AlphaScreen® *SureFire*®
ERK 1/2 (p-Thr202/Tyr204) Assay Kits

Manual

Assay Points	Catalog #
500	TGRESB500
10 000	TGRESB10K
50 000	TGRESB50K

For Research Use Only
Research Reagents for Research Purposes Only

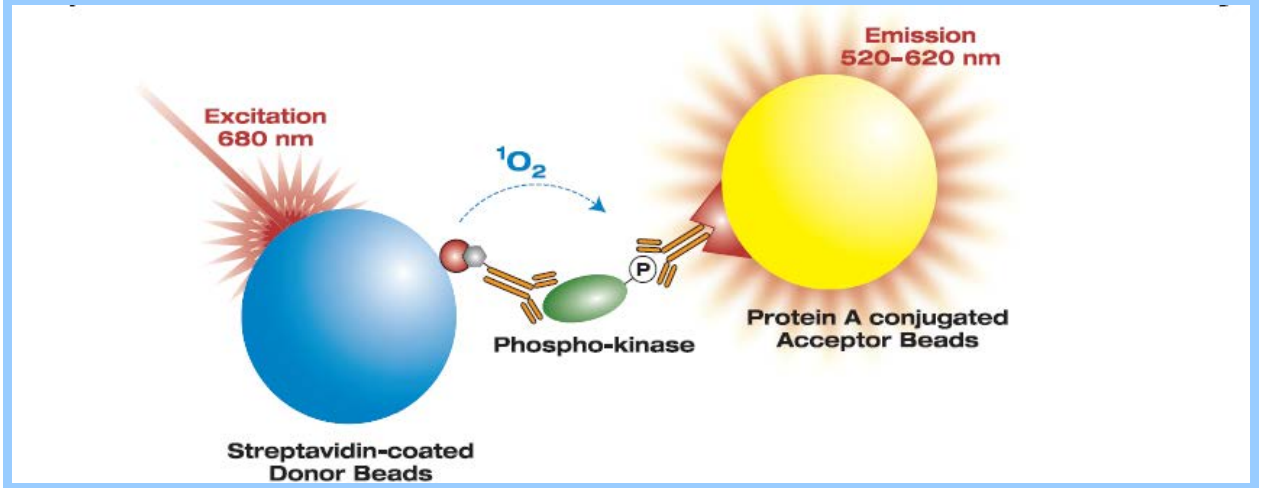
General Information on the AlphaScreen® SureFire® p-ERK 1/2 assay

The AlphaScreen® *SureFire*® 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. The assay is an ideal system for the screening of both 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 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.

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*® 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.

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		
	500 points	**10,000 points**	**50,000 points**
Lysis buffer (5X)	1 x 10 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	1 x 3.4 mL	2 x 35 mL	1 x 360 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_2O		
Negative Control Lysate	1 tube to be re-dissolved in 250 µL H_2O		

*Dilution buffer is only required for the high sensitivity protocol

Storage conditions upon receipt

The kit buffers, e.g. 5X Lysis buffer, Activation buffer, Dilution buffer and Reaction 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.

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.	6050185	100/box
Envision® or Enspire® Alpha-reader	PerkinElmer Inc.	-	-

Buffer preparation and subsequent storage conditions

5X Lysis buffer	Store 5X Lysis buffer at 4°C. For assay, dilute 5-fold in water immediately prior to use. Discard unused 1X Lysis 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*	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.
Reaction Mix** (Reaction buffer + Activation buffer and AlphaScreen beads)	Immediately prior to use, dilute Activation buffer **7-fold** in Reaction buffer (e.g. take 98 µL Activation buffer and dilute in 587 µL Reaction buffer). Under low-light conditions, dilute Acceptor beads **70-fold**, and donor beads **140-fold** in Reaction mix (e.g. add 10 µL Acceptor beads and 5 µL Donor beads to 685 µL of premixed Reaction buffer + Activation buffer). The Reaction mix should be used immediately for best results. Excess mix should be discarded.
Assay Control lysates	After reconstitution in 250 µL water, lysates should be frozen at -20°C in single use aliquots and to be used 1 month.

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

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

Control Lysate information

Control lysates are prepared at a concentration of approximately 1 mg/mL from flasks of A431 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.

Negative Lysate: Prepared from A431 cells treated with EGF receptor inhibitor (2 µM AG1478) for 2 hours prior to lysis.

Positive Lysate: Prepared from A431 cells treated with 200 ng/mL EGF for 10 minutes.

p-ERK AlphaScreen® *SureFire*® Assay – Standard protocols

A. 2-Plate Assay

This assay protocol is for adherent cells.

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.

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).

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). 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 4 µL Control lysates to separate wells if required*).

SureFire Assay

5. Prepare Reaction mix as described (page 4) and add 7 µL of mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for at least 2 hours at room temperature.

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

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

B. 1 Plate Assay

This assay protocol is for non-adherent cells, and for high-throughput applications. 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 µL of cells/well into a 384-well culture plate.

2. If using test agents/antagonists, add 2 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.

Cell Treatment

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

Lysate Preparation

5. To lyse the cells, add 2 µL/well 5X Lysis buffer. (*Add 10 µL Control lysates to separate wells if required*). Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.

SureFire Assay

6. Prepare Reaction mix as described (page 4) and add 10 µL of Reaction Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 2 hours at room temperature.

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.

High Sensitivity Protocols

ERK 1/2 is generally abundantly expressed in cells, and the standard protocol has sufficient sensitivity for many routine applications. However, there are applications where a high sensitivity assay protocol is useful e.g. for the use of less cells per assay, for analysis of diluted lysates, or for the detection of activation through low-abundance receptors, for example.

When performing high-sensitivity assays for phospho-ERK detection, make the adjustments detailed below to the standard protocols. All other steps in the protocol should remain the same.

! Assay reagents (Acceptor Mix and Donor Mix) should be prepared according to the guide on page 8.

For a 2-plate assay protocol (page 5), use the following amendments:

SureFire Assay

7. Add 5 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and incubate for 2 hours at room temperature.

8. Add 2 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 2 hours at room temperature.

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

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

For a 1-plate assay protocol (page 6), use the following amendments:

SureFire Assay

6. Add 8 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and incubate for 2 hours at room temperature.

7. Add 3 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 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.

Buffer preparation and subsequent storage conditions – High sensitivity protocols

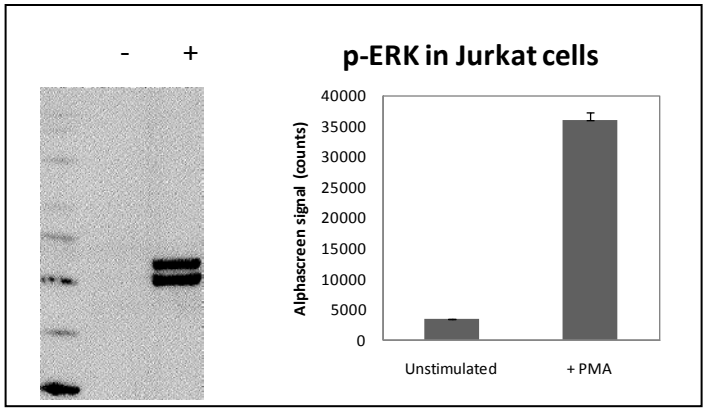
5X Lysis buffer	Store 5X Lysis buffer at 4°C. For assay, dilute **5-fold** in water immediately prior to use. Discard unused buffer.
Activation buffer	Precipitation will occur during storage 4°C. To re-dissolve, warm to 37°C and mix. Alternatively, Activation buffer can be stored at room temperature 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.
Acceptor Mix (Reaction buffer + Activation buffer + AlphaScreen® Acceptor beads)	Immediately prior to use, dilute Activation buffer **5-fold** in Reaction buffer (e.g. take 98 µL Activation buffer and dilute in 392 µL Reaction buffer). Dilute Acceptor beads **50-fold** 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.
Donor Mix** (Dilution buffer + AlphaScreen® Donor beads)	Immediately prior to use, dilute Donor beads **20-fold** in Dilution buffer (e.g. add 10 µL Acceptor 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.

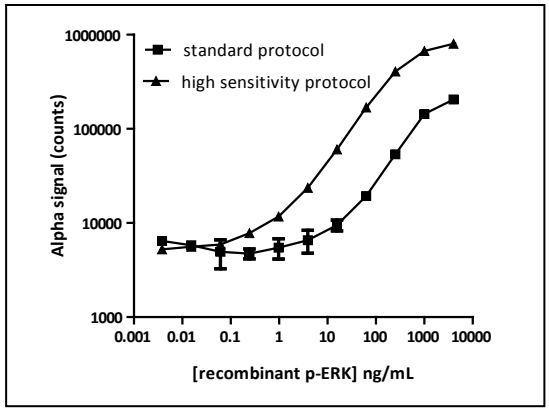
* Do not vortex the Reaction buffer, as vigorous mixing can damage some antibodies.

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

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, or the AlphaScreen *SureFire* assay (7.5 µg protein/well). Right panel: detection of recombinant phospho-ERK (Biaffin GMBH Cat# PK-ERK1-A010). Using the standard protocol, the limit of detection is around 10 ng/mL, while with the high sensitivity protocol, the limit of detection is less than 1 ng/mL.



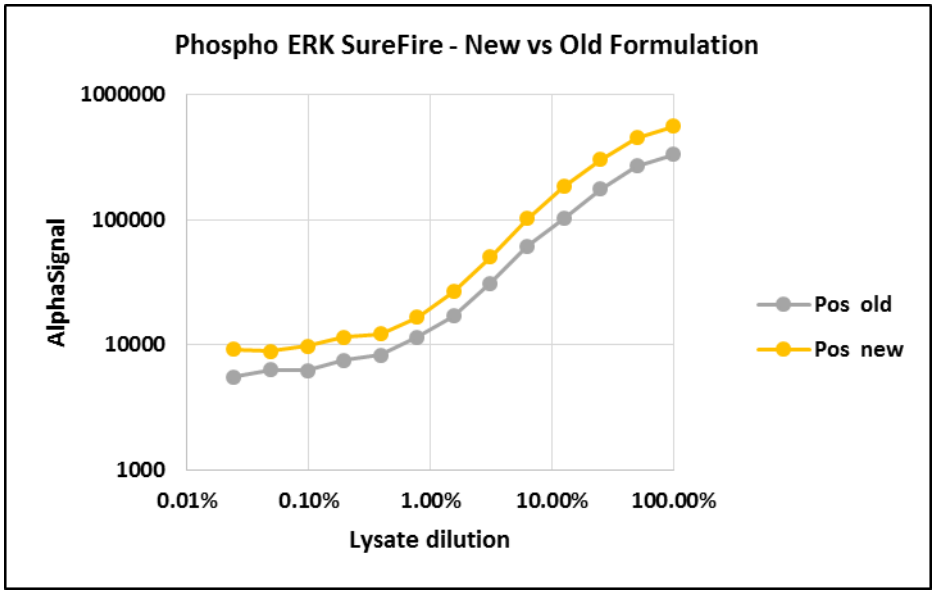


Irradiated CHO cells transfected with the NOP (ORL1) receptor (PerkinElmer Cat# ES-230-CF) were seeded at 40K cells/well in 96-well microplates for 6 hours in media containing 10% FBS, serum-starved overnight, and stimulated with nociceptin for 10 minutes at room temperature (left) or pre-treated with an ORL1 receptor inhibitor for 15 minutes, then stimulated (right). Cells were lysed with 50 µL 1X Lysis buffer with shaking at RT for 10 minutes and analyzed for phospho-ERK 1/2.

<u>Comparison of new SureFire p-ERK (TGRESB) vs old p-ERK (TGRES) Assay kit performance</u>

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 TGRESB kit was 64 vs TGRES of 59.



Frequently Asked Questions & Troubleshooting

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

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

To download these resources, and other related technical information, visit http://www.perkinelmer.com/category/alpha-surefire-kits
For general information on AlphaScreen *SureFire* assays, visit http://www.tgrbio.com

Customer Care

To contact the customer care team, please visit **www.perkinelmer.com/ServiceCall**

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

PerkinElmer web site: www.perkinelmer.com

TGR BioSciences website: www.tgrbio.com

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