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Digestion of PCR and RT-PCR Products With Restriction Endonucleases Without Prior Purification or Precipitation

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We have tested 33 commonly used Promega restriction endonucleases (REs) for their ability to digest DNA directly in polymerase chain reaction (PCR) amplification buffers. In total, 29 (88%) of the REs showed complete digestion of the target DNA after overnight incubation in PCR buffer without the requirement for the addition of any other component. The remaining 4 (12%) REs required additional magnesium or the addition of restriction endonuclease digestion buffer to function adequately. The composition of the PCR buffers tested did not affect the results. The REs also functioned equally well in an RT-PCR Buffer. The results show that digestion of PCR and RT-PCR products may often be performed directly in the PCR tube without the requirement for any precipitation or purification steps. However, we do not recommend the use of this procedure for cloning applications.

Introduction

A common procedure in molecular biology is to perform restriction enzyme (RE) digestion of PCR or RT-PCR products. This is required for restriction fragment length polymorphism (RFLP) analysis (1,2), to allow single-stranded conformational polymorphism (SSCP) analysis of large PCR products (3,4), as well as to allow cloning of the amplified DNA (5).

There are a wide variety of commercial kits and techniques available for purification of PCR products. However, these techniques may be time- consuming and expensive, particularly if large numbers of samples are to be analyzed. The purpose of this study was to determine if REs retained sufficient activity in PCR buffers to allow digestion of the PCR product by addition of the enzyme directly into the tube immediately after the amplification step, without the requirement for the addition of any other reagents or for purification of the PCR product.

Restriction digestion of lambda DNA

Our aim was to determine what conditions were required to allow REs to function adequately in PCR Buffer with minimal alteration to the buffer by addition of extra reagents (e.g., Mg^{2+}, NaCl, Tris-HCl, KCl). We considered that the enzyme functioned adequately for routine laboratory use if the target DNA was completely digested after overnight incubation. Obtaining a partial digest after 1 hour was not considered a problem because this could be readily overcome with the addition of more enzyme or by performing an overnight digestion.

We first examined the ability of 33 commonly used REs (Table 1), obtained from Promega Corporation, to digest unmethylated lambda DNA in various enzyme buffers. The enzymes were tested for their ability to digest the target DNA in 1 or 18 hours, under the standard set of conditions described in Figure 1. The digestion products were then resolved by agarose gel electrophoresis. The ability of the RE to function in different buffers was measured by comparing the resulting restriction pattern to the pattern obtained using the recommended restriction enzyme buffer.

Table 1. Restriction Enzymes Examined.

Aat II	*Bgl* II	*EcoR* I	*Hinf* I	*Msp* I	*Sal* I	*Taq* I
Alu I	*BstO* I	*Hae* III	*Hpa* II	*Pst* I	*Sau3A* I	*Xba* I
Ava I	*Bsp1286* I	*Hha* I	*Kpn* I	*Pvu* II	*Sma* I	*Xma* I
Ava II	*BstX* I	*Hinc* II	*Mbo* I	*Rsa* I	*Stu* I	
BamH I	*Dra* I	*Hind* III	*Mbo* II	*Sac* II	*Ssp* I	

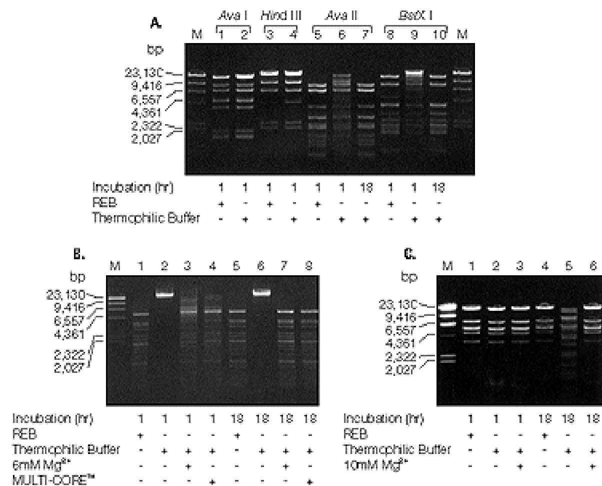

Figure 1. Representative digests of lambda DNA in various buffers. One microgram of unmethylated lambda DNA was digested with 1 unit of RE in 50µl of the indicated buffer at the appropriate assay temperature. After 1 hour, a 25µl aliquot was removed and added to 5µl of stop buffer (25% glycerol, 0.5% SDS, 0.2% Orange-G, 50mM EDTA). The remaining 25µl was incubated for an additional 17 hours, then 5µl of the stop buffer added to the reaction. All tubes were heated to 65°C for 15 minutes, then quick-chilled on ice immediately prior to agarose gel electrophoresis (1% agarose gels in 1X Tris-Acetate-EDTA buffer). The gels were visualized by staining with ethidium bromide. Lanes marked M contain molecular weight markers (*Hin*d III-digested lambda DNA). **Panel A:** Digestion of lambda DNA with the indicated REs in the recommended REB or Thermophilic Buffer supplemented to 2mM Mg^{2+}. **Panel B:** Lambda DNA digested with *Bsp*1286 I in the recommended REB, Thermophilic Buffer containing 2mM Mg^{2+}, Thermophilic Buffer supplemented to 6mM Mg^{2+} or Thermophilic Buffer containing 2mM Mg^{2+} and 0.5X MULTI-CORE™ Buffer. **Panel C:** Lambda DNA digested with *Eco*R I in the recommended REB, Thermophilic Buffer containing 2mM Mg^{2+} or Thermophilic Buffer supplemented to 10mM Mg^{2+}. Note the star activity (Lane 5) is abolished by the addition of extra Mg^{2+} (Lane 6).

The buffers analyzed were as follows: (i) the Promega Restriction Enzyme Buffer (REB) provided with each enzyme, (ii) Promega's Thermophilic Buffer (with a 1X composition of 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton® X-100; Union Carbide) supplemented with 0.2mM dNTPs and 2mM Mg^{2+}, (iii) Promega's Thermophilic Buffer supplemented with 0.2mM dNTPs and 6-10mM Mg^{2+} (to match the Mg^{2+} concentration of the recommended REB) and (iv) Promega's Thermophilic Buffer supplemented with 0.5X Promega MULTI-CORE™ Buffer [a restriction enzyme buffer designed for broad compatibility with many REs (12.5mM Tris-acetate (pH 7.8), 50mM potassium acetate, 5mM magnesium acetate and 0.5mM dithiothreitol)].

The results obtained for each enzyme are provided in Table 2. As indicated, 21 of 33 (64%) of the enzymes functioned as well in the Thermophilic Buffer as they did in the recommended REB, exhibiting complete digestion of lambda DNA in 1 hour (Figure 1A, Lanes 1-4). A further 8 (24%) of the enzymes exhibited slightly reduced activity, but completely digested the DNA if the incubation was extended to 18 hours (Figure 1A, Lanes 5-10). Only 4 (12%) enzymes required addition of other components to the Thermophilic Buffer to facilitate digestion (Figure 1B). The activity of *Bsp*1286 I and *Sal* I was significantly increased by the addition of magnesium to a final concentration of 6mM, with both showing complete digestion after 18 hours. The supplementation of Thermophilic Buffer with MULTI-CORE™ Buffer to half of its normal final concentration also dramatically improved the activity of *Bsp*1286 I, *Sal* I and *Sau*3A I, with the latter two enzymes showing complete digestion in 1 hour using this buffer combination. *Bsp*1286 I appeared to be the enzyme least active in Thermophilic Buffer, with complete digestion of the lambda DNA only attained after an 18 hour incubation in the modified buffers (Figure 1B). *Eco*R I was the only RE tested that exhibited detectable star activity in Thermophilic Buffer, which was observed after an 18 hour digestion. This was readily abolished by the addition of magnesium ions to a final concentration of 10mM (Figure 1C), or by the addition of MULTI-CORE™ Buffer to half of its normal final concentration. With either adjustment to the Thermophilic Buffer, the activity shown by *Eco*R I was equivalent to that in the REB recommended for the enzyme.

Table 2. Activity of Restriction Endonucleases in Promega's Thermophilic Buffer[a].

Restriction Enzyme	Enzyme Activity
Aat II, *Alu* I, *Ava* I, *Bam* H I, *BstO* I, *Dra* I, *Hae* III, *Hha* I, *Hinc* II, *Hind* III, *Hpa* II, *Kpn* I, *Msp* I, *Pvu* II, *Rsa* I, *Sac* II, *Sma* I, *Stu* I, *Taq* I, *Xba* I, *Xma* I	Full[b]
Ava II, *Bgl* II, *BstX* I, *Hinf* I, *Mbo* I, *Mbo* II, *Pst* I, *Ssp* I	Slightly Reduced[c]
*Bsp*1286 I[e], *Sal* I[f], *Sau*3A I[g]	Reduced[d]
EcoR I	Star Activity[h]

[a]Thermophilic Buffer containing 0.2mM dNTPs and 2mM Mg^{2+}.

[b]Activity equivalent to that seen in the appropriate REB after 1 hour incubation.

[c]Incomplete digestion after 1 hour incubation but complete digestion after 18 hour incubation.

[d]Incomplete digestion after 18 hour incubation.

[e]Cut lambda DNA completely after 18 hours if the Mg^{2+} concentration was increased to 6mM or 0.5X MULTI-CORE™ Buffer added.

[f]Cut lambda DNA completely after 18 hours if the Mg^{2+} concentration was increased to 6mM or after 1 hour if 0.5X MULTI-CORE™ Buffer was added.

[g]Cut lambda DNA completely after 1 hour if 0.5X MULTI-CORE™ Buffer was added.

[h]Star activity was seen only at 18 hours. The addition of extra Mg^{2+} to 10mM or 0.5X MULTI-CORE™ Buffer abolished the star activity and cut lambda DNA completely within 1 hour.

Restriction digestion of PCR and RT-PCR products

To determine whether other factors in a PCR reaction affected RE activity (e.g., the presence of *Taq* DNA polymerase or oligonucleotide primers), digestions were performed on PCR- and RT-PCR-amplified DNA. In the PCR experiments, a 549bp fragment from a cDNA clone of the T48 strain of Ross River virus (GenBank®Accession Number K00046; USDHHS) was amplified using primers described elsewhere (6). In the RT-PCR experiments, described in Figure 2, the same 549bp fragment of Ross River virus was generated from viral mRNA using the same set of primers. Following completion of the PCR, the amplified DNA fragments were digested with the same REs as described above. A comparatively large amount of enzyme (10 units) was added to each tube because a PCR reaction generates in the order of 5µg of DNA, compared to the 1µg of lambda DNA that was used to assess enzyme activity.

Figure 2. RT-PCR products digested with restriction enzymes. The RT-PCR reaction was performed in a final volume of 25µl containing 20pg Ross River viral RNA (6), 67mM Tris-HCl (pH 8.9), 50mM KCl, 6mM EDTA, 0.1% Triton® X-100, 0.5mM DTT, 2mM $MgCl_2$, 100µM dNTPs, 2µg tRNA, 5pmol of each primer, 1 unit *Taq* DNA polymerase and 0.5 unit AMV reverse transcriptase. First strand cDNA was synthesized in a preliminary incubation at 42°C for 30 minutes (6). Amplifications were performed on a MJ Research PTC-100 thermocycler fitted with a hot bonnet (Watertown, MA, USA). The thermocycle was 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, for a total of 35 cycles. The initial denaturation and final extension steps were extended to five minutes. Immediately after completion of the amplification, the tubes were chilled to 4°C and 10 units of each enzyme known to cut the 549bp fragment were added to the PCR tubes, one enzyme per tube. After incubating the samples for 18 hours at 37°C, the digestion was stopped as described in Figure 1. Samples were analyzed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. The amplification products were digested with the following REs (predicted fragment sizes (bp) are given in parentheses after each enzyme): Lane 1, *Alu* I (300, 219, 21, 10); Lane 2, *Ava* II (322, 228); Lane 3, *Bsp*1286 I (426, 124); Lane 4, *Hae* III (461, 89); Lane 5, *Hin*f I (345, 114, 79, 12); Lane 6, *Hpa* II (253, 174, 123); Lane 7, *Mbo* I (300, 172, 47, 31); Lane 8, *Msp* I (253, 174, 123); Lane 9, *Pvu* II (321, 229); Lane 10, *Rsa* I (339, 76, 64, 33, 30, 8); Lane 11, uncut (549); Lanes M, Promega PCR Markers (Cat.# G3161).

The 549bp fragment generated by PCR (data not shown) and RT-PCR (Figure 2) both digested exactly as predicted from the sequence, indicating that the components of the Thermophilic Buffer and RT-PCR Buffer do not significantly affect the activities or specificities of the REs. In some cases, a small proportion of the RT-PCR product was refractory to digestion, even after the addition of extra RE or prolonging the digestion time (Figure 2, Lanes 2-4). This appeared to be due to the amplification product itself, and not to the method employed, since some undigested product still remained even when the product was purified by ethanol precipitation or passage through Microcon™ 30 columns (Amicon, Beverly, MA, USA) before restriction digestion (data not shown).

For 29 of the 33 (88%) enzymes analyzed, the REB consisted of 6-10mM Tris-HCl (pH 7.5-7.9), 6-10mM $MgCl_2$, 50-150mM NaCl or KCl, and 1mM DTT. Direct comparison of the Thermophilic Buffer and the RT-PCR Buffer with the typical REB shows wide variation in the components, particularly with respect to the pH, Tris-HCl concentration, magnesium concentration and the presence of Triton® X-100. This indicates that many REs are able to retain their specificity and a significant degree of activity despite a wide variation in buffer composition. The four enzymes that required supplements to improve their activity to an acceptable level were all from different REB groups. Other enzymes from those REB groups functioned well in the Thermophilic Buffer, suggesting that there is no obvious way to determine in advance whether a RE will retain sufficient activity and specificity in PCR buffers.

Summary

All 33 REs tested were capable of digesting lambda DNA to completion after an 18 hour incubation in Thermophilic Buffer. Only 4 enzymes required any sort of addition to the buffer to obtain adequate activity. Identical results were obtained in a completely different PCR buffer (containing 6mM Tris-HCl (pH 8.8), 16.6mM $(NH_4)_2SO_4$, 0.45% Triton® X-100, 0.2mM/ml gelatin, 0.2mM dNTPs and 2mM magnesium ions; obtained from BioTech International, Western Australia) (data not shown), and with REs obtained from other sources, despite the sometimes considerable variation in storage and assay buffers (data not shown).

This research shows that PCR and RT-PCR products may be readily digested directly in the PCR tube immediately after the completion of the amplification. This eliminates the requirement for expensive and tedious purification and/or precipitation steps to ensure that the amplified DNA is resuspended in an appropriate REB. This may be of particular benefit if radiolabeled PCR fragments are being manipulated. Furthermore, in some instances, double digestion of DNA may be simplified. Rather than performing one RE digestion, precipitating the DNA and then doing the second digest, or attempting to find a common buffer where both enzymes may only function at significantly reduced activity, the DNA could be resuspended in PCR buffer and digested with both enzymes simultaneously. This simplified method for RE digestion of PCR products will have applications in RFLP analysis and SSCP analysis of large PCR products. However, the activity of *Taq* DNA Polymerase in RE buffers may result in the addition of a single A nucleotide to the ends of restriction fragments. Therefore, we do not recommend the use of this procedure for cloning applications. Finally, RE digestion of DNA prior to PCR can be better performed in PCR buffer, as the target DNA could then be transferred to the PCR reaction in any volume desired, without concern that components of the digestion buffer, particularly magnesium, carried over into the PCR reaction will affect the reaction.

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