

Embryo In-Situ

FIXATION

- 1) Collect embryos from o/n plate
- 2) Transfer to basket and dechorionate by soaking in 50% bleach solution for 5 minutes
- 3) Rinse with distilled water
- 4) Rinse with 70% ethanol for 30 seconds
- 5) Transfer to fix in scintillation vial for 40 minutes (8ml **First fix** + 8ml heptane)
- 6) Remove lower layer and add equal volume of methanol
- 7) Shake for 30 seconds to remove vitelline membrane (embryos will collect at the bottom of the vial after the membrane has been removed)
- 8) Remove all of the liquid and wash the fallen embryos 3 times with methanol.
- 9) Wash embryos in 100% ethanol. You can store over time at -20 in 100% ethanol.

STAINING

Turn on 55C water bath.

- 1) Put embryos in a total volume of 500ul of ethanol and add 500ul xylene, invert once.
- 2) Remove ethanol/xylene and add 1300ul xylene. Place on shaker for 2-3 hours.

Make **Second fix** and **Methanol: Second fix**

SECOND FIX

| <u>Final Conc.</u> | <u>Stock Conc.</u> | | <u>Amount</u> |
|---------------------|--------------------|---------------|---------------|
| ddH ₂ O | - | 12.16ml | 24.32ml |
| 0.1% Twn 20 | 20% | .08ml | 0.16ml |
| 1X PBS | 10X | 1.6ml | 3.2ml |
| 5% Formaldehyde | 37% | 2.16ml | 4.32ml |
| FINAL VOLUME | | 16.0ml | 32.0ml |

- 3) Remove 500ul of the xylene and add 800ul of 100% ethanol to get 1:1 mixture.
- 4) Rinse with 100% ethanol twice.
- 5) Rinse with methanol.
- 6) Rinse with **Methanol: Second fix**

7) Post-fix for 40min in **Second fix** on shaker.

Prepare 1x hybridization solution. Each sample will require at least 3ml of.

Prepare .5x hybridization solution (Dilute 1x in PBT). Each sample requires 1ml.

1x Hybridization Solution (3ml per sample including .5x)

| Final Conc. | Stock Conc. | Amount | Amount | Amount |
|----------------------|-------------|---------------|---------------|---------------|
| ddH ₂ O | - | 1.75ml | 3.5ml | 7ml |
| 50% Formamide | 100% | 5ml | 10ml | 20ml |
| 5x SSC | 20x | 2.5ml | 5ml | 10ml |
| 50ug/ml Heparin | 10mg/ml | 100ul | 200ul | 400ul |
| .1% Tween 20 | 20% | 50ul | 100ul | 200ul |
| 100ug/ml ssdna | 10mg/ml | 200ul | 400ul | 800ul |
| 100ug/ml yRNA | 5mg/ml | 400ul | 800ul | 1.6ml |
| FINAL VOLUME. | | 10.0ml | 20.0ml | 40.0ml |

.5x Hybridization Solution

Dilute 1x 1:1 in PBT

8) Rinse 5x with **PBT**

PBT

| Final Conc. | Stock Conc. | Amount | Amount |
|----------------------|-------------|--------------|-------------|
| ddH ₂ O | - | 447.5ml | 44.75ml |
| 0.1% Tween 20 | 20% | 2.5ml | 25ml |
| 1X PBS | 10X | 50ml | 5ml |
| FINAL VOLUME. | | 500ml | 50ml |

9) Rinse with 1ml of **.5x hybridization solution**

10) Rinse with 1ml of **1x hybridization solution**. Remove this and add another 1ml of **1x hybridization solution** and invert.

11) Prehybridize at 55C for 2-3 hours

-Invert the tube every 30 minutes

-When the 2-3 hours are almost up, prepare the probe.

-Heat probe in a siliconized microcentrifuge tube for 10 minutes with a cap lock

-Spin briefly in the 4C centrifuge

-Place tube on ice

12) After prehybridizing, remove as much prehyb as possible. Add appropriate amount of probe to the tube and put in back in the 55C with gentle shaking o/n.

Day 2

1) Prepare **1x Hybridization solution without salmon sperm or yeast tRNA**. Heat these solutions to 55C.

1x Hybridization Solution (2ml per sample including .5x)

| Final Conc. | Stock Conc. | Amount | Amount | Amount |
|-------------|-------------|--------|--------|--------|
|-------------|-------------|--------|--------|--------|

| | | | | |
|----------------------|---------|---------------|---------------|---------------|
| ddH ₂ O | - | 2.355ml | 4.7ml | 9.4ml |
| 50% Formamide | 100% | 5ml | 10ml | 20ml |
| 5x SSC | 20x | 2.5ml | 5ml | 10ml |
| 50ug/ml Heparin | 10mg/ml | 100ul | 200ul | 400ul |
| .1% Tween 20 | 20% | 50ul | 100ul | 200ul |
| FINAL VOLUME. | | 10.0ml | 20.0ml | 40.0ml |

.5x Hybridization Solution

Dilute 1x 1:1 in PBT

- 2)Preabsorb anti-DIG antibody at 1:1000 dilution against fixed embryos O/N.
- 3)Wash embryos with **1x Hybridization Solution** for 10 minutes
- 4)Wash 1 time in **.5x Hybridization Solution**
- 5)Wash 5x in **PBT** for 20 min each
- 6)Add room temperature **PBT** and transfer tube to benchtop to cool.
- 7)Remove PBT from tube and add preabsorbed anti-DIG at 1:2000 final concentration
- 8)Incubate 3 hrs.
- 9)Wash embryos 4x in **PBT** for 20 minutes.

10)Collect embryos and put in staining wells. Add **staining buffer**

| Staining buffer: | <u>50 ml of soln</u> | <u>Stocks</u> |
|--------------------------|----------------------|-----------------------|
| 100 mM NaCl | 1 ml | 5 M NaCl |
| 50 mM MgCl ₂ | 2.5 ml | 1 M MgCl ₂ |
| 100 mM Tris/HCl pH 9.5 | 2.5 ml | 2 M Tris |
| 1 mM Levamisol (Sigma) * | 0.5 ml | 100 mM Levamisol |
| 0.1% Tween 20 | 50 | 100% |

11)Rinse samples 2 times with staining buffer.

12)Add staining solution and let color develop in the dark—examine every 10-15 minutes.

| Color Solution: | <u>10 ml of soln</u> |
|------------------------|----------------------|
| Staining buffer | 10 ml |
| Tube 9 (NBT) | 70 |
| Tube 10 (X-phosphate) | 35 |

13)When done, wash 2x with PBT.

- 14) For Double stain with antibody, wash 2 more times with PBT.
- 15) Incubate in PBT + Goat serum (10%) for 30 minutes.
- 16) Incubate o/n in PBT + Goat + primary antibody
- 17) Rinse quickly three times with PBT. Wash three times 20min in PBT.
- 18) Incubate in PBT + Goat serum (10%) for 15 minutes.
- 19) Incubate in PBT + Goat + Secondary for 3 hours.
- 20) Rinse quickly three times with PBT. Wash three times 20min in PBT.
- 21) Perform color reaction.
- 22) To stop color reaction, rinse embryos in PBT.
- 23) Dehydrate for five minutes each in 50, 75, 95, 100% ethanol. Incubate for 10 minutes in acetone. Incubate o/n on benchtop in 1:1 acetone:plastic solution.

Solutions for In-situ

FIRST FIX

| <u>Final Conc.</u> | <u>Stock Conc.</u> | <u>Amount</u> | <u>Amount</u> | <u>Amount</u> |
|--------------------------|--------------------|---------------|---------------|---------------|
| ddH ₂ O | - | 6.65ml | 10.64ml | 21.3ml |
| 1X PBS | 10X | 1.0ml | 1.6ml | 3.2ml |
| 50 mM EGTA | 0.5 M | 1.0ml | 1.6ml | 3.2ml |
| 5% Formaldehyde | 37% | 1.35ml | 2.16ml | 4.32ml |
| FINAL VOLUME..... | 10.0ml | 20.0ml | 40.0ml | |

SECOND FIX

| <u>Final Conc.</u> | <u>Stock Conc.</u> | <u>Amount</u> |
|--------------------------|--------------------|---------------|
| ddH ₂ O | - | 24.32ml |
| 0.1% Tween 20 | 20% | 0.16ml |
| 1X PBS | 10X | 3.2ml |
| 5% Formaldehyde | 37% | 4.32ml |
| FINAL VOLUME..... | | 32.0ml |

1x Hybridization Solution (3ml per sample)

| <u>Final Conc.</u> | <u>Stock Conc.</u> | <u>Amount</u> | <u>Amount</u> | <u>Amount</u> |
|----------------------|--------------------|---------------|---------------|---------------|
| ddH ₂ O | - | 1.75ml | 3.5ml | 7ml |
| 50% Formamide | 100% | 5ml | 10ml | 20ml |
| 5x SSC | 20x | 2.5ml | 5ml | 10ml |
| 50ug/ml Heparin | 10mg/ml | 100ul | 200ul | 400ul |
| .1% Tween 20 | 20% | 50ul | 100ul | 200ul |
| 100ug/ml SSDNA | 10mg/ml | 200ul | 400ul | 800ul |
| 100ug/ml YeasttRNA | 5mg/ml | 400ul | 800ul | 1.6ml |
| FINAL VOLUME. | | 10.0ml | 20.0ml | 40.0ml |

.5x Hybridization Solution (1ml per sample)

Dilute 1x 1:1 in PBT

PBT

| <u>Final Conc.</u> | <u>Stock Conc.</u> | <u>Amount</u> | <u>Amount</u> |
|--------------------|--------------------|---------------|---------------|
| ddH ₂ O | - | 447.5ml | 44.75ml |
| 0.1% Tween 20 | 20% | 2.5ml | .25ml |
| 1X PBS | 10X | 50ml | 5ml |

FINAL VOLUME.....500ml 50ml

SSC

| <u>Final Conc.</u> | <u>Amount</u> | <u>Amount</u> |
|--|---------------|---------------|
| ddH ₂ O | 800ml | 160ml |
| NaCitrate | 88.2g | 17.64g |
| NaCl | 175.3g | 35.06g |
| adjust volume with ddH₂O | | |
| FINAL VOLUME..1L | | 200ml |

| Staining buffer: | <u>50 ml of soln</u> | <u>Stocks</u> |
|--------------------------|----------------------|-----------------------|
| 100 mM NaCl | 1 ml | 5 M NaCl |
| 50 mM MgCl ₂ | 2.5 ml | 1 M MgCl ₂ |
| 100 mM Tris/HCl pH 9.5 | 2.5 ml | 2 M Tris |
| 1 mM Levamisol (Sigma) * | 0.5 ml | 100 mM Levamisol |
| 0.1% Tween 20 | 50 | 100% |

| Color Solution: | <u>10 ml of soln</u> |
|------------------------|----------------------|
| Staining buffer | 10 ml |
| Tube 9 (NBT) | 70 |
| Tube 10 (X-phosphate) | 35 |