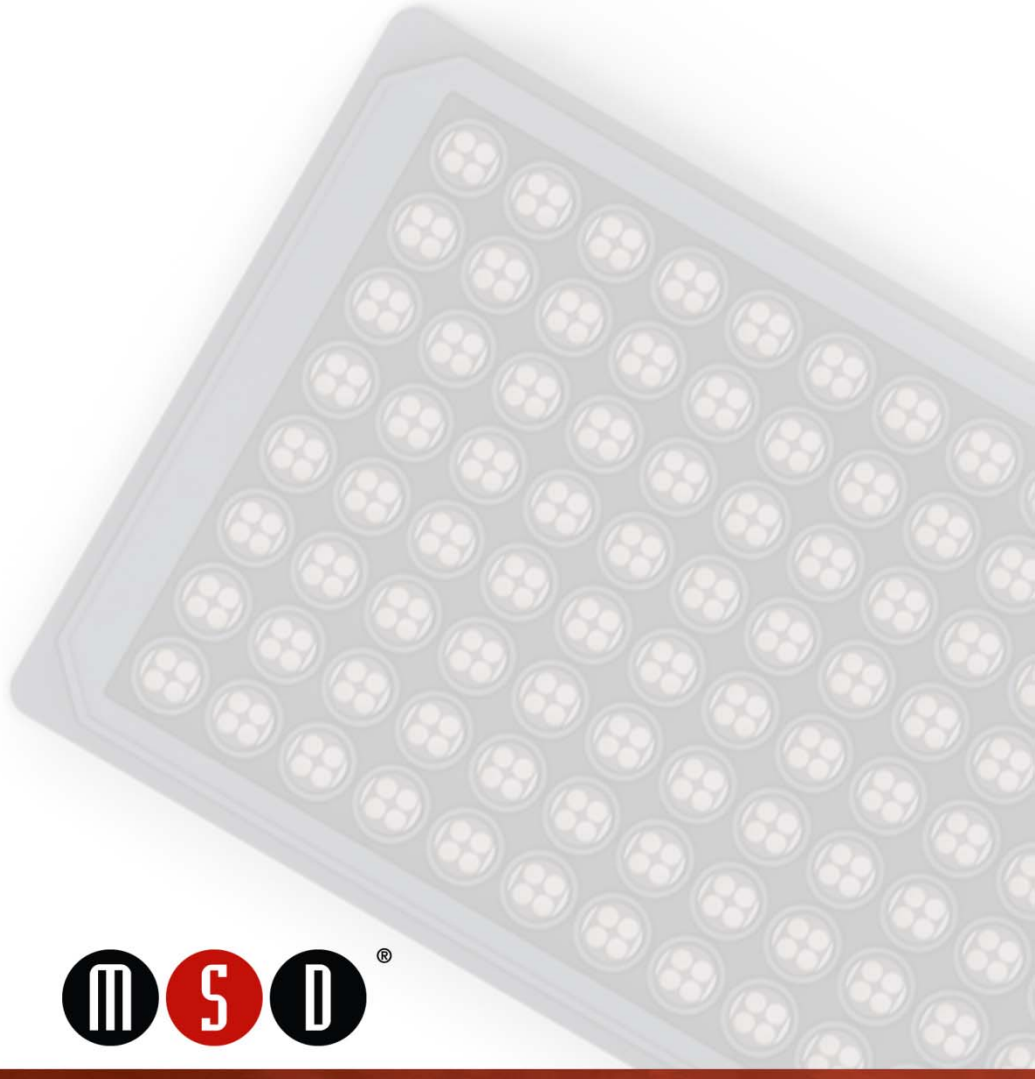
MSD® MULTI-SPOT Assay System

Total FOXO3a Kit

1-Plate Kit	K150SQD-1
5-Plate Kit	K150SQD-2
25-Plate Kit	K150SQD-3



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MSD Cell Signaling Pathway Assays

Total FOXO3a Kit

For use with human and mouse cell lysates.

This package insert must 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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Ordering Information

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Introduction

Forkhead box O3a (FOXO3a) belongs to the O subclass of the forkhead family of transcription factors (FOXO1, FOXO3a, FOXO4, FOXO6) which are characterized by a forkhead DNA binding domain. This transcription factor regulates multiple transcriptional targets involved in various cellular processes, including proliferation, stress resistance, apoptosis, metabolism, and longevity.[1,2] As a transcription factor, FOXO3a plays a key role in cellular senescence through regulation of genes promoting cell cycle arrest and apoptosis. Increased proliferation results when FOXO3a is inactivated through phosphorylation by Akt or phosphatidylinositide 3-kinase (PI3K) at Thr32, Ser253, and Ser315. This results in nuclear export and inhibition of transcription factor activity.[3] Other post-translational modifications, including acetylation, methylation, or interactions with reactive oxygen species (ROS), may also result in increased or abnormal FOXO3a activity.[1,2] Therefore, phosphorylation of FOXO3a is often associated with tumorigenesis and cancer development.[1] A better understanding of the regulation of FOXO3a activity and its specific transcriptional targets may provide strong insight into the mechanisms controlling cell fate decisions.

Principle of the Assay

MSD cell signaling pathway assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. Total FOXO3a is a sandwich immunoassay (Figure 1). MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots in the layout shown below. The user adds 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. The user adds an MSD buffer that provides the appropriate chemical environment for electrochemiluminescence and loads 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 to provide a quantitative measure of analytes in the sample.

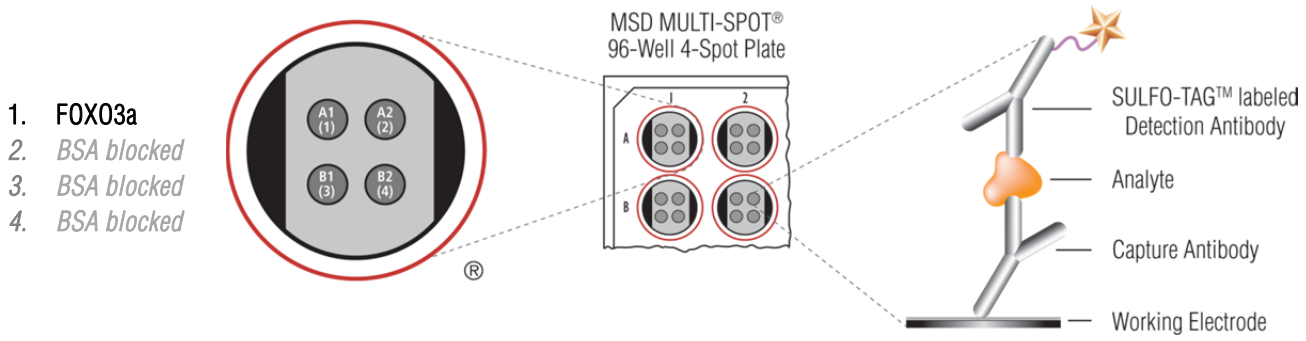

Figure 1. *Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.*

Reagents Supplied

Reagent	Storage	Catalog #	Size	Quantity Supplied			Description
				1 Plate Kit	5 Plate Kit	25 Plate Kit	
MULTI-SPOT® 96-well 4-spot Total FOXO3a Plate	2–8°C	N450SQA-1	4-spot	1 plate	5 plates	25 plates	96-well plate, foil sealed with desiccant.
Anti-Total FOXO3a Antibody (50X)	2–8°C	D20SQ-2	75 µL	1 vial			SULFO-TAG conjugated antibody
		D20SQ-3	375 µL		1 vial	5 vials	
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 protein protease inhibitors
			0.5 mL		1 vial	5 vials	
Blocker D–M (2%)	≤-10°C		0.2 mL	1 vial			Mouse gamma globulin solution
			1.8 mL		1 vial	5 vials	
Blocker D–R (10%)	≤-10°C		0.05 mL	1 vial			Rabbit gamma globulin solution
			1.0 mL		1 vial	5 vials	
Blocker A (dry powder)	RT	R93BA-4	15 g	1 bottle	1 bottle	5 bottles	Bovine serum albumin, reagent grade pure powder
Read Buffer T (4X)	RT	R92TC-3	50 mL	1 bottle	1 bottle	5 bottles	MSD buffer to catalyze the electro-chemiluminescence reaction

Additional Materials and Equipment

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Polypropylene microcentrifuge tubes for preparing dilutions
- ❑ Liquid handling equipment for desired throughput, capable of 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 300–1000 rpm
- ❑ Adhesive plate seals
- ❑ Deionized water

Optional Material

Akt Signaling Whole Cell Lysate Set (available for separate purchase from MSD, catalog # C1196-1)

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Additional safety information is available in the product safety data sheet, which can be obtained from MSD Customer Service.

Best Practices and Technical Hints

- Do not mix or substitute reagents from different sources or different kit lots. Lot information is provided in the lot-specific certificate of analysis (C of A).
- Complete lysis buffer should be kept on ice during the experiment.
- Dilute calibrators, samples, and controls in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution; vortex after each dilution before proceeding.
- Avoid prolonged exposure of detection antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results; bubbles introduced when adding read buffer may interfere with signal detection.
- Use reverse pipetting where necessary to avoid introduction of bubbles, and pipette to the bottom corner of empty wells.
- Shaking should be vigorous with a rotary motion between 300 and 1000 rpm.
- When using an automated plate washer, rotating the plate 180 degrees between wash steps may improve assay precision.
- Gently tap the plate to remove residual fluid after washing.
- Read buffer should be at room temperature when added to the plate.
- Keeping time intervals consistent between adding read buffer and reading the plate should improve inter-plate precision. Limit the time the plate is incubated with read buffer.
- No shaking is necessary after adding read buffer.
- If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the wells.
- 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 reagents. 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.
- Remove plate seals prior to reading the plate.

Reagent Preparation

Prepare Tris Wash Buffer

Dilute the 10X Tris Wash Buffer to 1X as shown below. Tris Wash Buffer (1X) will be used throughout the assay to make additional reagents and 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

Excess Tris Wash Buffer may be stored at room temperature in a tightly sealed container.

Prepare Blocker A Solution

For 1 plate, combine:

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

Prepare Antibody Dilution Buffer

For 1 plate, combine:

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

Set aside on ice.

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 and Dilute Samples

Most lysis buffers and sample matrices are compatible with MSD plates, although high concentrations of denaturing reagents should be avoided. Keep SDS and other ionic detergents to a concentration of 0.1% or less in the sample applied to the well. Avoid reducing agents and chaotropes such as DTT >0.1mM and urea >0.1M. Complete lysis buffer may be used to lyse many different cell lines, tumors, and tissues; however, specific sample types may benefit from alternative buffer components. See the Appendix for more detailed information on lysate preparation. Please contact MSD Scientific Support if you have any questions about lysate preparation options.

As a starting point, samples may be diluted to a working concentration of 6.25–800 µg/mL (0.156–20 µg/well with a 25 µL sample volume) using complete lysis buffer as prepared above.

Prepare Control Cell Lysate (Optional)

Akt Signaling Whole Cell Lysate Set is available for separate purchase from MSD, catalog # C1196-1. The set contains cell lysates from MCF7 cells that were treated to stimulate (positive) or inhibit (negative) FOX03a phosphorylation.

Thaw on ice and dilute to a working concentration of 6.25–800 µg/mL (0.156–20 µg/well with a 25 µL/well sample volume) using complete lysis buffer. The lysates can go through an additional freeze–thaw cycle without significantly affecting analyte levels.

Prepare Detection Antibody Solution

MSD provides detection antibody as a 50X stock solution. The working detection antibody solution is 1X. Prepare detection antibody solution immediately prior to use.

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-Total FOXO3a Antibody
- ❑ 2.94 mL of cold antibody dilution buffer

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 1X.

For 1 plate, combine:

- ❑ 5 mL of Read Buffer T (4X)
- ❑ 15 mL of deionized water

You may keep excess diluted read buffer in a tightly sealed container at room temperature for up to 1 month.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates may be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Protocol

1. **Add Blocker A Solution:** Add 150 µL of Blocker A solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 1 hour.

2. **Wash and Add Sample:** Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer. Add 25 µL of diluted sample or control per well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 1 hour.

3. **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer. Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 1 hour.

4. **Wash and Read:** Wash the plate 3 times with at least 150 µL/well of 1X Tris Wash Buffer. Add 150 µL of 1X Read Buffer T to each well. Read plate on MSD instrument. No incubation in read buffer is required.

Tested Samples

Whole cell lysates from the Akt Signaling Whole Cell Lysate Set (catalog # C1196-1) were titrated and then assayed with the Total FOXO3a Kit. The treatment conditions used for preparing the Akt Signaling Whole Cell Lysate Set are outlined below.

- Positive Akt Signaling Lysate: MCF7 cells were treated with 100 nM IGF-1 for 30 minutes to stimulate FOX3a phosphorylation.
- Negative Akt Signaling Lysate: MCF7 cells were treated with 50 µM LY294002 for 2.5 hours to inhibit FOX3a phosphorylation.

Data is shown below. Western blot analysis of the whole cell lysate set is shown for comparison.

Lysate	Positive			Negative			P/N
(µg)/well	Average Signal	StdDev	%CV	Average Signal	StdDev	%CV	
0	28	29	103.3	19	24	123.4	
0.6	1 817	545	30.0	2 893	138	4.8	1.6
2.5	11 775	2 065	17.5	17 743	1 806	10.2	1.5
10	68 177	4 414	6.5	84 512	5 990	7.1	1.2

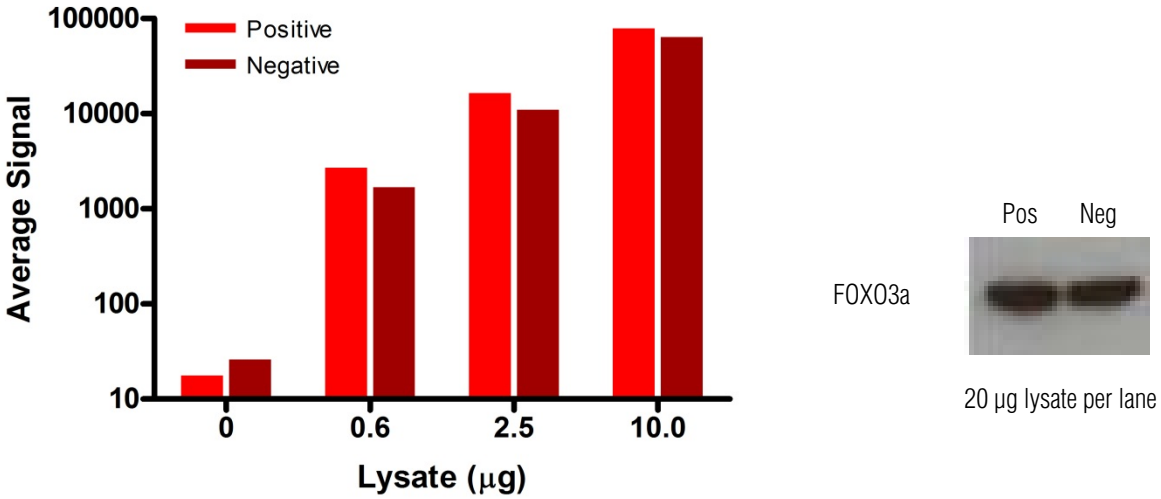

Figure 2: *Sample data generated with the Total FOXO3a Kit. Increased signal for total FOXO3a was observed with the titration of both phosphorylated FOXO3a positive and negative cell lysates. The Total FOXO3a Kit provides a quantitative measure of the information obtained with the traditional Western blot.*

Assay Components

The capture and detection antibodies used in this assay are listed below. The antibodies cross-react with human and mouse cell lysates.

	Source Species		Assay Generation
Analyte	MSD Capture Antibody	MSD Detection Antibody	
FOXO3a	Rabbit Monoclonal	Mouse Monoclonal	A

References

1. Fu Z, Tindall DJ. FOXOs, cancer and regulation of apoptosis. Oncogene. 2008;27:2312-9.
2. Miyamoto K, Miyamoto T, Kato R, Yoshimura A, Motoyama N, Suda T. FoxO3a regulates hematopoietic homeostasis through a negative feedback pathway in conditions of stress or aging. Blood. 2008;112:4485-93.
3. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96:857-68.

Appendix: Suggested Cell Lysis Protocols

You will need to determine the optimum 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 will vary by cell line. To get your desired final protein concentration, you will need to optimize the number of cells used and the amount of complete lysis buffer added. Depending on the stability of the target in the matrix, you may need additional protease and phosphatase inhibitors 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. Pellet cells by centrifugation at 500g for 3 minutes at 2–8°C. Discard supernatant and wash the pellet once with cold PBS. Pellet cells again, discard supernatant, and resuspend in 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.) Clear cellular debris from the lysate by centrifuging (≥10 000g) for 10 minutes at 2–8°C. Transfer cleared lysate to a fresh tube and discard the pellet. Determine the protein concentration in the lysate using a detergent-compatible protein assay such as a bicinchoninic acid (BCA) assay. Unused lysates should be aliquotted, quickly frozen in a dry ice-ethanol bath, and stored at ≤-70°C.

Adherent Cells. All volumes given are for cells plated on 15 cm dishes. Remove media from the dish and wash cells once with 5 mL cold PBS. Add 2 mL PBS to each dish, scrape the cells from the surface of the dish, and transfer into 15 mL conical tubes. Pellet the cells by centrifugation at 500g for 3 minutes at 2–8°C. 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.) Incubate on ice for 30 minutes. (A shorter incubation time of 15 minutes may be adequate for many targets.) Clear cellular debris from the lysate by centrifuging (≥10 000g) for 10 minutes at 2–8°C. Transfer cleared lysate to a fresh tube and discard the pellet. Determine protein concentration in the lysate using a detergent compatible protein assay such as BCA. Unused lysates should be aliquotted, 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 will vary depending on cell line used.

Suspension Cells. You may lyse many cell types 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. Plate cells on coated tissue culture plates to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. 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. Add 100 µL of 1X complete lysis buffer per well. You may modify lysis volume for different cell types or applications.

Carefully pipette cell lysate onto prepared assay plate and proceed with assay protocol. Note: It is important to transfer a constant volume and to avoid introducing air bubbles by pipetting too vigorously.

Summary Protocol

Total FOXO3a Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Total FOXO3a assay.

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 detection antibody solution by diluting 50X detection antibody 50-fold in antibody dilution buffer immediately prior to use.

Prepare 1X Read Buffer T by diluting 4X Read Buffer T 4-fold with deionized water.

Step 1: Add Blocker A Solution

Add 150 µL/well of Blocker A solution.

Incubate at room temperature with shaking for 1 hour.

Step 2: Wash and Add Sample

Wash plate 3 times with at least 150 µL/well of Tris Wash Buffer.

Add 25 µL/well of sample (samples or controls).

Incubate at room temperature with shaking for 1 hour.

Step 3: Wash and Add Detection Antibody Solution

Wash plate 3 times with at least 150 µL/well of Tris Wash Buffer.

Add 25 µL/well of 1X detection antibody solution.

Incubate at room temperature with shaking for 1 hour.

Step 4: Wash and Read Plate

Wash plate 3 times with at least 150 µL/well of Tris Wash Buffer.

Add 150 µL/well of 1X Read Buffer T.

Analyze plate on the MSD instrument.

Plate Diagrams