AlphaPlex quick start guide

A. AlphaPlex overview

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 proteins, molecules and biomarkers. Using a universal, streptavidin-coated Donor bead, multiple AlphaPlex Acceptor beads targeted to various analytes are combined in a single assay well. Figure 1 illustrates a typical AlphaPlex assay.

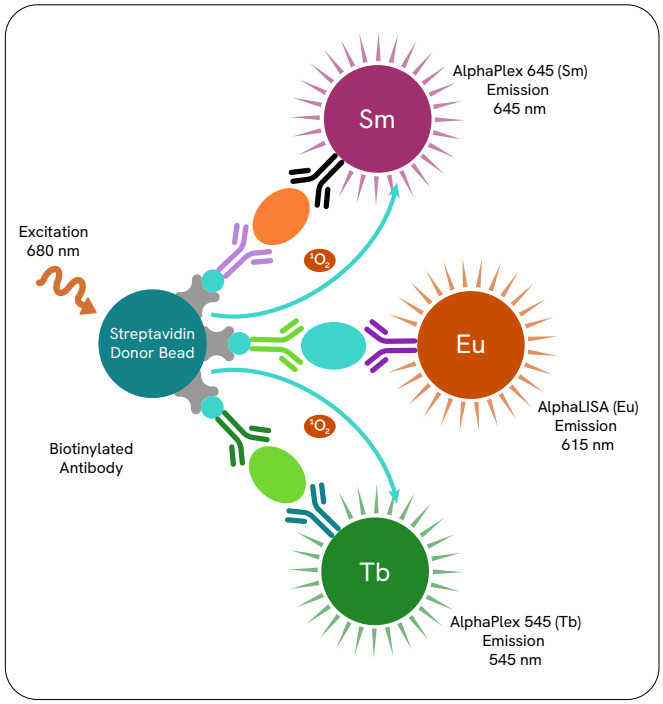

Figure 1: Schematic of an AlphaPlex triplex assay using streptavidin Donor beads with AlphaPlex 645 (Sm), AlphaLISA (Eu) and AlphaPlex 545 (Tb) Acceptor beads targeted toward three different analytes. Streptavidin Donor beads act as a source of singlet oxygen for all colors of bound AlphaPlex Acceptor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

Biotinylated anti-analyte antibodies bind the streptavidin Donor beads while complementary anti-analyte antibodies are conjugated to AlphaPlex or AlphaLISA® Acceptor beads. In the presence of the target analytes, the Acceptor beads come into close proximity to the Donor beads. Excitation of the Donor beads with 680 nm laser light provokes the release of singlet oxygen which triggers chemiluminescent light emission from the bound Acceptor beads. Each type of Acceptor bead emits light at specific wavelengths which are accurately resolved by AlphaPlex optics to independently quantify each analyte with minimal interference. Users can build multiplex assays from numerous commercial AlphaLISA and AlphaPlex detection kits for specific analytes or develop their own assays with toolbox beads. This Quick Start Guide is intended to provide the Alpha user with the knowledge and tools necessary to quickly and easily unleash the power of multiplexing using AlphaPlex.

B. Setting up an AlphaPlex assay

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.[1]
The recommended buffer is standard 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.[2]
Typical final concentrations in an assay well for an AlphaPlex duplex assay are shown in Table 1.

For an AlphaPlex multiplex assay, the various assay components including Acceptor beads and biotinylated antibodies are added in the same order as an AlphaLISA 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.

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

Calibration curves for AlphaPlex assays are generally 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, including appropriate correction of any optical crosstalk (see section D below). In the majority of cases, the calibration curves with and without the second analyte will be essentially identical. 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. Please refer to section III of the AlphaPlex Development Guide for advanced tips on multiplex assay optimization.

C. AlphaPlex optics

When reading an AlphaPlex assay, it is critical to make sure the right optics are in place. The optics required for AlphaPlex multiplexing with a Revvity EnVision Multilabel reader consist of a single or dual dichroic mirror block and emission filters specific for the emission of the AlphaPlex or AlphaLISA 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.

The major emission peaks of AlphaLISA (Eu), AlphaPlex 545 (Tb) and AlphaPlex 645 (Sm) Acceptor beads are centered at 615 nm, 545 nm and 645 nm, respectively. Eu is the strongest emitter, while Tb and Sm give somewhat lower signal (typically 12-20%), but sensitivity and signal to background ratios are comparable. The recommended emission filters for multiplexing AlphaLISA (Eu), AlphaPlex 545 (Tb) and AlphaPlex 645 (Sm) are shown in Table 2.

The standard AlphaScreen mirror, barcode 444, may be utilized for single and sequential multiplex reads of AlphaPlex 545 (Tb) and AlphaLISA (Eu) Acceptor beads, whereas the AlphaPlex single mirror, barcode 605, has been optimized for all three AlphaPlex Acceptor beads, including AlphaPlex 645 (Sm). Additionally, two dual mirror modules, barcodes 653 and 658, are available for simultaneous reads of AlphaLISA (Eu) and AlphaPlex 545 (Tb) or AlphaPlex 645 (Sm) and AlphaLISA (Eu), respectively. Emission filters for both of the AlphaPlex Acceptor beads under analysis must be adjacent to one another and in the correct order in the EnVision filter slide in order to perform a simultaneous AlphaPlex read using a Dual mirror.[3] A Sm compatible mirror (barcode 605 or 658) is required for all AlphaPlex 645 (Sm) applications due to its longer emission wavelength.

Table 2. AlphaPlex optics for EnVision Multilabel reader

	Description	Cat. #	Bar-code	Recommended use
Mirrors	AlphaScreen Single	2101-4010	444	Sequential duplexing of Eu and Tb
	AlphaPlex Single Tb-Eu-Sm	2102-5910	605	For all sequential AlphaPlex applications
	AlphaPlex Dual Tb-Eu	2102-5900	653	*Simultaneous* duplexing of Tb and Eu
	AlphaPlex Dual Sm-Eu	2102-5920	658	*Simultaneous* duplexing of Sm and Eu or Tb
Filters	AlphaScreen	2100-5710	244	Suitable for AlphaPlex single plexing; not for multiplexing
	Resorufine/Amplex Red	2102-5570	124	Suitable for Tb single plexing and Tb/Eu duplexing
	Europium	2100-5090	203	For all Eu applications and multiplexing
	AlphaPlex Tb	2100-5930	701	For all Tb applications and multiplexing
	AlphaPlex Sm	2100-5940	702	For all Sm applications and multiplexing

D. Crosstalk correction

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. Optical crosstalk is quantified as a percentage of the emission signal on one detection channel that is detected on a second channel (e.g. the percentage of 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 by running standard curves for each analyte with all components present. 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%. Once the magnitude of signal crosstalk of a given channel to another is determined, it can easily be subtracted from the signal on a second channel (i.e. by multiplying the signal obtained on the Eu channel by the percentage and subtracting that value from the Tb signal measured in the same well). Once 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 help automate the crosstalk correction process. See section V.E of the AlphaPlex Development Guide for more in depth information about optical crosstalk correction.

E. Summary

The beauty of AlphaPlex is the ease with which an established AlphaLISA assay can be extended to include the detection of other analytes of interest in the same well with minimal changes to experimental design. The guidelines here represent a useful starting point that will function well for the majority of multiplex assays. Each multiplex assay is unique, however, and will benefit from individual optimization. The full AlphaPlex Development Guide provides more in-depth information for advanced users who wish to further develop their own assays.

References

1. Complementary Acceptor beads are of different emission wavelengths and are specific for each target analyte relative to the other analyte(s).
2. 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 sufficient streptavidin binding sites; 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.
3. The primary channel filter for a dual mirror must be exactly one numbered slot higher in the filter slide than the secondary channel filter.

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