Krishnaja.
17-7-03

From: **Helen G Tempest <Helen.Tempest@brunel.ac.uk>**
To: <krishnaja11@vsnl.com>
Subject: **FISH protocol for sperm aneuploidy**
Date: Wed, 16 Jul 2003 09:29:04 +0100 (BST)

Dr Krishnaja,
Hi my name is Helen Tempest (i work in Darren Griffin's lab) i can only apologise for not getting back to you sooner about the FISH protocol. Darren asked me to send you the protocol i read the e-mail in the middle of an incubation and then it went to the back of my mind. We routinely carry out sperm FISH but do not do PRINS. I will track down my protocol this morning add all the extra bits of information and send this to you ASAP.
Once again apologies for the delay
With Best Regards,
Helen

From: **Helen G Tempest <Helen.Tempest@brunel.ac.uk>**
To: <krishnaja11@vsnl.com>
Subject:
Date: Wed, 16 Jul 2003 10:09:47 +0100 (BST)
Attachments: fish protocol.doc, Size: 31048 bytes.

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Dear Dr Krishnaja,
As promised here is the FISH protocol i have tried to put in the important points/extra information in bold within the text i hope it is not to confusing. In general terms it is a fairly straight forward procedure that is fairly reliable once set up.
If you are having technical difficulties or are not sure about what i have written please e-mail me and hopefully i will be able to help you out.
With Best Regards,
Helen

Helen G Tempest
Brunel University, UK
bipghgt@brunel.ac.uk

Krishnaja. 19-7-03.

(1) Preparation of sperm nuclei for FISH analysis

Semen samples were washed three times in 10mM Tris HCl, 10mM NaCl (pH 8.0), to remove the seminal fluid and centrifuged at 1200 rpm for 5 minutes after each centrifugation the pellet was carefully resuspended. Samples were then stored at 4oC until required for FISH analysis. Once ready for analysis the sample was briefly centrifuged and the majority of the supernatant was removed and the pellet resuspended to give a semi-translucent suspension. Slides were then prepared by spreading 10-20µl of the cell suspension onto a clean glass slide air-dried and then dehydrated through an ethanol series 70, 80 and 100% for 5 minutes in each and **air dried**. Age overnight at RT or for one hour at 70oC.

Decondensation and Denaturation of sperm nuclei

Prior to hybridisation sperm nuclei were decondensed for 30 minutes in 0.1M Tris buffer (pH 8.0) (40mls) containing 400µl of 1M DTT (Sigma D9779) **(make aliquots and store at –20Oc)**, slides were then incubated for 1 hour in 20mM LIS (Sigma D3635) in 0.1M Tris (pH 8.0) **(This step is optional it helps probes further penetrate highly condensed nuclei, I would suggest trying without this step if probe signal is weak/absent then carryout LIS incubation. I make up a small amount of the LIS and store at 4oC for up to a month)**. **(It is important to carry out decondensation incubations in the dark as they are light sensitive)**.After decondensation the slides were briefly rinsed in 2xSSC pH 7.0 **(about 1 min with gentle agitation)**, dehydrated in ethanol as above and **air-dried**. And subsequently denatured for 11 minutes in 70% formamide/2xSSC pH 7.0 at 72oC + 1oC per slide. After denaturation slides were quenched in ice cold 70% ethanol for 2 minutes, followed by 2 minutes each in 80% and 100% ethanol to dehydrate the slide. Slides were then air dried before the probe was applied.

Probe preparation and denaturation

Carry out according to probe manufacturer guidelines (or standard preparation used for FISH if non-commercial paints are used).

Post Hybridisation Washes

Carry out according to probe manufacturer guidelines (or standard washes used for FISH if non-commercial paints are used).

Microscopy and scoring criteria

Approximately 5000 sperm nuclei were scored per patient (or the maximum that could be counted in patients with severe oligozoospermia) triple (if analysing sex chromosomes)/dual colour FISH was always undertaken to enable disomic spermatozoa to be distinguished from diploid ones. For scoring slides a stringent criteria was applied: Sperm nuclei were only scored if spermatozoa were intact, displaying a similar degree of decondensation and clear hybridisation signals; disrupted or overlapping spermatozoa were excluded from analysis. Spermatozoa were regarded as abnormal if two copies of the same chromosome (disomic) were found within the sperm nuclei, in this case the two signals recorded for the same chromosome had to be equal in intensity and size found in normal haploid nuclei. Signals that were clear hybridisation signals, similar in size, and at least separated from each other by at least one signal domain and clearly positioned within the sperm nuclei, (therefore excluding divided (split) signals being wrongly scored as disomies). Spermatozoa exhibiting two signals (as above) for each chromosome 21 and the sex chromosomes were counted as diploid. Cells that presented with an absence of either a sex chromosome or chromosome 21 were not counted as nullisomic as it is not possible to distinguish whether this is true nullisomy or failure of the probe to hybridise, therefore nuliisomic spermatozoa were not included in analyses.