MANUAL

Technology: HTRF™ Biomarkers

HTRF Glucagon Kits

Part number:	62CGLPEG	62CGLPEH
Test size	500 tests	10,000 tests

Storage: ≤ 60°C

Assay volume : 20 µL

Version: 06 **Date:** November 2024

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of Glucagon in cell/tissue culture supernatants and offers a fast alternative to ELISA. The assay is compatible with human, mouse, rat, and porcine species and is highly specific for pancreatic Glucagon.

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

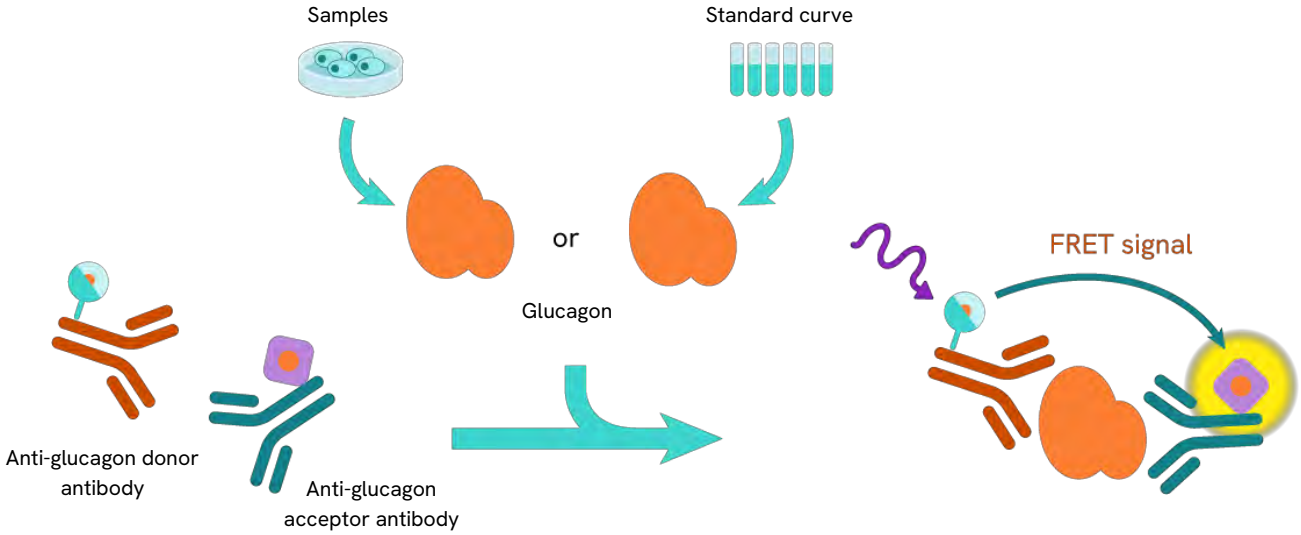
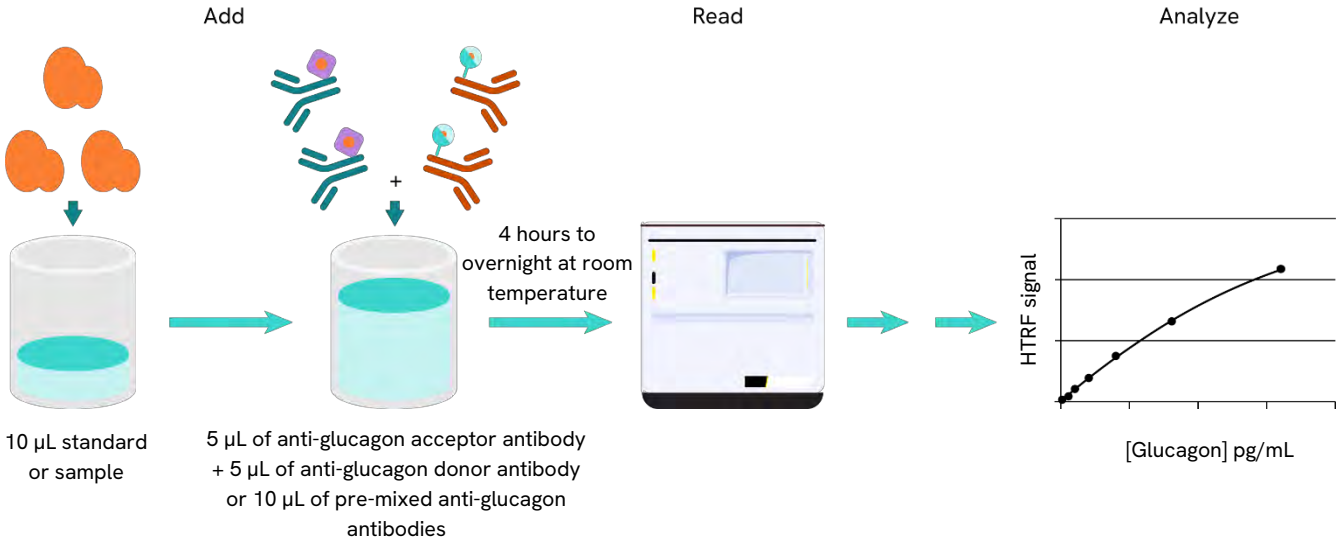


Figure 1: Principle of HTRF Glucagon sandwich assay

PROTOCOL AT A GLANCE



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

MATERIAL PROVIDED

KIT COMPONENTS	500 TESTS*			10,000 TESTS*		
Glucagon Standard Frozen		green cap	1 vial 100 ng/mL		green cap	1 vial 100 ng/mL
Glucagon Tb Cryptate Antibody Frozen 50X		orange cap	1 vial 50 µL		red cap	1 vial 1 mL
Glucagon d2 Antibody Frozen 50X		blue cap	1 vial 50 µL		purple cap	1 vial 1 mL
Diluent #5 Ready-to-use**		Please indicate the color	1 vial 10 mL		Please indicate the color	1 vial 100 mL
Detection Buffer* #6 Ready-to-use**		red cap	1 vial 7 mL		red cap	1 vial 105 mL

* 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 Tb Cryptate is used. For a list of HTRF-compatible readers and set-up recommendations, please visit our website.
- Small volume (SV) detection microplates. Use white plate only. For more information about microplate recommendations, please visit our website.

STORAGE AND STABILITY

Kit

- Store the kit at -60°C or below.
- Under proper storage conditions, reagents are stable until the expiry date indicated on the label.

Reagents

- Glucagon standard must not be aliquoted. The standard can be refrozen in the same vial and can be thawed multiple times.
- Thawed diluent and detection buffer can be stored at 2-8°C in your premises.

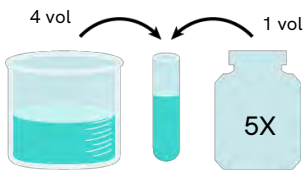
REAGENT PREPARATION

Before you begin

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- 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 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.
- Glucagon 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.

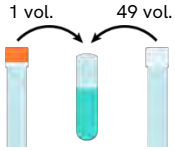
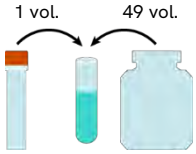
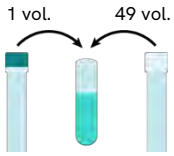
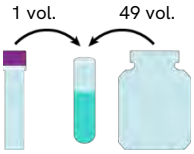
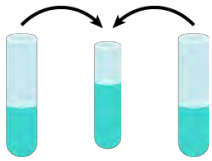
To prepare reagent stock solutions

500 TESTS			10,000 TESTS
Anti-Glucagon Tb Cryptate antibody			
Thaw the Glucagon Tb Cryptate antibody. Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.			Thaw the Glucagon Tb Cryptate antibody. Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.
Anti-Glucagon d2 antibody			
Thaw the Glucagon d2 antibody. Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.			Thaw the Glucagon d2 antibody. Mix gently. This 50X stock solution can be frozen and stored at -16°C or below
Glucagon Standard			
Thaw the Glucagon Standard in order to obtain a 100 ng/mL stock solution. Mix gently. This stock solution can be frozen and stored at -60°C or below			Thaw the Glucagon Standard in order to obtain a 100 ng/mL stock solution. Mix gently. This stock solution can be frozen and stored at -60°C or below.
Diluent			
Dilute 5-fold the 5X diluent #5 with distilled water: homogenize the 5X 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 5X diluent #5 with distilled water: homogenize the 5X 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.

To prepare antibody working solutions

Each well requires 5 µL of Glucagon-Tb Cryptate Antibody and 5 µL of Glucagon-d2 Antibody.
Prepare the two antibody solutions in separate vials.

<table>
<tr><th colspan="2">500 TESTS</th><th colspan="2">10,000 TESTS</th></tr>
<tr><td colspan="4">Glucagon Tb Cryptate antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of Glucagon Tb Cryptate antibody with the Detection buffer #6: add 1 volume of Tb Cryptate antibody stock solution in 49 volumes of Detection buffer #6 (e.g., 50 µL of reconstituted Tb Cryptate antibody stock solution + 2.45 mL of Detection buffer #6).</td><td> 
</td><td> 
</td><td>Dilute 50-fold the 50X stock solution (thawed reagent) of Anti Glucagon Tb Cryptate antibody with the Detection buffer #6: add 1 volume of Tb Cryptate antibody stock solution in 49 volumes of Detection buffer #6 (e.g., 1 mL of reconstituted Tb Crypate antibody stock solution + 49 mL of Detection buffer #6).</td></tr>
<tr><td colspan="4">Glucagon d2 antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of Glucagon d2 antibody with the Detection buffer #6: add 1 volume of d2 antibody stock solution in 49 volumes of Detection buffer #6 (e.g., 50 µL of reconstituted d2-antibody stock solution + 2.45 mL of Detection buffer #6).</td><td> 
</td><td> 
</td><td>Dilute 50-fold the 50X stock solution (thawed reagent) of Glucagon d2 antibody with the Detection buffer #6: add 1 volume of stock solution in 49 volumes of Detection buffer #6 (e.g., 1 mL of reconstituted d2 antibody stock solution + 49 mL of Detection buffer #6).</td></tr>
<tr><td colspan="4">Antibody Mix</td></tr>
<tr><td>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).</td><td colspan="2">  </td><td>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. 5 mL of d2 antibody + 5 mL of Cryptate antibody).</td></tr>
</table>

To prepare working standards solutions

- Each well requires 10 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) supplemented with 0.05% Tween 20.
- 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.

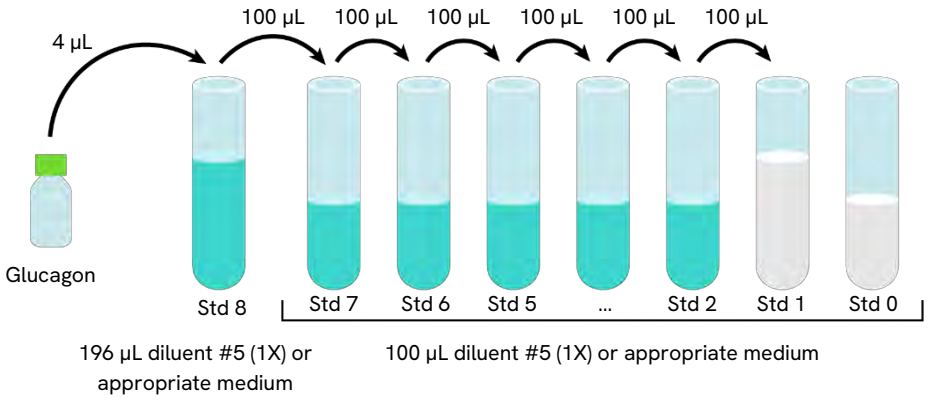
A recommended standard dilution procedure is listed and illustrated below:

Dilute the standard stock solution 50-fold with diluent #5 (1X) to prepare high standard (Std 8): e.g. take 4 µL of standard stock solution and add it to 196 µL of diluent #5 (1X). Mix gently

Use the high standard (Std 8) to prepare the standard curve using 1/2 serial dilutions as follows:

- Dispense 100 µL of diluent #5 (1X) in each vial from Std 7 to Std 0.
- Add 100 µL of standard to 100 µL of diluent #5 (1X), mix gently and repeat the 1/2.4 serial dilution to make standard solutions: std7, std6, std5, std4, std3, std2, std1.

This will create 8 standards for the analyte. Std 0 (Negative control) is diluent #5 (1X) or appropriate culture medium alone



STANDARD	SERIAL DILUTIONS	WORKING SOLUTIONS
Standard Stock solution	Thawed stock solution	100 000 pg/mL
Standard 8	4 µL stock solution + 196 µL Diluent #5 (1X)	2 000 pg/mL
Standard 7	100 µL standard 8 + 100 µL Diluent #5 (1X)	1 000 pg/mL
Standard 6	100 µL standard 7 + 100 µL Diluent #5 (1X)	500 pg/mL
Standard 5	100 µL standard 6 + 100 µL Diluent #5 (1X)	250 pg/mL
Standard 4	100 µL standard 5 + 100 µL Diluent #5 (1X)	125 pg/mL
Standard 3	100 µL standard 4 + 100 µL Diluent #5 (1X)	62.5 pg/mL
Standard 2	100 µL standard 3 + 100 µL Diluent #5 (1X)	31.25 pg/mL
Standard 1	100 µL standard 2 + 100 µL Diluent #5 (1X)	15.6 pg/mL
Standard 0	100 µL Diluent #5 (1X)	0

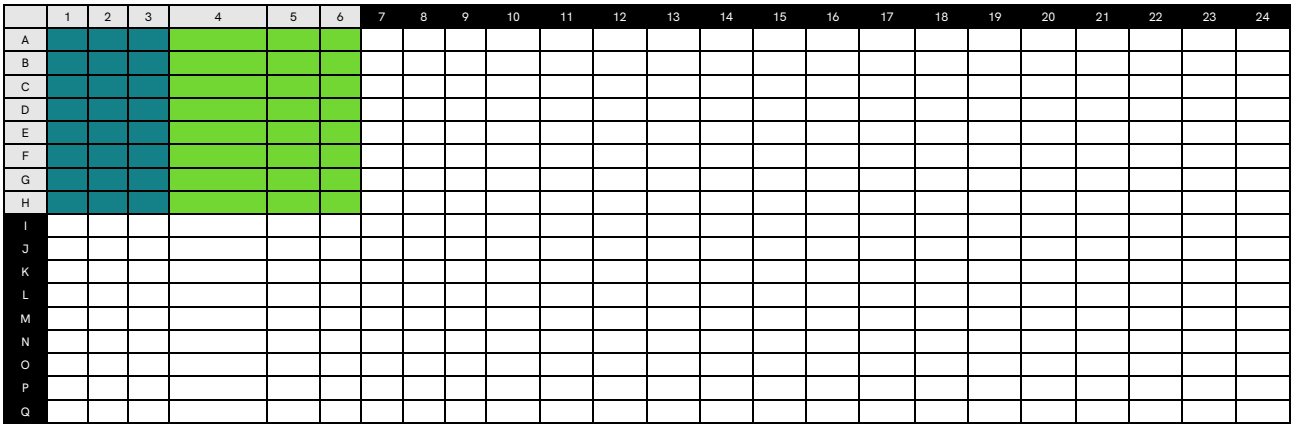
To prepare samples

- Each well requires 10 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.
- If preparing the standard curve in your own secretion assay buffer, we recommend to supplement it with 0.05% Tween 20: Tween 20 avoids sticking effect of glucagon during the preparation of the standard curve by serial dilutions
- Samples with a concentration above the highest standard (Std 8) must be diluted diluent #5 (1X)
- As Terbium Cryptate is sensitive to phenol red, it is mandatory to use medium without phenol red (e.g. KRB or HSBC).

ASSAY PROTOCOL

		STANDARD (STD 0 – STD 8)	SAMPLES
Step 1		Dispense 10 µL of each Glucagon standard (Std 0 - Std 8) into each standard well	Dispense 10 µL of each sample into each sample well
Step 2		Add 5 µL of Glucagon d2 antibody working solution to all wells	
Step 3		Add 5 µL of Glucagon Tb Cryptate antibody working solution to all wells	
Step 4		Seal the plate and incubate 4 hours to overnight @ 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	10 µL Std 0 (Negative control) 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well A1	Repeat Well A1	10 µL sample 1 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well A4	Repeat Well A4
B	10 µL Std 1 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well B1	Repeat Well B1	10 µL sample 2 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well B4	Repeat Well B4
C	10 µL Std 2 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well C1	Repeat Well C1	10 µL sample 3 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well C4	Repeat Well C4
D	10 µL Std 3 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well D1	Repeat Well D1	10 µL sample... 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well D4	Repeat Well D4
E	10 µL Std 4 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well E1	Repeat Well E1	10 µL sample... 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well E4	Repeat Well E4
F	10 µL Std 5 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well F1	Repeat Well F1	10 µL sample... 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well F4	Repeat Well F4
G	10 µL Std 6 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well G1	Repeat Well G1	10 µL sample... 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well G4	Repeat Well G4
H	10 µL Std 7 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well H1	Repeat Well H1	10 µL sample... 5 µL Glucagon-d2 5 µL Glucagon-Tb Cryptate	Repeat Well H4	Repeat Well H4



DATA REDUCTION & INTERPRETATION

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 our website.

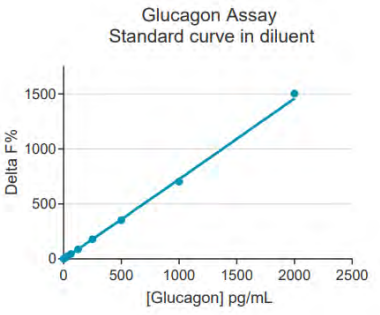
RESULTS

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 with $1/Y^2$) model

		Ratio (1)	CV% (2)	Delta F% (3)
Standard 0	Negative control	1 860	2.1%	-
Standard 1	15.6 pg/mL	2 054	2.4%	10%
Standard 2	31.25 pg/mL	2 301	1.5%	24%
Standard 3	62.5 pg/mL	2 674	5.7%	44%
Standard 4	125 pg/mL	3 486	1%	87%
Standard 5	250 pg/mL	5 162	1.3%	178%
Standard 6	500 pg/mL	8 399	1.1%	352%
Standard 7	1,000 pg/mL	14 909	0.4%	702%
Standard 8	2,000 pg/mL	29 824	1.1%	1,503%



ANALYTICAL CHARACTERISTICS

Assay performances

Assay range	15.6 to 2,000 pg/mL
Limit of detection (LoD*) = Std 0 Mean + 2 SD	6 pg/mL
incubation time	4h to overnight at RT

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

Specificity

The different peptides listed below were tested up to 10 µM

	Specificity (% of recognition)
Glucagon	100%
Glucagon fragment 1-18	<1.81%
Glucagon fragment 19-29	<0.03%
Oxyntomodulin	<0.07%
Glicentin	<0.07%
GLP-1 (7-36 amide)	<0.06%
GLP-1 (7-37)	<0.11%
GLP-2	<0.3%
GRPP (Glicentin-Related Pancreatic Peptide)	<0.01%

Dilution linearity

To assess the linearity of the assay, mouse samples with high concentration of glucagon were serially diluted with diluent # 5 (1X) to produce samples within the dynamic range of the assay. The insulin concentration measured was compared to the expected concentration.

Mouse a-cell supernatant	[Glucagon] measured (pg/mL)	[Glucagon] expected (pg/mL)	% of expected concentration
Undiluted	1 460		
1:2	733	730	100.4%
1:4	351	365	96.2%
1:8	167	182.5	91.5%
1:16	80.3	91.3	88.0%

Intra-assay variability (n=24)

Sample	CV
Standard 2 (31.25 pg/mL)	2.8%
Standard 6 (500 pg/mL)	2.4%
Standard 8 (2,000pg/mL)	3.0%

Inter-assay variability (n=4)

Sample	CV
Standard 2 (31.25 pg/mL)	8.1%
Standard 6 (500 pg/mL)	4.4%
Standard 8 (2,000pg/mL)	5.4%

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

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