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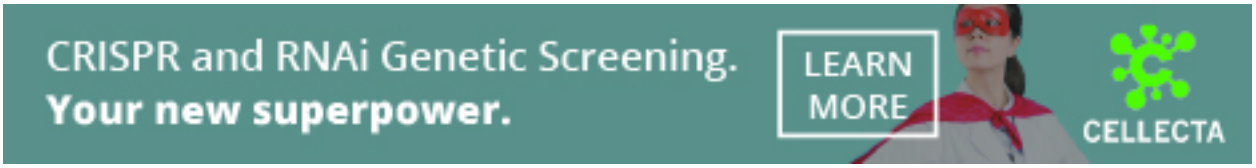

Quantitation of Specific Transcripts by RT–PCR SNuPE Assay

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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 [^{32}P]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 $MgCl_2$.
2. 10× SNuPE buffer (100 mM Tris-HCl at pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 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 H_2O from 100 mM stock solutions (Boehringer Mannheim, Indianapolis, IN).
11. [^{32}P]dNTPs 3000 Ci/mmole, corresponding to the mismatched bases, diluted to 2 μCi/μl in H_2O 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 $MgCl_2$, 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.

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3. Heat to 99°C for 5 min.
4. Cool to 50°C for hot start.[5]
5. To each tube add 80 μl of a prewarmed master mix containing RT–PCR buffer, $MgCl_2$, upstream primer, and AmpliTaq polymerase, so that the final reaction contains, in a volume of 100 μl, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM upstream and downstream primer and AmpliTaq polymerase, 2.5 units per reaction.
6. Add 100 μl 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 ethidum 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 μg, 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 μg, as a carrier if needed.
4. Resuspend the DNA in 10–20 μl of TE, and estimate the concentration of an aliquot on an ethidum bromide-stained gel.

Quantitative SNuPE Assay[1,2]

1. To each of two tubes, add 10 ng of template DNA, 1× SNuPE buffer, 1 μM SNuPE primer, 0.75 units of *Taq* polymerase, and 2 μCi of the [^{32}P]dNTP specific for one allele or the other (final volume, 10 μl).
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 [^{32}P]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 [^{32}P]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 [^{32}P]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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