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Success of the PCR-based replication assay depends on the number of methylation sensitive restriction sites in the PCR amplifying region

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Abstract

The PCR-based replication assay is one of the most simple, quick and economical methods for the analysis of episomal replication. However, in spite of its advantages the method has not been able to replace the southern-based replication assay, the latter of which is a tedious and time-consuming process. This is due to the generation of spurious amplification products in the PCR-based replication assay. The replication assay is based on the use of methylation-sensitive restriction endonucleases (eg. *DpnI, MboI*) to distinguish bacterial replicated (adenosine methylated) and mammalian replicated plasmids (adenosine non-methylated). In this work we addressed the problem by evaluating (a) restriction enzyme digestion and (b) the minimum number of restriction sites that are required in the amplifying region. The efficiency of restriction digestion was tested by subjecting the plasmid to one and two rounds of digestion. Multiple rounds of digestions were found to be inefficient in preventing false positives when the number of *DpnI* sites in the amplifying region is less than 8. However, use of a minimum of 15 *DpnI* sites in the amplifying region was found to overcome the false positives.

Key words: Episomal, PCR amplification, Replication, Restriction digestion, Southern Hybridization, and Plasmids.

Introduction

Expression systems have played a major role in life science research since the emergence of recombinant DNA technology in the mid 1970s. They facilitate the expression of recombinant protein in a desired host cell line such as those of bacteria, yeast, insect and mammalian cells. Expression of recombinant protein in mammalian cells is often challenged by low expression. Episomal replication plasmids which replicate in permissive mammalian cells (rodent or human) have shown promise by increasing the copy number and giving high expression in these cells (1), (8). Episomal replicating vectors have several other advantages besides high expression; First, the inserted gene of interest cannot be interrupted or subjected to regulatory constraints which often occur from integration into cellular DNA. Secondly, the presence of an inserted heterologous gene does not lead to rearrangement or interruption of the cells own important regions. Thirdly, they provide a means to isolate the mammalian gene by expression cloning which is not otherwise possible in other host cell lines (6) and finally, in stable transfection procedures, the use of episomal vectors often results in higher transfection efficiency than the use of chromosome-integrated plasmids (7).

Replication, which is the key function of episomal replicating vectors, has often been analyzed by the southern based replication assay (16), (3), (5). The assay is based on distinguishing the bacterial replicated plasmid from that of the mammalian replicated plasmid with the use of methylation sensitive restriction endonucleases such as *DpnI* and *MboI* (12). Plasmid DNA prepared in DNA adenine methylase (dam) - positive bacteria is methylated at the adenine nucleotide of the GATC site, the recognition sequence for both *DpnI* and *MboI*. In contrast, mammalian cells lack the dam methylase enzyme and do not methylate adenine nucleotide of the GATC site. Hence, *DpnI*, which cleaves GATC recognition sites only if adenine nucleotides on both strands are methylated (11), will digest plasmid DNA but not newly replicated episomal DNA. Similarly, *MboI* cleaves the same recognition site only if both strands are unmethylated, a characteristic of DNA having a eukaryotic methylation pattern. The restriction pattern of the digested samples is then analyzed by southern hybridization which is often a tedious and time consuming process.

To simplify the analysis Mark J Cooper et al. (1994) have introduced a PCR-based replication assay (3). The assay helps in distinguishing bacterial and mammalian replicated plasmids by providing amplified product with mammalian replicated plasmid digested with *DpnI* but not bacterial replicated plasmid. Similarly, it provides amplified product with bacterial replicated plasmid digested with *MboI* but not mammalian replicated plasmid. Such PCR-based replication assays are valuable tools in; (A) evaluation of the replication function of episomal replicating plasmids (10), (B) quantification of replicated DNA using Real-Time PCR (10), (C) identification and isolation of new human origin replication sequences (4), and (D) investigation of the mechanism of gene replication (10). In spite of its advantages, the method is not able to substitute for the southern-based replication assay. This could be due to the generation of spurious amplification products during PCR ampli-

fication which is often a result of incomplete digestion. In the current study we have evaluated the efficiency of restriction digestion and the number of *DpnI* sites that are required in the PCR amplifying region to prevent false positive results in PCR based replication assays, thereby making this assay widely applicable and reliable for episomal replication plasmids.

Materials and methods

Materials

Bacterial strains JM109 (dam$^+$) was purchased from Promega Life Sciences (San Luis, CA, USA) and ER2925 (dam$^-$) was purchased from New England Biolabs (Ipswich, MA, USA). pUB-C-O plasmid, 1kb ladder (consisting of 10, 7, 5, 4, 3, 2, 1, 0.5 kb fragments) and a UB-Plasmid Purification Kit were purchased from Usha Biotech Pvt Ltd (Hyderabad, India). *DpnI* was purchased from New England Biolabs (Ipswich, MA, USA) and Taq DNA Polymerase and Ethidium Bromide were purchased from Bangalore Gene (Bangalore, KA, India). Six oligonucleotide primers were designed using the Vector NTI software (Invitrogen BioServices India Pvt Ltd, Bangalore, KA, India): forward primers included Rep-F1 (5'-aggacgaggcagcgcggcta-3'), Rep-F2 (5'-acctgtccggtgccctgaat-3'), Rep-F3 (5'-ggatcccgtaagggtagacacttca-3') and Reverse primers included Rep-R1 (5'-attcatcgactgtggccggc-3'), Rep-R2 (5'-tctaaaacttcccagacaacggatcc-3') and Rep-R3 (5'-aactggcttcagcagagcgc-3'). These primers were synthesized by Sigma Aldrich (Bangalore, KA, India).

DpnI digestion of pUB-C-O plasmid

Digestions were carried out according to manufacturer's instructions. pUB-C-O plasmid isolated from JM109 (dam$^+$) and ER2925 (dam$^-$) strains were digested with *DpnI*. In the first round of digestion 0.5 µg of pUB-C-O plasmid was digested with 20 U of *DpnI* in a 20 µL reaction volume. The reaction was carried out at 37°C for 2 h. Following the first round of digestion, 10 µL of digested sample was subjected to a second round of digestion with 20 U of *DpnI*. The reaction was carried out at 37°C for 1 h. Digested samples were analyzed on 1% agarose gel in 1x TAE buffer. Ethidium bromide stained gels were visualized using Mini BIS Pro Gel Documentation system (Biotron Healthcare (India) P. Ltd, Mumbai, MA, India).

PCR-based Replication Assay

The PCR-based replication assay often involves the digestion of Hirt extracted samples using methylation sensitive restriction endonucleases (*DpnI* and *MboI*) followed by PCR amplification (2). However, to simplify the study we used plasmid isolated from JM109 (dam$^+$), which methylates adenosine residue of the GATC sequence on both strands, and plasmid isolated from ER2925 (dam$^-$), which does not methylate the adenosine residue of the GATC sequence. Plasmids isolated from ER2925 represented the mammalian replicated plasmid for the study. We designed four sets of primers (Table 1) to evaluate the minimum number of *DpnI* sites that were required to prevent the false positive results. Using the four sets of primers PCR amplifications were carried out on *DpnI* digested pUB-C-O/JM109 and pUB-C-O/ER2925 plasmids. Briefly, PCR reactions were carried out in 20 µL reaction volumes with 50 pmol of each primer, 1 µL of digested sample, 1.25 mM $MgCl_2$, 1 U Taq DNA polymerase, 0.4 mM of each dNTP's and 1x PCR buffer. Initial denaturations were carried out at 94°C for 2 min, 30 cycles of amplification were performed using cycle times (first set of primers: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C), (second set of primers: 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C), (third set of primers: 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C) and (fourth set of primers: 1 min at 94°C, 1 min at 60°C, and 4 min at 72°C). A 10 min extension step at 72°C followed the PCR amplification. Amplification products were analyzed on 1% agarose gel in 1x TAE buffer. Ethidium bromide stained gels were visualized using MiniBIS Pro Gel Documentation system (Biotron Healthcare (India) P. Ltd, Mumbai, MA, India).

Results

In order to address the problems of the PCR-based replication assay, this study focused on the evaluation of *DpnI* restriction digestion and the number of *DpnI* sites that were required in a PCR amplifying region. Figure 1 shows a region of pUB-C-O plasmid which was used to design PCR primers for the replication assay. Six oligonucleotides were designed to amplify four different lengths of PCR products consisting of 5, 8, 15 and 21 *DpnI* sites (Table 1). To minimize the variation in PCR amplification among different sets of primers we first optimized the PCR amplification conditions for each set of primers by varying the temperature and $MgCl_2$ concentration (13). The optimized conditions resulted in efficient amplification of pUB-C-O plasmid with all the

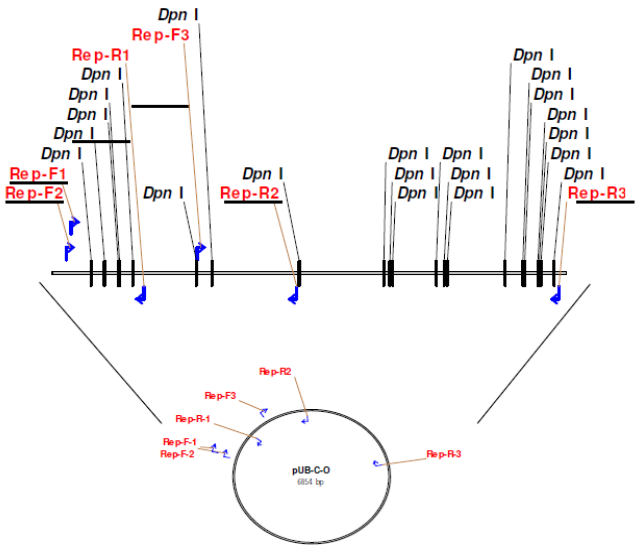

Figure 1. pUB-C-O plasmid map with the list of primers and *DpnI* sites: pUB-C-O is a mammalian replication plasmid. Primers shown here were designed to amplify four different lengths of PCR products spanning different numbers of *DpnI* sites. The first set of primers consists of Rep – F1 and Rep – R1, which amplifies the 453 bp fragment and contains 5 *DpnI* sites. The second set of primers consists of Rep – F2 and Rep – R2, which amplifies the 1381 bp fragment and contains 8 *DpnI* sites. The third set of primers consists of Rep – F3 and Rep – R3, which amplifies the 2138 bp fragment and contains 15 *DpnI* sites. The fourth set of Primers consists of Rep – F2 and Rep – R3, which amplifies the 2892 bp fragment and contains 21 *DpnI* sites.

Table 1. List of primer sets used to evaluate the number of *DpnI* sites required in the PCR amplifying region.

Primer Set	Forward primer	Reverse Primer	Length of product	Number of DpnI sites in the PCR amplifying region
First	Rep – F1	Rep – R1	453 bp	5 *DpnI*
Second	Rep – F2	Rep – R2	1381 bp	8 *DpnI*
Third	Rep – F3	Rep – R3	2138 bp	15 *DpnI*
Fourth	Rep – F2	Rep – R3	2892 bp	21 *DpnI*

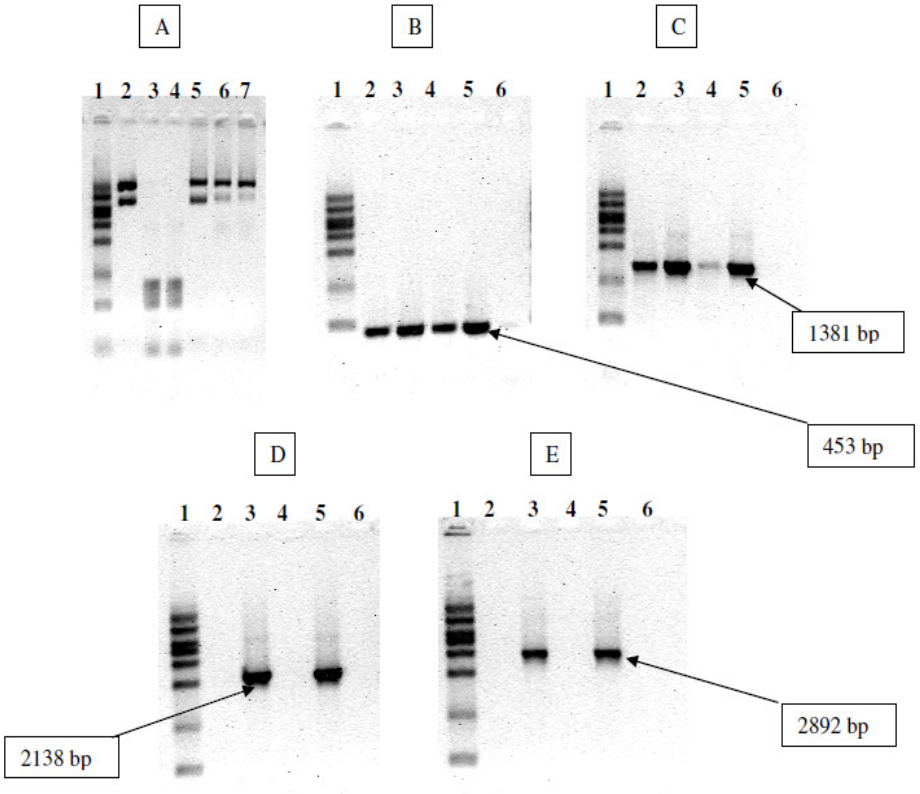

Figure 2. Evaluation of the effect of one and two rounds of *DpnI* digestion and the number of *DpnI* sites in a PCR amplifying region to minimize false positive during the PCR based replication assay: (A) First and second round of *DpnI* digestion of pUB-C-O plasmid isolated from JM109 (dam^+) and ER2925 (dam^-). Lane 1: 1 kb ladder, Lane 2: Supercoiled pUB-C-O/JM109, Lane 3: pUB-C-O/JM109-first digest, Lane 4: pUB-C-O/JM109-second digest, Lane 5: supercoiled pUB-C-O/ER2925, Lane 6: pUB-C-O/JM109-first digest, Lane 7: pUB-C-O/ER2925 second digest. (B-E) PCR amplification of *DpnI* digested pUB-C-O plasmid isolated form JM109 and ER2925. (B) PCR amplification of 453 bp fragment spanning 5 *DpnI* sites. (C) PCR amplification of the 1381 bp fragment spanning 8 *DpnI* sites. (D) PCR amplification of the 2138 bp fragment spanning 15 *DpnI* sites and (E) PCR amplification of the 2892 bp fragment spanning 21 *DpnI* sites. Figures B-E show, Lane 1: 1 kb ladder, Lane 2: pUB-C-O/JM109-first digestion, Lane 3: pUB-C-O/ER2925-first digestion, Lane 4: pUB-C-O/JM109-second digestion, Lane 5: pUB-C-O/ER2925-second digestion and Lane 6: negative control.

primer sets (Table 1) (Data not shown).

Restriction digestions using methylation sensitive restriction endonucleases play a vital role in PCR-based replication assays. Inefficiencies in restriction digestion could result in false positives due to the high sensitivity of PCR which amplifies even a single copy of undigested plasmid (9). To assess this pUB-C-O/JM109 and pUB-C-O/ER2925 were subjected to one and two rounds of *DpnI* digestion (Figure 2a). Following digestion, first and second round digested pUB-C-O/JM109 and pUB-C-O/ER2925 plasmids were used as templates for PCR amplification using four sets of primers. Figure 2B, C, D and E show that PCR amplification results vary between first and second round *DpnI* digested pUB-C-O/JM109 samples and four sets of primer amplifications using the same template. The first round of digested sample (pUB-C-O/JM109) gave false positive results with the first and second set of primers. However, the second round of digested sample (pUB-C-O/JM109) gave false positive results with the first set of primers indicating that multiple rounds of digestion are efficient in overcoming false positives to a certain extent. Comparison of different sets of primers using the same template showed that false positives could be minimized by increasing the number of *DpnI* sites in the PCR amplifying region. The minimum number of *DpnI* sites required in the PCR amplifying region was found to be 8 provided the digestion was efficient. This was shown by the lack of amplified product using a second round digested sample and a second set of primers. This could be one reason why Cooper et al. used a 1.6 Kb fragment containing 9 *DpnI* sites for their assay (15). The result of false positives due to inefficient digestion can be overcome by increasing the number of DpnI sites from 8 to 15. This was shown by PCR amplification of the first and second digested pUB-C-O/JM109 plasmid

using a third set of primers which gave no false positive results. The chances of other factors effecting PCR amplification of pUB-C-O/JM109 using a third and fourth set of primers was ruled out by the presence of pUB-C-O/ER2925 control which remained as an intact plasmid after *DpnI* digestion (Fig 2A) and gave PCR amplified product (Fig 2B, C, D and E). The presence of equally intense amplified product indicated that the PCR amplifications among different sets of primers were equally efficient.

The efficiency of restriction digestion is unpredictable as it can be affected by the template concentration and the time of restriction digestion. A study was carried out to evaluate the possibility of template concentration and the time of restriction digestion using third set of primers which showed promise in overcoming false positive results. For the template concentration related study 50 ng, 250 ng, 500 ng, 1000 ng of pUB-C-O/JM109 plasmid were digested with 20 U of *DpnI* for 2 h and digested samples were amplified using a third set of primers. For the time of restriction digestion study 500 µg of pUB-C-O plasmid was digested with 20 U of *DpnI* for 30, 60, 90 and 120 min and the digested samples were amplified using a third set of primers. Figure 3A and 3B show the digestion and amplification results of the pUB-C-O/JM109 plasmid for these two tests. Template concentration and time of restriction digestion did not give false positive amplified results with a third set of primers indicating that 15 *DpnI* sites can overcome the concentration and time dependent effects. The chance of other factors affecting PCR amplification was ruled out by the presence of amplified products with pUB-C-O/ER2925 plasmid which was subjected to similar reaction conditions.

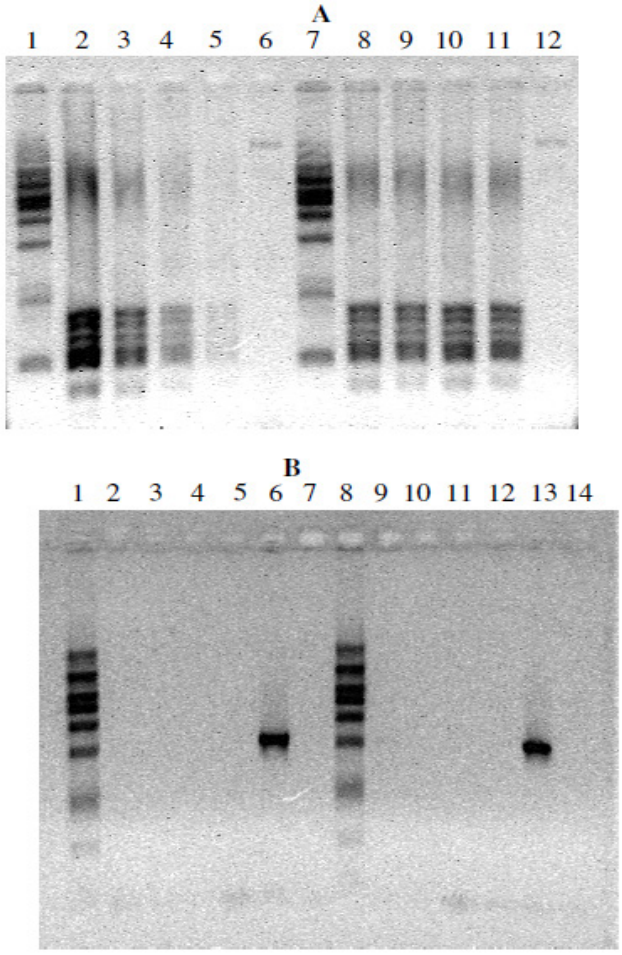

Figure 3. Effect of template concentration and restriction digestion time in minimizing false positives during the PCR based replication assay: (A) *DpnI* digested pUB-C-O/JM109 plasmids: Lane 1 and 6: 1 kb ladder, Lane 2 – 5: 1 µg, 500 ng, 250 ng, 50 ng pUB-C-O/JM109 digested with 20 U of *DpnI* for 2 h), Lane 8-11: 500 µg of pUB-C-O/JM109 digested for 30, 60, 90 and 120 min, Lane 6 and 12: 50 ng of pUB-C-O/ER2925 digested with 20 U of *DpnI*. (B) PCR amplification of *DpnI* digested pUB-C-O/JM109 plasmids using a third set of primers Rep – F3 and Rep – R3) which amplify the 2138 bp fragment and span 15 *DpnI* sites: Lane 1 and 8: 1 kb ladder, Lane 2–5: 1 µg, 500 ng, 250 ng, 50 ng of pUB-C-O/JM109 plasmid digested with 20 U of *DpnI*, Lane 6: 50 ng of pUB-C-O/ER2925 digested with 20 U of *DpnI*, Lane 7 and 14: negative control, Lane 9-12: 500 ng of pUB-C-O/JM109 plasmid digested for 30, 60, 90 and 120 min, and Lane 13: 50 ng of pUB-C-O/ER2925 digested for 120 min.

Discussion

Most of the studies related to the PCR based replication assay have made use of primers that amplify 1.5 to 2.5 kb fragments (3, 2, 14,15). However, none of them have provided clear evidence of why such large sequences have to be used in the assay. *DpnI* recognizes four nucleotides palindrome sequences (GATC) and it occurs quite frequently in any DNA sequence. Larger sequences will have a greater number of *DpnI* sites and the probability of restriction endonucleases cutting at least one site in the amplifying region is increased with an increase in the length of the PCR amplifying region. From our study it is clear that use of a minimum of 15 sites in the PCR amplifying region results in reproducible and reliable PCR based replication assays.

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