the present method; that is, allele frequency can be precisely estimated by analyzing pooled DNA samples.

REFERENCES


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3’ RACE Walking along a Large cDNA Employing Tiered Suppression PCR


ABSTRACT

Large genes present particular cloning difficulties, especially when expressed at relatively low levels. We describe a novel method, termed 3’ rapid amplification of cDNA ends (RACE) walking, for the rapid determination of unknown 3’ flanking sequence of a large cDNA. The technique is a derivative of the anchored PCR 5’ RACE procedure but includes a specific and limited second-strand cDNA synthesis and a tiered “panhandle” suppression of nonspecific products. The method generated 900 bp of new sequence for the large tammar wallaby ATRY gene in two easy steps, in which standard 3’ RACE and PCR-based cDNA library walking proved unsuccessful. This robust approach represents a new tool for isolating unknown sequence under challenging cloning scenarios such as poor library representation, long coding regions, long 3’ untranslated regions, and difficult template regions.

INTRODUCTION

Several rapid PCR-based methods have been described for determining unknown sequence flanking known regions, with no need of a library. These include various forms of one-sided PCR such as anchored PCR, 5’ or 3’ rapid amplification of cDNA ends (5’/3’ RACE) (1), cassette ligation anchored PCR (2), vectorette or bubble PCR (3), splinkerette PCR (4), panhandle PCR (5,6), Alu PCR (7), restriction site PCR (8), thermal asymmetric interlaced PCR (9), extensionprimer tag selection/ligase-mediated PCR (EPTS/LM PCR) (10), and the recently described universal fast walking method (11). Many of these methods have limitations such as low specificity, restriction enzyme site dependence, or the requirement for an efficient ligation step. Universal fast walking and anchored PCR 5’/3’ RACE address these issues. How-
ever, although the former is applicable to the generation of cDNA sequence in principle; to date, it has only been applied to genomic sequence. Anchored PCR 5' and 3' RACE were developed for the purpose of cloning cDNA ends. 5' RACE uses gene-specific reverse transcription that is primed from a known sequence region, followed by homopolymeric nucleotide tailing and a series of hemi-nested, anchored PCRs to generate 5' flanking sequence. 3' RACE is a simpler procedure that uses the naturally occurring poly(A) tail of mRNA as the common anchor site. However, 3' RACE is limited in cases when unknown coding regions are large, long 3' untranslated regions are present, or problematic template regions exist.

Here we describe 3' RACE walking (Figure 1), a derivative of 5' RACE, which includes a tiered panhandle suppression of nonspecific product amplification for the generation of a 3' flanking sequence of a large cDNA.

MATERIALS AND METHODS

RNA Extraction, DNase I Treatment, and Reverse Transcription

Total RNA was extracted from adult tammar wallaby (Macropus eugenii) testis tissue using the GenElute™ Mammalian Total RNA kit (Sigma, Castle Hill, NSW, Australia). Sample tissue was obtained during a previous study (12) using protocols approved by the Institutional Animal Ethics Committees (Melbourne University, Melbourne, Australia). Total RNA was treated with 1 µL DNase I, as described by the manufacturer’s instructions (Ambion, Geneworks, Thebarton, SA, Australia), except that a 15-min 65°C heat inactivation step was included after DNase I Inactivation Agent treatment. Reverse transcription of 500 ng RNA was catalyzed by SuperScript™ II Reverse Transcriptase (Invitrogen, Melbourne, VIC, Australia) and primed...
by 1 μM poly(T) primer (5′-TTTGTGTGTATGTGACA-GTTGCT-3′) in a 20-μL reaction. The product was then treated with 2 U RNase H at 37°C for 20 min before heat inactivation at 70°C for 10 min.

**Gene-Specific Second-Strand cDNA Synthesis and Poly(A) Tailing**

The single-stranded cDNA was used as template for gene-specifically primed limited extension second-strand cDNA synthesis. The limited extension is designed to generate templates that can be readily amplified without the challenge of long-range PCR. A Taq DNA polymerase (2.5 U) (Invitrogen) catalyzed hot-start procedure was performed using gene-specific primer 1 (GSP1: 5′-ATTGGTTGTGACTCTTG-CCAT-3′) and dNTPs as the final components of a thermally cycled linear amplification (94°C for 45 s, 58°C for 45 s, 72°C for 2 min, and then 30 cycles of 30 s). The product was buffer exchanged to 15 μL 5 mM Tris, pH 8.0, using the QIAEX® II bulk silica DNA purification system (Qiagen, Clifton Hill, VIC, Australia). This removed contaminating enzymes, primers, and dNTPs and allowed for the optimal buffer conditions for the subsequent poly(A) tailing reaction, which was performed using 20 U terminal transferase (New England Biolabs, Arundel, QLD, Australia) and a final dATP concentration of 200 μM. After a 15-min incubation at 37°C, the product was buffer exchanged to 10 μL 5 mM Tris, pH 8.0.

**Anchored Hemi-Nested PCRs**

PCR products were performed using 2.5 U Taq DNA polymerase and 250 nM of each primer in a 50-μL reaction volume. The first round PCR, primed by GSP2 (5′-GATTGCTGTATGTGACAGTTGCT-3′) and the poly(T) anchor primer T16ANC (5′/3′ RACE system; Roche Applied Science, Kew, VIC, Australia) (5′-GACCACGCTATCGAGTGCAGTTCTTTTTTTTTTTTTTTTTTTTV-3′) used 2 μL poly(A) tailed product as template in a hot-start procedure in which dNTPs were added as the final reaction component (94°C for 45 s, 56°C for 45 s, 72°C for 2 min, and 35 cycles of 30 s).

Second-round PCRs used the diluted product of the first-round PCR as template (1 μL of a 1:20 dilution). The reactions were primed by GSP3 (5′-CGTTTAAAGCACAAGGTAAGG-3′) or the second-step primer designed from the first-step generated sequence, GSP3B (5′-AGGCTCATAAGGTGTTAGAAAC-3′) (Figure 3, panels B and C) and the anchor primer ANC (5′-GACGACGCTATCGAGTGCAG-3′). A stepped hot-start strategy was employed in which the penultimate reagents, gene-specific primer, and dNTPs were added during an initial hold at 94°C. The reactions were then allowed to proceed through four complete cycles before the addition of the ANC primer as the final component at 94°C (94°C for 45 s, 65°C for 45 s, 72°C for 2 min, and 44 cycles of 30 s).

**Results and Discussion**

Using the 3′ RACE walking technique, we have been able to obtain sequence from a very large and complicated coding region. In contrast, attempts to cDNA library PCR walk along ATRY proved unsuccessful. Similarly, attempts to clone ATRY via long-range, direct 3′ RACE failed.

The starting point for the 3′ RACE walking procedure (Figure 1) was primer design that was based on the known coding sequence of the large gene, ATRY, of the tammar wallaby (12) (unpublished data) (see Figure 3A), according to principles described for the anchored PCR 5′/3′ RACE procedure (1,13).

Adult tammar wallaby testis total RNA was DNase I treated to remove genomic contamination that would otherwise increase template complexity and the potential to yield intronic rather than coding sequence. A standard reverse-transcription reaction at 42°C was
employed to generate single-stranded cDNA. Control PCR, using intron spanning primers for a housekeeping gene, indicated the success of this step. This overall method proved successful with no need for such measures as poly(A) RNA isolation, gene-specific capture (14), or thermostable reverse transcription. However, these remain considerations for method refinement.

Second-strand cDNA synthesis was gene-specifically primed using a hot-start, limited extension, thermal cycling approach, followed by the polyadenylation of 3′ termini. The hot start and thermal cycling offer stringency. The limited extension should result in a population of premature second-strand chains partly determined by the rate of polymerization. It is assumed that certain sites would favor chain termination and that resulting products would outperform other members during subsequent heminested PCRs. The choice of polyadeny-
The prominent 460-bp product ob-
aining the 3 from the first complete step us-
PCR primers has been demonstrated to
increased ratio of specific to general
conditions are obvious targets for fu-
protocol. Both first and second cDNA
reaction stringency and primer Tms. In
and stepped hot starts. Annealing tem-
PCRs were performed using hot starts
plate environment.
ndergo amplification in a selected tem-
first gene-specific primer would be
nonpaired sequences. However, paired
mung bean nuclease to break down
might be digestion with S1 nuclease or
lowing second-strand cDNA synthesis
hanced intra-strand annealed panhandle
primes nonspecifically in both orienta-
when the anchor primer T16ANC
amplified. Furthermore, in instances
ond-strand cDNAs do not form such
structures and are therefore more readily
absent from the second cDNA strands.
ranalization is important for several reasons.
Long internal homopolymeric runs of
adenines are relatively uncommon in the
eukaryotic genome. Additionally, the
lower relative hydrogen bonding
strength of A:T compared to G:C base
pairs reduces the chance of false priming
vents. An important additional benefit
is conferred in the 3 RACE walking
both first and second cDNA
strands will be poly(A) tailed at the 3′
termini. However, first cDNA strands
also have poly(T) 5′ termini, which are
absent from the second cDNA strands.
As such, the first-strand cDNA mole-
cules are able to form panhandle sup-
pression PCR structures that inhibit their
participation in an amplification reac-
tion. The gene-specifically primed sec-
ond-strand cDNAs do not form such
structures and are therefore more readily
amplified. Furthermore, in instances
library PCR walking and adaptor ligation-
generated PCR libraries. The approach
has been demonstrated in the cloning
unknown sequence from a so-called
problem gene, ATRY, where more conven-
tional methods had proved unsuc-
full. The method does not require a
library, ligation, cloning, fortuitous re-
striction enzyme or anchor sites, is rapid
and includes an built-in suppression
mechanism for enhanced specificity.

3′ RACE walking uses routine mol-
ecular biology laboratory enzymes and
reagents. As such, it represents an im-
mediately accessible in-house tool for
molecular biologists faced with chal-
lenging cloning scenarios.

REFERENCES

printing of a muscle specific enhancer by liga-
tion mediated PCR. Science 246:780-786.
4. Devon, R.S., D.J. Porteous, and A.J. Brookes. 1995. Splinkerettes—improved vec-
quenence specific generation of a DNA panhan-
dle permits PCR amplification of unknown
flanking DNA. Nucleic Acids Res. 20:595-600.
6. Mecgonigal, M.D., E.F. Rappaport, R.B. Wil-
son, D.H. Jones, J.A. Whitlock, J.A. Ortega,
dle PCR for cDNA: a rapid method for iso-
lation of MLL fusion transcripts involving un-
USA 97:9597-9602.
1994. Restricted PCR: amplification of an indi-
vidual sequence flanked by a highly repetitive
element from total human DNA. Nucleic Acids
Res. 22:3251-3252.
1993. Restriction site PCR: a direct method of
unknown sequence retrieval adjacent to a
known locus by using universal primers. PCR
Methods Appl. 2:318-322.
asymmetric interlaced PCR: automatable am-
plification and sequencing of insert end frag-
ments from P1 and YAC clones for chromo-
10. Schmidt, M., G. Hoffmann, M. Wissler, N.
Lemke, A. Mahig, H. Grimm, D.A. Williams,
S. Ragg, et al. 2001. Detection and direct ge-
nomic sequencing of multiple rare unknown
versal fast walking for direct and versatile de-
termination of flanking sequence. Gene
2000. The human sex-reversing ATRX gene
has a homologue on the marsupial Y chro-
some, ATRY: implications for the evolution of
Acad. Sci. USA 97:13198-13202.
amplification of cDNA ends using nested
primers. Technique 1:165-170.
1991. Capture PCR: efficient amplification of
DNA fragments adjacent to a known sequence
in human and YAC DNA. PCR Methods Appl.
1:111-119.
1998. 3′ RACE: skewed ratio of specific to
general PCR primers improves yield and speci-

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