Journal of Dairy Research (2002) **69** 645–649. © *Proprietors of Journal of Dairy Research 2002*
DOI: 10.1017/S0022029902005848 *Printed in the United Kingdom*

High quality genomic DNA extraction from large milk samples

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(*Received 29 December 2000 and accepted for publication 12 April 2002*)

Keywords: Cattle, milk, DNA, SCC, PCR, EDTA.

Recently, techniques have been reported which describe the isolation of genomic DNA from somatic cells of bovine milk (Lipkin *et al*. 1993, 1998), epithelial cells of human milk (Lindquist *et al*. 1994) and bovine and caprine milk (Amills *et al*. 1997). In each of these reports, somatic cell DNA was successfully amplified by polymerase chain reaction (PCR). However, none of these methods are suitable for large-scale genotyping projects, because consistent quantifiable amounts of good quality genomic DNA were not obtained from milk somatic cells, or because good quality PCR product could not consistently be amplified from genomic DNA.

The somatic cell count (SCC) in bovine milk increases in response to infection from a low range between 2×10^4 and 2×10^5 cells/ml from a healthy quarter, to a high range from 3×10^5 to 1×10^7 cells/ml from an infected quarter (Holmes & Wilson, 1984; Kehrli & Shuster, 1994). In whole blood, the numbers of white blood cells (nucleated cells which can yield genomic DNA) also vary in response to infection, ranging between 7×10^6 and 1×10^7 cells/ml (Swenson, 1993). Whole blood can contain up to 350 times more nucleated cells than milk, when comparing lower cell counts for the same volumes of whole blood and milk.

A volume of 500 μl whole blood can typically be used to provide adequate genomic DNA for PCR-based techniques. Conservatively, 500 μl whole blood should yield approximately $3{\cdot}5 \times 10^6$ copies of genomic DNA, which is equivalent to 11·7 μg mammalian genomic DNA (Innis & Gelfand, 1990). A 50-ml volume of milk from an uninfected quarter with a very low SCC should yield about 3·3 μg genomic DNA, providing the technique for isolating genomic DNA is of similar efficiency.

In a preliminary report (Murphy *et al*. 1995), we demonstrated that good quality genomic DNA could be isolated from 1·5-ml samples of milk collected from single quarters. However, this method was not reliable for isolating DNA for large-scale genotyping projects, because of the highly variable yields of genomic DNA. If DNA was isolated directly from 50 ml milk, then the resulting genomic DNA was severely degraded because casein interfered with Proteinase K digestion of enzymes that degrade somatic cell DNA. Good quality genomic DNA could be isolated from 50-ml samples only if the somatic cells were separated from casein prior to Proteinase K digestion. Previously, this involved laborious procedures of somatic cell aspiration and although the final yield of genomic DNA was of high quality, it was also highly variable in quantity. In the current report we demonstrate radical changes to the

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https://doi.org/10.1017/S0022029902005848 Published online by Cambridge University Press

previous protocol. Somatic cell aspiration has been excluded and instead casein micelles were dissolved with EDTA prior to digestion of somatic cells. In addition, variation in the yield of genomic DNA between cows has been reduced by sampling milk from all four quarters rather than from single quarters.

materials and methods

A pooled 50-ml sample of milk was collected equally from the four quarters of each cow by hand-stripping, just prior to machine milking. Although an alternative easy method of sampling pooled milk is to collect from the milking jar, this method was not used because of the risk of cross contamination. Milk samples were collected in 50-ml tubes (with V-shaped bottoms) and immediately placed into crushed ice in an enclosed polystyrene-insulated ice chest. Samples could be stored unfrozen in crushed ice for at least 48 h without noticeable deterioration of genomic DNA. Longer-term storage of whole milk without deterioration of genomic DNA, has been achieved for at least 2 months at −20 °C and for at least 18 months at −80 °C. Frozen samples were thawed at room temperature before isolating DNA.

Samples were centrifuged at 2200 ***g*** at room temperature for 5 min. Supernatant, including the hardened fat layer, was aspirated and discarded, except for the bottom 5 ml, which included somatic cells and the casein pellet. A simple aspirator connected to a water tap was used to remove the supernatant. The bottom 1 ml of the remaining supernatant (including the casein pellet) was transferred by pipette into a 1·5-ml tube.

To dissolve casein, 300 μl 0·5 M-disodium ethylenediamine-tetraacetate (EDTA), pH 8·0 and 200 μl 10 mM-Tris-HCl–1 mM-EDTA, pH 7·6 (TE) were added to the 1 ml supernatant. Somatic cells and casein micelles were resuspended by vortexing for 20 s. After standing for several minutes, the opaque milky appearance of the tube contents cleared as the casein micelles dissolved. Tubes were then centrifuged, 16000 ***g*** at room temperature for 20 s. Supernatant was removed, leaving the bottom 100 μl, which contained somatic cells. Cells were washed twice by refilling tubes to 1·5 ml with TE, vortexing and re-centrifuging.

Digestion of somatic cells was performed with addition of 100 μl digestion mix, containing 40 mM-Tris-HCl, pH 8·3, 50 mM-KCl, 3 mM-$MgCl_2$, 1 μl Tween 20 and 20 μl Proteinase K (PK; 20 mg/ml). Digestion was performed at 55 °C for 3 h. After digestion, 10 μl (from the total 200-μl volume) was analysed on a 0·7 % agarose gel containing ethidium bromide (20 μg/100 ml gel) for 20 min at 110 V. A known-concentration marker (5 μl of 0·1 μg λDNA/*Hin*dIII/μl) was loaded in an adjacent lane. DNA bands were visualised under UV light and photographed. Genomic DNA was quantified by comparing the band intensities of samples and marker. The quantity of DNA in each band of the marker is proportional to the DNA fragment size in that band. Fragment sizes measured in base pairs (bp) and corresponding DNA quantities in each band (in parentheses) in 500 ng λDNA/*Hin*dIII are 23130 bp (238 ng), 9416 bp (97 ng), 6557 bp (68 ng), 4361 bp (50 ng), 2322 bp (24 ng), 2027 bp (21 ng), 564 bp (6 ng) and 125 bp (1·3 ng). Phenol/chloroform extraction and ethanol precipitation were performed, as described by Sambrook *et al.* (1989), if the samples were to be stored for more than 1 month. After purification, genomic DNA was dissolved overnight in TE to obtain an approximate concentration of 20 ng/μl.

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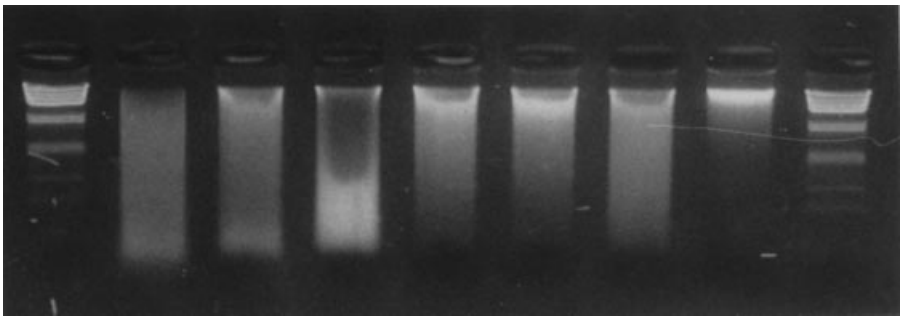


Fig. 1. Genomic DNA obtained from EDTA-treated milk samples, showing improved quality of genomic DNA with the increasing concentration of EDTA used to dissolve casein micelles. EDTA concentrations: nil, 5, 10, 25, 50, 75 and 100 mM (lanes 2–8). In each lane, 10 μl of a 200-μl DNA extract isolated from 50 ml milk, was analysed. λDNA/*Hin*dIII (5 μl of 100 ng/μl) was used for comparison (lanes 1 and 9).

RESULTS

Effects of a range of EDTA concentrations on the quality of isolated genomic DNA, are shown in Fig. 1. EDTA concentrations of up to 10 mM (lanes 2–4) showed significant smearing (indicating degraded DNA) and the genomic DNA was not suitable for PCR amplification. Concentrations between 25 and 100 mM (lanes 5–8) showed reduced smearing and the genomic DNA was satisfactory PCR template. Genomic DNA was of highest quality with 100 mM EDTA (lane 8).

Yields of genomic DNA from mixed-milk samples collected equally from all four quarters of each of five cows were compared with those collected from individual quarters of the same five cows (Fig. 2). Genomic DNA isolated from individual-quarter milk samples varied greatly. Two individual-quarter milk samples (top of lanes 14 and 18) yielded no detectable genomic DNA, while 12 out of the 20 individual-quarter samples (top of lanes 2, 5–13, 16 and 19) produced high yields of genomic DNA (at least 10 ng/μl digest, with a total volume of 200 μl). All mixed-quarter milk samples yielded quantifiable amounts of genomic DNA (at least 10 ng/μl digest).

DISCUSSION

Casein requires calcium to form micelles (Holt, 1992). When EDTA is added to milk, it chelates calcium, which is removed from casein causing micelles to dissolve (Owen & Andrews, 1984). Centrifugation of milk samples treated with EDTA, does not produce the typical 'fluffy' casein pellet, but sedimentation of somatic cells does not appear to be otherwise affected.

When casein was dissolved prior to PK digestion (e.g. Fig. 1, lane 8; 100 mM-EDTA treatment) the quality of genomic DNA was improved, compared with an untreated sample (Fig. 1, lane 2). Using EDTA to dissolve the casein micelles allowed for quicker isolation of genomic DNA, when compared with somatic cell aspiration.

The substantial variation between yields of genomic DNA, when milk was sampled from single quarters, was reduced if genomic DNA was isolated from mixed-quarter milk samples (Fig. 2). The chance of sampling an infected quarter, with substantial somatic cells, is increased fourfold and infected quarters have between 10 and 500 times more somatic cells than healthy quarters.

Agarose gel electrophoresis was used for DNA quantification and for assessing the

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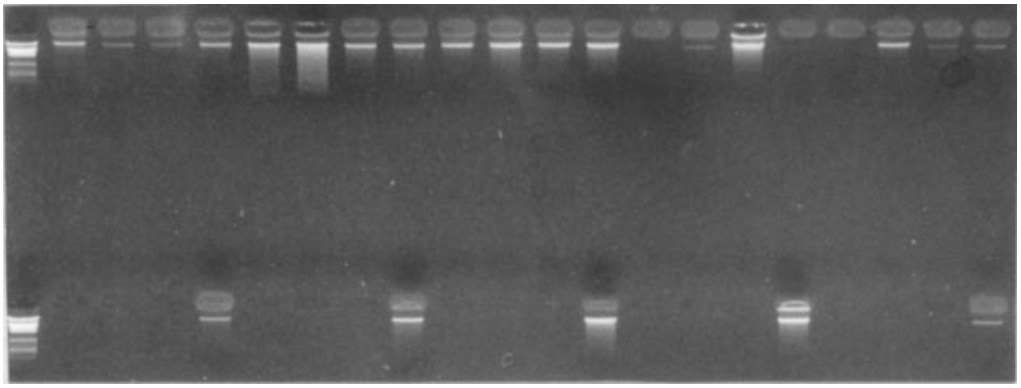


Fig. 2. Comparison of genomic DNA yields from individual-quarter milk samples (four samples for each of five cows, shown in the top row of lanes 2–5, 6–9, 10–13, 14–17, and 18–21) with genomic DNA yield from mixed-quarter milk samples (one sample for each of the same five cows, shown in the bottom row of lanes 5, 9, 13, 17 and 21, respectively). In each lane, 10 μl of a 200-μl DNA extract isolated from 40 ml milk, was analysed. λDNA/*Hin*dIII (5 μl of 100 ng/μl) was used for comparison (lane 1).

quality of DNA. Using this procedure, the mixed-quarter sample in lane 5 of Fig. 2 (one of the lowest yielding samples) was estimated to have 50 ng genomic DNA and the amount of genomic DNA in the total PK digest was therefore about 1 μg. A typical 50-μl PCR requires about 20 ng template DNA, so this digest could provide sufficient template DNA for about 50 PCR amplifications.

It should be noted that EDTA is also a chelator of magnesium, and magnesium concentration can affect several PCR properties (Innis & Gelfand, 1990). Also, EDTA is not removed by phenol/chloroform extraction or ethanol precipitation, as it co-precipitates with nucleic acid (Sambrook *et al.* 1989). However, in the current protocol, the final concentration of EDTA in the PK digest is not significantly higher than that of the TE buffer itself and there is no sign that it adversely affects the efficiency of PCR.

This work was partly funded by the Dairy Research and Development Corporation (DRDC), Australia. M. A. Murphy also held a research fellowship from the DRDC. M. R. Shariflou held a scholarship from MCHE of Iran.

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