MANUAL

AlphaLISA® Human Granzyme B Detection Kit

Product number: AL3170

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of human hGranzyme B using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 5 components: AlphaLISA Acceptor beads coated with anti-hGranzyme B Antibody, Streptavidin-coated Donor beads, Biotinylated anti-hGranzyme B antibody, Lyophilized hGranzyme B and 10X AlphaLISA Immunoassay Buffer.

Sensitivity: Lower Detection Limit (LDL): 145 pg/mL
Lower Limit of Quantification (LLOQ): 488 pg/mL
EC_{50}: 420 ng/mL

Dynamic Range: 145 – 1 000 000 pg/mL

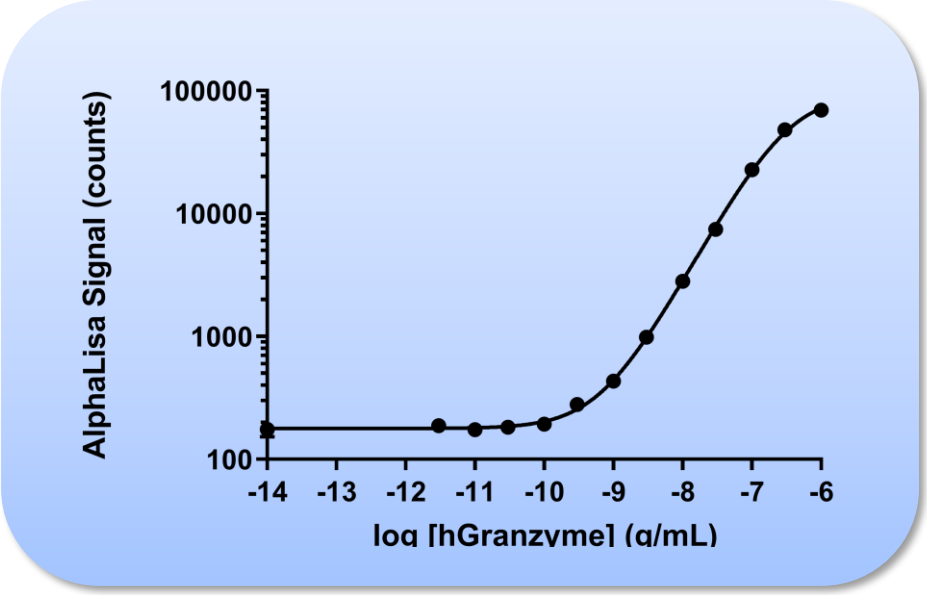


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -20 ºC for up to two weeks. **Avoid freeze-thaw cycles**.

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

Analyte of Interest

Human Granzyme B is a 35 kDa serine protease of the Granzyme family. It is mainly secreted by natural killer cells and will penetrate corrupted cells through pores created by Perforin. Once in the cell, it will cleave targets such as PARP, caspase 3 zymogen and BID to initiate apoptosis and targets such as tomoisomerase I and nucleolin to prevent viral nucleic acid replication.

The enzyme can also bind to heparan sulfate and serve as extracellular remodeling and inflammation.

Granzyme B has been found to be involved in autoimmune diseases and type I diabetes.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in culture medias (DMEM, MEM and RPMI 1640) in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-hGranzyme B antibody binds to the streptavidin coated AlphaLISA Donor beads, while another anti-hGranzyme B antibody is conjugated to AlphaLISA Acceptor beads. In the presence of hGranzyme B, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm (Figure 2).

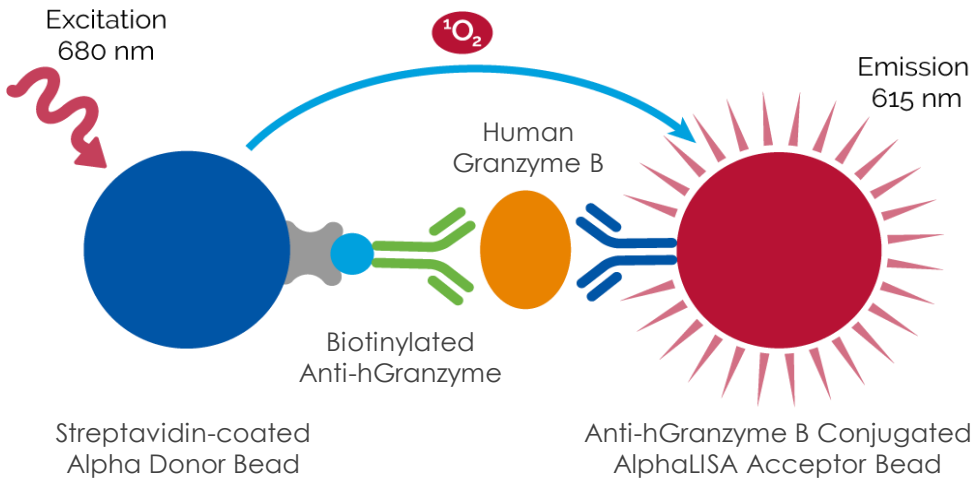


Figure 2. AlphaLISA Human Granzyme B Detection Assay Principle.

Precautions

- The Alpha Donor beads are light sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (Roscolux filters #389 from Rosco are recommended) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-hGranzyme B antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3170HV 100 assay points***	AL3170C 500 assay points***	AL3170F 5000 assay points***
AlphaLISA Anti-hGranzyme B Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Anti-hGranzyme B Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized hGranzyme B Analyte*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20ºC for future experiments. **The aliquoted analyte at -20ºC is stable up to 14 days. Upon unfreezing and usage of reconstituted analyte, discard excess material. Avoid freeze-thaw cycles**. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # ALAL3170S).

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the biotinylated anti-hGranzyme B antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity Inc.	-

Recommendations

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

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

Assay Procedure

- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA Acceptor Beads**	**Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL3170HV	100	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3170C	250	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	10 µL	10 µL	25 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3170F	5 000	50 µL	5 µL	10 µL	10 µL	25 µL	½ Area AlphaPlate-96 (cat # 6002350) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

The following 3-Step manual described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL Milli-Q® grade H_2O.

2) Preparation of hGranzyme B analyte standard dilutions:

   a. Reconstitute lyophilized hGranzyme B (1 µg) in 100 µL Milli-Q® grade H_2O.

b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of hGranzyme B (µL)	Vol. of diluent (µL) *	[hGranzyme B] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted hGranzyme B	90	1.00E-06	1 000 000
B	60 µL of tube A	140	3.00E-07	300 000
C	60 µL of tube B	120	1.00E-07	100 000
D	60 µL of tube C	140	3.00E-08	30 000
E	60 µL of tube D	120	1.00E-08	10 000
F	60 µL of tube E	140	3.00E-09	3 000
G	60 µL of tube F	120	1.00E-09	1 000
H	60 µL of tube G	140	3.00E-10	300
I	60 µL of tube H	120	1.00E-10	100
J	60 µL of tube I	140	3.00E-11	30
K	60 µL of tube J	120	1.00E-11	10
L	60 µL of tube K	140	3.00E-12	3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

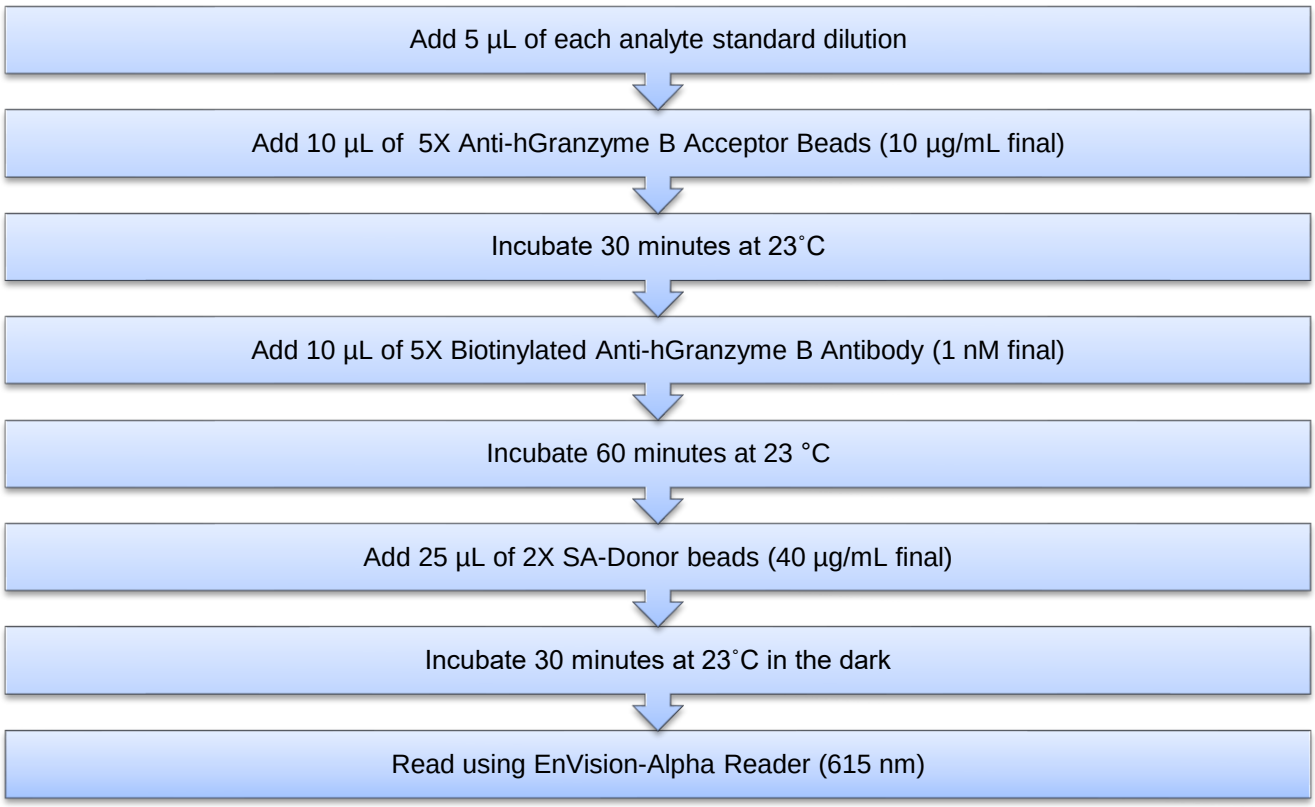
* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-hGranzyme B Antibody Acceptor beads (50 µg/mL):
   a. Prepare just before use.
   b. Add 10 µL of 5 mg/mL AlphaLISA Anti-hGranzyme B Antibody Acceptor to 990 µL of 1X AlphaLISA Immunoassay Buffer.
4) Preparation of 5X Biotinylated Anti-hGranzyme B Antibody (5 nM):
   a. Prepare just before use.
   b. Add 10 µL of 500 nM Biotinylated Anti-hGranzyme B Antibody to 990 µL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 32 µL of 5 mg/mL SA-Donor beads to 1968 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white OptiPlate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined to be a 3-step manual using AlphaLISA Immunoassay Buffer 1X as assay buffer. The analytes (standards) were prepared in Immunoassay Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, MEM, MEM + 10% FBS and 100% FBS. All other components were prepared in AlphaLISA Immuno assay Buffer 1X.

- Assay Sensitivity:

The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL sample using the recommended assay conditions.

LDL (pg/mL)	(Analyte diluent)
145	AlphaLISA Immunoassay buffer 1X
149	DMEM
224	DMEM + 10% FBS
98	RPMI
482	RPMI 1640 + 10% FBS
46	MEM
133	MEM + 10% FBS
77	100% FBS

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in Immunoassay Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, MEM, MEM + 10% FBS and 100% FBS. All other components were prepared in AlphaLISA Immunoassay Buffer 1X. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well plate format.

  - Intra-assay precision:

The intra-assay precision was determined using a total of 6 independent determinations in triplicate. Shown as CV%.

hGranzyme B	AlphaLISA Immunoassay Buffer 1X	DMEM	DMEM + 10% FBS	RPMI 1640	RPMI 1640 + 10% FBS	MEM	MEM + 10% FBS	100% FBS
Intra-CV (%)	12.9	5.0	1.7	10.3	2.1	1.7	10.5	3.8

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 determinations. Shown as CV%.

hGranzyme B	AlphaLISA Immunoassay Buffer 1X	DMEM	DMEM + 10% FBS	RPMI 1640	RPMI 1640 + 10% FBS	MEM	MEM + 10% FBS	100% FBS
Inter-CV (%)	9.5	6.2	8.4	12.1	7.6	4.6	11.3	6.8

- Spike Recovery:

Three known concentrations of analyte were spiked into Immunoassay Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, MEM, MEM + 10% FBS and 100% FBS. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in Immunoassay Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, MEM, MEM + 10% FBS and 100% FBS. All other assay components were diluted in AlphaLISA Immunoassay Buffer 1X.

Spiked hGranzyme B (ng/mL)	% Recovery							
	IAB 1X	DMEM	DMEM + 10% FBS	RPMI	RPMI 1640 + 10% FBS	MEM	MEM + 10% FBS	100% FBS
1 000	107	109	104	90	83	87	122	129
100	96	87	108	112	118	106	116	105
10	103	100	97	107	101	101	106	103

- Specificity:

Cross-reactivity of the hGranzyme B AlphaLISA Detection Kit was tested using the following proteins in AlphaLISA Immunoassay Buffer 1X. The cross reactivities were calculated using the signals of 100 ng/mL hGranzyme B as 100%.

Proteins	Cross Reactivity (%)
Granzyme H	5.7%
Granzyme A	0

- Human Cells Experiments:

Human **peripheral blood mononuclear cells** (**PBMC**) were used for testing Granzyme B secretion. PBMC cells differentiated with phytohemagglutinin (PHA) are known to secrete significant amounts of hGranzyme B.

Cell growth and treatment:

1. A tube of frozen THP-1 cells was unfrozen and added to a T-75 flask with RPMI 1640 + 10% FBS.
2. Cells were grown until confluence (about 4 days).
3. Cells were harvested by centrifugation 5 minutes at 1200g.
4. Cells were washed twice with 20 mL cold sterile PBS.
5. Cells were resuspended in RPMI + 10% FBS and counted by the Trypan blue method.
6. Cells were plated in duplicate in the wells of a 96 well culture-treated plate.
7. Cells were plated at 500 000 cells per mL in 100 uL
8. One series of cells was incubated alone.
9. One series of cells was incubated with 2 000 nM PHA
10. The cells were incubated for 3 days at 37ºC with an atmosphere of 5% CO_2.

Supernatant of each well was tested in triplicate using the assay described in this document, with RPMI 1640 + 10% FBS used as the diluent for the standard curve.

Granzyme B kit	Granzyme B levels in PBMC cell culture supernatant samples (ng/mL)
Un-Treated	0.32 ± 0.2*
PHA-Treated	125 ± 5.4**

* Average of 5 replicates of neat (1x) sample;
** Average of 3 (N=3) dilutions (1x, 5x, and 20x). Each dilution has 5 replicates in the assay.

Results show clear stimulation of secretion of hGranzyme B upon differentiation.

Troubleshooting Guide

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

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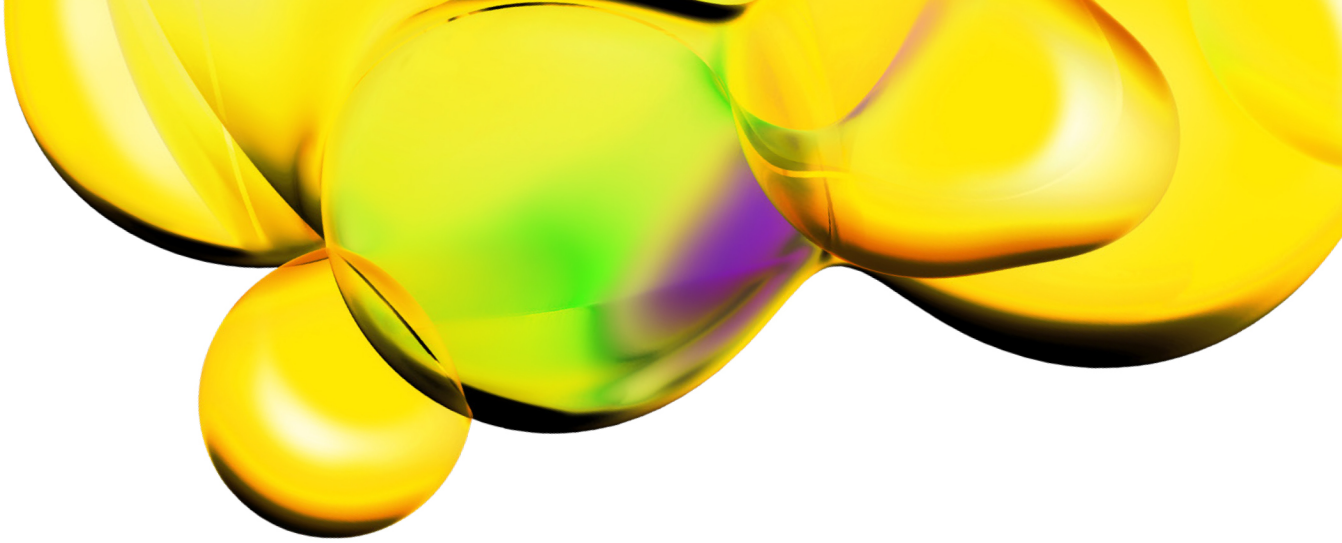

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