Quantitative Immunoassays to Measure Total Akt-1 and Phospho-Akt (Ser473) in Cell and Tissue Lysate Models

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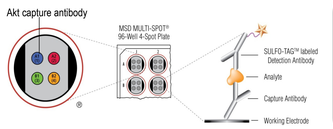
1 Abstract

Akt1, Akt2, and Akt3 kinases are critical effectors of the activated tyrosine receptor kinase/phosphoinositol 3-kinases, influencing cell growth, proliferation, and survival. Akt1-specific substrates have been implicated in cell cycle progression and cell motility, processes that underlie increased proliferation and metastatic spread of cancer cells. In this study, we report development and analytical validation of immunoassays for quantification of total Akt1 and phospho-Akt (Ser473) using fit-for-purpose and CLSI principles. We also demonstrate the ability of these assays to quantify total Akt1 and phospho-Akt (Ser473) in tumor-derived cell lines and mouse xenograft tumor lysates. These assays allow cancer researchers to correlate characteristics of cancer cells with absolute protein levels.

The Akt1 singleplex assays were developed using MULTI-ARRAY® technology. A monoclonal mouse antibody specific for total Akt1 was used to capture Akt1 in the solid phase, and SULFO-TAG™-labeled antibodies specific for an alternate epitope of total Akt1 or phospho-Akt (Ser473) were used as assay-specific reporters. Both assays were calibrated using full-length recombinant human Akt1 protein expressed in *baculovirus* and phosphorylated in vitro by sequential incubation with phosphoinositide-dependent protein kinase-1 (PDK1) and MAP kinase-activated protein kinase 2 (MAPKAPK2). Akt calibrator concentrations were assigned following multi-day testing. Akt1 concentrations were confirmed by quantitative amino acid analyses, and units of phosphorylation (U_{P473}) were assigned. The functional performance of the Akt1 calibrator was verified to be equivalent across 3 lots.

Both assays demonstrated sensitivity (lower limits of detection were 0.028 pg/well for the total Akt1 assay and 0.005 U_{P473}/well for the phospho-Akt (Ser473) assay) and inter-plate reproducibility (coefficients of variation were below 12.1% for both assays). Dilution linearity and spike recovery testing demonstrated minimal matrix effects and accurate quantitation (recovery values in the range of 75–120% of the expected values were observed). The assays showed no substantial cross-reactivity with Akt2 or Akt3 or with the active forms phospho-Akt (Ser472) and phospho-Akt (Ser474). Calibration with recombinant protein enabled the absolute quantification of both total and phospho-Akt (Ser473) levels in cultured cell lysates (including MCF-7, Jurkat, NIH3T3, rat L6, and COS7); epithelial, renal cell, and kidney carcinomas; and human tissue lysates.

2 Methods



Protocol

1. Add 150 µL Blocker A. Incubate for 1 hour at room temperature (RT).
2. Wash with TBS-T. Add 25 µL of calibrator, control, or diluted sample. Incubate for 1 hour at RT.
3. Wash with TBS-T. Add 25 µL of detection antibody. Incubate for 1 hour at RT.
4. Wash with TBS-T. Add 150 µL of 1X Read Buffer T. Read on MSD® imager.

Total Akt and phospho-Akt (Ser473) singleplex assays were developed and optimized for electrochemiluminescence detection. The assays use a common capture antibody; total Akt and phospho-Akt (Ser473) are detected using anti-total Akt and anti-phospho-Akt (Ser473) antibodies, respectively. Akt assay controls were prepared by diluting Jurkat cell lysates to 400 µg/mL and 25 µg/mL in lysis buffer supplemented with protease and phosphatase inhibitors. We performed a complete analytical validation of the kits.

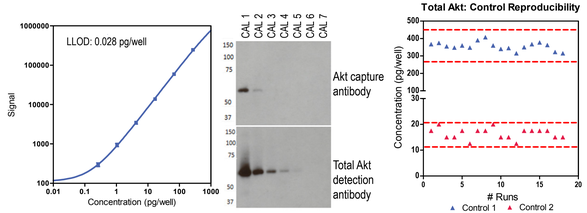
3 Isoform Specificity

Protein Conc. pg/well	Total Akt Isoform Specificity: Assay Signal Akt1	Akt2	Akt3	% Cross-reactivity Akt1	Akt2	Akt3
78	71 045	69	264	N/A	0.10	0.38

Protein Conc. U_{P473}/well	Phospho-Akt (Ser473) Isoform Specificity: Assay Signal pAkt1 (Ser473)	pAkt2 (Ser472)	pAkt3 (Ser474)	% Cross-reactivity pAkt1 (Ser473)	pAkt2 (Ser472)	pAkt3 (Ser474)
3.1	36 543	217	538	N/A	0.5	1.5

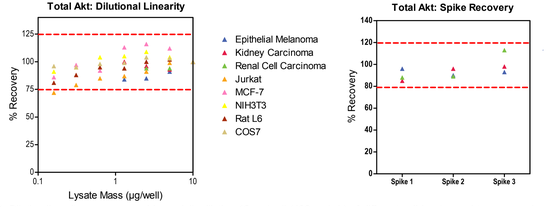
Assay specificity to Akt isoforms was assessed by assaying full-length, recombinant human phospho-Akt1 (Ser473), phospho-Akt2 (Ser472), and phospho-Akt3 (Ser474). Neither assay exhibited substantial reactivity with either phospho-Akt2 (Ser472) or phospho-Akt3 (Ser474). Total Akt and phospho-Akt (Ser473) assays are specific to the Akt1 isoform.

4 Total Akt: Assay Performance



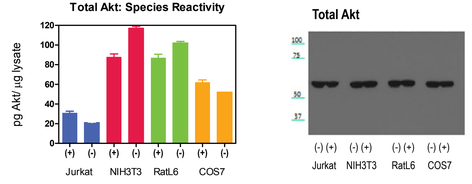
Left: Calibration curve for total Akt. The average lower limit of detection (LLOD) computed across 18 runs is shown.
Middle: Western blots of diluted Akt calibrators confirm antibody specificity of capture and detection antibodies. The Western blots are not as sensitive as the MSD immunoassay.
Right: Akt controls (Jurkat lysates) were assayed in 18 runs by 2 operators over 2 months. Dashed red lines represent +/-20% from the target control value.

5 Total Akt: Matrix Tolerance: Linearity and Spike Recovery



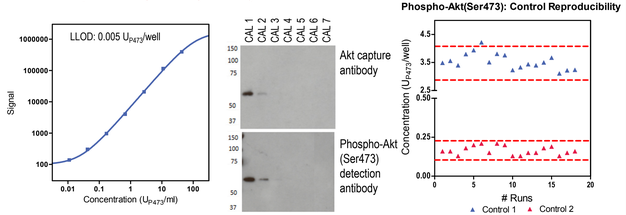
Left: Dilution linearity was assessed by serially diluting 10 µg/well (400 µg/mL) of different cell lysates and tumor samples. Average percent recoveries at each dilution factor are shown. Dashed red lines represent boundaries of +/-25% recovery.
Right: Spike recovery was assessed by diluting tumor xenograft lysate samples to 1.25 µg/well (50 µg/mL) and spiking with Akt calibrator at 3 levels throughout the assay range. Recoveries were within 15% of the expected concentrations. Dashed red lines represent boundaries of +/-20% recovery.

6 Total Akt: Species Reactivity



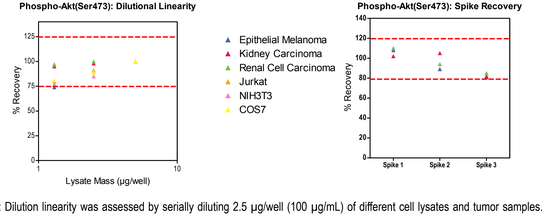
Species reactivity was evaluated by testing human (Jurkat), mouse (NIH3T3), rat (RatL6), and non-human primate (COS7) cell lysates in which Akt phosphorylation was either stimulated (+) or suppressed (-). Total Akt protein was quantifiable across species, showing modest changes in concentration with different treatment conditions. MSD assay and Western blot results are shown.

7 Phospho-Akt (Ser473): Assay Performance



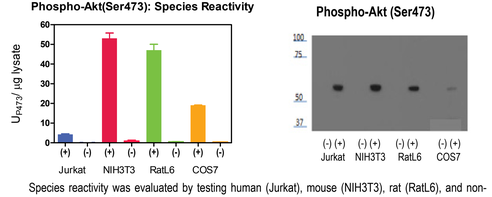
Left: Calibration curve for phospho-Akt (Ser473). The average LLOD computed across 18 runs is shown.
Middle: Western blots of diluted Akt calibrators confirm antibody specificity of capture and detection antibodies. The Western blots are not as sensitive as the MSD immunoassay.
Right: Akt assay controls (Jurkat lysates) were assayed on 18 plates by 2 operators over 2 months. Dashed red lines represent +/-20% from the target control value.

8 Phospho-Akt (Ser473): Matrix Tolerance: Linearity and Spike Recovery



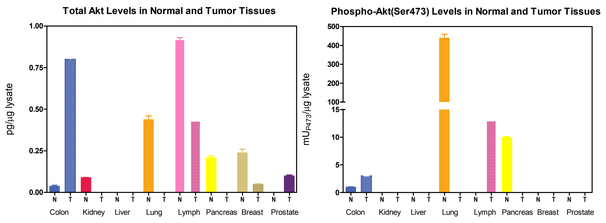
Left: Dilution linearity was assessed by serially diluting 2.5 µg/well (100 µg/mL) of different cell lysates and tumor samples. The average percent recoveries at each dilution factor are shown. Dashed red lines represent boundaries of +/-25% recovery.
Right: Spike recovery was assessed by diluting tumor xenograft lysate samples to 1.25 µg/well (50 µg/mL) and spiking with Akt calibrator at 3 levels throughout the assay range. Recoveries were within 20% of the expected concentrations. Dashed red lines represent boundaries of +/-20% recovery.

9 Phospho-Akt (Ser473): Species Reactivity



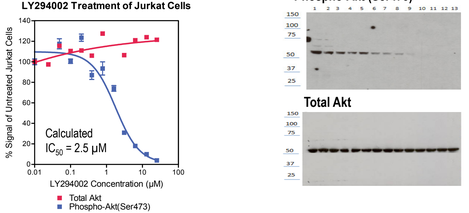
Species reactivity was evaluated by testing human (Jurkat), mouse (NIH3T3), rat (RatL6), and non-human primate (COS7) cell lysates in which Akt phosphorylation was either stimulated (+) or suppressed (-). Phospho-Akt (Ser473) protein was quantifiable across species and treatment conditions. MSD immunoassay and Western blot results are shown.

10 Akt Quantitation in Human Tissue Samples



Total and phospho-Akt (Ser473) were measured in normal (N) and tumor (T) human tissues. Akt levels varied across tissue types and N/T status indicating the significance of Akt expression and phosphorylation in these samples. Optimization of lysis conditions may improve analyte detection. Samples were not collected with optimal concentrations of phosphatase inhibitors.

11 Pharmacodynamics of LY294002



Human Jurkat cells were treated with serial dilutions of the PI3K inhibitor LY294002 for 30 minutes. ECL signals from treated samples were normalized to ECL signals in untreated samples. IC_{50} is the concentration that inhibited Akt phosphorylation at Ser473 by 50%. Total Akt levels were not affected by treatment. Assay data were consistent with Western blot results.

12 Conclusion

Total Akt and phospho-Akt (Ser473) immunoassays were developed and tested for suitability to measure total and phosphorylated protein in cell lysate and xenograft models. The assays were shown to have reproducible performance demonstrated by precision of calibration curves and controls, and proteins were quantitated in multiple matrices. These assays will support ongoing efforts to study intracellular post-translational modifications and may be expanded to accommodate additional proteins in the Akt signaling pathway.

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