Evaluation of native and denaturing polyacrylamide gel electrophoresis for short tandem repeat analysis.

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In recent years, one has been exposed to a number of advances in the methods of human identity analysis. Why, in this high-tech age, one may wonder, should time be devoted to another paper on polyacrylamide gel electrophoresis (PAGE)? The answer to this question is intimately associated with the increasing popularity of short tandem repeat (STR) analysis. Many laboratories are using PAGE to both optimize PCR conditions and to obtain databases, and there are almost as many PAGE systems being used as there are loci being analyzed! It is time to standardize the PAGE conditions, so that inter-laboratory variation in results can be minimized. In this communication, three PAGE systems are compared, in order to generate a discussion that may lead to a consensus towards the adoption of a single PAGE system for STR analysis.

First, however, we need to review some features of STRs, that cause one to adopt certain PAGE systems over others. STR-PCR generates amplimers in discrete size ranges between about 100 and 350 bp. The PCR cycling times are short, and the small PCR products do not require long PAGE runs for their separation. One caveat of STRs, however, is that there are few alleles per locus, a fact that makes it necessary to include many loci in the assay, in order to increase the probability that two randomly selected individuals in a population will differ in overall type. Co-amplification of different STR loci is often possible, and this feature allows the analyst to save preparative time. However, this also causes a problem in the analysis of the PCR products, because many of the loci yield amplimers that have similar mobility on PAGE. Thus, one needs to choose loci, whose alleles do not overlap, when silver staining is utilized for detection of the bands. This restricts the analysis to certain loci, and may exclude other loci that are just as informative. Of course, one can run the STRs in many different lanes of the gel, but that technique disallows rapid screening, unless the laboratory is set-up to run 20 or so gels a day. To do that, a very high degree of quality assurance would be necessary.

Since one of the advantages of STR analysis is high throughput, some investigators have concentrated on speed, and chosen to utilize native PAGE, because native gels run much faster than denaturing gels (Frégeau, C.J. & Fourney, R.M., BioTechniques 15, 100-119, 1993). In addition, time is saved by not having to perform the denaturation step. The samples from many PCRs can be combined, but they might need to be desalted before denaturing PAGE, since DNA in solutions of high salt may be refractory towards denaturation. Native gels, on the other hand, are not so sensitive to salt effects, as long as each sample is similar in its composition. Another characteristic of STRs that helped make them popular was the stability and reproducibility of their 3 and 4 basepair (bp) repeats. Nevertheless, some investigators have chosen sequencing-type denaturing gels, which, of course, provide single-base resolution (Kimpton, C.P., Gill, P., Walton, A., Urquhart, A., Millican, E.S. & Adams, M., PCR Methods and Applications 3, 13-22, 1993). These gels offer a high degree of accuracy and precision, but they require long run times, too. Thus, we have two things to consider in the adoption of a PAGE system: a low degree of variability on one hand, and speed, or throughput, on the other.

In this paper, data are reported for five, 4-bp repeat STRs utilizing three types of PAGE systems. The STRs chosen include the polymorphism at the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2), also known as "SE33" (Polymeropoulos, M.H., Rath, D.S., Xiao, H. & Merril, C.R. Nuc. Acids Res. 20, 1432, 1992), which is popular due to its high number and even distribution of alleles. The other four STRs derive from the "Multiplex 1" system described recently by Kimpton et al. (1993) and presented elsewhere in this volume. It includes HUMVWA31, HUMTHO1, HUMF13A1, and HUMFES/FPS. Since we are interested in adopting automation to STR analysis, we have utilized the Applied Biosystems model 373A DNA Sequencer with fluorescent detection of PCR products generated with fluorescently labeled primers. In this system, all bands traverse the same distance from the well to "reading" (WTR) region of the laser before they are detected. We utilized a WTR of both 12 and 24 cm, the latter distance being the typical one for DNA sequencing. Data for 6% T 5% C denaturing gels with 8M urea (12 and 24 cm WTR, 44V/cm) or 6% T 5% C native gels (24 cm WTR, 30V/cm) are reported here. Five gels of each type were compared. We also analyze the STR systems on the GIBCO BRL/Life Technologies SA32 apparatus with silver stain detection.

The time required for the separation of the STR bands could be greatly reduced when native gels were employed. A 24 cm WTR sequencing type gel required 5.5 h and a bit more than 7 h for separation of the multiplex and SE33 amplimers, respectively. The time necessary for the same degree of separation of the STR-PCR products on native gels was reduced to 3.5 h and 4.5 h, respectively. However, we obtained just as good results with the 12 cm WTR denaturing PAGE system, and this required less than 3 h for the multiplex and 3.5 h for the SE33 one.

Extra bands were sometimes detected in all three PAGE systems. When we utilized the PCR conditions of Kimpton et al. (1993), we found the system extremely robust. We found it advantageous not to include Triton X100 in the PCR buffer, because this led to extra bands in all PAGE systems and to a turbid solution following the PCR. The locus F13A1 often yielded faint extra bands on native gels, which were not present in denaturing gels. We attributed these extraneous bands to heteroduplex formation during the final stage of the PCR. Under different gel crosslinking conditions (10% T, 2.5% C), extra bands for vWF could be distinguished on native gels, which were absent in the standard PAGE crosslinking conditions. When the denatured PCR products were not loaded immediately, there was some degree of re-annealing, giving rise to extra bands on the denaturing gels. Sometimes, for reasons that could not be determined, the PCR samples did not denature, and gave rise to smears. Thus, a 95°C block containing oil in the wells and a careful sample treatment protocol did not always ensure denaturation.

Another problem giving rise to extra bands appeared in some cases and could be attributed to the non-templated addition of a nucleotide to the blunt ends of the amplimers by Taq DNA polymerase (Clark, J.M., Nuc. Acids Res. 9677-9686, 1988). Kimpton et al. (1993) described two enzymatic procedures to eliminate these extra bands. This approach introduces not only an extra step in the procedure, requiring time of the analyst, but provides another quality assurance consideration. We found a simpler procedure: the doublets one observes due to -A/+A in 24 cm WTR denaturing gels are absent on 12 cm WTR gels, and a single band appears. This not only makes the interpretation of the patterns simpler, but it also allows a more accurate measurement of mixed samples, because one is dealing with a single band, rather than a split one.

Obtaining the genotype when allelic ladders were utilized was a facile procedure in most cases. However, this required the use of multiple colors in the case of the multiplex system. For example, since the alleles of F13A1 and FES/FPS overlap, we used green for one and blue for the other. It also meant that the respective allelic ladders had to be

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run in adjacent lanes, a procedure which wasted area on the gel that could have been utilized for samples. In order to increase the throughput, we employed a sizing ladder that was labeled with a dye that fluoresces red and placed it in the lanes with the samples. A calibration curve for the internal standard was constructed by the software, and then the size of the bands was calculated based on their position on the curve. Rather than visually estimating the genotype, the analyst obtains the size of the bands.

The variation of the size calls was very low, when compared to the length of the bands under consideration. The ratio of highest standard deviation observed to the lowest band size was 0.1% (0.1 bp), 0.2% (0.2bp), and 0.3% (0.45 bp) for the 24 cm denat., 12 cm denat. and 24 cm native PAGE systems, respectively. The standard deviation for the STRs is given in Table 1. From these data, we see that the typical denaturing gel is indeed the most precise one. However, is such a degree of precision necessary, when one is dealing with alleles that are separated by only 3 to 4 bp?

Table 1. Average Standard Deviation (bp) for Multiplex and SE33 STR Systems

	24 cm denat.	12 cm denat.	native
vWA	0.08	0.32	0.15
THO1	0.07	0.27	0.40
F13A1	0.12	0.15	0.26
FES/FPS	0.10	0.15	0.30
SE33	0.27	0.32	0.54

To measure the resolution of a gel system, one can utilize the familiar graphic procedure, whereby the peak width at one-half peak height is related to the distance between the peak maxima. Another way is to perform an experiment with alleles that vary 1, 2, 3, and 4 bp from each other. We have in our database an allele that runs one bp in size slower than the 10th allele of the THO1 allelic ladder, which I will call 10*. Now, the 10th allele has recently been sequenced and is reported to have one repeat of 3 bp (Puers, C., Hammond, H.A., Jin, L., Caskey, C.T. & Schumm, Am. J. Hum. Genet., in press), explaining why it is 3 bp distant from allele 9 and 5 bp distant from allele 11. These results indicate that it might be difficult to obtain the correct genotype by comparing a band in an adjacent lane to one of an allelic ladder. It is possible to discern the mobility difference between allele 10 and 10* visually in *all* the PAGE systems examined here. However, without using the in-lane sizing ladder, we have no way of knowing whether the slight mobility decrease noted for such an allele is due to differences in the local field strength of the particular lanes, or to length differences in the amplimer size. Another case for which an allelic ladder led to problems was with F13A1 on native gels. These gels are more capable of detecting differences in conformation of the DNAs than denaturing gels. Thus, whilest the repeat between the F13A1 alleles was tight and centered at 4 bp in denaturing gels, we saw what appeared to be 2 bp repeats between some of the alleles run on native gels (Table 2). This extra sensitivity of native gels for conformation of the DNA will lead to problems in inter-laboratory comparisons for the F13A1 locus.

Table 2. F13A1 Genotyping Differences on Native and Denaturing PAGE

	Native PAGE		Denaturing PAGE	
ID	genotype (bp)	Δ (bp)	genotype (bp)	Δ (bp)
28	189.3, 192.6	3.3	183.3, 187.3	4.0
30	192.8, 198.3	5.5	187.3, 191.1	3.8
35	198.4, 200.9	2.5	191.1, 195.0	3.9
36	192.6, 198.0	5.4	187.3, 191.1	3.8
7	198.4, 200.8	2.4	191.1, 194.9	3.8

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