UAB Universitat Autònoma de Barcelona

Dipòsit digital de documents de la UAB

This is the **accepted version** of the journal article:

Anton, Ester; Sarrate Navas, Zaida. «Fluorescence in situ hybridization (FISH) protocol in human sperm». Journal of visualized experiments, Núm. 31 (2009), art. e1405. DOI 10.3791/1405

This version is available at https://ddd.uab.cat/record/289970

under the terms of the license

Title: Fluorescence *in situ* hybridization (FISH) protocol in human sperm

Authors: Zaida Sarrate and Ester Anton

Author affiliation:

Zaida Sarrate
Unitat de Biologia Cel·lular (Facultat de Biociències)
Universitat Autònoma de Barcelona.
08193-Cerdanyola del Vallès, SPAIN.
zaida.sarrate@uab.cat

Ester Anton
Unitat de Biologia Cel·lular (Facultat de Biociències)
Universitat Autònoma de Barcelona.
08193-Cerdanyola del Vallès, SPAIN.
ester.anton@uab.cat

Corresponding author:
Ester Anton

Keywords:

Fluorescence *in situ* hybridization, human, infertility, numerical chromosome abnormalities, spermatozoa

Short abstract: This video-article describes, step by step, how to process a semen sample to achieve good-quality fluorescence *in situ* hybridization on human spermatozoa. Preparations obtained can be used for aneuploidy screening in the context of clinical diagnosis.

Long abstract: Aneuploidies are the most frequent chromosomal abnormalities in humans. Most of these abnormalities result from meiotic errors during the gametogenic process in the parents. In human males, these errors can lead to the production of spermatozoa with numerical chromosome abnormalities which represent an increased risk of transmitting these anomalies to the offspring.
For this reason, the technique of fluorescence *in situ* hybridization (FISH) on sperm nuclei has become a protocol widely incorporated in the context of clinical diagnosis. This practice provides an estimate of the frequencies of numerical chromosome abnormalities in the gametes of the patients that seek for genetic reproductive advice.
To date, the chromosomes most frequently included in sperm FISH analysis are chromosomes X, Y, 13, 18 and 21.
This video-article describes, step by step, how to process and fix a human semen sample, how to decondense and denature the sperm chromatin, how to proceed to obtain sperm FISH preparations, and how to visualize the results at the microscope. Special remarks of the most relevant steps are given to achieve the best results.

Protocol:

I. Sample processing and cell fixation

1. Leave the semen sample in sterile containers at room temperature for 20 minutes until liquefaction.
2. Transfer the sample to a centrifuge tube and spin it at 1000g for 5 minutes.
3. Gently remove and discard the supernatant using a Pasteur pipette.
4. Add hypotonic solution (KCl, 0.075M) pre-heated at 37ºC, drop by drop, while mixing on a vortex to obtain a final volume of 10 ml.
5. Place the tube in a water bath at 37ºC for 30 minutes.
6. Centrifuge at 1000g for 5 minutes. Carefully discard the supernatant by decantation, without disturbing the pellet.
7. After resuspending the pellet, add freshly prepared Carnoy's fixative (3:1 methanol:acetic acid) drop by drop while mixing on a vortex to obtain a final volume of 8 ml.
8. Repeat points 6 and 7 as many times as necessary to obtain a white pellet.
9. Add freshly prepared methanol:acetic acid (3:1) drop by drop to the pellet. Adjust the final volume to the cellular concentration required for obtaining good cell extensions. A test slide can be made by dropping the resuspended sample onto an ungreased slide and examining it under a phase contrast microscope. This will let to check the cellular dispersion obtained and would allow correcting it, if it is necessary, in further slides.
10. To make the extensions, drop the cell suspension into the center of ungreased slides (previously stored in methanol at -20ºC). To facilitate prospect relocalizations, delimit the area containing the sperm extension on the opposite side of the slide using a diamond pencil.
11. Store the slides at -20ºC until further use.

Note: The addition of methanol:acetic acid (3:1) drop by drop while mixing is a very important step to avoid the formation of sperm aggregates.

II. Decondensation

1. The slides stored at -20ºC must be defrosted to continue with the protocol (leave them at room temperature).

2. Place the slide in two consecutive coplin jars with 2x saline-sodium citrate solution (2xSSC) for 3 minutes each.
3. Transfer the slide through a series of ethanol washes for 2 minutes in each coplin jar. Start with 70% ethanol, follow by 90% and finish with 100%. Dry out the slide leaving it at room temperature.
4. Incubate the slide in dithiothreitol solution (DTT) at 37ºC in the incubator to decondense the chromatin. This product acts by breaking the disulfur bridges of the protamines that coil the DNA in the spermatozoa nucleus. This is a very important step because the DNA in the sperm nucleus is highly compacted. The incubation time of the slides in DTT must be adjusted according to the sample's reactivity to this product. Usually, this time is around 8 minutes, although it can vary from 2 to 15 minutes.
5. Immediately, transfer the slide to two consecutive coplin jars with 2xSSC for 3 minutes in each coplin.
6. Continue by placing the slide through a series of ethanol washes for 2 minutes in each coplin jar (70%, 90% and 100% ethanol). Let the slide to dry out at room temperature.

Note: An excessive exposition to DTT would result in disperse FISH signals at the end of the procedure, whereas a too short exposition would result in a lack of some signals.

III. Hybridization

1. Denature the sperm DNA by incubating the slide in a coplin jar with formamide solution (70%) at 73ºC for 5 minutes.
2. Transfer the slide through ethanol solutions for 1 minute per coplin jar (start with 70% ethanol, 85% and finish with 100%). Leave the slide to dry out at room temperature.
3. Add directly 5 µl of the corresponding ready-to-use probe mixture to a 15x15 cover slip (AneuVysion Assay Multi-color Probe Panel: CEP 18/X/Y or LSI 13/21).
4. As it is shown in the video, carefully place the slide onto the cover slip to put together the target region and the probe mixture. Seal it with rubber cement.
5. Place the slide into a pre-warmed 37ºC hybridization chamber (HYBrite™) and incubate it at 37ºC for 6-24 hours.

Note: Whereas the denaturation of the sperm sample is mandatory, the probes denaturation depends on the requirements of the manufacturer's supplier. The probe

mixtures used in this protocol are ready to use and, in this case, probe denaturation is not required.

The volume of the probe mixture will vary according to the size of the hybridized area.

IV. Washes post-hybridization

1. Take out the slide from the hybridization chamber.
2. Remove the rubber cement and carefully pull out the cover slip stretching gently from the side.
3. To eliminate the unspecific hybridization signals place the slide for 2 minutes in a coplin jar with 0.4xSSC/0.3%NP-40 pre-warmed at 73ºC in a water bath.
4. Transfer the slide to a 2xSSC/0.1%NP-40 wash solution at room temperature for 1 minute. Let the slide to dry out at room temperature.
5. Add a counterstaining product to the target region (8 µl for an 18x18 cover slip). The most common product used is 4',6-diamidino-2-phenylindole (DAPI, Vysis Inc.).
6. Cover the hybridized region with a cover slip (to preserve the hybridization the cover slip can be sealed with nail varnish).
7. The slides can be stored at -20ºC until their prospect analysis.

Note: A higher temperature or an excessive wash time can result in the elimination of some signals, whereas the opposite would not wash out unspecific probe hybridizations.

The volume of the counterstaining product added will vary according to the size of the hybridization area to cover.

V. Visualization

The preparations can be evaluated under an epifluorescence microscope equipped with a triple-band filter for DAPI/Texas Red/FITC and single-band filters for Aqua, FITC and Texas Red. Standard assessment criteria must be followed for the correct evaluation of the sperm nuclei [1].

The preparation hybridized with probes for chromosomes X, Y and 18 should display signals for these three chromosomes. Every normal spermatozoa must show one blue signal (corresponding to the chromosome 18), and a green signal (X-bearing spermatozoa) or a red signal (Y-bearing spermatozoa).

In the preparation hybridized with probes for chromosomes 13 and 21, a green signal for chromosome 13 and a red signal for chromosome 21 should be distinguished in every normal spermatozoa.

Discussion:

This protocol describes how to process human semen samples for obtaining sperm FISH preparations. Using this protocol it is possible to analyze chromosomal abnormalities in male gametes. Spermatozoa aneuploidy screening has applications either in the context of basic research and in the reproductive advice given to infertile males. Although in clinical diagnosis the most widely studied chromosomes are X, Y, 13, 18 and 21, other commercial probes for a wide range of chromosomes and loci are available and can also be used in these experiments.

Acknowledgments: This work was supported by the Generalitat de Catalunya (2005-SGR00437). The authors thank to J. Blanco and F. Vidal for their supervising and encouragement, and J. Lucas for his technical assistance.

Disclosures: We have nothing to disclose

Materials:

Name	Type	Company	Catalog Number	Comments
1,4-Dithiothreitol (DTT)	Material	Roche	10197777001	
Acetic acid	Material	Merck	100.063	
AneuVysion® Multicolor DNA Probe Kit - 20xSaline-Sodium Citrate - 4,6-diamidino-2-phenylindole (DAPI II) - CEP 18/X/Y DNA probe - NP-40 - LSI 13/21 DNA probe	Material	Vysis Inc.	32-161075 30-805850 30-804941 30-171077 30-804820 30-171078	
Centrifuge 5804R	Tool	Eppendorf		
Centrifuge tube	Material	Nunc	347856	
Coplin jar (glass)	Material	Barloworld Scientific	Hellendahl (ZCT278)	
Coplin jar (plastic)	Material	Deltalab	191087	
Cover slips (15x15)	Material	Knittel gläser	4600115	
Cover slips (18x18)	Material	Knittel gläser	4600118	
Distilled water	Material			
Diamond pencil	Material	Hammacher	335117010	
Ethanol	Material	Merck	100.983	
Formamide	Material	Roche	11814320001	
Freezer	Tool	Zanussi		
Gloves	Material	Sanyc	101/3300	
HYBrite™	Tool	Vysis Inc.		
Immersion Oil	Material	Olympus	35505	
Incubator	Tool	Heraeus		
Methanol	Material	Merck	106.009	
Micropipette	Material	Labsystems	Finnpipette (4500000)	
Micropipette replacement tip	Material	Daslab	16-2001	
Nail varnish	Material	Quo-cosmetics		
Olympus BX60 epifluorescence microscope	Tool	Olympus		Equipped with a triple-band filter for DAPI/Texas Red/FITC and single-band filters for Aqua, FITC and Texas Red.
Pasteur Pipette	Material	Rubilabor	211.0230	
Phase contrast microscope	Tool	Nikon Diaphot		
Plastic pipette (3ml)	Material	Deltalab	200007	
Potassium chloride (KCl)	Material	Fluka	60128	
Rubber cement	Material	Best-test		
Slides	Material	Knittel gläser	4520022	
Slide Saver Boxes	Material	Deltalab	19276.1	
Sterile container	Material	Deltalab	409726	
Syringe	Material	PentaFerte	002022300	
Thermometer	Material	Comark Instruments, Inc	KM12	
Tweezers	Material	B/Braun	Aesculap (BD224R)	
Vertical laminar flow hood	Tool	Burdinola	OR-ST 1500	
Vortex mixer	Tool	Fisher Scientific	FB15012	
Water bath	Tool	Raypa®		

References:

1. Blanco, J., Egozcue, J. & Vidal, F. Incidence of chromosome 21 disomy in human spermatozoa as determined by fluorescent in-situ hybridization. Hum. Reprod. 11, 722–726 (1996).