Precision and Accuracy in the Analysis of VNTR Polymorphisms for Paternity Testing

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INTRODUCTION

Single locus VNTR DNA profiling has become an important technique in the identification of parentage. Due to the complex multi-step nature of the VNTR assay, a degree of variability exists in the determination of band sizes. Unacceptable levels of measurement error may adversely effect conclusions regarding inclusion and exclusion of alleged fathers, as well as the establishment of accurate population allele frequency data bases. Possible sources of error are poor separation of large bands, poor visualization of small bands, variation in electrophoretic mobility between gels (inter-assay error) or across a single gel (intra-assay error), and observer error in measurement of band migration distance. The recommended method for determining the level of error for a chosen VNTR assay system is repetitive analysis of human genomic DNA samples (Budowle 1991). Inter and intra-assay precision was determined by repetitive analysis of volunteer DNA using eight commercially available VNTR probes. In order to gauge the accuracy of band sizing techniques we performed repetitive analysis of DNA from the HeLa human cell line.

MATERIALS AND METHODS

DNA was extracted from peripheral blood leucocytes of eight volunteers, and digested with a range of enzymes. The digests were electrophoresed, Southern transferred and hybridized using standard methods, (Maniatis 1982) with the eight probes listed in Table 1. Of the enzymes tested, only Pvu II gave band sizes within a 2.0 to 15.0 kb. size range with all probes, and this enzyme was used throughout the remainder of the trial. For inter-assay precision, the eight volunteer DNA samples were electrophoresed on ten gels, and the membranes hybridized sequentially with the eight probes. For intra-assay precision, DNA from a single individual was electrophoresed in twenty wells of a single gel. This was repeated three times for three different individuals for a total of nine gel runs. These membranes were hybridized with the Mucin-HVR probe. In all gels, an analytical sizing marker (Promega) which displays 30 bands over a 30 kb. size range was included in every fifth well

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of each gel. In lane one of each of the gels used for inter-assay precision, 5ug of Puv II digested HeLa cell DNA (Amersham) was included. Band migration distance was determined by measurement with a ruler to the nearest 0.5 mm by two independent readers. Sizes of unknown DNA fragments were calculated according to the hyperbolic method as suggested by Southern (1979), using a spreadsheet program. Each band was reported as an average of the repeated measurements.

INTER- AND INTRA-ASSAY PRECISION

TABLE 1. Inter-assay Precision

PROBE[1]	MEAN RANGE[2]	CV(%) RANGE	PAIRED MEASUREMENTS[3]
D16S85	2.06-5.06	0.38-1.02%	0.60%
Mucin	3.47-5.92	0.36-0.92%	0.60%
Ha-Ras	2.73-4.18	0.36-0.93%	0.53%
DXYS14	3.65-15.7	0.57-2.42%	0.72%
D2S44	2.85-5.31	0.33-1.07%	0.61%
D14S13	3.93-5.97	0.45-1.02%	0.57%
D10S24	3.18-5.76	0.39-1.01%	0.66%
D17S26	6.40-12.7	0.77-3.72%	0.79%

[1]Probes D16S85, Mucin, Ha-Ras, and DXYS14 were supplied by Amersham; probes DS244, D14S13, D10S24, and D17S26 were supplied by Promega.
[2]The range is given for 16 means (2 bands reported per individual) from 10 repeated gel runs for each probe.
[3]The standard deviation was calculated from 160 repeated measurements per probe and expressed as a percentage of the mean band size.

TABLE 2. Intra-assay Precision

SUBJECT[1]	MEAN RANGE[2]	CV(%) RANGE	PAIRED MEASUREMENTS[3]
1a	5.10-5.12	0.61-0.68%	0.29%
1b	3.75-3.80	0.66-0.72%	0.31%
2a	4.16-4.30	0.68-0.84%	0.42%
2b	4.08-4.25	0.51-0.92%	0.55%
3a	5.64-5.85	0.88-0.92%	0.38%
3b	3.33-3.49	0.64-0.80%	0.56%

[1]All subjects were heterozygous, hybridization was with the mucin probe.
[2]The mean range is given for the 3 means for each band from each individual. For each mean n=20.
[3]The standard deviation was calculated from 60 repeated measurements per allele and expressed as a percentage of the mean band size.

Values for inter-assay precision are reported in Table 1. Two bands were sized for each of the eight individuals (single

homozygous band patterns were recorded as two bands of identical size), and means and standard deviations were calculated for each of the sixteen bands from the results of the ten gel runs. This was repeated for each of the eight probes tested using the same ten membranes for each probe (i.e. each of the ten membranes were stripped and re-hybridized eight times). Inter-assay precision is reported as a coefficient of variation (CV), and this was calculated from the means and standard deviations for each of the sixteen bands. It was arbitrarily decided that if a probe demonstrated a CV range greater than 2.0% then it would be rejected for use with this gel/enzyme system. The probes DXYS14 and D17S26 detected several bands greater than 12 kilobase pairs with corresponding CVs which exceeded the acceptable upper limit. This appeared to be due to inadequate separation of marker DNA in that size range, making it difficult to achieve consistent observer agreement on mobility measurements. For the other six probes, the CVs ranged from 0.33% to 1.07%. The percent error for the 160 repeated measurements is the standard deviation calculated from the difference in the two measurements, expressed as a percentage of the mean band size. Values ranged from 0.53% to 0.79% indicating a close agreement between the two readers. There was minimal deviation of band migration across a single gel as evidenced by the intra-assay precision values in Table 2. The CVs ranged from 0.51% to 0.92% for 20 measurements per allele, and the CVs for 60 repeated measurements per allele were all less than 0.6%.

INTER-ASSAY ACCURACY

TABLE 3. Inter-assay Accuracy [1]

PROBE[2]	MEAN[3]	S.D.	REPORTED[4]	DIFFERENCE[5]
D16S85	8.90	0.08	9.00	1.1%
D16S85	3.41	0.03	3.20	6.3%
Mucin	5.81	0.03	6.00	3.2%
Mucin	3.79	0.02	3.50	7.9%
Ha-Ras	2.94	0.02	2.80	4.9%
DXYS14	9.63	0.08	10.0	3.8%

[1]Band sizes detected for a Pvu II digest of the HeLa cell line were compared to band sizes reported for four probes.
[2]Probes listed twice detected heterozygous loci.
[3]The means are calculated from the average value for 10 repeated measurements.
[4]Band sizes reported by probe supplier (Amersham) to 100 base pairs.
[5]Size difference reported as a percentage of the average band size.

Bands detected for a Pvu II digest of the HeLa cell line for each of the probes tested are reported in Table 3. The percent differences between the mean values and reported values ranged from 1.1% to 7.9% of the average band size.

CONCLUSIONS

In estimating band sizing precision, it was found that the greatest source of error was in measuring identical bands in different gels (inter-assay error). Here the probes DXYS14 and D17S26 demonstrated CVs in excess of 2.0%, and they were excluded for further use with Pvu II. The remaining 6 probes produced inter-assay CVs less than 1.1%. Measurement error in sizing identical bands within a single gel (intra-assay precision), and the variation between repeated readings, were found to be comparatively low. Laboratories have reported precision values ranging from 0.6% to 2.0% of the measured band size (Baird, et al., 1986; Gjertson, et al., 1990). The methods described in this report have demonstrated a comparable level of precision. Furthermore, based on these results, an overall band sizing error of 2.0% would seem to be adequately conservative for use in determining the exclusion or non-exclusion of accused putative fathers, and in constructing band frequency binning charts. The determination of band sizing accuracy using the HeLa cell line revealed differences of 1.1% to 7.9% between the mean band sizes measured in this laboratory, and values reported by the supplier of four of the probes tested. This rather large discrepancy was most likely due to differences in analytical techniques. The results did however reveal that size estimates were similar (differences of 100 to 400 base pairs). Also the mean values determined by us will serve as an internal assay reference source in an ongoing quality control program.

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