Riboprobe Synthesis for *In Situ Hybridization*

Based on Andy McMahon Lab protocol (and references therein), modified by Karl Staser, M. Todd Valerius, and Jing Yu.
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The *in situ* hybridization methods were developed by Dave Wilkinson from protocols from Phil Ingham, Ron Conlon, Barry Rosen, and Richard Harland in David Wilkinson, ed., In Situ *Hybridization: A Practical Approach,* Oxford: IRL Press, 1992.

Plasmid Purification

The following details the protocol for isolation and purification of the plasmids that provide the template for riboprobe synthesis.

Colony Isolation

Obtain MSR and MTF clones from university and commercial sources.

Note: When a clone cannot be obtained from a library, we create our own bacterial clones. See the end of this document for the manual cloning protocol.

Streak ampicillin-resistant bacteria from frozen glycerol stock onto 50-100 µg/ml ampicillin/LB plates.
Re-streak three single colonies onto a "master plate" – one master plate contains 8 different clones, or 24 colonies.

- Pick **one** colony with pipette tip (record colony number).
- Inoculate into 2 ml 50-100 µg/ml ampicillin/liquid LB in a 15 ml conical tube.
- Grow overnight at 37°C with constant agitation.

Note: Creating a "master plate" of 8 clones with 3 colonies is more reliable than partially picking colonies from the original plate. It also condenses the number of plates needed.

Plasmid Purification

- Transfer inoculation product to 1.6 ml Eppendorf centrifuge tube.
- Centrifuge product at maximum speed for 30 seconds to obtain bacteria pellet.
- Decant supernatant. Vacuuming or pipetting out fluid is not necessary.
- Purify plasmid with Qiagen's QIAprep Spin Miniprep Kit (Qiagen 27106). Follow the manufacturer's standard protocol with the following notes:
    - Skip optional nuclease purification step.
    - Elute DNA with 50 µl of the provided elution buffer (EB Buffer).

- Dilute the plasmid prep 1:50 (2 µl DNA in 98 µl sterile water) and quantify with the spectrophotometer. A concentration of 100-400 ng/µl indicates high-quality plasmid isolation. Do not use plasmids with concentrations less than 60 ng/µl.
- Store at -80°C in 2 ml screw-top vial (Sarstedt 72.694.006).

Error Procedure: If the concentration is less than 60 ng/µl, re-perform mini-prep procedure on each of two remaining colonies. If each additional colony fails, another clone source must be found.

Note: Miniprep yields less than 60 ng/µl fail in subsequent steps (sequencing or PCR amplification). A low yield for three independent colonies suggests a problem with the vector or insert. A new clone source should be found.

Plasmid Sequencing and Analysis

Plasmid Sequencing

Mix the following at room temperature in a 0.2 ml tube; ensure homogeneity by mixing well and quickly spinning in a centrifuge:

Reagent	**Volume (µl)**	**Source**
10x Big Dye® v3.1 sequencing buffer	4.0	ABI*
10 µM T7 primer	0.5	Invitrogen
Big Dye® v3.1	1.0	ABI 4337455
Sterile water	11.5	
Purified plasmid (60+ ng/µl)	3.0	

*Provided with enzyme.

- Add one drop of sterile, filtered mineral oil to each tube.
- Use PCR machine that ramps temperature (do not use RoboCyclers).

PCR conditions: $96^{o}C$ 30 seconds
$50^{o}C$ 15 seconds
$60^{o}C$ 4 minutes **25 cycles**

- Use Edge BioSystems Performa spin columns (73328) for reaction purification.
    - Spin at 850g for 3 minutes to remove packing buffer.
        - Do **not** let columns stand dry for more than five minutes.
    - Add reaction product (16-20 µl) to the center of gel column. Do not touch pipette tip to gel column or sidewall.
    - Spin at 850g for 3 minutes to elute purified product.
    - Transfer to 8-tube PCR strips.
    - Submit to Harvard's MCB Sequencing Facility for analysis.

Sequence Analysis

- Analyze sequence with BLAT (http://genome.ucsc.edu/).
- Use Entrez-Gene to research alternate gene names.
- Use chromosome numbers to quickly identify suspected mismatches.
- Three possible outcomes of research are **match**, **no data**, and **mismatch**.

- **Match**: record anti-sense primer (e.g. T7, T3, SP6). This indicates the RNA polymerase to be used in the probe synthesis reaction.
- **No data**: Scrambled sequence information, a high frequency of null nucleotides, or no sequence data. The sequencing reaction may have failed, the plasmid DNA may be poor quality, or the plasmid may be cross-contaminated.
- **Mismatch**: positive identification different from the intended clone, indicating three possible sources of error:
    - In most cases the "mismatch" is a correct identification of the DNA. The clone source provided the wrong clone and a new source must be found.
    - In rare cases signal "bleed-through" of the sequencing machinery provides a weak signal when actual plasmid information is absent. Typically, the weak signal will match the identity of an adjacent plasmid. The plasmid contains little or no genetic information.

Error Procedure: If "no data" and it is suspected that the sequencing reaction failed (e.g. most results from one batch are "no data"), repeat sequencing reaction. If otherwise "no data" or "mismatch," re-perform purification and sequencing procedure with remaining two colonies from master plate.

Bacteria Storage

- Sequencing data **MUST** match supposed identity for permanent storage.
- Re-pick original colony from master plate with a pipette tip.
- Inoculate into 2 ml of 8% glycerol/LB/ampicillin in a 15 ml conical.
- Grow overnight at 37°C with agitation.
- Transfer to 2 ml screw-top vial (Sarstedt 72.694.006) and store at -80°C.

Probe Template PCR

Procedure generates DNA template for synthesis of riboprobe. We have experimented with various annealing temperatures; 46^{o}C yields robust product with high specificity.

- Use a 96-well PCR plate with cover.
- Dilute purified plasmid to 1.0-5.0 ng/µL (typically 1:100).
- Keep enzyme and plate on ice; mix the following:

Reagent	Volume (µl)	Source
10x Qiagen TAQ Buffer	5.0	Qiagen*
10 mM dNTPs	1.0	
10 µM T7/T3/SP6 primer	2.5	Invitrogen
10 µM T7/T3/SP6 primer	2.5	Invitrogen
Sterile water	36.75	
Qiagen TAQ	0.25	Qiagen 201443
Purified plasmid (1.0-5.0 ng/µL)	2.0	

*Provided with enzyme.

- Add one drop of sterile, filtered mineral oil to each well.

PCR conditions: 94^{o}C 3 minutes **1 cycle**

94^{o}C 40 seconds
46^{o}C 40seconds **35 cycles**
72^{o}C 2 minutes

72^{o}C 10 minutes **1 cycle**

- Analyze 7.5 µl by electrophoresis on a 1.0% agarose/TAE gel.
- One bright distinct band indicates robust product. Compare to control product.

Error procedure: If PCR fails to yield ample product, verify primer information and re-try reaction one time. If PCR fails again, clone should be alternatively sourced.

Digoxigenin-Labelled Riboprobe Synthesis from Template PCR

- Mix at room temperature in a 1.6 ml Eppendorf tube:

Reagent	Volume (µ1)	Source
0.1M DTT (freshly dilute in sterile water from 1M)	2.0	Roche 708992
10x Transcription Buffer	2.0	Roche*
RNAse Inhibitor (40 U/µ1)	0.3	Roche 799017

SP6, T7, or T3 RNA polymerase (10 U/µ1)	1.0	Roche±
10x Dig Labeling Mix (40 U/µ1)	2.0	Roche 1277073
Template PCR product	12.7	

*Provided with enzyme.
±SP6 Polymerase: Roche 10810274001. T7 Polymerase: Roche 881775. T3 Polymerase: Roche 1031171.

- Warm all reagents to room temperature before mixing. Ensure that 10x buffer is fully dissolved (requires vortexing).
- Incubate at 37°C for 2 hours.
- Add 2 µl DNase I (Roche 776785), mix.
- Incubate at 37°C, 15 minutes.
- Add 40 µl sterile water.
- Purify with Micro Bio-Spin 30 Columns (BioRad 732-6251).
- Analyze 1.5 µl of product by electrophoresis in 1% agarose/TAE gel. Ideal product shows distinct bands. Minimal band diffusion is acceptable.
- Dilute a sample of the probe 1:50 (2 µl DNA in 98 µl sterile water) and quantify with the spectrophotometer.
- Dilute to 20x (10 ng/µl) in **pre-hyb solution.**

    - **Pre-hyb solution:** 50% formamide, 5x SSC pH 4.5 (citric acid to pH, Sigma S-8015), 50 ug/ml yeast tRNA (Invitrogen 15401-011), 1% SDS, 50 µg/ml heparin (Sigma H8514).

Note: We tested a more stringent **pre-hyb solution** based on Henrique laboratory protocol but found no difference in appearance or timing of strong, average, and weak expression patterns (tested with Hmg1, Tcn2, Nr3b3, Nr1f3, and Tcea3 probes).

- Store at –80°C in 2 ml screw-top vial (Sarstedt 72.694.006).

Error procedure: Extensive band smearing on RNA gel indicates RNAse activity, and the absence of a band indicates a failed reaction. Re-perform reaction once. A low initial concentration does **not** indicate a failed reaction. Typical OD range is 100 to 400 ng/µl.

Manual Cloning

This section describes the methodology of selecting a gene region appropriate for *in situ* hybridization riboprobe construction. This methodology is based on the collective experience of the lab in designing probes. The goal is to select the appropriate region and design primers for amplification in an RT-PCR reaction.

Primer Design

The complete RefSeq file is imported into VectorNTI to include all annotation. The file is named by official gene symbol and RefSeq accession number (e.g. Hoxa11-NM_010450). This provides a permanent link to accession numbers for all the probes in case the embedded annotation is lost.

- Use coding regions for probe design, resulting in better probes than using 3' UTR.
- Probe length should be around 750bp and should fall between 500bp and 1000bp.
- Primer annealing temperatures should be 58-62°C for design purposes.
- The T7 promoter sequence should be included in the 3' primer for direct synthesis without cloning (this would therefore make the anti-sense probe with T7). This allows one to synthesize and use the probe immediately and then clone only those that are deemed useful.

Note: If highly conserved sequences are deemed a problem, attempt to use a region unique from other gene family members. The *in situ* protocol uses stringent conditions (70°C with formamide washes and an RNAse wash) so some sequence overlap is tolerated.

Cloning

- We primarily use a the pCR8/GW/TOPO vector from Invitrogen though other TOPO vectors are acceptable.
- Follow the manufacturer's protocol using 4ul of RTPCR product for TOPO ligation. We plate 100ul of the diluted transformation after 1 hour of growth.

Quality Control

- At this point the clones enter the same quality control system already established for the commercially purchased.
- Three colonies instead of one are initially mini-prepped (as correct sequence yield decreases with manually cloned bacteria).
- A single clone confirmed correct by sequence analysis is then entered into the permanent archive and a glycerol stock is made (refer to main protocol).