AlphaScreen® *SureFire* HV™
ERK 1/2 (p-Thr202/Tyr204) Assay Kit

Manual

Assay Points	Catalog #
100	TGRESHV100

For Laboratory Use Only
Research Reagents for Research Purposes Only

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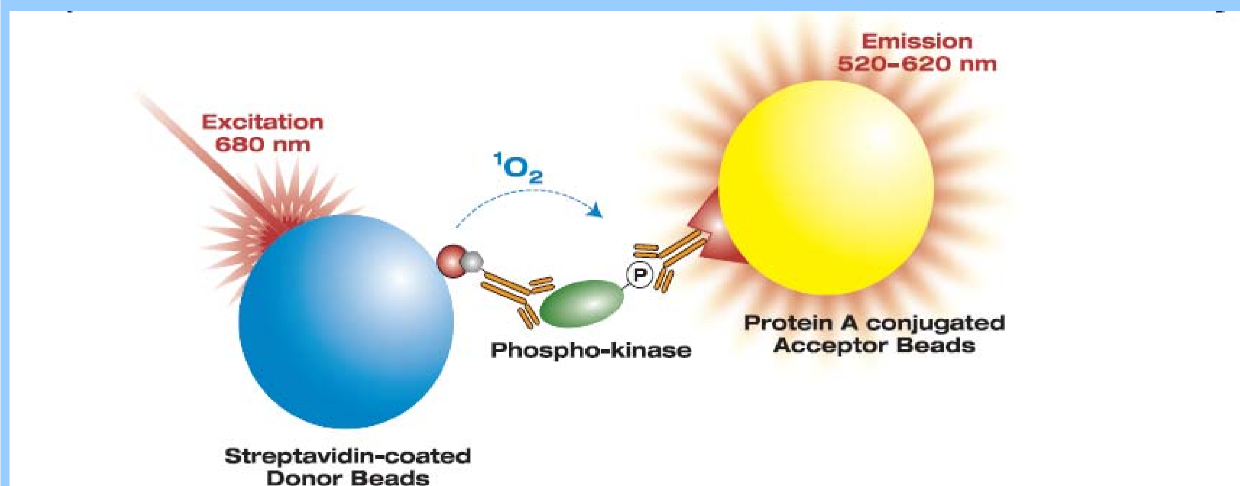
General Information on the AlphaScreen® *SureFire* HV™ p-ERK 1/2 assay

The AlphaScreen® *SureFire* HV™ p-ERK 1/2 assay is used to measure the phosphorylation of endogenous extracellular signal-regulated kinase 1 and 2 (ERK 1/2) in cellular lysates, using a convenient 96-well protocol for low throughput applications and assays with minimum volume requirements. The assay is an ideal system for interrogating modulators of receptor activation (e.g. agonists and antagonists) as well as agents acting intracellularly, such as small molecule inhibitors of upstream events. The assay will measure ERK 1/2 activation by either recombinant or endogenous receptors, and can be applied to primary cells.

This quantitative assay eliminates the need for laborious techniques, such as Western blotting or conventional ELISA. It is a homogeneous assay, in that no sample washing steps are required, which allows for minimal handling, less variation, and short assay times. The assay utilizes the bead-based Alpha Technology, and requires an Alpha Technology-compatible plate reader.

The p-ERK 1/2 AlphaScreen *SureFire* HV assay kits contain all reagents necessary to carry out the measurement of phospho-ERK 1/2 in cells, with the exception of AlphaScreen beads, which need to be ordered separately (see below). The number of assay points provided in the kit is based on a 50 μL protocol.

Alpha Technology AlphaScreen® *SureFire* HV™ Assay Principle



AlphaScreen *SureFire* technology allows the detection of phosphorylated proteins in cellular lysates in a highly sensitive, quantitative and user friendly assay. In these assays, sandwich antibody complexes, which are only formed in the presence of analyte, are captured by AlphaScreen Donor and Acceptor beads, bringing them into close proximity. The excitation of the Donor bead provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in the emission of light at 520-620nm.

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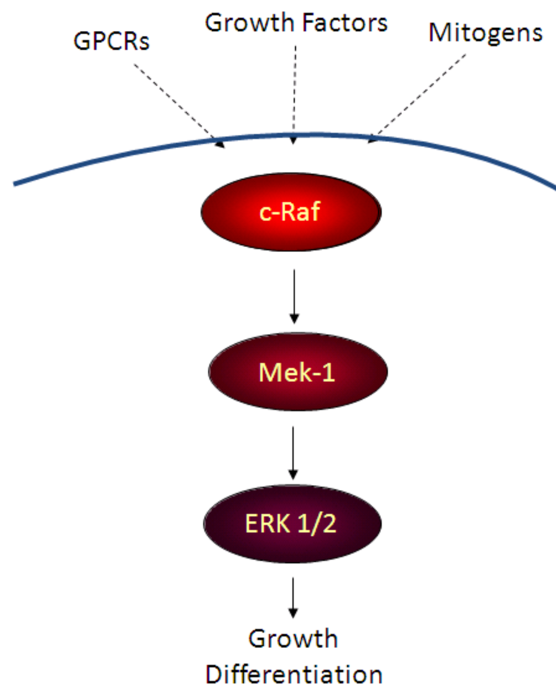
Background information on the detected analyte

Two closely related proteins, ERK1 and ERK2, belong to a widely conserved family of serine/threonine protein kinases. ERK 1/2 are involved in cellular signaling events associated with a range of stimuli, including mitogens, growth factors, and cytokines. The kinase activity of ERK proteins is regulated by dual phosphorylation at Threonine 202/Tyrosine 204 in ERK1, and Threonine 185/Tyrosine 187 in ERK2. MEK1 and MEK2 are the primary upstream kinases responsible for ERK 1/2 in this pathway. Many downstream targets of ERK 1/2 have been identified, including other kinases, and transcription factors. In addition to normal cellular processes, this pathway has been associated with signaling in many cancers.

Below is an overview of the classical MAPK signaling pathway, where the activation of a cell surface receptor triggers the classical MAPKKK/MAPKK/MAPK phosphorylation cascade.



Kit-Specificity information

This assay kit contains 2 antibodies; a biotinylated antibody which recognizes the phospho-Thr202/Tyr204-epitope, and a non-biotinylated antibody which recognizes a distal epitope on ERK 1/2. The proteins detected by this kit correspond to GenBank Accession NP 002737.2(ERK1); NP 620407(ERK2). Alternate Names include p44 MAPK, MAPK3 (ERK1), p42 MAPK, MAPK1 (ERK2).

These antibodies recognize ERK 1/2 of human, mouse, rat and hamster origin. Other species should be tested on a case-by-case basis.

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Kit Contents

	Kit Size
	100 points
Lysis buffer (5X)	4 x 2 mL
Activation buffer	1 x 2 mL
Reaction buffer	1 x 0.9 mL
Dilution buffer	1 x 1.5 mL
Assay Control Lysate	1 tube to be re-dissolved in 250 µL dd H_2O

Materials Required But Not Provided

The AlphaScreen® *SureFire* HV™ assay kits are optimized to work with AlphaScreen Protein A general IgG detection beads. These are available separately from PerkinElmer. The AlphaScreen Protein A general IgG detection kits contain a biotinylated rabbit IgG control, which can be used to test the instrument settings and bead performance.

Item	Suggested source	Catalog #	Size
Protein A general IgG detection kit (contains the Acceptor and Donor Beads)	PerkinElmer Inc.	6760617HV	100pt
1/2 AREAPLATE-96, White	PerkinElmer Inc.	6005560 6005569	50/box 200/box
TopSeal-A™ 96, clear adhesive sealing film	PerkinElmer Inc.	6005185	100/box
EnVision® or EnSpire® Alpha-reader	PerkinElmer Inc.	-	-

Complementary Products

Item	Suggested source	Catalog #	Size
5X AlphaScreen *SureFire* Lysis buffer	PerkinElmer Inc.	TGRLB10ML TGRLB100ML	10 mL 100 mL
AlphaScreen *SureFire* Activation buffer	PerkinElmer Inc.	TGRAB10ML TGRAB100ML	10 mL 100 mL
AlphaScreen *SureFire* Dilution buffer	PerkinElmer Inc.	TGRDB10ML TGRDB100ML	10 mL 100 mL

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Storage conditions upon receipt

The kit buffers (e.g. 5X Lysis buffer, Activation Buffer, Reaction Buffer and Dilution Buffer) should be stored at 4°C. DO NOT FREEZE the kit buffers – the Reaction Buffer contains antibodies and freeze/thaw cycles can lead to a loss of activity.

The Assay control lysates are supplied lyophilized. After reconstitution, control lysates should be frozen in single use aliquots kept at -20°C or -80°C, and unused portions discarded.

Buffer preparation and subsequent storage conditions

5X Lysis buffer	Store 5X Lysis buffer at 4°C. For assay, dilute **5-fold** in either dd H_2O or MilliQ® H_2O immediately prior to use. Discard unused buffer.
Activation buffer	Precipitation will occur during storage at 4°C. To re-dissolve, warm to 37°C and mix. Alternatively, Activation buffer can be stored at room temperature with no loss in activity.
Reaction buffer*	Store at 4°C. Keep on ice while in use. Do not freeze. Once diluted discard unused reaction buffer.
AlphaScreen® Protein A IgG Kit	Store at 4°C in the dark.
Acceptor Mix (Reaction buffer + Activation buffer + AlphaScreen® Acceptor beads)	Immediately prior to use, dilute Activation buffer **5-fold** in Reaction buffer (e.g. take 40 µL Activation buffer and dilute in 160 µL Reaction buffer). Dilute Acceptor beads **67-fold** in Acceptor Mix (e.g. add 3 µL Acceptor beads to 200 µL of premixed Reaction buffer + Activation buffer). The Acceptor mix should be used immediately for best results. Excess mix should be discarded.
Donor Mix** (Dilution buffer + AlphaScreen® Donor beads)	Immediately prior to use, dilute Donor beads **33-fold** in Dilution buffer (e.g. add 6 µL Donor beads to 194 µL Dilution buffer). The Donor mix should be used immediately for best results. Excess mix should be discarded.
Assay Control lysate	Stable while lyophilized at -20°C, to expiry date. After reconstitution in 250 µL water, lysates should be frozen at -20°C in single use aliquots and used within 1 month.

* Do not vortex the Reaction buffer, as vigorous mixing can damage some antibodies.

** Prepare and use Donor Mix under low-light conditions.

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Control Lysate information

Control lysates are prepared at a protein concentration of approximately 0.25 mg/mL from flasks of A-431 cells (ATCC #CRL-1555). The controls are supplied lyophilized, and should be reconstituted in either dd H_2O or MilliQ® H_2O. Once reconstituted, lysates should be stored frozen in single use aliquots.

Control Lysate: Prepared from A431 cells (no serum-starvation) treated with 200 ng/mL EGF for 10 minutes.

Protocol Overview

For AlphaScreen *SureFire* HV assays, a cellular lysate is generated in a flask or tissue culture plate, and transferred to an assay plate for analysis.

Protocol Overview

Prepare cells in a tissue culture plate, treating with agonists/antagonists as required

Prepare cellular lysates,

Transfer a portion of lysate to an assay plate

Add Acceptor Mix tolysate

Incubate plate

Add Donor Mix tolysate

Incubate plate

Read plate

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Assay optimization recommendations

There are several parameters that should be optimized to achieve the best possible assay performance. We advise that the following parameters are optimized during the early phase of assay validation, to ensure optimum assay performance.

1) Cell Culture

Adherent Cells: low passage cells should be maintained in full growth media, and split at 70-90% confluence. Cells should not be allowed to grow to confluence.

Non-Adherent Cells: low passage cells should be maintained in logarithmic growth phase, in full media. Do not allow cells to grow to stationary phase during maintenance. Follow manufacturer instructions for cell-line specific splitting conditions and media recommendations. Useful cell handling guides can be found at the ATCC website (http://www.lgcstandards-atcc.org).

2) Cell Seeding

Adherent Cells: cell seeding densities of 10,000-20,000 cells/well (96- well format) are generally sufficient for most cell lines, but optimization for individual cell lines is recommended to maximize signal. We recommend that adherent cells are used once they reach a confluent monolayer. Some applications may benefit from a serum-starvation step, where full media is removed and replaced with serum-free media. This step should be optimized on a case-by-case basis, but will generally be between 2 hours up to overnight.

Non-Adherent Cells: cells should be harvested from flasks and re-suspended in an assay buffer such as HBSS at an optimized density (10^7 cells/mL is the recommended starting point). Typically, after handling, cells are incubated at 37°C for 2 hours, prior to stimulation.

3) Cell Stimulation

The optimal time of stimulation can vary widely, from a few minutes to more than one hour, depending on the type of stimulation, temperature, and the target of interest. Because of this, we recommend a time course study be carried out by the end user to determine the optimal stimulation time. Useful cell handling and stimulation information can be found on the TGR website (http://www.tgrbio.com).

Please note that peptidic agonists and antagonists can often stick to plastic surfaces. To minimize this effect, dilute in serum-free media containing a suitable carrier protein (e.g. 0.1% IgG free BSA - Jackson Immunoresearch Cat #001-000-161).

4) Lysate Preparation

The Lysis buffer is supplied as a 5X concentrate, and should be diluted 5-fold with H_2O immediately prior to use. We recommend cells are lysed at room temperature with shaking (~350 rpm) for 10 minutes. Lysates can be frozen and stored at -80°C for analysis at a later time, although long-term storage of frozen lysates is not recommended. The amount of Lysis buffer can be varied to obtain more concentrated cell lysates, and higher signal. e.g. 50 µL of 1x Lysis buffer can be used to lyse adherent cells instead of 100 µL.

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p-ERK AlphaScreen *SureFire* HV™ assay protocols

I. Adherent Cells - Assay Protocol

Cell Seeding

1. Seed cells (10-20K cells/well for a 96-well plate is usually sufficient) in tissue culture plates. Incubate at 37°C overnight in serum-containing media.

Cell Treatment

2. Remove culture media, and stimulate the cells with 50 µL agonists prepared in serum-free media. (*If testing antagonists, prior to stimulation, remove culture medium and replace with 50 µL serum-free media containing antagonists*). Return cells to 37°C incubator for desired time. 1 hour is often sufficient for signal transduction inhibitors and 5 minutes for receptor agonists.

Lysate Preparation

3. To lyse cells, carefully remove medium from wells, and add freshly prepared 1X Lysis Buffer (use 50-100 µL/well for a 96-well plate). Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.

4. Take 30 µL of the lysate and transfer to a 96-well ½-area assay plate for assay. (*Add 30 µL Control lysate to separate wells if required, use 30 µL 1X Lysis buffer for negative control*).

SureFire Assay

5. Add 10 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Agitate on plate shaker for 2 minutes (~350 rpm), and then incubate for 2 hours at room temperature.

6. Add 10 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Agitate on plate shaker for 2 minutes (~350 rpm), and then incubate for 2 hours at room temperature.

Note: Longer incubation may give greater sensitivity. Plates can be incubated overnight if required.

7. Remove the Topseal and read plate on an Alpha Technology-compatible plate reader, using standard AlphaScreen® settings.

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II. Non-adherent Cells – Assay Protocol

<u>*Cell Seeding*</u>

1. Harvest cells by centrifugation, and re-suspend cells in HBSS at a suitable cell density. We recommend 10^7 cells/mL as a starting point. Seed 40 µL of cells/well into a tissue culture plate.

2. If using test agents/antagonists, add 20 µL/well of antagonists prepared in HBSS. (*If no inhibitors are used, proceed directly to step 3*).

Note: Peptidic agonists and antagonists can often stick to plastic surfaces. To minimize this effect, use a suitable carrier protein (e.g. 0.1% IgG free BSA - Jackson Immunoresearch Cat #001-000-161).

3. Return cells to incubator at 37°C for 1-2 hours.

<u>*Cell Treatment*</u>

4. Stimulate cells with agonists by addition of 20 µL/well of 4X agonist stock in HBSS containing 0.1% BSA. <u>The final volume in the wells should be 80 µL</u>. (*If no antagonists were used at step 2, stimulate the cells with 40 µL/well of 2X agonist, to give a final volume in the wells of 80 µL*.)

<u>*Lysate Preparation*</u>

5. To lyse the cells, add 20 µL/well 5X Lysis buffer. Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.

6. Take 30 µL of the lysate and transfer to a 96-well ½-area assay plate for assay. (*Add 30 µL Control lysate to separate wells if required, use 30 µL 1X Lysis buffer for negative control*).

<u>*SureFire Assay*</u>

6. Add 10 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature.

7. Add 10 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature.

Note: Longer incubation may give greater sensitivity. Plates can be incubated overnight if required.

8. Remove the Topseal and read plate on an Alpha Technology-compatible plate reader, using standard AlphaScreen® settings.

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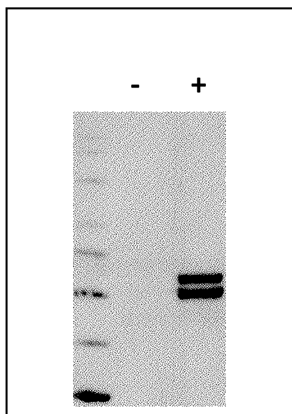
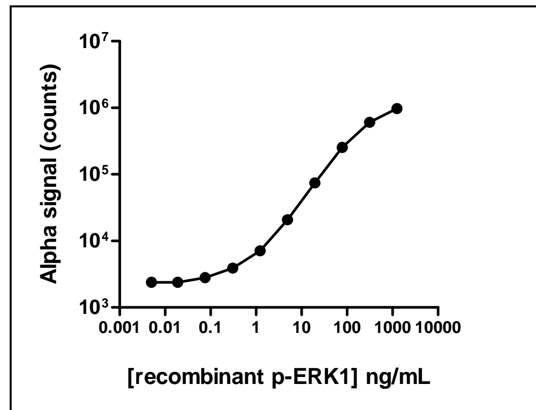
Data Analysis

Raw counts are used as the Y axis unit, which can be referred to as "AlphaScreen Signal (counts)". To analyze the data, calculate the averaged counts for untreated and treated cells. We recommend using at least 3 separate wells (n=3) to calculate an average response. Dose response and dose inhibition curves are readily analyzed using 4 parameter non-linear regression equation (e.g. sigmoidal dose-response curve with variable slope). These types of regression analyses output key parameters such as EC_{50} (or IC_{50}), Min and Max signals, and Hillslope factors. While absolute AlphaScreen counts will vary from reader to reader, and from day to day, the assay window (Stimulated/Basal) is expected be specific for a given cell type under selected assay conditions. Temperature has an impact on the signal, and the use of a 22-25°C incubator will help to generate a more consistent signal.

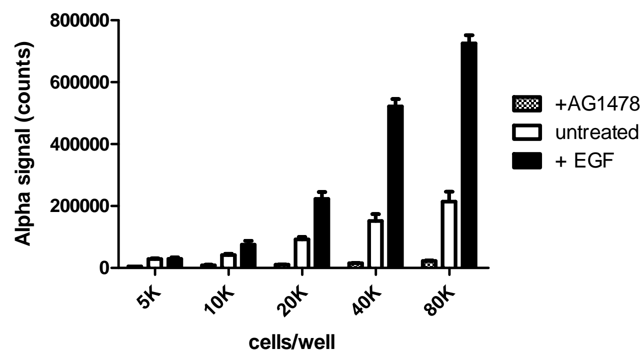
Representative Data

Left panel: Western blot analysis (30 µg protein/lane) of phospho-ERK 1/2 in lysates generated from either unstimulated (-) or PMA-stimulated (+) Jurkat cells. Right panel: detection of recombinant phospho-ERK (Biaffin GMBH Cat# PK-ERK1-A010). Using the standard protocol, the limit of detection is around 0.1 ng/mL.





A431 cells were seeded at various densities in 96-well microplates in media containing 10% FBS, and incubated overnight. The following day, the cells were either treated with 10 µM EGFR inhibitor AG1478 for 2 hours, or stimulated with 1 µg/mL EGF, or left untreated. The cells were lysed with 50 µL 1X Lysis buffer with shaking at RT for 10 minutes and analyzed for phospho-ERK 1/2 using the standard AlphaScreen *SureFire* HV protocol.



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Frequently Asked Questions

General cell handling

Cells should be harvested from flasks for seeding into microplates when approximately 70-90% confluent. The cells should be detached from the flasks using mild conditions, accurately counted, and diluted to the appropriate density in fresh media. If using adherent cells, allow to adhere in full media for at least 6 hours prior, allowing time for cells to regain full signaling capacity after harvesting.

Serum starvation requirement

Some applications may benefit from a serum-starvation step, where full media is removed and replaced with serum-free media. This can reduce the basal level of activity of certain signaling pathways, such as MAPK signaling. This step should be optimized on a case-by-case basis, but will generally be from 2 hours, up to overnight. In some cases, seeding the cells in low serum medium (e.g. 1% serum) may be more convenient than and as effective as a serum-starvation step.

Cell lysis

The standard Lysis buffer is of a gentle nature, and cells will often appear 'intact' when viewed with a microscope. However, the soluble components of the cells have been released. A more aggressive lysis formulation can be prepared by the addition of activation buffer to the lysis buffer formulation, which will solublize the cells more thoroughly and release proteins bound in protein complexes.

The more aggressive lysis buffer can be easily prepared prior to lysis by diluting Activation buffer 10-fold in 1X Lysis buffer (e.g. dilute 1 mL Activation buffer in 9 mL 1X Lysis buffer). The release of chromatin may be observed using this Lysis buffer, which may make the lysates more difficult to handle.

! Important: if Lysis buffer/Activation buffer mix is used to lyse the cells, ensure that no Activation buffer is added to the Acceptor mix during preparation (e.g. Acceptor mix should contain just Reaction buffer and AlphaScreen beads).

A low signal can often be improved by generating more concentrated lysates. In most cases, a typical adherent cell line grown in 96-well plates is readily detected in a lysis volume of 50-100 µL. However, for low abundance proteins, the lysis volume can be adjusted down to 25 µL, to increase the analyte concentration in the lysate. For cells that express very low levels of the target of interest (e.g. if immunoprecipitation is required to see a band on a Western blot) the amount of analyte may be below the detectable limit for *SureFire* HV assays.

The standard Lysis buffer supplied with the kits contains phosphatase inhibitors. The addition of protease inhibitors or EDTA may be beneficial in some cases.

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Assay incubation times

The general assay incubation times that are recommended are 2 hours for each assay reagent addition - 2 x 2 hours is recommended. Longer incubations (up to overnight) may be more convenient for certain assays, and can enhance sensitivity in some cases.

AlphaScreen bead concentrations

The standard concentration of AlphaScreen beads is provided. However, if poor sensitivity is observed, adjusting the bead concentrations in the Acceptor or Donor Mix may help.

Buffer compatibility

The AlphaScreen *SureFire* HV assays are compatible with most cell culture media and reagents, however there are some exceptions. Media that contain biotin (i.e. RPMI) will reduce assay sensitivity due to the interference of biotin on the antibody-streptavidin interaction. When it is necessary to use a media such as RPMI for growing cells, they should be harvested and re-suspended in HBSS or similar buffers for the assay. Phenol red can also quench AlphaScreen signal. This is not a problem when media is removed from the cells prior to lysis. For non-adherent cells that are resuspended in media rather than HBSS, use phenol-red free media where possible.

Common interfering compounds used in cell culture

Compound	Effect
Biotin (present in media such as RPMI)	Can interfere with immunoassay components
Serum	Can interfere with immunoassay components
SDS	Can denature streptavidin at low concentrations
Phenol Red	Quenches AlphaScreen signal.
Antibodies	Can interfere with immunoassay components

Cell types that can be used in the assay

The assay can be used for many adherent and non-adherent cell types, including transfected cell lines and primary cells. However, because kinase expression and phosphorylation conditions can vary from one cell line to another, some cells may be more amenable for particular assays than others. Parameters such as stimulation time and cell number can be optimized for each cell line used to optimize parameters such as assay window and EC_{50}.

Cells over-expressing a receptor of interest have been shown to elicit good phosphorylation responses. Cell lines expressing high levels of an intracellular kinase of interest can also be used, but should be the full-length kinase to ensure correct binding of assay antibodies. When using overexpressed intracellular targets, the concentration of cell lysate should be optimized to ensure the signal is within the working range of the assay.

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Subtracting a background control for data analysis

In most cases, we would not recommend the subtraction of buffer-only background during data analysis. For methods such as ELISA, subtraction of buffers-only controls is possible because cellular debris and interfering substances are washed away during the many wash steps involved in typical ELISA protocols. In contrast, *SureFire* assays are homogeneous, and the assays are performed and read in crude cellular lysates containing proteins, lipids, nucleic acids and other cellular debris. Therefore, in this homogeneous system, the most appropriate background control for subtraction is a cellular lysate that has no phosphorylated target. Because these cellular debris can impact the immunoassay, a "buffer-only" value is not an appropriate reference in this case. Subtraction of cellular background/basal phosphorylation prior to analysis may be useful in some instances.

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Troubleshooting

Low Signal

- Ensure the Activation buffer is properly re-dissolved prior to use.
- Ensure that all assay steps involving AlphaScreen beads are performed in a light-subdued environment. Exposure to bright light can permanently quench AlphaScreen beads. All bead handling should be done in either a green light environment, or under low light conditions. Attention: Dark rooms equipped with red lighting (for photographic/radiographic work) are NOT sufficient when working with AlphaScreen beads, as these beads are mostly sensitive to red light.
- Ensure incubation temperature for assay is at least 22°C – temperature can have a dramatic effect on both antibody performance, and AlphaScreen bead performance.
- Check that cell density is correct. Cell numbers that are too high or low can influence the activation of intracellular signaling pathways.
- Ensure cell passage number is not too high, and that cells have not lost responsiveness.
- During assay setup, a useful guide to the expected kit performance is Western blot analysis. If a target band is observed by Western blot, then a signal should be detected using the AlphaScreen *SureFire* HV assay.

High Background

- Check that cell density is correct. Cell numbers that are either too low or too high can affect basal kinase activation.
- Ensure cell passage number is not too high, and that cells are behaving as expected.
- Ensure that stimulation buffer does not contain serum if the pathway that is being monitored is activated by serum.
- Some pathways may have a high level of basal or constitutive activity in certain cells (e.g. AKT activation in HEK293 cells). An upstream pathway inhibitor is often useful to determine assay window for these targets.
- Ensure that AlphaScreen beads are in good condition, and have been stored and handled correctly.

Poor Assay Sensitivity

- Produce more concentrated lysates by either reducing lysis volume, or increasing the number of cells/well. Often endogenous targets are at low abundance in cells.
- A useful guide to expected kit performance is by Western blot analysis. If a target band is observed by Western blot, then a signal should be detected using the *SureFire* assay.
- Increase total incubation period (up to overnight incubation) of the reaction solution; this can increase assay sensitivity in some cases.

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Poor cell stimulation

- Check that the cells are confluent. When confluent, many signaling pathways – particularly those associated with growth such as ERK – can become quiescent and synchronized. When an agonist is introduced, the cells can often respond uniformly.
- Ensure cell passage number is not too high, and that cells have not lost responsiveness.
- Check cell harvesting conditions and ensure good cell viability after harvesting. Typically cells should be maintained in log-phase growth, and harvested when 70-90% confluent. Where possible use mild harvesting conditions, such as trypsin-free cell dissociation.
- Ensure the receptor and signaling pathway of interest is active in the cells, and is activated by the specific agonist that is used. This may vary depending on the cell line.
- Ensure that stimulant/agonist is not degraded. Prepare fresh prior to assay.
- Many agonists and antagonists can stick to plastic surfaces. To minimize this effect, dilute in buffer or serum-free media containing a suitable carrier protein
- Test stimulation of another kind of endogenous receptor of the cells to check that the poor stimulation observed is not specific to the receptor/pathway/cells looked at.

Day to Day Variation

- Check cell harvesting conditions, use a standard protocol for cell culture and harvesting. Pay attention not to aspirate cells in the medium removal step. A few µL of culture medium can remain in the wells without interfering with the assay.
- Check for variability in room temperature.
- Check for variation in stimulation times and assay incubation times.
- A useful control for assay variation is to use a standard lysate on all assay plates where possible.

To download these resources, and other related technical information, visit
http://las.perkinelmer.com/surefire

For general information on AlphaScreen *SureFire* assays, visit http://www.tgrbio.com

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USA and Europe

Phone: Please do not hesitate to contact PerkinElmer Customer Care for more information at toll free 1-800-762-4000 (US & Canada), 0800 111933 (AT), 0800 40858 (B), 800 26588 (L), 808 84236 (DK), 800 117186 (FI), 0805 111333 (F), 0800 1810032 (DE), 800 906642 (I), 0800 234490 (NL), 800 18854 (NW), 800 099164 (SP), 020 0887520 (SE), 0800 000015 (CH), 0800 896046 (GB), 81-45-314-8261 (JP) - **Prompt 1 all numbers**.

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