

Kit for DNA fragmentation analysis of *Equus caballus*. (10 determinations)

THE KIT CONTAINS

- 10 precoated slides
- 10 vials with low melting agarose
- 1 flask with 100 ml of base lysing solution
- 1 vial with reducing agent (**Work under continuous air removal or cover the trays during incubation step**)

BACKGROUND

The **SPERM-HALOMAX[®]** kit from **HALOTECH DNA SL** bursts into the market as a pioneering product for the analysis of fragmentation levels in spermatozoa both in domestic and experimental animals.

SPERM-HALOMAX[®] is based on the differential response of fragmented and unfragmented spermatozoa nuclei to a protein depletion treatment. The extraction of nuclear proteins from spermatozoa containing fragmented DNA releases DNA fragments between two breakage points. Sperm nuclei disperse chromatin around, forming a low stained peripheral halo, which is distinguishable under low magnification microscopy. These images contrast sharply with the intensely stained nuclei of the spermatozoa which occupies a centered position. On the contrary, those spermatozoa containing unfragmented DNA develop a very small dispersion halo, and appears as a very thin crown around the core. **SPERM HALOMAX[®]** allows the quick assessment of the proportion of spermatozoa with fragmented DNA.

MATERIAL AND EQUIPMENT REQUIRED BUT NOT PROVIDED

Fluorescence microscope, refrigerator at 4°C, water bath at 37°C and 90-100°C, latex gloves, glass coverslips (18x18 mm, 22x22mm, 24x60 mm), micropipettes, plastic tank for horizontal incubations, distilled water, 70%, 90% and 100% ethanol, staining solutions.

RECOMMENDED STAINING SOLUTIONS

For fluorescence microscopy: any DNA-specific binding fluorochrome could be used (DAPI, propidium iodide, ethidium bromide or synergy brand related fluorochromes). Antifading solution.

INSTRUCTIONS FOR USE

Preparing the final lysing solution:

1) The final lysing solution is unstable. Once the final solution has been reconstituted shall be use immediately.

10 ml of the final lysing solution are necessary to process one slide in a 9x3 cm box. This is the volume needed for one slide. Scale the volume depending on the number of slides to be processed.

Two slides could be processed in quick succession using the same solution.

To reconstitute the final lysing solution, add 1 µl of the reducing agent for each ml of base lysing solution which is required to process a certain number of slides. **Mix to homogenize the final lysing solution.** Add the solution to a covered box to avoid evaporation and work under continuous air removal.

Including the sperm sample in agarose microgel

1) Set the lysing buffer at room temperature (22°C)

2) Dilute the sperm sample in culture medium or Phosphate Buffered Saline (PBS), to give a final concentration of 5 – 10 million spermatozoa per millilitre. Either fresh or liquid nitrogen frozen samples can be used.

3) In order to melt the agarose, place vial with low melting agarose onto a float and both into a water bath at 90-100°C for 5 minutes.

4) Transfer vial together with the float to a thermostatic water bath at 37°C, and leave it for 5 min to equilibrate agarose temperature.

5) Once agarose is at 37°C, add 25 µl of semen solution to the vial and mix thoroughly.

6) Place a drop of the cell suspension in vial onto the treated face of the slide (marked surface) and cover with a glass coverslip. Avoid making air bubbles by gently pressing. We recommend a drop of 15, 25 or 50 microlitres to coverslips of 18x18 mm, 22x22 mm or 24x60 mm, respectively. Slides need to be always placed in horizontal position.

7) Place the slide on the cooled plate into the fridge and leave the sample to solidify for 5 minutes.

Processing the sperm sample

8) Remove the coverslip smoothly, and set the slide (in horizontal position) in 10 ml of the lysing solution. This is the volume needed for one slide. Scale the volume depending on the number of slides to be processed. Incubate for 5 min at room temperature (22°C). **Work under fume hood or cover the tray during incubation!**

9) Transfer the slide to another plastic tray with distilled water. Wash it for 5 min.

10) Dehydrate in sequential 70, 90 and 100% ethanol baths (2 min each) and air dry. Remember to always keep the slide in horizontal position.

11) Once the slides are dried, they can be stored for several months at room temperature in a dry place.

Staining the sample

Proceed to stain the slide just before visualization and analysis. For fluorescence microscopy any DNA-specific binding fluorochrome could be used (DAPI, Propidium Iodide, Ethidium Bromide or Synergy Brand related fluorochromes).

Concentrations of use are identical to those for routine fluorescence microscopy. (i.e. propidium iodide 2,5 µg/ml, and mounted in Vectashield antifading).

Once obtained the concentration, mix the fluorochrome with an antifading solution in proportion 1:1. A final volume of 5 µl is recommended for staining a 25x 25 coverslip.

UNDER THE MICROSCOPE AND CLASSIFYING THE NUCLEOIDS

Any magnification can be used, from 10x to 100x. 20x, 40x or 60x dry objectives are recommended. The nucleoid corresponding to the intensely protein depleted nuclei is made up of two parts: the *core*, which appears at central position, and the peripheral *halo* corresponding to the chromatin/DNA dispersion ringlets. The analysis of a minimum number of 500 spermatozooids per sample is recommended. The criteria for classification are as follows:

SPERMATOOZA WITH FRAGMENTED DNA:
Spermatozoa with a large and spotty halo of chromatin dispersion.

SPERMATOOZA WITH UNFRAGMENTED DNA:
Spermatozoa with a small and compact halo of chromatin dispersion.

SAFETY WARNINGS AND ENVIRONMENTAL PRECAUTIONS

Biological samples have to be handled as potentially infectious.
 Care should be taken to avoid contact with skin or eyes, and to prevent inhalation. Gloves should be worn to handle the products. Lysing solution contains β-mercaptoethanol. Work under air removal environment and please follow the manufacturer's Material Safety Data Sheet regarding safe handling.

Do not dispose waste products into the environment.
 Please follow the specific safety regulation of your Faculty or Research Center with respect to chemicals storage and toxic products disposal.

REAGENTS STABILITY AND STORAGE

The components of the kit are stable at room temperature; always keep in a dry and light protected place. Expiration: the reagents supplied are stable for a minimum period of one year. Keep fluorochrome at 4°C.

TROUBLESHOOTING THE PROBLEM – IT'S COMMON CAUSE – OUR SUGGESTED APPROACH

- 1) *Weakly stained halos – Too short colouring exposure time – Extend time exposure to dye.*
- 2) *Failure to differentiate halo and core - Too long colouring exposure time – Discolour by intense buffer washing and colour again but reducing time exposure to dye.*
- 3) *The halos appear displaced from the core in one-way direction – Slides were not kept in horizontal position during sample processing.*

Figure: Arrows show those with fragmented DNA

