

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

Manual

Assay Points	Catalog #
100	TGRESBHV100

For Research Use Only
Research Reagents for Research Purposes Only

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

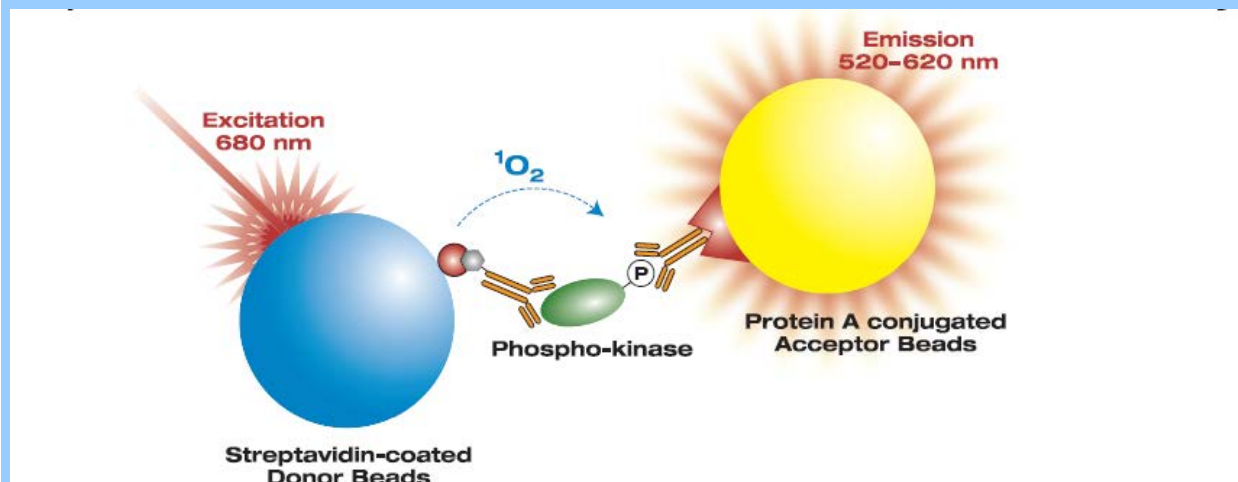
The AlphaScreen® *SureFire* HV™ p-ERK 1/2 assay is used to measure the phosphorylation of endogenous extracellular signal-regulated kinase 1 and 2 (ERK 1/2) in cellular lysates, using a convenient 96-well protocol for low throughput applications and assays with minimum volume requirements. The assay 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.

This new formulation (TGRESB) has a monoclonal total antibody replacing a polyclonal total antibody in the previous kit (TGRES), which delivers equivalent or better assay performance, and greater batch consistency. The monoclonal phospho antibody is the same in both versions.

Alpha Technology AlphaScreen® *SureFire* HV™ Assay Principle

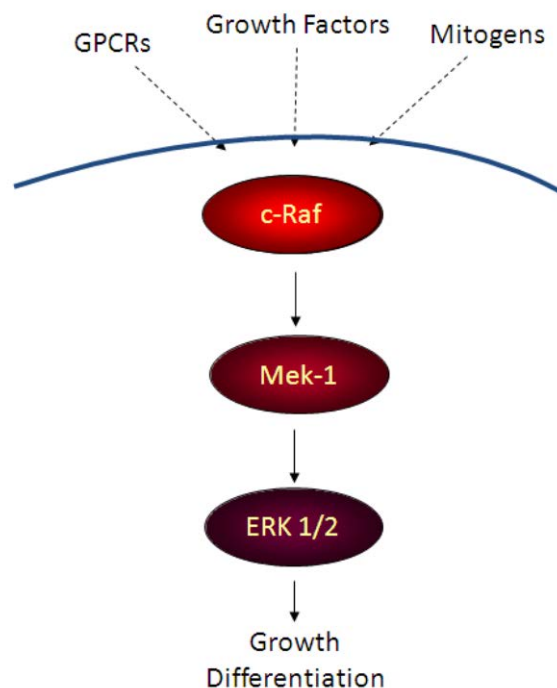


AlphaScreen *SureFire* technology allows the detection of phosphorylated proteins in cellular lysates in a highly sensitive, quantitative and user friendly assay. In these assays, sandwich antibody complexes, which are only formed in the presence of analyte, are captured by AlphaScreen Donor and Acceptor 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.

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.

Kit Contents

	Kit Size
	100 points
Lysis buffer (5X)	1 x 10 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 ₂ O

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.	6050185	100/box
EnVision® or EnSpire® Alpha-reader	PerkinElmer Inc.	-	-

Complementary Products

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

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 ₂ O or MilliQ® H ₂ O 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	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.

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 H₂O or MilliQ® H₂O. 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 to lysate



Incubate plate

Add Donor Mix to lysate



Incubate plate

Read 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.

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 H₂O 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.

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.

II. Non-adherent Cells – Assay Protocol

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 40 μ L of cells/well into a tissue culture plate.
2. If using test agents/antagonists, add 20 μ 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 20 μ L/well of 4X agonist stock in HBSS containing 0.1% BSA. The final volume in the wells should be 80 μ L. (*If no antagonists were used at step 2, stimulate the cells with 40 μ L/well of 2X agonist, to give a final volume in the wells of 80 μ L.*)

Lysate Preparation

5. To lyse the cells, add 20 μ L/well 5X Lysis buffer. Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.
6. Take 30 μ L of the lysate and transfer to a 96-well $\frac{1}{2}$ -area assay plate for assay. (*Add 30 μ L Control lysate to separate wells if required, use 30 μ L 1X Lysis buffer for negative control*).

SureFire Assay

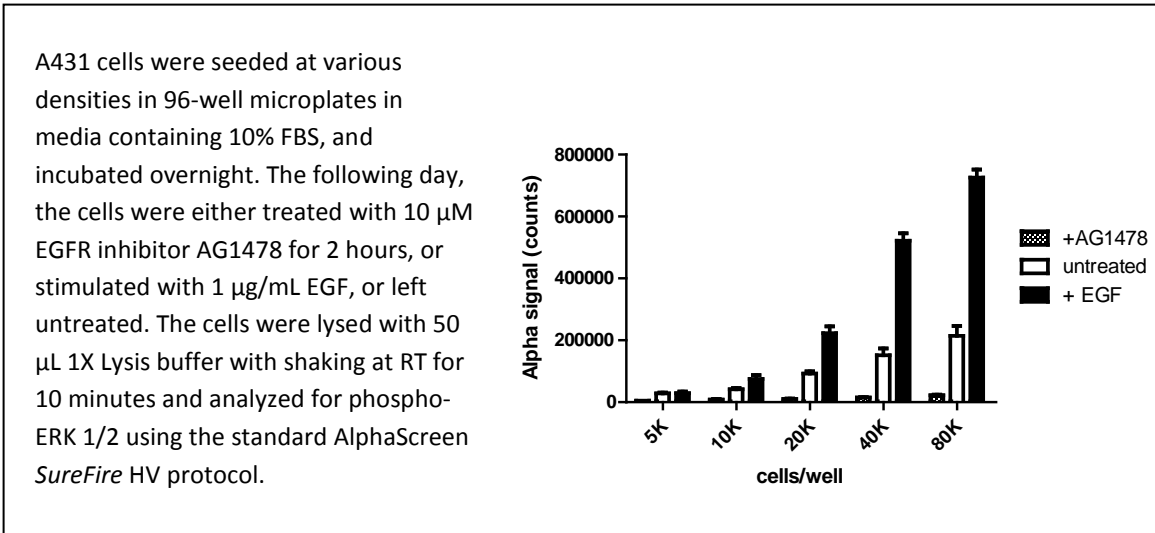
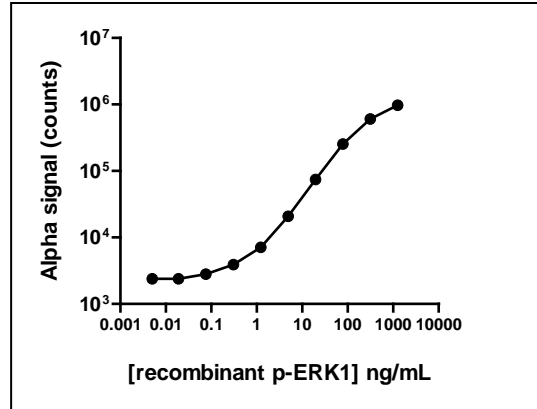
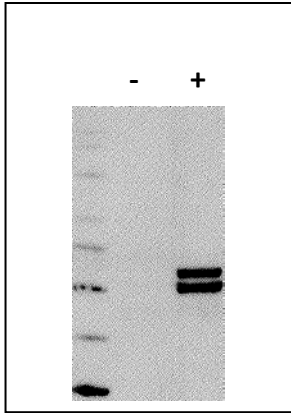
6. Add 10 μ L of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Agitate gently on plate shaker for 2 minutes, and then incubate for 2 hours at room temperature.
7. Add 10 μ L of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil.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

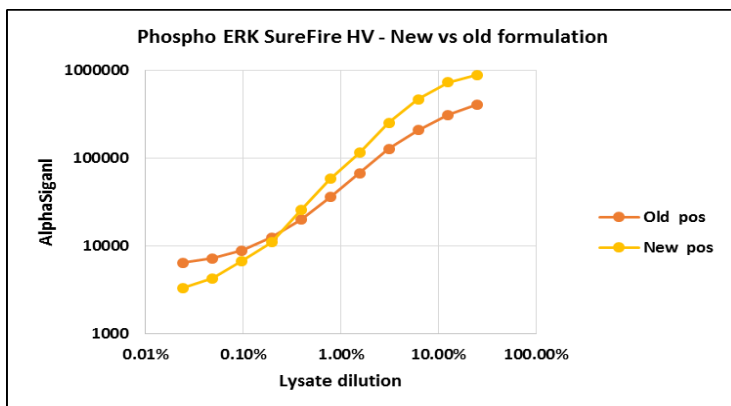
Representative Data

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.



Comparison of new SureFire p-ERK (TGRESBHV) vs old p-ERK (TGRESHV) Assay kit performance

Control lysates were diluted serially in 1X Lysis buffer and assayed with the respective kits in a 2-plate, 1-step format. S:B of the new TGRESBHV kit was 430 vs TGRESHV of 80.



Frequently Asked Questions

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 solubilize 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 μ L. However, for low abundance proteins, the lysis volume can be adjusted down to 25 μ L, 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.

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

Further Information:

To download these resources, and other related technical information, visit

<http://www.perkinelmer.com>

For general information on AlphaScreen *SureFire* assays, visit

<http://www.tgrbio.com>

To contact the customer care team, please visit

www.perkinelmer.com/ServiceCall

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