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Authors' Response

Sir:

The response by Melton et al. of Mitotyping Technologies, LLC to the Divne et al. paper, "Forensic casework analysis using the HVI/HVII LINEAR ARRAY™ assay," raises some interesting points but contains some confused arguments. Melton et al. urge caution for any method that "develops only a partial profile on evidentiary material." Any genotyping method short of whole genome sequencing provides only a "partial profile," including STR genotyping and sequencing portions of the mitochondrial DNA (mtDNA) D-loop (i.e., the HVI and HVII regions). Melton et al. express concern about a perceived limitation of the linear array probe based genotyping by noting the potential for "null" or "blank" results, based on polymorphisms present in the population that fail to hybridize to any of the probes. The likelihood of such a result is a function of the number of probes used in a given polymorphic region and the population distribution of polymorphic sequence motifs recognized by the panel of probes. Dr. Melton's experience was with a 23 probe dot blot system whereas Divne et al. used an immobilized probe LINEAR ARRAY™ system (Roche Applied Science, Indianapolis, IN) with 33 probes, which were chosen to minimize the number of such "null" results and maximize the informativeness of the typing system. For example, the frequency of null results or "0's" observed in the IIB region in the African American population was significantly reduced from 63.9% (using three IIB probes as with the 23 probe system) to 10.8% (using seven IIB probes as with the 33 probe LINEAR ARRAY™ system). Also, it should be noted that such a "null" result is still informative and becomes part of the "mitotype" for that polymorphic region.

The authors also express concern that evidentiary samples whose linear array genotype does not match a suspect would not be available for comparisons with future suspects or other reference samples. We fail to see the basis for this concern. The probe reactivity patterns are available for comparison as well as the remaining PCR product from the genotyping and usually 9/10 of the original extract. In addition, if only a 2 cm portion of the hair (or half of the hair for shorter hairs) is consumed during the initial extraction, then the remainder of the hair will be available for further analyses years later. Moreover, the use of real-time PCR to estimate nDNA (or preferably mtDNA) copy number before typing or sequencing will ensure that only a small fraction of the total DNA in the extract is used in the analysis as performed by Divne et al. (1). Melton et al. further say the argument that the linear array PCR product will be available over time "is not logical." First, the PCR product amplified by the duplex primers can be used for any kind of HVI/HVII mtDNA analysis, including sequencing; it is not "a linear array PCR product." Second, the stability is not an issue of *logic* but of empirical observation. Our data indicate that the PCR product generated by these primers can be stored frozen for at least 1 year. Melton et al. advocate for developing a "full mtDNA sequence profile" at the time when the DNA is first extracted from the samples; we assume that they are simply referring to the HVI and HVII regions. In any case, we feel that this is an unnecessarily expensive and time consuming first step.

In general, the authors seem to overestimate the increase in discrimination power over the linear array afforded by sequencing the HVI and HVII regions. For example, the estimated discrimination power of the LINEAR ARRAY™ assay for African Americans is ~ 0.9927 compared with 0.9977 for HVI/HVII sequencing estimated from a population database consisting of ~ 200 African Americans. The discrimination power is limited by the presence of some rather frequent D-loop sequences; the most effective way to increase discrimination power is to analyze polymorphisms, by sequencing OR probe hybridization, in the rest of the mtDNA genome. We, and others, are developing such systems (2–4).

Melton et al. suggest that the time and cost required for HVI/HVII sequence analysis is not substantially greater than for LINEAR ARRAY™ typing. This view is not consistent with our experience. LINEAR ARRAY™ typing results for a set of 48 samples can be obtained in a single day, allowing for approximately 6 h for extraction, amplification, and PCR quantification and 3 h for typing and analysis. Typing can be performed manually using a rotating water bath or can be "hands-off" using a system such as the Tecan ProfiBlot™ 48 in as little as 2 h. Sequencing requires PCR purification, cycle sequencing, sequencing product purification, sequence data collection with an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) or similar instrument, sequence alignment and analysis in addition to extraction, amplification, and PCR quantification. The time required to generate and analyze a HVI/HVII sequence profile will vary depending on the methods and instruments used. Melton et al. estimate that sequencing results for a *single* sample and set of controls can be obtained in 2 days using an ABI Prism® 310 Genetic Analyzer. However, to obtain full HVI/HVII sequence data for 48 samples/controls, accounting for eight reactions per sample or a total of 384 sequencing reactions, the sequence data collection time alone using a ABI Prism® 310 Genetic Analyzer as used by Melton et al. would take ~ 16 days. Although it could be argued that a typical case would not involve 48 samples, mass casualty cases certainly could have this many or more samples. Therefore, we feel that the LINEAR ARRAY™ typing will prove to be invaluable in any future mass casualty cases contrary to the views expressed by Melton et al.

Furthermore, we feel that the reagent cost comparison provided by Melton et al. is misleading as the authors compare amplification and typing using the LINEAR ARRAY™ HVI/HVII system to the reagent cost of sequencing only a single amplicon and only in one direction. The authors appear to recommend a screening method involving the sequencing of a single amplicon (*only* a portion of HVI or HVII), an approach that is less discriminating than the LINEAR ARRAY™ mtDNA HVI/HVII Region-Sequence Typing Kit, more time consuming and more expensive. Melton et al. only account for the reagent cost of sequencing a single amplicon for the "questioned sample" in their estimate and do not account for the reagent cost of generating a full HVI/HVII sequence profile for the "known sample"; the total reagent cost would be \$540 (\$60+\$480), not \$60 as estimated by Melton et al. A full HVI/HVII sequence profile would require four PCRs and eight sequencing reactions per sample or control as carried out by Melton et al. (5), yielding a reagent cost of \$480 for a sample and three controls compared with \$124 for duplex amplification and LINEAR ARRAY™ typing (see Table 1). In fact, LINEAR ARRAY™ typing *and* HVI/HVII forward and reverse sequencing of the duplex amplicon for the sample and three controls has an estimated reagent cost of \$364. Moreover, their estimate only

TABLE 1—*Cost comparison of different strategies for HVI/HVII typing and/or sequencing.*

Method	Per Sample				For Sample, Negative, Positive, Reagent Blank			
	#PCRs	#Typings	#Sequencing Reactions	Reagent Cost	#PCRs	#Typings	#Sequencing Reactions	Reagent Cost
LINEAR ARRAY™ Typing	1	1	0	$31	4	4	0	$124
LINEAR ARRAY™ Typing and HVI/HVII F+R sequencing (duplex PCR)	1	1	4	$91	4	4	16	$364
Melton et al. HVI/HVII F+R sequencing (four separate PCRs*)	4	0	8	$120	16	0	32	$480

*Two fragments to cover the entire HVI and two to cover HVII.

accounts for reagent cost and does not include the cost of labor or the cost of the instrumentation, which are significantly more for sequencing than for LINEAR ARRAY™ typing.

They acknowledge that the "need to perform DNA sequencing may prohibit the average lab from undertaking mtDNA analysis" but argue that it is unlikely that the extracted DNA and/or PCR products from a lab that performs screening with the linear array "will be acceptable to a majority of sequencing labs." In our experience sequencing PCR products previously genotyped by the LINEAR ARRAY™ has not been a problem and we see no reason why it should be, as long as the samples (extracted DNA and PCR product) are handled using the appropriate quality assurance and controls.

It is understandable, perhaps, that a commercial mtDNA sequencing lab might prefer that all analyses of mtDNA in evidentiary samples be carried out by sequencing but it is hardly a compelling argument for the rest of us.

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