Transfer 4 µl of lysate (or positive/negative control lysates) to white 384-well ProxiPlate™. Add 7 µl of Reaction Mix. Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥2 h, up to overnight (RT° or 22°C). Allow to equilibrate to plate reader temperature prior to reading.

### Adherent Cells
- Seed cells in 96 / 384 well plate, in 200 µl / 50 µl culture medium
- 6 h to Overnight (≥ 16h) adherence
- (4h to O/N Serum Starvation) ¹
- Remove medium
- Add inhibitor in 50 µl / 25 µl new medium and incubate 5 min to 1 hour ²
- Add 5 µl / 2.5 µl of 10x-concentrated stimulator and incubate for desired time ¹
- Remove medium (wash with PBS if using medium containing high biotin concentration, like RPMI)
- Add 50 to 100 µl / 20 to 25 µl of 1x Lysis Buffer and incubate for 10 min on plate shaker (~350 rpm). ³
- Transfer 4 µl of lysate (or positive/negative control lysates) to white 384-well ProxiPlate™

### Suspension Cells
- Seed cells in 96 / 384 well plate, in 50 µl / 10 µl HBSS
- (2 h equilibration at 37°C)
- Add 25 µl / 5 µl of 4x-concentrated inhibitor and incubate 5 min to 1 hour ²
- Add 25 µl / 5 µl of 4x-concentrated stimulator and incubate for desired time ¹
- Add 25 µl / 5 µl of 5x Lysis Buffer and incubate for 10 min on plate shaker (~350 rpm). ³

### Reaction Mix:
- **Typical volume for p-ERK1/2** ⁴
  - Reaction Buffer: 587 µL
  - Activation Buffer: 98 µL
  - Acceptor Beads: 10 µL
  - Donor Beads: 5 µL
- **Typical volume for p-MEK1** ⁴
  - Activation Buffer: 636 µL
  - Acceptor Beads: 10 µL
  - Donor Beads: 5 µL

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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.