Optimizing DpnI Digestion Conditions to Detect Replicated DNA

BioTechniques 33:316-318 (August 2002)

In vitro replication systems are powerful tools for studying DNA replication and repair. Replication competent cellular extracts can be used with reporter plasmids that contain the simian virus 40 (SV40) DNA replication origin (2,3). Replication is initiated by the addition of the large T antigen (8). By using plasmid DNA prepared in bacteria expressing adenosine methylase as substrate, processive eukaryotic replication involving polymerase α, δ, and ε can be distinguished from gap-filling reactions mediated by other DNA polymerases such as polymerase β by the methylation status of the resulting DNA (Figure 1) (7). The bacterially derived plasmid will contain methyladenosine at the DpnI restriction site, GATC, making the plasmid susceptible to DpnI cleavage. The complete absence of methylation profoundly suppresses DNA cleavage by DpnI. However, after a single round of semi-conservative replication, the DNA is only hemimethylated. The susceptibility of hemimethylated DNA has not been well studied, though previous work (12) suggests that replicated DNA also may be susceptible to cleavage. We demonstrate that under conditions of enzyme excess, long duration digestion, or small DNA amounts, the hemimethylated DNA is susceptible to DpnI cleavage. The successful schism of DNA by a restriction endonuclease is directly proportional to the amount of enzyme and the duration of digestion, or inversely proportional to amount of substrate DNA. The relationship can be expressed as:

\[ D = \frac{[DpnI]}{[DNA]} \]

where “D” is the overdigestion, “t” is time in hours, [DpnI] is in units, and [DNA] is in micrograms. For the following experiments, the time was set at 1 h, and the amount of DNA was set at 0.05 μg. The methylation status of the DNA prepared from bacteria was not manipulated before digestion. We reasoned that most studies do not treat DNA with Dam methylase before use but depend on the bacterial modification. Digestions using 0.6, 0.3, 0.15, 0.075, and 0.018 U DpnI (Invitrogen, Carlsbad, CA, USA) were conducted in 50 mM KCl.

We used the methylase producing strain DH5α and the methylase-deficient strain GM2163 (dam and dcm) to propagate the shuttle vector pZ189 (9,13). DNA was prepared by alkaline lysis and purified by CsCl isopycnic banding, and dialysis against 10 mM Tris, 1 mM EDTA, pH 7.6. DNA was linearized with a 30-fold overdigestion with EcoRI (11). In separate reactions, 100 ng unmethylated and methylated pZ189 were radiolabeled using 20 U T4 polynucleotide kinase (Invitrogen), 0.06 μCi [γ-32P]ATP (NEN Dupont, Boston, MA, USA) and exchange buffer, according to the manufacturer’s directions. Hemimethylated DNA was prepared by mixing radiolabeled unmethylated or methylated DNA in separate aliquots of 1 μg unlabeled, methylated DNA so that there was a 10-fold excess of unlabeled, methylated DNA in 50 mM imidazole-HCl, pH 6.4, 12 mM MgCl₂. The tubes were incubated at 94°C for 5 min and allowed to cool slowly so that the radiolabeled templates would anneal to the unlabeled, methylated DNA. For DpnI digestions, 50 ng prepared DNA were used.

In vitro replication reactions are extremely helpful tools used to understand both replication and mutagenesis. The reaction contains a mixture of products that includes fully replicated plasmid, making purification necessary before analysis by gel electrophoresis or transfection. The reaction product

![Figure 1. Model of possible outcomes of an in vitro replication assay using the SV40 shuttle vector pZ189 (13).](image)

Semi-conservative replication would yield two plasmids that are hemimethylated, depicted by using a dashed line to represent the radiolabeled, newly synthesized, unmethylated DNA. Alternative, gap-filling, and repair reactions would not be fully semi-conservative, and only selected regions would contain labeled, newly synthesized, unmethylated DNA. Upon DpnI digestion, under the right conditions, the fully methylated plasmid would digest, while the hemimethylated DNA would be preserved.
methylation status has been used to selectively remove unreplicated products with the restriction endonuclease \textit{DpnI}. There are some difficulties in using \textit{DpnI} for this purpose. Sanchez et al. (12) reported digestion inconsistencies and demonstrated that the use of sodium chloride could reduce the digestion efficiency. They reported the necessity of adding 300 ng additional DNA to the reaction, in addition to 100 mM sodium chloride, to get consistent digestion results. They do not report the reaction time of \textit{DpnI} digestion, but if one assumes an hour, their overdigestion factor would be in the 6.5 range. These results are consistent with our results that demonstrate that hemimethylated DNA

![Figure 2. Digestion of hemimethylated and fully methylated DNA by \textit{DpnI} and \textit{Sau3AI}.

The top panel contains the undigested controls (H, hemimethylated; F, fully methylated) in the left two lanes, followed by the hemimethylated substrate. The bottom panel contains the results of the reactions using fully methylated DNA. The numbers above the lanes indicate the digestion factor described in the text. Fully methylated DNA is more susceptible to \textit{DpnI} than hemimethylated DNA. This difference vanishes with the methyl-insensitive \textit{Sau3AI}.

![Figure 3. Densitometric analysis of \textit{DpnI}-treated DNA. Band intensity was normalized using the input substrate intensity. Solid bars represent the hemimethylated DNA, while the open bars depict the fully methylated DNA. Intensity is expressed on the ordinate as percent of intensity of the undigested DNA. The abscissa is labeled with the overdigestion factor. The data represent the $\bar{x} \pm$ so of three experiments.]

Vol. 33, No. 2 (2002)
also can be cleaved by DpnI. A 12-fold overdigestion with DpnI results in hemimethylated DNA digestion that could be interpreted as suggesting replication failure, where in fact significant replication actually took place. Three- or 6-fold overdigestion digested the fully methylated DNA but preserved approximately half of hemimethylated product (Figure 2). The differential DpnI digestion of fully methylated DNA compared to hemimethylated DNA is not present when the methyl insensitive Sau3A1 is used on the two substrates. Comparing the DpnI digestion intensity to undigested DNA, the 3–6-fold overdigestions cleave roughly half of the hemimethylated form while completely eliminating the fully methylated form (Figure 3).

Discriminating replicated plasmid from plasmid with radioactively incorporated DNA by repair or short primer extensions is critical for interpreting in vitro replication studies. Underdigestion with DpnI will result in bands that have not been fully replicated, and overdigestion will result in loss of signal from fully replicated plasmid. Rao and Martin (10) have suggested that only the supercoiled band of DpnI-resistant plasmid represents actual replication, while other non-supercoiled bands may not represent true replication. This observation also could be compatible with an underdigestion by DpnI, or the digestion was sufficient to cleave the hemimethylated strand, leading to only cleaved and open circular molecules, though the concise nature of the manuscript precludes a definitive conclusion on these possibilities.

The SV40 replication system has illuminated a great deal of information about the role of the large T antigen and human replication protein A in replication (5,6). However, this is a specific viral system and is not shared across all eukaryotes. Unlike the yeast origin of replication, which contains a recognizable consensus sequence, mammals appear to have a more complex mechanism of origin recognition and initiation of replication (4). Clarifying the digestion requirements for DpnI detection of replicated DNA may simplify the analysis of potential mammalian origins of replication using an in vitro system. By working out precise parameters of the interaction of DpnI with hemimethylated DNA, such a technique may be used as a screen for origin-like activity before going to more arduous techniques such as 2-D replication gels (1,14).

REFERENCES

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Received 26 November 2001; accepted 16 April 2002.

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Optimized Sample Preparation for Tandem Capillary Electrophoresis
Single-Stranded Conformational Polymorphism/ Heteroduplex Analysis

BioTechniques 33:318-325(August 2002)

ABSTRACT
Here we describe DNA sample preparation methods that allow the rapid, simultaneous generation of both single-stranded conformational polymorphism (SSCP) and heteroduplex DNA elements from a single sample in a single tube, which are suitable for direct injection into a capillary electrophoresis (CE) instrument with excellent sensitivity of genetic mutation detection. The p53 gene was used as a model DNA region for this study, which was performed on a high-throughput MegaBACE™96-capillary array electrophoresis instrument. We found that, contrary to the practice common in slab-gel SSCP analysis, denaturants such as formamide are incompatible with this novel technique because they result in homo- and heteroduplex peak broadening in