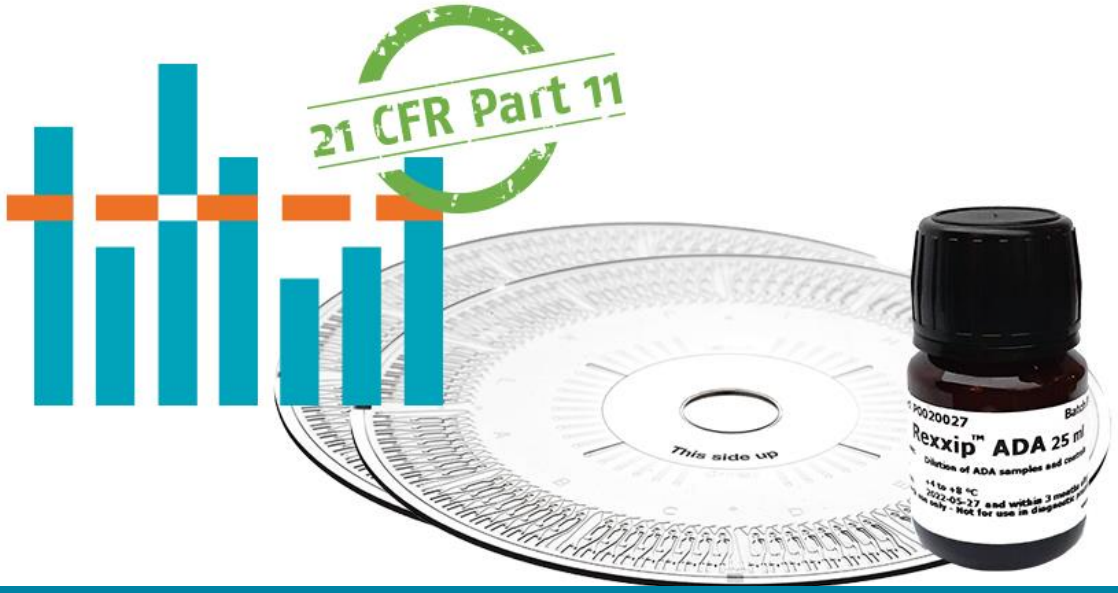
GYROS PROTEIN Technologies



Gyrolab ADA assay protocol

Contents

Abbreviations

Abbreviation	Explanation
ADA	Anti-Drug Antibody
CD	Compact Disc
FAS	Field Application Specialist
LOD	Limit of Detection
MM	Master Mix
NC	Negative Control
PC	Positive Control
PK	Pharmacokinetics
SD	Standard Deviation
S/B	Signal-to-Background ratio
SCP	Screening Cut Point
SCF	Screening Correction Factor
CCP	Confirmatory Cut Point

Introduction

Anti-Drug Antibodies (ADAs) may be formed in human patients or pre-clinical animal models, hereafter referred to as study objects, upon administration of a biotherapeutic. This immune response can be a safety concern, may neutralize the effect of the drug, and/or may mask epitopes on the drugs used by the PK assay, resulting in lower-than-expected exposure values [1]. An ADA assay must therefore be developed to monitor ADA levels during preclinical and clinical trials [2, 3]. Immunogenicity is monitored based on regulatory guidelines and involves a tiered approach that includes screening, with an assay cut point set to give a low level of false positives, and a confirmatory assay that confirms the true positives. The assays must be sufficiently sensitive to detect at least 100 ng/mL ADA in the presence of levels of free drug that can be expected in samples.

The ADA assay format we recommend for use on a Gyrolab® system is a homogeneous bridging format, which detects ADAs of all isotypes and is not species dependent. This means that it can be used for both pre-clinical and clinical studies. In this assay format, the drug is used as both capture (biotinylated) and detection (fluorescently labeled) reagent (combined to form a Master Mix) and the sample and the reagents are mixed before addition to the streptavidin columns on Gyrolab Mixing CD 96 or Gyrolab Bioaffy CD. A number of different complexes will be formed in this mixture. Complexes formed by ADA or the positive control (PC) bridging between the biotinylated drug molecule and the drug molecule labeled with Alexa Fluor® 647 will bind to the streptavidin coated beads in the CD column and be detected in the assay (see Figure 1).

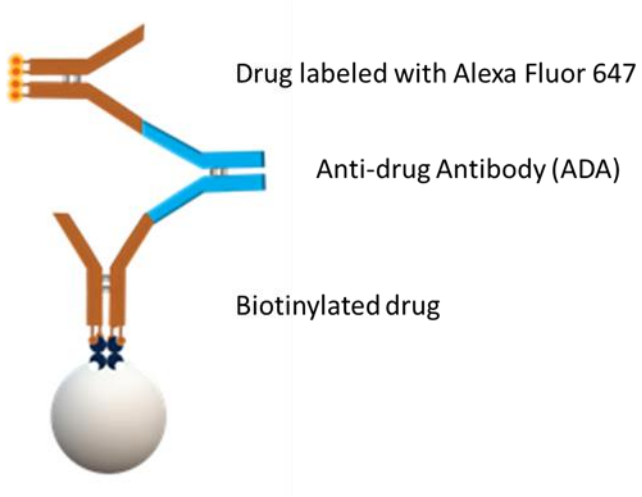


Figure 1. Detectable complex bound to a streptavidin-coated bead in a homogeneous bridging assay

Free drug in the sample will compete with labeled drug to build complexes with the ADA and reduce the observed signal. It is therefore necessary to disrupt any drug-ADA complexes in the sample before assaying to allow labeled drug to bind to the ADA. There are two common approaches to solving this problem:

- **Acid dissociation before analysis**: Acid is added to the sample to temporarily dissociate ADA-drug complexes. This acidified mixture is then neutralized in the presence of labeled reagents to enable the formation of detectable complexes. Using the Gyrolab Mixing CD 96 enables these steps to be automated.

- **Over-night incubation of the ADA assay reagents with the sample before analysis**: This allows sufficient time for ADA-drug complexes to dissociate and form detectable complexes with labeled reagents.

We recommend that both approaches are evaluated when running Experiment 1 and 2 to ensure that the most appropriate method for the detection of ADAs is chosen. Where possible, automated acid dissociation before analysis is preferable as this increases the likelihood of meeting regulatory guidelines, especially those relating to sensitivity and drug tolerance. The decreased hands-on time of the automated process using Gyrolab Mixing CD 96 also enables more data to be generated in less time. The high binding capacity of the particles in Gyrolab CDs supports higher concentrations of labeled reagents than normally used in other platforms, which may give a stoichiometric advantage regarding interference from the free drug.

Materials and Gyrolab Methods

Capture and detection reagents

Prior to ADA assay development, capture and detection reagents must be prepared by labeling the study drug with biotin and Alexa Fluor 647. For further labeling instructions, see Gyrolab User Guide. The degree of labeling for both capture and detection reagents may be optimized if needed, and most commonly a lower molar excess of labeling reagent is preferred to ensure optimal assay performance. Ideally, the binding characteristics of the labeled drug molecules should also be assessed to ensure that the capture and detection reagents have approximately the same affinity to maximize formation of the detectable complexes. In the homogeneous bridging assay format, both capture and detection reagent are added to the sample in a 1-to-1 ratio. This is done by preparing a Master Mix (MM) of equimolar concentrations of capture and detection reagents.

Positive Control

The choice of positive control (PC) is very important. The positive control should be as similar as possible to the actual ADA formed in study objects and preferably be polyclonal. Serum samples from previous studies shown to contain ADA are usually very good positive controls. Although a viable option, a high affinity monoclonal antibody is not recommended as this will have low similarity to the actual ADA and may bind too strongly to the drug, possibly resulting in interference from free drug.

Matrices and buffers

Matrix or buffer	Use
Naïve individual serum	Sufficient number of treatment-naïve individuals that represent the desired study object population (species, gender, disease state, comedications etc.). These can be pre-dose samples or commercially sourced.
Pooled naïve serum	Pooled serum that represents the desired study object population (species, gender, disease state, comedications etc.). This can be commercially sourced or prepared from prescreened individuals as above.
Rexxip ADA	Rexxip buffer used to dilute samples and Master Mix that will be used in the acid dissociation protocol. This may also be used in the overnight incubation protocol.
Rexxip H, Rexxip A, or Rexxip F	If desired, Rexxip H, Rexxip A, or Rexxip F buffers may be tested in place of Rexxip ADA for the dilution of samples and Master Mix preparation for the overnight protocol.

Matrix or buffer	Use
1 M Glycine	7.51 g glycine + 100 mL H_2O – used in the preparation of the Acidic Buffer
1 M HCl	12.1 mL 25% HCl (8.2 M) + 87.9 mL H_2O – used in the preparation of the Acidic buffer
Acidic Buffer (0.5 M Glycine-HCl, pH 2.6)	12.5 mL 1 M glycine + 6.34 mL 1 M HCl + 6.16 mL H_2O used to acidify sample to force dissociation
Neutralization buffer 2 M Tris-HCl, pH 8.0	12.11 g Tris + 30 mL H_2O. Adjust pH to 8.0 with 25% HCl. Make up to 50 mL with H_2O – used to dilute Master Mix prior to addition to the acidified sample

Gyrolab CDs for ADA analysis

Gyrolab Mixing CD 96 allows for an automated addition of the acid for the acid dissociation process. For overnight incubation analysis we recommend Gyrolab Bioaffy 200 CD. Other Gyrolab Bioaffy CD types may be tested but requires that the appropriate method is acquired from your local Field Application Specialist (FAS).

Gyrolab ADA analysis procedure overview

Acid dissociation using Gyrolab Mixing CD 96

1. Dilute samples, positive controls and negative controls in Rexxip ADA.
2. Prepare a 2x Master Mix solution.
3. Dilute 2x Master Mix in Neutralization Buffer.
4. Load PCR plate as per the loading list.
5. Analyze on Gyrolab Mixing CD 96 in duplicate.

Overnight incubation using Gyrolab Bioaffy CD

1. Dilute samples, positive controls and negative controls in chosen Rexxip buffer.
2. Prepare a Master Mix solution in chosen Rexxip buffer.
3. Mix each sample, positive control, and negative control in an equal volume of the Master Mix solution.
4. Load PCR plate as per the loading list.
5. Incubate overnight at +4°C without shaking.
6. Analyze using Gyrolab Bioaffy CD in duplicate.

Gyrolab instrument methods

Two Gyrolab methods for ADA analysis on Gyrolab are available for downloading from the Gyrolab User Zone if not already stored in your method database:

Table 1. ADA methods

Method Name	Description
Mixing96-1W-003-A	Homogeneous assay on Gyrolab Mixing CD 96 with acid dissociation inside the CD, designed for the ADA application
200-1W-004-A	Homogeneous assay on Gyrolab Bioaffy 200 CD without acid dissociation, designed for the ADA application

Please note that Gyrolab Control version 7.2 or higher is required to run these methods.

The methods in Table 1 use Gyrolab Wash Buffer pH 11 as a second wash solution for the needles. This procedure washes the needles with a high pH solution, which helps to prevent carry-over. This is particularly important when labeled reagents are loaded using sample needles as is the case for homogeneous bridging assays.

Gyrolab ADA Assay development

Optimizing an ADA assay differs from optimizing other assays. Instead of looking primarily at the Signal-to-Background ratio (S/B), it is more relevant to optimize for the most robust assay around the cut point, *i.e.* the level that determines whether a sample is ADA positive or ADA negative. We recommend that this is done by including the free drug at a concentration close to what can be expected in study samples (trough levels), or preferably slightly above. The presence of free drug in samples cannot normally be avoided, which means that optimizing the assay without adding free drug might be misleading and will result in an assay with sub-optimal drug tolerance. The level of drug to be used throughout this protocol should be determined based on the requirements of the study the assay aims to support.

For optimal assay development using the Gyrolab system, we propose a four-experiment approach, summarized in Table 2. The Run Designs for the ADA experiments in this protocol can be obtained from your local FAS or downloaded from Gyrolab User Zone (https://www.gyrosproteintechnologies.com/gyrolab-user-zone).

Table 2. ADA Experiment Principles

Experiment	Purpose	Aim	Expected outcome
Experiment 1	Initial Screen	To estimate sensitivity and drug tolerance, and assess blank suppression by drug levels used in the confirmatory assay Assess compatibility with acidification process	Confirm the efficacy of reagents Determine the expected range for the Master Mix concentration, which is confirmed in Experiment 2
Experiment 2	Assay optimization	Further optimization of Master Mix concentration Test sufficient blank replicates to estimate sensitivity	Finalize Master Mix concentration Determine estimated screening cut point level
Experiment 3	Cut point determination	Test sufficient number of individuals to determine the biological variability in the assay Determine suitability of ADA assay type (acid dissociation or overnight incubation)	Finalize screening correction factor and confirmatory cut point Set Assay Type for future analysis
Experiment 4	Assay characterization	Titrate drug in the presence of 100 ng/mL PC to determine where it crosses Screening Cut Point (SCP) Titrate PC to below SCP	Confirm drug tolerance level and screening assay sensitivity

Gyrolab ADA cut point evaluation

Determining the cut point is a vital part of assay validation and it is highly recommended that a floating cut point is chosen for Gyrolab ADA analysis [4]. The negative controls should be used to normalize the data in each run and a cut point factor should be determined based on the pre-study validation data (Experiment 3).

ADA Experiment 1 – Initial Screen

Aims

To perform an initial assessment of assay sensitivity and drug tolerance at two Master Mix concentrations. An assessment of non-specific inhibition caused by high concentrations of drug in the Negative Control. An acid tolerance test is also included to help troubleshoot an assay failure caused by acid denaturation of assay components.

Experimental design:

- A 5-step serial dilution of anti-drug antibody sample (PC's) in naïve serum pool is tested at:
    - Two Master Mix concentrations
    - With (Drug Spike) and without drug at, or slightly above, estimated trough levels of drug
    - Acid test for one Master Mix concentration
- Drug confirmatory test, *i.e.* two high concentrations of unlabeled drug to test for inhibition of negative control

A detailed protocol of this experiment can be found in the associated experimental sheet available in ADA Experiment 1 – Initial Screen on page 14 with suggested volumes, starting concentrations of positive control and Master Mix concentrations. A Run Design template for Experiment 1 may also be downloaded at Gyrolab User Zone.

Evaluation

- Decide at what PMT setting you will continue to evaluate your data at. Higher PMT settings, 5 and 25% are often preferential.
- Determine if the reagents perform as expected. Assess the sensitivity in the presence of drug. Note that final sensitivity will be based on the cut point calculations in Experiment 3 and will usually be at quite low S/B levels, so do not be discouraged if you do not have S/B ratios of lower than 2 at 100 ng/mL PC.
- Compare curves with and without drug to distinguish between sensitivity and drug tolerance.
- Compare curves with and without acid to assess acid tolerance.
- Determine which Master Mix delivers the best results in the presence of drug, in terms of the lowest LOD.
- If the experiment is run using both acid dissociation (Gyrolab Mixing CD 96) and overnight incubation (Gyrolab Bioaffy 200) the evaluations should be done independently to determine optimal assay conditions for each set-up, *i.e.* a higher Master Mix concentration for the acid dissociation protocol does not necessarily mean a that a higher Master Mix concentration should be used for the overnight incubation set-up.

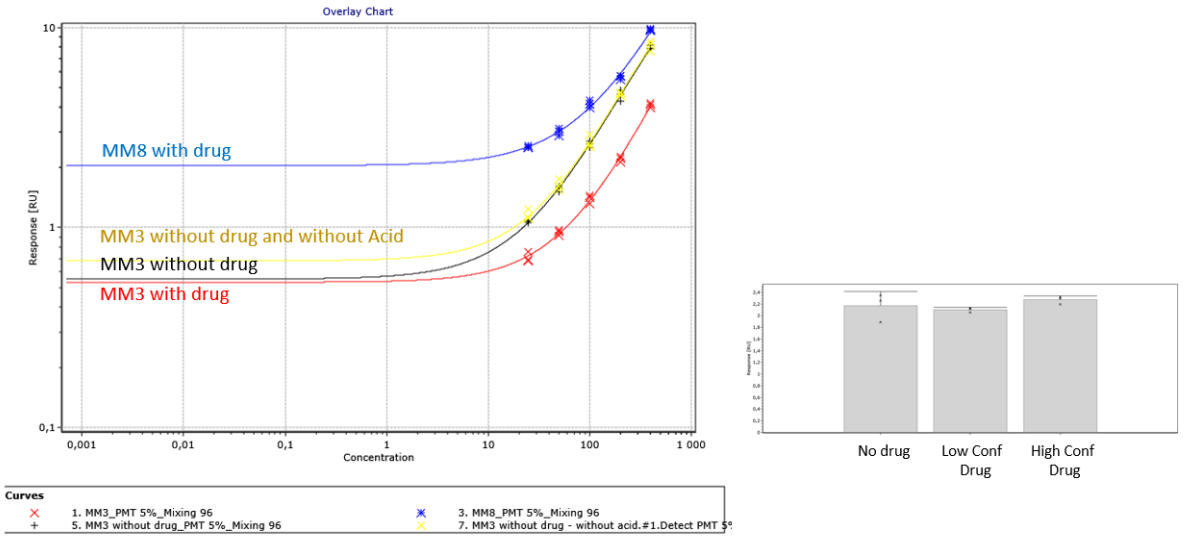


Figure 2. Example of data obtained from Experiment 1

ADA Experiment 2 – Optimization

Aims

To find the most suitable Master Mix concentration by utilizing the Limit of detection (LOD) function for each Master Mix. If required, Minimum Required Dilution may be further evaluated in this experiment by running additional matrix dilutions on parallel CDs.

Experimental design

Optimization should be performed in the presence of free drug. If a higher Master Mix concentration was more promising in the Initial Screen experiment, examine a series of higher Master Mix concentrations *e.g.* 8, 12, and 16 µg/mL. If the lower Master Mix concentration was more promising, examine a series of lower Master Mix concentrations *e.g.* 1, 2 and 3 µg/mL. A high number of blank replicates (17 replicates – sufficient to fill the CD) are included to include a determination of assay precision in the optimization. This enables both the blank precision and sensitivity to be taken into account in the optimization.

A detailed protocol of this experiment can be found in the associated experimental sheet available in ADA Experiment 2 – Optimization on page 18 with suggested volumes, starting concentrations of positive control and Master Mix concentrations. A Run Design template for Experiment 2 may also be downloaded from Gyrolab User Zone.

Evaluation

- Determine which Master Mix concentration to use based on the LOD obtained from the curve for each Master Mix concentration. The LOD should be as low as possible.
- Set the positive control concentrations to be assessed in Experiment 3. A low positive control concentration should be set at approximately three times the LOD but ≤ 100 ng/mL. If required, a high positive control can be included in subsequent experiments.

- If performed, MRD assessment should be evaluated and an MRD determined for subsequent experiments.

ADA Experiment 3 – Cut point setting

Aims

To set a screening cut point factor and confirmatory cut point using a large number of treatment-naïve individuals. This experiment will be repeated across multiple runs to ensure that sufficient individuals are assessed.

Experiment design

Use the assay parameters set in **ADA Experiment 2 – Optimization,** to test the expected biological variability of the study population. Individual serum samples are tested for ADA with and without drug.

A detailed protocol of this experiment can be found in the associated experimental sheet available in ADA Experiment 3 – Cut point determination on page 22 with suggested volumes, starting concentrations of positive control and Master Mix concentrations. A Run Design template for Experiment 3 may also be downloaded from Gyrolab User Zone.

Evaluation

- Set a floating screening cut point factor according to industry guidelines [4].
    - An example of the results obtained from a cut point run is shown in Figure 3
- Set a confirmatory cut point according to industry guidelines [5].
- An estimate of the assay sensitivity in the presence of drug can now be calculated using the standard curve from **ADA Experiment 2 – Optimization** and the correction factor set in this experiment. This will be confirmed in **ADA Experiment 4 – Performance**.

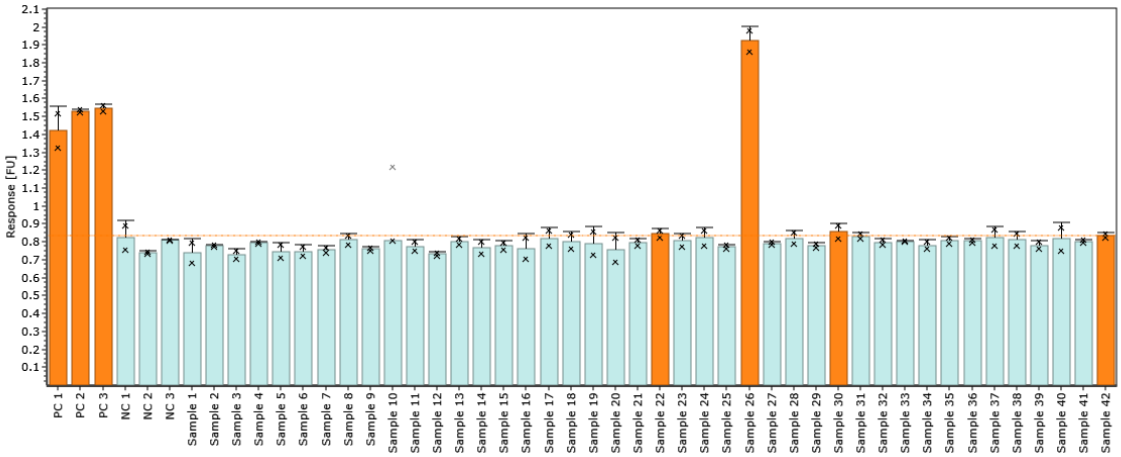


Figure 3. Example of a cut point run on Gyrolab Mixing CD 96. Samples with mean response levels above the cut point are colored orange. Sample 26 was found to be an outlier and was excluded from the cut point calculation.

ADA Experiment 4 – Performance

Aims

To calculate the maximum concentration of free drug a certain concentration of Positive Control can tolerate before being classified as negative in the screening ADA assay and to determine the minimum concentration of Positive Control required for a sample to be considered positive in the screening ADA assay.

Experimental design

Perform a dilution series of drug in the presence of known levels of Positive Control, ensuring that the levels of drug expected in associated studies are included in the drug curve. We recommend using 100 ng/mL and 800 ng/mL of positive control antibody, the latter of which is only used as a clear indicator that suppression has occurred.

Perform a dilution series of positive control with dilution steps no greater than 1 in 3, including a minimum of five dilutions.

A detailed protocol of this experiment can be found in the associated experimental sheet available in ADA Experiment 4 – Assay performance on page 25 with suggested volumes, starting concentrations of positive control and Master Mix concentrations. A Run Design template for Experiment 4 may also be downloaded from Gyrolab User Zone.

Evaluation

- Investigate the effect of high drug concentrations on the Negative Control. There may be an acceptable slight decrease in the background signals at high drug concentrations.
- Calculate the assay cut point by using the Cut Point Factor established according to industry guidelines [4] using the data from **ADA Experiment 3 – Cut point setting**
- Estimate the upper limit of drug tolerance for a given Positive Control concentration (*usually* 100 ng/mL) by identifying the **highest** concentration of drug that is **above** the screening cut point for this assay.
- Estimate the assay sensitivity by identifying the **lowest** concentration of Positive Control that is **above** the screening cut point for this assay.

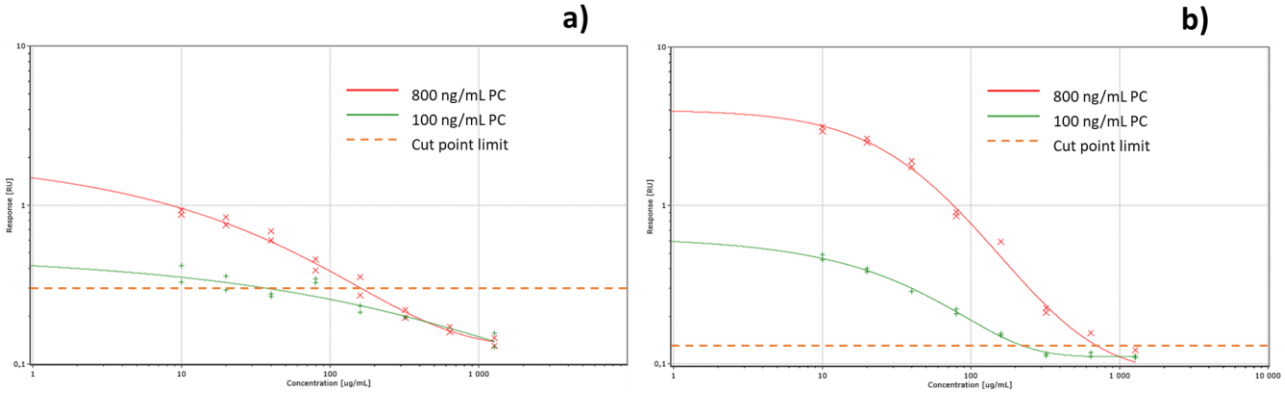


Figure 4. Example of drug tolerance for a) Bioaffy 200 CD with overnight incubation and b) Mixing CD 96 with automated acid dissociation.

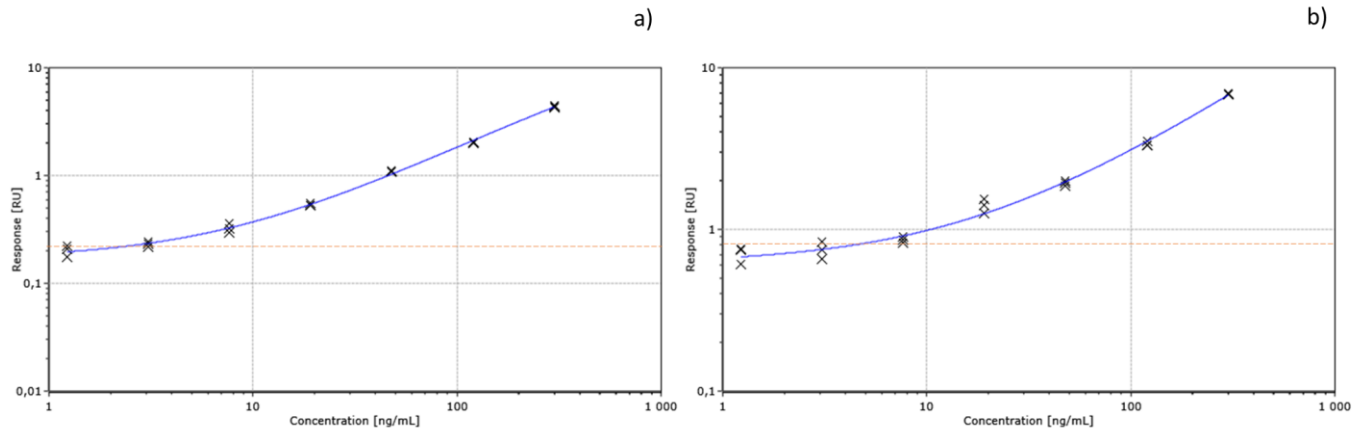


Figure 5. Example of sensitivity for a) Bioaffy 200 CD with overnight incubation and b) Mixing CD 96 with automated acid dissociation.

Further Characterization

The work performed by following this protocol is not sufficient for a formal validation of the assay. When an assay has passed through the four experiments described in this protocol, you have established that you have a working ADA assay. Here are some examples of experiments required for full validation, although it is vital that sufficient assays are performed to satisfy required regulatory guidelines:

- Hook effect (prozone effect). A homogeneous bridging assay will give lower signals at very high analyte concentrations. This is not a problem providing the assay does not give false negative responses at high ADA concentrations. Higher Master Mix concentrations will shift the hook to even higher analyte concentrations. Run a standard curve of PC starting with as high concentrations as possible to investigate the hook effect.
- Selectivity and Specificity. The assay should specifically detect anti-drug antibodies but not the drug product, soluble drug target, non-specific endogenous antibodies, or antibody reagents used in the assay. Testing of pooled matrix spiked with one or more of these should not result in a positive result. Selectivity may be determined by testing serum from naïve individuals spiked with the positive control antibody at the low positive control level, which should result in a positive result.
- Precision and Reproducibility. Results should be reproducible within and between assay runs to assure adequate precision. Demonstrating assay precision is critical to the assessment of ADA because assay variability is the basis for determining the cut points and ensuring that low positive samples are detected as positive. To provide reliable estimates, both intra-assay- (repeatability) and inter-assay (intermediate precision) variability of assay responses, should be evaluated.

References

1. Schellekens H, 2002. Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clinical Therapy 24(11), 1720-40
2. EMEA 2017. Guideline on Immunogenicity assessment of therapeutic proteins
3. FDA 2019. Guidance for Industry. Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products.
4. Shankar G, 2008. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. J Pharm Biomed Anal 48(5):1267-81
5. Jani D, 2015. Recommendations for the development and validation of confirmatory anti-drug antibody assays. Bioanalysis. 2015;7(13):1619-31. doi: 10.4155/bio.15.96. PMID: 26226311.

ADA Experiment 1 – Initial Screen

Notes before starting:

- Download the Run Designs for Experiment 1 from Gyrolab User Zone, "ADA Exp 1 – Initial Screen Mix96" and "ADA Exp 1 – Initial Screen BA200". The protocol below is for running both analyses in parallel.
    - If only performing the acid dissociation analysis using the Mixing CD 96, omit steps 11 and 13-14 and adjust the volumes for Master Mix preparations.
    - If only performing the overnight incubation assay, omit steps 10 and 12 and adjust the volumes for Master Mix preparations.
- Create Run Setups for the experiments and print the loading lists with the plate layouts
- Determine the estimated trough level concentrations of the test drug to be used in the experiment

Required materials

Required consumables	**Required Reagents**	
Gyrolab Mixing CD 96	Unlabeled drug	Rexxip ADA
Gyrolab Bioaffy 200 CD	Biotin labeled drug (capture)	Neutralization buffer
PCR Plate	Alexa labeled drug (detection)	Acidic buffer
Microplate foil	Positive Control antibody	PBS-T
General laboratory equipment	Pooled naïve serum	Wash Buffer 2 (pH 11)

Experiment design

- A serial dilution of positive control samples (PC's) in pooled serum is tested at;
    - Two Master Mix (MM) concentrations; in this protocol we will test MM3 and MM8, i.e. 3 µg/mL and 8 µg/mL
    - Testing with and without drug. Drug concentration at approx. 2x estimated trough levels of unlabeled drug (2x Drug)
    - Acid tolerance test for one Master Mix concentration
- Drug confirmatory assessment, *i.e.* two high concentrations of unlabeled drug to test for inhibition of negative control serum pool

Reagent preparations

The order of reagent additions has been optimized for the Gyrolab system and should not be deviated from without good cause.

Drug

1) Prepare High Confirmatory and Low Confirmatory unlabeled drug in **Rexxip ADA**. Both samples should contain an excess of free drug such that any ADA response would be inhibited. The concentrations used below are examples, additional drug titration experiments may be required for regulatory studies.

For example:
High Confirmatory: 1000 µg/mL
Low confirmatory: 50 µg/mL

2) Prepare 2x concentration of unlabeled drug in **serum pool** (2x Drug). The concentration should be approximately two times the trough levels of the unlabeled drug and should maintain a minimum of 95% serum.

For example:
Trough levels of test drug, estimated from literature: 5 µg/mL
Drug conc. for this experiment: 2x 5 µg/mL = 10 µg/mL
2x concentration of unlabeled drug: 2x 10 µg/mL = 20 µg/mL
Dilute 2 µL (High Confirmatory: 1000 µg/mL) in 98 µL pooled serum to reach 20 µg/mL

Negative control and confirmatory drug preparations

3) Prepare a Negative control, a high and a low confirmatory drug preparation by making a 1:10 dilution of serum pool in either Rexxip ADA, the high or the low confirmatory drug preparation from step 1, i.e. 10 µL serum pool + 90 µL Rexxip ADA or Conf. preparation.

Anti-drug antibody positive controls – Illustrated in *Figure 6*

4) Prepare an intermediate concentration of the positive control. Suggested concentration is 20 000 ng/mL in a total volume of 200 µL. This PC intermediate stock could be retained, stored refrigerated, and used throughout the experiment series.

5) Prepare a 2x concentrations titration curve of ADA positive controls (PC's) in serum pool. Suggested 2x concentrations of PC's are 800, 400, 200, 100 and 50 ng/mL.

For example, a dilution schedule when using an intermediate stock concentration of 20 µg/mL:

ID	Concentration	Volume	Stock to use	Volume pooled serum	Total volume
2 x PC_1	800 ng/mL	4 µL	PC Intermediate Stock (20 µg/mL)	96 µL	100 µL
2 x PC_2	400 ng/mL	45 µL	2 x PC_1	45 µL	90 µL
2 x PC_3	200 ng/mL	45 µL	2 x PC_2	45 µL	90 µL
2 x PC_4	100 ng/mL	45 µL	2 x PC_3	45 µL	90 µL
2 x PC_5	50 ng/mL	45 µL	2 x PC_4	45 µL	90 µL
2x PC_Blank	0 ng/mL	N/A		45 µL	45 µL

6) Dilute the 2x ADA PC dilution series 1:2 (15 µL + 15 µL) in either serum pool or 2x drug in serum (prepared in step 2) to prepare 1x ADA PC curves with and without drug.

7) Incubate for approximately 1 hour at room temperature to allow the formation of ADA complexes.

8) Dilute all ADA PC's 1:10 (5 µL + 45 µL) in Rexxip ADA.

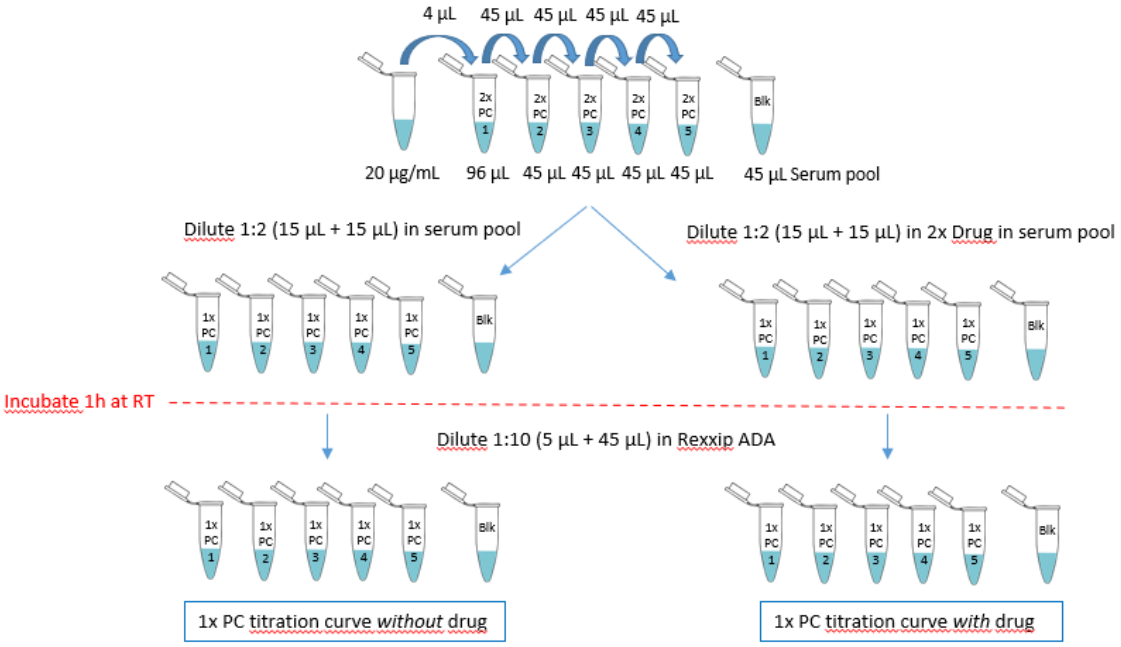


Figure 6. Schematic overview of preparation of positive control titration curve.

Master Mix preparations and plate preparation – Illustrated in *Figure 7*

9) Prepare 150 µL 2x of Low and High Master Mix (MM) concentrations (containing equimolar amounts of biotinylated drug and Alexa labeled drug) in Rexxip ADA. Suggested concentration for Low MM is 3 µg/mL and for High MM, 8 µg/mL.

   For Example:
   Stock Biotinylated drug: 150 µg/mL
   Stock Alexa labeled drug: 150 µg/mL
   For antibody therapeutic drugs, 150 µg/mL equals 1000 nM. Following volumes are for both acid dissociation analysis on Mixing CD 96 and overnight incubation analysis on BA200 CD.

ID	Concentration	Biotinylated Drug volume	Alexa labeled Drug volume	Volume of Rexxip ADA	Total Volume
2 x MM3	6 µg/mL	6.0 µL	6.0 µL	138 µL	150 µL
2 x MM8	16 µg/mL	16 µL	16 µL	118 µL	150 µL

10) Dilute each Master Mix concentration 1:2 (50 µL + 50 µL) in 2M Tris-HCl pH 8 (Neutralization buffer). ***This will be used for acid dissociation analysis on the Mixing CD 96***.

11) Dilute each Master Mix concentration 1:2 (90 µL + 90 µL) in Rexxip ADA. ***This will be used for overnight incubation and analysis using the Bioaffy 200 CD.***

12) **For acid dissociation analysis.** Pipet samples and reagents to a Microtiter plate according to the plate layout in the Gyrolab loading list for experiment “ADA Exp 1 – Initial Screen Mix96” and run the assay accordingly.

13) **For overnight incubation.** Mix 10 µL of the 1x PC titration curve with 10 µL 1 x MM of each concentration on a non-binding plate according to the Gyrolab loading list for the experiment “ADA Exp 1 - Initial Screen BA200”. Cover with plate foil.

14) Incubate plate overnight at +4°C (nominal), ***without shaking*** and run the assay accordingly.

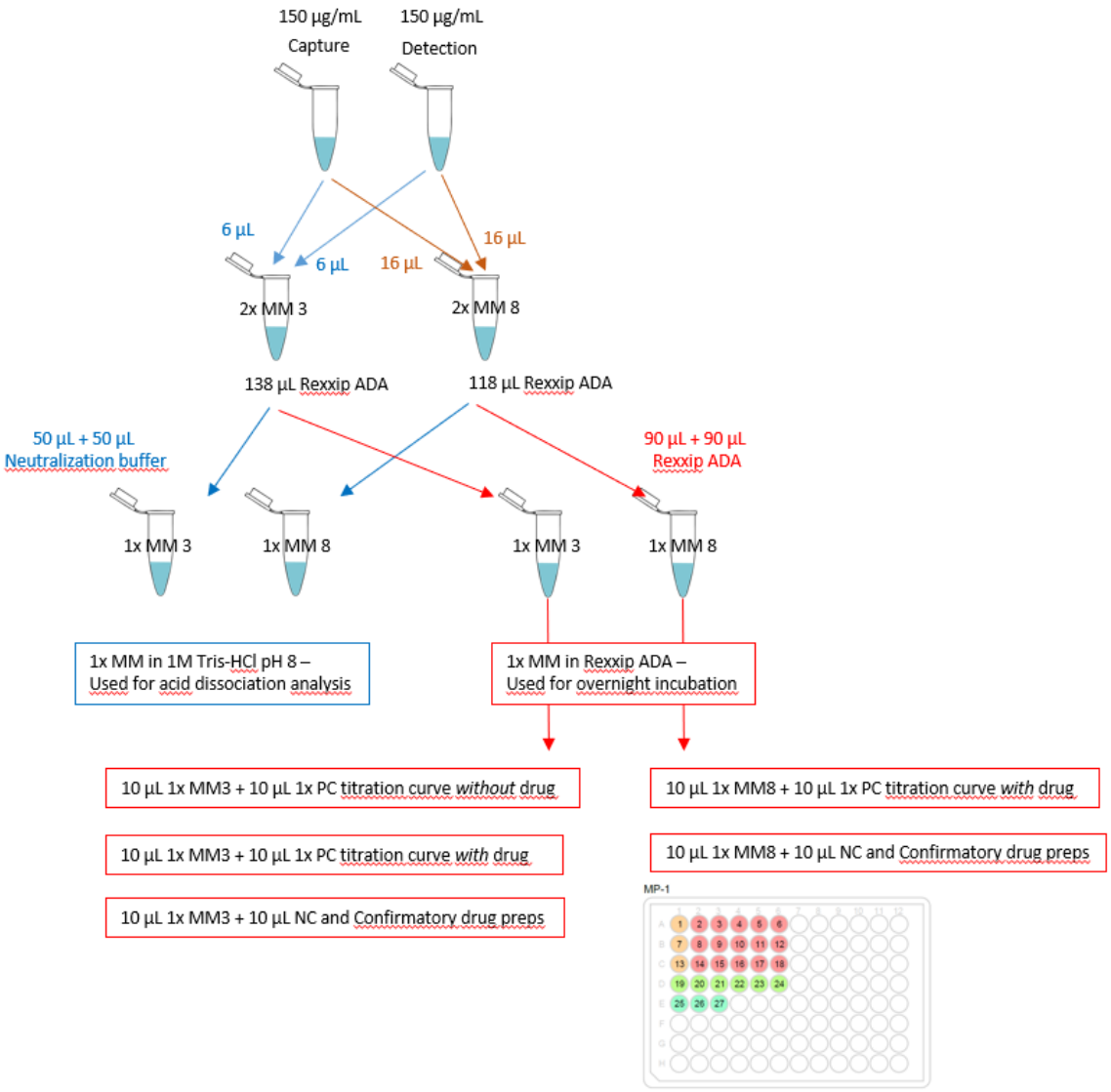


Figure 7. Schematic overview of steps 9-13.

ADA Experiment 2 – Optimization

Notes before starting:

- Download the Run Designs for Experiment 2 from Gyrolab User Zone, "ADA Exp 2 – Optimization Mix96" and "ADA Exp 2 – Optimization BA200". The protocol below is for running both experiments in parallel.
    - If only performing the acid dissociation analysis using the Mixing CD 96, omit steps 8 and 10-11 and adjust the volumes for Master Mix preparations.
    - If only performing the overnight incubation assay, omit steps 7 and 9 and adjust the volumes for Master Mix preparations.
- Create Run Setups for the experiments and print the loading lists with the plate layouts.

Required materials

Required consumables	**Required Reagents**	
Gyrolab Mixing CD 96	Unlabeled drug	Rexxip ADA
Gyrolab Bioaffy 200 CD	Biotin labeled drug (capture)	Neutralization buffer
PCR Plate	Alexa labeled drug (detection)	Acidic buffer
Microplate foil	Positive Control antibody	PBS-T
General laboratory equipment	Pooled naïve serum	Wash Buffer 2 (pH11)

Experiment design

- A serial dilution of positive control samples (PC's) in serum pool is tested at;
    - Three Master Mix concentrations; High, Mid and Low. The concentrations are determined from the best outcome in Experiment 1
    - Testing in the presence of drug. Drug concentration at approx. 2x estimated trough levels of unlabeled drug (2x Drug)
- 17 blank replicates for LOD assessment

Reagent preparations

The order of reagent additions has been optimized for the Gyrolab system and should not be deviated from without good cause.

Drug

1) Prepare 2x concentration of unlabeled drug in **serum pool** (2x Drug). The concentration should be approximately two times the through levels of the unlabeled drug and should maintain a minimum of 95% serum. A total volume of 200 µL should be sufficient for the experiment.

    For example:
    Trough levels of test drug, estimated from literature: 5 µg/mL
    Drug conc. for this experiment: 2x 5 µg/mL = 10 µg/mL
    2x concentration of unlabeled drug: 2x 10 µg/mL = 20 µg/mL

Anti-drug antibody positive controls (PC) – Illustrated in *Figure 8*

2) Prepare an intermediate concentration of the positive control, suggested concentration of 20 000 ng/mL, or use the intermediate positive control preparation retained from Experiment 1.

3) Prepare 2x concentrations titration curve of ADA positive controls (PC's) in serum pool. Suggested 2x concentrations of PC's are 800, 400, 200, 100 and 50 ng/mL.

For example, a dilution schedule when using an intermediate stock concentration of 20 µg/mL:

ID	Concentration	Volume	Stock to use	Volume pooled serum	Total volume
2 x PC_1	800 ng/mL	4 µL	PC Intermediate Stock (20 µg/mL)	96 µL	100 µL
2 x PC_2	400 ng/mL	45 µL	2 x PC_1	45 µL	90 µL
2 x PC_3	200 ng/mL	45 µL	2 x PC_2	45 µL	90 µL
2 x PC_4	100 ng/mL	45 µL	2 x PC_3	45 µL	90 µL
2 x PC_5	50 ng/mL	45 µL	2 x PC_4	45 µL	90 µL
2x PC_Blank	0 ng/mL	N/A		45 µL	45 µL

4) Dilute the 2x ADA PC dilution series 1:2 (15 µL + 15 µL) in 2x drug (prepared in step 1) to prepare 1x ADA PC curve.

5) Incubate for approximately 1 hour at room temperature to allow the formation of ADA complexes.

6) Dilute all ADA PC's 1:10 (5 µL + 45 µL) in Rexxip ADA.

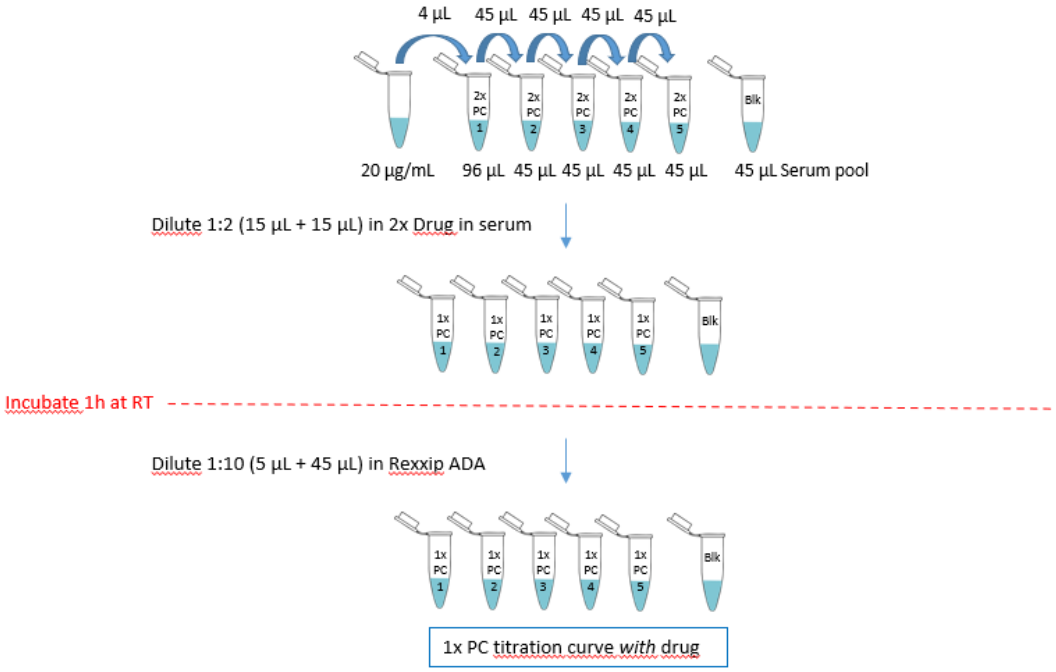


Figure 8. Schematic overview of steps 3-6, preparation of positive control titration curve with drug.

Master Mix and plate preparations – Illustrated in *Figure 9*

7) Prepare 100 µL 2x of the Master Mix (MM) concentrations (containing equimolar amounts of biotinylated drug and Alexa labeled drug). For example, if the 3 µg/mL MM concentration was deemed to look most promising in Experiment 1 we suggest testing the MM concentrations 1 µg/mL, 2 µg/mL and 3 µg/mL in this experiment.

   For Example:
   Stock Biotinylated drug: 150 µg/mL
   Stock Alexa labeled drug: 150 µg/mL
   For antibody therapeutic drugs, 150 µg/mL equals 1000 nM. Following volumes are for both acid dissociation analysis on Mixing CD 96 and overnight incubation analysis on BA200 CD.

ID	Concentration	Biotinylated Drug volume	Alexa labeled Drug volume	Volume of Rexxip ADA	Total Volume
2 x MM Low	2 µg/mL	1.3 µL	1.3 µL	97.3 µL	100 µL
2 x MM Mid	4 µg/mL	2.7 µL	2.7 µL	94.7 µL	100 µL
2 x MM High	6 µg/mL	4.0 µL	4.0 µL	92.0 µL	100 µL

8) Dilute each Master Mix concentration 1:2 (25 µl + 25 µl) in 2M Tris-HCl pH 8 (Neutralization buffer). ***This will be used for acid dissociation analysis on the Mixing CD 96***.

9) Dilute each Master Mix concentration 1:2 (60 µl + 60 µl) in Rexxip ADA. ***This will be used for overnight incubation and analysis using the Bioaffy 200 CD.***

10) **For acid dissociation analysis.** Pipet samples and reagents to a Microtiter plate according to the plate layout in the Gyrolab loading list for experiment “ADA Exp 2 – Assay optimization Mix96” and run the assay accordingly.

11) **For overnight incubation.** Mix 10 µL of the 1x PC titration curve with 10 µL 1 x MM of each concentration on a non-binding plate according to the Gyrolab loading list for the experiment “ADA Exp 2 – Assay optimization BA200”. Cover with plate foil.

12) Incubate plate overnight at +4°C (nominal), ***without shaking*** and run the assay accordingly.

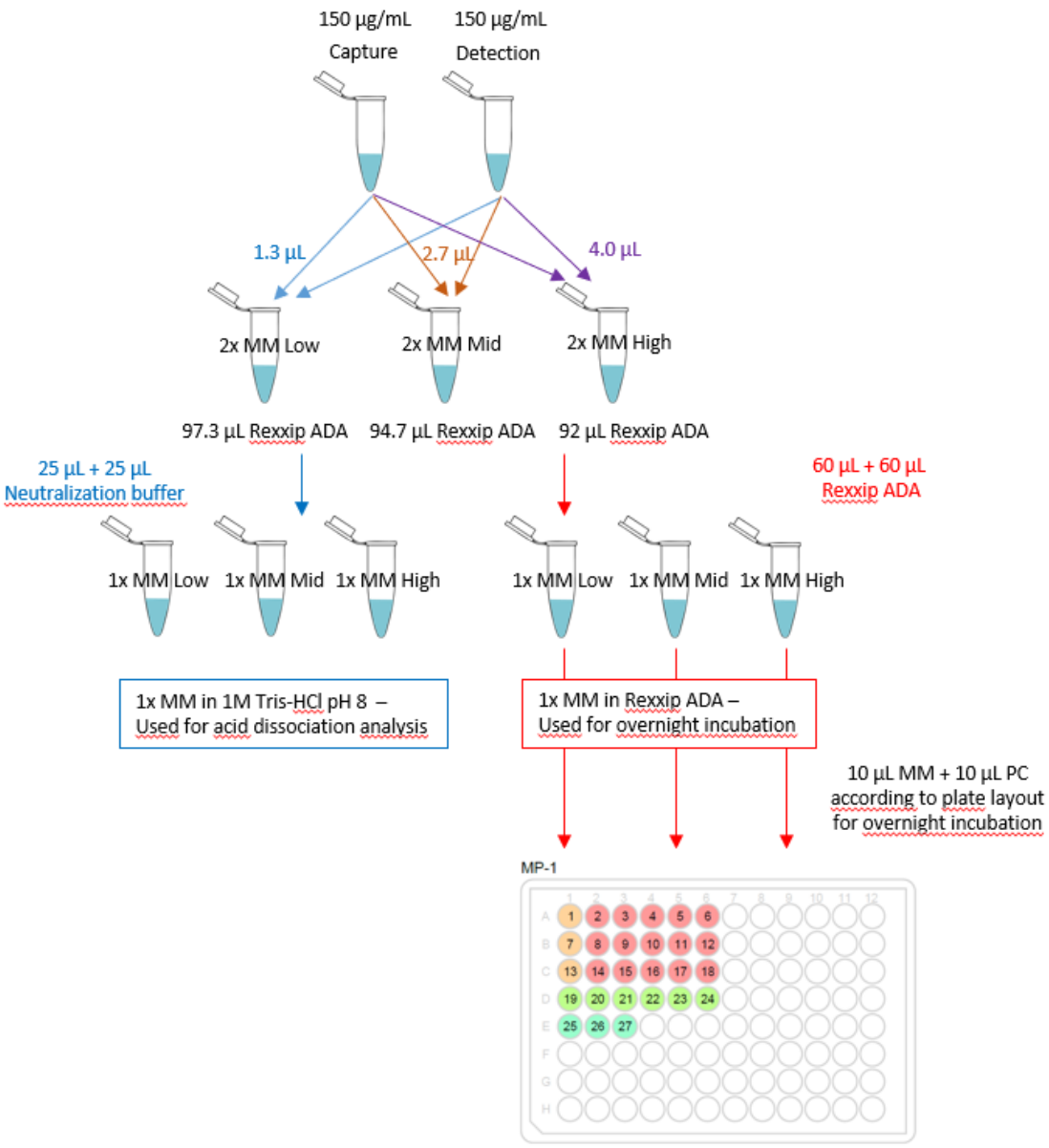


Figure 9. Schematic overview of steps 7-11.

ADA Experiment 3 – Cut point determination

Notes before starting:

- Download the Run Designs for Experiment 3 from Gyrolab User Zone, "ADA Exp 3 – Cut point Mix96" and "ADA Exp 2 – Cut point BA200". The protocol below is for running both experiments in parallel.
    - If only performing the acid dissociation analysis using the Mixing CD 96, omit steps 8 and 10-11 and adjust the volumes for Master Mix preparation.
        - One Mixing CD 96 analyzes 18 individual serum samples with and without confirmatory drug, *i.e.* running the experiment three times will yield results from 54 individuals.
    - If only performing the overnight incubation assay, omit steps 7 and 9 and adjust the volumes for Master Mix preparation.
        - One Gyrolab Bioaffy 200 CD analyzes 21 individual serum samples with and without confirmatory drug, *i.e.* running the experiment three times will yield results from 63 individuals.
- Decide what concentrations the positive controls should have. For help choosing positive control concentrations, please see the evaluation tips from Experiment 2 in the main document.
- Set confirmatory drug concentration based on expectation of inhibition of positive controls.
- Create Run Setups for the experiments and print the loading lists with the plate layouts.

Required materials

Required consumables	**Required Reagents**	
Gyrolab Mixing CD 96	Unlabeled drug	Rexxip ADA
Gyrolab Bioaffy 200 CD	Biotin labeled drug (capture)	Neutralization buffer
PCR Plate	Alexa labeled drug (detection)	Acidic buffer
Microplate foil	Positive Control antibody	PBS-T
General laboratory equipment	Pooled naïve serum	Wash Buffer 2 (pH11)
N/A	Individual serum samples	N/A

Experiment design

- Sufficient number of individual serum samples, three positive control and three negative control samples are tested with and without confirmatory drug

Reagent preparations

Anti-drug antibody positive controls (PC)

1) Prepare an intermediate concentration of the positive control, suggested concentration of 20 000 ng/mL, or use the intermediate positive control preparation retained from Experiment 1.

2) Prepare three positive control samples (PC1-PC3) in pooled serum, *e.g.* two Low positive controls and one High positive control, concentrations depending on the results from previous experiment.

   For negative controls, pipet pooled serum into three individual tubes, labelled NC1-NC3.

Confirmatory buffer preparation

3) Prepare 1200 µL of unlabeled drug in Rexxip ADA at the desired confirmatory buffer concentration.

Sample dilutions

4) Dilute 5 µL of the individual serum samples, the PC1-PC3 and the NC1-NC3 in 45 µL Rexxip ADA buffer. These individual samples and controls are set up as the *unspiked* samples in the Run Setup.

5) Dilute 5 µL of the individual serum samples, the PC1-PC3 and the NC1-NC3 in 45 µL Confirmatory buffer. These individual samples and controls are set up as the *spiked* samples in the Run Setup.

Master Mix and plate preparations

6) Prepare 200 µL 2x Master Mix (MM) concentration (containing equimolar amounts of biotinylated drug and Alexa labeled drug).

   For Example:
   Final MM concentration: 3 µg/mL
   Stock Biotinylated drug: 150 µg/mL
   Stock Alexa labeled drug: 150 µg/mL
   For antibody therapeutic drugs, 150 µg/mL equals 1000 nM. Following volumes are for both acid dissociation analysis on Mixing CD 96 and overnight incubation analysis on BA200 CD.

ID	Concentration	Biotinylated Drug volume	Alexa labeled Drug volume	Volume of Rexxip ADA	Total Volume
2 x MM	6 µg/mL	8.0 µL	8.0 µL	184 µL	200 µL

7) Dilute the 2x Master Mix 1:2 (50 µL + 50 µL) in 2M Tris-HCl pH 8 (Neutralization buffer) for a 1x Master Mix. ***This will be used for acid dissociation analysis on the Mixing CD 96***.

8) Dilute the 2x Master Mix 1:2 (140 µL + 140 µL) in Rexxip ADA for a 1x Master Mix. ***This will be used for overnight incubation and analysis using the Bioaffy 200 CD.***

9) **For acid dissociation analysis.** Pipet diluted samples, PC's, NC's and reagents to a Microtiter plate according to the plate layout in the Gyrolab loading list for experiment "ADA Exp 3 – Cut point Mix96" and run the assay accordingly.

10) **For overnight incubation.** Mix 10 µL of each diluted sample, PC's and NC's with 10 µL 1 x MM on a non-binding plate according to the Gyrolab loading list for the experiment "ADA Exp 3 – Cut point BA200". Cover with plate foil.

11) Incubate plate overnight at +4°C (nominal), ***without shaking*** and run the assay accordingly.

ADA Experiment 4 – Assay performance

Notes before starting:

- Download the Run Designs for Experiment 4 from Gyrolab User Zone, "ADA Exp 4 – Performance Mix96" and "ADA Exp 4 – Performance BA200". The protocol below is for running both experiments in parallel
    - If only performing the acid dissociation analysis using the Mixing CD 96, omit steps 8 and 10-11 and adjust the volumes for Master Mix preparations
    - If only performing the overnight incubation assay, omit steps 7 and 9 and adjust the volumes for Master Mix preparations
- Decide at what increments and concentration range the positive control titration curve should include based on the results from Experiment 2, *i.e.* if the results indicates that the sensitivity of the assay is around 20 ng/mL, choose to make smaller increments around this concentration
- Update the pre-set concentrations in the Run Designs if they are changed from the examples used in this protocol
- Create Run Setups for the experiments and print the loading lists with the plate layouts.
- If desired, download the protocol as an excel sheet with example calculations from Gyrolab User Zone

Required materials

Required consumables	**Required Reagents**	
Gyrolab Mixing CD 96	Unlabeled drug	Rexxip ADA
Gyrolab Bioaffy 200 CD	Biotin labeled drug (capture)	Neutralization buffer
PCR Plate	Alexa labeled drug (detection)	Acidic buffer
Microplate foil	Positive Control antibody	PBS-T
General laboratory equipment	Pooled naïve serum	Wash Buffer 2 (pH11)

Experiment design

- Drug tolerance. A dilution series of unlabeled drug in pooled serum is tested at;
    - Two concentrations, 100 ng/mL and 800 ng/mL, of the positive control antibody. 800 ng/mL is only included as a clear indicator that suppression has occurred
    - Without addition of positive control
- Sensitivity assessment. A dilution series of positive control samples in pooled serum

Reagent preparations

Drug tolerance (DT) curve

1) Prepare a 2x concentration dilution series of unlabeled drug in pooled serum. Suggested 2x concentrations are 2560, 1280, 640, 320, 160, 80, 40, 20 and 0 µg/mL.

For example, a dilution scheme when using a stock concentration of 10 mg/mL of unlabeled drug:

ID	Concentration	Volume	Stock used	Volume pooled serum	Total volume
2 x DT_1	2560 ng/mL	23 µL	Stock (10 mg/mL)	67 µL	90 µL
2 x DT_2	1280 ng/mL	45 µL	2 x DT_1	45 µL	90 µL
2 x DT_3	640 ng/mL	45 µL	2 x DT_2	45 µL	90 µL
2 x DT_4	320 ng/mL	45 µL	2 x DT_3	45 µL	90 µL
2 x DT_5	160 ng/mL	45 µL	2 x DT_4	45 µL	90 µL
2 x DT_6	80 ng/mL	45 µL	2 x DT_5	45 µL	90 µL
2 x DT_7	40 ng/mL	45 µL	2 x DT_6	45 µL	90 µL
2 x DT_8	20 ng/mL	45 µL	2 x DT_7	45 µL	90 µL
Blank	0 ng/mL	N/A		45 µL	45 µL

Anti-drug antibody positive controls (PC)

2) Prepare an intermediate concentration of the positive control, suggested concentration of 20 000 ng/mL, or use the intermediate positive control preparation retained from Experiment 1.

3) **For drug tolerance assessment**. Prepare 200 µL of 100 ng/mL and 800 ng/mL of positive control (PC) antibody at 2x concentration in serum pool.

For example, a dilution schedule when using an intermediate stock concentration of 20 µg/mL:

ID	Concentration	Volume	Stock used	Volume pooled serum	Total volume
2 x PC 800 ng/mL	1600 ng/mL	20 µL	PC Intermediate Stock (20 µg/mL)	230 µL	250 µL
2 x PC 100 ng/mL	200 ng/mL	25 µL	2 x 800 ng/mL	175 µL	200 µL
2 x PC 0 ng/mL	0 ng/mL	N/A		200 µL	200 µL

4) Dilute the 2x unlabeled drug DT curve 1:2 (10 µl + 10 µl) in either 2x PC 800 ng/mL, 2x PC 100 ng/mL or 2x PC 0 ng/mL to obtain 1x DT curves.

5) Incubate for approximately 1 hour at room temperature to allow the formation of ADA complexes.

6) **For sensitivity assessment**. Prepare a dilution series of positive controls (PC's) in pooled serum.

For example, a dilution schedule when using an intermediate stock concentration of 20 µg/mL:

ID	Concentration	Volume	Stock Used	Volume pooled serum	Total volume
Intermediate	1000 ng/mL	5 µL	PC Intermediate Stock (20 µg/mL)	95 µL	100 µL
PC_1	200 ng/mL	16 µL	Intermediate	64 µL	80 µL
PC_2	100 ng/mL	40 µL	PC_1	40 µL	80 µL
PC_3	50 ng/mL	40 µL	PC_2	40 µL	80 µL
PC_4	25 ng/mL	40 µL	PC_3	40 µL	80 µL
PC_5	12.5 ng/mL	40 µL	PC_4	40 µL	80 µL
PC_6	6.3 ng/mL	40 µL	PC_5	40 µL	80 µL
PC_7	3.1 ng/mL	40 µL	PC_6	40 µL	80 µL
Blank	0 ng/mL	N/A		40 µL	40 µL

7) Dilute the DT curves and the sensitivity curve 1:10 (5 µL + 45 µL) in Rexxip ADA.

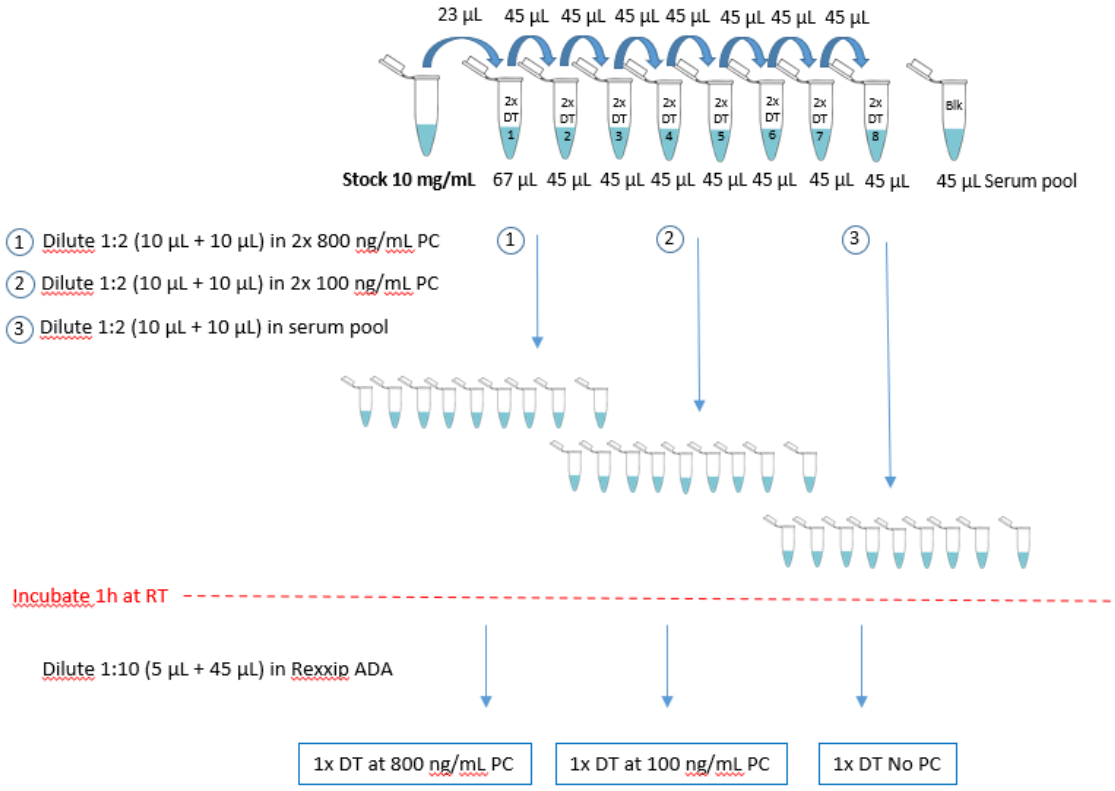


Figure 10. Schematic overview of drug tolerance curve preparations, step 1, 3-5 and 7.

Master Mix and plate preparations

8) Prepare 250 µL 2x Master Mix (MM) concentration (containing equimolar amounts of biotinylated drug and Alexa labeled drug).

For Example:
Final MM concentration: 3 µg/mL
Stock Biotinylated drug: 150 µg/mL

Stock Alexa labeled drug: 150 µg/mL
For antibody therapeutic drugs, 150 µg/mL equals 1000 nM. Following volumes are for both acid dissociation analysis on Mixing CD 96 and overnight incubation analysis on BA200 CD.

ID	Concentration	Biotinylated Drug volume	Alexa labeled Drug volume	Volume of Rexxip ADA	Total Volume
2 x MM	6 µg/mL	10 µL	10 µL	230 µL	250 µL

9) Dilute the 2x Master Mix 1:2 (50 µL + 50 µL) in 2M Tris-HCl pH 8 (Neutralization buffer) for a 1x Master Mix. ***This will be used for acid dissociation analysis on the Mixing CD 96***.

10) Dilute the 2x Master Mix 1:2 (190 µL + 190 µL) in Rexxip ADA for a 1x Master Mix. ***This will be used for overnight incubation and analysis using the Bioaffy 200 CD.***

11) **For acid dissociation analysis.** Pipet diluted DT curves, positive control titration curve and reagents to a Microtiter plate according to the plate layout in the Gyrolab loading list for experiment "ADA Exp 4 – Assay performance Mix96" and run the assay accordingly.

12) **For overnight incubation.** Mix 10 µL of each diluted sample with 10 µL 1 x MM on a non-binding plate according to the Gyrolab loading list for the experiment "ADA Exp 4 – Assay optimization BA200". Cover with plate foil.

13) Incubate plate overnight at +4°C (nominal), ***without shaking*** and run the assay accordingly.

Patents and disclaimer