Increased yield of PCR product from degenerate primers with nondegenerate, nonhomologous 5' tails

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BioTechniques 38:34-38 (January 2005)

PCR amplification of homologous genes using degenerate primers followed by direct sequencing is a standard approach to generate a data set for evolutionary analysis. In our laboratory, we are systematically designing and testing primers that can amplify by reverse transcription PCR (RT-PCR) a wide array of messenger RNA (mRNA) sequences across extant Panarthropoda, a diverse and ancient assemblage of taxa (i.e., tardigrades, onychophorans, crustaceans, hexapods, myriapods, and chelicerates) whose phylogenetic relationships are of interest (1). Primers (17–24 nucleotides in length) are made to protein coding-invariant regions of Basic Local Alignment Search Tool (BLAST)-aligned orthologs from human, fly, and roundworm with complete degeneracy added (typically ≤ 256 -fold) wherever synonymous changes might occur (Regier, Cunningham, Ganley, Shi, and Ball, unpublished data). Since before 1995 (2), we have used bipartite PCR primers that include a 5' tail of nondegenerate, nonhomologous M13REV

or M13(-21) sequence to facilitate direct sequencing of PCR products. In this report, we demonstrate that M13 or other nonhomologous sequences attached to the 5' ends of degenerate primers can also greatly improve the yield of PCR amplification. This finding expands on that of Rose et al. (3,4), who used bipartite primers with degenerate 3' ends (approximately 12 nucleotides in length) and nondegenerate but homologous 5' ends (approximately 20 nucleotides in length) to amplify homologous sequences.

Total nucleic acids were extracted using the SV Total RNA Isolation kit, with the DNase step omitted (Promega, Madison, WI, USA) and used as template for reverse transcription. Ten microliters reverse transcription reactions occurred at 42°C for 35 min and included 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM of each dNTP (optimal dNTP concentration varies with batch), 1 U/μL RNase inhibitor (Applied Biosystems, Foster City, CA, USA), 2.5 U/μL murine leukemia virus (MuLV) RTase (Applied Biosystems), and 4.0

pmol/μL gene-specific reverse primer or 2.5 pmol/μL dT₁₂₋₁₈. Fifty-microliter PCRs occurred in the same tube and included final concentrations of 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.4 mM of each dNTP, 0.025 U/μL AmpliTaq® DNA polymerase (Applied Biosystems), 0.035 μM Taq antibody (BD Biosciences Clontech, Palo Alto, CA, USA), 1.0 pmol/μL forward primer, and 0.4 pmol/μL gene-specific reverse primer (in addition to that already present) or 1.0 pmol/μL gene-specific reverse primer if dT₁₂₋₁₈ had been used for reverse transcription. In one set of experiments, the gene-specific primer concentrations were increased 3.9-fold. Gene-specific primers were made both with and without 5' tails (Tables 1 and 2).

PTC-200™ thermal cycler (MJ Research, Waltham, MA, USA) conditions for PCR were set for Touchdown (5) with the annealing temperature set to decrease uniformly from 55° to 45°C over 25 cycles, followed by 13 more cycles at 45°C. To visualize the PCR products, equal volume aliquots of the RT-PCRs were fractionated by electrophoresis on a 1.4% agarose gel containing 0.5 μg/mL ethidium bromide and 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, approximate pH 8.3). Semiquantification was performed by comparing band intensities over a range of photographic exposures.

Figure 1 is illustrative of our results. Addition of M13REV/M13(-21) 5' tails onto degenerate primers can increase the product yield well over 10-fold, making otherwise invisible bands visible [c.f., +M tails and -tails for 63fin2F_3R (Figure 1, upper panel), 44fin2F_3R (Figure 1, lower panel), and 226fin1F_4R (Figure 1, lower panel)]. This effect is not specific to M13 sequences, as their substitution with T3/T7 tails yields similar results [c.f., +M tails and +T tails for 63fin2F_3R (Figure 1, upper panel)]. Part of the 5' tail effect can be accounted for by the increased length of primers [a correlate of increased melting temperature (T_m)], as evidenced by the approximately 2-fold greater PCR product yield using nondegenerate and perfectly matched gene-specific primers with 5' tails than without [c.f., +M tails and -tails for 44Lpofin2F_5R (Figure 1, lower panel)]. However, a comparable comparison of degenerate primers that bind to the exact same region displays a

Table 1. Gene-Specific Primers and Sequences (Without 5' Tails)

Primer	Sequence (Degree of Degeneracy)
63fin2F	5'-GAYATHGAYTTYGGNCAYATG-3' (192-fold)
63fin3R	5'-ACRAARTARTANGTRTARTCYTG-3' (256-fold)
44fin2F	5'-TTYAAYATGGAYGARTAYGT-3' (32-fold)
44fin3R	5'-CCYTTRAARTAYTTNAC-3' (64-fold)
44fin4F	5'-AGYTAYCAYHMYTWYATGTGG-3' (384-fold)
44fin5R	5'-GTNGCNTCYTCRTCRCA-3' (128-fold)
44Lpofin4F	5'-AGTTATCATTTCTTCATGTGG-3' (nondegenerate)
44Lpofin5R	5'-GTGGCGTCCTCATCACA-3' (nondegenerate)
226fin1F	5'-CARTTYCAYCCNGARGT-3' (64-fold)
226fin4R	5'-ACYTCRTCYTTRTGRAARTC-3' (64-fold)

Individual genes are referred to by number at the front of the primer name. The 63, 44, and 226 genes correspond to homologs of α -adaplin, glucosamine-6-phosphate isomerase, and glutamine amidotransferase, respectively. Forward and reverse primers are indicated by F and R, respectively, at the end.

Table 2. Nondegenerate 5' Tails and Sequences

5' Tail	Sequence
M13REV	5'-CAGGAAACAGCTATGACC-3'
M13(-21)	5'-TGTAACGACGCGCCAGT-3'
T3	5'-ATTAACCTCACTAAAGGGA-3'
T7	5'-TAATACGACTCACTATAGGG-3'

M13REV and T3 sequences were added to forward gene-specific primers (see Table 1), while M13(-21) and T7 sequences were added to reverse gene-specific primers, as described in the text and in Figure 1.

greater than 10-fold difference [c.f., +M tails and -tails for 44fin4F_5R (Figure 1, lower panel)]. A reasonable, though as yet untested, hypothesis for this

additional effect is that 5' tails enhance amplification through incorporation of degenerate primers of greater mismatch than in the absence of 5' tails; that is, following the initial PCR cycle, the nondegenerate 5' tails increase the overall T_m of the bipartite primer in subsequent cycles and thereby allow a more diverse array of mismatched gene-specific primers to amplify.

Increasing the overall primer concentration (with or without 5' tails) might be another means of increasing

PCR product yield. Indeed, a 3.9-fold increase in primer concentration does result in an approximate 2-fold increase in product yield when using primers with 5' tails [c.f., +M tails and +M tails (3.9 $\times$) (Figure 1, middle panel)], although any increase with primers lacking 5' tails remains below the limit of detection [c.f., -tails and -tails (3.9 $\times$) (Figure 1, middle panel)]. This illustrates that adding 5' tails is likely to be a more effective means of increasing product yield than simply increasing primer concentration. Finally, the 5' tail effect is likely to be specific to the PCR, since the use of dT₁₂₋₁₈ as a primer in

the reverse transcription reaction (rather than the usual gene-specific degenerate primer) does not dampen the differential amplification during the subsequent PCR phase [c.f., dT/+M tails and dT/-tails (Figure 1, middle panel)].

Although this report illustrates the 5' tail effect with four degenerate primer pairs, we have performed equivalent direct comparisons between $\pm$ 5' tails for 49 primer pairs total, representing 25 genes. Thirty-seven of the 49 primer pairs with 5' tails amplified appropriately sized visible bands for a larger fraction of the six test taxa (see Figure 1 legend) than the same primers without 5' tails. For 11 primer pairs, the same number of taxa were amplified. Only one primer pair amplified more taxa without 5' tails. Averaged across all taxa, primers with 5' tails amplified 50% more specific bands than the same primers without tails. Furthermore, in most cases, the intensity and purity of the PCR products were greater with 5' tails than without. We conclude that the

Figure 1. Quantification of reverse transcription PCR (RT-PCR) products by agarose gel electrophoresis using primers with and without nondegenerate, nonhomologous 5' tails. RNA from six diverse panarthropod taxa (L, N, H, P, Q, T) plus a "minus template" negative control (0) were reverse-transcribed and then amplified by PCR. Sets of reactions are demarcated by horizontal lines above the three panels. The upper horizontal line in each panel identifies the gene-specific primer pair tested. Primer pairs were either degenerate (63fin2F_3R, 44fin2F_3R, 226fin1F_4R, 44fin4F_5R) or nondegenerate (44Lpofin4F_5R) in their 3' regions. 44Lpofin4F_5R primers were designed to be a perfect match with the corresponding cDNA sequence from *Limulus polyphemus* (L). The lower horizontal line in each panel identifies whether 5' tails were present and their type [+M tails = M13REV and M13(-21), +T tails = T3 and T7], whether 5' tails were absent (-tails), whether the primer concentration was increased 3.9-fold above normal [$\pm$ M tails (3.9 $\times$)], and whether dT₁₂₋₁₈ was used to prime the reverse transcription reaction followed by gene-specific primers with or without tails for PCR (dT/ $\pm$ tails). As a further negative control, RT-PCRs were primed with 5' tail sequences only, but no products of the expected size were observed (unpublished observations). L, *Limulus polyphemus* (Chelicerata); N, *Narceus americanus* (Myriapoda); H, *Nebalia hessleri* (Crustacea); P, *Podura aquatica* (Hexapoda: Collembola); Q, *Prodoxus quinquepunctella* (Hexapoda: Lepidoptera); T, *Thulina stephaniae* (Tardigrada); 0, negative control; m, length standard (300–1000 bp, every 100 bp).

addition of nondegenerate, nonhomologous 5' tails to degenerate gene-specific primers is a useful tool for improving the yield of PCR products and has the added benefit of allowing for convenient direct sequencing.

ACKNOWLEDGMENTS

We thank Austen Ganley and Cliff Cunningham for performing the gene alignments used in defining primers, Jeffrey Shultz and Diane Nelson for specimens, and David O'Brochta and Vikram Vakharia for helpful comments. This research was supported by the National Science Foundation (award no. DEB-0120635).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 7 July 2004; accepted 3 September 2004.

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Localization of transgenes and genotyping of *H-2K^b-tsA58* transgenic mice

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BioTechniques 38:38-42 (January 2005)

The *Immortomouse*TM (1) harbors a transgene that expresses the thermolabile simian virus 40 (SV40) large T antigen tsA58 under the control of the interferon-inducible murine *H-2K^b* promoter. This allows the isolation of conditionally immortalized cell lines directly from the transgenic mouse. These cells undergo reversible immortalization when grown under permissive conditions (33°C, with interferon- γ) and stop dividing and differentiate at higher temperatures in the absence of interferon. To date, cell lines derived from the *Immortomouse* include inner ear hair cells (2), kidney collecting tubuli cells (3), skeletal myoblasts (4), hepatocytes (5), and colonocytes (6). However, the exploitation of this mouse model—particularly for any purposes requiring crossbreeding with other transgenic mice—has been hampered by the fact that no molecular test existed for the distinction of hemizygous and homozygous *Immortomice*.

A single allele of the transgene is sufficient to immortalize cells (4), and the mice can be obtained from Charles River Laboratories (Wilmington, MA, USA) either as homozygotes or as F1-hemizygotes. Testing for the *H-2K^b-tsA58* transgene can easily be accomplished using PCR. However, because the localization of the transgene was not known, until now it was not possible to test for the corresponding wild-type allele and thus identify hemizygotes. The alternative approach, to determine the genotype by backcrossing, is cumbersome and inappropriate for this mouse model because *Immortomice* have a reduced fertility and lifespan due to thymic hyperplasia (1). Thus, homozygosity of the transgene could hardly ever be restored in a colony after crossbreeding with any other mouse strain.

To localize the transgene, we determined the 3'-flanking region using the

Universal GenomeWalkerTM Kit (BD Biosciences Clontech, Palo Alto, CA, USA). High-weight genomic DNA was isolated from mousetail clips and digested in aliquots with three blunt-end-cutting restriction enzymes (*EcoRV*, *PvuII*, *StuI*). The restriction fragments were ligated to blunt adaptors provided with the kit and used as templates for PCR. The forward primer was located near the 3' end of the tsA58 sequence, the reverse primer (AP1) in the adaptor sequence, and a long-distance polymerase with proofreading capability (AdvantageTM Polymerase; BD Biosciences Clontech) was used. The PCR products were reamplified in a second PCR with nested primers. The *StuI*-digested template DNA produced a single band of approximately 1.3 kb in length, which was sequenced from the 5' end (MWG-Biotech, Munich, Germany). A Basic Local Alignment Search Tool (BLAST) search (<http://www.ncbi.nlm.nih.gov/BLAST/>; mouse genome v22.32b1) localized the sequence corresponding to the PCR product in band B5 of mouse chromosome 16 (Figure 1B). The 3' end of the tsA58 gene is located 1.4 kb upstream of the chromosomal marker D16Mit30 (Figure 1A). Currently, no gene is predicted at this site that could have been disrupted by the insertion of the transgene.

Initial attempts to amplify the 5'-flanking region by PCR failed because part of the wild-type genome apparently had been deleted during the insertion of the transgene. We estimated the size of the deleted sequence by testing several primer pairs located upstream of the insertion site, using wild-type mouse DNA as a positive control. Next, the 5'-transition was identified with a primer pair spanning the sequence between the verified wild-type genomic region and the *H-2K^b* promoter. The PCR product