Congo Red Staining Protocol

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1. Overview
   1. The Amyloid Stain (Congo Red) is intended for use in the histological visualization of amyloid in tissue sections. Examination under a polarizing microscope results in green birefringence of amyloid.
   2. Staining Interpretation:

|  |  |
| --- | --- |
| Amyloid | Red to Pink |
| Erythrocytes | Light Orange |
| Eosinophil Granules | Orange to Red |
| Nuclei | Blue |
| (from Abcam Congo Red Stain Kit Protocol Booklet, [https://www.abcam.com/congo-red-stain-kit-amyloid-stain-ab150663.html#](https://www.abcam.com/congo-red-stain-kit-amyloid-stain-ab150663.html)) | |

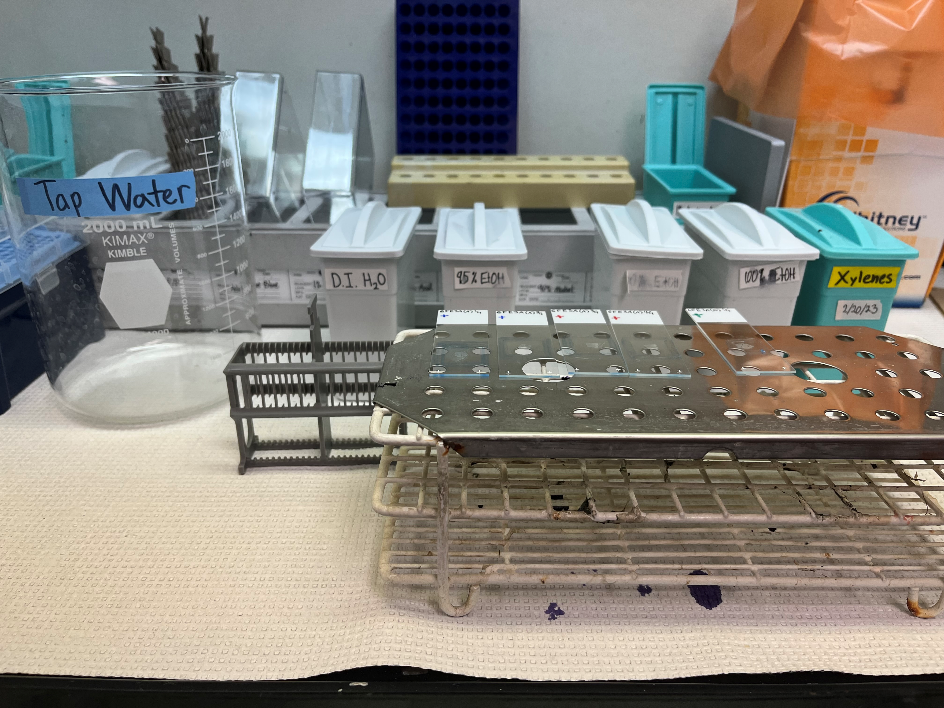
1. Required Materials
   1. Abcam Congo Red Stain Kit (Abcam, SKU#: [ab150663](https://www.abcam.com/congo-red-stain-kit-amyloid-stain-ab150663.html))
   2. Microscope slides with tissue section(s)
   3. Hydrophobic Barrier Pap Pen (Vector Labs, SKU#: [H-4000](https://vectorlabs.com/products/histology/immedge-hydrophobic-barrier-pen))



**Figure 1**: ImmEDGETM Hydrophobic Barrier Pap Pen

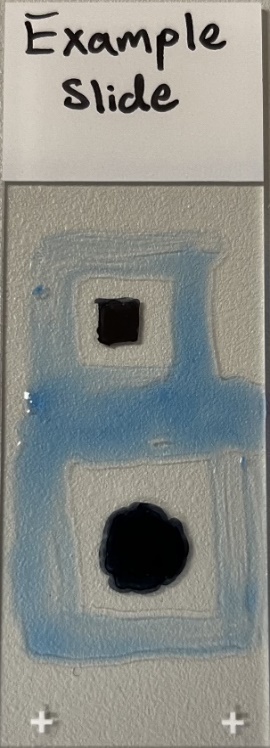
* 1. Solutions required but not supplied by Abcam (prepare beforehand):
     1. Tap Water (~1L/stain)
     2. D.I. H2O (~200mL/stain)
     3. 95% Ethanol (~200mL/stain)
     4. 2x 100% Ethanol (2x ~200mL/stain)
     5. Xylenes Solution (HistoPrep, SKU#: [HC700-1GAL](https://www.fishersci.com/shop/products/xylene-fisherbrand-histoprep/HC7001GAL))
     6. Permount Mounting Medium, Electron Microscopy Science (VWR, SKU#: [100496-550](https://us.vwr.com/store/catalog/product.jsp?catalog_number=100496-550))
  2. Other useful equipment:
     1. SHURStain Manual Stainer Rack, 12 position (VWR. SKU#: [89238-898](https://us.vwr.com/store/product?keyword=89238-898))
     2. Flat surface to lay slides on while they stain (since stains will be pipetted onto slides)

1. Setup:
   1. Have all reagents and washes pre-made before start. Keep reagent/washes for the next step close-to-hand so transition between staining and washing steps are as quick as possible. Once a reagent or wash is used, set it outside the work area (i.e., the fume hood) to signal it has been used and is queued for discard. Removing used chemicals also allows for more work room, reducing chance of spillage.
   2. An example image ([**Figure 2**](#Fig2)) of a past staining setup can be viewed below:



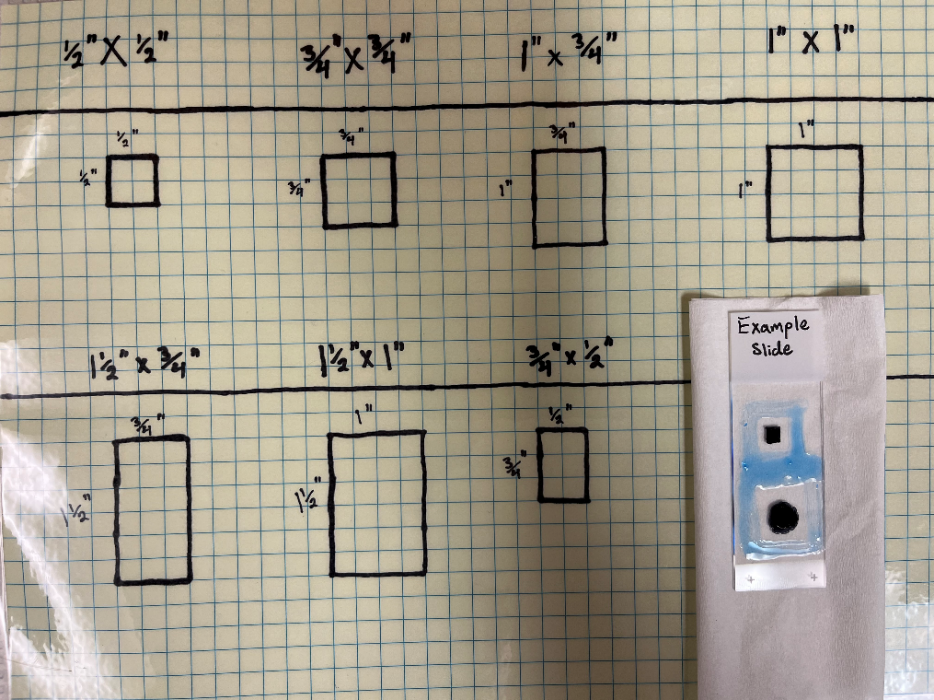
**Figure 2**: Assay setup prior to start

1. Assay Procedure
   1. Preliminary Steps – do BEFORE applying first stain:
      1. Equilibrate slides with frozen sections to room temperature for 30 mins
      2. Equilibrate all reagents to room temperature just prior to use.
         1. Abcam reagents should be stored at room temp already.
      3. While slides and reagents equilibrate, make/pour 200mL of D.I. H2O, 95% Ethanol, 100% Ethanol, and Xylene into the SHURStain wells.
         1. NOTE: The green SHURStain wells are reportedly “Xylene resistant”.
         2. NOTE: These wells are made to hold up to 250mL, but filling them to this volume will completely submerge the slides, which, when dunked in alcohol, will strip away any hand-written labels on the slides. Hence, the suggestion to only use 200mL to preserve labels and conserve reagents.
            1. Another solution is to label with pencil instead of marker. Pencil labels can withstand an alcohol wash, but are relatively faint to begin with.
      4. Gently agitate Abcam reagents before use.
         1. NOTE: Better to agitate reagents immediately prior to using
      5. Apply hydrophobic barrier around sections with Pap Pen ([**Figure 3**](#Fig3)):



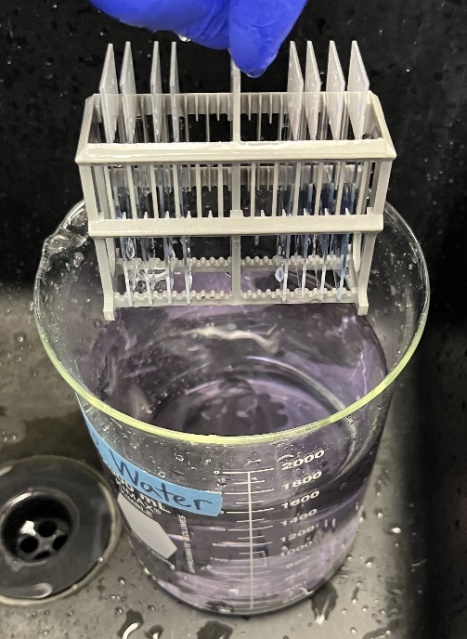
**Figure 3**: Example slide showing hydrophobic barriers drawn with dimensions (top) ½“ x ½“ and (bottom) ¾“ x ¾”.

* + 1. Using a pre-made template sheet ([**Figure 4**](#Fig4)), trace a box around tissue sections.
       - 1. ½” X ½” box is preferred (to concentrate solutions on tissue section and prevent run-off).
         2. Double layer PapPen around the tissue to reduce chances of solution run-off – i.e., increase the thickness of the hydrophobic cage around tissue.



**Figure 4**: Template sheet for tracing standardized hydrophobic cages around tissue sections with Pap Pen (Hydrophobic Barrier Pen).

* 1. Staining & Wash Steps
     1. Apply 4-6 drops (~160μL) of **Hematoxylin** for 5 seconds.
        1. Rinse/dunk in **stagnant tap water** – i.e., not running water; taken from any regular sink faucet ([**Figure 5**](#Fig5)).



**Figure 5**: Tap water rinse in 2L glass beaker.

* + - 1. Flick off as much water as possible before adding next reagent, *but do not allow to dry completely!!*
    1. Apply 4-6 drops (~160μL) of **Bluing Reagent** for 15 seconds.
       1. Rinse slides in **distilled water**.
       2. Dip slides in **95% Ethanol** for 5 seconds.
       3. LET TISSUE DRY COMPLETELY BEFORE NEXT STEP:
          1. Congo Red Solution will run off tissue very easily, even with the hydrophobic barrier. If it spreads over the slide, the solution may crystallize in tissue, resulting in a poor stain.
    2. Apply 4-6 drops (~160μL) of **Congo Red Solution** for 1 minute. ([**Figure 6A**](#Fig6A) & [**Figure 6B**](#Fig6B))

**NOTE:** The key with this step is to prevent Congo Red from evaporating while staining tissue, as this causes crystallization of the reagent and results in poor tissue differentiation and poor staining overall.

* + 1. Rinse in **100% Ethanol** (2x changes for 20 seconds each)
    2. Place slides in **Xylenes Solution** for 10-15 minutes (until all the hydrophobic pen residue has been stripped from the slide).
    3. Allow slides to dry before mounting coverslips. Use the air nozzle on the lab bench to speed up the drying process if desired.
  1. Mounting Coverslip
     1. Once slides have dried completely, dunk again in Xylene for 20 seconds and tap-off.
        1. NOTE: mount coverslip while the slide is still wet from Xylene. DO NOT allow to completely dry before applying Permount.
     2. Using Permount Mounting Medium, dab a line NEXT TO tissue (NOT directly on it). Take coverslip and touch the edge of it to side of slide where Permount was placed, creating a region of shared contact between the slide and coverslip with the Permount. Then, guide the body of the coverslip to gently “fall” across the tissue and body of the slide.
  2. Summary table of procedure:

|  |  |
| --- | --- |
| *Step (stain/wash)* | *Time (minutes)* |
| Hematoxylin | **5 seconds** |
| wash | Tap water |
| Bluing Reagent | **15 seconds** |
| wash | Rinse slides in distilled water, followed by 5 seconds of 95% Ethanol |
| Congo Red | **1 minute** |
| Final wash | *Tap off in between dunks:* 20 seconds in 2x changes of 100% Ethanol, followed by 10-15 minutes in Xylene. Air dry completely using air hose. Re-dunk in Xylene before applying mounting medium and cover slip. |

1. Appendix I: Safety Precautions & Disposal Instructions
   1. Xylene is extremely toxic!
      1. Always handle inside fume hood
      2. Always wear gloves and lab coat while handling; safety goggles probably wouldn’t be a terrible idea either.
      3. **DO NOT DISPOSE IN SINK**
         1. There should be a glass 4L bottle labeled for Xylene waste disposal.
   2. All other chemicals can be safely washed down the sink with running water
2. Appendix II: Troubleshooting Tips, Common Errors, & Helpful Info
   1. Running the procedure alone?
      1. Stain max 4 slides at once when by yourself. Step 1 takes 5 seconds (hematoxylin will overstain the tissue very quickly). For me, that is just enough time to dropper the stain on 4 slides, throw away the transfer pipette, and close the bottle before time’s up on whichever slide I stained first.
      2. Make sure that the 1st slide stained is the 1st slide tapped off once the timer goes off; this helps keep the application time relatively equal for all slides.
      3. I will try to optimize efficiency by making sure the stain, paper towels, containers with the next washes, etc. are ready for use before I start each step so everything is readily available and easily accessible before I need them. Some of these steps are so quick you won’t have time to setup after dropping the stain on the slide.
         1. Setting up like this also forces you to run through the next step in your head, and the rehearsal helps me increase efficiency.
   2. Congo Red Stain:
      1. NOTE: slides are covered to reduce airflow over tissue during this step. This is an attempt to prevent Congo Red from drying out on tissue, as this causes crystallization, resulting in poor tissue differentiation and a poor stain overall.
3. Appendix III: Website Links
   1. Abcam Congo Red Stain Kit: <https://www.abcam.com/congo-red-stain-kit-amyloid-stain-ab150663.html>
   2. Microscope slides:
   3. ImmEdge® Hydrophobic Barrier PAP Pen: <https://vectorlabs.com/products/histology/immedge-hydrophobic-barrier-pen>
   4. HistoPrep Xylenes: <https://www.fishersci.com/shop/products/xylene-fisherbrand-histoprep/HC7001GAL>
   5. Permount Mounting Medium: <https://us.vwr.com/store/catalog/product.jsp?catalog_number=100496-550>
   6. SHURStainTM Manual Stainer Rack, 12 position: <https://us.vwr.com/store/product?keyword=89238-898>