TECHNICAL DATA SHEET

AlphaPlex® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

α-tubulin AlphaPlex 545 Detection Kit

Product number: AP383Tb

Contents

Product Information

Application: This kit is designed for the quantitative determination of human α-tubulin in cell lysates using a homogeneous AlphaPLEX 545 assay (no wash steps). Other species of recombinant α-tubulin were not tested,

Sensitivity: Lower Detection Limit (LDL): 261 pg/mL

Lower Limit of Quantification (LLOQ): 785 pg/mL

EC_{50}: 311 ng/mL

Dynamic range: 261 – 1 000 000 pg/mL

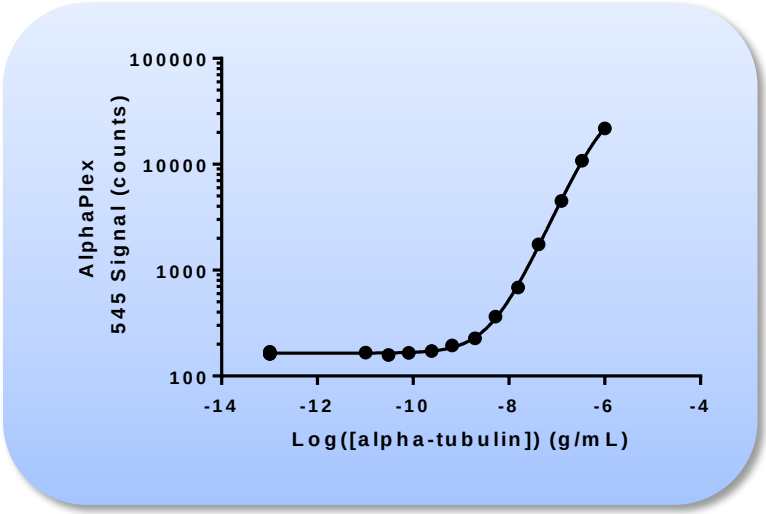


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. Analyte must be stored at -80 °C. **Avoid freeze-thaw cycles**.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Quality Control

Lot to lot consistency is confirmed in an AlphaPlex 545 assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on LDL measurement.

Analyte of Interest

α-tubulin is a 451 amino acid protein that is one of the components of the microtubule cytoskeleton of eukaryotic cells. Alpha and beta tubulin form the skeleton itself, while gamma tubulin is involved in its organization. Alpha-tubulin is ubiquitous in every cell and tissue of the human body, and is highly preserved at the eukaryotic level (human 95% with mouse and 74% with yeast). As such, detection of alpha tubulin in cell extracts is a good tool for normalization of the amount of cell proteins per well in a biochemical assay. The α-tubulin AlphaPlex 545 detection kit allows for the detection of α-tubulin in cell lysates.

Description of the AlphaPlex 545 Assay

AlphaPlex 545 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 AlphaPlex 545 assay, a biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaPlex 545 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 545 nm (Figure 2). Combining this assay with an AlphaLISA or AlphaPlex 645 - based kit will allow the quantification of 2 (or more) analytes in the same well.

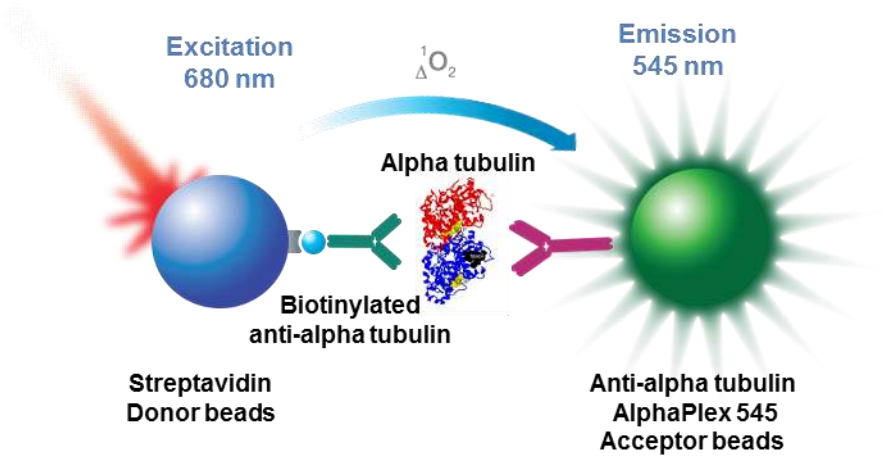


Figure 2. AlphaPlex 545 Assay Principle.

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	AP383Tb-HV (100 assay points***)	AP383Tb-C (500 assay points***)	AL383Tb-F (5000 assay points***)
AlphaPlex 545 Anti- α-tubulin Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, white cap)	100 µL @ 5 mg/mL (1 brown tube, white cap)	1 mL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, 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-α-tubulin Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	40 µL @ 500 nM (1 tube, black cap)	100 µL @ 500 nM (1 tube, black cap)	1 mL @ 500 nM (1 tube, black cap)
Human α-tubulin*	10 µL at 30µg/mL (1 tube, clear cap)	10 µL at 30µg/mL (1 tube, clear cap)	10µL at 30µg/mL (2 tubes, clear caps)
AlphaLISA Immunoassay Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Analyte is sold as aliquots of 10µL at 30µg/mL on dry Ice. Upon reception, store at -80°C. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 12 standard curves. Additional vials can be ordered separately (cat # AL383S).

** 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 AlphaPlex 545 signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaPlex 545 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	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer 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 Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Plus Film.
- AlphaPlex 545 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).
- AlphaPlex 545 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 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**	**AlphaPlex Acceptor beads and Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AP383Tb-HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP383Tb-C	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)
AP383Tb-F	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 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 α-tubulin analyte standard dilutions:
    a. Thaw analyte sample at room temperature.
    b. Do not vortex analyte!
    c. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of α-tubulin (µL)	Vol. of diluent (µL) *	[α-tubulin] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	3.3 µL of α-tubulin stock	96.7	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

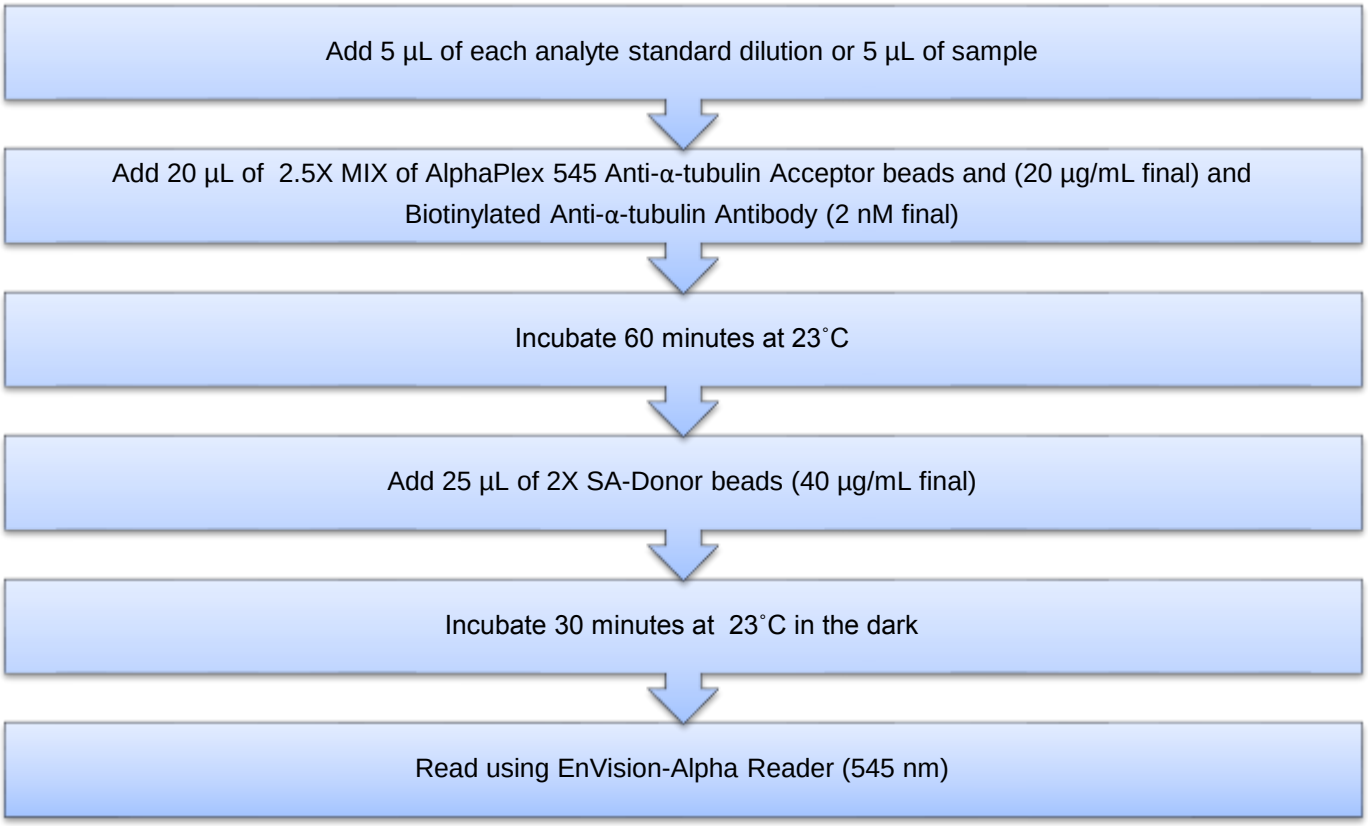
* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** 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 2.5X MIX AlphaPlex 545 Anti-α-tubulin Acceptor beads (50 µg/mL) and Biotinylated Anti-α-tubulin antibody (5 nM):
    a. Prepare just before use.
    b. Add 100 µL of 5 mg/mL AlphaPlex 545 Anti-α-tubulin Antibody Acceptor and 100 µL of Biotinylated Anti-α-tubulin antibody to 9800 µL of 1X AlphaLISA Immunoassay Buffer.

4) 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.

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 AlphaPlex 545 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

AlphaPlex 545 assay performance described below was determined using the 2 step protocol 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
261	IAB	6
250	AlphaLISA Lysis Buffer (ALB)	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 or ALB. 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%.

α-tubulin	IAB	ALB
CV (%)	3	3

- Inter-assay precision:

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

α-tubulin	IAB	ALB
CV (%)	9	8

- Spike Recovery:

Known concentrations of analyte were spiked into IAB or ALB. All samples, including non-spiked buffer were measured in

the assay. Note that the standard curves were prepared in either IAB or ALB. All other components were diluted in IAB.

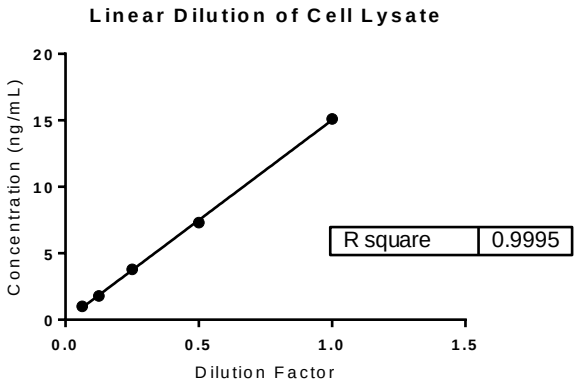
Spiked α-tubulin (pg/mL)	% Recovery	
	IAB	ALB
1000	93	95
300	103	102
100	91	99

Cell Lysate Experiments

- Dilution Linearity

To validate the assay kit, 100K cell lysate samples with unknown concentrations of α-tubulin were tested. The standard was prepared in the AlphaLISA Lysis Buffer and lysate samples were diluted with AlphaLISA Lysis Buffer. All other reagents were prepared in IAB. In the samples, 14.9 ng/mL α-tubulin was detected and excellent dilution linearity (R^2 > 0.995) was achieved. The results are summarized from 3 experiments and shown in table and figure below.

Cell Lysate Dilution Fold (DF)	α-tubulin detected in Positive Cell Lysate (ng/mL)	α-tubulin Positive Cell Lysate (ng/mL x DF)
1	15.1	15.1
2	7.3	14.6
4	3.8	14.4
8	1.8	14.4
16	1.0	16.0
Average ± SD	N/A	14.9 ± 0.7



Specificity Experiments

Cross-reactivity of the α-tubulin kit was tested using the following proteins up to 1 µg/mL in IAB.

Protein	% Cross-reactivity
Human beta-tubulin	0
Human gamma-tubulin	0

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaPlex 545 Assay kit at:

http://www.perkinelmer.com/askAlpha

For more information on utilizing you AlphaPlex 545 assay in duplexing applications please see the following tech note:

http://www.perkinelmer.com/tubulinduplex

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

This product is not for resale or distribution except by authorized distributors.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com