MANUAL

AlphaLISA™ *SureFire®* *Ultra™ HV* KRAS Total

Part Number	ALSU-TKRAS-A-HV
Assay Points	100 (96 well format)

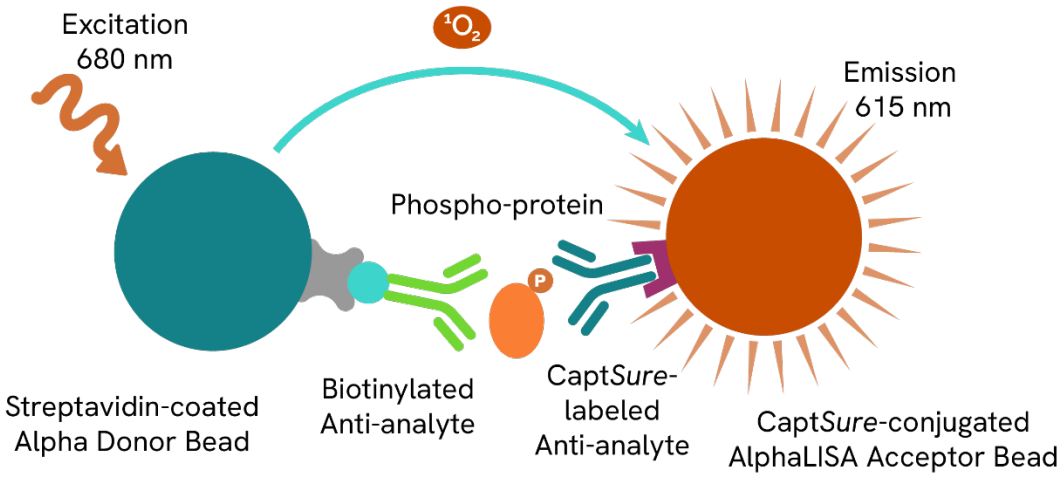
For assay-specific information, relating to Kit Specificity, Control Lysates and Representative Data, please refer to the Technical Data Sheet of the kit, available from www.revvity.com.

Note: For kit handling and disposal information see page 3 & 4 of this manual

ASSAY PRINCIPLE

The AlphaLISA™ *SureFire*® *Ultra*™ *HV* assay kits enable the rapid and sensitive detection of phosphorylated or total cellular proteins. The kit utilizes bead-based Alpha Technology. The Acceptor Bead is coated with a proprietary Capt*Sure*™ agent to specifically immobilize the assay specific antibody which is labeled with a Capt*Sure*™ tag. The Donor Bead is coated with streptavidin to capture the biotinylated antibody. In the presence of a target protein, the two antibodies bring the Donor and Acceptor Beads in close proximity. This enables the generation of an Alpha signal upon illumination of Donor Beads by an Alpha-enabled plate reader such as the EnVision™, EnVision Nexus™, Enspire™ and EnSight™ Multimode Plate Readers. The amount of light emission is directly proportional to the amount of target protein present in the sample.

SureFire assays have a simple and homogeneous no-wash assay format which make them highly amenable to automation and far less laborious than other techniques such as Western blotting or conventional ELISA. The workflow attributes and high performance characteristics (i.e. sensitivity, dynamic range and reproducibility) of our extensive *SureFire* assay offering are ideally suited to both high throughput screening in pharma drug discovery programs as well as lower throughput academic research applications.



MAIN FEATURES

The AlphaLISA™ *SureFire® Ultra™ HV* assays are used to measure a phosphorylated or total protein in cellular extracts. The assays are 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 upstream events and also for targeted protein degradation approaches such as PROTAC. The assays measure changes in levels of cellular proteins in endogenous or engineered systems and can be applied to all types of cellular extracts, including cultured primary/non-primary cell lines and tissue lysates. The kits are very sensitive and formulated to provide improved signal:noise assay windows and to perform without interference in the presence of extraneous antibodies

The AlphaLISA™ *SureFire® Ultra™ HV* assays are carried out using a higher volume of reagents than the standard AlphaLISA™ *SureFire® Ultra™* assays, and in 96 well plate format rather than 384 well format. These kits are useful for those laboratories where handling of the larger volumes is more appropriate for the laboratory equipment.

The kits are formulated to provide improved signal:noise assay windows, and to perform without interference in the presence of extraneous antibodies.

KIT-SPECIFICITY / CONTROL LYSATE / REPRESENTATIVE DATA INFORMATION

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

KIT CONTENTS

	100 points
Lysis Buffer (5X)	1 x 12 mL
Activation Buffer	1 x 0.3 mL
10X Reaction Buffer 1 – ALSU	1 x 0.09 mL
10X Reaction Buffer 2 - ALSU	1 x 0.09 mL
Dilution Buffer	1 x 3.6 mL
AlphaLISA™ Capt*Sure*™ Acceptor Beads (2mg/mL in PBS plus 0.03% Proclin-300)	1 x 0.045 mL
Alpha Streptavidin Donor Beads (2mg/mL in PBS plus 0.03% Proclin-300)	1 x 0.045 mL
Positive Control Lysate	1 X Lyophilized tube

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

STORAGE AND HANDLING CONDITIONS

Expiry date indicated on kit box.

Upon receipt		Remove 10X Reaction Buffers 1 and 2 from kit. **Store at -20°C** (formulation contains glycerol and will not freeze)
Opened kit	Lysis Buffer (5X) Dilution Buffer	Store at 4°C
	Activation Buffer	Precipitates at 4°C. To re-dissolve, warm to 37°C and mix before each use. Alternatively, once re-dissolved 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. Vortex vigorously for 5-10 seconds, then give a quick spin (e.g 500rpm for 1 second) to ensure bead suspension is at the bottom of the tube/bottle.
	Positive Control Lysate	Store at -20°C.

MATERIALS REQUIRED BUT NOT PROVIDED

Item	Suggested source	Part #	Size
Half AreaPlate-96, white Opaque assay plate [1]	Revvity Inc.	6002290	2X25
Half Area AlphaPlate-96, Light Gray Opaque assay plate [2]	Revvity Inc.	6002350	2X25
Half Area AlphaPlate-96, Light Gray Opaque, Sterile, TC-Treated assay plate [3]	Revvity Inc.	Custom product	1X50
Half Area ViewPlate-96, White with clear bottom, Sterile, TC-Treated assay plate [4]	Revvity Inc.	6005760	1X40
White adhesive seal for the bottom of microplates [5]	Revvity Inc.	6005199	1X55
Spectraplate-96, Clear, Sterile, TC-treated, plate [6]	Revvity Inc.	6005650	50/box
TopSeal-A PLUS, clear adhesive sealing film	Revvity Inc.	6050185	100/box
Envision™or Ensight™ Alpha-reader	Revvity 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) when using adherent cells, currently only available as a custom-made product; (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 clear bottom 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.revvity.com.

KIT CONTENT INFORMATION

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 **www.revvity.com**

Lysis Buffer (5X)
Lysis Buffer (5X) 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.

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.

Lysis Buffer color may vary from clear-yellow-green. The visual appearance has no impact on performance.

10X Reaction Buffer 1 and 10X Reaction Buffer 2
Dilute 10-fold in Dilution Buffer 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 must 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 COA for the recommended dilution in the linear range of the assay.

BUFFER PREPARATION AND SUBSEQUENT STORAGE CONDITIONS

Prepare volumes as needed and discard excess

1X Lysis Buffer	Dilute 5X Lysis Buffer **5-fold** in deionized water. For example: For 10 mL of 1X Lysis Buffer: Add 2 mL of 5X Lysis Buffer to 8 mL deionized water.
1X Reaction Buffer 1 +Dilution Buffer **1X Reaction Buffer 2** +Dilution Buffer	Dilute 10X Reaction Buffer **10-fold** in Dilution Buffer. For example: For 145 mL of 1X Reaction Buffer 1 and 2: Add 15 µL of 10X Reaction Buffer 1 to 135 µL of Dilution Buffer. Add 15 µL of 10X Reaction Buffer 2 to 135 µL of Dilution Buffer.
Acceptor Mix 1X Reaction Buffer 1 + 1X Reaction Buffer 2 + Activation Buffer + Acceptor Beads	Combine 1X Reaction Buffer 1 and 1X Reaction Buffer 2 in **equal parts**. Dilute Activation Buffer **25-fold** in final volume. Dilute Acceptor Beads **50-fold** in final volume. For example: For 300 µL of Acceptor Mix: Combine 141 µL of 1X Reaction Buffer 1 and 141 µL of 1X Reaction Buffer 2. Then add 12 µL of Activation Buffer. Then add 6 µL of Acceptor Beads. The Acceptor Mix should be made up and used within 30 minutes for best results.
Donor Mix Dilution Buffer + 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**
Positive Control Lysate	Reconstitute with 250µL deionized water. Reconstituted lysate can be stored effectively at -80°C in single use aliquots but should be tested on a case by case basis. Dilute as required with 1X Lysis Buffer.

AlphaLISA™ *SureFire® Ultra™* HV ASSAY PROTOCOL

A. 2-Plate Assay - protocol for adherent cells

Cell Seeding

1. Seed cells (200 µL of cells per well) in 96 well tissue culture treated 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.
   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. *If not testing antagonists, directly add 50 µL of* 1X agonists prepared in serum-free media.
   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 per well). Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.

6. Take 30 µL of the lysate and transfer to a 96-well 1/2AreaPlate™ for assay. Add 30 µL of Control lysate/ 1X Lysis Buffer 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.*

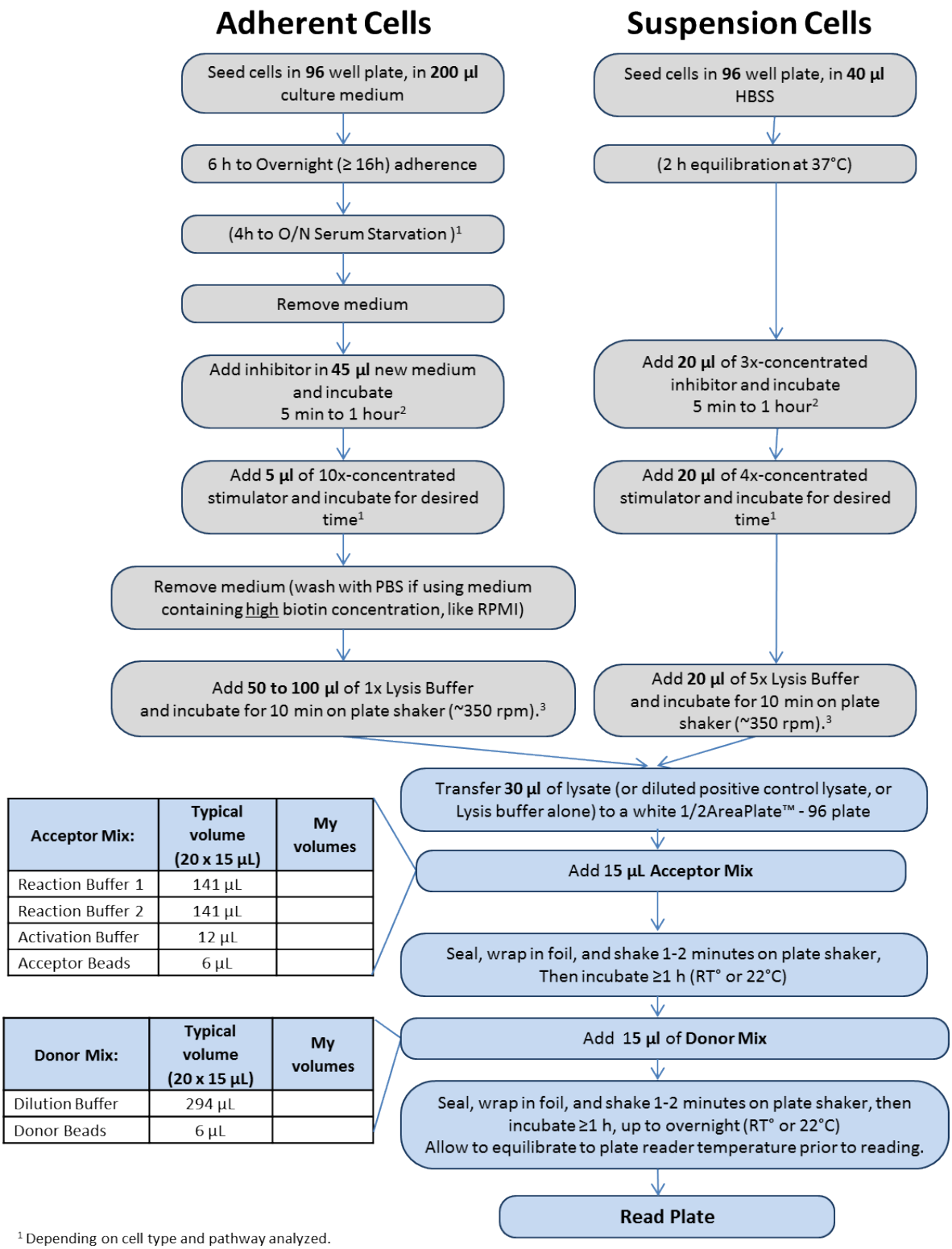
SureFire Ultra Assay

7. 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.

8. 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.

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

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



Acceptor Mix:	Typical volume (20 x 15 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 (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.

B. 1 Plate Assay - protocol for suspension cells, or for adherent cells with no transfer.

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 (e.g. 1/2Area ViewPlate-96).

   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 6 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. 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. *(If not testing antagonists, directly add 12 µL of* 2X agonists prepared in serum-free media).

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

Lysate Preparation

5. To lyse the cells, add 6 µL/well of 5X Lysis Buffer. Add 30 µL of Control lysates/ 1X Lysis Buffer 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.*

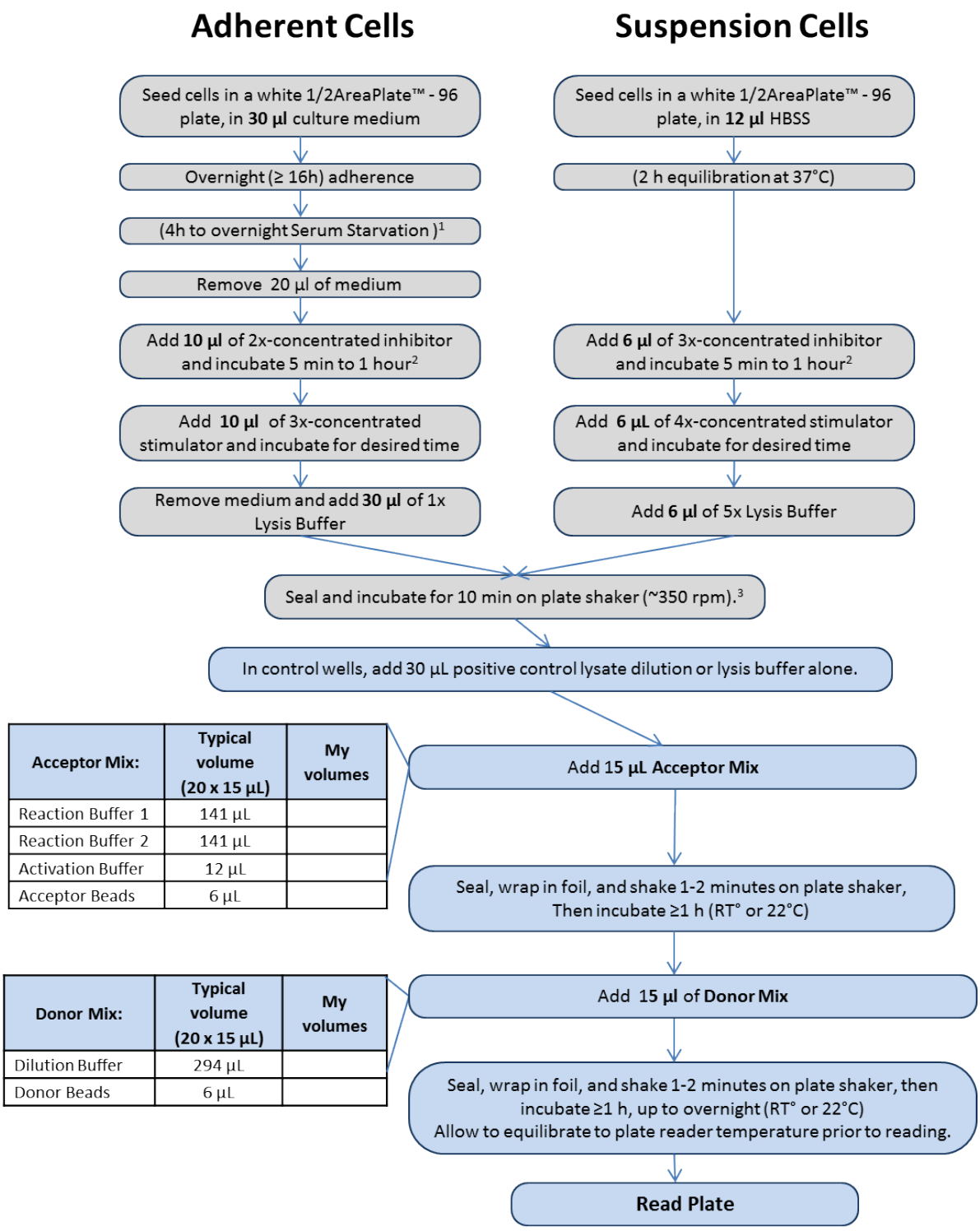
SureFire Ultra Assay

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.

   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™ HV*: 1-plate / 2-incubations assay flowchart



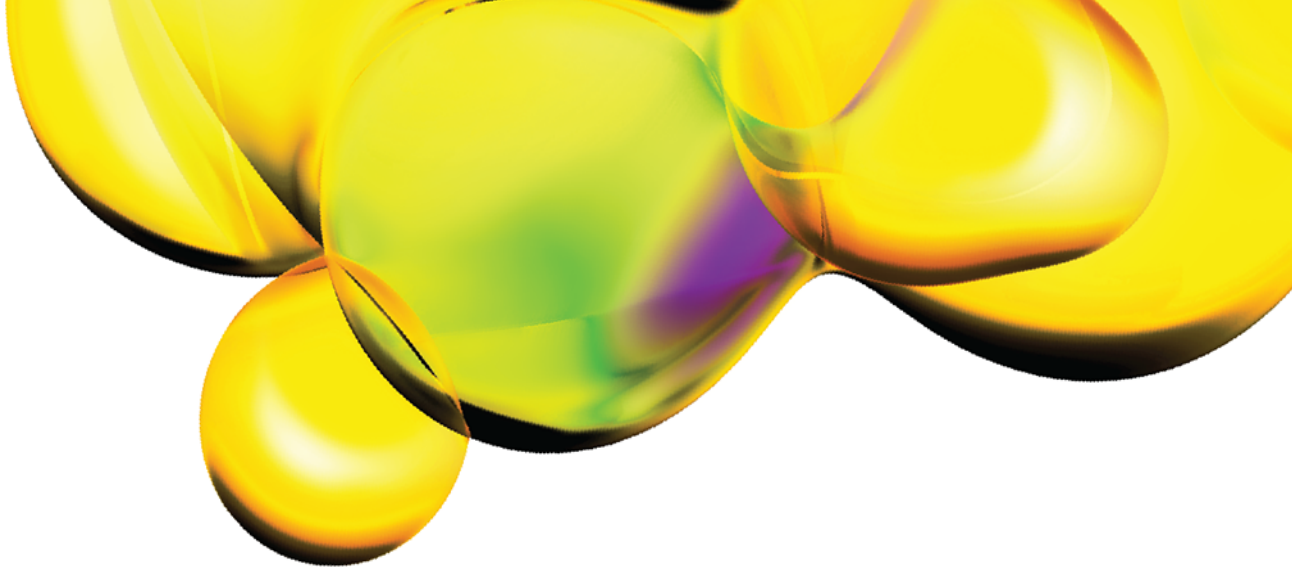
Acceptor Mix:	Typical volume (20 x 15 µL)	My volumes
Reaction Buffer 1	141 µL	
Reaction Buffer 2	141 µL	
Activation Buffer	12 µL	
Acceptor Beads	6 µL	

Donor Mix:	Typical volume (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.

If using the standard protocol, sufficient amount 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 part numbers:

Item	Suggested source	Part numbers	Size
Lysis Buffer (5X)	Revvity Inc.	ALSU-LB-10mL	10 mL
	Revvity Inc.	ALSU-LB-100mL	100 mL
	Revvity Inc.	ALSU-LBC-100mL	100 mL
Activation	Revvity Inc.	ALSU-AB-10mL	10 mL
	Revvity Inc.	ALSU-AB-100mL	100 mL
	Revvity Inc.	ALSU-ABC-100mL	100 mL
Dilution Buffer	Revvity Inc.	ALSU-DB-10mL	10 mL
	Revvity Inc.	ALSU-DB-100mL	100 mL
Alpha Streptavidin Donor Beads -2 mg/mL	Revvity Inc.	ALSU-ASDB-0.06mL	60 µL
	Revvity Inc.	ALSU-ASDB-1.2mL	1.2 mL
	Revvity Inc.	ALSU-ASDB-6mL	6 mL
AlphaLISA™ Capt*Sure*™ Acceptor Beads - 2 mg/mL	Revvity Inc.	ALSU-ACAB-0.06mL	60 µL
	Revvity Inc.	ALSU-ACAB-1.2mL	1.2 mL
	Revvity Inc.	ALSU-ACAB-6mL	6 mL



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