User Guide

AlphaPlex assay development guide

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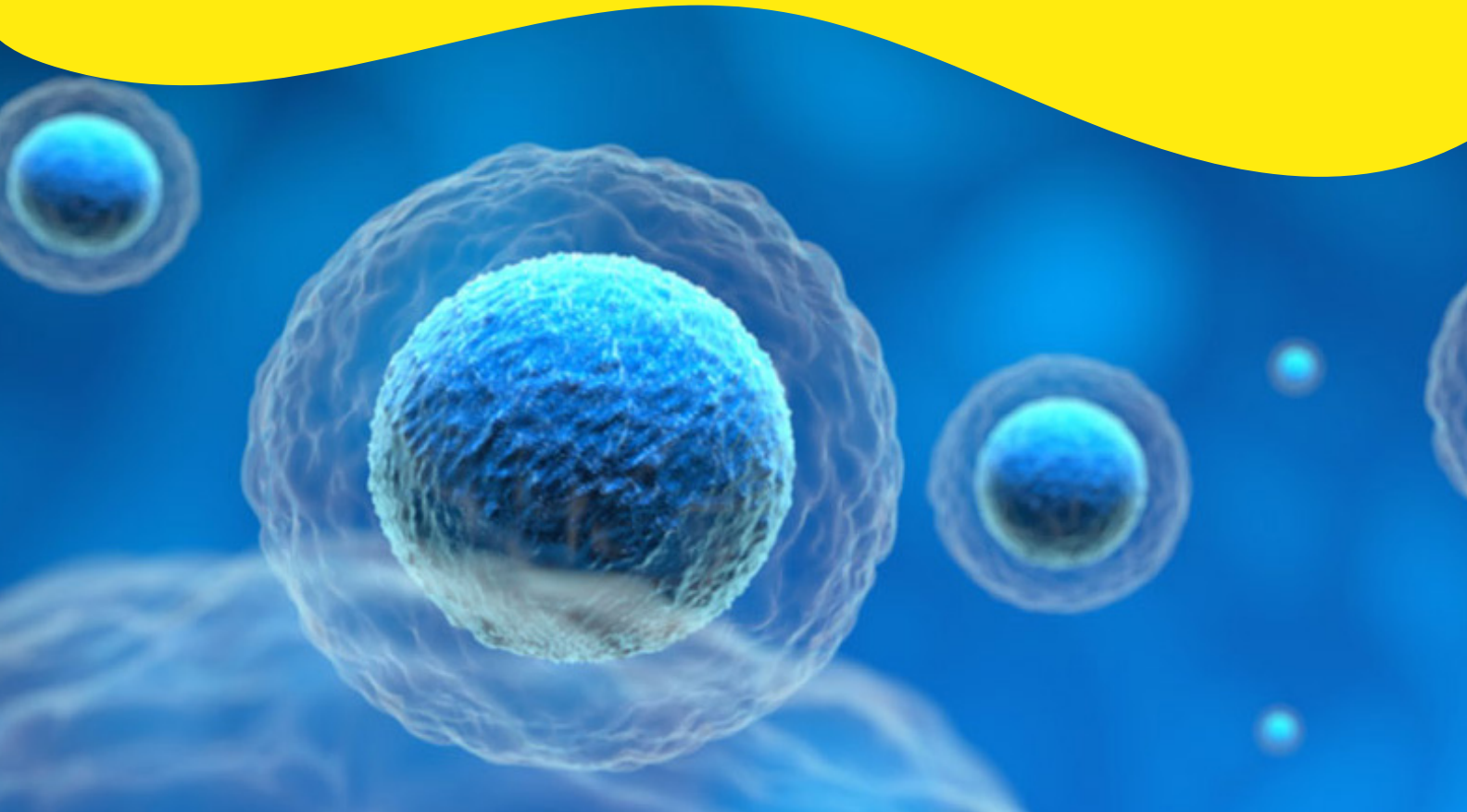


Notes and precautions

- In this guide, AlphaPlex™ Acceptor Beads refer to AlphaPlex 545 (terbium) and AlphaPlex 645 (samarium) beads and may also include AlphaLISA (europium) beads, unless specified otherwise.
- Alpha Donor beads, AlphaLISA™ and AlphaPlex Acceptor beads should be stored in the dark at 4 °C.
- AlphaLISA and AlphaPlex Acceptor or Alpha Donor beads may slightly sediment in the stock solution over several days. This is normal. It is advised to vortex the beads prior to use.
- Alpha Donor beads are light-sensitive. All additions to a plate involving the Donor beads should be performed under subdued laboratory lighting of less than 100 lux. Alternatively, green filters (e.g., Roscolux filters #389 from Rosco Laboratories, Inc., or equivalent) can be applied to light fixtures. Any incubation of streptavidin Donor beads should be performed in the dark.
- Due to the small volumes used in the assay, it is recommended to cover microplates with TopSeal-A™ adhesive sealing film to reduce evaporation during incubation periods (Revvity, Inc., Cat. No. 6005185). The microplates can be read with the TopSeal-A film in place, except if condensation is present.
- AlphaLISA and AlphaPlex products are intended for research purposes only and are not for use in diagnostic procedures.

Table of contents

I. Introduction 4

II. Principle of the assay 4

III. AlphaPlex assay development 5

A. Initial assay conditions: 5

B. Determination of assay performance from calibration curves 6

C. Antibody specificity 7

D. Assay optimizations: Buffers, volume, incubation time and order of addition 8

i. Assay buffer optimization 8

ii. Assay volume 8

iii. Incubation time 8

iv. Order of addition 9

v. Assay component concentrations. 9

E. AlphaPlex considerations 9

F. Determination of the level of analytes in unknown samples by interpolation from the calibration curves in an AlphaPlex assay 10

G. Set up of A calibrated multiplex assay for unknown samples 11

IV. Acceptor bead and optics selection 12

A. Acceptor bead selection 12

B. Optics selection 12

C. Optics and assay performance 14

V. Crosstalk 14

A. Introduction 14

B. Antibody crosstalk 14

C. Well-to-well crosstalk 16

D. Optical crosstalk overview 16

E. Optical crosstalk correction from standard curves 17

Appendix I: Summary of AlphaPlex assay development steps 20

Appendix II: New assay development 21

A. Selection of antibody pairs 22

B. Biotinylation of the antibody 22

C. Acceptor bead conjugation with the antibody 23

D. Determination of the best antibody pair 25

Appendix III: Multiplexing examples 27

A. Simultaneous detection of hIFN-γ (Tb) and hIL-1β (Eu) using AlphaPlex technology 27

I. Introduction

AlphaPlex™ is a homogeneous, all-in-one-well multiplexing reagent technology that provides highly sensitive detection of a wide range of analytes in a simple no-wash assay format based on Revvity's proven Alpha Technology. AlphaPlex assays can quantify multiple analytes in a single well for faster and more accurate results, even with low sample volumes. Three distinct AlphaPlex Acceptor beads are available with narrow emission spectra along with complementary optics for precise resolution of individual signals. Combining multiple AlphaPlex Acceptor beads in a multiplex assay can allow for a faster analysis times, smaller sample volumes, higher accuracy and reproducibility than single-plex assays. Users can build multiplex assays from numerous validated AlphaLISA™ and AlphaPlex detection kits for specific analytes or develop their own assays with unconjugated or toolbox beads. This guide is intended to provide the Alpha user with the knowledge and tools necessary to unleash the full power of multiplexing using AlphaPlex.

Before starting:

The protocols included in this manual are for OptiPlate-384 microplates (Catalog number 6007290). Other plates may be used with the appropriate assay volume. Assays must be read using an AlphaPlex compatible reader such as EnVision™ Multilabel plate readers with a standard Alpha option. Also required are the EnVision software version 1.13 and the appropriate mirror blocks and filters. For information on required optics, see section IV.B.

II. Principle of the assay

The AlphaPlex reagent system was designed to enable fast and easy transition from well-established AlphaLISA assays to multiplexed detection of a broad range of molecules, proteins and biomarkers.

AlphaPlex allows multiplexing in serum, plasma, cell culture supernatants or cell lysates in a very sensitive, quantitative, reproducible, and user-friendly assay. Using a single, universal streptavidin-coated Donor bead, multiple pairs of biotinylated antibodies and AlphaPlex Acceptor beads targeted to desired analytes are combined in a single assay well. Figure 1 illustrates a typical AlphaPlex assay: anti-analyte biotinylated antibodies bind the streptavidin Donor bead while complementary anti-analyte antibodies are conjugated to AlphaLISA and AlphaPlex Acceptor beads. In the presence of the analytes, the various Acceptor beads come into close proximity to the Donor beads. The excitation of the Donor beads with 680 nm laser light provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in the Acceptor beads, resulting in chemiluminescent light emission. Each type of Acceptor bead emits light at a specific wavelength (centered at 615 nm for AlphaLISA (Eu) beads, 545 nm for AlphaPlex 545 ((Tb)) beads and 645 nm for AlphaPlex 645 (Sm) beads). Specially designed optics then accurately resolve the resulting emission signals to independently quantify each analyte with minimal interference. In most cases, migrating an AlphaLISA assay to an AlphaPlex multiplex assay requires little more than combining complimentary Acceptor beads in the same well and employing the recommended optics.

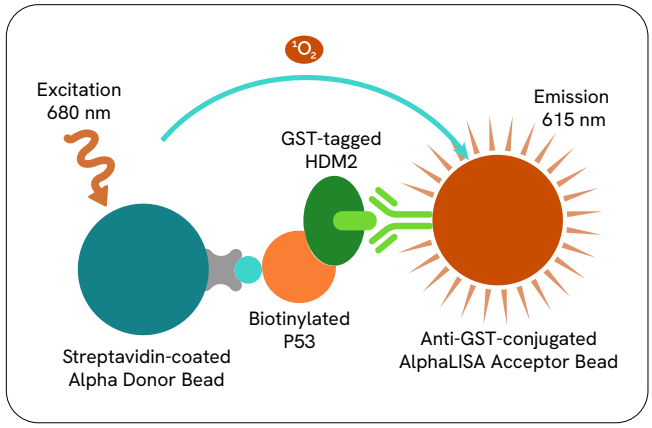


Figure 1: Schematic of an AlphaPlex triplex assay using streptavidin Donor beads with AlphaPlex 645 (Sm), AlphaLISA (Eu) and AlphaPlex 545 (Tb) Acceptor beads targeted toward three different analytes. Streptavidin Donor beads act as a universal source of singlet oxygen for all colors of bound AlphaPlex Acceptor beads.

III. AlphaPlex assay development

Generally, setting up an AlphaPlex multiplexing assay is as easy as combining two or three Alpha assays in the same well using complementary AlphaPlex Acceptor beads. Complementary Acceptor beads must be of different emission wavelengths (AlphaPlex 545 (Tb), AlphaPlex 645 (Sm) or AlphaLISA (Eu) and also be highly specific for one target analyte in the multiplex over all other analyte(s) present. Also, specific assay conditions such as buffer components, order of addition of components and incubation times must be compatible among all combined assays. A general overview of the process of evaluating AlphaLISA and AlphaPlex kits and combining into a multiplex assays is illustrated in Appendix I.

Revvity offers numerous validated AlphaLISA and AlphaPlex detection kits tailored to a variety of different applications. However, it is also possible to develop custom kits using unconjugated or Toolbox AlphaLISA and AlphaPlex beads. More information on developing custom AlphaLISA and AlphaPlex assays is provided in Appendix II. The remainder of this section describes the process involved in building an AlphaPlex multiplex assay from previously validated AlphaLISA and AlphaPlex assay kits.

A. Initial assay conditions:

Typical concentrations in an assay well for an AlphaPlex duplex assay are shown in Table 1. The recommended buffer is AlphaLISA Immunoassay Buffer (Product No. AL000C/F). The recommended bead concentration for each Acceptor bead is the same as that for single AlphaLISA assays. The concentration of Donor beads is set equal to or greater than the sum of the concentrations of Acceptor beads. In the unusual case where the total concentration of biotinylated antibodies expressed in nM equals or exceeds the concentration of the Donor beads expressed in µg/mL, the Donor bead concentration should be increased to ensure there are sufficient streptavidin binding sites available (e.g. if the total concentration of all biotinylated antibodies is 20 nM, the Donor beads should be >20 µg/mL, or at least 25 µg/mL).

Table 1: Typical AlphaPlex assay conditions for a duplex.

Component	Concentration
AlphaPlex Bead A	10 µg/mL
AlphaPlex Bead B	10 µg/mL
AlphaPlex biotinylated Ab A	1.0 nM
AlphaPlex biotinylated Ab B	1.0 nM
Streptavidin Donor Bead	40 µg/mL

For an AlphaPlex multiplex assay, the various assay components including Acceptor beads and biotinylated antibodies are added in the same order as an AlphaLISA or AlphaPlex single assay. Components targeted toward each analyte in the multiplex (e.g. AlphaPlex 645 (Sm) Acceptor beads and AlphaLISA (Eu) Acceptor beads) can be added at the same time. The streptavidin Donor beads are added last. Incubation times for AlphaPlex assays are the same as AlphaLISA assays.

These general guidelines are the starting point for the development of all AlphaPlex assays with commercial AlphaLISA kits and will work well in the majority of cases. Sometimes, however, certain combinations of AlphaLISA kits or custom assays may require modified protocols to optimize signal or minimize interference based on the specific nature of the analyte under investigation. It is particularly important to confirm that each antibody pair used in the assay is specific for its own target and will not recognize the other target that the user is attempting to multiplex. This can be tested by running each assay individually with the analytes of each other assay. See Section V.B for more information on antibody specificity.

B. Determination of assay performance from calibration curves

The most important parameters to determine the best combination of antibodies are the lower detection limit (LDL), the assay window (S/B) and the assay dynamic range. These parameters are determined by running a calibration curve over a wide range of analyte concentrations and performing a few simple calculations on the resulting data. For the purposes of determining the best conditions for a new AlphaPlex multiplex assay, the calibration curves should be run separately first as single-plex assays as described below. For calibration of an AlphaPlex multiplex assay, the calibration curve should be run in the presence of all assay components of the multiplex (i.e. both biotinylated antibodies and both Acceptor beads that will be used for a duplex).

The following conditions may be used for each analyte calibration curve in a single-plex assay set-up:

In an OptiPlate-384 microplate, add:

- 5 µL of the analyte diluted in assay buffer (use dilutions that cover the working range for the target detection plus a few points above and below the range of target detection as well as negative controls (background) consisting of 4 independent conditions without analyte in triplicate for a total of 12 points)
- 20 µL of biotinylated antibody (as a starting point, 2.5 nM for a 1 nM final concentration in each well)

  AND

  antibody-conjugated AlphaPlex Acceptor beads at 25 µg/mL (10 µg/mL final concentration in each well)

Incubate at room temperature for 1 h then add:

- 25 µL of streptavidin-Donor bead solution at 80 µg/mL prepared under subdued light conditions (40 µg/mL final concentration in the well)

Incubate at room temperature in the dark for 30 min and read on an Alpha reader with appropriate optics and protocol.

Example data are shown in Figure 2. The lower detection limit (LDL) is usually calculated as follows:

- Average the 12 background values and calculate the standard deviation (SD).
- Multiply the standard deviation (SD) by 3
- Add the 3X SD value calculated to the average background signal.
- On the graph, extrapolate the value obtained (AlphaPlex signal counts) to determine the corresponding analyte concentration. This concentration is the lowest the assay can detect and corresponds to the lower detection limit (LDL) when the analytes are diluted in assay buffer.

The assay dynamic range corresponds to the concentration window in the standard curve running from the lower detection limit to the maximum concentration up to (but excluding) the hook point.

Data can be analyzed using linear or nonlinear regression analysis. However, wider dynamic ranges are usually interpreted using nonlinear regression Eq.1. Four-Parameter Logistic Model also described as Sigmoidal Dose-Response (variable slope) with weight of 1/Y2 where the four parameters to be estimated are Top, Bottom, EC_{50} and Slope. For more details refer to the NIH Chemical Genomics Center manual.

(http://ncgc.nih.gov/guidance/manual_toc.html).

$$Response = Top + \frac{(Bottom - Top)}{1+\left(\frac{concentration}{EC_{50}}\right)^{Slope}} \quad \text{Eq. (1)}$$

Top' refers to the top asymptote, 'Bottom' refers to the bottom asymptote, and 'EC_{50}' refers to the concentration at which the response is halfway between Top and Bottom.

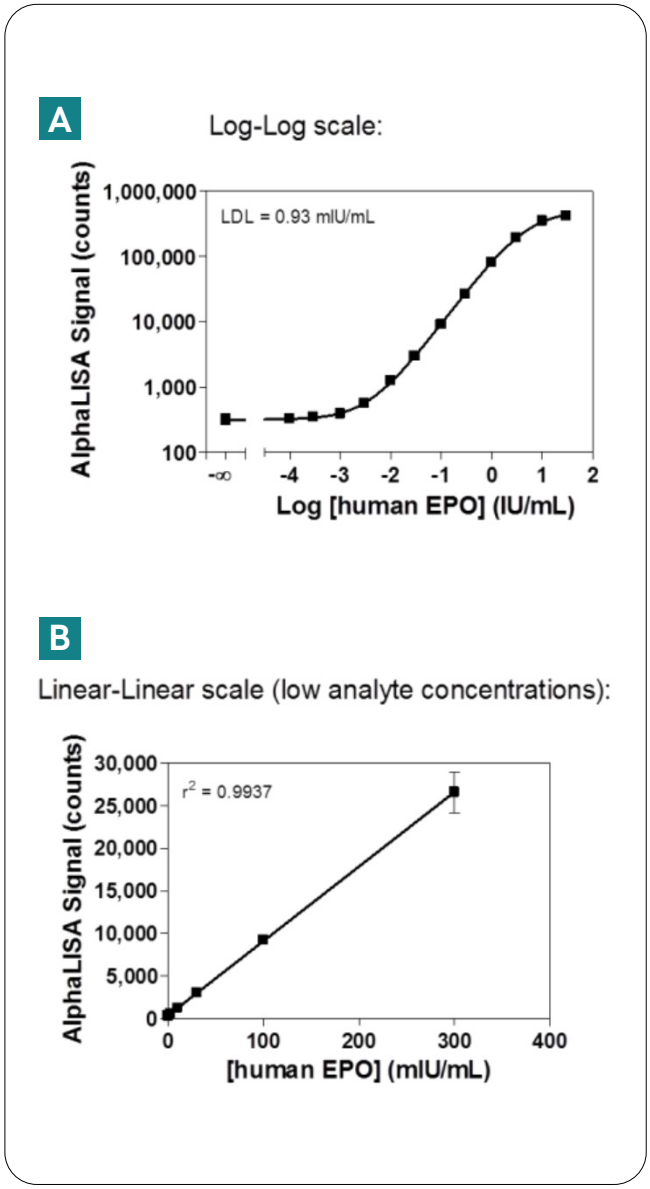


Figure 2: Calibration curve for a human EPO detection assay prepared in assay buffer. The graph can be analyzed using nonlinear (A) or linear (B) regression analysis. Note that nonlinear representations are more suitable for a wide range of analyses. The lower concentrations of the calibration curve (up to 300 mIU/mL) are shown as a linear plot (B). Reported concentrations are those in the samples.

The above assays will provide baseline curves and assay parameters to judge how each assay could perform under multiplex conditions. In addition, the data obtained in this way can be used to determine which assay of a multiplex would be most appropriate for each AlphaPlex or AlphaLISA Acceptor bead. Generally speaking, the assay which gives the least total signal is combined with the brightest emitting Acceptor bead, which is AlphaLISA (Eu). Thus, the assay with the highest total signal would generally be used with AlphaPlex 545 (Tb) or AlphaPlex 645 (Sm) Acceptor beads. While this is a good rule of thumb, it is recommended practice when developing a new multiplex assay to compare the performance of each AlphaPlex and AlphaLISA Acceptor bead targeted to each analyte in the desired multiplex and select pair that gives the best performance. It is also recommended to run both assays in the multiplex format prior to beginning assay optimizations to help determine assay compatibility and parameters that may need to be optimized in each single assay.

C. Antibody specificity

To ensure that the signal obtained in each channel is specific, the above experiments should be repeated by putting the AlphaLISA analyte in the AlphaPlex assay and the AlphaPlex analyte in the AlphaLISA assay. Specificity among the selected pair of assays is confirmed by the absence of signal response to the analyte of the other assay. If signal is generated, the assay with exchanged analyte should show an EC_{50} at least two logs shifted to the right compared with the regular assay. To further ensure the absence of interference, if a protein or large peptide is used for stimulating cells or is a key part of the assay, such as an enzyme, this element should also be tested for specificity by trying to detect it with both assays. See also Section V.B for more information about determination of antibody specificity.

D. Assay optimizations: Buffers, volume, incubation time and order of addition

Sometimes the parameters of a new assay may not meet the required levels for sensitivity, assay window, dynamic range, etc. for a particular application using the standard assay conditions. If this occurs, the assay may be further optimized by changing the following parameters. Often such optimizations can result in substantial improvements in assay performance. These optimizations are equally applicable to single-plex AlphaLISA assays and AlphaPlex multiplex assays. However, for a multiplex assay, all individual assays will eventually be run under the same final conditions, so each assay must be tested under all trial conditions. Sometimes the final optimized conditions may need to be a compromise to ensure that each assay component meets the required specifications. If calculated parameters of one of the assays of a multiplex is closer to the edge of the required range, then it is best to try the optimized conditions for that assay with the other assays of the multiplex first.

i. Assay buffer optimization

The standard assay buffer (AlphaLISA Immunoassay buffer, Product No. AL000C/F) is a useful starting point but may be optimized. The following buffers are also available and may improve some AlphaPlex assays: AlphaLISA HiBlock Buffer (Product No. AL004C/F), used in situations where high background from sample components could interfere with the assay, AlphaLISA Universal Buffer (Product No. AL001C/F) typically tested as an assay buffer for binding assays (e.g., protein-protein interactions)., and AlphaLISA NaCl Buffer (Product No. AL007C/F), which contains 0.5M NaCl to help reduce background or interference in certain assays. Additionally, the following custom parameters can be adjusted individually or in combination:

- Buffer type: Tris, HEPES, at different pH values (pH 6.8 to 8.2 for HEPES and pH 7.5 to 9.0 for Tris).
- The presence of Dextran 500: for some serum or plasma samples, it is important to add Dextran 500 at 1 mg/mL in order to prevent non-specific bead aggregation.
- The presence of detergent, like Tween-20, CHAPS, and Triton X-100, at a concentration between 0.01 to 1%.
- The presence of protein blockers, such as casein or BSA, at a concentration between 0.01 to 1%.

ii. Assay volume

Most analyte assays will perform best in 50-75 µL final volume in 96-well 1/2 AreaPlates, 25-50 µL in 384-well plates and 5-10 µL in 1536-well plates. However, the volume can be adjusted to fulfill specific user requirements. If working with serum or plasma samples, it is strongly recommended that the sample volume represents no more than 10% of the total volume in the well in order to reduce interference. Less interference will be observed using the lowest sample volume possible. It is always possible to pre-dilute the sample in the sample matrix solution if the concentration of the analyte in the sample permits.

iii. Incubation time

The incubation time at each step of an assay can have an impact on the assay performance. The presence of the components of a second assay in a multiplex can also alter the optimal incubation times. To determine the best incubation time, a time course can be performed following each addition step. A time course could also be performed after the addition of Donor beads. Usually the signal will reach a plateau after a few hours. It is recommended that each time be assigned its own set of wells to avoid having to read the same well repeatedly, which can lead to a small amount of signal decrease due to bead depletion.

iv. Order of addition

For the majority of analyte assays, the order of addition of reagents described above performs best (i.e., analyte>biotinylated antibodies + Acceptor beads>Donor beads). However, the addition steps can be changed in order to optimize the assay sensitivity. It is sometimes beneficial to add the biotin-antibodies and the Acceptor beads separately. In this case, incubation time is introduced between each addition step. Alternatively, the components from each Acceptor bead of a multiplex may be added separately. The best order of addition of components for a given multiplex must be determined empirically. From our experience, pre-mixing the biotinylated antibodies with the Donor beads lowers the sensitivity of the assay and should in most cases be avoided.

v. Assay component concentrations

Sometimes two or more well performing AlphaLISA assays may not perform as well when combined together in a multiplex format due to interactions between the components of the two assays. If this is the case, in addition to the optimizations above, it may be necessary to readjust the concentrations of one or more the components of a multiplex (Acceptor beads and/or biotinylated antibody). Specifically, if it is determined that one of the assays is negatively impacted by the presence of the components of the other in a duplex, the concentrations of the interfering assay (biotinylated antibody and Acceptor bead) can be reduced to minimize the interference. Often, lowering the concentration of the components by a factor of two or three will lower total signal but have little effect on sensitivity of the assay. Lowering the concentration of an interfering component will reduce the degree of interference and may restore sensitivity and other assay parameters to acceptable levels. For example, if an AlphaPlex 545 (Tb) assay has a higher LDL in the presence of 10 µg/mL of an AlphaLISA (Eu) bead in a multiplex, the concentration of the AlphaLISA bead can be lowered to 3 to 5 µg/mL.

E. AlphaPlex considerations

Once Alpha assay beads and optimal conditions have been chosen for each analyte of a new AlphaPlex multiplex assay, the beads and analytes can be tested together in a single well AlphaPlex assay format. Suitability of two or more assays for multiplexing is determined by running calibration curves for each of the analytes under multiplex conditions. Calibration curves for AlphaPlex multiplex assays are run over the same range of analyte concentrations as single assays, but with all of the assay components in place (i.e. including Acceptor beads and biotinylated antibodies of the other analytes of the multiplex). Each analyte is titrated individually. In addition, it is useful to run a calibration curve of each analyte in the presence of a fixed concentration of the other analytes of the multiplex, for example at a concentration of about 50% maximal signal for that analyte (corresponding to the third or fourth point on a typical dilution curve), including appropriate correction of any optical crosstalk (see Section V.E below). In the majority of cases, the calibration curves with and without the second analyte will be essentially identical to one another and also the individual single-plex assays of each target of the multiplex. However, if the curves are substantially different, it indicates that there is some interference between the assays of the multiplex. If the level of interference is unacceptable based on the characteristics of the standard curve, then further steps to optimize the multiplex, such as lowering the concentration of the interfering Acceptor bead, should be taken.

The following AlphaPlex standard curve conditions can be used to assess the performance of the assays as a multiplex. Dilutions of each analyte that cover the working range for the target detection plus few points above and below the range of target detection are prepared. Typically 12 dilution concentrations are used in half-log increments. Four additional wells with no analyte are prepared as negative controls (background). Each dilution and control is plated in triplicate for a total of three sets of 16 points per calibration curve.

In an OptiPlate-384 microplate, add:

5 µL of the analyte detected by AlphaLISA only diluted in assay buffer.
OR
5 µL of the analyte detected by AlphaPlex 545 (Tb) or AlphaPlex 645 (Sm) only diluted in assay buffer
OR
5 µL of assay buffer for the background wells
OR
5 µL of the dilution point of one analyte diluted in assay buffer with a *fixed* concentration of the other analyte (using, for example, the concentration from the third or fourth dilution point for that analyte). This should be performed for each analyte.

- 20 µL of both biotinylated antibodies (as a starting point, 2.5 nM for a 1 nM final concentration in each well)

  AND

  Both types of AlphaPlex / AlphaLISA Acceptor beads at 25 µg/mL (10 µg/mL final concentration of each bead in each well)

Incubate at room temperature for 1 h then add:

- 25 µL of streptavidin-Donor bead solution at 80 µg/mL prepared under subdued light conditions (40 µg/mL final concentration in the well)

Incubate at room temperature in the dark for 30 min and read on an Alpha reader with the appropriate optics and protocol.

The results of the above multiplex calibration curves are analyzed the same way as above. The last curves, performed with a fixed concentration of the opposite analyte, require crosstalk correction (see Section V.E) prior to analysis. Note that a slight loss of signal compared to individual curves can be seen due to the increase opacity of the well due to the presence of both Acceptor beads. Since background is similarly affected, signal to background and sensitivity is generally not affected. Curves where only one analyte is present should generate signal only with the assay for this analyte, while the assay buffer should not generate any significant signal above background. The calculated parameters from the calibration curves (such as LDL and EC_{50}) should be compared with and without the presence of a fixed concentration of the opposite analyte, after correction for optical crosstalk. If interference (such as a significantly higher LDL) by the presence of the other analyte is observed and deemed unacceptable, then further assay optimization outlined may be required, specifically lowering the concentration of the interfering Acceptor bead by several fold and running the curve again. If all curves are found to be within acceptable ranges, the performance of the multiplex assay standard curves in the sample matrix should be checked as described in the following section.

F. Determination of the level of analytes in unknown samples by interpolation from the calibration curves in an AlphaPlex assay

Determination of the concentration of multiple analytes in an unknown sample, such as a serum or plasma sample, by AlphaPlex is very similar as that for a standard AlphaLISA assay using calibration. Calibration curves for AlphaPlex are run as described above, but the calibrators (solutions containing known concentrations of analytes) should be prepared in a "matrix solution" representative of the samples under analysis, instead of Assay Buffer. We recommend using a serum or plasma (similar to the samples) first depleted of the analytes of interest by, for example, pretreatment with streptavidin-Sepharose beads and the biotinylated antibody then spiking with known concentrations of analyte. Alternatively, an artificial matrix solution can be prepared. Such a matrix should behave the same way in the AlphaPlex assay as the real samples (same level of signal interference).
In such instance, high concentrations of BSA or any other signal quencher could be used for this purpose. Finally, the concentrations of analyte used to perform the calibration curve must cover the range expected to be found in the samples.

G. Set up of a calibrated multiplex Assay for unknown samples

A general protocol to generate calibration curves in the matrix solution and determine the analyte concentrations of unknown samples is presented below. For most AlphaPlex assays, it is sufficient to combine both analytes together and perform a simultaneous dilution curve and applying optical crosstalk correction to each curve as described in Section V.E. However, if interference between the assays combined in the multiplex is suspected or is a concern, it may be preferable to perform the calibration curves as described previously, where each analyte is diluted individually in matrix solution with and without the presence of a fixed concentration of the other analyte. The simultaneous dilution is described below:

For duplex calibration curves:

- Add 5 µL of both analytes of the duplex diluted together in the matrix solution

For unknown samples:

- Add 5 µL of unknown samples

Then to calibration or unknown sample,

- Add 20 µL of both antibody-conjugated AlphaPlex Acceptor beads + both biotinylated antibody solutions

Incubate for 1 h at 23 °C.

- Add 25 µL of streptavidin-Donor bead solution

Incubate for 30 min at 23 °C, in the dark and read plate on an Alpha reader with appropriate optics and protocol.

Dilutions of each analyte that cover the working range for the target detection plus few points above and below the range of target detection are prepared. Typically 12 dilution concentrations are used in half-log increments, (from 3e-13 to 1e-7 g/mL in the example below).

Four additional wells with no analyte are prepared as negative controls (background). Each dilution and control is plated in triplicate for a total of three sets of 16 points per calibration curve. The analyte serial dilutions are prepared in the matrix solution, while the solutions of antibody-conjugated AlphaPlex Acceptor beads and of biotinylated antibodies are prepared in the assay buffer. The data are subjected to optical crosstalk correction as described in Section V.E.

Figure 3 demonstrates an example set of crosstalk corrected simultaneous calibration curves for a hIFN-γ (AlphaPlex 545 (Tb)) and IL-1β (AlphaLISA (Eu)) duplex assay also shown in Appendix III. Increasing concentrations of analytes are on the x-axis and the corrected Alpha signal on the y-axis. Both axes are shown on a log scale. The level of analyte in unknown samples is then determined by interpolation from the calibration curves thus generated.

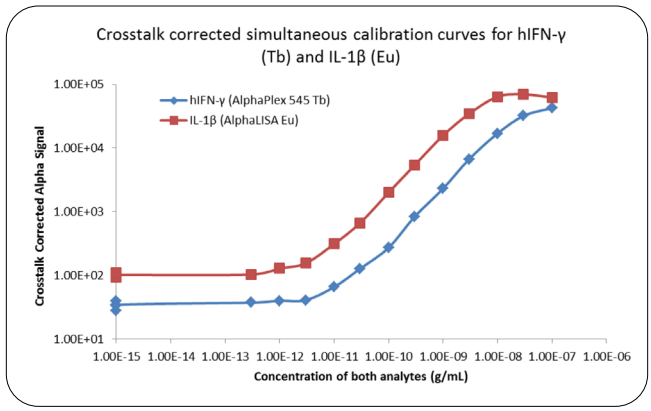


Figure 3: Calibration curves for a hIFN-γ (AlphaPlex 545 (Tb)) and IL-1β (AlphaLISA (Eu)) duplex assay (see Appendix III). The biotinylated antibodies were each used at a final concentration of 1 nM. The antibody-conjugated AlphaPlex and AlphaLISA Acceptor beads were each used at 10 µg/mL and the streptavidin-Donor beads were used at a final concentration of 40 µg/mL. The data were generated on an EnVision Multilabel reader equipped with a Tb/Eu simultaneous dual mirror and Tb and Eu filters. Optical crosstalk was corrected according to Section V.E.

IV. Acceptor bead and optics selection

A. Acceptor bead selection

AlphaPlex Acceptor beads contain one of three unique lanthanide chelates comprising either europium (Eu), terbium (Tb) or samarium (Sm) as the emitting species. These three lanthanides have narrow emission peaks at distinct wavelengths that can be independently detected and quantified using appropriate emission filters, allowing for highly sensitive detection of multiple analytes simultaneously.

All of the lanthanides used for AlphaPlex have a major emission peak, which is used for selective detection, but also some additional secondary emission peaks. The major emission peak of AlphaLISA (Eu) Acceptor beads is centered at 615 nm, while the major emission peaks of AlphaPlex 545 (Tb) and AlphaPlex 645 (Sm) Acceptor beads are centered at 545 nm and 645 nm, respectively. AlphaPlex emission filters were designed to isolate the individual signals from each of the lanthanides while minimizing any interference from these secondary emissions. It is for this reason that appropriate filter selection is critical for good multiplex assay performance.

The three lanthanides used in AlphaPlex technology also have different emission intensities. Eu is the strongest emitter, while Tb and Sm give somewhat lower signal (typically 12-20%). As the background of the Tb and Sm beads is also lower, assay sensitivity is usually comparable among the three AlphaPlex beads. When selecting beads for a multiplex, it is recommended to select Eu Acceptor beads for the least abundant analyte if possible, due to the inherently higher signal output, selecting Tb or Sm for the more abundant analyte. If more equal signal intensities are desired, a somewhat higher concentration of AlphaPlex 545 (Tb) or AlphaPlex 645 (Sm) beads than AlphaLISA (Eu) beads can be used (e.g. about 2-fold higher).

An additional consideration when selecting AlphaPlex Acceptor beads is potential interference from matrix components. Absorbing compounds sometimes present in sample media such as phenol red, hemoglobin or chlorophyll have a differential effect on AlphaPlex Acceptor bead emission depending on the wavelengths of absorption. For example, AlphaPlex 545 (Tb) beads might be preferred when working with chlorophyll or similar green pigments while the longer wavelength of emission of AlphaPlex 645 (Sm) might be preferred in the presence of hemoglobin.

B. Optics selection

The optics required for AlphaPlex multiplexing with a Revvity EnVision Multilabel reader consist of a single or double dichroic mirror block and one or more emission filters specific for the emission of the AlphaPlex Acceptor beads being used. A single dichroic mirror is used for single readings as well as sequential multiplexing (separate reads for each wavelength channel). A double dichroic mirror separates the emitted light into two distinct channels based on wavelength and allows simultaneous reading of two AlphaPlex Acceptor beads in a single measurement with an EnVision equipped with two detectors.

While the standard AlphaScreen mirror, barcode 444, will work fine for single and sequential multiplex reads of AlphaPlex 545 (Tb) and AlphaLISA (Eu) Acceptor beads, the preferred single dichroic mirror for all AlphaPlex is the AlphaPlex Single Tb-Eu-Sm mirror, barcode 605, which has been optimized for all three Acceptor beads. A Sm compatible mirror is required for all Sm applications due to its longer emission wavelength.

For simultaneous duplexing applications, two different duplexing mirror blocks are available, the AlphaPlex Dual Tb/Eu module, barcode 653, and the AlphaPlex Dual Eu/Sm module, barcode 658, which can also be used for Tb/Sm duplexing. Emission filters for both of the AlphaPlex Acceptor beads under analysis must be present in the instrument adjacent to one another and in the correct order in the filter slide in order to

perform a simultaneous AlphaPlex read using a Dual mirror. A summary of the recommended mirrors for use with AlphaPlex technology is shown in Table 2.

The primary differences between sequential read multiplexing and simultaneous read duplexing are the read time and the signal quality. Read time is substantially lower for simultaneous reads since each read generates twice as many data points. Signal quality is also higher for simultaneous reads since there is a slight degradation of signal after the first measurement due to oxidation of protein recognition elements. When the plate is read sequentially, a second reading may have a lower signal by a few percent relative to the first reading. For this reason it is recommended to read the Tb or Sm channel first and the generally stronger Eu signal second during a sequential duplexing assay. It should also be noted that during a simultaneous read, one of the lanthanide signals is directed to the secondary detector, thus following a longer path length. This results in a lower signal than would be obtained with the primary detector. For this reason, both of the AlphaPlex dual mirrors have been configured to direct the generally stronger Eu signal to the secondary detector. There are no significant differences in assay sensitivity between a sequential read duplex with a single mirror and a simultaneous read with a dual mirror.

To take full advantage of the multiplexing capability of AlphaPlex Acceptor beads, narrow band emission filters specific to the emission peak of each lanthanide must be used. Each AlphaPlex filter was designed to maximize the signal from the main emission peak of the target lanthanide while minimizing interference from other lanthanides used in a multiplex assay. In comparison to the standard wide band AlphaScreen filter (barcode 244), using narrow band filters will result in a somewhat lower signal, typically about one third of that obtained with the wide filter. A summary of the recommended filters for use with AlphaPlex technology is shown in Table 2. For optimal results, it is recommended to use the AlphaPlex Tb filter for detection of AlphaPlex 545 (Tb), the Europium filter for detection of AlphaLISA (Eu) and the AlphaPlex Sm filter for detection of AlphaPlex 645 (Sm).

Table 2: AlphaPlex Optics for EnVision Multilable Reader

	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 read AlphaPlex applications
	AlphaPlex Dual Tb-Eu	2102-5900	653	For **simultaneous** duplexing of Tb with Eu
	AlphaPlex Dual Sm-Eu	2102-5920	658	For **simultaneous** duplexing of Sm with Eu or Tb
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
	AlphaPlex Sm	2100-5940	702	Preferred filter for all Sm applications and multiplexing

C. Optics and assay performance

Using the appropriate mirrors and filters, AlphaPlex signals from the three AlphaPlex Acceptor beads should be well resolved. However, there will still be a small amount of signal "crosstalk" from one Acceptor bead emission to the detection channels of the other Acceptor beads due to minor secondary emissions from the lanthanide chelates. Crosstalk is typically quantified as a percentage of the signal of emission on the primary detection channel that is detected on the second channel (e.g. the percentage of Eu signal that is detected through a Tb filter relative to that detected through a Eu filter). This percentage will depend on the particular optics chosen for a multiplex experiment and therefore must be measured for each experimental setup. Typical observed values are in the range of 1% to 3%. Once the magnitude of signal crosstalk for a given experiment is determined, it can easily be subtracted from the signal on the second channel. See Section V for more information about crosstalk signal correction.

Signal intensity of AlphaPlex beads can be affected by a number of factors not related to the assay itself, including the emitting lanthanide, mirror configuration, path length to the detector and the filters used. However, a lower signal intensity due to the optical configuration does not translate into reduced sensitivity of the assay, as the background is generally lowered by an equal amount as the signal such that the S/N ratio remains unchanged.

V. Crosstalk

A. Introduction

Using the appropriate mirrors and filters, AlphaPlex signals from the three AlphaPlex Acceptor beads should be well resolved. However, there will still be a small amount of optical signal from one Acceptor bead emission detected on the channel(s) of the other Acceptor bead(s). This effect is called optical crosstalk and it can be easily corrected. In the context of Alpha, the term crosstalk can refer to several phenomena that can potentially make analysis of the data of an assay more complex than expected. In this section, the various forms of crosstalk will be distinguished and specific procedures for correcting optical crosstalk, a form specific to AlphaPlex, will be discussed.

B. Antibody crosstalk

Antibody crosstalk is an issue of antibody specificity, and is sometimes also referred to as cross-reactivity. Antibody crosstalk occurs when the antibodies used in an assay recognize components in the sample other than the desired analyte. Antibody specificity is critical for Alpha assays to ensure that assay signal response is due to the desired analyte rather than another component in the analysis sample. Revvity's validated AlphaLISA and AlphaPlex kits are screened for cross-reactivity against probable interfering sample components. However, users who develop new assays or combinations of assay kits should determine the specificity of assays they wish to combine in a multiplex. In particular, when performing an AlphaPlex assay using multiple pairs of antibodies each against their own analyte together in the same well, the user must ensure that each of these pairs are specific to their own analyte and will not recognize the other. If significant antibody crosstalk exists, the results of the assay may be inaccurate. While often not a major issue, specificity becomes more serious if the two analytes are closely related, such as modified vs. unmodified proteins (e.g. phosphorylation) where the difference between the two analytes is subtle.

As it is difficult to correct for low antibody specificity by adjustments to the assay itself, the following method is provided to verify specificity between two sets of biotinylated antibody and AlphaPlex Acceptor bead pairs. First, a standard curve is performed as described in Section III above with each biotinylated antibody / Acceptor bead pair with the targeted analyte and separately with the analytes of assays to be combined in the multiplex. The target analyte should provide a curve within specifications of the assay, while the other curve should not generate any signal. If signal is observed, this means the antibody lacks specificity. The process is then repeated with the other curve. The graphs in Figure 4 below show examples of assays with and without significant antibody crosstalk. The top graph (a) shows an example of a non-specific antibody. While the affinity of the non-targeted analyte is lower, the presence of significant signal could complicate analysis of the targeted analyte. At the bottom (b) is an example of a specific antibody. No signal can be seen in the non-targeted analyte. In this case, antibody crosstalk is not an issue.

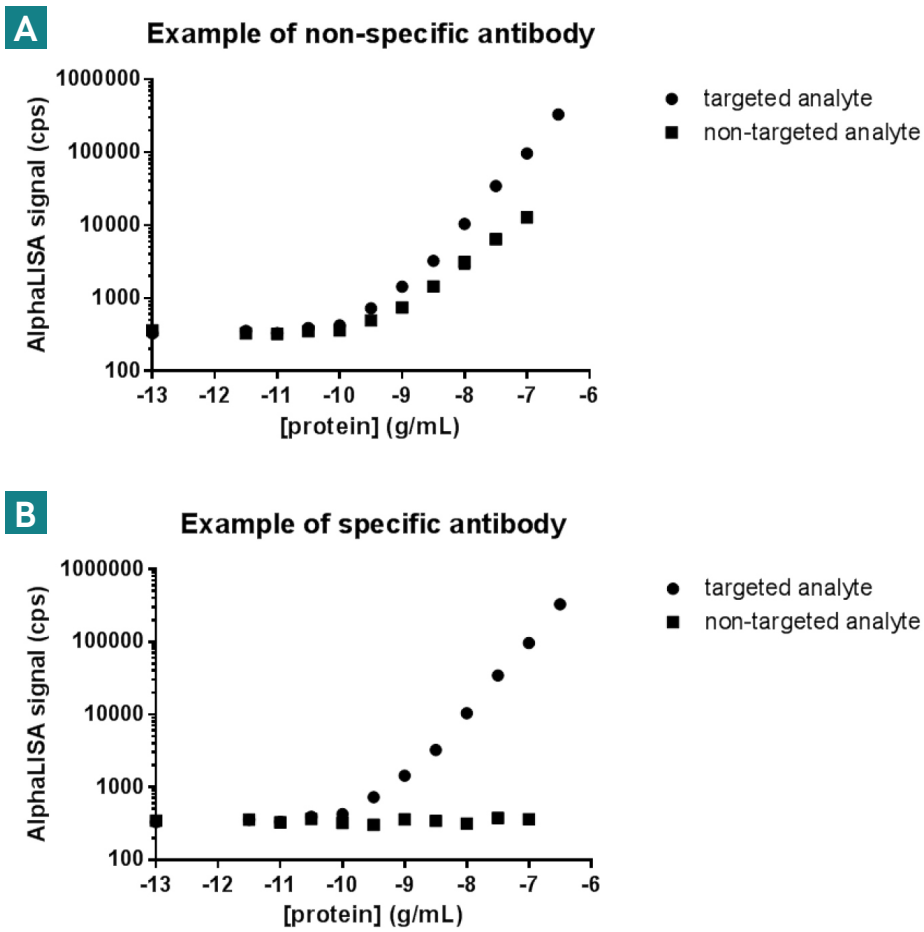


Figure 4: Titration curves for assays with non-specific (a) and specific (b) antibodies.

C. Well-to-well crosstalk

A second source of assay crosstalk occurs between individual wells in a microplate. Referred as well-to well crosstalk, such interference is usually very small and is directly related to light permeability of the walls of the well. Well-to-well crosstalk occurs either from signal from one excited well passing through to adjacent well or from laser excitation light exciting beads in an adjacent well. In both cases, a small amount of phantom signal in adjacent wells from a previously read well. Well-to-well crosstalk is mostly observed with white plates and is most pronounced in 1536 well plates with thinner walls than 384 or 96 well 1/2 AreaPlates.

While usually very small and not a particular concern, a simple solution to minimize well-to-well crosstalk is to use AlphaPlates, which have been doped with a darkening agent that substantially reduces light permeability of the well walls (http://www.revvity.com).

D. Optical crosstalk overview

Optical crosstalk is a phenomenon that is specific to AlphaPlex multiplexing and results from having two or more types of Acceptor beads in the assay. While the major emission lines of the lanthanide chelates used in AlphaLISA and AlphaPlex are generally narrow, small amounts of light from minor secondary emissions can pass the filters of the other channels in a multiplex assay. This effect is intrinsic to the nature of the chemicals and instrument optics and is not a function of the type of assay used or the plate. Since the behavior of the optics is very consistent, once the degree of optical crosstalk for a particular setup has been determined, it can be corrected very precisely.

Optical crosstalk is quantified as a percentage of the signal of emission on one detection channel that is detected on a second channel (e.g. the percentage of an AlphaLISA (Eu) signal that is detected through a Tb filter relative to that detected through a Eu filter). This percentage will depend on the particular optics chosen for a multiplex experiment and therefore must be measured for each new experimental setup. Optical crosstalk can be measured by running standard curves for each analyte with all components present—i.e. all Acceptor beads, all biotinylated antibodies, Donor beads and a titration of a single analyte. It is recommended to use the three highest signal points of the standard curve—e.g. simply divide the signal detected through secondary optical channels by that detected through primary channel for those data points and average the result. Typical observed values are in the range of 1% to 3%. This measurement process presupposes that the above forms of crosstalk are not present or have been minimized. If antibody crosstalk is present towards a particular analyte, then standard curves should be run in the presence of only the Acceptor beads targeted to that analyte.

For a given assay, the crosstalk needs to be determined only once as long as the instrument optics and settings are kept the same. Once the magnitude of signal crosstalk of a given channel to another is determined, it can easily be subtracted from the signal on the second channel by multiplying the signal obtained on one channel by the measured crosstalk percentage and subtracting that value from the signal on the second channel measured in the same well. After the optical crosstalk has been removed, each data set can be treated as if it were acquired as a single-plex AlphaLISA assay. An Excel worksheet is available to automate the calculation of crosstalk percentages and the crosstalk correction process on an entire plate of data at a time. More in depth examples illustrating the measurement and correction of optical crosstalk between two channels are provided in the next section.

E. Optical crosstalk correction from standard Curves

The magnitude of optical crosstalk is determined from the data in a typical standard curve. The following assumes that both antibody crosstalk and well-to-well crosstalk (sections V.B and V.C above) have been determined to be negligible or have been corrected for. If such crosstalk cannot be completely eliminated, it is recommended to measure crosstalk by preforming standard curves in the absence of the second Acceptor beads (in the case of antibody crosstalk) or in non-adjacent wells (in the case of well-to-well crosstalk). The standard curve for a given analyte is read on all channels to be used in the multiplex. The three highest signal data points are used, after subtracting the average background from a series of blank wells (Donor and Acceptor beads but no analyte). The optical crosstalk is then obtained by dividing the observed signal on the secondary channels used in the multiplex assay (e.g. Tb) by the signal on the primary channel (e.g. Eu) for each data point and averaging the result. The obtained fractional crosstalk can be multiplied by 100 to express as a percentage, if desired. The same is done for the other analyte(s) to obtain the crosstalk ratio in the reverse direction. This process can be performed using the available Excel worksheet as described below. The example provided is for an AlphaPlex 545 (Tb) and AlphaLISA (Eu) duplex, targeted to analyte A and analyte B, respectively. Standard curves, each in triplicate with 12 dilution points and 4 blanks are run as follows:

1. One standard curve in duplex using only analyte A
2. One standard curve in duplex using only analyte B

Each curve is read on both emission channels (e.g. 545 nm Tb and 615 nm Eu). The data thus obtained from the first two experiments are then entered into the worksheet as sets of triplicates as illustrated below:

	Data experiment 1 (analyte A)							Data experiment 2 (analyte B)						
Row	Read at 545 nm Tb			Read at 615 nm Eu				Read at 545 nm Tb			Read at 615 nm Eu			
1	31289	30139	30357	463	449	434		2335	2306	2442	108381	102163	107201	
2	19872	17423	19738	343	325	368		2318	2351	2442	110618	103941	109251	
3	8835	8670	7823	277	325	234		2029	2012	1962	91742	88229	85886	
5	1013	875	979	219	200	181		501	492	534	22609	21184	22213	
6	368	363	310	220	234	210		248	253	210	7975	7777	7856	
7	133	119	167	243	210	205		124	133	147	3758	3426	3488	
8	86	81	148	220	205	215		128	95	90	1841	1918	1913	
9	62	67	28	263	253	229		95	76	90	969	854	882	
10	43	47	66	224	205	239		71	71	71	911	792	781	
11	47	43	47	244	239	234		81	76	81	796	729	763	
12	38	38	28	234	229	220		85	109	56	700	725	781	
13	71	47	43	311	258	239	blank	71	47	43	311	258	239	
14	57	67	52	225	201	210	blank	67	57	47	311	277	296	
15	47	47	52	272	277	268	blank	57	52	52	306	296	267	
16	86	57	43	321	282	253	blank	47	47	52	268	277	325	

Each column of 16 values represents the 12 dilution points and 4 blanks (no analyte) with sets of three columns representing triplicate wells. For illustration purposes, triplicates read on the Tb channel are shaded light green and those read on the Eu channel are shaded light red. An average of each set of triplicates is executed by the Excel formula. Rows 13, 14 15 and 16, representing equivalent sets of triplicate blank wells, are all averaged for each reading channel to generate the average background for that channel. The resulting values are shown below:

	Experiment 1	
	Data points	
Row	545 Tb	615 Eu
1	30595	449
2	19011	345
3	8443	279
4	2889	242
5	956	200
6	347	221
7	140	219
8	105	213
9	52	248
10	52	223
11	46	239
12	35	228
	Background	
	56	260

Experiment 2	
Data points	
545 Tb	615 Eu
2361	105915
2370	107937
2001	88619
1133	47484
509	22002
237	7869
135	3557
104	1891
87	902
71	828
79	763
83	735
Background	
53	286

The averaged background from each measurement channel is then subtracted from the averaged data points of each curve to generate background subtracted data sets for each measurement channel.

Some of the lower concentration data points may have negative values due to the background correction. This is not a concern so long as the first three values, which will be used for the crosstalk correction, are positive.

	Experiment 1	
	Data points	
Row	545 Tb	615 Eu
1	30539	189
2	18955	86
3	8387	19
4	2833	-18
5	900	-60
6	291	-38
7	84	-40
8	49	-46
9	-3	-11
10	-4	-37
11	-10	-21
12	-21	-32

Experiment 2	
Data points	
545 Tb	615 Eu
2283	105108
2292	107130
1923	87812
1055	46677
431	21195
159	7062
57	2750
26	1084
9	95
-7	21
1	-44
5	-72

To determine the correction factor of the 545 nm Tb signal detected on the 615 nm Eu channel, the 3 highest concentration points measured in Experiment 1 on the 615 nm Eu channel (in red below) are divided by the signal obtained for the same wells on the 545 nm Tb channel (in green below). The three highest points are selected to avoid artifacts introduced from background correction with low signal. The reverse is applied to the data from Experiment 2 to obtain the crosstalk from the 615 nm Eu signal into the 545 nm Tb channel. Finally, these data are averaged to obtain the correction factors. The obtained correction factors are displayed as percent values. In the case shown below, the percentage of AlphaPlex 545 (Tb) signal that is read on the AlphaLISA 615 (Eu) channel is 0.43% and the percentage of AlphaLISA 615 (Eu) signal that is read on the Tb channel is 2.17%.

	Experiment 1	
	Data points	
Row	545 Tb	615 Eu
1	30539	189
2	18955	86
3	8387	19
4	2833	-18
5	900	-60
6	291	-38
7	84	-40
8	49	-46
9	-3	-11
10	-4	-37
11	-10	-21
12	-21	-32

Experiment 2	
Data points	
545 Tb	615 Eu
2283	105108
2292	107130
1923	87812
1055	46677
431	21195
159	7062
57	2750
26	1084
9	95
-7	21
1	-44
5	-72

Exp. 1
615/545
0.0062
0.0045
0.0023

Exp. 2
545/615
0.0217
0.0214
0.0219

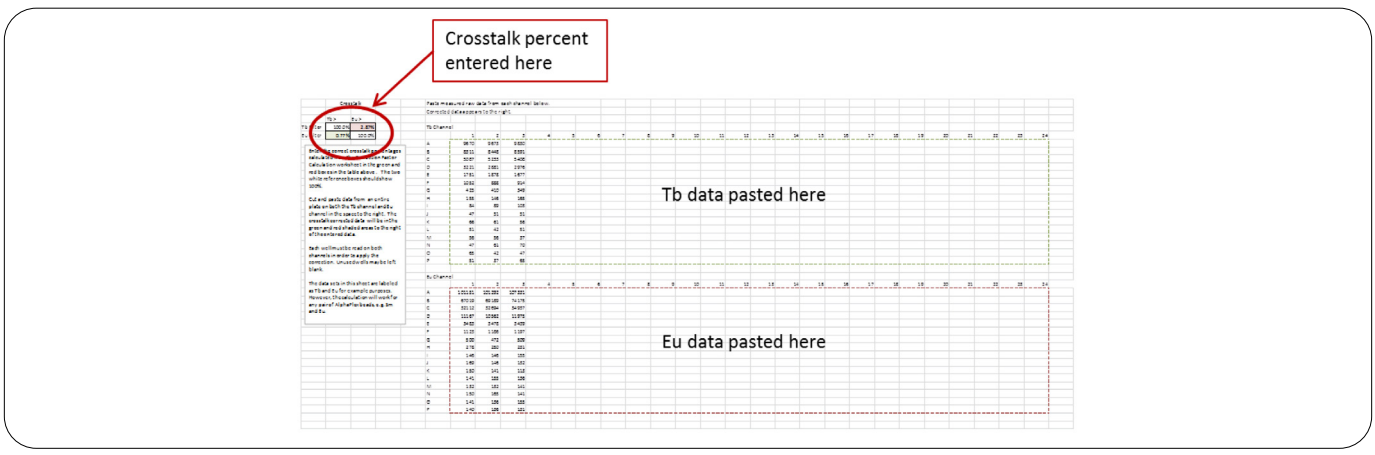
correction factor
545 > 615
0.0043

correction factor
615 > 545
0.0217

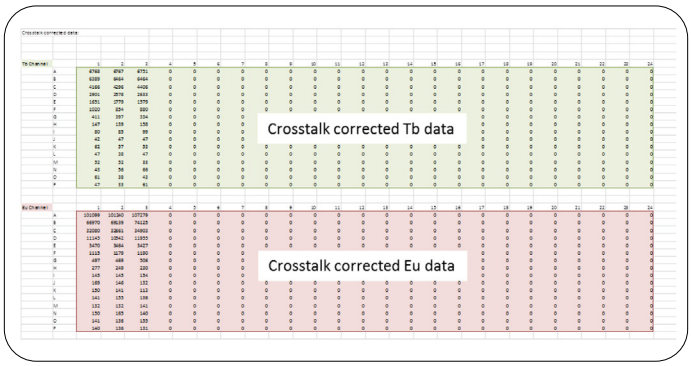
Once the correction factors have been identified, the crosstalk corrected data can be obtained by taking the counts at one wavelength and subtracting the counts from the other channel multiplied by their correction factor.

$$545_{corr} = 545 - (615 * \text{corr factor})$$
$$615_{corr} = 615 - (545 * \text{corr factor})$$

The process can be performed on an entire plate of data at a time using the crosstalk correction worksheet by entering the calculated crosstalk percentages in the color coded boxes in the upper left of the worksheet, then pasting up to an entire 384-well plate of data read on each of the two channels in the space provided as shown below:



The crosstalk corrected data appears in the shaded green and red regions to the right of the entered data:



As long as the assay is performed on the same instrument with the same optics (mirror block and filters) and with the same reagent batch, there is no need to measure the correction factor every time the experiment is performed. If the instrument and/or the optics (see Chapter V) are changed a new measurement of the correction factor is recommended.

Appendix I: Summary of AlphaPlex assay development steps

A Check compatibility of AlphaLISA and AlphaPlex kits and optics

Ensure that each assay to be combined is on a separate wavelength channel.

↓

Ensure the necessary optics are in place and configured for use in the instrument.

↓

Test each assay for cross-reactivity toward the analyte of each assay to be combined in the multiplex.

B Optimize assay conditions & Determine assay characteristics

Determine optimal assay conditions:

Assay buffer
Assay volume
Incubation time
Order of addition
Bead concentration

For each assay individually and select one set of conditions that works well for all assays.

↓

Determine assay characteristics from calibration curves performed with all multiplex components present. Determine the effect of a high concentration of each analyte on the calibration curve of the other assay(s).

C Determine analyte level in unknown samples

Perform calibration curves in matrix solution with all analytes presence and absence of other analytes for each analyte of the assay

↓

Determine the percent of optical crosstalk between each AlphaPlex channel from calibration data of each analyte alone and apply optical crosstalk correction to curves with multiple analytes.

↓

Interpolate the analyte concentration in unknown samples from the corrected calibration curves with all analytes present.

Appendix II: New assay development

In some cases, a validated assay kit toward an analyte of choice may not exist. This section describes the steps necessary for developing a new Alpha assay towards a desired target using Unconjugated AlphaLISA or AlphaPex Acceptor beads. The recommended reagents and materials are summarized in Tables 3 and 4 below. Once developed, such a custom assay can be paired with other kits or custom assays to establish a desired multiplex assay.

Table 3: Recommended reagents and materials.

Item	Selected Source	Catalog #
AlphaScreen Streptavidin-coated Donor beads	Revvity, Inc.	6760002S (1 mg) 6760002 (5 mg) 6760002B (50 mg)
Analyte of choice	N/A	N/A
Pairs of anti-analyte antibodies	N/A	N/A
Microplate	Revvity, Inc.	see Table 4
TopSeal-A Adhesive Sealing Film	Revvity, Inc.	6005185 (pack size of 100 pieces)
Single-channel pipettors	N/A	N/A
NHS activated biotinylating reagent (ChromaLink)	SoluLink Inc.	B1001-105
Carboxymethoxylamine hemihydrochloride (CMO)	Sigma-Aldrich Co.	C13408
Sodium cyanoborohydride	Sigma-Aldrich Co.	156159
Zeba desalting columns	Pierce (ThermoFisher Scientific Inc.)	89882 (0.5 mL) 89889 (2 mL) 89891 (5 mL) 89893 (10 mL)
Proclin-300	Sigma-Aldrich Co.	48912-U
Tween-20 (Surfact-Amps 20)	Pierce (ThermoFisher Scientific Inc.)	28320
Dextran 500 MW ~500000	Sigma-Aldrich Co.	D1037
Casein 5% Alkali-soluble solution	Novagen (EMD Chemicals Inc.)	70955
Triton-X100 (Surfact Amps X100)	Pierce (ThermoFisher Scientific Inc.)	28314
Streptavidin-Sepharose beads	GE Healthcare, Inc.	17-5113-01

Table 4: Microplates.

Assay Format	Microplate	Catalog #	Total well volume	Recommendations
96	OptiPlate™-96	6005290	400 µL	Ideal for assay volume ≥ 100 µL
	½ AreaPlate-96	6005560	180 µL	Ideal for assay volume of 50 µL in 96-well format
	ProxiPlate™-96	*6006290*	*100 µL*	***Not recommended***
384	OptiPlate-384	6007290	105 µL	Good for assays of 25-50 µL in 384-well format
	AlphaPlate™- 384	6005350	105 µL	Ideal for high-count assays of 25-50 µL in 384-well format (lower cross-talk than OptiPlate-384)
	ProxiPlate-384	6008280	30 µL	Good for assays of 10-25 µL in 384-well format

A. Selection of antibody pairs

To develop an AlphaPlex analyte detection assay, antibody pairs that recognize the targeted analytes are required. It is always preferable to find antibody pairs that have already been tested in a sandwich assay. Most of the identified pairs should perform well in AlphaPlex. If no identified pairs are available, different antibodies need to be tested to find pairs that will perform optimally in AlphaPlex. Each separate analyte in the AlphaPlex assay will require its own antibody pair.

- All antibodies must be selected according to the following guidelines:
- Each antibody should recognize a different epitope on the corresponding analyte.
- The two antibodies against a given analyte must be specific to that analyte.
- Monoclonal or purified polyclonal antibodies will perform best; avoid the use of anti-sera.

One of the two antibodies of each pair will have to be biotinylated and the other one should be conjugated to Alpha 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-AlphaPlex Acceptor beads
2. biotinylated antibody B + antibody A-AlphaPlex Acceptor beads

Therefore, it is recommended to biotinylate both antibodies of each pair as well as conjugating both to AlphaPlex Acceptor beads to eventually select the optimal assay configuration.

(Note: Alternatively, the antibodies can be captured in an indirect assay set-up using anti-species IgG AlphaLISA or AlphaPlex Acceptor beads and/or biotinylated anti-species IgG)

B. Biotinylation of the antibody

The following protocol is used for biotinylation of antibodies:

a. A preliminary check of the antibody to be biotinylated is mandatory. The User must check for the following:

- 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.

b. 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.

c. On the day of the assay, prepare a fresh solution of N-hydroxysuccinimido-ChromaLink-biotin (NHS-ChromaLink-biotin) 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.

d. 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.

e. Incubate for 2 hours at 21-23 °C.

f. 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).

C. Acceptor bead conjugation with the antibody

The following procedures may be applied to both AlphaLISA and AlphaPlex beads. Prior to performing a new conjugation, it is recommended to perform a preliminary check of the material to be conjugated for the following:

- The conjugation will perform best when the antibody concentration is at least 1 mg/mL (when conjugating 1-2 mg of beads) or 0.53 mg/mL (when conjugating 2.5 mg of beads or higher amounts). Lower concentrations of antibody yield lower coupling efficiency.
- 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. *(Note: Although both buffers can be utilized, for clarity purposes, phosphate buffer will be used in the protocol below).*
- Ideally, antibody solutions should not contain any protein or peptide-based stabilizers (such as BSA or gelatin). However in the presence of such substances, the conjugation process can still be performed, but may result in lower coupling efficiency in some cases.
- Glycerol will significantly impact coupling efficiency (10% glycerol final in the conjugation mix will cause approx. 50% signal reduction). Dialysis of the antibody is recommended prior to coupling.

The ratio of antibody to mg of beads is an important parameter for a successful assay development.

Typical coupling weight ratios (amount of beads to amount of antibody) vary from 10:1 to 50:1. When preparing low amounts of beads (1-2 mg), a 10:1 ratio is recommended (i.e. 1 mg of Acceptor beads to 0.1 mg of antibody), while a ratio of 50:1 is used with bead amounts equal to or higher than 2.5 mg to minimize the antibody consumption (i.e. 5 mg of Acceptor beads to 0.1 mg of antibody).

Protocol for conjugating 1 mg AlphaPlex Acceptor beads (10:1 coupling ratio)

This procedure is appropriate for a solution of antibody ≥ 1 mg/mL; if the concentration is below 1 mg/mL, the antibody solution must be concentrated, for example using an iCON Concentrator (Pierce Cat# 89886), Microcon or Centricon (Ultracell YM-30, Millipore, cat# 4208 or 42409).

Washing

- Wash AlphaPlex Acceptor beads (50 µL at 20 mg/mL) once with 50 µL PBS: centrifuge at 16,000 × g or maximum speed for 15 min and then discard the supernatant.

Conjugation

In an Eppendorf tube, add the following reagents:

- 1 mg of AlphaPlex Acceptor bead pellet (prepared as described above)
- 0.1 mg of antibody
- the appropriate volume of 0.13 M 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 25 mg/mL solution of $NaBH_3CN$ in water (prepare fresh as required).

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.

Blocking

- Prepare a fresh 65 mg/mL solution of carboxy-methoxylamine (CMO) in a 0.8 M NaOH solution.
- Add 10 µL of CMO solution to the reaction (to block unreacted sites).
- Incubate for 1 hour at 37 ° C.

Purification

- Centrifuge for 15 minutes at 16,000 × g (or maximum speed).
- Remove the supernatant with a micropipette and re-suspend the bead pellet in 200 µL of 0.1 M Tris-HCl pH 8.0.
- Centrifuge for 15 minutes at 16,000 × g (or maximum speed), then remove the supernatant.
- Repeat the washing steps (re-suspend the pellet and centrifuge) another time.
- After the last centrifugation, re-suspend the beads at 5 mg/mL in storage buffer (200 µL of PBS + 0.05% Proclin-300 as a preservative).
- Vortex, then briefly spin (e.g. 2000 g for one minute) and optionally sonicate the bead solution (up to 20 short pulses of 1 second using a probe sonicator).

Storage

- Store the conjugated Acceptor bead solution at 4 °C

Important note: always vortex conjugated AlphaPlex Acceptor beads before use, as beads tend to settle with time.

Protocol for conjugating 5 mg AlphaPlex Acceptor beads (50:1 coupling ratio)

This procedure is appropriate for a solution of antibody ≥ 0.53 mg/mL; if the concentration is below 0.53 mg/mL the antibody solution must be concentrated using an iCON Concentrator (Pierce Cat# 89886), Microcon or Centricon (Ultracell YM-30, Millipore, cat# 4208 or 42409).

Washing

- Wash AlphaPlex Acceptor beads (250 µL at 20 mg/mL) once with 250 µL PBS: centrifuge at 16,000 ×g or maximum speed for 15 min and then discard the supernatant.

Conjugation

In an Eppendorf tube, add the following reagents:

- 0.1 mg of antibody to 5 mg of bead pellet (prepared as described above)
- the appropriate volume of 0.13 M 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 25 mg/mL solution of $NaBH_3CN$ in water (prepare fresh as required).

Incubate for 18-24 hours at 37 °C with mild agitation (6-10 rpm) using a rotary shaker.

Blocking

- Prepare a fresh 65 mg/mL solution of carboxy-methoxylamine (CMO) in a 0.8 M NaOH solution.
- Add 10 µL of CMO solution to the reaction (to block unreacted sites).
- Incubate for 1 hour at 37 °C.

Purification

- Centrifuge for 15 minutes at 16,000 × g (or maximum speed).
- Remove the supernatant with a micropipette and resuspend the bead pellet in 1 mL of 0.1 M Tris-HCl pH 8.0 (200 µg per mg of beads).
- Centrifuge for 15 minutes at 16,000 × g (or maximum speed), then remove the supernatant.

- Repeat the washing steps (resuspend the pellet and centrifuge) another time.
- After the last centrifugation, resuspend the beads at 5 mg/mL in storage buffer (1 mL 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).

Storage

- Store the conjugated Acceptor bead solution at 4 °C.

Important note: always vortex conjugated AlphaPlex Acceptor beads before use, as beads tend to settle with time.

D. Determination of the best antibody pair

These procedures apply equally for AlphaLISA and AlphaPlex Acceptor beads

One of the first development steps is the selection of the antibody combination.

- If any information is available for AlphaLISA (or AlphaLISA kit) results, the assay giving the most signal should be the one adapted to AlphaPlex.
- If the information is unknown, the following procedure should be tested with AlphaLISA beads for both analytes. The assay giving the most signal should then be converted to AlphaPlex beads.
- After that, as previously mentioned, both combinations of antibody set-up must be tested for each analyte:

1. biotinylated antibody A + antibody B-AlphaPlex Acceptor beads
2. biotinylated antibody B + antibody A-AlphaPlex Acceptor beads

This section describes the method to be used to perform this selection.

Selection of optimal antibody pair(s) using a matrix assay

- A matrix assay should be performed using fixed concentrations of antibodies and 2-3 different concentrations of analyte (within the usual working range) and a negative control (no analyte).
- It is important to note that this method is a guideline; it will need to be optimized for every analyte studied.
- These preliminary conditions could be used for this experiment:

Typical assay buffer:

- 25 mM HEPES pH 7.4
- 0.5% Triton X-100
- 0.1% Casein
- 1 mg/mL Dextran 500
- Adjust pH to 7.4

This buffer is available from Revvity, Inc. as a 10X solution:

AlphaLISA ImmunoAssay Buffer (10X)
(Cat No. AL000C/F).

Dilute 10-fold with water prior to use.

In an OptiPlate-384 microplate, add:

- 5 µL of the analyte diluted in assay buffer (use various concentrations that are in the working range for the target detection)
- 10 µL of fixed amount of the biotinylated antibody (Use a final concentration between 0.3 nM and 3 nM of biotin-antibody. Often the optimal biotinylated antibody concentration is 1 nM.)
- 10 µL of antibody-coupled AlphaPlex Acceptor beads at 50 µg/mL (10 µg/mL final concentration in each well)

Incubate at 23 °C for 1 h then add:

- 25 µL of streptavidin Donor beads at 80 µg/mL prepared under subdued light conditions (40 µg/mL final concentration in each well)

Incubate at 23 °C in the dark for 30 min and read on an Alpha reader with the appropriate filter.

Select the pair(s) of antibodies providing the highest signal-to-background (S/B) ratio and the best sensitivity as defined as the highest S/B ratio for the condition with lowest concentration of analyte. Once selected, determination of optimal biotinylated antibody concentration can be performed. Sample data are shown in Figure 5.

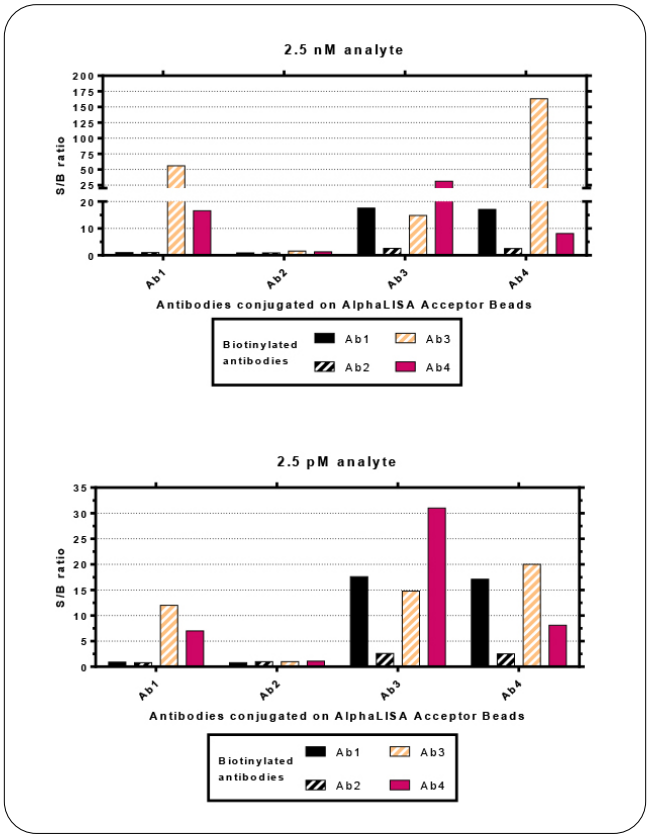


Figure 5: Determination of the best antibody pair (shown for AlphaLISA but also applies to AlphaPlex). S/B ratio obtained for each antibody pair (each permutation possible), with 2.5 nM and 2.5 pM of analyte (S/B ratio is calculated as AlphaLISA signal obtained for 2.5 nM or 2.5 pM analyte ÷ AlphaLISA signal obtained without analyte (background)). In this example, Antibody-Ab3-conjugated AlphaPlex Acceptor Beads and Biotinylated-Antibody-Ab4 were selected.

Determination of optimal biotinylated antibody concentration

An antibody titration curve should be performed using a fixed concentration of analyte (within the usual working range).

The following preliminary conditions may be used for this titration.

It is important to note that the method is a guideline; it will need to be optimized for every analyte studied.

In an OptiPlate-384 microplate, add:

- 5 µL of the analyte diluted in assay buffer (use a fixed concentration that is in the working range for the target detection)
- 10 µL of increasing amounts of the biotinylated antibody (as a starting point, use concentrations between 0.1 nM to 100 nM final concentration of biotin-antibody)
- 10 µL of antibody-coupled AlphaPlex Acceptor beads at 50 µg/mL (10 µg/mL final concentration in the well)

Incubate at 23 °C for 1 h then add:

- 25 µL of streptavidin-Donor bead solution at 80 µg/mL prepared under subdued light conditions (40 µg/mL final concentration in each well)

Incubate at 23 °C in the dark for 30 min and read on an AlphaScreen reader.

A bell-shaped curve should be obtained (Figure 6). The highest signal obtained indicates the hook point (highest signal before saturation of the bead binding capacity) of the biotinylated anti-analyte antibody. A sub-hooking concentration should be used for the next optimization steps. A lack of specific signal likely indicates that the selected antibodies are not able to capture the analyte simultaneously in this specific configuration.

For the selection of the best combination of antibody pair, the usual criterion is the highest S/B signal. However, in order to confirm the choice of the antibody pair, a calibration curve of the analyte should be performed. This will allow the measurement of the analyte detection limit and the dynamic range of the specific pair of antibody in the AlphaPlex assay.

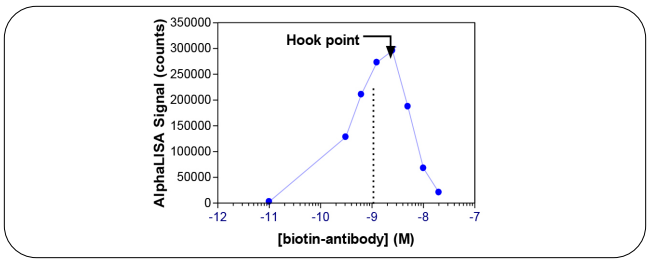


Figure 6: Titration curve for an insulin detection assay, prepared in assay buffer: in this particular example, the optimal antibody concentration was determined to be 1 nM.

Appendix III: Multiplexing examples

A. Simultaneous detection of hIFN-γ (Tb) and hIL-1β (Eu) using AlphaPlex technology

Summary: Immunoassays are the primary methods used to measure production and modulation of cytokines by cells. AlphaPlex technology allows the simultaneous analysis of two different cytokines in the same sample. Using a combination of AlphaPlex 545 (Tb) and AlphaLISA (Eu) beads sequential and simultaneous detection of both recombinant hIFN-γ and hIL-1β, has been demonstrated.

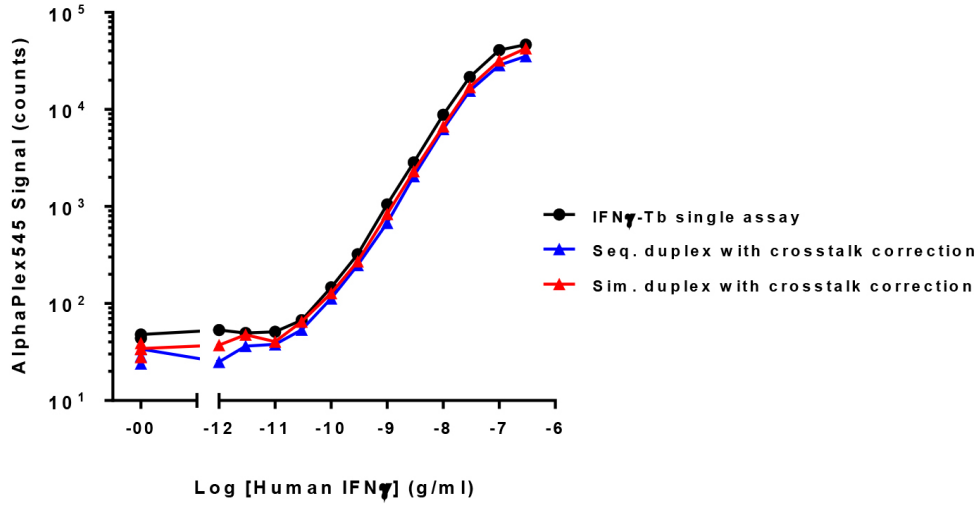
Methods: A standard curve was prepared using AlphaLISA (Eu) beads targeted to hIL-1β (kit cat # AL220) and AlphaPlex 545 beads targeted to hIFN-γ (kit cat # AP217TB) with individual analytes and simultaneous dilution of both analytes.
The biotinylated antibodies were each used at a final concentration of 1 nM. The antibody-conjugated AlphaPlex and AlphaLISA Acceptor beads were each at 10 µg/mL and the streptavidin-Donor beads were used at a final concentration of and 40 µg/mL.

The plates were read on an EnVision Multilabel Reader using standard Alpha optics for the single curves (Mirror barcode 444 and Alpha filter barcode 244 for Eu and Resorufine Filter barcode 124 for Tb), a narrow Eu filter (barcode 203) and a Resorufine filter (barcode 124) for sequential duplex read and a Tb/Eu dual mirror (AlphaPlex Tb/Eu barcode 653) with the same filters for simultaneous measurement of the two analytes. Crosstalk between Tb and Eu emission channels was corrected as specified in Section V. Sensitivity (LDL) was calculated according to Section III.

Results: Sensitivity curves for each optical configuration, as well as LDL and maximum observed counts and measured crosstalk percentages for each analyte of the duplex are shown below. The sensitivity of the assays in single, sequential duplex and simultaneous duplex, as measured by the calculated LDLs for hIL-1β and hIFN-γ were all comparable. The total counts for the AlphaPlex 545 (Tb) assay for hIFN-γ were similar with both the standard Alpha mirror (barcode 444) and the duplexing mirror (AlphaPlex Tb/Eu barcode 653) using the same filter (Resorufine barcode 124). For the AlphaLISA (Eu) assay for hIL-1β, the signal intensity (counts) for the three curves reflects the expected changes due to the different optics used for each curve. The single assay with the Alpha mirror (barcode 444) and wide filter (barcode 244) gave the highest counts, while the narrower Eu filter (barcode 203) required to isolate the Eu signal from the Tb signal gave about 3X lower counts. The duplexing mirror, which sends the Eu signal to the secondary detector along a longer path length, was also somewhat lower. It is important to note that sensitivity was comparable even though the signal intensity was lowered by the optics required for simultaneous multiplexing. Signal crosstalk between channels was in the expected range of 0.6% to 3%, depending on the optical configuration.

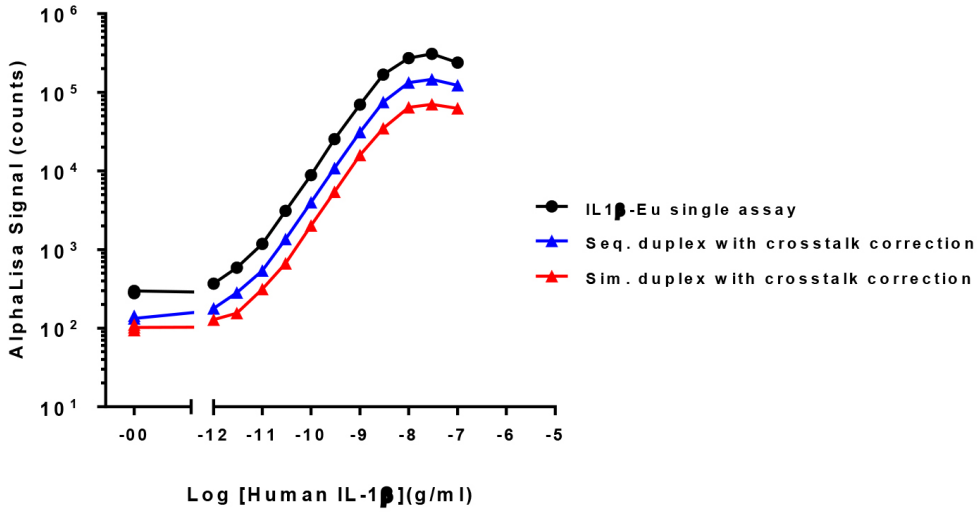
Conclusion: Both analytes could be detected successfully in duplex assays by both sequential reading with a standard mirror and simultaneous duplexing with an AlphaPlex dual mirror without compromising assay sensitivity.

hIFN-γ (Tb) Results Summary:

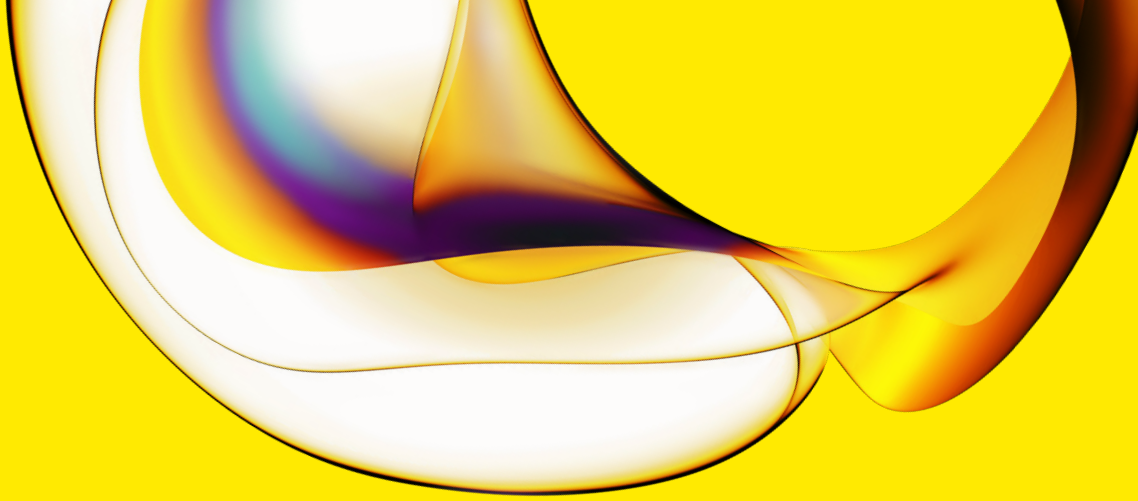


IFN-γ (Tb)	IFN-γ LDL pg/mL	Mirror	Eu filter	Tb filter	Max counts	Crosstalk Tb→Eu (%)
Single	12.9	444	n/a	124	46605	
Duplex seq	41.1	444	203	124	39940	1.25
Duplex sim	26.1	653	203	124	42686	0.59

IL-1β (Eu) Results Summary:



IL-1β (Eu)	IL-1β LDL pg/mL	Mirror	Eu filter	Tb filter	Max counts	Crosstalk Eu→Tb (%)
Single	0.60	444	244	n/a	322971	
Duplex seq	1.06	444	203	124	123113	1.25
Duplex sim	1.98	653	203	124	62359	0.59



www.revvity.com

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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