APPLICATION BRIEF

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Off-Rate Ranking & Epitope Binning using Octet®

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Successful monoclonal antibody (mAb) development requires more than identifying binders. For diagnostic and advanced research applications, antibody candidates must demonstrate appropriate affinity, dissociation kinetics, and epitope diversity to ensure downstream assay compatibility, stability, and reproducibility.

Traditional monoclonal antibody development programs often screen a limited number of clones and rarely differentiate relative affinity or epitope coverage across large cohorts. When relative affinity and binding relationships are critical, restricted screening increases development risk and may delay identification of fit-for-purpose reagents.

To address these limitations, Rockland has integrated high-throughput off-rate ranking and in-tandem epitope binning into its standardized antibody discovery workflow using the Sartorius Octet® Bio-Layer Interferometry (BLI) platform. This approach enables screening of dozens to hundreds of hybridoma clones within days, providing decision-ready kinetic and epitope characterization early in the development process.

This technical note highlights application of this workflow to rapid characterization of mAbs generated against Highly Pathogenic Avian Influenza (HPAI) H5N1 clade 2.3.4.4b hemagglutinin (HA).

Affinity Requirements Differ by Application

Basic research mAbs are widely used for screening, discovery, and structural studies. Affinity requirements for research antibodies are often undefined and less stringent. Animal-derived antibodies typically develop sufficient affinity (~1 × 10^{-5} M) through natural affinity maturation, and specificity is frequently the primary screening criterion.

In contrast, diagnostic and therapeutic mAbs require well-defined performance characteristics that include both specificity and binding kinetics. In diagnostic assays, the dissociation rate constant (k_{off}) plays a critical role in maintaining stability of the antibody-analyte complex. A slower k_{off} supports improved signal retention through wash steps and extended incubations.

Assay format directly influences optimal kinetic properties. ELISA and other plate-based assays involve multiple wash steps and benefit from low k_{off} values to preserve antibody binding. Immunohistochemistry (IHC) similarly favors slow dissociation during extended incubations and washing. Lateral flow assays operate under rapid flow conditions where interaction time is short; in these systems, moderate k_{off} values may be acceptable depending on assay design.

Therapeutic antibodies often require very slow dissociation rates to promote sustained receptor occupancy and enhanced pharmacodynamic response. Typical K_D values for therapeutic mAbs fall in the 10^{-9} to 10^{-11} M range. For example, the PD-1 checkpoint inhibitor Pembrolizumab (Keytruda®) has a reported K_D of approximately 29 pM (2.9×10^{-11} M), while Nivolumab (Opdivo®) exhibits a K_D of approximately 3.06 nM (3.06×10^{-9} M). In the case of Pembrolizumab, a k_{off} of approximately 1.0×10^{-5} s^{-1} contributes to sustained PD-1 engagement. These examples illustrate that overall affinity is defined by $K_D = k_{off} / k_{on}$, and that dissociation rate frequently contributes substantially to functional performance.

> To bridge the gap between large-scale clone generation and the need for precision kinetics, we developed methods that harness the speed and simplicity of Octet® BLI technology to rapidly evaluate large hybridoma cohorts prior to full kinetic characterization.

Table 1. Antibody Off-Rates by Use: General Requirements

k_{off} range (s^{-1})	Research	Diagnostic	Therapeutic
≤ 1×10^{-5}	Excellent	Excellent	Gold Standard
1×10^{-5} - 1×10^{-4}	Very Good	Very Good	Preferred
1×10^{-4} - 1×10^{-3}	Good	Acceptable	Needs Improvement
> 1×10^{-3}	Marginal	Risky	Not Acceptable

Off-Rate Ranking

Off-rate ranking by BLI enables rapid comparison of the relative binding behavior of antibody candidates against a common antigen. Because the k_{off} is independent of analyte concentration during the dissociation phase, reliable relative ranking can be achieved without precise antibody quantitation. Importantly, off-rate measurements can be performed directly in crude hybridoma supernatants obtained from conditioned tissue culture media. This eliminates the need for purification or full kinetic characterization during early screening and allows large antibody cohorts (e.g., >100 clones) to be evaluated rapidly while minimizing sample consumption.

Unlike traditional workflows that require purification and full 1:1 kinetic analysis of each clone, off-rate ranking significantly reduces sample preparation time, reagent consumption, and overall screening effort. As a result, large antibody discovery campaigns can be triaged efficiently prior to deeper biophysical characterization.

In this H5N1 project, a large cohort of monoclonal antibodies reactive with recombinant hemagglutinin (HA) protein from HPAI H5N1 clade 2.3.4.4b was evaluated using the Octet® BLI R8 system. Nearly 100 hybridoma clones were characterized and ranked within days.

Off-rate measurements were performed using the following workflow:

His-tagged HA protein was immobilized onto HIS1K biosensors (*Sartorius, #18-5120*).

↓

Biosensors were dipped into antigen-free media buffer (*Dulbecco's Modified Eagle Medium (DMEM), Gibco, #11995-040*) to establish a baseline signal.

↓

Sensors were then introduced into neat hybridoma supernatants containing candidate antibodies to allow association to near equilibrium.

↓

Biosensors were subsequently transferred back into antigen-free buffer to monitor dissociation of the antibody–antigen complex.

Using Octet® Analysis Studio software, BLI sensorgrams were generated for each antibody candidate (Figure 1). The dissociation phase of each curve was analyzed to calculate experimental k_{off} values. Antibodies were then ranked from slowest to fastest dissociation (Table 2), with slower k_{off} values interpreted as stronger relative HA binding under these assay conditions.

This strategy enabled rapid elimination of weaker binders, prioritizing high-value candidates for characterization without requiring full kinetic analysis of the entire cohort. At this scale, traditional purification-dependent workflows demand substantially more time and resources.

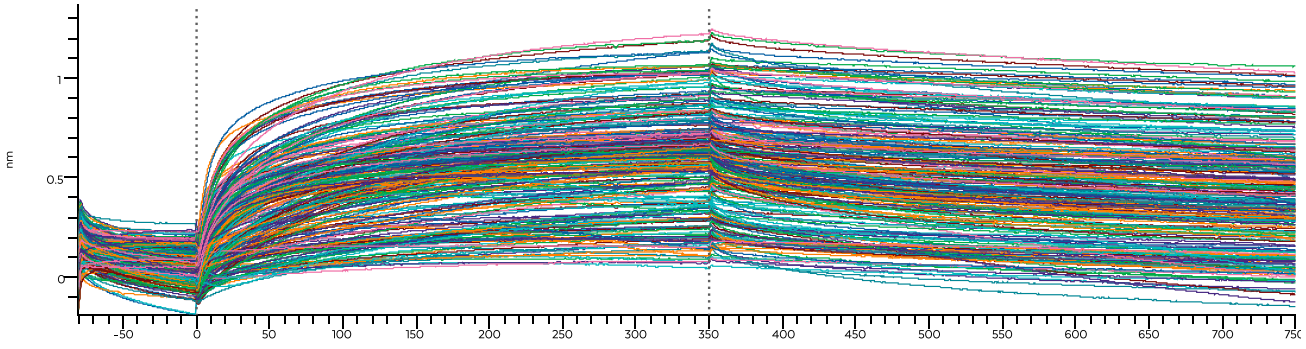

Figure 1. Sensorgram overlay of Off-Rate ranking data. An 80s baseline in buffer is followed by a 350s association step in antibody SUP before a 400s dissociation in the same buffer. Software-assisted curve fitting was performed on the dissociation step to calculate off-rate of each antibody against the common antigen HA protein.

Table 2. Sorted Off-Rate Ranking Data for 100 hybridoma subclones*

Antibody ID Set 1	K_D (s-1)	Antibody ID Set 2	K_D (s-1)	Antibody ID Set 3	K_D (s-1)
33F2.E4.F7	1.64E-05	1H11.H8.E2	3.33E-03	29C7	6.85E-03
28D5.F1.G3	2.40E-05	46C1.D10	3.40E-03	25A5.D5.E6	6.89E-03
12D5.E6.D2.F8	2.70E-05	23G2.B4	3.63E-03	9D5	6.89E-03
12B12.H10.D2	3.30E-05	28A4	3.82E-03	49F4	6.97E-03
43G4.E8.G8	3.82E-05	23F2.C1	3.89E-03	33F2.E4	7.04E-03
34A10.E5.D6	3.97E-05	27A8.E6	4.15E-03	6E9.E6.G3	7.23E-03
3B10.D10.B7	6.64E-05	3G1.E9	4.16E-03	30C5	7.66E-03
6F1	6.72E-05	20E8.H4.A9	4.38E-03	23D12	7.83E-03
1A5.D6.C9.E9	6.86E-05	12E4.G2.D6	4.40E-03	14D6	7.97E-03
3C1.G8.B8	7.47E-05	4D10.E10	4.42E-03	3C11.D1.C6	8.59E-03
28B3.C8	8.11E-05	1C10	4.53E-03	12D5	8.67E-03
10A7.F3.C2	9.93E-05	20G3.B11.F1	4.66E-03	29C12	8.73E-03
11F9.E1.A8	2.07E-04	5A5.F3.C7	4.80E-03	7G7.G12.H3	9.00E-03
47G3	2.14E-04	44B11	5.08E-03	29E8	9.12E-03
9G12.F11	2.23E-04	9E7.C6.F10	5.13E-03	22A8.D2.F5	9.55E-03
5F2.C9	4.29E-04	11F12	5.24E-03	17A10.G5.H1	9.69E-03
1H8.D3	5.91E-04	22D2.C2.F8	5.25E-03	20E10.B12	1.01E-02
1D4.G1	7.90E-04	8B2.F2.F3	5.44E-03	30F1.B8.F9	1.01E-02
17C5.E7	1.02E-03	5G2.F2.F6	5.50E-03	14E2.E11.F3	1.07E-02
18A4.E4.E3	1.06E-03	12B12	5.73E-03	2A2.E1	1.99E-02
14E11.A4	1.09E-03	20D5	5.75E-03	14F8.G4.E2	2.53E-02
22B9.E11.D3	2.00E-03	6G9	5.87E-03	9C6.G3	3.00E-02
1E1.C3.D4	2.27E-03	11D6.C11.F2.C7.F11	5.94E-03	24H5.C9.H10	3.65E-02
44D6	2.38E-03	3C3	5.95E-03	6E1.C4.F2	3.84E-02
17G6.C5	2.83E-03	28D5.C2	5.96E-03	30E10.F9	6.04E-02
24E4.G4.F4.C2	2.89E-03	8B5.D11	6.03E-03	28F5.F8	1.08E-01
29D11.D12	2.94E-03	16C2.E1.D4	6.04E-03	14E5.D8.C11	1.23E-01
22G11.C2.B9	2.95E-03	24H5.C9	6.10E-03	20D5.A5.C11	8.76E-01
9E7.C6.C4	3.01E-03	17A10.G5.B1	6.16E-03	13A6.A10.B5	2.26E+00
1D8.C7.D3.B11	3.16E-03	9A8.E6.E7.D10.B6	6.38E-03	8B5.D11.C10	2.32E+00
3A5.G3.A2	3.18E-03	25A9.H6.D3	6.44E-03	14D6.C3.E3.G5	2.38E+00
23A10.D5.C7	3.22E-03	10B4.D7.E7	6.46E-03	21A11	9.80E+01
1C5	3.27E-03	50B7	6.65E-03		
23A2.B4	3.29E-03	2G9	6.65E-03		

*In the table, the antibody ID corresponds to subclone identification.

In-Tandem Epitope Binning

While off-rate ranking identifies strong binders, it does not reveal whether antibodies recognize identical or distinct regions of the antigen. For many downstream applications, particularly sandwich ELISA, lateral flow assays, and other dual-antibody detection formats, epitope diversity is essential to enable compatible antibody pairing.

To address antibody pairing ability, in-tandem epitope binning was performed using the Octet® BLI system to characterize the binding relationships among mAbs generated against HPAI H5N1 clade 2.3.4.4b hemagglutinin (HA).

The in-tandem format uses a sequential binding assay on a biosensor surface, a format commonly employed on both SPR and BLI platforms. Following immobilization of antigen on the biosensor tip, a primary antibody is allowed to bind the antigen. A second antibody is then introduced to determine whether it can bind simultaneously. If the second antibody binds, the antibodies are considered non-competing and are inferred to recognize distinct epitopes. If binding is blocked or significantly reduced, the antibodies are interpreted as competing, suggesting that they bind overlapping or sterically hindered epitopes (Figure 2).

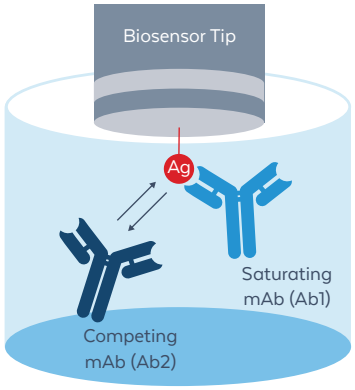

Figure 2. In-tandem epitope competition format where the antigen is immobilized onto the biosensor tip.

Similar to off-rate ranking, BLI-based epitope binning is inherently scalable. Current instrument configurations permit the screening of hundreds of antibodies over several days, thereby accelerating the characterization of binding relationships across extensive antibody cohorts without the need for structural data on the antigen.

Pairwise comparisons of antibody binding responses are compiled into a competition matrix and antibodies are grouped into discrete “bins” based on shared competition profiles (Table 3). Antibodies within the same bin compete with one another for binding, whereas antibodies assigned to different bins bind independently, indicating recognition of distinct antigenic regions.

Epitope binning experiments were performed using the following workflow:

Biosensors were pre-conditioned using three regeneration cycles in 10 mM glycine buffer (pH 1.7) followed by neutralization in 1×KB buffer (Octet® Kinetics Buffer).

↓

Recombinant HA antigen was immobilized onto the biosensor surface (300s loading step).

↓

A first antibody clone was introduced to allow antigen binding (300s association).

↓

A second antibody clone was subsequently introduced (420s association) to assess competitive or non-competitive binding.

↓

Sensors were regenerated using three cycles of glycine buffer (pH 1.7) and 1×KB prior to subsequent measurements.

BLI sensorgrams were analyzed to determine competitive versus non-competitive binding interactions, and percent inhibition values were calculated between antibody pairs. Based on these competitive binding profiles, antibodies were classified into distinct epitope bins representing groups of clones targeting the same or overlapping antigenic regions.

Table 3. Examples of in-tandem epitope binding results

Bin A	If Ab2 binds fully	Distinct epitope (no competition for binding)
Bin B	If Ab2 shows partial binding	Adjacent epitope (steric hindrance)
Bin C	If Ab2 cannot bind	Overlapping epitope (competitive)

Candidate Selection

In this study, four (4) unique epitope bins were identified among the anti-HA monoclonal antibody cohort. Each bin represents a class of antibodies that compete with one another for binding but do not compete with antibodies from other bins, suggesting recognition of distinct epitopes on the H5 antigen.

Integration of epitope binning with off-rate ranking enabled rational candidate selection from the larger hybridoma panel. Nine (9) monoclonal antibodies were selected for further characterization based on desirable combinations of relative affinity, epitope diversity, and specificity (Table 4). These antibodies were selected from an initial cohort of more than 100 clones.

Table 4 summarizes the selected candidates, including clone ID, assigned epitope bin, antibody isotype, relative specificity for H5 versus H3 hemagglutinin, performance in hemagglutination-inhibition (HI) or neutralization assays, and off-rate ranking determined using the Octet® binding assay.

Additional technical details describing BLI-based epitope binning workflows can be found in the Sartorius Octet® application note.

Table 4. High-value clones based on epitope binning and off-rate ranking

Final Clone ID	Epitope Bin	Isotype	HI	HA5 .2.3.4.4b Reactivity	HA3 (H3N2) Reactivity	Off-rank Rating (K_D)
1D4.G1	1	IgG1/Kappa	-	+	+	7.90E-04
13A6.A10.B5	1	IgG1/Kappa	-	+	-	2.26E+00
16E7.E6.D4	2	IgG1/Kappa	-	+	+	4.12E-02
22A8.D2.F5	2	IgG2b/Kappa	-	+	-	9.55E-03
23F2.C1	2	IgG1/Kappa	-	+	-	3.89E-03
18A4.E4.E3	3	IgG1/Kappa	-	+	+	1.06E-03
22D2.C2.F8	3	IgG1/Kappa	-	+	+	5.25E-03
17C5.E7	4	IgG1/Kappa	+	+	-	1.02E-03
20E8.H4.A9	4	IgG1/Kappa	-	+	-	4.38E-03

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Custom Monoclonal Antibody Production	Visit Webpage
Custom Polyclonal Antibody Production	Visit Webpage
Recombinant Antibody Services	Visit Webpage
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Recombinant Proteins from antibodies-online

Product	Item No.
Hemagglutinin (HA) (AA 17-338) Protein (His tag)	ABIN6386939
Hemagglutinin (HA) (AA 19-5) protein (His tag)	ABIN7825063
Influenza A NS1 Protein	ABIN572660
Influenza A Virus Neuraminidase (NA) (AA 30-4) Protein (His tag)	ABIN7825096

Influenza H5N1 Target Reagents

Product	Item No.
H5 HA 2.3.4.4b Antibody (13A6)	200-301-NH5
H5 HA 2.3.4.4b Antibody (16E7)	200-301-NH6
H5 HA 2.3.4.4b Antibody (23F2)	200-301-NH8
H5 HA 2.3.4.4b Antibody (17C5)	200-301-NJ1
H5 HA 2.3.4.4b Antibody (18A4)	200-301-NH9
H5 HA 2.3.4.4b Antibody (22D2)	200-301-NJ0
H5 HA 2.3.4.4b Antibody (1D4)	200-301-NH4

Detection Reagents

Product	Item No.
Mouse IgG (H&L) (Goat)	610-1102
Mouse IgG (H&L) (Goat) Peroxidase Conjugated	610-1302
Mouse IgG (H&L) (Goat) Biotin Conjugated	610-1602
Mouse IgG (H&L) Biotin Conjugated	610-106-121
Rabbit IgG (H&L) (Goat) Peroxidase Conjugated	611-1302
Human IgG (H&L) (Goat) Peroxidase Conjugated	609-1302
Human IgG (H&L) (Goat) Biotin Conjugated	609-1602
Streptavidin Peroxidase Conjugated	S000-03

Hemagglutination & RBC Reagents

Product	Item No.
Chicken Red Blood Cells 10% Washed Pools	R401-0050
Turkey Red Blood Cells 10% Washed Pools	R408-0050
Horse Red Blood Cells 10% Washed Pools	R409-0050
Chicken Red Blood Cell Antibody	103-4139

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