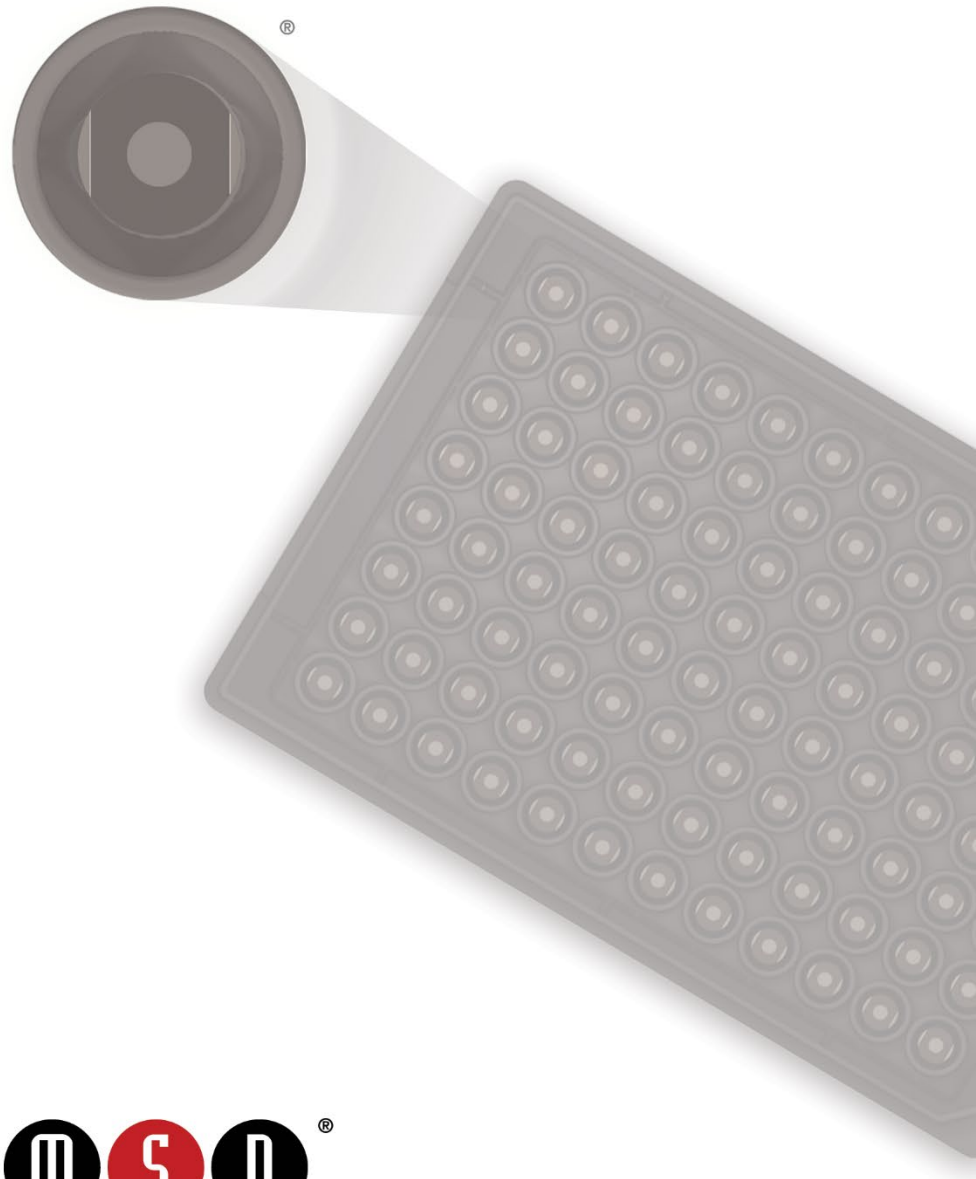
R-PLEX® Multiplex Assays



MSD R-PLEX Platform

R-PLEX Multiplex Assays

Use for the development of R-PLEX multiplex assays.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

MESO SCALE DISCOVERY® R-PLEX Antibody Sets provide an expanded menu of electrochemiluminescence assays for biomarker discovery and development. The sets include a matched, biotinylated capture and SULFO-TAG™ conjugated detection antibody pair and a calibrator for the quick and easy development of highly sensitive immunoassays on MSD plates.

R-PLEX Antibody Sets enable the development of singleplex and multiplex immunoassays:

- Singleplex assays on MSD GOLD™ Small Spot Streptavidin plates.
- Multiplex assays with combinations of R-PLEX Antibody Sets, as well as combining R-PLEX plus U-PLEX® Antibody Sets or your own antibody pairs. Multiplex assays use a 10-spot U-PLEX MULTI-SPOT® plate and unique Linkers.

This product insert provides the details for developing a multiplex assay on the MSD platform using R-PLEX Antibody Sets. During development, MSD observed improved performance for certain groups of assays when following a modified procedure. Thus, three assay protocols are provided in this product insert:

- Assay Protocol 1—R-PLEX standard procedure (used for most assays)
- Assay Protocol 2—requires coincubation of Calibrator or sample with detection antibody (or Tracer) for 2 hours, and optional blocking step
- Assay Protocol 3—uses 50 µL/well of Calibrator or sample and requires 1X MSD Tris Wash Buffer.

Refer to Table 1 to determine the optimal assay protocol for your R-PLEX assay.

Table 1. Recommended assay protocols for R-PLEX Antibody Sets

Protocol	R-PLEX Antibody Sets
Assay Protocol 1	This assay is recommended for most R-PLEX Antibody Sets. If your antibody set is not listed below, follow Assay Protocol 1.
Assay Protocol 2	This is a homogenous assay that is recommended for Human Aβ (all 6E10) (order Diluent 35 separately), CKBB, Rabbit IL-1β, and Rat Albumin Antibody Sets.
Assay Protocol 3	This assay is recommended for these Human Antibody Sets: Aiolos, Bcl-2, BIM/Bcl2-L-11, Hemoglobin alpha, Hemopexin, Ikaros, IRS-1, Lactotransferrin, LRRK2, LRRK2 (pS935), Mcl-1/Bcl2-L-3, Mcl-1/BAK Complex, Mcl-1/BIM Complex, and Serpin F2.

If you are combining R-PLEX with U-PLEX analytes, in addition to this Insert, refer to the U-PLEX Development Pack product insert that is supplied with the U-PLEX product or available at U-PLEX-Development-Pack.

A complete list of available R-PLEX Antibody Sets is available at www.mesoscale.com/R-PLEX. A representative data set for each R-PLEX Antibody Set is presented in the product-specific datasheets available at R-PLEX documents.

Principle of the Assay

Multiplex assays can be developed with either a combination of R-PLEX Antibody Sets or with a combination of R-PLEX and U-PLEX Antibody Sets. Biotinylated capture antibodies are coupled to U-PLEX Linkers that 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.

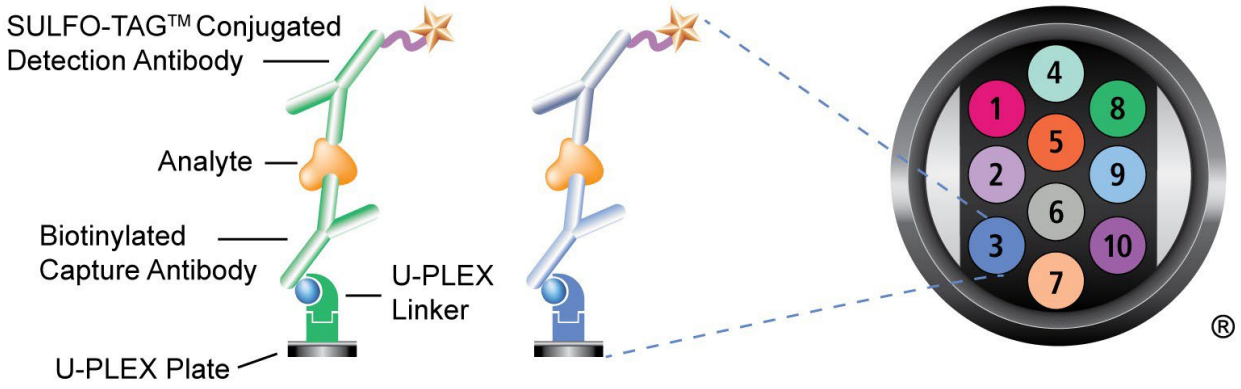

Figure 1. U-PLEX immunoassay on a U-PLEX 10-Assay Plate.

Components

Reagents Provided

Table 2 lists components needed for assay development with R-PLEX Antibody Sets.

The R-PLEX Antibody Set contains a biotinylated capture antibody, a SULFO-TAG conjugated detection antibody (or Tracer), and a frozen calibrator. The calibrator is provided at a 20-fold higher concentration than the suggested Calibrator Standard 1 (top of curve) concentration. The representative Calibrator Standard 1 concentration for each assay is shown in the product-specific datasheet.

Table 2. Contents of R-PLEX Antibody Sets

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 (or Tracer) (analyte-specific)	2–8 °C	5 Plates	1 vial	SULFO-TAG conjugated detection antibody (or Tracer) (100X). Provided as one vial per five plates.
Calibrator (analyte-specific)	≤–70 °C	5 Plates	5 vials	Calibrator (analyte-specific)

Dash (—) = not applicable

Assay-Specific Reagents

U-PLEX Development Packs

U-PLEX Development Packs provide the plates, linkers, Stop Solution, and read buffer required for multiplex assays. For a complete listing of assay development plates and reagents, visit www.mesoscale.com®.

Plates

R-PLEX multiplex assays are developed on 96-well U-PLEX plates, which are available as part of the U-PLEX Development Packs (Table 3). U-PLEX plates contain ten individual spots on the bottom of each well; the spots correspond to ten unique U-PLEX Linkers. The number and layout of active spots on the plate depend on the number of assays to be multiplexed. For example, if you plan to multiplex four assays together, you can choose the U-PLEX 4-Assay Development Pack.

Table 3. *Catalog numbers of U-PLEX Development Packs*

Name	1 Plate	5 Plates	25 Plates	Description
U-PLEX 2-Assay Development Pack	K15227N-21	K15227N-22	K15227N-24	Includes U-PLEX plate(s), Stop Solution, and read buffer
U-PLEX 3-Assay Development Pack	K15228N-21	K15228N-22	K15228N-24	
U-PLEX 4-Assay Development Pack	K15229N-21	K15229N-22	K15229N-24	
U-PLEX 5-Assay Development Pack	K15230N-21	K15230N-22	K15230N-24	
U-PLEX 6-Assay Development Pack	K15231N-21	K15231N-22	K15231N-24	
U-PLEX 7-Assay Development Pack	K15232N-21	K15232N-22	K15232N-24	
U-PLEX 8-Assay Development Pack	K15233N-21	K15233N-22	K15233N-24	
U-PLEX 9-Assay Development Pack	K15234N-21	K15234N-22	K15234N-24	
U-PLEX 10-Assay Development Pack	K15235N-21	K15235N-22	K15235N-24	

Linkers

Based on the number of assays you select for multiplexing, you will receive the corresponding number of unique Linkers (Figure 2; 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 2. *Unique color-coded Linkers (10), Antibody Sets (10), and Calibrator vials (5) as shipped in a U-PLEX box.*

Table 4. *Linkers supplied with each Development 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

Table 5. *Linker color coding, storage conditions, catalog numbers, and size*

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

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

Stop Solution and Read Buffer

Stop Solution and MSD GOLD Read Buffer B (Table 6) are provided with the Development Packs.

Table 6. Additional reagents provided in the Development Pack

Reagent	Storage	Part No.	Size	Quantity Supplied			Description
				1-Plate	5-Plate	25-Plate	
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*	15–30 °C	R60AM-1	18 mL	1 bottle	—	—	Buffer to catalyze the electro-chemiluminescent reaction
		R60AM-2	90 mL	—	1 bottle	5 bottles	

*Remove the bottle from the box and allow read buffer to equilibrate to RT before use.
RT = room temperature
Dash (—) = not applicable

General Reagents

Diluents

R-PLEX and U-PLEX assays may have specific diluents for sample and calibrator dilution as well as for preparing the detection antibody (or Tracer) solution. The catalog numbers for diluents commonly used in multiplex assays are provided in Table 7, but a range of diluents are available for purchase at www.mesoscale.com.

*Table 7. Catalog numbers of common diluents used in R-PLEX multiplex assays**

Name	Catalog No.	Size
Diluent 1	R50CK-2	50 mL
	R50CK-4	200 mL
Diluent 2	R51BB-4	8 mL
	R51BB-3	40 mL
	R51BB-2	200 mL
Diluent 3	R50AP-1	8 mL
	R50AP-2	40 mL
Diluent 5	R52BA-4	5 mL
	R52BA-5	25 mL
Diluent 6	R53BB-3	40 mL
Diluent 7	R54BB-4	5 mL
	R54BB-3	50 mL
Diluent 8	R54BA-3	50 mL
Diluent 10	R55BB-3	50 mL
Diluent 11	R55BA-4	5 mL
	R55BA-5	10 mL
	R55BA-3	50 mL
Diluent 12	R50JA-4	10 mL
	R50JA-3	50 mL
	R50JA-2	200 mL
Diluent 13	R56BB-4	10 mL
	R56BB-3	50 mL
Diluent 17	R50KA-4	6 mL
	R50KA-3	30 mL
Diluent 22	R50BB-4	40 mL

*Diluent 100 may be used in place of assay diluent for samples that require high dilution.

Name	Catalog No.	Size
Diluent 27	R500A-3	30 mL
Diluent 29	R50HA-4	15 mL
	R50HA-3	40 mL
Diluent 30	R50AB-4	25 mL
Diluent 35	R50AE-3	30 mL
	R50AE-2	150 mL
Diluent 37	R50AF-3	25 mL
	R50AF-6	125 mL
Diluent 39	R5ABB-2	50 mL
Diluent 40	R50AJ-1	5 mL
	R50AJ-2	40 mL
Diluent 41	R50AH-1	10 mL
	R50AH-2	50 mL
Diluent 42	R50AK-1	10 mL
	R50AK-2	50 mL
Diluent 43	R50AG-1	10 mL
	R50AG-2	50 mL
Diluent 45	R50AI-1	5 mL
	R50AI-3	8 mL
	R50AI-2	25 mL
	R50AI-4	40 mL
Diluent 57	R50BZ-1	10 mL
	R50BZ-2	50 mL
Diluent 58	R50CA-2	50 mL
Diluent 65	R50CJ-1	10 mL
	R50CJ-2	50 mL
Diluent 100	R50AA-4	50 mL
	R50AA-2	200 mL
	R50AA-3	1,000 mL
Diluent 101	R51AD-3	50 mL

Note: To run five plates, 50 mL of assay diluent and 40 mL of antibody diluent are required 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 different diluents can help optimize assays for specific experimental conditions.

Wash Buffer

Wash Buffers are provided in different volumes (Table 8).

Table 8. Catalog numbers of Wash Buffers

Name	Storage	Catalog No.	Size	Description
MSD Wash Buffer (20X)	RT	R61AA-1*	100 mL	Phosphate-buffered saline (PBS) plus surfactant
MSD Tris Wash Buffer (10X)	2–8 °C	R61TX-2*	200 mL	Tris-buffered solution with surfactant (required for Assay Protocol 3)
		R61TX-1	1000 mL	

*This size of Wash Buffer (Table 7) is sufficient for washing four plates manually or for washing two plates with an automated plate washer.

- ❑ Prepare a 1X working solution with MSD Wash Buffer or MSD Tris Wash Buffer.
  i. MSD Wash Buffer: for one plate, combine 15 mL of MSD Wash Buffer (20X) with 285 mL of deionized water.
  ii. MSD Tris Wash Buffer: for one plate, combine 30 mL of MSD Tris Wash Buffer (10X) with 270 mL of deionized water.

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
- ❑ 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 applicable safety data sheet(s) (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. Thaw the frozen calibrator (when applicable) on wet ice.
- For multiplex assays, 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 of the same assay, it is recommended that the same Linker be coupled with the same antibody for the duration of the study.
- Prepare Calibrator Standards and samples in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution, and mix by vortexing after each dilution.
- 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 as they may lead to variable results. Bubbles introduced when adding read buffer may interfere with signal detection.
- Use reverse pipetting when necessary to avoid the introduction of bubbles. For empty wells, pipette gently to the bottom corner.
- 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 at the middle of this range (~700 rpm) or above.
- When using an automated plate washer, rotate the plate 180 degrees between wash steps to improve assay precision.
- Gently tap the plate on a paper towel to remove residual fluid after washing.
- If an incubation step needs to be extended, leave the sample or detection antibody (or Tracer) 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 and follow the guidelines on how to read partial plates provided in the instrumental manual. Partially used plates may be stored for up to 30 days at 2–8 °C in the original foil pouch with desiccant. You may adjust volumes proportionally when preparing reagents.
- Calibrators for R-PLEX Antibody Sets whose representative curves surpass 1 million counts may be diluted an extra 4-fold to lower the top of curve signals.

Reagent Preparation

Bring all reagents to room temperature and refer to the Error! Reference source not found. section before beginning the protocol. Determine which assay protocol is optimal based on the R-PLEX analytes (see Table 1). Reagent preparation may vary based on the assay protocol.

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

To prepare supplemental reagents such as MSD Wash Buffer, please refer to the Components section.

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 in Figure 3 as an example 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. Also, see the Spot Map on page 22.

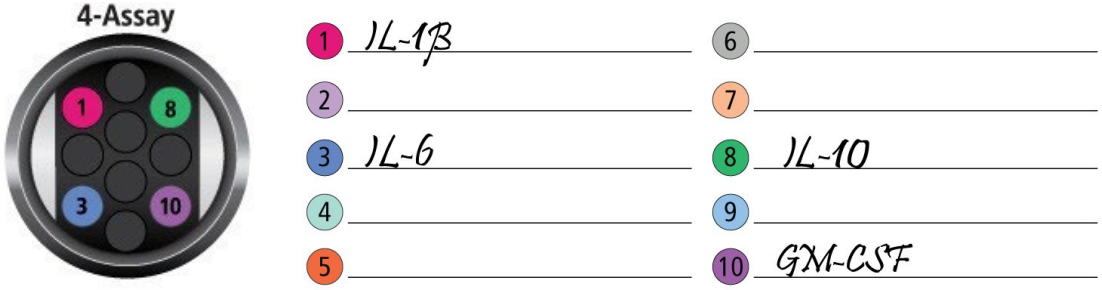

Figure 3. A U-PLEX 4-Assay Plate with recorded antibodies and assigned Linkers.

Preparation of Multiplex Coating Solution for 96-well Plate

STEP 1: Create Individual Linker-Coupled Antibody Solutions

A different Linker must be used for each unique biotinylated antibody. The number and layout of the active spots on the plate depend on the number of assays to be multiplexed. As an example, the steps to complete the coupling reactions for a 4-Assay plate are provided below.

- ❑ 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 22).
- ❑ 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 individual 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 Linker-coupled antibody solution into a single tube and vortex. Up to 10 Linker-coupled antibodies can be pooled. Do not combine 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 multiplex coating solution is at 1X.

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 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 1X MSD Wash Buffer. If following Assay Protocol 3, wash plates with at least 150 µL/well of 1X MSD Tris 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 (Table 9). If using a partial plate (fewer than 96 wells), refer to Table 10.

Table 9. 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

Table 10. Amount of each component needed for U-PLEX Coating Solution (partial 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

Prepare Calibrator Standards

MSD supplies Calibrator for R-PLEX Antibody Sets at a concentration that is 20-fold higher than the recommended highest standard. We recommend a 7-point calibration curve with 4-fold serial dilution steps and a zero calibrator blank. Thaw the stock calibrator on wet ice for at least 30 minutes and keep on ice, and then add it to the assay diluent at room temperature to make the calibration curve solutions.

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates (see Figure 4):

- ❑ Prepare the highest calibrator (Calibrator Standard 1) by adding 15 µL of each stock calibrator. Add assay diluent to bring the volume to 300 µL. Mix well.
- ❑ Prepare the next calibrator by transferring 100 µL of the highest calibrator to 300 µL of assay diluent. Mix well. Repeat 4-fold serial dilution 5 times to generate 7 calibrators
- ❑ Use assay diluent as Calibrator 8 (zero Calibrator). Discard any unused, diluted calibrators.

Notes:

- Assay Protocol 3 requires twice as much Calibrator Standard (50 µL/well) as Assay Protocols 1 and 2 (25 µL/well).
- Dilution volumes can be adjusted for fewer replicates.
- For the recommended Calibrator Standard 1 (top of curve) concentration, refer to the product-specific datasheet supplied with the R-PLEX Antibody Set. The datasheet is also available at R-PLEX documents.

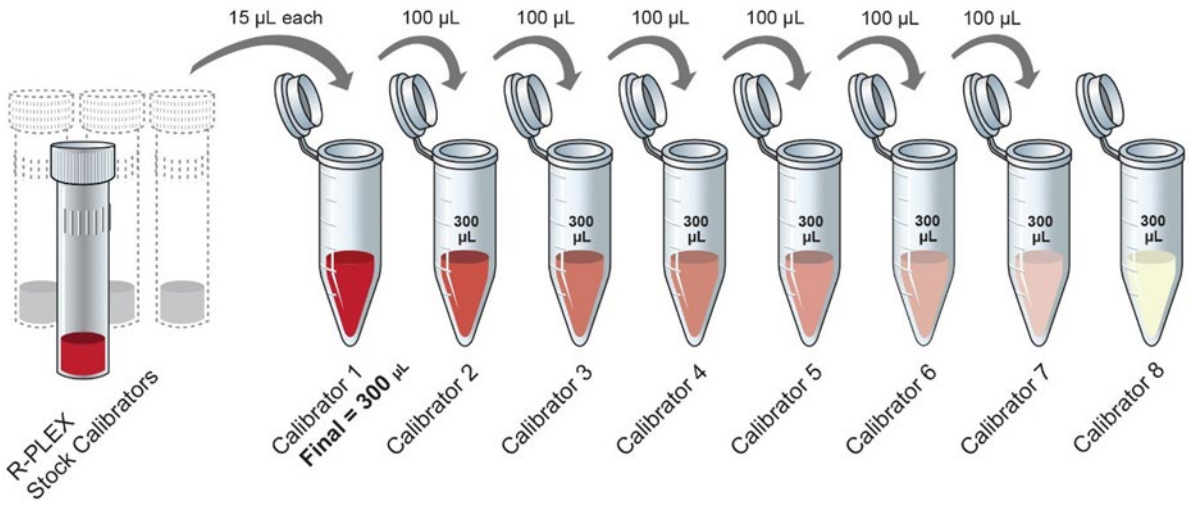

Figure 4. Dilution schema for Calibrator Standards for R-PLEX multiplex assays.

Prepare Detection Antibody (or Tracer) Solution

The detection antibody (or Tracer) is provided as a 100X stock solution. The working solution is 1X. Prepare the detection antibody (or Tracer) solution immediately before use.

For one plate, combine:

- ❑ 60 µL of each 100X detection antibody (or Tracer)
- ❑ Antibody diluent to bring the final volume to 6,000 µL

Note: Assay Protocol 2 uses 25 µL/well of 1X detection antibody (or Tracer) solution. Prepare 3,000 µL of 1X working solution for one plate.

Dilute Samples

- ❑ Depending on the sample set under investigation, a dilution may be necessary. Suggested dilution factors are provided in the R-PLEX product-specific datasheet. Assay diluent may be used for sample dilution. The dilution factor for a given sample type should be optimized.

Notes:

- The sample preparation for biomarkers listed in Table 1 for Assay Protocol 3 may not be compatible with secreted biomarkers. Refer to the R-PLEX product-specific datasheet for information on biomarker diluent/sample preparation.
- 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.

Read Buffer

MSD GOLD Read Buffer B is included in the U-PLEX Development Pack and is provided at the working concentration of the assay. Do not dilute.

Assay Protocol 1

Refer to the Introduction section (Table 1 on page 4) for the optimal assay protocol for your R-PLEX Antibody Sets.

Note: Before beginning STEP 1, prepare the plate as described on pages 13 and 14.

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of assay diluent 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 (or Tracer) Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of detection antibody (or Tracer) 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.

Assay Protocol 2

Assay Protocol 2 includes a blocking step. Refer to the Introduction section (Table 1 on page 4) for the optimal assay protocol for your R-PLEX Antibody Sets.

Note: Before beginning STEP 1, prepare the plate as described on pages 13 and 14.

STEP 1: Add Blocker and Wash (This step is NOT required for Human CKBB or Rat Albumin)

- ❑ Add 150 µL of Diluent 35 as a blocking solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.
- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.

STEP 2: Add Samples, Calibrators, and Detection Antibody (or Tracer) Solution

- ❑ Add 25 µL of detection antibody (or Tracer) solution to each well.
- ❑ 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 2 hours.

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.

Assay Protocol 3

Assay Protocol 3 requires 1X MSD Tris Wash Buffer. Refer to the Introduction section (Table 1 on page 4) for the optimal assay protocol for your R-PLEX Antibody Set.

Note: Before beginning STEP 1, prepare the plate as described on pages 13 and 14.

STEP 1: Add Samples and Calibrators*

- ❑ Add 50 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.

***Note:** For Human Aiolos, Ikaros, and IRS-1, add 25 µL of assay diluent and 25 µL of the prepared Calibrator Standard or sample to each well.

STEP 2: Wash and Add Detection Antibody (or Tracer) Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Tris Wash Buffer.
- ❑ Add 50 µL of detection antibody (or Tracer) 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 Tris 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 (or Tracer) 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 datasheet shipped with the product which is also available at Mesoscale Datasheets. The data represent the performance of the assay tested in singleplex 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, the assay may perform differently than the representative data shown.

Summary Protocol for R-PLEX Multiplex Assays

Refer to the Introduction section (Table 1 on page 4) for the optimal assay protocol for your R-PLEX Antibody Sets.

Gather Required Assay Components

- ❑ R-PLEX Antibody Set(s) with Calibrator
- ❑ U-PLEX plate, Linkers, and Stop Solution
- ❑ Coating diluent, assay diluent, and antibody diluent
- ❑ MSD GOLD Read Buffer B
- ❑ MSD Wash Buffers*

*Not included in R-PLEX Assays

U-PLEX Plate Preparation

STEP 1: Create Individual U-PLEX 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 (page 22).

- ❑ 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 should be used for each unique biotinylated antibody. Vortex. Incubate at room temperature for 30 minutes.
- ❑ Add 200 µL of Stop Solution. Vortex. Incubate at room temperature for 30 minutes.

STEP 2: Prepare Multiplex Coating Solution

- ❑ 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.

STEP 3: Coat U-PLEX Plate

- ❑ Add 50 µL of multiplex coating (page 14) 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 1X MSD Wash Buffer, or if following Assay Protocol 3, wash the plate with at least 150 µL/well of 1X MSD Tris Wash Buffer. The plate is coated and ready for use.

Summary Assay Protocol 1

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of assay diluent 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 (or Tracer) Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of detection antibody (or Tracer) 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.

Summary Assay Protocol 2

STEP 1: Add Blocker and Wash (this step is NOT required for Human CKBB or Rat Albumin).

- ❑ Add 150 µL of Diluent 35 as a blocking solution to each well. Seal the plate with an adhesive plate seal. Incubate at room temperature with shaking for 1 hour.
- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.

STEP 2: Add Samples, Calibrators, and Detection Antibody (or Tracer) Solution

- ❑ Add 25 µL of detection antibody (or Tracer) solution to each well.
- ❑ 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 2 hours.

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.

Summary Assay Protocol 3

STEP 1: Add Samples and Calibrators

- ❑ Add 50 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.

Note: For Human Aiolos, Ikaros, and IRS-1, add 25 µL of assay diluent and 25 µL of the prepared Calibrator Standard or sample to each well.

STEP 2: Wash and Add Detection Antibody (or Tracer) Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Tris Wash Buffer.
- ❑ Add 50 µL of detection antibody (or Tracer) 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 Tris 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.

Spot Map

Map your assay spot location by writing the name of each analyte next to its spot number.

1 _____ 6 _____

2 _____ 7 _____

3 _____ 8 _____

4 _____ 9 _____

5 _____ 10 _____

Figure 5: Spot Map

Plate Diagram