Medina Lab, Penn State University

Probe construction for in-situ hybridizations protocol
Last updated: 01/28/09 by Shini/Matt/Aubrie (to be completed)

1. PCR amplification of target sequence from plasmids (TOPO-pCRII)

For each probe, set up:

	1x (25 uL rxn)
PCR-grade H_2O	17.375 uL
10 x Ex Taq PCR buffer	2.5 uL
dNTPs (2.5 mM)	2 uL
Primer M13_fw (10 uM)	1 uL
Primer M13_rv (10 uM)	1 uL
Ex Taq polymerase (5U/uL)	0.125 uL
Plasmid DNA (~20 ng/uL)	1 uL

M13 Forward (-20) 5′-GTAAAACGACGGCCAG-3’ (Tm:51)

M13 Reverse 5′-CAGGAAACAGCTATGAC-3’ (Tm:50)

Run PCR

Hot-start: 94°C – 2’

30 cycles: 94°C – 30”

50°C – 30”

72°C – 30” (extension time depends on probe size)

1 cycle: 72°C – 8’

For ever: 4°C

Check PCR products

Run out 5 uL on a 1% agarose gel.

Clean up PCR products and nanodrop

Use the QIAquick PCR Purification Kit, elute in MiniElute columns:

Elute in 10 uL water

Nanodrop sample

Making the antisense (or sense-) probe

Make the (anti)sense probe
Use the following reaction for a total of 10 uL
1 uL T7 RNA polymerase (or SP6)
Roche, #
1 uL DIG DNA labeling

Roche, #11727073910
1 uL 10X Buffer (Buffer is the same for T7 or SP6)

Roche, #11465384001
Be sure to dissolve all the precipitate before using, i.e., vortex
0.25 uL RNasin (final 10 U)

3 uL PCR product (final 500 ng)

	1X (10 uL rxn)
RNase-free water	3.75 uL
10X Buffer	1 uL
DIG RNA labeling mix	1 uL
RNasin	0.25 uL (final 10 U)
Template DNA	3 uL (final 500 ng)
T7/SP6	1 uL

Incubate for 3.5 hours at 37 °C

Add 1 uL of DNase I and incubate for 15 min at 37 °C
place on ice

Precipitate RNA according to: http://www.ambion.com/techlib/tb/tb_160.html

- add 29 uL water
- add 10 uL $LiCl_2$ (12 M)
- chilled 30 min -20°C
- centrifuge for 30 min 16,000 x g at 4°C
- remove supernatant by aspiration
- 1 mL 70% EtOH
- centrifuge for 10 min 16,000 x g at 4°C
- remove supernatant and dry pellet for 10 min
- resuspend pellet in 22.5 µL of Nuclease-free water.

use 1 uL for nanodrop

use 1.5 uL for RNA-gel

pour 1% agarose gel

mix:

RNA-sample	1.5 uL
10x loading dye	1 uL
EtBr (1mg/mL)	1 uL
Formamide	6.5 uL
final vol.	10 uL

denature RNA for 5 min at 65°C

place on ice for 5 min

run out sample (at max. 5V/cm)

add 80 uL hybridization buffer and store probe until use

Materials

Takara Ex polymerase

Item: TaKaRa Ex Taq

Company: Takara Bio Inc.

Cat. #: RR001A (250 U)

Vector: pCRII-TOPO

Item: TOPO TA Cloning Kit Dual Promoter (20 rxns)

Company: Invitrogen

Cat. #: 45-0640

PCR-cleanup kit

Item: QIAquick PCR purification kit

Company: Qiagen

Cat. #: 28106 (250 rxns)

MiniElute kit

Item: MiniElute Reaction cleanup kit

Company: Qiagen

Cat. #: 28206 (250 rxns)

10X Buffer

Item: 10X Buffer

Company: Roche

Cat. #: 11465384001

DIG RNA labeling mix

Item: DIG RNA labeling mix

Company: Roche

Cat. #: 11277073910

T7 RNA polymerase

Item: T7 RNA polymerase

Company: Roche

Cat. #: 10881767001

SP6 RNA polymerase

Item: SP6 RNA polymerase

Company: Roche

Cat. #: 10810274001