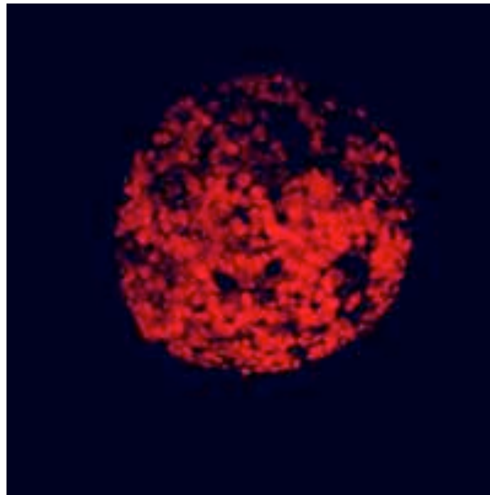
The Pancreatic ß-Cell Functional Differentiation Analysis Laboratory



Director – Dr. Christopher J. Rhodes
Manager – Dr. Cristina Alarcón

The Kovler Diabetes Center
The University of Chicago
5841 S. Maryland Ave., MC 1027
Chicago, IL 60637, USA.

Tel: 773-702-5828
Fax: 773-834-0486

JDRF Juvenile Diabetes Research Foundation International
dedicated to finding a cure

INTRODUCTION -

The Pancreatic ß-Cell Functional Differentiation Analysis Laboratory at the University of Chicago Kovler Diabetes Center (http://www.kovlerdiabetescenter.org/index.php) provides a service to *functionally* analyze candidate surrogate ß-cells derived from alternative renewable sources (*e.g.* stem cells), isolated human islets (± pharmacological or genetic manipulations), and regenerated or treated ß-cells for their ability to synthesize proinsulin, process proinsulin and appropriately secrete insulin in a physiological manner (http://www.kovlerdiabetes center.org/res_rhodes-stemcell.php).

This is intended to indicate whether candidate surrogate ß-cells are appropriately producing, processing and secreting (pro)insulin that might then be considered suitable for eventual ß-cell replacement therapies. In addition, the analysis evaluates the potency of various human islet preparations.

Among the basic questions that the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory addresses are: Do these cells synthesize proinsulin with appropriate regulation? If they do, do they efficiently process it properly to insulin? Do they store and secrete insulin in a correct regulated manner? Can stimulated insulin secretion, if it occurs, be rapidly stopped by physiologically relevant factors?

The Pancreatic ß-Cell Functional Differentiation Analysis Laboratory emphasizes a *functional* analysis, steering away for genomic and proteomic approaches that tend to be descriptive. It uses tried and tested HPLC and perifusion systems to assess dynamic (pro)insulin production and secretion in islets/cells, that may not be readily available to most diabetes researchers. As such, the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory offers a subsidized service to outside diabetes researchers for a diagnostic *functional* analysis of either their 'candidate surrogate ß-cells', human islet preparations and/or islets/ß-cells which have been subjected to various genetic or pharmacological manipulations. The overall intention is to assist in more effectively pushing forward therapeutic strategies for ß-cell replacement and/or ß-cell regeneration to treat type-1 diabetes. Funding of the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory is supported by the Juvenile Diabetes Research Foundation International (JDRF).

THE SERVICE –

Essentially, the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory's service asks three basic questions of appropriate ß-cell function conducted in order:

- Do the cells/islets synthesize proinsulin appropriately?
- If so, do the cells/islets convert proinsulin to insulin appropriately and efficiently?
- If so, do the cells/islets secrete insulin appropriately?

This is done in two steps.

***Step-1a*: Proinsulin biosynthesis protocol.** This follows a simple [^{3}H]leucine pulse radiolabeling protocol followed by HPLC analysis for proinsulin synthesis. Only about 50-100 human islets or 2x10^5 candidate ß-cells are required per analysis. At least two analyses are done, where the cells are incubated at a basal glucose or a stimulatory glucose. Both the media and acid-extract of the cells/islets are subjected to HPLC where the fractions are counted for radioactivity and subjected to radioimmunoassay (RIA) for insulin and C-peptide. This enables both glucose-regulated proinsulin synthesis and (pro)insulin secretion to be evaluated from the same cells.

Figure 1 depicts such an HPLC analysis. Because of a short 20 minute radiolabeling period used only proinsulin synthesis is observed (without conversion to insulin and C-peptide), as expected. In this example, more [^{3}H]proinsulin is detected at stimulatory glucose than at basal glucose indicating that these are good function human islets responsive to glucose for control of proinsulin biosynthesis. RIA for insulin and C-peptide of the same human islet acid-extract and media HPLC analyses can yield further information, about insulin content as well as basal and regulated insulin secretion.

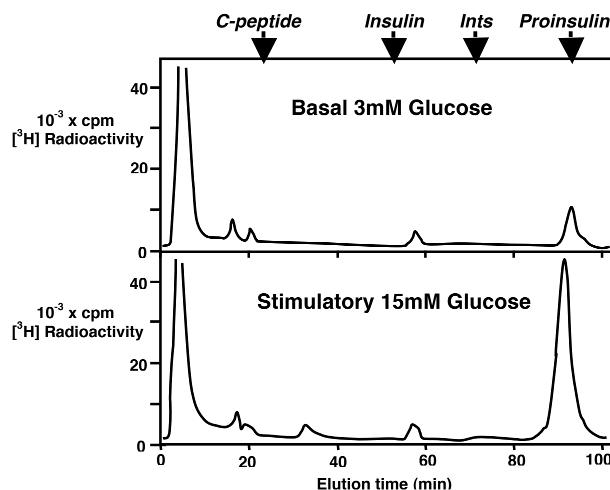


Figure 1: *An example HPLC analysis of [^{3}H]proinsulin synthesis in acid-extracts of good functioning isolated human islets.* The elution points of human C-peptide, human insulin, human split proinsulin intermediates ('Ints' - des 31, 32 proinsulin and des 64,65proinsulin) and intact proinsulin are indicated.

The information obtained from this 'Step-1a' analysis may be sufficient, especially if it is indicated that the cell preparation is not synthesizing sufficient proinsulin, not viable, and/or not glucose responsive. However, if proinsulin synthesis is detected then a second analysis can be applied to make sure proinsulin is appropriately processed to insulin and C-peptide, in Step-1b.

***Step-1b*: Proinsulin processing protocol.** The step-1b follows a simple [^{3}H]leucine pulse-*chase* radiolabeling protocol followed by HPLC analysis, similar to Step-1a. Around 100-200 human islets or 4x10^5 candidate ß-cells are required for this analysis. Essentially, the cells are 'pulse' radiolabeled for 30 minutes with [^{3}H]leucine at a stimulatory glucose concentration to ensure that a large amount of [^{3}H]proinsulin is synthesized. An aliquot of islets/cells are removed fro acid extraction and HPLC analysis. The remaining cells/islets are washed, then incubated for a second 'chase' period of 90 minutes at either basal 3mM or a stimulatory 15mM glucose concentration. At the end of the *chase* incubation period these cells/islets (~50 islets or 1x10^5 candidate ß-cells) are removed, washed and subjected to acid extraction and HPLC analysis.

Figure 2 depicts a [^{3}H]proinsulin processing HPLC analysis. In the acid extract of the 'pulsed islets' (*i.e.* immediately after the 30 minute [^{3}H]leucine pulse radiolabeling period) [^{3}H]proinsulin is predominately detected (Fig, 2). In islets incubated for a further 90 minute 'chase' period at either basal or stimulatory glucose about 50% of the [^{3}H]proinsulin had been processed to [^{3}H]insulin and [^{3}H]C-peptide in an equimolar ratio as would

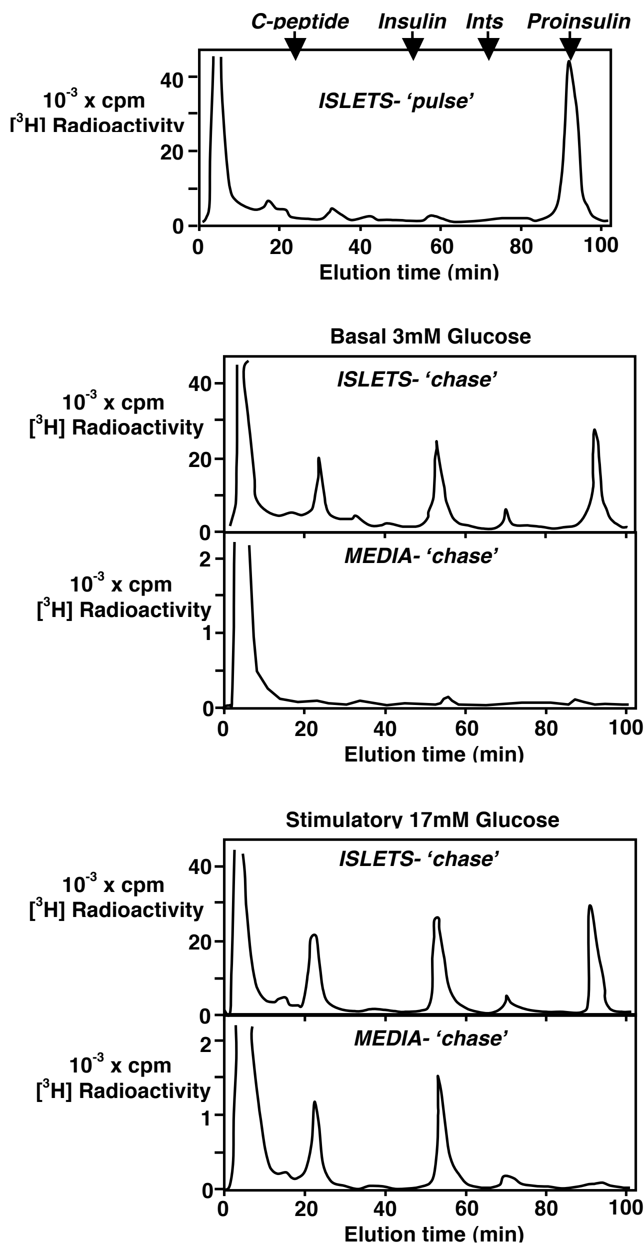


Figure 2: *An example of an HPLC analysis of [^{3}H]proinsulin processing in good functioning isolated human islets.* The elution points of human C-peptide, human insulin, human split proinsulin intermediates ('Ints' - des 31, 32 proinsulin and des 64,65proinsulin) and intact proinsulin are indicated.

be predicted for good functioning islets. HPLC analysis of the 'chase incubation media' reveals mostly [^{3}H]insulin and [^{3}H]C-peptide are secreted (in an equimolar ratio; Fig. 2). In this particular example of a good human islet preparation there is ~6 fold more [^{3}H]insulin/[^{3}H]C-peptide secreted at stimulatory glucose compared to basal glucose (Fig. 2). RIA for insulin and C-peptide of the same human islet acid-extract and media HPLC analyses can yield further information.

As to whether Step-1a and/or Step-1b is used will be dependent on the cell/islet preparation and requirements of the investigator. All options will be discussed with the investigator prior to the analysis. Of course, the HPLC analysis can be expanded, at the request of the 'customer' investigator, to include accumulating data on time courses and does response curves for glucose regulation of proinsulin synthesis, processing and secretion as well as the effect of other secretagogues on these parameters. However, although HPLC offers a powerful technique to gain much information about essential ß-cell function in relatively few analyses, it has its limitations and does leave a few important questions unanswered. If a cell/islet preparation is shown to synthesize and process proinsulin appropriately then the 'Pancreatic ß-Cell Functional Differentiation Analysis Laboratory' can provide deeper analysis of insulin/C-peptide secretion using a perifusion system.

Step-2*: *Perifusion secretion profiles - *In vitro* perifusion of isolated islets or 'candidate surrogate ß-cells' can yield valuable information about the kinetics of stimulating and inhibiting insulin secretion, as well as a quantitative measurement of insulin/C-peptide secretion.

Step-2a* *– The 'on switch' protocol. This examines the kinetics of glucose/secretagogue stimulated insulin secretion in perifused islets/cells. Around 100 isolated islets or 3-5x10^{5} 'candidate surrogate ß-cells' are sufficient for this analysis. Essentially, the islets/cells are perifused for 90 minutes at basal 3mM glucose. Then islets are perifused for a further 35 minutes at a stimulatory glucose (± secretagogues) then brought back to basal glucose and perifused for a further 15 minutes. One minute fractions are collected then assayed for insulin and C-peptide by RIAs. The islets/cells in the perifusion chambers are collected at the end of the analysis and assayed by RIA for insulin and C-peptide cellular content. The percentage of islet/cellular insulin/C-peptide content secreted can then be calculated. Figure 3 shows an example perifusion analysis for a batch of good functioning isolated human islets using this protocol.

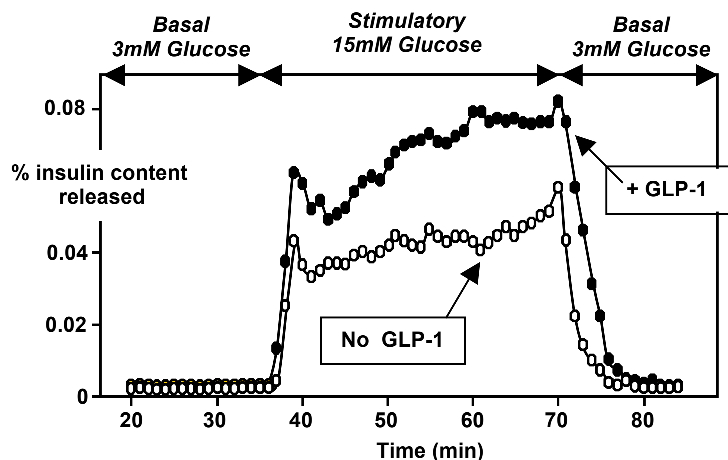


Figure 3: *An example perifusion analysis of glucose and glucose/GLP-1 stimulated insulin secretion in isolated human islets.*

The insulin secretory information that can be gathered from this sort of perifusion analysis are as follows: 1) A low percentage of insulin content released (>0.08% per minute at maximal stimulatory conditions in this example (Fig. 3)); 2) A low basal

insulin secretory rate; 3) A lag period for the insulin secretory response to a secretagogues (normally ~ 2 minutes for glucose-induced insulin secretion (Fig. 3)); 4) A kinetic biphasic stimulated insulin secretion response to glucose (normally ~10 min period for the 1st phase followed by a prolonged and sustained 2nd phase (Fig. 3)); 5) Potentiation of glucose-induced insulin secretion by relevant secretagogues such as GLP-1 (5nM) (Fig. 3; filled circles); 6) a rapid return to basal insulin secretion after removal of the stimulus (normally ~5 minutes (Fig. 3)).

***Step-2b* – The 'off switch' protocol.** This examines the kinetics for inhibition of glucose/secretagogue stimulated insulin secretion by physiologically relevant inhibitors (*e.g.* epinephrine) in perifused islets/cells. Essentially, the islet/cell perifusion system is set up as described above for the 'on switch protocol', except that after 25 minutes glucose stimulation an inhibitor is added to one set of perifused islets/cells at stimulatory glucose and not to a set of

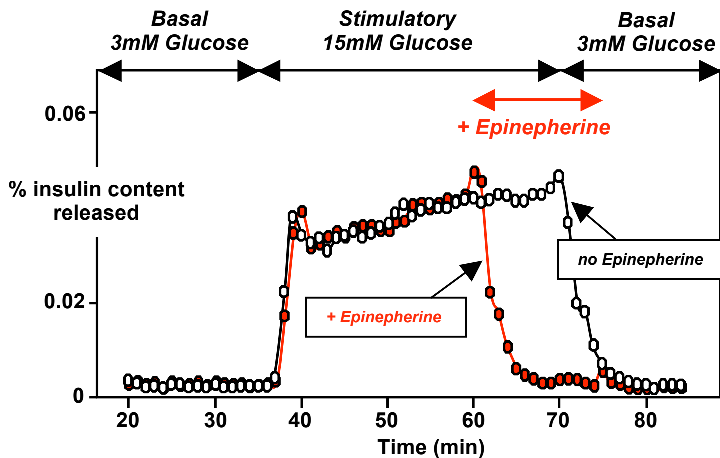


Figure 4: *An example perifusion analysis for epinephrine inhibition of glucose stimulated insulin secretion in isolated human islets.*

perifused islets/cells conducted in parallel. Then, after a further 15 minute incubation, the inhibitor is removed and perifused islets/cells are returned to basal glucose. Figure 4 shows an example perifusion analysis for a good batch of isolated human islets using this 'off switch' protocol. The same parameters for the kinetics of glucose-induced insulin secretion and basal insulin secretion rate can be ascertained from this protocol as mentioned previously for the 'on switch protocol' (Fig. 3). Also, the rapid inhibition of glucose-induced insulin secretion by an inhibitor (epinephrine here (red circles)) can be seen, that normally occurs ≤ 5 minutes in good functioning ß-cells (Fig. 4).

Using these perifusion protocols the kinetics of turning on and off regulated insulin secretion can be appreciated. Of course, these protocols can be expanded and adjusted to consider other secretagogues and inhibitors. We shall work with 'customer' investigators in the design of additional perifusion protocols as they wish.

EXTRA ß-CELL ANALYSES –

At this point, the HPLC and perifusion analyses in Steps -1 and –2 respectively, will give a very good indication for ß-cell functionality of human islet preparations, 'candidate surrogate ß-cells' or islets/cells undergone manipulation to promote survival/regeneration. However, some investigators utilizing the 'Pancreatic ß-Cell Functional Differentiation Analysis Laboratory' may wish to get a deeper functional analysis of their ß-cell preparations. This may be most pertinent to researchers who have generated promising 'candidate surrogate ß-cells' and wish to show how close they are to *bone fide* ß-cells. If so, some 'added extras' can be provided as an additional service using facilities and expertise available at the University of Chicago Comprehensive Diabetes Center. Such extra analyses include –

- Analysis of characteristic ß-cell metabolism.
- Analysis of the generation of secondary messengers (*e.g.* $[Ca^{2+}]_i$; $[cAMP]_i$; PIP3; *etc.*) in real time.
- Activation of protein kinase cascade signal transduction pathways.
- Analysis of insulin secretory granule trafficking and exocytosis in real time.

The details of extra ß-cell analyses are provided upon request.

OTHER ANALYSES –

1. In vivo serum analysis – Sometimes human 'candidate surrogate ß-cells' are transplanted into rodent animal models. We have developed an HPLC analysis of sera, where *human* proinsulin, insulin and C-peptide can be quantified separately from the endogenous rodent proinsulins, insulins and C-peptides in the very same HPLC analysis run.
2. Proglucagon peptides analysis – Because of the nature of *in vitro* stem/precursor cell differentiation protocols, sometimes a mixture of pancreatic endocrine cells are obtained. As such, we have also set up a 'pancreatic α-cell' analysis to examine proglucagon processing. Therapeutically glucagon production may be a concern since it counter regulates the effects of insulin, but glucagon-like peptide-1(GLP-1) production could be beneficial since it enhances ß-cell function. In a single HPLC analysis of serum or candidate surrogate cell extracts, we can quantitatively measure glucagon, active GLP-$1_{7\text{-}37}$, inactive GLP-$1_{9\text{-}37}$, and GLP-2 separately.

PRACTICALITIES –

1. All data gathered for each analysis is held and treated in strict confidence. It is considered HIGHLY CONFIDENTIAL and is only communicated to the 'customer investigator' or person(s) that he/she designates. Absolutely no data derived from this analysis will be published or publicly communicated without prior written permission from the 'customer investigator'.

2. For an optimal analysis, live cells/human islets are best shipped to the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory at the University of Chicago Comprehensive Diabetes Center. However, it is also possible to provide pulse-chase radiolabeling protocols/guidance to the interested investigator, and then the cell-lysates and incubation media shipped to the Pancreatic ß-Cell Functional Differentiation Analysis Laboratory for subsequent HPLC analysis.

3. The 'Pancreatic ß-Cell Functional Differentiation Analysis Laboratory' is willing to work together with interested investigators to adapt the functional analysis protocols to better suit the characteristics of their cell/islet preparations and intentions.

4. The cost of the analysis can be partly subsidized but there will be some moderate charge-back costs to the 'customer investigator' depending on the extent of the analysis requested. Please inquire and consult with Drs. Rhodes or Alarcón prior to an analysis.

ADDRESSES AND CONTACTS –

Director –

Christopher J. Rhodes Ph.D.
Research Director, The Kovler Diabetes Center
The University of Chicago
Knapp Center for Biomedical Discovery
900 E. 57th Street, Room 8130,
Chicago, IL 60637, USA.
Tel: 773-702-5828
Fax: 773-834-0851
Email: cjrhodes@uchicago.edu

Manager–

Cristina Alarcón Ph.D.
The Kovler Diabetes Center
The University of Chicago
Knapp Center for Biomedical Discovery
900 E. 57th Street, Room 8160,
Chicago, IL 60637, USA.
Tel: 773-702-2796/4019
Fax: 773-834-0851
Email: calarcon@medicine.bsd.uchicago.edu

Administration & Inquires –

San Juanita Mendez
The Kovler Diabetes Center
The University of Chicago
Knapp Center for Biomedical Discovery
900 E. 57th Street,
Chicago, IL 60637, USA.
Tel: 773-702-5828
Fax: 773-834-0851
Email: smendez@medicine.bsd.uchicago.edu