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AlphaScreen® SureFire®

Cellular Kinase Assays

User Guide

For Laboratory Use Only

RESEARCH REAGENTS FOR RESEARCH PURPOSES ONLY

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Introduction

The AlphaScreen® SureFire® range of cellular kinase assays have been designed to directly measure endogenous levels of phosphorylated proteins in a cell-based format. Combined with PerkinElmer's versatile and highly sensitive AlphaScreen® technology, the SureFire® cellular kinase assays provide an ideal, no-wash solution for measuring intracellular signaling events.

Flexible Homogeneous Assays

AlphaScreen SureFire cellular kinase assays measure endogenous levels of cellular proteins, and so can be used with many different types of cells, including primary cells, provided the cells have an active signaling pathway and express the particular protein of interest at sufficient levels. These assays can also be used on cell lines expressing transfected kinases or whole-protein biochemical assays.

There are several applications for detection of intracellular kinase activation. Assays can be used to monitor modulators of cellular receptors, including G protein-coupled receptors (GPCRs), cytokine receptors and receptor tyrosine kinases (RTKs), through specific agonist-mediated activation of intracellular kinase pathways. The SureFire range of cellular kinase assays includes targets activated by RTK signaling via PI3-kinase pathway signaling or MAPK-mediated pathways (i.e. insulin signaling), inflammatory receptor signaling via MAPK, TGFβ or NFκB phosphorylation, and cytokine receptor activation through JAK/STAT phosphorylation. The list of assays is rapidly expanding, and the latest product offerings can be found at the Perkin Elmer website (las.perkinelmer.com).

HTS compatible

Whether you are running single plates or hundreds of plates, AlphaScreen SureFire assays will work for you. These two advanced technologies provide the ultimate homogeneous non-radiometric assay for full-length activated phosphorylated kinases in cell lysates. Easily miniaturizable, AlphaScreen SureFire is the choice for rapid and accurate high throughput screening.

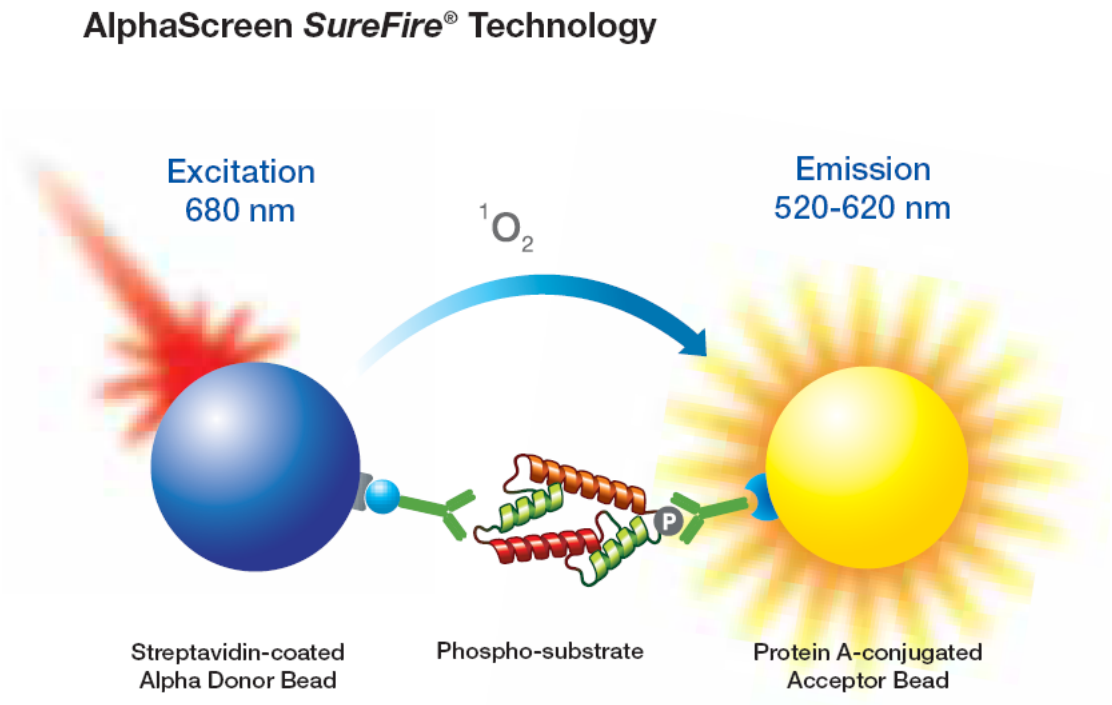
For rapid detection of full-length activated kinases in cell lysates — even with difficult targets…

AlphaScreen *SureFire* Cellular Kinase Assays

AlphaScreen® SureFire® Assay Principle

The SureFire family of optimized cellular kinase assays detect phosphorylated proteins by immunosandwich capture. One antibody is directed against a specific phospho-epitope on the target protein, while the other is directed against an epitope on a distal part of the protein. The distal antibody binds the target protein in cell lysates, independent of the phosphorylation state of the protein. However, the phospho-antibody can only bind when the phospho-epitope on the protein is formed – usually by a specific signaling cascade. Therefore, the amount of immuno-sandwich complexes that are formed is dependent on the amount of phosphorylated target in the cellular lysates.

SureFire assays are specifically designed to work with the AlphaScreen General IgG (Protein A) beads. AlphaScreen is a proximity-based technology that only emits a signal when donor and acceptor beads are brought into close proximity. Each antibody in the SureFire kits is specifically selected such that it will only bind either the donor or acceptor bead. Thus, only immuno-complexes that contain both antibodies can bind both beads, and are subsequently detected. The assay is efficient at measuring both agonist and antagonist activities that affect protein phosphorylation.



Kit Contents

The SureFire kits are comprised of several proprietary components designed for optimal performance across a number of cellular systems. The kits contain the following components:

- Control lysates
- Reaction buffer
- Lysis buffer
- Activation buffer
- Dilution buffer (only supplied with 2-step kits)

The amounts and recommended handling of each of the components that are supplied with each kit is detailed in Tables 1, 2, 3 and 4.

SureFire kits are designed to work with the AlphaScreen® General IgG (Protein A) Detection Kit (Catalogue 6760617M, 6760617R and 6760617C). These AlphaScreen products are supplied separately, and so need to be ordered along with the SureFire kits. When ordered together, these companion products are usually delivered together, but are in separate boxes. All kits are available in 500 pt, 10,000 pt and 50,000 pt pack sizes.

Control lysates

A small quantity of lyophilized cell lysates are included with each kit, and are intended for use as a control to ensure that all kit reagents are working as expected. Negative controls are cell lysates prepared from flasks of unstimulated, or sometimes inhibited, cells that contain low amounts of the phophorylated analyte. Positive controls are cell lysates prepared from flasks of cells stimulated with an agonist to promote phosphorylation of the phosphoprotein of interest. The cells used to prepare the control lysates vary from kit to kit, and are chosen because they express detectable levels of the phosphoprotein of interest. The signal that is achieved with these may be quite different from that observed in other cell lines, and is mainly dependent on expression levels in the cell line of interest.

Reaction buffer

Supplied at working concentration, the SureFire Reaction buffer is a proprietary mixture of antibodies and buffer components, pre-diluted to an optimal concentration for detecting cellular phosphoproteins in combination with the AlphaScreen Protein A IgG detection kits, which are sold separately by Perkin Elmer. As this reagent contains all of the antibodies required for the assay, this solution must be stored correctly at 4ºC, and not frozen. Vortexing is unnecessary and should also be avoided.

Lysis Buffer

Supplied as a 5X concentrate, the SureFire Lysis buffer is a proprietary mixture of buffers, detergents, and phosphatase inhibitors, optimised for lysis of a broad range of cells without releasing nuclear DNA. Additives can be supplemented to the Lysis buffer as required for particular cells, and may include excipients such as protease inhibitors or extra detergents. These will need to be checked on a case by case basis to ensure that they do not interfere with the assay.

Activation buffer

Supplied at working concentration, the SureFire Activation buffer is a proprietary formulation that acts to enhance the sensitivity of detection of many cellular phosphoproteins. Components of this reagent WILL precipitate during normal storage at 4ºC, and so this reagent MUST BE REDISSOLVED PRIOR TO EVERY USE. Often the easiest way to achieve this is to incubate Activation buffer in a 37ºC water bath for 5-10 minutes, and then mix thoroughly by vortex or immersion. If this is inconvenient, this reagent is quite stable and can be readily stored at room temperature. If precipitates still form, simply re-dissolve as described above.

Dilution buffer

Supplied at working concentration, the SureFire Dilution buffer is used to dilute AlphaScreen donor beads, and is only required for 2-step SureFire assay procedures. It is not supplied with 1-step assay protocols as it is not required. It is strongly recommended that this component is always stored correctly at 4ºC.

Precipitation

Any precipitates that form in 5X Lysis buffer or Activation buffer can be re-dissolved by warming to 37ºC and mixing. If precipitates form in the Reaction buffer, it should be discarded.

Shelf Life

All components should be stored as indicated until the expiry date stated. Kits have a guaranteed shelf life of between 6-9 months from the date of manufacture.

Table 1: Listing of components in SureFire® assay 1-step kits

Kit size	500 Point	10000 Point	50000 Point
Lysis Buffer (5X)	5 X 2 mL	4 X 60 mL	3 X 400 mL
Activation Buffer	1 X 2 mL	1 X 60 mL	1 X 300 mL
Reaction Buffer	2 X 1.7 mL	2 X 35 mL	1 X 360 mL
Assay Control Samples (lyophilized)			
Negative Cell Lysate	50 µL	250 µL	250 µL
Positive Cell Lysate	50 µL	250 µL	250 µL

Table 2: Listing of components in SureFire® assay 2-step kits

Kit size	500 Point	10000 Point	50000 Point
Lysis Buffer (5X)	5 X 2 mL	4 X 60 mL	3 X 400 mL
Activation Buffer	1 X 2 mL	1 X 60 mL	1 X 300 mL
Reaction Buffer	2 X 1.3 mL	1 X 45 mL	1 X 225 mL
Dilution Buffer	1 X 1.5 mL	1 X 25 mL	2 X 60 mL
Assay Control Samples (lyophilized)			
Negative Cell Lysate	50 µL	250 µL	250 µL
Positive Cell Lysate	50 µL	250 µL	250 µL

*Also required for running SureFire assays: Protein A general IgG detection kit. Available from Perkin Elmer in 500pt (Cat.# 6760617C), 10,000pt (Cat.# 6760617M), and 50,000pt (Cat.# 6760617R) pack sizes.

Table 3: Buffer preparation and Storage Conditions for 1-step assay kits

	Storage and preparation descriptions
5X Lysis buffer	Store 5X Lysis buffer at 4^{o}C. After dilution, excess 1X Lysis buffer can be frozen and thawed up to 5 times without loss in activity.
Activation buffer	Precipitation may occur at 4^{o}C. Warm slowly to 37^{o}C and gently mix to re-suspend. Activation buffer can be stored at room temperature with no loss in activity.
Reaction buffer	Keep at 4^{o}C while in use. Do not freeze. Once diluted discard unused reaction buffer.
AlphaScreen® Protein A IgG Kit	Store at 4^{o}C in the dark.
Reaction buffer + Activation buffer and AlphaScreen® beads	Mix Reaction buffer (60 parts), Activation Buffer (10 parts) and Donor and Acceptor beads (1 part each). Mix to be stored at room temperature and used the same day, excess mix should be discarded.
Assay control samples Negative cell lysate Positive cell lysate	Stable while lyophilized at 4^{o}C for life of kit. Reconstitute lysates in water for 15 min at room temperature and mix by pipetting. Once reconstituted, lysates should be frozen at -20^{o}C in single use aliquots.

Table 4: Buffer preparation and Storage Conditions for 2-step assay kits

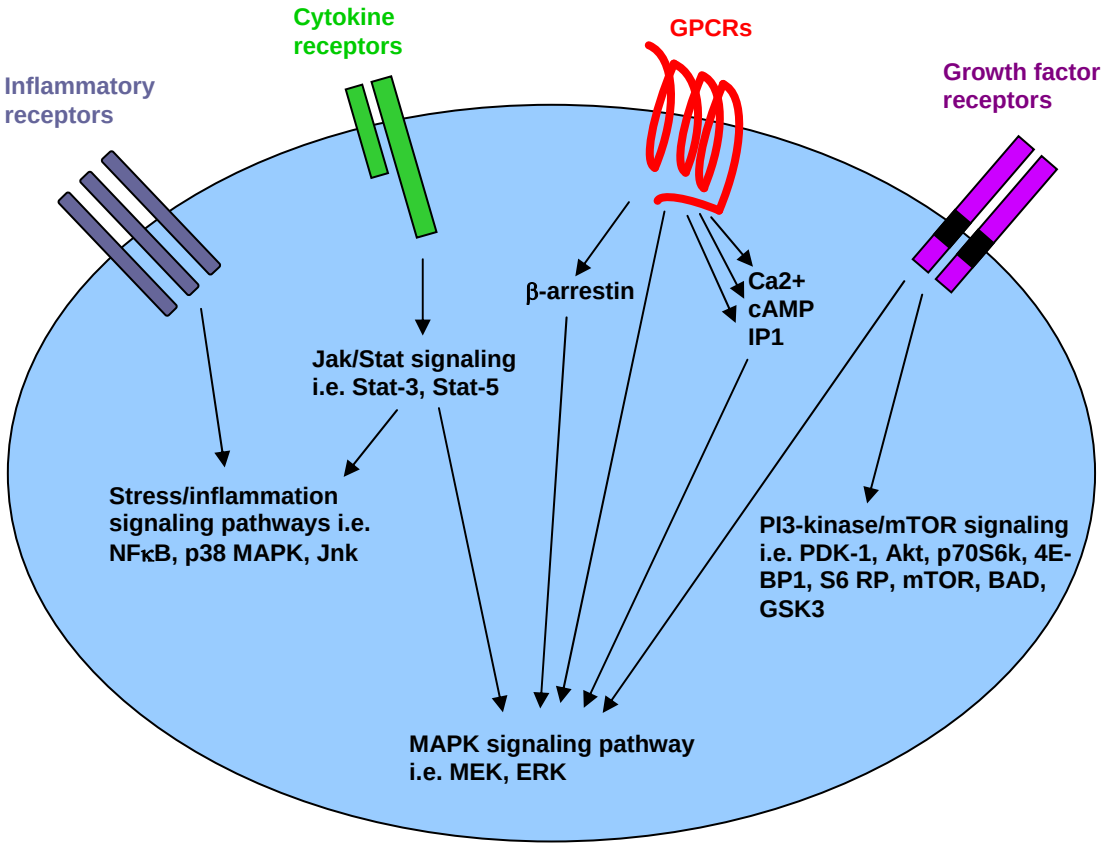
	Storage and preparation descriptions
5X Lysis buffer	Store 5X Lysis buffer at 4°C. After dilution, excess 1X Lysis buffer can be frozen and thawed up to 5 times without loss in activity.
Activation buffer	Precipitation may occur at 4°C. Warm slowly to 37°C and gently mix to re-suspend. Activation buffer can be stored at room temperature with no loss in activity.
Reaction buffer	Keep at 4°C while in use. Do not freeze. Once diluted discard unused reaction buffer.
AlphaScreen® Protein A IgG Kit	Store at 4°C in the dark.
Reaction buffer + Activation buffer and AlphaScreen® Acceptor beads	Mix Reaction buffer (40 parts), Activation Buffer (10 parts) and Acceptor beads (1 part). Mix to be stored at room temperature and used the same day, excess mix should be discarded.
Dilution buffer + AlphaScreen® Donor beads	Mix Dilution buffer (20 parts) and Donor beads (1 part). Mix to be stored at room temperature and used the same day, excess mix should be discarded.
Assay control samples Negative cell lysate Positive cell lysate	Stable while lyophilized at 4°C for life of kit. Reconstitute lysates in water for 15 min at room temperature and mix by pipetting. Once reconstituted, lysates should be frozen at -20°C in single use aliquots.

Target Selection

The SureFire range of assays can be used to assay for a number of cellular pathways, including:

- MAPK signaling
- Akt signaling
- Translational control
- NFκB signaling
- TGFβ/Smad signaling
- Apoptosis
- Tyrosine kinase receptor signaling
- GPCR signaling

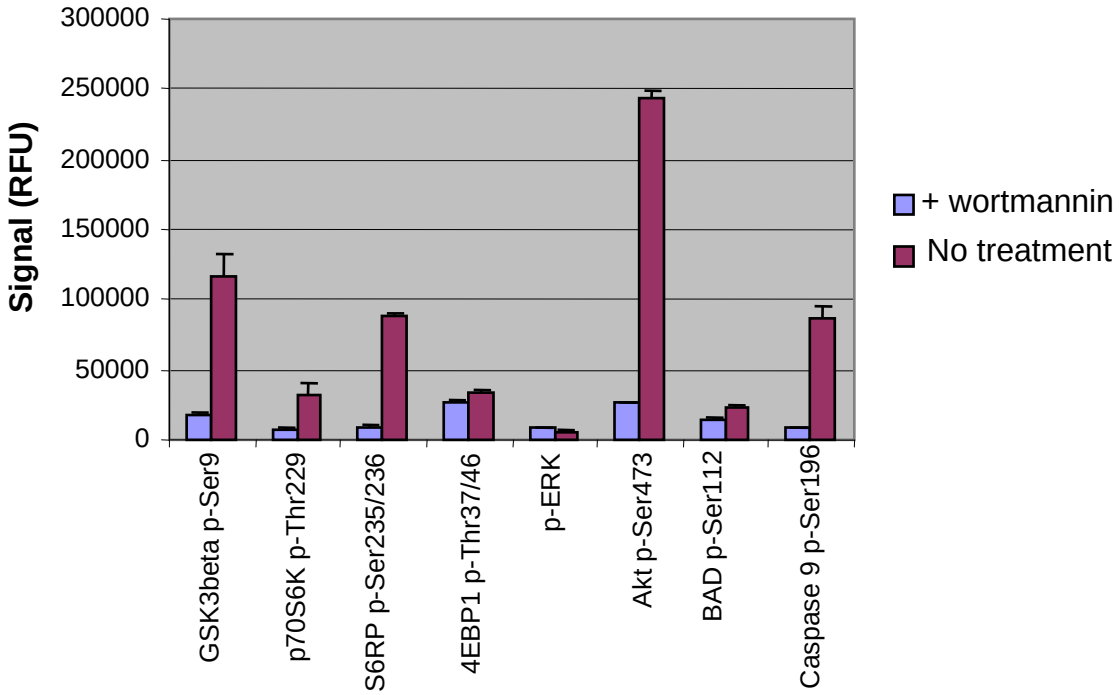
For each pathway, there are multiple SureFire kits that are available, each measuring a different target or phosphorylation site. This allows signaling pathways to be analysed in many cell lines, even if particular kinases in a signaling pathway are not well expressed. Furthermore, because of the low amounts of cell lysate used in the SureFire assays, important information regarding specificity and off-target compounds effects can be deduced from a single cell culture well through measurement of several target proteins from the same lysate.



The range of available assays is rapidly increasing, and an up to date list of assays is available at the Perkin Elmer website (las.perkinelmer.com).

The expression level of various targets can vary considerably between cell lines, as can the level of basal protein phosphorylation during normal cell growth. Because SureFire assays only use around 4μL of lysate per well, a typical 96-well or 384-well cell lysate from growing cells be readily assayed for many targets. Simply lysing a well of growing cells +/- a pathway inhibitor and analysing for targets of interest can quickly reveal which targets are detectable along the pathway of interest.

Detection of various phosphoproteins in LNCAP cells



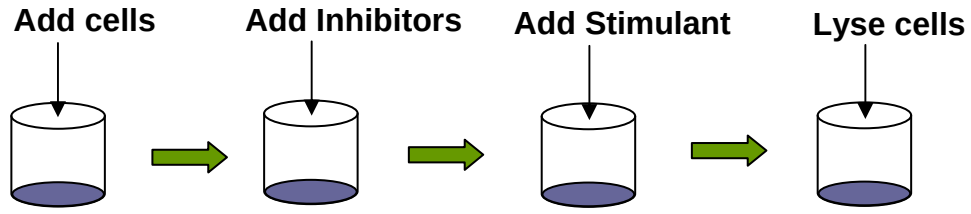
In the experiment above, LNCAP cells were seeded at a density of 25,000 cells per well in a 96-well microplate, and incubated overnight at 37°C/5% CO_2 overnight in RPMI containing 10% FBS, glutamine, sodium pyruvate and pen/strep. The following morning, the media in the wells was replaced with the same fresh media, either with or without 2 μM wortmannin, and the cells were returned to the incubator for a further 3 hours. The media was removed from the wells and the cells were washed with cold PBS to remove traces of biotin contained in the media. Finally, the cells were lysed with 50 μL 1X Lysis Buffer and agitated gently for 10 min. From each culture well, 4 μL lysate was transferred to 8 different wells of a proxiplate, and analysed for 8 separate phosphorylation events. Of the 8 targets, 5 showed a usable assay window for basal phosphorylation in LNCAP cells.

Common Protocols

General transfer procedure

The most common method for performing these assays is to grow and treat the cells in a cell culture plate, generate cell lysates, and analyse a portion of this cell lysate in a separate assay plate transferred from the culture plate. This 'transfer' procedure can be thought of in two stages; (1) the culture of cells and the stimulation and/or inhibition of particular cellular pathways, followed by preparation of cellular lysates, and (2) the examination of cellular lysates for particular phospho-targets of interest.

For adherent cells, there are a number of variations that can be used that are compatible with SureFire assays. The most common cell culture procedure is outlined below. Here, cells are plated into microplate wells for an optimal period of time, followed by the addition of inhibitors and or agonists as required for an optimal time period. The media can then be removed, and lysates prepared by the addition of 1X lysis buffer, or the media can remain on the cells and lysates prepared by the addition of 5X lysis buffer.



Adherent cells:
Incubate overnight

Non-adherent cells:
Assay immediately

Adherent cells:
Remove media first **or**
Add concentrated stock direct to wells

Non-adherent cells:
Add concentrated stock direct to wells

Adherent cells:
Add concentrated stock direct to wells

Non-adherent cells:
Add concentrated stock direct to wells

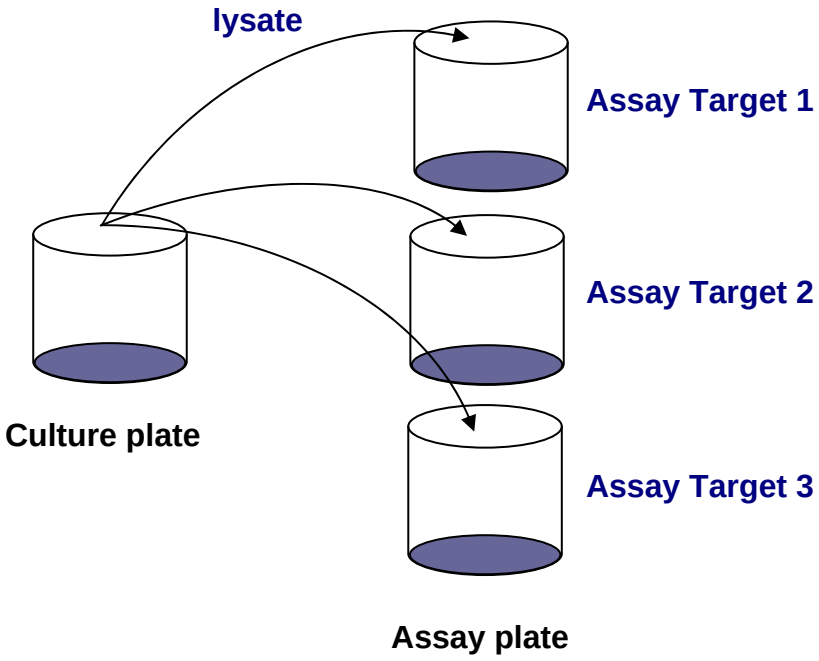
Adherent cells:
Remove media first and lyse with 1X Lysis buffer **or**
Add 5X Lysis buffer direct to wells

Non-adherent cells:
Add 5X Lysis buffer direct to wells

Where possible, we recommend removing the cell culture media prior to cell lysis. This allows the removal of any agents that are used during the cell culture experiments that may interfere with the subsequent SureFire assay. Interfering agents may include compounds that interfere with the antibody-target interaction or the antibody-protein A interaction, biotin or biotin mimetics that interfere with the biotin-streptavidin interaction, or free radical scavengers that interfere with the AlphaScreen bead signal generation.

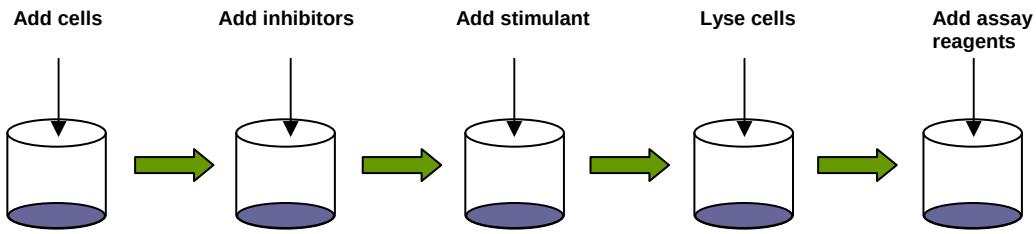
Once the lysates have been generated, a small aliquot of lysate is transferred to an assay plate for analysis. The standard protocol recommends transferring

4 μL of lysate to a low-volume 348-well proxiplate. Because of the low lysate usage, a typical cell lysate can be analysed for several different targets if desired.



Single well procedures

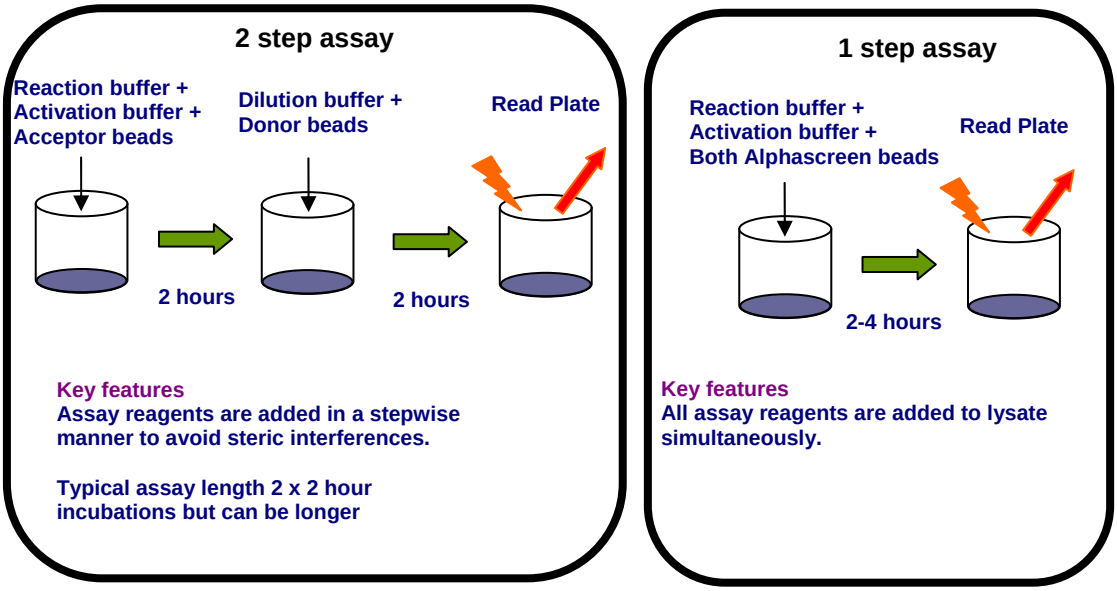
Single well procedures are intended for higher-throughput projects, where many wells are analysed for a single target. Here the cells are cultured in the same microplate that they are subsequently assayed in. These may be either standard white 384-well or 1536-well microplates, or low volume 384-well proxiplates.



Again, the media may either be removed prior to lysis, or the cells may be lysed in culture medium by the addition of an appropriate volume of 5X Lysis buffer. By removing media, the potential for assay interference is reduced.

Single reagent addition vs dual reagent addition procedures

All SureFire assay kits follow either a procedure with a single simultaneous reagent addition – the '1-step' procedure, or 2 sequential additions of assay reagents - the '2-step' procedure. For the 2-step assays, the sensitivity of the assay is enhanced by separating the reagent additions, a procedure that is likely to reduce steric constraints of the antibody-antigen complexes, thereby allowing a more optimal signal to be generated.



However, for high-throughput users particularly, the ability to add all reagents simultaneously has the potential to lower liquid handling requirements. For users that want to minimize liquid handling, most 2-step procedures can be modified to perform adequately in a one-step format. Here, all assay reagents - including dilution buffer and donor beads – are mixed together and added to the lysates simultaneously. A reduction in signal window may be alleviated by using a longer assay incubation time – for example, overnight incubations can be used to increase signal window.

Assay Optimization

There are several parameters that should be optimized to achieve the best possible assay performance. A useful tool when performing these assays is the Western blot with an appropriate phospho-antibody. This tool can be used to determine whether there is a problem with the cells, or with the assay when an experiment does not perform as expected. As a general guide, if a band can be seen on a Western blot, then it should be detected with a SureFire assay also. If not, it may indicate that there may be problems with assay procedures or kit components.

Selection of assay format

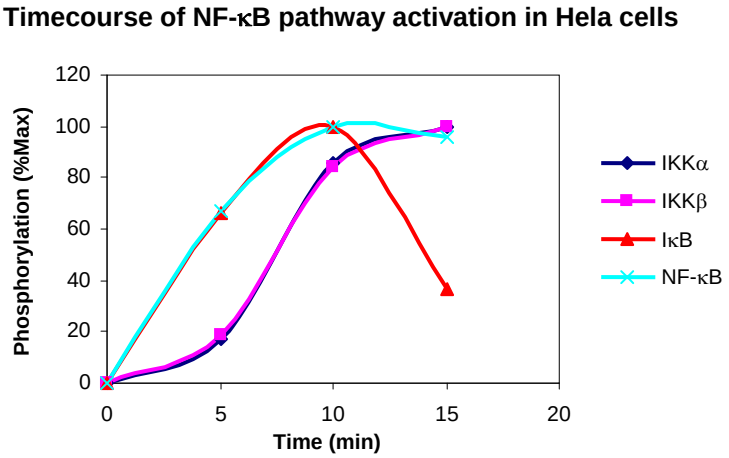
For first time users, we suggest using a 96-well format transfer protocol with adherent cells, whereas a 384-well single-well assay approach is best when using non-adherent cells. Once the cell response conditions have been optimized, it is usually straightforward to transfer the protocol to a more favourable configuration.

Cell seeding and density

The cell seeding can be very important, depending on which pathway is being analysed. For example, when looking at ERK phosphorylation, adherent cells must be seeded in serum-containing media for at least 15 hours (i.e. overnight) to allow for effective ERK signaling. If cells are seeded in serum-free media, or for shorter periods, the maximal ERK phosphorylation can be dramatically reduced. Similarly, cell seeding density is important. Typically, cell number should be titrated to determine an optimal balance between the assay window, and the amount of cell culture support that is required. The titration of cell number is also important to determine the responsive range of the assay. Optimizing cell culture conditions can also have a big influence on variation induced by factors such as edge-effects and cell clumping leading to variable cell numbers per well.

Timecourse of activation

Many signaling pathways are not activated in normal growing cells, or are only activated at low levels. For these pathways, an agonist is often used to stimulate the cells, inducing a receptor-mediated phosphorylation cascade. Various proteins that are activated as part of a cascade may take different times to reach maximal levels of phosphorylation. Further, the maximal phosphorylation response may be quite transient in some cases, while others may be induced in a more sustained manner. Therefore, the timecourse of activation for a particular target of interest must be determined experimentally. The timecourse will also be influenced on whether the assays are performed at room temperature or at 37°C.



In the example above, Hela cells were seeded in 96-well microplates at a density of 100,000 cells per well, and incubated overnight at 37°C/5% CO_2 overnight in MEM containing 10% FBS, sodium pyruvate, non-essential amino acids and pen/strep. The following morning, the cells were stimulated with TNFα for varying lengths of time prior to lysis in 50μL of Lysis Buffer. Lysates were transferred to a proxiplate and assayed for several different kinases involved in NFκB pathway activation. These experiments demonstrate that although the kinases analyzed here show quite different timecourse of phosphorylation, for these experiments a timepoint of 10 min gave a close to maximal response for all kinases that were analyzed.

Assay buffer system & DMSO tolerance

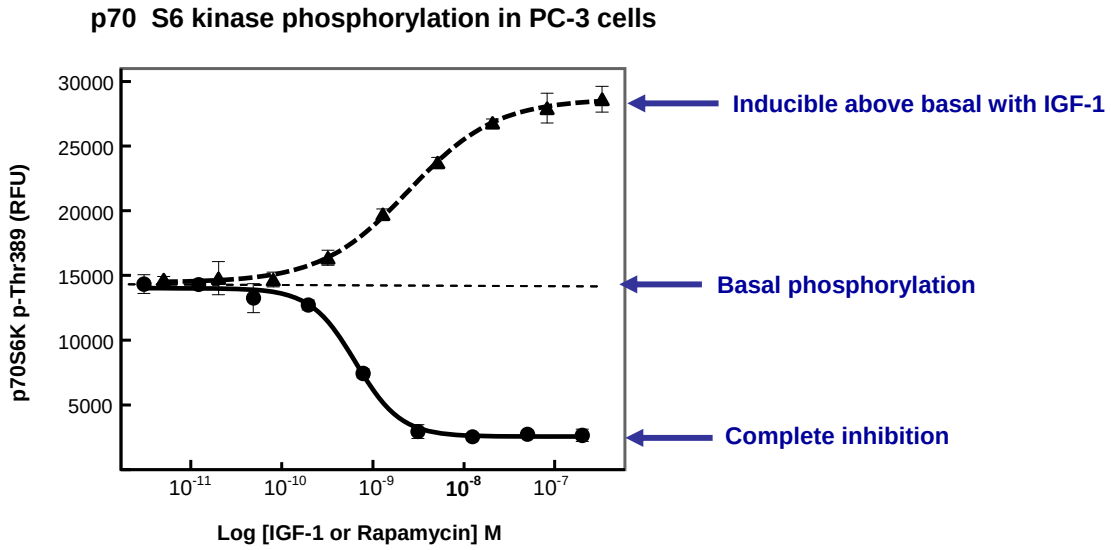
The SureFire assays are compatible with most cell culture media, however there are some exceptions. Media that contain biotin (i.e. RPMI) will reduce assay sensitivity due to the interference of biotin on the antibody-streptavidin interaction. When it is necessary to use a media such as RPMI for growing cells, they should be transferred to an alternative media for assaying. When using non-adherent cells, they can be harvested and resuspended in HBSS or similar buffers for the assay. For adherent cells this is less of a problem, because all media can be removed prior to lysis.

DMSO is a common diluent for inhibitor compounds, and is commonly present in assay buffers at concentrations up to 1%. Because cells can vary in their tolerance for DMSO, its effect on cell signaling must also be determined. For example, GPCR-mediated calcium signaling in CHO cells is normally tolerant of DMSO at 1%, whereas phospho-ERK signaling in the same cells is often lower than 0.5%.

Maximal response window and basal phosphorylation

For cells with quiescent pathways, the maximal signal window will be influenced primarily by cell number, lysate concentration, and culturing techniques such as serum-starvation prior to assay. However, many cells may

have signaling pathways that exhibit high basal levels of phosphorylation. For example, many cancer cells may have deregulated PI-3 kinase pathways that are not lowered by serum starvation or contact inhibition. For these situations, a pathway inhibitor may often provide a more accurate determination of the true assay window for a particular cell line. In these cases, an inhibitor will often drop the background signal to levels that are well below unstimulated controls.



In the example above, PC-3 cells were seeded in 96-well microplates at a density of 50,000 cells per well, and incubated overnight at 37°C/5% CO_2 overnight in F12 Kaighn's media containing 10% FBS, sodium pyruvate, and pen/strep. The following morning, the cells were either serum starved for 2h, or treated with various concentrations of rapamycin for 2 hours. Starved cells were stimulated with IGF-1 for 30 min, and then all wells were lysed in 25µL of Lysis Buffer. Lysates were transferred to a proxiplate and assayed for p70 S6 kinase phsophorylation at Thr389. Under these conditions, basal phosphorylation of p70 S6 kinase phsophorylation at Thr389 gives around a 5-fold signal window, which can be approximately doubled by receptor-mediated pathway activation, using a specific agonist such as IGF-1.

Troubleshooting

Low Counts

- Ensure the Activation buffer is properly redissolved prior to use.
- Ensure that all assay steps involving AlphaScreen® reagents 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.
- Ensure that white opaque 384-well low volume microplates (i.e. proxiplates) are used – the low total assay volume (11 μL) can cause problems in standard 384 well microplates.
- Ensure incubation temperature for assay is at least 22ºC – temperature can have a dramatic effect on both antibody binding performance, and AlphaScreen bead performance.
- Ensure that buffers are prepared correctly. In particular, ensure that 5X Lysis buffer is diluted to 1X Lysis buffer prior to use, and that the Activation buffer is fully re-dissolved prior to use.
- Ensure the correct amounts of AlphaScreen beads are used.
- Check that cell density is correct. Cell numbers that are too high or low can affect the activation of intracellular signaling pathways.
- Ensure cell passage number is not too high, and that cells have not lost responsiveness.
- A useful guide to the expected kit performance is by analysis using Western blot. If a target band is observed by Western blot, then a signal should be detected using the SureFire 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 did not contain serum if the kinase pathway that is being monitored is activated by serum.
- Some pathways may have a high level of basal or constitutive activity. 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

- Ensure the correct amounts of AlphaScreen beads are used.
- Ensure the Activation buffer was properly redissolved prior to use.
- Ensure cell passage number is not too high, and that cells have not lost responsiveness.
- Ensure incubation temperature for assay is at least 22ºC. Temperature has a large effect on both the performance of antibody binding, and also AlphaScreen bead signal.
- Reduce lysis volume to produce more concentrated lysates. Often endogenous phosphoprotein targets are at low abundance in cells. The lower the lysis volume, the higher the concentration of the final lysate.
- Use a single-plate method for assaying the target. Transfer methods typically use only a portion of the total amount of cells that are plated, whereas single well methods use all of the cells in a particular experiment.
- A useful guide to expected kit performance is by analysis using Western blot. If a target band is observed by Western blot, then a signal should be detected using the SureFire assay.

Poor Cell Stimulation

- Check that the cells are confluent. When confluent, many signaling pathways – particularly those associated with growth such as MAPK and PI3K pathways – can become quiescent and synchronized. When an agonist is introduced to quiescent and synchronized cells, they can respond uniformly.
- Check that cell density is correct. Too high or low cell numbers can affect basal kinase activation. Cell density should be such that the target phosphoprotein is within the responsive range of the assay.
- Ensure cell passage number is not too high, and that cells have 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.
- Ensure the kinase 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, and use a carrier protein such as BSA if necessary.

Day to Day Variation

- Check cell harvesting conditions, use a standard protocol for cell culture and harvesting.
- Check for variability in room temperature.
- Check for exposure to bright light sources.
- Check for variation in stimulation times and assay incubation times.
- A useful control for assay variation is to use a standard positive and negative lysate on all assay plates where possible.

FAQs

1. What types of cells 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, parameters such as stimulation time and cell number should be optimized for each cell line used.

2. What concentration of lysate is required for these assays?
These assays are optimized for the detection of endogenous levels of cellular kinases. Typically, the SureFire assays work well in the range of 0.2-0.5 mg/mL of lysate. Usually, if a band can be seen on a Western blot for a particular target, then it should be detectable with the corresponding SureFire assay. Cells that express low levels of the target of interest, for example if immunoprecipitation is required to see a positive band on a Western blot, then it may be below the detectable limit for SureFire assays.

3. Can cell lines with stable or transiently transfected kinase or receptors be used?
Both transient and stable cell lines have been shown to elicit good phosphorylation response, however we recommended using stable cell lines to enhance assay reproducibility. Stable cell lines expressing high levels of a kinase of interest should give strong signal. However, when using overexpression systems the concentration of cell lysate should be optimized to ensure the signal is within the working range of the assay.

4. How should the cells be handled?
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, plate for at least 16h prior to assaying, because adherent cells often require some time to regain full signaling capacity after harvesting.

5. What parameters require optimization for these assays?
For these assays, several parameters will generally require optimization to ensure robust and reproducible assay performance. These parameters include cell seeding density and culturing, whether cells require serum starvation, and agonist and time course of stimulation. Optimization of parameters are covered more fully in the optimization section of this document.

6. Are SureFire assays scalable?
The primary SureFire assay methodology is optimized for a low-volume 384-well microplate. It has a total of 11 μL per assay, 4 μL of which is cell lysate, and 7 μL of assay reagents. However, there are validated protocols that are

scalable down to 4-5μL total assay volume in 1536-well microplates, allowing a saving of both lysate and assay reagents. Although the assays can be scaled up, we do not recommend this due to the excessive use of reagents.

7. Why would I choose a particular assay protocol?
Transfer assay methods are those where the cells are grown in microplates, typically either 96-well or 384-well, stimulated/inhibited and lysed. A sample of this lysate is then transferred to an assay plate to analyse for a particular phosphoprotein. These assays are particularly useful for method development and optimization, low- to medium-throughput projects, and when assaying for multiple proteins from a single well. Single plate methods are usually for high-throughput projects, where wells are analysed for a single target, and minimal use of reagents and liquid handling equipment is essential.

8. Can I assay for multiple targets from a single lysate?
One of the unique features of SureFire protocols is the use of very small amounts of cell lysate. The standard protocol suggests the use of just 4μL of lysate per well, whereas a typical 96-well or 384-well cell culture microplate would use 20-50μL of lysis buffer per well. Therefore, a typical cell lysate can be assayed for many targets, given temporal and expression level constraints can vary from cell line to cell line.

9. What is the specificity of these assays?
All SureFire assays react with the human targets. These tend to be well conserved across species, and most kits can also be used to detect the corresponding mouse and rat phosphoproteins. Other species should be tested on a case-by-case basis.

10. Can I incubate my assays overnight?
The assay time is optimized such that the antibody binding is close to equilibrium after the specified incubation period – usually 2-4 hours when performing assays at room temperature. However, once equilibrium is reached, there is no problem in incubating for longer periods, up to overnight at room temperature, if that is convenient.

11. Can I subtract 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. Negative lysates can typically be generated either through the use of kinase inhibitors, and/or non-stimulation of the assay pathway.

Contact Details

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For more information regarding related SureFire products and protocols refer to the PerkinElmer web site: http://las.perkinelmer.com