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TITLE:

Measuring Relative Insulin Secretion using a Co-Secreted Luciferase Surrogate

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KEYWORDS:

Insulin, secreted luciferase, pancreatic beta cell, diazoxide paradigm, secretion assay, Gaussia

SUMMARY:

This protocol describes how to perform rapid low-cost luciferase assays at medium-throughput using an insulin-linked Gaussia luciferase as a proxy for insulin secretion from beta cells. The assay can be performed with most luminescence plate readers and multichannel pipettes.

ABSTRACT:

Performing antibody-based assays for secreted insulin post-sample collection usually requires a few hours to a day of assay time and can be expensive, depending on the specific assay. Secreted luciferase assays expedite results and lower the assay cost per sample substantially. Here we present a relatively underused approach to gauge insulin secretory activity from pancreatic β cells by using Gaussia luciferase genetically inserted within the C-peptide. During proteolytic processing of proinsulin, the C-peptide is excised releasing the luciferase within the insulin secretory vesicle where it is co-secreted with insulin. Results can be obtained within minutes after sample collection because of the speed of luciferase assays. A limitation of the assay is that it is a relative measurement of insulin secretion and not an absolute quantitation. However, this protocol is economical, scalable, and can be performed using most standard luminescence plate readers. Analog and digital multichannel pipettes facilitate multiple steps of the assay. Many different experimental variations can be tested simultaneously. Once a focused set of conditions are decided upon, insulin concentrations should be measured directly using antibody-based assays with standard curves to confirm the luciferase assay results.

INTRODUCTION:

The method presented here allows insulin secretion from a genetically-modified beta cell line to be assayed rapidly and affordably in 96-well-plate format. The key to this protocol is a modified version of insulin with the naturally-secreted Gaussia luciferase (GLuc, ~18 kDa) inserted (see **Figure 1**) into the C-peptide to generate insulin-Gaussia (InsGLuc)[1,2]. Other larger proteins, such as GFP (~25 kDa), have been successfully inserted into the C-peptide of insulin and exhibited the

expected post-translational processing from proinsulin-GFP to insulin and GFP-C-peptide[3,4]. For the assay in this protocol, GLuc has been codon-optimized for mammalian expression and two mutations have been introduced to enhance glow-like kinetics[5,6]. Multiple combinations and replicates of treatment conditions can be easily tested in 96-well-plate format and the secretion results can be obtained immediately following the experiment.

A major advantage, as previously noted[2], is the low cost of this luciferase-based secretion measurement (< $0.01/well) which differentiates it from the relatively higher costs and technical aspects of enzyme-linked immunosorbent assays (ELISAs) (> $2/well) and homogenous time-resolved fluorescence (HTRF) or other Förster resonance energy transfer (FRET)-based antibody (> $1/well) assays. In comparison to these antibody-based assays, which measure the concentration of insulin by referencing a standard curve, the InsGLuc assay measures secretory activity as a relative comparison to control wells on the plate. For that reason, every experiment requires the inclusion of proper controls. This distinction is a trade-off to allow rapid and inexpensive measurements. However, InsGLuc secretion has been demonstrated to be highly correlated with insulin secretion as measured by ELISA[1,2]. This technology has been scaled up for high-throughput screening[1,2,7] and has led to the identification of novel modulators of insulin secretion including a voltage-gated potassium channel inhibitor[7] as well as a natural product inhibitor of β cell function, chromomycin A_2[8]. The use of InsGLuc is most appropriate for researchers who plan to continually test many different treatment conditions for their impact on insulin secretion. In follow-up experiments it is necessary to repeat key findings in a parental β cell line, and optimally in murine or human islets, and measure insulin secretion using an antibody-based assay.

PROTOCOL:

1. Preparation of reagents, media and buffers (Table 1)

1.1. Prepare MIN6 complete media in 500 mL of high-glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) with the following additives: 15% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 292 µg/mL L-glutamine, and 50 µM β-mercaptoethanol.

NOTE: The stable cell line in this case is maintained in 250 µg/mL of G418 antibiotic.

1.2. Prepare Krebs-Ringer bicarbonate buffer (KRBH) by making a solution containing 5 mM KCl, 120 mM NaCl, 15 mM HEPES (pH 7.4), 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 mg/mL radioimmunoassay-grade bovine serum albumin (BSA). Glucose is to be added where specified from a 2 M stock.

NOTE: KRBH is used to incubate the cells with and without stimulation in order to assess insulin and/or Gaussia luciferase secretion.

1.3. Prepare coelenterazine (CTZ) stock solution as follows. Prepare acidified methanol by adding 106 µL of concentrated HCl to 10 mL of methanol. Next, dissolve lyophilized CTZ in acidified

methanol at 1 mg/mL and store at -80 °C in screw-cap tubes.

NOTE: These stocks retain sufficient activity in routine luciferase assays, even after 1 year of proper storage.

1.4. Prepare Gaussia luciferase (GLuc) assay buffer based upon the literature[9] as well as patent information[10] to aid in half-life of the Gaussia luciferase assay in 96 well plate format. Use the formula: 25 mM Tris pH 8, 1 mM EDTA, 5% glycerol, 1 mg/mL Na_2PO_4, 300 mM sodium ascorbate, 200 mM Na_2SO_3 in water. Freeze stocks at -20 °C. After thawing, store the buffer at 4 °C.

1.5. To prepare Gaussia luciferase working solution, add 4.2 µL/mL of the 1 mg/mL (2.36 mM) CTZ stock solution to GLuc assay buffer. This results in a 2x working solution of 10 µM CTZ which will have a 5 µM final concentration in the assay.

1.6. Prepare the viability assay buffer (relative [ATP]) according the manufacturer's instructions with some modifications.

1.6.1. Add 100 mL of manufacturer-provided buffer (see the **Table of Materials**) to the substrate and mix.

1.6.2. Dilute the 100 mL of the mixture by adding 300 mL of PBS containing 1% Triton X-100 and mix well.

1.6.3. Dispense into 40 mL aliquots and store at -20 °C. After thawing, the unused solution can be refrozen.

2. Culture of InsGLuc MIN6 cells and seeding for secretion assays

2.1. To culture MIN6 cells, trypsinize and seed the cells once per week using standard cell culture techniques. Change media on the cells every two to three days. Include appropriate selection antibiotic, such as 250 µg/mL of G418 in the media.

2.1.1. To provide a sufficient number of cells weekly for experiments, maintain the cells in T75 flasks. Seeding 6 x 10^6 cells per T75 in 10 mL of media will typically yield 30 x 10^6 to 40 x 10^6 cells total per T75 after 7 days of culture.

2.2. To prepare cells for plating into 96-well plates, wash a confluent T75 of InsGLuc MIN6 cells twice with PBS and add 2 mL of trypsin. Incubate at 37 °C for ~5 min or until the cells dissociate from the flask. Determine the cell concentration per milliliter.

2.2.1. Dilute the cells in complete media to 1 x 10^6 cells/mL to result in 1 x 10^5 cells in 100 µL per well in a 96-well plate. The cells should be sufficiently confluent for the assay after 3–4 days.

NOTE: To extend the culture period, half the cell concentration can be plated. Media changes are

not required prior to the day of the assay unless the cells are to be subjected to experimental treatments.

3. Glucose-stimulated Gaussia luciferase secretion assay

3.1. On the day of the assay prepare enough KRBH for the experiment (step 1.2). Typically, 50 mL of KRBH per 96-well plate is sufficient. Prepare extra buffer if different combinations of drug treatment conditions will require KRBH for dilution.

3.2. Prepare a reservoir with glucose-free KRBH. Decant the medium from 96-well plate(s) by quickly inverting the plate over a laboratory sink and then blot firmly on a stack of paper towels to remove excess medium.

3.3. Using either an electronic or manual 8-channel pipette, pipette 100 µL /well KRBH from the reservoir across the 96-well plate(s). Repeat for a total of two washes.

3.4. (Optional step of acute compound treatments) If not performing drug treatments, proceed with steps 3.5 and 3.6 without modification. To test the effects of small molecules on insulin secretion, compounds can be added to the cells during the preincubation period, stimulation period, or both.

3.4.1. One technique is to add compounds in batch to the KRBH in 1.5 mL tubes and use an adjustable 8-channel digital pipette to transfer the drug-KRBH from the tubes to cells in 96-well plates.

NOTE: If an adjustable pipette is not available, a replica 96-well plate of drug-KRBH can be made and a standard 8-channel pipette can be used to transfer buffer. Further modifications to the treatment paradigm can be made to treat cells for 24 h in media prior to the assay, as previously described[1,8].

3.5. Add 100 µL of KRBH containing the desired concentration of glucose or compounds and place the dish in the 37 °C incubator for 1 h.

NOTE: Depending on the experimental layout, it is extremely helpful to have an electronic multichannel pipette that allows transitioning the channel distances from a column of eight 1.5-mL tubes to the 8 rows of a 96-well plate.

3.6. After the 1 h of preincubation, decant the buffer into the sink and blot firmly on paper towel. Add 100 µL of glucose-free KRBH per well to wash away accumulated background of Gaussia luciferase. Decant the plate again and add control and stimulatory conditions to the plate at 100 µL per well. Place the dish in the 37°C incubator for 1 h.

3.7. Carefully collect 50 µL of supernatant using a multichannel pipette, changing tips between treatment conditions as necessary, and transfer the supernatant to a clean opaque white 96-well

assay plate.

NOTE: White-walled clear-bottom plates can be used if necessary, although a significant amount of luciferase signal will be lost.

3.8. After collection of 50 µL of KRBH supernatant, the sample can be assayed immediately. If necessary, seal and store samples at 4 °C for a few days (GLuc activity half-life ~6 days) or -20 °C for up to one month[11,12].

4. Secreted Gaussia luciferase assay

4.1. To prepare the GLuc assay working solution, pipette the required amount of CTZ stock solution (4.2 µL/mL) into GLuc assay buffer. To prevent warming the CTZ, pipette the CTZ quickly at the -80 °C freezer or keep the tube on dry ice.

4.2. Using an electronic multichannel pipette, quickly add 50 µL of the GLuc assay working solution per well across the 96-well dish containing the collected KRBH supernatants. If there are any droplets on the sides of any wells, briefly spin the plate in a table-top swing-bucket centrifuge.

4.3. Read the luminescence in a suitable plate reader within a few minutes and read each well with a 0.1 s integration time.

REPRESENTATIVE RESULTS:

To gauge the performance of the assay under control conditions, a simple glucose dose-response curve or a stimulation using the diazoxide paradigm can be completed. In the case of the former, pre-incubating the cells for 1 h in glucose-free conditions followed by treating for 1 h with increasing glucose concentrations should result in very little secretory activity at and below 5 mM, while increased secretion is observed above 8 mM glucose (**Figure 2**). Stimulation with 35 mM KCl also serves as a positive control for stimulated secretion. Inclusion of secretion-modulating drugs during the stimulation period should give the expected inhibition or potentiation of secreted GLuc activity (**Figure 3**). For example, diazoxide binds the K_{ATP} channel and prevents it from closing upon increased [ATP/ADP] ratio, blocking membrane depolarization and preventing secretion[13]. Phorbol esters like para-methoxyamphetamine (PMA) activate protein kinase C (PKC) and are known amplifiers of insulin secretion[14]. Finally, stimulation with 1 µM epinephrine activates α_{2A}-adrenergic receptors which in turn activate the heterotrimeric G-protein complex Gi, inhibiting membrane depolarization and insulin secretion[15]. It is important to recognize that while MIN6 cells are an immortal cell line, they start to lose proper glucose-induced insulin secretion responses (such as left-shifting of the response curve) after extended passaging[16]. For this reason, it is good practice to routinely culture all MIN6 cell lines for up to eight weeks (splitting once per week) before starting over from liquid nitrogen stocks.

FIGURE AND TABLE LEGENDS:

Figure 1: Description of the InsGLuc reporter. (**A**) First, a stable β cell line (in this case MIN6 cells) was generated expressing the insulin-Gaussia transgene from the rat insulin promoter. (**B**) The full protein is synthesized and packaged in insulin granules along with endogenous insulin. Prohormone convertases cleave the peptide, indicated by asterisks. (**C**) The processed insulin and Gaussia are co-secreted and the luciferase activity is detected by the addition of CTZ in an ATP-independent, oxygen-dependent reaction.

Figure 2: The InsGLuc reporter is a faithful proxy of insulin secretion from MIN6 beta cells. (**A**) Response of MIN6 InsGLuc cells to increasing glucose concentrations and KCl (35 mM) with Gaussia luciferase secretion. Data are the mean fold luciferase activity ± SE compared to 0 mM glucose conditions for three independent experiments. *, $P < 0.05$. The figure has been modified with permission from Kalwat et al. ACS Sensors 2016[1]. © 2016 American Chemical Society. (**B**) The InsGLuc reporter in MIN6 cells exhibits the expected secretory response to the diazoxide (Dz) paradigm where 250 μM Dz treatment holds the K_{ATP} channel open, blocking membrane depolarization unless extracellular KCl (35 mM) is provided to elicit the 'triggering' calcium influx. Further addition of glucose (20 mM) under the Dz + KCl condition reveals the metabolic amplification of secretion that occurs without further increases in calcium influx.

Figure 3: Inclusion of secretion-modulating compounds during glucose-stimulates InsGLuc secretion. InsGLuc MIN6 cells plated in 96-well format as described were preincubated in glucose-free KRBH for 1 h. Cells were then treated with or without 20 mM glucose in the presence of dimethyl sulfoxide (DMSO) (0.1%), KCl (35 mM), diazoxide (250 μM), PMA (100 nM), or epinephrine (1 μM) for 1 h. Bar graph represent the mean ± SE of at least 3 independent experiments.

Table 1: Buffer and stock solution recipes used to perform the presented assays.

DISCUSSION:

Herein we present a method to rapidly assess glucose-stimulated insulin secretion responses from MIN6 β cells. For the best responses in the assay it is important to seed the MIN6 cells at the proper density and allow them to become 85–95% confluent. This improves β cell responses to glucose because of improved cell-cell contacts and synchronization and occurs both in primary islets[17-21] as well as MIN6 cells[16,18]. To prevent losses in secretory response to glucose stimulation, it is important to maintain the cells at as low of a passage as possible and culture the cells for only 6–8 weeks prior to thawing a new vial from liquid nitrogen stocks. Modifications can be made to the plating strategy in protocol section 2 to adapt to the available equipment as necessary. Plating InsGLuc MIN6 cells into 96-well plates for secretion assays affords a large number of wells for experimental manipulations (including replicates) as well as maintaining accuracy of plating in a normal lab setting, as plating into dishes with higher well numbers often requires special equipment usually available in high-throughput screening cores.

Current assays for insulin secretion, other than the indirect luciferase assay described here, include: ELISAs that use colorimetric readouts (direct assay), radioimmunoassays (competition assay) which use radioactive readouts, FRET-based antibody competition assay[22] and HTRF[23]

which uses FRET between dye-linked antibodies to measure insulin directly, and DNA aptamers[24]. Each of these methods has its own advantages, but in general they are more expensive and/or time-consuming than a luciferase assay. One key limitation of the InsGLuc assay is the fact that luminescent activity of the co-secreted luciferase is only a proxy for actual insulin secretion. Additionally, theoretically there is no expected difference in luciferase activity between Gaussia with fragments of C-peptide on its N- and C-termini or Gaussia within the proinsulin protein, as Gaussia luciferase has been successfully used as a tag to measure the secretion of other proteins[25]. This highlights the requirement of confirmation studies using assays that measure processed insulin specifically. Alternatives to direct and indirect measurements of insulin secretion can also be used to assess β cell function. A variety of optical reporters exist for readouts including ATP:ADP ratio, calcium influx, NAD^+/NADH ratio, extracellular signal–regulated kinases (ERK) activation, or cyclic adenosine monophosphate (cAMP) levels[26].

The future applications of InsGLuc in particular appear to be in high-throughput screening. This assay has already been used in a handful of published small screens[1,2] and unpublished larger screens are either completed[7] or underway. Development of other iterations of this technology may involve tagging of other secreted islet hormones with luciferases to facilitate rapid measurements, such as for glucagon or somatostatin. Modifications could be made in any case where the cell line is regenerated using alternate approaches including CRISPR/Cas9, lentivirus, or transposase-mediated insertion in any suitable beta cell line. Additional possible modifications to the original reporter may include substituting alternate secreted luciferases for Gaussia or combining multiple different secreted luciferases linked to different hormones for a multiplexed assay. Beyond cell culture, CRISPR/Cas9 technology presents the possibility of generating a mouse model where a suitable luciferase is knocked in to the C-peptide coding region of Ins2 in the genome. Such a mouse would be feasible given that transgenic mice have been created with GFP knocked in to the same C-peptide site[27] and would allow measurement of endogenous β cell function with a luciferase assay in vivo or ex vivo.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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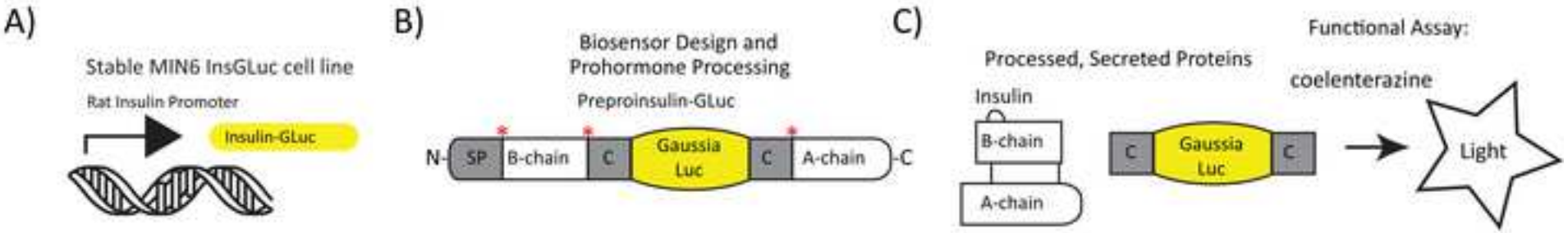

Figure 2

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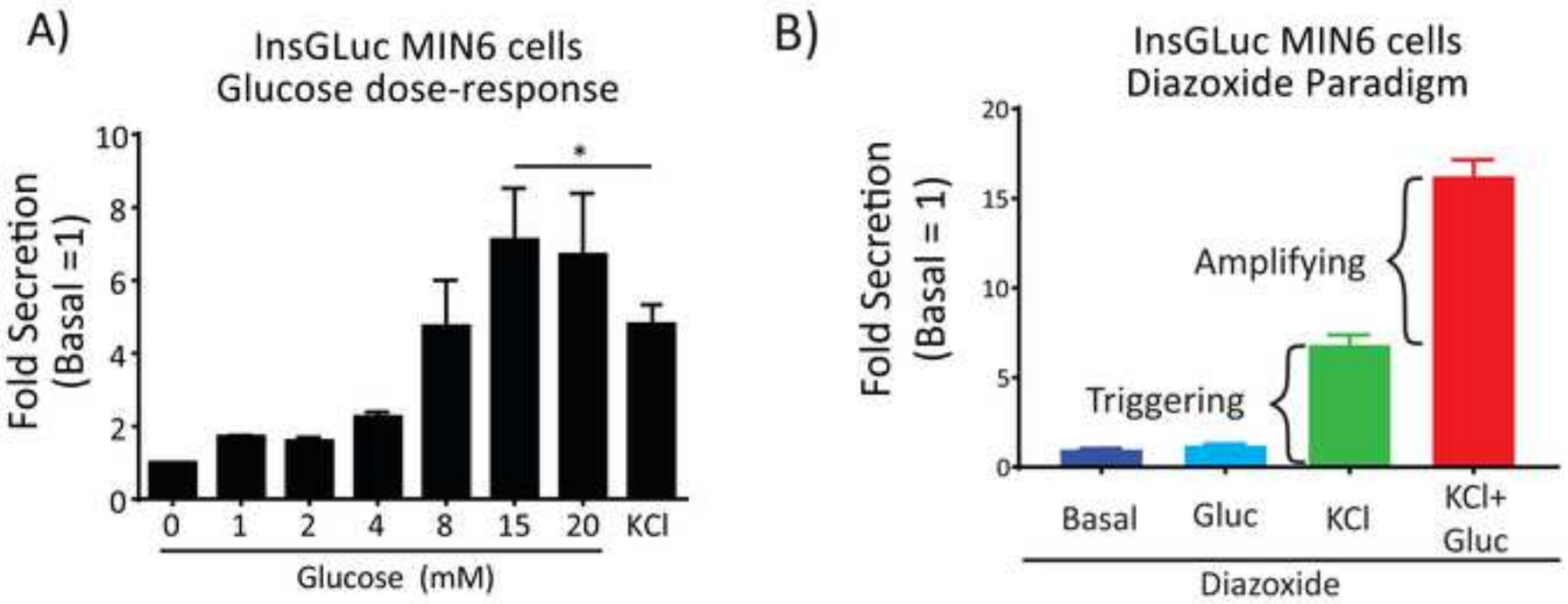

Figure 3

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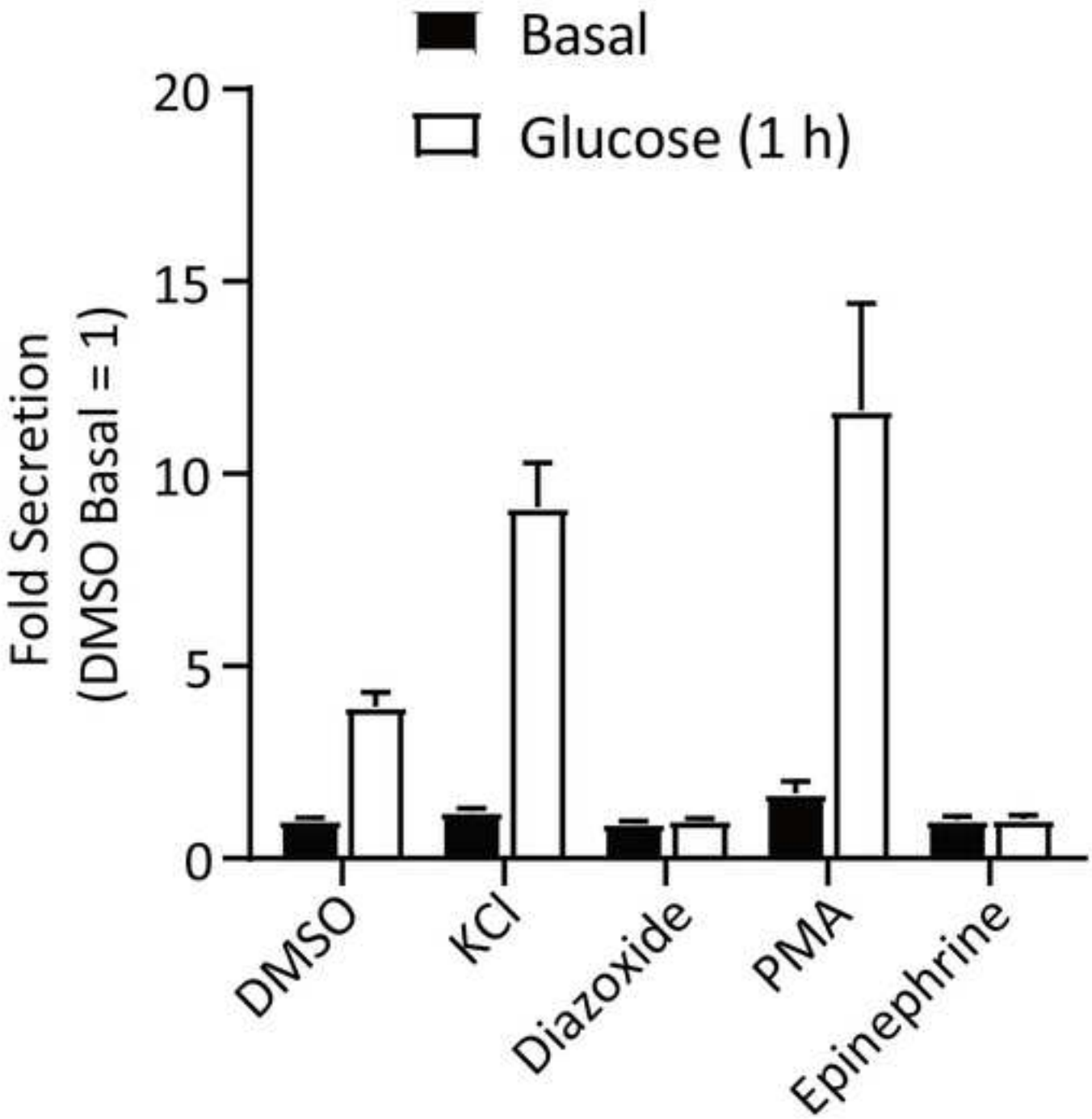

Table

Click here to access/download;Table;Table 1. Buffer and stock recipes.xlsx

Gaussia Assay Buffer*

Stock solution or powder	Stock	Final Concentration	50 mL
Disodium phosphate	powder	0.1% (1 mg/mL)	50 mg
Glycerol	40%	5%	6.25 mL
Sodium Bromide	powder	150 mM	772 mg
EDTA pH 8	0.5M	1 mM	100 µL
Tris-HCl pH 8	1M	25 mM	1.25 mL
Ascorbic Acid*	powder	300 mM	2.64 g
Na2SO3**	powder	200 mM	1.26 g
Water			up to 50mL
Store in aliquots at -20 °C, thaw one at a time and keep at 4 °C.			

*Modified recipe from Luft et al. BMC Biochemistry 2014, 15:14.

Acidified MeOH

Stock solution or powder	Stock	Final Concentration	10 mL
Methanol	100%		10 mL
HCl	11.65 M	1.06%	0.106 mL

Coelenterazine solution

Stock solution or powder	Stock	Final Concentration	1 mL
Coelenterazine	powder	1 mg/mL (2.36 mM)	1 mg
Acidified methanol			1 mL

4.2 µL of stock per 1 mL of Gaussia Assay Buffer results in 10 µM CTZ to be used as a 2x working soluti
For example, add 50 µL of 2x working solution to 50 µL of KRBH sample containing secreted Gaussia.

ɔn.

Click here to access/download;Table of Materials;Kalwat_2019_JoVE_Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Cell culture materials			
rIns-GLuc stable MIN6 cells			Parental MIN6 cell line stab
DMEM	Sigma	D6429	4.5 g/L glucose media
fetal bovine serum, heat-inactivated	Sigma	F4135	
Penicillin/Streptomycin	Thermo-Fisher Scientific	SV30010	
beta-mercaptoethanol	Thermo-Fisher Scientific	BP 176-100	
glutamine	Thermo-Fisher Scientific	BP379-100	
Trypsin-EDTA	Sigma	T3924-500	
G418	Gold Biotechnology	G418-10	Stock solution 250 mg/mL i
T75 tissue culture flasks	Fisher Scientific	07-202-000	
96 well tissue culture plates	Celltreat	229196	
Reagent reservoirs (50 mL)	Corning	4870	
Secretion assay reagents			
BSA (RIA grade)	Thermo-Fisher Scientific	50-146-952	
D-(+)-Glucose	Sigma	G8270-1KG	
KCl	Thermo-Fisher Scientific	P217-500	
NaCl	Thermo-Fisher Scientific	S271-3	
Hepes, pH 7.4	Thermo-Fisher Scientific	50-213-365	
$NaHCO_3$	Thermo-Fisher Scientific	15568414	
$MgCl_2$	Thermo-Fisher Scientific	M9272-500G	
$CaCl_2$	Sigma	C-7902	
Optional drugs for stimulation experiments			
Diazoxide	Sigma	D9035	Stock solution: 50 mM in 0.
epinephrine (bitartrate salt)	Sigma	E4375	Stock solution: 5 mM in wa
PMA (phorbol 12-myristate)	Sigma	P1585	Stock solution: 100 µM in D
Guassia assay materials			
Disodium phosphate (Na_2HPO_4)	Thermo-Fisher Scientific	S374-500	
Glycerol	Thermo-Fisher Scientific	G334	

Sodium Bromide	Thermo-Fisher Scientific	AC44680-1000	
EDTA	Thermo-Fisher Scientific	AC44608-5000	Stock solution: 0.5 M pH 8
Tris base	RPI	T60040-1000.0	Stock solution: 1 M pH 8
Ascorbic Acid	Fisher Scientific	AAA1775922	US Patent US7718389 sugg
Na_2SO_3	Sigma	S0505-250G	US Patent US8367357 sugg
Coelenterazine (native)	Nanolight / Prolume	3035MG	Stock solution: 1 mg/ml in a
OptiPlate-96, White Opaque 96-well Microplate	Perkin Elmer	6005290	Any opaque white 96 well p
Equipment			
Synergy H1 Hybrid plate reader or equivalent	BioTek	8041000	A plate reader with lumines
8-channel VOYAGER Pipette (50-1250 µL)	Integra	4724	An automated multichanne
8-channel 200 µL pipette	Transferpette S 20-200 µL	2703710	

ly expressing pcDNA3.1+rInsp-Ins-eGLuc and maintained in 250 ug/ml G418

n water. Freeze aliquots at -20C.

1N NaOH. Add equal amount of 0.1N HCl to any buffer where diazoxide is added.
ter
MSO

ested ascorbate can increase coelenterazine stability.
ested sulfite may decrease background due to BSA
acidified MeOH (2.36 mM)
plate should be sufficient. Clear bottom plates will also work, however some signal will be lost.

scence detection and 96-well plate capabilities is required.
l pipette is extremely useful for rapid addition of luciferase reagents and plating cells in 96 well format

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Author(s):	Michael A. Kalwat and Melanie H. Cobb

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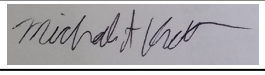
Signature:	
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Rebuttal Letter
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Dear Dr. Steindel,

Thanks to you and the reviewers for the helpful comments. We have addressed each comment in a point-by-point response below and amended the manuscript and figures accordingly. We hope the revised manuscript is now acceptable.

Best regards,

Michael Kalwat

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript.

2. Please include at least 6 key words or phrases.

We have now included the correct number of key words.

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We have removed commercial language from the manuscript. We took this to mean that including company names in the table of materials is acceptable. Please advise if this is incorrect and we can amend it.

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The Protocol appears to be under 2.75 pages, but we highlighted the essential parts in yellow.

2. For each protocol step/substep, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have checked each step and made any necessary changes as requested.

Specific Protocol steps:
1. 2.2: What volume of media (per well) is used?
Cells are plated in 100 µL. This text has been amended.

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2. Please cite Figure 1 in the main text of the manuscript (outside of the figure legends).

Thank you for pointing this out. We have now referenced Figure 1 in the introduction.

3. Please split the Figure file into 3 separate files, one per figure. Please remove 'Figure 1' etc. from the Figures themselves.

We have amended the figures as requested.

References:
1. Please do not abbreviate journal titles.

We have now listed the full journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have verified that the table of materials has all the necessary information.

2. Please remove the 'Recipes' table from the Table of materials and upload it as a separate 'Table' file (cited in the main text and with a legend).
We have separated the tables as requested.

Reviewers' comments:

Reviewer #1:

Minor Concerns:
1. in discussing the importance of confluence of MIN6 cells, it is alleged that at greater confluence the cultures are better synchronized due to increased cell cell contacts. All the references to back up this statement are to primary islets where gap junctions are indeed vital for efficient synchronization. However, gap junction-mediated synchronization has to my knowledge not proven to be at work in MIN6 cells.

We apologize for neglecting to reference additional work by others that demonstrated MIN6 cells express connexin 36 and have cell-cell contacts. For support of gap junctions mediated by connexin 36, please see Calabrese et al, Diabetes 2003. For support of EphA-Ephrin A cel-cell contact signaling in MIN6 cells please see Konstantinova, et al Cell 2007. We have now included these references in the discussion.

2. the discussion of alternative methods to study beta cell activity using proxies for insulin secretion is not complete without mentioning intracellular calcium, which can be detected using dyes or genetically encoded calcium sensors and generally tracks insulin secretion very closely.

We have now included additional text in the discussion to highlight using calcium influx as well as other optical measurements as readouts for beta cell function.

3. Future applications should list the possibility of making a reporter mouse line of this construct by introducing it into one of the insulin loci.

We have now mentioned this possibility in the discussion.

Reviewer #2:
Minor Concerns:
1) In figures 2& 3, the authors demonstrated fold stimulation of insulin secretion determined by GLuc assay. I wonder whether these relative stimulation data are correlated with released insulin assessed by specific ELISA or other assays.

Yes. We did not include the figure panel here, but we (Kalwat, et al. ACS Sensors 2016) and other (Burns, et al. Cell Metab 2015) have published that the co-secreted luciferase activity correlates well ($R^2 > 0.98$) with insulin secretion measured by ELISA.

2) Please provide information if incompletely processed insulin (including proinsulin) still exhibited GLuc activity, and if this may actually affect the relative assessment of insulin secretion.

Thank you for bringing up this point. We expect that Gaussia luciferase will retain activity even if it has not been processed out of the proinsulin molecule. Other researchers regularly use

Gaussia luciferase tagged to other much larger proteins to measure their secretion and the enzyme works as expected (Hulleman, et al. J Biomol Screen 2013). Additionally, other groups have knocked GFP into the insulin C-peptide between the exact same amino acids in the context of cell lines (Rajan S, et al AJP Endo Metab 2010) as well as a transgenic mouse (Zhu S, et al. Diabetes 2016), and this Insulin-GFP-C-peptide protein is processed properly and co-secreted. It is worth noting that GFP is ~25 kDa and Gaussia luciferase is ~18 kDa. Therefore, while we have not specifically measured the relative amount of processed vs. unprocessed InsGLuc, we think that the good correlation between secreted Gaussia activity and insulin measured by ELISA is sufficient for the purpose of this reporter assay. Of course we agree that to confirm results direct insulin measurements should be made and this is pointed out in the manuscript.

3) It is not clear whether Ref 7 has been already published. Please make this point clear.

Thank you for pointing that out. Ref 7 is a reference to a patent and the formatting needed to be corrected in Endnote. It is not yet published in a peer reviewed journal, but it is citable.

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Title: Insulin Promoter-Driven Gaussia Luciferase-Based Insulin Secretion Biosensor Assay for Discovery of β-Cell Glucose-Sensing Pathways

Author: Michael A. Kalwat, Chonlarat Wichaidit, Alejandra Y. Nava Garcia, et al

Publication: ACS Sensors

Publisher: American Chemical Society

Date: Oct 1, 2016

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