

Eurexpress II - SOP “*In Vitro*-Transcription”

(Preliminary version 4 Feb 2005)

Note: This is a preliminary version of the Eurexpress II SOP for riboprobe production from templates. A section about the use of the Agilent Bioanalyzer as an additional quality control (where available) will be added later.

For questions and comments, please contact:

Axel.Visel@mpihan.mpg.de

Ana.Martinez-Hernandez@mpihan.mpg.de

1 Materials

- DIG RNA Labelling Mix (Roche, Cat. No 11 277 073 910)
- Ribonuclease inhibitor (Fermentas, Cat. No. E00311)
- RNA polymerases (*suitable polymerases are also available from other manufacturers*)
 - Sp6 (New England Biolabs, Cat. No. M0207S)
 - T7 (New England Biolabs, Cat. No. M0251S)
 - T3 (Stratagene, Cat. No. 600112-51)
- 10x transcription buffer (supplied with the RNA polymerase enzyme)
- DNase I, RNase-free (Roche, Cat. No. 776785)
- 1.5ml safe-lock, RNase-, DNase-, ATPase-free microtubes (Eppendorf Biopur 0030 121.589)
- 0.3M MgCl₂ (it is practical to prepare a 3M stock solution and dilute 1:10 before using)
- 4M NH₄Ac, autoclaved (keep at –20°C)
- 100% Ethanol (keep at –20°C)
- 70% Ethanol (keep at –20°C)
- DEPC-treated water

2 Procedure

2.1 Preparations

- i. Turn on a water bath and set to 37°C.
- ii. Turn on a heat block and set to 50°C.
- iii. Ethanol clean lab bench, and set of pipettes to be used.
- iv. Arrange DNA templates according to their anti-sense primer (i.e. Sp6)
- v. Vortex all *In-Vitro* transcription reagents (except enzymes!) and pulse-spin to bring contents to bottom of tubes
- vi. Heat the transcription buffer to 50°C if necessary (if DTT has precipitated).

2.2 *In vitro* transcription

1. Prepare per reaction:

	Vol.
H ₂ O (DEPC-treated)	Add to 20 µl
10x transcription buffer	2 µl
10x DIG RNA labelling mix	2 µl
Rnase Inhib.	1 µl
RNA Polymerase (T3, T7 or SP6)	Sp6 (1µl) <u>or</u> T3 (1µl) <u>or</u> T7 (0.7µl)
Template *	0.5 µg

* Use 0.5µg of DNA if using PCR product, or 1µg if using linearized plasmid.

2. Incubate at 37°C for 2.5 hrs.
3. In the meantime, prepare the Stop solution as follows:

Stop Solution:

H ₂ O (DEPC-treated)	16.4 µl
MgCl ₂ (0.3 M)	1.6 µl
DNase I (10 U/µl)	2.0 µl

4. Add 1µl Stop solution. This stops IVT and removes the DNA template.
5. Incubated for 15 min at 37°C.

2.3 Precipitation of RNA

1. Add 72 μl ice-cold NH_4Ac (4 M, autoclaved) and 470 μl ice-cold 100% EtOH to precipitate the nucleic acids. Make sure to use RNase-free solutions only.
2. Vortex samples briefly and place at -80°C for 20 min.
3. Centrifuge at max. speed in a table-top microcentrifuge (e.g. 13,000 rpm) at 4°C for 20 min. It is always a good practice to position the tubes in a consistent manner in the centrifuge rotor (i.e. hinges out), so that you will know where the pellet should be even if it is not visible.
4. Carefully remove the supernatant with a pipette, making sure not to disturb the pellet.
5. Wash the pellet with 640 μl 70% EtOH.
6. Centrifuge at max. speed at 4°C for 20 min.
7. Dry the pellet in a vacuum centrifuge for approx. 6 min. The pellet should be dry, but do not dry longer than necessary (pellet may become difficult to re-suspend).
8. Re-suspend the pellet with 22 μl DEPC- H_2O and shake it (horizontal shaker) at 1150 rpm for 15 min (room temperature).

2.4 Quality control and quantitation

1. Prepare samples for loading on a 1% agarose gel as follows:
 - a) aliquot into clean 0.2 ml tubes 4.0 μl depc- H_2O + 1 μl probe and denature for 5 min at 70°C .
 - b) chill on ice for approx. 3 min, and add 1 μl 6x loading buffer.
2. Load on a 1% agarose gel and run an electrophoresis (suitable voltage and time will depend on your electrophoresis unit). Include a size marker and keep a photograph of the gel for documentation purposes. Ideally, one single strong band should be visible per lane. However, often times an upper faint band is also present. Smearing from the bottom up is a sign of total degradation (in most cases the main band is very faint or not even present). Smearing from the main band down is a sign of partial degradation. Unfortunately, an agarose gel cannot yield much information about the integrity or purity of a riboprobe. For more accurate information, samples can be injected into a bioanalyzer (if at all available).

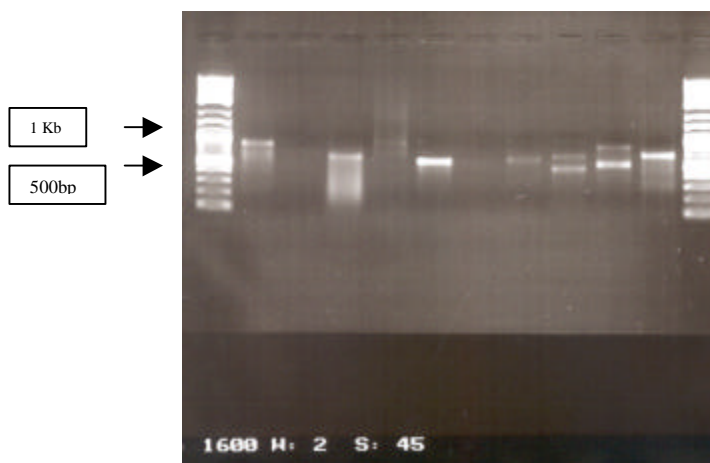
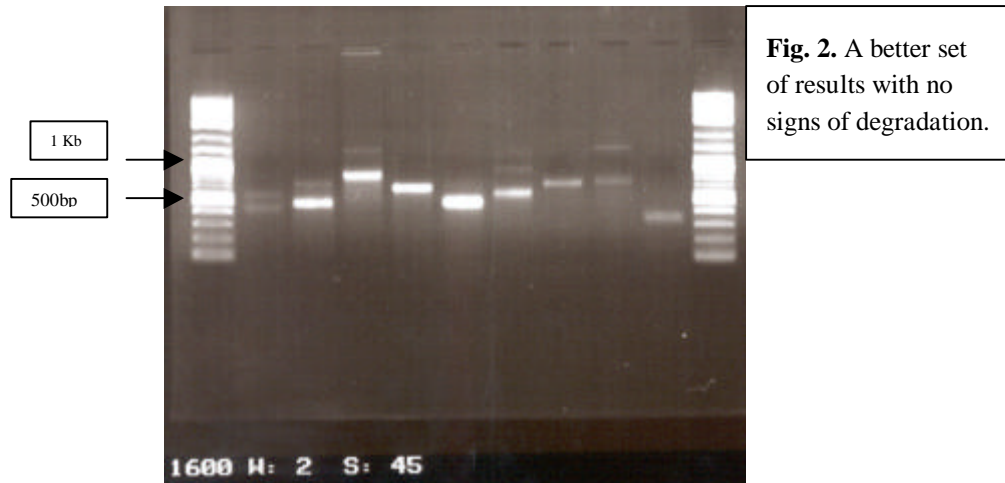


Fig. 1. Riboprobe #1 shows starting of degradation. #2, #4 and #6 are completely degraded. #3 seems partially degraded; however, after several trials with the same result, analysis of the covered region showed sequence-base problems (namely, a string of Cs). All other samples are acceptable.



3. Use 1 μ l of probe to make a dilution and determine the probe concentration using a spectrophotometer ($A_{260/280}$). For example, use a 1:100 dilution (1 μ l probe + 99 μ l Tris-buffer).
[ng/ μ l] = (A_{260})x(dilution factor)x(40). Concentrations between 500 – 850ng/ μ l are usual.
4. Adjust the volume to 150 ng/ μ l and store in 20 μ l aliquots.
5. Store at -80°C (stable up to 1 year).

Prior to use for *in-situ* hybridization, riboprobes are further diluted to a concentration of 30 ng/ μ l with hybridization-mix and stored at -20°C for a maximum of 2 months.