

Immunoperoxidase on Cultured Cells

DAY ONE:

1. Fix cells on dishes by replacing the media with either 4%-8% Para. for one hour, Nakane for 4 hours, or 0.05% glut + 3% Para. for one hour.
2. Wash for 15mins in PBS over multiple changes.
3. Permeabilise with PBS/ 10% FCS/ 10uM glycine/ 0.05-0.1% Saponin/ 0.1% NaN₃ . For 10-15 minutes Note Saponin lot number, use fresh, (Non-expired)
4. Wash with the Saponin buffer 1X.
5. Dilute primary antibodies in the Saponin buffer, azide may be used to prevent bacterial growth. Incubate primary antibody in a level humid chamber overnight. Optimally have the first two hours at room temperature and then put the chamber in the cold room for the evening wrapped around the sides with parafilm. (aprox 0.5 ml diluted antibody per 35mm dish is needed to cover cells and keep them from drying out)

DAY TWO: **NO AZIDE!!!!!!!!!!**

1. Wash cells completely free of primary antibody and azide. 20 minutes many changes.
2. If necessary for antibody detection incubate bridge one hour in saponin buffer.
3. Wash for 30 minutes in Saponin buffer over multiple changes.
4. Incubate HRP conjugate (1:50 dilution in saponin buffer) on cells for two hours.
5. Wash for 20 minutes multiple changes with Saponin buffer.
6. Wash for 20 minutes multiple changes in **0.1M PBS/ 0.5%BSA (or FCS)**.
7. Wash 10 minutes in **0.1M Sodium Cacodylate buffer + 7.5% sucrose**, (pH7.4) Multiple changes.
8. Fix cells with **2.5% gluteraldehyde, 3% para , 5% sucrose in 0.1M cacodylate** buffer at room temperature for one hour.

9. Make DAB. **WARNING** DAB is a very dangerous chemical, always wear gloves and treat any/all waste in a bleach solution. !! For Polyscience DAB add 5 ml of 0.05M Sternberger Tris buffer to a 10 mg vial with syringe. Shake to dissolve. Pull out 5 ml of DAB/Tris. In clean beaker pH to 7.4 with 1N NaOH. It important that you do not over shoot the 7.4 mark. store freshly made DAB on a syringe, covered with foil and placed on ice.

Sternberger Tris Buffer:

pH 7.6

Trizma HCL 6.06gm

Trizma Base 1.39gm

DI H₂O 1 liter

After fixation.

1. Wash cells for 30 minutes in 0.1M Sodium Cacodylate + 7.5% sucrose, pH 7.4, multiple changes.
2. Rinse cells with Tris buffer + 7.5% sucrose (pH 7.4) for five minutes through multiple changes.
3. Perform DAB reaction. Add freshly made and filtered DAB to cells through a 0.22 Millipore filter with a 10 ml syringe. The cells should be kept on ice.
4. Monitor the reaction with an inverted phase microscope and keep track of the time and amount of H₂O₂ added. No more than 5 drops at a time. **Make H₂O₂ by mixing 50ul of 30% H₂O₂ into 3ml of ddH₂O.** Keep on ice.Use fresh H₂O₂.
5. Start reaction by adding H₂O₂ to the cells, in a drop wise fashion. Wait and observe
6. Stop the reaction by rinsing the cells 2X fast with Tris/ sucrose buffer.
7. Wash cells with sodium cacodylate buffer 2X.
8. Pellet cells, gently scrape with a cell lifter, spin in microfuge for 10 min.
9. Post-fix cell pellet with **reduced OsO₄** for one hour on ice, light tight under the hood. For one hour.

reduced osmium= 5 ml 0.2M Cac
+ 2.5 ML 4% OsO₄
+ 0.1 g KFe(CN) (10 mg/ml---Add just prior to use, be sure it dissolves.
+ ddH₂O to 10 ml

=1% OsO₄ + 1% KFe(CN) in 0.1 M Cac, ph 7.4 (osmium will be black)

10. Rinse with Cac buffer until clear. Take resin out of the freezer, let it come to room temperature.

11. Dehydrate cells with a graded series of ethanol (70,90,95,100,100,100).

12. Place in 50/50 100% ETOH / Epon for 30 minutes rotating.

13. Infuse with 100% Epon, rotating for 1 hour. Change Epon if possible, infuse for one more hour. Embed in fresh Epon and place in 60 degree oven overnight.

DAY THREE:

Start cutting.

Protocol taken from the W.M. Keck Microscopy Facility at The Whitehead
Institute website