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High-throughput screening for insulin secretion modulators

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Abstract

The application of forward chemical genetics to insulin secretion in high-throughput has been uncommon because of high costs and technical challenges. However, with the advancement of secreted luciferase tools, it has become feasible for small laboratories to screen large numbers of compounds for effects on insulin secretion. The purpose of this chapter is to outline the methods involved in high-throughput screening for small molecules that chronically impact pancreatic beta cell function. Attention is given to specific points in the protocol that help to improve the dynamic range and reduce variability in the assay. Using this approach in 384-well format, at least 48 and as many as 144 plates can theoretically be processed per week. This protocol serves as a guideline and can be modified as required for alternate stimulation paradigms and improved upon as new technologies become available.

Keywords

high-throughput screening; insulin secretion; Gaussia luciferase; small molecules; pancreatic beta cells

1. Introduction

Diabetes afflicts over 425 million people worldwide and results from pancreatic islet dysfunction [1]. Blood glucose homeostasis is largely maintained by the concerted action of insulin secretion from islet beta cells. Consumption of nutrients leads to elevated blood glucose levels which are sensed by beta cells, stimulating them to release insulin. Insulin signals to peripheral skeletal muscle and adipose tissue to take up glucose, restoring euglycemia. In diabetes, the beta cells do not release enough insulin and eventually fail or perish. Chronically elevated blood glucose levels lead to disease pathologies including nephropathy, neuropathy and retinopathy. The inability of current therapies to preserve or fully restore diabetic islet function is directly tied to insufficient knowledge of nutrient-regulated secretion and limited pharmacological targets and interventions. Compounds that enhance or inhibit insulin secretion represent useful agents to identify important β cell regulatory pathways and provide novel pharmacological opportunities to stabilize β cell function in disease. However, high-throughput screens for chemical perturbagens of insulin secretion are rare [2–5], largely due to high cost and technical challenges.

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The goal of this methods chapter is to outline one successful high-throughput screening strategy and point out critical steps and places where the method may be improved upon. This approach relies on using a stable beta cell line expressing a luciferase reporter that is co-secreted with insulin [4, 5]. This system has been used at the bench in low to medium throughput experiments [6, 7]. Others have also used a similar system to discover compounds with short-term effects on glucose stimulated insulin secretion using beta cells in suspension [4, 8]. However, to discover compounds with more chronic, long-term effects, alternate technical methods are required.

2. Materials

1. Required equipment and high-throughput screening facility capabilities
    1. BioMek FX robotic liquid handler or suitable alternative.
    2. BioTek Multiflo FX liquid handler and 5μL cassette (see Note 1).
    3. Perkin Elmer EnVision multi-mode plate reader or equivalent.
    4. Table-top swing bucket centrifuge with collecting trays (see Note 2).
    5. Cell culture materials: T175 flasks, opaque white 384 well tissue culture-treated plates.
2. InsGLuc MIN6 cell culture
    1. InsGLuc-MIN6 cells (see Note 3).
    2. InsGLuc-MIN6 cell media: DMEM containing phenol red, 25 mM glucose, 0.11 g/L sodium pyruvate, 15 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 292 μg/mL L-glutamine, 50 μM β-mercaptoethanol, 250 μg/mL G418 (see Note 4).
3. InsGLuc secretion assay
    1. Stock solutions (see Note 5):
    2. Coelenterazine (CTZ): 1 mg/mL (2.36 mM) coelenterazine in acidified methanol. Keep stocks in screw-capped tubes sealed with parafilm at −80°C (see Note 6).
    3. Diazoxide: 11.53 mg/mL (50 mM) in 0.1 N NaOH. Freeze stocks at −20°C.
    4. 3 M KCl: 26.8 g in 120 mL water. Filter sterilize and store stock at room temperature.
    5. 2 M glucose: 36 g in 100 mL water. Filter sterilize and store stock at 4°C.
    6. Krebs-Ringer Bicarbonate Hepes (KRBH) buffer: 5 mM KCl, 120 mM NaCl, 15 mM Hepes pH 7.4, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$ (see Note 7).

7. Diazoxide-KRBH: KRBH with 250 μM diazoxide from the stock in 0.1 N NaOH. Add an equal amount of 0.1 N HCl to maintain the pH of the solution.
8. 2X KCl/glucose in Diazoxide-KRBH: 70 mM KCl, 40 mM glucose.
9. Ascorbate KRBH: 5 mM KCl, 15 mM Hepes pH 7.4, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 300 mM sodium ascorbate (see Note 8).
10. Gaussia luciferase working solution: Add 1.5 μL per mL of Ascorbate-KRBH to result in 3.54 μM CTZ. Prepared ~15 min before use.

3. Methods

1. Expansion and plating of InsGLuc MIN6 cells
   - **1.1.** On Day 1 (e.g. Monday), trypsinize 8–10 confluent T175 flasks. Suspend detached cells in media and spin down in swing bucket centrifuge at 800 rpm for 5 min to remove trypsin. Resuspend cell pellets in ~10mL per flask used and pass through a 45 μm cell strainer into a fresh 50ml conical tube.
   - **1.2.** Count the cells and dilute to 1.5×10^6 cells/mL in ≥500 mL of InsGLuc MIN6 cell media (see Note 9). Confirm diluted cell concentration on cell counter.
   - **1.3.** Plate 15–17 × 10^6 cells in each of 8–10 new T175 flasks with 20 mL media for plating/screening the following week (see Note 10).
   - **1.4.** Using BioTek Multiflo with the 5 μL cassette, prime at least 15–20ml through the tubing. Set the liquid handler to dispense 50 μL per well to each of 24 384 well opaque white cell culture dishes (see Note 11). Give the flask a gentle swirl in between each plating to ensure a homogenous cell slurry. Place the dishes without stacking in a 37°C tissue culture incubator for 24 h.
   - **1.5.** On Day 2 (after 24 h) the cells should be treated with control and test compounds on a liquid handling robot (see Note 12). The media does not need to be changed. Place the drug-treated cells back into the incubator for 24 h.
2. InsGLuc secretion assay
   - **2.1.** On Day 3 prepare the buffers needed for the screen: KRBH, Dz-KRBH, 2X KCl/Glucose-Dz-KRBH, and Asc-KRBH (see Note 13)
   - **2.2.** Prime the BioTek cassette on the Multiflo with KRBH (see Note 14).
   - **2.3.** Centrifuge plates upside down in collection trays at 30×g for 1min to remove the media. Wash the plate twice with KRBH buffer. First with 75 μL per well, then with 50 μL per well, centrifuging and blotting

on paper towels between each wash. After the final spin, add 25ul of KRBH-Dz buffer to all wells (see Note 15).

2.4. Incubate 60 min in tissue culture incubator. Note the time that particular set of plates was placed in the incubator.

2.5. Prime the BioTek cassette with 2X KCl/Glucose Dz-KRBH. After the 60 min incubation, remove the set of plates and add 25 μL of 2X KCl/Glucose Dz-KRBH to all wells. Note the time and return the set of plates to the incubator for 60 min.

2.6. During the incubation, prepare the Gaussia luciferase working buffer.

2.7. After the 60 min incubation, remove plates from incubator (see Note 16). Prime the cassette with Gaussia luciferase working solution and add 20 μL to each well.

2.8. Stack plates without lids and load into the Perkin Elmer EnVision plate reader (or suitable alternative). Read the plates at 0.1 s integration per well (see Note 17). An example of a 24 plate run is shown in Figure 1A. The summation of an entire 100,000 compound screen is shown as a plot in Figure 1B.

4. Notes

1. Carefully calibrate the cassette before starting a screen and always rinse it well with 70% ethanol. It is helpful to take apart the cassette every few weeks after heavy use and clean the nozzles out. Preferably, two cassettes should be used to reduce wear and tear during screening. One for plating cells and one for performing the washes and stimulations in the assay.

2. Access to two adjacent swing bucket centrifuges facilitates staggering plates in sets of four to allow a smooth 24 plate run. Beckman Coulter reservoirs (Cat # 372784) are a useful option for spinning 384-well plates upside-down to collect media or buffer. Centrifuging the media/buffer from plates was chosen subsequent to testing a liquid handler with a 96-tip vacuum manifold. Even on the gentlest setting, the vacuum manifold disrupted the MIN6 cell layer and led to variable Z-scores. Centrifuging the inverted plates at low speed was gentler, took a similar amount of time and led to higher and more consistent Z-scores.

3. InsGLuc MIN6 cells will be shared upon request. Others can also regenerate their own stable lines by transfecting the pcDNA3.1+rIns-hIns-eGLuc2.1 plasmid (Addgene #89928) into MIN6 or INS1 cells and selecting with G418. Alternatively, lentivirus can be made from the pLenti-rIns-hIns-eGLuc2.1 vector (Addgene #89927) and transduced cells can be selected with puromycin.

4. The stable InsGLuc MIN6 cell line was originally selected using G418 and so it is included in the media to help maintain expression of the transgene [5]. The line can be generated using lentivirus and puromycin selection as well [4].

5. When beginning a large screen, we found it helpful to make large stocks of all buffer components to last through the entire screen. This improves the stability and repeatability of the assay.

6. Acidified methanol is 1.06% HCl in methanol. Coelenterazine activity is highly batch dependent. Anecdotally, during these studies CTZ from RPI and NanoLight was tested. While the RPI CTZ had lower activity than the NanoLight CTZ, it was used for the screen because the slightly lower signal improved the dynamic range of the assay, leading to better Z-scores. It is recommended to test a small amount and then purchase enough of the same lot of CTZ to last for the entire screen in order to avoid switching lots/batches/vendors in the middle of the screen.

7. Normally, KRBH contains bovine serum albumin (BSA) at 1 mg/ml. We omitted BSA from the buffer to prevent bubbles and drips from occurring on the nozzles of the BioTek Multiflo cassette.

8. Sodium ascorbate is included to increase the stability of the Gaussia luciferase reaction with its substrate coelenterazine [9]. Sodium ascorbate contributes sodium ions, so NaCl is omitted from the buffer. The Ascorbate-KRBH buffer containing coelenterazine will eventually be diluted into KRBH (~1:3), reducing the final salt concentration. Coelenterazine can also be added to plain KRBH or PBS, however the half-life may be shorter. Avoid using any buffer that contains detergents which may lyse the cells and cause release of stored Gaussia luciferase.

9. 1.5E6 cells/mL results in a final concentration of 7.5e4 cells per 50 μL per well of a 384 well plate. This concentration was determined to result in the best Z-scores in this assay. Therefore, 28.8×10^6 cells per 384 well plate are needed. A confluent T175 yields ~120×10^6 cells. Because at least 7 T150s are needed to plate 24 × 384 well as well as plate new T175s for the following week, it is suggested to use 8–10 T175s and make >500 mL of cell suspension. This helps ensure there is sufficient volume to prime the BioTek cassette and account for the cassette dead volume.

10. Change media on the flasks one Wednesday and Friday if cells are plated on Monday. For cells plated on Tuesday, media can be changed on Friday only.

11. It is helpful when beginning this assay to also seed a clear bottom 384-well dish to monitor the density of the cells after plating and on the day of the assay, as well as confirm effects of any control compounds that cause visible changes to cell morphology.

12. MIN6 cells tolerate a 24 h treatment with 1–3% DMSO without impact on secretory response in this assay[5]. Therefore, 0.5 μL of DMSO (100%) or test compounds (0.5 mM) are typically added to the cells in 50 μL of media. The first or last column of the plate can be used for a positive control, such as thapsigargin (100 nM) or any chronic treatment that represses beta cell function.

13. Diazoxide targets the K_{ATP} channel, holding it open. In this state, glucose can only elicit exocytosis in the presence of depolarizing concentrations of KCl [10]. The diazoxide paradigm was chosen because it stimulates a large amount of secretion in response to nutrients without needing other drugs/hormones like GLP-1 or forskolin/IBMX. This generates a large dynamic range in the secretion assay, however a caveat is that inhibitors are much easier to detect than activators. To screen more specifically for chronic enhancers of beta cell function, a less potent stimulus (such as glucose alone) can be used in the assay. In that case, a stimulus like forskolin/IBMX or Dz/KCl/Glucose can be used in the positive control column for activation; glucose stimulation alone for compound-treated wells. The dynamic range may be smaller, but enhanced secretion should be more easily detected.

14. Excess primed buffer can be conserved by removing the nozzle/head portion of the cassette and aiming it into a 25 mL reservoir during the priming. The reserved buffer can be carefully added back to the source bottle.

15. With two centrifuges that can hold four plates each, a set of eight plates can be washed in ~15 min. Three sets of eight can be done in under and hour, allowing sufficient time to prime the 2X KCl/Glucose Dz-KRBH for stimulating the cells after 1 h of preincubation. This technical aspect is the main reason for choosing 24 plates in a run. Alternate methods or equipment may allow for increased numbers of plates to be screened at once.

16. Because the Gaussia luciferase working solution is at room temperature, cooling the plates to room temperature by placing them on a cool metal surface for 5 min prior to adding the substrate working solution will reduce variability in the luciferase reaction due to temperature changes.

17. Plates can be read within 5–10 min after substrate addition. Luciferase signal is still sufficient 20–30 min later as long as a sensitive reader is used.

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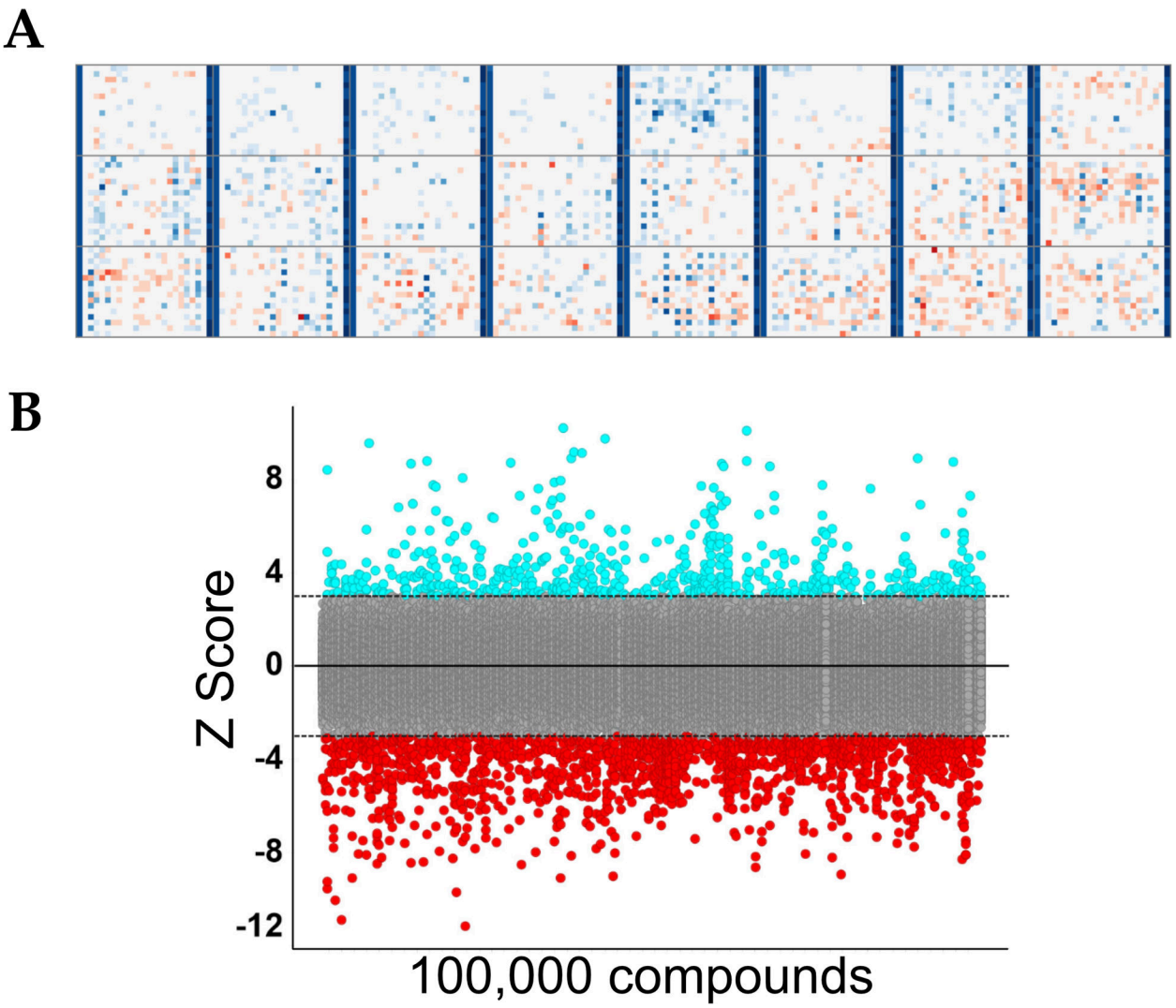

Figure 1. Example of high throughput screening results.

A) A representative example of a 24 plate run (7680 test compounds) with positive controls (low signal) in columns 1 and 24 and negative control DMSO (high signal) in columns 2 and 23. Columns 3 through 22 are all test compounds. Blue is suppressed and red is increased with respect to the negative control columns. **B)** Waterfall plot displaying overall results of an entire high throughput primary screen using the InsGLuc reporter in MIN6 cells. Hits with |Z-score| ≥ 3 are intended to be confirmed in triplicate followed by dose-response experiments and follow-up studies. Plot was generated in TIBCO Spotfire.