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Advancements in Genetic Engineering

Review Article

Recent developments in RACE-PCR for the full-length cDNA identification

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Abstract

Different novel strategies have been developed for the identification of full-length cDNA using bioinformatics tools and multiplexed PCR methods. Although high-throughput RNAseq has revolutionized the way transcript sequences are identified, many a time, the sequences are either incomplete and have missing UTR sequences. Hence, researchers still use the PCR based rapid amplification of cDNA ends (RACE) technique to obtain the full-length cDNA sequences. For the amplification of 3′ ends of cDNA, an efficient and effective oligo(dT)-anchor primer with hairpin structure is specially designed. Arbitrary degenerate (AD) and sequence-specific reverse (SPR) primers were also developed for the amplification of 5′ cDNA ends by two or three-round of TAIL-PCR or touch-down PCR. Tail PCR needs to be performed until the 5′ sequence of multi-assembled fragment reaches the exon 1 region, which can be identified by aligning these fragments to reference genome database. Two types of adapters (inhibitory and functional adapters) are designed for the attachment to the end of the 5' mRNA strands with specific design at different times (after the first-strand cDNA synthesis). It is a new procedure to obtain a full cDNA sequence with a partially known mRNA sequence. The inhibitory adapter is designed to attach to all mRNA molecules and unmethylated cap, however, due to the lack of phosphate at the end of the 5' of adapter, it can't attach to full-length mRNAs with cap structure. Therefore, degraded or uncapped mRNAs can be removed from the reaction. Thus, only 5' end of full-sequenced cDNAs can be selected and amplified. This technique is highly efficient for separation of the unknown regions of cDNA ends, when, only a short sequence of cDNA is available. Several different 5' RACE methods have been developed, and one particularly simple and efficient approach called CapFinder relies on the 5' cap-dependent template-switching that occurs in eukaryotes. However, most prokaryotic transcripts lack a 5' cap structure. Recently, a special technique is also reported to capture primary transcripts based on capping the 5' triphosphorylated RNA in prokaryotes. With the help of vaccinia capping enzyme, primary transcripts are first treated to add a 5' cap structure. Specific primers need to be designed for further processes to identify full cDNA of a gene (both 5' and 3' end).

Keywords: mRNA, RACE, Adapter, Tail-PCR, Cap-switching, Arbitrary degenerate (AD), and sequence-specific primers.

Introduction

Full-length cDNA sequence information is a prerequisite for the functional characterization of genes. Identification of an unknown gene sequence is done traditionally by degenerate primer PCR targeting evolutionarily conserved regions of the coding sequence. This often results in only partial cDNA sequence information with missing 5' and 3' sequence. RACE (Rapid amplification of cDNA ends) is a conventional PCR based technique, used to obtain the full-length sequence of an RNA transcript (Eyal et al., 1999 and Park et al., 2003). The transcriptomes of the increasing number of species have been sequenced by next-generation sequencing (NGS) technique. However, the transcriptomes are always incomplete in length, especially the ends of genes, and due to the complexity and high cost, the complete genome sequencing is only conducted in some limited species. Therefore, researchers still use the PCR based RACE technique to identify the full-length cDNA sequences (Chen et al., 2016). Also, RACE PCR is the most simple and cost-effective technique when the study involves a few targeted genes. Two general RACE strategies exist: one amplifies 5′ cDNA ends (5′ RACE) and the other captures 3′ cDNA end sequences (3′ RACE). Gene-specific primers

Correspondence to: A Gazi, Gadjah Mada, Indonesia, ~~E-mail: withanage@scamt-itmo.ru~~ ~~Citation: Withanage SR, Daniil K (2020) Genetically Engineered Biomaterials. Adv Genet Eng 9:161. doi: 10.35248/2169-0111.2020.9.161~~

Received date: July 01, 2020; Accepted date: : July 15, 2020; Published date:July 31, 2020

are designed within the known cDNA sequence oriented towards the unknown sequence. In 3' RACE, an oligo dT adaptor primer targeted against the universal polyA tail of mRNAs is used as a reverse primer along with the gene-specific forward primer. In 5' RACE, a known sequence is first incorporated into the 5' end of the mRNA sequence either through homopolymeric tailing or adaptor ligation. Then PCR is performed using a forward primer against the homopolymeric tail or adaptor sequence and a gene-specific reverse primer (Bower and Johnston, 2010; Fromont et al., 1993). Creating a small known region at the 5' cDNA end is a major issue (Edery et al., 1995; Bower and Johnston, 2010). Homopolymeric tailing by the terminal transferase enzyme is the common practice. Common approaches, such as homopolymeric tailing and ligation anchored tailing requires a lot of enzymatic reaction after completion of reverse transcription (1st strand cDNA synthesis). Each enzymatic reaction can potentially introduce failures and destroy the integrity of cDNA. However, the efficiency of TdT is low, resulting in poor yield (Schaefer, 1995). Also, homopolymeric tailing does not differentiate between full-length (5' cap intact) and 5' truncated transcripts. Hence, there is a higher representation of 5' partial cDNAs.

Methods

Nowadays, several RACE techniques including RLM-RACE and Cap-switching RACE have been developed, especially for the 5 RACE due to the difficulty in operation (Liu and Gorovsky,1993; Schmidt and Mueller, 1999; Schramm et al., 2000). In the new RACE or RLM-RACE, before conducting the reverse transcription (RT) of mRNA into the first-strand cDNA, an anchored RNA adaptor is ligated to the 5 ends of mRNA. Shrimp alkaline phosphatase (SAP) is used to remove a phosphate group, and then with the help of T4 RNA ligase, the anchor RNA adaptor is ligated to the 5 ends. However, the RNA truncated at the 5' end can also be ligated to the RNA adaptor in this method. To address this limitation, an alternate method, oligo-capping, was developed (Maruyama and Sugano, 1994).

The Cap-switching RACE (CapFinder) is performed by Moloney murine leukemia virus (MMLV) reverse transcriptase. The enzyme can add extra 2-4 cytosines to the 3 ends of newly synthesized first-strand cDNAs upon reaching the 5' cap structure of mRNAs (Schramm et al., 2000). A template-switching oligonucleotide (TSO) containing a 3' poly(rG) tail is added to base pair with the extra cytosine residues. Thus, introducing a known adaptor to the 3' end of cDNA (TSO serves as a template for reverse transcription). The method has additionally been developed into inverse PCR, T-RACE, and step-out PCR to enhance the specificity and efficiency (Matz et al., 1999; Huang & Chen, 2006).

Results

cDNA made of degraded mRNA or full-length segments are difficult to identify or recognize in conventional RACE technique. Therefore, an improved RACE technique has been developed to reproduce only 5' end of full-length cDNA, exclusively. With the help of two kinds of adapters (inhibitory and functional adapters) all degraded mRNA components and unmethylated cap can be omitted (after the first-strand cDNA synthesis). The inhibitory adapter is designed especially (without phosphate group) to attach all un-wanted mRNA components and unmethylated cap. Suppressor adapter can't attach to full-length mRNAs with cap structure due to the lack of phosphate at the end of the 5' of the adapter. Therefore, degraded or uncapped mRNAs can be easily removed from the reaction. Having phosphate at 5' end of the functional adapter helps binding to mRNA with the cap structure. Thus the fruitful result can be obtained after only one PCR; 1st step PCR is enough to get the result with the minimum background (Purfrad et al., 2017).

This is a modified RACE method to separate the 5' end of the cDNA without using specific enzymes. The modified RACE has several advantages, such as requires less time and lower costs related to the commercially existing procedures; other advantages are:

1) Fast and simple and requires a minimum amount of total mRNA (About 1 µg).
2) Only one PCR is enough to get the result with the minimum background.
3) Removal of degraded mRNA or other nucleotide strings without cap structure.
4) High repeatability for amplification of the desired region.
5) Having phosphate at 5' end of the functional adapter helps binding to mRNA with cap structure.
6) This binding is maintained during PCR and provides a binding site of the primers at the 5' end of mRNA.

Using this RACE technique, 5' end of the Aeluropus littoralis SOS1 gene was isolated and sequenced by Purfrad et al., 2017. Specific primers were designed to amplify 5' end of the gene (Table 1).

Name	Sequences (5' → 3')	Modification
P1	ACCTCGGCCG	-
P2	AGCGTGGTCGCGGCCGAGGT	-
P3	AAGGAGTAGTTT	-
P4	AAACTACTCCTTCAGTCCATGTCAGTGTCCTCGTGCTCCA	5' phosphate
FP1	CTGGAGCACGAGGACACTG	-
FP2	CTGACATGGACTGAAGGAGTA	-
GSP1	TGCCATGTCTCTCAAAATGG	-
GSP2	CAATGTAGGCAACCATTTCCC	-

Table 1. Primers sequence used in the new 5' RACE method

* P: Primer, FP: Functional primer, GSP: Gene- specific primer P4 is the functional adapter, having phosphate at 5' end helps binding to mRNA with cap structure. This binding is maintained during PCR and provides a binding site of the primers at the 5' end of mRNA. Alignment of the sequence with the NCBI data-

base confirmed the efficacy of the modified technique (Table 2).

In UK, consumption of AB decreased for 11.01%. The decreasing of consumption was in all groups of AB (Table 2).

Description	Max score	Total score	Query cover	E Value	Ident.	Accession
Aeluropus littoralis plasma membrane Na+/H+ antiporter (SOS1) mRNA, complete cds.	1639	1639	97%	0.0	100 %	JN936862.1
Aeluropus littoralis plasma membrane Na+/H+ antiporter mRNA, complete cds. Distichlis spicata plasma membrane	1520	1520	95%	0.0	98%	HQ329792.2
Distichlis spicata plasma membrane Na+/H+ antiporter (SOS1-1) mRNA, complete cds.	1360	1360	95%	0.0	95%	FJ865581.1

Table 2: Three items of the sequences in database of NCBI, which most closely resembled the 5' SOS1 sequence of the Aeluropus littoralis

Discussion

Often occurring problems in RACE-PCR are multiple bands and non-specific bands. Masamha and Todd (2018) suggested a few PCR cycling conditions to solve some problems in RACE-PCR. Those are:

1) Increasing of the initial denaturation length (up to 3 mins) and temperature (98 °C) breaks any secondary structures and helps yield the single specific band because the T7 oligo dT primer generally forms weak secondary structures.

2) The long annealing time (5 mins) and extension or elongation step (10-15 mins) allow complete synthesis of incomplete amplicons, enabling the full extension of the initial and final amplification products.

3) The non-specific bands are mostly lighter than the expected bands. Suggesting that it can appear at higher PCR cycles (eg., up to 35 cycles). Therefore, limiting the PCR cycles to only 20 reduces the amplification of the non-specific band.

4) Reverse transcription without MMLV-RT enzyme can be used as a negative control. Presence of a band, in negative control signifies the potential contamination with the genomic DNA.

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