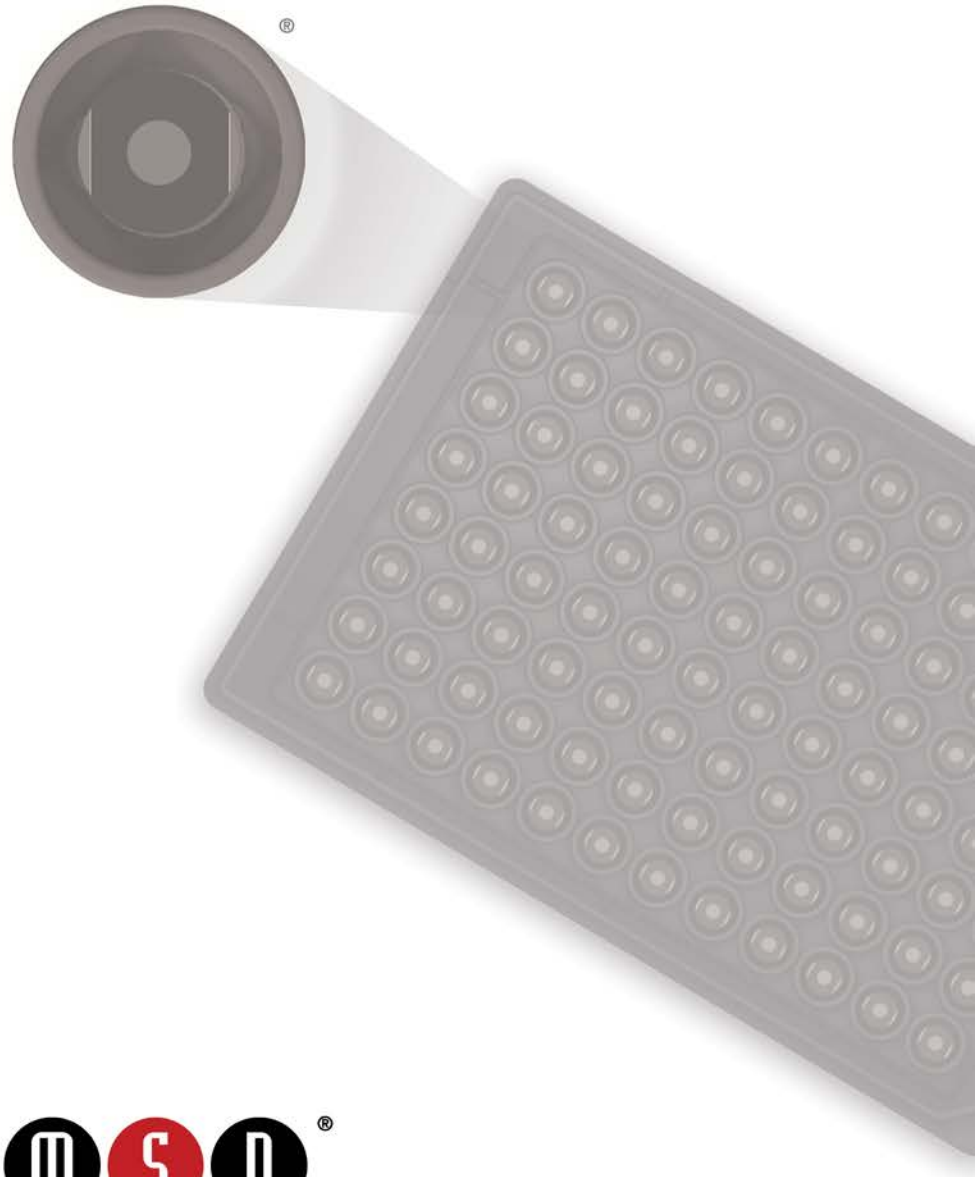
MSD Booster Pack™ A and B

Guidelines for Assay Development



Booster Pack A kit	Catalog No.
1-Plate Size Pack	K05728S-1
5-Plate Size Pack	K05728S-2
25-Plate Size Pack	K05728S-4

Booster Pack B kit	Catalog No.
1-Plate Size Pack	K15743S-1
5-Plate Size Pack	K15743S-2
25-Plate Size Pack	K15743S-4

MSD MULTI-ARRAY® Assay System

Booster Pack A and B

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

MESO SCALE DISCOVERY® MULTI-ARRAY technology forms the basis of an immunoassay system for measuring biomarkers using highly sensitive electrochemiluminescence (ECL) detection. Our immunoassay technology overcomes many challenges associated with other platforms, offering sensitivity and accuracy, superior result reliability, and a faster, simpler workflow.

The Booster Pack A and B assay development tools use MSD technology to provide a rapid and convenient method for developing signal-enhanced ECL assays on the MSD platform with users' antibody pairs and reagents. The Booster Pack reagents are compatible with assays run on streptavidin plates but not on pre-coated assay plates.

In some cases, the enhanced signal shifts the dynamic range of assays, increases sensitivity, and reduces the amount of sample required. This depends, in part, on the quality of reagents used, especially antibodies, calibrators, and diluents, and the sensitivity improvements vary by analyte (Figure 1). In most cases, enhanced assays demonstrate greater sensitivity than conventional assays, with significant improvements in assay signal.

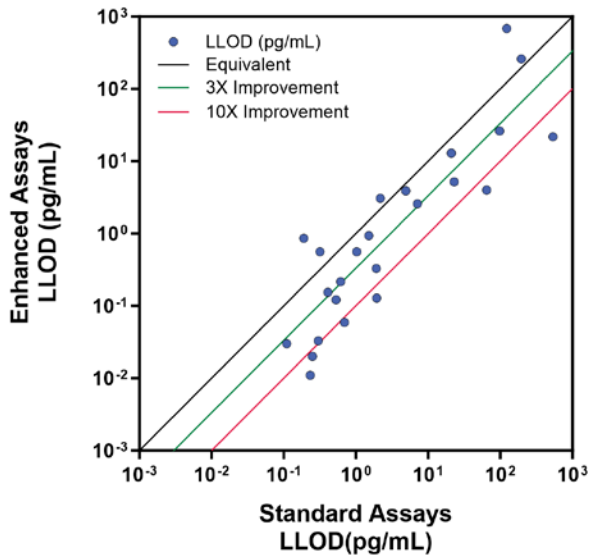


Figure 1. The graph shows the concordance between the LLOD computed from 24 assays run using the standard and enhanced protocols using the Booster Pack A assay development tool. The black line represents equivalent LLOD performance between the two assay formats. The green line represents 3X, and the red line represents 10X LLOD improvement over the standard format.

This product insert provides instructions for developing an immunoassay using the Booster Pack assay development tool and MSD technology.

Principle of the Assay

Using the Booster Pack A or B assay development tool to develop an immunoassay with enhanced signals requires biotinylated capture and SULFO-TAG™ detection antibodies. The biotinylated capture antibody binds to MSD GOLD™ Small Spot Streptavidin 96-well plates (Figure 2), where the analyte in the sample interacts with the capture reagent. The detection antibody, conjugated with electrochemiluminescent label (MSD GOLD SULFO-TAG), then binds to the analyte to complete the sandwich immunoassay.

Reagents in Booster Pack assay development tools facilitate signal enhancement from the SULFO-TAG antibody. When the plate is loaded into an MSD instrument, a voltage is applied to the plate electrodes, causing the capture labels to emit light. The instrument measures the intensity of emitted light, which is proportional to the amount of analyte present in the samples, providing a quantitative measure of analyte in each sample.

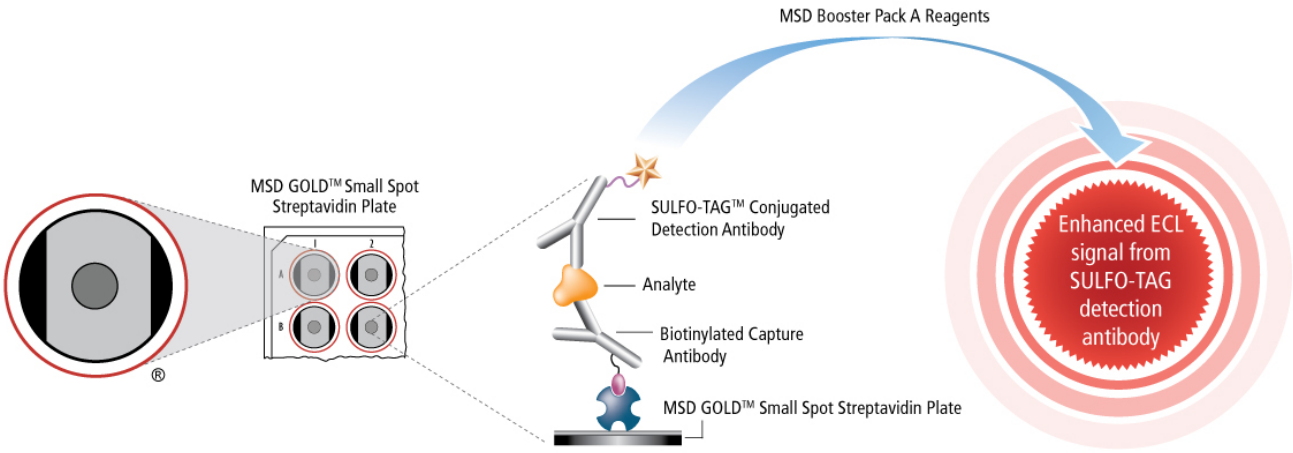


Figure 2. *Sensitive singleplex assay development on an MSD GOLD 96-well Small Spot Streptavidin Plate using Booster Pack A and B reagents.*

Pack Components

Reagents provided in Booster Pack A and B assay development tools are shown in Table 1. The Booster Pack assay development tools are available in 1-, 5- and 25-plate sizes.

Table 1. Reagents that are provided in the Booster Pack A and B assay development tools

Reagent	Storage	Catalog No.	Size	Quantity Supplied		
				1 Plate	5 Plates	25 Plates
BP Reagent A	≤–70 °C	R31AL-1	300 µL	1	5	25
BP Reagent B	≤–10 °C	R31AM-1	500 µL	1	5	25
BP Reagent C	2–8 °C	R31AN-1	45 µL	1	5	25
BP Reagent D	≤–10 °C	R31AP-1	1.7 mL	1	5	25
BP Reagent E	≤–10 °C	R31AQ-1	1.7 mL	1	5	25
BP Reagent F	≤–70 °C	R31AR-1	50 µL	1	5	25
BP Reagent G	≤–70 °C	R31AS-1	1.7 mL	1	5	25
BP Reagent H	≤–70 °C	R31AT-1	50 µL	1	5	25
Diluent 100	2–8 °C	R50AA-4	50 mL	1 bottle	—	—
		R50AA-2	200 mL	—	1 bottle	5 bottles

Dash (—) = not applicable

Additional Booster Pack B Components

Table 1. Reagents that are provided only in the Booster Pack B assay development tool

Reagent	Storage	Catalog No.	Size	Quantity Supplied		
				1 Plate	5 Plates	25 Plates
MSD GOLD 96-Well Small Spot Streptavidin Plate	2–8 °C	L45SA-1	1 plate	1	5	25
MSD GOLD Read Buffer B	RT	R60AM-1	18 mL	1	—	—
		R60AM-2	90 mL	—	1	5

Dash (—) = not applicable

Using Your Reagents

Table 2 lists the reagents required to develop an assay with user-provided antibodies, calibrator, and diluents on the MSD platform that is compatible for use with Booster Pack A and B reagents.

Table 2. Contents required to develop an immunoassay using your reagents

Reagent (analyte-specific)	Storage	Catalog No.	Size	Quantity Supplied			Description
				1 Plate	5 Plates	25 Plates	
Biotin Capture Antibody	Analyte-specific, user-provided reagents. Guidance on preparing antibodies and calibrators is provided in **Appendix A.**						Biotinylated capture antibody
SULFO-TAG Detection Antibody							SULFO-TAG conjugated detection antibody
Calibrator							Native or recombinant protein
Assay Diluent							Diluent for sample and calibrator preparation
Antibody Diluent							Diluent for detection antibody preparation
MSD GOLD 96-Well Small Spot Streptavidin SECTOR plate	2–8 °C	L45SA-1	1 plate	1	5	25	96-well plate, foil-sealed with desiccant (Provided in the Booster Pack B assay development tool)
MSD GOLD Read Buffer B	RT	R60AM-1	18 mL	1	—	—	Buffer to catalyze the electrochemiluminescent reaction (Provided in the Booster Pack B assay development tool)
		R60AM-2	90 mL	—	1	—	
		R60AM-2	1000 mL	—	—	1	

Dash (—) = not applicable; RT = room temperature

Additional Materials and Equipment

Materials

- Adhesive plate seals
- Micropipettes with filtered tips
- Tubes (polypropylene microcentrifuge tubes, conical tubes, library tubes)
- Serological pipettes and pipette controller
- Reagent reservoir
- Plastic bottles
- Wet ice and ice bucket
- Deionized water
- Molecular biology grade water
- MSD Wash Buffer (20X, 100 mL, catalog number R61AA-1) for plate washing.
- MSD Diluent 100 (50 mL, catalog number R50AA-4) for diluting samples which require high dilution
- Assay Diluent
- Biotin-labeled capture antibody
- SULFO-TAG labeled detect antibody

Equipment

- Microtiter plate shaker capable of shaking at 500–1,000 rpm and maintaining a controlled temperature of 27 °C (For example: BioSan PST-60HL-4)
- 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)
- Vortex mixer
- Water bath
- Microcentrifuge

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), which can be obtained from MSD Customer Service or at the www.mesoscale.com website.

Best Practices

- Bring frozen reagents (diluents, BP Reagents D, E, and G) to room temperature in a 22–25 °C water bath before use. If a controlled water bath is not available, thaw at room temperature. Ensure that frozen reagents are fully thawed and equilibrated to room temperature before use. Mix well after thawing and before use.
- Incubation temperatures below 22 °C for incubation steps can negatively affect assay signals and sensitivity. For best results, perform these incubation steps between 22–27 °C.
- Thaw frozen vials of BP Reagents F and H on ice until needed. Ensure that BP Reagents F and H are fully thawed before use. Mix well after thawing and before use.
- To avoid cross-contamination between vials, open vials for one protocol step at a time. Close the cap after use. Use filtered pipette tips, and use a fresh pipette tip for each reagent addition.
- Prepare calibrators and samples in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution and mix by vortexing after each dilution.
- 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. Do not touch the pipette tip on the bottom of the wells when pipetting into the MSD Plate.
- Plate shaking should be vigorous, with a rotary motion between 500–1,000 rpm. Binding reactions may reach equilibrium sooner if shaken in the middle of this range (~700 rpm) or above.
- Use a new adhesive plate seal for all incubation steps. Remove the plate seal before reading the plate.
- When washing assays, the best results are obtained by using a low dispense flow rate and by positioning dispenser tips at the outer edge of the well (e.g., horizontal dispense offset towards the left side of the well). See Appendix D for more information on plate washing recommendations.
- When performing manual plate washing using a multichannel pipette, plates should be washed using at least 150 µL of wash buffer per well. Excess residual volume after washing should be removed by gently tapping the plate on a paper towel.
- Do not allow plates to dry after washing steps. Solutions associated with the next assay step should be added to the plate immediately after washing.
- Make sure that the read buffer is at room temperature when adding to the plate.
- Do not shake the plate after adding read buffer.
- If the sample requires higher dilutions, Diluent 100 may be used in place of assay diluent.
- Avoid prolonged exposure of the BP Reagent G and detection solutions to light. Keep stocks of BP Reagent G in the dark.
- Intense light sources can affect assay performance. Plates should be protected from direct light during the plate shaking steps for optimal results.

Recommended Protocol

Before starting the assay, make sure to be aware of the following:

- Have a biotin-conjugated capture antibody, SULFO-TAG-conjugated detection reagent, and calibrator. See **Appendix A** for guidance on preparing antibodies and calibrator.
- Bring all reagents to room temperature and refer to the **Best Practices** section (above) before beginning the protocol.
- Vortex each vial to mix and spin down briefly before use. Prepare all working solutions immediately before use.
- Reagents prepared at each step below are sufficient for a one-plate experiment.
- Diluent 100 or other diluents may be used in place of Assay Diluent. The diluent choice should be established during assay development.

CRITICAL: Incubation temperatures can affect assay signals and sensitivity. For optimal results, follow the recommendations provided for each incubation step.

❖ Reagent Preparation: Capture Coating Solution

Preparation of the plate involves coating the MSD GOLD 96-well Small Spot Streptavidin plate with a biotin conjugated capture antibody in a buffer containing BP Reagent A. The typical working capture antibody concentration used for coating should be 0.25 µg/mL or lower.

The specific protocol in this section describes the preparation of coating solution for one 96-well plate with a stock biotin antibody concentration of 10 µg/mL.

Note: For use with Booster Pack A and B reagents, MSD recommends performing a 40-fold dilution in the working capture coating solution.

Prepare the capture coating solution immediately before use by combining the following reagents. Vortex briefly to mix.

- ❑ 5,820 µL Diluent 100
- ❑ 150 µL of Biotinylated Capture Antibody (10 µg/mL stock user-provided biotinylated capture antibody)
- ❑ 30 µL of BP Reagent A

Note: The unused BP Reagent A should be frozen immediately after use. The reagent is stable through 5 freeze-thaw cycles.

CRITICAL: Failure to add BP Reagent A in the coating solution will drastically reduce the assay signal.

STEP 1: Coat the Plate

- ❑ Add 50 µL of the capture coating solution to each well. Tap the plate gently. Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at room temperature for 1 hour.

Notes: While the coated plate is incubating, prepare the BP Reagent B working solution, calibrators, and samples.

❖ Reagent Preparation: BP Reagent B Working Solution

The choice of assay diluent should be established during assay development.

Prepare the BP Reagent B working solution by combining the following reagents. Vortex briefly to mix.

- ❑ 3,465 µL of Assay Diluent
- ❑ 35 µL of BP Reagent B

Note: The unused BP Reagent B should be frozen immediately after use. The reagent is stable through 5 freeze-thaw cycles.

❖ Reagent Preparation: Calibrator Standards

MSD recommends that the assay diluent be used for diluting calibrators and samples. If samples are used undiluted, an assay diluent that best represents the sample matrix can be used for diluting the calibrators. For most assays, 100-1,000 pg/mL may be an appropriate top calibration standard (Calibrator Standard 1). A typical calibration curve has 7 calibration points that are 4-fold serial dilutions from Calibrator Standard 1, and an 8th point is diluent only.

To prepare calibrator solutions for up to 4 replicates (Table 3):

- ❑ Prepare 300 µL of Calibrator Standard 1. Calculate the volume of calibrator and assay diluent to be combined based on the stock concentration of the calibrator and target Calibrator Standard 1 concentration. Mix by vortexing.
- ❑ For Calibrator Standard 2, add 50 µL of Calibrator Standard 1 to 150 µL of assay diluent. Mix by vortexing.
- ❑ Repeat 4-fold serial dilutions five additional times to generate Calibrator Standards 3–7. Mix by vortexing between each serial dilution.
- ❑ Use assay diluent as Calibrator Standard 8 (zero Calibrator).

Discard any unused, diluted calibrators.

Table 3. Serial dilutions to generate the standard curve

Calibrator Standard #	Tube #	Source of Calibrator	Volume of Calibrator (µL)	Assay Diluent (µL)	Total Volume (µL)
1	1	See above instructions			300
2	2	From tube 1	50	150	200
3	3	From tube 2	50	150	200
4	4	From tube 3	50	150	200
5	5	From tube 4	50	150	200
6	6	From tube 5	50	150	200
7	7	From tube 6	50	150	200
8	8	-	0	200	200

❖ Reagent Preparation: Samples

Depending on the sample and analyte abundance, sample dilution may be necessary. Sample dilution should be performed using the same assay diluent that is used for the calibrator. For samples requiring dilutions greater than 10-fold dilution, Diluent 100 may be used as a sample diluent. Assays require 25 µL/well of sample. We recommend running at least two replicates per sample.

STEP 2: Add Calibrators and Sample

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of BP Reagent B working solution to each well.
- ❑ Add 25 µL of calibrator or sample to each well.
- ❑ Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at room temperature for 1 hour.

Note: The BP Reagent B working solution should be added to the plate before sample and calibrator additions.

❖ Reagent Preparation: SULFO-TAG Detection Antibody Solution

Optimal SULFO-TAG detection antibody concentrations will be dependent on antibody affinity, but a working concentration of 0.33 µg/mL is recommended for use with the Booster Pack A or B reagents. This is lower than the typical working concentrations of 0.5–1 µg/mL in standard MSD assays.

Dilute the SULFO-TAG detection antibody to the final working concentration immediately prior to use. Prepare the SULFO-TAG detection antibody solution by combining the following reagents. Vortex briefly to mix.

- ❑ 5,980 µL of Antibody Diluent (Assay specific)
- ❑ 20 µL of SULFO-TAG Detection Antibody (user provided stock solution at ~100 µg/mL SULFO-TAG Detection Antibody)

STEP 3: Add SULFO-TAG Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of SULFO-TAG detection antibody solution at the working concentration to each well.
- ❑ Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at room temperature for 30 minutes.

❖ Reagent Preparation: BP Reagent C Working Solution

Stock BP Reagent C is provided at a stock concentration of 3.13 µg/mL. The final concentration of BP Reagent C will depend on the SULFO-TAG detection antibody concentration used in the assay, but the typical working concentration for the BP Reagent C is 0.015 µg/mL (200-fold dilution from the stock). Prepare the BP Reagent C working solution by combining the following reagents. Vortex briefly to mix.

- ❑ 5,970 µL of Diluent 100
- ❑ 30 µL of BP Reagent C

Note: To optimize performance, select the BP Reagent C working concentration that gives the desired signals and backgrounds for the assay. BP Reagent C may be titrated for each assay to determine the optimal concentration, which typically ranges from 0.03 µg/mL to 0.015 µg/mL. Adjust the Diluent 100 volume based on the BP Reagent C volume for a final volume of 6 mL.

STEP 4: Add BP Reagent C Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagent C working solution to each well.
- ❑ Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at room temperature for 30 minutes.

Notes:

- While the BP Reagent C working solution is incubating, thaw 1 vial each of BP Reagents D, E, and G in a 22–25 °C water bath. If a water bath is not available, thaw at room temperature. Thaw BP Reagents F and H on ice.

❖ Reagent Preparation: BP Reagents D, E, and F Working Solution

Prepare BP Reagents D, E, and F working solution up to 30 minutes before use by combining the following reagents. Vortex briefly to mix.

- ❑ 2,970 µL Molecular Biology Grade Water
- ❑ 1,500 µL of BP Reagent D
- ❑ 1,500 µL of BP Reagent E
- ❑ 30 µL of BP Reagent F

Note: BP Reagent F stock solution is viscous. Pipette slowly to avoid bubble formation in the pipette tip and to ensure accurate pipetting volume.

STEP 5: Add BP Reagent D, E, and F Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagents D, E, and F working solution to each well.
- ❑ Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at room temperature for 10 minutes.

Notes:

- CRITICAL: The next step (STEP 6) requires incubation at 27 °C. Upon completion of STEP 5, prepare a shaker at 27 °C. If you do not have access to a temperature-controlled shaker, a plate shaker can be placed inside an incubator maintaining 27 °C.

❖ Reagent Preparation: BP Reagents G and H Working Solution

Prepare the BP Reagents G and H working solution before use by combining the following reagents. Vortex briefly to mix.

- ❑ 4,470 µL Molecular Biology Grade Water
- ❑ 1,500 µL of BP Reagent G
- ❑ 30 µL of BP Reagent H

Notes:

- CRITICAL: Avoid prolonged exposure of the BP Reagent G and BP Reagents G and H working solution to light.
- BP Reagent H stock solution is viscous. Pipette slowly to avoid bubble formation in the tip and to ensure accurate pipetting volume.

STEP 6: Add BP Reagents G and H Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagents G and H working solution to each well.
- ❑ Seal the plate with an adhesive plate seal and incubate with shaking (~700 rpm) at 27 °C for 1 hour.

Note: CRITICAL: The incubation temperature for this step can affect the background and assay signals, thereby affecting the assay sensitivity. It is highly recommended that the STEP 6 incubation be performed at 27 °C. If you do not have access to a temperature-controlled shaker, a plate shaker can be placed inside an incubator maintaining 27 °C.

STEP 7: Add Read Buffer

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.

Note: Do not allow plates to dry after the final wash step. Proceed to add read buffer immediately after washing the plate.

- ❑ Add 150 µL of MSD GOLD Read Buffer B to each well and read on an MSD reader. Incubation in read buffer is not required before reading the plate.

Note: CRITICAL: SECTOR™ plates are compatible with SECTOR® and QuickPlex® SQ instruments.

Appendix A: Assay Optimization

Enhancement of an existing immunoassay may be achieved through use of the Booster Pack A or B assay development tools. The selection and performance of antibodies hinge on various characteristics, including affinity, purity, degradation and/or aggregation state, off-rate, and non-specific binding to other analytes, antibodies, and plate surfaces. For optimal immunoassays, choose high-affinity antibodies that are thoroughly characterized to ensure minimal degradation and aggregation. It is worth noting that in certain cases, the ideal antibody pair for one immunoassay format may not be the most suitable for another, necessitating the use of an alternative set of antibodies.

Table 4: A summary of assay parameters and optimization ranges for use with Booster Pack A and B assay development tools

Assay Step/Parameter	Recommended Condition	Optimization Range
Capture Antibody Concentration	0.25 µg/mL	The recommended concentration is the binding capacity for the plates. Increased capture concentration may improve signal but may introduce variability.
SULFO-TAG Antibody Concentration	0.33 µg/mL	Optimize from 0.3 to 1 µg/mL based on background and specific signals.
Calibrator	1,000 pg/mL	The optimal concentration at Calibrator Standard 1 (100–1,000 pg/mL) should generate a signal near but below 1,000,000 counts.
SE Reagent C	0.015 µg/mL	Optimize from 0.03 to 0.015 µg/mL based on background and specific signals.

Prepare Conjugated Capture and Detection Antibodies

The Booster Pack A and B assay development tools work with assays using a biotinylated capture antibody and a SULFO-TAG conjugated detection antibody. Therefore, when developing assays with user-specific antibody pairs, it is essential to biotinylate the capture antibodies before initiating the protocol. The detection antibody must be conjugated directly with SULFO-TAG.

Note: Refrain from using a biotinylated detection antibody and SULFO-TAG Streptavidin for detection. Their use may result in high backgrounds due to binding to the streptavidin plate or the biotinylated capture antibody, respectively.

Biotinylated Capture Antibody

The recommended working antibody concentration for the plate coating step is 0.25 µg/mL. This concentration corresponds to the binding capacity of the MSD GOLD 96-well Small Spot Streptavidin plate and has proven to be suitable for optimizing signal and minimizing background across various assays. However, it can be adjusted to meet specific assay requirements. To achieve this, prepare the stock solution of biotinylated capture antibody at a target concentration of 10 µg/mL, 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). It is recommended to start with a biotin challenge ratio of 10 biotins to 1 capture antibody. This ratio typically results in the conjugation of an average of 2–4 biotins per antibody. Refer to the MSD Biotin Conjugation Quick Guide at www.mesoscale.com.

Note: Free biotin can interfere with the assay signal. Therefore, after conjugation, it is advised to purify the biotinylated antibody from the free biotin reagent using desalting columns.

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

SULFO-TAG Conjugated Detection Antibody

The optimal working concentration of the SULFO-TAG conjugated detection antibody for use in the MSD platform-developed assay is typically within the range of 0.5–1 µg/mL (standard format), with 0.33 µg/mL as a recommended starting concentration for use with Booster Pack A and B reagents. Prepare a concentrated stock solution (ideally 100X) for each SULFO-TAG conjugated detection antibody by following the SULFO-TAG conjugation guidelines available at www.mesoscale.com. Refer to the MSD GOLD SULFO-TAG Conjugation Quick Guide or the MSD GOLD SULFO-TAG NHS-Ester Technical Note for detailed instructions. We recommend using a 20:1 challenge ratio for the SULFO-TAG conjugation of antibodies, resulting in a typical conjugation ratio of 10 SULFO-TAG labels per antibody molecule. Optimizing the SULFO-TAG challenge ratio may be necessary to reduce background and increase the assay signal.

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.

User-provided Calibrator

An ideal calibrator should represent the native protein. Choose either a purified native protein or a recombinant protein that closely resembles the native form whenever possible. 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 cases where they are not available, such as with intracellular signaling assays, an appropriate cell model may be developed to generate a native form of the protein.

Select a simple assay diluent, such as Diluent 100, containing a carrier protein to use as the calibrator diluent. If the samples to be tested are in a complex matrix, serum-containing diluents lacking the analyte of interest may be necessary to match the performance of the calibrator in the assay diluent with that of the analyte in the matrix being tested.

Choosing the optimal concentrations for the calibration curve is crucial for maximizing the dynamic range of the assay. Assay signals on MSD instruments can saturate above 1,000,000 counts. A good starting point for optimizing a biomarker assay is with the top calibrator at 10,000 times the expected sensitivity of the assay. For example, if the expected sensitivity is 0.01 pg/mL, the top of the curve should be 100 pg/mL. A four-fold dilution between calibrator points will provide a calibrator range of 16,384-fold between the first and 7th calibrator points. A blank should be run as part of the assay optimization. The top calibrator concentration can be adjusted to generate a signal near but below 1,000,000 counts. A good starting concentration is 100-1,000 pg/mL for the highest calibrator point (Calibrator Standard 1).

Avoid reagents that could denature the capture antibody. In general, ionic detergents such as SDS should be <0.1%, and 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 should be diluted in a suitable buffer lacking a denaturing agent before adding it to the antibody-coated plate.

BP Reagent C Concentration

The concentration of BP Reagent C can be titrated for optimal enhanced assay performance. The optimal range typically falls between 0.03 µg/mL and 0.015 µg/mL. In the absence of conducting a titration, we recommend using BP Reagent C at a concentration of 0.015 µg/mL.

Select the working concentration of BP Reagent C that yields the optimal balance of signal and background for the assay.

Data from a wide-range titration of BP Reagent C concentration for a representative assay is presented below (Figure 4). We suggest testing 3-4 BP Reagent C concentrations between 0.03 and 0.015 µg/mL to determine the appropriate final working concentration. When choosing a final concentration, consider both the signal and background for optimal sensitivity (i.e. signal:background ratio).

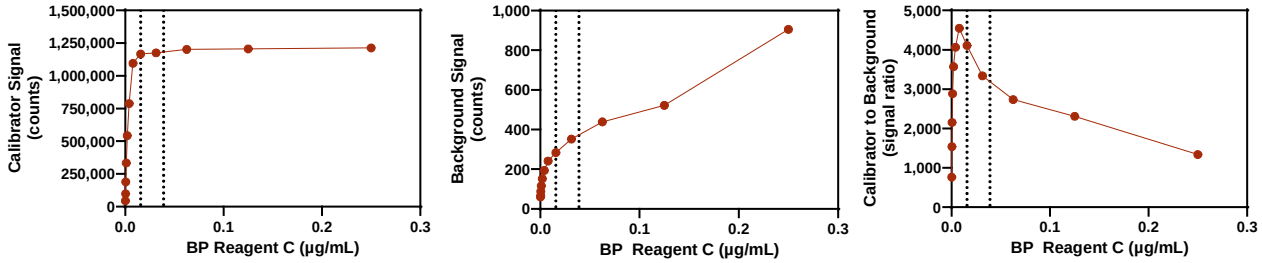


Figure 3. BP Reagent C titration for the human IL-10 assay for a) calibrator signals, b) background signals, and c) calibrator to background signal ratio. The dotted lines show the optimal concentration range between 0.03 µg/mL to 0.015 µg/mL.

Appendix B: Assay Performance

The performance of representative assays was tested in conjunction with Booster Pack A reagents. These tests evaluated sensitivity, dynamic range, and sample measurement. Increased sensitivity and improved sample measurement were observed in the enhanced assays.

Typical Calibration Curves

Representative calibration curves from four assays run using their standard assay protocols with and without the Booster Pack A reagents are presented below (Figure 5). The enhanced assays typically showed 4 logs of dynamic range with significant increases in signal.

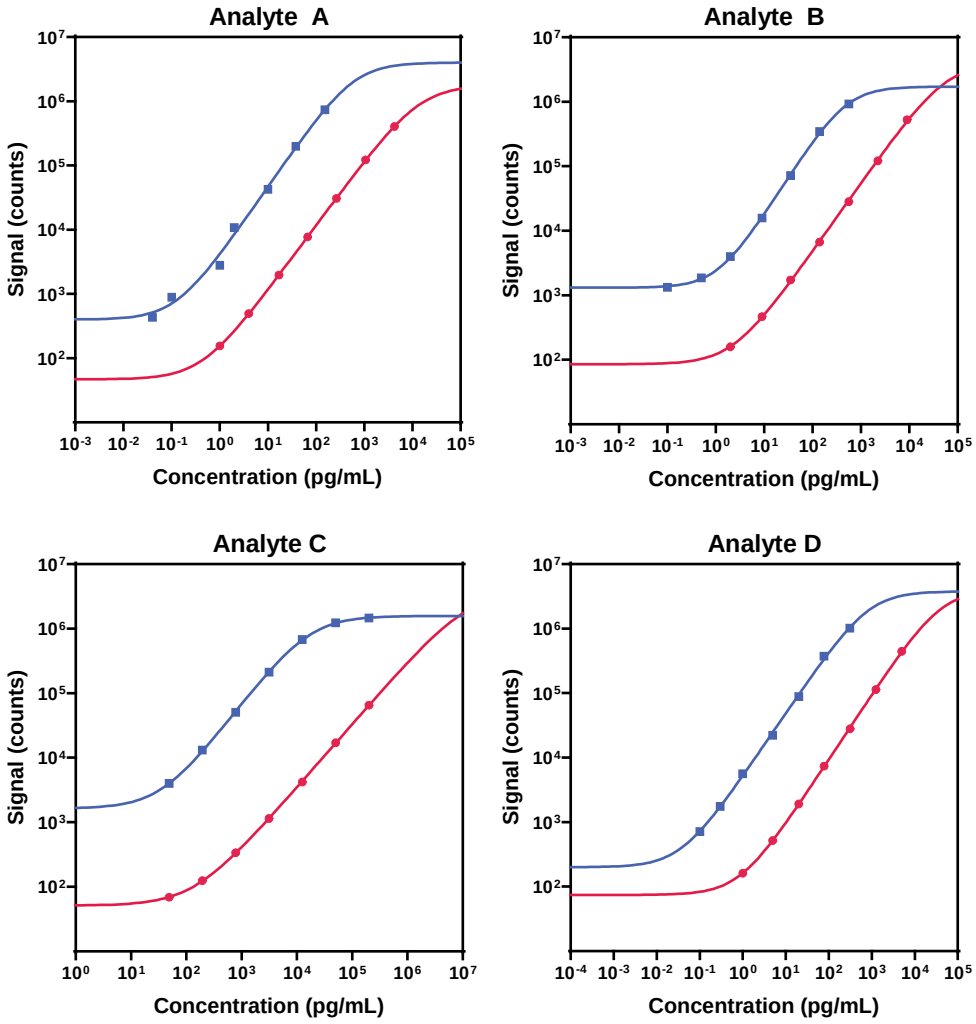


Figure 4: Representative calibration curves for a variety of assays using standard (red line) and Booster Pack A (blue line) protocols.

Sensitivity

For each assay, we measured the lower limit of detection (LLOD), which is the calculated concentration corresponding to the signal 2.5 standard deviations above the background. Increased sensitivity was observed for all four assays tested, ranging from 3- to 21-fold improvement (see table below).

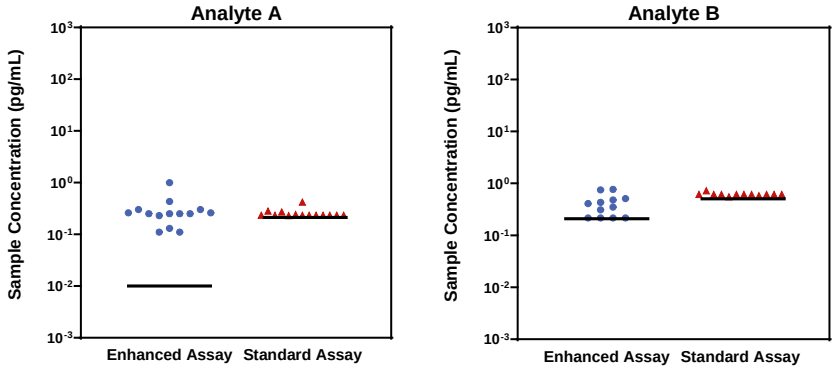
	LLOD (pg/mL)			
Assays	Analyte A	Analyte B	Analyte C	Analyte D
Enhanced Assay	0.011	0.216	4.0	0.02
Standard Assay	0.233	0.615	65	0.25
Sensitivity Fold Improvement	21	3	16	13

Sample Measurement

Five human serum and nine human plasma samples were commercially sourced and tested using both the standard and enhanced assay formats. For analyte A and B assays, many samples were not measurable with the standard assays (Figure 6a). In contrast, most of these samples were well above the LLOD of the enhanced assay, providing more reliable quantitation.

Additionally, in most cases the sample measurements are well correlated between the standard and enhanced formats. Figure 6b shows good correlation for the analyte E assay.

a)



b)

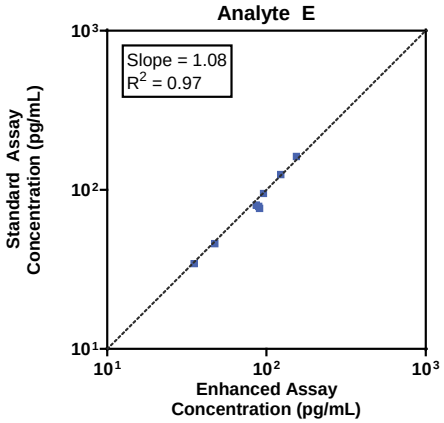


Figure 5. Comparison of native samples measurement between enhanced and standard assays for a) analyte A and B assays. Samples with undetectable levels of analyte are graphed at the LLOD. (b) Sample concordance using serum and plasma is shown for the analyte E assay.

Appendix C: Recommended Plate Washer Parameters

When using an automated plate washer, best results are obtained by using a low dispense flow rate and by positioning dispense tips at the outer edge of the well (e.g., horizontal dispense offset towards the left side of the well). This low flow rate dispense program is recommended for all assay wash steps.

Create a new program for your automated plate washer with the optimal settings before starting your assay. Example wash program settings for a common plate washer (Biotek Model 405 LS) that MSD recommends for use with Booster Pack A and B reagents are shown below (Table 7).

Table 5. Parameters for customized programs on the Biotek 405 LS microplate washer

Wash Program Parameters	Booster Pack Wash Program Settings
Plate type	96
CYCLES	
Wash cycles	3
ASPIRATION	
Aspirate Type	TOP
Travel Rate	1 (4.1% 1.0 mm/second)
Aspirate Delay	0500 milliseconds
Aspirate X-Position	49
Aspirate Y-Position	00
Aspirate Height	24
Secondary Aspirate?	NO
DISPENSE	
Dispense Rate	02
Dispense Volume	0300 µL/well
Vacuum Delay Volume	0010 µL/well
Dispense X-Position	-45 (1.600 mm)
Dispense Y-Position	00 (0.000 mm)
Dispense Height	120 (15.245 mm)
OPTS	
PRE	
Wash Pre dispense?	NO
Bottom Wash?	NO
MIDCYC	
Wash Shake?	NO
Wash Soak?	NO
Home Carrier?	NO
Between Cycle Pre Dispense?	NO
POST	
Final Aspirate?	YES
Aspirate Type	TOP
Travel Rate	1 (4.1% 1.0 mm/second)
Final Aspirate Delay	0500 milliseconds
Final Aspirate X-Position	-49
Final Aspirate Y-Position	0
Final Aspirate Height	24
Secondary Aspirate?	NO
Final Aspirate Secondary X-Position	
Final Aspirate Secondary Y-Position	
Final Aspirate Secondary Height	

Summary Protocol

STEP 1: Coat Plate with Biotin Capture Antibody

- ❑ Add 50 µL of coating solution containing biotinylated capture antibody and BP Reagent A to each well. Tap the plate gently. Seal the plate with an adhesive plate seal.
- ❑ Incubate at room temperature (22–27 °C) with shaking (700 rpm) for 1 hour.

STEP 2: Add Samples and Calibrators

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 25 µL of BP Reagent B working solution to each well. Tap the plate gently.
- ❑ Add 25 µL of calibrator or sample to each well. Seal the plate with an adhesive plate seal.
- ❑ Incubate at room temperature (22–27 °C) with shaking (700 rpm) for 1 hour.

STEP 3: Add SULFO-TAG Detection Antibody Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of SULFO-TAG detection antibody solution to each well. Tap the plate gently. Seal the plate with an adhesive plate seal.
- ❑ Incubate at room temperature (22–27 °C) with shaking (700 rpm) for 30 mins.

STEP 4: Add BP Reagent C Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagent C working solution to each well. Tap the plate gently. Seal the plate with an adhesive plate seal.
- ❑ Incubate at room temperature (22–27 °C) with shaking (700 rpm) for 30 minutes.

STEP 5: Add BP Reagents D, E, and F Mix Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagents D, E, and F mix working solution to each well. Tap the plate gently. Seal the plate with an adhesive plate seal.
- ❑ Incubate at room temperature (22–27 °C) with shaking (700 rpm) for 10 minutes.

STEP 6: Add BP Reagents G and H Mix Working Solution

- ❑ Wash the plate 3 times with at least 150 µL/well of 1X MSD Wash Buffer.
- ❑ Add 50 µL of BP Reagents G and H mix working solution to each well. Tap the plate gently. Seal the plate with an adhesive plate seal.
- ❑ Incubate at 27 °C temperature with shaking (700 rpm) for 1 hour.

STEP 7: Add Read Buffer

- ❑ 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. Read the plate on an MSD instrument. Incubation in MSD GOLD Read Buffer B is not required before reading the plate.

Plate Diagram

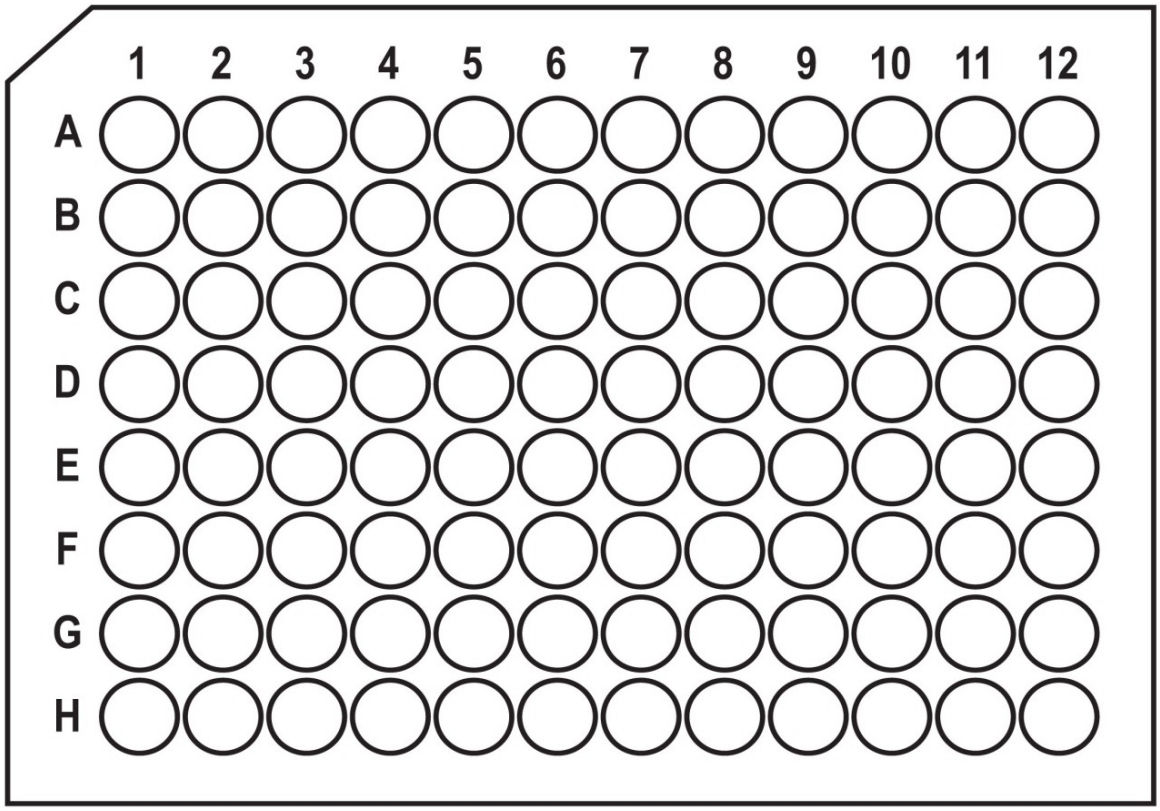


Figure 6. *Plate diagram provided for illustration.*