Bovine X-Y Sex Test FISH Protocol

(X- Biotin labelled / Y – Cy3 labelled)

(CA-1611)

Introduction

The FISH protocol is divided into two stages. Denaturation and Hybridisation are performed on **Day One**. Washing and Detection are performed on **Day Two**.

On day one, the DNA of the chromosomes and paints is denatured and the hybridisation process (reannealing) takes place overnight. On day two, the slides are washed to remove unbound DNA sequences followed by detection, counterstaining and mounting.

Kit Contents		
Product Code	*Description*	*Volume*
No Kit Required		

Requirements (not provided)

Equipment	Reagents
Ethanol cleaned slides	Sodium Chloride
Coverslips	Sodium Citrate
Eppendorf tubes	HCl
Coplin jars	Tris
Humidified chamber	Formamide
Micro-pipette 1µl, 10µl, 500µl	Pepsin
Pipette 10ml, 20ml	Dithiothreitol
Vortex	Di-sodium tetraborate
Parafilm	Sodium lauryl sulphate
Micro-centrifuge	Absolute Ethanol
65°C , 45°C Water bath	Double Distilled Water
37°C Incubator	Clear nail varnish

Approx time:

Slide preparation 30 min + overnight (or + 90 min)

Denaturation and hybridisation: 30 min + overnight

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Denaturation and Hybridisation – Day One

Solutions to be prepared:
20XSSC
2XSSC
Denaturation solution
Pepsin solution
Working Reagent A
Working Reagent B
Working Reagent C

Solution 20XSSC:
87.6g NaCl
44.1g Na Citrate
up to 500ml Deionised Distilled water

Adjust pH to 7.0 using concentrated HCl (before finalising water volume), aliquot and autoclave. Store at 4°C).

Solution 2XSSC:
50ml 20XSSC
450ml Double distilled water
500ml 2XSSC

Denaturation solution:
70ml Formamide
30ml 2XSSC
100ml Denaturation solution

Pepsin solution:
0.5ml Pepsin Solution (1% in water)
49.5ml 10mM HCl
50ml Pepsin solution

Note: *Stock solution can be stored at –20°C in small aliquots.*

Working Reagent A: 0.01 M Tris, 0.9% NaCl

Working Reagent B: 0.25M DTT in solution A

Working Reagent C: Sodium lauryl sulphate 1% (w/v), Di Sodium tetraborate 1.9% (w/v) in Distilled water

Note: *Ensure all solutions are mixed well.*

All solution volumes sufficient for 10 slides

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Procedure: Washing

1. Place 50µl of delivered sperm in a 0.5ml eppendorf
2. Add 100µl solution A
3. Spin 2000rpm (RCF 400) for 10 min
4. Remove supernatant
5. Add 200µl solution A
6. Determine sperm concentration
7. Dilute with solution A until sperm is at final concentration of is 2.5x 10^8 / ml

Procedure: Decondensation of sperm

8. Place 5µl sperm in 0.5ml eppendorf
9. Add 5µl of solution B
10. Incubate 2.5min
11. Add 5µl solution C
12. Incubate 10 sec
13. Add 50µl of 100% ethanol

Procedure: Slide Preparation

14. Place 2µl of decondensed sperm on slide
15. Dry slide at room temperature
16. Check sperm with phase contrast microscope; (enlarged intact grey sperm)
17. Incubate slide in 100% ethanol for 5 min
18. Dry at room temperature
19. Check sperm with phase contrast microscope; (clean enlarged intact grey sperm)

Procedure: Pepsin Treatment

20. Incubate slide in pepsin solution for 30min
21. Wash in 2XSSC for 1min. Repeat twice
22. Wash in distilled water for 2 sec. Repeat twice
23. Dehydrate by serial ethanol washing for 2 min each in 70% (v/v) ethanol, 70%, 90%, 90%, and 5 min 100%.
24. Dry at room temperature

25. Bake slide at 65ºC for 30 min.

Procedure: Probe preparation & denaturation

26. Warm chromosome paints to 37ºC, vortex and centrifuge for 1- 3 seconds.
27. Denature probe for 10 min at 65ºC, and hold at 37ºC for 30-60 min.

Procedure: Slide denaturation

28. Denature slide in Denaturation solution at 80ºC for 10 min.
29. Incubate slide in ice cold 70% ethanol for 5 min. Dehydrate by serial ethanol washing for 2 min each in 70% (v/v) ethanol, 70%, 90%, 90%, and 5 min 100%. Dry at room temperature.

Procedure: Hybridisation

30. Apply 10µl denatured chromosome paint sample on slide at sperm location
31. Cover with 22X22 coverslip

Note: *Use a total of 10µl for a 22X22 slide, 15µl for a 34X22 slide.*

32. Seal with rubber cement
33. Place slide in an air tight, prewarmed humidified chamber and incubate overnight in the dark at 37ºC.

Note: *Post hybridisation washes are incorporated in detection protocol as part of day 2*

34. Proceed with Detection Kit and Protocol (1089-KB-50 Biotin-FITC Detection)

References:

T Révay, A Kovács, W Rens, I Gustavsson (2002)

Simultaneous detection of viability and sex of bovine spermatozoa.

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