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HTRF® package insert

General information

Document reference: 62INSPEC rev08 (July 2008)

For in vitro research use only
 Storage temperature: 2–8°C

Packaging details:

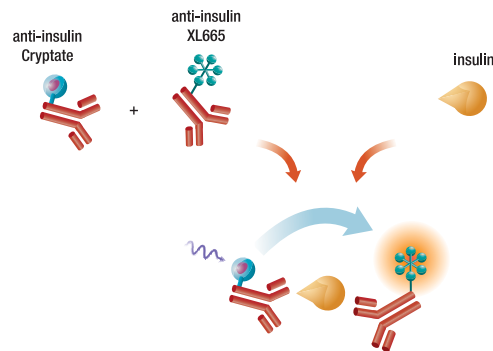
		384-well low volume plate (20 µL)
62INSPEC	Sensitive assay protocol	20,000 tests
	High Range protocol	4,440 tests

1. Assay description and intended use

This assay is intended to be used for the quantitative detection of rat or human insulin in buffered solution or cell-culture supernatants (for in-vitro research use only).

The assay involves two different anti-insulin antibodies, one being labelled with europium cryptate, and the other one with XL665.

When a sandwich is formed with insulin molecules, the two antibodies come into close proximity, allowing FRET to occur between the europium cryptate and the cross-linked allophycocyanin. This FRET phenomenon increases proportionally with the insulin concentration.



2. Background

Insulin, a polypeptidic hormone of 5800 Da, is synthesized from its precursors, proinsulin and proinsulin, in the β -cells of the islets of Langerhans. Within the β -cell granule proinsulin is converted by a process of enzymatic cleavage to insulin and C-peptide. Two endopeptidases, prohormone convertases 2 and 3 (PC2 and PC3), cleave the proinsulin molecule at two sites marked by pairs of dibasic amino-acids. The type –1 endopeptidase (PC3) cleaves at the Arg31-Arg32 site at the junction of the B/C chains of proinsulin, and the second endopeptidase (PC2) cleaves at the Lys64-Arg65 site, the proinsulin A/C junction. Finally, through the action of carboxypeptidase H there is a loss of basic amino-acids at each site to give the « des » forms of the partially processed proinsulins. When the cleavage at both sites is completed, C-peptide and insulin are produced.

Insulin possesses a wide spectrum of biological actions. It stimulates cellular glucose oxidation, glycogenesis, lipogenesis and proteogenesis, and plays a key role in the regulation of plasma glucose levels.

3. Reagent preparation and stability

3.1. Supplied reagents

Reagents should be allowed to warm up to room temperature for at least 30 mins before reconstitution.

Anti-insulin-Ab-cryptate	1 vial (lyophilized*)
Anti-insulin Ab-XL665	1 vial (lyophilized*)
Insulin standard	1 vial (lyophilized*)
Insulin control	1 vial (lyophilized*)
Reconstitution buffer (50 mM phosphate buffer, pH7.0, 0.8M KF, 0.2% BSA)	1 vial of 200 mL
Diluent (50 mM phosphate buffer, pH7.0, 0.2% BSA, 0.02% Na ₃ , preservatives)	1 vial of 20 mL

Note : Supplementary Insulin standard (ref 62INSCDA), Insulin control (ref 62INSTDA) and diluent (ref 62DL1DDD) can be obtained separately on request.

*** All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.**

Precaution: HTRF® reagent concentrations have been set for optimal assay performances. Note that any unforeseen dilution or improper use of the XL665 and Cryptate-conjugates will impair the assay's quality. For reagent reconstitution, please refer to attached protocols.

3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels. Once reconstituted, unused Cryptate and XL665 conjugates are stable up to two days at 4°C. They can be refrozen (at -80°C) and thawed once only. Insulin can be refrozen (at -20°C) and thawed once only.

4. Assay protocols

The insulin kit offers the possibility to accommodate two different assay protocols, a «Sensitive» and a «High Range» protocols described in appendix 1 and 2.

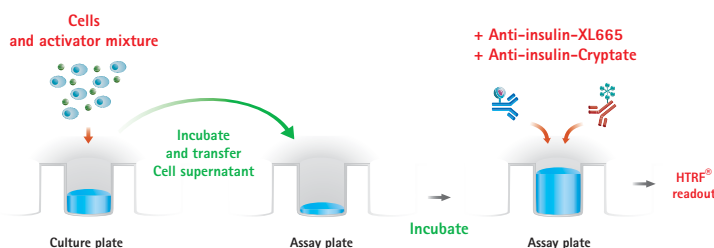
The choice of the protocol will depend on the nature of the samples (cell supernatants and serum samples or samples from glucose perfused pancreas).

The same kit is able to cover all the needs in terms of insulin quantitation, without the requirements for multiple dilutions.

The procedures for reagent reconstitution, standard curve preparation, and addition volumes are described for each assay in two separate attached sheets (refer to appendix 1 and 2).

5. Supernatant assay

The supernatant assay must be run in two distinct microplates, a culture plate for cell stimulation and an assay plate in which insulin detection is carried out.



5.1. Standard curve preparation

Depending on which protocol is chosen, standard curve preparation is detailed in §2.1. in each appendix.

Dilutions should however be carried out in cell-culture medium instead of kit diluent.

5.2. Assay protocol for 384-well low volume plate (20 µL total)

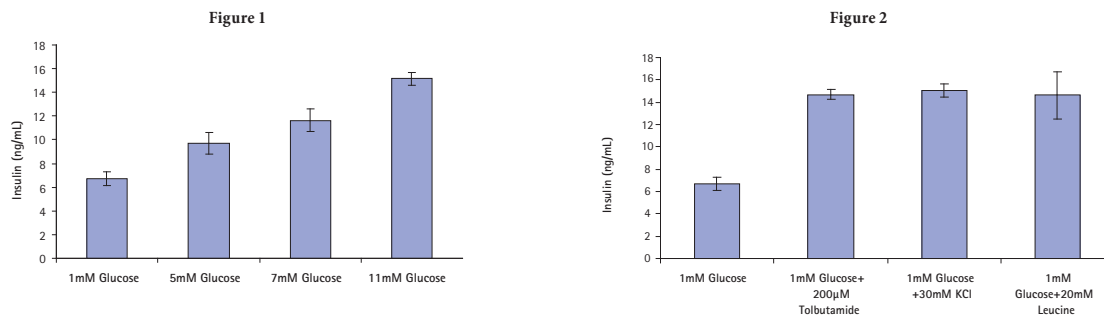
Depending on which protocol is chosen, dispensing steps are detailed in §2.3. in each appendix.

However, the negative control should include cell-culture medium instead of kit diluent for the first dispensing step.

5.3. Case study: insulin secretion in pancreatic β-cells INS-1E

INS-1E pancreatic β-cells were grown in complemented RPMI medium washed twice with KREBS-Ringer buffer and starved overnight in the same buffer. The same procedure of washing/ starving was applied again. Cells were finally washed with KREBS-Ringer buffer and insulin secretion was induced for 1h30 using increasing concentrations of glucose (Figure 1) or different insulinotropic molecules together with 1mM glucose in KREBS-Ringer buffer (Figure 2).

To assess insulin concentration, 10 µL of the different supernatants were transferred to a 384-well low volume plate, and insulin was measured by following the «Sensitive» assay protocol (see appendix 1).



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Appendix 1: Insulin «Sensitive» protocol

Document reference: 62INSPEC rev08 (July 008)

Packaging details:

384-well low volume plate (20 µL)		
62INSPEC	Sensitive assay protocol	20,000 tests

1. Reagent reconstitution and preparation

Reagents should be allowed to warm up to room temperature for at least 30 mins before reconstitution.

Supplied reagents	Working solutions
Anti-insulin Ab-cryptate** 1 vial (lyophilized*)	⇒ Reconstitute the lyophilized material with 5mL of distilled water. Mix gently. In a separate vial, make a further 1:20 dilution in reconstitution buffer (for example, mix 1mL of reconstituted reagent in 19 mL of reconstitution buffer).
Anti-insulin Ab-XL665 1 vial (lyophilized*)	⇒ Reconstitute the lyophilized material with 5 mL of distilled water. Mix gently. In a separate vial, make a further 1:20 dilution in reconstitution buffer (for example, mix 1mL of reconstituted reagent in 19 mL of reconstitution buffer).
Insulin standard 1 vial (lyophilized*)	⇒ See label indications for reconstitution volume. Mix gently. See standard curve preparation (§2.1.) for further dilution.
Insulin control 1 vial (lyophilized*)	⇒ Reconstitute with H ₂ O or cell culture medium (in case of cell supernatant assay). See label indications for reconstitution volume. Mix gently. In a separate vial make a further 1:10 dilution in the diluent or in cell culture medium (in case of cell supernatant assay).
Reconstitution buffer (1 vial of 200 mL) 50 mM Phosphate buffer, pH 7.0, 0.8M KF, 0.2 % BSA	
Diluent (1 vial of 20 mL) 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN ₃ , preservatives	

* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.

** The Ab-cryptate concentration was optimised for a maximum assay sensitivity and to ensure an average counting of 40,000 cps at 620 nm (384-well low volume plate) using the reference PHERAstar Plus reader (BMG Labtech).

HTRF® reagent concentrations have been set for optimal assay performances. Please note that any unforeseen dilution or improper use of the XL665 and cryptate antibodies will impair the assay's quality.

2. Assay protocol

2.1. Standard curve preparation

The dilution sequence shown in the table below should be followed to prepare the standard curve. Dilutions should be carried out with the kit diluent (or with cell culture medium in case of cell supernatant assay).

Standard	Initial concentration (ng/ mL)	Preparation
Standard 6	10	20 µL stock solution + 980 µL diluent*
Standard 5	5	300 µL standard 6 + 300 µL diluent*
Standard 4	2.5	300 µL standard 5 + 300 µL diluent*
Standard 3	1.25	300 µL standard 4 + 300 µL diluent*
Standard 2	0.625	300 µL standard 3 + 300 µL diluent*
Standard 1	0.312	300 µL standard 2 + 300 µL diluent*

*use culture medium instead of diluent for supernatant assay.

2.2. Sample preparation

All samples to be assayed should be diluted with the kit diluent (or with cell culture medium). Sample dilutions should fall in the 0-10 ng/mL range (initial concentration) of the standard curve.

2.3. Assay protocol for 384-well low volume plate (20 µL total)

Reagents should be dispensed in the following order:

- 10 µL insulin standard or sample*
- 5 µL anti-insulin Ab-cryptate
- 5 µL anti-insulin Ab-XL665

* For the negative control, the first reagent should be replaced by 10 µL of kit diluent or cell culture medium.

The plate should be covered with a plate sealer and incubation should take place 2 hrs at RT before readout on an HTRF® compatible reader (more information about compatible readers is available at www.htrf.com).

2.4. Assay flexibility and miniaturisation

When used as per instructions, the kit will provide sufficient reagents for 20,000 tests using a 384-well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

When moving to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted, in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final assay volume, half as much material per well is used, thereby allowing 2,000 tests to be run. The performances of the HTRF® assay will remain the same, whatever the level of miniaturisation.

Assay components	Volume proportion	Assay format		
		1536-well (10 µL)	384-well low volume (20 µL)	96 half-well (100 µL)
Sample	2 volumes	5 µL	10 µL	50 µL
Ab-XL665	1 volume	2.5 µL	5 µL	25 µL
Ab-cryptate	1 volume	2.5 µL	5 µL	25 µL
		40,000 tests	20,000 tests	4,000 tests

Plate references: 96 half-well plate (Costar #3694 or equivalent), 384-well low volume plate (Greiner #784076), 1536-well (Greiner #782086).

2.5. Data reduction and example of standard curve

[Insulin] ng/mL initial	665 nm	620 nm	Ratio	Mean ratio	CV%	DF %
0	1,828	47,148	388	387	0.35	
	1,805	46,787	386			
0.312	2,269	45,603	498	496	0.39	28
	2,401	48,526	495			
0.625	3,085	46,927	657	665	1.6	72
	3,166	47,080	672			
1.25	5,034	43,723	1,151	1,145	0.83	196
	5,774	48,107	1,138			
2.5	9,911	45,127	2,196	2,220	1.48	474
	10,002	44,596	2,243			
5	19,298	45,955	4,199	4,284	2.80	1,008
	19,411	44,430	4,369			
10	31,475	46,501	6,769	6,772	0.07	1,651
	29,999	44,276	6,775			

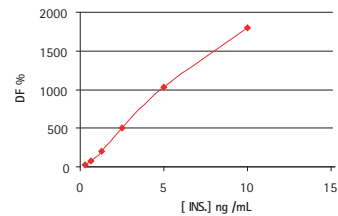
$$1. \text{Ratio} = \frac{A_{665\text{nm}}}{B_{620\text{nm}}} \times 10^4$$

$$2. \text{Mean Ratio} = \frac{\sum \text{ratios}}{2}$$

$$3. \text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$$

$$4. \text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

(Ratio_{neg} = negative control)



2.6. Analytical characteristics

Detection limit (dose of mean zero + 2SD)	Hook effect	Linear range
0.20 ng/mL	1250 ng/mL	Up to 10 ng/mL

The HTRF® Insulin assay was calibrated against the NIBSC international standard 66/304. The following equivalence was found:

1 ng Insulin HTRF® ↔ 1 ng Insulin NIBSC 66/304 (i.e.: 2.3x10⁻⁵ IU).

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Appendix 2: Insulin «High Range» protocol

Document reference: 62INSPEC rev08 (July 2008)

Packaging details:

384-well low volume plate (20 µL)		
62INSPEC	High Range protocol	4,440 tests

1. Reagent reconstitution and preparation

Reagents should be allowed to warm up to room temperature for at least 30 mins before reconstitution.

Supplied reagents	Working solutions
Anti-insulin Ab-cryptate** 1 vial (lyophilized*)	⇒ Reconstitute the lyophilized material with 5 mL of distilled water. Mix gently. In a separate vial, make a further 1:8 dilution in reconstitution buffer (for example, mix 1 mL of reconstituted reagent in 7 mL of reconstitution buffer).
Anti-insulin Ab-XL665 1 vial (lyophilized*)	⇒ Reconstitute the lyophilized material with 5 mL of distilled water. Mix gently. In a separate vial, make a further 1:8 dilution in reconstitution buffer (for example, mix 1 mL of reconstituted reagent in 7 mL of reconstitution buffer).
Insulin standard 1 vial (lyophilized*)	⇒ See label indications for reconstitution volume. Mix gently. See standard curve preparation (§2.1.) for further dilution.
Reconstitution buffer (1 vial of 200 mL) 50 mM Phosphate buffer, pH 7.0, 0.8M KF, 0.2 % BSA	
Insulin control 1 vial lyophilized	⇒ Reconstitute with H ₂ O or cell culture medium (in case of cell supernatant assay). (See label indications for reconstitution volume. Mix gently.)
Diluent (1 vial of 20 mL) 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN ₃ , preservatives	

* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.

** The Ab-cryptate concentration was optimised to allow a High Range assay. Therefore, 620 nm counts are much higher than in the sensitive assay, averaging around 250,000 cps (384-well low volume plate) using the reference PHERAstar Plus reader (BMG Labtech).

HTRF® reagent concentrations have been set for optimal assay performances. Please note that any unforeseen dilution or improper use of the XL665 and cryptate antibodies will impair the assay's quality.

2. Assay protocol

2.1. Standard curve preparation

The dilution sequence shown in the table below should be followed to prepare the standard curve. Dilutions should be carried out with the kit diluent (or with cell culture medium in case of cell supernatant assay).

Standard	Initial concentration (ng/mL)	Preparation
Standard 6	100	50 µL stock solution + 200 µL diluent*
Standard 5	50	30 µL standard 6 + 30 µL diluent*
Standard 4	25	30 µL standard 5 + 30 µL diluent*
Standard 3	12.5	30 µL standard 4 + 30 µL diluent*
Standard 2	6.25	30 µL standard 3 + 30 µL diluent*
Standard 1	3.12	30 µL standard 2 + 30 µL diluent*

*use culture medium instead of diluent for supernatant assay.

2.2. Sample preparation

All samples to be assayed should be diluted with the kit diluent (or with cell culture medium). Sample dilutions should fall in the 0-100 ng/mL range (initial concentration) of the standard curve.

2.3. Assay protocol for 384-well low volume plate (20 µL total)

Reagents should be dispensed in the following order:

- 2 µL insulin standard or sample*
- 9 µL anti-insulin Ab-cryptate
- 9 µL anti-insulin Ab-XL665

* For the negative control, the first reagent should be replaced by 2 µL of kit diluent or cell culture medium.

The plate should be covered with a plate sealer and incubation should take place 2 hrs at RT before readout on an HTRF® compatible reader (more information about compatible readers is available at www.htrf.com).

2.4. Assay flexibility and miniaturisation

When used as per instructions, the kit will provide sufficient reagents for 4,440 tests using a 384-well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

When moving to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted, in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final assay volume, half as much material per well is used, thereby allowing 440 tests to be run. The performances of the HTRF® assay will remain the same, whatever the level of miniaturisation.

Assay components	Volume proportion	Assay format		
		1536-well (10 µL)	384-well low volume (20 µL)	96 half-well (100 µL)
Sample	1 volume	1 µL	2 µL	10 µL
Ab-XL665	4.5 volume	4.5 µL	9 µL	45 µL
Ab-cryptate	4.5 volume	4.5 µL	9 µL	45 µL
		8,880 tests	4,440 tests	888 tests

Plate references: 96 half-well plate (Costar #3694 or equivalent), 384-well low volume plate (Greiner #784076), 1536-well (Greiner #782086).

2.5. Data reduction and example of standard curve

[Insulin] ng/mL initial	665 nm	620 nm	Ratio	Mean ratio	CV%	DF %
0	8711	207872	419	419	0.1	0
	9054	216453	418			
3.12	13752	242943	566	554	3.2	32
	12317	227509	541			
6.25	17360	250388	693	684	1.9	63
	17263	255779	675			
12.5	24914	243706	1022	1021	0.2	144
	24383	239212	1019			
25	41719	225473	1850	1812	3.0	333
	43394	244645	1774			
50	77810	229819	3386	3481	3.9	732
	91121	254721	3577			
100	180585	226195	7984	7725	4.7	1745
	166006	222350	7466			

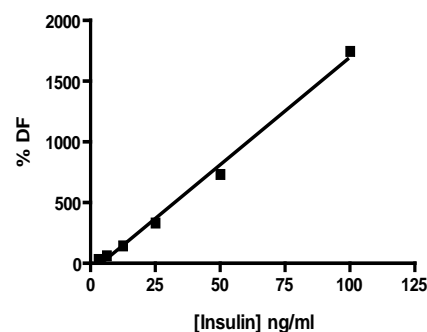
$$1. \text{ Ratio} = \frac{A_{665\text{nm}}}{B_{620\text{nm}}} \times 10^4$$

$$2. \text{ Mean Ratio} = \frac{\sum \text{ratios}}{2}$$

$$3. \text{ CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$$

$$4. \text{ Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

(Ratio_{neg} = negative control)



2.6. Analytical characteristics

Detection limit (dose of mean zero + 2SD)	Hook effect	Linear range
4 ng/mL	10 µg/mL	Up to 100 ng/mL

The HTRF® Insulin assay was calibrated against the NIBSC international standard 66/304. The following equivalence was found:

1 ng Insulin HTRF® ↔ 1 ng Insulin NIBSC 66/304 (i.e.: 2.3x10⁻⁰⁵ IU).