Nissl Counterstain Protocol for RNAscope

Solutions needed

100% Ethanol (EtOH)	*1.58 – 3.16 L*
17M Acetic Acid (CH_3COOH)	*7 drops*
Chloroform ($CHCl_3$)	*300 mL*
HemoDe	*800 mL*
MilliQ water (H_2O)	*620 mL – 1.24 L*
Permount	*a couple drops per stained slide*
Thionin Buffer	*300 mL*
0.1% Thionin Stain	*250 mL*

Materials needed

Absorbent bench liner
Coverslips
Glass slide racks (for staining slides)
Square glass containers (12)
Tissue-Tek® staining dishes (3)
Tissue-Tek® slide rack
Transfer pipette
Kim wipes
Cotton swabs

% of ethanol solution	Amount of 100% ethanol per container (mL)			Amount of MilliQ water per container (mL)			# of containers needed
	T	S	L	T	S	L	
100 %	200	300	400	0	0	0	3
95 %	190	285	380	10	15	20	2
70 %	140	210	280	60	90	120	2
50 %	100	150	200	100	150	200	2
30 %	60	90	120	140	210	280	2

*T= Tissue-Tek staining dish, S= small glass dish, L= large glass dish

Note: Do all steps under the fume hood. Steps are time-sensitive and will move quickly once the protocol starts. ***Run through the full protocol with one slide as a test before running the rest of the slides to ensure that all steps are correct.***

Protocol

Workspace setup

1. Prepare the work area by labeling each square glass container.
    a. Write each of the following labels on separate containers: Chloroform, Buffer, Thionin Stain, 70% EtOH, 70% EtOH + Acetic Acid.
    b. Label two containers each with the following: 50% EtOH, 30% EtOH, 95% EtOH, HemoDe.
    c. Label three containers with 100% EtOH.
    d. Order the containers so that they follow the staining protocol.
2. Prepare solutions for each corresponding container. Use the table above for making the different concentrations of ethanol. Add 7 drops of 17M acetic acid to one of the 70% EtOH solutions.

a. For larger containers, it may be necessary to add more than 200 mL so that the slide will be completely covered when the slide rack is lowered into the liquid (Tissue-Tek® dish = 200 mL, small glass dish = 300 mL, large glass dish = 400 mL).

3. Place slides (with brain sections to be stained) in a glass slide rack that can easily fit inside each square glass container.

Staining

For each step: Submerge the slides, held in a glass slide rack, in the specified solution for the required amount of time. Dry the slides off between each bath by dabbing the slide holder on absorbent bench liner.

1. Delipidizing:
   a. Chloroform for 40 minutes.
2. Rehydrating:
   a. 100% EtOH for 1 minute.
   b. 95% EtOH for 1 minute.
   c. 70% EtOH for 1 minute.
   d. 50% EtOH for 1 minute.
   e. 30% EtOH for 1 minute.
3. Staining:
   a. Buffer for 1 minute.
   b. Thionin stain for 3 minutes.
   c. Buffer for 15 seconds.
4. Dehydrating:
   a. 30% EtOH for 30 seconds.
   b. 50% EtOH for 30 seconds.
   c. 70% EtOH + acetic acid for ~30 seconds, up to ~2 minutes.
      - Watch the colour of the sections and adjust the time in the bath accordingly. Start checking the colour just before the 30 second mark, sections should appear blue and have minimal pink/purple colouration.
   d. 95% EtOH for 1 minute x1.
   e. 100% EtOH for 1 minute x2.
   f. HemoDe for 4 minutes x2.
      - Leave the slides in the last bath of HemoDe until coverslipping.
5. Coverslipping:
   a. Use kim wipes and bench liner to dab off excess* HemoDe. *Do NOT dry slides!
      - Make sure slides don't have any condensation on them before proceeding! If droplets or condensation is present, then return slides to HemoDe.
   b. Pour Permount into a falcon tube and use a transfer pipette to place a few drops on a slide. Lower the coverslip onto the slide at an angle in order to minimize the number of trapped bubbles.
   c. If necessary, use cotton swabs to gently press on cover slipped slide to move trapped bubbles away from tissue sections. Use kim wipes to absorb any excess Permount.
6. Once cover-slipped, lay the slides out on absorbent bench paper and cover with paper towel or foil.