INTRODUCTION

The Thermo Scientific™ FOXO3a Activation Assay measures activation of FOXO3a, a transcription factor involved in the direct measurement of molecular translocation of specific proteins using a fixed end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates. Quantitation is accomplished by using a monoclonal rabbit FOXO3a antibody, a Thermo Scientific™ DyLight 550-Conjugated Goat Anti-Rabbit Secondary Antibody and various other reagents and buffers required for immunofluorescent detection of FOXO3a for high-content screening (HCS) assays.

FOXO3a is a member of the forkhead box, class O transcription factors regulated by protein kinase B (AKT). FOXO3a is negatively regulated by phosphorylation. Active FOXO3a resides in the nucleus; inactivation and transport to the cytoplasm is facilitated through phosphorylation by AKT or IKK (IK kinase) and sequestration by 14-3-3σ. The inactive complex is then transported from the nucleus to the cytoplasm. The FOXO transcription factors promote cell survival, arrest and cell death, depending on the cell type and stimulus, and are potential targets for cancer therapeutics.

The FOXO3a Activation Assay, in combination with the Thermo Scientific™ ArrayScan™ HCS Reader and the Thermo Scientific™ Compartmental Analysis BioApplication™ Software Module, enable automated plate handling, focusing, cell image acquisition, analysis and quantification of FOXO3a activation.

MATERIALS REQUIRED

<table>
<thead>
<tr>
<th>Description</th>
<th>Recommended Product No.</th>
<th>Concentration / Units</th>
<th>Recommended Amount per 96-Well Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO3a Primary Antibody (rabbit)</td>
<td>MA5-14932</td>
<td>100µg/mL†</td>
<td>17-27µL</td>
</tr>
<tr>
<td>DyLight 550-Conjugated Goat Anti-Rabbit Secondary Antibody</td>
<td>84541</td>
<td>1mg/mL</td>
<td>10-15µL</td>
</tr>
<tr>
<td>High Content Analysis Buffer Kit #2</td>
<td>8422000</td>
<td>kit‡</td>
<td>–</td>
</tr>
<tr>
<td>Sealing Tape for 96-Well Plates, pre-cut</td>
<td>15036</td>
<td>100 sheets</td>
<td>1</td>
</tr>
<tr>
<td>16% Formaldehyde Solution</td>
<td>28906</td>
<td>10x1mL ampule</td>
<td>3mL</td>
</tr>
<tr>
<td>GSK-3 Inhibitor X</td>
<td>*</td>
<td>–</td>
<td>100µg/mL†</td>
</tr>
<tr>
<td>Lysine-coated, clear-bottom 96-well plate</td>
<td>*</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: This protocol provides general guidelines for the FOXO3a Activation Assay. Further optimization of the antibodies, reagents, their preparation and the overall protocol may be required. Other reagents and dyes may be used (e.g., other DyLight Dyes), but optimization is required for each specific change.

†Primary antibody is provided in a lot-specific concentration (~100µg/mL) for
optimal functionality per application.

†Kit components are also available separately in larger pack-sizes. Buffer Kit #2 includes Wash Buffer 10X (100mL); Wash Buffer II 10X (100mL); Permeabilization Buffer 10X (100mL); Blocking Buffer 10X (85mL); and Hoechst Dye (30μL).

*These products are available through multiple suppliers.

††Prepare activator when needed. Other agonist and antagonists of FOXO3a activation can be used and should follow preparation instructions provided by the supplier.
Cell Preparation

- This assay was performed using A549 cells.
- A549 cells were cultured using Thermo Scientific™ F-12K Complete Medium, supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin and 1.5 g/L sodium bicarbonate.
- Cells were diluted when they reached 70-80% confluence (2-3 times per week) at a ratio of 1:4 to 1:6. Cells were used at passage number ≤ 10.
- For FOXO3a activation, cells were harvested by trypsinization, diluted into F12-K Complete Medium and then cell density was determined.
- Cell density was diluted to 5 to 10 × 10⁴ cells/mL into F12-K Complete Medium and 100 μL of the cell suspension was added to each well of a 96-well microplate (= 5000 to 10,000 cells/well).
- Cells were grown for 18-20 hours at 37°C in 5% CO₂ before drug treatment. A549 cells were used at ~60-70% confluence before treatment.

Example Protocol for FOXO3a Activation

A. Materials Required

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Wash Buffer</td>
<td>Add 20 mL of 10X Wash Buffer to 180 mL of ultrapure water. Store buffer at 4°C for up to 7 days.</td>
</tr>
<tr>
<td>1X Wash Buffer II</td>
<td>Add 20 mL of 10X Wash Buffer II to 180 mL ultrapure water. Store buffer at 4°C for up to 7 days.</td>
</tr>
<tr>
<td>Fixation Solution</td>
<td>Add 3 mL of 16% formaldehyde to 9 mL of 1X Wash Buffer. Prepare solution just before each assay.</td>
</tr>
<tr>
<td>1X Blocking Buffer</td>
<td>Add 7.5 mL of 10X Blocking Buffer to 67.5 mL of 1X Wash Buffer. Store buffer at 4°C for up to 7 days.</td>
</tr>
<tr>
<td>1X Blocking Buffer with FBS</td>
<td>Add 10 mL of 10X Blocking Buffer to 88 mL of Wash Buffer. Supplement with 2 mL of FBS for a final volume of 100 mL. Store buffer at 4°C for up to 7 days.</td>
</tr>
<tr>
<td>1X Permeabilization Buffer</td>
<td>Add 10 mL of 10X Permeabilization Buffer to 90 mL of ultrapure water for a final volume of 100 mL. Store buffer at 4°C for up to 7 days.</td>
</tr>
<tr>
<td>Primary Antibody Solution</td>
<td>Add 17-27 μL of FOXO3A Primary Antibody to 6 mL of 1X Blocking Buffer. Prepare solution just before each assay.</td>
</tr>
<tr>
<td>Staining Solution</td>
<td>Add 0.6 μL of Hoechst Dye and 18 μL of DyLight 550-Conjugated Goat Anti-Rabbit to 6 mL of 1X Blocking Buffer. Prepare solution just before each assay.</td>
</tr>
</tbody>
</table>

B. Procedure

1. Dilute stock hydrogen peroxide to 1m in ultrapure water and keep on ice until treatment. Dilute hydrogen peroxide to 20mM into culture medium. Add 100 μL/well and incubate 1 hour at 37°C in 5% CO₂.
2. Aspirate culture medium and add 100 μL/well of Fixation Solution. Incubate plate in a fume hood at room temperature (RT) for 15 minutes.
3. Aspirate Fixation Solution completely and then wash plate twice with 100 μL/well of 1X Wash Buffer.
4. Aspirate Wash Buffer completely, add 100μL/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.

5. Aspirate Permeabilization Buffer and wash plate twice with 100μL/well of 1X Blocking Buffer.

6. Aspirate Blocking Buffer, add 100μL/well of 1X Blocking Buffer supplemented with 2% FBS and incubate for 15 minutes at RT.

7. Aspirate Blocking Buffer and add 50μL/well of Primary Antibody Solution. Incubate for 1 hour at RT.

8. Aspirate Primary Antibody Solution and wash with 100μL/well of 1X Wash Buffer II.

9. Aspirate Wash Buffer II and wash twice with 100μL/well of 1X Wash Buffer.

10. Aspirate Wash Buffer, add 100μL/well of 1X Blocking Buffer supplemented with 2% FBS and incubate for 15 minutes at RT.

11. Aspirate Blocking Buffer and add 50μL/well of Staining Solution containing the secondary antibody. Incubate for 45 minutes at RT.

12. Aspirate Staining Solution and wash plate twice with 100μL/well of 1X Wash Buffer II.

13. Aspirate Wash Buffer II and wash plate twice with 100μL/well of 1X Wash Buffer.

14. Aspirate Wash Buffer, add 100μL/well of 1X Blocking Buffer supplemented with 2% FBS and incubate for 15 minutes at RT.

15. Aspirate Blocking Buffer, add 50μL/well of Staining Solution and incubate for 45 minutes.

16. Aspirate Staining Solution and wash plate twice with 100μL/well of 1X Wash Buffer II.

17. Aspirate Wash Buffer II and then wash plate twice with 100mL/well of 1X Wash Buffer.

18. Aspirate and replace with 100μL/well of 1X Wash Buffer.

19. Seal plate and run on an ArrayScan HCS Reader.

20. Store sealed plates at 4°C.

Appendix A: Microscope Information

- Cells prepared and labeled according to these assay guidelines can be used and analyzed by confocal microscopy or fluorescence microscopy using the appropriate filter set(s).

- Optimization may be required when using slides, coverslips or multi-well chamber slides.

- Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are 550/568nm for DyLight 550 Conjugates and 350/461nm for Hoechst Dye.

Appendix B: Recommendation for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific™ Multidrop™ Combi or WellMate™ Dispensers.
• Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. When calculating your reagent requirements, base prime and rinse volumes on dead-volume limitations.

• Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.

• Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.

• Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.

• Incubation: Minimize the time when plates with live cells are out of a controlled CO2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.

• Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.

• Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed.

Cited References


