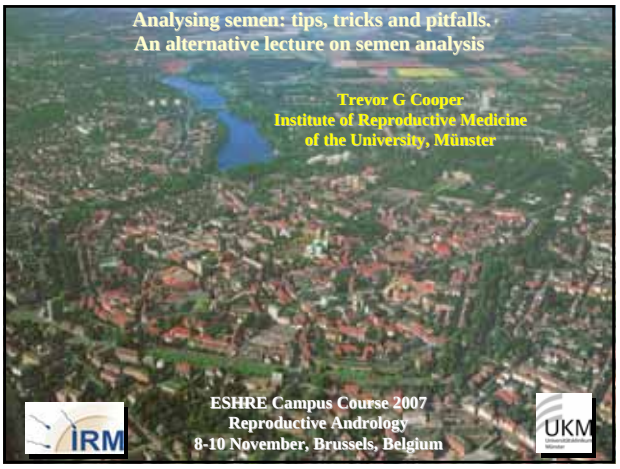


Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

Semen volume: WHO5-recommended methods

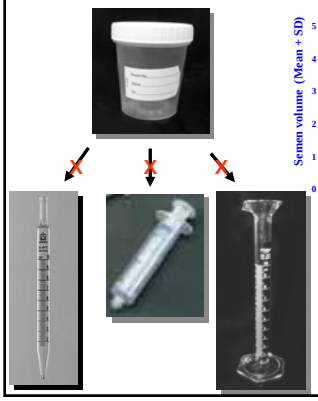
Direct volume measurement in collection vessel

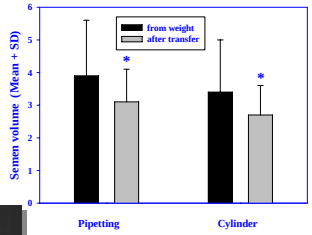
Indirect volume measurement from weight, assuming semen density of 1 g/ml (it is 1.014 g/ml)

Weigh the vessel (with label) (g)
Weigh the vessel with semen (g)
Calculate the weight difference (g = ml)

Semen volume: WHO5-not recommended methods

Transfer from the collection vessel





Transfer from the collection vessel by pipetting or decanting to a cylinder leads to:

- falsely low volumes
- -0.4 to -0.8 ml (14%)

First microscopic evaluation

Liquefaction at RT or 37°C (30 min)

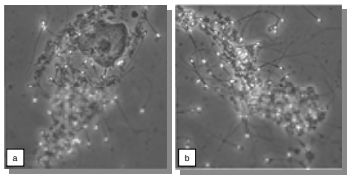
Positive-displacement pipette, 10 µl, 22 mm x 22 mm coverslip

Phase contrast microscope ± heated (37°C) stage

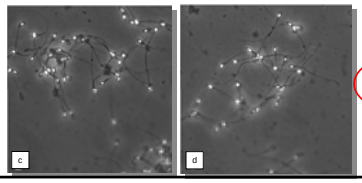
- *Aggregation, Agglutination*
- *Non-sperm cells*
- *Judge dilution for sperm concentration*
- *Sperm motility*

Sperm aggregation and agglutination

Aggregation **- immotile sperm bind to each other**
motile sperm bind to non-sperm cells or debris

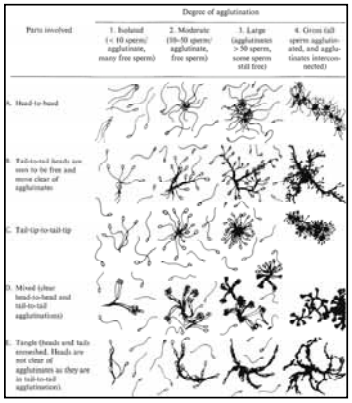


Agglutination **- motile sperm bind to other motile sperm**



Antisperm antibodies?

WHO5: Degrees of sperm agglutination

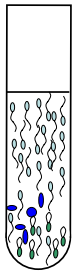


Semen analysis: representative aliquots

Semen is viscous

Area 1 high sperm concentration
fast sperm
good morphology

Area 2 low sperm concentration
slow sperm
bad morphology



- **Mix the sample well**
- **Take 2 aliquots**
- **Assess 2 x 200 sperm (when possible)**
- **Compare duplicates**
  - ***Poisson Distribution*** **(sperm/leukocyte numbers)**
  - ***Binomial Distribution*** **(% Motility, % Morphology, % Vitality)**

Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

Sperm motility: chamber depth

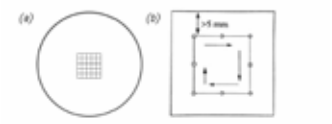
Assess at RT or 37°C in ~20 µm deep chambers, to permit free motion

Depth (D, µm) is volume (V, µl = mm^3) divided by area (A, mm^2)

22 x 22	18 x 18	21 x 26
10 µl	6.5 µl	11 µl

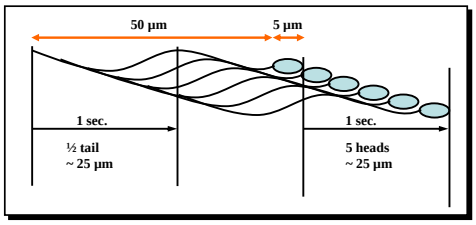
Assess 2 x 200 sperm (if possible) in at least 5 different fields

use an eyepiece graticule to limit the area examined
avoid the edges of the slide

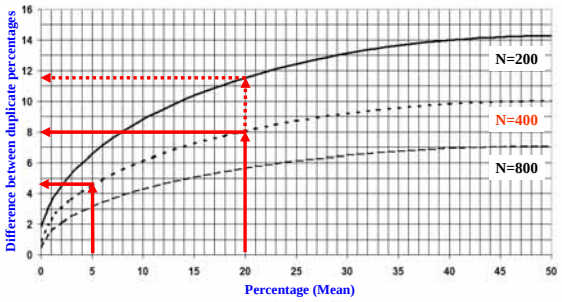


Sperm motility: grading

Is assessed as:	WHO4	WHO5
Immotile	Grade d	IM
Non-progressive	Grade c	NP
Progressive		PR
slow	Grade b	
fast (> 25 µm/s at 37°C)	Grade a	



% errors: Binomial Distribution



e.g. for 400 assessed sperm: the difference between duplicates
for a mean of 20% should be ≤8%; for a mean of 5% should be ≤5%
If >this, prepare and assess two more wet preparations

Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

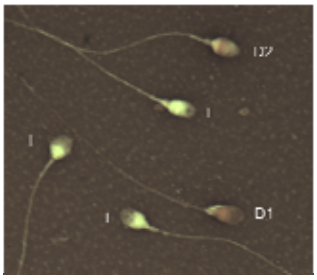
WHO5 sperm vitality: one-step method

If <40% PR, detection of intact sperm head membranes

Intact sperm head membrane: impermeant dyes excluded

e.g. Eosin-Nigrosin
Eosin = impermeant dye
Nigrosin = background

Red head	= Dead (D1)
Pink head	= Dead (D2)
White head	= Alive (L)
Pink neck	= Alive (L)



Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

WHO5: sperm counting chambers

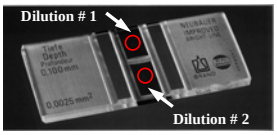
Not recommended:

shallow, small volume chambers: too few sperm
capillary-filled chambers: uneven filling
unfixed samples: motile sperm

Recommended:

haemocytometer (100 µm deep)
fixed, diluted samples

Sperm concentration: Neubauer Improved Chamber



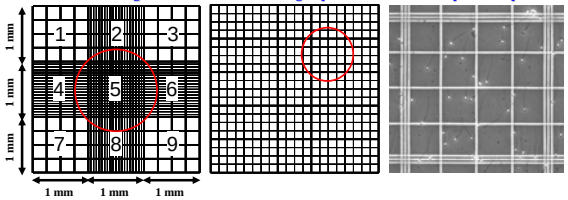
- Make duplicates from two separate dilutions
- Do not fill both chambers from one dilution
- Do not count the same chamber twice

- Sampling error
- Pipetting error
- Diluting error

100 µm deep chamber holds 9 x 100 nl grids

Central grid holds 25 x 4 nl large squares

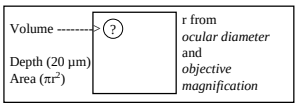
Each large square holds 16 x 250 pl small squares



Sperm concentration: semen dilutions

Dilution for the exact measurement is based on a rough assessment of the number of sperm in the volume observed

The volume observed depends on the area of the microscopic field and the depth of the chamber



For a x40 objective and x10 ocular of 20 mm aperture

The HPF has a diameter of 20 mm/40 = 500 µm, the radius r = 250 µm

So r^2 = 62500 μm^2 and the area = πr^2 = 196,375 μm^2

The volume = area x depth = 196,375 μm^2 x 20 µm = 4,064,962 μm^3 = 4 nl

Sperm concentration: semen dilutions

With 50 sperm per HPF (4 nl)
there are 12.5 per nl = 12,500 per µl = 12,500,000 per ml

Dilute 1:20 as recommended in WHO4 = 625,000 per ml

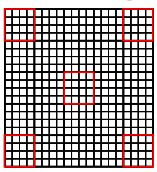
The central grid of the Neubauer chamber holds 100 nl
i.e. there are 63 sperm per grid
- not enough for an acceptable count (<200)

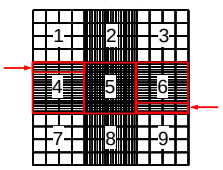
Dilute 1:5 as recommended in WHO5 = 2,500,000 per ml

The central grid of the Neubauer chamber holds 100 nl
i.e. there are 250 sperm per grid
- more than enough for an acceptable count (>200)

Sperm concentration: semen dilutions

	WHO4	WHO5
	More and higher	fewer and lower
Dilutions	1+49 1+19 1+9 1+4	1+19 1+4 1+1
N/x400HPF	>200 40-200 15-40 <15	>100 16-100 2-15
Examine:	Central grid (#5) 5, 10, 25 small squares	Central 3 grids (#4, 5, 6) one row (20 nl)





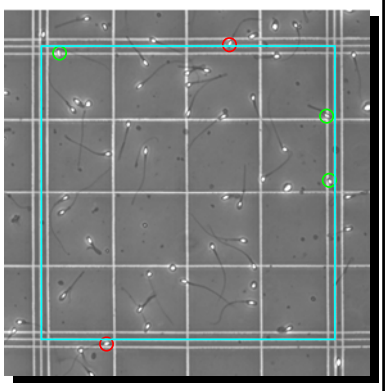
12 factors rarely 200 sperm	3 factors always 200 sperm

Which sperm to count?

Count all sperm within the central square

The middle line shows the relevant square

Preventing double counting



: counted

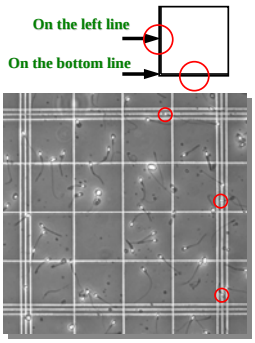
: not counted

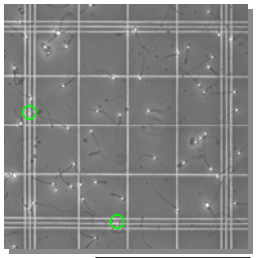
Which sperm to count?

Count sperm on lines only when they are the bottom or left lines ("L")

On the left line

On the bottom line

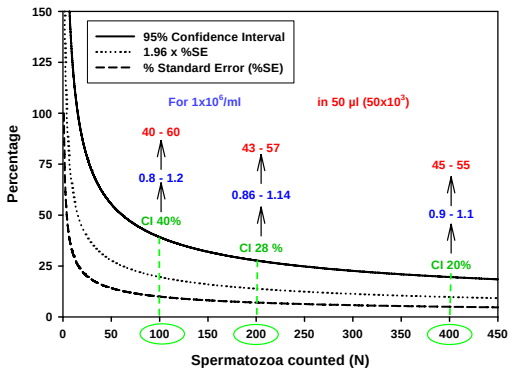




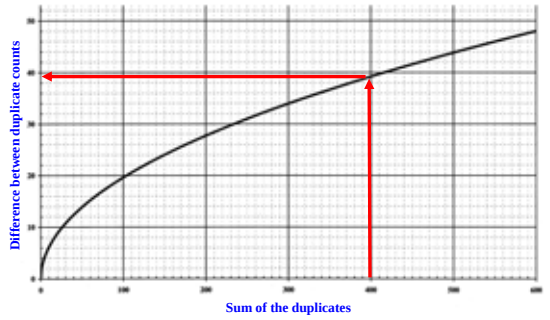
: counted

: not counted

How many sperm to count?

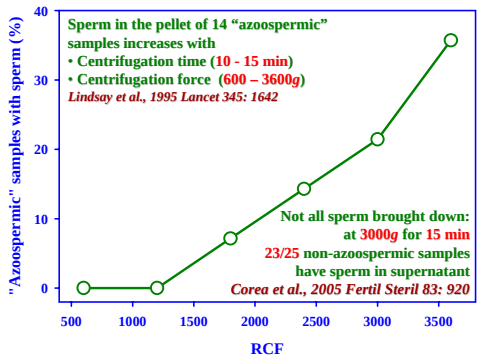


Counting error: Poisson Distribution



e.g. for 400 sperm assessed, acceptable difference is 39
If >39, make two more dilutions

Azoospermia: dependent on RCF



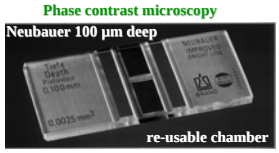
Centrifugation inaccurate!

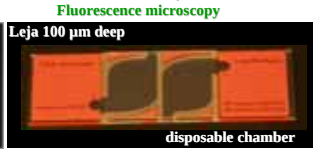
Low sperm numbers: accurate assessment required

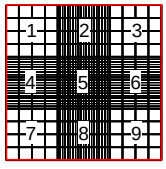
Dilute semen less and read larger volumes

Dilute semen 1+1 with Formalin
Phase contrast microscopy

Dilute semen 1+1 mit Formalin
+ Hoechst 33342 dye
Fluorescence microscopy







Read all 9 grids
= 900 nl

Sensitivity
56,000 per ml

Read all the chamber
= 25,000 nl

Sensitivity
2,000 per ml

If <25 spermatozoa observed, state:
"<56,000" per ml" or "<2,000 /ml"

Ejaculate analysis

Semen volume

Sperm motility

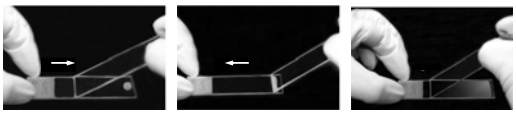
Sperm vitality

Sperm concentration

Sperm morphology

Reference values

Sperm morphology: semen smears



- Pull the semen droplet behind the slide
- Do not push it in front of it

Semen smear thickness depends on:

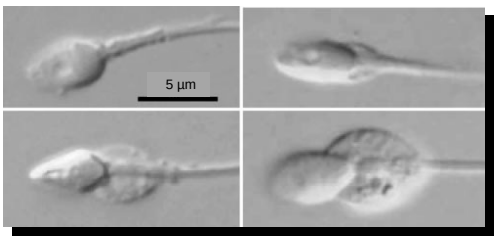
• aliquot volume (10 µl)	smaller → separated sperm
• angle of dragging slide (45°)	greater → thinner smear
• speed of smear (~1 sec)	higher → thicker smear

Disadvantages of fixing air-dried semen smears:

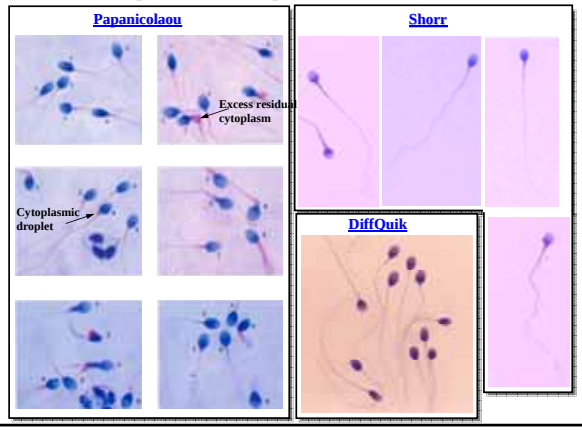
• dehydration	smaller than in wet preparations
• shearing forces	expansion of immature sperm heads
• osmotic insults	loss of cytoplasmic droplet
	retention of excess residual cytoplasm

Sperm morphology: cytoplasmic droplets

- Cytoplasmic droplets (midpiece vesicles)
- Present on motile sperm in semen, mucus, medium
- Osmotically sensitive: shrink on air-drying



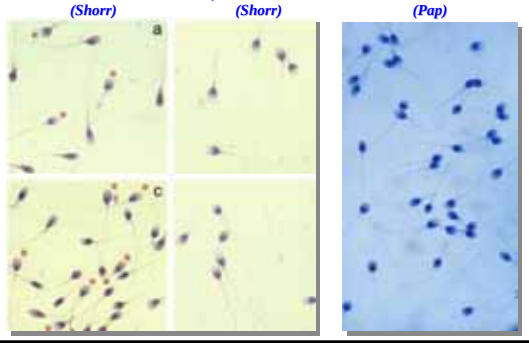
Sperm stains: Pap, Shorr, DiffQuik



"Normal Sperm": concept

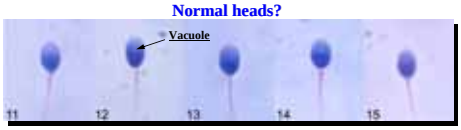
Identify a subpopulation of potentially fertilising spermatozoa (normal/ideal)
Biologically selected by cervical mucus or the zona pellucida

in semen (Shorr) | *from the Zona (Shorr)* | *in endocervical mucus (Pap)*

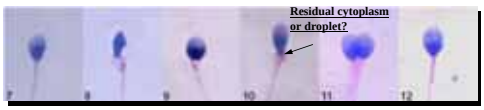


Sperm morphology

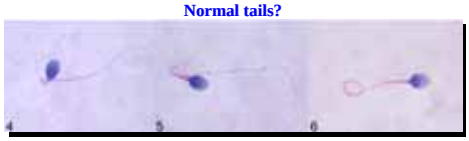
Normal heads?



Abnormal heads?



Normal tails?



WHO5: sperm micrographs

WHO5: sperm descriptions

	Sperm	Head	Other head	Midpiece	Principal	Overall	Comments
		Shape	comments		piece		
Plate 7	37	normal				normal	If PP OK
	38	abnormal	round			abnormal	
	39	normal				normal	
	40	normal				normal	
	41	normal				normal	
	42	normal		thick		abnormal	
	43	normal	<40 % acr			abnormal	
	44		out of focus				
	45	abnormal	round			abnormal	
	46	abnormal	round			abnormal	
	47	normal				normal	
	48	normal				normal	If PP OK

Spermatozoa and other non-sperm cells in semen

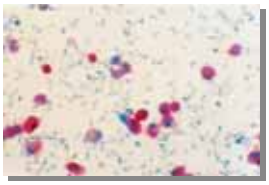
With Pap: immature germ cells **more** *pinkish*, polymorphs **more** *bluish*
Concentration: number relative to that of spermatozoa (conc. known)

Other cell types in semen

Leucocytes

Peroxidase-positive Leukocytes
Granulocytes

Pan Leukocyte marker
CD45 Antibody



Ejaculate analysis

Semen volume

Sperm motility

Sperm vitality

Sperm concentration

Sperm morphology

Reference values

WHO5: Reference values for human semen

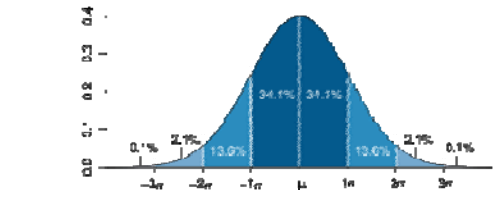
Reference Population
Fathers (Partners with time to pregnancy of ≤12 months)
N > 1600 3 continents 5 centres

Samples
Only 2-7 days of abstinence
one sample per man
complete samples

Methods
collection, preparation, analysis with WHO methodology
IQC + EQC-controlled labs.
only haemocytometer (not CASA, Makler chambers)
Tygerberg morphology

Reference Distribution and Intervals

It is accepted that 95% of the values should lie between the reference intevals



Two-sided: Serum 2.5% ⟷ 2.5%
95%

One-sided: Semen 5.0% ⟷
95%

One-sided Reference Intervals:

- higher values irrelevant
- lower reference limit is the 5th percentile

WHO2-4 "Normal", "reference" values und WHO5 "lower reference limits"*

WHO	2	3	4	5
	1987	1992	1999	2007/8
	normal	normal	reference	LRL (5 PC)
Semen Vol. (ml)	2.0	2.0	2.0	1.5
Total number (M)	40	40	40	39
Sperm concn. (M/ml)	20	20	20	15
Progr. Motility (%)	50	50	50	28
Vitality (%)	50	75	75	59
Normal Forms (%)	50	30	(15)	3

*subject to confirmation by WHO

Summary

Changes to the WHO laboratory manual for the examination of human semen are aimed at:

- increasing the accuracy of analytical results
- providing more experimental details of common methods
- giving hints and details of what to do when QC results are poor

These should help improve:

- standardisation between labs
- the diagnostic value of semen analysis results
- follow up of therapeutic treatments

Low sperm numbers: no accurate assessment required

Rough estimation:

- if <4 sperm per x400 HPF (<16 per x200 HPF)
- state "< $2x10^6$ sperm /ml"

Are any sperm present?

- Centrifuge 1 ml semen at 3000*g* for 15 min
- Leave ~50µl supernatant
- Place 2x10 µl pellet under 22mm x 22 mm coverslips (20 µm deep)
- Examine both coverslips entirely (~2x480 fields)
- If sperm found, state "cryptozoospermia"

Are any motile sperm present?

- Place 40 µl semen under 24mm x 50mm coverslip (33 µm deep)
- Examine coverslip entirely (~1200 fields)
- If motile sperm found, state how many

N.B. All these procedures are inaccurate

- Few sperm = large counting error
- No sperm seen ≠ no sperm present
- The 95% upper CI for 0 = 3.7