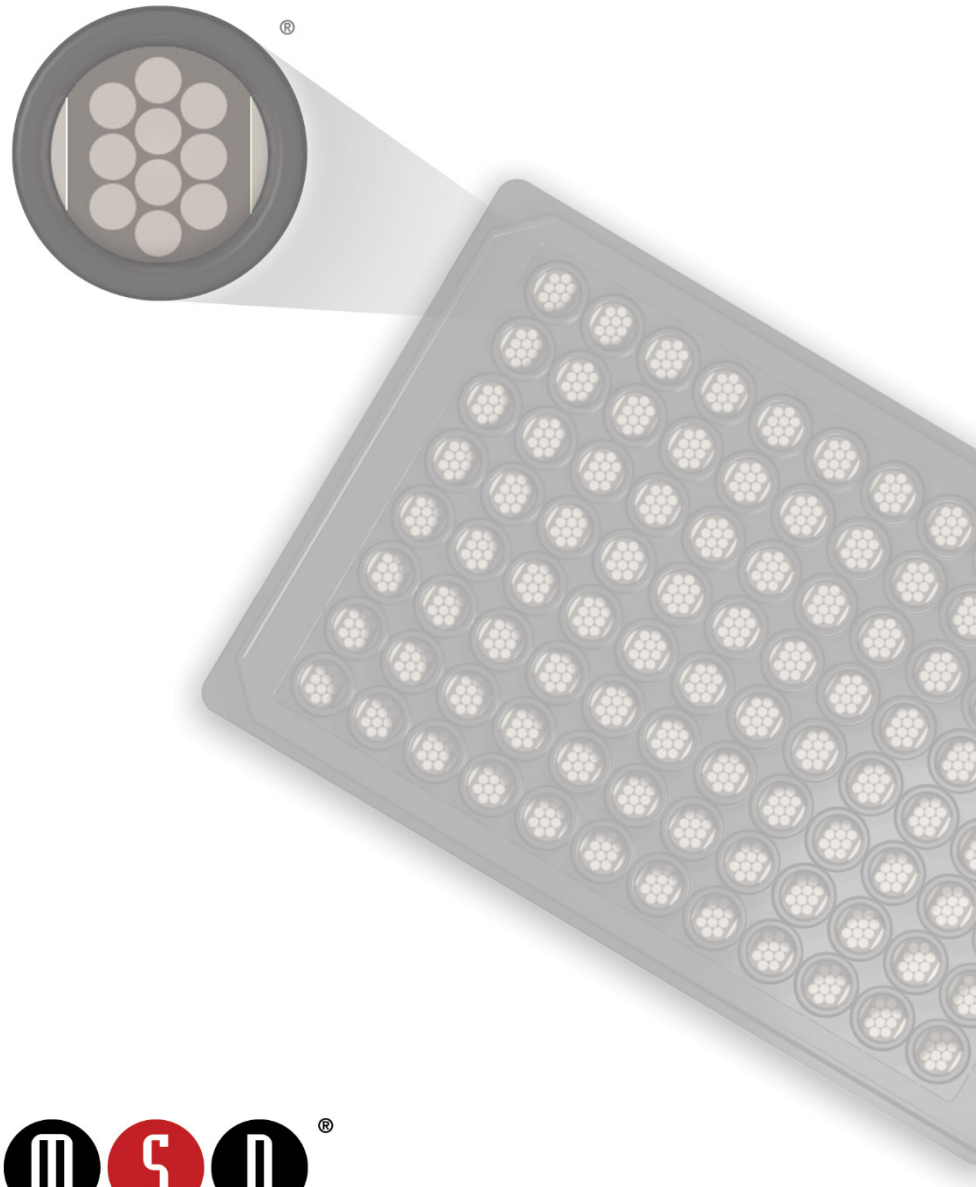
U-PLEX® Custom Biomarker Group 1 (NHP)

Multiplex Assays

U-PLEX®



MSD®

MSD U-PLEX Platform

U-PLEX Custom Biomarker (NHP) Multiplex Assays

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

Available as part of U-PLEX Custom Biomarker (NHP) Assays (catalog numbers: K15068M-1, K15068M-2, and K15068M-4).

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 two simple tools: a 10-Spot, U-PLEX plate and unique Linkers (Figure 1). The U-PLEX platform combines high sensitivity, up to 5 logs of linear dynamic range, a read time of less than 2 minutes, and the flexibility to create your own personalized multiplex assays.

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. To combine your assays with MSD U-PLEX assays, simply select the appropriate number of open spots for the number of assays you wish to add. The necessary number of Linkers and other reagents will be provided in your order. As many as 10 assays may be multiplexed on each plate for simultaneous measurement.

This product insert is for U-PLEX custom multiplex assays that contain a combination of assays from the U-PLEX Biomarker Group 1 (NHP) and open spots to enable you to use your antibody pairs. The U-PLEX Biomarker Group 1 (NHP) contains 60 analytes (Table 1) that are important in many biological processes. These assays can detect secreted biomarkers in a variety of body fluids and cell culture supernatants where over- or underexpression may indicate a shift in the biological equilibrium.

A representative data set for each of the assays in U-PLEX Biomarker Group 1 (NHP) is presented in the product-specific datasheets available at www.mesoscale.com/U-PLEX-documents. Performance of MSD assays may vary when tested in a 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.

Table 1. Assays included in U-PLEX Biomarker Group 1 (NHP)

Assays			
CTACK (CCL27)	IL-1RA	IL-17A/F	MDC (CCL22)
ENA-78 (CXCL5)	IL-2	IL-17B	MIF
Eotaxin (CCL11)	IL-4	IL-17C	MIP-1α (CCL3)
Eotaxin-2 (CCL24)	IL-5	IL-17D	MIP-1β (CCL4)
Eotaxin-3 (CCL26)	IL-6	IL-17F	MIP-3α (CCL20)
EPO	IL-7	IL-18	MIP-3β (CCL19)
FLT3L	IL-8 (CXCL8)	IL-22	MIP-5 (CCL15)
Fractalkine (CX3CL1)	IL-9	IL-23	SDF-1α (CXCL12)
G-CSF	IL-10	IP-10 (CXCL10)	TARC (CCL17)
GM-CSF	IL-12/IL-23p40	I-TAC (CXCL11)	TNF-α
GRO-α (CXCL1)	IL-12p70	MCP-1 (CCL2)	TNF-β
I-309 (CCL1)	IL-13	MCP-2 (CCL8)	TPO
IFN-γ	IL-15	MCP-3 (CCL7)	TRAIL
IL-1α	IL-16	MCP-4 (CCL13)	VEGF-A
IL-1β	IL-17A	M-CSF	YKL-40

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.

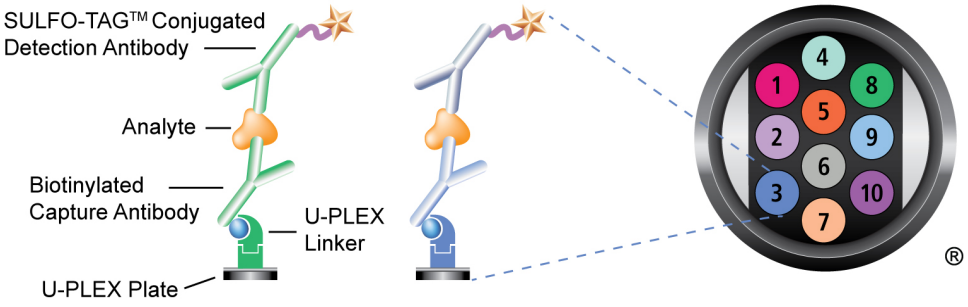

Figure 1. U-PLEX Immunoassay on a U-PLEX 10-Assay Plate.

Components

The U-PLEX Custom Biomarker (NHP) assay pack includes all the components required to complete your MSD U-PLEX assay plus sufficient U-PLEX reagents that will allow you to add your assays to the open spots provided. Table 2 and Table 3 list the components provided with U-PLEX Custom Biomarker (NHP) assays. You will only receive components relevant to the assays that you order.

Reagents Supplied With All U-PLEX Multiplex Assays

Table 2. Reagents that are supplied with all U-PLEX Custom Biomarker (NHP) assays

Reagent	Storage	Catalog No.	Size	Quantity Supplied			Description
				1 Plate	5 Plates	25 Plates	
Diluent 57	≤–10 °C	R50BZ-1	10 mL	1 bottle	—	—	Diluent for samples and Calibrators; contains serum, blockers, and preservatives
		R50BZ-2	50 mL	—	1 bottle	5 bottles	
Diluent 3	≤–10 °C	R50AP-1	8 mL	1 bottle	—	—	Diluent for detection antibody; contains protein, blockers, and preservatives
		R50AP-2	40 mL	—	1 bottle	5 bottles	
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 electro-chemiluminescent reaction. Use at room temperature
		R60AM-2	90 mL	—	1 bottle	5 bottles	

dash (—) = not applicable
RT = room temperature

Assay-Specific Reagents

10-Spot, 96-Well U-PLEX Plates

U-PLEX assays use MSD 96-well, 10-Spot plates, 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 depends on the number of assays to be multiplexed (Figure 2). For example, if 5 assays are being multiplexed, the U-PLEX 5-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.

Note: *In cases where there is a remainder of one assay, you will receive a U-PLEX 2-Assay plate. This will hold true for orders of 11, 21, 31 assays, and so on.*

Table 3. Part numbers and pack sizes for U-PLEX multiplex plates

SECTOR™ Plates	Part No.	Storage	Quantity Supplied		
			1 Plate	5 Plates	25 Plates
U-PLEX 2-Assay	N05227A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 3-Assay	N05228A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 4-Assay	N05229A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 5-Assay	N05230A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 6-Assay	N05231A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 7-Assay	N05232A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 8-Assay	N05233A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 9-Assay	N05234A-1/-2/-4	2–8 °C	1	5	25
U-PLEX 10-Assay	N05235A-1/-2/-4	2–8 °C	1	5	25

Linkers

Based upon the number of assays you select for multiplexing, you will receive the corresponding number of unique Linkers (Figure 3; Table 4; Table 5). 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 on 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.

Figure 3. Unique color-coded Linkers (10), Antibody Sets (10), and Calibrator vials (5) as shipped in a U-PLEX box.

Table 4. *U-PLEX Linker color coding, storage conditions, part numbers, and size*

Name	Color Coding	Storage	Part No.	Size	Quantity Supplied		
					1 Plate	5 Plates	25 Plates
U-PLEX Linker 1	1	2–8 °C	E2226-2	0.3 mL	1 vial	—	—
			E2226-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 2	2	2–8 °C	E2227-2	0.3 mL	1 vial	—	—
			E2227-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 3	3	2–8 °C	E2228-2	0.3 mL	1 vial	—	—
			E2228-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 4	4	2–8 °C	E2229-2	0.3 mL	1 vial	—	—
			E2229-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 5	5	2–8 °C	E2230-2	0.3 mL	1 vial	—	—
			E2230-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 6	6	2–8 °C	E2231-2	0.3 mL	1 vial	—	—
			E2231-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 7	7	2–8 °C	E2232-2	0.3 mL	1 vial	—	—
			E2232-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 8	8	2–8 °C	E2233-2	0.3 mL	1 vial	—	—
			E2233-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 9	9	2–8 °C	E2234-2	0.3 mL	1 vial	—	—
			E2234-3	1.8 mL	—	1 vial	5 vials
U-PLEX Linker 10	10	2–8 °C	E2235-2	0.3 mL	1 vial	—	—
			E2235-3	1.8 mL	—	1 vial	5 vials

dash (—) = not applicable

Table 5. *U-PLEX Linkers supplied with each pack*

Pack Name	Linker 1	Linker 2	Linker 3	Linker 4	Linker 5	Linker 6	Linker 7	Linker 8	Linker 9	Linker 10
U-PLEX 2-Assay	1	—	—	—	—	—	—	—	—	10
U-PLEX 3-Assay	1	—	3	—	—	—	—	—	—	10
U-PLEX 4-Assay	1	—	3	—	—	—	—	8	—	10
U-PLEX 5-Assay	1	2	3	—	—	—	—	8	—	10
U-PLEX 6-Assay	1	2	3	—	—	—	—	8	9	10
U-PLEX 7-Assay	1	2	3	4	—	—	—	8	9	10
U-PLEX 8-Assay	1	2	3	4	—	—	7	8	9	10
U-PLEX 9-Assay	1	2	3	4	5	—	7	8	9	10
U-PLEX 10-Assay	1	2	3	4	5	6	7	8	9	10

dash (—) = not applicable

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 containing the biotinylated capture antibody and the SULFO-TAG™ conjugated detection antibody (Table 6). 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 1 (NHP) and their respective catalog numbers is provided in the Appendix (Table 12).

Table 6. Contents of U-PLEX Antibody Set

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

dash (—) = not applicable

Calibrators

Calibrators (Table 7) provided with U-PLEX Custom Biomarker (NHP) assays are multianalyte blends, each containing multiple recombinant proteins lyophilized in a buffered diluent. Individual analyte concentrations are provided in the lot-specific certificates of analysis (COA) that are shipped with the product and are also available on the www.mesoscale.com® website. Depending on the specific assays requested, one or more of the following multianalyte Calibrators will be provided.

Table 7. Analytes included in the Calibrator blends available for U-PLEX Custom Biomarker (NHP) assays

Name	Storage	Catalog No.	Size	Quantity Supplied			Analytes
				1 Plate	5 Plates	25 Plates	
Calibrator 1	2–8 °C	C0060-2	1 vial	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	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	1 vial	5 vials	25 vials	G-CSF, 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	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	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	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	1 vial	5 vials	25 vials	Eotaxin-2, GRO-α, I-309, MCP-2, MCP-3, M-CSF, MIF, MIP-5, TRAIL, YKL-40

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 microtiter plate
- ❑ Plate-washing equipment: automated plate washer or multichannel pipette
- ❑ Microtiter plate shaker (rotary) capable of shaking at 500–1,000 rpm
- ❑ MSD GOLD SULFO-TAG NHS-Ester (catalog number 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 number 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 number R50AA-4) for diluting the capture antibody
- ❑ MSD Wash Buffer (20X, 100 mL, catalog number R61AA-1) or phosphate-buffered saline (PBS) plus 0.05% Tween-20 (PBS-T) for plate washing
  - This size of MSD Wash Buffer is sufficient for washing 4 plates manually or for washing 2 plates with an automated plate washer
  - Prepare a 1X working solution. For one plate, combine 15 mL of MSD Wash Buffer (20X) with 285 mL of deionized water. 1X MSD Wash Buffer can be stored at room temperature for up to two weeks.
- ❑ Adhesive plate seals
- ❑ Deionized water
- ❑ Vortex mixer

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 www.mesoscale.com.

Best Practices

- Bring frozen diluent to room temperature in a 22–25 °C water bath.
- Avoid cross-contamination between Linkers by following the techniques below:
    - Pulse centrifuge the vials to get all of the contents to the bottom of the vial.
    - Open one U-PLEX Linker 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, it is recommended that the same Linker be coupled with the same antibody for the duration of the study.
- While most lyophilized material is located at the bottom of the vial, some may be on the sides or cap. To ensure that all lyophilized powder is reconstituted, it is recommended to vortex the vial with 3 short pulses (upright, inverted, upright) after the solution sits at room temperature for 15–30 minutes.
- Prepare Calibrator Standards and samples in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution and mix by vortexing after each dilution.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette gently to the bottom corner.
- Avoid prolonged exposure of the detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light (except for direct sunlight).
- Avoid bubbles in wells during all pipetting steps because they may lead to variable results. Bubbles introduced when adding Read Buffer may interfere with signal detection.
- Plate shaking should be vigorous, with a rotary motion between 500 and 1,000 rpm. Binding reactions may reach equilibrium sooner if you use shaking in the middle of this range (~700 rpm) or above.
- Reversing plate orientation between wash cycles may improve assay precision. Gently tap the plate on a paper towel to remove residual fluid after washing.
- If you plan to coat U-PLEX plates for later use, keep each plate pouch and the desiccant that came with the plate. After the plates are incubated with the coating solution, wash them with MSD Wash Buffer or PBS-T, and then return each plate to its original packaging with the desiccant, and seal.
- If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
- Remove the plate seal before reading the plate.
- Make sure that the Read Buffer is at room temperature when added to a plate.
- Do not shake the plate after adding Read Buffer.
- To improve interplate precision, keep time intervals consistent between adding Read Buffer and reading the plate. Unless otherwise directed, read the plate as soon as possible after adding Read Buffer.
- If the sample results are above the top of the calibration curve, dilute the samples, and repeat the assay.
- 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 up to 30 days at 2–8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.

Reagent Preparation

Bring all reagents to room temperature and refer to the Best Practices section (page 11) before beginning the protocol.

Important: Upon the first thaw, aliquot Diluent 3 and Diluent 57 into suitable volumes before refreezing.

To prepare MSD Wash Buffer and other supplemental reagents, please refer to the Additional Materials and Equipment section (page 10).

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 own 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 background, as 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.

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 U-PLEX Plate

The preparation of a U-PLEX plate involves coating the provided plate with Linker-coupled capture antibodies. A U-PLEX, 4-Assay plate is shown below as an example. This kit includes a plate with four activated spots at locations 1, 3, 8, and 10. Assign each antibody to a unique Linker and record the antibody identity next to the assigned Linker, as shown in Figure 4.

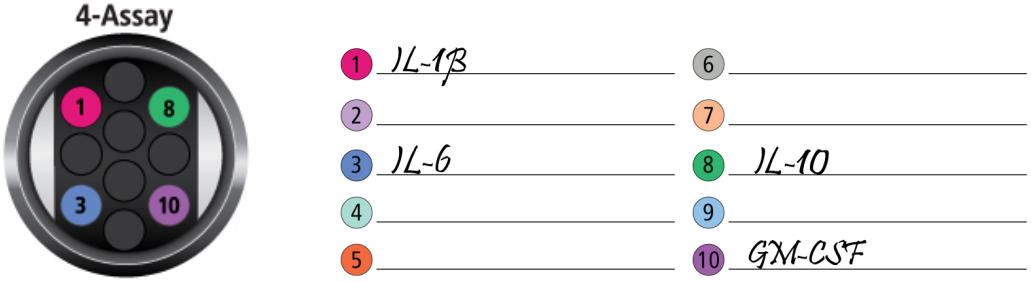

Figure 4. A U-PLEX, 4-Assay Plate, with recorded antibodies and assigned Linkers.

The protocol in this section describes the preparation of a multiplex coating solution for one 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 (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 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 is provided on page 25).

- ❑ The biotinylated capture antibody provided as a component of the U-PLEX Antibody Set is at a ready-to-use concentration. For your own capture antibodies, dilute each biotinylated antibody to 10 µg/mL in coating diluent for a final volume of ≥200 µL per plate.

Note: The antibody solution should not contain free biotin.

- ❑ 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. 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. The volumetric ratio of Linker:antibody:Stop Solution is 3:2:2.

STEP 2: Prepare the Multiplex Coating Solution

- ❑ Combine 600 µL of each U-PLEX Linker-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 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 to result in a final 1X concentration. Mix by vortexing.

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 3: Coat the U-PLEX Plate

- ❑ 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 one 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.

The recommended volumes of Linker, biotinylated capture antibody, and Stop Solution for coating one or multiple U-PLEX plates are provided below. If using a partial plate (fewer than 96 wells), refer to Table 14 in the Appendix.

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

Depending on the assays ordered, you may receive one or more multianalyte Calibrator vials with your order. For assays that are being developed with your own 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.

Guidance on using recombinant protein Calibrators can be found in the Development Pack Product Insert at www.mesoscale.com/U-PLEX-documents.

Note: A maximum of 5 Calibrator blends can be mixed in the Calibrator Standard 1 preparation step. It is recommended to mix the Calibrators for the assays that you are developing to prepare a single blend for ease in dilution. If you are using your own Calibrator, prepare 250 µL of a 5X concentrated blend of the calibrators in Diluent 57. Use this 5X concentrated stock to generate the Calibrator Standard 1 (Table 9; Table 10). The following instructions will enable you to prepare seven Calibrator Standard solutions and a zero Calibrator Standard for up to six replicates (Figure 5).

Bring the Calibrator vial(s) provided to room temperature. Reconstitute each vial of Calibrator by adding 250 µL of Diluent 57 to the glass vial. This will result in a 5X concentrated stock of each Calibrator, which will need to be diluted 5-fold (per the instructions given below) to generate the highest point in the standard curve (i.e., Calibrator Standard 1). 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. If you intend to use the remainder of the Calibrator later, 60 µL aliquots of the Calibrator must immediately be stored at ≤–70°C.

Depending on the number of Calibrator blends being used, prepare Calibrator Standard 1 (top of the curve) in a clean polypropylene tube by mixing and diluting the reconstituted stock Calibrator as indicated in Table 9. Mix by vortexing.

Table 9. Combining Calibrators to generate the Calibrator Standard 1 (top of the curve) level

No. of Calibrator Blends	Volume of Reconstituted Calibrator (µL)	Diluent 57 (µL)	Total volume (µL)
1	50	200	250
2	50 each	150	250
3	50 each	100	250
4	50 each	50	250
5	50 each	0	250

Prepare the subsequent 6 dilutions for the curve (4-fold serial dilutions) in Diluent 57 (Table 10). Use Diluent 57 for the Calibrator Standard 8 (zero Calibrator/blank). Mix by vortexing the tubes between each serial dilution.

Table 10. 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)	See Table 9		
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

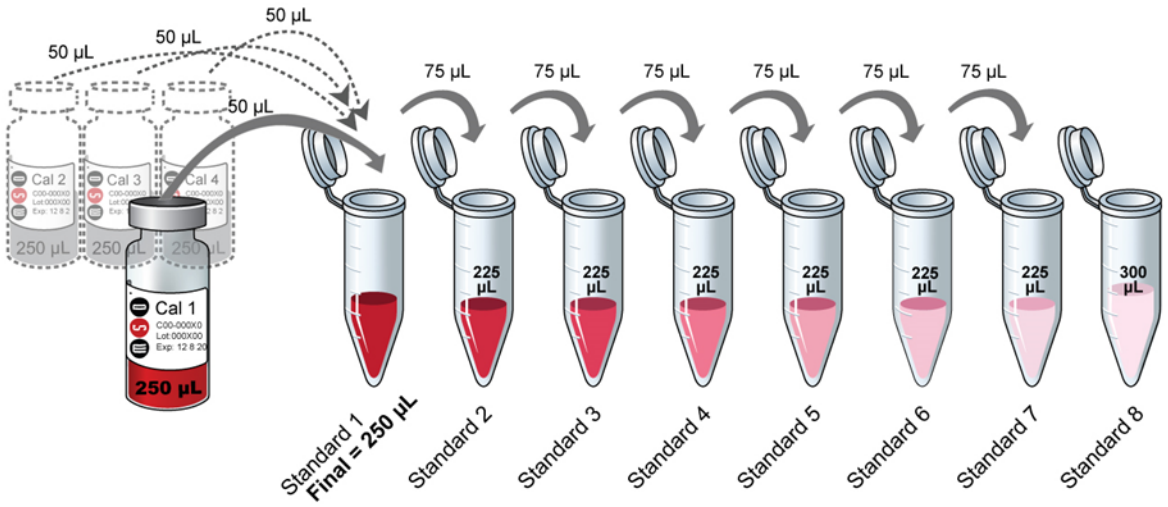

Figure 5. Dilution schema for preparation of Calibrator Standards for U-PLEX Custom Biomarker (NHP) Assays.

Alternate Calibrator handling procedures

If an assay needs more than 5 Calibrators blended together, reconstitute each Calibrator with 125 µL of Diluent 57. This will result in a 10X concentrated stock of the Calibrator. Take extra care that all of the lyophilized material is reconstituted. Follow the instructions in Table 9 but blend 25 µL of each Calibrator (rather than 50 µL) and add enough Diluent 57 to get a final volume of 250 µL.

Dilute Samples

Depending on the sample set under investigation, a dilution may be necessary. Diluent 57 may be used for sample dilution. The dilution factor for the given sample type may need to be optimized.

Note: For MIF, and YKL-40, in-house data indicate that a large sample dilution is required to generate optimal results. Refer to the product-specific datasheets for additional information.

Prepare Detection Antibody Solution

The detection antibody for each MSD assay is provided as a 100X stock solution. When using your own antibody pairs, a 100X concentrated stock of each detection antibody should be prepared. The working solution is 1X. Prepare the detection antibody solution immediately before use.

For one plate, combine:

- ❑ 60 µL of each 100X detection antibody
- ❑ Diluent 3 to bring the final volume to 6 mL

Read Buffer

MSD provides MSD GOLD Read Buffer B ready to use.

Important: Unlike Read Buffer T, which is provided at a 4X concentration, MSD GOLD Read Buffer B is provided at the working concentration of the assay. Dilution of MSD GOLD Read Buffer B may compromise the results of the assay.

Assay Protocol

Note: Follow Reagent Preparation before beginning this assay protocol.

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of Diluent 57 to each well. Tap the plate gently on all sides.
- ❑ Add 25 µL 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 µL/well of 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.

Alternate Protocols

The suggestions below may be useful for simplifying the protocol.

- ❑ **Alternate Protocol 1, Extended Incubation:** Incubating samples overnight at 2–8 °C may improve sensitivity for some assays.
- ❑ **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.

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 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 group was tested individually against a larger panel of antibodies and recombinant analytes for nonspecific binding (CTACK, ENA-78, Eotaxin, Eotaxin-2, Eotaxin-3, FLT3L, Fractalkine, G-CSF, GM-CSF, GRO-α, I-309, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17A/F, IL-17B, IL-17C, IL-17D, IL-17F, IL-18, IL-21, IL-22, IL-23, IP-10, I-TAC, MCP-1, MCP-2, MCP-3, MCP-4, M-CSF, MDC, MIF, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-5, SDF-1α, TARC, TNF-α, TNF-β, TPO, TRAIL, VEGF-A, and YKL-40).

Nonspecific binding was less than 0.5% for all assays included in the U-PLEX Biomarker Group 1 (NHP) using the following calculation.

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

Exceptions are noted below:

- IL-12p70 and IL-23 contain the p40 subunit and will cross-react with the IL-12/IL-23p40 assay.
- IL-17A cross-reacts with the IL-17A/F assay. IL-17A/F cross-reacts with the IL-17A assay.
- Eotaxin (Calibrator 2) nonspecifically binds MCP-2 (2.8%) and MCP-3 (3.7%) detection antibodies.

Appendix

Considerations for Assay Development

Choosing a Detection and a Capture Antibody

Sensitivity, specificity, and format of the assay are affected by selection of a capture antibody and a detection antibody, each of which must recognize a different nonoverlapping epitope.

For many assays, it is preferable to have both the capture antibody and detection antibody be monoclonal antibodies, each recognizing a unique epitope. Monoclonal antibodies are typically easier to reproduce from lot to lot and can be produced in large quantities leading to increased longevity of the assay. Selection of two monoclonal antibodies may not be possible because of reagent availability or desired sensitivity achieved with a polyclonal antibody. When both a monoclonal antibody and a polyclonal antibody are used, it is preferable to use the monoclonal antibody as the capture antibody and the polyclonal antibody as the detection antibody.

The advantage of using a polyclonal antibody is that it may contain multiple antibodies that recognize different epitopes, leading to higher avidity. When a polyclonal antibody is not affinity purified, it contains nonspecific antibodies that could lead to nonspecificity issues or reduced assay performance. An immuno-affinity purified polyclonal antibody has greater specificity than one purified by Protein A or G, but it may still exhibit lot-to-lot variability because each lot may be a different mixture of antibodies. When using a polyclonal antibody as a capture antibody, it could contain a population of antibodies that share or block the detection antibody epitope, leading to reduced signals or sensitivity.

Antibody Conjugation

Consistent conjugation of the capture antibody and detection antibody is important for good assay performance. Optimization of the conjugation ratios is not necessary for assay development, but it could improve assay performance in certain situations. At least one biotin must be present on the capture antibody for it to be coupled to the U-PLEX Linker. The signal from an assay is proportional to the number of SULFO-TAG molecules conjugated to the detection protein. Overconjugation of capture and detection antibodies can lead to reduced performance. In this protocol, we recommend typical challenge ratios for biotinylation and SULFO-TAG conjugation of antibodies that should work for most assays; however, if optimization is needed we recommend following the guidelines outlined in the product-specific protocols.

Recommendation for Calibrators

An ideal Calibrator should be representative of the native protein; select either a purified native protein or a recombinant protein that resembles the native form whenever possible. The antibodies may preferentially recognize the Calibrator or standard over the native protein, especially if the Calibrator was used to generate the antibody. Recombinant proteins or standards may not be available for all assays. In these cases, such as with intracellular signaling assays, an appropriate cell model may be developed to generate a native form of the protein.

Avoid reagents that could denature the capture antibody (general guidelines are: ionic detergents such as SDS should be <0.1%; reducing agents such as DTT should be <1 mM in the Calibrator when added to the well). If high concentrations of potentially denaturing agents are required for extraction, the Calibrator and samples should be diluted in a suitable buffer lacking denaturing agent before being added to the antibody-coated plate.

U-PLEX Combinations

U-PLEX Combinations include U-PLEX Plates, Linkers, Antibody Sets, Calibrators, Stop Solution, Diluents, and Read Buffer (Table 11).

Table 11. Catalog numbers of U-PLEX Biomarker Group 1 (NHP) multiplex combinations

Product	Analytes	Catalog Numbers (-1/-5/-25 Plate Size)
U-PLEX Biomarker Group 1 (NHP) 60-Plex, SECTOR	CTACK, ENA-78, Eotaxin, Eotaxin-2, Eotaxin-3, FLT3L, Fractalkine, G-CSF, GM-CSF, GRO-α, I-309, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17A/F, IL-17B, IL-17C, IL-17D, IL-17F, IL-18, IL-21, IL-22, IL-23, I IP-10, I-TAC, MCP-1, MCP-2, MCP-3, MCP-4, M-CSF, MDC, MIF, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-5, SDF-1α, TARC, TNF-α, TNF-β, TPO, TRAIL, VEGF-A, YKL-40	K15082K-1/-2/-4
U-PLEX TH1/TH2 Combo (NHP) SECTOR	IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, TNF-α	K15080K-1/-2/-4
U-PLEX TH17 Combo (NHP) SECTOR	IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17A, TNF-α	K15079K-1/-2/-4
U-PLEX Proinflam Combo 1 (NHP) SECTOR	IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α	K15070K-1/-2/-4
U-PLEX Cytokine Combo 1 (NHP) SECTOR	GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A	K15057K-1/-2/-4
U-PLEX Chemokine Combo 1 (NHP) SECTOR	Eotaxin, Eotaxin-3, IL-8, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC	K15055K-1/-2/-4
U-PLEX T-Cell Combo (NHP) SECTOR	GM-CSF, IFN-γ, IL-2, IL-4, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, MIP-3α, TNF-α	K15095K-1/-2/-4
U-PLEX Viral Combo 1 (NHP) SECTOR	G-CSF, GM-CSF, IFN-α2a, IFN-γ, IL-1β, IL-1RA, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IP-10, MCP-1, MIP-1α, VEGF-A, TNF-α	K15344K-1/-2/-4

U-PLEX Antibody Sets

Antibody Sets (Table 12; Table 13) include a biotinylated capture antibody and SULFO-TAG conjugated detection antibody.

Table 12. Catalog numbers of Antibody Sets available for the U-PLEX Biomarker Group 1 (NHP)

Product	Catalog Numbers (-1/-5 Plate Size)
U-PLEX NHP CTACK Antibody Set	B21VD-2/-3
U-PLEX NHP ENA-78 Antibody Set	B26VE-2/-3
U-PLEX NHP Eotaxin Antibody Set	B26UD-2/-3
U-PLEX NHP Eotaxin-2 Antibody Set	B21XQ-2/-3
U-PLEX NHP Eotaxin-3 Antibody Set	B21UE-2/-3
U-PLEX NHP FLT3L Antibody Set	B21XF-2/-3
U-PLEX NHP Fractalkine Antibody Set	B21VC-2/-3
U-PLEX NHP G-CSF Antibody Set	B21VG-2/-3
U-PLEX NHP GM-CSF Antibody Set	B21UM-2/-3
U-PLEX NHP GRO-α Antibody Set	B21UX-2/-3
U-PLEX NHP I-309 Antibody Set	B21UY-2/-3
U-PLEX NHP IFN-γ Antibody Set	B21TT-2/-3
U-PLEX NHP IL-1α Antibody Set	B26UN-2/-3
U-PLEX NHP IL-1β Antibody Set	B21TU-2/-3
U-PLEX NHP IL-1RA Antibody Set	B21XP-2/-3
U-PLEX NHP IL-2 Antibody Set	B21TV-2/-3
U-PLEX NHP IL-4 Antibody Set	B26TW-2/-3
U-PLEX NHP IL-5 Antibody Set	B21UO-2/-3
U-PLEX NHP IL-6 Antibody Set	B21TX-2/-3
U-PLEX NHP IL-7 Antibody Set	B21UP-2/-3
U-PLEX NHP IL-8 Antibody Set	B21TY-2/-3
U-PLEX NHP IL-9 Antibody Set	B21XK-2/-3
U-PLEX NHP IL-10 Antibody Set	B21TZ-2/-3
U-PLEX NHP IL-12/IL-23p40 Antibody Set	B21UQ-2/-3
U-PLEX NHP IL-12p70 Antibody Set	B26UA-2/-3
U-PLEX NHP IL-13 Antibody Set	B26UB-2/-3
U-PLEX NHP IL-15 Antibody Set	B21UR-2/-3
U-PLEX NHP IL-16 Antibody Set	B21US-2/-3
U-PLEX NHP IL-17A Antibody Set	B21UT-2/-3
U-PLEX NHP IL-17A/F Antibody Set	B21VY-2/-3

Product	Catalog Numbers (-1/-5 Plate Size)
U-PLEX NHP IL-17B Antibody Set	B21XN-2/-3
U-PLEX NHP IL-17C Antibody Set	B21WJ-2/-3
U-PLEX NHP IL-17D Antibody Set	B21XO-2/-3
U-PLEX NHP IL-17F Antibody Set	B21WA-2/-3
U-PLEX NHP IL-18 Antibody Set	B21VJ-2/-3
U-PLEX NHP IL-21 Antibody Set	B26WB-2/-3
U-PLEX NHP IL-22 Antibody Set	B21WI-2/-3
U-PLEX NHP IL-23 Antibody Set	B21WG-2/-3
U-PLEX NHP IP-10 Antibody Set	B21UF-2/-3
U-PLEX NHP I-TAC Antibody Set	B21UW-2/-3
U-PLEX NHP MCP-1 Antibody Set	B21UG-2/-3
U-PLEX NHP MCP-2 Antibody Set	B21XH-2/-3
U-PLEX NHP MCP-3 Antibody Set	B21XI-2/-3
U-PLEX NHP MCP-4 Antibody Set	B21UH-2/-3
U-PLEX NHP M-CSF Antibody Set	B21XR-2/-3
U-PLEX NHP MDC Antibody Set	B21UI-2/-3
U-PLEX NHP MIF Antibody Set	B21XJ-2/-3
U-PLEX NHP MIP-1α Antibody Set	B21UJ-2/-3
U-PLEX NHP MIP-1β Antibody Set	B21UK-2/-3
U-PLEX NHP MIP-3α Antibody Set	B26UZ-2/-3
U-PLEX NHP MIP-3β Antibody Set	B21VA-2/-3
U-PLEX NHP MIP-5 Antibody Set	B21XS-2/-3
U-PLEX NHP SDF-1α Antibody Set	B26VB-2/-3
U-PLEX NHP TARC Antibody Set	B21UL-2/-3
U-PLEX NHP TNF-α Antibody Set	B26UC-2/-3
U-PLEX NHP TNF-β Antibody Set	B21UU-2/-3
U-PLEX NHP TPO Antibody Set	B21VK-2/-3
U-PLEX NHP TRAIL Antibody Set	B21XT-2/-3
U-PLEX NHP VEGF-A Antibody Set	B21UV-2/-3
U-PLEX NHP YKL-40 Antibody Set	B21VL-2/-3

Table 13. Catalog numbers of Antibody Sets available for U-PLEX Biomarker Group 2

Product	Catalog Numbers (-1/-5 Plate Size)
U-PLEX TGF-β1 Antibody Set	B20XW-2/-3
U-PLEX TGF-β2 Antibody Set	B20XU-2/-3
U-PLEX TGF-β3 Antibody Set	B20XV-2/-3

Working with Partial Plates

A portion of a plate (fewer than 96 wells) may be used when developing assays (Table 14). 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 14. Amount of each component required for U-PLEX coating solution (partial plate)

No. of Wells	Individual Linker (µL)	Individual Biotinylated 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

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.

Multiplate Assays

Multiplex U-PLEX assays can occupy more than one plate, depending on the number and compatibility of the selected assays. Preconfigured multiplexes, including Combos and Biomarker Groups, are arranged in optimal layouts, with compatible assays and Calibrators packaged together along with adequate linkers and an activated U-PLEX plate. Components of a multiplate assay should not be mixed between boxes, except for Stop Solution, Diluents, and Read Buffer.

An example of a multiplate U-PLEX assay is the U-PLEX Biomarker Group 1 (NHP) 60-Plex (catalog number K15082K). The assay is supplied in eight separate U-PLEX boxes. Each box includes one 10-Spot, U-PLEX plate (with the appropriate number of activated spots), Linkers, antibody pairs, and Calibrators that run optimally together. Components should not be mixed between boxes, except for Stop Solution, Diluents, and Read Buffer.

To perform the U-PLEX Biomarker Group 1 (NHP) 60-Plex assay, we recommend that you position the seven boxes as shown in Table 15. When multiple Calibrators are in one box, they should be blended as instructed in this product insert. Do not combine with any other Calibrators from another box. There will be a unique Calibrator curve for each box.

Table 15. NHP 60-Plex Layout

Box 1 10-Activated Spots Plate	Box 2 10-Activated Spots Plate	Box 3 10-Activated Spots Plate	Box 4 9-Activated Spots Plate	Box 5 10-Activated Spots Plate	Box 6 9-Activated Spots Plate	Box 7* 2-Activated Spots Plate
Calibrators 1 and 4	Calibrators 2 and 6	Calibrator 3	Calibrator 9 and 6	Calibrator 1	Calibrators 1 and 10	Calibrator 10
IL-13	Eotaxin	G-CSF	FLT3L	GM-CSF	Eotaxin-2	MIF
IL-17A	Eotaxin-3	IFN-α2a	IL-9	IFN-γ	I-309	YKL-40
TNF-α	IP-10	IL-1α	IL-17B	IL-1β	M-CSF	—
SDF-1α	MCP-1	IL-7	IL-17C	IL-2	MCP-2	
CTACK	MCP-4	IL-12/IL-23p40	IL-17D	IL-4	MCP-3	
ENA-78	MDC	IL-15	IL-1RA	IL-5	MIP-5	
Fractalkine	MIP-1α	IL-16	IL-17A/F	IL-6	TRAIL	
I-TAC	MIP-1β	IL-18	IL-22	IL-8	GRO-α	
MIP-3α	TARC	TNF-β	IL-23	IL-10	VEGF-A	
MIP-3b	IL-17F	TPO	—	IL-12p70	—	

dash (—) = not applicable

*These analytes are expressed at much higher levels than the other analytes in the samples. They are separated to allow samples to be diluted specifically for these analytes. Internal testing suggests that normal serum and plasma should be diluted 100-fold.

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 own 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.

Summary Protocol

Prepare Conjugated Capture and Detection Antibodies

- ❑ For assays that are being developed with your own antibody pairs, conjugate the capture antibody with Sulfo-NHS-LC-Biotin by following the manufacturer's guidelines, and dilute each biotinylated antibody to 10 µg/mL in coating diluent for a final volume of ≥200 µL per plate.
- ❑ The antibody solution should not contain free biotin.
- ❑ For assays that are being developed with your own antibody pairs, conjugate the detection antibody with SULFO-TAG NHS-Ester 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. Prepare a 100X concentrated stock solution for each SULFO-TAG conjugated detection antibody.

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 6).

- ❑ 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

- ❑ 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 3: Coat the U-PLEX 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.

Spot Map

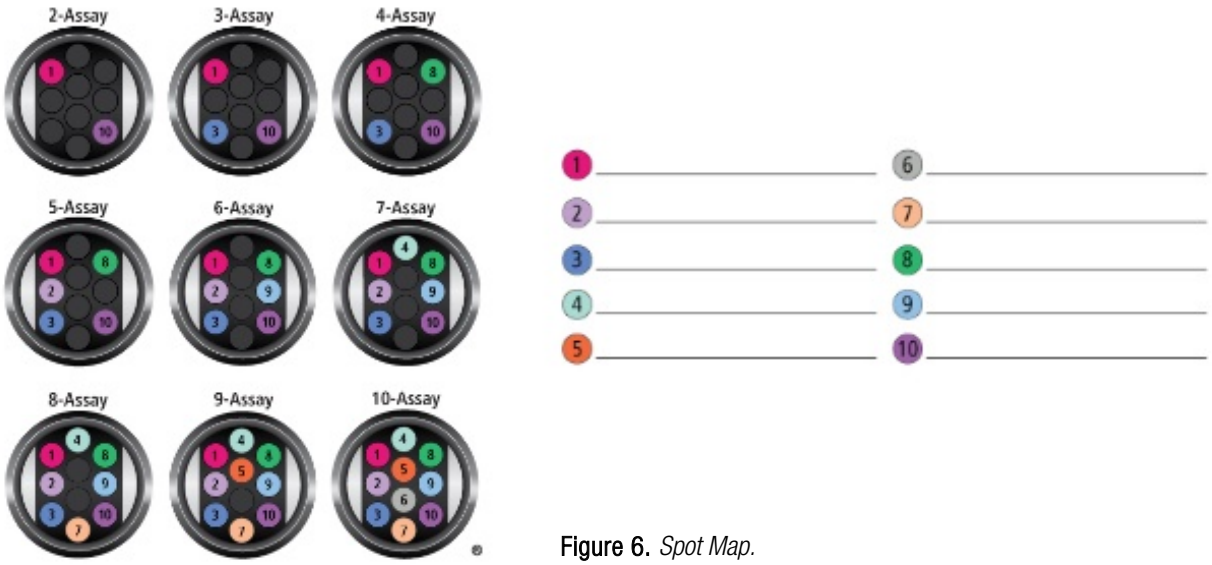

Figure 6. *Spot Map.*

Assay Protocol

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of Diluent 57 to each well. Tap the plate gently on all sides.
- ❑ Add 25 µL 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 µL/well of 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.

Plate Diagram

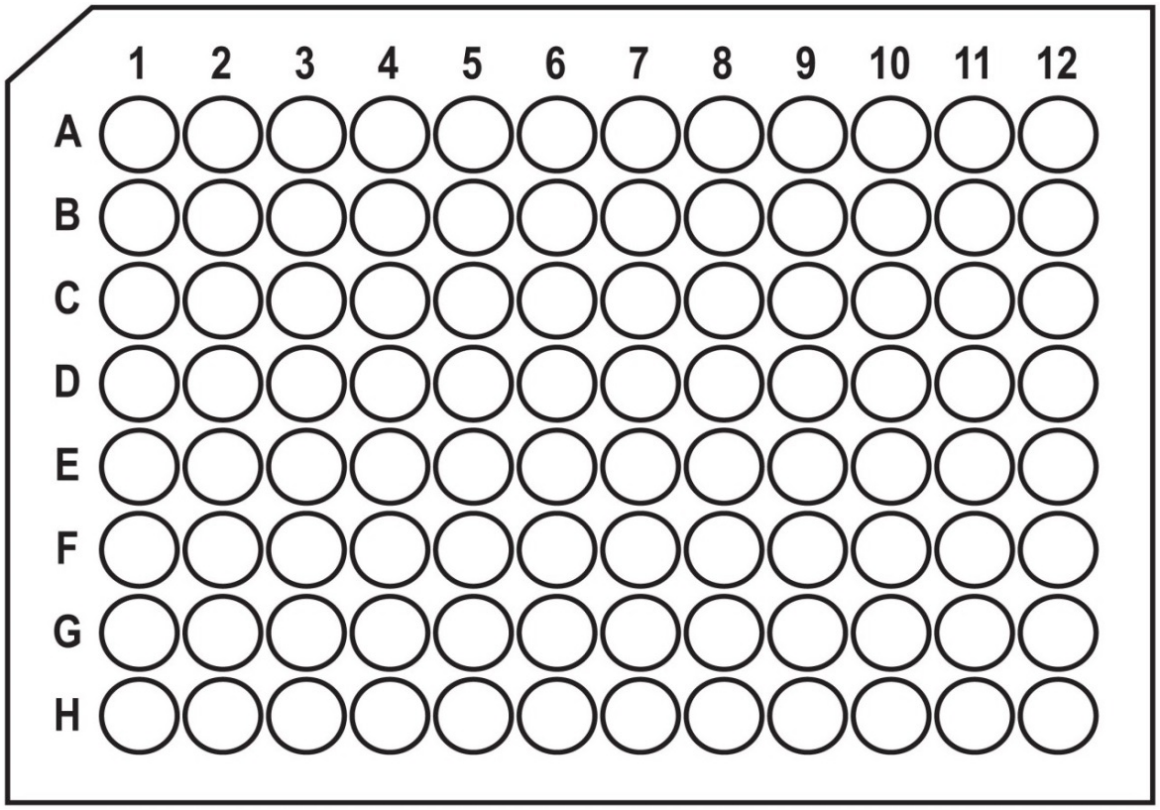

Figure 7. Plate diagram; a similar plate layout can be created in Excel and easily imported into DISCOVERY WORKBENCH® software.