The Examination for and of Spermatozoa

1 PURPOSE AND SCOPE

This method describes the microscopic examination of smears for the presence of spermatozoa. It includes the examination of items contained within the Sexual Assault Investigation Kits (SAIKS) that are assembled by either QHSS or other external companies such as Medi-Redi (owned by House with No Steps).

This SOP also includes workflow diagrams pertaining to examination of items in alleged sexual assault cases. These diagrams show the steps necessary in these examinations which include AP and PSA screening, along with microscopic examination for spermatozoa.

2 ACTIONS

2.1 Interpretation

1 The basophile haematoxylin stains the deoxyribonucleic-acid (DNA)/histone rich base of the sperm head deep purplish-blue. The acidophile eosin stains the acrosomal cap pink and, in intact-spermatozoa, also stains the tail pink.

2 The use of counterstaining differentiates spermatozoa from most cell debris and can assist in the differentiation of human spermatozoa from common animal spermatozoa.

3 Confusion with yeasts, especially monilia, can occur and extreme care must be taken when monilial infections such as thrush are suspected. With experience, spermatozoa and yeasts can be distinguished by size and/or the presence of cell walls.

2.2 Slide Preparation (for AP positive stains and Sexual Assault kits with no slides)

1 Use new slides and clean with ethanol. Label with the sample ID, date, case number and sampler's initials using a pencil only.

2 Use clean, flamed instruments.

3 Create a suspension from the exhibit by one of the following methods,

I. Scrape the stained area into a 1.5ml eppendorf tube. Add drops of distilled water to the tube until the scraping is covered. Vortex thoroughly.
II. Excise the stained area and cut into small pieces. Place pieces into a 1.5ml eppendorf tube and add drops of distilled water to the tube until the pieces are covered. Vortex thoroughly.
III. If slide is being prepared from a swab, excise the cotton from the swab and cut the cotton into small pieces. Place the pieces of cotton into a 1.5ml eppendorf tube and add drops of distilled water to the tube until the pieces are covered (approx 150-300µl). Vortex thoroughly.

4 Add a drop of the recently vortexed suspension to the labelled slide.

5 Dry the slide on a heat block. If a heat block is not available, heat-fix the slide by passing it over a flame with the material to be stained uppermost.

2.3 Slide Stainer

The slide can be stained in the automatic slide stainer in Histology. If this is not available, see manual staining procedure in Appendix 1.

2.4 Microscopic Examination

1 Examine slide using the x40 or x100 (oil immersion) objective. Score the number of spermatozoa observed (use the standard microscopy form, 17037 or the Sexual Assault Investigation Kit form, 17032).

0	(0)	None seen
<+	(<1+)	Very hard to find (Use vernier)
+	(1+)	Hard to find
++	(2+)	Easy to find
+++	(3+)	Very easy to find
++++	(4+)	Abundant

2 Note whether spermatozoa are intact (heads and tails) or non-intact (heads only). Look for epithelial cells and whether there are bacteria or yeast present. Human spermatozoa are distinguished from non-human mammalian sources by their morphology and by their behaviour toward HE, resulting in a purple base and clear cap (see Section 2.5).

3 If limited sperm are located, not the location on the slide as per the current laboratory procedure and/or take photographs.

2.5 Animal Semen

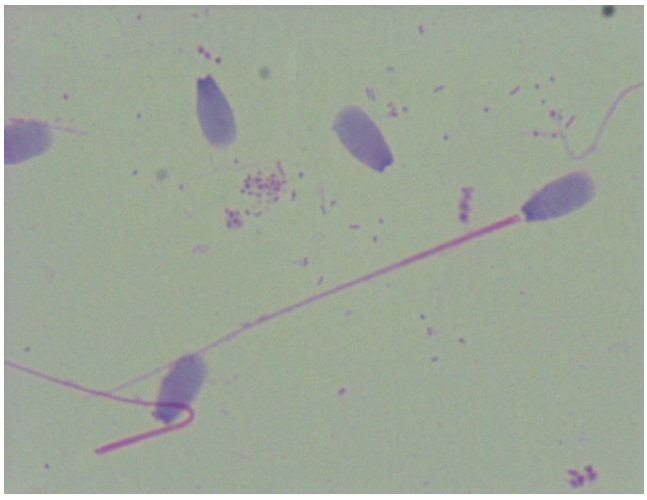
1 Identification or differentiation of animal spermatozoa is an extremely unusual request, however its possible presence should always be considered. Microscopy and our molecular biology protocols will differentiate human from animal spermatozoa.

2 Special stains are available to assist in the differentiation/identification of human/animal spermatozoa. However H/E and routine molecular biology techniques will resolve the issue, and if non-human reactions are obtained there will be support for an opinion.

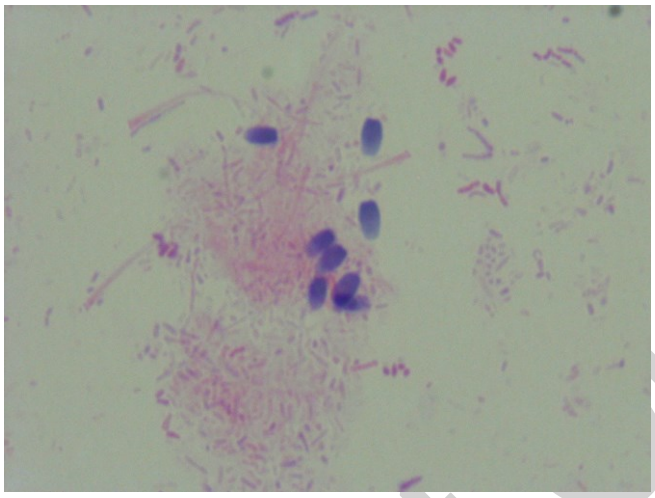
2.6 Animal Spermatozoa Images

1 The images of animal spermatozoa given below are observations from a limited number of samples. It must be borne in mind that there may be variations in shape and size according to age, breed, or individuality of animal concerned.

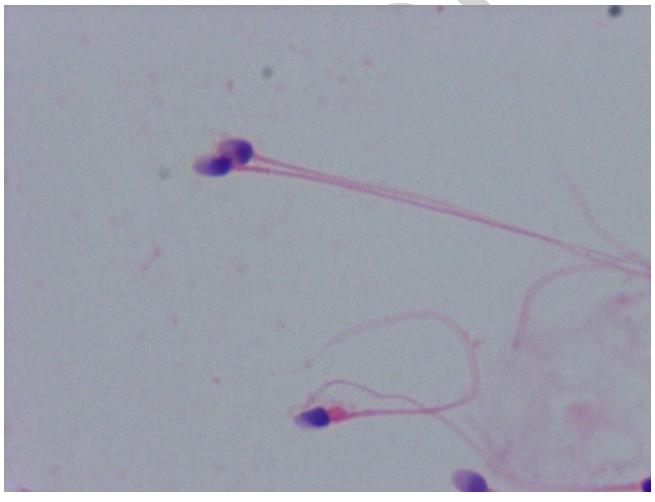
2 The following images are meant to assist in the formation of an opinion. Do not attempt to diagnose an animal spermatozoa species and do not rely on immune antisera. If animal non-human reactions (i.e. negative reactions) are given at DNA quantification stage, a typical opinion could be, “spermatozoa present did not appear to be of human origin. No human DNA was detected”.



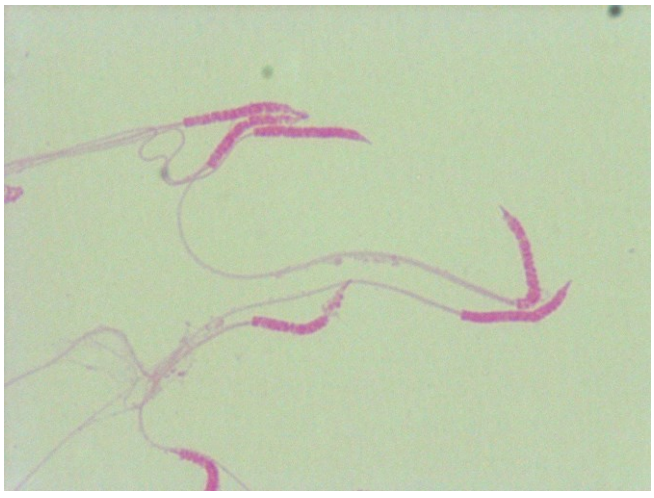
Bull Sperm at x1000 magnification. (Oil immersion) Sperm head lengths ~6-7µm.



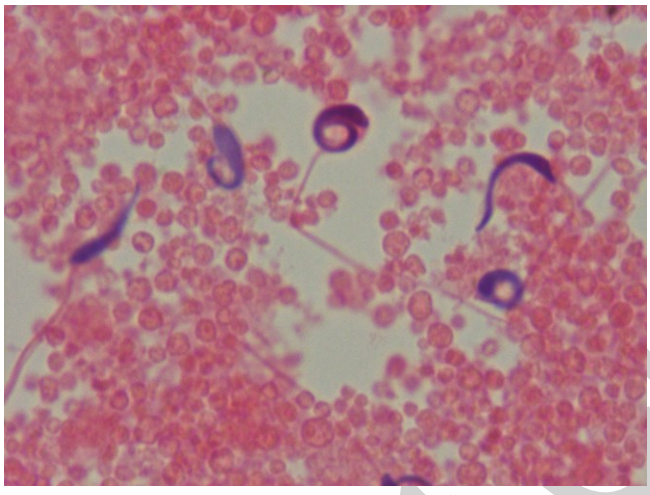
Cat Sperm at x1000 magnification. (Oil immersion) Sperm head lengths ~3-3.5µm.



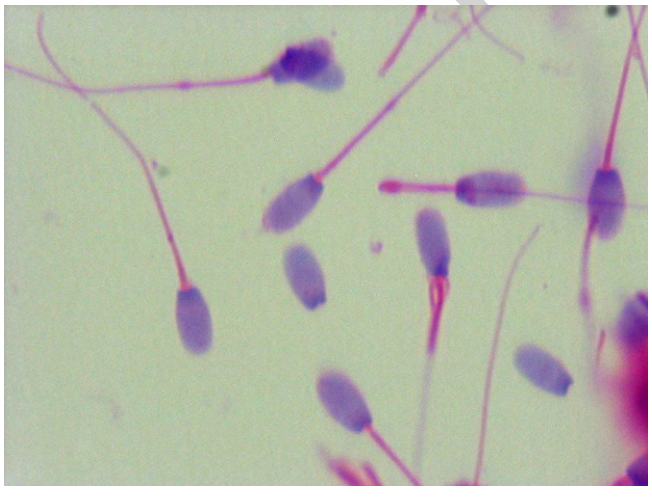
Human Sperm at x1000 magnification. (Oil immersion) Sperm head lengths ~3-4µm.



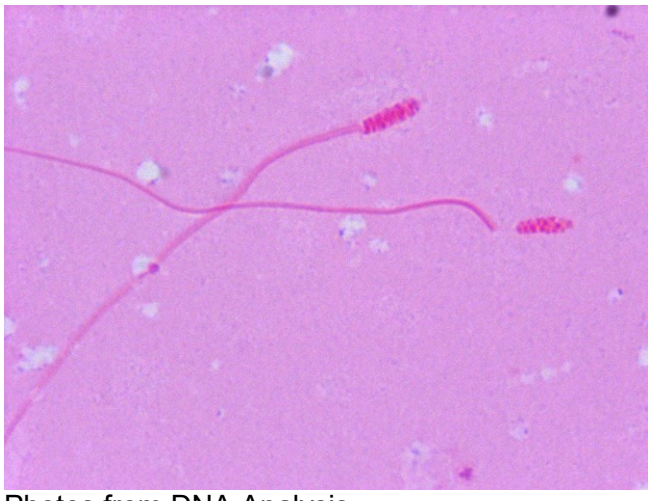
Kangaroo Sperm at x1000 magnification. (Oil immersion)
Sperm head lengths ~9-11µm.



Koala Sperm (uncurled) at x1000 magnification. (Oil immersion)
Sperm head lengths ~8-9µm.



Pig Sperm at x1000 magnification. (Oil immersion)
Sperm head lengths ~6-6.5µm.



Possum Sperm at x1000 magnification. (Oil immersion) Sperm head lengths ~4-5µm.

Photos from DNA Analysis.

2.7 Spermatozoa Interpretation

If slides are stained properly spermatozoa should be easily distinguished from epithelial cells, cellular debris, fibres etc. Spermatozoa heads can look similar in shape and colour to yeasts. If in any doubt consult an experienced examiner.

The recovery of semen is dependent on a number of factors but not limited to

I. The amount of spermatozoa in the ejaculate
II. The amount of ejaculate
III. The environment the ejaculate is deposited on
IV. Washing
V. Douching
VI. Menstruation
VII. Efficiency of the sampling process
VIII. Time between ejaculation and sampling
IX. Storage of the samples
X. Natural drainage or degradation of spermatozoa in certain environments

With respect to the above influences, the time since ejaculation has occurred can only be estimated. A number of studies have been conducted regarding the persistence of spermatozoa in the vagina. References to these studies can be found in Appendix 6.2.

2.8 Penile Swabs

Submit for DNA testing even if the semen on the penile swabs is of no evidentiary value. Unprotected sexual intercourse with no ejaculation may leave foreign cells (vaginal, anal, oral) on the penis. Depending on the time of swab collection and the effectiveness of the swab, female DNA on the penile swab may be successfully profiled from an unwashed penis.

Penile swabs may be submitted for Differential Lysis if the case history is such that the complainant had intercourse with another male prior to the alleged offence. Transfer of semen from the previous partner to the alleged offender may occur and DNA may be obtained in the sperm fraction.

2.9 Examination of Sexual Assault Swabs

1 If serum coated, charcoal swabs or other unsuitable swabs/media are submitted, the client must be notified. These swabs should still be examined. The serum coated or transport media swabs can be submitted for analysis. Add ‘Manual DNA IQ’ as the

processing comment and email the Analytical Senior Scientist with the barcode numbers. If the supernatant is to be retained the sample will be processed by off-deck lysis and Manual DNA IQ. Register as per normal and email the Analytical Senior Scientist with the numbers. No processing comment is required.

2 If a smear has not been received, one will need to be made. See Section 2.2. Stain the smears with the H&E stainer in Histology. Examine the smears for spermatozoa noting the presence or absence of intact sperm, sperm heads, epithelial cells, white blood cells, bacteria and any other cells seen.

3 If no spermatozoa are found, perform AP tests on suspensions made from the swabs.

4 If a smear is negative for spermatozoa and positive for AP it is necessary to perform a PSA test to characterize the biological material as semen.

5 If smears are received with paper labels attached, photograph the slide with the label, remove the label and relabel with the Statmark Pen or a diamond pencil and rephotograph the slide with the new labels. See 20080 Photography of Exhibits in DNA Analysis.

6 Submit swabs in separate sterile 1.5mL tubes for differential lysis extraction if not already in a tube as part of the slide preparation.

2.10 Examination of Items Previously Screened by QPS

QPS Scientific Officers will sometimes perform AP tests on items. Confirmatory testing should be performed in this case, and re-AP testing or feedback on screening results may be required. (See flow diagram in Appendix 6.3)

3 REFERENCES

1 *Biology Methods Manual*, Metropolitan Police Forensic Science Laboratory, Great Britain, 1978.

2 Allard, J.E (1997). “The collection of data from findings in cases of sexual assault and the significance of spermatozoa on vaginal, anal and oral swabs.” *Science and Justice* V37(2): April; 99-108.

3 Allery, J.P., Telmon, N., Mieuset, R., Blanc, A., Rouĝe, D. (2001). “Cytological Detection of Spermatozoa: Comparison of Three Staining Methods.” *Journal of Forensic Sciences* V46(2): 349-351.

4 Chiasson, D.A., Vigorito, R., Lee, Y.S., Smialek, J.E. (1994). “Interpretation of postmortem vaginal acid phosphatase determinations.” *American Journal of Forensic Medicine and Pathology* 15(3): 242-246.

5 Collins, K.A., Bennett, A.T. (2001). “Persistence of Spermatozoa and Prostatic Acid Phosphatase in Specimens from Deceased Individuals During Varied Postmortem Intervals.” *American Journal of Forensic Medicine and Pathology* 22(3): 228-232.

6 Khaldi, N., Miras, A., Botti, K., Benali, L., Gromb, S. (2004) "Evaluation of Three Rapid Detection Methods for the Forensic Identification of Seminal Fluid in Rape Cases." *Journal of Forensic Sciences* July; 49(4):749-753.

6 Maher, J., Vintiner, S., Elliot, D., Melia, L. (2002) "Evaluation of the BioSign PSA Membrane Test for the Identification of Semen Stains in Forensic Casework." *The New Zealand Medical Journal* Feb 8:115(1147):48-49.

8 Montagna, C.P. (1996). "The recovery of seminal components and DNA from the vagina of a homicide victim 34 days postmortem." *Journal of Forensic Sciences* July 41(4): 700-702.

9 Randall, B. (1987). "Persistence of vaginal spermatozoa as assessed by routine cervicovaginal (Pap) smears." *Journal of Forensic Sciences* May 32(3): 678-683.

10 Ricci, L. R., Hoffman, S.A., (1982). "Prostatic acid phosphatase and sperm in the post-coital vagina." *Annals of Emergency Medicine* 11(10): 530-534.

11 Silverman, E. M., Silverman, A.G. (1978). "Persistence of spermatozoa in the lower genital tracts of women." *JAMA: The Journal of the American Medical Association* 240(17): 1875-1877.

12 Willott, G.M. and Allard, J.E. (1982). "Spermatozoa - their persistence after sexual intercourse." *Forensic Science International* 19(2): 135-154.

4 ASSOCIATED DOCUMENTS

QIS: 20080 - Photography of Exhibits in DNA Analysis

QIS: 17185 - Detection of Aspermic Semen in case Work Samples using the Biosign PSA11-WB Rapid Test for Prostate Specific Antigen

QIS: 17186 - The Acid Phosphatase Screening Test for Seminal Stains

QIS: 17037 - Microscopy of Smears Form

5 AMENDMENT HISTORY

Version	Date Issue	Author/s	Comment
1	Unknown	Unknown	Unknown
2	Unknown	Unknown	Unknown
3	Unknown	Unknown	Unknown
4	27 Nov 2002	V Ientile	Format updated, manual staining to appendix. Removed notes on examination of swabs, removed unpublished paper, as work wasn't completed.
5	19 Nov 2003	L Freney	Updated references
6	12 Jul 2006	J Howes/A Williamson	"Reference" put after "Actions".
7	05 Aug 2006	J Howes	Added in Sexual Assault Investigation Flowcharts, examination of SAIK Swabs, Photograph or Witness required for ++ (1+) sperm and PSA test.
8	23 Oct 2006	J Howes	Reporting results Eg. ++ **or** 2+
9	25 Jun 2007	J Howes	Unified grading scale comments. Added Crimelite flowchart.
9	13 Mar 2008	QIS2 Migration	Headers and Footers changed to new CaSS format. Amended Business references from

		Project	QHSS to FSS, QHPSS to CaSS and QHPS to Pathology Queensland
10	16 July 2010	A Lloyd	Removal of Crimelite in scope and the Crimelite flowchart. Changed section 2.2 to include use of suspensions. Removal of section 2.8 – Vaginal Secretions. Changes to section 2.10 to remove AP testing on smears positive to spermatozoa. Photograph or locations required for smear with 1 or 2 sperm seen. Clarification of flowchart regarding previously screened items by QPS. Changes to SAIK flowchart. Removal of animal sperm diagrams and insertion of photographs of animal sperm.

6 APPENDICES

1 Preparation of H & E stain and manual staining procedure

2 How long do spermatozoa remain in the vagina?

3 Workflow Charts

6.1 Preparation of H & E Stain

CHEMICALS

Absolute alcohols
WARNING: Ethanol liquid and vapour are combustible. May irritate eyes and skin. Health effects well known – substance of abuse.

Eosin (yellowish)
WARNING: Eosin (yellowish) can cause serious damage to the eyes. Avoid contact, wear PPE.

Haematoxylin
WARNING: Haematoxylin: the toxicological properties have not been investigated. Prevent contact with skin and eyes. Do not inhale or ingest. Wear PPE.

Sodium iodate
WARNING: Sodium iodate causes burns and is harmful if inhaled or swallowed. Protect eyes and skin.

Chloral hydrate (SLR)
WARNING: Chloral hydrate causes burns and is harmful if inhaled or swallowed. Protect eyes and skin.

Citric acid
WARNING: May cause skin irritation. Inhalation may cause irritation to mucus membranes. Avoid skin contact

Acetic acid
WARNING: Acetic acid is extremely corrosive and is harmful if inhaled or swallowed. Protect eyes and skin.

Hydrochloric acid
WARNING: Hydrochloric acid causes burns and is harmful if inhaled or swallowed. Protect eyes and skin.

NOTE: All the above chemicals are available from the Histology Section

PREPARATION OF REAGENTS

NOTE: All reagents prepared in the laboratory shall bear a label:

….(*enter details eg* 10% NaOH)….
Prepd from Lot/batch:……..
Date: ../../.. Initials: ……..
Expires:../../.. Store at:….°C
WARNING: Contains ……

Mayer's Haematoxylin

Preparation of solution:

Haematoxylin	1g
Distilled water	1000mL
Potassium or ammonium alum	50g
Sodium iodate	0.2g
Citric Acid	1g
Chloral hydrate SLR	50g

The haematoxylin, potassium alum and sodium iodate are dissolved the distilled water by warming and stirring or by allowing to stand overnight. The chloral hydrate and citric acid are added and mixture is boiled for 5 minutes then cooled and filtered.
This mixture is then ready for use and has a shelf life for over one year at room temperature.

Eosin

Eosin Y (eosin yellowish, eosin water soluble) C. I. No 45380 (C. I. Acid Red 87)

Use as a 1% solution in distilled water. Add 0.5mL of acetic acid to 1000mL of eosin Y

- **NOTE: This method provides a useful stain for spermatozoa and vaginal and buccal epithelium. It results in a dark purple nucleus with pink cytoplasm.**
- Place slide on staining rack over sink, flood with haematoxylin and leave for 5-10 minutes.
- Wash well in running tap water until smear “blues” (5 minutes or less)
- Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 seconds.
- Wash well in tap water until smears are “blue” again (5 minutes or less).
- Counterstain in 1% eosin for 10 minutes.
- Wash in running tap water for 1-5 minutes.
- Allow slide to dry on hot plate or on filter paper on the bench
- Mount in depex if desired.

6.2 How long do Spermatozoa remain in the vagina?

6.2.1 Other references mentioning persistence of spermatozoa in the vagina -

1 O.J. Pollack. 1963 Arch. *Pathology* 35 p140-184
- non-motile sperm 3-24 hrs.

2 Noble Sharp 1963 Canada. *Med. Ass.* J. 89
- non-motile sperm 7-12 hrs, exceptionally 18-24 hrs, unique case 3-4 days.

3 Gordon, Turner and Price 1965 *Medical Jurisprudence*
- 3-4 days

4 Morrison 1972 *Brit. J. Vener. Dis* 48 p141
- up to 9 days or 12 days in the cervix, sometimes after menstruation.

6.2.2 How Long Do Spermatozoa Remain Alive (Motile) In the Vagina?

The period for which spermatozoa may remain alive after deposition in the vagina may best be reviewed by quoting from the following workers in this field.

O.J. Pollak: 'The number of motile spermatozoa discernible in the vagina may be normal after one hour and markedly decreased after 2 hours; after 3 hours normally no spermatozoa are found.
Menstruation often prolongs motility in the vagina to as long as 4 hours compared with the normal period of 30 to 45 minutes.
Concerning human spermatozoa Weisman in his book "Spermatozoa and Sterility" (1941) summarises the periods of motility as follows:

Vagina…2 to 3 hours. Cervix…48 to 110 hours.

6.2.3 **Semen and Seminal Stains**, Arch. Path., 1943, 35, 140.

Samuel L. Siegler: 'Normally 10% of the spermatozoa are alive in the vagina at the end of 2 hours post coitum. Variations in number and motility depend upon the pH of the vagina and semen, quantity of semen deposited, bacteria and flora of the vagina and the time examined post-coitally. The author has seen motile spermatozoa in the vaginal pool after 8 hours. "Fertility in Women" (Wm.Heinmann Medical Books Ltd., 1945).

Lane-Roberts, Sharman, Walker, Wiesner and Barton: 'In most cases all intra-vaginal spermatozoa cease to move irreversibly within a few hours of coitus (Seguy and Vimeux, 1933; Hartman, 1932). Huhner (1928, 1937), who has paid much attention to the problems involved, regards 30 minutes to 3 hours as a common measure of survival in the human subject, reduction in this period signifying an abnormal condition involving lowered fertility. Our own observations support the view that spermatozoa survive in the vagina for only a few hours and that even in fecund couples the variations are considerable.

In several cases in which repeated examinations were possible before conception occurred, all motility ceased within one hour after intercourse. A fall of motility to 10% within 30 minutes is compatible with fecundity. On the other hand, spermatozoa may continue to move for 3 hours in a normal untreated vagina.' (Sterility and Impaired Fertility, Hamish Hamilton Medical Books, 1948).

Sidney Smith: 'The evidence points to a comparatively short life of the spermatozoa in the female tract and the period appears to get shorter with the number of observations. It is at present believed that the life of the spermatozoa in the vagina is a matter of hours.' (Forensic Medicine, 1955).

Gonzales, Vance, Helpern and Umberger: 'The motility of the spermatozoa in the specimen may give a clue to their length of stay as they remain motile from 30 to 60 minutes after deposition in the vagina.' (Legal Medicine, 1954).

Louis Portnoy and Jules Saltman: 'As for motility, the sperms are mostly found non-motile or dead. This is to be expected because, after a lapse of one to three or more hours, all the sperms will normally have been killed by the acidity of the vaginal secretions. There may be live sperms but more likely not.' (Fertility in Marriage, Signet Book, 1951).

6.2.4 How Long Do Spermatozoa (Non-Motile) Remain In the Vagina

Pollak in his comprehensive paper "Semen and Seminal Stains", states:

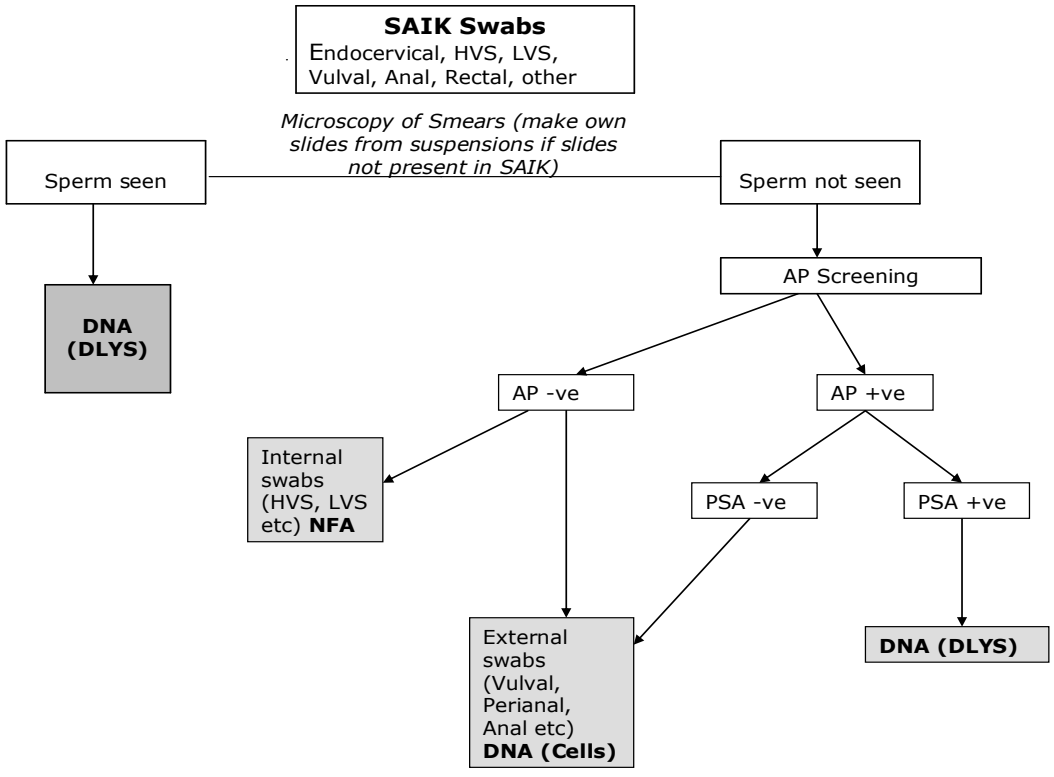
"Although the various authors give the period of their presence in the vagina as from 30 minutes to 17 days, one may safely consider the period for non-motile spermatozoa in the vagina after coitus to be 30 minutes to 24 hours."

Gonzales and others in their book "Legal Medicine" state:
"Some have claimed that spermatozoa may be demonstrated in the vaginal contents of non-fatal cases from 45 minutes to several days after the last coitus, but these considerations do not offer a satisfactory basis for estimating the duration of their residence in the vagina in as much as the time of the last coitus cannot be determined with precision. If they are non-motile, it might be difficult to determine the length of time they have been in the female tract."

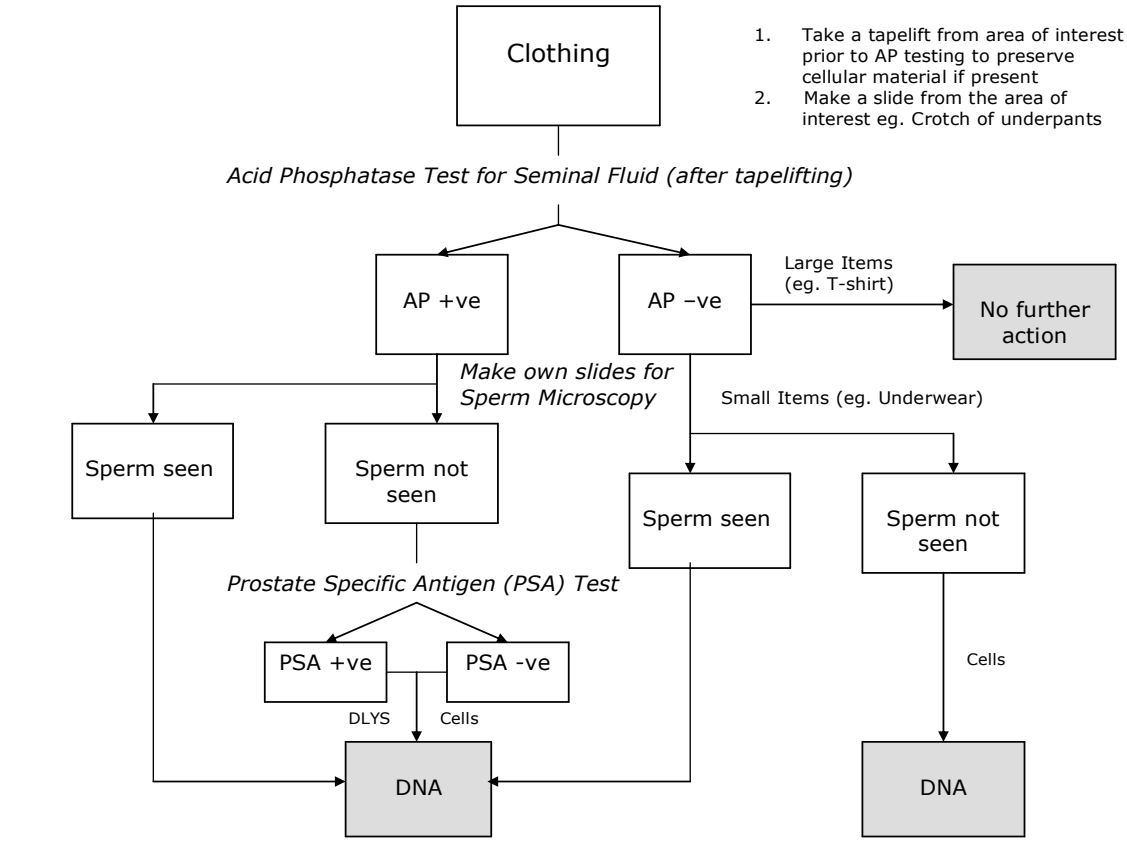
Gordon, Turner and Price in their book "Medical Jurisprudence" make this comment:
"The finding of spermatozoa on examination of a vaginal smear is indicative of an ejaculation into the vagina but it affords no evidence of the time of the ejaculation. In charges of rape, therefore, particularly in the case of married women, it becomes necessary to exclude the possibility of sexual intercourse having taken place before the assault. In this connection it should be noted that spermatozoa could be recovered from the vagina 3 to 4 days after their introduction. Some authorities claim that they may be recovered after a lapse of even longer periods."

6.3 Workflow Charts

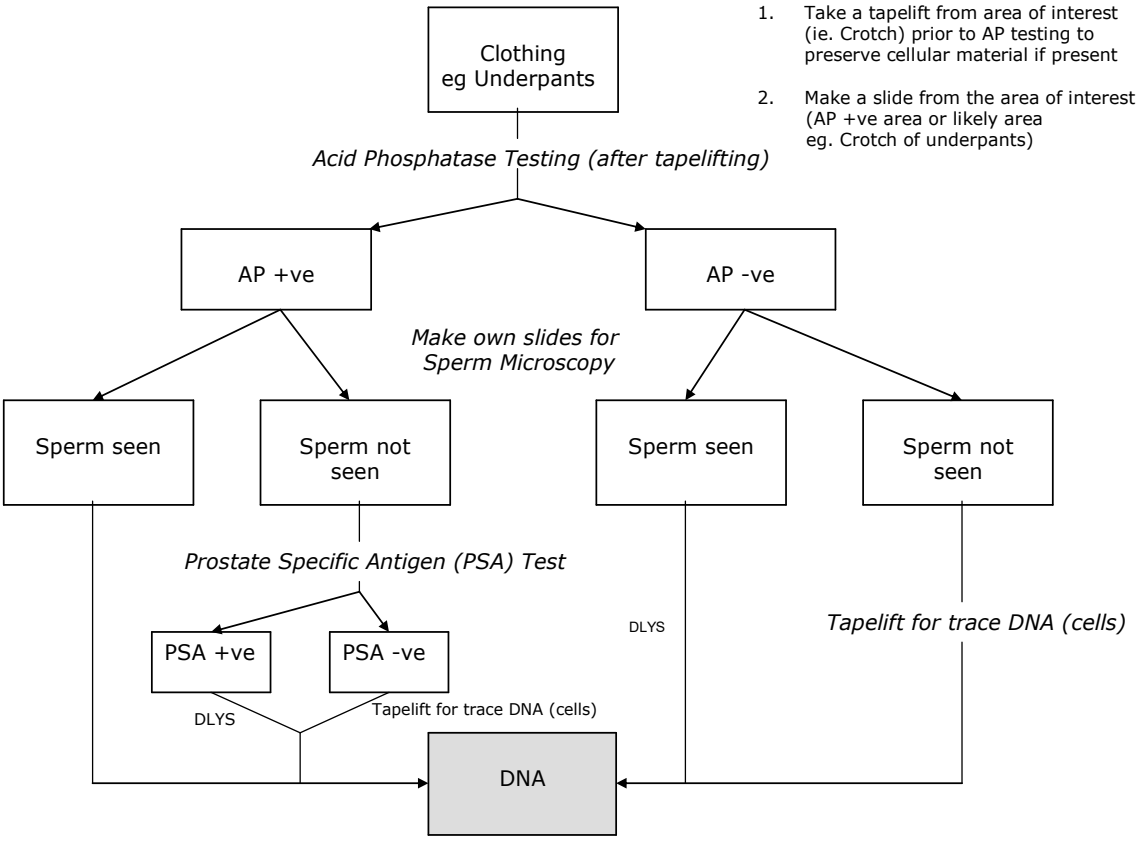
Examination of **Sexual Assault Investigation Kits** (SAIKs)



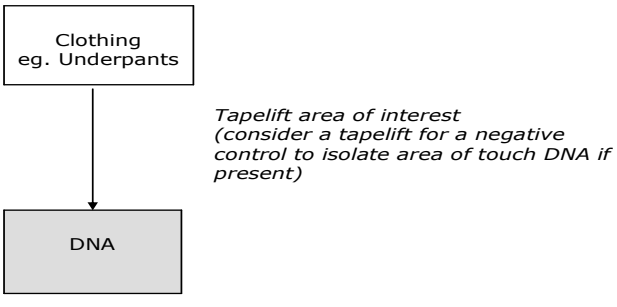
Examination of clothing in a **PENILE PENETRATION** case



Examination of a **DIGITAL and PENILE PENETRATION** case



Examination of clothing in a **DIGITAL PENETRATION ONLY** Case



Examination of Items previously AP screened by QPS Scientific Officers