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PhosphoSens AKT1/2/3 Cell Lysate Kinase Assay Kit Protocol & Validation Data

Determination of AKT Activity in Crude Lysates from PDGF-treated NIH-3T3 Cells Using the Selective AQT0982 Sensor Peptide Substrate

HGNC Name: AKT1 (PKB), AKT2 (PKBbeta), and AKT3 (PKBgamma)
Long Names: RAC-alpha serine/theonine-protein kinase, RAC-beta serine/theonine-protein kinase, RAC-gamma serine/theonine-protein kinase

PhosphoSens AKT1/2/3 Cell Lysate Kinase Assay Kit (AQT0982-KL-100)

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MATERIALS INCLUDED

TABLE 1		
Components	**Description**	**100 Assay Kit Volumes**
PhosphoSens Kinase-Selective Lysate Substrate, AQT0982, 1mM	AKT1/2/3-selective sensor peptide substrate for assaying kinase activity in complex biological samples	45 µL
ATP Solution, 100 mM	100 mM ATP in nuclease-free water	30 µL
DTT Solution, 1 M	1 M DTT in nuclease-free water	5 µL
Enzyme Reaction Buffer (ERB), 10X	500 mM HEPES, pH 7.5, 0.1% Brij-35, 100 mM MgCl2	300 µL
Enzyme Dilution Buffer (EDB), 5X Base	20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/mL Bovine Serum Albumin (BSA)	800 µL
EGTA Solution, 550 mM	550 mM EGTA in water (pH adjusted with NaOH)	30 µL
PhosphoPreserve Cell Extraction Buffer, 1X Base	Please reach out to support@assayquant.com to inquire	1,000 µL
PhosphoPreserve Protease Inhibitor Cocktail	Please reach out to support@assayquant.com to inquire	20 µL
PhosphoPreserve Phosphatase Inhibitor Cocktail	Please reach out to support@assayquant.com to inquire	20 µL
PhosphoSens PhosphoControl Peptide, AQT1146, 1 mM	Fully phosphorylated version of the AKT1/2/3-selective sensor peptide substrate, AQT0982.	10 µL
NIH3T3 Lysate (+PDGF)*	Crude lysate of NIH3T3 cells treated with 50 ng/mL of PDGF after an overnight serum depletion to make the cells quiescent. Lysate is in PhosphoPreseve CEB, supplemented with DTT, and PhosphoPreserve Protease and Phosphatase inhibitor cocktails.	25 µL
Pre-activated AKT1*	AKT activated with a lipid/detergent mix, MK2, and PDK1	2 µL
Rizavasertib (A-443654)	Rizavasertib (A-443654) resuspended in 100% DMSO	5 µL
Vevorisertib trihydrochloride (ARQ 751 trihydrochloride)	Vevorisertib trihydrochloride (ARQ 751 trihydrochloride) resuspended in 100% DMSO	5 µL

MATERIALS NOT INCLUDED

1. Ultra-pure deionized water
2. Dimethyl Sulfoxide (DMSO), $(CH_3)_2SO$
3. Precision pipettes capable of dispensing down to 0.5 µL and pipette tips. Having both single and multichannel pipettes is helpful
4. Plasticware: Low Protein Binding Microcentrifuge Tubes (0.5 and 1.5 mL), and materials to make your own cell or tissue lysates for a titration.
5. Centrifuge capable of spinning plates at 200xg and microfuge tubes at 10,000xg (standard microcentrifuge)
6. Fluorescence microplate reader capable of reading kinetically with filter setup for excitation (360 nm) and emission (485 nm) wavelengths. Alternatively, an instrument with a monochromator can be used to set the excitation (360 nm) and emission (485 nm) wavelengths, although this can reduce the assay sensitivity.

Lysate Assay Conditions and Reaction Setup

Assay Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/mL BSA

0.54 mM EGTA

10 mM $MgCl_2$

15 µM Sensor Peptide substrate, AQT0982 or Phosphopeptide control, AQT1146

Kinase Enzyme:

- 0.5 nM Preactivated AKT1 Recombinant Enzyme
- **For simple lysate assay** – 2.0 µg/well for NIH3T3 lysates from cells treated ± 50 ng/mL PDGF for 15 minutes following overnight serum starvation using 0.1% FBS to make cells quiescent. The extent to which quiescence is achieved will vary by cell line and the pre-treatment conditions used.
- **For Lysate titration** –***This test is performed with your cell lysates and not the samples provided. Lysates should be 1 mg/mL or higher total protein concentration.*** *Example experiments are provided for lysates from PDGF-treated NIH3T3 cells (slides 15). With NIH3T3 lysates we started at 10000 ng/well (final linear range was 78-2500 ng/well or 32-fold). This lysate titration should be determined empirically and will vary depending on the cell line and the treatment conditions.*

Notes:

1. Total protein concentration for cell lysates was determined using a modified Bradford assay (Cat # 5000006, BIO-RAD).
2. **PhosphoPreserve Cell Extraction Buffer:** Should be **supplemented with included protease and phosphatase inhibitors** as prepared on slides 5 and 11 **just before use**.
3. **Final 1X Enzyme Dilution Buffer (EDB):** 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/ml Bovine Serum Albumin (BSA), and supplemented with 0.2 mM EGTA and 1 mM DTT just before use.
4. Reactions were run in 25 µL final volume in Corning, low volume 384-well, white flat bottom polystyrene NBS microplates (Cat. #3824) after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185] or Dot Scientific [Cat. #T480]) in a Biotek Synergy Neo2 microplate reader with filter setup for excitation (360 nm) and emission (485 nm) wavelengths.
    - Alternatively, reactions can be run in 50 µL in Corning, half-area 96-well, white flat-bottom polystyrene NBS microplates (Cat. # 3642).

Reagent Preparation for a Low Volume 384-well Format (25 µL final reaction volume)*

*If you are working in a Corning 96-well plate (Cat. # 3642), multiply the volume of components by 2 for a final reaction volume of 50 µL per well

1. Using the stock solutions provided with the kit (Table 1), prepare the reagents shown in Table 2 and Table 3 (final concentrations shown in parentheses).
   - **10 mM ATP :** Make 90 µL of 10 mM ATP by adding 9 µL of 100 mM ATP to 81 µL of ultrapure deionized water.
   - **10 mM DTT:** Make 300 µL of 10 mM DTT by adding 3 µL of 1M (1000 mM) DTT to 297 µL of ultrapure deionized water.
   - **5.5 mM EGTA:** Make 300 µL of 5.5 mM EGTA by adding 3 µL of 550 mM EGTA to 297 µL of ultrapure deionized water.
   - **150 µM Sensor Peptide:** Make 90 µL of 150 µM AQT0982 by adding 13.5 µL of 1 mM AQT0982 to 76.5 µL of ultrapure deionized water. Make 14 µL of 150 µM AQT1146 by adding 2.1 µL of 1 mM AQT1146 to 11.9 µL of ultrapure deionized water.
   - **250 µM AKT 1/2/3 Inhibitors (Rizavasertib/ Vevorisertib) (50X):** Make 100 µL of 250 µM Rizavasertib/ Vevorisertib by adding 2.5 µL of each 10 mM stock into 95 µL DMSO.
2. Prepare final 1X *PhosphoPreserve* Cell Extraction Buffer by adding 16.7 µL of the provided Inhibitor Cocktails for Proteases and Phosphatases to the 1000 µL of *PhosphoPreserve* Cell Extraction Buffer, Base. Keep on ice prior to extracting cells.
3. Prepare '1.28X Master Mix' by combining volumes of the components listed in Tables 2 and 3. The volumes for a single well and 32 wells (Table 2) or 5 wells (Table 3) are shown.
4. Prepare 624 µL (sufficient for 32 wells but test requires only 28 wells and includes dead volume) of 1.28X Master mix per Table 2 and 97.5 µL of 1.28X Master mix per Table 3.
5. When adjusting the volume for a different number of wells, ensure that you include an additional 8% dead volume above the actual volume required.

Table 2 - Selective Sensor Peptide Substrate AQT1076

Components for 1.28X Master Mix:	For 1 Well:	For 32 Wells:
Enzyme Reaction Buffer (**10X**)	2.5 µL	80 µL
ATP (**10 mM**)	2.5 µL	80 µL
DTT solution (**10 mM**)	2.5 µL	80 µL
EGTA Solution (**5.5 mM**)	2.5 µL	80 µL
Selective Sensor Peptide Substrate ***AQT0982*** (**150 µM**)	2.5 µL	80 µL
Ultrapure deionized water	7.0 µL	224 µL
Total volume	**19.5 µL**	**624 µL**

Table 3 - Phospho-Peptide Control AQT1107

Components for 1.28X Master Mix:	For 1 Well:	For 5 Wells:
Enzyme Reaction Buffer (**10X**)	2.5 µL	12.5 µL
ATP (**10 mM**)	2.5 µL	12.5 µL
DTT solution (**10 mM**)	2.5 µL	12.5 µL
EGTA Solution (**5.5 mM**)	2.5 µL	12.5 µL
Sensor Phosphopeptide Control ***AQT1146*** (**150 µM**)	2.5 µL	12.5 µL
Ultrapure deionized water	7.0 µL	35.0 µL
Total volume	**19.5 µL**	**97.5 µL**

Step-by-Step Guide to Performing a Lysate Activity Assay in a 384-well Plate

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A plate map for a simple lysate assay is shown on the next slide, which serves as a guide for making additions to the plate as outlined below.

1. Prepare 1X EDB using the 5X stock of EDB, Base provided, and supplement with DTT and EGTA. For example, to make 5000 µL of 1X EDB, add 1000 µL 5X EDB Base along with 5 µL of 1M DTT, 5 µL of 550 mM EGTA, and 3990 µL of ultrapure deionized water to create the final composition shown on slide 5. **Keep on ice.**
2. Prepare 250 µL BLANK by adding 167 µL 1X EDB and 83 µL of 1X Final PhosphoPreserve Cell Extraction Buffer .
3. Prepare 250 µL of 2.5 nM recombinant AKT1 (5X) by adding 0.625 µL of the 1000 nM AKT1 stock to 249.375 µL of 1X EDB. **Keep on ice until needed.**
4. Prepare 35 µL of 0.20 mg/mL lysate by adding 1.82 µL of the 3.8 mg/mL PDGF treated HEK293 stock to 33.25 µL of 1X EDB, and 1.96 µL of the 3.6 mg/mL untreated HEK293 stock to 33.25 µL of 1X EDB. **Keep on ice until needed.**
5. Transfer 20 µL of stimulated lysate to a new tube and heat at 95 °C for 5 minutes to serve as a Heat Inactivated (HI) negative control. Remove the tube and cool to room temperature.
6. Add 0.5 µL of 250 µM AKT 1/2/3 Inhibitor Mix to all wells in columns 2, 5 and 7 (as shown on the plate map on the next slide). Add 0.5 µL of DMSO to all wells without the tool compound in columns 1, 3, 4, 6 and 8 (as shown on the plate map on the next slide).
7. Add 19.5 µL of 1.28X Master Mix for AQT0982 from Table 2 to all the wells except the wells labeled 'AQT1146'. For the wells labeled 'AQT1146', add 19.5 µL of 1.28X Master Mix for AQT1146 from Table 3.
8. Seal the plate using the plate seal supplied and press down with the supplied paddle. Incubate at 30 °C for 15 minutes to equilibrate the plate and Master Mix. This can be done by placing the plate inside a plate reader set at 30 °C. This step is important to prevent temperature changes that can create anomalies in the data at the beginning of the reaction.

   Note: The additions in step 8 and step 9 should be performed quickly since the reaction will start with these additions.

9. Source of Kinase Enzyme or control (BLANK or EDB, for background determination and the AQT1146 sensor phosphopeptide positive control):
   - Add 5 µL BLANK (prepared in step 2 above) to the wells labeled 'BLANK' or 1X EDB to wells labeled '1X EDB'
   - Add 5 µL 1X EDB to wells labeled 'AQT1146'
   - Add 5 µL of the Control (-PDGF) or stimulated (+PDGF) lysates to the corresponding wells labeled '1.0 µg Lysate'
   - Add 5 µL of 2.5 nM AKT1 (5X) to the wells labeled '0.5 nM AKT1'.
10. Re-seal the plate, centrifuge at 200xg for one minute, and place the plate in the microplate reader set at 30 °C.
11. Read the plate in kinetic mode with continuous fluorescence intensity detection (Ex/Em 360/485 nm) every 2 minutes for 1-4 hours. The frequency of the readings and the overall duration can be adjusted as needed.

Plate Additions and Plate Map For a Simple Lysate Activity Assay

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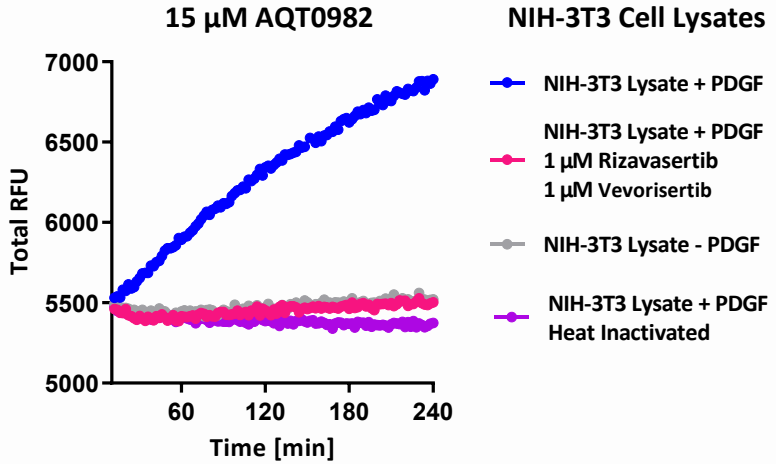
Component	Volume to add to wells
Tool Compound or 100% DMSO	0.5 µL
Master mix	19.5 µL
Lysate, AKT1, BLANK, or 1X EDB	5 µL

Total volume 25 µL

	Plate map for Lysate activity assay							
	Stimulated lysates +PDGF			With recombinant enzyme		Control lysates -PDGF		
	No tool compounds	With tool compounds (Rizavasertib/ Vevorisertib)	Heat inactivated lysate	No tool compounds	With tool compounds (Rizavasertib/ Vevorisertib)	No tool compounds	With tool compounds (Rizavasertib/ Vevorisertib)	Phosphopeptide control
	1	2	3	4	5	6	7	8
A	BLANK	BLANK	BLANK	1X EDB	1X EDB	BLANK	BLANK	AQT1146
B	BLANK	BLANK	BLANK	1X EDB	1X EDB	BLANK	BLANK	AQT1146
C	1.0 ug Lysate	1.0 ug Lysate	1 .0 ug HI lysate	0.5 nM AKT1	0.5 nM AKT1	1.0 ug Lysate	1.0 ug Lysate	AQT1146
D	1.0 ug Lysate	1.0 ug Lysate	1 .0 ug HI lysate	0.5 nM AKT1	0.5 nM AKT1	1.0 ug Lysate	1.0 ug Lysate	AQT1146

Progress Curves for Total Fluorescence with AQT0982



AQT1146 data can be found on slide 10

This test uses 32 wells (28 wells for the AQT0982 sensor peptide substrate and 4 wells for the AQT1146 phosphopeptide control). All conditions are tested in duplicate.

Data Analysis

1. Collect the data from the microplate reader. This is a complete time course (Progress Curve) for every well with values in Relative Fluorescence Units (RFU) for each time point for Total (for each experimental condition) and “EDB/blank” wells.

2. Take the average of duplicate “EDB/blank” wells for each condition at each time point. Subtract the average EDB/blank values from the corresponding Total RFU of individual wells for each condition at each time point to obtain the background-corrected RFU values. For example, take the average of A1 and B1, and subtract the value from the total RFU determined for individual wells C1 and D1 at each time point. You can then either plot these RFU (Corrected) values separately to assess individual wells or take the average of the RFU (Corrected) values at each time point and plot this data.

3. It is highly recommended to run the “EDB/blank" wells at each compound concentration to correct for tool compound autofluorescence, if any. Since this is a kinetic assay format, the background with compounds will not change over time and can be subtracted from the total.

4. From the plot of the RFU (Corrected) values, determine the slope from the points in the linear region. This is the “initial reaction rate” in RFU (Corrected)/min. We recommend using ~30 minutes of the linear region of the progress curve to determine the rate. This can be performed in Excel, Excel-Fit, GraphPad Prism, the software provided with your microplate reader, or any other suitable software package, such as DynaFit, GeneData Screener, KinTek, Mathematica, MATLAB, or SigmaPlot.

5. Compare the RFU (Corrected)/min values for the samples to evaluate the activity of the kinase in each sample.

6. Refer to slide 10 for representative validation data for this simple lysate assay.

Data for a Simple Activity Assay Validation

NIH-3T3 Cell Lysate or Recombinant AKT1 with AQT0982 Sensor Peptide Substrate or AQT1146 Control

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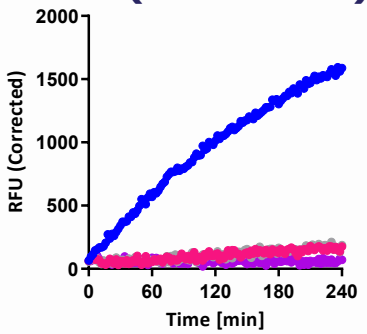
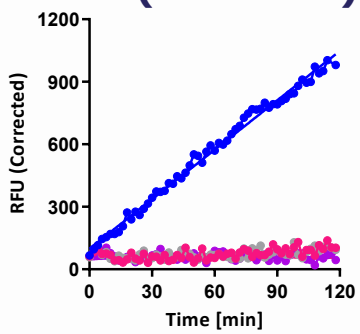
A. Crude Lysate Samples (2.0 µg/well)

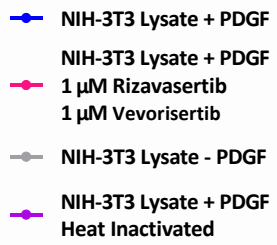
PROGRESS CURVES

1) Full Time Course (0-240 min.)



2) Linear Range (0-120 min)



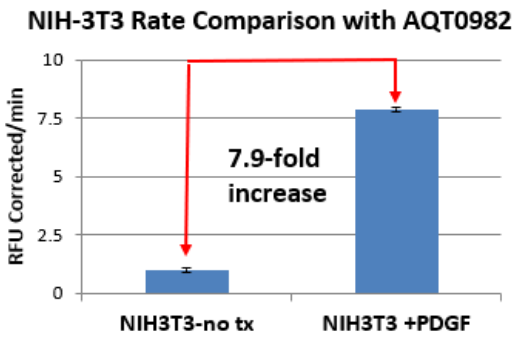


QUANTITATIVE ASSESSMENT

3) Reaction Rates to Assess Change

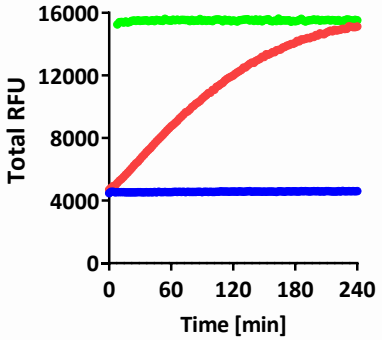
Description of Conditions for Crude Cell Lysates (2 µg/well)	Reaction Rate (RFU/min)	Change
NIH-3T3 Control lysate (-PDGF)	1.0 ± 0.07	
NIH-3T3 lysate (+50 ng/mL PDGF)	7.9 ± 0.10	8-fold increase above control
NIH-3T3 lysate (+50 ng/mL PDGF), Heat Inactivated	0	100% inhibition of PDGF Activation
NIH-3T3 lysate (+50 ng/mL PDGF) + 1.0 µM Rizavasertib & 1.0 µM Vevorisertib	0.41 ± 0.08	100% inhibition of PDGF Activation

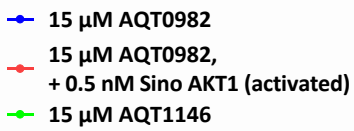
4) Histogram



B. Recombinant AKT1 & AQT1146 Control

1) Full Time Course (0-240 min.)





AQT1146 is a chemically synthesized phosphopeptide control

A. Crude lysate samples: The AQT0982 sensor peptide was used to generate **1)** Full progress curve time course (0-240 min.) and **2)** Linear range (0-120 min.). The reaction rates (RFU Corrected values [Total – Background]/min. +/- standard deviations) are the slope of the linear region of each progress curve, which are presented in the table in **3)** and as a histogram in **4)**, highlighting a 7.9-fold activation of AKT kinase activity in lysates from NIH3T3 cells treated with PDGF. The signal was eliminated by heat inactivation of the lysate or by adding the selective AKT inhibitors Rizavasertib (ATP Competitive) and Vevorisertib (Allosteric) to the reactions. **Note:** The amount of activation depends on several factors, including cell type, the serum deprivation pretreatment used to make cells quiescent, as well as the nature, concentration, and duration of the activating stimulus (in this experiment, we used 50 ng/mL PDGF for 15 minutes) as described on slide 7. These conditions can be varied to determine the effect on AKT activity. The total amount of AKT protein can be determined by Western Blotting or an ELISA; however, with the short stimulation times typically used, these levels are not expected to change.

B. Purified recombinant AKT1 enzyme & AQT1146 Control: The full-length and activated AKT1 protein (0.5 nM) fully phosphorylated the AQT0982 sensor peptide substrate by 240 min., as shown by convergence with the signal obtained with the AQT1146 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with AQT1146 is used to convert RFU (Corrected) values to nmoles of phosphopeptide product.

Reagent Preparation for a 384-well Format (25 µL final reaction volume) Lysate Titration*

*If you are working in a Corning 96-well plate (Cat. # 3642), multiply the volume of components by 2 for a final reaction volume of 50 µL per well

This test is performed with your cell lysates and not the samples provided. Lysates should be 1 mg/mL or higher total protein concentration

1. Using the stock solutions provided with the kit (Table 1), prepare the reagents shown in Table 4 (final concentrations shown in parentheses).
   - **10 mM ATP :** Make 120 µL of 10 mM ATP by adding 12 µL of 100 mM ATP to 108 µL of ultrapure deionized water.
   - **10 mM DTT:** Make 300 µL of 10 mM DTT by adding 3 µL of 1M (1000 mM) DTT to 297 µL of ultrapure deionized water.
   - **5.5 mM EGTA:** Make 300 µL of 5.5 mM EGTA by adding 3 µL of 550 mM EGTA to 297 µL of ultrapure deionized water.
   - **150 µM Sensor Peptide:** Make 105 µL of 150 µM AQT0982 by adding 15.75 µL of 1 mM AQT0982 to 89.25 µL of ultrapure deionized water.
2. Prepare final 1X *PhosphoPreserve* Cell Extraction Buffer by adding 16.7 µL of the provided Inhibitor Cocktails for Proteases and Phosphatases to the 1000 µL of *PhosphoPreserve* Cell Extraction Buffer, Base. Keep on ice prior to extracting cells.
3. Prepare lysate from cells following treatment with a stimulus to activate the AKT pathway (e.g., NIH3T3 + PDGF) using sufficient cells to achieve a final concentration of 1 mg/mL of total protein determined using a modified Bradford assay (Cat # 5000006, BIO-RAD). **See next slide for additional details.**
4. Prepare '1.25X Master Mix' by combining volumes of the components listed in Table 4. The volumes for a single well and 40 wells are shown.
5. Prepare 800 µL (sufficient for 40 wells but this test requires only 36 wells and includes dead volume) of 1.25X Master mix per Table 4.
6. When adjusting the volume for a different number of wells, ensure that you include an additional 8% dead volume above the actual volume required.

Table 4 – Selective Sensor Peptide Substrate AQT1076

Components for 1.25X Master Mix:	For 1 Well:	For 40 Wells:
Enzyme Reaction Buffer (**10X**)	2.5 µL	100 µL
ATP (**10 mM**)	2.5 µL	100 µL
DTT solution (**10 mM**)	2.5 µL	100 µL
EGTA Solution (**5.5 mM**)	2.5 µL	100 µL
Selective Sensor Peptide Substrate ***AQT0982*** (**150 µM**)	2.5 µL	100 µL
Ultrapure deionized water	7.5 µL	300 µL
Total volume	**20 µL**	**800 µL**

5.0 µL of crude lysate diluted in 1X EDB or BLANK (2:1 ratio of 1X EDB: 1X Final *PhosphoPreserve* Cell Extraction Buffer-A) alone for the blanks is added to each well (see slides 12 - 14 for detailed protocol).

Preparation of Crude Cell Lysates

Example: Preparing NIH3T3 lysates (+/- 50 ng/mL PDGF)

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Generate a lysate from cells following treatment with a stimulus to activate the AKT pathway (e.g., NIH3T3 cells + PDGF) using sufficient cells to achieve a final concentration of 1 mg/mL of total protein.

1. **Cell Culture and Stimulation:**
   a. 600,000 NIH-3T3 Cells were plated in 2 mL per well in 6-well tissue culture-treated plates and incubated at 37 °C in DMEM Medium with 10% FBS and 1% PenStrep in an atmosphere of 5% CO_2.
   b. Cells at ~90% confluency (~ 2.0 X 10^6 cells) were washed with PBS, the residual liquid aspirated, and the cells serum-starved in DMEM Medium with 0.1% FBS and 1% PenStrep for 24 hours.
   c. Cells were then incubated for 15 min with or without 50 ng/mL PDGF-bb (ThermoFisher, 100-14B-10UG) to stimulate the AKT pathway.

2. **Lysate Preparation:**
   a. After washing with PBS and the residual liquid aspirated, cells were lysed in 30 µL per well of ice-cold 1X *PhosphoPreserve* CEB supplemented with protease and phosphatase inhibitors added just before use per slide 11. Rotate the plate to cover the cells completely with CEB and then use a cell scraper to ensure all cells are detached, followed by a pipette tip to wash the surface several times and break up any clumps of cells.
   b. Collect the lysate into a 0.5 mL microcentrifuge tube and then break up the DNA strands, if necessary, by passing through a 22-gauge needle 3 times, adding DNAase, or briefly sonicating on ice for 2 seconds on low power, followed by a 5 min spin at 10,000xg in a microcentrifuge at 4 °C. Remove and retain the supernatant and keep on ice. Determine the protein concentration and then immediately set up a lysate activity assay to determine the linear range. Alternatively, make aliquots and then snap freeze in liquid nitrogen or using dry ice in ethanol, and store at -80 °C.

This procedure yielded 30 µL per well of ~1.1 mg/mL total protein. This can be scaled up or down with larger flasks or plates. The yield may vary slightly with cell size or passage number. The number of freeze-thaws should be minimized until the stability is demonstrated.

Step-by-Step Guide to Performing a Lysate Activity Assay Titration in a 384-well plate

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A plate map for lysate dose-dependent assay is shown on the next slide, which serves as a guide for making additions to the plate as outlined below.

This test is performed with your cell lysates and not the samples provided. Lysates should be 1 mg/mL or higher total protein concentration

1. Transfer 20 µL of 1.25X Master Mix per well to Rows A-C (triplicate samples) of the assay plate utilizing a multichannel pipette.
2. Seal the plate using the optically clear plate seal supplied and press down with the supplied paddle. Incubate at 30°C for 15 minutes to equilibrate the plate and Master Mix. This can be done by placing the plate inside a plate reader set at 30 °C. This preincubation is performed just prior to adding the 5 µL of sample.
3. Prepare 1X EDB using the 5X stock of EDB, Base provided, and supplement with DTT and EGTA. For example, to make 5000 µL of 1X EDB, add 1000 µL 5X EDB Base along with 5 µL of 1M DTT, 5 µL of 550 mM EGTA, and 3990 µL of ultrapure deionized water to create the final composition shown on slide 5. **Keep on ice.**
4. Prepare 60 µL of 0.50 mg/mL of your stimulated lysate (for example, combine 30 µL of a 1 mg/ml lysate stock with 30 µL of 1X EDB; more concentrated lysate can be used but using lysate at less than 1 mg/mL is not recommended due to possible interference of lysate buffer in the assay when using higher amounts of lysate). **Keep on ice until needed.**
5. Prepare a 1:1 mixture of 1X EDB and CEB-A (50% EDB/50% CEB) for diluting the lysate prepared above, by mixing 200 µL of CEB-A with 200 µL of 1X EDB (If the lysate is more concentrated, adjust this buffer to match the composition of the buffer used for the diluted lysate prepared in step 4).
6. Utilizing a separate lysate dilution plate, add 30 µL of 50% EDB/50% CEB per well to wells 1-11 in a single row. Add 30 µL of the stimulated lysate prepared in step 4 to well 11. Add the remainder of the stimulated lysate to well 12.
7. Mix the contents of well 11 and transfer 30 µL to well 10. Mix the contents of well 10 and transfer 30 µL to well 9. Repeat this procedure down to well 2. Well 1 will be used for the "no enzyme" blank and receive only the 50% EDB/50% CEB.
8. Transfer 5 µL per well from the lysate dilution plate to rows A-C of the assay plate.
9. Centrifuge the plate at 200 x g for one minute, reseal, and place in the microplate reader set at 30 °C.
10. Read the plate in kinetic mode with continuous fluorescence intensity detection (Ex/Em 360/485 nm) every 2 minutes for 1-4 hours.

Note: The additions in step 8 should be performed quickly since the reaction will start with these additions.

Data analysis is performed as described on slide 9.

Plate Additions and Plate Map for a Lysate Titration

Component	Volume to add to wells
Master Mix	20 µL
Lysate	5.0 µL
	Total volume 25 µL

Plate Map for Lysate Titration to Assess Dose Dependence

		1	2	3	4	5	6	7	8	9	10	11	12
+PDGF Cell Lysates (ng)/well	A	0	2.4	4.9	9.8	20	39	78	156	313	625	1250	2500
	B	0	2.4	4.9	9.8	20	39	78	156	313	625	1250	2500
	C	0	2.4	4.9	9.8	20	39	78	156	313	625	1250	2500

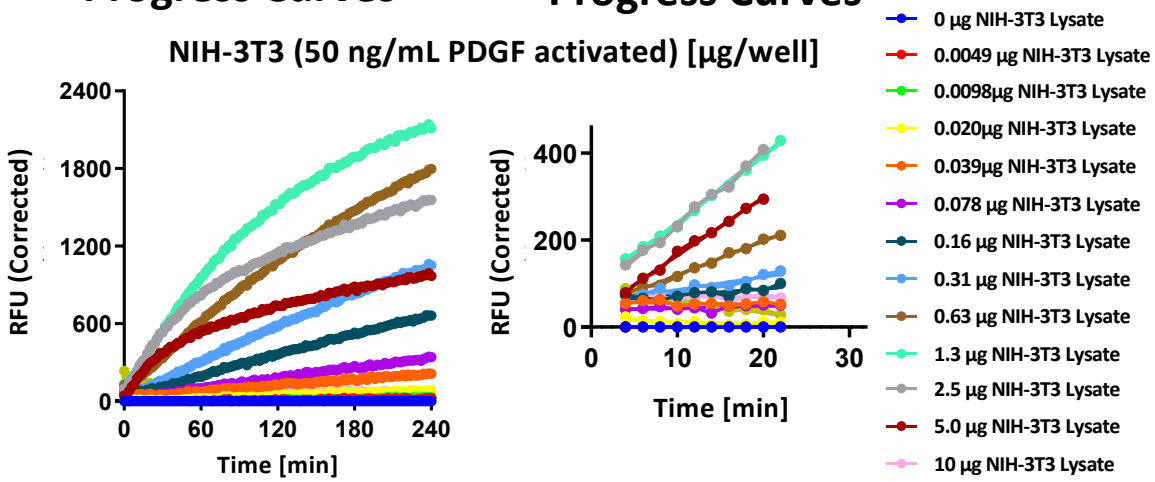
This test uses 36 wells and only the AQT0982 sensor peptide substrate and the lysate from NIH-3T3 cells stimulated with PDGF. Column 1 is the Blank. All conditions are tested in triplicate.

Lysate Titration for NIH-3T3 Cells Treated + PDGF and AKT 1/2/3 Activity Measured with AQT0982

AssayQuant TECHNOLOGIES INC.

1. Full Time Course Progress Curves

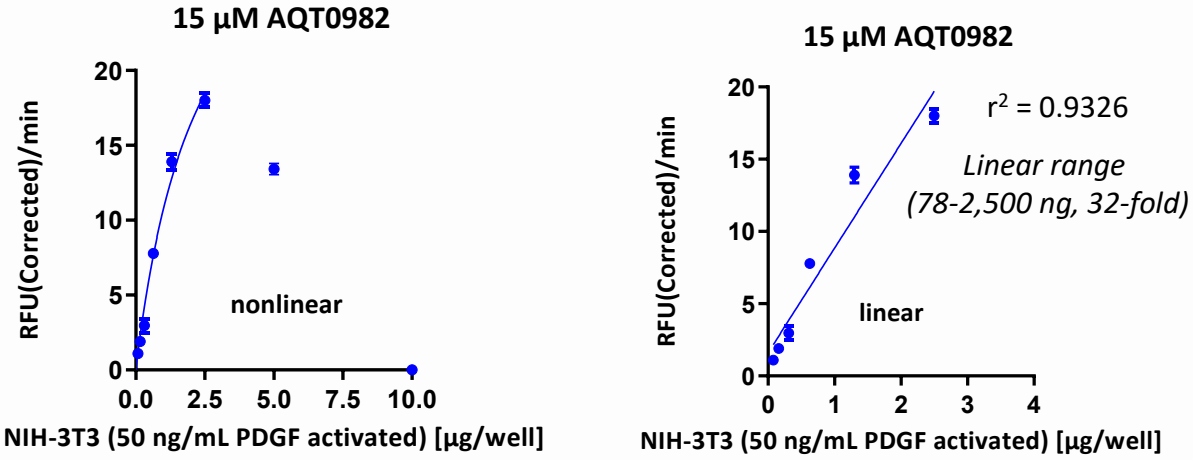
2. Linear Range of Progress Curves



3. Reaction Rate vs Amount of Lysate per Well

A. All Lysate Amounts

B. Lysate Amounts in Linear Range



The AQT0982 sensor peptide was used at 15 µM with an increasing amount of lysate from NIH-3T3 cells treated with PDGF to activate the AKT Pathway. RFU Corrected values (Total – Background) were determined for each condition. The results are presented for each amount of lysate for **1)** Full time course of each progress curve (0-240 min.), and **2)** Linear range of each progress curve, which was used to determine the slope for each amount of lysate. The results were then plotted as Reaction rates (RFU Corrected/min. +/- standard deviations) for **3A)** all lysate amounts, or **3B)** the linear range with an r^2 value > 0.93. Having the concentration of crude lysate samples at 1 mg/mL or higher, ensures that the amount of CEB-A in the reaction is minimized, even at the highest concentrations of lysate to avoid any inhibition of the kinase activity that can reduce the linear range (e.g., see 5 and 10 µg data points in **3A**).

The PhosphoSens-Lysate kinase activity assay for AKT 1/2/3 provides a selective, highly quantitative, and accurate measure of kinase activity in a complex sample.