2019

Immunohistochemistry Protocols

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Contents

Rabbit IHC

Day 1: Four Hours

1) Submerge slides in CCS for 4 min (Repeat 2x)[1]
2) Submerge slides in 100% ethanol for 4 min (Repeat 3x)
3) Submerge slides in 90% ethanol for 4 min
4) Submerge slides in 80% ethanol for 4 min
5) Submerge slides in 70% ethanol for 4min
6) Submerge slides in dH_2O for 5 min
7) Pressure boil slides in antigen retrieval buffer for 15min
8) Cool slides at room temperature for 15min
9) Wash slides in dH_2O for 5 min (Repeat 3x)
10) Submerge slides in 3% hydrogen peroxide for 10min
11) Wash slides in dH_2O for 5 min (Repeat 2x)
12) Prepare blocking solution
13) Place slides in staining setup[2]
14) Add 150-300µl blocking solution to each slide[3]
15) Incubate at room temperature for 30 min
16) Prepare diluted primary antibody
17) Add 150-300µl of primary antibody to the slides and incubate overnight at 4°C[4]

[1] When submerging, do not rapidly plunge the samples multiple times. The dunking motion can cause the sample to be washed off the cover slide. The time is optimized to allow for diffusion of each reagent.

[2] The best way to add slides to the coverplates is by submerging them both under water and pressing the slide to the coverplate. This is critical for pushing air bubbles out. If you skip this step or do it incorrectly you will bright blue spots that didn't receive primary.

[3] If you are not sure how much to add, use 200 µl. It may be beneficial to use 300µl if the tumor volume on the slide is excessive. The volume that you choose at this step should be conserved through the rest of the protocol.

[4] Refer to antibody data sheet for the suggested antibodies, use Signalstain® Antibody Diluent. Otherwise, dilute antibody in PBST-5% normal goat serum.

Day 2: Three Hours

1) Let slides come to room temperature for 10 min
2) Wash slides with the PBST (Repeat 3x)
3) Prepare biotinylated secondary antibody by diluting it in PBST and adding 150-300µl to each slide
4) Incubate for 30 min at room temperature.
5) Begin preparing the ABC reagent and let it sit at room temperature for 30 min before use
6) Wash with PBST (Repeat 3x)
7) Incubate slides in ABC reagent 150-300µl at room temperature for 30 min.
8) Wash with PBST buffer (Repeat 3x)[5]
9) Prepare DAB and 150-300µl of it into each slide[6]
10) As soon as the sections develop, immerse slides in dH_2O.
11) Stain with hematoxylin with 2-4 dunks[7]
12) Immediately gently rinse with water
13) Incubate sections in 95% ethanol for 5 min (Repeat 2x)
14) Incubate in 100% ethanol for 5 min (Repeat 3x)
15) Incubate in CCS for 5 min (Repeat 2x)
16) Using the cover slipping machine to mount Cytoseal® mount slides

[5] If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

[6] Timing of the DAB step can be subjective. Monitor your slides closely for signal development but if the proper primary concentration was chosen, you should not have to wait more than 5 min for it to develop. Longer development times leads to excessive background because, given enough time, DAB will develop independently of the primary.

[7] The number of dunks will determine how dark blue tissue is. For darker DAB, using more dunks. If hematoxylin is left out, it begins to crystallize. If you notice black clumps on your staining, you can filter your hematoxylin.

Reagents

- CitriSolv (CCS)
- Ethanol anhydrous denatured histological grade (100% EtOH)
- Deionized water (dH_2O)
- Harris Hematoxylin Stain
- Signalstain® Antibody Diluent: Antibody Diluent #8112
- Signalstain® Boost IHC Detection Reagent (HRP, Rabbit)
- Biotinylated secondary antibody
- ABC Reagent: (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA)
- DAB Reagent or suitable substrate

Recipes

- **TBST Wash buffer** (TBST for phospho-protein staining): Mix 0.2% Tween20 in 1L TBS
- **PBST Wash buffer**: Mix 0.2% Tween20 in 1L PBS
- **Blocking Solution**: Mix 5ml 1x TBST add 250µl normal goat serum
- **Blocking Solution**: Mix 5ml 1x PBST add 250µl normal goat serum
- **3% hydrogen peroxide**: Mix 10ml 30% H_2O_2 and 90ml dH_2O.
- **Sodium Citrate Buffer**: Mix 1 L add 5.88g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$), 2L dH_2O, and 1ml of HCL into 2L dH_2O (adjust pH to 6.0
- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH_2O. The pH will be around 9.0.

Mouse on Mouse (MOM) IHC

Day 1: Five Hours

1) Submerge slides in CCS for 4 min (Repeat 2x)[8]
2) Submerge slides in 100% ethanol for 4 min (Repeat 3x)
3) Submerge slides in 90% ethanol for 4 min
4) Submerge slides in 80% ethanol for 4 min
5) Submerge slides in 70% ethanol for 4min
6) Submerge slides in dH_2O for 5 min
7) Pressure boil slides in antigen retrieval buffer for 15min
8) Cool slides at room temperature for 15min
9) Wash slides in dH_2O for 5 min (Repeat 3x)
10) Submerge slides in 3% hydrogen peroxide for 10min
11) Wash slides in dH_2O for 5 min (Repeat 2x)
12) Place slides in staining setup[9]
13) Incubate in working solution of Mouse IgG Blocking Reagent
14) Incubate at room temperature for 1 hour
15) Wash with PBST (Repeat 3x)
16) Incubate for 5 min in working solution of M.O.M.™ Diluent
17) Prepare diluted primary antibody in M.O.M.™ Diluent
18) Add 150-300µl of primary antibody to the slides and incubate overnight at 4°C[10]

[8] When submerging, do not rapidly plunge the samples multiple times. The dunking motion can cause the sample to be washed off the cover slide. The time is optimized to allow for diffusion of each reagent.

[9] The best way to add slides to the coverplates is by submerging them both under water and pressing the slide to the coverplate. This is critical for pushing air bubbles out. If you skip this step or do it incorrectly you will bright blue spots that didn't receive primary.

[10] Refer to antibody data sheet for the suggested antibodies, use Signalstain® Antibody Diluent. Otherwise, dilute antibody in PBST-5% normal goat serum.

Day 2: Three Hours

1) Let slides come to room temperature for 10 min
2) Wash slides with the PBST (Repeat 3x)
3) Apply working solution of M.O.M.™ Biotinylated Antibody IgG Reagent
4) Incubate sections for 30 min
5) Begin preparing the ABC reagent and let it sit at room temperature for 30 min before use
6) Wash with PBST (Repeat 3x)
7) Incubate slides in ABC reagent 150-300µl at room temperature for 30 min.
8) Wash with PBST buffer (Repeat 3x)[11]
9) Prepare DAB and 150-300µl of it into each slide[12]
10) As soon as the sections develop, immerse slides in dH_2O.
11) Stain with hematoxylin with 2-4 dunks[13]
12) Immediately gently rinse with water
13) Incubate sections in 95% ethanol for 5 min (Repeat 2x)
14) Incubate in 100% ethanol for 5 min (Repeat 3x)
15) Incubate in CCS for 5 min (Repeat 2x)
16) Using the cover slipping machine to mount Cytoseal® mount slides

[11] If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

[12] Timing of the DAB step can be subjective. Monitor your slides closely for signal development but if the proper primary concentration was chosen, you should not have to wait more than 5 min for it to develop. Longer development times leads to excessive background because, given enough time, DAB will develop independently of the primary.

[13] The number of dunks will determine how dark blue tissue is. For darker DAB, using more dunks. If hematoxylin is left out, it begins to crystallize. If you notice black clumps on your staining, you can filter your hematoxylin.

Reagents

- CitriSolv (CCS)
- Ethanol anhydrous denatured histological grade (100% EtOH)
- Deionized water (dH_2O)
- Harris Hematoxylin Stain
- Biotinylated secondary antibody
- ABC Reagent: (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA)
- DAB Reagent or suitable substrate

Recipes

- **TBST Wash buffer** (TBST for phospho-protein staining): Mix 0.2% Tween20 in 1L TBS
- **PBST Wash buffer**: Mix 0.2% Tween20 in 1L PBS
- **Blocking Solution**: Mix 5ml 1x TBST add 250µl normal goat serum
- **Blocking Solution**: Mix 5ml 1x PBST add 250µl normal goat serum
- **3% hydrogen peroxide**: Mix 10ml 30% H_2O_2 and 90ml dH_2O.
- **Sodium Citrate Buffer**: Mix 1 L add 5.88g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$), 2L dH_2O, and 1ml of HCL into 2L dH_2O (adjust pH to 6.0
- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH_2O. The pH will be around 9.0
- **M.O.M.™ Mouse IgG Blocking Reagent**: Add 2 drops (90µl) of stock solution to 2.5ml of PBST
- **M.O.M.™ Diluent**: Add 600µl of protein concentrate stock solution to 7.5ml of PBST
- **M.O.M.™ Biotinylated Anti-Mouse IgG Reagent**: add 10µl of stock solution to 2.5ml of M.O.M.™ Diluent prepared as described above.

Cell Signaling Technology IHC

Day 1: Four Hours

1) Submerge slides in CCS for 4 min (Repeat 2x)[14]
2) Submerge slides in 100% ethanol for 4 min (Repeat 3x)
3) Submerge slides in 90% ethanol for 4 min
4) Submerge slides in 80% ethanol for 4 min
5) Submerge slides in 70% ethanol for 4min
6) Submerge slides in dH_2O for 5 min
7) Pressure boil slides in antigen retrieval buffer for 15min
8) Cool slides at room temperature for 15min
9) Wash slides in dH_2O for 5 min (Repeat 3x)
10) Submerge slides in 3% hydrogen peroxide for 10min
11) Wash slides in dH_2O for 5 min (Repeat 2x)
12) Prepare blocking solution
13) Place slides in staining setup[15]
14) Add 150-300µl blocking solution to each slide[16]
15) Incubate at room temperature for 30 min
16) Prepare diluted primary antibody in CST diluent
17) Add 150-300µl of primary antibody to the slides and incubate overnight at 4°C[17]

[14] When submerging, do not rapidly plunge the samples multiple times. The dunking motion can cause the sample to be washed off the cover slide. The time is optimized to allow for diffusion of each reagent.

[15] The best way to add slides to the coverplates is by submerging them both under water and pressing the slide to the coverplate. This is critical for pushing air bubbles out. If you skip this step or do it incorrectly you will bright blue spots that didn't receive primary.

[16] If you are not sure how much to add, use 200 µl. It may be beneficial to use 300µl if the tumor volume on the slide is excessive. The volume that you choose at this step should be conserved through the rest of the protocol.

[17] Refer to antibody data sheet for the suggested antibodies, use Signalstain® Antibody Diluent. Otherwise, dilute antibody in PBST-5% normal goat serum.

Day 2: Three Hours

1) Let slides come to room temperature for 10 min
2) Wash slides with the PBST (Repeat 3x)
3) Add CST secondary antibody
4) Incubate for 30 min at room temperature.
5) Wash with PBST buffer (Repeat 3x)[18]
6) Prepare DAB and 150-300µl of it into each slide[19]
7) As soon as the sections develop, immerse slides in dH_2O.
8) Stain with hematoxylin with 2-4 dunks[20]
9) Immediately gently rinse with water
10) Incubate sections in 95% ethanol for 5 min (Repeat 2x)
11) Incubate in 100% ethanol for 5 min (Repeat 3x)
12) Incubate in CCS for 5 min (Repeat 2x)
13) Using the cover slipping machine to mount Cytoseal® mount slides

[18] If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

[19] Timing of the DAB step can be subjective. Monitor your slides closely for signal development but if the proper primary concentration was chosen, you should not have to wait more than 5 min for it to develop. Longer development times leads to excessive background because, given enough time, DAB will develop independently of the primary.

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- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH_2O. The pH will be around 9.0