Kit for DNA fragmentation visualization in *Sus domesticus* spermatozoa (for the evaluation of 25x2 samples)

THE KIT CONTAINS

- 25 precoated slides
- 25 vials with low melting agarose
- 1 flask with 200 ml of lysing solution*

*** Lysing buffer contains β-mercaptoethanol. Work under extractor!**

BACKGROUND

The **SPERM-*Sus*-HALOMAX®** kit from **CHROMACELL SL** bursts into the market as a pioneering product for the analysis of fragmentation levels in spermatozoa both in domestic and experimental animals. **SPERM-*Sus*-HALOMAX®** is a simple test to study DNA fragmentation levels in *Sus domesticus* spermatozoa. The method is a variant of the Sperm Chromatin Dispersion protocol currently employed in humans (SCD; Fernández et al., 2003. J. Androl. 24: 49-66) which has been adapted for *Sus domesticus* spermatoza. This new methodology provides a simple way to distinguish, even at low magnification, spermatozoa with and without fragmented DNA, giving high quality images both under fluorescence and light microscopy.

SPERM-*Sus*-HALOMAX® is based on the differential response of fragmented and unfragmented *Sus domesticus* spermatozoa nuclei to a desproteinization treatment. The extraction of nuclear proteins from spermatozoa with fragmented DNA, releases the fragments between two breakage points. Sperm nuclei disperse chromatin around, forming a low tinted peripheral halo, distinguishable under low magnification microscopy. These images contrast sharply with the highly tinted nuclei of the spermatozoa (the core) in centered position. On the contrary, those spermatozoa with unfragmented DNA do not develop dispersion halo, or if the halo is developed, it appears as a very thin crown around the core, which is almost indistinguishable under low magnification. Thus, the **SPERM-*Sus*-HALOMAX®** kit allows estimating the proportion of spermatozoa with fragmented DNA as additional information for fertility evaluation.

NOT PROVIDED MATERIAL AND EQUIPMENT REQUIRED

Fluorescence or light microscope, refrigerator at 4ºC, water bath at 37ºC, latex gloves, glass coverslips (18x18 mm, 22x22mm, 24X60 mm), micropipettes, plastic tank for horizontal incubations, distilled water, 70%, 90% and 100% ethanol.

RECOMMENDED DYE SOLUTIONS

For light microscopy: Wright solution (Merck 1.01383.0500) in Phosphate Buffer pH 6.88 (Merck 1.07294.1000). Mounting media: Eukitt® (Panreac 253681).

INSTRUCTIONS FOR USE

Including the sperm sample in agarose microgel

Set the lysing buffer at room temperature (22ºC)

1) 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.
2) In order to melt the agarose, place vial with low melting agarose onto a float and both into a water bath at 90-100ºC for 5 minutes.
3) Transfer vial together with the float to a thermostatic water bath at 37ºC, and leave it for 5 min to equilibrate agarose temperature.
4) Once agarose is at 37ºC, add 25 microlitre of semen solution to the vial and mix thoroughly.
5) Place pretreated slides onto a metallic or glass plate which has been previously cooled at 4ºC.
6) Place a drop of the cell suspension in vial onto the treated face of the cooled slide (marked surface) and cover with a glass coverslip, avoiding making air bubbles. 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 promptly introduce 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 (22ºC). **Your attention, please! Work under extractor!**
9) Raise the slide with the aid of a lancet and wearing gloves. **Keeping the slide always in horizontal position**, the slide is trasferred to another plastic tank with plenty of distilled water, where the slide is washed for 5 min in order to remove what is left over of the lysing solution.
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 kept for several months at room temperature in a filing box.

Dying the sample

Proceed to dye the slide just before visualization and analysis.

12) 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 dying solution. Leave it for 5-10 min blowing on it from time to time. Remove the dying solution, wash briefly and smoothly in tap water and air dry.
13) Check colouring level under the microscope, verifying to clearly discriminate the peripheral halo. If colouration results very weak, especially on the region of chromatin dispersion halos, the slide can be retinted with Wright solution. If colouration is too strong, the slide can be discoloured by washing gently in tap water, or ethanol if preferred. After air dried, it can be dyed again but reducing colouring exposure time. Once the desired level of colouration is achieved and the slide is perfectly dried, it can be mounted in Eukitt.

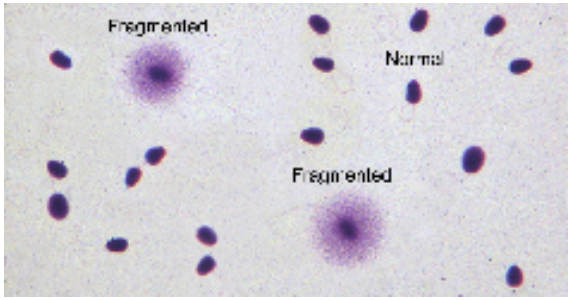
UNDER THE MICROSCOPE AND CLASIFYING THE NUCLEOIDS

Any magnification can be used, from 10x to 100x. 20x or 40x are recommended for counting purposes. The nucleoid, corresponding to the heavily desproteinized spermatozoid 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. Spermatozoid tail is visible.

The analysis of a minimum number of 500 espermatozoids per sample is recommended. The criteria for classification are as follows:

SPERMATOZOA WITH FRAGMENTED DNA: spermatozoa where the thickness of the halo is equal of greater to the length of the core minor diameter.

UNFRAGMENTED SPERMATOZOA: Halo-less spermatozoa or those with a halo thickness of less than a third of the core minor diameter.



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.

TROUBLESHOOTING

THE PROBLEM – ITS COMMON CAUSE – OUR SUGGESTED APPROACH

1) **There are no halos – Processing error. The lysing agent did not work – Try again but processing at the same time a control slide.**
2) **Weakly stained halos – Too short colouring exposure time – Extend time exposure to dye**.
3) **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.**
4) **The halos appear displaced from the core in one-way direction – Slides were not kept in horizontal position during sample processing – Try again and remember to keep the slides horizontally placed during incubation and when transferring from tank to tank**.

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