Alpha *SureFire*® *Ultra*™ *HV* Multiplex

Phospho (Eu) + Total (Tb) Target Detection Kit

Manual

Assay Points	Catalog #
100 (96 well format)	MPSU-XXXX-X-HV

This Manual is a generic manual for the Alpha *SureFire*® *Ultra*™ Multiplex HV Phospho + Total kits.

This manual does not apply to MPSU-PTERK-K-HV and MPSU-PTAKT-K-HV kits, which have specific manuals and slightly different protocol.

For assay-specific information, relating to Kit Specificity, Control Lysates and Representative Data, please refer to the Technical Data Sheet of the kit, also available from www.perkinelmer.com

For Research Use Only. Not for use in Diagnostic Procedures.

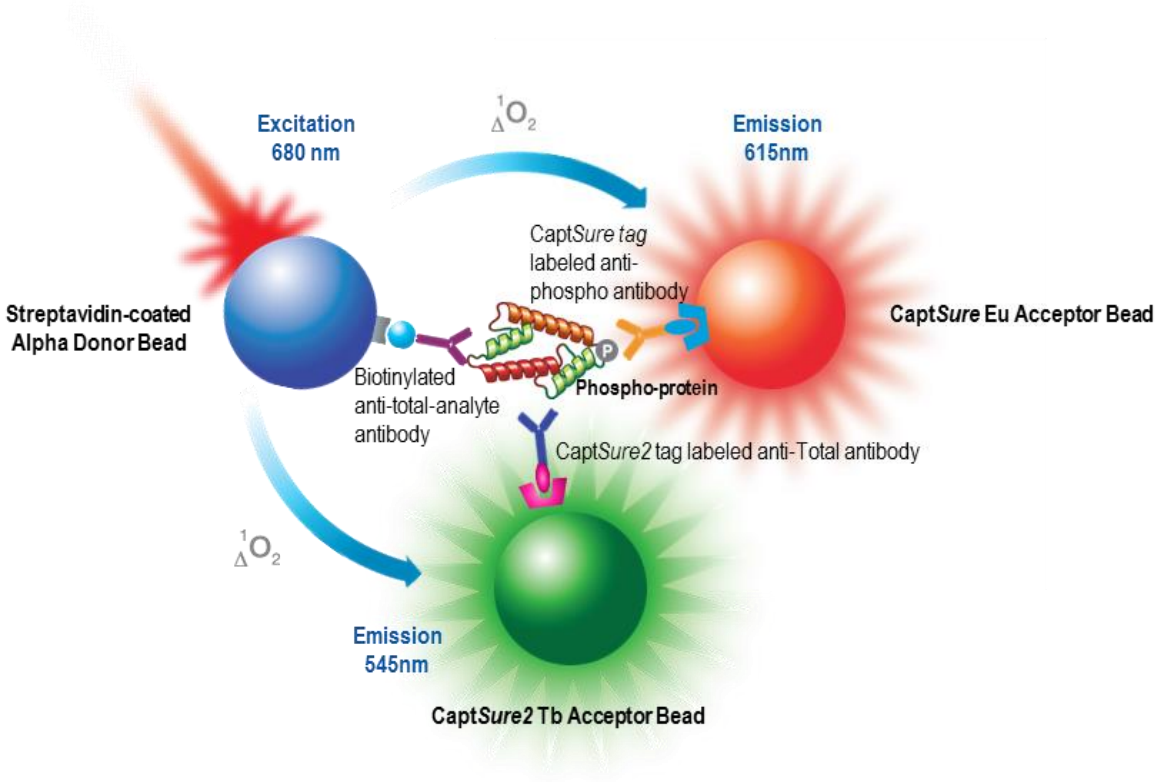
Note: See important kit disposal information on page 5 of this manual

Alpha *SureFire® Ultra™ HV* Multiplex

Assay Principle

The Alpha *SureFire Ultra HV* 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 coated with the Capt*Sure*™ antibody, which binds the Capt*Sure*-tagged anti-phospho target antibody. The Alpha 545 Acceptor bead is coated with the Capt*Sure2* antibody, which binds the Capt*Sure2* tagged anti-total target protein antibody. The Alpha Donor bead binds the biotinylated anti-total target protein antibody.



General Information on the Alpha *SureFire® Ultra™* Multiplex assays

The Alpha *SureFire Ultra* Multiplex assay kits are used to measure both the phosphorylation and total levels of endogenous signaling proteins 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 full length recombinant or endogenous proteins, and can be applied to primary cells.

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

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.

Kit-Specificity Information /Control Lysate Information / Representative Data

The assay specific Technical Data Sheet and Certificate of Analysis (COA) are available on the website. Search for Lot Specific COA's from **http://www.perkinelmer.com/COA**

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 antibody)*	1 x 0.6 mL
Reaction Buffer 2 – MPSU *(CaptSure™ tagged anti-phospho antibody)*	1 x 0.6 mL
Reaction Buffer 3 – MPSU *(CaptSure2™ tagged anti-Total antibody)*	1 x 0.6 mL
Dilution Buffer - *Ultra*	1 x 1.8 mL
Alpha 615 Capt*Sure*™ Acceptor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.045 mL
Alpha 545 Capt*Sure2*™ 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 lyophilized 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.

***** Some kits contain assay specific **Activation Buffer B - *Ultra***

Kit Content Handling Conditions

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are generic for all kits and available from **http://www.perkinelmer.com**

Lysis Buffer (5X) - *Ultra*

Lysis Buffer (5X) - *Ultra* is a proprietary mixture of buffers, detergents and generic phosphatase inhibitors (Orthovanadate, Pyrophosphate and sodium fluoride), optimized for lysis of a broad range of cells without the excessive release of nuclear DNA. It does not contain protease inhibitors. Additives can be supplemented to the Lysis Buffer as required for particular cell types and may include excipients such as protease inhibitors or extra detergents. These will need to be tested on a case-by-case basis.

All Lysis Buffers contain Triton X-100, otherwise known as p-tert-octylphenol ethoxylate, which must be disposed of as Controlled Waste in accordance with Local Regulations.

Activation Buffer

Activation Buffer precipitates at 4°C. To re-dissolve, warm to 37°C and mix before each use.

Alpha Streptavidin Donor Beads

Alpha Streptavidin Donor Beads are light-sensitive. 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. The Donor Beads should NOT be used under red/orange light as can be found in photographic work darkrooms because red light (680 nm) excites the beads. All other assay reagents can be used under normal light conditions.

Positive Control Lysate

The Positive Control lysates are prepared from various cell types, which have been cultured and prepared to optimize the activation of the intracellular pathway of interest. The Lysate is intended for use as an assay positive control only and should not be used for the absolute quantification of a particular protein or phosphorylated target. The Lysate can be further diluted with Lysis buffer (1X) and used to give an indication of the expected signal range for a given assay. See the Certificate of Analysis for the recommended dilution in the linear range of the assay.

Storage Conditions

Expiry date indicated on kit box.

Unopened kit		Store at 4°C. DO NOT freeze the kit. The Reaction Buffer contains antibodies and freeze/thaw cycles can lead to a loss of activity.
Opened kit	Lysis Buffer (5X) - *Ultra*	Store at 4°C
	Reaction Buffer 1 - *Ultra*	
	Reaction Buffer 2 - *Ultra*	
	Reaction Buffer 3 - *Ultra*	
	Dilution Buffer - *Ultra*	
	Activation Buffer - *Ultra*	Precipitates at 4°C. To re-dissolve, warm to 37°C and mix before each use. Alternatively, can be stored at room temperature with no loss in activity.
	Acceptor/Donor Beads	Store at 4°C, in the dark zip lock bag or box provided.
	Positive Control Lysate	Store at 4°C or for long term storage at -20°C.

Materials Required But Not Provided

Item	Suggested source	Catalog #	Size
Half AreaPlate-96, white Opaque assay plate (1)	PerkinElmer Inc.	6002290	2x25
Half Area AlphaPlate-96, Light Gray Opaque assay plate (2)	PerkinElmer Inc.	6002350	2x25
Half Area AlphaPlate-96, Light Gray Opaque, Sterile, TC-Treated assay plate (3)	PerkinElmer Inc.	Custom product	1x50
Half Area ViewPlate-96, White with clear bottom, Sterile, TC-Treated assay plate (4)	PerkinElmer Inc.	6005760	1x40
White adhesive seal for the bottom of microplates (5).	PerkinElmer Inc.	6005199	1X55
Spectraplate-96, Clear, sterile TC-treated plate (6)	PerkinElmer Inc.	6005650	50/box
TopSeal-A 384, clear adhesive sealing film	PerkinElmer Inc.	6050185	100/box
Envision® or Ensight™ Alpha-reader with adequate AlphaPlex filters (see table below)	PerkinElmer Inc.	-	-

(1) Plates used for the immunoassay or for the one-plate protocol (from cell seeding to immunoassay) using suspension cells; (2) Same as (1) but optimal if cross-talk needs to be reduced; (3) Plates for assays run in a 1-plate protocol (from cell seeding to immunoassay) using adherent cells; (4) Same as (3) but with the possibility to check cells by microscopy, in this case a white adhesive seal should be stuck to the bottom of the plate before plate reading; (5) This seal can be used to turn the clear bottom of microplates opaque; (6) Plates used to seed and stimulate cells before Lysis and transfer of lysate in an immunoassay plate. 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 Lysis buffer (5X) - Ultra in deionised 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 deionised water. Discard unused 1X buffer.
<u>Acceptor Mix</u> Reaction Buffer 1 - MPSU (31 parts or 31%) + Reaction Buffer 2 – MPSU (31 parts or 31%) + Reaction Buffer 3 – MPSU (31 parts or 31%) + Activation Buffer - *Ultra* (4 parts or 4%) + Alpha 615 Capt*Sure*™ Acceptor beads (2 parts or 2%) + Alpha 545 Capt*Sure2*™ Acceptor beads (2 parts or 2%) See flowchart for table	Mix equal volumes of Reaction Buffers 1, 2 and 3. Dilute Activation Buffer **25-fold** in combined Reaction Buffer 1 + Reaction buffer 2 + Reaction buffer 3. Dilute each Acceptor bead **50-fold** in combined Reaction Buffers plus Activation Buffer. <u>For example: for 324 µL of Acceptor Mix:</u> Combine 100µL Reaction buffer 1, 100µL of Reaction buffer 2 and 100µL of Reaction buffer 3, 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.
<u>Donor Mix</u>* 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 <u>For example: for 300 µL of Donor Mix, add:</u> 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 deionised 250µL water. Store at -20°C in single use aliquots and use within 3 months. Dilute as required.

Alpha *SureFire® Ultra™* Multiplex HV Phospho + Total protein Assay Protocol

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

Cell Seeding

1. Seed cells (200 µL of cells for 96 well plates, 50 µL for 384 well plates) 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 5-20 minutes for receptor agonists.

   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% BSA)

Lysate Preparation

3. To lyse cells, remove medium from wells, and add freshly prepared 1X Lysis Buffer - *Ultra* (50-100 µL for a 96 well plate, 25 µL for a 384 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 1/2AreaPlate™ for assay. Add 30 µL of Control lysates to separate wells. *We recommend testing a serial dilution of Control lysate in 1X Lysis Buffer. See the COA for recommended dilution in the linear range of assay.*

Alpha SureFire Ultra Multiplex HV Assay

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

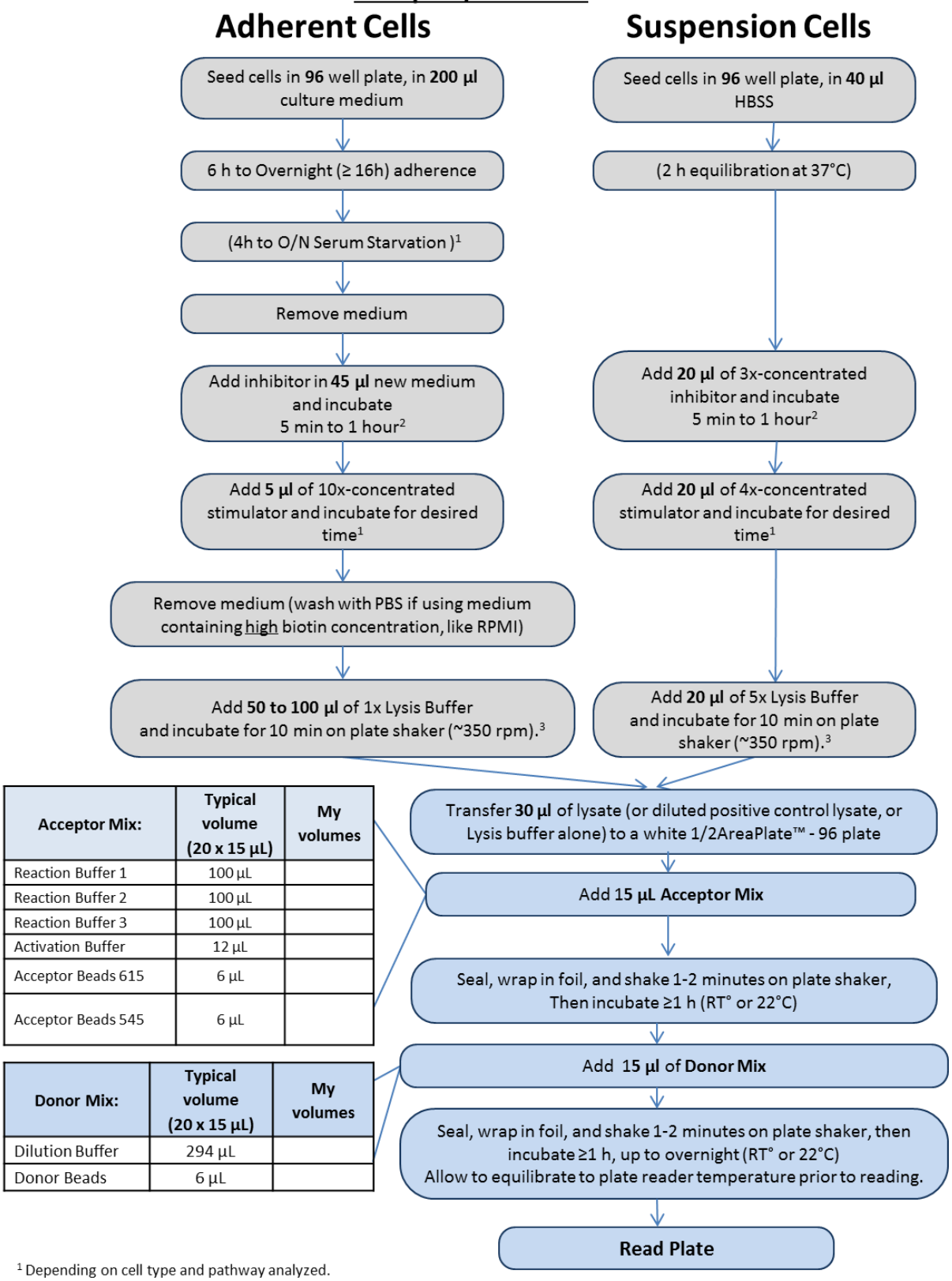
6. Add 15 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature in the dark.

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

7. Read plate on an AlphaPlex Technology-compatible plate reader, using standard AlphaPlex settings (see above).

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

Phospho / Total kits



Acceptor Mix:	Typical volume (20 x 15 µL)	My volumes
Reaction Buffer 1	100 µL	
Reaction Buffer 2	100 µL	
Reaction Buffer 3	100 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.

Alpha *SureFire® Ultra™* Multiplex Phospho + Total protein 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 4 µL of cells/well into a 384-well white opaque culture plate (eg PerkinElmer Cat # 6007680).

2. If using test agents/inhibitors, add 2 µL/well of 4X inhibitors prepared in 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% 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 in 1X Lysis Buffer. See the COA for recommended dilution in the linear range of assay.*

SureFire Ultra Assay

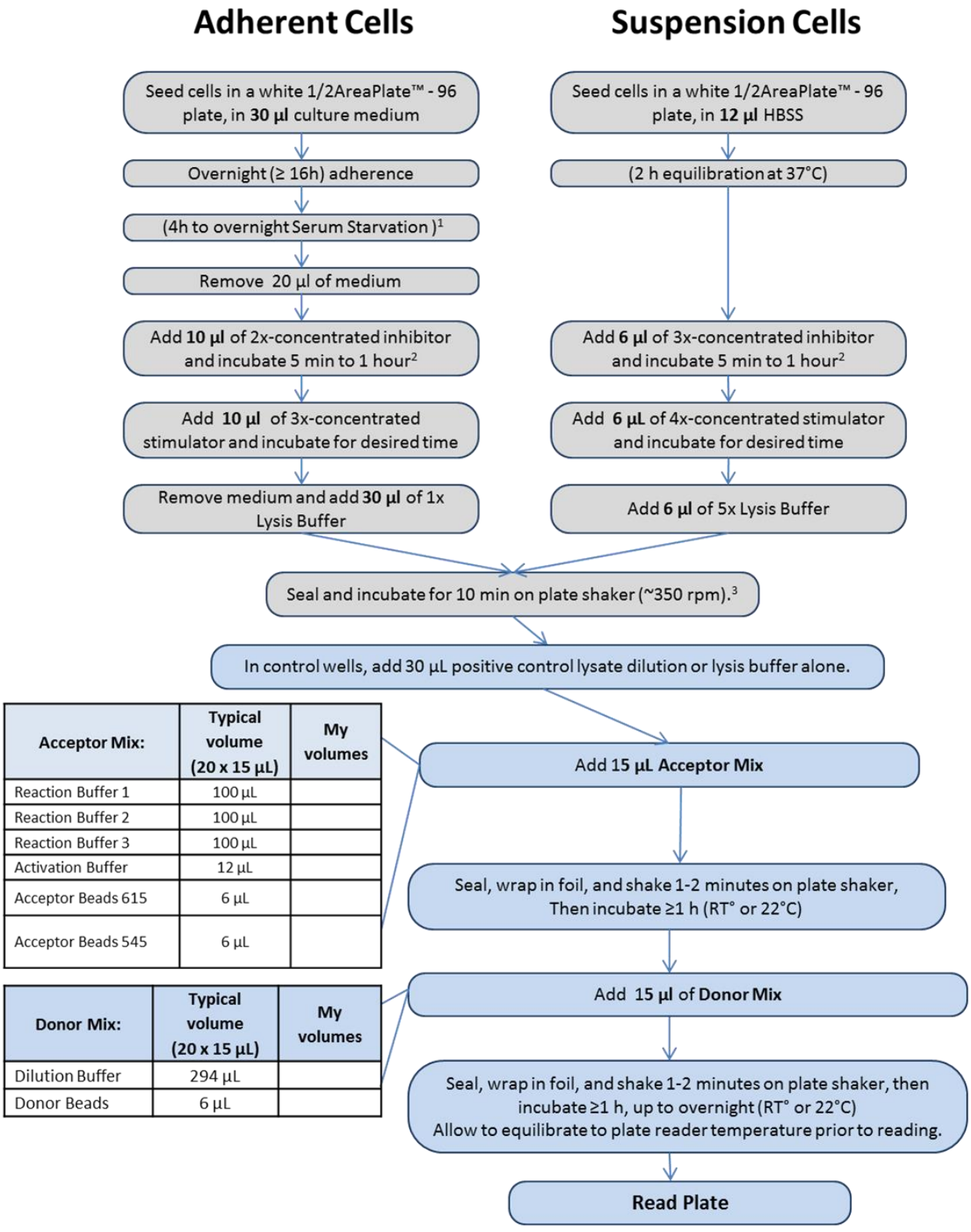
6. Add 15 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature.

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.

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

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



Acceptor Mix:	Typical volume (20 x 15 µL)	My volumes
Reaction Buffer 1	100 µL	
Reaction Buffer 2	100 µL	
Reaction Buffer 3	100 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.

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
AlphaLISA® Capt*Sure*™ 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
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) CaptSure2 Acceptor Beads -2mg/mL	PerkinElmer Inc.	MPSU-CS2B-0.06mL	60µL
	PerkinElmer Inc.	MPSU-CS2B -1.2mL	1.2mL
	PerkinElmer Inc.	MPSU-CS2B -6mL	6mL

Useful Links

For FAQ and troubleshooting, please go to: **www.perkinelmer.com/SureFireFAQ**
or the following Application Notes:
https://www.perkinelmer.com/lab-solutions/resources/docs/APP_SureFire_Multiplex_Cellular_Kinase.pdf
https://www.perkinelmer.com/lab-solutions/resources/docs/APP_Terbium_SureFire_Ultra_Multiplex.pdf

For a complete list of AlphaLISA *SureFire Ultra* and Alpha *SureFire Ultra* Multiplex kits, please go to:
www.perkinelmer.com/category/alpha-surefire-kits

For technical support please go to: **www.perkinelmer.com/ASK**

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