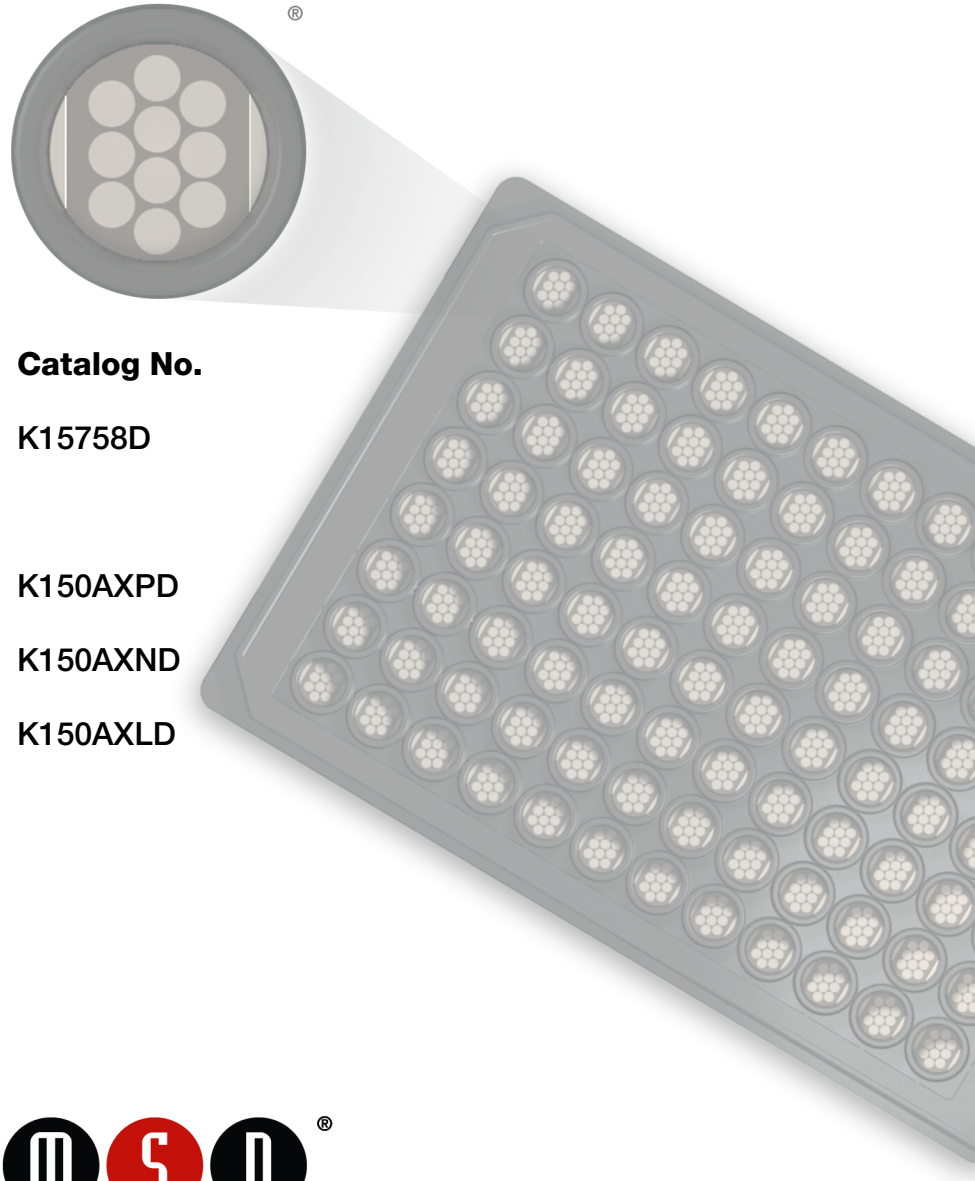
MSD® MULTI-SPOT Assay System

Phospho-STAT Kits

Phospho-STAT3 (Tyr705), Phospho-STAT4 (Tyr693), Phospho-STAT5a,b (Tyr694)

T-PLEX®



Multiplex Kit	Catalog No.
Phospho-STAT Panel	K15758D
Singleplex Kit	
Phospho-STAT3 (Tyr705) v2	K150AXPD
Phospho-STAT4 (Tyr693) v2	K150AXND
Phospho-STAT5a,b (Tyr694) v2	K150AXLD

MSD Cell Signaling Pathway Assays

Phospho-STAT Kits

For use with human lysates.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Introduction

This product insert describes the T-PLEX® Phospho-STAT Kits, lists the components, and provides instructions for use.

STAT Family

The **Signal Transducer and Activator of Transcription (STAT)** family consists of cytoplasmic transcription factors that are part of the JAK/STAT signal transduction pathway, and are activated by cytokines, growth factors, and hormones. When inactive, STATs are cytoplasmic. Upon ligand binding and activation of a cytokine receptor, the receptor binds to a member of the JAK family. The receptor is phosphorylated and recruits a member of the STAT family that is then phosphorylated. It then dimerizes and is transported into the nucleus, where it can act as a transcription factor.[1] There are about 38 cytokines that signal through their cytokine receptors and through the JAK/STAT pathway to cause downstream transcriptional effects. These effects have been reported to be cell-type specific and JAK/STAT specific.[2]

The T-PLEX Phospho-STAT Kits measure the levels of phosphorylated STAT3, STAT4, and STAT 5a and 5b.

STAT3

Phosphorylation of STAT3 on tyrosine 705 results in its activation and subsequent dimerization, nuclear translocation, and DNA binding.[3,4] In response to cellular stimulation by cytokines, STAT3 phosphorylation is mediated through the JAK family of receptor-associated tyrosine kinases, most notably JAK1.[3] Growth factor receptors with intrinsic tyrosine kinase activities may phosphorylate STAT3 directly, and the non-receptor tyrosine kinase SRC phosphorylates STAT3 as well.[4,5] Activated STAT3 plays a critical role in cellular processes including proliferation, tissue-dependent cell survival of apoptosis, and embryonic development and organogenesis.[3,6] Constitutively activated STAT3 has been observed in skin, prostate, lung, and breast cancers.[7]

STAT4

STAT4 is a 748 amino acid member of the STAT family expressed in myeloid cells, thymus, and testis. STAT4 has multiple functional domains, including an N-terminal interaction domain, a central DNA-binding domain, an SH2 domain, and the C-terminal transactivation domain. Activation of STAT4 is initiated by IL-12–mediated signaling. IL-12, a cytokine secreted by antigen-presenting cells, binds to transmembrane receptors, resulting in receptor heterodimerization and activation of the Janus kinases JAK2 and TYK2.[8,9] These tyrosine kinases activate STAT4 by phosphorylation of tyrosine 693; active STAT4 homodimerizes through the SH2 domain and translocates into the nucleus to activate gene transcription of cytokines as well as cytokine receptors and other proteins.[10] Haplotypes for STAT4 are associated with increased risk for rheumatoid arthritis and systemic lupus erythematosus.[11,12]

STAT 5a and 5b

STAT 5a and 5b are members of the STAT family of transcription factors and approximately 90% identical at the amino acid level. STAT5s respond to cellular stimulation by IL-2, IL-3, IL-5, IL-7, GM-CSF, IFN-γ, insulin, erythropoietin, growth hormone, and prolactin.[13] STAT5a and 5b are involved in different types of cancer (including breast and prostate), inflammatory responses, and allergic reactions.[14] STAT5s also prevent apoptosis in certain cell types and diseases based on their role as transcription factors and the genes they regulate.[15] Due to the transcriptional control provided by the STAT family of proteins, their role in intracellular signaling, and their significance in diseases such as cancer and inflammation, this important signaling pathway is the subject of a number of basic research and pharmacological studies.

Principle of the Assay

MESO SCALE DISCOVERY® (MSD) assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the T-PLEX Phospho-STAT Kits are sandwich immunoassays (Figure 1). MSD provides a plate pre-coated with capture antibodies on independent defined spots in the layout shown below. Add the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. Add an MSD read buffer that creates the appropriate chemical environment for electrochemiluminescence and load the plate 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.

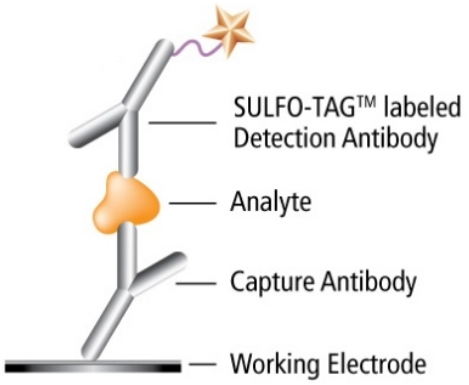

Figure 1. Principle of the T-PLEX sandwich immunoassays assay.

The T-PLEX Phospho-STAT Kits

MSD Phospho-STAT Kits antibodies are located in defined spots in each well, as shown in the layout below (Figure 2 for the Phospho-STAT Panel and Figure 3 for the singleplex plates). This assay is provided with 10-spot MULTI-SPOT® plates.

Spot	Analyte
1	Phospho-STAT3 (Tyr705)
3	Phospho-STAT4 (Tyr693)
10	Phospho-STAT5a,b (Tyr694)

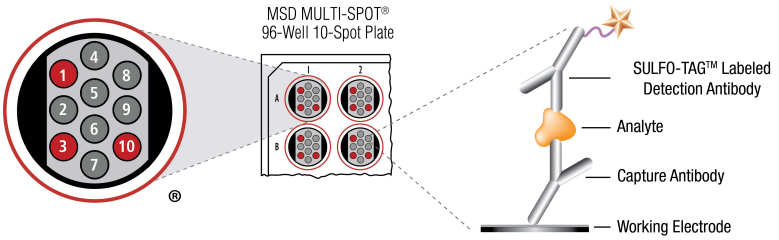

Figure 2. *Multiplex plate spot diagram showing spot locations for the Phospho-STAT Panel. Assay spots are colored red, and unused spots colored grey. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

Spot	Analyte
1	Phospho-STAT3 (Tyr705), Phospho-STAT4 (Tyr693), or Phospho-STAT5a,b (Tyr694)

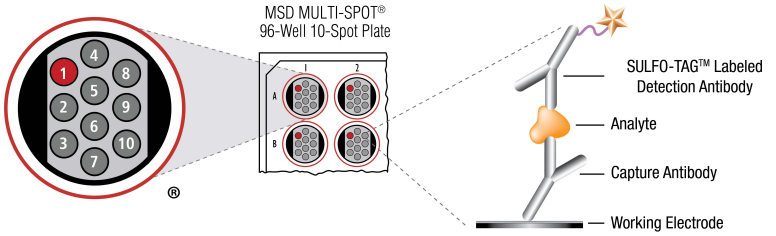

Figure 3. *Plate spot diagram showing the spot location for the singleplex plates. Singleplex kits are provided on 10-spot plates with analyte capture antibody coated on spot 1 (colored red). Unused spots are colored grey. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

Standard Workflow for T-PLEX Phospho-STAT Kits

An overview of the steps in the T-PLEX Phospho-STAT Kits workflow with incubation durations is outlined below. This is an overview for planning purposes; for the full workflow, see *Protocol* on page 10.

Step	Incubation
Add Blocker A solution	1 hr.
Add sample	1 hr.
Add detection antibody solution	1 hr.
Add read buffer	No incubation
Read plate	Read time is dependent on instrument model

Materials and Equipment

This section lists the components provided with the T-PLEX Phospho-STAT Kits.

Reagents Supplied With All Kits

Table 1. Reagents that are supplied with all T-PLEX Phospho-STAT Kits

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Tris Lysis Buffer (1X)	2–8 °C	R60TX-3	50 mL	1 bottle	1 bottle	5 bottles	Tris-based lysis buffer for preparing lysates and diluting samples.
Tris Wash Buffer (10X)	2–8 °C	R61TX-2	200 mL	1 bottle	1 bottle	5 bottles	10X Tris buffered solution with surfactant for washing plates.
Phosphatase Inhibitor I (100X)	2–8 °C	—	0.1 mL	1 vial	—	—	Cocktail of serine/threonine protein phosphatase inhibitors
			0.5 mL	—	1 vial	5 vials	
Phosphatase Inhibitor II (100X)	2–8 °C	—	0.1 mL	1 vial	—	—	Cocktail of tyrosine protein phosphatase inhibitors
			0.5 mL	—	1 vial	5 vials	
Protease Inhibitor Solution (100X)	2–8 °C	—	0.1 mL	1 vial	—	—	Cocktail of protease inhibitors
			0.5 mL	—	1 vial	5 vials	
Blocker A (dry powder)	RT	R93BA-4	15 g	1 bottle	1 bottle	1 bottle	Bovine serum albumin, reagent grade pure powder
MSD GOLD™ Read Buffer B	RT	R60AM-1	18 mL	1 bottle	—	—	MSD buffer to catalyze the electrochemiluminescence reaction
		R60AM-2	90 mL	—	1 bottle	5 bottles	

RT = room temperature
dash (—) = not applicable

Components Specific to Each Kit

Phospho-STAT 3

Table 2. Reagents that are supplied with the Phospho-STAT 3 Kit

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Phospho STAT3 (Tyr705) Gen. B Antibody (50X)	2–8 °C	D20AXP-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXP-3	375 µL	—	1	5	
STAT3 Gen. B 96-Well 10-Spot SECTOR™ Plate	2–8 °C	N050AXMA-1	1 plate	1	5	25	96-well plate, foil sealed, with desiccant

dash (—) = not applicable

Phospho-STAT 4

Table 3. Reagents that are supplied with the Phospho-STAT 4 Kit

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Total STAT4 Gen. B Antibody (50X)	2–8 °C	D20AXQ-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXQ-3	375 µL	—	1	5	
Phospho-STAT4 (Tyr693) Gen. B 96-Well 10-Spot SECTOR Plate	2–8 °C	N050AXNA-1	1 plate	1	5	25	96-well plate, foil sealed, with desiccant
Blocker D-M (2%)	≤-10 °C	R93BM-1	0.2 mL	1 vial	—	—	Reduces non-specific binding
		R93BM-3	1.8 mL	—	1 vial	5 vials	
Blocker D-R (10%)	≤-10 °C	R93BR-2	0.2 mL	1 vial	—	—	
		R93BR-3	1.0 mL	—	1 vial	5 vials	

dash (—) = not applicable

Phospho-STAT 5a,b

Table 4. Reagents that are supplied with the Phospho-STAT 5a,b Kit

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Phospho STAT5 (Tyr694) Gen. B Antibody (50X)	2–8 °C	D20AXR-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXR-3	375 µL	—	1	5	
STAT5a,b Gen. B 96-Well 10-Spot SECTOR Plate	2–8 °C	N050AXLA-1	1 plate	1	5	25	96-well plate, foil sealed, with desiccant

dash (—) = not applicable

Phospho-STAT Panel

Table 5. Reagents that are supplied with the Phospho-STAT Panel Kit

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1-Plate Kit	5-Plate Kit	25-Plate Kit	
Phospho STAT3 (Tyr705) Antibody (50X)	2–8 °C	D20AXP-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXP-3	375 µL	—	1	5	
Total STAT4 Antibody (50X)	2–8 °C	D20AXQ-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXQ-3	375 µL	—	1	5	
Phospho STAT5 (Tyr694) Antibody (50X)	2–8 °C	D20AXR-2	75 µL	1	—	—	SULFO-TAG conjugated antibody
		D20AXR-3	375 µL	—	1	5	
Phospho-STAT Panel Plate, Gen. B	2–8 °C	N05758A-1	1 plate	1	5	25	96-well plate, foil sealed, with desiccant
Blocker D-M (2%)	≤-10 °C	R93BM-1	0.2 mL	1 vial	—	—	Reduces non-specific binding
		R93BM-3	1.8 mL	—	1 vial	5 vials	
Blocker D-R (10%)	≤-10 °C	R93BR-2	0.2 mL	1 vial	—	—	
		R93BR-3	1.0 mL	—	1 vial	5 vials	

dash (—) = not applicable

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

Optional Materials

- STAT Panel Whole Cell Lysate Set is available for separate purchase from MSD, catalog # C1202-1. The set contains cell lysates from human T-cells that were starved for 30 minutes and then either left untreated (negative control) or treated for 30 minutes with both IL-12 (10 ng/mL) and IFN-α (1000 U/mL) to stimulate STAT3, STAT4, and STAT5a,b phosphorylation (positive control).
- JAK-STAT Whole Cell Lysate Set is available for separate purchase from MSD, catalog # C10KQ-1. The set contains cell lysates from HeLa cells that were either left untreated (negative control) or treated with sodium vanadate (1mM) for 4 hours followed by treatment with Oncostatin M (40 ng/mL) to stimulate STAT3 and STAT5a,b phosphorylation (positive control).

Safety

Use safe laboratory practices. Wear appropriate personal protective equipment, including 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.

Protocol

Summary Protocol

This summary protocol is for experienced users familiar with the assay only. For the detailed protocol, see pages 13–16.

Sample and Reagent Preparation

- ❑ Prepare Tris Wash Buffer.
- ❑ Prepare Blocker A solution.
- ❑ Prepare antibody dilution buffer.
- ❑ Prepare complete lysis buffer.
- ❑ Dilute samples to 6.25–800 µg/mL in complete lysis buffer.
- ❑ Prepare the detection antibody solution by diluting the stock detection antibody 50-fold in antibody dilution buffer.

STEP 1: Add Blocker A Solution

❑ 1. Add 150 µL of Blocker A solution to each well.
Incubate 1 hour at room temperature with shaking.

STEP 2: Wash and Add Sample

❑ 2. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 3. Add 25 µL of sample (controls or unknowns) to each well.
Incubate 1 hour at room temperature with shaking.

STEP 3: Wash and Add Detection Antibody Solution

❑ 4. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 5. Add 25 µL of 1X Detection Antibody Solution to each well.
Incubate 1 hour at room temperature with shaking.

STEP 4: Wash and Read

❑ 6. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 7. Add 150 µL of MSD GOLD Read Buffer B to each well.

❑ 8. Read the plate on an MSD instrument immediately.

*If you are using an automatic washer, use 450 µL/well for the wash steps. See *Appendix A: Recommended Plate Washer Parameters* on page 18

Best Practices

Read this product insert in its entirety before use. In addition, adhere to the following best practices:

Reagent Preparation

Do Not Mix Lots	Mixing or substituting reagents from different sources or different kit lots is not recommended.
Complete Lysis Buffer on Ice	Complete lysis buffer should be kept on ice during the experiment.

Samples Handling

Samples and Standards Dilution	Samples and standards cannot be serially diluted in the MSD plate. Use microcentrifuge tubes or a separate 96-well polypropylene plate to prepare dilutions.
Additional Inhibitors	Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent.
Cell LysateThawing	Due to the unstable nature of phosphoproteins, lysates should be thawed immediately prior to use on ice. Any remaining thawed material could be flash-frozen, but that might affect assay performance. We recommend to discard all thawed unused material.
Keep on Ice	Keep diluted samples on ice until use.
Quantifying Protein Concentration	We recommend quantifying the total protein concentration in a lysate detergent-compatible protein assay such as BCA to normalize assay results using predetermined sample concentrations.
Sample Matrix Compatibility	MSD plates are compatible with most sample matrices. Avoid reagents that will denature the capture antibodies. For example, avoid high concentrations of reducing agents in the lysis buffer, such as DTT. SDS and other ionic detergents should be 0.1% or less in the sample applied to the well. When using a new sample collection method or an alternative lysis buffer, confirm matrix compatibility with MSD plates and reagents.
Purified Protein	If working with purified protein, only a few nanograms per well will generally provide a strong assay signal. Purified recombinant proteins may exhibit differences in both signal and background as compared to native proteins in lysates.
Avoid Phosphate Buffers	All buffers containing phosphate should be avoided when detecting phosphoproteins.

Reagent Handling

Prepare in Polypropylene Tubes	Prepare controls and samples in polypropylene microcentrifuge tubes. Use a fresh pipette tip for each dilution and mix by vortexing after each dilution.
Protect Reagents from Light	Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be protected from light except for direct sunlight.
MSD Blocker A Storage	Solutions containing MSD Blocker A should be stored at 2–8 °C and discarded after 14 days.
Inhibitor Cocktails	Thaw or equilibrate inhibitor cocktails to room temperature, then vortex the vials before use to ensure a uniform suspension. Add inhibitors immediately to complete Lysis Buffer; do not keep at room temperature for extended periods.
Avoid Bubbles During Pipetting	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	Use reverse pipetting when necessary to avoid the introduction of bubbles. If pipetting into an empty well, pipette gently to the bottom corner. Do not touch the bottom of the wells with the pipette tip when pipetting into the MSD plate.

Plate Handling

Protect Plate from Sunlight	Protect plates from direct sunlight.
Plate Shaking Guidelines	Plate shaking should use a rotary motion between 500–1,000 rpm.
Rotate Plate	When using an automated plate washer, rotating the plate 180 degrees between wash steps may improve assay precision.
Incubation Temperature	Assay incubation steps should be performed between 20–26 °C to achieve the most consistent signals between runs.
Tap Plate	Tap the plate on a paper towel to remove residual fluid after washing. Make sure the plate does not dry out after the washing steps.
Incubation Extension	If an incubation step needs to be extended, leave the sample or detection antibody solution in the plate to keep the plate from drying out.
Partial Plate	When running partial plates, use the sector map in the instrument or software manual to select the wells to be used. Seal the unused portion of the plate with a plate seal to avoid contaminating unused wells. You may adjust volumes proportionally when preparing detection antibody solution. After reading a partial plate, remove fluid, reseal unused sectors, return plate to its original foil pouch with desiccant pack, and seal pouch with tape. Partially used plates may be stored for up to 14 days at 2–8 °C.
No-cross-aspiration Protocol	Low humidity might affect assay performance, we recommend a no-cross-aspiration protocol on the automated washer.

Plate Reading

Remove Plate Seal	Remove the plate seal before reading the plate.
Read Buffer at Room Temperature	Ensure that the read buffer is at room temperature (20–26 °C) before adding to the plate.
Do Not Shake Plate	Do not shake the plate after adding read buffer.
Time Intervals	Keep time intervals consistent between adding the read buffer and reading the plate to improve inter-plate precision. Prepare the MSD instrument to read a plate before adding read buffer. Unless otherwise directed, read the plate as soon as possible after adding the read buffer.

Sample Collection and Handling

Below are general guidelines for lysate sample handling. If possible, use published guidelines.[16,17] Evaluate sample stability under the selected method as needed.

- Keep lysates on ice.
- Flash-freeze lysates if not using immediately. Unused lysates should be aliquoted and quickly frozen in a dry ice-ethanol bath.
- Store at ≤-70 °C.
- After thawing, centrifuge samples at 2,000 × *g* for 3 minutes at 4 °C to remove particulates before sample preparation.
- Avoid multiple freeze-thaw cycles.
- We recommend to normalize samples by protein concentration.
- When using a new sample collection method or an alternative lysis buffer, assess sample stability and confirm matrix compatibility with MSD plates and reagents.

For molre information, see also *Samples Handling* on page 11 in *Best Practices* and *Appendix B: Suggested Cell Lysis Protocols* on page 19.

Reagent Preparation

Prepare Tris Wash Buffer

Dilute the 10X Tris Wash Buffer to 1X as shown below. Tris Wash Buffer (1X) is used throughout the assay to make additional reagents and to wash plates. Approximately 350 mL per plate is required—more if using an automated plate washer.

For 1 plate, combine:

- ❑ 35 mL of Tris Wash Buffer (10X)
- ❑ 315 mL of deionized water

It is best practice not to store Tris Wash buffer and use it the same day.

Prepare Blocker A Solution

For 1 plate, combine:

- ❑ 600 mg Blocker A (dry powder)
- ❑ 20 mL 1X Tris Wash Buffer

Filter Blocker A solution before use. Refer to the Blocker A instructions (MSD catalog number R93BA) for a detailed protocol.

Prepare Antibody Dilution Buffer

Phospho-STAT Panel Kit or Phospho-STAT4 (Tyr693) Kit

For the Phospho-STAT Panel Kit or Phospho-STAT4 (Tyr693) Kit, combine for 1 plate:

- ❑ 150 µL 2% Blocker D-M
- ❑ 30 µL 10% Blocker D-R
- ❑ 1 mL of Blocker A solution
- ❑ 1.82 mL of 1X Tris Lysis Buffer

Phospho-STAT3 (Tyr705) Kit or Phospho-STAT5a,b (Tyr694) Kit

For the Phospho-STAT3 (Tyr705) Kit or Phospho-STAT5a,b (Tyr694) Kit, combine for 1 plate:

- ❑ 1 mL of Blocker A solution
- ❑ 2 mL of 1X Tris Lysis Buffer

Prepare Complete Lysis Buffer

Prepare complete Lysis Buffer just prior to use. The working solution is 1X.

For 1 plate, combine:

- ❑ 50 µL of Protease Inhibitor Solution (100X stock)
- ❑ 50 µL of Phosphatase Inhibitor I (100X stock)
- ❑ 50 µL of Phosphatase Inhibitor II (100X stock)
- ❑ 4.85 mL of 1X Tris Lysis Buffer

Place the complete Lysis Buffer on ice; it should be ice cold before use.

Prepare Detection Antibody Solution

MSD provides each detection antibody as a 50X stock solution. The working detection antibody solution concentration is 1X.

Phospho-STAT Panel Kit

For 1 plate, add the following detection antibodies to antibody dilution buffer for a total of 3,000 µL:

- ❑ 2,820 µL antibody dilution buffer
- ❑ 60 µL of Phospho STAT3 (Tyr705) Antibody
- ❑ 60 µL of Total STAT4 Antibody (50X)
- ❑ 60 µL of Phospho STAT5 (Tyr694) Antibody

Singleplex Kits

For 1 plate, add 60 µL of the supplied 50X detection antibody to 2,940 µL of antibody dilution buffer for a total of 3,000 µL:

- ❑ 2,940 µL antibody dilution buffer
- ❑ 60 µL 50X detection antibody

Read Buffer

MSD GOLD Read Buffer B is provided ready to use. Do not dilute.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Assay Protocol

This section describes the assay protocol. Complete *Reagent Preparation* on page 13 before beginning this assay protocol.

STEP 1: Add Blocker A Solution

❑ 1. Add 150 µL of Blocker A solution to each well.
Seal the plate with an adhesive plate seal.
Incubate 1 hour at room temperature with vigorous shaking (500–1,000 rpm).

STEP 2: Wash and Add Sample

❑ 2. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 3. Add 25 µL of sample (controls or unknowns) to each well.
Seal the plate with an adhesive plate seal.
Incubate 1 hour at room temperature with vigorous shaking (500–1,000 rpm).

i You can prepare detection antibody solution during the incubation.

STEP 3: Wash and Add Detection Antibody Solution

❑ 4. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 5. Add 25 µL of 1X Detection Antibody Solution to each well.
Seal the plate with an adhesive plate seal.
Incubate 1 hour at room temperature with vigorous shaking (500–1,000 rpm).

STEP 4: Wash and Read

❑ 6. Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer.*

❑ 7. Add 150 µL of MSD GOLD Read Buffer B to each well.

❑ 8. Read the plate on an MSD instrument immediately. Incubation in read buffer is not required before reading the plate.

i Delays in reading the plate may result in lower assay signals for the Phospho-STAT3 (pY705) assay.

*If you are using an automatic washer, use 450 µL/well for the wash steps. See *Appendix A: Recommended Plate Washer Parameters* on page 18

Assay Characteristics

Typical Data

Representative results for the Phospho-STAT Kits are illustrated in Figure 4. The signal values provided are examples; individual results will vary depending upon the samples tested. Note that the amount of lysate required per well depends on the samples being tested and should be optimized for each different sample type. Western blot analysis for each analyte are shown for comparison.

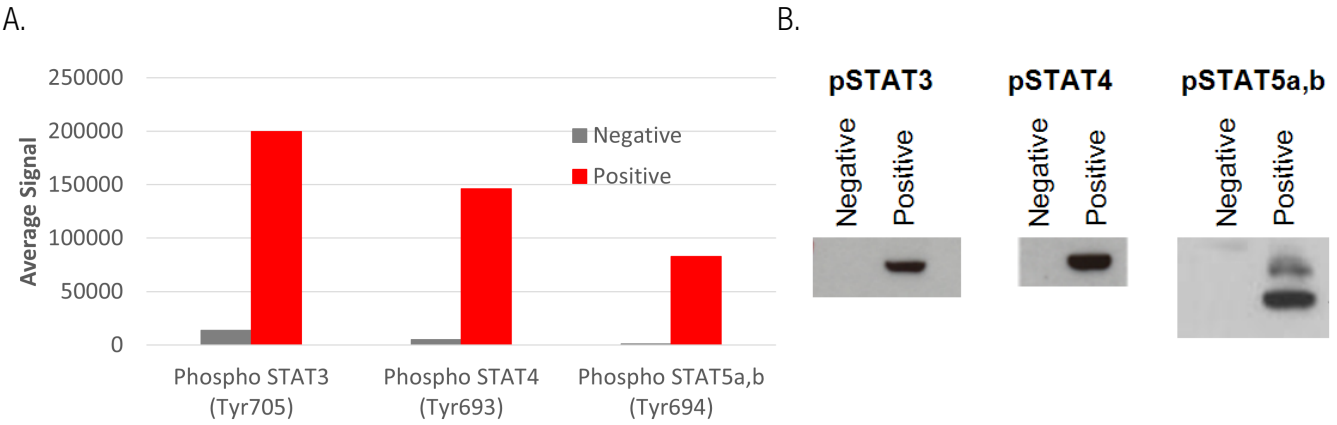

Figure 4. *Sample data generated with Phospho-STAT Kits.* ***A.*** *Average signal from the Phospho-STAT Kits using 2.5 µg/well of positive and negative control whole cell lysates (MSD catalog number C10KQ-1).* ***B.*** *Western blots with pSTAT3, pSTAT4, and pSTAT5a,b antibodies using 20 µg/lane of positive and negative control whole cell lysates.*

For additional data, see the Phospho-STAT3 (Tyr705), Phospho-STAT4 (Tyr693), and Phospho-STAT5a,b datasheets.

Additional Information

Appendix A: Recommended Plate Washer Parameters

Create a program for your automated plate washer with the optimal settings before starting your assay. Example settings for a typical (MSD-recommended) wash program are shown below for a common plate washer (Biotek Model 405 LS,Table 6).

Table 6. *Parameters for customized programs on the Biotek 405 LS microplate washer*

Wash Program Parameters		Typical 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	-35
	Aspirate Y-Position	-35
	Aspirate Height	22
	Secondary Aspirate?	NO
DISPENSE	Dispense Rate	05
	Dispense Volume	0450 µL/well
	Vacuum Delay Volume	0450 µL/well
	Dispense X-Position	00 (0.000 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	3
	Final Aspirate Delay	0500 milliseconds
	Final Aspirate X-Position	-35 (1.600 mm)
	Final Aspirate Y-Position	-35 (1.600 mm)
	Final Aspirate Height	22
	Secondary Aspirate?	YES
	Final Aspirate Secondary X-Position	35 (1.600 mm)
	Final Aspirate Secondary Y-Position	35 (1.600 mm)
	Final Aspirate Secondary Height	22

Appendix B: Suggested Cell Lysis Protocols

You need to determine the optimal cell lysis time. Some targets are immediately available for detection. Other targets may require an incubation step at room temperature or on ice with gentle agitation.

Perform all manipulations on ice; keep PBS wash buffer and complete lysis buffer ice cold. Cell concentrations for lysis can range from 0.5 to 5 x 10^7 cells per mL of lysis buffer. Protein yields vary by cell line. To get the desired final protein concentration, optimize the number of cells used and the amount of complete lysis buffer added. Depending on the stability of the target in the matrix, additional protease and phosphatase inhibitors may be required in the matrix or diluent. Additionally, specific cell types or targets may benefit from alternative buffer components or techniques, depending upon the particular research application.

Preparation in Culture Flask or Petri Dish

Suspension Cells.

1. Pellet cells by centrifugation at 500g for 3 minutes at 2–8 °C.
2. Discard supernatant and wash the pellet once with cold PBS.
3. Pellet cells again by centrifugation at 500g for 3 minutes at 2–8 °C.
4. Discard supernatant and resuspend in complete lysis buffer at 1–5 x 10^7 cells per mL.
5. Incubate on ice for 30 minutes.

   A shorter incubation time of 15 minutes may be adequate for many targets.
6. Clear cellular debris from the lysate by centrifuging (≥10,000g) for 10 minutes at 2–8 °C.
7. Transfer cleared lysate to a fresh tube and discard the pellet.
8. Determine the protein concentration in the lysate using a detergent-compatible protein assay such as a bicinchoninic acid (BCA) assay.

Aliquot unused lysates, freeze quickly in a dry ice-ethanol bath, and store at ≤-70 °C.

Adherent Cells.

All volumes given are for cells plated on 15 cm dishes.

1. Remove media from the dish and wash cells once with 5 mL cold PBS.
2. Add 2 mL PBS to each dish, scrape the cells from the surface of the dish, and transfer into 15 mL conical tubes.
3. Pellet the cells by centrifugation at 500g for 3 minutes at 2–8 °C.
4. Discard supernatant and resuspend cells in 0.5–2 mL of complete lysis buffer per dish.

   Alternatively, cells can be lysed by adding 1–2 mL of complete lysis buffer per 15 cm dish after completely removing the PBS wash buffer.
5. Incubate on ice for 30 minutes.

   A shorter incubation time of 15 minutes may be adequate for many targets.
6. Clear cellular debris from the lysate by centrifuging (≥10 000g) for 10 minutes at 2–8 °C.
7. Transfer cleared lysate to a fresh tube and discard the pellet.
8. Determine protein concentration in the lysate using a detergent-compatible protein assay such as BCA.

Unused lysates should be aliquoted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70 °C.

Preparation in 96-well Culture Plate

Successful adaptation to a 96-well culture format depends on cell type and target. MSD generally recommends plating concentrations ranging from 1 x 10^4 to 10^5 cells per well; however, the optimal concentrations varies depending on cell line used.

Suspension Cells.

Many cell types can be lysed without removing growth medium.

- For **flat bottom plates**, design the experiment so that the final suspension cell volume per well is such that a concentrated complete lysis buffer (prepared by the user) can be added to the well to achieve a final 1X lysis buffer concentration in the well. For example, 40 µL of 5X complete lysis buffer added to a well containing 160 µL of cell culture medium would provide a 1X concentration of complete lysis buffer.
- 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.

1. Plate cells on coated tissue culture plates to reduce variability due to cells lost as growth medium is removed.
2. Treat cells as desired.
3. Gently aspirate growth medium from the microwell plate to avoid disrupting the cell monolayer. A PBS wash step is not required and can introduce variability as cells may detach during the wash step.
4. Add 100 µL of 1X complete lysis buffer per well. You may modify lysis volume for different cell types or applications.
5. Carefully pipette cell lysate onto prepared assay plate and proceed with assay protocol.

It is important to transfer a constant volume and to avoid introducing air bubbles by pipetting too vigorously.

Plate Diagram

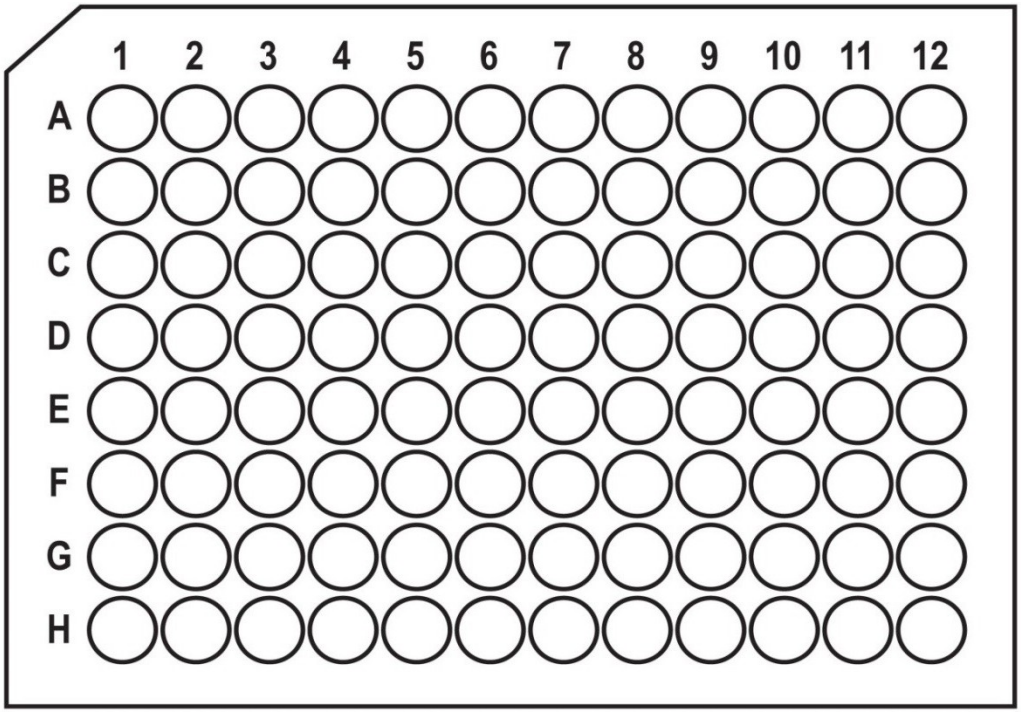

Figure 5. Plate diagram.

Catalog Numbers

Table 7. Catalog numbers associated with the T-PLEX Phospho-STAT Kits

Kit Name	SECTOR Plate		
	1-Plate Kit	5-Plate Kit	25-Plate Kit
Phospho-STAT Panel Kit	K15758D-1	K15758D-2	K15758D-4
Phospho-STAT3 (Tyr705) v2 Kit	K150AXPD-1	K150AXPD-2	K150AXPD-4
Phospho-STAT4 (Tyr693) v2 Kit	K150AXND-1	K150AXND-2	K150AXND-4
Phospho-STAT5a,b (Tyr694) v2 Kit	K150AXLD-1	K150AXLD-2	K150AXLD-4

Table 8. Instrument compatibility for plate type

Plate Type	Instrument Compatibility
SECTOR Plate	MESO® SECTOR S 600, MESO SECTOR® S 600MM, MESO QuickPlex® SQ 120, MESO QuickPlex SQ 120MM

Ensure the plate type is compatible with your MSD instrument.

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