Krishnaja. 17-11-99.

Protocols

Slide treatment with pepsin (for ageing fresh made slides)

- incubate for 30' at 37oC in '0.005% pepsin/0.01M HCl'*** (stock)**
- wash **1xPBS** at RT for 5'
- 5' at RT in **buffer*** for 50ml*: 5ml 10xPBS
5ml 0.5M MgCl2
40ml H2O)
- 5' in **paraformaldehyde 4%** at RT* for 50ml: 5ml 10xPBS
5ml 0.5M MgCl2
15ml H2O
25ml paraformaldehyde 8%
- wash **1xPBS**, 5' at RT
- 3 wash in **ethanol** series (70%, 90%, and 100%)
- air drying

Rapid ageing of slides:

- prepare slides as usual
- ON incubation at 37oC
- 2h in oven at 80-90oC
- pepsin treatment

DNA extraction from YACs (CEPH megalibrary)

- seed the YAC in 5-10ml of YPD (with ampicillin)
- incubate 2 days at 30°C in a stirrer
- fuge 5' a 3500RPM; discard the SN
- suspend in 0.5ml 1M sorbitol-0.1M EDTA (pH7.5);
- transfer in eppendorf tube
- add 7ul of lyticase (stock solution: 10.000U/ml).
- incubate at 37°C for 30'
- fuge for 1', discard the SN.
- suspend in 0.5ml of 50mM Tris-HCl (pH7.4) - 20mM EDTA
- add 50ul of SDS 10%; mix

- at 65°C for 30'
- add 0.2ml of 5M KAc; in ice for 60'
- fuge for 5'
- transfer the SN in a eppendorf tube
- add 1 vol isopropanol at RT
- mix; leave at RT per 5'
- fuge for 10 seconds, discard the SN, let the pellet dry
- suspend in 0.3ml of TE (pH7.4)
- add 15µl of RNAse A 1mg/ml; at 37°C for 30'
- add 30µl of Na Acet., mix
- precipitate with 0.2ml of isopropanol
- fuge briefly; discard the supernatant
- dry the pellet in Savant for 5'
- suspend in 0.1-0.3ml TE (pH7.4)
- fuge briefly to eliminate insoluble material

YPD: (1 liter): 10gr yeast extract
& nbsp; &n bsp; 20gr peptone

Autoclave for 30'; when the temperature is around 50C add 50ml of glucose (40%, sterilized by filtration)

PAC-BAC Miniprep

NOTE: Resistance: BAC: Cloramphenicol; PAC: Kanamycin

1. Grow bacteria in 10 ml bacterial medium containing the right antibiotic in50 ml falcon tubes for 16/20 hours.
2. Centrifuge 10' at 4000 rpm.
3. Add 300µl of GTE (Glucose 50mM,Tris pH=8 25mM, EDTA 10Mm)
4. Resuspend pellet completely.
5. Trasfer the cell suspension into 2.2ml eppendorf tubes.
6. Add 600 µl of denaturation solution freshly made; do not vortex, mix by inverting several times,do not lyse for more than 5'. The lysate should appear viscous.
7. Add 500µl of 7.5M Ammonium Acetate;do not vortex,mix immediately by inverting several times; leave on ice for 10'; invert several times during the incubation period.
8. Centrifuge at 13000 rpm for 20'
9. Pour the supernatant into fresh 2.2ml eppendorf tubes; the supernatant is most often not clear and a second centrifuge at 14000 rpm for 10' needs to be carried out.
10. Pour the supernatant into fresh 2.2ml eppendorf tubes.
11. Add 700µl isopropanol; mix by inverting several times.
12. Centrifuge at 14000 rpm for 20'.
13. Discard supernatant;the pellet should be barely visible
14. Wash the pellet with 500µl of 70%EtOH.
15. Centrifuge at 14000 rpm for5'
16. Discard the supernatant; don't let the pellet dry
17. Resuspend the pellet in 100µl of TE by taping the tubes
18. Treat with RNAse f.c. 100ug/ml: at 37C for 30'

19. Precipitate the DNA with 1/10 vol NaAc and 3 vol Ethanol
20. Incubate at -20C for 20'
21. Fuge for 15' at 14000RPM; wash with 70% Ethanol
22. Resuspend in TE (appropriate volume)
23. Check on gel; store at 4 degrees

Before labeling: fuge for 15' at 14.000RPM

Alu-PCR amplification

PCR amplifications are performed using the following Alu primers (Liu et al., 1993):

5' GGATT ACAGG YRTGA GCCA 3';
5' RCCAY TGCAC TCCAG CCTG 3' (Y=C/T; R=A/G),

50-100ng of genomic DNA from the hybrid is amplified in a volume of 50ul. Cycles: 5 min at 95°C; then 30 cycles as follows: 1 min at 95°C, 1 min at 65°C, 4 min at 72°C, with a final extension of 10 min at 72°C.

Check the concentration of amplified products on gel. A good amplification yields about 200ng/ul
Label the products using Nick-translation as described (see below).

Reagents for PCR (store frozen):
- 10x dNTPs mix 2mM each, pH7.
- 10x reaction buffer (usually comes with the Taq).

Probe labelling with Biotin by nick-translation

1. Add to a microfuge tube,on ice:
- 2µg of amplification products (not purified)
- 10µl 10x nick translation buffer*
- 10µl dNTPs mix*
- 5µl biotin mix* or 0.5ul dUTP-Cy3 1mM NOTE
- 10µl 0.1M beta-mercaptoethanol
- dilute (immediately before use) 1µl of DNAseI (2Uul)
in 1ml distilled water;
add 20µl to the nick-tranlation mix:
the DNAseI should be calibrated to give
fragments of 100-500bp
- 10U DNA polymerase I
- distilled sterile water to 100µl

2. incubate at 15°C for 2h
3. place at 4°C until it is checked on gel.

4. Take 5µl aliquot of each sample, add 4µl H2O and
1µl of 10x loading buffer. Run on 1% agarose gel.
Load also a suitable MW marker (as PhiX-HaeIII).
Inspect the gel on UV transilluminator:
fragments should be between 100 and 500bp.
If fragments are larger, add additional DNAseI to the samples,
incubate for additional 30' and check again.
5. Stop reaction adding 4µl 0.5M EDTA

* nick translation buffer:
10x buffer:
0.5M Tris-HCl pH 7.8-8
50mM MgCl2
0.5mg/ml BSA

* dNTPs mix:
0.5mM dATP
0.5mM dCTP
0.5mM dGTP

* biotin-16-dUTP mix
0.5mM dTTP
0.5mM bio-16-dUTP (Boehringer Mannheim)

NOTE: Direct labeling with dUTP-Cy3 (Amersham) can be used as an efficient alternative to biotin labeling. If co-hybridization are performed we usually label the first probe with dUTP-Cy3
and the second probe with biotin (detected with avidin-FITC).

Fluorescence in situ hybridization (FISH)

Slide denaturation

1. Prepare 200ul/slide of denaturing solution (70% deionized formamide/2xSSC).
2. Pre-warm slides at 60°C in dry oven.
3. Put 200ul of denaturation solution on each slide and incubate for exactly 2 min at 80°C in a dry oven or on a thermoblock plate.
4. Dehydrate slides in 70%, 90% and 100% ethanol, 3min each time (70% ethanol at -20°C).
5. Dry slides after dehydratation.

Probe denaturation

1. Precipitate 20µl labelled DNA (400ng) with 5µg human COT1 DNA (BRL), 3µg salmon sperm DNA, 2.8µl 3M Na acetate and 3 vol cold (-20°C) ethanol. Leave at -80°C for 15 minutes. Spin 15' (14,000RPM) at 4°C. Dry **completely** the pellet on a

Savant fuge for few minutes.

2. Prepare hybridization mix (10 µl per slide). Add to a test tube: 5µl of deionized formamide, 2µl dextran sulphate (50% in distilled water, autoclaved), 2µl distilled water and 1µl 20xSSC. If more slides have to be hybridized, a master mix can be prepared.
3. Resuspend pellet in 10µl hybridization mix, by vortexing accurately;
4. Denature DNA mix at 80°C for 8 min; transfer to 37°C for 20 min. Place on ice until used.
5. NOTE: the amount of probe depends on the type of probe: 20-50 ug for repetitive DNA; 500-600 for painting probes.

Hybridization

1. Apply 10µl hybridization mix to denatured slides, avoiding air bubbles.
2. Cover with 24x24mm clean coverslip; seal with rubber cement.
3. Incubate in a moist chamber at 37°C overnight.

Post-hybridization washing and detection.

Do not allow slides to dry at any passages! All washings are done in Choplin jar.

1. Remove coverslips and wash 3 times for 5min in prewarmed solution (50% form. / 2xSSC) in a Choplin jar in a shaking waterbath at 42°C (we usually skeep this first washing)
2. Wash 3 times for 5' in prewarmed 0.1xSSC in waterbath at 60°C, **then go to 6 if direct labeling has been performed.**
3. Apply 200µl of blocking solution per slide (3% BSA/4xSSC/0.1 Tween 20); cover with 24x60mm coverslip; transfer the slides in a moist chamber; incubate for 30' at 37°C.
4. Dilute stock solution of avidin-Cy3 in detection buffer (1%BSA/1xSSC0/0.1 Tween 20) (Cy3 is from Amersham; Cy3 is stronger and more stable than other fluorochromes; use the same filter for rhodamine, or a specific one). Let coverslips slide off; apply 200µl detection solution per slide. Cover with 24x60mm coverlips. Transfer the slides in a dark moist chamber. Incubate at 37°C for 30 min.
5. Remove the coverslips; rinse the slides 3 times for 5 min in pre-warmed washing solution (4xSSC / 0.1 Tween 20) in waterbath at 42°C.
6. Counterstain with DAPI (200ng/ml in 2xSSC) or with propidium iodide (200ng/ml in 2xSSC).
7. Rinse 2 min in 2xSSC 0.05% Tween 20 at room temperature.
8. Apply 30µl of antifade-mounting medium* and cover with 24x60mm coverslip; slides can be stored for weeks in the dark at 4°C.

* Antifade-mounting medium (for 10ml):
- 0.233gr of DABCO (1,4-diazabicyclo-(2.2.2)octane, Sigma)
- 800µl H2O
- 200µl 1M Tris-HCl
- 9ml glycerol

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FISH protocol 1st day
for two-three chromosomes with pan-centromeric probe

Pretreatment:

✓ 1	Wash slides	PBS	5 min	RT
✓ 2	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
3	Air dry			RT
4	Pipette 100 ul of RNase A (100 ug/ml/2xSSC) per slide, overlay with coverslip			
	Incubate in the moist chamber		60 min	37oC

For 5 slides:
- 445 ul H_2O
- 50 ul 20xSSC
- 5 ul RNase 10 mg/ml

prepare in advance keep at -20°C .

50 ul RNase .
222 . 5 ml H_2O
25 ml 20xSSC
2 . 5 ml . RNase . 10mg/ml.

✓ 5	2xSSC		3 x 5 min	RT

During 1st washing remove coverslips
For 3 washes I usually prepared 400ml (40 ml 20xSSC + 360 ml water)

✓ 6	PBS		5 min	RT
7	Pepsin (0.005% / 10mM HCl)		8-10 min	37oC

44 . 5 ml
0 . 5 ml
25 ml .
- 99 ml H_2O
- 1 ml 1N HCl
- 50 ul 10% pepsin

Prepare in advance . keep -20° c

NB:solution should be pre-warmed in water-bath at least 30 min before use;
I usually prepared 100 ml 10mM HCL, pre-warmed it and added pepsin 5-10 min before use;
(if pepsin will stay for longer time at 37oC - it can lose activity)

8	PBS		5 min	RT
9	50mM $MgCl_2$ / PBS		5 min	RT

- 5 ml 1M $MgCl_2$
- 95 ml PBS

10	1% formaldehyde/PBS/$MgCl_2$		10 min	RT

1 . 5 ml .
2 . 5 ml .
46 ml .
- 3 ml 37% formaldehyde (commercial)
- 5 ml 1M $MgCl_2$
- 92 ml PBS

or in 2xSSC Twice 5 min RT

✓ 11	PBS		2 x 5 min	RT
✓ 12	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
	Air dry			RT

NB: for steps - 1, 2, 5, 6, 11, 12 - use shaker
NB: steps - 1, 2, 5, 6, 7, 8, 9, 10, 11, 12 - slides are washing in solutions in coplin jar

Probe denaturation and competition:

For "Cambio" concentrated paints (chromosome-specific probes) and pan-centromeric probe (CP)
Paints and hybridization buffer (HB) for paints, CP and HB for CP should be store at -20oC

A Paints for specific chromosomes

* take paints and HB from -20oC and keep at RT 5 min (in dark)
* warm paints at 42oC in water bath 10-15 min
* mix well (vortex could be used) and spin dawn for 2-3 seconds
* mix sufficient amount of every paints (in separate Eppendorf) with hybridization buffer (HB)
* mix very well, spin down for few seconds
* denature paints by incubation at 65oC for 10 min (or - 70oC for 8 min) in water bath
* briefly put on ice for 2-3 min (not very necessary)
* transfer to water bath at 37oC and incubate for 60 to 90 min for competition

B Pan-centromeric probe (CP)
(start 30 min before the end of paint`s competition)

* take CP and HB from -20oC and keep at RT 5 min (in dark)
* warm CP and HB at 37oC for 5-10 min
* take sufficient amount of CP plus HB and place in Eppendorf tube
* mix very well, spin down for few seconds
* denature CP by incubation at 85oC for 10 min in water bath
* immediately put on ice for 2-3 min - than spin down for few seconds and keep on ice

For FISH (paints with pan-centromeric probes) use final volume 18-20 ul of hybridization mixture per slide

Cambio protocol recommends to use per one slide: 3 ul of concentrated paint
2-3 ul of concentrated CP

For example:

Cocktail: #1 Bio + #4 Bio / FITC + #8 FITC + CP FITC
color red + yellow + green + green

#1 Bio	3 ul +	HB 1.6 ul
#4 Bio	1 ul +	HB 1.6 ul
#4 FITC	2 ul +	HB 1.6 ul
#8 FITC	3 ul +	HB 1.6 ul
CP FITC	3 ul +	HB 1.6 ul
	Total volume = 20 ul	

#1 Bio + #4 FITC + CP BIO /FITC
red +green +yellow

#1 Bio	3 ul +	HB 2.5 ul
#4 FITC	3 ul +	HB 2.5 ul
CP BIO	2.5 ul +	HB 2.5 ul
CP FITC	1.5 ul +	HB 2.5 ul
	Total volume = 20 ul	

Note 1 Bio-labeled paints look usually more brig if signal is very 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 an Eppendorf from Cambio, sometimes it is written **NO Cot 1**), in this case you should add Cot 1 DNA

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

1 Prepare in Eppendorf tube (for 5 slides)

350 ul deionize 100% formamide
50 ul 0.5 M phosphate buffer (PB) 50mM
50 ul 20xSSC
50 ul H_2O

2 Pipette 100 ul of 70% formamide/2xSSC/50mM phosphate buffer per slide and overlay with coverslip 24 x 60 mm

3 Denature slides at 80oC for 2.5-3 min on the hot plate

4 Wash slides with ethanol series (during 1st wash remove coverslip)

70% ethanol	5 min	(should be kept at -20oC before use) ✓
90% ethanol	2-5 min	RT
100% ethanol	2-5 min	RT

5 air dry

Hybridization

4 Mix all paints and CP in one Eppendorf tube
mix very well, spin down for few seconds (several times !!!)

5 Pipette 20 ul of the mix solution onto each slide,
overlay with coverslip 24 x 50 mm
seal with Cow Gum, give time to glue to dry

6 Incubate overnight at 42oC in the moist chamber
(incubation could be 2 overnights, if necessary)

Detection:

I *Make solutions for washing:*

1 50% formamide / 2xSSC for 3 washing

200 ml 100% formamide + 20 ml 20xSSC + 180 ml water
adjust pH 7.0

2 0.1xSSC for 3 washing

3 0.05% Tween / 4xSSC (2-2,5 L)

200 ml	20xSSC
5 ml	10% Tween 20
800 ml	H2O
1 L	

Pre-warm all solutions to 42-45oC in water bath

II *Make antybodies for amplification:*

Dilute Blocking Protein (BP from Cambio) to 10-15% (v/v) in wash solution from step 3.
This is solution A.
NB: sometimes 10-15% is too strong and could give big background, you can go down to 7%

Use solution A for dilution of antibodies:

I layer	B 3	1 : 500	1 ul B 3 in 500 ul A	B 3 - Texas-Red Avidin
II layer	B 4	1 : 250	2 ul B 4 and 2.5 ul F 1 in 500 ul A	B 4 - Biotinylated goat anti-Avidin
	F 1	1 : 200		F 1 - Rabbit anti-FITC
III layer	B 3	1 : 500	1 ul B 3 and 5 ul F 2 in 500 ul A	F 2 - FITC goat anti-rabbit IgG
	F 2	1 : 100		

Incubate in the dark for 10 min at RT
Microfuge at 11,000g for 10 min and use supernatants

Stringency washes:

Carefully removed dried Cow Gum and wash slides

1	50% formamide / 2xSSC	3 x 5 min	42oC	(45oC - Optional)
	During 1st washing remove coverslips			
2	0.1xSSC	3 x 5 min	42oC	(60oC - Optional)
3	0.05% Tween / 4xSSC	5 min	42oC	
4	Pipette 100 ul of solution A per slide and overlay with coverslip 24x50 mm Incubate in moist chamber for 15-20 min at 37oC			
	NB: incubation can be up to 30 min			
5	Wash slides 0.05% Tween / 4xSSC	2 x 5 min	42oC	
6	Pipette 100 ul of I layer of antibody per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
7	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
8	Pipette 100 ul of II layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
9	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
10	Pipette 100 ul of III layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
11	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	

Repeat steps 8-11

Alcohol	70%	2-5 min	RT
	90%	2-5 min	RT
	100%	2-5 min	RT
Air dry			RT

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

Cover with coverslip

Note: Do not allow slides to dry out at any stage until last step - Air dry

Steps 5, 7, 9, 11 - washing with buffer - during 1st washing remove coverslip

Prepare all solutions with double-distilled, deionized water

PBS (phosphate-buffer saline)

for 10 L:	NaCl	80 g
	$Na_2HPO_4 . 2H_2O$	12 g
	KCl	2g
	KH_2PO_4	2g
	add water till 10 L	

mix on the magnetic stir (approximately 1/2 hour)
adjust pH 7.2-7.4 (with 1M HCl)
put in 1L bottles and sterilize
store near 1 month at room temperature (RT)

8 g
1.2 g
.2
.2

20 x SSC

NaCl	175.3 g
sodium citrate	88.24 g
add 950 ml water	

mix on the magnetic stir (approximately 1/2 hour)
adjust pH 7.0 (with 1M or 5 M HCl)
add water till 1 L and sterilize
store near 1 month at RT

1 M $MgCl_2$

M.v. 203.3

for 250 ml	$MgCl_2$	50.82g

add water till 250 ml
mix on the magnetic stir
sterilize
store near 1 month at RT

10% Tween

100% Tween 20	10 ml
water	90 ml

mix well
sterilize by filtration through a 0.2-micro m filter
store near 1 month at RT

Fluorescent antibody enhancer for DIG detection

10 X PBS. pH 7.4.

1 L. 80 g NaCl
2 g KCl.
12.4 g Na_2HPO_4.
2.4 g KH_2PO_4. (Boehringer)

0.5 M PB (phosphate buffer)

prepare solution 1 0.5 M Na_2HPO_4
solution 2 0.5 M NaH_2PO_4

solution 1 Na_2HPO_4 7.1 g in 100 ml water
should be dissolved on the magnetic stir
pH will be approximately 9

solution 2 $NaH_2PO_4 . 1 H_2O$ 6.9 g in 100 ml water
pH will be approximately 4

in full volume of solution 1 (which is still on the magnetic stir)
add solution 2 to adjust pH till 7.0
(when I did it, for 100 ml solution 1 approximately - 40 ml of solution 2)
dispense into 1 ml aliquots and store at -20oC

100% deionized formamide

mix 50 ml of 100% (commercial) formamide and 5 g Serdolit MB
stir on the magnetic stir - 1,5-2 hours at RT
filter twice trough Whatman No.1 filter paper
dispense into 1 ml aliquots and store at -20oC

Serdolit MB - ion-exchange resin; SERVA (company), cat.number 45500

10% pepsin

1 g pepsin dissolve in 10 ml of 10 mM HCl
mix well on vortex
dispense into 1 ml aliquots and store at -20oC

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FISH protocol 1st day
for two-three chromosomes with pan-centromeric probe

Pretreatment:

1	Wash slides	PBS	5 min	RT
2	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
3	Air dry			RT
4	Pipette 100 ul of RNase A (100 ug/ml/2xSSC) per slide, overlay with coverslip			
	Incubate in the moist chamber		60 min	37oC
	For 5 slides:			
	445 ul H_2O			
	50 ul 20xSSC			
	5 ul RNase 10 mg/ml			
5	2xSSC		3 x 5 min	RT
	During 1st washing remove coverslips			
	For 3 washes I usually prepared 400ml (40 ml 20xSSC + 360 ml water)			
6	PBS		5 min	RT
7	Pepsin (0.005% / 10mM HCl)		8-10 min	37oC
	99 ml H_2O			
	1 ml 1N HCl			
	50 ul 10% pepsin			
	NB:solution should be pre-warmed in water-bath at least 30 min before use;			
	I usually prepared 100 ml 10mM HCL, pre-warmed it and added pepsin 5-10 min before use;			
	(if pepsin will stay for longer time at 37oC - it can lose activity)			
8	PBS		5 min	RT
9	50mM $MgCl_2$ / PBS		5 min	RT
	5 ml 1M $MgCl_2$			
	95 ml PBS			
10	1% formaldehyde/PBS/$MgCl_2$		10 min	RT
	3 ml 37% formaldehyde (commercial)			
	5 ml 1M $MgCl_2$			
	92 ml PBS			
11	PBS		2 x 5 min	RT
12	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
	Air dry			RT

NB: for steps - 1, 2, 5, 6, 11, 12 - use shaker
NB: steps - 1, 2, 5, 6, 7, 8, 9, 10, 11, 12 - slides are washing in solutions in coplin jar

Probe denaturation and competition:

For "Cambio" concentrated paints (chromosome-specific probes) and pan-centromeric probe (CP)
Paints and hybridization buffer (HB) for paints, CP and HB for CP should be store at -20oC

A Paints for specific chromosomes

* take paints and HB from -20oC and keep at RT 5 min (in dark)
* warm paints at 42oC in water bath 10-15 min
* mix well (vortex could be used) and spin dawn for 2-3 seconds
* mix sufficient amount of every paints (in separate Eppendorf) with hybridization buffer (HB)
* mix very well, spin down for few seconds
* denature paints by incubation at 65oC for 10 min (or - 70oC for 8 min) in water bath
* briefly put on ice for 2-3 min (not very necessary)
* transfer to water bath at 37oC and incubate for 60 to 90 min for competition

B Pan-centromeric probe (CP)
(start 30 min before the end of paint`s competition)

* take CP and HB from -20oC and keep at RT 5 min (in dark)
* warm CP and HB at 37oC for 5-10 min
* take sufficient amount of CP plus HB and place in Eppendorf tube
* mix very well, spin down for few seconds
* denature CP by incubation at 85oC for 10 min in water bath
* immediately put on ice for 2-3 min - than spin down for few seconds and keep on ice

For FISH (paints with pan-centromeric probes) use final volume 18-20 ul of hybridization mixture per slide

Cambio protocol recommends to use per one slide: 3 ul of concentrated paint
2-3 ul of concentrated CP

For example:

Cocktail: #1 Bio + #4 Bio / FITC + #8 FITC + CP FITC
color red + yellow + green + green

#1 Bio	3 ul +	HB 1.6 ul
#4 Bio	1 ul +	HB 1.6 ul
#4 FITC	2 ul +	HB 1.6 ul
#8 FITC	3 ul +	HB 1.6 ul
CP FITC	3 ul +	HB 1.6 ul

Total volume = 20 ul

#1 Bio + #4 FITC + CP BIO /FITC
red +green +yellow

#1 Bio	3 ul +	HB 2.5 ul
#4 FITC	3 ul +	HB 2.5 ul
CP BIO	2.5 ul +	HB 2.5 ul
CP FITC	1.5 ul +	HB 2.5 ul

Total volume = 20 ul

Note 1 Bio-labeled paints look usually more brigh if signal is very 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 an Eppendorf from Cambio, sometimes it is written **NO Cot 1**),
in this case you should add Cot 1 DNA

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

1 Prepare in Eppendorf tube (for 5 slides)

350 ul deionize 100% formamide
50 ul 0.5 M phosphate buffer (PB)
50 ul 20xSSC
50 ul H_2O

2 Pipette 100 ul of 70% formamide/2xSSC/50mM phosphate buffer per slide and overlay with coverslip 24 x 60 mm

3 Denature slides at 80oC for 2.5-3 min on the hot plate

4 Wash slides with ethanol series (during 1st wash remove coverslip)

70% ethanol	5 min	(should be kept at -20oC before use)
90% ethanol	2-5 min	RT
100% ethanol	2-5 min	RT

5 air dry

Hybridization

4 Mix all paints and CP in one Eppendorf tube
mix very well, spin down for few seconds (several times !!!)

5 Pipette 20 ul of the mix solution onto each slide,
overlay with coverslip 24 x 50 mm
seal with Cow Gum, give time to glue to dry

6 Incubate overnight at 42oC in the moist chamber
(incubation could be 2 overnights, if necessary)

Detection:

I *Make solutions for washing:*

1 50% formamide / 2xSSC for 3 washing

200 ml 100% formamide + 20 ml 20xSSC + 180 ml water
adjust pH 7.0

2 0.1xSSC for 3 washing

3 0.05% Tween / 4xSSC (2-2,5 L)

200 ml	20xSSC
5 ml	10% Tween 20
800 ml	H2O
1 L	

Pre-warm all solutions to 42-45oC in water bath

II *Make antybodies for amplification:*

Dilute Blocking Protein (BP from Cambio) to 10-15% (v/v) in wash solution from step 3.
This is solution A.
NB: sometimes 10-15% is too strong and could give big background, you can go down to 7%

Use solution A for dilution of antibodies:

I layer	B 3	1 : 500	1 ul B 3 in 500 ul A	B 3 - Texas-Red Avidin
II layer	B 4	1 : 250	2 ul B 4 and 2.5 ul F 1	B 4 - Biotinylated goat anti-Avidin
	F 1	1 : 200	in 500 ul A	F 1 - Rabbit anti-FITC
III layer	B 3	1 : 500	1 ul B 3 and 5 ul F 2	F 2 - FITC goat anti-rabbit IgG
	F 2	1 : 100	in 500 ul A	

Incubate in the dark for 10 min at RT
Microfuge at 11,000g for 10 min and use supernatants

Stringency washes:

Carefully removed dried Cow Gum and wash slides

1	50% formamide / 2xSSC	3 x 5 min	42oC	(45oC - Optional)
	During 1st washing remove coverslips			
2	0.1xSSC	3 x 5 min	42oC	(60oC - Optional)
3	0.05% Tween / 4xSSC	5 min	42oC	
4	Pipette 100 ul of solution A per slide and overlay with coverslip 24x50 mm Incubate in moist chamber for 15-20 min at 37oC			
	NB: incubation can be up to 30 min			
5	Wash slides 0.05% Tween / 4xSSC	2 x 5 min	42oC	
6	Pipette 100 ul of I layer of antibody per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
7	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
8	Pipette 100 ul of II layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
9	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
10	Pipette 100 ul of III layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
11	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	

Repeat steps 8-11

Alcohol	70%	2-5 min	RT
	90%	2-5 min	RT
	100%	2-5 min	RT
Air dry			RT

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

Cover with coverslip

Note: Do not allow slides to dry out at any stage until last step - Air dry

Steps 5, 7, 9, 11 - washing with buffer - during 1st washing remove coverslip

Prepare all solutions with double-distilled, deionized water

PBS (phosphate-buffer saline)

for 10 L:		
	NaCl	80 g
	$Na_2HPO_4 . 2H_2O$	12 g
	KCl	2g
	KH_2PO_4	2g
	add water till 10 L	

mix on the magnetic stir (approximately 1/2 hour)
adjust pH 7.2-7.4 (with 1M HCl)
put in 1L bottles and sterilize
store near 1 month at room temperature (RT)

20 x SSC

	NaCl	175.3 g
	sodium citrate	88.24 g
	add 950 ml water	

mix on the magnetic stir (approximately 1/2 hour)
adjust pH 7.0 (with 1M or 5 M HCl)
add water till 1 L and sterilize
store near 1 month at RT

1 M $MgCl_2$

M.v. 203.3
for 250 ml $MgCl_2$ 50.82g
add water till 250 ml
mix on the magnetic stir
sterilize
store near 1 month at RT

10% Tween

100% Tween 20	10 ml
water	90 ml

mix well
sterilize by filtration through a 0.2-micro m filter
store near 1 month at RT

0.5 M PB (phosphate buffer)

prepare solution 1 0.5 M Na_2HPO_4
solution 2 0.5 M NaH_2PO_4

solution 1 Na_2HPO_4 7.1 g in 100 ml water
should be dissolved on the magnetic stir
pH will be approximately 9

solution 2 $NaH_2PO_4 . 1 H_2O$ 6.9 g in 100 ml water
pH will be approximately 4

in full volume of solution 1 (which is still on the magnetic stir)
add solution 2 to adjust pH till 7.0
(when I did it, for 100 ml solution 1 approximately - 40 ml of solution 2)
dispense into 1 ml aliquots and store at -20oC

100% deionized formamide

mix 50 ml of 100% (commercial) formamide and 5 g Serdolit MB
stir on the magnetic stir - 1,5-2 hours at RT
filter twice trough Whatman No.1 filter paper
dispense into 1 ml aliquots and store at -20oC

Serdolit MB - ion-exchange resin; SERVA (company), cat.number 45500

10% pepsin

1 g pepsin dissolve in 10 ml of 10 mM HCl
mix well on vortex
dispense into 1 ml aliquots and store at -20oC

Krishnaja, A.P.
1-8-2000
(J)

FISH protocol **1st day**

short version for one chromosome and pan-centromeric probe

Pretreatment:

*	PBS	5 min	RT
*	70% ethanol	5 min	RT
	90% ethanol	5 min	RT
	100% ethanol	5 min	RT
*	Air dry		RT
*	100 ul of RNase A (100 ug /ml / 2xSSC) (stock RNase 10 mg / ml)	60 min	37oC
*	2xSSC	3 x 5 min	RT
*	PBS	5 min	RT
*	Pepsin (0.005% / 10mM HCl) (stock 10% pepsin)	10 min	37oC
*	PBS	5 min	RT
*	50mM MgCl2 / PBS	5 min	RT
*	1% formaldehyde/PBS/MgCl2	10 min	RT
*	PBS	5 min	RT
*	70% ethanol	5 min	RT
	90% ethanol	5 min	RT
	100% ethanol	5 min	RT
*	Air dry		RT

Probe denaturation and competition:

A Paint for specific chromosome
warm paint to 42oC
mix 3 ul of paint with 7 ul of hybridization buffer (HB)
denature paint at 65oC for 10 min
incubate for 60 to 90 min at 37oC

B Pan-centromeric probe (CP)
warm CP and HB at 37oC for 5-10 min
mix 2 ul of CP with 7 ul of HB
denature CP at 85oC for 10 min
put on ice for 2-3 min - than spin down for few seconds and keep on ice

Prehybridization of slides:

1	100 ul/slide of denaturation solution (70% formamide / 2xSSC / 50mM phosphate buffer)		80oC	2.5-3 min
2	70% ethanol	5 min	(kept at -20oC before use)	
	90% ethanol	5 min	RT	
	100% ethanol	5 min	RT	
3	Air dry			

Hybridization

4 Mix paint and CP in one Eppendorf tube
5 Pipette 20 ul of the mix solution on slide,
overlay with coverslip, seal with Cow Gum
6 Incubate overnight at 42oC in the moist chamber

Detection:

1 Make a wash solution 0.05% Tween / 4xSSC

2 Dilute Blocking Protein (BP) to 10-15% (v/v) in wash solution
This is solution A.

3 Use solution A for dilution of antibodies:

I layer	B 3	1 : 500	B 3 - Texas-Red Avidin
II layer	B 4	1 : 250	B 4 - Biotinylated goat anti-Avidin
	F 1	1 : 200	F 1 - Rabbit anti-FITC
III layer	B 3	1 : 500	F 2 - FITC goat anti-rabbit IgG
	F 2	1 : 100	

Keep antibodies in the dark for 10 min at RT
Microfuge at 11,000g for 10 min and use supernatants

4 Pre-warm to 45oC:

50% formamide / 1xSSC, pH 7.0 for 3 washing
0.1xSSC for 3 washing

5	50% formamide / 1xSSC	2 x 5 min	45oC
6	0.1xSSC	3 x 5 min	45oC
7	0.05% Tween / 4xSSC	5 min	45oC
8	100 ul / slide of solution A	20 min	37oC
9	0.05% Tween / 4xSSC	2 x 5 min	45oC
10	100 ul / slide of I layer of antibody	20 min	37oC
11	0.05% Tween / 4xSSC	3 x 5 min	45oC
12	100 ul / slide of II layer of antibodies	20 min	37oC
13	0.05% Tween / 4xSSC	3 x 5 min	45oC
14	100 ul / slide of III layer of antibodies	20 min	37oC
15	0.05% Tween / 4xSSC	3 x 5 min	45oC

Repeat steps 12-15

16	Alcohol	70%	5min	RT
		90%	5min	RT
		100%	5min	RT
17	Air dry			RT
18	Counterstain with DAPI			

Krishnaji.
25-4-2000.
(From Jamma).

FISH protocol

Pretreatment:

1	Wash slides	PBS	5 min	RT
2	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
3	Air dry			RT
4	Pipette 100 µl of RNase A (100 µg/ml) per slide, overlay with coverslip			
	Incubate in the moist chamber		60 min	37oC

445 µl H_2O
50 µl 20xSSC
5 µl RNase 10 mg/ml (store at -20oC)

5	2xSSC		3 x 5 min	RT
	During 1st washing remove coverslips			
6	PBS		5 min	RT
7	Pepsin (0.005% / 10mM HCl)		8-10 min	37oC

99 ml H_2O
1 ml 1N HCl
50 µl 10% pepsin (store at -20oC)

NB:solution should be pre-warmed in water-bath before use
at least 30 min before use; I usually prepair 10mM HCl, pre-warmed it
and add pepsin 10 min before putting slides in solution
(if pepsin will stay for longe time at 37oC - it can lose activity)

8	PBS		5 min	RT
9	50mM $MgCl_2$ / PBS		5 min	RT

5 ml 1M $MgCl_2$
95 ml PBS

10	1% formaldehyde/PBS/$MgCl_2$		10 min	RT

3 ml 37% formaldehyde
5 ml 1M $MgCl_2$
92 ml PBS

11	PBS		2 x 5 min	RT
12	Alcohol	70%	2-5 min	RT
		90%	2-5 min	RT
		100%	2-5 min	RT
	Air dry			RT

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

Probe denaturation and competition:
For "Cambio" concentrated paints and pan-centromeric probe (CP)

A Paints for specific chromosomes

a warm paints to 42oC and mix well before use
b mix sufficient amount of every paints (in separate Eppendorf) with hybridization buffer (HB)
mix very well, spin down for few seconds (see Note 1)
c denature paints by incubation at 65oC for 10 min (or - 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 (CP)
(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 denature 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

Note: for triple-colours FISH with pan-centromeric probe
use final volume 18-20 µl of hybridization mixture per slide

Cambio protocol recommends to use per one slide: 3 µl of concentrated paint
2-3 µl of concentrated CP

For example:

Cocktail: #1 Bio + #4 Bio / FITC + #8 FITC + CP FITC
colour *red* + *yellow* + *green* + *green*

For 1 slides:	#1 Bio	3 µl + HB 1.6 µl	
	#4 Bio	1 µl + HB 1.6 µl	(see Note 2)
	#4 FITC	2 µl + HB 1.6 µl	
	#8 FITC	3 µl + HB 1.6 µl	
	CP FITC	3 µl + HB 1.6 µl	
	Total volume = 20 µl		

Note 1 Bio-labeled paints look usually more bright;
if signal is very strong and gives high background
it is possible to add Cot 1 DNA to paint before denaturation (not more than 1 µl per slide)
(also, see an Eppendorf from Cambio, sometimes it is written **NO Cot 1**)

Note 2 Optional: Mix paints #4 Bio and #4 FITC in one Eppendorf and add HB

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

1 Pipette 100 µl of 70% formamide/2xSSC/50mM phosphate buffer per slide and overlay with coverslip

{
350 µl deionize 100% formamide (store at -20oC)
50 µl 0.5 M phosphate buffer (store at -20oC)
50 µl 20xSSC
50 µl H_2O
}

2 Denature slides at 80oC for 2.5-3 min on the hot plate

3

70% ethanol	5 min	should be kept at -20oC before use
90% ethanol	2-5 min	RT
100% ethanol	2-5 min	RT

air dry

Hybridization

4 Mix all paints and CP in one Eppendorf tube
Note: mix very well (several times), spin down for few seconds

5 Pipette 20 µl 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 overnights, if necessary)

Detection:

1 Make a wash solution:

a. add 0.5 ml of detergent to 1L of 4xSSC (Cambio)

b. 0.05% Tween / 4xSSC

200 ml 20xSSC
5 ml 10% Tween 20
800 ml H_2O

NB: I am ussualy use - b.

2 Dilute Blocking Protein (BP) to 10-15% (v/v) in wash solution from step 1.
This is solution A.
NB: sometimes 10-15% is too strong and give big background, you can go down to 7%

3 Use solution A for dilution of antibodies:

I layer	B 3	1 : 500	B 3 - Texas-Red Avidin
II layer	B 4	1 : 250	B 4 - Biotinylated goat anti-Avidin
	F 1	1 : 200	F 1 - Rabbit anti-FITC
III layer	B 3	1 : 500	F 2 - FITC goat anti-rabbit IgG
	F 2	1 : 100	

NB: 100 µl per hybridisation area plus 50 µl excess

4 Incubate in the dark for 10 min at RT
Microfuge at 11,000g for 10 min and use supernatants

5 Pre-warm to 42-45oC:

2xSSC	for 1 washing
50% formamide / 2xSSC, pH 7.0	for 2-3 washing
0.1xSSC	for 3 washing

6 Carefully removed 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	(60oC - Optional)
	0.05% Tween / 4xSSC	5 min	42oC	
8	Pipette 100 µl of solution A per slide and overlay with coverslip Incubate in moist chamber for 15-20 min at 37oC			
	NB: incubation can be up to 30 min			
9	Wash slides 0.05% Tween / 4xSSC	2 x 5 min	42oC	
10	Pipette 100 µl of I layer of antibody per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
11	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
12	Pipette 100 µl of II layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
13	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
14	Pipette 100 µl of III layer of antibodies per slide and overlay with coverslip Incubate in moist chamber for 20-30 min at 37oC			
15	Wash slides 0.05% Tween / 4xSSC	3 x 5 min	42oC	
	Repeat steps 12-15			
16	Alcohol 70%	2-5 min	RT	
	90%	2-5 min	RT	
	100%	2-5 min	RT	
17	Air dry		RT	
18	Counterstain with DAPI (0.15 µg/ml in Vectashield) 25 µl per slide under coverslip			
19	Cover with coverslip,			

Krishnaji 25-4-2000

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Protocol A

Approx. time: ***Slide Preparation: 30+overnight (or +90 min) + 30 min***
Probe preparation: 75 min

IN SITU HYBRIDISATION PROTOCOL

This protocol is for use with all human STAR★FISH chromosome paints.

Also for use with the Biotin (FITC) Painting Kit (1089-KB), Biotin (Texas Red) Painting Kit (1082-KT) and the FITC Amplification Kit (1084-KF).

SLIDE PREPARATION

1. One day before needed, prepare fresh slides from 3:1 methanol:acetic acid fixed preparations. Air dry overnight. (Alternatively, prepare fresh slides from 3:1 methanol:acetic acid and age slides for 1.5 hours on a hot plate at 65°C).
2. Dehydrate by serial ethanol washing (70%(v/v), 70%, 90%, 90%, 100%) and air dry.
3. Denature slides by incubating in 70% (v/v) formamide (35ml formamide plus 15ml of 2X SSC) at 65°C for 1.5-2 min. Ensure that the 70% (v/v) formamide has reached temperature before use and do not exceed this time. For best results use fresh deionised formamide.
4. Quench in ice-cold 70% (v/v) ethanol and dehydrate using ethanol as in 2 above. Air dry.

MEANWHILE

5. Warm paints to 42°C and mix well before use. Take sufficient probe (3µl of probe, plus hybridisation buffer to a total of 15µl per slide) and place in a small Eppendorf tube. Denature paint by incubating at 65°C for 10 mins then at 37°C for 60 to 90 mins. **Note:** Paints may be prediluted (1 part paint:4 parts hybridisation buffer) and stored in hybridisation buffer if so desired.
6. Pipette 15µl of probe onto each slide, overlay with cover slips, seal with Cow Gum and incubate overnight at 42°C.

1 X SSC Recipe:
0.015 M Na Citrate, pH 7.0 containing 0.15 M NaCl.

Note: *These products are not for use in humans and are for research purposes only.*

Revised 9th March 1998

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Protocol F ***Approx. time: Slide Preparation: 30 min***
Detection: 140 min

DETECTION PROTOCOL

Dual Colour Painting Kit (Texas Red & FITC) (1090-KD)
for use with biotin and FITC labelled STAR*FISH chromosome paints.

1. Make a Wash Solution: add 250µl of Detergent to 500ml of 4X SSC.
2. Dilute Blocking Protein to 15% in the 4X SSC/Detergent Wash Solution from step 1. This is solution A.
3. Dilute detection reagents using Solution A:

a) Make a 1:500 dilution of Detection Reagent B3 (100µl per slide plus 50µl excess).
b) Make a mix of 1:250 final dilution of Detection Reagent B4 and a 1:200 final dilution of Detection Reagent F1 (100µl per slide plus 50µl excess).
c) Make a mix of 1:500 final dilution of Detection Reagent B3 and a 1:100 final dilution of Detection F2 (100µl per slide plus 50µl excess).

4. Incubate in the dark for 10 min at room temperature. Microfuge at 11,000g for 10 min and use supernatants.
5. Pre-warm to 45°C: one Coplin jar of 2X SSC, two Coplin jars of 50% (v/v) formamide/1X SSC (ie: 1:1 mix of formamide and 2X SSC) and two Coplin jars of 0.1X SSC.
6. Carefully remove dried Cow Gum and rinse off cover slips in the jar of 2X SSC for 5 mins.

NOTE: DO NOT ALLOW SLIDES TO DRY OUT AT ANY STAGE UNTIL STEP 16.

7. Stringency Washes: Wash slides by incubating for 5 min at 45°C in each of the two jars containing formamide.
8. Wash slides twice by incubating for 5 min at 45°C in each of the two jars containing 0.1X SSC.
9. Pipette 100µl of the diluted Detection Reagent B3 onto a slide and cover with a Parafilm cover slip. Incubate at 37°C for 15-20 min in a humidified box.
10. Wash 3 times in Wash Solution at 42°C for 5 min each time.
11. Pipette 100µl of diluted Detection Reagent B4/F1 supernatant onto each slide and overlay with Parafilm cover slip. Incubate in a humidified box for 15-20 min at 37°C.
12. Wash 3 times in Wash Solution at 42°C for 5 min each time.
13. Pipette 100µl of diluted Detection Reagent B3/F2 supernatant onto each slide and overlay with a Parafilm cover slip. Incubate in a humidified box for 15-20 min at 37°C.
14. Wash 3 times in wash solution at 42°C for 5 min each time.
15. Dilute 1µl of Counterstain 1 with 9µl of distilled water. Add 6µl of the diluted Counterstain 1 to 200µl of Mountant. Mix well. Discard unused diluted counterstain.
16. Briefly dehydrate slides using an ethanol series as in Step 3 (from slide preparation protocol), air dry and mount by adding 20µl of the Mountant/Counterstain mixture. Overlay with cover slip and seal with nail varnish.
Store slides in the dark at 4°C.
17. View slides using standard epifluorescence filters for FITC and Texas Red and for DAPI.

1 X SSC Recipe:
0.015 M Na Citrate, pH 7.0 containing 0.15 M NaCl.

Note: *These products are not for use in humans and are for research purposes only.*

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Protocol G ***Approx. time : Probe Preparation: 20 min + 16 hours (overnight) + 30 min***

IN SITU HYBRIDISATION PROTOCOL

STAR★FISH Pan Centromeric Chromosome Paint (1141).
To be used with the Biotin (Texas Red) Painting Kit (1082-KT) or Dual Colour Painting Kit (1090-KD)

1. Warm probe to 37°C for 5 min and mix well.
2. Add 25µl of probe to a microcentrifuge tube.
3. Denature probe for 10 min at 85°C. Immediately chill on ice.
4. Apply 25µl of probe to slide.
5. Hybridise for 16 hours at 37°C.
6. **POST HYBRIDISATION WASH:**
   Remove cover slip in 2X SSC at 37°C. 5 min.
   Wash slides in 50% formamide/2X SSC 37°C, 2X 5 min.
   Wash slides in 2X SSC, 2X 5min.
7. Detect the probe using Cambio's Biotin (Texas Red) Painting Kit (1082-KT) or Dual Colour Painting Kit (1090-KD).

1 X SSC RECIPE:
0.015 M Na Citrate, pH 7.0 containing 0.05 M NaCl.

Note: *These products are not for use in humans and are for research purposes only.*

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