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Impact of Mutation Type and Amplicon Characteristics on Genetic Diversity Measures Generated Using a High-Resolution Melting Diversity Assay

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We adapted high-resolution melting (HRM) technology to measure genetic diversity without sequencing. Diversity is measured as a single numeric HRM score. Herein, we determined the impact of mutation types and amplicon characteristics on HRM diversity scores. Plasmids were generated with single-base changes, insertions, and deletions. Different primer sets were used to vary the position of mutations within amplicons. Plasmids and plasmid mixtures were analyzed to determine the impact of mutation type, position, and concentration on HRM scores. The impact of amplicon length and G/C content on HRM scores was also evaluated. Different mutation types affected HRM scores to varying degrees (1-bp deletion < 1-bp change < 3-bp insertion < 9-bp insertion). The impact of mutations on HRM scores was influenced by amplicon length and the position of the mutation within the amplicon. Mutations were detected at concentrations of 5% to 95%, with the greatest impact at 50%. The G/C content altered melting temperature values of amplicons but had no impact on HRM scores. These data are relevant to the design of assays that measure genetic diversity using HRM technology. (*J Mol Diagn* 2013, 15: 130—137; http://dx.doi.org/10.1016/j.jmoldx.2012.08.008)

High-resolution melting (HRM) technology has been widely used to detect specific mutations in DNA.[1,2] Mutations and polymorphisms in DNA amplicons affect the melting profile of DNA duplexes.[3] In HRM assays, DNA melting is observed as a sample is warmed over a range of temperatures; duplex melting is visualized as declining fluorescence due to the release of a saturating duplex-dependent fluorescent dye.[2] In HRM assays, mutation detection usually relies on changes in the temperature at which the peak melting rate is achieved (Tm) or on other features of melting curve shape.[4] Assays based on HRM of DNA duplexes have been developed to detect mutations associated with cancer[5] and genetic disease.[6] HRM technology is also being developed for analysis of specific mutations in bacterial, viral, and parasitic pathogens[7] that might harbor resistance mutations[8] or mutations conferring virulence.[9]

We developed a rapid assay for measuring nucleic acid diversity that is based on HRM technology.[10] This assay is based on the premise that the magnitude of the temperature range required to melt a particular DNA pool increases with the diversity of molecules in that pool. In the HRM diversity assay, the level of genetic diversity in a pool of DNA amplicons is reported as a single numeric HRM score. This assay has been successfully used to quantify genetic diversity in plasma samples from HIV-infected individuals.[10—12] The results of the assay are highly reproducible[10,13] and are significantly associated with sequence-based diversity measures.[12]

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HIV diversity may have important clinical, demographic, or subtype-specific correlates. Historically, analysis of HIV diversity has required costly and time-consuming sequencing analysis of individual HIV variants (eg, using cloning, single-genome sequencing, or next-generation sequencing). Although next-generation sequencing is a powerful tool for evaluating HIV diversity, the methods are expensive and require specialized equipment and complex data-handling protocols.[14,15] The HRM diversity assay offers a simple, less expensive, and scalable method for quantifying HIV diversity. HRM scores are associated with HIV disease stage in adults, suggesting that the HRM diversity assay may be useful for cross-sectional analysis of HIV incidence.[11] In pediatric populations, the HRM diversity assay has revealed associations between HIV diversity and duration of HIV infection,[13,16] infant survival,[13] and response to antiretroviral therapy.[16] The HRM diversity assay has also been used to document bottlenecking of viral populations in children exposed to nonsuppressive antiretroviral therapy.[16]

Previous studies have evaluated the impact of amplicon characteristics and mutation types on Tm. Herein, we characterized the impact of mutation type, amplicon size, mutation location within the amplicon, concentration of mutant DNA in a DNA mixture, and G/C content on HRM score. These data provide a foundation for understanding the output of the HRM diversity assay for analysis of HIV and for other applications.

Materials and Methods

Generation of HIV Plasmids

Three regions of the HIV genome, each approximately 1000 bp, were ligated into the EcoRV site of vector pUC57, and plasmids were propagated in the *Escherichia coli* strain DH5α. These regions correspond to the following nucleotide positions in HXB2 (accession number K03455): GAG, 1117 to 2106; POL, 4353 to 5342; and ENV, 7425 to 8411. The plasmid inserts [wild type (WT)] were then subjected to site-directed mutagenesis to generate a series of mutant plasmids with single-base change mutations. These mutations were introduced at the following nucleotide positions in the HXB2 sequences: GAG, 1614; POL, 4847; and ENV, 7916. Additional plasmids were generated by introducing a single-base deletion, a 3-bp insertion, or a 9-bp insertion at the same nucleotide positions. The sequence of each plasmid insert was verified by sequencing (Supplemental Table S1). WT plasmid inserts each matched the relevant region of GenBank (*http://www.ncbi.nlm.nih.gov/genbank*; accession number K03455). GenBank accession numbers for mutagenized plasmids are as follows: GAG, JX472242 to JX472247; POL, JX472248 to JX472253; and ENV, JX472254 to JX472259. Plasmids were diluted to a concentration of 5 ng/μL, and artificial diverse populations were generated from the diluted plasmid preparations. WT and mutant plasmids were mixed at the following ratios (WT:mutant): 0:100, 1:99, 5:95, 10:90, 25:75, 50:50, 75:25, 90:10, 95:5, 99:1, and 100:0, yielding populations that were 0%, 1%, 5%, 10%, 25%, 50%, 75%, 90%, 95%, 99%, and 100% WT, respectively. These plasmids and plasmid mixtures were analyzed using the HRM diversity assay, as described later.

Clade B Clones

A panel of 11 clade B molecular clones was obtained from the NIH AIDS Research and Reference Reagent Program (Pathogenesis and Basic Research Branch, Division of AIDS NIAID). This panel included transmitted/founder HIV-1 infectious molecular clones,[17–20] HXB2-gpt,[21] and HXB2-env.[21] These plasmids were diluted to approximately 5 ng/μL and analyzed with the HRM diversity assay, as described later.

Design of Primers Used in the HRM Diversity Assay

HRM primers ($n = 42$) were designed to anneal to each set of plasmids (Supplemental Table S1) at approximately the same distances (25, 50, 100, 150, 200, 300, and 400 bp) upstream and downstream of the mutation site (Tables 1–3). These primers were used to generate amplicons that varied in size and in the position of the mutation within the amplicon. Additional HRM primers ($n = 24$) were designed to amplify 12 different regions of the HIV genome using infectious molecular clones as templates; these regions were selected based on variable G/C content (Table 4 and Figure 1).

Analysis of Plasmids and Plasmid Mixtures with the HRM Diversity Assay

Regions of the HIV genome were amplified using plasmids as templates in the presence of LCGreen Plus dye (Idaho Technology Inc., Salt Lake City, UT) (Tables 1–4 and Figure 1). PCRs (10 μL) included the following components: 4.6 μL of H_2O, 4 μL of Idaho Technology Mastermix (Idaho Technology Inc.), 0.2 μL each of 10 μmol/L forward and reverse primer, and 1 μL (5 ng) of template DNA. Cycling conditions were as follows: a 2-minute hold at 95°C, followed by 45 cycles of 94°C for 30 seconds and 63°C for 30 seconds, followed by two sequential 30-second holds at 94°C and 28°C, and a terminal hold at 4°C. The resulting amplicons were melted using the LightScanner Instrument (model HR 96; Idaho Technology Inc.), and release of the dye was quantified as a function of temperature (melting range for amplicons derived from HIV *gag* and *pol* amplicons, 68°C to 98°C with a 65°C hold; melting range for amplicons derived from HIV *env* amplicons, 60°C to 98°C with a 57°C hold). Melting curves were used to determine HRM scores (the diversity output of the assay; melting peak width) using an automated software tool (DivMelt: HRM Diversity Assay Analysis Tool[12,22]) with