

Methods and Protocols for Decalcification of Bone Material

(from IHC world)

Fixation: 10% Buffered Neutral Formalin for up to 5 days.

All fixed specimens are washed in slowly running tap water for a minimum of 30 minutes. Larger specimens are washed up to a maximum of 1 hour. Avoid rinsing in rapidly running water.

Solutions:

8% Hydrochloric Acid Stock Solution:

Hydrochloric acid, concentrated ----- 40 ml
Distilled water ----- 460 ml
Mix well and store at room temperature.

8% Formic Acid Stock Solution:

Formic acid ----- 40 ml
Distilled water ----- 460 ml
Mix well and store at room temperature.

Hydrochloric Acid/Formic Acid Working Solution:

Combine equal parts of the 8% hydrochloric acid solution and the 8% formic acid solution before use.

Ammonia Solution:

Ammonia, Concentrated ----- 5 drops
Distilled water ----- 100 ml
Mix well

Procedure:

1. Specimens should be decalcified in hydrochloric acid/formic acid working solution 20 times their volume.
2. Change to fresh solution each day until decalcification is complete. It may take 24 hours up to days or months depending on size of the specimens. See below for the testing procedures **(Decalcification for rabbit spine using this protocol for overnight about 20 hours and it showed very good result)**
3. Once the decalcification is complete, rinse specimens in water briefly and transfer to ammonia solution to neutralize acids left in specimens for 30 minutes.
4. Wash specimens in running tap water thoroughly up to 24 hours.
5. Routine paraffin embedding.

Specimens should **NOT** be crowded together and should **NOT** contact the bottom of container in order to provide for complete decalcification.

Overdecalcification can also permanently damage a specimen. The following procedure help determine the correct end-point of decalcification.

End-Point of Decalcification:

- X-ray (the most accurate way)
- Chemical testing (accurate)
- Physical testing (less accurate and potentially damage of specimen)

Chemical Test:

The following solutions are needed to chemically test for residual calcium.

5% Ammonium Hydroxide Stock:

- Ammonium hydroxide, 28% ----- 5 ml
- Distilled water ----- 95 ml
- Mix well

5% Ammonium Oxalate Stock:

- Ammonium oxalate ----- 5 ml
- Distilled water ----- 95 ml
- Mix well

Ammonium Hydroxide/Ammonium Oxalate Working Solution:

Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution.

Procedure:

1. Insert a pipette into the decalcifying solution containing the specimen.
2. Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube.
3. Add approximately 10 ml of the ammonium hydroxide/ammonium oxalate working solution, mix well and let stand overnight.
4. Decalcification is complete when no precipitate is observed on two consecutive days of testing. Repeat this test every two or three days.

Physical Tests:

The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue. The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears and pinhole artifacts. Slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

Note: If paraffin embedded bones were not decalcified fully, one can soak the paraffin blocks in the same decalcification solution for a few minutes before cutting. This is usually helpful.