PROTOCOL FOR MCFISH MULTICOLOR FLUORESCENCE *IN SITU* HYBRIDIZATION

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STOCK SOLUTION RECIPES.

For all stock solutions to be prepared, use chemicals with highest grade available. All stock solutions must be prepared with deionized distilled H_2O (ddH_2O). For most steps in the DNA handling, it is necessary to autoclave the ddH_2O for 20 min on a liquid cycle in order to destroy any DNase activity and assure sterility. Note, the water quality in these procedures has a stronger effect on the signal-to-noise ratio than the purity grade of the chemicals.

A. Stock solutions stored at room temperature (RT).

50% DS Dissolve 5 g of dextran sulfate in 5 ml ddH_2O.

4 M NaOH Dissolve 16 g of NaOH in ddH_2O to a final volume of 100 ml.

1x PBS pH 7.4 Dilute 26 ml of 5 M NaCl + 20 ml of 500 mM PB pH 7.4 with 954 ml ddH_2O to a final volume of 1,000 ml.

2 x SSC pH 7.0 Dilute 100 ml of 20 x SSC pH 7.0 with 900 ml ddH_2O to a final volume of 1,000 ml.

1x TAE buffer pH 8.0 Dilute 20 ml of 50 x TAE buffer pH 8.0 with 980 ml ddH_2O to a final volume of 1,000 ml..

B. Stock solutions stored at 4ºC (refrigerator).

1% acetocarmine Dissolve 10 g of carmine (Fisher) in 1,000 ml of 45% glacial acetic acid. Add boileezers and reflux (in hood) for 24 hr. Filter into a dark bottle.

Antifade Use Vectashield (Vector Laboratories, H-1000) for mounting slides..

100 mM citric acid Dissolve 21.0 g of citric acid in ddH_2O to 1,000 ml.

FA Use formamide with high grade (e.g., Merck or Fisher).

50% FA in 2x SSC Dilute 50 ml of formamide + 10 ml of 20 x SSC with 40 ml ddH_2O.

LB-amp broth, pH 7.0 (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, 50 μg/ml ampicillin), sterile
Dissolve 4 g of Bacto-tryptone (Difco) + 2 g of Bacto-yeast extract (Difco) + 4 g of NaCl in 380 ml ddH_2O. Titrate pH to 7.0 with NaOH and bring volume to 400 ml with ddH_2O. Autoclave for 20 min at 121°C, cool to room temperature, add 400 μl of 50 mg/ml ampicillin under sterile conditions.

McIlvaine buffer pH 7.0 Dilute 17.65 ml of 100 mM citric acid + 32.94 ml of 500 mM Na_2HPO_4 with 49.41 ml ddH_2O

1 M $MgCl_2$ Dissolve 20.33 g of $MgCl_2 \cdot 6H_2O$ in ddH_2O to a final volume of 100 ml.

5 M NaCl Dissolve 29.22 g of NaCl in ddH_2O to a final volume of 100 ml.

500 mM NaH_2PO_4 Dissolve 68.99 g of $NaH_2PO_4 \cdot H_2O$ in ddH_2O to a final volume of 1,000 ml.

500 mM Na_2HPO_4 Dissolve 88.99 g of $Na_2HPO_4.2H_2O$ in ddH_2O to a final volume of 1,000 ml.

500 mM PB (phosphate buffer) pH 7.4 (350 mM Na_2HPO_4, 150 mM NaH_2PO_4)
Add 500 mM Na_2HPO_4 + 500 mM NaH_2PO_4 to pH 7.4.

10x PBS (phosphate buffered saline) pH 7.4 (1.3 M NaCl, 70 mM Na_2HPO_4, 30mM NaH_2PO_4)
Dilute 26 ml of 5 M NaCl + 20 ml of 500 mM PB pH 7.4 with 54 ml ddH_2O.

PFA Use high-grade paraformaldehyde (e.g., Merck or Fisher).

20 x SSC (saline sodium citrate) pH 7.0 (3 M NaCl, 0.3 M Na_3 citrate)
Dissolve 175.32 g of NaCl + 88.23 g of Na_3 citrate·$2H_2O$ in 850 ml ddH_2O, titrate pH to 7.0 with NaOH/HCl, add ddH_2O to a final volume of 1,000 ml.

50 x TAE buffer pH 8.0 (2 M Tris acetate pH 8.0, 50 mM Na_2EDTA)
Dissolve 18.6 g of Na_2EDTA + 242.24 g of Tris base in 700 ml ddH_2O, titrate pH to 8.0 with glacial acetic acid (~ 57 ml), add ddH_2O to a final volume of 1,000 ml.

1 M Tris.Cl buffer pH 7.5 Dissolve 121.12 g of Tris base in 800 ml ddH_2O, titrate pH to 7.5 with concentrated HCl (~ 65 ml), and add ddH_2O to a final volume of 1,000 ml.

1 M Tris.Cl buffer pH 8.0 Dissolve 121.12 g of Tris base in 800 ml ddH_2O, titrate pH to 8.0 with concentrated HCl (~ 42 ml), and add ddH_2O to a final volume of 1,000 ml.

1 M Tris.Cl buffer pH 8.5 Dissolve 121.12 g of Tris base in 800 ml ddH_2O, titrate pH to 8.5 with concentrated HCl (~ 28 ml), add ddH_2O to a final volume of 1,000 ml.

1 M Tris.Cl buffer pH 9.5 Dissolve 121.12 g of Tris base in 800 ml ddH_2O, titrate pH to 9.5 with concentrated HCl (~ 20 ml), add ddH_2O to a final volume of 1,000 ml.

C. Stock solutions stored at –20ºC (freezer).

10 μg/μl carrier DNA Use sonicated, denatured DNA from salmon testes (Sigma D-7656) and store in aliquots.

Counterstains

100 μg/ml DAPI [$Excitation_{max}$: 344 nm, $Emission_{max}$: 449 + 488 nm]
Dissolve 1 mg of 4',6-diamidino-2-phenylindole.2HCl in 10 ml ddH_2O and store in 1-ml aliquots.

100 μg/ml PI [$Excitation_{max}$: 340 + 530 nm, $Emission_{max}$: 615 nm]
Dissolve 1 mg of propidium iodide in 10 ml ddH_2O and store in 1-ml aliquots.

5 U/μl DNA Pol I Use *E. coli* DNA Polymerase I (Kornberg enzyme) (Boehringer # 642711) and store in 10-μl aliquots.

30 U/μl DNase I Use pancreatic DNase I solution (*e.g.* Boehringer # 776785) and store in 10-μl aliquots.

10x NT buffer (Nick Translation buffer) pH 7.5 (500 mM Tris.Cl pH 7.5, 50 mM $MgCl_2$, 0.5 mg/ml BSA)
Dilute 1 ml of 1 M Tris.Cl pH 7.5 + 100 μl of 1 M $MgCl_2$ + 10 μl of 10% BSA with 890 μl ddH_2O and store in 100-μl aliquots.

100 mM DTT Dissolve 155 mg of 1,4-dithiothreitol + 33 μl of 3 M Na-acetate pH 5.2 in ddH_2O to 10 ml and store in 1-ml aliquots.

1.3 mM dNTP mix for nick translation (400 μM dATP, 400 μM dGTP, 400 μM dCTP, 100 μM dTTP)
Use 100 mM solutions of the lithium salts of the dNTPs (Boehringer). Dilute 4 μl of dATP + 4 μl of dGTP + 4 μl of dCTP + 1 μl of dTTP with 987 μl ddH_2O and store in 100-μl aliquots.

1 mM F-x-dUTP Use 1 mM solution of an appropriate Fluorophore-x-dUTP (Amersham, Boehringer, DuPont, Enzo, or Molecular Probes; see Table on p. 5).

1% RNase A in 10 mM Tris.Cl pH 7.5, 15 mM NaCl (DNase free)

Dissolve 10 mg of RNase A (Sigma) + 10 µl of 1 M Tris.Cl pH 7.5 + 3 µl of NaCl in 987 µl ddH_2O, incubate in boiling waterbath for 15 min, cool slowly, and store in aliquots.

Alternative counterstains

Counterstain	Catalog #	Excitation$_{max}$ nm	Excitation$_{max}$ color	Emission$_{max}$ nm	Emission$_{max}$ color
POPO-1	Molecular Probes P-3580	434	Violet	456	Blue
DAPI	Molecular Probes D-1306	359	Ultraviolet	461	Blue
Hoechst 33342	Molecular Probes H-3570	355	Ultraviolet	465	Blue
Hoechst 33258	Molecular Probes H-3569	365	Ultraviolet	480	Blue
BOBO-1	Molecular Probes B-3582	462	Blue	481	Blue
YOYO-1	Molecular Probes Y-3601	491	Blue	509	Green
SYTOX Green	Molecular Probes S-7020	504	Green	523	Green
TOTO-1	Molecular Probes T-3600	514	Green	533	Green
Lucifer Yellow VS	Sigma L-3510	428	Violet	540	Green
SYTO 15	Molecular Probes S-7577	515	Green	546	Yellow
Acridine Yellow	Sigma A-2520	470	Blue	550	Yellow
POPO-3	Molecular Probes P-3584	534	Green	570	Orange
BOBO-3	Molecular Probes B-3586	570	Orange	604	Red
Etbr	Molecular Probes E-3565	518	Green	605	Red
PI	Molecular Probes P-1304	535	Green	617	Dark red
YOYO-3	Molecular Probes Y-3606	612	Dark red	631	Dark red
TOTO-3	Molecular Probes T-3604	642	Dark red	660	Far red

Alternative F-X-dUTPs

F-x-dUTP	Catalog #	Excitation$_{max}$ nm	Excitation$_{max}$ color	Emission$_{max}$ nm	Emission$_{max}$ color
ChromaTide Cascade Blue-7-dUTP	Molecular Probes C-7612	400	Violet	420	Blue
FluoroBlue Coumarin-4-dUTP	Amersham RPN 2123	350	Ultraviolet	440	Blue
Coumarin-5-dUTP	DuPont NEL-415	402	Violet	443	Blue
Amino-methylcoumarin-6-dUTP	Boehringer 1 534 386	342	Ultraviolet	450	Bright blue
Amino-methylcoumarin-6-dUTP	Enzo 42851	342	Ultraviolet	450	Bright blue
Amino-methylcoumarin-13-dUTP	Enzo 42852	342	Ultraviolet	450	Bright blue
ChromaTide BODIPY FL-14-dUTP	Molecular Probes C-7614	505	Green	513	Green
Fluorescein-12-dUTP	Enzo 42831	495	Blue	517	Green
Fluorescein-12-dUTP	DuPont NEL-413	494	Blue	517	Green
Fluorescein-12-dUTP	Boehringer 1 373 242	494	Blue	517	Green
ChromaTide Fluorescein-12-dUTP	Molecular Probes C-7604	494	Blue	518	Green
ChromaTide Alexa 488-5-dUTP	Molecular Probes C-11397	490	Blue	519	Green
FluoroGreen Fluorescein-11-dUTP	Amersham RPN 2121	490	Blue	520	Green
ChromaTide Oregon Green 488-5-dUTP	Molecular Probes C-7630	496	Blue	524	Green
ChromaTide Rhodamine Green-5-dUTP	Molecular Probes C-7629	502	Green	527	Green
ChromaTide Alexa 532-5-dUTP	Molecular Probes C-11398	525	Green	550	Yellow
Tetramethylrhodamine-6-dUTP	DuPont NEL-416	550	Yellow	570	Orange
ChromaTide Tetramethylrhodamine-6-dUTP	Molecular Probes C-7606	550	Yellow	570	Orange
ChromaTide Alexa 546-5-dUTP	Molecular Probes C-11401	555	Yellow	570	Orange
FluoroLink Cy3-dUTP	Amersham PA 53022	550	Yellow	570	Orange
ChromaTide BODIPY TMR-14-dUTP	Molecular Probes C-7616	542	Green	574	Orange
FluoroRed Rhodamine-4-dUTP	Amersham RPN 2122	545	Yellow	575	Red
Tetramethyl-rhodamine-6-dUTP	Boehringer 1 534 378	550	Yellow	575	Red
Tetramethylrhodamine-5-dUTP	Enzo 42841	551	Yellow	575	Red
ChromaTide Tetramethylrhodamine-5-dUTP	Molecular Probes C-7606	555	Yellow	580	Red
Lissamine-5-dUTP	DuPont NEL-418	570	Orange	588	Red
ChromaTide Alexa 568-5-dUTP	Molecular Probes C-11399	575	Red	600	Red
Texas Red-dUTP	DuPont NEL-417	593	Red	612	Dark red
ChromaTide Alexa 594-5-dUTP	Molecular Probes C-11400	590	Red	615	Dark red
ChromaTide Texas Red-12-dUTP	Molecular Probes C-7631	595	Red	615	Dark red
ChromaTide Texas Red-5-dUTP	Molecular Probes C-7608	595	Red	615	Dark red
ChromaTide BODIPY TR-14-dUTP	Molecular Probes C-7618	589	Red	617	Dark red
ChromaTide BODIBY 630/650-14-dUTP	Molecular Probes C-11395	632	Dark red	648	Dark red
ChromaTide BODIPY 650/665-14-dUTP	Molecular Probes C-11396	650	Dark red	668	Far red
FluoroLink Cy5-dUTP	Amersham PA 55022	649	Dark red	670	Far red

PREPARING CLONED DNA FOR MCGISH

The DNA to be used as a FISH probe had been inserted into the **M**ulti **C**loning **S**ite (MCS) of plasmid vectors. The vector possesses the gene for resistance to antibiotics. The recombinant plasmid can be propagated in the *E. coli* strain DH5α.

A. Culturing bacteria.

1. Inoculate 3 ml of LB-amp media with a single white colony using a sterile toothpick.
2. Shake the LB-amp bacteria culture at 225 rpm for 12–16 h at 37ºC (Optical density $_{600\ nm}$: 1.5–1.7).

B. Isolating plasmids.

QIAprep Spin Miniprep Kit (QIAgen #27104 or 27106).

1. Centrifuge sample at 13,000 rpm for 3 min at 4ºC to collect bacteria; decant the supernatant.
2. Resuspend pelleted bacterial cells in 250 μl of Buffer P1 and transfer to a microfuge tube.
3. Add 250 μl of Buffer P2 and gently invert the tube 4–6 times to mix.
4. Add 350 μl of Buffer N3 and invert the tube immediately but gently 4-6 times.
5. Centrifuge for 15 min. During centrifugation, place a QIAprep spin column in a 2-ml collection tube.
6. Apply the supernatant from step 5 to the QIAprep column by decanting or pipetting.
7. Centrifuge 60 sec. Discard the flow-through.
8. Wash QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuge for 60 sec. Discard the flow-through.
9. Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuge for 60 sec.
10. Discard the flow-through and centrifuge for an additional 1 min to remove residual wash buffer.
11. Place the QIAprep column into a clean, 1.5-ml microfuge tube. To elute DNA, add 50 μl of Buffer EB to the center of each QIAprep column, let stand for 1 min, and centrifuge for 2 min.

F-LABELING OF CLONED DNA FOR MCFISH

The DNA to be used as a FISH probe had been inserted into the **M**ulti **C**loning **S**ite (MCS) of plamids. The whole recombined plasmid is labeled by the method of nick translation in presence of F-x-dUTP. Nick translation (Rigby *et al.* 1977: J. Mol. Biol. 113, 237-251) is based on the introduction of random single-stranded nicks in double-stranded DNA by pancreatic DNase I. The three activities of the *E. coli* DNA polymerase I then catalyze the addition of nucleotide residues to the 3'-hydroxyl terminus of a nick (5'→3' polymerase activity), with the simultaneous elimination of nucleotides from the 5'-phosphoryl terminus (5'→3' exonuclease activity). As nucleotides are removed and the new ones are added, the nick is linearly moved, or "translated" along the DNA strand. In the presence of F-x-dUTP, a dTTP analog, about 50 or 60 % of the dTTP residues are replaced with F-x-dUTP. The use of low reaction temperature (15ºC) avoids synthesis of snap back DNA structures by optimal proofreading (3'→5' exonuclease activity). After denaturation, the final probe length (ssDNA) should be about 400 nucleotides for best penetration of the labeled probe into the condensed structure of a mitotic or meiotic plant chromosome or chromatin of an interphase nucleus.

A. Diluting cloned DNA.

1. Dilute the DNA with ddH_2O or Buffer EB (10 mM Tris.Cl, pH 8.5) to a concentration of lower than 1 μg/μl.

B. Diluting DNase I.

1. **Immediately prior to use**, remove an aliquot of DNase I stock from –20ºC freezer and put on ice.

2. Dilute (1/500*) the thawed DNase I aliquot on ice: 1 μl of DNase I (30 U/μl) + 499 μl ddH_2O

* The dilution must effect a nicking activity for a final probe length of 400 nucleotides (ssDNA), i.e., about 600 or 700 basepairs (dsDNA). Depending on the purity and medium length of the cloned DNA and the enzyme activity of the DNase I stock solution, the needed dilution can vary from about 1/50 (for BACs or YACs) to about 1/2,000 (for microcloned DNA of about 2.5 kbp).

→ 1/50 dilution	»	600 mU/μl	→ 1/500 dilution	»	60 mU/μl
→ 1/100 dilution	»	300 mU/μl	→ 1/1,000 dilution	»	30 mU/μl
→ 1/300 dilution	»	100 mU/μl	→ 1/2,000 dilution	»	15 mU/μl

C. Nick-translation.

1. Set up the reaction mix (50 µl) in a sterile 200-µl safe-lock tube on ice.

Component	Volume (µl)	Mass per reaction	Final concentration
ddH_2O	30–x		
10x NT buffer	5	2.5 µmol Tris.Cl pH 7.5	50 mM
		250 nmol $MgCl_2$	5 mM
		2.5 µg BSA	50 ng/µl
100 mM DTT	5	500 nmol	10 mM
1.3 mM dNTP mix, equimolar(*)	5	2 nmol dATP	40 µM
		2 nmol dGTP	40 µM
		2 nmol dCTP	40 µM
		500 pmol dTTP	10 µM
1 mM F-x-dUTP, equimolar *	2	2 nmol	40 µM
DNA	x	1 µg	20 ng/µl
DNase I, diluted	1	60 mU	1.2 mU/µl
DNA Pol I	2	10 U	0.2 U/µl

* 2.5 nmol (dTTP + F-x-dUTP) for mol fraction T takes into account the > 25% lower incorporation rate of fluorochrome labeled dUTPs into the DNA than that of unmodified dTTP.

2. Thoroughly, but carefully, mix the solutions and incubate for 2 h at 15ºC.

3. Stop incubation by placing the tube on ice, then incubate at 65ºC for 7 min to inactivate the enzymes.

4. Purify the probe using a QIAquick Nucleotide Removal Kit (QIAgen 28304).

   a. Add 500 µl Buffer PN to the reaction mixture and mix.

   b. Place a QIAquick spin column in a 2-ml collection tube.

   c. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min at 6,000 rpm.

   d. Discard the flow-through and place QIAquick column back into the same tube.

   e. Wash QIAquick column by adding 750 µl of Buffer PE and centrifuge for 1 min at 6,000 rpm.

   f. Discard the flow-through and place the QIAquick column back in the same tube, which should be empty. Centrifuge for an additional 1 min at 10,000 x g.

   g. Place the QIAquick column in a clean 1.5-ml microcentrifuge tube.

   h. Elute DNA by adding 50 µl of Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min at 10,000 x g.

5. Store the probe at –20ºC.

D. Minigelelectrophoresis.

1. Mix samples (5 μl):

   a. 2 μl of F-probe (~40 ng) + 2 μl ddH_2O + 1 μl of 5x DNA loading buffer and
   b. 5 μl of DNA 1-kb ladder with loading buffer.

2. Load samples into the wells and electrophorese in 1x TAE buffer pH 8.0 for 1 h at RT.

3. Check the gel on a UV light transilluminator for the band of the probe at about 600 to 700 bp.

E. Spot test.

1. Spot 1 μl of F-probe (~20 ng) onto a small sheet (2 cm x 3 cm) of nylon membrane and air dry for ~10 min.

2. Examine fluorescence intensity under light at appropriate wavelength.

PREPARING AND PRETREATING SLIDES FOR MCFISH

Among plant tissues containing actively dividing cells, root-tip meristems are the most commonly used for making mitotic chromosome preparations. Other tissues may be used, such as leaf meristems, calli, or protoplasts. Root-tip meristem cells may be obtained from germinating seeds, newly formed roots in soil or hydroponics, or root tips from tissue-culture-grown plants. Root tip collection includes a pretreatment in order to arrest as many metaphase cells as possible. The common method is the so-called ice-water treatment followed by the fixation in modified Carnoy's fixative (absolute ethanol : glacial acetic acid = 3:1).

A. Preparation of slides.

1. Germinate seeds in distilled water on filter paper in a petridish at RT for 2–3 days (2 cm long roots).
2. Cut roots and pretreat in distilled water in tubes on ice for 24 h.
3. Fix roots in fresh fixative (3 volumes of 100% EtOH + 1 volume of glacial acetic acid) at RT.
4. Store roots at 4ºC until ready for use.
5. Put roots in 1% acetocarmine for a few min or until the tip of the root is dark red.
6. Cut the very tip of the root and gently squeeze out meristem cells from the root tip onto a slide.
7. Squash meristem cells in a drop of 45% acetic acid under a cover slip (18 x 18 mm). Examine mitotic index under the phase-contrast microscope.
8. Freeze the slide upside down on dry ice. Remove cover slip with the edge of a double-sided razor blade and incubate in 100% EtOH for 5 min at RT.
9. Air dry.

B. RNase A treatment.

1. **Immediately prior to use**, remove an 8-μl aliquot of RNase A stock from the –20ºC freezer and thaw on ice.
2. Dilute (1/100) the thawed RNase A aliquot on ice: 8 μl of 1% RNase A + 80 μl of 20 x SSC + 712 μl ddH_2O.
3. Add 100 μl of diluted RNase A onto one slide, cover with a plastic cover slip (20 x 20 mm), and incubate in a wet chamber for 45 min at 37ºC.
4. Wash slides 3x by gently shaking in 2x SSC for 5 min each at RT.

C. Postfixation.

1. **Immediately prior to use**, prepare 100 ml of 4 % PFA:
   Warm 80 ml ddH_2O to 60–70ºC
   Add 4 g of PFA
   Add 100 µl of 4 M NaOH
   Add 10 ml of 10 x PBS
   Cool to RT and add ddH_2O to 100 ml.

2. Incubate slides in PFA for 10 min at RT in a fume hood.

3. Wash slides 3x by gently shaking in 2x SSC for 5 min each at RT.

D. Dehydration.

1. Incubate slides in a 70%, 95%, and 100% EtOH series, for 3 min each at RT.

2. Air dry slides at RT.

MULTICOLOR FLUORESCENCE *IN SITU* HYBRIDIZATION (MCFISH) WITH F-DNA

A. Denaturing slides.

1. Set the temperature of a hot plate to 80ºC.
2. Denature slides in 100 µl of 70% FA / 2 x SSC for 2 min at 80ºC.
3. Dehydrate slides in 70% EtOH (–20ºC) for 5 min, 95% EtOH (RT) for 3 min, and 100% EtOH (RT) for 3 min.
4. Air dry slides and prepare for GISH.

B. Fluorescence in situ *hybridization.*

1. Prepare 30 µl of hybridization solution (HS) per slide (final concentration in the hybridization solution): 50% FA, 2 x SSC, 10% dextran sulfate, 0.3 mg/ml of salmon testes DNA, and 40–60 ng of labeled probe.

   For example (for 4 slides): 120 µl of HS in a sterile 1.5-ml save-lock tube

Component	Volume (µl)
ddH_2O	20–x
dFA	60.0
20 x SSC	12.0
50% dextran sulfate	24.0
carrier DNA (10 µg/µl)	4.0
probe DNA (20 ng/µl)	x (8.0–12.0)

2. Boil the HS for 5 min.
3. Chill on ice for 5 min and spin down condensed water.
4. Add 30 ml of HS onto each slide and cover with a plastic cover slip (20 x 20 mm).
5. Incubate in a wet chamber overnight at 37°C.

C. Post-hybridization washes.

1. Wash slides 2x by gently shaking in 2 x SSC for 5 min at 42°C.
2. Wash slides 1x by gently shaking in 50% FA in 2x SSC for 10 min at 42°C.
3. Wash slides 2x by gentle shaking in 2 x SSC for 5 min at 42°C.
4. Wash slides by gentle shaking in 2 x SSC for 5 min at RT.

MCFISH stringency (%) according to:

T_m = melting temperature (ºC) **= 81.5** (ºC) **+ 16.6 $\log_{10}$M + 0.41 (% G+C) – 500/n – 0.61 (% formamide)**

M: 2.0 x SSC = 0.3900 M Na^+
1.5 x SSC = 0.2925 M Na^+
1.0 x SSC = 0.1950 M Na^+
0.5 x SSC = 0.0975 M Na^+

(% G+C) = 45.5 %
n ~ 400 (probe)
n ~ 200 (competitor)
n ~ 150 (carrier)

(% formamide) = 50 %
(% formamide) = 40 %
(% formamide) = 30 %

on the assumption that **f_m** = mismatch factor = 1

stringency (%) **= 100 + hybridization temperature – melting temperature = 100 + T_h - T_m**

T_h	50% FA/2x SSC			50% FA/1.5x SSC			50% FA/1x SSC			50% FA/0.5x SSC		
(ºC)	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp
37	75 %	75 %	77 %	77 %	77 %	79 %	80 %	80 %	82 %	85 %	85 %	87 %
42	80	80	82	82	82	84	85	85	87	90	90	92
47	85	85	87	87	87	89	90	90	92	95	95	97
52	90	90	92	92	92	94	95	95	97	100	100	D
57	95	95	97	97	97	99	100	100	D	D	D	D
62	100	100	D	D	D	D	D	D	D	D	D	D
67	D	D	D	D	D	D	D	D	D	D	D	D
72	D	D	D	D	D	D	D	D	D	D	D	D

T_h	40% FA/2x SSC			40% FA/1.5x SSC			40% FA/1x SSC			40% FA/0.5x SSC		
(ºC)	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp
37	69 %	69 %	71 %	71 %	71 %	73 %	74 %	74 %	76 %	79 %	79 %	81 %
42	74	74	76	76	76	78	79	79	81	84	84	86
47	79	79	81	81	81	83	84	84	86	89	89	91
52	84	84	86	86	86	88	89	89	91	94	94	96
57	89	89	91	91	91	93	94	94	96	99	99	D
62	94	94	96	96	96	98	99	99	D	D	D	D
67	99	99	D	D	D	D	D	D	D	D	D	D
72	D	D	D	D	D	D	D	D	D	D	D	D

T_h	30% FA/2x SSC			30% FA/1.5x SSC			30% FA/1x SSC			30% FA/0.5x SSC		
(ºC)	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp	800 bp	400 bp	200 bp
37	63 %	63 %	64 %	65 %	65 %	67 %	68 %	68 %	69 %	73 %	73 %	74 %
42	68	68	69	70	70	72	73	73	74	78	78	79
47	73	73	74	75	75	77	78	78	79	83	83	84
52	78	78	79	80	80	82	83	83	84	88	88	89
57	83	83	84	85	85	87	88	88	89	93	93	94
62	88	88	89	90	90	92	93	93	94	98	98	99
67	93	93	94	95	95	97	98	98	99	D	D	D
72	98	98	99	100	100	D	D	D	D	D	D	D

COUNTERSTAINING (BLUE OR RED)

DAPI: Excitation$_{max}$: 344 nm (**ultraviolet**) Emission$_{max}$: 449 nm (**blue**) + 488 nm (**blue**)

PI: Excitation$_{max}$: 340 nm (**ultraviolet**) + 530 nm (**green**) Emission$_{max}$: 615 nm (**red**)

for 4 slides: 2 ml of counterstaining solution

DAPI counterstain (blue)

1. **Immediately prior to use**, remove an aliquot of DAPI stock from a –20ºC freezer and thaw on ice.
2. Dilute (1/250) DAPI on ice: 8 µl of DAPI (100 ng/µl) + 1,992 µl of McIlvaine buffer.
3. Add 500 µl of diluted DAPI onto each slide and incubate for 2 min at RT.
4. Wash slides with ~ 3 ml of McIlvaine buffer from a pipette.
5. Mount slides with a small drop of Vectashield antifade and cover with cover glass (24 x 30 mm).
6. Store slides in the dark at 4ºC.

PI counterstain (red).

1. **Immediately prior to use**, remove an aliquot of PI stock from a –20ºC freezer and thaw on ice.
2. Dilute (1/2,500) PI on ice: 0.8 µl of PI (100 ng/µl) + 2,000 µl of 1 x PBS.
3. Add 500 µl of diluted PI onto each slide and incubate for 2 min at RT.
4. Wash slides with ~ 3 ml of 1 x PBS from a pipette.
5. Mount slides with a small drop of Vectashield antifade and cover with cover glass (24 x 30 mm).
6. Store slides in the dark at 4ºC.