



# Tissue Clearing Reagents and Protocols for CUBIC Method

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The following CUBIC-R+ can achieve clearing using either CUBIC-R+(M) (TCI Product No. **T3741**) or CUBIC-R+(N) (TCI Product No. **T3983**).

Prepare the 1% formalin solution used in each protocol as follows:

	Volume
<a href="#">Formalin (37%)</a> (TCI Product No. <b>F0622</b> )	1 mL
PBS	36 mL

### [Method for Mouse Whole-Organ Clearing]

#### Required Reagents:

- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. **M3294**)

#### Procedure:

1. Tissue Excision: Perfuse mouse transcardially and excise organs.
2. Fixation: Fix sample in 4% paraformaldehyde solution diluted with PBS overnight at 4°C.
3. Wash: Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
4. (Optional Step): Pre-delipidate sample in 50% CUBIC-L (equal volumes of CUBIC-L and ion-exchanged water) with gentle shaking overnight at 37°C.
5. Delipidation: Immerse sample in CUBIC-L with gentle shaking for 2-5 days at 37°C. Refresh CUBIC-L on days 1, 2, and 4. (\*1)
6. Wash: Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
7. (Optional Staining): Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 3+ days.
8. (Optional Wash, if staining performed): Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
9. (Optional Post-fixation, if staining performed): Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
10. (Optional Post-fixation, if staining performed): Transfer step 9 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
11. (Optional Wash, if staining performed): Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.

12. Pre-replacement: Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
13. RI Adjustment: Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
14. Observation: Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

\*1

Delipidation completion indicator light transmits through organ or used CUBIC-L shows no coloration. Excessive delipidation may make tissues fragile.

\*2

Use tube positioned horizontally with organ nearly submerged. Use tube slightly larger than organ diameter. For adult mouse brain in 5 mL tube: ~14 mL CUBIC-L and ~6 mL CUBIC-R+ required. Volume varies by brain size.

## [Method for Mouse Whole-Body Clearing]

### Required Reagents

- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. **M3294**)

### Procedure:

1. Perfusion: Perfuse mouse in sequence with PBS, 4% paraformaldehyde solution diluted with PBS, PBS, and 50% CUBIC-L (equal volumes of CUBIC-L and ion-exchanged water).
2. (Optional Step): Pre-delipidate sample in 50% CUBIC-L with gentle shaking overnight at 37°C.
3. Delipidation: Immerse sample in CUBIC-L with gentle shaking for 5+ days at 37°C. Refresh CUBIC-L on days 1, 2, and every 2 days thereafter.
4. Wash: Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
5. (Optional Staining): Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 3+ days.
6. (Optional Wash, if staining performed): Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
7. (Optional Post-fixation, if staining performed): Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
8. (Optional Post-fixation, if staining performed): Transfer step 7 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
9. (Optional Wash, if staining performed): Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
10. Pre-replacement: Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
11. RI Adjustment: Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
12. Observation: Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

## [Method for Clearing Mouse Tissue with Expansion]

### Required Reagents

- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-X1 \[for tissue expansion\]](#) (TCI Product No. **T3866**)
- [Tissue-Clearing Reagent CUBIC-X2 \[for RI matching while keeping the expanded size\]](#) (TCI Product No. **T3867**)
- [Mounting Solution \(RI 1.467\) \[for CUBIC-X2\]](#) (TCI Product No. **M3292**)

### Procedure:

1. Tissue Excision: Perfuse mouse transcardially and excise tissues.
2. Fixation: Fix sample in 4% paraformaldehyde solution diluted with PBS overnight at 4°C.
3. Wash: Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
4. Pre-delipidation: Pre-delipidate sample in 50% CUBIC-L (equal volumes of CUBIC-L and ion-exchanged water) with gentle shaking for 3 hours at 37°C.
5. Delipidation: Immerse sample in CUBIC-L with gentle shaking at 37°C. 1-week-old mouse brain 5 days; 3-week-old mouse brain: 7 days; 8-week to 6-month-old mouse brain 14 days. Refresh CUBIC-L every 4 days.
6. Wash: Wash sample with PBS with gentle shaking overnight at room temperature.
7. Staining: Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 3+ days.
8. Wash: Wash sample with PBS with gentle shaking overnight at room temperature.
9. Post-fixation: Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
10. Post-fixation: Transfer step 9 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
11. Wash: Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
12. Expansion: Immerse sample in CUBIC-X1 with gentle shaking for 2.5 days at 4°C.
13. RI Adjustment: Immerse sample in CUBIC-X2 with gentle shaking for 1.5 days at room temperature. Refresh CUBIC-X2 every 12 hours.
14. Observation: Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.467) and observe under microscope.

**Note on Reagent Volume:** For adult mouse brain: ~10 mL CUBIC-L, ~30 mL CUBIC-X1, ~120 mL CUBIC-X2 required. Volume varies by brain size.

## [Method for Smoother Clearing of Adult Mice (6+ Weeks Post-birth)]

### Required Reagents:

- [Tissue-Clearing Reagent CUBIC-P \[efficiently aids perfusion fixation\]](#) (TCI Product No. **T3782**)
- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. **M3294**)

### Procedure:

1. **Sacrifice:** Sacrifice mouse with pentobarbital overdose.
2. **Perfusion:** Perfuse mouse in sequence with 15 mL PBS, 20 mL 4% paraformaldehyde solution diluted with PBS, 15 mL PBS, and 100 mL CUBIC-P. Excise tissues afterward.
3. **Delipidation:** Immerse sample in CUBIC-L with gentle shaking for 3-7 days at 37°C.
4. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
5. **(Optional Staining):** Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 5-7 days.
6. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking overnight at room temperature.
7. **(Optional Post-fixation, if staining performed):** Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
8. **(Optional Post-fixation, if staining performed):** Transfer step 7 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
9. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
10. **Pre-replacement:** Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
11. **RI Adjustment:** Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
12. **Observation:** Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

## [Method for Clearing Mouse Tissues Including Bone]

### Required Reagents:

- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-B \[for decalcification\]](#) (TCI Product No. **T3780**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. **M3294**)

### Procedure:

1. **Organ Excision:** Perfuse mouse transcardially and excise organs.
2. **Fixation:** Fix sample in 4% paraformaldehyde solution diluted with PBS overnight at 4°C.
3. **Wash:** Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
4. **Delipidation:** Immerse sample in CUBIC-L with gentle shaking for 3-7 days at 37°C.
5. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
6. **Decalcification:** Immerse sample in CUBIC-B with gentle shaking for 5-7 days at 37°C. Refresh CUBIC-B at least once.
7. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
8. **Delipidation:** Immerse sample in CUBIC-L with gentle shaking for 2-4 days at 37°C.
9. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
10. **(Optional Staining):** Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 5-7 days.
11. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking overnight at room temperature.
12. **(Optional Post-fixation, if staining performed):** Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
13. **(Optional Post-fixation, if staining performed):** Transfer step 12 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
14. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
15. **Pre-replacement:** Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
16. **RI Adjustment:** Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
17. **Observation:** Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

## [Method for Human Brain Clearing]

### Required Reagents:

- [Tissue-Clearing Reagent CUBIC-L \[for delipidation and decoloring\]](#) (TCI Product No. **T3740**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. **M3294**)

### Procedure:

1. **Fixation:** Store sample immersed in formalin until use.
2. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
3. **Delipidation:** Immerse sample in CUBIC-L with gentle infiltration for 1-2 weeks at 45°C. Refresh CUBIC-L at least once.
4. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
5. **Pre-replacement:** Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
6. **RI Adjustment:** Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
7. **Observation:** Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

\*Autofluorescence of brain cells decreases as delipidation progresses. If preserving autofluorescence signal, delipidation within 1 week is recommended.

## [Method for Human Tissue Clearing]

### Required Reagents:

- [Tissue-Clearing Reagent CUBIC-HL \[for highly fatty tissue and quenching autofluorescence\]](#) (TCI Product No. : **T3781**)
- [Tissue-Clearing Reagent CUBIC-R+\(M\) \[for RI matching\]](#) (TCI Product No. : **T3741**)
- [Tissue-Clearing Reagent CUBIC-R+\(N\) \[for RI matching\]](#) (TCI Product No. : **T3983**)
- [Mounting Solution \(RI 1.520\) \[for CUBIC-R+\]](#) (TCI Product No. : **M3294**)

### Procedure:

1. **Fixation:** Store sample immersed in formalin until use.
2. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
3. **Delipidation:** Immerse sample in CUBIC-HL with gentle infiltration for 1-2 weeks. Human brain and heart at 37°C; human heart, liver, lung, spleen at 45°C. Refresh CUBIC-HL at least once. \*
4. **Wash:** Wash sample with PBS with gentle shaking overnight at room temperature.
5. **(Optional Staining):** Perform nuclear staining with any nuclear staining agent or immunostaining with antibodies. Room temperature for 5-7 days.
6. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking overnight at room temperature.
7. **(Optional Post-fixation, if staining performed):** Fix sample with gentle shaking in 1% formalin solution overnight at 4°C.
8. **(Optional Post-fixation, if staining performed):** Transfer step 7 sample in 1% formalin solution to 37°C. Gently shake for 1 hour.
9. **(Optional Wash, if staining performed):** Wash sample with PBS with gentle shaking for 2+ hours × 3 times, total ~1 day at room temperature.
10. **Pre-replacement:** Pre-replace sample in 50% CUBIC-R+ (equal volumes of CUBIC-R+ and ion-exchanged water) with gentle shaking overnight at room temperature.
11. **RI Adjustment:** Immerse sample in CUBIC-R+ with gentle shaking for 1+ day at room temperature.
12. **Observation:** Remove sample, wipe surface reagent with Kimwipes, transfer to microscope chamber. Fill chamber with Mounting Solution (RI 1.520) and observe under microscope.

\*Delipidation time varies by sample size. As delipidation progresses, opaque parts inside sample decrease. For delipidation >2 weeks, lower temperature (room temperature etc.) for subsequent steps or delipidation with CUBIC-L is recommended.

## [Q&A]

Q What kind of tissue staining reagents can be used with CUBIC?

A When immunostaining cleared samples, it is possible to use only fluorescent-labeled primary antibodies in the vast majority of cases, i.e. secondary antibodies are usually not required. Exact dilutions must be determined on a case-by-case basis. Dilute antibodies in PBS containing 0.5% Triton™ X-100 and 0.01% NaN<sub>3</sub>. For nuclear staining, use propidium iodide diluted to 10 µg/mL in 0.1 M phosphate buffer (pH 7.4) with 0.5 M NaCl.

Q What is the appropriate antibody concentration for immunostaining?

A It is recommended to use a concentration slightly higher than that used for immunostaining on tissue sections.

Q Can I use fluorescent-labeled secondary antibodies?

A As primary antibodies are generally sufficient, we do not have information / procedures regarding the use of secondary antibodies. However, considering the time required for equilibration of each antibody within samples, we highly recommend labeling your primary antibody with fluorescent reagents in lieu of using secondary antibodies.

Q Why won't my specimen clear?

A Find common troubleshooting methods below.

a) PFA fixation solution pH too high:

A pH of greater than 8 may result in over-fixation, making it harder for samples to be cleared; try adjusting the pH to between 7 – 7.5.

b) Incomplete delipidation:

Try extending delipidation time or refreshing CUBIC-L more frequently. We recommend shaking samples immersed in CUBIC-L at 37°C for at least 2 - 5 days and replacing CUBIC-L with fresh reagent daily.

c) Incomplete clearing:

Try extending clearing time and/or consider changing out CUBIC-R+ reagent for fresh reagent partway through RI matching.

Q Can cleared samples be sectioned?

A Cleared samples may become fragile, so thin sectioning should be avoided. If sectioning is desired, section the tissue before clearing.

Q Can cleared samples be subjected to in situ hybridization?

A In situ hybridization cannot be performed on cleared samples because CUBIC-R+ has a pH of 10 or higher (basic), and the nucleic acids in the sample are degraded by the basic conditions.