Hybridization Buffer (Hyb+) for *in situ* Hybridization

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| **Reagents** | **Volume Added** | **Final Concentration** |
| Formamide | 25 mL | 50% Formamide |
| 20x SSC buffer | 12.5 mL | 5x SSC |
| 5 mg/mL Heparin | 500 µl | 50 µg/mL |
| 25 mg/mL tRNA | 1 mL | 500 µg/mL |
| Tween-20 | 50 µL | 0.10% |
| 1M Citric Acid | 460 µL | → pH 6 |
| RNase-free H2O | 10.49 µL | → 50 mL |

1. Collect all materials necessary to make Hybridization buffer (+):

**Formamide**

**20x SSC**

**5 mg/mL Heparin**

**25 mg/mL tRNA**

**Tween-20**

**1M Citric Acid**

**RNase-free H2O**

Bucket of ice (*Formamide must remain cold!*)

Automated pipettor w/ three 10 mL and two 25 mL serological pipettes

Sterile 300 mL glass beaker

P1000 pipet w/ sterile-filter tips

P200 pipet w/ sterile-filter tips

Two sterile 50mL conical vials w/ holders

Repeat pipettor w/ 10 mL tips

50 sterile Eppendorf tubes

Make up these reagents prior to Step 2:

* Pour roughly 250 mL 20x SSC buffer into a sterile 300 mL glass beaker. Sterile filter and label “Sterile 20x SSC buffer, name, date.”
* Take a new 50 mL conical and add 50 mL RNase-free H2O, then add 10.51 g Citric Acid Monohydrate (F.W. 210.14) to make 1M Citric Acid. Sterile filter and label “Sterile 1M Citric Acid, name, date.”

2. Clean inside the fume hood. Bring all necessary items into the fume hood once they are wiped down with 70% ethanol. Close the hood and UV sterilize for 15 seconds.

3. Using a serological pipette with three 10 mL tips, add 25 mL formamide to a new sterile 50 mL conical vial.

4. Using a serological pipette with one 25 mL tip and a p1000 set to 500 µL, add 12.5 mL 20x SSC buffer to the 50 mL conical vial.

5. The concentration of heparin in the -20°C freezer is 5 mg/mL. Using a p1000, add 500 µL Heparin to the 50 mL conical vial.

6. Use a p1000 for this step. Add 1 mL RNase-free H2O to a new vial of 25 mg yeast tRNA to bring the concentration in the vial to 25 mg/mL. Add the full amount (1 mL) to the 50 mL conical vial.

7. Using a p200 – over the course of ~1 minute – slowly pipet up 50 µL of Tween-20 and dispense it into the 50 mL conical vial.

8. Using a p1000, add 460 µL Citric Acid to the 50 conical vial.

9. Using a serological pipette with one 25 mL tip and a p1000 set to 490 µL, add 10.49 mL RNase-free H2O to the 50 mL conical vial.

10. 50 sterile Eppendorf tubes should be placed in a clean rack with the lids open prior to pipetting.

11. Insert a 10 mL tip into the repeat pipettor and set the dial to 1 mL. Aliquot 1 mL of HYB+ into 50 sterile Eppendorf tubes. *(Be sure to mix the solution between each few aliquots, this is important for ensure equal concentrations will be distributed to each tube.)*

12. Label and parafilm each tube. Store in a box labeled “HYB+” in -20°C freezer.