U-PLEX®

Immunogenicity Assay Application Note

Measuring anti-drug antibodies (ADAs) to two different drugs using a multiplex bridging assay

The use of biotherapeutics, biosimilars, and combination biotherapies require the study of immunogenicity, or the study of anti-drug antibodies (ADAs). While it is possible to measure ADAs to a single drug using MSD GOLD™ Streptavidin plates (see MSD's Bridging Immunogenicity Assays Guidelines for Assay Development available at www.mesoscale.com), there is an increasing need to measure ADAs to multiple drugs in a single sample due to the combined use of multiple biotherapeutics, or, as in the case of multi-domain therapeutics, measure ADAs in a single sample to different domains of a drug. MSD's U-PLEX® platform allows for the simultaneous measurement of multiple ADAs in a single well with a simple, two-step protocol, resulting in reduction of both time and sample volume.

This Application Note serves two purposes:

Section A provides a general overview of our multiplex screening immunogenicity assays and how they detect and measure ADAs in a single sample.

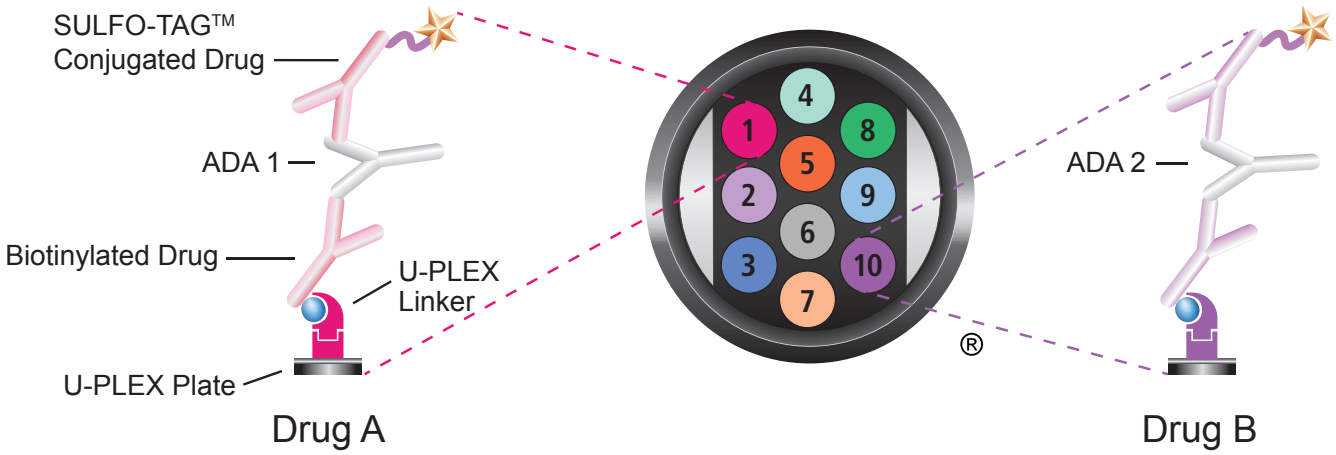
Section B provides a detailed protocol, workflow, and representative data that serve as guidelines for successfully developing, optimizing, and running an assay.

It's All About U™

A. General Overview

MSD's U-PLEX technology allows for the creation of multiplex assays through the use of U-PLEX assay development packs. These packs are available in different configurations of activated spots and U-PLEX Linkers, giving you the flexibility to develop custom multiplexes ranging from two to ten assays per well. Immunogenicity assays designed to detect anti-drug antibodies (ADAs) can be developed for different biologic-based platforms including antibodies, proteins, and peptides, on the U-PLEX platform. Figure 1 illustrates the principle of a multiplex immunogenicity assay as developed on our U-PLEX platform.

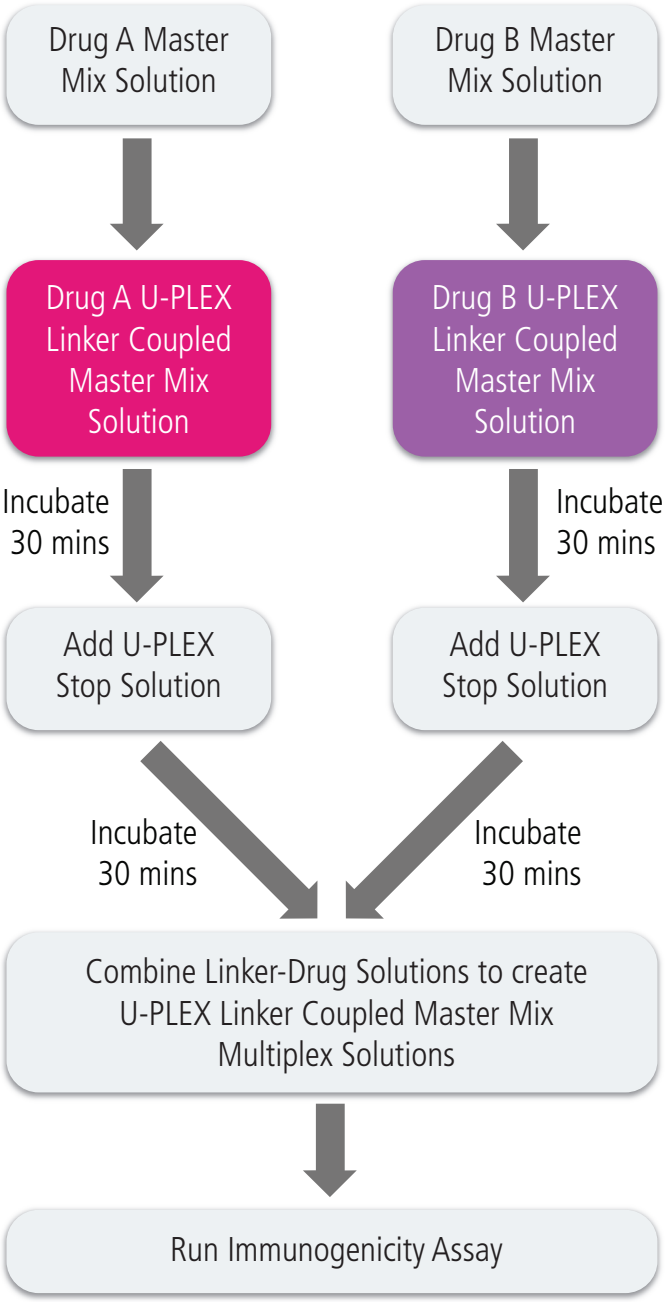
Figure 1: Multiplex immunogenicity assay on the U-PLEX platform.



To illustrate the principle of the assay, two bridging immunogenicity assays (one to detect ADA to Drug A and one to detect ADA to Drug B) were combined. Biotinylated Drug A was coupled to U-PLEX Linker 1 and Biotinylated Drug B was coupled to U-PLEX Linker 10. Both drugs were also conjugated with SULFO-TAG™. Spot 1 on the U-PLEX plate measured the signals generated from the bridging assay for Drug A and Spot 10 measured the signal generated from the bridging assay for Drug B. Two controls were also tested: An antibody with dual specificity that binds both Drug A and Drug B and a negative antibody that does not bind to either drug.

Protocol at a Glance

Figure 2: Overview of protocol for multiplex screening immunogenicity assays for two antibody drugs with MSD's U-PLEX technology.



A. Create a master mix for each drug (biotinylated drug and SULFO-TAG conjugated drug).

B. Add a unique U-PLEX Linker to each master mix.

C. Add Stop Solution.

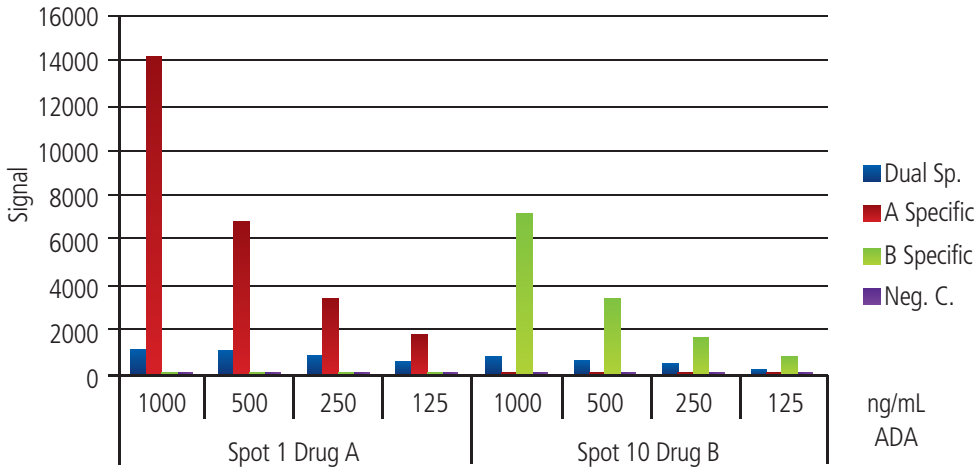
D. Combine the individual master mix solutions to prepare the multiplex master mix solution.

E. Add the multiplex master mix solution to the sample and incubate. Add the solution to the U-PLEX plate. Incubate for one hour, then add read buffer and read.

Figure 3: The data below represent 4 ADAs: dual specificity for Drug A and Drug B (Dual Sp.), specific for Drug A (A Specific), specific for Drug B (B Specific), and the negative control ADA (Neg. C.).

Spot 1 Drug A	Test ADA			
ng/mL	Dual Sp.	A Specific	B Specific	Neg. C.
1000	1149	14219	52	77
500	1060	6917	62	84
250	887	3391	58	58
125	562	1716	81	79

Spot 10 Drug B	Test ADA			
ng/mL	Dual Sp.	A Specific	B Specific	Neg. C.
1000	843	92	7212	75
500	698	53	3424	62
250	533	46	1694	71
125	288	28	850	77



Summary

As the field of biotherapeutics expands and the use of biosimilars and combination therapies becomes more prevalent, there is an increasing need to measure multiple ADAs in a single sample. The following sections of this Application Note provide detailed protocols, guidelines, and a representative workflow for the rapid development of sensitive, multiplexed immunogenicity/autoantibody assays[1-3]. The U-PLEX platform also offers multiplexing options for PK and biomarker assays to conserve sample volume, streamline workflows, and increase throughput.

1. Cludts I, et al. Detection of neutralizing interleukin-17 antibodies in autoimmune polyendocrinopathy syndrome-1 (APS-1) patients using a novel non-cell based electrochemiluminescence assay. Cytokine 50. 2010: 129-137.

2. Miao D, et al. GAD65 Autoantibodies detected by electrochemiluminescence assay identify high risk for Type 1 Diabetes. Diabetes. 2013 Dec; 62: 4174-4178.

3. Yu L, et al. Distinguishing persistent insulin autoantibodies with differential risk: Nonradioactive bivalent proinsulin/insulin autoantibody assay. Diabetes. 2012 Jan; 61: 179-186.

B. Developing a Multiplexed Immunogenicity Assay for Two Biotherapeutic Drugs

Optimizing Master Mix Concentrations

Optimization of the master mix concentrations is an essential step in the development of an immunogenicity assay. The next section describes the detailed protocol for this optimization step.

Reagent Preparation

The guidelines below are designed to help you prepare reagents that will optimize the master mix concentrations of each drug in the U-PLEX multiplex assay. The quantities listed are sufficient for the plate layout described on page 8.

A. Preparation of Master Mix Solutions

Prepare a separate series of master mix solutions for each drug to be multiplexed:

1. Prepare a master mix of 10 µg/mL biotinylated antibody drug and 10 µg/mL of MSD GOLD SULFO-TAG conjugated antibody drug in the assay buffer.
    a. Mix 120 µL of biotinylated drug (20 µg/mL) with 120 µL of MSD GOLD SULFO-TAG-drug (20 µg/mL).

2. Create 5 µg/mL, 2.5 µg/mL, and 1.25 µg/mL master mix solutions by performing three separate two-fold serial dilutions of the 10 µg/mL master mix:
    a. Drug master mix 5 µg/mL = 120 µL 10 µg/mL master mix + 120 µL assay buffer.
    b. Drug master mix 2.5 µg/mL = 120 µL 5 µg/mL master mix + 120 µL assay buffer.
    c. Drug master mix 1.25 µg/mL = 120 µL 2.5 µg/mL master mix + 120 µL assay buffer.

Note: The protocol above describes the use of equimolar biotinylated antibody drug and SULFO-TAG-antibody drug master mix solutions. To optimize the ratio of biotinylated antibody drug and SULFO-TAG-antibody drug in the master mix, please see the "Data Illustrating the Process for Selecting the Optimal Master Mix Concentration" section on page 9 of this Note, and refer to page 11 of the MSD Bridging Immunogenicity Assay Application Note for more detailed instructions. Once the optimal ratio of biotinylated antibody drug and SULFO-TAG conjugated antibody drug has been identified, the different master mix concentrations can be coupled to U-PLEX Linkers as described below. Use the concentrations of antibody drug described, and adjust the concentration of the SULFO-TAG antibody if the optimal master mix is not an equimolar mixture of biotinylated and SULFO-TAG drug. If required, please consult your local MSD Field Application Scientist for assistance.

B. Preparation of U-PLEX Linker-Coupled Master Mix Solutions

1. For Drug A: Transfer **120 µL** of **Linker 1** into four polypropylene microcentrifuge tubes. Label the tubes as '**Linker 1 Drug A**' and write the master mix concentration on each individual tube (e.g., tubes 1, 2, 3, and 4 should be labeled 'Linker 1_10 µg/mL', 'Linker 1_5 µg/mL', 'Linker 1_2.5 µg/mL' and 'Linker 1_1.25 µg/mL', respectively).

2. Add **80 µL** of **Drug A master mix (10 µg/mL)** to the '**Linker 1_10 µg/mL**' tube. Vortex to mix, and then spin down briefly. Repeat this step for the remaining Drug A master mix concentrations:
    a. Add 80 µL of Drug A master mix (5 µg/mL) to the 'Linker 1_5 µg/mL' tube.
    b. Add 80 µL of Drug A master mix (2.5 µg/mL) to the 'Linker 1_2.5 µg/mL' tube.
    c. Add 80 µL of Drug A master mix (1.25 µg/mL) to the 'Linker 1_1.25 µg/mL' tube.

3. For Drug B: Transfer **120 µL** of **Linker 10** into four polypropylene microcentrifuge tubes. Label the tubes as '**Linker 10 Drug B**' and write the master mix concentration on each individual tube (e.g., label the tubes 'Linker 10_10 µg/mL', 'Linker 10_5 µg/mL', 'Linker 10_2.5 µg/mL' and 'Linker 10_1.25 µg/mL', respectively).

4. Add **80 µL** of **Drug B master mix (10 µg/mL)** to the '**Linker 10_10 µg/mL' tube**. Vortex to mix, and then spin down briefly. Repeat this step for the remaining Drug B master mix concentrations:

    a. Add 80 µL of Drug B master mix (5 µg/mL) to the 'Linker 10_5 µg/mL' tube.
    b. Add 80 µL of Drug B master mix (2.5 µg/mL) to the 'Linker 10_2.5 µg/mL' tube.
    c. Add 80 µL of Drug B master mix (1.25 µg/mL) to the 'Linker 10_1.25 µg/mL' tube.

5. Incubate the solutions at room temperature (RT) for 30 minutes.

6. Add 80 µL of U-PLEX Stop Solution to all the "Linker 1" and "Linker 10" tubes. Use a fresh pipette tip for each addition[4]. Vortex and then spin down briefly. Incubate the solutions at RT for 30 minutes.

4. Use filter tips when pipetting U-PLEX Linkers. Open U-PLEX Linker vials one at a time to avoid the possibility of contamination.

C. Preparation of the U-PLEX Linker-Coupled Master Mix Multiplex Solution

1. Label four polypropylene tubes (15 mL) with the following concentrations: '10 µg/mL', '5 µg/mL', '2.5 µg/mL', and '1.25 µg/mL'. These tubes will contain the U-PLEX Linker-coupled master mix multiplex solutions.

2. Add 1.6 mL of U-PLEX Stop Solution into each tube.

3. Add 200 µL of each U-PLEX Linker-coupled antibody drug solution into its respective tube (i.e., add 200 µL 'Linker 1_10 µg/mL' and 200 µL 'Linker 10_10 µg/mL' into 1.6 mL of U-PLEX Stop Solution). Vortex to mix.

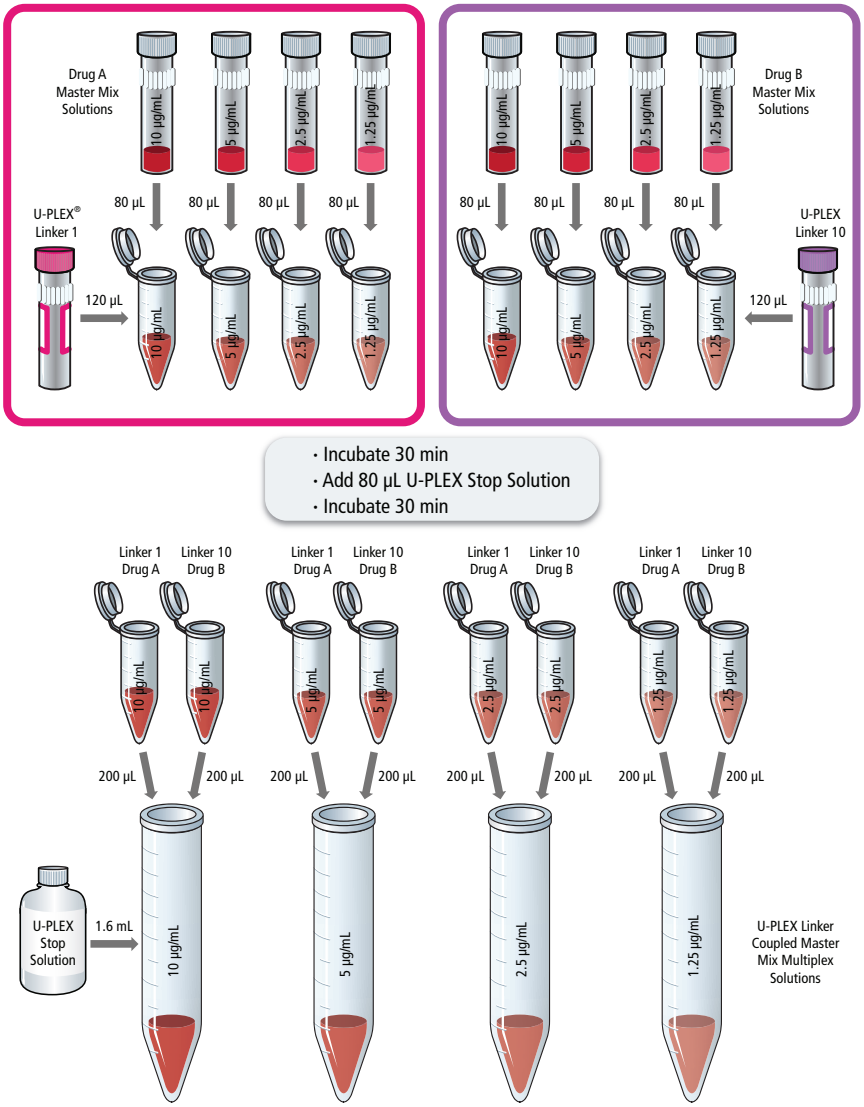


Figure 4: Schematic diagram illustrating the reagent preparation steps for optimizing a multiplex of two antibody drug screening immunogenicity assays using MSD's U-PLEX technology.

Volumes shown are sufficient for four master mix concentrations of biotinylated and SULFO-TAG conjugated antibody drug to coat 32 wells per master mix.

Table 1: Example plate layout

Anti-Drug Antibody	Master Mix Concentration (µg/mL)											
	1.25			2.5			5			10		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10,000 ng/mL			10,000 ng/mL			10,000 ng/mL			10,000 ng/mL		
B	2,500 ng/mL			2,500 ng/mL			2,500 ng/mL			2,500 ng/mL		
C	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		
E	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		
G	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		

U-PLEX Assay Protocol for Optimization of Master Mix

1. Add 50 µL of U-PLEX Linker-coupled master mix multiplex solution and 25 µL of positive control anti-drug antibody samples to each well of a round-bottom 96-well polypropylene plate. Seal the plate and incubate for one to two hours at RT with moderate shaking, or shake for one hour at RT and then incubate overnight at 4°C.

2. During the master mix incubation, add 150 µL per well of Blocking Solution (e.g., 3% [w/v] MSD Blocker A in PBS or PBS-T) to the U-PLEX plate. Seal the plate and incubate for one to two hours at RT with moderate shaking. Remove the Blocking Solution from the U-PLEX plate. Wash the plate with wash buffer (e.g., PBS-T). Transfer 50 µL from each well of the polypropylene plate to the corresponding well of the U-PLEX plate. Seal the plate and incubate for one hour at RT with moderate shaking.

3. Wash the plate with wash buffer. Add 150 µL per well of 2X Read Buffer T and read on an MSD instrument. Use appropriate reverse pipetting techniques when adding read buffer, to avoid introducing bubbles.

Select the master mix concentration that provides the best signal to background for each drug. For further guidance, please consult Table 2 on page 9, or see our Bridging Immunogenicity Application Note.

Data Illustrating the Process for Selecting the Optimal Master Mix Concentration

Representative data illustrating the optimization of master mix concentrations for Drug A and Drug B using positive control ADA 1 for Drug A and positive control ADA 2 for Drug B following the protocol described in this Application Note are shown below. Master mix concentrations generating optimal signal to background ratios were 5 µg/mL for Drug A (Spot 1) and 1.25 µg/mL for Drug B (Spot 10).

Table 2: Optimization of Master Mix Solutions (designated by a red outline in the tables below)

SPOT 1 Drug A Average Signal

ADA 1 (ng/mL)	Master Mix Concentration (µg/mL)			
	1.25	2.5	5	10
10,000	3449	18666	21750	19771
2500	3032	14908	19843	15234
625	1506	10818	13439	10260
156.3	998	5302	8509	7883
39.1	379	2270	4992	6167
9.8	143	849	1462	2421
2.4	137	460	643	711
0	89	95	98	123

SPOT 1 Drug A Signal: Background

ADA 1 (ng/mL)	Master Mix Concentration (µg/mL)			
	1.25	2.5	5	10
10,000	38.8	196.5	221.9	160.7
2500	34.1	156.9	202.5	123.9
625	16.9	113.9	137.1	83.4
156.3	11.2	55.8	86.8	64.1
39.1	4.3	23.9	50.9	50.1
9.8	1.6	8.9	14.9	19.7
2.4	1.5	4.8	6.6	5.8
0	1.0	1.0	1.0	1.0

SPOT 10 Drug B Average Signal

ADA 1 (ng/mL)	Master Mix Concentration (µg/mL)			
	1.25	2.5	5	10
10,000	346609	278851	254985	186764
2500	220387	199161	134013	123937
625	89091	48577	45103	36842
156.3	35860	12227	12274	10805
39.1	3327	2807	1974	2365
9.8	441	543	428	483
2.4	114	132	125	138
0	85	109	124	134

SPOT 10 Drug B Signal: Background

ADA 1 (ng/mL)	Master Mix Concentration (µg/mL)			
	1.25	2.5	5	10
10,000	4077.8	2558.3	2056.3	1393.8
2500	2592.8	1827.2	1080.8	924.9
625	1048.1	445.7	363.7	274.9
156.3	421.9	112.2	99.0	80.6
39.1	39.1	25.8	15.9	17.6
9.8	5.2	5.0	3.5	3.6
2.4	1.3	1.2	1.0	1.0
0	1.0	1.0	1.0	1.0

Preparation of U-PLEX Linker-Coupled Master Mix Solutions for Non-Antibody Drugs

For non-antibody capture concentrations, use a starting concentration of 66 nM of biotinylated drug in the master mix solution.

Formula to convert nM to µg/mL: **µg/mL = nM Concentration x MW (Da)/1,000,000**

Example for a drug of 50,000 MW µg/mL = [66 x 50,000]/1,000,000 = 3.3 µg/mL

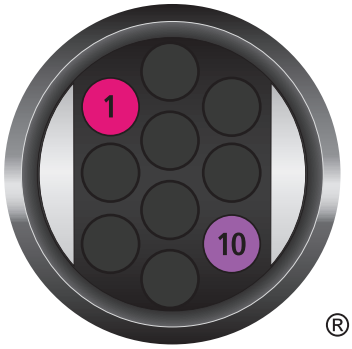
Immunogenicity Assay Protocol (with Optimized Master Mix Concentrations)

The above section describes the steps for optimizing master mix concentrations and guidelines for choosing the master mix concentration that provides the best signal to background for each drug.

Following optimization of the master mix concentrations for each drug, a multiplex immunogenicity assay can be performed. The section below provides an example protocol for a multiplex immunogenicity assay with two drugs.

For this assay, use MSD's U-PLEX 2-Assay SECTOR® Plate (Cat No. K15227N-1) [Figure 5] to perform the multiplex bridging immunogenicity assay.

Figure 5: Activated U-PLEX 2-Assay Plate.



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Multiplexing of More Than Two Antibody Drugs

In the current protocol, the total volume of the U-PLEX Linker-coupled master mix multiplex solution will be 2 mL. This is sufficient to coat 32 wells per plate for each master mix. If additional wells are to be coated, adjust the volumes but keep the ratios of each component consistent.

If multiplexing of more than two antibody drugs is desired, combine 200 µL of each U-PLEX Linker-coupled antibody drug to every master mix concentration that is going to be tested. Make sure the final volume is 2 mL (with U-PLEX Stop Solution). This will be sufficient to coat 32 wells per plate for each master mix. For example, if four antibody drugs are to be tested, add 200 µL of each U-PLEX Linker-coupled antibody drug to 1.2 mL of U-PLEX Stop Solution.

Note: The volumetric ratio of Linker: Antibody Drug: Stop Solution is 3:2:2. Recommended volumes of Linker, biotinylated antibody, and Stop Solution for coating partial plates, one plate, or multiple U-PLEX plates are provided on page 11 of the U-PLEX Development Pack Product Insert.

Preparation of U-PLEX Linker-Coupled Master Mix Multiplex Solutions

1. For each drug, prepare a master mix of 200 µL biotinylated drug and MSD GOLD SULFO-TAG conjugated drug. Make sure you use the appropriate optimized master mix concentrations (see "Preparation of Master Mix Solutions" on page 5). 200 µL of each master mix solution will be sufficient to coat one U-PLEX plate.

2. Add **200 µL** of each **master mix solution** to **300 µL** of **U-PLEX Linker**. Vortex to mix:
    a. 200 µL Drug A master mix + 300 µL of U-PLEX Linker 1.
    b. 200 µL Drug B master mix + 300 µL of U-PLEX Linker 10.

3. Incubate the solutions at RT for 30 minutes.

4. Add 200 µL of U-PLEX Stop Solution to each tube. Vortex to mix, then incubate at RT for 30 minutes.

5. Add 600 µL of each U-PLEX Linker-coupled master mix solution into a single tube. Vortex to mix the combined solution. The total volume (including U-PLEX Stop Solution) should be 6 mL.

	600 µL	Linker 1 Drug A master mix
+	600 µL	Linker 10 Drug B master mix
+	4800 µL	U-PLEX Stop Solution
	6000 µL	

Multiplex Immunogenicity Assay with U-PLEX Linker-Coupled Master Mix Multiplex Solution

1. Add 50 µL of U-PLEX Linker-coupled master mix multiplex solution and 25 µL of sample to each well of a round-bottom 96-well polypropylene plate. Double the volumes when testing in duplicate. Seal the plate and incubate for one to two hours at RT with moderate shaking, or shake for one hour at RT and then incubate overnight at 4°C.

2. During the master mix incubation, add 150 µL per well of Blocking Solution (e.g., 3% (w/v) MSD Blocker A in PBS or PBS-T) to the U-PLEX plate. Seal the plate and incubate for one hour at RT with moderate shaking. Remove the Blocking Solution from the U-PLEX plate. Wash the plate with wash buffer (e.g., PBS-T). Transfer 50 µL from each well of the polypropylene plate to the corresponding well of the U-PLEX plate. Seal the plate and incubate for one hour at RT with shaking.

3. Wash the plate with wash buffer. Add 150 µL per well of 2X Read Buffer T and read on an MSD instrument. Use appropriate reverse pipetting techniques when adding read buffer to avoid introducing bubbles.

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