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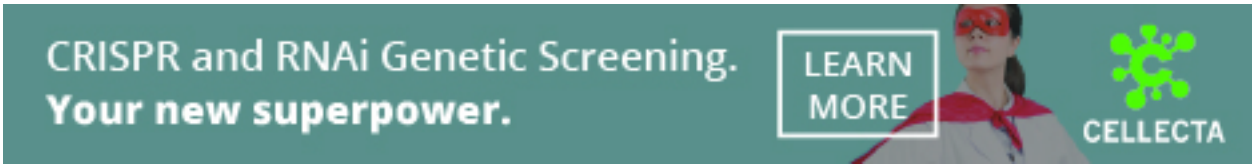

Template Integrity Is Essential for PCR Amplification of 20- to 30-kb Sequences from Genomic DNA

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The feasibility of amplifying genomic DNA targets up to 20 kb long by PCR has been demonstrated.[1] Amplifying even longer sequences and making such long PCR amplifications routine depend on recognizing the critical role played by template integrity. Here, we demonstrate that a highly intact template is essential for amplification of 20- to 30-kb sequences from total human genomic DNA and compare methods to prepare genomic DNA templates for long PCR.

With increasing target size, there is an increasing likelihood that DNA sample preparation will have rendered a template strand unusable by randomly introducing a single-stranded (ss) nick or double-stranded break within the target sequence. To compare methods for preparing long PCR templates, genomic DNA was isolated from cultured cells by boiling in the presence of a chelating resin,[2] phenol extraction,[3] or high-salt precipitation[4] of digested/denatured proteins, dialysis through a membrane[5] or across an agarose matrix,[6] or passage across an anion-exchange resin.[7] Each DNA preparation was then evaluated by estimating the ssDNA integrity with alkaline agarose gel electrophoresis[8] and by amplifying targets of 1–30 kb.

The amplification of sequences of 24–30 kb was absolutely dependent on the integrity of the initial template. These targets were readily amplified only from those DNA preparations that had an average ssDNA size of >25 kb. Template preparations of the highest average ssDNA size, agarose-embedded DNA excepted, yielded several hundred nanograms of 30-kb product from an initial ~10^4 genome copies, but no products of >30 kb were detected. Optimized template preparation is clearly necessary, although not always sufficient, for successful amplification of increasingly longer targets from genomic DNA.

MATERIALS AND METHODS

Nucleic Acids

Solutions of dNTPs (at pH 7) were obtained from either Perkin-Elmer (Applied Biosystems Division, Foster City, CA) or Pharmacia Biotech (Piscataway, NJ). Human placental DNA was purchased from Sigma Chemicals (St. Louis, MO). Total human genomic DNA was also isolated from an SV-40-transformed normal fibroblast (SVNF) cell line (IMR70, SV-40 cell line from the NIGMS Human Genetic Mutant Cell Respository) and the KASO11 β-lymphoblastoid cell line [no. 9009 from the 10th International Histocompatibility Workshop[9]]. DNA molecular weight markers were from GIBCO BRL/Life Technologies (Gaithersburg, MD).

Primer sequences are listed in Table 1. These primers were selected using RightPrimer 1.01 (BioDisk Software, San Francisco, CA), which screens GenBank sequences from the target genome (in this case, the human genome) for the "uniqueness" of primer sequences. Program parameters were set to screen for primers of 18–24 nucleotides, with a minimum G+C content of 60% and a minimum melting temperature of 60°C. Many of these selected primers did work at an annealing temperature of 68°C, as described below. Primer sequences were also screened against the human β-globin gene cluster sequence (GenBank accession no. J00179) using Oligo 4.0 (National Biosciences, Plymouth, MN) to identify homologous primer annealing sites within the targeted sequence that could result in truncated PCR products. All primers were synthesized using the cyanoethoxyphosphoramidite method (1-μm scale) on an Applied Biosystems 394 DNA Synthesizer. Primers RH1059 and RH1064 were purified on a polyacrylamide gel, but this did not appear to be generally necessary.

Proteins

GeneAmp XL PCR buffer and r*Tth* DNA polymerase, XL were from Perkin-Elmer. *Asp*718 restriction endonuclease was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); *Mlu*I, *Sfi*I, and nonacetylated bovine serum albumin (BSA) were from New England Biolabs (Beverly, MA).

Preparation of Total Genomic DNA

Cells were rinsed once in Mg^{2+}-free phosphate-buffered saline (PBS) before each DNA isolation procedure. DNA concentrations were determined by absorbance readings at 260 nm. All genomic DNA stocks were stored at 4°C.

Method I: Phenol Extraction

KASO11 DNA was isolated using phenol

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TABLE 1 Primer Sequences (Listed 5′ → 3′)

Left-hand primers for the human β-globin gene cluster (accession no. J00179)		
RH1017	ACAGACACCCAGGCCTACTTG	pos. 58221–58242
RH1019	CTGCTGAAAGAGATGCGGTGG	pos. 54529–54549[10]
RH1024	TTGAGACGCATGAGACGTGCAG	pos. 44348–44369
RH1063	CACAAGGGCTACTGGTTGCCGATT	pos. 37830–37853
RH1087	CACTTGTTTAGGCCTTAGCGGGCT	pos. 32736–32759
RH1064	TGCTGCTCTGTGCATCCGAGTG	pos. 31193–31214
Right-hand primers for the human β-globin gene cluster		
RH1053	GCACTGGCTTAGGAGTTGGACT	complements pos. 62007–61986[10]
RH1059	AGCTTCCCAACGTGATCGCCTT	complements pos. 61091–61070
Primers for the human mitochondrial DNA genome[11]		
L15996	CTCCACCATTAGCACCCAAAGC	pos. 15975–15996
H408	CTGTTAAAAGTGCATACCGCCA	complements pos. 429–408
Primers for the human hprf *gene*[12]		
b	TGGGATTACACGTGTGAACCAACC	pos. 14578–14601
e	TGTGACACAGGCAGACTGTGGATC	complements pos. 17310–17287

essentially as described in Sambrook et al.[13] Cells were resuspended in 1×TEN (10 mM Tris-HCl at pH 8, 150 mM NaCl, and 10 mM EDTA) and incubated overnight at 50°C with 0.1 mg/ml of proteinase K and 0.5% SDS. Digested proteins were removed by extraction with Tris-buffered phenol. The DNA was precipitated with NaOAc/ethanol, treated with RNase A and extracted one or two times with phenol/chloroform and dialyzed against TE (10 mM Tris-HCl at pH 8 and 1 mM EDTA). KASO11 DNA prepared in this manner was a gift of the laboratories of A. Begovich and H. Erlich, Roche Molecular Systems; this DNA was not prepared with particular precautions for highly intact DNA, such as use of freshly distilled, oxidant-free phenol and very gentle inversions during the extractions.

Method II: High-salt Methods

A. The procedure of Miller et al.[4] was adapted for isolation of DNA from cultured cells. Two pellets of 3×10^6 to 5×10^6 KASO11 cells were each resuspended using 0.6 ml of nuclei lysis buffer (10 mM Tris-HCl at pH 8.2, 0.4 M NaCl, and 2 mM EDTA) and mixed with 40 μl of 10% SDS, 100 μl of 2 mg/ml proteinase K (freshly prepared in 1% SDS and 2 mM EDTA), and 7.5 μl of 10 mg/ml RNase A. Each sample was incubated overnight in a shaking water bath at 50°C. To precipitate the proteins, each digest was shaken vigorously for 15 sec with 0.2 ml of saturated NaCl (~6 M) and centrifuged at 10,000 rpm for 15 min to yield a tight, white pellet. The clear supernatant containing DNA was carefully removed and divided across two tubes. Two volumes of room temperature 95% ethanol were added, and the tube inverted several times. The DNA aggregate was recovered using a glass pipette (reshaped to form a hook), rinsed in 70% ethanol and air-dried briefly and resuspended with 0.2–0.5 ml of TE.

Digestion of a genomic DNA template with restriction enzymes has been observed to improve yields of a 2.7-kb PCR product.[12] One explanation may be that a fraction of the template from highly intact genomic DNA is effectively sequestered within a DNA network and thus incompletely denatured during the early cycles of PCR. Because convenient restriction sites may not be identified easily for all genomic targets, SVNF DNA prepared by this method was gently sheared by passage through a 26-gauge needle up to four times. Modest improvements in yields were observed: up to 50% more of a 2.7-kb PCR product (*hprt* gene) and up to 25% more of a 24.2-kb product (β-globin gene cluster). More extensive shearing led to decreased yields. Some improvement in PCR product yields was also seen following an additional dialysis step against water or TE.

B. KASO11 DNA was also prepared from each of two pellets of 3×10^6 to 5×10^6 cells using the Puregene (Gentra Systems, Minneapolis, MN) DNA Isolation Kit, which is based on high-salt extraction of the proteins.

A recently reported variation of the Miller et al.[4] protocol that uses successive low-speed centrifugations[14] was not evaluated.

Method III: Megapore Dialysis

KASO11 DNA was isolated by dialysis using the Megapore method of Monforte et al.[5] At least two pellets each of 10^3, 10^4, 10^5, or 10^6 KASO11 cells were resuspended with 75 μl of PBS, mixed with 75 μl of 2 mg/ml proteinase K (freshly prepared in 2% SDS and 0.1 M EDTA), and incubated at 50°C for 20–60 min. With a wide-bore 1-ml pipette tip, each digest was carefully pipetted onto a 47-mm type HA 0.45-μm membrane (Millipore, Bedford, MA) that was floating on 35 ml of TE in a 100-mm petri dish. Alternatively, 30-mm Millicell–HA culture plate inserts (Millipore) were suspended (to prevent sinking) at the surface of TE in a Pyrex crystallizing dish. Samples were dialyzed at room temperature for at least 18 hr. DNA concentrations of the dilute preparations were estimated based on the starting cell number.

If more than ~10^6 cells were used, the high viscosity of the final DNA solutions made accurate pipetting of small volumes difficult. Consequently, these concentrated DNA preparations were either digested with rare-cutting restriction endonucleases that would leave the target region intact (*Asp*718, *Mlu*I, or *Sfi*I) or gently sheared by passage through a 21-gauge syringe needle four times. For the digestions, 30 units of enzyme and 30 μl of 10× buffer (supplied by the manufacturer) were stirred into aliquots of ~300 μl of dialyzed DNA and incubated for at least 3 hrs at the recommended temperature. *Sfi*I digestion or shearing did, however, appear to contribute preferentially to the accumulation of a secondary product in amplifications of the 29.9-kb target.

Method IV: Chelex 100

KASO11 DNA was extracted using the Chelex chelating resin, essentially as described in Walsh et al.[2] Duplicate pellets containing as few as ~10^3–10^4 KASO11 cells were tested. For recovery of amplifiable DNA, an upper limit of

~10^5–10^6 cells per 0.2 ml of 5% Chelex (Perkin-Elmer) was noted. Successful long PCR amplifications also required that these resuspended cells be incubated at 50–56°C in the presence of 0.2 mg/ml of proteinase K before being boiled.

Method V: Agarose-embedded Cells

KASO11 DNA was isolated from agarose-embedded cells essentially as described in Gardiner et al.[15] A pellet of ~10^7 KASO11 cells was resuspended with 0.4 ml of buffer L (10 mM Tris-HCl at pH 7.5, 20 mM NaCl, and 0.1 M EDTA), then mixed with an equal volume of molten (45–50°C) 1% LMP agarose (GIBCO BRL) in buffer L, and immediately pipetted into 8-inch sections of Tygon tubing (1/16-inch inner diam.; 3/32-inch outer diam.). The sections were clamped at both ends, cooled at 4°C for 20 min, emptied into a petri dish, and sliced into 1-cm plugs. The plugs were incubated in a 2× volume of 1 mg/ml of proteinase K and 1% *N*-laurylsarcosine (Sigma) in buffer L at 50°C for 3 hr then in a fresh 3× volume at 50°C overnight. Plugs were dialyzed (4–6 hourly changes of TE) and stored in TE. The final DNA concentration was estimated at 5×10^4 genome copies per millimeter of plug.

Plug DNA was digested by equilibrating a plug in 1× buffer, then replacing this with fresh buffer and adding 15 units of *Asp*718 and 30 units of *Mlu*I, and incubating overnight. Digested plugs were reequilibrated in TE for storage.

Method VI: QIAGEN Anion-exchange Resin

KASO11 DNA was isolated using a QIAGEN (Chatsworth, CA) Genomic-tip 20/G or 100/G (one cell pellet each) with supplied buffers, according to the manufacturer's protocol. Following precipitation with isopropanol, any visible DNA aggregate was removed using a Pasteur pipette (end melted shut). The precipitation mixture was then centrifuged, and the pellet was recovered as a separate DNA sample.

Alkaline Agarose Gels

Agarose (SeaKem GTG or SeaKem Gold, FMC BioProducts) gel solutions were prepared in 50 mM NaCl and 1 mM EDTA; a 0.1× volume of 10× alkaline running buffer (0.3–0.5 N NaOH and 10 mM EDTA) was added just before the gel was poured. Gels were presoaked in 1× running buffer for 30 min before samples were loaded and run at 1.5–1.8 V/cm for 3.5–5 hr, with buffer circulation. Gels were neutralized in 0.1 M Tris-HCl (pH 8) and 1 mM EDTA for 30 min, with gentle shaking, and then stained with 0.5 μg/ml of ethidium bromide in TAE.

PCR and Product Analysis

PCR amplifications (50-μl) were performed in GeneAmp PCR System 9600 thermal cyclers using ~37 ng of template DNA in 1× GeneAmp XL PCR buffer (Perkin-Elmer) with 0.2 mM each dNTP, 50 μg/ml of nonacetylated BSA, 1–1.15 mM $Mg(OAc)_2$, and 1 unit of r*Tth* DNA polymerase, XL. The $Mg(OAc)_2$ was withheld until all reaction tubes were at 78°C, as a "hot start". For targets of <10 kb, samples were amplified using 32 cycles of 94°C for 10 sec, 68°C for 10 min. Targets of 15–25 kb were amplified using 20 cycles of 94°C for 10 sec, 68°C for 12 min; then 16 cycles of 94°C for 12 sec, 68°C for 12.5 min with a 15 sec/cycle autoextension. Yields of the 28- to 30-kb products were improved by using an initial 13-min extension time for 10 cycles, then 13.25 min with the above autoextension for 27 cycles. For lower copy number reactions, cycle numbers were increased to 18 plus 24, for a total of 42 cycles. All runs finished with a final extension step of 10 min at 72°C.

PCR products were initially analyzed on 0.6% SeaKem GTG agarose gels. For greater resolution of these products, smaller aliquots were run on 0.3% SeaKem Gold (FMC BioProducts, Rockland, ME) agarose gels run in 1× TAE (40 mM Tris-acetate and 2 mM EDTA at pH ~8.5) at 7 V/cm for 2 min and then at 0.8 V/cm for 15 hr. One set of PCR products (targets of 23, 28, and 29.9 kb amplified from DNA purified by Method IIB) was analyzed by restriction digestion with *Bam*HI (GIBCO BRL) and with *Bgl*II (New England Biolabs) to confirm the product identities (data not shown).

RESULTS AND DISCUSSION

Total human genomic DNA was isolated from cultured cells using six different approaches: boiling in the presence of a chelating resin,[2] phenol extraction[3] or high-salt precipitation[4] of digested/

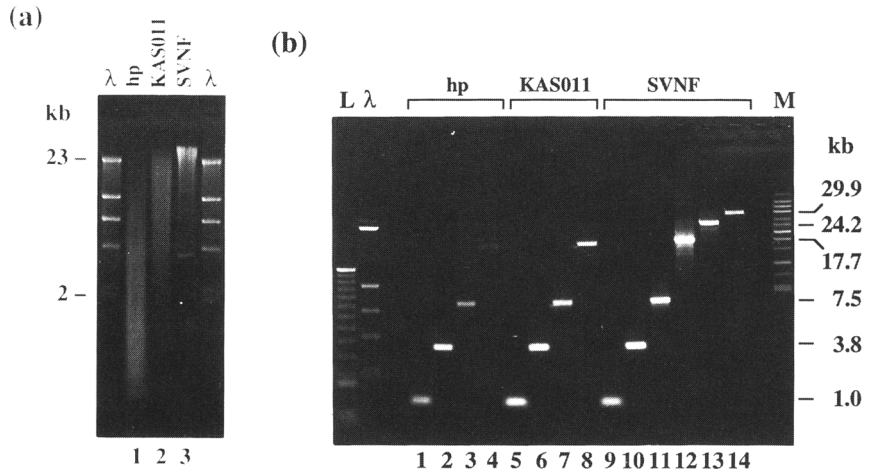

FIGURE 1 (*a*) Comparison of three templates by alkaline agarose gel electrophoresis. SeaKem GTG agarose gel (0.7%) stained with ethidium bromide. (Lane *1*) 1.3 μg of hp DNA; (lane *2*) 1.2 μg of phenol-extracted KASO11 (Method I); (lane *3*) 1.2 μg of high-salt-extracted SVNF DNA (Method IIA). The marker lane (λ) contains *Hin*dIII-digested λ DNA. (*b*) Analysis of PCR products after 36 cycles. SeaKem Gold agarose gel (0.3%) stained with ethidium bromide. (Lanes *1–4*) Amplifications from hp DNA; (lanes *5–8*) from phenol-extracted KASO11 DNA, Method I; (lanes *9–14*) from high-salt-extracted SVNF DNA, Method IIA. Aliquots from each 50-μl PCR sample were 3 μl of each 1-kb product (primers L15996 and H408; lanes *1,5,9*), 1 μl of each 3.8-kb product (primers RH1017 and RH1053; lanes *2,6,10*) and 7.5-kb product (primers RH1019 and RH1053; lanes *3,7,11*), 1.8 μl of each 17.7-kb product (primers RH1024 and RH1053; lanes *4,8,12*), 2 μl of 24.2-kb (primers RH1063 and RH1053; lane *13*) and 8 μl of 29.9-kb product (primers RH1064 and RH1059; lane *14*). Marker lanes are 50 ng of λ/*Hin*dIII digest (λ), 100ng of 1-kb DNA ladder (L), and 70 ng of high-molecular-weight DNA marker (M).

denatured proteins, dialysis through a membrane[5] or across an agarose matrix,[6] and passage across an anion-exchange resin.[7] The overall integrity of each DNA preparation was characterized using alkaline agarose gel electrophoresis.[8] Each DNA sample was then evaluated as a template for long PCR by amplifying targets of up to 30 kb.

Alkaline agarose gel electrophoresis revealed significant differences in ssDNA integrity amongst commercially available human placental (hp) DNA, phenol-extracted (Method I) KASO11 DNA, and high-salt-extracted (Method IIA) SVNF DNA samples. The average ssDNA sizes of these templates appeared to be ~2 kb for hp DNA (Fig. 1a, lane 1), 15–20 kb for KASO11 (lane 2), and >25 kb for SVNF DNA (lane 3).

Equivalent amounts (37 ng) of these genomic DNA samples were used for amplification of a 1-kb mitochondrial DNA target and targets of 3.8–29.9 kb within the β-globin gene cluster. As shown in Figure 1b, template-dependent differences in product yields were discernible beginning with the 7.5-kb target. These differences became even more apparent with longer targets. Total product yields were estimated by comparing the band intensities with those of the molecular weight marker λ/*Hin*dIII: From the hp DNA with an average ~2 kb ssDNA size, a low level of 17.7-kb product was obtained (Fig. 1b, lane 4; estimated total 50-ng product per 50-μl reaction). By comparison, the KASO11 DNA yielded 0.6–0.7 μg (lane 8) and SVNF DNA yielded 2–3 μg (lane 12) of 17.7-kb product per reaction. (This phenol-extracted KASO11 DNA is the same template used previously to achieve a low yield of 22-kb product from the β-globin gene cluster).[1] These results could not be simply explained by many-fold errors in concentration determinations for the hp, KASO11, and SVNF DNA stocks. Rather, the data show the significant impact that initial ssDNA integrity has on the success of PCR amplification for increasingly longer targets.

The high-salt-extracted SVNF template further proved to be of sufficient integrity that 24.2- and 29.9-kb sequences (Fig. 1b, lanes 13,14) could be effectively amplified. The 29.9-kb sequence was also amplified from high-salt-extracted KASO11 DNA (included in Fig. 2, below), suggesting that the result was independent of the cell line. This is the longest single-copy gene sequence that has been amplified from a complex genomic DNA.

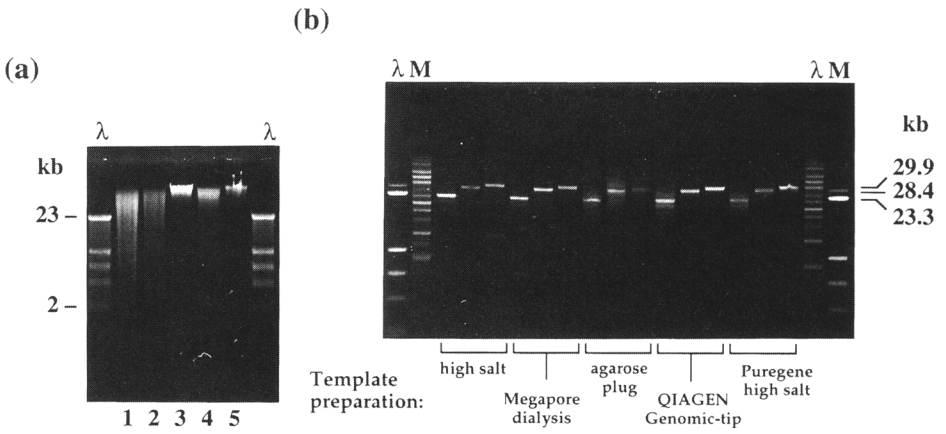

FIGURE 2 (*a*) Comparison of several highly intact templates by alkaline agarose gel electrophoresis. SeaKem GOLD agarose gel (0.35%) stained with ethidium bromide. Samples are 0.8 μg of KASO11 DNA purified by high-salt Method IIA (lane *1*); 1.2 μg of SVNF DNA purified by high-salt Method IIA (lane *2*); 0.2 μg of KASO11 DNA purified by the Puregene kit (Method IIB) (lane *3*); 1.6 μg of KASO11 DNA purified by QIAGEN Genomic-tip (Method VI) (lane *4*); ~1 μg of KASO11 DNA purified by Megapore dialysis (Method III) (lane *5*). DNA isolated in agarose plugs (Method V) was too high in molecular weight to be electrophoresed in this manner. (*b*) Amplification of 23.3-, 28.4-, and 29.9-kb targets within the β-globin gene cluster, from various preparations of KASO11 DNA as in *a*. SeaKem Gold agarose gel (0.3%) stained with ethidium bromide. (Set 1) High-salt method IIA (~37 ng or 10^4 genome copies per PCR sample, 37 cycles of amplification); (set 2) Megapore isolation from 10^4 cells (estimated 10^{2-3} copies per PCR, 42 cycles); (set 3) *Asp*718/*Mlu*I-digested plug slices (estimated 5×10^4 copies per PCR, 37 cycles); (set 4) QIAGEN Genomic-tip (~7.5 ng per PCR, 42 cycles); (set 5) Puregene kit (~12 ng per PCR, 37 cycles). Equivalent volumes were loaded in sets 1, 2, and 4. In set 3, volumes were reduced by 67–90% to match band intensities with the remaining lanes; in set 5, volumes were reduced by 50–67%. Marker lanes are as in Fig. 1.

Several samples of total genomic DNA isolated by boiling cells in the presence of the Chelex chelating resin (Method IV) were also evaluated, although yields were insufficient to permit analysis by alkaline gel electrophoresis. Such DNA has been used successfully for amplification of 16.3 kb from the 16.6-kb human mitochondrial DNA genome[16] that is present at several hundred copies per cell.[17] PCR products of up to 28 kb were occasionally detected after 42 cycles of amplification, but this upper size limit varied even among samples of equivalent input cell number. The reproducible target size limit for single-copy genomic targets from Chelex-extracted DNA appeared to be ~15 kb (data not shown).

KASO11 DNA was also prepared using either Megapore dialysis (Method III), agarose-embedded cells (Method V), or a QIAGEN Genomic-tip (Method VI). The first method was developed for generating large DNA inserts for cloning; the second is routinely used to prepare high molecular weight DNA for pulsed-field gel electrophoresis. DNA was also prepared using the Puregene kit, a version of the high-salt extraction approach (Method IIB). As judged by alkaline agarose gel electrophoresis, all of these methods yielded genomic DNA of much higher molecular weight than the initial high-salt extraction method (Fig. 2a). These DNA samples were therefore promising templates for the amplification of targets 30 kb and longer.

Sequences of up to 30 kb were readily amplified from all of these highly intact templates (Fig. 2b). From the more intact samples, such targets could be amplified from lower template copy numbers, with or without additional cycles of amplification. For example, after 37 cycles, DNA prepared with the Puregene kit yielded an estimated 500 ng of 29.9-kb product from $\sim3\times10^3$ genome copies (50-μl reaction). DNA prepared by Megapore dialysis or QIAGEN Genomic-tip also yielded up to several hundred nanograms of 29.9-kb product from $\sim10^4$ genome copies; in contrast, the other high-salt preparations yielded ~100 ng at

most. However, attempts to amplify 34- and 36-kb targets from these templates were unsuccessful. Further analysis is needed to distinguish the roles of primer design, reaction buffer, polymerase characteristics, and thermal cycling parameters for these longer amplifications.

Conclusions

Successful long PCR amplifications depend on a number of factors. As shown here, attention to the preparation of DNA templates is critical for the highest product yields, particularly for amplifications of single-copy sequences within complex genomic DNA. These data illustrate that at least three methods are currently available to generate the highly intact DNA that is most favorable for long PCR. Given a high-quality template, greater reaction efficiency and better primer design for reaction specificity may be the next most critical parameters for routine amplification of increasingly longer targets.

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