RFLP TYPING: A NEW HIGHLY POLYMORPHIC VNTR LOCUS AND CHEMILUMINESCENT DETECTION

Bruce Budowle[1], Leonard Klevan[2], and Arthur J. Eisenberg[3]

1) FSRTC, FBI Academy, Quantico, VA 22135
2) Life Technologies, Gaithersburg, MD 20884
3) Texas College of Osteopathic Medicine (TCOM), Fort Worth, TX 76107

INTRODUCTION

There are two basic DNA typing methodologies available to the forensic scientist for characterizing biological evidence. The first technology to gain wide use in the forensic arena was typing of DNA for variable number of tandem repeat (VNTR) loci by restriction fragment length polymorphism (RFLP) analysis. RFLP typing is well-defined, provides a high degree of discrimination, and can be accomplished, at times, with less than 50 ng of high molecular weight genomic DNA. This methodology has been validated for forensic applications (Budowle and Baechtel 1990). The other strategy for typing DNA is based on increasing the number of copies of a target sequence of DNA by amplification using the polymerase chain reaction (PCR) (Saiki, et al. 1985). Since the number of target sequences of interest is increased dramatically by PCR, simplified typing methods can be used for determining DNA polymorphisms in a sample. The advantages a PCR-based technology offers, compared with the currently employed RFLP approach, are: 1) augmented sensitivity and specificity, 2) decreased assay time and labor, 3) absence of an isotopic label, and 4) many degraded DNA samples can be amplified by PCR and subsequently typed because alleles amenable to PCR are much smaller in size compared with alleles detected by RFLP analysis. These qualities combine to make PCR-based technologies extremely useful tools for analyzing biological material found at crime scenes.

While the application of PCR-based technology to the genetic characterization of forensic evidence is coming to fruition, the RFLP method currently in use provides a higher degree of discrimination, and the molecular biology aspects of the RFLP technique are better understood. Therefore, research to facilitate RFLP typing of DNA still should be continued. This paper discusses two such aspects: additional VNTR loci to augment the discrimination power of RFLP typing of forensic biological evidence and the use of chemiluminescence, instead of ^{32}P, as a detection component of the probe-target DNA sequence hybridization.

D5S110 - A HIGHLY POLYMORPHIC VNTR LOCUS

Of the genetic markers used for forensic analyses, the most informative for discrimination among individuals are the highly polymorphic VNTR loci. By using a panel of DNA probes, sufficient data can be obtained to produce a composite profile that is unique to an individual (excluding monozygotic twins). More importantly, typing VNTR loci currently provides the forensic scientist the best avenue to exclude a suspect who has been falsely associated with an evidentiary sample.

Two factors which influence the effectiveness of RFLP analysis are the availability of well-characterized VNTR loci and the quantity and quality of DNA in evidentiary material. RFLP analysis can be made more effective by identifying other VNTR loci, compatible with the restriction enzyme utilized for RFLP analysis (for our laboratories that would be HAE III), whose alleles generally fall in a size range that is greater than 1000 base pairs and less than 10,000 base pairs, are highly polymorphic, and have a high degree of sensitivity of detection. The VNTR locus D5S110 (Amour, et al. 1990) meets these criteria.

The histograms displaying allele frequencies in 31 fixed bin formats for Caucasians, Blacks, southeastern Hispanics, and southwestern Hispanics are shown in Figure 1 for locus D5S110. The D5S110 locus is highly polymorphic in all sample populations studied, with the Black population sample demonstrating the highest degree of variation. Most alleles are less than 10,000 base pairs in length. Furthermore, the probe for D5S110

provides a sensitivity of detection similar to pH30/D4S139 (approximately 10-20 ng of human genomic DNA may be typeable) (Fig. 2).

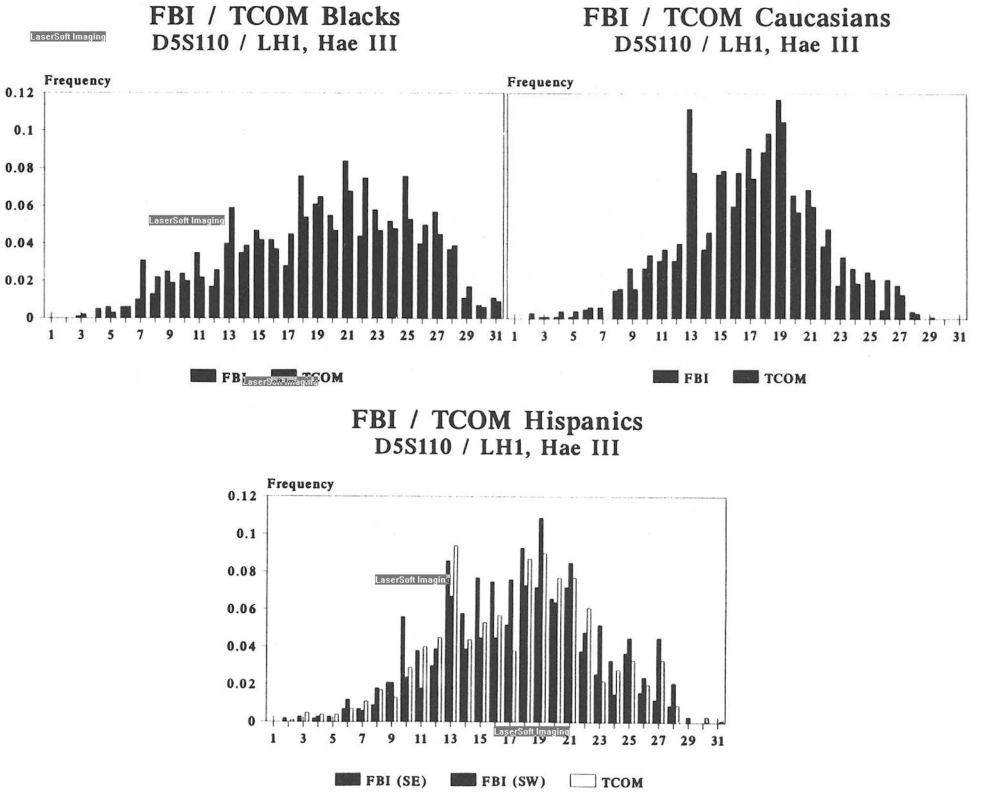

Figure 1. Histograms of binned allele distributions for Caucasians, American Blacks, and Hispanics from the FBI and TCOM.

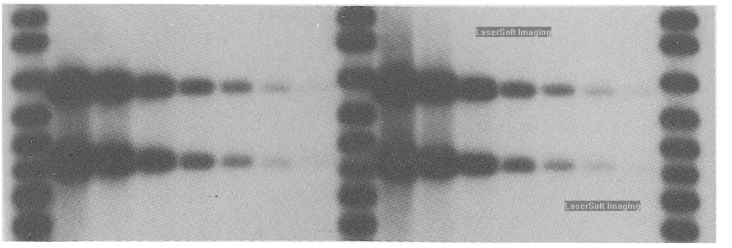

Figure 2. An Autoradiogram displaying a dilution series of Hae III digested human genomic DNA probed for the D5S110 locus. The quantities of DNA from left to right are: 1000 ng, 500 ng, 250 ng, 100 ng, 25 ng, and 10 ng.

The Caucasian, Black, southeastern Hispanic, and southwestern Hispanic population samples (FBI databases) were tested for independence using the exact test described by Guo and Thompson (1992); thus, all satisfy Hardy-Weinberg expectations for D5S110 (p=0.554; p=0.850; p=0.475; and p=0.163, respectively). In addition, D5S110 allele distributions from the FBI were compared with those obtained by TCOM. Although the FBI and TCOM

laboratories employ RFLP methodologies which vary to a degree (e.g., organic vs. non-organic extractions, ME vs. LE agarose, ethidium bromide vs. no ethidium bromide in the resolving gel, and different size standards) the D5S110 allele distribution appear similar for the same populations between the two laboratories (χ^2 p=0.053 and G_{stat} p=0.062 for rebinned Caucasian data; χ^2 p=0.083 and G_{stat} p=0.090 for Black data; and χ^2 p=0.110 and G_{stat} p=0.108 for southwestern Hispanic data).

Adams, et al. (in preparation) recently have evaluated the forensic utility of D5S110 for analyzing environmentally-insulted biological samples. The results were similar to that observed for other forensically-validated VNTR loci (Adams, et al. 1991). Based on these data, it is recommended that laboratories consider D5S110 as a useful locus for the forensic characterization of human biological samples.

CHEMILUMINESCENCE

PROBE LABELING

Hybridization of a DNA target sequence with its complementary DNA probe is a rapid, sensitive, and highly specific method for identifying a specific DNA component amongst a myriad of unrelated DNA sequences. Typically, a labeled nucleic acid probe is used to identify the position of a target DNA molecule which has been immobilized on a solid support. The strong affinity of the DNA probe for its target sequence results in a high degree of selectivity and precision in the hybridization reaction. The level of sensitivity of detection of target DNA is dependent upon the quantity of probe, hybridization conditions, and the procedure used to visualize the annealed probe/target hybrid, as well as the sequence of the probe. The quantity of probe and hybridization conditions required for augmenting sensitivity of detection are well-described and are used routinely in forensic RFLP analyses (Budowle and Baechtel 1990).

The ease of incorporation into DNA probes and the sensitivity of autoradiographic procedures have made radioisotopes (specifically ^{32}P) the most widely used detection component of hybridization systems. However,

there are inherent limitations associated with the use of radioisotopes, which include the instability of ^{32}P-labeled probes, disposal problems, and health concerns. The latter two can impact negatively on the transfer of RFLP typing to some application-oriented laboratories.

An alternative to incorporation of radioisotopes into probes is the attachment of enzymes to DNA probes. Enzymes provide catalytic activity that amplifies the primary hybridization signal, and a wide variety of substrates for subsequent detection are available. The enzymes most often employed in DNA detection strategies are alkaline phosphatase and horseradish peroxidase.

Alkaline phosphatase and horseradish peroxidase can be attached to a DNA probe either after (i.e., indirect assay) or before the hybridization reaction (i.e., direct assay). In indirect protocols, a probe is labeled with a ligand, such as biotin or digoxigenin; following hybridization, the probe-target DNA complex is recognized by a streptavidin or anti-hapten conjugated enzyme (Klevan and Gebeyehu 1990). This method is often employed for DNA probes obtained from cloned inserts from which little sequence information is available. Probes from several hundred to several thousand nucleotides in size can be labeled indirectly, and hybridization times ranging from several hours to overnight are common. In contrast, a direct assay employs synthesized oligonucleotides covalently linked to an enzyme. Several methods to prepare probes in this manner have been described (Jablonski et al. 1986, Mackey et al. 1992), and commercial probes and labeling kits are now available. The use of direct labeled probes reduces the number of development steps, as well as the overall time required to process the filter prior to the detection procedure. The annealed probe-target hybrid can be detected using a variety of reagents, such as chemiluminescence substrates.

CHEMILUMINESCENT DETECTION BY STABILIZED DIOXETANES

Chemiluminescence is the production of electromagnetic radiation as a result of a chemical process. Although many systems have been developed which produce light through a variety of chemical reactions (for a review see Campbell 1988), only a few have found application in DNA detection protocols. Many chemiluminescent assays were designed so that, upon activation, they emit radiation very rapidly; however, specialized instrumentation was required to detect the single "flash" of light emitted. Application of chemiluminescent detection to Southern blotting procedures used by the forensic scientist requires a system with continuous light output so that signal can be collected over time (for increased sensitivity) and multiple detections are possible. The most sensitive chemiluminescent system which emits a continuous "glow" and has found widespread application in genetic research and biomedical analysis involves the selective cleavage of stabilized 1,2-dioxetanes.

1,2-Dioxetanes are 4-member ring peroxides which decompose to form products which may be in an excited electronic state. These species then decompose further and emit light. The most familiar demonstration of dioxetane decomposition is the yellow flash of light observed from the firefly, *Photinus pyralis*. In this multistep bioluminescent reaction, the molecule luciferin reacts with ATP and molecular oxygen to form a substituted dioxetane intermediate which undergoes further rearrangement and light emission. The dioxetane is a short-lived unstable intermediate in the process, the half-life of emission for the reaction is approximately 40 msec (Handley et al. 1990). The first example of a dioxetane reagent which was stable at room temperature was the compound bisadamantyl 1,2-dioxetane (Wieringa et al. 1972). The molecule consisted of a dioxetane ring surrounded by two adamantyl groups and was found to be stable up to 240 degrees. More importantly, the reaction of this compound provided further insight into the mechanism for dioxetane decomposition. The mechanism involves stretching of the oxygen-oxygen bond followed by ring cleavage to produce two carbonyl species, one of which may be in an electronic excited state. The decomposition of the peroxide ring is sterically constrained by the presence of the bulky adamantyl groups which confer stability to the compound. This observation led to the development of the first enzyme-triggerable substrate.

Schaap, et al. (1987) developed a stable phosphate-substituted dioxetane, which was the first chemiluminescent substrate compatible with alkaline phosphatase. Upon removal of the phosphate stabilizing group, a reactive aryl oxide was generated which decomposed with low overall efficiency of light output. Dioxetane chemistry was advanced further when Schaap (1988) synthesized the phenylphosphate substituted dioxetane: 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane]. This compound, termed Lumigen® PPD, has a calculated half-life at 25 degrees of 19 years and is extremely stable in aqueous solutions (Schaap, et al. 1987). Schaap et al. (1989) also reported on the development of system for chemiluminescent energy transfer which greatly increased the quantum efficiency of the light producing reaction with Lumigen PPD. Removal of the phosphate group from PPD by alkaline phosphatase at high pH generates a highly reactive phenoxide dioxetane which associates with a surfactant preparation composed of cetyltrimethylammonium bromide (CTAB) and 5-(N-tetradecanoylaminofluorescein). The phenoxide derivative decomposes in the hydrophobic environment of the micelle to give an activated ester product which transfers its energy to the fluorescein acceptor so light can be emitted. The rate of dephosphorylation of PPD is not effected by the presence of the CTAB preparation and the observed efficiency of the light producing reaction is increased 400 fold over that observed when no modified surfactant is present. Further details on the chemical nature of dioxetane stabilization and activation have been described by Beck and Koster (1990). The PPD and surfactant in 2-amino-2-methyl-1-propanol buffered solution at pH 9.6 is the reagent termed Lumi-Phos® 530. Alternatively, Voyta, et al. (1988) reported the synthesis of a similar substrate termed AMPPD. AMPPD also has been demonstrated to be very stable with a half-life in water of 142 hours at 30°C and an indefinite shelf-life in solid form at 4°C (Bronstein, et al. 1989a, 1989b).

An alternate chemiluminescent system which has found application in DNA detection protocols is the enzyme catalyzed oxidation of luminol in the presence of enhancers (Mathews, et al. (1985). Luminol is modified by complexes formed between oxidants and horseradish peroxidase to form luminol radicals which decompose through an endoperoxide intermediate to form the excited state 3-aminophthalate dianion (Thorpe and Kricka 1986). When the excited state decays into its ground state, it emits light. This detection system has been used with Southern blot analysis (Polliard-Knight, et al. 1990: 84-89) and a human quantitation slot blot method (Walsh, et al 1992). However, it appears that alkaline phosphatase, with its ability to turn-over more substrate for a longer period of time, may be a better enzyme for a chemiluminescent assay (Polliard-Knight, et al. 1990:353-358). Furthermore, while luminol-based systems have faster ramp times than dioxetane-based systems, the signal from luminol also tends to decay more rapidly. These two factors make the use of alkaline phosphatase/dioxetane systems more desirable than horseradish peroxidase/luminol systems for RFLP typing.

Forensic Applications of Chemiluminescent DNA Detection

Giles, et al. (1989) were the first to apply alkaline phosphatase-labeled probes and lumigen as a detection substrate for human DNA identity typing. They found that the technology provided sensitivity with sharp bands, enabled sequential probing, and was relatively simple. Our laboratories are developing an assay system, using Lumigen PPD because of its demonstrated effectiveness in various research applications, for detection and analysis of DNA samples under conditions relevant to forensic use of chemiluminescent technology. Our results are similar to those of Giles, et al. (1989).

Dioxetane-based chemiluminescent detection was evaluated by us for quantitation of human DNA with the alphoid probe D17Z1 by a slot blot hybridization assay and for detection of human genomic DNA in a Southern blot format using oligonucleotide probes to the VNTR loci D1S7, D2S44, D4S139, D5S110, D10S28, and D17S79. The conditions for hybridization and detection of alkaline phosphatase-labeled probes have been optimized in our laboratories to facilitate use, speed, and sensitivity. A protocol using ACES 2.0 (Advanced Chemiluminescent Enhancement System) (BRL, Gaithersburg, MD) has been evaluated by us. This protocol has been developed for use with the non-charged nylon membrane Biodyne® A which (after UV-crosslinking of the target DNA to the filter) yields good results with low background staining. The same buffer (0.5M $NaPO_4$, pH7.2, 1% casein, 0.5% Tween 20) is used for prehybridization (20 min. at 55°C) and hybridization (20 min. at 55°C) of the membrane. This is followed by two 10 min. washes at 55°C in a solution containing 0.05M $NaPO_4$, pH7.2, 0.5% Tween 20 and two final washes (5 min. each) at ambient temperature in a solution containing 0.01M Tris-HCl, pH 7.5, and 0.15M NaCl (for human quantitation by slot blotting the temperature is at 50°C). Approximately 0.01 ml/cm^2 Lumi-Phos 530 is added to the membrane and the solution is incubated to allow the gradual increase in light intensity (termed "ramping") before exposure to XAR film (Kodak) at room temperature. The ramp-up of light intensity is often allowed to proceed for 3 hours or overnight. Kinetic studies have shown that 70% of the maximum light intensity output of the reaction is achieved within 3 hours of incubation and maximum light intensity generally is reached in 5 hours. The light intensity remains stable for at least 21 hours after ramping (Carlson et al. 1990). Multiple exposures with X-ray film may then be taken over a 24 hour period. Typical exposure times range from 30 minutes to 5 hours.

Klevan, et al. (1992, 1993) demonstrated that this detection system, employing an enzyme-triggerable dioxetane and chemiluminescence enhancers, provides a high degree of sensitivity in forensic protocols with a significant savings in overall filter processing time and development. The sensitivity of detection for human genomic DNA quantitation can be as little as 10-20 pg with the alphoid probe D17Z1 (Fig. 3). Moreover, they found that three probes for the VNTR loci D2S44, D4S139 and D1S7 enabled detection of DNA bands from 50-100 ng genomic DNA with a high degree of consistency and reproducibility. Furthermore, the nonisotopic chemiluminescent assay and the isotopic ^{32}P detection procedure behaved

similarly in that larger alleles (i.e., those containing more repeat sequences) were easier to detect than smaller alleles (Fig. 4).

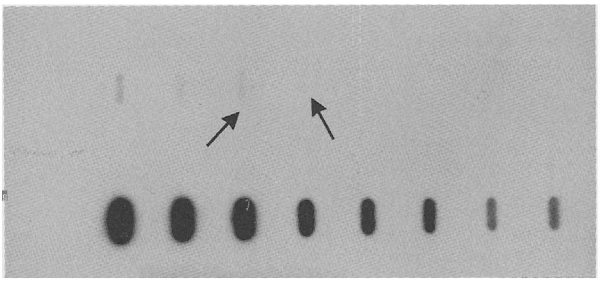

Figure 3. A two-hour exposure chemiluminescent slot blot quantitation of a dilution series of human genomic DNA displaying detection of as little as 10-20 pg of DNA (arrows).

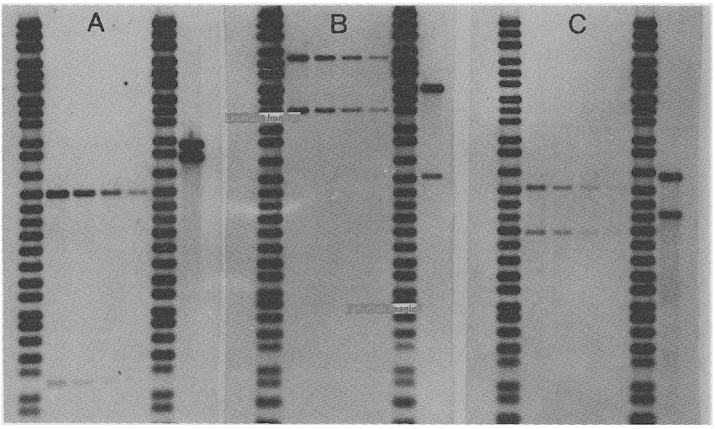

Figure 4. Lumigrams for three VNTR loci: D1S7 (A), D4S139 (B), and D5S110 (C). Each lumigram contains a dilution series of Hae III digested human genomic DNA. The quantities of DNA from left to right are: 400 ng, 200 ng, 100 ng, and 50 ng.

We also have observed that the membranes can be stripped at least six times with no apparent loss of bound DNA. Therefore, the analysis of forensic samples is similar to the ^{32}P-based system in that one membrane can be analyzed sequentially for several loci. This reduces the total amount of DNA required for a forensic analysis.

Chemiluminescence detection of alkaline phosphatase-labeled VNTR probes provides a rapid, sensitive technique for visualization of small quantities of DNA under conditions relevant for forensic analyses. An ancillary benefit of directly-labeled probes is their inherent stability. A batch of probe can be quality checked and used over a long period of time (unlike isotopically-labeled probes). Thus, quality control for RFLP typing can be improved.

While it may be that on average ^{32}P labeled probes provide greater sensitivity than non-isotopic systems, this should not present a problem for the majority of DNA human identification analyses. For paternity testing, the limitation of the quantity of DNA is rarely an issue. For forensic cases that yield conclusive results, there generally is sufficient DNA to be detected using a chemiluminescence-based detection

system. It can be envisioned that for those cases where the quantity of DNA is insufficient for non-isotopic RFLP analyses, a simple strategy can be developed to accommodate characterization of the evidentiary materials.

Once the DNA has been extracted, the quantity can be estimated using a chemiluminescent-based slot blot system (such as ACES 2.0 Human Quantitation System, BRL, Gaithersburg, MD). If, for example, the amount of human genomic DNA were greater than 100 ng, then it could be decided to proceed with analysis using the non-isotopic RFLP approach; however, if the total recovered human DNA were less than 100 ng, then the sample can be analyzed with any of a number of PCR-based assays, such as HLA-DQα (AmpliType HLA DQα kit), AmpliType PM, D1S80, or short tandem repeat loci. The actual quantity of human DNA necessary to proceed with either RFLP or PCR-based typing will have to be determined, once the chemiluminescent-based RFLP system is optimized. Regardless, this strategy of employing chemiluminescent-based RFLP assays (or PCR-based testing when the DNA is limiting) enables the forensic laboratory to characterize evidentiary samples with a high degree of discrimination in an efficient, effective manner without compromising quality and without exposure to some potentially hazardous chemicals.

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