Manual Hematoxylin and Eosin Staining of Mouse Tissue Sections

Robert D. Cardiff, Claramae H. Miller and Robert J. Munn

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot073411

Protocol

Manual Hematoxylin and Eosin Staining of Mouse Tissue Sections

Robert D. Cardiff,[1] Claramae H. Miller, and Robert J. Munn

Center for Comparative Medicine and Center for Genomic Pathology, University of California, Davis, Davis, California 95616

The hematoxylin and eosin (H&E) stain is the standard used for microscopic examination of tissues that have been fixed, processed, embedded, and sectioned. It can be performed manually or by automation. For economic reasons, the manual technique is generally the method of choice for facilities with a low sample volume. This protocol describes manual H&E staining of fixed, processed, paraffin-embedded, and sectioned mouse tissues. In H&E-stained tissues, the nucleic acids stain dark blue and the proteins stain red to pink or orange. For accurate phenotyping and delineation of tissue detail, the protocol must be adhered to rigorously. This includes frequent reagent changes as well as the use of "in-date" reagents. Appropriate color in a good H&E stain allows for identification of many tissue subtleties that are necessary for accurate diagnosis.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Eosin Y solutions <R>
Ethanol (100%, 95% and 70%)
Hematoxylin solution (Mayer's) <R>
Permount mounting medium
Samples to be stained

Before beginning this procedure, the tissues of interest should be embedded in paraffin, sectioned to 3–5 µm, floated on a water bath, picked up onto glass slides, and placed in slide racks. Mouse tissues tend to need more time in staining solutions than human tissues, so do not process them in the same rack.

Xylene

Equipment

Coverslips
Fume hood

[1]Correspondence: rdcardiff@ucdavis.edu

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot073411

Glass slides
Glass (or metal) staining dishes and racks
Microscope

METHOD

In all of the timed steps below, the slide racks should be placed into separate staining dishes. The solutions should be changed frequently because there is some carryover as slide racks are moved through the containers. How often reagents must be changed depends on how many samples are stained. Room temperature should be used for all steps and solutions.

1. Place the glass slides that hold the paraffin sections in staining racks. Clear the paraffin from the samples in three changes of xylene for 2 min per change.
2. Hydrate the samples as follows.
   i. Transfer the slides through three changes of 100% ethanol for 2 min per change.
   ii. Transfer to 95% ethanol for 2 min.
   iii. Transfer to 70% ethanol for 2 min.
   iv. Rinse the slides in running tap water at room temperature for at least 2 min.
3. Stain the samples in hematoxylin solution for 3 min.
4. Place the slides under running tap water at room temperature for at least 5 min.
5. Stain the samples in working eosin Y solution for 2 min.
6. Dehydrate the samples as follows.
   i. Dip the slides in 95% ethanol about 20 times.
   ii. Transfer to 95% ethanol for 2 min.
   iii. Transfer through two changes of 100% ethanol for 2 min per change.
7. Clear the samples in three changes of xylene for 2 min per change.
8. Place a drop of Permount over the tissue on each slide and add a coverslip. View the slides using a microscope.

   Stain intensity will vary with species and fixative. The eosin stain will be more intense with alcohol-based fixatives than with strong cross-linker fixatives. Weak cross-linker fixatives tend to have poor uptake of hematoxylin stains. See Troubleshooting.

TROUBLESHOOTING

Problem (Step 8): Hematoxylin is too light (the nuclei are very pale).
Solution: The hematoxylin staining time may have been inadequate, or the hematoxylin solution may have been overoxidized. Discard the hematoxylin solution and start over. It is also possible that the differentiation step was too long.

Problem (Step 8): Hematoxylin is too dark (the nuclei are overstained).
Solution: Check the staining time and the section thickness. The hematoxylin staining time may have been too long, or the sections may have been too thick. Alternatively, the differentiation step may have been too short.

Problem (Step 8): Hematoxylin is staining red-brown nuclei.
Solution: The hematoxylin is exhausted and no longer usable. Prepare a new solution.

Problem (Step 8): Eosin staining is too pale.
Solution: Check the pH of the eosin; it may have been >5. It is also possible that the slides were left in the dehydrating solutions too long.

 Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot073411

Problem (Step 8): Eosin is overstaining (the cytoplasm is too dark).
Solution: Check the concentration of eosin; the eosin reagent may have been too concentrated. Alternatively, the slides may have passed through the dehydrating steps too quickly for good eosin differentiation to occur.

Problem (Step 8): Slides have a blue-black deposition product on top of the tissue.
Solution: Filter the hematoxylin stain.

DISCUSSION

Most microtomes are set to cut paraffin sections at a thickness of 5 µm. With proper training and attention to detail, this thickness or less is achieved. The quality of the stains is also critical. We very frequently receive poorly stained slides. In some cases, the coverslip can be removed and the slide restained for adequate quality. However, a knowledgeable professional should take responsibility for the quality of the product. The lack of knowledgeable professionals leads to poor products that plague publications. Quality control is essential.

Most laboratories use an automated tissue processor and an automated immunocytochemistry stainer, both of which are major monetary investments. A given institution could have several different brands or models. This fact makes it almost impossible to dictate a single protocol because each machine has different specifications. However, there are several processing errors that can be avoided by all investigators. If using a core laboratory for processing, the researcher should inform the laboratory of the species being processed. The researcher should inquire about the processing schedules and the frequency at which the different reagent baths are changed. The type of the paraffin is also a critical variable, because melting points can vary. When we receive paraffin-embedded blocks from other institutions, frequently a sunken tissue face is found, indicating a poorly dehydrated and embedded specimen. This sample must be reembedded if a high quality or even interpretable preparation is to be obtained. If the reagent containers in the processor or stainer are not changed regularly, the different solutions become contaminated downstream and inhibit proper processing. Most processors use a formalin bath for storage of the blocks before processing. All subsequent solutions are then contaminated by formalin. In turn, this can inhibit certain cytochemical reactions if the reagents are not regularly changed.

RELATED INFORMATION

Excellent publications on histologic techniques are available from the Armed Forces Institute of Pathology (U.S.) (1968) and Carson and Hladik (2009). Full details on H&E staining are available in a guideline published as a PDF document by the National Society for Histotechnology (2001).

RECIPES

Eosin Y Solutions

1. Prepare eosin Y stock solution. Add 2.0 g of water-soluble eosin Y to 40 mL of double-distilled H_2O, and mix until dissolved. Then add 160 mL of 95% ethanol, and mix. Store at room temperature.
2. Prepare eosin Y working solution. Add 200 mL of eosin Y stock solution to 600 mL of 80% ethanol and mix well. While working in a fume hood, add 4 mL of glacial acetic acid and mix well. Store covered at room temperature.

Hematoxylin Solution (Mayer's)

1. Add 50.0 g of aluminum ammonium sulfate to 800 mL double-distilled H_2O. Stir, but do not use heat to assist dissolving.
2. Add 1.2 g of hematoxylin crystals and stir until dissolved.
3. Add 0.2 g of sodium iodate and 1.0 g of citric acid. Stir until dissolved.
4. Add 50 g of chloral hydrate and stir overnight at room temperature.

   Chloral hydrate is a controlled substance; check with your laboratory safety officer regarding purchase and appropriate/safe storage.
5. Bring the total volume up to 1000 mL (this usually takes ~100 mL H_2O). Filter and store in a dark bottle at room temperature.

   Mayer's hematoxylin keeps well for several months. This recipe is based on one described by the Armed Forces Institute of Pathology (1968).

ACKNOWLEDGMENTS

The protocol presented here is from the Mutant Mouse Pathology Laboratory at the Center for Comparative Medicine, University of California, Davis.

REFERENCES

Armed Forces Institute of Pathology (U.S.). 1968. *Manual of histologic staining methods of the Armed Forces Institute of Pathology*, 3rd ed (ed. Luna LG). Blakiston Division, McGraw-Hill, New York.

Carson FL, Hladik C. 2009. *Histotechnology: A self-instructional text*, 3rd ed. ASCP Press, Chicago.

National Society for Histotechnology (U.S.). 2001. *Guidelines for hematoxylin and eosin staining*, http://bit.ly/SQWR0w.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot073411