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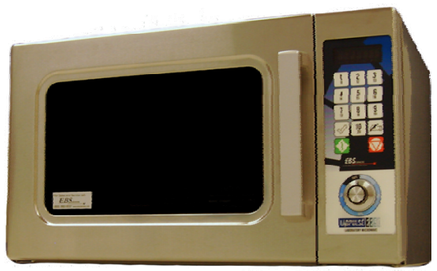
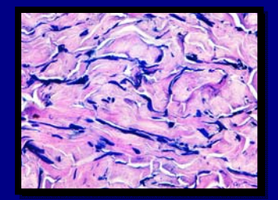
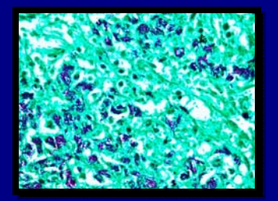
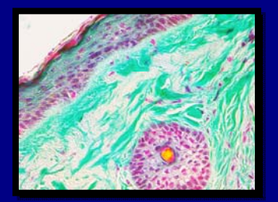
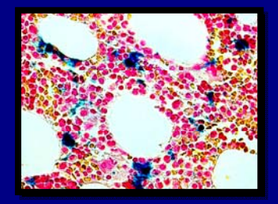


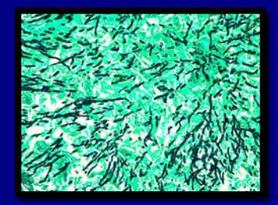
Microwave Companion for Histology

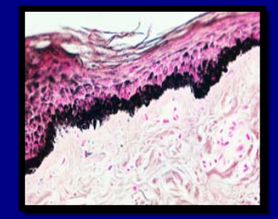












LABPULSE® MEDICAL

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MICROWAVE COMPANION FOR HISTOLOGY

Third Edition

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Extra Thanks To

Marcia L. Dunne, Clinical Laboratory Partners, Hartford Hospital, Hartford, CT
For her proofreading, and editing skills as well as her patience and understanding.

Preface

The new environment in which we operate places an ever increasing demand on technologists for improved sample quality, faster diagnostic turn-around times, greater efficiency, lower consumable costs, and reductions in hazardous material use. For all these reasons, the use of microwave technology is becoming more essential to an ever increasing number of laboratories.

With this in mind, we have revised, and added content to the Microwave Companion for Histology, including a section containing useful terms pertaining to microwave technology. Many of the procedures that were developed for the first 2 editions are included in this booklet with the edition of the theory behind those applications. This was done with the desire to retain the basic goals of the first edition, expand on the operation of laboratory microwaves and explain how they are used to meet these needs of our ever changing environment. We have used much of the theory and concepts of histology techniques that I have acquired through my career as a histologist and educator. Also throughout this booklet is valuable information I have learned from Michael Whittlesey and Bill Zimmerman. Michael Whittlesey, our engineer at Energy Beam Sciences, is a wealth of information and has been invaluable to my learning of microwave technology. I can't say enough about his expertise! Bill Zimmerman is our service technician and has been extremely helpful and supportive, while teaching me the hands on operations. Bill has explained his testing methods and shown me helpful methods for running verification and maintenance procedures, which I have included in this booklet. I would like to thank both Mike and Bill for their contribution to this booklet and to my education.

It is my hope that the contents of this publication will give you an understanding of microwave technology that goes above and beyond just how to push the "start" button. I believe that becoming familiar with the workings of a microwave in relation to theory and concepts in histology will translate directly to improved efficiency and Patient care on the part of your Lab.

Zoe Ann Durkin
Zoe Ann Durkin
Histotechnology Manager
LabPulse® Medical

Table of Contents

Section VI – Reference Information

Terms

Polar – Molecules with a positive charge on one end and a negative charge on the other end. Microwaves cause polar molecules to align to the wave as it passes. Once the wave stops, the molecules go back to the direction they want. This occurs with every wave that passes thus the aligning and un-aligning of the molecules cause them to bump into each other which creates friction or heat. Examples of polar substances are water and alcohol.

Ramp time – Is the time in which the microwave is heating prior to reaching the desired set-point temperature. When the Time at Temperature (Time @) mode is used the timer starts counting at the end of the ramp time.

UL – Underwriters Laboratories

CAP – College of American Pathologist.

CSA – Canadian Standards Association

NRTL – Nationally Recognized Testing Laboratories (UL, CSA, ETL, etc..)

IVDD – In Vitro Diagnostic Directive (Europe)

Validation – as defined by CAP "A defined process by which a laboratory confirms that a laboratory developed or modified FDA-cleared/approved test performs as intended or claimed."

Verification - as defined by CAP "The process by which a laboratory determines that an FDA-cleared/approved test performs according to the specifications set forth by the manufacturer."

Watchdog Timer – a software timer that generates an error message if the set-point temperature is not reached within 10 minutes. Designed to prevent runaway heating of microwave contents if temperature probe is improperly installed or has failed.

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Also See

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Section VI – Reference Information

Microwave Q&A

Isn't 82°C too hot for tissue and paraffin?
Not with the vast majority of tissue types, and not for the relatively short time frames involved. Also, the tissue has already been fixed, dehydrated, and defatted; while you certainly don't want to burn or cook the tissue, 82°C isn't really extreme in this context. However, some finicky tissues like brain can develop clefts with excessive temperature (even if your water bath is too hot!); alternate lower-temperature protocols are available. As for paraffin, usually it's not a problem, but check with the manufacturer. If there's an issue it's probably with additives, not the paraffin itself.

Can I use zinc formalin in the microwave?
Doing so is not advisable. EBS strongly discourages microwaving formalin in any form; there are serious health-and-safety issues involved. However, we recognize that safe and effective alternatives to reagents like zinc formalin have yet to be proven in some cases, so formalin can be a "necessary evil." If you insist on using zinc formalin, make sure that the temperature probe is kept clean of zinc build-up or arcing may result. And NEVER breathe warm formalin fumes!

Why can't I use xylene in the microwave?
For starters, it produces hazardous vapors at room temperature, and gets worse when heated. Eliminating xylene should be a goal; it's now an officially recognized hazard to pregnant women and unborn babies. Next, it is as non-polar as paraffin, and wouldn't heat anyway. Finally, there is no value to using it a microwave, because isopropanol clears tissue in the microwave as well as, or better than xylene in any Processor.

We use "100% Dehydrate. This is the same as ethyl alcohol, right?
Not necessarily. For example, a commonly-used dehydrant is actually a blend of methyl, ethyl, and isopropyl alcohol. While it does an excellent job, it's not safe to assume that it's a "drop in" replacement for EtOH, and some "tweaking" of time and temperature may be necessary. Never make assumptions.

Can I re-use alcohol?
Sometimes. If you're not processing a lot of cassettes, it's OK; it's a question of contamination, not microwave exposure. Even with a full load, it's often OK to re-use once. Cloudiness can be a giveaway that it's time to discard (or recycle), but not always. It can be a sign of water and/or lipid contamination.

Alcoholic fixation produces different morphology and sometimes "crunchy" sections. Does this happen with use of ethyl and isopropyl alcohols in the microwave?
Not as long as the tissue been properly fixed before the alcohol steps. Ethyl is a dehydration step only, and isopropyl is a clearing step only.

When should I use vacuum?
Vacuum can be used for any paraffin step. Vacuum can be especially beneficial when processing thick (>4mm), fatty, or thick and fatty samples. It is used to help lower the boiling point of isopropyl alcohol remaining from the previous step, and assist its replacement by paraffin.

What is the proper amount of vacuum, and why?
Too little vacuum will result in inadequate infiltration; too much can result in tissue damage like clefts. 15" Hg is usually adequate, although up to 20" Hg can usually be used.

Can my microwave share a circuit with my paraffin pot?
Ideally, a laboratory microwave should have its own dedicated circuit, with nothing else plugged in. A paraffin pot is one of the worst things to plug into the same circuit, due to the high demand of its heating element. This can cause a severe drop in magnetron output of the microwave, producing inconsistent results, or trip the circuit breaker in the middle of a run.

When should I use agitation?
Almost always. Agitation promotes even heating of reagent, preventing uneven distribution of solutes, suspensions, etc. A good example is the pink meniscus ring that forms in microwave PAS staining procedures, when agitation is omitted. This layer rises to the top due to vaporization of SO_2, and agitation helps prevent this. Rarely, you may find a protocol that warns against the "damaging effects of bubbling," for example, but we have seen no evidence supportive of this claim and much evidence to the contrary.

Section I – Considerations and Tests

Objectives

Upon completion of this section the reader will be able to:
a. Consider the applicable features when purchasing a laboratory microwave
b. Incorporate the verification process for a new laboratory microwave
c. Perform the following tests:
   i. Microwave Transparency
   ii. Power Output Test
   iii. Temperature Measurement Calibration Check

Microwave Uses

Laboratory microwaves are multifunctional and used for more than just simply heating reagents. They may be used for a variety of procedures to improve sample quality, provide faster diagnostic turn-around times and greater efficiency. Although simple to operate, microwaves do require the technician or operator to pay attention to detail and follow operating protocols to ensure proper operation of the machine and to produce quality results. As a rule, laboratory equipment requires monitoring and should not be left unattended. With proper use, microwaves in the laboratory have the ability to lower consumable costs and reduce the use of hazardous materials along with the need for them to be hauled away for disposal. Some of the different applications that may be incorporated into the laboratory using microwave technology are:

- Fixation
- Antigen retrieval
- Special staining
- Tissue processing
- Decalcification
- Slide drying
- Deparaffinize & hydrate slides prior to staining using a xylene free, nonhazardous solution

Section I – Considerations and Tests

Selecting a Laboratory Microwave

When choosing a laboratory microwave, there are several key aspects to consider. For example, using a dedicated laboratory microwave instead of a kitchen microwave is essential. Kitchen microwaves state in their manuals "Do not use corrosive chemicals or vapors". They also state that they are specifically designed for food, not industrial or laboratory use. Therefore, kitchen microwaves are not CAP, CLIA or OSHA compliant when used for laboratory applications. Laboratory microwaves are designed for chemical exposure and to eliminate hot spots, unlike kitchen microwaves. In addition, they are vented to remove harmful and flammable vapors and the electrical systems are protected from exposure to those vapors. Here are some features that are essential for even the simplest application:

- External chamber venting of fumes
- Appropriate seal around the door to contain fumes while in use
- Insulation of electrical system from flammable vapors
- Waveguide antenna or stirrer to minimize hotspots
- Adequate chamber size to accommodate large containers and accessories
- Accurate timer & power settings
- Agitation
- Safety controls appropriate to application
- CLIA, CAP compliant

If tissue processing is a consideration, you will need:

- Temperature probe or sensor
- High precision temperature control (± 1°C or better)
- Short magnetron cycle time or continuous power
- A "time at temperature" mode that does not start the timer until the reagent reaches set point temperature
- Programmability
- Available vacuum, for thick and/or fatty samples

An appropriate microwave that has been selected for the laboratory will have been tested in the factory prior to being shipped. When a new microwave is received in the lab, the performance should be verified upon installation and before use. Along with the verification, laboratory microwaves should have preventive maintenance procedures performed routinely. The equipment must also be re-verified after:

- Major repairs
- Equipment is relocated

To test the performance of the microwave: use microwave transparent containers to run a power output test and to check temperature calibration, if appropriate. This will be the baseline for future readings which should be recorded monthly and maintained in Quality Control (QC) records.

Microwave Transparency

Microwave transparent materials are non-polar, which means they allow microwave energy to pass through without significant absorbing the energy, and therefore not heating up. These Materials are not necessarily optically transparent.

Testing for Microwave Transparency

Fill a glass container with approximately 50ml of water. Place in the microwave next to the (empty) container in question. Set microwave on maximum power and heat for one minute. If the new container is warm or hot, it is absorbing microwave energy; if it remains at or near room temperature, it is microwave transparent.

Use caution when handling any container that has been in a microwave. Even though a container may be microwave transparent, the heated solution inside can still heat the container by conduction, causing burns if touched.

NOTE: Containers with some amount of microwave absorbance may be acceptable, even desirable for specific applications that require heating of non-liquids or non-polar materials. Examples of these applications are paraffin infiltration or drying a rack of sectioned paraffin slides.

Section VI – Reference Information

Microwave Q&A

Should containers be covered, or uncovered?
With the exception of a pressure/vacuum vessel specifically designed for your microwave application, what's important is that you prevent pressurization. Containers with vented covers are ideal, but if not available, leave containers open. Paper towels can be used as a light covering to prevent splatter and to absorb moisture. Waxed paper helps to retain heat and moisture.

Our lab in Colorado is at a high altitude. Anything we need to know?
Solutions may require a slightly longer heating time, since water boils at a lower temperature.

I've heard that microwaves can "superheat" reagent. How?
This isn't exclusive to microwaves; it's possible with many heating methods when a solution is undisturbed. That's why boiling chips are used to prevent "bumping" (sudden, violent boiling of superheated liquid) when heating via sand bath or Bunsen burner. The best way to mitigate this risk in a Lab microwave is through the use of the agitation system

Terms

Additive fixatives - link to reactive groups of tissue. They may or may not become permanently attached.

Coagulants - precipitate proteins, changes sponge work of proteins into meshes through which paraffin can pass easily. A disadvantage is they alter the fine structure of tissue, causes more artifacts than non-coagulants due to the formation of artificial structures. An advantage is they penetrate tissue rapidly, strengthening protein linkages against breaking during processing.

Load – A microwave energy absorbing material (e.g. a water load). Insufficient, improper or no loads or improper containers (metal) will cause the magnetron or parts of the microwave's interior to overheat. A thermal switch will eventually turn the oven off so the magnetron may cool.

Magnetron - Is a specialized component that utilizes a strong magnetic field to create a circulating electron current, resulting in the conversion of electrical energy into microwave radiation. This radiation mechanically excites the water molecules in solutions or specimens resulting in an increase of their thermal activity, i.e., their temperature.

Microwave Transparent – refers to (non-polar) materials which do not absorb microwave energy and therefore are not heated in a microwave processor. For example, process containers must allow microwave energy to pass through without the container heating up significantly. Microwave transparent containers are not necessarily optically transparent.

Waveguide – a metallic duct of very specific dimensions that transports microwave energy from a magnetron to an entry point in the cavity of a microwave. A rotating antenna then distributes this microwave energy in overlapping patterns that provide even heating within the cavity, i.e., it reduces the formation of hot and cold spots.

NBF – Neutral buffered formalin

Non-additive fixatives - do not link to tissue. They act solely on basis of physical presence

Non-coagulants - These do not precipitate proteins, they increase the number of cross-linkages between reactive groups (form bridges). An advantage is they preserve fine structure

Non-Polar – Material that has covalent bonds. The electrons are shared evenly resulting in no net charge, so they remain neutral and are not affected by microwaves. Consequently there is no movement, hence, no friction or warming. Examples of non-polar substances are olive oil, paraffin and xylene.

Section VI – Reference Information

Objectives

Upon completion of this section the reader will be able to:

a. Gain informational facts about microwaves
b. Use appropriate terms relating to microwaves

Microwave Q&A

Do microwaves heat all material equally?
No. Microwaves heat polar material like water and alcohol, and don't heat non-polar material like paraffin and fats. (Some fats are actually emulsions or other associations with polar components, so they can heat.) Materials like Pyrex can be slightly polar and can absorb microwave energy. And metal can cause "arcing" (sparking).

My "microwave transparent" containers are white plastic. Is this right?
"Microwave transparent" isn't synonymous with "optically transparent." It means the material allows the passage of microwaves without converting a significant portion to heat. Many materials that we wouldn't think of as "transparent," like opaque white Teflon are, in fact, "microwave transparent."

I know never to put metal in a microwave, but are staples OK?
Yes, usually. Larger amounts of metal are to be avoided. With staples, do ensure that multiple staples aren't right next to each other, and that no staple is close to a metal temperature probe, or arcing can occur. Occasionally staples can overheat, however. Make very sure they stay immersed in solution to minimize this effect.

Can I use biopsy sponges in the microwave?
Doing so is not advisable. They tend to contain air, which can cause inconsistent results and tissue damage. If the specimens are tiny and there is a need to use biopsy sponges, presoak the sponges in water.

Why do we get different readings on the power output when different people perform the test?
When performing the power output test it is critical to be consistent. This means using exactly 1L of water at room temperature. If one person who performs the test measures exactly to 1L and the next person fills the container above or below the 1L mark it will skew the results. Also, if you use cold water one time and room temperature the next time the results will be different. You may get accurate results if everyone pays attention to details and runs the power output test the same each time.

I use a microwave all day; don't I need a radiation badge or lead apron?
No. Microwave radiation is not ionizing radiation like X-Rays. Microwave energy is more akin to radio waves, is not mutagenic, and effects are not cumulative. Equipment standards have been developed to assure safety, assuming that your microwave has been properly maintained, regularly inspected, and is not damaged.

My protocol calls for 82°C paraffin, but we can only pre-heat it to 60°C. Is it OK to heat the paraffin that extra 20°C in the microwave?
Yes, if one is using PolarHeat® Sheets (or other alternative paraffin heating technology), paraffin can safely be heated in the microwave. In cases where PolarHeat® Sheets are not used, paraffin heating in the microwave is generally not recommended. The reason paraffin should not be heated by conventional microwave heat is simple. Paraffin is non-polar and will not absorb microwave energy, therefore it doesn't heat in the microwave. If conventional microwave heating of paraffin is attempted, damage to the unit, accessories, and tissue samples can result, since they are absorbing all available microwave energy. When performing microwave infiltration of tissue samples, it is essential that paraffin is pre-heated to within several degrees of the proper temperature or one uses an alternative paraffin heating technology.

Section I – Considerations and Tests

The Automation Argument

Automation can be a great help to Histology Labs in their quest to improve efficiency, however, it is not always the answer. To determine what is best for your Lab, you should carefully identify your specific needs, then analyze and compare the features and capabiltiies of each and every technology and device that is available for the purpose. On more occasions that you might think, truly objective analysis will show that the convenience frequently offered by automated systems comes at a high cost in efficiency, which is made up of one or several of the following important areas of consideration.

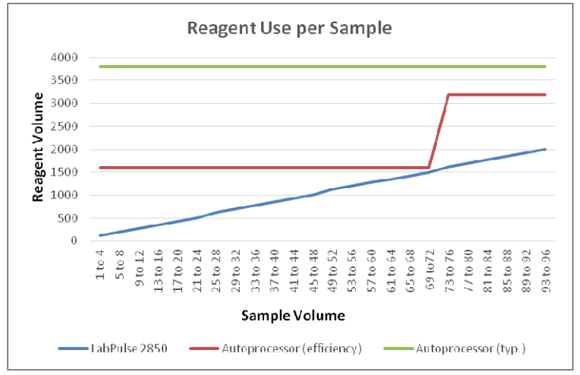
- **Efficiency**
- **Workflow**
- **Dependability**
- **Purchase cost**
- **Operating cost**
- **Flexibility**
- **Speed**

Why aren't EBS, LabPulse Microwave Processors automated?

SPEED & THROUGHPUT

EBS Microwave Processors are uniquely capable of:

- Processing 74 biopsies and <1mm Samples in under 20 minutes.
- Processing biopsies and >1mm Samples at a rate of 220 per hour
- Performing ultra-fast Microwave paraffin infiltration
- Decalcifications in under 2 hours



Samples	LabPulse Microwave	Conventional Processing
Small Biopsies	16-35 Min	2-4 Hours
1-3 mm Sections	1 Hour	4-6 Hours
Thick Fatty Sections	2-3 Hours	8+ Hours (Overnight)

Section I – Considerations and Tests

Power Output Test

Periodic monitoring of the lab microwave's power output is a good idea for many reasons; among them are assurance of consistency and repeatability, internal quality control, and compliance with laboratory accreditation programs such as that administered by the College of American Pathologists (CAP). There should be a written procedure for the Power Output test and the results should be logged and maintained in laboratory records. Some microwaves feature a built-in power output test, but many others do not, either way, it's important to understand what the maximum power is a function of. The maximum power consists of 3 elements (power line voltage, temperature of the magnetron and the age of the unit):

- *Power line voltage* (when operating at full power). If the microwave has been operating in one spot in the lab and is moved to a different location the Power Output Test should be performed. Variations in power line voltage can often occur from outlet to outlet in the same room.. This will affect the power output of the unit.
- *Maximum power is influenced by the temperature of the magnetron.* As equally important, when the power output test is performed it should be done at a time when the microwave has not been in use for several hours. Extended use of a microwave will cause the magnetron temperature to rise. This heating is a normal occurrence, but if the test is performed after the microwave has been used extensively ,the heat retained in the magnetron will skew the test results.
- *Maximum power is also influenced by some extent to the age of the unit.* Over time it is normal for the wattage of the microwave to go down as a result of magnetron wear. Tracking a unit's output power using the power output test will help you to determining when it is time to service or replace the unit. As a rule, a test result of 560 watts or more is acceptable.

The following procedure is for all microwaves, including those with built-in output testing, "power only" microwaves, and those featuring a temperature probe.

For the Power Output test use a microwave transparent container sufficient to hold 1 liter. The choice of the container <u>does</u> matter.

- Round is best
- The depth of the water should be approximately the width of the container.
  - A narrow, deep container may skew results low
  - A shallow, wide container may skew results high
- 1L- beaker
- Room-temperature water (exactly 1L for each test)
- Non-slip thermal mitts
- Calibrated Thermometer
- Log book

Power Output Test

The Power Output Test measures the maximum available power: To perform the test:

- Connect the unit to an appropriate power outlet. It is recommended that this be on a dedicated electrical circuit.
- Depress the power switch on and allow the vent system to come up to speed
- Set up the microwave timer for a 2 minute run
- Make sure the power output is set to 100% (high power setting)
- Place a glass or plastic container with 1L of room temp water in the center of chamber (ex. 19°C).
- Measure & record temperature of water. *It's important to always begin with the same water temperature to provide consistency to the test results*
- Close door & run microwave at full power for 2 minutes
- Open door & immediately note temperature (ex. 42°C)

To calculate the total watts subtract the initial temperature from the final temperature and multiply the result by 35.
(Final temp – Initial temp) X 35 = Power Output.
(42 - 19) X 35 = 805 Watts

Note: A more accurate measurement may be obtained by stirring the water to thoroughly mix it before taking each reading. The final reading must be taken very quickly.

Tracking power output is important, so it is recommended this test be performed and results be recorded monthly. This data can also be helpful to a Technician who is troubleshooting a unit in, case a service issue should arise.

Section V – Staining Protocols without Temperature Control

Pigments & Minerals Gomori's Iron

The Gomori's Iron Stain uses the Prussian Blue Reaction to demonstrate ferric ions in formalin-fixed paraffin tissue. The positive staining target or Prussian Blue reaction is achieved via treatment with an acid solution of ferrocyanide . Hydrochloric acid (HCl) is used to release iron bound to tissue and falcilitates demonstration with potassium ferrocyanide. Together HCl and potassium ferrocyanide creates the formation of a bright blue pigment called Prussian blue. Nuclear fast red solution provides an excellent counterstain. The resulting staining pattern of positive samples exhibits a blue staining of ferric ions (+3) with a pink/red counterstain demonstrating nuclei and cytoplasm.

Working Iron Staining Solution

10% aqueous potassium ferrocyanide 20ml
20% aqueous hydrochloric acid 20ml
Mix equal parts just before use. Use chemically clean glassware.

Important Notes and Hints

Discard working solution after use
Do not use metal forceps with the Iron solution
Rinse the slides well after NFR to prevent cloudiness

Iron Staining Procedure

Step	Detail	Power	Time
1	Prepare Working Iron Staining solution		
2	Deparaffinize & hydrate slides to DI water		
3	Stain in Working Iron Staining Solution *	Full	45 seconds
4	Rinse slides in DI water		5 minutes
5	Counterstain in Nuclear fast red **	Full	30 seconds
6	Rinse slides in tap water		5 minutes
7	Dehydrate, clear, coverslip		

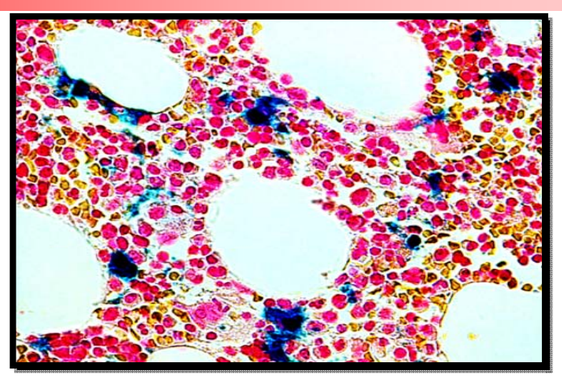
*Ferric ions in tissue turn blue during this step
**Sections should turn pink during this step

Iron Control Tissue

Spleen or liver tissue sections containing iron

Interpretation of Staining Procedure

Iron (ferric ions) — Bright Blue
Nuclei — Red
Cytoplasm — Pink



Section V – Staining Protocols without Temperature Control

Pigments & Minerals Fontana Masson

The Fontana Masson Stain is used to demonstrate argentaffin granules such as melanin. The steps involved are impregnation of argentaffin granules with silver nitrate, toning with gold chloride, removal of unreacted silver and gold with sodium thiosulfate (Hypo) and nuclear fast red is used as the counterstain. Positive staining of melanin is due to the argentaffin reaction (melanin is impregnated with silver and reduces it to a visible metallic state without the use of a reducing agent). The resulting staining pattern of positive samples exhibits black staining of target regions with a pink/red counterstain demonstrating nuclei.

Stock Silver Solution

10% Silver Nitrate	95ml
Ammonium Hydroxide	(drop by drop) Add to the 95mls of sliver nitrate until solution is clear, with no precipitate obtained (solution turns dark brown prior to clearing)
10% Silver Nitrate	4 to 5ml

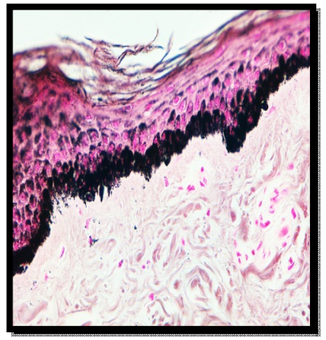
Add drop by drop to the above mixture to cause the clear solution to become slightly cloudy. Let solution stand overnight prior to use. Stock solution is stable for approximately 1 month. Store the stock silver solution in the refrigerator in a dark bottle.

Working Silver Staining Solution

Stock Sliver Solution	12.5ml
DI water	37.5ml
Filter	

Fontana Masson Staining Procedure

Step	Detail	Power	Time
1	Prepare Working Silver Staining solution		
2	Deparaffinize & hydrate slides to DI water		
3	Place slides in Silver nitrate solution	Full	45 seconds
4	Agitate and let stand in hot solution		10 minutes
5	Rinse slides well in DI water		4 changes
6	Tone in Gold chloride		5 minutes
7	Rinse slides well in DI water		2 changes
8	Place slides in Sodium thiosulfate	Full	30 seconds
9	Rinse slides well in tap water		
10	Counterstain in nuclear fast red *	Full	30 seconds
11	Rinse slides well in tap water		5 minutes
12	Dehydrate, clear, coverslip		



* Sections should turn pink during this step

Fontana Masson Staining Procedure — Interpretation of Staining Procedure

Skin or small intestine

Melanin & Argentaffin Granules — Black
Nuclei — Pink

Section I – Considerations and Tests

Temperature Measurement Calibration Check

The temperature measurement system is calibrated at the factory to be within +/- 1 degree of the actual temperature as measured with a certified thermometer. It is recommended to perform the temperature measurement calibration check in the laboratory bi-annually. If the difference is greater than +/- 2 degrees, the unit needs to be re-calibrated or repaired as necessary. Below is the procedure to check the temperature measurement.

- A 1L microwave-transparent container that is taller than it is wide
- An accurate hand-held thermometer that is calibrated in one degree increments from 0-100°C
- Power-up unit and wait for the Welcome screen to appear
- Set up a process, or a process step, for heating a container of water to 100°C.
- Depending on the model that you have, move to the first screen (usually the Start Screen) that displays the measured temperature but does not run the magnetron heating system.
- Place a container of water at or below room temperature (a 1L load is desirable) in the microwave.
- Place the microwave temperature probe in the container of water which is at room temp (~20°C) or lower. Try to keep the probe tip about halfway through the water.
- Place the tip of the thermometer (calibrated from 0-100°C) in contact with the tip of the temperature probe.
- This physical contact insures that they are sensing the same volume of solution for this comparative temperature test.

Record the displayed microwave temperature reading and the reading taken from the thermometer.

Microwave Display Low Temp _____
Thermometer Low Temp _____

Remove the thermometer and heat the container of water to boiling, or near boiling.

Be sure to leave the microwave probe in the water if you heat the container using the microwave itself (you could use hot water from another source if desired).

Repeat the temperature reading process as described earlier. Keep in mind that the temperature of the water will fall rapidly this time, so it is important to try to get the readings as closely together in time as possible.

Microwave Display High Temp _____

Thermometer High Temp _____

The temperature displayed by the microwave should be within +/- 1 degree of the "actual" temperature as measured with the calibrated thermometer.

If the difference is greater than +/- 2 degrees, the unit needs to be re-calibrated or repaired as necessary.

If a test result is questionable, especially at the high temperature end of the range, a re-test is recommended.

Section I – Considerations and Tests

Verifying Protocols and New Microwaves

It is a requirement, as well as good practice to validate all new procedures or programs and verify equipment in the laboratory. In some cases the validation or verification is performed to duplicate an existing procedure or incorporate one that has been acquired from another lab to incorporate into your lab. This is done by comparing the old and the new methods and evaluating the results. Sometimes the process is done through trial and error since there are variables such as differences in the types of equipment and reagents. Upon completion of the testing the final results are presented to a Pathologist or lab medical director for approval.

When validating new procedures or verifying Instruments, record keeping is important. An example of the process is to run some test slides or cassettes (depending on what is being tested), made up of several types of tissues. If the Instrument from which the Protocol was derived is still available, run two sets of samples, one on the original Instrument and a duplicate set on the new one. For the validation or verification documentation sheet, start with a chart that lists all the reagents, time, temperature, and power settings used. Keep in mind, when testing two microwaves, a lower power setting may be needed on the new unit to produce the desired results.
Record the following:

1. Date and test being performed
2. Number of cassettes in the carousel or slides in each rack
3. Time spent in each step/reagent
4. Power setting
5. Temperature
6. Results
7. The technician's initials performing the testing, and pathologist who is assessing the final results or slides upon staining

Next, record any problems and include the observations. For example, if processing tissue and the specimens are brittle upon embedding this could indicate that the specimens are over processed or were *not* left in fixative long enough. First try to pinpoint the problem then adjust the times, power and possibly temperatures accordingly.

There may be the need to make changes to the protocol, run additional specimens, and record the results. Continue doing this, until the desired quality and results are achieved. This now becomes the new processing schedule. Keep in mind, that there should be different processing protocols for different sizes and types of specimens. For example, there are different protocols for biopsies verses large specimens and using the appropriate one for the specimen size will ensure quality results.

Knowing how to adapt old microwave protocols to new equipment can be an important skill. Some old protocols were developed for an obscure "niche," repurposed for mainstream applications, then used daily for many years. This happens in countless clinical labs all across the Country. Unfortunately, many of those old protocols were developed for use with kitchen microwaves, which lacked features now known to be essential in a laboratory environment. Due to the variability of consumer-grade microwaves, and their inadequate features, many creative work-arounds or modifications were developed that appeared to work for the lab with the particular non-laboratory grade microwave that was in use. Unfortunately, those work-arounds often resulted in additional processing steps, questionable settings, odd instructions or all of the above. It is often impossible to track down the Technician who developed the Protocol to ask "why?", which means sorting out the necessary steps from unclear or unnecessary steps can be extremely difficult or impossible.

When faced with the task of updating an old protocol, try and determine:

Context: What are they really trying to achieve and why?

Wattage: What is the wattage of the oven in question?

Power setting: Is it specific (e.g., 60%), or something almost useless like "Medium?"

Times: What times are specified, and why?

Temperature: What temperatures are specified, and why? Are they trying to maintain a particular temperature, or achieve a temperature only at the end of the process?

If you arrive at a point where you have reached the limit of your frustration, please remember that yours is most likely not the only Lab that requires a protocol for the application at issue. Check the list of pre-defined protocols in your new Instrument and you will most likely find one for that very application that is simple, straightforward and has been meticulously validated.

Section V – Staining Protocols without Temperature Control

Connective Tissue Masson Trichrome

The Masson Trichrome Stain is used to identify connective tissue in paraffin embedded tissue samples. The Trichrome Stain uses Bouin's Fluid as a mordant and to decrease pH levels in the tissue to enhance subsequent reagent-protein interaction. The Biebrich Scarlet-Acid Fuchsin Stain is used to identify plasma regions and the Aniline Blue Stain demonstrates collagen. The resulting staining pattern exhibits red staining of muscle fibers and cytoplasm, blue staining of collagen, and blue/black staining of nuclei.

Working Weigert's Staining Solution

Weigert's Hematoxylin Solution A	25ml
Weigert's Mordant Solution B	25ml

Masson Trichrome Staining Procedure

Step	Detail	Power	Time
1	Deparaffinize & hydrate slides		
2	Mordant slides with Bouin's Fluid	Full	15 seconds
3	Agitate and let stand in hot solution		10-20 minutes
4	Rinse slides well in running tap water		5 minutes
5	Stain in Working Weigert's Hematoxylin solution		15 to 20 minutes
6	Rinse slides well in running tap water		5 minutes
7	Stain in Biebrich Scarlet-Acid Fuchsin	Full	20 seconds
8	Rinse slides well in DI water		
9	Treat with PMA/PTA	Full	20 seconds
10	Agitate and let stand in hot solution		2 minutes
11	Rinse slides well in DI water		
12	Stain slides with Aniline Blue	Full	20 seconds
13	Agitate and let stand in hot solution		1 minute
14	Rinse slides in Water		
15	Place in 1% Acetic Acid		4 minutes
16	Dehydrate, clear, coverslip		

Masson Trichrome Control Tissue

Skin, small intestine uterus, muscle

Interpretation of Staining Procedure

Muscle & Cytoplasmic Regions — Red
Collagen — Blue
Nuclei — Black

Section V – Staining Protocols without Temperature Control

The following protocols were tested using LP2250, 100% power, agitation on, LabPulse Medical's plastic Coplin jar with vented lid, with slides immersed in reagent (~ 40 ml).

Connective Tissue Gomori Trichrome

The Gomori's Trichrome Stain Kit is used to identify connective tissue in paraffin embedded tissue samples. The Trichrome Stain uses Bouin's Fluid as a mordant and to decrease pH levels in the tissue to enhance subsequent reagent-protein interaction. The Trichrome Stain is used to identify plasma regions (chromotrope 2R) as well as collagen (light green SFY) within the tissue sample. The resulting staining pattern exhibits red staining of muscle fibers and cytoplasm, green/blue staining of collagen, and blue staining of nuclei.

Gomori Trichrome Staining Procedure

Step	Detail	Power	Time
1	Deparaffinize & hydrate slides		
2	Stain slides with Harris Hematoxylin		10 minutes
3	Rinse slides well in running tap water		5 minutes
4	Mordant slides with Bouin's Fluid	Full	15 seconds
5	Agitate and let stand in hot solution		10 minutes
6	Rinse slides well in running tap water		5 minutes
7	Stain slides with Trichrome Stain	Full	15 seconds
8	Agitate and let stand in hot solution		5 minutes
9	Rinse slides in Water		
10	Dehydrate, clear, coverslip		

Gomori Trichrome Control Tissue

Positive Controls: Skin, small intestine uterus, muscle

Interpretation of Staining Procedure

Muscle & Cytoplasmic Regions — Red
Collagen — Green
Nuclei — Blue

Section I – Considerations and Tests

Verifying Protocols and New Microwaves

At LabPulse Medical we often are faced with something like this: "I have a very specific protocol that I want to use with my new lab microwave: Bring slides to boiling in a plastic Coplin jar. We use plastic containers that hold 24 slides, placed in vertically. We fill the container with 10mM Citrate pH 6.0 buffer past the sections, almost to the top, leaving enough room so that the buffer will not boil over. We bring the slides to boiling. It takes 3 minutes on high power for us to reach this point. We want the temperature to just reach boiling. We then keep them at a sub-boiling temperature in the microwave for 10 minutes. To do this, we heat them at 30% power for 10 minutes (to keep them at 98 - 100°C). We then remove the container from the microwave, leaving the top slightly ajar, we let them cool on the bench top."

This "very specific" protocol is, in fact, anything but when it comes to the microwave:
Container: They recommend a Coplin jar, but then talk about 24 slides.
Time and temperature: The desired temperature may be 99°C, although 100°C, "boiling," "sub-boiling" and 98°C are all mentioned. Furthermore, we're not talking about boiling water, but buffer. What is the boiling point of the buffer? The first 3-minute setting appears to be merely "ramp up" time, the time required to reach the buffer's boiling point.
Bottom line: apparently this microwave does not have true temperature control, and they have worked around this by adjusting the power.
Power: Since we don't know the wattage of the microwave used, we cannot determine the 30% power component to maintain the 98 - 100°C for 10 minutes.

Here's how to approach this particular protocol. Primarily, work to eliminate as many variables as possible, run controls in the old unit if possible (and/or via "traditional" means), then run tests and "tweak" the protocol in the new microwave as necessary.

First, unknowns and contradictions aside, what are they trying to achieve? Overall, everything can probably be reduced to the following*: it's critical that the sections remain immersed in buffer that's just shy of boiling for 10 minutes*. That's it. The first 3-minute step appears to be simple ramp-up time; since a good lab microwave with temperature control and a "time at temperature" mode can compensate for ramp-up time, this can (probably) be safely disregarded. Power appears to be important only insofar as it's being adjusted to maintain (probably) 99°C, assuming that's almost at the boiling point of the buffer. Since a modern microwave processor measures temperature and adjusts its power output to achieve a given temperature, power per se is (probably) unimportant during the run. During ramp-up, however, excessive power might cause tissue damage, so it's a good idea to start off with low to moderate power settings. Ignore the Coplin jar recommendation and use an appropriate container to hold 24 slides. Finally, it's (probably) safe to assume that agitation was unavailable; while agitation is usually recommended, in the initial tests omit it to try and be true to the original protocol. Agitation can always be added later for comparison.

If there is access to the old microwave, establish its wattage and calculate proper power settings. In this case, it's an unknown, so assume the original microwave was an 800-watt model. 30% of 800 watts is 240 watts. If the new lab microwave is 1000 watts, 24% power gives the same 240 watts. (Since we're dealing with an unknown, "trial and error" comes into play here.)

For this protocol, first run controls and use non-critical samples, try 10 minutes @ 99°C, start timer at set-point temperature, 24% power, agitation off. To replicate the original protocol, apply 80% power during ramp-up rather than 24% (this may require two preset steps).

Section I – Considerations and Tests

Verifying Protocols and New Microwaves

Other variables possibly encountered in old protocols could be:

Carousel: If the lab microwave has one, fine; if not, it probably uses an antenna or "waveguide stirrer," or multiple magnetrons instead: any one of these produce the desired results. If none of these features are present, it's probably not a laboratory microwave and uneven heating is a fact of life.

Dummy load: If it's specified, use it, for example a load may be, "500 ml water in vented, covered container, placed at right rear corner of chamber."

When replicating protocols developed for microwaves without a temperature probe, putting the new lab microwave into "power" mode should help. For example, special stain protocols often require less critical temperature control than microwave fixation, unmasking, and tissue processing. Or there may be an unmasking protocol that was designed for a pressure cooker with no provision for a temperature probe. Usually an ending temperature is specified, or can be found with some research.

So, via trial and error, initially using water and then moving on to reagent and *non-critical* samples, put the laboratory microwave in "power control" mode and come up with a power setting that provides the endpoint temperature at the proper time.

Then either continue to run the protocol in "power" mode, or if a probe can be used, change to "temperature" mode, retain the power setting just tested, and program the endpoint temperature. This should ensure that the desired temperature would not be exceeded.

Below are two examples of QC records to document validation or verification of changes in equipment, methods or procedures:

Validation/Verification of Methods—Documentation Sheets

Method to be validated: _____
Reason for validation: _____
Procedure for validation: _____
Tech: _____ Date: _____
Reviewed/Approved by: _____ Date: _____
Director Approval: _____ Date: _____

Or you may prefer this type to use or modify:

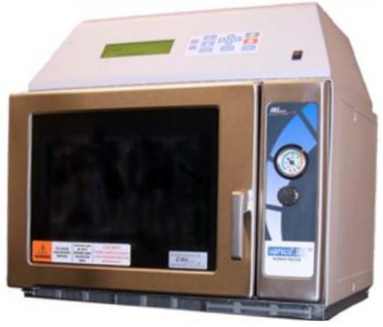
New

☐ Reviewed ☐ Revised _____ _____

☐ Reviewed ☐ Revised _____ _____

☐ Reviewed ☐ Revised _____ _____

☐ Reviewed ☐ Revised _____ _____



Tech: _____ Date: _____
Reviewed/Approved by: _____ Date: _____
Director Approval: _____ Date: _____

Section V – Staining Protocols without Temperature Control

GMS Staining Procedure

Step	Detail	Power	Time
1	Prepare Working Staining solution		
2	Deparaffinize & hydrate slides to DI water		
3	Oxidize in 5% Chromic acid	Full	15 seconds
4	Agitate and let stand in hot solution		15 minutes
5	Rinse slides in tap water		
6	Place slides in 1% Sodium bisulfite		1 minute
7	Rinse slides in tap water, then DI water		4 changes
8	Stain in Working Methenamine Sliver Sol	Full	30 seconds
9	Agitate and let stand in hot solution		1 minute
10	Microwave again	Full	15 seconds
11	Agitate and let stand in hot solution		5-15 minutes
12	Place slides in heated DI water to check silver impregnation with the microscope *		
13	Rinse slides in DI water		4 changes
14	Tone in 0.2% Gold chloride		5 minutes
15	Rinse slides in DI water		2 changes
16	Place slides in 5% Sodium thiosulfate		3 minutes
17	Rinse slides well in tap water		
18	Counterstain in 1% light green		30 seconds
19	Go directly into 95% alcohol, dehydrate, clear, coverslip		

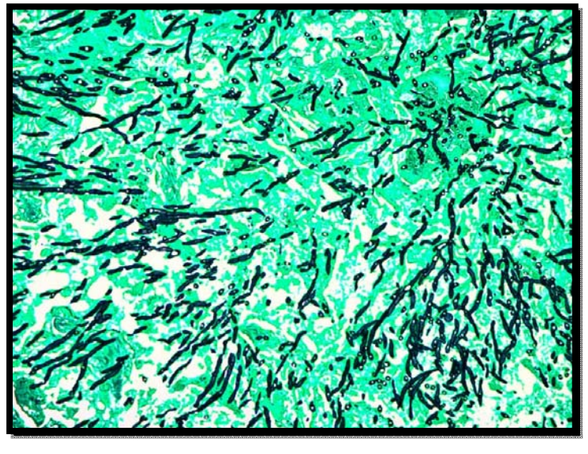
* Grossly the sections will be the color of a paper bag

GMS Control Tissue

Tissue sections containing fungus

Interpretation of Staining Procedure

Fungal Walls — Black
Mucin — Taupe to Dark Gray
Background — Green



Section V – Staining Protocols without Temperature Control

Fite's

The Fite's Staining Method works the same as the AFB with the exception of paraffin oil to deparaffinize. The resulting staining pattern of positive samples exhibits red staining of target (acid-fast positive) bacterium with a green (negative) background stain.

Fite's Staining Procedure

Step	Detail	Power	Time
1	Deparaffinize with xylene-petrolatum mixture		12 minutes
2	Second xylene-petrolatum mixture		12 minutes
3	Drain slides and wipe off excess oil, blot well*		
4	Stain slides with Carbol-Fuchsin	Full	20 seconds
5	Rinse slides in running tap water		5 minutes
6	Differentiate with Acid Alcohol until runoff is clear		
7	Rinse slides in running tap water		5 minutes
8	Counterstain in 1% light green		1 dip
9	Go directly into 95% alcohol, dehydrate, clear, coverslip		

* It is important to remove oil

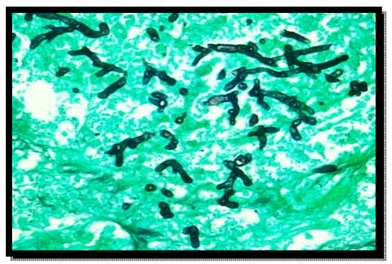
Control Tissue	Interpretation of Staining Procedure
Slides containing acid-fast bacterium such as Mycobacterium or Nocardia	Acid-Fast organisms — Red Background — Green

Grocott Methenamine Silver (GMS) Staining Procedure

The GMS Stain is used to demonstrate fungus in formalin-fixed paraffin tissue. Chromic acid is used to oxidize the fungal walls. Sodium bisulfite removes traces of chromic acid. A buffered Methenamine Silver solution is very alkaline and impregnates the oxidized fungal walls. Gold Chloride is used to tone the fungi from a brown/black color to purple/black. Sodium thiosulfate fixes the silver staining in the fungal walls and removes unreacted silver and gold to prevent nonspecific staining. Light green provides an excellent counterstain. The resulting staining pattern of positive samples exhibits a black staining of target regions with a light green background.

Working Methenamine Silver Solution

3% Methenamine	20ml
10% Silver Nitrate	1ml
Add 21ml of the following solution to the above (Discard the remaining 6ml)	
5% Sodium Borate	2ml
DI water	25ml



Section I – Considerations and Tests

Microwave Tips—Always

- use a laboratory microwave for laboratory work
- connect chamber venting system to external fume removal system
- use manufacturer-approved containers
- use vented or uncovered containers when not using vacuum
- handle containers with potholders or thermal mitts
- use a fume hood to work with hazardous reagents such as formalin
- run controls

Microwave Tips—Periodically

- use a microwave leakage detector to check for leakage
- inspect/clean chamber and hinges
- inspect/clean air intakes and filters (if so equipped); replace filter(s) as necessary
- inspect/clean temperature probe (if equipped) and probe mount
- inspect/clean door seal (if so equipped) and area where door and cabinet meet
- check samples (staining, processing, unmasking, etc.) against controls

Microwave Tips—NEVER...

- use a consumer-grade microwave for laboratory work
- cover non-vacuum or pressure approved containers tightly
- operate the microwave without a load
- use metal accessories inside the unit
- heat food in a microwave oven used for laboratory procedures
- breathe warm reagent fumes, especially formalin

Section II – Theory & Concepts

Objectives

Upon completion of this section the reader will be able to:

a. Relate the concepts of fixation, dehydration, clearing and infiltration to microwave processing of tissue specimens
b. Apply processing principals using microwave technology
c. Compare the steps in traditional tissue processing to those in microwave tissue processing

Processing Principles

Tissue processing prepares the specimen to be infiltrated with paraffin wax. It involves 4 main steps for all types of processors:

1. Completion of fixation
2. Dehydration
3. Clearing
4. Paraffin infiltration

Fixation

Fixation stabilizes the proteins and is an important step in preparing a high quality microscopic slide for diagnosis. Specimens should be thoroughly fixed prior to dehydration and there are a couple of considerations to be aware of to insure fixation is complete.

One is the rate of fluid penetration into the tissue and the amount of fixative in the container verses the size of the tissue. Smaller specimens such as a 4mm piece of tissue in a greater volume of fixative will allow neutral buffered formalin (NBF) to penetrate relatively fast. However, another factor to consider is the binding time of NBF. NBF is a non-coagulant and it works by increasing the number of cross links or by forming bridges between reactive groups. These links occur slowly and enough time must be allotted for formalin to bind with the tissue in order to stabilize the proteins. Another factor is water and alcohol may remove the links because formalin is an additive fixative. Therefore, fixation must be complete prior to dehydration to retain the formation of cross links between the tissue and NBF. Otherwise, the alcohol in the dehydration steps will complete the fixation process and alcohol's mechanism of action is via coagulation which precipitates or gels proteins thus altering the fine structure of the tissue.

Contrary to popular belief, over fixation of tissue takes a long time to occur, therefore the focus here will be on under fixation of specimens. If the tissue has not had enough time in a non-coagulant fixative to create cross linkages the final product may result in hard and brittle tissue which is often mistaken as over-processing the tissue. In this case the hard or brittle tissue occurs by going into the dehydrating alcohols too soon and the alcohol continues the fixation process instead of the desired noncoagulant fixative, i.e. NBF. Alcohol works differently than formalin, it is a coagulant, a non-additive fixative and its characteristics are hardening and shrinking tissue excessively. Alcohol alters the fine structure within the tissue due to being a coagulant because it precipitates proteins or gels them within the tissue.

Section V – Staining Protocols without Temperature Control

Microorganisms **Acid Fast (AFB)**

The AFB procedure is used to stain acid-fast bacterium in tissue samples. The acid-fast staining method uses Carbol-Fuchsin Stain (Ziehl Neelsen) to target acid-fast bacterium by adhering to their lipid-rich cell walls. The acid alcohol decolorizing agent removes unbound stain from the tissue and enhances the distinction between the counterstain and acid-fast stained bacteria. The resulting staining pattern of positive samples exhibits red staining of target (acid-fast positive) bacterium with a green (negative) background stain.

Reagents

Carbol-Fuchsin Stain (Ziehl Neelsen)
Acid Alcohol Decolorizer
1% Light Green Stain

AFB Staining Procedure

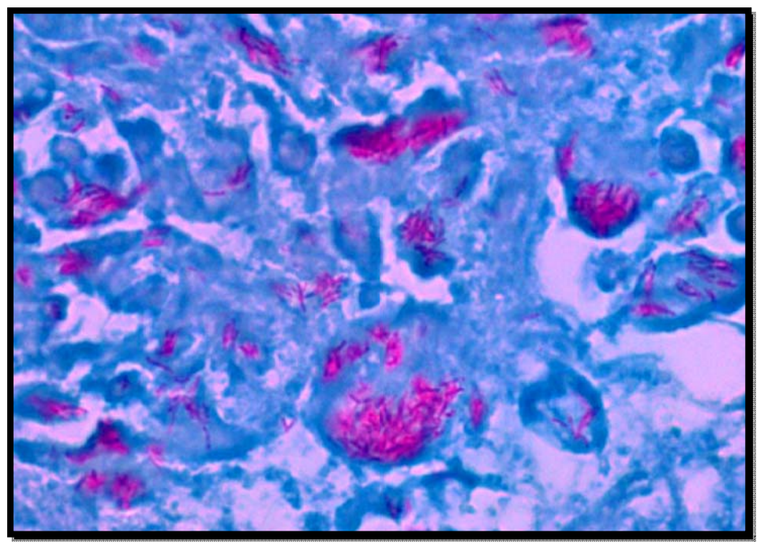
Step	Detail	Power	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Stain slides with Carbol-Fuchsin	Full	20 seconds
3	Agitate and let stand in hot solution		3-5 minutes
4	Rinse slides in running tap water		5 minutes
5	Differentiate with Acid Alcohol Decolorizer until slide runoff is clear		
6	Rinse slides in running tap water		5 minutes
7	Counterstain in 1% light green		1 dip
8	Go directly into 95% alcohol, dehydrate, clear, coverslip		

AFB Control Tissue

Slides containing acid-fast bacterium such as Mycobacterium or Nocardia

Interpretation of Staining Procedure

Acid-Fast organisms — Red
Background — Green



Section V – Staining Protocols without Temperature Control

Objectives

Upon completion of this section the reader will be able to:

a. Reduce time of special stains and turnaround time.
b. Apply staining protocols without the use of a probe to control the solution temperature
c. Adapt traditional staining methods to ones using microwave technology

Staining Procedures using Microwaves without a Probe

The following protocols were tested using LP2250 and LP1850, using LabPulse Medical' plastic Coplin jars with vented lid, with slides immersed in reagent (~ 40 ml) or plastic slide mailers with the lid open, slides immersed in reagent (~ 20 ml) may be used. Times need to be adjusted when using different volumes of solutions. The LP2250 has a built-in agitation mechanism that may be used during staining to eliminate hot stops in the solutions.

Carbohydrates Periodic Acid Schiff (PAS)
PAS Staining Procedure

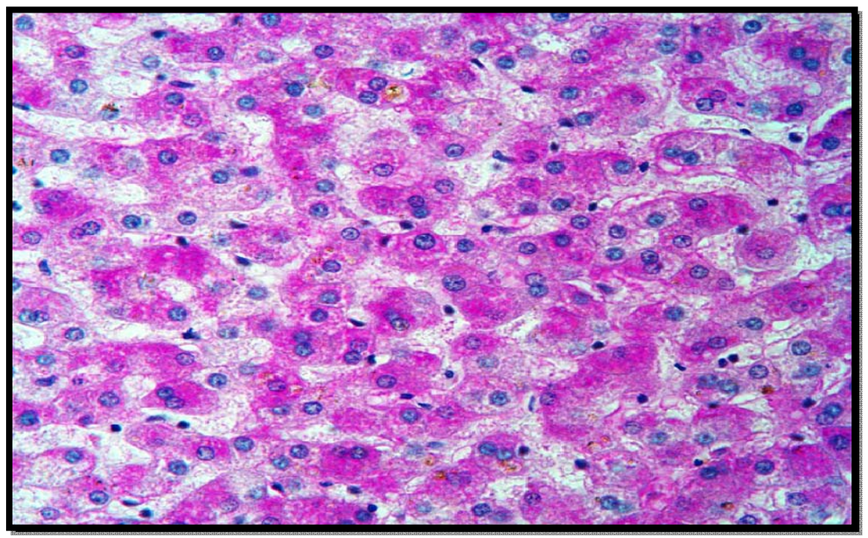
Step	Detail	Power	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Oxidize with Periodic Acid Solution		5-10 minute
3	Rinse in DI water		5 minute
4	Stain with Schiff reagent *	Full	45-60 seconds
5	Rinse slides in running tap water **		5 minutes
6	Counterstain with light green		1 dip
7	Go directly into 95% alcohol, dehydrate, clear, coverslip		

* Positive sections should turn light pink

** Sections should turn dark pink during this step

Interpretation of Staining Procedure

Glycogen — Rose to Purplish Red.
Mucopolysaccharides, Fungal Walls — Rose to Purplish Red.
Nuclei, Background — Green



Section II – Theory & Concepts

Completion of Fixation

Factors to consider when ensuring complete fixation.

1. Allow enough time for the penetration rate of fixatives especially if cross linking needs to occur.

2. Ensure the correct volume ratio of tissue to fixative was used. Remember the tissues contain water and will dilute the fixative. Therefore, a large enough volume of fixative is required to offset this from happening. (At least 10-20 volumes of fixative should be used for every volume of tissue).

3. Size of tissue samples, is it small or thin enough? Fixative will penetrate more quickly into smaller pieces of tissue.

Avoid Over and Under Fixation:

1. Formalin: over or under fixation causes shrinkage and hardening of tissue.
2. Aldehydes in general: over fixation causes inhibition of enzyme activity and under fixation will inhibit cross linking.

Formalin Alternatives

Many labs want to eliminate or reduce their use of Formaldehyde because it is dangerous, a carcinogen and a combustible liquid. Formaldehyde is a skin and respiratory sensitizer, it is toxic and may affect the CNS, eyes, heart, kidneys or liver. Some labs are using a glyoxal based fixative which is a less toxic alternative, it takes less time to fix tissue, and glyoxal based fixatives are more compatible with IHC staining.

Microwave processing of tissue using Preserve™, LabPulse Medical's glyoxal-based fixative, can mean the process takes under 2 hours from taking the specimen from the patient to embedding the tissue in paraffin. The tissues may be received fresh, or fixed in most common fixatives. Initial fixation for 30 minutes at room temperature in LabPulse Medical' Preserve™, Anatech's Prefer™, or Shandon Glyo-Fixx™ is recommended.

> Anatech's (1998) study found the following:
>
> Preserve is compatible with a wide range of fixatives, including Preserve, NBF, and zinc formalin, so nearly any specimen received in your lab can be treated with this program. Final appearance of tissues will depend upon how long they have been in the primary fixative. Those fixed for hours in NBF, for instance, will look like high quality formalin fixed specimens. The same is true for the other fixatives. Tissues only lightly fixed, or unfixed, will resemble those exposed to Preserve for several hours. Red blood cells will be lysed, but nuclear chromatin patterns will be very sharp, cell membranes will be conspicuous and immunoreactivity will be retained very well. (p. 2)

Dehydration

Dehydration after fixation is necessary to remove water from the tissue because water is not miscible with most clearing agents and paraffin. The dehydrating agents used in tissue processing must be compatible with water and also with the clearing agent. As with fixation, dehydration must be complete to ensure a high quality microscopic slide for diagnosis. If dehydration is incomplete the tissue retains water and prevents the clearing agent from penetrating the tissue completely and then in the next step, there will be improper infiltration of paraffin. Incomplete dehydration may be apparent to the technician in a couple of ways; one is after the blocks have been sectioned and the other may be during sectioning. The signs are:

1. Once an incompletely dehydrated specimen is embedded and sectioned, the tissue may sink or depress into the paraffin block upon standing.
2. The other sign of incomplete dehydration is while sectioning. The section may disintegrate on the water bath when it is laid out as a ribbon. To obtain a section with this scenario, turn the temperature of the water bath down.

The majority of dehydrating agents are alcohols. In traditional tissue processing dehydration occurs by using several changes of alcohol, beginning with a lower percent such as 70% or 80% alcohol, moving through changes of 95% alcohol up to additional changes of 100% alcohol to facilitate the removal of water. Using microwave technology the dehydration step has been reduced to one or two changes only and begins with 90% or more often 100% alcohol. This reduces the time and amount of reagents used in the process.

Section II – Theory & Concepts

Dehydration Principles

General principles:

1. The purpose is to remove aqueous fluids in the tissue. Most fixatives are water based and are not miscible with the embedding media and clearing agents.
2. Reagents used in the dehydration process must be miscible with water and also with the clearing agent. The majority of dehydrating agents are alcohols.
3. Mode of action:
   a. Dehydrants that are hydrophilic draw out water
   b. Dehydrants may act to dilute aqueous tissue fluids.

Clearing

Clearing the tissue during processing prepares it for paraffin infiltration, and is necessary because, as mentioned above, most dehydrants are not miscible with paraffin. A commonly used clearing agent in traditional tissue processing is xylene. Xylene is an aromatic hydrocarbon that is bad for the environment and is a health hazard which needs to be hauled away for waste removal. Additionally, xylene is non-polar and should never be used in the microwave.

It is good laboratory work practice to decrease exposure of hazardous material. One way to do this is to alter the manner in which the technique is performed, such as using a fixative alternative or eliminating the use of xylene in tissue processing via microwave processing of specimens. When using microwave technology xylene is replaced with isopropanol alcohol which is better for the environment, staff and laboratory budget.

Infiltration

Paraffin infiltration removes the clearing agent and facilitates embedding and sectioning by giving support to the tissue in order to produce thin sections during microtomy.

The use of vacuum during this step is beneficial and reduces the time in the infiltrating paraffin as well as aids in removing air bubbles from the tissue while in the tissue processor. The use of vacuum during paraffin infiltration also helps in preventing tissue hardening and vacuum is recommended for tissue specimens such as lung, heart, spleen, bloody organs, decalcified bone and skin.

Tissue Processing Considerations

There are a variety of tissue processors to choose from. Considerations in choosing processors include: turnaround time, type of specimens, workload, and how many cassette the processor holds, laboratory space, budget, and versatility of processor.

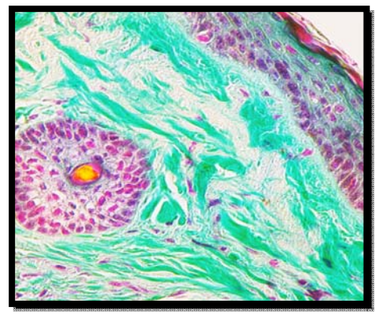
Tissue processing is rapid and efficient with Microwave Technology. This fast and easy use of microwave technology processes tissue without the use of xylene as the clearing agent. Microwave processing incorporates processing anything from rush biopsies to routine work in a reduced time frame and in some cases, the potential of eliminating longer overnight runs without the loss of quality.

Section IV – Staining Protocols with Temperature Control

Connective Tissue Gomori Trichrome

The Gomori Trichrome method is a time-saving procedure with color differentiation similar to Masson's. This method distinguishes connective tissue structures in paraffin embedded tissue samples. This staining method uses Bouin's Fluid as a mordant and to decrease pH levels in the tissue to enhance subsequent reagent-protein interaction. The mordant is an important step and the slides may need extra time in Bouins to produce red staining of muscle. If the muscle is staining purple then the slides have not been left in Bouin's long enough and the samples should be allow to stand in the hot Bouin's solution for 5 minutes prior the step 3, rinsing in running water. The Trichrome Stain identifies plasma regions and muscle by using chromotrope 2R and collagen with light green. The resulting staining pattern exhibits red muscle fibers and cytoplasm, green collagen, and blue nuclei.

Gomori Trichrome Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate		
2	Mordant in Bouin's	@ 60°C	5 minutes
3	Rinse in running water until yellow disappears		~5 minutes
4	Stain with Gill 3 hematoxylin	@ 60°C	5 minutes
5	Rinse in running water		1 minute
6	Stain with Gomori Trichrome	800 watts	15 seconds
7	Incubate or let stand in Gomori solution		1 minute
8	Rinse in 1% Acetic Acid		0.5 -1 minutes
9	Dehydrate, clear, coverslip		

Gomori Trichrome Control Tissue

Striated muscle, Appendix, Fallopian tube, Uterus

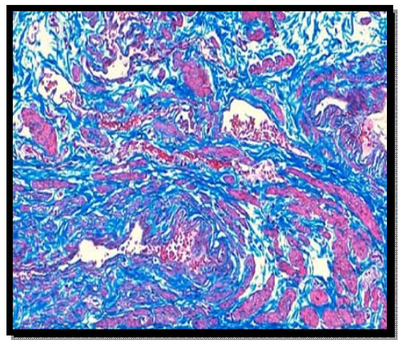
Interpretation of Staining Procedure

Muscle fibers, cytoplasm — Red
Collagen — Green
Nuclei — Blue

Connective Tissue Masson Trichrome

The Masson Trichrome staining method is used to distinguish certain connective tissue components in paraffin embedded tissue samples. As with the Gomori Trichrome Staining method Masson's Trichrome uses Bouin's Fluid as a mordant and to decrease pH levels in the tissue to enhance subsequent reagent-protein interaction. The Biebrich Scarlet-Acid Fuchsin Stain is used to identify muscle and plasma regions and the Aniline Blue Stain demonstrates collagen. The resulting staining pattern exhibits red muscle fibers and cytoplasm, blue collagen, and blue/black nuclei.

Masson Trichrome Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate slides		
2	Mordant in Bouin's	@ 60°C	5 minutes
3	Let stand in warm Bouin's solution		5 minutes
4	Rinse in running water until yellow disappears		~5 minutes
5	Stain with Gill 3 hematoxylin	@ 60°C	5 minutes
6	Rinse in running water		5 minute
7	Stain with Biebrich Scarlet-Acid Fuchsin	@ 60°C	10 seconds
8	Rinse in DI		3 changes
9	Stain in Aniline Blue	@ 60°C	10 seconds
10	Rinse in 1.0% Acetic Acid		0.5 -1 minute
11	Dehydrate, clear, coverslip		

Masson Trichrome Control Tissue

Striated muscle, Appendix, Fallopian tube, Uterus

Interpretation of Staining Procedure

Muscle fibers, cytoplasm — Red
Collagen — Blue
Nuclei — Dark Blue/Purple

Section IV – Staining Protocols with Temperature Control

Microorganisms **Warthin-Starry**

The GMS demonstrates fungus in formalin-fixed paraffin tissue. Chromic acid is used to oxidize the fungal walls. Sodium bisulfite removes traces of chromic acid. A buffered Methenamine Silver solution that is very alkaline impregnates the oxidized fungal walls. Gold Chloride is used to tone the fungi from a brown/black color to purple/black. Sodium thiosulfate fixes the silver staining in the fungal walls and removes unreacted silver and gold to prevent nonspecific staining. Light green provides an excellent counterstain.

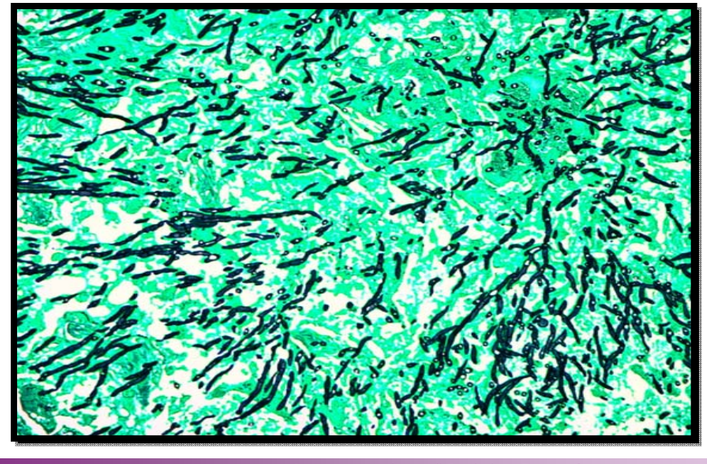
Working Methenamine Silver Solution

3% Methenamine	20ml
10% Silver Nitrate	1ml
Add 21ml of the following solution to the above (Discard the remaining 6ml)	
5% Sodium Borate	2ml
DI water	25ml

GMS Staining Procedure

Step	Detail	Temp	Time
1	Prepare Working Staining solution		
2	Deparaffinize & hydrate, rinse in DI water		
3	Oxidize in 5% Chromic acid	@ 60°C	2 minutes
4	Rinse slides in tap water		
5	Place slides in 1% Sodium bisulfite		1 minute
6	Rinse slides in tap water, then 4 changes of DI		
7	Stain in Working Methenamine Sliver Solution*	@ 60°C	6 minutes
8	Rinse slides in DI water		4 changes
9	Tone in 0.2% Gold chloride		5 minutes
10	Rinse slides in DI water		2 changes
11	Place slides in 5% Sodium thiosulfate		3 minutes
12	Rinse slides well in tap water		
13	Counterstain in 1% light green		1 minute
14	Go directly into 95% alcohol, dehydrate, clear, coverslip		

* Grossly the sections will be the color of a paper bag



GMS Control Tissue

Tissue sections containing fungus

Interpretation of Staining Procedure

Fungal walls: Black
Mucin: Taupe to Dark Gray
Background: Green

Section II – Theory & Concepts

Reducing Processing Time

There are different reasons why reducing processing time is beneficial. Whether it is to provide better patient care or to improve outcomes for a research project, us of microwave processing can speed up results for almost any procedure. Microwave tissue processing is used in a wide variety of applications.

Rohr's (2001) study found the following:

We believe that routinely received, formalin-fixed, small and large tissue specimens may be processed rapidly by microwave irradiation and sectioned and stained without compromising quality of the histologic sections. We also believe that this microwave processing technique can serve as the routine method for tissue processing and preparation of histologic specimens in an active university hospital histology laboratory. This technique has been used routinely in our daily workload for the past 3 years. The use of commercially available microwave ovens for the routine rapid processing of small biopsy specimens has not been associated with problems in tissue quality, as evidenced by our quality assurance and quality control records. We believe that rapid microwave-assisted tissue processing is the optimal method for substantially reducing turnaround time and permitting the histopathology laboratory to consistently provide same-day diagnosis for a variety of types of tissue biopsy specimens. (p. 5)

Emerson's (2006) study found the following:

Our data show that immunohistochemical analysis performed on tissue processed by microwave-assisted rapid tissue processing of previously formalin-fixed large surgical specimens is of comparable quality to that performed on tissue processed by conventional methods for a large battery of antibodies commonly used in diagnostic surgical pathology. These findings support the growing evidence that microwave-assisted rapid tissue processing is a desirable and reliable alternative to conventional tissue processing in the diagnostic surgical pathology laboratory. (p. 8)

Toledo-Hernandez's (2014) study found the following:

The use of the accelerated microwave procedure assures the antigenicity of proteins, enabling us to perform fluorescence immunohistochemistry. (p. 4)

This technique not only accelerated the chemical processing of tissue, but also provided sections of high histological quality and resolution and, thereby, the capacity to distinguish between tissues and structures that are in close proximity. The microwave-accelerated tissue processing procedure also has several advantages over the conventional methods. (p.6)

Traditional Tissue Processing

In traditional tissue processors the specimens are immersed in a series of solutions. Below is an example of traditional processing steps:

2 changes of Fixatives
1 step in 70% alcohol
2 changes of 95% alcohol
2 changes of 100% alcohol
2 changes of clearing agent
3 to 4 changes of paraffin

Agitation, heat, and/or vacuum are applied. Many of the traditional processors have a 12-container processing cycle. The processing cycle can be set for a schedule of 24 or 48 hours, or longer.

Section II – Theory & Concepts

Microwave Tissue Processing

Microwave tissue processing uses the same processing principals with fewer steps and less reagent. Example:

10% Neutral Buffered Formalin or Formalin Alternative
1 step in 100% alcohol
1 step in Isopropyl alcohol (Clearing agent)
2 steps in Paraffin

Microwave processors often use 3 to 4 steps verses 10 to 12 steps used in traditional processing. This is due to microwave energy accelerating tissue processing of specimens. Agitation, heat, and/or vacuum are also applied. The process provides quality results and excellent patient care.

Facts

Following are some facts to assist in understanding how and why microwave processing works:

1. Microwave energy penetrates from the outside in.
2. Due to the size of tissue specimens the samples heat at the same rate throughout.
3. Microwaves heat polar material (water, alcohol)
4. Microwaves do not heat non-polar material (paraffin, xylene). Xylene is not used in microwave technology and paraffin requires the use of a PolarHeat® disk or heat shield to melt the paraffin.

Following are some things to avoid:

a. Large amounts of metal, such as numerous staples right next to each other or in close proximity to the metal temperature probe. If staples are present, they must be submersed in solution, away from other metal.
b. Biopsy sponges tend to contain air, presoak in water prior to using.
c. Xylene produce hazardous fumes at room temperature, which are magnified when heated.

Section IV – Staining Protocols with Temperature Control

Microorganisms Warthin-Starry

The following Warthin-Starry procedure provides times that have been adjusted to LabPulse Medical' plastic Coplin jar with vented lid holding approximately 40ml of solution. The LP2850, time@temp mode, 80% power, agitation on was used

1% Silver Nitrate (Impregnation)

Silver nitrate C.P. crystals 0.5 g Acidulated water 50.0 ml

Working Developing Solution

2% Silver Nitrate (Developer)

Silver nitrate C.P. crystals 0.5 g
Acidulated water 25.0 ml

0.5% Hydroquinone

Hydroquinone crystals, photographic quality 0.35 g
Acidulated water 25.0 ml

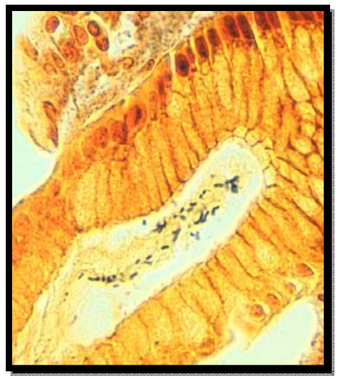
5% Gelatin

Gelatin 1.5 g
Acidulated water 25.0 ml

Acidulated Water

Acidulate 1 liter distilled water with 0.1 g citric acid until pH of 3.8-4.4 is reached; pH 4.0 is ideal for staining spirochetes, pH 3.6 is ideal for demonstrating Donovan Bodies of granuloma inguinale.

Warthin-Starry Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Impregnate in 1% silver nitrate	@ 60°C	5 minutes
3	Pre-heat developing ingredients separately *		45 seconds
4	Remove slide(s) from silver solution, do not rinse, place slide(s) flat, cover with developer		
5	Develop slides **	@ 60°C	20 seconds
6	Rinse slides in 50 ml of DI water	@ 56°C	
7	Tap water		3 changes
8	Dehydrate, clear and coverslip		

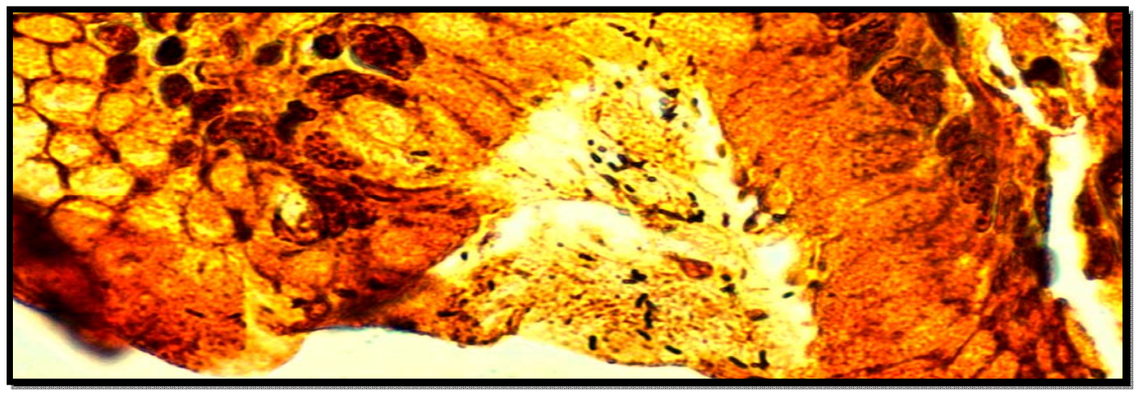
* Using a warm empty flask mix 1.5 ml developer, 3.75 ml gelatin, 2 ml hydroquinone, rotating flask after each addition
** Sections should be light golden brown or yellow

Warthin-Starry Control Tissue

Tissue infected with Spirochetes

Interpretation of Staining Procedure

Spirochetes — Black
Background — Pale Yellow to Light Brown



Section IV – Staining Protocols with Temperature Control

Microorganisms **Warthin-Starry**

The LabPulse Medical Warthin-Starry Staining method is used to demonstrate spirochetes in formalin-fixed paraffin tissue. Silver nitrate impregnates argyophilic substance such as spirochetes. Hydroquinone is used as the reducing agent to make the spirochetes visible. Spirochetes are demonstrated black with a yellow background.

Below are two procedures for the Warthin Starry technique to demonstrate spirochetes. They provide examples of how the timing and/or heat varies when using different volumes of solutions.

Important Notes and Hints

- If spirochetes appear yellow, they are underdeveloped
- Spirochetes are tiny and difficult to see when they are next to other argyophilic substances
- Never use metal forceps or probe with silver or gold solutions

1% Silver Nitrate

10% Silver Nitrate	6ml
DI	54ml

2% Silver Nitrate

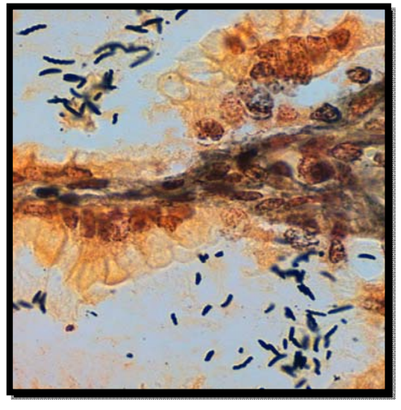
10% Silver Nitrate	2ml
DI	8ml

Working Developing Solution

2% Silver Nitrate	1.5ml
5% Gelatin	3.75ml
0.15% Hydroquinone	2.0ml

The times in the procedure below have been adjusted to slide mailers with approximately 20ml of solution, holding 2 to 3 slides. The LP2850, time@temp mode, 80% power, agitation on was used.

Warthin-Starry Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Impregnate in 1% silver nitrate	@ 60°C	3 minutes
3	Pre-heat developing ingredients separately *	@ 55°C	3 minutes
4	Remove slide(s) from silver solution, do not rinse, place slide(s) flat, cover with developer		
5	Develop slides **	@ 55°C	2-3 minutes
6	Rinse slides in DI water		3 changes
7	Rinse slides in tap water		
8	Dehydrate, clear and coverslip		

* Using a warm empty flask add the ingredients in the order given above, rotating flask after each addition
** Sections should be light golden brown or yellow

Warthin-Starry Control Tissue	Interpretation of Staining Procedure
Tissue infected with Spirochetes	Spirochetes — Black Background — Pale Yellow to Light Brown

Section III – Procedures & Protocols

Objectives

Upon completion of this section the reader will be able to:

a. Select appropriate processing protocols to incorporate into the lab environment
b. Utilize laboratory microwaves for procedures other than processing
c. Reduce turnaround time for tissue specimens of all types

Tissue Processing Protocols

EBS recommends using the following settings when processing tissue specimens: "Time @ Temperature" mode (this means the process timer starts after ramp-up, or heating of the solution to the set-point temperature), 80% power, and agitation "on."

Below are examples of schedules comparing traditional and microwave processing. The charts compare times and solutions. Some of the examples are after fixation is complete:

"Presets" refer to EBS LP2850 units after 12-01-2005, and may be disregarded for other microwaves.

Small Biopsies (GI, Prostate, Cervical Material, etc.)

Traditional Tissue Processor			Microwave Tissue Processor		
Solution	Time		Solution	Temperature °C	Time
80% alcohol	20 minutes		100% alcohol	67	5 minutes
80% alcohol	20 minutes		Isopropanol	74	3 minutes
95% alcohol	20 minutes		Paraffin	74	3 minutes
95% alcohol	20 minutes		Paraffin	82	5 minutes
100% alcohol	20 minutes			**TOTAL**	**16 minutes**
100% alcohol	20 minutes				
100% alcohol	20 minutes				
100% alcohol	20 minutes				
Xylene	20 minutes				
Xylene	20 minutes				
Paraffin	20 minutes				
Paraffin	20 minutes				
TOTAL	**3 hours 40 minutes**				

Routine Specimens (1—3 mm thick)

Traditional Tissue Processor			Microwave Tissue Processor		
Solution	Time		Solution	Temperature °C	Time
Fixative	2 hours		Preserve	55	16 minutes
95% alcohol	1 hours		100% alcohol	67	8 minutes
95% alcohol	1 hours		Isopropanol	74	8 minutes
100% alcohol	1 hours		Paraffin	74	8 minutes
100% alcohol	1 hours		Paraffin	84	28 minutes
Xylene	1 hours			**TOTAL**	**68 Minutes**
Xylene	1 hours				
Paraffin	1 hours				
Paraffin	1 hours				
Paraffin	1 hours				
Paraffin	20 minutes				
TOTAL	**11 hours 20 min**				

Section III – Procedures & Protocols

Below are additional schedules for Microwave processing. All settings are "Time @ Temperature" mode (timer starts after desired temperature is reached), 80% and power, agitation "on."

Fatty Tissue

Solution	Temperature °C	Time
90% alcohol	67	30 minutes
100% alcohol	67	30 minutes
Isopropanol (or PreSolve®)	74	30 minutes
Paraffin	82	30 minutes
Paraffin	82	45 minutes

Fatty tissue 4 mm thick. Tissue pre-fixed in 10% NBF. Change solution between each step except paraffin.

Non Fatty Tissue

Non-Fatty tissue ≤ 4mm such as colon, uterus, gallbladder, uterine content, cervical cones, prostate, and skin with deep tissue.

Solution	Temperature °C	Time
100% alcohol	67	30 minutes
Isopropanol	74	30 minutes
Paraffin	74	30 minutes
Paraffin	82	45 minutes

Skin, Endocervical Tissue

Small amounts of skin or endocervical material.

Solution	Temperature °C	Time
100% alcohol	67	10 minutes
Isopropanol (or PreSolve®)	74	8 minutes
Paraffin	74	5 minutes
Paraffin	82	10 minutes

≤ 1 mm Biopsies

Solution	Temperature °C	Time
Preserve™ or formalin	55	4 minutes
100% alcohol	67	4 minutes
Isopropanol	74	4 minutes
Paraffin	74	4 minutes
Paraffin	84	7 minutes

≤ 3 mm Biopsies

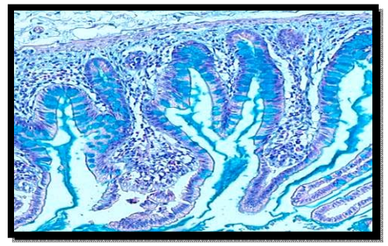
Solution	Temperature °C	Time
Preserve™	55	16 minutes
100% alcohol	67	8 minutes
Isopropanol	74	8 minutes
Paraffin	74	8 minutes
Paraffin	84	28 minutes

Section IV – Staining Protocols with Temperature Control

Carbohydrates — Alcian Blue pH 2.5 (AB)

The Alcian blue method demonstrates acid mucopolysaccharides and can distinguish sulfated mucins from nonsulfated mucins by controlling the pH. Alcian blue pH 2.5 demonstrates both carboxylated and sulfated acidic mucosubstances blue. To demonstrate only sulfated acidic mucins use Alcian blue pH 1.0. The procedure below uses eosin as a counterstain NFR may also be used as a counterstain. To distinguish neutral mucins from acidic mucins PAS may be used as the counterstain because the Schiff's in the PAS method demonstrates neutral mucins whereas Alcian blue does not. Start with formalin-fixed, 4µM paraffin sections (or 8µM eye sections).

AB Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Stain in Alcian Blue pH2.5 *	@ 60°C	45 seconds
3	Rinse in water		
4	Counterstain with eosin **		1 minute
5	Dehydrate, clear and coverslip		

*Due to temperature, the acetic acid "buffer" solution can drift in pH; therefore, it is helpful to set the pH at 60° C. (positive areas should turn blue during this step)

**To counterstain, dehydrate, and clear you may place the slides in the 2nd eosin in automatic stainer.

AB Control Tissue

Appendix, GI specimens

Interpretation of staining Procedure

Acid Mucopolysaccharides — Blue
Background — Pink

Carbohydrates — Rapid Mucin

The Rapid mucin method demonstrates acid mucin in epithelial and connective tissue specimens. Basic Fuchsin is used to demonstrate mucin, cartilage and mast cell granules. The Fast Green demonstrates cytoplasm and the nuclei are stained with Weigert's Iron Hematoxylin.

Working Weigert's Staining Solution

Weigert's Hematoxylin Solution A	25ml
Weigert's Mordant Solution B	25ml

Mucin Staining Procedure

Step	Detail	Temp	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Stain in working Weigert's Iron Hematoxylin		1 minute
3	Rinse in running water		1 minute
4	Stain in Fast Green FCF (FD&C Green #3) sol	@ 60°C	30 seconds
5	Rinse in 0.1% glacial acetic acid		1-2 seconds
6	Stain in 0.5% Basic Fuchsin	@ 60°C	30 seconds
7	Dehydrate, clear and coverslip		

Mucin Control Tissue

Intestinal goblet cells

Interpretation of staining Procedure

Nuclei — Blue-Black
Mucin, Cartilage and Mast Cells —Pink
Cytoplasm — Yellow-Green

Section IV – Staining Protocols with Temperature Control

Objectives

Upon completion of this section the reader will be able to:

a. Reduce time required for special stains/diagnostic turnaround time.
b. Apply staining protocols incorporating the use of a probe to control the solution temperature
c. Adapt traditional staining methods to microwave methods

Microwave Special Staining

Staining methods that normally take minutes can often be done in a microwave oven in seconds, and those that take hours, in minutes. Ideally, it's best to use a microwave featuring a temperature probe or sensor, but "power only" units do an good job, provided care is taken to optimize protocols and periodically monitor performance. Agitation is preferred for even staining; some microwaves such as the LP2850 and LP2250 have an air agitator built in and the LP2850 is equipped with a temperature probe. When using a probe for staining, you may place it in a container of water of equal temperature, size and volume as the dye or reagent.

For microwaves without these features protocols may be adapted. The optimal temperature for most non-metallic stains is ~ 60° C, and for metallic stains ~ 95° C. An alternative microwave method to immersion involves covering the slide with a few drops of staining solution, placing the slide on a platform in the microwave oven, and microwaving for 20-30 seconds.

Most procedures start with formalin-fixed, 4µM paraffin sections. Protocols will also need to be adjusted to the power settings and volume of solutions used in the staining containers. The following protocols were tested using the LP2850, time@temp mode, 80% power, agitation on. Some of the examples use LabPulse Medical's plastic Coplin jars with vented lid, with slides immersed in reagent (~ 40 ml). There are also examples included that use a plastic slide mailer with the lid open, slides immersed in reagent (~ 20 ml).

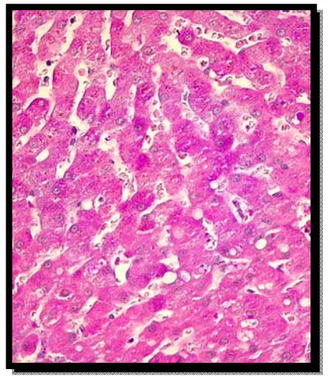
Staining Procedures using Microwaves with a Probe

Carbohydrates Periodic Acid Schiff (PAS)

Use tissue containing PAS positive material as a control. Positive staining targets would include such regions as glycogen bodies, basement membranes, cartilage, fungus, and neutral muccopolysaccharides in tissue samples. This staining procedure uses Periodic Acid Solution to oxidize specific target regions of the tissue which subsequently react with the Schiff reagent developing a purplish-magenta or rose to purple color in positive staining tissue regions. Light Green Stain Solution provides excellent counterstaining. Harris Hematoxylin may be substituted for Light Green. The resulting staining pattern of positive samples exhibits a purplish-magenta staining of target regions with a green or blue counterstain.

If PAS reaction for glycogen with digestion is desired, after step 1, deparaffinization and hydration, place sections to be digested in a Coplin jar with 0.5% diastase for 45 seconds in microwave. (Parallel, "non-digestion" slides should be placed in distilled water in Coplin jar and microwaved for 45 seconds at the same time as the digested slides.) Rinse the slides in deionized water (DI) and begin the procedure at step 2, the oxidation step.

PAS Staining Procedure



Step	Detail	Temp	Time
1	Deparaffinize & hydrate, rinse in DI water		
2	Oxidize with 0.5% Periodic Acid Solution	@ 60°C	1 minute
3	Rinse slides in DI water		5 minutes
4	Stain with Schiff reagent *	@ 60°C	1 minute
5	Rinse in 55% K2S2O5 (optional)		30 seconds
6	Rinse in 55% K2S2O5 (optional)		30 seconds
7	Rinse slides in running tap water		5 minutes
8	Counterstain with Harris Hematoxylin		30 seconds
9	Rinse slides in running tap water		5 minutes
10	Dehydrate, clear, coverslip		

* Positive sections should turn light pink

PAS Control Tissue

Appendix, skeletal muscle, kidney for basement membrane, tissue containing fungus, or liver for glycogen.

Interpretation of Staining Procedure

Glycogen — Rose to Purplish Red
Neutral Mucins, Thyroid Colloid, Fungal walls — Rose to Purplish Red
Nuclei, Background — Blue

Section III – Procedures & Protocols

Antigen Retrieval

Formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining due to the mechanism of action of Formalin. As a non-coagulant, formalin forms methylene bridges during fixation and these cross-links may mask the antigenic sites. Heat is then used in order to retrieve antigens, which is known as heat-induced epitope retrieval, or HIER. Antigen retrieval works by breaking the methylene bridges and exposing the antigenic sites allowing the antibodies to bind. Below is an example of Antigen retrieval that may be used with microwave technology.

Buffer Solutions

Sodium Citrate Buffer (pH 6.0)

- Tri-sodium citrate (dihydrate) 2.94 g
- Distilled water 1000 ml
- Mix to dissolve. Adjust pH to 6.0 with 1N HCl
- Add 0.5 ml of Tween 20, mix well
- Store at room temp for 3 months or at 4°C for a longer shelf life

1 mm EDTA (pH 8.0)

- EDTA 0.37 g
- Distilled water 1000 ml
- Store at room temp for 3 months

Tris-EDTA (pH 9.0)

- Tris 1.21 g
- EDTA 0.37 g
- Distilled water 1000 ml (100 ml to make 10x, 50 ml to make 20x)
- Mix to dissolve. pH is usually at 9.0
- Add 0.5 ml of Tween 20 and mix well.
- Store at room temp for 3 months or at 4°C for a longer shelf life

Important Notes and Hints

- Use plastic slide racks and containers. Standard glass staining racks and containers may crack when heated.
- Monitor the retrieval buffer for evaporation. Add more buffer if necessary. Do not allow the slides to dry out.
- 20 minutes is a suggested antigen retrieval time. Less than 20 minutes may produce a weak stain due to the antigens being under-retrieved. More than 20 minutes may produce nonspecific background staining and also increases the chances of the sections falling off the slides due to over-retrieved antigens.
- A validation run is recommended first before instituting a set protocol. Slides of the same tissue section are retrieved for 5, 10, 15, 20, 25 and 30 minutes before IHC staining to evaluate optimum antigen retrieval time for the particular antibody being used.
- Use care with hot solution to avoid burns.

Procedure using the LP1850 or LP2250 Staining Microwave

Step	Detail	Power	Time
1	Deparaffinize & hydrate slides		
2	Place hydrated slides in antigen retrieval buffer in a microwaveable container		
3	Microwave slides	Full	Until boils
4	Microwave at a boil	Full	20 minutes
5	Remove the container and run cold tap water into it *		10 minutes
6	Continue with the IHC staining protocol		

* Allows the antigenic site to re-form after being exposed to high temperatures

Procedure—LP2850 Processing Microwave (Time @ Temperature Setting)

Step	Detail	Temp	Time
1	Deparaffinize & hydrate slides		
2	Place hydrated slides in antigen retrieval buffer in a microwaveable container		
3	Microwave slides	@ 98°C	20 minutes
4	Remove the container and run cold tap water into it *		10 minutes
5	Continue with the IHC staining protocol		

* Allows the antigenic site to re-form after being exposed to high temperatures

Decalcification

Decalcification is the process of removing calcium salts from tissue and is necessary for sectioning paraffin embedded specimens. Advantages of decalcification via the aid of the microwave are that it is extremely fast, economical and reliable, provided that the end point is not exceeded. As with any decalcification method, exceeding the end point or over-decalcifying will cause a decrease in staining, most obvious in the nucleic acids. Traditional decalcification methods are typically performed at room temperature and may take days or weeks to complete depending on the density of the bone. Microwave decalcification reduces the time to hours and provides superior results.

> Gruntz's (2011) study found the following:
> Significantly reduced processing times were achieved when bone samples, especially from large animals, were decalcified in a microwave processor. Tissue quality following microwave decalcification was determined to be comparable or superior to the results obtained using traditional decalcification methodology. Microwave processing proved to be an efficient and reliable procedure for the decalcification of bones from laboratory animal species. (p. 4)

With any type of decalcification, specimens should be thoroughly fixed prior to beginning the decalcification process to prevent soft tissue damage. Once the fixation is complete rinse the specimens with water then begin the decalcification process. Ensure that the fluid is approximately 100 times the volume of the tissue during the decalcification process. Once the decalcifying process is complete wash the specimens well in running water to stop further decalcification from occurring. The following are two examples of microwave decalcification protocols.

Bone Marrow Decalcification < 3 mm

Step	Detail	Temp, °C	Time, min.	Preset
1	Zinc Formalin	40	30	12
2	Wash in water		10	
3	Decal (Biocare)	40	30	12

Bone Marrow Decalcification 3-5 mm

Step	Detail	Temp, °C	Time, min.	Preset
1	Zinc Formalin	40	60	13
2	Wash in water		10	
3	Decal (Biocare)	40	30*	12

*Decalcification time may vary depending on sample thickness or bone density; up to 2 hours.

Slide Drying

Drying slides prior to staining is important because water left on the slides from the water bath during sectioning may cause incomplete deparaffinization and impair the staining of the tissue.

Deparaffinization is the first step in the staining process and its purpose is to remove paraffin from the tissue sections and surrounding area. Deparaffinization is most often done by using xylene in the first few stations of the staining set-up. If all of the paraffin is not removed, incomplete deparaffinization, creates uneven staining or patches of unstained areas in the tissue sample.

One cause of incomplete deparaffinization is due to water being left on the slide prior to being placed into the deparaffinization solution. This most often is because the slides were not dried completely before being placed in xylene. Water and clearing agents such as xylene do not mix. The water droplets create a barrier that the xylene cannot penetrate so it is unable to reach the paraffin to remove it. This problem may appear as white patches in the tissue while slides are in xylene.

Ovens have been traditionally used in histology to dry slides prior to staining. While drying the slides in the oven the slides heat up and also melt the paraffin in the process. Melting of the paraffin is not a necessary step prior to staining, but the removal of water is. Remember, the purpose of the xylene in the staining set up is to remove the paraffin. Using microwaves to dry slides works because the water is polar and is removed because the molecules are excited or heated. Paraffin, on the other hand, is non polar and typically is not affected by the microwaves so it is not melted unless the container holding the slides is not microwave transparent. Then the container will be heated by the microwave energy and some melting of the paraffin will occur. Many labs have had great success with drying slides in the microwave in approximately 45 seconds to one minute. The timing depends on how many slides are being dried at a time, the power setting used in the microwave and the type slides and container being used. Using charged or plus slides is preferable because they help the tissue adhere to the slides.

To incorporate microwave slide drying into the lab validation of the new procedure should be performed. The validation process will produce a working protocol that will drastically speed up the drying process, decreasing turnaround time. Test slides should be sectioned in order to experiment with the power setting and different times in the microwave.

Deparaffinization and Hydration in the Microwave

The purpose of staining slides is to make cell structures visible. This is accomplished through the use of various dyes that stain certain tissue components. The slides must be deparaffinized and hydrated to accommodate aqueous dyes, i.e. nuclear (Hematoxylin) and cytoplasmic (Eosin) solutions. Using EBS' PreStain® Solution these two steps may be done in the microwave without the use of xylene and alcohol.

Traditional Method of Deparaffinization

Xylene is a common solvent used to remove the paraffin from the embedded and sectioned tissue samples. Once the paraffin is removed, the sections are ready to be treated with alcohol to bring water back into the tissue. Below are examples of ways to deparaffinize sections prior to staining.

Different labs use different time sequences:
- 3 changes xylene over 10-minute period or
- 2 changes xylene for 2 minutes each or
- 3 changes xylene for 5 minutes each

A minimum of 2 changes of xylene is typically used because the 2nd and/or 3rd changes of xylene prevent paraffin from contaminating subsequent reagents.

Traditional Method for Hydration

Since water does not mix with xylene, absolute alcohol is used as the first step in the hydration process. The slides then proceed through 95% alcohol, 80% or 70% alcohol and down to water so that the sections may be treated with aqueous dyes. As a troubleshooting note, if you see clear patches after hydration this is an indication that the deparaffinization is incomplete.

Microwave Method for Deparaffinization and Hydration

Eliminating or reducing the use of xylene and alcohol is value added for the lab and histologist. Using PreStain®, a biodegradable solution, to deparaffinize and hydrate sections in the microwave is easy and safe. Because PreStain in nonhazardous it eliminates the hazardous disposal costs (~$25 savings) associated with xylene and alcohol while reducing the exposure risk for Lab personnel.

PreStain

PreStain is a biodegradable Anionic Surfactant. It works by using 3 types of energy: chemical, thermal and mechanical. The solution is light green and is a water-soluble solution containing potassium salts of fatty acids. The advantages are:

- deparaffinizes and hydrates at the same time
- not toxic
- stable
- nonhazardous waste according to Federal RCRA regulations (40 CFR 261)
- good for routine work
- non-corrosive, biodegradable
- pleasant odor
- not carcinogenic
- no special precautions necessary for handling or storage

PreStain Procedure with Temperature Sensing Capable Microwaves

1. Place nonmetallic slide rack with sectioned, dried slides into the working PreStain solution in the microwave
2. Place another microwave save container filled with tap water in the microwave
3. Submerge temperature probe, if applicable, into PreStain solution
4. Set microwave for 1 minute at 60^0C (using time-at-temperature control)
5. Use the built in agitation system or manually
6. Next move the slide rack into the heated water, microwave for 2 minutes at 60^0C (again using time-at-temperature control)
7. Agitate
8. Remove slides and stain