



Short Communication

Band-cutting no more: A method for the isolation and purification of target PCR bands from multiplex PCR products using new technology

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ABSTRACT

A procedure for the isolation of target PCR bands from multiplex PCR products using the E-Gel[®] iBase Power System, an E-Gel[®] Safe Imager[™] Real-Time Transilluminator, and E-Gel[®] CloneWell 0.8% SYBR Safe[™] agarose cassettes is presented. The collected isolates are suitable for direct use in sequencing reactions without need of further purification. Reductions in time spent per sample and cost per sequence produced compared to traditional “band-cutting” methods are demonstrated. An added benefit is that the new procedure does not require exposure to ethidium bromide or ultraviolet radiation.

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1. Introduction

The utilization of DNA sequences as characters in phylogenetic analysis has become commonplace among systematists. The availability of thousands of informative characters for a wide range of taxa makes DNA sequences a valuable source of information. PCR amplification followed by direct Sanger sequencing of PCR products (Sanger et al., 1977) is the most common protocol in molecular phylogenetic projects. In molecular phylogenetic research, the vast majority of specimens represent species that are being sequenced for the first time. Sequencing specimens from widely separate evolutionary lineages necessitates the use of universal primers (Palumbi, 1996). While these primers allow the amplification and sequencing of a target gene region for never-before-sequenced taxa, they introduce the possibility of producing non-specific PCR products. Also, unidentified introns in protein-coding genes and expansion segments in ribosomal genes can result in target gene regions that vary greatly in size between specimens (Palumbi, 1996). For this reason, the sequence length of the target gene segment may not be known for a particular specimen. To facilitate sequencing, it is necessary to isolate only the target PCR product from non-specific, multiplex PCR products. This process usually involves “cutting bands”; extracting PCR product of inter-

est from an agarose gel, followed by purification and sequencing (Whiting, 2002). This process often results in low concentration of amplified DNA and subsequently low sequencing success. In addition, isolation and sequencing of specific PCR bands from multiplex PCR products via traditional band-cutting is often cost-prohibitive for a large number of specimens. An alternate method has been proposed which makes this process much simpler by using a custom-made combination of regular and low-melting point (LMP) agarose (Ma and DiFazio, 2008). This alternate protocol, however, does not reduce exposure to ethidium bromide and ultraviolet radiation, both known carcinogens.

We have developed a procedure using existing technology that removes the necessity of cutting bands or fabricating custom-made gels. The procedure involves the use of the E-Gel[®] system (Invitrogen[™], Carlsbad, CA, USA). This system uses pre-cast E-Gel[®] CloneWell 0.8% SYBR Safe[™] agarose gels, an E-Gel[®] iBase[™] Power System, and an E-Gel[®] Safe Imager[™] Real-Time Transilluminator. This system was initially designed for the isolation of cloning products. We have found that it is possible to load the products of PCR amplification reactions into the gels and run them using the pre-programmed CloneWell voltage program. The system allows migrating DNA product to be constantly monitored via SYBR Safe[™] DNA staining and an LED transilluminator. As the PCR product migrates through the gel, multiple bands of DNA product can be determined in real-time. A lower well in the gel is filled with water when the PCR product is loaded. It is from this lower well that the desired DNA band can be extracted directly. It is possible to stop the running program at any time in order to extract from the lower well. It is also possible to reverse the voltage with a reverse

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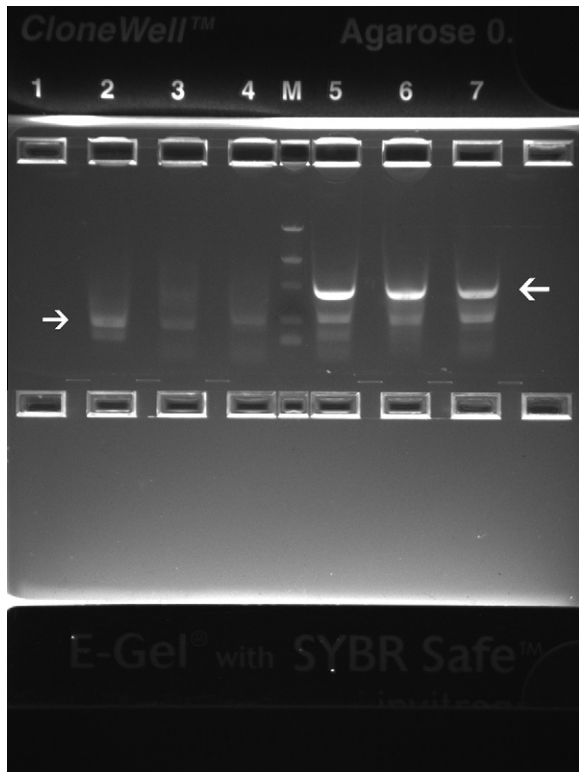


Fig. 1. PCR reaction products loaded on a pre-cast E-Gel[®] CloneWell 0.8% SYBR Safe[™] agarose gel and run on the E-Gel[®] iBase[™] Power System and E-Gel[®] Safe Imager[™] Real-Time Transilluminator using the CloneWell program setting. Lane components (product/template): 2, AATS/Chloropidae; 3, AATS/Pipunculidae; 4, AATS/Syrphidae; M, DNA ladder; 5, CAD/Chloropidae; 6, CAD/Pipunculidae; 7, CAD/Syrphidae. Note: lanes 1 and 8 are left blank for picture clarity. Arrows indicate the target PCR product extracted and used for sequencing reactions.

program should the desired product migrate beyond the lower well. The extract from the lower well can be used as template in a sequencing reaction without need of further purification. The method also allows for the isolation and sequencing of more than

one band from multiplex PCR products as each band can be extracted separately as it passes through the lower well. To reduce the potential of contamination of previous bands when extracting multiple bands from the same multiplex PCR product, the product could be divided amongst many different lanes in the gel. The purpose of the present research is to determine the viability of the new procedure compared to conventional band-cutting protocols.

2. Materials and methods

We tested our procedure using pre-cast 8-well cassettes. Primer pairs for two different gene regions with a history of producing multiplex PCR products (*alanyl-tRNA synthetase* (AATS) and the *carbamoyl phosphate synthetase region of CAD* (CAD)) were tested for each of three specimens representing different fly families (Chloropidae, Pipunculidae, and Syrphidae). For each combination of primer and taxon, a 50 μ L PCR reaction using 4 μ L of genomic template was completed. The resulting PCR product was divided in half. Twenty-five microliters of PCR product was loaded into pre-cast E-Gel[®] CloneWell 0.8% SYBR Safe[™] agarose gels. The CloneWell program was selected and run until the band of interest had migrated to the lower well, where it was extracted (Fig. 1). The remaining 25 μ L of each PCR product was loaded onto 2% LMP agarose gel for visualization. Desired bands were extracted using Gene Catcher gel excision tips (Diamed Lab Supplies, Mississauga, ON, Canada) under UV exposure. Extracted gel bands were purified using QIAquick Gel Extraction kit[®] protocols (Qiagen Inc., Santa Clara, CA, USA). Four microliters of either E-Gel extract or Qiaquick Gel purified product were used as template in a 10 μ L sequencing reaction with a BigDye Terminator v3.1 Cycle Sequencing kit on an ABI 3130x DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed at the Agriculture & Agri-Food Canada Eastern Cereal and Oilseed Research Centre Core Sequencing Facility (Ottawa, ON, Canada).

3. Results and discussion

Five of six combinations of gene and specimen produced readable sequence (Table 1). In four out of five combinations, the

Table 1

Summary of sequencing results for three Diptera specimens and two gene segments using conventional band-cutting and E-Gel protocols.

Specimen	Specimen number	Treatment	#bp sequenced	
			AATS	CAD
Chloropidae	JSM3847	Band-cutting	560	1400
		E-Gel	560	1500
Pipunculidae	JSM3921	Band-cutting	570	1500
		E-Gel	570	1500
Syrphidae	JSM3934	Band-cutting	N/A	1500
		E-Gel	N/A	900

N/A – Sample did not sequence successfully.

Table 2

Summary of sequencing success with conventional band-cutting and E-Gel protocols for six different gene segments.

Gene segment	E-gel success (#)	E-gel failure (#)	E-gel success rate (%)	Band-cutting success (#)	Band-cutting failure (#)	Band-cutting success rate (%)
<i>cytochrome b</i>	6	0	100	10	2	83
<i>wingless</i>	7	4	64	34	153	18
<i>elongation factor-1 alpha</i>	16	8	67	48	57	46
<i>alanyl-tRNA synthetase</i>	6	0	100	30	14	68
<i>CAD</i>	161	95	63	113	443	20
<i>28S rDNA</i>	18	0	100	48	49	49
Mean success rate		67			28	

new protocol produced sequence as long as or longer than conventional band-cutting protocols. The time required to complete the new protocol for eight specimens is 30 min. The time required to complete the conventional band-cutting protocol for eight specimens is 130 min. The cost of each 8-well pre-cast E-Gel® CloneWell 0.8% SYBR Safe™ agarose gel is \$13.78 with no further purification costs. The cost of LMP agarose, gel-cutting pipette tips, and spin column gel extraction kits averages to \$21.60 for eight specimens. There is an initial cost of \$1104 for the E-Gel® iBase™ Power System and E-Gel® Safe Imager™ Real-Time Transilluminator.

We have successfully used this new protocol to recover sequence data from six different genes that have proven difficult to sequence in the past due to multiplex PCR products (*28S rDNA*, *cytochrome b*, *AATS*, *wingless*, *elongation factor-1 α* , *CAD*). We have made over 1300 attempts at isolating and sequencing PCR products using either conventional band-cutting or our new protocol (Table 2). The overall average sequencing success rate has increased from 28% using conventional band-cutting to 67% using our new protocol. The sequencing success rate has improved for all six individual genes. The sequencing success rate for individual genes has improved by as much 46% (*wingless*).

Our new procedure allows sequencing of target gene segments with a substantially improved success rate compared to conventional band-cutting protocols. The use of SYBR Safe™ DNA staining and LED transillumination means that no exposure to ethidium bromide or UV is necessary. The time required to produce template

suitable for sequencing is reduced by approximately 77% compared to conventional band-cutting protocols. The cost to process eight samples from PCR product to readable DNA sequence is reduced by approximately 36%. If labour costs are included, the cost savings per sample are even greater. For labs seeking to obtain a large number of sequences for phylogenetic study, our new method presents considerable time and financial savings.

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