	NATIONAL CENTRE FOR ANIMAL HEALTH **LABORATORY SERVICES UNIT**	**Second edition**
	STANDARD OPERATING PROCEDURE	**Version 2018.1**
	HISTOPATHOLOGY	

Laboratory SOP	***Method Number***
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TITLE: Histological tissue processing

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. PURPOSE
To provide guidelines for processing of histopathological tissue for different staining techniques.

2. APPARATUS
2.1 Tissue Processor Machine – Shadon Citadel 1000
2.2 Wax dispenser (0-100 ^{0C}) – J.P.SELECTA
2.3 Microtome (0-60) microns) – Micro Tech CUT 4060
2.4 Microtome blade – Feather safety razor Co. Ltd, Medical Division, Japan
2.5 Circular water bath (0-100 ^{0}C) – J.P.SELECTA
2.6 Labelling sticker
2.7 Incubator (for drying slide) – Joun
2.8 Vaccum Pump – Rocker 300/K 17
2.9 Fume hood – Esco

3. REAGENTS, SOLUTION AND BUFFER
3.1 Paraffin wax
3.2 Iso-propyl alcohol
3.3 Xylene
3.4 Ethyl alcohol

4. PROCEDURE

4.1 Removal of Fixative

Fixative is removed from tissues by keeping them overnight in running water.

4.2 Dehydration

Remove water by passing the tissues in ascending grades of alcohol to prevent undue shrinkage of tissues.

Ethyl alcohol 50%	8 hours
Ethyl alcohol 70%	2 hours
Ethyl alcohol 90%	2 hours

Absolute alcohol - I 1 hour
Absolute alcohol - II 1 hour
Dioxane can be used instead of alcohol. Here shrinkage is minimum and dehydration is quick.

4.3 **Clearing**

Removes alcohol from the tissues and prepare them for penetration by paraffin during embedding.

Xylene - I 30 min
Xylene- II 30 min

Xylene is cheap and quick in action - makes tissues transparent - causes shrinkage and hardening if tissues are kept longer. Other clearing agents are: Cedar – wood oil, toluene, benzene and chloroform but are expensive.

4.4 **Infiltration**

Impregnating the tissues completely with paraffin (melting point 50 to 56^{o}C) kept in 3 cups and melted in a paraffin oven. The tissue is kept for 30 minutes in each cup. Celloidin is also used.

4.5 **Tissue processor**

4.5.1 The above process can be carried out in Tissue Processor. Close the lid of the retort chamber. Press MODULE button, select appropriate PROGRAM number from the screen and press that number on the Program Panel. Press START. The first step on the screen is highlighted in white. The cassettes are processed overnight by (1) dehydration in a series of alcohol solutions of increasing concentration, (2) clearing with Xylene to remove alcohol, (3) infiltrated with molten paraffin wax.

4.5.2 Then next morning the cassettes are taken out of the tissue processor, and the tissues are embedded into metal moulds (with their respective cassette base) in paraffin wax on the tissue Embedding System. Wax embedding provides support to the tissues during sectioning.

4.5.3 Embedding: Two L shaped moulds are arranged in the form of a rectangle over a porcelain slab. Melted paraffin is poured into the mould and the tissue is so oriented that the cutting surface of the tissue faces the porcelain slab. The moulds are removed as soon as paraffin sets and the block is ready for sectioning.

4.5.4 Once all the blocks are removed from the moulds, excess paraffin is removed from the block by running the sides of the block on the hot plate. This procedure allows the block to fit squarely into the chuck of the microtome.

4.5.5 The blocks are faced down on the Microtome, using an old microtome blade, at 20-30 microns to expose the entire surface of the tissue for final sectioning.

The tissues are placed on the Tissue Cool Plate for approx.10 minutes to cool, so they are able to be cut at 5 microns (in hot places).

4.5.6 Cut sections on the Microtome and float tissue sections onto the water surface of the water bath set at 40-45 0C. Once the section has sufficiently flattened, pick up the section with the Superfrost glass slide (or glass slide coated with egg albumen) being careful to position section in the middle of the slide.

4.5.7 Once all the tissue blocks are cut, place slides into the slide rack. Keep any special stain slides separate from the H&E slides. Include 1 control slide with each set of special stains. Place into the Slide Dryer for 10 minutes set at 37-50^{0}C so tissues heat-fix onto the slides.

4.5.8 Take the slides out of the slide dryer (they will be hot, so use gloves and paper wadding) and place the slide rack onto the Slide stainer.

5. WASTE DISPOSAL

5.1 Xylene and the Formalin should be disposed as per the guideline of chemical waste of the waste management guidelines.

5.2 Xylene is highly inflammable hence should be properly disposed.

5.3 Clear off the paraffin waste in a paraffin collection tray. Never use sharp cleaning tool since, it will damage the coating of the work spaces.

5.4 Staining solutions are collected in plastic cans and handled as hazardous waste.

5.5 Used xylene is collected in its original packaging or in plastic cans and handled as hazardous waste.

5.6 Used ethanol is collected in plastic bottles and is handled as hazardous waste.

5.7 Antibody solutions that contain 0.1% Na-azide or more are collected and disposed of in yellow containers for toxic waste.

6. RISK ASSESSMENT

6.1 Paraffin may burn in fire conditions hence, should not be used near open flames/heat/spark/hot surfaces. Avoid contact with hot paraffin since it can cause burn.

6.2 Since, Xylene and the Formalin are harmful to human, processing should be done in fume hood.

6.3 In case of contact in eye, should be washed thoroughly with water.

7. TROUBLESHOOTING

Problem	Possible Causes	Corrections
Tissue feels soft or mushy during embedding	• Tissue may have been grossed in too thick • Tissue may have been processed on a program that was too short for that tissue type • Processing reagents may be saturated with water • Paraffin may be saturated with xylene or isopropanol	• Reprocess tissue on proper program • Reprocess tissue on correct processing protocol • Change reagents and reprocess tissue • Change paraffin and reprocess tissue
Tissue bounces out of paraffin block during microtomy or tissue does not adhere to block or slides (Commonly experienced with uterus and prostate tissue, as well as dense organ core samples)	• Poor dehydration and paraffin infiltration due to water left in the tissue	• Change reagents and reprocess tissue on proper processing protocol
Tissue looks greasy and "explodes" or separates rapidly when ribbon is placed on water bath	• If the temperature of the water bath is between 45-50° C, then the tissue is under-processed • Tissue may have been grossed in too thick • Tissue may have been processed on a program that was too short for that tissue type • Processing reagents may be saturated with water • Paraffin may be saturated with xylene or	• Reprocess tissue on correct processing protocol • Reprocess on proper program • Reprocess tissue on correct processing protocol • Change reagents and reprocess • Change paraffin and reprocess tissue

	isopropanol	
Tissue does not adhere to slide or falls off easily	• If tissue slides are placed in oven prior to deparaffinization in xylene, tissue is under-processed • Reagents saturated with water or contaminated with the preceding reagent	• Reprocess tissue on correct processing protocol • Change reagents and paraffin and reprocess tissue on proper processing protocol

8. REFERENCES

Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology Lee G. Luna, Third Edition, 1968

Theory and Practice of Histotechnology Sheenan DC. and Hrapchak BB., Second Edition 1980. Publisher: The C.V. Mosby Company

Cellular Pathology Technique. Culling CFA. Allison RT. and Barr WT. Fourth Edition 1985. Pub. Butterworth and Company

Anderson G. And Gordon K. C (1999). Tissue processing microtomy and paraffin sections. Theory and practice of Histological techniques. PP 47-67. Published by Churchill Living stone Ltd. London, UK.

Manual of Histological Staining Methods of the Armed Forces Institute of Pathology Techniques. Lee G. Luna (Editor) Third Edition.

TITLE: Haematoxylin and Eosin (H & E) staining

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION
In order to visualise detail tissue structure at light microscope level; it is necessary to impart colour to element being studied in the tissue sections.

2. PRINCIPLES
Haematoxylin is not considered as a dye as it lacks a chromphoric group. When oxidised by sodium iodate, haematin is produced. Haematin is an acidic dye which has a poor affinity for nuclear material but, when combine with salts of aluminium, a dark blue-black complex known as a lake is formed which binds to cell nuclei very strongly. Eosin Y, being an anionic dye, stains cationic tissue components.

Haematoxylin component stains the cell nuclei blue/black, with good intra-nuclear details and eosin stains cell cytoplasm.

3. APPLICATION
For Histopathological studies.

4. OBJECTIVE
To describe the staining procedure of H & E which will aid in identifying the cellular structural changes due to diseases or other conditions.

5. APPARATUS
5.1 Floatation Bath/water bath
5.2 Slide Dryer
5.3 Glass slide
5.4 Fume hood

6. REAGENTS, SOLUTION AND BUFFER
For preparation, (refer appendix)
6.1 Haematoxylin
6.2 Eosin
6.3 Xylene
6.4 Absolute alcohol
6.5 DPX mountant
6.6 Egg albumin

7. PROCEDURE

7.1 Deparaffinise the section by Xylol 5-10 minutes

7.2 Remove Xylol by absolute alcohol 85 % & 95 %

7.3 Wash in tap water

7.4 Stain with haematoxylin for 2 minutes

7.5 Wash in tap water

7.6 Wash in 70 % alcohol

7.7 Counter stain with eosin for 30 seconds

7.8 Dehydrate in alcohol grade of 95 % & 100 %

7.9 Clear with xylene (15 to 30 seconds)

7.10 Mount in Canada balsam or DPX Mountant

7.11 Keep slides dry and remove air bubbles, if any

8. RESULT INTERPRETATION

8.1 Nuclei -Blue, with some metachromasia.
8.2 Cytoplasm-Various shades of pink, identifying different tissue components.

9. WASTE DISPOSAL

9.1 Xylene should be disposed as per the guideline of chemical waste of the waste management guidelines.
9.2 Xylene is highly inflammable hence should be properly disposed.

10. RISK ASSESSMENT

10.1 Since, Xylene are harmful to human, processing should be done in fume hood. In case of contact in eye, should be washed thoroughly with water.
10.2 Ethyl alcohol is flammable so use with proper care.

11. TROUBLESHOOTING

Problems	Possible causes	Corrections
Hematoxylin and eosin (H&E) stained tissue section shows uneven nuclear staining and "blue blobs" lacking distinct chromatin patterns	If tissue was fixed properly, then sample was improperly dehydrated and infiltrated with paraffin	Change reagents and reprocess tissue on proper processing protocol
Presence of a fine black precipitate on the slides, often with no relationship to the tissue (i.e., the precipitate appears adjacent to tissues or within interstices or vessels)	Suggestive of formation of formalin-heme pigment. When the formalin buffer is exhausted, tissue becomes acidic, promoting formation complex of haem and formalin	Make the sections thin and use of enough neutral buffered formalin. If the tissue in vial appears murky brown to red, place the tissue in new fixative
Tearing artifacts, holes in the sections	Insufficient dehydration before clearing and infiltration with paraffin wax	Tissue processor should allow sufficient time for dehydration and ethanol final dehydrant should be 100% Covering and sealing of dehydrants in humid areas
Bubbles under the cover slip	Too thin mounting media	Use appropriate concentration

12. REFERENCES

https://www.labce.com/spg572653_troubleshooting_processing_problems.aspx accessed at 2Pm 09/02/2018

Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. Lee Luna (Editor) Third Edition. (1968) Pages 32-37

Stevans A. and Wilson I (1999). The haematoxylin and eosin. Theory and practice of Histological techniques. PP 99-112. Published by Churchill Living stone Ltd.London, UK.

13. APPENDIX

Reagents for haematoxylin & eosin staining

SOLUTIONS
NOTE: All reagent expiry dates are 12 months from date of preparation.
Except: Stock Eosin –

a) Mayer's Haematoxylin

- Aluminium ammonium sulphate
  or Aluminium potassium sulphate ..... 100.0 gm
- Deionised water ..... 2000.0 ml
- Haematoxylin ..... 2.0 gm
- Sodium Iodate ..... 0.4 gm
- Citric acid ..... 2.0 gm
- Chloral hydrate ..... 100.0 gm

Dissolve the Aluminium sulphate in the deionised water using the aid of the magnetic stirrer and large stir bar. Do not heat. When completely dissolved add the Haematoxylin. Once the Haematoxylin is completely dissolved add in the following order- Sodium Iodate, Citric acid, Chloral hydrate. Ensure that all chemicals are in complete solution. The final colour of the stain is reddish violet and shelf life is 12 months.

b) Harris's haematoxylin

- Haematoxylin 2.5g
- Absolute alcohol 25ml
- Potassium alum 50g
- Distilled water 500ml
- Mercuric oxide 1.25g
- Or Sodium iodate 0.5g
- Glacial acetic acid 20ml

The haematoxylin dissolved in the absolute alcohol and is then added to the alum which has previously dissolved in the warm distilled water in a flask. The mixture is rapidly brought to boiling and the mercuric oxide is then slowly added. The stain rapidly cooled by plunging the flask to the cold water. When the solution is cold add acetic acid. Filter the stain and is ready for use.

c) Eosin-Phloxine stain

Stock Eosin

- Eosin Y 1.0 gm
- Deionised water 100.0 ml

NOTE: Prepare fresh

d) Stock Phloxine

- Phloxine B 1.0 gm
- Deionised water 100.0 ml

e) Working Eosin-Phloxine

- Stock Eosin 100.0 ml
- Stock Phloxine 10.0 ml
- 95% alcohol 780.0 ml
  (740ml absolute alcohol + 40ml deionised water)
- Glacial acetic acid 4.0 ml

STANDARD OPERATING PROCEDURE
NATIONAL VETERINARY LABORATORY
NATIONAL CENTRE FOR ANIMAL HEALTH, SERBITHANG

Number: HP 03 **Version:** 2018.1 **Print Date: 11 Mar. 19**

TITLE: Gram's staining

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

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1. INTRODUCTION
When bacteria are present in large numbers as in abscess or on vegetation on a heart valve, they appear ass blue-grey granular mass in H & E stain often. However, organisms are invisible or obscured by cellular debris. For most of the pyogenic bacteria their reaction with Gram stain, together with morphological characters helps in classification.

2. PRINCIPLES
The tissue sections are over stained by the Crystal violet solution. Iodine's purpose in staining is to precipitate the crystal violet. This makes the stain less soluble in alcohol and water so that it is resistant to extraction during the subsequent processing steps.
Differentiation with alcohol/acetone is achieved because Gram-negative bacteria are de-stained more quickly than Gram-positive bacteria.
The tissue sections are then over-stained with New Fuchsin Working Solution. The Gram-negative bacteria take up this dye.
Differentiation and background staining are achieved by the Picric acid/ Acetone solution.

3. APPLICATION
To detect the gram positive and negative bacteria in the tissue sections.

4. OBJECTIVE
This procedure is for the demonstration of Gram positive and Gram negative bacteria.

5. APPARATUS
- 5.1 Magnetic stirrer/hotplate
- 5.2 Staining rack
- 5.3 Fume hood
- 5.4 Glass slide
- 5.5 Microscope

6. REAGENTS, SOLUTION AND BUFFER
For preparation, refer appendix
- 6.1 Crystal Violet
- 6.2 Amonium Oxalate
- 6.3 New Fuchsin
- 6.4 Potassium iodide

6.5 Fuchsin
6.6 Acetone
6.7 Picric acid
6.8 Tolouene

7. PROCEDURE

7.1	Deparaffinise and hydrate to deionised water.	
7.2	Stain with Modified Hucker-Conn cystal violet	2 min.
7.3	Quickly rinse in tap water	1-2 dips.
7.4	Stain with Modified Gram's iodine solution	1 - 2 min.
7.5	Quickly rinse in tap water	1-2 dips
7.6	Decolourise with Acetone/Alcohol mixture until no more colour runs off. Approx.	10 - 20 dips
7.7	Without rinsing, place in Working New fuchsin	1 min.
7.8	Dip several times before timing	
7.9	Quickly rinse with acetone	3 dips.
7.10	Differentiate in Picric acid/acetone	10 dips.
7.11	Quickly rinse with Acetone/Toluene	3 dips
7.12	Rinse in Toluene x 2 and cover slip in DPX.	

8. RESULT INTERPRETATION

8.1	Gram positive	Deep Blue
8.2	Gram negative	Red
8.3	Background	Yellow

9. WASTE DISPOSAL

9.1 Alcohols, acids and staining solutions are collected in plastic cans and handled as hazardous waste.
9.2 Used slides should be disposed after autoclaving.

10. RISK ASSESSMENT

As with all techniques involving pathogenic and potentially pathogenic microorganisms, established aseptic practices should be consistently applied throughout his procedure.

These reagents are harmful or fatal if swallowed and can cause eye irritation if contact is made. In event of eye contact, flush eyes with an eye wash system or tap water for 15 minutes. Gram decolorizer solution is flammable and its vapors; may be harmful; use in a well-ventilated area away from open flames. Directions for use and interpretation should be read and followed carefully.

11. TROUBLESHOOTING

Problem	Probable cause /solution
Gram negative microorganism appearing grams positive	• The slide was over-decolorized • Failure to include the mordanting (iodine) step
Gram positive appearing gram negative	• The slide was under-decolorized • The decolorization step was too short or was omitted • The gram stain dried on the smear

12. REFERENCES

Churukian, CK, Schenk, EA. A method for demonstrating gram-positive and Gram-negative bacteria. J Histotech. Vol 5 (3) Sept 1982: Pages 127 - 128.

Stevans A. and Francis J.R. (1999). Microrganisms. Theory and practice of Histological techniques. PP 291-308. Published by Churchill Living stone Ltd. London, UK.

13. APPENDIX: Reagents for grams staining

NOTE: All reagent expiry dates are 12 months from date of preparation.
Except: 1. Modified Hucker-Conn Crystal Violet
2. New Fuchsin Working solution

a) Modified Hucker-Conn Crystal Violet
- 10% Alcoholic crystal violet ..... 4.2 ml
- 1% Aq. Ammonium oxalate ..... 210.0 ml

NOTE: Change monthly

b) 10% Alcoholic Crystal Violet
- Crystal Violet ..... 10.0 gm
- Absolute alcohol ..... 100.0 ml

c) 1% Ammonium Oxalate
- Ammonium oxalate ..... 10.0 gm
- Deionised water ..... 1000.0 ml

d) Modified Gram's Iodine Solution

- Dissolve 5 g of Potassium iodide in 20 ml of deionised water, then add 2.5 g of Iodine to the solution and mix very well with the aid of magnetic stirrer. Add extra deionised water to make it to 500 ml total and mix well.

NOTE: Change monthly.

e) 0.5% New Fuchsin Stock Solution

- New Fuchsin ..... 0.5 g
- Deionised water ..... 100.0 ml

NOTE: Use the magnetic stirrer and apply heat until dissolved.

f) New Fuchsin Working Solution

- New Fuchsin stock solution..... 21.0 ml
- Deionised water ..... 189.0 ml

NOTE: Change monthly.

g) Absolute Alcohol - Acetone 1:1

- Absolute alcohol..... 1000.0 ml
- Acetone..... 1000.0 ml

NOTE: Change weekly.

h) Picric Acid in Acetone 0.1%

- Weigh 2.0 g of Picric acid powder on filter paper, (Dry Picric acid with absorbant paper), then dissolve into 2000.0 ml of acetone.

NOTE: Change weekly.

i) Acetone – Toluene 1:1

- Acetone..... 1000.0 ml
- Toluene..... 1000.0 ml

NOTE: Change weekly.

TITLE: Ziehl Neelson's acid fast staining

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION
Mycobacterium organisms are difficult to demonstrate by the grams technique because they possess a capsule containing a long chain fatty acid, mycolic acid which makes them hydrophobic. This fatty capsule influences the penetration and resistance to removal of stain by acid and alcohol.

2. PRINCIPLES
Tubercle bacilli (mycobacteria) have a lipid rich wall, containing a waxy substance composed of mycolic acid. The mycolic acid (and other cell wall lipids) present a barrier to dye entry as well as elution and this is overcome by adding phenol to a concentrated aqueous solution of the Fuchsin (hence Carbol Fuchsin). The phenol in Carbol Fuchsin gives an increased lipophilia and aids the passage of the dye through lipids. During differentiation with acid and alcohol, mycobacteria show an "acid fastness" (strictly acid-alcohol). This is due to the high lipid content of mycobacteria. Other bacteria and tissue elements stain with the counter-stain.

3. APPLICATION
To detect the acid fast organisms in the tissue sections.

4. OBJECTIVE
This procedure is for the demonstration of Acid-fast organisms in the tissue sections.

5. APPARATUS
5.1 Magnetic stirrer/hotplate
5.2 Staining rack
5.3 Fume hood
5.4 Glass slide
5.5 Microscope

6. REAGENTS, SOLUTION AND BUFFER

For preparation please refer appendix

6.1 Basic fuchsin
6.2 Hydrochloric acid

6.3 Phenol crystals
6.4 Alcohol 100%
6.5 Methylene blue

7. PROCEDURE

NOTE: Run a positive control slide. All times are approximate, except where stated.

7.1 Deparaffinise and hydrate to deionised water.

7.2 Stain slides with filtered Carbol Fuchsin. 30 min.

7.3 The staining with Carbol Fuchsin solution is carried out in the fume hood due to the staining solution containing Phenol.

7.4 Set up the same number of plastic Petri plates as there are slides. Cut the same number of "orange sticks" into pieces so that they fit in to the top section of the Petri plates. Place one slide in each plate so that the section is facing down and the super frosted end is on the orange stick.

7.5 With a plastic transfer pipette place a small amount of the filtered stain at the junction of the slide and the plastic plate. Capillary action allows the stain to move up the underside of the slide to cover the section.

7.6 Place the lids on the Petri plates, as to avoid evaporation of the stain.

7.7 Rinse well in tap water.

7.8 Differentiate with 1% Acid Alcohol until section is pale pink.

7.9 Wash in running tap water. 8 min.

7.10 Place slides on staining rack.

7.11 Counter stain with Methylene Blue Working Solution.20 to 30 sec.

NOTE: Stain no longer than 30 sec.

Dehydrate in 95% Alcohol x 1, Absolute Alcohol x 2, clear in Toluene x 2 and coverslip with DPX.

8. RESULT INTERPRETATION

Acid-fast organisms' ..... Bright red
Other tissue elements ..... Pale blue

9. WASTE DISPOSAL

9.1 Alcohols, acids and staining solutions are collected in plastic cans and handled as hazardous waste.

9.2 Used slides should be disposed after autoclaving.

10. RISK ASSESSMENT

10.1 Since the acid fast organisms like mycobacterium species are of zoonotic nature hence, safety precaution should be taken to avoid contamination.

10.2 Phenol crystals and its vapour are poisonous and caustic. Melt the crystals in the fume hood. Only gentle heat is required, as the melting point of Phenol is 43^0C. This is done by placing the crystals in a test tube then placing this into a beaker of very hot water.

11. TROUBLESHOOTING

Inadequate stain or faint staining of acid fast bacilli can occur due to insufficient staining with Carbol Fuchsin during the Ziehl-Neelsen staining procedure and/or insufficient flaming of the Carbol Fuchsin while on the slide. This could be reported as false negative result.

Positive and negative control slide should be included with each run of stains. This will verify the correct performance of the procedure as well as the staining intensity of the acid-fast organisms. Control slides should be reviewed before samples are read to confirm that the mycobacteria stain acid-fast.

12. REFERENCES

Manual of Histologic Staining Methods of the Armed Forces Institute, 3rd edition. Lee Luna (Editor).

Theory and Strategy in Histochemistry. Hans Lyon (Editor).

Stevans A. and Francis J.R. (1999). Microrganisms. Theory and practice of Histological techniques. PP 291-308. Published by Churchill Living stone Ltd.London, UK.

APPENDIX: Reagents for Ziehl Neelson's Acid Fast stain

NOTE: All reagent expiry dates are 5 years from date of preparation.

Except:
1. Conc. Carbol Fuchsin– see expiry date on container.
2. Methylene Blue Working Solution – 2 years from date of preparation.

1% Acid-Alcohol

70% Alcohol	1000.0 mL
Hydrochloric Acid, concentrated	10.0 mL

Carbol Fuchsin Solution – *Alternate stain solution*

Phenol crystals (melted)	5.0 mL
100% Alcohol	10.0 mL
Basic Fuchsin	1.0 g
Deionised water	100.0 mL

CAUTION: Phenol crystals and its vapour are poisonous and caustic. Melt the crystals in the Hamilton fume hood. Only gentle heat is required, as the melting point of Phenol is 43^0C. This is done by placing the crystals in a test tube then placing this into a beaker of very hot water.

Methylene Blue Stock Solution

Methylene Blue	1.4 g
95% Alcohol	100.0 mL

Methylene Blue Working Solution

Methylene Blue Stock Solution	1.0 mL
Deionised water	40.0 mL

NOTE: Measure and mix in a 50 mL red-capped centrifuge tube.

TITLE: Perls Prussian blue staining (Minerals & Pigments)

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION
Haemosiderin released during haemolysis is not visible by ordinary H&E staining techniques. Hence, require special staining to examine in the tissue.

2. PRINCIPLES
The basis for the method is the release of ferric iron from haemosiderin by acid treatment where it is loosely bound to proteins. The ferric iron reacts with potassium ferrocyanide to form ferric ferrocyanide. This is an insoluble blue compound known as Prussian blue. The intensity of the colour gives some indication as to amount, but it is qualitative only.

3. APPLICATION
To detect hemosiderin in macrophages in the tissues.

4. OBJECTIVE
This procedure is to describe the method to demonstrate hemosiderin pigment in the tissue sections.

5. APPARATUS
5.1 Magnetic stirrer/hotplate
5.2 Staining rack
5.3 Fume hood
5.4 Glass slide
5.5 Microscope
5.6 Forceps (non-metalic)

6. REAGENTS, SOLUTION AND BUFFER
For preparation, (refer appendix)

6.1 Potassium Ferrocyanide
6.2 Hydrochloric acid
6.3 Nuclear Fast Red
6.4 Aluminium sulphate

7. PROCEDURE
7.1 Deparaffinise and hydrate to deionised water.

7.2 Place into 10% Potassium ferrocyanide stock solution 5 min.

7.3 Place into freshly prepared Potassium Ferrocyanide - Hydrochloric Acid Working Solution 20 min.

7.4 Rinse well in deionised water.

7.5 Counter stain with Nuclear Fast Red solution 5 min.

7.6 Wash well in running tap water.

7.7 Dehydrate in 95% alcohol x1, absolute alcohol x 2, clear in Toluene x 2 and cover slip with DPX.

8. RESULT INTERPRETATION

- Haemosiderin Iron Pigments Bright blue
- Other tissue component Pink to Red.

9. WASTE DISPOSAL

9.1 Alcohols, acids and staining solutions are collected in plastic cans and handled as hazardous waste.

9.2 Used slides should be disposed after autoclaving.

10. RISK ASSESSMENT

Wear gloves, goggles and lab coat. Avoid contact and inhalation. Potassium ferrocyanide; Low toxicity as long as it is not heated, it will release cyanide gas. Hydrochloric acid; target organ effects on reproductive system and fetal tissue. Irritant to skin eyes and respiratory system.
Acids are corrosive, avoid contact and inhalation.

11. TROUBLESHOOTING

Problem	Probable cause & solution
False positive	Due to washing in tap water before placing in working solution. So wash well

12. REFERENCES

Manual of Histological Staining Methods of the Armed Forces Institute of Pathology. Lee Luna Editor. 3rd edition. Page 184.

Stevans A. and Chalk B.T. (1999). Microrganisms. Theory and practice of Histological techniques. PP 243-267. Published by Churchill Living stone Ltd.London, UK.

13. APPENDIX: Reagents for Perl's stain

NOTE: All reagent expiry dates are 12 months from date of preparation
Except: Potassium Ferrocyanide - Hydrochloric Acid Working Solution

a) 10% Potassium Ferrocyanide Stock Solution

- Potassium ferrocynide..... 10.0 g
- Deionised water 100.0ml

b) 10% Hydrochloric Acid Stock Solution

- Hydrochloric acid conc..... 10.0 ml
- Deionised water ..... 90.0 ml

CAUTION: Always add the concentrated acid to the water.

c) **Potassium Ferrocyanide - Hydrochloric Acid Working Solution**

- 10% Potassium Ferrocyanide Stock Solution .....35.0 ml
- 10% Hydrochloric Acid Stock Solution ..... 15.0 ml

NOTE: Prepare fresh.

d) **Nuclear Fast Red**

Dissolve 0.1 gm of Nuclear Fast Red in 100.0 ml 0f 5.0% solution of Aluminium sulphate with the aid of heat. Cool, filter and add a grain of thymol as a preservative.

TITLE: Periodic Acid Schiff (PAS) Staining Technique for Carbohydrates and fungi

PREPARED BY: Histopathology Section

REVISED BY: Histopathology

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION
Periodic Acid Schiff (PAS) staining is one of the most commonly performed special staining technique in histopathology laboratory which is used to highlight molecules with high percentage of carbohydrate content such as mucin, glycogen, fungi and basement membrane in skin.

2. PRINCIPLES
The PAS stain is a histochemical reaction in that the periodic acid oxidizes the carbon to carbon bond forming aldehydes which react to the fuschi-sulfurous acid which form the magenta color.

3. APPLICATION
To detect fungal infections

4. OBJECTIVE
This procedure describes the method to demonstrate fungal structures in tissues

5. APPARATUS
5.1 Magnetic stirrer/hotplate
5.2 Staining rack
5.3 Fume hood
5.4 Glass slide
5.5 Microscope
5.6 Whatman #2 filter paper
5.7 500 ml graduated cylinder

6. REAGENTS, SOLUTION AND BUFFER
For preparation, refer appendix

6.1 Lillie's Cold Schiff's Reagent
6.2 Activated charcoal
6.3 0.5% Periodic Acid
6.4 Hematoxylin

7. PROCEDURE

7.1 Deparaffinize and hydrate to distilled water.
7.2 Place slides into 0.5% Periodic acid for 5 minutes.
7.3 Rinse in distilled water.
7.4 *Schiff's Reagent, microwave for 45-60 seconds, until the solution is a deep magenta.
7.5 Wash in running tap water for 5 minutes.
7.6 Counterstain in hematoxylin for 3 minutes.
7.7 Wash in tap water, blue hematoxylin, rinse in distilled water.
7.8 Dehydrate in alcohols, clear, and coverslip. * Conventional method: d. Schiff's reagent, room temperature for 30 minutes.

8. RESULT INTERPRETATION

Glycogen, fungus:	magenta
Nuclei	blue

9. WASTE DISPOSAL

9.1 Alcohols, acids and staining solutions are collected in plastic cans and handled as hazardous waste.
9.2 Used slides should be disposed after autoclaving.

10. RISK ASSESSMENT

Hydrochloric acid is caustic use caution, flush with water. Avoid breathing the basic fuchsin, carcinogen. Wear gloves, goggles and lab coat.

11. TROUBLESHOOTING

Examination of the control slides is essential to make sure that everything worked like they were supposed to.

11.1 Causes for poor glycogen digestion (persistent glycogen after digestion):

11.2 inactivation of diastase reagent (fresh solution works)

11.3 failure to warm the solutions to 37°C prior to the digestion

11.4 concentration of diastase too low

11.5 digestion period too short

11.6 failure to prepare in a phosphate buffer at pH 6.0

12. REFERENCES

https://laboratoryinfo.com/periodic-acid-schiff-pas-staining-technique-for-carbohydrates/ accessed on4PM on 09/02/2018

Stanforth DA. Staining methods: Carbohydrates and amyloid. Pages 48-59. IN: Wulff S (editor). Guide to Special Stains. Dako. 2004.

Stevans A. and Francis J.R. (1999). Microrganisms. Theory and practice of Histological techniques. PP 291-308. Published by Churchill Living stone Ltd. London, UK.

1. APPENDIX: Preparation of staining solutions

a) Lillie's Cold Schiff's Reagent:

Basic fuchsin 10.0 gm
Sodium metabisulfite 18.0 gm
Distilled water 1000.0 ml
Hydrochloric Acid 10 0 ml
Stir solution for 2 hours, set in a dark cool place overnight. The solution is now a clear light brown to yellow color.

Add: Activated charcoal 500.0 gm (or two heaping spoons) Stir.

Filter through Whatman #2 filter paper into a 500 ml graduated cylinder. Change the filter paper often.
Restore volume to 500 ml with distilled water. Store in Refrigerator, solution is stable for 6 months.

Basic fuchsin 10.0 gm
Sodium metabisulfite 18.0 gm
Distilled water 1000.0 ml
Hydrochloric Acid 10 0 ml
Stir solution for 2 hours, set in a dark cool place overnight. The solution is now a clear light brown to yellow color.

Add: Activated charcoal 500.0 gm (or two heaping spoons) Stir. Filter through Whatman #2 filter paper into a 500 ml graduated cylinder. Change the filter paper often. Restore volume to 500 ml with distilled water.

Store in Refrigerator, solution is stable for 6 months.

CAUTION: **Carcinogen, corrosive**.

b. Hematoxylin, GILL-3:

c. *0.5% Periodic Acid*:

Periodic acid 0.5 gm
Distilled water 100.0 ml
Mix well, label with date and initial. Stable for 1 year.

STANDARD OPERATING PROCEDURE
NATIONAL VETERINARY LABORATORY
NATIONAL CENTRE FOR ANIMAL HEALTH, SERBITHANG

Number: HP 07 **Version:** 2018.1 **Print Date: 11 Mar. 19**

TITLE: Geimsa staining for parasites

PREPARED BY: Histopathology Section

REVISED BY: Histopahology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

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1. INTRODUCTION
This procedure is for demonstration of parasites (Babesia, Protoza, Cryptospordia)

2. PRINCIPLES
Giemsa is a Romanowsky type stain. It contains both basic (i.e. the azures – mono, di, tri and tetramethyl thionin. Tetramethyl thionin is commonly called methylene blue) and acidic dyes (i.e. the eosins – eosin Y, eosin B, erythrosin B and phloxine B) in the same solution and in principle all structures with ionized groups should be stained. By using Giemsa dye, one can see a wide variation in colour when staining parasites. This is referred to as the Romanowsky colour range

3. APPLICATION
To detect parasitic infections in tissue sections

4. OBJECTIVE
This procedure is for demonstration of parasites (Babesia, Protoza, Cryptospordia)

5. APPARATUS
5.1 Magnetic stirrer/hotplate
5.2 Staining rack
5.3 Fume hood
5.4 Glass slide
5.5 Microscope

6. REAGENTS, SOLUTION AND BUFFER
For preparation, refer appendix
6.1 Giemsa powder
6.2 Glycerin (AR)
6.3 Methanol
6.4 Glacial acetic acid
6.5 Absolute alcohol
6.6 Hydrochloric acid

7. PROCEDURE

NOTE: Run a positive control slide. All times are approximate, except where stated.

7.1 De-paraffinise and hydrate to de-ionised water.

7.2 This step is optional Place into 1.0% Acid alcohol for 5 minutes and wash well with distilled water

7.3 Place the slides in Working Giemsa solution ----------60 min.

7.4 Without washing, decolourise in 1% Acetic acid/alcohol until no more stain streams from the tissue and tissue assumes a pink colour.

7.5 Dehydrate in 80% alcohol x1, 95% alcohol x1, Absolute alcohol x2, clear in Toluene x2 and cover slip with DPX.

8. RESULT INTERPRETATION

Parasites	Dark Blue	
	Nuclei	Blue
	Cytoplasm	Pink

9. WASTE DISPOSAL

9.1 Alcohols, acids and staining solutions are collected in plastic cans and handled as hazardous waste.

9.2 Used slides should be disposed after autoclaving.

10. RISK ASSESSMENT

Hydrochloric acid is caustic use caution, flush with water. Wear gloves, goggles and lab coat.

11. TROUBLESHOOTING

-NA

12. REFERENCES

Stevans A. and Francis J.R. (1999). Microorganisms. Theory and practice of Histological techniques. PP 291-308. Published by Churchill Living stone Ltd. London, UK.

13. APPENDIX: Reagents for Geimsa staining

NOTE: All reagent expiry dates are 12 months from date of preparation.
Except: Working Giemsa solution

a) Stock Giemsa Solution

- Giemsa powder ..... 2.0 gm
- Glycerin (AR) ..... 132.0 ml
- Methanol ..... 132.0 ml

NOTE: Mix Glycerin and Giemsa powder with gentle heat (Hot plate magnetic stirrer for one hour with heat setting on low). Then add methanol and mix for another 10 minutes.

b) Working Giemsa Solution

- Stock Giemsa ..... 5.0 ml
- Deionised water ..... 45.0 ml

NOTE: Prepare fresh

c) 1.0% Acetic Acid/Alcohol

- Glacial acetic acid ..... 10.0 ml
- Absolute alcohol..... 1000.0 ml

d) 1.0% Acid Alcohol

- Hydrochloric acid ..... 10.0 ml
- 70% Alcohol..... 1000.0 ml

TITLE: Immuno-histochemistry for Canine Distemper

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION

Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific antigens by utilizing a specific antigen/antibody reaction tagged with a visible label. IHC allows visualization of the localization and distribution of specific antigenic components, including pathogens, within a cell or tissue.

2. PRINCIPLES

The principle of immunohistochemistry is to localize antigens in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.

3. APPLICATION

VECTASTAIN kit 3ABC is used to detect Canine distemper viral antigen.

4. OBJECTIVE

To describe the protocol for the detection of CDV using the VECTASTAIN kit 3ABC Elite PK1602

5. APPARATUS

5.1 Microtome with blades
5.2 Tissue processor
5.3 Cryostat for sectioning the frozen sections
5.4 Microscope
5.5 Incubator
5.6 Freezing medium

6. REAGENTS, SOLUTION AND BUFFER

6.1 0.9% Saline (PBS) (10 mM sodium phosphate, pH 7.5),
6.2 Positive and negative control sections
6.3 Xylene
6.4 H_2O_2
6.5 VECTASTAIN kit 3ABC Elite PK1602
6.6 VECTOR SG Peroxidase (HRP) Substrate kit BK4700
6.7 Antidistemper mouse monoclonal MAI 82327

6.8 PIMA 182327 Fischer scientific
6.9 DPX mount media

7. PROCEDURE

7.1 Staining procedure for paraffin sections

7.1.1 Deparaffinize and hydrate tissue sections through xylenes or other clearing agents and graded alcohol series.
7.1.2 Rinse for 5 minutes in tap water.
7.1.3 If quenching of endogenous peroxidase activity is required, incubate the sections for 30 minutes in 0.3% H_2O_2 in either methanol or water. Incubation times may be shortened by using higher concentrations of H_2O_2. If endogenous peroxidase activity does not present a problem, step 3 may be omitted.
7.1.4 Wash in buffer for 5 minutes.
7.1.5 Incubate sections for 20 minutes with diluted normal blocking serum which was prepared from the species in which the secondary antibody is made. (In cases where non-specific staining is not a problem, Steps 5 and 6 may be omitted).
7.1.6 Blot excess serum from sections.
7.1.7 Incubate sections for 30 minutes with primary antiserum diluted in buffer.
7.1.8 Wash slides for 5 minutes in buffer.
7.1.9 Incubate sections for 30 minutes with diluted biotinylated secondary antibody solution.
7.1.10 Wash slides for 5 minutes in buffer.
7.1.11 Incubate sections for 30 minutes with VECTASTAIN® Elite ABC Reagent.
7.1.12 Wash slides for 5 minutes in buffer.
7.1.13 Incubate sections in peroxidase substrate solution until desired stain intensity develops (see Note 2).
7.1.14 Rinse sections in tap water.
7.1.15 Counterstain, clear and mount.

7.2 Staining procedure for frozen sections

This procedure is generally appropriate for frozen sections, cell smears or cytocentrifuge preparations.

7.2.1 Sections are air dried.
7.2.2 Immediately before staining, fix sections with acetone or the appropriate fixative for the antigen under study.
7.2.3 Transfer slides into buffer.
7.2.4 If quenching of endogenous peroxidase is required , use gentle H_2O_2 blocking to reduce the risk of antigen destruction or tissue loss: 0.3% H2O2 in 0.3% Normal Sera in PBS for 5 minutes; or 0.3% H_2O_2 in methanol for 30 minutes, If necessary, H_2O_2 treatment may also be performed after the biotinylated secondary antibody step.

7.2.5 Follow steps 4-15 of the pro c e d u re recommended for paraffin sections.

8. RESULT INTERPRETATION

If the antigen is present, it will stain light pink with light purple stained background.

9. WASTE DISPOSAL

- Dispose properly.

10. RISK ASSESSMENT

- NA

11. TROUBLESHOOTING

11.1 Drying (most common)
11.2 Retrieval pH
11.3 Water bath contaminates
11.4 Tissue thickness- 3-4 microns
11.5 Slide handling
11.6 Counter stain

12. REFERENCES

https://www.vectorlabs.com/catalog.aspx?catID=42
http://www.vectorlabs.com/catalog.aspx?prodID =36&locID=609997
http://www.pierceantibodies.com/Distemper-Virus-antibodyclone-DV2-12-monoclonal--MA182327.html

13. Annexure: Immunoperoxidase Detection of CDV

Preparation of PBS

1. Dist. H2O = 600ml
2. PBS tablet = 6 tablets

Citrate Buffer Solution

1. Tris Sodium citrate = 2.9gm
2. D.H2O = 100 ml
3. Mix thoroughly

3% H2O2

1. Hydrogen peroxide = 3ml
2. D.H2O = 97 ml

Normal Serum (Diluted)

1. 3 drops of horse normal serum
2. 10 ml of PBS

Biotinylated Antibody

1. 3 drops of Anti mouse IgG
2. 10ml of diluted normal serum

Avidin Biotin Complex (ABC reagent)

1. 2 drops of VECTASTAIN ABC
2. 5ml of PBS

Peroxidase Substrate Solution

1. Chromogen =3 drop
2. Hydrogen peroxide (substrate reagent) =3 drop
3. PBS = 5ml

TITLE: Fluorescent Antibody Technique (FAT) for Classical Swine Fever

PREPARED BY: Histopathology Section

REVISED BY: Histopathology Section

APPROVED BY: Head LSU

DATE: 11.06.2018

1. INTRODUCTION
Classical Swine Fever (CSF) in pigs is caused by virus of genus *Pesti virus* under Family *Flaviviridae*. The virus is closely related to ruminant pesti viruses causing bovine virus diarrhoea and border disease. FAT is simple test used for diagnosis of CSF and requires less time.

2. PRINCIPLES
Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to Fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC conjugate and examined by fluorescence microscopy.

3. APPLICATION
The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum.

4. OBJECTIVE
To describe the procedures to detect CSF virus antigen in tonsils, kidney, lymph nodes or ileum.

5. APPARATUS
5.1 Cryostat for sectioning the frozen sections – LEICA CM 1850
5.2 Compound Microscope - Olympus

6. REAGENTS, SOLUTION AND BUFFER
6.1 Phosphate buffered saline
6.2 Anti-CSF immunoglobulin
6.3 Fluorescein isothiocyanate (FITC)
6.4 Positive and negative control sections

7. PROCEDURE
7.1 Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.

7.2 Freeze the piece of organ on to the cryostat table.

7.3 Cut sections not more than 4–8 µm thick and mount these on to 10 × 32 mm grease-free cover-slips with one corner cut-off. All sections are mounted with this corner in the same position (e.g. top right).

7.4 After drying, fix the mounted sections for 10 minutes at room temperature in acetone (analytical grade) or air-dry for 20 minutes at 37°C.

7.5 Immerse the sections briefly in phosphate buffered saline (PBS), remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in an incubation chamber humidified with a small volume of water placed in the bottom of the chamber.

7.6 Dispense the anti-CSF immunoglobulin at working dilution on to the entire section and incubate in the closed chamber for 30 minutes at 37°C. If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.

7.7 Wash the sections five times for 2 minutes each in PBS at room temperature.

7.8 Remove the remaining PBS by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.

7.9 Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope.

7.10 Include positive and negative control sections in each series of organ samples to be examined.

Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs. The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from freshly dead animals, as autolysis and bacterial contamination can often result in high background staining.

8. RESULT INTERPRETATION

A CSF-positive section shows brilliant green fluorescing cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla.

In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühn glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

9. WASTE DISPOSAL

Proper disposal of the slides should be done.

10. RISK ASSESSMENT

- N/A

11. TROUBLESHOOTING

11.1 The FAT involves the use of an anti-CSF immunoglobulin prepared from a polyclonal antibody to CSFV that will not distinguish between the antigens of different pestiviruses.

11.2 Pigs infected with ruminant pestiviruses can give false-positive FAT reactions. Infections by CSFV or ruminant pestiviruses can be differentiated by testing sera from the dam and litter mates, or from other contacts of an FAT-positive piglet, for neutralising antibodies to each virus.

11.3 If the virus was isolated, or viral nucleic acid can be detected, using RT-PCR, subsequent sequencing provides a rapid and accurate tool to distinguish ruminant pesti viruses from CSFV.

12. REFERENCES

OIE Terrestrial Manual (2008) classical swine fever. PP 1092-1106

TEST CATEGORIZATION

Histopathology section

Sl. No.	Procedure / SOP	DVL	SVL/TVH	RLDC/ NVH	NCAH
1	Histopathology - Processing - H & E staining - Gram's staining - Acid fast staining - Pigment staining- Pearls staining - Fungal staining Parasites staining techniques in tissues				X
2	Immuno-histochemistry tests: Immuno-diagnosis for Canine distemper Immuno-diagnosis for Classical Swine fever				X