APPLICATION NOTE

Alpha Technology

Quick Guide to AlphaScreen® *SureFire*® Assay Optimization

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 biological context. However, getting initial results with the AlphaScreen® *SureFire*® assay 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 2 culture conditions).

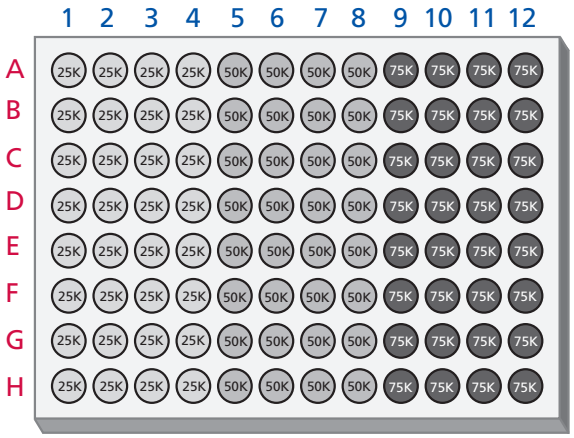
The assays as described in the kit manuals can be performed as either two-plate transfer protocols, or as a single-plate assay. We recommend beginning with the two-plate protocol.

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.

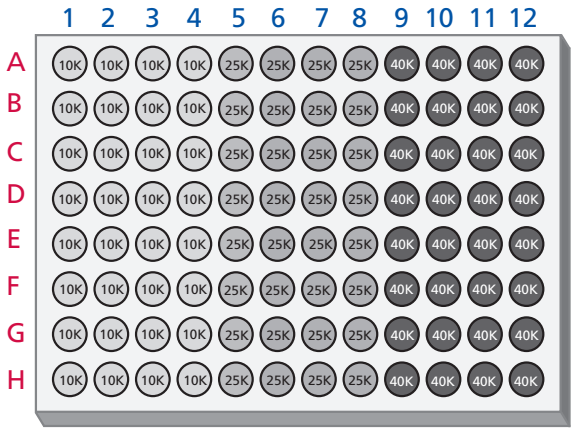
Day 0 (Adherent cells)

Plate 1 – to be assayed on Day 1



For adherent cells, seed Plate 1 (96 wells) using 3 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 3 different densities of adherent cells: 10K, 25K and 40K cells per well. Incubate plate two days.

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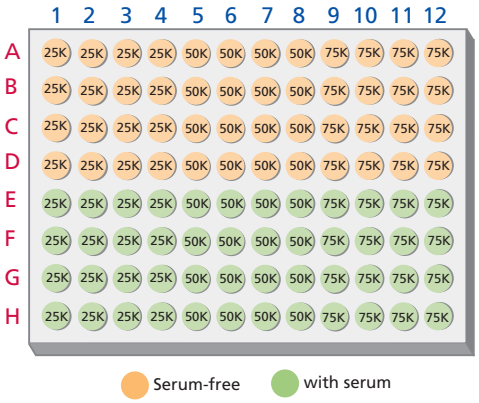
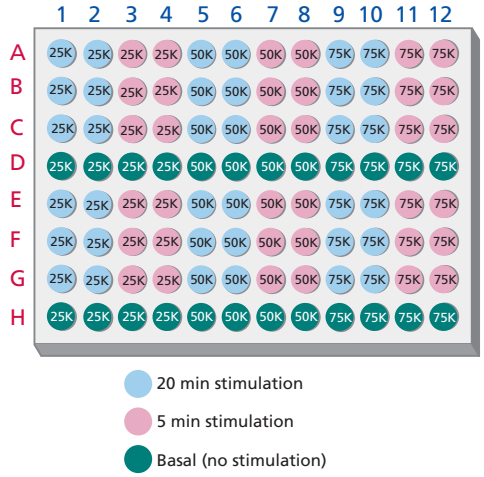
Day 1 (Plate 1, adherent cells)

- Remove medium from half of Plate 1.
- Replace with serum-free medium.
- Incubate 3 hours.

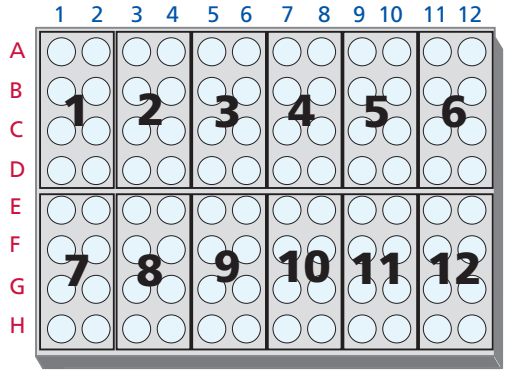


- Add agonist to "20 min stimulation" wells.
- Incubate 15 minutes.
- Add agonist to "5 min stimulation" wells.
- Incubate 5 minutes.
- Lyse cells 10 min with shaking.



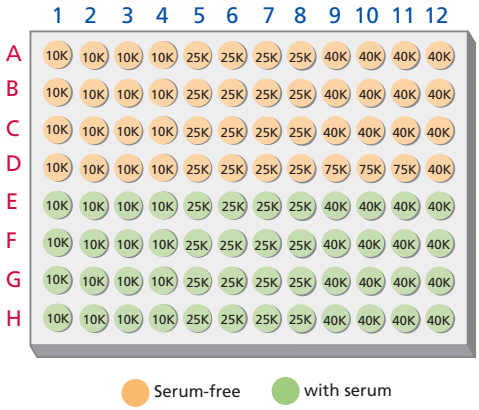
- Transfer to 384-well ProxiPlate.
- Perform AlphaScreen assay incubations.

Results will be obtained for 12 different conditions, with 6 replicate and 2 basal samples for each.



Day 2 (Plate 2, adherent cells)

- Remove medium from half of Plate 2.
- Replace with serum-free medium.
- Incubate 3 hours.



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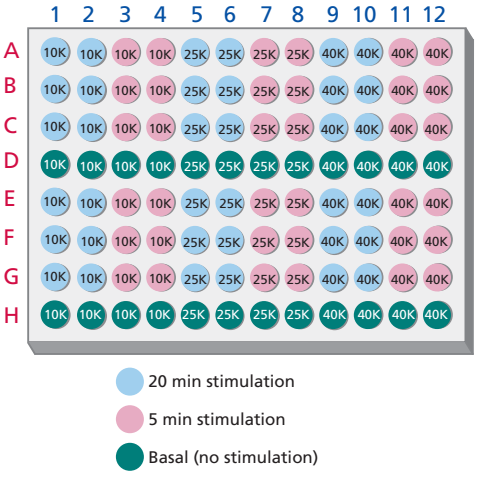

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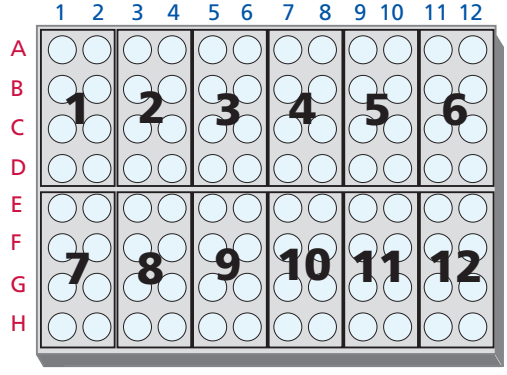
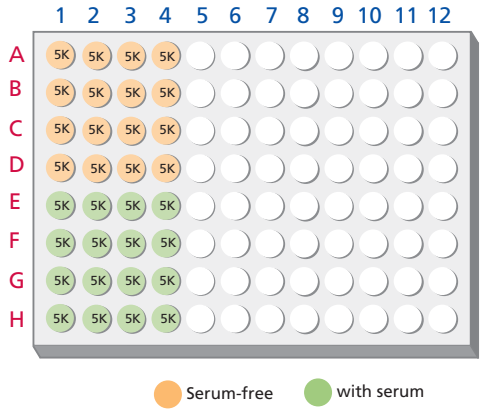


Non-adherent (suspension) cells

- For non-adherent cells, culture cells at a density between 10^6 and 10^7 cells/mL.
- On the assay day, resuspend in HBSS media plus or minus serum at 10^6 cells/mL. Plate in a 384-well OptiPlate at 5000 cells (5 µL) per well.
- Incubate 2 hours.



- Add agonist to "20 min stimulation" wells.
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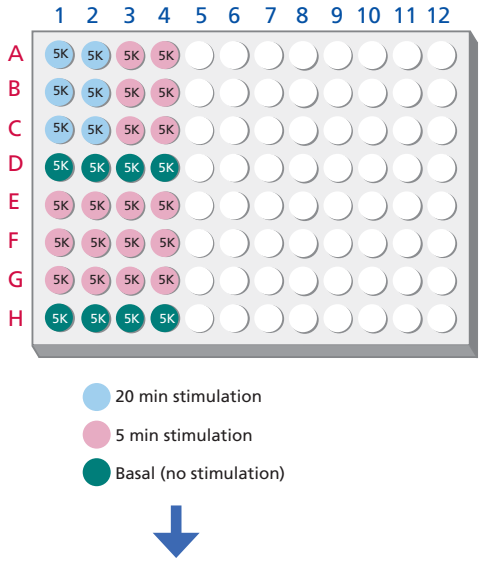
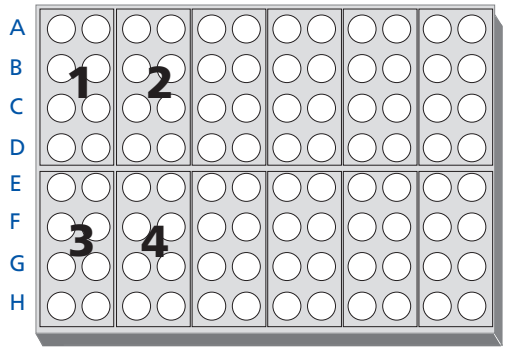
- Lyse cells 10 min with shaking.



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

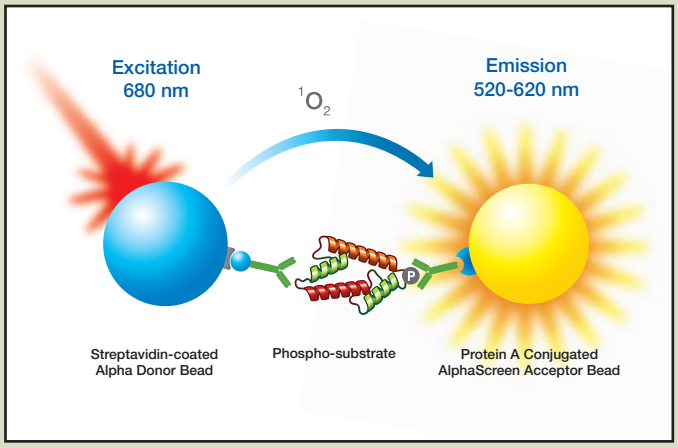
Results will be obtained for 4 different conditions, with 6 replicate and 2 basal samples for each.



Mechanism of Action

1. In the cellular kinase assay, the first antibody is captured by the Protein A-coated Acceptor beads, but only recognizes the phosphorylated format of the substrate of interest
2. The second antibody is biotinylated and is captured by the streptavidin-coated Alpha Donor bead, which in turn captures the endogenous substrate
3. The two beads are only brought into close proximity in the presence of the phosphorylated substrate. Because the technology is extremely sensitive, it can detect endogenous levels of substrate in cells

AlphaScreen® *SureFire*® Technology



AlphaScreen® *SureFire*® kits are used in conjunction with AlphaScreen Protein A kits, which include the streptavidin-coated Alpha Donor bead and the Protein A-coated Acceptor bead.

Representative data: Initial assay conditions with good S:B ratios were identified using a single multi-variable experiment.

Day 1 (Plate 1, adherent cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A	68988	121352	59060	67236	171172	202870	93184	96212	245812	271276	169176	179952
B	113392	47592	68084	65620	111812	208396	102096	110804	255168	251636	152044	166496
C	43300	48440	57196	58724	86824	125928	88328	83776	262336	172100	120692	161268
D	25484	23720	22936	21636	27536	28680	27732	28160	39544	28556	38604	40088
E	29420	25664	47884	51576	76548	42764	53860	54584	78560	146212	92020	115128
F	34860	32584	54092	41780	46004	50232	60408	58944	61880	64724	91696	102152
G	30348	38356	77424	68700	91764	54324	78192	88716	82444	89568	105404	112796
H	21904	19552	18776	19540	23140	29328	22292	21044	32400	29524	39424	37012

Day 2 (Plate 2, adherent cells)

	1	2	3	4	5	6	7	8	9	10	11	12
A	66684	131348	53508	55604	301420	326768	95080	101500	353064	446272	134660	154824
B	125396	154044	67868	66152	262624	325160	121056	122732	399456	416948	151392	196304
C	96096	112324	43268	43500	224084	242140	85908	89520	330452	307676	131208	144392
D	13860	14720	14216	14204	25608	21356	27016	25460	32780	30860	30796	31568
E	19148	24336	44304	31836	116040	93028	116948	90776	130356	127996	163588	122024
F	22924	50772	40428	41616	122624	91248	85960	94852	146216	129680	105160	98308
G	30152	23604	65824	62628	141724	156224	100912	129504	155132	168164	167672	186012
H	256	12792	13456	10880	21740	29572	29248	26480	33040	35428	30284	29732

Figure 1. The two images show the results of a multi-variable experiment performed as described above. The assay results are visualized as false-color images from the EnVision multilabel detection reader, where red wells have generated the highest signals. Although initial AlphaScreen® *SureFire*® assay results can vary widely, this approach quickly identifies those assay conditions (circled in green) that can best serve as a starting point for further assay optimization. In this experiment, the level of ERK phosphorylation was measured in an adherent CHO cell line expressing the muscarinic M4 receptor stimulated with 1 µM acetylcholine. S:B values for the circled values were 7 for Day 1 and 13 for Day 2.

Step 2: Further optimize the AlphaScreen *SureFire*® assay using the parameters in this table . For more information please consult the AlphaScreen® *SureFire*® Cellular Kinase Assays User Guide.

AlphaScreen® *SureFire*® Optimization Parameter	Recommendations and Comments
Seeding density for adherent cells in microplates	Suggested range: 10,000-75,000 cells per well Start with: Try 3 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 adherent cells in assay plates	Suggested range: 1-2 days 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 but cells should be seeded for assay in phenol red free medium, since phenol red quenches the AlphaScreen® *SureFire*® signal.
Serum starvation requirement	Suggested range: none to overnight Start with: 2-3 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.

AlphaScreen® *SureFire*® Optimization Parameter	Recommendations and Comments
Cell stimulation time course	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: EC100 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 during stimulation	Suggested range: room temperature or 37 °C Start with: room temperature AlphaScreen® *SureFire*® 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	Options: Standard or more aggressive lysis buffer Start with: Standard lysis buffer Cell lysis for 10 minutes with gentle shaking (350 rpm) is usually sufficient for complete lysis. The AlphaScreen® *SureFire*® lysis buffer is a very mild formulation, so cells will generally appear intact when viewed under a microscope post-lysis. However, all of the soluble components of the cell will have been released. The lysis buffer contains phosphatase inhibitors; additional protease inhibitors or EDTA may be beneficial in individual cases. A more aggressive lysis solution** may be used for certain cell lines or for nuclear-associated kinases, but this will release chromatin and result in a more viscous solution. **(1 part activation buffer + 4 parts 1X lysis buffer)
Lysis buffer volume	Suggested range: 25–100 µL for a 96-well plate Start with: 100 µL In most cases, 100 µL of lysis buffer is satisfactory. Reducing the lysis to 50 µL may give an improved signal for targets present in low abundance. Shaking (350 rpm) or other mixing is important to reduce assay variability.
AlphaScreen® *SureFire*® assay: 1-step vs. 2-step	Start with: 2-step protocol In general, AlphaScreen® *SureFire*® assays can be formatted as 2-step bead additions or a single addition of a mixture of both beads. The 2-step protocol gives a higher signal than the 1-step protocol. The data sheet for each AlphaScreen SureFire assay will indicate if a 1-step assay is an option. If the assay yields satisfactory results using the 1-step bead addition it gives the benefit of one less liquid handling step.
AlphaScreen® *SureFire*® assay Incubation times	Suggested range: Acceptor bead step: 2 hours, Donor bead step: 2 hrs to overnight Start with: 2-hours for each step The AlphaScreen® *SureFire*® assay signal is usually sufficiently developed so that the plate can be read after a donor bead incubation of 2 hours. In some cases a 4 hour or even an overnight incubation with donor beads may give a higher signal window.
Donor and acceptor bead concentrations	Suggested range: 0.5-1X of donor and acceptor bead concentrations Start with: 1X (recommended) bead concentrations If signal:background ratios or Z′ values are low, try adding a 2-fold lower concentration of the donor beads and/or acceptor beads. This may be helpful with some combinations of cell lines and assay targets.

References

AlphaScreen® S*ureFire*® Cellular Kinase Assays User Guide.

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008829_01 Printed in USA