4

Detection of Insulin Production by Immunohistochemistry

Susan C. Campbell and Wendy M. Macfarlane

1. Introduction

Detection of insulin by immunocytochemistry is one of the most powerful and sensitive techniques available to monitor levels of expression in islets ***(1)***, β-cells in culture ***(2)***, tissue samples ***(3)***, and cells transgenically expressing the insulin gene ***(4)***. Like all immunocytochemistry techniques, much depends on the availability of a high-quality primary antibody. Species-specific insulin antibodies are now available from a number of commercial sources (including Santa Cruz), and these allow sensitive and specific detection of even very low levels of expression. Described below is the protocol for detection of insulin in β-cell lines in culture, using a FITC-coupled secondary antibody. A broad range of secondary antibodies are also available, including fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), and biotin-labeling, among others. For cells in culture, two-well, four-well, or eight-well chamber slides are recommended (available from several commercial sources, including Nunc), as these allow rapid and simple processing of large numbers of replicate samples, with small volumes of antibody. However, the following protocol can also be applied to cells grown on coated cover slips. The methods described here are for analysis by fluorescence microscopy. For analysis of insulin expression in intact islets, confocal microscopy would be required ***(1)***.

2. Materials

1. Blocking buffer: 0.7% Glycerol, 0.2% Tween-20, 2% bovine serum albumin (BSA). Store at 4°C.

From: *Methods in Molecular Medicine, vol. 83: Diabetes Mellitus: Methods and Protocols*
Edited by: S. Özcan © Humana Press Inc., Totowa, NJ

2. Chamber slides.
3. Cover slips.
4. FITC-coupled secondary anti-mouse antibody (Santa Cruz; Molecular Probes).
5. Anti-insulin primary antibody
6. Methanol (ice cold).
7. Phosphate-buffered saline (PBS): 150 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
8. Vectashield mounting medium with DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories). Store at 4°C in the dark
9. Wash buffer: 0.7% Glycerol, 0.4% Tween-20, 2% BSA. Store at 4°C.

3. Methods

1. Split insulin-producing cells into eight-well chamber slides and allow to grow until they are approx 50% confluent (*see* **Note 1**).
2. Wash cells four times in 1X PBS.
3. Fix cells by adding a large volume of ice-cold methanol and incubating at 4°C for 10 min (*see* **Note 2**).
4. Remove methanol and wash once in 1X PBS.
5. Add 200 µL blocking buffer and incubate at room temperature for 15 min (*see* **Note 3**).
6. Discard blocking buffer and add insulin primary antibody, which has been diluted 1:400 in 200 µL blocking buffer (*see* **Notes 4–6**).
7. Incubate at 4°C overnight.
8. Remove primary antibody and wash three times in blocking buffer.
9. Add FITC-coupled secondary antibody, which is diluted 1:400 in 200 µL blocking buffer, and incubate in the dark at room temperature for 1 h, with gentle agitation (*see* **Notes 7** and **8**).
10. Remove secondary antibody and rinse in wash buffer for 1 h with gentle agitation, in the dark.
11. Mount cells in vectashield with DAPI and affix cover slips (*see* **Note 9**).
12. Examine by fluorescent microscopy.

4. Notes

1. Cells can be plated onto coated cover slips (glass cover slips coated with 100 m*M* poly-L-lysine; Sigma) in six-well tissue culture dishes. However, chamber slides (available from Nunc) allow two, four, or eight replicate samples to be processed simultaneously and are easier to handle.
2. As an alternative to methanol, the cells can also be fixed in 4% *p*-formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Quench in 100 m*M* glycin in PBS for 15 min at room temperature.
3. For four-well chamber slides, adjust volume to 300 µL, for two-well chamber slides, adjust volume to 500 µL, and for six-well plates, adjust volume to 1 mL.
4. Insulin antibodies are available from a number of commercial sources depending on requirements (Linco Research, Santa Cruz).

5. Because the quality of the primary antibody depends on the source, initial titration of the primary antibody concentration will be required for each new antibody. However, 1:200 to 1:400 is a good starting point for initial experiments.
6. For each primary antibody, replicate control samples are essential, using primary antibody only, secondary antibody only, and preimmume serum (species specific) at the same dilutions as the specific primary/secondary antibodies.
7. FITC-coupled secondary antibodies are available from a number of commercial sources (Santa Cruz, Molecular Probes). Initial titration of antibody concentration will be required for each new antibody; however 1:400 is a good starting point for initial experiments. TRITC- and biotin-labeled secondary antibodies are available from several commercial sources and work equally as well as FITC using this protocol.
8. FITC and TRITC antibodies are light sensitive. Therefore, to decrease background, wrap slides in aluminum foil or incubate in a light proof box.
9. Use 15 µL vectashield with DAPI per well of an eight-well chamber slide and affix cover slips. DAPI forms flourescent complexes with double-stranded DNA and is used for staining of the nucleus.

References

1. Shih, D. Q., Heimesaat, M., Kuwajima, S., Stein, R., Wright, C. V. E., and Stoffel, M. (2002) Profound defects in pancreatic β-cell function in mice with combined heterozygous mutation in PDX-1, HNF-1α, and HNF-3β. *Proc. Natl. Acad. Sci. USA* **99**, 3818–3823.
2. Macfarlane, W. M., Campbell, S. C., Elrick, L. J., et al. (2000) Glucose regulates islet amyloid polypeptide gene transcription in a PDX1- and calcium-dependent manner. *J. Biol. Chem.* **275**, 15,330–15,335.
3. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **13**, 606–609.
4. Shaw, J. A., Delday, M. I., Hart, A. W., Docherty, H. M., Maltin, C. A., and Docherty, K. (2002) Secretion of bioactive human insulin following plasmid-mediated gene transfer to non-neuroendocrine cell lines, primary cultures and rat skeletal muscle in vivo. *J. Endocrinol.* **172**, 653–672.