FluoAcro Protocol

ACROSOME INTACT AND ACROSOME REACTED SPERM AS IMPORTANT SPERM FUNCTIONAL TEST

Background and rationale:

Once capacitation of sperm has taken place in the female reproductive system, the next important step is the acrosome reaction in order for fertilization to take place. However, the acrosome reaction can only take place if sperm arriving at the fertilization site has good intact acrosomes. Moreover, it does not help sperm have good acrosomes but they cannot undergo the acrosome reaction in order to attach to the oocyte and penetrate the zona pellucida of the oocyte.

Accordingly, this sperm functional test measures in the first instance the percentage intact acrosomes of washed/diluted sperm samples. The next step is that we use a biological substitute to induce the acrosome reaction such as Ca++ ionophore.

The outer acrosome membrane is detected by using a lectin based substance, called Peanut agglutinin (PNA). Using this lectin and fluorescence microscopy and a FITC filter the acrosomes appears bright green. However, the sperm without acrosomes are invisible. It is therefore necessary to use a counterstain such as Hoechst based in combination with the correct fluorescence filter cube (Dualband) to visualize both the green fluorescence (intact acrosomes) and blue fluorescence of sperm heads only (reacted acrosomes).

Please note that the original PNA/Hoechst procedure also involved detecting live and dead sperm and acrosome reacted live and acrosome reacted dead. We designed this test in its most basic form of either acrosome reacted or intact.

Description of kit reagents for Acrosome reaction (for performing 300 tests):

Solution 1 (S1): Ca based medium for acrosome reaction (3 mL): On arrival from supplier divide in 10 µL aliquots and store frozen at -20°C (or divide aliquots as 20 or 30 µL depending on number of tests you may do per day). **Note that you need to use a control here without Ca Solution.**

Solution 2 (S2): PNA staining solution (565 µL): Provided as stock solution: dilute the 565 µL of stock PNA in **14.5 mL** of **PBS (in dark)** and make 0.5 mL aliquots and freeze at –20°C. Each 0.5 mL of this working solution is sufficient for 10 Acrosome tests.

Solution 3 (S3): Basic Hoechst type working solution (150 µL) – keep at 4°C (in dark): Provided as stock solution. Make pre-working solution by taking 1 µL of stock into 1mL of PBS (stable for 5 days at 4°C).

Solution 4 (S4): Anti-fading fluorescence medium (5 mL)

2 x Slide templates for drop location

Other media not provided:

- Ham's F-10 medium or **preferred capacitating medium** of laboratory containing 3.0% (3.0 g/100mL) **human serum albumin** (HSA).
- Ethanol 70% (v/v) needed to stop reaction.
- Phosphate buffered saline (PBS) or pH balanced salt tablets.
- Distilled water.
- Fixing solutions: 95% ethanol (4°C) and 4% paraformaldehyde - (optional).

Acrosome reaction protocol

Acrosome reaction is induced only in sperm suspension exposed to Ca++ based substance. We recommend to perform an additional acrosome intact suspension (control assay) when semen parameters are below WHO cut-off points for potentially infertile, but particularly when idiopathic male infertility is suspected.

Step 1: Wash semen sample with phosphate buffer (PBS).

Step 2: Add in an empty vial **1 ml** of preferred capacitating medium (e.g. HAMS F10, HTF capacitating medium or other) with 0.03 g/mL Human Serum Albumin and preheat at 37°C.

Step 3: Dilute washed sperm into capacitating medium to have a working concentration of 4 to 8 million sperm/ml. Incubate for **25 minutes at 37°C**.

Step 4: Add **10 µl** of **Solution 1** for acrosome reaction only **(not for control)**.

Step 5: Incubate for **15 minutes at 37°C (also control)**.

Step 6: Stop the reaction by adding **100µl** of 70% ethanol to the vial **(also add to control)**.

Step 7: Take **5 µL** of sperm suspension and make one drop on slide (add one drop of control and one drop of reaction vial per slide by using the provided template).

Step 8: Dry **for 10 min at 37°C**.

Step 9: Immerse slides in a Coplin jar with pre-cooled **95% ethanol.** Incubate at 4°C for **30 minutes (in dark)** and Air dry.

Acrosome Staining procedure

Use the provided template to locate dye solutions on each sample spot:

Step 1: Pipette **20 µL** of **Solution 2** on each sperm spot and leave for **5 minutes** at room temperature **(in dark)**.

Step 2: Drain drops.

Step 3: Add another **30 µL** of **Solution 2** on each drop and leave slides for **75 minutes** in a wet chamber (large **Petri dish** with **wet filter paper** under slide; Otherwise fluid evaporates) **in dark**. (40 to 50 µL Solution 2 may be needed if found that drop evaporates too fast).

Step 4: Immerse briefly twice in **PBS (in dark)**.

Step 5: Add **10 µL** of final working **Solution 3** on each dried sample spot for **5-10 minutes (in dark).**

Step 6: Immerse slides slowly for **one second** in H2Od **(in dark)**.

Step 7: Fix slides by immersion in **4% paraformaldehyde** at room temperature for **15 minutes** (in dark). This step is **optional** and only if slides cannot be analysed on same day. Immerse briefly in **distilled water (in dark)**.

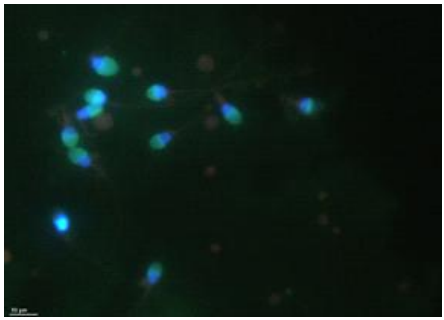
Step 8: Allow slides to **dry completely (in dark)**.

Step 9: Mount cover slips with **one drop** of 15 µL of **Solution 4 (in dark)**. View within 48 hours if fixed in paraformaldehyde; otherwise, on the same day (in dark).

Step 10: View slides using 40X Plan Fluor objective and DualBand Semrock DAPI & FITC DA/FI-A-000 (EX 387/480, EM 433/530, DM 403/502) which will cover range of DAPI (Hoechst) and FITC and Texas Red – here **intact** acrosomes will show bright **green**/blue and **only blue** if no acrosome present (**reacted**). The most typical patterns are the full acrosome (bright green) or a green band at equatorial segment (reacted) and blue (reacted). (View using fluorescent microscope also in dark).

Acrosome reaction interpretation:

The relation of intact versus reacted acrosomes can be used to evaluate sperm ability to undergo acrosome reaction as an important additional sperm functional test. There should be typically a **15% difference** between acrosome reacted control and induced acrosome reacted.



Green fluorescence show acrosome intact and blue only acrosome reacted with Dualband filter using x40 objective

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