

Subject: protocol, page 1

Date: Mon, 28 Feb 2000 12:01:23 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Dear Krish,

I hope everything is OK. I will try to send FISH-protocol. I think it will take several messages.

FISH protocol
Preatreatment

- 1 Wash slides in PBS 5 min at room temperature (RT)
- 2 Alcohol 70%, 90%, 100% - each for 2 or 5 min, RT
- 3 Air dry
- 4 Pipette 100 microl of RNase A
(concentration 100 microgramm/ml),
overlay with coverslip.
Incubate in moist chamber at 37oC 45-60 min
NB: work solution of RNase should be prepared from stock
solution - concentration 10 mg/ml (store at -20oC):
890 microl H2O
100 microl 20xSSC
10 microl RNase stock (10 mg/ml)
- 5 Wash slides
2xSSC 3 times for 5 min, RT, (during 1st washing - remove
coverslip)
- 6 PBS 5 min, RT
- 7 pepsin treatment - final concentration 0.005%/10mM HCl
8-10 min at 37oC
99 ml H2O + 1 ml 1N HCl + 50 microl 10% pepsin (stock
solution of pepsin store at -20oC)
(work solution should be pre-warmed in water-bath before use)
- 8 PBS 5 min, RT
- 9 50mM MgCl2 / PBS 5 min, RT
(95 ml PBS + 5 ml 1M MgCl2)
- 10 1% formaldehyde/PBS/MgCl2 10 min, RT
(92 ml PBS + 5 ml 1M MgCl2 + 3 ml 37% formaldehyde)
- 11 PBS 2 times for 5 min, RT
- 12 Alcohol 70%, 90%, 100% - each for 2 or 5 min, RT
- 13 Air dry

NB: for steps - 1,2, 5, 6,11,12 - use shaker

Dear Krish, I also will send everything by mail.

With best wishes, yours Janna

Subject: protocol, page2

Date: Mon, 28 Feb 2000 12:28:47 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Probe denaturation and competition:
for "Cambio" **concentrated** paints and pan-centromeric probe

A: Paints for specific chromosomes

- a warm paints to 42oC and mix well before use
- b mix sufficient amount of every paints (in separate Eppendorfs) with hybridization buffer (HB - Cambio)
mix very well, spin down for few seconds (see Note 1)
- c denaturate paints by incubation at 65oC for 10 min
(or at 70oC for 8 min) in water bath
- d briefly put on ice for 2-3 min
- e transfer to water bath at 37oC and incubate for 60 to 90 min

B Pan-centromeric probe (PC)

(start 30 min before the end of paint's competition)

- a warm CP and HB at 37oC for 5-10 min
- b take sufficient amount of CP plus HB and place in Eppendorf tube
mix very well, spin down for few seconds
- c denaturate CP by incubation at 85oC for 10 min in water bath
- d immediately put on ice for 2-3 min - than spin down for few seconds and keep on ice

For triple-colours FISH with pan-centromeric probe use final volume 18-20 ul (microliters) of hybridization mixture per slide.

Cambio protocol recommends to use per one slide:

3ul of each concentrated paints and 2-3 ul of concentrated CP.

For example:

Coctail: #1 Bio + #4 Bio/FITC + #8 FITC + CP FITC

<i>colour</i>	<i>red</i>	<i>yellow</i>	<i>green</i>	<i>green</i>
For 1 slide:	#1 Bio	3ul	+	1.6ul HB
	#4 Bio	0.75ul	+	1.6ul HB (see Note 2)
	#4 FITC	2.25 ul	+	1.6ul HB
	#8 FITC	3 ul	+	1.6ul HB
	CP FITC	3 ul	+	1.6ul HB
Total volume	-	20 ul		

Note 1. Bio-labelled paints look usually more bright; if signal is too strong and gives high background it is possible to add Cot 1 DNA to paint before denaturation (not more than 1 ul per slide); also, see label on Eppendorf with probe from Cambio, sometimes it is written "NO Cot1". In these case you should add Cot 1 DNA.

Note 2. Mix paints #4 Bio and #4 FITC in one Eppendorf and add HB (Optional)

Dear Krish, it will be 2 or 3 E-mail more.

Subject: protocol, 3

Date: Mon, 28 Feb 2000 12:48:31 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Prehybridization of slides

(start 30 min before the end of paints competition)

- 1 Pipette 100ul of 70% formamide/2xSSC/50 mM phosphate buffer per slide and overlay with coverslip
(350ul deionized 100% formamide + 50ul 0.5 M phosphate buffer + 50ul 20xSSC + 50 ul H₂O)
- 2 Denature slides at 80oC for 2.5-3 min on hot plate
- 3 70% ethanol 5 min (should be kept at -20oC before use)
90%, 100% ethanol - 2-5 min, RT.
Air dry

Hybridization

- 4 Mix all paints and CP in one Eppendorf tube
Note: mix very-very well and spin down for few seconds
(several times)
- 5 Pipette 20ul of the mix solution onto each slide, overlay with coverslips, seal with Cow Gum, give time to glue to dry
- 6 Incubate overnight at 42oC in the moist chamber
(incubation could be 2 overnight, if necessary)

Subject: protocol, 4

Date: Mon, 28 Feb 2000 13:13:37 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Detection

- 1 Make a wash solution (WS) - 0.05% Tween / 4xSSC
(200ml 20xSSC + 5 ml 10% Tween 20 + 800 ml H2O)
- 2 Dilute Blocking Protein (BP - Cambio) to 10-15% in wash solution from step 1.
This is solution A.
- 3 Use solution A for dilution of antibodies
(Antibodies are from Cambio, too)

*100ml : 20xSSC.
2.5ml 10% Tween 20
400ml : DW water.*

- I layer B3 1 : 500
- II layer B4 1 : 250
- F1 1 : 200
- III layer B3 1 : 500
- F2 1 : 100

- 4 Incubate in the dark for 10 min at RT
Microfuge at 11.000g for 10 min and use supernatant

- 5 Pre-warm to 42-45oC:

- 2xSSC for 1 washing
- 50% formamide/2xSSC for 2-3 washing
(pH 7,0)
- 0.1xSSC for 3 washing

- 6 Carefully remove dried Cow Gum and rinse off coverslips in the jar of 2xSSC

NOTE: Do not allow slides to dry out at any stage until step 16

Stringency washes:

- 7 Wash slides

- 50% formamide/2xSSC 2-3 x 5 min 42oC (45oC - Optional)
- 0.1xSSC 3 x 5 min 42oC (45oC - Optional)
- Wash Solution (WS) 5 min 42oC

- 8 Pipette 100ul of solution A per slide and overlay with coverslip
Incubate in moist chamber for 15-20 min at 37oC

- 9 Wash slides with WS 2 x 5 min 42oC

- 10 Pipette 100ul of I layer of antibody per slide and overlay with coverslip

Incubate in moist chamber for 20-30 min at 37oC

- 11 Wash slides with WS 3 x 5 min 42oC

- 12 Pipette 100ul of II layer of antibody per slide and overlay with coverslip

Incubate in moist chamber for 20-30 min at 37oC

- 13 Wash slides with WS 3 x 5 min 42oC

14 Pipette 100ul of III layer of antibody per slide and overlay with coverslip

Incubate in moist chamber for 20-30 min at 37oC

15 Wash slides with WS 3 x 5 min 42oC

Repeat steps 12-15

16 Alcohol 70, 90, 100% 2-5 min RT

17 Air dry

18 Counterstain with DAPI (0.15 ug/ml in Vectashield) - 25ul per slide under coverslip

Dear Krish, I will write more explanation about solution etc. if you will need it.

With love Janna

P.S.I hope the protocol will be readable

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Note 2. Mix paints #4 Bio and #4 FITC in one Eppendorf and add HB (Optional)

Dear Krish, it will be 2 or 3 E-mail more.

Subject:

Date: Tue, 14 Mar 2000 16:42:07 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Dear Krish,

I will try to put protocol about sperm.

We worked with frozen samples of sperm, but the same can be done for fresh one (we did, too).

0.2 ml of sperm put in the centrifuge tube

add 10 ml of PBS (gently shake, by hands)

spin 10 min 3000 rpm

take out supernatant

resuspend pellet in small amount of PBS, first, than add 10 ml PBS

(pellet is very hard, if you will add 10 ml of PBS at once - it will be difficult to resuspend it)

Wash with PBS 3 times.

Fresh fixative 3:1 methanol:acetic acid is used for fixation.

Fixation should be very gentle, first 20 drops - drop by drop and after

every drop = shake. Total 10 ml of fixative for 5min. Spin 10 min 3000 rpm.

Air-dried preparation.

For FISH:

washing with 2xSSC for 5 min and with ethanol (50, 70, 80 and 100%), air dry. 50, 70, 90,

Slides are treated with 25 mM DDT in 0.1% trypsin for 12 min. 100.

Wash with 2xSSC two times for 3-5 min and with ethanol (50, 70, 80 and 100%), air dry.

Hybridization mix (probes with hybridization buffer) put on the slide and cover with cover slip.

Together put on hot plate 80°C for 5 min.

Hybridize over night at 37°C.

Next day: washing with 50% formamide in 2xSSC 3 times at 45°C,

washing with buffer 3 times for 5 min. Amplification with anty-bodies (depends, which probes you will use). Ethanol series, air dry.

Counterstaine with DAPI or Propidium Iodine (depends, which probes you will use).

With love Janna

Subject:

Date: Wed, 22 Mar 2000 16:17:08 +0100

From: Janna Fomina <J.N.Fomina@lumc.nl>

To: "krishnja@bom7.vsnl.net.in" <krishnja@bom7.vsnl.net.in>

Dear Krish,

I will write about solutions.

All work solutions should be made before use and not kept for another day.
Stock-solutions could be kept.

PBS (we use these prescription)

NaCl - 160g; Na₂HPO₄ · 2H₂O - 24g; KCl - 4g; KH₂PO₄ - 4g

dissolve in 20 L (liter) of water with magnetic stir. pH should be between 7.2-7.4 Put in the 1 L bottles and sterilize (autoclave). You could prepare 10 or 5 L. We prepared 20 L for whole lab. You can use it 2-3 weeks, better to store at 4°C (for long use). For 1 week we store at room temperature (RT).

20xSSC

NaCl - 175,3g; sodium citrate - 88,24g

add 900 ml of water (you have to stir on the magnetic stir to dissolve); adjust pH to 7 with 5N HCl (be careful, can drop pH very quickly). Add water to 1 L, and sterilize (autoclave). Can be store at RT for month.

2xSSC, 1xSSC, 0.1xSSC - work-solutions, should be made before use and not kept for another day. Don't need to adjust pH.

1M MgCl₂

for 1 L - 203.3g; for 250 ml - 50.825g (I am preparing 250 or 500 ml),

Weight 50.825g and add water till 250 ml (no problem with dissolving). Sterilize and keep at RT.

Formamide 2

I use deionized formamide only for denaturation of slides (when I prepare hybridization mixture: formamide/2xSSC/phosphate buffer;

this is usually small amount, you don't need to check pH).

For the washing step, after hybridization, when 50% (or 60%,65% formamide needed), I adjusted pH 7 with 12N HCl (or 10N HCl), for approximately 400ml - 8-10 drops (!) of 12 N HCl needed.

RNase

stock-solution 10mg/ml we keep in Eppendorf (approximately 50 microL per Eppendorf) at -20°C for a long time.

Work-solution should be prepared every time fresh

Pepsin

stock-solution 10% we keep in Eppendorf (approximately 50 microL per Eppendorf) at -20°C for a long time.

Work-solution should be prepared every time fresh

PBS/MgCl₂; 1% formaldehyde/PBS/MgCl₂

should be prepared every time fresh.

Pretreatment: I prefer to do pretreatment all time, when cells are clean from cytoplasm it is easier for probes to bind to DNA of chromosomes.

Incubation: for Cambio probes it is better to incubate at 42°C, and very often I am doing this for 2 days. For example, in the evening I put slides in the oven and start in the morning day after tomorrow.

Dear Krish, I will be glad to answer on other questions, too. Do write it more specifically.

Love to everybody Janna