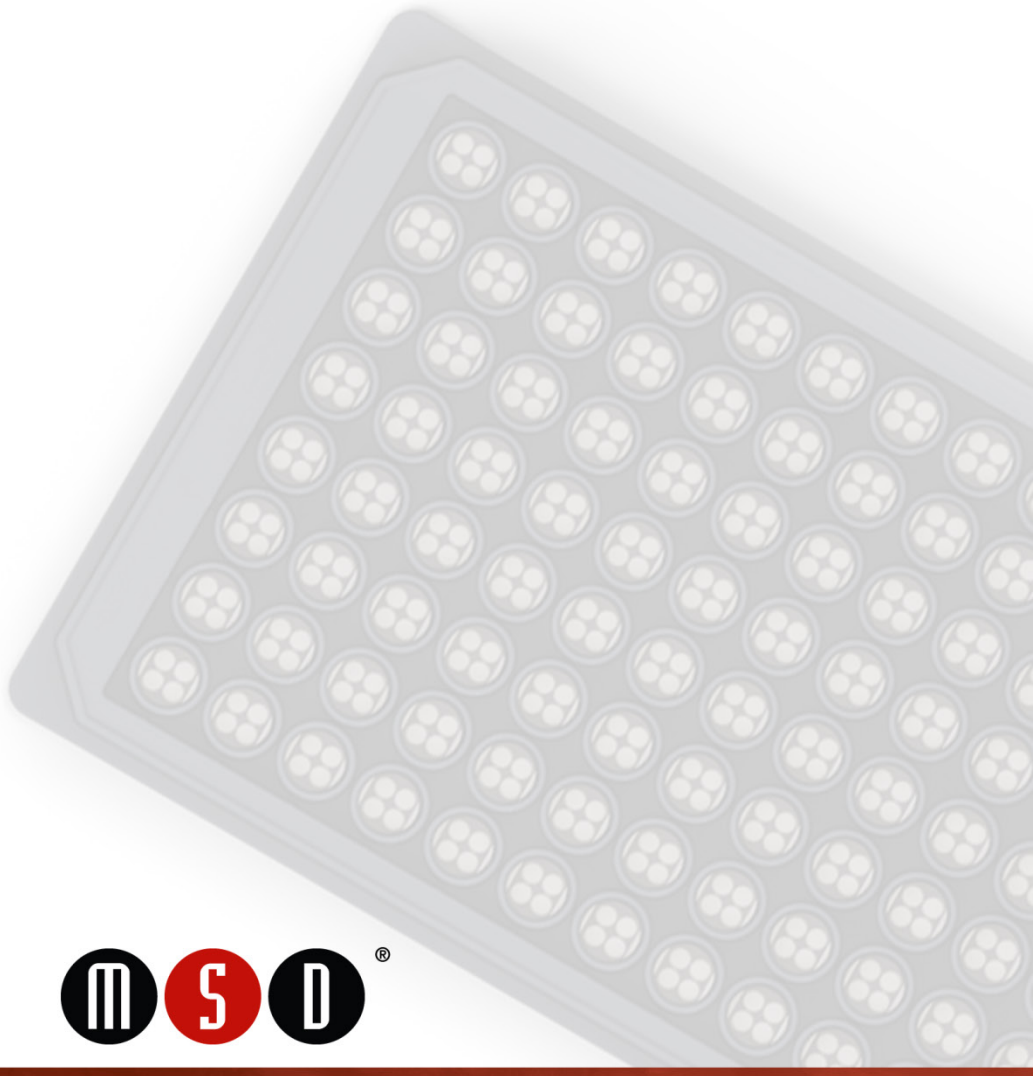
MSD® MULTI-SPOT Assay System

Total Akt Kit

1-Plate Kit	K150MOD-1
5-Plate Kit	K150MOD-2
25-Plate Kit	K150MOD-4
25-Plate Base Kit	K150MOA-4



MSD Cell Signaling Assays

Total Akt Kit

For use with human, mouse, rat, and non-human primate cell lysates and tissue 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

The Akt family (three isoforms denoted as Akt1, Akt2, and Akt3) are serine/threonine kinases that transmit cellular growth and survival signals in response to growth factors and nutrient availability. The mammalian Akt isoforms share a common structure that consists of three conserved domains: an amino-terminal pleckstrin homology (PH) domain, a central catalytic region, and a carboxy-terminal regulatory domain containing the hydrophobic motif phosphorylation site (FXXF(S/T)Y).[1,2] In response to receptor tyrosine kinase activation, phosphoinositide 3-kinase generates the lipid product PI(3,4,5)P_3, a docking site for Akt PH domains.[3] The resultant translocation of Akt to the plasma membrane enables phosphorylation of Akt (Thr308) by phosphoinositide-dependent protein kinase-1 (PDK1) and a subsequent conformational change that allows phosphorylation of Akt (Ser473) by mTORC2, leading to full Akt activation.[4]

Akt inhibits glycogen synthesis by inactivating glycogen synthase kinase 3α and β and increases glucose uptake by stimulating GLUT4 translocation, supplying cells with constant energy.[5] Activated Akt upregulates protein synthesis and cell growth by inactivation of tuberin (TSC2) GTPase activity, contributing to mTOR activation.[6] In turn, activated mTOR regulates translational control over proteins required for cell growth and division. Activated Akt has multiple anti-apoptotic functions, including inactivation of pro-apoptotic BAD and activation of anti-apoptotic MDM2.[7,8] Activated Akt also translocates to the nucleus to phosphorylate the FOXO3 transcription factor, resulting in secretion of FOXO3 into the cytoplasm.[9] This transcriptional regulation reduces pro-apoptotic signals and drives cell cycle progression. Cell lines that lack functional PTEN, a negative regulator of Akt, show delayed cell death attributed to constitutively activated Akt. This phenomenon has been observed in breast, pancreatic, and ovarian cancer cells.[10] Levels of both total and activated Akt are of significant interest to diabetes and cancer researchers due to its crucial regulatory roles in nutrient availability, cell growth, and cell survival processes.[11-13]

The MSD Total Akt Kit can be used to quantify levels of Akt1 in human, mouse, rat, and non-human primate cell lysates and tissue lysates.

Principle of the Assay

MSD assays for intracellular signaling pathways provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. Total Akt 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 a SECTOR® Imager 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.

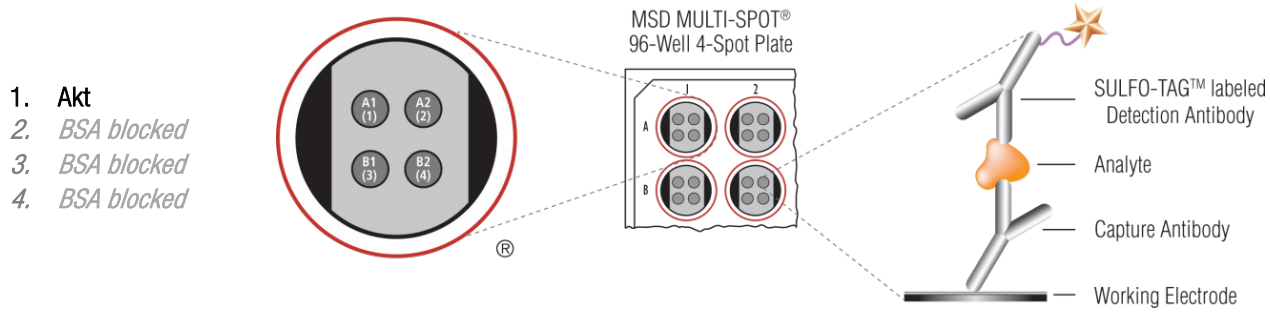


Figure 1. Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files.

Kit Components

Reagents Supplied

Reagent	Storage	Catalog #	Size	Quantity Supplied				Description
				1 Plate Kit	5 Plate Kit	25 Plate Kit	Base Kit	
MULTI-SPOT® 96-well 4-Spot Akt Plate	2–8°C	N450MQA-1	4-Spot	1	5	25	25	96-well plate, foil sealed with desiccant.
Anti-Total Akt Antibody (50X)	2–8°C	D20MO-2	75 µL	1				SULFO-TAG–conjugated antibody
		D20MO-3	375 µL		1	5	5	
Akt Calibrator (20X)	≤-70°C	C00MN-2	20 µL	1 vial	5 vials	25 vials		Recombinant human Akt1 protein in a buffered protein diluent. See the lot-specific certificate of analysis (C of A) for analyte concentration.
Diluent 39	2-8°C	R5ABB-2	50 mL	1 bottle	1 bottle	5 bottles		Assay diluent for the dilution of samples and calibrator; Tris Lysis Buffer supplemented with carrier protein.
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 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
		R92TC-2					1 bottle	

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 Materials

- ❑ Akt Control Pack I, catalog #C4115-1; frozen Jurkat cell lysates in 2 concentrations.
- ❑ Tris Lysis Buffer, catalog #R60TX-3, 50 mL; cell lysis buffer for lysate preparation.
- ❑ Inhibitor Pack, catalog #R70AA-1, phosphatase and protease inhibitor solutions for use with Tris Lysis Buffer for preparation of lysates or with Diluent 39 for preparation of additional assay diluent.
- ❑ Recommended for base kit: Phosphoprotein Reagent Support Pack, catalog #K0000D-3; cell lysis reagents, wash buffer, and additional blockers.

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.

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).
- Assay diluent and 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.
- Shaking should be vigorous with a rotary motion between 300 and 1000 rpm.
- Use reverse pipetting where necessary to avoid introduction of bubbles, and pipette to the bottom corner of the well when pipetting into empty wells.
- When using an automated plate washer, rotating the plate 180 degrees between wash steps may improve assay precision.
- 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.
- Remove plate seals prior to reading the plate.
- 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, 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 using a partial plate, seal unused sectors with a plate seal. Return plate to its original foil pouch with desiccant pack and seal with tape. Partially used plates may be stored for up to 14 days at 2–8°C.
- If assay results are above the top of the calibration curve, dilute samples and repeat the assay.

Reagent Preparation

Bring all reagents to room temperature. Thaw the stock calibrator on ice.

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 to wash plates. Approximately 350 mL per plate are 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 3% (w/v) Blocker A Solution

For 1 plate, combine:

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

Prepare Antibody Dilution Buffer

For 1 plate, combine:

- ❑ 1 mL Blocker A solution
- ❑ 2 mL 1X Tris Wash Buffer

Prepare Assay Diluent

Assay diluent is prepared using Diluent 39, with Phosphatase Inhibitor I, Phosphatase Inhibitor II, and Protease Inhibitor Solution included to maintain phosphatase and protease inhibition throughout the sample incubation

For 1 plate, combine:

- ❑ 100 µL Phosphatase Inhibitor I (100X)
- ❑ 100 µL Phosphatase Inhibitor II (100X)
- ❑ 100 µL Protease Inhibitor Solution (100X)
- ❑ 9.7 mL Diluent 39

NOTE: The presence of carrier protein in the assay diluent makes it unsuitable for use as a cell lysis buffer in situations where you need to quantify total lysate protein concentration for normalization purposes prior to performing the Total Akt assay. MSD Tris Lysis Buffer (catalog #R60TX-3) and Inhibitor Pack (catalog #R70AA-1) are available for separate purchase for preparation of lysate samples. See Appendix A for more information on cell lysing protocols.

Prepare Calibrator Dilutions

Thaw the stock calibrator and keep on ice, then add to assay diluent to make the calibrator solutions as described below.

MSD supplies calibrator for the Total Akt Kit at a concentration 80-fold higher than the recommended highest calibrator concentration. Prepare an intermediate solution by diluting the calibrator stock 20-fold. Perform the 4-fold dilution series beginning with the intermediate solution.

To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates:

1) Prepare an intermediate solution by adding 10 µL of stock calibrator to 190 µL of assay diluent. Mix well.
2) Prepare the highest calibrator by transferring 50 µL of the intermediate solution to 150 µL of assay diluent. Mix well.
3) Repeat 4-fold serial dilutions 6 additional times to generate 7 calibrators.
4) Use assay diluent as the zero calibrator.

For the actual concentration of the calibrator, refer to the C of A supplied with the kit. You may also find a copy of the lot-specific C of A at www.mesoscale.com.

Prepare and Dilute Samples

Experiment materials should be prepared in a user-specified lysis buffer prior to dilution in assay diluent to create assay samples. For the most accurate quantification of Total Akt, we recommend quantifying the total protein concentration in the cell lysate by BCA assay and then performing the Total Akt assay using predetermined sample concentrations.

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. MSD Tris Lysis Buffer supplemented with the inhibitors from the MSD Inhibitor Pack (catalog #R70AA-1) may be used to lyse many different cell lines, tumors, and tissues; however, specific sample types may benefit from alternative buffer components. Assay diluent may be used to lyse samples only if cellular protein quantification is not desired. See Appendix A 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 3.0-800 µg/mL (0.078-20 µg/well) using assay diluent as prepared above. Adjust the dilution for the sample set under investigation.

Prepare Controls (Optional)

Two levels of controls are included in the Akt Control Pack, Catalog #C4115-1, available for separate purchase from MSD. The controls are Jurkat cell lysates at 2 concentrations. Thaw on ice and use without dilution. The controls can go through 2 freeze-thaw cycles without significantly affecting analyte levels.

An additional control (Control 3) may be prepared by diluting Control 1 in assay diluent 100-fold to a final concentration of 4 µg/mL, which equates to 0.10 µg/well.

Prepare Detection Antibody Solution

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

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-Total Akt Antibody
- ❑ 2.94 mL of 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 can 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 with shaking for 1 hour at room temperature.

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, calibrator, or control per well. Seal the plate with an adhesive plate seal and incubate with shaking for 1 hour at room temperature.

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 with shaking for 1 hour at room temperature.

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. No incubation in read buffer is required before reading the plate. Read plate on MSD instrument.

Alternate Protocols

The suggestions below may be useful for simplifying the protocol; however, they are not used in the kit verification process. See Appendix B for assay performance using these protocols.

Steps	Reference	Alternate 1	Alternate 2
Block	◆		
Wash	◆		
Add sample	◆	◆	◆
Incubate	◆		
Wash	◆		
Add Detection Ab	◆	◆	◆
Incubate	◆	◆	◆
Wash	◆	◆	
Add Read Buffer	◆	◆	◆
Read	◆	◆	◆

- **Alternate Protocol 1 (homogeneous, one wash step):** You may simplify the protocol by removing the blocking step and removing all wash steps except the one prior to adding read buffer. Sample and detection antibody solution are incubated in a homogenous format for 2 hours.
  1) Add diluted sample, calibrator, or control and detection antibody solution to the plate and incubate for 2 hours.
  2) Wash the plate. Add 150 µL of 1X Read Buffer T to each well, and read plate.
- **Alternate Protocol 2 (homogeneous, no wash steps):** Same as alternate protocol 1 without the wash step.
  1) Add diluted sample, calibrator, or control and detection antibody solution to the plate and incubate with shaking for 2 hours at room temperature.
  2) Add 100 µL of 1.5X Read Buffer T to each well, and read plate.
- **Extended sample incubation:** You may modify the sample incubation time by incubating samples in the plate for 3 hours (with shaking) at room temperature or overnight at 4ºC.

Verification

MSD's verification testing is conducted following fit-for-purpose principles,[14] and product development follows MSD design control policies. Performance of the Total Akt Kit was evaluated by assaying calibrators, controls, and cell lysates across multiple days and multiple runs. Two independent analysts assayed 3 plates per day across 3 days to confirm kit performance prior to release. Data analysis methods and representative data are presented below.

- **Sensitivity**

  The lower limit of detection (LLOD) is established based on runs throughout assay development. It is a calculated concentration corresponding to the signal 2.5 standard deviations above the average reading from the zero calibrator.

- **Precision**

  Cell lysates from Akt Control Pack I (catalog #C4115-1) were tested over multiple days using a total of 18 runs to measure intra- and inter-run accuracy and precision. Coefficient of variance (CV) is presented as a measure of precision in this product insert. Precision and accuracy were verified for each lot as part of the lot verification and quality control release process.

  The typical specification for precision is a concentration CV of less than 20% for controls on both intra- and inter-day runs.

- **Robustness and Stability**

  Freeze–thaw testing and accelerated stability studies for calibrators and controls were performed during assay development are augmented with real-time stability studies on complete kits out to 30 months from the date of manufacture.

- **Specificity, Interferences, Spike and Recovery, and Dilutional Linearity**

  Isoform specificity was tested in assay diluent. Tolerance to common lysis buffer additives was tested in cell lysates. Spike and recovery and dilutional linearity were tested across the assay range to evaluate sample matrix effects.

- **Tested Samples**

  Multiple immortalized mammalian cell lysates and mouse xenograft models were tested to determine the suggested lysate concentration ranges detected with the assay. Cells derived from xenografts and cell lysates were prepared by lysing cells in MSD Tris Lysis Buffer supplemented with inhibitors from the Inhibitor Pack. Protein concentrations in each lysate sample were determined by BCA assay.

Representative data from this kit's verification studies are presented below. The lot-specific calibration curve and measured limits of detection can be found in the C of A enclosed with each kit. You may also find a copy of the lot-specific C of A at www.mesoscale.com.

Analysis of Results

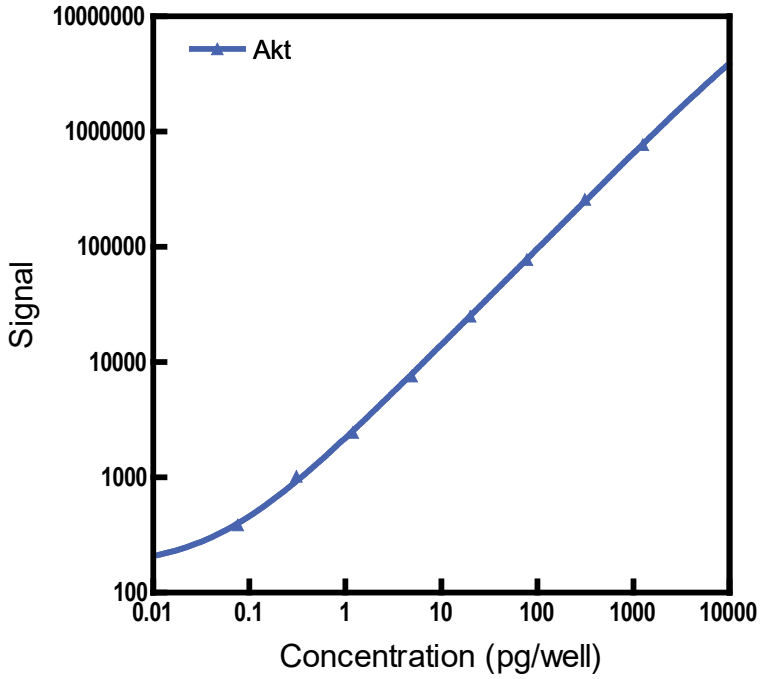
Run at least 1 set of calibrators in duplicate to generate the calibration curve. The calibration curve is modeled using least squares fitting algorithms so that signals from the calibrators can be used to calculate the concentration of analyte in the samples. The assays have a wide dynamic range (4 logs), which allows for accurate quantification of samples without the need for multiple dilutions or repeated testing. The data displayed below were generated by DISCOVERY WORKBENCH® analysis software using a 4-parameter, logistic curve-fitting model (sigmoidal dose-response) with a $1/Y^2$ weighting function. The weighting function provides a better fit of data over a wide dynamic range, particularly at the low end of the calibration curve.

Total Akt calibration curves are constructed based on the concentration of Akt calibrator added to each well. Total Akt protein present in experimental samples is quantified by interpolation from the standard curve and then normalized to the mass of cell lysate loaded into each well (0.078-20 µg). DISCOVERY WORKBENCH reports sample results from this assay in pg of Akt per well (pg/well). To convert this value to pg/µg lysate, you must divide the reported value by the lysate mass loaded into the well in µg units. The table below illustrates this calculation using Control 1 and Control 2 (Akt Control Pack, MSD catalog #C4115-1).

Sample	µg Lysate/well	pg/well	pg/µg Lysate
Control 1	10	353	35.3
Control 2	0.63	18	28.6

Typical Data

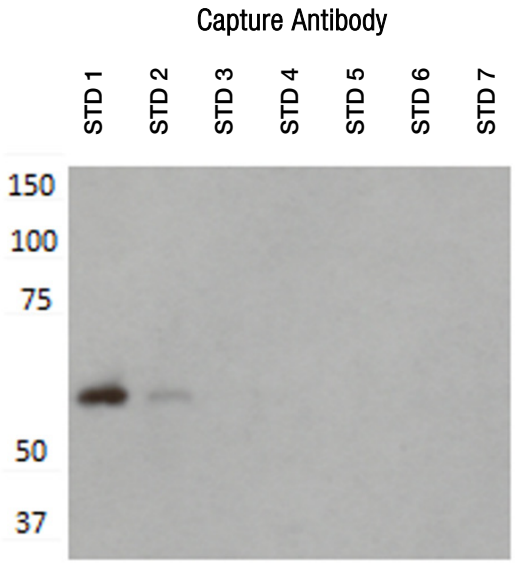
The following standard curve graph illustrates the dynamic range of the assay. Actual signals will vary. Best quantification of unknown samples will be achieved by generating a standard curve for each plate using a minimum of 2 replicates of each standard. For each kit lot, refer to the C of A for the actual calibrator concentration.

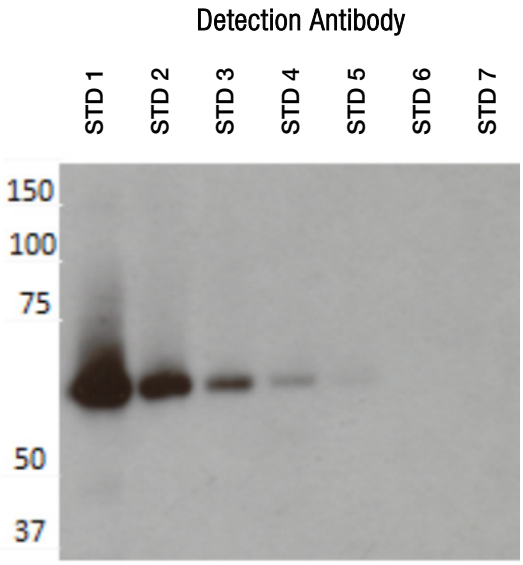


Total Akt		
pg/well	Average Signal	%CV
0	46	2.8
0.076	388	1.3
0.31	1023	3.0
1.2	2459	5.2
4.9	7574	1.1
20	25 036	0.8
78	77 677	2.3
313	258 478	0.2
1250	772 333	1.0

Calibrator Western Blots

Calibration curves (STD 1-7) were evaluated by Western blot using the capture and detection antibodies.





Sensitivity

The lower limit of detection (LLOD) is a calculated concentration corresponding to a signal 2.5 standard deviations above the background (zero calibrator). The LLOD shown below was calculated based on 18 runs.

	Total Akt (pg/well)
Average LLOD	0.028
LLOD Range	0.018-0.033

Precision

The precision of the Total Akt Kit was measured using Akt Control Pack 1 (MSD catalog #C4115-1), which includes frozen lysates from untreated exponentially growing Jurkat cells in 2 concentrations: Control 1 (total protein: 400 µg/mL; 10 µg/well) and Control 2 (total protein: 25 µg/mL; 0.63 µg/well). Control 3 was prepared by diluting Control 1 in assay diluent 100-fold to a final concentration of 4 µg/mL (0.10 µg/well). Analyte levels were measured using a minimum of 3 replicates on 18 runs over 3 days.

Average intra-run %CV is the average %CV of the signal from control replicates within an individual run.

Inter-run %CV is the variability of controls across 18 runs.

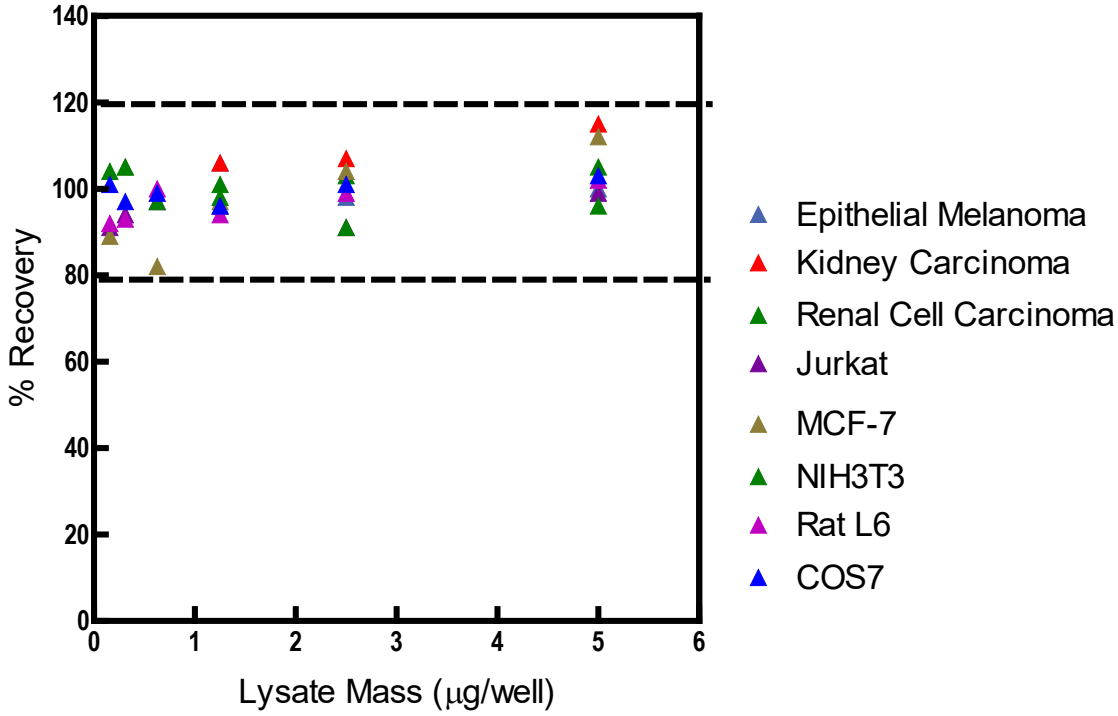
	Control	Runs	Average Signal	Average pg Akt per µg of Lysate	Average Intra-run %CV	Inter-run %CV
Total Akt	Control 1	18	238 356	35	3.2	2.8
	Control 2	18	13 000	29	3.6	12.1
	Control 3	18	2375	30	3.3	8.6

Dilution Linearity

To assess linearity, tumor xenograft and cell lysate samples were diluted to a concentration of 400 µg/mL (10 µg/well) of total protein, then serially diluted 2-fold before testing. Percent recovery at each dilution was calculated by dividing the calculated concentration by the expected concentration. The expected concentration compares the sample to the 10 µg/well of total protein condition. The average percent recovery shown below and in the table on the following page is based on samples within the quantitative range of the assay.

% Recovery=measured levels/expected levels∗100

Dotted lines in the graph below represent acceptable ranges of accuracy (+/- 20% of measured Total Akt). Dilutional linearity results may be dependent on the sample tested.



		Total Akt		
Sample Type	µg Lysate/well	Signal	pg/µg Lysate	% Recovery
Epithelial Melanoma (Human)	1.3	7258	8.4	84
	2.5	15 096	8.5	85
	5	32 827	9.1	91
	10	71 525	10	
Kidney Carcinoma (Human)	1.3	2255	2.7	100
	2.5	4498	2.6	96
	5	8880	2.5	93
	10	19 503	2.7	
Renal Cell Carcinoma (Human)	1.3	14 684	17	94
	2.5	30 917	17	94
	5	63 478	17	94
	10	130 700	18	
Jurkat (Human)	0.16	5330	22	72
	0.31	10 845	25	79
	0.63	21 782	26	85
	1.3	42 453	27	87
	2.5	83 704	28	91
	5.0	172 799	31	99
	10	329 914	31	
MCF-7 (Human)	0.16	24 095	114	86
	0.31	50 372	128	97
	0.63	90 994	122	92
	1.3	207 038	149	113
	2.5	401 144	154	116
	5	726 580	148	112
	10	1 217 969	132	
NIH3T3 (Mouse)	0.16	13 089	80	91
	0.31	26 103	84	95
	0.63	54 664	91	104
	1.3	106 952	93	105
	2.5	211 148	96	109
	5	384 424	91	104
	10	699 180	88	
Rat L6 (Rat)	0.16	10 982	71	81
	0.31	23 229	77	88
	0.63	48 909	83	95
	1.3	95 291	82	94
	2.5	196 163	88	100
	5	378 791	89	102
	10	694 030	87	
COS-7 (Non-Human Primate)	0.16	9635	59	96
	0.31	18 436	58	95
	0.63	36 996	60	98
	1.3	72 726	61	99
	2.5	146 767	64	104
	5	277 684	63	103
	10	507 433	61	

Spike Recovery

Tumor xenograft lysate samples were diluted to 50 µg/mL of total protein (1.25 µg/well) and spiked with Akt calibrator at 3 concentrations throughout the assay range.

% Recovery=(measured levels-endogenous levels)/expected levels∗100. Spike recovery results may be dependent on the sample tested.

Xenograft Sample Type	Average Signal	Akt Spike (pg/µg Lysate)	Measured Akt (pg/µg Lysate)	% Recovery
Epithelial Melanoma (human) (N=3)	3632	0	4.8	
	15 345	14	18	93
	66 944	70	68	90
	323 073	360	340	96
Kidney Carcinoma (human) (N=3)	2390	0	3.4	
	14 354	14	17	98
	71 519	70	72	96
	297 644	360	300	85
Renal Cell Carcinoma (human) (N=3)	11 422	0	14	
	26 633	14	29	113
	74 413	70	76	89
	311 713	360	320	88

Specificity

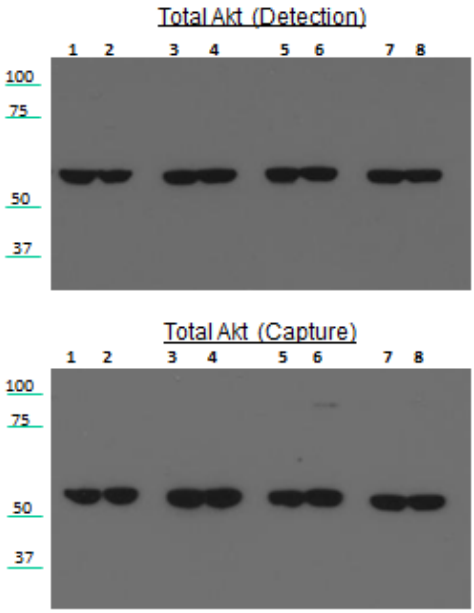
Akt Isoforms Specificity

To assess the assay specificity to different Akt isoforms, 1250 pg/well of full-length, recombinant human Akt1, Akt2, and Akt3 proteins were serially diluted 4-fold prior to testing.

	Akt1		Akt2		Akt3	
pg/well	Average Signal	%CV	Average Signal	%CV	Average Signal	%CV
0	80	45	61	0	61	22
1.2	1380	1.5	60	5.9	104	1.4
4.9	5052	3.6	59	12.0	74	1.9
20	19 328	1.7	63	6.7	122	1.2
78	71 789	1.5	70	1.0	275	5.4
313	246 134	2.3	95	7.4	768	3.6
1250	768 752	1.4	180	4.3	2841	5.8

Species Reactivity

To assess the species reactivity of the capture and detection antibodies, human, mouse, rat, and non-human primate cell lysates were examined by using 20 µg of cell lysates in Western blots and probing blots with the capture or detection antibody. Concentrations derived from the MSD assay are reported in the Tested Samples section below.



	Species	Result	Cell line
Lane 1	Human	negative	Jurkat LY924002 treated
Lane 2	Human	positive	Jurkat
Lane 3	Mouse	negative	NIH3T3 starved
Lane 4	Mouse	positive	NIH3T3 stimulated
Lane 5	Rat	negative	Rat L6 starved
Lane 6	Rat	positive	Rat L6 stimulated
Lane 7	Monkey	negative	COS-7 starved
Lane 8	Monkey	positive	COS-7 stimulated

Analyte Specificity

To assess the reactivity of the Total Akt assay with signaling proteins in the PI3K-Akt pathway, recombinant 4EBP1, GSK3β, p70s6K, FOX03a, PTEN, S6RP, and PRAS40 were run using the Total Akt Kit. No significant level of crossreactivity was observed (<0.04%).

Stability

Kit components were tested for freeze–thaw stability. Results (not shown) demonstrated that the MSD Akt calibrator and controls from the MSD Akt Control Pack can undergo 2 freeze-thaw cycles without significantly affecting the performance of the assay. The stability of experimental cell lysates should be determined by the end user for each cell model.

Partial Plate Stability

Assay plates were tested for stability following partial use. Partially used plates were subsequently sealed and stored in the original packaging (plate pouch with desiccant). Unused plate portions were stable for at least 14 days when stored at 2–8°C after initial use. Calibration curves should be prepared for each plate or partial plate run.

Tested Samples

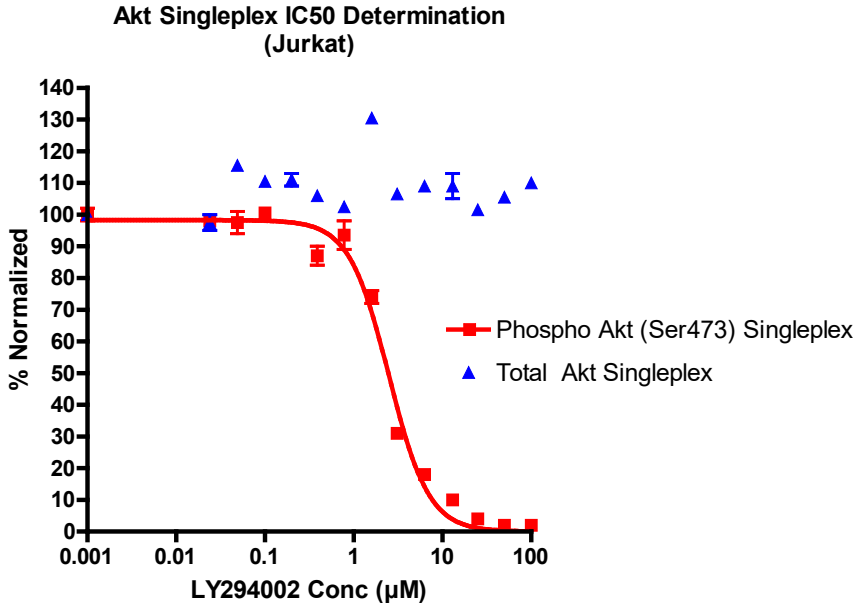
Single Point Treatments of Multiple Cell Lines

Test samples of immortalized mammalian cell lysates (10 µg/well) were characterized as being either positive or negative for phosphorylated Akt at serine 473. These lysate sets were assayed in triplicate with the Total Akt Kit. For each lysate set, catalog number, treatment, average pg/µg lysate, and %CV are displayed below. Akt was quantifiable in human, mouse, rat, and non-human primate cell lysates.

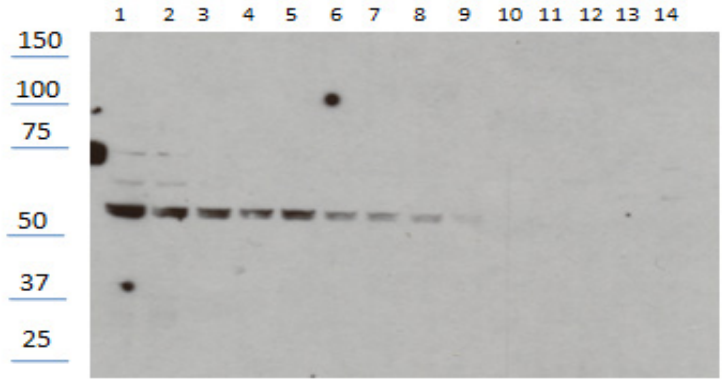
Sample Type	Positive Lysate				Negative Lysate			
	Treatment	Average Signal	pg/µg Lysate	%CV	Treatment	Average Signal	pg/µg Lysate	%CV
Jurkat (human) (Cat#C11CA-1)	Growing cells	329 914	31	5.4	50 µM LY294002 (2.5 hours)	222 984	21	3.0
MCF-7 (human) (Cat#C10JZ-1)	100 nM IGF-1 (20 minutes)	1 217 969	133	7.7	50 µM LY294002 (2.5 hours)	1 095 801	117	3.5
NIH3T3 (mouse)	1 mM sodium vanadate pretreatment (4 hours) followed by 33.5 nM Calyculin A (30 minutes)	699 180	88	3.4	Growing cells	896 334	117	1.8
Rat L6 (rat)	1 mM sodium vanadate pretreatment (4 hours) followed by 33.5 nM Calyculin A (30 minutes)	694 030	87	4.2	Growing cells	882 616	102	1.7
COS7 (non-human primate)	1 mM sodium vanadate pretreatment (4 hours) followed by 33.5 nM Calyculin A (30 minutes)	507 433	62	4.2	Growing cells	436 837	52	3.5

Dose Response Treatment of Jurkat Cells

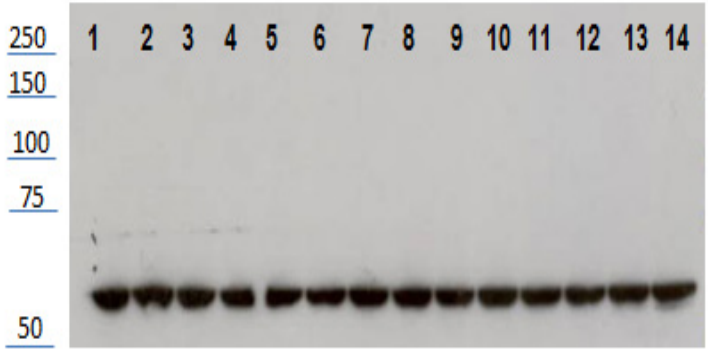
Human Jurkat cells were treated with serial dilutions of the PI3K inhibitor LY294002 for 30 minutes. In the graph below, average ECL signals from treated samples were normalized to the ECL signal from untreated Jurkat cells. The IC_{50} of LY294002 was defined as the concentration that suppressed phosphorylation of Akt (Ser473) by 50%. Levels of total Akt were not affected by treatment. (Western blots are shown below).



Phospho-Akt (Ser473)



Total Akt



	LY294002 (µM)
Lane 1	0
Lane 2	0.024
Lane 3	0.049
Lane 4	0.10
Lane 5	0.20
Lane 6	0.39
Lane 7	0.78
Lane 8	1.6
Lane 9	3.1
Lane 10	6.3
Lane 11	13
Lane 12	25
Lane 13	50
Lane 14	100

Tissue Samples

Tumor xenograft lysate samples were diluted to 400 µg/mL of total protein (10 µg/well) and tested using the Total Akt Kit.

Sample Type	Signal	pg/µg Lysate
Epithelial Melanoma (Human)	71 525	10
Kidney Carcinoma (Human)	19 503	2.7
Renal Cell Carcinoma (Human)	130 700	18

Assay Components

Calibrator

The Total Akt assay was calibrated using full length recombinant human Akt1 protein expressed in *baculovirus* and phosphorylated in vitro using phosphoinositide-dependent protein kinase-1 (PDK1) and MAP kinase-activated protein kinase 2 (MAPKAPK2). Akt concentrations in the reference lot were confirmed by amino acid analyses then used to bridge calibrator lots.

Antibodies

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

	Source Species		Assay Generation
Analyte	MSD Capture Antibody	MSD Detection Antibody	
Total Akt	Mouse Monoclonal	Mouse Monoclonal	C

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Appendix A: Suggested Cell Lysis Protocols

Most lysis buffers are compatible with MSD plates. Assay diluent, which shares some of the components of lysis buffer, should not be used to lyse cells unless you do not need to quantify lysate protein. MSD Tris Lysis Buffer (catalog #R60TX-3) and Inhibitor Pack (catalog #R70AA-1) are available for separate purchase. Together they contain reagents for preparation of complete lysis buffer.

For 10 mL of complete lysis buffer, combine:

- ❑ 100 µL Phosphatase Inhibitor I (100X)
- ❑ 100 µL Phosphatase Inhibitor II (100X)
- ❑ 100 µL Protease Inhibitor Solution (100X)
- ❑ 9.7 mL Tris Lysis Buffer (150 mM NaCl; 20 mM Tris, pH 7.5; 1 mM EDTA; 1 mM EGTA; and 1% Triton X 100)

Good practices:

- Inclusion of PMSF is protocol-dependent. PMSF should be slowly added to room temperature buffer solution to prevent precipitation.
- Lysis buffer should be ice-cold prior to cell lysis.

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 to add additional protease and phosphatase inhibitors to the matrix or diluent.

MSD provides these suggested cell lysis protocols; however, 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 aliquoted, 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 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. First, determine the number of cells of each cell type to be plated per well. 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.

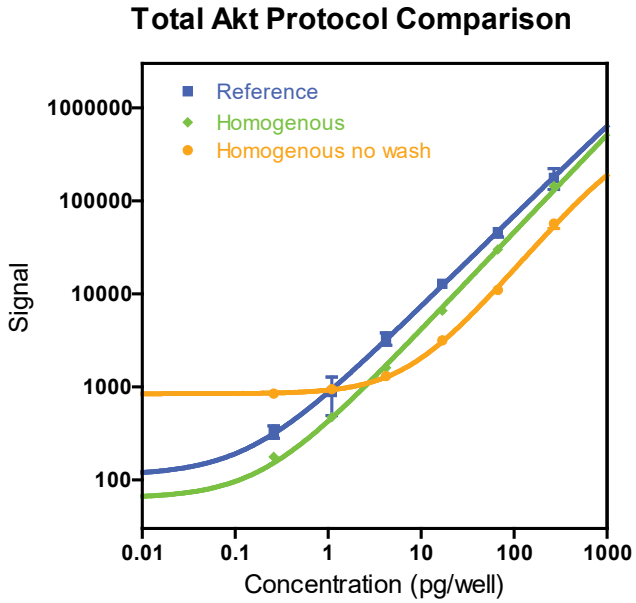
Adherent Cells. Plate cells on coated tissue culture plates to reduce variability due to cells lost as growth medium is removed. Treat cells as desired. Gently aspirate growth medium from the microwell plate to avoid disrupting the cell monolayer. A PBS wash step is not required and can introduce variability 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.

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.

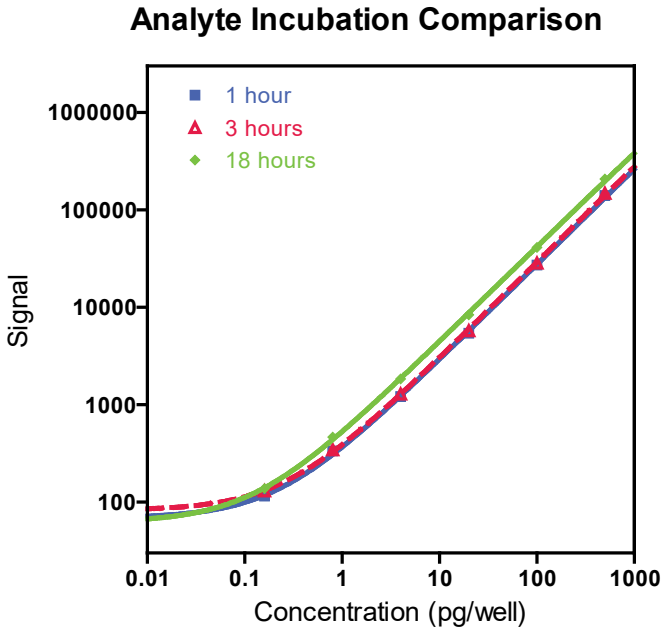
Carefully pipette cell lysate onto prepared plate and proceed with assay protocol. Note: It is important to transfer a constant volume and to avoid introducing air bubbles by pipetting too vigorously.

Appendix B: Performance of Alternate Protocols

Standard curves below illustrate the relative sensitivity for the reference protocol (blue curve, standard protocol), Alternate Protocol 1 (green curve, homogeneous protocol) and Alternate Protocol 2 (yellow curve, homogeneous, with no wash steps).



Standard curves below illustrate the relative sensitivity for a 1-hour sample incubation time (recommended protocol) compared to 3 hours or overnight (18 hours).



Summary Protocol

Total Akt Kits

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

Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the calibrator on ice.

Prepare Blocker A solution.

Prepare calibrator solutions:

- Dilute the stock calibrator 20-fold in assay diluent to prepare an intermediate solution.
- Prepare the highest calibrator by diluting the intermediate solution 4 fold.
- Perform a series of 6 additional 4-fold dilution steps and prepare a zero calibrator.

Dilute samples to 3.0-800 µg/mL in assay diluent before adding to the plate.

Prepare detection antibody solution by diluting stock detection antibody 50-fold in antibody dilution buffer.

Prepare 1X Read Buffer T by diluting stock 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 (calibrators, 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.

Read plate on MSD instrument.

Plate Diagram