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SmartLabel Pro: Labeling Protocol Quick Guide

Recommended set/limit values for SmartLabel:

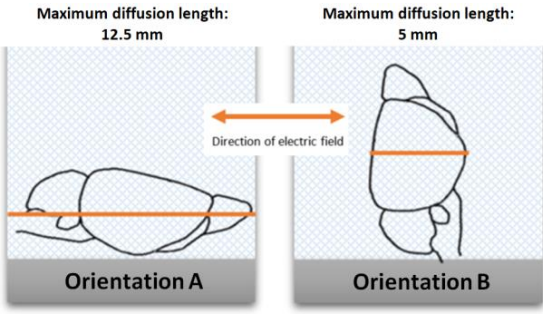
- Voltage = 90 V; Current = 1000 mA (500 mA if only using one labeling chamber); Reservoir A & B Temperatures = 25° C; Sample Cup Rotation = 0.01 Rpm

Reagents (volumes; *depends on sample size and cup used):

- Labeling-OFF buffer (one bottle, 500 mL total; Step 1 = 25 mL; Step 2 = 475 mL)
- Labeling-ON buffer (one bottle, 1 L total; Steps 3 & 4 = 500 mL each)
- Working volumes of *Sample* (~1.5-4 mL*) & *Sample-WASH* (~7-15 mL*) *buffers* with 2% BSA added
- 20% Triton-X 100 in Labeling-ON buffer solution (prepare a small amount of stock solution)
- Labeling reagents, such as primary & secondary antibodies or fluorescent nuclear dyes
- 10 mM SDS + 0.02% sodium azide in DI water solution, for rinsing and storing sample cups
- Optional: thin strips of plastic mesh (e.g., ~5 mm wide by ~40-50 mm long, cut from McMaster cat# 9318T22) to prevent small samples from moving or rotating within the sample cup

Key considerations relating to sample preparation, size, and orientation in the sample cup:

- SmartLabel is designed for use with samples preserved using a method such as SHIELD, CLARITY, or SWITCH and that have been delipidated using an SDS-based clearing protocol such as by using LifeCanvas's SmartClear 2 Pro.
- Minimize sample size, to both limit the number of antigenic sites and keep labeling reagent cocktail volume low. If the olfactory bulb, cerebellum, and brainstem are not of interest, it is recommended to remove them.
- Labeling efficiency is greatly improved by orienting the sample so that its shortest dimension is parallel with the direction of the electric field, as shown in Orientation B below. Orienting the sample in this manner should be of primary concern versus minimizing overall solution volume.
  - If you are labeling a few-millimeter -thick brain slice, it can be laid at an angle on the bottom of the insert, such that the maximum diffusion length through the tissue is only slightly greater than it would be if the slice were positioned vertically and standing on a narrow edge. If positioning a slice this way, make sure that it will not move or fall flat during the experiment.



- The sample must be placed at the bottom of the sample cup insert so that it is as close as possible to the cup's floor.
- The sample must not be able to move or rotate on its own within the sample cup, i.e. independently of the desired 0.01 Rpm sample cup rotation speed. If the sample is too small or light and is not held in place by the sample cup insert, weigh it down and secure it in place by laying thin strips of plastic mesh on top of it. In one configuration, the strips may form a U-shape when viewed from the side of the cup, such that the base of the U touches the top of the sample and the arms of the U are arrayed vertically along the walls of the insert.

High-level overview of SmartLabel labeling protocol: (total protocol length = 42-48 hours)

	Labeling-OFF step	Labeling-ON step	WASH step
Reservoir	Labeling-OFF buffer	Labeling-ON buffer	Fresh Labeling-ON buffer
Sample Cup	*Sample buffer*	Add 2% Triton-X 100	*Sample-WASH buffer*

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1. **Before labeling, samples must be equilibrated in ~25 mL Labeling-OFF buffer overnight / until sinking (some sample types may not sink). Equilibration occurs quickest by using a shaker and by increasing temp. to 37° C.**
2. **Labeling-OFF step: (time = 21 hours)**
   a. If compatible with your sample size, use the small sample cup whenever possible to conserve reagents and make it easier to position and stabilize the sample. Before each experiment, pre-treat the sample cup with ~2 mL of BSA-containing *Sample buffer* by tilting and rotating the cup to coat all inner surfaces of the membrane. Discard this solution but do not rinse the cup.
   b. Prepare labeling reagent cocktail in a prep tube containing ½ of the volume of *Sample buffer* needed to submerge sample. Add this volume to the remaining ½ when it is already present in the sample cup. In practice, the overall volume needed for a mouse brain hemisphere is typically 2-4 mL.
   c. Before and after loading the sample cup insert and sample, remove any bubbles in the solution using a pipetter, especially those adjacent to the sample and in the compartment containing the stir bar.
   d. Load the side A reservoir with 500 mL Labeling-OFF buffer and place the cup in the side A labeling chamber.
   e. Remember to turn on both sample cup rotation (0.01 Rpm; verify proper motor functionality by temporarily setting speed to ~2-3 Rpm and observing rotation by eye) and the sample cup stir bar when starting the experiment, in addition to the side A pump and electrode power. Set side-A temperature to 25°C.
   f. Set the timer to the recommended duration for the Labeling-OFF step (21 hours) and turn it on. *Note that the concluded timer turns off sample cup rotation, which needs to be turned back on for subsequent steps.*
3. **Labeling-ON step: (time = 21 hours)**
   a. Remove the sample cup and place it on the benchtop. Wash side A with deionized (DI) water while it is wet.
   b. Add Triton-X 100 to the sample cup at a concentration of 2%, using [20% TX-100 in Labeling-ON buffer]. For example, if sample cup volume is 3 mL, add 300 µl of 20% TX-100 solution. (Since during the Labeling-ON step a ~100% increase in sample cup volume typically occurs due to electro-osmotic flow, an initial concentration of 2% TX-100 is targeted to account for this, so that the final concentration stays > ~1%.)
   c. Load the B side reservoir, which should have been previously washed (and free of all residual SDS if it was at some point used for an OFF-step), with 500 mL Labeling-ON buffer. Place the cup in the side B chamber.
   d. Remember to turn on both sample cup rotation (0.01 Rpm) and the sample cup stir bar when starting the experiment, in addition to the side B pump and electrode power. Set the timer to 21 hrs, and side-B temperature to 25°C.
4. **Post-labeling WASH step: (time = 6 hours)**
   (This step may not be needed if using fluorescent nuclear dyes or antibody-fluorophore conjugates.)
   a. Remove the sample cup and place it on the benchtop. Wash side B with 250 mL DI water, two times.
   b. Transfer the sample to a petri dish and remove the antibody solution from the sample cup and rinse it extensively under DI water. Shake it dry. Briefly pre-treat the cup with a few mL of *Sample-WASH buffer*.
   c. Load a few mL of fresh *Sample-WASH buffer* into the sample cup, load the sample, and then fill up the cup to within ~2-3 mm of the top with *Sample-WASH buffer* (up to ~10 mL total volume if using the large sample cup). Remove any bubbles, especially those near the sample.
   d. Load the B side reservoir with 500 mL Labeling-ON buffer. Place the cup in the side B chamber.
   e. Remember to turn on both sample cup rotation (0.01 Rpm) and the sample cup stir bar when starting the experiment, in addition to the side B pump and electrode power. Set the timer to 6 hrs.
5. **Labeling complete; transfer sample to refractive index (RI) matching solution:**
   a. Remove the sample cup and place it on the benchtop. Wash side B with 250 mL DI water, two times.
   b. Remove the sample from the cup, place in a clean container with a few mL of RI matching solution so that the buffer can begin to diffuse out, and then transfer the sample to ~25 mL of solution for longer incubation.
   c. Rinse the sample cup with DI water, and then soak it briefly in DI water with 10 mM SDS & 0.02% sodium azide to remove any residual labeling reagents. Rinse the sample cup under DI water and then store it in a sealed container with 10 mM SDS & 0.02% sodium azide.