**AlphaScreen® SureFire®: 1-plate/1-step assay flowchart (p-ERK1/2 and p-MEK1 kits only)**

### Adherent Cells
- Seed cells in 384 well TC-Proxiplate, in 20 µl culture medium
- 6h to overnight (≥ 16h) adherence
- (4h to overnight Serum Starvation) \(^1\)
- Remove 10 µl of medium
- Add 5 µl of 4x-concentrated inhibitor and incubate 5 min to 1 hour \(^2\)
- Add 5 µl of 4x-concentrated stimulator and incubate for desired time \(^1\)
- Remove medium and add 4 µl of 1x Lysis Buffer

### Suspension Cells
- Seed cells in 384 well plate, in 4 µl HBSS
- (2 h equilibration at 37°C)
- Add 2 µl of 4x-concentrated inhibitor and incubate 5 min to 1 hour \(^2\)
- Add 2 µl of 4x-concentrated stimulator and incubate for desired time \(^1\)
- Add 2 µl of 5x Lysis Buffer

In control wells, add positive or negative control lysates. Incubate for 10 min on plate shaker (~350 rpm). \(^3\)

**Reaction Mix:**

<table>
<thead>
<tr>
<th></th>
<th>Typical volume for p-ERK1/2 (^4)</th>
<th>Typical volume for p-MEK1 (^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Buffer</td>
<td>587 µL</td>
<td>636 µL</td>
</tr>
<tr>
<td>Activation Buffer</td>
<td>98 µL</td>
<td>49 µL</td>
</tr>
<tr>
<td>Acceptor Beads</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Donor Beads</td>
<td>5 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

\(^1\) Depending on cell type and pathway analyzed.

\(^2\) Depending on type of inhibitor used: 5 min is generally enough for receptor antagonists; more time is needed to block intracellular targets.

\(^3\) May stop and freeze lysates at -20°C if desired. If doing this, re-shake after thawing to ensure homogeneity of solution.

\(^4\) Important note: only the p-ERK and p-MEK1 assays can be run using a single step immunoassay. For other kits; a separate Acceptor Mix and Donor Mix procedure needs to be performed.

OR

Add 7 µl Reaction Mix (adherent cell protocol)

Seal, wrap in foil, and (shake 1-2 min on plate shaker), then incubate ≥2 h (RT° or 22°C)

OR

Add 12 µl Reaction Mix for p-ERK1/2, or 10 µl Reaction Mix for p-MEK1 (suspension cell protocol)

Read Plate