

# AlphaScreen® *SureFire*® VEGFR2 (p-Tyr1175) Assay Kits

## Manual

Assay Points	Catalog #
500	TGRVRS500
10 000	TGRVRS10K
50 000	TGRVRS50K

For Laboratory Use Only  
Research Reagents for Research Purposes Only

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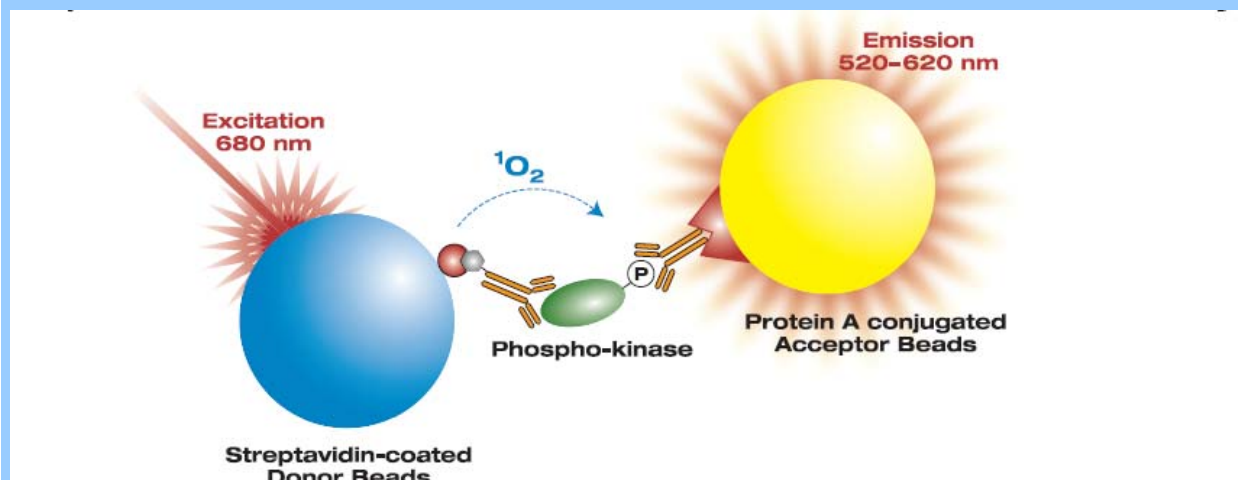
### General Information on the AlphaScreen® *SureFire*® VEGFR2 p-Tyr1175 assay

The AlphaScreen® *SureFire*® VEGF Receptor 2 p-Tyr1175 assay is used to measure the phosphorylation of endogenous VEGFR2 in cellular lysates. The assay is an ideal system for the screening agents acting at the cell surface, and can be applied to primary cells if expression levels are sufficient.

This 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, short assay times, and robotic operation if desired. The assay utilizes the bead-based Alpha Technology, and requires an Alpha Technology-compatible plate reader.

The VEGF Receptor 2 p-Tyr1175 AlphaScreen *SureFire* assay kits contain all the reagents necessary to carry out the measurement of VEGF Receptor 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 the standard, 2-plate protocol.

### Alpha Technology AlphaScreen® *SureFire*® 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.

### Background information on the detected analyte

Vascular endothelial growth factor receptor 2 (VEGFR2) is a receptor for VEGF, and is one of the major routes for VEGF-mediated signaling in endothelial cells. Upon ligand binding, VEGFR2 becomes activated via autophosphorylation events in its kinase and catalytic domains. Once activated, several adaptor proteins can be recruited to VEGFR2 to effect downstream signaling. The phosphorylation of Tyr1175 mediates binding with the p85 subunit of PI-3 kinase, PLC $\gamma$ , and Shb. VEGFR2 functions as one of the main mediators of VEGF-induced endothelial proliferation, survival, migration, and sprouting.

### Kit-Specificity information

This assay kit contains antibodies which recognize the phospho-Tyr1175 epitope, and a distal epitope, on vascular endothelial growth factor receptor 2 (VEGFR2). The protein detected by this kit corresponds to GenBank Accession NP\_002244. VEGFR2 is also known as FLK1, CD309, VEGFR, and KDR.

These antibodies recognize VEGFR2 of human origin. Other species should be tested on a case-by-case basis.

### Control Lysate information

Control lysates are prepared from confluent flasks of human umbilical vascular endothelial cells (LONZA Cat# CC-2519) at a concentration of approximately 1 mg/mL. The controls are supplied lyophilized, and should be reconstituted in either dd H<sub>2</sub>O or MilliQ<sup>®</sup> H<sub>2</sub>O. Once reconstituted, lysates should be stored frozen in single use aliquots.

Negative Lysate: Prepared from serum-starved HUVEC cells.

Positive Lysate: Prepared from serum-starved HUVEC cells, treated with 100 ng/mL rhVEGF for 10 minutes.

## Kit Contents

	Kit Size		
	500 points	10,000 points	50,000 points
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
Positive Control Lysate	1 tube to be re-dissolved in 250 µL H <sub>2</sub> O		
Negative Control Lysate	1 tube to be re-dissolved in 250 µL H <sub>2</sub> O		

## Materials Required But Not Provided

The AlphaScreen *SureFire* 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.	6760617C 6760617M 6760617R	500 pt 10,000 pt 50,000 pt
Proxiplate™-384 Plus, white, shallow well assay plate	PerkinElmer Inc.	6008280 6008289	50/box 200/box
Optiplate™-384 Plus, white, assay plate	PerkinElmer Inc.	6007290 6007299	50/box 200/box
TopSeal-A 384, clear adhesive sealing film	PerkinElmer Inc.	6005250	100/box
Envision® or Enspire® Alpha-reader	PerkinElmer Inc.	-	-

### 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 and should be stored at -20°C upon receipt of kit. After reconstitution, control lysates should be frozen in single use aliquots, and unused portions discarded.

### Buffer preparation and subsequent storage conditions

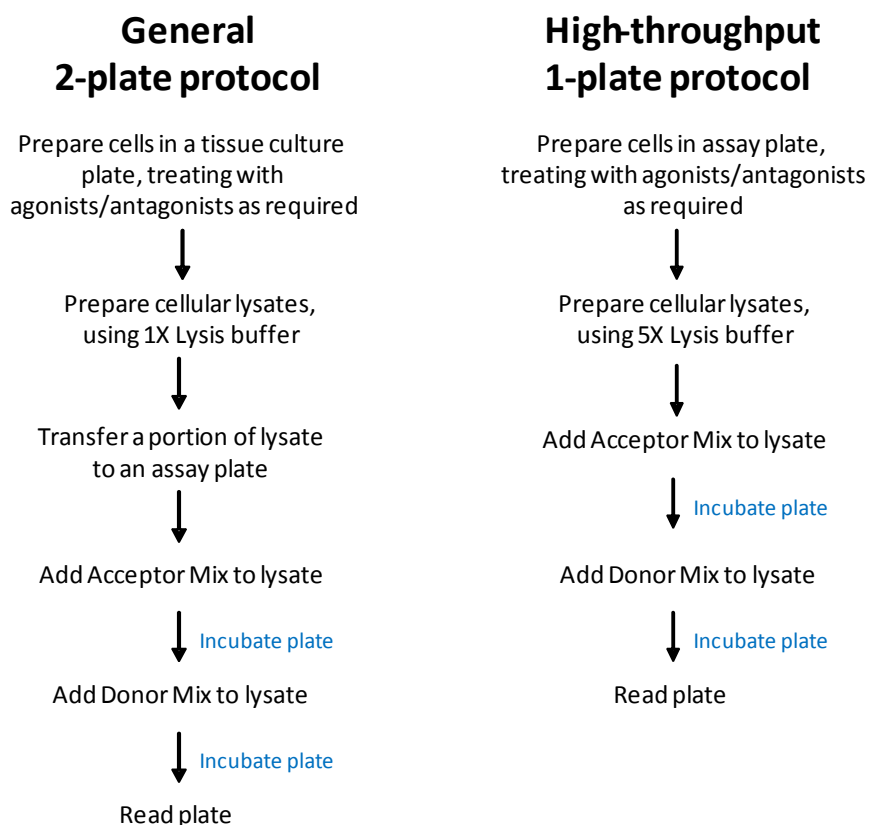
5X Lysis buffer	Store 5X Lysis buffer at 4°C. For assay, dilute <b>5-fold</b> in water immediately prior to use. Discard unused buffer.
Activation buffer	Precipitation will occur during storage 4°C. To re-dissolve, warm to 37°C and mix. Alternatively, Activation buffer can be stored at room temperature with no loss in activity.
Reaction buffer*	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.
<b>Acceptor Mix</b> (Reaction buffer + Activation buffer + AlphaScreen® Acceptor beads)	Immediately prior to use, dilute Activation buffer <b>10-fold</b> in Reaction buffer (e.g. take 49 µL Activation buffer and dilute in 441 µL Reaction buffer).  Dilute Acceptor beads <b>50-fold</b> in Acceptor mix (e.g. add 10 µL Acceptor beads to 490 µL of premixed Reaction buffer + Activation buffer).  The Acceptor mix should be used immediately for best results. Excess mix should be discarded.
<b>Donor Mix**</b> (Dilution buffer + AlphaScreen® Donor beads)	Immediately prior to use, dilute Donor beads <b>20-fold</b> in Dilution buffer (e.g. add 10 µL Donor beads to 190 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.

## SureFire® Protocol Overview

AlphaScreen *SureFire* cellular assays can be set up in a number of different configurations, depending on the requirements of the assay. For general applications, a cellular lysate is generated in a flask or tissue culture plate, and transferred to an assay plate for analysis. For high-throughput applications, cells can be stimulated, lysed and assayed in a single plate.



## 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. For a more detailed list of assay optimization recommendations, see the troubleshooting on page 18 and FAQ section on page 15, or use the Quick Guide to AlphaScreen *SureFire* Assay Optimization: <http://las.perkinelmer.com/surefire>.

### 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 40,000 cells/well (96- well format) or 10,000 cells/well (384-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 ( $5 \times 10^6$  cells/mL is the recommended starting point). Typically, cells are seeded into an assay plate and 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<sub>2</sub>O 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.



## VEGFR2 p-Tyr1175 AlphaScreen® SureFire® Assay Protocols

### I. Adherent Cells

#### A. 2-Plate Assay

##### Cell Seeding

1. Seed cells (40K 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 (25 µL for 384-well plates). (*If testing antagonists, prior to stimulation, remove culture medium and replace with 50 µL serum-free media containing antagonists (25 µL for 384-well plates)*). 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, remove medium from wells, and add freshly prepared 1X Lysis Buffer (use 50-100 µL for a 96 well plate; 384-well plate volume). Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.

4. Take 4 µL of the lysate and transfer to a 384-well Proxiplate for assay. Avoid bubbles. (*Add Control lysates to separate wells if required*).

##### SureFire Assay

5. Add 5 µL of Acceptor mix. Seal plate with TopSeal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature.

6. Add 2 µL of Donor mix under subdued light. Seal plate with TopSeal-A adhesive film, and cover plate with foil. Agitate gently on plate shaker for 2 min, and then incubate for an additional 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).

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

7. Read plate on an Alpha Technology-compatible plate reader, using standard AlphaScreen settings.

## B. 1 Plate Assay

This assay protocol is for screening antagonists in high throughput laboratories.

### Cell Seeding

1. Plate 20  $\mu\text{L}$  of cells into a 384-well Proxiplate in appropriate medium and incubate overnight. Cell density should be optimized by end user ( $10^5$  cells/mL = 2000 cells per well is the recommended starting point).

### Cell Treatment

2. If testing antagonists, remove 10  $\mu\text{L}$  of medium, and pre-treat with 5  $\mu\text{L}$ /well of antagonist diluted in serum-free culture medium. Well volume should be 15  $\mu\text{L}$ . Return cells to 37°C incubator for desired time (1 hour is often sufficient for signal transduction inhibitors, and 5 minutes for receptor antagonists).

3. Stimulate the cells with 5  $\mu\text{L}$  4X agonists in serum-free media. Final volume in the well is 20  $\mu\text{L}$ .

### Lysate Preparation

4. Remove medium from cells. (A small volume of residual medium is acceptable for the assay).

5. Add 4  $\mu\text{L}$  of freshly prepared 1X Lysis Buffer to wells. (*Add 4  $\mu\text{L}$  control lysates to separate wells if required.*)

### SureFire Assay

6. Add 5  $\mu\text{L}$  of Acceptor mix. Seal plate with TopSeal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).

7. Add 2  $\mu\text{L}$  of Donor mix 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 an additional 2 hours at room temperature.

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

8. Read plate on an Alpha Technology-compatible plate reader, using standard AlphaScreen settings.

## II. Non-Adherent Cells

### A. 2-Plate Assay

This assay format is useful if multiple analytes require testing in parallel from the same lysate. If testing for a single analyte, a 1-plate assay format is often more practical.

#### Cell Seeding

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 10  $\mu$ L of cells/well into a 384-well culture plate.
2. If using test agents/inhibitors, add 5  $\mu$ L/well of 4X inhibitors prepared in HBSS.
3. Return cells to incubator at 37°C for 1-2 hours.

#### Cell Treatment

4. Stimulate cells with agonists by addition of 5  $\mu$ L/well of 4X agonist stock in HBSS. The final volume in the wells should be 20  $\mu$ L. *(If no antagonists are used at step 2, stimulate the cells with 10  $\mu$ L/well of 2X agonist stock in HBSS. The final volume in the wells should be 20  $\mu$ L.)*

#### Lysate Preparation

5. To lyse the cells, add 5  $\mu$ L/well 5X Lysis buffer, and agitate on a plate shaker (~350 rpm) for 5-10 minutes.
6. Take 4  $\mu$ L of the lysate and transfer to a 384-well Proxiplate for assay. Avoid bubbles. *(Add 4  $\mu$ L control lysates to separate wells if required.)*

#### SureFire Assay

7. Add 5  $\mu$ L of Acceptor mix. Seal plate with TopSeal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).
8. Add 2  $\mu$ L of Donor mix 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 an additional 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).

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

9. Read plate on an AlphaScreen-compatible plate reader, using standard AlphaScreen settings.

## B. 1 Plate Assay

**Note:** the larger volumes required using this assay will result in achieving less assay points per kit.

### Cell Seeding

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 4  $\mu$ L of cells/well into a 384-well culture plate.
2. If using test agents/antagonists, add 2  $\mu$ L/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.

### Cell Treatment

4. Stimulate cells with agonists by addition of 2  $\mu$ L/well of 4X agonist stock in HBSS containing 0.1% BSA. The final volume in the wells should be 8  $\mu$ L. (*If no antagonists were used at step 2, stimulate the cells with 4  $\mu$ L/well of 2X agonist, to give a final volume in the wells of 8  $\mu$ L.*)

### Lysate Preparation

5. To lyse the cells, add 2  $\mu$ L/well 5X Lysis buffer. (*Add 10  $\mu$ L Control lysates to separate wells if required*).

### SureFire Assay

6. Add 8.5  $\mu$ L of Acceptor mix. Seal plate with TopSeal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).
7. Add 3.5  $\mu$ L of Donor mix 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 an additional 2 hours at room temperature (an incubator set for 22°C may offer greater assay reproducibility).

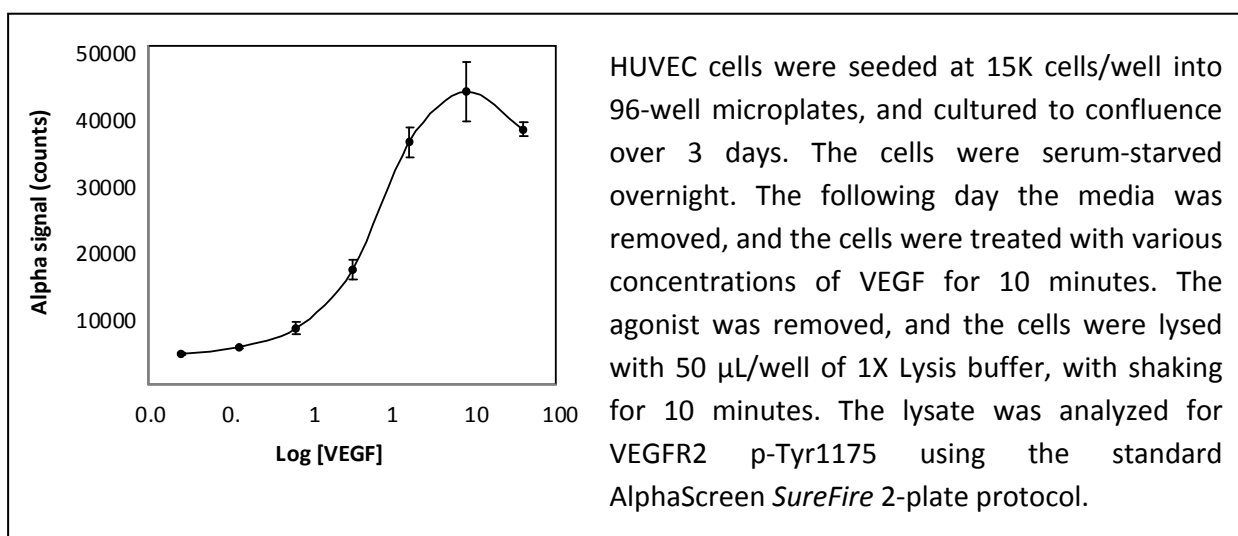
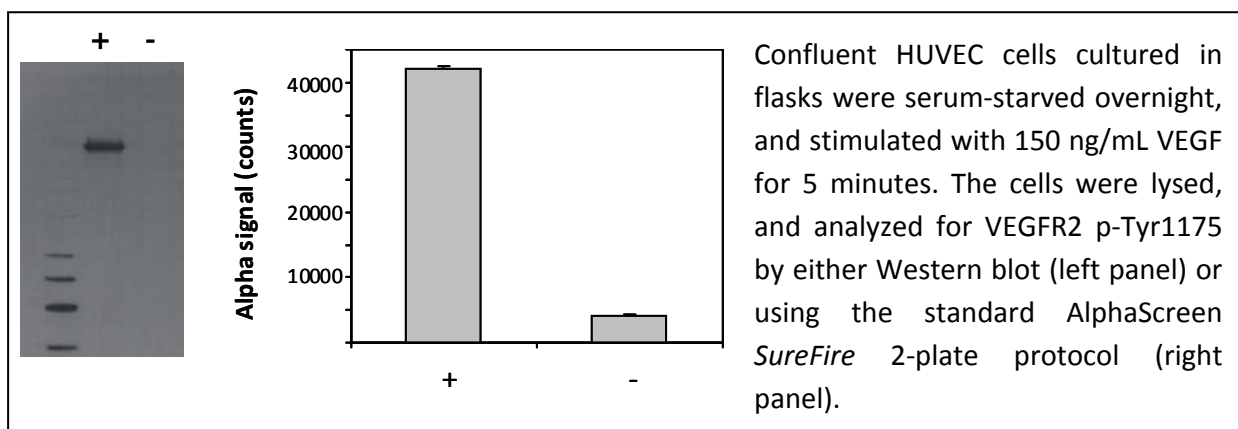
**Note:** Longer incubation may give greater sensitivity. Plates can be incubated overnight if required.

8. Read plate on an Alpha Technology-compatible plate reader, using standard AlphaScreen settings.

## 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 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<sub>50</sub> (or IC<sub>50</sub>), 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 (S/B) 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



## Frequently Asked Questions

Some commonly asked questions and troubleshooting parameters are outlined below.

### 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.

### 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 be 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 Reaction mix during preparation (e.g. Reaction 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  $\mu$ L. However, for low abundance proteins, the lysis volume can be adjusted down to 25  $\mu$ L, to increase the analyte concentration in the lysate. 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) then it may be below the detectable limit for *SureFire* 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.

### Assay incubation times

The general assay incubation times that are recommended are 2 hours for each assay reagent addition. For assays that require 1 reagent addition (1-step) the recommended incubation is 2 hours, and for assays that require 2 reagent additions (2-step), and 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 Reaction Mix may help. In particular, decreasing the concentration of the Donor bead can often help with assay sensitivity, particularly for 1-step assay configurations.

### Buffer compatibility

The AlphaScreen *SureFire* 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 should be optimized for each cell line used.

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 full-length 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.

### **Assay scalability**

The primary *SureFire* assay methodology is optimized for a low-volume 384-well microplate. It has a total of 11  $\mu\text{L}$  per assay (4  $\mu\text{L}$  of cell lysate and 7  $\mu\text{L}$  of assay reagents). However, the assays are scalable down to 4-5  $\mu\text{L}$  total assay volume in 1536-well microplates, allowing a saving of both lysate and assay reagents.

### **Choosing an 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 analyze for a particular phosphoprotein. This format is 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 analyzed for a single target, and minimal use of reagents and liquid handling equipment is essential.

### **Assaying 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 4  $\mu\text{L}$  of lysate per well, whereas a typical 96-well or 384-well cell culture microplate would use 20-50  $\mu\text{L}$  of lysis buffer per well. Therefore, a typical cell lysate can be assayed for many targets, given that temporal and expression level constraints can vary from cell line to cell line.

### **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. Subtraction of cellular background/basal phosphorylation prior to analysis may be useful in some instances.



## Troubleshooting

### Low Signal

- Ensure the Activation buffer is properly re-dissolved 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 assay volume (11  $\mu$ L) is not well suited to standard 384 well microplates.
- 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 *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 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.
- Use a single-plate method for assaying the target. Transfer methods typically use only a portion of the total amount of cells that are used, whereas single well methods use all of the cells in a particular experiment.
- 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.

### 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

### 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 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.

For comprehensive information on assay optimization and troubleshooting, please refer to the following resources:

- AlphaScreen® *SureFire*® full manual
- Guide to AlphaScreen® *SureFire*® assay optimization
- AlphaScreen® *SureFire*® user guide

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>

## Customer Service

### 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.**

#### Email:

[CustomerCareUS@perkinelmer.com](mailto:CustomerCareUS@perkinelmer.com) (US and Canada)

[cc.nordic@perkinelmer.com](mailto:cc.nordic@perkinelmer.com) (Norway, Sweden, Denmark and Finland)

[cc.uk@perkinelmer.com](mailto:cc.uk@perkinelmer.com) (UK and Ireland)

[cc.germany@perkinelmer.com](mailto:cc.germany@perkinelmer.com)

[cc.austria@perkinelmer.com](mailto:cc.austria@perkinelmer.com)

[cc.switzerland@perkinelmer.com](mailto:cc.switzerland@perkinelmer.com)

[cc.france@perkinelmer.com](mailto:cc.france@perkinelmer.com)

[cc.italy@perkinelmer.com](mailto:cc.italy@perkinelmer.com)

[cc.spain@perkinelmer.com](mailto:cc.spain@perkinelmer.com)

[cc.benelux@perkinelmer.com](mailto:cc.benelux@perkinelmer.com) (Belgium, Luxembourg and The Netherlands)

[cc.export@perkinelmer.com](mailto:cc.export@perkinelmer.com) (All others)

**For more information regarding related AlphaScreen® SureFire® products and protocols refer to:**

**PerkinElmer web site:** <http://las.perkinelmer.com/surefire>

**TGR BioSciences website:** <http://www.tgrbio.com>

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