Multi-Kinase ELISA Array

Human/Rat/Mouse

Akt1 (pS473), Akt2 (pS474), GSK3α (pS21) & GSK3β (pS9)

Instruction Manual

MKA 010

Version 2

For Research Use Only
The kit should be stored at 4°C upon receipt.

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Introduction

Symansis Multi-Kinase ELISA Array allows the researcher to simultaneously analyze activation of multiple target proteins on a single ELISA plate.

Symansis have developed a unique sandwich ELISA that allows the researcher to study key components of the PI3-Kinase/Akt signal transduction pathway.

Symansis have formulated a **Denaturing Cell Lysis buffer** containing 6 M Urea to maximize assay performance.

Materials provided

Components	Quantity	Storage
Antibody-Coated Microwells. 2 x 8-microwell strips (colour coded)	6 pouches	4°C
Biotin-Conjugated Detection Antibody (colour coded caps). Contains 0.05% w/v Sodium Azide	6 x 1.7 mL	4°C
Positive Control (Assay QC)	1 x 1.4 mL	4°C
Streptavidin Horseradish Peroxidase (SAV-HRP). Contains 3.3 mM Thymol	11 mL	4°C
Tetramethylbenzidine (TMB) Substrate	11 mL	4°C
20 x Wash Buffer Concentrate. Contains 3.3 mM Thymol	25 mL	4°C
5 x Sample Dilution Buffer Concentrate. Contains 0.05% w/v Sodium Azide	25 mL	4°C
Stop Solution	5.5 mL	4°C
Plate Cover	3 sheets	
Microwell Strip Holder	1	

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Notes

Sodium Azide reacts with lead and copper plumbing to form explosive metals azides. Upon disposal, flush drains with copious amount of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water.

Colour Coding and Number of Vials and Microwells Provided

Target Protein	Detection Antibody: Cap Colour and Number of vials provided	Microwell : Rim Colour and Number of wells provided
Akt1 (pS473)	Green (2)	Green (16)
Akt2 (pS474)	Orange (1)	Orange (8)
GSK3α (pS21)	Pink (2)	Pink (16)
GSK3β (pS9)	Blue (1)	Blue (8)

Additional Material Required (Not Supplied)

- Microtitre plate reader capable of measurements at or near 450nm
- Calibrated pipettes with disposable pipette tips. A manifold multichannel pipette is useful for large assays and to minimize pipetting errors.
- Cell Lysis Buffer Preparation for the recommended buffer formulation.
- Wash bottle, manual dispenser or automated plate washer
- Lint-free tissue
- Absorbent paper tissue
- Microcentrifuge Tubes
- Distilled or de-ionised Water

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Procedural Notes

- Ensure all kit components are refrigerated when not in use. All reagents should be allowed to equilibrate to room temperature before use.
- Antibody-Coated microwell strips should be allowed to equilibrate to room temperature before opening the pouch. Any unused strips can be sealed and stored in the provided pouch (containing desiccant) at 4^{o}C for 3 months.
- Cell Lysates samples must be prepared using the **denaturing** cell lysis buffer containing **6 M Urea** and protease and phosphatase Inhibitors for optimal assay performance **(see Cell Lysis Buffer Preparation).**
- Samples should be frozen if not analyzed. Avoid multiple freeze/thaw cycles of frozen samples. Samples must be allowed to thaw on ice prior to analysis.
- Cell lysate samples must be diluted to the desired test concentration with 1x diluted Sample Dilution Buffer **(see Reagent Preparation).**
- It is recommended that all test samples be run in duplicate.
- When pipetting reagents, maintain a consistent order of addition well to well. This ensures equal incubation times for all wells.
- Cover or cap all reagents when not in use.
- Do not mix reagents from other kits.
- Do not use reagents after the expiry date of the kit.
- Read Absorbances at 450 nm within 30 minutes of assay completion at room temperature.
- All residual wash liquid must be drained from the wells by decanting or aspiration followed by forceful tapping of the plate on absorbent paper tissue. Never insert absorbent paper into the wells. Ensure there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results **(see Directions for Washing).**
- The TMB substrate is light-sensitive, avoid prolonged exposure to light. Avoid contact of TMB with metal, or colour may develop.

Safety Precautions

- TMB is toxic if inhaled or swallowed. Avoid contact with skin. Keep container tightly closed when not in use.
- Stop Solution is an acidic solution. Appropriate personal protection equipment must be worn (laboratory coat, gloves, eye protection).

Directions for Washing

- Incomplete washing will adversely affect the test outcome. All washing must be performed with wash buffer provided.

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- Completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of the well. Care must be taken not to scratch the inside of the well.
- After aspiration, fill the wells with 0.3 mL of 1 x Wash Buffer **(see Reagent Preparation)**. Let soak for 15 to 30 seconds, and then aspirate the liquid. Repeat as directed in the **Assay Procedure**. After washing, invert the plate and forcibly tap on absorbent paper tissue.
- If a wash bottle is used, flood the plate with wash buffer, completely filling all the wells. After washing, to remove residual solution, invert the plate and forcibly tap on absorbent paper tissue. Do not allow the wells to dry at any time.
- Clean the underside of the wells with lint-free tissue.
- If an automated plate washer is used, follow the operational procedure and programme for 30 second soak cycles.

Reagent Preparation

- **1x Wash Buffer** : Allow 20x Wash Buffer Concentrate to equilibrate to room temperature. Mix to ensure any precipitated salt have re-dissolved. Dilute 25 mL 20x Wash Buffer Concentrate into de-ionised or distilled water to a total volume of 500 mL. The diluted Wash Buffer is stable at 4^{o}C for up to 14 days.
- **1x Sample Dilution Buffer :** Allow 5x Sample Dilution Buffer Concentrate to equilibrate to room temperature. Mix to ensure any precipitated salts have re-dissolved. Dilute 25 mL 5x Sample Dilution Buffer Concentrate into de-ionised or distilled water to a total volume of 125 mL. The diluted Sample Buffer is stable at 4^{o}C for up to 14 days.

Cell Lysis Buffer Preparation (denaturing conditions)

Denaturing Cell Lysis Buffer, containing **6 M Urea must** be used for optimal assay performance. **Symansis cannot guarantee optimal performance of the assay, if the recommended denaturing cell lysis buffer is not used.**

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Denaturing Cell Lysis Buffer

10 mM Tris, pH 7.4
150 mM NaCl
1 mM EDTA
1 mM EGTA
0.5% (v/v) Triton X®-100
6 M Urea
10 µg/mL Leupeptin
10 µg/mL Pepstatin
3 µg/mL Aprotonin
100 µM PMSF
1 mM Sodium Orthovanadate (Na_3VO_4)
2 mM Sodium Pyrophosphate ($Na_4P_2O_7$)
5 mM Sodium fluoride (NaF)

Cell Lysate Preparation (adherent cells)

The researcher is recommended to optimize the cell extraction procedures for their own applications.

1. Aspirate media. Treat cells by adding fresh media containing regulator for the desired time.
2. Rinse once with ice cold PBS on ice.
3. Remove PBS and add 0.5 mL of **Denaturing** Cell Lysis Buffer **(see Cell Lysis Buffer Preparation)** containing protease/phosphatase inhibitors and PMSF to each plate (10cm diameter, 70% - 80% confluent). Incubate for 10 minutes on ice.
4. Gently scrape cells off the plate and transfer to chilled tubes/vials. Keep on ice
5. Vortex briefly and Microcentrifuge at 14,000 rpm for 10 minutes at 4°C.
6. Aliquot the clear lysate to clean tubes. These samples are ready for testing. Lysates can be stored at -80°C. Multiple freeze/thaw cycles must be avoided.

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Assay Procedure

The following procedure is for 12 x 8 microwell antibody coated strips. The researcher is advised to scale down or up appropriately, if processing less or more strips.

1. 2x Antibody-Coated Microwell Strips are provided for each target protein (colour coded, 8 microwell/strip).
2. Gently, place the strips in the mircowell strip holder. Unused strips can be resealed and stored at 4^{o}C. See **Procedural Notes** for equilibrating the microwell strips to room temperature.
3. Add 100 µL of each diluted **cell lysate (sample)** and **positive control (Assay QC)** into the appropriate well. 100 µL of 1x Sample Dilution Buffer alone should be added to **negative** control/blank wells.
    a. **Positive Control** (Assay QC) allows the researcher to assess the performance of the assay
    b. As a guide, a minimum concentration of 20 µg/well of cell lysate can be used as a starting concentration. However, the researcher may adjust the test concentration for specific treatments or cell lines if desired.
4. Seal with plate cover and press firmly onto top of microwells. Incubate for 2 hours at room temperature.
5. Gently remove the plate cover and wash wells. Thoroughly decant or aspirate solution from wells and discard the liquid. Wash wells 3 times with 0.3 mL of diluted 1x Wash Buffer **(see Directions for Washing)**.
6. Add 100 µL of the appropriate Biotin-Conjugated Detection Antibody to each well. **Be sure to match the cap colour of the Detection Antibody with the corresponding colour coded Microwell Strips.** Seal with plate cover and press firmly onto top of microwells. Incubate for 2 hours at room temperature.
7. Wash wells 3 times as directed in Step 5.
8. Add 100 µL SAV-HRP **(equilibrated to room temperature)** to each microwell. Seal with plate cover and press firmly onto top of microwells. Incubate for 30 minutes at room temperature.
9. Wash wells 3 times as directed in Step 5.
10. Add 100 µL of TMB substrate solution **(equilibrated to room temperature).** Incubate the plate for 20 minutes or until the **strongest cell lysate sample** turns **dark** blue at room temperature in the dark.
    a. The rate of colour development is dependant on amount of target protein in each sample and thus varies for each sample preparation.
    b. Colour development for each target protein must be individually monitored. Consequently, it may be necessary to stop the

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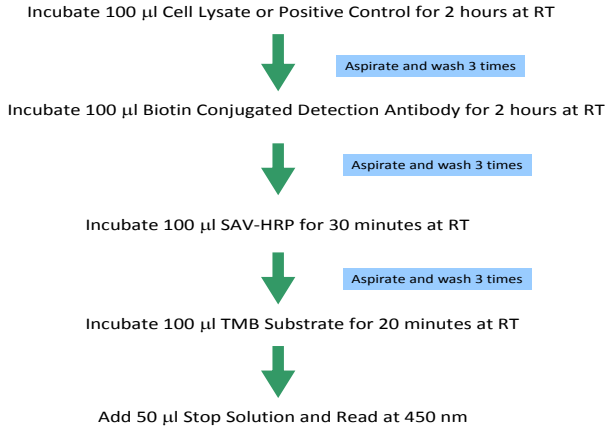
reaction for each target protein separately, within the same plate.

11. Add 50 µL of Stop Solution to each microwell (see section 10 a, b). Gently tap the microtitre plate for a few seconds. The colour in each well should change from blue to yellow.
12. Wipe the underside of the microwells with lint free tissue and read absorbance at 450 nm **within 30 minutes** of stopping the reaction.

Assay Summary



- Total Assay Time : Approximately 5 hours including washing

Optional Assay Procedure

Step 3, (addition of sample) and Step 6 (addition of detection antibody) incubation times can be shortened to **30 minutes** each with the use of an orbital shaker. (100 rpm recommended)

Step 8, (addition of SAV-HRP) should be incubated on the orbital mixer for **30 minutes**. Step 10, (addition of TMB) should be incubated on a benchtop for **30 minutes**.

To minimize assay drift, it is essential to add samples and detection antibody in as short a time as is practical. This shortened time procedure may result in lower OD values compared to the standard procedure.

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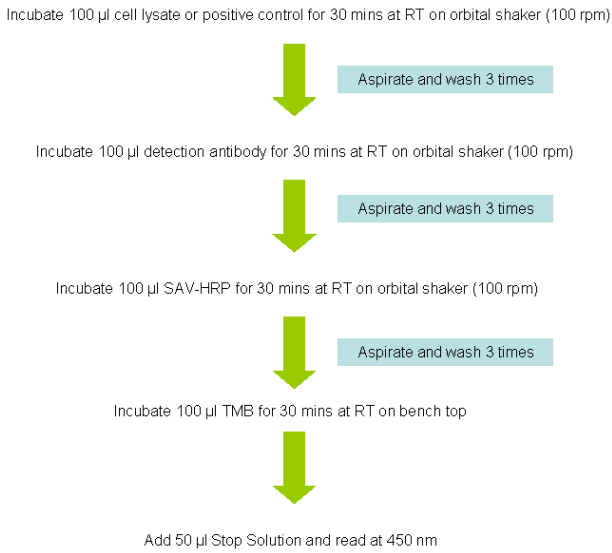
Optional Procedure Summary



- Total Assay Time : Approximately 2.5 hours including washing

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