Int J Leg Med (1992) 105:31–34

International Journal of
Legal Medicine

Some technical parameters influencing the precision and accuracy of fragment size determination for RFLP's

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Received February 6, 1992 / Received in revised form April 14, 1992

Summary. A comparison was carried out between 3 computer-assisted systems for the estimation of DNA fragment length: (1) the "Digitab" system (developed at our institute), (2) the FBI analysis system (FBI Quantico, USA) and (3) the BioImage system (Waters/Millipore, USA). Both the FBI system and the BioImage system were found to be much more precise than the manual Digitab system. In an additional experiment, a possible influence of the field strength on the "accuracy" of fragment size measurement was checked. Lowering the field strength to approx. 1 V/cm led to a statistically significant increase in the measured fragment size.

Key words: DNA fragment size estimation – Field strength – Computer-assisted analysis systems – RFLP

Zusammenfassung. Drei computer-unterstützte Systeme zur DNA-Fragmentlängenbestimmung wurden verglichen: (1) Das „Digitab-System" (entwickelt im Institut für Rechtsmedizin, Münster, FRG), (2) das „FBI-Analysis-System" (FBI, Quantico, USA) und (3) das BioImage-System (Waters/Millipore, USA). Das FBI-Analysis-System und das BioImage-System arbeiteten präziser als das halbautomatische Digitab-System. In einem zusätzlichen Experiment wurde der mögliche Einfluß der Feldstärke auf die „Richtigkeit" der Fragmentlängenbestimmung untersucht. Eine Reduzierung der Feldstärke auf etwa 1 V/cm führte zu signifikant höheren Werten bei der Fragmentlängenbestimmung.

Schlüsselwörter: DNA-Fragmentlängenschätzung – Feldstärke – Computer-unterstützte Analysesysteme – RFLP

Introduction

Precision and accuracy of fragment length determination are important factors which have an influence on the biostatistical procedure and are key factors for comparability and reproducibility of results [1]. Collaborative studies have been carried out [8, 12, 16] with VNTR systems (variable number of tandem repeats [9, 11]) to define the precision of fragment length estimation within and between laboratories.

The aim of these collaborative studies was to minimize measurement errors and the interlaboratory variation. The aim of the present study was to further evaluate the influence of the system for fragment length determination.

Material and methods

DNA from 2 persons was extracted according to Gill et al. [7] and the concentration was measured fluorimetrically according to Brunk et al. [3]. The extraction and the subsequent restriction digests with *Hin*fI (Gibco BRL) were checked on mini gels [14].

Samples (0.5 µg of each human DNA and 0.5 ng of the standard marker DNA) were inserted into 5 mm × 1.5 mm slots of a 1% horizontal agarose gel (20 cm × 23 cm × 1 cm) together with standard marker DNA fragments according to the application scheme shown in Fig. 1.

Electrophoresis was performed at 4°C in boric acid buffer (134 m*M* Tris[1]-HCl, 75 m*M* boric acid, 2.55 m*M* EDTA, pH 8.8).

Electrophoresis was stopped when the 2027 bp fragment of the DNA standard marker (Lambda restriction fragments, Drigest III, Pharmacia) had migrated approx. 18 cm. Repeat experiments were carried out as follows:

- 3 runs were performed at 1 V/cm for approximately 60 h and
- 6 runs were performed at 4 V/cm for approximately 24 h

using the same electrophoretical equipment for each run.

The DNA was transferred to a nylon membrane (Hybond N™, Amersham) using the blotting technique described by Southern [17] and fixed by heating at 80°C for 6 h.

The VNTR probe YNH24 [11] and the analytical marker DNA, wide range (#DG1931, Promega) were labelled by random priming according to Feinberg and Vogelstein [5] using α-^{32}P-dCTP.

Hybridization and visualization was carried out as previously described [2].

Three systems of measurement were used to determine fragment sizes:

1) "Digitab" system:

- *Hardware:* Digitizing tablet (SummaSketch II™) together with digitizing magnifier, light box, IBM™ compatible computer

[1] Tris-(hydroxymethyl)aminomethane

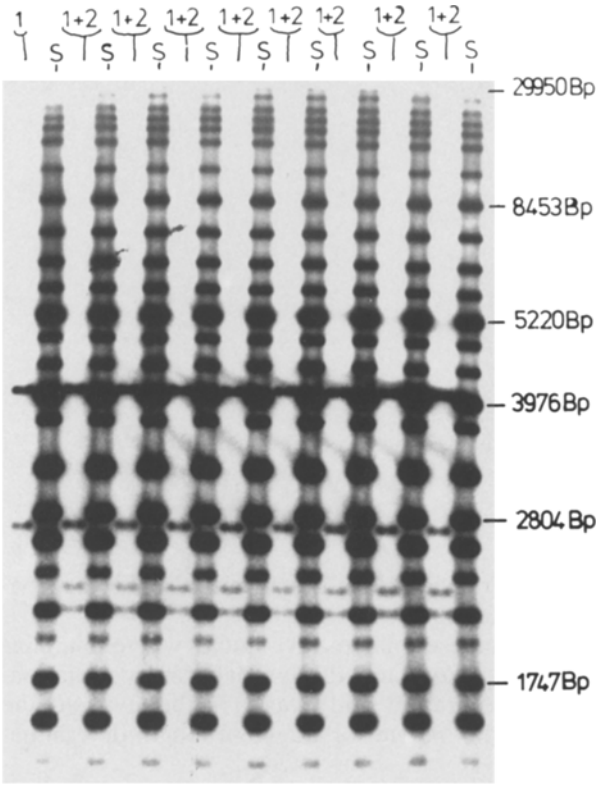

Fig. 1. Autoradiography of single locus probe YNH24, digestion with *Hin*fI. *S* = Marker lanes (Analytical Marker, wide range, Promega) 1+2: Sample lanes containing 4 human fragments (1:1 mixture from 2 persons)

(processor 80286, coprocessor 80287, 20 Mbyte hard disk, floppy disk, VGA[2] colour monitor and VGA[2] colour graphic card (512 kByte monitor memory)), printer.
- *Software:* MS-DOS[3] (version 3.3), AutoCAD[4] (version 10.0), Analysing module Autorad.exe[5], user-programmed standard marker files.
- *Mathematics:* Second degree polynomial [13] for correction of gel inhomogeneities; global reciprocal method [4, 15] for correlation of migration distances with fragment lengths; the band centre was estimated visually.

2) *FBI-analysis system* [10]:
- *Hardware:* This system employs a 512 × 512 Pixel CCD[6] video camera (Sony CCD XC 77), a frame grabber (Data Translation 2853 SQ 60, 60 Hz) and a video monitor (Sony GVM 2000, over scan). The rest of the hardware does not differ from the Digitab system.
- *Software:* MS-DOS (from version 3.3), FBI analysis software [10], user-programmed standard marker files.
- *Mathematics:* Linear correction of gel inhomogeneities; local logarithmic correlation of migration distances [4, 6] for fragment lengths; automatic estimation of band center by creating an intensity profile and sub-pixel determination of intensity maxima in descending sequence.

3) *BioImage system (Waters/Millipore, USA):*
- *Hardware:* This system employs a 1024 × 1024 Pixel CCD video camera and a Sun workstation.
- *Software:* Unix operating system (version 4.3), BioImage software (version 4.0.3c).
- *Mathematics:* Linear correction of gel inhomogeneities; local logarithmic or reciprocal[7] [4, 6, 15] or user-defined correlation of migration distances with fragment lengths; estimation of band centers is made automatically by correlation of intensity profiles.

Results and discussion

1) *Efficiency – Ease of use and reliability*

Efficiency is mainly related to 3 factors: applicability of the software, time needed for the whole procedure, recognition and correction precautions to avoid mistakes.

The "Digitab" system uses easily expandable program parts, but the "manual" digitizing of band positions is laborious and mistakes can be overlooked. Duplicate measurements are therefore absolutely necessary.

The FBI analysis software is more economical, the whole procedure is much faster but could be improved using a faster computer. Mistakes can be recognized and therefore avoided.

The BioImage system uses a software which offers more specifications in each step of the analysis procedure than the FBI system (e.g. different intensity values for band determination, contrast enhancement etc.). The whole evaluation procedure is slightly faster than with this early version of the FBI software (an update of the FBI software is now available, which has improved the operation time).

2) *Comparability of results*

To compare the precision and accuracy of the 3 systems in calculating fragment lengths, 9 autoradiograms from 9 different blots were prepared an example of which is shown in Fig. 1. All 3 methods gave virtually identical mean fragment sizes and is therefore an indication that the degree of accuracy is similar. Fragment 2A exhibited the maximum variation of 14 bp but this is too small to be able to draw any conclusions on systematic influences. This negligible difference is astonishing because there was not only a considerable difference in the hardware but the mathematical approaches also differed.

3) *Precision*

The measurement error differed only slightly between both video-imaging analysis systems, the sigma value being in the range of 0.25%, but this value increased up to 6-fold using the manual method.

Since the mean values did not differ between the 2 video-based approaches we are of the opinion that the automatic ascertainment of the band centre accounts for the improvement in precision over the manual system. A decrease in precision can, of course, grossly impair the

[2] Video graphics array

[3] MS-DOS = Microsoft disk operating system, Microsoft Corp. (USA)

[4] Trade-mark of the firm Autodesk Inc.

[5] Programme developed in cooperation with the Institute of Mathematics, University Münster

[6] CCD = Charge coupled diode

[7] The local reciprocal correlation was used for this study

Table 1. Differences in the mean values for the analysis of blots 1–3 (voltage gradient 1 V/cm) and blots 4–9 (voltage gradient 4 V/cm) for the FBI analysis system. The lengths of the 4 human DNA fragments were measured 24 times in the first 3 blots and 47 times in the second 6 blots. MV = mean value, SD = standard deviation

	Blot 1–3	Blot 4–9	Blot 1–3	Blot 4–9	Blot "1–9"	Blot "1–9"
Human DNA fragment	MV (bp)	MV (bp)	SD (%)	SD (%)	MV(1–3)–MV(4–9) (bp)	MV(1–3)–MV(4–9) (%)
1A	4033	3974	0.18	0.25	59	1.47
1B	2705	2661	0.23	0.28	44	1.64
2A	2245	2220	0.18	0.30	25	1.12
2B	2126	2086	0.15	0.31	40	1.90

statistical values because the window widths must be considerably enlarged, and search procedures will also become more difficult with increasing measurement variations.

4) Field strength

The DNA fragment lengths were estimated by comparing the mobility of human DNA fragments with the mobility of standard marker fragments (phage DNA) in agarose gels under non-denaturing conditions.

It was astonishing to find that the measurement of fragment length was obviously influenced by the field strenght.

The different voltage gradients for the first 3 blots (1 V/cm) and the second 6 blots (4 V/cm) led to substantially different mean values for all 3 measuring systems.

An example using the FBI analysis system is shown in Table 1 listing the mean values and standard deviations.

The differences between both groups were statistically significant. Since the standard deviations (SD) for individual fragments were always below 0.32% (Table 1), the differences between the mean values of both collectives ranged between approx. 6-fold and 12-fold of the SD-value. In other words, there was no overlap between the single values contained in both groups (i.e. 1–3 and 4–9).

If this influence could be confirmed for other kb-ranges and other systems, and between laboratories, it could become an important tool if the aim is to fix fragment length values for controls and/or cell lines. If all laboratories employed a common control sample, a positive deviation could possibly be corrected by increasing the field strength and vice versa.

Standardization of the field strength during gel electrophoresis experiments could be improved for the analysis of VNTR DNA fragments and especially for comparison of different blots. This means that the field strength could be an important tool to minimize the inter and intra lab variation. As indicated before the field strenght has an important influence on the accuracy of the analysis.

To check this parameter for all 3 analysis systems, all measurements of the fragment lengths in blots 1–3 (1 V/cm) were corrected with respect to the values for the blots 4–9 (4 V/cm). The correction factors used were the differences between the mean values (see for example Table 1). The corrected values for 1–3 were combined with the original values for blot 4–9 (Fig. 2).

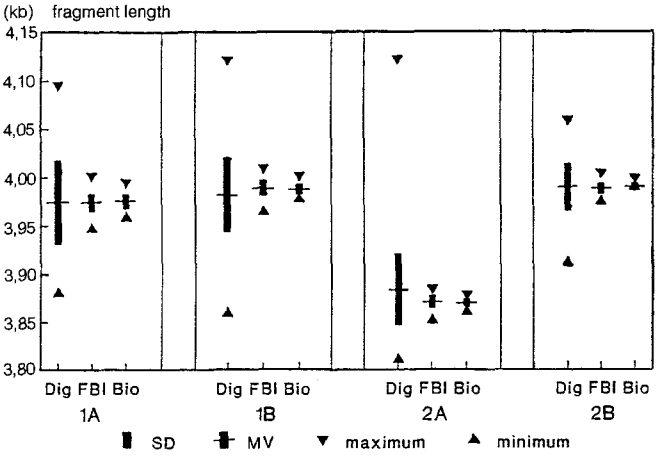

Fig. 2. Graphical representation of the combined analysis after correction of the values for blot 1–3 for the 3 different analysis systems. *Dig* = Digitab system, *FBI* = FBI analysis system, *Bio* = BioImage system. *SD* = standard deviation, *MW* = mean value. Correction factor: differences between the mean values (see Table 1)

Since we do not know the correct size of a given fragment we cannot actually deal with the accuracy of our estimation procedure.

The only indication of this is the comparison of the mean values calculated for the different analysis systems.

To fulfill the requirements of quality assurance, results of participating laboratories must fall within a given window. This can be achieved by harmonizing on a standard protocol, but even deviations can still occur. These can be reduced by minimizing the measurement error. Further reduction in cross-laboratory variation may result by employing the calibration for field strength as tentatively proposed here.

Acknowledgement. The construnction of the Digitab system was only possible with the help of the following persons: G. Kohlruss, A. Bornstedt, A. Braun.

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