MANUAL

AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV

Phospho (Eu) + Total (Tb) Target

Part Number	MSBF-PTXXXX-X-HV
Assay Points	100 (96 well format)

This is a generic manual for the AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV Phospho + Total kits.

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.revvity.com.](http://www.revvity.com)

Note: For kit handling and disposal information see pages 4-6 of this manual

For research only. Not for use in diagnostic procedures

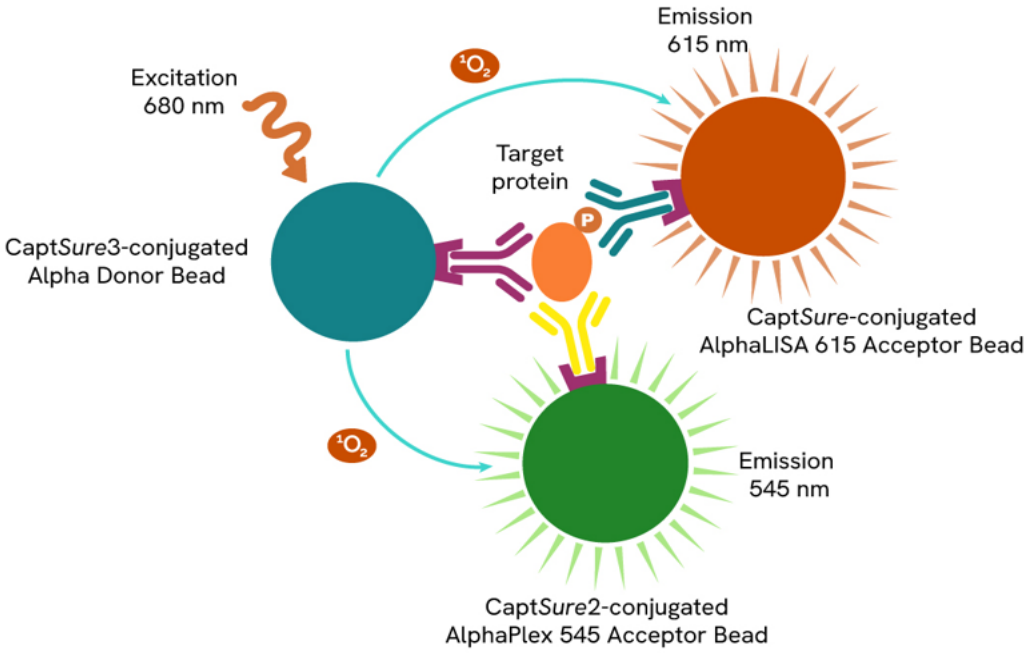
Confidential - Company Proprietary

ASSAY PRINCIPLE

The AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV Phospho + Total assay kits enable rapid and sensitive detection of both phosphorylated and total cellular proteins simultaneously. The measurement is performed in the same well of an assay plate from a single biological sample (e.g. cell lysate). The assay uses three antibodies that recognize the phospho epitope and two distal epitopes on the target protein. Detection is achieved using three bead types, two distinct Acceptor Beads and one Donor Bead, as described below.

Single target – Phospho + Total Assay kits

The Alpha 615 Acceptor Bead is conjugated with a proprietary Capt*Sure*™ agent, which binds the Capt*Sure*-tagged anti-phospho target antibody. The Alpha 545 Acceptor Bead is conjugated with a proprietary Capt*Sure2* agent, which binds the Capt*Sure2* tagged anti-total target protein antibody. The Donor Bead is conjugated with a propriety Capt*Sure*3 agent, which binds the Capt*Sure*3 tagged anti-total target protein antibody.



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MAIN FEATURES

The AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV assay kits are used to simultaneously measure the phosphorylated and total protein in biological samples (e.g. cell lysate).
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. The assays measure changes in levels of cellular proteins in endogenous or engineered systems and can be applied to all type of cellular extracts, including cultured primary/non-primary cell lines and tissue lysates.

The AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV platform eliminates the need to consider biotin free media while maintaining high performance. 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™* Multiplex Biotin Free HV assays are carried out using a higher volume of reagents than the standard Multiplex assays, and in 96 well plate format rather than 384 well format. These kits are useful for laboratories where the handling of larger volumes is more appropriate for the laboratory equipment.

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

The assay utilizes the bead-based Alpha Technology and requires an Alpha Technology-compatible plate reader capable of reading dual emission wavelengths. See www.revvity.com 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 / 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.**

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KIT CONTENTS

	100 points
Lysis Buffer (5X)*	1 x 12 mL
Supplement** (only in kits with Lysis Buffer B or C (5X))	1 x 1.5 mL
Activation Buffer *	1 x 0.3 mL
Reaction Buffer 1 – MSBF (Capt*Sure3*™ *anti-Total antibody*)	1 x 0.6 mL
Reaction Buffer 2 – MSBF (*CaptSure™ tagged anti-phospho antibody*)	1 x 0.6 mL
Reaction Buffer 3 – MSBF (*CaptSure2™ tagged anti-Total antibody*)	1 x 0.6 mL
Dilution Buffer	1 x 1.8 mL
Alpha 615 Capt*Sure*™ Acceptor Beads (2mg/mL in PBS plus 0.03% Proclin-300)	1 x 0.045 mL
Alpha 545 Capt*Sure2*™ Acceptor Beads (2mg/mL in PBS plus 0.03% Proclin-300)	1 x 0.045 mL
Alpha Capt*Sure3*™ Donor Beads (2mg/mL in PBS plus 0.03% Proclin-300)	1 x 0.045 mL
Positive Control Lysate	1 lyophilized tube

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

* **Some kits contain assay-specific Lysis Buffer B or C (5X) and Activation Buffer B or C.**

** **An additional Supplement B or C is provided in kits with Lysis Buffer B or C (5X) only. This supplement must be added to the respective Lysis Buffer B or C (5X) before use. See Storage and Handling Conditions and Buffer Preparation sections for details.**

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STORAGE AND HANDLING 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) (including B and C) Reaction Buffer 1 – MSBF Reaction Buffer 2 – MSBF Reaction Buffer 3 - MSBF Dilution Buffer	Store at 4°C
	Supplement B or C (only in kits with Lysis Buffer B or C (5X))	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.
	Activation Buffer (including B and C)	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. Before use, mix well by vortexing 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.

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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 384, clear adhesive sealing film	Revvity Inc.	6050185	100/box
Envision™ or Ensight™ Alpha-reader with adequate AlphaPlex filters (see table below)	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) 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.revvity.com.

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.revvity.com.

	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

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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.

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.

Lysis Buffers (5X) B and C are assay-specific and must not be inter-changed.

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

Supplement B or C

An additional Supplement B or C is provided in kits with Lysis Buffer B (5X) or Lysis Buffer C (5X). This supplement must be added to respective Lysis Buffer B or C (5X) before use.

Activation Buffer

Activation Buffers B and C are assay-specific and must not be interchanged.

Alpha Capt*Sure*3 Donor Beads

Alpha Capt*Sure*3 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.

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BUFFER PREPARATION

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.
5X Lysis Buffer B or C	**Supplement must be added before use.** Dilute Supplement B or C **10-fold** in respective 5X Lysis Buffer B or C. For example: For 2 mL of 5X Lysis Buffer B or C: Add 0.2 mL of Supplement B or C to 1.8 mL of respective 5X Lysis Buffer B or C.
1X Lysis Buffer B or C	Dilute 5X Lysis Buffer B or C (with supplement) **5-fold** in deionized water. For example: For 10 mL of 1X Lysis Buffer B or C: Add 2 mL of 5X Lysis Buffer B or C (with supplement) to 8 mL deionized water.
Acceptor Mix Reaction Buffer 1 + Reaction Buffer 2 + Reaction Buffer 3 + Activation Buffer + 615 Acceptor Beads + 545 Acceptor Beads	Combine Reaction Buffers 1, 2 and 3 in equal parts. Dilute Activation Buffer **25-fold** in final volume. Dilute each Acceptor bead **50-fold** in final volume. For example: For 300 µL of Acceptor Mix: Combine 92µL Reaction Buffer 1, 92 µL of Reaction Buffer 2 and 92 µL of Reaction Buffer 3. Then add 12µL Activation Buffer. Then add 6µL 615 Acceptor Bead and 6µL 545 Acceptor Bead. The Acceptor mix should be made up and used within 30min 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.

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AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV PHOSPHO + TOTAL ASSAY PROTOCOL

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

Cell Seeding

1. Seed cells in cell culture plates (200 µL for 96 well plates). Incubate at 37°C overnight in serum-containing media.

Cell Treatment and Lysis

2. Remove culture media and treat the cells with the corresponding agonist, antagonist, degrader or any other chemical compound. These compounds can be prepared in complete cell culture media, serum-free media or HBSS + 0.1% BSA, depending on the experiment. Return cells to 37°C incubator for desired time. Treatment times vary and need to be tested on case-by-case basis. Two hours are often sufficient for signal transduction inhibitors, and 5-20 minutes for receptor agonists.

   Please refer to the TDS for more experimental details.

   Note: Peptidic agonists and antagonists can often stick to plastic surfaces. To minimize this effect, dilute in culture media or HBSS containing a suitable carrier protein (e.g. 0.1% BSA).

3. To lyse cells, remove medium/treatment 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. Lysis volume may vary depending on the cell system, seeding cell density and protein expression levels.

   Please refer to the TDS for more experimental details.

Assay

4. Take 30 µL of the cell lysate and transfer to a 96-well 1/2 AreaPlate™ for assay. Add 30 µL of Control lysates and 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.

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.

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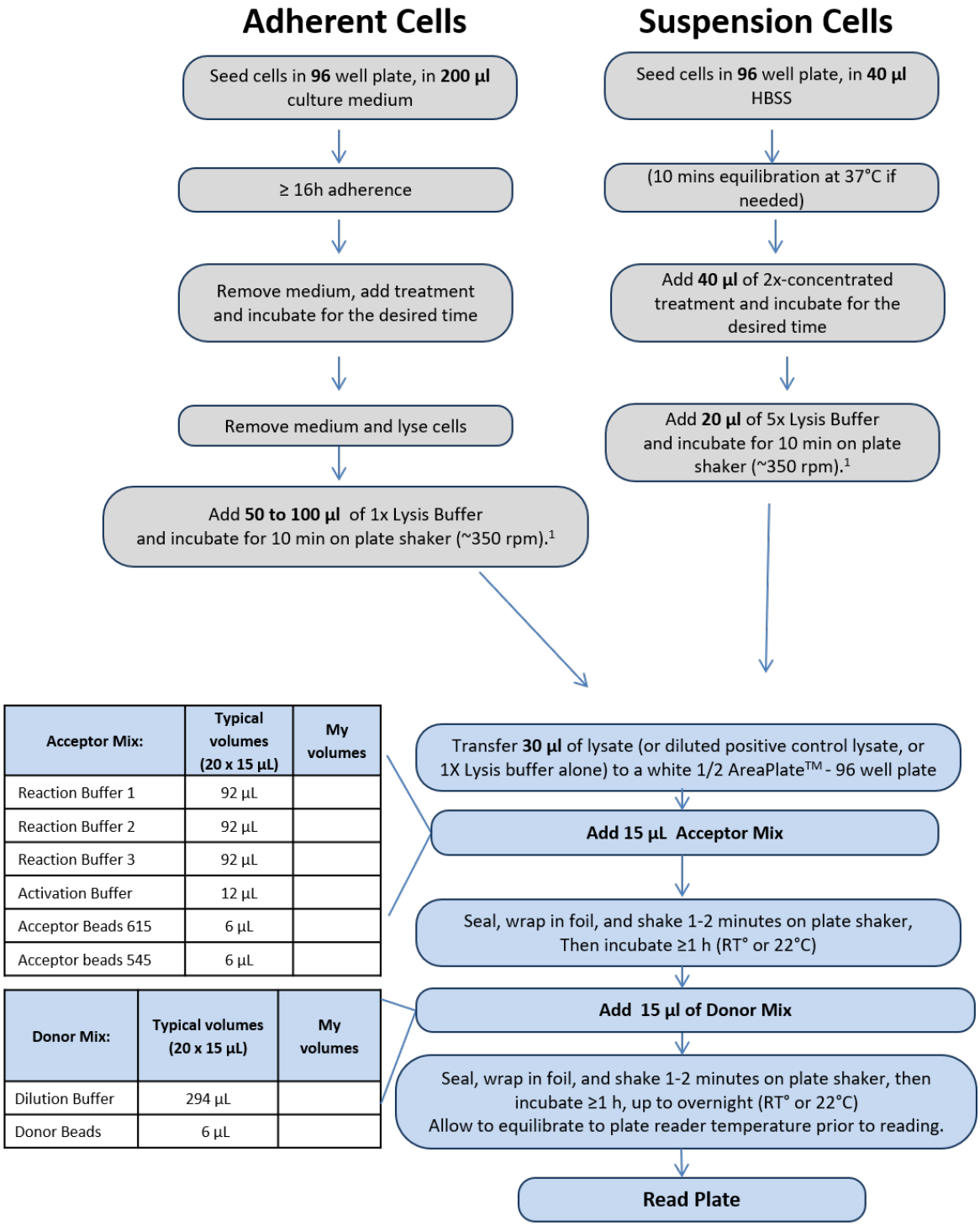
AlphaLISA™ *SureFire® Ultra*™ Multiplex Biotin Free HV- Phospho/Total: 2-plate / 2-incubation assay flowchart



Acceptor Mix:	Typical volumes (20 x 15 µL)	My volumes
Reaction Buffer 1	92 µL	
Reaction Buffer 2	92 µL	
Reaction Buffer 3	92 µL	
Activation Buffer	12 µL	
Acceptor Beads 615	6 µL	
Acceptor beads 545	6 µL	

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

[1] May freeze lysates at -80°C if desired. If doing this, re-shake after thawing to ensure homogeneity of lysate before pipetting.

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AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV PHOSPHO + TOTAL ASSAY PROTOCOL

B. 1-Plate Assay - assay protocol for suspension 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 4 x 10^6 cells/mL as a starting point. Seed 12 µL of cells/well into a 96-well 1/2 AreaPlate™. If needed, return plate to the incubator for around 10 minutes to let the cells settle and reach equilibrium.

Cell Treatment

2. Treat cells with the corresponding agonist, antagonist, degrader or any other chemical compound. It is recommended to prepare these compounds in HBSS + 0.1% BSA. Return cells to 37°C incubator for desired time. Treatment times vary and need to be tested on a case-by-case basis. Two hours are often sufficient for signal transduction inhibitors, and 5-20 minutes for receptor agonists.

   During treatment the final volume in the wells should be 24 µL. Therefore add 12 µL/well of 2X treatment, to give a final volume in the wells of 24 µL.

   See flow diagram for more details.

   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).

Cell Lysis

3. To lyse cells, add 6 µL of 5X Lysis Buffer. Add 30 µL of Control lysates and 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.

Assay

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

5. 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.

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

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AlphaLISA™ *SureFire® Ultra™* Multiplex Biotin Free HV - Phospho/Total: 1-plate / 2-incubation assay flowchart

Adherent Cells

Seed cells in a white 1/2 AreaPlate™ - **96 well plate**, in **30 µl** culture medium

↓

≥ 16h adherence

↓

Remove 15 µl of medium

↓

Add **15 µl** of 2x-concentrated treatment and incubate for the desired time

Remove medium and add **30 µl** of 1x Lysis Buffer

Suspension Cells

Seed cells in a white 1/2 AreaPlate™ - **96 well plate**, in **12 µl** HBSS

↓

(10 mins equilibration at 37°C if needed)

↓

Add **12 µL** of 2x-concentrated treatment and incubate for desired time

↓

Add **6 µl** of 5x Lysis Buffer

↓

Seal and incubate for 10 min on plate shaker (~350 rpm)

↓

In control wells, add 30 µL positive control lysate dilution or 1x lysis buffer alone.

Acceptor Mix:	Typical volumes (20 x 15 µL)	My volumes
Reaction Buffer 1	92 µl	
Reaction Buffer 2	92 µl	
Reaction Buffer 3	92 µl	
Activation Buffer	12 µl	
Acceptor Beads 615	6 µl	
Acceptor beads 545	6 µl	

↓

Add 15 µL Acceptor Mix

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, Then incubate ≥1 h (RT° or 22°C)

↓

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

↓

Add 15 µl of Donor Mix

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h, up to overnight (RT° or 22°C)
Allow to equilibrate to plate reader temperature prior to reading.

↓

Read Plate

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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)	Revvity Inc.	ALSU-LB-10mL	10mL
	Revvity Inc.	ALSU-LB-100mL	100mL
Lysis Buffer B (5X)*	Revvity Inc.	ALSU-LBB-10mL	10 mL
	Revvity Inc.	ALSU-LBB-100mL	100 mL
Lysis Buffer C (5X)*	Revvity Inc.	ALSU-LBC-10mL	10 mL
	Revvity Inc.	ALSU-LBC-100mL	100 mL
Activation Buffer	Revvity Inc.	ALSU-AB-10mL	10mL
	Revvity Inc.	ALSU-AB-100mL	100mL
Activation Buffer B	Revvity Inc.	ALSU-ABB-10mL	10 mL
	Revvity Inc.	ALSU-ABB-100mL	100 mL
Activation Buffer C	Revvity Inc.	ALSU-ABC-10mL	10 mL
	Revvity Inc.	ALSU-ABC-100mL	100 mL
Dilution Buffer	Revvity Inc.	ALSU-DB-10mL	10mL
	Revvity Inc.	ALSU-DB-100mL	100mL
AlphaLISA™™ Capt*Sure*™ Acceptor Beads -2mg/ml	Revvity Inc.	ALSU-ACAB-0.06mL	60µL
	Revvity Inc.	ALSU-ACAB-1.2mL	1.2mL
	Revvity Inc.	ALSU-ACAB-6mL	6mL
Alpha 545 (Tb) CaptSure2 Acceptor Beads -2mg/mL	Revvity Inc.	MPSU-CS2B-0.06mL	60µL
	Revvity Inc.	MPSU-CS2B -1.2mL	1.2mL
	Revvity Inc.	MPSU-CS2B -6mL	6mL
Alpha CaptSure3 Donor Beads -2mg/mL	Revvity Inc.	ASBF-CS3DB-0.06mL	60µL
	Revvity Inc.	ASBF-CS3DB-1.2mL	1.2mL
	Revvity Inc.	ASBF-CS3DB-6mL	6mL

***Includes respective Supplement B or C.**

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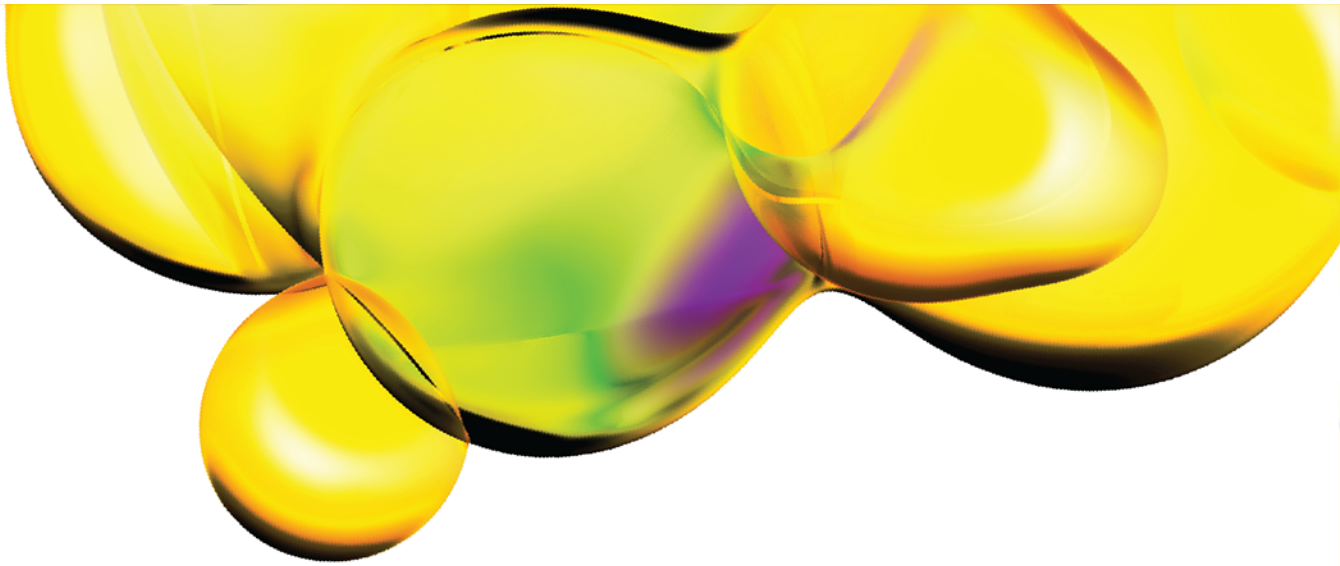

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