

Double Immunohistochemistry of Cultured Cells

This protocol is designed for using two detection methods, in this case alkaline phosphatase and horse radish peroxidase, but can be modified for using only one method of detection. If only doing AP follow the black and red text, for HRP follow the black and blue text.

- Remove media from cultured cells and wash them with PBS
- Fix the cells with 4% Paraformaldehyde for 10 minutes on ice. If the cells to be stained are EBs or have large detailed structures fixation might need to be done for 20 minutes.
- Wash the cells 2X with PBS to remove the paraformaldehyde.
- Hydrogen peroxide treatment for 30 minutes at 4°C or on ice.
 - Hydrogen peroxide solution
50% Methanol, 10% H₂O₂ (from 30% stock), 40% PBS
- Wash with PBS two times for 5 minutes
- Block with blocking solution for at least one hour at room temperature or a couple of hours at 4°C
- Incubate with primary antibodies (in blocking solution) at 4°C overnight
- Wash with PBST (with 2mM levamisole) 3 X 10 minutes
- Add secondary antibodies (in blocking solution) and incubate for at least one hour at room temperature
- Wash with PBST (with 2mM levamisole) 3 X 10 minutes

When using multiple detection methods I find that doing the HRP detection first works better.

- Incubate samples in the appropriate detection buffer for 5-10 minutes
- Mix the detection solutions in the appropriate buffer making sure to follow the substrate directions, mix well.
- Add appropriate detection solution and incubate with samples until the desired colour is obtained.
- Wash samples with appropriate buffer to remove detection solution

Follow above instructions again for the second detection method

- Post-fix the samples with 4% PFA overnight at 4°C

Solutions and Reagents

4% Paraformaldehyde

Dissolve 2g of paraformaldehyde in 50ml of PBS without Ca and Mg at 65°C, shaking the tube as frequent as possible. Powder should dissolve within 2-3 hours. Filter using a 0.45um syringe filter. Can be stored at 4°C for about 1 week. Older solutions have worked but if using for embryos fresh should be made.

Blocking solution

1% serum from the animal that generates the secondary (usually conjugated) antibody
0.2% BSA (Sigma A9418, or any fraction V BSA)
2% skim milk (Difco/fisher 0032-17-3)

PBST

PBS with Ca and Mg with 0.05% Tween20 added to it.

For AP detection Levamisol (Sigma L-9756) should be added to this solution with a concentration of 2mM.

Detection Methods

I use detection solutions from Vector Labs. By choosing a secondary antibody conjugated to HRP or AP there are many options available for different colour detections.

The best AP colour that I've used is the Vector Red AP substrate kit 1 (Cat# SK-5100, about \$125 for at least 100mls of detection solution). This colour is great and does not fade with time but seems to get darker. You can also use a filter and see this colour under fluorescence and apparently it doesn't fade although I haven't tried this.

There are a number of different kits for the HRP. You can always use the DAB detection kit (Vector labs SK-4100, or Sigma D7304). I've also found that the Vector SG kit (Vector labs SK-4700) works and produces a blue/grey colour however the Vector VIP (SK-4600) seems to end up looking red in colour so it doesn't work well with the Red AP mentioned above.

**Make sure to follow the directions since some of these substrates need a specific buffer in order for the reaction to work well.