MSD® Phosphoprotein Assay Development: Materials List and Preparation Guide

Material	Vendor	Product	Catalog Number
Blocker A	SeraCare Life Sciences, Inc.	Bovine Serum Albumin, Reagent Grade Pure Powder	AP4510
Blocker B	Amersham Biosciences	Membrane Blocking Agent	RPN2125
Blocker D-M	Rockland	Mouse Gamma Globulin	D609-0100
Blocker D-R	Rockland	Rabbit Gamma Globulin	D610-1000
Blocker D-G	Equitech-Bio, Inc.	Goat IgG	GGG-0010
Blocker D-B	Rockland	Bovine Gamma Globulin	D600-0100
Phosphatase Inhibitor I	Sigma-Aldrich, Inc.	Phosphatase Inhibitor I	P-2850
Phosphatase Inhibitor II	Sigma-Aldrich, Inc.	Phosphatase Inhibitor II	P-5726
Protease Inhibitor Solution	Roche Applied Science	Protease Inhibitor Tablets	1873580 1836170 (mini)
Spin Columns	Roche Applied Science	Quick Spin™ High Capacity Columns	100965

Buffer Preparation Instructions

Tris Wash Buffer (10X)	500 mM Tris, pH 7.5 1.5 M NaCl 0.2% Tween-20 Store at 4ºC.
Tris Lysis Buffer (1X, Incomplete)	150 mM NaCl 20 mM Tris, pH 7.5 1 mM EDTA 1 mM EGTA 1% Triton-X-100 Store at 4ºC.
Complete Lysis Buffer	10 mL 1X Tris Lysis Buffer 1 Protease Inhibitor Tablet * (Complete Mini Protease Inhibitor Tablet, Roche # 1836170) 100 µL Phosphatase Inhibitor I 100 µL Phosphatase Inhibitor II 100 µL 1 M NaF * *In the reagents supplied by MSD, the protease inhibitor tablet and the NaF have been combined into a 50X protease inhibitor solution.

Buffer Preparation Instructions (continued)

p38 Incomplete Dilution Buffer (1X) **(also p38 Lysis Buffer, Incomplete)**	50 mM NaCl 20 mM Tris, pH 7.5 1 mM EDTA 1 mM EGTA 1% Triton X-100

Reagent Preparation Instructions

Blocker A	*General Notes:* Blocker A is added to Tris Wash Buffer (1X) to prepare Blocking Solution A and Antibody Dilution Buffer. Please see individual assay inserts for specific preparation instructions.
Blocker B	*General Notes:* Blocker B solution is required for the dilution of cell lysates only for certain targets. Please see individual assay inserts for specific preparation instructions.
Blocker D-M	*General Notes:* Store Blocker D-M for up to 1 year at -20ºC, or up to 30 days at 4ºC. The MSD working assay concentration is 0.1%. *Reagents Required:* Sterile H_20 + 0.05% Sodium Azide *Protocol:* Reconstitute Mouse Gamma Globulin (Rockland D609-0100) by adding 5 mL H_20 + 0.05% Sodium Azide to one 0.1 g vial to prepare a 2% stock solution. Aliquot and store at -20ºC or 4ºC.
Blocker D-R	*General Notes:* Store Blocker D-R for up to 1 year at -20ºC, or up to 30 days at 4ºC. The MSD working assay concentration is 0.1%. *Reagents Required:* Sterile H_20 + 0.05% Sodium Azide *Protocol:* Reconstitute Rabbit Gamma Globulin (Rockland D610-1000) by adding 10 mL H_20 + 0.05% Sodium Azide to one 1.0 g vial to prepare a 10% stock solution. Aliquot and store at -20ºC or 4ºC.

Reagent Preparation Instructions (continued)

Blocker D-B	*General Notes:* Store Blocker D-B for up to 1 year at -20ºC, or up to 30 days at 4ºC. The MSD working assay concentration is 0.7% for MSD Mouse Cytokine Assays, and 0.1% for MSD Alzheimer's Disease Assays. Preparation for MSD Alzheimer's Disease Assay Reagent is described below. *Reagents Required:* Sterile H_20 + 0.05% Sodium Azide *Protocol:* Reconstitute Bovine Gamma Globulin (Rockland D600-0100) by adding 4 mL H_20 + 0.05% Sodium Azide to one 0.1 g vial to prepare a 2.5% stock solution. Aliquot and store at -20ºC or 4ºC.
Blocker D-G	*General Notes:* Store Blocker D-G for up to 1 year at -20ºC, or up to 30 days at 4ºC. The MSD working assay concentration is 0.1%. *Reagents Required:* Sterile H_20 + 0.05% Sodium Azide *Protocol:* Reconstitute Goat IgG (Equitech GGG-0010) by adding 18 mL H_20 + 0.05% Sodium Azide to 2 g to prepare a 10% stock solution. Aliquot and store at -20ºC or 4ºC.
Protease Inhibitor Solution	*General Notes:* Store Protease Inhibitor Solution for up to 4 months at -20ºC. The MSD working assay concentration is 50X. *Reagents Required:* Sterile H_20, 1 M Sodium Fluoride (NaF), Protease Inhibitor Solution Tablets - EDTA free *Protocol for 1M NaF:* Add 8.4 g NaF (Sigma S-7920) to 200 mL sterile H_20. Aliquot (10 mL volumes) and freeze at -20ºC. *Protocol for Protease Inhibitor Solution:* Add 10 Protease Inhibitor Solution Tablets (Roche 1873580) to 10 mL sterile H_20 and 10 mL 1 M NaF. Mix, aliquot, and store at -20ºC.

Whole Cell Lysate Preparation Instructions

General notes:

All manipulations should be performed on ice. Prepare desired amount of Complete Lysis Buffer immediately prior to use. Lysis volumes will vary between cell types. Larger cells (such as NIH3T3, HeLa) should be lysed at concentrations of 1-5 x 10^6 cells per mL of Lysis Buffer. Smaller cells (such as Jurkat) should be lysed at concentrations of 1-5 x 10^7 cells per mL of Lysis Buffer.

Reagents required:

Tris Lysis Buffer (1X, Incomplete)	150 mM NaCl 20 mM Tris, pH 7.5 1 mM EDTA 1 mM EGTA 1% Triton-X-100
Complete Lysis Buffer	10 mL 1X Tris Lysis Buffer 1 Protease Inhibitor Tablet * 100 µL Phosphatase Inhibitor I 100 µL Phosphatase Inhibitor II 1% Triton-X-100 100 µL 1 M NaF* *In the reagents supplied by MSD, the protease inhibitor tablet and the NaF have been combined into a 50X protease inhibitor solution. ***NOTE:*** *For assays that require PMSF, a final concentration of 2 mM in Complete Lysis Buffer is recommended.* For 10 mL Complete Lysis Buffer, add 40 µL PMSF (from 500 mM stock prepared in DMSO) (Sigma, Catalog # P-7626). When adding PMSF to the buffer, all reagents should be at room temperature prior to mixing. The Complete Lysis Buffer should be mixed at room temperature on a rotator for 5 minutes (with no obvious precipitates), and should then be thoroughly chilled prior to use for lysate dilution or preparation.
When analyzing cells for p38 alone, the following Lysis Buffer affords optimal performance:	
p38 Lysis Buffer (1X)	50 mM NaCl 20 mM Tris, pH 7.5 1 mM EDTA 1 mM EGTA 1% Triton X-100 Prepare Complete p38 Lysis Buffer as shown above, substituting the 10 mL of Tris Lysis Buffer (1X) with p38 Lysis Buffer (1X).

Whole Cell Lysate Preparation Instructions (continued)

Protocol:

Cells should be prepared as desired to activate target protein.

Suspension cells	Pellet cells (500 x g, 3 minutes at 4ºC) and wash one time with cold 1X PBS. Pellet cells again and resuspend in 1X Complete Lysis Buffer at 1-5 x 10^7 cells per mL. Incubate on ice for 30 minutes (a shorter incubation time of 15 minutes may be adequate for many targets). Centrifuge lysates at greater than or equal to 10,000 x g, 4ºC for 10 minutes to clear cellular debris from the lysate. Lysates can be quantitated using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and snap frozen and stored at -80ºC.
Adherent cells	All volumes are determined for cells plated in 15 cm dishes. Remove media from the plates and wash cells one time with 5 mL cold 1 X PBS. Add 2 mL 1 X PBS to the plates and scrape the cells from the surface of the dish and transfer into 15 mL conical tubes. Pellet the cells at 500 x g for 3 minutes at 4ºC. Resuspend the cells in 0.5-2 mL of Complete Lysis Buffer per dish. Alternatively after medium removal, cells can be washed one time with PBS including a careful aspiration of residual PBS and lysed directly on the dish by adding 1-2 mL (depending on cell type) of Complete Lysis Buffer per dish. Incubate on ice for 30 minutes (a shorter incubation time of 15 minutes may be adequate for many targets). Centrifuge lysates at greater than or equal to 10,000 x g, 4ºC for 10 minutes to clear cellular debris from the lysate. Lysates can be quantitated using a detergent compatible protein assay such as BCA. Unused lysates should be aliquoted and snap frozen and stored at -80ºC.

96 well format modifications	Successful adaptation to a 96 well format is cell type and target-dependent. The number of cells to be plated per well should be determined per cell type. General recommended plating concentrations for adherent cells range from 1 x 10^4-5 x 10^4 cells per well, and approximately 2 x 10^6 cells per mL (50-75 µL per well) for suspension cells. These numbers are provided as a guide and the optimal concentrations will vary depending upon cell line used.
Suspension cells	For flat bottom plates, experiments should be designed such that the final volume per well is 50-75 µL. Perform cell lysis using a 4X Complete Lysis Buffer concentrate, supplemented with protease and phosphatase inhibitors at 4X concentrations. Add 4X Complete Lysis Buffer directly to cells in the growth medium for a final 1X concentration in the well. NOTE: With some effort, a 10X Complete Lysis Buffer can also be prepared. *(For conical microwell plates, perform lysis by pelleting the cells, removing most of the growth medium and adding a constant amount of 1X Complete Lysis Buffer.)*
Adherent cells	Plate cells on biologically treated tissue culture ware (such as BIOCOAT™) to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from microwell plate. A PBS wash step is not required and can introduce variability. Add 50-100 µL 1X Complete Lysis Buffer per well. Cell lysis time should be determined by the end user. Some targets are immediately available for detection, while others may require an incubation step at room temperature, 4ºC, or on ice with gentle agitation. Carefully pipet 25-90 µL cell lysate onto prepared capture plate. It is important to transfer a constant volume and avoid pipeting too vigorously, as the introduction of air bubbles may result.