2nd Symposium of the Croatian Society of Clinical Embryologists and Andrology Workshop	**Workstation** **4**
SPERM VITALITY and HOS TEST	

Source: WHO laboratory manual for the examination and processing of human semen, Fifth edition (WHO5)

WHO parameters
1. >40% progressively motile spermia
2. ≥58% lower reference limit for vitality

Asthenozoospermia

IMMOTILE SPERM

VITAL (LIVE) SPERM or NON-VITAL (DEAD) SPERM

Sperm vitality is test which is performed to distinguish immotile but live spermatozoa from dead cells.
Vitality is estimated by assessing the membrane integrity of the cells.
Live spermatozoa with an intact cell membrane are identify with dye exclusion or by hypotonic swelling.

DYE EXCLUSION METHOD

The dye exclusion method is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-impermeant stains.
Coloured cells are non-vital. Damaged plasma membranes allow entry of membrane-impermeant stains.
- diagnostic purpose

1. Vitality test using eosin alone (WHO manual 5ed)
2. Vitality test using eosin–nigrosin (WHO manual 5ed)
3. VitalScreen™ (FertiPro N.V., Belgium) is commercial test based on eosin-nigrosin

HYPO-OSMOTIC SWELLING TEST

The hypo-osmotic swelling test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions.
Live sperm cells with intact membranes will swell in hypotonic solutions. Swollen spermatozoa are identified by coiling of the tail.
-diagnostic and terapeutic purpose

1. Swelling solution for diagnostic purposes (WHO manual 5ed)
2. Water test (Fuse H, 1993, Arch Androl.)
3. Swelling solution for therapeutic use (WHO manual 5ed)
4. HOS test for the ICSI procedure (Sanja Vujisic, PhD, senior embryologist, BetaPlus)

Lower reference limit

The lower reference limit for vitality (membrane-intact spermatozoa) is 58%
Comment: The total number of membrane-intact spermatozoa in the ejaculate is of biological significance. This is obtained by multiplying the total number of spermatozoa in the ejaculate by the percentage of membrane-intact cells.

The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum (Chemes & Rawe, 2003);
a high percentage of immotile and non-viable cells (necrozoospermia) may indicate epididymal pathology (Wilton et al., 1988; Correa-Perez et al., 2004).

Vitality test using eosin alone

Source: WHO5, Moderator: Linda Panic Horvat, Mr.Sc., KBC Rijeka

This method is simple and rapid, but the wet preparations cannot be stored for quality control purposes.

Reagents

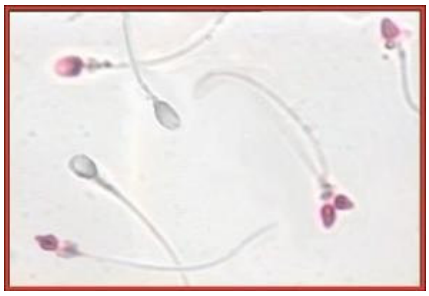
- Eosin Y, 0.5% (w/v):
- Dissolve 0.5 g of eosin Y (colour index 45380) in 100 ml of 0.9% NaCl.

Procedure

- Remove an aliquot of 5µl of semen and combine with 5µl of eosin solution on a microscope slide. Mix with a pipette tip, swirling the sample on the slide.
- Cover with a 22 mm x 22 mm coverslip and leave for 30 seconds.
- Examine each slide, preferably with negative-phase-contrast optics (200 or 400 magnification).
- Evaluate 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error
- Calculate the average and difference of the two percentages of vital cells from the replicate preparations.

Scoring

Live spermatozoa have white or light pink heads and dead spermatozoa have heads that are stained red or dark pink.



Vitality test using eosin–nigrosin

Source: WHO5, Moderator: Gabrijela Kirinec, MSc in molecular biology, KBC Zagreb

This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes (Björndahlet al., 2003).

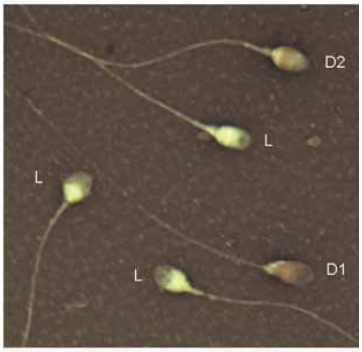
Reagents: Sigma Aldrich # HT110232 Eosin Y solution aqueous
Sigma Aldrich # N4754 Nigrozin water soluble

Preparing the reagents:

- Eosin Y: dissolve 0.67 g of eosin Y (colour index 45380) and 0.9 g of sodium chloride (NaCl) in 100 ml of purifi ed water with gentle heating.
- Eosin–nigrosin: add 10 g of nigrosin (colour index 50420) to the 100 ml of eosin Y solution.
- Boil the suspension, then allow to cool to room temperature.
- Filter through fi lter paper (e.g. 90 g/m2) to remove coarse and gelatinous precipitates and store in a sealed dark-glass bottle.

Procedure:

- Remove an aliquot of 10 µl of semen and combine with 10 µl of eosin solution on a microscope slide and mix with a pipette tip
- Wait for 30 - 60 seconds to dry the sample
- Take 10uL of nigrosine solution and mix it with sample of semen and eosine solution on a microscope slide using a pipette tip. Repeat this step.
- For each suspension make a smear on a glass slide and allow it to dry in air.
- Examine immediately after drying, or later after mounting with a permanent non-aqueous mounting medium
- Examine each slide with brightfi eld optics at ×1000 magnifi cation and oil immersion



Scoring:

Spermatozoa with red (D1) or dark pink (D2) heads are considered dead (membrane-damaged)

Andrology Workshop

Vitality test – VITALSCREEN

Source: FertyPro N.V. Belgium, Moderator: Sonja Šogorić, MSc in molecular biology, Poliklinika „Škvorc“

VitalScreen™ by FertyPro

Nigrosin contains 0.05% Na-azide

- Material included in the kit: Reagent 1 - 20ml of 1% Eosin Y in saline, Reagent 2 - 30ml of 5% Nigrosin in saline
- Material not included in the kit: Light microscope (400 - 600x magnification), Microscope glasses, Cover glasses, Pipettes, Test tubes (sterile)

The eosin-nigrosin staining technique is based on the principle that dead cells will take up the eosin and as a result stain red. The nigrosin provides a dark background which makes it easier to assess the slides. The VitalScreen™ is based on the test procedure described in the WHO laboratory manual (2010).

GENERAL INFORMATION

Sperm vitality is reflected in the proportion of spermatozoa that are “alive”.
Sperm vitality should be determined in semen samples with less than about 40% progressive motile spermatozoa.
VitalScreen uses the eosin-nigrosin staining technique to establishes the percentage of live spermatozoa.
The technique is based on the principle that dead cells will take up the eosin, and as a result stain red. The nigrosin provides a dark background which makes it easier to assess the slides.
VitalScreen provides an accuracy check of the motility evaluation since the percentage dead spermatozoa should not exceed the percentage immotile spermatozoa.
The VitalScreen kit may help in assessing the diagnosis and the management of male infertility.

PREPARATIONS

Shake reagent 2 (Nigrosin stain) before use.

METHOD

1. Mix 50 µL of semen with 2 drops of reagent 1 in a sterile test-tube.
2. After 30 seconds, add three drops of reagent 2 and mix thoroughly.
3. Within 30 seconds of adding reagent 2, place a drop of semen-stain mixture on a microscope slide and make a thin smear using a cover glass.
4. Cover the smear with a cover glass before the smear is dry* and read immediately under the microscope.

* When the smear is allowed to dry, crystals of nigrosin will form which can interfere with the interpretation of the results.

INTERPRETATION

- Colourless spermatozoa: live spermatozoa
- Red stained spermatozoa: dead spermatozoa

Count between 100 and 200 cells and differentiate the living from the dead spermatozoa.
Read results immediately, waiting too long will yield lower vitality percentages.
It is clinically important to know whether immotile spermatozoa are alive or dead. Vitality results should be assessed in conjunction with motility results from the same semen sample.

LIMITATIONS OF THE METHOD

Spermatozoa stained with VitalScreen cannot be used for any further procedures.

STORAGE

Suitable for transport or short term storage at elevated temperatures (up to 5 days at 37°C). Store reagents between 2°C and 25°C.

Vitality test using hypo-osmotic swelling

Source: WHO5, Moderator: Linda Panic Horvat, Mr.Sc., KBC Rijeka

As an alternative to dye exclusion, the hypo-osmotic swelling (HOS) test may be used to assess vitality (Jeyendran et al., 1984). This is useful when staining of spermatozoa must be avoided, e.g. when choosing spermatozoa for ICSI. Spermatozoa with intact membranes swell within 5 minutes in hypo-osmotic medium and all flagellar shapes are stabilized by 30 minutes (Hossain et al., 1998).
Thus, use:

- **30 minutes** incubation for routine diagnostics;
- **5 minutes** incubation when spermatozoa are to be processed for therapeutic use.

Reagents

1. Swelling solution for diagnostic purposes:
   Dissolve 0.735 g of sodium citrate dihydrate and 1.351 g of D-fructose in 100 ml of purified water.
   Freeze 1-ml aliquots of this solution at –20°C.
2. For therapeutic use:
   Dilute the medium to be used 1 + 1 (1:2) with sterile, purified water.

Procedure

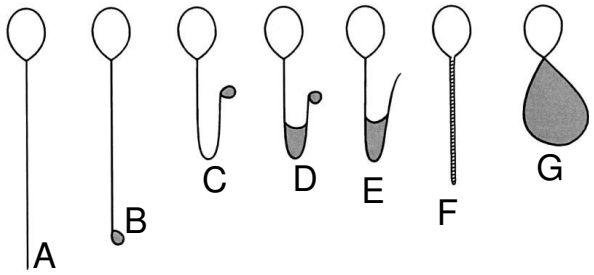
- Warm 1 ml of swelling solution or 1 ml of 1 + 1 (1:2) diluted medium in a closed microcentrifuge tube at 37°C for 5 minutes.
- Remove a 100µl aliquot of semen and add to the swelling solution.
- Incubate at 37°C for exactly 5 minutes or 30 minutes (see above), then transfer a 10µl aliquot to a clean slide and cover with a 22 mm x 22 mm coverslip.
- Examine each slide with phase-contrast optics at 200x or 400x magnification.
- Evaluate 200 spermatozoa

Scoring

1. Swollen spermatozoa are identified by changes in the shape of the cell, as indicated by coiling of the tail
2. Live cells are distinguished by evidence of swelling of the sperm tail; score all forms of swollen tails as live spermatozoa.

Schematic representation of
typical morphological changes in human spermatozoa subjected to hypo-osmotic stress
(A) No change
(B)–(G) Various types of tail changes.

Swelling in tail is indicated by the grey area.



Reproduced from Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. (1984) Journal of Reproduction and Fertility, 70: 219–228. © Society for Reproduction and Fertility (1984).

Lower reference limit

HOS test values approximate those of the eosin test (Carreras et al., 1992).
The lower reference limit for vitality (membrane-intact spermatozoa) is 58%

Water test

Source: original paper Fuse et al., Moderator: Linda Panic Horvat, Mr.Sc., KBC Rijeka

Hypoosmotic swelling test with a medium of distilled water.

Fuse H1, Ohta S, Sakamoto M, Kazama T, Katayama T.

Arch Androl. 1993 Mar-Apr;30(2):111-6.

Abstract

The functional competence of the human sperm membrane is assessed by studying the swelling reaction of sperm when suspended in a medium of distilled water (water test). Eighty-seven patients with idiopathic infertility were investigated by the water test and the results were compared with various semen parameters. High correlations with the percentage of b-g type swollen sperm in the water test were observed for sperm concentration ($r = .53$, $p < .05$) and percent motility ($r = .62$, $p < .01$). The sperm swelling values obtained by the water test correlated well with those obtained by the hypoosmotic swelling test (HOST). Correlation coefficients in b-g and g swelling pattern were 0.89 and 0.71, respectively. The percentage of g-type swelling obtained by the water test was significantly larger than that obtained by the HOST ($p < .05$), although the two hypoosmotic procedures gave similar percentages of b-g swelling pattern. The percentage of g swelling pattern obtained by the water test correlated well with percent motility ($r = .70$, $p < .001$). These findings suggest that the water test is more useful for assessing the integrity of the sperm membrane than the HOST. The relationship between this test and subsequent fertility, as well as to other sperm function tests, needs further investigation.

Reagents

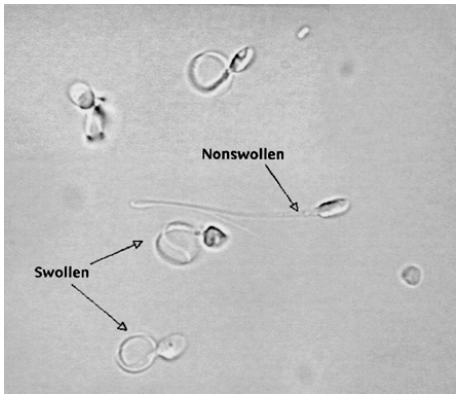
distilled water

Procedure

- Warm 1 ml of distilled water in a closed microcentrifuge tube at 37°C.
- Remove a 100µl aliquot of semen and add to the warmed water
- Incubate at 37°C for 30 minutes, then transfer a 10µl aliquot to a clean slide and cover with a 22 mm x 22 mm coverslip.
- Examine each slide with phase-contrast optics at 200x or 400x magnification.
- Evaluate 200 spermatozoa

Scoring

Live cells are distinguished by evidence of swelling of the sperm tail.

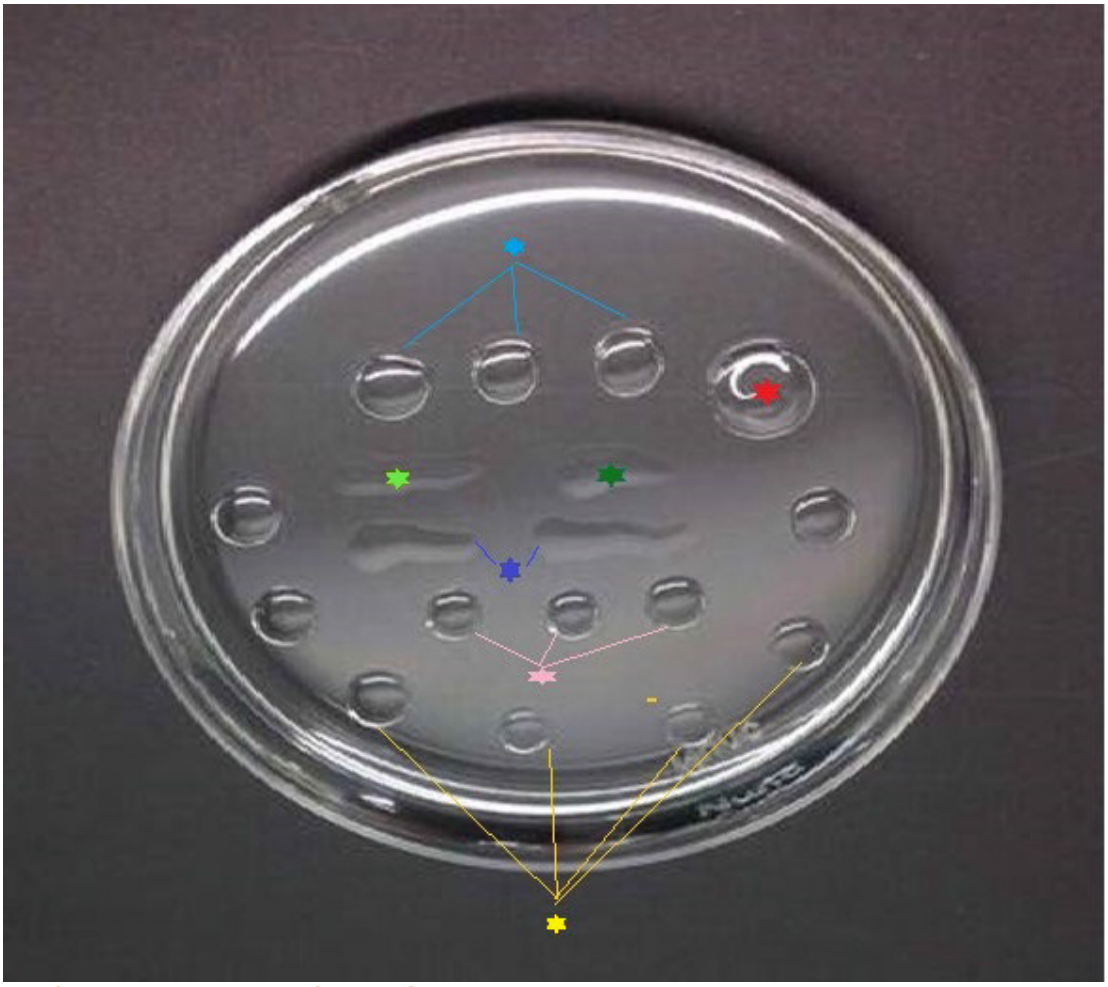


HOS test for the ICSI procedure

- intended for immotile sperm obtained by microsurgical procedure (TESA, TESE, PESA, MESA),
and for immotile sperm from ejaculated semen sample.
- preparing HOS solution: in a sterile, non-toxic test tube place 500 µl of fertilisation medium (you
can replace this medium with any medium intended for keeping oocytes during ICSI procedure)
and add 500 µl of pro-injection water, mix the solution.
- preparing ICSI dish: use a sterile, non-toxic 60 mm Petri dish. Near the dish wall, first place a 50 µl
drop of hyaluronidase, followed by at least six 50 µl drops of fertilization medium (rinsing drops). In
the center, place an elongated, 10 µl drop of HOS solution followed by an elongated, 10 µl drop of
fertilization medium. Below these, place two elongated drops of PVP. Above the HOS drop, place
three 20 µl drops of fertilization medium (for sperm suspension), and do the same below the PVP
drops (for oocytes prepared for ICSI procedure, ICSI drops). Cover with oil. Mark the type of each
different drop solution with a number.

PROCEDURE:

1. Denude oocytes by exposing them to hyaluronidase solution no more than 30 seconds. Rinse
t h e m t r o u g h rinsing drops. In the same time continue denuding oocytes mechanically using
denuding pipette,
2. Place denuded oocytes into the ICSI drop,
3. Place the pre-washed semen sample into the center of the drops prepared above the HOS
solution
4. Choose morphologically normal sperm from the suspension and move it into the HOS solution
u s i n g ICSI pipette. Observe the sperm tail. If it appeares HOS test positive (you will see after
approximately 10 seconds), pick it up with the pipette,
5. Move the HOS positive sperm to the fertilization medium, prepared behind the drop for HOS
test, and leave it. Observe the recovery carefully. Gently rinse sperm a few times using ICSI pipette,
6. Move the sperm by ICSI pipette into the PVP solution and proceed with standard procedure for ICSI.



DROP WITH HYALURONIDASE
DROPS FOR RINSING AND DENUDATION OF OOCYTES
DROP WITH SPERM SUSPENSION
DROP FOR THE HOS TEST
DROP FOR THE SPERM RECOVERY
PVP
DROPS FOR OOCYTES INJECTION

Moderators:	Linda Panić Horvat, MrSc (KBC Rijeka) Sonja Šogorić, MSc (Poliklinika „Škvorc“) Sanja Vujisić, PhD, (Poliklinika BetaPlus) Gabrijela Kirinec, MSc (KBC Zagreb)