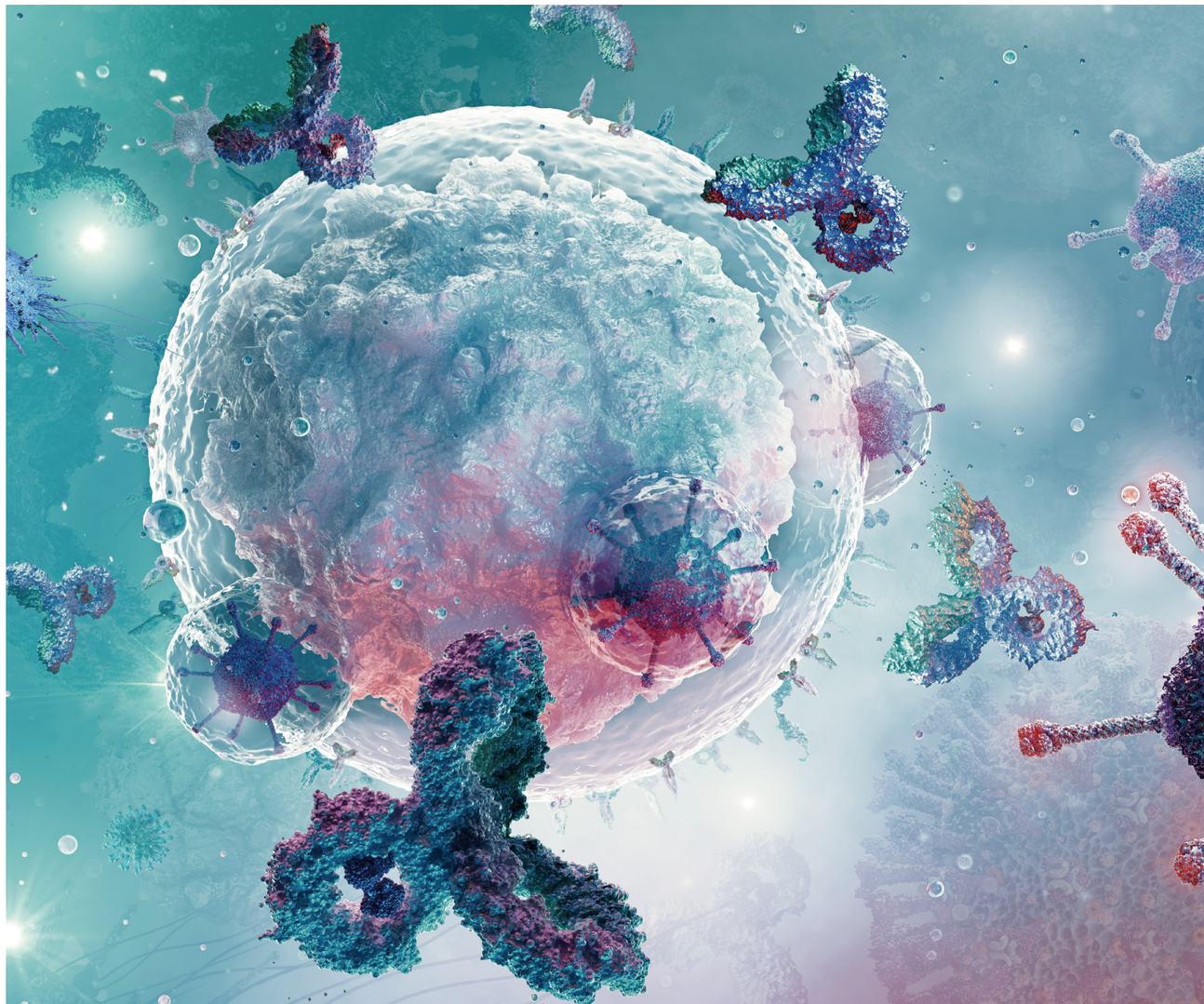
ProBio

Discovery of Functional Antibody Leads at Early Stage of Lead Screening

Our Values

Customer Centric · Innovation · Integrity



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Limitations of a conventional "binder-focused" workflow in therapeutic antibody discovery

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Functional screening is the most crucial step for therapeutic antibody discovery, providing key evidence to justify the necessity for further development of an Ab candidate. However, currently most antibody lead discovery strategies are limited by a "binder focused" screening workflow, in which at early stages of lead discovery, only binding assays can be performed to identify binders to the target, while functional screening can be performed only at a very late stage, using recombinantly expressed and purified Ab candidates. Such a workflow provides a comprehensive screening funnel to narrow down the candidate list in a step-by-step fashion, but frequently with a very low efficiency, especially when the functional binders only take a very small fraction among all binders, since most of the budget and time will be spent on the identification of undesirable, non-functional binders.

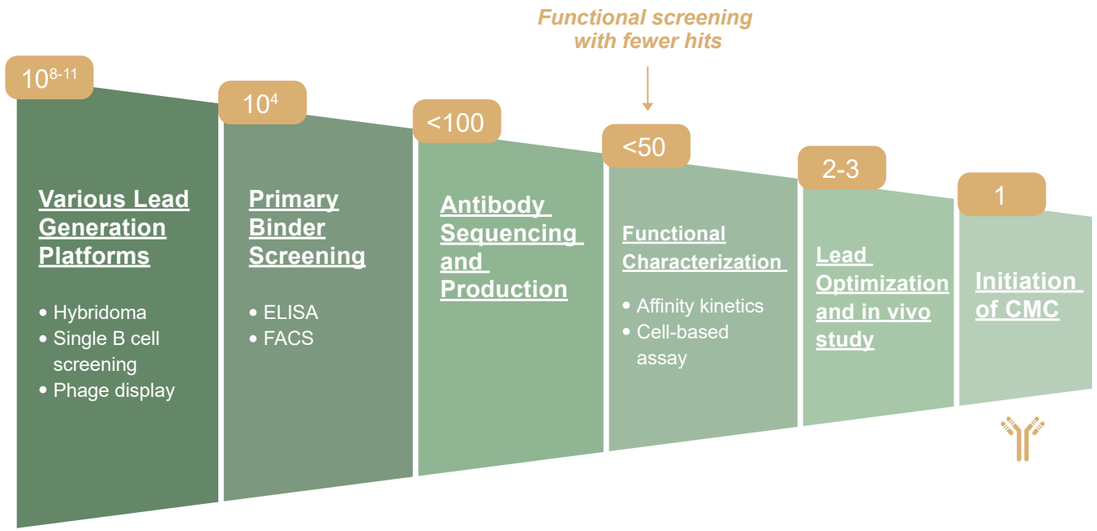

Figure 1: Low efficiency is the major bottleneck of a conventional "binder-focused" antibody discovery workflow. Massive resources are invested into the time-consuming, step-by-step screening schema based mostly on binding assays, resulting in a possible loss of diversity and lower chance of identifying functional Ab hits.

Therefore, in the next chapter, we will focus on the discussion of solutions that can be applied onto various antibody discovery platforms, to overcome these limitations and significantly improve the efficiency of Ab lead discovery.

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Discovery of functional antibody leads using various antibody generation platforms

Discovery of functional antibody leads using various antibody generation platforms

Functional lead discovery via hybridoma platform

The core concept of conventional hybridoma technology (figure 2, right panel) is to culture the cells in polyclonal condition initially, to maximally promote the survival of newly fused cells, followed by repeated subcloning step to recover stabilized monoclones. This strategy achieves a compromise between preservation of candidate hybridoma clones for further screening and prolonged timeline, but still, leading to the following limitation:

1. First of all, in cultured hybridoma clone of polyclonal nature, most of hybridoma cells do not secret antigen-specific antibody. Thus frequently the antigen-specific IgG in the supernatant is diluted and of low concentration, **making it difficult to run cell-based functional assays.**
2. Alternatively, if the hybridoma cells are cultured for an extended period to enrich IgG in the supernatant, the longer they are cultured, there may be a higher chance that those negative cells will outgrow those positive ones, **leading to loss of positive hits.**
3. Moreover, the subcloning process may take about 6~8 weeks, during which many hybridoma cells may not **survive due to their intrinsic nature of instability, again resulting in loss of positive hits**.

To address these limitations, an upgraded hybridoma platform, Powerdoma™ was developed (figure 2, left panel), in which the tedious and time-consuming process of subcloning is skipped , leading to shortened timeline and earlier achievement of monoclonal nature to allow functional screening to be performed at early stage of lead idenyification.

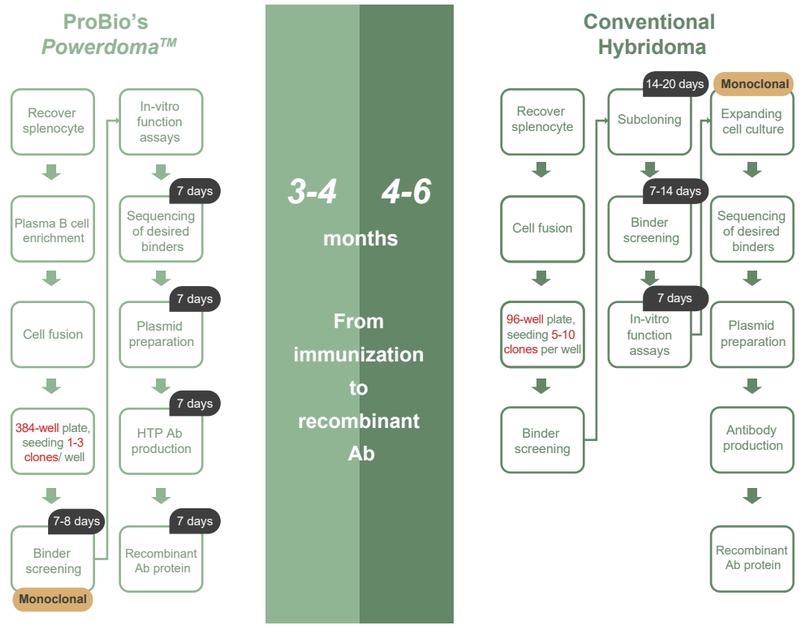


Figure 2: Conventional hybridoma vs. Powerdoma™

Discovery of functional antibody leads using various antibody generation platforms

Project statistics of Powerdoma™ platform

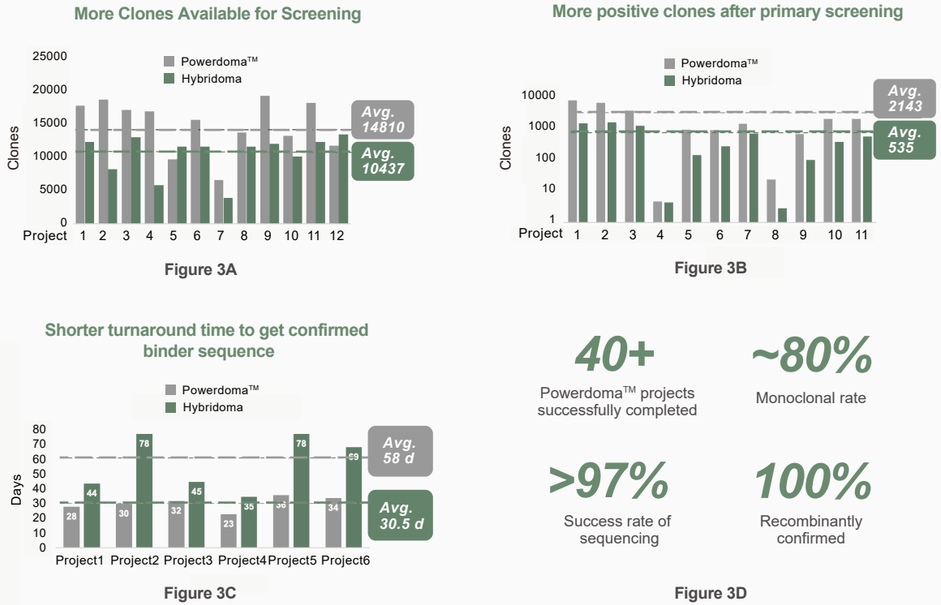


Figure 3 Project statistics of Powerdoma™ platform

Comparing to conventional hybridoma workflow, Powerdoma allows ~40% more hybridoma clones to be screened (figure 3A), with a shortened timeline decreasing from 58 to 30 days in average (figure 3B), and a four-fold higher positive hit rate (2000+ vs. 500+) in average from 11 projects with head-to-head comparison (figure 3C). Based on 40+ previously delivered projects using the Powerdoma workflow, a monoclonal rate of 80%, sequencing success rate of 97%, and 100% confirmation rate by recombinant expression, was observed (figure 3D).

Affinity ranking by KD ELISA using Powerdoma™ supernatant

Affinity ranking is a common practice to evaluate antibody kinetics with high throughput fashion. However, conducting such assay using Biacore or Octet can be very expensive, especially when it comes to hundreds of samples. ELISA, on the contrary, is a much cheaper way to assess relative affinity, but lacks the kinetics information. Moreover, the readout of a conventional ELISA assay based on a single dose of supernatant is determined by both affinity and IgG concentration, therefore, cannot be taken as a measurement to rank the affinity of Ab hits. To address this issue a **KD ELISA assay is developed as a cost-effective way to gain insight into the affinity ranking data.**

Discovery of functional antibody leads using various antibody generation platforms

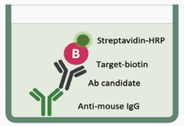


Figure 4A

KD ELISA utilize anti-mouse IgG secondary antibody to capture hits (antibody candidates) in the supernatant, followed by detection with biotinylated antigen (figure 4A). The concentration of anti-mouse IgG is set to a relatively low level, to make sure roughly the same amount of hits across all testing samples is captured.

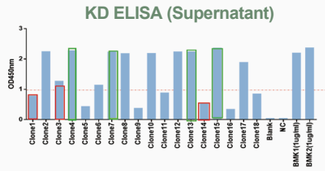


Figure 4B

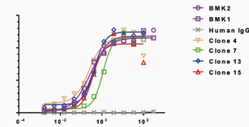


Figure 4C

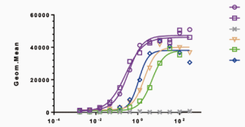


Figure 4D

In a primary screening assay using Powerdoma supernatant based on kD-ELISA, a number of potentially strong binders (green box) and weak binders (red box) were identified (figure 4B). Then these selected candidates were recombinantly expressed and characterized by full curve ELISA. Consistently, the previously identified strong binders (clone 4, 7, 13, 15 in green box) showed a high binding affinity similar to benchmarks (figure 4C), while those weak binders (clone 1, 3, 14) showed much lower affinity than benchmarks (figure 4D).

Reporter Gene Assay (RGA) using Powerdoma™ supernatant

Reporter Gene Assay (RGA) is widely used in therapeutic antibody discovery as a powerful tool to identify candidates with desirable biological function. Comparing to primary cell based assay, RGA usually comes with better assay window and good intra- and inter-assay consistency, making it an ideal tool for high throughput screening at early stage of Ab discovery.

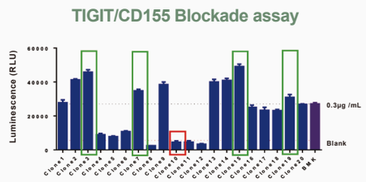


Figure 5A

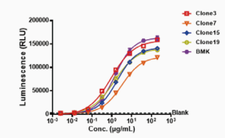


Figure 5B

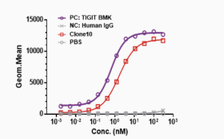


Figure 5C

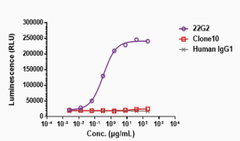
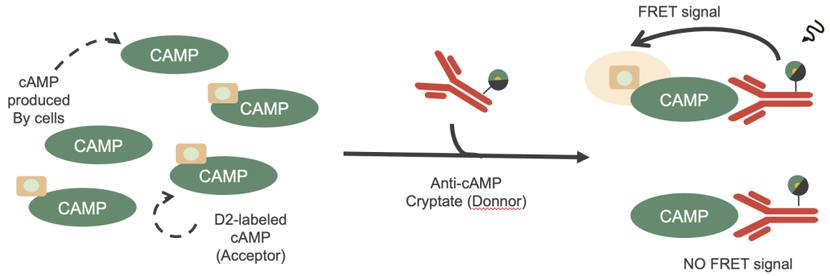


Figure 5D

TIGIT reporter gene assay was conducted with powerdoma™supernatant (figure 5A), identifying four potential functional blockers of TIGIT/CD155 signaling (clone 3, 7, 15, 19, in green box), and one non-functional binder (clone 10, in red box). These candidates were recombinantly expressed and the purified Abs subjected to full curve blocking assays. As shown in figure 5B, these previously identified functional blockers are confirmed to potently block TIGIT/CD155 signaling, while the non-functional binder (clone 10) binds to TIGIT (figure 5C) without signaling blockade (figure 5D).

Discovery of functional antibody leads using various antibody generation platforms

GPCR antagonist/agonist assay using Powerdoma™ supernatant



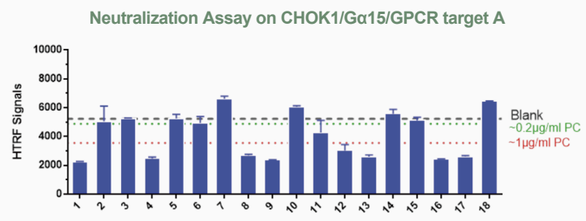


Figure 6A

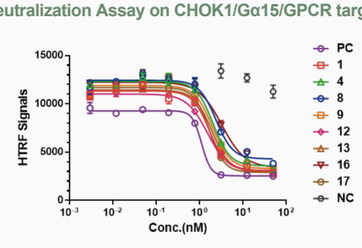


Figure 6B

cAMP assay is a well-recognized approach to evaluate the agonist/antagonist activity of antibody against GPCR targets. By such an assay using powerdoma™ supernatant (figure 6A), a number of potential antagonist Abs against target A were identified. Consistently, after recombinant expression and purification, these Abs were confirmed to block target A mediated cAMP production in a dose-dependent manner (figure 6B)

Discovery of functional antibody leads using various antibody generation platforms

Functional lead discovery via single B cell screening platform

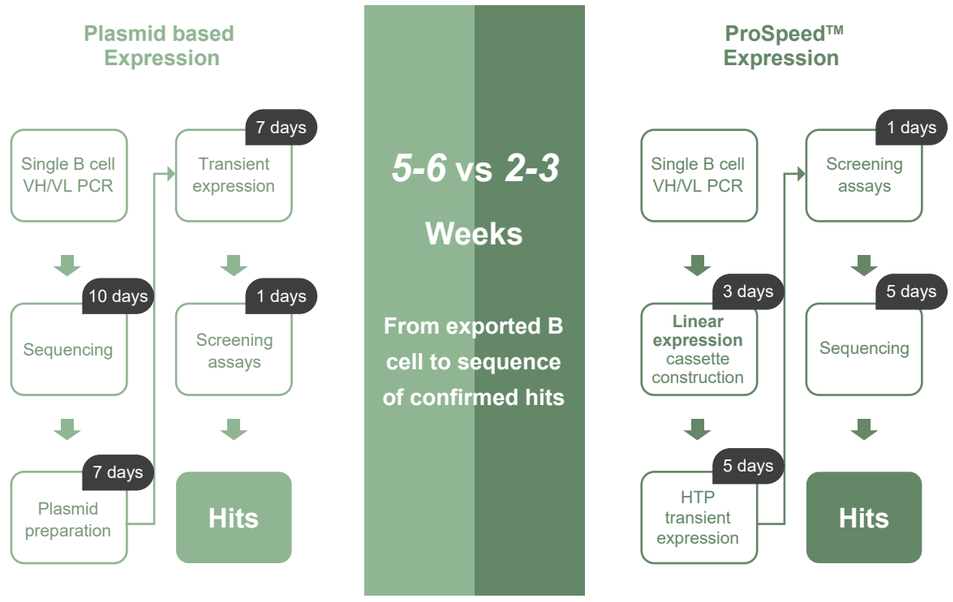
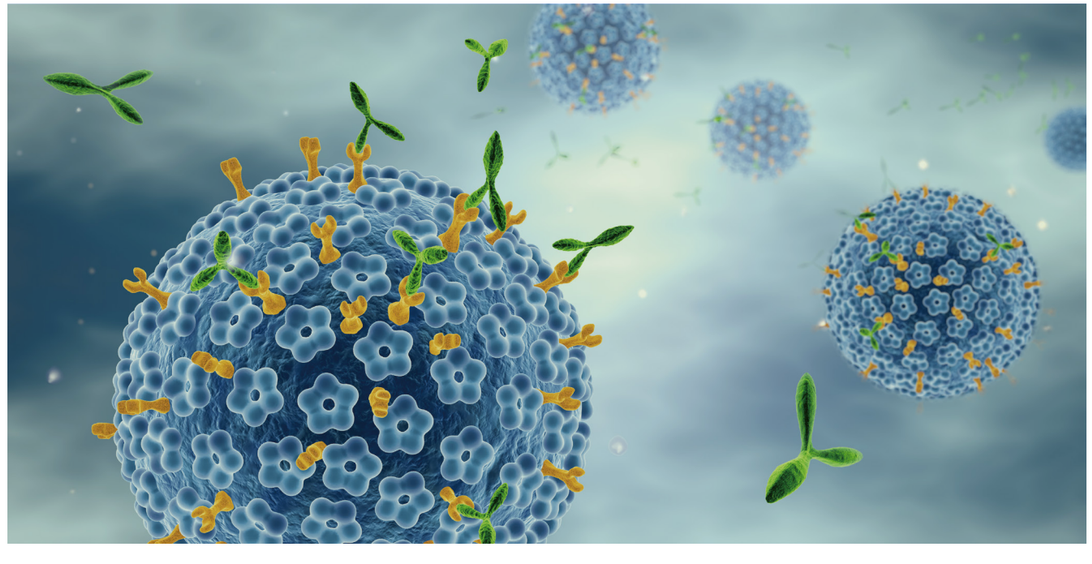


Figure 7. Workflow of plasmid-based and ProSpeed™ expression



Discovery of functional antibody leads using various antibody generation platforms

To confirm the binding & function of antibody sequences derived from recovered positive B cells, conventional work-flow (figure 7, left panel) relies on costly and time-consuming sequencing and gene syntheses/plasmid preparation, followed by transient transfection and antibody purification.
In contrast, in the ProSpeed™ workflow (figure 7, right panel), the DNA fragment of variable region from antibody heavy chain and light chain is amplified by PCR, and cloned into the linear expression cassette, which already contains the antibody constant region and all necessary expression module. **Without any need of plasmid preparation,** the reaction mixture is used for transient transfection. The antibody expression is usually conducted in 96-deep well plate in a high throughput fashion, and the typical range of IgG concentration in the supernatant is 10μg/ml to 100μg/ml, which is usually sufficient **for binding confirmation and cell-based functional characterization afterward.**

In general, the removal of laborious single B cell sequencing and gene synthesis from the workflow greatly **reduces the turnaround time from exported B cell to sequence of confirmed hits, from 5-6 weeks to 2-3 weeks, and with a substantial reduction of cost as well.**

Functional Screening with ProSpeed™ Supernatants

Similar to Powerdoma™ platform, ProSpeed™ expression supernatant can also be applied to RGA functional assay.

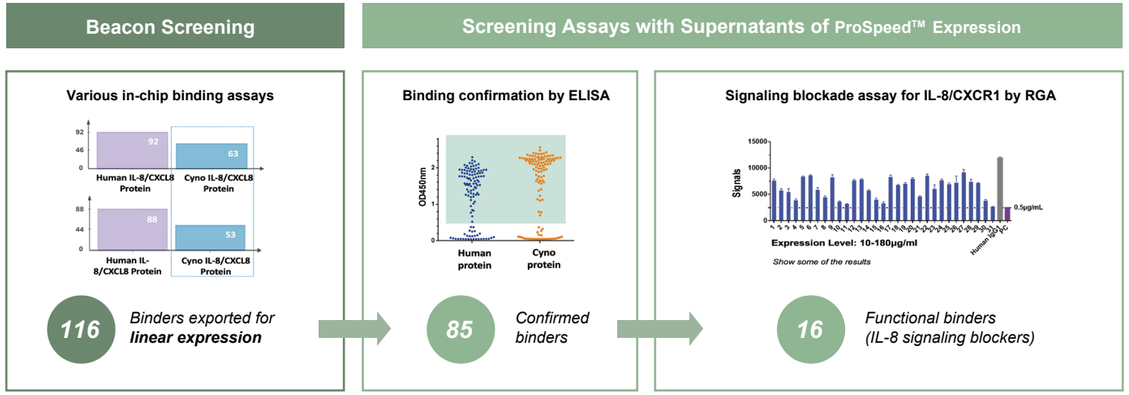


Figure 8A | Figure 8B | Figure 8C

In the case study of IL-8 antagonist antibody discovery (figure 8), 116 positive B cells were identified by Beacon on-chip screening with human and cyno IL-8 proteins, followed by ProSpeed™ linear expression (figure 8A). The supernatant of ProSpeed™ expression were first tested by ELISA for binding to human and cyno IL-8, confirming 85 hits were dual binders (figure 8B), among which 16 candidates were shown to block IL-8/CXCR1 signaling by reporter gene assay (figure 8C)

Multiple Rounds of Screening Beyond Beacon's Limits

In addition to cell-based functional assay, ProSpeed™ expression supernatant also enables identification of specific binders against a matrix of positive and negative antigens by multiple rounds of screening.

Discovery of functional antibody leads using various antibody generation platforms

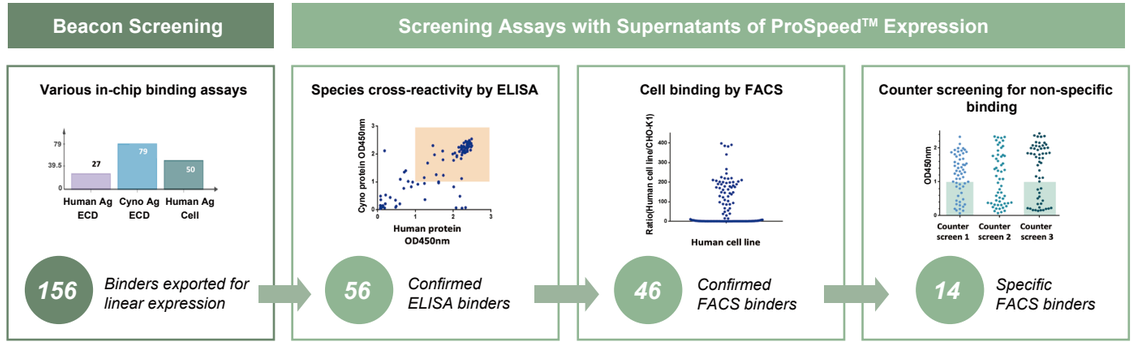


Figure 9A | Figure 9B | Figure 9C | Figure 9D

In this case study, 156 positive B cells were identified by Beacon on-chip screening with binding to human, cyno ECD proteins and human antigen cell line, followed by ProSpeed™ linear expression (figure 9A).
Using this ProSpeed™ expression supernatant, 56 candidates were confirmed to be binders to human and cyno Ag proteins by ELISA (figure 9B), and 46 candidates specific binder to human Ag cell line by FACS (figure 9C), among which only 14 showed minimal cross binding to 3 undesired Ags (with high homology to the target Ag) in additional counter-screen assays (figure 9D).

Improved Assay Sensitivity to Reduce False Negatives

Due to the usually higher concentration of Abs in ProSpeed™ expression supernatant, the sensitivity of assays using such supernatant is usually improved with reduced false negative results (figure 10).

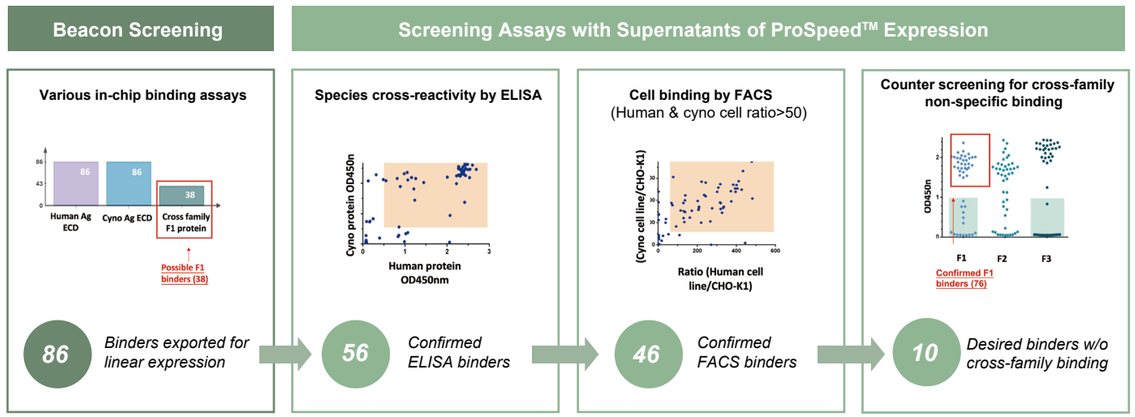


Figure 10A | Figure 10B | Figure 10C | Figure 10D

The initial on-chip Beacon screening identified 86 positive B cells binding to human and cyno antigen proteins, among which only 38 hits shown undesirable binding to cross-family Ag F1.
However, in the follow-up screening using supernatant of ProSpeed™ expression with improved assay sensitivity, 76 of them showed cross bind to family Ag F1, and only 10 hits were confirmed to be the desirable non-cross binders to all three family Ags F1, F2 and F3.

Discovery of functional antibody leads using various antibody generation platforms

Functional lead discovery via phage display platform

Unlike the hybridoma and single B cell approaches based on cultured mammalian cells, a phage display approach for Ab discovery relies on a prokaryotic system. As a result, phage supernatant containing secreted Ab fragment is usually contaminated with components that are harmful to the health of cultured mammalian, thus incompatible with most cell based function assays. Additionally, the concentration of secreted Ab in a phage supernatant is usually too low for many assay formats that require a minimal concentration of Abs. Therefore, it is usually very challenging to perform functional screening using a phage supernatant.

However, customized panning schemes for a phage library can still be developed as a functional screening approach to enrich antibodies with desired functional characteristics at early phase of lead discovery.

Customized panning scheme for a phage library to enrich antibodies capable of mediating internalization

In an ADC (antibody drug conjugate) drug, the cytotoxic payload is internalized together with the conjugated Ab upon binding to its target, usually a Tumor-Associated Antigen (TAA), to kill the cancer cells. Therefore, the potency of the targeting Ab in mediating internalization is a key determinants of a ADC drug, and the discovery of such a TAA Ab is highly desirable

In order to improve the success rate in identifying antibody candidates capable of effectively mediating internalization, a panning scheme **is designed to enrich for antibodies with such features, as depicted in figure 11.** Briefly, the initial binding (step 2) and wash (step 3) is conducted at 4° C to prevent internalization. Then the culture is transferred to 37° C to allow the endocytosis of phage particles (step 4), but only for a very short period of time to avoid degradation of phage in lysosome. After the wash of the remaining surface-bound phage (step 5), the internalized phage is recovered from cell lysate (step 6). This cycle of panning can be repeated for several times to improve the effectiveness of enrichment.

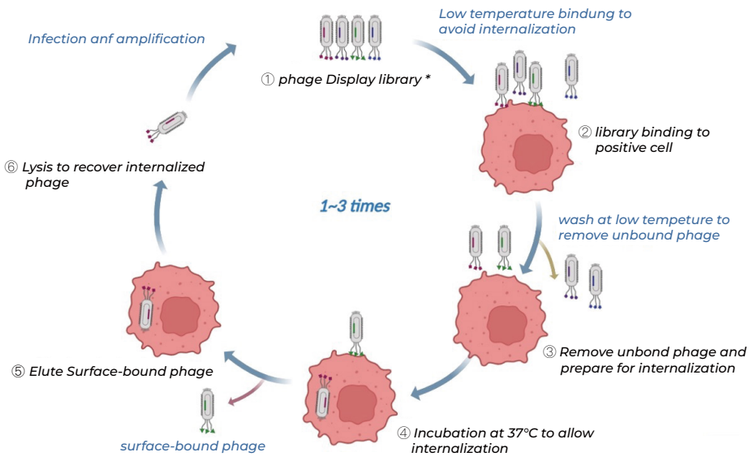


Figure 11: Panning process to enrich for antibodies with internalization capability

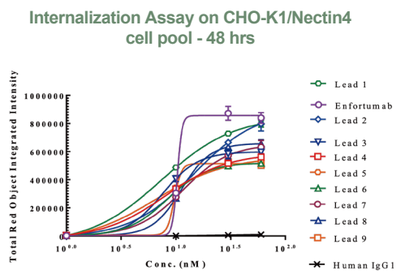


Figure 12: Confirmation of effective internalization mediated by purified Nectin4 Abs discovered via a panning scheme described herein

Discovery of functional antibody leads using various antibody generation platforms

Customized panning scheme for a phage library to enrich antibody with higher affinity

For some targets/indications, higher affinity of an Ab lead is desired. To more effectively enrich such Ab leads with a higher affinity, a customized panning scheme employing competition with an free target antigen can be used, as described in figure 13.

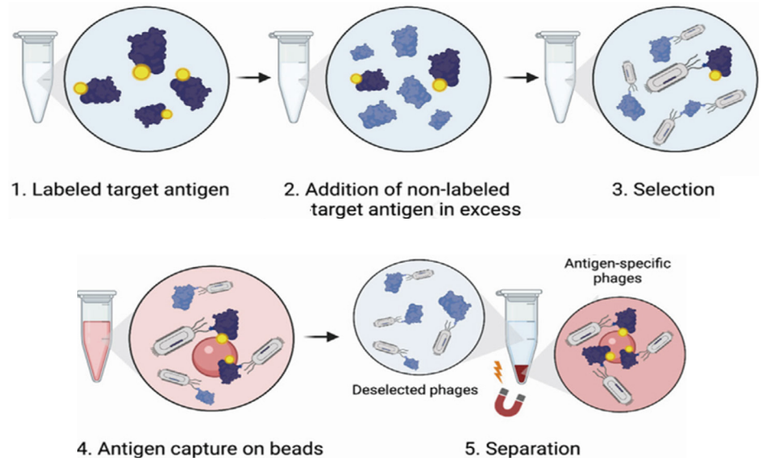


Figure 13: A competition panning scheme to enrich for antibodies with higher affinity

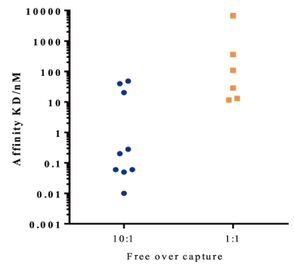


Figure 14: Affinity distribution of purified Abs discovered using different stress of competition panning. A higher stress of competition applied during library panning resulted in the identification of Ab leads with higher affinity

Discovery of functional antibody leads using various antibody generation platforms

In order to enrich for antibodies with higher affinity, competitive panning can be applied (figure 13). Briefly, the phage library is incubated with biotinylated antigen captured to beads in the presence of excessive amount of non-biotinylated, free antigen (step 2, 3). Thus, only Ab candidates with a higher affinity can resist the competition with free antigen and remain bound to the captured antigen after extensive wash. By repeating such a competition panning for multiple rounds, there is a good chance that binders with higher affinity will be enriched. As a result, Ab leads identified under a higher stress condition (higher "free" vs "capture" ratio) showed a high affinity (figure 14).

Customized panning scheme for a phage library to enrich pH selective antibodies

pH-selective antibody shows differential binding affinity to its target at different pH, namely it binds to the target with strong/normal affinity at one pH, while the affinity is greatly reduced under another pH condition. Such a selectivity in target binding at different pH may be a desirable feature under certain circumstances. For example, Abs with a low affinity at neutral pH (7.4) may not bind to the target substantially in the peripheral (pH7.4) to avoid the antigen sink effect and fast clearance, while in the acidic tumor micro environment, the same Ab with a high affinity at acidic pH 6.0 can strongly bind to the target and achieve an improved tumor selectivity .

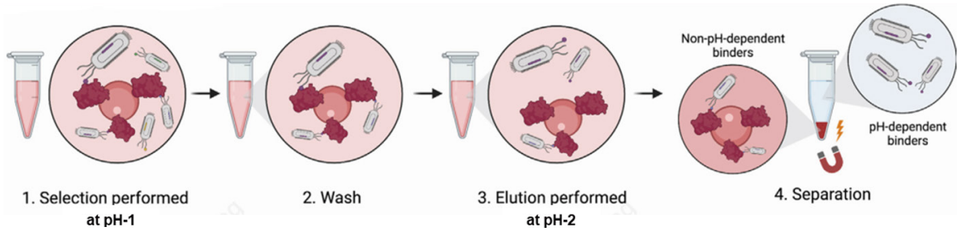


Figure 15: Panning process to enrich for pH-selective antibodies

To obtain antibody with pH-selective binding, a customized panning scheme can be designed as depicted in figure 15. The initial binding (step 1) and wash (step 2) are carried out under certain pH-1, while the elution (step 3) is done at different pH-2. After repeating this panning schedule for multiple rounds, pH dependent hits with higher affinity at pH1 but low affinity at pH-2 can be enriched (step 4).

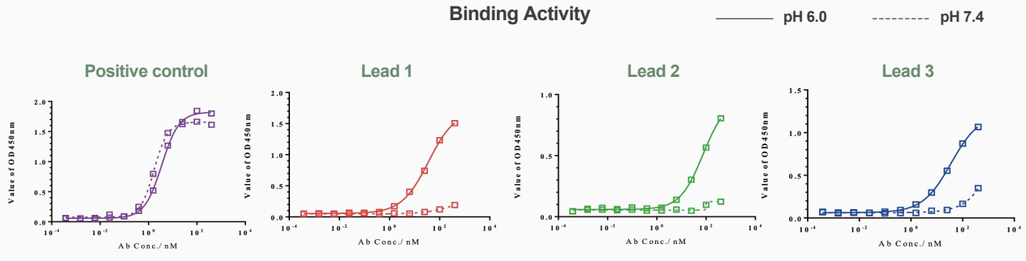


Figure 16: Differentiated affinity of pH-selective antibody

Following such a pH selective panning scheme, as shown in 16, three leads with selective binding at pH6.0 but low/no binding at pH7.4 were identified, comparing with a reference Ab (Batocilmab) which shows no pH selectivity.

Discovery of functional antibody leads using various antibody generation platforms

Customized panning scheme for a phage library to enrich antibody with desired epitope

The potency or functionality of a therapeutic Ab is determined by not only its affinity, but also the binding epitope, or where it binds on a target protein. For instance, antagonist antibodies aimed to block the cell signaling mediated by the interaction of a cytokine with its receptor, have to bind to the right place or epitope to completely block the signaling.

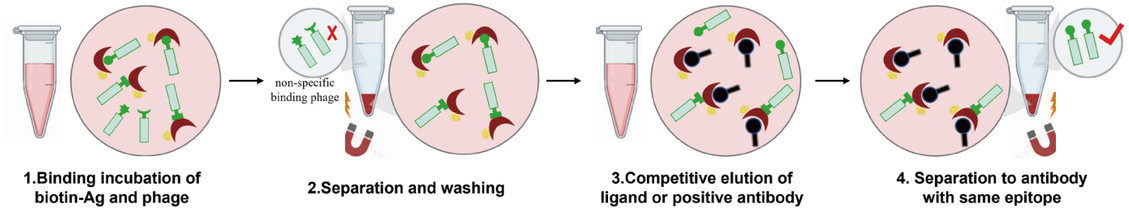


Figure 17: Panning process to enrich for antibodies with alternative epitope via epitope masking

In order to steer the direction of antibody enrichment towards desired epitope, masking antibody is co-incubated with phage in the competitive panning process (figure 17). Those that bind to the same epitope will be retained in suspension, while those bind to alternative epitope will be captured by antigen (step 2). These two fractions can easily be separated via streptavidin conjugated magnetic bead coupled with biotinylated antigen.

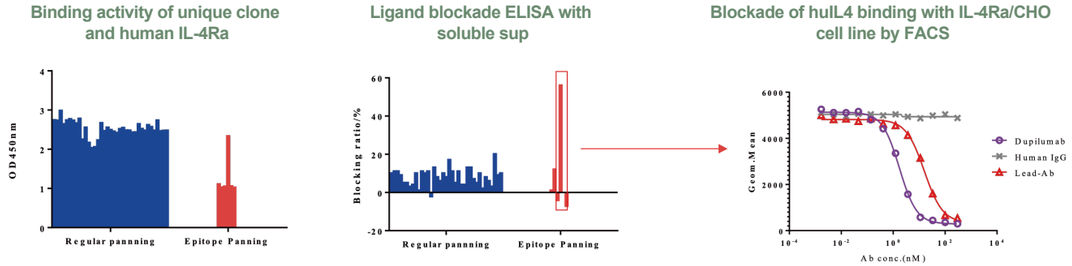


Figure 18: Antibody affinity distribution under different competitive panning stress

In the case study of IL4Ra neutralizing antibody, both regular and epitope panning are applied as comparison. Regular panning generates a wide range of candidates but none of them shows significant blockade against IL4Ra and IL4 interaction (figure 18A, B, blue bar). Alternatively, epitope panning leads to much narrowed diversity but the most dominant one gives relatively stronger blocking capability (figure 18A, B, red bar). The top candidates is further analyzed by dose-dependent competition assay against IL4 in flow cytometry assay, along with clinical benchmark Dupilumab, both of which show robust inhibition of IL4Ra/IL4 interaction (figure 18C).

03

The selection of a suitable partner to discover functional antibody leads

The selection of a suitable partner to discover functional antibody leads

To improve the chance of discovering Ab leads with the desired functionality, suitable Ab lead generation platforms compatible with function screening at early stage of discovery will be critical. Equally important, working with an experienced partner capable of providing such a customized, functional screening based solution will greatly improve the chance of success.

With 20 years of experience in biological drug discovery and development, ProBio is such an ideal partner for therapeutic antibody discovery , thanks to its extensive expertise and integrated solutions tailored to client's project needs.

Tailored Discovery Strategies for Functional Leads , Ensuring the Success Rate!

An Upgrade of Conventional Hybridoma Technology

- Powerdoma™ **vs Hybridoma**: **1-1.5 month saved**
- **Various functional screening feasible at very early stage of discovery:**
  Affinity ranking by KD ELISA, reporter gene assay, GPCR antagonist/agonist assay, etc.

A Perfect combination of Beacon® and ProSpeed™ expression

- Breaking-through the technical limitations of single B cell screening technology:
  - Improved assay sensitivity
  - Compatibility with multiple rounds of various functional assays
  - Better cost-effectiveness
- **1 month** to get confirmed functional antibody sequences

Various libraries for different Ab formats

- Naïve/immunized library
- Human naïve library

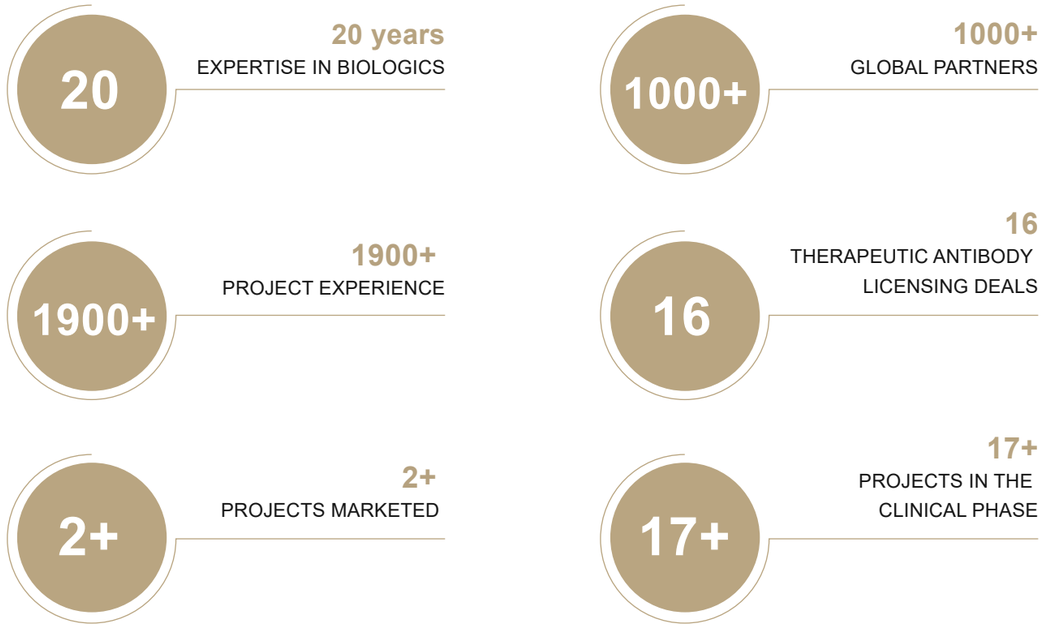
Various functional screening feasible at panning and screening process

How to select a suitable partner to discover functional leads?

Highly Integrated Discovery Platforms for Faster Advancement to the Clinical Stage!

Ab lead Optimization from developability and functionality:	Well-established pharmacology platforms in house:
Ab humanization: Industry-leading timeline **in 2.5 weeks**	Abundant Ready-to-Use assays & animal models
Ab affinity maturation: Guarantee **10-fold** affinity improvement	Tailored full-service capability in cell lines, disease models and method development
Ab developability: prediction, optimization, and assessment in early stage	

Excellence Proven by Impressive Track Record



Discovery of Functional
Antibody Leads at Early Stage
of Lead Screening