APPLICATION NOTE

Performing AlphaLISA assays with RPMI-1640.

Authors

Jessica Suschak
Jason Hu
Roger Bossé
Sylvie Kossodo

Revvity, Inc.

Introduction

AlphaLISA™ Technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly manner. As shown in Figure 1, a biotinylated anti-analyte antibody binds to streptavidin-coated donor beads while another anti-analyte antibody is conjugated to AlphaLISA acceptor beads. The analyte is recognized by both antibodies, thus allowing the beads to come into close proximity. The excitation of the donor beads at 680 nm induces the release of singlet oxygen molecules that trigger an energy transfer cascade in the acceptor beads. Consequently, a sharp peak of light emission is generated at 615 nm, which can be detected using the appropriate instrumentation, such as Revvity EnSpire™ Multimode Plate Reader and EnVision™ Multilabel Plate Reader.

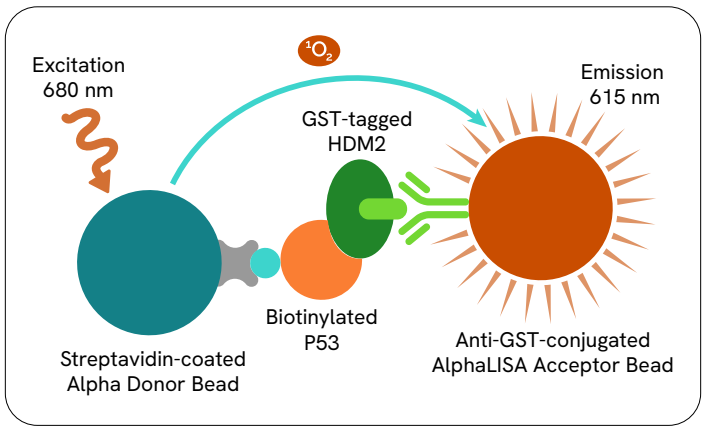


Figure 1: Principle of AlphaLISA technology

Quantitation of analyte in buffer and cell culture media samples can simply be performed by preparing a standard curve using the analyte diluted in analyte-free buffer or cell culture media and interpolating the unknown sample concentration (in buffer or cell culture media) from the standard curve. However, some cell culture media, such as RPMI, contain high levels of biotin (Table 1) which can interfere with AlphaLISA by competing for the streptavidin sites on the donor beads. Biotin, also known as vitamin H or B7, is a water-soluble B-complex vitamin produced by a variety of cells. Biotin plays a vital role in the metabolism of fatty acids and leucine, gluconeogenesis, the citric acid cycle, and the regulation of transcription and DNA repair. Kidney, liver and spleen cells usually contain high levels of endogenous biotin. The presence of high levels of free biotin can result in a decrease in total counts and lower signal to background ratios when performing AlphaLISA assays. Also, the presence of free biotin might affect the LDL (lower detection limit).

This application note provides alternatives that can be used when working with samples in RPMI medium.

Table 1: Biotin content in commonly used cell culture media.

Medium	Biotin Content (µg/mL)
FreeStyle CHO Expression Medium	1760
BME Eagle	1000
RPMI 1640	200
McCoy's	200
MEM a	100
Iscove's MDM	13
Grace's Insect Medium	10
DMEM	0
DMEM/F12	0
HAT	0
EMEM	0
FBS	47

Materials and methods

Reagents

The AlphaLISA Human IgG[1] (Revvity AL307C), Mouse IgG_{2b} Isotyping kits (Revvity AL524C), and Human IL-2 (Revvity AL221C), and Transferrin (Revvity AL311C), kits were used. All media used are commercial formulations sold by Mediatech. Custom biotin-free RPMI media was also prepared by Mediatech.

Instruments

A JANUS® Automated Workstation equipped with WinPREP™ 4.8 software, an 8-tip Varispan™ arm with Versatip® adaptors and Modular Dispense technology arm was used. Plates were read on an EnVision Multilabel Plate Reader.

Assay

Stock solutions of all required reagents (analyte, acceptor beads, biotinylated antibody, donor beads) were prepared. JANUS was used to combine 5 µL of analyte with 10 µL of AlphaLISA anti-analyte acceptor beads and 10 µL of biotinylated anti-analyte antibody. After 60 minutes incubation at 23 °C, the JANUS was used to add 25 µL streptavidin alpha donor beads to each well in a single step. After incubation for 30 min at 23 °C in the dark, the plates were removed and read using EnVision Multilabel Plate Reader. Data was analyzed and graphs were generated using GraphPad Prism®.

Results

The majority of AlphaLISA assays can be performed effectively with samples in cell culture media, even those in RPMI (Table 2, top 3). However, in certain instances (e.g. transferrin), the lower level of sensitivity can be significantly reduced in the presence of RPMI.

Table 2: Examples of AlphaLISA assays performance in buffer and media.

	Recommended Buffer	DMEM + 10% FBS	RPMI + 10% FBS
Human lgG1	47	47	63
Mouse lgG2b	61	27	52
Human lL-2	8	7	7
Human Transferrin	29	38	143

**AlphaLISA Immunoassay or HiBlock buffers*

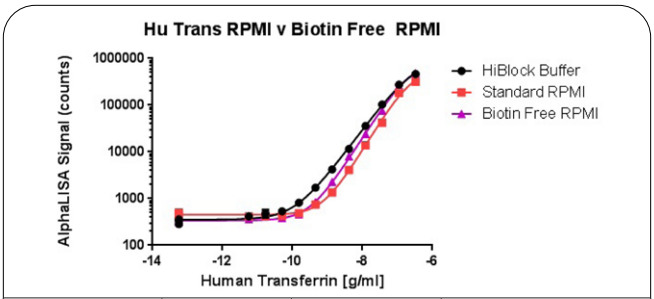
1. At first glance, the easiest approach to circumvent sensitivity issues with RPMI is to switch cell culture media. Human lymphoid cells (either peripheral blood monocytes or cell lines such as Jurkat) are traditionally grown in RPMI, but Iscove and DMEM also work well. In addition, Hybridomas are commonly grown in DMEM/F12 and Iscove.

However, if the protocol used requires RPMI, there are a few alternatives that can be tested:

2. Commercially available biotin blocking solutions (i.e.Vector Avidin/Biotin blocking kit Cat # SP-2001, Zymed Avidin/ Biotin blocking kit Cat # 00-4303 or DAKO Biotin Blocking System Cat # X0590). These solutions are based on their ability to irreversibly mask biotin by the addition of avidin. Briefly, blocking solution is added to the samples in cell culture media, incubated (generally for 15 min), centrifuged (at 10,000 g). The supernatants are then run through gravity columns.

It should be noted that this approach is hardly compatible with large numbers of samples or instances in which chromatography might affect the analyte.

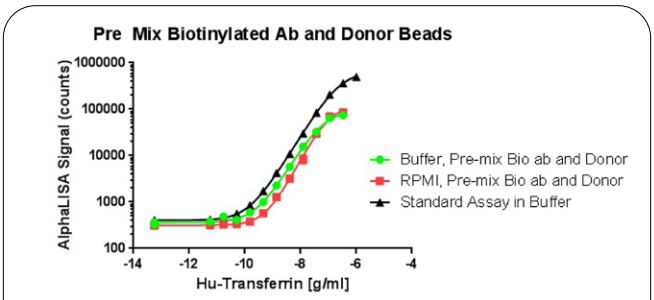
3. Clearly, culturing cells in biotin-free RPMI can also be a viable alternative. We performed the assay using either the recommended buffer (HiBlock Buffer), standard RPMI or biotin-free RPMI (custom-prepared by Mediatech, not yet available commercially) to generate the standard curves. As shown in Figure 2, using biotin-free RPMI greatly improved the sensitivity of the assay, from a 15 fold loss in sensitivity using RPMI to only two fold using the biotin-depleted alternative, as compared to buffer only.



	HiBlock	Std RP MI	Biotin Free RP MI
LDL (pg/mL)	24.8	388.1	51.7
LLOQ (pg/mL)	103.3	961.0	188.7

Figure 2: Depleting the cell culture medium of biotin greatly improves sensitivity of the assay.

4. An easy alternative is to pre-mix the biotinylated antibody with the streptavidin-coated donor beads before adding the sample (analyte). Given the strength of the streptavidin-biotin interaction this step should allow to block as many streptavidin sites on the donor beads as possible with the biotinylated antibody, thus decreasing competition with free biotin present in the media.



	Standard Protocol in Buffer	Pre-mix Biotinylated Antibody with Donor Beads (Buffer)	Pre-mix Biotinylated Antibody with Donor Beads (RPMI)
LDL (pg/mL)	29	78	143
LLOQ (pg/mL)	102	294	424

Figure 3: Pre-mixing donor beads and biotinylated antibody improves sensitivity of the assay.

Figure 3 shows the results of this study. Using the Transferrin kit, donor beads were premixed with the biotinylated antibody for 30 min at room temperature before adding the analyte (in either buffer or standard RPMI) and proceeding with the assay as recommended. In this case, pre-mixing decreased the loss in sensitivity to five fold as compared to buffer only. While not as efficient as using biotin-free media, this approach still improved sensitivity compared to no pre-mixing (five fold from 15 fold loss in sensitivity). Interestingly, pre-mixing itself led to a 2.6 loss in sensitivity, independently of the media used.

Conclusions

The use of the AlphaLISA technology offers significant advantages over current platforms for screening assays: 1) Fully homogeneous, simple protocol, 2) Excellent sensitivity, 3) Very good reproducibility. Very importantly, the fully homogeneous AlphaLISA format eliminates the requirement for wash steps, shortening hands-on time to execute the assay. However, detection of analytes present in biotin-rich culture media such as RPMI 1640 can potentially limit this otherwise powerful screening platform.

This application note provides alternatives to improve the sensitivity of RPMI-sensitive AlphaLISA assays. Based on the data shown,the following recommendations are made. Run the calibration curve in the recommended buffer versus RPMI. In many cases, there will be no significant differences in sensitivity. If a loss of sensitivity is observed, changing to a biotin-poor medium such as DMEM or custom-made biotin-free RPMI will eliminate the problem. Otherwise, pre-incubating streptavidin-coated donor with the biotinylated antibody for 30 min before addition of the analyte will significantly improve the sensitivity of the assay.

References

1. http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/biotin.html
2. AlphaLISA™ Assay Development Guide, Revvity, Inc.

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

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