

Benchmarks

5' RACE by Tailing a General Template-Switching Oligonucleotide

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Rapid amplification of cDNA ends (RACE) is a convenient way to isolate a specific cDNA without building a phage library. RACE kits are available commercially but are expensive and may not capture rare transcripts. We describe a method for isolating 5' RACE clones based on Step-out PCR (7) using Molony murine leukemia virus reverse transcriptase (MMLV-RT) and controlled ribo-tailing (8). MMLV-RT (e.g., SUPERSCRIPT™ II; Life Technologies, Rockville, MD, USA) can add a few nontemplate nucleotides (generally 3 or 4 Cs) to the 3' end of newly synthesized first-strand cDNAs (1,7,9). This terminal transferase activity is strongly dependent on the 5' end of mRNA (1,9). Furthermore, MMLV-RT has "template switching" activity, meaning that it can switch from transcribing a strand of mRNA to transcribing an appropriate oligonucleotide, thereby extending cDNA synthesis to include a specific sequence (1,10). The template switching oligonucleotide (TSO) is designed to be complementary to the newly added Cs on the 3' end of the first-strand cDNA and can have PCR priming and restriction endonuclease sites. These properties of MMLV-RT make it possible to engineer a common priming site at the 5' end of a collection of first-strand cDNAs. A general primer compatible with the TSO is used with gene-specific primers (GSPs) to amplify 5' cDNA ends. Based on these features of MMLV-RT, protocols such as Step-out (7) and CapSelect (9) PCR have been developed for 5' RACE.

For CapSelect PCR, the first-strand cDNA is synthesized, tailed with ribonucleotide and then ligated to an adaptor oligonucleotide. Step-out PCR exploits the template switching activity of MMLV-RT by adding the TSO to the first-strand cDNA synthesis reaction. Although using a single step to create cDNAs with a common 5' end is efficient, there is a practical difficulty with Step-out PCR. To get template switch-

ing, the TSO has a ribo-G tail that can base pair with the newly added Cs on the first-strand cDNA. The TSO in commercial RACE kits typically contains three ribo-Gs that can rescue only a portion of first-strand cDNAs because MMLV-RT terminal transferase activity is not uniform (9). We describe a method of ribo-tailing custom DNA oligonucleotides to generate TSOs that are suitable for use in Step-out PCR. Nonspecific amplification was suppressed using a carrier oligonucleotide (7).

The *Aspergillus nidulans* cell morphogenesis gene, *hypA*, was identified by Kaminskyj and Hamer (5). The genomic region of *hypA* contains two open reading frames (ORFs) with a possible intron between them (GenBank® accession no. AF001273). To clarify this, *hypA* cDNA was screened for in three cDNA libraries, but this was unsuccessful, which was consistent with *hypA* having a 5.4-kb low-abundance message (not shown). We decided to isolate *hypA* cDNA clones using 5' RACE. RNA was isolated from *A. nidulans* vegetative mycelium using Tri Reagent® (Molecular Research Center, Cincinnati, OH, USA) or Oligotex™ (Qiagen, Valencia, CA, USA) and treated with DNase I (Amersham Pharmacia Biotech, Piscataway, NJ, USA). 5' RACE, using two different commercial kits, did not give a clonable product, even with recently devised optimization strategies (e.g., see Reference 6). We reasoned that rare messages might be amplified if we modified aspects of the RACE procedure as described below.

We adapted the controlled ribonucleotide tailing method of Schmidt and Mueller (8) using terminal deoxynucleotidyl transferase (TdT) and rGTP to ribo-tail, a custom DNA oligonucleotide (5'-TTACCACTCATGCTTCGGAGACTAGCG-3'), creating the TSO. When designing the TSO, it was important to ensure that its 3' end could not form a hairpin loop that would reduce ribotailing efficiency. Ribo-tailing with TdT is self-limited (mostly 2–4 nucleotides) and greater than 98% efficient (7). A collection of TSOs tailed with different numbers of ribo-Gs is more likely to rescue cDNAs that have three or four Cs added by MMLV-RT. Ribo-tailing used a 25- μ L reaction containing 15 U TdT (Life Technologies), 1 \times original TdT

buffer [5 \times buffer: 0.5 M potassium cacodylate (pH 7.2), 10 mM CoCl₂, 1 mM DTT] or self-made TdT buffer [10 \times buffer (3): 0.1 M Tris-HCl (pH 7.2), 0.5 M KCl, 15 mM MgCl₂, 0.4 mM rGTP, 25 pmol custom oligonucleotide and 10 U SuperASE-IN™ RNase inhibitor (Ambion, Austin, TX, USA). The mixture was incubated at 37°C for 30–60 min, and then the TdT was inactivated at 65°C for 15 min.

First-strand cDNA synthesis used SUPERSCRIPT II essentially as described by the manufacturer, except that MgCl₂ concentration was raised to 6 mM from the original 3 mM in supplied buffer. Briefly, 10 pmol gene-specific 5' *hypA* cDNA synthesis primer (CSP; 5'-CACCTCACAGGAGAACG-3') was mixed with 5 μ g RNA, and the volume was brought to 10 μ L with water. After denaturing at 70°C for 10 min, the mixture was chilled on ice for 5 min, and the condensate was collected by a brief centrifugation for 1–2 s at 1000 rpm. We added 4 μ L 5 \times first-strand buffer, 1 μ L 0.1 M DTT, 1 μ L dNTP mixture (10 mM each dNTP; Life Technologies), 1 μ L TSO (10 pmol/ μ L), 1 μ L 60 mM MgCl₂ and 1 μ L SuperASE-IN. These were gently mixed and incubated at 42°C for 2 min before adding 1 μ L SUPERSCRIPT II (200 U). cDNA synthesis

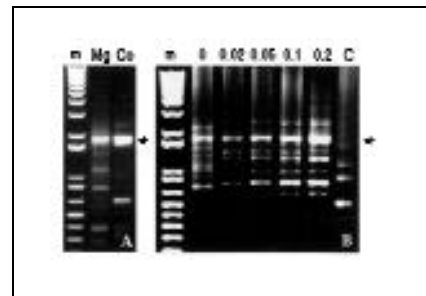


Figure 1. PCR amplification of the 5' end of *A. nidulans hypA* cDNA. (A) Comparison of template switching oligonucleotides that were ribo-tailed in Mg²⁺-containing buffer (Mg), or in Co²⁺-containing buffer, which was desalted before use (Co), on first-round 5' RACE PCR. To reduce nonspecific amplification, 0.02 μ M PA5-CO was added to each reaction. Arrow represents the expected size of the target band; m, 1-kb Plus DNA ladder (Life Technologies). (B) Optimizing amplification of the target fragment in second-round PCR using 0–0.2 μ M PA5-CO. Best amplification of the target fragment was obtained using 0.2 μ M PA5-CO (lane 0.2). C, control using an unrelated gene-specific primer, lacks the target band; m, 1-kb DNA ladder (Life Technologies); arrow, expected size of target band.

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was allowed to proceed at 42°C for 60 min and then was stopped by a 10-min heat inactivation at 70°C. This reaction was diluted fivefold in water before PCR amplification.

TSO made with either kind of TdT buffer (original with Co²⁺ or self-made with Mg²⁺) worked well for cDNA synthesis (not shown), but they did not perform equally in 5' RACE. Co²⁺ inhibits *Taq* DNA polymerase (Reference 3 and this study, not shown), whereas cDNA from TSO ribo-tailed in Mg²⁺-TdT buffer resulted in higher nonspecific amplification. For optimum results, we used a TSO that had been ribo-tailed in Co²⁺-containing buffer and desalted before cDNA synthesis (Figure 1A). The Co²⁺-buffered tailing reaction was transferred to a Microcon YM-3 (3000 Da mwco; Millipore, Mississauga, ON, Canada) and centrifuged at 14000× *g* until less than 10 μL retentate remained, approximately 1 h. The

retentate was washed twice by adding 600 μL RNase-free water to the YM-3, and spinning again until about 10 μL remained, approximately 2 h per wash. The final product was brought up to 25 μL with RNase-free water.

5' RACE amplification used touch-down PCR (2,4) with slight modifications. A 50-μL PCR contained 1.25 U Platinum *Pfx* DNA polymerase (Life Technologies), 1× *Pfx* amplification buffer, 1 mM MgSO₄, 0.2 mM each dNTP, 0.2 μM general primer, PA5 (5'-GCTCTAGAGCGGGTGAGCATGA-3'), 0.2 μM GSP1 (5'-GCCCTCTTCTTGTCGATAC-3') for first-round PCR or 0.2 μM GSP2 (5'-GGTACACCTCGCTCTCAAG-3') for second-round PCR. One microliter of fivefold diluted first-strand cDNA or tenfold diluted first-round PCR product was used as template. Suppression of nonspecific amplification in first-round PCR used 0.02 μM of the carrier oligonu-

cleotide (7), PA5-CO (5'-GCTCTAGAGCGGGTGAGCATGATTACCACTCATGCTTCGGAGACTA-3'). Various concentrations of PA5-CO were tested for second-round PCR (Figure 1B). To prevent degradation of PA5, PA5-CO, GSP1 and GSP2 by the proofreading activity of Platinum *Pfx* DNA polymerase, these primers were modified by having the 3' nucleotide attached with a nonhydrolyzable phosphorothioate ester (Cortec Laboratories, Kingston, ON, Canada).

Our 5' RACE amplification touch-down regime was one cycle of 2.5 min at 94°C; one cycle of 0.5 min at 92°C and 3 min at 70°C; two cycles of 0.5 min at 92°C and 3 min at 68°C; three cycles of 0.5 min at 92°C, 1 min at 67°C and 3 min at 68°C; four cycles of 0.5 min at 92°C, 1 min at 66°C and 3 min at 68°C; five cycles of 0.5 min at 92°C, 1 min at 65°C and 3 min at 68°C; four cycles of 0.5 min at 92°C, 1 min at

64°C and 3 min at 68°C; three cycles of 0.5 min at 92°C, 1 min at 63°C and 3 min at 68°C; twenty cycles of 0.5 min at 92°C, 1 min at 65°C and 3 min at 68°C, programmed on a Hybaid Omne thermal cycler (InterScience, Markham, ON, Canada).

First-round amplification using PA5, PA5-CO and GSP1 gave multiple bands, including a doublet that was close to the expected size (Figure 1A, arrow), requiring that we use seminested PCR to isolate the proper band. Using PA5 and GSP2 alone to reamplify the first-round product did not reduce the band complexity. However, using 0.2 µM each PA5-CO, PA5, and GSP2 (Figure 1B, lane 0.2), the expected 1.8-kb band (Figure 1B, arrow) was strongly amplified. Comparison with lower concentrations of PA5-CO (Figure 1B, lanes 0.02–0.1) showed that this was a single band rather than the doublet seen after first-round PCR (Figure 1A). This product was gel-purified and cloned and confirmed by restriction enzyme analysis.

Sequencing showed that *hypA* contains a 57-bp in-frame intron, which removes the two stop codons after message maturation. The *hypA* translation start site is presumed to be the first ATG in the ORF, although this has yet to be confirmed by expression and N-terminal sequencing of the gene product; the predicted peptide has methionine at the first and twentieth residues. Consistent with this, there is a TATAA site at -12 in the genomic sequence and a CCAAT site at -108.

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Corrigendum

In an article published in January (Atienzar, F., A. Evenden, A. Jha, D. Savva and M. Depledge. 2000. Optimized RAPD analysis generates high-quality genomic DNA profiles at high annealing temperature. *BioTechniques* 28:52-54), an error was made in units of concentration. The dNTP concentration in Table 1 should be mM rather than µM.