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A Rapid and Automated Method For High-Throughput Nanobody Discovery

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Abstract

Biopanning methods to select target-specific Nanobodies® (Nbs) often involve presenting the antigen, immobilized on plastic plates, to Nb libraries displayed on phage. Most routines are operator-dependent, labor-intensive and often material- and time-consuming. Here we describe the use of the Octet® Bio-Layer Interferometry (BLI) platform for Nanobody discovery. Presenting the antigen to phage libraries by immobilizing it to automated Octet® biosensors enables high throughput and precise control over each step. By adjusting association and dissociation times, one can efficiently decrease the background of nonspecific and low-affinity Nbs, reducing the rounds of panning needed for the enrichment of high-affinity binders. Octet® panning also enables the use of unpurified target proteins and unpurified phage from a bacterial culture supernatant. Moreover, enrichment of binders can be quantified by monitoring phage binding to the target by biolayer interferometry, omitting additional phage titration steps. Routinely, up to three rounds of Octet® panning can be performed in only five days to deliver target-specific binders, ready for screening and characterization using the same Octet® system.

Introduction

Nanobodies® (Nbs), the variable domains of heavy-chain-only antibodies that naturally occur in camelids¹, are valuable research tools to study proteins due to their small size, high solubility and stability.

Most pipelines for the discovery of target-specific Nbs involve the immunization of a llama with the target protein to create an immune library. Target-specific binders from these immune libraries are next enriched using biopanning. In conventional biopanning, the target is immobilized in the wells of a plastic microtiter plate and presented to phage-displayed Nbs (Figure 1)². Next, the plate is manually washed repeatedly to remove aspecific phage. Phage that are specifically bound to the target are eluted typically by changing the pH. Since the elution step also releases aspecific phage, all eluted phage, enriched for target-specific binders, are amplified to use as input for a following round of selection. The whole procedure is time-consuming and labor-intensive and the washing steps, which define the stringency of the panning, are operator-dependent.

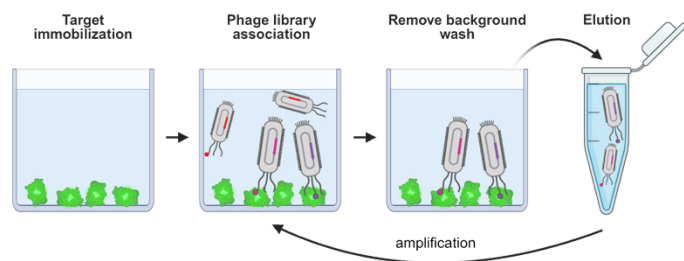


Figure 1: Conventional biopanning. First, the target is immobilized in a microtiter plate. Then, it is presented to a phage display library. Next, the background of nonspecific phage is removed by washing. Finally, target-specific phage are eluted and amplified to use as input for a subsequent panning round.

To overcome these shortcomings, an automated biopanning method using the Octet^{®3}, a biolayer interferometry (BLI)-based system that dips biosensors in consecutive wells of a plate and monitors biomolecule interactions on the biosensor surfaces (Figure 2) was developed. Presenting the target on automated biosensors to phage-displayed Nbs in a well ensures robustness, reproducibility and precise control over each step. Since most Nbs have similar association rate constants while their dissociation rate constants vary, most affinity can be gained by selecting Nbs that dissociate slowly from the target. Additionally, instead of a titration of eluted phage to follow enrichment, phage binding to the immobilized target can be directly monitored by interferometry.

Time-consuming purification steps can be reduced as well, since this method enables the use of nanogram quantities of unpurified target and unpurified phage from a bacterial culture supernatant. As a result, three consecutive rounds of panning can be performed in only five days. To further increase the throughput, 8 (Octet[®] Red96 (Octet[®] R8)), 16 (Octet[®] Red384 (Octet[®] RH16)) or even 96 (Octet[®] HTX (Octet[®] RH96)) pannings can be done in parallel.

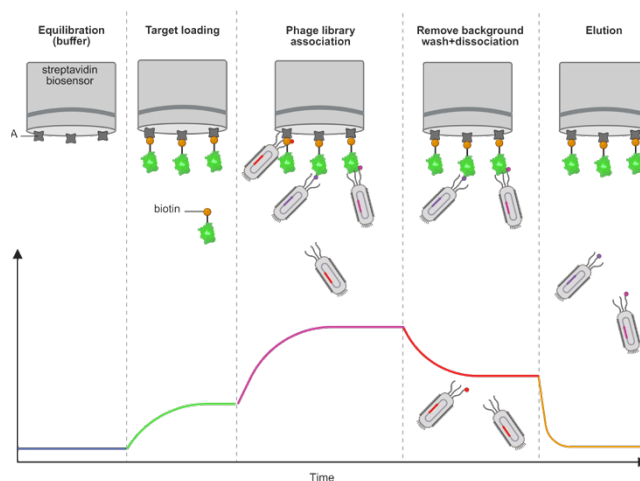


Figure 2: Schematic overview of Octet[®] panning.

Materials and Methods

Experiment 1:

Selection on purified target with purified phage

For an Octet® panning on purified Green Fluorescent Protein (GFP), a GFP immune library in *Escherichia coli* cells was prepared as described in Pardon et al.². During Octet® panning, the purified phage library was used at 1 nM in assay buffer.

Materials and equipment:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® Streptavidin (SA) Biosensors (Sartorius, cat. no. 18-5019)

Target: Biotinylated GFP

Phage library from immunization with: GFP

Assay buffer: PBS pH 7.4, 0.005% Tween 20, 0.01% BSA

Elution buffer: 200 mM glycine at pH 2.3

The assays steps of the Octet® panning were defined in the assay method settings (Table 1) from the Octet® BLI Discovery Software.

Table 1: Assay method settings of experiment 1.
Instrument temperature: 30 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Target	until 1 nm increase	1,000
Assay buffer	30	1,000
Assay buffer	30	1,000
Phage library	600	1,000
Assay buffer	180	1,000
Assay buffer	600	1,000
Assay buffer	5,400	1,000
Assay buffer	30	1,000
Elution buffer	60	1,000

As the negative control, this panning was mirrored with a sensor that was loaded with an irrelevant biotinylated protein. For the second panning round, 50 µL of eluted phage were recovered overnight by incubation with *E. coli* cells, rescued with helper phage, amplified and prepared as described in Pardon et al.².

Experiment 2:

Selection on unpurified target with purified phage

For an Octet® panning on an unpurified target, yeast 1,6-bisphosphate aldolase (FBA1, Systematic Name YKL060C) was expressed as a GFP-fused protein. FBA1-GFP was captured on the sensor from yeast lysate diluted fourfold in assay buffer by Nb207, a pM-affinity GFP binder discovered previously via conventional panning⁴. During Octet® panning (Table 2), the purified phage library was used at 1 nM in assay buffer.

Materials and equipment

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® SA Biosensors (Sartorius, cat. no. 18-5019)

Target: FBA1-GFP

Phage library from immunization with: the soluble yeast proteome of *Saccharomyces cerevisiae* strain EBY100 (ATCC® MYA-4941™)

Assay buffer: PBS pH 7.4, 0.005% Tween 20, 0.01% BSA

Elution buffer: 200 mM glycine at pH 2.3

Table 2: Assay method settings of experiment 2.
Instrument temperature: 30 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Biotinylated Nb207	until 1 nm increase	1,000
Target	until 1 nm increase	1,000
Assay buffer	30	1,000
Assay buffer	30	1,000
Phage library	600	1,000
Assay buffer	180	1,000
Assay buffer	600	1,000
Assay buffer	5,400	1,000
Assay buffer	30	1,000
Elution buffer	60	1,000

Lysate from the EBY100 strain was used to prepare control sensors. For this selection, three rounds of panning were performed.

Experiment 3:

Selection on purified target with unpurified phage

To select GFP-specific Nbs starting from an unpurified phage library, a 20 mL culture of the GFP immune library was infected with helper phage and grown overnight at 37 °C in a shaking 50 mL tube. The culture supernatant was collected by spinning down the cells for 15 min at 3,200 rcf. During Octet® panning (Table 3), 200 µL of the supernatant containing the phage library was used.

Materials and equipment

Round 1:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL

Round 2:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® Red384 (Octet® RH16)
- Flat Bottom 384-well plates (Greiner, cat. no. 781076): wells of 80 µL
- Octet® Red384 (Octet® RH16)
- Tilted Bottom 384-well plates (Sartorius, cat. no. 18-5080): wells of 40 µL
- Octet® SA Biosensors (Sartorius, cat. no. 18-5019)

Target: Biotinylated GFP

Phage library from immunization with: GFP

Assay buffer: PBS pH 7.4, 0.005% Tween 20, 0.01% BSA

Elution buffer: 200 mM glycine at pH 2.3

Table 3: Assay method settings of experiment 3.

Instrument temperature: 30 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Target	until saturation	1,000
Assay buffer	30	1,000
Assay buffer	30	1,000
Phage library	600	1,000
Assay buffer	180	1,000
Assay buffer	600	1,000
Assay buffer	5,400	1,000
Assay buffer	30	1,000
Elution buffer	60	1,000

For the second panning round, 50 µL of eluted phage were recovered overnight, rescued with helper phage and amplified, but not purified.

Experiment 4:

Selection on unpurified target with unpurified phage

To select on multiple unpurified targets in parallel, 1,6-bisphosphate aldolase (FBA1, YKL060C), pyruvate decarboxylase isozyme (PDC1, YLR044C), HSP70 type SSA1 (SSA1, YAL005C), phosphogluco-isomerase (PGI1, YBR196C) and 14-3-3 protein (BMH1, YER177W) were expressed as GFP-fused proteins and captured from lysate on five sensors using GFP-specific Nb207 (Table 4).

Materials and equipment:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® SA Biosensors (Sartorius, cat. no. 18-5019)

Target: FBA1-GFP, PDC1-GFP, SSA1-GFP, PGI1-GFP, BMH1-GFP

Phage library from immunization with: the soluble yeast proteome of *S. cerevisiae* strain EBY100 (ATCC® MYA-4941™)

Assay buffer: PBS pH 7.4, 0.005% Tween 20, 0.01% BSA

Elution buffer: 200 mM glycine at pH 2.3

Table 4: Assay method settings of experiment 4.

Instrument temperature: 30 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Biotinylated Nb207	until saturation	1,000
Target	until saturation	1,000
Assay buffer	30	1,000
Assay buffer	30	1,000
Phage library	600	1,000
Assay buffer	180	1,000
Assay buffer	600	1,000
Assay buffer	30	1,000
Elution buffer	60	1,000

To proceed with the second round the next morning, 100 µL of eluted phage were recovered during 5 h, rescued and amplified overnight.

Experiment 5:

Selection on membrane protein

Mouse brain membrane protein IgSF8 fused to GFP was expressed in HEK cells, solubilized in DDM and immobilized on a Nb207-coated sensor. During Octet® panning (Table 5), this sensor was incubated with culture supernatant containing phage from a mouse brain membrane immune library, supplemented with protease inhibitors.

Materials and equipment:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® SA Biosensors (Sartorius, cat. no. 18-5019)

Target: IgSF8-EGFP

Phage library from immunization with: the membrane fraction of mouse brain extracts enriched in synaptic vesicles

Assay buffer: 20 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol, 0.3% DDM/0.03% CHS

Elution buffer: 200 mM glycine at pH 2.3

Table 5: Assay method settings of experiment 5.

Instrument temperature: 15 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Biotinylated Nb207	until saturation	1,000
Target	until saturation	1,000
Assay buffer	30	1,000
Assay buffer	30	1,000
Phage library	7,200	1,000
Assay buffer	60	1,000
Assay buffer	60	1,000
Elution buffer	60	1,000

Screening of Nanobodies selected by Octet® panning

Single colonies picked from the recovery cultures were grown, induced to express Nbs and lysed by freezing and thawing, based on the protocol in Pardon et al.². For screening by BLI on an Octet® (Table 6), these lysates were diluted fourfold in assay buffer (PBS pH 7.4, 0.005% Tween 20, 0.01% BSA). We employed lysates containing previously discovered target-specific binders as positive controls and a lysate containing an irrelevant Nb as a negative control.

Materials and equipment:

- Octet® Red96 (Octet® R8)
- Flat Bottom 96-well plates (Greiner, cat. no. 655076): wells of 200 µL
- Octet® SA Biosensors (Sartorius, cat. no. 18-5019)

Table 6: Assay method settings of Octet® screening.

Instrument temperature: 30 °C.

Assay step	Step time (s)	Shake speed (rpm)
Assay buffer	240	1,000
Target	until 1 nm increase	1,000
Assay buffer	30-60	1,000
Lysate	600	1,000
Assay buffer	600	1,000

Association and dissociation sensorgrams were analyzed in MATLAB, first smoothing the data and subtracting a reference curve (PBS instead of lysate) before aligning all curves at the start of association. By convention, Nbs were classified as antigen-specific binders when their signal was > 0.06 nm higher compared to the negative control at the end of association. Antigen-specific binders were ranked based on the time required for 50% dissociation. A weak binder has a half-life shorter than 1 min. A medium binder has a half-life between 1 and 10 min, whereas a strong binder has a half-life exceeding 10 min.

Results and Discussion

Octet® pannings can be used to timely recover phage that remained bound to the immobilized target during passive dissociation. Octet® pannings on different targets showed remarkably low backgrounds of nonspecific phage (Figure 3). The panning on FBA1 was performed by capturing GFP-tagged FBA1 from a crude yeast lysate with GFP-specific Nb207 that was immobilized on the sensor. It thus appears that Octet® panning can be used for (parallel) high-throughput selections starting from unpurified targets that can be captured on the sensor via a (fusion-)tag. Additionally, we performed successful Octet® pannings with unpurified phage contained in the supernatant of a bacterial culture, omitting the labor-intensive phage purification step before each round of selection. Moreover, pannings on more challenging targets such as membrane proteins were successful.

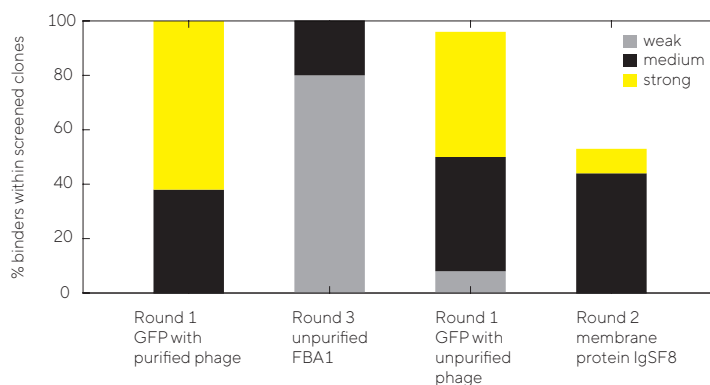


Figure 3: Summary of screening results for different Octet® pannings.

Though we used sensors that are saturated with the target protein, we found that the number of phage that binds to the sensor in a first round is too low to detect association by biolayer interferometry. When panning an unpurified phage library on GFP, phage binding could only be monitored in the second round, causing a significant increase upon binding and an decrease back to baseline upon dissociation and elution of the remaining phage (Figure 4).

We found that differences in amplitudes amongst similar experiments correlates with the amount of phage that binds and elutes. Accordingly, the enrichment of binders can be followed over panning rounds via association curves to omit time-intensive titration steps. We also found that the amount of target needed for selections can be reduced by panning in 384-well plates (Figure 4).

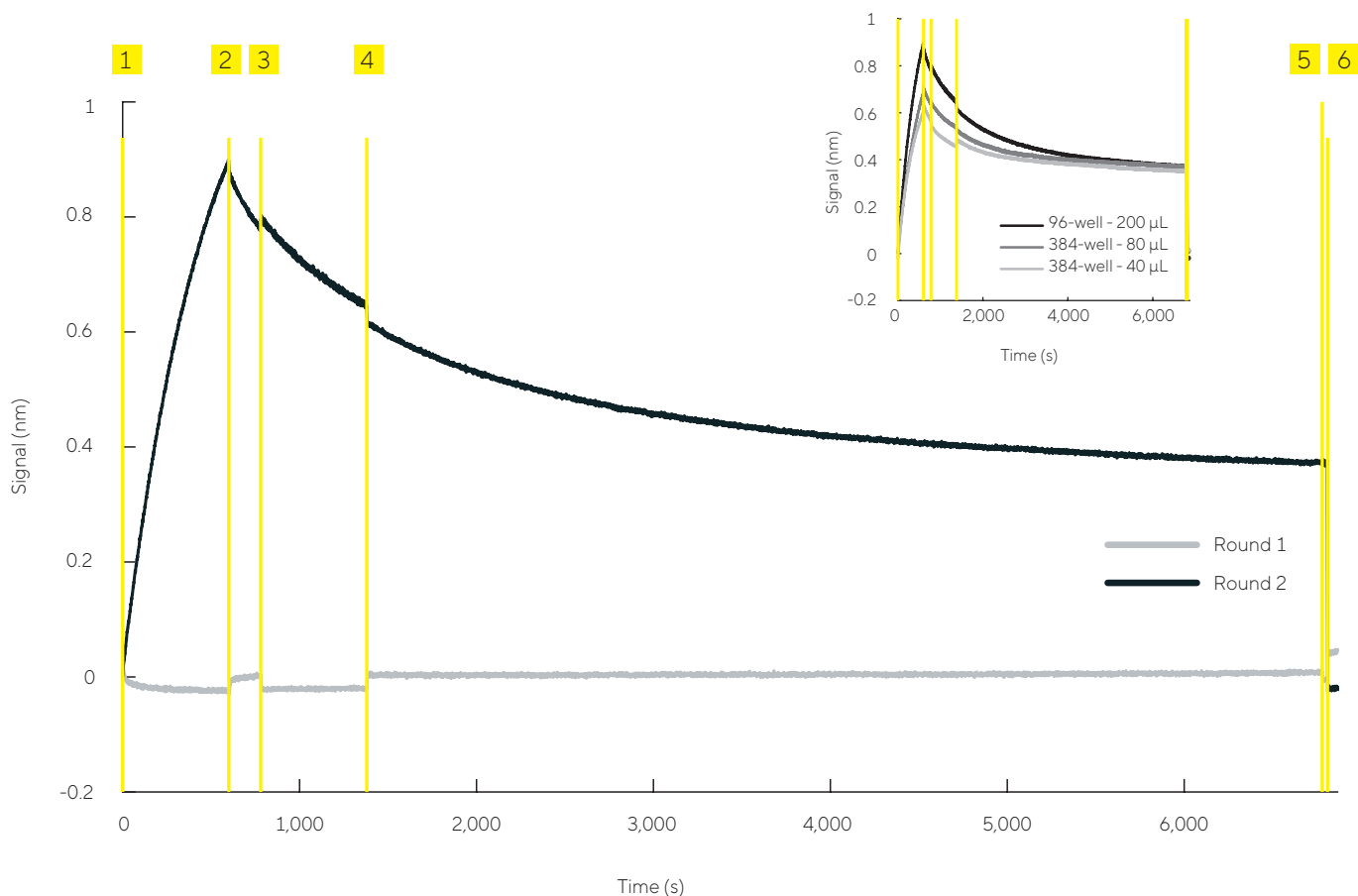


Figure 4: Sensorgrams during Octet® panning on GFP with unpurified phage for round 1 and round 2. Biotinylated GFP was loaded on a streptavidin sensor and the following steps were done in PBS/Tween/BSA: (1) 10 min association with culture supernatant containing the GFP phage library, (2) 3 min wash, (3) 10 min dissociation, (4) 90 min dissociation, (5) 0.5 min wash, (6) 1 min elution with 200 mM glycine pH 2.3. Eluted phage were rescued and amplified for a second round. As a negative control, a sensor was incubated with an irrelevant Nb-phage culture supernatant. The panning curves were analyzed in MATLAB, first smoothing the data and subtracting the irrelevant Nb-phage curves before aligning all curves at the start of association. Round 2 was repeated in 80 µL wells of a flat bottom 384-well plate and in 40 µL wells of a tilted bottom 384-well plate.

To showcase the throughput of Octet® panning, two rounds were performed in parallel on five different yeast proteins. A llama was immunized with the soluble fraction of a crude yeast lysate containing thousands of unpurified proteins. Five unpurified GFP-tagged yeast proteins were loaded on separate sensors using Nb207.

Figure 5 compares the association curves of phage during the first round and the second round for the five proteins. Remarkably, these curves appear to mirror the enrichment of target-specific phage for the different antigens. Indeed, the larger amplitudes for PDC1 and PGI1 coincide with screening at least 50% strong binders compared to 0% strong binders for the other targets.

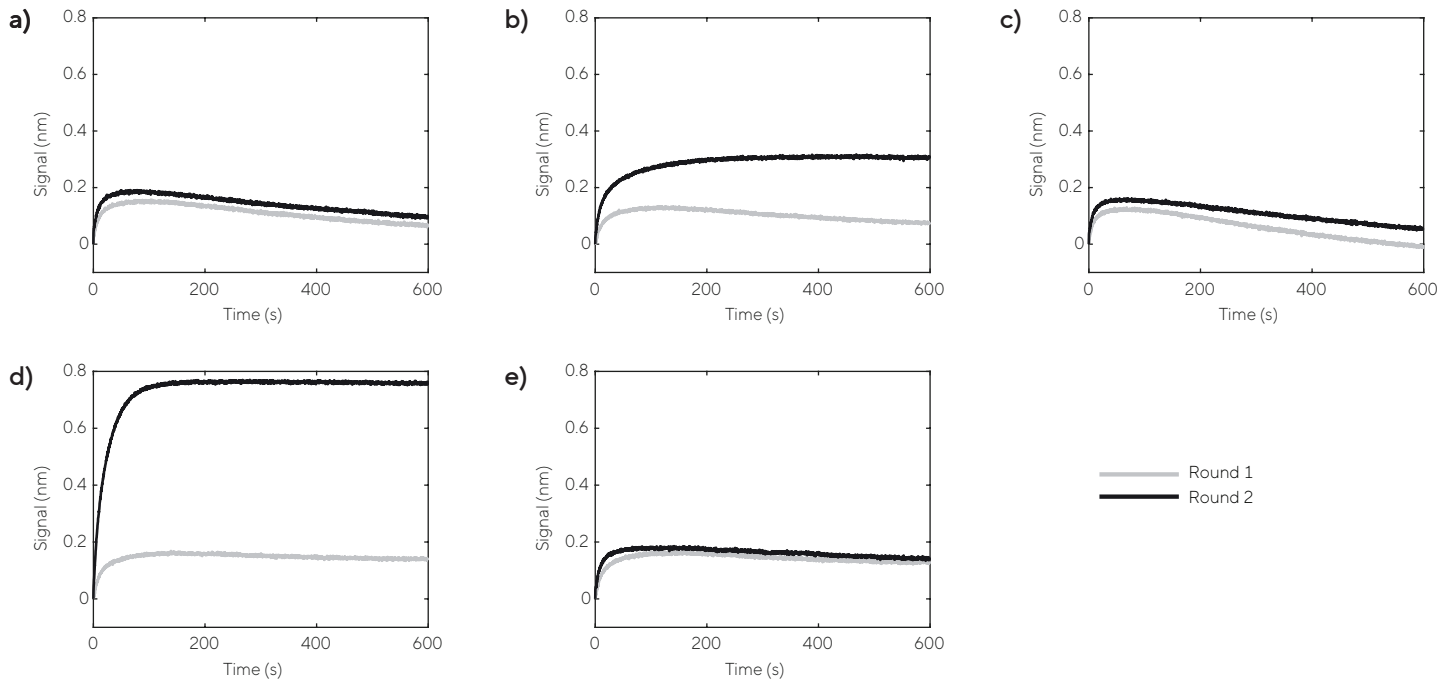


Figure 5: Association curves during Octet® panning on GFP-fused yeast proteins with unpurified phage for round 1 and round 2. Biotinylated Nb207 was loaded on streptavidin sensors to capture a) FBA1-GFP, b) PDC1-GFP, c) SSA1-GFP, d) PG11-GFP, e) BMH1-GFP from yeast lysate in PBS/Tween/BSA. The curves show the subsequent 10 min association with unpurified phage culture supernatant. After a 10 min dissociation and 1 min elution with 200 mM glycine pH 2.3, 100 μ L of eluted phage were rescued and amplified overnight to proceed to a second round the next morning. The curves were aligned at the start of association in MATLAB.

Summary

Throughout this study we showed that immobilizing a target on an Octet® biosensor and using the Octet® platform to transfer it to different wells of a microtiter plate allows for perform phage display selections under well-controlled regimes with passive dissociation and active elution. By omitting phage purification and titration steps, we can complete three rounds of Octet® panning in five consecutive days. On an Octet® Red96 (Octet® R8) system, one can easily perform up to eight selections in parallel to compare different experimental conditions or to discover binders for different antigens. We anticipate that similar experiments can be scaled up to 96 parallel pannings on an Octet® HTX (Octet® RH96).

In conclusion, we show that Octet® selections are a full-fledged alternative to conventional pannings to discover Nanobodies. Experiments can easily be automated to perform tunable pannings under precisely controlled conditions and are amenable to throughput approaches or parallel selections where one parameter is varied.

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