



# Immunohistochemistry Protocols

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# Rabbit IHC

## Day 1: Four Hours

- 1) Submerge slides in CCS for 4 min (Repeat 2x)<sup>1</sup>
- 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<sub>2</sub>O 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<sub>2</sub>O for 5 min (Repeat 3x)
- 10) Submerge slides in 3% hydrogen peroxide for 10min
- 11) Wash slides in dH<sub>2</sub>O for 5 min (Repeat 2x)
- 12) Prepare blocking solution
- 13) Place slides in staining setup<sup>2</sup>
- 14) Add 150-300μl blocking solution to each slide<sup>3</sup>
- 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<sup>4</sup>

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<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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.

<sup>4</sup> 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)<sup>5</sup>
- 9) Prepare DAB and 150-300µl of it into each slide<sup>6</sup>
- 10) As soon as the sections develop, immerse slides in dH<sub>2</sub>O.
- 11) Stain with hematoxylin with 2-4 dunks<sup>7</sup>
- 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

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<sup>5</sup> If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

<sup>6</sup> 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.

<sup>7</sup> 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<sub>2</sub>O)
- 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<sub>2</sub>O<sub>2</sub> and 90ml dH<sub>2</sub>O.
- **Sodium Citrate Buffer**: Mix 1 L add 5.88g sodium citrate trisodium salt dihydrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> • 2H<sub>2</sub>O), 2L dH<sub>2</sub>O, and 1ml of HCL into 2L dH<sub>2</sub>O (adjust pH to 6.0)
- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH<sub>2</sub>O. The pH will be around 9.0.

# Mouse on Mouse IHC

## Day 1: Five Hours

- 1) Submerge slides in CCS for 4 min (Repeat 2x)<sup>8</sup>
- 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<sub>2</sub>O 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<sub>2</sub>O for 5 min (Repeat 3x)
- 10) Submerge slides in 3% hydrogen peroxide for 10min
- 11) Wash slides in dH<sub>2</sub>O for 5 min (Repeat 2x)
- 12) Place slides in staining setup<sup>9</sup>
- 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.<sup>™</sup> Diluent
- 17) Prepare diluted primary antibody in M.O.M.<sup>™</sup> Diluent
- 18) Add 150-300µl of primary antibody to the slides and incubate overnight at 4°C<sup>10</sup>

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<sup>8</sup> 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.

<sup>9</sup> 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.

<sup>10</sup> Refer to antibody data sheet for the suggested antibodies, use Signalstain<sup>®</sup> 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.<sup>™</sup> 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)<sup>11</sup>
- 9) Prepare DAB and 150-300µl of it into each slide<sup>12</sup>
- 10) As soon as the sections develop, immerse slides in dH<sub>2</sub>O.
- 11) Stain with hematoxylin with 2-4 dunks<sup>13</sup>
- 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<sup>®</sup> mount slides

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<sup>11</sup> If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

<sup>12</sup> 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.

<sup>13</sup> 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<sub>2</sub>O)
- 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<sub>2</sub>O<sub>2</sub> and 90ml dH<sub>2</sub>O.
- **Sodium Citrate Buffer**: Mix 1 L add 5.88g sodium citrate trisodium salt dihydrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> • 2H<sub>2</sub>O), 2L dH<sub>2</sub>O, and 1ml of HCL into 2L dH<sub>2</sub>O (adjust pH to 6.0)
- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH<sub>2</sub>O. The pH will be around 9.0
- **M.O.M.<sup>™</sup> Mouse IgG Blocking Reagent**: Add 2 drops (90µl) of stock solution to 2.5ml of PBST
- **M.O.M.<sup>™</sup> Diluent**: Add 600µl of protein concentrate stock solution to 7.5ml of PBST
- **M.O.M.<sup>™</sup> Biotinylated Anti-Mouse IgG Reagent**: add 10µl of stock solution to 2.5ml of M.O.M.<sup>™</sup> Diluent prepared as described above.



# Cell Signaling Technology IHC

## Day 1: Four Hours

- 1) Submerge slides in CCS for 4 min (Repeat 2x)<sup>14</sup>
- 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<sub>2</sub>O 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<sub>2</sub>O for 5 min (Repeat 3x)
- 10) Submerge slides in 3% hydrogen peroxide for 10min
- 11) Wash slides in dH<sub>2</sub>O for 5 min (Repeat 2x)
- 12) Prepare blocking solution
- 13) Place slides in staining setup<sup>15</sup>
- 14) Add 150-300µl blocking solution to each slide<sup>16</sup>
- 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<sup>17</sup>

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<sup>14</sup> 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.

<sup>15</sup> 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.

<sup>16</sup> 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.

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

## Day 2: Two 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)<sup>18</sup>
- 6) Prepare DAB and 150-300µl of it into each slide<sup>19</sup>
- 7) As soon as the sections develop, immerse slides in dH<sub>2</sub>O.
- 8) Stain with hematoxylin with 2-4 dunks<sup>20</sup>
- 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

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<sup>18</sup> If you forget this wash step, the entire slide will stain dark because the ABC is what reacts with the DAB

<sup>19</sup> 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.

<sup>20</sup> 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

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## Recipes

- **TBST Wash buffer** (TBST for phospho-protein staining): Mix 0.2% Tween20 in 1L TBS
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- **Blocking Solution**: Mix 5ml 1x PBST add 250µl normal goat serum
- **3% hydrogen peroxide**: Mix 10ml 30% H<sub>2</sub>O<sub>2</sub> and 90ml dH<sub>2</sub>O.
- **Sodium Citrate Buffer**: Mix 1 L add 5.88g sodium citrate trisodium salt dihydrate (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> • 2H<sub>2</sub>O), 2L dH<sub>2</sub>O, and 1ml of HCL into 2L dH<sub>2</sub>O (adjust pH to 6.0)
- **TE Buffer**: Mix 2.42g Tris base, 0.74g EDTA, 2L of dH<sub>2</sub>O. The pH will be around 9.0

## My Antibody Suggestions

*These are the IHC approved antibodies that I have optimized for staining in the Oliver Lab. Some of them are ideal and others may need further optimization. I have indicated the quality of the current conditions as either poor, fair, excellent.*

### Rabbit

- **Adipophilin** (Thermo Fisher PAI-16972, Rabbit) **1:200**, DAB 3:00 min, citrate buffer · Fair
- **Axin2** (Abcam ab109307, Rabbit) **1:100**, DAB 5:00 min, TE buffer · Good
- **Ccsp** (Millipore ab07623, Rabbit) **1:1000**, DAB 5:00 min, citrate buffer · Fair
- **CGRP** (Sigma C8198, Rabbit) 1:250, DAB 5:00 min, citrate buffer · Good
- **ΔNp63** (Novus NBP2-29467, Rabbit) **1:200**, DAB 6:30 min, citrate buffer · Fair
- **FoxA2** (Abcam ab108422, Rabbit) **1:1000**, DAB 3:00 min, citrate buffer · Excellent
- **Krt5** (Covance PRB-160P, Rabbit) **1:2000**, DAB 4:00 min, citrate buffer · Excellent
- **Krt13** (Abcam ab92551, Rabbit) **1:250**, DAB 3:00 min, citrate buffer · Excellent
- **Ly6g** (BioXCell BE0075-1, Rabbit) **1:1000**, DAB 5 min, citrate buffer · Fair
- **Mpo** (Novus NBP1-42591, Rabbit) **1:250**, DAB 4:30 min, citrate buffer · Fair
- **Myc** (Santa Cruz sc-764, Rabbit) **1:150**, DAB 2:00 min, citrate buffer · Excellent
- **NeuroD1** (Abcam ab109224, Rabbit) **1:150**, DAB 5:00 min, citrate buffer · Fair
- **Nfib** (Sigma HPA0D3956, Rabbit) **1:250**, DAB 5:00 min, citrate buffer · Excellent
- **Ngfr** (Abcam ab52987, Rabbit) **1:50**, DAB 13:00 min, citrate buffer · Poor
- **Nkx2-1** (Abcam ab76013, Rabbit) **1:250**, DAB 4:00 min, citrate buffer · Excellent
- **Nqo-1** (GenTex 40135, Rabbit) **1:500**, DAB 3:00 min, citrate buffer · Fair
- **Pou2f3** (Sigma HPA019652, Rabbit) **1:300**, DAB 5 min, citrate buffer · Good
- **Pou2f3** (Invitrogen PA5-38028) **1:250**, DAB 3:00 min, citrate · Fair
- **Psca** (Fisher Scientific PIPA511463, Rabbit) **1:100**, DAB 10:00 min, citrate buffer · Fair
- **Rest** (Abcam ab202962, Rabbit) **?:??**, DAB 5:00 min, citrate buffer · fair
- **Runx2** (Abcam ab192256, Rabbit) **1:1000**, DAB 2:50 min, TE buffer · Excellent
- **Sox9** (Abcam ab185966, Rabbit) **1:1000**, DAB 6:00 min, citrate buffer · Excellent
- **Sox6** (Abcam ab30455, Rabbit) **1:200**, DAB 7:00 min, citrate buffer · N/A
- **Spc** (Millipore ab3786, Rabbit) **1:1000**, DAB 10:00 min, citrate buffer · Poor
- **Sp7** (Abcam ab209484, Rabbit) **1:1000**, DAB 2:50 min, TE buffer · Excellent
- **Syp** (Thermo-fisher RB1461P1) **1:200**, DAB 5:00 min, citrate buffer · Good
- **Tgfb $\beta$ 2** (Santa Cruz sc-220, Rabbit) **1:100**, DAB 5:00 min, citrate buffer · Excellent
- **Uchl1** (Sigma hpa005993, Rabbit) **1:300**, DAB 5:00 min, citrate · Good
- **8ohdg** (Santa Cruz sc-66036, Rabbit) **1:400**, DAB 5:00 min, citrate buffer · Good

### Mouse on Mouse

- **Ascl1** (Fisher Sci BDB556604, Rabbit) **1:200**, DAB 7:00 min, TE buffer · Good
- **Insm1** (sc-271408 sc-271408, Rabbit) **1:100**, DAB 5:00 min, citrate buffer · Fair

### Cell Signaling

- **Ctnnb1** (CST #9587S, Rabbit) **1:250**, DAB 5:00 min, citrate buffer · Excellent
- **Gfp** (CST #2956S) **1:100**, DAB 10:00 min, citrate buffer · Fair

## My Antibody Suggestions

- **Hes1** (CST #11988S) **1:600**, DAB 5:00 min, citrate buffer · *Fair*
- **Hnf4 $\alpha$**  (CST #3113) **1:300**, DAB 5 min, citrate buffer · *Fair*
- **H3k27me3** (CST #9733S) **1:200**, DAB 3:30 min, citrate buffer · *Excellent*
- **Notch1** (CST #4147S) **1:100**, DAB 12 min, citrate buffer · *Fair*
- **Yap** (CST #14074S) **1:300**, DAB 5 min, citrate buffer · *Fair*