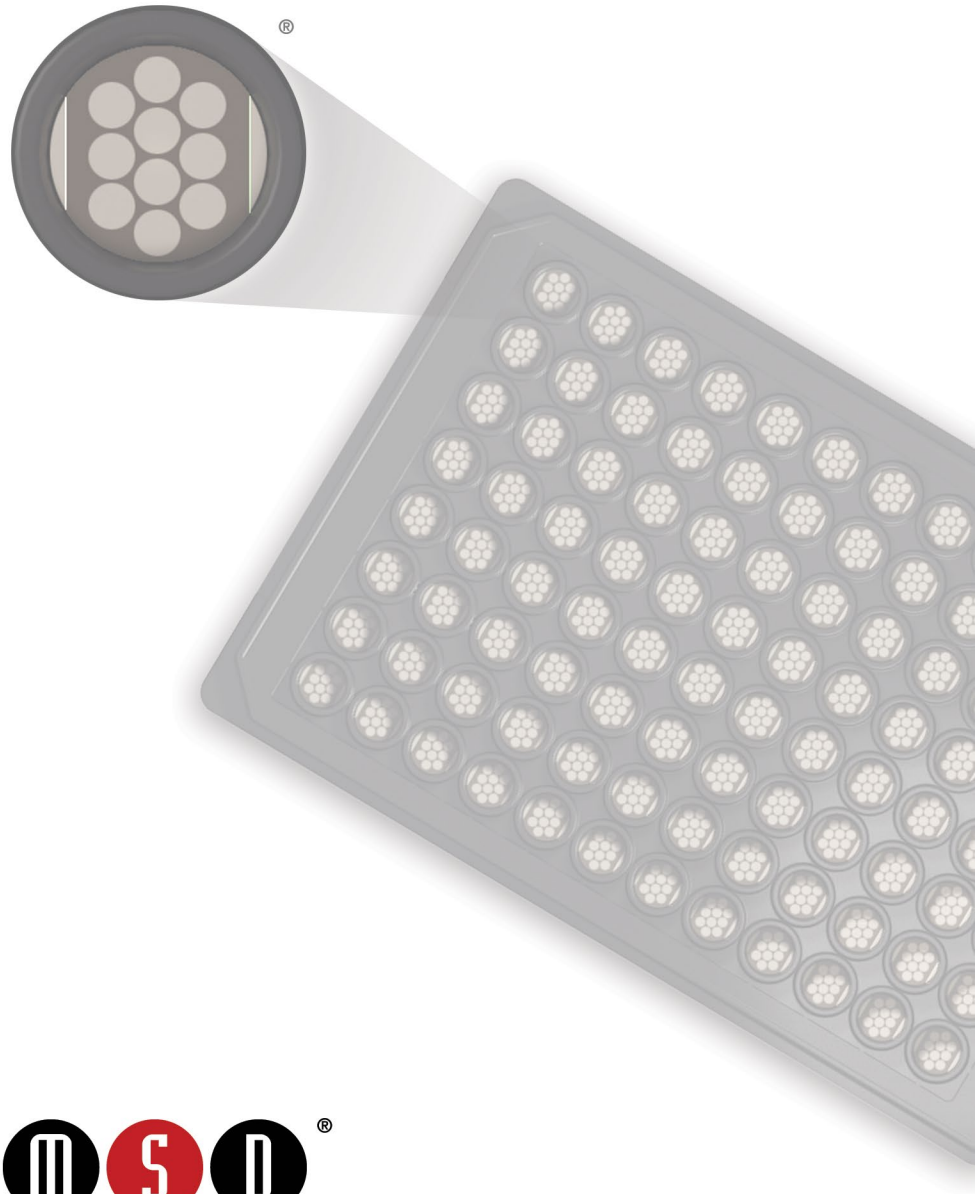
U-PLEX® Biomarker Group 2 (human, mouse, NHP) Multiplex Assays



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MSD U-PLEX Platform

U-PLEX Biomarker Group 2 (human, mouse, NHP) Multiplex Assays

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

For use with:

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

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

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

Catalog numbers for U-PLEX Biomarker Group 2 Combos are available at https://www.mesoscale.com/en/products_and_services/assay_kits/u-plex_gateway/u-plex-combinations)

This product insert should be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY
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Introduction

The MESO SCALE DISCOVERY® U-PLEX platform allows the creation of custom multiplex assays for any combination of analytes in the same Group.

This product insert is for U-PLEX Combos and multiplex assays that contain any number of the assays in the U-PLEX Biomarker Group 2 (human, mouse, or NHP). Using open spots, custom combinations can include R-PLEX® Antibody Sets or your antibodies for other analytes.

Representative data for each U-PLEX assay is presented in the product-specific datasheets available at the www.mesoscale.com® website.

Principle of the Assay

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

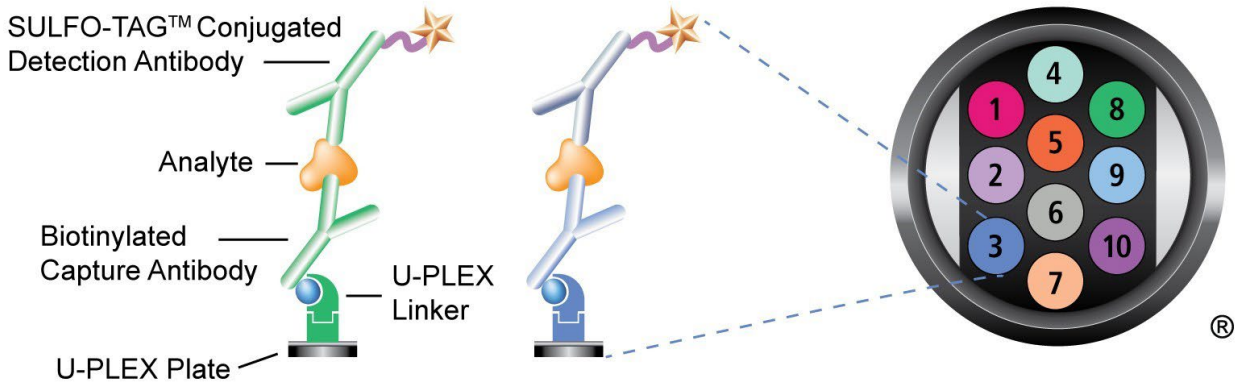


Figure 1. U-PLEX multiplex immunoassay on a U-PLEX 96-well 10-Assay Plate. U-PLEX 384-well 4-Assay plates are similar.

Components

Tables 1, 2, and 3 list the components provided with multiplex U-PLEX Biomarker Group 2 Assays. You will only receive components relevant to the assays that you order.

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

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

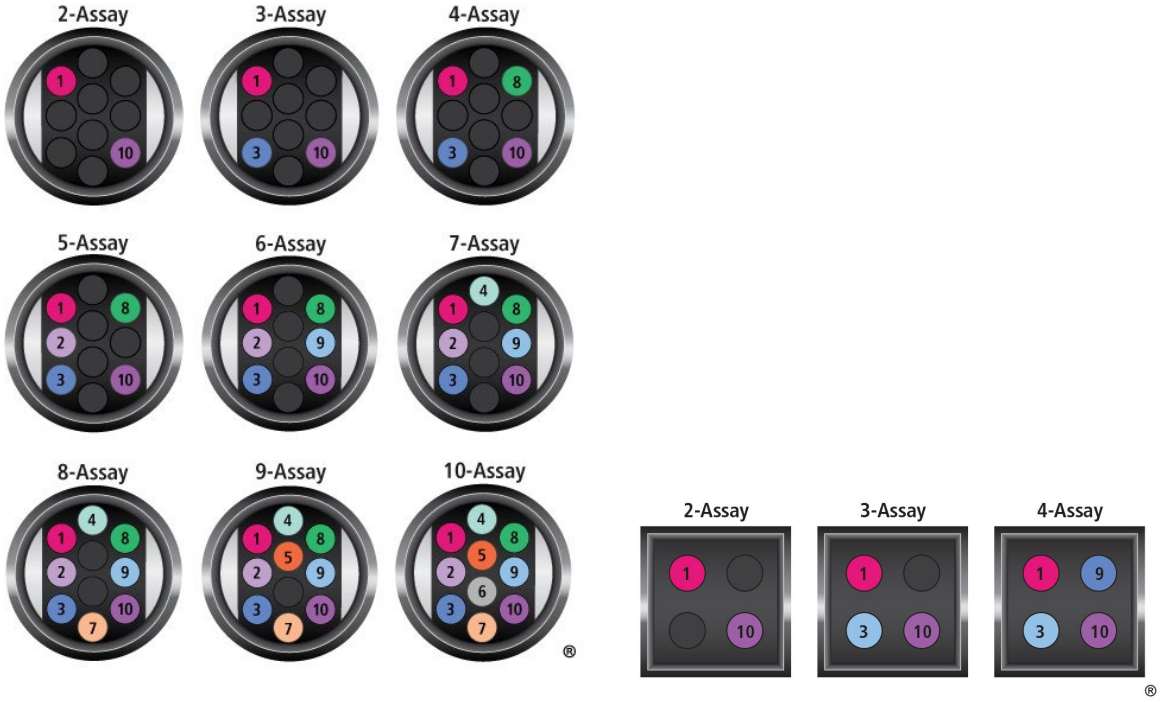
RT = room temperature
Dash (—) = not applicable

Assay-Specific Reagents

U-PLEX plates are provided in a sealed foil pouch with desiccant. The spots correspond to unique U-PLEX Linkers. The number and layout of the active spots on the plate depend on the plate well density (96 vs 384) and the number of assays to be multiplexed (Figure 2). For example, if 4 assays are to be multiplexed, either a U-PLEX 96-well or 384-well 4-Assay Plate will be provided.

U-PLEX Plates

U-PLEX plates are specific to the assays with which they are provided. Do not separate plates from the other refrigerated components.



A. 96-well spot maps B. 384-well spot maps

Figure 2. Spot Maps 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 and in the data files.

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 on the plate.

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

U-PLEX Antibody Sets

You will receive U-PLEX Antibody Sets containing the biotinylated capture antibody and the SULFO-TAG™ conjugated detection antibody (Table 2).

Table 2. Contents of U-PLEX Antibody Set

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

Dash (—) = not applicable

U-PLEX Calibrators

Calibrators may be either lyophilized or frozen. Individual analyte concentrations are provided in lot-specific certificates of analysis (COA). Depending on the specific assays requested, one or more of the following Calibrators will be provided (Table 3).

Table 3. Analytes included in the Calibrators available for U-PLEX Biomarker Group 2

Name	Storage	Catalog No.	Analytes
Calibrator 11	2–8 °C	C0244-2	TGF-β1, TGF-β2, TGF-β3

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 (1,000–1,500 for 384-well plates).
- ❑ MSD Wash Buffer (20X, 100 mL, catalog number R61AA-1) for plate washing. The standard protocol uses a minimum of 415 mL of 1X Wash Buffer for a 384-well plate and 130 mL for a 96-well plate. Automated plate washers may need overage added to these volumes.
- ❑ Adhesive plate seals.
- ❑ Deionized water.
- ❑ Vortex mixer.
- ❑ 1 M HCl.
- ❑ 1.2 M NaOH in 0.5 M HEPES.
- ❑ pH paper to confirm neutralization of samples (optional).
- ❑ MSD Diluent 100 (50 mL, catalog number R50AA-4) may be needed to dilute samples.

Note: If including Open Spots, 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 for diluting the capture antibody.

Instrument Compatibility

MSD offers U-PLEX Assays designed for use on specific instrument platforms (Table 4).

Table 4. Instrument compatibility

Instrument	Assays on U-PLEX 96-well SECTOR™ Plate	Assays on U-PLEX 96-well QuickPlex Ultra™ Plates	Assays on U-PLEX 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	—	Y	—

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.

Reagent Preparation for 96-well Plates

Important: Upon the first thaw, aliquot diluents into suitably sized aliquots before refreezing.

Prepare U-PLEX Plate

The preparation of a U-PLEX plate involves coating the provided plate with Linker-coupled capture antibodies. Assign each antibody to a unique Linker and record the antibody identity next to the assigned Linker, as shown in the examples below.

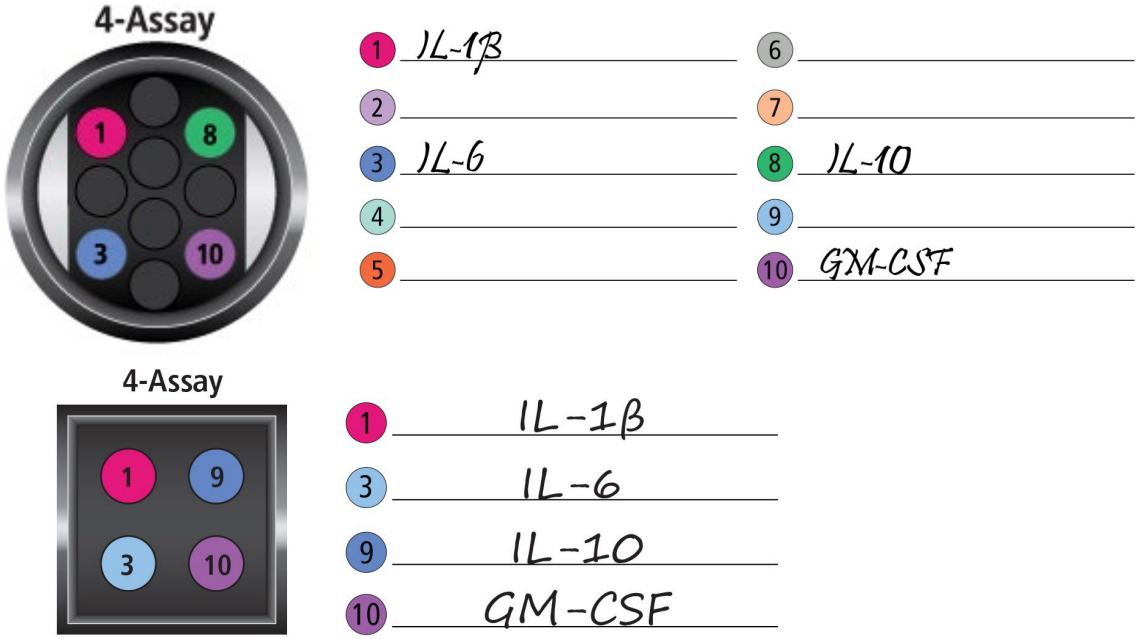


Figure 3. U-PLEX 4-Assay Plates and assigned Linkers: (top) 96-well plate, (bottom) 384-well plate.

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 5).

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

A different Linker is used for each unique biotinylated antibody. Below are the steps to couple linkers with capture antibodies.

- ❑ 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 21).
- ❑ 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 individual 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 5). The volumetric ratio of Linker:antibody:Stop Solution is 3:2:2.

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

- ❑ 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 3: Coat U-PLEX 96-well Plates

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

Note: The plate is now coated and ready for use. Sealed plates may be stored in the original pouch 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 5. If using a partial plate, adjust the volumes used proportionally.

Table 5. Amount of each component required for U-PLEX coating solution per plate

No. of Plates	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

Bring the Calibrator to room temperature. Reconstitute by adding 250 µL of Assay Diluent to the vial. This will result in a 10X concentrated stock of each Calibrator. 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.

Note: We recommend that reconstituted or thawed Calibrators be used immediately. If storage is necessary, divide Calibrators into suitably sized aliquots (60 µL aliquots are recommended) and store immediately at ≤–70 °C.

Prepare Calibrator Standard 1 (top of the curve) in a polypropylene tube by diluting the reconstituted Calibrator as indicated in Row 1 of Table 6. Mix by vortexing.

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

Table 6. 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)	25	225	250
2	2	From tube 1	75	225	300
3	3	From tube 2	75	225	300
4	4	From tube 3	75	225	300
5	5	From tube 4	75	225	300
6	6	From tube 5	75	225	300
7	7	From tube 6	75	225	300
8 (zero Calibrator)	8	—	0	300	300

Dash (—) = not applicable

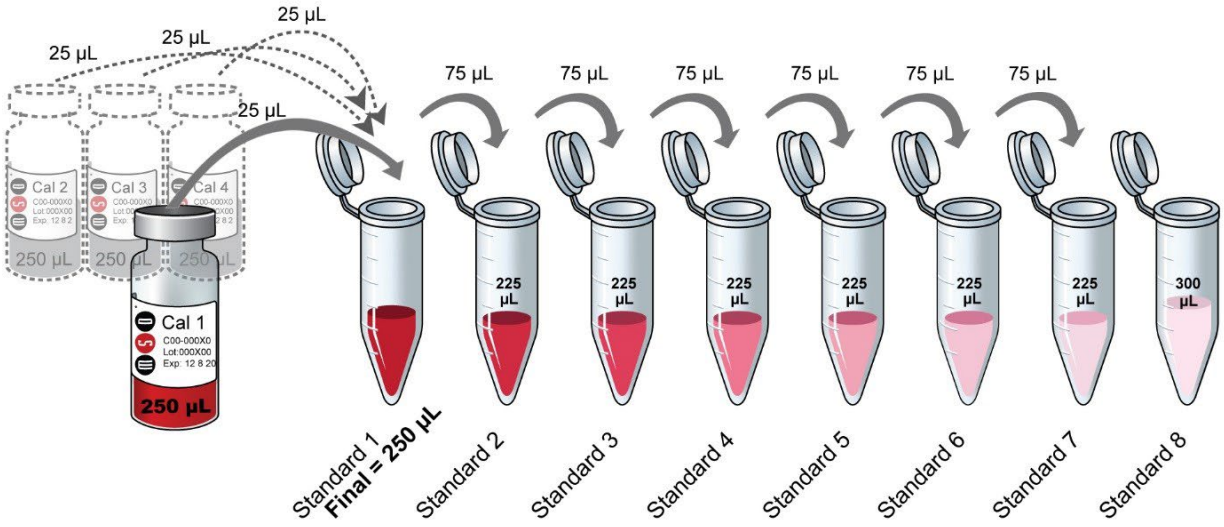


Figure 4. Dilution schema for preparation of Calibrator Standards for U-PLEX Biomarker Group 2 Assays.

Prepare Samples

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

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

Note: The pipetted volumes noted above can be reduced by half to 10 µL, 50 µL, and 7 µL. if pipettes are available to accurately transfer these volumes.

Dilute samples at least two-fold using Assay Diluent. The dilution factor may need to be optimized for the given sample type. Consult MSD technical support if assistance or additional information is required.

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

Prepare Detection Antibody Solution

The detection antibody is provided as a 100X stock solution. The working solution for 96-well plates is 1X. Prepare the detection antibody solution immediately before use.

For one plate, combine:

- ❑ 60 µL of each 100X detection antibody
- ❑ Antibody Diluent3 to bring the final volume to 6 mL

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.

Assay Protocol (96-well plates)

Note: Follow Reagent Preparation before beginning this assay protocol.

STEP 1: Add Sample or Calibrator Standard

- ❑ 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 2 hours.

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.

Alternate Protocols

The suggestions below may be useful for simplifying the protocol.

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

Assay Performance

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

Specificity

To assess specificity, each Antibody Set in the Group was tested individually against all of the analytes for nonspecific binding. Nonspecific binding was less than 2.0% for all assays in the Group using the following calculation.

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

Appendix A

Best Practices

- Equilibrate all assay components to room temperature before use. Mix well before use. Bring plates to room temperature before opening the packet.
- Avoid bubbles at each stage of reagent addition because they can lead to variable results. This is very important when adding Read Buffer (just prior to reading the plate).
- 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. Keep the shaking speed and shaker model consistent for long-term studies.
- Tap the plate on a paper towel after washing to ensure the removal of residual fluid.
- Avoid excessive drying of the plate during washing steps, especially if working inside a laminar flow hood or another high-airflow environment. Cover the plate with a new plate seal immediately after washing to protect it from airflow, and add solutions to the plate as soon as possible.
- Use a new adhesive plate seal for all incubation steps. Avoid re-using plate seals.
- Dispense reagents and wash fluids at the side of the well towards the bottom corner away from the coated spots.
- Remove the plate seal before reading the plate in the instrument.
- Keep time intervals consistent between the addition of Read Buffer and reading the plate to improve interplate precision. Prepare an MSD instrument before 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.
- 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 a new filtered pipette for each reagent addition.
- Do not mix components between boxes of multiplex U-PLEX assays except for Stop Solution, Diluents, and Read Buffer. There will be a unique calibrator curve for each plate.

Working with Partial Plates

A portion of a plate may be used when developing assays. Volumes should be adjusted proportionally when preparing reagents for partial plates.

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

Working with Multiplate Assays

U-PLEX assays can occupy more than one plate, depending on the number and compatibility of the selected assays. In such cases, each box contains components that are intended to be run together. Do not mix components between boxes, and do not separate the plate from the antibody sets. There will be a unique Calibrator curve for each box.

Open Spots

Prepare Conjugated Capture and Detection Antibodies

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.

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

Prepare Biotinylated Capture Antibody

The working concentration of 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 desalting columns.

For long-term storage, 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 in 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 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 signals.

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

Appendix B

Components for 384-well Assays

Table 8. Reagents that are supplied with all U-PLEX Biomarker Group 2 384-well Assays

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

Reagent Preparation for 384-well Plates

Important: Upon the first thaw, aliquot diluents into suitably sized aliquots before refreezing.

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

A different Linker is used for each unique biotinylated antibody. Below are the steps to couple linkers with capture antibodies.

- ❑ 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 21).
- ❑ 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 individual 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 5). The volumetric ratio of Linker:antibody:Stop Solution is 3:2:2.

STEP 2: 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 2, 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 3: Coat U-PLEX 384-well Plates

- ❑ Wash the plate 3 times with 80 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 80 µL/well of 1X Wash Buffer.

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

Prepare Detection Antibody Solution

The detection antibody is provided as a 100X stock solution. The working solution for 384-well plates is 0.5X. 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 12 mL

Assay Protocol (384-well plates)

STEP 1: Add Samples and Calibrators

- ❑ Add 25 µL of the prepared Calibrator Standard or sample to each well of a prepared plate. 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 80 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 80 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 suggestion below may be useful for simplifying the protocol.

- ❑ **Alternate Protocol, Shortened Incubations:** Some assays in 384-well plates may achieve acceptable performance with shorter incubations. Consider incubating samples in the plate for 2 hours.

Spot Maps

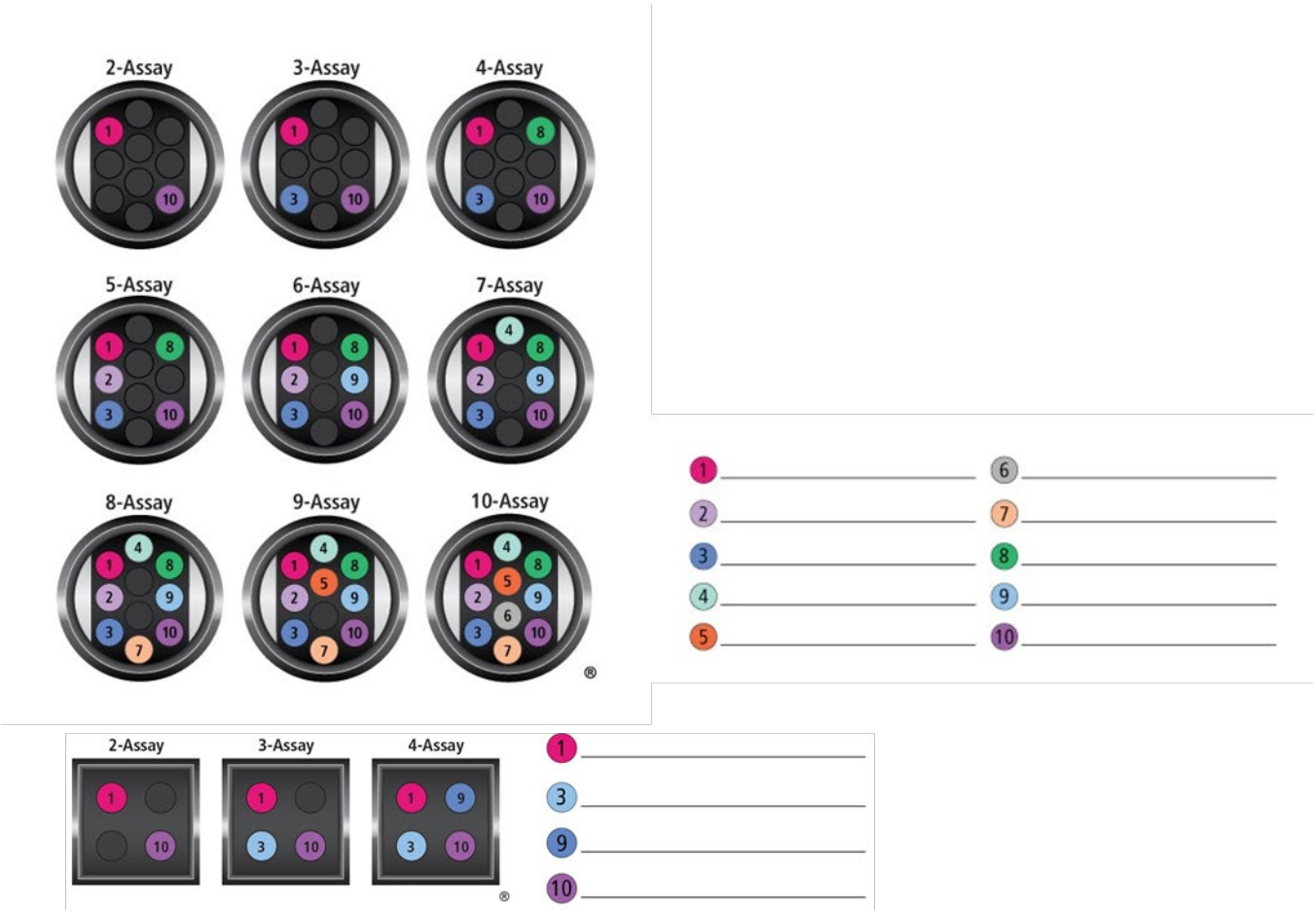


Figure 5. Spot maps. ***(top)*** 96-well plate, ***(bottom)*** 384-well plate.

Plate Diagrams

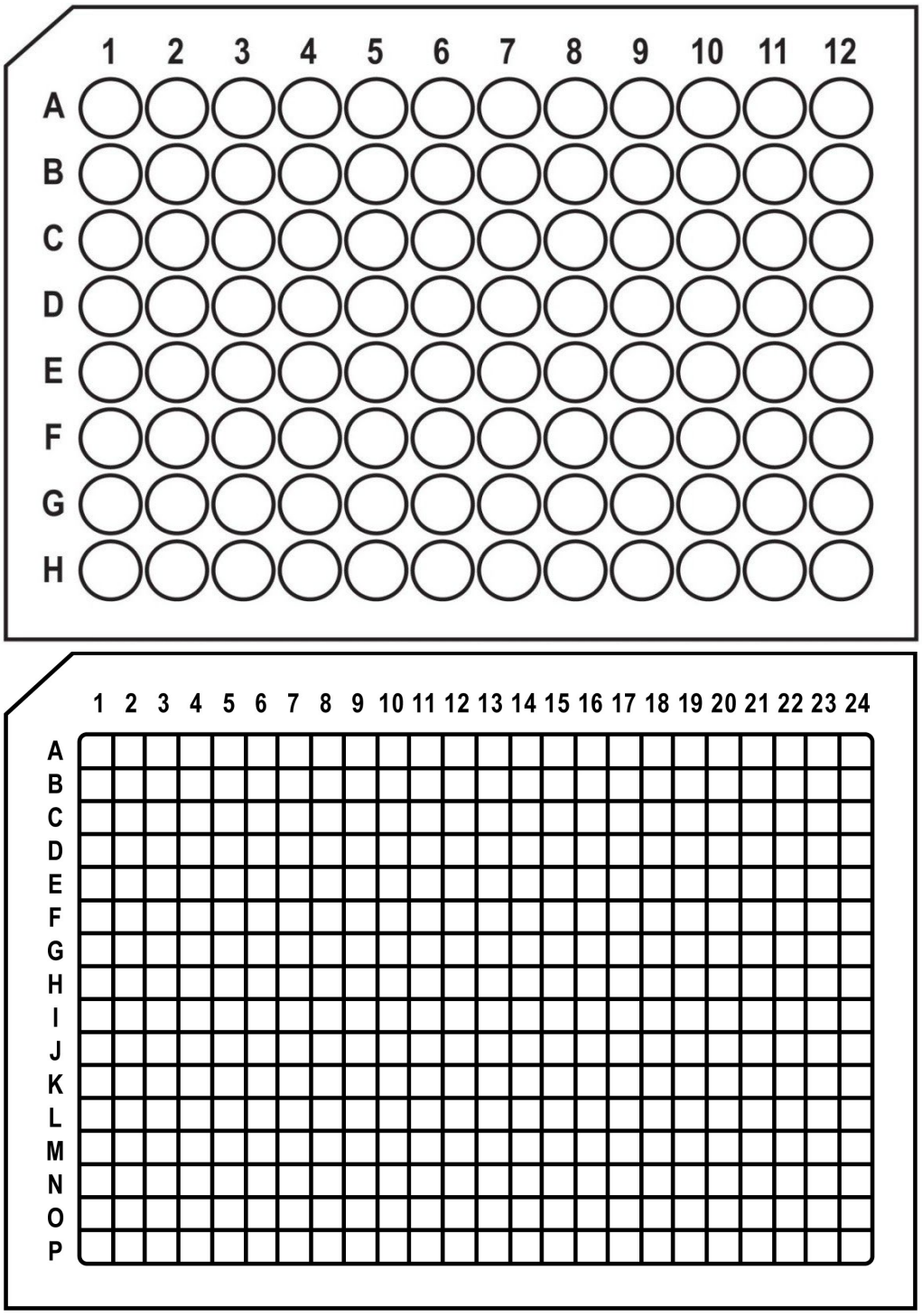


Figure 6. *Plate diagrams; similar plate layouts can be created in Excel and in the DISCOVERY WORKBENCH® software.*