MSD Application Note

MSD® High Throughput Western Blot Replacement Assays

This document describes how to quantify a protein of interest or a specific post-translational state of a protein, such as phosphorylation, in total cell lysates using Meso Scale Discovery™ technology.

There are two approaches. The first involves direct immobilization of the lysate onto the working electrode at the bottom of MSD MULTI-ARRAY™ plates and detection with either a directly labeled primary or labeled secondary antibody. This method offers a very rapid protocol with minimal manipulations. The sensitivity of the assay will be dependent on the primary antibody used but is typically equal to the sensitivity of conventional western blots.

The second approach uses plates pre-coated with a suitable capture antibody. This approach enables the detection of smaller amounts of the target protein since the capture antibody enriches for the target at the electrode. Also, greater amounts of lysate may be used. Combined, these features afford detection of low abundance proteins and phosphoproteins in cell lysates. The captured target protein is then detected through a directly labeled primary or labeled secondary antibody.

Method 1. Direct Lysate Immobilization

MSD Catalog Items

- MSD MULTI-ARRAY 96-well High Bind Plate
- MSD Read Buffer T (4x)
- MSD Blocker A Kit

> **Note:**
>
> *It is critical that High Bind plates be used. Standard plates have lower binding capacity and require much longer immobilization times.*

R&D Custom Materials

- MSD SULFO-TAG™ labeled secondary antibody

Other Materials (not supplied)

- Primary antibody
- Cell lysate
- Cell lysis buffer containing appropriate protease/phosphatase inhibitors for making further lysate dilutions
- Deionized water for diluting Assay Buffer and Read Buffer
- Automated or manual multi-channel liquid handling equipment. It must accurately deliver 5 µL in XYZ coordinates and dispense 150 µL into a 96-well microplate.

Preparation & General Notes

MSD Blocker A solution

1. Set aside 100-200 mg of dry MSD Blocker A powder.
2. Prepare as directed in included instructions.

Lysate

Prepare 2-5 µL per well:

1. Prepare a stock of fresh cell lysis buffer suitable for diluting the lysate of interest. It may be appropriate to add fresh protease or phosphatase inhibitors.
2. It is recommended that one titrate lysate over a range from 0.1 µg/µL to 2 µg/µL in fresh lysis buffer. (Eight two-fold serial dilutions are recommended.)
3. Keep on ice until ready to dispense.

Detection Antibody solution

Prepare 25 µL per well:

1. Add 1 mg/mL MSD Blocker A to a stock of lysis buffer.
2. It is recommended to titrate the antibodies over a range. Listed below are final concentrations commonly used with tight-binding antibodies.
   - primary antibody 5-40 nM
   - MSD SULFO-TAG labeled secondary Antibody. 5-40 nM
3. If using a secondary antibody, combine both antibodies in lysate dilution buffer with MSD Blocker A added.
4. Keep on ice until ready to dispense.

> **Note:**
>
> *A simpler buffer such as TBS or PBS may be used to dilute antibodies if MSD Blocker A and appropriate protease and/or phosphatase inhibitors are added.*

Read Buffer

Prepare 150 µL per well:

1. Dilute 4X MSD Read Buffer T to 1X with deionized water.
2. Diluted read buffer may be stored at room temperature for later use.

Assay Protocol

This method describes the most conservative approach toward first achieving the best results possible. Once desirable results are achieved, it is possible to develop a more streamlined workflow, omitting the dedicated blocking step or simply adding read buffer and analyzing the plate without washing out the free detection antibody.

Begin with a MSD MULTI-ARRAY High Bind Plate. No pre-treatment is necessary.

1. Carefully deliver 2-5 µL of lysate directly to the center of each working electrode trying to contain the droplet within the dielectric barrier. If the lysate buffer contains detergent, larger volumes will spread over the entire bottom of the well. This is sub-optimal. Choose a volume that covers but can be contained on the working electrode.

   Incubate uncovered for 1 hour at room temperature.

2. Dispense 150 µL/well MSD Blocker A solution.

   Incubate for 1 hour at room temperature.

3. Wash plate 3 times with PBS.
4. Dispense 25 µL/well antibody solution.

   Incubate for 1-4 hours shaking at room temperature.

5. Wash plate 3 times with PBS.
6. Dispense 150 µL/well of diluted MSD Read Buffer T.
7. Analyze with a SECTOR™ Imager or a SECTOR PR™ Reader.

   Note: Plate types are not interchangeable between readers).

> **Note:**
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> *Add-only, no-wash assays have been developed after optimization. In the add-only protocol, keep the volumes of the lysate (2-5µL) and antibodies(20-50µL) low to speed formation of the complex at the electrode. Raise the volume to 150µL with the appropriate dilution of Read Buffer just before analysis.*
>
> *The goal of step 1 is to passively immobilize the lysate on the working electrode of the MULTI-ARRAY plate. The capacity of the electrode in a large spot 96 well plate is 1-2 µg of total protein. MSD MULTI-ARRAY plates are compatible with most lysis buffers. A variety of buffers have been tested.*
>
> *Unlike conventional westernblots, this method does not denature proteases and phosphatases as in SDS-PAGE. Thus, extra effort should be made to inhibit these enzymes. We recommend the use of cocktails of protease inhibitors (e.g. Roche, cat. nos. 1-873-580 or 1-836-580) and phosphatase inhibitors (e.g. Sigma cat. nos. P-2850 and/or P-5726) in addition to the commonly used NaFl and sodium orthovanadate.*
>
> *The binding of certain phospho-specific antibodies is inhibited by PBS. TBS or a Hepes-buffered solution can be substituted.*
>
> *The primary antibody chosen for detection may be one that detects the entire pool of the target protein (pan) or it may detect a specific post-translation state of a protein. Anti-phospho- and cleavage-specific antibodies are examples of the latter. Users seldom know the K_d of the antibody for the substrate. Thus, part of assay optimization is to examine the specific signal obtained as a function of various concentrations of primary antibody. If a secondary antibody is employed, keep the*
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Method 2. Capture Antibody Pre-coated Plate

MSD Catalog Items

- MSD Read Buffer T (4x)
- MSD Blocker A Kit

R&D Custom Materials

- Custom MSD MULTI-ARRAY 96-well plate coated with capture antibody
- MSD SULFO TAG labeled secondary antibody

Other Materials (not supplied)

- Primary antibody
- Cell lysate
- Cell lysis buffer containing appropriate protease/phosphatase inhibitors for making further lysate dilutions
- Deionized water for diluting Assay Buffer and Read Buffer.
- Automated or manual multi-channel liquid handling equipment. It must accurately deliver 5 µL in XYZ coordinates and dispense 150 mL into a 96-well micro plate.

Preparation & General Notes

MSD Blocker A solution

1. Set aside 100-200 mg of dry MSD Blocker A powder.
2. Prepare as directed in included instructions

Lysate

Prepare 25 µL per well:

1. Prepare a stock of fresh cell lysis buffer suitable for diluting the lysate of interest. It may be appropriate to add fresh protease or phosphatase inhibitors.
2. It is recommended to titrate the lysate over a range from 0.005 µg/µL to 1.6 µg/µL in fresh lysis buffer. (Ten, two-fold serial dilutions work well).
3. Keep on ice until ready to dispense.

Detection Antibody solution

Prepare 25 µL per well:

1. Add 1 mg/mL MSD Blocker A to a stock of lysis buffer.
2. It is recommended to titrate the antibodies over a range. Listed below are final concentrations commonly used with tight-binding antibodies.
   - primary antibody 5-40 nM
   - MSD SULFO-TAG labeled secondary Antibody. 5-40 nM
3. If using a secondary antibody, combine both antibodies in lysate dilution buffer with MSD Blocker A added.
4. Keep on ice until ready to dispense.

Read Buffer

Prepare 150 µL per well:

1. Dilute 4X MSD Read Buffer T to 1X with deionized water.
2. Diluted read buffer may be stored at room temperature for later use.

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molar ratio of the two antibodies as close to 1:1 as possible. During development of the assay, one may wish to wash the plate between the addition of the primary and secondary antibody with at least one-hour incubations for each. Once the ideal primary antibody concentration has been established, the primary and secondary antibodies can be introduced simultaneously at the 1:1 ratio.

The primary antibody can be directed at a post-translational modification shared by many proteins in the cell. For example, an antibody against ubiquinylated proteins that does not recognize free ubiquitin has been used to quantify total ubiquinylation in cell lysates.

Note:

MSD provides a variety of plates that have capture antibodies immobilized on the working electrode. The cognate target proteins of these plates are common in drug discovery efforts and biomedical research including cytokines, kinases and their substrates, and proteins involved in apoptosis.

MSD will also prepare pre-coated plates on a custom order basis.

Users may wish to coat their own plates, especially for low volume applications and in 96-well formats where coating of the electrode is less challenging. See separate literature for passive immobilization of antibodies and purified proteins in MULTI-ARRAY plates.

Note:

When immobilizing a target protein through a capture antibody already coated on a MULTI-ARRAY plate, the addition of carrier protein (i.e. BSA, IgG, gelatin, etc) to the lysate dilution can enhance the performance of the assay by keeping the lysate from sticking to side walls and the counter electrode in the well. MSD MULTI-ARRAY plates are compatible with most lysis buffers. A wide variety of buffers have been tested.

A simpler buffer such as TBS or PBS may be used to dilute antibodies if MSD Blocker A and appropriate protease and/or phosphatase inhibitors are added.

Assay Protocol

This method describes the most conservative approach toward first achieving the best results possible. Once good results are achieved it is possible to more toward a more streamlined workflow, omitting the dedicated blocking step or simply adding read buffer and analyzing the plate without washing out the free detection antibody.

Begin with a Custom MSD MULTI-ARRAY plate pre-coated with capture antibody. No pre-treatment is necessary.

1. Dispense 150 µL/well MSD Blocker A solution.
   Incubate for 1 hour at room temperature.
2. Wash plate 3 times with PBS.
3. Dispense 25 µL/well lysate.
   Incubate for 1 hour shaking at room temperature.
4. Wash plate 3 times with PBS.
5. Dispense 25 µL/well antibody solution.
   Incubate for 1-4 hours shaking at room temperature.
6. Wash plate 3 times with PBS.
7. Dispense 150 µL/well of diluted MSD Read Buffer T.
8. Analyze with a SECTOR Imager or a SECTOR PR Reader.
   Note: Plate types are not interchangeable between readers.

Note:

Unlike conventional westernblots, this method does not denature proteases and phosphatases as in SDS-PAGE. Thus, extra effort should be made to inhibit these enzymes. We recommend the use of cocktails of protease inhibitors (e.g. Roche, cat. nos. 1-873-580 or 1-836-580) and phosphatase inhibitors (e.g. Sigma cat. nos. P-2850 and/or P-5726) in addition to the commonly used NaFl and sodium orthovanadate.

The binding of certain phospho-specific antibodies is inhibited by PBS. TBS or a Hepes-buffered solution can be substituted.

See the notes on detection antibody usage on page 3.

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