TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

IP-One AlphaLISA® Detection Kit

Product No.: AL3145 D/M/R

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Product Information

Application: This kit is designed to measure myo-Inositol 1 phosphate (IP1) accumulation in cells. It enables the direct pharmacological characterization of compounds acting on Gαq-coupled receptors in either adherent or suspension cells using a homogeneous no wash AlphaLISA assay.

Sensitivity: IC_{50}: 82 nM (average of standard curve using IP1 standard).

Signal to background ratio: 53 (average of standard curve using IP1 Standard, assay window may vary from a reader to another).

Kit contents: The kit contains 6 components: AlphaLISA anti-IP1 Acceptor Beads, AlphaLISA Streptavidin Donor beads, Biotinylated IP1, IP-One Gq Standard, Stimulation Buffer 2 (5X), and AlphaLISA Immunoassay buffer (10X)

Storage: The kit components must be stored at 4˚C in the dark. Reconstituted reagents can be stored at -20 °C for 1 month.

Stability: This kit is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum and minimum counts may vary between bead lots and the instrument used**.**

Analyte of Interest

The metabolic inositol phosphate cascade often results from Gαq protein coupled receptors signaling pathway. Following GPCR activation, Gαq subunit of heterotrimeric G-protein acts to positively stimulate the activity of PLC-β resulting in an increase in cellular IP1. It is well known that measuring an important mediator such as D-myo-Inositol 1-Phosphate (IP1) can be used as a surrogate for IP3. Lithium chloride (LiCl) leads to IP1 accumulation by inhibiting inositol monophosphatase, responsible of its rapid degradation to myo-inositol.

Description of the AlphaLISA Assay

The AlphaLISA® IP-One detection assay uses AlphaLISA anti-IP1 Acceptor beads and AlphaLISA Streptavidin-coated Donor beads to capture the biotinylated IP1 analog. Donor beads and Acceptor beads come into proximity through biotinylated IP1 binding. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1). Native IP1 produced by cells or unlabeled IP1 (standard curve) compete with biotinylated IP1 for binding to AlphaLISA anti-IP1 Acceptor beads. Based on competitive assay, the specific signal is thus inversely proportional to the concentration of IP1 in the standard or sample (Figure 2).

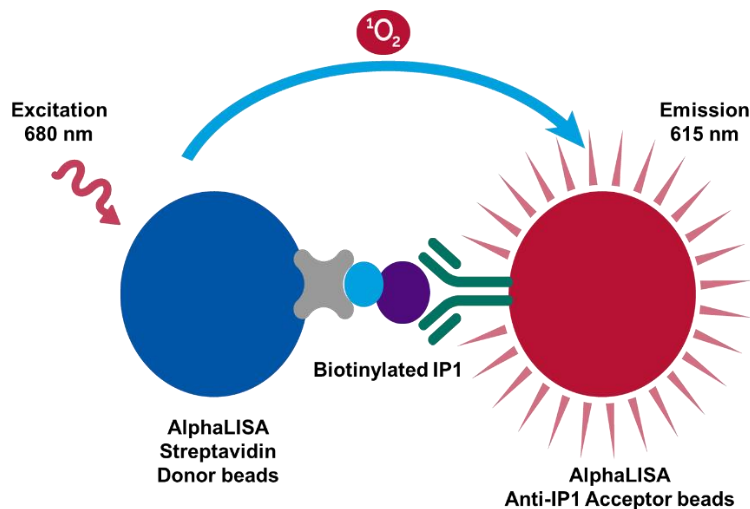


Figure 1. AlphaLISA Assay Principle

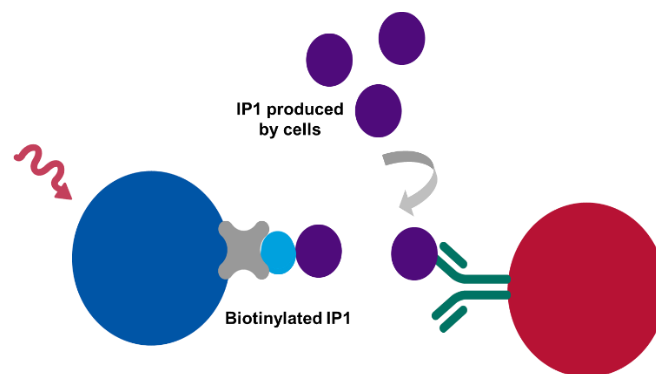


Figure 2. IP1 measurement with the AlphaLISA IP-One Competitive assay

Precautions

- 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 (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous.

Kit Content: Reagents and Materials

Kit components	AL3145D *** (1,000 assay points)	AL3145M *** (10,000 assay points)	AL3145R *** (50,000 assay points)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	1 vial of 400 µL @ 5 mg/mL (brown tube, black cap)	4 vials of 1 mL @ 5 mg/mL (brown tube, black cap)	2 vials of 10 mL @ 5 mg/mL (brown vial, brown Cap)
AlphaLISA Anti-IP1 Acceptor Beads in PBS, 0.05% Kathon CG/ICP II, pH 7.2	1 vial of 200 µL @ 5 mg/mL (brown tube, white cap)	2 vials of 1 mL @ 5 mg/mL (brown tube, white cap)	1 vial of 10 mL @ 5 mg/mL (brown vial, brown cap)
Biotinylated IP1* (Lyophilized)	1 vial (brown vial, red cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.	1 vial (brown vial, red cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.	5 vials (brown vial, red cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.
IP-One Gq standard* (Lyophilized)	1 vial (clear vial, green cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.	1 vial (clear vial, green cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.	1 vial (clear vial, green cap) Reconstitute using distilled water. See instructions on vial label for reconstitution volume.
Stimulation Buffer 2 (5X)	2 bottles - 8 mL	2 bottles - 100 mL	7 bottles - 100 ml
AlphaLISA Immunoassay buffer (10X)**	1 bottle - 10 ml	1 bottle - 100 mL	3 bottles - 100 ml

* Reconstitute the IP-One Gq standard and biotinylated IP1 using distilled water. Mix gently after reconstitution. The Standard and the biotinylated IP1 concentrate are stable frozen for 3 months at -20°C. Avoid multiple freeze-thaw cycles.

** The AlphaLISA is used 3X to dilute detection reagents. Extra buffer can be ordered separately (cat # AL000C : 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 50 µL in 384 well plates (i.e OptiPlate™-384 #6007290 or AlphaPlate-384 Light gray #6005350 for suspension cells, and white CulturPlate 384-well, TC-treated #6007680 for adherent cells) using the kit components at the recommended concentrations.

Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050185
AlphaPlate-384 Light gray or OptiPlate™-384 for suspension cells protocol	PerkinElmer Inc.	6005350 or 6007290
CulturPlate 384-well, TC-treated for adherent cells protocol	PerkinElmer Inc.	6007680
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend the beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute AlphaLISA Immunoassay buffer and to reconstitute the lyophilized reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal™-A Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

Competition Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an ***example*** for generating 250 assay points in a 50 µL final assay volume per point. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes. If a different number of samples are tested, the volumes of all reagents must be adjusted accordingly.
- The dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

Reagents preparation:

1) Preparation of 3X AlphaLISA Immunoassay Buffer (for 5 mL):

Add to 1.5 volume of 10X Immunoassay Buffer to 3.5 volumes of MilliQ distilled water (i.e to prepare 5 ml of 3X Immunoassay Buffer, Add 1.5 ml of 10X Immunoassay Buffer and 3.5 ml of MilliQ distilled water).

2) Preparation of 1X Stimulation Buffer 2 (for 15 mL):

Add to 1 volume of 5X Stimulation Buffer 2 to 4 volumes of MilliQ distilled water. Adjust volumes for the required amount of buffer (i.e to prepare 15 ml of 1X Stimulation Buffer 2, Add 3 ml of 5X Stimulation Buffer 2X and 12 ml of MilliQ distilled water).

3) Preparation of serial dilutions of IP-One Gq standard as follows:

- For accurate quantification of IP1 in the cell-based assay, a standard curve must be performed at the same time than cell-based using the same working reagents solution.
- Change tips between each dilution:

Reconstitute lyophilized IP1-Standard using distilled water. See instructions on vial label for reconstitution volume.

a. Add distilled Water to make 220 µM of IP1 Standard stock solution. Homogenize gently.
b. Prepare serial dilutions working solution of the IP1 Standard (5X) in Stimulation Buffer (1X) as follow:

Tube	Volume of IP1 Standard	Volume of stimulation buffer (1X)	[IP1 Standard] (nM) (5X)	[IP1 Standard] (nM) (1X)
A (STD 12)	70 µL of 220 µM stock	215 µL	61600	12320
B (STD 11)	30 µL of tube A	70 µL	18665	3733
C (STD 10)	30 µL of tube B	60 µL	6220	1244
D (STD 9)	30 µL of tube C	70 µL	1885	377
E (STD 8)	30 µL of tube D	60 µL	630	126
F (STD 7)	30 µL of tube E	70 µL	190	38
G (STD 6)	30 µL of tube F	60 µL	63	13
H (STD 5)	30 µL of tube G	70 µL	19.25	3.8
I (STD 4)	30 µL of tube H	60 µL	6.5	1.3
J (STD 3)	30 µL of tube I	70 µL	2.0	0.4
K (STD 2)	30 µL of tube J	60 µL	0.65	0.13
L (STD 1)	30 µL of tube L	70 µL	0.2	0.04
M (STD 0)	0 µl	100 µl	0	0

4) Recommendation for cells and compounds preparation (cell-based assay):

Note:

- The kit is designed to work with the provided stimulation buffer, which we recommend using.
- Cell density must be also optimized to ensure that IP1 levels of unstimulated and stimulated cells fall within the linear dynamic range of the assay (IC_{10} to IC_{90}). If results fall outside the assay's linear range, the data will be inaccurate.
- Stimulation time needs to be optimized (It is not recommended to keep cells in the stimulation buffer more than 2H).
- Reagents kit concentrations have been set for optimal assay performances. Note that any dilution or improper use of reagents will impair the assay quality.
- Suspension cells and compounds working solutions are prepared 3X (1X final in the well during stimulation step).
- Prepare cells and compounds just before use.

a) Cell solution preparation:

- Suspension cells protocol: After cell medium removal, dissociate cells from the flask and prepare cells solution at the desired density (3X working solution) in the stimulation buffer 1X. Cell density is 1X in stimulation step (30 µl).
- Adherent cells protocol: Dispense cells in the cell culture plate at the final desired cell density in the appropriate cell culture medium. After cell adhesion (recommended 24h), remove cell culture medium (carefully to not remove cells) and add 10 µl/well of stimulation buffer 1X.

b) Recommendation for compounds preparation:
Compounds must be prepared in the stimulation buffer (1X) at the desired working concentration (3X).

5) Preparation of biotinylated-IP1 stock solution:

a. Reconstitute lyophilized biotinylated-IP1 following the instructions below:

Conditioning	Volume distilled water	Stock concentrations
1,000 tests	See instructions on vial label for reconstitution volume	1250 nM
10,000 tests		1250 nM
50,000 tests		1250 nM

b. Homogenize gently.

6) Preparation of Biotinylated-IP1 + Anti-IP1 AlphaLISA Acceptor beads Mix working solution:

Note

- Prepare working solution just before use.
- Stock solution of biotinylated-IP1 is at 1250 nM.
- Stock solution of Anti-IP1 AlphaLISA acceptor beads is at 5 mg/ml.

a. Keep the beads under subdued laboratory lighting.
b. Dilute the reconstituted biotinylated-IP1 and the Anti-IP1 AlphaLISA Acceptor beads 50X in the 3X Immunoassay buffer as follow: 1 Volume of biotinylated IP1 stock solution, and 1 Volume of Anti-IP1 AlphaLISA Acceptor beads into 48 volumes of 3X AlphaLISA Immunoassay buffer. Adjust volumes for the required amount of working solution. (i.e add 50 µl of biotinylated-IP1 stock solution and 50 µl of Anti-IP1 AlphaLISA Acceptor beads stock solution to 2400 µl of 3X AlphaLISA Immunoassay buffer).
c. Homogenize well and gently.
d. Mix working solution (5X) contains 25 nM of biotinylated-IP1 and 100 µg/ml of Anti-IP1 AlphaLISA Acceptor beads.
e. Prepare just before use.

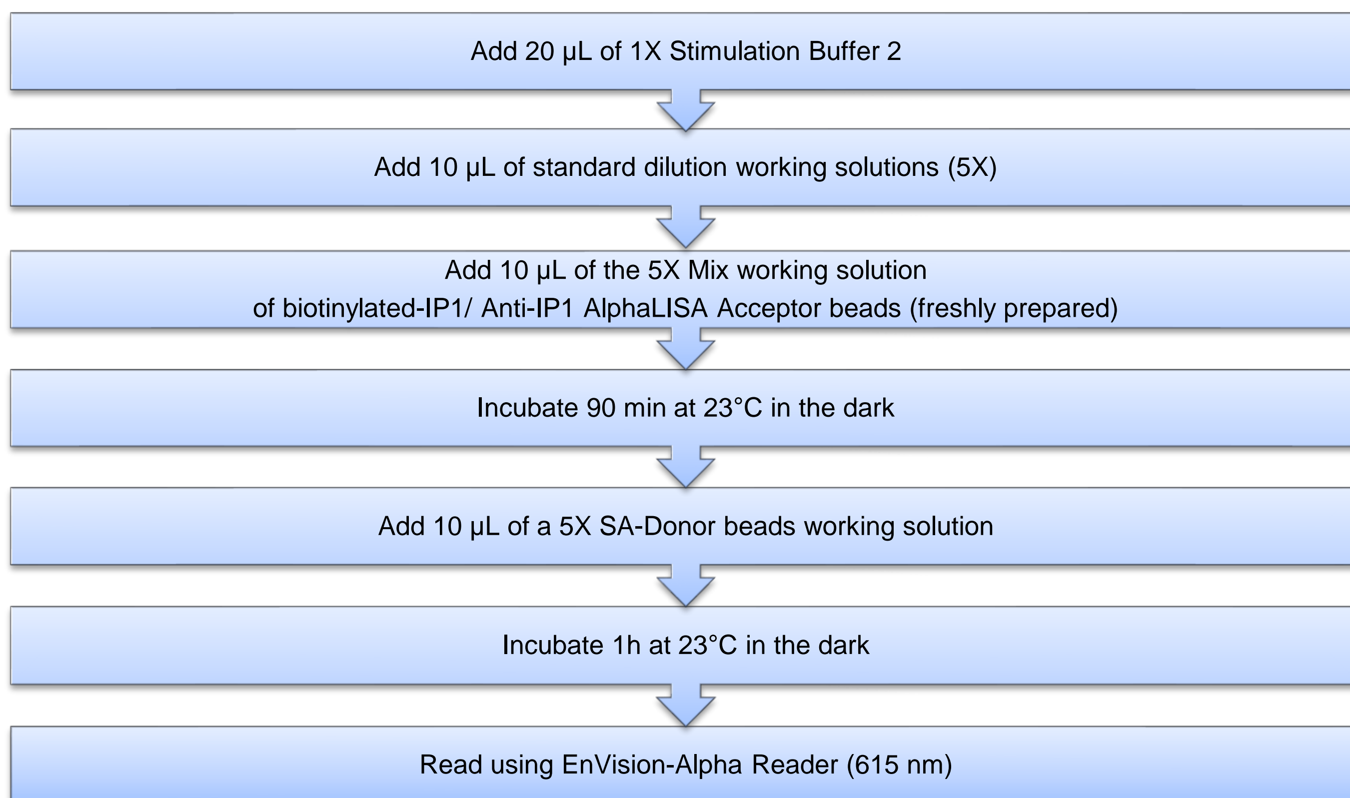
7) Preparation of 5X AlphaLISA Streptavidin-coated Donor beads working solution:

Note
- Prepare working solution just before use.
- Stock solution of AlphaLISA Streptavidin-coated Donor beads is at 5 mg/ml.

a. Keep the beads under subdued laboratory lighting.
b. Dilute Strepatvidin-coated donor beads stock solution 25X: Add 1 Volume of Streptavidin-coated Donor beads stock solution to 24 volume of 3X AlphaLISA Immunoassay buffer. Adjust volumes for the required amount of working solution (i.e add 100 µl of streptavidin-coated donor beads to 2400 µl of 3X AlphaLISA Immunoassay buffer. Adjust volumes for the required amount of working solution).
c. Homogenize well and gently
d. Working solution 5X is at 200 µg/ml.
e. Prepare just before use.

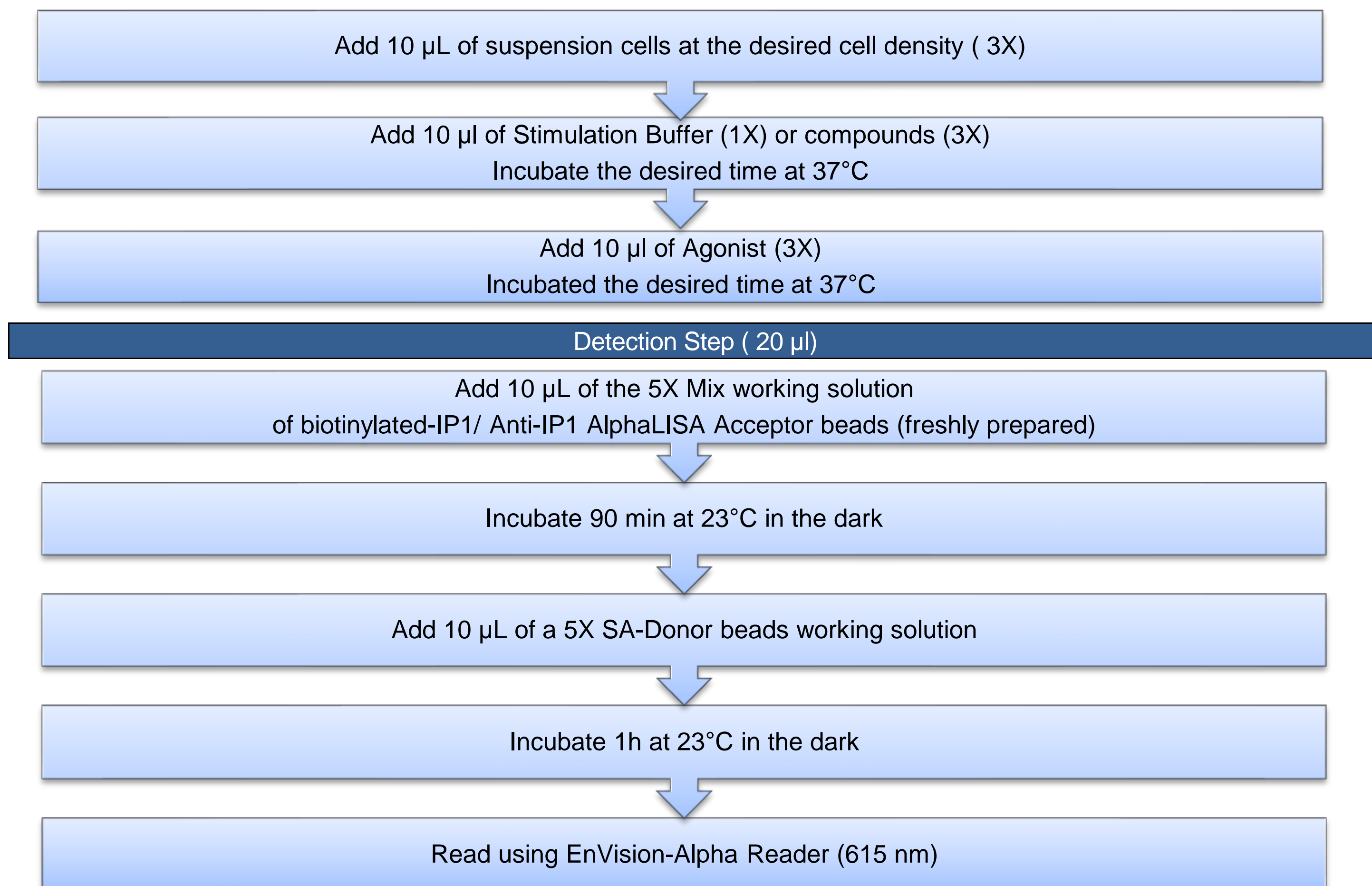
8) Reagents dispensing in the 384 appropriate plate for a 50 µl / well final assay volume (see plate recommendation section for suspension or adherent cells protocol):

a) Standard curve:



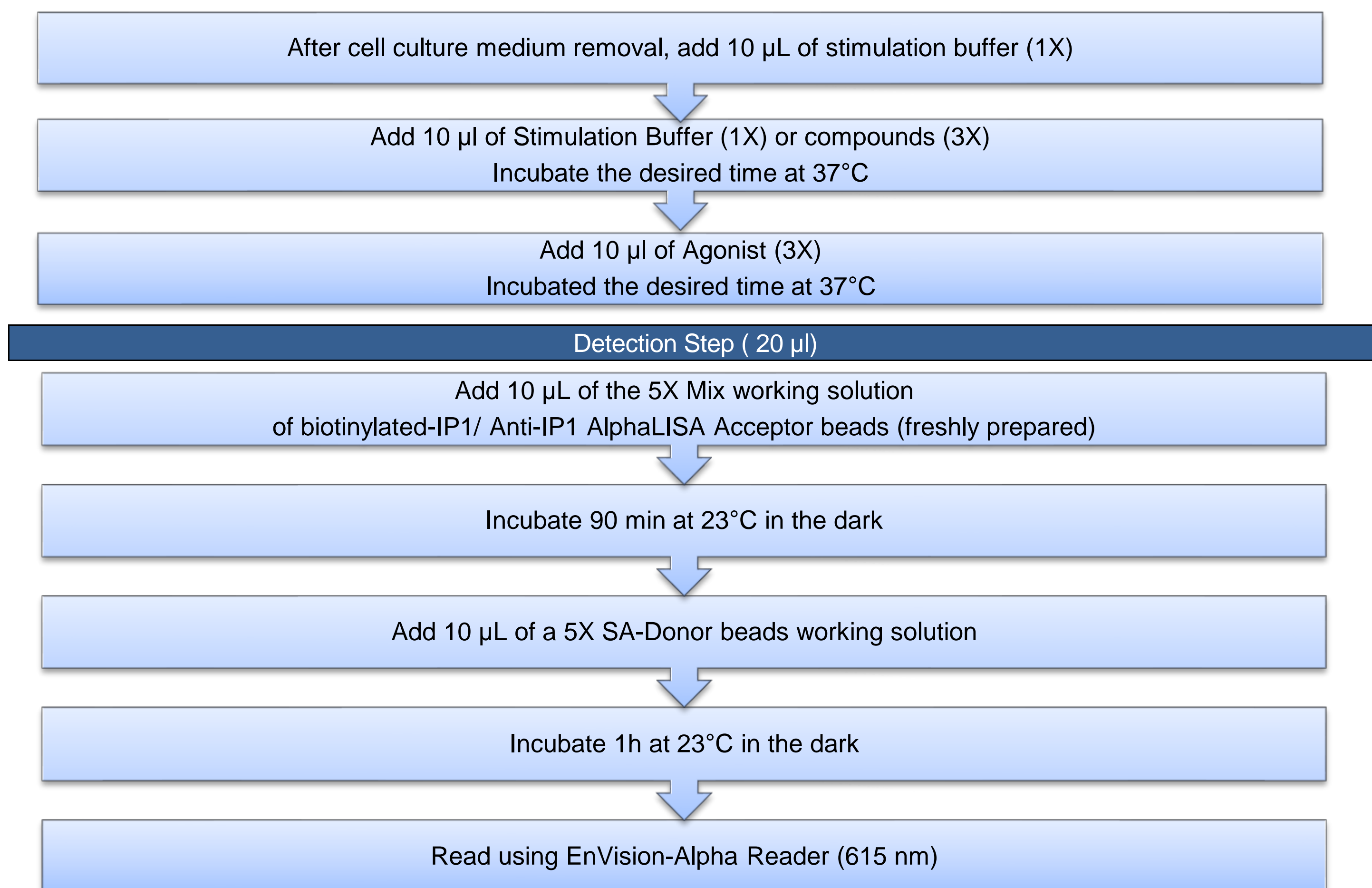
b) Suspension cells protocol:

Stimulation Step (30 µl)



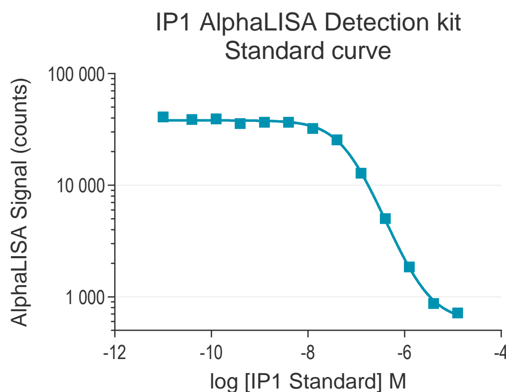
c) Adherent cells protocol:

Stimulation Step (30 µl)



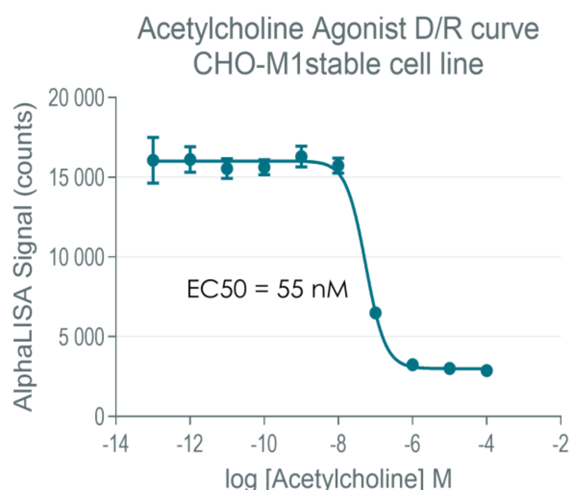
Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

Typical competitive binding data (standard curve):



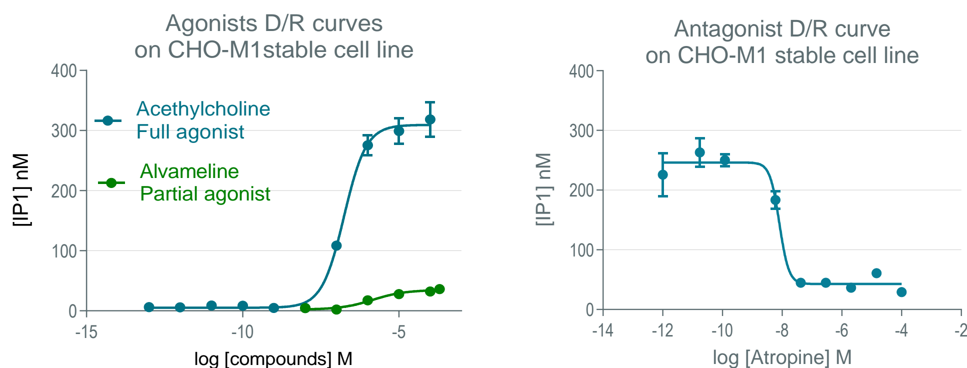
IP1 AlphaLISA standard curve: Data generation using AlphaPlate-384 Light gray and reading with the EnVision Reader. IP1 Standard IC_{50} (80 nM) was calculated by using GraphPad Prism software (nonlinear regression fitting, log(inhibitor) vs. response - Variable slope).
IC_{10}-IC_{90} values of the standard curve are corresponding to the dynamic range of the kit. Cell density should be optimized to work within this range for accurate pharmacology.

Typical compounds dose-response curve (cell-based assay):



Agonist characterization: CHO cells stably expressing the muscarinic M1 receptor (20 kcells/well) were stimulated 45 min with the Acetylcholine full agonist in a dose-dependent manner. Potency. Data generation using AlphaPlate-384 Light gray and reading with the EnVision Reader. Potency (EC50) was calculated by using GraphPad Prism software (nonlinear regression fitting, log(inhibitor) vs. response - Variable slope). If desired, and thanks to the standard curve, Alpha signal can be converted into IP1 concentration.

Typical data processing for pharmacological compounds characterization (cell-based assay):



Compounds	Potency EC50 or IC50 (nM)	Efficacy (%)
Acetylcholine (Full)	176	100%
Alvameline (Highly Partial)	1490	10%
Atropine (Antagonist)	12	-

Data processing by converting Alpha signal in IP1 concentration for compounds characterization: Alpha signal can be converted in IP1 concentration thanks to the standard curve (cell density must be optimized to obtain compounds dose response curves within the dynamic range of the standard curve). In the examples presented here, CHO cells stably expressing the muscarinic M1 receptor were treated with different pharmacological class of compounds (full agonist, partial agonist and antagonist). Potencies (EC50 and IC50) and efficacies were reported in the table and agreed with published values.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:

https://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/alphalisa-alphascreen-no-wash-assays/alpha-troubleshooting.html

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