

# Mouse Embryo

*Which RapiClear reagent is right for your mouse embryo samples?*

| Sample            |            | RapiClear (RC)       | Clearing Time | FPs preservation <sup>(1)</sup> | Antibody penetration | Lipid dye | Nucleic acid dye | Method   |
|-------------------|------------|----------------------|---------------|---------------------------------|----------------------|-----------|------------------|----------|
| Slice (thickness) | <500um     | RC147                | hours         | ★★★★                            | ✓                    | ✓         | ✓                | Page 1~2 |
|                   | 500~1000um | RC149/152            | hours         | ★★★★                            | ✓                    | ✓         | ✓                | Page 1~2 |
| Whole embryo      | <E12.5     | RC149                | days          | ★★★★                            | ✓ <sup>(2)</sup>     | ✓         | ✓                | Page 3~4 |
|                   | >E12.5     | RC155 <sup>(3)</sup> | week          | ★★★                             | ✓ <sup>(2)</sup>     | N.D.      | N.D.             | Page 5~6 |
|                   |            | RCCS <sup>(4)</sup>  | weeks         | ★★★                             | ✓ <sup>(2)</sup>     | ✗         | ✓                | Page 7~8 |

















<sup>(1)</sup>FPs, Fluorescent Proteins

<sup>(2)</sup>Immunostaining for whole mouse embryo may have limited antibody penetration depth

<sup>(3)</sup>RapiClear 1.55 should work with ScaleB4 solution

<sup>(4)</sup>RapiClear CS should work with CLARITY™ technology

# Slice RapiClear 1.47/1.49/1.52

|   |  |   |
|---|--|---|
| <p><b>1</b>  room temp.  1hr</p> <p><b>Perfusion:</b><br/>Animal fixed with 4% PFA via cardiac perfusion</p>                                    | <p><b>4</b>  room temp.  1hr</p> <p><b>Section:</b><br/>Embryo can be sectioned manually (e.g., 500<math>\mu</math>m or 1000<math>\mu</math>m)</p>                               | <p><b>7</b>  room temp.  O/N</p> <p><b>Clear:</b><br/>Slices were incubated in 1ml RapiClear solution. Reagent can be reused for no more than 3 times (<i>on an orbital shaker</i>)</p> |
| <p><b>2</b>  4°C  O/N</p> <p><b>Fixation:</b><br/>Embryo subjected to 10~20ml PFA solution in a 50ml tube<br/><i>(on an orbital shaker)</i></p> | <p><b>5</b>  room temp.  2hr</p> <p><b>Fixation :</b><br/>Slices fixed with 4% PFA again<br/><i>(on an orbital shaker)</i></p>   | <p><b>8</b>  room temp.</p> <p><b>Mount:</b><br/>Slices placed in fresh RapiClear between two coverslips separated with iSpacer</p>  |
| <p><b>3</b>  room temp.  1hr</p> <p><b>Wash:</b><br/>Wash with PBS 3 times, 20min/time<br/><i>(on an orbital shaker)</i></p>                | <p><b>6</b>  room temp.  O/N</p> <p><b>Permeabilization:</b><br/>Wash with PBS 3 times and then transfer samples into 2% PBST solution<br/><i>(on an orbital shaker)</i></p> | <p><b>9</b>  room temp.</p> <p><b>Image:</b><br/>Confocal or 2-photon<br/><b>Option:</b><br/><i>Staining of antibody or nucleic acid dyes should follow the protocol in next page</i></p>  |

# Protocol:

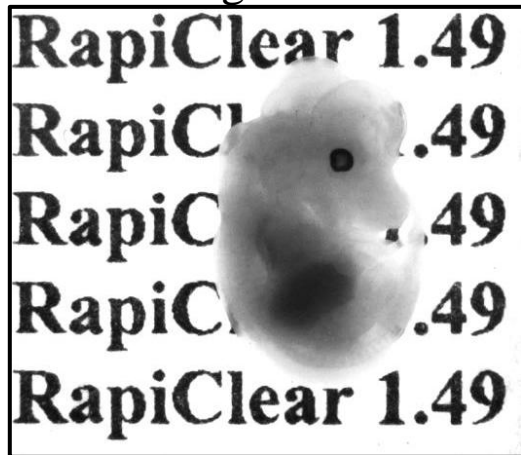
# Slices RapiClear 1.47/1.49/1.52

**\*\*\*To prepare any type of chemical, please read the MSDS book to determine what types of safety clothing and equipment you should wear\*\*\***

1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with ice-cold 4% PFA at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml PFA solution. (Note: PFA is toxic. Perform all procedures in a fume hood.)
3. Incubate the embryos in the PFA solution overnight at 4°C with gentle shaking.
4. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
5. Embryos can be sectioned manually (e.g., 500µm for RC147 clearing or 1000µm for RC149/RC152 clearing) using either vibratome, cryo-section, or a tissue matrix with razor blades.
6. Fix the sample slices in a 12-well plate with 4% PFA solution on an orbital shaker or rocker for 2h at RT.
7. Wash the samples three times with PBS (10min/time) at RT with gentle shaking.
8. Transfer samples into 2% PBST (2% Triton-X100) solution overnight at RT for permeabilization.
9. Option: Immunostaining
  - 9-1. Keep the specimen in blocking buffer on an orbital shaker or rocker at 4°C overnight. Blocking buffer (10% normal goat serum, 2% Triton-X 100, and 0.2% sodium azide in PBS): Store this solution for only a short period of time (overnight at most) at 4°C.
  - 9-2. Incubate the specimen with primary antibody in a 24-well plate (500 µl/well) on an orbital shaker or rocker at 4°C for 3~5 days. Ab dilution buffer (1% normal goat serum, 0.2% Triton-X 100, and 0.2% sodium azide in PBS): Store this solution for only a short period of time (overnight at most) at 4°C.
  - 9-3. Wash the specimen twice with washing buffer for >1hr at room temperature. Then keep the specimen in washing buffer on an orbital shaker or rocker at 4°C, overnight. (Note: washing step is quite important for immunostaining!) Washing buffer (3% NaCl and 0.2% Triton-X 100 in PBS): Store this nonhazardous buffer at 4°C.
  - 9-4. Incubate the specimen with secondary antibody on an orbital shaker or rocker at 4°C for 1~2 days.
  - 9-5. Wash the specimen twice with washing buffer for >1hr at room temperature. Then keep the specimen in washing buffer on an orbital shaker or rocker at 4°C, overnight. (Note: washing step is quite important for immunostaining!)
10. Option: DAPI staining
  - 10-1. Incubate the sample with DAPI on an orbital shaker or rocker at 4°C, overnight. DAPI (Invitrogen D3571, preparing 1mg/ml for stocks) working solution, 1:2000 of stocks.
  - 10-2. Wash the sample three times with PBS (20min/time) at RT. Then keep the sample in fresh PBS on an orbital shaker or rocker at 4°C, overnight.
11. Kimwipes can be used to remove the residual PBS from the sample surface.
12. Incubate samples in 1ml RapiClear solution (it is reusable, but no more than three times) with gentle shaking and periodically check the clarity of the sample over the next few hours. Pre-warm the RC reagent to 37°C before mounting can facilitate solution penetration. Once the sample is fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear for several weeks at room temperature without affecting imaging quality.
13. Mount the sample in fresh RapiClear reagent between two coverslips separated by iSpacer. Press gently around the sticker to seal the coverslips.
14. Remove the extra solution at the edges with Kimwipes.
15. Fill the space outside the iSpacer with clear nail polish to seal the edges between the two coverslips.
16. Before RapiClear (or after), the sample may be stored indefinitely in PBS [with 0.1% (wt/vol) sodium azide] at 4°C.
17. Confocal or 2-Photon microscopy imaging.

### E12.5 embryo

#### Pre-clearing



#### Post-clearing



**1** room temp. 1hr

**Perfusion :**  
Animal fixed with 4% PFA via cardiac perfusion

**4** 4°C 3 days

**Permeabilization:**  
Add 3ml 2% Triton X-100, refresh after 24hr  
*(on an orbital shaker)*

**7** room temp.

**Mount:**  
Mount the sample in fresh RC149 between two coverslips separated with iSpacer

**2** 4°C O/N

**Fixation:**  
Embryo subjected to 3ml 4% PFA with 2% Triton X-100 in a 12-well plate  
*(on an orbital shaker)*

**5** room temp. 1hr

**Wash:**  
Wash with PBS 3 times, 20 min/time, and then remove excess PBS from sample  
*(on an orbital shaker)*

**8** room temp.

**Image:**  
Confocal or 2-photon microscopy

**3** room temp. 1hr

**Wash:**  
Wash with PBS 3 times, 20 min/time  
*(on an orbital shaker)*

**6** room temp. 2 days

















**Clear:**  
Add 2ml of RapiClear 1.49 (RC149). Reagent can be reused for no more than 3 times  
*(on an orbital shaker)*

**Option:**  
*Staining of antibody or nucleic acid dyes should follow the protocol in next page*

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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with ice-cold 4% PFA at ~10 ml/min. Recover the tissues from the mice and place it in the 10~20 ml PFA solution. (Note: PFA is toxic. Perform all procedures in a fume hood.)
3. Embryos were left to incubate in the PFA solution containing 2% Triton X-100 overnight at 4°C with gentle shaking.
4. Wash the samples three times with PBS (20min/time) at RT.
5. Transfer the sample into 2% PBST (2% Triton-X100 and 0.2% sodium azide in PBS) solution three days at 4°C with gentle shaking for permeabilization.
6. Wash the samples three times with PBS (20min/time) at RT.
7. Option: Immunostaining
  - 7-1. Keep the specimen in blocking buffer on an orbital shaker or rocker at 4°C for 2 days. Blocking buffer (10% normal goat serum, 2% Triton-X 100, and 0.2% sodium azide in PBS): Store this solution for only a short period of time (overnight at most) at 4°C.
  - 7-2. Incubate the specimen with 2.5ml of primary antibody in a small tube or bottle on an orbital shaker or rocker at 4°C for 5~7 days. Ab dilution buffer (1% normal goat serum, 0.2% Triton-X 100, and 0.2% sodium azide in PBS) : Store this solution for only a short period of time (overnight at most) at 4°C.
  - 7-3. Wash the specimen twice with washing buffer for >2hr at room temperature. Then keep the specimen in washing buffer on an orbital shaker or rocker at 4°C, overnight. (Note: washing step is quite important for immunostaining!) Washing buffer (3% NaCl and 0.2% Triton-X 100 in PBS): Store this nonhazardous buffer at 4°C.
  - 7-4. Incubate the specimen with secondary antibody on an orbital shaker or rocker at 4°C for 3 days.
  - 7-5. Wash the specimen twice with washing buffer for >2hr at room temperature. Then keep the specimen in washing buffer on an orbital shaker or rocker at 4°C, overnight. (Note: washing step is quite important for immunostaining!)
8. Option: DAPI staining
  - 8-1. Incubate the sample with DAPI on an orbital shaker or rocker at 4°C, overnight. DAPI (Invitrogen D3571, preparing 1mg/ml for stocks) working solution, 1:2000 of stocks.
  - 8-2. Wash the sample three times with PBS (20min/time) at RT. Then keep the sample in fresh PBS on an orbital shaker or rocker at 4°C for overnight.
9. Kimwipes can be used to remove the residual PBS from the sample surface.
10. Incubate the samples in 2~3ml RapiClear1.49 solution (it is reusable, but no more than three times) and periodically check the visual clarity of the sample over the next few hours. An intact mouse embryo will typically complete the refractive index (RI) homogenization process in 24~48hr. Once the sample has been fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear1.49 for several weeks at 4°C without affecting imaging quality.
11. Mount the sample in fresh RapiClear1.49 reagent between two coverslips separated by iSpacer. Press gently around the sticker to seal the coverslips.
12. Remove the extra solution at the edges with Kimwipes.
13. Fill the space outside the iSpacer with clear nail polish to seal the edges between the two coverslips.
14. Before RapiClear1.49 (or after), the sample may be stored indefinitely in PBS [with 0.1% (wt/vol) sodium azide] at 4°C.
15. Confocal, 2-photon or Light-sheet microscopy imaging.

# RapiClear 1.55

|  |   |  |
|--|---|--|
| <p><b>1</b>  room temp.  1hr</p> <p><b>Perfusion:</b><br/>Mouse fixed by 4% PFA via cardiac perfusion</p>  | <p><b>4</b>  -20°C  O/N</p> <p><b>OCT embedding:</b><br/>Embed the embryo in OCT compound and freeze</p>  | <p><b>7</b>  room temp.  2~4 days</p> <p><b>Clear:</b><br/>Subject the embryo in 15ml of working solution prepared by diluting RapiClear 1.55 with ScaleB4</p> |
| <p><b>2</b>  4°C  O/N</p> <p><b>Fixation:</b><br/>Subject the embryo in 20ml 4% PFA with 2% Triton X-100 solution in a 50ml tube<br/><i>(on an orbital shaker)</i></p> | <p><b>5</b>  room temp.  2hr</p> <p><b>Fixation:</b><br/>Subject the embryo in 20ml 4% PFA again in a 50ml tube</p>   | <p><b>8</b>  room temp.</p> <p><b>Mount:</b><br/>Mount the embryo in fresh working solution between two coverslips separated with iSpacer</p>   |
| <p><b>3</b>  4°C  24hr</p> <p><b>Cryoprotection:</b><br/>Subject the embryo in 50ml 20% sucrose/PBS solution</p>   | <p><b>6</b>  room temp.  5~10 days</p> <p><b>ScaleB4:</b><br/>Subject the embryo in 50ml ScaleB4 solution, refresh ScaleB4 every two days<br/><i>(on an orbital shaker)</i></p> | <p><b>9</b>  room temp.</p> <p><b>Image:</b><br/>Confocal, 2-photon, or light-sheet microscopy</p>   |



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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with ice-cold 4% PFA at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml PFA solution. (Note: PFA is toxic. Perform all procedures in a fume hood.)
3. Embryos is left to incubate in the PFA solution containing 2% Triton X-100 overnight at 4°C with gently shaking.
4. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
5. Transfer the samples to 20% sucrose/PBS (wt/vol) at 4°C for 1 day.
6. Embed the samples in OCT compound and freeze overnight.
7. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
8. Samples are fixed again with 4% PFA solution for 2hr at RT with gentle shaking.
9. Wash the samples three times with PBS (20min/time) at RT with gentle shaking.
10. Treat the samples with 50ml ScaleB4 solution (8M Urea with 0.1% Triton X-100) for 5~10 days at RT with gentle shaking. Refresh ScaleB4 solution every two days. (Note: During the preparation of the ScaleB4 solution, solution will initially become cold to the touch. A 25~28 °C water bath will aid in the dissolution of the powdered urea. Solution can be stirred with a heavy magnetic bar and the temperature should never exceed 30°C. Do not allow the temperature to rise above 30 °C, since urea may begin to breakdown into isocyanate, which is detrimental to the proteins.)
11. Option: Immunostaining.
12. Kimwipes can be used to remove the residual solution from the tissue surface.
13. Samples were transferred to 15ml working solution prepared by 1:1 diluting RapiClear® 1.55 with ScaleB4 in a 50ml tube on to a rotator (Elmi Intelli-Mixer, Rose Scientific Ltd) for 2~4 days at RT. (Note: Pre-warm the RapiClear® to 40°C before mounting can facilitate solution penetration.)
14. Once the sample is fully clarified, proceed with imaging preparations. Sample can be stored in working solution for several weeks at room temperature without affecting imaging quality.
15. Mount the sample in fresh working solution between two coverslips separated by iSpacer. Press gently around the sticker to seal the coverslips.
16. Remove the extra solution at the edges with Kimwipes.
17. Before working solution prepared by 1:1 diluting RapiClear® 1.55 with ScaleB4 (or after), the sample may be stored indefinitely in PBS [with 0.1% (wt/vol) sodium azide] at 4°C.
18. Confocal, 2-photon or Light-sheet microscopy imaging.

## >E12.5

# RapiClear CS

E14.5 embryo

Pre-clearing



Post-clearing



|   |  |  |
|---|--|--|
| <p><b>1</b>  room temp.  1hr</p> <p><b>Perfusion:</b><br/>Animal fixed by HM solution via cardiac perfusion</p>   | <p><b>4</b>  39°C  10~21 days</p> <p><b>Passive clarification:</b><br/>8% SDS is used in clarification</p>                               | <p><b>7</b>  room temp.</p> <p><b>Mount:</b><br/>Mount the sample in fresh RCCS solution between two coverslips separated with iSpacer</p> |
| <p><b>2</b>  4°C  3 days</p> <p><b>Fixation:</b><br/>Embryo subjected to 20ml HM solution in a 50ml tube, refreshed HM everyday<br/><i>(on an orbital shaker)</i></p> | <p><b>5</b>  37°C  2 days</p> <p><b>Wash:</b><br/>Wash with 0.2% PBST and 1X PBS<br/><i>(on an orbital shaker)</i></p>                   | <p><b>8</b>  room temp.</p> <p><b>Image:</b><br/>Confocal, 2-photon, or light-sheet microscopy</p>   |
| <p><b>3</b>  37°C  5hr</p> <p><b>Degassing &amp; Polymerization:</b><br/>Oxygen must be removed from the tissue sample before polymerization</p>                      | <p><b>6</b>  room temp.  1~3 days</p> <p><b>Clear:</b><br/>Add 5~10ml RapiClear CS (RCCS) solution<br/><i>(on an orbital shaker)</i></p> | <p><b>Option:</b><br/><i>Staining of antibody or nucleic acid dyes should follow the protocol in next page</i></p>                         |



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1. Anesthetize and perfuse the mice with ice-cold 1× PBS at ~10 ml/min. Make sure that the PBS becomes colorless by the end of perfusion.
2. Perfuse the mice with hydrogel solution (HM solution) [mixture of ice-cold 4% PFA, 2% acrylamide, 0.125% Bis, 0.025% VA-044 initiator, in 1X PBS]] at ~10 ml/min. Recover the tissues from the mice and place it in the 20 ml HM solution. (Note: PFA and acrylamide monomer are toxic. Perform all procedures in a fume hood.)
3. Embryos were left to incubate in the HM solution at 4°C for 3 days with gentle shaking. (Note: HM solution need to be refreshed everyday.)
4. Place sample in the 50-ml conical tube filling with 15ml HM solution
5. Open the tube cap and place a plastic tube connecting with nitrogen tank main valve into the tube above HM solution (do not touch with HM solution).
6. Open the nitrogen tank main valve and adjust the control valve to fill the tube with nitrogen gas for >2min. Make sure to remove as much residual oxygen as possible, importantly.
7. Remove the nitrogen plastic tube and close the tube cap as soon as possible.
8. Transfer the closed conical tube to a 37°C water bath for 3~4hr. Make sure that the hydrogel has solidified before proceeding to the next step. If not, repeat the degassing section and transfer the tube to 37°C again. Gentle shaking helps in heat transfer for achieving robust and uniform polymerization.
9. Do not leave the sample in the HM (at 37°C) for more than 5hr. Over-polymerization of the hydrogel will make tissue clearing and staining very difficult.
10. Carefully extract tissue samples from solidified hydrogel. Kimwipes can be used to remove the residual gel from the tissue surface. (Note: residual gel is toxic. Perform all procedures in a fume hood.)
11. Wash the sample twice with 50 ml 200mM NaOH-Boric Buffer (pH=8.5) containing 8% SDS for 12~24hr at RT to dialyze the remaining PFA, initiator and monomer.
12. Passive clearing of hydrogel-embedded tissue.
13. Place samples in a simple combination of 50ml tube and heated stirring plate. 100mM Tris-Boric Buffer (pH=8.5) containing 8% SDS is used to accelerate the clarification (at 39°C). Detailed setup is available online at <http://capture-clarity.org/optimized-clarity/>
14. With this setup, embryos can be cleared in 10~21 days. The 8% SDS/100mM Tris-Boric Buffer (pH=8.5) needs to be refreshed once the pH goes below 7.5 or clearing efficiency will drop. Therefore, the buffer may need to be changed every 3~4 days.
15. Check for completion of sample clearing by assessing for transparency (easy visualization of high-contrast signals such as a black-and-white grid or printed text through the tissue) and homogeneity (even distribution of transparency across the tissue). Once the tissue is clearer, proceed to the next step.
16. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN<sub>3</sub>) for 12hr at 37 °C.
17. Wash the samples twice with 50ml 0.2% PBST (0.2% Triton-X100 and 0.1% sodium azide, NaN<sub>3</sub>) for 12hr at 37°C.
18. Wash the samples twice with 50ml PBS (containing 0.1% sodium azide, NaN<sub>3</sub>) for 1 day at 37 °C.
19. Option: Molecular labeling [following the descriptions in the Nature Protocols 9, 1682–1697 (2014): Advanced CLARITY for rapid and high-resolution imaging of intact tissues]
20. Incubate the samples in RapiClear CS mounting solution and periodically check the visual clarity of the sample over the next few hours. An intact mouse embryo will typically complete the refractive index (RI) homogenization process in 1~3 days. Once the tissue has been fully clarified, proceed with imaging preparations. Sample can be stored in RapiClear CS for several weeks at room temperature without affecting imaging quality.
21. Before RI homogenization (or after), the tissue may be stored indefinitely in 0.2% PBST [with 0.1% (wt/vol) sodium azide] at room temperature.
22. Confocal, 2-photon or Light-sheet microscopy imaging (for Ultramicroscope imaging setup is available online at <http://capture-clarity.org/imaging/>).