DNA Extraction from small samples using a microextraction method with low gelling temperature (LGT) agarose

M. Prinz, H. Haas, M. Staak and G. Berghaus

Institute for Forensic Medicine, University of Cologne, Melatenguertel 60, 5000 Koeln 30, FRG

The method of embedding biological material in low gelling temperature agarose, as is employed for pulsed field gel electrophoresis, does not require further transfer steps of the DNA, so that the unavoidable loss of DNA with the conventional methods is reduced to a great degree. Consequently, different authors have employed the LGT agarose method for working with minimal numbers of cells (Williams 1987; Mage et al. 1988; Feddersen and Van Ness 1989). After having presented technical modifications for the application on dried bloodstains (Prinz et al. 1989), our goal in this work was a quantitative and qualitative comparison of the LGT agarose method to the frequently used phenol extraction.

MATERIALS AND METHODS

1,5,10, and 20 µl amounts of blood, as well as 1,5, and 10 µl of semen were spotted on glass and cotton fabric, and after two days storage used for DNA extraction. Anagen hair roots were cut off freshly plucked hairs and DNA was isolated immediately.

DNA extraction using LGT agarose: Stains were shredded or scraped off and presoaked in 10-25 µl of 0.001M EDTA at 4°C, for 2h in 0.7 ml eppendorf tubes. A 2% low gelling temperature agarose (type VII, Sigma) solution was prepared in DNA-lysis buffer (0.05M TrisHCl pH 8.0, 0.01M EDTA, 0.5% sodium lauroyl sarcosinate) and after the solution cooled down to 50°C, 500 µg/ml proteinase K was added. 20-50 µl of this solution and 10-25 µl of double concentrated lysis buffer were given to the samples and thoroughly mixed in. DTT in a final concentration of 0.039M was added only to the semen samples. Incubation took place at 50°C for 2h, for hair root samples the incubation time was increased to 4h. For stains on cotton the stain carrier had to be removed prior to dialysis. This was achieved by melting the samples at 65°C, piercing the bottom of the tube and centrifuging the extraction liquid down to a second tube (5 min, 3000xg). The agarose was then allowed to solidify. Samples without stain carrier were briefly centrifuged and allowed to solidify on ice for 10 min. Dialysis was carried out by covering the agarose with 500 µl of cold TE buffer and incubation at 4°C for two days (6 buffer changes).

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DNA extraction using phenol/chloroform: DNA was extracted according to Gill et al. (1985). The stain carrier was removed as above. DNA was precipitated by the addition of 0.1 volume of 4M lithiumchloride, 10 µg of glycogen (Boehringer Mannheim) and 2.5 volumes of ice cold ethanol (-20°C, overnight, 25 min,14000 xg). The samples were dialysed as described by Gill (1987).

The amounts of recovered DNA were estimated by comparison with ethidiumbromide stained lambda DNA markers of 20, 50, 100, 300, and 600 ng DNA after minigel electrophoresis.

Samples prepared by phenol/chloroform extraction were digested with 20 U of restriction enzyme (Hae III, Taq I and Pst I, Boehringer Mannheim) according to the instructions. The LGT agarose samples were melted at 65 °C after the dialysis buffer had been carefully removed. After adding reaction buffer and 40 U of restriction enzyme the samples were vortexed, briefly centrifuged, and incubated with the other samples. Before electrophoresis the LGT agarose samples were melted at 65 °C, 4 µl of gel loading buffer were added, and each sample was briefly vortexed, centrifuged and loaded onto the gel using a micro- pipette. Each slot was filled to the top with 0.6% melted LGT agarose. The samples were allowed to solidify for 5 min before the gel was placed in the electrophoresis chamber, and covered with TBE buffer. Electrophoresis was carried out for 20-28 h at 40 V. The DNA was blotted on nylonmembranes (Hybond N, Amersham) following the original method by Southern (1975).

DNA probes YNH24 (Nakamura et al,. 1987) and ps194 (Barker et al. 1987) were labelled with digoxigenin using the Nonradio-active DNA Labeling and Detection Kit (Boehringer Mannheim) and hybridization was carried out following the Boehringer manual. Reaction was scaled up so that the final concentration of labelled probe in the hybridization mix was 200 ng/ml.

RESULTS

For comparison of the DNA yields the results from 45 sets (2 per set) of performed DNA extractions from bloodstains of various sizes and/or donors were evaluated. The amount of DNA that could be recovered using the LGT agarose method was in 24 cases higher, in 19 cases equal and in 2 cases lower than that of the conventional method. For both types of stain carrier the percental difference in yields was higher for the 10 and 20 µl stains. An example for a concentration test gel is shown in fig 1. The samples in lane 1-6 have been extracted using the LGT agarose method and show a distinctly higher intensity of fluorescence.

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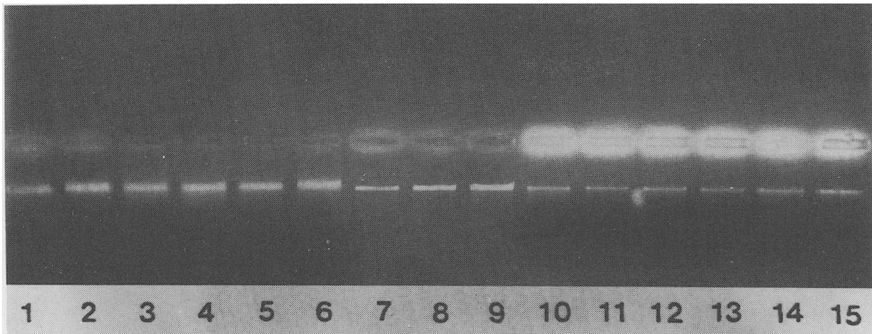

Figure 1. Concentration test gel for DNA extraction from 10 µl blood on cotton fabric, lane 1-6: LGT agarose samples, lane 7-9: 50, 100, 300 ng lambda DNA, lane 10-15: phenol extraction samples

The LGT samples were suitable for restriction enzyme digestion and RFLP typing. Different restriction enzymes show different degrees of sensitivity to the presence of LGT agarose in the reaction. While TaqI and HaeIII were unproblematical, the samples could not be completely digested with PstI, despite controlled dialysis (compare Feddersen and Van Ness 1989).

Compared to the conventionally extracted DNA samples on the same blot, the intensity of hybridization signals was increased for the LGT agarose samples (fig 2). For DNA concentrations close to the detection limit, which for the digoxigenin system is stated to be 0.1pg target sequence the rate of successful RFLP typing was increased in the LGT agarose samples. For 3 kb long DNA fragments, 0.1pg target sequence correspond to 200 ng of genomic DNA. Approximately this amount could be extracted from 1 hair root, 5 µl semen stains, and 10 µl blood stains. For 10 µl blood stains on cotton fabric it was for example possible to type all of 8 samples using the LGT agarose method and only 6 of 8 samples using phenol/chloroform extraction. Testing 4 samples of 5 µl semen stains on cloth and glass, hybridization signals were visible for all the LGT agarose samples and 3 of the conventional method.

A problem connected with DNA extraction in agarose is the occurrence of band shifting as on the membrane shown in fig. 2. The positions of the DNA fragments differ not only compared to the conventionally extracted samples (lane 1 and 2 to lane 9 and 10), but also between different extractions in LGT agarose (lane 5 to 6). To achieve more homogenous electrophoretic qualities two modifications have been tested. Firstly, the reaction volume for the restriction enzyme digestion was increased to lower the final agarose concentration of the sample. Secondly, a different slot-forming comb was used to get thinner slots. The first modification did not influenc the band shifting, while using the different comb had a positive effect.

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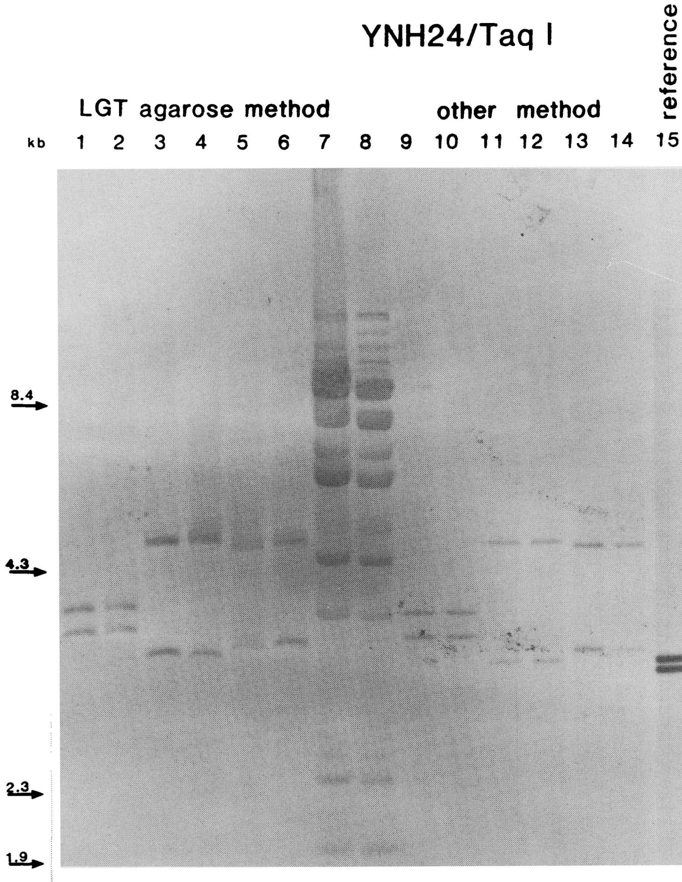
Figure 2. Hybridization result of DNA extracted from 20 µl bloodstain on glass (lane 7,8 lambda DNA)



DISCUSSION

The comparison of two methods presented here confirmed the theoretical expectations concerning the quantity of DNA. The DNA extraction in LGT agarose showed higher yields. The rate of severe loss of DNA using the conventional method however, turned out to be lower than had been expected after the initial experiences with phenol/chloroform extraction of minute samples. This can be attributed to improved handling through practical experience and due to the introduction of modifications to increase the efficiency of DNA recovery, as adding glycogen as inert carrier to the ethanol precipitation and prolonged centrifugation times (Zeugin and Hartley 1985).

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Extraction time being the same, the LGT agarose method has the advantage of involving less work-consuming steps, so that it is possible to handle many samples simultaneously. Since the band shifting is not that severe, the accuracy should be sufficient for certain clinical applications of DNA typing, although for forensic use even the smallest degree of band shifting has to be avoided or at least controlled. Till now this problem has not been satisfactorily solved. There are several improvements to be considered. A binning marker can be added to the samples as a control. To improve the mobility of DNA embedded in agarose variations of different electrophoretical parameters have to be tested. Using thinner slots already had a positive effect, and other possibilities are e.g. the gel concentration and the applied voltage. Another approach is the embedding of the material using insert moulds, and thereby avoiding the addition of enzymes and reagents, which may have an adverse effect on the mobility.

To summarize the results achieved so far, the LGT agarose method still has deficiencies in the quality of the resulting RFLP typing, but the quantity of recovered DNA is higher than that of conventional phenol/chloroform extraction.

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