

AlphaLISA[®] SureFire[®] Ultra[™] HV

p-VEGF Receptor 2 (Tyr1175) Assay Kit

Manual

Assay Points	Catalog #
100 (96 well format)	ALSU-PVGFR-A-HV

For Research Use Only. Not for use in Diagnostic Procedures

For a full, electronic, version of this manual, please go to:

www.perkinelmer.com/pVEGFR2

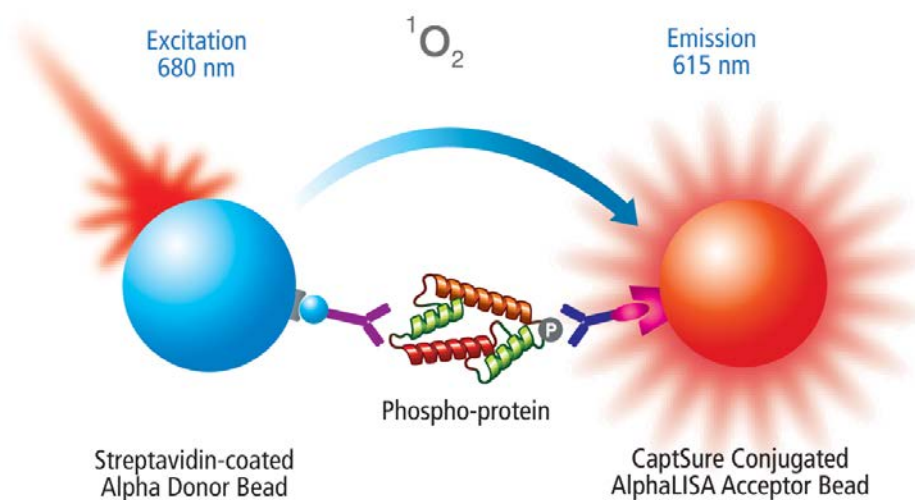
AlphaLISA[®] SureFire[®] Ultra[™] HV

Assay Principle

The AlphaLISA[®] SureFire[®] Ultra[™] assay kits allow the rapid, sensitive, and quantitative detection of phosphoproteins from cells. The kits utilize Alpha beads that are each coated to specifically capture one of the assay antibodies. The Donor bead is coated with streptavidin to capture the biotinylated antibody. The Acceptor bead is coated with a proprietary “CaptSure[™]” agent that immobilizes the other assay antibody which is labeled with a CaptSure[™] tag. As such, this assay system performs well in the presence of extraneous antibodies, such as antibody biotherapeutics, and can be used to screen such reagents. In the presence of phosphorylated protein, the two antibodies bring the Donor and Acceptor beads close to each other, enabling the generation of an Alpha signal upon illumination of Donor beads by the Alpha-enabled plate reader, such as the EnVision[®] Multilabel Plate Reader or EnSpire[®] and EnSight[™] Multimode Plate Readers. The amount of light emission is directly proportional to the amount of phosphoprotein present in the sample.

The AlphaLISA[®] SureFire[®] Ultra[™] assay kits are also optimized for enhanced signal-to-noise windows, while using shorter incubation times and larger volumes for pipetting compared to the AlphaScreen[®] SureFire[®] kits.

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, better well-to-well reproducibility (lower CV%), and robotic operation if desired. The assay utilizes the bead-based Alpha Technology, and requires an Alpha Technology-compatible plate reader.



General Information on the AlphaLISA® SureFire® Ultra™ HV p-VEGF Receptor 2 (Tyr1175) assay

The AlphaLISA® SureFire® Ultra™ HV p-VEGF Receptor 2 (Tyr1175) assay is used to measure the phosphorylation of endogenous VEGF Receptor 2 at Tyrosine 1175 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 receptor kinase activity. The assay will measure VEGF Receptor 2 activation of either recombinant or endogenous receptors, and can be applied to primary cells.

This kit has been formulated to provide improved signal:noise assay windows, and to perform without interference in the presence of extraneous antibodies.

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.

Kit Contents (store at 4°C)

Kit Size	100 points
Lysis Buffer (5X) – <i>Ultra</i>	1 x 12 mL
Activation Buffer – <i>Ultra</i>	1 x 0.3 mL
Reaction Buffer 1 – <i>Ultra</i>	1 x 0.71 mL
Reaction Buffer 2 – <i>Ultra</i>	1 x 0.71 mL
Dilution Buffer – <i>Ultra</i>	1 x 1.47 mL
AlphaLISA® CaptSure™ Acceptor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 30 µL
AlphaScreen® Streptavidin Donor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 30 µL
Positive Control Lysate (Note: remove and store at -20°C or -80°C)	1 tube to be re-dissolved in 250 µL H ₂ O

Storage Conditions Upon Receipt

The kit should be placed at 4°C upon receipt. **DO NOT** freeze the kit buffers or beads – the Reaction buffer contains antibodies and freeze/thaw cycles can lead to a loss of activity.

AlphaScreen Donor Beads need to be stored at 4°C in the dark, and should be returned to the kit box after use.

The Activation Buffer precipitates at 4°C. To re-dissolve, warm to 37°C and mix before each use. Alternatively, Activation buffer can be stored at room temperature with no loss in activity. All other components to be returned to 4°C after each use.

The Positive control lysate tube should be placed at -20°C or -80°C for long term storage.

This product is stable for at least 9 months from the manufacturing date if used and stored under recommended conditions.

Materials Required But Not Provided

Item	Suggested source	Catalog #	Size
1/2AreaPlate™ - 96 assay plate	PerkinElmer Inc.	6005560	50/box
TopSeal-A 384, clear adhesive sealing film	PerkinElmer Inc.	6050185	100/box
Envision®, Enspire® or EnSight™ Alpha-reader	PerkinElmer Inc.	-	-

Precautions

*Only the AlphaScreen® Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco, or the equivalent) can be applied to light fixtures.

Buffer Preparation and Subsequent Storage Conditions

<p>1X Lysis Buffer</p>	<p>Dilute 5X Lysis buffer in MilliQ water to a final concentration of 1X</p> <p>For example: for 10 mL of 1X Lysis Buffer, add: 2 mL of 5X Lysis Buffer to 8 mL MilliQ water. Discard unused 1X buffer.</p>
<p>Acceptor Mix (Reaction buffer 1 + Reaction buffer 2 + Activation Buffer + AlphaLISA® CaptSure™ Acceptor beads)</p>	<p>Dilute Activation Buffer 25-fold in combined Reaction Buffer 1 and Reaction buffer 2 Dilute Acceptor beads 50-fold in combined Reaction Buffers</p> <p>For example: for 300 µL of Acceptor Mix: Combine 141µL of Reaction Buffer 1 and 141µL of Reaction buffer 2, and to this add 12µL Activation Buffer and 6µL Acceptor Beads</p> <p>The Acceptor mix should be made up and used immediately when required for best results. Excess mix should be discarded.</p>
<p>Donor Mix* (Dilution buffer + AlphaScreen® Donor beads)</p>	<p>Dilute Donor beads 50-fold in Dilution buffer</p> <p>For example: for 300 µL of Donor Mix, add: 6µL Donor Beads to 294 µL of <u>Dilution Buffer</u></p> <p>The Donor mix should be made up and used immediately when required for best results. Excess mix should be discarded.</p>
<p>Positive control lysate</p>	<p>Stable while lyophilized at -20°C to expiry date. After reconstitution in 250 µL of water, lysate should be frozen at -20°C or -80°C in single use aliquots and used within 1 month.</p>

* Prepare and use under low-light conditions.

Note: the buffers (lysis, activation, reaction, dilution) in the AlphaLISA *SureFire Ultra* kits have a different formulation compared to the buffers from the AlphaScreen *SureFire* kits, and buffers from the two types of kits should not be interchanged.

AlphaLISA® SureFire® Ultra™ HV p-VEGF Receptor 2 (Tyr1175) Assay Protocols

A. 2-Plate Assay - assay protocol for adherent cells

Cell Seeding

1. Seed cells (200 µL of cells per well) in 96 well 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 5-20 minutes for receptor antagonists).*

Note: 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% protease-free BSA)

Lysate Preparation

3. To lyse cells, remove medium from wells, and add freshly prepared 1X Lysis Buffer (50-100 µL per well). 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 1/2AreaPlate™ for assay. *(Add 30 µL Control lysate to separate wells if required).*

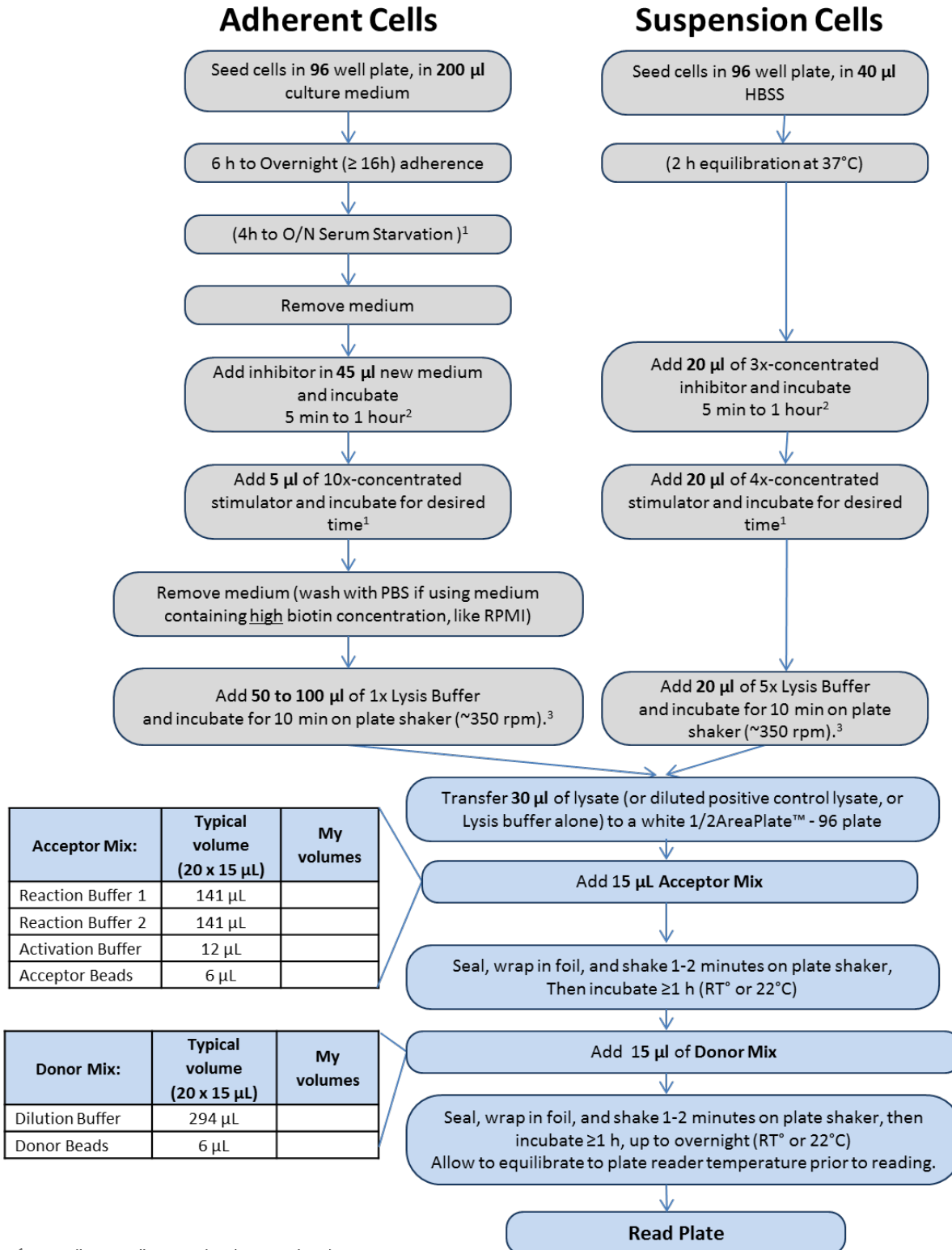
SureFire Ultra Assay

5. Add 15 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature.

6. Add 15 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature in the dark.

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

AlphaLISA® SureFire® Ultra™ HV: 2-plates / 2-incubations assay flowchart



¹ Depending on cell type and pathway analyzed.

² Depending on type of inhibitor used: 5 min is generally enough for receptor antagonists; more time is needed to block intracellular targets.

³ May stop and freeze lysates at -20°C if desired. If doing this, re-shake after thawing to ensure homogeneity of lysate before pipetting.

AlphaLISA® SureFire® Ultra™ HV p-VEGF Receptor 2 (Tyr1175)

B. 1 Plate Assay - assay protocol for non-adherent cells, and for high-throughput applications.

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 12 μ L of cells/well into a 96-well white opaque culture plate (eg 1/2AreaPlate™ - 96). Note: as engaging less cells per well can result in increased signal to background ratios, it is important to optimize this factor.

2. If using test agents/inhibitors, add 6 μ L/well of 4X inhibitors prepared in HBSS. Otherwise add 6 μ L/well of HBSS.

Note: Peptidic agonists and antagonists can often stick to plastic surfaces. To minimize this effect, dilute in HBSS containing a suitable carrier protein (e.g. protease-free BSA).

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

Cell Treatment

4. Treat cells with agonists/buffer by addition of 6 μ L/well of 4X agonist stock/buffer in HBSS containing 0.1% BSA. The final volume in the wells should be 24 μ L.

Lysate Preparation

5. To lyse the cells, add 6 μ L/well of 5X Lysis Buffer.
(Add 30 μ L control lysates to separate wells if required)

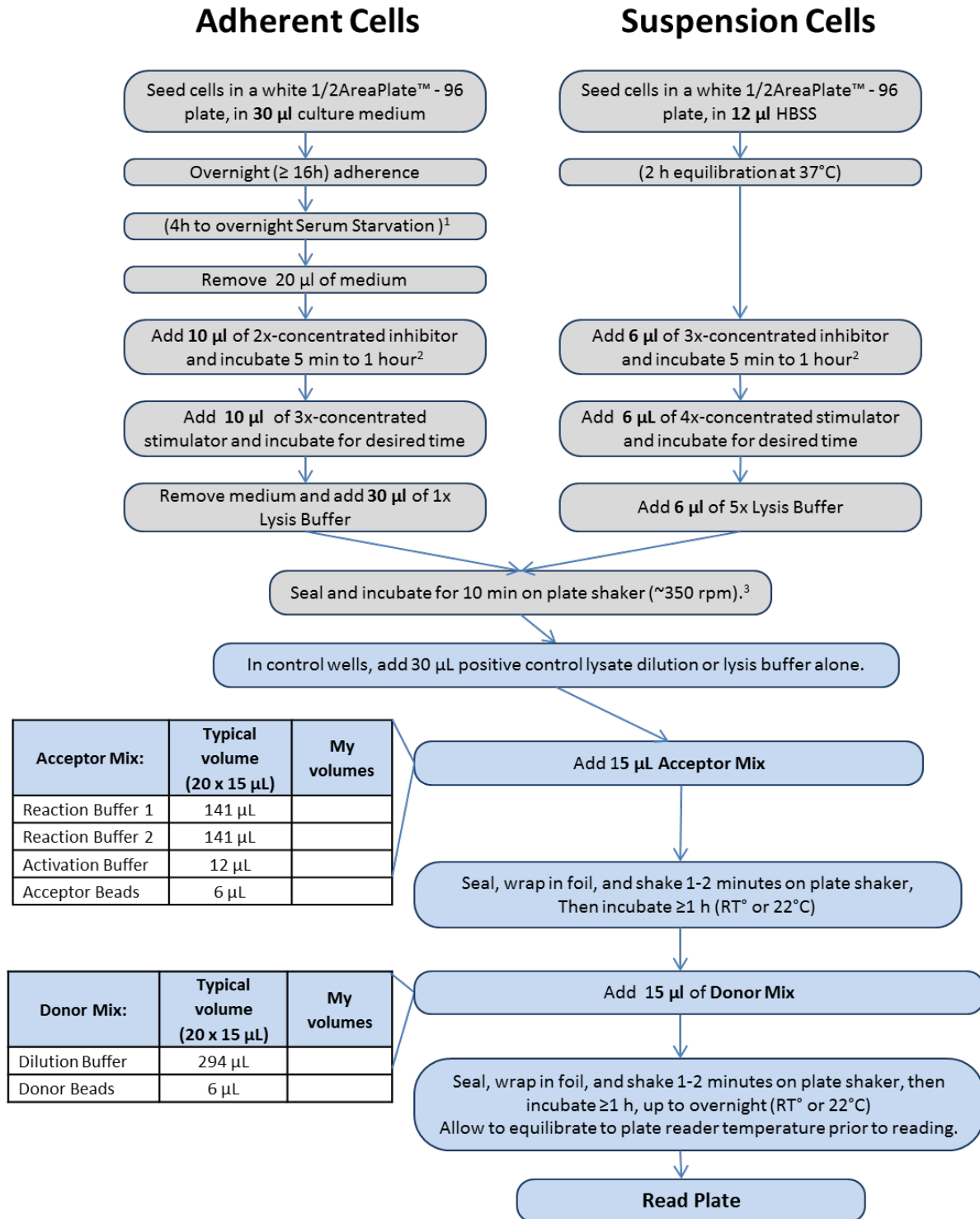
SureFire Ultra Assay

6. Add 15 μ L of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature.

7. Add 15 μ L of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature in the dark.

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

AlphaLISA® SureFire® Ultra™ HV: 1-plate / 2-incubations assay flowchart



¹ Depending on cell type and pathway analyzed.

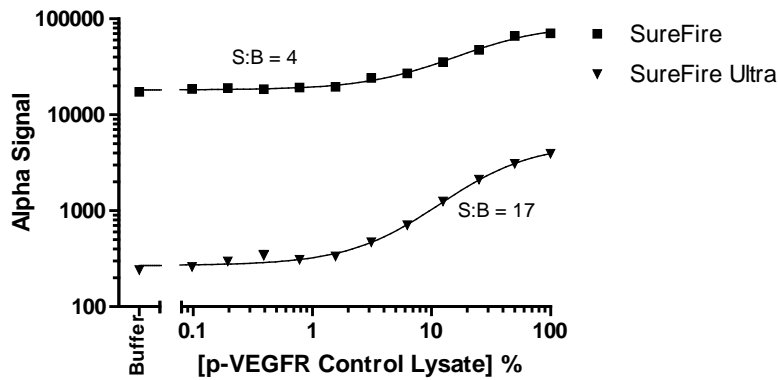
² Depending on type of inhibitor used: 5 min is generally enough for receptor antagonists; more time is needed to block intracellular targets.

³ May stop and freeze lysates at -20°C if desired. If doing this, re-shake after thawing to ensure homogeneity of lysate before pipetting.

Control Lysate Information

Positive Control Lysate: HUVEC cells were grown to confluence in T175 flasks, and then serum-starved overnight. The following day the media was removed, and the cells were treated with 100ng/mL VEGF for 10 minutes. The agonist was removed, and the cells were lysed with 4mL of freshly prepared 1X Lysis buffer, with shaking for 10 minutes.

Representative Data



Data obtained with 2-incubation protocol.

Supplementary Buffers and Beads

If using the standard protocol, sufficient amounts of buffers and beads are provided in the kit. However in case the standard protocol would be modified, more buffers or beads may be needed. In this case, you can order additional buffers and beads using the following catalog numbers:

Item	Suggested source	Catalog #	Size
Lysis Buffer (5X) - <i>Ultra</i>	PerkinElmer Inc.	ALSU-LB-10mL	10mL
	PerkinElmer Inc.	ALSU-LB-100mL	100mL
Activation Buffer - <i>Ultra</i>	PerkinElmer Inc.	ALSU-AB-10mL	10mL
	PerkinElmer Inc.	ALSU-AB-100mL	100mL
Dilution Buffer - <i>Ultra</i>	PerkinElmer Inc.	ALSU-DB-10mL	10mL
	PerkinElmer Inc.	ALSU-DB-100mL	100mL
AlphaScreen® Streptavidin Donor Beads -2mg/mL	PerkinElmer Inc.	ALSU-ASDB-0.06mL	60µL
	PerkinElmer Inc.	ALSU-ASDB-1.2mL	1.2mL
	PerkinElmer Inc.	ALSU-ASDB-6mL	6mL
AlphaLISA® CaptSure™ Acceptor Beads - 2mg/mL	PerkinElmer Inc.	ALSU-ACAB-0.06mL	60µL
	PerkinElmer Inc.	ALSU-ACAB-1.2mL	1.2mL
	PerkinElmer Inc.	ALSU-ACAB-6mL	6mL

My Assay Notes

Useful Links

For FAQ and troubleshooting, please go to:

www.perkinelmer.com/SureFireFAQ

For a complete list of AlphaLISA *SureFire Ultra* kits, please go to:

www.perkinelmer.com/SureFire

or

www.tgrbio.com

For technical support please go to:

www.perkinelmer.com/ASK

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