MANUAL

Technology: AlphaLISA®

AlphaLISA Human Interferon Beta (hIFNβ) Detection Kit

Part number:	AL3191HV	AL3191C	AL3191F
Assay points:	100	500	5,000

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

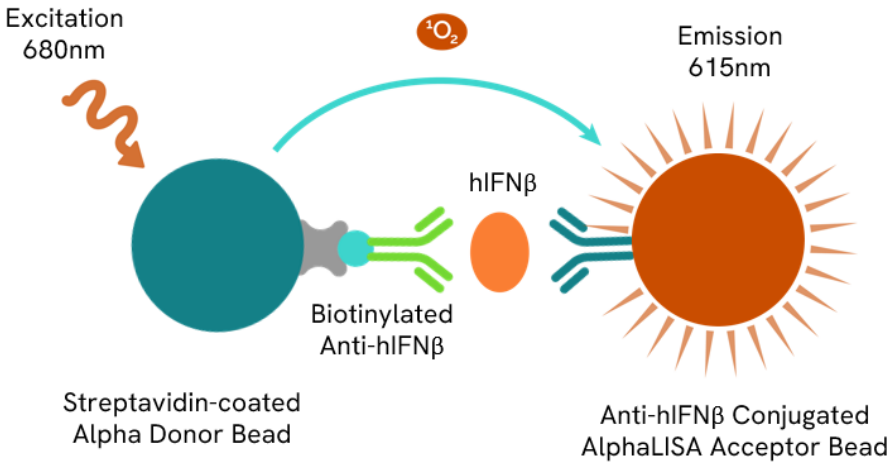
Version: 1 Date: August 2023

ANALYTE OF INTEREST

Mature Interferon-beta (IFN-ß) contains 166 amino acids with a molecular weight of 22 kDa and is produced in large quantities by fibroblasts in response to pathogens. IFN-ß is a cytokine involved in the regulation of unspecific humoral immune responses as well as in immune responses against viral infections. Only small amounts of IFN-ß are produced under healthy conditions. However, levels can dramatically increase during viral infection, inflamation, and autoimmunity. IFN-ß exhibits several biological effects including antiviral and antiproliferative activities. IFN-ß has been used, with some success, as a therapeutic agent in the treatment of multiple sclerosis, as well as in chronic active hepatitis B where it appears to be most promising if the disease has not lasted longer than 5 years.

DESCRIPTION OF THE ALPHALISA ASSAY

AlphaLISA technology allows the detection of molecules of interest in cell culture medium and serum in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-hIFN ß antibody binds to the streptavidin coated AlphaLISA Donor beads, while an anti-hIFN ß antibody is conjugated to AlphaLISA Acceptor beads. In the presence of hIFN ß, 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.



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) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.

- The biotinylated anti- hIFN ß antibody contains sodium azide. Contact with skin or inhalation should be avoided.

KIT CONTENT: REAGENTS AND MATERIALS

Kit components	AL3191HV***	AL3191C****	AL3191F****
AlphaLISA Anti-hIFN ß Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, 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)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	75 µL @ 5 mg/mL (1 brown tube, black cap)	150 µL @ 5 mg/mL (1 brown tube, black cap)	1.5 mL @ 5 mg/mL (1 brown tube, black caps)
Biotinylated Anti-hIFN ß Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	100 µL @ 500 nM (1 tube, black cap)	1 mL @ 500 nM (1 tube, black cap)
Lyophilized Recombinant Anti-hIFN ß *	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Assay 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_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 4 weeks. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3191S).

** 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.

**** 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 biotinylated anti-hIFN ß antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Additional reagents and materials:

The following materials are recommended but not provided in the kit:

Item	Suggested source
Light gray AlphaPlate™-384	Revvity Inc.
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.
EnVision®-Alpha Reader	Revvity Inc.

RECOMMENDATONS

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 the Donor and Acceptor beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute buffers 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 Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode 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. It is recommended to avoid multiple reads of the same well of the assay plate.
- The standard curves shown in this manual are provided for information only. A standard curve must be generated for each experiment.

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 amounts of samples are tested, the volumes of all reagents must 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.

Format	# of data points	*Volumes for 2-Step High Concentration Protocol*				Plate recommendation
		Final	Sample	AlphaLISA Acceptor Beads + biotinylated Ab Mix	SA-Donor beads	
AL3191HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 White ½ AreaPlate-96
AL3191C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96
	500	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate™-384
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 ProxiPlate™-384 Plus White OptiPlate-384
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536
AL3191F	5 000	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate-384
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 ProxiPlate-384 Plus White OptiPlate-384
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536

The 2-Step High Concentration protocol 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 must be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 2.5 mL of 10X AlphaLISA Immunoassay Buffer to 22.5 mL Milli-Q® grade H_2O.

2) Preparation of hIFN ß analyte standard dilutions:

   a. Reconstitute lyophilized hIFN ß (0.1µg) in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 °C for future assays (see page 2 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 (change tip between each standard dilution).

Tube	Vol. of hIFN ß (µL)	Vol. of diluent (µL)*	[hIFN ß] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted hIFNß	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 appropriate diluent (e.g. 1X AlphaLISA Immunoassay buffer 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 10X MIX AlphaLISA Anti hIFN ß Acceptor beads (100 µg/mL) + Biotinylated Anti hIFN ß Antibody (20 nM):

   a. Prepare just before use.
   Add 5 µL of 5 mg/mL AlphaLISA Anti hIFN ß Acceptor Bead and 10 µL of 500 nM Biotinylated Anti hIFN ß Antibody to 235 µL of 1X AlphaLISA Immunoassay Buffer.

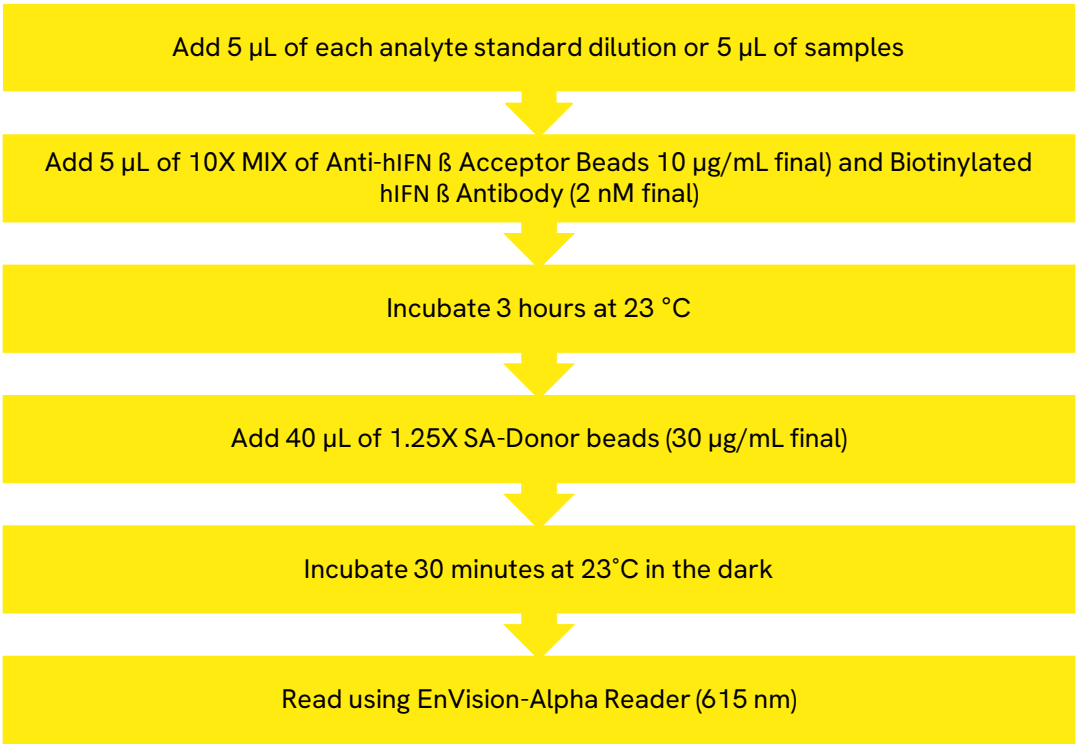
4) Preparation of 1.25X Streptavidin (SA) Donor beads (37.5 µg/mL):

a. Prepare just before use.

b. Keep the beads under subdued laboratory lighting.

c. Add 15 µL of 5 mg/mL SA-Donor beads to 1985 µL of 1X AlphaLISA Immunoassay Buffer.

5) 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).

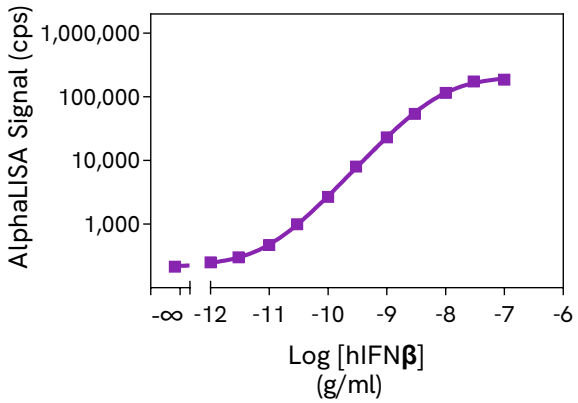
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

ASSAY PERFORMANCE CHARACTERISTICS

AlphaLISA assay performance described below was determined by using a 2-step high concentration protocol using 1X AlphaLISA Immunoassay Buffer as an assay buffer. The analytes (standards) were prepared in different matrix diluents depending on sample type. All other components were prepared in 1X AlphaLISA Immunoassay Buffer.

Standard curve:

A typical sensitivity curve is shown below, using the 2-step high concentration protocol described on page 4, using 1X AlphaLISA Immunoassay Buffer to dilute the standard.



Assay sensitivity:

The LDL was 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 values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL of sample using the recommended assay conditions.

Analyte diluent	*LDL (pg/mL)*	LLOQ (pg/mL)
Immunoassay Buffer	1.05	3.85
RPMI + 10% FBS	1.68	9.47
DMEM + 10% FBS	0.48	3.39

Assay precision:

The following assay precision data were calculated from 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. 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 a 384-well plate format.

Intra-assay precision:

The intra-assay precision was determined using a total of 24 independent determinations. Shown as CV% of measured concentration.

hIFN ß CONCENTRATION	Immunoassay buffer	RPMI + 10% FBS	DMEM + 10% FBS
Sample at 397 pg/mL	4.0%	6.8%	4.9%

Inter-assay precision:

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

hIFN ß	Immunoassay buffer	RPMI + 10% FBS	DMEM + 10% FBS
Sample at 100 pg/mL	2.5%	5.9%	3.5%

Spike and recovery:

Three known concentrations of analyte were spiked into RPMI + 10% FBS, DMEM + 10% FBS and human serum. 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, DMEM + 10% FBS and human serum. All other assay components were diluted in AlphaLISA Immunoassay Buffer.

Spiked hIFN ß (pg/mL)	% Recovery	
	RPMI + 10% FBS	*DMEM + 10% FBS*
30 000	91%	88%
3 000	88%	96%
300	85%	108%

Specificity:

Cross-reactivity of the AlphaLISA human IFN ß Detection Kit was tested using recombinant IFN ß from mouse and rat as analytes in an assay using the protocol as described above. Human isoforms were also tested (IFNγ, IFNα 2 and IL6). The cross reactivities were established using the hIFN ß related proteins below at a test concentration from 300 000 to 1pg/mL. Percentage recovery was computed by comparing the measured Interpolated concentration versus the theoretical one.

Proteins	Cross reactivity (%)
Mouse IFN ß	0.00
Rat IFN ß	0.00
*Human IFN**γ***	0.00
*Human IFN**α** 2b*	0.00
Human IL6	0.00

Calibration:

Target NIBSC/WHO International Standard (code 01/420) was tested using the AlphaLISA hIFN ß detection kit: 1 unit of Standard NIBSC 01/420 corresponds to 42 pg/mL of AlphaLISA hIFN ß respectively.

Cell experiments:

Cell culture conditions

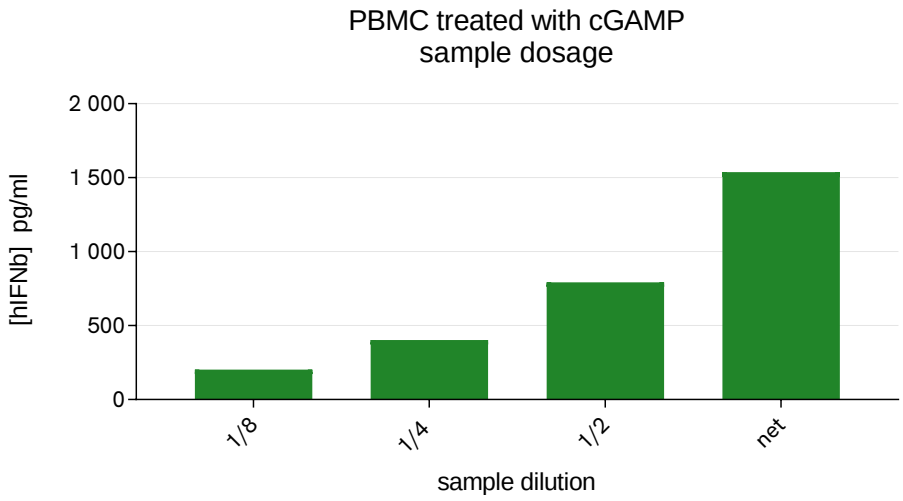
PBMC cells were collected following a Ficoll gradient treatment of whole blood.

Cells were prepared at 30 millions cells In 1mL.

Pharmacological rreatment

A cGAMP treatment was done on cells In order to induce cytokine release. A -ènM of cGAMP was added on top of the cells during 16 hours.

Supernatant are then collected and diluted In a 2-fold serial dilution and tested In HP hIFN ß kit.



Human serum experiments:

Dilution linearity

Normal human serum and hIFN ß spiked (7 600 pg/mL) normal human serum samples were diluted with 100% FBS and the assay was performed along with a standard curve prepared in 100% FBS. Concentrations of hIFN ß in diluted samples were determined by interpolating from the standard curve. The other components (anti-hIFN ß Acceptor beads, biotinylated anti-hIFN ß antibody, and SA-Donor beads) of the assays were prepared in 1X AlphaLISA ImmunoAssay Buffer.

Basal levels of hIFN ß was not detected in human serum from presumably health subjects. Excellent dilution linearity (R^2 >0.99, slope=0.98) and dilution recovery was achieved in the hIFN ß spiked human serum samples (in the range of 2- down to 16-fold dilution. The results are shown in the table below.

Serum dilution factor (x)	Expected hIFN ß (pg/mL)	Observed hIFN ß (pg/mL)	*Dilution Recovery (%)*
neat	7633	7633	-
2	3817	4118	108
4	1908	1829	96
8	954	972	102
16	477	348	73

Spike recovery

Three known amounts of human IFN ß were spiked into normal human serum (30 000, 3 000 and 300 pg/mL). The samples were assayed along with the standard prepared in 100% FBS. Other components (anti- hIFN ß Acceptor beads, biotinylated anti- hIFN ß antibody, and SA-Donor beads) of the assays were prepared in AlphaLISA ImunoAssay Buffer. The spike recoveries of human IFN ß were determined. The results shown in the table below indicate that excellent recoveries were achieved for all three spikes tested.

hIFN ß	Diluent: 100% FBS	
	Spiked sample (normal human serum)	
Spike (pg/mL)	Concentration (pg/mL)	Recovery (%)
30 000	29 453	98%
3 000	3 386	113%
300	309	103%

TROUBLESHOOTING

To find detailed recommendations for common situations you might encounter with your AlphaLISA assay kit, please visit our website at www.revvity.com.

www.revvity.com

revvity

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