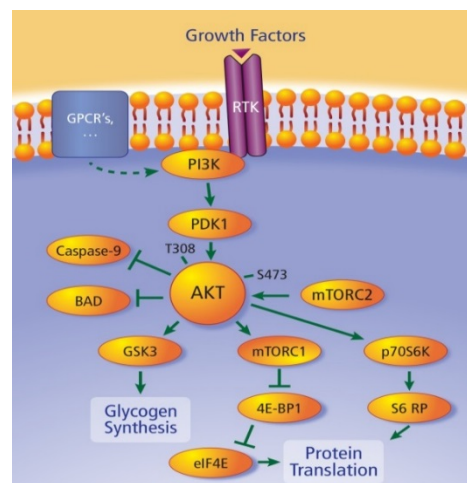


Alpha SureFire® Ultra™ Multiplex

p-Akt 1/2/3 (Ser473) 615 (Eu) + Total Akt 1 545 (Tb) Assay Kit

Manual

Assay Points	Catalog #
500	MPSU -PTAKT-M500
10 000	MPSU -PTAKT-M10K
50 000	MPSU -PTAKT-M50K



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

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

Alpha SureFire® Ultra™ Multiplex

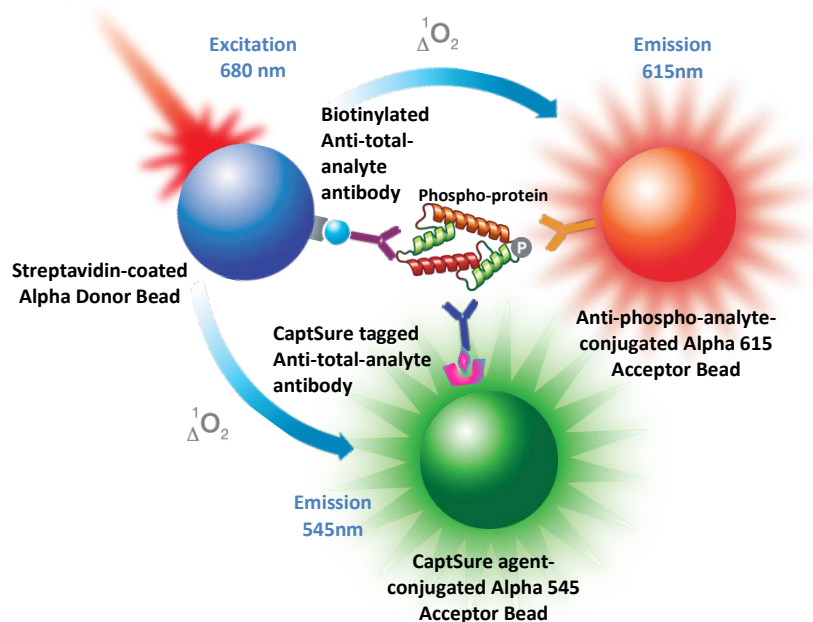
Assay Principle

The Alpha SureFire Ultra Multiplex 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.

Single target – Phospho + Total Assay kits

The Alpha 615 Acceptor bead is directly-conjugated with an antibody to the phosphorylated site on the target protein. The Alpha 545 Acceptor bead is coated with the CaptSure agent, which binds the CaptSure tagged anti-total target protein antibody. The Alpha Donor bead binds the biotinylated anti-total target protein antibody.

The antibodies used in this kit are identical to those used in the individual phospho- (Ser473) and total- Akt AlphaLISA SureFire Ultra kits (ALSU-PAKT-B and ALSU-TAKT1-A catalog numbers), although their orientation of bead attachment may vary between kits.



General Information on the Alpha *SureFire*[®] *Ultra*[™] Multiplex p-Akt 1/2/3 (Ser473) + Total Akt 1 assay

The Alpha *SureFire Ultra* Multiplex p-Akt 1/2/3 (Ser473) + Total Akt 1 assay kit is used to measure both the phosphorylation (Ser473) and total levels of endogenous Akt in cellular lysates. The kit measures phosphorylation on Ser473 of Akt 1/2/3, and total levels of Akt 1 only. 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 Akt (Ser473) activation by either recombinant or endogenous receptors, and can be applied to primary cells.

The 615nm (Eu) signal corresponds to the phosphorylated Akt (Ser473) analysis, and the 545nm (Tb) signal corresponds to the total Akt 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.

Kit-Specificity Information

This assay kit contains three antibodies which recognize Akt. Two antibodies recognize the three forms of Akt (1/2/3), one at the phospho-Ser473 epitope, and one at a distal epitope. The third antibody recognizes Akt1 only, and provides the total signal on the Tb Acceptor bead. The proteins detected by this kit correspond to GenBank Accessions NP_001014431, NP_001617, and NP_859029. Akt proteins are also known as PKB or RAC.

These antibodies recognize Akt of human, mouse and rat origin. Other species should be tested on a case-by-case basis.

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.

Note: the buffers (lysis, activation, reaction, dilution) in the Alpha *SureFire Ultra* Multiplex kits have a different formulation compared to the buffers from the AlphaScreen *SureFire* kits, and buffers from the two types of kits should not be interchanged.

Kit Contents

	Kit Size		
	500 points	10,000 points	50,000 points
Lysis Buffer (5X) - <i>Ultra</i>	1 x 12 mL	4 x 60 mL	3 x 400 mL
Activation Buffer - <i>Ultra</i>	1 x 0.8 mL	1 x 10 mL	1 x 50 mL
Reaction Buffer 1 – MPSU (<i>Biotinylated anti-Total AKT (1/2/3) antibody – rIgG</i>)	1 x 0.13 mL	1 x 2.4 mL	1 x 12 mL
Reaction Buffer 2 –MPSU (<i>CaptSure™ tagged anti-Total AKT1 antibody – mIgG1</i>)	1 x 2.8 mL	1 x 52 mL	1 x 260 mL
Dilution Buffer - <i>Ultra</i>	1 x 3 mL	1 x 60 mL	2 x 150 mL
Alpha 615 anti-p-AKT(1/2/3) (Ser473) (mIgG1) 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 545 CaptSure™ 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 ₂ O		

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

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 use.

The Activation Buffer 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 -20°C or -80°C for long term storage.

See kit box label for expiry date.

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

<p>1X Lysis Buffer</p>	<p>Dilute 5X Lysis buffer in MilliQ water to a final concentration of 1X</p> <p>For example: for 10 mL of 1X Lysis Buffer, add: 2 mL of 5X Lysis Buffer – <i>Ultra</i> to 8 mL MilliQ water. Discard unused 1X buffer.</p>
<p>Acceptor Mix</p> <p>Alpha Reaction Buffer 1 - <i>Ultra</i> (4 parts or 4%) + Alpha Reaction Buffer 2 - <i>Ultra</i> (88 parts or 88%) + Activation Buffer - <i>Ultra</i> (4 parts or 4%) + Alpha 615 anti-p-Akt 1/2/3 Acceptor beads (2 parts or 2%) + Alpha 545 CaptSure™ Acceptor beads (2 parts or 2%)</p> <p>See flowchart for table</p>	<p>Dilute Reaction Buffer 1 25-fold in Reaction Buffer 2. Dilute Activation Buffer 25-fold in combined Reaction Buffer 1 and Reaction buffer 2. Dilute each Acceptor bead 50-fold in combined Reaction Buffers plus Activation Buffer.</p> <p><u>For example: for 300 µL of Acceptor Mix:</u> Add 12µL of Reaction Buffer 1 to 264µL of Reaction Buffer 2, and to this add 12µL Activation Buffer and 6µL Acceptor Bead 615 and 6µL Acceptor Bead 545.</p> <p>The Acceptor mix should be made up and used within 30min for best results. Excess mix should be discarded.</p>
<p>Donor Mix*</p> <p>Dilution buffer - <i>Ultra</i> (98 parts or 98%) + Alpha Donor beads (2 parts or 2%)</p> <p>See flowchart for table * Prepare and use under low-light conditions.</p>	<p>Dilute Donor beads 50-fold in Dilution buffer</p> <p><u>For example: for 300 µL of Donor Mix, add:</u> 6 µL Donor Beads to 294 µL of Dilution Buffer</p> <p>The Donor mix should be made up and used within 30min for best results. Excess mix should be discarded.</p>
<p>Positive control lysate</p>	<p>Reconstitute with 250µL water. Store at -20°C in single use aliquots and use within 3 months. Dilute as required.</p>

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.

Alpha SureFire® Ultra™ Multiplex p-Akt 1/2/3 (Ser473) + Total Akt 1 Assay Protocol

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

Cell Seeding

1. Seed cells (200 μ L of cells for 96 well plates, 50 μ L 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 μ L agonists prepared in serum-free media (25 μ L for 384-well plates). (*If testing antagonists, prior to stimulation remove culture medium and replace with 50 μ L serum-free media containing antagonists (25 μ L 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 μ L for a 96 well plate, 25 μ L for a 384 well plate). Agitate on a plate shaker (~350 rpm) for 10 minutes at room temperature.
4. Take 10 μ L of the lysate and transfer to a 384-well Optiplate™ for assay. *Add 10 μ L 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).*

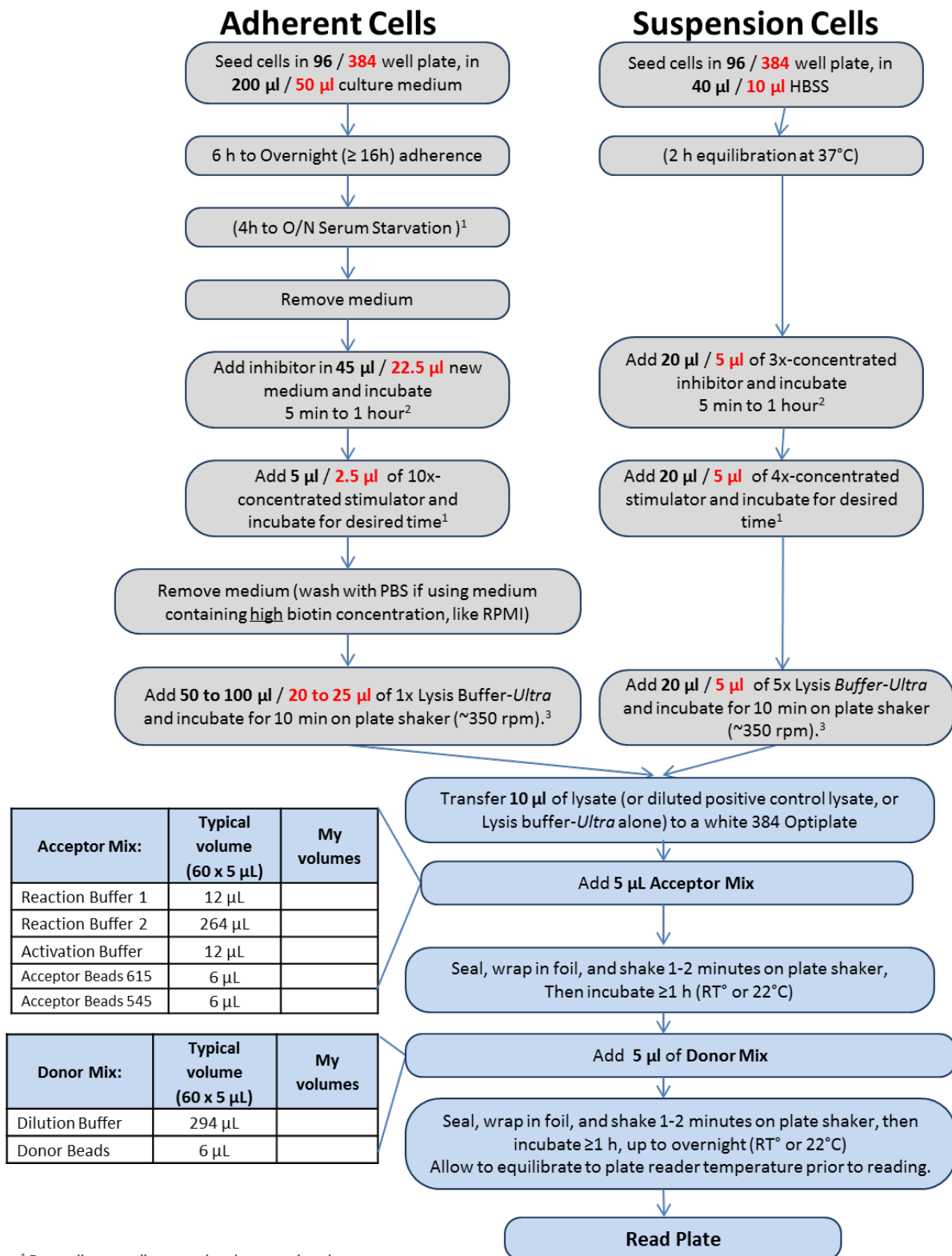
Alpha SureFire Ultra Multiplex Assay

5. Add 5 μ L of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Incubate for 1 hour at room temperature.
6. Add 5 μ L 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 Single target – Phospho/Total



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 p-Akt 1/2/3 (Ser473) + Total Akt 1 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 μ L of cells/well into a 384-well white opaque culture plate (eg PerkinElmer Cat # 6007680).
2. If using test agents/inhibitors, add 2 μ L/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 μ L/well of 4X agonist stock in HBSS containing 0.1% BSA. The final volume in the wells should be 8 μ L. (if no antagonists were used in step 2, stimulate the cells with 4 μ L/well of 2X agonist, to give a final volume in the wells of 8 μ L.)

Lysate Preparation

5. To lyse the cells, add 2 μ L/well of 5X Lysis Buffer - Ultra. Add 10 μ L 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).

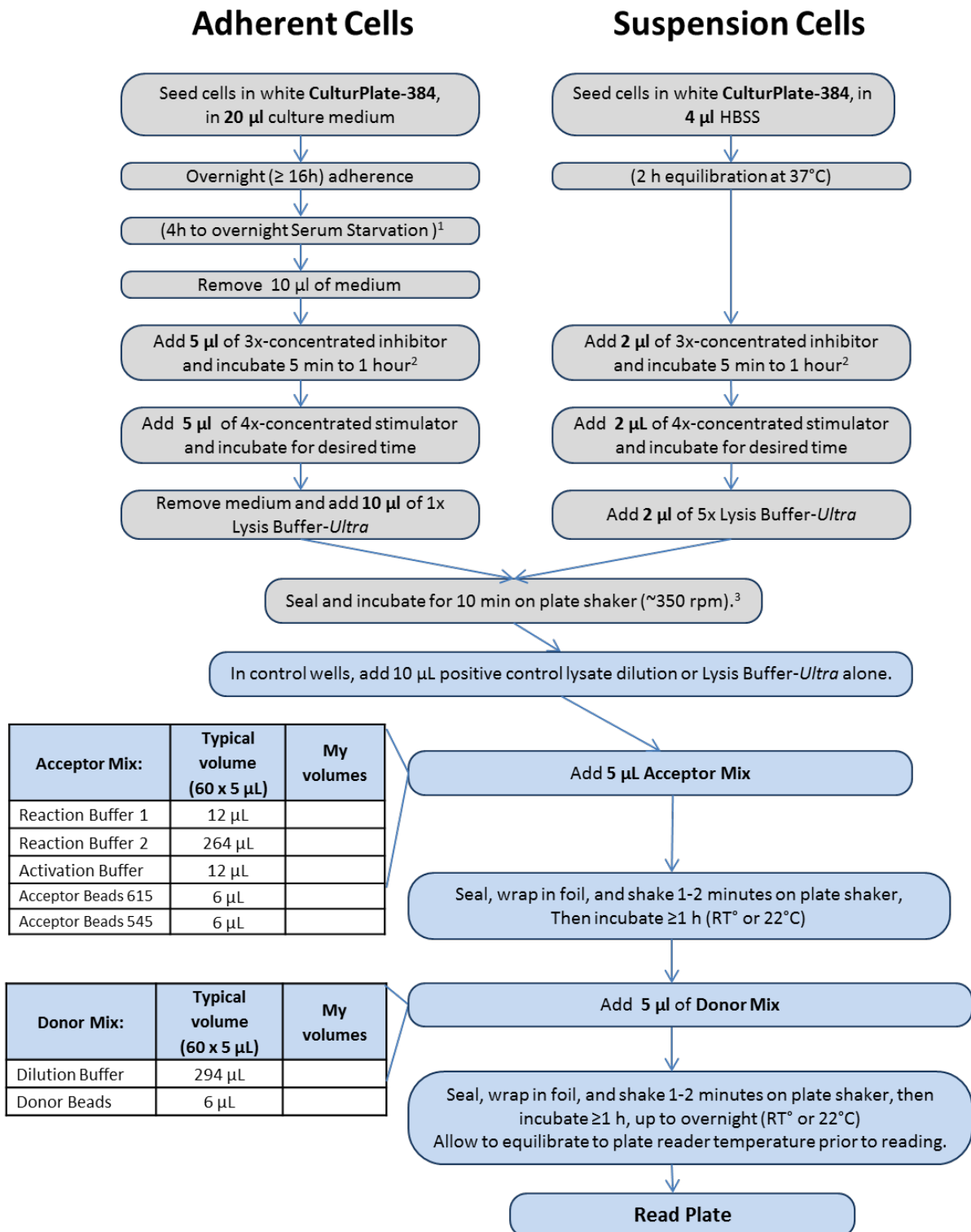
Alpha SureFire Ultra Multiplex Assay

6. Add 5 μ L of Acceptor Mix to wells. Seal plate with Topseal-A adhesive film. Incubate for 1 hour at room temperature.
7. Add 5 μ L 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 Single target – Phospho/Total



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.

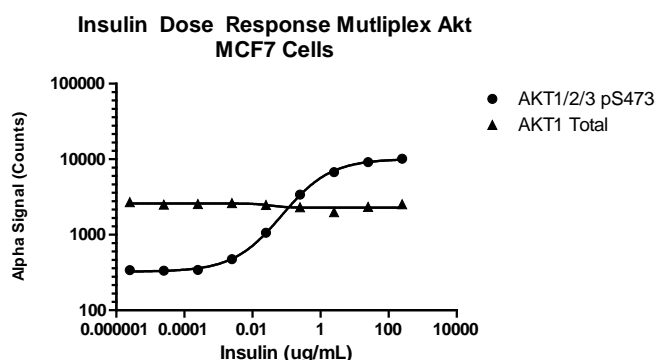
³ May stop and freeze lysates at -20°C if desired. If doing this, re-shake after thawing to ensure homogeneity of lysate before pipetting.

Control Lysate Information

Positive Control Lysate: Prepared from HEK293 cells, cultured to confluence in T175 flasks in 10% FBS containing medium for 3 days, then treated with 20% FBS for 15min and lysed in 10mL of 1X *SureFire Ultra* Lysis buffer.

Representative Data: Phospho @ 615 nm (Eu), Total @ 545 nm (Tb)

Data obtained with a 2-plate, 2-incubation protocol. MCF7 cells were plated at 100K cells per mL, incubated for 2 days, starved for 4 hours, then stimulated with insulin for 30 minutes at the indicated concentrations. Cells were lysed with 100µL of 1X Lysis buffer and assayed simultaneously for phospho Akt1/2/3 (Ser473) and total Akt1 as detailed in the manual.



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) - <i>Ultra</i>	PerkinElmer Inc.	ALSU-LB-10mL	10mL
	PerkinElmer Inc.	ALSU-LB-100mL	100mL
Activation Buffer - <i>Ultra</i>	PerkinElmer Inc.	ALSU-AB-10mL	10mL
	PerkinElmer Inc.	ALSU-AB-100mL	100mL
Dilution Buffer - <i>Ultra</i>	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
Alpha 545 (Tb) CaptSure™ Acceptor Beads - 2mg/mL	PerkinElmer Inc.	MPSU-ACAT- 0.06mL	60µL
	PerkinElmer Inc.	MPSU-ACAT- 1.2mL	1.2mL
	PerkinElmer Inc.	MPSU-ACAT-6mL	6mL

Using the Alpha SureFire® Ultra™ Multiplex p-Akt 1/2/3 (Ser473) + Total Akt 1 to measure only one of these targets

You may occasionally want to use this kit to measure only one of the analytes (either measuring only phospho-Akt (Ser473), or only total Akt). In such a case you can replace the reagents corresponding to the non-measured analyte by an equivalent volume of dilution buffer. For example, to use this kit to measure only p-Akt (Ser473), the preparation of the Acceptor Mix on page 6 would become:

<p><u>Acceptor Mix</u></p> <p>Multiplex Reaction Buffer 1 - <i>Ultra</i> (4 parts or 4%) + Dilution Buffer (88 parts or 88%)+ Activation Buffer (4 parts or 4%) + AlphaLISA™ 615 anti-p-AKT (Ser473) Acceptor beads (2 parts or 2%) + Dilution Buffer (2 parts or 2%)</p>	<p>Dilute Reaction Buffer 1 25-fold into Dilution Buffer.</p> <p>Dilute Activation Buffer 25-fold in combined Reaction Buffer 1 and Dilution Buffer .</p> <p>Dilute the Acceptor bead 50-fold in combined Reaction Buffers plus Activation Buffer.</p> <p>For example: for 300 µL of Acceptor Mix: Add 12µL of Reaction Buffer 1 to 264µL of Dilution Buffer, and to this add 12µL Activation Buffer and 6µL Acceptor Bead 615 and 6µL Dilution Buffer.</p> <p>The Acceptor mix should be made up and used immediately when required for best results. Excess mix should be discarded.</p>
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Please be aware that in this case, there will not be sufficient amount of Donor Beads or Dilution Buffer to measure in separate assays both analytes. (Beads and Dilution buffer can be re-ordered separately).

The reagents to read only one of the analytes (using Alpha 615 Acceptor beads) are available as different kits:

To measure only phospho-Akt (Ser473): ALSU-PAKT-B500 / 10K / 50K / -HV

To measure only total-Akt (Ser473): ALSU-TAKT-A500 / 10K / 50K / -HV

[Useful Links](#)

For FAQ and troubleshooting, please go to:

www.perkinelmer.com/SureFireFAQ 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/SureFire or www.tgrbio.com

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

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