

101 STEPS TO BETTER HISTOLOGY



Knowledge
Pathway

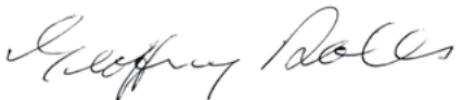
Advancing Cancer Diagnostics
Improving Lives

Leica
BIOSYSTEMS

From patient to pathologist, preparing tissue specimens for histological examination requires care, skill and sound procedures. This guide provides practical advice on best-practice techniques and simple ways to avoid common errors.

Each aspect of the histology process is covered: specimen collection, grossing, processing, embedding, sectioning and staining (routine, special, immunohistochemistry and *in situ* hybridization).

We hope each step provides a valuable reminder of good histology practice and also helps with troubleshooting when unacceptable results do occur.

A handwritten signature in black ink, appearing to read "Geoffrey Rolls".

Geoffrey Rolls
Leica Biosystems

Contents

Specimen Collection and Transport

- Step 1 Avoid Mechanical Trauma
- Step 2 Prevent Specimen Drying
- Step 3 Avoid Heat Damage
- Step 4 Avoid Chemical Damage
- Step 5 Label Specimens Properly
- Step 6 Ensure Prompt Fixation
- Step 7 Use Sufficient Fixative and a Suitable Container
- Step 8 Check Fixative pH
- Step 9 Expedite Large Specimen Fixation
- Step 10 Avoid Unnecessary Delays
- Step 11 Handle Specimens Gently

Grossing

- Step 12 Check Fixation Status
- Step 13 Prepare Thin Slices
- Step 14 Avoid Specimen Trauma
- Step 15 Avoid Cross-contamination
- Step 16 Take Care with Biopsy Pads
- Step 17 Choose Appropriate Cassettes
- Step 18 Avoid Overloading Cassettes
- Step 19 Clearly Label Cassettes

Processing

- Step 20 Use an Appropriate Schedule
- Step 21 Provide Additional Fixation
- Step 22 Maintain Reagent Quality
- Step 23 Use High Quality Wax
- Step 24 Avoid Hazardous Reagents

Embedding

- Step 25 Orientate Specimens Carefully
- Step 26 Choose an Appropriate Mold
- Step 27 Handle Specimens Gently
- Step 28 Avoid Excessive Heat
- Step 29 Check Temperatures Regularly
- Step 30 Do Not Over-fill Molds

Microtomy

- Step 31 Use High Quality Blades
- Step 32 Optimize Knife Tilt Angle
- Step 33 Carefully Trim Blocks
- Step 34 Avoid Freezing Damage
- Step 35 Use Cold Blocks
- Step 36 Cut Sections Slowly

Flotation

- Step 37 Use Clean Water
- Step 38 Ensure Slides are Clean
- Step 39 Avoid Cross-contamination
- Step 40 Avoid Contamination with Squames
- Step 41 Don't Float from Multiple Blocks
- Step 42 Check Water Temperature
- Step 43 Avoid Wrinkles in Sections
- Step 44 Avoid Over-expanding Sections
- Step 45 Don't Damage Floating Sections
- Step 46 Carefully Choose Sections
- Step 47 Prevent Bubbles Under Sections
- Step 48 Prevent Section Lifting

Section Drying

- Step 49 Drain Before Drying
- Step 50 Monitor Drying Temperature
- Step 51 Dry for Appropriate Time

Routine Staining (H&E)

- Step 52 Use Accurate Timing
- Step 53 Regularly Monitor Quality
- Step 54 Standardize Staining Conditions
- Step 55 Ensure Complete Dewaxing
- Step 56 Renew Reagents Regularly
- Step 57 Hydrate Sections Thoroughly
- Step 58 Monitor Hematoxylin Quality
- Step 59 Ensure Complete Nuclear "Blueing"
- Step 60 Avoid Uneven Eosin Staining
- Step 61 Monitor Eosin pH

Coverslipping

- Step 62 Thoroughly Dehydrate Before Clearing and Coverslipping
- Step 63 Avoid Drying and Crystal Formation

Special Stains

- Step 64 Understand the Stain
- Step 65 Use a Positive Control
- Step 66 Use Accurate Timing
- Step 67 Consider Reagent Stability
- Step 68 Store Reagents Correctly
- Step 69 Adhere to the Method
- Step 70 Record any Changes
- Step 71 Standardize Washing Steps
- Step 72 Set Up Microscope Carefully

Immunohistochemistry

- Step 73 Use High Quality Sections
- Step 74 Ensure Optimal Fixation
- Step 75 Avoid Section Adhesion Problems
- Step 76 Optimize Wax Removal and Reagent Application
- Step 77 Avoid Concentration Gradients
- Step 78 Choose Antibody Carefully
- Step 79 Read Specification Sheets
- Step 80 Optimize Retrieval Methods
- Step 81 Consider Antibody Cross-reactivity
- Step 82 Block Endogenous Peroxidase
- Step 83 Avoid Background Staining
- Step 84 Use an Appropriate Detection System
- Step 85 Standardize Washing Steps
- Step 86 Optimize Counterstaining
- Step 87 Use Appropriate Controls
- Step 88 Evaluate Results Carefully

In Situ Hybridization

- Step 89 Use High Quality Sections
- Step 90 Ensure Optimal Fixation
- Step 91 Avoid Section Adhesion Problems
- Step 92 Optimize Wax Removal and Reagent Application
- Step 93 Choose Probe Carefully
- Step 94 Read Specification Sheets
- Step 95 Optimize Pretreatment Conditions
- Step 96 Handle Tissue Carefully
- Step 97 Use Appropriate Detection System
- Step 98 Avoid Reagent Evaporation
- Step 99 Standardize Washing Steps
- Step 100 Use Appropriate Controls
- Step 101 Evaluate Results Carefully



- Step 1 Avoid Mechanical Trauma
- Step 2 Prevent Specimen Drying
- Step 3 Avoid Heat Damage
- Step 4 Avoid Chemical Damage
- Step 5 Label Specimens Properly
- Step 6 Ensure Prompt Fixation
- Step 7 Use Sufficient Fixative and a Suitable Container
- Step 8 Check Fixative pH
- Step 9 Expedite Large Specimen Fixation
- Step 10 Avoid Unnecessary Delays
- Step 11 Handle Specimens Gently

Specimen Collection
and Transport

Step 1

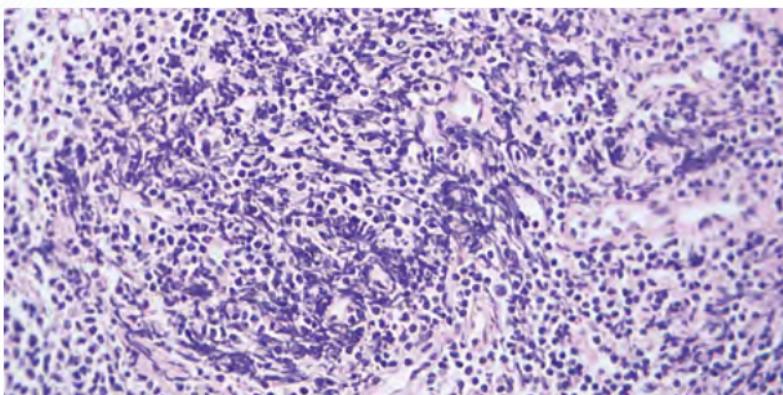
Avoid Mechanical Trauma



Tissue is removed gently to avoid trauma to the specimen caused by crushing or tearing. This applies both during surgery and during any further dissection that may be required of a fresh specimen.



Specimen is damaged before fixation by crushing or tearing during removal.



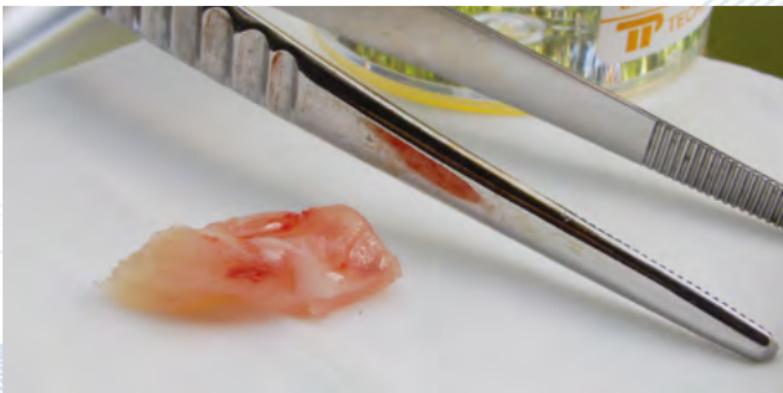
Typical crush artifact is shown in this section of lymphoid tissue. It is characterized by dark, distorted cell nuclei, some of which are extremely elongated and intensely basophilic.

Specimen Collection
and Transport

Step 2

Prevent Specimen Drying

- ✓ Specimen is not allowed to dry out prior to fixation. If immediate fixation is not practicable, gauze moistened with saline can be used to prevent this.
- ✗ Specimen is left on absorbent surface for some time prior to fixation.



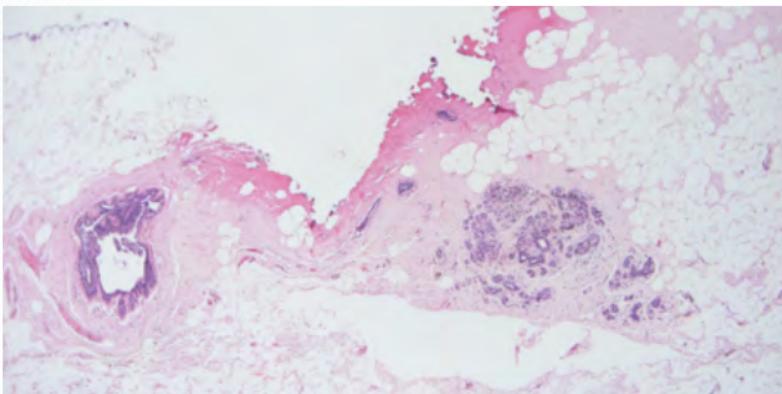
This fresh specimen has just been removed from a patient during surgery. Because it is resting on an absorbent surface and the theatre is quite warm it will rapidly dry out unless it is immediately placed in fixative.

Specimen Collection and Transport

Step 3

Avoid Heat Damage

- ✓ As far as possible avoid local heat damage to specimens (some damage by cautery may be unavoidable).
- ✗ Any unnecessary local heat applied to tissue will cause damage.
Fresh tissue is particularly susceptible.



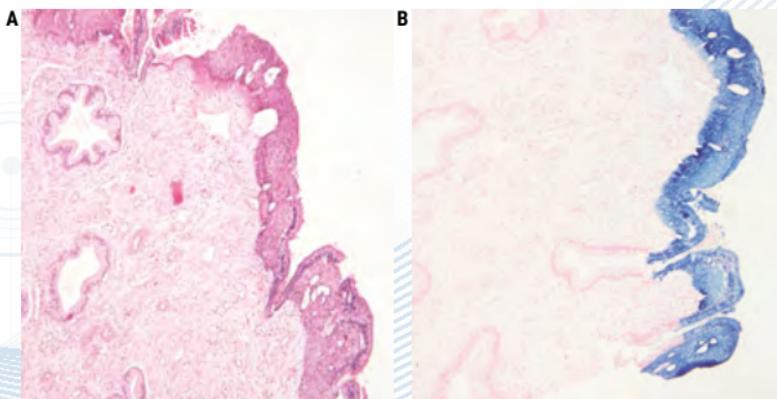
A localized area at the edge of this breast specimen exhibits strong acidophilia with a loss of nuclear and cytoplasmic detail. These effects are the result of heat damage caused when cautery was used during the removal of the specimen. Adjacent glandular tissue is unaffected.

Specimen Collection
and Transport

Step 4

Avoid Chemical Damage

- ✓ Avoid contaminating fresh specimens with foreign chemicals or substances such as disinfectants.
- ✗ The surface of unfixed tissue can be easily penetrated and damaged by foreign reagents or substances.



Monsel's solution (ferric subsulphate solution) is a topical hemostatic agent used to control bleeding following mucosal biopsy. It causes coagulation and necrosis of the mucosal surface. If it is applied before a biopsy is taken it causes local basophilia and signs of early necrosis, masking pathological changes that may be present. Monsel's solution artifact is most commonly seen when the patient is rebiopsied or wider excision is done later. The effects are seen in micrograph A of a H&E stained cervical biopsy. Micrograph B, stained with Perl's method, shows the extensive deposition of iron on the specimen surface.

Specimen Collection and Transport

Step 5

Label Specimens Properly



Each specimen should be properly identified and all details recorded as soon as possible.



Recording of specimen details is delayed and the information provided is incomplete.



Specimens with incomplete labels such as these, should not be accepted by a laboratory. A procedure must be in place to deal with specimens that arrive at the lab inadequately labeled or accompanied by incomplete or inconsistent documentation.

Specimen Collection
and Transport

Step 6

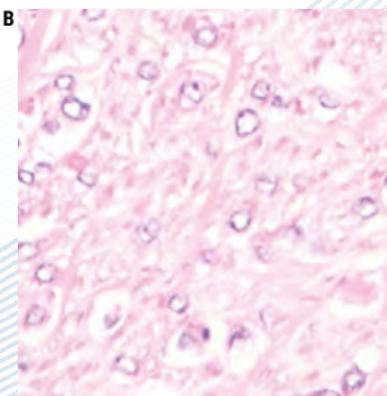
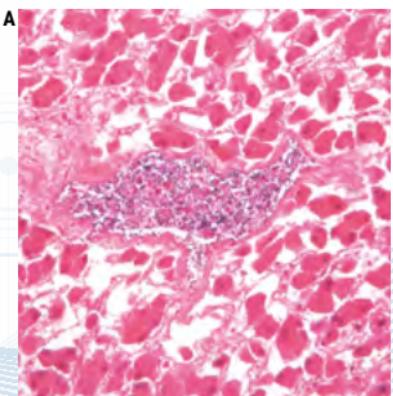
Ensure Prompt Fixation



Fixation is always carried out promptly. If it is necessary that a specimen remains unfixed for a short period of time, it should be refrigerated at 4 °C.



Fixation is delayed (degeneration of tissue elements commences as soon as the specimen is deprived of a blood supply).



- A This autopsy liver specimen (H&E) shows the result of an extended delay before fixation. Note the poorly defined nuclei and imprecise cytoplasmic detail. Many bacteria are present within the central blood vessel.
- B In this section of fibro-muscular tissue the nuclear chromatin is poorly preserved due to an extensive delay prior to fixation.

Specimen Collection and Transport

Step 7

Use Sufficient Fixative and a Suitable Container



An adequate volume of fixative (ratio of at least 20:1) is used in a container of an appropriate size. This avoids distortion of the fresh specimen and ensures good quality fixation.



Specimens are sometimes squashed into a small container with insufficient fixative to cover the specimen surface.



This container is too small for the mass of tissue it contains. There is insufficient fixative present and the specimen may well have been distorted as it was pushed into the container.

Specimen Collection
and Transport

Step 8

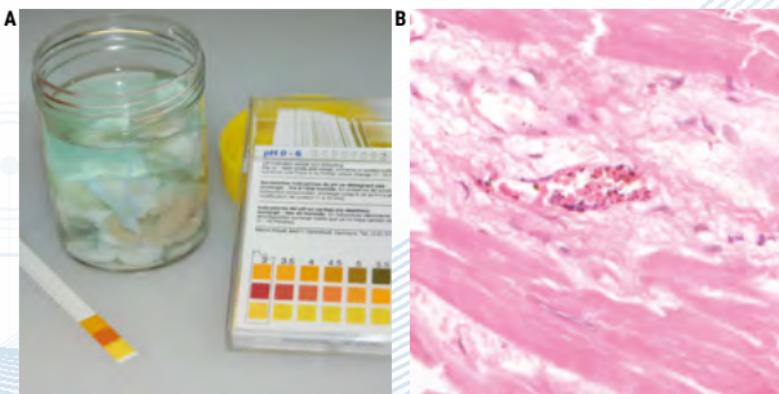
Check Fixative pH



The fixative is of high quality and at the optimal pH.



The fixative is of poor quality and unknown pH. If formalin is used at acid pH it rapidly produces "formalin pigment" by reaction with hemoglobin. Near neutral solutions will still produce the pigment but much more slowly. In good histological preparations formalin pigment should be removed prior to staining.



- A The fixative used here has an unsatisfactory pH of 4.5. Buffered formalin solutions should have a pH of 6.8–7.0.
- B The brownish-black granular deposit seen in the blood vessel in the center of this field is formalin pigment (acid formaldehyde hematin). It readily forms when tissue is fixed in acidic formalin and is usually seen in association with red blood cells.

Specimen Collection and Transport

Step 9

Expedite Large Specimen Fixation



The specimen dimensions allow rapid penetration of the fixative. Large specimens should be rapidly transported to the lab to allow grossing (tissue slices can be prepared to allow proper fixation to occur).



Large specimens are left in fixative for an extended time prior to grossing. The center of the specimen may remain unfixed and the tissue can become markedly distorted.



This large specimen (pig heart) has been sliced to allow the fixative access to all parts of the tissue. The slices are approximately 4–5 mm thick.

Specimen Collection
and Transport

Step 10

Avoid Unnecessary Delays

- ✓ No unnecessary delays – specimen reaches lab in minimum time.
- ✗ Specimens are sometimes delayed – specimen transport has a low priority and is not well organized.



Priorities are important when delivering specimens to the laboratory. This is particularly so when frozen sections are involved.

Specimen Collection and Transport

Step 11

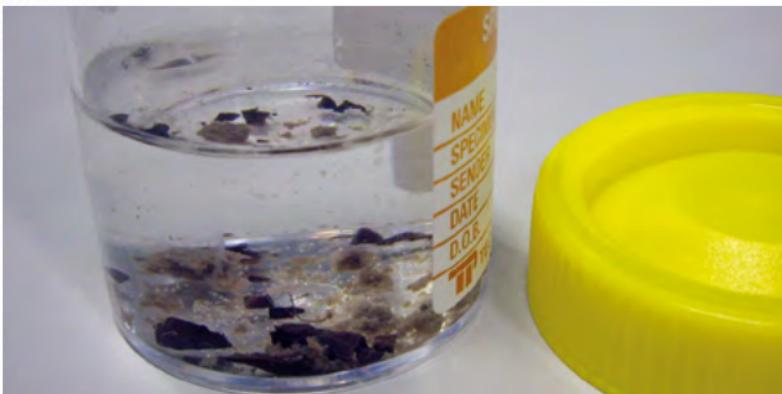
Handle Specimens Gently



Specimens handled gently – fragile specimens remain intact.



Specimens handled roughly – delicate friable specimens can be damaged.



Some of the tissue fragments seen at the bottom of this container have been produced by excessively rough handling during specimen transport. What was originally a cohesive specimen now consists of tissue fragments.

Specimen Collection
and Transport



- Step 12 Check Fixation Status
- Step 13 Prepare Thin Slices
- Step 14 Avoid Specimen Trauma
- Step 15 Avoid Cross-contamination
- Step 16 Take Care with Biopsy Pads
- Step 17 Choose Appropriate Cassettes
- Step 18 Avoid Overloading Cassettes
- Step 19 Clearly Label Cassettes

Grossing

Grossing

Step 12

Check Fixation Status



Specimens are dealt with promptly (especially large specimens that may otherwise be inadequately fixed).



No consideration given to optimizing the fixation of problem specimens.



Specimens in the large containers should be checked as soon as possible to ensure they will be adequately fixed.

Grossing

Step 13

Prepare Thin Slices

- ✓ Care is always taken to prepare uniform, thin slices from large specimens (3-4 mm maximum thickness). This is particularly important with dense tissues.
- ✗ Slices are sometimes 6 mm (or more) thick and are often uneven.



Shown here are uniform, thin (2-3 mm) slices of a tumor ready for processing. They should process effectively and should section without difficulty.

Grossing

Step 14

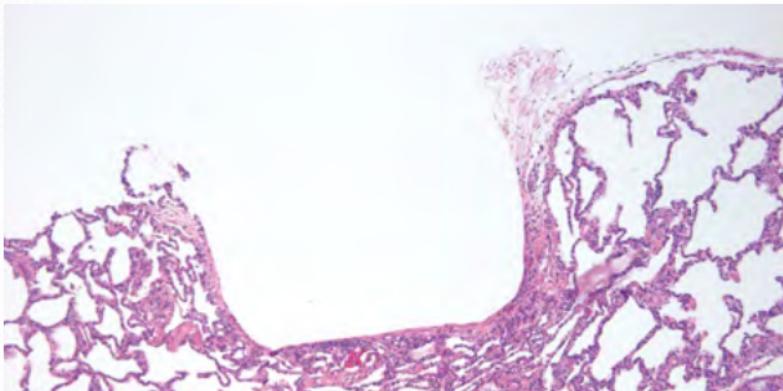
Avoid Specimen Trauma



Care is taken to avoid traumatizing delicate specimens, particularly those that are incompletely fixed (handle carefully, do not crush, always use sharp blades).



Specimens are handled roughly without any consideration as to their state of fixation. Sometimes blunt blades are used in dissection.



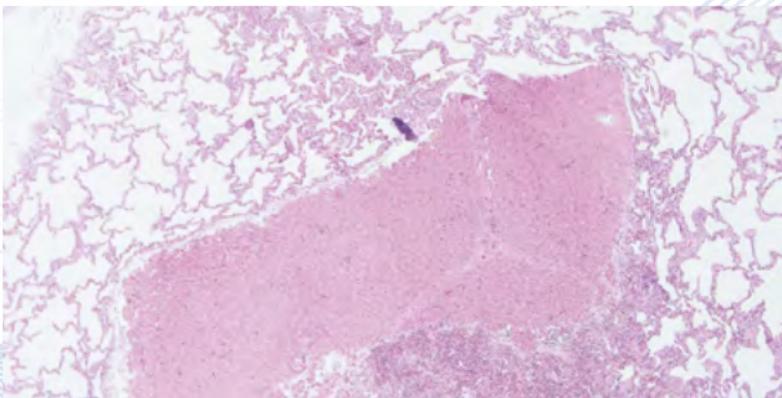
Section of H&E stained lung showing obvious local trauma due to very forceful grasping with forceps. Fresh or partly fixed tissue is most susceptible to damage but even well-fixed tissue can be damaged by rough handling.

Grossing

Step 15

Avoid Cross-contamination

- ✓ Each specimen is handled on a clean surface avoiding the possibility of specimen-to-specimen contamination.
- ✗ Sometimes the surface of the cutting board is not properly cleaned between specimens. This is of particular concern when the same specimen types are cut up one after the other. You do not want to have carryover from a specimen that is malignant to one that is benign.



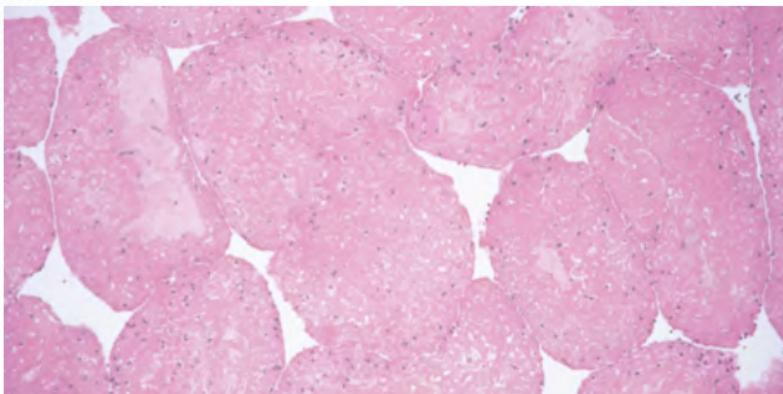
Section of H&E stained lung containing a piece of foreign tissue (liver) impacted into the surface at cut-up.

Grossing

Step 16

Take Care with Biopsy Pads

- ✓ Fresh or incompletely fixed specimens are not placed between foam biopsy pads, particularly needle-core specimens (biopsy pad artifact is avoided).
- ✗ Sometimes small, fresh or incompletely fixed specimens are placed between biopsy pads, put into a cassette and then fixed. This can produce a characteristic artifact.



The triangular spaces visible in this section result from local pressure effects caused by the cellular structure of the foam pads when applied to fresh or very briefly fixed tissue.

Grossing

Step 17

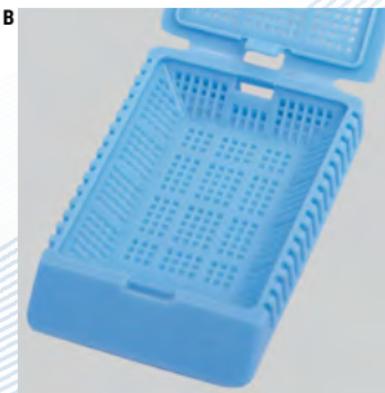
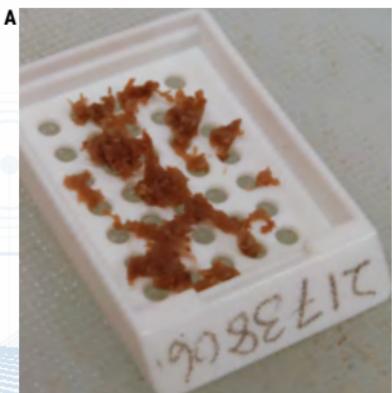
Choose Appropriate Cassettes



Choose appropriate cassettes for the specimen type being processed. Tissue fragments shrink during processing and, if cassette perforations are too large, fragments may escape into processing reagents or, worse still, transfer over to another specimen.



A “one size fits all” approach is used when placing specimens into cassettes.



- A Some of the smaller tissue fragments seen here may escape through the holes in the cassette. This will become even more likely as the tissue shrinks during processing.
- B Cassettes with fine perforations are available for small tissue fragments.

Grossing

Step 18

Avoid Overloading Cassettes



Cassettes are never overloaded with tissue thus allowing ready access to processing reagents and preventing distortion of specimens. If the volume of tissue is too great a second cassette is used.



Cassettes are often crammed full of tissue thus preventing access of processing reagents. Sometimes specimens are distorted in the process.



These cassettes are over-loaded. If processing goes ahead the specimens will be distorted and it is likely that the processing will be incomplete.

Grossing

Step 19

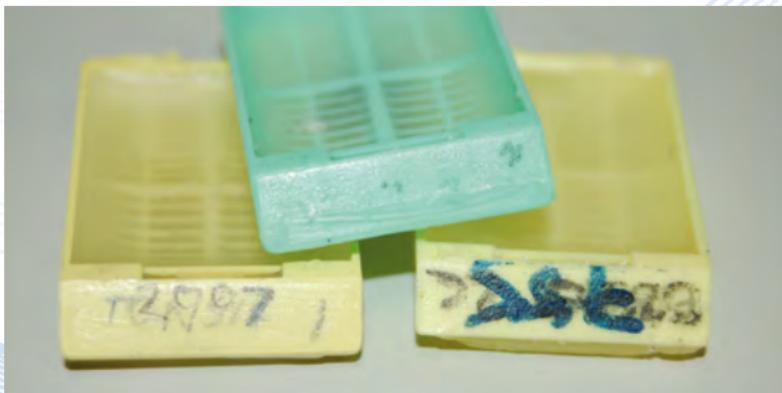
Clearly Label Cassettes



Cassettes are always clearly labeled. Accurate identification of specimens is of paramount importance.



Sometimes it is difficult to read the labels on cassettes. A bit of guesswork may be required.



These illegible cassette labels are totally unacceptable.

Grossing



- Step 20 Use an Appropriate Schedule
- Step 21 Provide Additional Fixation
- Step 22 Maintain Reagent Quality
- Step 23 Use High Quality Wax
- Step 24 Avoid Hazardous Reagents

Processing

Step 20

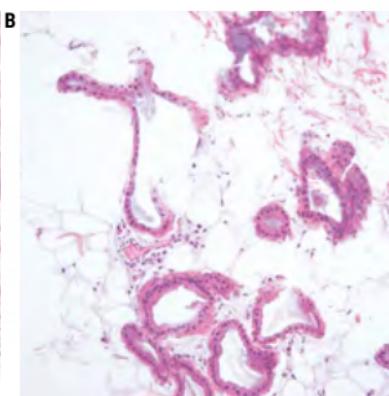
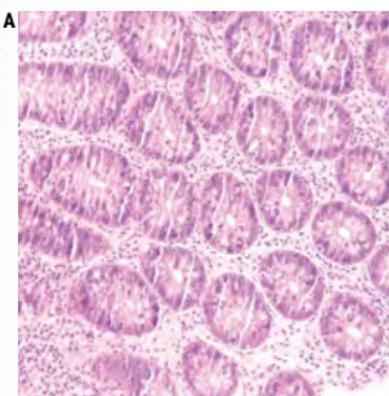
Use an Appropriate Schedule



An appropriate schedule is chosen for the tissue type and size.



An inappropriate schedule is chosen. For example, a very long schedule for a small endoscopic biopsy or a very short schedule for a large, fatty breast specimen.



- A This endoscopic biopsy has been over-processed and has become very brittle. As a consequence many fine cracks are visible throughout the section. Poor microtomy technique will exacerbate the problem (H&E).
- B This micrograph of a small area of subcutaneous tissue from a large, fatty specimen shows the effects of under-processing. The fibro-fatty tissue is poorly supported and therefore fragmented while the epithelial tissue of the glands shows a lack of nuclear definition and peculiar staining due to retained solvent (H&E).

Processing

Step 21

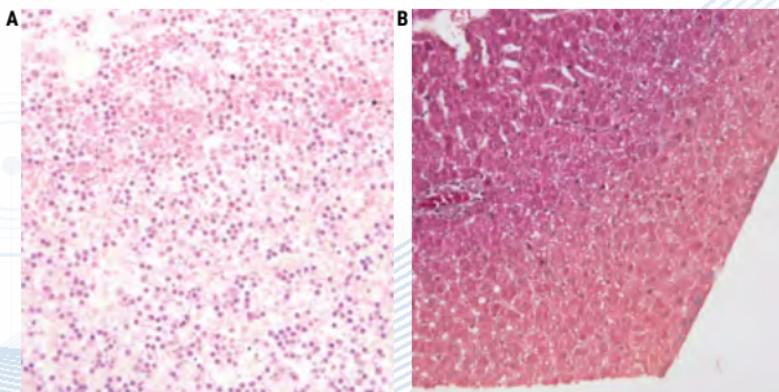
Provide Additional Fixation



For optimal processing and good morphology tissue should be well fixed before processing. Where specimens are incompletely fixed additional formalin fixation is provided in the processing schedule.



Incompletely fixed specimens go directly into alcohol producing zonal fixation (formalin fixation for the outside of the specimen, alcohol fixation for deeper areas).



- A This micrograph shows the effects of zonal fixation on a section of a marrow aspirate (H&E). In the upper left portion the red cells are intact whereas in the lower part they are hemolyzed.
- B This micrograph shows a low power view of liver stained with a trichrome stain. The staining result in the outer zone of the specimen is different to that of the inner.

Processing

Step 22

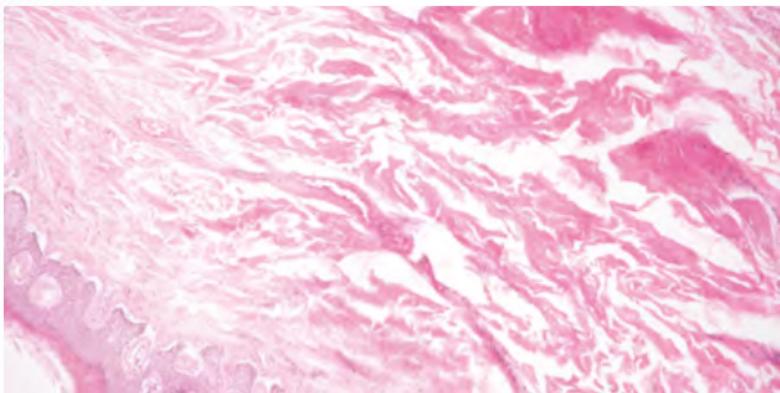
Maintain Reagent Quality



Processing reagents are replaced strictly according to established guidelines (ideally using a reagent management system in an advanced tissue processor such as Leica Biosystems' PELORIS).



Guidelines for the replacement of processing reagents are ignored, meaning that ineffective, contaminated or diluted reagents are used (eg "out-of-threshold" warnings from the PELORIS reagent management system are ignored). This can cause poor processing quality.



In this section – from a large skin specimen – the poor preservation of the dense collagen is due to inadequate processing. In this case we believe it was due to the use of heavily contaminated reagents well "out-of-threshold".

Processing

Step 23

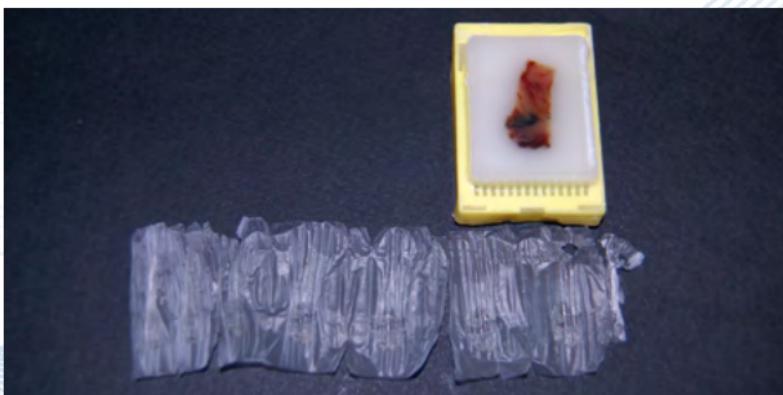
Use High Quality Wax



High quality wax is used for infiltration and especially for embedding (blocking out) to ensure high quality blocks that are easy to cut.



Cheap, poor quality wax from little-known sources is used for infiltration and embedding. Poor quality wax produces blocks that are difficult to cut.



A ribbon of sections was slowly cut from this block while the block was cold. The sections show considerable compression despite the low temperature used. Here the poor quality wax failed to properly support the tissue.

Processing

Step 24

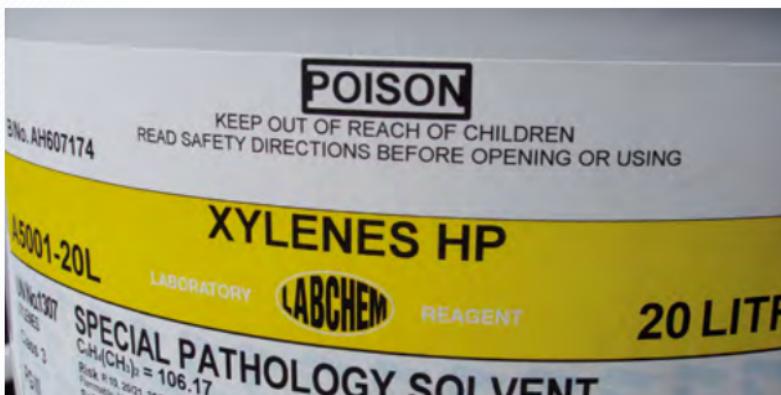
Avoid Hazardous Reagents



Where possible, xylene-free protocols are used (such as those available when using Leica Biosystems' PELORIS). This provides a safer laboratory environment without compromising processing quality.



No consideration is given to the health effects of xylene use. The possibility of using alternatives has not been considered.



Xylene-free processing can improve laboratory safety while maintaining quality.

Processing



- Step 25 Orientate Specimens Carefully
- Step 26 Choose an Appropriate Mold
- Step 27 Handle Specimens Gently
- Step 28 Avoid Excessive Heat
- Step 29 Check Temperatures Regularly
- Step 30 Do Not Over-fill Molds

Embedding

Step 25

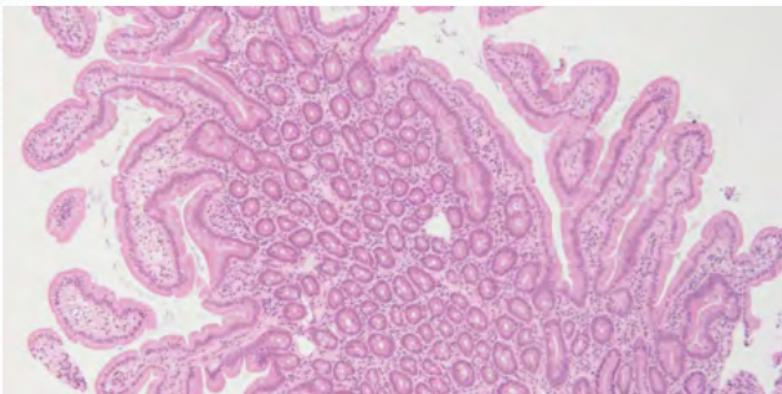
Orientate Specimens Carefully



Specimens are carefully orientated. Competent grossing ensures flat surfaces on most specimens. Staff performing embedding have ready access to each specimen description and are appropriately trained.



Orientation is incorrect. This can result in loss of tissue as re-embedding is required. Some poorly prepared specimens require extensive trimming on the microtome to obtain a full-face section.



This endoscopic biopsy has been orientated incorrectly and shows only the superficial level of the mucosa.

Embedding

Step 26

Choose an Appropriate Mold



A mold of suitable size is always chosen for each specimen.

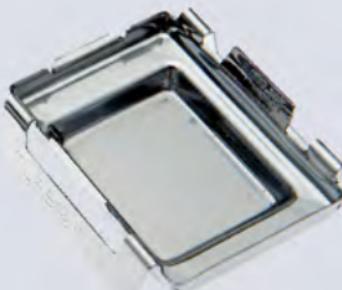


The same mold size is used for every specimen. Often the tissue touches the edge of the mold.

A



B



- A The mold used for this specimen was too small. The specimen is in contact with the edges of the block and may therefore be difficult to section.
- B Molds of different sizes are available for a variety of specimen sizes.

Embedding

Step 27

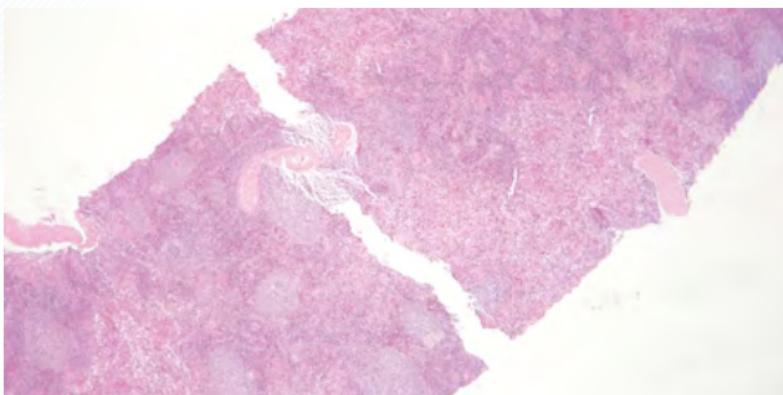
Handle Specimens Gently



Specimens are handled gently during embedding.



Specimens are handled forcefully during embedding to make them lie flat in the mold. Some tissue can be fractured by this process.



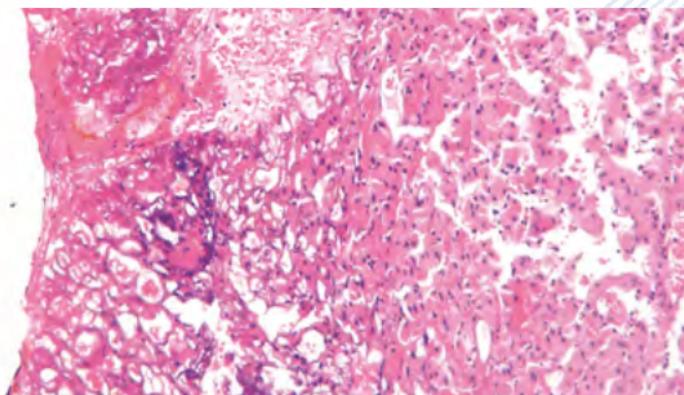
An H&E stained section of spleen which was fractured during embedding in an attempt to make the specimen lie flat on the base of the mold.

Embedding

Step 28

Avoid Excessive Heat

- ✓ Before handling tissue, forceps are heated to the point where the wax just melts.
- ✗ Forceps are heated well beyond the melting point of wax. This can cause local heat damage and a change in morphology in the area close to the contact point.



This micrograph shows the surface of a section of liver (H&E). Extreme local damage (making the tissue almost unrecognizable) has been caused by the application of heat to the tissue during embedding.

Embedding

Step 29

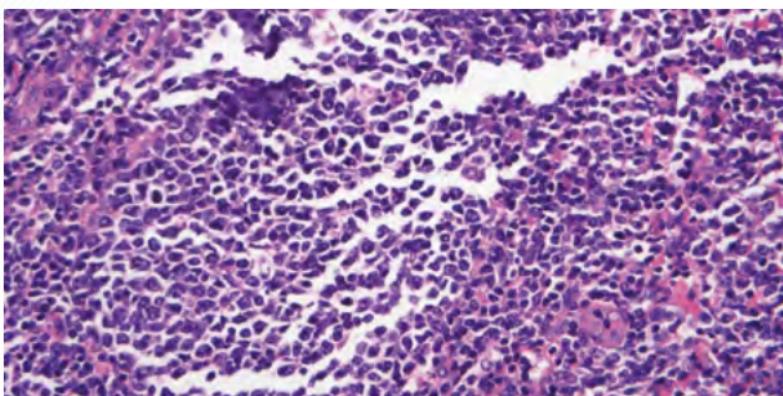
Check Temperatures Regularly



The temperature of the embedding center hot plate and wax reservoir is regularly checked.



The temperature of the embedding center hot plate is never checked. Even at this stage of processing specimens can be damaged by excessive local heat.



This lymph node was damaged by over-heating of the embedding center hot plate. Note the shriveled, pyknotic nuclei and extensive cracking. Cracking like this can also be caused by flotation on a water bath that is too warm, or by drying on a hot plate without sufficient draining.

Embedding

Step 30

Do Not Over-fill Molds



Molds are filled to an optimum level and do not overflow.



Molds are over-filled, requiring scraping of the back and edges of the cassette prior to microtomy. Over-filled blocks may sit unevenly in the microtome chuck causing instability that may lead to the tissue becoming damaged during microtomy.



Blocks that have resulted from over-filling the molds during embedding.

Embedding



- Step 31 Use High Quality Blades
- Step 32 Optimize Knife Tilt Angle
- Step 33 Carefully Trim Blocks
- Step 34 Avoid Freezing Damage
- Step 35 Use Cold Blocks
- Step 36 Cut Sections Slowly

Microtomy

Step 31

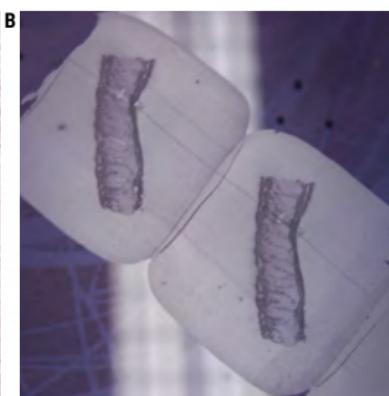
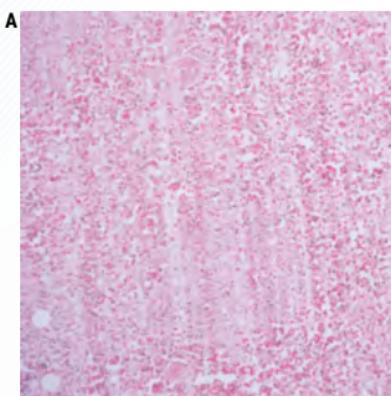
Use High Quality Blades



High quality, sharp blades are always used for cutting.



Blades are used for as long as possible – a few “train lines” are considered acceptable.



- A A section of spleen (H&E) showing many fine lines due to a defective blade.
- B Skin sections undergoing flotation. A severe knife line can be seen running right through the tissue. Defects such as this can be easily seen during flotation.

Microtomy

Step 32

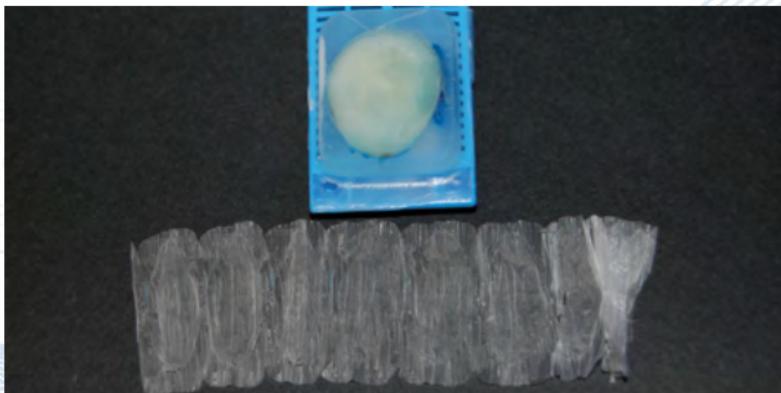
Optimize Knife Tilt Angle



Knife tilt angle is always optimized for each microtome and blade type



Knife tilt angle is never adjusted when conditions are changed (different microtome, new blade type, different wax etc).



This short ribbon of sections that was cut from a cold block shows considerable compression (30–40%). In this case re-setting the knife tilt angle overcame the problem.

Microtomy

Step 33

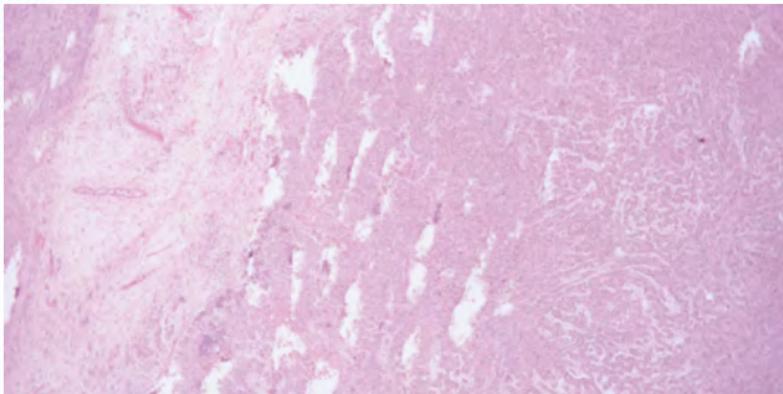
Carefully Trim Blocks



Blocks are carefully trimmed to expose tissue. The last few sections are always cut at what will be the final thickness to polish the block face.



Blocks are roughly trimmed to save time. The surface is not polished before taking final sections. This often produces a "moth-eaten" appearance in the final section which is full of small, ragged holes.



Initial exposure of the tissue (roughing) in this block has pulled fragments from the block surface which has resulted in numerous holes in the final section (H&E).

Microtomy

Step 34

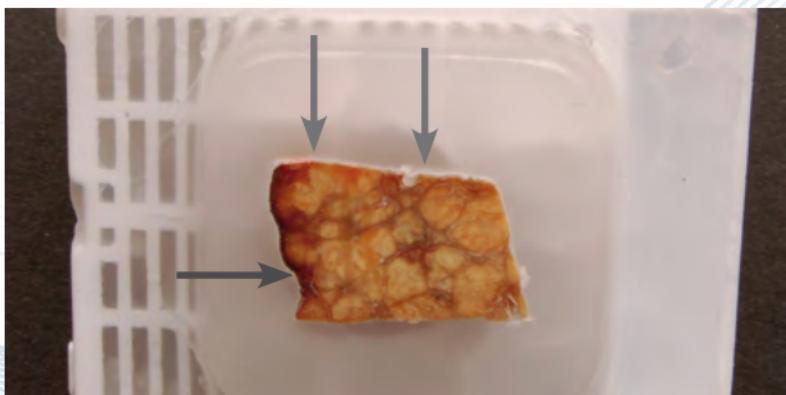
Avoid Freezing Damage



Blocks are chilled on a cold wet surface and are always cold when cut (the surface of melting ice is excellent).



Blocks are frozen before cutting. This sometimes causes the blocks to crack.



This block face has cracked because it was frozen to -15°C in a freezer prior to cutting. The cracks may make sectioning and flotation difficult because the wax is no longer bound to the tissue.

Microtomy

Step 35

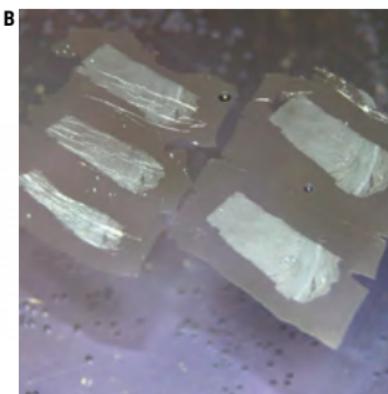
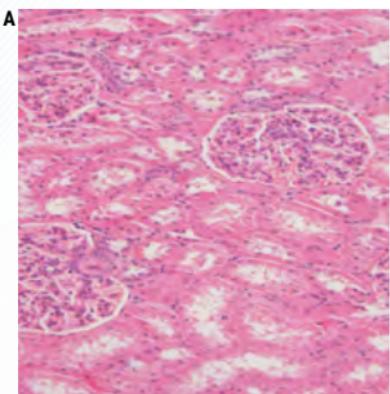
Use Cold Blocks



Blocks are always cold when cut.



There is sometimes a delay before final sections are cut from a block. The block may be warm and this may result in excessive compression of sections.



- A The distortion of the glomeruli in this kidney section is due to excessive compression when the section was cut (H&E).
- B Sections from the same block undergoing flotation. The sections on the left were cut without chilling the block while those on the right were cut when the block was cold.

Step 36

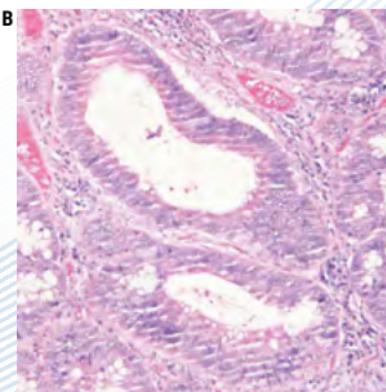
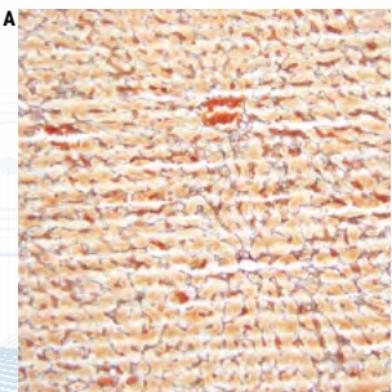
Cut Sections Slowly



The final sections from each block are cut gently with a uniform, slow rotation.



Sections are cut as quickly as possible with a rapid rotation, in the belief that "any section compression will be overcome on the flotation bath".



- A Rodent liver (reticulin stain) showing fine chatter due to cutting a cold block of brittle tissue too fast. In this case the problem was overcome by allowing the block to warm slightly then cutting very slowly. Chatter can also result if the paraffin block or blade is poorly secured in the microtome.
- B This H&E stained section of mucosal tissue shows fine chatter due to cutting a very cold block very quickly.

Microtomy



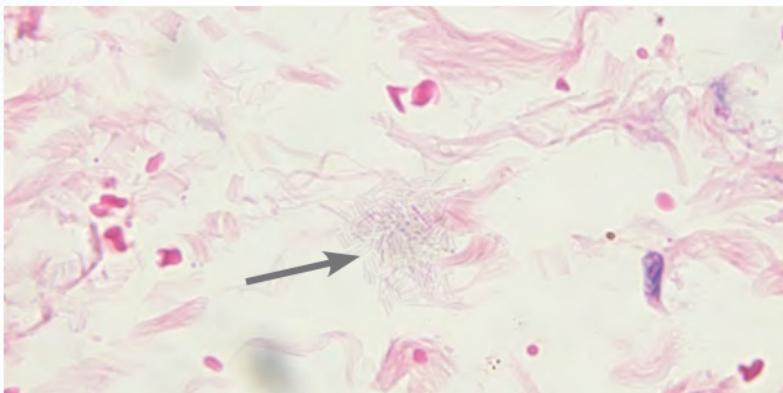
- Step 37 Use Clean Water
- Step 38 Ensure Slides Are Clean
- Step 39 Avoid Cross-contamination
- Step 40 Avoid Contamination with Squames
- Step 41 Don't Float from Multiple Blocks
- Step 42 Check Water Temperature
- Step 43 Avoid Wrinkles in Sections
- Step 44 Avoid Over-expanding Sections
- Step 45 Don't Damage Floating Sections
- Step 46 Carefully Choose Sections
- Step 47 Prevent Bubbles Under Sections
- Step 48 Prevent Section Lifting

Flotation

Step 37

Use Clean Water

- ✓ The water in the flotation bath is replaced regularly.
- ✗ The water in the flotation bath is topped up regularly but replaced only occasionally. Any contaminants in the bath may end up on the slide under the section (fungi, molds, etc).



Section from serosa of gastrointestinal tract (H&E). Clusters of weakly staining microorganisms are present within the tissue but could also be seen on the slide, outside the section. The likely source of these contaminating organisms was the flotation bath.

Flotation

Step 38

Ensure Slides are Clean

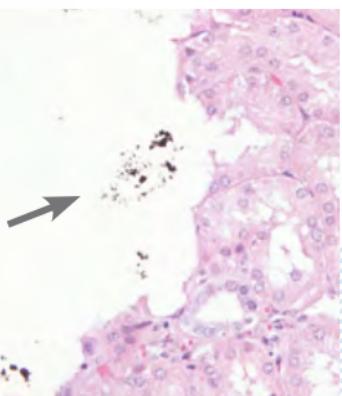


The cleanliness of slides is always checked before they are used. Handling of slides is kept to a minimum to avoid contamination with squamous cells prior to flotation.

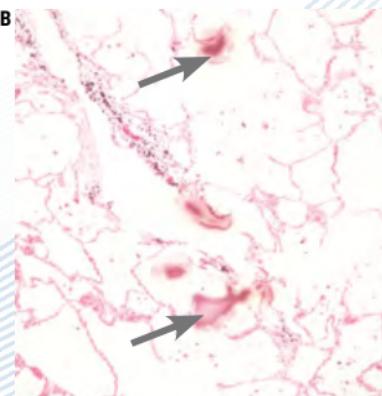


Slides are not checked for cleanliness: "As long as the sections stay on the slide during staining we consider they are satisfactory". Dust, organisms and other contaminants can spoil an otherwise good slide.

A



B



- A This section of kidney is spoilt by a black contaminant that was present on the slide prior to use. The deposit could be seen under the section in other parts of the slide.
- B A section of lung containing stained adhesive "pools" which have been formed as the section dried. The adhesive (probably gelatin based) was present in the flotation bath. The protein content of the adhesive has been concentrated as the water evaporated. Proper draining of the section prior to drying may have avoided the problem.

Flotation

Step 39

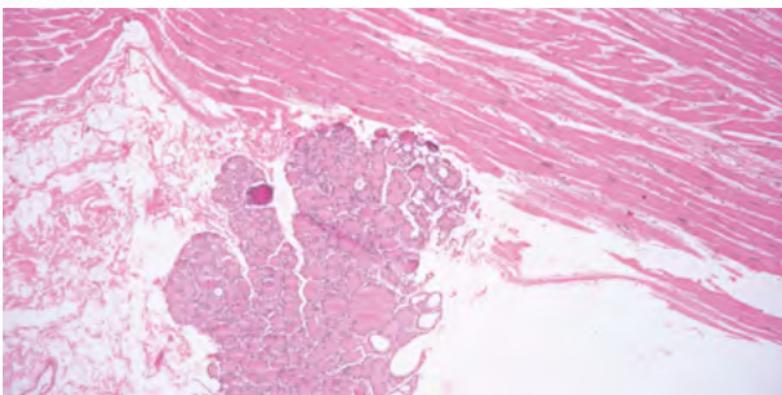
Avoid Cross-contamination



The water surface is always skimmed between specimens to avoid contamination of one section with cells from another.



The water surface is not skimmed between every block. This can result in specimen-to-specimen contamination which can cause confusion and even an inaccurate diagnosis.



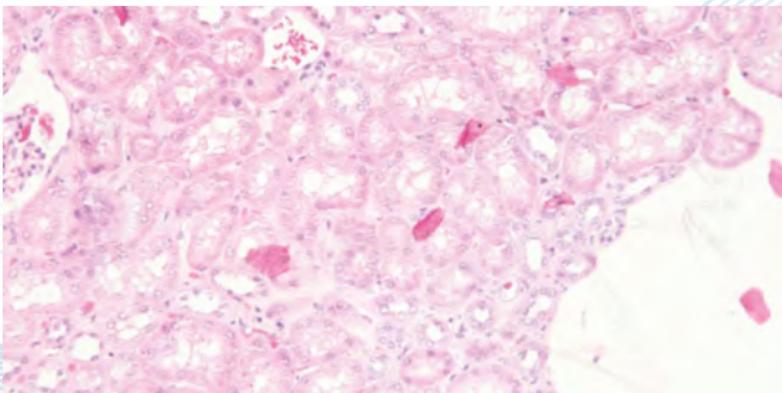
A section of cardiac muscle has been contaminated with a fragment of thyroid from another case. This example of specimen-to-specimen transfer occurred on the flotation bath.

Flotation

Step 40

Avoid Contamination with Squames

- ✓ Care is taken not to brush hair or hands whilst floating-out sections (squames can contaminate sections).
- ✗ "Some of our staff produce slides containing many squames. They seem unaware that this can be avoided."



A kidney section containing many extraneous squames that were deposited on the surface of the section while it was on the flotation bath. They adhere firmly and are subsequently stained with eosin.

Flotation

Step 41

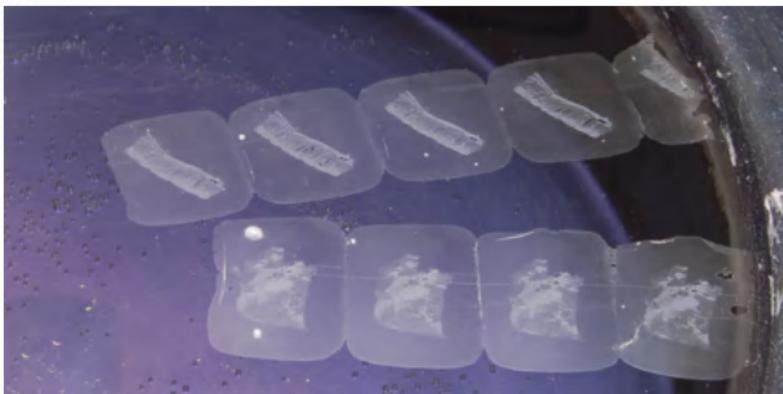
Don't Float from Multiple Blocks



Sections from more than one block (case) are never simultaneously floated on the water bath.



Sometimes sections from two or more blocks (cases) are left floating-out simultaneously. This is a dangerous practice that can lead to inaccurate identification of specimens. There is a particular risk when the sections happen to be from the same type of specimen.



Here sections from two different cases are being floated-out simultaneously. This practice can result in confusion and lead to inaccurate identification of sections.

Flotation

Step 42

Check Water Temperature



Flotation bath temperature is carefully checked. A temperature 4–5 °C below the melting point of the wax is optimal. Sections should readily flatten but the wax should not melt.



If sections are left on the flotation bath for more than 15 seconds the wax melts. Although this may seem to make the process faster it can rapidly cause over-expansion and tissue and cell damage.



These sections of skin clearly show cracks and excessive separation of layers, the typical effects of over-expansion. Poorly processed tissue is very prone to this problem.

Flotation

Step 43

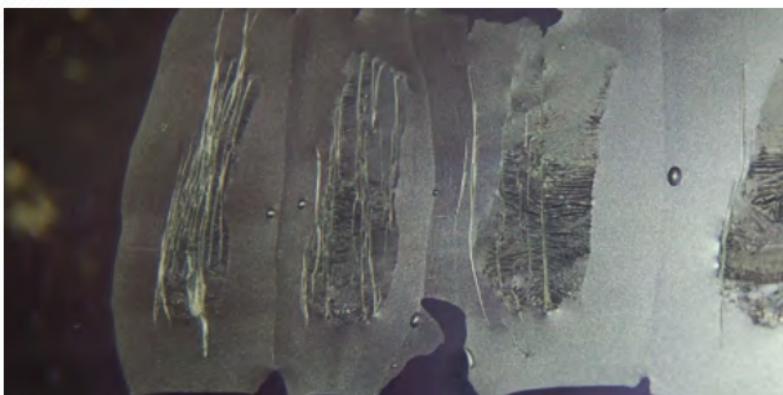
Avoid Wrinkles in Sections



Sections flatten readily on the flotation bath.



Sections never quite flatten on the flotation bath. The bath may be too cold and the sections may remain wrinkled when picked up on the slide.



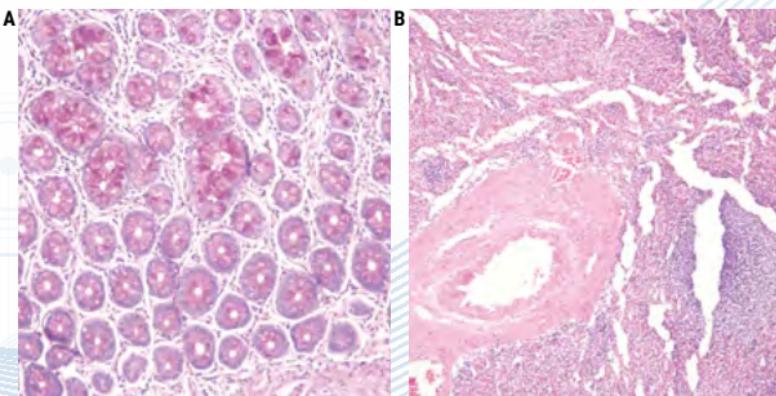
In this case flotation has not overcome the wrinkles produced during the cutting of these sections. Better cutting technique and slightly warmer water would overcome this problem.

Flotation

Step 44

Avoid Over-expanding Sections

- ✓ Sections are left on the flotation bath for just long enough to flatten then promptly picked up on a slide.
- ✗ For convenience, some sections are left for extended periods on the flotation bath. This can cause over-expansion and tissue damage (particularly to delicate specimens such as lymphoid tissue).



- A A section of intestinal mucosa stained with PAS shows a lamina propria that is over-expanded (shows excessive separation from the intestinal glands). In this case the section was floated for too long on a bath that was too hot.
- B A section of lymphoid tissue that has cracked due to over-expansion on the flotation bath. Lymphoid and hematopoietic tissues are particularly prone to damage in this way.

Flotation

Step 45

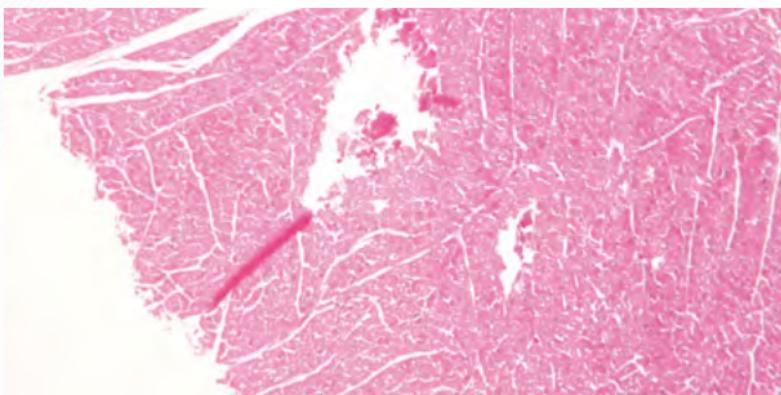
Don't Damage Floating Sections



Extreme care is taken to avoid damaging floating sections when mechanically removing wrinkles with a brush or forceps.



Wrinkles are vigorously removed from floating sections with a brush or forceps. Macroscopic and microscopic damage can easily be caused by this procedure.



A section of cardiac muscle shows mechanical damage (gouging), caused when attempting to remove a fold in the section (heart, H&E).

Flotation

Step 46

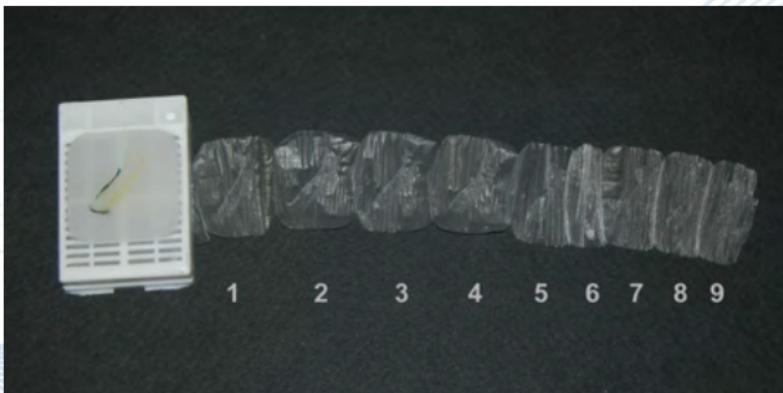
Carefully Choose Sections



The first one or two sections in a ribbon are never picked up on slides.



The first and second sections in a ribbon are selected for mounting because they look better than the later sections. They look better because they are invariably thicker due to the expansion of the cold block during the first couple of passes across the knife.



The sections in the ribbon prepared from this block are numbered in the order in which they were cut. Note that the first couple of sections are widest (least compressed) but as the block warmed the sections got narrower (more compressed). Although the microtome was set on 3 μm the first couple of sections would be 4–5 μm thick due to thermal expansion.

Flotation

Step 47

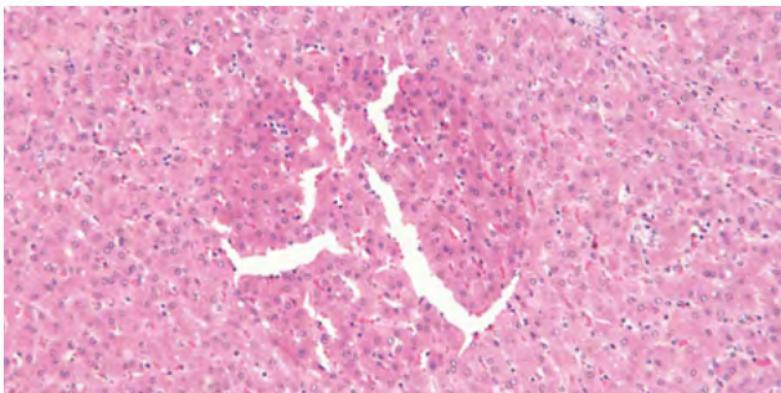
Prevent Bubbles Under Sections



Care is taken to avoid the formation of air bubbles in the flotation bath. Any visible bubbles are dislodged before the sections are laid on the water.



Small air bubbles in the flotation bath are ignored. Any bubbles that are trapped under the section apparently disappear as the section dries. Although the bubbles may apparently disappear, the areas in the section above the bubble are often distorted and are likely to float off during staining.



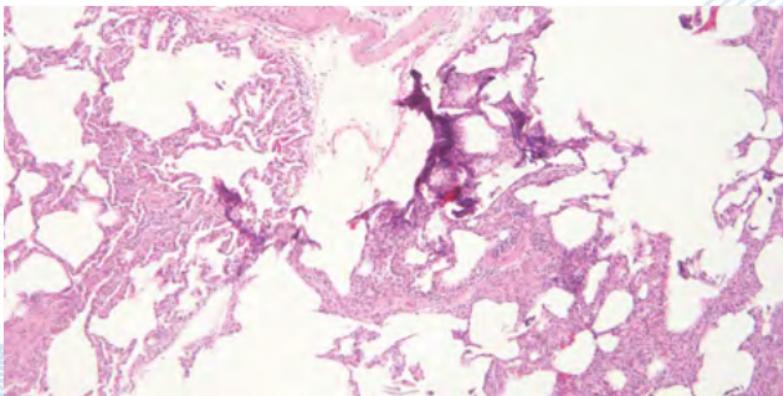
A liver section stained H&E, showing a circular, cracked area where the section has lifted. The cause was a bubble that lodged under the section during flotation and prevented proper flattening and adhesion.

Flotation

Step 48

Prevent Section Lifting

- ✓ The use of "sticky" (charged) slides or section adhesives such as AAS is considered and used appropriately.
- ✗ Sometimes sections float off during staining (particularly during antigen retrieval for IHC, or when methods require the use of heat). Charged slides or section adhesives are required in these circumstances.



This slide shows an area where the section has lifted and been deposited on adjacent tissue (lung, H&E).

Flotation



- Step 49 Drain Before Drying
- Step 50 Monitor Drying Temperature
- Step 51 Dry for Appropriate Time

Section Drying

Step 49

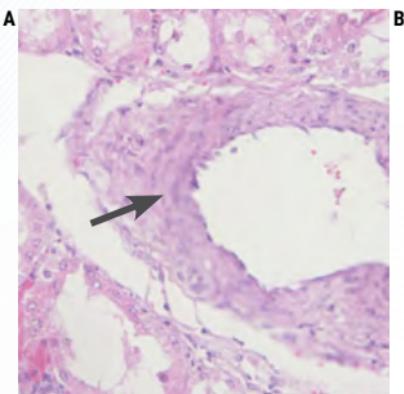
Drain Before Drying



Sections are drained briefly before being placed in the slide drier or onto a hotplate.



Sections are not drained properly before being dried horizontally. Sections move on the slide and sometimes do not dry flat.



B



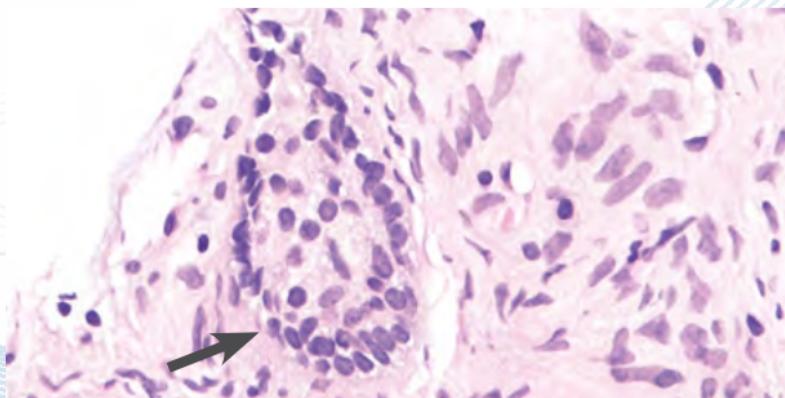
- A This section was dried horizontally without effective preliminary draining. As a result, a raised out-of-focus area is visible in the center of the field.
- B This section has just been picked up from the flotation bath and will be drained vertically for a brief time before being placed in the slide drier. This will avoid the problem shown in A.

Section Drying

Step 50

Monitor Drying Temperature

- ✓ The temperature of the slide drier is carefully monitored.
- ✗ Sometimes the slide drier is very hot. Excessive heat can produce hot-spots in sections and cause uneven staining.



This section of prostate shows the features of "nuclear meltdown", one possible cause being excessive heat when drying slides. Faulty tissue processing can produce a similar effect. Nuclear meltdown is typically seen at the perimeter of specimens and usually affects epithelial tissue. Nuclei show uneven staining, sometimes appearing pink or very blue and completely lacking detail.

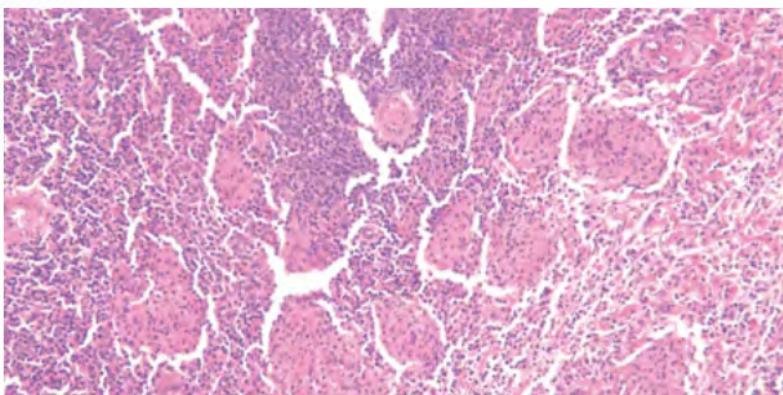
Section Drying

Step 51

Dry for Appropriate Time

✓ The minimum and maximum slide drying time is monitored.

✗ Slide drying times vary considerably. Extended drying at higher temperatures may be detrimental to sections.



A section of lymph node H&E that shows extensive cracking due to prolonged drying at too high a temperature. Cracking like this can also be caused by other factors including over-processing.

Section Drying



- Step 52 Use Accurate Timing
- Step 53 Regularly Monitor Quality
- Step 54 Standardize Staining Conditions
- Step 55 Ensure Complete Dewaxing
- Step 56 Renew Reagents Regularly
- Step 57 Hydrate Sections Thoroughly
- Step 58 Monitor Hematoxylin Quality
- Step 59 Ensure Complete Nuclear "Blueing"
- Step 60 Avoid Uneven Eosin Staining
- Step 61 Monitor Eosin pH

Routine Staining
(H&E)

Step 52

Use Accurate Timing



Each step in the staining protocol is accurately timed.



Step times in staining are approximate and "if we are in a hurry" some steps are skipped. This can produce inconsistent results.



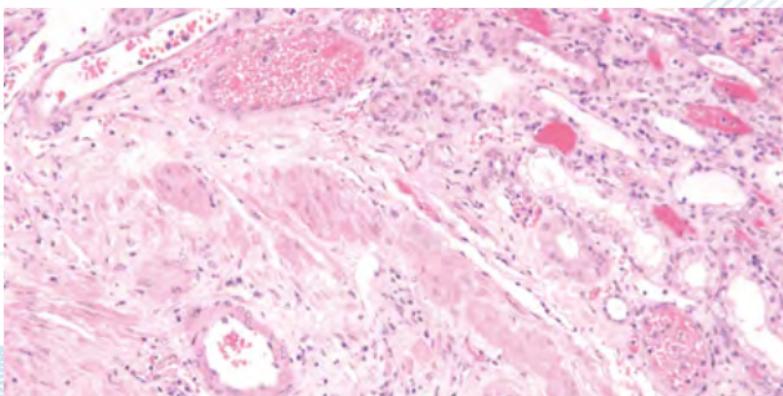
These sections were cut from the same block at the same thickness and manually stained H&E by different staff members using what was supposed to be the same method. Even macroscopically the inconsistency of the stain can be seen.

Routine Staining (H&E)

Step 53

Regularly Monitor Quality

- ✓ Control slides are regularly stained to monitor stain quality.
- ✗ Control slides are never used for H&E stains. This can make it very difficult to determine whether a staining problem is due to poor reagents, an inappropriate protocol or poor fixation.



This section of kidney includes a variety of eosinophilic tissue elements and well-preserved nuclei that allows a reliable assessment of the quality of H&E staining. Placenta is another specimen type that can be used as a useful control.

Routine Staining (H&E)

Step 54

Standardize Staining Conditions



Agitation, wash and drain times are optimized for all steps during staining.



Agitation, wash and drain times are inconsistent. Solvents and reagents rapidly become contaminated. Staining becomes inconsistent.



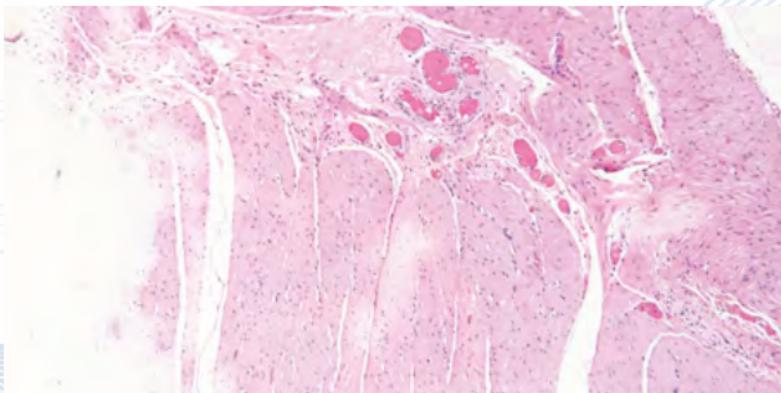
One of the benefits of using an automated staining instrument is that agitation, wash and drain times are consistent. Providing other variables are properly controlled, this will ensure good, consistent results.

Routine Staining (H&E)

Step 55

Ensure Complete Dewaxing

- ✓ Slide dewaxing is optimized.
- ✗ Slide dewaxing is sometimes incomplete and slides contain patches of residual wax. This produces unstained, or unevenly stained areas in sections.



This H&E stained section shows a large unstained area on the left and several smaller areas that are either partially stained or unstained. This is due to incomplete wax removal prior to staining.

Routine Staining (H&E)

Step 56

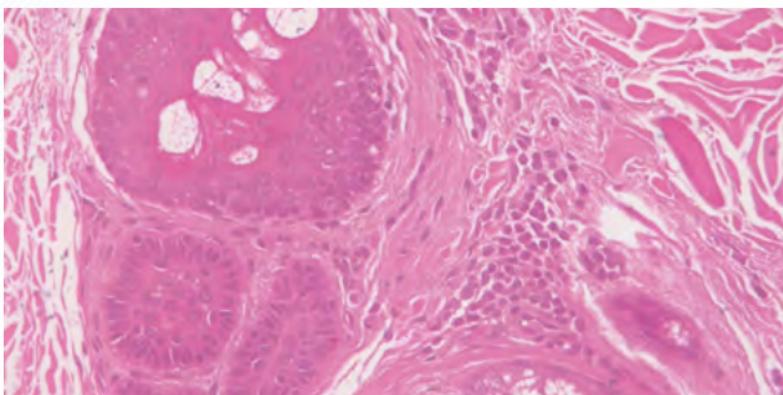
Renew Reagents Regularly



Solvents and staining reagents are regularly replaced based on the number of slides stained or racks processed.



Replacement of solvents and staining reagents is haphazard. They are not replaced until stain quality declines.



This section shows poor quality, muddy hematoxylin staining. This reagent should be replaced immediately.

Routine Staining (H&E)

Step 57

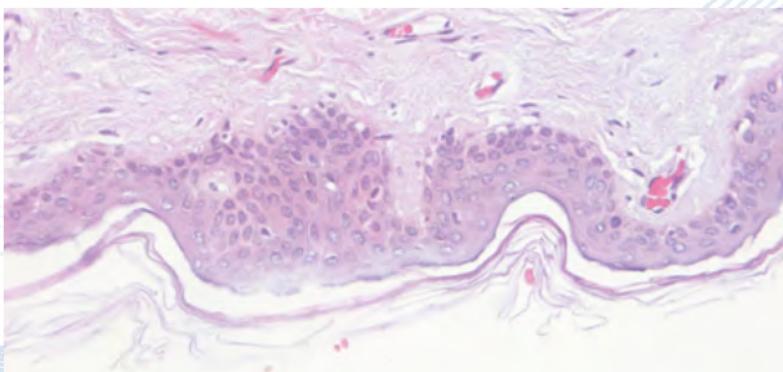
Hydrate Sections Thoroughly



Slides are thoroughly hydrated prior to hematoxylin staining.



Hematoxylin solution rapidly becomes contaminated with alcohol and sometimes xylene. This causes uneven staining.



The uneven hematoxylin staining visible in the epidermis in this skin section was caused by residual xylene (and traces of wax) present when the hematoxylin was applied.

Routine Staining (H&E)

Step 58

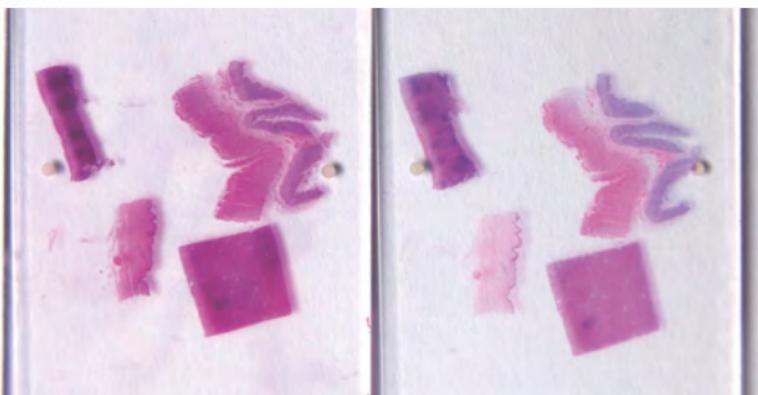
Monitor Hematoxylin Quality



The performance of hematoxylin solutions is carefully monitored. During their working life hematoxylin solutions are progressively diluted by carryover from slides and racks and also affected by continuing oxidation.



Hematoxylin staining is variable from day-to-day and no attempt is made to understand why. For example, the staining bath surface area, the extent of aeration during staining, and the ambient temperature can all affect the oxidation rate.



Two slides from the same control block are shown. They were stained H&E using identical protocols on an automated stainer but with an interval of seven days between runs. Even macroscopically the variation in the level of staining is obvious.

Routine Staining (H&E)

Step 59

Ensure Complete Nuclear “Blueing”

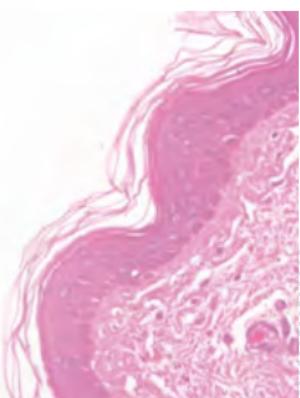


Thorough “blueing” of nuclei with Scott’s alkaline tap water substitute or ammonia water is always performed after hematoxylin staining. This requirement is influenced by the natural pH of the local tap water.

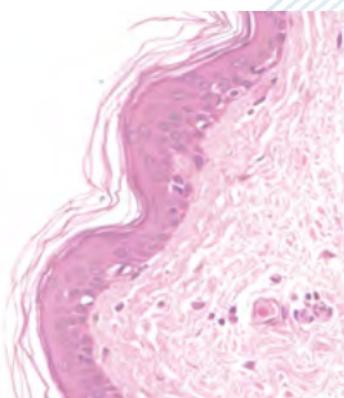


Sometimes nuclei appear pinkish in completed sections due to incomplete “blueing” in alkaline tap water after hematoxylin staining. Nuclei that are under-stained with hematoxylin (or over-differentiated) and over-stained with eosin also appear pink.

A



B



- A In this section the epidermal nuclei are poorly defined and are pinkish in color. This section was not properly “blued” in alkaline water after hematoxylin staining (skin, H&E).
- B This shows another section that was properly “blued” after the nuclear stain. Here the nuclei are much better defined (skin, H&E).

Routine Staining (H&E)

Step 60

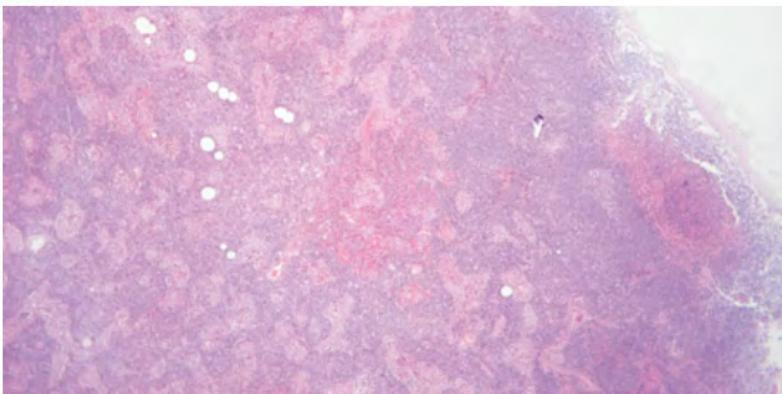
Avoid Uneven Eosin Staining



"Blueing" is followed by a very thorough wash in tap water to remove residual alkali that can impede eosin staining and cause weak and uneven staining.



Inefficient washing after "blueing" (leaving residual alkali) causes eosin staining to be weak and uneven.



This section demonstrates the effect of residual alkali on eosin staining. Note the patchy nature of the stain (spleen, H&E).

Routine Staining (H&E)

Step 61

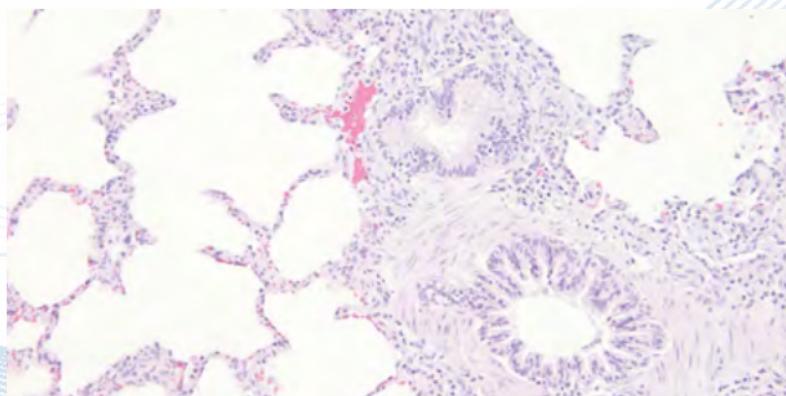
Monitor Eosin pH



The pH of the eosin solution is monitored. It is kept close to pH 5.0 to maintain optimal staining. The addition of a couple of drops of acetic acid can be used as a convenient means of lowering pH.



No attempt is made to monitor the pH of eosin. When staining intensity falls away the solution is replaced (carryover of alkaline tap water can cause the pH of eosin solutions to rise).



A section of lung stained H&E. The eosin stain is uniformly very weak and quite unacceptable. Note that the only components stained with eosin are the red blood cells.

Routine Staining (H&E)



- Step 62 Thoroughly Dehydrate Before
Clearing and Coverslipping
- Step 63 Avoid Drying and Crystal Formation

Coverslipping

Coverslipping

Step 62

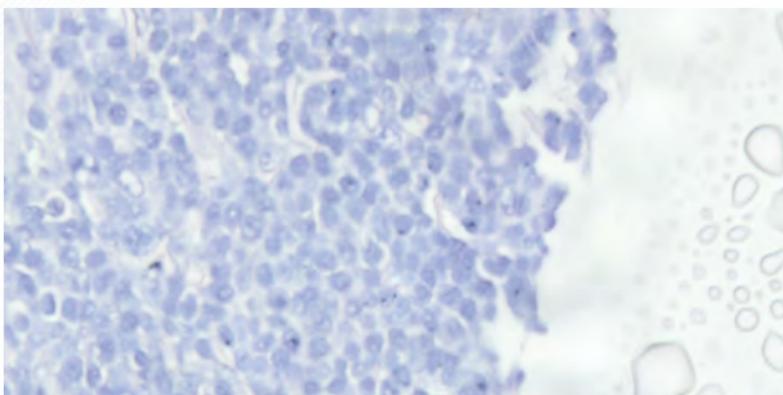
Thoroughly Dehydrate Before Clearing and Coverslipping



Sections are thoroughly dehydrated before being placed in xylene for clearing.



Sections are sometimes rushed through alcohol to xylene. Clearing in xylene contaminated with water can result in the presence of tiny water droplets in the tissue that are seen microscopically as opaque areas lacking detail.



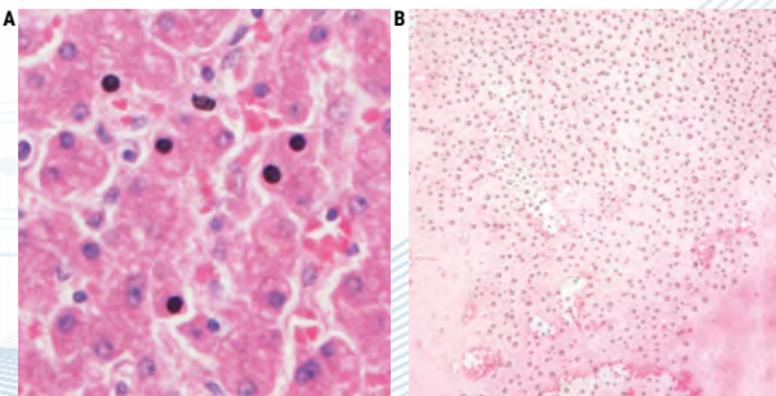
This section lacks clarity (it appears opaque to the naked eye). Careful examination reveals tiny water droplets to be present throughout.

Coverslipping

Step 63

Avoid Drying and Crystal Formation

- ✓ The coverslip is always applied before the section has a chance to dry and a high quality mountant is used. The long-term storage qualities of the mountant must be known because crystals can appear in poor quality mountant – sometimes after a long period (months or years).
- ✗ Sections are allowed to partially dry before the coverslip is applied causing some nuclei to appear black. Mountant chosen on the basis of price alone may develop crystals during long-term storage and coverslips may lift.



- A This section was allowed to partially dry before coverslipping. This has caused tiny air bubbles to be trapped over some nuclei making them appear black (sometimes referred to as "corn-flaking").
- B A coverslipped section stained H&E showing a multitude of refractile spherocrystals that developed from poor quality mountant within six months of mounting.

Coverslipping



- Step 64 Understand the Stain
- Step 65 Use a Positive Control
- Step 66 Use Accurate Timing
- Step 67 Consider Reagent Stability
- Step 68 Store Reagents Correctly
- Step 69 Adhere to the Method
- Step 70 Record any Changes
- Step 71 Standardize Washing Steps
- Step 72 Set Up Microscope Carefully

Special Stains

Step 64

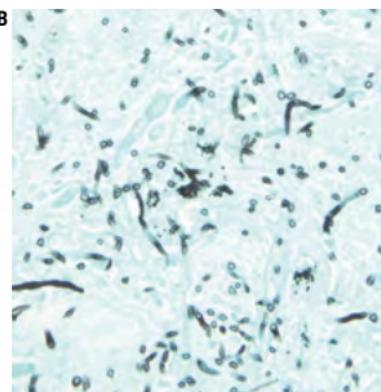
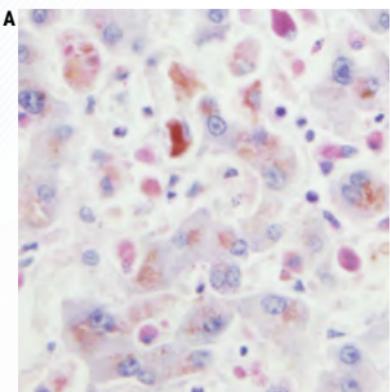
Understand the Stain



Know what you are trying to demonstrate with the stain you are performing.



Just "following the method" and not really knowing what should be seen in the finished section will lead to poor results.



- A A section of liver stained with PAS. Lipofuscin and glycogen are PAS positive while traces of bile and hemosiderin are PAS negative and appear in their natural colors (yellow and brown respectively).
- B This section shows an opportunistic fungal infection in lung (*Aspergillus*) stained with the Grocott-Gomori method. Fungal hyphae are black as is unstained carbon, a common feature in the lungs of smokers and most city dwellers.

Special Stains

Step 65

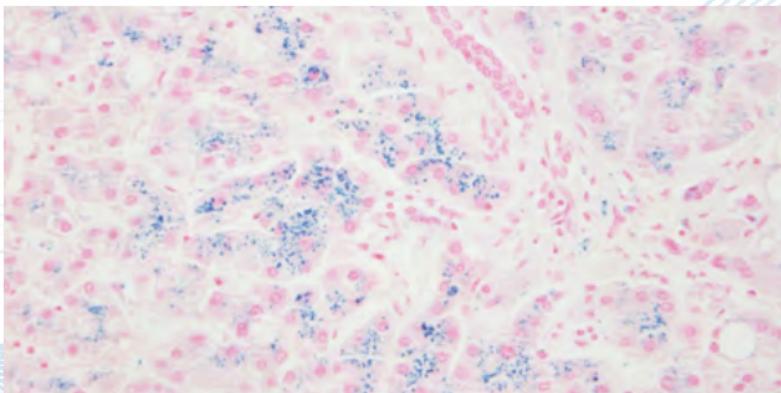
Use a Positive Control



Always use a control slide known to contain the structure/substance you are trying to demonstrate.



"If the structure/substance we are staining for is not visible in a slide we assume it is not present."



A section of cirrhotic liver stained with Perl's method to demonstrate iron-containing hemosiderin (blue). This would make a satisfactory control block for iron stains.

Special Stains

Step 66

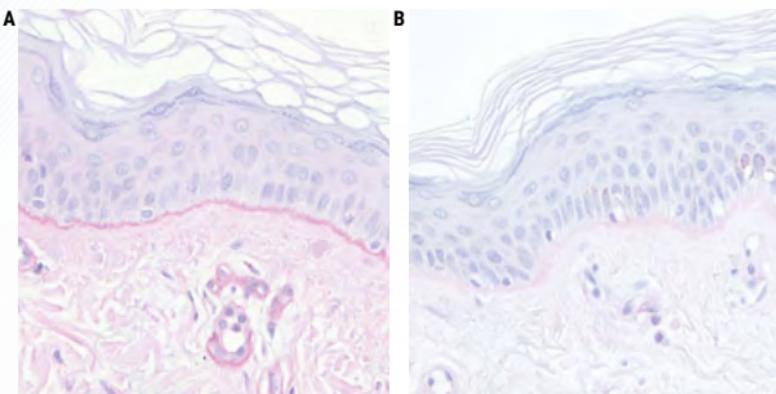
Use Accurate Timing



Use accurate timing.



Timing is always approximate. Inaccurate timing produces inconsistent results.



Both these sections of skin from the same block have been stained with the PAS method. Section A was treated with periodic acid (oxidation step) for 5 minutes whereas section B had only 30 seconds (a mistake). Note that the basement membrane is very poorly stained in section B as a consequence.

Special Stains

Step 67

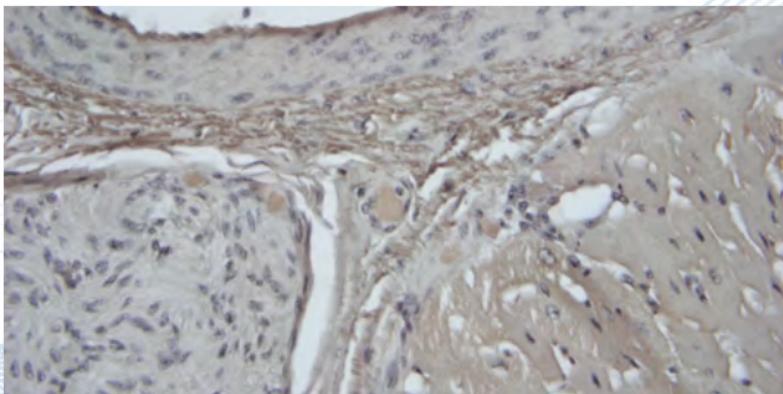
Consider Reagent Stability



Be aware of the shelf life of the reagents you are using. Some reagents or dye solutions deteriorate slowly while others are very unstable and must be made up fresh and used immediately. Others have to be left for some time to oxidize (ripen) before they can be used at all.



We assume all reagents can be used for an indefinite period.



Muddy Weigert's hematoxylin due to overoxidation. Note the brown staining of collagen.

Special Stains

Step 68

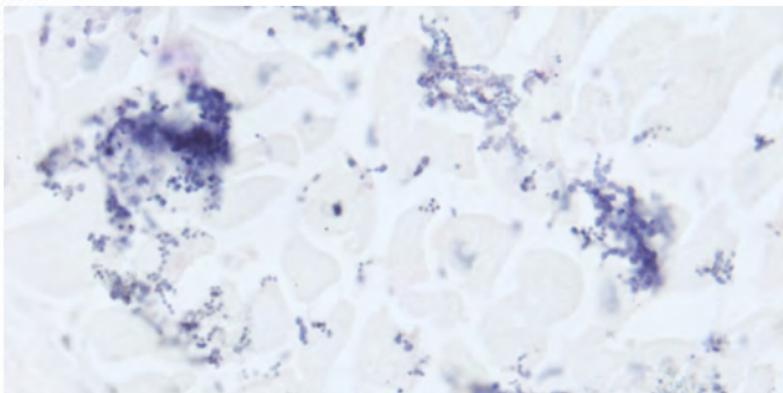
Store Reagents Correctly



Store reagents correctly. Some require refrigeration because they are inclined to support the growth of fungi or molds. Others are light sensitive and require storage in the dark.



"All our reagents are stored on the shelf above the staining bench. Sometimes we see stray organisms in our sections."



This section shows large deposits of extraneous microorganisms which have grown in the staining solution (in this case hematoxylin) then been deposited on top of the section.

Special Stains

Step 69

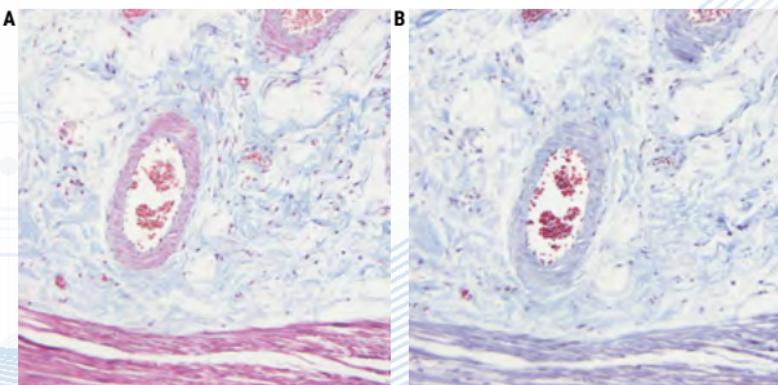
Adhere to the Method



Follow the protocol exactly.



Staff members achieve different results when supposedly using the same protocol.



These sections of formalin-fixed submucosa have been stained with Masson trichrome stain. Section A shows red smooth muscle. In this case the stain was performed correctly following the lab protocol and including a preliminary chromic acid step (sensitization or secondary mordanting). This step was overlooked when section B was being stained. Note the lack of differential coloration of muscle in section B (intestine).

Special Stains

Step 70

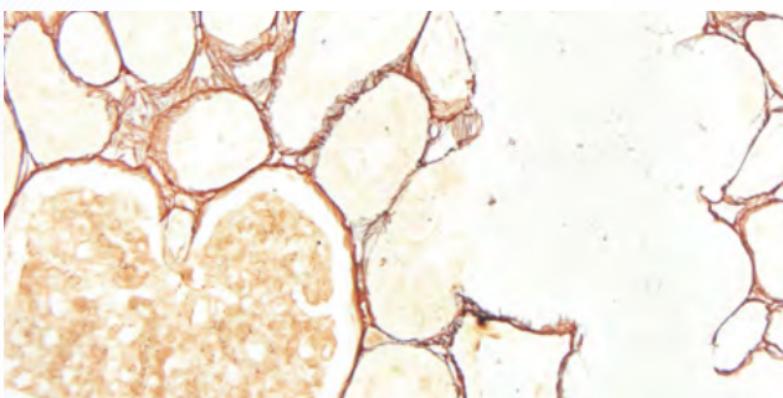
Record Any Changes



Document any departure from the method you are using.



Sometimes when results are poor it is difficult or impossible to work out why because protocol changes have not been recorded.



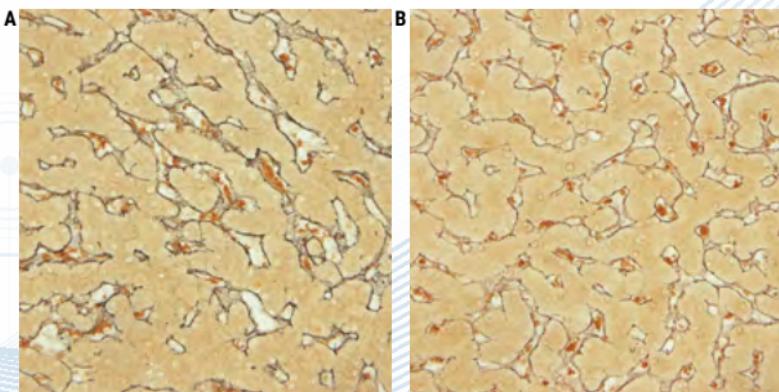
In this silver impregnation stain for reticulin the fibers are poorly demonstrated and there is a background scum (precipitate) on the slide. It is very difficult to determine the cause of such a problem if the method has not been followed exactly (Gordon & Sweets method, kidney).

Special Stains

Step 71

Standardize Washing Steps

- ✓ Take particular care with washing steps. Standardize them as far as possible as they are frequently the cause of variable results.
- ✗ Lab staff members use different washing techniques – some use vigorous agitation, others are much more gentle.



These liver sections were stained by the same method. The only difference between them was the technique by which they were rinsed between impregnation and reduction. The reticulin fibers are black and better defined in section A (Gordon & Sweets method).

Special Stains

Step 72

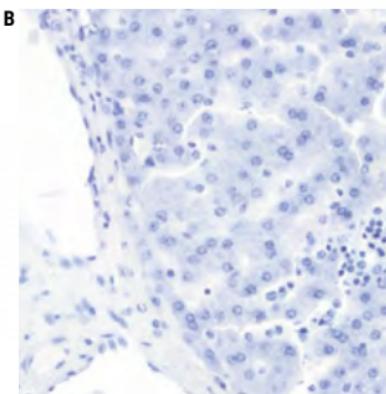
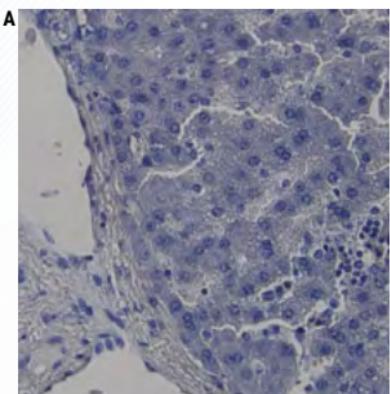
Set Up Microscope Carefully



Use microscopic control at crucial stages such as differentiation steps. Be aware of the effect of the microscope setup on the appearance of un-coverslipped (wet) sections; it can produce the appearance of false background staining.



For all methods the level of staining is assessed by looking at the slide with the naked eye.



- A. Wet section (no coverslip) viewed under a microscope with closed condenser diaphragm. Note the false background.
- B. Wet section (no coverslip) viewed under a microscope with open condenser diaphragm. Note the clear background.

Special Stains



- Step 73 Use High Quality Sections
- Step 74 Ensure Optimal Fixation
- Step 75 Avoid Section Adhesion Problems
- Step 76 Optimize Wax Removal and Reagent Application
- Step 77 Avoid Concentration Gradients
- Step 78 Choose Antibody Carefully
- Step 79 Read Specification Sheets
- Step 80 Optimize Retrieval Methods
- Step 81 Consider Antibody Cross-reactivity
- Step 82 Block Endogenous Peroxidase
- Step 83 Avoid Background Staining
- Step 84 Use an Appropriate Detection System
- Step 85 Standardize Washing Steps
- Step 86 Optimize Counterstaining
- Step 87 Use Appropriate Controls
- Step 88 Evaluate Results Carefully

Immunohistochemistry

Step 73

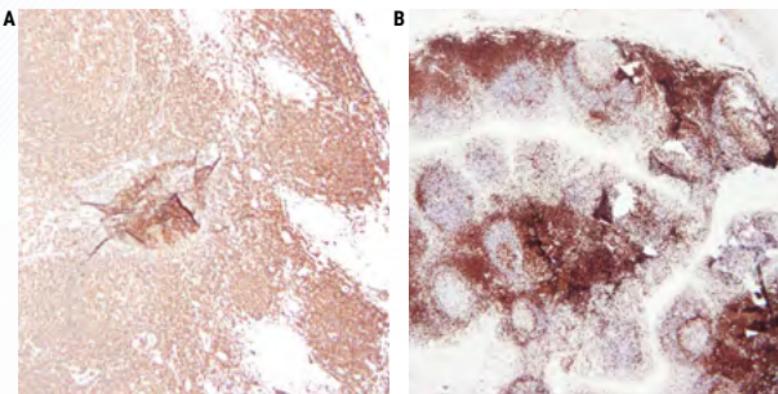
Use High Quality Sections



Take particular care to use thin, flat sections that have been thoroughly dried onto the slide. Preferably use charged slides or APES coated slides for IHC.



Uneven, poorly-adhering sections stain unevenly with variable background staining.



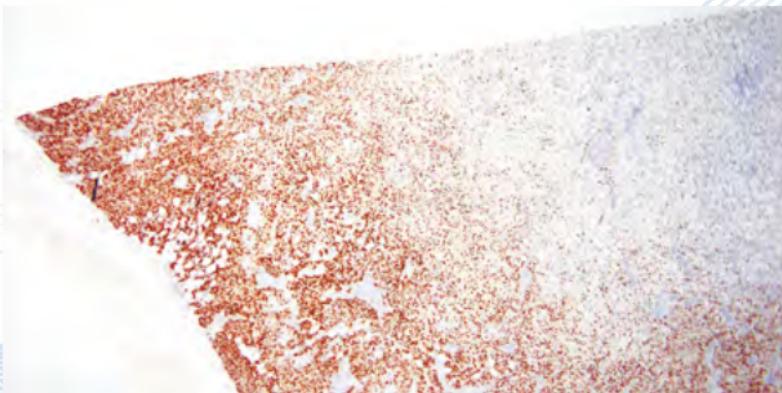
- A A bubble under the section (from mounting) has resulted in subsequent detachment of the section during staining (tonsil, CD45).
- B A poor quality section, that has not been properly flattened and dried before staining, has lifted making the slide unsatisfactory (tonsil, CD3).

Immunohistochemistry

Step 74

Ensure Optimal Fixation

- ✓ Good quality fixation using known and consistent fixation conditions (fixative type, pH, temperature, time) produces the best results. Specimens should be checked prior to processing to determine if further fixation is required.
- ✗ Inconsistent fixation conditions, producing under-fixed or over-fixed tissues, produce variable results and make troubleshooting difficult.



Uneven fixation (zonal fixation) has resulted in uneven staining in this section (breast tumor, ER).

Immunohistochemistry

Step 75

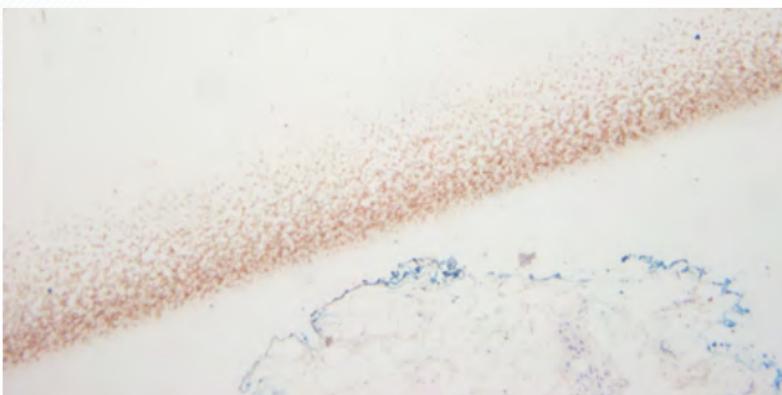
Avoid Section Adhesion Problems



Avoid the use of protein-based section adhesives in the flotation bath (glue, starch, or gelatin), particularly on charged slides.



Protein-based adhesives can block the surface of the charged slide. This causes inconsistent adhesion and leads to uneven staining due to pooling of IHC reagents beneath lifting sections.



A line of thick protein-based section adhesive has stained adjacent to the section (breast, PR).

Immunohistochemistry

Step 76

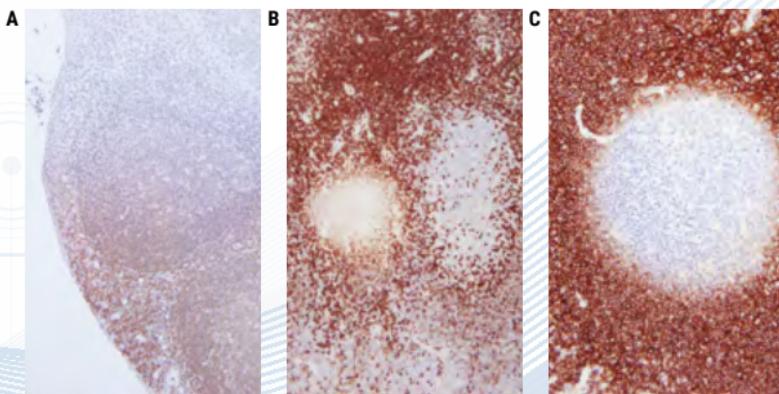
Optimize Wax Removal and Reagent Application



Take particular care with dewaxing and hydration of sections as well as efficient and uniform distribution of reagents on the specimen surface. This ensures even staining and consistent results.



Incomplete removal of wax or uneven distribution of reagents on the specimen surface can produce unstained or poorly stained areas in sections.



- A Poor reagent flow has produced uneven staining (tonsil, CD45).
- B Residual wax has resulted in an unstained area (tonsil, CD5).
- C A bubble in the primary antibody has prevented uniform staining (tonsil, CD20).

Step 77

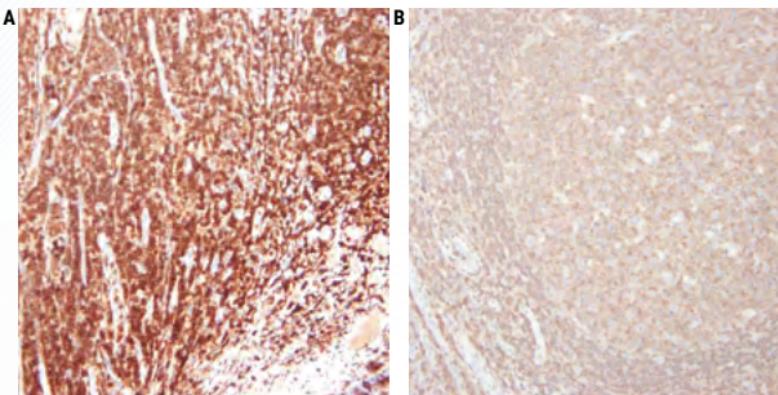
Avoid Concentration Gradients



Concentration gradients are avoided by careful application of reagents.



"We sometimes see strong staining at one end of the slide progressing to weak staining at the other."



A and B are micrographs taken from opposite ends of the same slide. One end of the slide shows strong staining (A) whereas at the other end (B) the staining was very weak. This is an extreme example of a concentration gradient created during staining. (tonsil, CD45).

Immunohistochemistry

Step 78

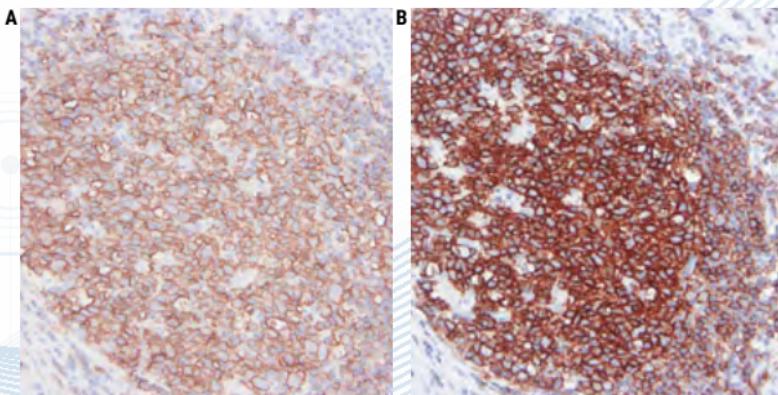
Choose Antibody Carefully



Choose your primary antibody carefully with regard to its sensitivity and specificity. Be aware that antibodies sold by different suppliers often come from the same source and are repackaged/branded for sale. It is important to use the clone name when assessing an antibody.



"We buy our antibodies based on price alone."



These sections of human tonsil from the same block have been stained with the B cell marker CD20 using primary monoclonal antibodies from different sources (suppliers). In each case the recommended pretreatment and optimized dilution was used. There is an obvious difference in the quality of the results achieved.

Immunohistochemistry

Step 79

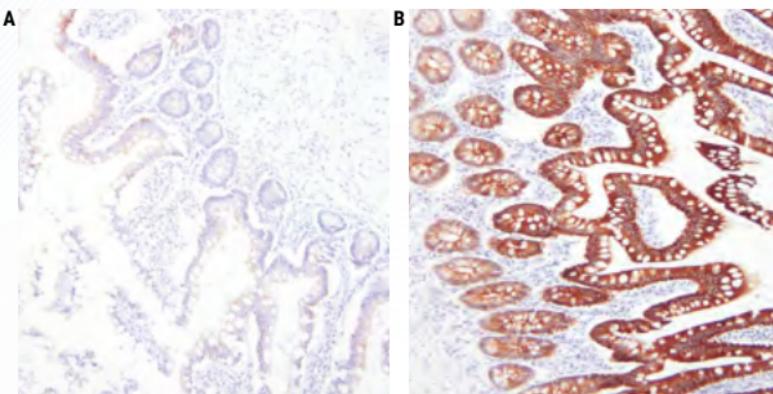
Read Specification Sheets



Know your primary antibody. Always check the specification sheet to determine the suitability of your method for a particular antibody. Specification sheets should be updated when a new batch of antibody is purchased.



"We don't have access to the antibody specification sheets in our laboratory."



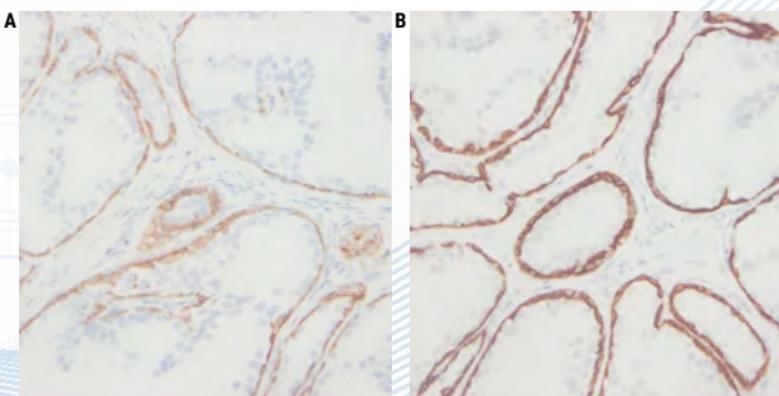
These sections of intestine have been stained for Cytokeratin AE1/AE3. Different retrieval conditions were used for each section. Section A shows unacceptable weak staining, while section B shows strong precise staining.

Immunohistochemistry

Step 80

Optimize Retrieval Methods

- ✓ Choose appropriate unmasking conditions for the primary antibody being used, the tissue being stained and the fixation employed (pH, reagent, reaction conditions).
- ✗ The same retrieval technique is used for all primaries on the assumption that there is a successful universal HIER method.



Prostate sections stained for Cytokeratin 34 β E12. Section A shows weak staining while section B is stronger and sharper. The only difference between the two was the retrieval method used.

Immunohistochemistry

Step 81

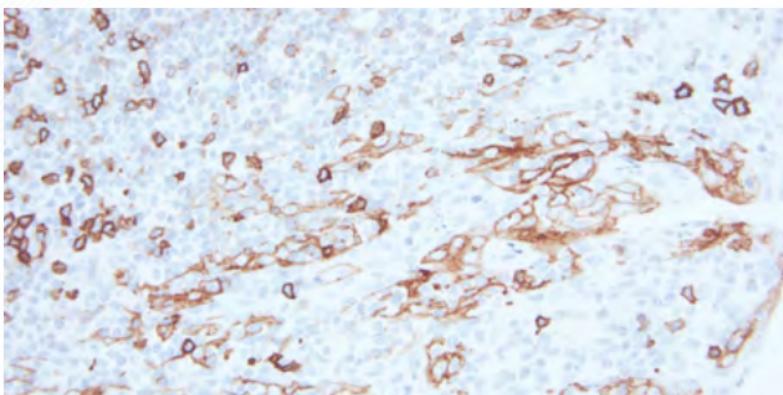
Consider Antibody Cross-reactivity



Be aware of any potential problems with antibody cross-reactivity (read the specification sheet).



No attempt is made to explain unexpected positive staining.



Palatine tonsil showing the base of a tonsillar crypt stained for CD5, a lymphocyte marker that stains mainly T cells. This particular clone (4C7) cross-reacts with epithelial cells deep in the crypt.

Step 82

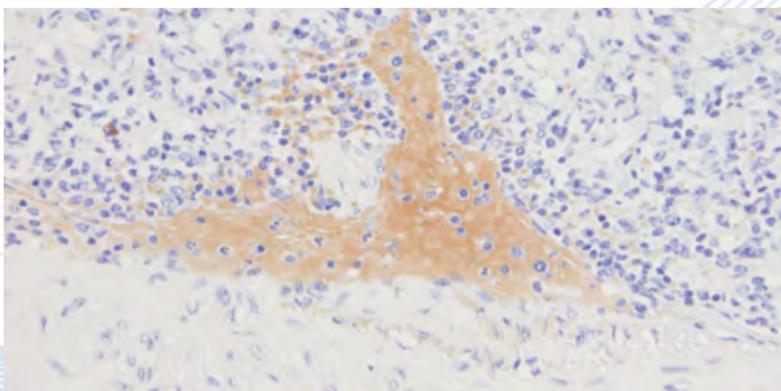
Block Endogenous Peroxidase



For peroxidase-based detection systems, always use a peroxidase-blocking step.



Non-specific staining is often seen in erythrocytes, granulocytes, monocytes, and in muscle. This is due to incompletely-blocked endogenous peroxidase.



Spleen showing typical, non-specific staining of erythrocytes due to incomplete blocking of endogenous peroxidase. Here the natural peroxidase present in the red cells has reacted with the DAB chromogen.

Immunohistochemistry

Step 83

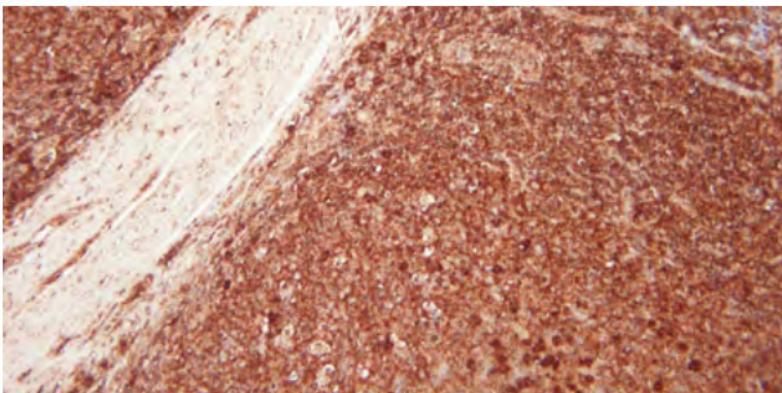
Avoid Background Staining



Appropriate protein block is always used.



Generalized background staining is sometimes seen due to ineffective protein block.

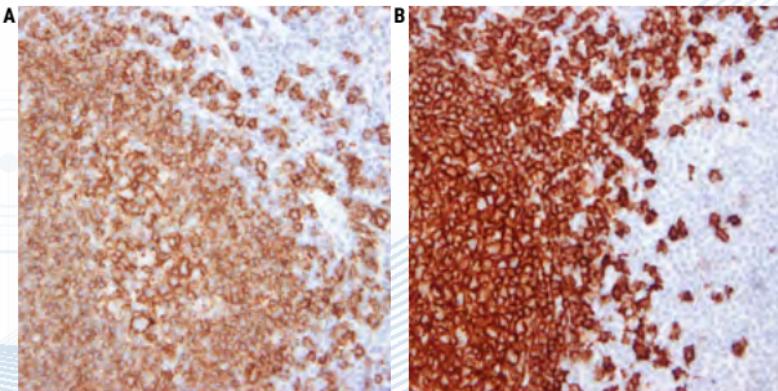


Normal tonsil stained for Kappa light chain showing a heavy back-ground stain due to ineffective protein block.

Step 84

Use an Appropriate Detection System

- ✓ Choose an appropriate detection system that will provide precise, specific staining with adequate sensitivity.
- ✗ "We have been using the same detection system for a long time and see no reason to change. Sometimes our stains are weak and are not as sharp as we would expect."



Sections A and B are from the same specimen but have been stained using different detection systems. Note the difference in the intensity and precision of the stains. (tonsil, CD20).

Immunohistochemistry

Step 85

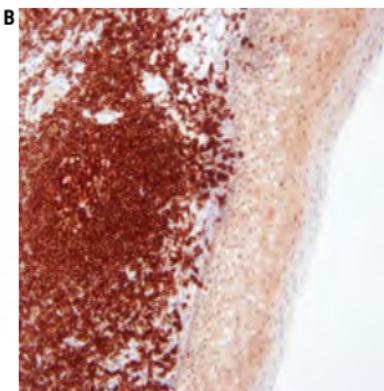
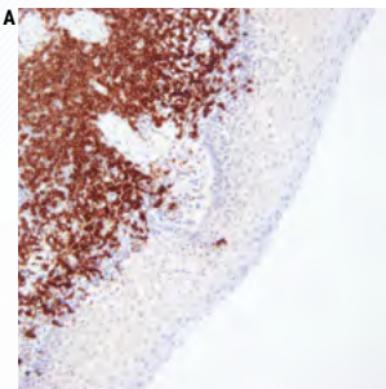
Standardize Washing Steps



Use standardized washing steps throughout (duration, volume and form of agitation). This will ensure consistency of results.



Results are very variable within runs with the same antibody and between runs on different days. This can be due to different washing techniques used by different operators.



Sections A and B are from the same specimen and have been stained manually using the same reagents. Note the difference in the level of background staining in the stratified epithelium. This is probably due to a difference in the efficiency of the washing technique used. (tonsil, CD20).

Immunohistochemistry

Step 86

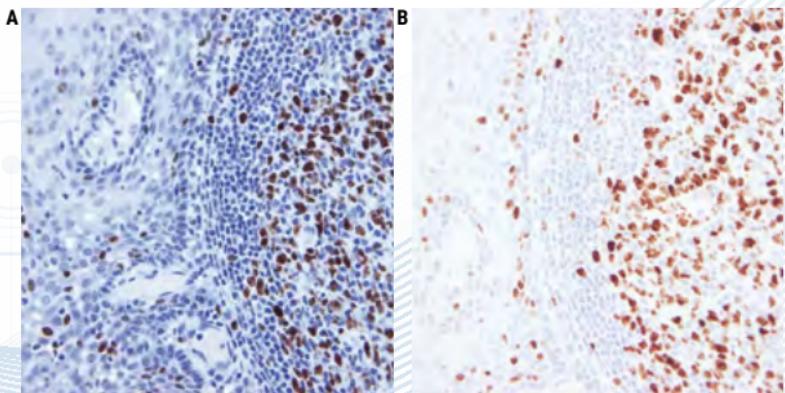
Optimize Counterstaining



The level of nuclear counterstain is carefully regulated and standardized so as not to obscure positive staining. The counterstain should provide the best possible contrast between chromogen and background tissue elements. An appropriate counterstain is chosen for the chromogen used.



Nuclear counterstain is sometimes very strong. This can obscure weak specific staining.

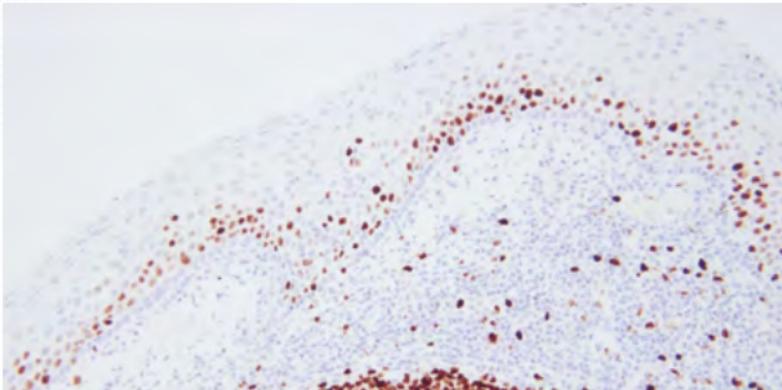


Tonsil stained for Ki67, a nuclear marker for proliferating cells. The two sections are from the same specimen showing different levels of hematoxylin counterstaining. Slide A shows staining that is too strong and would obscure a weak positive reaction. Slide B shows a better level of staining.

Step 87

Use Appropriate Controls

- ✓ Always use appropriate positive and negative controls that are carefully examined to validate results. Internal positive and negative controls are also important and provide an excellent means of ensuring quality assurance in IHC.
- ✗ "We only do controls when our method doesn't seem to work. If we did them for every run people wouldn't bother to look at them."



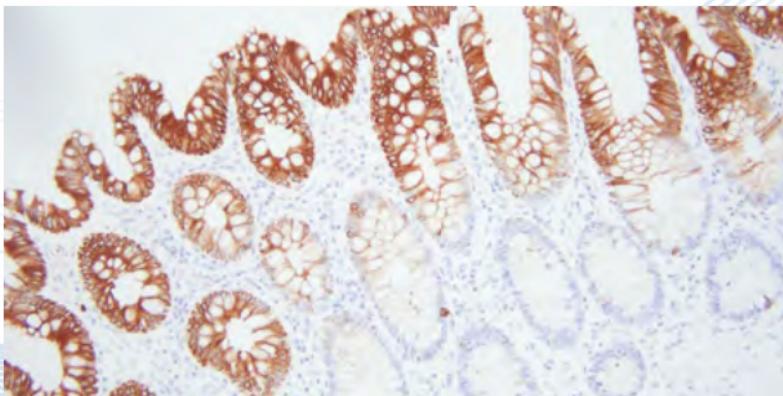
Tonsil stained for Ki67. This was the negative control slide and the nuclei should not be stained. The primary antibody was mistakenly applied to this slide instead of the negative control reagent.

Immunohistochemistry

Step 88

Evaluate Results Carefully

- ✓ Know what to look for and where to look when evaluating your test sections and controls after staining.
- ✗ If staining is observed in test sections it is assumed the stains are satisfactory.



Intestine stained for AE1/AE3. Unexpected weak staining of the crypt epithelium has occurred. On investigation it was found that CK20 had been wrongly used as the primary antibody.

Immunohistochemistry



- Step 89 Use High Quality Sections
- Step 90 Ensure Optimal Fixation
- Step 91 Avoid Section Adhesion Problems
- Step 92 Optimize Wax Removal and Reagent Application
- Step 93 Choose Probe Carefully
- Step 94 Read Specification Sheets
- Step 95 Optimize Pretreatment Conditions
- Step 96 Handle Tissue Carefully
- Step 97 Use Appropriate Detection System
- Step 98 Avoid Reagent Evaporation
- Step 99 Standardize Washing Steps
- Step 100 Use Appropriate Controls
- Step 101 Evaluate Results Carefully

Step 89

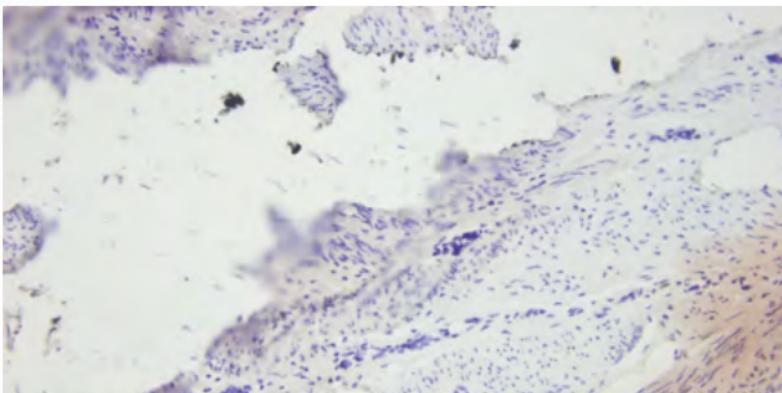
Use High Quality Sections



Take particular care to use thin, flat sections that have been thoroughly dried onto the slide. Use charged slides for ISH.



Uneven, poorly-adhering sections stain unevenly with variable background staining.



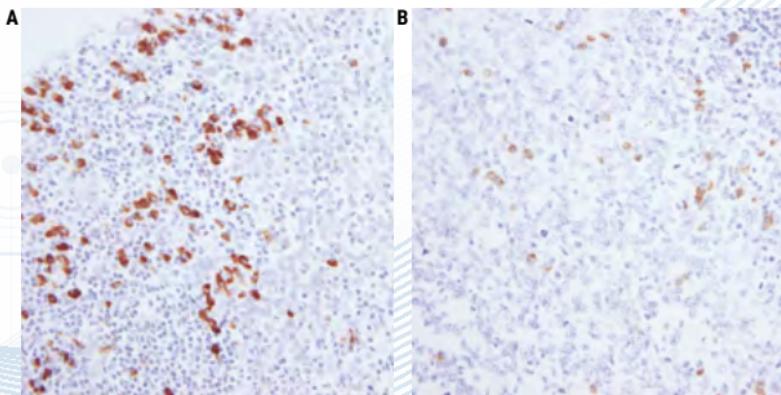
This poor quality section shows lifting and background staining (HPV).

In Situ Hybridization

Step 90

Ensure Optimal Fixation

- ✓ Good quality fixation using known and consistent fixation conditions (fixative type, pH, temperature, time) produces the best results.
- ✗ Inconsistent fixation conditions, producing under-fixed or over-fixed tissues, produce variable results and make troubleshooting difficult.



- A ISH for kappa light chain mRNA on well-fixed tonsil shows a sharp, strong reaction.
- B ISH for kappa light chain mRNA on poorly-fixed tonsil shows a weak reaction.

In Situ Hybridization

Step 91

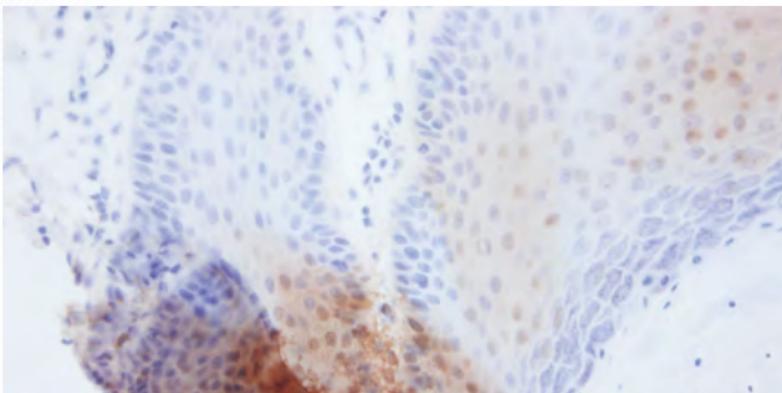
Avoid Section Adhesion Problems



Avoid the use of protein-based section adhesives in the flotation bath (glue, starch, or gelatin), particularly on charged slides.



Protein-based adhesives can block the surface of the charged slide. This causes inconsistent adhesion and leads to uneven staining due to pooling of ISH reagents beneath lifting sections.



Poor adhesion and folds have resulted in uneven staining (HPV).

In Situ Hybridization

Step 92

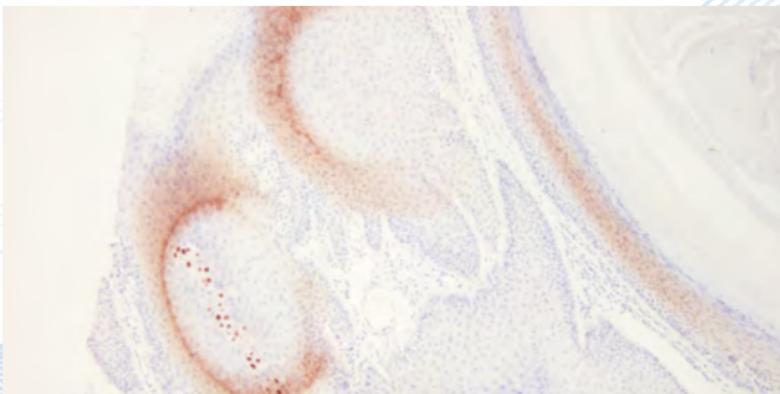
Optimize Wax Removal and Reagent Application



Take particular care with dewaxing and hydration of sections as well as efficient and uniform distribution of reagents on the specimen surface. This ensures even staining and consistent results.



Incomplete removal of wax can produce unstained or poorly-stained areas in sections. Bubbles retained on the section surface during pretreatment or staining can cause problems.



Bubbles formed during pretreatment at 95 °C have caused uneven staining (HPV).

In Situ Hybridization

Step 93

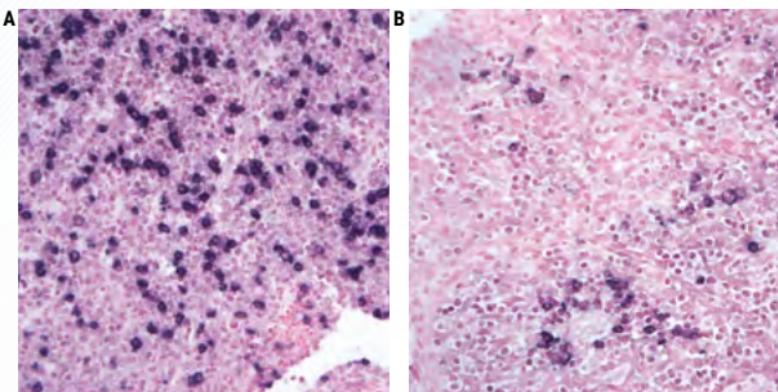
Choose Probe Carefully



Choose your probe carefully with regard to its sensitivity and specificity.



"We buy our probes based on price alone."



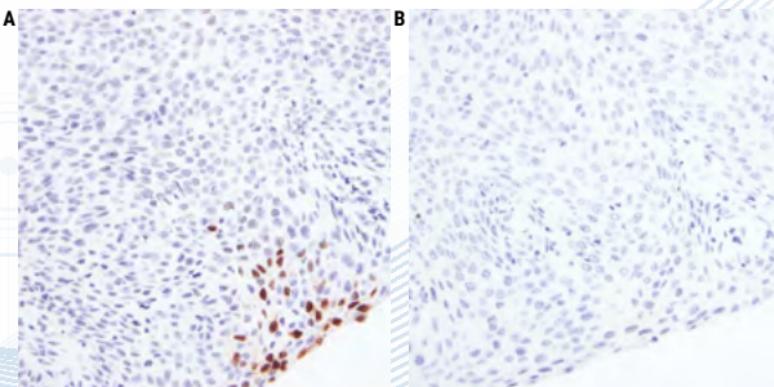
Both sections of tonsil were stained using ISH (BCIP/NBT) for kappa light chain mRNA using oligonucleotide probes from different sources. Section A shows strong staining while section B demonstrates weaker staining with fewer cells stained.

In Situ Hybridization

Step 94

Read Specification Sheets

- ✓ Know your probe. Always check the specification sheet to determine the suitability of your method for a particular probe. Carefully control temperature and time to provide optimal hybridization conditions. These must be exactly right to ensure that maximum specific binding occurs.
- ✗ No access to the probe data sheets in the laboratory: "We just follow the standard method".



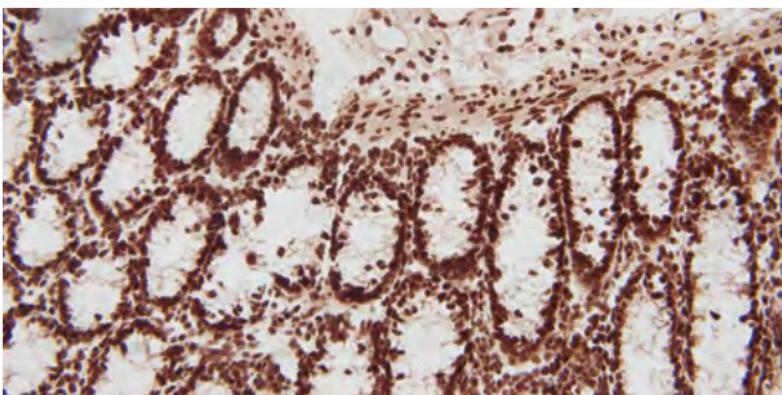
Both sections of condyloma were stained using ISH for HPV using the same DNA probe but different hybridization conditions. Section A shows strong staining while in section B staining is unsatisfactory.

In Situ Hybridization

Step 95

Optimize Pretreatment Conditions

- ✓ Choose appropriate pretreatment and optimization conditions. These will depend on fixation and tissue type.
- ✗ Use of the same enzyme pretreatment conditions for different probes may sometimes produce poor results.



This section of colon has been stained with a Poly d(T) positive control probe. The section demonstrates the result of over-digestion with Proteinase K. Note the loss of cytoplasmic structure in the mucosal epithelium and heavy background staining.

In Situ Hybridization

Step 96

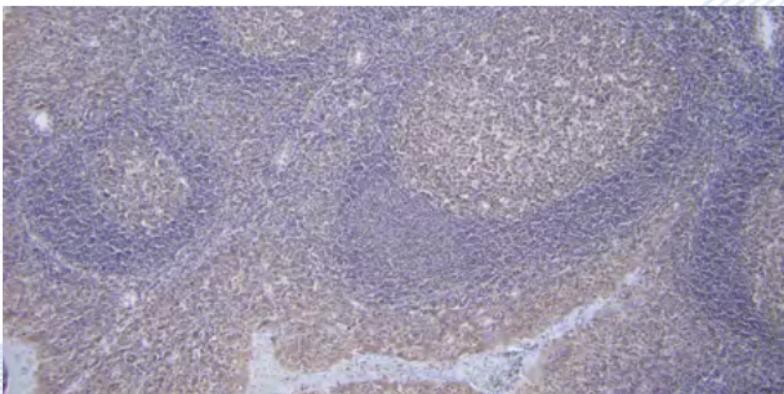
Handle Tissue Carefully



Careful handling of tissue specimens and prompt fixation will limit the loss of RNA by the action of endogenous RNases.



Careless handling of tissue specimens and delayed fixation will encourage the loss of RNA by the action of endogenous RNases.



Weak staining due to the breakdown of nuclear RNA by RNases. Tonsil stained with a Poly d(T) positive control probe.

In Situ Hybridization

Step 97

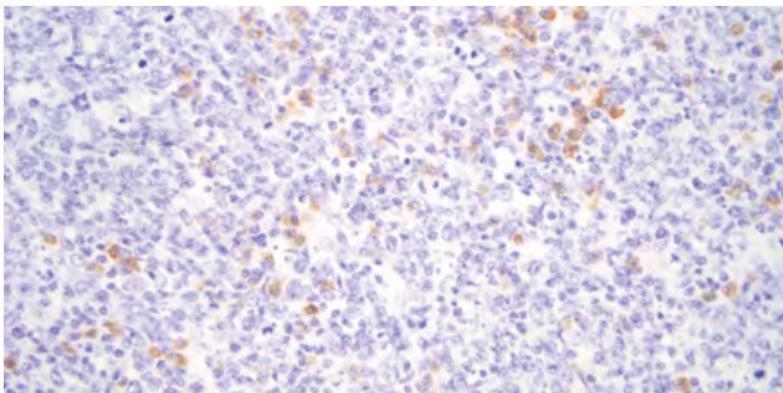
Use Appropriate Detection System



Choose a sensitive detection and visualization system and optimize incubation conditions.



A lack of sensitivity in the detection and visualization system can result in very weak or even negative staining even though the probe is bound to a target.



The weak staining in this section of tonsil is due to a lack of sensitivity in the detection system. ISH for lambda light chain using a non-polymer detection kit.

In Situ Hybridization

Step 98

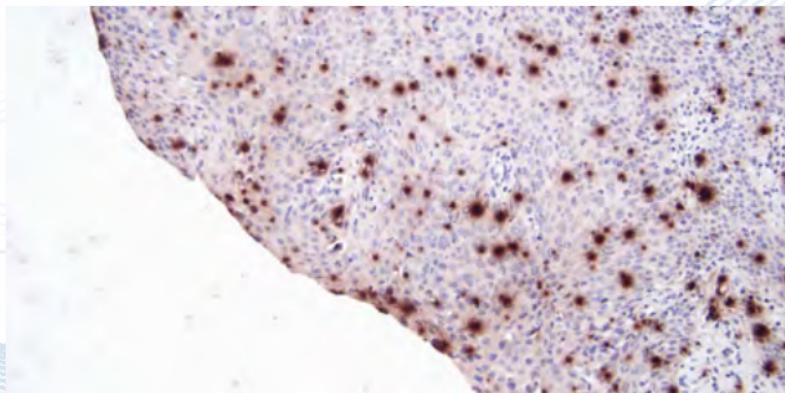
Avoid Reagent Evaporation



Prevent evaporation of the probe solution and other reagents during incubation. Because of the need for long incubation times drying of the reagents is a common problem. The use of good quality equipment is essential.



If the probe or other reagents dry out on the section (usually at the edges) it can cause heavy, non-specific staining in areas.



This section of tonsil stained with ISH for kappa light chain has dried out during the formamide post-hybridization wash causing inconsistent, non-specific staining.

In Situ Hybridization

Step 99

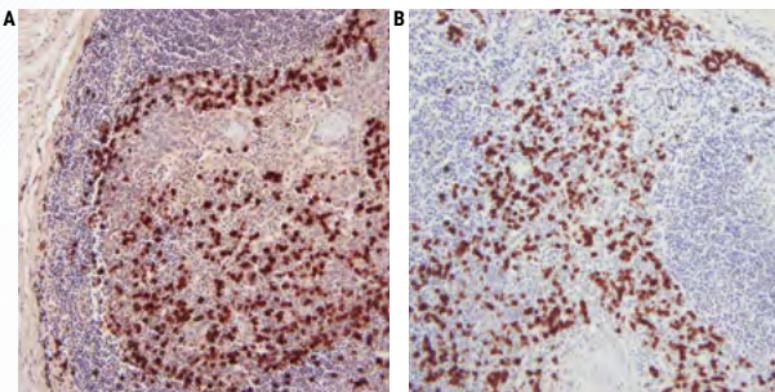
Standardize Washing Steps



Use standardized washing steps throughout (duration, volume and form of agitation). This will ensure consistency of results.



Results are very variable within runs with the same probe and between runs on different days. This can be due to different washing techniques used by different operators.



These sections of tonsil stained with ISH for kappa light chain show how washing affects the final result. Section A shows excessive background staining due to poor washing technique while section B was properly washed and has no background staining.

In Situ Hybridization

Step 100

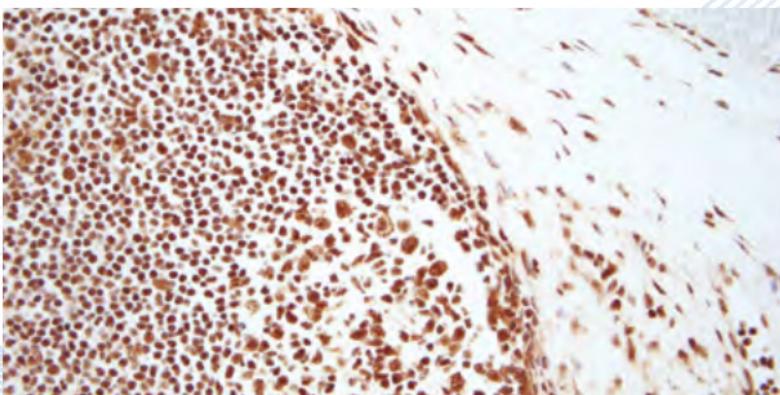
Use Appropriate Controls



Use appropriate controls with every run. This should include known positive tissue and a negative control using a non-specific probe.



"We only do controls when our method doesn't seem to work. If we did them for every run people wouldn't bother to look at them."



Section of tonsil stained with Poly d(T) positive control probe. The precise staining indicates that the tissue is well fixed and that RNA sequences will be well preserved.

In Situ Hybridization

Step 101

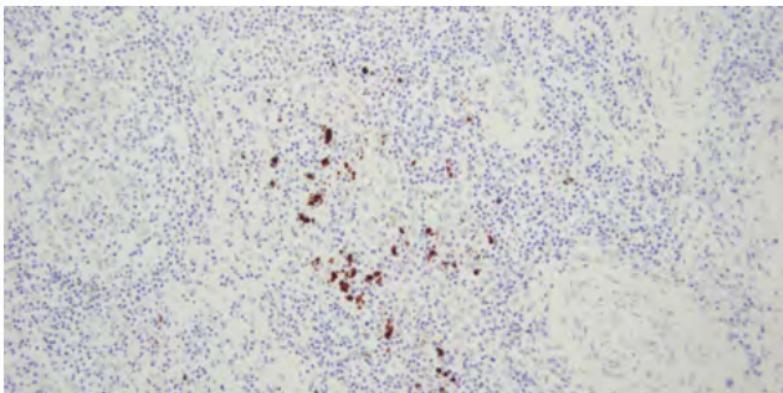
Evaluate Results Carefully



Know what to look for and where to look when evaluating your test sections and controls after staining. Anyone undertaking ISH should have a fundamental knowledge of the underlying theory of the technique and where to find positive staining.



If any staining is observed in test sections it is assumed the stains are satisfactory.



This negative control section of tonsil has gone through all steps of ISH but without the application of a probe. It shows hemosiderin, which has a natural brown color and additionally binds DAB which intensifies the color. This does not represent positive staining.

In Situ Hybridization



**Knowledge
Pathway**

Authors

Geoffrey Rolls (principal author)

Simon Davies (immunohistochemistry)

Aodin Gallagher (in situ hybridization)

Contributors

Kerrie Scott-Dowell

Annette Mullane

Neville Farmer

Fiona Tarbet



Knowledge Pathway

Knowledge Pathway is a scientific and educational resource for professionals in the Anatomic Pathology field. Built by a steadily growing community of authors and experts, this lively science portal continuously offers you new and relevant information, ranging from the basics to specific application know-how.

knowledgepathway.com

LEICA BIOSYSTEMS

Leica Biosystems is a global leader in workflow solutions and automation, integrating each step in the workflow. As the only company to own the workflow from biopsy to diagnosis, we are uniquely positioned to break down the barriers between each of these steps. Our mission of "Advancing Cancer Diagnostics, Improving Lives" is at the heart of our corporate culture. Our easy-to-use and consistently reliable offerings help improve workflow efficiency and diagnostic confidence. The company is represented in over 100 countries and is headquartered in Nussloch, Germany.