AlphaLISA® *SureFire® Ultra™*

Assay Kits Manual

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

This Manual is a generic manual for all the AlphaLISA® *SureFire® Ultra™* 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.

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

AlphaLISA® *SureFire® Ultra™*

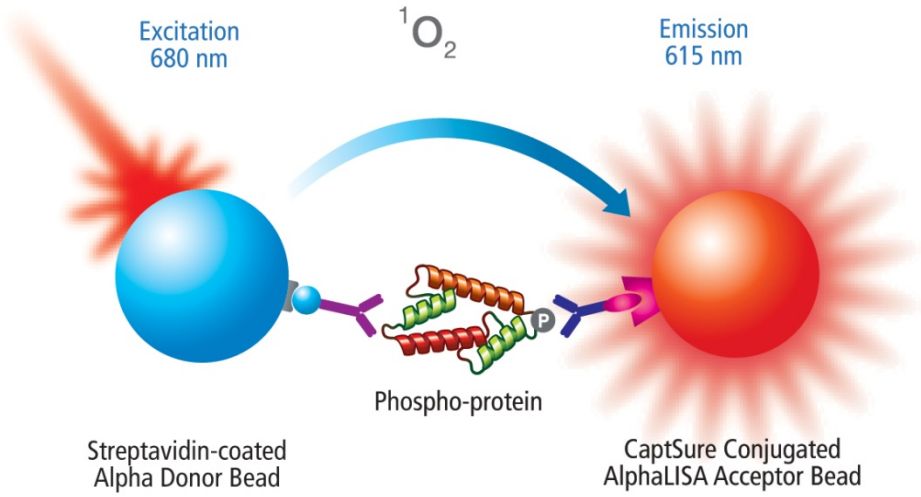
Assay Principle

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 Capt*Sure*™ agent that immobilizes the other assay antibody which is labeled with a Capt*Sure*™ 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™* assay

The AlphaLISA® *SureFire®* *Ultra™* assays are used to measure a phosphorylated or total protein in cellular lysates. The assays are 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 assays measure the activation of recombinant or endogenous proteins, and can be applied to primary cells.

The kits are formulated to provide improved signal:noise 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 included in assay kit box.
Technical Data Sheets are also available as pdf file from
http://www.perkinelmer.com/category/alpha-surefire-kits.

Kit Contents

	Kit Size		
	500 points	**10,000 points**	**50,000 points**
Lysis Buffer (5X) - *Ultra*	1 x 12 mL	4 x 60 mL	3 x 400 mL
Activation Buffer - *Ultra*	1 x 0.8 mL	1 x 10 mL	1 x 50 mL
Reaction Buffer 1 - *Ultra*	1 x 1.5 mL	1 x 28 mL	1 x 140 mL
Reaction Buffer 2 - *Ultra*	1 x 1.5 mL	1 x 28 mL	1 x 140 mL
Dilution Buffer - *Ultra*	1 x 3 mL	1 x 60 mL	1 x 300 mL
AlphaLISA® Capt*Sure*™ 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 X Lyophilized tube (to be re-constituted with 250 µL H_2O)		

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

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

All AlphaLISA® *SureFire® Ultra™* kits contain the same formulations of **Lysis Buffer (5X) - *Ultra***, **Dilution Buffer - *Ultra***, **Acceptor** and **Donor Beads** and <u>are</u> interchangeable between kits. The **Activation Buffer** is also interchangeable, with the <u>exception</u> of the GAPDH Total and the p-STAT4 (Tyr693) assay kits which specifically require Activation Buffer –B and –C respectively. The **Reaction Buffers** contain assay-specific antibodies and the **Lysates** are assay specific and <u>are not</u> interchangeable.

The AlphaLISA® *SureFire® Ultra™* kit components (buffers, beads and lysates) are different formulations to the AlphaScreen® *SureFire®* kits and <u>are not</u> interchangeable.

Storage Conditions (See kit box label for expiry date)

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	Lysis Buffer (5X) - *Ultra*	Store at 4°C
	Reaction Buffer 1 - *Ultra*	
	Reaction Buffer 2 - *Ultra*	
	Dilution Buffer - *Ultra*	
	Acceptor Beads	
	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.
	Donor Beads	Store at 4°C in the dark, and should be returned to the kit box after use.
	Positive Control Lysate	Store at 4°C or for long term storage at -80°C

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 Opaque assay plate [(2)]	PerkinElmer Inc.	6005350	50/box
CulturPlate-384, White Opaque, Sterile, TC-Treated [(3)]	PerkinElmer Inc.	6007680	50/box
ViewPlate-384, White with clear bottom, Sterile, TC-Treated [(4)]	PerkinElmer Inc.	6007480	40/box
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	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

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 to 8 mL MilliQ water. Excess 1X buffer should be discarded.
Acceptor Mix (Reaction buffer 1 + Reaction buffer 2 + Activation Buffer + AlphaLISA® Capt*Sure*™Acceptor beads)	Dilute Activation Buffer **25-fold** in combined Reaction Buffer 1 and Reaction buffer 2. Dilute Acceptor beads **50-fold** in combined Reaction Buffers. 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. The Acceptor mix should be made up and used within 30 minutes for best results. Excess mix should be discarded.
Donor Mix (Dilution buffer + Alpha Streptavidin Donor beads)	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 30 minutes for best results. Prepare and use under low light conditions. 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

The Alpha Streptavidin 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. The Donor beads should NOT be used under red/orange light as can be found in photographic work darkrooms, as red light (680nm) excites the beads.

AlphaLISA® *SureFire® Ultra™* Assay Protocols

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 replace with 45 µL of antagonists/inhibitors prepared 1X in serum-free media (22.5 µL for 384-well plates).
   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).

3. Return cells to incubator at 37°C for 5 min to 2 hours.
   Note: 1 hour is often sufficient for signal transduction inhibitors, and 5 - 20 minutes for receptor antagonists. For short incubation times, the plate can stay at room temperature.

4. Stimulate the cells by the addition of 5 µL of 10X agonist prepared in serum-free media (2.5 µL for 384-well plates). *If not testing antagonists, directly add 50 µL of 1X agonists prepared in serum-free media (25 µL for 384-well plates).*
   Note: Optimal agonist stimulation time is often between 5 and 20 minutes.

Lysate Preparation

5. To lyse cells, remove medium from wells, and add freshly prepared 1X Lysis Buffer (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.

6. 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).*

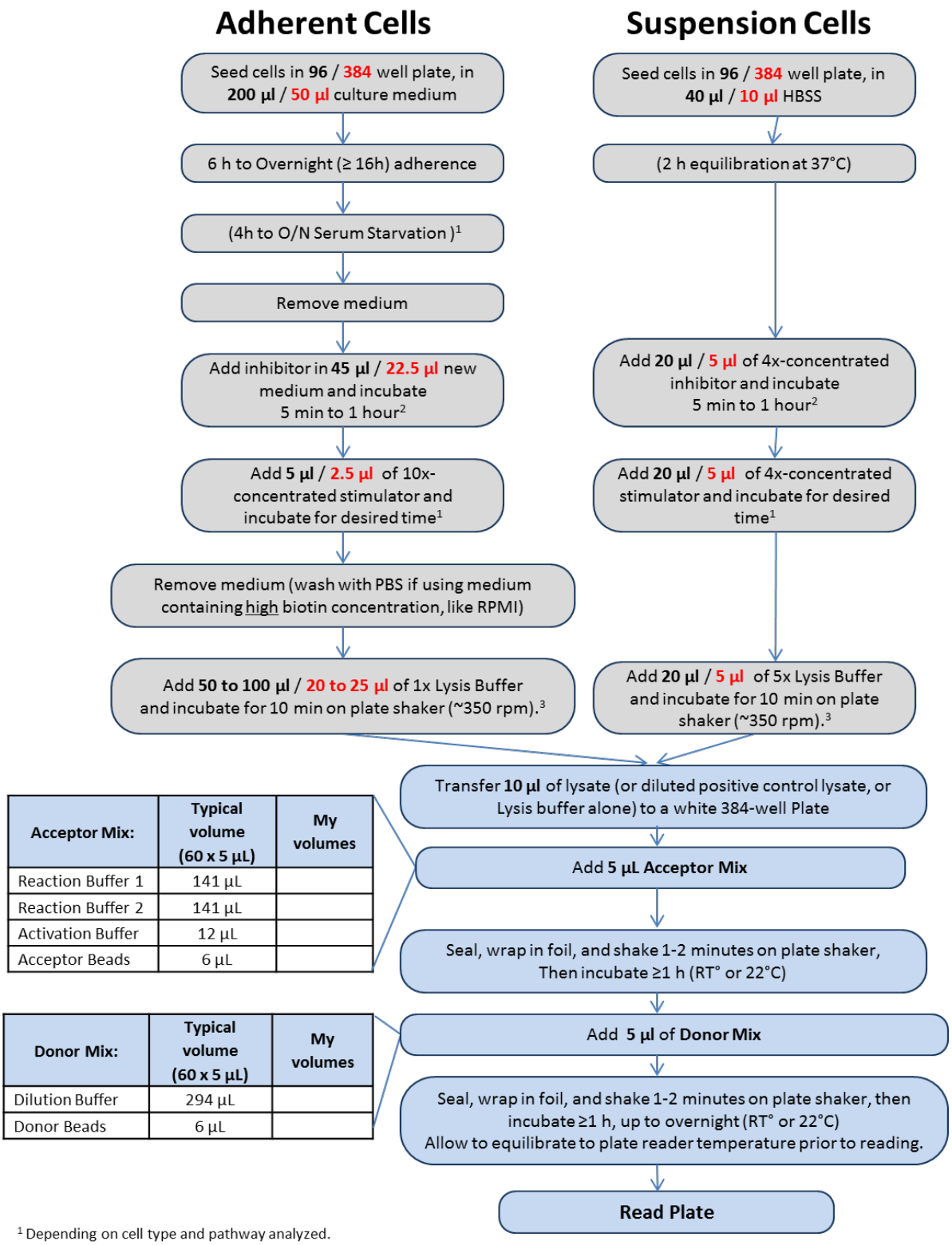
SureFire Ultra Assay

7. Add 5 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.

8. 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.

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

AlphaLISA® *SureFire® Ultra™*: 2-plates / 2-incubation assay flowchart



Acceptor Mix:	Typical volume (60 x 5 µL)	My volumes
Reaction Buffer 1	141 µL	
Reaction Buffer 2	141 µL	
Activation Buffer	12 µL	
Acceptor Beads	6 µL	

Donor Mix:	Typical volume (60 x 5 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™* Assay Protocols

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 (e.g. PerkinElmer Cat # 6007680).

   Note: As engaging less cells per well can result in increased signal to background ratios, it is important to optimize this factor.

Cell Treatment

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% protease-free BSA).

3. Return cells to incubator at 37°C for 5 min to 2 hours.

   Note: 1 hour is often sufficient for signal transduction inhibitors, and 5- 20 minutes for receptor antagonists. For short incubation times, the plate can stay at room temperature.

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*).

   Note: Optimal agonist stimulation time is often between 5 - 20 minutes.

Lysate Preparation

5. To lyse the cells, add 2 µL/well of 5X Lysis Buffer. *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).*

SureFire Ultra Assay

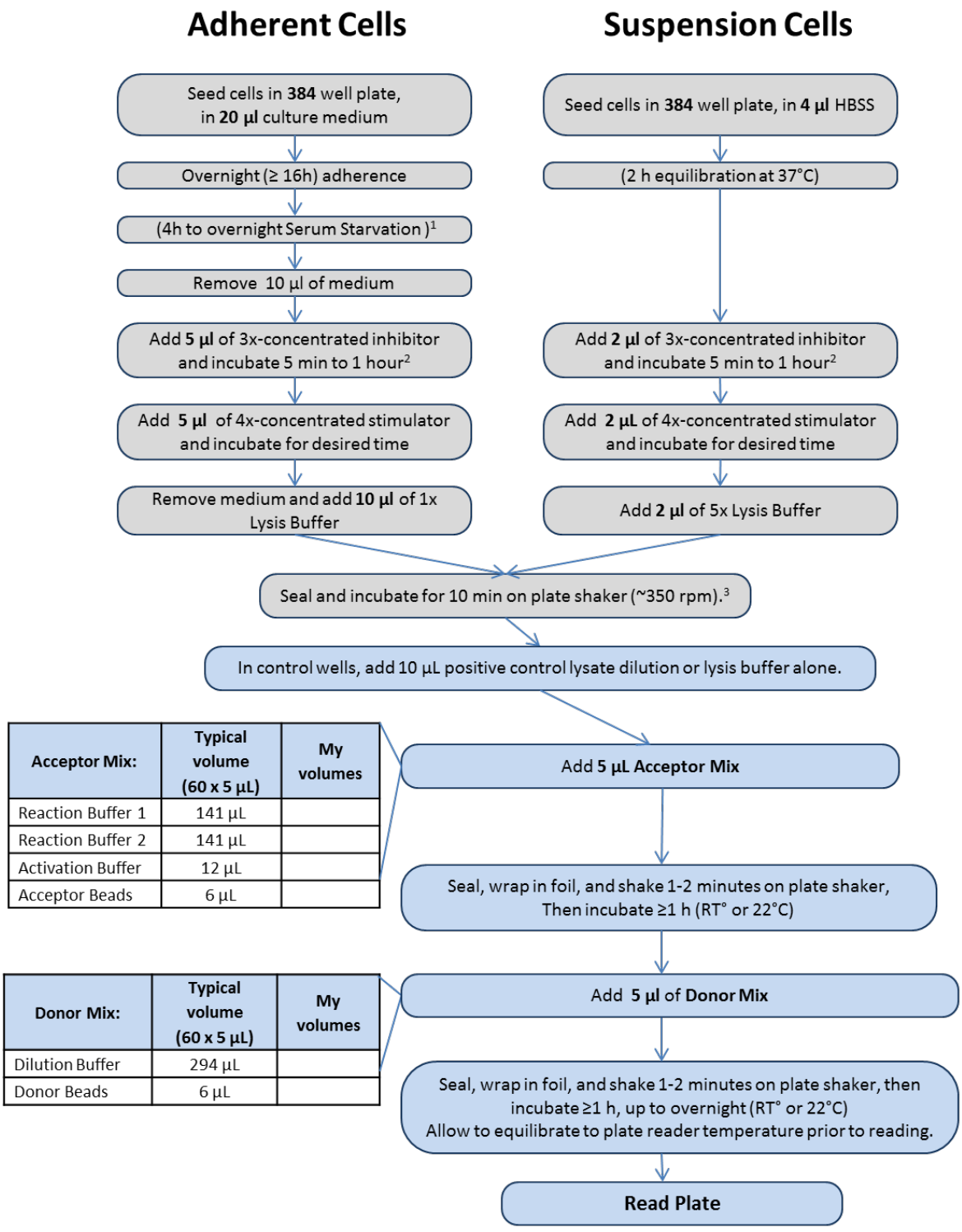
6. Add 5 µL of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film, and cover plate with foil. Incubate for 1 hour at room temperature.

7. Add 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.

   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 AlphaLISA settings.

AlphaLISA® *SureFire® Ultra*™: 1-plate / 2-incubation assay flowchart



Acceptor Mix:	Typical volume (60 x 5 µL)	My volumes
Reaction Buffer 1	141 µL	
Reaction Buffer 2	141 µL	
Activation Buffer	12 µL	
Acceptor Beads	6 µL	

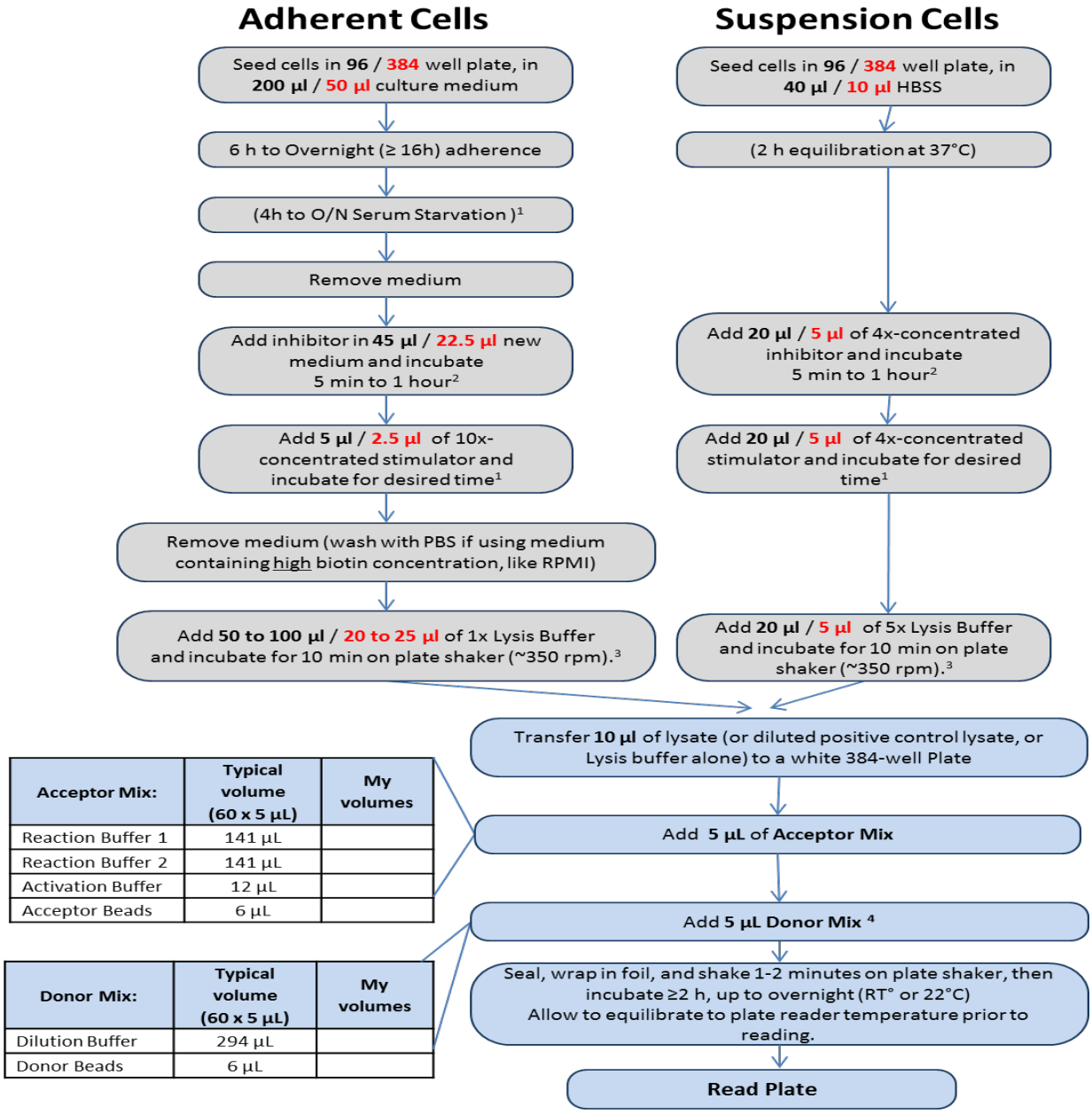
Donor Mix:	Typical volume (60 x 5 µL)	My volumes
Dilution Buffer	294 µL	
Donor Beads	6 µL	

[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.

Note:

The assay can also be carried out as a single 2 hour incubation after separate additions of Acceptor Mix then Donor Mix, as indicated below in the flow chart. This can be applied to either 1-plate or 2- plate assays. Shown below is the 2-plate protocol in this format. This assay format may result in a slightly reduced sensitivity. Do NOT premix the Acceptor and Donor mixes before adding to the sample lysates as this is expected to result in a significant sensitivity loss.

AlphaLISA® *SureFire® Ultra™*: 2-plates / 1-incubation assay flowchart



Acceptor Mix:	Typical volume (60 x 5 µL)	My volumes
Reaction Buffer 1	141 µL	
Reaction Buffer 2	141 µL	
Activation Buffer	12 µL	
Acceptor Beads	6 µL	

Donor Mix:	Typical volume (60 x 5 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.
[4] We recommend adding Acceptor Mix and Donor Mix in 2 sequential steps to maximize assay performance.

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
Activation Buffer B – *Ultra* (used in GAPDH Total assay kits)	PerkinElmer Inc.	ALSU-ABB-10mL	10mL
	PerkinElmer Inc.	ALSU-ABB-100mL	100mL
Activation Buffer C – *Ultra* (used in p-STAT4(Tyr693) assay kits)	PerkinElmer Inc.	ALSU-ABC-10mL	10mL
	PerkinElmer Inc.	ALSU-ABC-100mL	100mL
Dilution Buffer - *Ultra*	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® 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

Useful Links

For FAQ and troubleshooting, please go to:
www.perkinelmer.com/SureFireFAQ
For a complete list of AlphaLISA® *SureFire® Ultra*™, please go to:
www.perkinelmer.com/category/alpha-surefire-kits or **www.tgrbio.com**
For technical support please go to:
www.perkinelmer.com/ASK