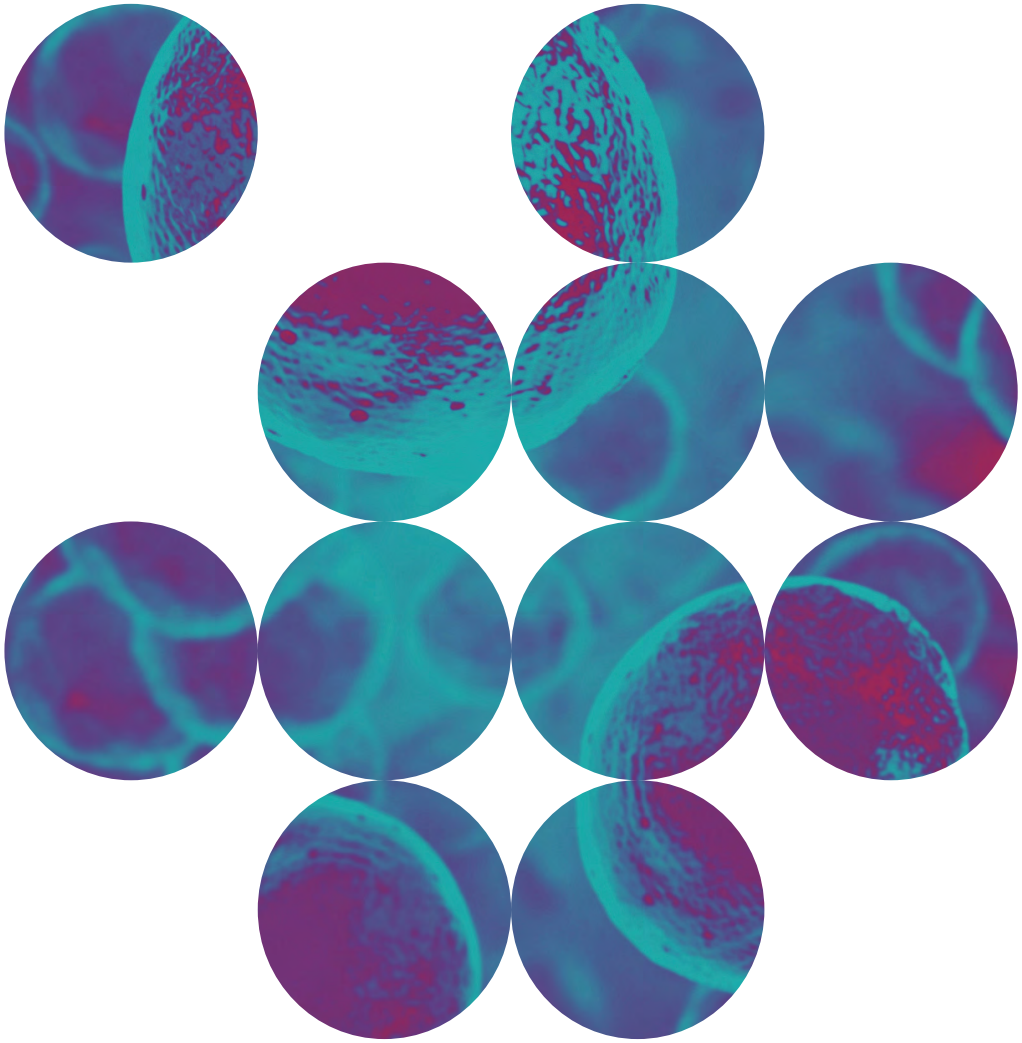
HUMAN CELL DESIGN
ACCELERATING DISCOVERY



USER GUIDE

ENDOC-βH5®

THE ULTIMATE HUMAN
PANCREATIC BETA CELL MODEL

NOTES

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EndoC-βH5® cells are for life science research use only and are subject to the use restrictions contained in the license agreement or the material transfer agreement. You are responsible for understanding and performing the protocols described within. Human Cell Design does not guarantee any results you may achieve. These protocols are provided as Human Cell Design's recommendations based on its own use and experience with EndoC-βH5® cells.

ORIGIN

EndoC-βH5® cells are manufactured in France.

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REVISION HISTORY

Version 1.0, February 2021.

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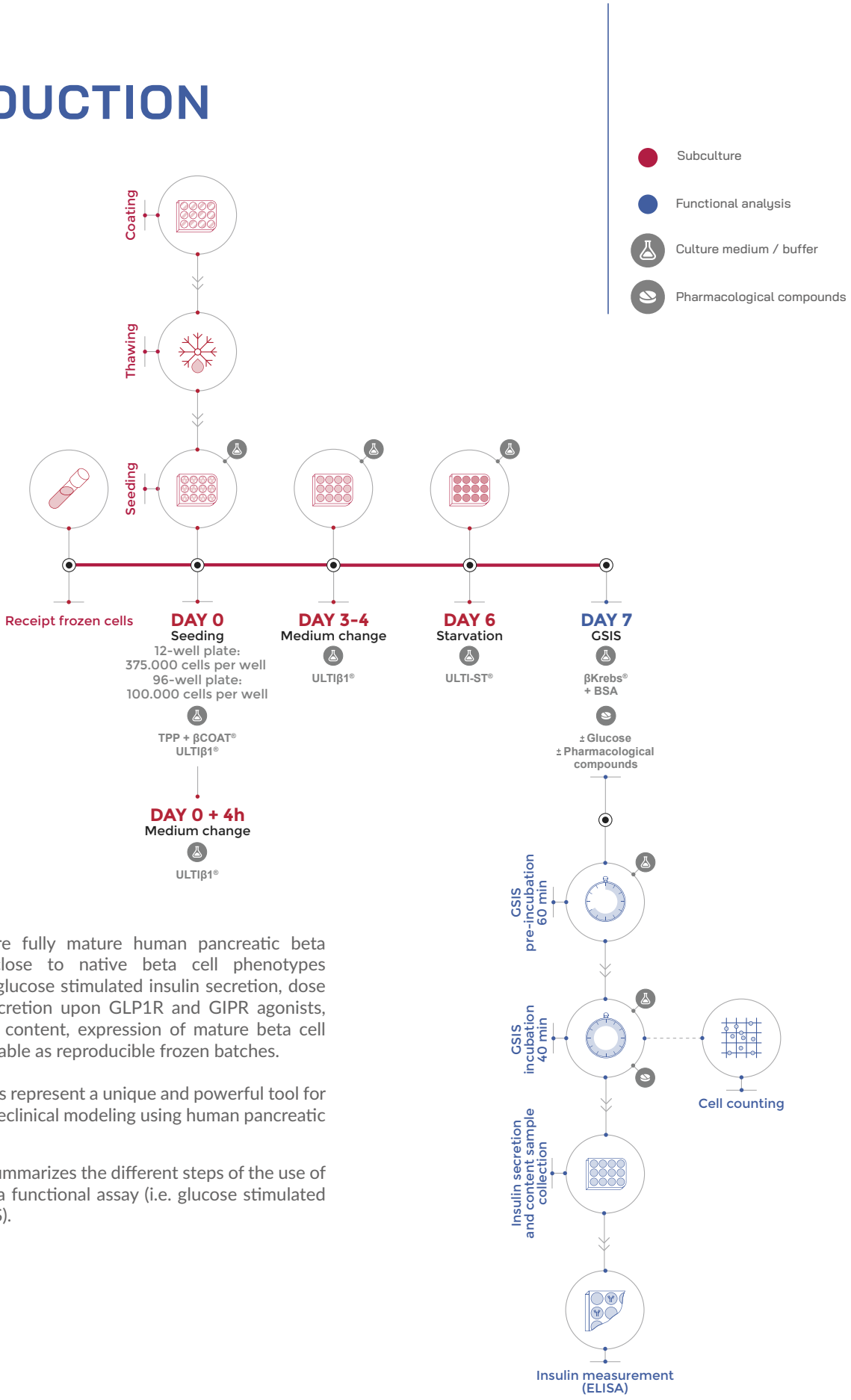
INTRODUCTION



EndoC-βH5® cells are fully mature human pancreatic beta cells that exhibit close to native beta cell phenotypes (i.e. dose dependent glucose stimulated insulin secretion, dose dependent insulin secretion upon GLP1R and GIPR agonists, elevated total insulin content, expression of mature beta cell markers) and are available as reproducible frozen batches.

EndoC-βH5® cells thus represent a unique and powerful tool for drug discovery and preclinical modeling using human pancreatic beta cells.

The diagram beside summarizes the different steps of the use of EndoC-βH5® cells in a functional assay (i.e. glucose stimulated insulin secretion, GSIS).

02
REQUIRED MATERIALS AND PRODUCTS

I - COMPONENTS SUPPLIED BY HUMAN CELL DESIGN

Upon receipt, immediately transfer cryovials to a liquid or vapour phase nitrogen storage tank.

Cell line	Cryopreserved EndoC-βH5® cells
Fraction	Cryovial 1 mL
Characterization	see Batch Release Certificate
Shipping	Dry ice
Storage	Nitrogen

It is critical to avoid temperature variations when handling cryopreserved EndoC-βH5® cells. When transferring vials to nitrogen storage tank, restrict exposure to room temperature to a minimum.

II - COMPONENTS TO BE ORDERED SEPARATELY

Upon receipt, immediately proceed to appropriate storage of media, protected from light.

Component	Use	Size	Provider	Reference	Storage
ULTIβ1®	Complete culture medium	100 mL	Human Cell Design	ULTIβ1®	-80 °C
βCOAT®	Coating Matrix aliquots	80-130 µL	Human Cell Design	βCOAT®	-80 °C
ULTI-ST®	Medium for starvation	50 mL	Human Cell Design	ULTI-ST®	-80 °C
βKREBS®	Buffer for GSIS	250 mL	Human Cell Design	βKREBS®	+4 °C

Once thawed, these products must be used or discarded, but never refrozen.

III - REQUIRED EQUIPMENT

Equipment	Provider	Recommendations
Water bath (37 °C)	Multiple	-
Laminar flow hood (class II safety cabinet)	Multiple	-
Centrifuge	Multiple	Refrigerated
Phase-contrast microscope with camera	Multiple	-
Material for cell counting (hemocytometer or automated cell counter)	Multiple	Ability to count cell aggregates
Pipet-aid, pipettes and micropipettes	Multiple	-
Timer	Multiple	hh:mm:ss

IV - REQUIRED CONSUMABLES AND REAGENTS

The following product references are those used at Human Cell Design and provide excellent results in experiments using EndoC-βH5® cells. In particular, the use of the following plastic culture dishes is highly recommended.

Cell Culture consumables	Reference used at UB	Other references
Sterile polypropylene tubes (15 & 50 mL)	Multiple	-
Microtubes (1.5 mL)	Multiple	-
Multi-well cell culture plates	TPP (exclusively used at Human Cell Design)	Other references that appear compatible but are not fully validated : - PureCoat™, CellBIND®, Corning; - Cell+, Yellow, Sarstedt.

Use of TPP plastic plates and Mercodia human insulin ELISA kit is recommended as they are the only plates and ELISA kit that have been validated for the use of EndoC-βH5® cells in functional assays so far.

Reagents	Provider	Reference	Storage
DMEM (1X) High glucose (4.5 g/L)	ThermoFisher Scientific	41965-039	4 °C
P/S Penicillin/Streptomycin (100X solution)	ThermoFisher Scientific	15140-122	-20 °C
Trypsin 0.05%, EDTA 0.02%	ThermoFisher Scientific	25300054	-20 °C
DPBS$^-$ 1X (w/o Ca^{2+} and Mg^{2+})	ThermoFisher Scientific	14190094	RT
Fetal Bovin Serum (FBS, US origin)	Sigma-Aldrich	F2442	-20 °C
IBMX	Sigma-Aldrich	I-5879	-20 °C
DMSO	Sigma-Aldrich	D2650-100ML	RT
D-(+)-glucose	Sigma-Aldrich	G-8270	RT
BSA Fraction V FFA	Sigma-Aldrich	10775835001	4 °C
NaCl	Sigma-Aldrich	S9888	RT
Tris pH 8.0	ThermoFisher Scientific	BP1758-500	RT
Triton X-100 (diluted 10% in sterile water)	Sigma-Aldrich	X-100	RT
Glycerol	Sigma-Aldrich	10675872	RT
EGTA	Sigma-Aldrich	E-4378	RT
Complete™ Mini, EDTA-free Protease Inhibitor Cocktail (Anti-protease tablets)	Sigma-Aldrich	04693159001	4 °C
Human insulin Kit	Mercodia	10-1113-01	4 °C
Sample dilution buffer	Mercodia	10-1195-01	4 °C
Exendin-4	Sigma-Aldrich	F7144	-20 °C

03

SEEDING OF ENDOC-βH5® FOR FUNCTIONAL ANALYSIS

The following protocols present 1) thawing and seeding of EndoC-bH5® cells and 2) their use in a glucose stimulated insulin secretion (GSIS) assay. We describe experiments performed in 12-well and 96-well plates as 12-well setting is slightly easier to set up and can be used as a positive control for new users whereas 96-well setting allows the most efficient use of your EndoC-bH5® cells. In general, please reduce mechanical stress to a minimum when collecting thawed cells from cryovials, resuspending them after centrifugation or homogenizing suspension for seeding by using recommended material and handling recommendations.

Regarding medium and buffer changes during culture and functional assays using EndoC-bH5® cells, it is highly recommended, in the 96-well setting and at all steps, to:
- Remove old medium/buffer using a multichannel pipette and avoid vacuum aspiration,
- Distribute fresh medium/buffer using a multichannel pipette, electronic pipette with combitip set up to minimal speed or micropipette.

In the 12-well setting:
- Old medium/buffer can be removed using adapted pipette or vacuum aspiration,
- While distribution of fresh medium/buffer can be done using a multichannel pipette, an electronic pipette with combitip set up to minimal speed or a micropipette.

To avoid any contamination, the following steps are to be performed in a laminar flow hood using appropriate sterile techniques.

I - THAWING CULTURE MEDIA

ULTIβ1® and ULTI-ST® bottles have to be thawed at 4 °C overnight at least one day before use. Protect from light while thawing and waiting to be used.

Once thawed, ULTIβ1® and ULTI-ST® media are kept at 4 °C and can be used for a maximum of 14 days.

II - COATING OF MULTI-WELL PLATES (ALWAYS MADE FRESHLY)

During coating medium preparation, strictly maintain all reagents at 4°C.

1 | Transfer a βCOAT® aliquot from -80 °C freezer to a container with ice or a refrigerated rack.

2 | Wait until aliquot is completely thawed (typically a few minutes).

3 | Prepare 9.9 mL of cold (4 °C) DMEM 4.5 g/L glucose in a 15 mL sterile tube.

4 | Add 100 µL of Penicillin/Streptomycin (P/S) 100X solution to the DMEM medium and mix.

5 | Add 300 µL of this medium to the βCOAT® vial.

6 | Pipet slowly up and down to mix βCOAT® and the medium.

Since βCOAT® is very viscous, so fill the pipette tip slowly to avoid the formation of bubbles and completely empty the tube. Slowly empty the tip and rinse it with the DMEM P/S solution.

7 | Transfer into the remaining 9.7 mL of DMEM/P/S solution.

8 | Homogenize by pipetting up and down.

9 | Add coating solution to culture plates.

Never let βCOAT® stand at temperature above 4 °C to avoid any polymerization before use.

Culture plate	Coating medium / well
12-well plate	500 µL
96-well plate	100 µL

10 | Gently tilt the culture plates to homogeneously spread coating solution over the entire surface of the wells. Make sure that the coating solution covers the whole surface of each well.

11 | Place culture plates in an incubator at 37 °C.

12 | Incubate for minimum 1 hour and maximum 6 hours.

13 | Just before seeding the cells, remove coating solution.

Do not store unused coated plates at 4 °C for later use. Discard unused plates or plates presenting inhomogeneous coating or dry areas. Coating solution is removed from the wells right before seeding. Do not let coating surface dry before adding cell suspension.

DAY 0

III - SEEDING OF ENDOC-βH5® CELLS

Carefully plan your experiment, especially the number of vials that will be thawed in parallel, before proceeding to thawing. Thawing is a critical step in the use of EndoC-βH5® cells in functional studies. Failure to respect recommendations will compromise EndoC-βH5® cell function and their responses in various assays.

1 | Prepare three aliquots of ULTIβ1® medium in sterile conical tubes and let them warm to room temperature before use (at least 20 minutes):

a | an aliquot of 2mL (per vial that will be thawed) to harvest thawed cells,

b | an aliquot of 7 mL (per vial that will be thawed) to collect thawed cells,

c | an aliquot of medium to seed the cells (volume dependent on the amount of cells and density of seeding).

Recommended density of platting of EndoC-βH5® cells for GSIS assay is 375.000 cells in 1mL of medium per well in 12-well plates and 100.000 cells in 100µL of medium per well in 96-well plates.

Culture plate	Medium volume	Number of cells
12-well plate	1 mL	375.000
96-well plate	100 µL	100.000

2 | Remove the cryovials from their liquid nitrogen storage and transfer to the culture facility on ice or using a refrigerated rack. Under a laminar flow hood, slightly open their cap in order to release pressure. Close the vials and transfer them to a 37 °C water-bath. Thaw cells for approximately 1-2 min.

Thawing is critical for the quality of EndoC-βH5® cells. Following temperature and handling protocols is critical. Noncompliance to these protocols may irreversibly damage the cells and dramatically compromise the experiment. In order to avoid contamination, make sure water level does not reach the cap of the vial. Do not completely thaw the cell suspension before adding medium.

3 | Remove the cryovials from the water-bath before the whole cell suspension has thawed.

4 | Wipe the cryovials with 70 % ethanol in order to reduce risk of contamination, and carefully open them under the laminar flow hood.

5 | Take 1 mL from the first ULTIβ1® aliquot (2mL/vial aliquot) and slowly add it to the first cryovial.

6 | Gently transfer the cell suspension into the tube containing the second ULTIβ1® aliquot (7mL/vial).

7 | Wash the cryovial once by repeating step 5 and 6.

8 | Proceed with next cryovial. It is critical that every vial is treated before it has completely thawed.

In order to guarantee quality of the cells, it is critical to proceed quickly with thawing in order to reduce exposure to DMSO. It is, however, required to proceed gently as EndoC-βH5® cells are sensitive to mechanical stress induced by harsh pipetting. For these reasons, it is highly recommended to carefully evaluate the number of cryovials that can be thawed together.

9 | Centrifuge the cell suspension for 5 min at 500 x g at room temperature.

10 | Discard the supernatant and add 1 mL ULTIβ1® medium taken from the third aliquot.

11 | Resuspend the pellet and break-up cell aggregates with gentle pipetting. Resuspension should only take 2 to 3 pipetting movements.

12 | Add 1 to 3 mL ULTIβ1® medium per thawed vial and gently homogenize using adapted pipette (2-3 pipetting).

13 | Count live cells using the method available in your laboratory (e.g., hemocytometer or electronic cell counter).

Calibration parameters for electronic cell counters are: min size - 5 µm, max size - 20 µm, circularity - 60 %.

14 | Prepare cell suspension for platting following the table below. Homogenize by inverting the tube or with gentle pipetting.

Culture plate	Concentration of cell suspension	Volume of cell suspension per well
12-well plate	375.000 cells/ml	1 mL
96-well plate	1.000.000 cells/mL	100 µL

15 | Remove coating medium from the wells.

16 | Transfer cell suspension to the wells. If further homogenization is needed, proceed by inverting the tube.

17 | It is recommended to treat a maximum of 6 wells at a time in order to prevent drying of the coated surface. If you are using an electronic pipette with combitip, which allows fast and reproducible distribution of the cells, it is possible however to seed 12 wells at a time. It is also possible to use a multichannel pipette to distribute the cells in the 96-well setting.

18 | If 12-well plates are used, gently shake the plates back-and-forth and side-to-side in order to evenly distribute the cells. Carefully place the plate in an incubator at 37 °C, 5 % CO_2 and saturated humidity. Gently shake the plate back-and-forth and side-to-side again.

19 | If 96-well plates are used, directly place the plates in the incubator while avoiding swirling or shaking them, in order to do not disturb the uniform platting of the cells.

20 | After 4 h, check cell adhesion under a microscope and replace medium with fresh ULTIβ1® medium. If using 96-well setting, it is strongly recommended to use a multi-channel pipette to remove old medium and avoid vaccum aspiration.

21 | Perform microscopic observation regularly to verify correct adhesion and distribution of the cells. Refer to figure 1A/B on page 15 to evaluate morphology of EndoC-βH5® cells at later time point (day 6).

Make sure to gently handle the cells during the entire thawing/seeding process (homogenization, pellet resuspension, cell suspension distribution) as EndoC-βH5® cells are sensitive to mechanical stress and damaging them can greatly impair functional results.

It is critical that the amount of cells that is platted is highly reproducible from well to well.

It is recommend to verify even distribution of the cells under a microscope.

DAY 3 OR DAY 4

IV - ULTIβ1® MEDIUM CHANGE

1 | Warm ULTIβ1® medium to room temperature in a sterile container.

2 | Proceed to medium change, gently removing old medium and adding fresh medium.

3 | Place plate back to incubator.

It is highly critical not to disturb the cell layer at this step. For this reason, it is very highly recommended to use a micropipette or multichannel pipette rather than vacuum pump to remove old medium, especially in the 96-well setting.

DAY 6

V - STARVATION - MEDIUM CHANGE TO ULTI-ST®

One day before starvation, place ULTI-ST® at 4 °C in order to allow for slow thawing.

1 | Warm ULTI-ST® medium to room temperature in a sterile container.

2 | Proceed to medium change, gently removing old medium and adding fresh medium.

3 | Place plate back to incubator.

It is highly critical not to disturb the cell layer at this step. For this reason, it is very highly recommended to use a micropipette or multichannel pipette rather than vacuum pump to remove old medium, especially in the 96-well setting. Starvation must be performed 24h before beginning GSIS assay.

Once ULTI-ST® is thawed, it can be stored at 4 °C for 14 days maximum.

04

PROTOCOL FOR GLUCOSE STIMULATED INSULIN SECRETION (GSIS)

In this chapter is presented a GSIS experimental design example using stimulation conditions known to work on EndoC-βH5® cells and that can thus be used as control conditions for your EndoC-βH5® cells. GSIS is performed here in 12-well plates. For GSIS in 96-wells, use 100 µL media and buffers per well.

I - PREPARATION OF STOCK SOLUTIONS FOR GSIS

The following solutions can be prepared in advance.

1 | Prepare aliquots of 1 M Glucose dissolved in sterile water.

2 | Prepare aliquots of 45 mM IBMX dissolved in DMSO.

3 | Prepare aliquots of 10 µM Exendin-4 dissolved in H20/1%BSA.

4 | Prepare TETG solution (anti-protease free) by adding reagents in the following order:

Reagent	H_2O	5 M NaCl	0.2 M EGTA	1M Tris pH 8.0	Glycerol	Triton X-100 10 % in water	H_2O
Volume	35 mL	1.37 mL	500 µL	1 mL	5 mL	5 mL	complete to 50 mL

5 | If you need to count cells at the end of the assay in order to express results per number of cells, prepare trypsin neutralization solution as follow:

a | Inactivation of fetal bovine serum (FBS)
Heat inactivate FBS for 30 min at 56 °C and store aliquots at -20 °C.

b | Preparation of 100 mL of trypsin neutralization solution
Mix 80 mL DPBS 1X and 20 mL inactivated FBS. Filter (0.22 µm). Store at 4 °C and use up to a maximum of 30 days.

⚠ To avoid any contamination, the following steps are to be performed in a laminar flow hood using appropriate sterile techniques.

Store at -20 °C for 12 months maximum.

Store at -20 °C for 3 months maximum. Discard thawed aliquots.

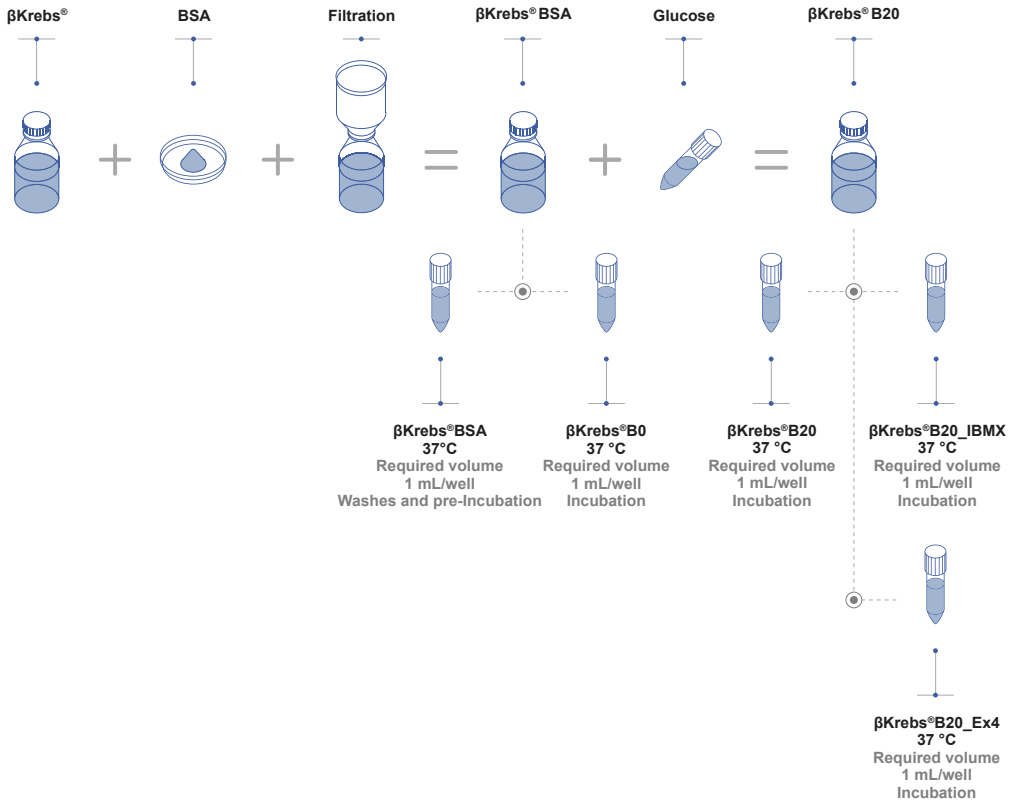
TETG solution (anti-protease free) can be stored at room temperature for 3 months.

Incubation time starts when entire volume of FBS reaches 56°C.

II - PREPARATION OF SOLUTIONS ON THE DAY OF GSIS

Seven days after seeding the cells, calculate the required volume of GSIS solutions and proceed to their preparation extemporaneously and as described below.
GSIS conditions below (βKrebs_B0 = no glucose; βKrebs_B20 = 20 mM glucose; βKrebs_IBMX = 45 µM IBMX; βKrebs_Ex4 = 1 nM Exendin-4) are given as examples. Please note that βKrebs®BSA and βKrebs®B0 are the same solution.

Solutions for GSIS must be prepared on the day of GSIS then discarded.



It is highly recommended to:
- use multichannel pipette to remove old medium/buffer in the 96-well setting,
- use multichannel or electronic pipette with combitip set up to minimal steep to rapidly and reproducibly distribute fresh medium/buffer.

βKREBS®BSA	
Reagent	**Quantity**
BSA	0,10 g
βKREBS®	100 mL

1. Place βKREBS® in a sterile bottle and add BSA.
2. Allow dissolution of BSA at least 15 min at 4°C without any stirring.
3. Filter at 0,22 µm.
4. Transfer volume required for washes and preincubation to a tube.
5. Place it in a water-bath at 37 °C.

βKREBS®B0

1. Place another aliquot of βKrebs®BSA in a water-bath at 37 °C.

Buffers for stimulation step are prepared and placed in a water bath at 37 °C during pre-incubation step.

βKREBS®B20	
Reagent	**Quantity**
Glucose 1 M	1 mL
βKREBS®BSA	49 mL

1. Transfer the appropriate volume of βKrebs®BSA.
2. Add the required volume of 1 M Glucose (20 mM is the final concentration used in this example).
2. Proceed to gentle stirring.
3. Divide into three aliquots.
4. Place the aliquots in a water-bath at 37 °C.

βKREBS®B20_IBMX	
Reagent	**Quantity**
IBMX 45 mM	10 µL
βKREBS®B20	10 mL

1. Just before delivering it to the cells, add the required volume of 45 mM IBMX to the corresponding aliquot of βKrebs®B20.
2. Proceed to gentle stirring.

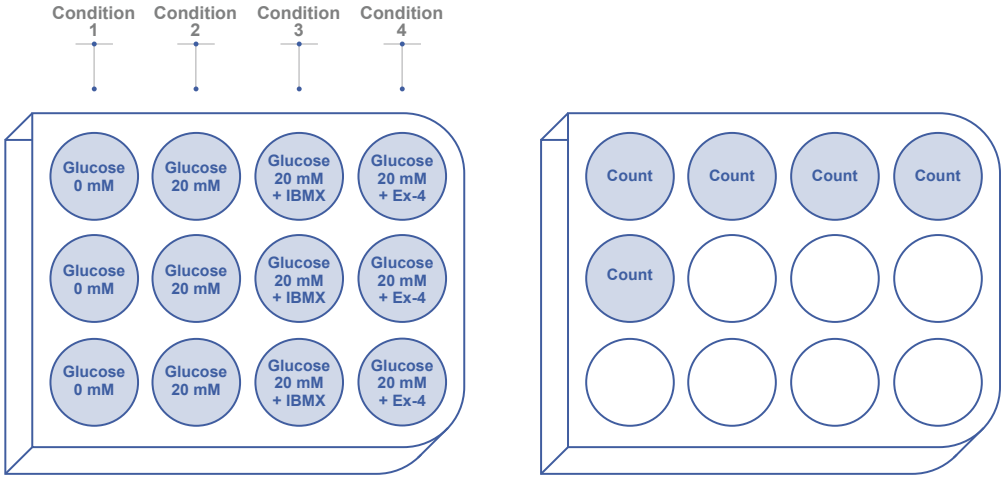
βKREBS®B0_Ex4	
Reagent	**Quantity**
Exendin-4 10 µM	1 µL
βKREBS®B20	10 mL

1. Proceed to pre-dilution of Exendin-4 in cold βKrebs®BSA (example 1/100).
2. Store at 4 °C.
3. Just before delivering it into the wells, add the required volume of pre-diluted Exendin-4 to the corresponding aliquot of βKrebs®B20.
4. Proceed to gentle stirring.

Volumes are given as examples. All of these five solutions must be discarded at the end of the experiment.

III - GLUCOSE STIMULATED INSULIN SECRETION ASSAY

Seven days after seeding, proceed with GSIS.
Example of template for an experimental design testing 4 conditions:



SECRETION PLATE COUNTING

For the insulin secretion part of the assay, only treat plates dedicated to the secretion assay.
It is recommended to treat a maximum of 3 to 6 wells at a time in order to prevent cells from drying (unless you are using an electronic pipet with combitip set up to slow mode, 12 wells at a time).

1 | Place βKrebs®BSA in a water-bath at 37 °C for 15 min.

2 | After 24 h of starvation, remove ULTI-ST®.

3 | Wash with 1 mL/well of βKrebs®BSA. Repeat once.

4 | Deliver 1 mL/well of βKrebs®BSA. Start the timer (60 min) after treating the last well of the last plate.

5 | Place secretion plate(s) in an incubator at 37 °C, 5 % CO_2 and saturated humidity for 60 min.

It is usually possible to proceed to cell counting from the dedicated plate during this incubation.

6 | Meanwhile, prepare lysis solution by adding 1 tablet of protease inhibitor / 10 mL TETG solution and keep it at 4 °C until the end of the experiment.

7 | Prepare the other incubation buffers without adding pharmacological compounds, aliquot them then place them in a water bath at 37 °C. For example :
- βKrebs®B0 = 0mM Glucose, already ready since it corresponds to βKrebs®BSA,
- βKrebs®B20 = 20 mM Glucose,
- βKrebs®B20 + IBMX 45 µM,
- βKrebs®B20 + Exendin-4 1 nM.

It is highly recommended to add Exendin-4 to warm βKrebs®B20 solution just before to add it to the cells.

8 | After 60 min of pre-incubation, proceed with stimulation.

9 | Homogenize the solutions and treat the cells with 1 mL/well of βKrebs®B0, βKrebs®B20, βKrebs®B20_IBMX and βKrebs®B20_Ex4 (or βKrebs® + pharmacological compounds following your plate template).

10 | Start the timer (40 min) while treating the first well of the first plate.

11 | Place the plate(s) in an incubator at 37 °C, 5 % CO_2 and saturated humidity for 40 min.

12 | Meanwhile, prepare two 1.5 mL microtubes per well (or two deepwell plates) for sample collection.

It is recommended to cool down the microtubes in a -20°C freezer or on ice.

13 | After 40 min of incubation, carefully collect 800 µL of supernatant from each well and transfer to microtubes or deepwell plates. Avoid disrupting the cell layer and change tip between wells.

14 | Store these samples at 4 °C until centrifugation.

These samples are dedicated to the measurement of insulin secretion.

15 | Remove remaining medium from each well.

16 | Deliver 1 mL/well of cold lysis solution and incubate for minimum 2 min at room temperature.

17 | Check cell lysis under microscope. Wait maximum 5 more minutes if lysis is not complete.

18 | Pipet cell lysate up and down to complete lysis, then collect to microtubes or a deepwell plate.

These samples are dedicated to the measurement of insulin cell content.

19 | Place these samples at 4 °C until centrifugation.

20 | Centrifuge all samples (incubation medium and lysate) at 700 x g, 5 min at 4 °C.

Centrifugation at low temperature is important to prevent insulin degradation.

21 | Transfer 300 µL from each sample to new microtubes or deepwell plates.

22 | Store samples according to the following recommendation:

If it is planned to repeat measures, make sample aliquots in order to avoid thawing/freezing.

ELISA performed on the same day	Store at 4 °C
ELISA performed later	Store at -20 °C for 4 weeks maximum. Thaw at 4 °C.

Thawing samples stored in deepwell plates may take several hours.

IV - CELL COUNTING

Cell count can be performed during the 60 min pre-incubation or the 40 min incubation.

1 | Remove ULTI-ST® medium from each well.

2 | Wash the cells with 1X DPBS once.

3 | Add trypsin/EDTA (500 µL/well) and place the cells in an incubator at 37 °C, 5 % CO_2 for 3 min.

If cells are not completely detached, put dishes back to the incubator for 2 more minutes. Detachment can be helped by gently tapping the plates.

4 | Check for complete cell detachment by examining under microscope.

5 | Add neutralizing solution in order to inhibit trypsin (volume to volume).

Minimize contact time with neutralizing solution since it may decrease cell survival.

6 | Harvest the cells in each well: tilt the plate, pipet cell suspension in order to collect the cells that are still attached, and cautiously break up cellular aggregates by pipetting up and down.

7 | Count cells in each well individually.

8 | Assess the number of live and dead cells, and calculate proliferation index.

Your notes

05

INSULIN CONCENTRATION MEASUREMENT AND ANALYSIS

Insulin concentration is measured using the ELISA method. It is recommended to use Mercodia's Insulin ELISA kit (10-1113-01, cf. chapter 02-III) and to perform analysis following manufacturer's recommendations.

I - ELISA MEASUREMENT

1 | Thaw samples at 4°C.

Thawing of samples stored in deep well plates may take several hours.

2 | Homogenize then dilute samples using Mercodia' dilution buffer (10-1195-01).

3 | Proceed with ELISA according to manufacturer's recommendations.

For GSIS ran in 12 well plates using 375.000 cells per well, 1mL buffer volume and 1mL lysis buffer volume (cf. chapter 05-V) recommended sample dilutions are: insulin content, 1/3000 - 1/5000; basal secretion, 1/20 - 1/50; 20mM Glucose secretion, 1/50 - 1/100; 20mM Glucose + pharmacological compound, 1/100 - 1/250.
These dilution factors, however, are indicative and must be evaluated by the operator for each independent experiment.

II - ANALYSIS

1 | Calculate insulin concentration in secretion samples (ng/40 min/million cells or ng/hour/million cells).

2 | Calculate insulin concentration in content samples (ng/million cells).

3 | Calculate cellular capacity to secrete insulin (%). It corresponds to the proportion of total insulin that is secreted.

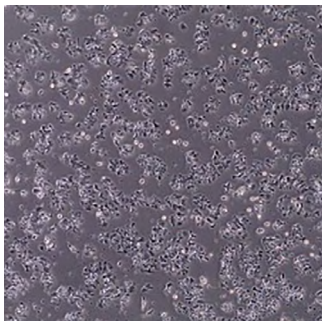
Your notes

06
ENDOC-βH5® CHARACTERISTICS

I - CELL MORPHOLOGY

Morphology of EndoC-βH5® cells 6 days after thawing and seeding which corresponds to the recommended time point for initiating starvation before GSIS assay. EndoC-βH5® cells form small adherent clusters of functional pancreatic beta cells.

6 days after thawing, 10x magnification **1A**



6 days after thawing, 20x magnification **1B**

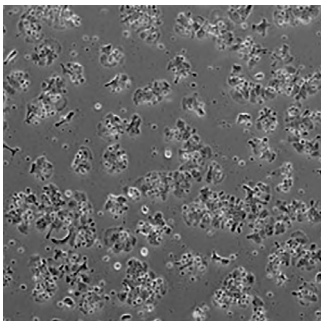


Figure 1
EndoC-βH5® cell morphology 6 days after thawing (12-well plate, 375.000 cells per well). Pictures at x10 and x20 magnification are shown.

II - PHENOTYPIC ANALYSIS BY FLOW CYTOMETRY

EndoC-βH5® cells form an homogenous population of functional human pancreatic beta cells expressing markers of mature human beta cells among which Insulin (>99% insulin high cells) as well as PDX1 and NKX6.1 (>90% expressing cells) (Figure 2).

2

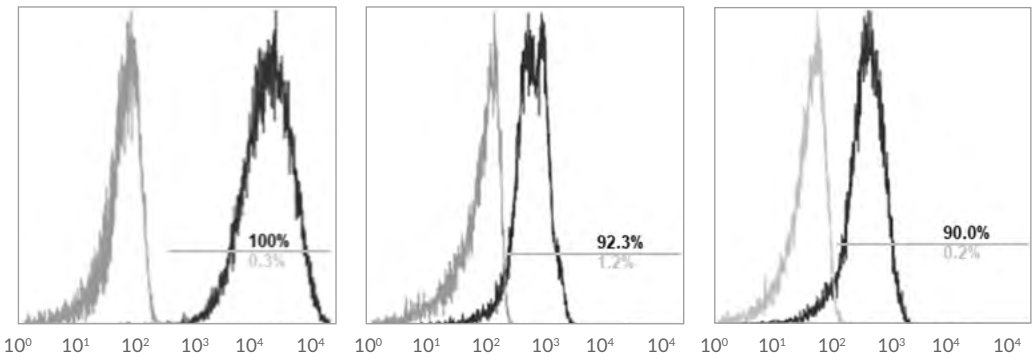


Figure 2
Flow cytometry analysis in EndoC-βH5® cells showing expression of Insulin, PDX1 and NKX6.1.

III - GLUCOSE STIMULATED INSULIN SECRETION

EndoC-βH5® cells secrete insulin very efficiently when stimulated with 20mM Glucose (Figure 3).
Insulin secretion is further potentiated by low concentration of GLP1R agonist Exendin-4 (1nM) (Figure 3).

3

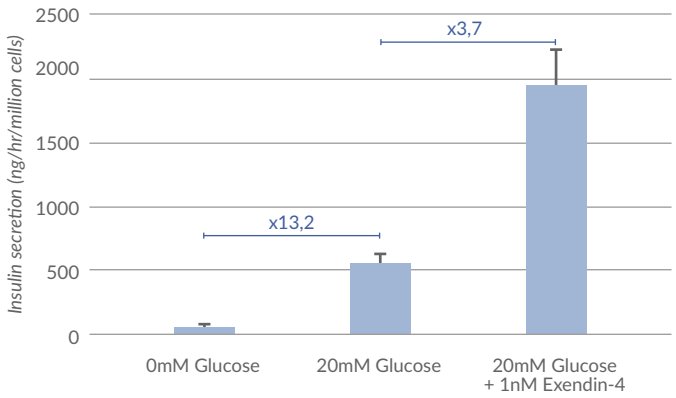


Figure 3
Average insulin secretion in response to Glucose (20mM) and Exendin-4 (1nM) in EndoC-βH5® cells. Results are expressed as ng secreted insulin/hour/million cells.

EndoC-βH5® cells respond to physiological concentrations of glucose in a dose dependent manner and with maximum increase in insulin secretion between 5,5 and 11mM Glucose (Figure 4).

4

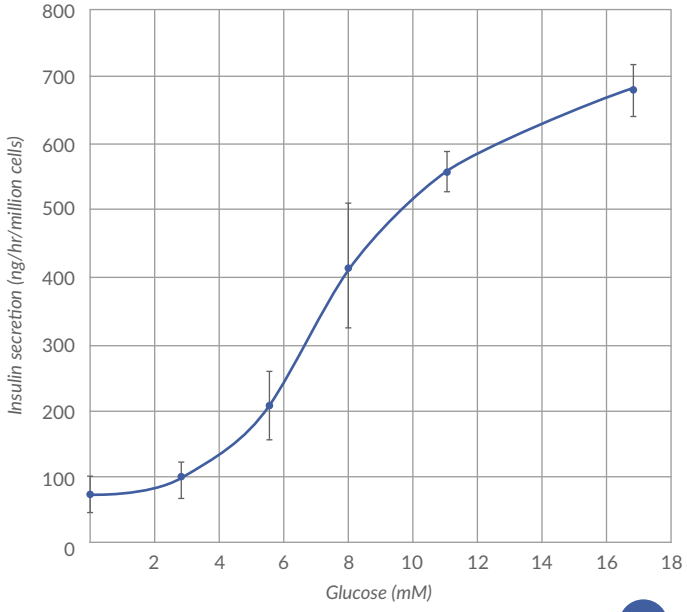


Figure 4
Glucose dose response in EndoC-βH5® cells (12-well plate, 375.000 cells per well). Results are expressed as ng secreted insulin/hour/million cells.

EndoC-βH5® cells dose dependently respond to incretin analogs Exendin-4, an agonist of GLP1R (Figure 5), and [D-Ala^2]-GIP, an agonist of GIPR (Figure 6).

5

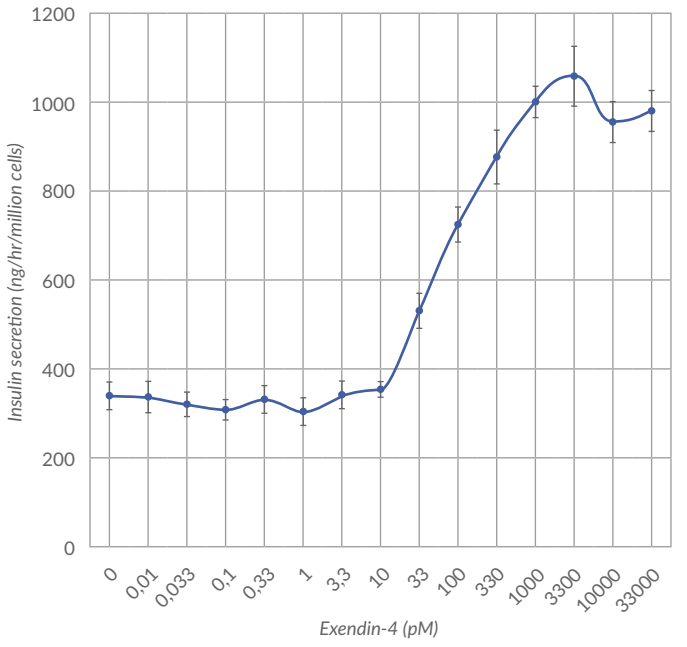


Figure 5
Exendin-4 dose response in EndoC-βH5® cells. Results are expressed as ng secreted insulin/hour/million cells (96-well plate, 100.000 cells per well).

11mM Glucose

6

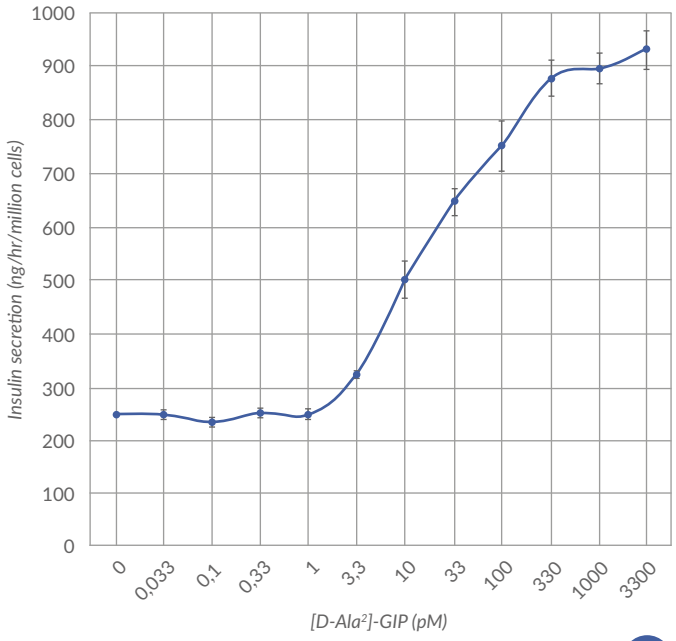


Figure 6
[D-Ala^2]-GIP dose response in EndoC-βH5® cells. Results are expressed as ng secreted insulin/hour/million cells (12-well plate, 375.000 cells per well).
11mM Glucose

GSIS in EndoC-βH5® cells can be performed in 96-well plates, thus reducing the required amount of cells for a given experiment (Figure 7).

7

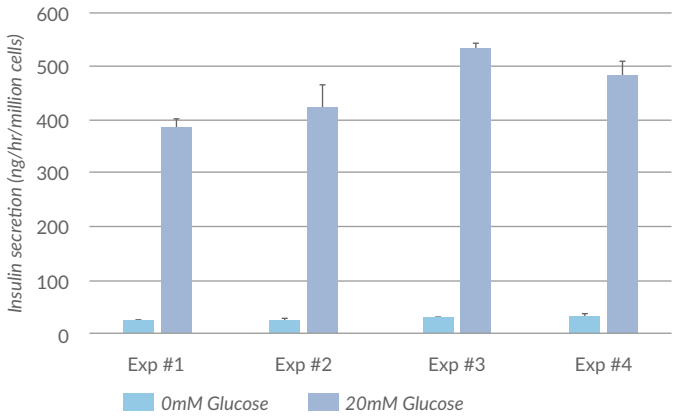


Figure 7
GSIS experiments were performed in EndoC-βH5® cells plated in 96-well plates. Results are expressed as ng secreted insulin/hour/million cells (96-well plate, 100.000 cells per well).
Exp = experiment.

EndoC-βH5® cells maintain stable responses to Glucose and to GLP1R agonist Exendin-4 for 4 weeks post thawing and seeding (Figure 8).

8

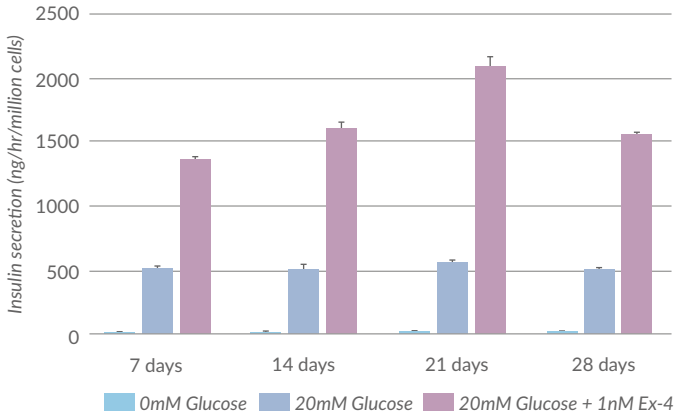


Figure 8
GSIS experiments were performed 7 days to 28 days after thawing and seeding of EndoC-βH5® cells (12-well plate, 375.000 cells per well). Results are expressed as ng secreted insulin/hour/million cells.

07
SAFETY PRECAUTIONS

BIOSAFETY QUALITY CONTROL

EndoC-βH5® cells are free of human pathogen.
EndoC-βH5® cells are free of HBV, HIV1, HIV2, HTLV1 and negative for mycoplasma.
EndoC-βH5® cells are biological in nature and should be used with appropriate caution, since not all of their characteristics are known and they may have hazardous properties.
It has been recommended by the French «Haut conseil pour les Biotechnologies» to cultivate EndoC-βH5® cells in BSL2 confinement.

Biosafety classification is based on U.S. Public Health Service Guidelines. It is the responsibility of the customers to ensure that their facilities comply with biosafety regulations for their own country.

PROCEDURE

All culture procedures must be performed under a class II safety cabinet.
When handling EndoC-βH5® cells, treat all biological materials according to BSL2 guidelines.
Use safety precautions when working with liquid nitrogen.

Use at least approved Biological Safety Level 2 (BSL-2) facilities and procedures.

Your notes

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PITFALLS AND FREQUENTLY ASKED QUESTIONS

COATING MATRIX

Coating is a KEY factor in the success of EndoC-βH5® cell culture.
A few points are of utmost importance:
• Never let the Coating Matrix aliquot remain at a temperature above 4 °C.
• Aliquots should stay on ice. Not doing that, the matrix starts to polymerize, inducing a non-uniform coating and preventing cells to adhere correctly to the support. Never use polymerized Coating Matrix.
• During coating medium preparation, use cold DMEM (4.5 g/L glucose) and place all the reagents on ice.
• Do not use DMEM if it is not ice cold.
• Place coated supports in a 37 °C incubator for a maximum duration of 6 h.
• Make sure that coating medium covers the whole surface of the culture support.

CELL THAWING

It is a critical step. Thawing must be performed quickly, with no more than 2 min in the water-bath at 37 °C. Not respecting this step would decrease cell quality and number of live cells.

CELL VIABILITY

It is very important to check viability on the day of seeding. Cell density is important and should be calculated on the basis of the number of viable cells.

CELL SEEDING

Cell count must be performed after centrifugation, after cells are re-suspended in ULTIβ1®.
Respecting cell density (nb cells/cm^2) for seeding is important.
Check the cells under the microscope frequently.
Avoid opening/closing their incubator since EndoC-βH5® cells are very sensitive to CO_2 levels.

COLLECTING ENDOC-βH5® CELLS FROM CRYOVIAL AND RESUSPENDING THEM

Contact time with freezing medium should be minimized since longer exposure will decrease viability.
EndoC-βH5® cells should be centrifuged at a maximum of 500 x g since higher speed would damage them.
Do not “vortex” cell pellet after centrifugation.
Re-suspend cell pellet using adapted pipet (see recommendations) and by gently pipetting up and down to ensure homogeneity of the suspension. Later on, when possible, invert the tube rather than further pipetting the suspension. carefully pipetting up and down to ensure homogeneity of suspension.

Your notes

09
APPENDICES

I - APPENDIX A. LIMITED WARRANTY

A | Human Cell Design S.A.S. warrants that its Products conform to the specifications contained in the Certificate of Analysis for the Product shipped to Customer. Customer's sole and exclusive remedy (and Human Cell Design S.A.S.'s sole and exclusive liability) under this limited warranty shall be replacement of the defective Products by Human Cell Design S.A.S.

B | Customer accepts the Original Material «as is» and acknowledges that it is experimental in nature and that it should be used with care and appropriate caution, since not all of its characteristics are known and it may have hazardous properties. Human Cell Design S.A.S. reserves the right to make changes in design, production, manufacture, or characteristics of the Products or to improve on the Products at any time and in any way, without incurring any obligations to replace or modify any Products previously sold.

C | Under no circumstances shall Human Cell Design S.A.S.' liability to Customer exceed the amount paid by Customer for the Products to Human Cell Design S.A.S. Human Cell DesignS.A.S. will bear all reasonable shipping costs if the Products are replaced pursuant to this warranty. This warranty does not apply to any defect or nonconformance caused by (i) Customer's use of EndoC-βH5 User's Guide of the Products for a purpose or in a manner other than that for which they were designed or that is permitted or in breach of this User's Guide, (ii) the failure by Customer to follow Human Cell Design S.A.S.' User's Guide for use, storage, and handling of the Products; or (iii) as a result of any other abuse, misuse or neglect of the Products by Customer. This warranty applies only to Customer and not to third parties. This warranty is not assignable.

D | TO THE FULLEST EXTENT PERMITTED BY APPLICABLE LAW, HUMAN CELL DESIGN S.A.S. DISCLAIMS ALL OTHER REPRESENTATIONS, AND WARRANTIES, EXPRESS OR IMPLIED, WITH RESPECT TO THE PRODUCTS, INCLUDING BUT NOT LIMITED TO, ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE OR NON-INFRINGEMENT. CUSTOMER'S SOLE REMEDY FOR BREACH OF WARRANTY IS STATED ABOVE.

E | Within five (5) business days of thawing the Product but prior to the expiration date of the Product as listed on the Certificate of Analysis and/or label, Customer must notify Human Cell Design S.A.S. in writing of any nonconformity of the Product, describing the nonconformity in detail; otherwise all Products shall be conclusively deemed accepted without qualification. Customer's failure to notify Human Cell Design S.A.S. in such time period voids the limited warranty described above. Customers who believe they have a warranty claim should email at contact@humancelldesign.com to request replacement Product based on a breach of the above limited warranty. Any action by Customer for Human Cell Design S.A.S.' breach of this limited warranty must begin within 18 months following the date of such breach.

F | Customer acknowledges that the Products are subject to E.U. and French export control laws and regulations. Customer represents and warrants that it is the ultimate end-user of the Products, and further represents and warrants that it will not knowingly sell, export, re-export, transfer, divert, or otherwise dispose of the Products (including other materials or goods derived from or based on the Products) to any other destination, entity, or person without the prior authorization of any relevant E.U. or French government agency and Human Cell Design S.A.S. Customer represents and warrants that it will not use the Products for any purpose prohibited by the laws or regulations of the E.U., French and/or other government authorities to which Customer is subject without the prior authorization from any government entity whose laws and regulations may apply to the use of the Products.

G | Human Cell Design S.A.S. makes no warranty of any kind or nature, neither express nor implied, for any Products or part of the Products that is not manufactured by Human Cell Design S.A.S. Any Products, or other such part or accessories to the Products shall have the warranty, if any, that is offered and granted by the manufacturer of such other products and accessories.

H | Customer acknowledges and agrees that Human Cell Design S.A.S. may fill Customer's order with any number of units of Products. Such units may be more units than Customer ordered. Customer will not be charged extra for any adjustments made by Human Cell Design S.A.S. The number of cells in a unit is determined by the Product's Certificate of Analysis. The number of cells that are contained in a unit accounts for both viability and plating efficiency percentages. Because this may vary from lot to lot, Human Cell Design S.A.S. reserves the right to fill the order with that number of units which is sufficient to fill Customer's order and such adjustments shall not constitute a breach of the warranty herein.

II - APPENDIX B. LIMITED LIABILITY

TO THE FULLEST EXTENT PERMITTED UNDER APPLICABLE LAW, HUMAN CELL DESIGN SHALL NOT HAVE ANY LIABILITY FOR INCIDENTAL, COMPENSATORY, PUNITIVE, CONSEQUENTIAL, INDIRECT, SPECIAL OR OTHER SIMILAR DAMAGES, HOWEVER CAUSED AND REGARDLESS OF FORM OF ACTION WHETHER IN CONTRACT, TORT (INCLUDING NEGLIGENCE), STRICT PRODUCT LIABILITY OR OTHERWISE, EVEN IF HUMAN CELL DESIGN HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. CUSTOMER UNDERSTANDS THAT ANY RISKS OF LOSS HEREUNDER ARE REFLECTED IN THE PRICE OF THE PRODUCTS AND THAT THESE TERMS WOULD HAVE BEEN DIFFERENT IF THERE HAD BEEN A DIFFERENT ALLOCATION OF RISK.