A RAPID AND SIMPLE TEMPLATE PREPARATION METHOD FOR DIRECT SEQUENCING OF RESOURCE LIMITED SPECIMENS

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Application of molecular techniques has greatly improved our ability to detect the presence of pathogenic microorganisms in patients specimens. The RT-PCR and direct sequencing of the amplification products for example has enabled enteroviruses detection from patients tissues. For direct sequencing of the PCR products, the template purity and amount are important factors which can affect the accuracy and throughput of the sequencing result. Consequently, more starting materials (DNA or RNA) are often needed for the RT-PCR because substantial amount of the amplified DNA products are lost during purification. Problem arises in dealing with clinical specimens where the resources are limited especially in cases of sudden death or specimens available are to be distributed between units and diagnostic centers. Sufficient DNA for sequencing can be obtained by cloning the initial amplified DNA products after purification into suitable cloning vectors, transforming bacteria, screening and isolating the recombinant plasmids. However, this method is laborious and time consuming. We report here application of a simple procedure to overcome this problem. Briefly, purified PCR products from the initial amplification were ligated into a suitable vector and then reamplified to obtain adequate DNA for direct sequencing. This method was successfully employed to detect the presence of Enterovirus 71 (EV71) in tissues of children who died from brainstem encephalomyelitis (1).

The post-mortem tissues (~ 100 µg each of brain material from patient 1 and spinal cord from patient 2) of patients who succumbed to an acute childhood viral infection were homogenized and total RNAs were isolated using TRIzol™ Reagent (GIBCO BRL, Life Technologies Inc., USA). The reverse transcription polymerase chain reaction (RT-PCR) was performed using Access RT-PCR System (Promega, USA) and the RT-PCR parameters were 42°C for 90 minutes, 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 5 minutes at the end of 30 cycles. The primers, 5' GGCGTTAGCACACTGGTATCAC 3' and 5' CTAGCTCAATAGACTCTTCGCA 3', annealed to positions 44 to 65 and 418 to 440 of the Coxsackievirus A9 genome respectively (2). The RT-PCR reagents were used following the manufacturer's protocols. Amplified

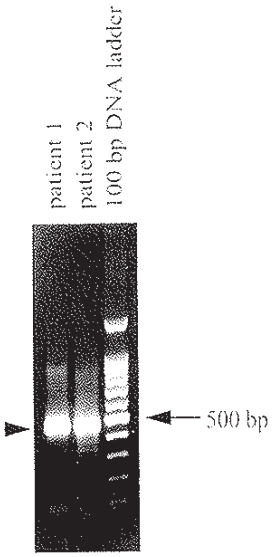

Figure 1. Amplification of enterovirus 5' UTR sequence from the ligation mix. The sequence was amplified from 1 µl of ligation mix using T7 and reverse primers. The amplified DNA fragment (8 µl) was electrophoresed in a 1.5% agarose gel. 100 bp DNA ladder was used for size indication. Arrow head indicates the amplified DNA fragments of about 460 bp.

DNA fragments (~400 bp) were separated on 1% agarose gel and purified using silica particles (3). The purified DNA fragments (4 µl) were ligated into pGEM-T PCR cloning vector (Promega, USA), which contained T7 and SP6 promoter sequences flanking the multiple cloning sites, for 16 hours at 15°C. Following day after, 1 µl of the ligation mix was used for a second step amplification using T7 universal primer and the Coxsackievirus reverse primer (Figure 1). The amplified DNA fragments (~460 bp, including 60 flanking nucleotides from the vector) were purified from agarose gel using silica particles, followed by column purification using Wizard™ PCR Preps DNA Purification System (Promega, USA) prior to sequencing. Using this method, the ligation mix could be fully utilized to prepare enough

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DNA fragments for direct sequencing (manual or automated) instead of the viral RNA which was limited. Furthermore, the use of T7 primer in the PCR enabled some of the vector sequences to be amplified together with the insert DNA and when the T7 primer was used in sequencing, full length nucleotide sequence of the gene of interest was obtained (4). In addition, the T7 primer used for sequencing was provided free by the automated sequencing service center (ACGT Inc., USA), therefore, preclude the need to synthesize expensive sequencing primers. In some cases, when sequencing were needed from the reverse end, the SP6 primer, another universal primer given free by the automated sequencing service centers was used. The method as outlined in Figure 2 is rapid and simple where only 2 days are needed in comparison to 3-5 days for cloning, transformation, and the messy screening steps. The saved viral RNA can also be used for other investigations.

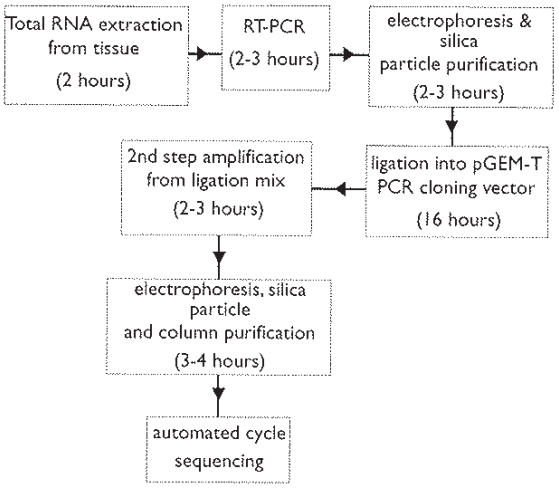

Figure 2. A rapid and simple method for preparation of template for automated cycle sequencing of resource limited specimens.

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