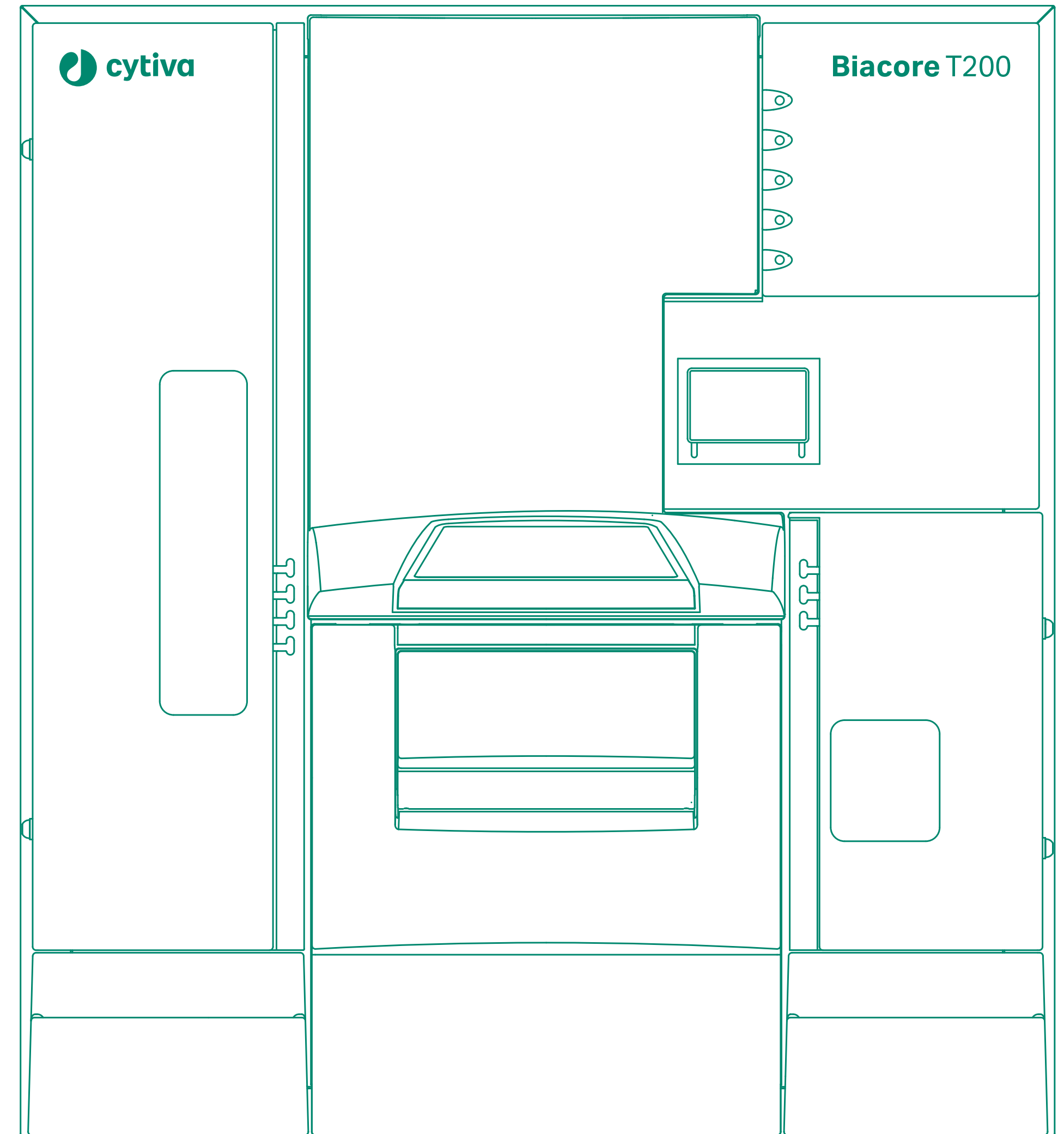


Handbook

# Biacore T200

## Immunogenicity



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# 01

# Introduction

Biacore™ T200 provides support for applications in the area of immunogenicity screening, confirmation and characterization. This Handbook provides some background to immunogenicity applications and describes the functionality in Biacore T200. Familiarity with the operation of Biacore T200 and use of the Control and Evaluation Software is assumed.

## 1.1 Additional documentation

Description of the Biacore T200 instrument and basic software functionality may be found in the Biacore T200 Operating Instruction and Biacore T200 Software Handbook respectively.

General information about sensor chips for use in Biacore systems, including details of ligand immobilization chemistry and surface regeneration procedures can be found at [cytiva.com/biacoregetstarted](http://cytiva.com/biacoregetstarted).

## 1.2 Support for regulated environments

Biacore T200 offers optional support for work in regulated environments in the Biacore T200 GxP Package<sup>1</sup>. This support encompasses use of the immunogenicity applications when the GxP Package is installed.

Functionality in the Biacore T200 GxP Package is described in the Biacore T200 GxP Handbook.

<sup>1</sup> GxP: Collective abbreviation for GLP (Good Laboratory Practice), GCP (Good Clinical Practice) and GMP (Good Manufacturing Practice).

# 02

# Immunogenicity background

This chapter gives a general background to the issues of immunogenicity in relation to the functionality provided by Biacore T200.

## 2.1 What is immunogenicity?

In a broad sense, immunogenicity refers to the ability of a substance to induce a humoral or cell-mediated immune response, expressed as production of specific antibodies. The immunogenic substance is by definition an antigen. The immune reaction may be desirable (as in the natural reaction of the immune defense system to infection, or in the provoked reaction to vaccines) or unwanted (as when induced antibodies counteract the effect of administered drugs or appear as part of an allergic reaction).

In recent years, immunogenicity has gained increasing focus in the context of immune reaction to biotherapeutic agents. As more biomolecules such as natural or engineered inhibitors, effectors or antibodies are used as therapeutics, issues related to the immunogenicity of the molecules become more important. The functionality in Biacore T200 focuses primarily on the (usually unwanted) immune response induced by biotherapeutic drugs or biologics.

In this Handbook, the term drug is used to refer to the antigenic therapeutic agent responsible for eliciting the immune response.

### 2.1.1 Effects of an immune response

Both the occurrence and the effects of an immune response to biologics vary widely. Administration of a biotherapeutic drug does not always elicit an immune response in the patient, and the same drug may cause a response in some patients but not in others. Clinically relevant consequences of an immune response include direct neutralization of the therapeutic effect, influence on the pharmacokinetic properties of the drug and potentially serious side effects. Not all immune responses are however clinically relevant: in many cases, the antibodies have no clinical effect on the patient's condition or on the effect of the treatment. Functions in Biacore T200 are aimed at detecting and characterizing the immune response itself and do not address the question of clinical relevance.

### 2.1.2 Immunogenicity in drug development and clinical situations

Testing drugs for immune response is an essential part of drug development, clinical trials and follow-up tests in therapeutic use. There are a number of challenges involved in detecting and characterizing an immune response and its clinical relevance:

- Testing drug candidates for potential immunogenicity, in either animal models or cell-based assays, can give some indication of the risks that may be met in clinical situations. Mere production of antibodies in a model system does not however necessarily indicate that the immune response will be clinically relevant.
- Model systems, using either laboratory animals or cell-based assays, can never completely mimic the effect of an immune response in human patients. Regardless of the extent of investigation during development, immunogenicity testing in human patients is necessary both during clinical trials and in treatment follow-up using approved drugs.
- Characterization of drug-specific antibodies produced during an immune response can give important clues to the possible clinical significance of the immune response.
- The length of time that the drug remains in circulation after administration (pharmacokinetics) varies between different drugs, and the presence of excess drug in samples can lead to drug interference with the assay. For full confidence in immunogenicity testing, the assay format should support the detection of antibodies in the presence of excess drug.
- The time course of the immune response (immunokinetics) is important, with respect to both antibody class and subclass (different antibody classes represent different physiological aspects of the immune response) and antibody maturation (the drug-binding properties of antibodies within a given class can change over time).
- Measurements are made in complex sample matrices (most commonly serum), and individual variation both between laboratory animals and patients and between samples taken at different times from the same subject complicates the analysis. Statistical techniques need to be applied to the data in order to establish reliable results.

### 2.1.3 Immunogenicity in vaccine development

Development of vaccines that are designed to elicit a long-lived immune defense reaction against the pathogen also relies on characterization of immunogenicity, but from a different perspective than biotherapeutic development. For biotherapeutic drugs, the goal is usually to avoid or minimize the immune response, at least in terms of clinically relevant antibodies, so that assays used need to be able to detect low levels of antibody production, and also need to be complemented with studies of the clinical consequences of the immune response. Vaccines, on the other hand, are intended to elicit a response, and high levels of persistent antibody production are usually desirable. Any clinical side-effects of the antibody production need to be studied, but the primary characteristic of the antibody is that it should lead to elimination of or neutralize the effect of the pathogen.

## 2.2 Support for immunogenicity studies

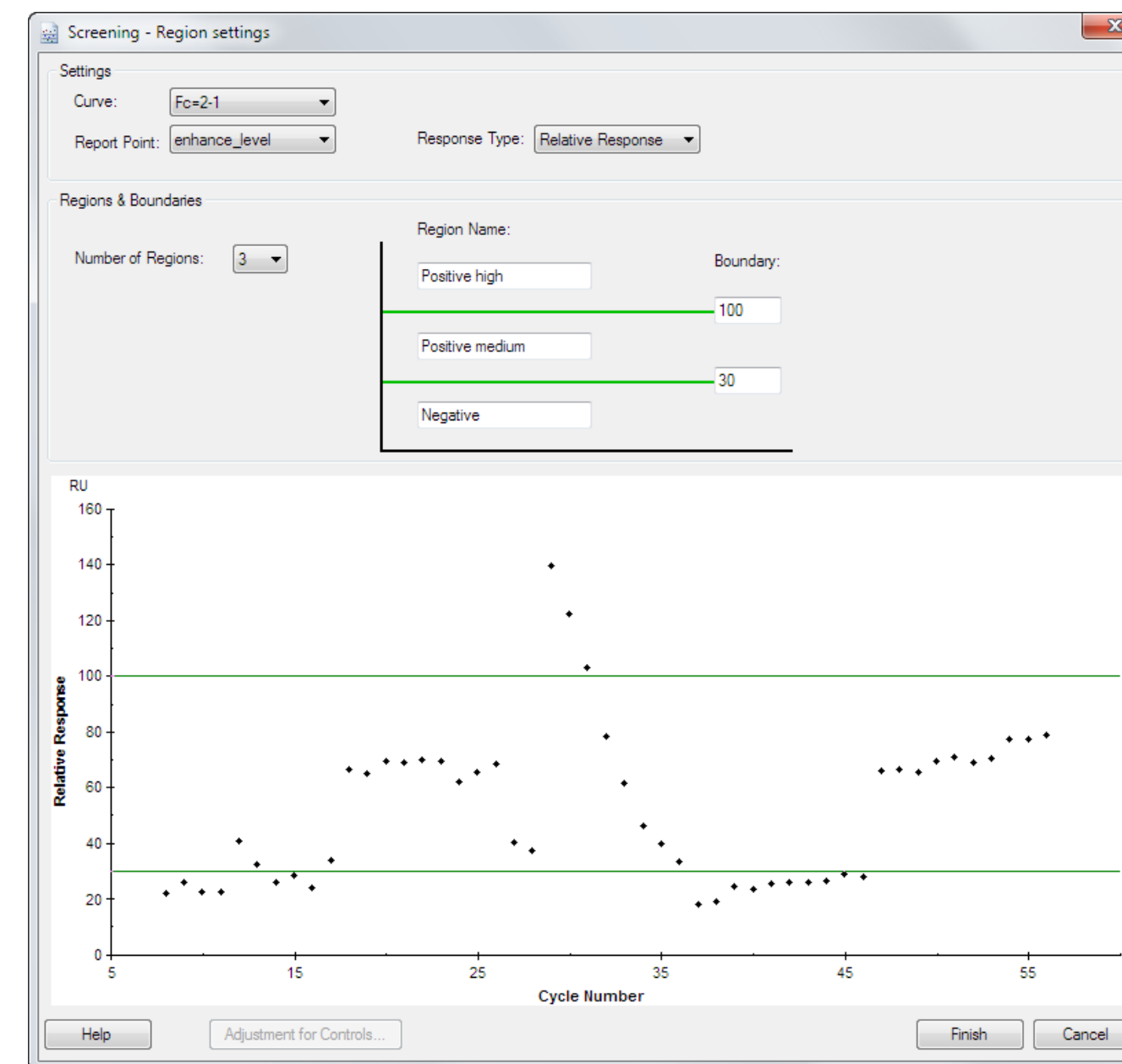
Biacore T200 supports four elements in immunogenicity characterization, through measurement of antibody binding to drug immobilized on the sensor surface. Binding responses are measured directly in serum or plasma (if necessary after appropriate dilution).

- Screening for the presence of antibodies.
- Confirmation that detected antibodies are directed against the drug.
- Characterization of antibodies in terms of isotype (class) and subtype (subclass).
- Characterization of antibodies in terms of the stability of binding to drug.

In addition, Biacore T200 can be used for epitope mapping studies for more detailed characterization of drug binding and competitive ligand binding (CLB) assays which present an alternative to cell-based assays for neutralizing antibodies. These assay formats are not supported by specific functions in Biacore T200, and details of the assays are outside the scope of this Handbook.

### 2.2.1 Screening for immune response

As a first step in immunogenicity measurements, samples are screened for the presence of antibodies specific for the drug. In simplified terms, the response level obtained when samples are injected over immobilized drug reflects the amount of antibody in the sample. Samples are judged positive when the response exceeds a cut-off level normally determined from the response from a number of negative serum samples (Figure 2-1).



**Figure 2-1.** Screening is evaluated by ranking the sample responses relative to a cut-off level. Samples can be ranked in up to 4 regions.

Screening for antibody response in samples is described in more detail in Chapter 5.

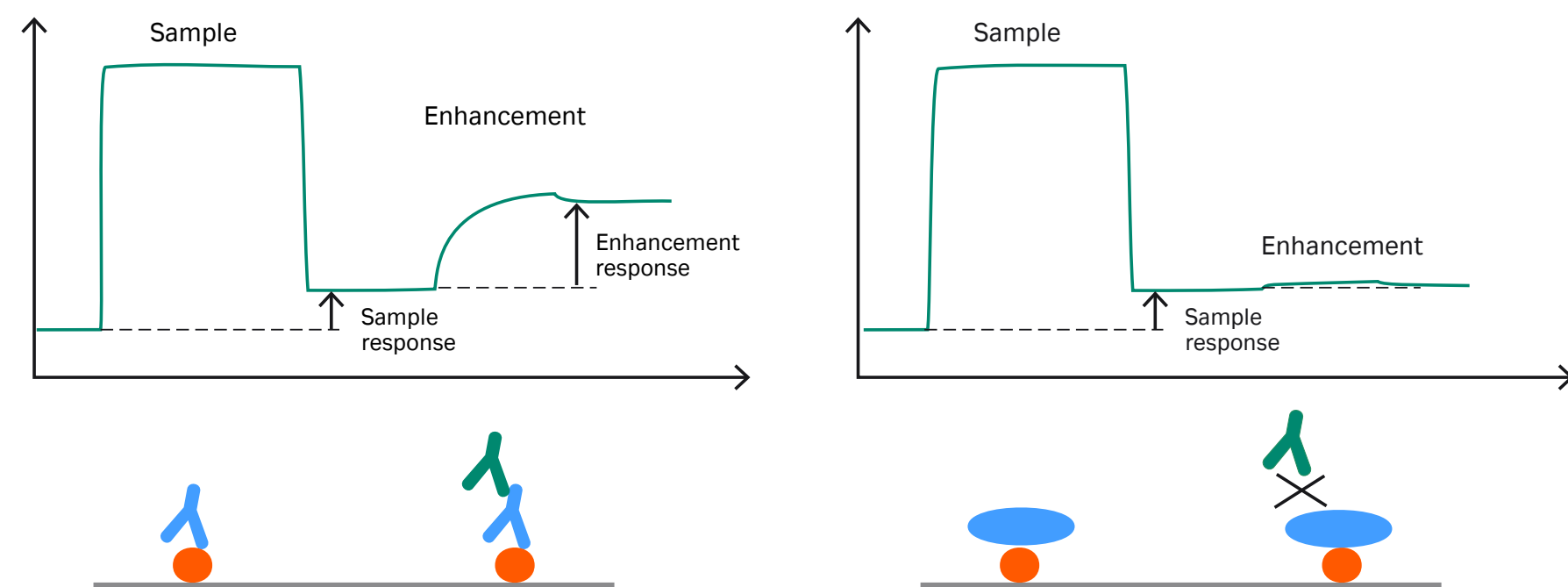
## 2.2.2 Antibody confirmation

A typical cut-off level for identifying positive samples in a screening experiment will usually give about 5% false positive identifications. Two kinds of confirmatory measurement help to eliminate these false positive samples:

- Injection of an enhancement reagent directed against common antibody structures after the sample injection reveals whether the response derives from an antibody. This measurement gives no information on the specificity of the antibody.
- Addition of excess drug to the sample to compete with binding to the drug on the sensor surface reveals whether the response derives from specific binding to the drug. This measurement only confirms the specificity of the response, and does not identify the binding molecule as an antibody.

### Confirmation by enhancement – antibody identity

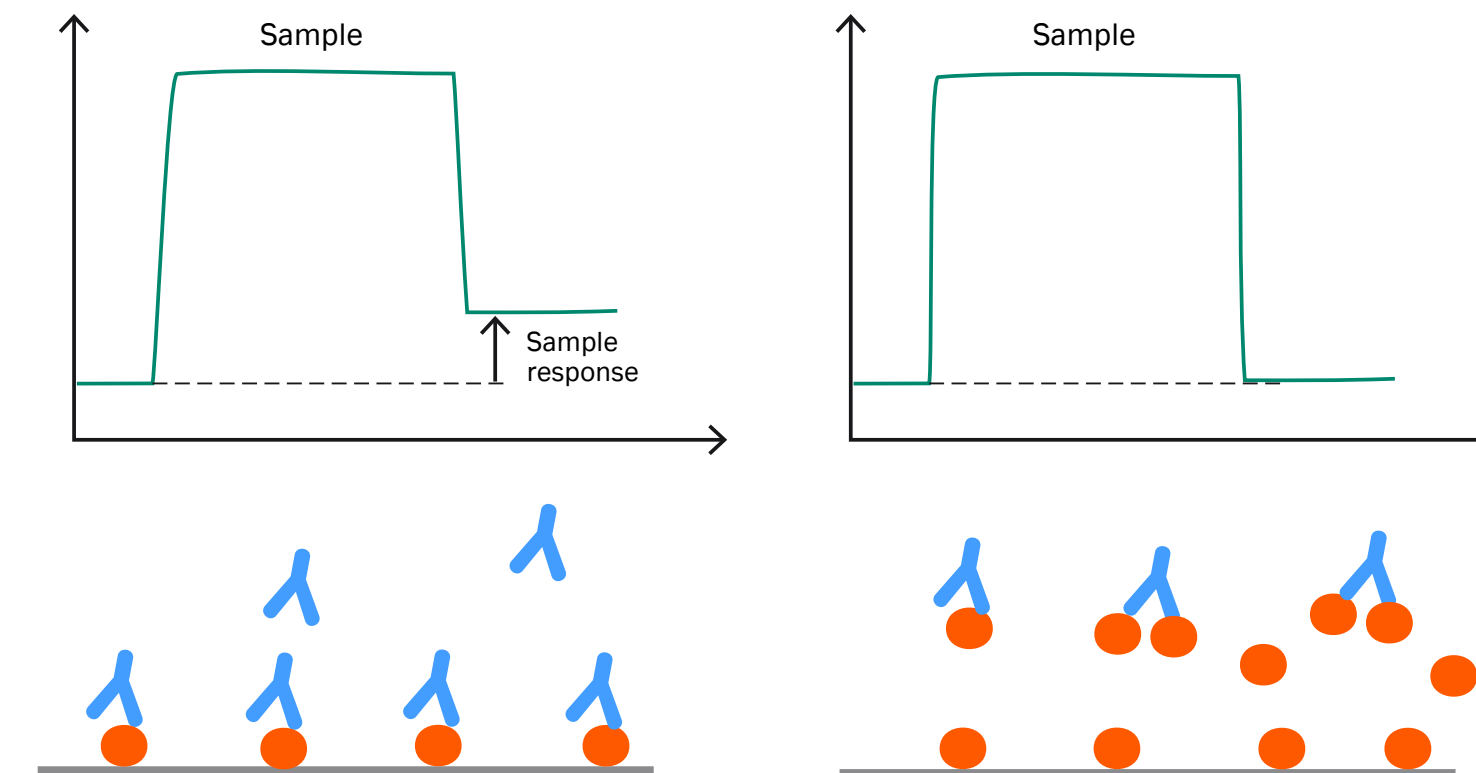
Enhancement reagents can be injected directly after the sample, either to amplify the response or to confirm the identity of the bound analyte (Figure 2-2). In immunogenicity screening, an antibody-specific reagent (e.g. anti-human IgG specifically for IgG antibodies, or anti-kappa or anti-lambda chains as more general reagents) can be used to confirm that the response seen in the sample injection represents bound antibody rather than other sample constituents.



**Figure 2-2.** Principle of antibody identity confirmation by enhancement. Left: Binding of antibody to the drug gives a primary sample response, and subsequent binding of antibody-specific enhancement reagent gives a secondary enhancement response. Right: Non-antibody molecules that bind to the drug give a primary sample response, but the secondary enhancement response is not observed since the enhancement reagent does not bind.

### Confirmation by inhibition – antibody specificity

Excess drug added to samples will compete with drug-specific antibodies for binding to drug on the sensor surface and thereby inhibit the specific response (Figure 2-3).

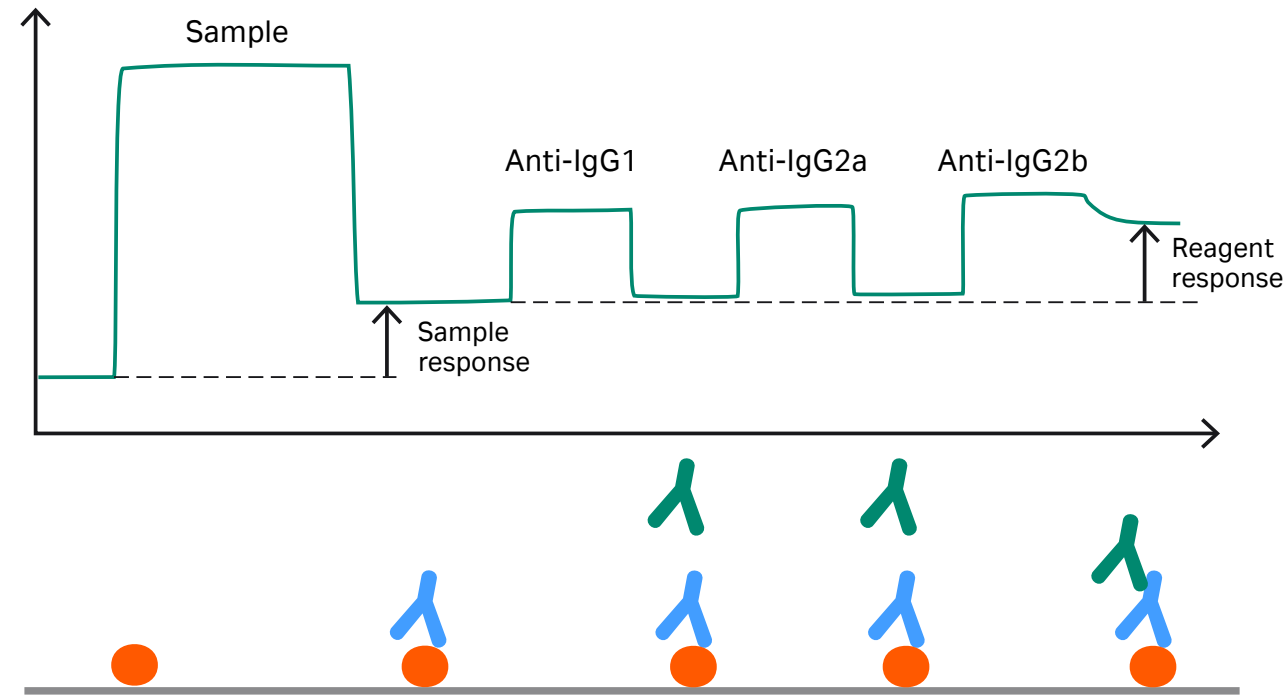


**Figure 2-3.** Principle of antibody specificity confirmation by inhibition. Left: When the sample does not contain excess drug, antibody can bind to the surface. Right: Addition of excess drug to the sample blocks the antibody binding to the surface.

Confirmation by inhibition is described in more detail in Chapter 6.

## 2.2.3 Isotype determination

Biacore T200 supports characterization of isotype and subtype (class and subclass) for antibodies detected in the sample. The antibodies from the sample that bind to the sensor surface are challenged with a series of anti-isotype reagents (typically antibodies), and the resulting responses indicate the relative amounts of different isotypes in the sample antibody population (see Figure 2-4). Since the antibodies from the sample are selected first by their ability to bind to the drug on the sensor surface, the characterization determines the isotype distribution of the specific antibodies involved in the immune response.



**Figure 2-4.** Principle of antibody isotype characterization. Antibody bound to the surface is challenged with isotype-specific reagents. This illustration shows sequential challenges with reagents specific for IgG1, IgG2a and IgG2b. Only the third reagent (anti-IgG2b) gives a significant response, identifying the bound antibody as subtype IgG2b.

**Note:** The term **isotype** is used generically in this handbook and in Biacore T200 software to cover both isotypes and subtypes. The terms **class** and **subclass** may be regarded as more correct in formal usage, but isotype and class are often used interchangeably and isotype is chosen here as a more clearly distinguishing term.

Antibody isotyping is described in more detail in Chapter 7.

### 2.2.4 Characterization of antibody binding stability

The antibody population in samples can be characterized in terms of the stability of binding to the drug on the sensor surface. The population may represent a broad spectrum of affinities within and between different antibody classes, and the dissociation behavior of antibody from the sensor surface is correspondingly complex. Evaluation of the dissociation in terms of a simple exponential decay (representing a 1:1 binding model) usually suffers from poor fitting quality, partly because of the heterogeneous antibody population in the sample and partly because antibodies are bi- or multivalent. In Biacore T200, binding stability is assessed in terms of a two-component dissociation model.

**Note:** It is important to appreciate that this evaluation approach does not identify or imply the existence of two specific antibody populations with the respective dissociation properties and proportions. Classification in terms of only two apparently discrete populations is a compromise that describes most experimental data adequately in mathematical terms and provides manageable data output.

Evaluation of antibody binding stability is described in more detail in Chapter 8.

### 2.2.5 Dealing with drug interference

If the serum samples contain drug as well as drug-specific antibody (as may be expected if samples are taken before the drug has been cleared from the bloodstream), some or all of the antibodies may be bound to drug in the sample and may not be detected in the assay (this is analogous to the situation where excess drug is deliberately added to the sample, Figure 2-3).

**Note:** Antibodies are bi- or multivalent, so some antibodies bound to drug in the sample may still be detected if there are free drug-binding sites available.

Most antibody-drug complexes dissociate in acidic conditions, so that antibodies present in complexes may be released by acidification and detected after appropriate neutralization of the samples. (Acidified samples cannot be analyzed directly since the antibody will not bind to the surface-attached drug under these conditions.) If the drug withstands the acidification without losing antibody-binding activity, measurements must be made as soon as possible after neutralization, since antibody-drug complexes will start to re-form when the samples are neutralized. On the other hand, if the drug is destroyed by acidification, complexes will not re-form on neutralization and measurements can be made at any time after the samples are neutralized. Analysis immediately after neutralization is supported in Biacore T200 by injecting the acidified sample together with neutralization solution (called a merged injection), so that there is a minimum delay between mixing and measurement. For assays where the time between neutralization and analysis is not critical, both the acidification and neutralization steps can be performed in a microplate well before the sample is injected.

Details of procedures for dealing with drug interference are described in more detail in Chapter 4.

# 03

# Assay design and development

This chapter considers common aspects of assay design for immunogenicity work with Biacore T200. Special considerations for dealing with drug interference are discussed in Chapter 4. General recommendations for design and optimization of immunoassays have been discussed by Mire-Sluis *et al*<sup>1</sup>.

<sup>1</sup> Mire-Sluis, A. R. *et al.* (2004), *J. Immunol. Methods* **289**, 1-16.

## 3.1 Sensor surface

### 3.1.1 Chip type

Sensor Chip CM5 is recommended for all immunogenicity applications, providing a high surface binding capacity and relatively low levels of non-specific binding. Sensor chips designed for specific capture applications (Sensor Chip SA, Sensor Chip NTA) and for use with membranes and hydrophobic proteins tend to give high levels of non-specific binding and are not suitable. The high capacity chip Sensor Chip CM7 is designed for work with low molecular weight analytes and is not suitable for immunogenicity studies.

### 3.1.2 Ligand attachment methods

Many protein ligands can be attached to the sensor surface with amine coupling chemistry, which gives efficient and stable immobilization of most proteins. Other immobilization chemistries may be used if appropriate. Ligand immobilization techniques can be found at [cytiva.com/biacoregetstarted](http://cytiva.com/biacoregetstarted).

High affinity capture approaches are generally not recommended since it is often difficult to achieve sufficiently high ligand levels using capture, and total consumption of ligand is high since fresh ligand is captured for every analysis cycle. In addition, there is a risk that serum components may show non-specific binding to the capturing molecule. Capture approaches may however be considered if immobilization or regeneration of the ligand is problematical. If you use a high affinity antibody as a capturing molecule, bear in mind that any reagents used for enhancement and characterization of detected immune responses must not interact with the capturing antibody.

If you are using merged injections to deal with drug interference (Section 4.1), do not immobilize ligand in flow cells 1 or 2. Since mixing occurs as the solution passes over the flow cells, the response from these flow cells is not reliable with merged injections. Flow cell 3 is recommended as the active surface: flow cell 4 may also be used but responses tend to be lower than in flow cell 3.

### 3.1.3 Establish ligand immobilization procedures

Test immobilization of the drug using amine coupling (or another suitable chemistry according to the properties of the drug). Use positive control antibodies in buffer (not serum) to determine the antibody binding capacity of the immobilized drug.

Detailed discussion of methods for immobilizing molecules on the sensor surface can be found at [cytiva.com/biacoregetstarted](http://cytiva.com/biacoregetstarted).

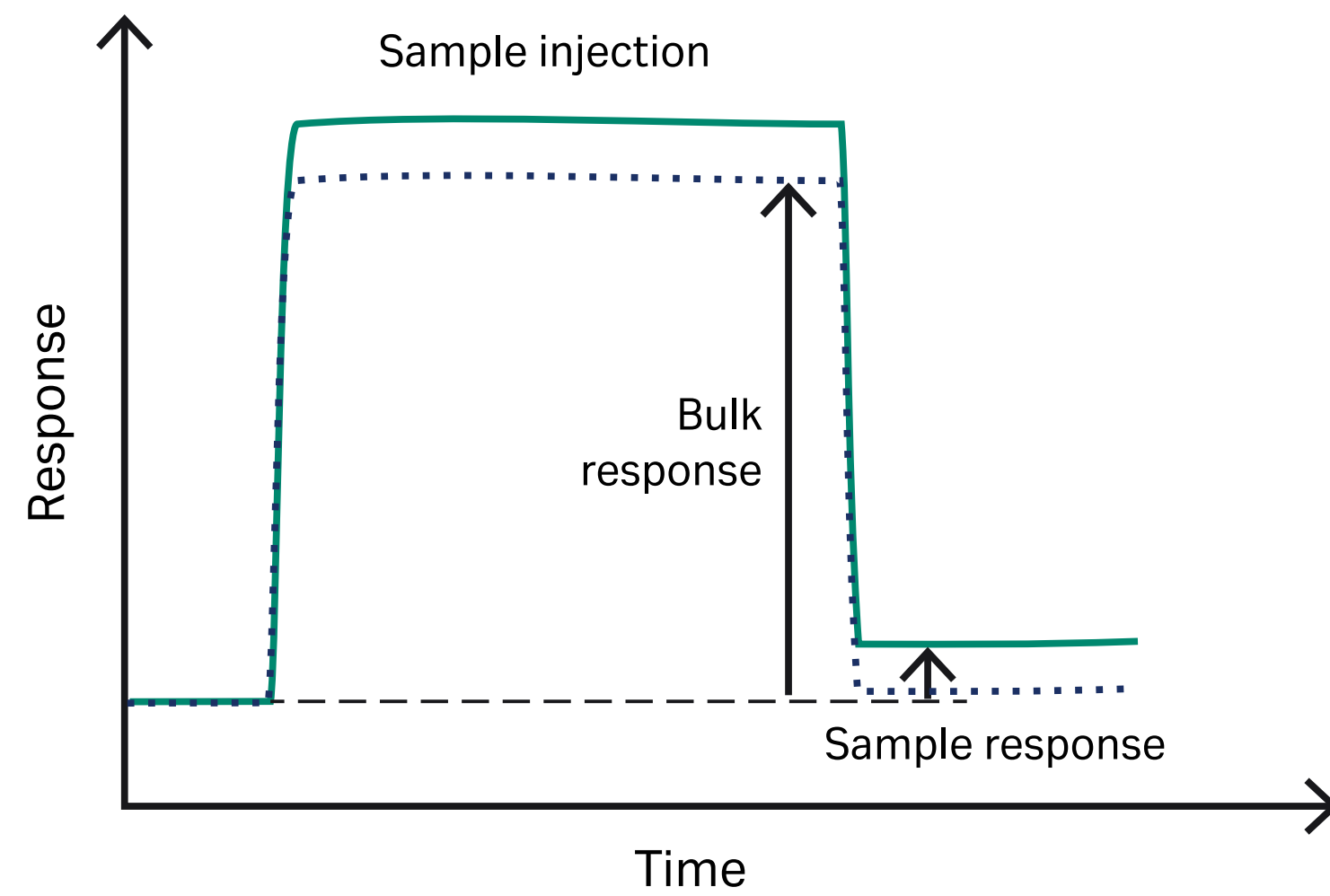
### 3.1.4 Ligand levels

The sensor surface should have a high binding capacity for specific antibodies, to ensure adequate sensitivity for detection of antibodies in serum. Typical levels for ligands with molecular weight 150,000 Da are usually in the range 7,000–15,000 RU. Aim for proportionately lower levels for smaller ligands or higher levels for larger ligands to maintain an approximately constant number of binding sites on the surface. Bear in mind that the ligand may not be fully active, so that the theoretical analyte binding capacity is seldom achieved. The assay sensitivity will also be a function of the affinity of the antibodies in the sample: antibodies with higher affinity are more easily detected. It is worth spending some effort on finding robust conditions for attaching fairly high levels of ligand to the surface with retained activity, to maximize the chances of detecting low affinities and low concentrations of antibodies. Further guidance on ligand attachment considerations can be found at [cytiva.com/biacoregetstarted](http://cytiva.com/biacoregetstarted).

### 3.1.5 Reference surface

In general, reference subtraction is not relevant to immunogenicity studies, since responses are normally measured after the end of the sample injection when bulk refractive index effects of the sample no longer apply. Binding responses are often observed with serum samples on a blank surface, and reference subtraction of the sensorgrams cannot normally be used to correct for non-specific binding, since the characteristics of the immobilized and blank surfaces will probably differ in this respect.

Bear in mind that it is seldom possible to match the refractive index of serum samples with running buffer, so that there will usually be a significant bulk response contribution during sample injection. Response levels cannot be usefully measured during the injection. The bulk shift from sample to running buffer will also obscure rapid binding events (Figure 3-1).



**Figure 3-1.** Response levels cannot usefully be measured during sample injection because of high bulk contributions from the sample.

## 3.2 Buffers and reagents

### 3.2.1 Running buffer

Hepes-buffered saline containing detergent (HBS-EP+ from Cytiva) is recommended as both running buffer and dilution buffer for serum samples. If you wish to use a different buffer, bear the following points in mind:

- Keep the ionic strength at approximately physiological levels (0.15 M salt) or higher to help reduce non-specific binding of serum components to the sensor surface.
- Include non-ionic detergent (HBS-EP+ contains 0.05% Surfactant P20), to help reduce deposition of serum components in the flow system tubing and IFC.
- Use pH values close to physiological (pH 7.4). Non-specific binding increases markedly under more acidic conditions.
- Make sure that the buffer is compatible with other solutions used in the assay (acidification, neutralization and regeneration reagents), so that precipitation does not occur at the interface between solutions in the flow system.
- Buffers should always be filtered to remove particles.

### 3.2.2 Wash solution

Extra wash with a solution of 20% isopropanol in 40 mM NaOH (final concentrations) is recommended at the end of each analysis cycle to keep the flow system clean. Prepare this solution by mixing 1 part 100% isopropanol with 4 parts 50 mM NaOH.

## 3.3 Samples

Assays supported in Biacore T200 have been developed with serum and plasma samples. Many of the assays should be applicable to detection of antibodies in other body fluids, although additional optimization of assay conditions may be necessary. Methods for detecting antibodies in the presence of potential drug interference (Chapter 4) are designed for undiluted serum or plasma only and should not be used with other types of sample.

All serum and plasma samples should be centrifuged or filtered before analysis if possible to remove particles and large aggregates. This may be particularly important if the samples have been frozen.

### 3.3.1 Sample dilution

Direct analysis of undiluted serum samples is generally not recommended except when using merged injections. Samples should be diluted in running buffer.

**Note:** *Samples for assays that use merged injections (Section 4.1) should not be diluted.*

Test dilution series of positive control samples in serum to establish that the assay performance is consistent over the dilution range that will be used. You may also want to test some unknown samples to establish appropriate dilution levels. A 2-fold dilution is a suitable starting point for finding appropriate dilution factors for unknown samples.

### 3.3.2 Control samples

Control samples provide a check on assay performance, and response levels for negative and positive controls analyzed at intervals during the assay may be used to adjust the measurements for drift in response levels arising from changes in ligand activity and/or background binding (see for example Section 5.2.2). Negative or pre-treatment serum (pooled if desired) is typically used as a negative control, and may be spiked with specific antibody for the positive control.

**Note:** *A set of individual negative serum samples (typically 10-20 samples) is generally used to establish the cut-off level for positive responses. These do not need to be analyzed repeatedly, and are not treated as controls in Biacore T200 software.*

### 3.3.3 Dealing with non-specific binding

Since all molecules that bind to the surface in Biacore systems are detected indiscriminately, contributions from irrelevant molecules can become an issue when complex samples such as serum are analyzed. This non-specific binding may arise from interaction of non-antibody components with the immobilized drug, or from binding of generally “sticky” proteins to the ligand or to the dextran matrix on the sensor surface. The extent of non-specific binding may be expected to vary for different drugs and between different individual serum samples.

The following measures can help to minimize non-specific binding and are generally recommended:

- Include detergent (recommended 0.05% Surfactant P20) in buffers and diluted samples.
- Maintain at least physiological ionic strength (0.15 M monovalent cations) in running buffer and samples.
- Addition of soluble dextran to a final concentration of 1 mg/mL to the sample can sometimes help to reduce unwanted binding to the dextran surface in samples that are analyzed without acidification and neutralization. Soluble dextran for this purpose is available as NSB Reducer from Cytiva. NSB Reducer has however generally little effect in samples that are acidified and neutralized for dealing with drug interference (Chapter 4).
- Start-up cycles using a positive control antibody in serum are recommended, so that the surface is exposed to both specific and non-specific binding followed by regeneration. Do not use buffer as sample in start-up cycles. The number of start-up cycles needed may vary according to the nature of the drug immobilized on the surface: 3–5 cycles are sufficient in most cases.
- Optimization of regeneration procedures can help to keep levels of non-specific binding low. If regeneration is not complete, material that accumulates on the surface between cycles can provide sites for additional binding as the assay progresses.
- Pay particular attention to cleaning and maintenance of the Biacore T200 instrument. Include an extra wash injection with 20% isopropanol in
- 40 mM NaOH at the end of each cycle, and run the maintenance tool **Desorb** after every assay using serum samples (in addition to the regular cleaning and maintenance procedures described in the Biacore T200 Operating Instruction).

Subtracting the response from a reference surface may in principle correct to some extent for non-specific binding to the dextran surface. However, constructing a reference surface that exactly mimics the active surface with respect to all non-specific binding and at the same time does not bind the specific antibody is practically impossible with samples such as serum, and reference subtraction is not a recommended approach to dealing with non-specific binding for immunogenicity work.

### 3.4 Enhancement reagents

Enhancement reagents are used to amplify the primary response and to identify response resulting specifically from antibody binding against a background of non-specific binding (Section 2.2.2).

The primary requirement for an enhancement reagent is that it should interact specifically with the bound antibody and not with any other components on the sensor surface. Commercial or custom-produced anti-immunoglobulins are generally suitable as enhancement reagents in immunogenicity screening. IgG fractions can often be used: affinity-purified antibodies may give better performance but are more expensive. (Note that some suppliers refer to IgG fractions purified on Protein A as "affinity-purified".) Some important considerations in using enhancement reagents are:

- In order to amplify the primary response, the enhancement reagent must bind to give a greater mass concentration than the analyte on the surface. This can be achieved by using an enhancement reagent either that is larger than the analyte or that binds to multiple sites on the analyte molecule. To amplify an antibody response, polyclonal antibodies that bind to multiple epitopes on the target antibody are most suitable.
- Some reagents may be specific for certain antibody classes or subclasses. This can be exploited if the screening is directed towards identifying class-specific immune responses. Broad-specificity reagents or mixtures of class-specific reagents should be used for detection of general responses.

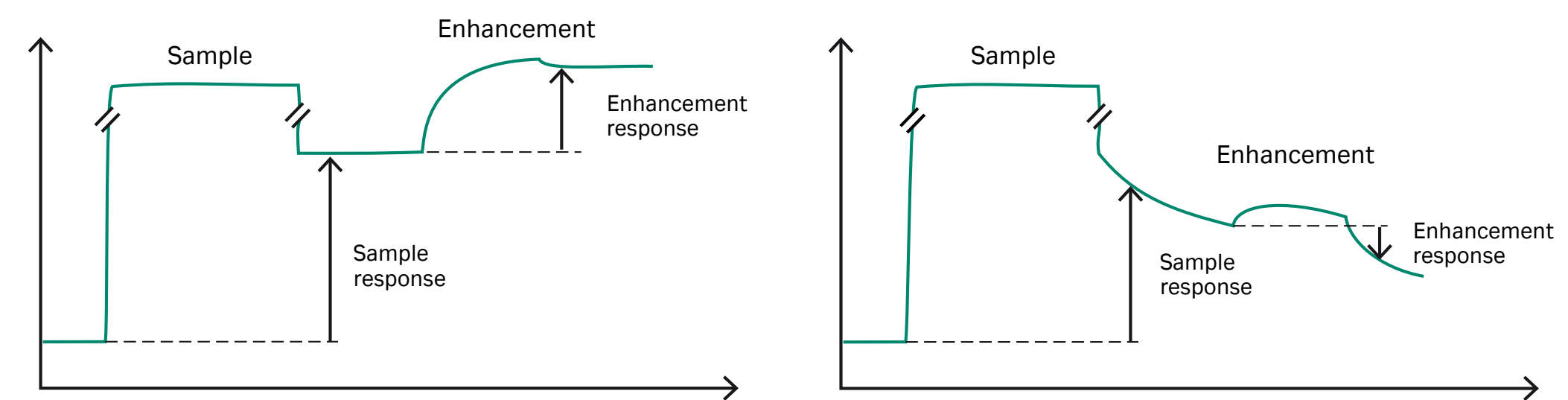
- If the drug is itself an antibody or is captured on the surface using an immobilized antibody as a capturing molecule, the enhancement reagent must be chosen with care so that it does not bind to the drug or the capturing antibody. Reagents directed towards kappa or lambda light chains, chosen to avoid binding to the light chains of the drug or capturing antibody, may be useful in such situations.

Using a fairly high concentration of enhancement reagent and a short contact time will help to keep the cycle time short without compromising enhancement response levels. As a starting point for optimizing conditions, a reagent concentration of 50–100 µg/mL and a contact time of 60 seconds are generally recommended. Run pilot experiments with positive control antibodies in serum to optimize the concentration and contact time for enhancement reagents.

Include samples with negative control serum to ensure that the enhancement reagents are sufficiently specific for anti-drug antibodies (some enhancement of non-specific binding may be expected if the non-specifically bound material includes other antibodies).

Note that enhancement reagents can give misleadingly low or even negative responses if the antibody dissociates significantly from the surface (Figure 3-2).

Always check the sensorgram appearance in assessing enhancement response levels.



**Figure 3-2.** The response from an enhancement reagent may be misleadingly low or even negative if the antibody dissociates significantly from the surface. Left: Enhancement response with no dissociation of antibody. Right: Rapid antibody dissociation can in extreme cases give a negative enhancement response even though the enhancement reagent binds to the surface. Dissociation is exaggerated in this diagram for illustrative purposes.

## 3.5 Regeneration

### 3.5.1 General principles

Many antibody-drug interactions can be regenerated using acidic conditions provided that the drug withstands exposure to low pH. A 30-second injection of glycine-HCl at pH 1.5–2 is often adequate for regeneration. It may be necessary to optimize the pH or to explore alternative regeneration conditions if the drug is sensitive to low pH.

A second regeneration injection at high pH (for example 10–50 mM NaOH) may be used if the ligand also withstands alkaline conditions, to remove other serum components that have bound to the surface and that are not removed by acidic regeneration.

Exposure of the surface to some regeneration solutions can lead to transient changes in the baseline. If this is observed, include a stabilization time after regeneration to allow the response to stabilize before the next cycle.

Principles of regeneration and scouting procedures for establishing optimal regeneration conditions can be found at [cytiva.com/biacoregetstarted](http://cytiva.com/biacoregetstarted).

### 3.5.2 Establishing regeneration procedures

Establish regeneration conditions first using positive control antibodies in buffer to establish that the ligand can be satisfactorily regenerated, then use these conditions as the starting point for optimization with positive control antibodies in serum. If you perform the initial scouting with serum samples, it may be difficult to distinguish between specific and non-specific binding in the regeneration scouting results and difficult to determine confidently whether the ligand retains full activity after regeneration.

To establish that regeneration also removes non-specifically bound material adequately, run separate regeneration scouting experiments using negative control serum.

If enhancement or isotyping reagents will be used in the assay, confirm that regeneration conditions established using only samples are also appropriate for the additional reagents.

Remember to optimize regeneration conditions for the temperature that will be used in the assay. Regeneration conditions that are established at one temperature may not be optimal at another. Recommended temperatures are 10°C for assays using merged injections (Section 4.1) and 25°C for all other assays (see Section 3.6).

Acceptance criteria for the regeneration conditions will depend on the demands of the assay in terms of reproducibility and expected number of cycles. Progressive decrease in positive control responses and an increase in baseline during the course of the assay is not a problem provided that sufficient response capacity remains after the expected number of cycles. Drift in both positive and negative control responses during the course of the assay can be corrected using **Adjustment for controls** in the evaluation procedures.

## 3.6 Sample compartment and analysis temperature

In general, the recommended temperature settings are 25°C for the analysis temperature and 10°C for the sample compartment temperature. Samples and reagents kept at 10°C in the sample compartment equilibrate to the analysis temperature during passage through the IFC and injection into the flow cell.

Samples that are acidified and neutralized using merged injections (Section 4.1) may show increased levels of non-specific binding with the analysis temperature set to 25°C. This effect is markedly reduced at lower temperatures, and an analysis temperature of 10°C is recommended for merged injections. Expected sample responses are reduced slightly in comparison with 25°C but the assay is more robust in overall terms.

Some regeneration solutions (for example guanidine-HCl) precipitate at 10°C and thus require a higher sample compartment temperature. Serum samples can normally be kept at 25°C in the sample compartment for the duration of an assay with no deleterious effects.

Remember that BIA Desorb Solution 1 (0.5% SDS) precipitates at low temperatures. The maintenance tool **Desorb** must be run with the sample compartment and flow cell at 20°C or higher.

## 3.7 Assay-specific development aspects

### 3.7.1 Confirmation assays

#### Establish levels of added drug for confirmation assays

Use positive control antibodies in serum in the concentration range expected in samples to establish a suitable concentration of added drug for confirmation assays. The assay should be run with a large excess of added drug to maximize inhibition of the antibodies, but you may want to optimize the concentration if the drug is expensive.

Use drug samples in buffer and negative control samples to test that the drug itself does not bind to the surface.

### 3.7.2 Isotyping assays

The general discussion of enhancement reagents (Section 3.4) also applies to isotyping reagents.

#### Establish choice of reagents and assay format

Isotyping reagents, whether commercially available or custom-developed, should be tested for performance with the serum samples that will be used in the assay. Run pilot experiments with positive control antibodies in buffer using one reagent per cycle (Section 7.2.2) to establish reagent concentration and suitable contact time, then check that the conditions are appropriate for control samples in serum. As a starting point for conditions with reagents of high quality, use concentrations in the range 5–20 µg/mL and a contact time of 60 seconds. Reagents should also be tested for cross-reactivity between isotypes and subtypes, and for binding to each other if the reagents are to be injected in sequence in the same analysis cycle.

When suitable conditions have been established, test the assay with a number of negative control samples to establish typical levels of background binding. Remember that any antibodies that are included in non-specific binding will be identified by the isotyping reagents.

You may also want to run pilot experiments with mixtures of positive control antibodies of different subtypes to optimize the assay setup if you want to use multiple reagents per cycle. Bear in mind that placing the reagent for the expected isotype last in the injection sequence can reduce problems of interference between reagents.

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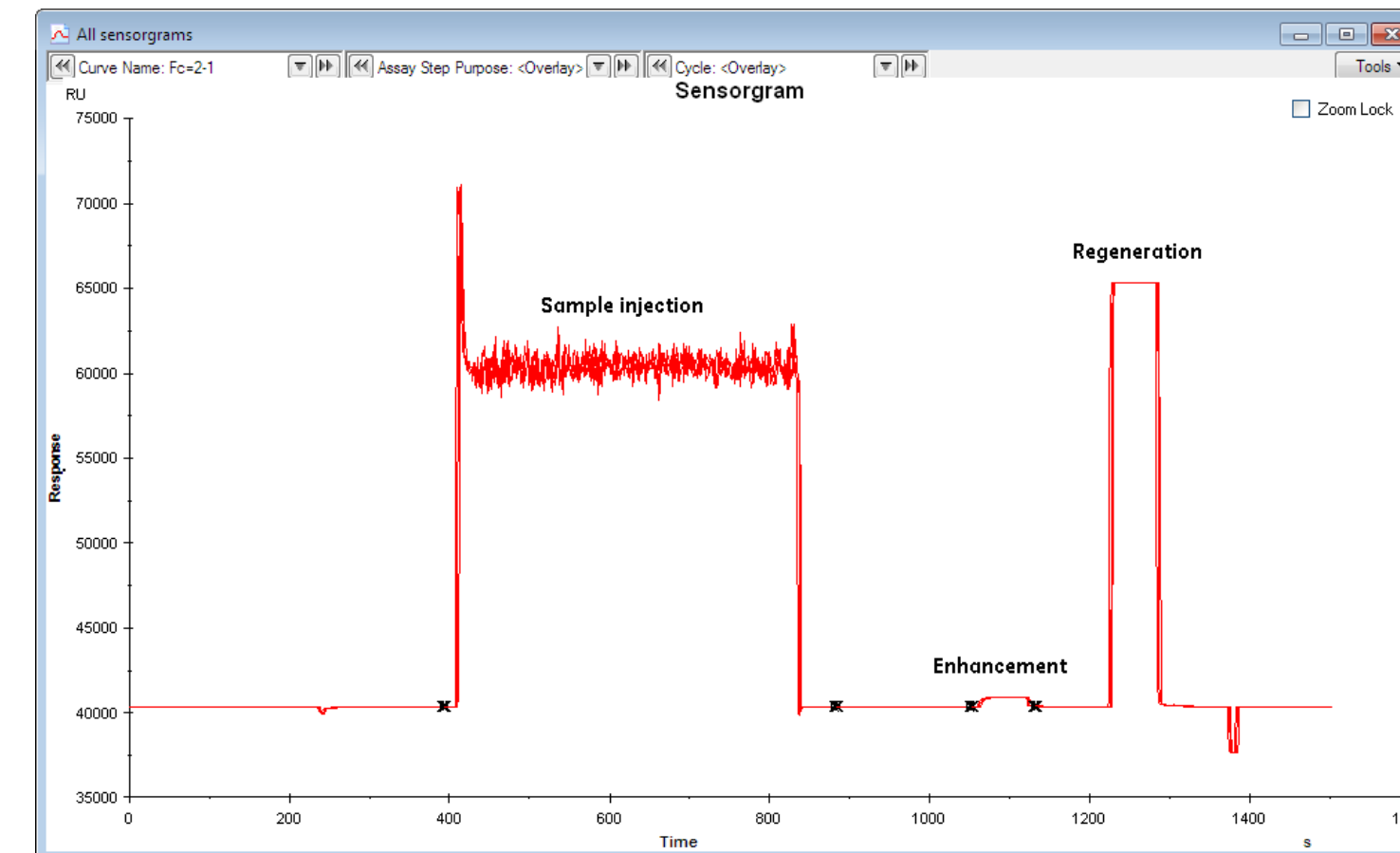
# Dealing with drug interference

Biacore T200 supports two modes of acidification and neutralization of samples for dealing with drug interference in screening and isotype characterization assays:

- Acidification of the sample in microplate wells followed by a merged injection of sample and neutralization solution, so that the neutralization occurs immediately before the sample is analyzed. This approach carries some limitations in terms of assay flexibility and evaluation (see Section 4.1.3), but will detect antibodies that are complexed in the original sample and that re-associate with the drug directly when the sample is neutralized. This mode is supported in the application wizards for screening (Chapter 5) and isotyping (Chapter 7) and in Method Builder (Appendix A).
- Acidification and neutralization in microplate wells, followed by analysis of the neutralized sample. This procedure is referred to as double mix, and may be used to detect complexed antibodies when the time between neutralization and analysis is not critical, because either re-formation of complexes after neutralization is slow or the drug is destroyed by acidification so that the complexes do not re-form at all. This mode is supported in Method Builder (Appendix A).

The rate of rebinding of antibodies to drug after neutralization will depend on the amount of excess drug in the sample and the kinetic properties of the interaction. As an example, an antibody with association rate constant  $10^6 \text{ M}^{-1}\text{s}^{-1}$  and dissociation rate constant  $10^{-3} \text{ s}^{-1}$  (affinity 1 nM) will re-form complexes involving 90% of the total antibody within 2 minutes in the presence of a 10-fold excess of drug. Using the merged injection approach is therefore preferable if you are not certain that the drug is destroyed by acidification. Using merged injection also improves the chances of detecting antibodies that deteriorate under acidic conditions.

The acidification and neutralization procedures result in samples containing significant concentrations of Tris-HCl in addition to serum components. Bulk responses during sample injection will be correspondingly high (see for example Figure 4-1).



**Figure 4-1.** Sensorgram appearance for merged injection (without enhancement). The high and uneven response during sample injection is caused by mixing of the acidified sample and neutralization solution in the flow cell.

## 4.1 Neutralization in the flow cell (merged injection)

Merged injections, whereby serum samples are acidified in a microplate well and then neutralized by simultaneous injection of acidified sample and neutralization solution, are supported in the application wizards for immunogenicity screening and isotyping. Merged injections may also be used in methods constructed with Method Builder (see Appendix A). Use of merged injections places some restrictions on other aspects of assay design (Section 4.1.3).

### 4.1.1 Solutions and reagents for merged injection

#### Buffer compatibility

Make sure that the running buffer is compatible with acidification and neutralization solutions so that precipitation does not occur in connection with aspiration and dispensation of the solutions.

#### Samples

Use undiluted serum with no added detergent as sample. Detergent is included in the acidification and neutralizing solutions. The acidification and neutralization procedure will result in dilution of the samples by a factor of approximately 4.

Dilution of samples before acidification and neutralization is not recommended. However, if circumstances require that you use samples diluted with buffer, you Neutralization in the flow cell (merged injection) should use more dilute HCl for acidification (see below). The neutralization solution does not need to be modified.

#### Acidification solution

The standard acidification solution for merged injections is 0.12 M HCl containing 0.1% Surfactant P20. This solution is suitable for most situations. Bear the following considerations in mind if you prepare custom acidification solutions:

- Aim for a pH value in the acidified sample between 2 and 3. If the pH lies outside this range, the neutralization step may not be satisfactory. Laboratory indicator paper provides sufficient accuracy in checking the pH of the acidified samples.
- The volumes of sample and acidification solution are fixed in both application wizards and Method Builder methods. Optimize your acid concentration for addition of 65  $\mu$ L acidification solution to 50  $\mu$ L sample (see Section 4.1.2).
- Include 0.1% Surfactant P20 in the acidification solution.

Do not store acidification solution for more than one week.

#### Neutralization solution

Neutralization solution for merged injection procedures is 1.3 M Tris-HCl pH 8.5 containing 0.05% Surfactant P20. This solution may be conveniently prepared by dissolving solid Tris-HCl in water to about 50% of the final volume and adjusting the pH to 8.5 with 4 M NaOH before adding Surfactant P20 and making up to the final volume with water.

Preparation of custom neutralization solution is not recommended. Neutralization in the flow cell is achieved by partial mixing of the solution with acidified sample, and custom neutralization solutions can only be optimized by empirical tests using merged injections with known antibodies and drug in serum samples. Insufficiently neutralized samples will bind significantly to an unmodified dextran surface.

Do not store neutralization solution for more than one week.

### 4.1.2 Merged injection procedure

The acidification and neutralization procedure using merged injection is detailed in Table 4-1. The time between addition of acid to the sample and injection into the flow cell is approximately 5 minutes in the application wizards. Additional incubation time between acidification and sample injection can be specified in Method Builder, but is generally not recommended. The procedure dilutes the original sample by a factor of approximately 4.

**Table 4-1.** Procedure for merged injection.

Step	Comments
Microplate prepared with exactly 50 $\mu$ L undiluted serum sample in each specified sample position	Use a calibrated micropipette to ensure accurate volume.
65 $\mu$ L acid added by the autosampler	Final concentration 68 mM HCl (pH 2–3).
Sample injected together with neutralization solution	Sample neutralized in the flow cell.

### 4.1.3 Notes on using merged injection

The following special considerations and restrictions apply to all assays using merged injections:

- Samples and reagents pass over all four flow cells in series, and the flow path is fixed in both application wizards and Method Builder. Mixing and neutralization occurs in the flow path, and responses from flow cells 1 and 2 are not reliable for assay purposes. Measurements should be made in flow cell 3 or 4. Responses in flow cell 4 are generally slightly lower than those in flow cell 3.
- The flow rate for merged injections is fixed at 10  $\mu\text{L}/\text{min}$ .
- Enhancement and isotyping reagents are injected only over flow cells 3 and 4 in application wizards. If ligand is captured on the surface for these applications, the ligand injection also passes only over flow cells 3 and 4 (a capture approach is however not recommended for assays using merged injections).
- An analysis temperature of 10°C is recommended for merged injections. Increased non-specific binding to the sensor surface is commonly observed with merged injections at higher analysis temperatures.
- Use serum samples (not buffer) for start-up cycles to mimic the acidification and neutralization conditions in the samples.
- Include negative controls in the assay as a check on non-specific binding levels. The baseline response may show an upward drift during the course of the assay, but this can generally be ignored as long as relative responses from positive control samples are acceptable.
- Merged injections require exactly 50  $\mu\text{L}$  of undiluted serum sample in the microplate wells. The maximum contact time for a merged injection is 600 seconds.
- Samples that are to be analyzed using merged injections must be placed in microplate wells and must not be pooled in the **Rack Positions** dialog.
- Reference subtraction is not supported in application wizards using merged injections. Reference subtraction can be used in Method Builder but is not generally recommended.
- Assays using merged injections do not support a dissociation time after the injection.

## 4.2 Neutralization in the microplate (double mix)

The double mix procedure mixes acidification solution with sample in a microplate well, then adds neutralization solution in the same well after a specified incubation time. The acidified and neutralized sample is injected directly after neutralization. This procedure is only available in methods constructed with Method Builder (see Appendix A).

### 4.2.1 Solutions and reagents for double mix

#### Buffer compatibility

Make sure that the running buffer is compatible with acidification and neutralization solutions so that precipitation does not occur in connection with aspiration and dispensation of the solutions. The recommended running buffer (HBS-EP+, see Section 3.2.1) is fully compatible with standard acidification and neutralization solutions.

#### Samples

Use undiluted serum with no added detergent as sample. Detergent is included in the acidification and neutralizing solutions. The acidification and neutralization procedure will result in dilution of the samples by a factor of 2.7.

Dilution of samples before acidification and neutralization is not recommended. However, if circumstances require that you use samples diluted with buffer, you should use more dilute HCl for acidification (see below). The neutralization solution may also need to be modified as described below.

#### Acidification solution

The standard acidification solution for double mix is 0.12 M HCl containing 0.1% Surfactant P20. This solution is suitable for most situations. Bear the following considerations in mind if you prepare custom acidification solutions:

- Aim for a pH value in the acidified sample between 2 and 3. If the pH lies outside this range, the neutralization step may also need to be optimized. Laboratory indicator paper provides sufficient accuracy in checking the pH of the acidified samples.

- The volumes of sample and acidification solution are fixed. Optimize your acid concentration for addition of 97  $\mu\text{L}$  acidification solution to 75  $\mu\text{L}$  sample (see Section 4.2.2).
- Include 0.1% Surfactant P20 in the acidification solution.

### Neutralization solution

The standard neutralization solution for double mix procedures is 1.0 M Tris-HCl pH 8.0 containing 0.05% Surfactant P20.

If you need to prepare custom neutralization solution, bear the following considerations in mind:

- Aim for a final pH value close to neutrality (pH 7–7.5). Non-specific binding tends to increase if samples are not sufficiently neutralized. Laboratory indicator paper provides sufficient accuracy in checking the pH of the neutralized samples.
- Use a moderately high concentration of neutralization solution to provide sufficient buffering capacity without unnecessary dilution of the sample. High ionic strength in the final neutralized sample will help to counteract non-specific binding, but may reduce the sensitivity of the assay for low affinity antibodies. Using standard solutions, the neutralized sample contains about 0.18 M Tris.
- The volumes of acidified sample and neutralization solution are fixed. Optimize your neutralization solution for addition of 27  $\mu\text{L}$  solution to 154  $\mu\text{L}$  acidified sample (see Section 4.2.2).
- Include 0.05% Surfactant P20 in the neutralization solution.

### 4.2.2 Double mix procedure

The double mix procedure is described in Table 4-2. With no additional wait periods specified in the method (see Section A.2), the time from addition of acid to the sample to addition of neutralization solution is about 4 minutes, and from addition of neutralization solution to the start of injection about 3 minutes. Each mixing operation takes about 3 minutes. The procedure dilutes the original sample by a factor of 2.7.

**Table 4-2.** Procedure for double mix.

Step	Comments
Microplate prepared with exactly 75 $\mu\text{L}$ sample in each specified sample position	Use a calibrated micropipette to ensure accurate volume.
97 $\mu\text{L}$ acid added by the autosampler	Final concentration 68 mM HCl (pH 2–3). Mixing consumes approximately 18 $\mu\text{L}$ , leaving 154 $\mu\text{L}$ acidified sample in the microplate well.
27 $\mu\text{L}$ neutralizing solution added by the autosampler	Sample neutralized in the microplate well.
Injection of neutralized sample	

### 4.2.3 Notes on using double mix

- Use serum samples (not buffer) for start-up cycles to mimic the acidification and neutralization conditions in the samples.
- Include negative controls in the assay as a check on non-specific binding levels. The baseline response may show an upward drift during the course of the assay, but this can generally be ignored as long as relative responses from positive control samples are acceptable.
- The double mix procedure requires exactly 75  $\mu\text{L}$  of serum sample in the microplate wells. The maximum contact time corresponds to injection of 105  $\mu\text{L}$  sample at the selected flow rate (e.g. 630 seconds at 10  $\mu\text{L}/\text{min}$ ).
- Samples that are to be analyzed using double mix must be placed in microplate wells and must not be pooled in the **Rack Positions** dialog.
- Reference subtraction is not recommended for double mix.

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# Screening for immune response

Screening for immune response involves analyzing serum samples for the presence of specific antibodies, by injecting the samples over a sensor surface carrying immobilized drug. The application wizard supports direct assay for detecting free antibodies and acidification in the microplate followed by neutralization at the flow cell dealing with drug interference (Chapter 4).

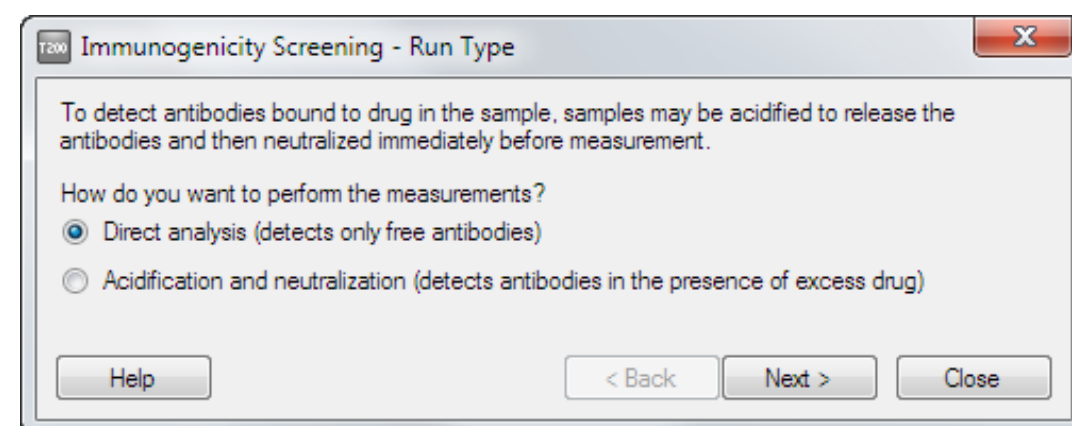
Screening results may be evaluated in terms of the primary response obtained from the sample. Injection of enhancement reagent and evaluation in terms of the enhanced response can give both improved confidence in detection of antibodies against a background of non-specific binding and improved sensitivity for detection of low antibody levels.

## 5.1 Setting up the assay

Screening for immune response is supported in the **Immunogenicity Screening** wizard. Wizard templates can be opened and modified in Method Builder if additional options are needed. General descriptions of application wizards and Method Builder functionality may be found in the Biacore T200 Software Handbook. This section considers only aspects specific to setting up an immunogenicity screening assay.

### 5.1.1 Run type

Choose whether you want to analyze the samples directly for free antibody or after acidification and neutralization to reduce drug interference (see Section 4.1). Acidification and neutralization is performed using merged injection.



### 5.1.2 Injection sequence

Choose the flow path and sensor chip for the run. If you have chosen to use sample acidification and neutralization, the flow path is fixed as **1,2,3,4** and enhancement injections pass only through flow cells 3 and 4. Sensor Chip CM5 is generally recommended for immunogenicity assays.

Choose the injection sequence that will be used in each cycle. Include an enhancement reagent if required to amplify the primary response and/or to confirm that the observed response derives from bound antibody and not from other components in the sample (see Section 3.4).

### 5.1.3 Setup

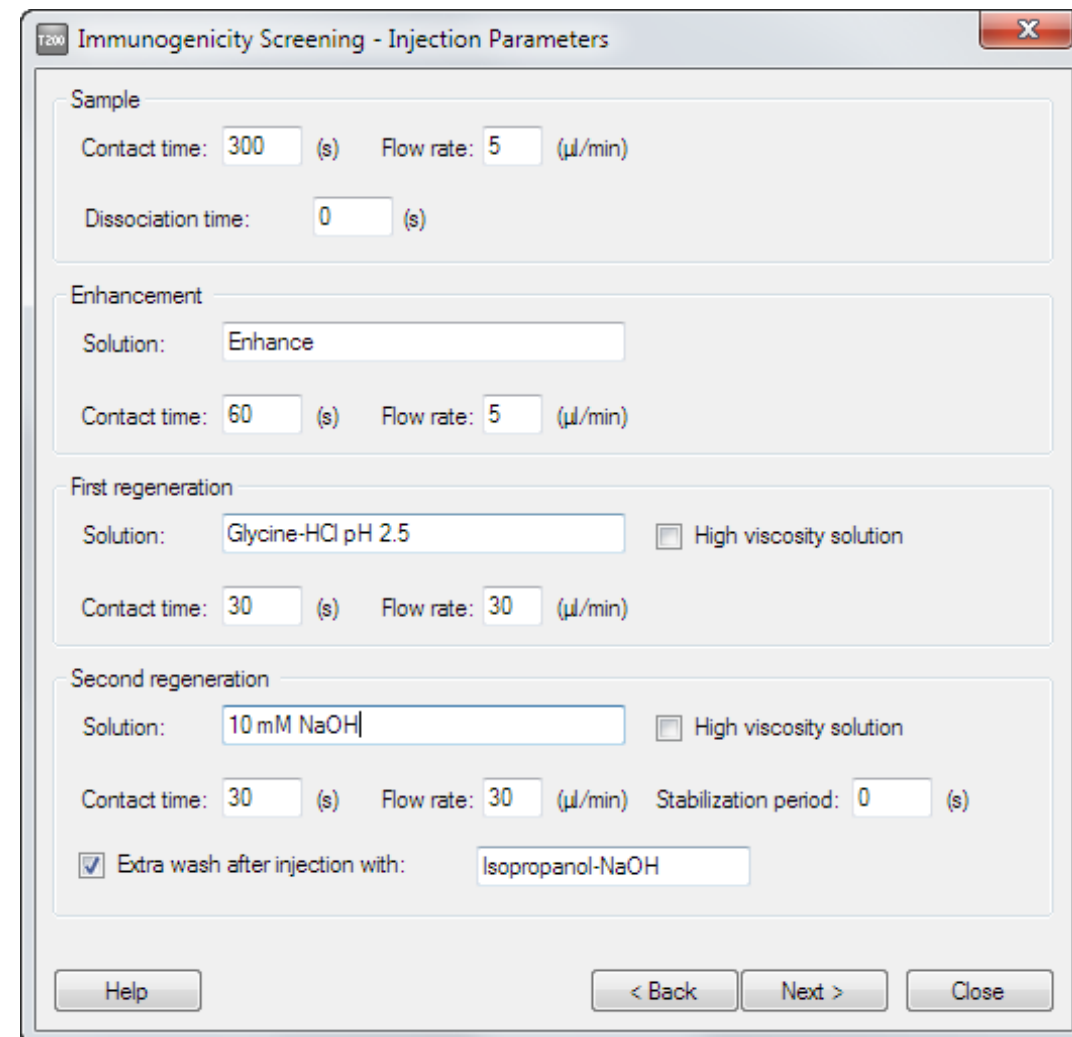
Conditioning cycles are not required for Sensor Chip CM5.

Start-up cycles with a positive control in serum as sample are recommended (see Section 3.6).

### 5.1.4 Injection parameters

Enter the details for the injections in the analysis cycle.

If you are analyzing samples directly without acidification, you may specify a dissociation time after the sample injection to monitor the stability of the antibody-drug complexes on the surface (see Chapter 8). A dissociation time of at least 120 seconds is required for evaluation of antibody binding stability.



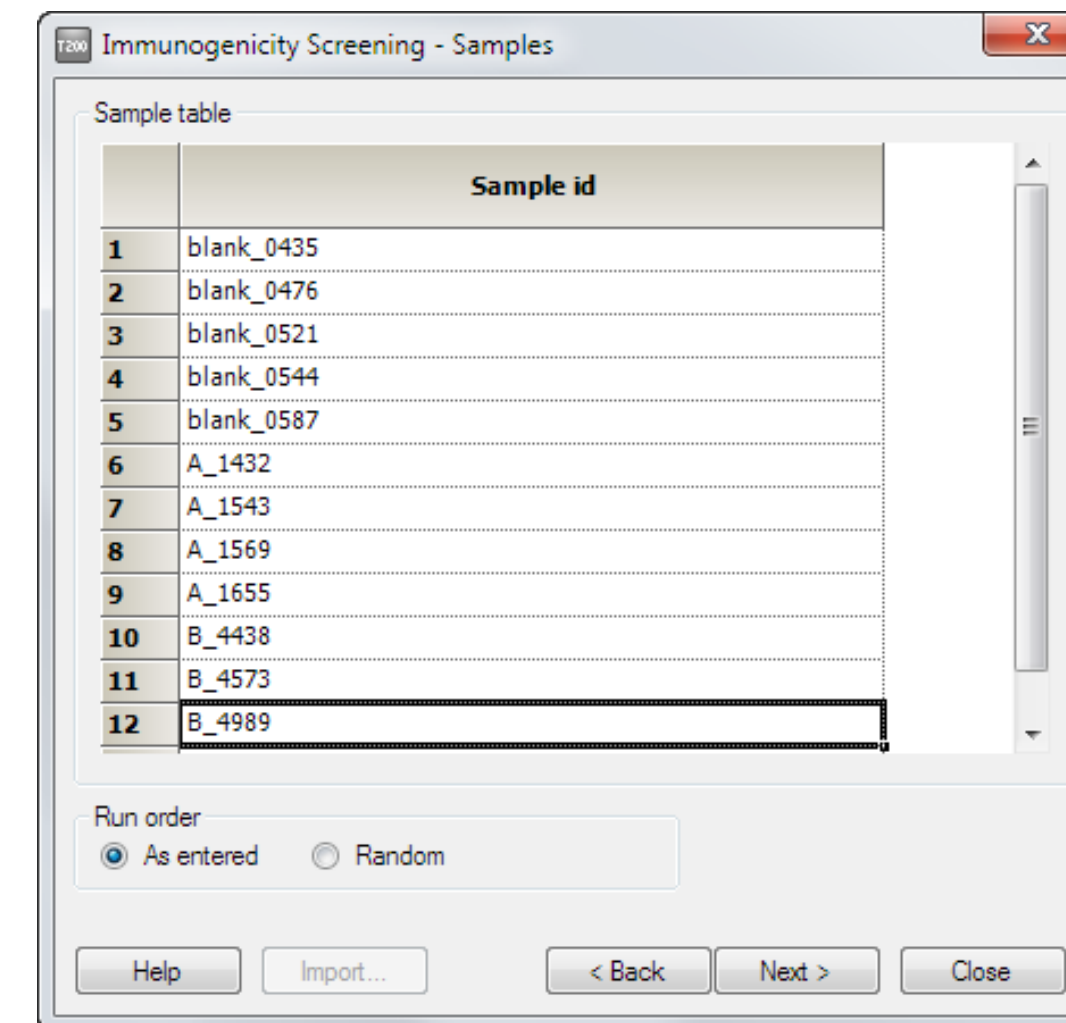
If you are using merged injection to deal with drug interference, the standard acidification and neutralization solutions are entered by default (see Section 4.1).

An extra wash with 20% isopropanol in 40 mM NaOH (Section 3.2.2) is strongly recommended after the last regeneration injection to help keep the flow system clean.

### 5.1.5 Samples

Enter the samples to be analyzed in this dialog, or use the **Import** function to import sample lists from external sources.

If you use a set of negative serum samples to establish a cut-off response for antibody detection, include these in the **Samples** table. Do not use the **Control Samples** table (Section 5.1.6) for this purpose. Name your negative samples with a common prefix (e.g. **blank\_**) so that you can easily identify them during evaluation. You may include negative samples in each run or establish a cut-off response during assay development and use the same value for each screening run during evaluation.

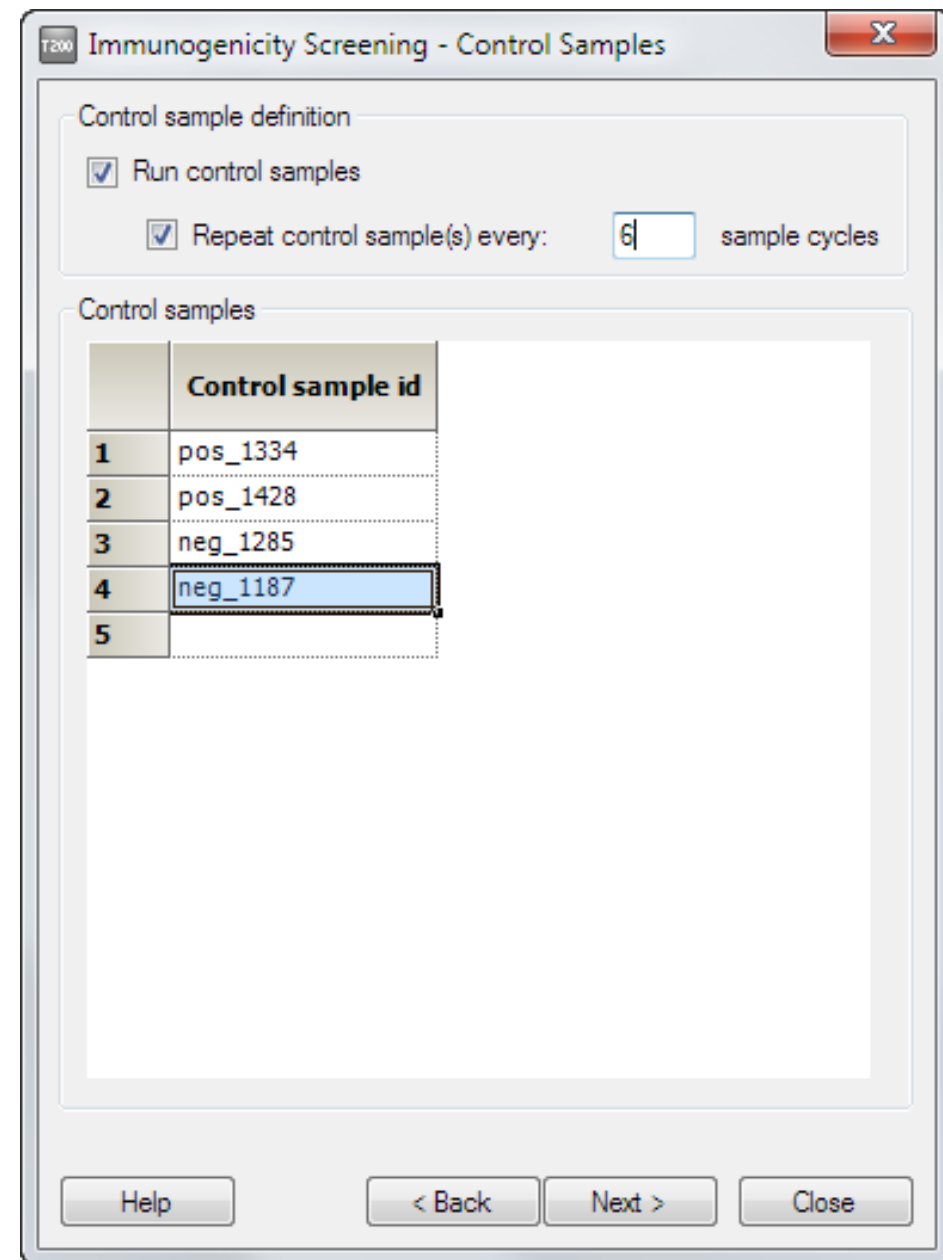


Choose whether to run the samples in random order or in the order entered. Using a random order is a convenient way of spreading negative serum samples throughout the run even if they are entered on consecutive rows in the table.

**Note:** Choosing **Random** order generates a pseudo-random run order for the samples in the table. The order listed in the table and the positioning in the microplate remain unchanged. Open the **Cycle Run List** from the **System Preparations** step of the wizard to check the order in which the samples will be run. The same pseudo-random order will be used each time the wizard is run with a given sample table.

### 5.1.6 Control samples

Control samples are intended as a check on the consistency of performance throughout the assay, and are typically run as one or two positive and negative controls at regular intervals. Do not confuse negative controls intended for performance consistency check with negative serum samples used to establish the cut-off response level (Section 5.2.2).



Control samples should be repeated at intervals throughout the run. The control sample responses can be used to adjust the results for performance drift (see Section 5.2.2).

### 5.1.7 System preparations

Check **Prime before run** and **Normalize detector** as required (see the Biacore T200 Software Handbook for details).

For samples that are analyzed directly, the default temperature settings are 10°C in the sample compartment and 25°C at the flow cell. When acidification and neutralization with merged injection is used, default settings are 10°C for both sample compartment and analysis. Temperature settings are discussed in more detail in Section 3.6.

### 5.1.8 Remaining wizard steps

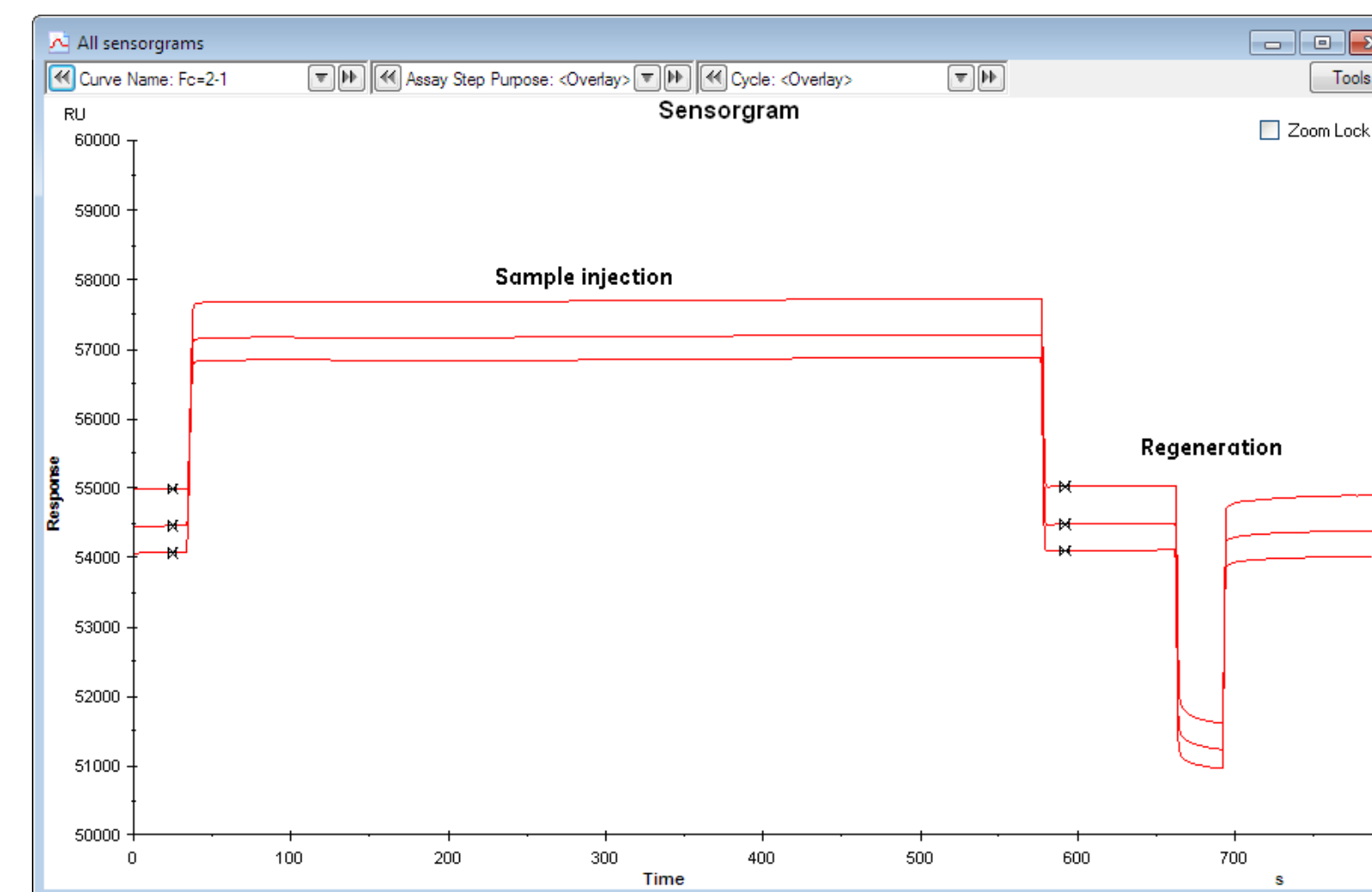
The remaining steps in the wizard follow standard procedures as described in the Biacore T200 Software Handbook.

## 5.2 Evaluating screening results

Use the **Immunogenicity:Screening** function in the Evaluation Software to evaluate screening results.

### 5.2.1 Prepare the data

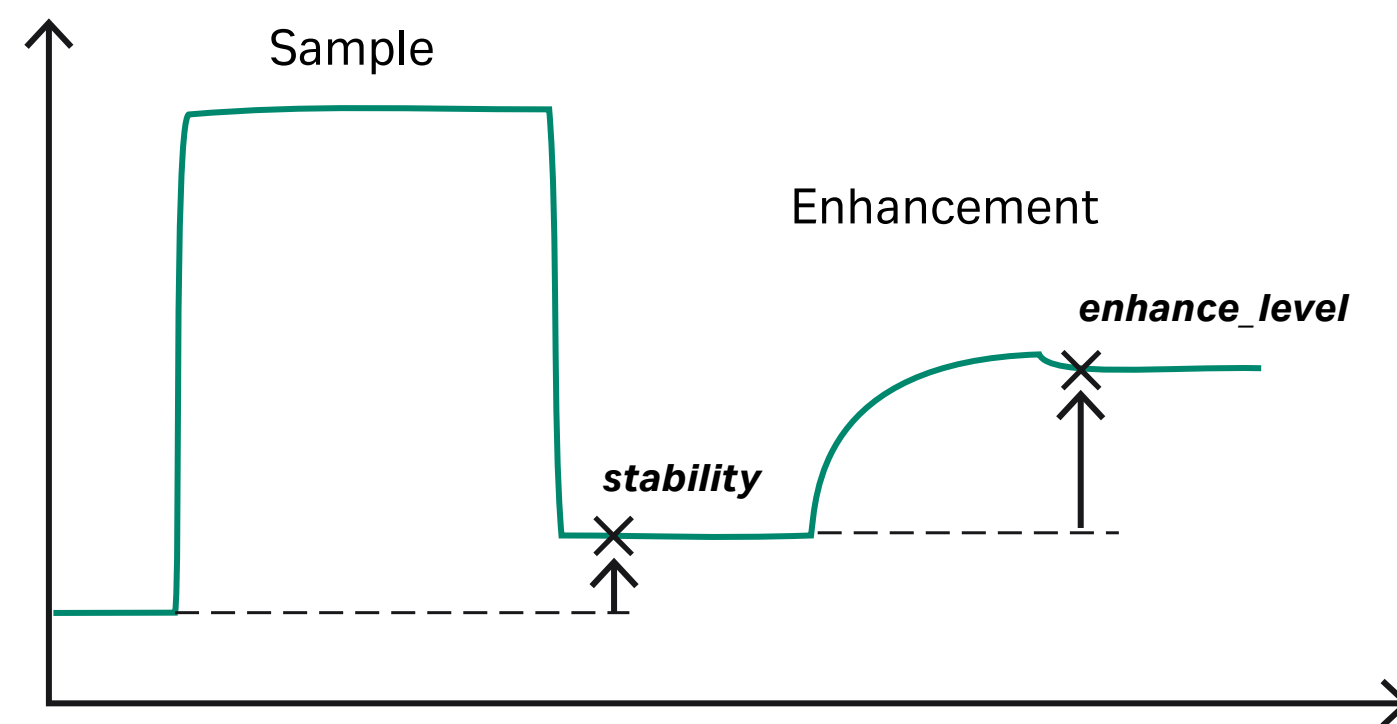
Examine the sensorgrams and pre-defined plots for general data quality (see the Biacore T200 Software Handbook for more detailed information). Exclude any cycles that are clearly disturbed.



**Figure 5-1.** Sensorgrams from a screening assay (without enhancement). See Figure 4-1 for an example of sensorgrams from screening using merged injection.

Decide which report point you will use to evaluate the results:

- **stability** (placed shortly after the end of the sample injection) is recommended for runs that do not use an enhancement reagent
- **enhance\_level** (placed shortly after the end of the enhancement injection) is recommended for runs that use an enhancement reagent



**Figure 5-2.** Placing of report points for evaluation of screening.

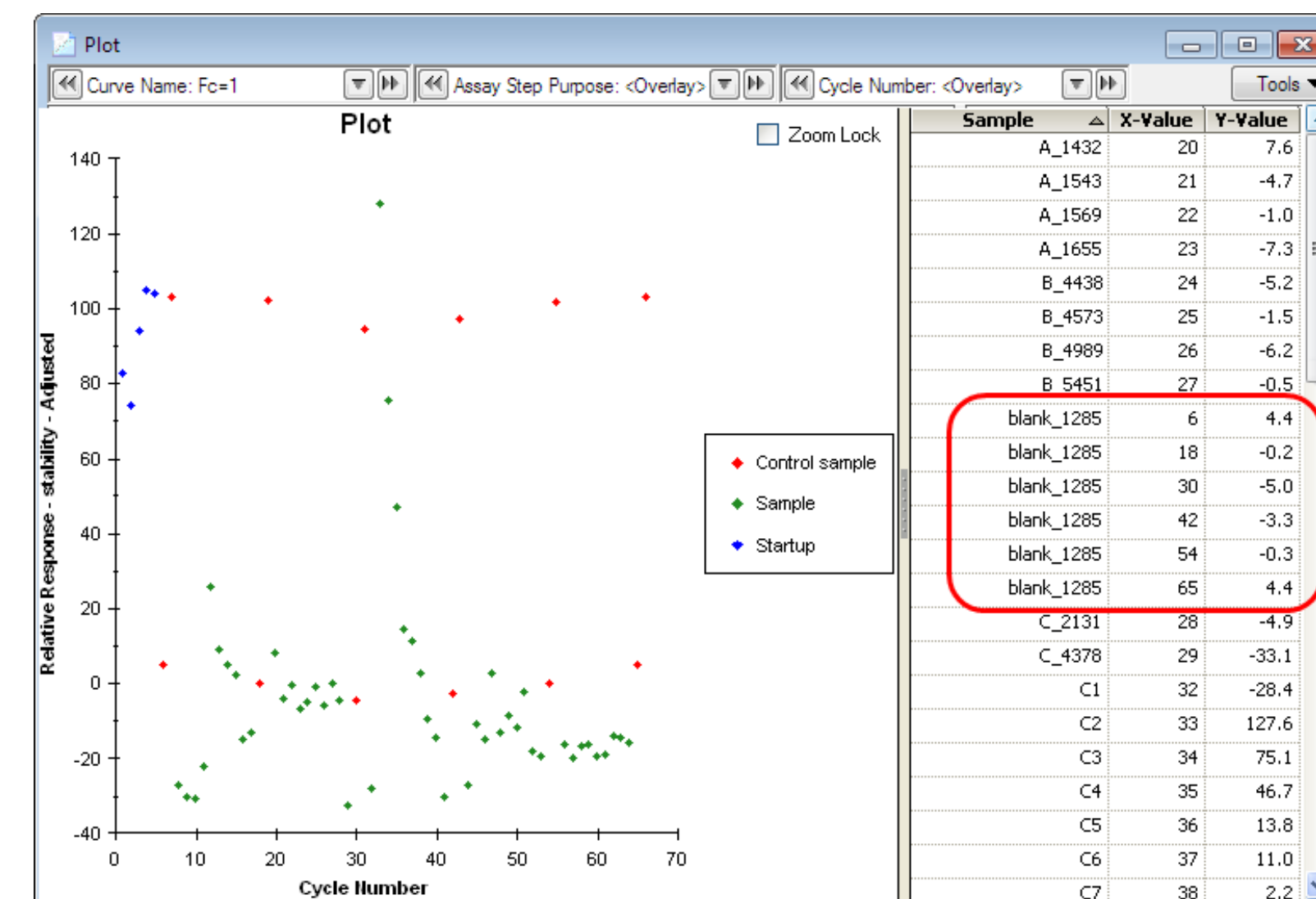
### 5.2.2 Establish the cut-off response level

If you have included negative serum samples, follow the procedure below to establish the cut-off response for antibody detection:

1. Create a plot of the relative response at your chosen report point against cycle number. (Depending on the report point you want to use, an appropriate plot may be created by default when you open the result file.)
2. Filter the plot or use the **Tools:Color by** function to identify the control samples. If there is a significant drift in the positive and/or negative control responses, apply **Tools:Adjustment for controls** to correct for the drift. Adjustment for controls can also be used as a way to normalize the response levels between different assay runs, provided that the same control

samples are used in each run. See the software help or the Biacore T200 Software Handbook for details of adjustment for controls. Bear in mind that if you apply adjustment for controls in determining the cut-off level, you must apply the same adjustment to the sample responses in the screening evaluation.

3. Use the **Tools:Table columns** function to include the sample name in the plot table, then click on the sample name header to sort the table by sample name. This will group your negative serum samples together if you have followed the naming recommendation in Section 5.1.5.

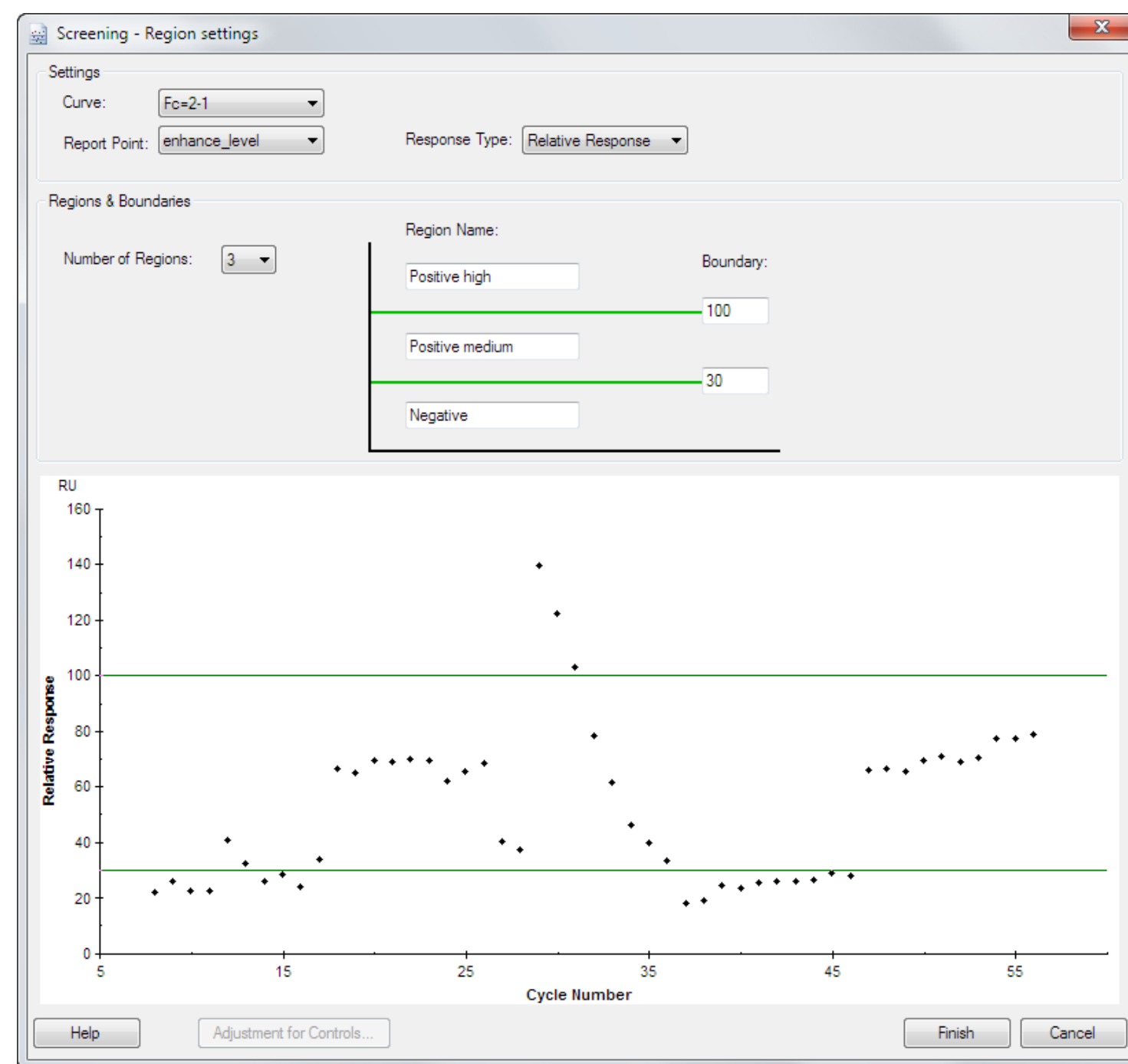


4. Calculate the average response and standard deviation for the negative samples. To simplify this if desired, select the table rows for the negative serum samples and copy the data to an external program such as Microsoft® Excel®. The cut-off level is usually determined from the average response and the standard deviation for the negative samples<sup>1</sup>.

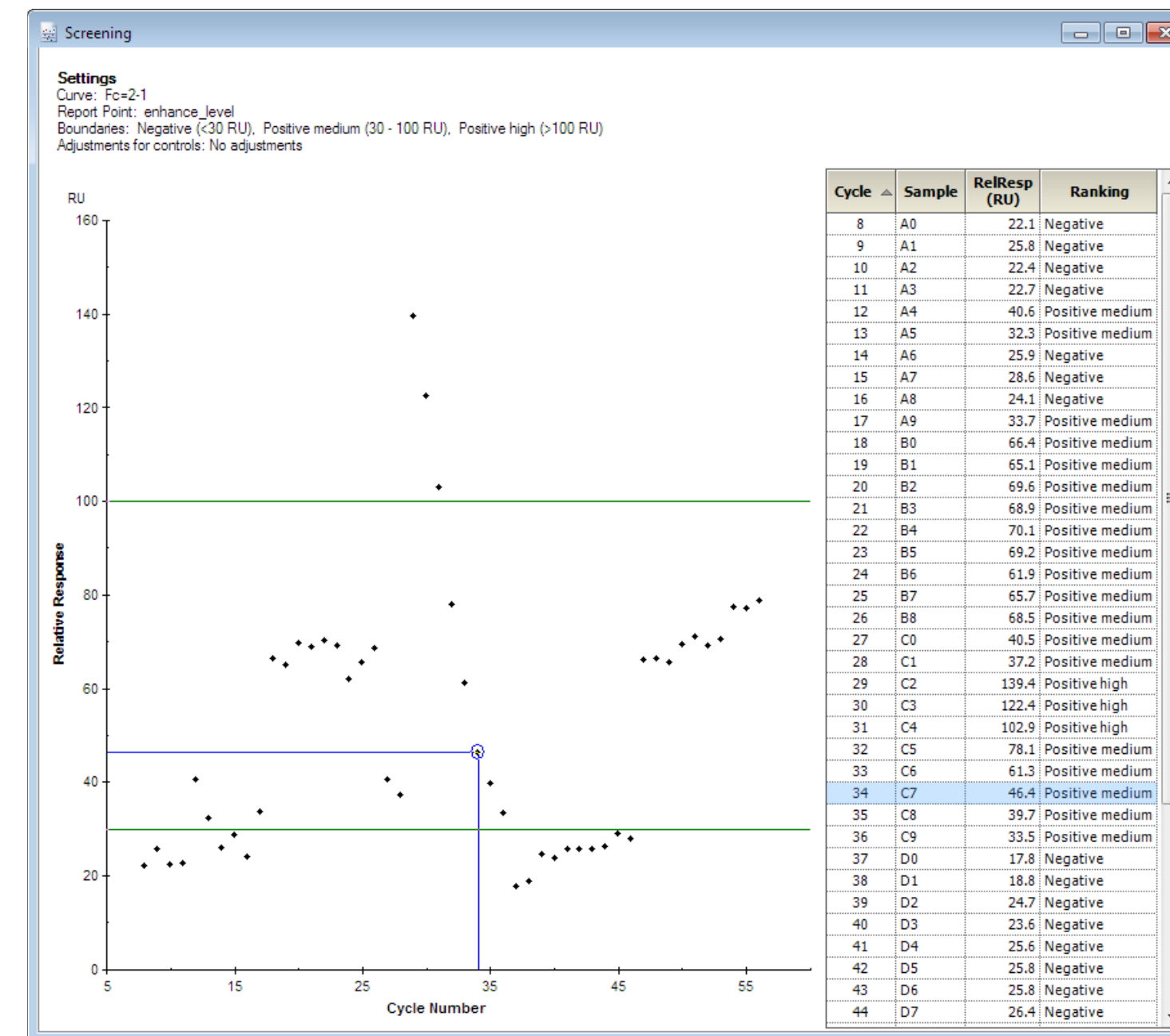
<sup>1</sup> See for example Mire-Sluis, A. R. *et al* (2004), *J. Immunol. Methods* **289**, 1-16.

### 5.2.3 Evaluate the screening

1. Choose **Immunogenicity:Screening** from the toolbar or the **Evaluation** menu.
2. Choose the same curve, report point and response type that you used in establishing the cut-off level (Section 5.2.2).
3. Apply **Adjustment for controls** if you have determined the cut-off level from adjusted responses.
4. Choose the number of regions you want to use (maximum 4) and enter region names and boundary response values. Samples will be ranked by region in the final results. You will typically set a negative region with an upper boundary at the cut-off level: the samples above this boundary may be divided into regions as required.



5. Click **Finish**. The results are presented as a ranking plot and table of response values or adjusted response values with ranking regions. The point corresponding to the currently selected row in the table is highlighted in the plot.



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# Confirming antibody specificity

The cut-off level for positive samples in a screening assay is often deliberately set to include a certain proportion of false positive results (e.g. 5% of the total samples), to reduce the risk that samples containing low antibody levels will be missed in the initial screen. As a second step in immunogenicity determination, it is important to establish that positive results from the screen are true positives. Enhancement with a generic antibody reagent can confirm that the response derives from antibody molecules: inhibition of the response by added excess drug can confirm that the response derives from specific binding to the drug (see Section 2.2.2).

The **Immunogenicity confirmation** wizard tests the specificity of the response by analyzing samples with and without addition of excess drug. Added drug will compete with the surface-bound drug for antibody binding and will inhibit the specific response without affecting the level of non-specific binding (see Figure 2-3).

**Note:** *Strictly, this competitive inhibition approach only confirms that the response derives from specific binding of some sample component to the drug, without actually establishing that the component is an antibody. To confirm that the response in fact derives from antibodies in the sample, use an enhancement injection with antibody specific reagent in the screening assay setup (see Section 5.1.2).*

## 6.1 Setting up the assay

### 6.1.1 Injection sequence

Choose the flow path and sensor chip for the run. Sensor Chip CM5 is generally recommended for immunogenicity assays.

Choose the injection sequence that will be used in each cycle. Include an enhancement reagent if required to amplify the primary response and/or to confirm that the observed response derives from bound antibody and not from other components in the sample (see Section 3.4).

### 6.1.2 Setup

Conditioning cycles are not required for Sensor Chip CM5.

Start-up cycles with a positive control in serum as sample are recommended (see Section 3.6). Samples for the start-up cycles will be mixed with drug-free solution only.

### 6.1.3 Injection parameters

Enter the details for the injections in the analysis cycle.

Each sample will be run in two separate cycles, one mixed with drug-free solution (buffer) and one mixed with an excess of drug. Specify the names of the mix solutions and the volume fraction to mix (a fraction of 25% will mix 1 part of mix solution with 3 parts of sample). The same fraction setting is used for sample with and without drug. The actual volumes used are determined from the required injection volume according to the specified contact time and flow rate.

The screenshot shows the 'Immunogenicity Confirmation - Injection Parameters' dialog box. The 'Sample' section has 'Contact time' set to 300 (s) and 'Flow rate' set to 5 (µl/min). The 'Cycles without drug' section has 'Mix solution' set to 'Buffer' and 'Fraction' set to 50 (%). The 'Cycles with drug' section has 'Mix solution' set to 'Drug' and 'Fraction' set to 50 (%). The 'First regeneration' section has 'Solution' set to 'Glycine-HCl pH 2.5', 'Contact time' set to 30 (s), 'Flow rate' set to 30 (µl/min), and the 'High viscosity solution' checkbox is unchecked. The 'Second regeneration' section has 'Solution' set to '10 mM NaOH', 'Contact time' set to 30 (s), 'Flow rate' set to 30 (µl/min), 'Stabilization period' set to 0 (s), and the 'High viscosity solution' checkbox is unchecked. The 'Extra wash after injection with' section has the checkbox checked and the solution set to 'Isopropanol-NaOH'. At the bottom are buttons for 'Help', '< Back', 'Next >', and 'Close'.

An extra wash with 20% isopropanol in 40 mM NaOH (Section 3.2.2) is strongly recommended after the last regeneration injection to help keep the flow system clean.

Set up a custom method using Method Builder if you want to run the assay using only samples mixed with drug (i.e. excluding the samples mixed with drug-free solution).

#### 6.1.4 Samples

Enter the sample names in the table. Each row in the table will generate two analysis cycles, one with and one without drug.

If you want to run known samples to check the drug inhibition performance or to establish cut-off levels for evaluation, include these in the sample table.

Samples entered in the **Control Samples** step (Section 6.1.5) will not be mixed with drug.

**Note:** *Each sample requires 3 positions in the microplate (one for the untreated sample, one for mixing with drug-free solution and one for mixing with drug). Mixing is not recommended in 384-well microplates, so that the maximum number of samples that can be analyzed in one run is 32.*

#### 6.1.5 Control samples

Control samples are intended as a check on the consistency of performance throughout the assay, and are typically run as one or two positive and negative controls at regular intervals. Control samples in the **Immunogenicity Confirmation** wizard are mixed with drug-free solution using the same settings as for samples. Control samples are not run mixed with drug.

Control samples should be repeated at intervals throughout the run. Use an even number for the interval so that control samples will always be run between and not within the pairs of sample cycles.

#### 6.1.6 System preparations

Check **Prime before run** and **Normalize detector** as required (see the Biacore T200 Software Handbook for details).

Default temperature settings are 10°C in the sample compartment and 25°C at the flow cell. Temperature settings are discussed in more detail in Section 3.6.

#### 6.1.7 Rack positions

The wizard sets up 3 positions for each sample (1 source position for untreated sample and 2 mixing positions for mixing with drug-free solution and drug respectively). Do not use 384-well microplates: mixing in small wells is not recommended.

## 6.2 Evaluating confirmation results

There are two approaches to evaluating the results of a confirmation assay:

- Compare the response with and without addition of drug to establish the degree of inhibition. This approach handles samples with widely different response levels assuming that background responses are comparable between samples. Data for this approach can be obtained from an **Immunogenicity Confirmation** assay, but must be exported to third-party software (e.g. Microsoft Excel) for calculation of the degree of inhibition.
- Set a simple cut-off response level for the response from samples mixed with drug. Samples below the cut-off are confirmed positive (addition of drug inhibits binding to the surface). This approach presupposes that the samples without addition of drug give responses above the cut-off level so that samples with low binding levels are not falsely classed as positive.

## 6.2.1 Prepare the data

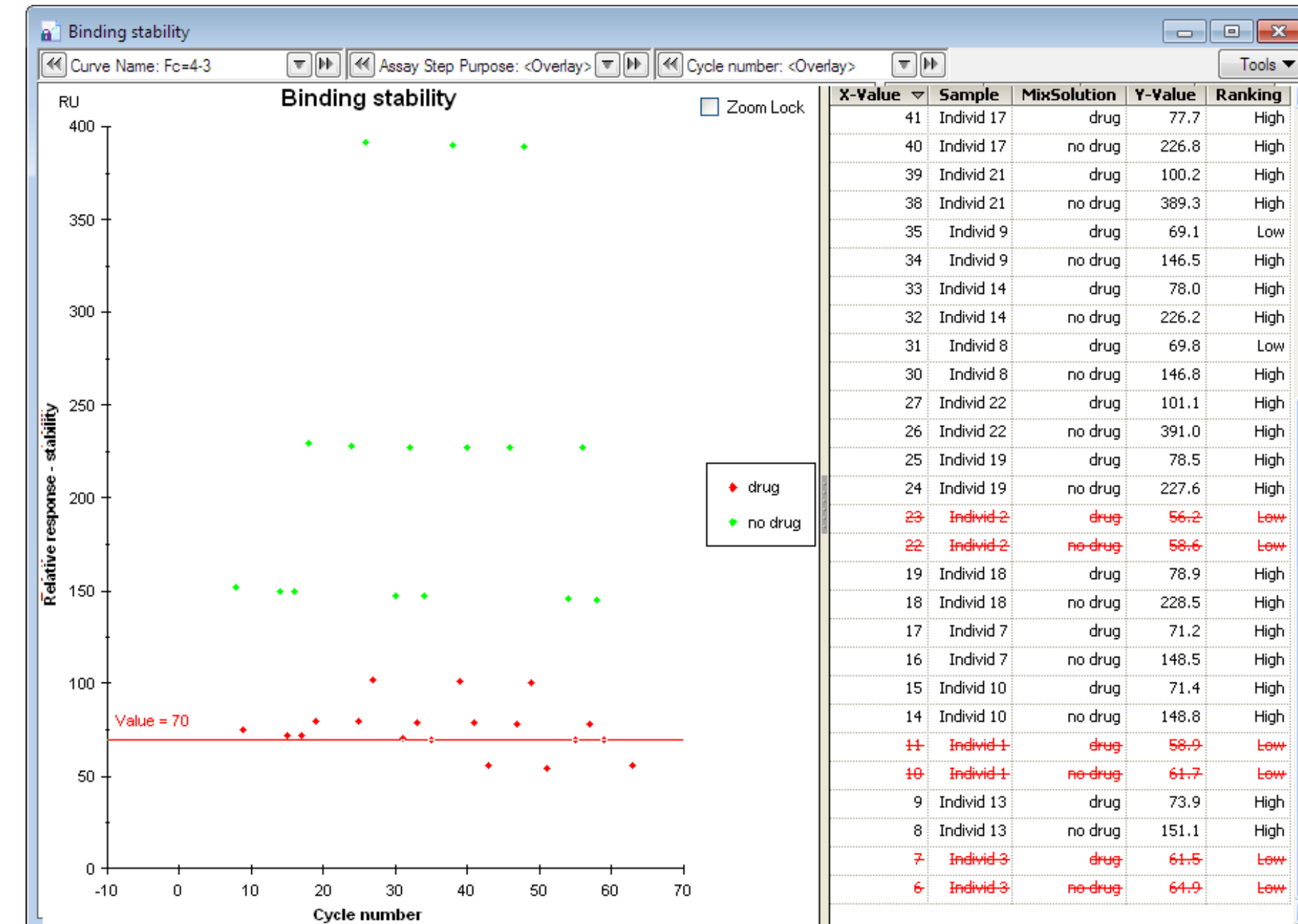
Before starting the **Confirmation** evaluation, check the sensorgrams and pre- defined plots for quality of the results. Exclude any cycles with clearly disturbed sensorgrams (see Section 5.2.1), and decide which report point you will use for evaluating the assay.

Open a plot window with the curve and report point you will use for the evaluation. The table of plot values for pre-defined plots includes the sample name and the name of the solution mixed with the samples: if you define your own plot, use **Tools:Table Columns** in the plot window to add the keywords **Sample** and **MixSolution** to the table. Sort the table and/or color the plot points by this keyword to simplify identification of samples mixed with and without drug.

Determine a cut-off level for samples without drug and exclude samples where the response without added drug is below this level. If the response without added drug is low, it will be difficult to establish inhibition by added drug with confidence. Note that you should exclude two cycles (one with and one without drug) for each sample.

**Notes:** Select **Assay Step Purpose:Sample** in the plot before excluding low response samples, to avoid excluding negative control samples that are analyzed without drug.

To streamline the procedure for excluding low response samples, use the ranking function in the plot together with the table sorting functions to find pairs of cycles with the same sample name and a low response without added drug.



**Figure 6-1.** Using the plot tools, exclude samples (two cycles for each sample) where the response without added drug is below a cut-off level.

## 6.2.2 Set up the evaluation

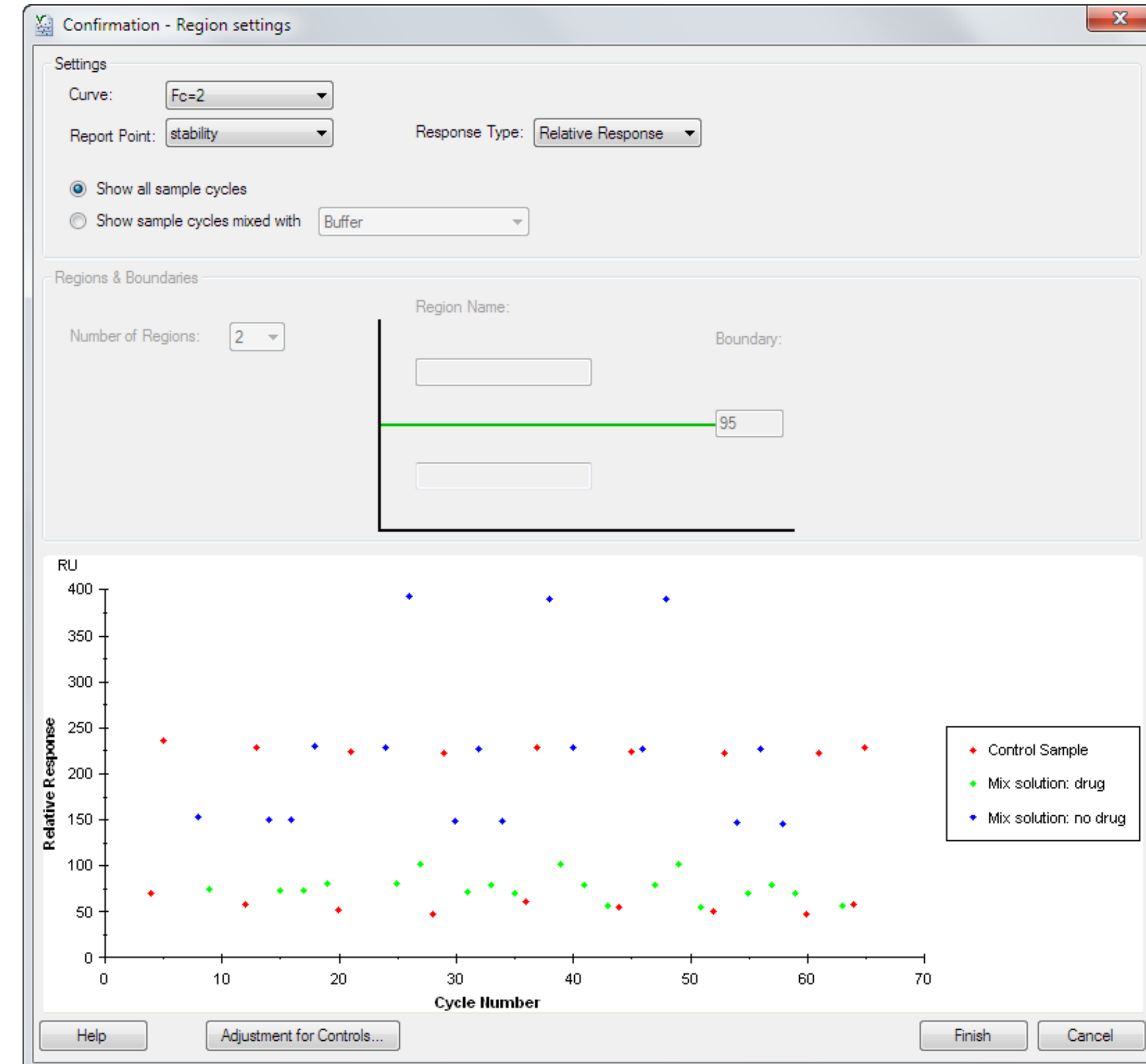
Choose **Immunogenicity:Confirmation** from the toolbar or the **Evaluation** menu to start the evaluation.

Examine the control sample responses (identified as red points in the plot). If there is significant drift in the positive and/or negative control responses, click **Adjustment for controls** to correct for the drift (see Section 5.2.2). Details of the adjustment for controls procedure may be found in the software help or in the Biacore T200 Software Handbook.

## 6.2.3 Evaluating by degree of inhibition

1. Choose the option **Show all sample cycles** in the **Confirmation** evaluation. The regions and boundary settings will be grayed out and all sample cycles will be displayed. Cycles where the sample is mixed with drug and drug-free solution are distinguished by different colors.
2. Click **Finish** to save the item.
3. In the finished item, right-click on the table and choose **Copy Table**. The table contents will be copied to the Windows® clipboard in tab-separated format, and can be pasted into third-party software (e.g. Microsoft Excel) for calculation and presentation of the degree of inhibition.

**Note:** Complete inhibition of the specific binding response may not reduce the observed response to baseline if there is a significant contribution from non-specific binding. Bear this in mind in setting up decision criteria for assessing the final results.



## 6.2.4 Evaluating by cut-off response level

### Establishing the cut-off level

In establishing a cut-off level for evaluation of confirmation assays, remember that a positive result is represented by inhibition of binding, i.e. by a low response in the presence of added drug. A low response will also be obtained if the sample does not contain antibodies in the first place. These situations can only be distinguished by examining the response obtained without added drug. A simplified scheme for interpreting the results is given in Table 6-1.

**Table 6-1.** Guide to interpreting response levels from confirmation assays.

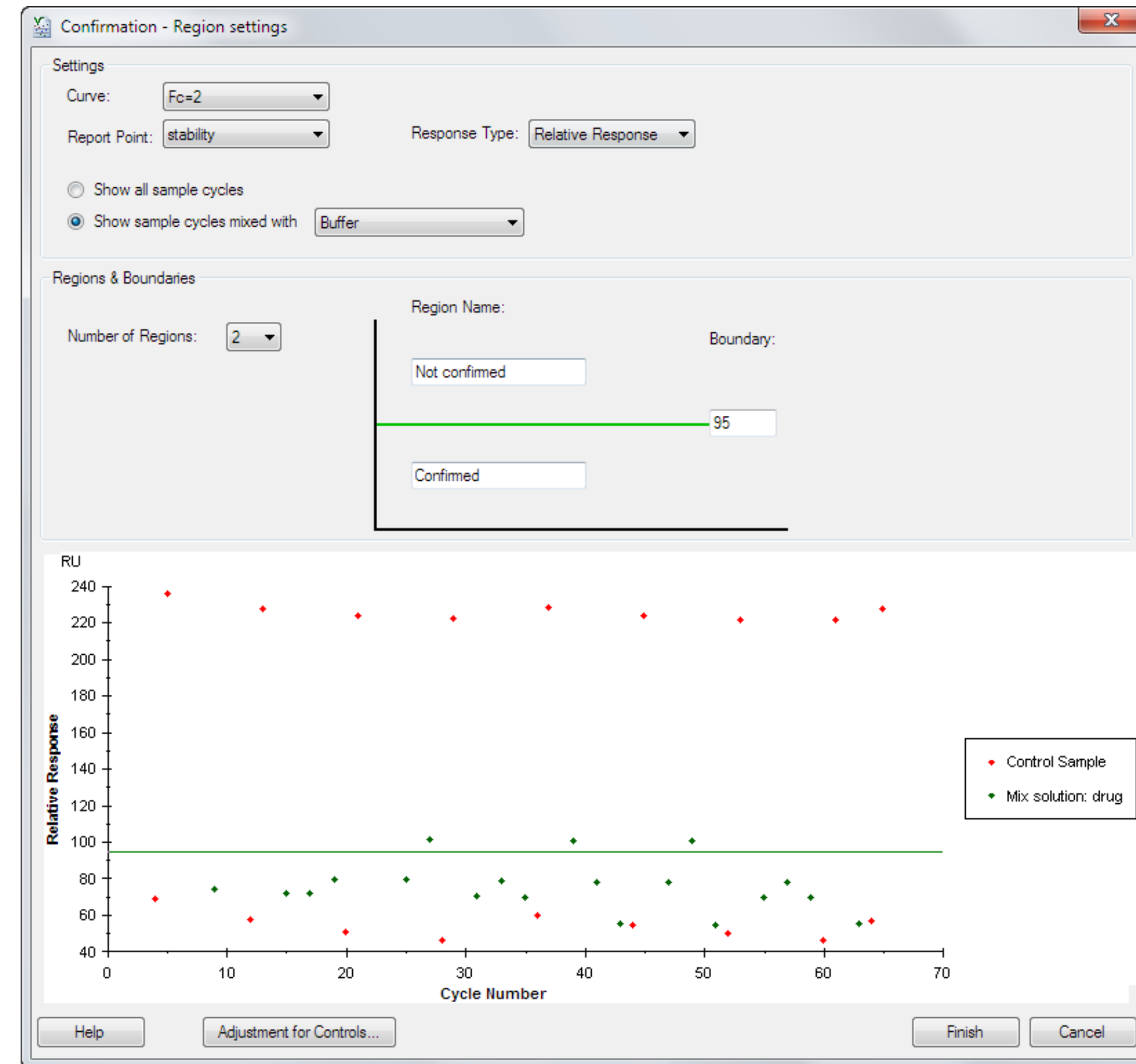
Response with added drug	Response without added drug	
	High	Low
<b>High</b>	Screened positive Not confirmed (false positive)	May indicate drug binding to the surface*
<b>Low</b>	Screened positive Confirmed positive	Screened negative

\*This situation should not arise if the drug itself does not bind to the surface.

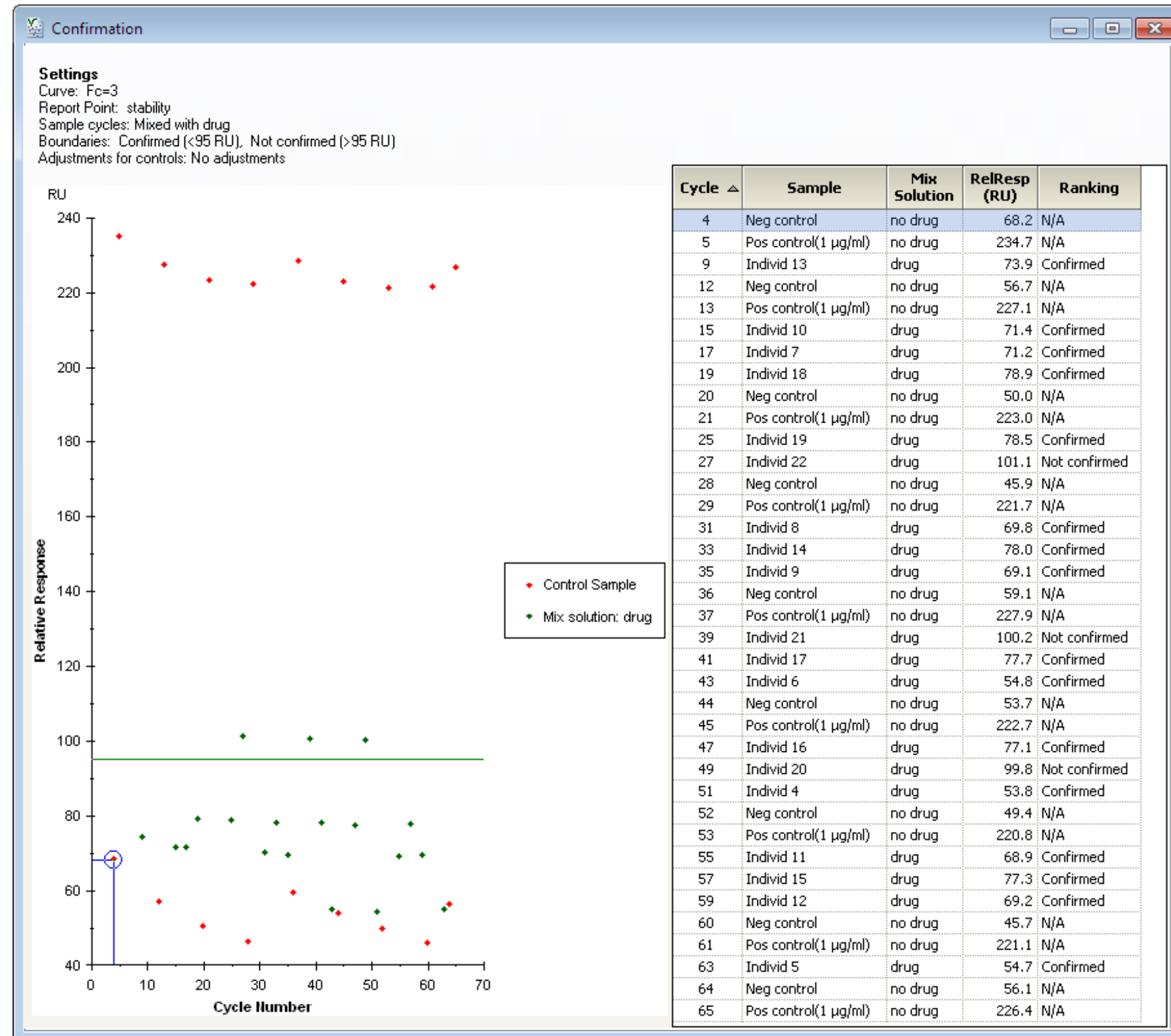
### Applying the evaluation

1. Choose the option **Show samples mixed with...** in the **Immunogenicity Confirmation** evaluation and choose the drug solution. Samples mixed with the chosen solution are distinguished from control samples by color. (Control samples are always shown regardless of the selected mixing solution, but they will be marked as N/A in the Ranking column of the finished evaluation.)

2. Enter region names and response boundary for the cut-off.



3. Click **Finish** to save the item.



# 07

# Antibody characterization – isotyping

*The isotype and subtype (class and subclass) of antibodies detected in samples can be determined by injection of isotype-specific reagents directly after the sample injection.*

## 7.1 Assay formats

The **Immunogenicity Isotyping** wizard offers two modes for this kind of assay:

- Using one isotyping reagent in each analysis cycle. Up to 20 reagents can be set up within the same run: each sample will be run in multiple separate cycles corresponding to the number of reagents used, as illustrated in the table below.

Samples	Reagents	Generated cycles
S1	R1	S1 + R1
S2	R2	S1 + R2
S3	R3	S1 + R3
S4		S2 + R1
		S2 + R2
		S2 + R3
		S3 + R1
		...

- Using multiple isotyping reagents in each sample cycle. Each sample is only analyzed once, and the same isotyping reagents are used in every cycle as illustrated in the table below. Up to 10 reagents can be used in one run.

Samples	Reagents	Generated cycles
S1	R1	S1 + R1 + R2 + R3
S2	R2	S2 + R1 + R2 + R3
S3	R3	S3 + R1 + R2 + R3
S4		S4 + R1 + R2 + R3

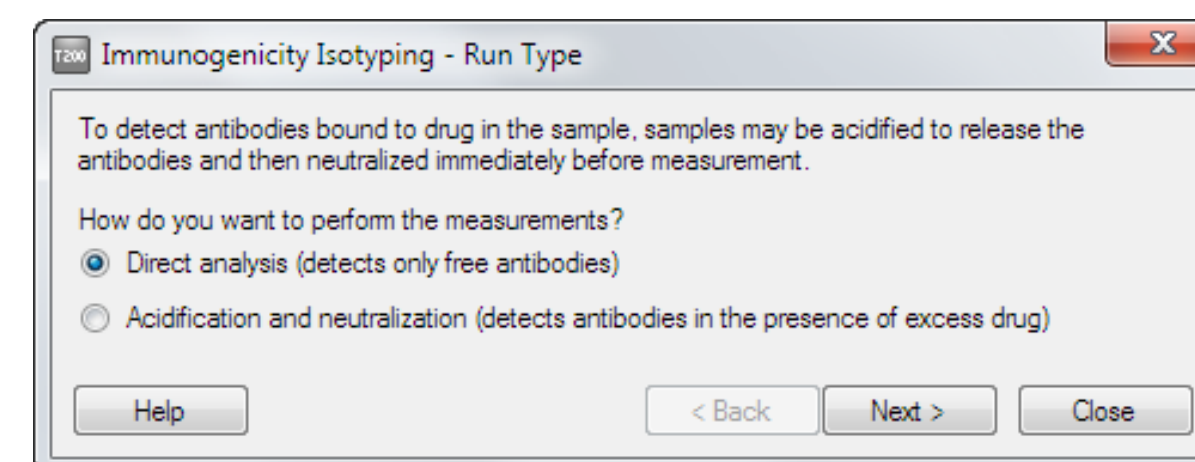
Using multiple reagents per cycle consumes less sample and takes less analysis time for the same number of cycles, but the results can be a little difficult to interpret if there is interference or cross-reactivity between reagents or if the detected antibody dissociates significantly from the surface (see Figure 3-2). Note also that low affinity antibodies dissociate from the surface in a relatively short time, and may have fallen to undetectable levels by the time the last isotyping reagents in a series are injected.

Run the assay with one reagent per cycle for best results, if time and material resources allow.

## 7.2 Setting up the assay

### 7.2.1 Run type

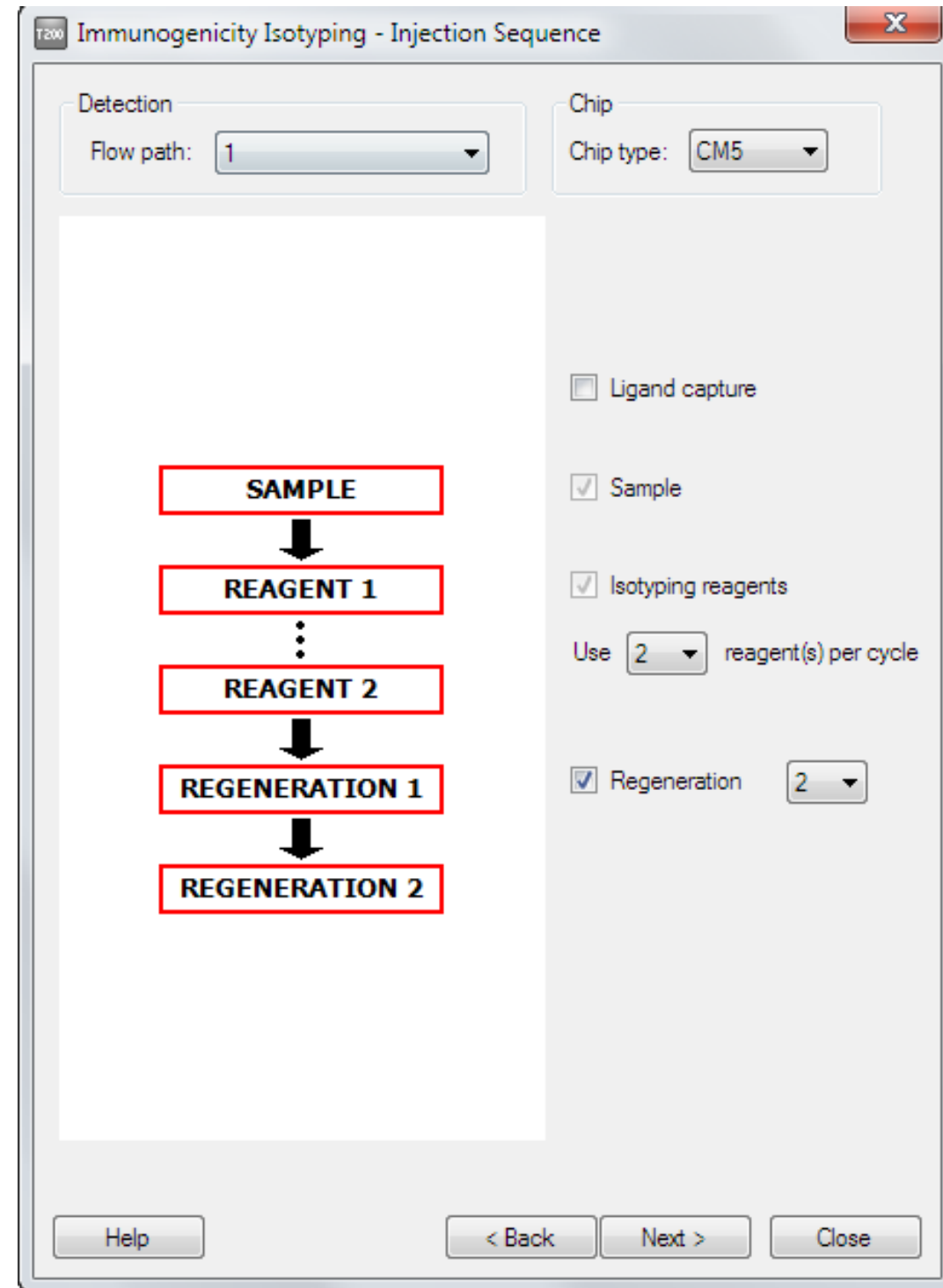
Choose whether you want to analyze the samples directly for free antibody or after acidification and neutralization to reduce drug interference (see Section 4.1).



### 7.2.2 Injection sequence

Choose the flow path and sensor chip for the run. If you have chosen to use sample acidification and neutralization, the flow path is fixed as **1,2,3,4** and isotyping injections pass only through flow cells 3 and 4. Sensor Chip CM5 is generally recommended for immunogenicity assays.

Choose the injection sequence that will be used in each cycle, including the number of isotyping reagents per cycle. If you choose one reagent per cycle, each sample will be analyzed repeatedly according to the number of reagents you enter in the reagent table (Section 7.2.6).



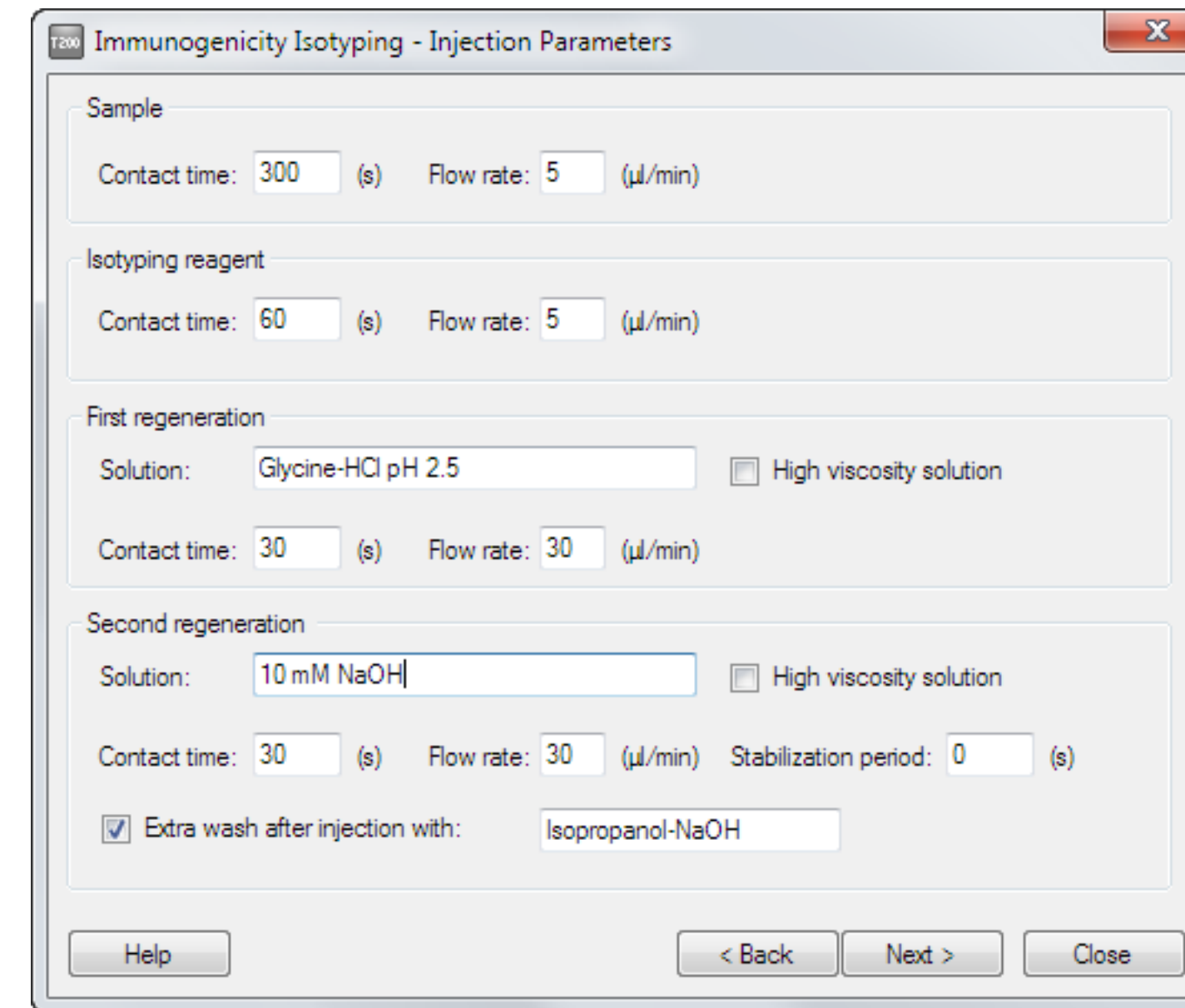
### 7.2.3 Setup

Conditioning cycles are not required for Sensor Chip CM5.

Start-up cycles with a positive control in serum as sample are recommended (see Section 3.6). With one isotyping reagent per cycle, startup cycles will be run with the first reagent only in the **Isotyping reagent** list.

### 7.2.4 Injection parameters

Enter the details for the injections in the analysis cycle.



If you are using merged injection to deal with drug interference, the standard acidification and neutralization solutions are entered by default (see Section 4.1).

An extra wash with 20% isopropanol in 40 mM NaOH (Section 3.2.2) is strongly recommended after the last regeneration injection to help keep the flow system clean.

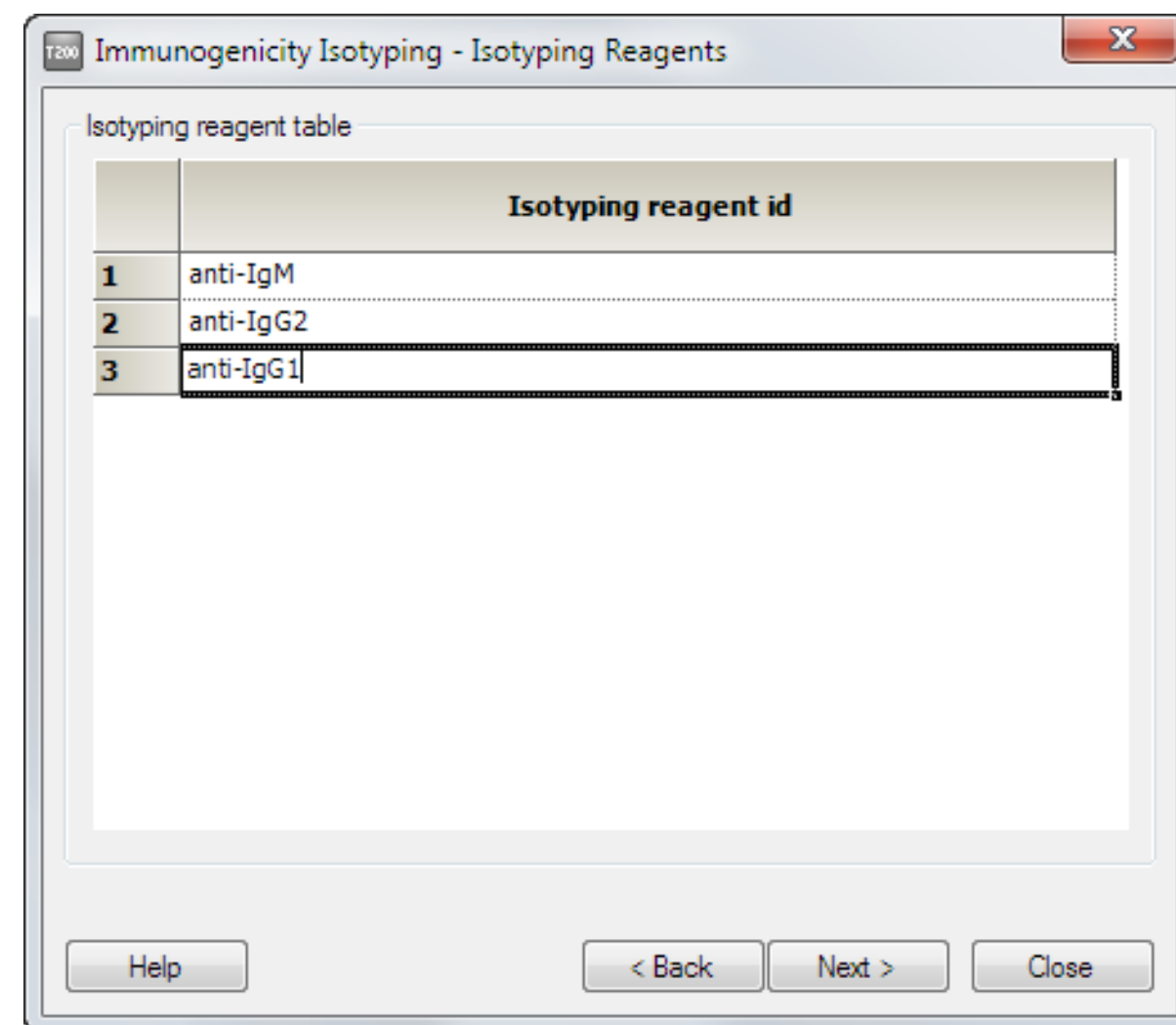
### 7.2.5 Samples

Enter the samples to be analyzed in this dialog, or use the **Import** function to import sample lists from external sources.

Any control samples intended to check the performance of the isotyping reagents or surface activity should be included in the sample table. The **Immunogenicity Isotyping** wizard does not support separately entered control samples analyzed at intervals throughout the assay.

### 7.2.6 Isotyping reagents

Enter the names of the isotyping reagents you want to use.



### One reagent per cycle

If you have chosen to use one isotyping reagent per cycle, you can enter up to 20 reagents in the table. Each sample will be run in a separate cycle for each reagent, so the number of sample analysis cycles will be the product of the number of samples and the number of reagents.

### Multiple reagents per cycle

If you have chosen to use more than one isotyping reagent per cycle, the reagent table contains a fixed number of rows, determined by the number of reagents you have chosen. All rows must be completed. Each sample will be analyzed in one cycle with reagents injected sequentially in the order listed in the table (Section 7.1).

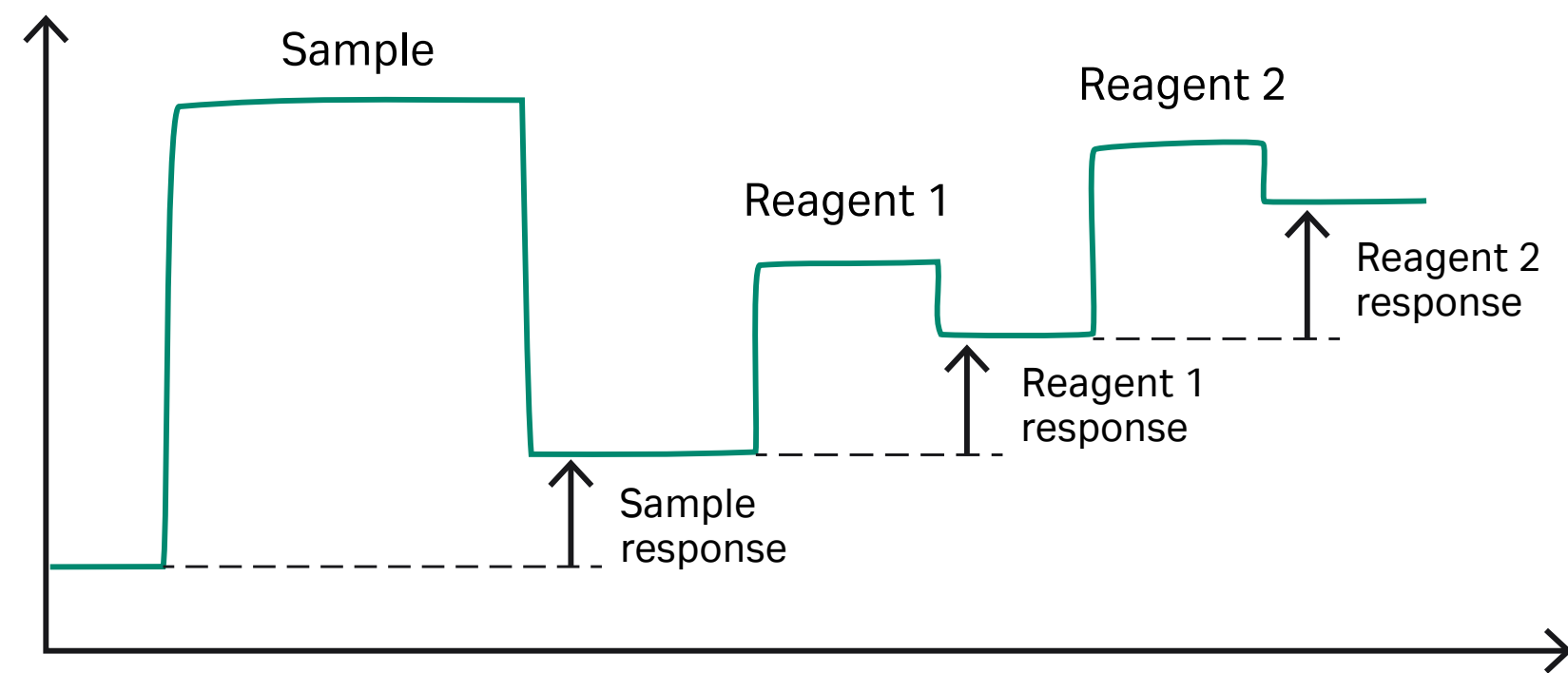
The order in which the reagents are tested can in some circumstances affect the results. If you expect one isotype or subtype to predominate in the immune response, place the corresponding reagent last in the series to minimize the risk of steric interference between multiple positive responses.

### 7.2.7 Remaining wizard steps

The remaining steps in the wizard follow standard procedures as described in the Biacore T200 Software Handbook.

## 7.3 Evaluating isotyping results

Isotyping assay results are presented as a bar chart of isotyping reagent levels, expressed either as response or response relative to sample response. Reagent response is measured directly after the reagent injection, relative to a baseline set just before the same injection (Figure 7-1). Cut-off levels are set for sample response (to eliminate samples with intrinsically low binding levels) and for the response level for each isotyping reagent.



**Figure 7-1.** Report points for isotyping reagent response levels measure the response for each reagent relative to the baseline just before the respective injections. Responses are exaggerated for clarity in this illustration.

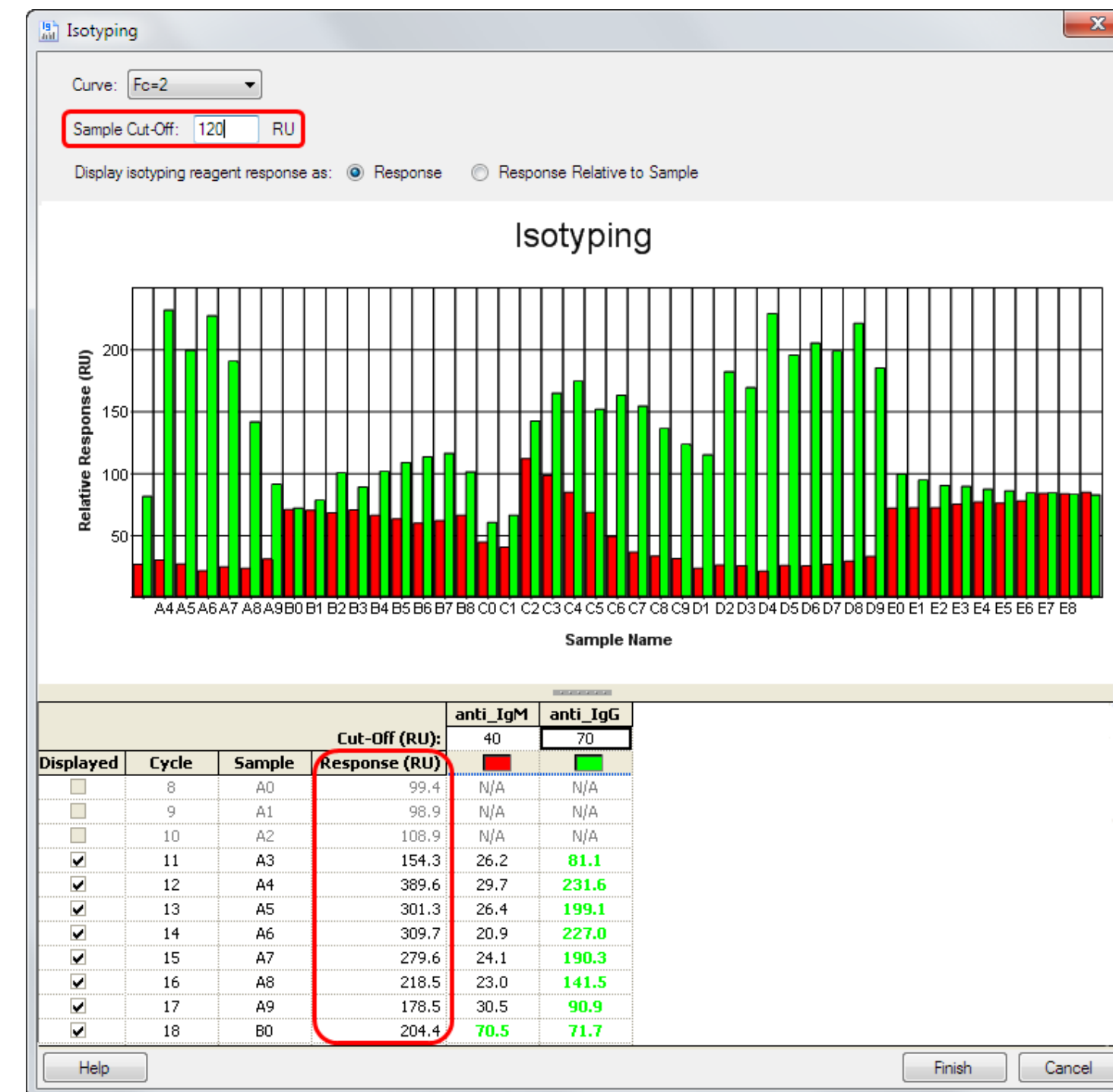
### 7.3.1 Set up the evaluation

Examine the sensorgrams and pre-defined plots before starting the evaluation, and exclude any cycles that are clearly disturbed.

Set up the evaluation as follows:

1. Select the curve from which the responses should be measured. If you change the curve setting, any cut-off values you have entered will be cleared.

2. Set a sample cut-off, based either on a negative control serum or on the sample responses listed in the table. Procedures for calculating the cut-off level from negative control responses are described in Section 5.2.2. If you do not want to exclude samples on the basis of a cut-off response, set a value below the lowest sample response.



Cycles with sample responses below the cut-off level will be listed in gray in the table and excluded from the bar chart. Reagent responses for these cycles will be listed as **N/A** (not applicable).

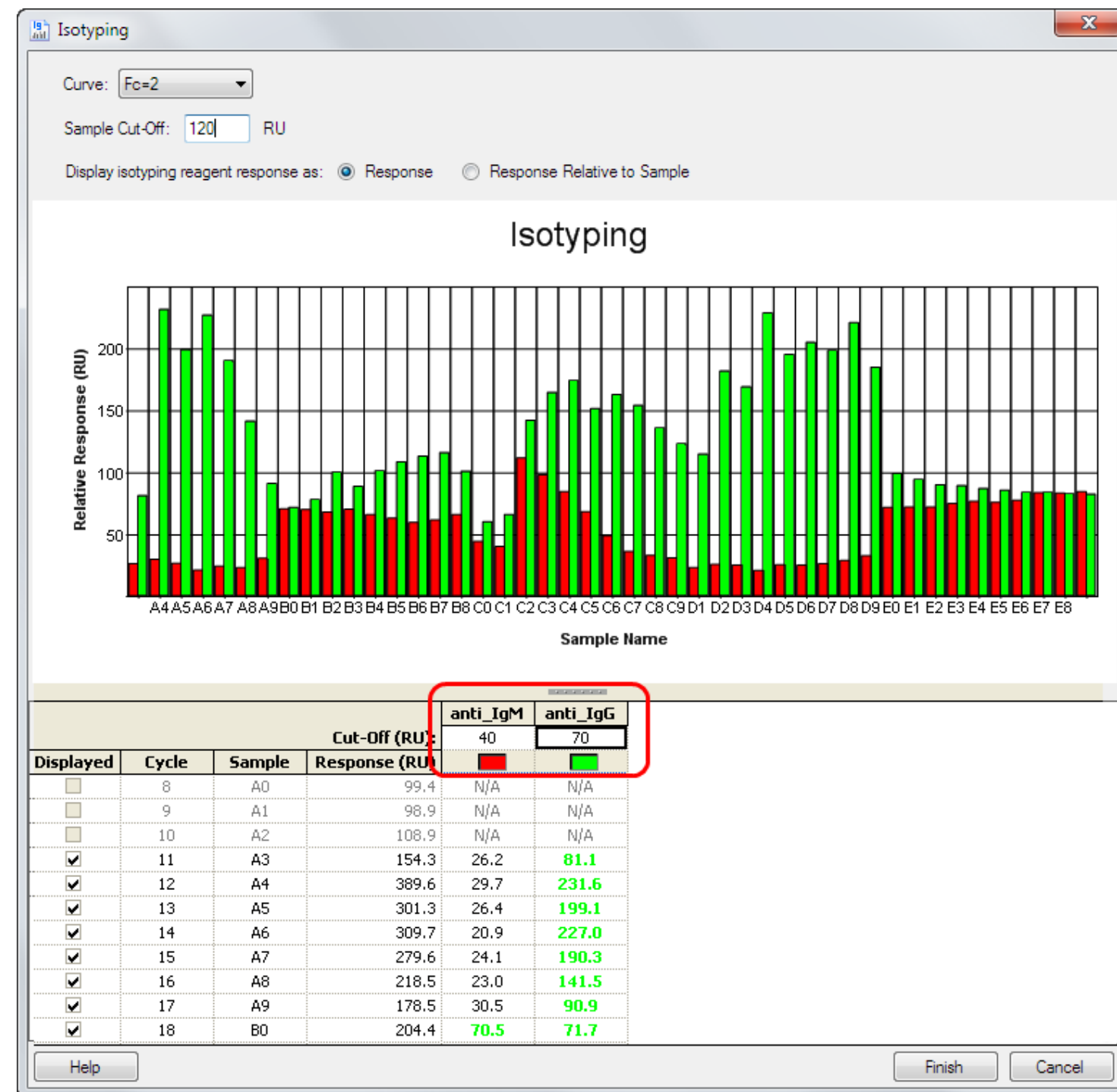
3. For each isotyping reagent, enter a cut-off response in RU in the column header. Reagent responses above the cut-off level will be shown in green in the table (and flagged as above cut-off in exported data). The reagent cut-off levels do not affect the bar chart presentation.

4. If required, change the **Display** setting to **Response Relative to Sample**. This will change the bar chart and the table to show reagent responses divided by sample responses. In this mode, reagent responses below the reagent cut-off are listed as **low** and are represented by zero-height bars in the chart.

**Note:** Reagent cut-off values are entered and displayed in RU even when the response values are shown as response relative to sample.

5. You can choose to hide or display cycles in the bar chart using the **Displayed** check-box in the table. Cycles that are hidden because the sample response is below the cut-off cannot be displayed in this way.

6. Click Finish to save the item.



# 08

# Antibody characterization – binding stability

Biacore T200 supports estimation of the stability of antibody binding to drug on the sensor chip surface, so that the immune response can be characterized in terms of antibody fractions or populations with rapid and slow dissociation rates. The dissociation rate is an approximate indicator of the binding affinity, and maturation of antibodies towards higher affinity binding is often reflected in slower dissociation rates.

## 8.1 Principles of stability determination

### 8.1.1 Analyzing the dissociation behavior

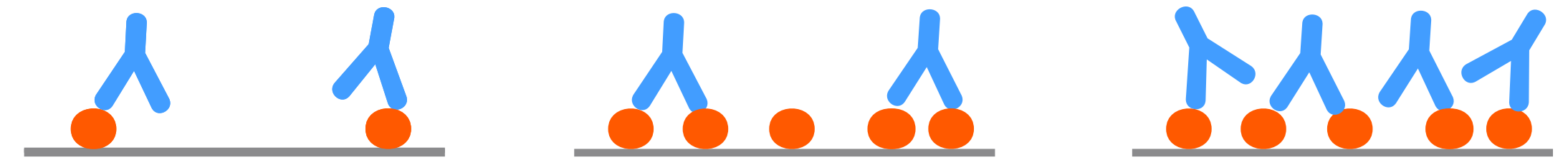
Antibodies produced in an immune response can usually be expected to represent a heterogeneous population with a spectrum of different kinetic properties. Attempting to describe the dissociation behavior of such a population in terms of a single interaction does not give satisfactory results, and it is not practicable to resolve the behavior into components that represent individual antibody species. As a compromise, the dissociation phase of the sensorgram is fitted to a model describing dissociation from two independent monovalent sites. Monovalent dissociation is described by an exponential equation (Section 8.4), and results are expressed in terms of the half-life of the complex at each site (i.e. the time taken for the amount of complex to be reduced by 50%).

It is important to realize that evaluating the data in terms of a two-site dissociation model does not imply that there are two distinct populations of antibodies in the sample. The two-site model represents an empirical compromise that describes most observed sensorgrams satisfactorily, and can help to classify the immune response in terms of predominance of rapidly or slowly dissociating antibody fractions.

### 8.1.2 Effects of antibody multivalence

The model used to analyze dissociation behavior treats the sensorgrams as the sum of two simple exponential functions describing dissociation of 1:1 complexes. In reality, antibodies are bi- or multivalent, and simultaneous attachment of multiple binding sites to drug immobilized on the surface reduces the dissociation rate compared with a 1:1 complex. The term avidity is used to refer to the enhanced affinity resulting from multivalent attachment of antibodies.

The opportunity for multivalent binding to the sensor surface is influenced by both the amount of drug immobilized on the surface and the amount of antibody bound in relation to the maximum binding capacity. If the drug molecules are widely spaced on the sensor surface (at very low levels of immobilized drug), there will be fewer opportunities for multivalent binding, while at high levels of saturation antibody molecules will compete for available sites. In both situations, the binding behavior will tend more towards single-site binding and the apparent dissociation rate will be higher (Figure 8-1).



**Figure 8-1.** Antibody binding behavior varies with level of immobilized drug and level of binding saturation. Left: At extremely low immobilization levels, drug molecules are not close enough to allow multivalent binding. Center: Under conditions normally used in immunogenicity studies, most antibody molecules will attach to immobilized drug at multiple binding sites, giving avidity effects that reduce the observed dissociation rate. Right: At high levels of saturation, antibody molecules may compete with each other for binding to drug and the behavior may tend more towards single-site binding.

For this reason, comparisons of the binding stability in different samples should be made between analyses performed under the same conditions of immobilized drug and antibody binding levels.

**Note:** *Extremely low levels of immobilized drug giving a maximum antibody binding capacity of less than 5 RU are required to eliminate avidity effects. Such low levels are not useful in immunogenicity studies.*

## 8.2 Setting up a stability assay

Evaluation of binding stability requires a dissociation time of at least 2 minutes after the sample injection. Set up the assay using the **Immunogenicity Screening** wizard (see Section 5.1), with a dissociation time after sample injection in the **Injection Parameters** step. This option cannot be used together with acidification and neutralization for detection of antibody complexes.

It is not generally appropriate to include enhancement injections together with a dissociation time, since the enhancement reagent will be injected when a significant proportion of the antibodies have dissociated from the surface. Normally, a stability assay will be run on samples that are judged positive in a screen and confirmed as drug-specific antibodies.

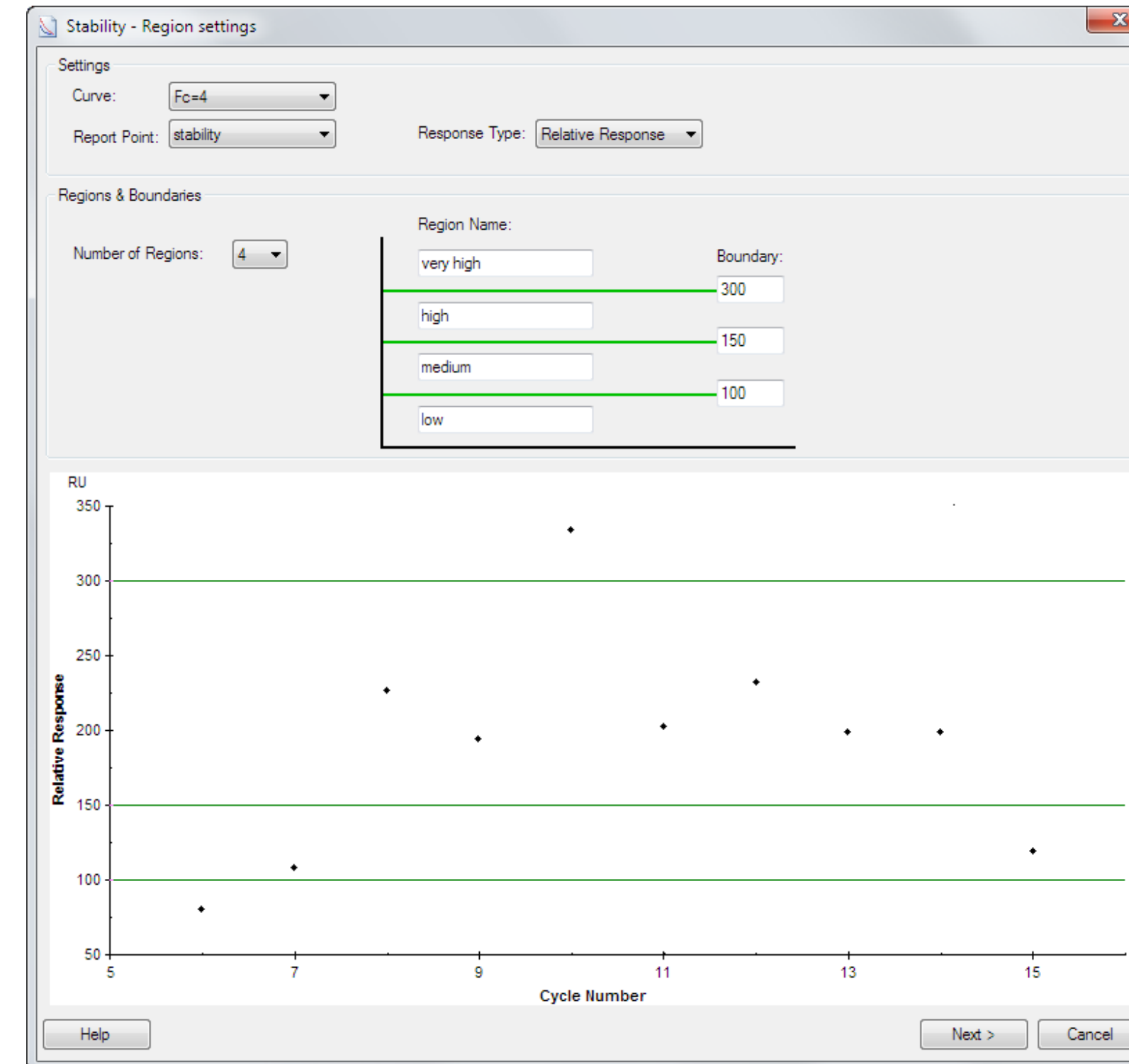
## 8.3 Evaluating the results

Choose **Immunogenicity:Stability** to evaluate the assay. Control samples are not included in the evaluation.

### 8.3.1 Region settings

Select the curve, report point and response type for dividing the responses into regions. The **Curve** setting also determines which curve will be used for fitting the stability data. Choice of report point and response type affect the ranking regions but not the stability fitting.

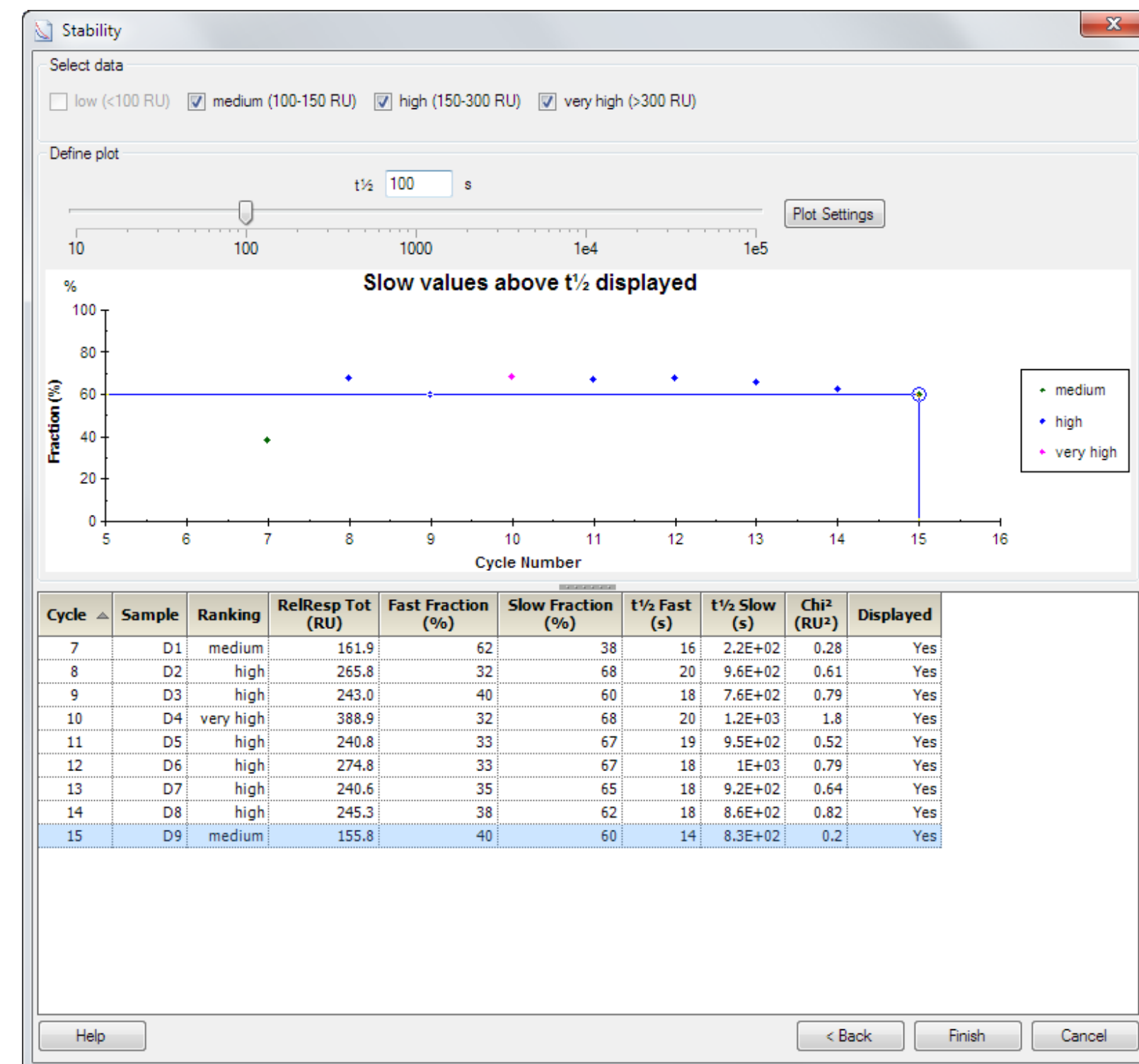
Set region names and boundaries in the same way as described for screening (Section 5.2.3). Samples in the lowest region will be excluded from the stability evaluation: set the lowest boundary to exclude samples that do not show significant binding. Using more than two regions allows you to filter the stability results on the basis of response level.



Click **Next** to continue with the evaluation. The dissociation phase of the sensorgrams in samples above the lowest region boundary will be fitted to the two-site model.

### 8.3.2 Stability

Results of the fitting are reported in the table as the half-lives of the fast and slow antibody fractions and the proportion of the total response assigned to each fraction. The fast fraction is reported as 0% and the slow as 100% if the calculated percentage in one fraction is less than 1% or the calculated  $t_{1/2}$  - values differ by less than 10%. The plot shows points for samples filtered as described below.



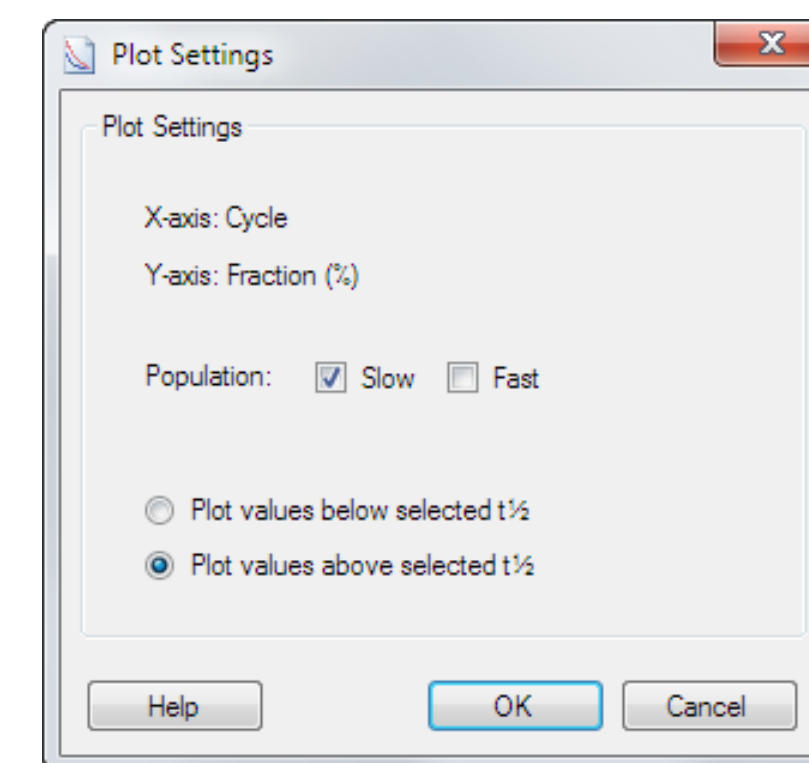
Check the regions you want to display under **Select data**. Points in the lowest region are automatically excluded and cannot be displayed. Regions that are excluded are not listed in the table or shown in the plot.

By default, the plot shows the fraction of the total response represented by the slow antibody fraction. The slider scale is determined by the range of measured  $t_{1/2}$  values and the slider is positioned at the low end of the scale so that all points are included.

In the table, the column **RelResp Tot** lists the relative response at the start of dissociation, determined by extrapolation of the fitted dissociation curve. The column **Chi<sup>2</sup>** shows the chi-squared value for the fitting (see the Biacore T200 Software Handbook for details). High chi-squared values (above about 5% of the reported total binding level) indicate a poor fit. Examine the sensorgrams and fitted curves carefully if the chi-squared value is high, to determine whether the results are trustworthy.

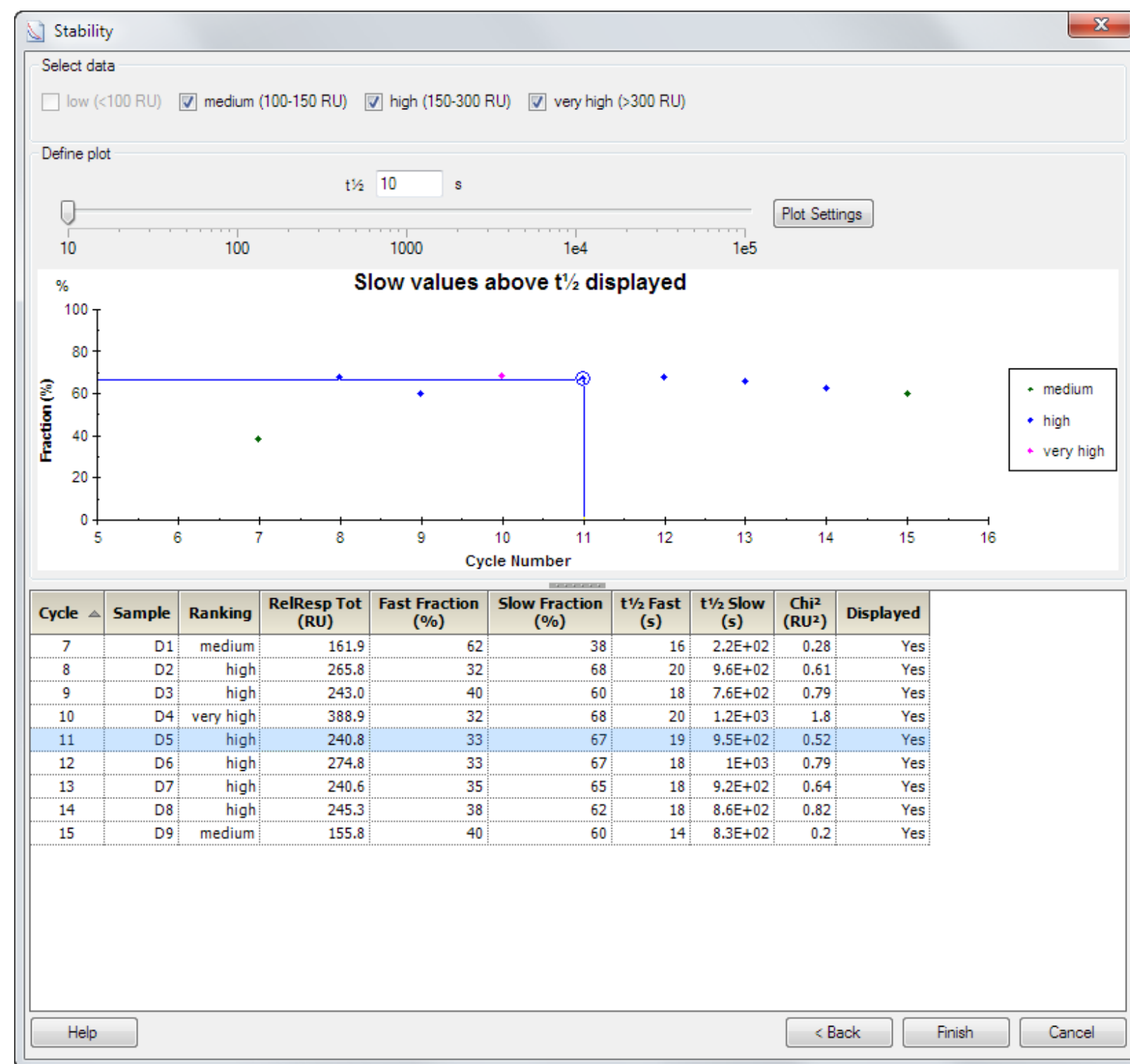
Drag the slider or enter a value for  $t_{1/2}$  to filter the plot. For example, moving the  $t_{1/2}$  slider to 1000 seconds with the setting **Slow values above  $t_{1/2}$  displayed** will result in a plot that shows points for the slow fraction with  $t_{1/2}$  greater than 1000 seconds. Samples where the slow fraction has a  $t_{1/2}$  less than 1000 seconds will not be shown on the plot. All samples that meet the **Select data** criteria are however still listed in the table.

Click **Plot settings** to change the way the points are filtered in the plot.



You can choose to display points for the slow or fast population or both. If you choose both, samples for which both  $t_{1/2}$  values are accepted by the slider limit will be shown as two points in the plot, with fraction values that add up to 100%.

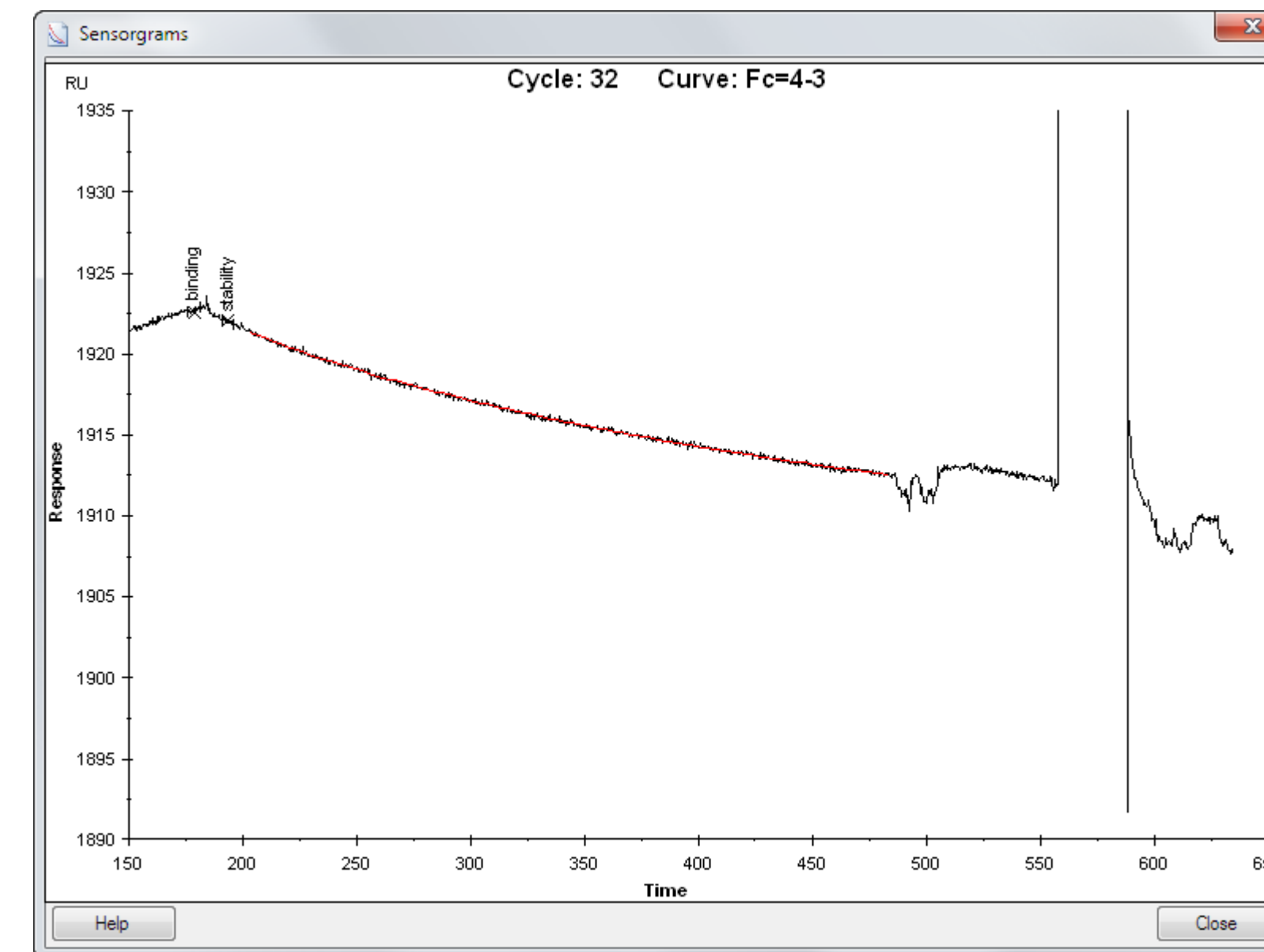
**Note:** The terms fast and slow do not refer to any absolute values for  $t_{1/2}$ , but are applied individually to the fitted results for each separate sample.



You can also choose to plot values above or below the selected  $t_{1/2}$  value (i.e. slow or fast fractions respectively).

Right-click on a point in the plot or select a row in the table and right-click on the row for options to exclude or include the corresponding cycle from the evaluation. You can select multiple rows in the table to manage multiple cycles in one operation. Excluded cycles are not shown in the plot and are struck out in the table with **N/A** listed for all results.

Choose **Show Sensorgram(s)** from the right-click menu to examine the sensorgrams for the selected cycles. Sensorgrams are shown in black with the fitted curve overlaid in red.



## 8.4 Curve fitting procedure

Binding stability is evaluated by fitting the dissociation phase of the sensorgrams to an equation describing simultaneous dissociation of two independent monovalent populations:

$$R = R_1 \times e^{-k_{d1}(t-t_0)} + R_2 \times e^{-k_{d2}(t-t_0)}$$

where R = response at time t

$R_1$  and  $R_2$  are the response contributions from the two populations at the beginning of the dissociation (time  $t_0$ )

$k_{d1}$  and  $k_{d2}$  are the dissociation rate constants for the two populations

**Note:** *This equation is based on simple monovalent dissociation kinetics for the two populations. Antibodies are bi- or multivalent, which complicates the dissociation behavior further. It is important to be aware that binding stability characteristics reported by the evaluation do not represent formal kinetic parameters.*

The data selected for fitting starts a few seconds after the end of the sample injection, to minimize interference from bulk refractive index contributions in the sample which may otherwise be interpreted as rapid dissociation events and contribute to the reported fast fraction of antibodies.

For presentation and evaluation of the results, dissociation rate constants determined by the fitting procedure are converted to half-lives ( $t_{1/2}$ , the time required for the amount of complex to be reduced by 50%).

$$t_{1/2} = \frac{\ln 2}{k_d}$$

**Note:** *These values are an artificial construction of the data fitting procedure and do not represent true half-lives of the antibody-drug complexes.*

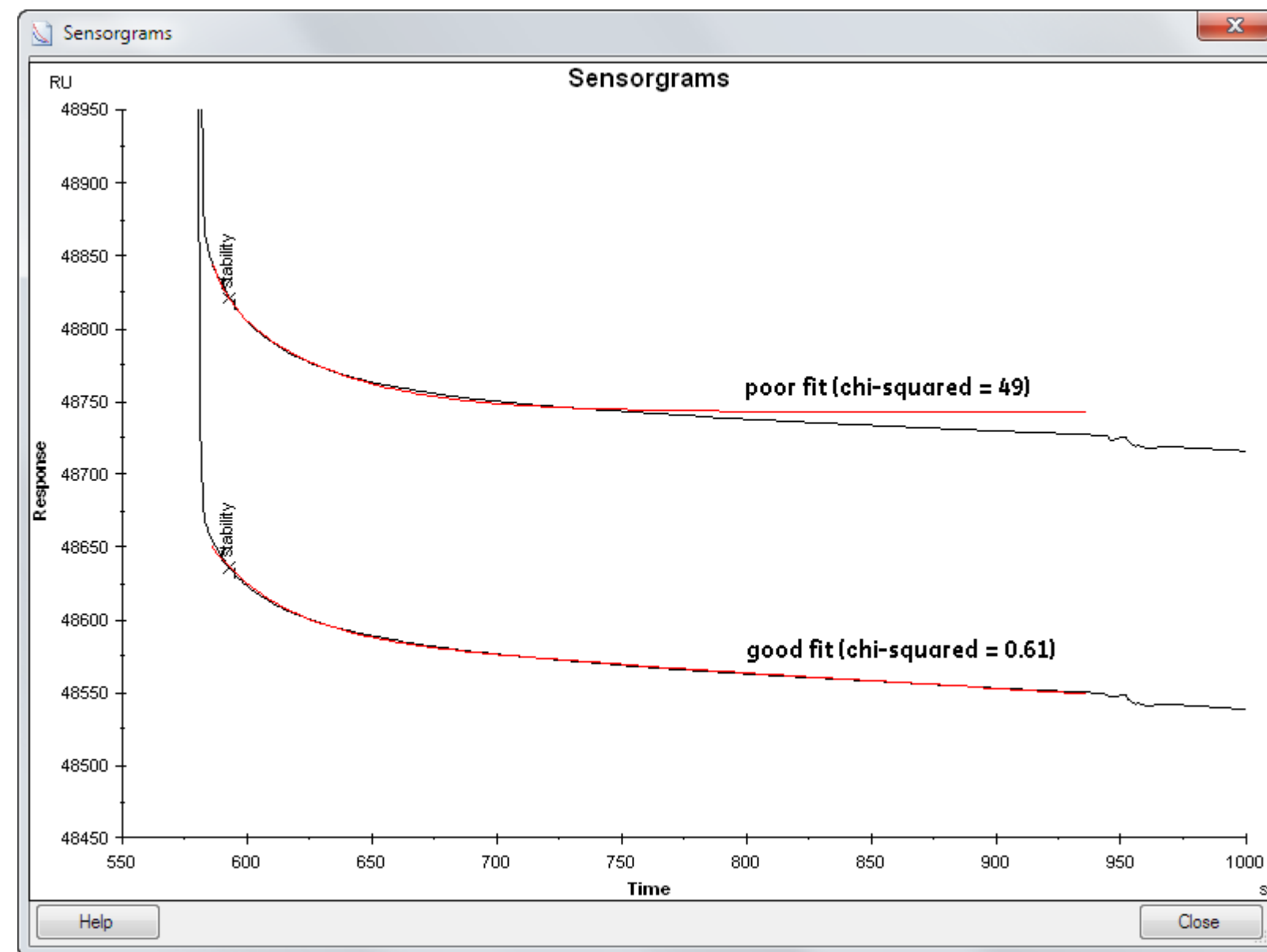
If the dissociation behavior describes a true exponential curve and can therefore in principle be analyzed with a single site model, the two-site fitting procedure will return either one population with a very low fraction and a non-significant value for  $t_{1/2}$  or two populations with closely similar values for  $t_{1/2}$  and an unpredictable division of the response between the populations. If either one fraction is calculated as less than 1% or the values for  $t_{1/2}$  in the two populations differ by less than 10%, the evaluation will assign the whole antibody population to the slow fraction and report the fast fraction as 0% with  $t_{1/2}$  **N/A**.

## 8.5 Points to watch

- Use consistent levels of immobilized drug if you want to compare stability data between different assay occasions.
- Include NSB Reducer in samples for stability determination, to minimize non-specific binding that otherwise can contribute to the evaluated binding stability.
- Evaluate stability only for samples that are confirmed positive for antibody content. Negative samples will give meaningless results in the evaluation.
- Examine the reported  $t_{1/2}$  values. Each sample is analyzed in terms of slow and fast fractions on its own merits with no absolute assessment of what constitutes slow and fast, and in some cases both reported fractions may have similar  $t_{1/2}$  values. In the example below, cycle 6 is reported with two fractions with  $t_{1/2}$  values that would normally both be classed as "fast" in terms of absolute dissociation rates, and cycle 10 with fractions that would normally both be classed as "slow".

Cycle $\Delta$	Sample	Ranking	RelResp Tot (RU)	Fast Fraction (%)	Slow Fraction (%)	$t_{1/2}$ Fast (s)	$t_{1/2}$ Slow (s)	Chi <sup>2</sup> (RU <sup>2</sup> )	Displayed
6	test	high	210.5	50	50	3.9	30	49	Yes
8	test	high	265.8	32	68	20	9.6E+02	0.61	Yes
10	test	high	388.9	32	68	1.0E+02	1.2E+03	1.8	Yes

- Always check the quality of the fitted curves. The chi-squared value gives an indication of fitting quality: visual inspection of the sensorgrams and fitted curves can help to identify fitting deviations. Do not trust stability results for sensorgrams where the fitting is poor. The illustration below shows examples of poor and good fits.



- The reported value for **RelResp Tot** is determined by extrapolation of the fitted curve to the start of dissociation, and does not correspond to the response level shown in a screening evaluation or used in defining regions. In particular, if there is a significant fast antibody fraction in the sample, the extrapolated **RelResp Tot** value does not necessarily provide a reliable indication of the amount of antibody in the sample.

# Appendix A

# Immunogenicity functions in Method Builder

Use Method Builder if you want assay flexibility that is not supported in the application wizard. Method Builder is described in detail in the Biacore T200 Software Handbook.

Two injection types are included to the **Sample** command in cycle type definitions to support acidification and neutralization of samples:

- **Merged injection** for acidification in the microplate and neutralization at the flow cell.
- **Double mix** for acidification and neutralization in the microplate followed by injection of the neutralized sample.

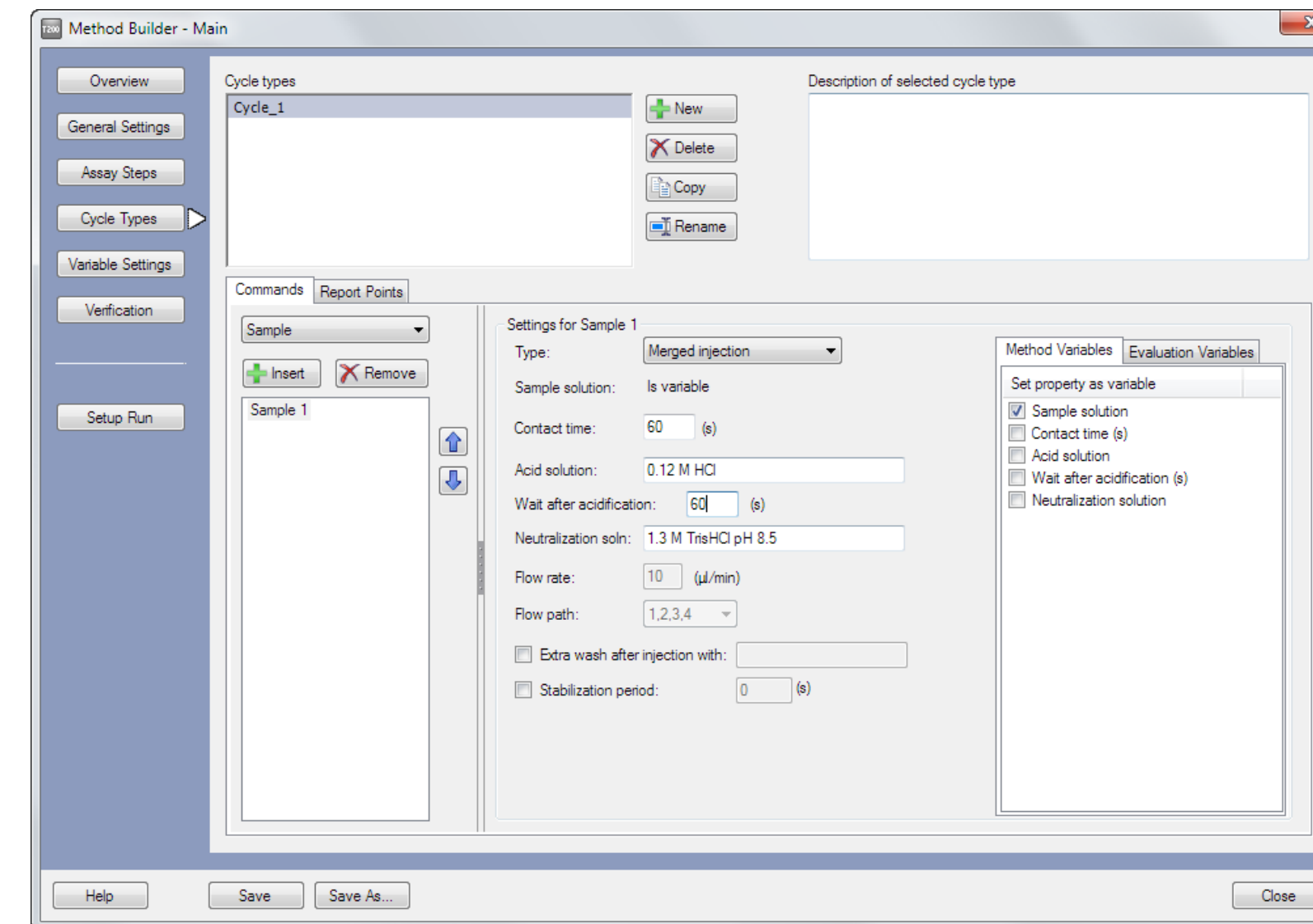
All other functions in the immunogenicity application wizards are implemented using Method Builder options in the standard Biacore T200 Software. To use application wizard functionality as the starting point for a customized method, save the wizard template and open it in Method Builder as described in the Biacore T200 Software Handbook.

Remember to include an extra wash with 20% isopropanol in 40 mM NaOH (Section 3.2.2) after the last injection in the cycle to help keep the flow system clean.

## A.1 Merged injection

The injection type **Merged injection** performs the mixing and injection steps for acidification in the microplate well followed by neutralization at the flow cell as described in Section 4.1. Volumes of sample and reagents are fixed.

A wait time can be specified between acidification and injection of the sample. This time will be added to that required to mix the sample with acid and prepare the injection.



The flow path for a merged injection is fixed as **1,2,3,4**, which requires that **Detection** is set to **Multi** in the **General Settings** workspace, and the flow rate is fixed at 10 µL/min. These limitations apply only to sample injections using the **Merged injection** mode: other injections in the method may use different settings. A merged injection has a maximum contact time of 600 seconds.

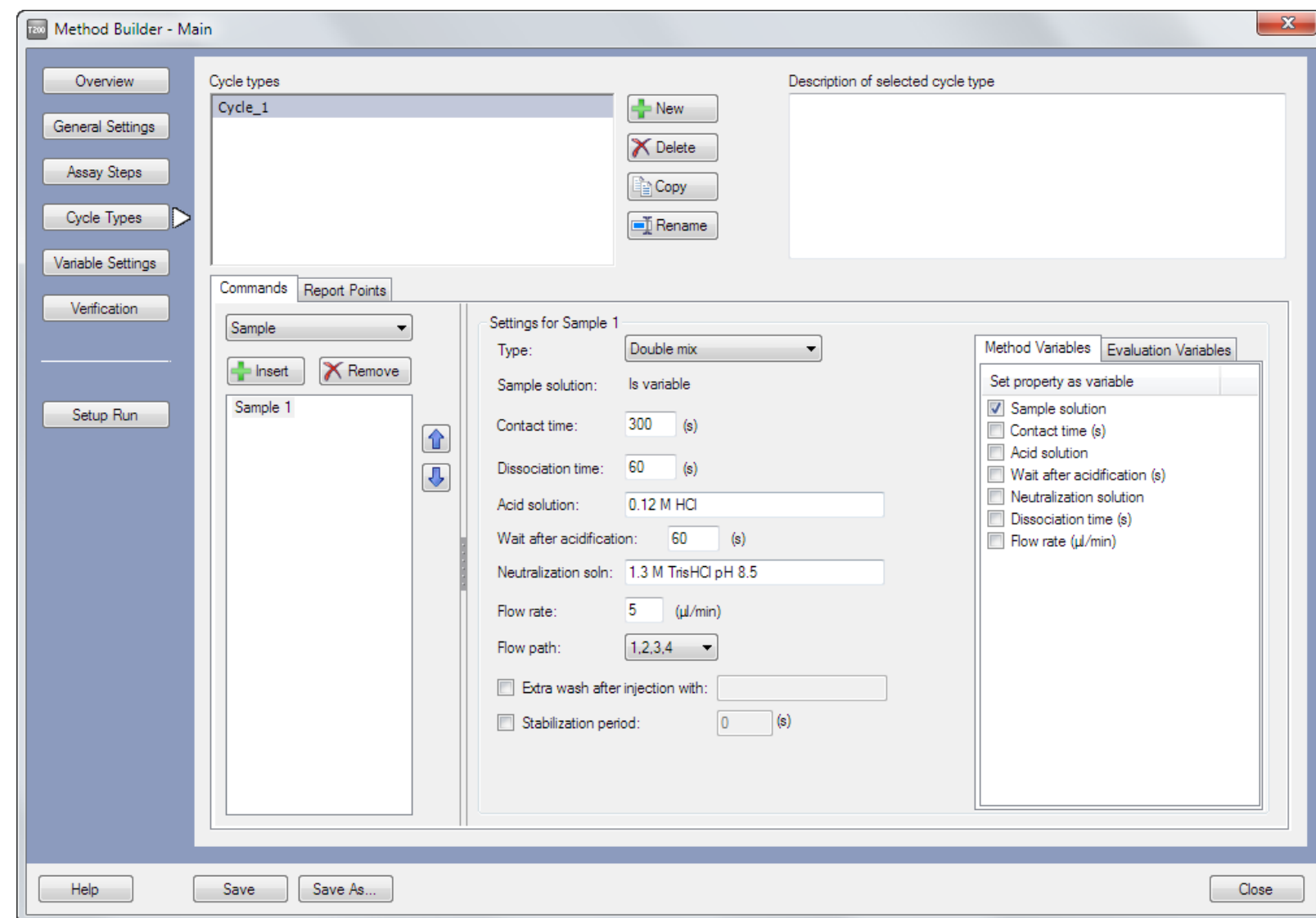
Acid solution, wait time after acidification and neutralization solution can be set as method variables.

Reference subtraction may be chosen in the **Detection** step when the run is set up. Remember however that the sensorgrams from flow cells 1 and 2 do not contain meaningful data when merged injection is used (see Section 4.1), and reference subtraction is not recommended together with merged injections.

Remember to set the analysis temperature to 10°C for methods that use merged injections (Section 3.6).

## A.2 Double mix

The injection type **Double mix** acidifies and neutralizes the sample in the microplate as described in Section 4.2. For double mix, you specify the incubation time between acidification and neutralization of the sample. The neutralized sample is injected as soon as possible after neutralization.



The flow path can be chosen for double mix within the limits of the Detection setting.

The maximum contact time for a double mix injection corresponds to injection of 105 µL sample at the specified flow rate (the default flow rate is 5 µL/min, giving a maximum contact time of 21 minutes).

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