TECHNICAL NOTE

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The Use of Ultrathin-Layer Agarose Gels for Phenotyping Erythrocyte Acid Phosphatase by Isoelectric Focusing

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ABSTRACT: A method is described for the use of ultrathin-layer agarose gels in phenotyping erythrocyte acid phosphatase (EAP) by isoelectric focusing (IEF). The results obtained using ultrathin-layer agarose gels are shown to be equally reliable and reproducible in comparison to established ultrathin-layer polyacrylamide gels. IEF of EAP on 0.168-mm agarose gels took place in 90 min using the LKB Multiphor system. The technique described allows for both time and cost efficient phenotyping of EAP.

KEYWORDS: forensic science, genetic typing, phosphatases, isoelectric focusing

Erythrocyte acid phosphatase (E.C. 3.1.3.2.) (EAP) is a protein of approximately 15 to 20 000 dalton molecular weight governed by three codominant autosomal alleles P^a, P^b, and P^c located on Chromosome 2. Methods for separating the phenotypes of EAP have been described for conventional electrophoresis [*1,2*] and ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) [*3-5*]. Diffusion and a lack of clarity can cause difficulty with subjective interpretation of relative band intensities in conventional electrophoretic phenotyping of EAP. ULPAGIF helps in resolving the issues of band intensity, diffusion, and clarity. In focusing the proteins on a narrow pH gradient, the proteins are concentrated in narrow bands, thus minimizing diffusion and subjectivity based on band intensity. Ultrathin-layer polyacrylamide gels also provide the option of preparing a number of plates in advance. However, in the situation where advance preparation is not feasible, ultrathin-layer agarose gels provide another option. Unlike overnight chemical polymerization or 3-h, long wave ultraviolet (UV) polymerization, an ultrathin-layer agarose gel can be ready for IEF in 1 h. Agarose gels also provide the advantage of working with a nontoxic gel medium rather than a neurotoxin such as acrylamide.

The modified technique also affords the same advantages of ULPAGIF in the interpretation of EAP phenotypes over the conventional method of electrophoretic analysis of EAP.

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Materials and Methods

Bloodstain samples were provided from the collection of standards and rare variants of the Illinois Department of State Police Training and Applications Laboratory. The samples had been stored in liquid nitrogen. The effect of age on band patterns was observed using stains that had been prepared on washed cotton cloth and stored at room temperature.

A sample measuring 1 by 5 mm is cut from each stain and extracted for 30 min in one drop from a double-drawn pipette of 0.05*M* DL-dithiothreitol (Cleland's reagent). The sample is then further diluted with an additional drop of Cleland's reagent. Scotch brand electrical (isolierband) tape is used to form the gasket on which the gels are cast. Three layers of 8-mm wide strips of tape are carefully placed on the long borders of a 259- by 124-mm glass plate. Each layer of tape was found to be 0.0056 cm thick. The gasket formed is, therefore, 0.168 mm deep. GelBond® NF film (FMC) is used as a support for the gel.

A minimum volume for comfortably producing a 0.168-mm-thick gel was found to be 9.2 mL. LKB ampholines pH 4 to 6 and 6 to 8 were selected. The gel is made with 0.3-mL ampholine pH 4 to 6, 0.3-mL ampholine pH 6 to 8 (5.4% ampholytes), 1.74-g sucrose (15.5%), 0.0964-g Agarose IEF (Pharmacia) (0.9%), and 8.6-mL distilled water (77.9%). The ampholytes are initially mixed together. The bottom gasket plate is placed in a 56°C oven until the gel solution is ready to pour. Omission of this step in the procedure will cause

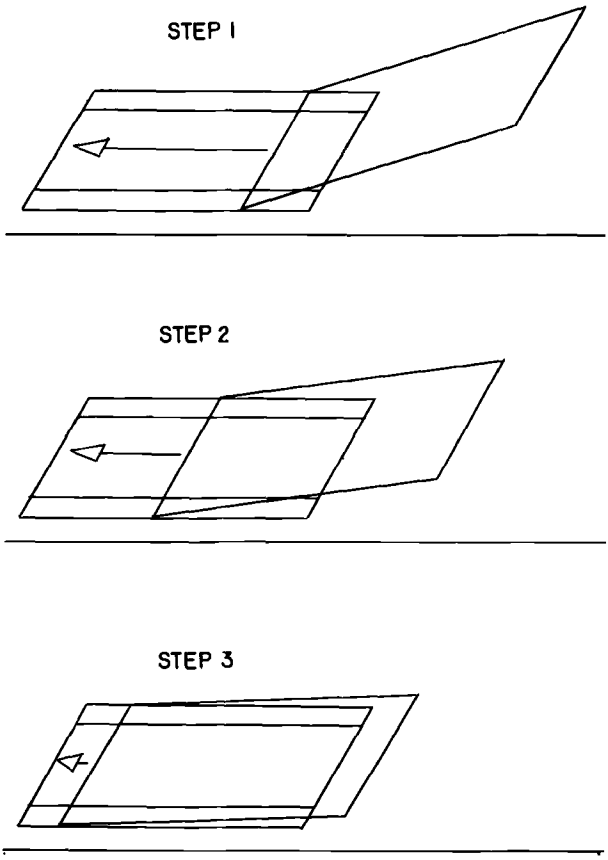

FIG. 1—*A diagram of the gel casting procedure used in both ULAGIF and ULPAGIF.*

rapid cooling of the gel solution when poured and difficulty in producing a gel of uniform thinness. The hydrophobic side of the gelbond film is placed on a glass plate of equal dimensions to the bottom gasket plate. A small amount of water is used to make the gelbond adhere to the plate.

The sucrose, agarose, and water are mixed together and boiled until all products are dissolved. The mixture is then degassed. The ampholytes are added to the solution when it cools to 75°C. The solution is then gently swirled and poured along one of the short edges of the bottom gasket plate. The short edge of the top plate is placed in the solution and the plate is lowered and simultaneously slid forward in the solution, trapping no air bubbles between the bottom plate and the gel bond (Fig. 1). The gel is allowed to polymerize at room temperature for 10 min and, on a 10°C cooling plate, for 50 min. After the gel cools, a scalpel blade is used to lift off the top plate. The gelbond is then lifted off the gasket with the gel adhering to its hydrophilic side. Approximately 8 mm of the gel is then trimmed off the short edges of the gel, creating a uniform border around the gel.

Treated stains are centrifuged and the liquid extract absorbed onto 5- by 5-mm squares of Whatman #1 filter paper. The samples are applied approximately 30 mm from the anode. The anodal electrolyte solution is $2M$ phosphoric acid (H_3PO_4). The cathodal electrolyte solution is $0.84N$ sodium hydroxide (NaOH).

No prefocusing is required. The voltage is set at 1600 V and the power at 5 W for the first 15 min. The power is set at 10 W for the final 75 min of the run. The voltage remains set at 1600 V for the entire 90-min run. The plate is developed with a solution of 4-methylumbelliferyl phosphate, according to Randall et al. [*4*].

Results and Discussion

Figure 2 shows an ultrathin-layer agarose gel isoelectric focusing (ULAGIF) run. Figure 3 shows an ULPAGIF run. Band intensities of the two isozymes of the EAP CB phenotype are relatively equal. Previous runs with thicker gels, 0.5 mm or more, presented the problem of weak C bands in heterozygous CB individuals, giving the appearance of a Phenotype B. Ultrathin-layer agarose gels overcome this problem.

Stains stored at room temperature up to six months were typed by conventional electrophoresis. The five CB samples identified were run by ULAGIF and no problems in interpretation were observed. Blind trials were also conducted from the 50 samples aged up to 6 months and no mistypings or inconclusive results were produced.

As noted by Divall [*3*] and Dykes,[2] the isoelectric points of the P^d and P^a allelic products are very close, the D bands being only slightly more cathodal than the A bands. The same result is produced in both ULAGIF and ULPAGIF. Conventional electrophoretic typing is required for clear separation of D and A isozyme bands.

ULAGIF is suggested as a reproducible and reliable alternative to ULPAGIF.

Upon completion of this paper, the authors were notified that FMC has discontinued production of GelBond NF film. Standard gelbond may be substituted with a few minor procedural changes. First, when the gelbond is applied to the top plate, all excess water should be squeezed out. The water may be displaced using a roller or by wiping across the hydrophilic surface of the gelbond with a Kimwipe®, expelling the excess water. This procedure is complete when the gelbond firmly adheres to the top plate. Another change to be noted concerns reading the developed plate. Standard gelbond is fluorescent. Therefore, the cellulose acetate membrane (CAM) should be peeled away from the gel, placed on a glass plate, and then read under long wave UV.

[2]D. Dykes, personal communication, 30 Jan. 1985.

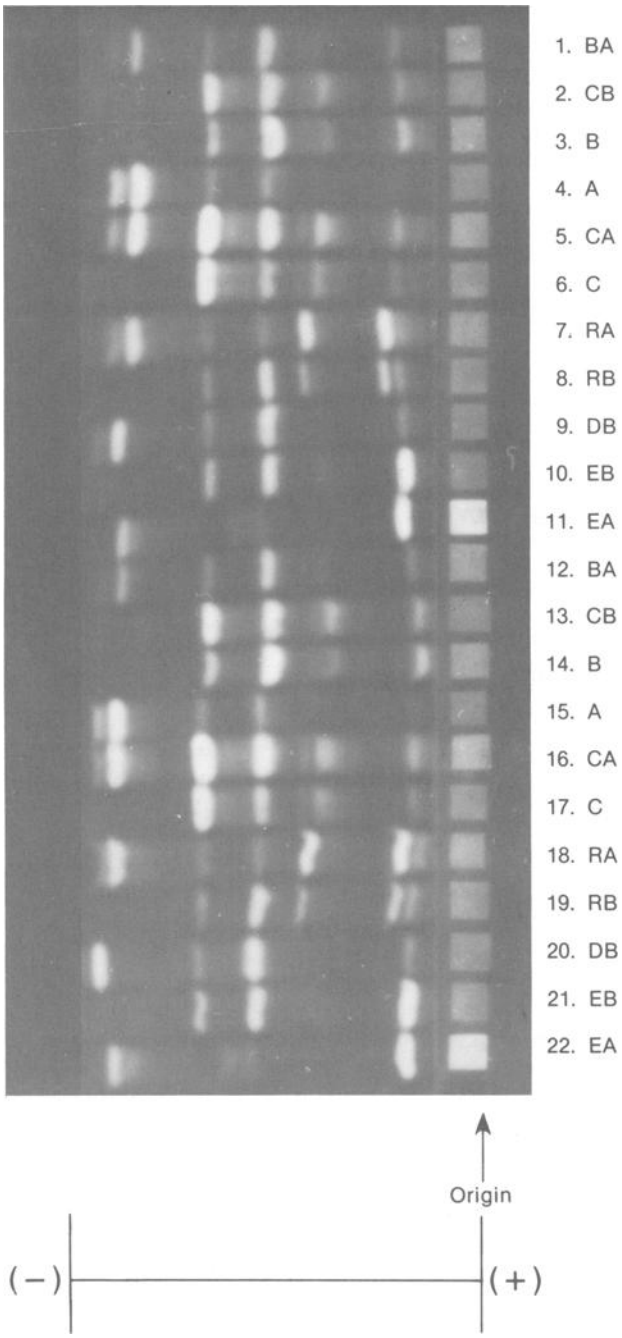

FIG. 2—*Phenotyping EAP by ULAGIF: a comparison of common phenotypes and rare variants.*

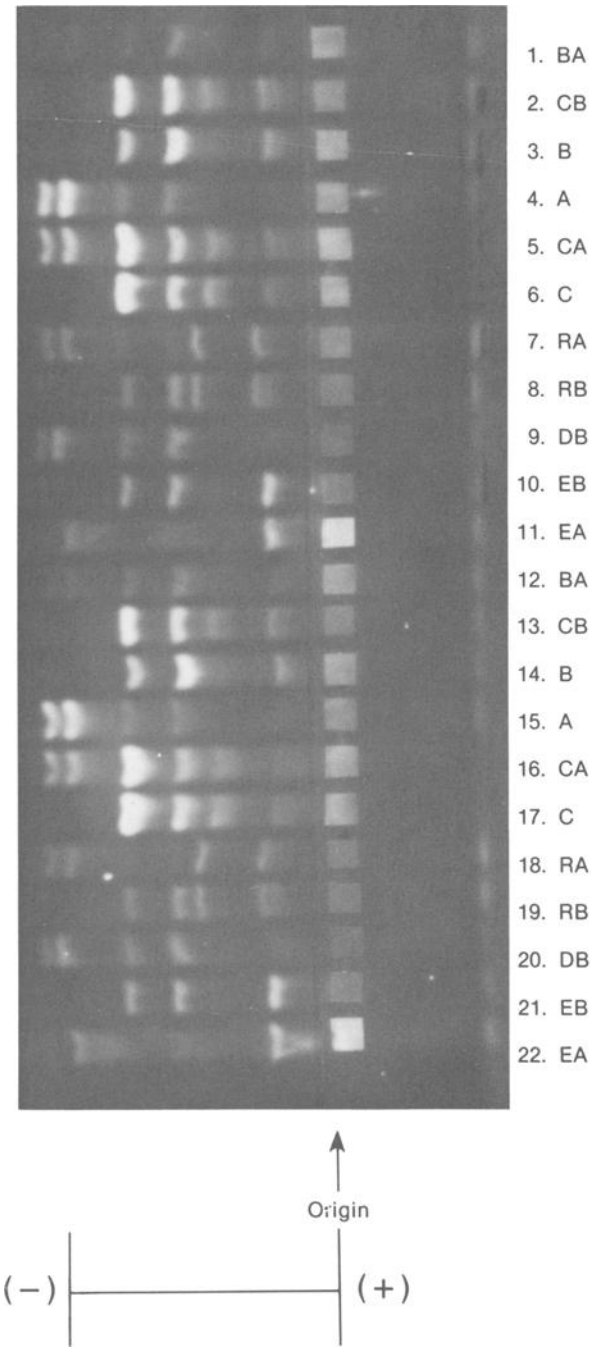

FIG. 3—*Phenotyping EAP by ULPAGIF: a comparison of common phenotypes and rare variants.*

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