MANUAL

AlphaLISA™ Human and Mouse TOM20 Detection Kit

Product number: AL3178

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of **human and mouse TOM20** using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 6 components: AlphaLISA Acceptor beads coated with anti-TOM20 Antibody, Streptavidin-coated Donor beads, Biotinylated anti-TOM20 antibody, Lyophilized human TOM20 AlphaLISA HiBlock Buffer and 5X AlphaLISA Lysis buffer.

Sensitivity: Lower Detection Limit (LDL): **5.2 pg/mL**
Lower Limit of Quantification (LLOQ): **15.24 pg/mL**
EC_{50}: 300 ng/mL

Dynamic Range: **5.2 – 30 000 pg/mL**

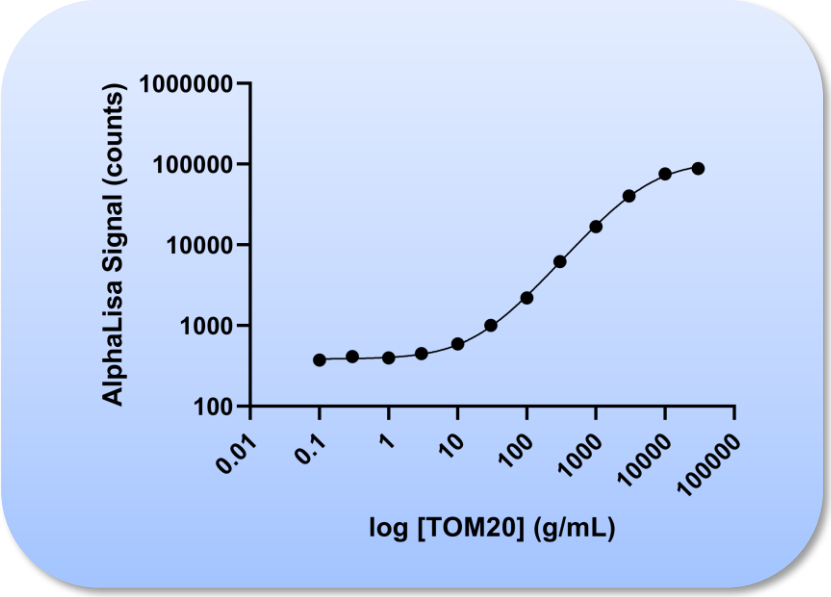


Figure 1. Typical sensitivity curve in AlphaLISA HiBlock Buffer. The data was generated using a grey AlphaPlate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -20 ºC for up to 1 month. Avoid freeze-thaw cycles.

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

Analyte of Interest

Mitochondria are essential organelles that provide cellular energy and contribute to cell death. This organelle is continuously exposed to intra and extra mitochondrial threats including reactive Oxygen species. The removal of damaged mitochondria is critical for maintaining cellular homeostasis. In damaged depolarized mitochondria, PINK1 accumulates on the mitochondrial outer membrane. Upon activation, PINK1 activates Parkin which will ubiquitinate many targets at the Mitochondrial Outer Membrane (MOM). One of these targets is TOM20. Polyubiquitinated mitochondrial substrates bind to LC3 adapters such as p62SQSTM1, OPTN or NDP52 and will be degraded by lysosomal enzymes. TOM20 degradation is a biomarker of Mitophagy activation. TOM20 has be shown to bind a-Synuclein in PD, playing a role in Mitophagy dysregulation.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in culture medias (DMEM, and RPMI 1640), in human serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-TOM20 antibody binds to the streptavidin coated AlphaLISA Donor beads, while another anti-TOM20 antibody is conjugated to AlphaLISA Acceptor beads. In the presence of TOM20, 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).

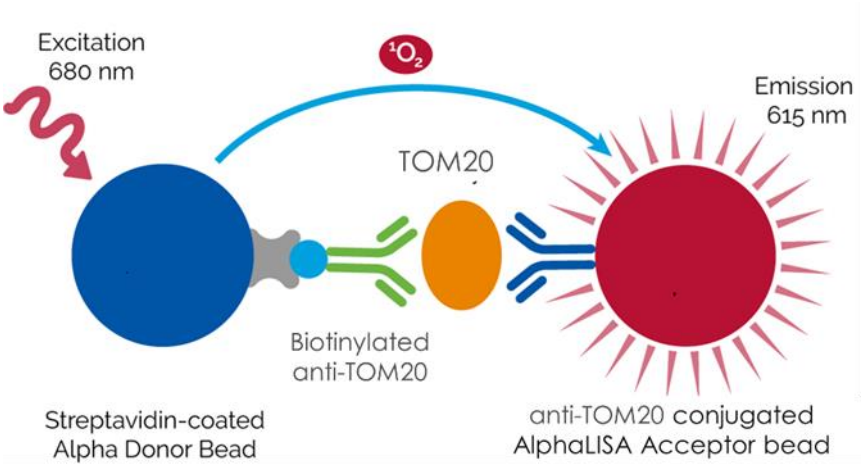


Figure 2. AlphaLISA Human and Mouse TOM20 Detection Assay Principle.

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 (Roscolux filters #389 from Rosco are recommended) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-TOM20 antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3178HV (100 assay points)	AL3178C (500 assay points)	AL3178F (5000 assay points)
AlphaLISA Anti-Total TOM20 Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, 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 CG/ICP II, 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-Total TOM20 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 TOM20	13.5 ng (1 tube, clear cap)	13.5 ng (1 tube, clear cap)	13.5 ng (1 tube, clear cap)
AlphaLISA HiBlock Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle
AlphaLISA Lysis Buffer (5X)	2 mL, 1 small bottle	5 mL, 1 small bottle	50 mL, 1 medium bottle

* Reconstitute lyophilized analyte in 450 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 ºC for future experiments. The aliquoted analyte at -20 ºC is stable up to 1 month. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3178S).

** 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 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 anti-TOM20 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 HiBlock 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.

Assay Procedure

- 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 different amounts 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/biotin antibody mix	SA-Donor beads	
AL3178HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3178C	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)
AL3178F	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 following 2-Step manual described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1. Preparation of 1X Alpha HiBlock Buffer (for 10 mL)
Add 1 mL of 10X AlphaLISA HiBlock Buffer and 9 mL of MilliQ water.

2. Preparation of the 1X AlphaLISA Lysis Buffer (for 10mL)
Add 2mL of 5X AlphaLISA Lysis buffer and 8mL of MilliQ water.

3. Preparation of human TOM20 analyte standard dilutions (tips may be changed for each dilution):

- **Make a short spin to collect to full pellet** (few seconds).
- Reconstitute lyophilized human TOM20 (13.5 ng) in 450 µL dH2O.

Tube	Vol. of	Vol. of	[Human TOM20] in standard curve	
	Human TOM20 (µL)	Lysis Buffer (µL) *	(g/mL in 5 µL)	(pg/mL in 5 µL)
A	20 µL of reconstituted human TOM20		3.00E-08	30 000
B	30 µL of reconstituted human TOM20	60	1.00E-08	10 000
C	30 µL of tube B	70	3.00E-09	3 000
D	30 µL of tube C	60	1.00E-09	1 000
E	30 µL of tube D	70	3.00E-10	300
F	30 µL of tube E	60	1.00E-10	100
G	30 µL of tube F	70	3.00E-11	30
H	30 µL of tube G	60	1.00E-11	10
I	30 µL of tube H	70	3.00E-12	3
J	30 µL of tube I	60	1.00E-12	1
K	30 µL of tube J	70	3.00E-13	0.3
L	30 µL of tube K	60	1.00E-13	0.1
M ** (background)	0	50	3.00E-15	0
N ** (background)	0	50	3.00E-15	0
O ** (background)	0	50	3.00E-15	0
P ** (background)	0	50	3.00E-15	0

* 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).

4. Preparation of 2.5X AlphaLISA Anti-TOM20 Acceptor beads + Biotinylated Antibody Anti-TOM20 MIX (25 µg/mL / 2.5nM):
   Add 50 µL of 5 mg/mL AlphaLISA Anti-TOM20 Acceptor beads and 50 µL of 500 nM Biotinylated Antibody Anti-TOM20 to 9 900 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

5. Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
   Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA HiBlock Buffer.

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

7. In a Light Gray AlphaPlate (384 wells):

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

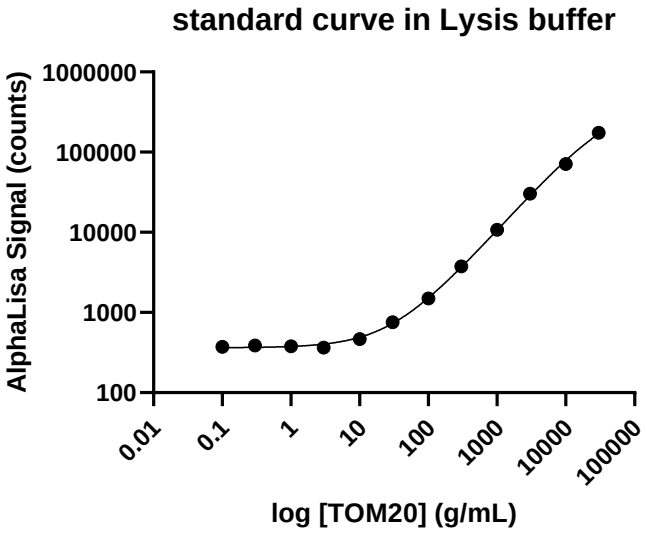
Add 20 µL of a 2.5 MIX (freshly prepared)
AlphaLISA Anti-Analyte Acceptor beads (10 µg/mL final) and Biotinylated Antibody Anti-Analyte (1 nM final)

Incubate 3 hours 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/Nivo-Alpha Reader (615 nm)



Typical data obtained for the standard curve prepared in 1X lysis buffer in a grey 384w AlphaPlate.

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.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined to be a 2-step manual using AlphaLISA HiBlock Buffer as assay buffer. The analytes (standards) were prepared in HiBlock buffer and lysis buffer. All other components were prepared in HiBlock.

- 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)	Human TOM20	# of experiments
HiBlock Buffer	5.43	15
Lysis Buffer	8.03	9

- 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 HiBlock and Lysis Buffer. All other components were prepared in HiBlock Buffer. Each assay consisted of one standard curve comprising 3 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. Each triplicate is interpolated in concentration, data of variability are shown in CV% on concentrations.

Human TOM20 (pg/mL)		HiBlock Buffer	Lysis Buffer
CV (%)	200	5%	6%
	2,000	8%	8%
	15,000	4%	6%

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 12 measurements for 3 different sample. Each measurement is interpolated in concentration, data of variability are shown in CV% on concentrations.

Human TOM20 (pg/mL)		HiBlock Buffer	Lysis Buffer
CV (%)	200	8%	12%
	2,000	9%	13%
	12,000	9%	9%

- Spike Recovery:

Three known concentrations of analyte were spiked into HiBlock and Lysis Buffer. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in HiBlock and Lysis buffer. All other assay components were diluted in HiBlock Buffer.

Spiked Human TOM20 (pg/mL)	% Recovery	
	HiBlock Buffer	Lysis Buffer
200	94%	93%
2,000	95%	89%
12,000	86%	97%

- Specificity:

Cross-reactivity of the TOM20 AlphaLISA Detection Kit was tested using the following proteins at various concentrations in HiBlock. The cross reactivities were calculated using the signals of corresponding concentration of human TOM20 as 100%.

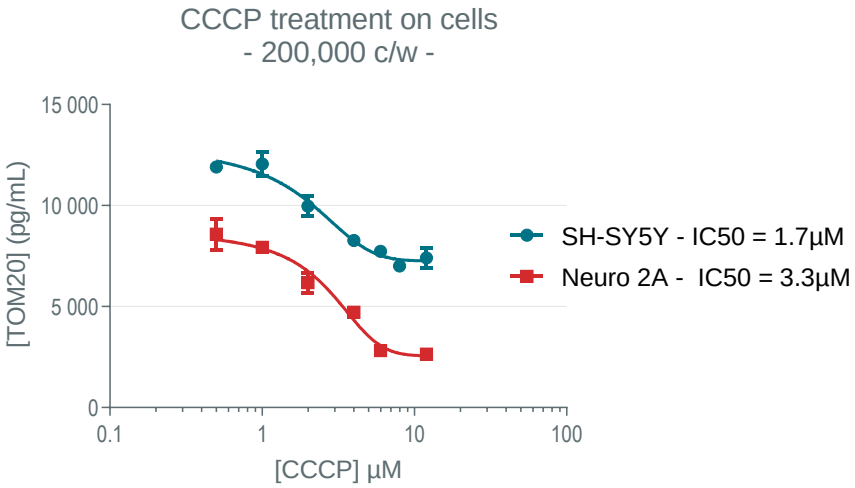
Proteins	Cross Reactivity (%)
Mouse TOM20	100.00

Human and mouse cells experiment

Human **SHY5Y** and mouse Neuro 2A neuronal cells were used for testing TOM20 secretion. Cells treated with CCCP induces mitophagy and TOM20 is decreased.

- Cell culture
  - SHSY5Y cells were cultured in a T175 flask, in DMEM/F12 + 10% FBS in a 5% CO_2 37C incubator.
  - Neuro 2A cells were cultured in a T175 flask, in MEM + 10% FBS in a 5% CO_2 37C incubator.
  - Cells were harvested using cell dissociation buffer.
  - Cells were washed with sterile PBS.
  - Cells were counted on a CellEx counter and were plated in 8-times replicas in a 96-well CulturPlate at 200,000 cells per well in 100 µL additions in appropriate cell culture medium.
  - Cells were incubated for 24 hours.

- Pharmacological treatment
    - CCCP serial dilution (12 to 0.5 µM) was prepared in appropriate cell culture medium.
    - Supernatant from plate was removed and discarded.
    - 100µL of CCP solution was added on top of the cells under 100µL, a negative control (cell culture medium without CCCP) was also done
    - Cells were incubated for 16 hours.
- Supernatant was removed and discarded.
- 100 µL of 1X AlphaLISA Lysis buffer was added to each well.
- The plate was incubated at 23°C for 30 minutes with agitation.
- Lysate were transfer to a 384-well plate for detection.



Results show that CCCP induce a decrease on TOM20 in SH-SY5Y and Neuro 2A cells.

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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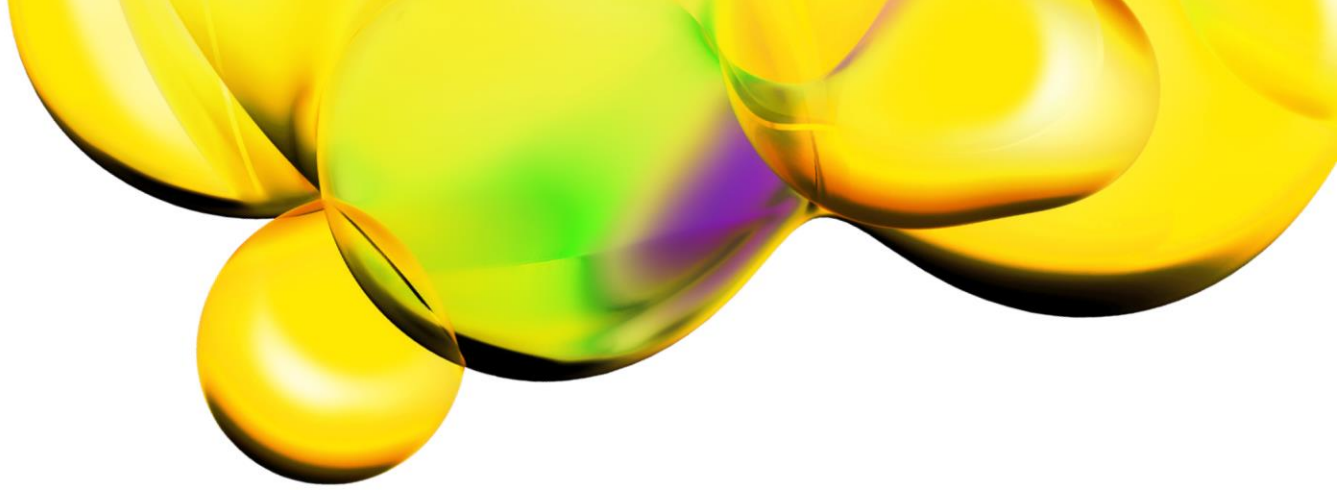

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