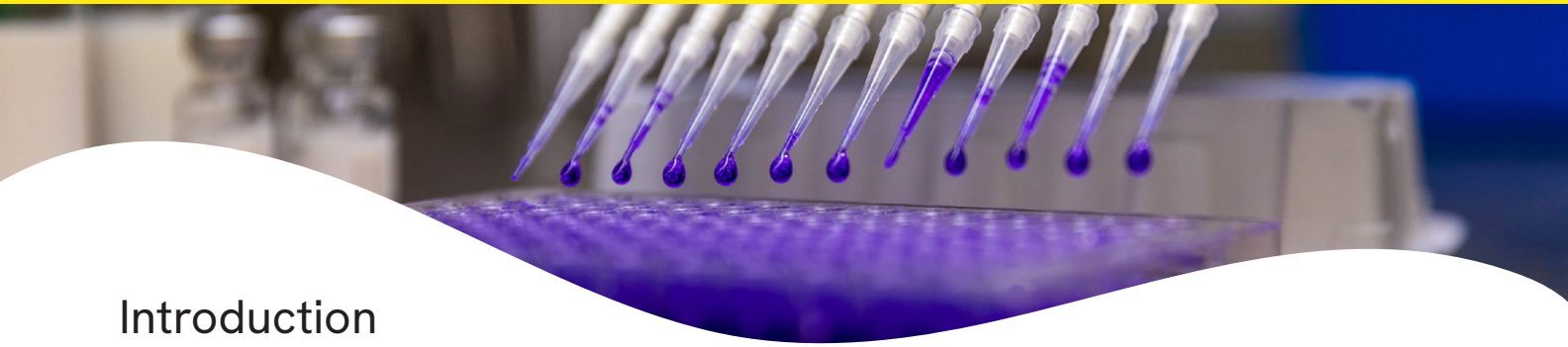
LANCE *Ultra* TR-FRET Cellular Phosphorylation.



Introduction

Screening kinase activities in cell-based assays offers advantages over the more traditional biochemical approach of using a purified recombinant enzyme to phosphorylate a substrate, since a cell-based assay gives information on a compound's activity in a more biologically relevant context. However, getting initial results with the LANCE® *Ultra* TR-FRET Cellular Phosphorylation assay (Figure 1) is highly dependent on optimal cell culture conditions, and often requires that multiple parameters be optimized in the first set of experiments. Certain parameters are more important to optimize initially in order to obtain a sufficient assay window for further study. This quick guide illustrates an approach that can help accomplish that goal. Results will be obtained for up to 24 different conditions (12 variables with each of two culture conditions).

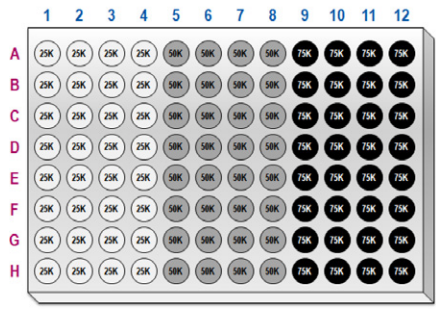
The assays as described in the kit manuals can be performed as either two-plate transfer protocols, or as a single-plate assay. We recommend beginning with the two-plate protocol.

Step one: Perform a multi-variable experiment to find initial conditions that give a sufficient stimulated: Basal response ratio.

This workflow illustrates how to test the four variables of cell seeding density, recovery time after cell seeding, serum starvation, and stimulation time in one experimental protocol. These parameters can be critical when setting up an assay for the first time. Once an initial signal above basal is achieved, subsequent assay optimization is easier. Separate work flows are shown for adherent cells and for non-adherent (suspension) cells.

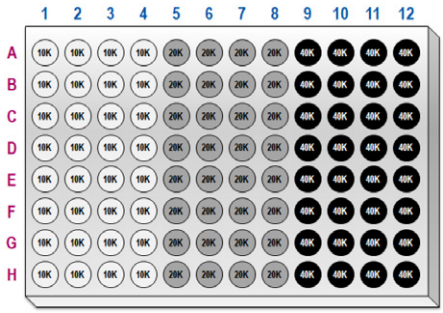
Day 0 (adherent cells)

Plate 1 - To be assayed on day 1



For adherent cells, seed Plate 1 (96 wells) using three different cell densities: 25K, 50K and 75K cells per well. Incubate plate overnight.

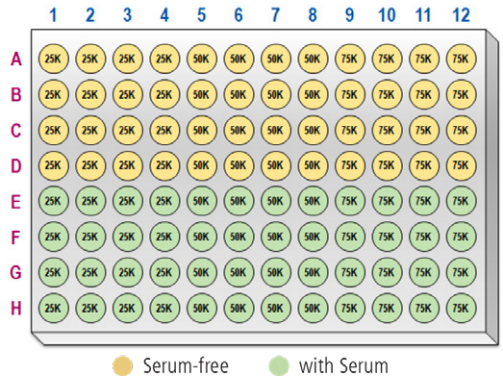
Plate 2 - To be assayed on day 2



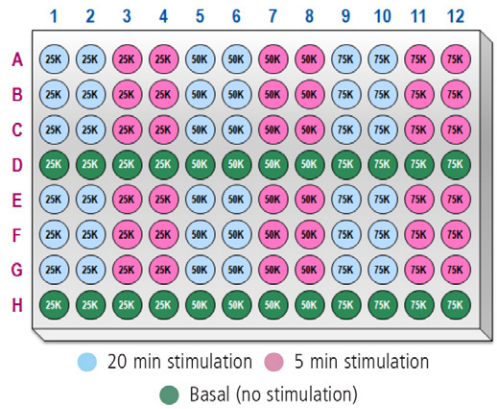
Seed Plate 2 (96 wells) using three different densities of adherent cells: 10K, 20K and 40K cells per well. Incubate plate two days.

Day 1 (Plate 1, adherent cells)

- Remove medium from half of Plate 1.
- Replace with serum-free medium.
- Incubate 4 hours.

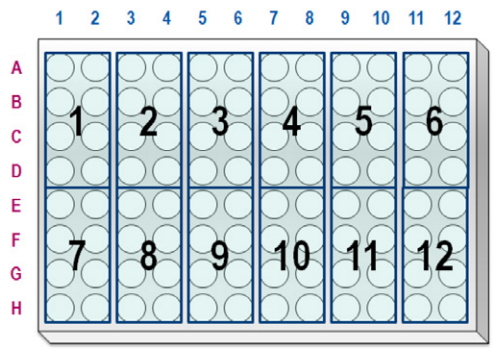


- Add agonist to “20 min stimulation” wells.
- Incubate 15 minutes.
- Add agonist to “5 min stimulation” wells.
- Incubate 5 minutes.
- Lyse cells 30 min with shaking.



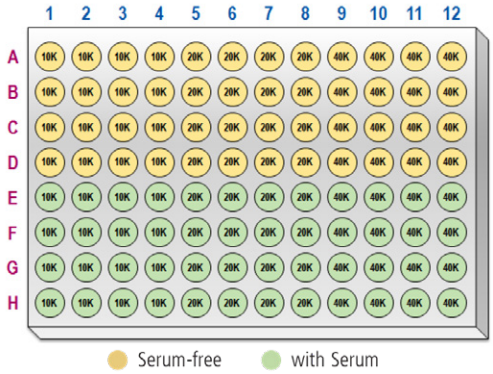
- Transfer to 384-well OptiPlate™.
- Perform LANCE assay incubations.

Results will be obtained for 12 different conditions, with six replicate and two basal samples for each.



Day 2 (Plate 2, adherent cells)

- Remove medium from half of Plate 2.
- Replace with serum-free medium.
- Incubate 4 hours.



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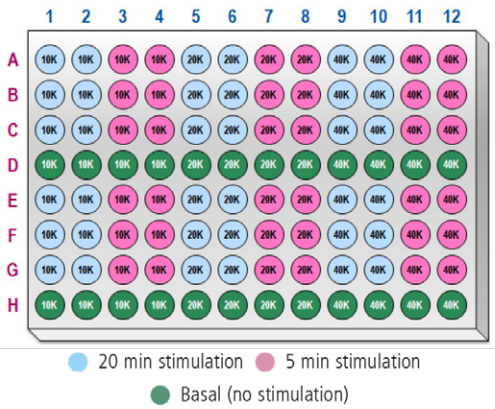

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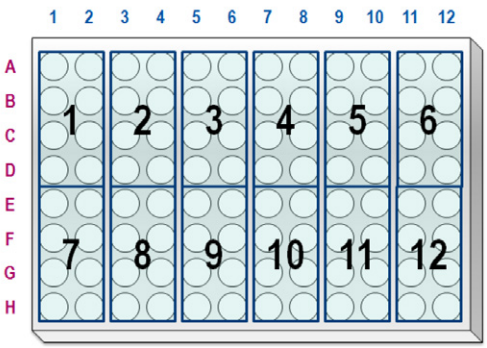
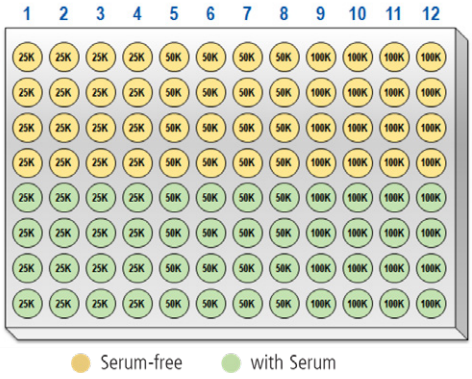


Non-adherent (suspension) cells

- For non-adherent cells, culture cells at a density between 10^7 and 10^8 cells/mL.
- On the assay day, resuspend in cell culture media plus or minus serum at 10^7 cells/mL. Plate in a 384-well OptiPlate at 50,000 - 150,000 cells (5 µL) per well.
- Incubate 2 hours.



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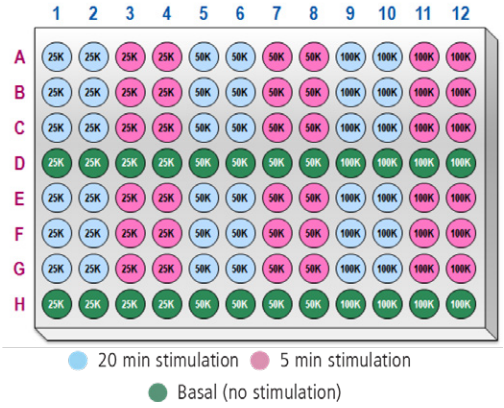
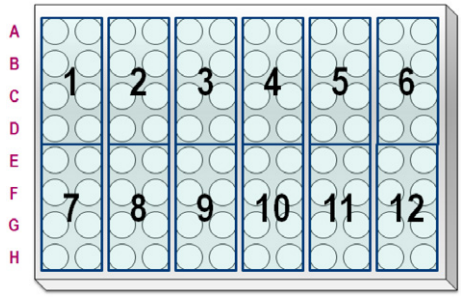


- Perform LANCE assay incubations in the 384-well OptiPlate.

Results will be obtained for four different conditions, with six replicate and two basal samples for each.



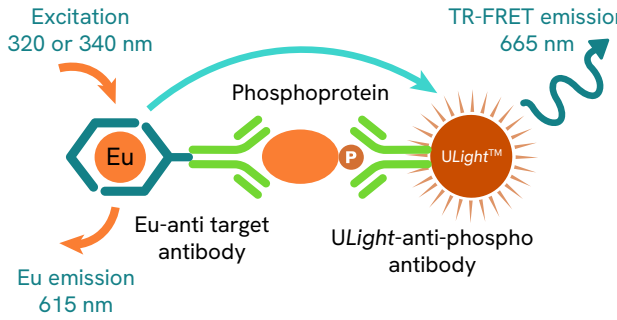


Figure 1: LANCE *Ultra* Cellular Phosphoprotein Assay Principle. In the cellular kinase assay, the first antibody is captured by the *ULight*™ anti-phospho antibody, but only recognizes the phosphorylated form of the substrate of interest. The second antibody is biotinylated and is captured by the Eu-anti-target antibody, which in turn captures the endogenous substrate. The *ULight* fluorophore and Europium chelate are only brought into close proximity in the presence of the phosphorylated substrate.

Representative data

Initial assay conditions with good S:B ratios were identified using a single multi-variable experiment (Figure 2).

Day 1 (Plate 1, adherent cells)

Serum Starved											
25K				50K				75K			
20 min		5 min		20 min		5 min		20 min		5 min	
1401	1076	2206	2265	2401	2327	4322	4376	3284	3086	**5173**	**5334**
1309	1024	2213	2292	2448	2264	4570	4548	3323	3102	**5243**	**5309**
1348	952	2282	2442	2426	2376	4795	4809	3329	3204	**5273**	**5370**
273	263	222	214	358	391	272	240	478	499	**474**	**223**
Serum											
1079	768	1937	2142	2218	2314	4695	4575	3629	3576	5296	5254
1064	830	2003	2093	2346	2420	4582	4616	3659	3664	5493	5379
1120	849	2007	2172	2353	2419	4523	4663	3671	3639	5370	5243
537	441	431	437	619	648	480	500	850	831	515	539

Day 2 (Plate 2, adherent cells)

Serum Starved											
10K				20K				40K			
20 min		5 min		20 min		5 min		20 min		5 min	
821	714	1461	1465	1006	1159	2761	2689	1612	1454	**3886**	**4449**
699	578	1470	1324	968	1013	2693	2852	1805	1701	**4233**	**3788**
670	588	1455	1314	977	1145	2841	2804	2108	1914	**4056**	**3691**
		334	245			392	243			**490**	**398**
Serum											
646	600	1324	1243	943	1048	2716	2873	2116	2196	4017	3745
604	557	1149	1221	894	1056	2788	2830	2176	2223	4027	3714
736	564	1200	1320	912	971	2159	2532	2061	2092	3932	3650
		397	419			496	467			434	440

Figure 2: Results from a multi-variable experiment for optimization of the phospho-ERK LANCE *Ultra* Cell-Based Kit. Various cell densities of A431 cells were plated and incubated for either 1 day or 2 days. Half the plates were serum-starved for four hours prior to stimulation with 100 nM (final) EGF for either 20 minutes or 5 minutes. Data were collected after overnight incubation with detection reagents. The data here are expressed as the ratio of the signal at 665 nM to the signal at 615 nM multiplied by 10,000. In this experiment, serum starving lowered the background levels of pERK and short (5 min) stimulation gave higher signal than longer (20 min) stimulation. The S:B for the best assay on Day 1 was 12.6 and for Day 2, it was nine.

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.

Table 1: LANCE *Ultra* TR-FRET Cell Signaling assay optimization parameters. (Continued)

LANCE *Ultra* TR-FRET cell signaling optimization parameter	Recommendations and comments
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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