Step Two: Assay Optimization

Further optimize the LANCE *Ultra* TR-FRET Cell Signaling assay using the parameters in Table 1.

Table 1. LANCE *Ultra* TR-FRET Cell Signaling assay optimization parameters.

LANCE *Ultra* TR-FRET Cell Signaling Optimization Parameter	Recommendations and Comments
Seeding Density for Adherent Cells in Microplates	**Suggested Range:** 10,000-120,000 cells per well. **Start With:** Try three different densities for the initial experiments. The initial screening experiments are designed to identify a cell seeding density that gives a signal window that is satisfactory to proceed with optimizing additional parameters. Once the assay has been optimized more fully, a cell titration study should be repeated to determine the optimal balance between cell culture requirements and assay performance.
Incubation Time After Plating	**Suggested Range**: 1-2 days adherent cells in assay plates. **Start With:** Try both 1 day and 2 days. Adherent cells need sufficient time after plating to recover and express the kinase activity of interest. This is particularly the case for cells that have been harvested using trypsin. With adherent cells a minimum of 15 hours of incubation is necessary to achieve maximal activity of the ERK pathway. For non-adherent cells, no recovery time is needed. Cells can be seeded for assay in either culture media or HBSS.
Serum Starvation Requirement	**Suggested Range:** none to overnight. **Start With:** 2-4 hours. Serum starvation may be necessary to reduce high basal levels of phosphorylation. Serum starvation may be beneficial or detrimental, depending on the pathway and cell line studied.
Cell Stimulation Time	**Suggested Range:** 5-60 minutes. **Start With:** 5 and 20 minutes. The time course for agonist stimulation varies depending on the specific pathway and cell line being studied. For some pathways the signal peaks within a few minutes and then declines rapidly. In other cases, the signal is maintained at a high level for up to an hour. Final optimization should include a detailed determination of the stimulation kinetic profile.
Pathway Inhibitor Addition to Reduce Basal Activity	In some cases, a high basal or constitutive activation of a pathway cannot be reduced by serum starvation. In this circumstance, an improved assay window may be achieved by the addition of a known pathway inhibitor to produce a lower signal for comparison to the stimulated response.
Agonist Dose Response	**Start With:** EC_{100} For the initial experiments, we recommend adding the agonist at a concentration that would be expected to elicit a maximal signal. Once the cell culture and cell plating parameters have been optimized and standardized, a full dose response curve should be generated.
Incubation Temperature	**Suggested Range:** Room temperature or 37 °C during stimulation. **Start With:** Room temperature. LANCE *Ultra* TR-FRET assays can generally be performed by stimulating the cells at room temperature. Certain cell lines may respond better to stimulation at 37 °C. The stimulation time course will vary depending on the temperature.
Cell Lysis Buffer	**Cell Lysis Buffer Options:** Lysis buffer provided in the kit or a more aggressive lysis buffer. **Start With:** Lysis buffer provided with the kit. Cell lysis for 30 minutes with gentle shaking (350 rpm) is usually sufficient for complete lysis. Generic protease inhibitors and phosphatase inhibitors such as NaF and activated Na_3VO_4 can be added to lysis buffers to protect kinases without affecting LANCE detection. Additional protease inhibitors may be beneficial in individual cases. If a more aggressive lysis solution is needed for a particular cell line, the lysis buffer should be tested to be sure it does not interfere with the LANCE *Ultra* TR-FRET signal.
Lysis Buffer Volume	**Suggested Range:** 25–100 µL for a 96-well plate. **Start With:** 50 µL In most cases, 50 µL of lysis buffer is satisfactory. Reducing the lysis to 25 µL may give an improved signal for targets present in low abundance. Shaking (350 rpm) or other mixing is important to reduce assay variability.
LANCE *Ultra* Assay Incubation Time	**Suggested Range:** 4 hours to overnight. **Start With:** 4 hours. Plate can be re-read at multiple time points, up to overnight.

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