SPOTTING THE HAYSTACKS WITH THE RIGHT NEEDLES

Harald Niederstätter[a], Petra Kralj[a,b], Christiane Maria Bauer[a] & Walther Parson[a]

[a]Institute of Legal Medicine, Innsbruck Medical University, Austria
[b]Faculty of Criminal Justice and Security, University of Maribor, Ljubljana, Slovenia

Introduction

PCR is sometimes called the method that turns the proverbial needle in a haystack into a needle stack. Its specificity and sensitivity paved the way for the revolution witnessed in forensic individualization of biological material. STR markers are the "stars amongst the needles" and their multiplexed typing represents the gold standard in human identification, but the analysis of other genetic markers, such as mitochondrial or Y-chromosomal DNA, fills vital niches. The speed and simplicity of these analyses enable the typing of large sample numbers e.g. in population studies or DNA mass screenings. However, testing the entire sample set becomes highly uneconomical when the investigation aims only at a particular part of the sample. Under such framework conditions affordable and reliable pre-screening assays for the high throughput exclusion of e.g. innocents in a DNA dragnet or samples not attributable to the mitochondrial or Y-chromosomal haplogroup under study are desirable to avoid unnecessary STR genotyping or sequencing analyses.

Materials and methods

Multiplexed fluorescent allele-specific PCR and subsequent mtDNA SNP allele calling based on melting curve analysis (ARMS-DCA) as well as non-allele-specific mtDNA amplification and allele scoring by high resolution melting curve analysis (HRM) were used for the homogeneous pre-screening of a large population sample from Tyrol (Austria; ~ 3,700 specimen). For ARMS-DCA, the Power SYBR Green PCR master mix and ABI 7700 or 7500 Fast real-time PCR instruments (all Applied Biosystems) were used. HRM analysis of short amplicons was run on a Rotor-Gene Q machine using the Eva Green containing Type-it HRM PCR kit (both Qiagen).

Fig. 1) alternate states of two signatory SNPs typed

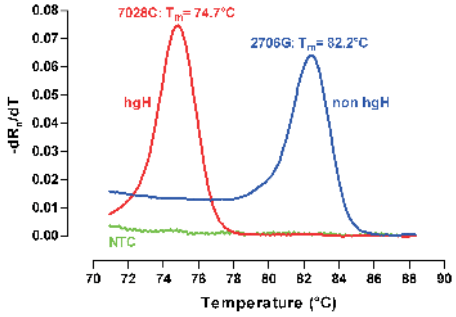


Fig. 2) hgH/nonH mixture detection

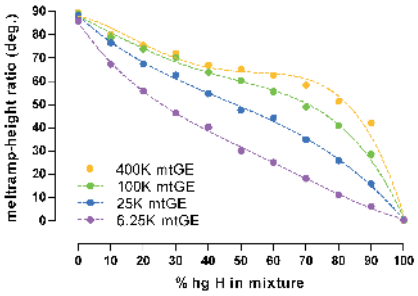


Fig. 3) both states of single SNP interrogated

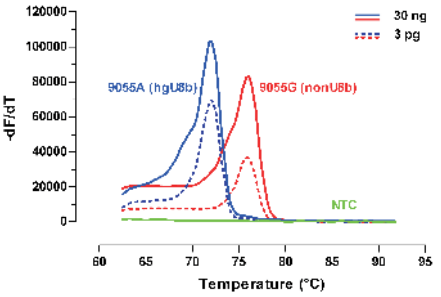
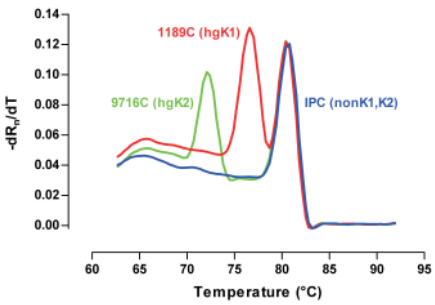


Fig. 4) derived states of two SNPs & IPC typed



Results and remarks

Both SNP-typing approaches fulfilled the requirements for a low-cost, reliable and high throughput pre-screening technique. Using a hierarchical approach, a large sample set was dissected into the mitochondrial haplogroups (hgs) H, J, T, U, U5, U8a, U8b, K1, K2, and the rest, setting the stage for economical in-depth genotyping experiments (data not shown).

HRM is an extension of dsDNA melting curve analysis. Consequently, both ARMS-DCA and (short amplicon) HRM feature a series of similarities, such as:

- simple design considerations
- end-point methods
- test execution is straightforward
- closed tube assay format
- quantitative information can be obtained when the pre-melt PCR is run on-line
- direct molecular haplotyping is possible; ARMS-DCA: 2 SNPs; HRM: 2 or more SNPs
- ability to detect of all four SNP classes as well as in/dels; for ARMS-DCA the intentional introduction of an additional mismatch is frequently required for reliable typing e.g. of a 3' terminal A>G transition; HRM: see Fig. 5
- mixtures can be quantitatively detected; during the temperature ramp, the dynamic redistribution of SYBR Green dye molecules to the non-denatured high T_m variant can result in complex melt-peak height ratio pattern (Fig. 2)
- small multiplexes are possible (Figs. 1, 4, 6, 8)
- choice between different assay layouts; ARMS-DCA: Figs. 1, 3, 4; HRM: short amplicon melting (~100 bp amplicons), probe melting, gene scanning for unknown mutations (~300-400 bp amplicons)
- instrumentation and optimized chemistries are readily available
- high amenability to automation

However, the two methods are also characterized by a list of differences from each other:

- with ARMS only the 3' terminally matching allele-specific PCR primer yields product; thus, only known sequence variants can be typed
- HRM genotyping uses two non-allele-specific primers
- ARMS-DCA genotyping is based on product characteristic T_m values (Figs. 1, 3, 4)
- for HRM analysis, the product characteristic relative position (Fig. 5) and shape (Figs. 6, 7, 8) of the normalized melt curves are considered, unknown samples are tested relative to reference samples with known genotype
- additional sequence variation is identified by atypical melt profiles
- dedicated algorithms allow for automated cluster assignments and the calculation of typicality values and class probabilities (Reja *et al*., Methods 50 (2010) S10-S14)
- ARMS-DCA shows a dynamic range typically spanning several orders of magnitude (Fig. 3), quantification and normalization of the DNA concentrations is not needed
- the concentrations of the DNA samples used in HRM genotyping should be within one order of magnitude, quantification/ sample normalization is recommended

Fig. 5) HRM of SNP classes I - IV (5 replicates each)

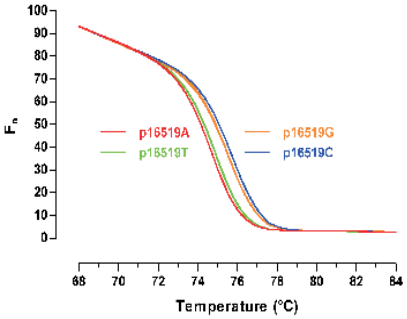


Fig. 6) triplex HRM genotyping

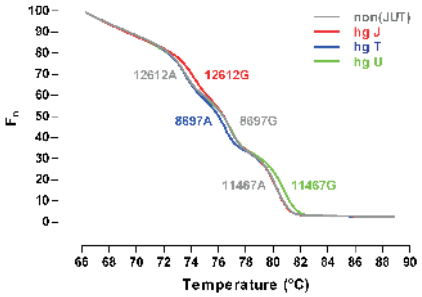


Fig. 7) difference to median plot

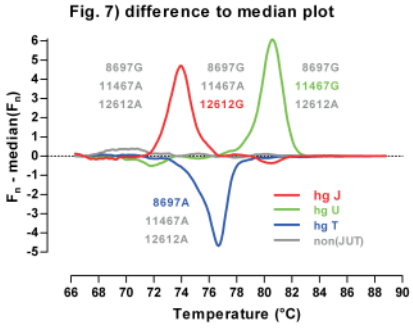
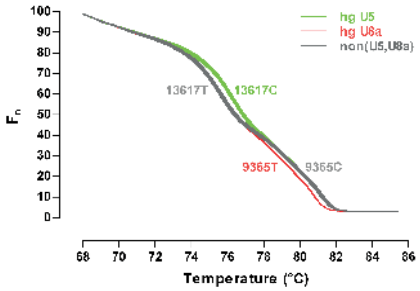


Fig. 8) hg U5, U8a genotyping (66 samples)



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