An assay for small scale screening of candidate β cell proliferative factors using intact islets

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Protocol For:

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Procedure:

1. Islets were isolated by the Islet Procurement and Analysis Core of the Vanderbilt Diabetes Research and Training Center as described previously (1).
2. Islets cultured in islet media were divided into even islet equivalents (IEQs) across a 96-well plate. (Not all wells need to be filled with islets.) 20 IEQs works well.
3. Incubate overnight at 37°C and 5% CO₂ in islet culture media.
4. Gently remove media from wells without disturbing the islets by using a multi-channel or single pipette.
5. Add 100 µl 0.1 mM EGTA supplemented media. In respective wells, include experimental mitogens and control vehicles in the 100 µl total volume.
6. Incubate at 37°C and 5% CO₂ for 48 hours.
7. Remove media as before and add fresh 0.1 mM EGTA and mitogen supplemented media to the correct wells.
8. Incubate at 37°C and 5% CO₂ for another 48 hours.
9. Add 10 µl resorufin to each well. Rotate plate to mix wells.
10. Incubate at 37°C and 5% CO₂ for 4 hours. This time was optimized for 20 IEQs per well in a 100 µl volume. More or less time may be needed depending on changes in IEQs or volume.
11. Read fluorescence with a plate reader (λex = 535±9 / λem 595±9) and determine which wells have elevated fluorescence emission in comparison with controls.
12. Remove media from wells of interest.
13. Gently rinse islets with PBS, taking care to not dislodge any islets from the wells.
14. Add 100 µl of a 0.025% Trypsin, 2 mM EDTA solution and incubate at RT, pipetting up and down every 2 minutes until islets are in single cells. (Confirm visually using a microscope.)
15. Add another 200 µl of media into the well and transfer to a 1.5 ml Eppendorf tube. Sometimes additional media is added to the wells to ensure all of the dissociated islets are transferred to the tube.
16. Centrifuge for 5 minutes at 1000 rpm and 4°C. If cells do not pellet due to the trypsin that remains, remove as much media as possible and dilute trypsin further by adding more media before centrifuging again.
17. Once pelleted, remove media and resuspend cells in 200 µl media.
18. Transfer resuspended dispersed islets to EZ cytofunnel that has been fitted with a charged microscope slide, following the manufacturer’s instructions.
19. Cytospin centrifuge for 5 minutes at 800 rpm.
20. Remove slides and let air dry at RT for 10 minutes.
21. Use a PAP pen around cells.
22. Add 200 µl of 4% paraformaldehyde (PFA) and incubate at RT for 10 minutes. Be sure to always gently add and remove solutions from the cells adhered to the slides.
23. Remove PFA and rinse with PBS for 5 minutes at RT (200 µl × 3).
24. Incubate adhered cells with 200 µl 0.2% Triton X-100 for 10 minutes.
25. Remove Triton X-100 and add 200 µl of Block Buffer [5% normal donkey serum (NDS) in PBS] and incubate 1 hour at RT.
26. Remove Block Buffer and incubate overnight at 4°C with 200 µl primary antibodies (1:500 dilution of both guinea pig anti-insulin and rabbit anti-Ki67).
27. The next morning, wash slides with PBS at RT (200 µl × 3).
28. Add 200 µl of secondary antibodies (1:300 dilution of both Cy2 anti-Guinea Pig and Cy3 anti-Rabbit and incubate at RT for 1–2 hours.
29. Remove antibody and rinse with PBS.
30. Incubate with 200 µl DAPI solution (1ug/ml) for 2 minutes.
31. Rinse slide with PBS, mount, and coverslip.

Reagents

- Resazurin or Alamar Blue (Invitrogen, catalog # D1025 Grand Island, NY)
- EGTA: Sigma, catalog # E4378, St. Louis, MO)
- Paraformaldehyde (Sigma, catalog # P6148)
- Triton-X100 (Fischer Scientific, catalog # BP151500, Fair Lawn, NJ)
- Trypsin/EDTA solution (Gibco, catalog # 25300, Grand Island, NY)
- Normal Donkey Serum (Jackson ImmunoResearch Laboratories, catalog # 117-000 West Grove, PA)
- Guinea Pig anti-Insulin (AbCam DAKO, Carpinteria, CA)
- Rabbit anti-Ki67 (AbCam, ab15580, Cambridge, MA)
- Cy2 anti-Guinea Pig (Jackson ImmunoResearch, Inc., catalog # 706-545-152)
- Cy3 anti-Rabbit (Jackson ImmunoResearch, catalog # 711-165-152)
- DAPI (Life Technologies, catalog # D1306, Grand Island, NY)
- Islet Culture Media: RPMI 1640, 10% horse serum, 11 mM glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin (Vanderbilt Molecular Cell Biology Resource Core, Nashville, TN)

Equipment:

- EZ cytofunnel (Thermoscientific, catalog # A78710003, Ashville, NC)
- Cytospin centrifuge (Thermoscientific, Shandon 4)
- 96-well tissue culture plates (Corning, catalog # 3603, Corning, NY)
- Synergy H4 plate reader (BioTek, Winooski, VT)