Protocol

Competitive RT-PCR: Preparation of Competitor RNA

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INTRODUCTION

The first step in competitive RT-PCR is the synthesis and purification of the synthetic competitor. This is an RNA molecule designed to be reverse-transcribed and PCR-amplified with the same efficiency as the endogenous transcript of interest. Once the competitor molecule has been prepared, as described in this protocol, competitive PCR can be carried out, as described in Competitive RT-PCR: Estimation of Copy Number.

MATERIALS

Reagents

Ethanol, 70% and 100%

DEPC-treated H₂O

DNA template (0.5-1.0 µg of linearized plasmid template or 0.1-0.5 µg of PCR template, see Steps 1 through 4 for template design and preparation)

[^32P]ATP, 10 mCi/ml (see Radioactive Substances)

T7 RNA polymerase (e.g., Ambion)

Transcription buffer 10X (as supplied by the enzyme manufacturer)

rNTP solution (2.5 mM GTP, ATP, CTP, and UTP)

DNase I, RNase-free, 5 units/µl (e.g., Ambion)

RNA gel loading buffer II

Probe elution buffer

SUPERaseIn RNase inhibitor, 20 U/µl (Ambion)

Equipment

Equipment for polyacrylamide gel electrophoresis and autoradiography (see Preparation of Denaturing Polyacrylamide Gels, Loading and Running DNA-Sequencing Gels, and Autoradiography and Reading of Sequencing Gels).

Equipment for Isolation of DNA Fragments from Polyacrylamide Gels by the Crush and Soak Method

Equipment for scintillation counting

Incubator, preset to 37°C
Heating block, preset to 95°C

METHOD

Competitor Design
1. Design the competitor RNA to include elements for efficient transcription by T7 RNA polymerase, as well as regions homologous to the endogenous target. The inclusion of a GCG clamp (or a restriction site for cloning purposes) on the 5' end of the forward primer may stabilize the promoter domain and provides increased transcription efficiency. Include 10 nucleotides 3' of the T7 promoter to ensure that transcription is initiated at the correct nucleotide. These 10 nucleotides must include only G and A residues.

2. Include two 18-20 nucleotide sequences that are homologous to the endogenous target. To facilitate separation of the competitor and target by gel electrophoresis in later steps, these two regions should flank a 10% deletion of the target. The total length of this primer should be approximately 70 nucleotides.

3. Place the downstream primer (3' reverse) 50 nucleotides or more downstream of the reverse primer-binding site used in the competitive RT-PCR experiment.

These extra sequences are necessary to ensure equivalent transcription of the competitor construct and the endogenous transcript.

4. Amplify the competitor construct by PCR to create enough template to use directly in a T7 RNA polymerase transcription reaction, or, if restriction sites have been included in the forward and reverse primers, ligate it into an appropriate vector.

Competitor RNA Synthesis
5. Prepare the transcription reaction at room temperature by assembling the reagents in the following order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC-treated H2O</td>
<td>for a final volume of 20 µl</td>
</tr>
<tr>
<td>10X Transcription buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>rNTP solution (each at 2.5 mM)</td>
<td>4 µl</td>
</tr>
<tr>
<td>DNA template (0.5-1.5 µg plasmid DNA or 0.1-1.0 µg PCR product)</td>
<td>1-5 µl</td>
</tr>
<tr>
<td>[α^{-32}P]ATP (10 µCi/ml)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>T7 RNA polymerase</td>
<td>2 µl</td>
</tr>
<tr>
<td>SUPERaseIn (20 U/µl)</td>
<td>0.5-1 µl</td>
</tr>
</tbody>
</table>

If a plasmid vector is used as the template for transcription, it should first be linearized. Trace amounts of [α^{-32}P]ATP are added to the transcription reaction to aid in the purification and quantitation of the competitor.

6. Flick the tube to mix the reagents, and centrifuge briefly to collect the reaction mixture the bottom of the tube. Incubate the transcription reaction for 2-4 hours at 37°C.

7. Remove a 1-µl aliquot for later use in the determination of the competitor concentration.

8. Add 40 µl of probe elution buffer and 200 µl of ethanol to the transcription reaction. Incubate at -80°C for 20 minutes.

9. Centrifuge at maximum speed in a microcentrifuge at 4°C to precipitate the transcription reaction products.

10. Aspirate the supernatant, wash the pellet with 70% ethanol, and air-dry the pellet.

11. Dissolve the pellet (containing the DNA template and the transcribed RNA) in 16 µl of DEPC-treated H2O
and incubate at 95°C for 2 minutes.

12. Add 2 µl of 10X transcription buffer and 2 µl of RNase-free DNase I (5 units/µl).

13. Flick the tube to mix the reagents, and briefly centrifuge to collect the reaction mixture at the bottom of the tube. Incubate at 37°C for 1 hour.

**Competitor Purification**

14. Add 22 µl of RNA gel loading buffer II to the DNase I reaction and denature the samples at 95°C for 3 minutes.

20. Run the sample on a 6% acrylamide/8 M urea, denaturing polyacrylamide gel until the bromophenol blue approaches the bottom of the gel (see Preparation of Denaturing Polyacrylamide Gels and Loading and Running DNA-Sequencing Gels).

21. Remove one glass plate from the gel, cover the gel with plastic wrap, and locate the full-length transcript by autoradiography (expose to X-ray film for 30 minutes to 2 hours). See Autoradiography and Reading of Sequencing Gels.

22. Develop the film and align it under the gel. Cut the full-length RNA transcript out of the gel with a scalpel or razor blade.

(See Troubleshooting)

23. Purify the RNA from the gel slice using the crush-and-soak method according to Isolation of DNA Fragments from Polyacrylamide Gels by the Crush and Soak Method. Store the RNA at -70°C until needed.

**Competitor RNA Quantitation**

24. Transfer 1 µl of the transcription reaction and 1 µl of the purified RNA competitor to scintillation vials and measure the counts using standard techniques.

25. Use the resulting counts in the following equations to determine the competitor concentration in micromolar or in copies/µl.

\[
\mu\text{M competitor RNA} = \left[\frac{\text{cpm/µl of purified competitor RNA}}{\text{cpm/µl of transcription reaction}}\right] \times [500 \mu\text{M ATP/#A's in the competitor RNA}]
\]

Copies of competitor RNA/µl = \[
\left[\frac{\text{cpm/µl of purified competitor}}{\text{cpm/µl of transcription reaction}}\right] \times [3 \times 10^{14} \text{ molecules of ATP/µl/#A's in the competitor RNA}].
\]

(See Troubleshooting)

26. The competitor RNA should be stored at -20°C at concentrations greater than \(6 \times 10^{10}\) copies/µl or 0.1 µM and should be stable for at least one year.

**TROUBLESHOOTING**

**Problem:** Competitor transcript product is longer or shorter than expected

[Step 22]

**Solution:** If the transcription product of the competitor is only slightly longer than expected, it is most likely because of an undetected cloning problem or salt effect. In the case of a salt effect, the transcription product should be reprecipitated and washed in 70% ethanol. If the transcription product of the competitor is much larger than expected, incomplete digestion of plasmid template may have occurred. Small amounts of supercoiled uncut plasmid can result in large amounts of RNA because of continuous transcription of template. In this case, the plasmid template
should be redigested.

**Problem:** Low yields of RNA competitor transcript

**[Step 25]**

**Solution:** If low quantities of RNA competitor template are obtained, first increase the amount of template in the T7 RNA polymerase reaction (up to 2.5 µg). Also, confirm that there are no C or U bases in the first 10 nucleotides after the T7 promoter. The presence of these bases in this position will decrease the efficiency and yield of the competitor RNA transcription reaction. The addition of 1-3 mM final concentration of MnCl₂ to the transcription reaction, and increasing the incubation time to 8-16 hours, may aid in increasing the yield. To prevent precipitation of DNA, MnCl₂ should not be added to a cold reaction tube. Instead, first heat the transcription reaction to 37°C for 2 minutes. Poor yields may also be obtained from GC-rich transcription templates. In this case, the addition of single-stranded binding protein (2.6 µg/µg of template) may increase transcription efficiency.

**ACKNOWLEDGMENTS**

I thank Ellen Prediger, Lori Martin, and Heath Thomas for supplying materials used in this protocol. I also thank James W. Horner II for critical reading of the manuscript.

**REFERENCES**


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**Caution**

**Radioactive substances**

Radioactive substances: When planning an experiment that involves the use of radioactivity, consider the physico-chemical properties of the isotope (half-life, emission type, and energy), the chemical form of the radioactivity, its radioactive concentration (specific activity), total amount, and its chemical concentration. Order and use only as much as needed. Always wear appropriate gloves, lab coat, and safety goggles when handling radioactive material. X rays and gamma rays are electromagnetic waves of very short wavelengths either generated by technical devices or emitted by radioactive materials. They might be emitted isotropically from the source or may be focused into a beam. Their potential dangers depend on the time period of exposure, the intensity experienced, and the wavelengths used. Be aware that appropriate shielding is usually made of lead or other similar material. The thickness of the shielding is determined by the energy(s) of the X rays or gamma rays. Consult the local safety office for further guidance in the appropriate use and disposal of radioactive materials. Always monitor thoroughly after using radioisotopes. A convenient calculator to perform routine radioactivity calculations can be found at: [http://www.graphpad.com/calculators/radcalc.cfm](http://www.graphpad.com/calculators/radcalc.cfm).

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**Caution**

[γ-³²P]ATP

[γ-³²P]ATP, see Radioactive substances
Recipe

**Diethyl pyrocarbonate (DEPC)-treated H\_2O**

Distilled H\_2O

Add 1 mL of fresh DEPC to 1 L of H\_2O. Shake well to disperse the DEPC through the H\_2O. Incubate at 37°C for at least 12 h and/or autoclave at 15 psi on liquid cycle for 20 min to inactivate the remaining DEPC.

Recipe

**Probe Elution Buffer**

0.5 M ammonium acetate

0.02% SDS

1 mM EDTA

Recipe

**RNA gel loading buffer II**

95% deionized formamide

0.025% (w/v) bromophenol blue

0.025% (w/v) xylene cyanol FF

5 mM EDTA

0.025% (w/v) SDS

Recipe

**rNTP**

Dissolve each NTP (ribonucleoside triphosphates) in H\_2O at an approximate concentration of 100 mM. Use 0.05 M Tris base (or 0.1 N NaOH as appropriate) and a micropipette to adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized NTP appropriately, and read the optical density at the wavelengths given in the table below. Calculate the actual concentration of each NTP. Dilute the solutions with H\_2O to a final concentration of 50 mM NTP. Store each separately at -70°C in small aliquots.

<table>
<thead>
<tr>
<th>Base</th>
<th>Wavelength (nm)</th>
<th>Extinction Coefficient (E)(M^{-1}cm^{-1})</th>
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<tbody>
<tr>
<td>A</td>
<td>259</td>
<td>1.54 x 10^4</td>
</tr>
<tr>
<td>G</td>
<td>253</td>
<td>1.37 x 10^4</td>
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</tbody>
</table>
For a cuvette with a patch length of 1cm, absorbance = EM.

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<tbody>
<tr>
<td>C</td>
<td>271</td>
<td>$9.10 \times 10^3$</td>
</tr>
<tr>
<td>T</td>
<td>267</td>
<td>$9.60 \times 10^3$</td>
</tr>
</tbody>
</table>