MANUAL

AlphaLISA® Placental Growth Factor (Human) Detection Kit

Product number: AL3030 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of placental growth factor (PlGF) in serum, plasma, CSF and cell lysates and cell culture supernatants using a homogeneous AlphaLISA assay (no wash steps). The assay shows no cross reactivity with VEGF (see page 10).

Sensitivity: Lower Detection Limit (LDL): 1.8 pg/mL

Lower Limit of Quantification (LLOQ): 5.9 pg/mL

EC_{50}: 12.6 ng/mL

Dynamic range: 1.8 – 100 000 pg/mL (Figure 1).

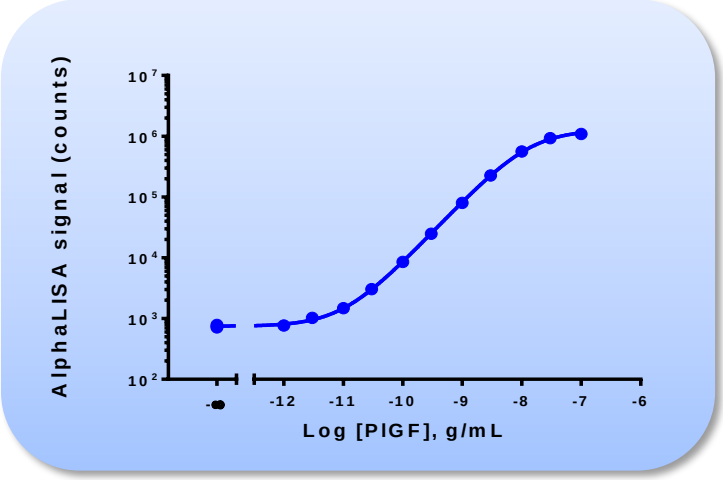


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Placental growth factor (PlGF) is a protein encoded by the PGF gene and is recognized as a member of the vascular endothelial growth factor sub-family. PlGF has been identified as a pro-angiogenic factor where it is primarily secreted in the placenta as a homodimer. Further, PlGF has been found in other tissues of the heart, lungs, and eyes to name a few. Studies using gene knockout models have demonstrated that PlGF plays a major role in the regulation of inflammatory processes associated with neo-angiogensis via PlGF/VEGFR-1. These studies identified relations between aberrant PlGF levels and atherosclerosis, hypertension, bone and collagen repair, and arthritis. This AlphaLISA kit allows for the detection and quantification of PlGF in human serum, plasma, CSF, cell culture media and cell lysates.

Description of the AlphaLISA Assay

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 mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

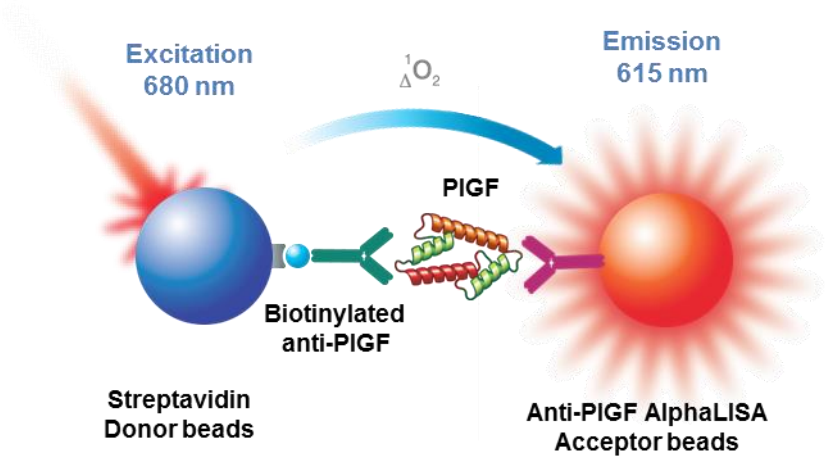


Figure 2. AlphaLISA Assay Principle.

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 (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3030HV (100 assay points***)	AL3030C (500 assay points***)	AL3030F (5000 assay points***)
AlphaLISA Anti-PlGF Acceptor beads stored in PBS, 0.05% Kathon, 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, 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-PlGF 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 PlGF Analyte*	0.3 µg (1 tube, clear cap)	0.3 µg (1 tube, clear cap)	0.3 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute PlGF in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20□C for further experiments. Avoid multiple freeze-thaw cycles. One vial contains an amount of PlGF sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3030S).

** 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 96- or 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 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

- 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 H2O (18 MΩ•cm) 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.
- The AlphaLISA signal is detected with an EnVision Multilabel 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. The standard curve should be performed in the FBS for serum and/or plasma samples.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If a different amount 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 protocol 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**
AL3030HV	100	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3030C	250	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) 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)
AL3030F	5 000	50 µL	5 µL	10 µL	10 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) 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)

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

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 10 mL of 10X AlphaLISA Immunoassay Buffer to 90 mL H_2O.

2) Preparation of PlGF analyte standard dilutions:

   a. Reconstitute lyophilized PlGF (0.3 µg) in 100 µL H_2O.
   b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of PlGF (µL)	Vol. of diluent (µL) *	[PlGF] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted PlGF	90	3.0E-07	300 000
B	60 µL of tube A	120	1.0E-07	100 000
C	60 µL of tube B	140	3.0E-08	30 000
D	60 µL of tube C	120	1.0E-08	10 000
E	60 µL of tube D	140	3.0E-09	3 000
F	60 µL of tube E	120	1.0E-09	1 000
G	60 µL of tube F	140	3.0E-10	300
H	60 µL of tube G	120	1.0E-10	100
I	60 µL of tube H	140	3.0E-11	30
J	60 µL of tube I	120	1.0E-11	10
K	60 µL of tube J	140	3.0E-12	3
L	60 µL of tube K	120	1.0E-12	1
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.

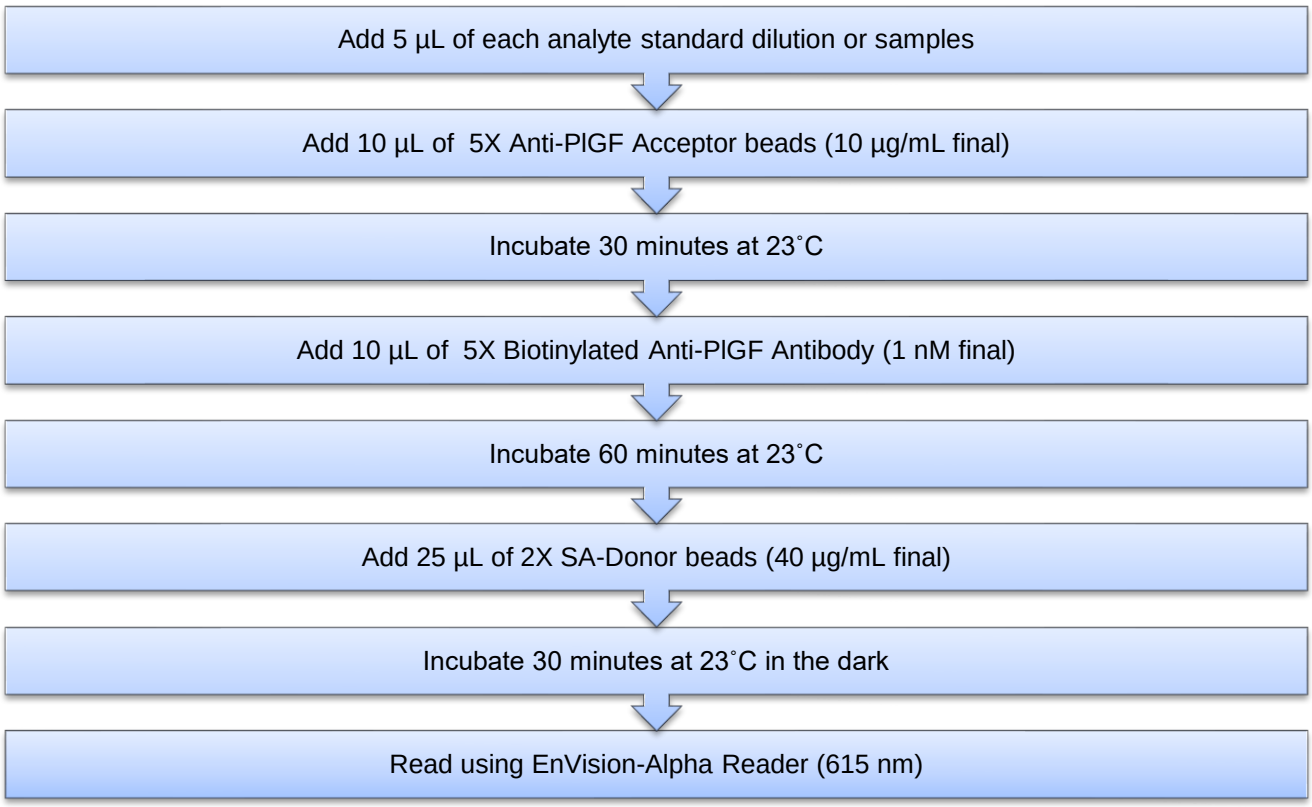
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-PlGF Antibody Acceptor beads (50 µg/mL):
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-PlGF Antibody Acceptor to 4950 µL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Biotinylated Anti-PlGF Antibody (5 nM):
   a. Prepare just before use.
   b. Add 50 µL of 500 nM Biotinylated Anti-PlGF Antibody to 4950 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 200 µL of 5 mg/mL SA-Donor beads to 12 300 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 using the 3 step protocol using AlphaLISA Immunoassay Buffer (IAB) and cell culture medium containing 10% FBS. In all cases assay components other than analyte were always prepared in IAB.

- 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 using the recommended assay conditions.

LDL (pg/mL)	Buffer/Serum/Medium	# of experiments
1.8	IAB	6
1.2	DMEM	6
1.6	RPMI	6
1.1	FBS	2

* The standard was prepared in these diluents. Note that LDL can be decreased (i.e. sensitivity increased) by preparing standards in different matrixes.

- 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 IAB, DMEM, or RPMI. 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 format using IAB.

- Intra-assay precision:

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

PIGF	IAB	DMEM	RPMI
CV (%)	4	5	6

- Inter-assay precision:

The inter-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

PIGF	IAB	DMEM	RPMI
CV (%)	8	9	11

- Spike Recovery:

Three known concentrations of analyte were spiked in IAB, or in cell culture media. All samples, including non-spiked buffer or media were measured in the assay. The average recovery from three independent measurements is reported. Note that the standard curves were prepared in IAB, DMEM, and RPMI.

Spiked PlGF (ng/mL)	% Recovery		
	IAB	DMEM	RPMI
10	106	105	110
3	100	102	102
1	98	98	102

- Specificity:

Cross-reactivity of the PlGF kit was determined by testing human, mouse and bovine IL18. Cross reactivity was quantified at the Ec50 of the PlGF standard curve.

Protein	% Cross-reactivity
VEGF	0
PlGF2	55

Human Serum, Plasma, and CSF Experiments

Dilution linearity was tested to determine the required dilution factor for accurate quantification in serum, plasma, and CSF samples. Commercially available normal human samples were spiked with a known concentration of PlGF and diluted in 2-fold increments with IAB.

Dilution Factor	Serum (% recovery)	Plasma (% recovery)	CSF (% recovery)
1	12	12	48
2	29	27	88
4	42	50	92
8	54	64	82
16	59	78	78
32	59	80	73
64	59	79	75
128	57	77	73

Dilution linearity was tests were repeated and diluted in ½ Log increments with fetal bovine serum (FBS) instead of IAB to determine the required dilution factor for accurate quantification in serum, plasma, and CSF samples. Commercially available normal human samples was spiked with a known concentration of PlGF then diluted in FBS. Sufficient recovery was observed for all sample types tested in the range of 3- to10-fold dilutions. Note: all bead and antibody dilutions were prepared in IAB.

Dilution Factor	Serum (% recovery)	Plasma (% recovery)	CSF (% recovery)
1	66	46	-
3	93	61	121
10	104	85	102
30	106	96	104
100	108	108	103

Cell Lysate Experiments

Commercially available control and PlGF overexpressed HEK293 cell lysates were tested. The lysates were prepared using RIPA buffer. AlphaLISA lysis buffer (ALB) can also be used for cell lysates. Cell lysates were diluted in 2-fold increments in AlphaLISA Lysis buffer (IAB can also be used as the diluent). Note: all beads and antibodies were diluted in IAB.

Dilution Factor	Total Protein (µg/mL)	PlGF detected in Control Lysate (pg/mL)	PlGF detected in overexpression Lysate (pg/mL)
1	100	60.8	14679
2	50	37.2	8233
4	25	18.2	4760
8	12.5	9.6	2548
16	6.3	5.5	1350
32	3.1	2.6	701
64	1.6	1.6	349
128	0.8	0.7	173

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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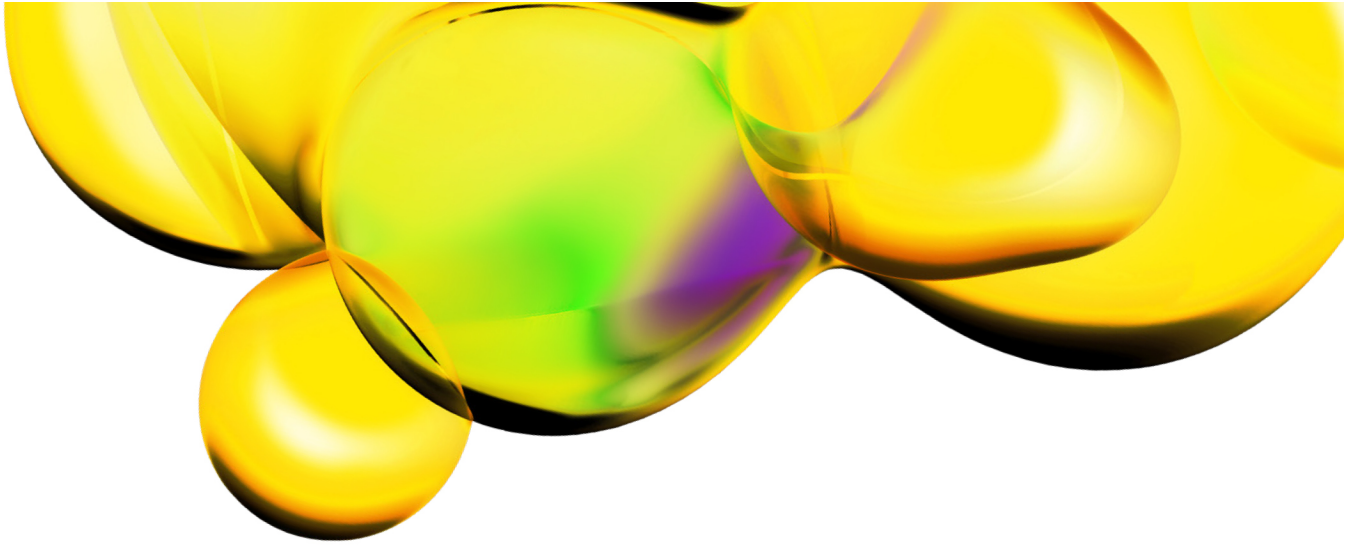

www.revvity.com

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

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