Alpha *SureFire® Ultra™* Multiplex

Terbium *SureFire® Ultra™* Assay Kit

For multiplexing with AlphaLISA® *SureFire® Ultra™* (ALSU) assay kits

Assay Kits Manual

Assay Points	Catalog #
500	TBSU-XXXX-X500
10 000	TBSU-XXXX-X10K
50 000	TBSU-XXXX-X50K

This Manual is a generic manual for all the kits. For target-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 3 of this manual

For an electronic version of this manual, please go to:
http://www.perkinelmer.com/category/alpha-surefire-kits

Alpha *SureFire® Ultra™* Multiplex

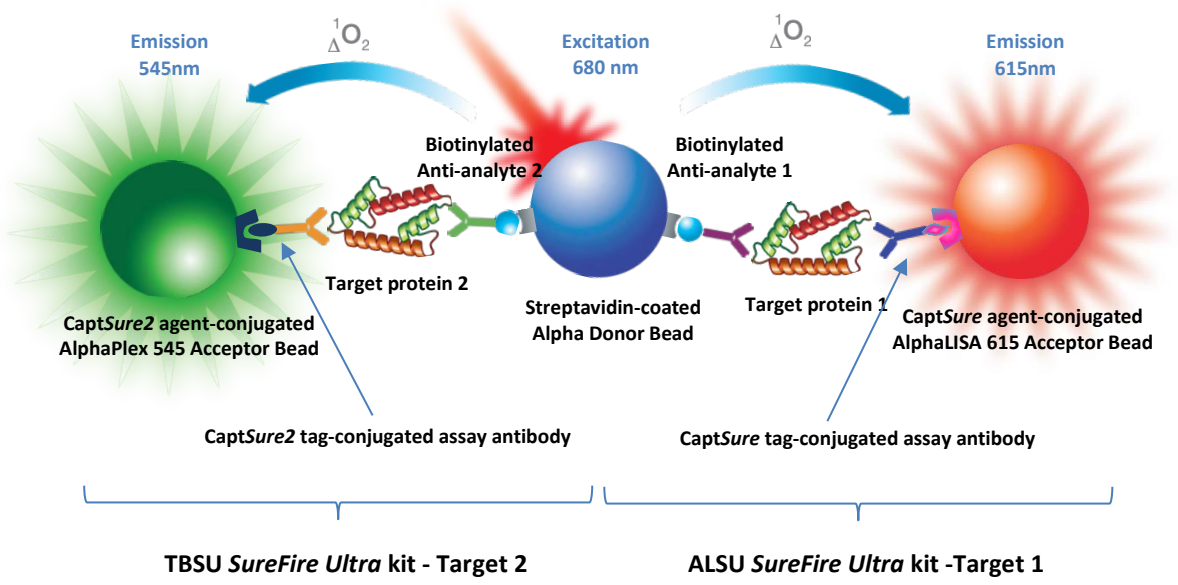
Assay Principle

The Alpha *SureFire Ultra* Multiplex kits allow the rapid, sensitive, and quantitative detection of two phosphoprotein targets in each well of an assay plate. This Multiplex measurement is achieved by the use of two types of Alpha Acceptor beads that emit at distinct wavelengths (Terbium, 545nm and Europium, 615nm).
The two distinct Alpha Acceptor beads report their binding to distinct antigens through their association with specific assay antibodies, as indicated below.

Terbium *SureFire Ultra* Assay kit for multiplexing with an AlphaLISA *SureFire Ultra* assay kit:
The Terbium *SureFire Ultra* (TBSU) assay kits contain reagents to be combined with those of an AlphaLISA *SureFire Ultra* (ALSU) assay kit, to allow multiplexed measurement of two different phosphoprotein targets. Antibodies in each TBSU or ALSU kit measure a single target. Combining two such kits allows the highly flexible selection of two phosphoprotein targets to be measured.

The terbium "Alpha 545 Acceptor Beads" in the TBSU kit are conjugated with a "Capt*Sure2*" agent, which binds a specific Capt*Sure2* tag on one of the assay antibodies. In combination with the biotinylated antibody provided, the 545 nm signal generated will report levels of the phosphoprotein of interest (Target 2). The TBSU kit is to be used in conjunction with the reagents in an ALSU kit, such that the ALSU kit will report that target of interest by Eu emission at 615nm (Target 1). This is shown diagrammatically below.
The protocol details how to combine these kits to allow multiplexed measurement of the desired targets.

Alpha *SureFire® Ultra™* Multiplex Technology



General Information on the Terbium *SureFire® Ultra™* Assay kit

The Terbium *SureFire Ultra* Assay (TBSU) kit is used to measure the phosphorylation of a target of interest plus another target of choice from the AlphaLISA *SureFire Ultra* (ALSU) list of kits. The TBSU kit must be used in conjunction with an ALSU kit, and can not be used alone to measure a single target.
Multiplexing is achieved through the use of two Alpha Acceptor beads. The first (545nm Tb) in this TBSU kit will report on the levels of the designated target of interest. The second (615nm Eu) Acceptor bead from another AlphaLISA *SureFire Ultra* kit reports on the protein designed to be measured using that kit.
The Donor beads in this kit are identical to those in a standard AlphaLISA *SureFire Ultra* kit.

Control lysates are provided in each kit to allow testing of the signal generated in the two channels (545 and 615 nm).

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

Kit-Specificity Information / Control Lysate Information / Representative Data

See Technical Data Sheet in assay kit box.

Unless otherwise indicated, the antibodies in each TBSU kit are identical to the antibodies to the same target in the corresponding ALSU kit, except for the Capt*Sure* or Capt*Sure2* tag.

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.

Note: the buffers in the *SureFire Ultra* kits have a different formulation compared to the buffers from the AlphaScreen *SureFire* kits, and buffers from the latter type of kit should not be interchanged.

Important disposal information

The Lysis Buffer (5X) – *Ultra* in this kit contains Triton X-100, otherwise known as p-tert-octylphenol ethoxylate.
p-tert-octylphenol ethoxylate must be disposed of as Controlled Waste in accordance with Local Regulations.

Kit Contents

	Kit Size		
	500 points	**10,000 points**	**50,000 points**
Reaction Buffer 3 – TBSU *(Biotinylated anti-target antibody)*	1 x 0.15 mL	1 x 2. 8 mL	1 x 14 mL
Reaction Buffer 4 – TBSU *(CaptSure2-tagged anti-target antibody)*	1 x 0.15 mL	1 x 2. 8 mL	1 x 14 mL
Alpha 545 Capt*Sure2* Acceptor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.06 mL	1 x 1.1 mL	1 x 5.5 mL
Alpha Streptavidin Donor Beads (2mg/mL in PBS plus 0.05% Proclin-300)	1 x 0.06 mL	1 x 1.1 mL	1 x 5.5 mL
Positive Control Lysate	1 lyophilized tube to be re-dissolved with 250 µL H_2O		

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

IMPORTANT: The components of this kit will be used in conjunction with the components of another standard AlphaLISA *SureFire Ultra* (ALSU) kit to provide multiplexing. On page 6 we detail how to set up the assay solutions from both kits.

Storage Conditions Upon Receipt

The kit should be placed at 4°C upon receipt. DO NOT freeze the kit buffers or beads. The Reaction buffers contain antibodies and freeze/thaw cycles can lead to a loss of activity. Alpha Donor Beads need to be stored at 4°C in the dark, and should be returned to the kit box after each use.

The Activation Buffer (use the one from the AlphaLISA *SureFire Ultra* kit) 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 -20ºC or -80ºC for long term storage.

See kit box label for expiry date

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.

Materials Required But Not Provided

Item	Suggested source	Catalog #	Size
Optiplate™-384, White Opaque assay plate (1)	PerkinElmer Inc.	6007290	50/box
AlphaPlate-384, Light gray assay plate (2)	PerkinElmer Inc.	6005350	50/box
CulturPlate-384, white, sterile, with lid, 384-well (3)	PerkinElmer Inc.	6007680	50/box
SpectraPlate-96, TC, clear, 96-well, tissue culture treated, sterile, with lids (4)	PerkinElmer Inc.	6005650	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.	-	-

(1) Plates used for the immunoassay in the 2-plates assay; (2) Same as (1) but optimal if cross-talk needs to be reduced; (3) plates for assays run in a 1-plate assay (from cell seeding to immunoassay); (4) plates used to seed and stimulate cells before Lysis and transfer of lysate in an immunoassay plate in the 2-plates assay.

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

1. Prepare sufficient Acceptor Mix from the ALSU AlphaLISA *SureFire Ultra* kit as you would for a standard single target assay, according to that kit manual.
2. The Terbium *SureFire Ultra* Assay kit reagents will be added to the pre-prepared ALSU Acceptor mix from step 1 to complete the Multiplex Acceptor Mix.
3. We recommend that you combine the Donor beads from both ALSU and TBSU kits into a single tube prior to use. Donor Mix will then be made up as indicated below.

Components	Preparation
1X Lysis Buffer Note: Lysis Buffer from ALSU kit	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.
Multiplex Acceptor Mix Prepare Acceptor Mix as normal from ALSU *SureFire Ultra* kit. To this: Add TBSU Reaction Buffer 3 (1:20) Add TBSU Reaction Buffer 4 (1:20) Vortex Add Capt*Sure2* Alpha 545 Acceptor Beads (1:50) Vortex See flowchart for table	**Important: combine the reagents in the order indicated below** For example: for 60 samples (require minimum 300 µL of Duoplex Acceptor Mix): Make up 300 µL of ALSU kit Acceptor Mix, according to that kit's protocol. To this ALSU Acceptor Mix add 15 µL of Reaction Buffer 3 and 15 µL of Reaction Buffer 4. Mix combined reagents by vortex. Then add 6µL Capt*Sure2* Alpha 545 Acceptor Beads. Mix again by vortex. The Multiplex Acceptor mix should be made up and used within 30min for best results. Excess mix should be discarded.
Donor Mix* Alpha Donor beads (1:25) (4 parts or 4%) Note: Dilution buffer from ALSU kit See flowchart for table * Prepare and use under low-light conditions.	Dilute Donor beads **25-fold** in Dilution buffer For example: for 60 samples (require minimum 300 µL of Donor Mix): Add 12 µL Donor Beads to 288 µL of Dilution Buffer. Note: Dilution Buffer from ALSU kit. 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.

Dual target measurement using combined Terbium *SureFire® Ultra™* Assay kit + AlphaLISA® *SureFire® Ultra™* target assay kit

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 10 µL of the lysate and transfer to a 384-well Optiplate™ for assay. *Add 10 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)*.

Alpha SureFire Ultra Multiplex Assay

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

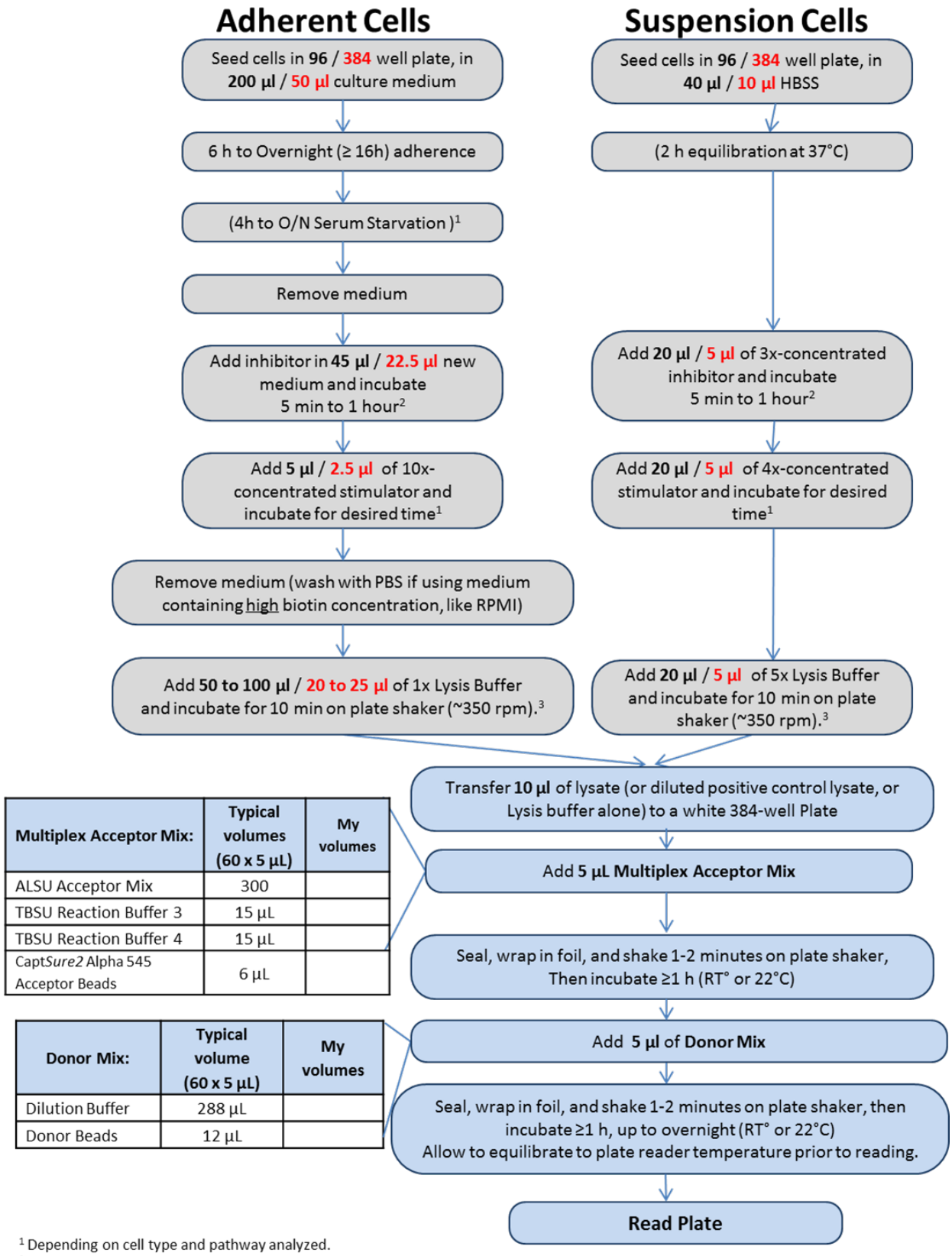
6. Add 5 µL of Donor Mix to wells under subdued light. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature in the dark.

   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™* Multiplex: 2-plates / 2-incubation assay flowchart

Dual Targets



Multiplex Acceptor Mix:	Typical volumes (60 x 5 µL)	My volumes
ALSU Acceptor Mix	300	
TBSU Reaction Buffer 3	15 µL	
TBSU Reaction Buffer 4	15 µL	
Capt*Sure2* Alpha 545 Acceptor Beads	6 µL	

Donor Mix:	Typical volume (60 x 5 µL)	My volumes
Dilution Buffer	288 µL	
Donor Beads	12 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.

Dual target measurement using combined Terbium *SureFire® Ultra™* Assay kit + AlphaLISA® *SureFire® Ultra™* target assay kit

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. 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 in 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 of 5X Lysis Buffer - *Ultra*. *Add 10 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).*

Alpha SureFire Ultra Multiplex Assay

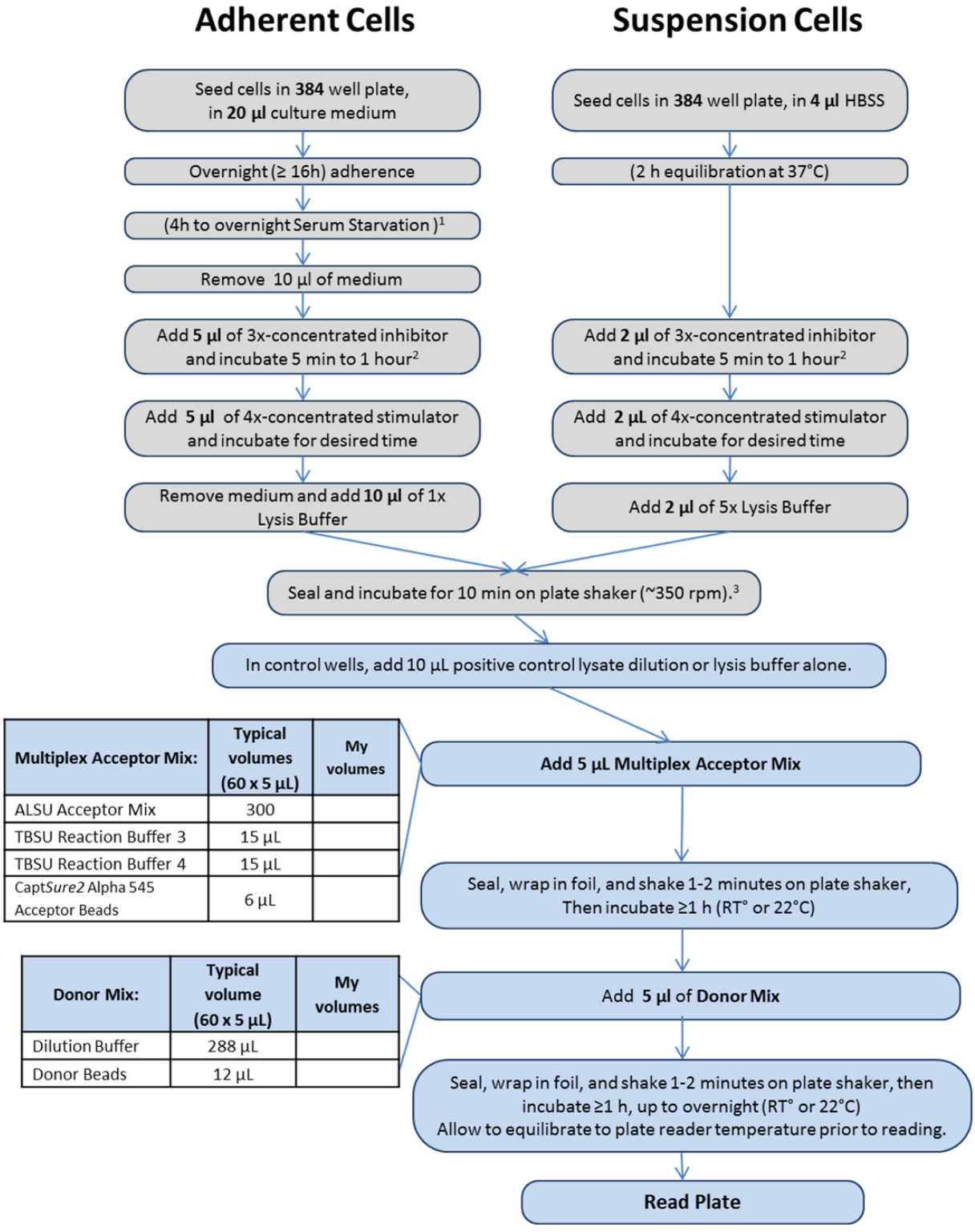
6. Add 5 µL of Multiplex Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Incubate for 1 hour at room temperature.

7. Add 5 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.

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

Alpha *SureFire® Ultra™* Multiplex: 1-plate / 2-incubation assay flowchart
Dual Targets



Multiplex Acceptor Mix:	Typical volumes (60 x 5 µL)	My volumes
ALSU Acceptor Mix	300	
TBSU Reaction Buffer 3	15 µL	
TBSU Reaction Buffer 4	15 µL	
Capt*Sure2* Alpha 545 Acceptor Beads	6 µL	

Donor Mix:	Typical volume (60 x 5 µL)	My volumes
Dilution Buffer	288 µL	
Donor Beads	12 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 kits. 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

Useful Links

For FAQ and troubleshooting, please go to:
www.perkinelmer.com 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/category/alpha-surefire-kits or **www.tgrbio.com**

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

List of Available TBSU kits

	Catalogue Numbers		
Assay	**500 point**	**10,000 point**	**50,000 point**
p-4E-BP1 (T37/46)	TBSU-P4EBP-A500	TBSU-P4EBP-A10K	TBSU-P4EBP-A50K
p-Akt1/2/3 (T308)	TBSU-PAKT-A500	TBSU-PAKT-A10K	TBSU-PAKT-A50K
p-Akt1/2/3 (S473)	TBSU-PAKT-B500	TBSU-PAKT-B10K	TBSU-PAKT-B50K
Total Cofilin	TBSU-TCOF-A500	TBSU-TCOF-A10K	TBSU-TCOF-A50K
p-CREB (S133)	TBSU-PCREB-A500	TBSU-PCREB-A10K	TBSU-PCREB-A50K
p-EGF Receptor (Y1068)	TBSU-PEGFR-A500	TBSU-PEGFR-A10K	TBSU-PEGFR-A50K
p-eIF2α (S51)	TBSU-PEIF2-B500	TBSU-PEIF2-B10K	TBSU-PEIF2-B50K
p-eIF4E (S209)	TBSU-PEIF4-A500	TBSU-PEIF4-A10K	TBSU-PEIF4-A50K
p-ERK1/2 (T202/Y204)	TBSU-PERK-A500	TBSU-PERK-A10K	TBSU-PERK-A50K
p-GSK3β (S9)	TBSU-PGS3B-A500	TBSU-PGS3B-A10K	TBSU-PGS3B-A50K
p-IGF-1 Receptor β (Y1135/1136)	TBSU-PIGFR-B500	TBSU-PIGFR-B10K	TBSU-PIGFR-B50K
p-IKKα (S176/180)	TBSU-PIKKA-A500	TBSU-PIKKA-A10K	TBSU-PIKKA-A50K
p-Insulin Receptor β (Y1150/1151)	TBSU-PINR-A500	TBSU-PINR-A10K	TBSU-PINR-A50K
p-JNK1/2/3 (T183/Y185)	TBSU-PJNK-A500	TBSU-PJNK-A10K	TBSU-PJNK-A50K
p-MEK1 (S218/222)	TBSU-PMEK1-A500	TBSU-PMEK1-A10K	TBSU-PMEK1-A50K
p-mTOR (S2448)	TBSU-PMTOR-A500	TBSU-PMTOR-A10K	TBSU-PMTOR-A50K
p-NF-κB p65 (S536)	TBSU-PNFKB-A500	TBSU-PNFKB-A10K	TBSU-PNFKB-A50K
p-p38 MAPK (T180/Y182)	TBSU-PP38-B500	TBSU-PP38-B10K	TBSU-PP38-B50K
p-p70 S6K (T389)	TBSU-PP70-A500	TBSU-PP70-A10K	TBSU-PP70-A50K
p-Ribosomal Protein S6 (S240/244)	TBSU-PS6R-A500	TBSU-PS6R-A10K	TBSU-PS6R-A50K
p-SLP-76 (S376)	TBSU-PSLP-A500	TBSU-PSLP-A10K	TBSU-PSLP-A50K
p-SMAD1 (S463/465)	TBSU-PSM1-A500	TBSU-PSM1-A10K	TBSU-PSM1-A50K
p-SMAD3 (S423/425)	TBSU-PSM3-A500	TBSU-PSM3-A10K	TBSU-PSM3-A50K
p-STAT1 (S727)	TBSU-PST1-B500	TBSU-PST1-B10K	TBSU-PST1-B50K
p-STAT1 (Y701)	TBSU-PST1-A500	TBSU-PST1-A10K	TBSU-PST1-A50K
p-STAT3 (Y705)	TBSU-PST3-A500	TBSU-PST3-A10K	TBSU-PST3-A50K
p-STAT4 (Y693)	TBSU-PST4-A500	TBSU-PST4-A10K	TBSU-PST4-A50K
p-STAT5 (Y694/699)	TBSU-PST5-B500	TBSU-PST5-B10K	TBSU-PST5-B50K
p-STAT6 (Y641)	TBSU-PST6-A500	TBSU-PST6-A10K	TBSU-PST6-A50K
p-SYK (Y525/526)	TBSU-PSYK-A500	TBSU-PSYK-A10K	TBSU-PSYK-A50K
p-VEGF Receptor 2 (Y1175)	TBSU-PVGFR-A500	TBSU-PVGFR-A10K	TBSU-PVGFR-A50K