SARTORIUS

A Rapid Method to Quantitatively Screen Bispecific Antibodies Using Protein A and Octet® His1K Biosensors

Hongshan Li[1], Sriram Kumaraswamy[1], Eva Rubio-Marrero[2], Danyang Huang[2], Girish Pendse[2]
1. Sartorius, Fremont, CA
2. Celgene Corporation, Summit, NJ

Correspondence
Email: octet@sartorius.com

Abstract

Recent advances in biotechnology have driven the development of bispecific antibodies. But for those working in bioprocessing or cell line screening, there still remains a number of development challenges. Many traditional technologies for characterizing antibodies are limited in their ability to provide quantitative functional assessment of two interactions to one bispecific molecule, and commonly used assay platforms like ELISA and SPR are time-intensive.

Scientists at Celgene Corporation developed a screening process incorporating the Octet® platform. Now they can identify pools and clones with higher concentrations of bispecific antibodies. High-throughput was achieved from the multiple simultaneous measures possible on the system and rapid assays times, such as binding optimization in only 10 minutes.

Find out more: www.sartorius.com

Introduction

Bispecific antibodies (bsAbs) were first reported by Nisonoff and Rivers in 1961[1], however the engineering challenges associated with bsAbs have limited their development. Recent advances in biotechnology have fueled development in the last 10 years. To date, two bispecific antibody drugs (blinatumomab and emicizumab) are on the market, and over 85 are in clinical development. Because this wide array of bsAbs is populated by many different species, comprising many different formats (including various engineering of antibody fragments in a symmetric or asymmetric manner), functional evaluation of bsAbs during bioprocessing is critical and challenging. ELISA and SPR are currently the most commonly used methods for bsAbs evaluation, they are both, however, time consuming. Therefore, there is high demand for a simple method for the functional assessment of two or more interactions of the complex bispecific therapeutics[2]. The production of bsAbs adds a challenge for scientists in the cell line screening process since many other conformations of antibodies can be produced by the cells. If screening is based only on Protein A titer, good pools and clone candidates might be discarded. For this reason, we developed a high-throughput assay using the Octet® RH96 system to identify pools and clones with higher concentrations of bispecific antibodies.

The ease of use and rapid processing of samples makes the Octet® RH96 system an excellent option for higher throughput applications such as expression clone screening and bispecific molecule evaluation. For example, bsAb binding optimization only takes 10 min (Figure 3). By combining the power of Dip and Read biosensors with the Octet® RH96 system, one can minimize time, effort, and costs in the assay development of bispecific therapeutics. Here we demonstrate the simple design for a bsAb quantitative assay on the Octet® system (Figure 1). The high-throughput Octet® platform allows for rapid screening of cell lines expressing the target bsAb. This assay consists of capturing bsAb by each antigen-binding site region sequentially using the correspondent antigen loaded onto the biosensor surface. For each molecule antigen, a reference material is required to develop the assay, as well as positive and negative controls. The developed BLI method offers an easy screening method and workflow that assesses bsAb interactions in a versatile, label-free, and easy-to-use format.

Materials and Reagents

- Octet® instrument with Octet® BLI Discovery Software and Octet® Analysis Studio Software
- HIS1K Biosensor, Sartorius Part No. 18-5120
- Protein A (ProA) Biosensor, Sartorius Part No. 18-5010
- 96-well black plate, flat bottom, polypropylene microplate, Greiner Bio-One, Sartorius Part No. 655209
- 384-well, black, flat bottom, polypropylene microplate. Greiner Bio-One, Sartorius Part No. 781209
- Buffer for sample dilution and biosensor hydration
- His-tagged antigens specific for each arm of the bsAb
- bsAb reference material (positive control)
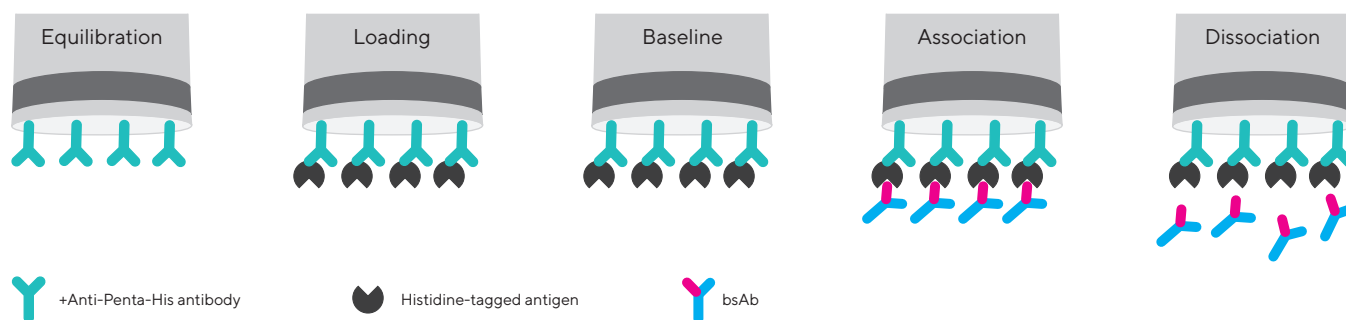- Regeneration buffer 10 mM Glycine pH 1.5

Figure 1: Example workflow for a bsAb quantitative assay. The assay consists of five assay steps. Step 1: equilibration, Step 2: loading and capture of His-tagged antigen, Step 3: baseline, Step 4. bsAb association, Step 5: bsAb dissociation.

Assay Development

The principal steps required for assay development are the following:

- Biosensor selection
  - Considerations: what tags are available on the antigen?
- Determine concentration of antigen A and antigen B to be loaded on biosensors
  - Considerations: molecular weight of antigens
- Antibody binding to both antigens separately (specificity)
- Standard curve generation (Technical Note 15 for details[3])
- Comparison of results with reference standard (antibody concentrations measured by ProA Biosensors)

Biosensor Selection

Sartorius biosensors are coated with a proprietary biocompatible matrix that is uniform and non-denaturing with minimal non-specific binding. The tips are derivatized and ready for use in a diverse set of biomolecular applications. The choice of biosensors depends on the assay format. For example, in these studies, HIS1K Biosensors were selected for antigen immobilization due to the availability of His-tagged antigens. Protein A Biosensors were used for antibody quantitation. An advantage of these biosensors is that they come ready to be used and can be regenerated easily.

Antigen Loading Optimization

Since antigens have different molecular weights compared to antibodies, different concentrations of antigens are tested first to obtain optimal response signals when loading onto the biosensors. For this assay, four concentrations of antigen were tested: 3, 6, 12 and 25 µg/mL. The antigens were diluted in the same buffer used for the reference material. The assay optimization was done in kinetics mode. Since this assay is designed for bsAb, the same concentrations were tested for antigen A and B for a loading step of 300 seconds after a 30-second baseline step (Figure 2).
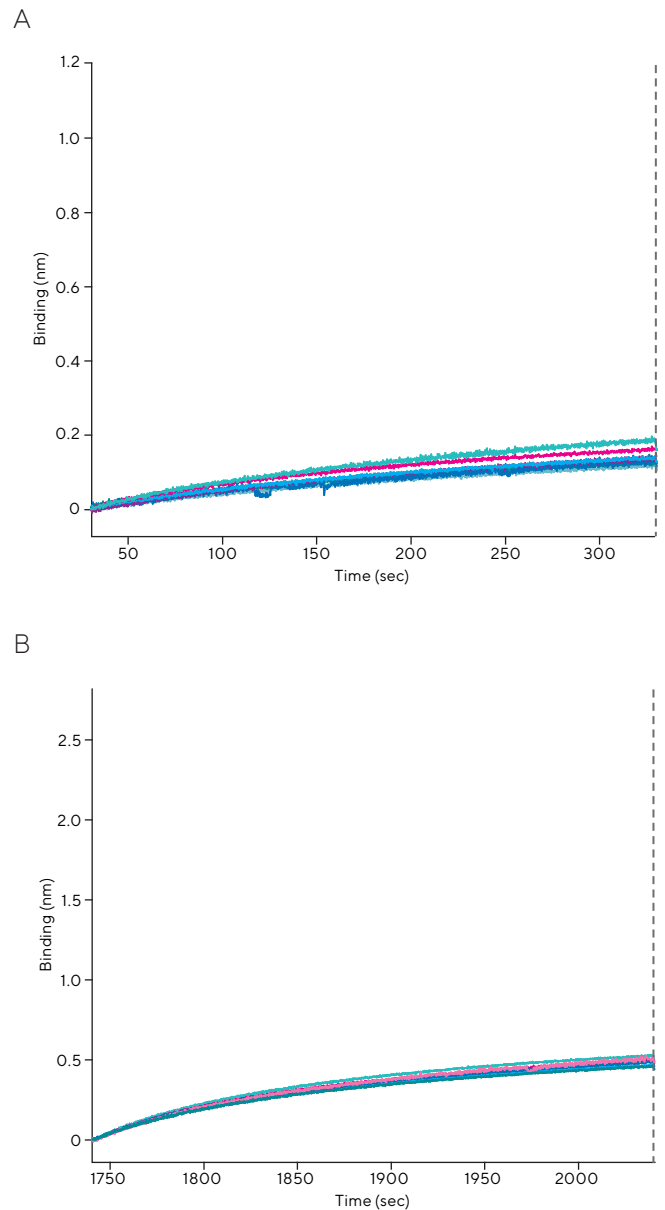
A

B

Figure 2: A) Loading curve for an antigen at 3 µg/mL. B) Loading curve of the same antigen at 12 µg/mL. Both antigen concentrations were evaluated sequentially in the same assay. Typically, a signal of ~0.2 nm is good for the loading step and capture of bispecific antibody using HIS1K Biosensor.

BsAb Binding Optimization

To test if the bsAb binds to antigen on the biosensor and to determine the optimal concentration of antigen to be loaded on the biosensors, the reference material was bound in a dose response assay with bsAb concentrations ranging from 100 µg/mL to 3 µg/mL. The binding of the bsAb was tested for ~120 seconds with all four concentrations of loaded antigen on the biosensors followed by a dissociation step of 60 seconds (Table 1 and Figure 3).

The results were analyzed using the Octet® Analysis Studio Software. The optimal loading concentration of antigen was selected based on good separation of curves between the different concentrations of bsAb. The binding response signals (step 4 in Figure 3) can be exported and plotted as a function of bsAb concentration. Linearity in the resultant curve can be used to identify the optimal antigen loading concentration. The minimum concentration that results into the widest dynamic linear range should be selected. In addition, the best response without binding saturation at the highest concentration of bsAb should be targeted. A negative control to verify the absence of non-specific binding from the bsAb should also be performed using biosensors that are not loaded with antigen (Figure 4). Typically, a signal shift of 0.2 nm is a good starting point for the loading step and capture of the bispecific antibody.

Step	Step type	Time (s)	Shaker speed (rpm)
1	Baseline	30	300
2	Loading	300	300
3	Baseline 2	30	300
4	Association	120	300
5	Dissociation	60	300

Table 1: Octet® assay steps. The loading and association time can be modified for each binding assay.

Standard Curve Generation

Once the antigen loading concentration, loading time, and association time are known, a standard curve can be generated for each antigen using the resultant dose response curves. Typically, samples are run in triplicate for the standard curve generation. A variation of less than 10% CV should be expected (Figure 5). Additional to the binding assay, a standard curve can be generated using the ProA Biosensors to calculate the total protein concentration in the sample. This concentration of total protein can be used in combination with binding results to rank the best pools and clones expressing bsAb.
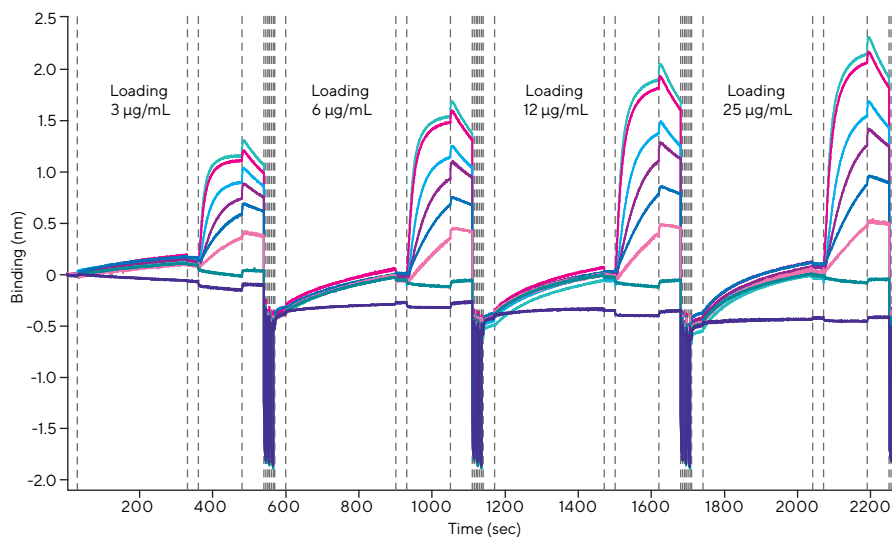

Figure 3: Example of complete Octet® assay run for the association of bsAb to a specific antigen. Lower yellow curve represents negative control where antigen was not loaded to the biosensor and 100 µg/mL of bsAb tested for binding.
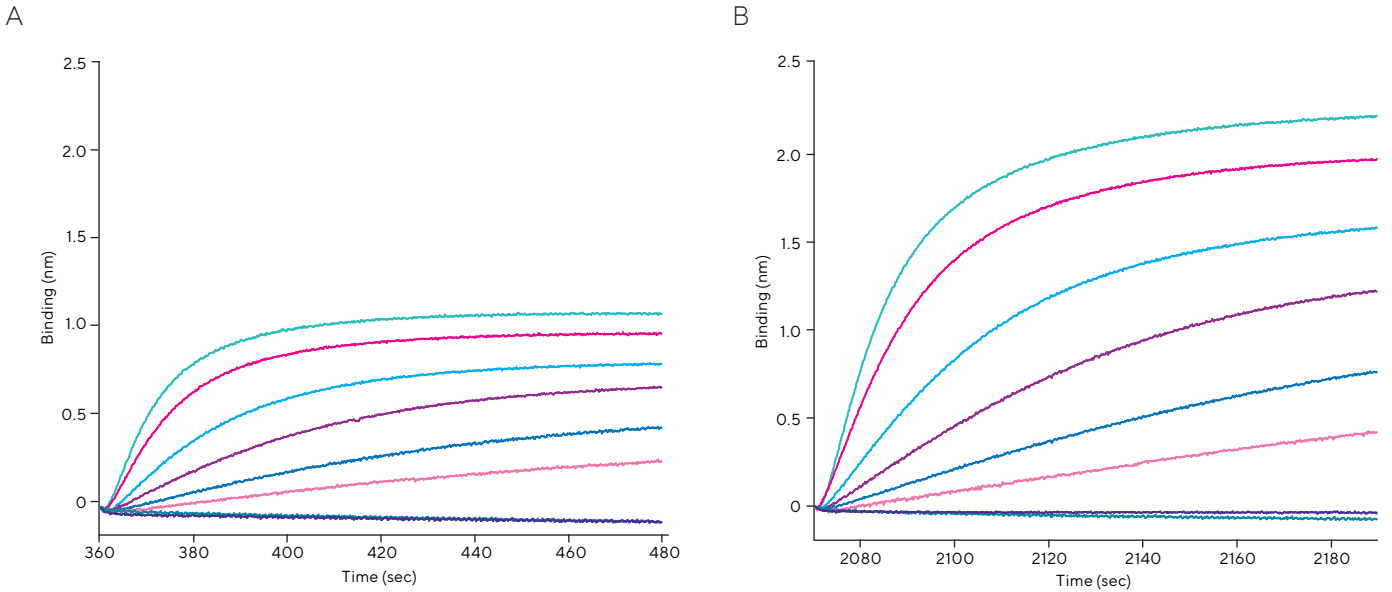
A

B

Figure 4: Dose response binding of the bsAb to different loading antigen concentration; A) 3 µg/mL of antigen. B) 12 µg/mL of antigen. Data was generated sequentially in the same experiment. The 12 µg/mL concentration of antigen was chosen for the loading step.
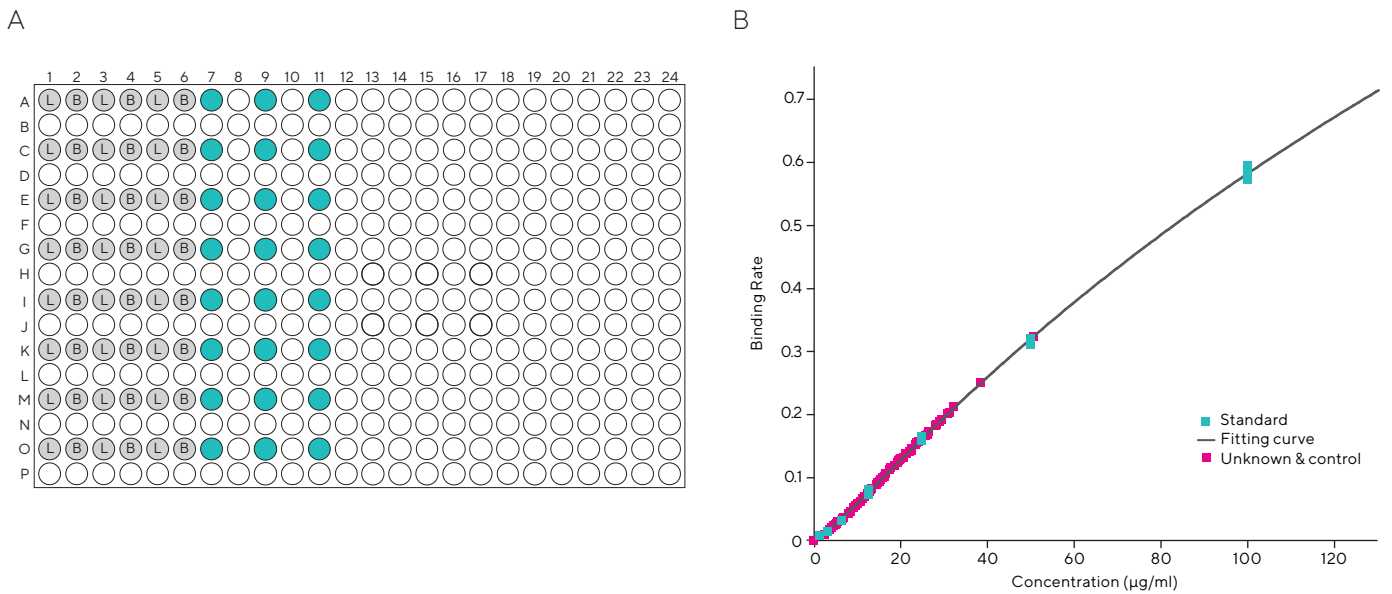
A

B

Figure 5: A) Plate layout to generate standard curve following the steps shown in Table 1 using triplicate of reference material at different concentrations (teal wells). B represents buffer well and L represents loading well. B) Standard curve prepared using triplicate samples of reference material from 3–100 µg/mL (teal squares). Magenta squares represent unknown samples tested in the assay showing different concentrations based on the standard curve generated.

Analysis and Results Using Reference Material

To verify that the binding assay is consistent, the reference material was tested at different concentrations in duplicate using the assay parameters tested before. As previously described, the antigen was loaded after a baseline step, and after a second baseline, the bsAb was associated to the antigen followed by a dissociation step (see Table 1). This assay was performed with binding assays where each antigen is loaded to the HIS1K Biosensors. Antigen binding assay specification can be verified using half molecule controls if these reagents are available (data not shown). Buffer was used as a negative control.

To analyze the results, the Octet® Analysis Studio Software was used and the Association step was selected[3]. To obtain the concentrations of the samples, the curves were analyzed using the standard curve generated previously for each antigen. The concentrations obtained should be close to the known concentration of the samples (could have small variation) and similar between duplicates. Having the concentration from both binding assays, a ratio can be calculated. Since the samples used here are reference material from the molecule of interest and is mostly pure bsAb, the calculated ratio is close to 1 but this may vary from molecule to molecule (Table 2). For pool or clone screening purposes, this reference material should be used in every assay and top pools/clones should be chosen with a similar ratio to the reference material. A ratio of 1 to 1.3 over five different dilutions were obtained and the assay variation of <30% was used for the ranking assay.

Since the overall titer is important when choosing the top pools/clones, the concentrations of the samples can be obtained by performing a ProA titer assay with ProA Biosensors and corresponding standard curve. Table 3 shows an example of results from a mini pool screening in a 96-well plate. The Octet® bsAb binding assay as well as ProA titer was performed using the pooled supernatant after incubation of seven days at 36.5°C. If the top six pools were chosen based on total ProA titer, most of the pools were expressing low levels of bsAb. Taking in to consideration the binding ratio for the antigens and the overall ProA titer could help in selecting the top pools with higher bsAb expression levels at earlier stages of the cell line development process.

Sample ID	Pro A		Antigen 1		Antigen 2		Ratio antigen 1/ antigen 2 – Rep. #1	Ratio antigen 1/ antigen 2 – Rep. #2
	Rep. #1	Rep. #2	Rep. #1	Rep. #2	Rep. #1	Rep. #2		
RS 80 µg/mL	75.2	73.2	89.4	86.2	83.2	82	1.1	1.1
RS 40 µg/mL	36.3	35.9	39.8	39.3	39	38.6	1.0	1.0
RS 20 µg/mL	18.7	18	21	21.8	19.3	19.5	1.1	1.1
RS 10 µg/mL	9.48	9.4	10.1	10.5	8.47	8.74	1.2	1.2
RS 5 µg/mL	Too low	Too low	4.87	5.51	4.17	4.09	1.2	1.3
RS 0 µg/mL	0	0	0	0	0	0	N/A	N/A

Table 2: Results of binding assay using bsAb reference material and His-tag antigens corresponding to both antigen-binding sites. Consistency is shown in replicates demonstrating the accuracy of the standard curve.

Mini pool ID	ProA titer (µg/mL)	Antigen 1 binding (µg/mL)	Antigen 2 binding (µg/mL)	Ratio antigen 1/ antigen2
1	116.5	59.8	68.4	0.9
2	91.9	54.6	18.3	3.0
3	88	38.5	84.3	0.5
4	85.1	18.6	58.5	0.3
5	80.4	50.8	16.8	3.0
6	57.7	22.3	27.2	0.8
7	56.5	39	2.04	19.12
8	56.3	2.9	48.4	0.06
9	51.7	23.4	29.7	0.8
10	51.5	36.1	3.05	11.8
11	45.9	20.8	27.9	0.75
12	45.7	21.2	28.8	0.7
13	45.6	11.6	22	0.53
14	39.8	2.68	4.89	0.5
15	36.9	17.8	23	0.8
Reference material	53.5	24.4	32	0.8

Table 3: Results from a mini pool screening in 96-well plate. Mini pools highlighted were not taken into consideration for further expansion due to the poor binding to either antigen, suggesting low levels of bsAb expression. Mini pools without highlighting represent the best pools with high titer and good binding to both antigens.

Discussion

There is currently a limitation in technologies that allow for quantitative functional assessment of two interactions to one bispecific molecule in the market. Here we have shown an easy method for bsAb evaluation. Utilizing Protein A and HIS1K Biosensors, we were able to identify conditions quickly that are suitable for quantitative measurement of bispecific binding.

By analyzing specific binding results in addition to IgG titer in a single high-throughput instrument, we are able to assess the binding activity of a bispecific molecule to both antigens to rank the top pools/clones during cell line development. The results obtained from the Octet® binding assay shown here have been validated using other analytical methods such as RP-HPLC, CE-SDS, SPR, and LC-MS to show that binding results can be correlated to the purity of the bsAb from the samples tested.

References

1. Recombination of a mixture of univalent antibody fragments of different specificity, Nisonoff A, Rivers MM, *Arch Biochem Biophys,* 1961; May;93:460-2.
2. Bispecific antibodies: a mechanistic review of the pipeline, Labrijn AF, Janmaat ML, Reichert JM and Parren PWHI, Nature Reviews Drug Discovery, 2019 Aug;18(8):55-608.
3. Technical Note 15, High Sensitivity Detection of Human IgG Using Protein A Biosensors.