SpermBlue® Protocol

STAIN FOR SPERM MORPHOLOGY ASSESSMENT IN HUMAN AND ANIMAL SPECIES

Background:

The stain has been developed to stain all components of sperm (acrosome, head, midpiece, principal piece of tail and end piece) differentially in different intensities of blue. The staining procedure is very simple and only involves two main steps, fixing/staining in one medium (less than a minute) and dipping in water for three to six seconds.

Contents of SpermBlue®:

All **Sperm Blue®** packages contain one bottle with 250ml combined fixative and stain sufficient to stain about 500 sperm smears on slides.

It is recommended that the staining of smears is performed in standardized containers, e.g. plastic/glass Coplin jars.

If **SpermBlue®** is stored at 4ºC it will last for at least one year or longer. Room temperature storage (20 – 25ºC) not guaranteed but normally lasts one year. Take note of expiry date.

Staining Procedure:

For getting optimal results, we recommend washing semen samples before starting procedure (ex: mix 200 μl raw sample with 400 μl PBS and centrifuge at 300g for 10 min. Remove supernatant and dilute the pellet with PBS to have a working concentration of approximately 50 million sperm/ml, or the one which is comfortable for the person performing the analysis).

Step 1: prepare a semen smear using 10μl of semen or 10 to 15μl of swim-up sperm/washed sperm (adapt volume to sperm concentration) and allow to air dry. Ideal angle of slide which is used to make smear is about 45º. *For further information, please check WHO 6th ed. Manual.*

If sperm concentration in semen is less than 20 million/ml, 1) use 15μl of semen for smear and 2) decrease angle of slide which is used to make smear to about 20º. A larger volume of sperm will accordingly be dragged behind moving slide resulting in more sperm on slide. Ensure sperm smear is totally dry before next step.

Step 2: carefully place dried smear vertically into staining tray (Coplin-type jar) containing **SpermBlue®**. Take care to slowly immerse slide in the solution at 20 to 25ºC.

Reference staining times for different species:

Human/Primate	50 secs
Boar/Horse/Rat	1 min
Bull/Dog/Ram	2 min

Alternative: immerse the slide for 45 secs in SpermBlue® and wash it for 5 secs in distilled H_2O.

Note that these staining periods could vary according to the laboratory temperature, humidity or pH of the deionized water used for washing slides. Find the best times for your lab.

Step 3: carefully remove slide from staining tray and hold it at an angle of 60º to 80º to drain off excess fixative/stain.

Step 4: dip slowly in container/Coplin jar containing distilled water, dip two times for three seconds/each and let excess fluid run off on paper. Allow it to dry in vertical position. Make sure angle of slide about 60º and ensure all stain is not "sucked" from slide by the paper.

Step 5: if blue color is not intense enough, stain for another 5-10 seconds. If blue is consistently too intense, stain for 40 seconds only and dip in deionized water for 3 seconds (performing very soft circular movements with the Coplin jar to drain excess of colorant).

Step 6: ensure slide is entirely dry and then mount slide with DPX, Eukitt or equivalent synthetic medium for making permanent slides. The steps to follow are:

1. Put 2 drops of Eukitt on the coverslip.

2. Then place the slide, smear-slide down, onto the coverslip.

3. Press on the slide spread the mountant.

4. Allow the mounted smear to dry horizontally, coverslip side up.

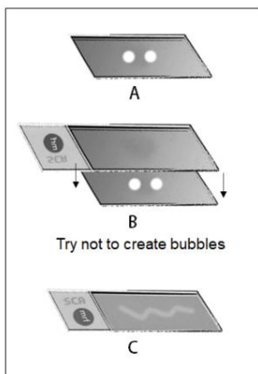


Figure 2: Mounting with Eukitt.

Important comments:

Initial staining results may suggest either too little staining of some sperm as well as differences in staining intensity on the same slide. Each researcher has to experiment to optimize her/his results in this context. Try and adapt staining times at temperature conditions between 20 and 25ºC.

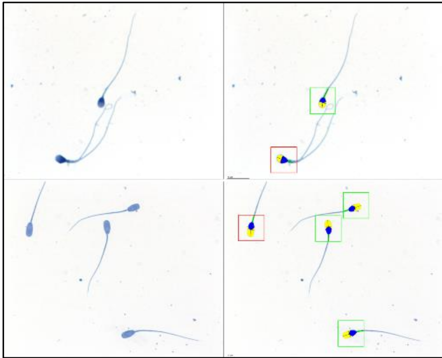
Many existing sperm staining techniques rely on "sperm painting" which is not cytologically acceptable. **SpermBlue®** clearly differentiates all sub-divisions of the sperm accurately and is particularly good in the identification of the sperm acrosome (van der Horst and Maree, (2009) **SpermBlue®**: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis, Biotechnique and Histochemistry 84:299-308).

Example:

With human and sub-human primate, horse, dolphin sperm the acrosome stains light blue and the head dark blue. Midpiece stains distinctly dark blue, rest of tail slightly lighter blue and end piece even lighter blue.

In domestic animals such as bull, boar and ram: Acrosome stains dark blue, post acrosomal area and particularly the equatorial zone stains light blue. Midpiece stains darker blue and rest of tail slightly less dark blue.

On the left, Human (above) and Boar (below) sperms stained with SpermBlue®. On the right, corresponding fields analyzed with the Sperm Class Analyzer (SCA).



Safety Datasheet for SpermBlue®:

SpermBlue® contains toxic components like all cytological stains but is not hazardous. The main active component is a slight skin, oral/nasal irritant and staining should preferably take place in a fume hood. If skin contact has occurred, wash affected area thoroughly with water.

Precautions:

All cytological stains are toxic and have to be handled with care. Always work with gloves and preferably in a fume cupboard. Only stain when sperm are fixed (dead). Do NOT use for live unfixed cells.

References:

WHO laboratory manual for the examination and processing of human semen. 2021, Sixth edition. Geneva: World Health Organization All rights reserved. Publications of the World Health Organization Press, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

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