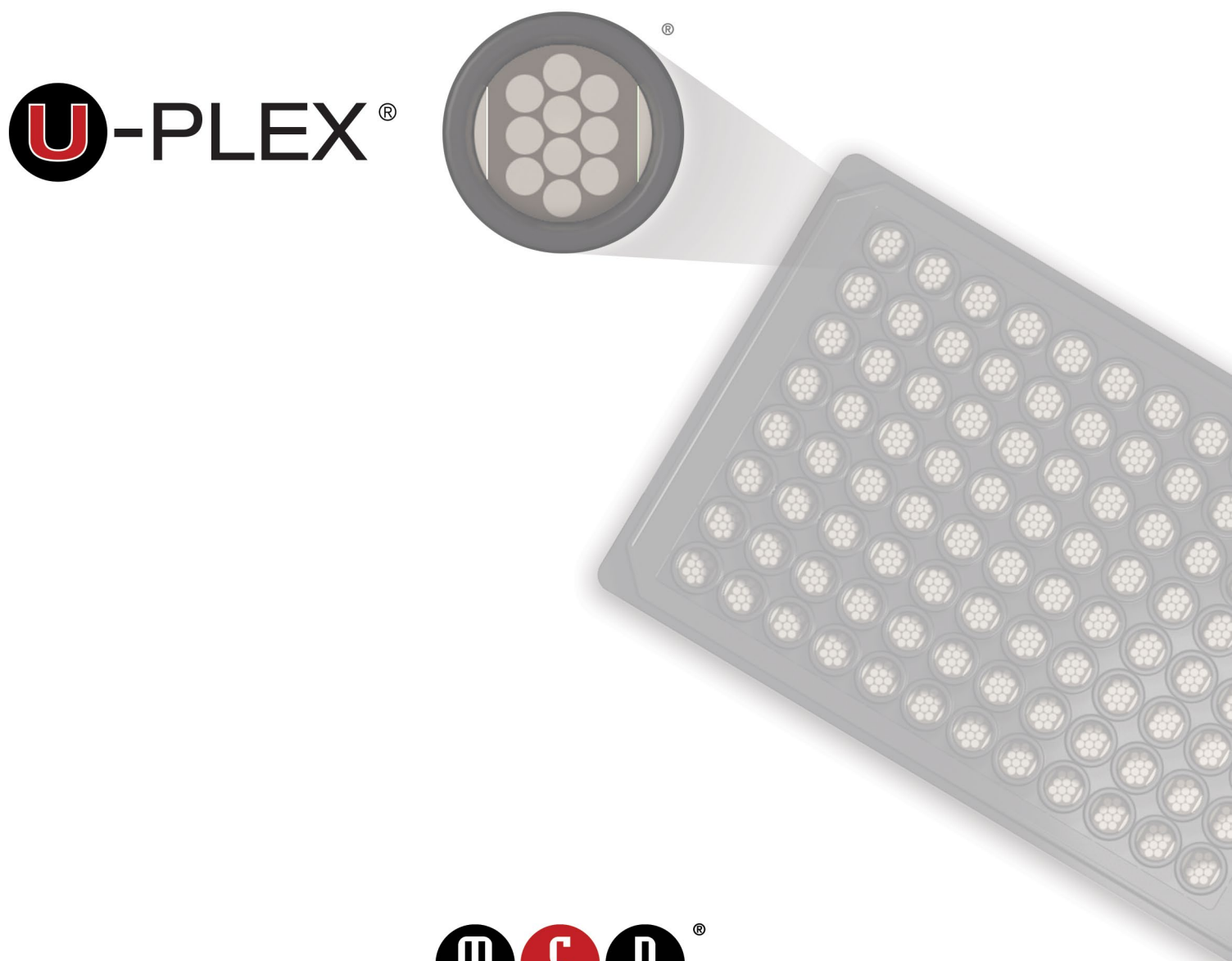


U-PLEX[®] Biomarker Group 2 (Human, Mouse, and NHP) Multiplex Assays



MSD U-PLEX Platform

U-PLEX Biomarker Group 2 (Human, Mouse, and NHP) Multiplex Assays

For use with serum, EDTA plasma, and cell culture supernatants.

For use with:

U-PLEX Biomarker Group 2 (human) Assays (K151ADM-1, K151ADM-2, K151ADM-4, K251ADM-2, K251ADM-4)

U-PLEX Biomarker Group 2 (mouse) Assays (K152ADM-1, K152ADM-2, K152ADM-4, K252ADM-2, K252ADM-4)

U-PLEX Biomarker Group 2 (NHP) Assays (K156ADM-1, K156ADM-2, K156ADM-4, K256ADM-2, K256ADM-4)

Catalog numbers for U-PLEX Biomarker Group 2 Combos are provided in Table 8 on page 18.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

U-PLEX technology permits the creation of custom multiplex assays for any combination of analytes by using 96- or 384-well U-PLEX plates and unique Linkers (Figure 1).

The U-PLEX assay menu is organized into groups, which include a broad menu of analytes assembled by species and analytical compatibility. For ultimate flexibility, custom combinations can be created from a selection of MSD U-PLEX assays, your antibodies, or a combination of both.

This product insert is for U-PLEX multiplex assays that contain a combination of assays from the U-PLEX Biomarker Group 2 on 96- and 384-well multiplexes.

A representative data set for each of the assays in U-PLEX Biomarker Group 2 is presented in the product-specific datasheets available at www.mesoscale.com/U-PLEX-documents. The performance of MSD assays may vary when tested in combination with your own assays. The data presented in the datasheets were generated during the development of the assays and do not represent the product specifications.

Principle of the Assay

Biotinylated capture antibodies are coupled to U-PLEX Linkers, which self-assemble onto unique spots on the U-PLEX plate. Analytes in the sample bind to the capture reagents; detection antibodies conjugated with electrochemiluminescent labels (MSD GOLD™ SULFO-TAG) bind to the analytes to complete the sandwich immunoassay (Figure 1). Once the sandwich immunoassay is complete, the U-PLEX plate is loaded into an MSD® instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample.

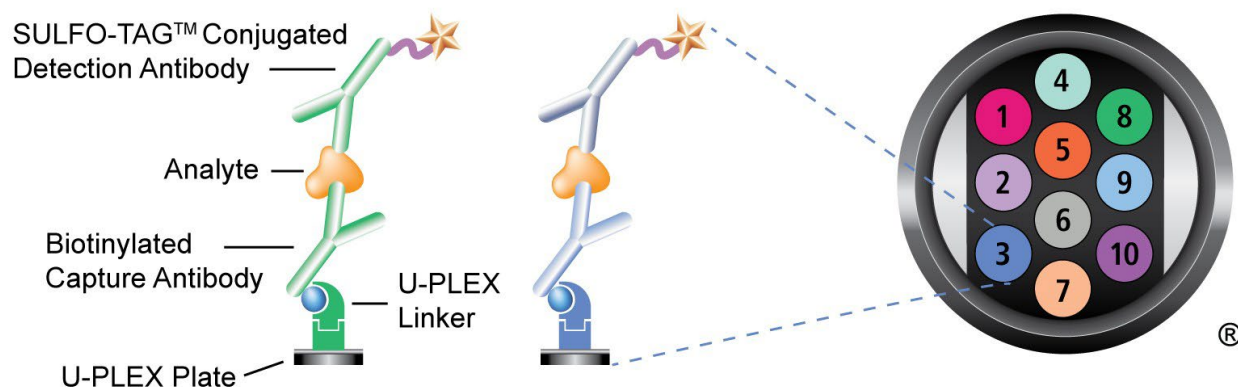


Figure 1. U-PLEX multiplex immunoassay on a U-PLEX 96-well 10-Assay plate. U-PLEX 384-well 4-Assay plates are similar.

Components

Reagents Supplied With All U-PLEX Multiplex Assays

Table 1. Reagents that are supplied with all U-PLEX Biomarker Group 2 96-well Assays

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1 plate	5 plates	25 plates	
Stop Solution	2–8 °C	R50AO-1	40 mL	1 bottle	1 bottle	5 bottles	Biotin-containing buffer to stop Linker-antibody coupling reaction
MSD GOLD Read Buffer B	RT	R60AM-1	18 mL	1 bottle	—	—	Buffer to catalyze the electrochemiluminescent reaction.
		R60AM-2	90 mL	—	1 bottle	5 bottles	
Human or NHP Assays							
Diluent 57	≤−10 °C	R50BZ-1	10 mL	1 bottle	—	—	Diluent for samples and Calibrators
		R50BZ-2	50 mL	—	1 bottle	5 bottles	
Diluent 3	≤−10 °C	R50AP-1	8 mL	1 bottle	—	—	Diluent for detection antibody
		R50AP-2	40 mL	—	1 bottle	5 bottles	
Mouse Assays							
Diluent 41	≤−10 °C	R50AH-1	10 mL	1 bottle	—	—	Diluent for samples and Calibrators
		R50AH-2	50 mL	—	1 bottle	5 bottles	
Diluent 45	≤−10 °C	R50AI-3	8 mL	1 bottle	—	—	Diluent for detection antibody
		R50AI-4	40 mL	—	1 bottle	5 bottles	

RT = room temperature

Dash (—) = not applicable

Table 2. Reagents that are supplied with all U-PLEX Biomarker Group 2 384-well Assays

Reagent	Storage	Catalog No.	Size	Quantity Supplied		Description
				5 Plates	25 Plates	
Stop Solution	2–8 °C	R50AO-1	40 mL	2 bottles	10 bottles	Biotin-containing buffer to stop Linker-antibody coupling reaction
MSD GOLD Read Buffer B	RT	R60AM-2	90 mL	1 bottle	5 bottles	Buffer to catalyze the electrochemiluminescent reaction
Human or NHP Assays						
Diluent 57	≤–10 °C	R50BZ-2	50 mL	2 bottles	10 bottles	Diluent for samples and Calibrators
Diluent 3	≤–10 °C	R50AP-2	40 mL	2 bottles	10 bottles	Diluent for detection antibody
Mouse Assays						
Diluent 41	≤–10 °C	R50AH-2	50 mL	2 bottles	10 bottles	Diluent for samples and Calibrators
Diluent 45	≤–10 °C	R50AI-4	40 mL	2 bottles	10 bottles	Diluent for detection antibody

RT = room temperature

Assay-Specific Reagents

10-Spot, 96-Well U-PLEX Plates

U-PLEX assays plates are provided in a sealed foil pouch with desiccant. The spots correspond to 10 unique U-PLEX Linkers. The number and layout of the active spots on the plate depend on the plate well density (96 vs 384) and the number of assays to be multiplexed (Figure 2). For example, if 3 assays are being multiplexed, the U-PLEX 3-Assay Plate will be provided.



Figure 2. Spot Map of the different U-PLEX multiplex plates showing the placement of Linkers within a well. The colored spots represent the active U-PLEX binding spots. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. *(top)* 96-well, *(bottom)* 384-well.

Linkers

Based upon the number of assays you select for multiplexing, you will receive the corresponding number of unique Linkers. Each Linker has a biotin-binding domain that couples to the biotinylated capture antibody as well as a domain that binds to its matching spot on the U-PLEX plate. The Linkers are color-coded and numbered with the spot to which they attach to the plate. 1-Plate packs include 300 μ L of each Linker. 5-Plate packs include 1.8 mL of each Linker. 25-Plate packs include 5 vials of 1.8 mL of each Linker.

We recommend recording which antibody is coupled to each Linker when performing the coupling step (as described in the Reagent Preparation section).

U-PLEX Antibody Sets

Based upon the analytes selected, you will receive U-PLEX Antibody Sets (Table 3) containing the biotinylated capture antibody and the SULFO-TAG™ conjugated detection antibody. The biotinylated capture antibody is provided at a ready-to-use concentration, and the SULFO-TAG conjugated detection antibody is provided at a 100X concentration. A complete list of all Antibody Sets available for U-PLEX Biomarker Group 2 and their respective catalog numbers is provided in the Appendix (Table 9).

Table 3. Contents of U-PLEX Antibody Set

Name	Storage	Size	Quantity Supplied			Description
			1 Plate	5 Plates	25 Plates	
U-PLEX Group 2 Analyte-Specific Antibody Set	2–8 °C	1-Plate	1	—	—	Set containing biotinylated capture antibody and SULFO-TAG conjugated detection antibody
		5-Plate	—	1	5	

Dash (—) = not applicable

Calibrators

Calibrators are multianalyte blends, each containing multiple recombinant human proteins lyophilized in a buffered diluent. Individual analyte concentrations are provided in the lot-specific certificates of analysis (COA). Table 4 lists the multianalyte Calibrator that will be provided with all Group 2 assays.

Table 4. Calibrator 11 is used for all U-PLEX Biomarker Group 2 assays

Name	Storage	Catalog No.	Size	Quantity Supplied			Analytes
				1 Plate	5 Plates	25 Plates	
Calibrator 11	2–8 °C	C0244-2	1 vial	1 vial	5 vials	25 vials	TGF-β1, TGF-β2, TGF-β3

Instrument Compatibility

MSD offers U-PLEX Assays designed for use on specific instrument platforms (Table 5).

Table 5. Instrument compatibility

Instrument	Assays on 96-well SECTOR™ Plate	Assays on 384-well SECTOR Plate
MESO® QuickPlex SQ 120	Y	—
MESO QuickPlex® SQ 120MM	Y	—
MESO SECTOR® S 600	Y	Y
MESO SECTOR S 600MM	Y	Y
MESO QuickPlex Q 60MM	—	—

Dash (—) = not applicable

Additional Materials and Equipment

- ☐ Appropriately sized tubes for reagent preparation
- ☐ Polypropylene microcentrifuge tubes for preparing dilutions
- ☐ Liquid-handling equipment suitable for dispensing 10 to 225 μL /well into a 96-well or 384-well microtiter plate
- ☐ Plate-washing equipment: automated plate washer or multichannel pipette
- ☐ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm (1,500 rpm for 384-well plates)
- ☐ MSD Wash Buffer (20X, 100 mL, catalog number R61AA-1) for plate washing. The standard protocol uses a minimum of 415 mL of 1X Wash Buffer for a 384-well plate and 130 mL for a 96-well plate. Automated plate washers may need overage added to these volumes.
- ☐ Adhesive plate seals
- ☐ Deionized water
- ☐ Vortex mixer
- ☐ 1 M HCl
- ☐ 1.2 M NaOH in 0.5 M HEPES
- ☐ pH paper to confirm neutralization of samples (optional)
- ☐ MSD Diluent 100 (50 mL, catalog number R50AA-4) may be needed to dilute samples.

Note: If including user-supplied antibody pairs, you will also need:

- ☐ MSD GOLD SULFO-TAG NHS-Ester (catalog No. R91AO-1) for conjugating detection reagents or SULFO-TAG conjugated antispecies antibodies for use as reporters with unconjugated detection antibodies
- ☐ Sulfo-NHS-LC-Biotin for biotinylation of the capture reagents (e.g., EZ-Link Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific, catalog No. 21327, or equivalent)
- ☐ Zeba Desalting Columns (Thermo Fisher Scientific, catalog numbers 87766-87773)
- ☐ Coating diluent such as 0.5% bovine serum albumin in PBS, or MSD Diluent 100 (50 mL, Catalog No. R50AA-4) for diluting the capture antibody

Safety

Use safe laboratory practices: wear 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 safety data sheet (SDS), which can be obtained from MSD Customer Service or at the www.mesoscale.com[®] website.

Best Practices

- Bring frozen diluents to room temperature in a 20–26 °C water bath before use. If a controlled water bath is not available, thaw at room temperature. Diluents may also be thawed overnight at 2–8 °C.
- Ensure that diluents, Wash Buffer, and Read Buffer are equilibrated to room temperature before use. Mix well before use. Plates should be brought to room temperature before opening the foil packet.
- To avoid cross-contamination between vials, open vials for one protocol step at a time. Use filtered pipette tips and use a fresh pipette tip for each reagent addition.
- MSD assays are tested and characterized between 21–26 °C; testing outside this temperature range may result in increased variability.
- Prepare calibrators, samples, and controls in a polypropylene container of sufficient volume.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates should not be exposed to direct sunlight.
- To ensure that all lyophilized powder is reconstituted, it is recommended that vials be inverted 3 times to distribute the diluent inside the vial. Then vortex the vial with 3 short pulses (upright, inverted, upright) after the solution sits at room temperature for the recommended amount of time in the product protocol.
- Ensure that all reagents are within their expiration date at the time of the test.
- For additional accuracy and precision, pre-wet pipette tips before transferring reagents and samples. Avoid pipetting bubbles while doing so.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm for 96-well plates and 1,000–1,500 rpm for 384-well plates. Binding reactions may reach equilibrium sooner if you use shaking at the middle of the range or above. For long-term studies, the shaking speed and shaker model be kept consistent.
- Tap the plate on a paper towel after washing to ensure the removal of residual fluid.
- Consistent incubation times will improve the reproducibility of test results.
- Ensure that all necessary instruments, equipment, and reagents for the next step are prepared before washing the plates to prevent the plates from drying out.
- Avoid excessive drying of the plate during washing steps, especially if working inside a laminar flow hood or another high airflow environment. Cover the plate with a new plate seal immediately after washing to protect it from airflow, and add solutions to the plate as soon as possible.
- Use a new adhesive plate seal for all incubation steps. Avoid re-using plate seals.
- Avoid creating bubbles in wells during all pipetting steps as they may lead to variable results.
- Use reverse pipetting when necessary and do not blow out residual liquid to avoid the introduction of bubbles. For empty wells, pipette gently to the bottom corner.
- Dispense reagents and wash fluids at the side of the well towards the bottom corner away from the coated spots.
- Protect plates from sources of heat such as vents, sunlight, etc., which may introduce variability across the plate surface. Some models of shakers generate heat that may affect plates on the platform.
- Ensure that all equipment is serviced and calibrated on a routine basis.
- Remove the plate seal before reading the plate.

- Read Buffer should be at room temperature (20–26 °C) before adding it to the plate.
- Keep time intervals consistent between the addition of Read Buffer and reading the plate to improve interplate precision. It is recommended that an MSD instrument be prepared to read a plate before adding Read Buffer. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer.
- Do not shake the plate after adding Read Buffer.
- Do not obscure or damage the plate barcode; it is required for the plate reader.
- Only use the Read Buffer and Wash Buffer recommended for use with this kit.
- Avoid cross-contamination between Linkers and antibodies by following the techniques below:
 - Pulse centrifuge the vials to get all of the contents to the bottom of the vial.
 - Open one vial at a time. Close the cap after use.
 - Each Linker vial is color-coded; ensure that each cap and tube have matching colors when opening and closing vials.
 - Use filtered pipette tips.
 - Use a fresh pipette tip after each reagent addition.
- For long-term studies using multiple plates of the same assay, it is recommended that the same Linker be coupled with the same antibody for the duration of the study.
- For multiplex U-PLEX assays that are provided in more than one box, each box is assembled with antibody pairs and calibrators for optimal performance. Components should not be mixed between boxes except for Stop Solution, Diluents, and Read Buffer.
- For 384-well assays, the protocol assumes the use of automated plate washers that can begin to aspirate before the total 90 µL is dispensed. If this ability is not present, reduce the wash volume to 80 µL to avoid overflowing the wells.
- Aliquot and freeze Diluent 100 to prevent contamination after opening.

Reagent Preparation

Bring all reagents to room temperature and refer to the Best Practices section (page 9) before beginning the protocol.

Important: Upon the first thaw, aliquot Assay Diluents into suitable volumes before refreezing.

To prepare MSD Wash Buffer and other supplemental reagents, please refer to the Additional Materials and Equipment section (page 8)

Prepare U-PLEX Plate

The preparation of a U-PLEX plate involves coating the provided plate with Linker-coupled capture antibodies. U-PLEX 4-Assay plates are shown below as an example. This graphic shows plates with four activated spots. Assign each antibody to a unique Linker and record the antibody identity next to the assigned Linker, as shown in the examples below.

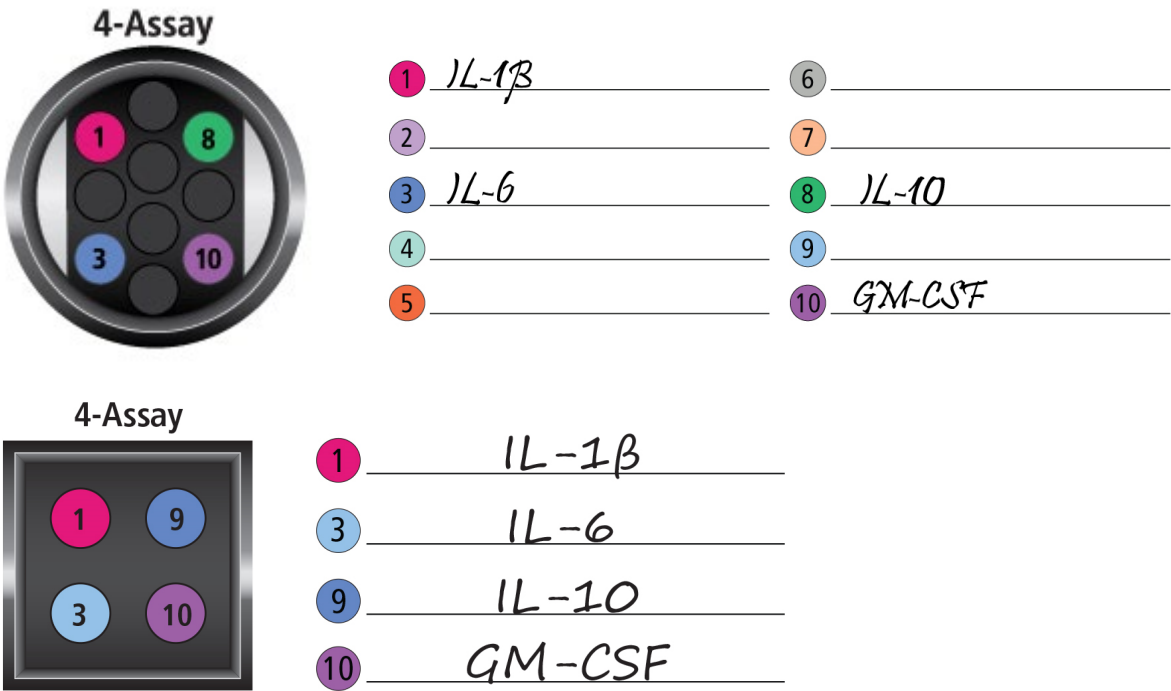


Figure 3. U-PLEX 4-Assay Plates, with recorded antibodies and assigned Linkers, **(top)** 96-well plate, **(bottom)** 384-well plate

The protocol in this section describes the preparation of a multiplex coating solution for one plate. The volumes can be adjusted depending on the number of plates or wells, but the ratios of the reagents should remain the same (Table 6).

STEP 1: Create Individual U-PLEX-Coupled Antibody Solutions

A different Linker must be used for each unique biotinylated antibody. Below are the steps to complete the coupling reactions for the above example of a 4-Assay plate.

Couple each biotinylated capture antibody to a unique Linker and record the antibody identity next to the Linker number on the Spot Map (a blank Spot Map image is provided on page 23).

- ☐ Add 200 μ L of each biotinylated antibody to 300 μ L of the assigned Linker in the Linker vial. Mix by vortexing. Incubate at room temperature for 30 minutes. Do not shake. If using a 5-plate Linker vial, combine each Linker with the biotinylated antibody in a new clean tube.

Notes:

- Each Linker vial has a matching colored cap and label.
- To remove liquid from the cap, briefly centrifuge the Linker vial and open the cap gently.
- ☐ Add 200 μ L of Stop Solution. Mix by vortexing. Incubate at room temperature for 30 minutes.

Note: At the end of STEP 1, each U-PLEX-coupled antibody solution is at 10X the coating concentration and can be stored at 2–8 °C. Do not store for more than 7 days.

Adjust the volumes for multiple plates (see Table 6). The volumetric ratio of Linker: antibody: Stop Solution is 3:2:2.

STEP 2a: Prepare the Multiplex Coating Solution for 96-well Plates

- ☐ Combine 600 μ L of each U-PLEX-coupled antibody solution (10X) into a single tube and vortex. Up to 10 U-PLEX-coupled antibodies can be pooled. *Do not combine U-PLEX-coupled antibody solutions that share the same Linker.*
- ☐ When combining fewer than 10 antibodies, bring the solution up to 6 mL with Stop Solution. This will result in a final 1X concentration. Mix by vortexing. For example, for a 4-Assay coating solution, add 3.6 mL of Stop Solution to the 2.4 mL of combined antibodies.

Note: At the end of Step 2a, the U-PLEX multiplex coating solution is at 1X and can be stored at 2–8 °C. Do not store for more than 7 days.

STEP 2b: Prepare the Multiplex Coating Solution for 384-well Plates

- ☐ Combine 600 μ L of each U-PLEX Linker-coupled antibody solution into a single tube and mix by vortexing. Up to 4 U-PLEX Linker-coupled antibodies can be pooled. Do not combine U-PLEX Linker-coupled antibody solutions that share the same Linker.
- ☐ Bring the solution up to 12 mL by mixing with Stop Solution. Mix by vortexing.

Note: At the end of Step 2b, the U-PLEX multiplex coating solution is at 0.5X and can be stored at 2–8 °C. Do not store for more than 7 days.

STEP 3a: Coat U-PLEX 96-well Plates

- ❑ Add 50 µL of multiplex coating solution to each well. Seal the plate with an adhesive plate seal and incubate with shaking at room temperature for 1 hour.
- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.

The plate is now coated and ready for use. Coated plates may be stored in the original pouch with desiccant and sealed for up to 7 days at 2–8 °C.

STEP 3b: Coat U-PLEX 384-well Plates

- ❑ Wash the plate 3 times with 90 µL/well of 1X Wash Buffer.
- ❑ Add 25 µL of the 0.5X multiplex coating solution to each well. Seal the plate with an adhesive plate seal and shake for 4 hours at room temperature.
- ❑ Wash the plate 3 times with 90 µL/well of 1X Wash Buffer.

The recommended volumes of Linker, biotinylated capture antibody, and Stop Solution for coating one or multiple U-PLEX plates are provided below (Table 6). If using a partial plate, refer to Tables 10 and 11 in the Appendix.

Table 6. Amount of each component required for U-PLEX coating solution per plate

No. of Plate(s)	Individual Linker (µL)	Individual Biotinylated Antibody (µL)	Stop Solution (µL)
1	300	200	200
2	600	400	400
3	900	600	600
4	1,200	800	800
5	1,500	1,000	1,000
N	300 × N	200 × N	200 × N

Prepare Calibrator Standards

The following instructions will enable you to prepare seven Calibrator Standard solutions plus a zero Calibrator Standard for up to six replicates (Table 7; Figure 4).

- ❑ Bring the Calibrator vial to room temperature. Reconstitute each vial of Calibrator by adding 250 µL of Assay Diluent to the glass vial. Do not acid-treat the Calibrator.
- ❑ Invert the reconstituted Calibrator at least 3 times. Do not vortex. Let the solution equilibrate at room temperature for 15–30 minutes and then vortex briefly. This will result in a 5X concentrated stock of the Calibrator.
- ❑ Dilute the 5X Calibrator stock solution. Add 50 µL of the 5X Calibrator stock to 200 µL of Assay Diluent to generate the highest point in the standard curve (i.e., Calibrator Standard 1). Calibrator Standard 1 is now ready for use. Keep the dilutions at room temperature.
- ❑ Prepare the subsequent 6 dilutions for the curve (4-fold serial dilutions) in Assay Diluent (Figure 4; Table 7). Use Assay Diluent for the Calibrator Standard 8 (zero Calibrator/blank). Mix by vortexing the tubes between each serial dilution.

Note: We recommend that reconstituted Calibrators be used immediately. If storage is necessary, divide into 60 µL aliquots and store immediately at ≤–70 °C.

Table 7. Serial dilution to generate the standard curve

Calibrator Standard No.	Tube No.	Source of Calibrator	Volume of Reconstituted Calibrator (μL)	Assay Diluent (μL)	Total volume (μL)
1	1	Calibrator Standard 1 (top of curve)	50	200	250
2	2	From tube 1	75	225	300
3	3	From tube 2	75	225	300
4	4	From tube 3	75	225	300
5	5	From tube 4	75	225	300
6	6	From tube 5	75	225	300
7	7	From tube 6	75	225	300
8 (zero Calibrator)	8	—	0	300	300

Dash (—) = not applicable

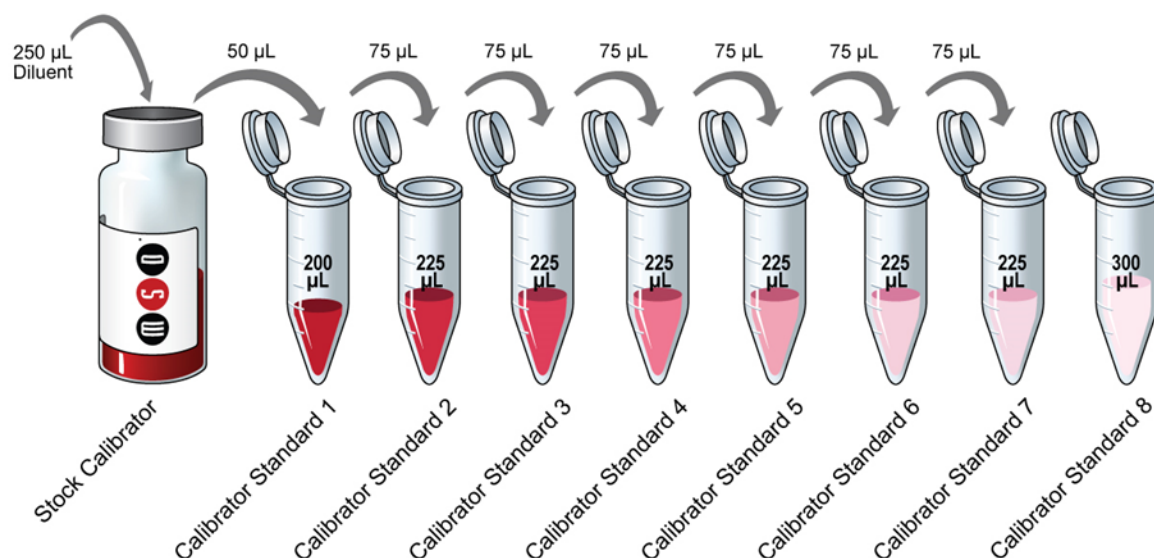


Figure 4. Dilution schema for preparation of Calibrator Standards for U-PLEX Biomarker Group 2 assays.

Prepare Samples

Note: TGF-β samples typically require an acid treatment for activation. Prepare TGF-β samples as follows:

- ☐ Add 20 μL of 1 M HCl per 100 μL of neat sample volume. Vortex briefly.
- ☐ Incubate the sample for 10 minutes at room temperature.
- ☐ Neutralize the sample by adding 14 μL of 1.2 M NaOH in 0.5 M HEPES per 100 μL of sample volume. Vortex briefly. Samples are ready to use. Use immediately.

Depending on the assay and sample set under investigation, a dilution may be necessary. Assay Diluent may be used for sample dilution. The dilution factor for the given sample type may need to be optimized.

Note: If samples require additional dilution, please dilute only after the acid neutralization step.

Prepare Detection Antibody Solution

The detection antibody is provided as a 100X stock solution. The working solution is 1X for 96-well assays and 0.5X for 384-well assays. Prepare the detection antibody solution immediately before use. Adjust the volumes proportionally for partial plates.

For one plate, combine:

- ☐ 60 μ L of each 100X detection antibody
- ☐ Antibody Diluent to bring the final volume to 6 mL (12 mL for 384-well assays)

Prepare Wash Buffer

Prepare a 1X working solution by diluting the 20X stock with deionized water

Prepare Read Buffer

MSD provides MSD GOLD Read Buffer B ready for use. Do not dilute.

Assay Protocols

96-well Plate Assays

Note: Follow Reagent Preparation (starting on page 11) before beginning this assay protocol.

STEP 1: Add Samples and Calibrators

- ☐ Add 25 μ L of Assay Diluent to each well. Tap the plate gently on all sides.
- ☐ Add 25 μ L of the prepared Calibrator Standard or sample to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.

STEP 2: Wash and Add Detection Antibody Solution

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 50 μ L of detection antibody solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.

STEP 3: Wash and Read

- ☐ Wash the plate 3 times with at least 150 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 150 μ L of MSD GOLD Read Buffer B to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

384-well Plate Assays

STEP 1: Add Samples and Calibrators

- ☐ Add 25 μ L of the prepared Calibrator Standard or sample to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 4 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ☐ Wash the plate 3 times with 90 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 25 μ L of detection antibody solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read

- ☐ Wash the plate 3 times with 90 μ L/well of 1X MSD Wash Buffer.
- ☐ Add 40 μ L of MSD GOLD Read Buffer B to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

Alternate Protocols

The suggestions below may be useful for simplifying the protocol.

- ❑ **Alternate Protocol 1, Co-incubation:** Co-incubating samples and detection antibody solution may improve the sensitivity for some assays. Note that the use of the co-incubation protocol may result in sample concentrations that vary from concentrations obtained with the standard protocol. If this protocol is chosen, we recommend that this protocol be used for the entirety of the research project.
- ❑ **Alternate Protocol 2, Reduced Wash:** For cell culture supernatants, you may simplify the protocol by eliminating one of the wash steps. After incubating the Calibrator Standard or sample, add detection antibody solution to the plate without decanting or washing the plate.
- ❑ **Alternate Protocol 3, Shortened Incubations:** Some assays in 384-well plates may achieve acceptable performance with shorter incubations. Consider incubating samples in the plate for 2 hours and detection antibody for 1 hour.

Assay Performance

A representative data set for each assay is presented in the product-specific datasheets available at www.mesoscale.com/U-PLEX-documents. The data represent the performance of the assay tested in multiplex format on U-PLEX plates. The data were generated during the development of the assay and do not represent the product specifications. Under your experimental conditions and with your specific multiplex, the assay may perform differently than the representative data shown.

Specificity

To assess specificity, the Antibody Set for each assay in the human, mouse, and NHP group was tested individually against the panel of antibodies and recombinant analytes for nonspecific binding (TGF- β 1, TGF- β 2, and TGF- β 3).

Nonspecific binding was less than 0.5% for all assays included in the U-PLEX Biomarker Group 2 using the following calculation.

$$\% \text{ nonspecificity} = \frac{\text{nonspecific signal}}{\text{specific signal}} \times 100$$

Appendix

U-PLEX Combos

U-PLEX Combos (Table 8) are preconfigured multiplex assays that include U-PLEX 96-well Plates, Linkers, Antibody Sets, Calibrators, Stop Solution, Diluents, and MSD GOLD Read Buffer B.

Table 8. Catalog numbers of U-PLEX Biomarker Group 2 Combos

Product	Analytes	Catalog Numbers (-1/-5/-25 Plate Size)
U-PLEX TGF- β Combo (human) SECTOR	TGF- β 1, TGF- β 2, TGF- β 3	K15241K-1/-2/-4
U-PLEX TGF- β Combo (mouse) SECTOR	TGF- β 1, TGF- β 2, TGF- β 3	K15242K-1/-2/-4
U-PLEX TGF- β Combo (NHP) SECTOR	TGF- β 1, TGF- β 2, TGF- β 3	K15243K-1/-2/-4

U-PLEX Antibody Sets

Antibody Sets (Table 9) include a biotinylated capture antibody and SULFO-TAG conjugated detection antibody.

Table 9. Catalog numbers of Antibody Sets available for U-PLEX Biomarker Group 2

Product	Catalog Numbers (-1/-5 Plate Size)
U-PLEX TGF- β 1 Antibody Set	B20XW-2/-3
U-PLEX TGF- β 2 Antibody Set	B20XU-2/-3
U-PLEX TGF- β 3 Antibody Set	B20XV-2/-3

Working with Partial Plates

A portion of a plate may be used when developing assays (Table 10; Table 11). Volumes should be adjusted proportionally when preparing reagents for partial plates.

For convenience, the recommended volumes of Linker, biotinylated capture antibody, and Stop Solution for coating partial plates are provided below.

Table 10. Amount of each component required for U-PLEX coating solution (partial 96-well plate)

No. of Wells	Individual Linker (μ L)	Individual Biotinylated Antibody (μ L)	Stop Solution per Reaction (μ L)	Vol. to Pull from Each Reaction (μ L)
16	60	40	40	100
32	120	80	80	200
48	150	100	100	300
64	210	140	140	400
80	240	160	160	500
96	300	200	200	600

Table 11. Amount of each component required for U-PLEX coating solution (partial 384-well plate)

No. of Wells	Individual Linker (μL)	Individual Biotinylated Antibody (μL)	Stop Solution per Reaction (μL)	Vol. to Pull from Each Reaction (μL)	Add Stop Solution and bring Vol to (μL)
64	60	40	40	100	2,000
128	120	80	80	200	4,000
192	150	100	100	300	6,000
256	210	140	140	400	8,000
320	240	160	160	500	10,000

When running a partial plate, seal the unused sectors to avoid contaminating unused wells. Remove all seals before reading. The uncoated wells of a partially used plate may be sealed and stored for up to 30 days at 2–8 °C in the original foil pouch with desiccant.

Open Spots

The U-PLEX platform allows users to add other analytes besides those that are available in the U-PLEX assay menu, such as R-PLEX® antibody sets or your antibodies, to a U-PLEX assay. This is enabled when open spots are included in a U-PLEX assay order. For more information about diluents when combining U-PLEX assays and R-PLEX Antibody Sets, refer to the U-PLEX Development Pack product insert or the R-PLEX Multiplex Assays product insert available at www.mesoscale.com.

Prepare Conjugated Capture and Detection Antibodies

The U-PLEX platform uses a biotinylated capture antibody and a SULFO-TAG conjugated detection antibody. Therefore, for assays that are being developed with your antibody pairs, the capture antibodies (or other suitable capture reagents) must be biotinylated before starting the U-PLEX protocol. Similarly, the detection antibody must be conjugated with SULFO-TAG; however, you may choose to use a SULFO-TAG conjugated secondary detection antibody that is raised against the host of the detection antibody. In such cases, the detection antibody should be raised in different host species than the capture antibodies in the U-PLEX assay to avoid cross-reactivity. For example, if the capture antibody is raised in a rabbit, choose a detection antibody raised in a different host species than rabbit (e.g., mouse).

Note: Since the capture antibody is always biotinylated, do not use a biotinylated detection antibody or SULFO-TAG Streptavidin as a method for detection. SULFO-TAG Streptavidin will cause high backgrounds as it will bind to the biotin on the capture antibody.

Prepare Biotinylated Capture Antibody

The working concentration of a biotinylated capture antibody needed to prepare the multiplex coating solution for the U-PLEX Plate is 10 μg/mL. Prepare a stock solution of the biotinylated capture antibody by following the manufacturer's guidelines for the conjugation of an antibody to Sulfo-NHS-LC-Biotin (such as EZ-Link Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific, or an equivalent product). At least one biotin must be present on the capture antibody for it to be coupled to the U-PLEX Linker. We recommend starting with a biotin challenge ratio of 10 biotins to 1 capture antibody. This challenge ratio typically leads to the conjugation of an average of 2–4 biotins per antibody.

Note: Free biotin will interfere with the U-PLEX assay signal. Therefore, after conjugation, it is recommended to purify the biotinylated antibody from the free biotin reagent by using Zeba Desalting Columns.

For long-term storage, it is recommended that you perform a buffer exchange to store the final biotinylated antibody in the Conjugate Storage buffer.

Prepare SULFO-TAG Conjugated Detection Antibody

The optimal concentration of the SULFO-TAG conjugated detection antibody for use in the U-PLEX assay is typically within the range of 0.5–1 µg/mL. Prepare a concentrated stock solution of 100X for each SULFO-TAG conjugated detection antibody by following the guidelines for SULFO-TAG conjugation available at www.mesoscale.com. Please refer to the MSD GOLD SULFO-TAG Conjugation Quick Guide or the MSD GOLD SULFO-TAG NHS-Ester Technical Note. We recommend using a 20:1 challenge ratio for SULFO-TAG conjugation of antibodies. This challenge ratio leads to a typical conjugation ratio of 10 SULFO-TAG labels per antibody molecule. Optimization of the SULFO-TAG challenge ratio may be necessary to reduce background and increase assay signal. To find out more details on optimizing the SULFO-TAG conjugation of the detection antibody, please refer to the MSD GOLD SULFO-TAG Conjugation Quick Guide or the MSD GOLD SULFO-TAG NHS-Ester Technical Note available at www.mesoscale.com. For long-term storage, purify the SULFO-TAG conjugated antibody to remove the unconjugated SULFO-TAG NHS-Ester. Antibody conjugates are typically stable for at least 1 year in conjugation storage buffer at 2–8 °C. Protect from direct exposure to light.

Prepare non-MSD Calibrator

For assays that are being developed with your antibody pairs, a recombinant protein that is representative of the native protein can be used for the calibration curve. A good starting concentration is 10 ng/mL for the high Calibrator and 0.001 ng/mL for the low Calibrator. We recommend testing an 8-point titration curve and optimizing the Calibrator diluent if required.

Prepare 250 µL of 10X concentrated blend of the calibrators in Metabolic Assay Working Solution. Use this 10X concentrated stock to generate the Calibrator Standard 1 (Table 7).

Summary Protocol

Prepare U-PLEX 96-well Plates

STEP 1: Create Individual U-PLEX Linker-Coupled Antibody Solutions

Couple an individual biotinylated antibody to a unique Linker, and record the antibody identity next to the Linker number on the Spot Map (Figure 5).

- ❑ Add 200 μL of each biotinylated antibody to 300 μL of the assigned Linker. Refer to the U-PLEX plate Spot Map to determine which Linkers can be combined. A different Linker must be used for each unique biotinylated antibody. Mix by vortexing. Incubate at room temperature for 30 minutes.
- ❑ Add 200 μL of Stop Solution. Mix by vortexing. Incubate at room temperature for 30 minutes.

STEP 2: Prepare the Multiplex Coating Solution for a 96-well Plate

- ❑ Combine 600 μL of each U-PLEX Linker-coupled antibody solution into a single tube and mix by vortexing. Up to 10 U-PLEX Linker-coupled antibodies can be pooled. Do not combine U-PLEX Linker-coupled antibody solutions that share the same Linker.
- ❑ When combining fewer than 10 antibodies, bring the solution up to 6 mL by mixing with Stop Solution to result in a final 1X concentration. Mix by vortexing. Coat plates the same day. Do not store overnight.

STEP 3: Coat a U-PLEX 96-well Plate

- ❑ Add 50 μL of the 1X multiplex coating solution to each well. Seal the plate with an adhesive plate seal and shake for 1 hour at room temperature.
- ❑ Wash the plate 3 times with at least 150 μL /well of 1X Wash Buffer. The plate is now coated and ready for use. It can be stored for up to 7 days at 4 $^{\circ}\text{C}$.

96-Well Assay Protocol

STEP 1: Add Sample or Calibrator Standards

- ❑ Add 25 μL of Assay Diluent to each well. Tap the plate gently on all sides.
- ❑ Add 25 μL of prepared Calibrator Standard or sample to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.

STEP 2: Wash and Add Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 μL /well of 1X Wash Buffer.
- ❑ Add 50 μL of detection antibody solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.

STEP 3: Wash and Read

- ❑ Wash the plate 3 times with at least 150 μL /well of 1X Wash Buffer.
- ❑ Add 150 μL of MSD GOLD Read Buffer B to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

Prepare U-PLEX 384-well Plates

STEP 1: Create Individual U-PLEX Linker-Coupled Antibody Solutions

Couple an individual biotinylated antibody to a unique Linker, and record the antibody identity next to the Linker number on the Spot Map (Figure 5).

- ☐ Add 200 μ L of each biotinylated antibody to 300 μ L of the assigned Linker. Refer to the U-PLEX plate Spot Map to determine which Linkers can be combined. A different Linker must be used for each unique biotinylated antibody. Mix by vortexing. Incubate at room temperature for 30 minutes.
- ☐ Add 200 μ L of Stop Solution. Mix by vortexing. Incubate at room temperature for 30 minutes.

STEP 2: Prepare the Multiplex Coating Solution for a 384-well Plate

- ☐ Combine 600 μ L of each U-PLEX Linker-coupled antibody solution into a single tube and mix by vortexing. Up to 4 U-PLEX Linker-coupled antibodies can be pooled. Do not combine U-PLEX Linker-coupled antibody solutions that share the same Linker.
- ☐ Bring the solution up to 12 mL by mixing it with Stop Solution to result in a final 0.5X concentration. Mix by vortexing. Coat plates the same day. Do not store overnight.

STEP 3: Coat a U-PLEX 384-well Plate

- ☐ Wash the plate 3 times with 90 μ L of 1X Wash Buffer.
- ☐ Add 25 μ L of the 0.5X multiplex coating solution to each well. Seal the plate with an adhesive plate seal and shake for 4 hours at room temperature.
- ☐ Wash the plate 3 times with 90 μ L/well of 1X Wash Buffer. The plate is now coated and ready for use. It can be stored for up to 7 days at 4 °C.

384-well Assay Protocol

STEP 1: Add Sample or Calibrator Standards

- ☐ Add 25 μ L of prepared Calibrator Standard or sample to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 4 hours.

STEP 2: Wash and Add Detection Antibody Solution

- ☐ Wash the plate 3 times with 90 μ L/well of 1X Wash Buffer.
- ☐ Add 25 μ L of detection antibody solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 2 hours.

STEP 3: Wash and Read

- ☐ Wash the plate 3 times with 90 μ L/well of 1X Wash Buffer.
- ☐ Add 40 μ L of MSD GOLD Read Buffer B to each well. Analyze the plate on an MSD instrument. Incubation in Read Buffer is not required before reading the plate.

Spot Maps

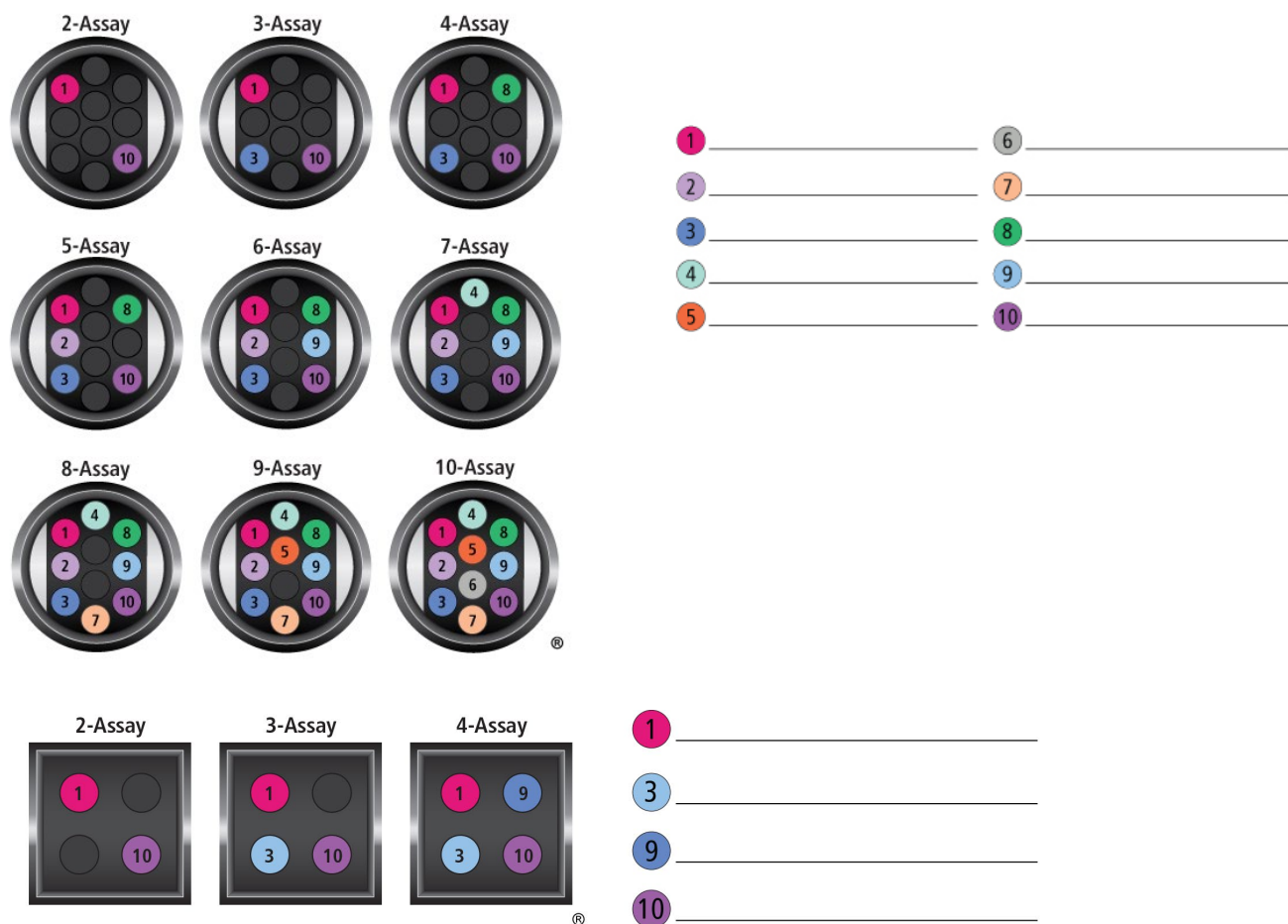


Figure 5. Spot maps. 96-well (top), 384-well (bottom)

Plate Diagrams

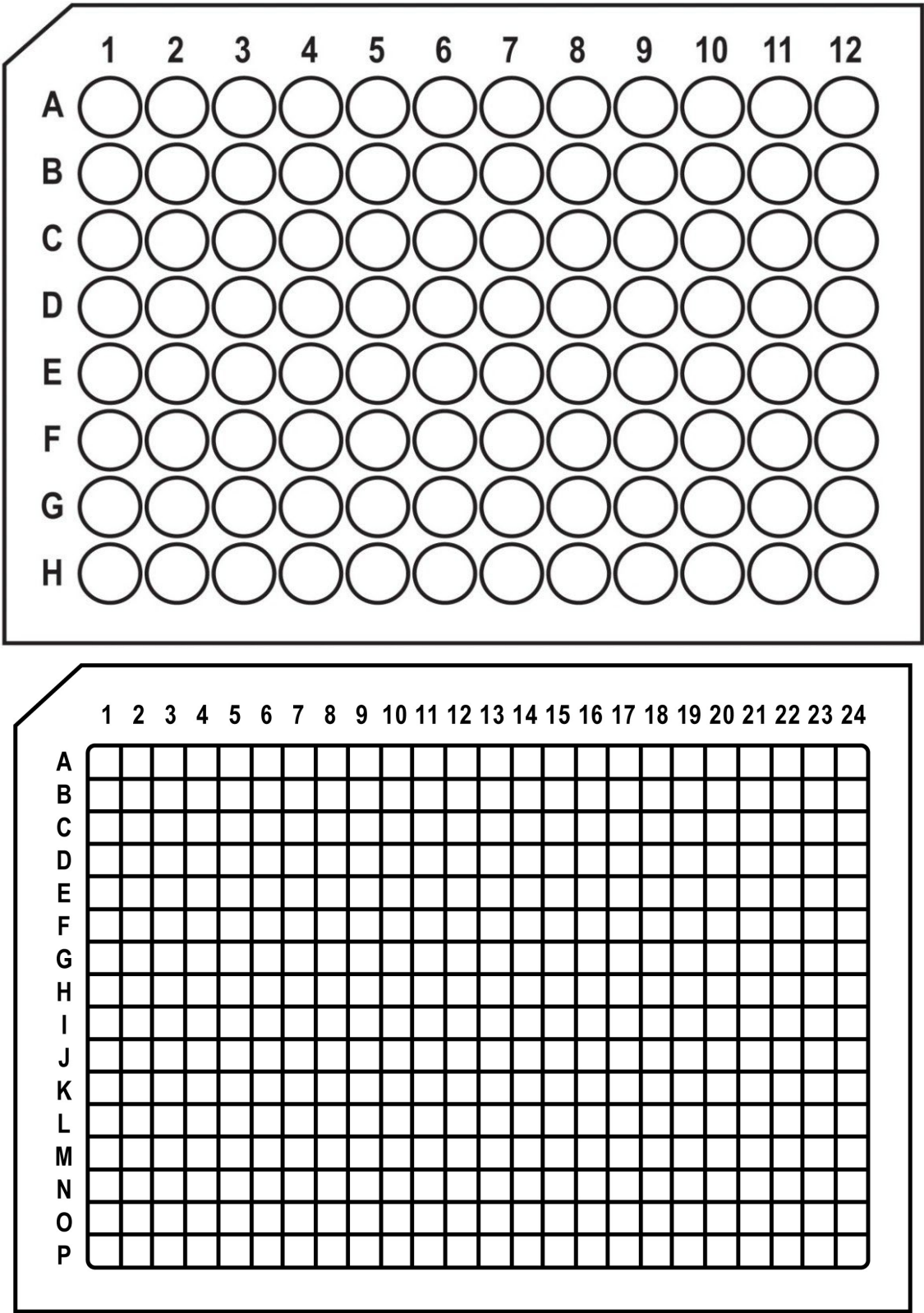


Figure 6. Plate diagrams. Similar plate layouts can be created in Excel and easily imported into DISCOVERY WORKBENCH® software.