MANUAL

AlphaLISA® High Performance (HP) Biotin-Free Human Interleukin 17A (IL17A) Detection Kit

Product number: AL3162HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of Human Interleukin 17A using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 5 components: AlphaLISA Acceptor beads coated with Anti-Human Interleukin 17A Antibody, Anti-digoxigenin Fab fragment Donor beads, Digoxignenin coupled Anti-Human Interleukin 17A, Lyophilized Human Interleukin 17A and 10X AlphaLISA Immunoassay Buffer.

Sensitivity: Lower Detection Limit (LDL): 4.58 pg/mL
Lower Limit of Quantification (LLOQ): 19.42 pg/mL
EC_{50}: 25.51 ng/mL

Dynamic Range: 4.58 – 300 000 pg/mL

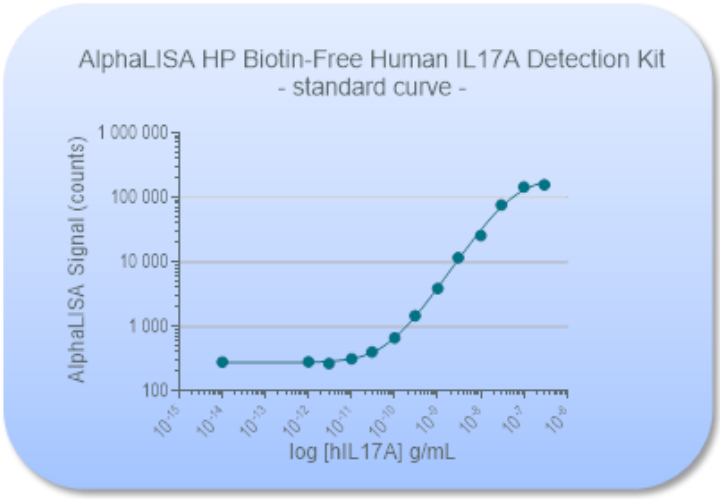


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a light gray AlphaPlate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

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

Stability: This kit is stable for at least 24 months from the date of manufacture when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Human Interleukin 17 (IL17 or IL17A) is a homodimer formed of two ~15 kDa subunits produced by a subset of T helper cells named Th17. It is a proinflammatory cytokine that enhances T cell priming and stimulates macrophages, fibroblasts, endothelial and epithelial cells to produce multiple mediators of inflammation like IL1, IL6, TNF-α, NOS-2, metalloproteases, and chemokines. IL17 has been implicated in the proinflammatory patterns associated with joint inflammation and rheumatoid arthritis (RA) in mouse and human models. It is also critical for neutrophil activation and migration, and induces IL8, a key chemokine for neutrophils. IL17 signals through IL-17R, which in mice has at least two members, IL-17RA, and IL-17RC. Recent studies suggest that the IL17 pathway may be a novel therapeutic target for the treatment of chronic inflammatory diseases like asthma and RA.

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 digoxigenin coupled anti-human interleukin 17A antibody binds to the Anti-digoxigenin Fab fragment coated AlphaLISA Donor beads, while the anti-human interleukin 17A antibody is conjugated to AlphaLISA Acceptor beads. In the presence of human interleukin 17A, 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).

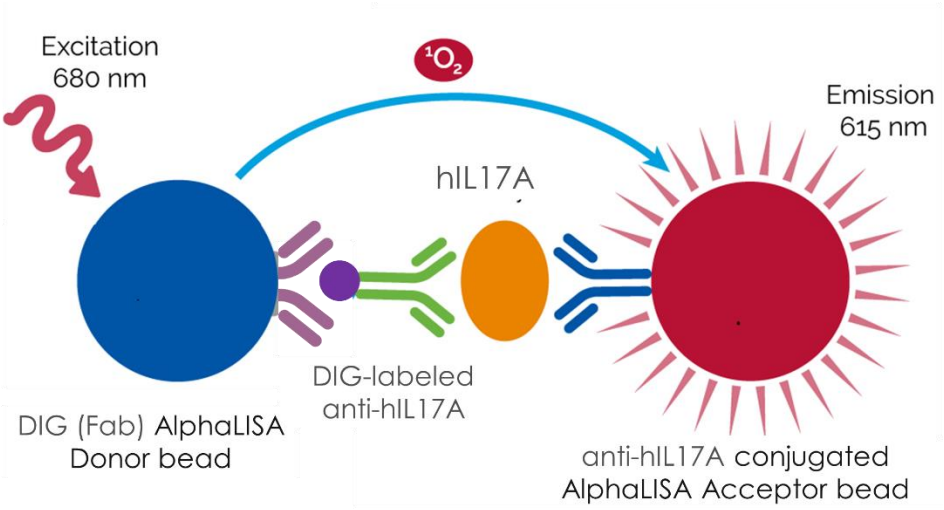


Figure 2. AlphaLISA HP Biotin-Free Human Interleukin 17A Detection Assay Principle.

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 (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 DIG labeled anti-Human Interleukin 17A antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3162HV (100 assay points***)	AL3162C (500 assay points***)	AL3162F (5000 assay points***)
AlphaLISA Anti-hIL17A Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, pH 7.2	25 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 CG/ICP, pH 7.4	100 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 cap)
Digoxigenin Anti-hIL17A Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	25 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA hIL17A Lyophilized 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 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 -20 C for future experiments. The aliquoted analyte at -20 C is stable up to 28 days. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3162S).

** 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 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 DIG labeled anti-human Interleukin 17A 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 (2000*g*, 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. The recommended fitting model is a 4-Parameter Logistic regression with a $1/Y^2$ weighting.

Assay Procedure

- 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 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**	**MIX AlphaLISA AccBeads + DIG Ab**	**SA-Donor beads**	**Plate recommendation**
AL3162HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3162C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	20 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	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)
AL3162F	5 000	50 µL	5 µL	20 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	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)

The 2-Step protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q® grade H_2O.

2) Preparation of human Interleukin 17A analyte standard dilutions:

a. Reconstitute lyophilized human Interleukin 17A (0.3µg) in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 ºC for future assays (see page 3 for more details).
b. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix diluent as the samples (e.g. cell culture media for cell supernatant samples, FBS for serum samples). Use of the 1X AlphaLISA Immunoassay Buffer is recommended as a diluent to confirm assay performance.
c. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of Human IL17A (µL)	Vol. of diluent (µL) *	[human IL17A] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human IL17A	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.

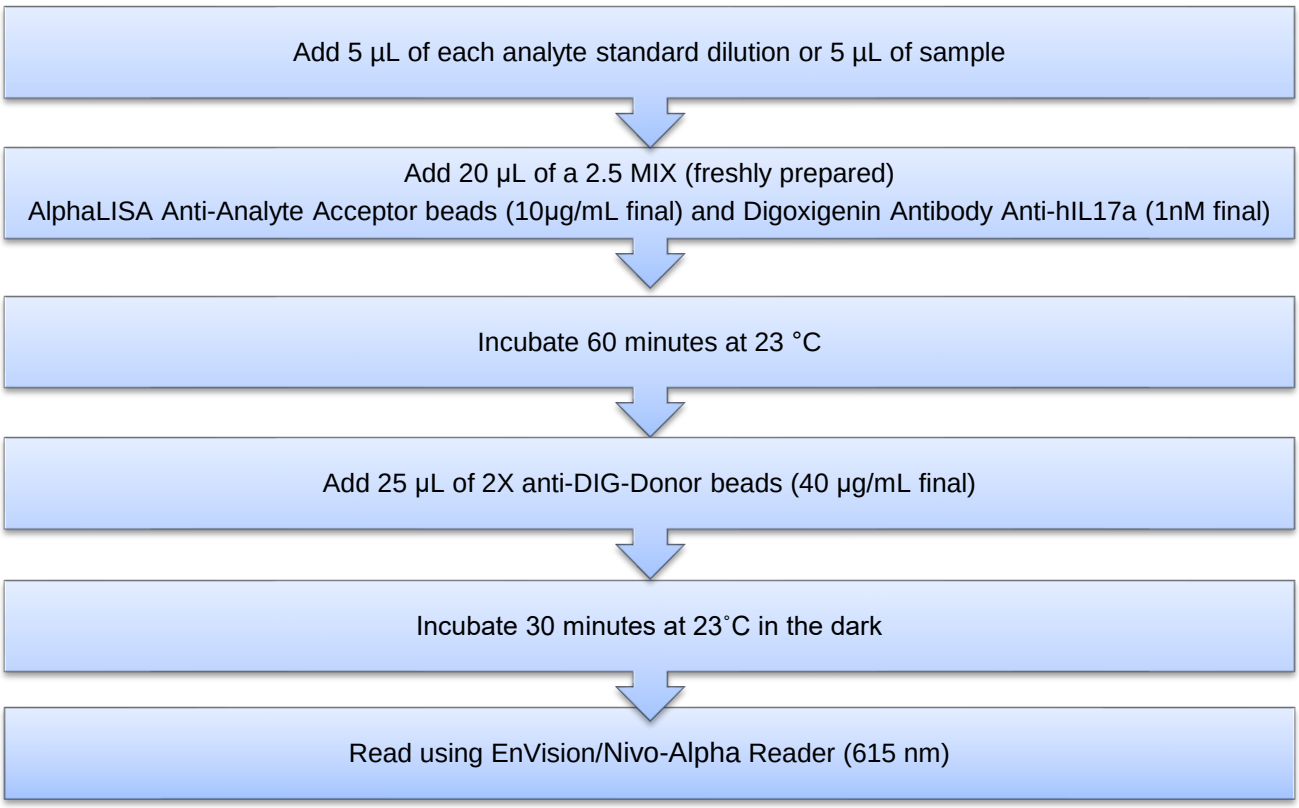
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3. Preparation of 2.5X AlphaLISA Anti-hIL17A Acceptor beads + DIG labeled Antibody Anti-hIL17A MIX (25 µg/mL / 2.5nM):
Add 50 µL of 5 mg/mL AlphaLISA Anti-hIL17A Acceptor beads and 50 µL of 500 nM DIG labeled Antibody Anti-hIL17A to 9 900 µL of 1X AlphaLISA Immunoassay Buffer. Prepare just before use.

4. Preparation of 2X anti-digoxigenin Fab fragment Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL anti-digoxigenin Fab fragment Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

5. Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA Immunoassay Buffer, cell culture medium or FBS).

6. In a Light Gray AlphaPlate (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 2-step protocol using AlphaLISA 1X AlphaLISA Immunoassay Buffer as assay buffer. The analytes (standards) were prepared in AlphaLISA Immunoassay Buffer, Cell culture medium and human serum. All other components were prepared in AlphaLISA Immunoassay Buffer.

- 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)	# of experiments
4.58	IAB	9
14.80	RPMI + 10% FBS	5
13	DMEM + 10% FBS	3
25.5	Human serum	3

- 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 RPMI + 10% FBS, DMEM + 10% FBS and Serum. All other components were prepared in AlphaLISA Immunoassay Buffer 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 plate format.

  - Intra-assay precision:

The intra-assay precision was determined using a total of 3 independent determinations in triplicate. Shown as CV%.

Human IL17A	IAB	RPMI + 10% FBS	DMEM + 10% FBS	Human serum
CV (%)	2%	4%	3%	5%

  - Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 6 measurements for 100 pg/mL sample. Shown as CV%.

Human IL17A	IAB	RPMI + 10% FBS	DMEM + 10% FBS	Human serum
CV (%)	5%	7%	5%	9%

- Spike Recovery:

Six known concentrations of analyte were spiked into PBMC supernatant samples produced in RPMI + 10% FBS. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in RPMI + 10% FBS. All other assay components were diluted in IAB.

Spiked Human IL17A (pg/mL)	Recovery (%)
7.5	102%
15	90%
30	88%
60	94%
120	97%
240	85%

- Specificity:

Cross-reactivity of the Human IL17A AlphaLISA Detection Kit was tested using the following proteins at 30000 to 0.1 pg/mL in IAB. The cross reactivities percentage were calculated using the signals of 30 000 pg/mL Human IL17A as 100%. No unwanted cross-reactions with mouse and rat IL17A proteins were observed. Human analog proteins were also tested, IL17B, IL17C, IL17D, IL17E and IL17F, no signal was observed.

Proteins	**Cross Reactivity (%)**
Mouse IL17A	0.00
Rat IL17A	0.00
Human IL17B	0.00
Human IL17C	0.00
Human IL17D	0.00
Human IL17E	0.00
Human IL17F	0.00

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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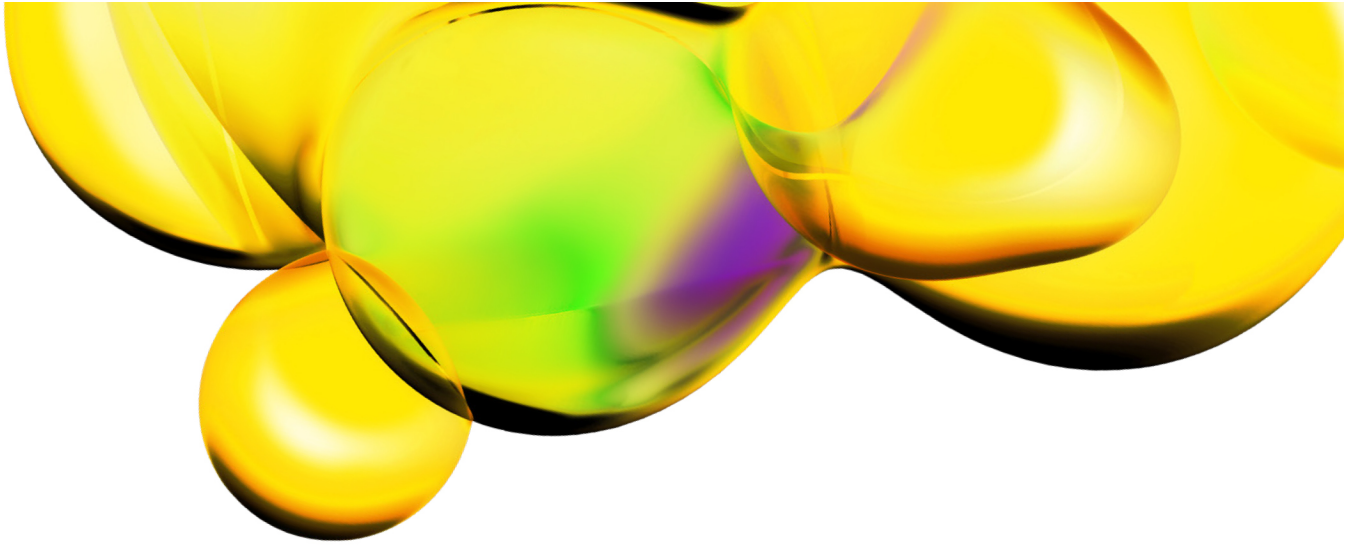

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