Making RNA probes for *in situ* hybridization

Reagents

Nuclease free water
Sigma W4502

NTPs
Roche order #1277057

DIG NTP mix
Roche order #1277073

Biotin NTP mix
Roche order #1685597

Fluorescein NTP mix
Roche order #1685619

DNP-11-UTP
Perkin Elmer NEL555001EA

Template
via PCR, purified and resuspended in nuclease free water

RNA polymerases
T3 - Ambion order #2062
T7 - Ambion order #2073
SP6 - Ambion order #2082

RNAse inhibitor
SUPERase-In Ambion #2694

RNAse free DNAse I
Boehringer Mannheim 0776785

RNAse free EtOH

RNAse free 3M NaOAc pH5.2
Dissolve NaOAc into ddH20 and adjust pH with HCl

Make DNA templates via PCR

Day 1

It's preferable to start with constructs that contain RNA polymerase start sites (T3, T7, or SP6) which allow use of primers outside the start-point. This gives the enzyme some docking space, improving transcription efficiency. If this is not possible, use extended primers with RNA-polymerase start sites in their 5' tails.

Purify PCR products with QiaQuick PCR purification kit and resuspend in nuclease free water.

Make DNP-NTP mixture (if needed)

Day 1

	Amount	Final concentration
Sigma H20	5.7 µl	
ATP 100mM	2 µl	10 mM
CTP 100mM	2 µl	10 mM
GTP 100mM	2 µl	10 mM
UTP 100mM	1.3 µl	6.5 mM
DNP-11-UTP 10mM	7.0 µl	3.5 mM

In vitro transcription reaction

Day 1

	10 µl reaction	20 µl reaction
Sigma H20 to final vol		
10X Buffer	1 µl	2 µl
10X NTP mix	1 µl	2 µl
RNAse inhibitor	0.7 µl	1.3 µl
template DNA	>150ng	>300ng
RNA polymerase	1 µl	2 µl

Heat 10X Buffer to dissolve salts before using.

Incubate at 37ºC for 2-4 hours.

Eliminate template

Day 1

Add 1 µl DNAseI (RNAse free) to each reaction.

Incubate for 15 min at 37ºC. *This is a time sensitive step.*

Precipitate probes

Day 1 (immediately after eliminating the template)

Bring reaction to 50µl with RNAse free water.

Add 1/10th volume 3M NaOAc pH5.2 (5µl)

Add 2.5 volumes of 100% EtOH (125µl)

Store at -20ºC for at least 2 hours.

Spin 20 min at 4ºC at 13200 rpm.

Remove supernatant.

Pellet for DIG/biotin reactions will be white, DNP reactions will be yellow and FITC reactions will be orange.

Add 250-400µl 70% EtOH and spin 20 min at 4ºC at 13200 rpm.

Remove supernatant and repeat wash with 100% EtOH.

For a 20µl reaction with a visible pellet, it is usually sufficient to dissolve the pellet directly in 100µl 50% formamide.

If necessary: resuspend probes in 50 µl of RNAse free water. Take 5 µl for quantitation. Then add 45 µl 100% formamide and store at -20ºC.

For 10µl reaction : with visible pellet, it is sufficient to dissolve them directly in 50µl 50% formamide in RNAse free water.

Properly dissolved probes that have sufficiently high concentrations typically foam a lot. It will take a couple of minutes to dissolve a probe.
