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TECHNICAL NOTE

Universal protocol for generating 100bp size standard for endless usage

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Abbreviations: AFLPs: Amplified Fragment LengthPolymorphisms
EDTA: Ethylene-Diamine-Tetra-Acetic acid
MCS: Multiple Cloning Site
PCR: Polymerase Chain Reaction
RAPD: Random Amplified Polymorphic DNA
SNPs: Single Nucleotide Polymorphisms

Developing countries are facing severe bottlenecks in the technological advancement in biotechnology, due to restrictions imposed by patent protected products and protocols. This calls for designing of simple and cost-effective alternatives for the indispensable products like DNA molecular weight markers. We demonstrate a novel, rapid and cost-effective method of making in-house 100bp ladder for routine use. In our method we use a single forward primer and five reverse primers designed on the backbone sequence of a commonly used vector template. These primers are used at a universal annealing temperature to amplify ten DNA fragments of accurate size ranging from 100bp to 1000bp. Our PCR-based method can provide size standards for an endless usage.

The prospect of high-throughput tools in the post-genomic era has revolutionized the field ofmolecular biology. DNA chip-based assays, micro arrays, sequencing and TaqMan probes have rapidly increased the pace of genomic research. Developing countries have not been able to keep up the same pace in scientific growth as compared to the developed countries, due to the technological gap. Microsatellites, AFLPs, SNPs and RAPDs are some of routine molecular techniques that are extensively used in genotyping. The developed countries have transformed from routine gel electrophoresis to capillary platforms while developing countries still use routine gel electrophoresis for their genotyping activities. In any molecular biology laboratory the use of size standard for gel electrophoresis is a prerequisite. Most of the research groups involved in large-scalemolecular biology studies needs to spend a sizable fraction of their research funds in acquiring these size standards.

Most of the conventional methods of generating size standards either require tedious cloning with several tandem repeats to construct special vectors followed by partial digestion of these vectors or restriction digestion of Lambda phage viruses (Sambrook et al. 1989). These restriction fragments require specialized treatments to dephosphorylate the ends to prevent self-ligation. These enzymatic treatments are prone to failures for various reasons. Therefore, in order to minimize the problems of reproducibility and minimize thecost on size standards we have developed a rapid and cost effective method which can provide us endless usage of size standards for routine laboratory use.

MATERIALS AND METHODS

We demonstrate an authentic method for generating DNA fragments that can be used as size standards for routine gel electrophoresis. In order to make our system more accessible to researchers we selected a universal TA cloning vector, the pGEM®-T vector system (Promega) as our template, so that the PCR products of required sizes can be cloned. We generated 100 - 1000bp fragment sizes using two templates. 100 - 500bp fragment sizes were generated using the self-ligated pGEM®-T vector template while 600 - 1000bp fragment sizes were generated from the recombinant pGEM®-T vector template containing a 500bp insert. The self ligated pGEM®-T vector was prepared by end filling the 3' overhang of linearized vector by adding 5U of T4 DNA Polymerase (NEB), 1X T4 DNA polymerase buffer along with 100 µM of each dNTP in a total reaction volume of 30 µl. Mixture was incubated in 37ºC for 5 min and the reaction was terminated by adding 2 µl of 0.5M EDTA (Sambrook et al. 1989). The vector was purified using GFX column and self-ligated. The second recombinant pGEM®-T vector template was prepared by cloning a 504 bp PCR product (hereinafter mentioned as approximate 500bp fragment) obtained from our earlier study into pGEM®-T system (Chandrasekhar et al. 2005). The recombinant plasmid was isolated from the positive clone and subsequently sequenced to confirm the exact insert size.

The primers were designed on the pGEM®-T vector sequence backbone, based on the anticipated product sizes. We consecutively reduced the cost by selecting a single common forward T7 primer along with five reverse primers. All these primers were used for both the vector templates, *i.e.* the self ligated template and the recombinant plasmid with approximate 500bp insert. These five primers were designed strictly based on the product size (Figure 1a). The primer sequences areshown in Table 1. The 100R primer was designed carefully flanking the MCS, without hampering the T overhangs. While designing the reverse primers, the primer length was adjusted to get an optimum annealing at universal temperature condition.

PCR amplification was carried in a total volume of 30 µl.Each reaction consisted of 1X Taq buffer with 1.5 mM $MgCl_2$, 1.2 U of Taq polymerase (BG), 0.25 mM of dNTPs (Amersham) and 10 pM of primer per reactions. Amplifications were performed in similar cycling conditions in thermocycler (BioRad) and programmed as follows: initial denaturation at 95ºC for 3 min, followed by 45 cycles of at 95ºC for 30 sec, annealing at 50ºC for 30 sec, extension at 72ºC for 30 sec, and final extension at 72ºC for 5 min, and held at 4ºC. The amplification products were separated using 1.5% agarose gel in 0.5X TBE buffer and stained with ethidium bromide visualized using a Fluor-S Multi Imager (BioRad). The molecular weight of each band was estimated by comparing with a co-migrating 1 kb plus ladder (Invitrogen) and 100bp ladder (NEB).

RESULTS AND DISCUSSION

The crux of this method is the amplification of ten DNA fragments of accurate size ranging from 100bp to 1000bp using two vector templates with minimal primers at universal PCR conditions. Among the two vector template,

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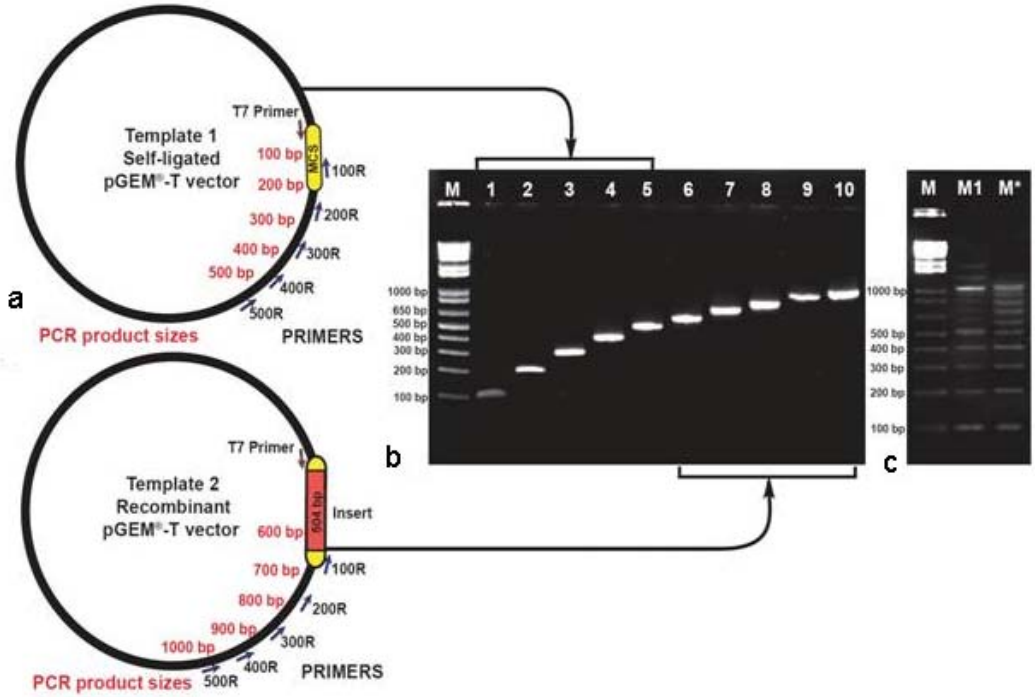

Figure 1. Schematic representation of generating 100bp ladder.
Figure 1a. Schematic representation of primers located on pGEM®-T vector system. Arrow indicates primers and its distance in the vector backbone while the bp indicates the corresponding fragment sizes that are generated using the corresponding primers.
Figure 1b. Agarose gel picture showing the amplified PCR products of the in house ladder.
Figure 1c. Agarose gel picture showing the commercial ladder Lane M (1kb plus ladder Invitrogen) lane M1 (100bp ladder NEB) and lane M*- (In-house 100bp ladder).

one was a commercial pGEM®-T vector which was modified to form a self-ligated vector while the second vector template was a recombinant pGEM®-T vector having an approximate 500bp insert. The self-ligated vector was used to generate five fragments ranging from 100 to 500bp and the second recombinant template which had 500bp insert was used to generate 600bp to 1000bp size. We were able to optimize the PCR amplification for both the templates at similar cycling condition using a common forward T7 primer and five different reverse primers namely 100R, 200R, 300R, 400R and 500R (Figure 1b) to generate fragment sizes of 100bp to 1000bp fragments. A total of ten fragments were amplified with their respective primer pairs in separate tubes in a single shot and the PCR products were mixed together to create the DNA ladder. The intensity of banding patterns was similar for all bands irrespective of the sizes. These PCR amplicons were quantified by spectrophotometer and reconstituted to suite our convenient concentration. The band sizes were estimated by gel electrophoresis along with a co-migrating commercial 100bp ladder from NEB and1kb plus ladder from Invitrogen in 1.5% agarose gel (Figure 1c).

As per our method once the self ligated pGEM®-T vector and the recombinant pGEM®-T vector is prepared, then using the described primers, the 100bp size standard can be generated in-house for endless usage. This method can be developed and used by any individual with their respective inserts in their own laboratory. The vectors can be propagated whenever required. Thus by adopting this method, researcher can be ensured of an unlimited supply of 100bp ladder at low cost. Additionally, customized ladder can be obtained by varying the size of insert fragment as well as varying the primer combinations according to the need of the researcher. However, the most advantageous part is that, one requires the same set of primers and similar PCR conditions for generating the ladders by varying the recombinant clones with 500+, 1000+bp insert fragments.

We found our system to be very simple and cost effective when compared to other commercial systems. Endless usage of size standards is available from only Seegene (www.seegene.com) which uses 12 different templates and two primers for each template. While conventional size standards available from Sigma, Promega, New England Biolabs, Amersham, Boerhinger Mannheim, Stratagene and Invitrogen require tedious cloning with several tandem repeats to construct special vectors. These vectors are fragmented to required size with specific restriction enzymes. This is prone to errors leading to poor reproducibility. We calculated the projected amount of money spent on procuring the commercial size standards for a period of ten years and found our size standard system to be the most economical and less labor intensive when compared with other systems (data not shown). Our system

Table 1. Primer sequences used in the study.

Primer name	Sequence 5'-3'
100R	5' GCGGCCGCGAATTCACTAG 3'
200R	5' CAGCTATGACCATGATTACG 3'
300R	5' GTTAGCTCACTCATTAGGCA 3'
400R	5' CAAACCGCCTCTCCC 3'
500R	5’ CGTATTACCGCCTTTGAG 3’
T7	5’ TAATACGACTCACTATAGGG 3’

happens to be 4-fold cheaper than a similar kind of product available from Seegene. We would like to emphasize the utility of our work and its impact on the overall budget of a typical molecular biology laboratory in developing countries and make them available through open access to all researchers. This work is an outcome of our realization to self-sufficiency which prompted us to devise this method through innovative thinking. Our aim was not to compete with the existing companies, instead to encourage underprivileged institutions to carry forward their research by generating their own size standards.

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