A Reliable and Quick Method for Screening Alternative Splicing Variants for Low-abundance Genes

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Research Article

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Abstract

Alternative splicing (AS) is an universal phenomenon in eukaryote and it's still challenge to identify AS events. Several methods have been developed to identify AS events, such as Expressed Sequence Tags (EST), microarray and RNA-seq. However, EST has limitation on identifying low-abundance genes, while microarray and RNA-seq are high-throughput technologies and they also need PCR-based technology to validate. To overcome the limitations of EST and shortcomings high-throughput technologies, we established a method to identify AS events, especially for low-abundance genes, by reverse transcript (RT) PCR with gene specific primers (GSPs) followed by nest-PCR. And it includes two major steps: 1) using GSPs to amplify as long as the specific gene segment; 2) multiple rounds of nest-PCR were used to screen the AS and confirm the unknown splicing variants. With this method, we successfully identified three new splicing variants, namely (GenBank Accession No. HM623886) for gene *bdnf* (GenBank GeneID: 12064), (GenBank Accession No. JF417977) for gene *trkc* (GenBank GeneID: 18213) and (GenBank Accession No. HM623888) for gene *glb-18* (GenBank GeneID: 172485). Besides the reliability and simplicity, the method is also cost-effective and labor intensive. In conclusion, we established a reliable and quick method for screening alternative splicing variants for eukaryote genes, especially for low-abundance genes.

1. Introduction

Alternative splicing (AS) is an universal phenomenon in eukaryote. Some studies have revealed that more than 80%[1] of the eukaryotic genes were alternatively spliced and even 95% [2] in human genes. AS, which could increase the diversity and complexity of genes and proteins, was closely associated with the development of oncology [3; 4; 5] and nervous system diseases [6; 7; 8]. Therefore, to establish a valuable and high efficient approach for identifying splice variants was important for studying the splicing-related diseases. Previous study has demonstrated several techniques for novel splice variant identification, including bioinformatics and molecular methods, such as Expressed Sequence Tags (EST)[9; 10], microarray[11; 12; 13], RNA-seq[14; 15], 5’ RACE and genomic mapping[16]. But there are several disadvantages in EST data, for instance, 3’ deflection, genomic contamination, low-abundance genes insensitivity. Microarray technologies also have their inevitable drawbacks, including limited array coverage degree, non-specific array hybridization and so on. Presently, RNA-seq has been generally used in AS studies, but this technique is very expensive.

In a word, these approaches were enormously limited due to low reliability, poor positive rate, needing experimental validation, expensive and tedious operation. To address these issues, we established a method for screening AS variants, especially for low-abundance genes, using GSPs for RT followed by nested PCR (nPCR), which can overcome these difficulties and provides an advantageous combination of specificity, efficiency, accuracy and usability. This method is also important for the function studies of eukaryotic genes, especially for low-abundance genes and understanding the mechanism of aberrant splicing-related diseases.

2. Description Of The Method

2.1 Getting the longest transcript RNAs by GSPs which located in the 3’-end of the gene

In order to amplify as long as the specific gene segment, GSPs as RT-primers, rather than universal primers (Random Primer and Oligo(dT)$_{20}$), were used and were designed in the distal end of gene sequence, which were located in constrution exons. The good points of GSPs usage were the enrichment of target gene RNA transcript, which provide the following nPCR with templates.

2.2 Checking the reducing pattern of the size of amplicons by multiple rounds of nest-PCR

Multiple upstream and downstream GSPs of every splice variant of target genes were designed according to its known gene structure. The upstream and downstream primers (2-3 primers each terminal) were devised from outer to inner in 5’- and 3’- extremitates, and it’s better to across introns in case of genomic contamination. Take the *bdnf* splice variant 1 in *Balb/c* mouse brain tissue as an example to describe nPCR. There are five rounds of PCR in identifying splice variant 1 of *bdnf*. The most of the lateral GSPs (tv1-5’GSP1 and 3’GSP1) were used to amplify specified splicing variants with cDNA as templates in the first round nPCR. Then, 5’-GSPs was locked, while 3’-GSPs were chosen from outer to inner gradually. At last, immobilizing 3’-GSPs, 5’-GSPs were selected step by step to amplification. Every round of PCR was performed using the same program but with a 1:10 dilution of the former round products as the template, other than the template of the first round cDNA. Eventually, each round of PCR amplicons were separated on 1.5% agarose gels supplemented with ethidium bromide. At this time, specific and unspecific bands were observed under a UV light, and we can determine preliminarily whether new variants were existed.

2.3 Validating the new variants by sequencing

Electrophoresis strips with diminishing pattern were identified whether or not known and unknown variants by T-A cloning, sequencing, bioinformatics analysis, and further determining AS forms of the new variants.

3. Validation Of The Methods

3.1 Culture and synchronization of *C. elegans*

The wild-type N2 strains of *C. elegans* (Caenorhabditis Genetics Center (CGC), USA) were cultured in a 90 mm petri dish with nematode growth medium (NGM). Adult nematodes were collected and treated with a 5 ml nematode lysate containing 400 µl 8% sodium hypochlorite, 80 µl 10 M NaOH and 4.52 ml M9-buffer. The solution was shaken vigorously by hand for about 10 min. At the same time, the state of the lysate was observed. The solution of lysate was centrifuged for 1 min at 1200 rpm to collect eggs, and washed three times with M9-buffer. The eggs were inoculated on NGM agar plates without *E. coli* OP50

for 16 h at 20°C (± 0.1°C) to hatch L1 larvae. The synchronized L1 larvae were then collected and placed on 90 mm NGM agar plates with *E. coli* OP50 seeded.

3.2 Preparation of the total RNA in different species

The *Balb/c* and *C57BL/6J* mice (18-20 g, Vital River Laboratories, China) were sacrificed and their brain tissues were prepared according to previous described[17]. When the worms grows to L4 stage after synchronization, they were washed and collected from the plates with M9 buffer and were subsequently cleaned three times[18]. The total RNA was extracted using the Total RNA kit (OMEGA, Japan).

3.3 Design of GSPs

According to the principle of our method, to aim directly at *bdnf* of *Balb/c* mouse, *trkc* of *C57BL/6J* mouse and *glb-18* of *C. elegans*, Multiple pairs of GSPs for RT and nPCR were designed by MPprimer (http://biocompute.bmi.ac.cn/MPprimer/)[19] and evaluated by MFEprimer (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/)[20] as shown in Table 1.

Table 1
GSPs primers

Gene		Primers(5’-3’)
bdnf in Balb/c mouse	RT-primer	AGCAACAGGCCTGCTGCCAT
	tv1-5’GSP1	CAGTAGCCGGCTGGTGCAGA
	tv1-5’GSP2	TGCTTCAGGAAACGCCCGCT
	tv1-5’GSP3	ACGTGTCTCTCAGAATGAGGGCGT
	tv2-5’GSP1	TGGCAAAGCCATCCACACGTG
	tv2-5’GSP2	CGAGGTTCGGCTCACACCGA
	tv2-5’GSP3	AGCCCCAGTTTGGTCCCCTC
	tv3-5’GSP1	AGAGGACTGCTCTCGCTGCC
	tv3-5’GSP2	GCTTCTCGCTGAAGGCGTGC
	tv3-5’GSP3	CCACCAAAGACTCGCCCCCT
	3’GSP1	ATGCCCCTGCAGCCTTCCTT
	3’GSP2	TGGGCCGAACCTTCTGGTCC
	3’GSP3	GAGTCCCATGGGTCCGCACA
trkc in *C57BL/6J* mouse	RT-primer	5’-AGCACGGGCCACAGCTTAAGT-3’
	5’-GSP1	5’-CCCTCTCCTGGAAGGGCAGG-3’
	5’-GSP2	5’-AGAGAACTGGCGAGGCCTGC-3’
	3’-GSP1	5’-ACACGGCCTTGGGTGATGCA-3’
	3’-GSP2	5’-CATCCAGCGGATGGGGAGCA-3’
glb-18 in *C. elegans*	RT-primer	GGCGGAAAACGATTGACACCTTTCA
	5’GSP1	TGCCGTCTGCTGCTCGTCAA
	3’GSP1	GGCGGAAAACGATTGACACCTTTCA
	3’GSP2	TCCTCCACACCGTCACTGCG
	3’GSP3	TCGGTCATAATGGAGAGACGGTGTT

3.4 GSP-dependent reverse transcription

The cDNA was prepared by RT using Rever Tra Ace -α- (Code No. FSK-100) kit (TOYOBO, Japan) in Professional Thermocycler (Biometra, Germany) as follows: adding 0.5 nM of GSPs (Table 1), 1 μg of

total RNA, RNase Free H_2O up to 12 µl in PCR tube for 5 min at 65°C. When the above procedure is over, put it on ice and add immediately 5×RT Buffer ($MgCl_2$ plus), 1 mM of dNTP Mixture, 10 unit of RNase Inhibitor and 1 µl of ReverTra Ace. The running program is 42°C for 40 min, 85°C for 5 min, 4°C for 5 min. The cDNA was stored at 4°C for usage.

3.5 nPCR

nPCR was operated to screen AS events after RT procedure. There were five rounds of nPCR in *bdnf* gene detection (Fig. 1). And the combination manner of variant 1 primers were tv1-5'GSP1+3'GSP1, tv1-5'GSP1+3'GSP2, tv1-5'GSP1+3'GSP3, tv1-5'GSP2+3'GSP3, tv1-5'GSP3+3'GSP3. The variant 2 primers were tv2-5'GSP1+3'GSP1, tv2-5'GSP1+3'GSP2, tv2-5'GSP1+3'GSP3, tv2-5'GSP2+3'GSP3, tv2-5'GSP3+3'GSP3. The variant 3 primers were tv3-5'GSP1+3'GSP1, tv3-5'GSP1+3'GSP2, tv3-5'GSP1+3'GSP3, tv3-5'GSP2+3'GSP3, tv3-5'GSP3+3'GSP3. There were three rounds of nPCR in *trkc* detection, and the assemble mode of primers were 5'GSP1+ 3'GSP1, 5'GSP1+3'GSP2, 5'GSP2+3'GSP2. There were three rounds of nPCR in *glb-18* detection, and the assemble mode of primers were 5'GSP1+3'GSP1, 5'GSP1+3'GSP2, 5'GSP1+3'GSP3.

In the *bdnf* gene detection, following reverse transcription using GSP, 0.5 µl of the cDNA was used for the first round amplification of 35 cycles of melting (45 sec at 95°C), annealing (45 sec at 62°C) and extension (1 min at 72°C) in the Professional Thermocycler (Biometra, Germany).

The 12.5 µl reaction mixture contained 10×Ex Taq Buffer (Mg^{2+} plus), 200 µM of each dNTP, 0.2 nM of forward primer tv1-5'GSP1, 0.2 nM of reverse primer 3'GSP1, 0.315 unit of TaKaRa Ex Taq, 0.5µl of cDNA and 9.187 µl of double distilled water (ddH_2O). The second round reaction was performed using the same program but with a 1:10 dilution of the first round reaction as the template, and the primers were tv1-5'GSP1 and 3'GSP2. Similarly, the third, fourth, and fifth round of PCR was performed using the same program but with a 1:10 dilution of the second, third, and fourth round reaction as the template, and the primers were corresponding.

The inspection of other variant of *trkc* and *glb-18* was performed using the same program but the primers were adjustment. The PCR products were separated on 1.5% agarose gels supplemented with ethidium bromide. DNA was visualized under a UV light. All of the experiments were repeated at least three times.

3.6 Verification of variants by sequencing

For the purpose of verification of splice variants, the sequencing results were analyzed after removing vector sequence using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene structure of *bdnf* in *Balb/c* mouse, *C57BL/6J* mouse and *glb-18* in *C. elegans* were then verified according to the blast results and GenBank information in NCBI RefSeq database.

4. Result

4.1 Identification of known splice variants of *bdnf* gene in *Balb/c* mouse

To validate the feasibility of splice variants by using RT and nPCR with GSPs, the known splicing variant 1 (Fig. 2A) and variant 3 (Fig. 2B) of the *bdnf* gene in *Balb/c* mouse were identified. Results showed that this method can identify these two variants (Fig. 2). As expected, there was one specific electrophoresis band with products diminishing pattern.

4.2 Screening of a new splice variant of *bdnf* gene in *Balb/c* mouse

The nPCR results of variant 2 displayed three DNA bands with diminishing pattern (Fig. 3A). Direct sequencing results and blast analysis displayed that the second band which arrow directed was the known variant, while the third band was the unknown variant, which has steep homology with *bdnf* gene, particular variant 2 (Fig. 3A). We mapped the gene structure of every *bdnf* variant according to their GenBank information in NCBI database as shown in Fig. 3B. In addition, the unknown variant was deposited in the GenBank database under Accession No. HM623886.

4.3 Screening of a new splice variant of *trkc* gene in *C57BL/6J* mouse

The identification results of *trkc* gene are shown in Fig. 4A. It can be seen that three target bands with decreasing characteristics of 2160 bp, 2051 bp and 1957 bp can be obtained by nPCR (as shown by the arrow in Fig. 4A, Lane 2, Lane 3 and Lane 4 respectively). It shows that the known variant 1 of *trkc* gene can also be identified by using RT and nPCR with GSPs.

For the nucleic acid fragments lower than 500 bp in Fig. 4 (marked by solid frame and dotted box in Lane 3), the strip sequencing result in the dotted box was 335 bp. Compared with mouse RefSeq database, it was found that the nucleic acid fragment sequence has high similarity with *trkc* gene (99%), especially with variant 1. Through in-depth analysis, it is found that the sequence of 1-298 bp is consistent with 222-519 bp in *trkc* gene splice variant 1and 299-334 bp is consistent with 2189-2224 bp in *trkc* gene splice variant 1, indicating that the sequence is missing 1669 bp between 520-2188 bp in *trkc* gene splice variant 1. Therefore, it is a new splice variant of *trkc* gene and is determined as variant 3. The nucleic acid fragment in the solid frame of Fig. 4 was identified as a non-specific amplification band by sequencing.

We mapped the gene structure of every *trkc* variant according to their GenBank information in NCBI database as shown in Fig. 4B. The sequenced cDNA in this variant has been submitted to GenBank database, and the sequence acceptance number is JF417977.

4.4 Screening of a new splice variant of *glb-8* gene in *C. elegans*

We further asked whether our approach worked fine for other species. Likewise, in *C. elegans*, *glb-18* also had strips with diminishing pattern, and other than expected strips, there were unexpected strips with the same diminishing pattern, which suggested that it was probably a new variant(Fig. 5A). Sequencing results and blast analysis manifested that the band in solid line was the known variant, while the band in dashed line has lots of homology with *glb-18*, particular variant 1. Analogously, we mapped the gene structure of every *glb-18* variant according to their GenBank information in NCBI database as shown in Fig. 5B. The unknown variant is missing exon 2, exon 3, exon 4, and part of exon 1 and exon 5, which was deposited in GenBank (Accession No. HM623888).

5 Discussion

AS, as a research hot spot all the time, is widely existence in organism and is closely associated with kinds of diseases. Currently, existing methods for identifying splice variants, such as EST[9; 10], microarray[11; 12; 13], RNA-seq[14; 15], 5'RACE and genomic mapping[16], have their defects. Besides of low reliability, poor positive rate, experimental validation deficiency, genomic contamination, low-abundance genes insensitivity, expensive and tedious operation of these methods, the universal primers (Random Primer and Oligo$(dT)_{20}$) are used for RT and routine PCR reaction is used for splice variants verification. Therefore, the emphasis of this study established a convenient and feasible method for alternative splice variants screening, especially for low-abundance genes. Because Random Primer and Oligo$(dT)_{20}$ are used for RT experiment, it can be combined with all mRNA or non coding RNA in RNA template and reverse transcribed into cDNA[21]. It is difficult to capture low-abundance mRNA and the alternative exons are hardly found using routine PCR. Nevertheless, we established method for screening gene splice variants using GSP for RT and nPCR techniques in this study just can redeem this fault and provide experimental testing for bioinformatics approaches. The key technology of this study is to use the primer design software (MPprimer and MFEprimer) developed by our laboratory to design GSPs instead of ordinary Random Primer and Oligo$(dT)_{20}$. In addition, multiply rounds nPCR using GSPs can quickly screen splice variants and find unknown variants, and electrophoresis strips with diminishing pattern also greatly raise amplification products specificity. For example, through the above methods, we have screened three new variants, namely *bdnf* and *trkc* gene variants from mouse and *glb-18* variants from *C. elegans*, all of which belong to low abundance genes. The splice variants is of significance for the mechanism research of aberrant splicing-related diseases, which can also supply diagnosis, treatment of these diseases with reliable technology evidence. Therefore, the method of screening splicing variants in this study has great application prospects. However, this method has inevitable disadvantages, for example, only can fraction of subsequences be gained, rather than their total length and it is not possible for high-flux. At present, our laboratory is being engaged in mPCR, including primer design[19] and evaluation[20], as well as specific experiment operation. In the future, the multiplex PCR (mPCR) is applied to this method, multiple variants of one gene or several genes could be identified simultaneously.

Taken together, the current method is a quick, reliable, low-abundance genes sensitive, cost-effective and labor intensive approach in screening alternative splicing variants of genes, which can cover known and unknown splice variants. It is expected that our approach will be valuable in AS related disease studies to disclose the aberrant splicing events.

Declarations

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Conflicts of Interest:

The authors declare no conflicts of interest.

Ethics approval and consent to participate:

Experiments involving animals were approved by the Animal Ethics Committee of the National Beijing Center for Drug Safety Evaluation and Research, ethical review number: IACUC-2019-025.

Informed consent:

No human experiments were involved.

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Figures

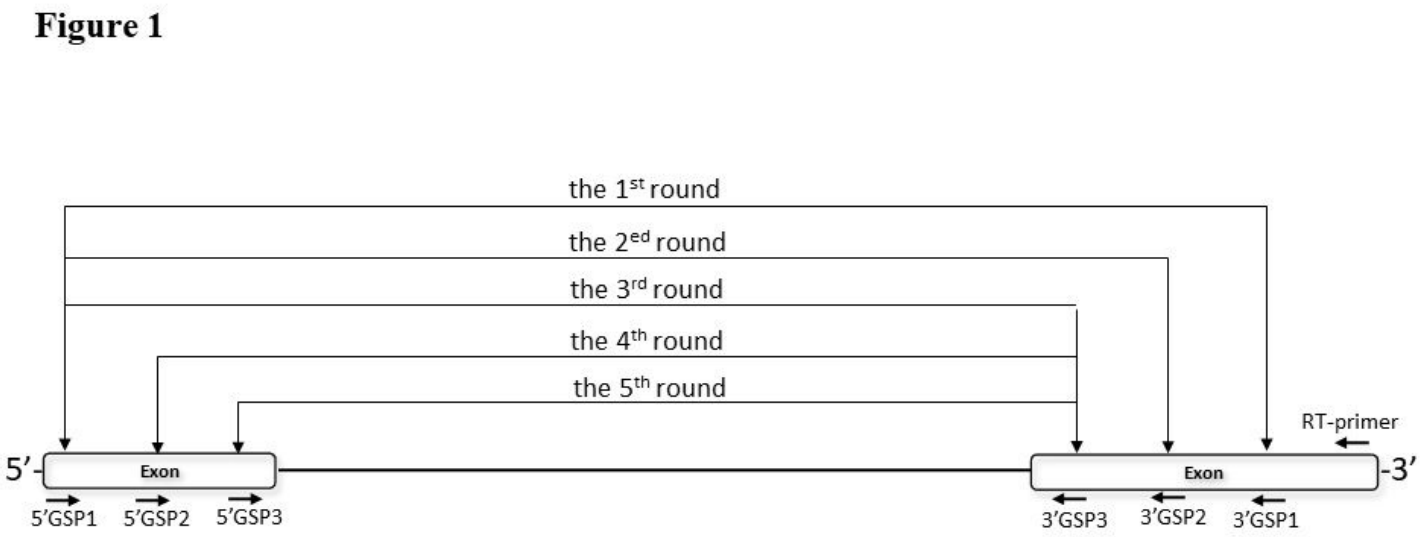

Figure 1

Schematic diagram of nPCR. In the figure, all GSPs are used in *bdnf* splice variant 1. RT-primer is only used for reverse transcription. There were five rounds of PCR, which produced 1258bp, 900bp, 783bp, 523bp and 430bp respectively. Their primer combinations were 5'GSP1+3'GSP1, 5'GSP1+3'GSP2, 5'GSP1+3'GSP3, 5'GSP2+3'GSP3, 5'GSP3+3'GSP3.

Figure 2

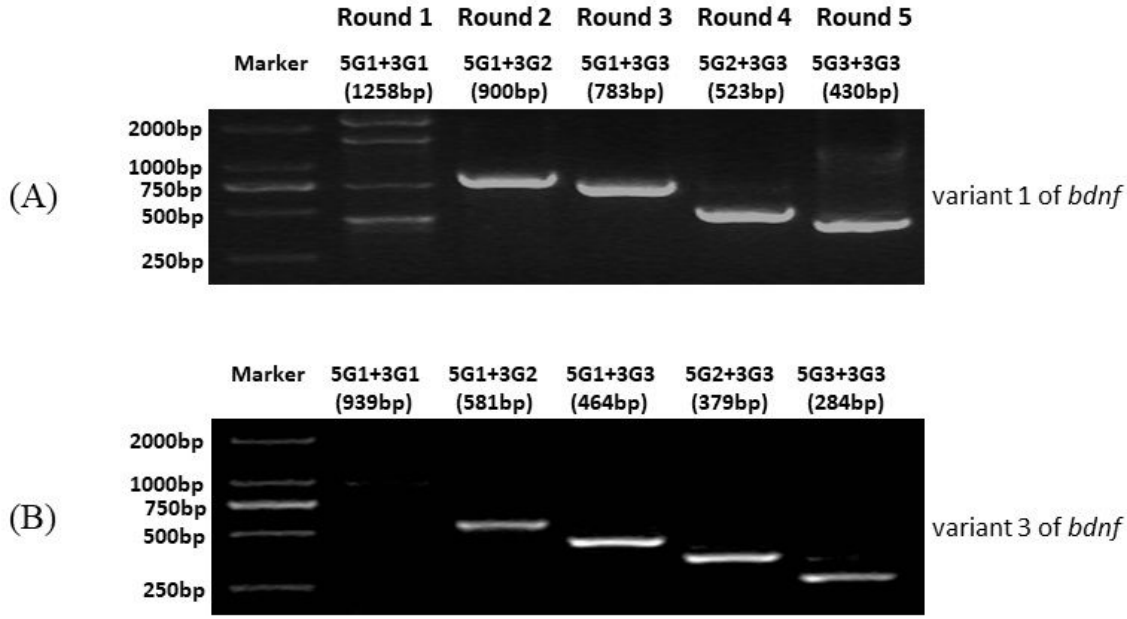

Figure 2

nPCR results of *bdnf* splice variant 1 and variant 3 in *Balb/c* mouse. (A) nPCR results of *bdnf* splice variant 1. (B)nPCR results of *bdnf* splice variant 3.

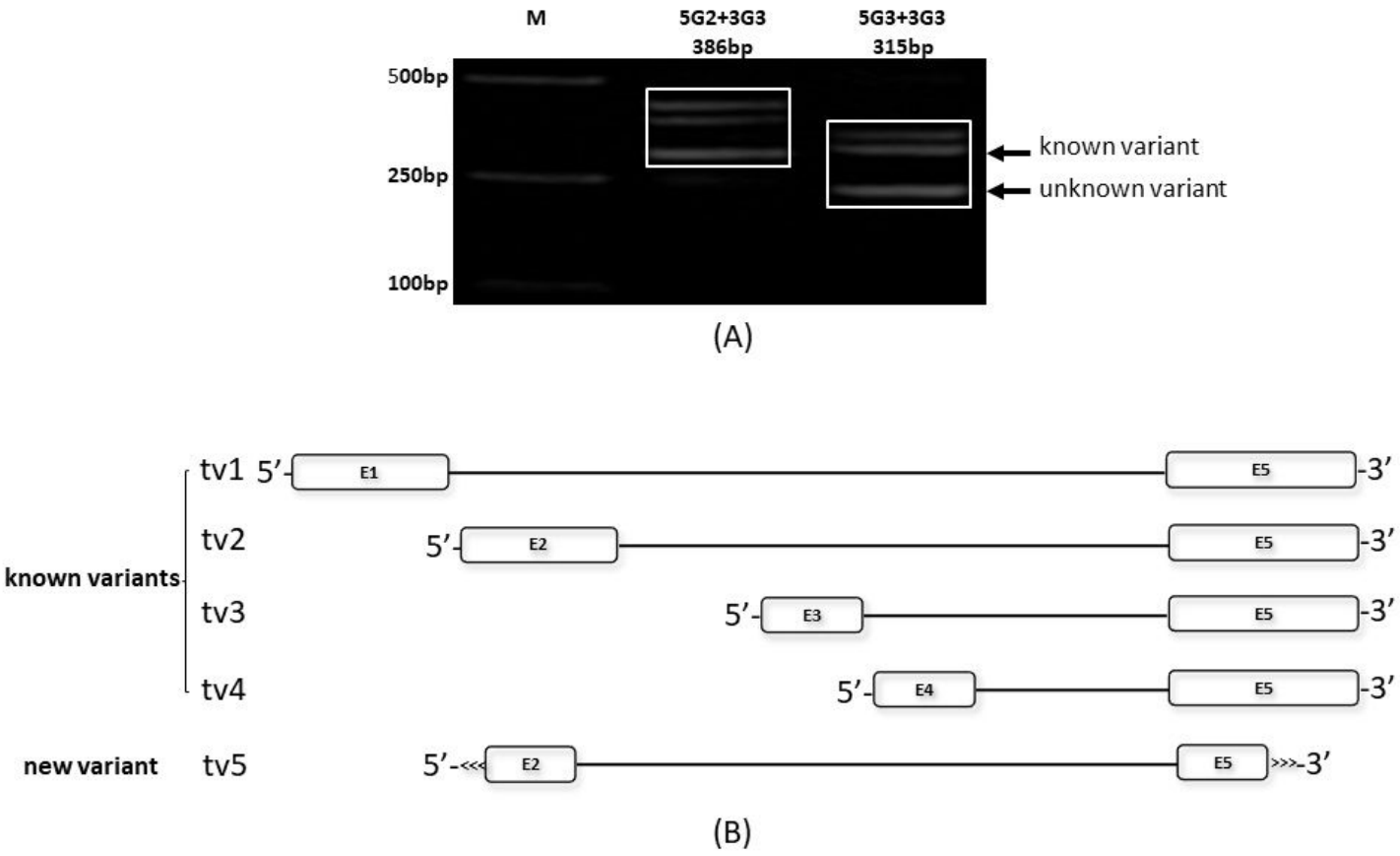

Figure 3

(A) nPCR results of *bdnf* variant 2 in *Balb/c* mouse. Sequencing and analysis results showed the second was the known variant, while the third was a new variant. (B) Gene structure of *bdnf* in *Balb/c* mouse.

Figure 4

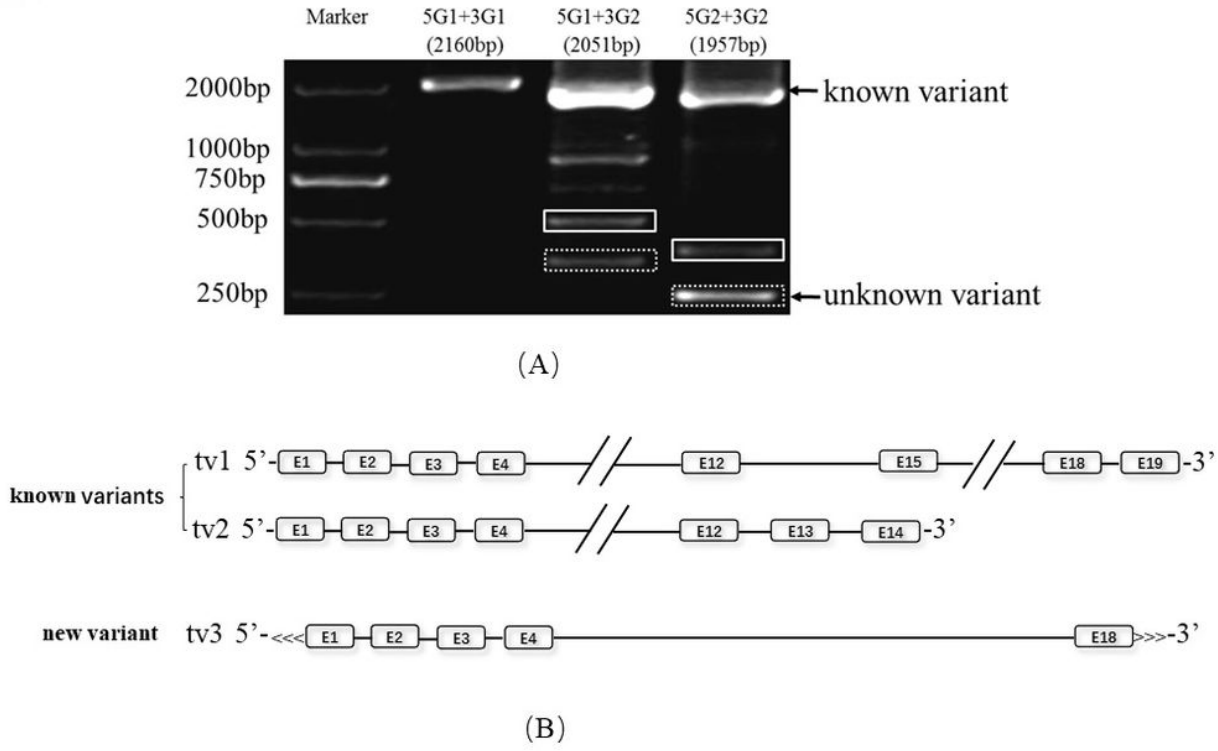

Figure 4

(A) nPCR results of *trkc* in *C57BL/6J* mouse. In addition to the expected bands, two bands showed a decreasing pattern. Sequencing and analysis showed that the bands in the dot box region was a new variant. (B) Gene structure of *trkc* gene in *C57BL/6J* mouse.

Figure 5

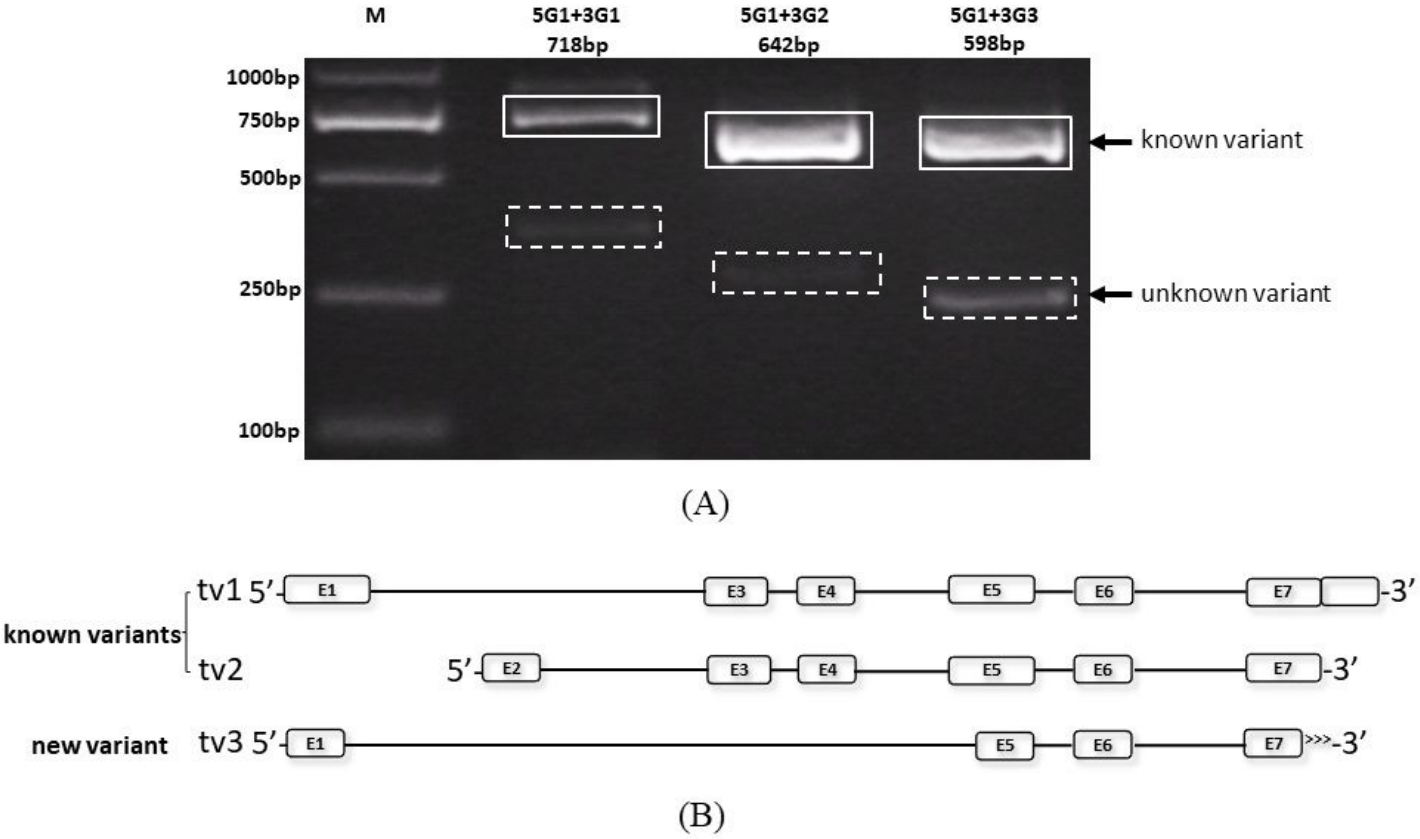

Figure 5

(A) nPCR results of *glb-18* in *C. elegans*. In addition to the expected bands, there is a band decreasing pattern. Sequencing and analysis results show that it is a new variant. (B) Gene structure of *glb-18* gene in *C. elegans*.