Science of Decalcification

For Routine, Advanced and Molecular Analysis

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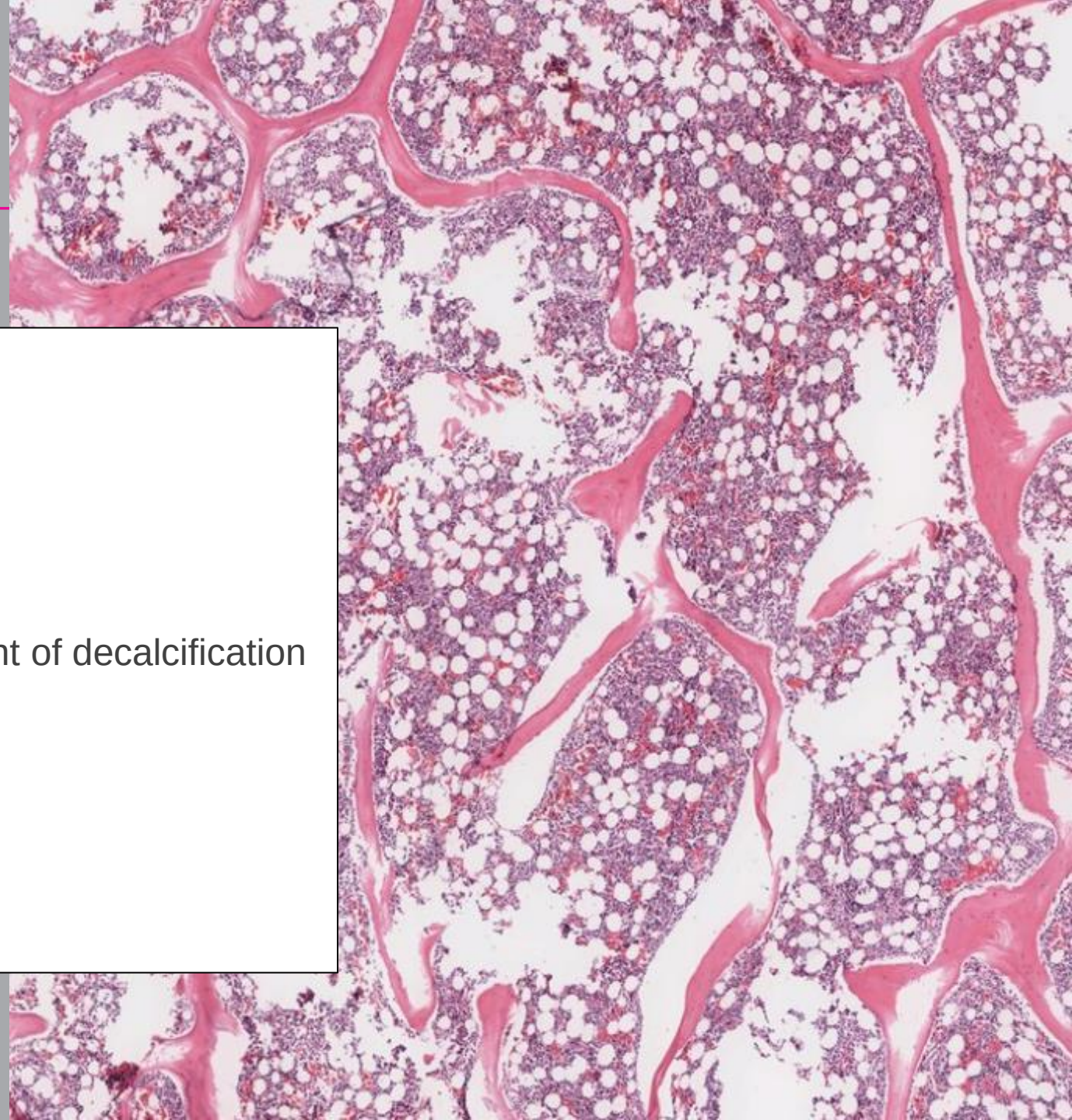
Sr. Technical Content Global Marketing Manager

epredia
Enhancing precision cancer diagnostics

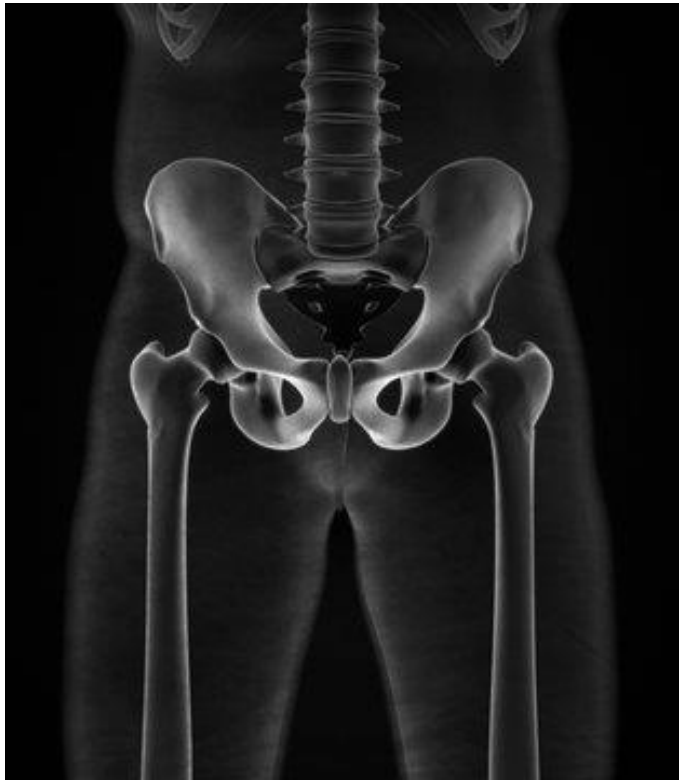
Agenda

- What is decalcification
- Why tissues need to be decalcified
- Decalcification methods
- Determining acid strength
- Chemical and physical tests to determine endpoint of decalcification
- Troubleshooting
- Decalcifying for advanced staining and analyses
- Epredia decalcifiers



What is Decalcification?

- Decalcification describes the technique for removing calcium minerals from bone or other calcified tissue so paraffin sections can be sectioned.
- Decalcification is carried out between fixation and processing steps.



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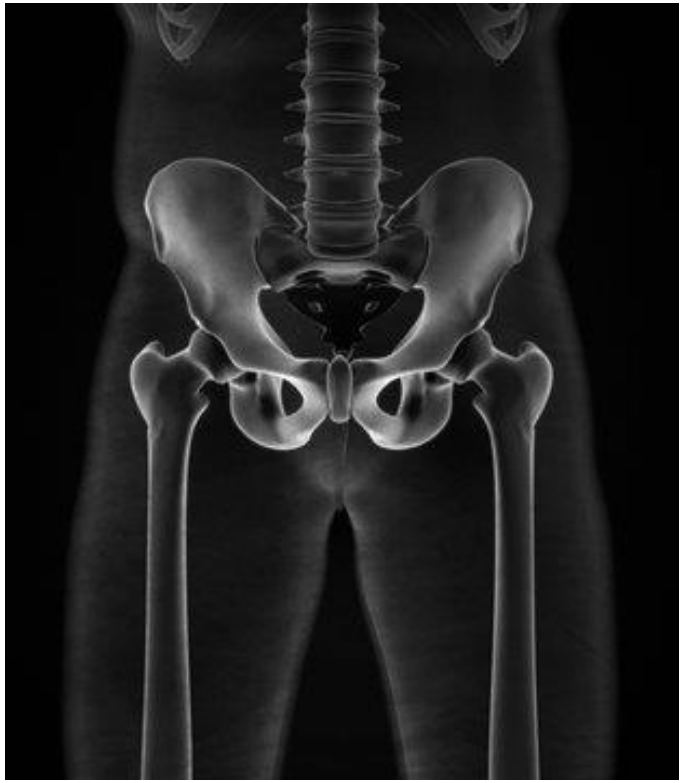
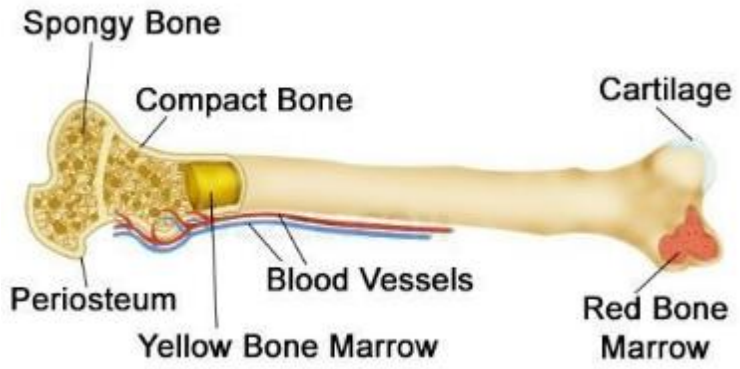
- Decalcification is carried out between fixation and processing steps.
- Amount of unwanted calcium salts determines the decalcification method that must be used.
- Calcification is a regulated physiological process occurring in bones and teeth. However, calcification is commonly found in soft tissues in association with aging and in a variety of diseases. Calcium deposits can form all over your body, including in soft tissues, arteries and organs.



Decalcification Background

- Decalcification is usually performed by immersing the hard tissue in different decalcification fluids with various properties.
- These decalcification fluids typically include inorganic and organic acids, a neutral fluid containing a chelating agent, or a mixture of solutions.



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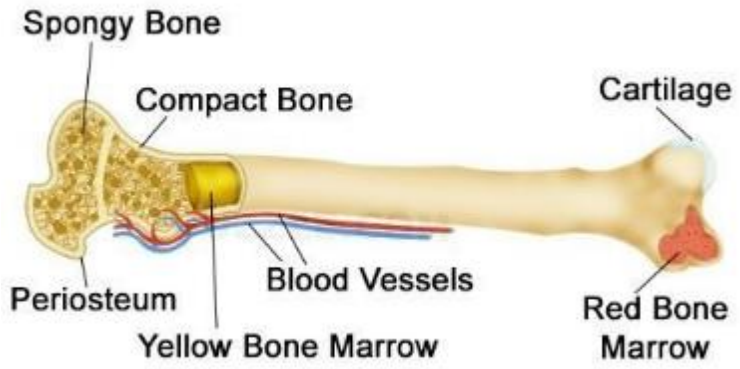
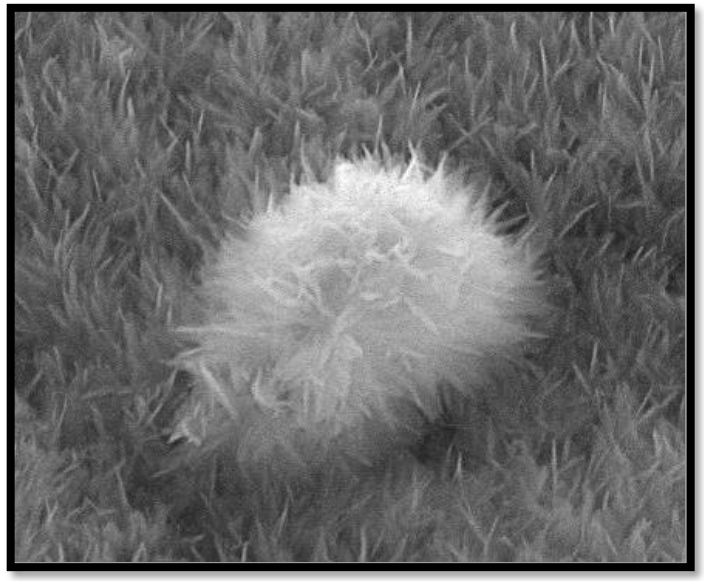


- Unfortunately, there is no universal decalcification fluid that satisfies all the requirements of pathologists such as rapid decalcification, easy handling, and minimal tissue damage.
- Different staining techniques (routine, IHC, ISH) or molecular assays usually require different decalcification methods.

Why Tissue Needs to Be Decalcified?

- Any paraffin embedded specimen that is sectioned on a microtome needs to be soft enough to be cut by a microtome blade. Some tissues, however, are made up from calcium containing material that is too hard for a disposable microtome blade to cut through.

- Such tissues include bones and any organs that contain **calcium ions**. These ions form hydroxyapatite crystals, which are deposited between the fibrous elements of the tissue.
- Removing the mineral from bone or other calcified tissue is necessary so that good quality paraffin sections can be prepared.
- Calcium salts, or hydroxyapatite crystals, are dissolved or reduced in size by chemicals.



Needle-like hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$.
Scanning electron microscope picture from University of Tartu.

Poll Question # 1

Typically, decalcification process is done_____

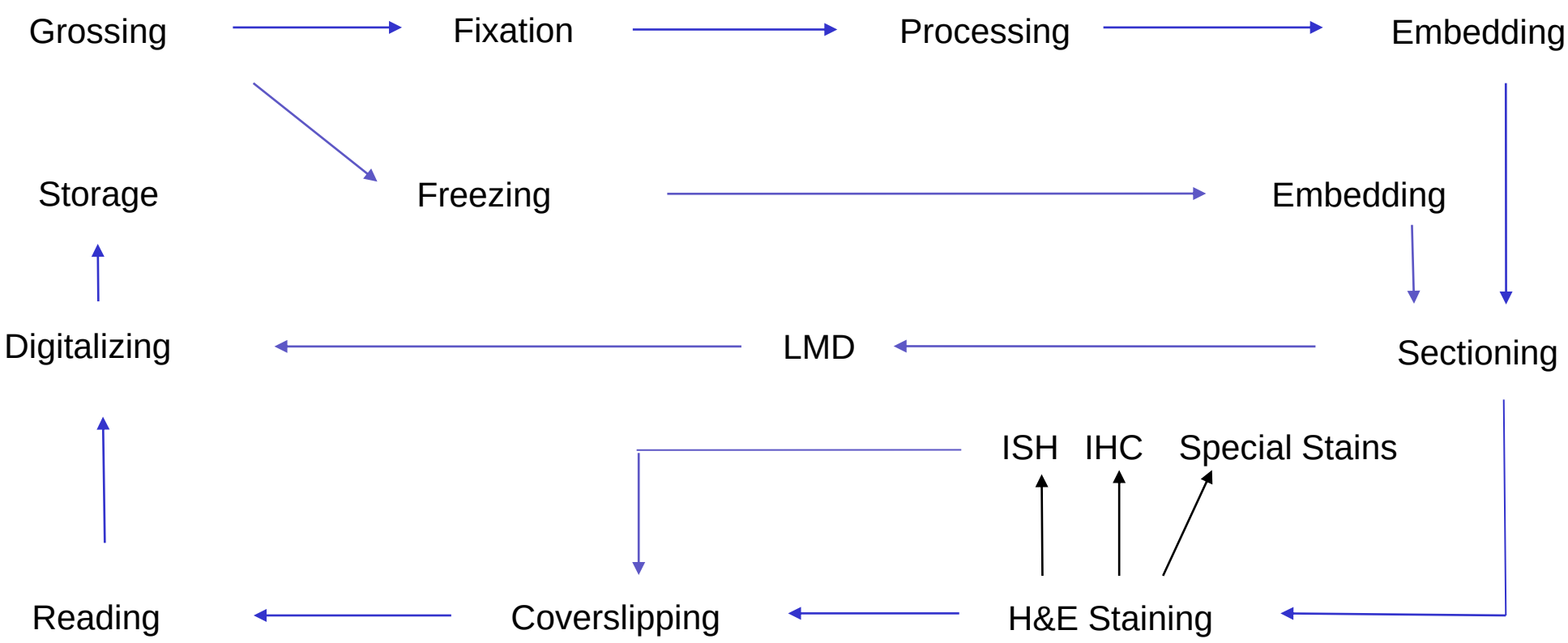
A. together with fixation.

B. after specimens are fixed.

C. prior to a fixation step.

D. after samples are processes to maintain morphology integrity.

Histology Workflow



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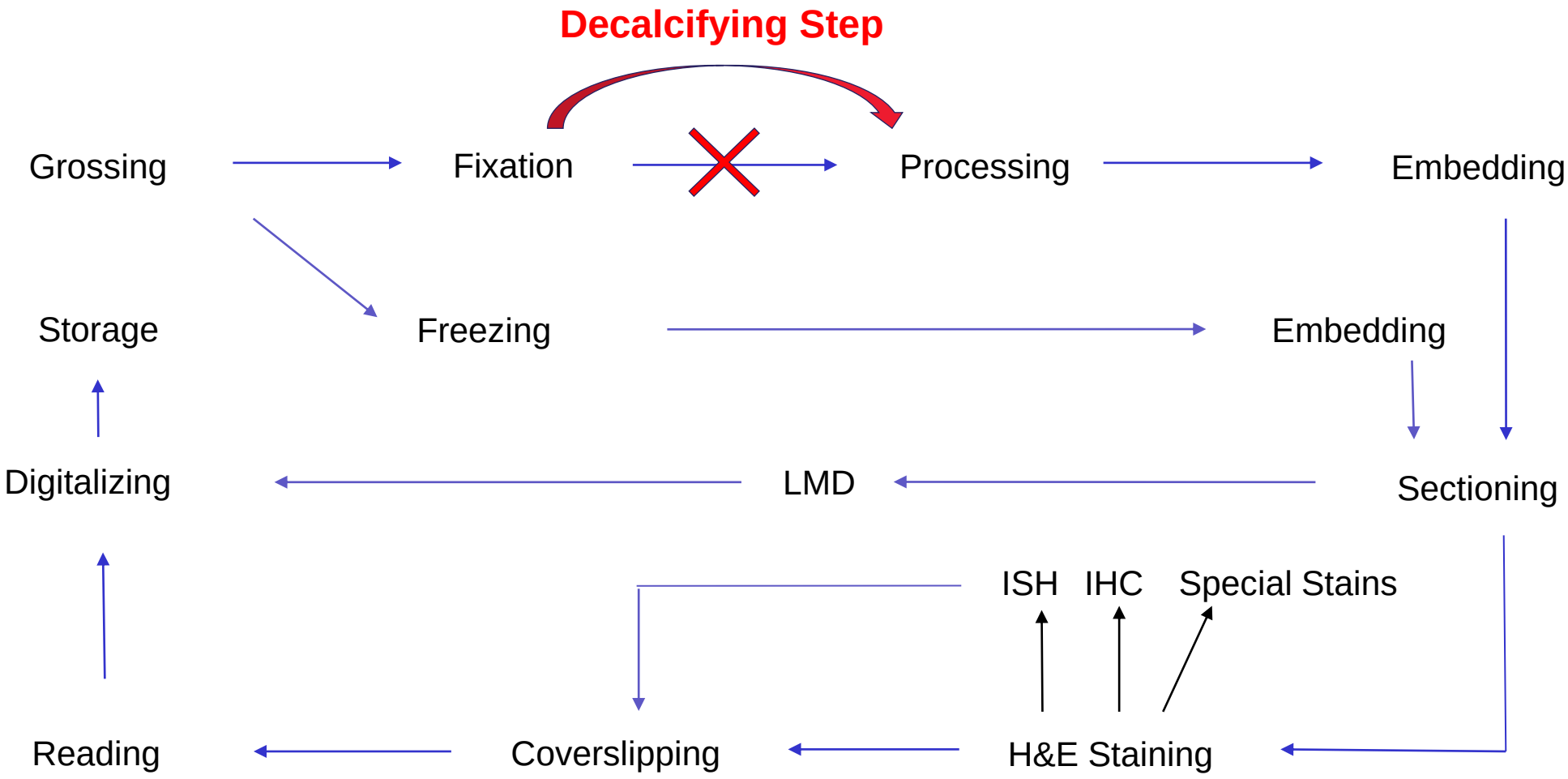

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Decalcification Methods

Acids

Strong

Weak

Chelating Agents

Surface

Clinical set-up

Ion-Exchange

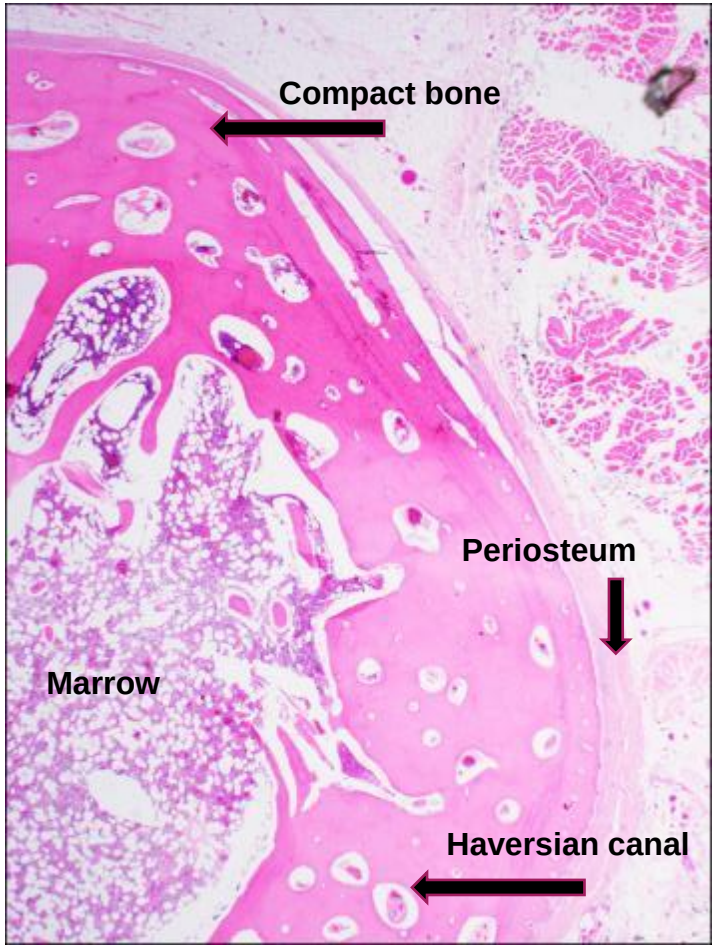
Electrolytic Ionization

Ultrasonic

Basic and clinical research

Fixation of Bone

- It is particularly important to **thoroughly** fix bone specimens prior to decalcification, it is therefore common practice for laboratories to extend fixation times.
- Poorly-fixed specimens become **macerated** during decalcification and **stain poorly** afterwards.
- It is important to provide **ready access** for the fixative to penetrate the bone, so skin and soft tissue should be removed, if possible.



Bone, normal histology, H&E stain.

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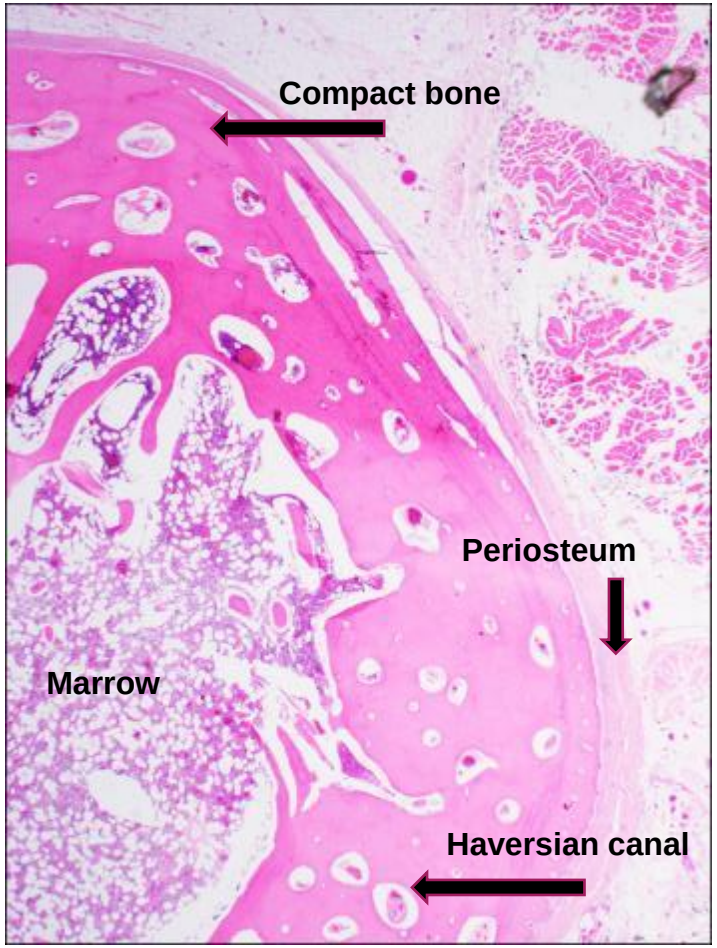
- It is important to provide **ready access** for the fixative to penetrate the bone, so skin and soft tissue should be removed, if possible.
- Bone specimens should be grossed into **smaller pieces** as soon as possible to enhance fixation and an adequate volume of fixative provided.
- Buffered formalin is a satisfactory fixative for bone, but where the preservation of bone marrow is important, some laboratories will use **alternatives** such as one of the Zinc formalin mixtures, B5, formol acetic alcohol (Davidson's fixative), or Bouin.



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Acid Decalcification

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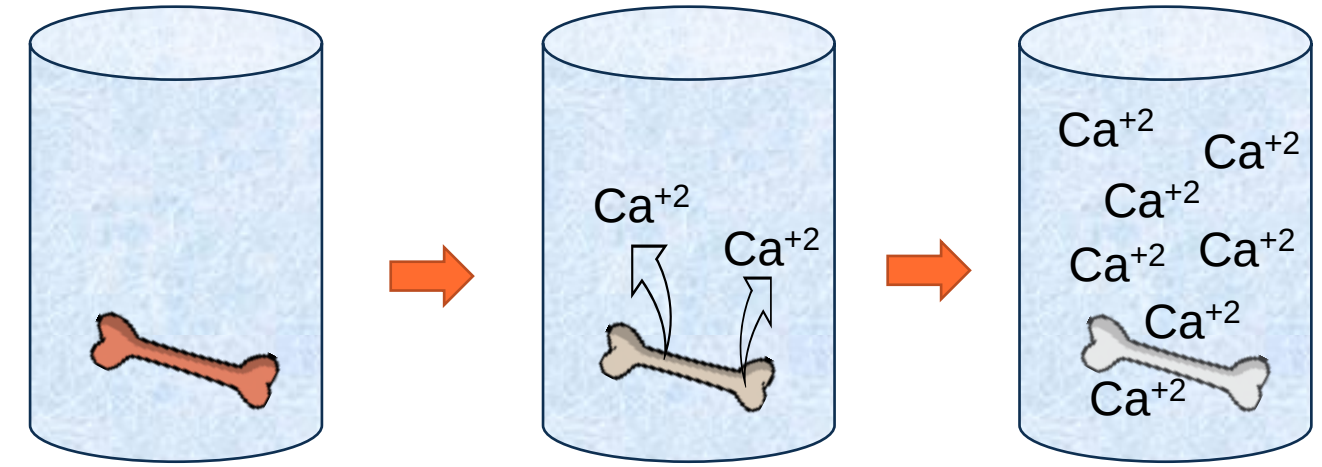
- At the proper pH, calcium ions migrate out of the tissue into the surrounding solution.



Decalcifying solution
pH 0.5 - 3.0

Ca ions are dissolved
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Ca^{+2} saturated
solution

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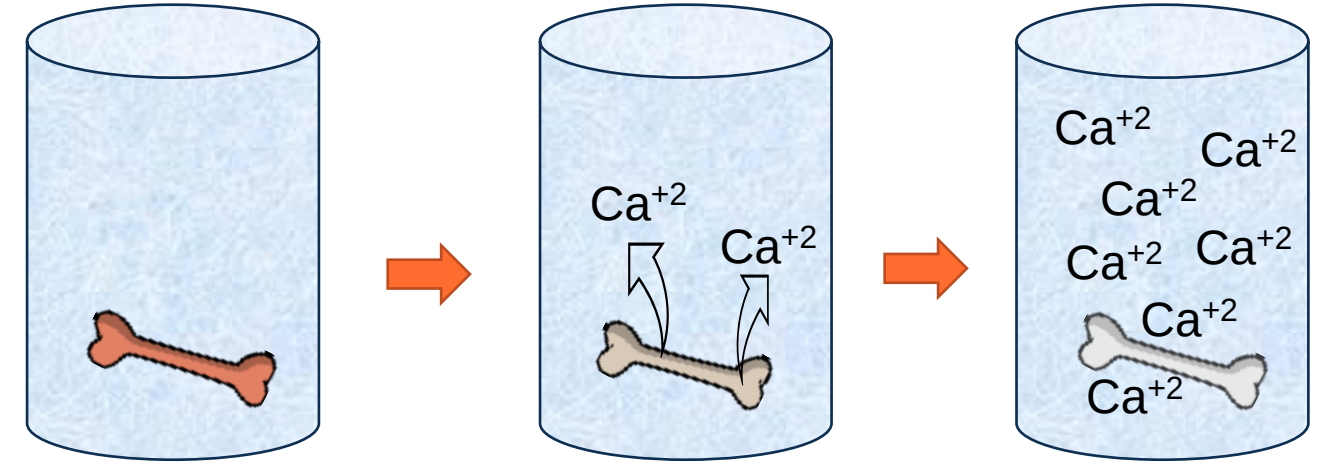
- The solution may become saturated with calcium ions and for this reason the solution should be changed frequently.
- Any acid, well buffered, will have some effect on the stainability of tissue.



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Decalcifiers Based on Strong Acids

- Acids commonly are used in 5% to 10% solutions.
- Hydrochloric and nitric acids decalcify rapidly, but the process must be **carefully monitored**.
- Nitric acid can cause serious deterioration of tissue stainability if decalcification is prolonged beyond 48 hrs.

Decalcifier	Composition
Nitric acid	~ 5% in dH_20
Perenyi's fluid	10% Nitric acid + chromic acid + alcohol
Hydrochloric acid	~ 5-10% in dH_20
Von Ebner's solution	8% Hydrochloric acid + sodium chloride + dH_20

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- If hydrochloric acid is used after formaldehyde fixation, specimens need to be **washed** out before placing it in the acid.
- **Heat** should never be used to speed up decalcification with acid, because heat also increases the effects of the decalcifying fluid on other tissue components, most likely resulting in swelling and maceration.

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- Any decalcifier, based on a weak inorganic acid is **ideal for small** calcified specimens.
- Formic acid is a slower acting acid and afford the user more latitude.
- **Mixtures of formaldehyde and formic acid** are excellent for simultaneous fixation and decalcification.

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Evans and Krajian	25% formic acid + sodium citrate + dH_20
Kristensen	8% formic acid + sodium formate + dH_20
Gooding and Stewart	5 - 25% formic acid + formaldehyde + dH_20
Trichloroacitic acid	~ 5% in formal saline

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- **Mixtures of formaldehyde and formic acid** are excellent for simultaneous fixation and decalcification.
- It is rare for tissue to lose staining ability after formic acid decalcification even after remaining in the solution for 2 weeks.
- Needle biopsies may be completely fixed and decalcified in one hour or less.
- Small bone specimens and calcified arteries may be completely fixed and decalcified in approx. 2-4 hours.

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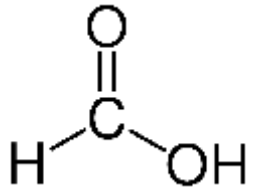
Formic Acid Story

- By far, one of the most common weak acids used in histology is formic acid.
- Formic acid (systematically called methanoic acid) is the simplest carboxylic acid containing a single carbon.



Formic Acid Story

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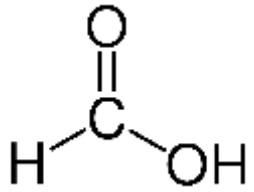
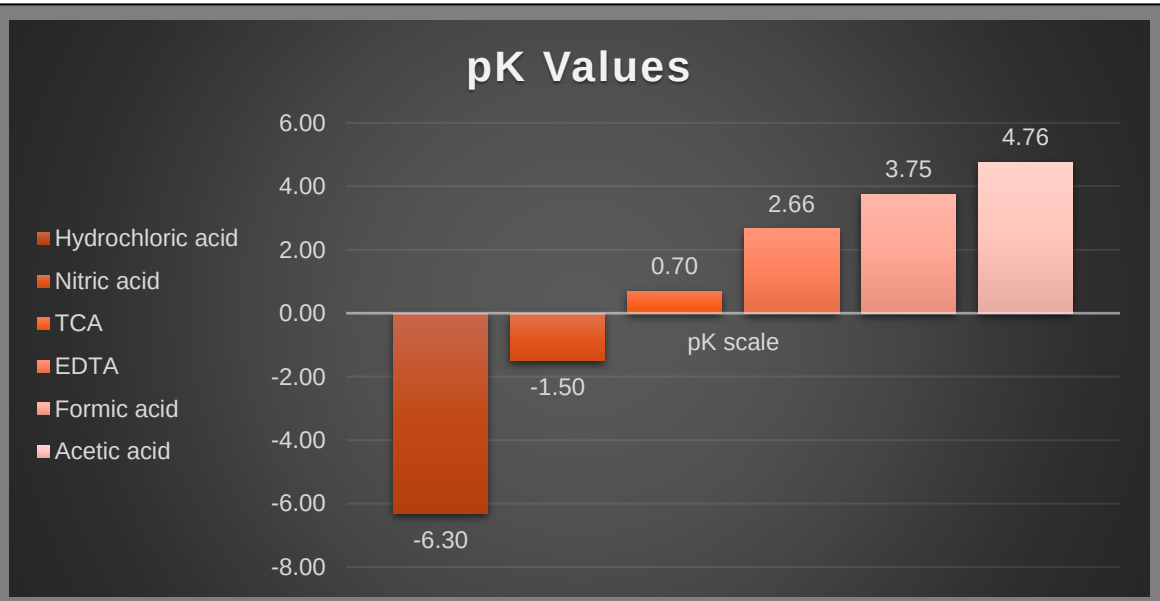
- Formic acid (systematically called methanoic acid) is the simplest carboxylic acid containing a single carbon.
- Formic acid got its name from “Formica”, the Latin name for an ant.
- An English naturalist named John Ray was the first person to isolate an acid from ants. In 1671 he distilled the crushed bodies of dead ants to extract the acid, which was eventually named formic acid.
- Ants produce and store formic acid in their glands as a weapon against predators.
- When bitten by an ant, we feel formic acid being injected under our skin!



Choosing Optimal Acid

- Acid is the main and key component of a decalcifier
- Acidity or strength of acids is measured by “pK” value
- The lower pK value, the stronger is the acid
- By choosing the right acid for a decalcification method we can optimize the results

- Hydrochloric acid pK -6.30
- Nitric acid pK -1.50
- TCA pK 0.70
- EDTA pK 2.66
- Formic Acid pK 3.75
- Acetic Acid pK 4.76



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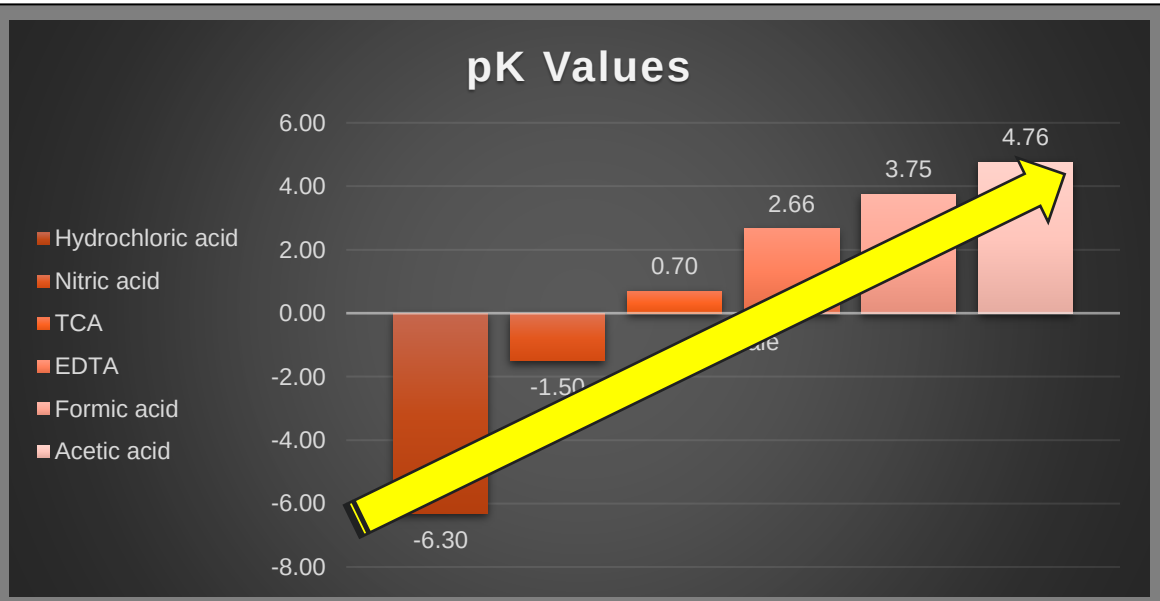

Chelating agents

- Chelating agents such as EDTA work by **capturing the calcium ions** from the surface of the apatite crystal, slowly reducing its size.
- Because the process is very **slow but very gentle** this method is not appropriate for urgent specimens.
- Popular in research where very high-quality morphology is required or molecular elements must be preserved for techniques such as IHC, ISH or PCR.

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- Popular in research where very high-quality morphology is required or molecular elements must be preserved for techniques such as IHC, ISH or PCR.
- It is used at a concentration of approximately 14%.
- Chelating agents do not affect tissue staining.
- Complete decalcification is achieved for dense bones at approx. 6-8 weeks and at 1 week for small specimens.

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Surface Decalcification

- This is a method of dealing with small **unexpected** deposits of calcium (e.g., calcified vessels) that may be encountered in paraffin blocks.
- Surface decalcification is also employed when the time allocated for decalcification was too short and not all calcium salts were dissolved.
- Normally, it is after trimming the block on the microtome that the calcium deposits are discovered.

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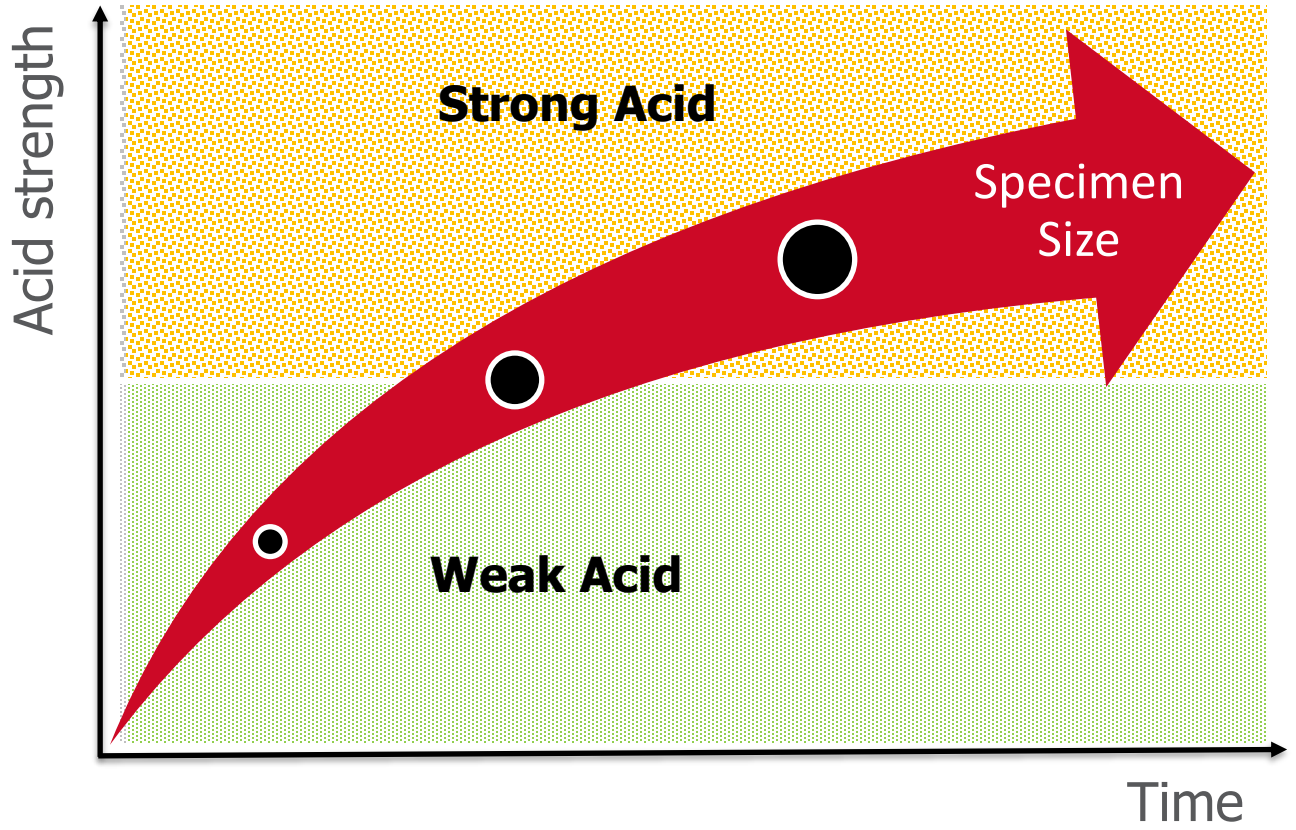
- Normally, it is after trimming the block on the microtome that the calcium deposits are discovered.
- After the tissue has been exposed, a couple of drops of decalcifier are placed over the tissue for 15 - 60 min.
- This surface treatment will allow the decalcifier to penetrate a small distance into the block and dissolve the calcium. The block can then be thoroughly rinsed in water to remove residual acid, chilled and re-sectioned.

Poll Question # 2

The optimal decalcifier for biopsy-sized specimen to be routinely stained is:

A. EDTA solution
B. Weak acid with formaldehyde
C. Strong acid with formic acid
D. Weak acid solution without formaldehyde

Relationship Between Specimen Size, Acid Strength and Time Needed for Decalcification



Specimen size usually dictates choice of acid and time allowed for decalcification.

Suggested Decalcifiers for Best Results

- Acid based decalcifiers are used when Hematoxylin and Eosin and Special Stains are performed.
- For Immunohistochemistry and In Situ Hybridization assays, decalcifier containing a weak acid is recommended only when targeted antigen or nucleic acid is known to survive acid treatment.

Specimen size	**Application**	
	Hematoxylin & Eosin Special Staining	Immunohistochemistry *In Situ* Hybridization
Biopsy to small	Formic acid based decalcifier	EDTA solution
Medium to large	Hydrochloric acid based decalcifier	EDTA solution

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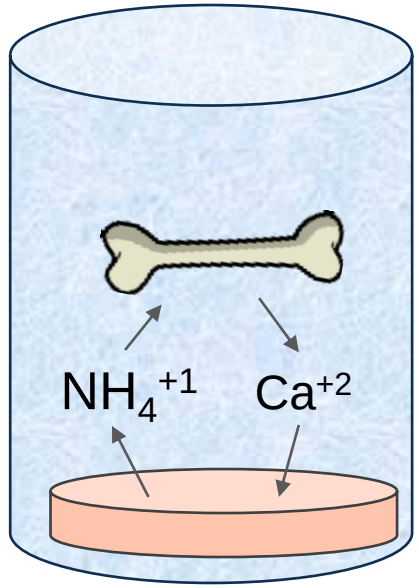
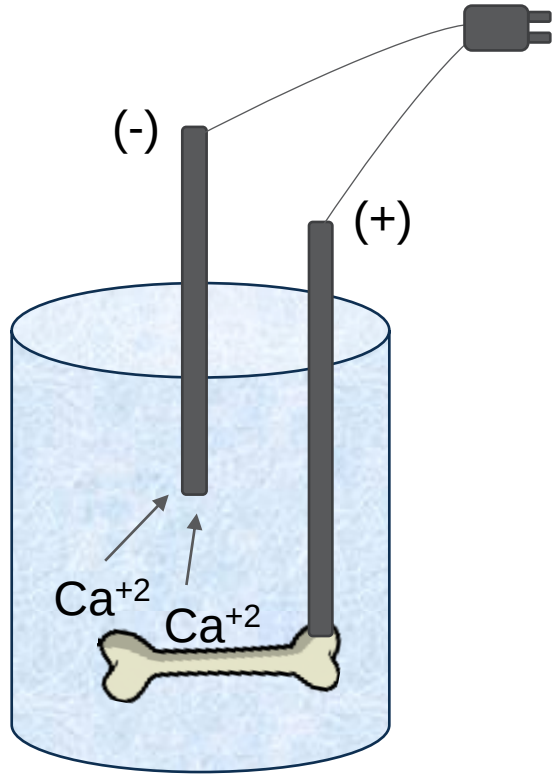
Ion-Exchange Resins Method

- This method involves the use of formic acid and ammoniated resin salt.
- Ammonium ions (NH_4^{+1}) from the resin are exchanges for calcium ions.
- This keeps the solution free of calcium ions and speeds up the reaction. Because the solution remains relatively free of calcium ions it does not need to be changed frequently.
- Staining is excellent; specimen shows superior cellular detail after application of this method.



Electrolytic Method

- Formic acid or HCl are used as electrolytic medium.
- The bone is attached to anode (+) and the electrical current is applied.
- (+) Ca ions are attracted to (-) cathode.
- The calcium ions move towards the cathode.
- Rapid decalcification is achieved but heat produced may damage the morphological structure affecting cellular details and stainability.
- Most bone specimens can be decalcified within 2 to 6 hours.



Electrolytic apparatus

Ultrasonic Decalcification

- Techniques involving use of ultrasonic apparatus have been shown to be useful for shortening the time for decalcification procedures.
- Decalcification of bone specimens of 2-5 mm thickness can be achieved in 5 hours or less when the decalcifying fluids are agitated by ultrasonic waves.
- Acid, chelating agent, or mixture of both may be used as decalcification fluids.
- Commercially available ultrasound decalcifiers with working temperature at 30-45°C do not affect histological and immunohistochemical analysis and do not cause any heat-related artefacts.

Ultrasonic instrument

Factors Influencing the Rate of Decalcification

Concentration

Decalcifying agent loses its potency as it removes calcium from the tissue and therefore needs to be replaced.

Temperature

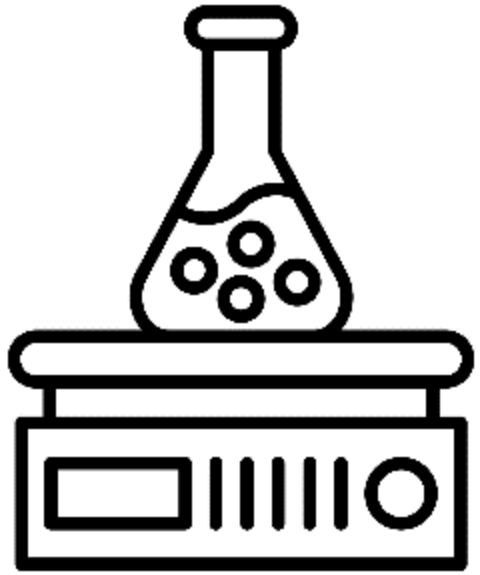
Increased temperature will speed up the decalcification, but also will increase the rate of tissue damage so must be applied with great care.

Agitation

Gentle agitation increases the rate of decalcification.

Access to mineralized tissue

Decalcifier needs exposure to all surfaces of the bone for better diffusion, penetration and removal of calcium.

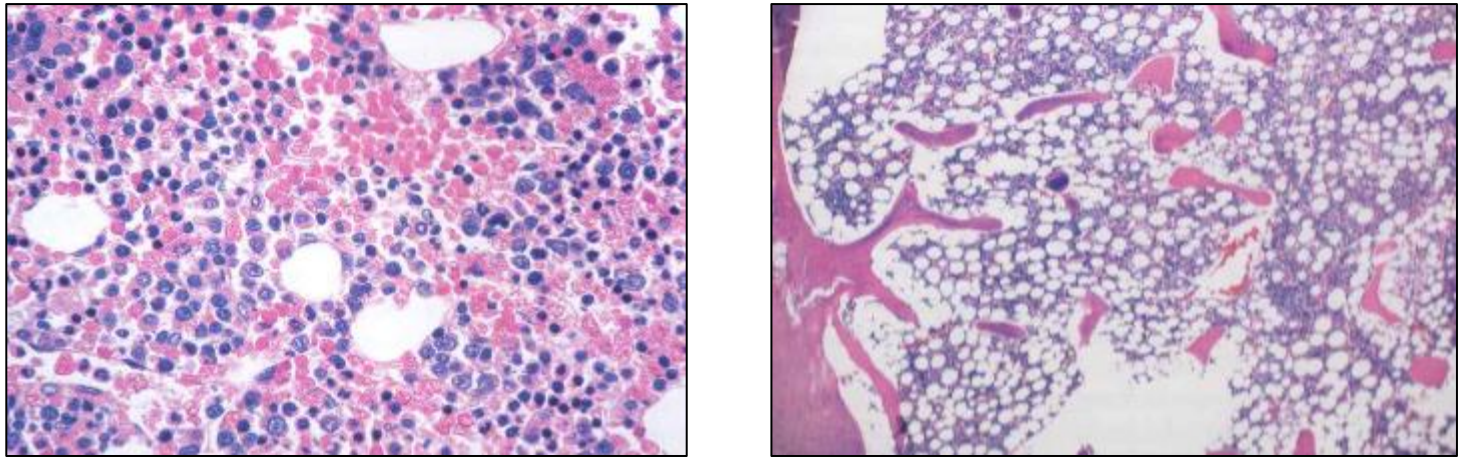


End Point of Decalcification

This is the most important step in the decalcification process, because underdecalcified tissue will section with difficulty and overdecalcified tissue most likely will stain very poorly.

The end point can be determined using 3 basic methods:

- Radiographic
- Chemical
- Mechanical



An H&E sections of bone that have been properly decalcified. The nuclear basophilia is preserved and cellular details can be distinguished easily.

End Point of Decalcification Tests

Radiographic Test

- Radiography is the most accurate method followed by chemical and physical testing procedures which are potentially damaging to the specimens.
- Good-quality X-ray will clearly reveal tiny residual calcium deposits and allow further treatment if required. It is an excellent method for following the process of decalcification of large specimens such as femoral heads.

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Chemical Test

- A simple chemical test can be applied when some acid decalcifiers are used (particularly formic acid).
- Ammonium oxalate solution is added to a sample of the final change of decalcified that has been neutralized with ammonium hydroxide.
- If calcium is present, a precipitate of calcium oxalate will form indicating that decalcification is probably incomplete and a longer time in decalcified is required.

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Mechanical Test

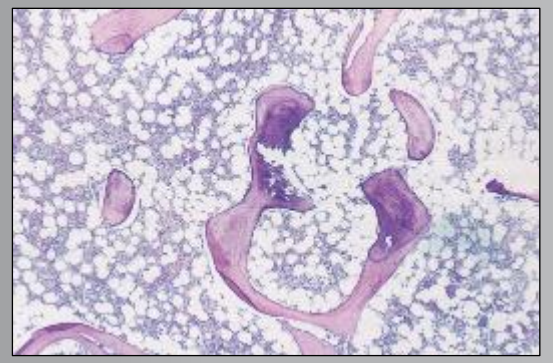
- Physical tests require manipulation: bending, probing, or trimming of the specimen to “feel” for remaining calcified areas. This method is generally considered to be unreliable.
- For apparent reasons, tears and pinhole artifacts can be expected with insertion of pins and scalpels. Bending the specimen might not be conclusive of complete decalcification.
- Moreover, small deposits of calcium can easily be missed.

Troubleshooting

The most common problems encountered on decalcified tissues are underdecalcification, and overdecalcification.

Underdecalcification

- This most often occurs of the necessity of rapid turnaround when appropriate decalcification times are not provided
- Choosing wrong decalcifier or wrong decalcification method
- Sectioning is difficult if not impossible



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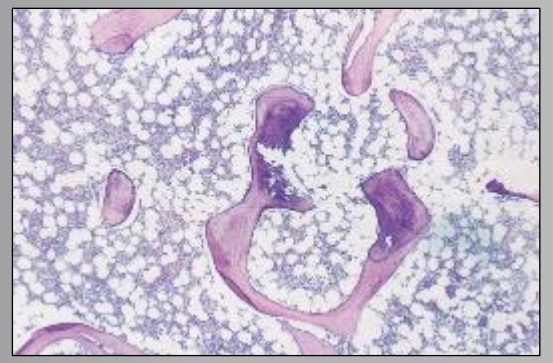
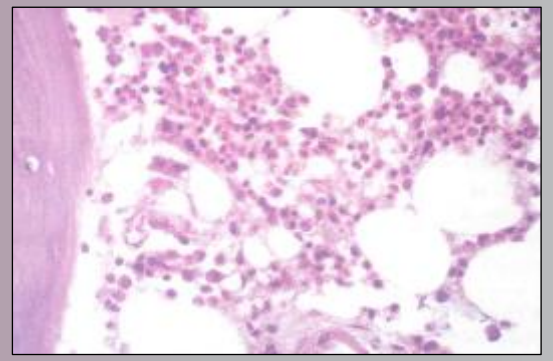


Overdecalcification

- When the end point of decalcification is not carefully checked overdecalcification may be the result
- Choosing wrong decalcifier or wrong decalcification method
- Poor nuclear staining, or loss of morphology



Effects of Decalcification Protocols on IHC and Molecular Assays Study

The objective of this study was to compare the effect of different decalcifying solutions and decalcification protocols (various number and duration of cycles) on immunohistochemistry, molecular and nucleic acid integrity in a large group of bone specimens.

Study set-up:

- 35 tissue samples, fixed in 10% NBF, each specimen was sampled in equal-sized fragments
- Decalcification procedures with hydrochloric acid, formic acid, and EDTA (six commercial decalcifying agents)
- Short, overnight and long cycles (4h, 6h, 8h, 12h, and 24h)
- Protein integrity was examined by immunohistochemistry (12 antibodies)
- Quantity and quality of nucleic acids was evaluated

This article was published in Modern Pathology, Vol. 33.
Miquelestorena-Standley, E., Jourdan, ML., Collin, C. et al. Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples, 1505–1517, Copyright Elsevier (2020).

Testing Methods

IHC Methods

Antibodies against PAX8, P63, Ki-67, INI1, MDM2, S100, EMA, CK7, MUC4, HER2 and ER

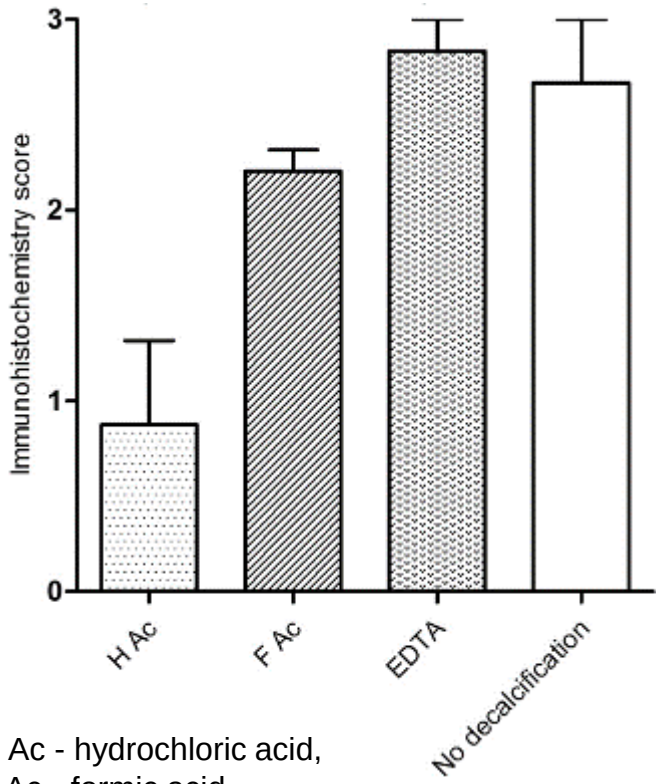
Molecular analysis (DNA, RNA)

DNA/RNA extraction
High-resolution melting (HRM)
Pyrosequencing
Real-time quantitative PCR (DNA)
Reverse transcription and real-time quantitative PCR (RNA)
DNA sequencing
RNA sequencing

In situ hybridization

Fluorescence in situ hybridization (FISH)
Chromogenic in situ hybridization (CISH)

Results: Immunohistochemistry



H Ac - hydrochloric acid,
F Ac - formic acid

The staining intensity was semi-quantitatively scored as 0 (no signal), 1 (weak), 2 (moderate), and 3 (intense signal).

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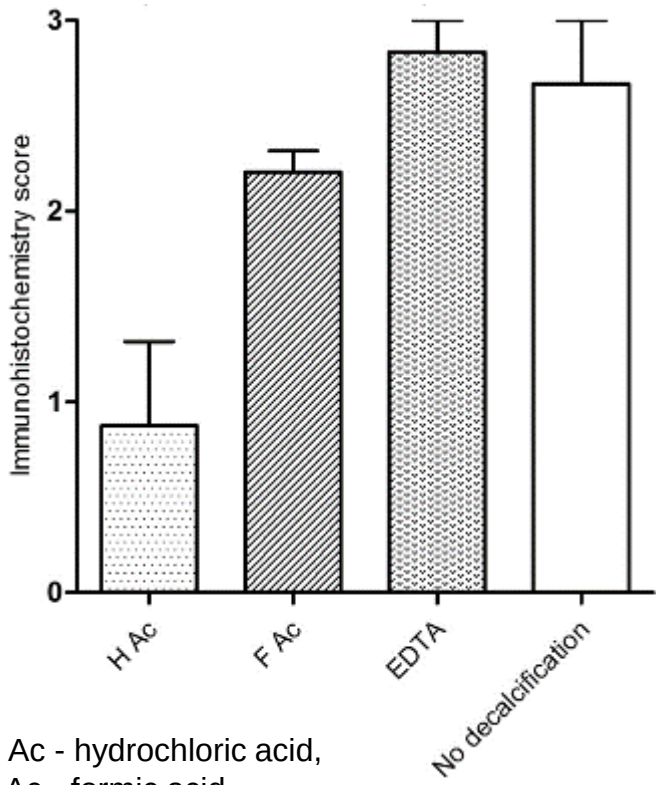

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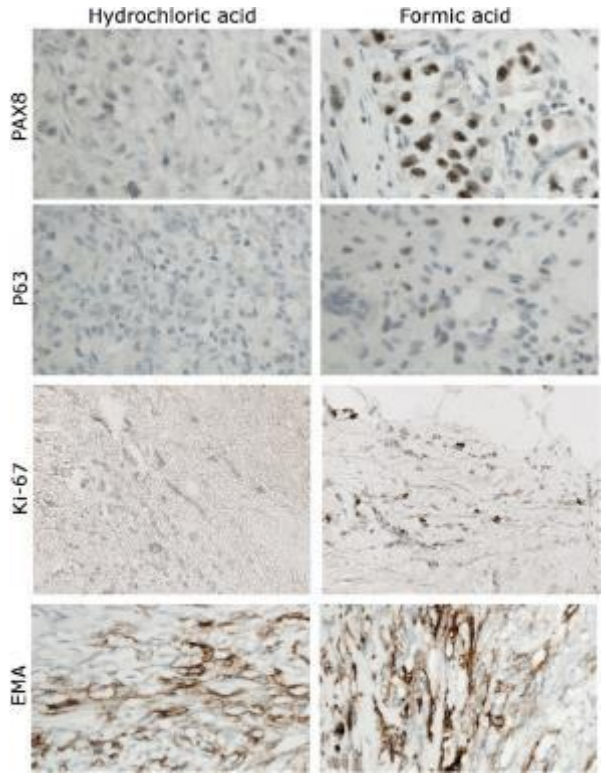

Immunohistochemistry of PAX8, p63, Ki-67, and EMA in bone samples decalcified with hydrochloric acid or formic acid.

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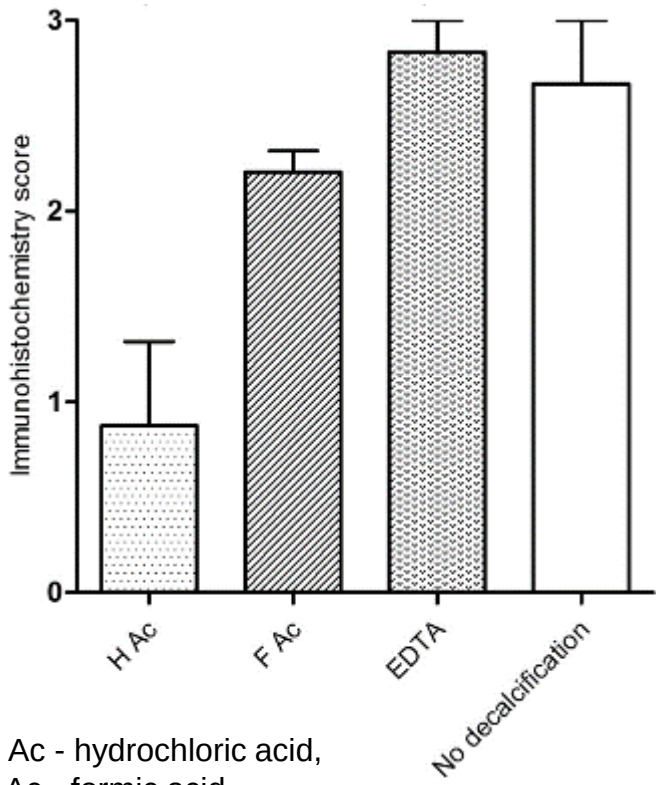
Results: Immunohistochemistry



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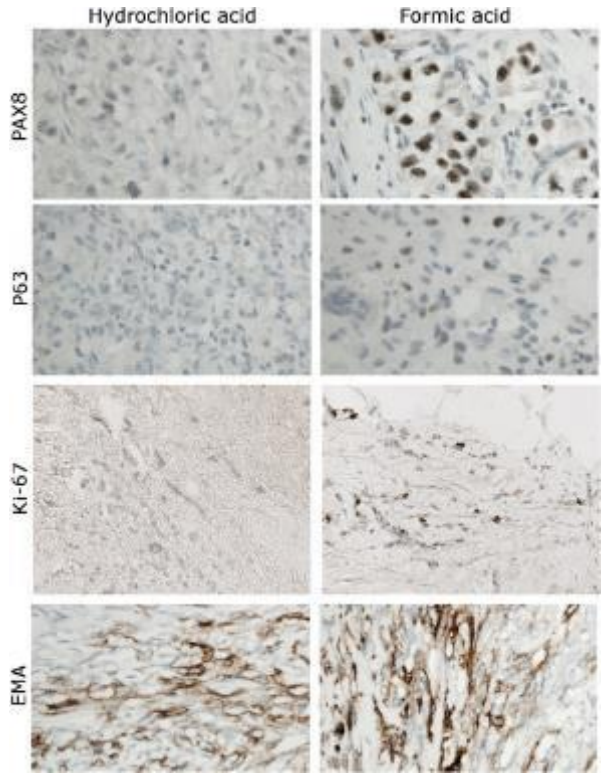

Immunohistochemistry of PAX8, p63, Ki-67, and EMA in bone samples decalcified with hydrochloric acid or formic acid.

Results

Hydrochloric acid- and long-term formic acid-based decalcification induced false-negative results on immunohistochemistry and molecular analysis.

EDTA and short-term formic acid-based decalcification (<5 cycles of 6 h each) did not alter antigenicity (IHC), showed superiority for in situ hybridization techniques.

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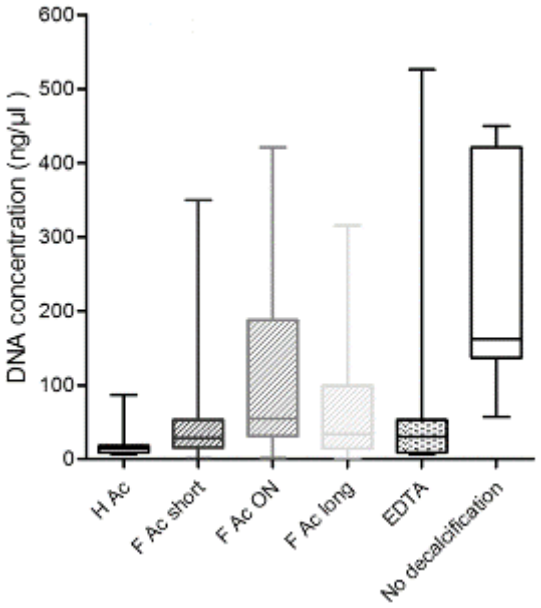
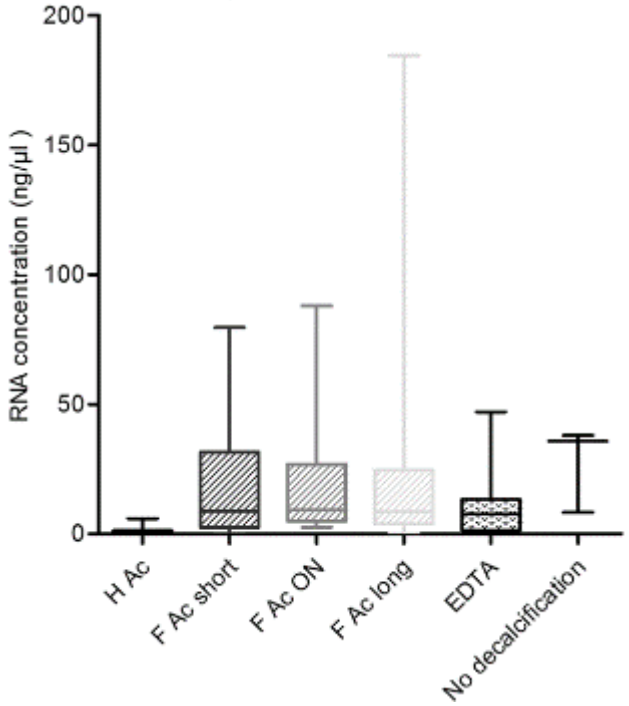
Results: DNA and RNA Concentration





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F Ac ON - formic acid 12h
F Ac long - formic acid 24h

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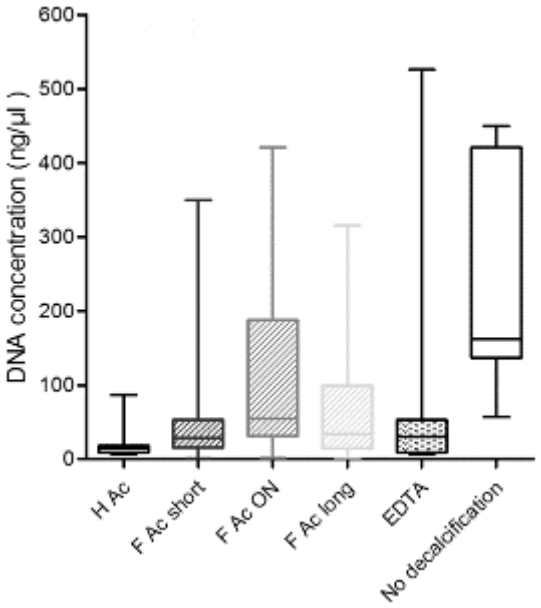

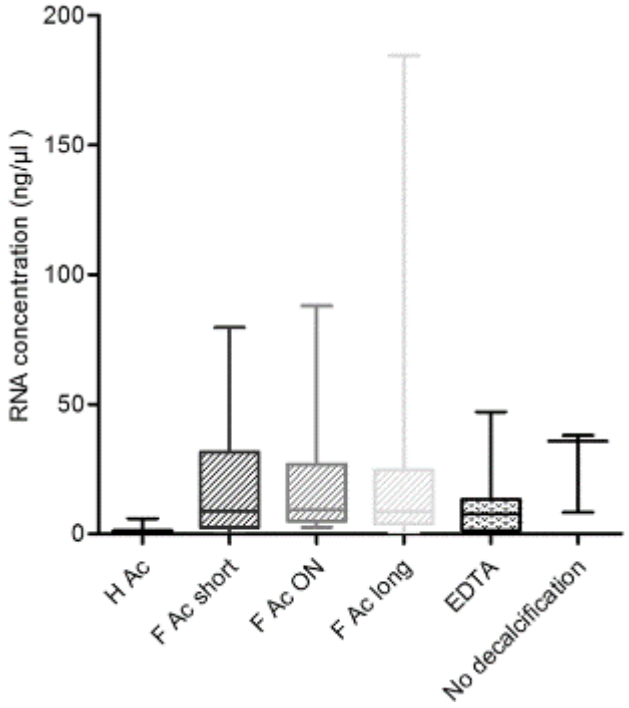

H Ac - hydrochloric acid,
F Ac short - formic acid 4h
F Ac ON - formic acid 12h
F Ac long - formic acid 24h

Concentration (ng/µl)	Hydrochloric acid	Formic acid	EDTA
DNA	20.4	71.6	96.7
RNA	1.2	21.2	10.7

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Overview of IHC and Molecular Analysis Results

		Hydrochloric acid	Formic acid	EDTA
Stain quality		• altered		
Nucleic acids		• damaged integrity • decrease in DNA and RNA yield		
IHC	**Nuclear antigens**	• altered antigenicity • false-negative results		
	Cytoplasmic antigens	• false-negative results		
ISH		• no signal or false-negative results		

Overview of IHC and Molecular Analysis Results

		Hydrochloric acid	Formic acid	EDTA
Stain quality		• altered	• satisfactory	
Nucleic acids		• damaged integrity • decrease in DNA and RNA yield	• non damaging changes	
IHC	**Nuclear antigens**	• altered antigenicity • false-negative results	• antigenicity preserved	
	Cytoplasmic antigens	• false-negative results		
ISH		• no signal or false-negative results	• weak or no detection reported	

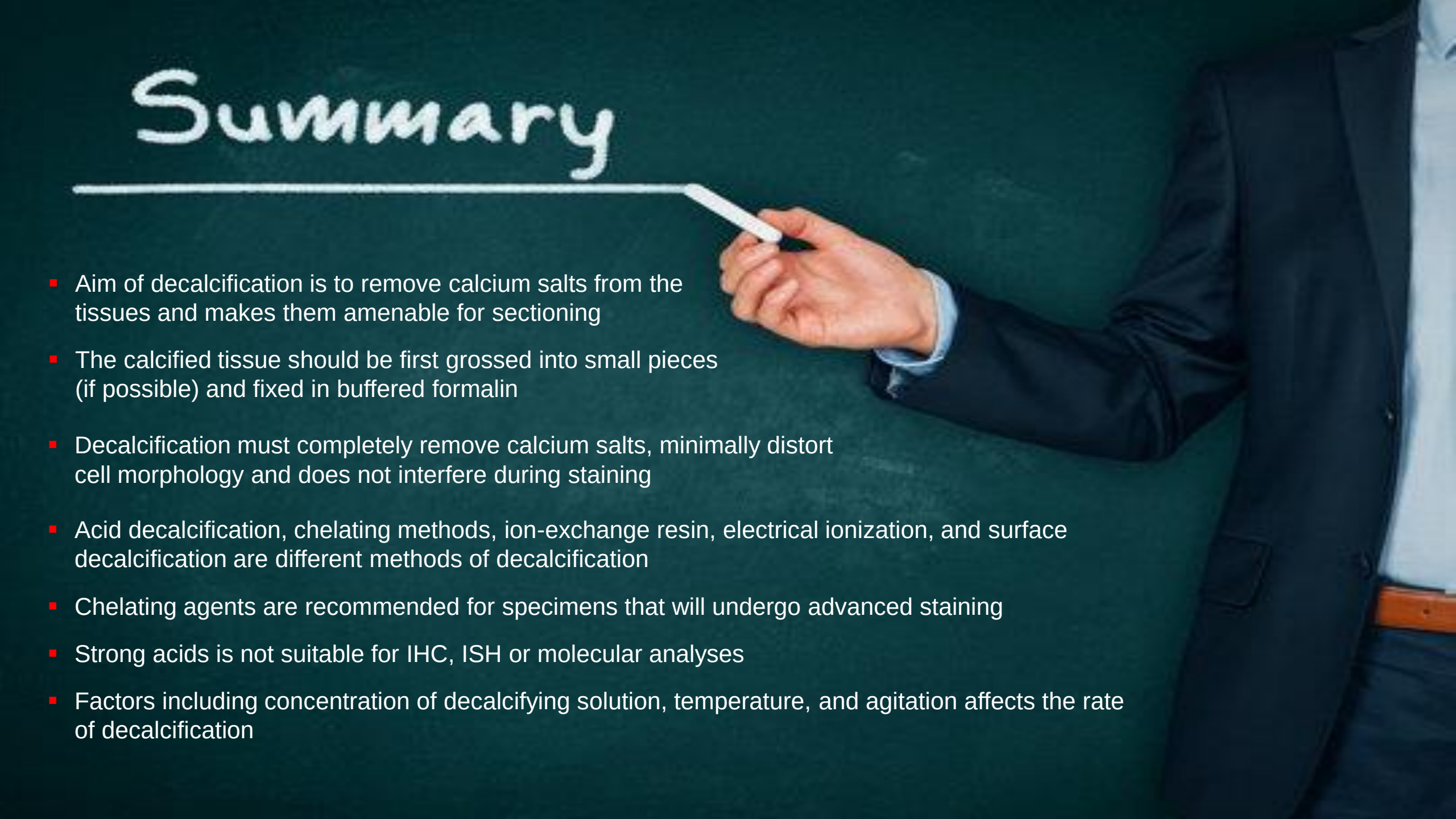
Overview of IHC and Molecular Analysis Results

		Hydrochloric acid	Formic acid	EDTA
Stain quality		• altered	• satisfactory	• satisfactory
Nucleic acids		• damaged integrity • decrease in DNA and RNA yield	• non damaging changes	• good results for DNA/RNA NGS
IHC	**Nuclear antigens**	• altered antigenicity • false-negative results	• antigenicity preserved	• antigenicity preserved • morphology preserved
	Cytoplasmic antigens	• false-negative results		
ISH		• no signal or false-negative results	• weak or no detection reported	• good results with small samples, short cycles

Epredia Decalcifying Agents

Decalcifying Solution	• Strong Decalcifier • Mixture of EDTA and hydrochloric acid
Cal-Rite	• Weak decalcifier • Mixture of formic acid and formaldehyde
Shandon TBD-1 Decalcifier	• Strong Decalcifier • Rapid hydrochloric acid decalcifier
Shandon TBD-2 Decalcifier	• Weak decalcifier • Slow formic acid decalcifier

Epredia Decalcification and Tissue Processing Options



- Aim of decalcification is to remove calcium salts from the tissues and makes them amenable for sectioning
- The calcified tissue should be first grossed into small pieces (if possible) and fixed in buffered formalin
- Decalcification must completely remove calcium salts, minimally distort cell morphology and does not interfere during staining
- Acid decalcification, chelating methods, ion-exchange resin, electrical ionization, and surface decalcification are different methods of decalcification
- Chelating agents are recommended for specimens that will undergo advanced staining
- Strong acids is not suitable for IHC, ISH or molecular analyses
- Factors including concentration of decalcifying solution, temperature, and agitation affects the rate of decalcification

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