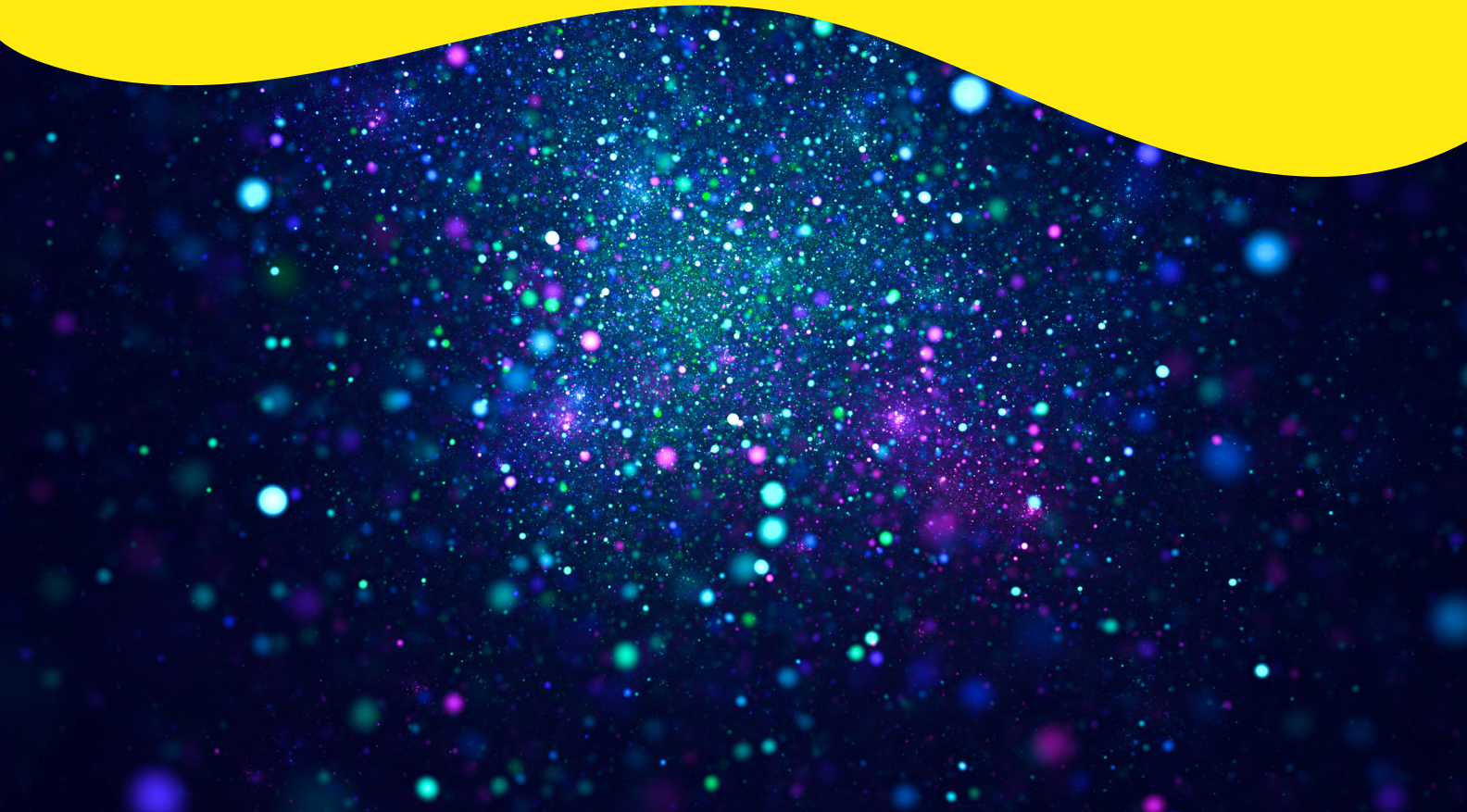
ELISA to Alpha

Immunoassay conversion guide



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Immunoassay conversion

This guide presents the simple conversion of an ELISA or other immunoassay to an AlphaLISA™ immunoassay. AlphaLISA is a highly sensitive, no-wash alternative to your existing immunoassay. In the AlphaLISA assay, a biotinylated antibody and an antibody-conjugated AlphaLISA Acceptor bead are used to capture the target analyte. The biotinylated antibody associates with an Alpha streptavidin-coated Donor bead. When the analyte is present in the sample, the Donor and Acceptor beads are brought together. Upon excitation, a photosensitizer inside the Donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen diffuses up to 200 nm to produce a chemiluminescent reaction in the Acceptor bead, leading to light emission. The amount of light is proportional to the amount of analyte present in your sample, and the assay can be quantitated by running against a standard curve.

The main differences between an AlphaLISA assay and other immunoassay formats are:

- A reduced number of assay steps offers maximum precision and improved CVs while simplifying workflow.
- With 100s of peer-reviewed citations available, Alpha technology is tested and proven for your application.
- Virtually anything can be measured with Alpha Technology using our off-the-shelf kits or custom solutions.
- Alpha is the versatile choice, allowing you to measure even large biomolecules (up to 2000 kDa) and very weak interactions (mM).

For specific information on your application, as well as detailed assay principles, please visit: www.revvity.com

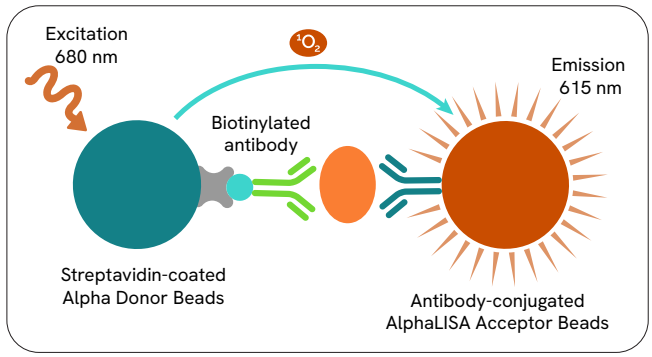


Figure 1: AlphaLISA immunoassay principle

Simple protocol: Just 4 steps to results

Table 1: A workflow comparison between AlphaLISA and standard immunoassays.

Alpha technology

Standard immunoassay

Assay design

The most common AlphaLISA immunoassay format is based on traditional "sandwich" configuration, which uses a streptavidin-coated Alpha Donor bead and an antibody-conjugated AlphaLISA Acceptor bead. One antibody is biotinylated and will associate with the streptavidin-coated Donor bead. The other antibody is unlabeled and can either be directly conjugated to an AlphaLISA Acceptor bead (for a direct assay format ***(Refer to figure 2a)***, or can indirectly associate with a Protein A, Protein G, Protein L, or anti-species AlphaLISA bead (for an indirect assay format) ***(Refer to figure 2b)***. The direct conjugation format is preferable if you will be working with serum or plasma samples, as the direct format is less sensitive to interference from IgG antibodies in the serum or plasma sample itself.

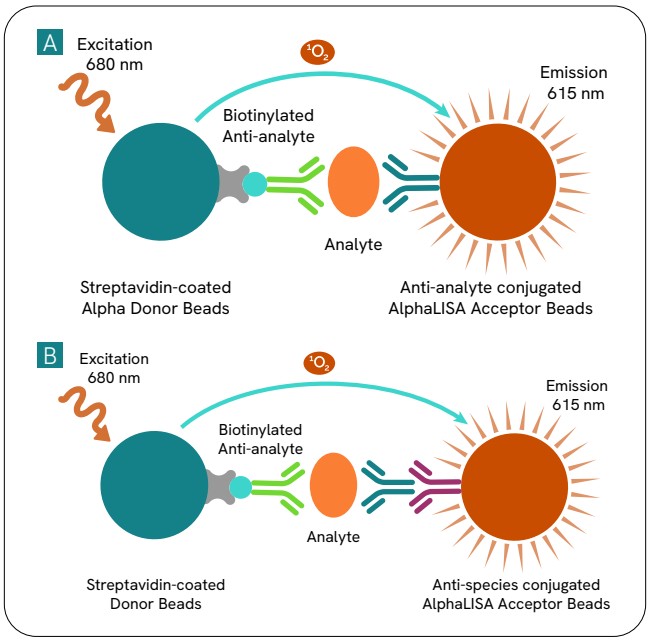


Figure 2: AlphaLISA sandwich immunoassay formats. a) Direct assay format: one of the antibodies is directly-conjugated to the AlphaLISA Acceptor beads. In the example above, the second antibody is biotinylated, to associate with a streptavidin Alpha Donor bead. b) Indirect assay format: each antibody associates with a streptavidin-coated, anti-species antibody-coated, or Protein A, Protein G, or Protein L-coated bead.

A variety of unconjugated and pre-coated bead products are available for your assay. If you will be using an indirect assay format, there are two main considerations:

1. You need to make sure that each antibody can only associate with either the Alpha Donor bead, or the AlphaLISA Acceptor bead. If one of your antibodies can strongly bridge both the Donor and Acceptor bead in your assay, you may see high background.

2. You need to make sure that your Donor and Acceptor beads cannot bind each other (in the absence of analyte and sandwiching antibodies). For more information on bead selection, including bead pairings that can crossreact, please refer to www.revvity.com.

Table 2: Alpha Donor and AlphaLISA Acceptor beads for immunoassay design.

Bead coating	Toolbox Alpha donor beads	Toolbox AlphaLISA Acceptor beads
Streptavidin	6760002	AL125
Strep-Tactin®	AS106	AL136
Protein A	AS102	AL101
Protein G		AL102
Protein L		AL126
Anti-rabbit IgG	AS105*	AL104*
Anti-mouse IgG	AS104*	AL105*
Anti-human IgG		AL103*
Anti-rat IgG		AL106*
Anti-goat IgG		AL107*
Anti-sheep IgG		AL132*
Anti-mouse IgM		AL130
Anti-chicken IgY		AL131
Anti-IgG1 **Coming soon!		AL141
Anti-IgG4 **Coming soon!		AL142
Lens culinaris agglutinin **Coming soon!		AL140
Unconjugated	6762013	6772001

* Fc-specific antibody

Biotinylation and direct bead conjugation protocols

The most common AlphaLISA immunoassay design involves a streptavidin-coated Donor bead, one biotinylated antibody, and a second antibody directly-conjugated to an AlphaLISA Acceptor bead. One of the two antibodies will have to be biotinylated and the other one should be conjugated to AlphaLISA Acceptor beads. It is recommended to test the two possible combinations as the sensitivity and the level of counts may differ dramatically depending on the set-up. The two combinations are as follows:

1. Biotinylated antibody A + antibody B-AlphaLISA Acceptor beads
2. Biotinylated antibody B + antibody A-AlphaLISA Acceptor beads

Therefore, it is recommended to biotinylate both antibodies of the pair as well as conjugating both to AlphaLISA Acceptor beads to eventually select the optimal assay configuration. If your current immunoassay already uses a biotinylated antibody and you want a quick assessment, it is possible to test a single orientation by using your biotinylated antibody on one side and to conjugate the second antibody to the Acceptor beads (or to capture the second antibody using a Toolbox Acceptor bead).

Antibody biotinylation protocol

- Many antibodies can be purchased in biotinylated form. These should perform well in AlphaLISA assays.
- The biotinylation will perform best when the antibody concentration is at least 0.5 mg/mL.
- The antibodies must not be in any amine-based buffer, including Tris, glycine, bicine, tricine, etc. If buffer exchange is necessary, the buffer should be replaced with a neutral to slightly alkaline buffer, such as PBS or carbonate buffer pH 8. Optimal performance will be obtained in sodium azide-free conditions.
- Antibody solutions must not contain any protein or peptide-based stabilizers (such as BSA or gelatin).
- The antibody will be labeled at slightly alkaline pH values (7.0-8.0) in aqueous buffer. Ensure that the antibody is soluble in these conditions.

Recommended reagents:		
NHS activated biotinylating reagent	(ChromaLink) SoluLink Inc.	#B1001-105
Zeba desalting columns	Pierce (ThermoFisher Scientific Inc.)	#89882 (0.5 mL) #89889 (2 mL) #89891 (5 mL) #89893 (10 mL)

1. Prepare antibody solution. If the antibody is provided in a powder form, resuspend at 5 mg/mL in PBS. If already in solution at neutral to slightly alkaline pH (pH ≥ 7.0), use as provided.
2. On the day of the assay, prepare a fresh solution of N-hydroxysuccinimido-ChromaLink-biotin (NHS-ChromaLink-biotin, SoluLink Inc. Catalog number B1001-105) at 2 mg/mL in PBS. Alternatively, other NHS reagents such as NHS-biotin, NHS-LC-biotin or NHS-LC-LC-biotin can also be used.
3. Add NHS-ChromaLink-biotin to the antibody solution. A 30:1 molar ratio of biotin to antibody is recommended. This represents using 7.6 µL of a 2 mg/mL NHS-ChromaLink-biotin solution for 100 µg of antibody. Adjust the volume to 200 µL with phosphate buffer pH 7.4.
4. Incubate for 2 hours at 21-23 °C.
5. When using NHS-ChromaLink-biotin, a purification step using Zeba columns is required to remove free biotin. To evaluate biotinylation efficiency, refer to SoluLink's website (http://solulink.com).

Protocol for direct conjugation of an antibody to an AlphaLISA Acceptor bead:

Protocol for conjugating 1 mg AlphaLISA Acceptor beads (10:1 coupling ratio). This procedure is appropriate for an antibody solution of ≥ 0.5 mg/mL. For conjugating larger amounts of beads, please refer to the technical brief, "Antibody conjugation to AlphaLISA™ Acceptor beads -*detailed protocol*".

Recommended reagents:		
Carboxymethylamine hemihydrochloride (CMO)	Sigma-Aldrich Co.	#C13408
Sodium cyanoborohydride ($NaBH_3CN$)	Sigma-Aldrich Co.	#296945
Unconjugated AlphaLISA Acceptor beads	Revvity	#6772001 (1 mg)

1. **Bead washing.** In a 1.5 mL Eppendorf tube, wash AlphaLISA Acceptor beads (50 µL at 20 mg/mL) once: add 50 µL PBS, centrifuge at 16,000 × ***g*** (or maximum speed) for 15 min and then discard the supernatant using a pipette tip. (Do not tilt the tube to prevent the pellet from coming off).

2. **Conjugation.** Prepare a fresh working $NaBH_3CN$ solution at 400 mM by diluting the 5 M stock solution 12.5X in water. In the Eppendorf tube containing 1 mg of AlphaLISA Acceptor bead pellet (prepared as described above), add:

   - 0.1 mg of antibody
   - the appropriate volume of 130 mM sodium phosphate buffer pH 8.0 to obtain a final reaction volume of 200 µL
   - 1.25 µL of 10% Tween-20
   - 10 µL of a 400 mM solution of $NaBH_3CN$ in water
   - Resuspend pellet by vortexing and/or pipetting and incubate for 18-24 hours at 37 °C with mild agitation (6 – 10 rpm) using a rotary shaker. Longer incubation times up to 48 hours might be used, which could result in higher conjugation yields.

3. **Blocking** (optional, may improve long term stability of beads)

   - Prepare a fresh 65 mg/mL solution of carboxymethoxylamine (CMO) in 800 mM NaOH.
   - Add 10 µL of CMO solution to the reaction (to block unreacted sites).
   - Incubate for 1 hour at 37 °C using a rotary shaker (6 – 10 rpm).

4. **Washing**

   - Centrifuge for 15 minutes at 16,000 × ***g*** (or maximum speed) at 4 °C.
   - Remove the supernatant with a micropipette and resuspend the bead pellet in 200 µL of 100 mM Tris-HCl pH 8.0.
   - Centrifuge for 15 minutes at 16,000 × ***g*** (or maximum speed) at 4 °C, and then remove the supernatant.
   - Repeat the washing step (resuspend the pellet and centrifuge) another time.
   - After the last centrifugation, resuspend the beads at 5 mg/mL in storage buffer (200 µL of PBS + 0.05% Proclin-300 as a preservative).
   - Vortex, briefly spin down and sonicate the bead solution (20 short pulses of 1 second using a probe sonicator; the sonicator power should not exceed 20% of the maximal power). This step is recommended but not mandatory – the sonicator helps resuspend the bead pellet.

5. **Storage**

   - Store the conjugated Acceptor bead solution at 4 °C in an opaque vial.
   - Important note: always vortex conjugated AlphaLISA Acceptor beads before use, as beads tend to settle with time.

Before you begin

- Alpha assays require a special reader capable of measuring an Alpha assay, such as an EnVision™ or EnSpire™ Multimode plate reader. Many standard time-resolved fluorimeters and luminometers cannot read Alpha assays.
- We recommend preparing only what you need for the day's experiments. Do not store working dilutions of beads for more than one day.
- You should vortex and centrifuge (< 1 minute) your beads prior to use, as beads may settle over extended storage times. A quick spin will not pellet your beads - Alpha, AlphaScreen, and AlphaLISA beads are made of latex with a dextran coating, are only 200 nm in size, and have a density close to 1. If you do need to pellet your beads, we recommend spinning at 16,000 x g for 15 minutes.
- Order-of-addition can have a dramatic effect on assay signal. We recommend that you follow the suggested protocols as indicated for your initial optimization.
- The Donor beads used in Alpha assays are somewhat light sensitive. We recommend working under subdued lighting conditions when working with the beads (less than 100 Lux - the level of light produced on an overcast day). For example, you can turn half of the laboratory lights off and work at a bench away from windows and where the overhead light is not on. Incubate the plate in the dark (for example, placing the covered or sealed plate in a drawer).
- Such as with most assay platforms, the Alpha signal is temperature-dependent. If you will be performing incubations at 37 °C or other temperatures, we recommend that you equilibrate the plate back to room temperature before reading to ensure signal uniformity across the plate.

Optimization

Step 1: testing of antibody configuration/biotinylated antibody titration

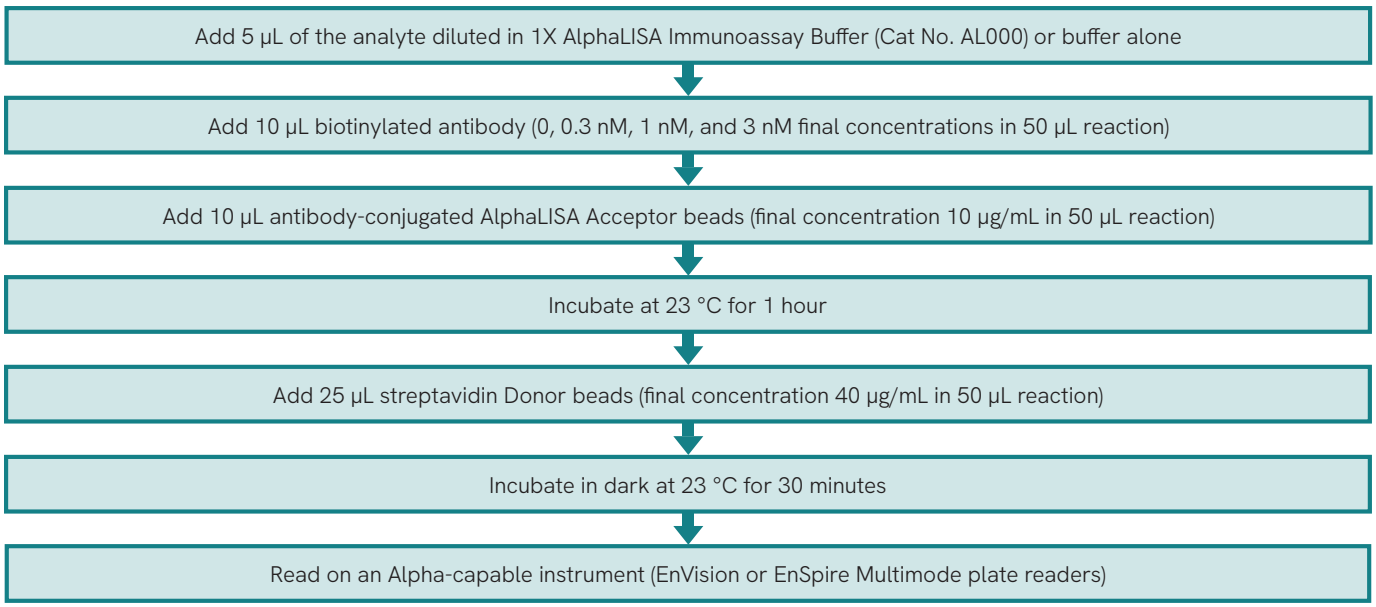
For the direct assay format, an assay should be performed using three concentrations of biotinylated antibody with and without analyte. For this step, a mid-range level of analyte must be set and held constant. From our experience of developing hundreds of assays, 3 ng/mL should be adequate for most assays.

It is recommended to test both possible assay configurations as the sensitivity and the level of counts may differ dramatically depending on the set-up. The two configurations are as follows:

1. Biotinylated antibody A + antibody B-AlphaLISA Acceptor beads
2. Biotinylated antibody B + antibody A-AlphaLISA Acceptor beads

Protocol for 50 µL assay in white 96-well ½ AreaPlate™

(*all reagents diluted in 1X AlphaLISA Immunoassay Buffer, Cat. No. AL000)



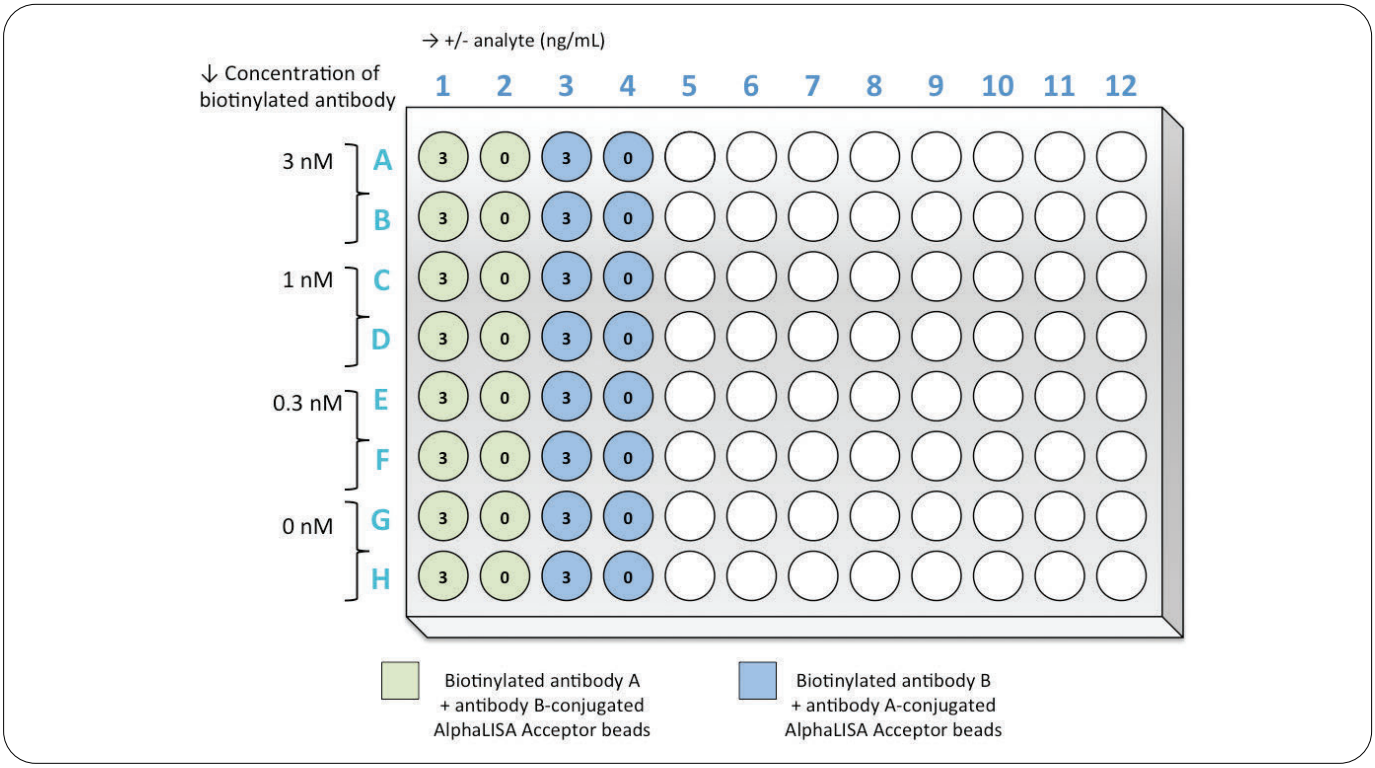


Figure 3: Plate map to test antibody configuration in a direct assay format. Two configurations are tested. The concentration of biotinylated antibody is titrated down the rows of the plate. Columns for analyte (3 ng/mL final concentration) or no analyte.

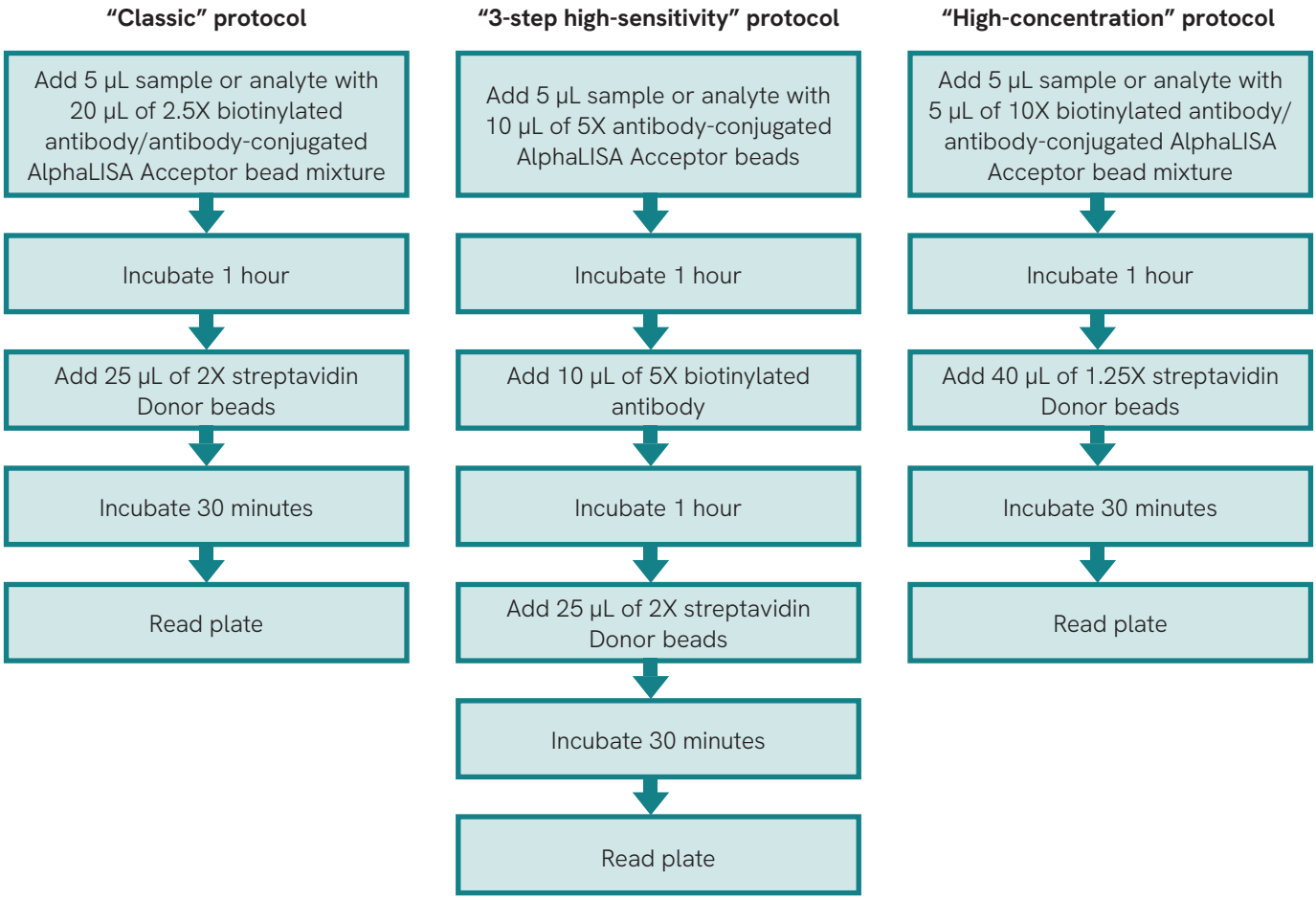
If you will be using an indirect assay format, both antibodies will need to be titrated in a cross-titration experiment. For more information, refer to the "AlphaLISA Immunoassay Conversion Quick Start Guide."

Step 2: protocol variations

The classic AlphaLISA immunoassay protocol involves adding sample with biotinylated antibody and antibody-conjugated AlphaLISA Acceptor beads first, incubating, then adding streptavidin Donor beads for a final incubation before measuring the plate. For indirect assay formats, the antibodies are typically added first (followed by an incubation step), then the Acceptor beads are added (followed by an incubation step), then the Donor beads (followed by a final incubation step). Order-of-addition can influence the sensitivity of the assay, the working dynamic range of the assay, and the assay signal. Assay components can be added one-at-a-time, in mixtures, or all in one step. The optimal order in which assay components interact should always be determined empirically for your particular assay.

Another protocol variation involves altering the binding equilibrium by changing the volumes of each intermediate step. The classic AlphaLISA immunoassay protocol first incubates 5 µL of sample with 20 µL biotinylated antibody/AlphaLISA Acceptor bead mixture for one hour. A "high concentration" protocol involves mixing 5 µL of sample with of a more-concentrated biotinylated antibody/AlphaLISA Acceptor bead mixture. This protocol changes the equilibrium of binding during the first incubation step while keeping the final concentration of all reagents the same, and can lead to greater assay sensitivity.

Table 3: Various AlphaLISA immunoassay order-of-addition protocols[1].



1 Total assay volume will be 50 µL in a white 96-well ½ AreaPlate (Cat. No. 6005560). Final concentration of biotinylated antibody as determined from previous experiment. Final concentration of AlphaLISA Acceptor beads = 10 µg/mL. Final concentration of Streptavidin Donor beads = 40 µg/mL. Analyte standard should be diluted in a matrix that closely matches your sample matrix. Beads and biotinylated antibody should be diluted in 1X AlphaLISA Immunoassay Buffer, Cat. No. AL000.

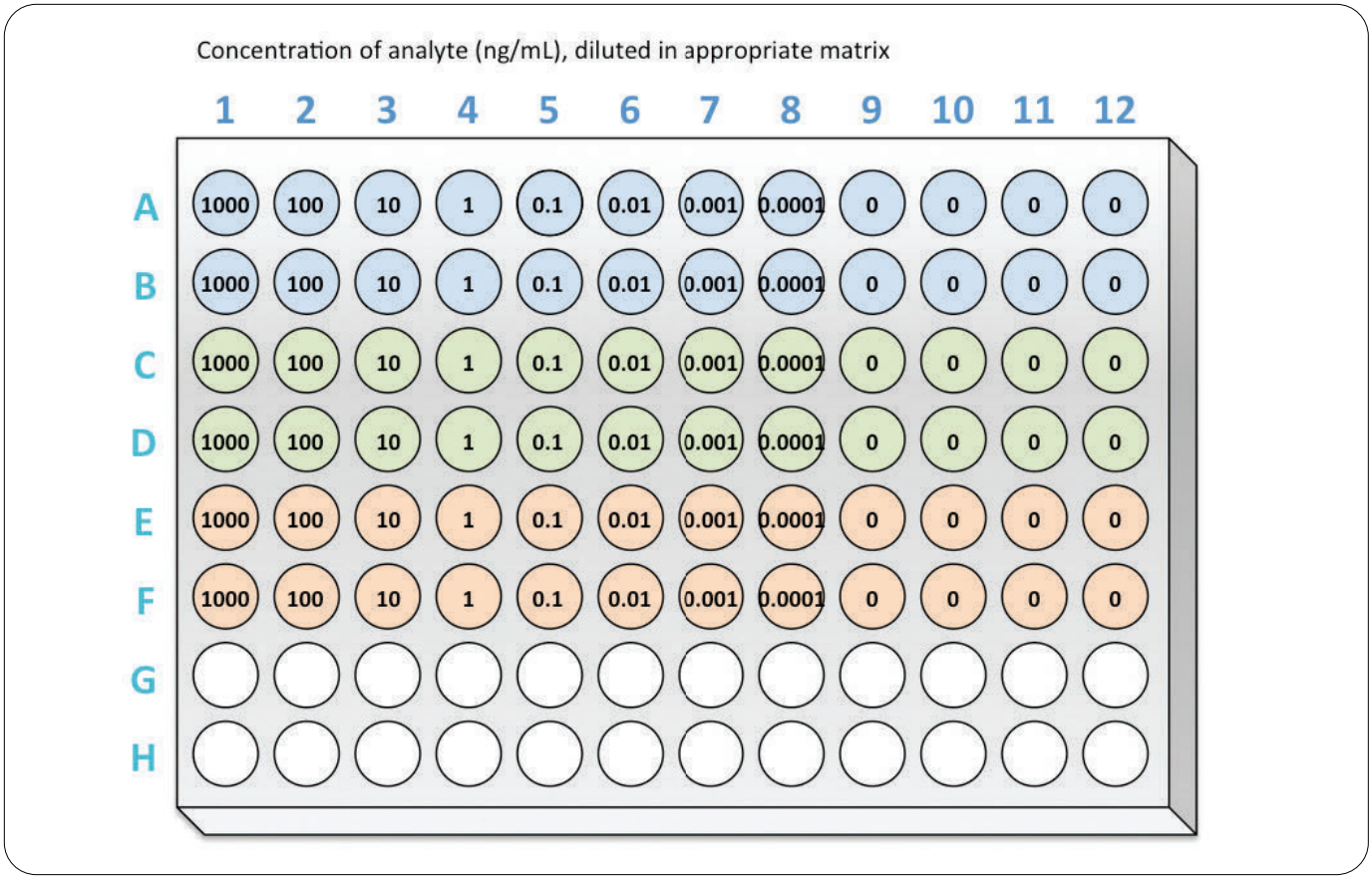


Figure 4: Plate map to test various order-of-addition protocols. Standard curves ranging from 0 to 1000 ng/mL analyte are set up in duplicate for each protocol tested. It may be easier to set up three plates, each with a different protocol.

1. For this optimization step, you will evaluate the sensitivity and dynamic range of the assay using various order-of-addition protocols to create a standard curve. For information on how to process your data, refer to Appendix A.

2. You will need to make your analyte serial dilutions (ranging from 1 µg/mL to 0.1 pg/mL) in a diluent that most-closely matches your sample matrix. For example, if you will be using serum samples for your assay, you could dilute your analyte in fetal bovine serum (FBS). If you will be using cell supernatants for your assay, you should dilute your analyte in the same type of culture media in which your cells are grown.

3. All other reagents (beads and biotinylated antibody) should be diluted in 1X AlphaLISA Immunoassay Buffer (Cat. No. AL000).

Possible further optimizations for AlphaLISA immunoassays

It is possible to further optimize your assay by titrating your beads and changing the incubation times. These optimizations are optional. In cases of high background, the assay buffer can also be optimized.

Table 4: Other parameters that may be optimized for an AlphaLISA immunoassay. These optimization steps are optional.

AlphaLISA optimization parameter	Recommendations and comments
Bead titration	Suggested range: 10 µg/mL to 40 µg/mL of Donor or Acceptor bead Start with: A cross-titration matrix, varying the concentration of Donor bead across each column (10, 20, 30, and 40 µg/mL) and Acceptor bead down each row (10, 20, 30, and 40 µg/mL). This will create 16 combinations of Donor and Acceptor beads.
Incubation time	Suggested range: 30 minutes – 2 hours per incubation step Start with: 60 minute incubation steps Incubation times may need to be lengthened (or can sometimes be shortened) depending on the kinetics of binding of your antibody to the analyte. Quick interactions (for example, binding of streptavidin to biotin) may only require a 15 or 30 minute incubation to reach equilibrium. Slower interactions may require longer times for association.
Buffer for diluting beads and biotinylated antibody	Start with: 1X AlphaLISA Immunoassay Buffer (Cat. No. AL000) and biotinylated antibody For most assays, 1X AlphaLISA Immunoassay Buffer will result in best performance. This buffer contains casein, dextran, and detergent. If your background is high following assay optimization, you may try 1X HiBlock Buffer (Cat. No. AL004) instead. This buffer contains BSA and gelatin as additional buffer components.
Assay volume	Start with: 50 µL final volume in white 96-well ½ AreaPlate (Cat. No. 6005560) The assay can easily be miniaturized to 384-well or 1536-well format by simply proportionally decreasing the volumes of each addition step. The concentrations of each reagent should remain the same.

Standard curve diluents and assay buffers

Standard curve diluents

For quantitation of analyte in your samples, it is necessary to create a standard curve of your analyte in a diluent that closely matches your samples. For example, if you are working with serum samples, you will need to create a standard curve of your analyte in analyte-depleted serum or something similar, such as fetal bovine serum (FBS). The use of FBS as a diluent for your standard curve can be very convenient when working with serum samples (as long as no component of the FBS can cross-react with your antibodies or otherwise interfere with the assay).

Table 5: Examples for possible diluents for your standard curve, for various sample types. Your standard curve diluent should closely match your sample matrix.

Sample type	Possible diluents for standard curve
Serum	FBS (or analyte-depleted serum)
Cell supernatant	The same culture media as used to treat your cells
Cell lysate	Lysis buffer (We recommend AlphaLISA Lysis Buffer, Cat. No. AL003, for creating cell lysates.)
Unusual sample types (cerebrospinal fluid, amniotic fluid, etc.)	Various diluents should be tested. In some cases where a perfect match for the fluid cannot be found, or where the sample matrix interferes with the assay, sample dilution may be required. For example, you may need to dilute your sample 2X in PBS + 0.1% BSA, then run your standard curve in PBS + 0.1% BSA.

A linearity experiment and a spike-and-recovery experiment should be performed to assess whether a proposed diluent is suitable for your sample type. For more information on how to perform these experiments, refer to Appendix B.

Assay buffers

The assay buffer refers to the buffer used to dilute your Donor beads, Acceptor beads, and antibodies. We offer multiple buffers for AlphaLISA immunoassays. Most assays will work best using 1X AlphaLISA Immunoassay Buffer for dilution of your beads and antibodies.

Table 6: Assay buffer options for dilution of antibodies and beads.

Assay buffer	Catalog number	Key buffer components	Recommended for:
1X AlphaLISA Immunoassay Buffer	AL000C/F	HEPES buffer pH 7.4, casein, Dextran-500, Triton X-100	Most AlphaLISA immunoassays
1X AlphaLISA HiBlock Buffer	AL004C/F	HEPES buffer pH 7.4, casein, Dextran-500, Triton X-100, BSA, and gelatin	Optimized assays that exhibit high background due to sample matrix
1X AlphaLISA NaCl Buffer	AL007C/F	High concentration of NaCl with HEPES buffer pH 7.4, casein, Dextran-500, Triton X-100, and gelatin	Optimized assays that exhibit high background due to sample matrix, but where it is not allowable for BSA to be present in the buffer (alternative to AL004)

Tips and troubleshooting

- Alpha assays require a special reader capable of measuring an Alpha assay, such as our EnSpire or EnVision Multimode Plate Readers. Many standard time-resolved fluorimeters and luminometers cannot read Alpha assays.
- We recommend preparing only what you need for the day's experiments. Do not store working dilutions of beads for more than one day.
- The Donor beads used in Alpha assays are somewhat light sensitive. We recommend working under subdued lighting conditions when working with the beads (less than 100 Lux - the level of light produced on an overcast day). For example, you can turn half of the laboratory lights off and work at a bench away from windows and where the overhead light is not on. Incubate the plate in the dark (for example, placing the covered or sealed plate in a drawer).
- The Alpha signal is temperature-dependent. If you will be performing incubations at 37 °C or other temperatures, we recommend that you equilibrate the plate back to room temperature before reading to ensure signal uniformity across the plate.
- We recommend using white plates, or light grey AlphaPlates™, for these assays.

Problem	Cause	Solution
High background	Bead concentration is too high	We recommended using 40 µg/mL Donor beads and 10 µg/mL Acceptor beads (final concentration). Higher levels of beads may lead to high background.
	One of the antibodies can bridge both the Donor and Acceptor beads simultaneously	Re-evaluate bead selection. Example: Use of a biotinylated rabbit IgG antibody in an assay using Streptavidin Donor beads and Protein A Acceptor beads. Protein A can bind strongly to rabbit IgG antibodies. Because of this, one single biotinylated rabbit IgG antibody can bring together both a Streptavidin Donor bead and a Protein A Acceptor bead in the absence of analyte.
	Buffer selection	We recommend using AlphaLISA Immunoassay Buffer (Cat. No. AL000) for dilution of your beads and antibodies. If this buffer still gives high background, you may need to try AlphaLISA HiBlock Buffer (Cat. No. AL004) or AlphaLISA NaCl Buffer (Cat. No. AL007).
	Bead selection	Certain bead pairs can associate with themselves in the absence of other assay components. For more information, refer to www.revvity.com.
	Pre-mixture of components	Pre-mixing reagents that may cross-react could result in high background.
	Contamination	You may be inadvertently introducing target analyte from your skin, saliva, etc. while preparing your reagents. If you are measuring a human analyte, take appropriate measures to prevent contamination.
	Interference by diluent used	Change or evaluate other buffers.
	Assay configuration	Switch antibody configuration and evaluate alternate protocols.

Problem	Cause	Solution
Low maximal signal	Signficant exposure of Donor beads to light	Alpha Donor beads are somewhat light-sensitive. If your beads have been exposed to light for a prolonged period, you may need to use fresh beads.
	Matrix interference	Some components of cell culture media may interfere with the AlphaLISA signal. If possible, try switching to a different medium.
	Order-of-addition	Some binding partners may interfere with the association of other binding partners if allowed to interact in the wrong order. Try an alternate order-of-addition protocol.
	Incubation time	You may need to extend the incubation time of your protocol steps to allow more time for antibody to bind target.
	Hook effect	The signal should increase with increasing concentrations of analyte, until you reach saturation (the Hook Point). At analyte concentrations above the Hook point, signal will begin to decrease. Make sure you are in the right range of your assay. The easiest way is to perform a full titration curve of the analyte.
	Instrument settings	Verify instrument settings and ensure instrument is working properly
	Verify concentrations of your reagents	If possible, take analyte OD measurements, and double-check calculations for preparation of working solutions.
	Antibody selection	You may need to test other antibodies in your assay.
Poor sensitivity	Order-of-addition	Try an alternate order-of-addition protocol (3-step protocol) – order-of-addition can influence assay sensitivity.
	Matrix interference	Some complex matrices may contain components that can interfere with the assay. For example, biotin present in culture media may interfere with binding of a biotinylated antibody to streptavidin Donor beads. You may need to dilute your samples in a buffer that will not interfere with the assay.
	Antibody selection	Try a different assay configuration (switch biotinylated and bead-conjugated antibody), or try a different antibody.
	Analyte selection	Make sure you have chosen an appropriate standard analyte for your antibodies and your assay. Recombinant versions of your analyte may be available in full-length or cleaved form, in active or inactive form, etc.
Cannot fit a standard curve	Trying to fit sigmoidal data to a linear curve	AlphaLISA data should be plotted to fit a dose response (or sigmoidal, 4PL) curve. If you would prefer to use just the linear portion of the curve, you will need to omit some of the higher and lower concentrations of analyte from your data.
	Hook effect	The signal should increase with increasing concentrations of analyte, until you reach saturation (the Hook Point). At analyte concentrations above the Hook point, signal will begin to decrease (resulting in a bell-shaped/parabolic curve). Omit higher concentrations of standard and re-fit the data to a sigmoidal curve.

Problem	Cause	Solution
Shifted curve (curve shifts right or left)	Order-of-addition	Your order-of-addition must stay consistent from assay-to-assay. Changing the order in which assay components are added can shift your standard curve.
	The diluent used for the standard curve was changed	The diluent used for your standard curve must closely match your sample type, and must be consistent from assay-to-assay. If your diluent was changed, this would likely cause a shift in your standard curve. If you are measuring different sample types, you need to use different diluents for each of your standard curves.
	Change in analyte	If your analyte has changed (for example, you changed lots and the new lot has a different level of glycosylation) or become degraded, your curve may shift.
	Sticky analyte	If your analyte is sticky and can stick to the walls of your tubes or pipette tips, you will see a curve shift reflecting the lower soluble concentration of analyte. You may need to use low-bind tubes and tips.
	Change in antibody lot	If you are working with a polyclonal antibody, a change in antibody lot may result in a curve shift due to differences in affinity.
% recovery too low or too high	Spike-in concentration is not within the dynamic range of the assay	Try using a different spike-in concentration for assessing % recovery.
	Selection of diluent for standard curve.	Try using an alternate diluent for your standard curve. Your diluent for the standard curve must match your sample matrix as closely as possible. You might also need to dilute your samples to decrease matrix interference.
AlphaLISA results do not match ELISA results	***It is important to determine which assay is giving accurate results. If possible, use a third method to assess which assay is accurate.**	
	Standard curve is not run in a diluent similar to the sample type	The standard curve should be run in a diluent that closely resembles your sample type. For example, if you are working with cell culture supernatant samples, you should run your standard curve in the same culture media used to treat your cells.
	Change in antibody	If you use different antibodies, you may see different selectivity. Different results may be obtained.
	Change in analyte	If you use a different standard for your standard curve, different results may be obtained.
	Standard curve is not being fit properly (e.g., sigmoidal data is being fit to a linear curve)	AlphaLISA data should be plotted to fit a sigmoidal curve. Data weighting is recommended ($1/Y^2$) to get the best curve fitting for lower concentrations. If you would prefer to use just the linear portion of the curve, you may need to omit some of the higher and lower concentrations of analyte from your data.
	Buffer selection	Your target analyte may exist in a state bound to another molecule in your sample and cannot be recognized by the antibodies. If this is a possibility, we recommend using AlphaLISA Dissociation Buffer (Cat. No. AL006) to disrupt the interaction within your sample.
	Concentration of analyte in your samples is too high or too low (beyond the dynamic range of the assay)	Sample dilution may be required to adjust your samples to be within the dynamic range of the assay. Refer to Hook effect – concentrations of analyte above the Hook point can give low signal.

Appendix A: AlphaLISA immunoassay data analysis

AlphaLISA immunoassay data can be fit to either a linear curve (using just the linear portion of your data) or a dose-response curve. A dose response curve (or sigmoidal or 4PL curve) is typically used to process AlphaLISA immunoassay data in order to take advantage of the full dynamic range of the assay. These types of curves can be fit using standard statistical software, such as GraphPad Prism® or Microsoft® Excel® with Solver plug-in. After fitting a standard curve, you can interpolate the concentration of analyte for your unknowns.

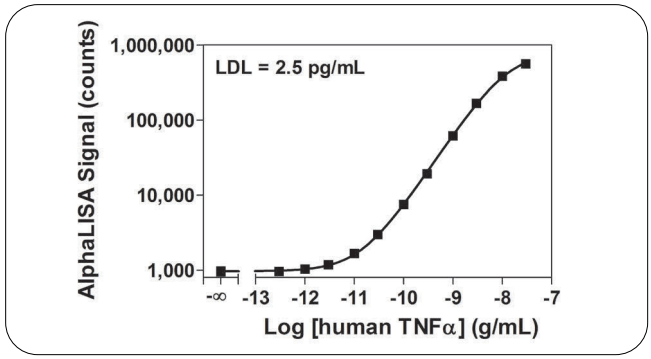


Figure 5: Standard curve for an AlphaLISA TNFα assay. The standard curve was run in AlphaLISA Immunoassay Buffer for these experiments. The data were fit in GraphPad Prism® using a dose-response curve.

If your standard curve begins to decrease at the higher concentrations of analyte tested (see Fig. 6), you may be seeing a “hook effect”. The hook effect occurs when you have saturated your beads with analyte. Excess analyte disrupts associations between Donor and Acceptor beads beyond the hook point.

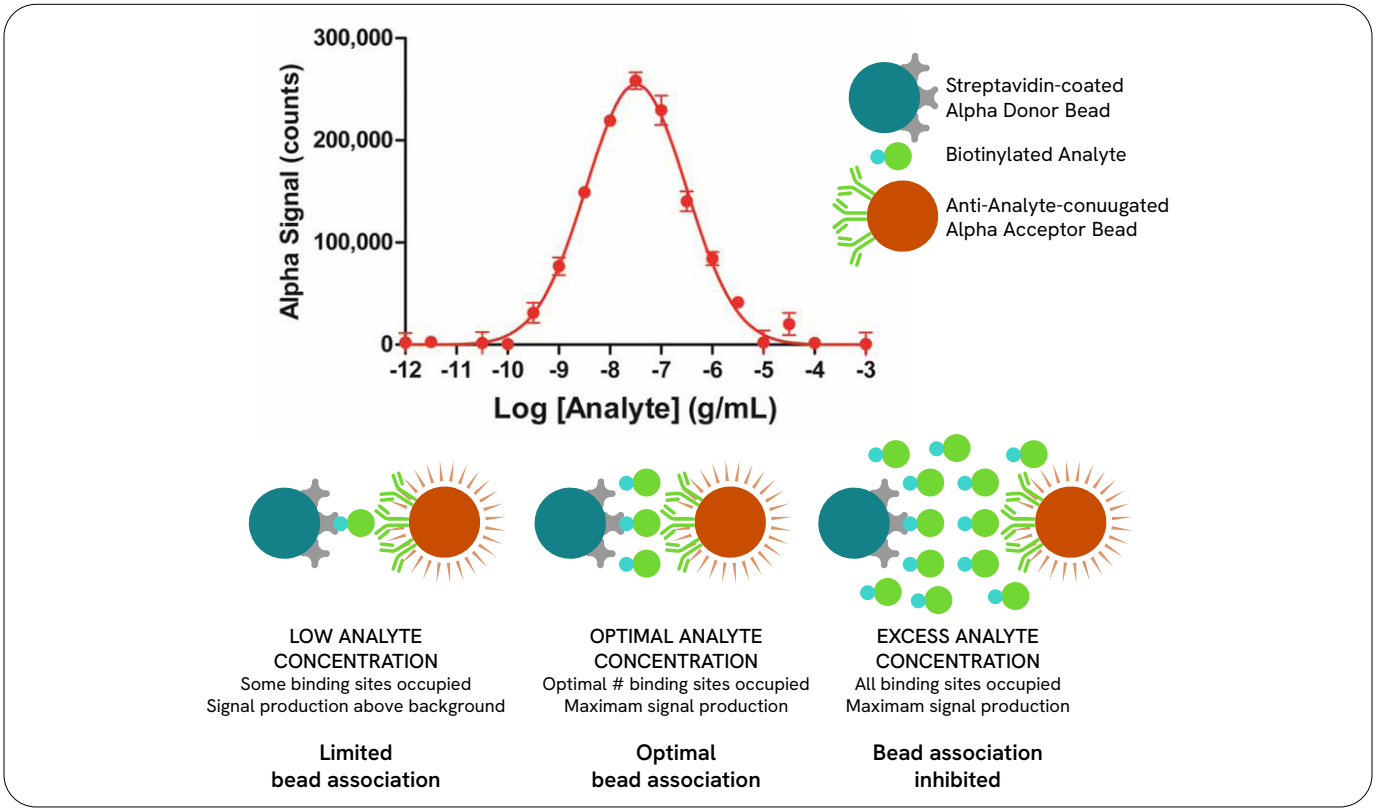


Figure 6: The hook effect. The top graph shows data for an AlphaLISA immunoassay. The signal increases with increasing concentrations of analyte, until you reach the hook point. At this point, the signal begins to decrease with increasing concentrations of analyte. The illustration underneath shows how increasing concentrations of analyte can disrupt interactions between Donor and Acceptor bead when the system is saturated.

If you see a hook effect, simply omit the standards with concentrations beyond the hook point from your data before fitting your standard curve.

Using your standard curve, you can calculate the sensitivity (lower detection limit, LDL) for your assay using the following equation:

LDL = mean (zeroes) + 3 SD

The lower detection limit (LDL or LLD) is equivalent to the concentration interpolated from a signal corresponding to the mean of your “zero analyte” signals + 3 standard deviations.

Appendix B: linearity and spike-and-recovery experiments

For quantitation of an analyte in your samples, it is necessary to create a standard curve of your analyte in a diluent that matches your samples. In order to assess whether a proposed diluent is suitable for your sample type, a linearity experiment and a spike-and-recovery experiment should be performed.

If you are testing more than one proposed diluent, repeat the experiment(s) as many times as needed for each proposed diluent. A suitable diluent will show good linearity and good percent recovery.

Linearity experiment

1. Spike one of your experimental samples with a high concentration (e.g., 3 ng/mL) of your standard analyte.
2. Perform a 2-fold serial dilution of this spike-in using your proposed diluent to dilute the spike-in. You will want at least five dilutions.

Table 8: Preparation of spike-in dilution series.

Tube	Volume of standard analyte	Volume of diluent to add to sample	Concentration analyte in tube*	Dilution
1	60 µL of prepared high-concentration spike-in (3 ng/mL)	0	3 ng/mL	1
2	30 µL of prepared high-concentration spike-in (3 ng/mL)	30 µL proposed diluent being tested	1.5 ng/mL	0.5
3	30 µL of tube 2	30 µL proposed diluent being tested	0.75 ng/mL	0.25
4	30 µL of tube 3	30 µL proposed diluent being tested	375 pg/mL	0.125
5	30 µL of tube 4	30 µL proposed diluent being tested	187.5 pg/mL	0.0625
6	30 µL of tube 5	30 µL proposed diluent being tested	93.75 pg/mL	0.03125

*Disregards existing level of analyte in the sample used to create your spike-in, as this is expected to be low in comparison with the high, spiked concentration.

3. In a separate set of tubes, create a standard curve of your standard analyte in the proposed diluent. If you are using an AlphaLISA immunoassay kit, refer to the standard curve concentrations provided in the protocol/tech data sheet. If you are designing your own AlphaLISA immunoassay, you can use concentrations in the range assessed from experiments run in AlphaLISA Immunoassay Buffer. An example is shown below, for the AlphaLISA TNFα kit standard curve.

Table 9: Example for preparation of a TNFα standard curve.

			[TNFα] in standard curve	
Tube	**Vol. of TNFα (µL)**	**Vol. of diluent (µL) ***	**(g/mL in 5 µL)**	**(pg/mL in 5 µL)**
A	100 µL human TNFα standard (100 ng/mL)		1E-07	100 000
B	60 µL of tube A	140	3E-08	30 000
C	60 µL of tube B	120	1E-08	10 000
D	60 µL of tube C	140	3E-09	3 000
E	60 µL of tube D	120	1E-09	1 000
F	60 µL of tube E	140	3E-10	300
G	60 µL of tube F	120	1E-10	100
H	60 µL of tube G	140	3E-11	30
I	60 µL of tube H	120	1E-11	10
J	60 µL of tube I	140	3E-12	3
K	60 µL of tube J	120	1E-12	1
L	60 µL of tube K	140	3E-13	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

4. Run your AlphaLISA experimental protocol with your standards, spike-in, and spike-in dilutions (in triplicate). Fit your standard data to a curve and interpolate the concentration of your spike-in and spike-in dilutions (for more information, refer to Appendix A).
5. To assess linearity, plot the interpolated concentrations of your spike-in and spike-in dilutions versus the "Dilution" listed in Table 8 (1, 0.5, 0.25, 0.125, 0.0625, 0.03125). Perform a linear regression and assess linearity by correlation coefficient.

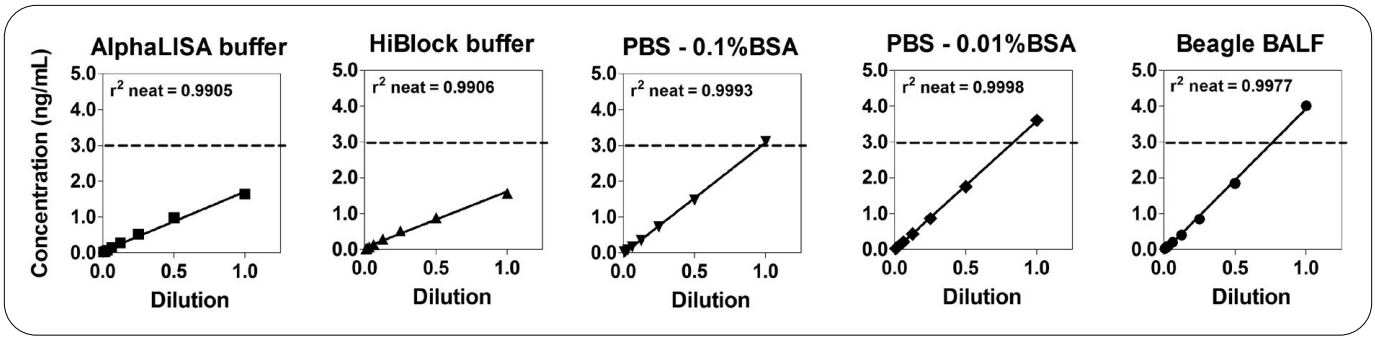


Figure 7: Linearity experiments for an AlphaLISA immunoassay to measure the amount of an analyte in bronchial lavage fluid (BALF) samples. Five linearity experiments were performed to assess the validity of using AlphaLISA Immunoassay Buffer, HiBlock Buffer, PBS + 0.1% BSA, PBS + 0.01% BSA, or Beagle BALF as a diluent for the standard curve. From these experiments, PBS + 0.1% BSA and Beagle BALF were chosen to be further assessed in spike-and-recovery experiments.

If good linearity (>0.995) is not seen with any of the proposed diluents, you may need to dilute your experimental samples 2-fold in order to decrease any interference from the sample matrix. You would adjust your calculated final sample concentrations by multiplying by the dilution factor, 2. The standard curve would be tested in the same diluent used for sample dilution. For more information, refer to the poster "Low volume, highly sensitive immunoassays for detecting cytokines in animal fluids".

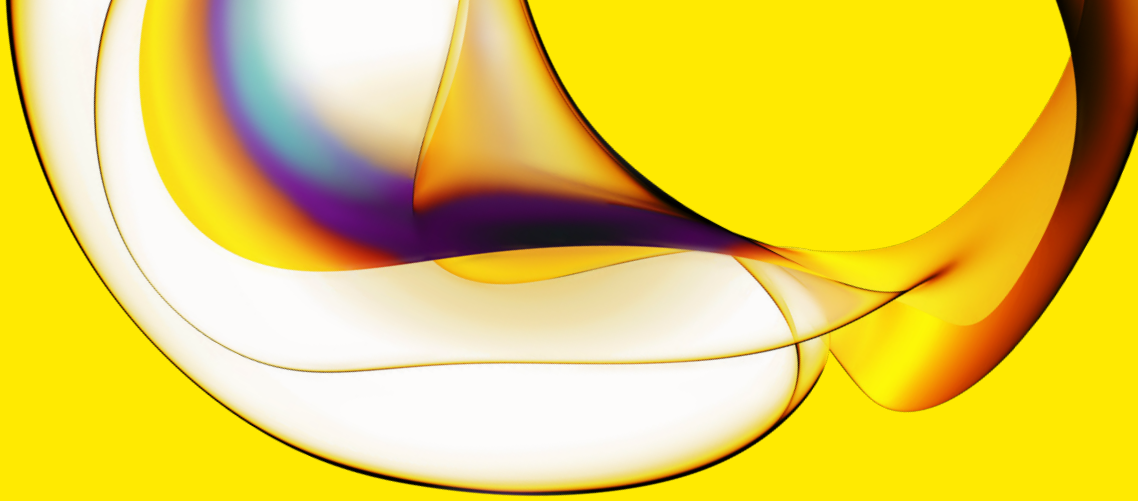
Spike-and-recovery experiments

1. In one set of tubes, spike one of your experimental samples with a low, a medium, and a high concentration of your analyte (include one tube that contains sample only – no spike-in). You should choose the low, medium, and high concentrations of analyte based on the dynamic range of your assay (refer to your previous standard curve data).
2. In a second set of tubes, take your proposed diluent (from linearity experiment) and spike with the same concentrations of analyte (include one tube that contains diluent only - no spiked analyte).
3. In a third set of tubes, create a standard curve of your standard analyte in the proposed diluent (refer to step 3 in Linearity Experiments, above).
4. Run your AlphaLISA experimental protocol with your standards and spike-ins. Fit your standard data to a curve and interpolate the concentration of your spike-ins.
5. Compare the interpolated concentrations of your sample spike-ins and diluent spike-ins. You will need to correct the interpolated concentrations of your spiked sample by subtracting the amount of analyte measured from your "No spike" sample from the low, medium, and high spike-in samples. Percent recovery is calculated using the following equation:

$$\text{Percent recovery} = \frac{\text{Calculated concentration of analyte in spiked sample}}{\text{Calculated concentration of spiked diluent}} \times 100$$

Table 10: Percent recovery data for an AlphaLISA immunoassay to measure TNFα in mouse bronchial lavage fluid (BALF) samples. PBS + 0.1% BSA was tested as a diluent for the standard curve. *Concentration for 10-3000 pg/mL spikes is equal to the measured concentration minus the no spike value (in this case, 17.1 pg/mL). Excellent recovery was achieved for all four spikes tested.

	Diluent: PBS + 0.1% BSA		
	Spiked diluent	Spiked sample (mouse BALF)	
Spike (pg/mL)	Concentration (pg/mL)	Concentration (pg/mL)*	Recovery (%)
No spike	0	17.1	n/a
10	12	9.2	76
30	35.2	32.3	92
300	300.2	292.6	97
3000	3141.2	2952.1	94



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