MESO SCALE DISCOVERY

MULTI-ARRAY Assay System

Bridging Immunogenicity Assays
Guidelines for Assay Development

Guidelines for Assay Development

Bridging Immunogenicity Assays

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY
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Ordering Information

ordering information

MSD Customer Service

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MSD Scientific Support

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Introduction

introduction

MESO SCALE DISCOVERY® offers a range of assay development materials and kits suitable for the implementation of immunogenicity assays, neutralization assays (binding, cell-based, and cytokine assays) and PK assays. The MSD® 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 drug types including antibodies, humanized antibodies, 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 since only one wash step is required. The development of MSD bridging immunogenicity assays is rapid and these assays exhibit a better tolerance to the presence of drug in test samples than ELISA and other platforms. The assay does not require any species-specific reagents and is thus independent of species and isotype allowing the same assay format to be used for both preclinical and clinical assays.

This product insert will focus on the development of bridging immunogenicity assays (shown in Figure 1) utilizing MSD Assay Development Pack and MSD technology. The note provides lists of reagents, reagent preparation, protocols, suggested plate layouts and sample data. Assay troubleshooting information is also included, as well as a discussion section. A drug tolerance experiment is discussed to provide a practical example of a bridging immunogenicity assay.

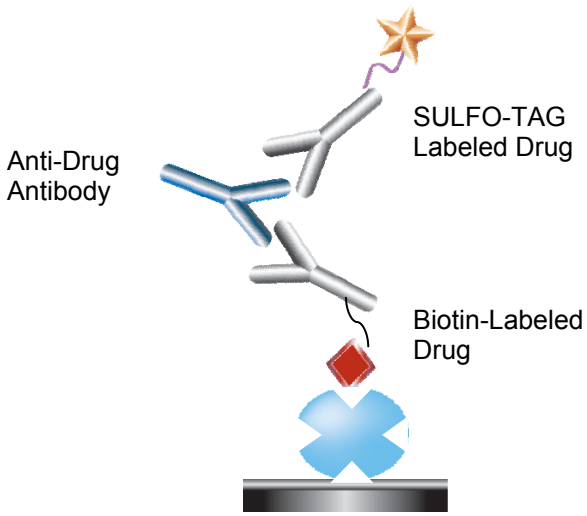

Figure 1. Bridging Immunogenicity Assay on Streptavidin/High Bind Avidin Gold Plate.

Recommended MSD Products

Recommended MSD Products

Immunogenicity Development Pack for Sector Imager K11A04-1 (SI6000), K15A04-1 (SI2400)	**Storage**
MULTI-ARRAY® 96-well Streptavidin Gold Plate[1] L15SA-1	2–8°C
MULTI-ARRAY 96-well High Bind Avidin Gold Plate[1] L15AB-1	2–8°C
MULTI-ARRAY 96-well High Bind Plate[1] L15XB-3	2–8°C
SULFO-TAG™ NHS Ester (150 nMoles)[1] R91AN-1	≤-10°C
SULFO-TAG Streptavidin (50 µg)[1] R32AD-5	2–8°C
SULFO-TAG Anti-Human Antibody (Goat) (50 µg)[1] R32AJ-5	2–8°C
Blocker A Kit[1] R93AA-1 (1 L)	RT
Blocker B (2 g)	RT
Spin Columns, 40K MWCO 2 mL	2–8°C
Read Buffer T (4X)[1] R92TC-2 (200 mL)	RT

Immunogenicity Development Pack for Sector PR K13A04-1 (PR400, PR100)	**Storage**
M ULTI-ARRAY® 96-well Streptavidin Gold Plate[1] L13SA-1	2–8°C
MULTI-ARRAY 96-well PR High Bind Avidin Plate[1] L13AB-1	2–8°C
MULTI-ARRAY 96-well High Bind Plate[1] L11XB-3	2–8°C
SULFO-TAG™ NHS Ester (150 nMoles)[1] R91AN-1	≤-10°C
SULFO-TAG Streptavidin (50 µg)[1] R32AD-5	2–8°C
SULFO-TAG Anti-Human Antibody (Goat) (50 µg)[1] R32AJ-5	2–8°C
Blocker A Kit[1] R93AA-1 (1 L)	RT
Blocker B (2 g)	RT
Spin Columns, 40K MWCO 2 mL	2–8°C
Read Buffer T (4X)[1] R92TC-2 (200 mL)	RT

Note:

For additional assay development reagents and plate types, please visit us at www.mesoscale.com

[1] Also available for purchase as a separate item.

III Required Reagents - not supplied

required reagents — not supplied

- Additional Desalting columns can be purchased from the following vendors:
  - ✓ Thermo Scientific (Pierce Protein Research Products) Zeba™ Spin Desalting columns catalog numbers 87768 and 87769 for 200 – 450 µL samples, catalog numbers 87772 and 87773 for 700 – 4000 µL samples, catalog numbers 87766 and 87767 for 7 – 100 µL
  - ✓ Roche Diagnostics Quick Spin™ High Capacity Columns catalog number 03117928001
- Conical tubes for use with ZEBA Spin Desalting columns
- Unlabeled Drug
  The drug should be in PBS or HEPES buffered saline at 1-2 mg/mL at pH 7.4 – 7.9 and free of any carrier proteins.
- Biotin Labeled Drug
  If the drug is not biotinylated, biotinylation reagents such as EZ-Link® SULFO-NHS-LC-Biotin or EZ-Link SULFO-NHS-LC-LC-Biotin (Thermo Scientific (Pierce Protein Research Products)) will be required.
- Polypropylene plates or tubes
  A number of vendors supply 96-well polypropylene plates, few examples are given below:
  Corning®: catalog number 3365
  Nunc™: catalog number 267245
  Greiner: catalog number 650201 or 651201
  Note: polystyrene plates should not be used.
- Biotin Quantification Kit
  A number of vendors supply Biotin Quantification Kits, few examples are given below:
  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)
- Adhesive plate seals
- Microtiter plate shaker

IV Safety

safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines. Material Safety Data Sheets are available upon request.

V Preparation and Choosing Reagents

preparation and choosing reagents

Protocol at a Glance

1. Combine 50 µL of master-mix containing biotin labeled drug and SULFO-TAG-drug and 25 µL sample in polypropylene plate, seal and incubate 1 hour to overnight.
2. Transfer 50 µL of the solution to pre-blocked Streptavidin Gold plate. Seal and shake for 1 hour.
3. Wash assay plate; add 150 µL 2X Read Buffer T; read plate.

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 will serve as the capture molecule and the SULFO-TAG labeled drug will be the reporter in the bridging assay.

If the drug requires biotin conjugation, biotinylate the drug using a biotinylation reagent such as Pierce EZ-Link Sulfo-NHS-LC-Biotin or Sulfo-NHS-LC-LC-Biotin. Typical starting conjugation ratios are 10:1 or 20:1 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. If precipitation occurs upon labeling with biotin, use a lower conjugation ratio. 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.

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. 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.

If the drug requires SULFO-TAG conjugation, label the drug following the protocol outlined in the MSD SULFO-TAG NHS-Ester Application note, found at
www.mesoscale.com/CatalogSystemWeb/WebRoot/literature/notes/pdf/MSD_Sulfo_Tag_NHS_Ester.pdf

Important Notes for SULFO-TAG Labeling

- 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.
- Minimum drug concentration for good incorporation of SULFO-TAG is 1 mg/mL.
- For antibody drugs suggested starting conjugation (challenge) ratios are 12:1 and 6:1 SULFO-TAG: drug. If only one ratio is tested, a 10:1 challenge ratio is recommended.
- Lower challenge ratios may be required for lower molecular weight drugs.
- If precipitation occurs upon labeling with SULFO-TAG, use a lower challenge ratio e.g. 2:1 SULFO-TAG: drug.
- Measure SULFO-TAG incorporation with absorbance at 455 nm, and measure protein concentration with a BCA assay using an IgG standard when determining concentration of antibody therapeutics, or 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 significant light absorption at this wavelength.
- Lower incorporation ratios of SULFO-TAG will yield lower background and lower signals.
- 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.

- If initial experiments indicate that increased incorporation of SULFO-TAG is required, the following parameters may be tested: use a higher conjugation ratio (e.g. 20:1), increase pH of the drug solution to 8.2 before labeling, and increase the drug concentration before labeling.
- Store SULFO-TAG labeled drug in a dark environment.

To ensure long-term stability, buffer exchange the labeled drug into formulation buffer. Labeled drug can be stored under the same conditions as those optimized for long-term stability of unlabeled drug.

Plate Types

We recommend the following two plate types for developing a bridging immunogenicity assay:

- MULTI-ARRAY 96-well Streptavidin Gold Plate
- MULTI-ARRAY 96-well High Bind Avidin Gold Plate *(for Sector Imager instruments only)*

Begin assay development on a Streptavidin Gold plate, and use optimized assay conditions to test other plate types and assess any improvement in assay performance.

Assay sensitivity is usually better for Standard plates than for the corresponding High Bind plates. High Bind plates have a greater binding capacity and therefore a larger dynamic range allowing higher ADA concentrations to be measured.
Avidin and Streptavidin have different isoelectric points. The net charge of the biotinylated capture molecule in the sample matrix may therefore influence its binding to avidin and streptavidin. The optimal plate choice should be empirically determined.

Blocking Solution

The choice of blocking solution will be driven by assay performance in the initial experiments. A good starting point is 3% (w/v) MSD Blocker A in PBS-T or PBS. To prepare this solution 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 and discard after 14 days.

Other blocking solutions that can be tested if required include casein blocker and 1% MSD Blocker B. 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 solution 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

Prepare a 2X solution of MSD Read Buffer T by combining equal volumes of 4X MSD Read Buffer T and distilled or deionized H_2O. Each plate will require 20 mL 2X Read Buffer T.

Read Buffer concentration and temperature can influence absolute ECL signals therefore, for assays where signals are compared across multiple plates; a large batch of diluted Read Buffer T can be prepared and stored in tightly sealed containers. Diluted Read Buffer will have the same expiration date as the stock as long as it is stored correctly. Read Buffer should be stored in a tightly capped container at room temperature.

VI Preliminary Experiments

preliminary experiments

In this section, preliminary experiments designed to optimize Bridging Immunogenicity Assay are described. The parameters to be optimized include the concentration of SULFO-TAG labeled and biotinylated drug and the minimum required sample dilution.

1. Optimization of Reagent Concentrations and Testing Sensitivity
    a. Testing Sensitivity using equimolar amounts of biotin labeled and SULFO-TAG labeled drug.
    b. Checkerboard optimization of concentrations of biotin labeled and SULFO-TAG labeled drug.
2. Testing Matrix Tolerance
3. Testing Free Drug Tolerance
4. Acid Dissociation to Improve Drug Tolerance

1. Optimization of Reagent Concentrations and Testing Sensitivity

a. Testing sensitivity using a master mix containing equal concentrations of biotinylated and SULFO-TAG labeled drug.

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

Reagent Preparation

Anti-Drug Antibody Samples

Prepare 100% and 25% normal or naïve serum or plasma. Dilute serum or plasma in assay diluent e.g. 1% (w/v) Blocker A in PBS-T.

Prepare a dilution series of anti-drug antibody (ADA) in 100% and 25% normal or naïve serum or plasma.
Recommended test concentrations of ADA are 10000; 2500; 625; 156.3; 39.1; 9.8; 2.4 and 0 ng/mL. Each well will receive 25 µL of ADA sample. The range of test concentrations of ADA can be adjusted if required.

If following the plate layout shown below in Figure 2, the following dilution scheme can be followed:

Prepare 400 µL of 10000 ng/mL ADA in 100% and 25% serum or plasma

Perform a 1 in 4 serial dilution in 100% and 25% serum or plasma respectively:

2500 ng/mL = 100 µL 10000 ng/mL ADA + 300 µL serum or plasma
625 ng/mL = 100 µL 2500 ng/mL ADA + 300 µL serum or plasma
156.3 ng/mL = 100 µL 625 ng/mL ADA + 300 µL serum or plasma
39.1 ng/mL = 100 µL 156.3 ng/mL ADA + 300 µL serum or plasma
9.8 ng/mL = 100 µL 39.1 ng/mL ADA + 300 µL serum or plasma
2.4 ng/mL = 100 µL 9.8 ng/mL ADA + 300 µL serum or plasma
0 ng/mL = 100% and 25% serum or plasma

		Biotinylated and SULFO-TAG labeled Drug											
		0.25	µg/mL	0.5	µg/mL	1	µg/mL	0.25	µg/mL	0.5	µg/mL	1	µg/mL
		1	2	3	4	5	6	7	8	9	10	11	12
Anti-Drug Antibody	A	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL
	B	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL
	C	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL
	D	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL
	E	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL
	F	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL
	G	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL
	H	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL
		ADA in 25 % serum/plasma						ADA in 100 % serum/plasma					

Figure 2. Sample plate layout for testing sensitivity using equal concentrations of biotinylated and SULFO-TAG labeled antibody drug using a Streptavidin Gold plate.

SULFO-TAG and Biotinylated Drug Mastermix

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 will receive 50 µL Master Mix; prepare 2 mL of each concentration if following the layout shown in Figure 2.
For antibody therapeutic drugs, recommended amounts labeled drug to test in master mix are:

- Streptavidin Gold plates: 1.0; 0.5; 0.25 and 0.125 µg/mL biotinylated and SULFO-TAG labeled antibody drug.
- High Bind Avidin Gold plates: 2.0; 1.0; 0.5 and 0.25 µg/mL biotinylated and SULFO-TAG labeled antibody drug.

Note:

Do not exceed the recommended amounts of biotinylated drug specified for the plate type.

For Streptavidin Gold plates, do not exceed 0.3 pmoles of biotinylated drug per well.
For High Bind Avidin Gold plates, do not exceed 0.6 pmoles of biotinylated drug per well.

For example, a 1 µg/mL concentration of biotinylated antibody drug corresponds to 0.33 pmoles in 50 µL of mastermix. The mastermix is incubated with 25 µL of sample in a polypropylene plate and 50 µL of the incubation mixture is transferred to the Streptavidin Gold plate, therefore only two thirds of the biotinylated drug in the incubation mixture or 0.22 pmole biotinylated drug is transferred to the Streptavidin Gold 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 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.

Please refer to Appendix Section (page 26) for conversion of pmoles to µg/mL.

Protocol

1. Add 50 µL of Master Mix (containing biotinylated drug and SULFO-TAG labeled drug mixture), and 25 µL of sample or standard to each well of a round-bottom 96-well polypropylene plate. Refer to the suggested plate layout in Figure 2. Seal the plate and incubate for 1-2 hours at room temperature with moderate shaking or overnight at 4˚C.
2. During the Master Mix incubation, add 150 µL per well of Blocking Solution (e.g. 3% Blocker A in PBS-T) to the Streptavidin Gold plate. Seal the plate and incubate at least 30 minutes at room temperature.
3. Remove the Blocking Solution from Streptavidin Gold plate. Wash the plate with wash buffer e.g. PBS-T. Transfer 50 µL from each well of the polypropylene plate to the plate. Seal the plate and incubate with shaking for 1 hour at room temperature at 300-700 rpm.
4. Wash the plate 3 times with at least 200 µL per well of wash buffer. Add 150 µL per well of 2X Read Buffer T and read on SECTOR® instrument. Avoid introducing bubbles when adding Read Buffer. This can easily be achieved using reverse pipetting techniques.

Example Data

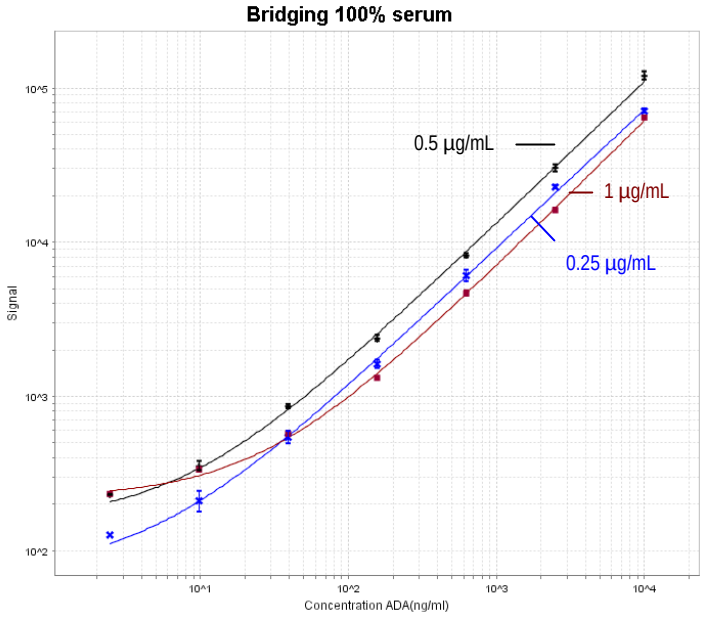

Figure 3. Example data from sensitivity experiment using equal amounts of biotinylated and SULFO-TAG labeled drug using a Streptavidin Gold plate. In this example, further studies were performed using a master mix where the final amount of biotinylated and SULFO-TAG labeled drug added per well of the plate was 0.5 µg/mL each. LLOD of the assay under these conditions was 3 ng/mL.

b. Checkerboard optimization of concentrations of biotin labeled and SULFO-TAG labeled drug.

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 will be tested in a checkerboard layout with a mid-range and zero anti-drug antibody (ADA) concentration.

Reagent Preparation

Anti-Drug Antibody Samples

Prepare a 500 ng/mL standard of the anti-drug-antibody (ADA) in normal or naïve serum to mimic the sample. Prepare an equal volume of normal or naïve serum with no anti-drug antibody added to serve as a 0 ng/mL control. Initial experiments can be done with 50% serum and the appropriate dilutions made in later experiments if required. Each well will receive 25 µL sample; prepare 1500 µL of 500 ng/mL ADA in 50% serum and 1500 µL 50% serum if following the layout shown in Figure 4.

			Biotinylated Drug											
			2 µg/mL	1 µg/µL	0.5 µg/mL	0.25 µg/mL	0.125 µg/mL	0 µg/mL	2 µg/mL	1 µg/µL	0.5 µg/mL	0.25 µg/mL	0.125 µg/mL	0 µg/mL
			1	2	3	4	5	6	7	8	9	10	11	12
SULFO-TAG Drug	4 µg/mL	A	500 ng/mL Anti-Drug Antibody in Serum						0 ng/mL Anti-Drug Antibody in Serum					
	2 µg/mL	B												
	1 µg/mL	C												
	0.5 µg/mL	D												
	0.25 µg/mL	E												
	0.125 µg/mL	F												
	0.062 µg/mL	G												
	0 µg/mL	H												

Figure 4. Sample plate layout for testing sensitivity using a checkerboard titration of biotinylated and SULFO-TAG labeled drug using a Streptavidin Gold plate.

Biotinylated Drug

Prepare 6 different concentrations of biotinylated drug in Assay Diluent (e.g. 1% Blocker A in PBS-T). Each well will receive 25 µL; prepare 500 µL of each concentration if following the layout shown in Figure 4.
Recommended concentrations of biotinylated drug for antibody therapeutics:

- Streptavidin Gold plates: 2; 1; 0.5; 0.25; 0.125; 0 µg/mL
- High Bind Avidin Gold plates: 4; 2; 1.0; 0.5; 0.25; 0 µg/mL

For example:
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 µg/mL: 500 µL Assay Diluent

SULFO-TAG Labeled Drug

Prepare 8 different concentrations of SULFO-TAG labeled drug in Assay Diluent. Each well will receive 25 µL; prepare 500 µL of each concentration if following the layout shown in Figure 4.
Recommended concentrations of SULFO-TAG labeled drug for antibody therapeutics:

- Streptavidin Gold plates: 4; 2; 1; 0.5; 0.25; 0.125; 0.62; 0 µg/mL
- High Bind Avidin Gold plates: 8; 4; 2; 1.0; 0.5; 0.25; 0.125; 0 µg/mL

For example:
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

Note:

Do not exceed the recommended amounts of biotinylated drug specified for the plate type.

For Streptavidin Gold plates, do not exceed 0.3 pmoles of biotinylated drug per well.
For High Bind Avidin Gold plates, do not exceed 0.6 pmoles of biotinylated drug per well.

For example, 25 µL of biotinylated antibody drug at 2 µg/mL corresponds to 0.33 pmoles. 25 µL of the biotinylated drug is incubated with 25 µL SULFO-TAG labeled drug and 25 µL sample in a polypropylene plate. 50 µL of the incubation mixture is transferred to the Streptavidin Gold plate, therefore only 0.22 pmole or two thirds of the biotinylated drug in the original 25 µL of 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 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.

Please refer to Appendix Section (page 26) for conversion of pmoles to µg/mL.

Protocol

1. Add 25 µL of biotinylated drug and 25 µL of SULFO-TAG labeled drug to each well of a round-bottom polypropylene 96-well plate according to the plate layout shown in Figure 4. Add 25 µL of standard (500 or 0 ng/mL anti-drug antibody in serum) to each well. Refer to suggested plate layout in Figure 4. Seal plate and incubate for 1-2 hours at room temperature with moderate shaking.
2. During the incubation of the sample and the biotinylated and SULFO-TAG labeled drug, add 150 µL per well of Blocking Solution to the Streptavidin Gold plate. Seal the plate and incubate with shaking at least 30 minutes at room temperature.
3. Remove Blocking Solution from the plate. Wash once with PBS-T, if desired. Transfer 50 µL from each well of the polypropylene plate to the Streptavidin Gold plate. Seal plate and incubate for 1 hour at room temperature with shaking (300-700 rpm).
4. Wash plate 3 times with at least 200 µL per well of wash buffer. Add 150 µL per well of 2X Read Buffer T and read on SECTOR instrument. Avoid introducing bubbles when adding Read Buffer. This can easily be achieved using reverse pipetting techniques.

Example Data

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

Figure 5. Example Data from a checkerboard optimization experiment for 500 and 0 ng/mL of anti-drug antibody. Since background signal did not change much with varying levels of biotinylated and SULFO-TAG labeled drug, signal was used as the indicator of optimal labeled drug concentration. 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.

2. Testing Matrix Tolerance

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

Reagent Preparation

Mastermix

Prepare 6 mL per plate of master mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in Assay Diluent. Each well will receive 50 µL of Master Mix.

Note:

If the concentration of SULFO-TAG and biotinylated drug was optimized using a checkerboard experiment as described above, the optimal concentrations of biotinylated and SULFO-TAG labeled drug selected from the checkerboard should be halved 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 or plasma diluted in Assay Diluent.
2000 µL of each serum or plasma concentration will be required if following the suggested layout in Figure 6.

Samples

Prepare a dilution series of anti-drug antibody (ADA) in each serum or plasma concentration. Recommended concentrations of ADA are 10000; 2500; 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. Each well will receive 25 µL of ADA sample.

For example:
10000 ng/mL – Prepare 500 µL10000 ng/mL (10 µg/mL) control ADA in sample matrix
2500 ng/mL – 50 µL 10000 ng/mL ADA + 150 µL sample matrix
625 ng/mL – 50 µL 2500 ng/mL ADA + 150 µL sample matrix
156.6 ng/mL – 50 µL 625 ng/mL ADA + 150 µL sample matrix
39.1 ng/mL – 50 µL 156.6 ng/mL ADA + 150 µL sample matrix
9.8 ng/mL – 50 µL 39.1 ng/mL ADA + 150 µL sample matrix
2.4 ng/mL – 50 µL 9.8 ng/mL ADA + 150 µL sample matrix
0 ng/mL – 150 µL sample matrix

		3.12% Serum		6.25% Serum		12.50% Serum		25% Serum		50% Serum		100% Serum	
		1	2	3	4	5	6	7	8	9	10	11	12
Anti-Drug Antibody	A	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL
	B	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL
	C	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL
	D	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL
	E	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL
	F	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL
	G	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL
	H	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL

Figure 6. Sample plate layout for testing matrix tolerance.

Protocol

1. Add 50 µL of Master Mix (containing biotinylated drug and SULFO-TAG labeled drug mixture), and 25 µL of sample or standard to each well of a 96-well round-bottom polypropylene plate. Refer to the suggested plate layout in Figure 6. Seal the plate and incubate for 1-2 hours at room temperature with moderate shaking or overnight at 4˚C.
2. During the Master Mix incubation, add 150 µL per well of Blocking Solution to the Streptavidin Gold plate. Seal the plate and incubate with shaking at least 30 minutes at room temperature.
3. Remove Blocking Solution from the plate. Wash once with PBS-T, if desired. Transfer 50 µL of the incubation mixture from each well of the polypropylene plate to the Streptavidin Gold plate. Seal the plate and incubate for 1 hour at room temperature with shaking (300-700 rpm).
4. Wash the plate 3 times with at least 200 µL per well of wash buffer. Add 150 µL per well of 2X Read Buffer T and read on SECTOR instrument. Avoid introducing bubbles when adding Read Buffer. This can easily be achieved using reverse pipetting techniques.

Example Data

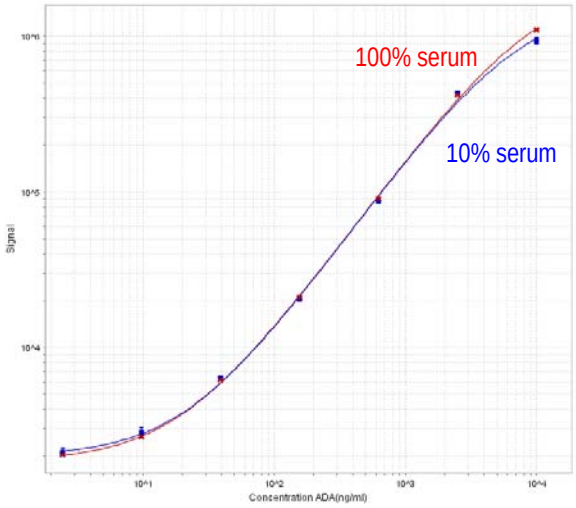

Figure 7. Example data from matrix tolerance experiment. In this example, there is little difference between the assay carried out in 100% and 10% serum.

3. Testing Free Drug Tolerance

MSD bridging assays are more tolerant of drug interferences which can cause false negatives and suppressed signal especially when high affinity immune complexes are formed between the drug and the anti-drug antibodies in the sample. The MSD bridging assay described in this insert utilizes 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 anti-drug antibodies 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 anti-drug antibody immune complex and enables the biotinylated and SULFO-TAG labeled drug present in excess to associate with the anti-drug antibody 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 anti-drug antibody 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.

Reagent Preparation

Mastermix

Prepare 6 mL per plate of Master Mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in Assay Diluent. Each well will receive 50 µL of master mix.

Anti-Drug Antibody Samples

Prepare a dilution series of anti-drug antibody at 2X final concentration in serum. Recommended 2X concentrations of ADA are 20000; 5000; 1250; 312.5; 78.1; 19.5; 4.8 and 0 ng/mL. Each well will receive 25 µL of ADA sample. 800 µL of each solution will be required if following the suggested layout in Figure 8.

For example:
20000 ng/mL – Prepare 1.2 mL 20000 ng/mL (20 µg/mL) solution of control ADA in serum
5000 ng/mL – 300 µL 20000 ng/mL ADA + 900 µL serum
1250 ng/mL – 300 µL 5000 ng/mL ADA + 900 µL serum
312.5 ng/mL – 300 µL 1250 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

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.
800 µL of each solution will be required if following the suggested layout in Figure 8.
Other concentrations of unlabeled drug based on the results of PK assays or expected circulating concentrations may also be tested.

For example:
100 µg/mL – Prepare 1.2 mL 100 µg/mL solution of unlabeled drug in serum
25 µg/mL – 300 µL 100 µg/mL drug + 900 µL serum
6.25 µg/mL – 300 µL 25 µg/mL drug + 900 µL serum
1.56 µg/mL – 300 µL 6.25 µg/mL drug + 900 µL serum
0.39 µg/mL – 300 µL 1.56 µg/mL drug + 900 µL serum
0 µg/mL - 900 µL serum

Free Drug		0	µg/mL	0.19	µg/mL	0.78	µg/mL	3.125	µg/mL	12.5	µg/mL	50	µg/mL
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Anti-Drug Antibody	**A**	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL
	B	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL
	C	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL
	D	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL
	E	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL
	F	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL
	G	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL
	H	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL

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

1. In a polypropylene plate, mix 50 µL 2X anti-drug antibody and 50 µL 2X free drug, seal and incubate with moderate shaking for 1h.
2. In a new polypropylene plate, add 50 µL of Master Mix (containing biotinylated drug and SULFO-TAG labeled drug mixture), and 25 µL of the anti-drug and unlabeled drug mixture to each well of a round-bottom polypropylene 96-well plate. Refer to suggested plate layout in Figure 8. Seal plate and incubate with moderate shaking overnight at 4˚C.
3. Add 150 µL per well of Blocking Solution to the Streptavidin Gold plate. Seal the plate and incubate with shaking at least 30 minutes at room temperature.
4. Remove Blocking Solution from the plate. Wash the Streptavidin Gold plate with wash buffer e.g. PBS-T. Transfer 50 µL from each well of the polypropylene plate to the Streptavidin Gold plate. Seal the plate and incubate for 1 hour at room temperature with shaking (400-700 rpm).
5. Wash plate 3 times with at least 200 µL per well of wash buffer. Add 150 µL per well of 2X Read Buffer T and read on SECTOR instrument. Avoid introducing bubbles when adding Read Buffer. This can easily be achieved using reverse pipetting techniques.

Example Data

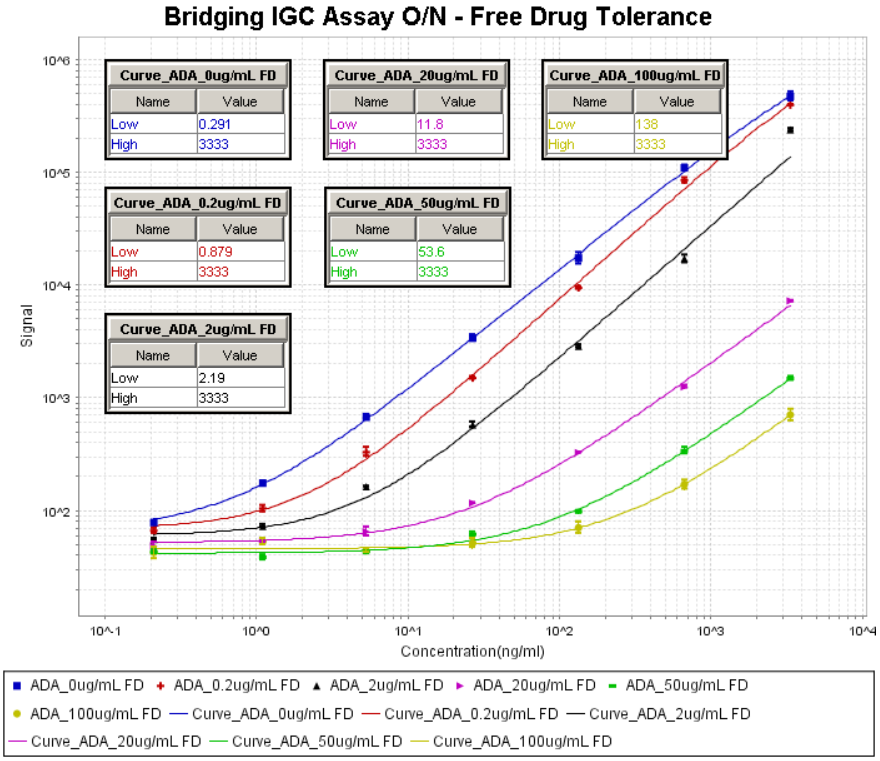

Figure 9. Example data from free drug tolerance experiment. The concentration of ADA (ng/mL) is indicated on the x-axis. In this example 150 ng/mL ADA is still detectable in the presence of 100 µg/mL unlabeled drug.

4. 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 [1,2]. In this protocol, the sample is treated with acid to dissociate ADA complexed to drug and then neutralized in the presence of excess SULFO-TAG labeled and biotinylated drug.

[1.] A. Patton, et. al. (2005) Journal of immunological methods 304:189-195.
[2.] J. Lofgren et al. (2006) Journal of immunological methods 308: 101-108.

Additional Reagent Required

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

Reagent Preparation

Mastermix

Prepare 12 mL per plate of Master Mix containing optimized concentrations of biotinylated and SULFO-TAG labeled drug in Assay Diluent. Each well will receive 90 µL of Master Mix.

Anti-Drug Antibody Samples

Prepare a dilution series of anti-drug antibody at 2X final concentration in serum. Suggested 2X concentrations of ADA are 20000; 5000; 1250; 312.5; 78.1; 19.5; 4.8 and 0 ng/mL. Each well will receive 25 µL of ADA sample. 800 µL of each solution will be required if following the suggested layout in Figure 10.

For example:
20000 ng/mL – Prepare 1.2 mL 20000 ng/mL (20 µg/mL) solution of control ADA in serum
5000 ng/mL – 300 µL 20000 ng/mL ADA + 900 µL serum
1250 ng/mL – 300 µL 5000 ng/mL ADA + 900 µL serum
312.5 ng/mL – 300 µL 1250 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

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.

800 µL of each solution will be required if following the suggested layout in Figure 10.
Other concentrations of unlabeled drug based on the results of PK assays or expected circulating concentrations may also be tested.

For example:
100 µg/mL – Prepare 1.2 mL 100 µg/mL solution of unlabeled drug in serum
25 µg/mL – 300 µL 100 µg/mL drug + 900 µL serum
6.25 µg/mL – 300 µL 25 µg/mL drug + 900 µL serum
1.56 µg/mL – 300 µL 6.25 µg/mL drug + 900 µL serum
0.39 µg/mL – 300 µL 1.56 µg/mL drug + 900 µL serum
0 µg/mL – 900 µL serum

Free Drug		0	µg/mL	0.19	µg/mL	0.78	µg/mL	3.125	µg/mL	12.5	µg/mL	50	µg/mL
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Anti-Drug Antibody	**A**	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL	10000	ng/mL
	B	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL	2500	ng/mL
	C	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL	625	ng/mL
	D	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL	156.3	ng/mL
	E	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL	39.1	ng/mL
	F	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL	9.8	ng/mL
	G	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL	2.4	ng/mL
	H	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL	0	ng/mL

Figure 10. Sample plate layout for testing free drug tolerance with acid dissociation. The concentrations listed are the final concentrations of ADA and free drug in the sample before addition of the Master Mix reagents.

Protocol

1. In a polypropylene plate, mix 50 µL 2X anti-drug antibody and 50 µL 2X free drug, incubate with moderate shaking for 1h. Refer to the suggested plate layout in Figure 10.
2. Prepare new polypropylene plates with 180 µL per well of 300 mM Acetic Acid for acid treatment plate or PBS for the control plate. Transfer 20 µL of sample (ADA + Free Drug) from the first polypropylene plate to the treatment plates. Incubate shaking for 40min-45min at RT.
3. Add 150 µL per well of Blocking Solution to the Streptavidin Gold plate. Seal the plate and incubate with shaking at least 30 minutes at room temperature.
4. In a new polypropylene plate, add 90 µL per well of Master Mix (containing biotinylated drug and SULFO-TAG labeled drug mixture), and just before use, add 12 µL of 1.5 M Trizma Base pH 10 (or other neutralizing solution) for the acid treatment plate or PBS for the control plate. Transfer 50 µL of sample from the treatment plate to the Master Mix plate. Incubate for 1-2 hr shaking at RT.
5. Remove Blocking Solution from the Streptavidin Gold plate. Wash with the plate with wash buffer. Transfer 50 µL from each well of the polypropylene plate to the Streptavidin Gold plate. Seal the plate and incubate for 1 hour at room temperature with shaking (300-700 rpm).
6. Wash plate 3 times with at least 200 µL per well of wash buffer. Add 150 µL per well of 2X Read Buffer T and read on SECTOR instrument. Avoid introducing bubbles when adding Read Buffer. This can easily be achieved using reverse pipetting techniques.

Notes:

- 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 antibody controls.

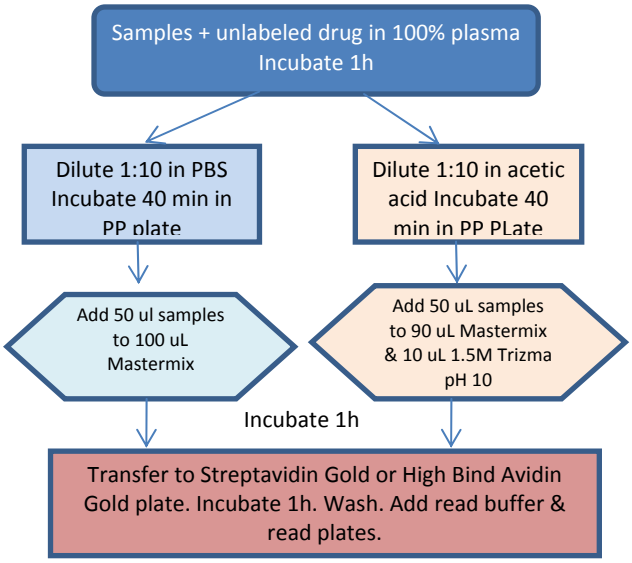

Figure 11. Overview of drug tolerance experiment with and without acid dissociation.

Example Data

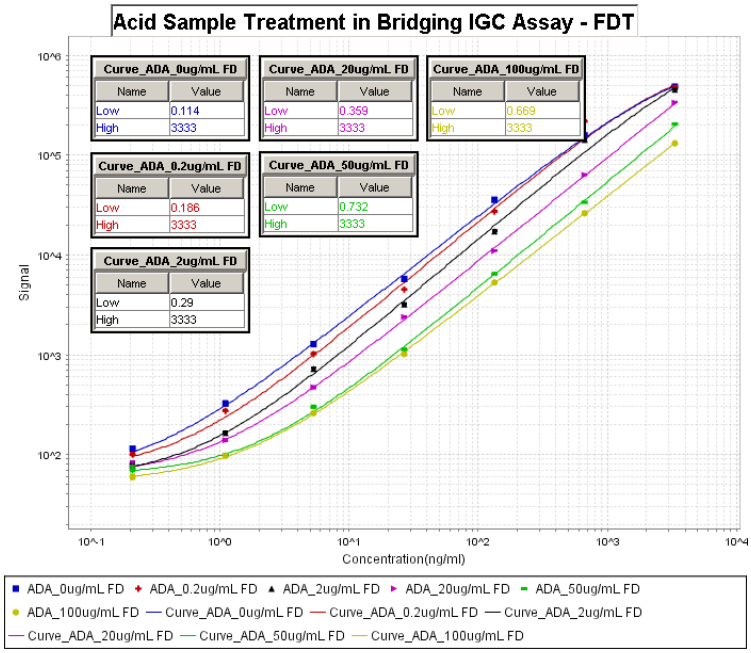

Figure 12. Example data from free drug tolerance experiment with acid dissociation. The concentration of ADA (ng/mL) is indicated on the x-axis. In this example 1 ng/mL ADA is still detectable in the presence of 100 µg/mL unlabeled drug.

VII Discussion and Troubleshooting

discussion and troubleshooting

MSD immunogenicity assays can be adjusted to improve performance in several different ways. In bridging assays, drug tolerance is improved by extending incubation of the sample, biotinylated drug and SULFO-TAG labeled drug 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.

- **Streptavidin Gold and High Bind Avidin Gold Plates**
  - The choice of plate is empirical. Streptavidin Gold plates are recommended for initial assay development after which other plate types may be tested.
  - *Streptavidin Gold Plates*: keep the final amount of biotinylated reagent added to Streptavidin Gold plates less than 0.3 pmole per well.
  - *High Bind Avidin Gold plates*: keep the final amount of biotinylated reagent added to High Bind Avidin Gold plates less than 0.6 pmole per well.

- **Signal levels**
  - Expected background signals levels for the SECTOR Imagers are around 60 to 100 counts (when using 2X Read Buffer T), and 200-300 counts on the SECTOR PR instruments.
  - The maximum signal for the assay should be less than 1 million counts as the top of the dynamic range of all of the SECTOR instruments is reached with maximum signals of 1 to 1.5 million counts for single spot plates.

- **Shaking During Incubation**
  - MSD recommends shaking the plates during incubation steps at > 300 rpm. Shaking increases diffusion kinetics and allows the binding equilibrium to be reached in a shorter period. Shaking speed should be kept consistent to minimize day to day variability.

- **Read Buffer concentrations**
  - MSD recommends using Read Buffer T at 2X concentration to prevent the background signals from being too close to 0 and to reduce variability in absolute signal when using different preparations of diluted Read Buffer.

Troubleshooting

High Backgrounds

Non-specific binding of SULFO-TAG labeled drug to the plate can lead to elevated background signals, this non-specific interaction can be identified by carrying out the assay in the absence of biotinylated drug. Control experiments should be performed to compare signals generated with SULFO-TAG labeled drug in matrix (e.g. serum or plasma) and in assay diluent to determine if the non-specific binding is linked to the drug or if it is a matrix-induced effect. Alternative assay diluents and/or blocking solutions should be tested to reduce non-specific binding.

Another potential source of high background is an interaction between the biotinylated and SULFO-TAG labeled drug in the absence of serum or plasma. 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.

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.

Low Assay Signals

Low assay signals may be indicative of inefficient labeling or inability of the biotinylated and SULFO-TAG labeled drug to bridge with the anti-drug antibody.

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, azide. Ensure that the drug is in an amine-free, carrier-free buffer prior to labeling.

Limited availability of reactive primary amine groups on the drug, either 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 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 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 Streptavidin Gold or Avidin Gold 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:

- Exceeding recommended concentrations of biotinylated reagent
- Read Buffer concentrations
- Variability introduced through pipetting
- Inconsistent shaking speed
- Variability introduced through plate washing equipment
- Dissociation rates
- Loss of specific activity of biotinylated and/or SULFO-TAG labeled drug

Exceeding the recommended capacity of the plates can lead to higher signal variability. The final amount of biotinylated drug loaded per well should be less than 0.3 pmole for Streptavidin Gold plates and less than 0.6 pmole for High Bind Avidin Gold plates. Refer to Appendix for formulas to convert pmole per well to µg/mL.

Higher concentrations of Read Buffer will lead to higher signals. The recommended Read Buffer concentration for immunogenicity assays is 2X MSD Read Buffer T. Read Buffer should be diluted in deionized water. Differences in preparation of diluted Read Buffer or evaporation of Read Buffer stocks can lead to differences in absolute signal values between plates. Read Buffer should be stored and used at room temperature.

Assay variability is often linked to pipetting differences due to equipment or differences between operators. Ensure that pipettes are calibrated and that the correct pipette tips are used. Repeater pipettes should be checked for accuracy for each dispense step.

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.

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.

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. MSD plates should therefore not be left in wash buffer and read time after addition of Read Buffer should be kept consistent.

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 the section on troubleshooting high background.

False positives

False positives in bridging experiments can result from matrix-induced bridging of biotinylated and SULFO-TAG labeled drug by soluble receptors, dimeric or bivalent target proteins, heterophilic antibodies or rheumatoid factor. 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 bivalent target proteins, if an antibody is available against the target protein, assay signals can be compared with and without immunodepletion the target protein from serum samples to confirm specificity.

VIII Reading an MSD Plate

reading an MSD plate

After addition of Read Buffer, avoiding any bubbles, analyze the plate with SECTOR Imager or SECTOR PR® instrument.

1. Place plate on single plate adapter or load into a stacker.
2. Double click on DISCOVERY WORKBENCH® icon on computer desktop (if not already open).
3. Click on the instrument icon in the upper left corner of the screen (if not already open to plate reading screen).
4. From the plate type pull down menu select “Read from Barcode “ if reading a whole plate (please refer to the instrument manual for partial plate instructions)
5. Check the “Read” box and enter the number of plates to be read.
6. Click the “Run” button.
7. Check the boxes for the desired export formats (if required, please refer to the instrument manual for instructions on editing export formats)
8. Select the location that the results file will be saved to in the “Output Path” dialog field.
9. Click OK to initiate the run.
10. The data will automatically be saved in the Plate Data History database and text versions of the results will be saved in the folder designated in the Output Path.

The introduction of bubbles during Read Buffer addition to the wells can interfere with reliable imaging of the plate. Reverse pipetting techniques are recommended for the addition of Read Buffer.

Plates can be imaged immediately after addition of Read Buffer. To ensure consistency in immunogenicity assays, the time between addition of Read Buffer and imaging of the plate should be consistent.

An all inclusive indelible copy of the data and associated meta data will be saved in the internal database regardless of the selections made for data file export. If required, additional copies of the data file may be exported from the Plate Data History database in any layout at any time. Consult the instrument manual for details.

IX Appendix

appendix

Equation 1

Converting pmole biotinylated drug added per well to drug concentration:

pmole per well x Drug MW (Da) / (µL biotinylated drug added to MSD plate x 1000) = µg/mL drug

Example 1
What should the concentration of a 150 kD biotinylated drug in a 25 µL volume be so that 0.25 pmole of the drug is added to the Streptavidin Gold plate?

pmole per well = 0.25
Drug MW = 150000
µL biotinylated drug added to MSD plate = 25

µg/mL drug = 0.25 x 150000 / (25 x 1000) = 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 pmole is added to the Streptavidin Gold 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 Streptavidin Gold plate. Therefore only two thirds or 33 µL of the original master mix is added to the Streptavidin Gold plate.

pmole per well = 0.25
Drug MW = 75000
µL biotinylated drug added to MSD plate = 33

µg/mL drug = 0.25 x 75000 / (33 x 1000) = 0.5 µg/mL

Equation 2

Converting nM drug concentration to µg/mL drug concentration:

nM concentration of drug x Drug MW (Da) / 1000000 = µg/mL drug

Examples:

For an antibody drug (MW = 150000 Da)
To prepare a 15 nM solution of labeled drug = (15 nM x 150000 Da)/ $1e^6$ ~ 2.2 µg/mL

For a protein drug (MW = 75000 Da)
To prepare a 15 nM solution of labeled drug = (15 nM x 75000 Da)/ $1e^6$~ 1.1 µg/mL

Equation 3

Converting µg/mL drug concentration into pmole.

µg/mL concentration of drug x vol (µL) x 1000 / drug MW (Da) = pmole drug

For an antibody drug (MW = 150000 Da)
To prepare 25 µL solution of drug at 1 µg/mL = (1µg/mL x 25 µL x 1000) / 150000 Da = 0.167 pmole

For a protein drug (MW = 75000 Da)
To prepare 25 µL solution of drug at 1 µg/mL = (1µg/mL x 25 µL x 1000) / 75000 Da = 0.333 pmole

X References

references

MSD recommends the following publications for supplemental reading on immunogenicity assay development. The first two references 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 the third and fourth references respectively. The fifth and sixth references describe the use of MSD technology for immunogenicity applications. The use of High Bind Avidin plates to address rheumatoid factor interference in a bridging immunogenicity assay is described in reference 7.

1. Mire-Sluis, A.R., et al. (2004) J. Immunol. Methods 289, 1-16.
2. Shankar, G., et al., (2008) J. Pharm. Biomed. Anal. 48 (5), 1267-1281.
3. EMEA guidelines on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins. http://www.emea.europa.eu/pdfs/human/biosimilar/1432706en.pdf
4. Guidance for Industry, Assay Development for Immunogenicity Testing for Therapeutic Proteins. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf
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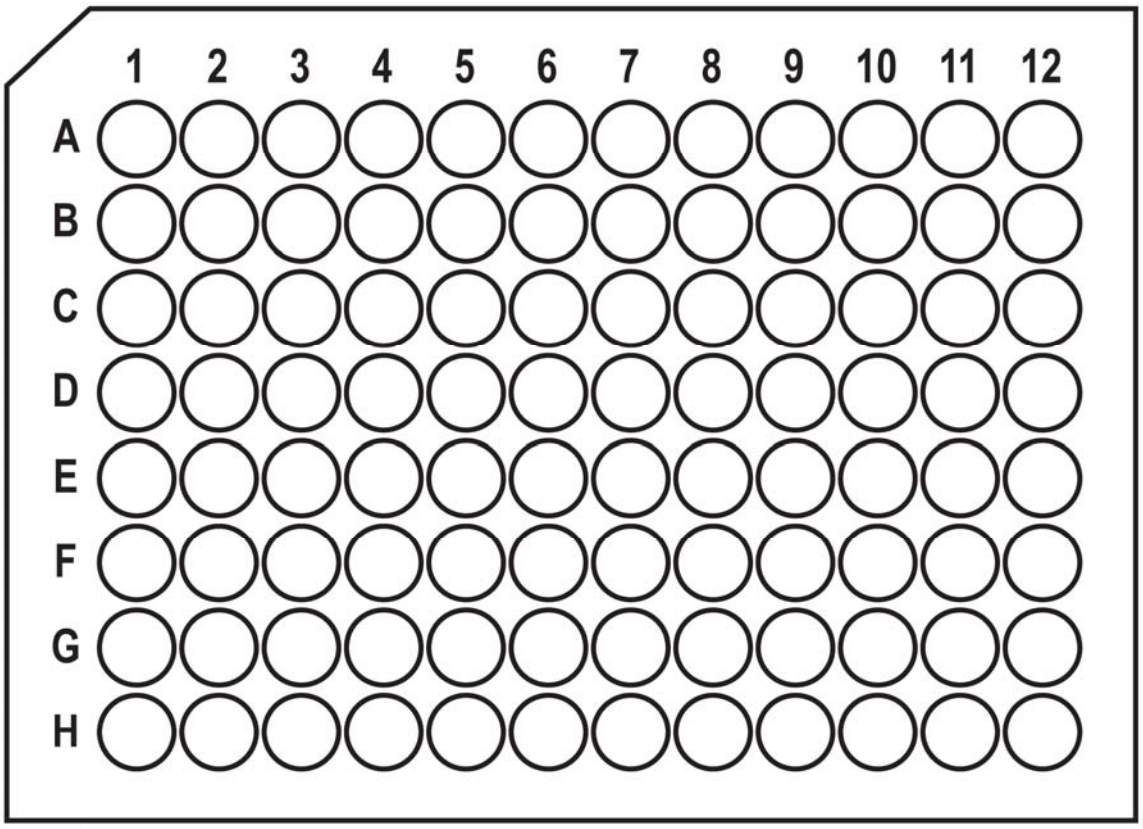
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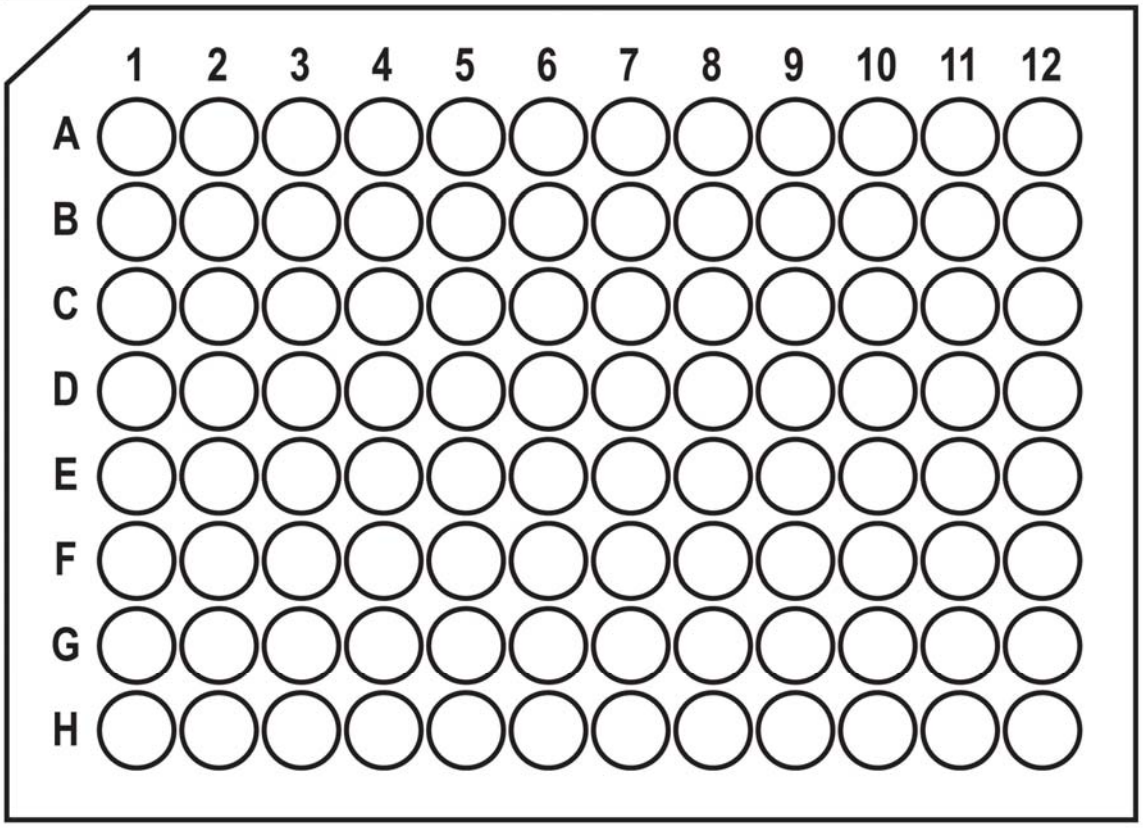
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