ADAPT-3D™ Tissue Clearing Kit Product Insert

Leinco Technologies, Inc.™
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Catalog Number A630

Product Information:

ADAPT-3D (Accelerated Deep Adaptable Processing of Tissue for 3-Dimensional Imaging)

The acronym ADAPT-3D reflects the adaptable and versatile nature of this protocol to work in a wide range of tissues and model systems. The outlined ADAPT-3D protocol is a guide with recommended incubation times for fixation, decolorization, delipidation, and refractive index matching (RIM) included at the end. If the sample contains bone, a decalcification step is also required.

NOTE: Incubation times vary depending on tissue type or size. Reference tables 1-4 for approximate incubation times and suggested volumes.

WARNING: *Read the Safety Datasheet (SDS) and the following handling instructions. Wear appropriate protective eyewear, clothing and gloves.*

Kit Components Include:

Product	Cat. No.	Size	Notes	Storage
Decolorization Buffer	**B671**	50 ml		• Store at room temperature in a dry environment. • Do not freeze. • When stored as directed, the products are stable for 6 months
Partial Delipidation Buffer (Component A)	**B672**	50 ml	Mix with Component B at 1:1 ratio in glass only container.	
Partial Delipidation Buffer (Component B)	**B676**	50 ml	Mix with Component A at 1:1 ratio in glass only container.	
1X ADAPT-3D Staining Buffer	**B673**	25 ml	0.1 mM Glycine, 0.167% Tween-20, 0.33% Triton X-100, 1% Donkey Serum, 1% Alpaca Serum, 1% BSA, 0.05% Hydrogen peroxide, 5% v/v DMSO + 0.09% Azide	• Store at 2–8**°C**. • Follow sterile techniques while using. • When stored as directed, the products are stable for 6 months from the date of receipt.
Refractive Index Matching (Clearing Solution)	**B675**	25 ml		• Store at room temperature. • Do not freeze. • When stored as directed, the products are stable for 6 months
Wash Buffer (5X)	**B674**	50 ml	PBS + 1.5M Glycine + 5U/mL heparin; 0.09% Azide at 1X	• Store at room temperature. • Do not freeze.

Note: ADAPT-3D is shipped and stored at multiple temperatures. Store according to product labeling.
Note: When stored as directed, the products are stable for 6 months from the date of receipt.

Products are for research use only. Not for use in diagnostic or therapeutic procedures.

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Required Buffers and Materials Not Included:

1. Cell Lines for CD cultures, organoids, tissue or animal organs (Use Positive and Negative controls as needed)
2. Slips, coverslips, containers, scintillation vials
3. Primary and Secondary Antibodies
4. Decalcification Buffer (Leinco SKU: B670)
5. Fixative Solution – 4% paraformaldehyde (PFA) in PBS with 10% sucrose at pH 9.0

Note: To search through the Leinco Technologies primary antibody collection, use our Antibody Search tool at www.leinco.com.

Recommended Workflows

- **Time Savings:** This protocol can achieve uniform RIM in minutes to hours for most tissues, significantly faster than other methods.
- **Tissue size and type:** The specific durations and volumes of the steps may vary depending on the tissue type and size. Thin tissues like mouse colon respond more quickly and completely.
- **Transparency:** Pigments from heme and lipids can prevent reaching full transparency. Decolorization and delipidation steps are crucial for optimal clearing.
- **Compatibility with all light microscopy (e.g., widefield, confocal):** With cell chamber (Example ibidi 81158 or ThermoFisher Atto Chamber A7816).
- **Mounting:** The cleared samples can be imaged in any appropriate container, such as mounted slides or light sheet microscope chambers.

Procedural guidelines

- For first-time users, we highly recommend cutting thicker tissues into ≤1-mm thick sections
- All steps in this procedure should be performed at room temperature with gentle agitation, except where noted.
- For liver, kidney, and lymphatic tissues, you may need to extend incubation times by 30–50%, depending on the degree of fixation.
- Use glass tubes with ADAPT-3D delipidation buffer. Do not use polystyrene, PEG or HDPE.
- For optimal results, tissues should be fixed by perfusion with 4% paraformaldehyde. However, tissues larger than 6 mm (e.g., whole brains) require perfusion with ice-cold 4% paraformaldehyde.
- It is recommended to carry out cardiac perfusion on animal models to remove the blood from the vasculature, as residual blood will block/obscure light during microscopy

Prior to Beginning this protocol:

1. 5X Wash Buffer is provided at a 5X concentration. Dilute the 5X Wash Buffer to 1X with PBS, pH 7.4 before use.
2. For samples with extensive pigmentation or fluorescent proteins –
   a. Extensive pigmentation and heme: good perfusion; if not, extended decolorization and delipidation.
   b. Fluorescent proteins: adjusted fixative along with delipidation and decolorization.
3. For samples containing bone, immerse the samples in an excess volume of Decalcification Buffer (catalog# B670) at room temperature. Change the buffer daily until the tissue is soft to the touch. For example, calvarium typically requires around 3 days.

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Standard Protocol

1. **Fixation:** Fix the tissue in 4%PFA (w/ sucrose) at 4°C for 4 hours to overnight depending on the size of the sample. Overnight is recommended for most size samples. For tissues with endogenous fluorescent reporter proteins, the ADAPT-3D fixative helps retain the intensity of sensitive endogenous fluorescent reporters. *NOTE: Fixation solution is not included in the ADAPT-3D kit (i.e., 4% PFA with 10% w/v sucrose at pH 9.0).*
2. **Wash:** Rinse the samples in 1X wash buffer diluted from 5X concentrate (catalog # B674; using at least 5 times the volume of the tissue and let rock/shake at RT for 30min. Change wash after 30 min and repeat (total incubation 1hr).
3. **Optional Decalcification (if bones are included):** Immerse the samples in an excess volume of Decalcification Buffer (catalog# B670) at room temperature. Change the buffer daily until the tissue is soft to the touch. **Wash (if decalcification is needed):** Rinse the samples with 1X Wash buffer diluted from 5X concentrate (catalog # B674) and rock/shake at RT for 30min. Change wash after 30min and repeat (total incubation 1hr).
4. **Decolorization:** Incubate the samples in Decolorization Buffer (catalog# B671) until partial transparency is achieved. This step removes light-interfering substances like heme and lipids.
5. **Washing:** Wash the samples in 1X Wash buffer (catalog # B674) when visible transparency appears to reverse, which occurs within minutes. Continue washing up to about 1 hour at room temperature.
6. **Delipidation:** Mix 1:1 ADAPT-3D Partial Delipidation Buffer A (catalog# B672) with Partial Delipidation Buffer B (catalog# B676) in a glass vessel. Incubate the samples in ADAPT-3D Delipidation Buffer until moderate transparency is observed. *NOTE: Partial delipidization B is corrosive to most plastic types. Read the Safety Datasheet (SDS) and the following handling instructions. Wear appropriate protective eyewear, clothing and gloves.*
7. **Washing:** For the first wash immediately after delipidation, rinse the samples with 0.2X wash buffer for 30 minutes. For the second wash step, change wash and rinse samples with 1X Wash buffer diluted from 5X concentrate (catalog# B674) and rock/shake at RT for 30min. Change wash after 30min and repeat (total incubation 1hr).
8. **Immunolabeling (if planned):** Incubate samples in 1X ADAPT-3D Staining Buffer (catalog# B673) for at least an hour, for smaller sized tissues, or overnight if whole tissues followed by incubation with buffer including primary and/or secondary antibodies.
9. **Washing:** Rinse the samples with 1X Wash buffer diluted from 5X concentrate (catalog# B674) and rock/shake at RT for 30min. Change wash after 30min and repeat (total incubation 1hr).
10. **Refractive Index Matching (RIM):** Immerse the samples in Refractive Index Matching Solution (catalog# B675) until transparent. *Note: For intestine and brain tissues, incubate tissues in a mixture of RIM with PBS, pH 7.4 in a 1:1 ratio for about 30 minutes.*
11. **Your sample is now ready for your imaging modality of choice.** *Note: This protocol is optimized for staining with samples with 500 µm thickness. For samples with a different thickness, the end user may need to perform further testing. Use 1% PFA if best clearing effect is desired, or 4% PFA for optimal epitope preservation and immunostaining. For additional support, please contact our technical support team or refer to the tables in the product insert for guidelines using other tissue samples and thinness.*

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Table 1: Suggested Incubation times for mouse tissue (can be significantly shortened depending on size)

Tissue	Fixation	Decolorization	Decalcification	Delipidation	Labeling	RI Matching
Skull/Brain	Overnight	1 day	3 days	2 days	3 days	6 hours
Skull	Overnight	1 day	3 days	1 day	2 days	2 hours
Brain (Whole)	Overnight	2 days	-	2 days	3 days	4 hours
Lung (Whole)	Overnight	1 day	-	1 day	3 days	4 hours
Heart (Whole)	Overnight	2 days	-	1 day	2 days	6 hours
Lymph Node	Overnight	4 hours – 1day	-	1 day	2 days	2 hours
Gut	Overnight	1 day	-	1 day	1 day	2 hours
Liver (Whole)	Overnight	3 days	-	2 days	3 days	6 hours
Kidney (Whole)	Overnight	3 days	-	1 day	2 days	6 hours
Spleen (Whole)	Overnight	2 days	-	1 day	2 days	6 hours
Hind leg	Overnight	3 days	3 days	3 days	4 days	Overnight
Embryo (~E15)	Overnight	1 day	1 day	1 day	1 day	6 hours
Tumor (~1 mm)	Overnight	2 days	-	-	-	-

Table 2: Suggested incubation times for human tissue. Age of 30-50 years

Tissue	Fixation	Decolorization	Decalcification	Delipidation	Labeling	RI Matching
Intestine (~ 2 mm thick)	Overnight	3 days	-	2 days	3 days	6 hours
Kidney (Wedge < 1 mm)	Overnight	1 day	-	1 day	1 day	0.5 hours

Table 3: Suggested incubation times for piglet (< 28 days old) tissue

Tissue	Fixation	Decolorization	Decalcification	Delipidation	Labeling	RI Matching
Intestine (~2 mm thick)	Overnight	3 days	-	2 days	3 days	6 hours
Lung	Overnight	1 day	-	1 day	1 day	4 hours

Table 4: Reagent Volume Guide for Immunolabeling and Tissue Clearing
The suggested incubation times, volumes, and considerations for your particular tissue of interest.

Thickness	Decolorization	Decalcification	Delipidation (to fill vial)	Immunostaining	RI Matching
4-10 mm (e.g. whole mouse brain)	20 mL	-	25 mL	5 mL	10 mL
1-2 mm	10 mL	-	20 mL	2 mL	5 mL
≤ 500 µm (usually in 12 well)	3 mL	-	10 mL	1 mL	2 mL

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Additional Considerations and Troubleshooting:

Problem	Problem Cause	Solution
Organ contains blood	Inefficient heparin/PBS perfusion wash	Increase heparin/PBS units/mL. Use non-expired heparin. Chill the mouse to 4°C during perfusion.
	Perfusion was carried out too fast	Perfuse with chilled heparin/PBS solution at a pump speed of 0.5 mL per minute.
Not enough fixation	Expired or old fixative solution	Prepare the fixative solution just before use. Protect PFA from light and store it in the dark.
	Insufficient fixation	Perfuse with at least 25 mL of chilled fixative solution per mouse. 4% PFA is recommended.
Agarose embedding of the sample failed	The organ surface was wet when embedded in agarose.	Gently pat the organ dry with tissue to minimize concentration changes from water content. Avoid over-drying.
		Use optimal temperature (touchable bottle) and the specified agarose (1.5%) or phytagel (1%) concentration is recommended.
Tissue Dried	Insuffcient permeabilization and blocking	Increase tissue permeabilization and blocking time with staining buffer.
Tissues did not clear	Strong or Over Fixation	Perfuse and fix on ice and with chilled bufers. Note: Use 1% PFA instead of 4% PSA as a fixative solution.
	Plastic incompatibility	Delipidation buffer degrades polystyrene. Check to see if plastic looks deformed and/or melted.
		Use glass in your workflow, where possible. Plastic leaching into your sample can affect the clearing ability of Tissue Clearing Pro reagents.
	Incomplete dehydration/clearing	Ensure you are using fresh reagents and water-free ethanol or methanol for dehydration. If not stored properly, methanol/ethanol will absorb water from the air. Methanol or ethanol that contains water will not remove all water from the tissue, resulting in cloudiness
Antibody did not label the tissue	Antibody is not compatible with 3D immunolabeling	• Verify antibody specificity on small tissue sections before using larger samples. Contact Technical Support with antibody-specific questions. • Only use antibodies that have been validated for use in IHC. If IHC validated antibody is not available, then IF/ ICC validated antibody might also work.
	Poor antibody labeling	Antigen retrieval might be needed to improve tissue permeability and antibody access

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