HealthSupport
Queensland
Forensic and Scientific Services

The Examination for and of Spermatozoa

1 Purpose

The presence of spermatozoa is a confirmatory test for the presence of semen. This document describes the method by which a scientist performs microscopic examination for the presence of spermatozoa which includes the preparation of microscopic slides from exhibits, staining of slides and interpretation of the microscopic smears/slides for spermatozoa and other cellular material.

2 Scope

This Standard Operating Procedure (SOP) applies to all scientists performing the examination of items for the presence of semen.

3 Definitions

In this document, where reference is made to spermatozoa, it refers to human spermatozoa unless otherwise specified.

4 Background

The investigation of sexual assault cases may require the testing of exhibits collected as part of a forensic medical examination or scene examination for the presence of semen. Within the laboratory the detection of spermatozoa confirms the presence of semen. A reliable and accurate staining method is essential to aid the examining scientist the ability to differentiate between cellular types; most significantly spermatozoa from epithelial, yeast and white blood cells.

Currently the Haematoxylin and Eosin (H&E) stain is adopted for this process. The H&E staining method has been used for this purpose within the laboratory for many years. The haematoxylin (basic stain) stains the deoxyribonucleic-acid (DNA)/histone rich base of the spermatozoa head deep purplish-blue. The eosin (acidic stain) stains the acrosomal cap pink and the tail pink if the spermatozoa are intact (N.B. because Forensic DNA Analysis uses a water based eosin stain, the acrosomal cap often appears very light pink or clear). The use of counterstaining differentiates spermatozoa from most cellular debris.

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.

Haematoxylin is a natural dye. Its active colouring agent is haematin, which is formed by the oxidation of haematoxylin. This oxidation process or “ripening” occurs when haematoxylin solutions are allowed to stand for several days. However, the process can be accelerated with the introduction of an oxidising agent such as sodium iodate. During

oxidation the haematoxylin loses two atoms of hydrogen, and its formula changes from $C_{16}H_{14}O_6$ to $C_{16}H_{12}O_6$. Sufficient haematoxylin should be left unoxidized in the solution so that natural oxidation can continue thus prolonging the shelf life and useability of the stain. Completely oxidized haematoxylin becomes colourless. As the oxidation process occurs when haematoxylin is exposed to light and continues over the life of the solution, haematoxylin should be stored in dark bottles until ready for use. Haematoxylin is an excellent nuclear stain. Haematin, via the aluminium ion mordant, binds to the anionic sites in the nuclei (a mordant is a substance that causes certain staining reactions to take place by forming a link between the tissue and the stain). At this stage the nuclei stain red, which is then converted to the blue-black colour when the pH is raised (by a weak alkali wash such as Scott's tap water substitute in some H&E staining methods) known as "blueing".

To avoid stain precipitation on the slide, the haematoxylin solution must be filtered. It should be changed immediately if staining quality deteriorates.

Eosin is an acid dye which combines electrostatically with the acidophilic tissue components such as cytoplasm. (an anionic dye that stains the cationic tissue components). Alcoholic and acidified solutions of eosin tend to stain much more vividly than do the aqueous solutions. With water soluble eosin, rinse in water very quickly or else eosin will wash out.

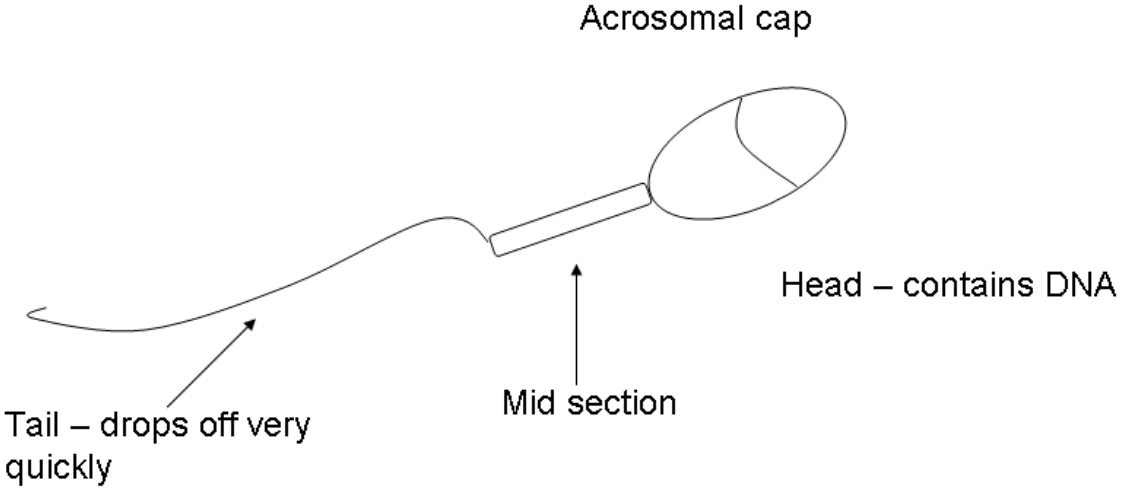


Figure { SEQ Figure * ARABIC } Spermatozoa

5 Actions- Staining procedure

5.1 Slide Preparation

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

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

1. Scrape the stained area into a 1.5/2ml tube. Add between100-300µl nanopure water with a POVA pipette to the tube until the scraping is moist. Vortex thoroughly.

2. Excise the stained area and cut into small pieces. Place pieces into a 1.5/2ml tube and add between100-300µl nanopure water with a POVA pipette to the tube until the pieces are moist. Vortex thoroughly.

3. If slide is being prepared from a swab, excise the material from the swab and cut the material into small pieces. Place the pieces of material into a 1.5/2ml tube and add between100-300µl nanopure water with a POVA pipette to the tube until the pieces are covered (approx 200µl). Vortex thoroughly.

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

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 once the suspension has dried.

5.2 Slide Staining

Microscopic slides are stained using Haematoxylin and Eosin (H&E). The method for preparing the staining reagents, and performing manual staining is as per procedure detailed in Appendix 1.

Note: registration of the reagent and association to individual exhibits is detailed in QIS 24469 (Batch Functionality in AUSLAB)

5.3 Microscopic Examination

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

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

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 H&E, resulting in a purplish/blue head and light pink/clear cap (see Section 5.4).

If less than ten spermatozoa are located, for at least one spermatozoa, note the location on the slide with the use of the England Finder Graticule (see Appendix 3).

5.4 Animal Semen

Animal spermatozoa are morphologically different to human spermatozoa and react differently to staining. Where suspected spermatozoa are located which are morphologically different to human spermatozoa, the examining scientist should consider the possible presence of animal spermatozoa. N.B. Forensic DNA Analysis does not identify or characterise animal spermatozoa.

5.5 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

- The amount of spermatozoa in the ejaculate
- The amount of ejaculate
- The environment the ejaculate is deposited on
- Washing
- Douching
- Menstruation
- Efficiency of the sampling process
- Time between ejaculation and sampling
- Storage of the samples
- 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 2.

6 Records

Nil

7 Quality assurance/acceptance criteria

Controls are used to test the quality and validity of the staining reagents prior to use. A positive control slide should be tested prior to the staining of slides for microscopy, (once daily), each time a new batch of Haematoxylin and Eosin solution is prepared and when positive control slides are prepared.

A positive control slide is a known sample of human semen, which has been diluted.

7.1 Creation of H&E control slides

Collect human semen in a sterile green-capped “Falcon” tube. The tube is to be labelled with the following information:

- Sperm donor number
- Date and time of collection

The semen is to be stored within a freezer until required to create H&E positive control slides using the following process:

1. Clean heating block using bleach and 70% ethanol solution.
2. Clean frosted microscope slides with ethanol and label with white label (H&E Pos Ctrl: Sperm donor number; Lot No.).
3. Spread slides out on heating block to heat before use.
4. Clean automatic pipette with bleach and 70% ethanol solution.
5. Using a new filtered pipette tip, add 20µL of the neat semen to 10.0ml nanopure water using a clean 10ml tube. Vortex.
6. Add 20 µL of the diluted semen solution to each slide.

7. Heat fix the slides on a heating block at 50ºC for approximately 30 minutes.
8. Store the slides in labelled plastic slide box “Unstained H&E Positive Control Slide Storage”, and store the box in Rm 6124.

7.2 Testing and interpretation of control slides

The following process is used to test, interpret and record control slide results:

1. Remove a H&E control slide from slide box , label with date, initials, lot number and batch, and stain with H&E using the method in Appendix 1.
2. Dry slide on heating block at 50ºC.
3. Coverslip slide using mounting medium.
4. Examine slide microscopically in Rm 6119 as per section 5.3.
5. Completed control slides get transferred to a plastic box labelled H&E Control Slide Storage box #
6. Once a slide box is full of completed positive control slides, transfer the box to Rm 6106B, ‘Exhibit Room’ for long term storage.
7. Add audit entry to document that the control slide has passed control and what box it has been stored in (i.e. 2)

Acceptance of the reagents is based on the interpretation of the Positive control slide. The following criteria must be met before passing the reagent for use:

1. Spermatozoa head stains a deep purplish-blue.
2. Acrosomal cap stains light pink/clear.
3. Tail stains pink.

In the event the control slide fails the following process is to be completed:

1. Repeat the staining procedure with a new control slide and assess as above.
2. If the control slide fails again then discard the in-use staining batch and stain a new slide using a fresh batch from the stock solutions and assess slide as above.
3. If the control slide fails then a new batch of stock solutions must be prepared and the old solutions discarded, and assess the validity of the reagents as per this section.

8 Associated Documentation

QIS: 17037 Microscopy of Smears Form

QIS: 17142 Examination of Items

QIS: 17185 Detection of Azoospermic Semen in Casework Samples

QIS: 17186 The acid Phosphatase Screening Test for Seminal Stains

QIS: 20080 Digital Imaging in Forensic DNA Analysis

QIS: 23849 Common Forensic DNA Analysis Terms and Acronyms

QIS: 30800 Investigating Adverse Events in Forensic DNA Analysis Unit

QIS: 24469 Batch Functionality in AUSLAB

QIS: 32106 Examination of Sexual Cases

9 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 Brown, G. (1978) “An Introduction To Histo Technology”

5 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.

6 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.

7 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.

8 Leong, A S-Y. (1996)” Principles And Practice of Medical Laboratory Science Volume 1 Basic Histotechnology”

9 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.

10 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.

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

12 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.

13 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.

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

10 Amendment History

Revision	Date	Author/s	Amendments
0	Unknown	Unknown	Unknown
1	Unknown	Unknown	Unknown
2	Unknown	Unknown	Unknown
3	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.
4	19 Nov 2003	L Freney	Updated references
5	12 Jul 2006	J Howes/A Williamson	"Reference" put after "Actions".
6	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.
7	23 Oct 2006	J Howes	Reporting results Eg. ++ **or** 2+
8	25 Jun 2007	J Howes	Unified grading scale comments. Added Crimelite flowchart.
Version	**Date**	**Updated By**	**Amendments**
9	13 Mar 2008	QIS2 Migration Project	Headers and Footers changed to new CaSS format. Amended Business references from 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.
11	03 Feb 2011	A Lloyd	Amended use of vernier for slides to use of the England Finder Graticule.
12	31 Oct 2013	A Lloyd J Seymour-Murray	Removed animal sperm photos. Amended workflow charts, changed headings from CASS to HSSA. Change H&E solutions and staining, add England Finder information. Updated some hyperlinks.
13	03 July 2015	J Seymour-Murray	New template, update hyperlinks, some formatting updates and minor wording changes.

11 Appendices

1 Appendix 1: Preparation of H & E Stain and Manual Staining Procedure
2 Appendix 2: Persistence of Spermatozoa in the Vagina
3 Appendix 3: England Finder Package Insert

11.1 Appendix 1: Preparation of H & E Stain and Manual Staining Procedure

11.1.1 Chemical Hazards

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

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

Sodium iodate (NaIO3)
WARNING: Sodium iodate causes burns and is harmful if inhaled or swallowed. Protect eyes and skin.
Wear PPE and eye protection.

Glacial acetic acid
WARNING: Acetic acid is extremely corrosive and is harmful if inhaled or swallowed. Protect eyes and skin. Wear PPE and eye protection.

Pertex Mounting medium
WARNING: Pertex is irritating to eyes, respiratory system and skin.

Xylene
WARNING: Flammable. Harmful by inhalation and in contact with skin. Irritating to skin.

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:….ºC
WARNING: Contains ……

Or an individual label printed by the Bar-One Lite system (B1Lite on short cut).

11.1.2 Preparation of Eosin

Eosin –Water soluble (CI 45380)

- Eosin 5.0g
- Nanopure water 500mL

Procedure

1. Weigh 5.0g Eosin and add 500mL nanopure water in a flask.
2. Mix on the magnetic stirrer until completely dissolved.
3. Label the reagent bottle according to laboratory standards.

11.1.3 Preparation of Haematoxylin

Haematoxylin (CI 75290)

- Haematoxylin 2.5g

- Sodium iodate (NaIO3) 0.2g (Must be accurate)
- Potassium aluminium sulphate dodecahydrate (KAl(SO4)2.12H2O (potassium alum) 25.0g
- Nanopure water 350mL
- Glycerol 150mL
- Glacial Acetic acid 10mL

Procedure

1. Weigh 2.5g Haematoxylin and dissolve in 350mL nanopure water in a flask. Mix on the magnetic stirrer. (Haematoxylin must be fully dissolved before adding the other reagents)
2. Weigh 25.0g potassium aluminium sulphate dodecahydrate and add to haematoxylin solution, continue stirring.
3. Weigh 0.2g sodium iodate and add to Haematoxylin solution, continue stirring until reagents are dissolved.
4. Measure 10mL glacial acetic acid and add to haematoxylin solution, continue stirring.
5. Measure 150mL glycerol and add to haematoxylin solution, continue stirring for 5 minutes.
6. Filter into a clean dark reagent bottle. (Haematoxylin oxidises)
7. Label the reagent bottle according to laboratory standards.

11.1.4 Register lot details of reagent

- Register lot details using QIS 24469 Batch Functionality in AUSLAB and QIS 17165 Receipt, Storage and Preparation of Chemicals, Reagents & Kits (Appendix 3).

11.1.5 Manual Staining Procedure

Staining is performed in the staining fumehood in Rm 6124, main laboratory, Forensic DNA Analysis.
Procedure:

1. Place slide on staining rack over sink, stain with haematoxylin for five minutes (add one volume and let rest)
2. Wash with nanopure water.
3. Stain with eosin for one minute.
4. Wash with nanopure water (quick wash).
5. Allow to dry on hot plate.
6. Mount coverslip in Pertex

11.1.6 Staining Quality Controls

The following quality steps should be implemented:

- Haematoxylin should be filtered before use as the crystals in solution can result in stain deposit on the slides which affects the reading of the slide. (Once a week)
- Haematoxylin differentiates better if it is matured for 3 to 4 days before use.
- Eosin should be filtered once a week.

When stored in dark bottles, haematoxylin may keep for up to 12 months and eosin for up to 12 months (dependent on control slide result).

11.2 Appendix 2: Persistence of Spermatozoa in the Vagina

Literature provides a range of time periods for the persistence of non-motile spermatozoa in the vagina:

- Up to 24 Hours[1]
- Up to 3-4 days[2]
- Up to 9 days or 12 days in the cervix, sometimes after menstruation[3]
- Up to 3 to 4 days, but may be longer[4]

Literature provides a range of time periods for the persistence of motile spermatozoa in the vagina:

- 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.[5]
- Spermatozoa remain motile in the vagina for 2 to 3 hours and in the cervix for 48 to 110 hours[6]
- 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.[7]
- 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.[8]
- 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.[9]

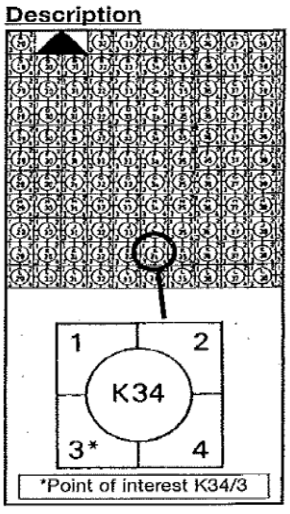
[1] O.J. Pollack. 1963 Arch. *Pathology* 35 p140-184
[2] Gordon, Turner and Price 1965 *Medical Jurisprudence*
[3] Morrison 1972 *Brit. J. Vener. Dis* 48 p141
[4]Gordon, Turner and Price 1965 Medical Jurisprudence
[5] O.J. Pollack. 1963 Arch. *Pathology* 35 p140-184
[6] Weisman 1941 *Spermatozoa and Sterility*
[7] Wm.Heinmann Medical Books Ltd 1945 *Fertility in Women*
[8] Hamish Hamilton Medical Books 1948 *Sterility and Impaired Fertility*
[9] Gonzales, Vance, Helpern and Umberger 1954 *Legal Medicine*

11.3 Appendix 3: England Finder Package Insert

The England Finder

The England Finder is a glass slide marked over the top surface in such a way that a reference position can be deduced by direct reading, the relationship between the reference pattern and the locating edges being the same in all finders. The object of the Finder is to give the microscopist an easy method of recording the position of a particular field of interest in a specimen mounted on a slide, so that the same position can be re-located using any other England Finder on any microscope.

Description



The England Finder, a section of which is illustrated, consists of a glass slide 3" x 1" marked with a square grid at 1mm intervals. Each square contains a centre ring bearing reference letter and number, the remainder of the square being subdivided into four segments numbered 1 to 4. Reference numbers run horizontally 1 to 75, and letters vertically A-Z (omitting I). The main locating edge is the bottom of the slide which is used in conjunction with either the left or right vertical edge of the slide, according to the fixed stops of the stage of the microscope, all three locating edges being marked with arrow heads. The label on the finder should always appear visually at the bottom left corner when through most microscopes the reference image will appear correct.

In the illustration (part shown), the point of interest is marked with a cross, and will be seen to lie in the third segment of the square of reference K34, hence the England Reference is K34/3.

Method of Use

1. Mark the specimen slide with a label on the left indicating with arrows which sides are to be used for location. Place the slide on the stage of the microscope bringing the bottom long edge in contact with the base stops of the stage and then sliding either left or right into contact with the vertical fixed stops as appropriate. It is important always to obtain the main location of the slide and finder on the base stops first.

2. Having examined the specimen in the normal way and found a point of interest, bring this to the centre of the field of view (a crosswire in the eyepiece is useful in this respect).

3. Taking care not to alter the position of the fixed stops of the stage, remove the slide and replace with the England Finder, again bringing the bottom edge in contact first and sliding to the appropriate vertical stop, the label of the Finder being at the bottom left corner.

4. The reference pattern of the Finder will now be seen through the microscope (adjusting the focus if necessary). The reference number of the main square is recorded followed by an oblique stroke and the number of the segment in which the centre of the field of view lies (1 to 4 or 0 if in the centre circle). The boundary lines of the main squares are easily distinguishable as these are the only continuous straight lines of the pattern.

5. The reverse procedure is adopted to re-locate the point of interest, The England Finder is placed on the stage as outlined above and the stage is adjusted until the appropriate reference square and segment appear in the centre of the field of view. Remove the finder and replace with the specimen slide with label to left and appropriate vertical slide in contact with the fixed stop, when the point of interest will appear in the centre of the field of view.

11.3.1 England Finder Graticule Use

Before use ensure stage slide holder is in correct position (slide holder should be in the correct position as it is not removed on cleaning but if not-hold with one hand push holder back to full extent against the screws, tighten screws while holding and check for correct positioning).

1. Place graticule on stage with labelled corner at LHS front and clear edge against back of slide holder

2. Using the 10x objective (and Kohler illumination) – locate co-ordinates

3. Proceed to 40x objective and adjust focus as required (using oil if applicable). Locate co-ordinates and revert back to the 10x objective.

4. Taking care not to alter the position of the fixed stops of the stage, remove the graticule and replace with the slide of interest.

5. Proceed stepwise to 40x objective (oil or dry as applicable)

6. Adjust focus and locate sperm

NB: If the stage has moved repeat from step 1.