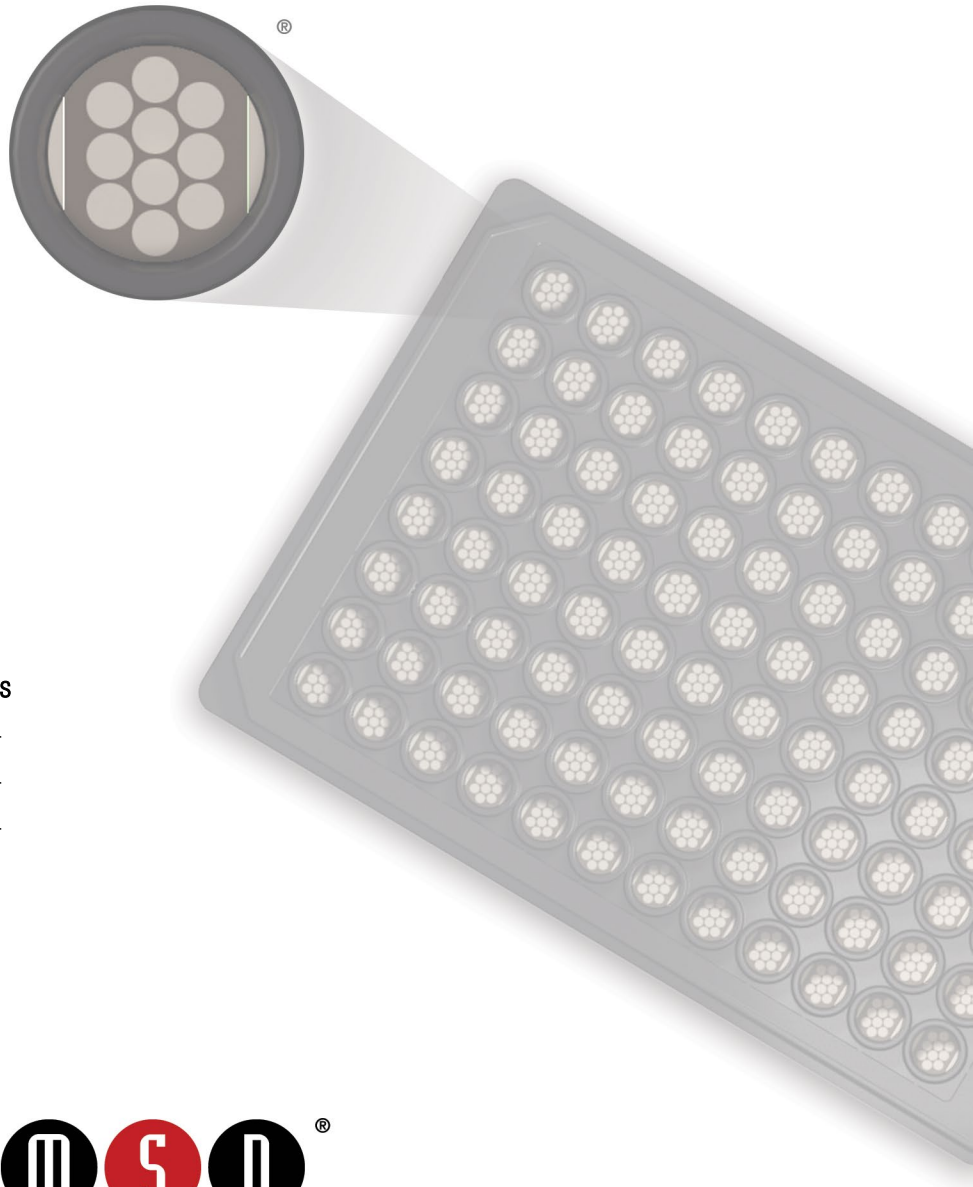
U-PLEX® Development Packs

For the Development of Multiplex Assays



Catalog Numbers

	96-well -1/-5/-25 Plate	384-well -1/-5/-25 Plates
2-Assay	K15227N-1/-2-/4	K25227N-1/-2-/4
3-Assay	K15228N-1/-2-/4	K25228N-1/-2-/4
4-Assay	K15229N-1/-2-/4	K25229N-1/-2-/4
5-Assay	K15230N-1/-2-/4	–
6-Assay	K15231N-1/-2-/4	–
7-Assay	K15232N-1/-2-/4	–
8-Assay	K15233N-1/-2-/4	–
9-Assay	K15234N-1/-2-/4	–
10-Assay	K15235N-1/-2-/4	–

MSD U-PLEX Platform

U-PLEX Development Pack

For the development of multiplex assays using U-PLEX Antibody Sets, R-PLEX® Antibody Sets, and/or your antibody pairs.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®

A division of Meso Scale Diagnostics, LLC.

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Rockville, MD 20850 USA
www.mesoscale.com

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Introduction

U-PLEX technology allows the creation of custom multiplex assays for any combination of analytes by using 384-well 4-spot or 96-well 10-spot U-PLEX plates and unique Linkers (Figure 1). Custom multiplex assays can be created with compatible combinations of R-PLEX or U-PLEX Antibody Sets or your own antibody pairs.

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.

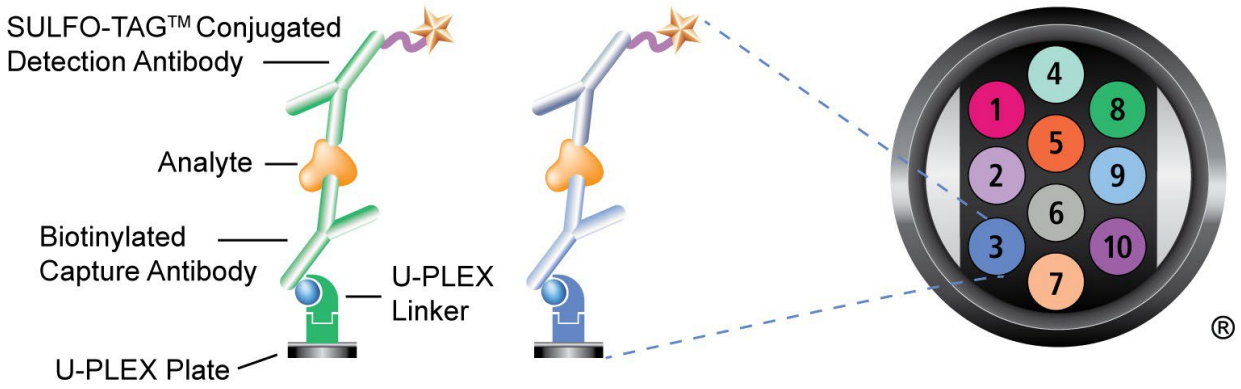

Figure 1. A sandwich immunoassay on a U-PLEX 96-well 10-Assay Plate. The 384-well 4-spot plate is similar.

Assay Formats

The U-PLEX platform enables you to build assays in several different formats. The most common format is the sandwich immunoassay (Figure 2A). Once the analyte is captured, the immunoassay can be completed using a SULFO-TAG™ conjugated detection antibody (Figure 2B). Guidelines on antibody selection are provided in Appendix A. For your convenience, MSD has developed a large, expanding suite of R-PLEX and U-PLEX Antibody Sets that can be used in combination with U-PLEX plates to create custom multiplex immunoassays (see page 14 for detailed protocols).

Immunoassays can also be performed as bridging assays, as is the case for immunogenicity assays, and can be done in either stepwise or homogenous formats.

Additionally, the U-PLEX platform can be used to measure antibodies against proteins, antibodies, peptides, carbohydrates, or polysaccharides (Figure 2C). In this assay, the antigen is biotinylated and then coupled to the U-PLEX Linker. The sample containing the antibody can be added either simultaneously with the U-PLEX-coupled Linker or after the antigen is coated on the plate. In this assay format, a SULFO-TAG antispecies antibody is used for detection.

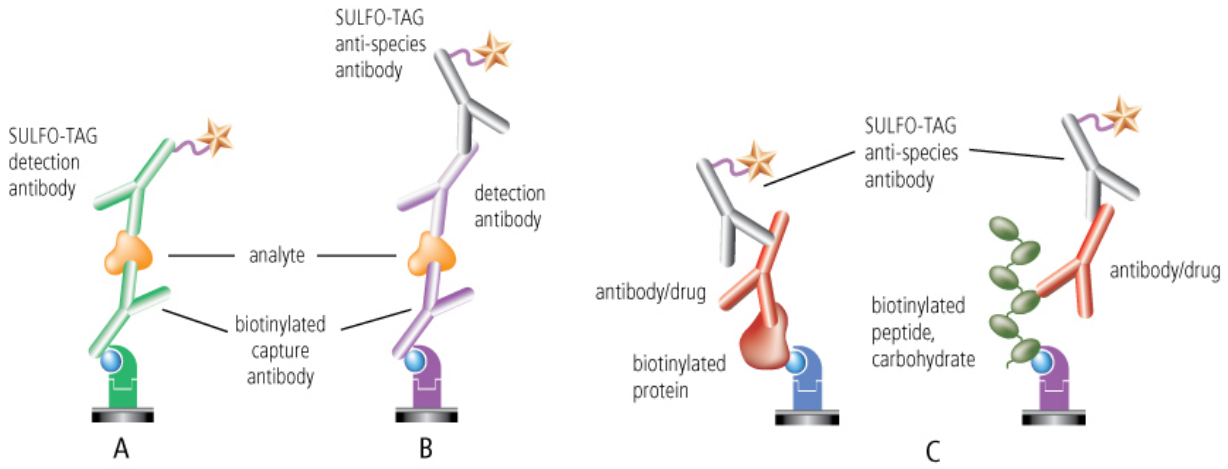

Figure 2. (A) Biotinylated capture antibody used with SULFO-TAG conjugated detection antibody. (B) Biotinylated capture antibody used with unlabeled primary detection antibody and SULFO-TAG conjugated antispecies antibody as a secondary reporter. (C) Auto-antibody or serology assay format to detect the presence of antibodies against proteins, peptides, or carbohydrates.

Components

U-PLEX Plates

U-PLEX plates contain spots on the bottom of each well; the spots correspond to unique U-PLEX Linkers. The number and layout of active spots on the plate depend on the plate well density (96 vs 384) and the number of assays to be multiplexed (Figure 3). U-PLEX 96-well Development Packs are available in 2 to 10 assay formats; U-PLEX 384-well Development Packs are available in 2 to 4 assay formats. Both come with corresponding plates.

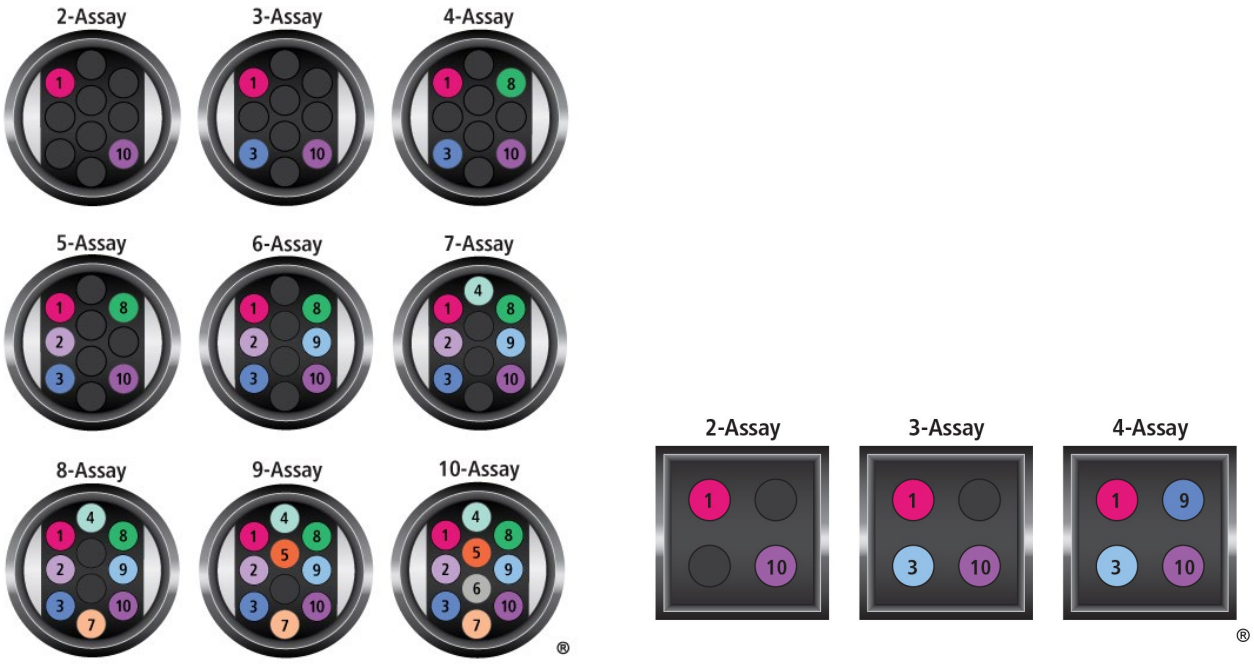

*Figure 3. Spot maps of the different U-PLEX multiplex plates **(left)** 96-well, **(right)** 384-well showing the placement of Linkers within a well. The colored spots represent the active U-PLEX binding spots. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. You can choose the appropriate Development Pack according to the number of assays you plan to multiplex. For example, if you plan to multiplex 4 assays together, you can choose either the U-PLEX 96-well or 384-well Development Pack 4-Assay.*

Linkers

Based upon the Development Pack you select, 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 µL 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).

Reagents Provided with Development Pack

Multiple reagents are provided in Development Packs (Table 1 and Table 2).

Table 1. Additional reagents provided in the Development Pack for 96-well Plates

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-plate pack	5-plate pack	25-plate pack	
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	

RT = room temperature
Dash (—) = not applicable

Table 2. Additional reagents provided in the Development Pack for 384-well Plates

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1-plate pack	5-plate pack	25-plate pack	
Stop Solution	2–8 °C	R50AO-1	40 mL	1 bottle	2 bottles	10 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	

RT = room temperature
Dash (—) = not applicable

Additional Components

The following is a list of additional components needed for creating R-PLEX and U-PLEX custom multiplexes in combination with U-PLEX Development Packs. These may be purchased separately.

U-PLEX Reagents

U-PLEX Antibody Sets

U-PLEX Antibody Sets (Table 3) contain a biotinylated capture antibody and a SULFO-TAG conjugated detection antibody.

*Table 3. Contents of U-PLEX Antibody Sets**

Name	Storage	Size	Quantity Supplied	Description
U-PLEX Biotin Capture Antibody (analyte-specific)	2–8 °C	1 Plate	1 vial	Biotinylated capture antibody
		5 Plates	5 vials	
U-PLEX SULFO-TAG Detection Antibody (analyte-specific)	2–8 °C	1 Plate	1 vial	SULFO-TAG conjugated detection antibody (100X)
		5 Plates	5 vials	

*U-PLEX Antibody Sets do not include calibrators. Calibrators can be purchased separately.

U-PLEX Calibrators

For multiplexing in combination with U-PLEX assays, one or more of the following Calibrators (Table 4) are required, depending on the U-PLEX assays of interest. Calibrators for U-PLEX assays contain one or more analytes and may be either lyophilized or frozen in a buffered diluent. Individual analyte concentrations are provided in the lot-specific Calibrator certificates of analysis (COA) received with your order and available at the www.mesoscale.com® website.

Table 4. Analytes included in the Calibrator blends available for U-PLEX Assays

Species	Name	Storage	Catalog No.	Quantity Supplied			Analytes
				1 plate	5 plates	25 plates	
Human/NHP	Calibrator 1	2–8 °C	C0060-2	1 vial	5 vials	25 vials	GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, TNF-α, VEGF-A
	Calibrator 2	2–8 °C	C0061-2	1 vial	5 vials	25 vials	Eotaxin, Eotaxin-3, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC
	Calibrator 3	2–8 °C	C0062-2	1 vial	5 vials	25 vials	G-CSF, IFN-α2a, IL-1α, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-18, TNF-β, TPO
	Calibrator 4	2–8 °C	C0063-2	1 vial	5 vials	25 vials	CTACK, ENA-78, Fractalkine, I-TAC, MIP-3α, MIP-3β, SDF-1α
	Calibrator 6	2–8 °C	C0072-2	1 vial	5 vials	25 vials	IL-17A/F, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-29/IFN-λ1, IL-31, IL-33, TSLP
	Calibrator 9	2–8 °C	C0090-2	1 vial	5 vials	25 vials	EPO, FLT3L, IFN-β, IL-1RA, IL-2Rα, IL-3, IL-9, IL-17B, IL-17C, IL-17D
	Calibrator 10	2–8 °C	C0091-2	1 vial	5 vials	25 vials	Eotaxin-2, GRO-α, I-309, MCP-2, MCP-3, M-CSF, MIF, MIP-5, TRAIL, YKL-40
Human	Calibrator 13	2–8 °C	C0271-2	1 vial	5 vials	25 vials	FGF-21, Ghrelin (total), GIP (inactive), GIP (total), GLP-1 (inactive), GLP-1 (total), Glucagon, Leptin, PP, PYY (total)
	Calibrator 14	2–8 °C	C0272-2	1 vial	5 vials	25 vials	BAFF, BDNF, FGF-23, FSH, LH, β-NGF
	Calibrator 15	2–8 °C	C0273-2	1 vial	5 vials	25 vials	C-Peptide, Insulin
	Human Clusterin Calibrator	2-8 °C	C01B9-2	1 vial	5 vials	25 vials	Clusterin
	Ghrelin (active) Calibrator^	2–8 °C	C016K-2	1 vial	5 vials	25 vials	Ghrelin (active)
	Human GIP (active) Calibrator	2–8 °C	C016N-2	1 vial	5 vials	25 vials	GIP (active)
	GLP-1 (active) Calibrator^	2–8 °C	C016L-2	1 vial	5 vials	25 vials	GLP-1 (active)
	Human Proinsulin Calibrator	2–8 °C	C016M-2	1 vial	5 vials	25 vials	Proinsulin
	Calibrator 20	2–8 °C	C0328-1	1 vial	5 vials	25 vials	CD276/B7-H3, CD20, CD27, CD28, CTLA-4, GITRL, OX40, PD1, TIGIT, TLR1
	Calibrator 21	2–8 °C	C0329-2	1 vial	5 vials	25 vials	BAFF-R/TNFRSF13C, CD40L (soluble), FGF (basic), PD-L1, PD-L2, PIGF, RANKL/TNFSF11, VEGF-D
	Calibrator 22	2–8 °C	C0330-2	1 vial	5 vials	25 vials	BCMA, gp130 (soluble), HAVCR2/TIM-3, Tie-2
	Calibrator 23*	≤–70 °C	C0331-2	1 vial	5 vials	25 vials	Granzyme A, Granzyme B, LAG3
	Calibrator 24	2–8 °C	C0351-2	1 vial	5 vials	25 vials	DPPIV, ICAM-1, SAA, SHBG, VCAM-1
	Calibrator 25	2–8 °C	C0352-2	1 vial	5 vials	25 vials	CA1, Complement Factor D, CRP, Cystatin C, Factor VII, NGAL/LCN2, sTfR-1
	Calibrator 26	2–8 °C	C0353-2	1 vial	5 vials	25 vials	A2M, Adiponectin, ApoA1, ApoC3, Complement C9, RBP4, Serpin A1
	Calibrator 27	2–8 °C	C0410-2	1 vial	5 vials	25 vials	APRIL/TNFSF13, E-Selectin, Galectin-9, HVEM/TNFRSF14, MIG, Pentraxin 3, RAGE (soluble)
	Calibrator 28	2–8 °C	C0411-2	1 vial	5 vials	25 vials	ICOS, LIGHT/TNFSF14, MMP-1, MMP-7, Nectin-4, Perforin, VEGFR-1/Flt-1

Species	Name	Storage	Catalog No.	Quantity Supplied			Analytes
				1 plate	5 plates	25 plates	
	Calibrator 29	2–8 °C	C0412-2	1 vial	5 vials	25 vials	ICOS-L/B7-H2, MMP-2, MMP-9 (total), proMMP-9, P-Selectin, RANTES, S100A12, TNF-RI, TNF-RII
	Human vWF Calibrator	2–8 °C	C01C9-2	1 vial	5 vials	25 vials	vWF
Mouse	Calibrator 5	2–8 °C	C0065-2	1 vial	5 vials	25 vials	EPO, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, KC/GRO, TNF-α, VEGF-A
	Calibrator 7	2–8 °C	C0073-2	1 vial	5 vials	25 vials	IL-16, IL-17A, IL-17C, IL-17E/IL-25, IL-21, IL-22, IL-23
	Calibrator 8	2–8 °C	C0074-2	1 vial	5 vials	25 vials	IL-15, IL-17F, IL-31, IL-33, IL-27p28/IL-30
	Calibrator 12	2–8 °C	C0092-2	1 vial	5 vials	25 vials	IL-9, IL-17A/F, IP-10, MCP-1, MIP-1α, MIP-1β, MIP-2, MIP-3α
	Calibrator 16*	≤–70 °C	C0295-2	1 vial	5 vials	25 vials	6CKine/CCL21, BAFF, BCA-1/BLC, CD40, IFN-β, MCP-5/CCL12, MDC
	Calibrator 17	2–8 °C	C0296-2	1 vial	5 vials	25 vials	Eotaxin, MMP-9 (total), NGAL/LCN2, RANTES, SDF-1α, TARC, TNF-RI
	Mouse IFN-α Calibrator	2–8 °C	C02W1-2	1 vial	5 vials	25 vials	IFN-α
Mouse/Rat	Calibrator 18	2–8 °C	C0292-2	1 vial	5 vials	25 vials	BDNF, C-Peptide, FGF-21, Ghrelin, GLP-1 (inactive), GLP-1 (total), PYY (total)
	Calibrator 19	2–8 °C	C0293-2	1 vial	5 vials	25 vials	Glucagon, Insulin, Leptin
Human/NHP /Mouse	Calibrator 11	2–8 °C	C0244-2	1 vial	5 vials	25 vials	TGF-β1, TGF-β2, TGF-β3

*Calibrators 16 and 23 are liquid blend calibrators that can be blended with other calibrators once thawed.
^Calibrators used for Human, Mouse, and Rat.

R-PLEX Reagents

R-PLEX Antibody Sets

The R-PLEX Antibody Set contains a biotinylated capture antibody, a SULFO-TAG conjugated detection antibody, and a frozen calibrator (Table 5). The top-of-the-curve concentration is shown in the datasheet.

Table 5. Contents of R-PLEX Antibody Set

Name	Storage	Size	Quantity Supplied	Description
Biotin Capture Antibody (analyte-specific)	2–8 °C	5 Plates	1 vial	Biotinylated capture antibody, provided as one vial per five plates
SULFO-TAG Detection Antibody (analyte-specific)	2–8 °C	5 Plates	1 vial	SULFO-TAG conjugated detection antibody (100X), provided as one vial per five plates
Calibrator (analyte-specific)	≤–70 °C	1 Plate	5 vials	Native or recombinant protein provided frozen in a buffered diluent, provided as one vial per plate

General Reagents

Diluents

R-PLEX and U-PLEX assays may have specific diluents for sample and calibrator dilution as well as for the preparation of the detection Antibody Solution. The catalog numbers for diluents commonly used in multiplex assays are available for purchase at www.mesoscale.com

To run five plates, 50 mL of assay diluent and 40 mL of antibody diluent are required for a 96-well plate when assaying samples that are diluted up to 10-fold (40 samples per plate, run in duplicate). Additional assay diluent is necessary for samples that are diluted greater than 10-fold. Diluent 100 may be used in place of assay diluent for samples that require high dilution. Testing of different diluents can help optimize assays for specific experimental conditions.

For further guidance on choosing diluents, please contact our Scientific Support team at 240-314-2798 or scientificsupport@mesoscale.com.

Wash Buffer

Wash Buffer (Table 6) may be ordered separately.

Table 6. Catalog number of MSD Wash Buffer

Name	Storage	Catalog No.	Size	Description
MSD Wash Buffer (20X)	RT	R61AA-1	100 mL	Phosphate-based buffer; for plate washing

RT = room temperature

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid-handling equipment suitable for dispensing 10 to 150 µL/well into a 96-well or 384-well microtiter plate
- ❑ Plate-washing equipment: automated plate washer or multichannel pipette
- ❑ MSD Wash Buffer (20X, 100 mL, catalog number R61AA-1) for plate washing.
- ❑ The standard U-PLEX 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.
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm (1,500 rpm for 384-well plates)
- ❑ Adhesive plate seals
- ❑ Deionized water
- ❑ Vortex mixer
- ❑ MSD Diluent 100 (50 mL, catalog number R50AA-4) for diluting samples that need high dilution.

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 biotinylating 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

Instrument Compatibility

MSD offers U-PLEX Assays designed for use on specific instrument platforms (Table 7).

Table 7. 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

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 applicable safety data sheet(s) (SDS), which can be obtained from MSD Customer Service or at www.mesoscale.com.

Best Practices

- Bring frozen diluents to room temperature in a 20–26 °C water bath prior to 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 prior to 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 polypropylene containers 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 prior to 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 should 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 other 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 ºC) prior to adding it to the plate.
- Keep time intervals consistent between the addition of Read Buffer and reading the plate to improve inter-plate 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 Prepartion

Follow the protocol provided here for R-PLEX and U-PLEX assays. See the “Using Your own Reagents” section for non-MSD components.

Using MSD reagents

Bring all reagents to room temperature and refer to the Best Practices (page 12) section before beginning the protocol.

Note: Upon the first thaw, aliquot diluents into suitable volumes before refreezing.

To prepare supplemental reagents such as MSD Wash Buffer, please refer to page 19.

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 examples (Figure 4). Assign each antibody to a unique Linker and record the antibody identity next to the assigned Linker, as shown in the examples below.

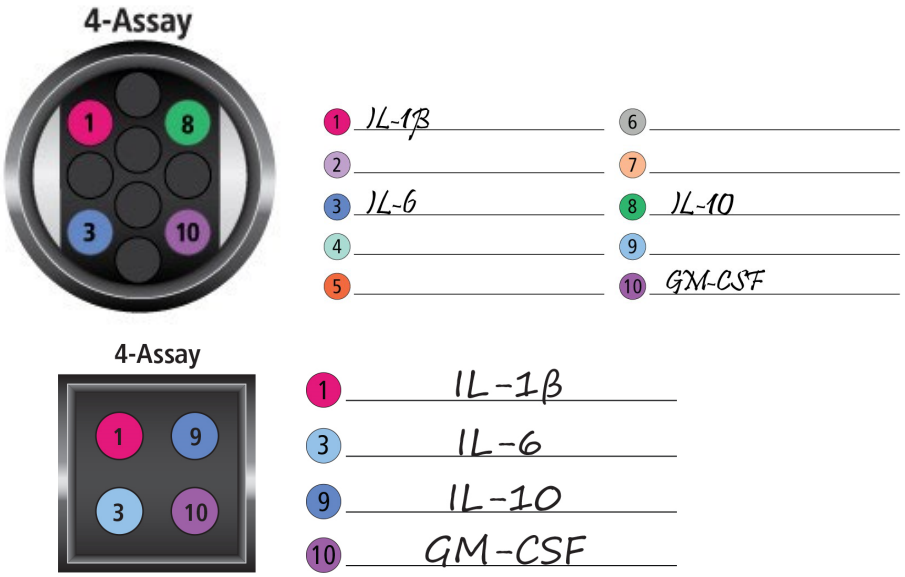

*Figure 4. U-PLEX 4-Assay plate maps with recorded antibodies and assigned Linkers (**top**) 96-well, (**bottom**) 384-well.*

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 8).

STEP 1: Create Individual U-PLEX Linker-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 (blank Spot Maps are provided on page 33).

- ❑ Add 200 µL of each biotinylated antibody to 300 µL of the assigned Linker. Mix by vortexing. Incubate at room temperature for 30 minutes. Do not shake.

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.
- Open one Linker at a time and close its cap as soon as you are done using it. Take precautions to avoid reagent contamination.
- For 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.

- ❑ Add 200 µL of Stop Solution, then mix by vortexing. Incubate at room temperature for 30 minutes.

Note: At the end of step 1, each U-PLEX Linker-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 8). The volumetric ratio of Linker:Antibody:Stop Solution is 3:2:2.

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

- ❑ Combine 600 µL of each U-PLEX Linker-coupled antibody solution into a 15 mL 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 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 2, 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 µL 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. 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 the U-PLEX 96-well Plate

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

STEP 3b: Coat the 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 plate is now coated and ready for use. Plates may be stored in the original pouch with desiccant and sealed for up to 7 days at 2–8 °C.

The recommended volumes of Linker, biotinylated capture antibody, and Stop Solution for coating one or multiple U-PLEX plates are provided below in Table 8. If using a partial plate, refer to Table 11 or Table 12. Also, see Table 10.

Table 8. 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

U-PLEX calibrators are provided as either frozen or lyophilized formulations. COAs for each calibrator are available at www.mesoscale.com and provide specific analyte amounts and concentrations for the recommended stock solutions.

R-PLEX calibrators are single analyte preparations that are provided as frozen formulations.

1. To prepare R-PLEX calibrator solutions for up to four replicates:

Thaw the R-PLEX assay calibrator(s) on wet ice for at least 30 minutes and keep on ice. The Calibrator will need to be diluted 40-fold (per the instructions in step 4) to generate the highest point in the standard curve (i.e., Calibrator Standard 1).

Note: R-PLEX calibrators are labeled as 20X, however, they are used at 40X in this protocol.

If only R-PLEX calibrators are being used in the assay, continue to Step 4.

2. To prepare all lyophilized U-PLEX calibrators:

a) Add 250 µL of assay diluent to each of the lyophilized calibrator vials supplied. This will result in 10X Calibrator stock concentration, which will need to be diluted 10-fold (per the instructions in step 4) to generate the highest point in the standard curve (i.e., Calibrator Standard 1).

b) Invert the reconstituted Calibrator at least 3 times. Do not vortex. Let the reconstituted solution equilibrate at room temperature for 15–30 minutes and then vortex briefly. The Calibrator is now ready for use. Keep the dilutions at room temperature.

3. To prepare liquid U-PLEX calibrators (calibrators 16 and 23):

a) Thaw the stock Calibrator(s) and keep on ice. The Calibrator will need to be diluted 10-fold (per the instructions in step 4) to generate the highest point in the standard curve (i.e., Calibrator Standard 1).

b) Once thawed, the Calibrator is ready to use. Keep dilution(s) at room temperature.

4. Prepare eight Calibrator Standards (7 calibrator solutions plus a zero calibrator) for R-PLEX and/or U-PLEX calibrators (Figure 5, Table 9):

a) Combine 10 µL of each R-PLEX calibrator and 40 µL of each U-PLEX calibrator, as needed. Add assay diluent to bring up the volume to 400 µL. Mix by vortexing.

b) For Calibrator Standard 2, add 50 µL of Calibrator Standard 1 to 150 µL of the assay diluent. Mix by vortexing.

c) Repeat 4-fold serial dilutions five additional times to generate a total of seven Calibrator Standards. Mix by vortexing between each serial dilution.

d) Use assay diluent as Calibrator Standard 8 (zero Calibrator).

Discard any unused diluted calibrators.

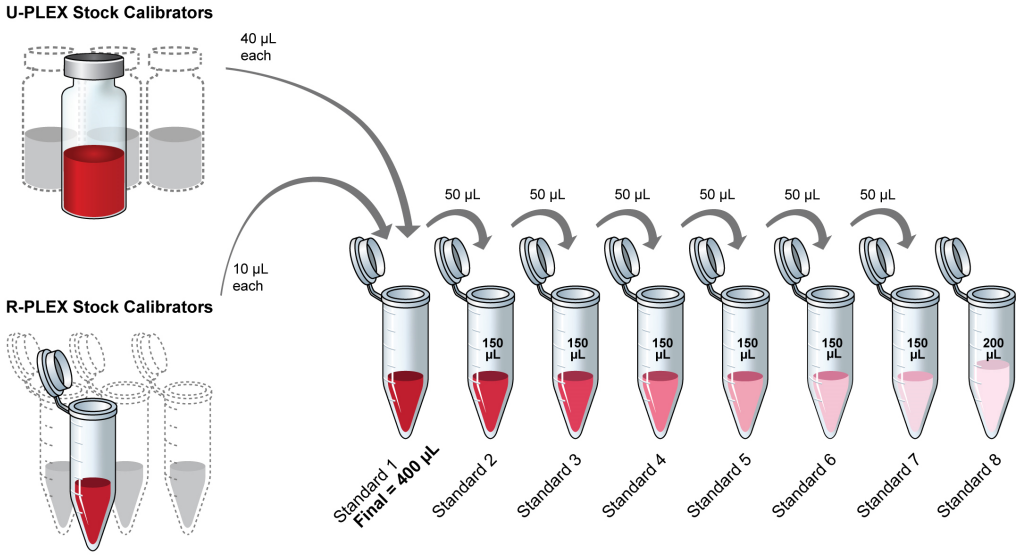

Figure 5. Dilution schema for preparation of Calibrator Standards for multiplex assays.

Note: For the lot-specific concentration of the analytes in U-PLEX Calibrators, refer to the COA received with your order and available at www.mesoscale.com. The concentration for liquid U-PLEX calibrator(s) is 10X and no adjustment is needed. For the R-PLEX analyte, refer to the datasheet supplied with the product for calibrator stock concentration and divide by 40 to calculate the top of curve concentration.

Table 9. 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)	varies		400
2	2	From tube 1	50	150	200
3	3	From tube 2	50	150	200
4	4	From tube 3	50	150	200
5	5	From tube 4	50	150	200
6	6	From tube 5	50	150	200
7	7	From tube 6	50	150	200
8 (zero Calibrator)	8	—	0	300	200

Dash (—) = not applicable

Sample Collection and Handling

Samples with hemolysis or significant lipemia may hinder accurate measurements.

Repeated freeze/thaw cycles of samples is not recommended. After thawing, centrifuge samples at 2,000 × *g* for 3 minutes to remove particulates before using them in the assay. If the samples are clear and no particulates are visible, you may not need to centrifuge. Hold at 2–8 °C until ready to use in the assay.

Below are general guidelines for sample collection, storage, and handling for metabolic markers. We strongly suggest following these procedures if measuring the active forms of metabolic analytes. If other methods are used, evaluate sample stability under the selected method as needed.

Sample Collection for Metabolic Assays

Samples (serum or plasma that is not from mouse) should be collected using the BD P800 Collection and Preservation System, which contains protease inhibitors (Product Number 366420 or 366421). The alternative collection method described below with K_2EDTA tubes can also be used.

Non-P800 collection method

Collect blood in BD Vacutainer K_2EDTA Tubes (Product Number 367841 or 366643). Immediately add a dipeptidyl peptidase IV (DPP-IV) inhibitor (0.1 mM final concentration of diprotin A is recommended, not provided) and aprotinin (1,000 KIU/mL final concentration) and mix to avoid cleavage/degradation of metabolic peptides.

For BD tubes, process as follows:

- ❑ In a swing-out rotor centrifuge, spin the blood collection tubes as follows;
  - For 2 mL tubes—10 minutes at 1,000 × *g* (2–8 °C).
  - For 8.5 and 10 mL tubes—20 minutes at 1,300 × *g* (2–8 °C).

Use the plasma immediately or the samples can be stored at 2–8 °C if used within 3 hours. For future use, aliquot the plasma and freeze it in suitably sized aliquots at ≤–70 ºC.

If not using the method above, add a DPP-IV inhibitor (0.1 mM final concentration, not provided) and aprotinin (1,000 KIU/mL final concentration) and use immediately or freeze at ≤–70 °C.

Dilute Samples

Depending on the sample set under investigation, dilution may be necessary. For complex matrices including plasma and serum, a minimum 2-fold dilution is recommended to avoid matrix effects. Assay Diluent may be used for sample dilution. The dilution factor for the given sample type may need to be optimized.

Notes:

- Additional assay diluent is necessary for samples that are diluted greater than 10-fold. Diluent 100 may be used in place of assay diluent for samples that require high dilution.
- Certain human assays (BCMA/TNFRSF17, gp130 (soluble), HAVCR2/TIM-3, ICOS-L/B7-H2, MIF, MIP-5, MMP-2, MMP-9 (total), proMMP-9, P-Selectin, RANTES, S100A12, Tie-2, TNF-RI, TNF-RII, and YKL-40), NHP assays (MIF, and YKL-40), and mouse assays (6CKine/CCL21, BAFF, and NGAL/LCN2) may need greater sample dilution to generate optimal results. Refer to the product-specific datasheets for additional information.
- For CD20, it is strongly recommended that samples be diluted in Assay Diluent supplemented with 0.5% Triton X-100 (catalog number R94AA-1). Triton X-100 was found to improve the recognition of analytes in samples.
- For PD-L1 (epitope 1), PD-L1 (epitope 2), and GITR, samples may optionally be diluted in Assay Diluent supplemented with 0.5% Triton X-100. Triton X-100 was found to improve analyte recognition in some samples.
- For Metabolic Group 1 assays, see the relevant Metabolic Group 1 Insert for preparing Metabolic Assay Working Solution.

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.

For one plate, combine:

- ❑ 60 µL of each 100X detection antibody
- ❑ Antibody diluent to bring the final volume to 6 mL (12 mL for 384-well assays)

Wash Buffer

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

Read Buffer

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

Using Your Own Reagents

Follow this protocol when developing assays with your reagents. Guidance on preparing calibrators and controls is provided in Appendix A.

Coating with Antibodies

Multiplexing assays on U-PLEX plates is achieved through specific capture material on each spot and coupling with a unique Linker. All capture antibodies (or other suitable capture reagents) should be biotinylated. Guidance on biotin conjugation is provided in Appendix A.

When coating a multiplex plate, it is important to couple each biotin antibody to a unique Linker.

The protocol in this section describes the preparation of a multiplex coating solution for a single 96-well plate. The volumes can be adjusted depending on the number of plates or wells, but the ratios of the reagents should remain the same. You may adjust the capture antibody concentration and the final coating buffer concentration, but most assays do not benefit from increasing the capture antibody concentration.

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

Couple individual biotinylated antibodies (or another suitable capture reagent) to a unique Linker and record the antibody identity next to the Linker number on the Spot Map (a copy is provided on page 33).

- ❑ Dilute each biotinylated antibody to 10 µg/mL in coating diluent for a final volume of at least 200 µL per plate.

Notes:

- The Antibody Solution should not contain free biotin.
- Coating diluents can be simple diluents using 0.5% bovine serum albumin in PBS or MSD Diluent 100. MSD offers a variety of diluents that could be used to dilute the capture antibody. One percent Blocker D-M, D-R, D-B, and/or D-G could be added to the capture antibody diluent to reduce antibody-antibody nonspecific interactions.

- ❑ Add 200 µL of each biotinylated antibody to 300 µL of the assigned Linker. A different Linker should be used for each unique, biotinylated antibody. Vortex. Incubate at room temperature for 30 minutes.

Notes:

- Refer to the U-PLEX plate Spot Maps to determine which Linkers can be combined. For example, when coating 4 assays on a 4-Assay, U-PLEX plate, the antibodies should be coupled to Linkers 1, 3, 8, and 10 respectively to match the Spot Map.
- 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.
- Each Linker vial has a matching colored cap and label. Open one Linker at a time and close its cap before opening another Linker to avoid reagent contamination.

- ❑ dd 200 µL of Stop Solution. Vortex. Incubate at room temperature for 30 minutes.

Note: At the end of STEP 1, each individual U-PLEX-coupled Antibody Solution is at 10X the coating concentration and can be stored for up to 7 days at 2–8 °C.

Adjust the volumes for multiple plates as shown in Table 10. The volumetric ratio of Linker:antibody:Stop Solution is 3:2:2.

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

- ❑ Combine 600 µL of each U-PLEX-coupled Antibody Solution 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 to result in a final 1X concentration. Vortex. For example, for a 4-assay coating solution, add 3.6 mL of Stop Solution to the 2.4 mL of combined antibodies.

The multiplex coating solution can be stored at 2–8 °C. Do not store for more than 7 days. If using a partial plate, refer to Table 11.

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

- ❑ Combine 600 µL 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.should be used the same day. Do not store.

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

- ❑ Add 50 µL of multiplex coating solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature for 1 hour. Shaking the plate during incubation is required.
- ❑ Wash the plate 3 times with at least 150 µL/well of MSD Wash Buffer.
- ❑ The plate is coated and ready for use. Plates may be stored in the original pouch with desiccant for up to 7 days at 2–8 °C. If using a partial plate, refer to Table 11.

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

- ❑ 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 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. If using a partial plate, refer to Table 12.

The recommended volumes of Linker, biotinylated antibody, and Stop Solution for coating one or multiple U-PLEX plates are provided below (Table 10). The volumetric ratio of Linker:antibody:Stop Solution is 3:2:2.

Table 10. Amount of each component needed for U-PLEX Coating Solution (multiple plates)

No. of Plates	Individual Linker (µL)	Individual Biotinylated Antibody (µL)	Stop Solution (µL) per reaction
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

The recommended volumes of Linker, biotinylated capture antibody, and Stop Solution for coating partial plates are provided below (Tables 11 and 12).

Table 11. Amount of each component needed for U-PLEX Coating Solution (partial 96-well plate)

No. of Wells	Individual Linker (µL)	Individual Biotinylated Capture Antibody (µL)	Stop Solution per reaction (µL)	Vol. to Pull from Each Reaction (µL)
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 12. 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

Coating with Non-Antibody Molecules

Non-antibody molecules, such as biotinylated antigens and peptides, can also be immobilized on the U-PLEX plate. To optimize the coating concentrations, we recommend preparing the biotin molecules at 66, 33, and 16.7 nM. The equation for calculating nM and µg/mL concentrations is available in the MSD Biotin Conjugation Quick Guide found at www.mesoscale.com.

Assay Protocols

Note: Follow Reagent Preparation before beginning this assay protocol.

96-well Plate Assays

STEP 1: Add Sample or Calibrator Standard

- ❑ Add 50 µL of the prepared Calibrator Standard or diluted 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 1X MSD 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 MSD 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.

384-well Plate Assays

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of the prepared Calibrator Standard or diluted 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 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL 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 µL/well of 1X MSD Wash Buffer.
- ❑ Add 40 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.

Alternate Protocols

The suggestions below may be useful for improving sensitivity or 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, Shortened Incubation:** Some 384-well assays may achieve acceptable performance with shorter incubations. Consider reducing the incubation time of samples in the plate and of detection antibody each to 1 hour.
- ❑ **Alternate Protocol 3, 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.

Appendix A

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 because it will bind to the biotin on the capture antibody.

Prepare Biotinylated Capture Antibody

The working concentration of 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. The MSD Biotin Conjugation Quick Guide may be found at www.mesoscale.com.

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 concentration 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 backgrounds 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 9).

U-PLEX is an assay development tool that enables you to coat multiplex plates with ease and flexibility. It is an excellent platform for creating assays of your choice, screening a large number of antibodies, or testing the feasibility of multiplexing assays together. You may need to optimize your assays once the capture antibodies are coated on the U-PLEX plate. The assay performance and quality will vary depending on several factors, including antibody affinity and concentration, diluent selection, incubation time, and the combination of multiplexed assays.

Appendix B

To evaluate the quality of the U-PLEX platform, we tested over 175 assays that were already optimized and validated, the majority of which share the same antibodies and reagents as the V-PLEX® product line. These assays were tested for sensitivity, dynamic range, precision, and sample measurement on U-PLEX.

Typical Calibration Curves

Representative calibration curves from 2 sets of multiplexed assays on U-PLEX plates are presented in Figure 6. Assays performed on U-PLEX typically show a 3- to 4-log dynamic range. Wide dynamic range allows simultaneous measurement of normal and diseased/stimulated samples with the same sample dilution.

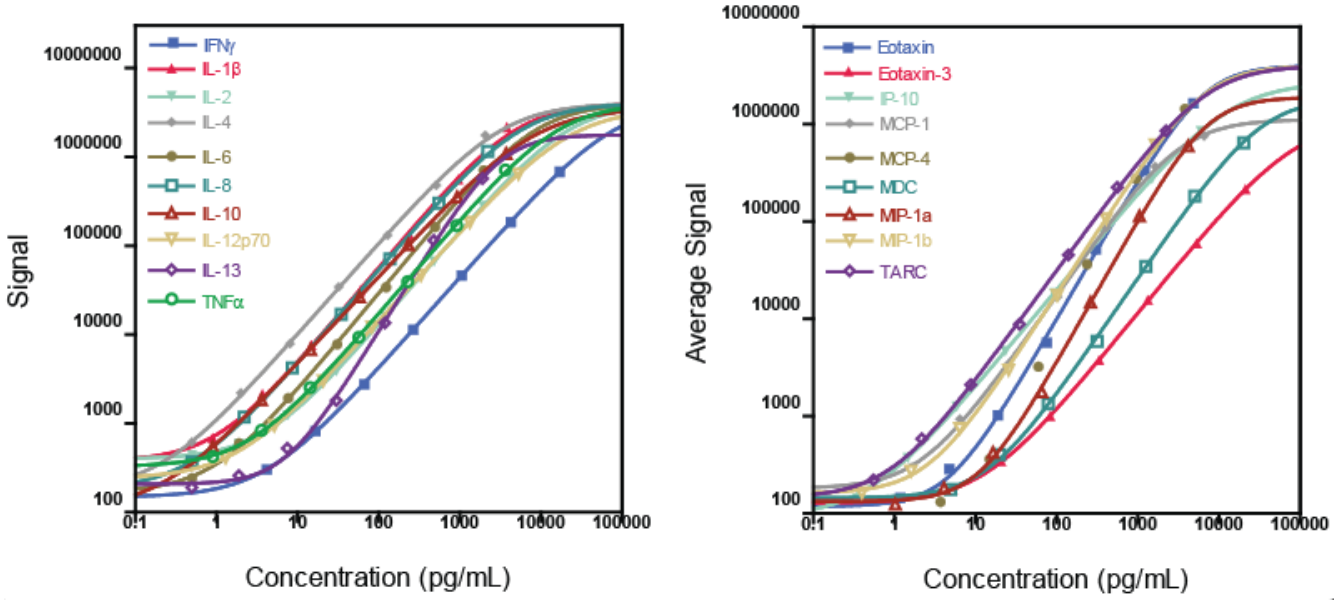

Figure 6. Representative calibration curves.

Sensitivity

For each assay, we measured the lower limit of detection (LLOD), which is the calculated concentration corresponding to the signal 2.5 standard deviations above the background (Table 13; Table 14). The median LLODs on U-PLEX were evaluated based on at least 17 plates across multiple independent runs. Representative LLODs are presented below. LLODs may vary depending on the antibodies used in developing the assays.

Table 13. LLOD, U-PLEX, selected analytes

	LLOD (pg/mL)									
Assay	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-13	TNF-α
U-PLEX	1.7	0.15	0.7	0.08	0.33	0.15	0.14	0.69	3.1	1.7

Table 14. LLOD, U-PLEX, selected analytes

	LLOD (pg/mL)							
Assay	Eotaxin	IP-10	MCP-1	MCP-4	MDC	MIP-α	MIP-β	TARC
U-PLEX	3.2	0.49	0.74	7.5	8.4	7.7	1.5	0.51

Reproducibility

We evaluated the reproducibility of the U-PLEX platform by measuring the intraplate, inter-plate, and interlot %CVs of calibrators and controls. Representative data from 8 assays are shown in Table 15. The same assays were tested across 3 lots of U-PLEX plates and 3 lots of Linkers. Calibrators within the measurable range of each assay were evaluated. The average intraplate and interplate %CVs for both the calibrator signals and the back-fitted calculated concentrations for most assays were less than 10% in all the lots tested (data not shown). Reproducibility of controls at 3 different levels is shown below. The average intra-plate and inter-plate %CVs for most assays were below 7%.

Table 15. U-PLEX platform reproducibility

Analyte	Control	Lot 1 (N = 9 Plates)			Lot 2 (N = 6 Plates)			Lot 3 (N = 9 Plates)			Interlot 11	
		Avg. Conc. (pg/mL)	Avg. Intra-Plate %CV	Inter-Plate %CV	Avg. Conc. (pg/mL)	Avg. Intra-Plate %CV	Inter-Plate %CV	Avg. Conc. (pg/mL)	Avg. Intra-Plate %CV	Inter-Plate %CV	Avg. Inter-lot Conc.	Inter-lot %CV
Eotaxin	High	924	3.4	3.8	991	3.4	3.4	997	2.3	2.8	971	4.2
	Mid	500	2.6	2.9	531	3.2	3.9	540	2.2	2.9	524	4.0
	Low	179	3.4	2.8	190	5.4	2.0	198	2.9	3.6	189	5.0
MIP-1β	High	167	4.5	2.6	162	6.0	7.3	155	6.9	2.3	161	3.7
	Mid	82	7.2	3.7	80	6.2	8.8	77	6.3	2.6	80	3.2
	Low	25	4.8	2.8	25	4.6	7.7	24	8.1	4.6	25	3.0
TARC	High	296	3.4	2.9	312	3.3	2.5	300	4.0	2.8	303	2.8
	Mid	146	3.1	2.7	153	3.0	4.1	148	4.6	2.0	149	2.3
	Low	47	3.2	2.9	49	4.3	2.7	49	4.6	2.4	49	2.2
IP-10	High	1,800	7.4	18.1	1,810	6.7	14.9	2,230	8.3	5.0	1,940	12.7
	Mid	896	5.6	11.8	955	4.0	8.5	1,050	4.2	4.9	967	8.1
	Low	296	3.5	8.9	319	4.6	5.1	339	4.9	6.5	318	6.6
MIP-1α	High	812	2.8	1.6	866	2.1	3.3	857	2.6	1.9	845	3.4
	Mid	417	3.0	1.2	444	2.6	2.4	435	3.3	2.5	432	3.1
	Low	136	2.8	1.8	144	3.5	3.0	140	2.9	3.1	140	2.6
MCP-1	High	642	4.8	2.4	722	4.1	1.9	681	3.9	5.6	682	5.9
	Mid	289	7.3	2.5	329	5.3	3.7	314	4.5	6.0	311	6.4
	Low	81	6.1	2.1	90	4.5	4.2	86	6.8	7.0	86	5.3
MDC	High	4,000	3.7	2.9	3,880	3.5	3.1	3,640	14.0	6.9	3,840	4.8
	Mid	2,100	3.3	2.8	2,042	3.0	4.4	1,880	12.2	7.8	2,010	5.7
	Low	683	3.1	2.8	672	2.5	6.1	608	15.6	8.0	654	6.2
MCP-4	High	576	2.0	1.7	647	2.4	3.2	616	2.2	4.2	613	5.9
	Mid	308	1.6	1.4	346	2.0	3.4	333	2.0	4.0	329	5.8
	Low	109	2.3	1.9	121	4.8	3.8	118	3.0	4.5	116	5.1

Sample Measurement

Forty human serum and EDTA plasma samples were tested on both U-PLEX and V-PLEX using the same antibodies, calibrators, and diluents (Table 16). Overall, there is a strong correlation between the V-PLEX assays and the same assays performed on U-PLEX as shown in the table.

Table 16. Comparison of U-PLEX sample measurement with V-PLEX

Sample Type	Statistic	Eotaxin	MIP-1β	TARC	IP-10	MIP-1α	MCP-1	MDC
Serum	r^2 Value	0.98	0.95	0.85	0.99	0.97	0.86	0.83
	Slope	0.81	0.83	0.92	0.90	1.19	1.08	0.71
EDTA Plasma	r^2 Value	0.84	0.95	0.93	0.97	0.99	0.78	0.86
	Slope	0.76	0.82	0.99	0.80	0.94	1.47	0.70

Summary Protocol

Prepare U-PLEX Plate

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 below (Figure 7).

- ❑ If using your antibodies, dilute each biotinylated antibody to 10 µg/mL in coating diluent for a final volume of at least 200 µL per plate.
- ❑ 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 2a: 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.

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

- ❑ Combine 600 µL 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.

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

- ❑ Add 50 µL of 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.

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

- ❑ 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 plate is now coated and ready for use.

Summary 96-well Assay Protocol

STEP 1: Add Sample or Calibrator Standards

- ❑ Add 50 µL of prepared Calibrator Standard or diluted 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.

Summary 384-well Assay Protocol

STEP 1: Add Sample or Calibrator Standards

- ❑ Add 25 µL of prepared Calibrator Standard or diluted 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 µL/well of 1X Wash Buffer.
- ❑ Add 25 µL 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 µL/well of 1X Wash Buffer.
- ❑ Add 40 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.

Spot Maps

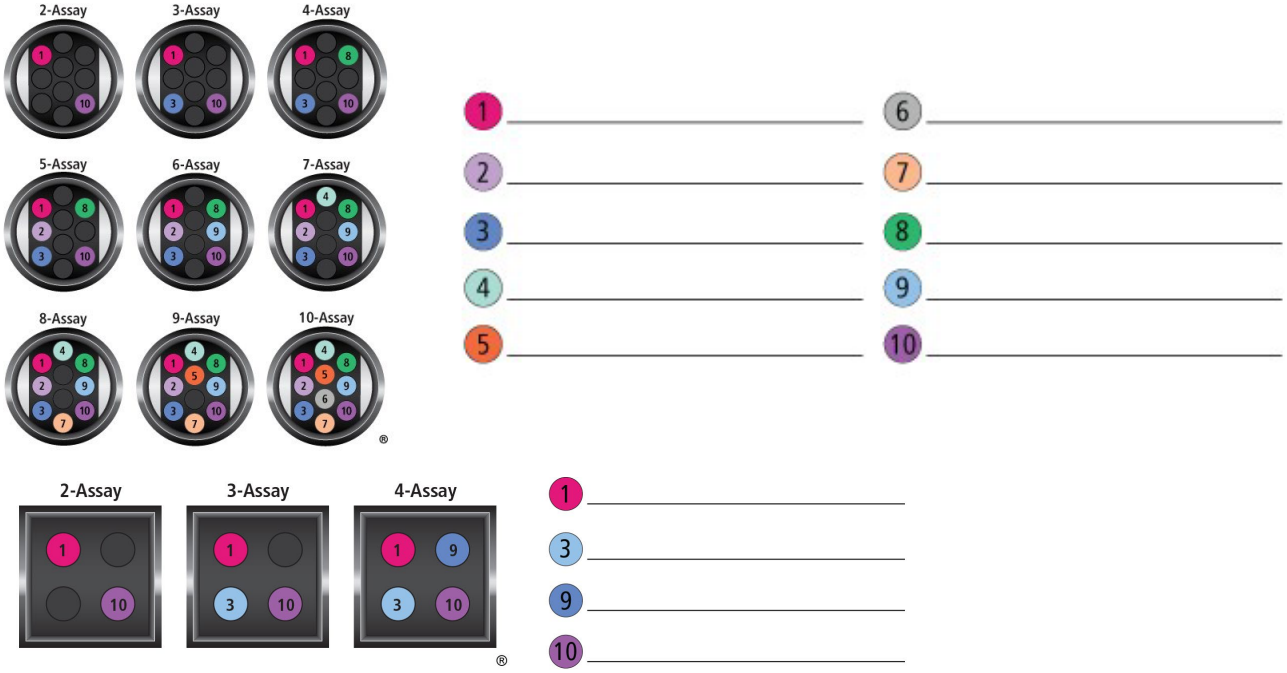

*Figure 7. Spot maps; **(top)** 96-well plate, **(bottom)** 384-well plate, for labeling linkers and spots with analytes.*

Plate Diagrams

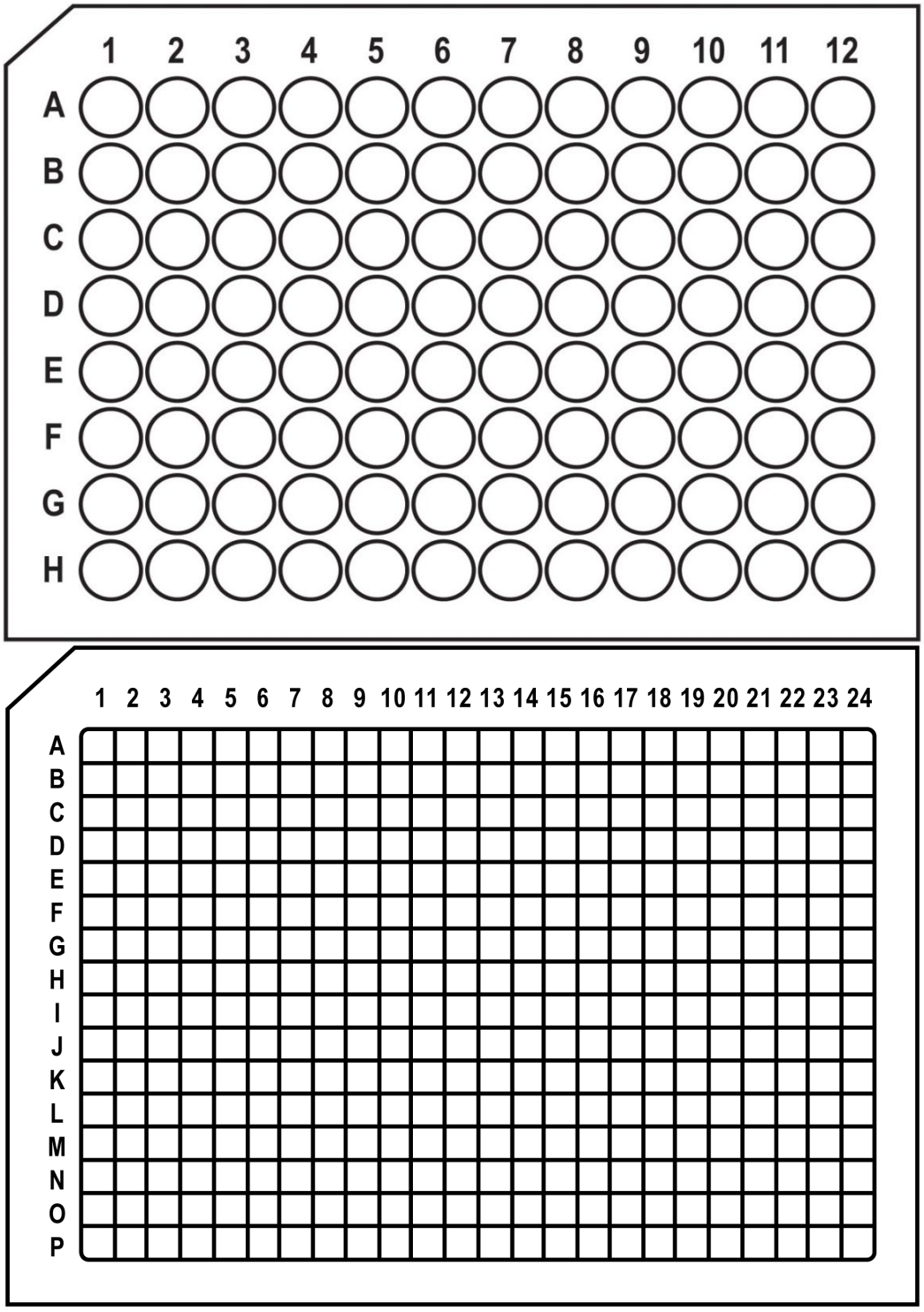

Figure 8. Plate diagrams; similar plate layouts can be created in Excel and easily imported into DISCOVERY WORKBENCH® software.