# AlphaLISA® TNFα (Cyno) Detection Kit

**Product number:**  AL562HV/C/F

Research Use Only. Not for use in diagnostic procedures.

## Product Information

**Application:**  This kit is designed for the quantitative determination of TNFα in cynomolgus and rhesus monkey serum and cell culture supernatants using a homogeneous AlphaLISA assay (no wash steps).

**Sensitivity:**  Lower Detection Limit (LDL): 5.1 pg/mL

Lower Limit of Quantification (LLOQ): 16.7 pg/mL

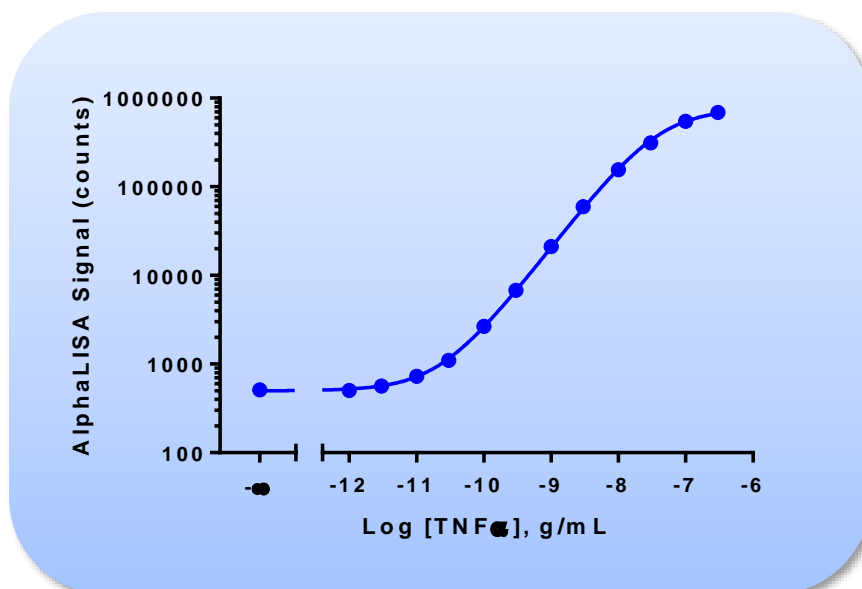$EC_{50}$: 28.7 ng/mL

**Dynamic range:**  5.1 – 300 000 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 2103 with Alpha option.

**Storage:**  Store kit in the dark at +4˚C. For reconstituted analyte aliquot and store at -20 °C. Avoid 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

Tumor necrosis factor alpha (TNFα) is a multifunctional proinflammatory cytokine produced primarily by activated macrophages and T cells. TNFα is primarily produced as 233 Amino-Acid long TNF type II transmembrane proteins. The soluble form of TNFα can be generated from its transmembrane form via proteolytic cleavage by ADAM17. Released TNFα self-associates to form a soluble complex that exerts its function by binding the TNFR1 and TNFR2 receptors. TNFα has been observed to play a role in inflammation, apoptotic cell death, and as an important regulator of tumorigenesis and infection. TNFα dysregulation has been observed in several major diseases such as Inflammatory Bowel Disease, Depression, Cancer, and Neurological Diseases. This kit has been designed for the detection of TNFα in cynomolgus and rhesus monkey serum and cell culture supernatants.

## 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).
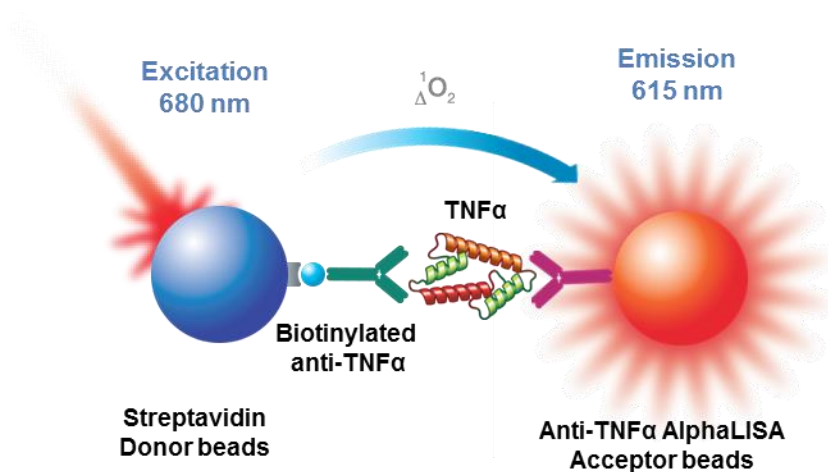


Figure 2. AlphaLISA Assay Principle.

## 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.
- 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 | AL562HV (100 assay points***) | AL562C (500 assay points***) | AL562F (5000 assay points***) |
|---|---|---|---|
| AlphaLISA Anti-TNFα Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2 | 20 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap) | 50 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap) | 500 µL @ 5 mg/mL (1 brown tube, <u>white</u> 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, <u>black</u> cap) | 200 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap) | 2 x 1 mL @ 5 mg/mL (2 brown tubes, <u>black</u> caps) |
| Biotinylated Anti- TNFα Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN$_3$, pH 7.4 | 20 µL @ 500 nM (1 tube, <u>black</u> cap) | 50 µL @ 500 nM (1 tube, <u>black</u> cap) | 500 µL @ 500 nM (1 tube, <u>black</u> cap) |
| Lyophilized Cyno TNFα* | 0.3 µg (1 tube, <u>clear</u> cap) | 0.3 µg (1 tube, <u>clear</u> cap) | 0.3 µg (1 tube, <u>clear</u> 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$_2$O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for future experiments. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL562S).

\*\*	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 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.


## 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). The manuals 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 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.

| Format | # of data points | Volume | | | | | Plate recommendation |
|---|---|---|---|---|---|---|---|
| | | Final | Sample | AlphaLISA Acceptor beads | Biotinylated Antibody | SA-Donor beads | |
| AL562HV | 100 | 100 µL | 10 µL | 20 µL | 20 µL | 50 µL | White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560) |
| AL562C | 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) |
| AL562F | 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 Manual 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:
   a. Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL $H_2O$.

2) Preparation of cyno TNFα analyte standard dilutions:
   a. Reconstitute lyophilized cyno TNFα (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):
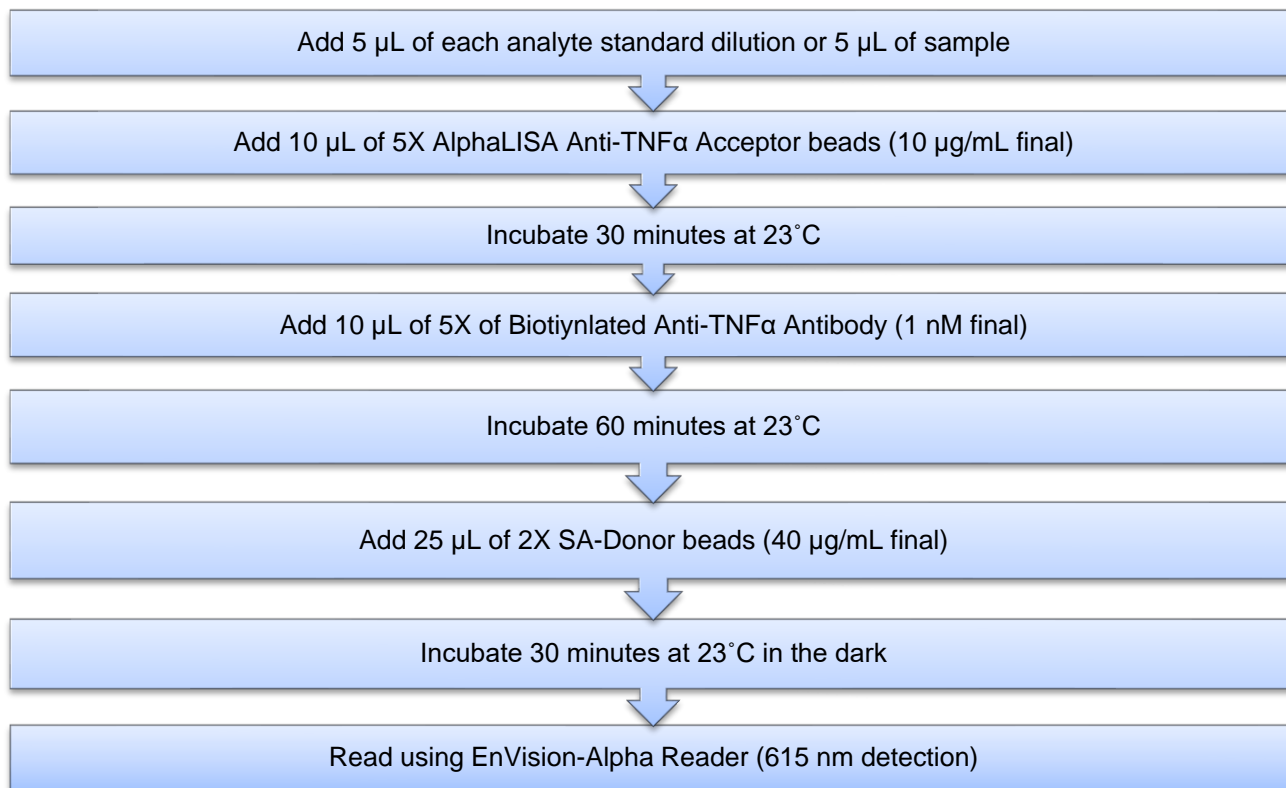
| Tube | Vol. of TNFα (µL) | Vol. of diluent (µL) * | [TNFα] in standard curve | |
|---|---|---|---|---|
| | | | (g/mL in 5 µL) | (pg/mL in 5 µL) |
| A | 10 µL of reconstituted TNFα | 90 | 3.00E-07 | 300 000 |
| B | 60 µL of tube A | 120 | 1.00E-07 | 100 000 |
| C | 60 µL of tube B | 140 | 3.00E-08 | 30 000 |
| D | 60 µL of tube C | 120 | 1.00E-08 | 10 000 |
| E | 60 µL of tube D | 140 | 3.00E-09 | 3 000 |
| F | 60 µL of tube E | 120 | 1.00E-09 | 1 000 |
| G | 60 µL of tube F | 140 | 3.00E-10 | 300 |
| H | 60 µL of tube G | 120 | 1.00E-10 | 100 |
| I | 60 µL of tube H | 140 | 3.00E-11 | 30 |
| J | 60 µL of tube I | 120 | 1.00E-11 | 10 |
| K | 60 µL of tube J | 140 | 3.00E-12 | 3 |
| L | 60 µL of tube K | 120 | 1.00E-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 Anti-TNFα AlphaLISA Acceptor beads (50 µg/mL)
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-TNFα Acceptor Beads to 4950 µl of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Anti-TNFα Biotinylated antibody (5 nM)
   a. Prepare just before use.
   b. Add 50 µL of 500nM Anti-TNFα biotinylated 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):

Add 5 µL of each analyte standard dilution or 5 µL of sample

↓

Add 10 µL of 5X AlphaLISA Anti-TNFα Acceptor beads (10 µg/mL final)

↓

Incubate 30 minutes at 23˚C

↓

Add 10 µL of 5X of Biotiynlated Anti-TNFα Antibody (1 nM final)

↓

Incubate 60 minutes at 23˚C

↓

Add 25 µL of 2X SA-Donor beads (40 µg/mL final)

↓

Incubate 30 minutes at 23˚C in the dark

↓

Read using EnVision-Alpha Reader (615 nm detection)

## 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 using AlphaLISA Immunoassay Buffer (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 * | # of experiments |
|:-----------:|:--------:|:----------------:|
| 5.1 | IAB | 6 |
| 8.7 | DMEM + 10% FBS | 6 |
| 18.5 | RPMI + 10% FBS | 6 |

\* The standard was prepared in these diluents and all other components were diluted in IAB. Note that LDL 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).

- 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. Cell culture media was supplemented with 10% FBS. All other components were prepared in IAB. 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.

- Intra-assay precision:

The intra-assay precision was determined by averaging 6 experiments each with 12 independent determinations in triplicate. Shown as CV%.

| TNFα | IAB | DMEM | RPMI |
|:----:|:---:|:----:|:----:|
| CV (%) | 5 | 5 | 8 |

- Inter-assay precision:

The inter-assay precision was determined comparing 6 experiments each with 12 independent determinations in triplicate. Shown as CV%.

| TNFα | IAB | DMEM | RPMI |
|:----:|:---:|:----:|:----:|
| CV (%) | 9 | 10 | 10 |

- Spike Recovery:

Known concentrations of analyte were spiked into IAB, DMEM and RPMI. Cell culture media was supplemented with 10% FBS. All samples, including non-spiked buffer were measured in the assay. Note that the standard curves were prepared in either IAB, or DMEM or RPMI. All other components were diluted in IAB.

| Spiked Cyno TNFα (pg/mL) | % Recovery | | |
|---|---|---|---|
| | IAB | DMEM | RPMI |
| 300 | 101 | 100 | 99 |
| 100 | 99 | 100 | 94 |
| 30 | 97 | 104 | 91 |

## Serum Experiments

To validate the assay kit, commercially available normal cyno serum was used. The standard curve is prepared in the AlphaLISA Immunoassay Buffer. The serum was diluted with or without a known concentration (300 ng/mL) Cyno TNFα.

In normal serum, 100 to 120 pg/mL Cyno TNFα was detected when serum were diluted up to 33 folds.

| Dilution Factor | Serum cyno TNFα, pg/mL |
|---|---|
| 1 | 108 |
| 3.3 | 117 |
| 10 | 103 |
| 33 | 122 |

In serum with a known spiked-concentration (300 ng/mL) of Cyno TNFα were diluted, 70 to 100 % of spiked Cyno TNFα was recovered with dilutions greater than 10-fold.

| Dilution Factor | % recovery |
|---|---|
| 1.0 | 13 |
| 3.3 | 45 |
| 10 | 68 |
| 33 | 89 |
| 100 | 89 |
| 333 | 100 |
| 1000 | 88 |
| 3332 | 92 |
| 9996 | 86 |
| 33317 | 81 |

## 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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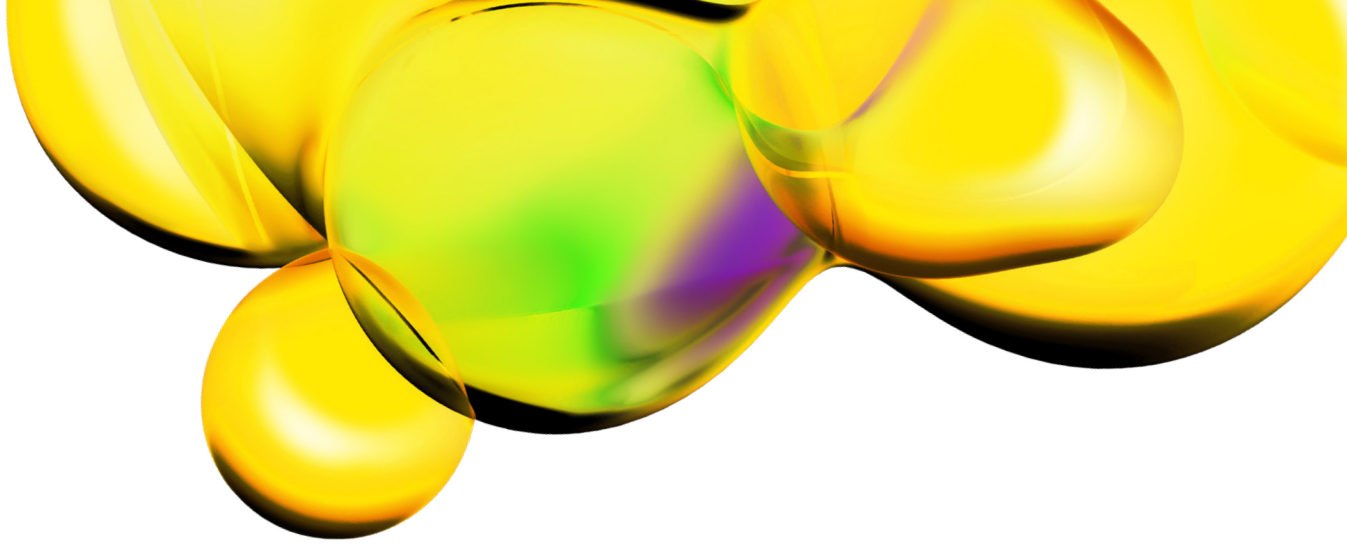
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