MANUAL

HTRF Insulin High Range Detection Kit

Part # 62IN1PEG & 62IN1PEH

Test size#: 500 tests (62IN1PEG) and 10,000 tests (62IN1PEH) - assay volume: 80 µL

Revision: #06 of September 2023

Store at: 2-8°C (62IN1PEG); 2-8°C (62IN1PEH)

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

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of Insulin in highly concentrated pancreatic beta cell supernatants/lysates, islet supernatants/lysates and pancreas perfusates and offers a fast alternative to ELISA. The assay is compatible with human, mouse, rat, porcine and bovine species, but is not designed for use with plasma or serum samples.

The detection principle of this kit is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, Insulin is detected in a sandwich assay by using anti Insulin antibody labeled with Europium cryptate (donor), and anti Insulin antibody labeled with XL665 (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 Insulin concentration.

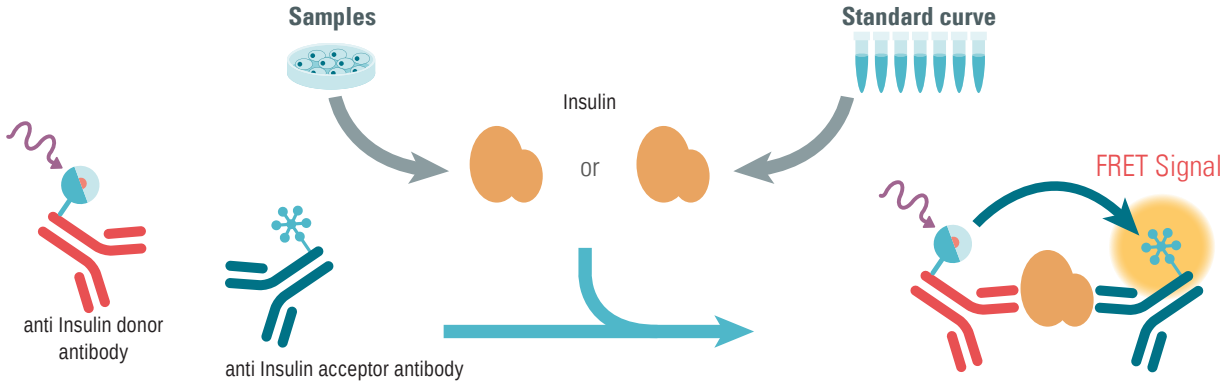
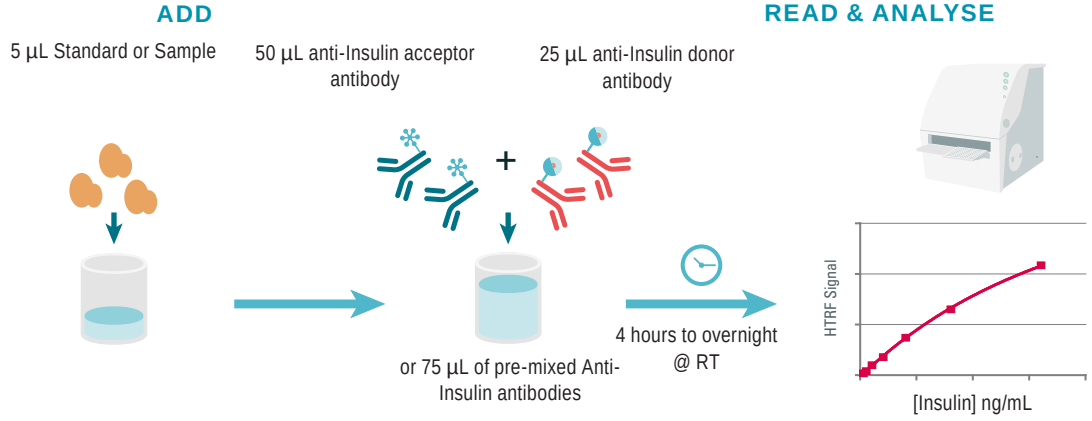


Figure 1: Principle of HTRF Insulin sandwich assay.

MANUAL AT A GLANCE



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

MATERIALS PROVIDED:

KIT COMPONENTS	500 TESTS * CAT # 62IN1PEG	10,000 TESTS * CAT # 62IN1PEH
Insulin Standard Lyophilized	1 vial	2 vials
Insulin Eu Cryptate Antibody	1 vial Lyophilized	2 vials Lyophilized
Insulin XL665 Antibody	1 vial Lyophilized	2 vials Lyophilized
Diluent #5 ** 5X	1 vial 10 mL	2 vials 100 mL
Detection buffer *** ready-to-use	1 vial 55 mL Detection Buffer #7	6 vials 130 mL Detection Buffer #7

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 tests and 10,000 tests respectively in 80 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

- For information about microplate recommendations, please visit our website at: revvity.com

STORAGE AND STABILITY

Store the kit at 2-8°C.

Under proper storage conditions, reagents are stable until the expiry date indicated on the label.

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 -60°C or below .

Volume of Insulin High Range standard aliquots should not be under 20 µL.

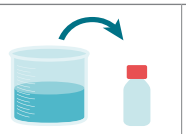
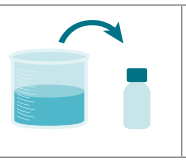
REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Allow the lyophilized reagents to warm up to room temperature for at least 30 mins before reconstitution
- Before use, allow Diluent 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.
- Insulin standards (for standard curve) must be prepared in diluent 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.

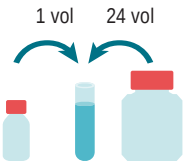
TO PREPARE REAGENT STOCK SOLUTIONS:

500 TESTS KIT - 62IN1PEG		10,000 TESTS KIT - 62IN1PEH
Anti-Insulin Eu Cryptate antibody		
Reconstitute the Insulin Eu Cryptate antibody with 0.5 mL distilled water. Mix gently. This 25 X stock solution can be frozen and stored at -60°C or below.		Reconstitute the Insulin Eu Cryptate antibody with 5 mL distilled water. Mix gently. This 25 X stock solution can be frozen and stored at -60°C or below.
Anti-Insulin XL665 antibody		
Reconstitute the Insulin XL665 antibody with 1 mL distilled water. Mix gently. This 25 X stock solution can be frozen and stored at -60°C or below.		Reconstitute the Insulin XL665 antibody with 10 mL distilled water. Mix gently. This 25 X stock solution can be frozen and stored at -60°C or below.
Insulin Standard		
Reconstitute the Insulin Standard with distilled water in order to obtain a 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 -60°C or below.		Reconstitute the Insulin Standard with distilled water in order to obtain a 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 -60°C or below.
Diluent		
Dilute 5-fold the 5 X diluent #5 with distilled water: homogenize the 5 X diluent #5 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water (e.g., 1 mL of diluent + 4 mL of distilled water). Mix gently after dilution. This 1X diluent can be frozen and stored at -60°C or below.		Dilute 5-fold the 5 X diluent #5 with distilled water: homogenize the 5 X diluent #5 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water (e.g., 10 mL of diluent + 40 mL of distilled water). Mix gently after dilution. This 1X diluent 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 25 µL of Insulin-Eu Cryptate Antibody and 50 µL of Insulin-XL665 Antibody.

Prepare the two antibody solutions in separate vials.

500 TESTS KIT - 62IN1PEG		10,000 TESTS KIT - 62IN1PEH
Insulin Eu Cryptate antibody		
Dilute 25-fold the 25 X stock solution (reconstituted reagent) of Insulin Eu Cryptate antibody stock solution with the Detection buffer #7: add 1 volume of Eu Cryptate antibody stock solution in 24 volumes of Detection buffer #7 (e.g., 100 µL of reconstituted Eu Cryptate antibody stock solution + 2.4 mL of Detection Buffer #7).		Dilute 25-fold the 25X stock solution (reconstituted reagent) of Insulin Eu Cryptate antibody stock solution with the Detection buffer #7: add 1 volume of Eu Cryptate antibody stock solution in 24 volumes of Detection buffer #7 (e.g., 1 mL of reconstituted Eu Cryptate antibody stock solution + 24 mL of Detection Buffer #7).
Insulin XL665 antibody		
Dilute 25-fold the 25 X stock solution (reconstituted reagent) of Insulin XL665 antibody stock solution with the Detection buffer #7: add 1 volume of XL665 antibody stock solution in 24 volumes of Detection buffer #7 (e.g., 200 µL of reconstituted XL665 antibody stock solution + 4.8 mL of Detection Buffer #7).		Dilute 25-fold the 25 X stock solution (reconstituted reagent) of Insulin XL665 antibody stock solution with the Detection buffer #7: add 1 volume of XL665 antibody stock solution in 24 volumes of Detection buffer #7 (e.g., 2 mL of reconstituted XL66-antibody stock solution + 48 mL of Detection Buffer #7).
Antibody mix		
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 2 volumes of XL665 antibody solution to 1 volume of Cryptate antibody solution (e.g. 2 mL of XL665 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 2 volumes of XL665-antibody solution to 1 volume of Cryptate antibody solution (e.g. 10 mL of XL665-antibody + 5 mL of Cryptate antibody).

TO PREPARE STANDARD WORKING SOLUTIONS:

- Each well requires 5 µL of standard.
- Dilute the standard stock solution serially with diluent #5 (1X) or with the cell culture medium used to culture the cells (e.g. KRB, KRHB, DMEM, MEM, RPMI+SVF).
- 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 diluent #5 (1X).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.
- However, if sticking standard is noticed, it is recommended to supplement your assay buffer with 0.05% Tween 20

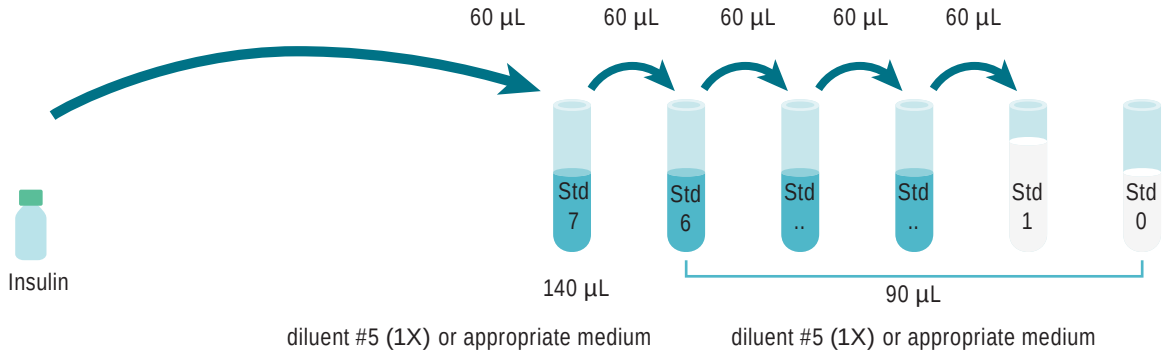
A recommended standard dilution procedure is listed and illustrated below:

Dilute the standard stock solution 3.33-fold with diluent #5 (1X) to prepare high standard (Std 7): e.g. take 60 µL of standard stock solution and add it to 140 µL of diluent #5 (1X). Mix gently.

Use the high standard (Std 7) to prepare the standard curve using 1/2.5 serial dilutions as follows:

- Dispense 90 µL of diluent #5 (1X) in each vial from Std 6 to Std 0.
- Add 60 µL of standard to 90 µL of diluent #5 (1X), mix gently and repeat the 1/2.5 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 diluent #5 (1X) or appropriate culture medium alone.



STANDARD	SERIAL DILUTIONS	INSULIN WORKING SOLUTIONS (ng/mL)
Standard Stock solution	Reconstituted lyophilisate	500
Standard 7	60 µL stock solution + 140 µL Diluent #5 (1X)	150
Standard 6	60 µL standard 7 + 90 µL Diluent #5 (1X)	60
Standard 5	60 µL standard 6 + 90 µL Diluent #5 (1X)	24
Standard 4	60 µL standard 5 + 90 µL Diluent #5 (1X)	9.6
Standard 3	60 µL standard 4 + 90 µL Diluent #5 (1X)	3.84
Standard 2	60 µL standard 3 + 90 µL Diluent #5 (1X)	1.54
Standard 1	60 µL standard 2 + 90 µL Diluent #5 (1X)	0.61
Standard 0	90 µL Diluent #5 (1X)	0

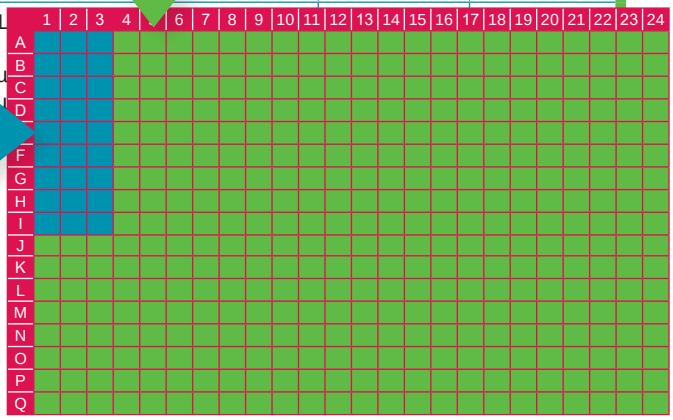
TO PREPARE SAMPLES:

- Each well requires 5 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.
- In order to measure the total Insulin content in cell or islet lysates, we recommend an acid-ethanol extraction step
- Samples with a concentration above the highest standard (Std 7) must be diluted diluent #5 (1X)
- Before measurement, cell or islet lysates must be diluted at least 20-fold in the used secretion assay buffer

ASSAY MANUAL

	Standard (Std 0 - Std 7)	Samples
Step 1 	Dispense 5 µL of each Insulin standard (Std 0 - Std 7) into each standard well	Dispense 5 µL of each sample into each sample well
Step 2 	Add 50 µL of Insulin XL665 antibody working solution to all wells	
Step 3 	Add 25 µL of Insulin Eu Cryptate antibody working solution to all wells	
Step 4 	Seal the plate and incubate 4 hours to overnight @ RT The best sensitivity is achieved after overnight incubation time. 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	5 µL Std 0 (Negative control) 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well A1	Repeat Well A1	5 µL Sample 1 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well A4	Repeat Well A4
B	5 µL Std 1 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well B1	Repeat Well B1	5 µL Sample 2 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well B4	Repeat Well B4
C	5 µL Std 2 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well C1	Repeat Well C1	5 µL Sample 3 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well C4	Repeat Well C4
D	5 µL Std ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well D1	Repeat Well D1	5 µL Sample ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well D4	Repeat Well D4
E	5 µLStd ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well E1	Repeat Well E1	5 µL Sample ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well E4	Repeat Well E4
F	5 µL Std ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well F1	Repeat Well F1	5 µL Sample ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well F4	Repeat Well F4
G	5 µL Std ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well G1	Repeat Well G1	5 µL Sample ... 50 µL Insulin-XL 25 µL Insulin-Eu C	Repeat Well G4	Repeat Well G4
H	5 µL Std ... 50 µL Insulin-XL665 25 µL Insulin-Eu Cryptate	Repeat Well H1	Repeat Well H1	5 µL 50 µ		



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.

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

3. Calculate the % delta F which reflects the signal to background of the assay. The negative control (Standard 0) plays the role of an internal assay control. Delta F is used for the comparison of day to day runs of the same assay.

$$\text{delta F (\%)} = \frac{\text{Ratio Standard or sample - Ratio Negative Control}}{\text{Ratio Negative Control}} \times 100$$

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

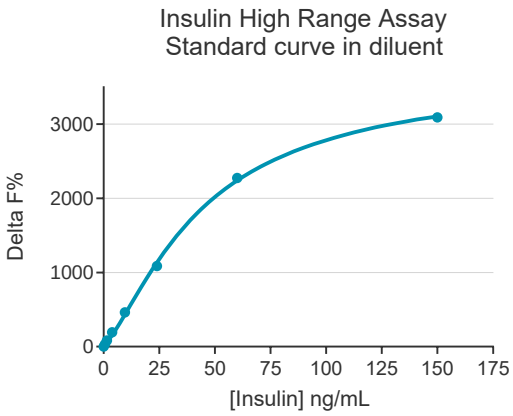
RESULTS

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:

	Ratio (1)	CV (2)	Delta F% (3)
Standard 0 - Negative control	728	3.1%	-
Standard 1 - 0.61 ng/mL	972	2.6%	34%
Standard 2 - 1.54 ng/mL	1,325	1.7%	82%
Standard 3 - 3.84 ng/mL	2,130	0.8%	193%
Standard 4 - 9.6 ng/mL	4,095	1.4%	462%
Standard 5 - 24 ng/mL	8,640	2.6%	1,087%
Standard 6 - 60 ng/mL	17,280	3.9%	2,273%
Standard 7 - 150 ng/mL	23,230	2%	3,091%

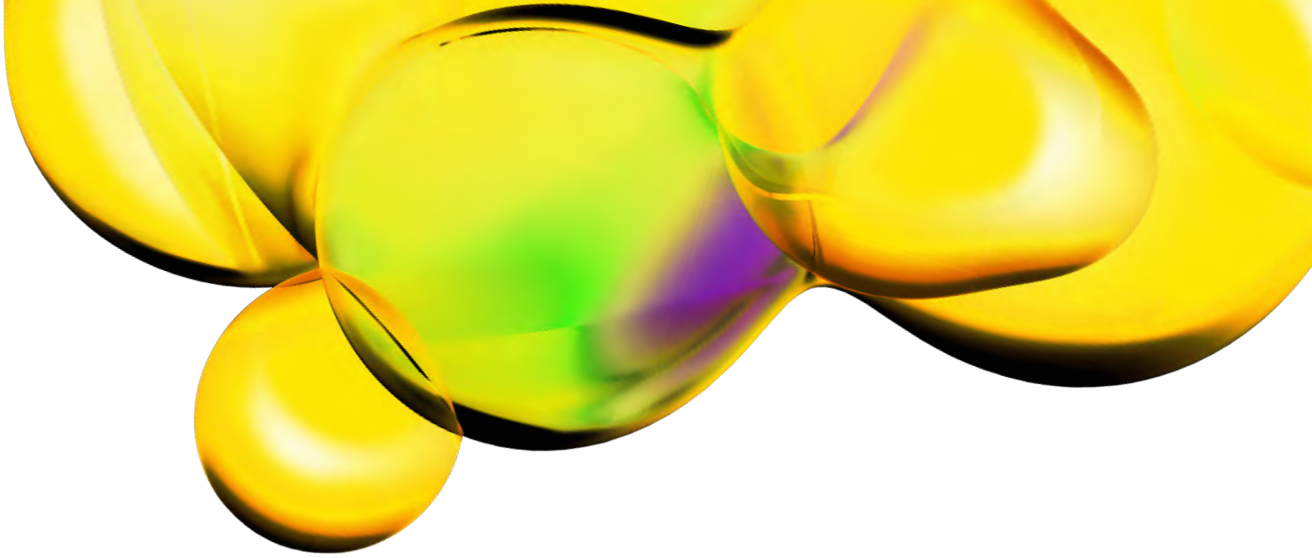


ANALYTICAL CHARACTERISTICS

ASSAY PERFORMANCES AND CALIBRATION

Calibration	NIBSC International standard 66/304 - 1 ng Insulin HTRF ⟷ 1 ng Insulin NIBSC 66/304 (i.e.: 2.3 x 10-05 IU)
Assay range	0.61 to 150 ng/mL
Limit of detection (LoD*) = Std 0 Mean + 2 SD	0.036 ng/mL
incubation time	4h to overnight at RT

*The LoD was calculated from data obtained with the PHERAstar Plus reader (flash lamp excitation) after overnight incubation. This may vary from one HTRFcompatible reader to another.



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