

Kit to analyze sperm dna fragmentation



application

Halosperm[®] Kit from Halotech Dna, SL is a simple test that allows assessment of sperm DNA fragmentation in humans.

Principle of the method:

The method is based on the Sperm Chromatin Dispersion (SCD) test (Fernández et al., J. Androl 24: 59-66, 2003; Fertil Steril 84: 833-842, 2005). Intact unfixed spermatozoa (fresh, frozen/unthawed, diluted samples) are immersed in an inert agarose microgel on a pretreated slide. An initial acid treatment denatures DNA in those sperm cells with fragmented DNA. Following this, the lysing solution removes most of the nuclear proteins, and in the absence of massive DNA breakage produces nucleoids with large halos of spreading DNA loops, emerging from a central core. However, the nucleoids from spermatozoa with fragmented DNA either do not show a dispersion halo or the halo is minimal.

Description of kit reagents:

Each kit is sufficient for 10 tests:

- SCS: 10 Super coated slides.
- ACS: 10 Eppendorf tubes with low-melting-point agarose (Agarose Cell Support).
- AD: 1 Tube with acid denaturation solution (Tube labeled AD), 1 ml.
- LS: 1 Bottle of lysis solution, 100 ml.

Material and equipment required not provided with the kit:

Bright field or fluorescence microscope, 4°C fridge, 90-100°C and 37°C incubation bath(s), Plastic gloves, Glass slide covers (18 x 18 mm or 22 x 22 mm), Micropipettes, Trays for horizontal incubations, Distilled water, Ethanol 70%, 90%, 100%. Microwave oven and fume hood.

Recommended solution for microscopy visualization:

- Bright field microscopy: Diff Quick stain (several trademarks) or Wright solution (Merck 1.01383.0500).
- Fluorescence microscopy: Fluorochromes for DNA staining.
- Phosphate buffer solution pH 6.88 (Merck 1.07294.1000).
- Mounting medium: Eukit[®] (Panreac 253681).

Specimen:

Samples should be collected in clean containers and the assay should be done as soon as possible. Cryopreserved and frozen samples at -20°C could be also processed. In this case, the sample will be totally thawed, and brought to room temperature before testing, which should be done right after thaw out.

instructions for use

1. Set the lysis solution (LS) at room temperature (22°C).
2. Dilute the semen sample in culture medium or sperm extender or PBS to a concentration of 5-10 million per milliliter. Be careful that solvent is not extremely dense.
3. Put the agarose eppendorf (ACS) provided through a float; the float should be at the level of the top of the tube. Leave floating in water 5 minutes at 90°-100°C, until the agarose dissolves. Alternatively, the agarose can be melted in a microwave oven.
4. Transfer the agarose eppendorf, with the float, to a temperature controlled water bath maintained at 37°C and leave for 5 minutes until the temperature is even.
5. Add 25 microliters of the semen sample to the agarose eppendorf and mix well. Place the cell suspension from the agarose eppendorf (SCS) on to the treated side of the slide and cover with a glass coverslip, being careful to avoid air bubbles. A drop of 14 or 20 µl for an 18 x 18 mm or 22 x 22 mm coverslip respectively, is recommended. If liquid does not spread till the edge of the coverslip, press gently with the tip of the micropipette.
6. Take care to keep the slide in a horizontal position throughout the entire process.
7. Place the slide on a cold surface (for example, a metal or glass plate pre-cooled at 4°C). Put the cold plate with the slide/s into the fridge at 4°C and leave the sample to gel for 5 minutes.
8. Prepare the denaturant solution (AD). To do this, add 80 microlitres of the contents of the acid denaturation solution (Tube labelled AD) to 10 millilitres of distilled water, mix and place in the incubation tray.
9. Remove the slide cover by sliding it off gently. From now on, wear gloves and use slide horizontally with the help of a lancet.
10. Immediately, immerse the slide into the acid denaturation solution from step 8 in a horizontal position, leaving it to incubate for 7 minutes.
11. Afterwards, place it in another incubation tray containing 10 ml of tempered lysis solution. Incubate for 25 minutes.
12. Pick the slide up and set up horizontally into a tray containing abundant distilled water in order to wash the lysis solution. Leave to incubate for 5 minutes.
13. Place the slide horizontally into a tray with 70% ethanol (2 minutes), followed by 90% ethanol (2 minutes) and finally, 100% ethanol (2 minutes).
14. Leave to dry at room temperature and stain.
15. After drying, the processed slides may be kept in archive boxes at room temperature, in the dark, for months.

microscopic visualization

For bright field microscopy, it is recommended two types of stain:

- Diff-quick stain, through the following guide: incubate the slide in horizontal position in the Eosin solution, red colour, for 6 minutes. Afterwards, incubate the slide in horizontal position in the Azur B solution, blue colour, for 6 minutes.
- Wright stain, prepare the Wright solution by mixing it in phosphate buffer (1:1). While keeping the slide in horizontal position, cover the sample with a gentle layer of the dyeing solution. Leave it for 10-15 min blowing on it from time to time. Remove the dyeing solution, wash briefly and carefully in tap water and air dry.

Check colouring level under the microscope. Strong staining is preferred, to clearly discriminate the peripheral border of the halo. If staining results are very weak, especially on the region of chromatin dispersion halos, the slide can be re-stained. If colouration is too strong, the slide can be discoloured by washing gently in tap water, or 10% ethanol if preferred. After air dried, it can be dyed again with reduced colouring exposure time. Once the desired level of colouration is achieved and the slide is perfectly dried, it can be mounted in a permanent mounting medium such as Eukit®.

Visualization under fluorescence microscopy is also possible using standard DNA directed fluorochromes.

20x and 40x objectives are the optimum for visualization and classification of spermatozoa.

sperm classification

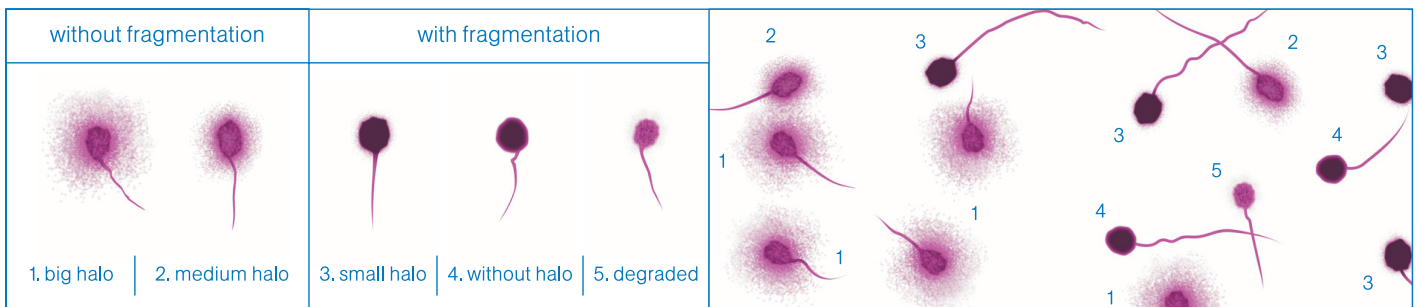
Removal of sperm nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. The sperm tails remain preserved. For sperm classification we must take into account that sperm DNA fragmentation is a continuous process which produces a series of different halo sizes. Initially, the study of a minimum of 500 spermatozoa per sample is recommended, adopting the criteria of Fernández et al. (Fertil Steril 84: 833-842, 2005). Avoid scoring cells close to the edge of the microgel. Classification:

• Spermatozoa without dna fragmentation:

- **spermatozoa with big halo:** those whose halo width is similar or higher than the minor diameter of the core (Figure 1).
- **spermatozoa with medium-sized halo:** their halo size is between those with large and with very small halo (Figure 2).

• Spermatozoa with fragmented dna:

- **spermatozoa with small halo:** the halo width is similar or smaller than 1/3 of the minor diameter of the core (Figure 3).
- **spermatozoa without halo:** (Figure 4).
- **spermatozoa without halo and degraded:** those that show no halo and present a core irregularly or weakly stained (Figure 5).
- **"others":** cell nuclei which do not correspond to spermatozoa. One of the morphological characteristics which distinguish them is the absence of tail.



positive and negative controls

Positive control: sperm cells with halo. Follow the instructions for use, steps 1 to 9. Add 50 µl of H₂O₂ (300µM) covering the entire gel surface. Incubate for 5 minutes in the fridge maintaining the slide in horizontal position. Continue following the protocol as instructed.

Negative control: sperm cells without halo. Follow the instruction for use, omitting steps 8 and 10.

limitations

This test provides presumptive quantitative information of DNA fragmentation in spermatozoa. This parameter should be analysed by a specialist. The results should be evaluated taking into account all clinical and laboratory findings related to the same sample.

safety and the environment

Attention! Slide processing must be performed under fume hood.

Avoid inhalation and contact with the solutions supplied. The acid solution (AD) contains Hydrochloric acid, and the lysing solution (LS) contains Dithiothreitol and Triton X-100. Consult specifications supplied by manufacturers.

Do not release the products used into the environment. Follow center guidelines for the storage and disposal of toxic substances.

Biological samples must be handled as potentially infectious.

precautions

For professional use only. For research use only.

1. All patient samples and reagents should be treated as potentially infectious and the user must wear protective gloves, eye protection and laboratory coats when performing the test.
2. The test should be discarded in a proper biohazard container after testing.
3. Do not eat, drink or smoke in the area where specimens and kit reagents are handled.
4. Do not use beyond the expiration date which appears on the package label.
5. It is recommended the use of gloves and face mask.

storage conditions

The kit should be stored at room temperature (2-30°C). The reagents have an expiry date of 1 year.

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