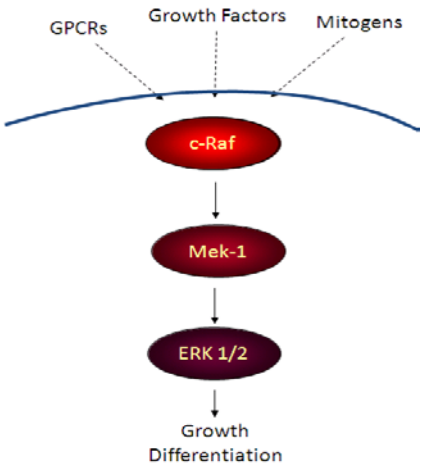
Alpha *SureFire® Ultra™ HV* Multiplex

p-ERK 1/2 (Thr202/Tyr204) 615 (Eu) + Total ERK 545 (Tb) Assay Kit

Manual

Assay Points	Catalog #
100 (96 well format)	MPSU-PTERK-K-HV



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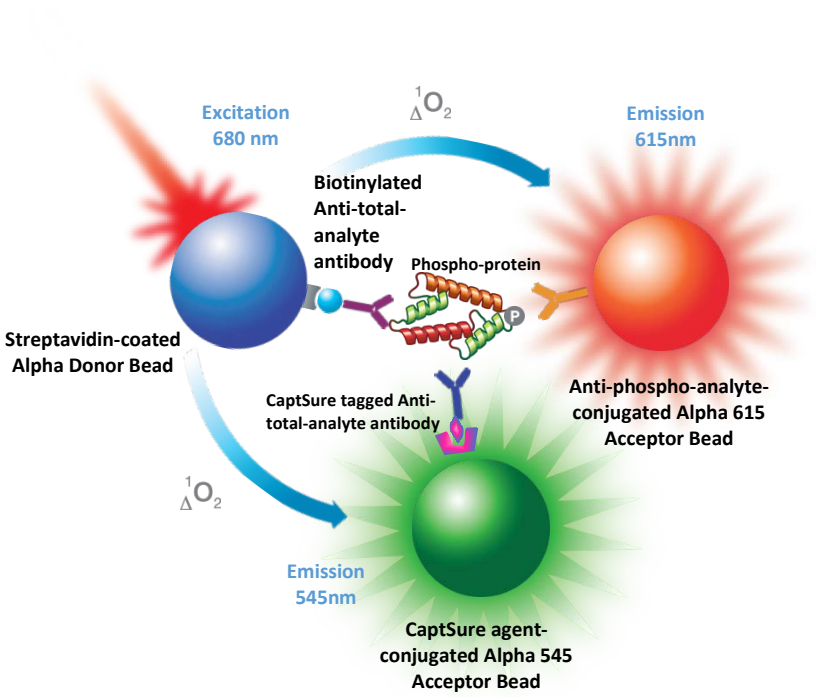
For a full, electronic, version of this manual, please go to:
www.perkinelmer.com/pERK or www.perkinelmer.com/tERK

Alpha *SureFire® Ultra™ HV* Multiplex

Assay Principle

The Alpha *SureFire Ultra* Multiplex Phospho + Total assay kits allow the rapid, sensitive, and quantitative detection of phosphoproteins from cells, combined with the measurement of the total amount of the same protein. This Alpha Multiplex measurement is carried out in the same assay plate well from a single sample of cell lysate, and is achieved by the use of two types of Alpha Acceptor beads that emit at distinct wavelengths (545nm and 615nm).
The two distinct Alpha Acceptor beads report their binding to a distinct antigen through their association with specific assay antibodies, as indicated below.

<u>Single target – Phospho + Total Assay kits</u>
The Alpha 615 Acceptor bead is directly-conjugated with an antibody to the phosphorylated site on the target protein. The Alpha 545 Acceptor bead is coated with the Capt*Sure* agent, which binds the Capt*Sure* tagged anti-total target protein antibody. The Alpha Donor bead binds the biotinylated anti-total target protein antibody.
The antibodies used in this kit are identical to those used in the individual phospho- and total- ERK AlphaLISA *SureFire Ultra* kits (ALSU-PERK-A and ALSU-TERK-A catalog numbers), although their orientation of bead attachment may vary between kits.



General Information on the AlphaLISA® *SureFire® Ultra™ HV* Multiplex p-ERK 1/2 (Thr202/Tyr204) + Total ERK assay

The Alpha *SureFire® Ultra™ HV* Multiplex p-ERK 1/2 + Total ERK assay kit is used to measure both the phosphorylation (Thr202/Tyr204) and total levels 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 modulators of receptor activation (e.g. agonists and antagonists) as well as agents acting intracellularly, such as small molecule inhibitors of signal transduction. The assay will measure ERK 1/2 activation by either recombinant or endogenous receptors, and can be applied to primary cells.

The 615nm (Eu) signal corresponds to the phosphorylated ERK analysis, and the 545nm (Tb) signal corresponds to the total ERK analysis.

This kit has been formulated to provide improved signal:background (i.e. S:B) assay windows, and to perform without interference in the presence of extraneous antibodies.

Kit-Specificity Information

This assay kit contains 3 antibodies; one that recognizes the phospho-Thr202/Tyr204-epitope, and two which recognize distinct distal epitopes 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.

The assay utilizes the bead-based Alpha Technology, and requires an Alpha Technology-compatible plate reader capable of reading dual emission wavelengths. See www.perkinelmer.com/AlphaPlex for more information about the AlphaPlex technology and download the "AlphaPlex Quick Start Guide" and the "AlphaPlex Assay Development Guide" to find guidance about filters and mirrors selection, instrument protocol and channels crosstalk correction. It is to be noted that, as the analytes recognized by both assays (i.e. the phosphorylated protein and the total protein) cannot be dissociated, it is not possible to omit one or the other analyte for the establishment of the channels crosstalk correction, but one or the other type of acceptor beads needs to be omitted instead. i.e. all the assay components but the Alpha 615 beads must be assembled to establish the crosstalk of the Alpha 545 beads into the 615 nm channel, and all the assay components but the Alpha 545 beads must be assembled to establish the crosstalk of the Alpha 615 beads into the 545 nm channel.

Note: the buffers (lysis, activation, reaction, dilution) in the Alpha *SureFire Ultra* Multiplex 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.

Kit Contents

Kit Size	100 points
Lysis Buffer (5X) - *Ultra*	1 x 12 mL
Activation Buffer - *Ultra*	1 x 0.3 mL
Reaction Buffer 1 – MPSU *(Biotinylated anti-Total ERK antibody – rIgG)*	1 x 0.08 mL
Reaction Buffer 2 – MPSU *(CaptSure™ tagged anti-Total ERK antibody – mIgG1)*	1 x 1.7 mL
Dilution Buffer - *Ultra*	1 x 1.8 mL
Alpha 615 anti-p-ERK (mIgG1) Acceptor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.045 mL
Alpha 545 Capt*Sure*™ Acceptor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.045 mL
Alpha Streptavidin Donor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.045 mL
Positive Control Lysate	1 tube to be re-dissolved in 250 µL H_2O

The above volumes supplied are in excess to the actual volume required to perform assay.

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.

See kit box label for expiry date.

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® Alpha-reader with adequate AlphaPlex filters (see table below)	PerkinElmer Inc.	-	-

For more assay plates options, please go to www.perkinelmer.com/microplates

Table : AlphaPlex Optics for EnVision Multilabel Reader – for complete information about how to set an AlphaPlex reading, please refer to the AlphaPlex Guides available at www.perkinelmer.com/AlphaPlex

	Description	Catalog #	Barcode	Recommendations
Mirrors	AlphaScreen	2101-4010	444	For Tb and Eu single and sequential reading ; not for Sm
	AlphaPlex Single Tb-Eu-Sm	2102-5910	605	Preferred mirror for all sequential AlphaPlex applications
	AlphaPlex Dual Tb-Eu	2102-5900	653	For **simultaneous** duplexing of Tb with Eu
Filters	AlphaScreen	2100-5710	244	Suitable for AlphaPlex single plexing, not for multiplexing
	Resorufine/ Amplex Red	2100-5570	124	Suitable for Tb single plexing and Tb/Eu duplexing.
	Europium	2100-5090	203	Preferred filter for all Eu applications and multiplexing
	AlphaPlex Tb	2100-5930	701	Preferred filter for all Tb applications and multiplexing

Buffer Preparation and Subsequent Storage Conditions

1X Lysis Buffer	Dilute 5X Lysis buffer in MilliQ water to a final concentration of 1X For example: for 10 mL of 1X Lysis Buffer, add: 2 mL of 5X Lysis Buffer – Ultra to 8 mL MilliQ water. Discard unused 1X buffer.
Acceptor Mix Alpha Reaction Buffer 1 - *Ultra* (4 parts or 4%) + Alpha Reaction Buffer 2 - *Ultra* (88 parts or 88%) + Activation Buffer - *Ultra* (4 parts or 4%) + Alpha 615 anti-p-ERK Acceptor beads (2 parts or 2%) + Alpha 545 Capt*Sure*™ Acceptor beads (2 parts or 2%) See flowchart for table	Dilute Reaction Buffer 1 **25-fold** in Reaction Buffer 2. Dilute Activation Buffer **25-fold** in combined Reaction Buffer 1 and Reaction buffer 2. Dilute each Acceptor bead **50-fold** in combined Reaction Buffers plus Activation Buffer. For example: for 300 µL of Acceptor Mix: Add 12µL of Reaction Buffer 1 to 264µL of Reaction Buffer 2, and to this add 12µL Activation Buffer and 6µL Acceptor Bead 615 and 6µL Acceptor Bead 545. The Acceptor mix should be made up and used within 30min for best results. Excess mix should be discarded.
Donor Mix* Dilution buffer - *Ultra* (98 parts or 98%) + Alpha Donor beads (2 parts or 2%) See flowchart for table * Prepare and use under low-light conditions.	Dilute Donor beads **50-fold** in Dilution buffer For example: for 300 µL of Donor Mix, add: 6 µL Donor Beads to 294 µL of Dilution Buffer The Donor mix should be made up and used within 30min for best results. Excess mix should be discarded.
Positive control lysate	Reconstitute with 250µL water. Store at -20°C in single use aliquots and use within 3 months. Dilute as required.

Precautions

Only the Alpha 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 (filter #90 from LEE Filters (preferred) or Roscolux filters #389 from Rosco, or the equivalent) can be applied to light fixtures.

AlphaLISA® *SureFire® Ultra™ HV* Multiplex p-ERK 1/2 (Thr202/Tyr204) + Total ERK Assay Protocol

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 of Control lysates to separate wells. We recommend testing a serial dilution of Control lysate (eg 100, 50, 25, 12.5, 6.25 and 0% diluted in 1X Lysis Buffer).*

SureFire Ultra Assay

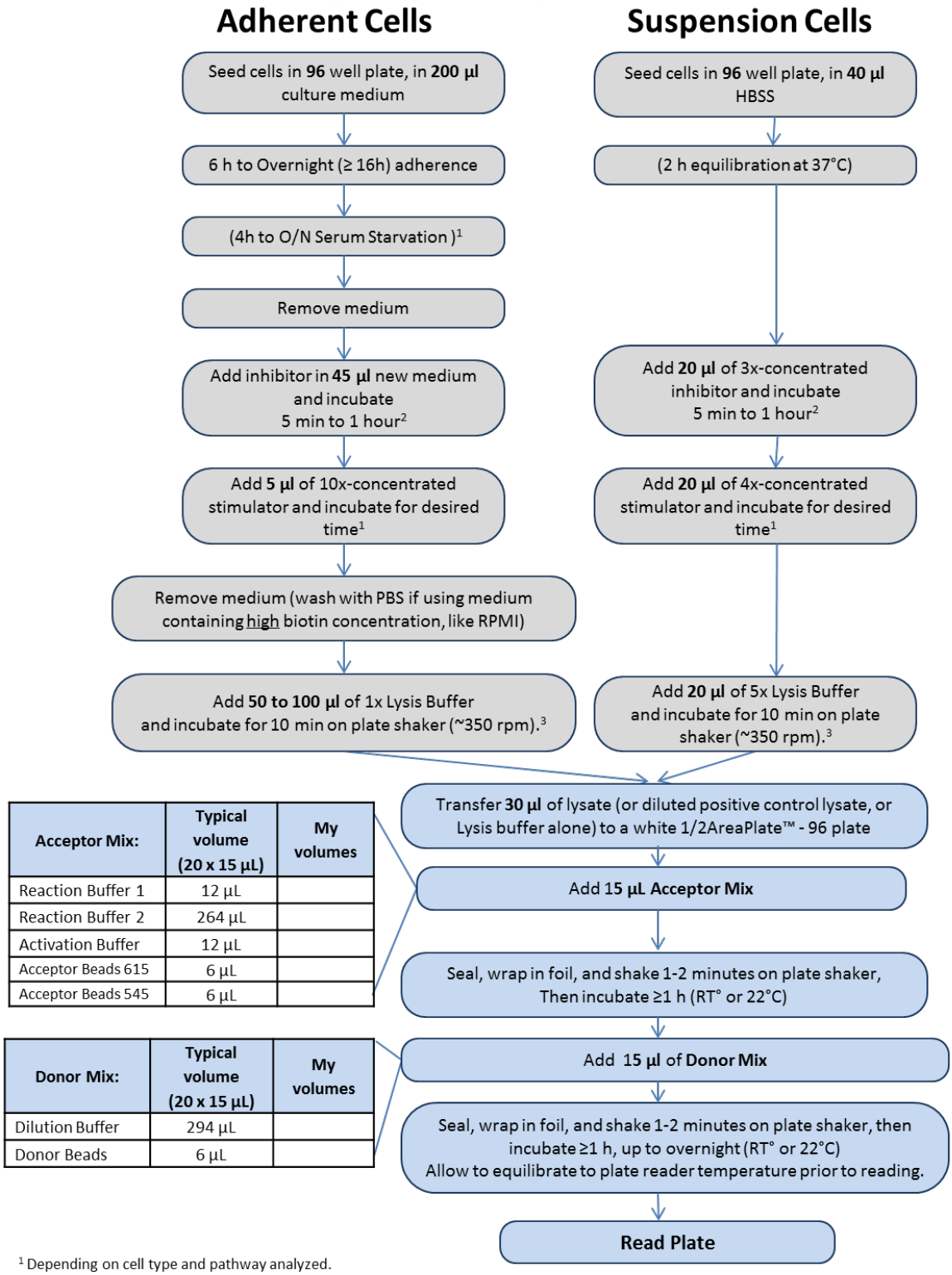
5. Add 15 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature.

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 AlphaPlex Technology-compatible plate reader, using standard AlphaPlex settings.

Alpha *SureFire® Ultra™ HV* Multiplex: 2-plates / 2-incubation assay flowchart

Single target – Phospho/Total



Acceptor Mix:	Typical volume (20 x 15 µL)	My volumes
Reaction Buffer 1	12 µL	
Reaction Buffer 2	264 µL	
Activation Buffer	12 µL	
Acceptor Beads 615	6 µL	
Acceptor Beads 545	6 µL	

Donor Mix:	Typical volume (20 x 15 µL)	My volumes
Dilution Buffer	294 µL	
Donor Beads	6 µL	

[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* Multiplex p-ERK 1/2 (Thr202/Tyr204) + Total ERK Assay Protocol

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 µL 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 µL/well of 4X inhibitors prepared in HBSS. Otherwise add 6 µL/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. 0.1% 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 µL/well of 4X agonist stock/buffer in HBSS containing 0.1% BSA. The final volume in the wells should be 24 µL.

Lysate Preparation

5. To lyse the cells, add 6 µL/well of 5X Lysis Buffer. *Add 30 µL of Control lysates to separate wells. We recommend testing a serial dilution of Control lysate (eg 100, 50, 25, 12.5, 6.25 and 0% diluted in 1X Lysis Buffer).*

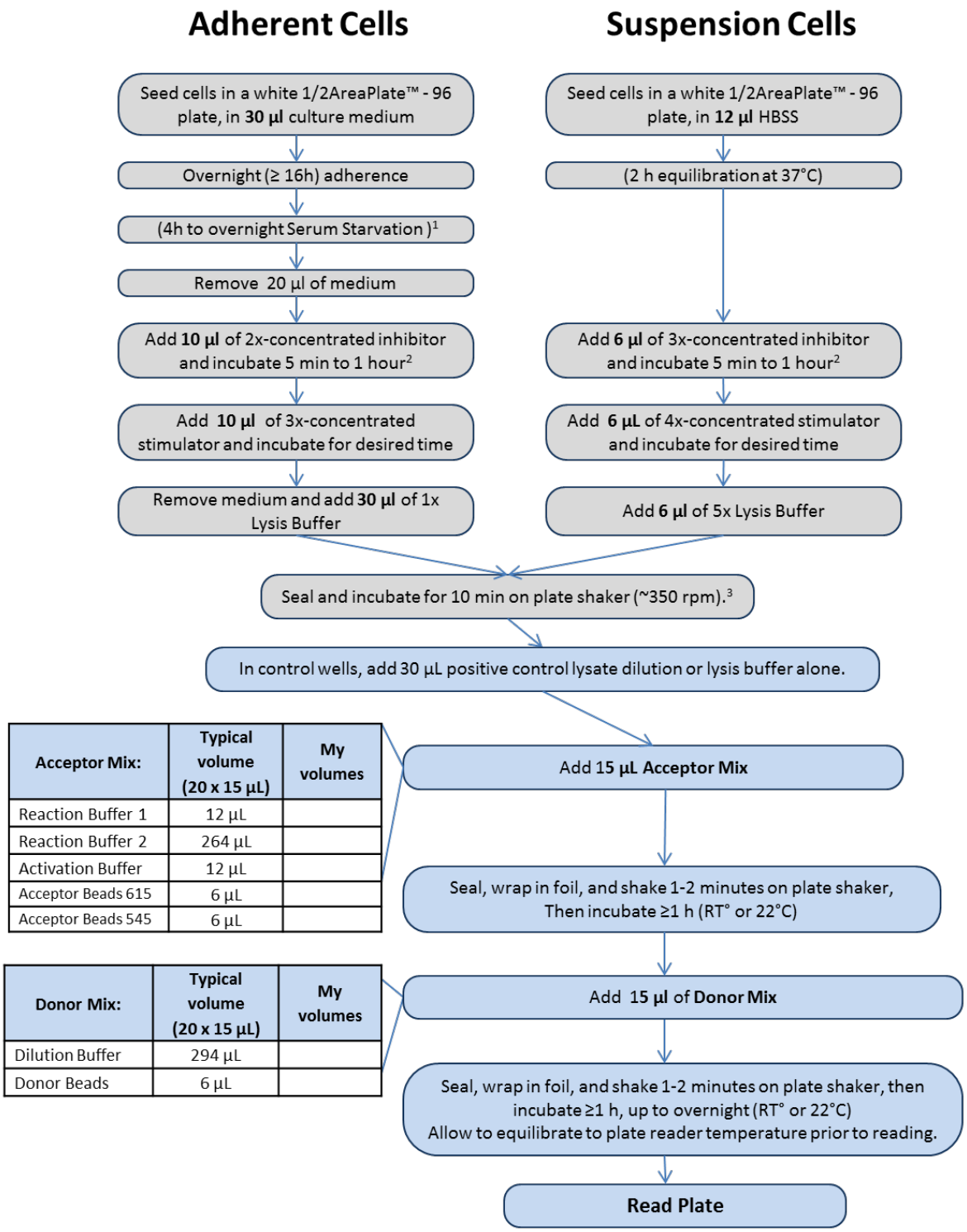
SureFire Ultra Assay

6. 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.

7. 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.

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

Alpha *SureFire® Ultra™ HV* Multiplex: 1-plate / 2-incubation assay flowchart
Single target – Phospho/Total



Acceptor Mix:	Typical volume (20 x 15 µL)	My volumes
Reaction Buffer 1	12 µL	
Reaction Buffer 2	264 µL	
Activation Buffer	12 µL	
Acceptor Beads 615	6 µL	
Acceptor Beads 545	6 µL	

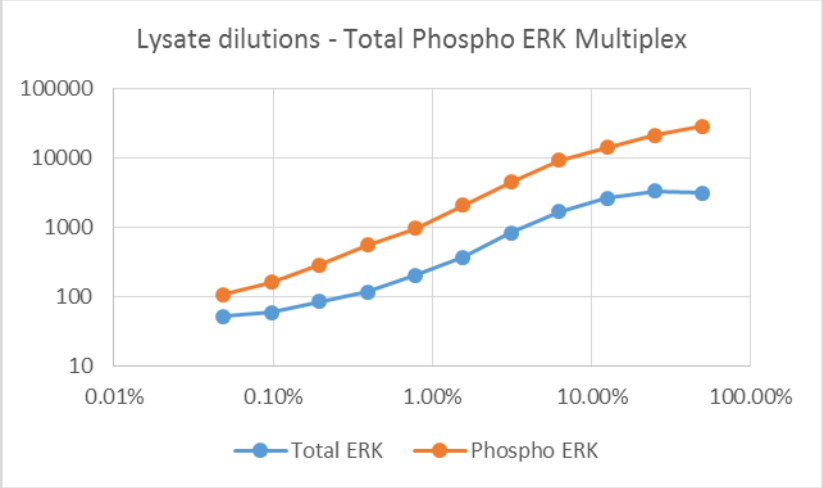
Donor Mix:	Typical volume (20 x 15 µL)	My volumes
Dilution Buffer	294 µL	
Donor Beads	6 µL	

[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: Prepared from A431 cells, cultured to confluence in T175 flasks in 10% FBS-containing medium for 3 days, then treated with EGF (200ng/mL) for 10min and lysed in 20mL of 1X *SureFire Ultra* Lysis buffer.

Representative Data: Phospho @ 615 nm (Eu), Total @ 545 nm (Tb)



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) - *Ultra*	PerkinElmer Inc.	ALSU-LB-10mL	10mL
	PerkinElmer Inc.	ALSU-LB-100mL	100mL
Activation Buffer - *Ultra*	PerkinElmer Inc.	ALSU-AB-10mL	10mL
	PerkinElmer Inc.	ALSU-AB-100mL	100mL
Dilution Buffer - *Ultra*	PerkinElmer Inc.	ALSU-DB-10mL	10mL
	PerkinElmer Inc.	ALSU-DB-100mL	100mL
Alpha 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
Alpha 545 (Tb) Capt*Sure*™ Acceptor Beads - 2mg/mL	PerkinElmer Inc.	MPSU-ACAT-0.06mL	60µL
	PerkinElmer Inc.	MPSU-ACAT-1.2mL	1.2mL
	PerkinElmer Inc.	MPSU-ACAT-6mL	6mL

Using the Alpha *SureFire*® *Ultra*™ Multiplex p-ERK 1/2 (Thr202/Tyr204) + Total ERK to measure only one of these targets

You may occasionally want to use this kit to measure only one of the analytes (either measuring only phospho-ERK, or only total ERK). In such a case you can replace the reagents corresponding to the non-measured analyte by an equivalent volume of Dilution buffer. For example, to use this kit to measure only p-ERK, the preparation of the Acceptor Mix on page 6 would become:

Acceptor Mix Alpha Multiplex Reaction Buffer 1 - *Ultra* (4 parts or 4%) + Dilution Buffer - *Ultra* (88 parts or 88%)+ Activation Buffer - *Ultra* (4 parts or 4%) + Alpha 615 anti-p-ERK Acceptor beads (2 parts or 2%) + Dilution Buffer - *Ultra* (2 parts or 2%)	Dilute Reaction Buffer 1 **25-fold** into Dilution Buffer. Dilute Activation Buffer **25-fold** in combined Reaction Buffer 1 and Dilution Buffer. Dilute the Acceptor bead **50-fold** in combined Reaction Buffers plus Activation Buffer. For example: for 300 µL of Acceptor Mix: Add 12µL of Reaction Buffer 1 to 264µL of Dilution Buffer, and to this add 12µL Activation Buffer and 6µL Acceptor Bead 615 and 6µL Dilution Buffer. The Acceptor mix should be made up and used immediately when required for best results. Excess mix should be discarded.

Please be aware that in this case, there will not be sufficient amount of Donor Beads or Dilution Buffer to measure in separate assays both analytes. (Beads and Dilution buffer can be re-ordered separately).

The reagents to read only one of the analytes (using Alpha 615 Acceptor beads) are available as different kits:

To measure only phospho-ERK: ALSU-PERK-A500 / 10K / 50K / -HV

To measure only total-ERK: ALSU-TERK-A500 / 10K / 50K / -HV

Useful Links

For FAQ and troubleshooting, please go to:
www.perkinelmer.com/SureFireFAQ or **www.perkinelmer.com/AlphaPlex**
For a complete list of AlphaLISA *SureFire Ultra* and Alpha *SureFire Ultra* Multiplex 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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