Quantitation of Specific Transcripts by RT-PCR SNUPE Assay

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The single nucleotide primer extension assay (SNUPE) provides a means of identifying and quantifying allelic variants in DNA and RNA.^(1,2) It has many uses, including the measurement of specific RNA levels, as well as the study of allelic variation and allele-specific expression. The SNUPE assay is done after amplification by PCR of a segment containing the allelic difference, which may be even a single nucleotide. The assay consists of the enzymatic extension by 1 base of a primer just 5' to the position of mismatch in the presence of a [³²P]dNTP specific for either the wild-type or variant sequence. The ratio of radioactivity in the primer products of these two reactions is then determined after denaturing gel electrophoresis.

A major advantage of the method is its usefulness for quantitative measurement over a wide range. The method allows detection of a given transcript in up to 1000-fold excess of RNA from the other allele, depending on which nucleotides differ. The method can be readily adapted for quantitation of absolute amounts of a specific transcript by addition of an internal standard.⁽³⁾ The protocol described here is the one we use for quantitation of the ratio of *Pgk-1a/Pgk-1b* transcripts in total RNA.

REAGENTS

Reagents are as follows:

1. 10× RT-PCR buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl). 25 mM MgCl₂.
2. 10× SNUPE buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM MgCl₂, 0.01% gelatin).⁽²⁾
3. TE (10 mM Tris-HCl, 1 mM EDTA at pH 8.0).
4. RNasin, 40 U/μl (Promega, Madison, WI).
5. Moloney murine leukemia virus reverse transcriptase, 200 U/μl (BRL, Gaithersburg, MD).
6. AmpliTaq polymerase 5 U/μl (Cetus, Norwalk, CT).
7. Gelase, 1 U/μl (Epicentre Technologies, Madison WI).
8. Upstream and downstream primers yielding an amplified RT-PCR product, which includes the region of mismatch (20 μM).
9. SNUPE primer, 18-mer or longer, with the 3' end just 5' to mismatched base (20 μM).⁽²⁾
10. 2.5 mM dNTPs diluted in H₂O from 100 mM stock solutions (Boehringer Mannheim, Indianapolis, IN).
11. [³²P]dNTPs 3000 Ci/mole, corresponding to the mismatched bases, diluted to 2 μCi/μl in H₂O just before incubation.
12. 20 mg/ml of mussel glycogen (Sigma, St. Louis, MO).
13. Phenol.
14. Chloroform-isoamyl alcohol (24:1).
15. 7.5 M ammonium acetate.
16. Low melting agarose, ultra pure (BRL).
17. 15% polyacrylamide-7 M urea gel. Ethanol.

PROTOCOL

RT-PCR⁽⁴⁾

1. Mix together at 4°C in a final volume of 20 μl: 1–100 ng of RNA, 1× RT-PCR buffer, 5 mM MgCl₂, 20 units of RNasin, 1 μM downstream primer, 1 mM each dNTP, and 50 units of reverse transcriptase. (For more than one reaction, master mix containing all reagents may be used).
2. Incubate for 15–30 min at 42°C.

3. Heat to 99°C for 5 min.
4. Cool to 50°C for hot start.⁽⁵⁾
5. To each tube add 80 μ l of a prewarmed master mix containing RT-PCR buffer, MgCl₂, upstream primer, and AmpliTaq polymerase, so that the final reaction contains, in a volume of 100 μ l, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2 mM MgCl₂, 200 μ M dNTPs, 0.2 μ M upstream and downstream primer and AmpliTaq polymerase, 2.5 units per reaction.
6. Add 100 μ l of mineral oil to each tube.
7. Carry out PCR in a thermal cycler for a sufficient number of cycles to give a strong band on an ethidium bromide-stained gel after amplification.

Purification of AmpliTaq Product

1. Extract RT-PCR products with phenol and chloroform/isoamyl alcohol, and precipitate with ethanol after addition of ½ volume 7.5 M ammonium acetate.⁽⁶⁾ Add glycogen, 20–40 μ g, if needed to obtain a visible pellet.
2. After resuspension of the pellet in TE, run samples on low melting agarose gel, and extract with Gelase, following instructions of the manufacturer.
3. Precipitate with ½ volume of 7.5 M ammonium acetate and 2 volumes of ethanol. Add glycogen, 20–40 μ g, as a carrier if needed.
4. Resuspend the DNA in 10–20 μ l of TE, and estimate the concentration of an aliquot on an ethidium bromide-stained gel.

Quantitative SNUPE Assay^(1,2)

1. To each of two tubes, add 10 ng of template DNA, 1 $\times$ SNUPE buffer, 1 μ M SNUPE primer, 0.75 units of *Taq* polymerase, and 2 μ Ci of the [³²P]dNTP specific for one allele or the other (final volume, 10 μ l).
2. Incubate the samples for one round of denaturation, annealing and synthesis (example: 95°C for 1 min, 42°C for 2 min, 72°C for 1 min).
3. As controls for background and maximum incorporation, incubate 10 ng of amplified product from each allele with the “incorrect” and appropriate [³²P]dNTP, respectively.
4. Electrophorese the samples on a 15% polyacrylamide–urea gel.⁽⁶⁾
5. Determine the amount of radioactivity in each ($n + 1$) band (n = length of SNUPE primer). Occasionally, the base following the incorporated [³²P]dNTP is identical to it, and one sees ($n + 2$) products. In that case, the contribution of both ($n + 1$) and ($n + 2$) products should be included.
6. After subtraction of background, the signal obtained with the [³²P]dNTP is then proportional to the relative amount of each allelic transcript. The background varies with different mismatches. We have found measurement of C in an excess of A (C/A) to be sensitive down to the 0.001% level; other mismatches giving a low background level include C/T, T/C, and A/G. G/C, C/G, and G/A mismatches give a background of ~1%.

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