CLARITY Tissue Clearing & Immunostaining Kit

Product Overview

CLARITY is a tissue clearing method used to render tissues optically transparent, allowing for non-destructive tissue processing and 3D imaging of both normal and diseased soft tissues. In the CLARITY tissue clearing method, the tissue is first embedded with hydrogel monomers to create a matrix scaffold, then the light scattering lipids are removed. After lipid clearing, the tissue-hydrogel hybrid is both transparent to light and permeable to molecules, enabling staining and imaging using a confocal or light-sheet microscope.

This kit is intended for Research Use Only.

Kit Contents

The CLARITY Tissue Clearing Kit components:

- CLARITY Lipid Embedding Solution (High Lipid or Low Lipid) – Store at 4°C
  - High Lipid (90 mL)
  - Low Lipid (62.5 mL)
- Thermal Initiator (1 g) – Store at room temperature
- Wash Buffer 1 (101 mL) – Store at room temperature
- CLARITY Lipid Clearing Solution (1000 mL) – Store at room temperature

- **IMPORTANT:** Do not store below ≤ 22°C or precipitation may occur.

- Wash Buffer 2 (101 mL) – Store at room temperature

Please inspect the kit contents upon receipt to confirm all parts have been included and ensure that no damage occurred during shipment. Check that all containers are sealed and that none of the seals have been broken. The contents of this kit should be shipped on ice. Any damage occurring as a result of shipping and handling should be filed with the carrier.

Sold separately:

- Immunostaining Buffer (not included in CLARITY Tissue Clearing Kit) (105 mL) – Store at room temperature

Safety Warning and Precautions

All reagents within this kit should be considered hazardous. Appropriate personal protective equipment (PPE, i.e., lab coat, gloves, goggles) should always be worn when using this kit.

Certain steps of the CLARITY process should also be performed under a fume hood to prevent inhalation. CLARITY tissue clearing should only be performed by personnel trained with good laboratory practices and techniques.

Please review the Safety Data Sheets for safe handling, disposal, and storage conditions for all reagents listed herein prior to use.

Procedural Guidelines

- This kit has been optimized for the lipid clearing of soft tissues. Tissue that are highly pigmented (*i.e.* melanin-rich) or mineralized (*i.e.* hard tissues: bone, teeth, etc.) will require additional sample processing beyond the scope of this kit.
- Perform all steps at room temperature (22-25°C) unless otherwise noted.
- Wear disposable gloves and appropriate PPE while handling the reagents and samples.
- Use disposable, sterile plasticware, pipette tips, and tubes.
- Lipid clearing performed in conical tubes should be placed in secondary containers (*i.e.*, trays) to limit any potential spills.
- Samples with fluorescently labeled endogenous markers should be protected from light throughout the entire procedure.
- Samples that have been previously embedded into paraffin blocks will need to undergo deparaffinization prior to embedding in the hydrogen monomer solution.

Required Equipment and Materials (not supplied)

- ddH_2O
- Stir bar
- Desiccation chamber
- Vacuum pump
- Nitrogen gas
- Water bath
- Incubator/shaker
- Spatula(s)
- Delicate task wipes
- 1X PBS
- 1X PBS + NaN_3 (0.01%, w/v)
- Pipettes and pipette tips (various sizes)
- Multi-well plates (various sizes)
- Conical tubes (various sizes)
- Plate sealing tape
- Blocking buffer, if preferred
- Antibodies of interest (primary and secondary), if necessary
- Nuclear counterstain, if necessary
- Refractive Index matching solution
- Imaging platform
    - Confocal or light-sheet microscope

Before you Begin – Preparatory Work

Tissue/sample collection and fixation

Use your standard tissue/sample collection preparation and perfusion method. Postfix the tissue using either 4% paraformaldehyde (PFA) at 4°C for 24 hours or 10% neutral buffered formalin (NBF) at 4°C for 24 hours.

- Note: We recommend and can only confirm results for tissues that have been fixed in 4% PFA or 10% NBF. Samples prepared with other fixatives have not been validated or have already been determined to interfere with the CLARITY protocol.
- Prolonged storage in fixative solutions can result in an increased autofluorescence background.
- When possible, the tissue should be perfused, but is not a requirement.
- Samples that have been or will be stored for prolonged periods (>1 week) of time should be stored in PBS+NaN_3 (0.01%) at 4°C.

*Reagent preparations (provided at **10X concentrated solutions**)*

The CLARITY Lipid Embedding Solution (High Lipid or Low Lipid).

The Lipid Embedding Solution will prepare 400 mL of embedding solution, and subsequently be prepared into smaller aliquots for future use.

1. Place at least a 500 mL beaker with a stir bar on ice.
2. Add the provided 10X Lipid Embedding solution, the Thermal Initiator, and ddH_2O (see below for specific Lipid Kit purchased) to the beaker:

- **High Lipid**: Add Thermal Initiator and **310 mL ddH_2O**.
- **Low Lipid**: Add Thermal Initiator and **337.5 mL ddH_2O**.

3. Place the beaker on a stir plate and allow the contents to dissolve and thoroughly mix.
    - Note: The Lipid Embedding Solution should remain cool during this step, so it may be necessary to place the beaker on ice while mixing to keep the solution cold.
4. Once the solution has been thoroughly mixed, the Lipid Embedding solution should be stored in smaller aliquots (*e.g.* 25 mL or 50 mL). The relevant aliquot size will depend on the sample size you plan on embedding.
    - Aliquots should be stored at ≤-20°C in sealed conical tubes.
    - The prepared aliquots are stable for 3 months.
    - Note: Avoid repeated freeze/thaw cycles, which may affect the product performance.

Wash Buffer 1 is provided at a 10X concentration. Dilute Wash Buffer 1 with 899 mL of ddH_2O to a total of 1000 mL of solution before use.

Wash Buffer 2 is provided at a 10X concentration. Dilute Wash Buffer 2 with 899 mL of ddH_2O to a total of 1000 mL of solution before use.

If purchased, Immunostaining Buffer is provided at a 10X concentration. Dilute Immunostaining Buffer with 895 mL of ddH_2O to a total of 1000 mL solution before use.

Procedures

Hydrogel Embedding

1. Defrost Lipid Embedding Solution on ice (same day embedding) or overnight at 4°C (next day embedding).
2. Place each fixed sample into a separate conical tube aliquot (one sample per tube) of prepared Lipid Embedding Solution maintained at ≤4°C.
3. Incubate the sample(s) at 4°C for 48 hours to allow for complete diffusion of Lipid Embedding solution into the tissue.
4. Remove the sample(s) from 4°C and place the conical tube(s) containing the embedded sample(s) on a rack in the desiccation chamber.
    - Note: Depending on the size of your desiccation chamber, it is recommended to limit the number of conical tubes per run to minimize excess oxygen exposure.
5. Slightly unscrew the cap of the conical tube to promote gas exchange, without removing the cap completely.
6. Place the sample(s) in the desiccation chamber and flood the chamber with N_2 gas for at least 2 minutes.
    - Note: The removal of oxygen from the submerged sample(s) promotes efficient hydrogel polymerization.
7. Place the degassed sample(s) under vacuum for at least 10 minutes.

- Note: We recommend the vacuum set for a minimum of 10 minutes and a maximum of 30 minutes.

8. Stop the vacuum and flood the desiccation chamber once more with N_2 gas, allowing it to fill the chamber for at least 10 seconds.
9. Carefully lift the desiccation chamber lid and quickly tighten the conical tube cap(s) on while minimizing exposure to air.
10. Turn off the N_2 gas tank and remove the conical tube(s) from the chamber.

Proceed to hydrogel polymerization.

Hydrogel Matrix (HM) Polymerization

1. Submerge the sealed tissue sample container(s) in a temperature-controlled 37°C water bath and incubate for 3 hours.
2. Remove the HM embedded sample from the conical tube using a spatula.

   For **High Lipid HM**:

   - Check all samples for solidification of the hydrogel.
   - Remove excess bulk hydrogel using a spatula and/or delicate task wipes under a fume hood.
   - Use a delicate task wipe, remove any excess gel on the tissue sample surface.
   - Discard of hydrogel using proper disposal procedures.

For **Low Lipid HM**:

- The hydrogel will not completely solidify.
- Pour off excess hydrogel under a fume hood using proper disposal procedures.
- Use a delicate task wipe, remove any excess gel on the tissue sample surface.

3. Place the embedded sample(s) into a new 50 mL conical tube and add at least 25 mL of Wash Buffer 1 (WB1).
4. Seal the tube and allow the sample to wash for 24 hours at 37°C with gentle shaking.
5. After 24 hours, replace the wash buffer in the tube with a fresh 25 mL of WB1.
6. Continue washing for 24 hours at 37°C with gentle shaking.

Note: Used WB1 solution may still contain toxic hydrogel monomers and must be disposed of in an appropriate hazardous liquid chemical waste container.

STOP/PAUSE POINT: At this point, after removing the samples from washing buffer, you may do one of the following:

- Store the samples in PBS + NaN_3 solution,
  - Sample(s) will need to be washed with 1X PBS or WB1 before proceeding to Tissue Lipid Clearing
- Section the tissue(s), if appropriate, or
- Proceed to the lipid clearing step.

Tissue Lipid Clearing

1. Remove tissue sample(s) from WB1 solution.
2. Place the sample(s) in your clearing chamber of choice:
    - Conical tube(s), well plate(s), or any container that allows for sealing.
3. Add Lipid Clearing Solution to the sample chamber(s) until almost filled.
4. Place clearing sample chamber(s) into an incubator at 37-45°C and gently shake for the entire clearing process.
5. Samples should be monitored regularly throughout the tissue clearing process (at least once a day) for clearing progress.
6. Change the lipid clearing solution at least every other day until the lipids have been removed and tissue becomes visually “clear”.

Note: Tissue samples that are rich in vasculature, extracellular matrix, and other non-lipid components will demonstrate varying levels of clearing due to different tissue compositions. We highly recommend you first determine the baseline transparency for your specific tissues with a test sample. Cancerous tissues are heterogeneous; therefore, lipid-clearing times should be established on a case-by-case basis.

Recommended starting time points for select tissues using the appropriate lipid content kit:

Tissue/Organ	<1 mm thick	1-2 mm thick	½ organ	Whole organ
Brain	1 day	2-3 days	7 days	14 days
Lung	1 day	1- 2 days	3 days	7 days
Kidney	1 day	1-2 days	7-8 days	15 days

7. Place the lipid-cleared samples in a new sample chamber of your choice.
8. Cover the samples with WB1 and proceed to wash your samples in a shaking incubator at 37°C or on a shaking platform at room temperature for 24 hours.
    - For whole organs or thick tissue sections (>3 mm thickness) use 40-50 mL in a 50-mL conical tube for washing.
    - For tissue sections (<3 mm thickness), wash in a 15 mL conical tube or a 6-well plate (for multiple samples) with 10 mL.
9. Remove WB1 and replace with Wash Buffer 2 (WB2), and continue washing your sample for another 24 hours.

Note: The samples should be washed for at least 48 hours after passive clearing is complete to remove any residual lipid clearing solution in the tissue. The lipid clearing

solution will crystalize at temperatures cooler than room temperature. Improper washing can cause damage to the tissue integrity.

STOP/PAUSE POINT: At this point, after removing the samples from washing buffer, you may do one of the following:

- Store the samples in PBS+NaN_3 solution at 4°C in a sealed container for prolonged storage (>1 week), or
- Proceed to immunostaining, if necessary, or
- Proceed to your preferred refractive index (RI) matching solution prior to sample imaging on your preferred imaging platform.

Immunostaining (Specific to Immunostaining Buffer)

1. Blocking samples: Block the sample(s) using your preferred blocking solution.
    - Blocking buffer should be prepared ahead of time.
    - Note: Not all sample types will require blocking, but we recommend this step to reduce non-specific binding.
2. In a new multi-well plate, use a spatula to place each washed lipid-cleared sample into one well in an appropriately sized multi-well plate (*i.e.*, 1 section per well).
    - Size-appropriate means that the volume of each well should be as small as possible, while still enabling the sample to lay flat.

This allows use of smaller volumes of blocking and staining buffers.

3. Tissue sections should be fully covered by blocking buffer in each well.
    - Note: We recommend filling any empty wells remaining with H_2O to minimize evaporation.
4. Cover plate with sealing tape.
5. Place in a shaking incubator at 37°C for 18-24 hours.
6. Immunostaining samples: Prepare antibody dilutions using the Immunostaining Buffer.
    - We recommend starting at ~10 µg/mL, if possible, to test feasibility for a thick tissue section.
    - Previously tested antibodies should be used at previously assessed optimal dilutions/concentrations.
    - Prepare enough antibody solution to fully cover each tissue sample in the well-plate. 200 µL is typically enough to cover a 1 mm thick sample in a well of a 48-well plate; however, more solution may be needed for thicker sample sections or wells of larger volumes.
7. Gently lift the sealing tape from the plate.
8. Carefully remove the blocking buffer solution from each sample well using a pipette.
9. Add the prepared antibody dilutions to each sample well until the sample is fully covered.
10. Reseal the plate with sealing tape.

- We recommend that all remaining empty wells be filled with H_2O to minimize evaporation.
- Note: **If using directly conjugated Ab°s**, protect the plate from light exposure to preserve the fluorophores.

11. Place sealed plate into a shaking incubator for 1-7 days at 37°C.
    - The duration of the immunostaining process is dependent upon the thickness of the sample.
    - Recommendation based on sample section thickness:
        - <1 mm: 1-2 days
        - 1-2 mm: 3 days
        - 3-5 mm: 5 days
        - >5 mm: 1 week
12. Carefully remove primary Ab° solution from the sample well(s).
13. Wash tissue section(s) with your preferred washing solution by adding the wash buffer to each sample and gently shaking for 24 hours.
    - We recommend using WB2, and exchanging the wash buffer multiple times.
14. **Indirect secondary Ab° conjugation only**: Prepare secondary Ab°(s) and nuclear counterstain dilutions in Immunostaining Buffer.
    - We recommend an initial secondary Ab° concentration of ~10 µg/mL.
    - Prepare enough Ab° and counterstain solution to fully cover each tissue sample in the well-plate.

15. Add the secondary Ab° and counterstain solutions to each sample well so that it covers the entire sample.
16. Reseal the plate with the sealing tape.
    - We recommend that all remaining empty wells be filled with H_2O to minimize evaporation.
    - Note: Protect the plate from light exposure to preserve the fluorophores.
17. Place the sealed plate into a shaking incubator for 1-7 days at 37°C.
    - The duration of the immunostaining process is dependent upon the thickness of the sample.
    - Recommendation based on sample section thickness:
        - <1 mm: 1-2 days
        - 1-2 mm: 3 days
        - 3-5 mm: 5 days
        - >5 mm: 1 week
18. Carefully remove secondary Ab° solutions from each sample well using a pipette.
19. Wash tissue sections with preferred washing solution by adding wash buffer to each sample and gently shaking for 24 hours.
    - We recommend using WB2 and exchanging the wash buffer multiple times.

Proceed to your preferred RI matching solution and imaging protocol for the samples.

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