Bridging Biochemistry and Biology: Direct and Continuous Kinase Activity Assays with Complex Samples

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ABSTRACT & INTRODUCTION

The human kinome contains 538 protein kinases implicated in over 400 diseases. Protein kinases form a network of signaling pathways, and are modified by multiple post-translational modifications, including phosphorylation, which can positively or negatively regulate the kinase enzymatic activity, the protein complexes that form, and their location in the cell. Given this rich biological complexity, over 30% of all drug development is focused on kinases. While kinase inhibitors are central to modern therapeutics, early discovery often relies on assays with limited mechanistic or physiological insight—creating a disconnect between biochemical results and biological complexity. AssayQuant® has combined chelation-enhanced fluorescence with high-throughput peptide synthesis methods to create optimized physiological sensor-based substrates that directly quantify kinase activity in crude cell lysates and tissue homogenates. This innovative continuous format delivers real-time insights under more physiological conditions to probe the complexity of kinase signaling and identify more effective drugs earlier to improve translational success in clinical trials with patients. Our initial development has successfully targeted kinase activity for multiple MAPKs, including pro-growth (ERK1/2) and stress (JNK, p38) pathways, AKT1-3, GSK3a/b, DNA-PK, and several CDKs (not shown). We used these assays to measure kinase activity in lysates from multiple cell models and in response to different treatments.

The PhosphoSens® Lysate Kinase Activity Assay – Continuous & Endpoint/Red Formats

Study Kinase Enzyme Activity Where Biology Happens

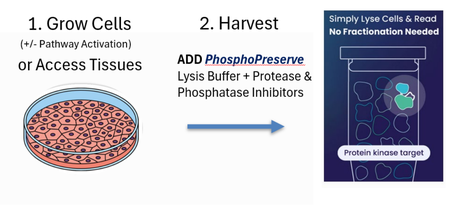
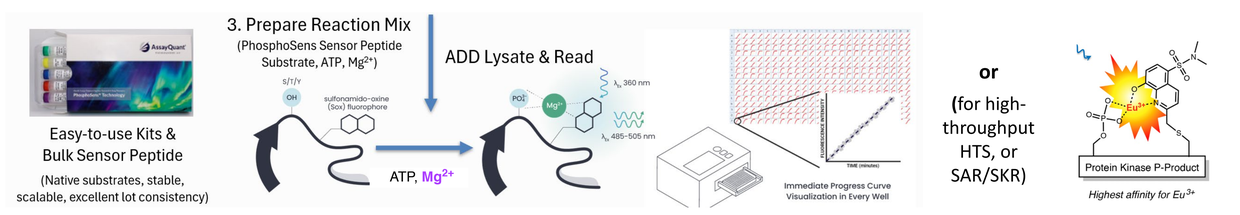


Figure 1. We combined innovative PhosphoSens detection technology, invented at MIT, with high-throughput peptide synthesis methods to design sensor peptide substrates that are highly selective for the target of interest. This development integrates the advantages of the PhosphoSens platform (activity-based, direct, homogeneous, continuous/kinetic, quantitative) with the enabling capability of measuring endogenous target kinase activity in unfractionated cell or tissue lysates.



All assays used 384- or 96-well white plastic flat-bottom treated microtiter plates, run at 30 °C with signals measured in a Biotech/Agilent Synergy Neo2 multi-mode plate reader

PhosphoSens® Assay – Selective Sensor Peptide Development Workflow

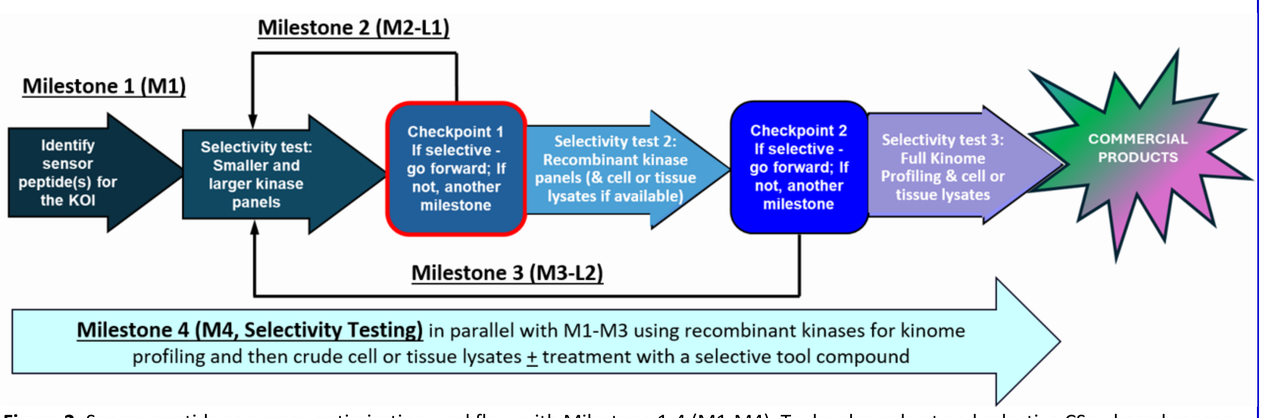
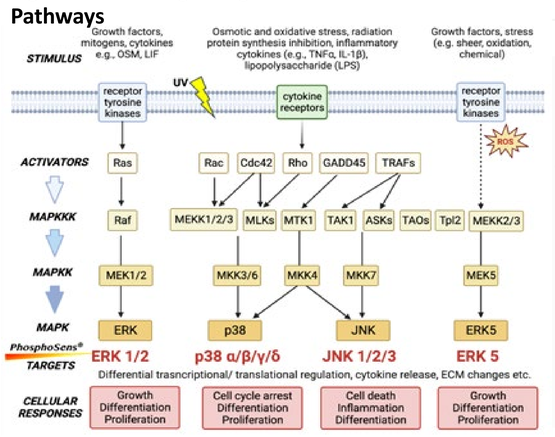


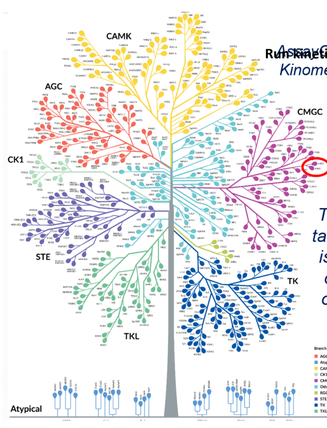
Figure 2. Sensor peptide sequence optimization workflow with Milestone 1-4 (M1-M4). To develop robust and selective CSox-based sensor peptides for profiling the activity of specific kinases in cell lysates. AQT's high-throughput Milestone-based kinetic screening process to efficiently identify sensor peptide substrates with high activity, selectivity, and signal-to-noise in the fluorescence-based readout. The innovative mechanism of the PhosphoSens assay technology enables the selection of sensor peptides with optimal performance using a functional and direct readout of kinase activity.

RESULTS & DISCUSSION: MAP Kinase (MAPK) Signaling & Sensor Peptide Development

Fig 3: A. MAPK Signaling Pathways

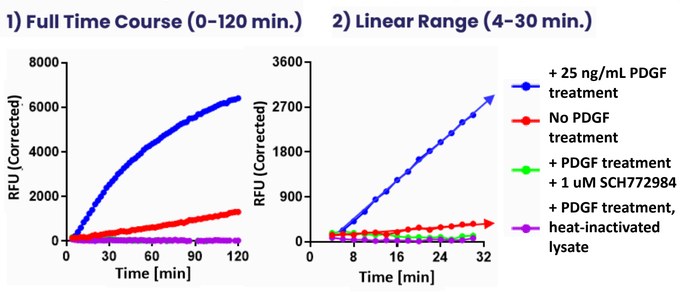


B. AQT's KinSight© Profiling with >400 Wild-type Kinases



Kinase target	Enzyme conc. (nM)	Average (RFU/pmol/min)	Rank	Selectivity Ratio	% Activity
ERK2	0.4	8,808	1	1.0	100.0
ERK1	0.4	8,109	2	1.1	92.1
P38G	0.4	93.6	3	94.1	1.1
NEK2	0.5	43.7	4	201.5	0.5
MK2	1	40.2	5	218.9	0.5
DYRK3	5	40.0	6	220.2	0.5
P38D	0.4	38.5	7	229.0	0.4
NEK9	0.5	31.0	8	284.1	0.4
SNRK	10	29.7	9	296.1	0.3
AKT3	1	25.6	10	344.0	0.3
MAP3K9	3	24.7	11	357.1	0.3
AURC	2.7	24.0	12	367.1	0.3
ERK5	5.3	23.8	13	370.1	0.3
PKACA	0.5	22.0	14	399.5	0.3
PKCQ	0.75	21.6	15	407.9	0.2
CAMK2B	0.5	20.8	16	424.3	0.2
CK1D	1	20.1	17	437.7	0.2
JNK3	5	19.7	18	446.5	0.2
PRKX	2	19.7	19	446.8	0.2
MARK2	1	19.0	20	463.2	0.2
DCAML1	3.6	17.0	21	517.1	0.2
AMPK223	2	16.2	22	543.3	0.2
AMPK213	1	15.2	23	579.1	0.2
CDK2/A2	2	14.5	24	606.2	0.2
MAP3K11	2.5	14.0	25	631.1	0.2

C. ERK1/2 Activity with AQT1076 (15 µM) Using 1.3 µg of Crude Cell Lysate Samples



D. Purified ERK2 w/AQT1076 & AQT1107

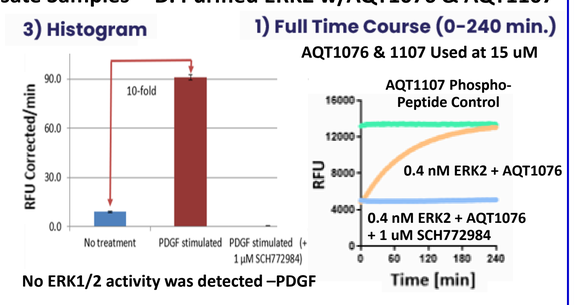
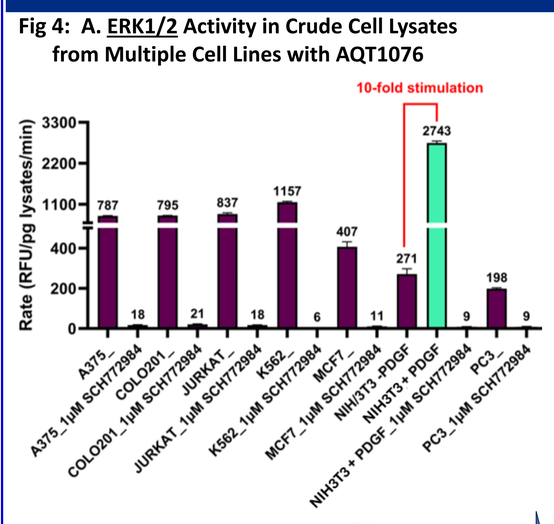
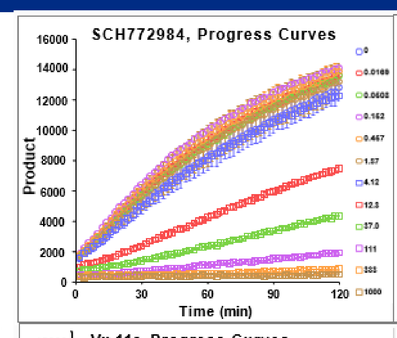


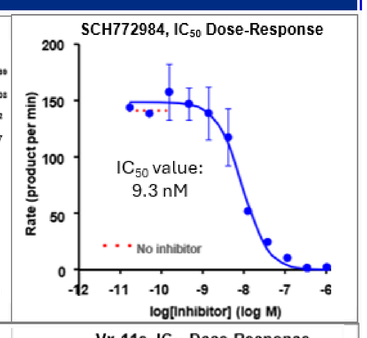
Figure 3. A. MAPK Signaling Pathways (Figure adapted from Tomida, 2015). These 10 MAPKs are a family of interrelated serine/threonine kinases that execute major cellular signaling pathways. The most actively studied MAPKs are associated with four pathways: 1) Extracellular-signal-regulated kinase 1 and 2 (ERK1/2), 2) c-Jun N-terminal kinase (JNK1-3), 3) p38α/β/γ/δ, and 4) ERK5, where different stimuli activate these individual MAPK subfamilies. Several genetic aberrations, including mutations and copy number variations of MAPK pathway genes, have been identified in human cancers, and many of these proteins are promising drug targets. Combined with the fact that pTXpY phosphorylation is often measured with phospho-specific antibodies as a surrogate measure of activation (which does not always correlate with activity), this emphasizes the urgent need for quantitative activity-based assays that can be used with cell lysates and tissue homogenates to understand the intricate dynamics of kinase activation and signaling networks. **3-B. AQT's KinSight© profiling results** with >400 wild-type kinases highlight the exquisite selectivity of the AQT1076 sensor peptide for ERK1/2. **3-C. Lysate quantitation:** AQT1076 enables sensitive quantitation of ERK1/2 activity in crude lysates from NIH3T3 cells ± PDGF. The signal is eliminated with the selective ERK1/2 inhibitor (SCH772984) or heat-inactivation of the lysate, and no ERK1/2 activity was detected without PDGF treatment of the NIH3T3 cells. **3-D. AQT1107 Control versus AQT1076 + purified ERK2:** The ERK2 protein (0.4 nM) fully phosphorylates the AQT1076 sensor peptide substrate (15 µM) by 240 min., with the signal converging with that obtained with the AQT1107 phosphopeptide positive control (15 µM). AQT1107 allows for absolute quantitation (moles of product/min) for comparison with different sensor peptide assays.

Cell Lysate Activity Assays for MAPKs: ERK1/2 (AQT1076), JNK (AQT1196) & p38 (AQT0648)

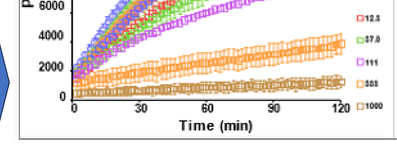
Fig 4: A. ERK1/2 Activity in Crude Cell Lysates from Multiple Cell Lines with AQT1076

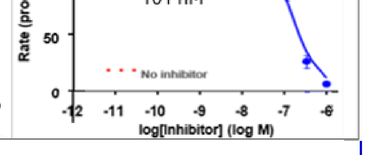






B. IC_{50} Dose Response for SCH772984 and Vx-11e Measuring ERK1/2 Activity in Crude Lysates from NIH3T3 Cells + PDGF. Inhibitors were titrated in the final reaction. Lysate was 1 µg/well.





C. Activity for JNK1-3 (AQT1196) & p38α/β/γ/δ (AQT0648) in Crude Lysates from Cells Treated with Anisomycin (stress stimulus) HEK293 or HeLa cells were grown and assayed in the same white plastic, 96-well plates

1) JNK lysates from HEK293 cells ± anisomycin (3 µM, 15 min), assayed with AQT1196 (15 µM) over 2-4 hours at 30 °C

2) p38 lysates from HeLa cells ± anisomycin (1 µM, 15 min), assayed with AQT0648 (15 µM)

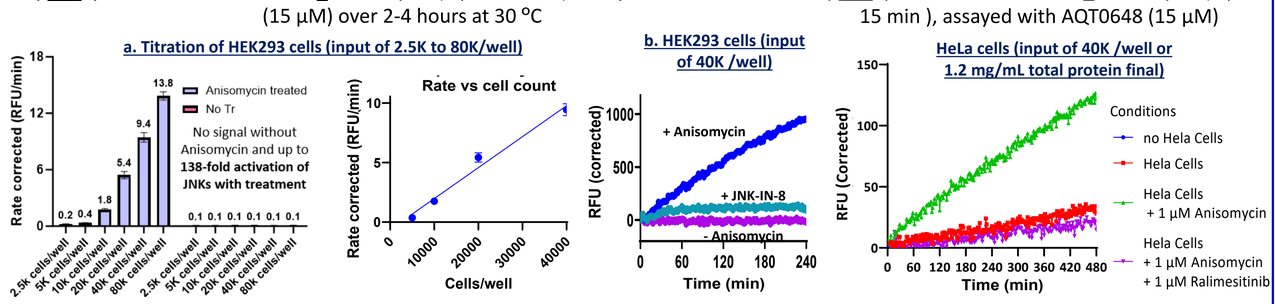
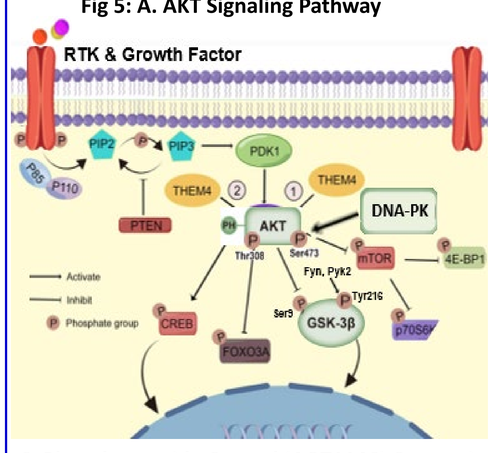


Figure 4-A. Multi-cell model kinase activity analysis: AQT1076 was used to assay ERK1/2 activity in lysates (1-5 µg of total protein per 384-well) from 7 cell lines. Reaction rates (RFU Corrected/pg of total lysate protein/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of duplicate reactions +/- the standard deviation. A tenfold increase in the rate of AQT1076 phosphorylation versus basal activity was seen with PDGF-stimulated NIH-3T3 cells (Green bar). In all cases, the ERK1/2-selective inhibitor, SCH77984 (1 µM), blocked the signal, confirming the selectivity of the AQT1076 sensor peptide for evaluating ERK1/2 activity in complex samples. **4-B. IC_{50} determination** for SCH772984 (9.3 nM) and Vx-11e (101 nM) in crude lysates from NIH3T3 cells +PDGF, with inhibitors titrated. **4-C.1.a. Titration of HEK293 cells** (input of 2.5K to 80K cells/well) grown for 48 hours, serum starved overnight, with or without anisomycin treatment (stress stimulus). JNK activity with AQT1196 was increased up to 138-fold, with a linear increase in JNK activity with 5K to 40K cells/well, and inhibited entirely with JNK-IN-8 inhibitor (**4-C.1.b.**). Similar results were obtained for p38 activity in HeLa cells (**4-C.2**).

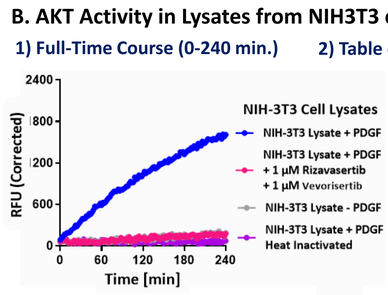
Cell Lysate Activity Assays for AKT1-3 (AQT0982), GSK3α/β (AQT1211) & DNA-PK (AQT440)

Fig 5: A. AKT Signaling Pathway



B. AKT Activity in Lysates from NIH3T3 cells + PDGF Using AQT0982

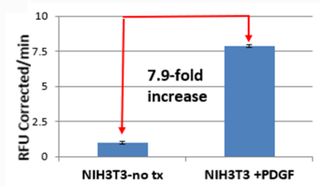
1) Full-Time Course (0-240 min.)



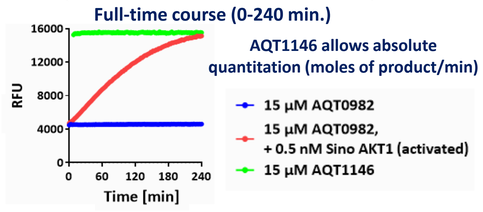
2) Table of Reaction Rates from Linear Range (0-85 min.)

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

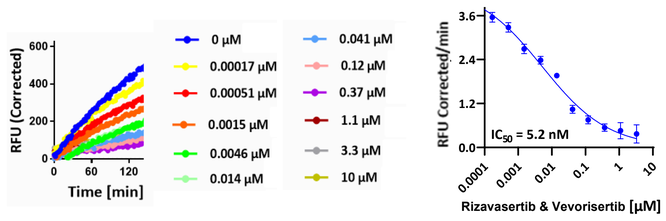
3) Histogram Showing Fold Activation of AKT with AQT0982 in Lysates of NIH3T3 Cells + PDGF Treatment



C. Phosphopeptide Control (AQT1146) Comparison of AQT1146 to Purified AKT1 with AQT0982



D. AKT IC_{50} for with Rizavasertib (A-443654) & Vevorisertib (ARQ 751)



E. AKT Pathway Kinase Activity Using 1-4 µg of Crude Lysate/well from Multiple Cell Lines with 15 µM Sensor Peptide

1. AKT1-3 Activity with AQT0982 **2. GSK3α/β Activity with AQT1211** **3. DNA-PK Activity with AQT0440**

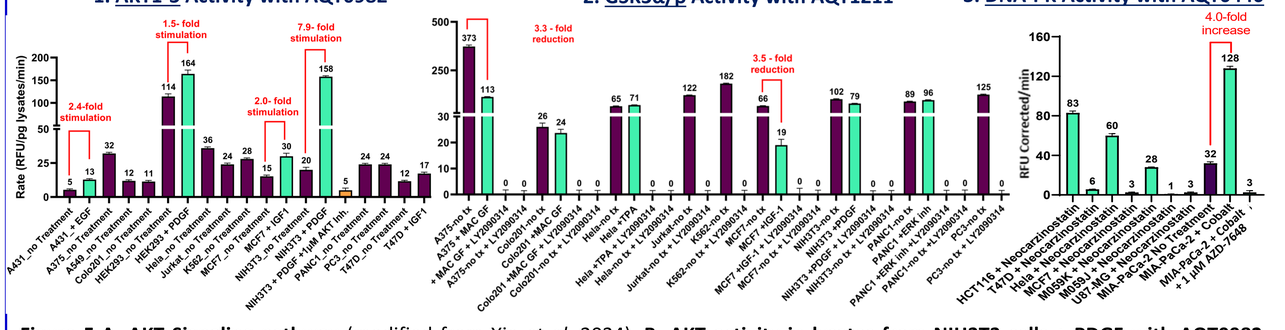


Figure 5-A. AKT Signaling pathway (modified from Xie *et al.* 2024). **B. AKT activity in lysates from NIH3T3 cells + PDGF with AQT0982** shows an 8-fold stimulation with treatment, and the signal is eliminated with addition of selective AKT inhibitors (1 µM of Rizavasertib [A-443654] & Vevorisertib [ARQ 751]) or heat-inactivation of the lysate (**5.B.1-3**). **5-C. AQT1146 Control versus AQT0982 + purified AKT1:** The AKT1 protein (1 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. This allows for absolute quantitation (moles of product/min) for comparison across different sensor peptide assays and samples. **5-D. AKT IC50 for with Rizavasertib (A-443654) & Vevorisertib (ARQ 751):** The IC_{50} of this inhibitor combination was 5.2 nM, which is more potent than either alone (data not shown). **5-E. Multi-cell model kinase activity analysis** for **AKT1-3 (1)**, **GSK3α/β (2)**, and **DNA-PK** (3) using the corresponding selective sensor peptide with lysates (1-4 µg of total protein per 384-well) from each cell line. Stimulation of AKT activity (1.5-7.9-fold) was seen with several cell treatments and for DNA-PK (4-fold with Cobalt). With GSK3, some treatments (but not PDGF) reduced activity, consistent with our understanding of these signaling networks.

CONCLUSIONS & REFERENCES:

We have created a systematic workflow that identifies highly selective sensor-based substrates, enabling direct quantitation of kinase activity in crude cell lysates or tissue homogenates, as demonstrated for multiple MAPKs (e.g., ERK1/2 pro-growth and JNK & p38 stress) pathways, AKT1-3, GSK3α/β, and DNA-PK, allowing activity in lysates from multiple cell models to be determined for advancing kinase biology, drug development, and personalized medicine. We are actively extending this approach to create a portfolio of assays to support this opportunity.

References:

1. Tomida, T., Visualization of the spatial and temporal dynamics of MAPK signaling using fluorescence imaging techniques, J Physiol Sci 2015, 65, 37-49.
2. Xie et al. 2024: Akt phosphorylation regulated by THEM4 in a positive/negative manner in various tumors. J. Zhejiang U.-SCIENCE B (Biomedicine & Biotechnology) 25(7):541-556.