Bridging Immunogenicity Assays

Guidelines for Assay Development

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Introduction

MSD offers a range of assay development materials and kits suitable for the implementation of immunogenicity assays, neutralizing antibody assays (ligand binding or cell-based), and pharmacokinetics (PK) assays. The MESO SCALE DISCOVERY® platform, based on electrochemiluminescence, provides excellent sensitivity, a large dynamic range, and flexibility. Immunogenicity assays to detect anti-drug antibodies can be developed using MSD® technology for many biotherapeutics including antibodies, antibody-drug conjugates, proteins, and peptides. Detection of anti-drug antibodies using the MSD bridging format follows a simple protocol with a homogenous solution phase incubation step, while direct assays afford a streamlined protocol with a reduced number of washes as compared to ELISA. The MSD bridging format enables detection of low affinity anti-drug antibodies because only one wash step is required. The development of MSD bridging immunogenicity assays is rapid, and these assays can be adapted to tolerate the presence of drug in test samples. The assays do not require any species-specific reagents and are thus independent of species and isotype.

Immunogenicity testing for anti-drug antibodies (ADAs) typically follows a three-tiered approach. The screening assay (Tier 1) detects antibodies that arise as a result of an immune response to the therapeutic drug. Samples that test positive in the screening assay are evaluated in a confirmatory assay (Tier 2) to verify that the response is specific to the drug. Confirmed positive samples then undergo characterization assays (Tier 3) to evaluate characteristics such as antibody titer, isotype, and whether the antibodies are neutralizing.

This guide provides recommendations for the development of Tier 1 assays to screen for anti-drug antibodies using a bridging assay format (shown in Figure 1). It provides lists of reagents, reagent preparation, protocols, suggested plate layouts, and sample data. Additionally, guidance is provided to optimize the bridging assay format to enhance the drug tolerance of the assay using acid dissociation. Assay troubleshooting information and a discussion section are provided at the end of the guide.

> **i** While this guide was written for measuring anti-drug antibodies, similar techniques and protocols are used to measure circulating levels of antibodies against endogenous proteins. Examples of auto-antibody detection via bridging assay are provided for anti-IL-17[16], anti-insulin[17], and anti-BARD1[18].

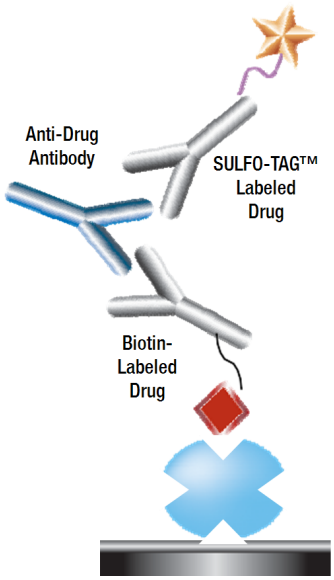

Figure 1. *Bridging Immunogenicity Assay on MSD streptavidin plates.*

Reagents and Equipment

Order the following reagents from MSD:

Reagent or Equipment	Product ID	Note
MSD GOLD™ SULFO-TAG NHS-Ester Conjugation Pack	R31AA-1 R31AA-2	Sufficient for conjugating 5 × 200 µg of IgG Sufficient for conjugating 5 × 1 mg of IgG
MSD 1-spot 96-well Streptavidin plates	SECTOR™ plates: L15SA QuickPlex® plates: L55SA QuickPlex Ultra™ plates: L4BLA	Compatible with S600 and SQ120 Compatible with SQ120 and Q60 Compatible with Q60
MSD GOLD Read Buffer B	R60AM	
MSD Blocker A	R93BA	

Procure the following reagents and equipment:

Reagent or Equipment	Description
Unlabeled Drug	Drug concentration should be ≥ 1–2 mg/mL in a buffer free of primary amines and free of carrier proteins
Biotin Labeled Drug	If the drug is not biotinylated, use biotinylation reagents such as: EZ-Link SULFO-NHS-LC-Biotin (Thermo Scientific: 100 mg 21335, 10 x 1 mg A39257) See *Biotin Conjugation* on page 8
SULFO-TAG™ Conjugated Drug	If the drug does not have a SULFO-TAG label, use kits such as the MSD GOLD SULFO-TAG NHS-Ester Conjugation Pack (R31AA) See *SULFO-TAG Conjugation* on page 7
Positive control anti-drug antibody(ies)	Purified antibody(ies) specific for the drug
Polypropylene plates or tubes	Suitable examples: • Millipore Sigma: CLS3365 • Corning: 3365 • Millipore Sigma: P6866 • Nunc: 267245 • Greiner: 650201 or 651201 Do not use polystyrene plates.
Biotin Quantification Kit	Suitable examples: • Pierce: EZ Biotin Quantification Kit • Invitrogen FluoReporter Biotin Quantitation Assay Kit
Base Buffer	PBS or other suitable base buffer
Wash Buffer	PBS + 0.05% Tween-20 or other suitable wash buffer
300 mM Acetic Acid	Only if using acid dissociation protocol

Reagent or Equipment	Description
Neutralizing solution	1.5M Trizma Base pH 10 or other suitable neutralizing solution, for example, 1M Tris-HCl pH 9.5–10 Only if using acid dissociation protocol
Sample Library /Cluster polypropylene tubes (1.1 mL – 1.2 mL) and compatible rack	Suitable examples: • VWR: 93000-042 • Corning: 4413
Adhesive plate seals	General laboratory vendor
Microtiter plate shaker	Capable of shaking a microtiter plate at 500–1000 rpm

Safety

Use safe laboratory practices. Wear appropriate personal protective equipment, including gloves, safety glasses, and lab coats, when handling assay components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional product-specific safety information is available in the applicable safety data sheet(s) (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Preparation of Reagents

Preparation of SULFO-TAG and Biotin-Labeled Drug

The bridging assay format requires the drug to be labeled with biotin and labeled with SULFO-TAG. Biotinylated drug serves as the capture molecule and the drug labeled with SULFO-TAG is the reporter in the bridging assay. If preparation of SULFO-TAG labeled or biotin-labeled drug is required, see recommendations below.

SULFO-TAG Conjugation

If the drug requires SULFO-TAG conjugation, label the drug following the protocol outlined in the MSD SULFO-TAG NHS-Ester Technical Note, found at www.mesoscale.com using the recommendations specific to immunogenicity assays outlined below.

> MSD also offers custom conjugation services for large scale conjugations of reagents with biotin or SULFO-TAG.

Topic	Description
Antibody drugs	For antibody drugs, the suggested starting conjugation ratio is 10:1.
Non-antibody drugs	Lower challenge ratios may be required for lower molecular weight drugs.
Measure SULFO-TAG incorporation	Measure SULFO-TAG incorporation with absorbance at 455 nm, and measure protein concentration with a BCA assay using an IgG standard instead of a bovine serum albumin (BSA) standard when determining concentration of antibody therapeutics, or use another suitable protein quantitation assay. The protein concentration of SULFO-TAG labeled drug should not be determined from absorbance at 280 nm as SULFO-TAG has light absorption at this wavelength.
Incorporation ratios	Incorporation ratios of 2-6 SULFO-TAG molecules per protein are sufficient to generate good signal levels and reduce the possibility of masking antigenic sites in immunogenicity assays. Lower incorporation ratios of SULFO-TAG will yield lower background and lower signals.
Minimum drug concentration	Minimum drug concentration for good incorporation of SULFO-TAG is 1 mg/mL.
Drug requirements	For preliminary experiments, obtain at least 50 to 100 µL of drug at 1–2 mg/mL in PBS or HEPES buffered saline, pH 7.4–7.9, free of any carrier proteins and without Tris, glycine, histidine, sodium azide, or glycerol.
Dark storage environment	Store SULFO-TAG labeled drug in a dark environment for longer term stability.
Buffer exchange	To ensure long-term stability, buffer exchange the labeled drug into formulation buffer. Labeled drug can often be stored under the same conditions as those optimized for long-term stability of unlabeled drug.

Biotin Conjugation

If the drug is not biotinylated, use biotinylation reagents such as EZ-Link SULFO-NHS-LC-Biotin (Thermo Scientific: 100 mg 21335, 10 x 1 mg A39257).

Follow the protocol provided by the manufacturer using the recommendations specific to immunogenicity assays outlined below.

Topic	Description
Starting conjugation ratios	Typical starting conjugation ratio is 10:1 biotin:drug. The efficiency of biotinylation will depend on the number of available lysine residues, the concentration of the drug, and the pH of the labeling reaction.
Biotin labels per drug	Unlike many other applications, only a few biotin labels per drug molecule are required in this format. Typically, 2-4 biotins per drug are sufficient. Too many incorporated biotins or no biotin incorporation could lead to reduced performance.
Purify biotin-labeled drug	Biotin-labeled drug should be completely purified from unincorporated biotin; desalting columns or extensive dialysis are suitable for this purpose. Buffer exchange using microconcentrators can also be used to remove unincorporated biotin.
Measure biotin incorporation	The degree of biotin incorporation can be measured with commercial assays such as a HABA assay, Pierce EZ Biotin Quantitation Kit, or Invitrogen FluoReporter Biotin Quantitation Assay Kit.

Plate Types

Select a plate type that is compatible with your MSD Instrument (see *Reagents and Equipment* on page 5). We recommend one of the following plate types:

- MSD GOLD 96-well 1-Spot Streptavidin SECTOR (L15SA) plates
- MSD GOLD 96-well 1-Spot Streptavidin QuickPlex (L55SA) plates
- MSD 96-well 1-Spot Streptavidin QuickPlex Ultra (L4BLA) plates

Blocking Solution

The choice of blocking solution will be driven by assay performance in the initial experiments. A good starting point is the following:

- 3% (w/v) MSD Blocker A in PBS-T or PBS.

  Weigh out 1.5 g MSD Blocker A and add PBS or PBS-T to 50 mL. Mix by gentle inversion until dissolved, filter sterilize, and store at 4˚C. Discard after 14 days.

Other blocking solutions that can be tested include:

- 1% MSD Blocker B
- Casein blocker

IMPORTANT

Solutions containing biotin should be avoided as free biotin can interfere with the binding of biotinylated reagents to streptavidin- and avidin-coated plates. Examples of solutions containing biotin are milk-based blockers and certain cell culture media (particularly RPMI 1640 media).

Assay Diluent

Prepare a solution of 1% (w/v) MSD Blocker A in PBS-T or PBS. For example, combine 10 mL 3% (w/v) Blocker A blocking solution and 20 mL PBS or PBS-T. Alternative assay diluents can also be tested if required.

Read Buffer

We recommend MSD GOLD Read Buffer B. This buffer is provided ready to use. Do not dilute.

Experiments

This section describes preliminary experiments for optimizing a Tier 1 screening assay to detect anti-drug antibodies using a bridging assay format.

The parameters to be optimized include:

- The concentration of SULFO-TAG labeled drug
- The concentration of biotinylated drug
- The minimum required sample dilution
- The drug tolerance of the assay, if required

Workflow for Assay Development

The optimization workflow for bridging immunogenicity assay development is as follows:

The optimization experiments are explained below.

Optimization of Reagent Concentrations

There are two approaches to evaluate the optimal concentrations of biotinylated drug and SULFO-TAG labeled drug in anti-drug antibody assays:

- Checkerboard optimization: Tests varying concentrations of biotinylated drug and SULFO-TAG labeled drug in combination to identify the optimal ratio. This experiment is the preferred approach, especially when their relative affinities differ or are unknown. Note that the affinity is relative to the positive controls used in the assay, not the test sample. See *Checkerboard Optimization of Concentrations* on page 12.
- Sensitivity testing with similar affinities: Evaluates assay performance across a range of ADA concentrations. If the biotinylated drug and SULFO-TAG labelled drug are known or expected to have similar affinities for the positive control ADA, then this approach can be used to evaluate the assay's sensitivity without first doing a checkerboard optimization. See *Testing Sensitivity* on page 15.

Testing Matrix Tolerance

Matrix tolerance testing assesses the impact of the sample matrix on assay performance. See *Testing Matrix Tolerance* on page 20.

Testing Free Drug Tolerance

Testing free drug tolerance evaluates the assay's ability to detect anti-drug antibodies in the presence of free or circulating drug. See *Testing Free Drug Tolerance* on page 22.

Acid Dissociation to Improve Free Drug Tolerance

Acid-induced dissociation of high affinity immune complexes can improve the drug tolerance of immunogenicity assays. This experiment is typically performed if the standard assay does not provide sufficient drug tolerance. See *Acid Dissociation to Improve Drug Tolerance* on page 27.

Overview of Experiment Steps

A general overview of the steps in the bridging immunogenicity experiments is outlined below. For specifics, see the experimental protocols.

Step	Incubation
Incubate sample with a master mix of SULFO-TAG labeled drug and biotinylated drug in a polypropylene plate.	60–120 min / overnight
Block Streptavidin plate.	≥30 min During previous step
Add the combined sample and master mix to an MSD Streptavidin plate.	60 min
Read.	Read time dependent on instrument model

Checkerboard Optimization of Concentrations

The relative affinities of the biotinylated drug and SULFO-TAG labeled drug for the anti-drug antibody may differ, in which case the optimal ratio of biotinylated and SULFO-TAG labeled drug in the master mix may not be 1:1. In this experiment, different concentrations of biotinylated and SULFO-TAG labeled drug are tested in a checkerboard layout with a negative control and with a mid-range concentration of positive control anti-drug antibody (ADA).

If the affinities of the SULFO-TAG conjugated drug and biotinylated drug are expected to be similar, you can also perform *Testing Sensitivity* on page 15 to determine optimal reagent concentrations.

Reagent Preparation

Anti-Drug Antibody Samples

For the anti-drug antibody (ADA) samples, note the following:

- Prepare a 500 ng/mL solution of a suitable positive control anti-drug antibody (ADA) in assay diluent.
- Use assay diluent with no ADA added to serve as a 0 ng/mL negative control.
- Use 25 µL sample per well.
- You need 1500 µL of 500 ng/mL ADA in assay diluent and 1500 µL assay diluent without ADA as a negative control if following the *Recommended Plate Layout* on page 13.

Biotinylated Drug

For the biotinylated drug, note the following:

- Prepare 6 different concentrations of biotinylated drug in assay diluent (e.g., 1% Blocker A in PBS-T).
- Use 25 µL per well.
- Prepare 500 µL of each concentration if following the *Recommended Plate Layout* on page 13.
- Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps.

Recommended concentrations of biotinylated drug for antibody therapeutics:

- For MSD 96-well 1-Spot Streptavidin plates: 2; 1; 0.5; 0.25; 0.125; 0 µg/mL

  Prepare these dilutions the following way:

Target Concentration	Biotinylated Drug	Add Diluent
2 µg/mL	Prepare 1 mL 2 µg/mL biotinylated drug in assay diluent	
1 µg/mL	500 µL 2 µg/mL biotinylated drug	500 µL assay diluent
0.5 µg/mL	500 µL 1 µg/mL biotinylated drug	500 µL assay diluent
0.25 µg/mL	500 µL 0.5 µg/mL biotinylated drug	500 µL assay diluent
0.125 µg/mL	500 µL 0.25 µg/mL biotinylated drug	500 µL assay diluent
0 µg/mL	-	500 µL assay diluent

SULFO-TAG Labeled Drug

For the SULFO-TAG labeled drug, note the following:

- Prepare 8 different concentrations of SULFO-TAG labeled drug in assay diluent.
- Use 25 µL per well. Prepare 500 µL of each concentration if following the *Recommended Plate Layout* on page 13.
- Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps.

Recommended concentrations of SULFO-TAG labeled drug for antibody therapeutics:

- For MSD 96-well 1-Spot Streptavidin plates: 4; 2; 1; 0.5; 0.25; 0.125; 0.062; 0 µg/mL.

  Prepare these dilutions the following way:

Target Concentration	SULFO-TAG Labeled Drug	Add Diluent
4 µg/mL	Prepare 1 mL 4 µg/mL SULFO-TAG labeled drug in assay diluent	
2 µg/mL	500 µL 4 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
1 µg/mL	500 µL 2 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
0.5 µg/mL	500 µL 1 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
0.25 µg/mL	500 µL 0.5 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
0.125 µg/mL	500 µL 0.25 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
0.062 µg/mL	500 µL 0.125 µg/mL SULFO-TAG labeled drug	500 µL assay diluent
0 µg/mL	-	500 µL assay diluent

Recommended Plate Layout

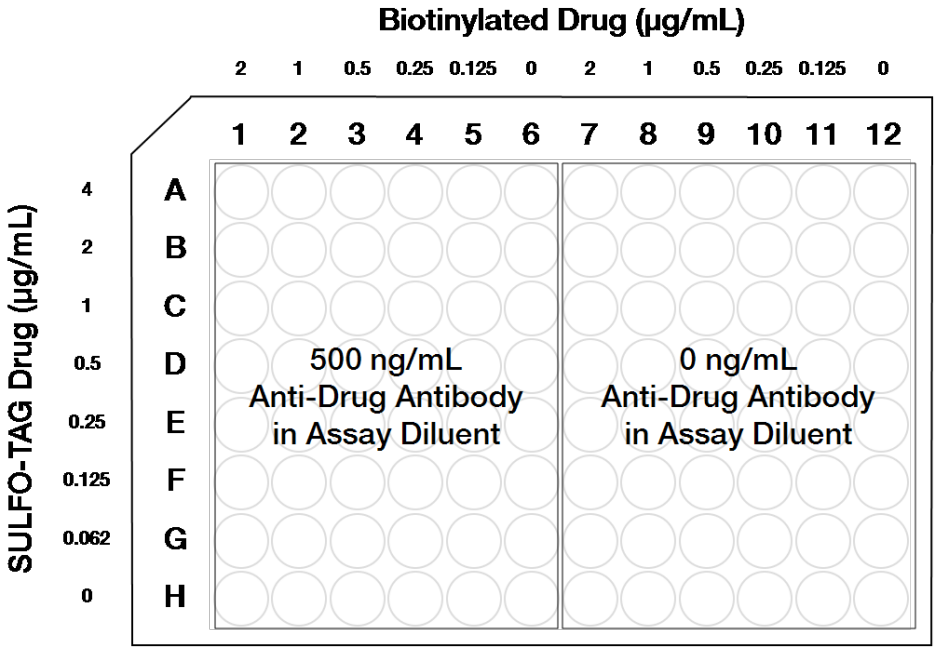

Figure 2. Sample plate layout for testing sensitivity using a checkerboard titration of biotinylated and SULFO-TAG labeled drug using an MSD 96-well 1-Spot Streptavidin plate.

Protocol Checkerboard Optimization

Refer to the recommended plate layout in Figure 2 on page 13, and example data in *Example Data Checkerboard Optimization* on page 15.

❑ 1. Combine biotin labeled drug, SULFO-TAG drug, and sample according to the plate layout:
Add 25 µL of the specified concentration of biotinylated drug to each well of a round-bottom polypropylene 96-well plate.
Add 25 µL of the specified concentration of SULFO-TAG labeled drug to each well of the 96-well plate.
Add 25 µL of sample (500 or 0 ng/mL anti-drug antibody) to each well of the 96-well plate.
Seal the plate and incubate for 1–2 hours at room temperature with moderate shaking at 300–500 rpm.

Moderate shaking should not result in splashing or condensation on the plate seal. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene 96-well plates.

❑ 2. Prepare the Streptavidin plate during the incubation in step 1:
During Step 1, add 150 µL of Blocking Solution per well to the MSD 96-well 1-Spot Streptavidin plate.
Seal the plate and incubate at least 30 minutes at room temperature with shaking at 500–1000 rpm.

❑ 3. Incubate the MSD Streptavidin plate:
Remove Blocking Solution from the MSD Streptavidin plate.
Wash the plate 3 times with at least 150 µL of wash buffer per well.
Transfer 50 µL from each well of the polypropylene plate to the MSD Streptavidin plate.
Seal plate and incubate for 1 hour at room temperature with shaking at 500–1000 rpm.

❑ 4. Read the plate:
Wash the plate 3 times with at least 150 µL of wash buffer per well.
Add 150 µL of MSD GOLD Read Buffer B per well.
Read the plate on an MSD instrument.

Avoid introducing bubbles when adding read buffer. This can be achieved using reverse pipetting techniques.

Additional Considerations

- Do not exceed the recommended amounts of biotinylated drug specified for the plate type. For MSD 96-well 1-Spot Streptavidin, do not exceed 0.3 pmols of biotinylated drug per well. For example, 25 µL of biotinylated antibody drug at 2 µg/mL corresponds to 0.33 pmols. In this experiment, 25 µL of the biotinylated drug is mixed with 25 µL SULFO-TAG labeled drug and 25 µL of sample in a polypropylene plate. Then 50 µL of the incubation mixture is transferred to the MSD 96-well 1-Spot Streptavidin plate; therefore 0.22 pmol or two thirds of the original 25 µL of biotinylated drug is transferred to the MSD plate and is within the binding capacity of the plate.
- If the assay is being developed for a protein therapeutic other than an antibody, the µg/mL concentration of biotinylated and SULFO-TAG labeled drug listed above should be adjusted according to the molecular weight of the drug; e.g., for a 75 kD protein therapeutic, which is half the molecular weight of a 150 kD antibody, the recommended concentrations of SULFO-TAG labeled and biotinylated drug would be half of those listed above for an antibody therapeutic.
- Refer to the Appendix *Equation for Converting pmol/well to µg/mL* on page 37 for conversion of pmols to µg/mL.

Example Data Checkerboard Optimization

A.

SULFO-TAG Drug (µg/mL)	Biotinylated Drug (µg/mL)											
	2	1	0.5	0.25	0.125	0	2	1	0.5	0.25	0.125	0
4	109537	95713	64793	30893	17669	158	1413	1266	747	452	272	161
2	194825	207001	172389	91267	56678	127	897	813	489	308	207	117
1	225982	290904	303258	205492	140786	124	538	504	312	215	154	101
0.5	170673	265677	286052	234022	155428	112	322	324	216	158	120	91
0.25	111044	177569	172617	143014	91683	100	213	219	160	134	110	96
0.125	63972	100861	93518	76086	50045	191	153	155	125	112	100	89
0.062	34248	53615	47154	39280	24296	107	117	122	102	101	92	87
0	107	116	117	104	87	91	79	79	81	88	82	82
	500 ng/mL Anti-Drug Antibody in Assay Diluent						0 ng/mL Anti-Drug Antibody in Assay Diluent					

B.

SULFO-TAG Drug (µg/mL)	Biotinylated Drug (µg/mL)					
	2	1	0.5	0.25	0.125	0
4	78	76	87	68	65	1
2	217	255	353	296	274	1
1	420	577	972	956	914	1
0.5	530	820	1324	1481	1295	1
0.25	521	811	1079	1067	833	1
0.125	418	651	748	679	500	2
0.062	293	439	462	389	264	1
0	1	1	1	1	1	1
	Signal/Background					

Figure 3. Example checkerboard optimization experiment for 500 and 0 ng/mL of anti-drug antibody.
A. Signal values for the experiment. Since background signal did not change significantly with varying levels of biotinylated and SULFO-TAG labeled drug, signal was used as the indicator of optimal labeled drug concentration.
B. Signal to background values for the experiment. The experiment illustrates that the best signal-to-background in this example is achieved near the 0.5 µg/mL concentration of both biotinylated drug and SULFO-TAG labeled drug.

Testing Sensitivity

In this experiment, three or more different concentrations of biotinylated and SULFO-TAG labeled drug are tested with a titration series of positive control anti-drug antibody.

This experiment can be used as an alternative to the checkerboard optimization to determine optimal reagent concentration if the affinities of the SULFO-TAG conjugated drug and biotinylated drug are expected to be similar. If affinities are not similar, perform *Checkerboard Optimization of Concentrations* on page 12.

This experiment provides initial insights into the matrix tolerance of the assay from the comparison of the results obtained in assay diluent versus those obtained in serum. A serum concentration of 25% serum is suggested to conserve the sample matrix. Other serum concentrations may be used as required.

Reagent Preparation

Anti-Drug Antibody Samples

For the anti-drug antibody (ADA) samples, note the following:

- Prepare 25% sample matrix (e.g., normal or naïve serum). Dilute serum in assay diluent (e.g., 1% (w/v) Blocker A in PBS-T).
- Prepare a dilution series of a positive control anti-drug antibody (ADA) in assay diluent and in 25% serum. Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps. Recommended test concentrations of ADA are 10,000; 2,500; 625; 156.3; 39.1; 9.8; 2.4 and 0 ng/mL.
- Use 25 µL of ADA sample per well.
- The range of test concentrations of ADA can be adjusted if required.

If using the plate layout shown in Figure 4 on page 17, prepare the dilution series in assay diluent or 25% serum the following way:

Target Concentration	Anti-Drug Antibody	Add Assay Diluent or 25% Serum
10,000 ng/mL	Prepare 400 µL of 10,000 ng/mL ADA in assay diluent or 25% serum	
2,500 ng/mL	100 µL 10,000 ng/mL ADA	300 µL assay diluent or 25% serum
625 ng/mL	100 µL 2,500 ng/mL ADA	300 µL assay diluent or 25% serum
156.3 ng/mL	100 µL 625 ng/mL ADA	300 µL assay diluent or 25% serum
39.1 ng/mL	100 µL 156.3 ng/mL ADA	300 µL assay diluent or 25% serum
9.8 ng/mL	100 µL 39.1 ng/mL ADA	300 µL assay diluent or 25% serum
2.4 ng/mL	100 µL 9.8 ng/mL ADA	300 µL assay diluent or 25% serum
0 ng/mL	-	300 µL assay diluent or 25% serum

SULFO-TAG and Biotinylated Drug Master Mix

For the SULFO-TAG and Biotinylated Drug master mix, note the following:

- Prepare 3-4 different concentrations of a master mix containing an equal concentration of both the SULFO-TAG labeled drug and biotinylated drug diluted in assay diluent (e.g., 1% Blocker A in PBS-T).
- Each well gets 50 µL master mix; prepare 2 mL of each concentration if using the plate layout shown in Figure 4 on page 17
- For antibody therapeutic drugs, recommended amounts of labeled drug to test in master mix for MSD 96-well 1-Spot Streptavidin plates are 1.0; 0.5; and 0.25 µg/mL biotinylated and SULFO-TAG labeled antibody drug.

- This experiment can also be performed using the optimal ratio of SULFO-TAG: Biotinylated drug determined from a checkerboard experiment if the optimal ratio is not 1:1.
- For MSD 96-well 1-Spot Streptavidin plate: Do not exceed 0.3 pmols of biotinylated drug per well. For example, a 1 µg/mL concentration of biotinylated antibody drug corresponds to 0.33 pmols in 50 µL of master mix. The master mix is incubated with 25 µL of sample in a polypropylene plate and 50 µL of the incubation mixture is transferred to the plate. Therefore, only two thirds of the biotinylated drug in the incubation mixture or 0.22 pmol biotinylated drug is transferred to the plate.
- If the assay is being developed for a protein therapeutic other than an antibody, the µg/mL concentration of biotinylated and SULFO-TAG labeled drug listed above should be adjusted based on the molecular weight of the drug. For example, for a 75 kD protein therapeutic, which is half the molecular weight of a 150 kD antibody, the recommended concentrations of SULFO-TAG labeled and biotinylated drug would be half of those listed above for an antibody therapeutic.
  Refer to *Appendix: Converting Concentrations* on page 37 for conversion of pmols to µg/mL.

Recommended Plate Layout

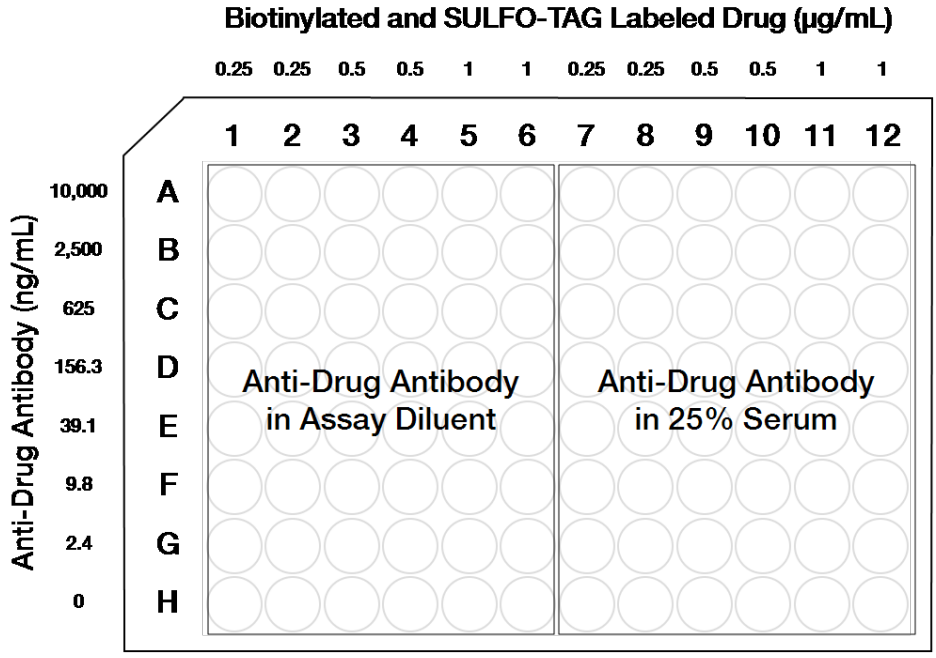

Figure 4. Sample plate layout for testing sensitivity for similar affinities of biotinylated and SULFO-TAG labeled drug using an MSD 96-well 1-Spot Streptavidin plate.

Protocol Testing Sensitivity

Refer to the recommended plate layout in Figure 4 on page 17, and example data in *Example Data Testing Sensitivity* on page 19.

❑ 1. Combine master mix and sample according to the plate layout:

Add 50 µL of SULFO-TAG and Biotinylated Drug master mix to each well of a round-bottom 96-well polypropylene plate.

Add 25 µL of the specified anti-drug antibody concentration to each well of the 96-well plate.

Seal the plate and incubate for 1–2 hours at room temperature or overnight at 4˚C with moderate shaking at 300–500 rpm.

Moderate shaking should not result in splashing or condensation on the plate seal. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene 96-well plates.

❑ 2. Prepare the Streptavidin plate during the incubation in step 1:

During Step 1, add 150 µL of Blocking Solution (e.g., 3% Blocker A in PBS-T) per well to the MSD 96-well 1-Spot Streptavidin plate.

Seal the plate and incubate at least 30 minutes at room temperature with shaking at 500–1000 rpm.

❑ 3. Incubate the MSD Streptavidin plate:

Remove the Blocking Solution from the MSD Streptavidin plate.

Wash the plate 3 times with at least 150 µL of wash buffer per well.

Transfer 50 µL from each well of the polypropylene plate to the MSD plate.

Seal the plate and incubate for 1 hour at room temperature with shaking at 500–1000 rpm.

❑ 4. Read the plate:

Wash the plate 3 times with at least 150 µL of wash buffer per well.

Add 150 µL of MSD GOLD Read Buffer B per well.

Read the plate on an MSD instrument.

Avoid introducing bubbles when adding read buffer. This can be achieved using reverse pipetting techniques.

Example Data Testing Sensitivity

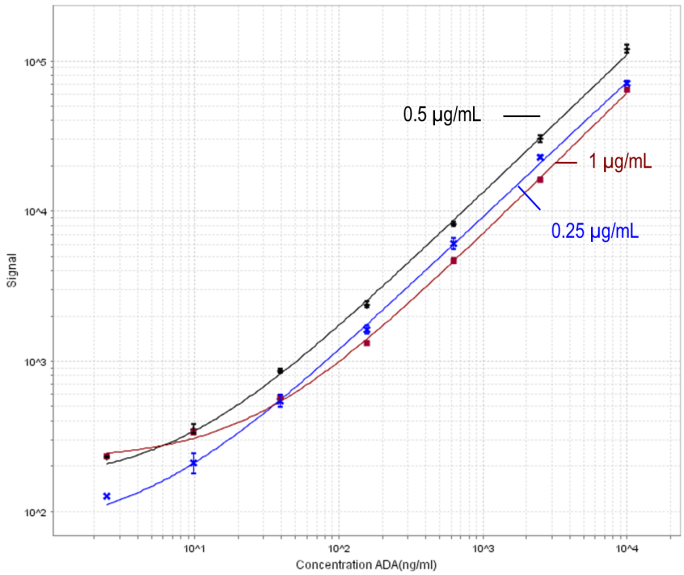

Figure 5. Example data from an experiment to evaluate assay sensitivity using equal amounts of biotinylated and SULFO-TAG labeled drug using an MSD 96-well 1-Spot Streptavidin plate. The plot represents the results of the experiment with master mix concentration of 1 µg/mL (red), 0.5 µg/mL (black), and 0.25 µg/mL (blue). In this example, further studies were performed using a master mix where the final amount of biotinylated and SULFO-TAG labeled drug added per well of the plate was 0.5 µg/mL each.

Testing Matrix Tolerance

Once the optimal concentrations of biotinylated drug and SULFO-TAG labeled drug have been determined, the matrix tolerance of the assay can be tested.

Reagent Preparation

Master Mix

Prepare 6 mL per plate of master mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in assay diluent. Use 50 µL of master mix per well.

> **i** If the concentrations of SULFO-TAG drug and biotinylated drug were optimized using a checkerboard experiment (see *Checkerboard Optimization of Concentrations* on page 12), halve the optimal concentrations of biotinylated and SULFO-TAG labeled drug selected from the checkerboard to prepare a master mix.

Matrix Dilution

Prepare a dilution series of the sample matrix, e.g., 100%; 50%; 25%; 12.5%; 6.25% and 3.125% serum diluted in assay diluent.

If following the suggested layout in Figure 6 on page 21, 1 mL of each serum concentration is required.

Samples

Prepare a dilution series of positive control anti-drug antibody (ADA) in each serum concentration. Recommended concentrations of ADA are 10,000; 2,500; 625; 156.3; 39.1; 9.8; 2.4 and 0 ng/mL or a suitable concentration range suggested by the results of the previous sensitivity experiments. Use 25 µL of ADA sample per well.

If using the plate layout shown in Figure 6 on page 21, prepare the dilution series in each serum concentration the following way:

Target Concentration	Anti-Drug Antibody	Add Serum
10,000 ng/mL	Prepare 100 µL of 10,000 ng/mL control ADA in sample matrix	
2,500 ng/mL	30 µL 10,000 ng/mL ADA	90 µL sample matrix
625 ng/mL	30 µL 2,500 ng/mL ADA	90 µL sample matrix
156.3 ng/mL	30 µL 625 ng/mL ADA	90 µL sample matrix
39.1 ng/mL	30 µL 156.3 ng/mL ADA	90 µL sample matrix
9.8 ng/mL	30 µL 39.1 ng/mL ADA	90 µL sample matrix
2.4 ng/mL	30 µL 9.8 ng/mL ADA	90 µL sample matrix
0 ng/mL	-	90 µL sample matrix

Recommended Plate Layout

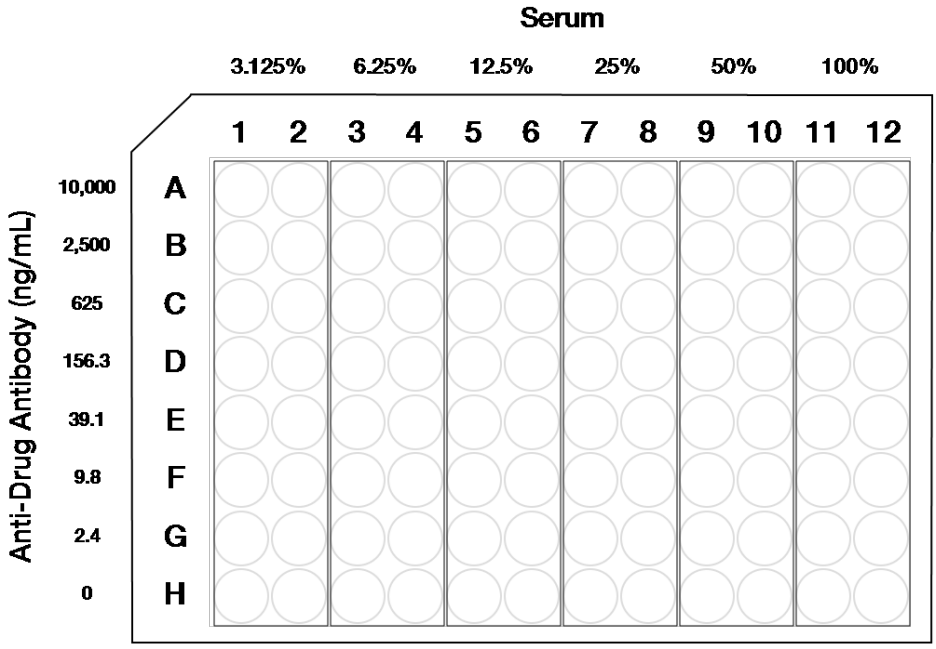

Figure 6. Sample plate layout for a matrix tolerance experiment.

Protocol Matrix Tolerance

Refer to the recommended plate layout in Figure 6 on page 21, and example data in *Example Data Matrix Tolerance* on page 22.

❑ 1. Combine the following reagents according to the plate layout:

Add 50 µL of master mix containing biotinylated drug and SULFO-TAG labeled drug to each well of a 96-well round-bottom polypropylene plate.

Add 25 µL of sample to each well of the 96-well plate according to the plate layout.

Seal the plate and incubate for 1–2 hours at room temperature or overnight at 4˚C with moderate shaking at 300–500 rpm.

Moderate shaking should not result in splashing or condensation on the plate seal. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene 96-well plates.

❑ 2. Prepare the Streptavidin plate during the incubation in step 1:

During Step 1, add 150 µL per well of Blocking Solution to the MSD 96-well 1-Spot Streptavidin plate.

Seal the plate and incubate at least 30 minutes at room temperature with shaking at 500–1000 rpm.

❑ 3. Incubate the MSD Streptavidin plate:

Remove Blocking Solution from the plate.

Wash the plate 3 times with at least 150 µL of wash buffer per well.

Transfer 50 µL of the incubation mixture from each well of the polypropylene plate to the MSD Streptavidin plate.

Seal the plate and incubate for 1 hour at room temperature with shaking at 500–1000 rpm.

❑ 4. Read the plate:

Wash the plate 3 times with at least 150 µL wash buffer per well.
Add 150 µL per well of MSD GOLD Read Buffer B.
Read the plate on an MSD instrument.

Avoid introducing bubbles when adding read buffer. This can be achieved using reverse pipetting techniques.

Example Data Matrix Tolerance

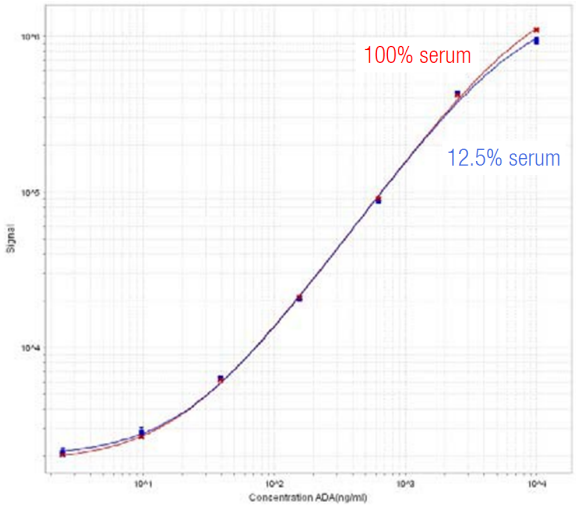

Figure 7. Example data from matrix tolerance experiment. In this example, there is little difference between the assay carried out in 100% and 12.5% serum.

Testing Free Drug Tolerance

Drug interference can result in false negatives and suppressed signal in immunogenicity assays, especially when high affinity immune complexes are formed between the drug and the anti-drug antibodies (ADAs) in the sample. The MSD bridging assay format described in this Assay Development Guide uses a homogenous, solution phase incubation that, when allowed to incubate for an extended time (overnight), can significantly reduce drug interference effects. Circulating or free drug has the potential to bind to ADAs in samples and prevent detection of ADAs in an immunogenicity assay. The longer, homogenous incubation in this format allows unlabeled drug to dissociate from the ADA immune complex and enables the biotinylated and SULFO-TAG labeled drug present in excess to associate with the ADA in the MSD bridging assay.

Tolerance to free drug can be enhanced by using a larger dilution of the sample, which will bias the binding equilibrium such that the ADA in the sample is more likely to interact with the labeled drug. The assay is tolerant to sample pre-treatments including acid/base neutralization and agents commonly used to reduce drug interference. Compare results from this experiment to expected drug levels in test samples to determine if the assay needs to be further optimized to include a sample pre-treatment step to further enhance the drug tolerance of the assay.

For details of alternative assay formats, see *Improving Performance* on page 32.

Reagent Preparation

Master Mix

Prepare 6 mL per plate of master mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in assay diluent. 50 µL of master mix is used per well.

Anti-Drug Antibody Samples

Prepare a dilution series of positive control anti-drug antibody (ADA) at 2X final concentration in serum. Recommended 2X concentrations of ADA are 20,000; 5,000; 1,250; 312.5; 78.1; 19.5; 4.8 and 0 ng/mL. Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps. Use 25 µL of ADA sample per well.

If using the plate layout shown in Figure 8 on page 24, prepare the dilution series the following way:

Target Concentration	Anti-Drug Antibody	Add Serum
20,000 ng/mL	Prepare 1.2 mL of 20,000 ng/mL control ADA in serum	
5,000 ng/mL	300 µL 20,000 ng/mL ADA	900 µL serum
1,250 ng/mL	300 µL 5,000 ng/mL ADA	900 µL serum
312.5 ng/mL	300 µL 1,250 ng/mL ADA	900 µL serum
78.1 ng/mL	300 µL 312.5 ng/mL ADA	900 µL serum
19.5 ng/mL	300 µL 78.1 ng/mL ADA	900 µL serum
4.8 ng/mL	300 µL 19.5 ng/mL ADA	900 µL serum
0 ng/mL	-	900 µL serum

Unlabeled Drug

Prepare a dilution series of unlabeled drug at 2X the final concentration in serum. Suggested 2X concentrations of unlabeled drug are 100; 25; 6.25; 1.56; 0.39 and 0 µg/mL for antibody drugs or 600; 150; 37.5; 9.3, 2.3 and 0 nM for other therapeutics. Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps. Use 25 µL of ADA sample per well.

Other concentrations of unlabeled drug based on the results of pharmacokinetics assays or expected circulating concentrations may also be tested.

If using the plate layout shown in Figure 8 on page 24, prepare the dilution series the following way:

Target Concentration	Unlabeled Drug	Add Serum
100 µg/mL	Prepare 1.2 mL of 100 µg/mL unlabeled drug in serum.	
25 µg/mL	300 µL 100 µg/mL unlabeled drug	900 µL serum
6.25 µg/mL	300 µL 25 µg/mL unlabeled drug	900 µL serum
1.56 µg/mL	300 µL 6.25 µg/mL unlabeled drug	900 µL serum
0.39 µg/mL	300 µL 1.56 µg/mL unlabeled drug	900 µL serum
0 µg/mL	-	900 µL serum

Recommended Plate Layout

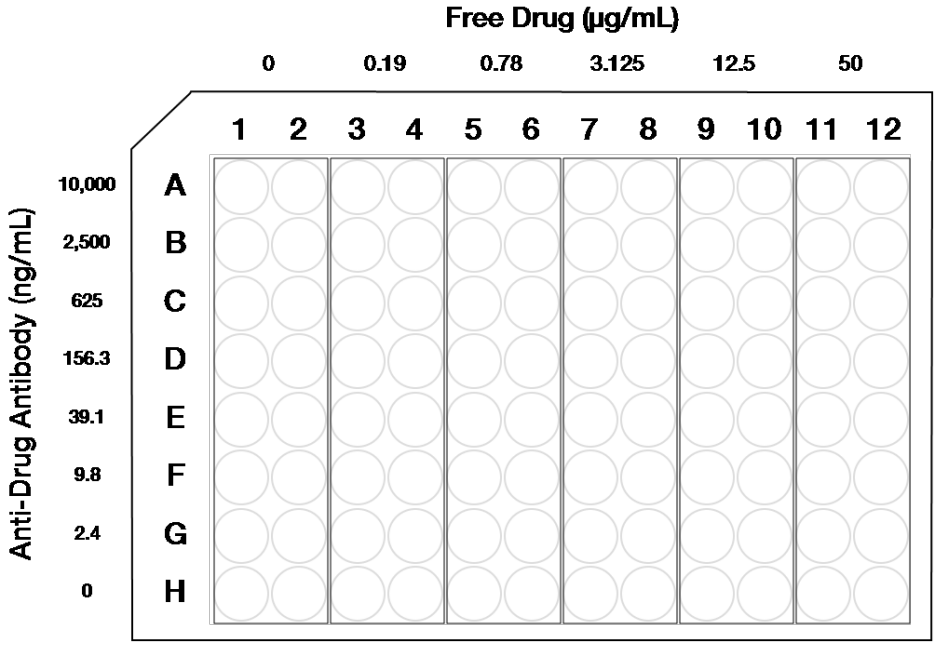

Figure 8. Sample plate layout for testing free drug tolerance. The concentrations listed are the final concentrations of ADA and free drug in the sample before addition of the master mix reagents.

Protocol Free Drug Tolerance

Refer to the recommended plate layout in Figure 8 on page 24, and example data in *Example Data Free Drug Tolerance* on page 26.

❑ 1. Combine ADA and free drug according to the plate layout:
Add 50 µL of 2X anti-drug antibody and 50 µL of 2X free drug to each well of a round-bottom polypropylene 96-well plate.
Seal the plate and incubate for 1 hour at room temperature with moderate shaking at 300–500 rpm.

Moderate shaking should not result in splashing or condensation on the plate seal. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene 96-well plates.

❑ 2. Combine master mix and the mixture of anti-drug antibody and unlabeled drug according to the plate layout:
Add 50 µL of master mix (containing biotinylated drug and SULFO-TAG labeled drug) to each well of a new round-bottom polypropylene 96-well plate.
Add 25 µL of the mixture of ADA and unlabeled drug to each well of the 96-well plate according to the plate layout.
Seal the plate and incubate overnight at 4˚C with moderate shaking at 300–500 rpm.

❑ 3. Prepare the Streptavidin plate:
Add 150 µL of Blocking Solution per well to the MSD 96-well 1-Spot Streptavidin plate.
Seal the plate and incubate at least 30 minutes at room temperature with shaking at 500–1000 rpm.

❑ 4. Incubate the MSD Streptavidin plate:
Remove Blocking Solution from the MSD Streptavidin plate.
Wash the plate 3 times with at least 150 µL of wash buffer per well.
Transfer 50 µL from each well of the polypropylene plate to the MSD Streptavidin plate.
Seal plate and incubate for 1 hour at room temperature with shaking at 500–1000 rpm.

❑ 5. Read the plate:
Wash the plate 3 times with at least 150 µL of wash buffer per well.
Add 150 µL of MSD GOLD Read Buffer B per well.
Read the plate on an MSD instrument.

Avoid introducing bubbles when adding read buffer. This can be achieved using reverse pipetting techniques.

Example Data Free Drug Tolerance

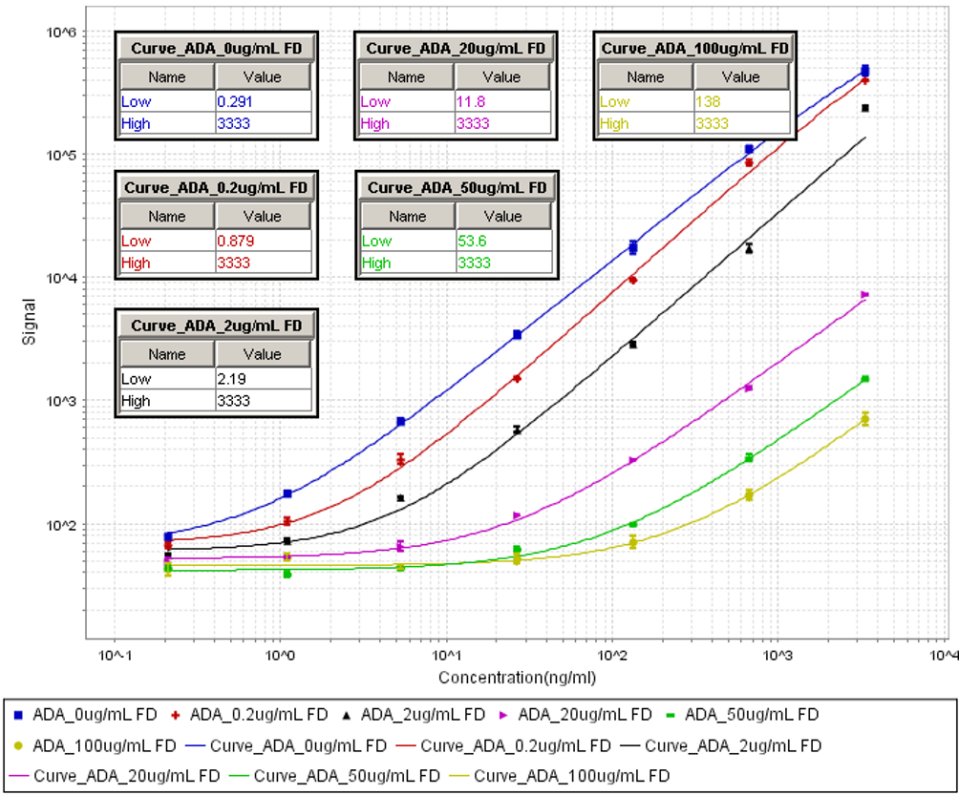

Figure 9. Example data from free drug tolerance experiment. The concentration of ADA (ng/mL) is indicated on the x-axis. In this example, 150 ng/mL ADA is still detectable in the presence of 100 µg/mL unlabeled drug.

Acid Dissociation to Improve Drug Tolerance

Acid-induced dissociation of high affinity immune complexes is a technique that has successfully been implemented to improve the drug tolerance of immunogenicity assays[11,12] . In this protocol, the sample is treated with acid to dissociate anti-drug antibody (ADA) complexed to drug and then neutralized in the presence of excess SULFO-TAG labeled and biotinylated drug.

> **i** This protocol uses acetic acid, but other acids can be used. Reference [15] has examples using 300 mM Glycine-HCl and 125 mM HCl for acidification.

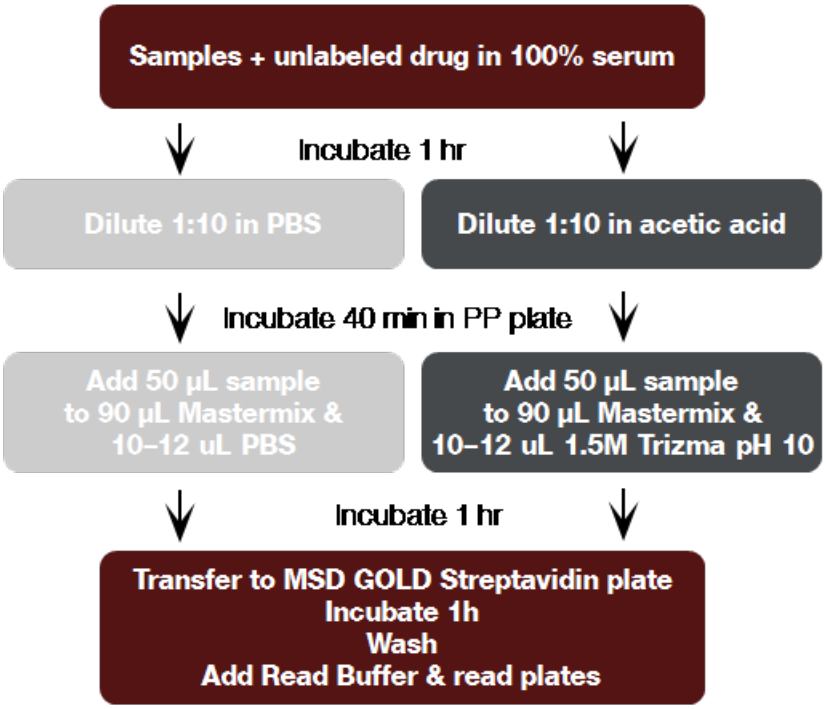

Figure 10. Overview of drug tolerance experiment with (dark gray) and without (light gray) acid dissociation.

Additional Reagents Required

Acid dissociation experiments need the following additional reagents:

- 300 mM Acetic Acid
- 1.5 M Trizma Base pH 10 or other suitable neutralizing solution, e.g., 1 M Tris-HCl pH 9.5-10.

Reagent Preparation

Master Mix

Prepare 12 mL per plate of master mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in assay diluent. Use 90 µL of master mix per well.

Anti-Drug Antibody Samples

Prepare a dilution series of positive control anti-drug antibody (ADA) at 2X final concentration in serum. Recommended 2X concentrations of ADA are 20,000; 5,000; 1,250; 312.5; 78.1; 19.5; 4.8 and 0 ng/mL. Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps. Use 30 µL of ADA per well.

If using the plate layout shown in Figure 11 on page 29 (top plate), prepare the dilution series the following way:

Target Concentration	Anti-Drug Antibody	Add Serum
20,000 ng/mL	Prepare 800 µL of 20,000 ng/mL positive control ADA in serum	
5,000 ng/mL	200 µL 20,000 ng/mL ADA	600 µL serum
1,250 ng/mL	200 µL 5,000 ng/mL ADA	600 µL serum
312.5 ng/mL	200 µL 1,250 ng/mL ADA	600 µL serum
78.1 ng/mL	200 µL 312.5 ng/mL ADA	600 µL serum
19.5 ng/mL	200 µL 78.1 ng/mL ADA	600 µL serum
4.8 ng/mL	200 µL 19.5 ng/mL ADA	600 µL serum
0 ng/mL	-	600 µL serum

Unlabeled Drug

Prepare a dilution series of unlabeled drug at 2X the final concentration in serum. Suggested 2X concentrations of unlabeled drug are 100; 25; 6.25; 1.56; 0.39 and 0 µg/mL for antibody drugs or 600; 150; 37.5; 9.3, 2.3 and 0 nM for other therapeutics. Sample library tubes are recommended for the dilution series to facilitate the use of a multi-channel pipette in subsequent transfer steps. Use 30 µL of free drug per well.

i Other concentrations of unlabeled drug based on the results of PK assays or expected circulating concentrations may also be tested.

If using the plate layout shown in Figure 11 on page 29, prepare the dilution series the following way:

Target Concentration	Unlabeled Drug	Add Serum
100 µg/mL	Prepare 1.2 mL of 100 µg/mL unlabeled drug in serum.	
25 µg/mL	200 µL 100 µg/mL unlabeled drug	600 µL serum
6.25 µg/mL	200 µL 25 µg/mL unlabeled drug	600 µL serum
1.56 µg/mL	200 µL 6.25 µg/mL unlabeled drug	600 µL serum
0.39 µg/mL	200 µL 1.56 µg/mL unlabeled drug	600 µL serum
0 µg/mL	-	600 µL serum

Recommended Plate Layout

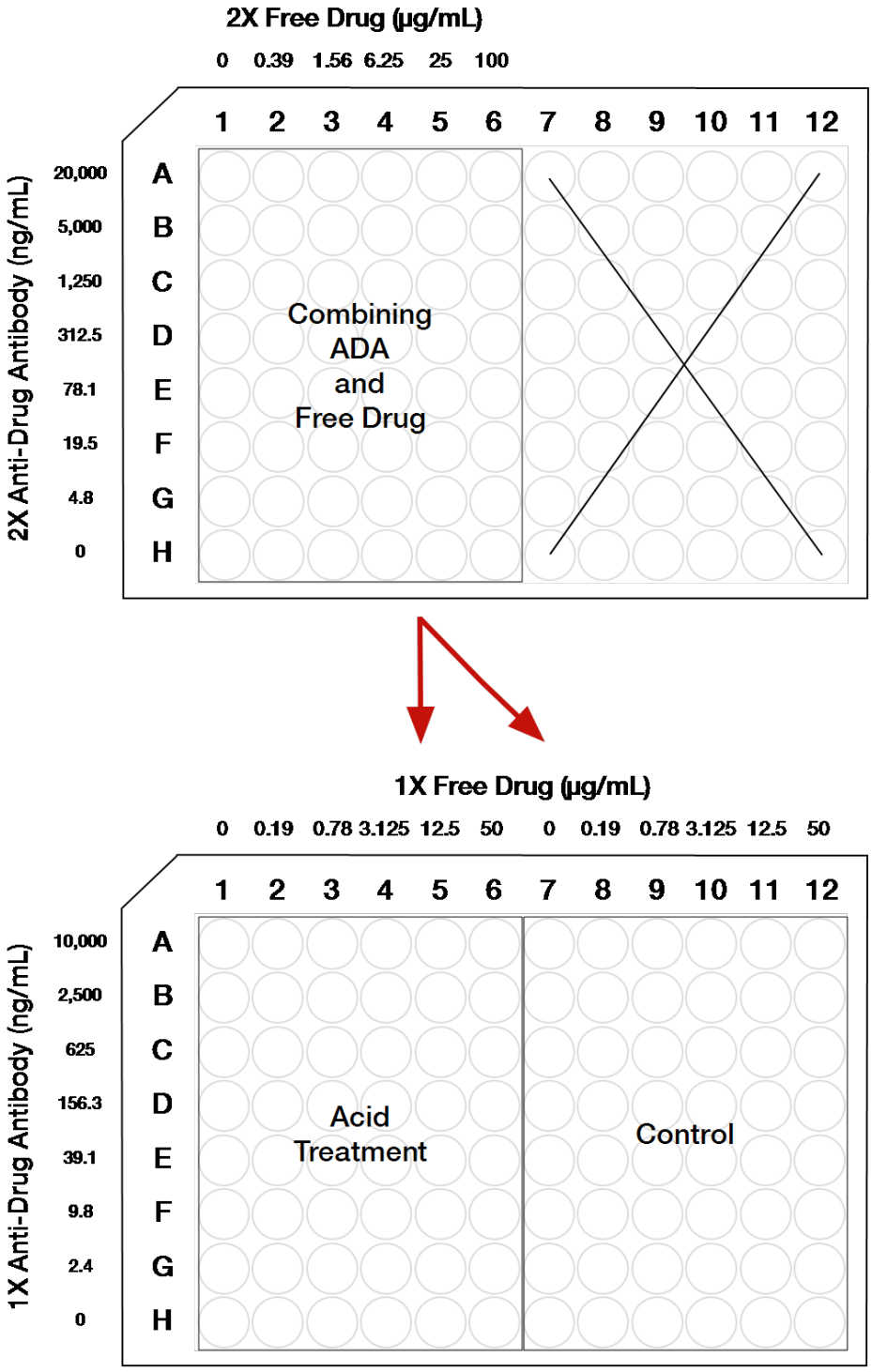

Figure 11. Sample plate layout for combining ADA and free drug (top) and acid treatment and control (bottom).

Protocol Acid Dissociation

Refer to the recommended plate layout in Figure 11 on page 29. Top plate refers to the plate layout when combining ADA and free drug, bottom plate refers to the plate layout when performing acid dissociation. For example data, see *Example Data Acid Dissociation* on page 31.

❑ 1. Combine ADA and free drug according to the plate layout:

Add 30 µL of 2X anti-drug antibody to 30 µL of 2X free drug to each well of a round-bottom polypropylene 96-well plate.

Seal the plate and incubate for 1 hour at room temperature with moderate shaking at 300–500 rpm.

Moderate shaking should not result in splashing or condensation on the plate seal. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene 96-well plates.

❑ 2. Perform acid dissociation according to the plate layout:

Add 180 µL of 300 mM Acetic Acid for acid treatment or PBS for the control to each well of a new round-bottom polypropylene 96-well plate.

Add 20 µL of the mixture of ADA and unlabeled drug from step 1 to each well of the 96-well plate.

Seal the plate and incubate 40–45 minutes at room temperature with moderate shaking at 300–500 rpm.

❑ 3. Add master mix and neutralize:

Add 90 µL of master mix (containing biotinylated drug and SULFO-TAG labeled drug) to each well of a new round-bottom polypropylene 96-well plate.

Just before use, add 12 µL/well of 1.5 M Trizma Base pH 10 (or other neutralizing solution) for the acid treatment wells or PBS for the wells with the control.

Starting recommendation is 12 µL of 1.5 M Trizma Base pH 10, but the actual volume needs to be optimized as it is dependent on the pH of the acid solution, the neutralizing solution, and the samples.

Transfer 50 µL of sample from the treatment plate to the master mix plate.

Seal the plate and incubate 1–2 hours at room temperature or overnight at 4˚C with moderate shaking at 300–500 rpm.

❑ 4. Prepare the Streptavidin plate:

During Step 3, add 150 µL of Blocking Solution per well to the MSD 96-well 1-Spot Streptavidin plate.

Seal the plate and incubate at least 30 minutes at room temperature with shaking at 500–1000 rpm.

❑ 5. Incubate the MSD Streptavidin plate:

Remove Blocking Solution from the MSD Streptavidin plate.

Wash the plate 3 times with at least 150 µL of wash buffer per well.

Transfer 50 µL from each well of the polypropylene plate from step 3 to the corresponding well of the MSD Streptavidin plate.

Seal plate and incubate for 1 hour at room temperature with shaking at 500–1000 rpm.

❑ 6. Read the plate:

Wash the plate 3 times with at least 150 µL of wash buffer per well.

Add 150 µL of MSD GOLD Read Buffer B per well.

Read the plate on an MSD instrument.

Avoid introducing bubbles when adding read buffer. This can be achieved using reverse pipetting techniques.

Additional Considerations

- Other acid-base combinations can be tested to further optimize the assay[15].
- Check the pH of the neutralized samples during assay development to ensure that neutral pH has been reestablished following acid dissociation and neutralization. If necessary, adjust the volume of the neutralizing solution.
- In some cases, acid dissociation treatment may cause suppression of the positive control signal and has the potential to abrogate the response from some monoclonal anti-drug antibody controls.
- Check the stability of the acidification and neutralizing solutions, since this assay is pH dependent.
- The control sample dilution in PBS is for development and is not required for routine sample testing in a Tier 1 screening assay. Use this experiment format to evaluate the levels of unlabeled drug required for a Tier 2 confirmatory assay.

Example Data Acid Dissociation

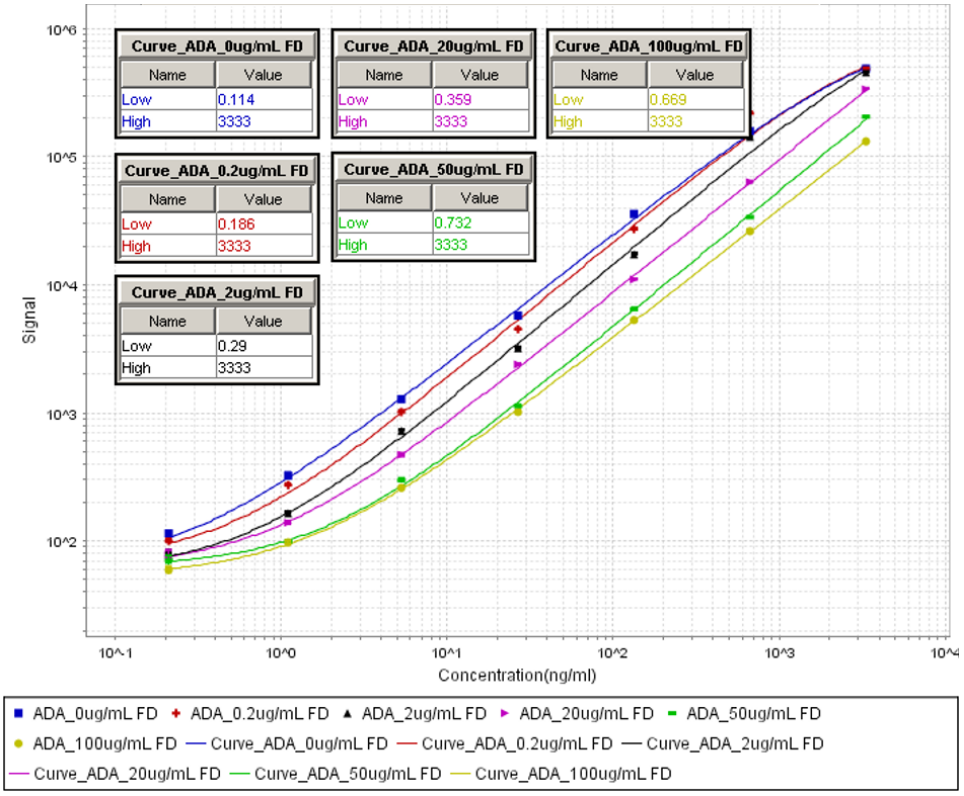

Figure 12. Example data from free drug tolerance experiment with acid dissociation. The concentration of ADA (ng/mL) is indicated on the x-axis. In this example 1 ng/mL ADA is still detectable in the presence of 100 µg/mL unlabeled drug.

Discussion and Troubleshooting

Improving Performance

MSD anti-drug antibody assays for unwanted immunogenicity can be adjusted to improve performance in several different ways:

- In bridging assays, drug tolerance is improved by extending the incubation of sample, biotinylated drug, and SULFO-TAG labeled drug to overnight, without sacrificing assay sensitivity.
- Increased levels of biotinylated and SULFO-TAG labeled drug, or larger sample dilutions, can be used to bias the kinetics of the binding events to reduce drug interferences. Do not exceed the recommended plate capacity when adjusting concentrations.
- Alternative assay designs or sample pre-treatment steps, such as heating the sample, have been used to further enhance the drug tolerance of immunogenicity assays with the MSD platform. These are represented in references for SPEAD[7], BEAD[8], ACE[9], PandA[10], SPECL[13], and heat treament[14].

In addition, note the following recommendations:

Topic	Recommendation
MSD 96-well 1-Spot Streptavidin Plates	MSD 1-Spot Streptavidin plates are recommended for initial assay development. Keep the final amount of biotinylated reagent added to MSD 96-well 1-Spot Streptavidin plates to less than 0.3 pmol per well.
Signal Levels	Expected background signal levels for the MSD instruments are around 60 to 100 counts (when using MSD GOLD Read Buffer B). The maximum signal for the assay should be less than 1 million counts, as the top of the dynamic range of the MSD instruments for single spot plates is reached at maximum signals of 1 to 1.35 million counts depending on the instrument model.
Shaking During Incubation	Moderate shaking should not result in splashing or condensation on the plate seal. MSD recommends shaking the streptavidin plates during incubation steps at 500–1000 rpm. Typically, moderate shaking is 300–500 rpm for round-bottom polypropylene. Shaking increases diffusion kinetics and allows the binding equilibrium to be reached in a shorter period. The shaking speed for each step should be kept consistent to minimize day-to-day variability.

High Background

The following conditions can lead to high background:

Cause	Description
Storage conditions	Storage conditions can affect the propensity of a drug to aggregate; labeled drug should preferably be stored at the temperature and in the formulation buffer optimized to ensure drug stability. Further optimizations to the buffer that help mitigate aggregation include pH (slightly acidic can help) and increased sodium chloride in combination with lower protein concentrations.
Interaction between the biotinylated and SULFO-TAG labeled drug	Another potential source of high background is an interaction between the biotinylated and SULFO-TAG labeled drug in the absence of sample matrix or anti-drug antibodies. Drug aggregates may be more prone to non-specific interactions. The aggregation state of the unlabeled, biotinylated and SULFO-TAG labeled drug can be verified through a number of biophysical techniques including but not restricted to non-denaturing gel electrophoresis, size-exclusion chromatography and dynamic light scattering. In some cases, this phenomenon has been linked to a particular batch or lot of drug and was not observed when a new batch or lot was relabeled with SULFO-TAG and/or biotin.
Non-specific binding of SULFO-TAG labeled drug	Non-specific binding of SULFO-TAG labeled drug to the plate can elevate background signals; this non-specific interaction can be identified by carrying out the assay in the absence of biotinylated drug. Perform control experiments to compare signals generated with SULFO-TAG labeled drug in sample matrix (e.g., serum) and in assay diluent to determine if the non-specific binding is linked to the drug or if it is a matrix-induced effect. Test alternative assay diluents and/or blocking solutions to reduce non-specific binding. Another cause is unoptimized Biotin and SULFO-TAG drug concentrations. Repeat the titration with a lower concentration of SULFO-TAG drug.

Low Assay Signals

The following conditions can lead to low assay signals:

Cause	Description
Poor labeling with biotin or SULFO-TAG	Incomplete labeling leads to free drug in the master mix. This will compete with binding of the anti-drug antibodies and therefore lower the signal. Poor labeling with biotin or SULFO-TAG is often linked to the presence of substances interfering with the labeling reaction, e.g., Tris, glycine, histidine, and azide. Ensure that the drug is in an amine-free, carrier-free buffer prior to labeling. Optimize labeling with different conjugation (challenge) ratios. For example, for SULFO-TAG labeled antibody drugs, try ratios of 12:1 and 6:1 SULFO-TAG:drug instead of the starting 10:1 challenge ratio. For biotin-labeling, try ratios of 20:1 or 40:1 instead of the starting 10:1 challenge ratio.
Inability to bridge	Low assay signals may be indicative of the inability of the biotinylated and SULFO-TAG labeled drug to bridge with the anti-drug antibody.
Limited availability of reactive primary amine groups	Limited availability of reactive primary amine groups on the drug, because these are limited in the protein sequence or blocked due to extensive modifications such as exhaustive PEGylation or linkage to polysialic acid, can also lead to poor or low incorporation of biotin and/or SULFO-TAG. In these cases, higher labeling ratios and/or increasing the pH of the labeling reaction to pH 7.9 – 8.2 may be required.
Over-labeling	Over-labeling of drug with biotin and/or SULFO-TAG, especially when working with lower molecular weight, highly positively charged proteins can result in drug precipitation as a result of charge neutralization. If precipitates are evident in the labeling reactions, use lower labeling ratios.
Incomplete removal of biotin	Incomplete removal of biotin after the drug has been biotinylated can lead to poor assay performance since the free biotin will compete with the biotinylated drug for binding to the MSD 96-well 1-Spot Streptavidin plate. Ensure that all unincorporated biotin is removed once the biotinylation reaction is complete.

Assay Variability and Signal Reproducibility

A number of factors can affect both intra- and inter-plate signal reproducibility. These include:

Cause	Description
Exceeding recommended concentrations of biotinylated reagent	Exceeding the recommended capacity of the plates can lead to higher signal variability. This can also lead to lower signals or lower sensitivity. The final amount of biotinylated drug loaded per well should be less than 0.3 pmol for MSD 96-well 1-Spot Streptavidin plates. Refer to the Appendix for formulas to convert pmol per well to µg/mL.
Read buffer temperature	Read Buffer B is designed to be used at room temperature. Read buffer that is below 18 °C will result in signal suppression.
Variability through pipetting	Assay variability is often linked to pipetting differences due to equipment or differences between operators. Follow best practices and use calibrated pipettes and the correct pipette tips. Repeater pipettes should be checked for accuracy for each dispense step.
Inconsistent shaking speed	Differences in plate shaking speed can affect absolute signals since shaking increases diffusion rates and hence binding kinetics of the assay components. Shaking conditions should be kept consistent to ensure optimal signal reproducibility.
Variability introduced through plate washing equipment	Automated plate washers can lead to signal inconsistencies if some pins are blocked or contaminated. Ensure that plate washers are kept clean and well maintained. Flipping the plate orientation during plate washing can be useful to troubleshoot plate washer related problems. Ensure that there is no liquid remaining in the wells of the MSD plate following a wash step.
Dissociation rates	In MSD assays, the signal is generated from electrically stimulated SULFO-TAG labeled molecules that are in close proximity (1 to 10 µm) to the bottom of the well of the MSD plate. Before the final wash step, the assay components are at or close to equilibrium; however, if the plate is left in wash buffer or read buffer, the assay components may start to dissociate and re-associate to reestablish a new binding equilibrium. Since the MSD assay is a proximity assay, signal will decrease if SULFO-TAG labeled drug dissociates from the other assay components on the surface. Signal decrease will not be significant for high affinity interactions with slow off-rates (k_{off}); however, interactions with rapid dissociation rates can result in a time-dependent signal decrease. Do not leave MSD plates in wash buffer and keep read time after addition of read buffer consistent.
Loss of specific activity	Loss of specific activity of biotinylated and/or SULFO-TAG labeled drug can lead to assay variability.
Uneven or excessive heat distribution	Uneven or excessive heat distribution across the bottom of the plate during shaking can lead to assay variability. Make sure the shaker placard does not get warm during shaking. Allow cold reagents and plates to equilibrate to room temperature. Uneven heat distribution can result in assay variability.
Critical reagents	All assay components critical to the assay's performance characteristics should be identified, well characterized and controlled through life cycle management. For example, differences in the conditions used for labeling the drug that impact efficiency of conjugation reactions can result in signal differences between different batches of labeled drug.

Drug Precipitation

Topic	Recommendation
SULFO-TAG Labeling	If precipitation occurs upon labeling with SULFO-TAG, use a lower challenge ratio, e.g., 2:1 SULFO-TAG:drug, or a different pH buffer. Ensure that the drug has, on average, at least 1 SULFO-TAG per drug molecule otherwise it will behave like free drug and will decrease the sensitivity of the assay.
Biotinylation	If precipitation occurs upon labeling with biotin, use a lower conjugation ratio.

Background Decreases in the Presence of Unlabeled Drug

Assay background can decrease in the presence of unlabeled drug if the unlabeled drug competes with SULFO-TAG labeled drug in an interaction with the biotinylated drug or with non-specific binding of the SULFO-TAG labeled drug to the plate.

Control experiments should be carried out to determine if the cause is binding of the SULFO-TAG labeled drug to the biotinylated drug (in diluent or matrix) in the absence of ADA or to the plate (in diluent or matrix). Refer to *High Background* on page 33.

False Positives

False positives in bridging experiments can result from matrix-induced bridging of biotinylated and SULFO-TAG labeled drug by dimeric or multimeric drug targets such as soluble receptors or bivalent target proteins, as well as other potential sources of interference including heterophilic antibodies or rheumatoid factor that can bind to antibody based therapeutics.

Control experiments for heterophilic antibodies or rheumatoid factor can be carried out using a molecule that is related but different from the drug, e.g., for an antibody therapeutic, a biotinylated or SULFO-TAG labeled antibody of the same isotype raised in the same species can be substituted for the biotinylated or SULFO-TAG labeled drug in the bridging assay.

In the case of dimeric or multimeric target proteins, if an antibody is available against the target protein, assay signals can be compared with and without immunodepleting the target protein from serum samples to confirm specificity.

The levels of potentially interfering soluble drug target should be evaluated and compared to the expected levels in test samples to determine if the assay needs to be optimized for target interference. Several strategies can be explored to overcome soluble drug target interference. These include higher sample dilutions, pre-treatment of the sample with anti-target proteins that do not compete with the drug for target binding, pre-treatment of the sample to selectively denature the drug target, solid-phase extraction of the target or polyethylene glycol (PEG) precipitation.

Appendix: Converting Concentrations

Equation for Converting pmol/well to µg/mL

Convert amount of drug in pmol/well to drug concentration in µg/mL with the following information:

- Amount of drug per well in pmol
- Drug molecular weight (MW) in Da
- Volume biotinylated drug added per well in µL

The formula is as follows:

```
drug concentration (µg/mL) =
amount of drug per well (pmol) x MW (Da) / (volume (µL) x 1,000)
```

Example 1

What should the concentration of a 150 kD biotinylated drug in a 25 µL volume be so that 0.25 pmol of the drug is added to a well of the MSD 96-well 1-Spot Streptavidin plate?

- Amount of drug per well: 0.25 pmol
- Drug MW: 150,000 Da
- Volume: 25 µL

Use the equation as follows:

```
drug concentration (µg/mL) =
0.25 pmol x 150,000 Da / (25 µL x 1,000) =
1.5 µg/mL
```

Example 2

What should the concentration of a 75 kD biotinylated drug be in a master mix so that 0.25 pmol is added to a well of the MSD 96-well 1-Spot Streptavidin plate? In this example, 50 µL of master mix is added to 25 µL of sample in a polypropylene plate, and after incubation, 50 µL of the reaction mixture is transferred to a blocked MSD 96-well 1-Spot Streptavidin plate. Therefore, only two thirds or 33 µL of the original master mix is added to a well of the MSD 96-well 1-Spot Streptavidin plate.

- Amount of drug per well: 0.25 pmol
- Drug MW: 75,000 Da
- Volume biotinylated drug added to well: 33 µL

Use the equation as follows:

```
drug concentration (µg/mL) =
0.25 pmol x 75,000 Da / (33 µL x 1,000) =
0.5 µg/mL
```

Equation for Converting nM to µg/mL

Convert drug concentration in nM into µg/mL with the following information:

- Concentration of drug in nM
- Drug molecular weight (MW) in Da

The formula is as follows:

```
drug concentration (µg/mL) =
drug concentration (nM) x MW (Da) / 1,000,000
```

Example Antibody Drug

To prepare

- 15 nM solution of labeled drug
- for an antibody drug with molecular weight of 150,000 Da

Use the equation as follows:

```
(15 nM x 150,000 Da) / 1,000,000 = 2.2 µg/mL
```

Example Other Protein Drug

To prepare

- 15 nM solution of labeled drug
- for an antibody drug with molecular weight of 75,000 Da

Use the equation as follows:

```
(15 nM x 75,000 Da) / 1,000,000 = 1.1 µg/mL
```

Equation for Converting µg/mL to pmol

Convert drug concentration in µg/mL into amount of drug in pmol with the following information:

- Concentration of drug in µg/mL
- Volume in µL
- Drug molecular weight (MW) in Da

The formula is as follows:

```
amount of drug (pmol) =
drug concentration (µg/mL) x volume (µL) x 1,000 / MW (Da)
```

Example Antibody Drug

To prepare

- 25 µL solution of drug at 1 µg/mL
- for an antibody drug with molecular weight of 150,000 Da

Use the equation as follows:

```
(1 µg/mL x 25 µL x 1,000) / 150,000 Da = 0.167 pmol
```

Example Other Protein Drug

To prepare

- 25 µL solution of drug at 1 µg/mL
- for a protein drug with molecular weight of 75,000 Da

Use the equation as follows:

```
(1 µg/mL x 25 µL x 1,000) / 75,000 Da = 0.333 pmol
```

References

MSD recommends the following publications for supplemental reading on immunogenicity assay development. References [1] and [2] are white papers containing assay development and validation guidelines for immunogenicity assays. Guidelines for immunogenicity assays from EMEA and draft guidance from the FDA are described in references [3] and [4] respectively. References [5] and [6] feature the use of MSD technology for immunogenicity applications. Alternative assay designs to further enhance the drug tolerance of immunogenicity assays with the MSD platform are represented in references for SPEAD[7], BEAD[8], ACE[9], PandA[10], SPECL[13], and heat treatment[14]. Reference[15] has examples using 300 mM Glycine-HCl and 125 mM HCl for acidification.

While this guide was written for measuring anti-drug antibodies, similar techniques and protocols are used to measure circulating levels of antibodies against endogenous protein. Examples of auto-antibody detection via bridging assay are provided for anti-IL-17[16], anti-insulin[17], and anti-BARD1[18].

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