MANUAL

AlphaLISA® Interleukin 6 (Human) AlphaLISA Biotin-Free Detection Kit

Product number: AL3025

Caution: For Laboratory Use. A research product for research purposes only.

Product Information

Application: This kit is designed for the quantitative determination of human Interleukin 6 (IL6) in cell culture supernatants using a homogeneous AlphaLISA assay (no wash steps). The kit utilizes a Digoxigenin (DIG) / Anti-DIG interaction as opposed to the traditional Streptavidin/Biotin interaction. This enables optimal performance when working with biotin-rich media (e.g. RPMI) or samples containing endogenous biotin (e.g. milk, brain extracts).

Sensitivity: Lower Detection Limit (LDL): 12 pg/mL

EC_{50}: 17.7 ng/mL

Dynamic range: 12 – 100 000 pg/mL (Figure 1).

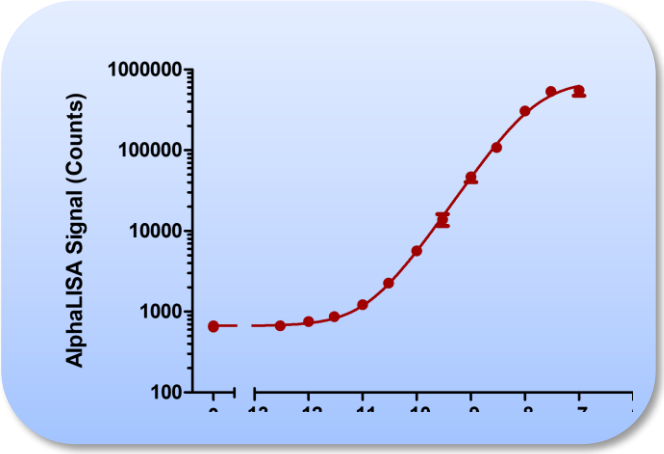


Figure1. Typical sensitivity curves in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2103.

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

Stability: This product is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the human IL6 analyte is stable for at least 60 days at -20°C.

Analyte of Interest

Interleukin 6 (IL6) is a ~22 kDa pleiotropic cytokine that acts not only on the immune system, but also affects many physiological events in various organs. IL6 exerts pro- or anti-inflammatory effects, depending on the target cell analyzed and the in vivo environmental circumstances. IL6 is a differentiation and proliferation factor for B and T cells, and acts as a migration factor on monocytic cells. It is the major activator of acute-phase protein expression in the liver, a hematopoietic factor, and acts as a survival factor on neuronal cells. IL6 signals through binding to the gp130/ IL-6R receptor complex, leading to the activation of JAK/STAT, MAPK and PI3K cascades.

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 this AlphaLISA assay, a DIG-labeled Anti-Analyte Antibody binds to the anti-DIG 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).

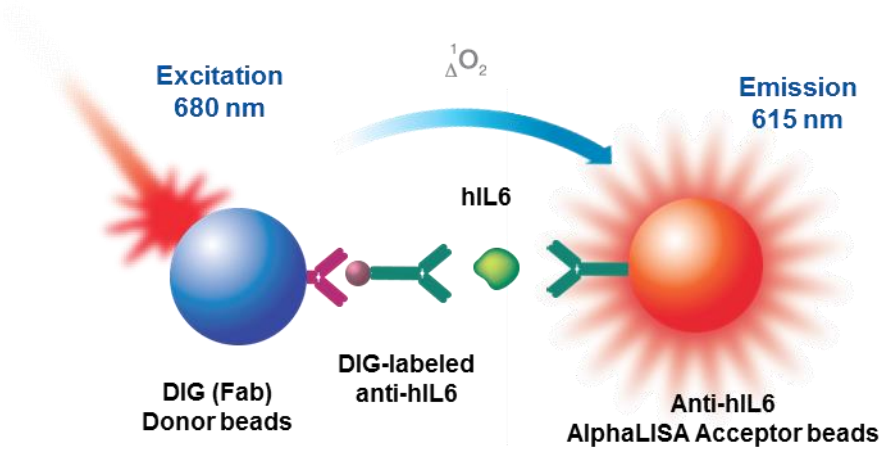


Figure 2. AlphaLISA Assay principle.

Precautions

- Anti-Digoxigenin Fab Fragment 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 Mouse source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The DIG labeled anti-analyte antibody is toxic. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3025HV (100 assay points***)	AL3025C (500 assay points***)	AL3025F (5000 assay points***)
AlphaLISA Anti-hIL6 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)
Anti-Digoxigenin Fab Fragment 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)
DIG labeled Anti-hIL6 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)
AlphaLISA human IL6 (0.1 µg), lyophilized Analyte*	0.1 µg 1 tube, clear cap	0.1 µg 1 tube, clear cap	0.1 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 human IL6 in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes, if possible or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted human IL6 is stable for at least 60 days at -20°C. One vial contains an amount of human IL6 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL223S).

** 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.

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 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 Immunoassay buffer 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 beads / Anti-DIG Antibody MIX**	**Donor beads**	**Plate recommendation**
AL3025HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3025C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	20 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	8 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	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3025F	5 000	50 µL	5 µL	20 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	8 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	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

2-Step Protocol - The 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 human IL6 analyte standard dilutions:

a) Reconstitute lyophilized human IL6 (0.1 µg) in 100 µL of H_2O.
b) Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of human IL6 (µL)	Vol. of diluent (µL) *	[human IL6] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human IL6	90	1.00E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-09	3 000
E	60 µL of tube D	120	1.00E-09	1 000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	60 µL of tube K	140	3.00E-13	0.3
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).

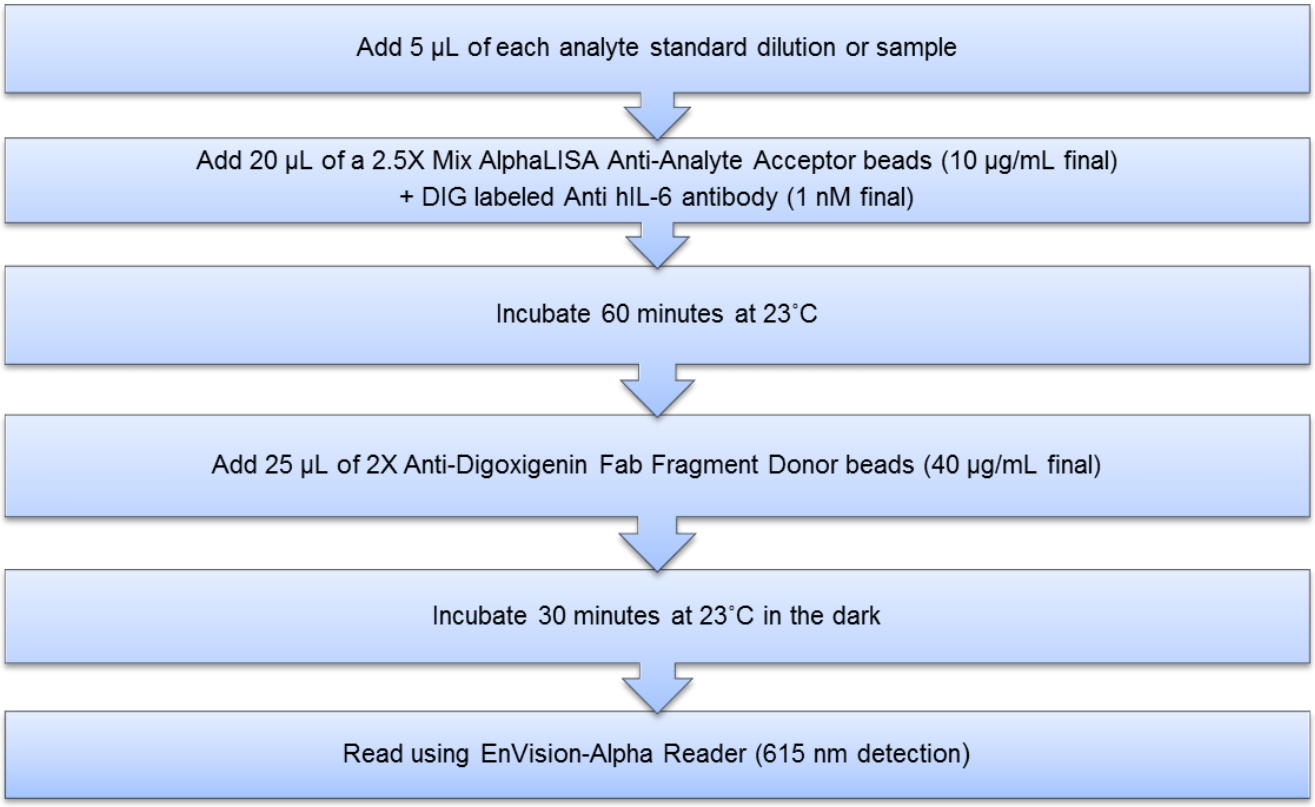
3) Preparation of 2.5X AlphaLISA Anti-hIL6 Acceptor beads (25 µg/mL) + DIG labeled Anti-hIL6 Antibody (2.5 nM) MIX:

a. Add 50 µL of 5 mg/mL AlphaLISA Anti-hIL6 Acceptor beads and 50 µL of 500 nM Anti-hIL6 Antibody to 9900 µL of 1X AlphaLISA Immunoassay Buffer.
b. Prepare just before use.

4) Preparation of 2X Anti-Digoxigenin Fab Fragment Donor beads (80 µg/mL):

a. Keep the beads under subdued laboratory lighting.
b. Add 200 µL of 5 mg/mL Anti-Digoxigenin Fab Fragment Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.
c. Prepare just before use.

5) 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) + 3x 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.

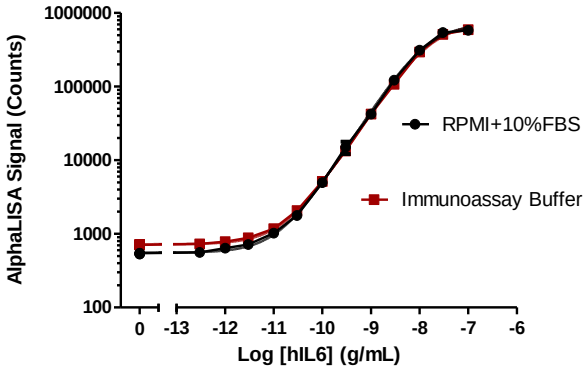
Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 2-step protocol.

- Assay Sensitivity:

The LDL was calculated as described in the data analysis section. The values correspond to the lowest concentration of analyte that can be detected using the recommended assay conditions.

LDL (pg/mL)	Buffer/Media	# of experiments
0.7	Immunoassay Buffer	6
0.9	RPMI with 10% FBS	6



* 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 AlphaLISA Immunoassay Buffer, 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 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%.

hIL6	Immunoassay Buffer	RPMI
CV%	4%	6%

- 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 was then averaged. Shown is the inter-experimental CV%.

hIL6	Immunoassay Buffer	RPMI
CV%	8%	9%

- Spike Recovery:

Four known concentrations of analyte were spiked in AlphaLISA Immunoassay Buffer and cell culture media supplemented with 10% FBS. The spiked samples were referenced to the analyte curve produced in AlphaLISA Immunoassay Buffer and cell culture media.

Spiked hIL6 (ng/mL)	% Recovery	
	Immunoassay Buffer	RPMI
1	97	87
0.3	102	82
0.1	99	94
0.03	94	80

Calibration: Human IL6 (NIBSC/WHO First International Standard (code 89/548)) was tested using this kit: 1 unit of Standard corresponds to 22.0 pg of AlphaLISA IL6.

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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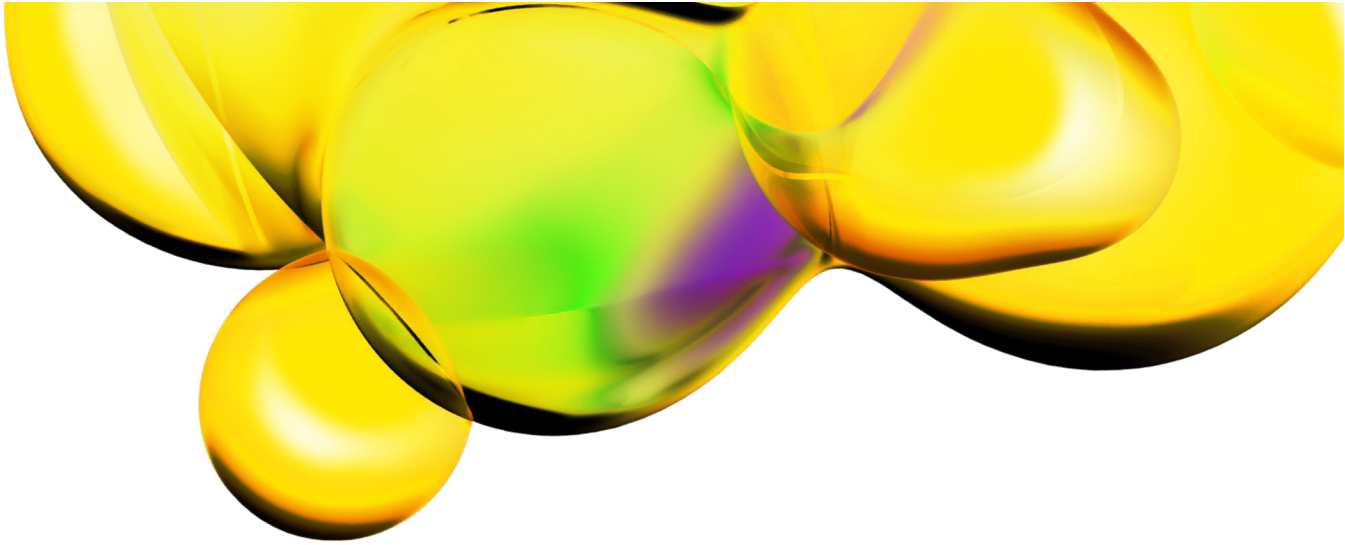

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