DDK Italia

IVD dispositivo medico-diagnostico in vitro

CE

Eosin vitality test technical information
Technical card code 13-102
Product code 13-102
Pack 1x100 ml or on request
Stability of product properly conserved at 15-20°C 12 months
CND code W01030799

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in case of emergency UE number	☎	112
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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 (Björndahl et al., 2003).
For estimates of sperm motility, or vitality by eosin alone and for the hypo-osmotic swelling (HOS) test, prepare fresh replicates from new aliquots of semen. For estimates of vitality from eosin–nigrosin smears and sperm morphology, reassess the slides in replicate

Method

1. Mix the semen sample well

Before removing an aliquot of semen for assessment, mix the sample well in the original container, but not so vigorously that air bubbles are created. This can be achieved by aspirating the sample 10 times into a wide-bore (approximately 1.5 mm diameter) disposable plastic pipette (sterile when necessary).
Do not mix with a vortex mixer at high speed as this will damage spermatozoa.

2. Remove a 50-µl aliquot of semen and mix with an equal volume of eosin solution on a microscope slide. Mix with a pipette tip, swirling the sample on the slide.
3. Cover with a 22 mm × 22 mm coverslip and leave for 30 seconds. For each suspension make a smear on a glass slide

Preparation of semen smears. Rapid addition of fixative to semen does not permit adequate visualization of spermatozoa, as they are obscured by denatured seminal proteins.
For morphological analysis, it is customary to prepare semen smears that are air-dried before fixation and staining. However, such a process leads to morphological artefacts, since airdrying of semen smears is associated with:

- *changes in sperm dimensions: dried, fixed and stained spermatozoa are smaller than live spermatozoa visualized in semen (Katz et al., 1986);*
- *expansion of immature sperm heads (Soler et al., 2000); and*
- *loss of osmotically sensitive cytoplasmic droplets (Abraham-Peskir et al., 2002; Cooper et al., 2004), although large amounts of excess residual cytoplasm are retained.*

Two or more smears should be made from the fresh semen sample in case there are problems with staining or one slide is broken.
Assessment is performed in replicate, preferably on each of the two slides, because there may be significant between-slide variation in sperm morphology and allow it to dry in air.

5. Examine each slide, preferably with negative-phase-contrast optics (positive phase- contrast makes faint pink heads difficult to discern) at ×200 or ×400magnification.

Mounting the stained semen smears
1. Add two or three small drops of mounting medium to the slide.
2. Place a coverslip (24 mm × 50 mm or 24 mm × 60 mm are most convenient) directly on the smear.
3. Position the coverslip so that contact with the mounting medium begins from one long side, in order to prevent air bubbles being trapped.

4. If necessary, press gently on the top of the coverslip to help move bubbles to the edge of the slide.
5. Wipe off excess xylene (if used) from underneath the slide.
6. Allow the mounted smear to dry horizontally in a slide drying rack or on absorbant paper for 24 hours in a fume cupboard).

6. Tally the number of stained (dead) and unstained (vital) cells with the aid of a laboratory counter.
7. Evaluate 200 spermatozoa in each replicate, in order to achieve an acceptably low sampling error.
8. Calculate the average and difference of the two percentages of vital cells from the replicate preparations.
Errors in estimating percentages.
How certain your estimate of a percentage is depending not only on the number (N) of spermatozoa counted but also on the true, but unknown, percentage (p) (binomial distribution).
The approximate standard error (SE) is √((p(100–p))/N) for percentages between 20 and 80. Outside this range, a more appropriate method to use is the angular transformation (arc sin square root), z = sin–1√(p/100), with a standard deviation of 1/(2√N) radians, which depends only on the number of spermatozoa counted and not the true percentage).

9. Determine the acceptability of the difference (table below). (Each shows the maximum difference between two percentages that is expected to occur in 95% of samples because of sampling error alone.).

Average (%)	*Difference**
0	*2*
1	*3*
2	*4*
3	*5*
4	*6*
5-6	*7*
7-9	*8*
10-12	*9*
13-15	*10*
16-19	*11*
20-25	*12*
26-33	*13*
34-66	*14*

Average (%)	*Difference**
67-74	*13*
75-80	*12*
81-84	*11*
85-87	*10*
88-90	*9*
91-93	*8*
94-95	*7*
96	*6*
97	*5*
98	*4*
99	*3*
100	*2*

**Based on rounded 95% confidence interval.*

10. If the difference between the percentages is acceptable, report the average percentage vitality. If the difference is too high, make two new preparations from two new aliquots of semen and repeat the assessment.

Comparison of replicate percentages
Percentages should be rounded to the nearest whole number. The convention is to round 0.5% to the nearest even number, e.g. 32.5% is rounded down to 32% but 3.5% is rounded up to 4%. Note that the rounded percentages may not add up to 100%.
If the difference between the replicate percentages is less than or equale, the estimates are accepted and the average is taken as the result.
Larger than acceptable differences suggest that there has been miscounting or errors of pipetting, or that the cells were not mixed well, with non-random distribution in the chamber or on the slide. When the difference between percentages is greater than acceptable, discard the first two values and reassess.
(Do not count a third sample and take the mean of the three values, or take the mean of the two closest values).
For estimates of sperm motility, or vitality by eosin alone and for the hypo-osmotic swelling (HOS) test, prepare fresh replicates from new aliquots of semen. For estimates of vitality from eosin–nigrosin smears and sperm

morphology, reassess the slides in replicate. With these 95% CI cut-off values, approximately 5% of replicates will be outside the limits by chance alone. Exact binomial confidence limits can now be computer-generated, and these are used in this manual for the graphs and tables provided to assess agreement of replicates.

11. Report the average percentage of vital spermatozoa to the nearest whole number.

Result
1. Live spermatozoa have white or light pink heads and dead spermatozoa have heads that are stained red or dark pink.
2. If the stain is limited to only a part of the neck region, and the rest of the head area is unstained, this is considered a "leaky neck membrane", not a sign of cell death and total membrane disintegration. These cells should be assessed as alive.
3. If it is difficult to discern the pale pink-stained head, use nigrosin to increase the contrast of the background

Lower reference limit
The lower reference limit for vitality (membrane-intact spermatozoa) is 58% (5th centile, 95% CI 55–63).
(*Errors in estimating numbers. The precision of the estimate of sperm number depends on the number of spermatozoa counted. In a Poisson distribution, the standard error (SE) of a count (N) is its square root (√N) and the 95% confidence interval (CI) for the number of spermatozoa in the volume of semen is approximately N ± 1.96 × √N (or N ± approximately 2 ×√_N). If 100 spermatozoa are counted, the SE is 10 (√100), and the 95% CI is 80–120 (100 ± 20). If 200 spermatozoa are counted, the SE is 14 (√200), and the 95% CI is 172–228 (200 ± 28). If 400 spermatozoa are counted, the SE is 20 (√400) and the 95% CI is 360–440 (400 ± 40).*
The sampling errors can be conveniently expressed as a percentage of the count (100×(√N/N)).

Reagent	
Eosine (WHO modified) solution ready to use	100 ml

References

WHO laboratory manual for the examination and processing of human semen, 5th edition, WHO, 2010