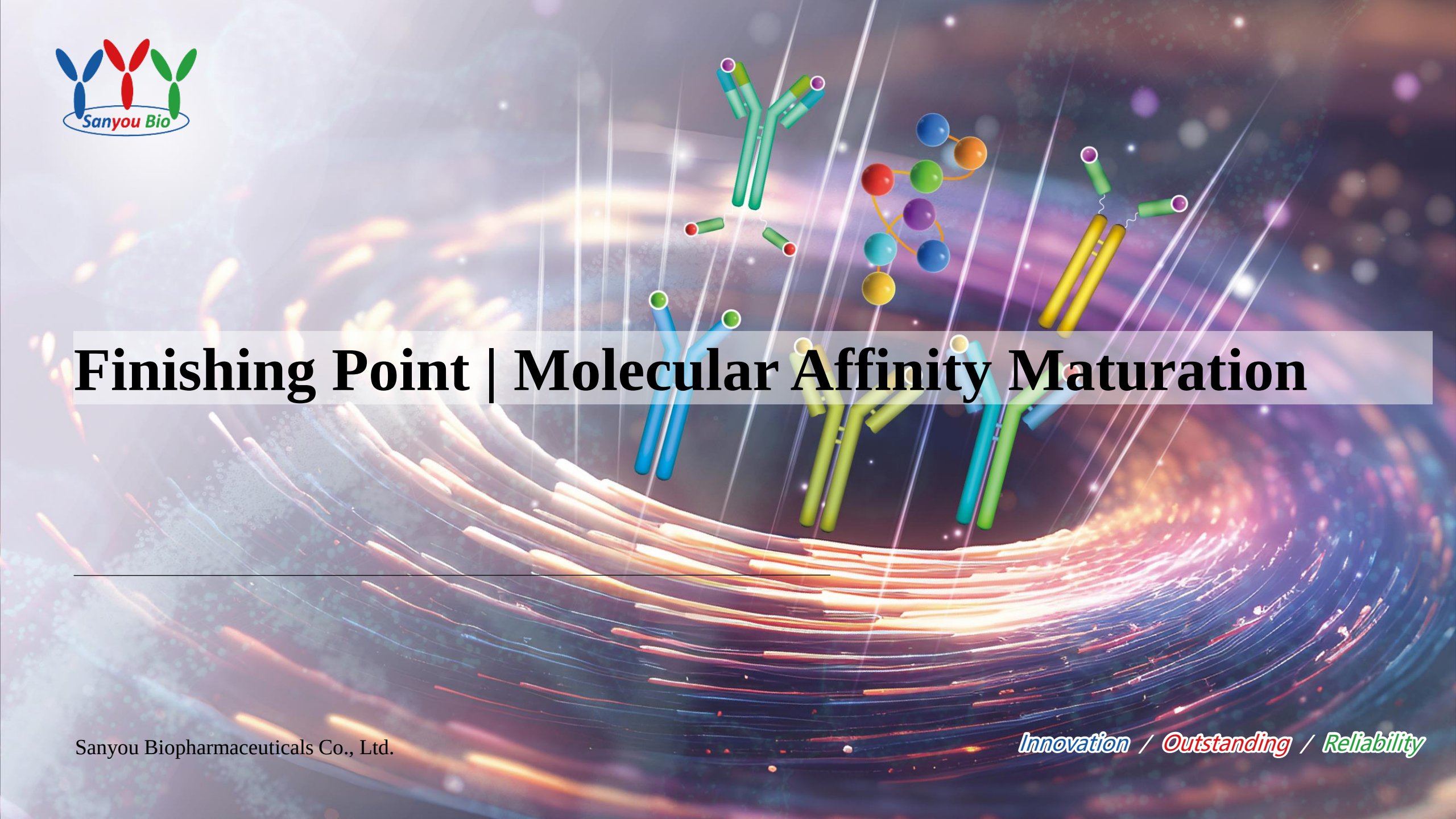


Finishing Point | Molecular Affinity Maturation

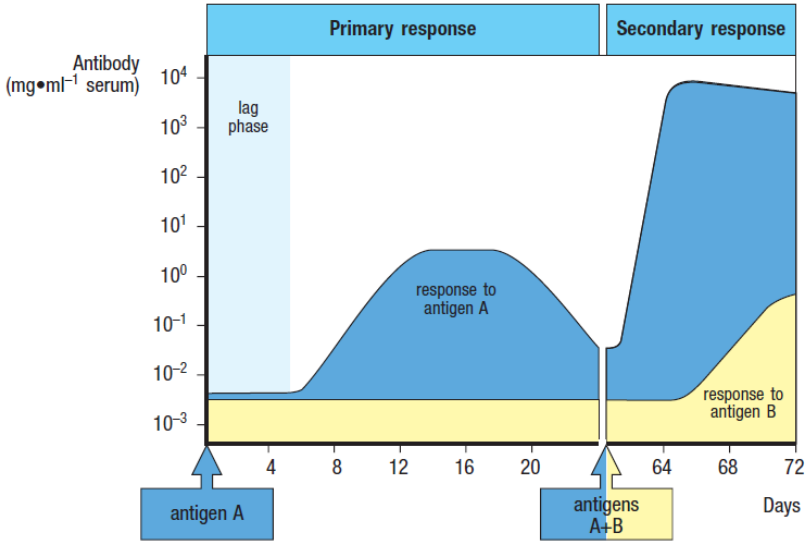
Sanyou Biopharmaceuticals Co., Ltd.

Innovation / Outstanding / Reliability

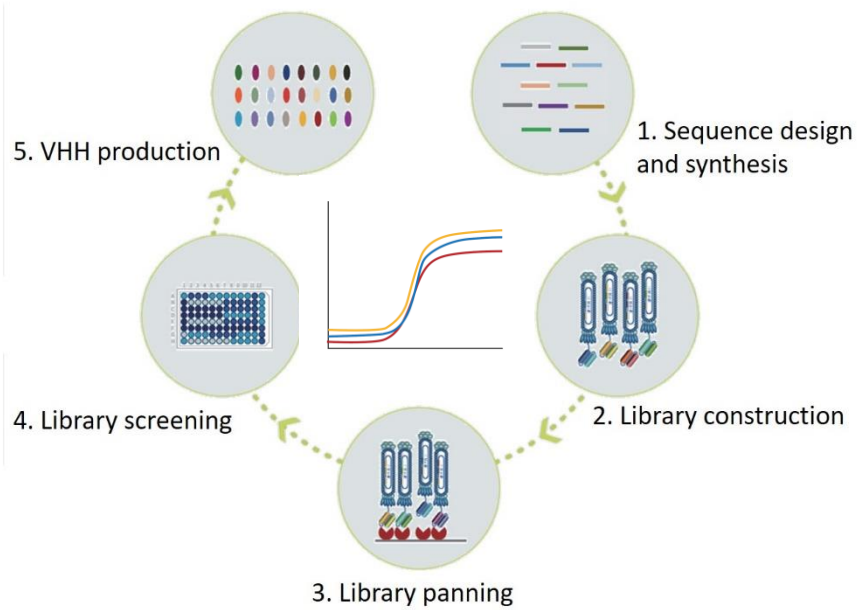
Introduction to and Application of Affinity Maturation

Technical Background:

- After the primary immune response in animals, the antibodies produced by innate immunity present low affinity and require *in vivo* affinity maturation to effectively respond to external antigen invasion, improve affinity for the antigen, and ultimately clear the antigen.
- Antibodies obtained from the immune antibody library or natural antibody library present lower affinity, especially antibodies derived from the natural library, which typically exhibit affinity comparable to innate immunity at the micromolar level. Therefore, it is necessary to simulate the process of *in vivo* affinity maturation and engineer the antibodies *in vitro*, so as to obtain antibodies that meet the expectations.



Typical Course of Antibody Response *In Vivo*
The secondary response to antigen A is stronger than the primary response.
(Janeway's Immunobiology 9th)



Improvement Process of *In Vitro* Affinity
The affinity of nanobodies is improved significantly after modification.

Principles of *In Vivo/Vitro* Affinity Maturation

Principles of *In Vivo* Affinity Maturation

- Antibody gene rearrangement

During the development of B cells, diverse antibody molecules are produced by recombining immunoglobulin gene fragments.

- Somatic hypermutation

After gene rearrangement, mature B cells undergo point mutations during differentiation and development upon antigen stimulation.

- Immunological memory

A second exposure to the same antigen triggers a stronger immune response.

In Vivo Affinity Maturation

01 Diversity of antibody gene rearrangement

02 Somatic hypermutation

03 Presence of immunological memory

Principles of *In Vitro* Affinity Maturation

- Simulation of *in vivo* gene rearrangement

During *in vitro* affinity improvement, CDR recombination, strand displacement and DNA recombination all involve the stimulation of antibody gene rearrangement *in vivo* to improve antibody diversity, thus increasing the possibility of screening high-affinity antibodies.

- Simulation of somatic hypermutation

The screening of candidate antibodies after introducing point mutations is a process that mimics somatic hypermutation *in vivo*. Mutations can be directly introduced at key functional sites of antibodies or introduced randomly.

- In silico

In silico allows for theoretical analysis to directly determine specific mutation strategies for antibodies.

In Vitro Affinity Maturation

01 Point mutations

02 CDR recombination

03 Strand displacement

04 DNA recombination

05 In silico

Methods for *In Vitro* Affinity Maturation

During in vitro antibody affinity maturation, the selection of mutation regions and the introduction of mutations are crucial issues. The current mutation strategies are mainly divided into four categories: random mutation, displacement, directed mutation and DNA recombination.

SOE-PCR

- By introducing mutations using primers, the codon corresponding to the target amino acid is replaced with NNK (degenerate amino acid codon). The target fragment is amplified by PCR for SOE PCR to obtain the mutation sequences.
- Random mutation and primers designed at specific positions can trigger mutants. Various combinations can be explored based on the strategy, enabling mutations and recombination to occur simultaneously.

Site-directed mutagenesis

- During affinity maturation of natural antibodies, the regions with somatic hypermutation are not evenly distributed but are primarily concentrated in CDRs directly contacting with the antigen. During in vitro affinity maturation, CDRs are the most commonly selected sites for site-directed mutagenesis, so as to obtain sufficient sequence diversity while avoiding disruption of the protein structure.

Strand displacement

- The heavy or light strand of a particular antibody is retained, and the other strand is combined with a randomized complementary strand from which mutants with higher activity are screened.
- One of the two strands of the antibody is immobilized, and a displacement library with sufficient diversity is constructed for the other. Random combinations may yield the best strand combination, and the strand displacement strategies often involve light-strand displacement.

DNA recombination

- It is a technique that involves using deoxyribonuclease I to cleave DNA into fragments of ≤ 50 bp, then randomly recombining these fragments and performing PCR amplification to generate a complete antibody gene. It involves the randomized cleavage, recombination and screening of antibody fragments, mimicking the affinity maturation process of natural antibodies to a certain extent and accelerating the in vitro directed evolution.

Strategies for *In Vitro* Affinity Maturation

Strategy 1

Antibody library with sequential mutations involving displacement in key CDRs

Note: The library construction strategy is for reference only, and is based on actual communication

Strategy 2

Antibody library with point mutations at each amino acid site covering 3 CDRs

VH-CDR1 single-site saturation mutagenesis

Parental	o	o	o	o	o	
Primer 1	NNK	o	o	o	o	20
Primer 2	o	NNK	o	o	o	20
Primer 3	o	o	NNK	o	o	20
Primer 4	o	o	o	NNK	o	20
Primer 5	o	o	o	o	NNK	20
SUM						100

VH-CDR2 single-site saturation mutagenesis

Parental	o	o	o	o	o	o	20
Primer 1	NNK	o	o	o	o	o	20
Primer 2	o	NNK	o	o	o	o	20
Primer 3	o	o	NNK	o	o	o	20
Primer 4	o	o	o	NNK	o	o	20
Primer 5	o	o	o	o	NNK	o	20
Primer 6	o	o	o	o	o	NNK	120
SUM							

VH-CDR3 single-site saturation mutagenesis

Parental	o	o	o	o	o	o	o	o	o	o	
Primer 1	NNK	o	o	o	o	o	o	o	o	o	20
Primer 2	o	NNK	o	o	o	o	o	o	o	o	20
Primer 3	o	o	NNK	o	o	o	o	o	o	o	20
Primer 4	o	o	o	NNK	o	o	o	o	o	o	20
Primer 5	o	o	o	o	NNK	o	o	o	o	o	20
Primer 6	o	o	o	o	o	NNK	o	o	o	o	20
Primer 7	o	o	o	o	o	o	NNK	o	o	o	20
Primer 8	o	o	o	o	o	o	o	NNK	o	o	20
Primer 9	o	o	o	o	o	o	o	o	NNK	o	20
Primer 10	o	o	o	o	o	o	o	o	o	NNK	20
SUM											200

Strategy 3

Antibody library with sequential mutations at multiple amino acid sites in key CDRs

VH-CDR1 saturation mutagenesis at 3 consecutive sites

Parental	o	o	o	o	o	o	o	o	o	o	o	o	
Primer-1	NNK	NNK	NNK	o	o	o	o	o	o	o	o	o	8.00E+03
Primer-2	o	NNK	NNK	NNK	o	o	o	o	o	o	o	o	8.00E+03
Primer-3	o	o	NNK	NNK	NNK	o	o	o	o	o	o	o	8.00E+03
Primer-4	o	o	o	NNK	NNK	NNK	o	o	o	o	o	o	8.00E+03
Primer-5	o	o	o	o	NNK	NNK	NNK	o	o	o	o	o	8.00E+03
Primer-6	o	o	o	o	o	NNK	NNK	NNK	o	o	o	o	8.00E+03
Primer-7	o	o	o	o	o	o	NNK	NNK	NNK	o	o	o	8.00E+03
Primer-8	o	o	o	o	o	o	o	NNK	NNK	NNK	o	o	8.00E+03
Primer-9	o	o	o	o	o	o	o	o	NNK	NNK	NNK	o	8.00E+03
Primer-10	o	o	o	o	o	o	o	o	o	NNK	NNK	NNK	8.00E+03
SUM													8.00E+04

VH-CDR saturation mutagenesis at 5 consecutive sites

Parental	o	o	o	o	o	o	o	o	o	o	o	o	
Primer-1	NNK	NNK	NNK	NNK	NNK	o	o	o	o	o	o	o	3.20E+06
Primer-2	o	NNK	NNK	NNK	NNK	NNK	o	o	o	o	o	o	3.20E+06
Primer-3	o	o	NNK	NNK	NNK	NNK	NNK	o	o	o	o	o	3.20E+06
Primer-4	o	o	o	NNK	NNK	NNK	NNK	NNK	o	o	o	o	3.20E+06
Primer-5	o	o	o	o	NNK	NNK	NNK	NNK	NNK	o	o	c	3.20E+06
Primer-6	o	o	o	o	o	NNK	NNK	NNK	NNK	NNK	o	o	3.20E+06
Primer-7	o	o	o	o	o	o	NNK	NNK	NNK	NNK	NNK	o	3.20E+06
Primer-8	o	o	o	o	o	o	o	NNK	NNK	NNK	NNK	NNK	3.20E+06
SUM													2.56E+07

Business Process for *In Vitro* Affinity Maturation

Technical Process

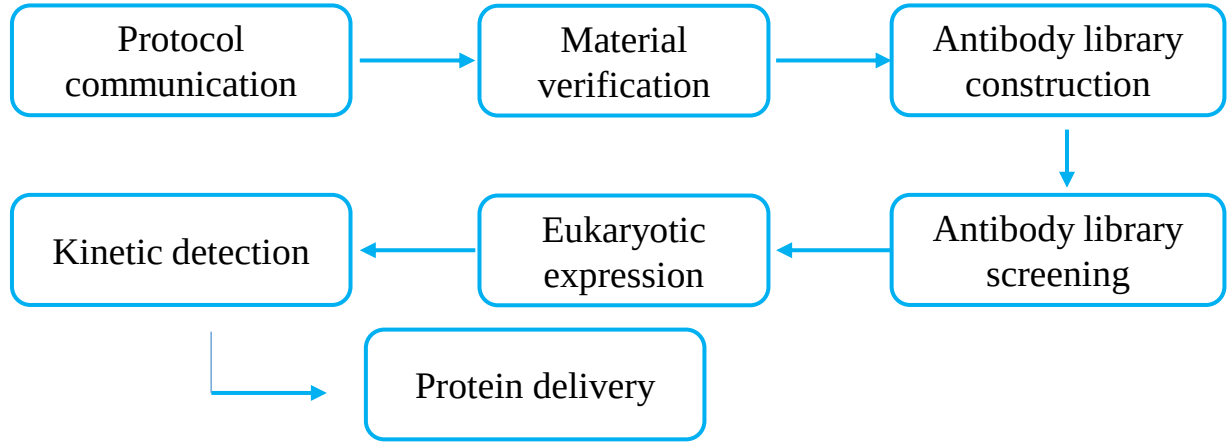
Library Design
- CDR walking
- CDR saturation mutations
- CDR residue scanning

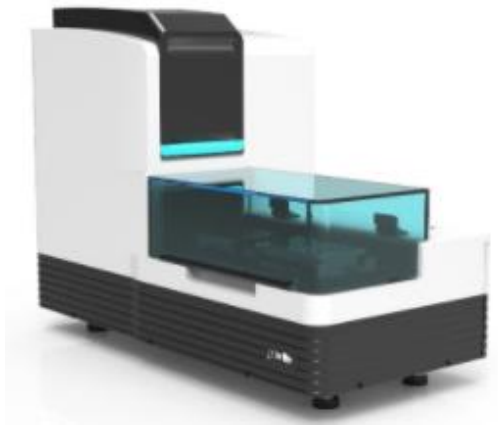
Library Construction
- Template preparation
- Molecular construction
- Library construction & QC

Biopanning
- Material preparation
- Off-rate panning
- Each round of sequencing analysis

Screening
- Preliminary screening & affinity ranking
- Expression and purification
- QC & Kinetics

Service Process

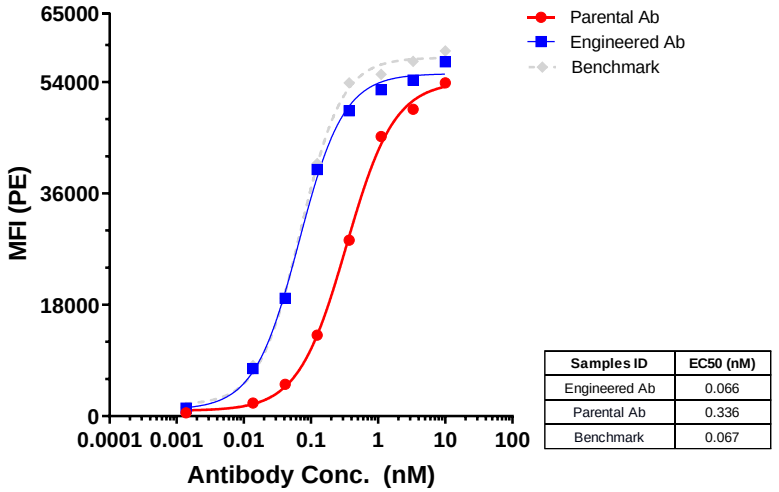




Representative Case 1

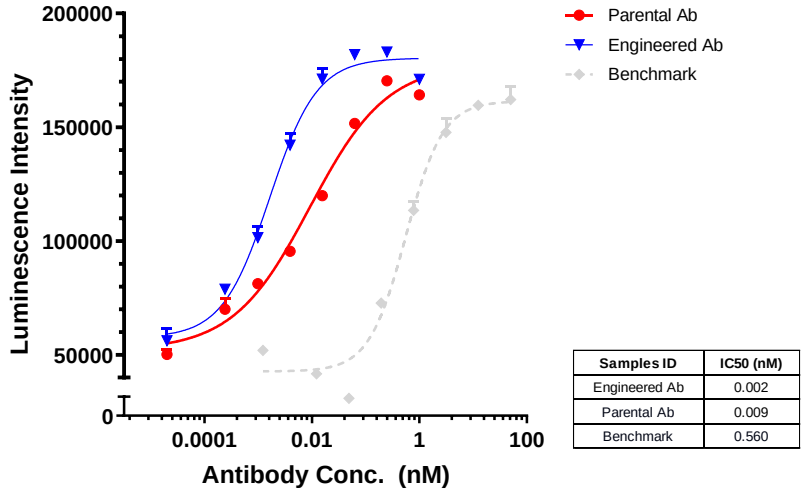
280-fold increase in antibody affinity and synchronized improvement in function

- **Background:** The antibody (Parental Ab) has better functional activity than the control antibody (Benchmark), but there is still a gap in affinity activity.
- **Purpose:** To increase the affinity of maternal antibody and maintain its functional activity.
- **Method:** Sanyou Bio employed the single and double-site saturation mutagenesis strategy for library construction combined with the targeted washing strategy for affinity improvement to obtain the preferred antibody with equivalent affinity activity to the control antibody (Benchmark).
- **Results:** As shown in Fig. 1A, the affinity of the pre-engineering antibody (Parental Ab) was increased by 5-fold after affinity maturation, which was comparable to that of Benchmark. As shown in Fig. 1B, the functional activity of the engineered preferred antibody (Engineered Ab) was increased by 280-fold compared with that of Benchmark. Both the affinity and functional activity were improved, so the engineering for affinity maturation was successful.
- **Conclusion:** The ultimate affinity maturation platform can simultaneously increase affinity by 280-fold and improve functional activity.



Samples ID	EC50 (nM)
Engineered Ab	0.066
Parental Ab	0.336
Benchmark	0.067

Fig. 1A Binding Affinity



Samples ID	IC50 (nM)
Engineered Ab	0.002
Parental Ab	0.009
Benchmark	0.560

Fig. 1B Functional Activity

Representative Case 2 - 276-fold increase in antibody affinity

- **Background:** The dissociation rate (kd) of Parental Ab is relatively fast, leading to unsatisfactory pharmacokinetic results *in vivo*.
- **Purpose:** To increase the antibody affinity by over 200-fold.
- **Method:** Sanyou Bio constructed a library with multi-point combination mutations, conducted 4 rounds of off-rate screening, and carried out the Fortebio affinity kinetic detection on the engineered antibody to obtain antibody molecules with low dissociation rate.
- **Results:** The affinity kinetic detection results of Engineered Ab are shown in Fig. 2. The KD value of Parental Ab was increased from 6.53 × 10^{-8} M to 2.37 × 10^{-10} M for Engineered Ab, indicating 276-fold improvement.
- **Conclusion:** The ultimate affinity maturation platform can simultaneously increase affinity by nearly 280-fold.

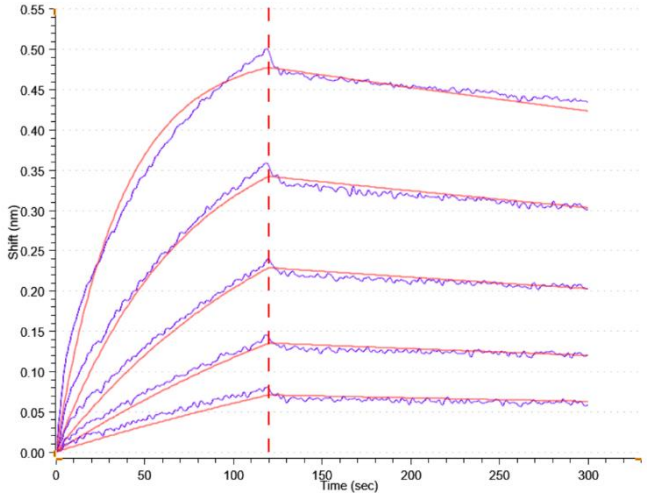


Fig. 2A Parental Ab

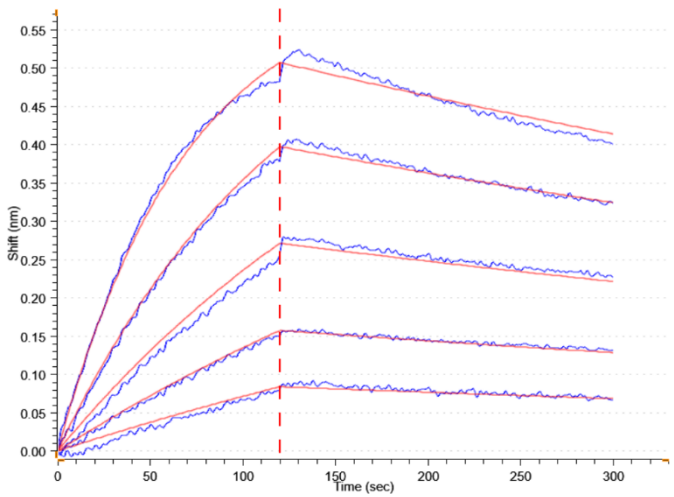


Fig. 2B Engineered Ab

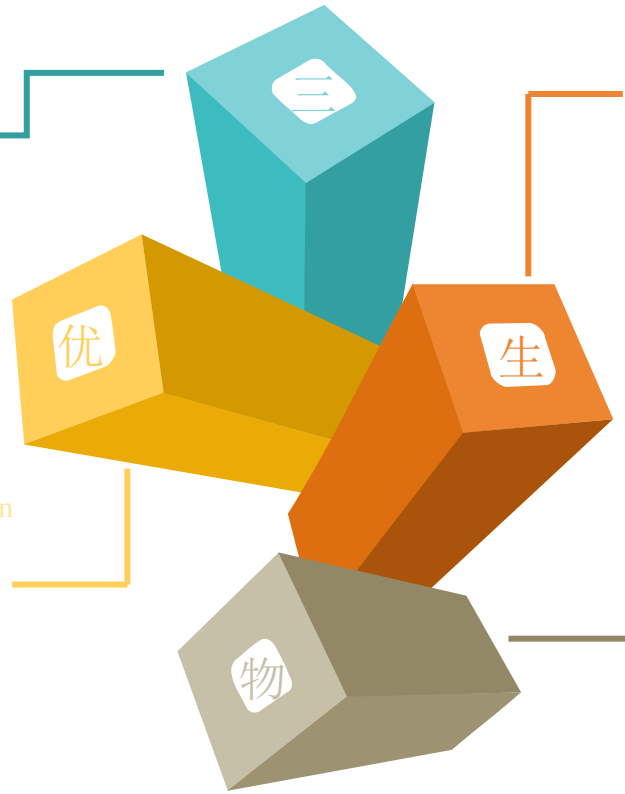
Samples ID	KD (M)	ka (1/Ms)	kd (1/s)
Parental Ab	6.53E-08	3.94E+04	2.57E-03
Engineered Ab	2.37E-10	3.70E+04	8.77E-06

Samples ID	R^2	Rmax (nm)	Ratio (WT/Ab)
Parental Ab	0.99	1.28	1
Engineered Ab	0.99	1.22	276

Preview of Next Chapter - Drug Optimization for Antibody Humanization

With computer-assisted deep simulation, high-precision structural simulation and site prediction, as well as comprehensive and systematic quality control verification, the degree of humanization reaches over 95%, with fewer than 6 reverse mutations.

- ✓ Humanization of murine antibodies
- ✓ Humanization of alpaca antibodies
- ✓ Humanization of rabbit antibodies



- ✓ Affinity improvement
- ✓ Functional activity modification
- ✓ Cross-species modification

- ✓ Druggability modification
- ✓ Half-life modification
- ✓ Blocking improvement

- ✓ Formulation modification

THANKS

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