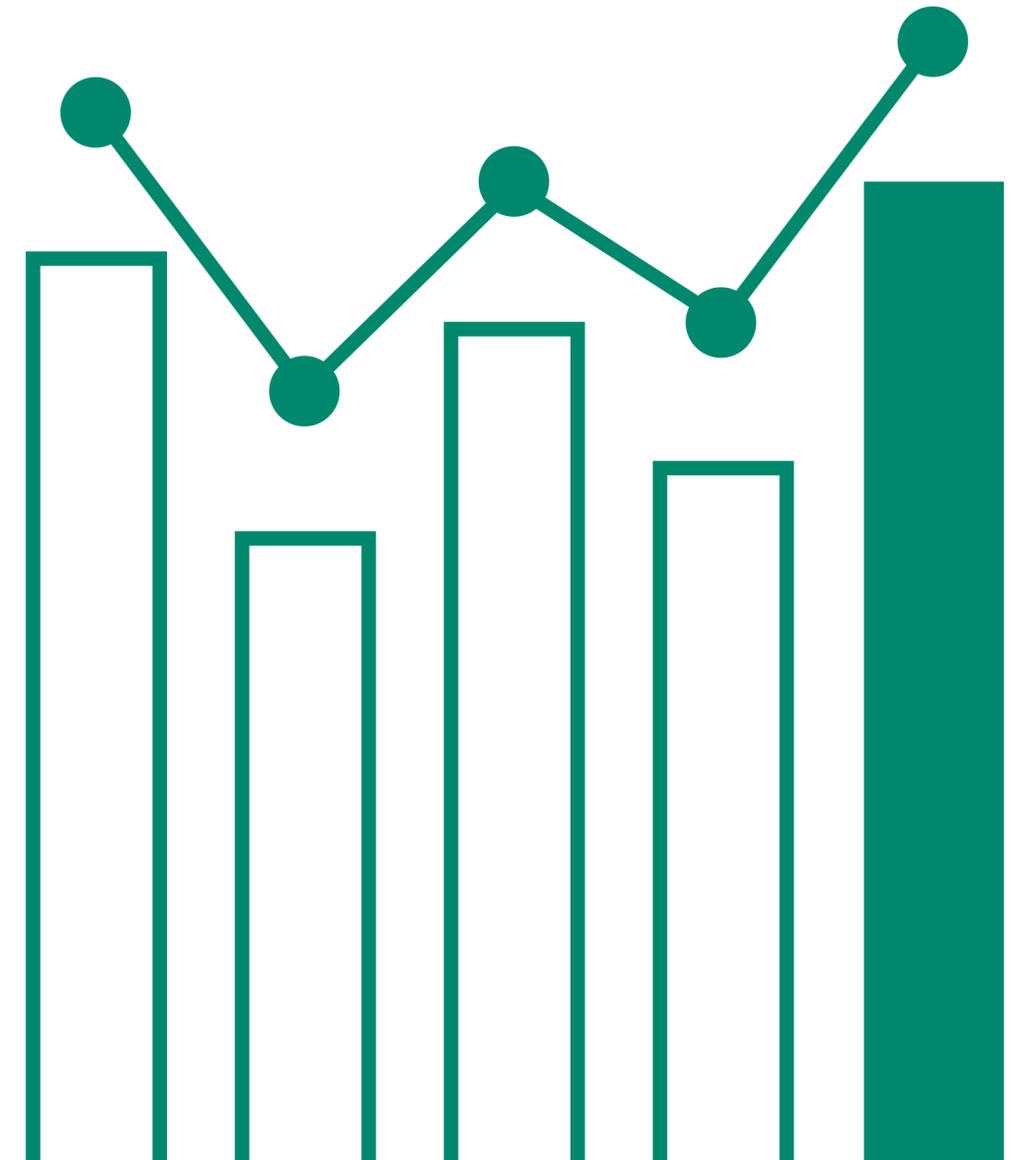# Fc receptor binding assays using surface plasmon resonance

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Introduction

Monoclonal antibodies (mAb) are the fastest growing subclass of biopharmaceuticals and mAb therapies have been successfully demonstrated to treat a variety of diseases, mainly in oncology and in the autoimmune disease and infectious disease segments. They are responsible for recognition of and binding to pathogens such as parasites, bacteria, and viruses and for detecting transformed cells via recognition of tumor-associated antigens. The antibodies form an immune complex with the pathogen/antigen, which induces effector responses such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) in innate immune cells through engagement with membrane-bound Fc receptors on the cell surface (1, 2).

Fc receptors recognize and interact with the Fc region of the antibodies and play a critical role in the immune response.

Fc receptor binding to effector cells

Fc receptors are glycoproteins found on the surface of effector cells such as B lymphocytes, natural killer cells, macrophages, human platelets, and mast cells. When Fc receptors interact with antigen-bound antibodies and form a membrane complex, information is communicated to the innate and adaptive immune system via tyrosine-based activation and inhibition motifs (Fig 1).

Phagocytic or cytotoxic cells are stimulated to destroy microbes, or infected cells, by antibody-dependent cell-mediated cytotoxicity. Some viruses, such as flaviviruses, also use Fc receptors to infect cells through a mechanism known as antibody-dependent enhancement of infection. Together, Fc receptors and antibodies contribute to the control of both inflammatory and anti-inflammatory responses of the innate immune system.

Fc receptors are divided into different classes based on the type of antibody they recognize:

*   Fc receptors that bind to immunoglobulins or IgG antibodies are called Fc gamma receptors (FcγR).

*   Fc receptors that bind IgA or IgE are called Fc alpha (Fcα) receptors and Fc epsilon (Fcε) receptors, respectively.

*   Another class of Fc receptor is the neonatal Fc receptor for IgG (FcRn), which plays a major role in the transport of the immunoglobulin (antibody) IgG across epithelial barriers at mucosal surfaces and in the regulation of IgG half-life.

This document will mainly focus on Fcγ receptors. However, recommendations on assay formats and experimental setups can to a large extent be applied also to other Fc receptors.

The Fcγ receptors can be classified into three families with respect to their binding affinity for IgG.

1. FcγRI (CD64) binds IgG with high binding affinity.

2. FcγRIIa (CD32a) and FcγRIIIa (CD16a) are medium affinity binders.

3. Low-affinity binding is seen for FcγRIIb (CD32b) and FcγRIIIb (CD16b).

Critical quality attributes (CQA), such as Fc receptor binding, are important for the safety and efficacy of a biopharmaceutical drug and monitoring of Fc receptor binding is crucial.
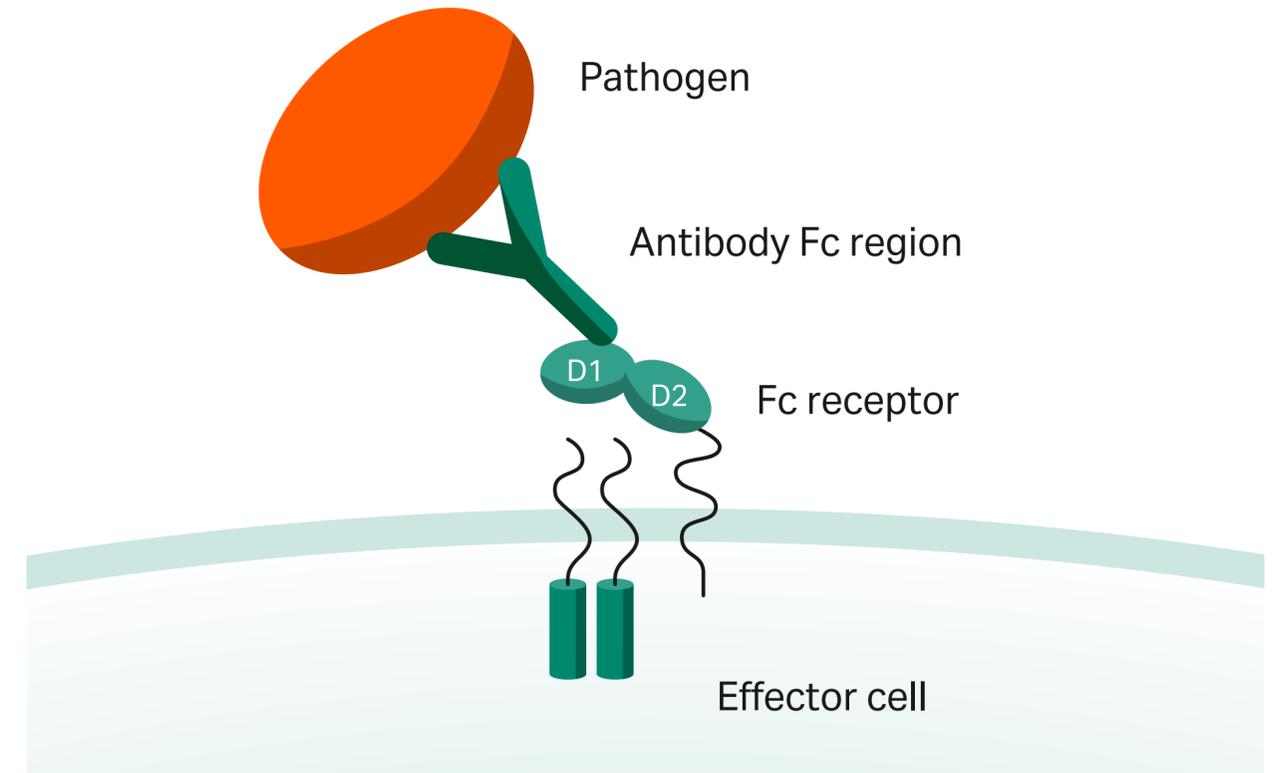
Fig 1. Interaction of Fc receptors on the cell surface of effector cells such B lymphocytes, natural killer cells, macrophages, human platelets, and mast cells with antigen-bound antibodies.

IgG-FcγR binding mechanisms have been the subject of multiple studies. However, reported affinities of the same IgG-FcγR interaction may differ as much as 10-fold depending on the source of the reagents and the experimental setups. There is also lack of consensus concerning optimal assay setup and assessment of data. In addition, there is significant heterogeneity found in both Fcγ receptors and antibodies due to glycosylation which adds to the complexity in analyzing the kinetics of the interactions (2).

This document describes how to set up, run, and evaluate Fcγ receptor assays using surface plasmon resonance (SPR) on Biacore™ systems to establish standardized guidelines.

Assay formats

Assays for analyzing Fcγ receptor-antibody interactions can be set up in different orientations:

- Fcγ receptor as ligand and the antibody injected as analyte.
- Antibody as ligand and the Fcγ receptor injected as analyte.

Regardless of assay orientation, capture of either the antibody or FcγR is the preferred method. Direct coupling of Fcγ receptor or the antibody to the sensor chip surface has been known to result in poor inter- and intra-assay reproducibility. In addition, a capture approach omits the need for optimization of immobilization and regeneration conditions. These time-consuming steps would need to be performed for each receptor/antibody combination.

Fc gamma receptor as ligand

The main recommendation for Fcγ receptors is to use capture. A method that has proven to work well is to capture the receptors via histidine (His) tag to an anti-His antibody covalently coupled to Sensor Chip CM5. After coupling, the Fcγ receptor is captured to the anti-His antibody. Concentration series of the antibodies are then run over the captured receptor. This approach can be used with either a single cycle or a multicycle kinetics setup.

An alternative capture method is to use biotinylated Fcγ receptors and capture to Sensor Chip CAP from Biotin CAPture Kit. Capture to Sensor Chip SA[1] is also an alternative.

Assay formats using Fc receptors as ligands are beneficial when there is low availability of receptors but requires that the active concentration of the antibodies is known to be able to assess kinetics and affinity of the interaction.

[1] Note: Capture of Fcγ receptor to Sensor Chip SA is not regenerable.

Tips for Fc receptor binding assays

- Choose assay format depending on your needs and preferences.
- Choose antibody as ligand if the active concentration of your antibodies is unknown.
- Choose Fcγ receptor as ligand when there is low availability of receptors.
- Choose a capture format over direct coupling as this will result in higher repeatability and a more stable assay.
- Check for nonspecific binding from the sample matrix by injecting blank samples (sample matrix without antibody) and check for binding to both the active and reference surfaces.
- Establish that the assay is suitable for purpose using a few samples and well-characterized control samples—characterized by orthogonal means—before you start extended runs with many samples.
- Prepare samples in running buffer to minimize bulk shift differences between sample and running buffer.
- When working with low concentration of antibodies, care must be taken that the carryover of excipient substances does not cause refractive index (RI) effects. Spin columns or dialysis should be used for preparation of such samples.
- Consider using SPR sensorgram comparison (available for Biacore T200, not for other Biacore systems) when facing heterogeneity.
- Include the reference sample as a control when you are using sensorgram comparison to ensure consistency of the assay.

Antibody as ligand

There are several ways to capture the antibodies to a sensor chip for analysis of Fcγ receptor binding. One approach that works for all Fcγ receptors is to biotinylate the antibodies and capture them on Sensor Chip CAP from Biotin CAPture Kit.

An alternative is to capture the antibodies to an anti-Fab surface using Human Fab Capture Kit. This product should, however, be avoided for FcγRI as this receptor is known to bind nonspecifically to the anti-Fab surface.

Sensor Chip Protein A is also known to work well for Fcγ receptor assays.

After capture of the antibody, concentration series of the Fcγ receptors are injected using either single cycle or multicycle kinetics setup. Assay formats using antibodies as ligands are suitable when there is high supply of Fcγ receptors as this format requires large amounts of Fcγ receptor. An advantage with this format is that the antibody concentration does not need to be known for assessment of kinetics and affinity. However, a suitable capture level must be determined in advance to ensure precise inter-assay variables.

Running buffer

Phosphate or HEPES saline buffers, such as PBS-P+, HBS-P+, and HBS-EP+ are recommended as running and sample dilution buffers.

Sensor chip surface preparation

For capture assays, the recommendation is to immobilize approximately 5000 RU of the capture molecule. Ready-to-use capture kits are available (*see* Table 1 and Table 2). These kits come with suggested immobilization and run condition protocols and, thus, reduce the need for immobilization, regeneration, and buffer optimization.

Table 1. Products for capture of Fcγ receptors

Product	Order code	Specific to
His Capture Kit	28995056	Histidine tagged molecules
Biotin CAPture Kit	28920233	Reversible capture of biotinylated molecules
Sensor Chip SA	BR100398	Permanent capture of biotinylated molecules

Table 2. Products for capture of antibodies

Product	Order code	Specific to
Biotin CAPture Kit	28920233	Reversible capture of biotinylated molecules
Human Fab Capture Kit	28958325	Human Fab fragments (kappa and lambda)
Sensor Chip Protein A	29127558	Antibodies according to the binding profile of Protein A

Sample preparation

Prepare the samples in the same buffer as the running buffer to minimize any bulk shift differences between sample and running buffer. It is important here to ensure that consideration has been given to the excipients in the antibody or FcγR buffers as cyclics such as sucrose, histidine, and trehalose since these are known to absorb in the spectrum used by Biacore SPR systems.

SPR binding assay temperature

Default sample compartment and run temperature for Fcγ receptor assays run on Biacore systems is 25°C. To conserve sensitive samples the sample compartment temperature can be set to 15°C or lower.

Biacore run setup

Fc gamma receptor as ligand

The general approach for assays using Fcγ receptor as ligand is described to the right using two different capture products: His Capture Kit and Biotin CAPture Kit. It is highly recommended that the instruction manual supplied in the kit is read and understood before starting.

Step	Action
1	Immobilize the anti-histidine antibody[1] on Sensor Chip CM5. Perform the immobilization in both active and reference flow cells. Alternatively dock Sensor Chip CAP and inject Biotin CAP Reagent on active and reference flow cells[2].
2	Run conditioning according to Instructions (Biotin CAPture Kit only).
3	Run at least three startup cycles to equilibrate the system. Include Fcγ receptor in the capture step (active surface only) and antibody replaced with buffer in the analyte step (reference and active surface). Ensure that regeneration solutions used during sample testing are included in the start-up cycles.
4	Capture Fcγ receptor on the active surface.
5	Inject a concentration series of the antibody over both active and reference surfaces. Include blank cycle(s).
6	Perform a regeneration of the surface.
7	Evaluate the results.

[1] = *See* Instructions for His Capture Kit
[2] = *See* Instructions for Biotin CAPture Kit

Conditions using His Capture Kit

Recommendations for assays using Fcγ receptor as ligand with His Capture Kit are listed in Table 3.

Table 3. Recommendations for assays using Fcγ receptor as ligand with His Capture Kit

Parameter	Recommended value	Comments
Immobilization capture molecule		
Immobilization level	~ 5000 RU	Use conditions specified in Instructions for His Capture Kit
Sensor surface	Sensor Chip CM5	
Capture injection (Fcγ receptor)		
Capture level	70–100 RU for kinetics 300–400 RU for affinity	Active flow cell only
Concentration	0.1–0.5 µg/mL	
Flow rate	5 µL/min	To minimize reagent consumption
Contact time	60–180 s	
Pooling	Yes	Capture solution can be pooled to minimize consumption
Molecular weight	N/A	Not needed in run method
Analyte injection (antibody)		
Flow rate	30 µL/min	To minimize mass transport effects
Contact time	60 s	
Dissociation time	≤ 600 s	Shorter dissociation time required for low-affinity interactions
Pooling	No	Pooling is not recommended for analyte injections
Molecular weight	Required if analyte concentrations are in weight units	Molar analyte concentrations required for evaluation of association rate constants

Parameter	Recommended value	Comments
Antibody concentrations	FcRI: 1–300 nM FcRIIIa: 25–2000 nM Other FcR: 0.5–8 µM	Use molar units
Number of analyte concentrations	Typically 5–9	
Startup cycles	≥ 3	Sufficient to stabilize the surface
Regeneration		
Flow rate	30 µL/min	
Solution	10 mM Glycine, pH 1.5	
Contact time	60 s	

Conditions using Biotin CAPture Kit

Recommendations for assays using Fcγ receptor as ligand with Biotin CAPture Kit are listed in Table 4.

Table 4. Recommendations for assays using Fcγ receptor as ligand with Biotin CAPture Kit

Parameter	Recommended value	Comments
Conditioning		
Flow rate	10 µL/min	
Solution	6 M Guanidine-hydrochloride/250 mM NaOH	
Contact time	3 × 60 s	
Injection of Biotin CAPture Reagent		
Sensor surface	Sensor Chip CAP	
Response level	2500 – 5000 RU	Use conditions specified in Instructions for Biotin CAPture Kit
Flow rate	2 µL/min	In order to minimize consumption
Contact time	300 s	
Capture injection (Fcγ receptor)		
Capture level	70–180 RU for kinetics	Active flow cell only
	300–400 RU for affinity	
Concentration	0.1–0.5 µg/mL	
Flow rate	5 µL/min	To minimize reagent consumption
Contact time	60–180 s	
Pooling	Yes	Capture solution can be pooled to minimize consumption
Molecular weight	N/A	Not needed in run method

Parameter	Recommended value	Comments
Analyte injection (antibody)		
Flow rate	30 µL/min	To minimize mass transport effects
Contact time	60 s	
Dissociation time	≤ 600 s	Shorter dissociation time required for low-affinity interactions
Pooling	No	Pooling is not recommended for analyte injections
Molecular weight	Required if analyte concentrations are in weight units	Molar analyte concentrations required for evaluation of association rate constants
Antibody concentrations	FcRI: 1–300 nM	Use molar units
	FcRIIIa: 25–2000 nM	
	Other FcR: 0.5–8 µM	
Number of analyte concentrations	Typically 5–9	
Startup cycles	≥ 3	Sufficient to stabilize the surface
Regeneration		
Flow rate	30 µL/min	
Solution	6 M Guanidine-hydrochloride/250 mM NaOH	
Contact time	60 s	

Antibody as ligand

The general approach for assays using antibody as ligand is described below using three different capture products: Biotin CAPture Kit, Human Fab Capture Kit, and Sensor Chip Protein A. It is highly recommended that the instruction manual supplied in the kit is read and understood before starting.

Step	Action
1	Dock Sensor Chip CAP in your Biacore instrument and inject Biotin CAP Reagent on active and reference flow cells[1]. For Human Fab Capture Kit, immobilize Human Fab Binder in active and reference flow cells according to instructions[2]. Alternatively dock Sensor Chip Protein A[3].
2	Run conditioning according to Instructions (Biotin CAPture Kit only).
3	Run at least three startup cycles to equilibrate the system. Include Fcγ receptor in the capture step (active surface only) and antibody replaced with buffer in the analyte step (reference and active surface). Ensure that regeneration solutions that are to be used during sample testing are included in the start-up cycles.
4	Capture antibody on the active surface.
5	Inject a concentration series of the Fcγ receptor over both active and reference surfaces. Include blank cycle(s).
6	Perform a regeneration of the surface.
7	Evaluate the results.

[1] = *See* Instructions for Biotin CAPture Kit
[2] = *See* Instructions for Human Fab Capture Kit
[3] = *See* Instructions for Sensor Chip Protein A

Conditions using Biotin CAPture Kit

Recommendations for assays using antibodies as ligand with Biotin CAPture Kit are listed in Table 5.

Table 5. Recommendations for assays using antibodies as ligand with Biotin CAPture Kit

Parameter	Recommended value	Comments
Conditioning		
Flow rate	10 µL/min	
Solution	6 M Guanidine-hydrochloride/250 mM NaOH	
Contact time	3 × 60 s	
Injection of Biotin CAPture Reagent		
Sensor surface	Sensor Chip CAP	
Response level	2500–5000 RU	Use conditions specified in Instructions for Biotin CAPture Kit
Flow rate	2 µL/min	In order to minimize consumption
Contact time	300 s	
Capture injection (antibody)		
Capture level	200-400 RU for kinetics	Active flow cell only
	900–1200 RU for affinity	
Concentration	0.1–0.5 µg/mL	
Flow rate	5 µL/min	To minimize reagent consumption
Contact time	60–180 s	
Pooling	Yes	Capture solution can be pooled to minimize consumption
Molecular weight	N/A	Not needed in run method

Parameter	Recommended value	Comments
Analyte injection (Fcγ receptor)		
Flow rate	30 µL/min	To minimize mass transport effects
Contact time	60 s	
Dissociation time	≤ 600 s	Shorter dissociation time required for low affinity interactions
Pooling	No	Pooling is not recommended for analyte injections
Molecular weight	Required if analyte concentrations are in weight units	Molar analyte concentrations required for evaluation of association rate constants
Antibody concentrations	FcRI: 1–300 nM	Use molar units
	FcRIIIa: 25–2000 nM	
	Other FcR: 0.5–8 µM	
Number of analyte concentrations	Typically 5–9	
Startup cycles	≥ 3	Sufficient to stabilize the surface
Regeneration		
Flow rate	30 µL/min	
Solution	6 M Guanidine-hydrochloride/250 mM NaOH	
Contact time	60 s	

Conditions using Human Fab Capture Kit

Recommendations for assays using antibodies as ligand with Human Fab Capture Kit are listed in Table 6.

Table 6. Recommendations for assays using antibodies as ligand with Human Fab Capture Kit

Parameter	Recommended value	Comments
Immobilization capture molecule		
Immobilization level	5000 RU	Use conditions specified in Instructions for Human Fab Capture Kit[1]
Sensor surface	Sensor Chip CM5	
Capture injection (antibody)		
Capture level	200–400 RU for kinetics	Active flow cell only
	900–1200 RU for affinity	
Concentration	0.1–0.5 µg/mL	
Flow rate	5 µL/min	To minimize reagent consumption
Contact time	60–180 s	
Pooling	Yes	Capture solution can be pooled to minimize consumption
Molecular weight	N/A	Not needed in run method
Analyte injection (Fcγ receptor)		
Flow rate	30 µL/min	To minimize mass transport effects
Contact time	60 s	
Dissociation time	≤ 600 s	Shorter dissociation time required for low affinity interactions

Parameter	Recommended value	Comments
Pooling	No	Pooling is not recommended for analyte injections
Molecular weight	Required if analyte concentrations are in weight units	Molar analyte concentrations required for evaluation of association rate constants
Antibody concentrations	FcRI: 1–300 nM	Use molar units
	FcRIIIa: 25–2000 nM	
	Other FcR: 0.5–8 µM	
Number of analyte concentrations	Typically 5–9	
Startup cycles	≥ 3	Sufficient to stabilize the surface
Regeneration		
Flow rate	30 µL/min	
Solution	10 mM Glycine, pH 2.1	
Contact time	2 × 60 s	

[1] = Do not deviate from recommended immobilization conditions as this will result in an uneven distribution of Fab binders on the surface.

Conditions using Sensor Chip Protein A

Recommendations for assays using antibodies as ligand with Sensor Chip Protein A are listed in Table 7.

Table 7. Recommendations for assays using antibodies as ligand with Sensor Chip Protein A

Parameter	Recommended value	Comments
Immobilization capture molecule		
Immobilization level	N/A	Prefunctionalized surface
Sensor surface	Sensor Chip Protein A	
Capture injection (antibody)		
Capture level	200–400 RU for kinetics 900–1200 RU for affinity	
Concentration	0.1–0.5 µg/mL	
Flow rate	5 µL/min	To minimize reagent consumption
Contact time	60–180 s	
Pooling	Yes	Antibody solution for capture can be pooled to minimize consumption
Molecular weight	N/A	Not needed in run method
Analyte injection (Fcγ receptor)		
Flow rate	30 µL/min	To minimize mass transport effects
Contact time	60 s	
Dissociation time	≤ 600 s	Shorter dissociation time required for low affinity interactions
Pooling	No	Pooling is not recommended for analyte injections

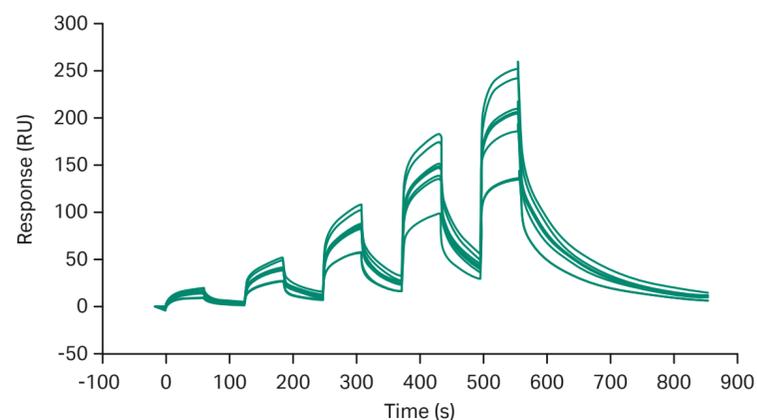| Parameter	Recommended value	Comments
Molecular weight	Required if analyte concentrations are in weight units	Molar analyte concentrations required for evaluation of association rate constants
Fc receptor concentrations	FcRI: 1–300 nM FcRIIIa: 25–2000 nM Other FcR: 0.5–8 µM	Use molar units
Number of analyte concentrations	Typically 5–9	
Startup cycles	1 to 3	Sufficient to stabilize the surface
Regeneration		
Flow rate	30 µL/min	
Solution	10 mM Glycine, pH 1.5	
Contact time	30 s	

Evaluation tools and options

Results from Fcγ receptor analysis are often reported as affinity data. Kinetic data is less common due to heterogeneity of the interactants. For example, it has been shown that the glycosylation state of both antibody and FcγR1 (CD64) has a direct impact on the kinetics (2). Thus, kinetics becomes very complex and kinetic and affinity constants cannot always be reliably determined, only estimated.

Evaluation of binding kinetics and steady-state affinity

Estimates of kinetics and/or affinity constants from Fcγ receptor experiments data can be obtained using standard procedure for kinetics and affinity assessment. For detailed information *see Biacore Application Guide Kinetics and affinity measurements with Biacore systems* (3).

Evaluation using sensorgram comparison with Biacore T200

FcγR-antibody interactions can also be evaluated using sensorgram comparison available on Biacore T200. Sensorgram comparison is a statistical approach that uses the full binding profile and compares the shape of the binding curve with that of a reference sample (4). Sensorgram comparison is setup as a standard kinetics experiment but does not involve fitting of the curve.
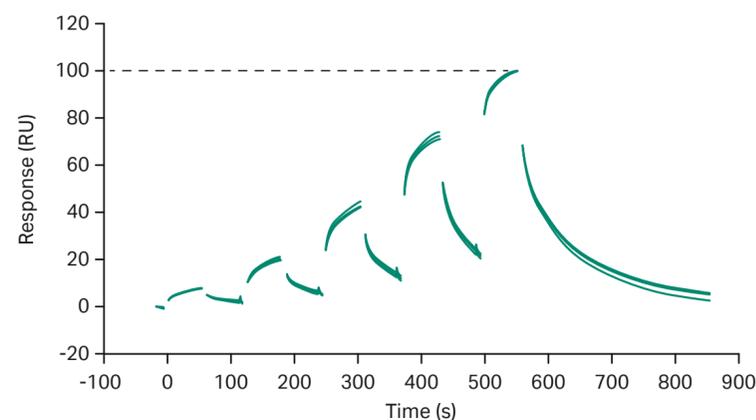
As in any kinetic experiment, the data set needs to contain kinetic information to enable use of sensorgram comparison, that is, there should be curvature and a visible dissociation (> 5%). The procedure for Fcγ receptor analysis using sensorgram comparison is described below.
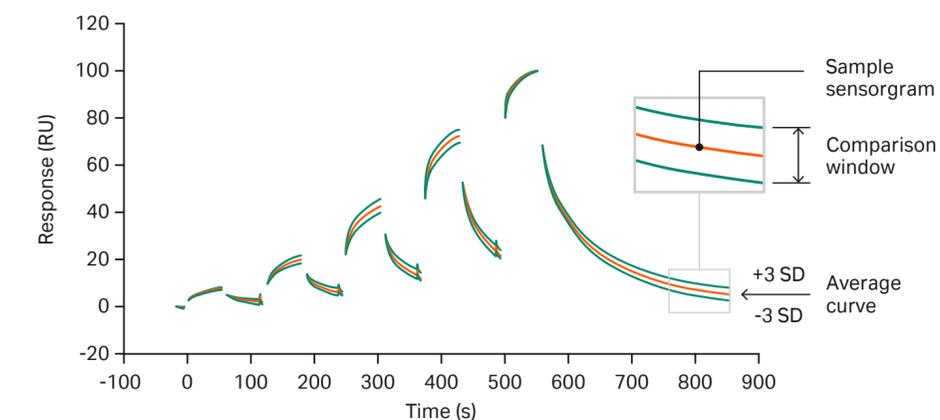
Establishing experimental variation of reference

The first step when working with sensorgram comparison is to establish the experimental variation of the reference. This is done by running experiments on different dates, with different preparations of the reagents and, if possible, with different operators and instruments. Data can also come from different batches of the reference sample. This will reveal the natural variation of the reference sample. Figure 2 shows repeated runs of Rituximab binding to FcγRIIIa V158.

The reference samples are collated and globally normalized to compensate for inter-assay variability in capture/immobilization level. Data is displayed on a normalized scale of 0–100 as seen in Figure 3.

From this data, an average curve is calculated. In this example, three standard deviations were applied to the experimental data (upper and lower curve in Fig 4). This is used to calculate a comparison window (*see* the enlarged window below in Fig 4). Samples with similar curve shapes, and thus similar kinetics, are expected to fall within this window. Comparison can be made using the entire binding curve or association/dissociation only.

Fig 2. Sensorgram of repeated runs of the chimeric monoclonal antibody biotherapeutic, Rituximab, binding to FcγRIIIa V158 ligand.

Fig 3. Normalized sensorgram of chimeric monoclonal antibody biotherapeutic, Rituximab, binding to FcγRIIIa.

Fig 4. Average sensorgram curve from which a comparison window is calculated; samples with similar curve shapes, and thus similar kinetics, are expected to fall within this window.
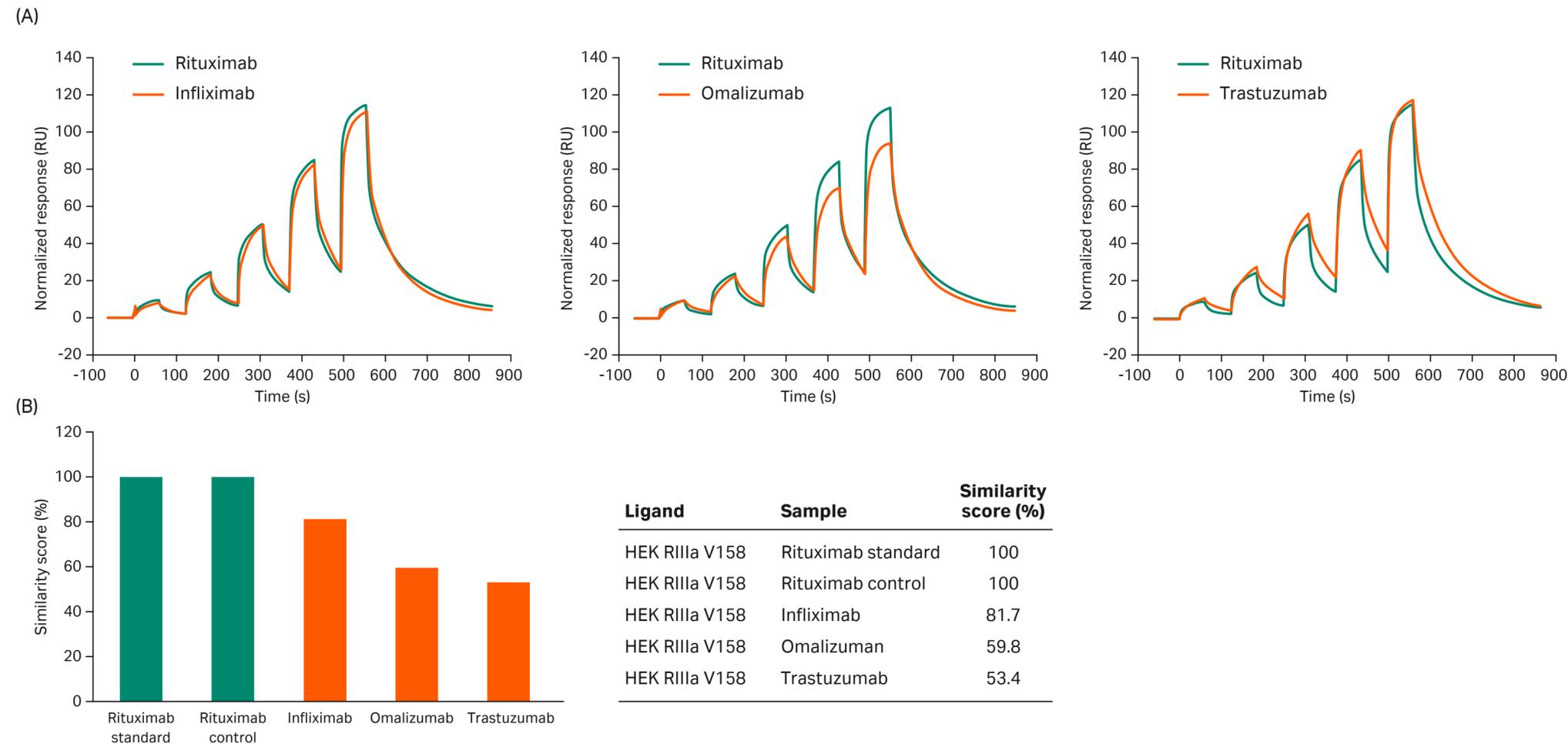
Assessment of similarity

Samples are run using the same settings as for the reference sample. The similarity of the interactions is quantitated by a similarity score (0% to 100%), which is calculated from the number of data points within and outside of the comparison window. A sample with all data points within the comparison window will have a similarity score of 100% while a sample with data points outside the comparison window will have a similarity score less than 100%.

Figure 5 shows three antibodies binding to FcγRIIIa V158. The reference sample in this example is Rituximab. Rituximab was also included in the experiment as a control to check consistency of the assay. As expected, all data points for the Rituximab control fall within the comparison window and the similarity score for the Rituximab control is 100%. The Infliximab curve (left sensorgram) is also relatively similar to the reference curve and the similarity

score is ~ 82%. For Omalizumab and Trastuzumab (middle and right sensorgrams, many of the data points are outside the comparison window and consequently the similarity scores are lower (60% and 53% respectively).

For more detailed information on sensorgram comparison, *see* reference 5.

(A)

(B)

Ligand	Sample	Similarity score (%)
HEK RIIIa V158	Rituximab standard	100
HEK RIIIa V158	Rituximab control	100
HEK RIIIa V158	Infliximab	81.7
HEK RIIIa V158	Omalizuman	59.8
HEK RIIIa V158	Trastuzumab	53.4

Fig 5. (A) Sensorgrams and (B) similarity scores in comparison to a Rituximab standard for the binding of three mAb biotherapeutics—Infliximab, Omalizumab, and Trastuzumab— to Fc FcγRIIIa V158 ligand.

Summary

In this document, we have presented generally applicable methods for assessment of Fcγ receptor-antibody interactions using Biacore systems. The proposed methods enable experiments using the antibodies either as ligands or as analytes. However, heterogeneity in both antibodies and Fcγ receptors can hinder reliable determination of binding kinetics and binding affinity (measured by the equilibrium dissociation constant, K_D). An alternative evaluation approach is to use sensorgram comparison, a statistical approach that compares the entire binding profile of the samples with a reference standard as a mean to assess Fcγ receptor-antibody interactions.

References

1. Hayes, J. M. *et al*. Identification of Fc gamma receptor glycoforms that produce differential binding kinetics for Rituximab. *Mol. Cell Proteomics* **16(10)**, 1770–1788 (2017).

2. Hayes, J. M. *et al*. Fc gamma receptor glycosylation modulates the binding of IgG glycoforms: a requirement for stable antibody interactions. *J. Proteome Res.* **13(12),** 5471–5485 (2014).

3. Biacore Application Guides: Kinetic and affinity measurements with Biacore systems, Cytiva, 29360778 Edition AA.

4. Karlsson R. *et al.* Comparison of surface plasmon resonance binding curves for characterization of protein interactions and analysis of screening data. *Anal. Biochem.* **502**, 53–63 (2016).

5. Gassner C. *et al*. Beyond conventional dose-response curves: Sensorgram comparison in SPR allows single concentration activity and similarity assessment. *J. Pharm. Biomed. Anal.* **154**, 57–65 (2018).

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