# PROTOCOL FOR IMMUNOCYTOCHEMISTRY

## Solutions

1. 4% paraformaldehyde (PFA) solution in PBS
   Mix 0.4 g PFA in 1.0 mL $dH_2O$ and add 100 µL 1N NaOH, and then dilute to 10mL with PBS. (Heat this mixture until the PFA has dissolved)
2. 100 mL 0.05% (v/v) Tween-20 in PBS (PBT)
3. 10 mL 1% (w/v) BSA in PBT
4. 20 mL 0.1% BSA in PBT (diluted from the 1% solution)

## Protocol

1. Plate cells in a 4-chamber glass slide. (The detail can be seen in cell culture protocol.)

2. Pour off the media from the wells, wash each chamber twice quickly with 0.5 mL PBS, and then fix the cells by adding 0.5 mL of 4% PFA to each chamber and incubating for 15 min.

3. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each, and then incubate the cells for 30 min. in 0.5 mL of 1% BSA.

4. Wash cells in PBT three times as previously described, then incubate cells in 0.2 mL of the working concentration of the primary antibody (about 1:200), diluted in 0.1% BSA, for 60 minutes at room temperature.

5. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each.

6. Incubate cells in 0.2 mL of the working concentration of the second antibody (about 1:500), diluted in 0.1% BSA, for 40 minutes at room temperature.

7. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each.

8. Incubate cells in the Streptavindin-HRP (three drops) for 40 minutes at room temperature.

9. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each.

10. Incubate the cells in 0.2 mL of ABS for 1 minutes at room temperature.

11. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each.

12. Add three drops of Heamatoxylin for 5 minutes at room temperature.

13. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each. Once again fix cells for 15 min. in 0.5 mL of 4% PFA.

14. Remove the plastic chamber piece and sealer holding it in place completely. And then place one drop of mounting solution (Permount) on each sheet of cells, and add coverslips to each sheet.

## IMMUNOHISTOCHEMISTRY PROCEDURES

### FIRST DAY

1. Deparaffination

Heat slides on warmer that has been preheated at 65°C - 70°C until wax melts (~15 min) (this is heat induced epitope retrieval- HIER). Prepare 40 mL (plastic bottle) or 45 mL (glass bottle for xylene) of each of the following solutions and place slide in bottle for specified amount of time (*keep solutions fresh), using the following sequence.

| | | | |
|---|---|---|---|
| Xylene 1 | 5 min | | |
| | Xylene 2 | 5 min | |
| | 100% EtoH | 5 min | |
| | 95% EtoH | 5 min | |
| 70% EtoH | 5 min | | |
| | 30% EtoH | 5 min | |
| | $H_2O$ | 2 min | |
| | $H_2O$ | | 2 min |

2. Put slides in beaker of 0.01M citrate buffer (pH=6.0), and place this beaker in a separate, larger beaker of boiling water. Citrate buffer should be 92°C-98°C when slide placed in it.

To prepare citrate buffer, use 18 mL buffer A, 82 mL buffer B, 900 mL water.

Buffer A: 0.1 M sodium citrate (29.41 g sodium citrate/1000 mL H2O)

Buffer B: 0.1 M citric acid (9.56 g citric acid/500 mL H2O).

Keep slides in citrate buffer (over boiling water) at this temperature for 20 minutes. Allow slides to cool in citrate buffer to room temperature.

3. Wash slide with 3% $H_2O_2$ (1.5 mL $H_2O_2$ in 48.5 mL MeOH) for 10 minutes.

4. Obtain container to hold slides and place wet paper towel in bottom to keep tissue environment moist at all times. Draw around the tissue pieces on the slide with pap pen to prevent leaking. Wash with PBS (rinsing buffer) for 2 minutes, twice.

To prepare rinsing buffer, follow kit instructions (p. 8) for specific volume.

In between and after washes dab back of slide, and front of slide around tissue to dry.

5. Incubate in block serum (is the same as the secondary antibody) for 30 minutes. Use 2 drops per tissue section.

6. Incubate with the primary antibody at 4°C overnight. Dilute primary antibody at 1:50. Keep primary antibody on ice at all times. Use 100 µL per slide (so 2 µL primary antibody diluted with 98 µL PBS). Put 50 µL of this solution on each tissue section (or enough to cover).

### SECOND DAY

7. Wash with PBS for 5 minutes, twice.

8. Incubate with the secondary antibody for 30 minutes at room temperature. Use 2 drops for each tissue section.
9. Wash with PBS for 5 minutes, twice.
10. Incubate with streptavidin-HRP for 30 minutes at room temperature.
11. Wash with PBS for 5 minutes, twice.
12. Add 50 µL of DAB (chromagen reagent) to each slide. For preparation of DAB solution, see kit instructions
(p. 8). Keep container of DAB solution covered in foil because it is light sensitive. Look at slide under microscope. When you see the backgroud turn brown, wash in $H_2O$ for 10 seconds.
13. Counterstain with Heamatoxylin for 5 minutes. Use 50 µL for each tissue section (or enough to cover). Observe slide under microscope.
14. Wash with $H_2O$ for 5 seconds, twice.
15. Place in 95 % EtoH for 15 seconds.
16. Place in 100 % EtoH for 15 seconds.
17. Place in Xylene for 15 seconds.
18. Add 2 drops mounting solution (Permount) to slide and add coverslips to each tissue section to save slides.

## IMMUNOFLUORESCENT STAINING PROCEDURES

**FIRST DAY**

1. Deparaffination

Heat slides on warmer that has been preheated at 65°C - 70°C until wax melts (~15 min). Prepare 40 mL (plastic bottle) or 45 mL (glass bottle for xylene) of each of the following solutions and place slide in bottle for specified amount of time (*keep solutions fresh weekly), using the following sequence.

| | | |
|---|---|---|
| Xylene 1 | | 5 min |
| Xylene 2 | 5 min | |
| 100% MeoH | | 5 min |
| 95% MeoH | 5 min | |
| 70% MeoH | 5 min | |
| 30% MeoH | | 5 min |
| $H_2O$ 1 | | 2 min |
| $H_2O$ 2 | | 2 min |

2. Put slides in beaker of 0.01M citrate buffer (pH=6.0), and place this beaker in a separate, larger beaker of boiling water. Citrate buffer should be 92°C-98 °C when slide placed in it.

Keep slides in citrate buffer (over boiling water) at this temperature for 20 minutes. Allow slides to cool in citrate buffer to room temperature.

4. Obtain container to hold slides and place wet paper towel in bottom to keep tissue environment moist at all times. Draw around the tissue pieces on the slide with pap pen to prevent leaking. Wash with PBST (PBS with 0.1% Tween 20) for 10 minutes, twice.

To prepare rinsing buffer, follow kit instructions (p. 8) for specific volume.

In between and after washes dab back of slide, and front of slide around tissue to dry.

5. Incubate in block serum (is the same as the secondary antibody) for 30 minutes. Dilute to 10% with 1% milk.
6. Incubate with the primary antibody at 4°C or Room temperature overnight. Dilute primary antibody at 1:50 with 1.5% serum and 0.5% milk. Keep primary antibody on ice at all times. Put 50 µL of this solution on each tissue section.

**SECOND DAY**

7. Wash with PBST for 10 minutes, thrice.
8. Incubate with the FITC-conjugated secondary antibody for 30 minutes at room temperature. Dilute 1:200 in PBST. Put 50µL of this solution on each tissue section.
9. Wash with PBST for 10 minutes, thrice.
10. Place the slides on the towel and use the cotton tip dipping with xylene cleaning the pep pen trace carefully. Don't touch any tissue section edge.
11. Cover with Vecta shield for delay fluorescence quenching.

**Negative Control (of the Primary antibody):**

Incubate with the block serum (the same as the secondary antibody).