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

SOP No: NCAH/LSU/HISTO 01
Title: SOP on Sample Collection for Histopathology
Version No: 3, Page: 4
Issue Month/Effective Date: May 2026
Revision Summary: NA
Supersedes Version No: 2
Prepared by: Histopathology Section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, SVL, DVL, BFDA, DoFPS

1. Scope

This SOP describes the proper procedures for collecting and preserving the tissue specimens collected during necropsy for evaluation by light microscopic histopathological evaluation.

2. Objective

To guide the laboratory technicians in collecting appropriate organs/tissue samples sizes using the proper preservatives.

3. Equipment and Consumables

3.1. Scalpel with blade
3.2. Scissors
3.3. HP vial
3.4. Sharp and blunt tip scissors
3.5. 10% Buffered Neutral Formalin or 10% buffered formalin

4. Procedure

4.1. The portion of normal tissue adjacent to the characteristic lesions should be included.
4.2. Fresh squashed tissues should be placed in fixative immediately after they have been removed from an animal.
4.3. The tissue samples meant for the histopathological examination should be less than 5cm thick to ensure thorough fixation.
4.4. The volume of 10% formalin should be at least 10-20 times the total volume of all the tissues collected. (Different tissues from the same animal can be collected in the same container provided the ratio of preservative to tissue is maintained).
4.5. The size of the container should be appropriate to the specimen (i.e. no tissue squashing).
4.6. This should be forwarded to the laboratory with a letter giving detailed clinical history, post-mortem report and other general information.
4.7. Do not over pack the container and do not freeze the tissue.
4.8. If tissues could not be submitted to the lab for more than 2 weeks, change the fixative every 2 weeks.

Note Recommendations for optimal tissue fixation

4.9. Tissue specimens should be placed in fixative immediately after they have been removed from an animal carcass and the fixative should be changed after the first hour of fixation.
4.10. The tissue section should be cut such that the thinnest dimension is no greater than 4- 5mm in thickness.
4.11. Initial fixation should be at room temperature since the penetration of formalin is related to the temperature of the solution.
4.12. The formalin should be gently shaken before use to avoid a concentration gradient in the bottle.
4.13. Tissues and organs should be fixed (depending on their size) for 2 hours to a maximum of 24 hours.

4.14 Once fixed, tissues can be removed from formalin and, as long as they are kept moist and

protected (e.g. by wrapping in formalin-soaked paper towels, then sealed in screw-capped jars), they can be forwarded to the laboratory without formalin.

5. Safety

Formalin is a flammable liquid and vapour, toxic if swallowed, toxic in contact with skin. It is considered hazardous and should be handled only after reviewing the MSDS, while wearing gloves, and under a fume hood. Formalin vapor is an irritant to the eyes and nasal passage and prolonged exposure to the skin can cause dermatitis.

6. Waste Disposal

All the waste generated during sample collection should be disposed appropriately based on the zoonotic and non-zoonotic nature of the cases.

7. References

7.1. Bancroft, J.D. and Stevens, A.: theory and practice of histological techniques ed.3, Churchill Livingstone inc. 1990. Edinburgh. London, Melbourne and New York.
7.2. USAID PREDICT. Marcela Uhart, University of California, Davis (2016) Livestock Sampling Methods: Cattle, Sheep, Goats, Camels, and Swine.
7.3. University of South Florida. (2002) Standard Operating Procedures. Histopathology. SOP#: 019.1

8. Annexure

A. 10% Buffered Neutral Formalin

100ml Formalin (37-40% stock solution) 900ml Water
4g/L NaH_2PO_4 (monobasic)
6.5g/L Na_2HPO_4 (dibasic/anhydrous)

- Formaldehyde is a pungent, colorless gas which dissolves in water and reaches saturation at 37-40% formaldehyde.
- This can therefore be regarded as 100% formalin. Usually with 10-15% methanol added to prevent polymerization to a solid (paraformaldehyde).
- This commercial preparation is regarded as 100% formalin and the 10% solution contains approx. 4% formaldehyde.

The final stock solution required is (in ml) =Required concentration (%) X Final diluted solution (ml)
Stock solution concentration (%)

Recommendation for 37-40% stock solution:
If a solution of formaldehyde is clear, colorless and has no precipitate, and has been stored at room temperature in a tightly sealed bottle that has not been exposed to sunlight, it is

considered good, however it is not recommended for using a stock solution older than 1 year.

Bottles of 37% formaldehyde that are already opened should not be used more than six months. Hence, it is recommended to procure formaldehyde more frequently in smaller quantities than large volume jars.

B. 10% buffered formalin

- The formaldehyde has a greater chance for oxidation in this concentration of tissue fixative and eventually the solution will start to drop in pH, in spite of the buffer.
- It is recommended that 10% buffered formalin solutions be used no longer than 3 months after they were initially mixed. The solution should be clear, colorless, with no precipitate and the pH should not be below 6.5.

SOP No: NCAH/LSU/HISTO 02
Title: SOP on Sample Collection by Fine Needle Aspiration (FNA)
Version No: 1, Page: 4
Issue Month/Effective Date: May 2026
Revision Summary: New
Supersedes Version No:
Prepared by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL, BFDA, DoFPS

1. Scope

Collecting tissue samples from cutaneous and sub-cutaneous masses for cytological analysis

2. Objective

To guide the laboratory personnel in proper collection and handling of tissue samples using fine needles for cytology.

The procedure is simple, accurate, fast, and economical. It is minimally invasive and usually does not typically require general anesthesia or sedation.

3. Personal Protective Equipment & Hygiene

3.1. Wash hands thoroughly or sanitize prior to and after FNA

3.2. Personal protective equipment appropriate to the setting should be used but is optional (sterile gloves, mask, drapes, etc. if in the operating room. Gloves outside of operating room).

4. Materials

4.1. Sterile hypodermic needle, 22-25 gauge, ¾ to 1½ inch length

4.2. Syringe(s), 3-12ml

4.3. Glass microscope slides

5. Procedure

5.1. **Lymph node**

5.1.1. ***Position***

- To collect cells from a lymph node or superficial mass via FNA, restrain the animal in an appropriate position allowing for adequate access to the sampling site.
- Isolate the desired lymph node in the non-dominant hand.

5.1.2. ***Preparation of the site***

- No preparation is necessary in most cases but in immunocompromised animals, clip and prepare the site with chlorhexidine scrub or betadine scrub according to the Sterile Scrub SOP or wipe with alcohol.

5.1.3. ***Sampling methods:***

a. *Needle-on method for sample collection*

- Carefully introduce the needle with an attached syringe into the lymph node.
- Apply negative pressure by withdrawing the syringe plunger.
- This step may need to be repeated in a brisk fashion to gather a
- sufficient amount of sampling material.
- Partially withdraw the needle and redirect it into the lymph node, repeating
- the aspiration process.
- Release the negative pressure by allowing the syringe plunger to retract on
- its own.
- Withdraw the needle and syringe from the lymph node. Separate the
- needle from the syringe.

b. *Needle-off method for sample collection*

- Introduce a hypodermic needle into the desired lymph node without a syringe attached.
- Partially withdraw needle and redirect into target in multiple directions (manipulate in a cutting motion radially throughout the lesion to ensure adequate sampling).
- Remove the needle from the lymph node.

5.2. **Fine needle aspiration in dogs and cats**

5.2.1. Draw air into a syringe, attach the needle used for sampling, and expel the contents of the needle in one swift motion onto a ready microscope slide or slides.

5.2.2. Oppose the slides deposited tissue with another glass slide and with a smooth, gentle motion, guide the new slide across the sample, creating even distribution of cells on the slide.

5.2.3. Allow slides to air dry or first fix the slides with alcohol or another fixative and allow the air to dry.

5.2.4. Stain slides (Diff-Quik or other) or submit to pathology unstained.

5.2.5. Dispose of the needle in a sharps container.

Refer the annexure

Potential Adverse Effects, Mitigation, or Treatment

a. Pain

i. Pain secondary to fine needle aspirates is typically tolerable.

ii. Most patients do not require pain medications for this procedure.

b. Bleeding

i. Bleeding and bruising are the most common complications of FNA.

ii. Bleeding should decrease over time.

Continue to monitor the site until bleeding subsides.

c. Infection

i. Infection is rare as sterility is maintained throughout the procedure.

Avoidance measures:

ii. Clean site if dirty

iii. Withdraw needle out of the mass but not out of the skin before re-directing

iv. Use small gauge needles

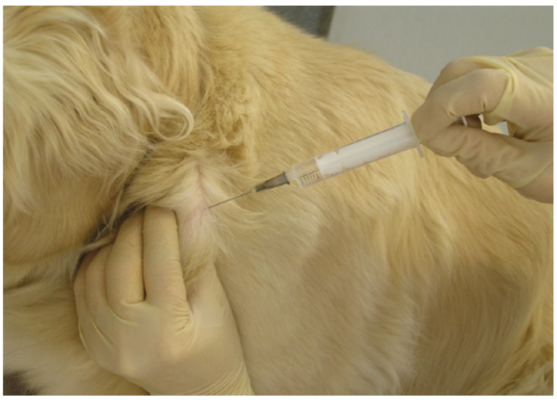
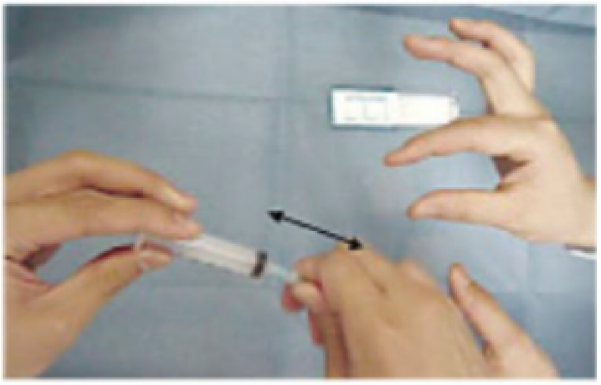
v. Apply direct pressure to the site if bleeding occurs

6. References

- http://veterinarymedicine.dvm360.com/vetmed/Medicine/Skills-Laboratory-How-to-make-
- high-quality-slide/ArticleStandard/Article/detail/773852?contextCategoryId

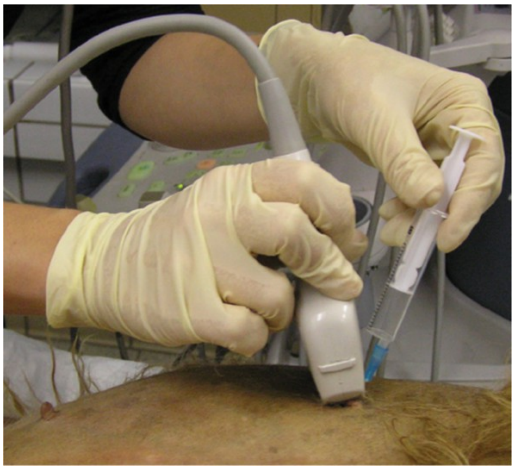
7. Annexure

7.1.Steps for FNA without ultrasound

The preparation of the smear is done immediately after the sample is taken. The sample is in the needle, so the syringe is removed, air is sucked into the syringe, and the needle is reattached. From this state, the sample is gently blown onto a slide or cover glass to prepare the smear.

7.2.Ultrasound guided FNA



SOP No: NCAH/LSU/HISTO 03
Title: SOP on Tissue Processing
Version No: 3, Page: 6
Issue Month/Effective Date: May 2026
Revision Summary: Tissue Processing
Supersedes Version No: 2
Prepared by: Histopathology Section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, RLDC, RVH & EC

1. Scope

This SOP describes the steps required to take an animal tissue from fixation to the state where it is completely infiltrated with a suitable histological wax and can be embedded ready for section cutting on the microtome.

2. Objective

To transform fresh biological samples into thin sections for staining and microscopic examination for diagnosis.

3. Principles

For microscopic analysis of cells and tissues requires the preparation of very thin, high-quality sections (slices) mounted on glass slides and appropriately stained to compare normal and abnormal structures. Usually, tissues are very delicate and easily get distorted or damaged, hence it is thus impossible to prepare thin sections from it unless it is chemically preserved or "fixed" and supported in some way whilst it is being cut. Tissue samples get infiltrated with a paraffin wax and subsequently be converted into a solid that has appropriate physical properties, which will allow thin sections to be cut from it.

4. Equipment and Consumables

4.1. Tissue Processor Machine – Bioevopeak
4.2. Wax dispenser (0-100 0C) – J.P.SELECTA
4.3. Microtome (0-60) microns) – Micro Tech CUT 4060
4.4. Microtome blade – Feather safety razor Co. Ltd, Medical Division, Japan
4.5. Circular water bath (0-100 0C) – J.P.SELECTA
4.6. Labelling sticker
4.7. Incubator (for drying slide) – Joun
4.8. Vacuum Pump – Rocker 300/K 17
4.9. Fume hood – Esco
4.10. Paraffin wax
4.11. Iso-propyl alcohol
4.12. Xylene
4.13. Ethyl alcohol

5. Procedure

5.1. Grossing

Tissues are trimmed in a 3mm section and placed into the tissue cassette.

5.2. Dehydration

Remove water by passing the tissue in ascending grades of alcohol to prevent undue shrinking of

tissues in the Auto processing machine.

- Ethyl alcohol 90% 2 hours
- Ethyl alcohol 70% 2 hours
- Absolute alcohol- I 1 hour
- Absolute alcohol –II 1 hour

5.3.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.

5.4.*Tissue Processing:* The above process is carried out in Tissue Processor.

5.4.1. Close the lid of the retort chamber.
5.4.2. Press MODULE button, select appropriate PROGRAM number from the screen and press that number on the Program Panel.
5.4.3. Press START.
5.4.4. The first step on the screen is highlighted in white.
5.4.5. 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.
5.4.6. Then next morning the cassettes are taken out of the tissue processor, and the tissues are embedded into metal mould.

5.5.Tissue embedding

5.5.1. **The paraffin is melted at 65^{0} C to 70^{0} C temperature and poured to mould base, immediately place impregnated tissue on melted paraffin filled in the mould base and allowed to cool the mould base.**
5.5.2. The processed tissue is orientated within a metal mould which is then filled with molten paraffin wax.
5.5.3. A labelled cassette is then placed on top and also filled with wax in the form of block and allowed to set solid in a cool chamber for sectioning the tissue.

(Refer SOP for tissue embedding)

5.6.Sectioning

5.6.1. Once all the blocks are removed from the molds, 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.
5.6.2. 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.
5.6.3. 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).
5.6.4. 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 Super frost glass slide (or glass slide coated with egg albumen) being careful to position the section in the middle of the slide.

5.6.5. 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.

5.6.6. 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.

6. Quality Control

The quality control reference and the troubleshooting guidelines are described in the annexure.

7. Waste Disposal

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

7.2. Xylene is highly inflammable hence should be properly disposed of.

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

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

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

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

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

All the waste generated is disposed of in a waste container and autoclaved.

8. References

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

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

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

8.4. 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 Livingstone Ltd. London, UK.

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

9. Annexure

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 doesnot 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 isopropanol	• 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

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

SOP No: NCAH/LSU/HISTO 04
Title: SOP on Tissue Embedding
Version No: 3, Page: 4
Issue Month/Effective Date: May 2026
Revision: Summary: New Inclusion
Supersedes Version No: 2
Prepared by: Histopathology Section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, RLDC, RVH & EC

1. Scope

This SOP describes the procedures for carrying out the tissue embedding manually using paraffin before sectioning. Embedding tissues to make paraffin blocks enables comparatively thin sections to be cut with good definition.

2. Objective

Paraffin wax is kept molten at 60°C; and poured on the mould containing tissue samples.

3. Principles

Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould. actual embedding takes place when the paraffin- infiltrated tissue is placed in fresh paraffin and the latter allowed to cool.

4. Equipment and Consumables

4.1.Paraffin
4.2.Dry oven at 58°C
4.3.Fixed, cell laden construct.
4.4.Embedding cassettes
4.5.70% Ethanol
4.6.96% Ethanol
4.7.100% Ethanol
4.8.Xylene or xylene substitute

5. Procedure

5.1.Preparation of paraffin

- Paraffin
- Dry oven at 58°C

Fill ¾ of a suitable container with paraffin and put in the 58°C dry oven to melt.

Note: This may take several hours to melt. Do not increase the temperature of the oven, higher temperatures will make the paraffin hard and brittle.

5.2.Preparation of constructs

- Fixed, cell laden constructs
- Embedding cassettes

Put fixed, cell laden constructs in embedding cassettes and label the cassette properly with a pencil.

5.3.Dehydration

- Embedding cassettes with constructs
- 70% ethanol
- 96% ethanol
- 100% ethanol
- Xylene or xylene substitute
- Glass breakers

Follow following dehydration series either through 1) moving the cassettes with constructs between beakers with the different reagents or 2) by adding and removing the different reagents of the dehydration series to a beaker with the cassettes.

Handle both xylene and ethanol with care inside a fume hood with proper PPE.

1. 70% ethanol: 2 x 30 min
2. 96% ethanol: 2 x 30 min
3. 100% ethanol: 2 x 30 min
4. Xylene or xylene substitute: 3 x 30 min

Note 1: If fixed samples have been stored in 70% ethanol before embedding only 1 x 30 min of 70% is necessary.

Note 2: The constructs might shrink after the dehydration procedure depending on the bioink type.

5.4. Paraffin infiltration

- Paraffin at 58°C

Transfer cassettes with constructs to the beaker with melted paraffin. Let sit in the oven for 45 min.

Note 1: The transfer of the cassettes to the melted paraffin must be done quickly since the paraffin solidifies under 56°C.

5.5.Paraffin embedding

- Tissue embedding machine

Add the infiltrated samples to the cassette container of the tissue embedding machine.

If samples have been stored at room temperature before embedding let the excess paraffin melt away from the cassettes by leaving them in the cassette holder pocket for ~20 min. If proceeding directly from infiltration, step 4, you can start embedding after a few minutes since the paraffin is already warm.

5.6. To embed samples:

5.6.1. Open the cassette.
5.6.2. Fill a metallic embedding mould with paraffin.
5.6.3. With warm tweezers; transfer the constructs. from the cassette to the mould with paraffin and push the constructs (cross-section down) to the bottom of the mould.
5.6.4. Let it stiffen slightly on a cold plate so the sample stays at the bottom of the mould.
5.6.5. Add the lid of the embedding cassette on the top of the mould before the paraffin stiffens completely. Throw the bottom.
5.6.6. Leave the embedded construct at the cooling plate until it easily can be removed rom the mould (~20 min).

6. Quality Control

Tissue orientation should be proper

7. Waste Disposal

All the waste generated is disposed of in a waste container and autoclaved.

8. References

Paraffin Embedding - Protocol - CELLINK

SOP No: NCAH/LSU/HISTO 05
Title: SOP on Hematoxylin and Eosin (H & E) staining
Version No: 3, Page: 5
Issue Month/Effective Date: May 2026
Revision: Summary: Hematoxylin stain time increased to 4 min and eosin to 1 min.
Supersedes Version No: 2
Prepared by: Histopathology section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL

1. Scope

This SOP describes the process for staining of thin tissue sections to demonstrate the cell architecture.

2. Objective

To provide a clear contrasting view of the cell/tissue structure at light microscope level by imparting colour to elements being studied in the tissue sections for diagnosis.

3. Principles

Hematoxylin highlights nuclei in shades of blue to purple, revealing chromatin and nuclear organization, while eosin stains the cytoplasm and extracellular matrix in pink, offering contrast that makes cellular components easily distinguishable.

4. Equipment and Consumables

4.1.Apparatus

4.1.1. Floatation Bath/water bath
4.1.2. Slide Dryer
4.1.3. Glass slide
4.1.4. Fume hood

4.2.Reagents, solution and buffer

4.2.1. Haematoxylin
4.2.2. Eosin
4.2.3. Xylene
4.2.4. Absolute alcohol
4.2.5. DPX mountant
4.2.6. Egg albumin

5. Procedure

5.1. Deparaffinize the section by Xylol 5-10 minutes
5.2. Remove Xylol by absolute alcohol 85 % & 95 %
5.3. Wash in tap water
5.4. Stain with haematoxylin for 4 minutes
5.5. Wash in tap water
5.6. Wash in 70 % alcohol
5.7. Counter stain with eosin for 60 seconds
5.8. Dehydrate in alcohol grade of 95 % & 100 %
5.9. Clear with xylene (15 to 30 seconds)
5.10. Mount in Canada balsam or DPX Mountant
5.11. Keep slides dry and remove air bubbles, if any

Nuclei -Blue, with some metachromasia.

Cytoplasm-Various shades of pink, identifying different tissue components.

6. Quality Control

Control Slides

- Use a **known positive control tissue** (e.g., normal liver, kidney, or tonsil).
- Stain the control slide **with every batch** of tissue sample slides.
- Expected result:
  - **Nuclei:** crisp blue-purple
  - **Cytoplasm:** pink
  - **RBCs:** bright red/pink

Details in annexure 9

7. Waste Disposal

7.1. Xylene should be disposed as per the guideline of chemical waste of the waste management guidelines.

7.2. Xylene is highly inflammable hence should be properly disposed of.

8. 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.

9. Annexure

9.1.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

9.2.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	500 ml
Mercuric oxide	1.25 g
Or Sodium iodate	0.5g
Glacial acetic acid	20ml

The haematoxylin is 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 into 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
- De-ionised 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

SOP No: NCAH/LSU/HISTO 06
Title: SOP on Gram's staining
Version No: 3, Page: 4
Issue Month/Effective Date: May 2026
Revision: Summary:
Supersedes Version No: 2
Prepared by: Histopathology Section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL

1. Scope

The test is for bacteria which are obscured or invisible caused by cellular debris.

2. Objective

The procedure is for demonstration of gram positive and Gram-negative bacteria in the tissue sections.

3. 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.

4. Equipment & Reagents

4.1.Apparatus

a. Magnetic stirrer/hotplate
b. Staining rack
c. Fume hood
d. Glass slide
e. Microscope

4.2.Reagents, solution and buffer

- For preparation, refer appendix
- Crystal Violet
- Ammonium Oxalate
- New Fuchsin
- Potassium iodide
- Fuchsin
- Acetone
- Picric acid
- Tolouene

5. Procedure

5.1.	Deparaffinise and hydrate to deionised water.	
5.2.	Stain with Modified Hucker-Conn cystal violet	2 min.
5.3.	Quickly rinse in tap water	1-2 dips.
5.4.	Stain with Modified Gram's iodine solution	1 - 2 min.
5.5.	Quickly rinse in tap water	1-2 dips

5.6.	Decolourise with Acetone/Alcohol mixture until no more colour runs off. Approx.	10 - 20 DIPS
5.7.	Without rinsing, place in Working New fuchsin	1 min.
5.8.	Dip several times before timing	
5.9.	Quickly rinse with acetone	3 dips.
5.10.	Differentiate in Picric acid/acetone	10 dips.
5.11.	Quickly rinse with Acetone/Toluene	3 dips
5.12.	Rinse in Toluene x 2 and cover slip in DPX.	

Result interpretation

5.13.	Gram positive	Deep Blue
5.14.	Gram negative	Red
5.15.	Background	Yellow

6. Waste disposal

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

6.2. Used slides should be disposed of after autoclaving.

7. 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

8. 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.

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

9. 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 1000.0ml

. 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 a magnetic stirrer. Add extra deionised water to make it to 500 ml total and mix well.

NOTE: Change monthly.

d. 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.

e. New Fuchsin Working Solution
- New Fuchsin stock solution 21.0 ml
- Deionised water 189.0 ml

NOTE: Change monthly.

f. Absolute Alcohol - Acetone 1:1
- Absolute alcohol 1000.0 ml
- Acetone 1000.0 ml

NOTE: Change weekly.

g. 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.

h. Acetone – Toluene 1:1
- Acetone 1000.0 ml
- Toluene 1000.0 ml

NOTE: Change weekly

SOP No: NCAH/LSU/HISTO 07
Title: SOP on Ziehl Neelson's Acid Fast Staining
Version No: 3, Page: 4
Issue Month/Effective Date: May 2026
Revision: Summary:
Supersedes Version No: 2
Prepared by: Histopathology Section, LSU, NCAH
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL

1. Scope

To detect the acid-fast organisms in the tissue samples

1. Objective

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

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. Equipment & reagents

3.1.Apparatus

1. Magnetic stirrer/hotplate
2. Staining rack
3. Fume hood
4. Glass slide
5. Microscope
6.

3.2.Reagents, solution and buffer

For preparation please refer appendix

1. Basic fuchsin
2. Hydrochloric acid
3. Phenol crystals
4. Alcohol 100%
5. Methylene blue

4. Procedure

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

4.1.Deparaffinise and hydrate to deionised water.
4.2.Stain slides with filtered Carbol Fuchsin. 30 min.
4.3.The staining with Carbol Fuchsin solution is carried out in the fume hood due to the staining solution containing Phenol.
4.4.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 into 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.

4.5. 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.
4.6. Place the lids on the Petri plates, as to avoid evaporation of the stain.
4.7. Rinse well in tap water.
4.8. Differentiate with 1% Acid Alcohol until the section is pale pink.
4.9. Wash in running tap water. 8 min.
4.10 Place slides on a staining rack.
4.11 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.

Result interpretation

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

5. Waste disposal

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

6. 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 a false negative result.

Positive and negative control slides 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.

7. References

Manual of Histologic Staining Methods of the Armed Forces Institute, 3rd edition. Lee Luna (Editor).
Theory and Strategy in Histochemistry. Hans Lyon (Editor).

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

8. Appendix:

8.1.Reagents for Ziehl Neelson's Acid Fast stain NOTE: All eagent

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^{0}C. 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.

SOP No: NCAH/LSU/HISTO 08
Title: SOP on Giemsa staining for Cytology
Version No: 1, Page: 4
Issue Month/Effective Date: May 2026
Revision Summary: NEW
Supersedes Version No: 1
Prepared by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL

1. Scope

For Giemsa staining of the FNA or direct impression smear samples for cytology.

2. Objective

To identify or differentiate the tumor cells or inflammatory cells in the fine needle aspirated samples from the swellings or lymph nodes.

3. Principle

Giemsa solution is a mixture of methylene blue, eosin, and Azure B. The stain is usually prepared from commercially available Giemsa powder/Readymade Concentrated solution

4. Procedure:

- Fix the smear in methanol for 2-3mins.
- Put the prepared giemsa (1drop giemsa in 2ml of buffer solution or dist.water.
- Keep for 30 mins.Wash with running water and air dry.
- View under oil immersion at X100.

5. References

Trevor JW. Cytology (2025). MSD Veterinary Manual
Standard operating procedures for Laboratories. (2023). Sanjay Gandhi Post graduate Institute of medical sciences 2023

6. Annexure:

6.1.Reagents and working solutions

Stock reagent: Giemsa powder or commercially prepared stock solution (contains azure, methylene blue, eosin).

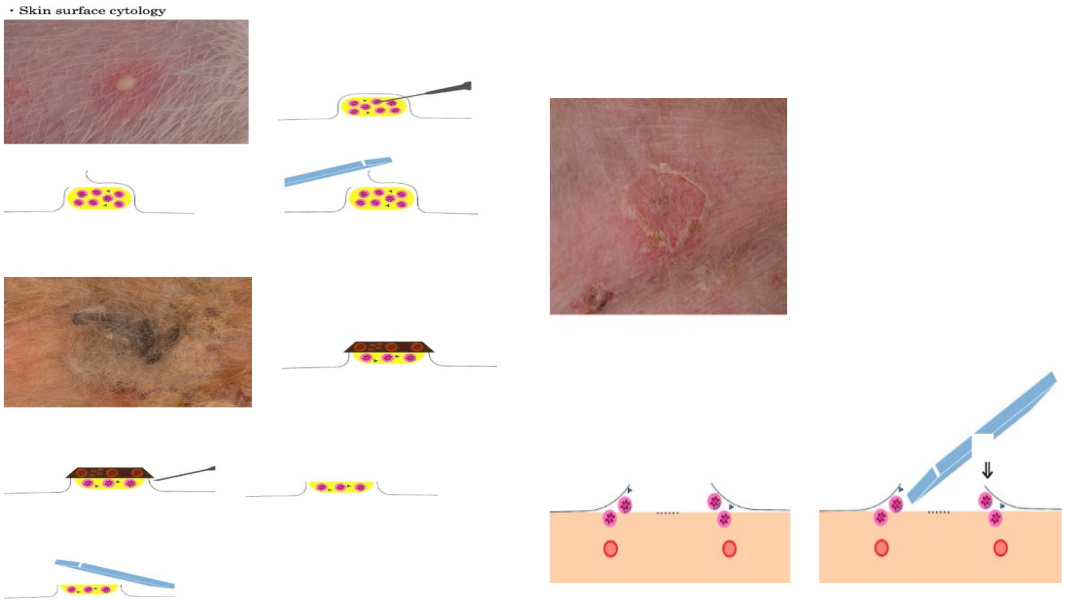
- Buffer: pH 6.8 phosphate buffer is commonly used to obtain the correct Romanowsky effect.

Working solution:

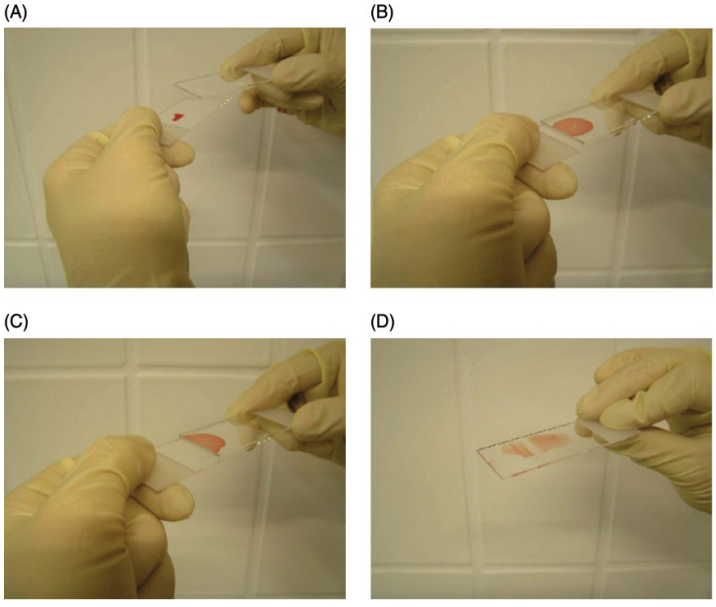
Dilute stock with a buffer (typical dilutions 1:10 to 1:20 depending on product) and let sit 10–15 minutes before use for best consistency.

6.2. Techniques

6.2.1. Technique of taking Impression Smear

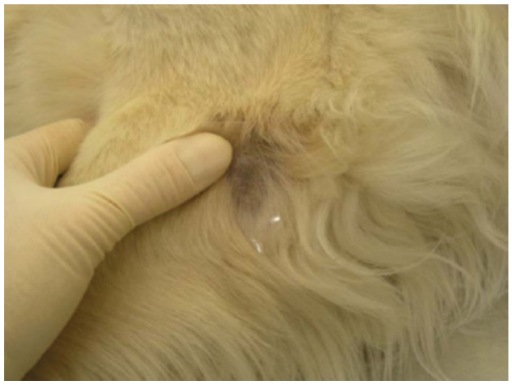


6.2.2.Technique for making smear (squashed, cello tape)

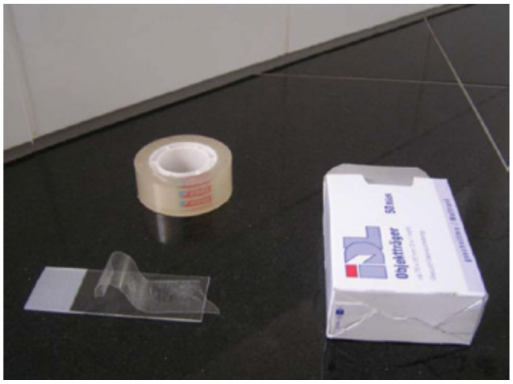


The squash preparation technique. (A) The fine-needle aspirate is placed on the glass slide by ejecting 3–5 ml air through the syringe and needle. (B) A second slide is gently placed on top of the first one. (C) The top slide is gently drawn over the bottom slide on which the aspirate has been deposited. (D) The top (spreader) slide is removed once it reaches the end of the bottom slide which can then be submitted for cytological examination.

(A)

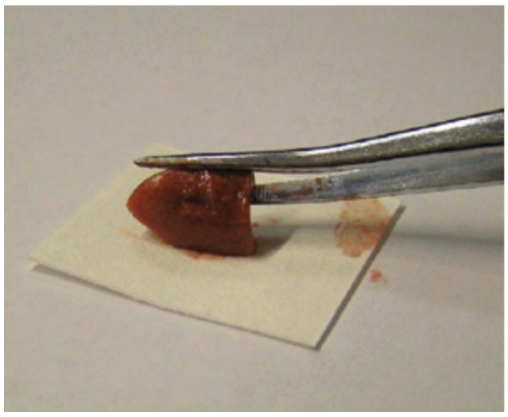


(B)

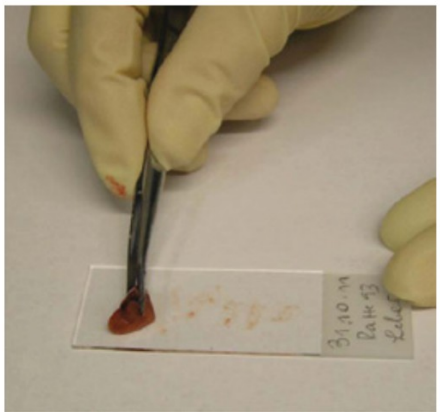


Sellotape imprint. (A) A strip of Sellotape is pressed several times onto the skin lesion. This technique is ideal to show micro-organisms (bacteria, yeasts) on the skin surface. (B) For preparation of a Romanowsky- stained Sellotape imprint, the piece of Sellotape is firstly put on the slide like an upturned 'u' (sticky side down), and the imprint can then be stained without fixation. After staining, the strip of Sellotape is put flat on the slide and can be then evaluated microscopically.

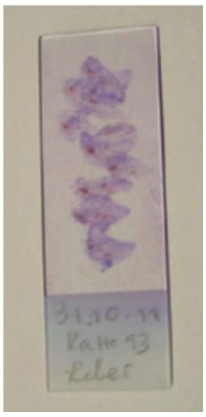
(A)



(B)



(C)



Impression smear of a liver biopsy. (A) Prior to preparing the impression smear, blood contamination is minimised by pressing the biopsy gently on a filter paper. (B and C) The imprint smear is prepared by touching the slide with the surface of the biopsy in several areas.

SOP No: NCAH/LSU/HISTO 09
Title: SOP on Trichogram
Version No:1, Page: 4
Issue Month/Effective Date: May 2026
Revision Summary: NEW
Supersedes Version No: 1
Prepared by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Reviewed by: Dr. N.K Thapa, Dr. Karma Choezang, Mr. Thrinang Wangdi, Mr. Rinzin Dorji, Ms. Punya Mata, Mr. Sonam Wangchuk
Approved by:
Application/Distribution: NCAH, NVH, RLDC, RVH & EC, DVL

1. Scope

To examine hair samples microscopically for diagnosis of dermatological conditions, including parasitic, fungal, and structural abnormalities.

2. Objective

To assess **hair shaft, root condition, and presence of pathogens** for accurate diagnosis of skin diseases.

3. Equipment

- Clean glass slides and coverslips
- Forceps
- Microscope
- 10% KOH (if required)
- Gloves

4. Procedure

4.1.Sample Collection

1. Select affected area (alopecia, lesions, scaling).
2. Pluck 5–10 hairs around the affected are with roots using forceps.
3. Ensure minimal contamination.

4.2.Slide Preparation

4. Place hair samples on a clean slide.
5. Add a drop of 10% KOH and mix
6. Place the coverslip gently over the sample.

4.3.Microscopic Examination

7. Examine under low power (10×), then high power (40×).
8. Observe:
    - Hair shaft structure
    - Hair root (anagen/telogen)
    - Presence of fungi, mites, or eggs
    - Breakage or abnormalities

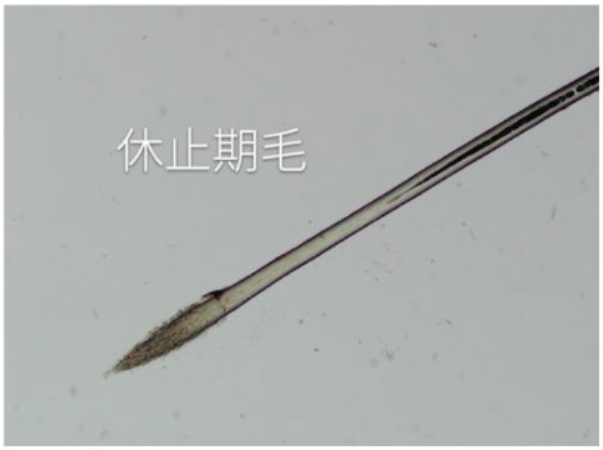
4.4.Result and Interpretation

1. Hair loss
   Hair splitting (+)
   - Hair splitting diseases

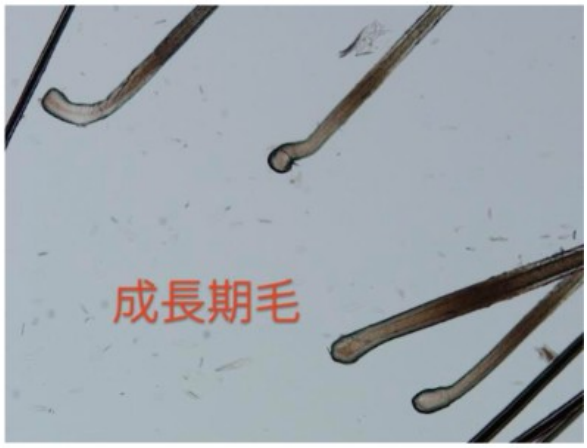
- Itching (+)
  Itchy diseases Parasitic, Allergic, Inflammatory, Neurological diseases
- Itching (-)
  Hair abnormalities: Dermatophytoses, Color dilution alopecia, Black hair dysplasia

Hair splitting (-) ・Hair loss diseases

2. Inflammation (+) Folliculitis
   - Pyoderma, Dermatophytoses, demodicosis, Granulomatous sebaceous adenitis .
3. Inflammation (-)
   - Hair growth cycle abnormalities Hair cycle arrest / Alopecia X, Hypothyroidism,
   - Hyperadrenalism, Sex hormone-related Cutaneous
4. Genetic disorders
   - Pattern hair fall, Familial dermatomyositis, Hair follicle dysplasia, Ectodermal Dysplasia
   - Others
     Flank alopecia, Alopecia after injection, Cutaneous lymphoma, Paraneoplastic alopecia Others



Resting phase



Growing phase

5. Waste Disposal

All the waste generated is disposed of in a waste container and autoclaved.

6. Reference

https://www2.zoetis.ca/content/_assets/PDF/Dermatology/Diagnostic-Techniques/Zoetis-Trichogram.pdf

7. Annexure

7.1.Trichogram Technique and reference

7.2.Lab Request Form

NATIONAL VETERINARY HOSPITAL
MOTITHANG, THIMPHU

Laboratory Request Form

PID: _____ Date: _____

Owner's Name: _____ CID no: _____

Address: _____ Phone No: _____

Pet Name: _____

Species: _____ Breed: _____ Sex/Age: _____

Requesting Dr: _____ SRN: _____ Ref. No: _____

Sample submitted(please tick)

BloodEDTA / Plain / Sodium Citrate / Heparin

Urine Voided / Catheterization / Cystocentesis

Faeces Milk Swab

Smear FNATissue

Body fluidSkin scrapping

Others _____

Tests required (please tick)

Haematology and Biochemistry

Complete Blood Count (CBC) Blood smear exam Blood parasite

Serum Biochemistry

Cytology

Skin scrapping exam KOH Trichogramimpression/Swab cytology

Urine sediment exam Faecal exam FNA cytology

Body fluid cytology

Others

UrinalysisBody fluid biochemistry Fungal Culture

Culture & sensitivity testing (CST) Rapid tests(specify) Histopathology

Others _____