MANUAL

AlphaLISA® NGAL (Human) Detection Kit

Product number: AL364 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This AlphaLISA kit is designed for the quantitative determination of Neutrophil Gelatinase Associated Lipocalin (NGAL) also known as lipocalin 2 in cell culture media and human serum using a homogeneous AlphaLISA assay (no wash steps). The assay shows no cross-reactivity with mouse NGAL, rat NGAL, bovine NGAL and chicken NGAL

Sensitivity: Lower Detection Limit (LDL): 64.5 pg/mL

Lower Limit of Quantification (LLOQ): 310 pg/mL

EC_{50}: 66.2 ng/ml

Dynamic range: Kit designed to detect [NGAL] between: 100 – 300,000 pg/mL (Figure 1).

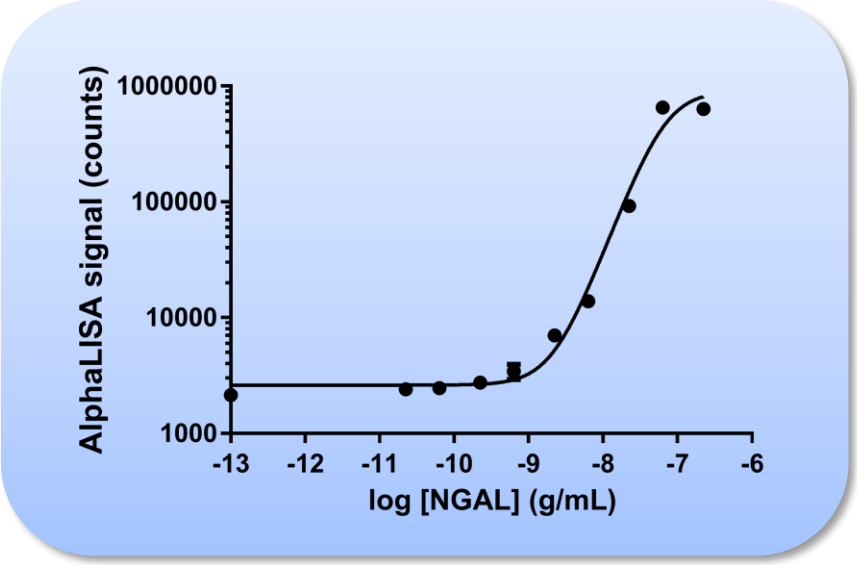


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. The human NGAL analyte is stable for at least 12 months at 4°C. Store reconstituted analyte at 4°C short term or aliquot and store at -20°C for long term storage. Limit the number of freeze-thaw cycles.

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

Analyte of Interest

NGAL/lipocalin-2 is a 25 kDa protein that is generated by inflammatory cells when encountering bacteria. Binding of bacteria to toll-like receptors generate the secretion of NGAL, which binds to bacterial iron-binding proteins and deactivates them. It is also secreted upon stimulation of immune cells by inflammatory cytokines such as TNFα. NGAL shows elevated levels in many pathologies related to inflammation such as inflammation, pneumonia, kidney failure, obesity, cancer, meningitis, bladder infection, Alzeimer's disease and others. As such, it is a valuable marker in all sort of pathologies in medias such as serum, plasma, urine and even saliva. It is also secreted in large amounts by some cancer cells.

Description of the AlphaLISA Assay

AlphaLISA technology allows for 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 transfers in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2). Combining this assay with an AlphaPlex 545 or AlphaPlex 645 based kits will allow for the quantification of 2 (or more) analytes in the same well.

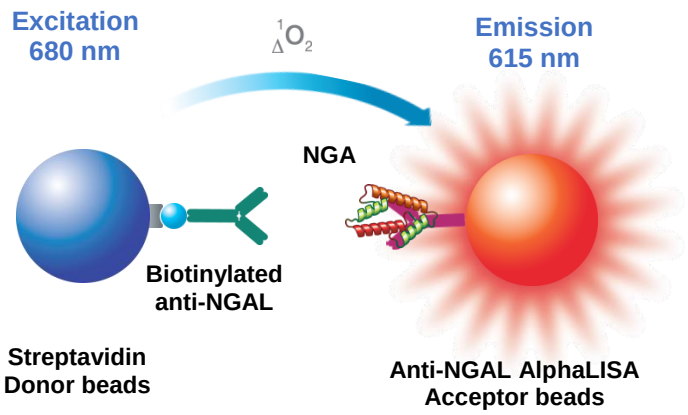


Figure 2. AlphaLISA assay principle.

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 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	AL364HV (100 assay points***)	AL364C (500 assay points***)	AL364F (5000 assay points***)
AlphaLISA Anti-NGAL 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-NGAL 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 Human NGAL *	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 small bottle	100 mL, 1 large bottle

* Please note that one NGAL analyte vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL364S).

** 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 H_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer.
- 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.
- AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).
- 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 AlphaLISA Immunoassay buffer.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations) and 452 samples. The manuals also include testing samples in 384 well plates. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. *These calculations do not include excess reagents 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 beads**	**Biotin Antibody**	**SA-Donor beads**	**Plate recommendation**
AL364HV	100	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL364C	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 µLs	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)
AL364F	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)

Manual for NGAL AlphaLISA Assay

3 Step Manual – Dilution of standards in 1X AlphaLISA Immunoassay Buffer. The manual described below is for one standard curve (48 wells) and 452 sample wells. *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

Steps for Preparing Reagents

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

2) Preparation of NGAL analyte standard dilutions:

   a. Reconstitute 1 ug lyophilized NGAL with 100 µl of water and vortexing briefly.
   b. Store unused analyte at 4°C or aliquot and place at -20°C for long term storage.
   c. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of NGAL (µL)	Vol. of diluent (µL) *	[NGAL] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted NGAL	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.

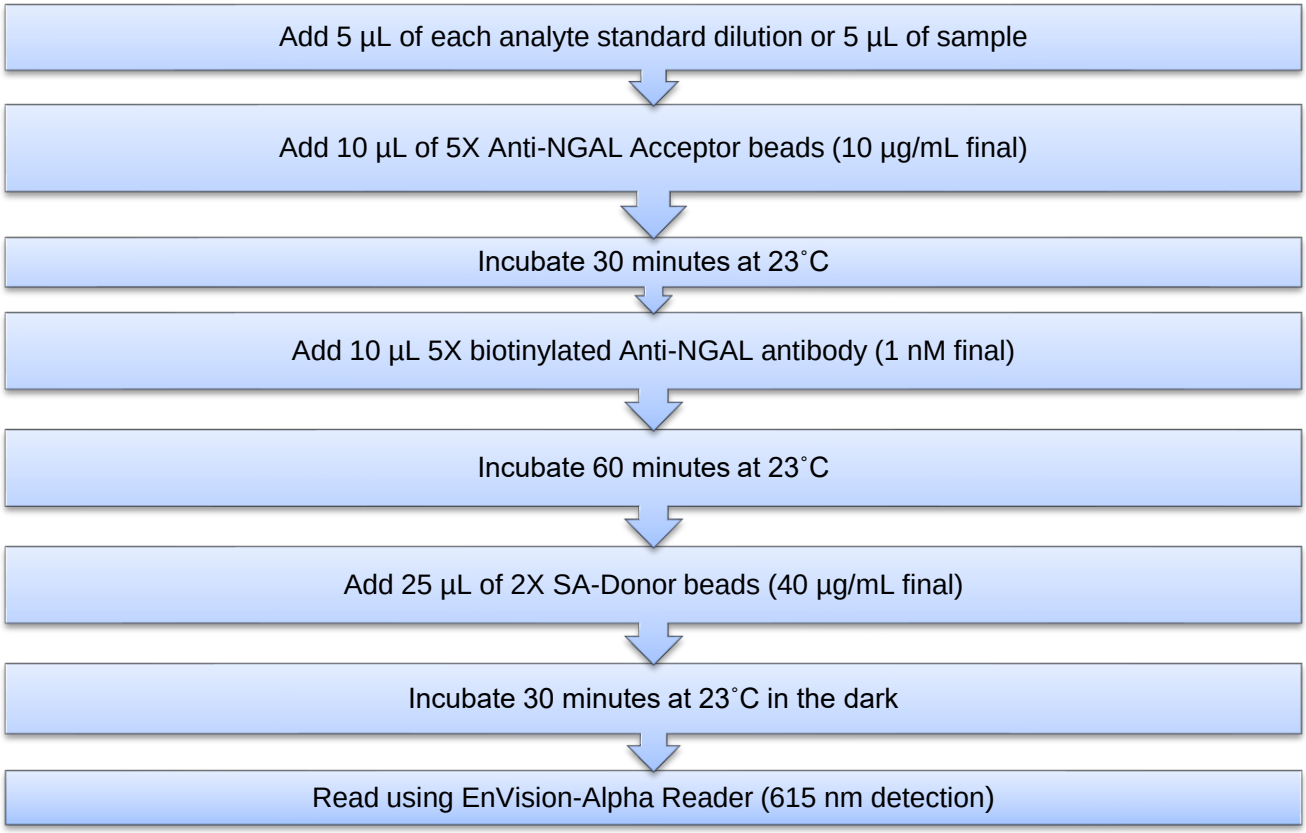
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

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

4) Preparation of 5X anti-NGAL biotinylated antibody (5 nM)
   a. Prepare just before use
   b. Add 50 µL of 500 nM biotinylated Anti-NGAL 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 12300 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white Optiplate (384 wells):



Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

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 manual.

Assay Sensitivity:

The LDL and LLOQ were 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/Cell culture media	# of experiments
64.5	AlphaLISA Immunoassay Buffer	8
106	DMEM	2
251	RPMI	2
482	DMEM+ 10% FBS	2
627	RPMI+ 10% FBS	2

* Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

** Only the analytes were prepared in Cell Culture or serum. All of other components were prepared in Immunoassay Buffer.

Note: FBS intereferes with this assay. Assays should be performed in cell culture media deprived of serum, or with cells washed with an appropriate buffer such as PBS.

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 AlphaLISA Immunoassay Buffer (IAB), DMEM medium, or RPMI medium supplemented with 10% FBS. Each assay consisted of one standard curve comprising 12 data points in triplicate and 12 background wells containing no analyte. The assays were performed in a 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra-experimental CV%.

NGAL	IAB	DMEM	RPMI
CV%	3.7	2.9	6.7

- Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 measurements in triplicate. CV% was calculated by comparing the same measurement in each experiment. The CV% for all 16 measurements were then averaged. Shown is the inter-experimental CV%.

NGAL	IAB	DMEM	RPMI
CV%	12.4	4.3	5.6

- Spike Recovery:

Three known concentrations of NGAL were spiked into AlphaLISA Immunoassay Buffer (IAB), DMEM medium or RPMI medium supplemented with 10% FBS. All samples, including non-spiked Immunoassay Buffers were measured in the assay. The average recovery was reported and compared to a standard prepared in the respective diluent

Spiked NGAL (ng/mL)	% Recovery		
	IAB	DMEM	RPMI
100	111	118	112
10	81	83	82
1	129	125	102

Human Serum Experiments

Pooled normal Human Serum (HS) was utilized and AlphaLISA Immunoassay Buffer (IAB) was used as the diluent**.** NGAL was detected in the normal Human Serum at a concentration of 54 ng/mL. NGAL is expected to be present at detectable levels in HS from normal healthy subjects.

Specificity

Cross-reactivity to other similar proteins was assessed for the AlphaLISA NGAL Kit by performing full 12 point curves in triplicate using animal sera.

Protein	% Cross Reactivity
Rat NGAL	0
Mouse NGAL	0
Bovine NGAL	0
Chicken NGAL	0

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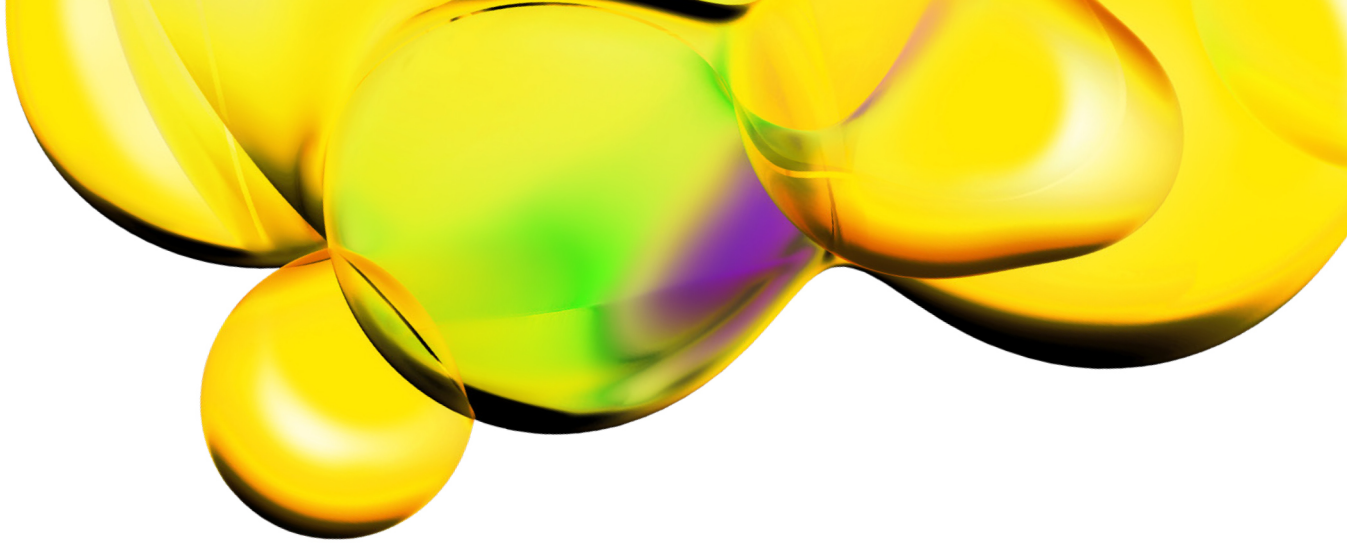

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www.revvity.com

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

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