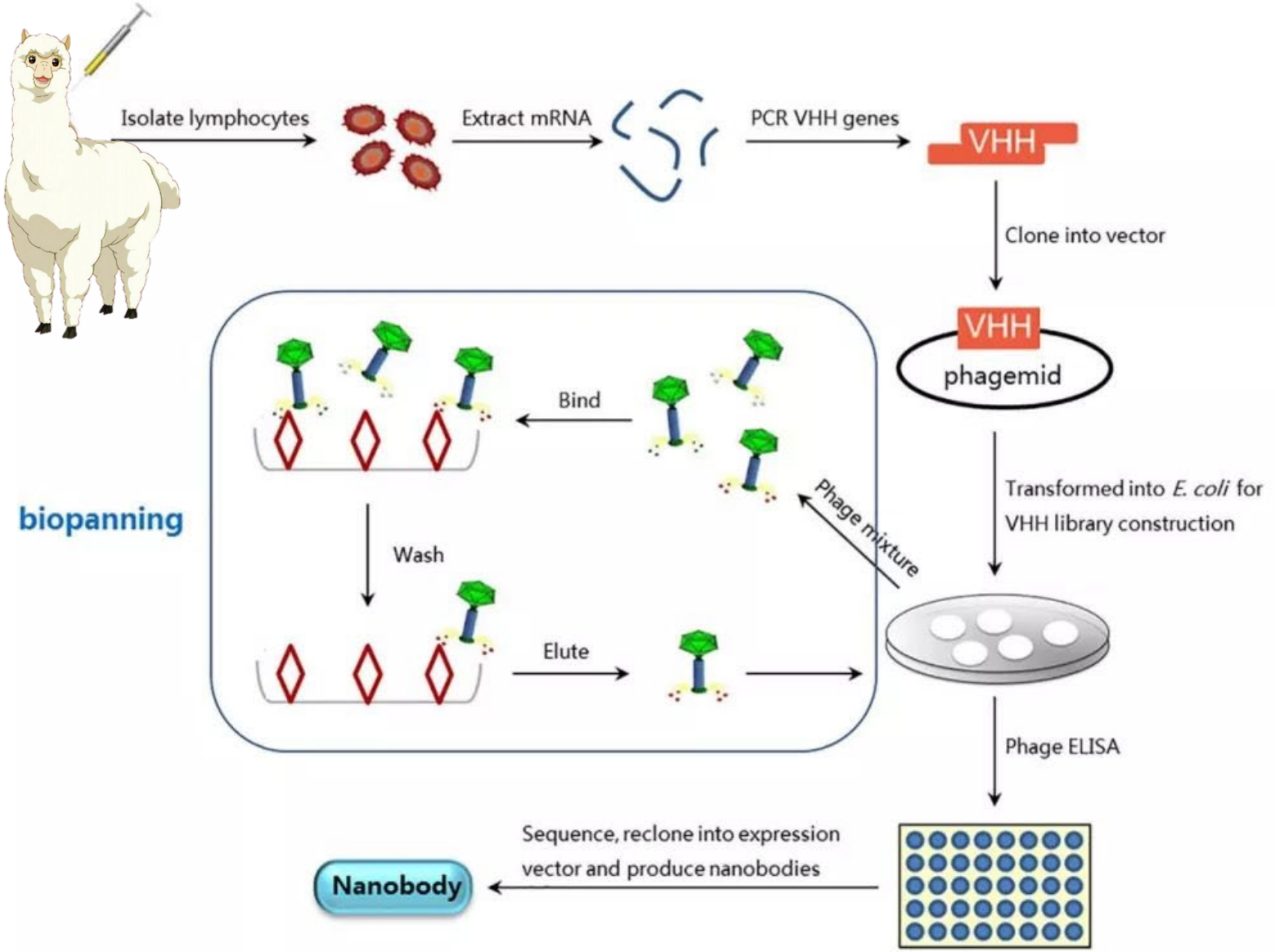


百英生物
BIOINTRON

Phage Display

VHH Discovery Platform

Phage Display as a Reliable Method for VHH Discovery

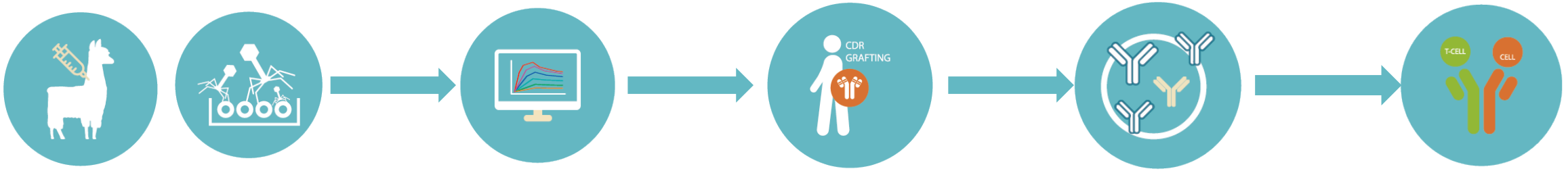


Adapted from Microbial Biotechnology, 10, 1468–1484

Anti-X Nanobody Discovery Case

Target X, a cell surface receptor expressed on T cells, is a co-stimulator for T cell activation.

Hit Generation & Screening → Hit Characterization → Lead Humanization → Lead Affinity Maturation → Construction of Bispecific Antibody

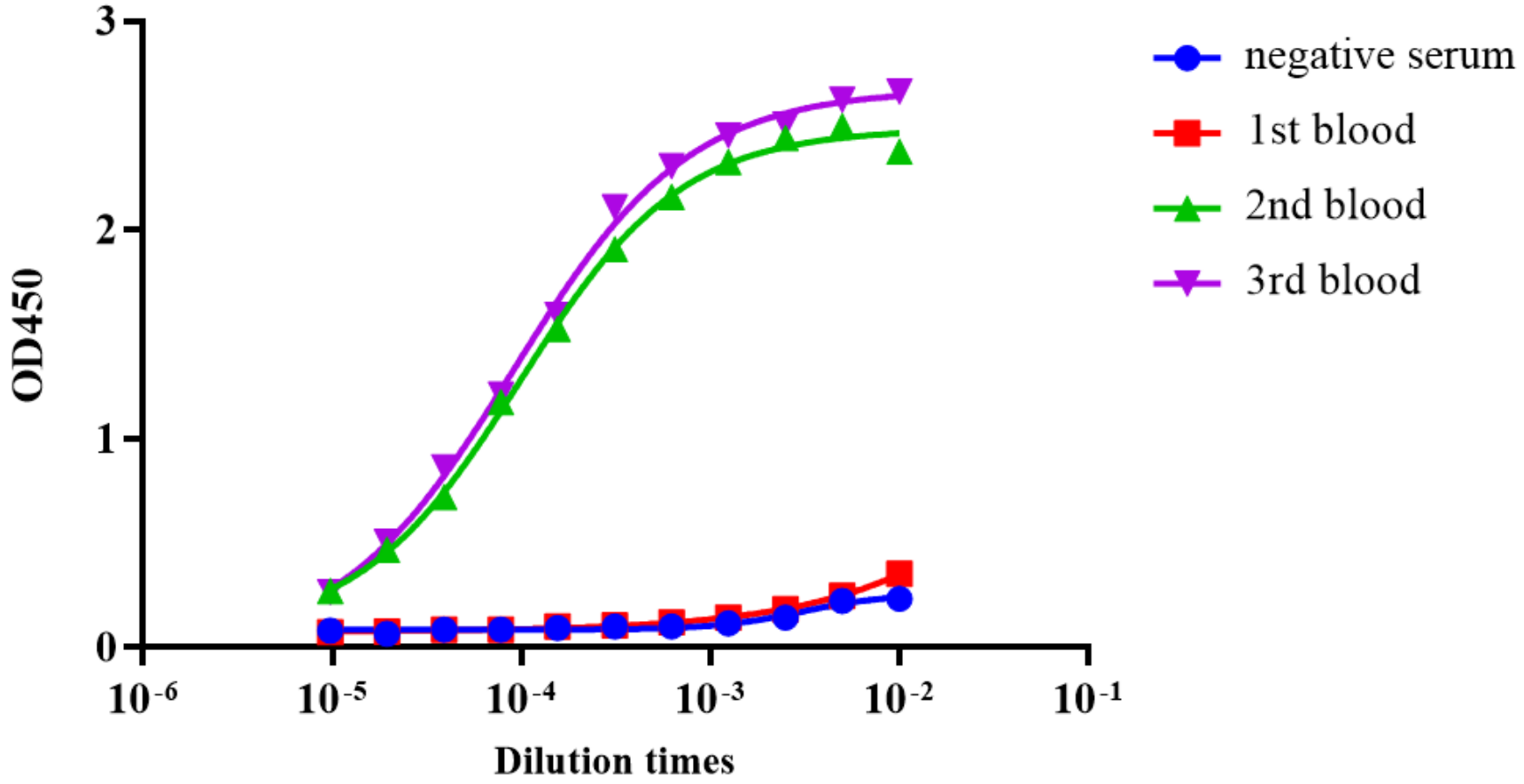


From the alpaca immune antibody library, **phage display** tech was used to screen the nanobodies targeting **X**.

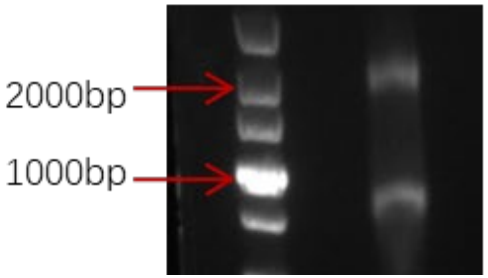
The selected hits were functionally characterized & validated.

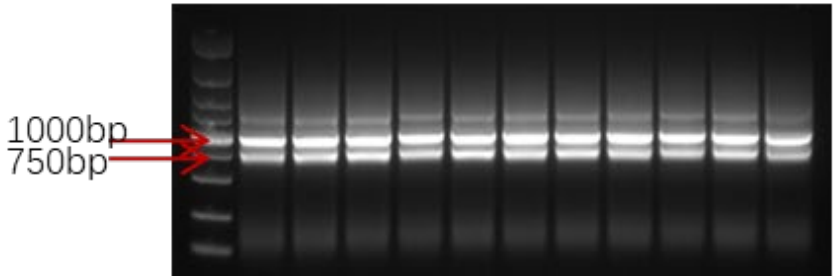
The candidate sequences have been selected. **Humanization** & **affinity maturation** are being carried out in preparation for the final construction of the **bispecific antibody**.

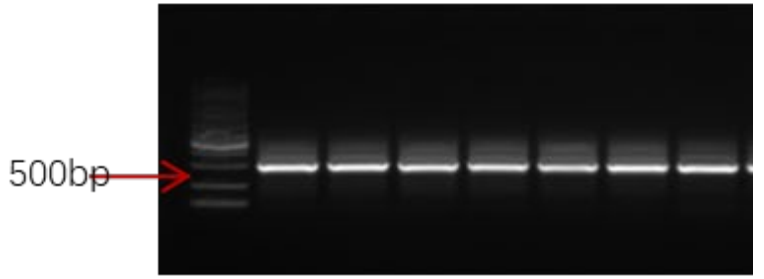
Immunization of Alpaca with Antigen X

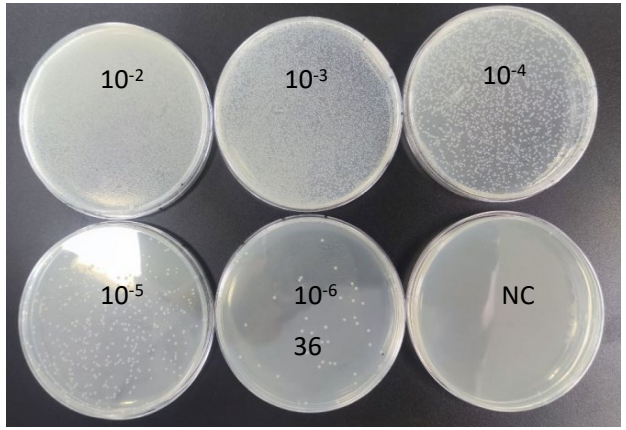


VHH Phage Display Library Construction









The library size = 5.18x10^9

Library Diversity Evaluation

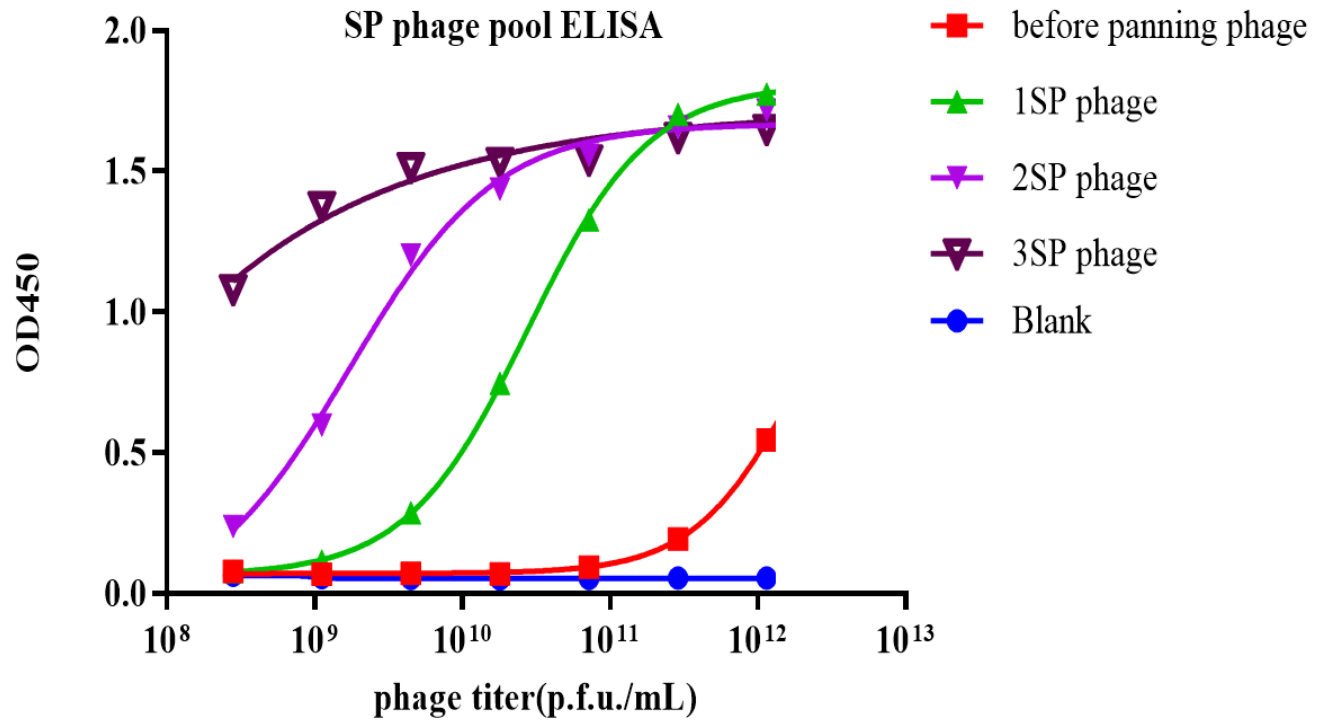
Clone #	CDR1	CDR2	CDR3	Clone #	CDR1	CDR2	CDR3
Diversity-A-3M-1	GFNL		GFDDY	Diversity-Z045-3、4M-16	GFTF		
Diversity-A-3M-25	GFNL		GFDDY	Diversity-Z045-3、4M-17	GISV		
Diversity-A-3M-2	GSPF			Diversity-Z045-3、4M-18	GSIS		
Diversity-A-3M-3	GDTM		DY	Diversity-Z045-3、4M-19	GFNV		GSYAY
Diversity-A-3M-4	GFTF		AYGMDY	Diversity-Z045-3、4M-20	GFIL		
Diversity-A-3M-5	GIVF			Diversity-Z045-3、4M-21	RSGF		
Diversity-A-3M-6	GSIF		DY	Diversity-Z045-3、4M-22	GSTF		
Diversity-A-3M-7	GFAF			Diversity-Z045-3、4M-23	GFTF		
Diversity-A-3M-8	GFTF			Diversity-Z045-3、4M-24	GVPS		KY
Diversity-A-3M-9	GRSI			Diversity-Z045-3、4M-25	GTTL		FGS
Diversity-A-3M-10	GFTL		GPEYNF	Diversity-Z045-3、4M-26	GGTF		
Diversity-A-3M-28	GFSL		SHYNDMDY	Diversity-Z045-3、4M-27	GGGF		
Diversity-A-3M-18	GFTL		TYDY	Diversity-Z045-3、4M-28	GGMY		
Diversity-A-3M-21	ETML		FHGVKY	Diversity-Z045-3、4M-29	GFTL		
Diversity-A-3M-24	GFTS			Diversity-Z045-3、4M-30	GDIF		

2 repetitive sequences in 48 clones sent for sequencing。

Solid Phase Panning

Output/Input

Solid phase panning (SP)	Input	Output	Output/Input
Round 1 SP	2.00E+12	1.20E+07	6.00E-06
Round 2 SP	1.53E+12	1.50E+08	9.78E-05
Round 3 SP	1.15E+13	1.17E+10	1.02E-03

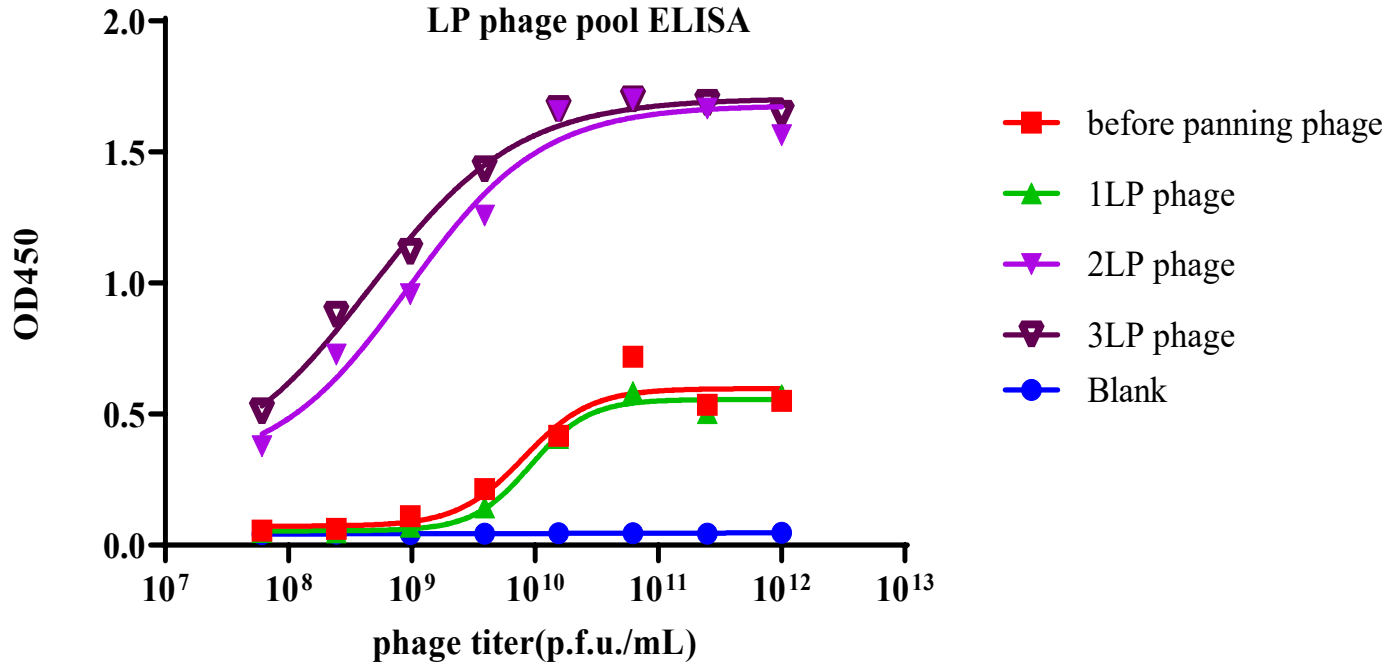


360 positive clones were obtained, **11** unique sequences were obtained & expressed in HTP system (CHO).

Liquid Phase Panning

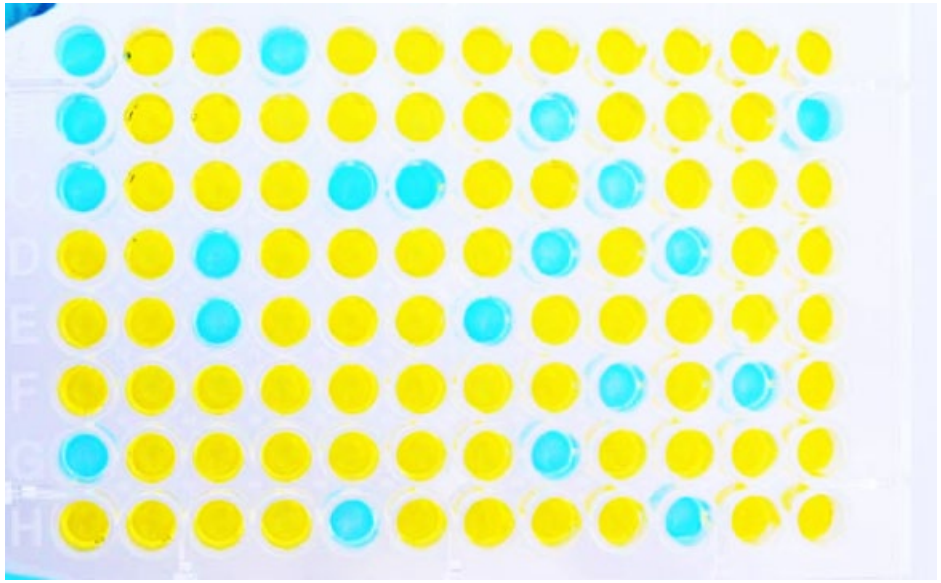
Output/Input

Liquid phase panning (LP)	Input	Output	Output/Input
Round 1 LP	2.00E+12	2.40E+06	1.20E-06
Round 2 LP	3.33E+11	4.20E+09	1.26E-02
Round 3 LP	4.10E+12	7.50E+09	1.83E-03



952 positive clones were obtained, **43** unique sequences were obtained & expressed in HTP system (CHO).

11 + **43** = **54** unique sequences

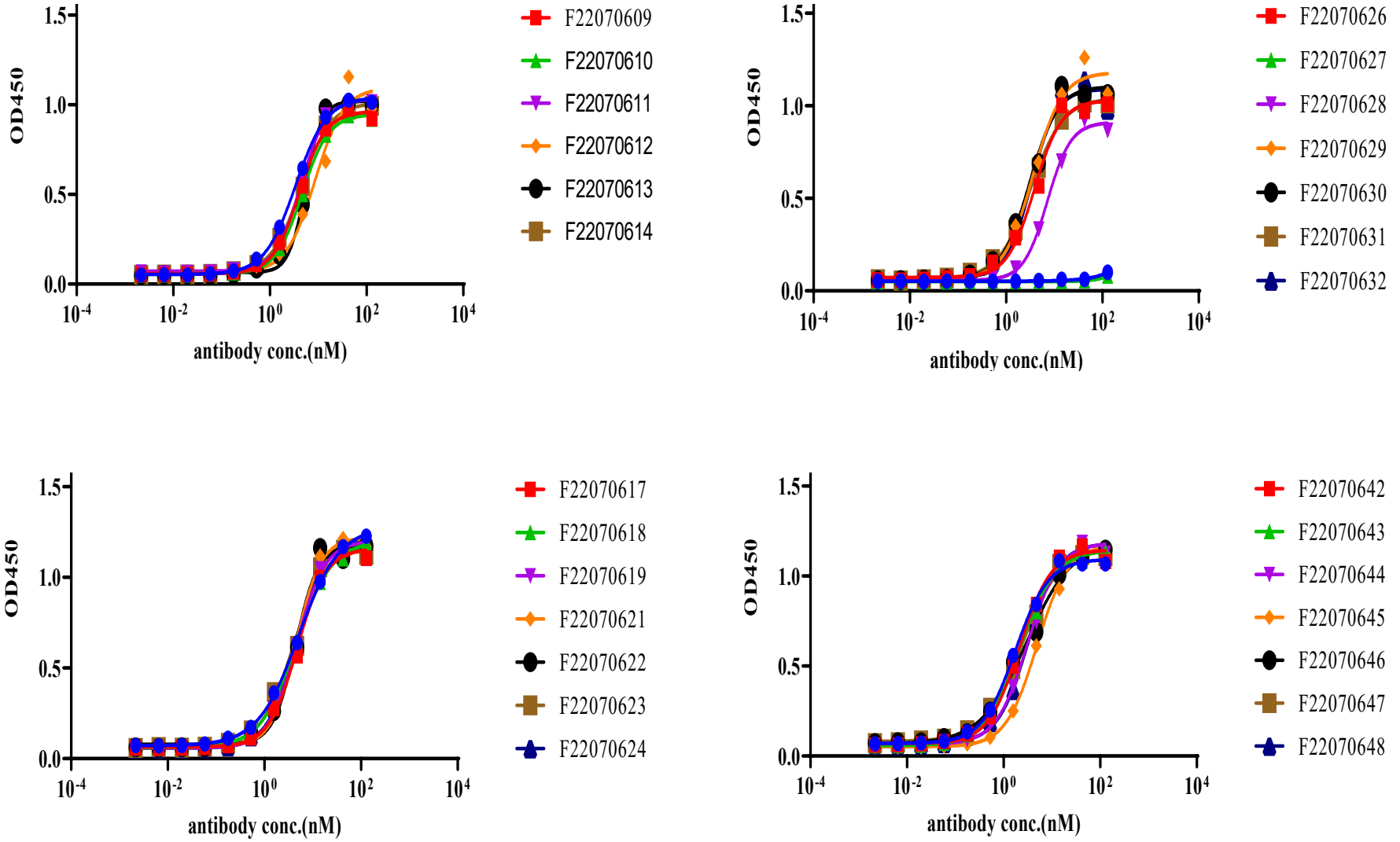


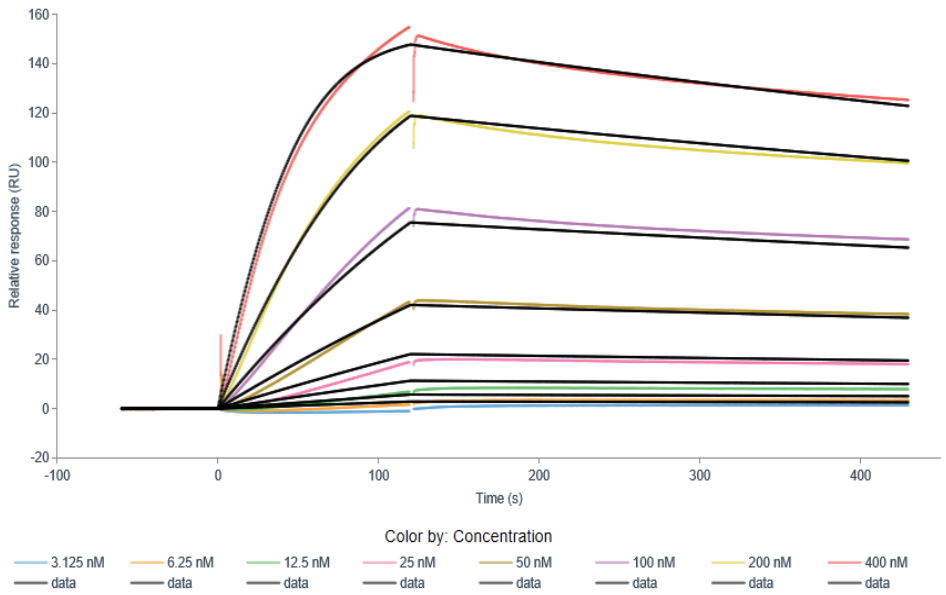
Antibody Characterization

ELISA BINDING ASSAY

54 VHH antibodies were expressed in HTP CHO expression system, purified, & validated by ELISA against Antigen X.

35 of 54 VHH Antibodies Bound to X in ELISA Assay



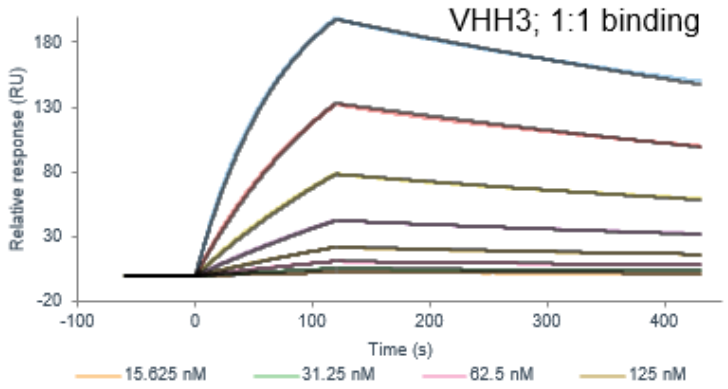


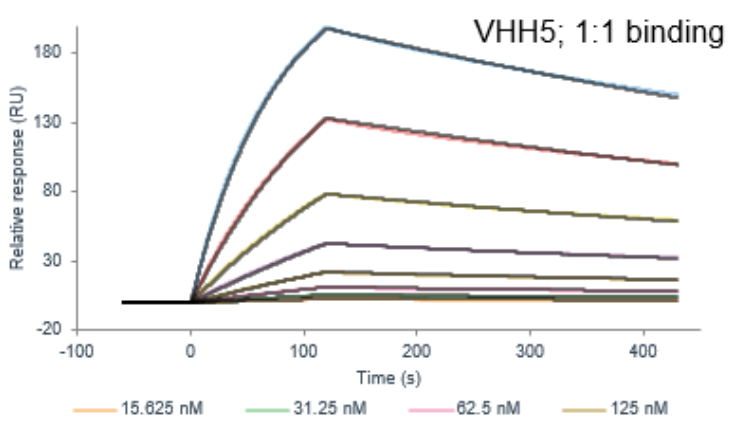
Antibody Characterization

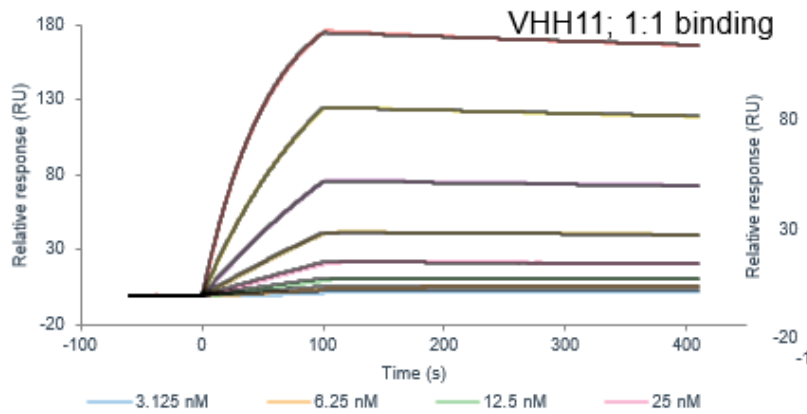
SPR AFFINITY MEASUREMENT

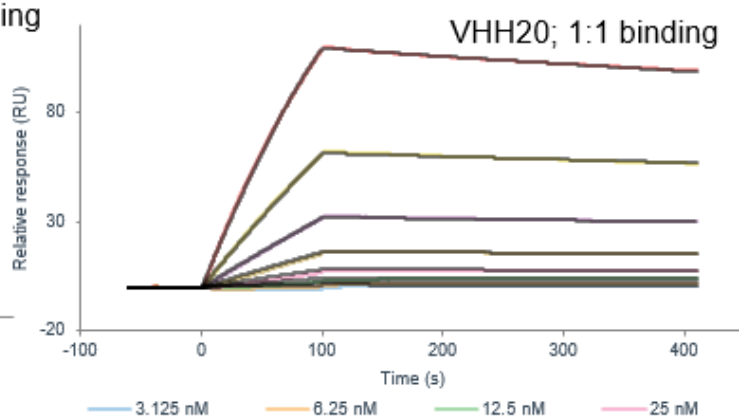
54 VHH antibodies were expressed in HTP CHO expression system, purified, & subjected to SPR test on BIACore 8k system against Antigen X.

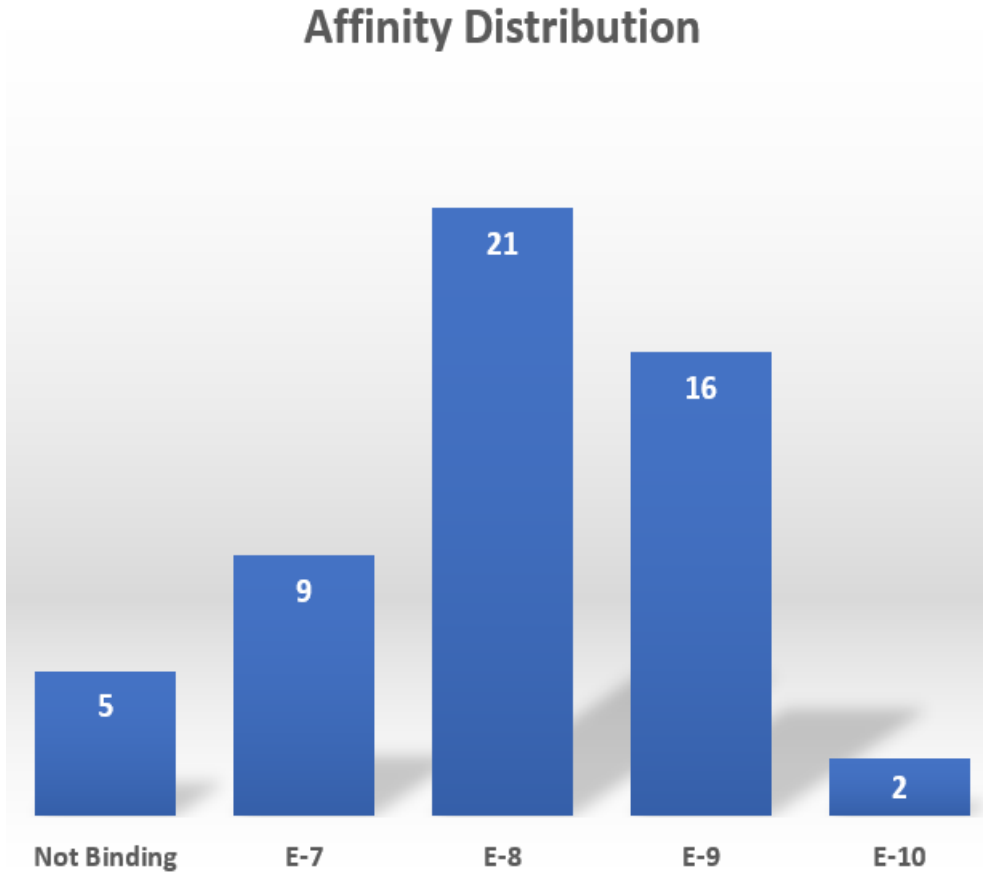
49 of 54 VHH Antibodies Bound to X in SPR Tests



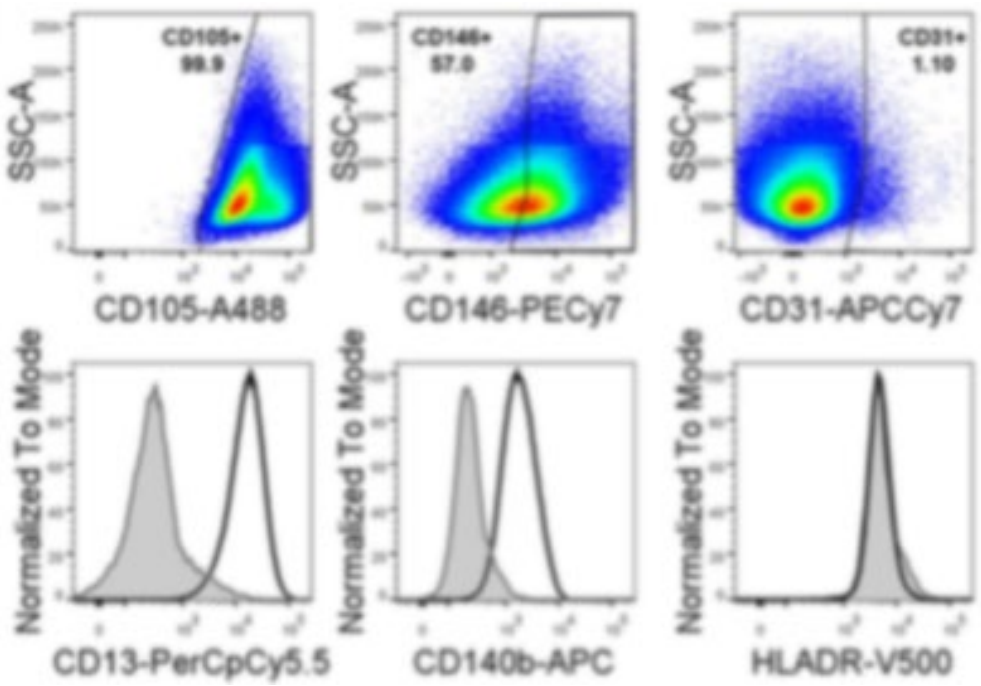








Affinity range 5.36×10^{-7} M to 7.66×10^{-10} M.

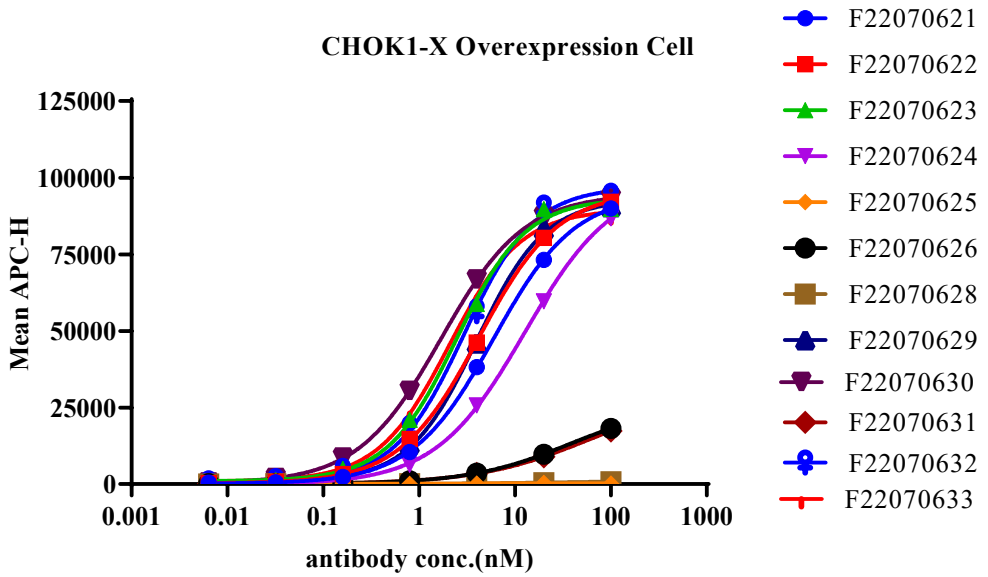


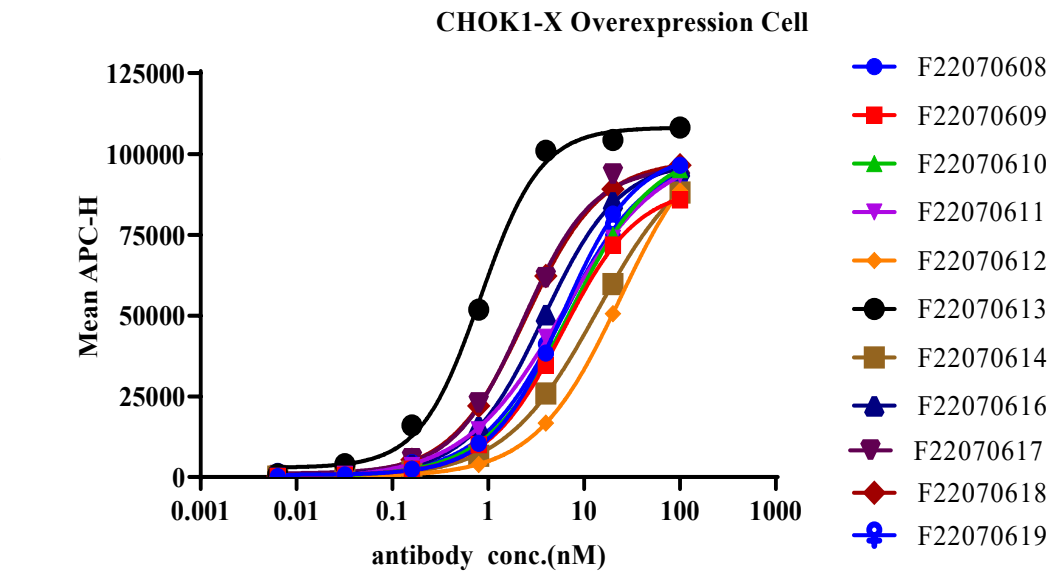
Antibody Characterization

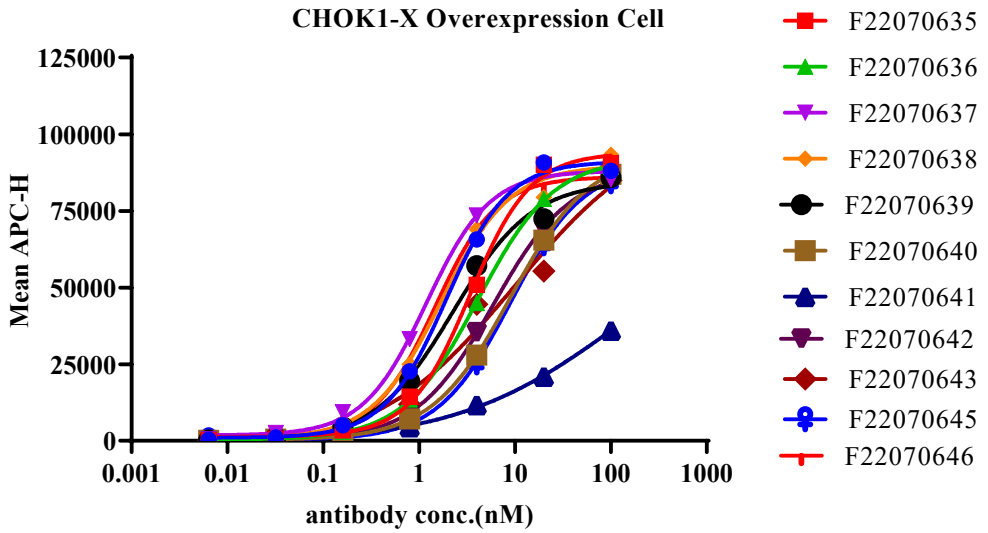
CELL-BASED BINDING ASSAY BY FLOW CYTOMETRY

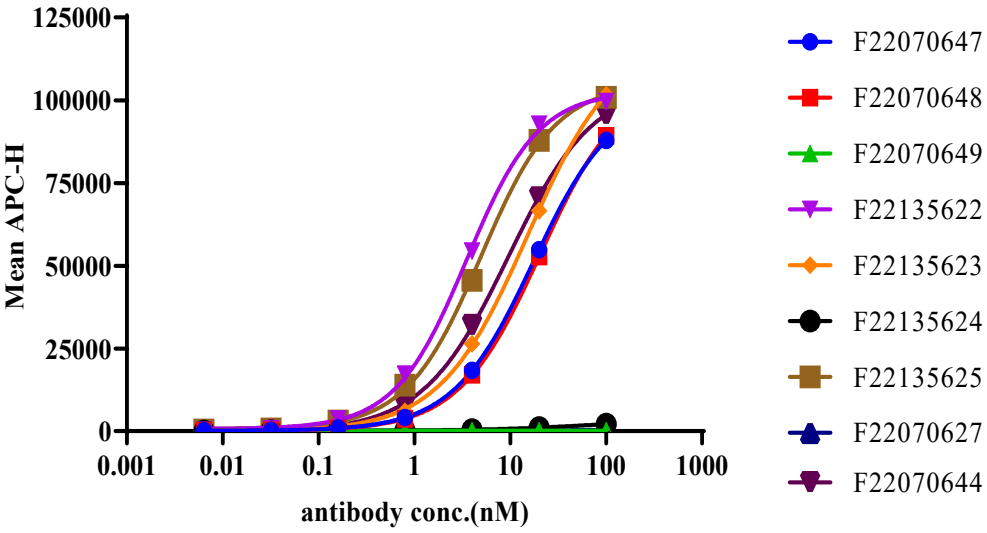
54 VHH antibodies were examined by flow cytometry against CHO-K1/X overexpression cell.

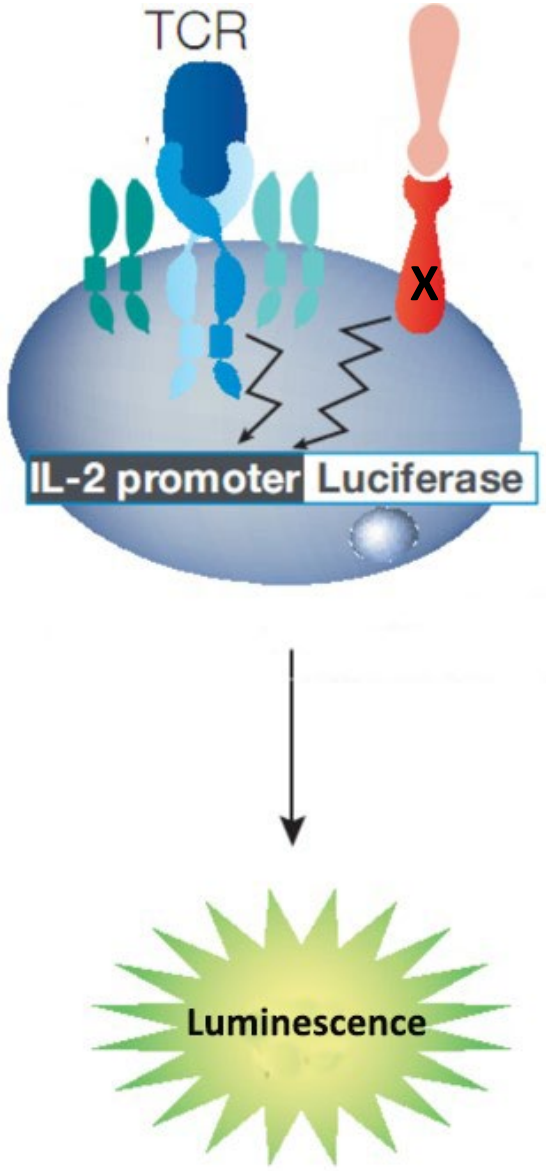
43 of 54 VHH Antibodies Bound against CHO-K1/X Cell











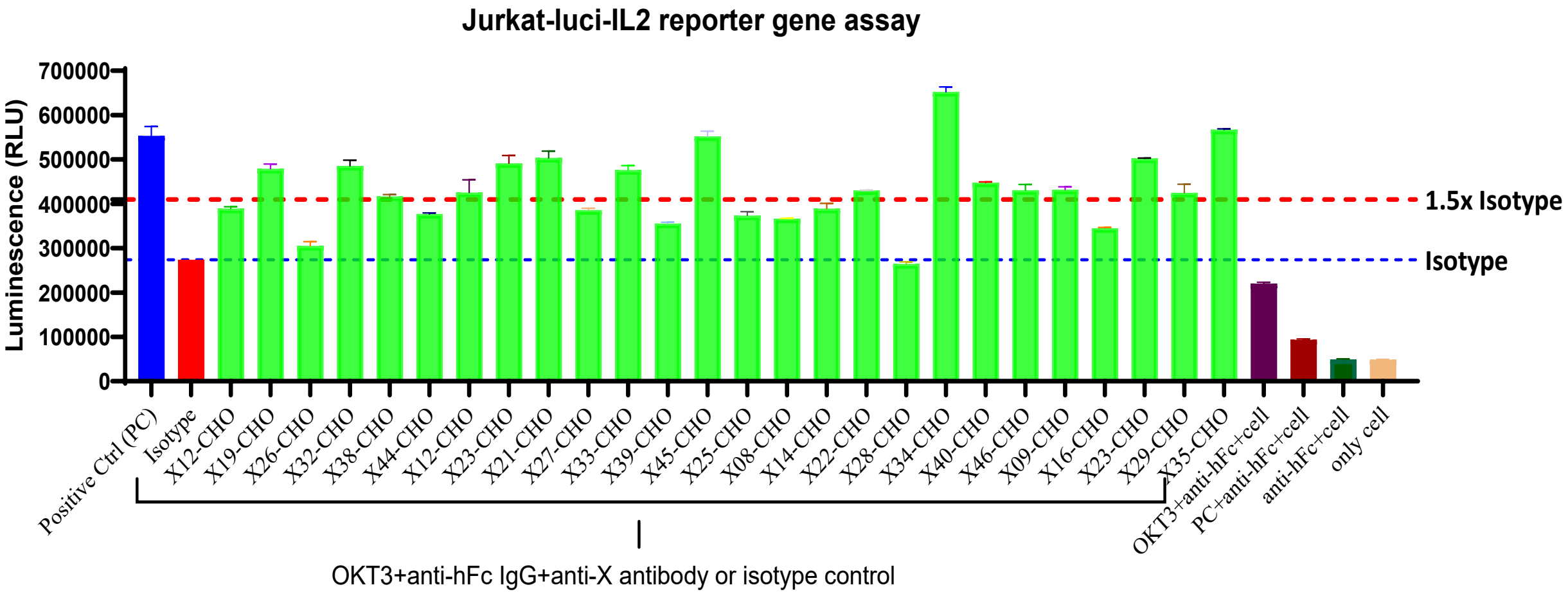
Antibody Characterization

AGONIST ACTIVITY ASSAY

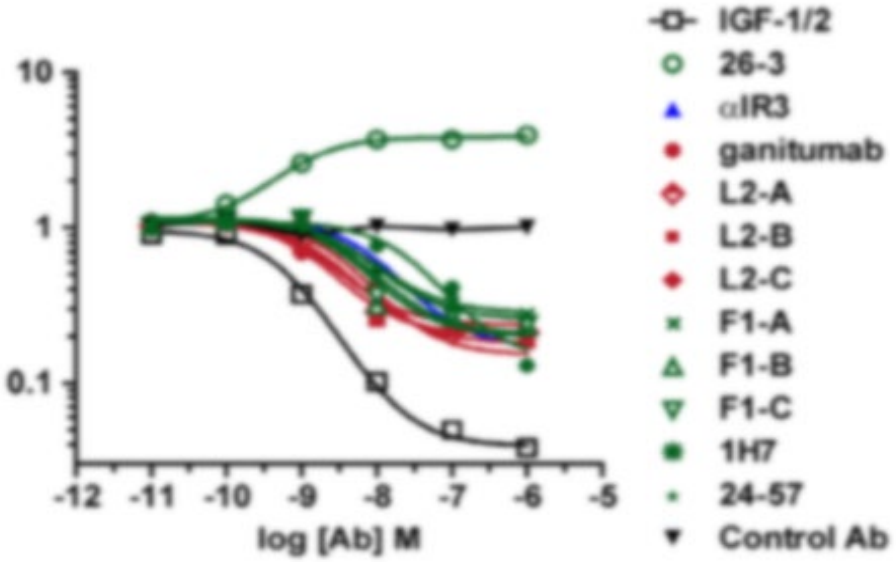
53 VHH antibodies were subjected to agonist activity assay with Jurkat-luci-IL2 reporter system.

Adapted from www.promega.com

31 of 53 Hits Showed Agonist Activity



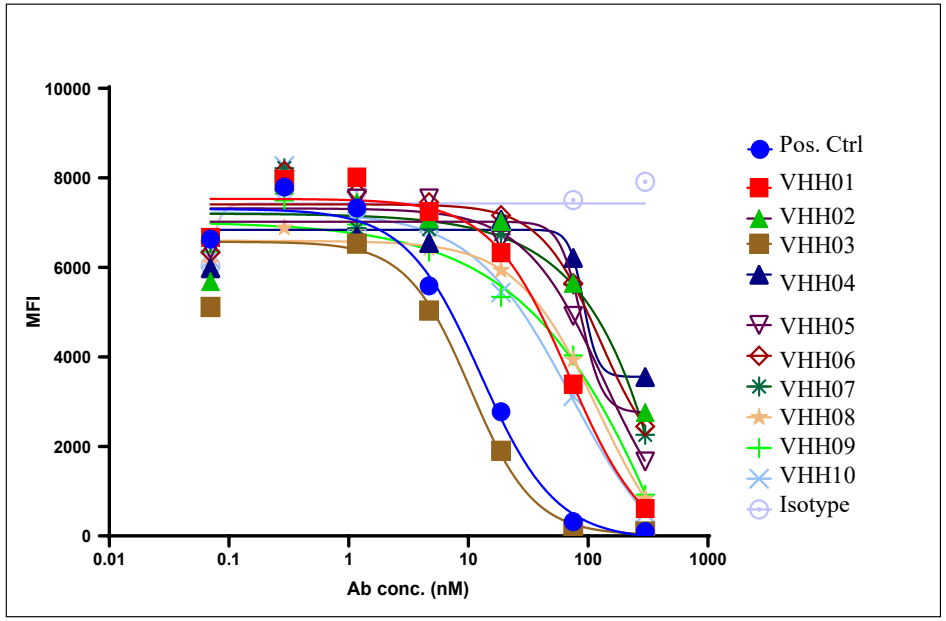
Antibody Characterization

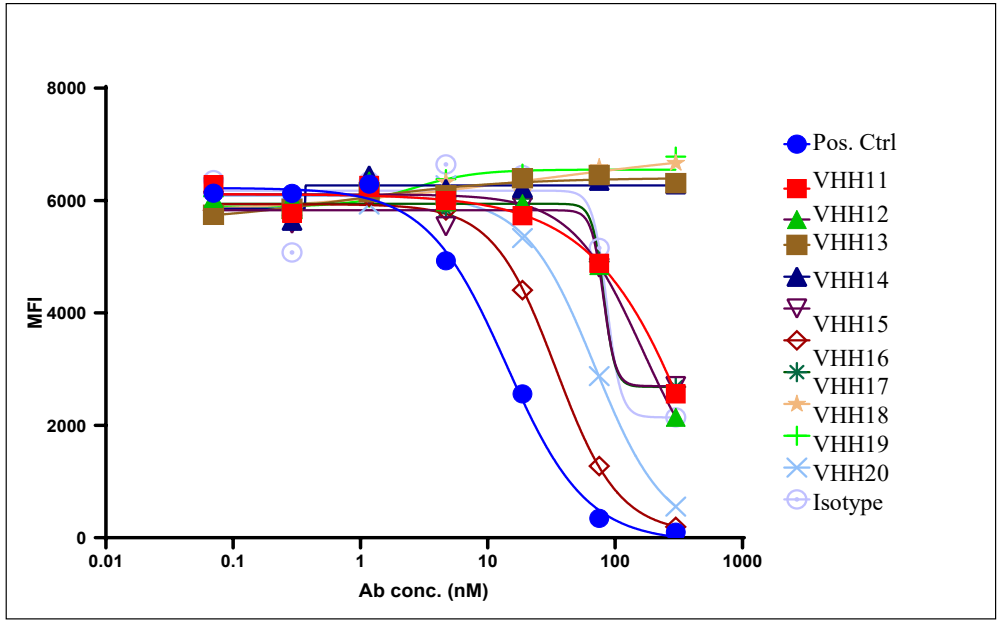


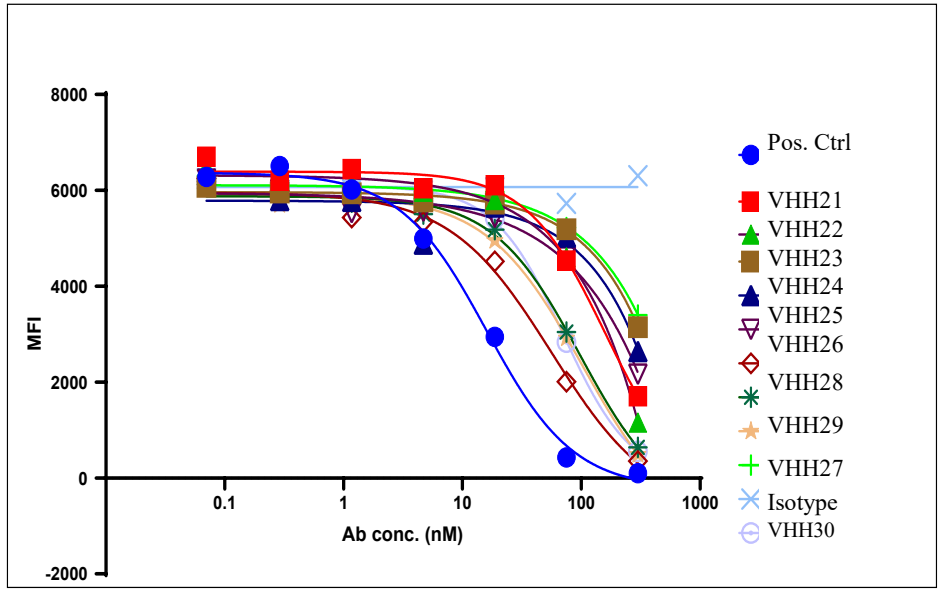
LIGAND BLOCKING ASSAY

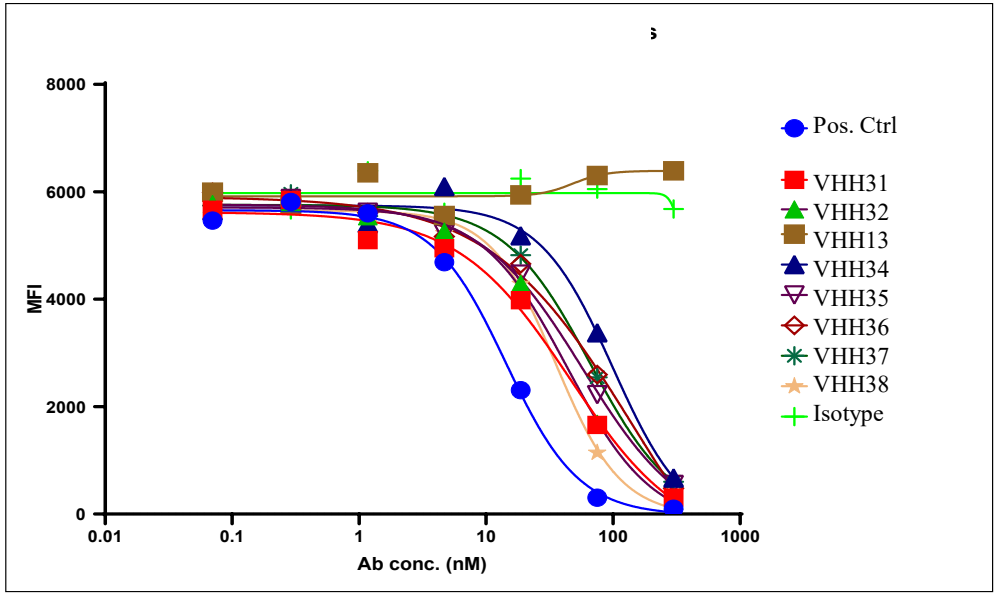
51 VHH antibodies were subjected to blocking assay to determine the ability of VHH-hFc to block interaction between ligands and cell membrane-bound Antigen X.

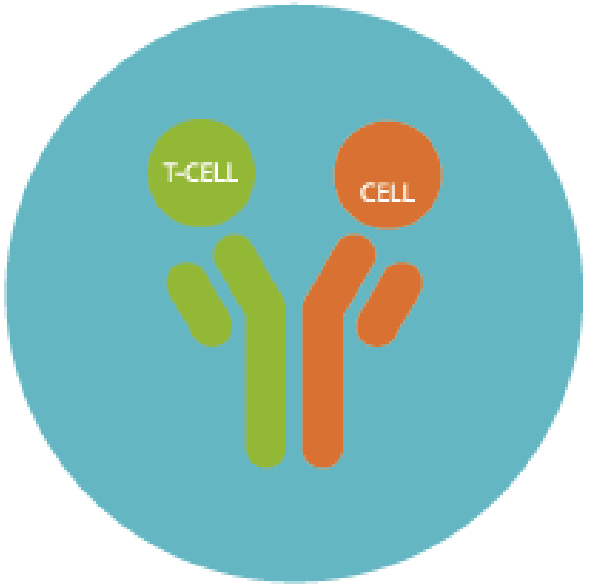
44 of 51 VHH Antibodies Showed Blocking Effect











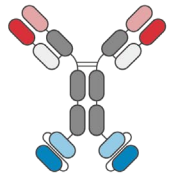
Antibody Application

BISPECIFIC ANTIBODIES

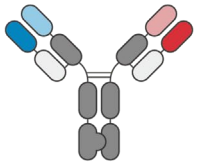
After VHH antibodies are screened, characterized and optimized to meet the requirements, they are ready for applications, such as the construction of bispecific antibodies.

Construction of Common Formats of Bispecific Antibodies

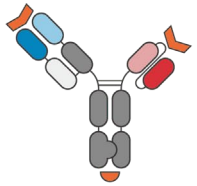
百英生物 BIOINTRON



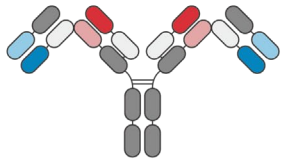
IgG-scFv



Knobs-into-holes



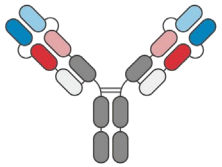
Y-body



FIT-Ig

Cross MAB

Ortho-Fab IgG



DVD-Ig

bi-Nanobody

BiTE

CH CL V_AH V_AL V_BH V_BL

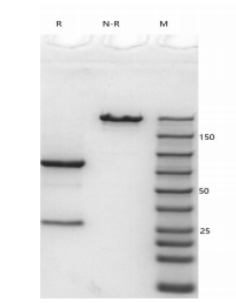
CERTIFICATE OF ANALYSIS

Name		**Label Type**	Human IgG1-Kappa
Project ID		**Host**	HEK293
Delivery Amount	86.42mg	**Lot. No.**	
Buffer	10mM NaAC-HAc , pH5.2	**Expression yield**	43.3mg/L
Delivery format	Liquid	**Concentration**	4.69mg/ml
Molecular characterization	The predicted MW is 74/23Kda and the experimental MW is about 70-100/25-35 kDa in SDS-PAGE under reducing condition.		

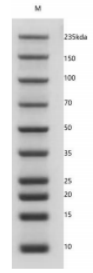
QC Results

Test Items	**Specifications**	**Results**
Purity	SDS-PAGE under reducing conditions.	>95%
	SEC-HPLC test.	92.476%
Endotoxin	LAL test.	<1EU/mg
Storage	sterile filtered, no preservatives.The product should be stored at -20℃ or lower upon receipt, and avoid repeated freeze-thaw cycles.	
	Storage condition suggestion: 1. 2°C to 8 °C for 2 weeks under sterile conditions; 2. -20°C for 3 months under sterile conditions; 3. -70°C for 36 months under sterile conditions.	

SDS-PAGE



LaneM：Marker
LaneR：Reducing
LaneN-R：Non-Reducing



SEC-HPLC

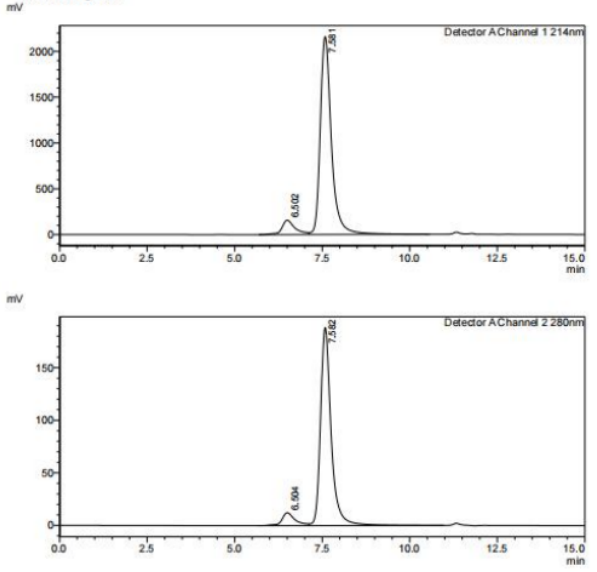
06/28/2021 13:58:26 Page 1 / 2

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

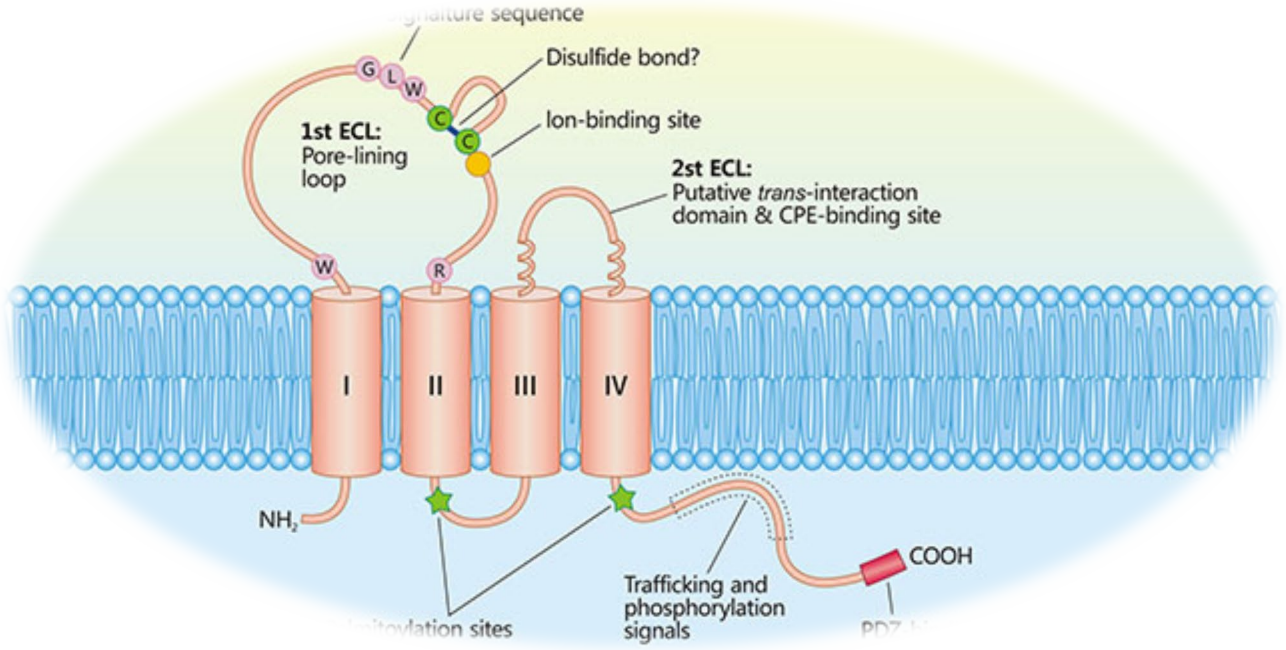
Sample Name	:		
Sample ID	:		
Data Filename	:		
Method Filename	: SEC.lcm		
Batch Filename	: 2021.06.28.lcb		
Vial #	: 1-6	Sample Type	: Unknown
Injection Volume	: 20 uL		
Date Acquired	: 06/28/2021 12:07:17	Acquired by	: System Administrator
Date Processed	: 06/28/2021 13:58:18	Processed by	: System Administrator

<Chromatogram>



Challenge 1. Transmembrane Targets

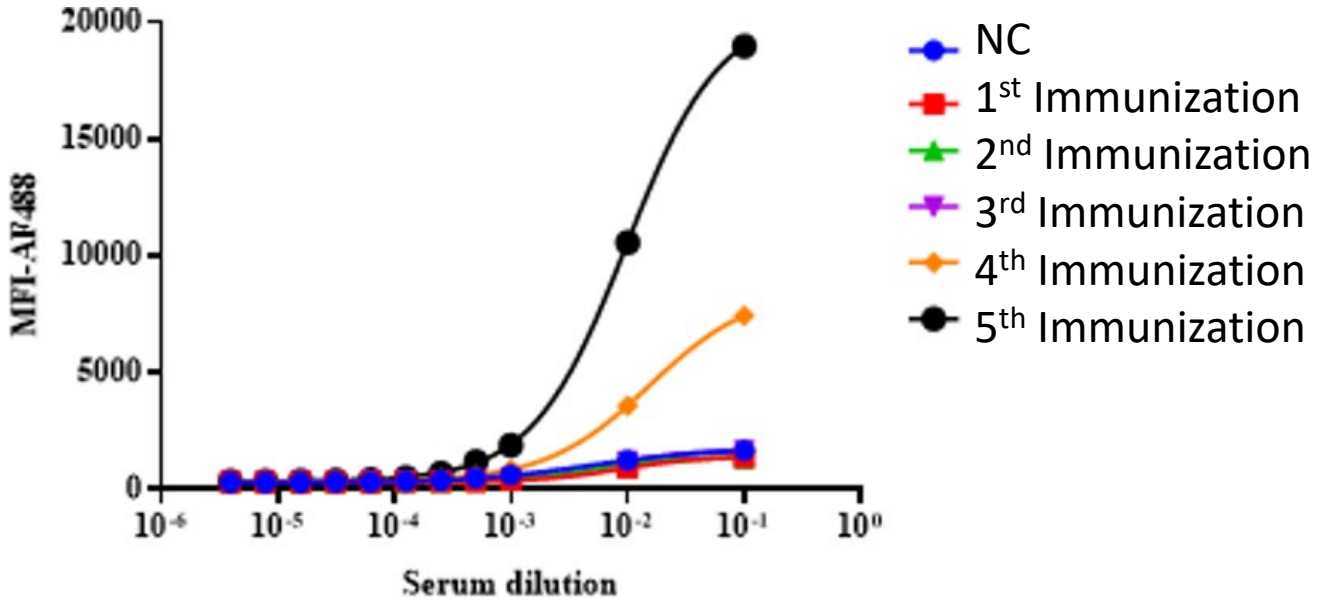
What if it is difficult to prepare soluble protein as antigen?



Adapted from Physiological Reviews. 2013, 93(2), 525–569

Human Claudin 18.2 Case

Antigen	Immunization
DNA encoding Claudin 18.2	1, 2
CHO-K1-Claudin 18.2	3, 4, 5



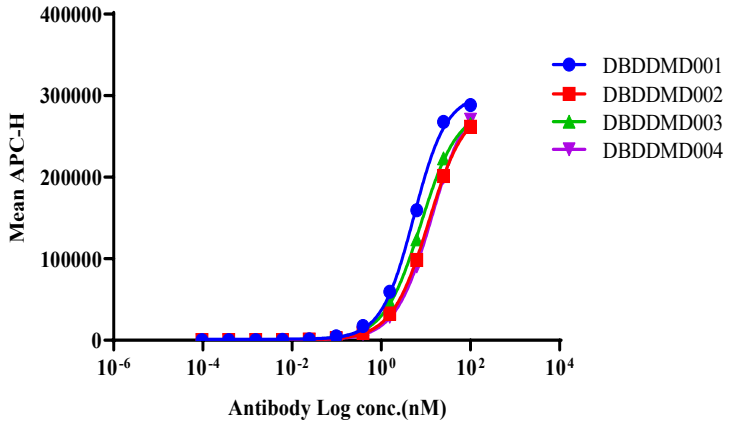
Cell Panning using CHO-K1-Claudin 18.2

Cell panning	Input (CFU/ml)	Output	Output/Input
1	2.00E+12	9.00E+07	4.50E-05
2	1.33E+12	2.10E+08	1.58E-04
3	2.07E+12	4.20E+08	2.03E-04

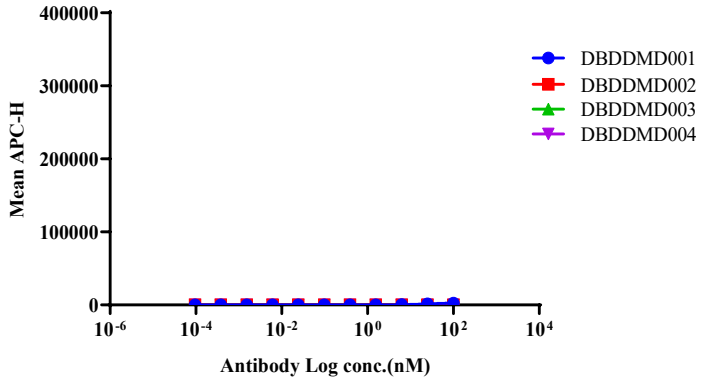
4 VHH Antibodies Specifically Bound Claudin 18.2 But Not 18.1

	Selected Clones	Positive Clones	Positive%	Unique Sequences
0635-Human C2-1CP	88	2	2.27%	1
0635-Human C2-2CP	88	6	6.82%	2
0635-Human C2-3CP	88	14	15.91%	10

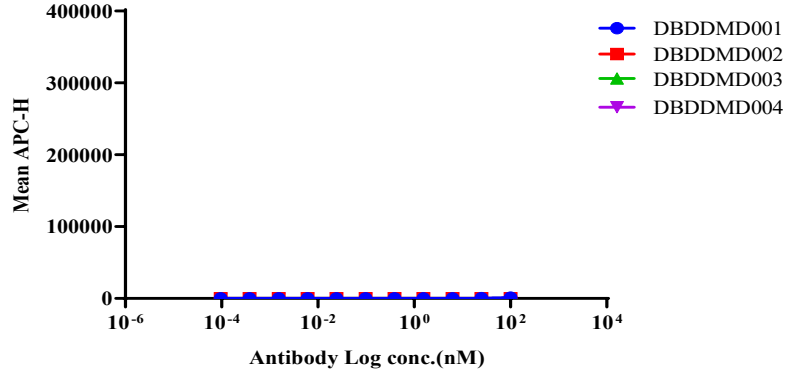
293T (Claudin 18.2)



293T (Blank)



293T (Claudin 18.1)



Challenge 2. Naïve Phage Display Library

Naïve phage display lib. instead of alpaca immune antibody lib.

300 Alpacas'PBMC

phage display & production

Naïve Phage Display Library
Library Size: 10^{12}

Monoclonal Phage ELISA Screening after Liq. Phase Panning

Plate ID	Total colnes	OD450 > 0.5	Ratio	OD450(BSA) > 0.1	Ratio	OD450 > 0.5&OD450(BSA)<0.1	Ratio
X/his -1LP	88	6	6.8%	6	6.8%	0	0.0%
X/his -2LP	88	29	33.0%	0	0.0%	29	33.0%
X/his -3LP	88	72	81.8%	1	1.1%	71	80.7%

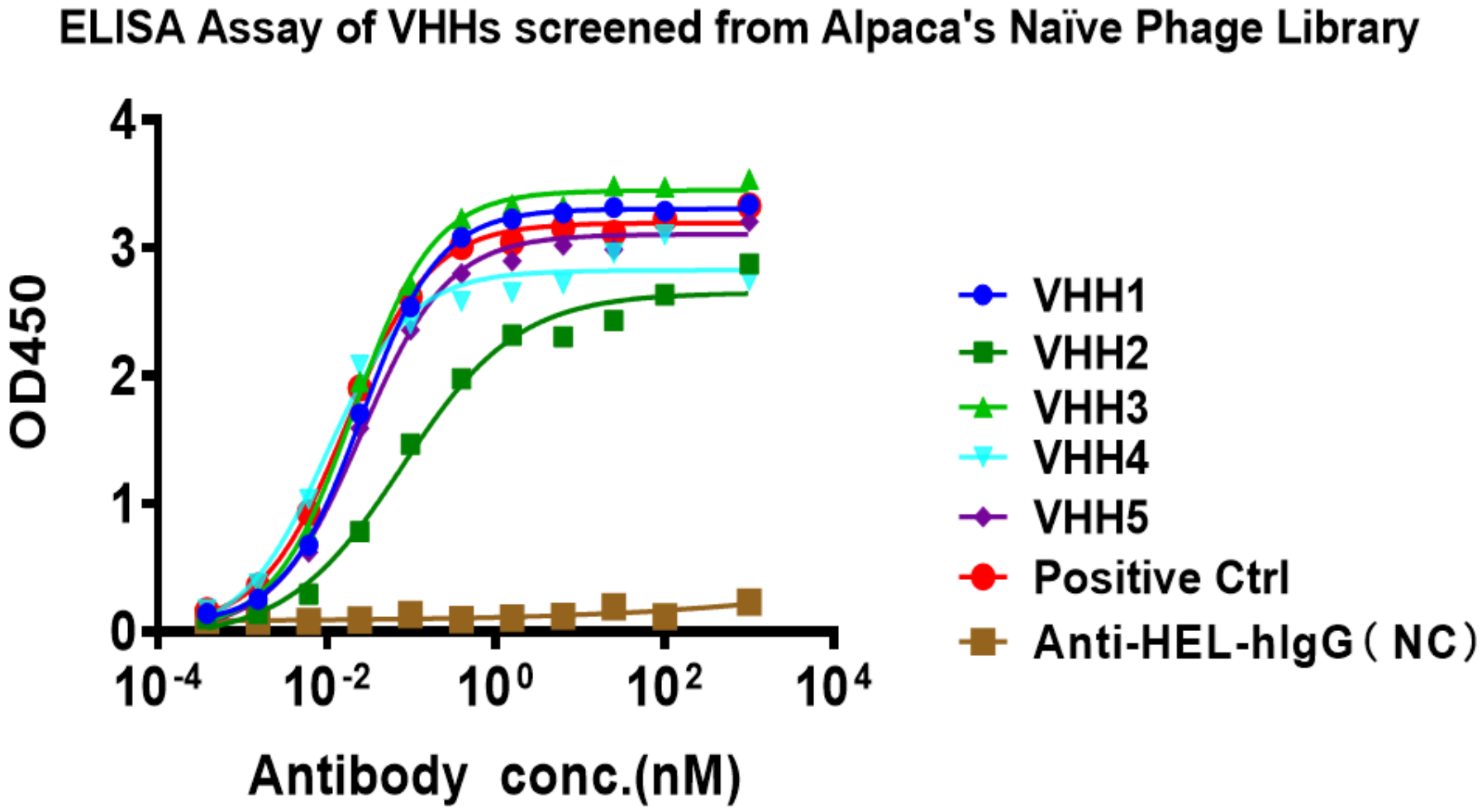
	Total clones	X-specific binder	X-specific binder /total clone	Unique seq.	Unique seq. /total clones
X/his -LP	264	100	37.8%	18	6.82%

100 positive clones identified, resulting in **18** unique sequences,

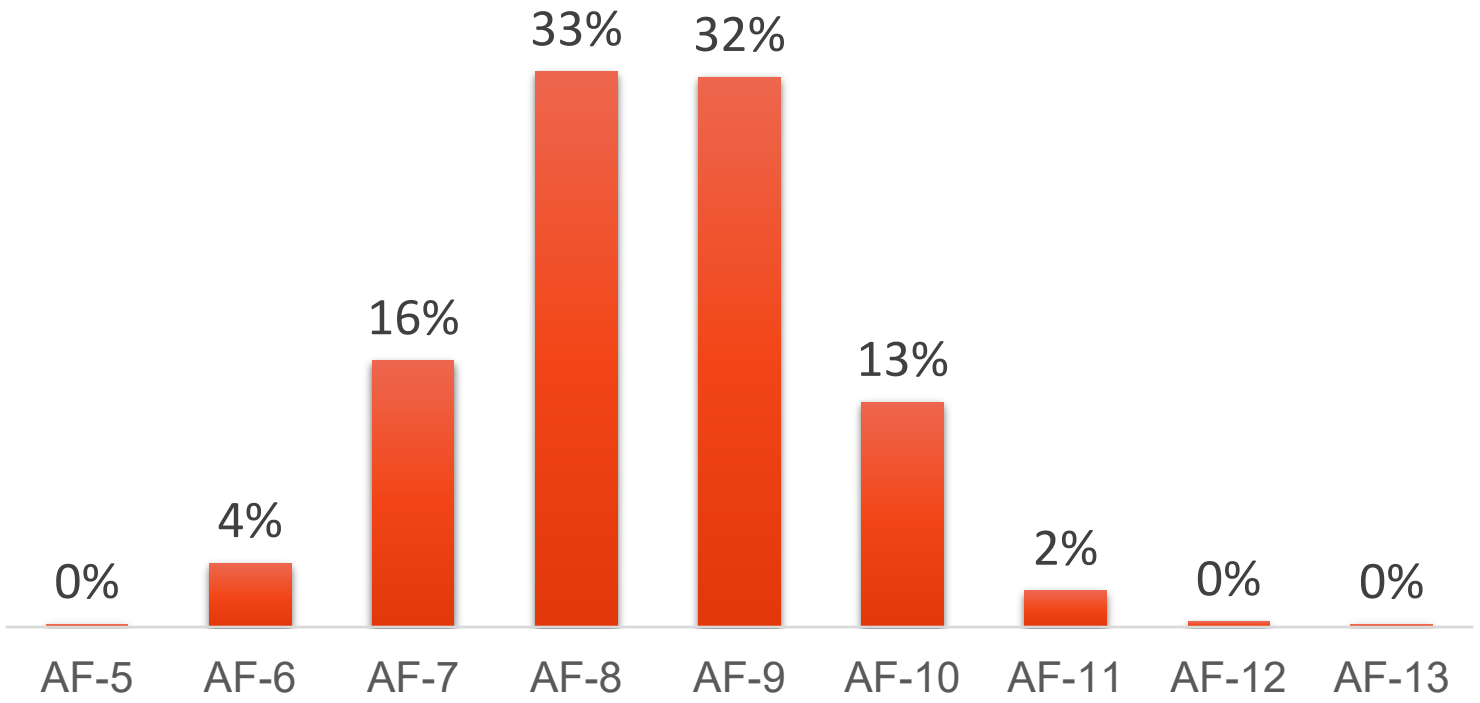
followed by VHH antibody express & characterization.

ELISA Binding Assay of Top 5 VHH Antibodies

Antibody	EC50
VHH1	0.02569
VHH2	0.07843
VHH3	0.02072
VHH4	0.009848
VHH5	0.02563
Positive Ctrl	**0.01688**
Anti-HEL-hIgG (NC)	~ 1.335e+15



Affinity Distribution of 615 Delivered VHH sequences



Data from 615 unique sequence of VHH

$$\frac{\text{Total number of VHH from different targets with an affinity of } 10^{-8} \text{ M or better}}{\text{Total number of VHH from different targets (615 unique sequence)}} = 80\%$$

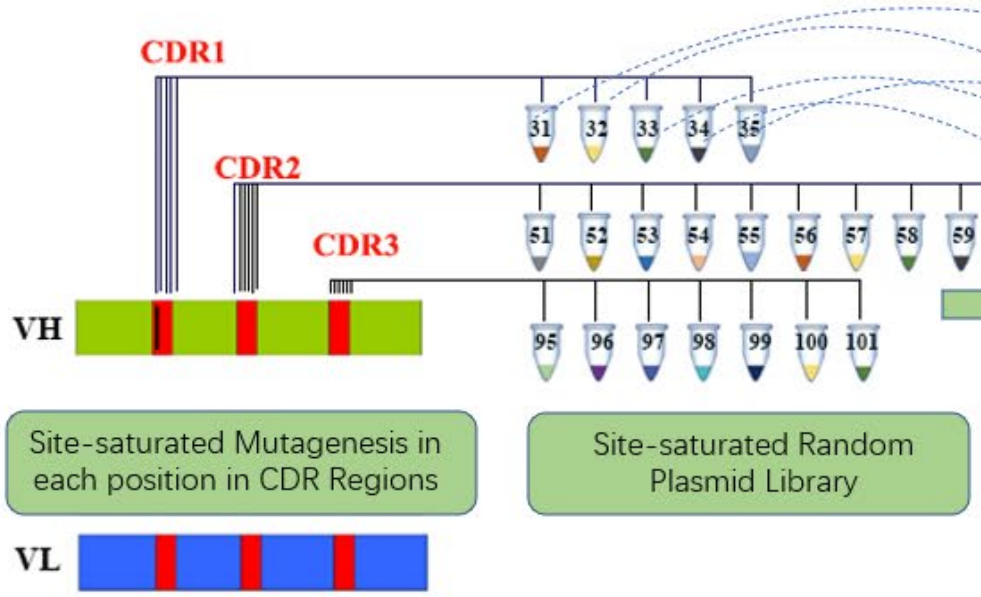
FCMES-AM

Affinity Maturation Platform for Antibody Optimization

FCMES-AM™

百英生物 BIOINTRON

Full Coverage Mammalian Expression System For Affinity Maturation



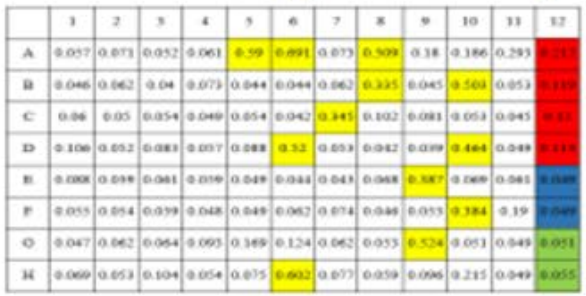
Site-saturated Mutagenesis in each position in CDR Regions

Site-saturated Random Plasmid Library

88 Colonies picked randomly from each dish

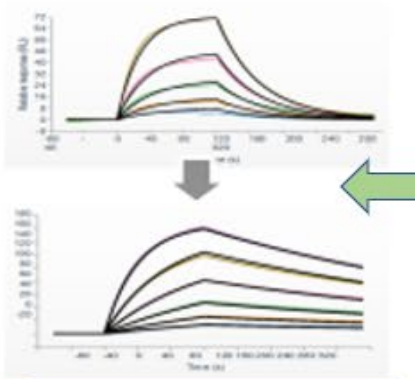
HT Mammalian Cell System Expression

AA at the mutation site		Occur.	Freq. of occur.
Gly (WT)	G	0	0
Ala	A	3	3.41%
Asp	D	3	3.41%
Glu	E	7	7.95%
Phe	F	3	3.41%
His	H	4	4.55%
Ile	I	6	6.82%
Lys	K	5	5.68%
Leu	L	8	9.09%
Met	M	3	3.41%
Asn	N	3	3.41%
Pro	P	3	3.41%
Gln	Q	5	5.68%
Arg	R	4	4.55%
Ser	S	7	7.95%
Thr	T	4	4.55%
Val	V	5	5.68%
Trp	W	4	4.55%
Tyr	Y	4	4.55%
Others		6	7.95%
Total Clones		88	100.00%



ELISA HTS

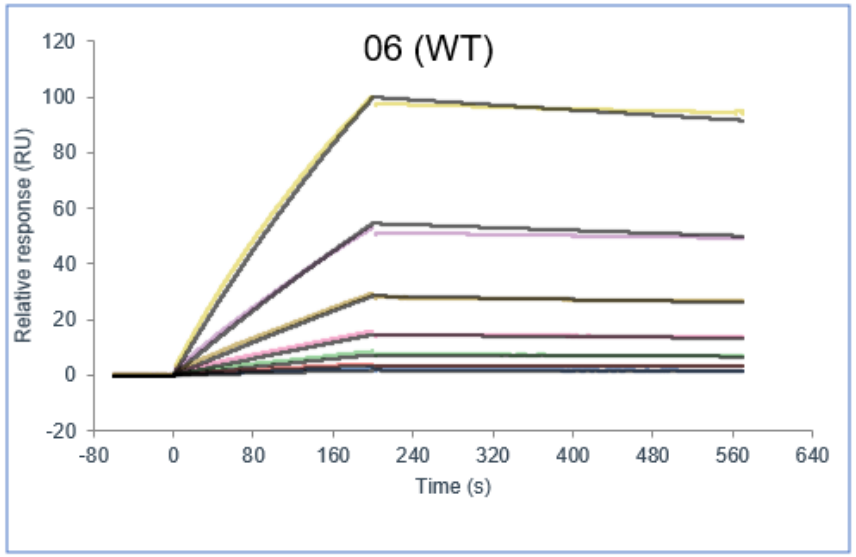
Expression of hot spot combinations in mammalian cell system



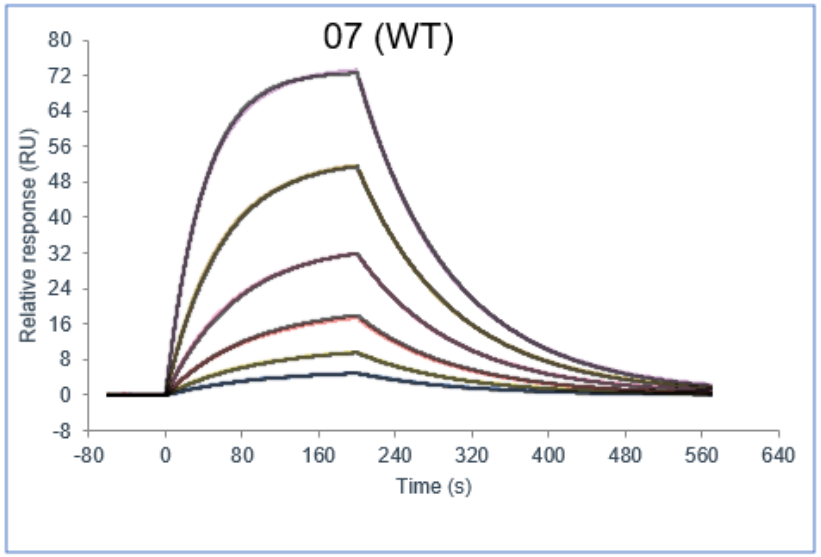
SPR Tests

Challenge - Nanobodies

Possible to improve low-affinity nanobodies?
Possible to improve K_{on} or K_{off} specifically?



$2.50x10^{-8}$ M

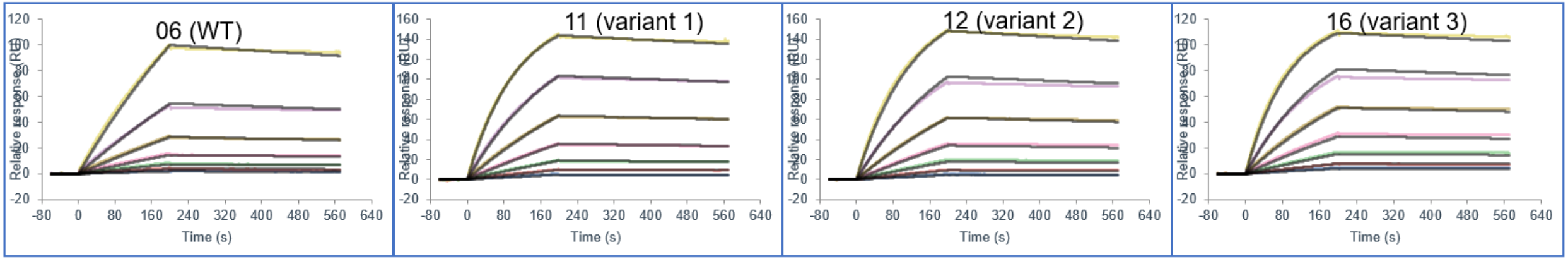


$3.03x10^{-8}$ M

Case 1. VHH (K_{on} improved)

	AA position	Original AA	Mutated AA	OD450 (mutant)	OD450 (parental)
06 (VHH)	32	*	Y	0.434	0.143
	52	*	D	0.851	0.143
	105	*	E	0.482	0.143
	109	*	D	0.512	0.143
	111	*	D	0.419	0.143

Ligand	ka (1/Ms)	kd (1/s)	KD (M)	Increased fold
11 (variant 1)	4.76E+04	1.66E-04	3.49E-09	7.16
12 (variant 2)	4.09E+04	1.89E-04	4.61E-09	5.42
16 (variant 3)	5.37E+04	1.57E-04	2.93E-09	8.53
06 (WT)	9.42E+03	2.36E-04	2.50E-08	1

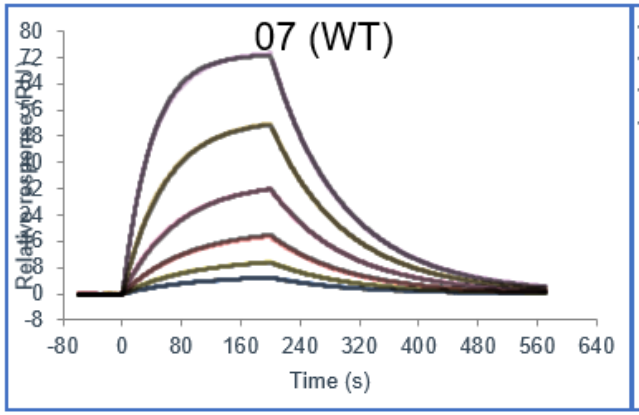


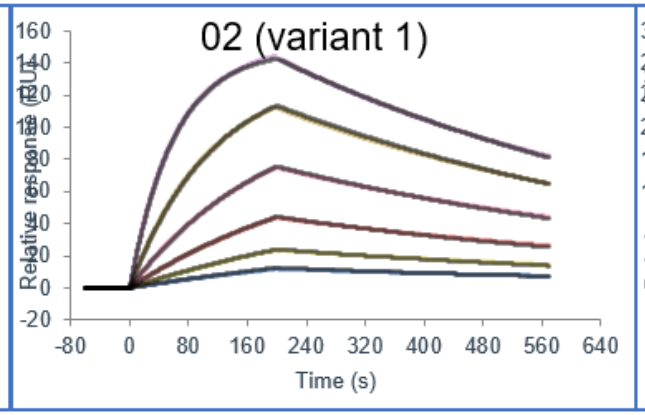
Parental **Affinity 7-fold ↑** **Affinity 5-fold ↑** **Affinity 8-fold ↑**

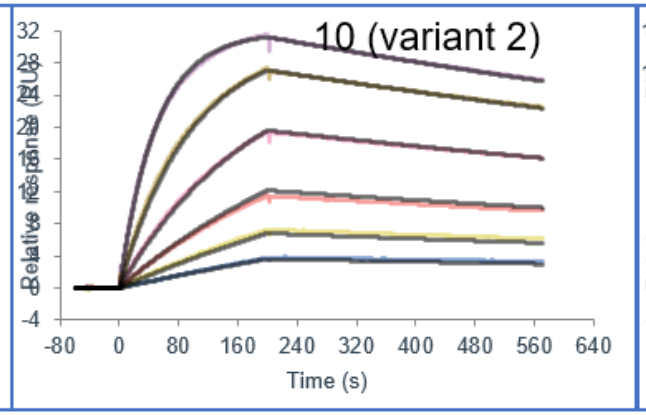
Case 2. VHH (K_{off} reduced)

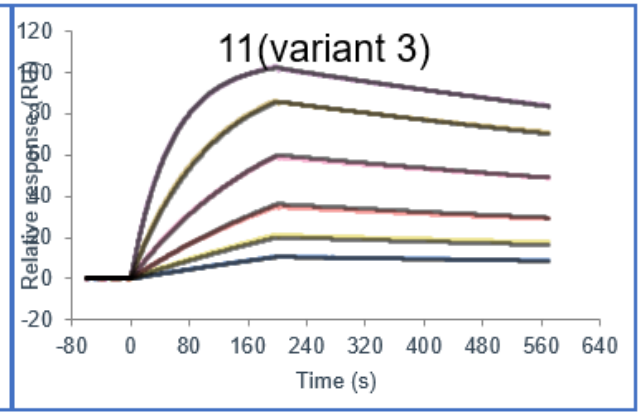
	AA position	Original AA	Mutated AA	OD450 (mutant)	OD450 (parental)
07 (VHH)	40	*	A	0.363	0.134
	59	*	Q	1.647	0.173
	106	*	I	0.957	0.173
	108	*	T	0.543	0.173

Ligand	ka (1/Ms)	kd (1/s)	KD (M)	Increased fold
02 (variant 1)	3.09E+05	1.55E-03	5.01E-09	6.05
10(variant 2)	3.96E+05	5.22E-04	1.32E-09	22.95
11(variant 3)	3.47E+05	5.48E-04	1.58E-09	19.18
07 (WT)	3.73E+05	1.13E-02	3.03E-08	1.00



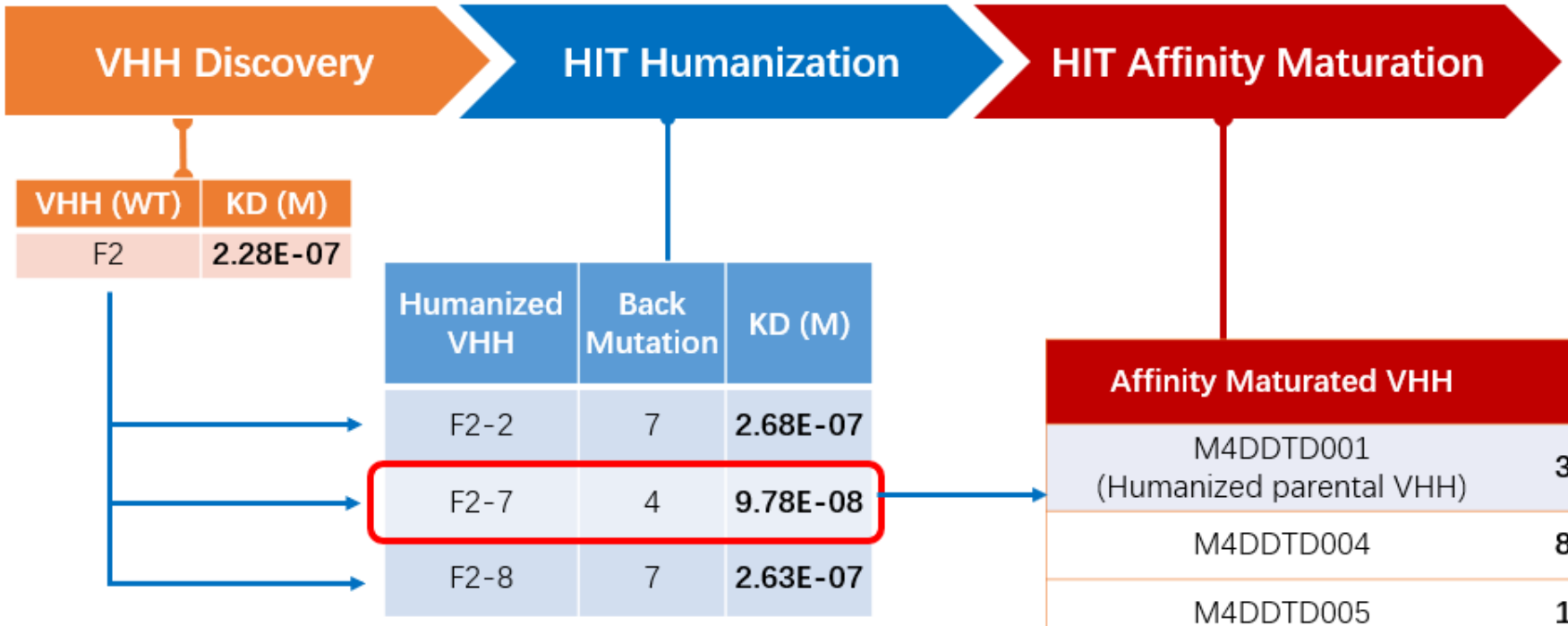






Parental **Affinity 6-fold ↑** **Affinity 23-fold ↑** **Affinity 19-fold ↑**

Case 3. VHH Affinity Maturation after Humanization



VHH (WT)	KD (M)
F2	2.28E-07

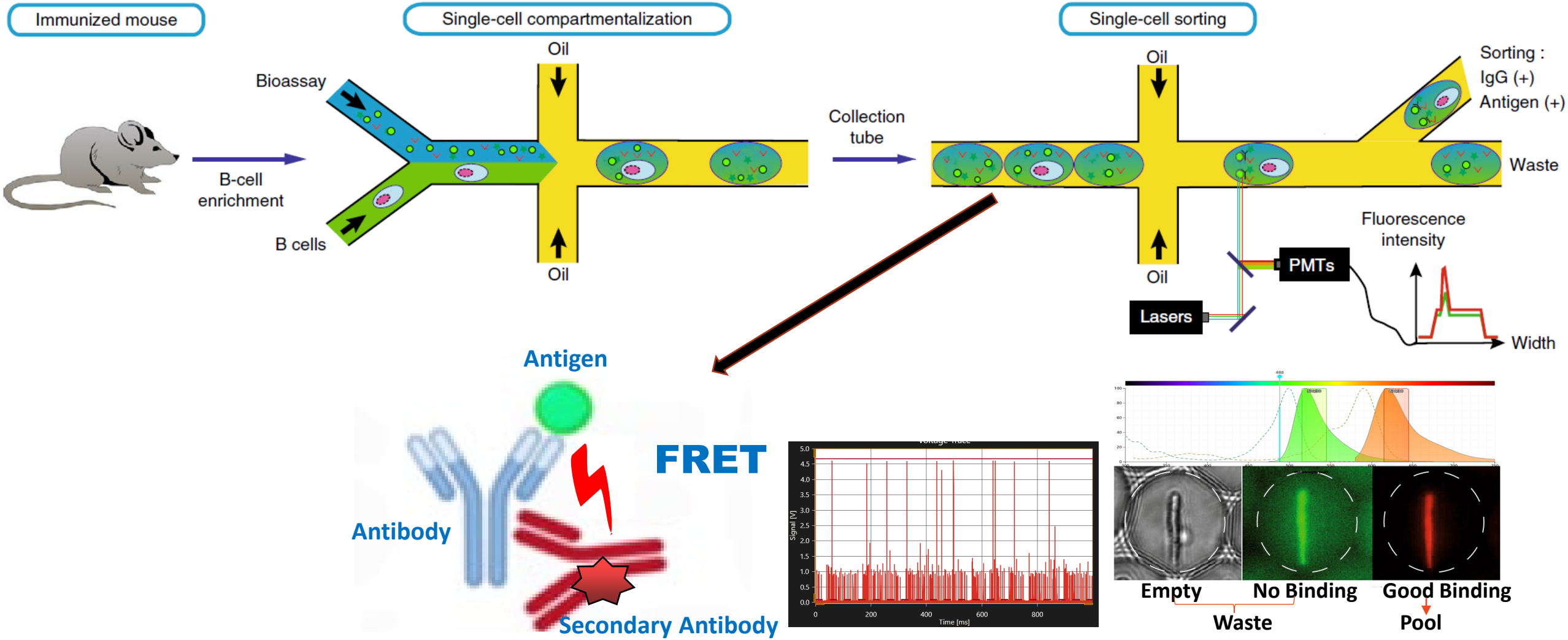
Humanized VHH	Back Mutation	KD (M)
F2-2	7	2.68E-07
F2-7	4	9.78E-08
F2-8	7	2.63E-07

Affinity Maturated VHH	KD (M)	Fold Increase
M4DDTD001 (Humanized parental VHH)	3.47E-07	-
M4DDTD004	8.88E-09	39.1
M4DDTD005	1.30E-09	266.9
M4DDTD008	3.36E-08	10.3
M4DDTD013	1.34E-08	25.9
M4DDTD016	2.45E-09	141.6
M4DDTD017	9.93E-09	34.9
M4DDTD021	1.48E-09	234.5

Single B Cell Platform

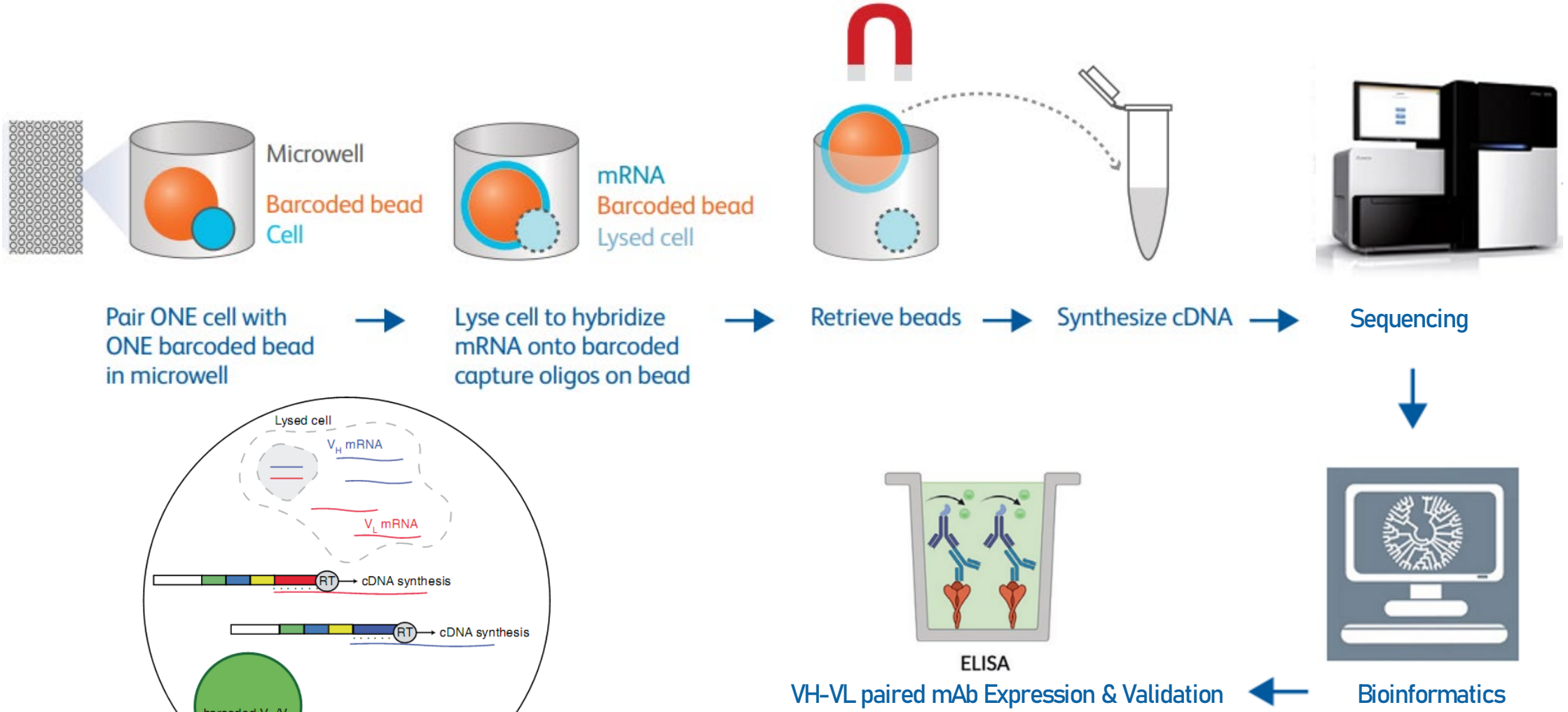
Ultra-fast Antibody Sorting Platform

Single B Cell Sorting

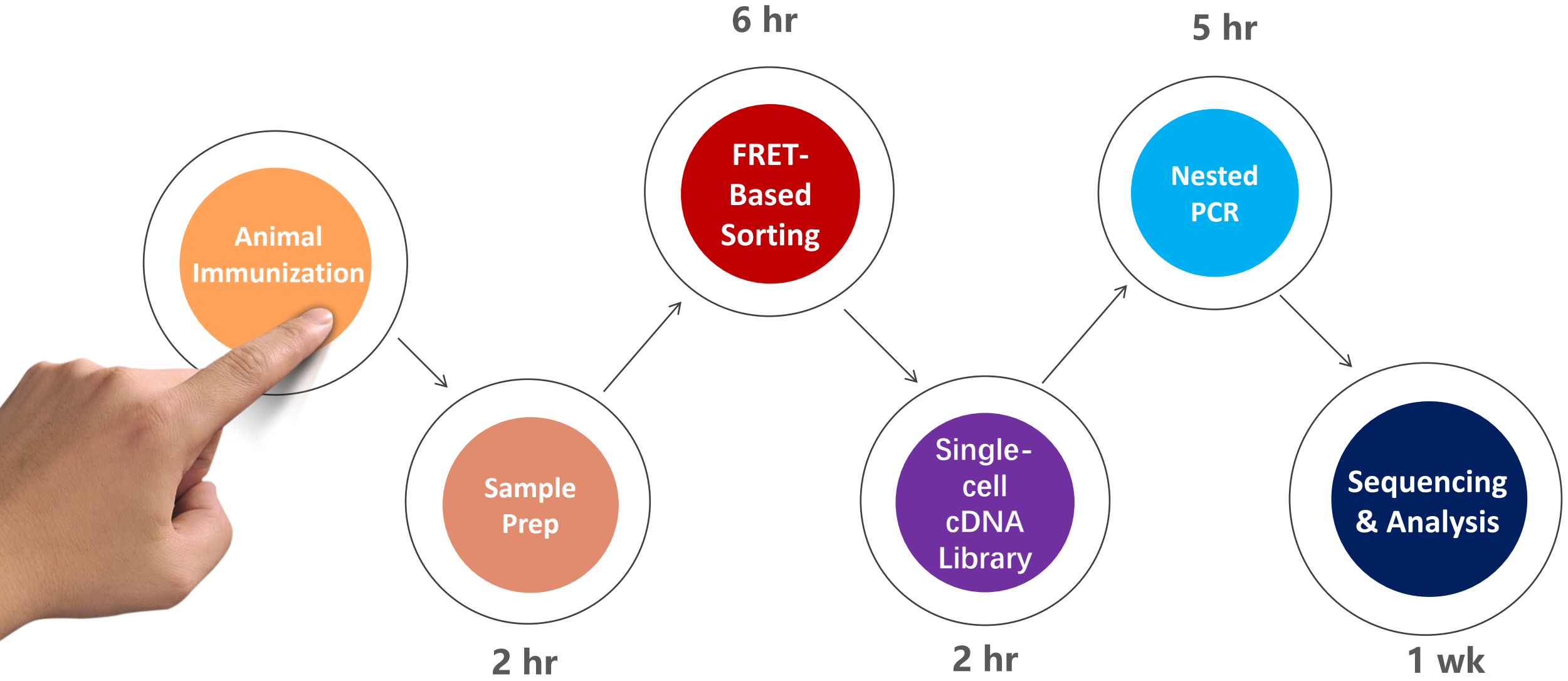


https://doi.org/10.1038/s41587-020-0466-7

Single B Cell Analysis & Validation



Single B cell Antibody Discovery

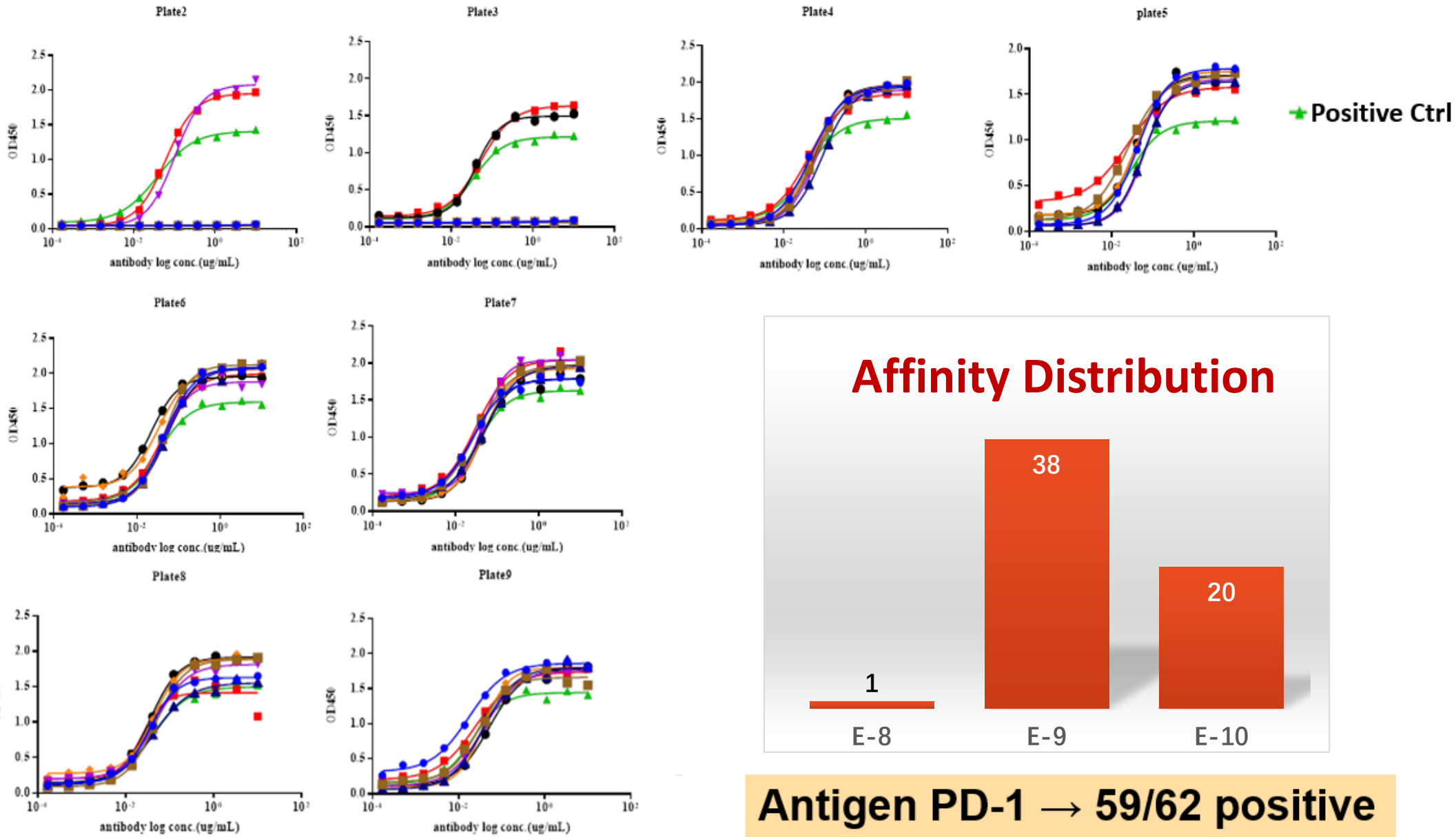


Paired Heavy & Light Chains

百英生物 BIOINTRON

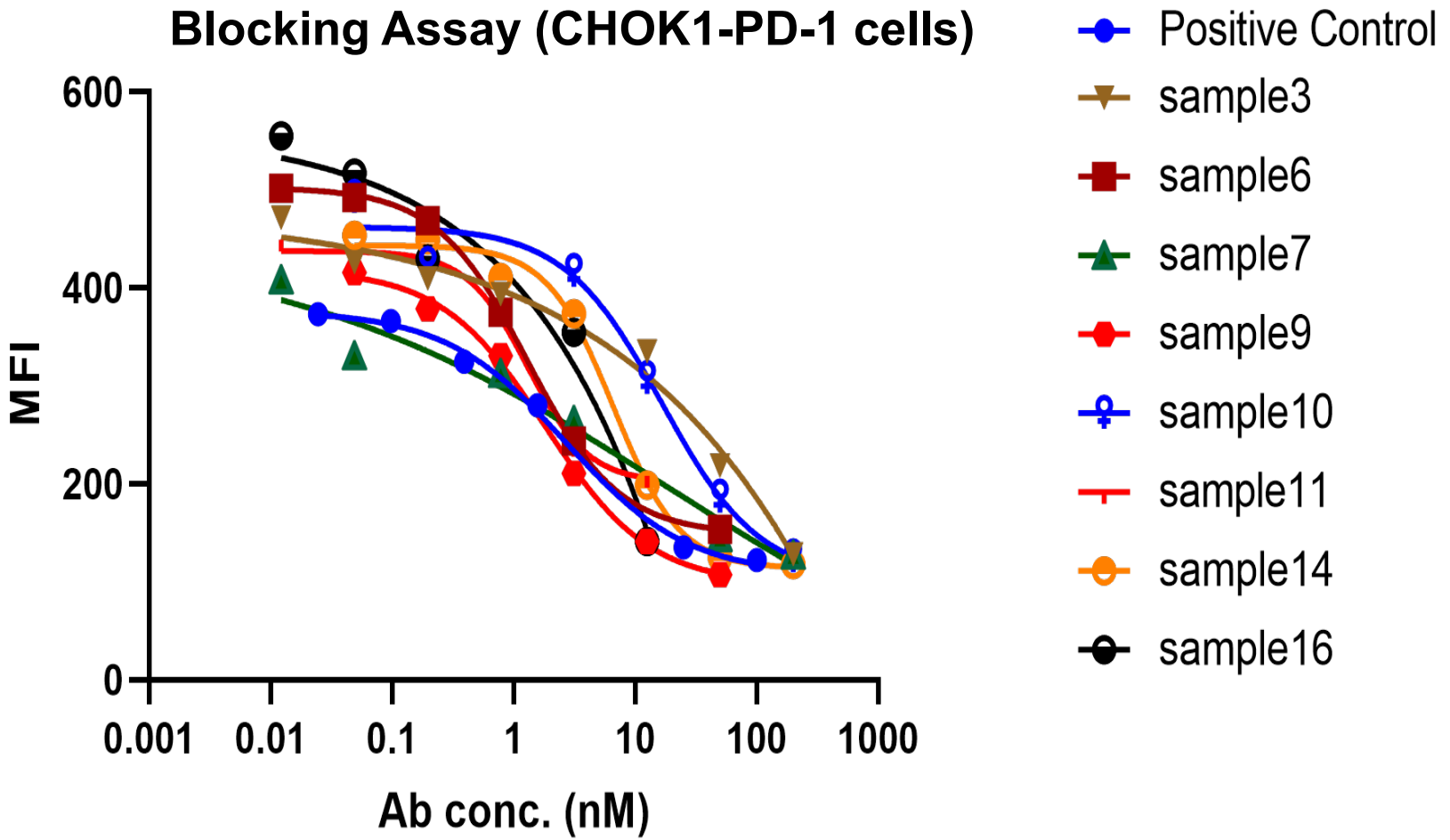
Unique Barcode	Locus	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3	FWR4
AACCGCCTC.ATGAGTTAC.AAATGGAGG	IGH							
AACCGCCTC.ATGAGTTAC.AAATGGAGG	IGK							
AACTGTATT.TAGTCTTGA.CTATTAGCC	IGH							
AACTGTATT.TAGTCTTGA.CTATTAGCC	IGK							
ACAAGGATC.ACCAAAATG.AGTTGCATC	IGK							
ACAAGGATC.ACCAAAATG.AGTTGCATC	IGH							
ACACACAAA.AGGAGGCGC.TGTCTATCA	IGH							
ACACACAAA.AGGAGGCGC.TGTCTATCA	IGK							
ACCAAGGAC.AATCAGTTT.ATATTGTAA	IGH							
ACCAAGGAC.AATCAGTTT.ATATTGTAA	IGK							
AGAAGCACT.TTCCATTGA.CAACCTCCA	IGK							
AGAAGCACT.TTCCATTGA.CAACCTCCA	IGH							
AGCACACAG.TAGGGATAC.CAATAGGGT	IGH							
AGCACACAG.TAGGGATAC.CAATAGGGT	IGK							
AGCACACAG.TTCCATTGA.CAACCTCCA	IGK							
AGCACACAG.TTCCATTGA.CAACCTCCA	IGH							
AGCCCAATC.ACTTTAACT.GAACGACAA	IGH							
AGCCCAATC.ACTTTAACT.GAACGACAA	IGK							
ATGTAATGG.ACCAGGTGT.ATCGAGTCT	IGH							
ATGTAATGG.ACCAGGTGT.ATCGAGTCT	IGK							
CAAACCGCC.TCGTTAGCA.GAACGACAA	IGH							
CAAACCGCC.TCGTTAGCA.GAACGACAA	IGK							
CAGCAAGAT.TTCCATTGA.GGTGAGTTA	IGK							
CAGCAAGAT.TTCCATTGA.GGTGAGTTA	IGH							
CTACAGAAC.TACTAGTCA.CGTCTAGGT	IGK							
CTACAGAAC.TACTAGTCA.CGTCTAGGT	IGH							
GACGGATTA.AGATAGTTC.TGTATGCGA	IGH							
GACGGATTA.AGATAGTTC.TGTATGCGA	IGK							
GACGGATTA.AGCGACACC.AAGCCTTCT	IGH							
GACGGATTA.AGCGACACC.AAGCCTTCT	IGK							
GACGGATTA.TTCCATTGA.GAACGACAA	IGK							
GACGGATTA.TTCCATTGA.GAACGACAA	IGH							
GAGCCAATA.CACCCAAAG.CAACGATCT	IGH							
GAGCCAATA.CACCCAAAG.CAACGATCT	IGK							
GAGCCAATA.GAGTGCGAA.ACAAACTTT	IGH							
GAGCCAATA.GAGTGCGAA.ACAAACTTT	IGK							
GAGTACATT.TACAGGATA.TGCATAGTA	IGK							
GAGTACATT.TACAGGATA.TGCATAGTA	IGH							
GCACTGTCA.AGCGACACC.CTAAGCTTC	IGK							
GCACTGTCA.AGCGACACC.CTAAGCTTC	IGH							
GCACTGTCA.AGCGACACC.CTAAGCTTC	IGK							
GGCAAGCAA.CACCCAAAG.ACAAACTTT	IGH							
GGCAAGCAA.CACCCAAAG.ACAAACTTT	IGK							
TCCAGTCGA.GTGTGTCGA.CAACCTCCA	IGK							
TCCAGTCGA.GTGTGTCGA.CAACCTCCA	IGH							
TCGAAGTGA.TTCCATTGA.CGTCTAGGT	IGK							
TCGAAGTGA.TTCCATTGA.CGTCTAGGT	IGH							
TGGCTCAGA.CACCCAAAG.CAACGATCT	IGH							
TGGCTCAGA.CACCCAAAG.CAACGATCT	IGK							
TGTACCTTA.TTCCATTGA.CAACCTCCA	IGK							
TGTACCTTA.TTCCATTGA.CAACCTCCA	IGH							

Case: High Affinity PD-1 Antibodies Identified



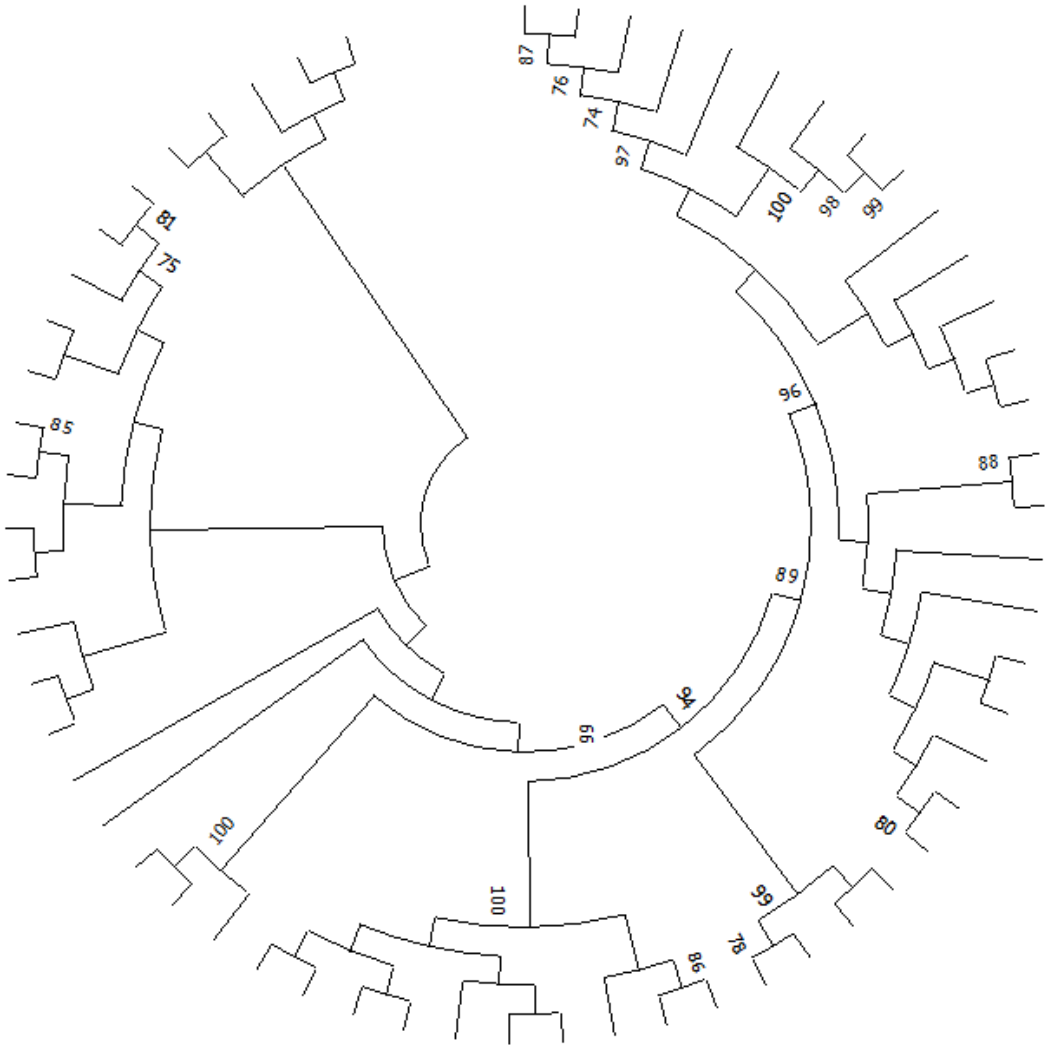
Antigen PD-1 → 59/62 positive

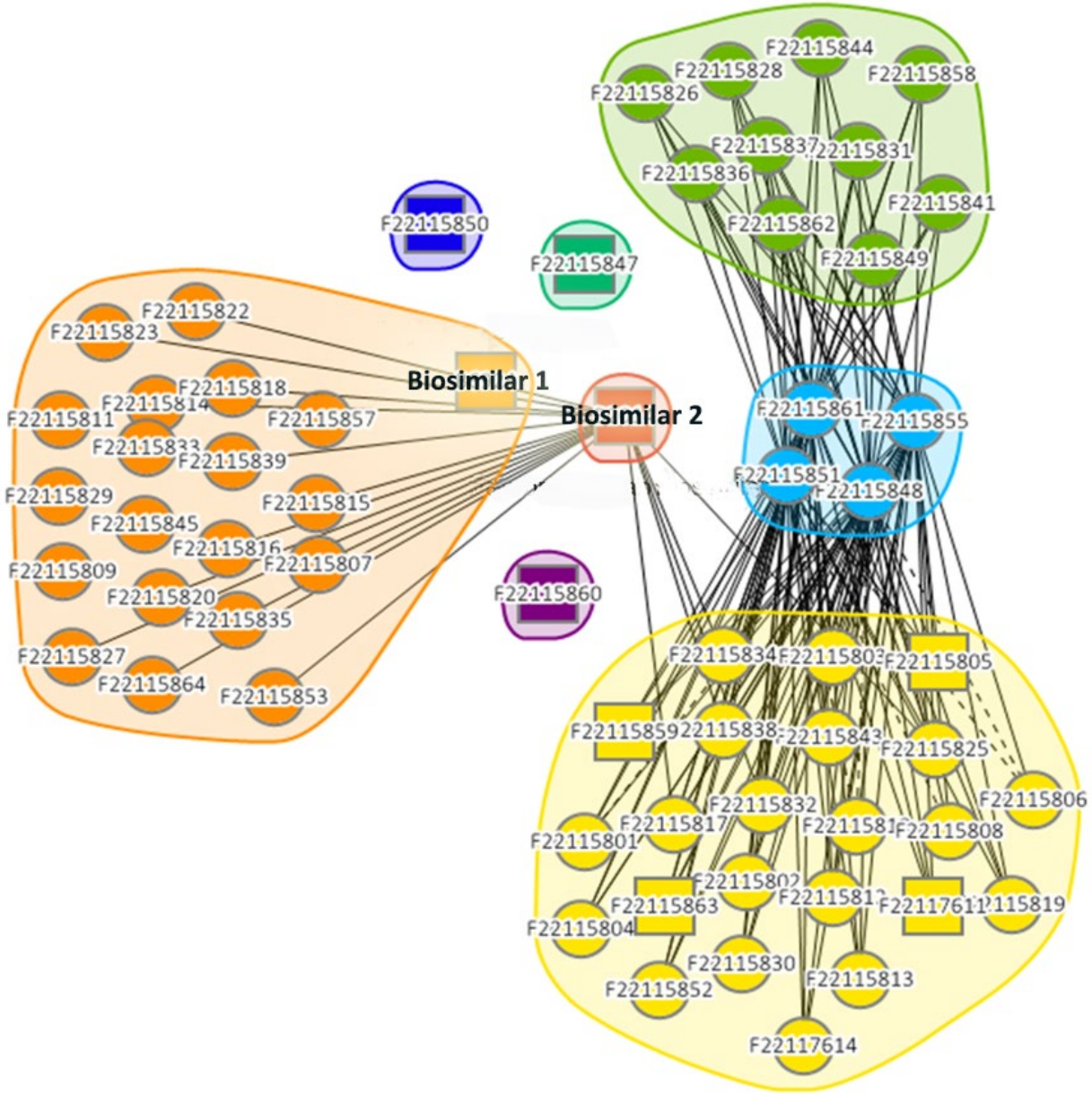
8 PD-1 Antibodies with Blocking Effect Identified



Blocking: 8 positive

Case: HTP SPR-based Epitope Binning by Caterra





百英生物
BIOINTRON

Thank You!

400-828-8830
xulong@biointron.com
info@biointron.com
www.biointron.com