MANUAL

HUMAN MOUSE FIBRONECTIN DETECTION KITS

Part # 64HMFNPEG & 64HMFNPEH

Test size#: 500 tests (64HMFNPEG) and 10,000 tests (64HMFNPEH) - assay volume: 20 µL

Revision: #02 of September 2023

Store at: -16°C or below (64HMFNPEG); -16°C or below (64HMFNPEH)

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of Human/mouse Fibronectin in supernatant or cell lysate and offers a fast alternative to ELISA.

The detection principle of this kit is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, Human/Mouse Fibronectin is detected in a sandwich assay by using anti Human/Mouse Fibronectin labeled with Europium cryptate (donor), and anti Human/Mouse Fibronectin labeled with d2 (acceptor).

When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the Human/Mouse Fibronectin concentration.

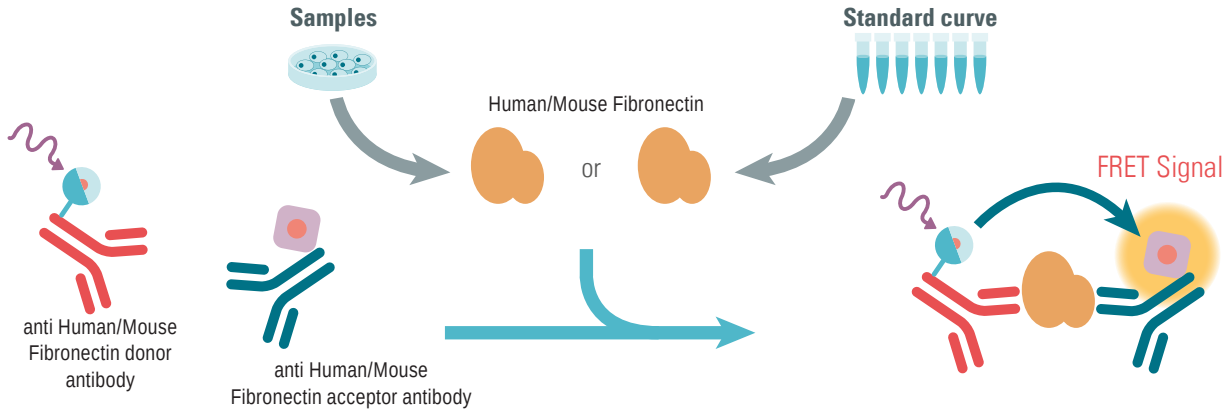
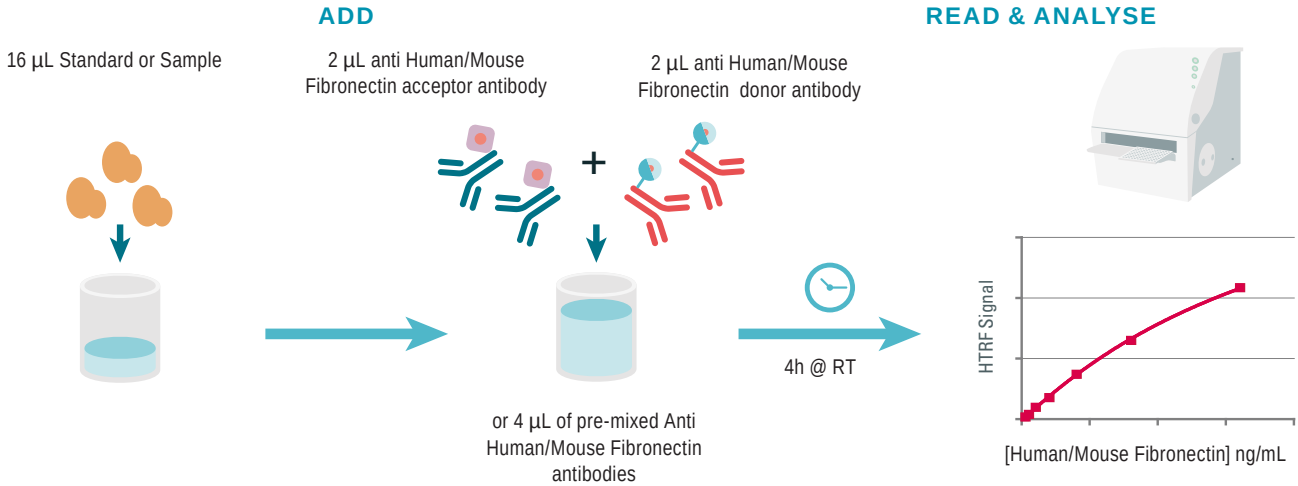


Figure 1: Principle of HTRF Human/Mouse Fibronectin sandwich assay.

MANUAL AT A GLANCE



Make sure to use the set-up for Eu Cryptate.

MATERIALS PROVIDED:

KIT COMPONENTS	500 TESTS * CAT # 64HMFNPEG	10,000 TESTS * CAT # 64HMFNPEH
Human/Mouse Fibronectin Standard Lyophilized	1 vial 4,500 ng/mL	2 vials 4,500 ng/mL
Human/Mouse Fibronectin Eu Cryptate Antibody	1 vial - 50 µL Frozen - 20X	1 vial - 1 mL Frozen - 20X
Human/Mouse Fibronectin d2 Antibody	1 vial - 50 µL Frozen - 20X	1 vial - 1 mL Frozen - 20X
Lysis buffer #3 ** 4X	1 vial 130 mL	1 vial 130 mL
Detection buffer *** ready-to-use	2 vials 1.5 mL Detection Buffer #3	1 vial 50 mL Detection Buffer #3

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 tests and 10,000 tests respectively in 20 µL final volume.. Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
** Medium like cell culture medium can be an alternative to the diluent.
*** The Detection buffer is used to prepare working solutions of acceptor and donor reagents.

PURCHASE SEPARATELY:

- HTRF®-Certified Reader. Make sure the setup for Eu Cryptate is used.

For a list of HTRF-compatible readers and set-up recommendations, please visit www.revvity.com

- Small volume (SV) detection microplates. For information about microplate recommendations, please visit our website at: www.revvity.com

STORAGE AND STABILITY

Store the kit at -16°C or below.

Under proper storage conditions, reagents are stable until the expiry date indicated on the label. Lysis buffer and detection buffer are shipped frozen, but can be stored at 2-8°C in your premises.

If lyophilized, reconstituted reagents, antibodies, and standard stock solutions may be frozen and thawed only once. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below .

Volume of Human/Mouse Fibronectin standard aliquots should not be under 100 µL.

Thawed buffer and detection buffer can be stored at 2-8°C in your premises.

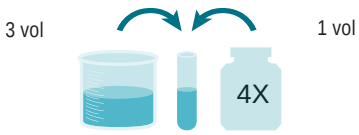
REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect lysis buffer may affect reagent stability and assay results.
- Avoid freeze thawing cycles.
- Thaw the frozen reagents at room temperature, allow them to warm up to room temperature for at least 30 mins before use
- Before use, allow Lysis buffer and Detection buffer to warm up at room temperature and homogenize them with a vortex.
- It is recommended to filter buffers.
- Antibody solutions must be prepared in individual vials and can be mixed prior to dispensing.
- Human/Mouse Fibronectin standards (for standard curve) must be prepared in lysis buffer or in the same medium as the samples.

TAKE CARE TO PREPARE STOCK AND WORKING SOLUTIONS ACCORDING TO THE DIRECTIONS FOR THE KIT SIZE YOU HAVE PURCHASED.

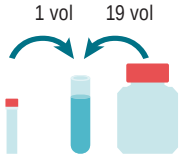
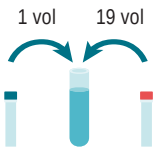
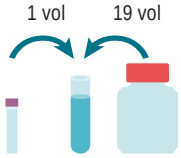
TO PREPARE REAGENT STOCK SOLUTIONS:

500 TESTS KIT - 64HMFNPEG		10,000 TESTS KIT - 64HMFNPEH
Anti-Human/Mouse Fibronectin Eu Cryptate antibody		
Thaw the Human/Mouse Fibronectin Eu Cryptate antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.		Thaw the Human/Mouse Fibronectin Eu Cryptate antibody . Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
Anti-Human/Mouse Fibronectin d2 antibody		
Thaw the Human/Mouse Fibronectin d2 antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.		Thaw the Human/Mouse Fibronectin d2 antibody. Mix gently. This 20X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
Human/Mouse Fibronectin Standard		
Reconstitute the Human/Mouse Fibronectin Standard with distilled water in order to obtain a 4,500 ng/mL stock solution. See instructions on vial label for reconstitution volume. Mix gently after reconstitution. This stock solution can be frozen and stored at -16°C or below.After use, the reconstituted standard solution must be frozen and stored at -16°C or below		Reconstitute the Human/Mouse Fibronectin Standard with distilled water in order to obtain a 4,500 ng/mL stock solution. See instructions on vial label for reconstitution volume. Mix gently after reconstitution. This stock solution can be frozen and stored at -16°C or below.After use, the reconstituted standard solution must be frozen and stored at -16°C or below
Lysis buffer		
Dilute 4-fold the 4 X lysis buffer #3 with distilled water: homogenize the 4 X lysis buffer #3 with a vortex and add 1 volume of stock solution in 3 volumes of distilled water (e.g., 1 mL of lysis buffer + 3 mL of distilled water). Mix gently after dilution. This 1X buffer can be frozen and stored at -60°C or below.		Dilute 4-fold the 4 X lysis buffer #3 with distilled water: homogenize the 4 X lysis buffer #3 with a vortex and add 1 volume of stock solution in 3 volumes of distilled water (e.g., 10 mL of diluent + 30 mL of distilled water). Mix gently after dilution. This 1X buffer can be frozen and stored at -60°C or below.
Detection buffer		
The Detection buffer is ready-to-use.		The Detection buffer is ready-to-use.

TO PREPARE ANTIBODY WORKING SOLUTIONS:

Each well requires 2 µL of Human/Mouse Fibronectin-Eu Cryptate Antibody and 2 µL of Human/Mouse Fibronectin-d2 Antibody.

Prepare the two antibody solutions in separate vials.

500 TESTS KIT - 64HMFNPEG		10,000 TESTS KIT - 64HMFNPEH
Human/Mouse Fibronectin Eu Cryptate antibody		
Dilute 20-fold the 20X stock solution (thawed reagent) of Human/Mouse Fibronectin Eu Cryptate antibody with Detection buffer #3: add 1 volume of Eu Cryptate antibody stock solution in 19 volumes of detection buffer (e.g. 50 µL of Eu Cryptate antibody stock solution + 950 µL of detection buffer).	 	Dilute20-fold the 20X stock solution (thawed reagent) of Human/Mouse Fibronectin Eu Cryptate antibody with Detection buffer #3: add 1 volume of Eu Cryptate antibody stock solution in 19 volumes of detection buffer (e.g. 1 mL of Eu Cryptate antibody stock solution + 19 mL of detection buffer).
Human/Mouse Fibronectin d2 antibody		
Dilute 20-fold the 20X stock solution (thawed reagent) of Human/Mouse Fibronectin d2 antibody with Detection buffer #3: add 1 volume of d2 antibody stock solution in 19 volumes of detection buffer (e.g. 50 µL of d2-antibody stock solution + 950 µL of detection buffer).	 	Dilute 20-fold the 20X stock solution (thawed reagent) of Human/Mouse Fibronectin d2 antibody with Detection buffer #3: add 1 volume of d2 antibody stock solution in 19 volumes of detection buffer (e.g. 1 mL of d2 antibody stock solution + 19 mL of detection buffer).
Antibody mix		
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Cryptate antibody).		It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 20 mL of d2 antibody + 20 mL of Cryptate antibody).

TO PREPARE STANDARD WORKING SOLUTIONS:

- Each well requires 16 µL of standard.
- Dilute the standard stock solution serially with lysis buffer #3 (1X) . Please note: For all samples to test even if are cell supernatant, it is mandatory to use lysis buffer #3 only as diluent to allow better detection of Human/Mouse Fibronectin
- For supernatant, we recommend to use medium supplemented with BSA (0.2 to 1%) instead of FCS, in order to avoid interferences or sticking to assay plates.
- In order to check for a potential interference effect from your own assay buffer when using the assay for the first time, we highly recommend the parallel preparation of a standard curve in your own supplemented cell culture medium and in lysis buffer #3 (1X).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended standard dilution procedure is listed and illustrated below:

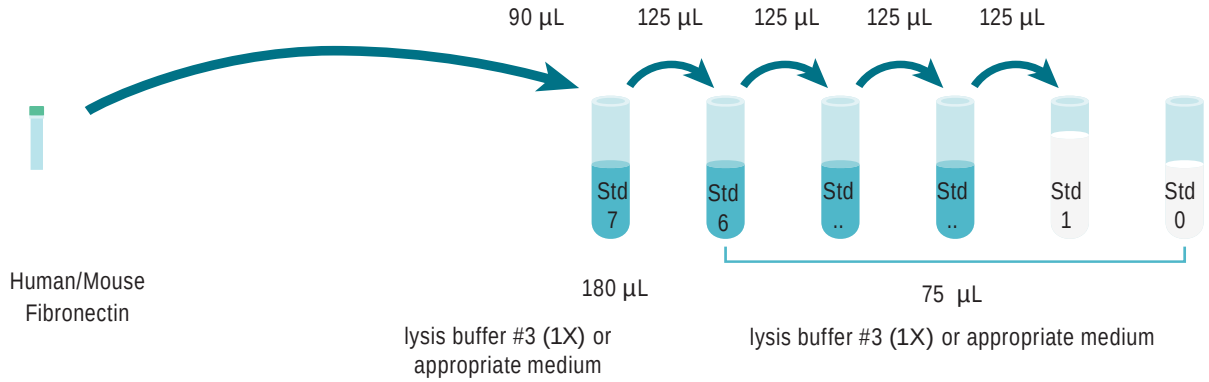
Dilute the standard stock solution 3-fold with diluent; this yields the Standard Max solution (1,500 ng/mL)

Dilute the standard stock solution 3-fold with lysis buffer #3 (1X) to prepare high standard (Std 7): e.g. take 90 µL of standard stock solution and add it to 180 µL of lysis buffer #3 (1X). Mix gently.

Use the high standard (Std 7) to prepare the standard curve using 1/1.6 serial dilutions as follows:

- Dispense 75 µL of lysis buffer #3 (1X) in each vial from Std 6 to Std 0.
- Add 125 µL of standard to 75 µL of lysis buffer #3 (1X), mix gently and repeat the 1/1.6 serial dilution to make standard solutions: std6, std5, std4, std3, std2, std1.

This will create 7 standards for the analyte. Std 0 (Negative control) is lysis buffer #3 (1X) or appropriate culture medium alone.



STANDARD	SERIAL DILUTIONS	TOTAL FIBRONECTIN WORKING SOLUTIONS (NG/ML)
Standard Stock solution	Reconstituted lyophilisate	4 500
Standard 7	90 µL stock solution + 180 µL lysis buffer #3 (1X)	1 500
Standard 6	125 µL standard 7 + 75 µL lysis buffer #3 (1X)	938
Standard 5	125 µL standard 6 + 75 µL lysis buffer #3 (1X)	586
Standard 4	125 µL standard 5 + 75 µL lysis buffer #3 (1X)	366
Standard 3	125 µL standard 4 + 75 µL lysis buffer #3 (1X)	229
Standard 2	125 µL standard 3 + 75 µL lysis buffer #3 (1X)	143
Standard 1	125 µL standard 2 + 75 µL lysis buffer #3 (1X)	89
Standard 0	100 µL Lysis buffer #3 (1X)	-

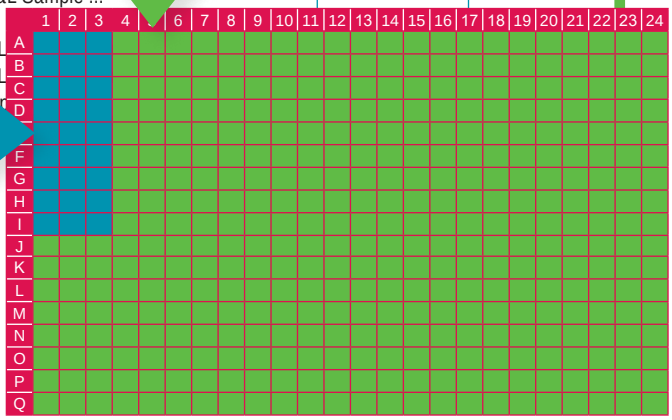
TO PREPARE SAMPLES:

- Each well requires 16 µL of sample.
- Just after their collection, put the samples at 4°C and test them immediately. For later use, samples should be dispensed into disposable plastic vials and stored at -60°C or below. Avoid multiple freeze/thaw cycles.
- Cell supernatants must be prepared using a culture medium supplemented with BSA (0.2 to 1%) instead of FCS to avoid interferences and Human/Mouse Fibronectin sticking to culture vessels. As Fibronectin is prone to degradation, addition of antiprotease inhibitor cocktail can be beneficial.
- Samples with a concentration above the highest standard (Std 7) must be diluted lysis buffer #3 (1X) necessarily in lysis buffer#3 as diluent, prepared, as recommended above.
- In order to measure Human/Mouse Fibronectin in cell lysates, cells must be lyzed with Lysis Buffer #3 (1X) for 30 min at RT under gentle shaking. Then cell lysates and cell supernatant must be diluted ysis Buffer #3 (1X) as diluent to allow better detection of HUman/Mouse Fibronectin. Please note that the 4X stock solution of Lysis Buffer #3 must be ordered separately (Ref# 64KL3FDF, 130 mL) and 4-fold diluted with distilled water before use.
- To obtain additional information or support, please contact the HTRF technical support team at www.revvity.com

ASSAY MANUAL

	Standard (Std 0 - Std 7)	Samples
Step 1	Dispense 16 µL of each Human/Mouse Fibronectin standard (Std 0 - Std 7) into each standard well	Dispense 16 µL of each sample into each sample well
Step 2	Add 2 µL of Human/Mouse Fibronectin d2 antibody working solution to all wells	
Step 3	Add 2 µL of Human/Mouse Fibronectin Eu Cryptate antibody working solution to all wells	
Step 4	Seal the plate and incubate 4h @ RT Following incubation, the signal remains stable over a period of 48 hours.	
Step 5	Remove the plate sealer and read on an HTRF® compatible reader	

	1	2	3	4	5	6
A	16 µL Std 0 (Negative control) 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well A1	Repeat Well A1	16 µL Sample 1 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well A4	Repeat Well A4
B	16 µL Std 1 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well B1	Repeat Well B1	16 µL Sample 2 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well B4	Repeat Well B4
C	16 µL Std 2 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well C1	Repeat Well C1	16 µL Sample 3 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well C4	Repeat Well C4
D	16 µL Std ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well D1	Repeat Well D1	16 µL Sample ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well D4	Repeat Well D4
E	16 µLStd ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well E1	Repeat Well E1	16 µL Sample ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well E4	Repeat Well E4
F	16 µL Std ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well F1	Repeat Well F1	16 µL Sample ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well F4	Repeat Well F4
G	16 µL Std ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well G1	Repeat Well G1	16 µL Sample ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well G4	Repeat Well G4
H	16 µL Std ... 2 µL Human/Mouse Fibronectin-d2 2 µL Human/Mouse Fibronectin-Eu Cryptate	Repeat Well H1	Repeat Well H1	16 µL Sample ... 2 µL 2 µL		



DATA REDUCTION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit www.revvity.com

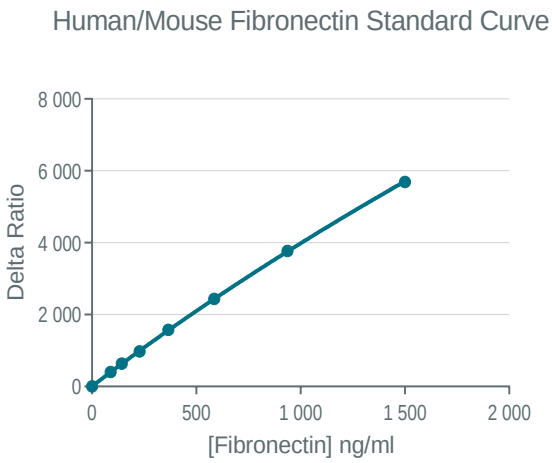
RESULTS

This data must not be substituted for the data obtained in the laboratory and should be considered only as an example.

Results may vary from one HTRF® compatible reader to another.

Standard curve fitting with the 4 Parameter Logistic (4PL) model (with 1/Y² weighting):

	Ratio	CV	DELTA RATIO
Standard 0 - Negative control	685	0%	0
Standard 1 - 89 ng/ml	1086	2%	401
Standard 2 - 143 ng/ml	1321	0%	636
Standard 3 - 229 ng/ml	1661	3%	976
Standard 4 - 366 ng/ml	2263	2%	1 578
Standard 5 - 586 ng/ml	3123	1%	2 438
Standard 6 - 938 ng/ml	4453	1%	3 768
Standard 7 - 1,500 ng/ml	6377	1%	5 692

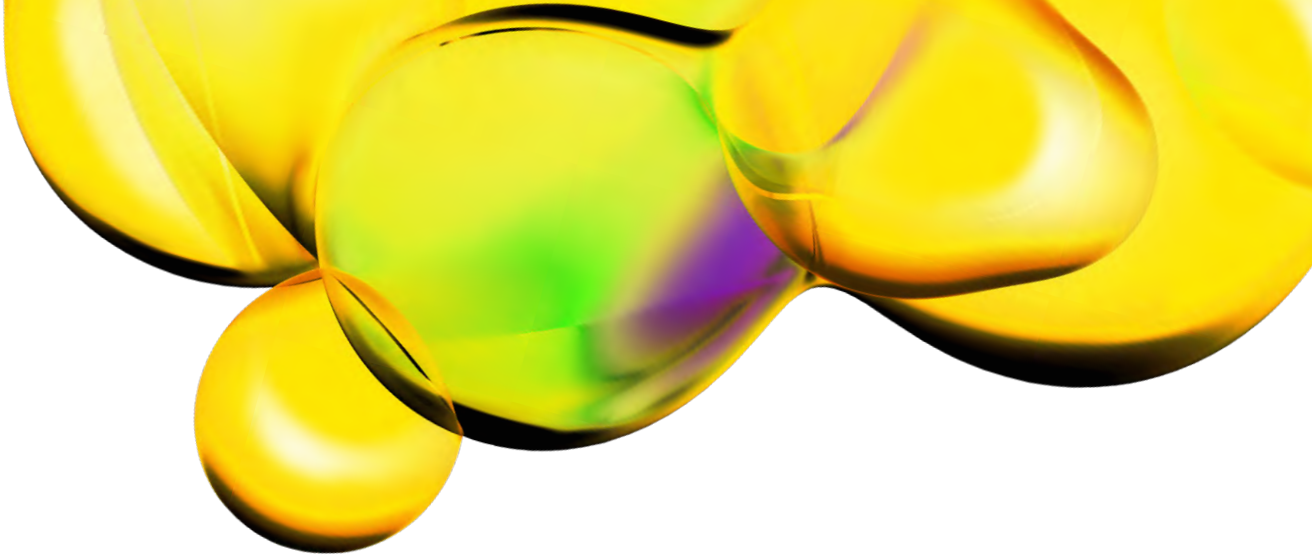


ANALYTICAL CHARACTERISTICS

ASSAY PERFORMANCES

Assay range (LOQ* to Std max)	29 - 1,500 ng/mL
Limit Of Detection (LOD)* = Mean Std 0 + 2 SD	5.6 ng/ml
Incubation time	4h at RT

*The LOD and LOQ were calculated from data obtained in diluent with the PHERAstar FS reader (flash lamp excitation) after overnight incubation. These values may vary from one HTRF compatible reader to another.



Manufactured by Cisbio Bioassays - Parc Marcel Boiteux - 30200 Codolet – FRANCE

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