# TECHNICAL NOTE

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# A Hybrid Ampholyte Focusing Technique for Esterase D Subtyping of Evidentiary Material

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**ABSTRACT:** An ultrathin-layer polyacrylamide gel isoelectric focusing technique that uses a composite of ampholytes from three commercial sources is described for subtyping esterase D. All common allelic products of esterase D were separated clearly. The technique described in this paper provides a higher conclusive call rate on known blood specimens (95.8%) and questioned bloodstains (69.7%) compared with continuous zone electrophoresis in agarose gels (89.9 and 37.6%, respectively).

**KEYWORDS:** forensic science, esterase D, genetic typing, isoelectric focusing, bloodstains, ultrathin-layer polyacrylamide gel isoelectric focusing, agarose gel continuous zone electrophoresis, ampholytes, evidentiary material

Esterase D (EsD; E.C. 3.1.1.1) is an erythrocyte-borne polymorphic enzyme used for the characterization of liquid blood and bloodstained materials. Continuous zone electrophoresis (CZE) of EsD results in three common phenotypes (1, 2-1, and 2) which are governed by two autosomal codominant alleles, designated EsD 1 and EsD 2 [*1*]. Martin [*2*], using high voltage agarose gel CZE, discovered that the EsD2 allele could be subtyped into an additional allele, EsD5. The capability of detecting the EsD5 allelic product increased the discriminating probability of EsD from 0.35 to 0.43 in Caucasians [*3*]. However, the high voltage agarose CZE approach did not separate clearly the EsD2 and EsD5 allelic products. Subsequently, several investigators used isoelectric focusing (IEF) in an attempt to increase the separation distance between the EsD2 and EsD5 bands. The first reports [*4-8*] showed that when EsD was separated by IEF, the EsD2 and EsD5 allelic products could be readily distinguished, but the EsD1 and EsD2 allelic products could not be differentiated clearly. Thus, reliable typing of EsD subtypes required that samples be analyzed by both CZE and IEF.

In 1984, Divall [*9*] and Budowle [*10*] reported on IEF methods for resolving the six subtypes of EsD. Since then, additional IEF techniques have been developed for typing the

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complete set of common EsD phenotypes [*11-16*]. Budowle [*10,16*] has developed two ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) techniques for subtyping EsD. The first approach used Serva ampholytes (pH 4.5 to 5.0 and 5.0 to 5.5; 1:3.4) [*10*]. This method yielded good separation of all common EsD allelic products, but EsD1 bands were somewhat broader than would be expected for a ULPAGIF technique (see Ref *10*). In contrast, the ULPAGIF procedure with Pharmacia ampholytes (pH 4.5 to 5.4) [*16*] produced zymograms with sharp bands but resulted in a decreased separation distance of the EsD1 and EsD2 allelic products. Thus, the EsD2 band and the middle dimer band of an EsD 2-1 phenotype could not be resolved.

This technical note describes a hybrid ampholyte ULPAGIF method that produces zymograms with sharp, linear band patterns and resolves all common allelic products of EsD.

## Materials and Methods

Blood samples were obtained from 20 donors of known phenotype at the FBI Academy by finger prick. In addition, 386 known liquid bloods and 221 questioned dried bloodstains deposited on a variety of strata submitted to the FBI Laboratory were analyzed. Bloodstains were prepared as described previously [*17*].

Cuttings of the stains (2 by 5 mm) were extracted in 20 to 40 uL of 0.05*M* dithiothreitol for 30 min. The extracts were absorbed into 10-by 5-mm applicator tabs (LKB), lightly blotted, and applied 1 cm from the cathode.

Polyacrylamide gels (5% T, 3% C, 125 by 110 by 0.2 mm) were cast using the flap technique [*18,19*]. The gels contained the synthetic carrier ampholytes pH 4.5 to 5.0 (Serva), pH 5.0 to 5.5 (Serva), pH 4.5 to 5.4 (Pharmacia), and pH 4 to 6 (LKB) in a ratio of 1.1:3.9:5:1. The total ampholyte concentration in the gel was 4% (w/v). The catholyte and anolyte were 0.20*M* sodium hydroxide and saturated L-aspartic acid, respectively. The distance between the electrodes was 9.5 cm. The ULPAGIF was performed on the Ultrophor (LKB) at 4°C using the conditions outlined in Table 1. After ULPAGIF, the EsD zymograms were developed according to the method of Hopkinson et al. [*1*].

For comparative purposes, EsD subtyping also was performed as described previously [*10,16*], and the anolyte was either saturated L-aspartic acid or 0.05*M* phosphoric acid.

## Results and Discussion

Figure 1 shows that the allelic products of EsD in bloodstains can be separated clearly without distortion by hybrid ampholyte ULPAGIF. The bands are sharp and linear, and the

TABLE 1—*Runnings conditions for EsD subtyping on hybrid ampholyte ultrathin-layer polyacrylamide gels.*

| Comments[a] | Initial Voltage | Time, min |
|---|---|---|
| Prefocus | 250 | 25 |
| Apply samples 1 cm from cathode, adjust settings | 500 | 10 |
| Adjust settings | 1100 | 10 |
| Remove sample tabs, adjust settings | 1500 | 10 |
| Adjust settings | 2000 | 10 |
| Adjust settings | 2500 | 10 |
| Adjust settings | 3000 | 10 |

[a]Always focus with constant power; adjust power to obtain voltage given in table (see Refs *17* and *20*).

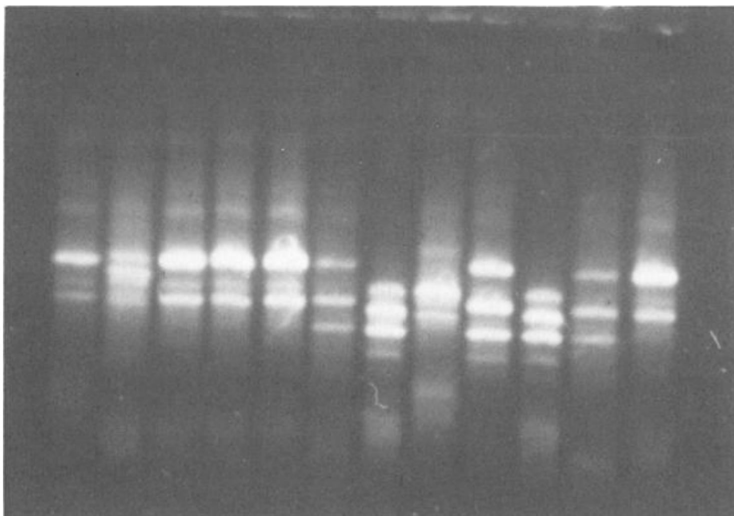

FIG. 1—*A zymogram of EsD subtypes derived from bloodstains. The phenotypes from left to right are: 1, 2-1, 1, 1, 1, 5-1, 5-2, 2, 5-1, 5-2, 5-1, and 1. The cathode is at the top.*

middle dimer band of the EsD 2-1 phenotype and the EsD2 band are resolved distinctly. Thus, this hybrid ampholyte gel method combined the positive aspects of both the Servalyt [*10*] and the Pharmalyte [*16*] ULPAGIF techniques.

Table 2 displays the effects that different ampholyte compositions and different anolytes can have on the separation distances of the EsD allelic products. The hybrid ampholyte gel (F) separated the EsD1 and EsD5 bands by 9.0 cm and EsD1 and EsD2 bands by 3.0 cm. The separation distance of the EsD1 and EsD2 bands was further than was possible using Gels A (Pharmalyte, no BES [*N, N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid]) and B (Pharmalyte and BES), and the EsD2-1 and EsD2 bands were separated clearly. Gel F also yielded zymograms with sharper, more linear band patterns than Gels D and E. While Gel C gave separation results comparable to Gel F, this approach is not recommended. As reported previously [*16,20*], a phosphoric acid anolyte at times can produce wavy, distorted patterns in gels containing Pharmalyte. Although Gel C produced good results in terms of separation distances of the EsD allelic products, reproducible linear patterns were not obtainable. The comparison of Gels D and E also suggests that using phosphoric acid as the anolyte can have a positive effect on the separation of the EsD allelic products. These observations support the findings of McCormick et al. [*21*] who suggested that different electrolytes can have varying effects on flattening the pH gradient.

Over a 2-month period, 386 known liquid blood specimens and 221 questioned dried bloodstains obtained from cases submitted to the FBI Laboratory were typed by ULPAGIF and agarose gel CZE [*22*]. The results are shown in Table 3. There was a slight increase in the number of conclusive calls for known blood samples using ULPAGIF (95.8%) compared with CZE (89.9%). However, there was a dramatic increase in conclusive determinations on questioned bloodstains (69.7 versus 37.6%). There were no phenotypic discrepancies between the two electrophoretic methods. The data is similar to that reported by Divall [*11*]. The greater success rate by ULPAGIF can be attributed to the concentrating effect of IEF, which provided more readily distinguishable patterns and greater sensitivity of detection.

TABLE 2—*Comparison of gels containing different ampholyte compositions and different electrolytes.*

| | | Separation Distance in mm Between | | |
|---|---|---|---|---|
| Ampholyte | Anolyte | EsD1 and EsD5 | EsD1 and EsD2 | Comments |
| (A) pH 4.5 to 5.4 (Pharmalyte) | aspartic acid | 9.0 | 1.0 | linear, sharp bands, but EsD1 and EsD2 bands do not separate effectively |
| (B) pH 4.5 to 5.4 (Pharmalyte) plus BES | aspartic acid | 10.0 | 1.5 | linear, sharp bands, separator improves separation distances but EsD2 and EsD2-1 bands do not separate |
| (C) pH 4.5 to 5.4 (Pharmalyte) | phosphoric acid | 11.0 | 3.0 | sharp bands with good separation; but phosphoric acid will yield undesirable wavy patterns |
| (D) pH 4.5 to 5.0 and pH 5.0 to 5.5 (1:3.4) (Servalyt) | phosphoric acid | 11.0 | 7.0 | resolves all EsD bands; EsD1 band is broad; patterns can appear scalloped |
| (E) pH 4.5 to 5.0 and pH 5.0 to 5.5 (1:3.4) (Servalyt) | aspartic acid | 10.0 | 5.0 | Same as D; demonstrates flattening effect of anolyte |
| (F) hybrid ampholyte gel described in materials and methods | aspartic acid | 9.0 | 3.0 | linear, sharp bands resolves all EsD bands |

TABLE 3—*Casework evaluation of EsD typing comparing ULPAGIF and CZE methods.*

| | | Conclusive | | Inconclusive | | No Activity | |
|---|---|---|---|---|---|---|---|
| Samples | No. | ULPAGIF | CZE | ULPAGIF | CZE | ULPAGIF | CZE |
| Known bloods | 386 | 370 | 347 | 12 | 35 | 4 | 4 |
| Questioned dried bloodstains | 221 | 154 | 83 | 33 | 79 | 34 | 59 |

## Conclusion

In conclusion, a method for subtyping EsD in evidentiary material which offers an improvement over the currently used technique has been described. The technique is reproducible, sensitive, and simple. A hybrid ampholyte gel provided a system for analyzing EsD with the positive qualities of the Servalyt and Pharmalyte gels. The EsD zymograms displayed sharp, linear bands, and all allelic products were resolved clearly. This procedure has been implemented in the FBI Laboratory.

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