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Full Length Research Paper

Genotyping the NAT2 341T>C SNP Using High-Resolution Melting

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Genotyping N-acetyltransferase 2 (NAT2) for acetylation status of its enzyme is very important for personalized medicine, especially individualized dosing of anti-tuberculosis drugs and in bladder cancer epidemiology. Human NAT2 gene with at least 359 single nucleotide polymorphism (SNP) accessions is highly polymorphic which makes it difficult to design specific primers for allele specific PCR. Because of this, sequencing is the preferred choice to genotype for NAT2. Currently, a common tag SNP (rs1495741) and 7 SNPs (rs1801279, rs1041983, rs1801280, rs1799929, rs1799930, rs1208 and rs1799931) are found to be highly relevant with slow acetylation phenotypes. One of the latest contributions to related work is genotyping NAT2 with only two SNPs (rs1041983 and rs1801280) that outperforms the tagging SNP and is equivalent to the conventional 7-SNP NAT2 genotyping strategy. The aim of our work is to decrease the number of samples to be sequenced for one of the aforementioned SNPs (rs1801280 341T>C) using high-resolution melting analysis sequence matching function. It enables a prior elimination of the samples to be sequenced that are above a user-defined confidence threshold when genotypes are auto-called in comparison with sequencing and KASP confirmed reference samples. The workflow in screening type 1 SNP (341T>C) was shown with various remarks to experimental flow, such as improving the usability by gradient facility of thermal cyclers, HRM availability of Rotor-Gene 6000™ real-time PCR instrument and its software with auto-calling genotypes function, the commercially available EvaGreen™ based HRM kits and challenges of method.

Key words: NAT2, SNP, rs1801280, 341T>C, High-resolution melting, sequence matching, 5 × HOT FIREPol® EvaGreen® HRM Mix, SsoFast^{TM} EvaGreen™ Supermix, Type-it® HRM™ PCR Mix, Rotor-Gene 6000™.

INTRODUCTION

High-resolution melting (HRM) analysis is a PCR-based new technology that has applications in genotyping for known sequence variants, such as mutations and poly-morphisms and identifying similarities or dissimilarities in DNA as sequence matching. HRM can discriminate nucleotide sequence differences by comparing DNA melting behavior of different samples with high accuracy. No end-point detection such as gel/matrix separations or processing of the samples is required as it is in alter-native methods such as single- strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE), denaturating high-performance liquid chromatography (DHPLC), and temperature gradient capillary electrophoresis (TGCE). After PCR amplification, melting curves are generated by monitoring the fluorescence of a saturating dye that does not inhibit PCR. The third generation fluorescent dsDNA binding dyes with higher saturation capacities and low PCR inhibitory effect and the HRM channel assisted spe-cialized real-time PCR instruments with HRM analysis compatible softwares with different levels of usability and still the commercial availability of HRM kits contribute a lot for SNP work. It is a cheap, fast and simple method enabling both PCR amplification and genotyping to be performed within the same reaction tube making the

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Table 1. Complex diseases and disorders found to be associated with rs1801280 and related references' PMIDs.

No	Associated complex disease/disorder	PMID
1	Breast cancer	11291049, 12163321, 9829711, 17535831
2	Bladder cancer	15122594, 11431340, 15725609, 15312364, 12552951, 11431340
3	Cholangiocarcinoma	15901993
4	Lung cancer	11219770, 15808404, 15890241, 14648207, 9731715, 19789190
5	Prostate cancer	15717312,12355549, 12552951
6	Cervical cancer	12474054
7	Colorectal cancer	11245417, 16981843
8	Laryngeal cancer	12015038
9	Haematological neoplasias	10383893
10	Oral squamous cell cancer	11751430
11	Nasopharyngeal cancer	16567317
12	Stomach cancer	16615268
13	Asthma	11927838
14	Crohn's disease	16097053
15	Adverse effects of Sulfasalazine	12465141
16	Rheumatoid arthritis	12465141
17	Periodontitis	11846845
18	Contact sensitization	10848734
19	Plasma glucose concentration	10803679
20	Parkinson's Disease	10634239, 9343502
21	Cytogenetic studies	15450429
22	Asbestos-associated pulmonary disorders	8961976
23	Endometriosis	11675475
24	Urinary mutagenicity	12067576
25	Ulcerative colitis	16097053

method a good fit especially in molecular diagnostics and in personalized medicine to predict therapeutic responses (Herrmann et al., 2006; Mao et al., 2007; Reed et al., 2007; Gudnason et al., 2007; Eischeid, 2011; Marashil et al., 2012). N-acetyltransferase 2 (arylamine N-acetyl-transferase) (NAT2) enzyme plays a crucial role in the metabolism, both activation and deactivation, of arylamine and hydrazine drugs. NAT2 appears to code for the genetically variant NAT enzymes responsible for N-acetylation polymorphism in which human populations segregate into rapid, intermediate, and slow acetylator phenotypes involved in the etiology of many human diseases and drug toxicity (Hein et al., 1993; Hein, 2002; Garcia-Closas et al., 2005; Borlak and Reamon-Buettner, 2006; Khelil et al., 2010). NAT2 gene has at least 359 accessions of single nucleotide changes and 68 prevalent haplotype groups with 31 prevalent nucleotide changes as given in Consensus Human Arylamine N-Acetyltransferase Gene Nomenclature (http://n-acetyltransferasenomenclature.louisville.edu/). Therefore, it is very difficult to assess the acetylation status of the enzyme by genotyping methods. However, recently a common tag SNP (rs1495741) and seven SNPs (rs1801279, rs1041983, rs1801280, rs1799929, rs1799930, rs1208 and rs1799931) were found to be highly relevant with slow acetylation phenotypes. The latest contributions to related work is genotyping NAT2 with only two SNPs (rs1041983 and rs1801280) that outperforms the tagging SNP and is equivalent to the conventional 7-SNP *NAT2* genotyping strategy in determining the acetylation status (Kuznetsov et al., 2009; Garcia-Closas and Hein, 2011; Selinski et al., 2011). rs1801280 is found to be the signature SNP for 16 over 68 haplotype clusters. It is a clinically important one, with many associated complex diseases and disorders (Table 1). The risk allele for rs1801280 is rs1801280(C). Full details for this SNP can be obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/SNP/ snp_ref.cgi?searchType=adhoc_search&type=rs&rs=rs1801280).

In this case, we propose an extension of HRM analysis for identification of the similarities and dissimilarities in DNA using the sequence matching and genotype auto-call functions that uses the changes in the melting profiles of amplicon which can be in two forms: a shift in melting temperature and an obvious difference in the shape of the melt curve. Both of these parameters are a function of the amplicon sequence. Although rs1801280 is a type 1 SNP (T/C) easy to genotype using HRM, presence of 10 SNPs neighbouring within 100 bp of it (Table 2), makes it more difficult. For prescreening of this

Table 2. List of other SNPs that neighbor within 100 bases of rs1801280.

Distance (base)	rs
-59	rs1041983
0	rs1801280
4	rs45532639
5	rs183409091
13	rs146405047
23	rs4986996
40	rs140983217
46	rs144828000
62	rs12720065
70	rs4986997
93	rs72554616

SNP using HRM, in order to reduce the load of sequencing, in this work, we have designed specific primers amplifying a short 83 bp amplicon to minimize the number of chances of mistake one SNP for others in the vicinity (Ebeshil et al., 2011; Liew et al., 2004; Wittwer, 2009). Melt curve analysis to verify amplification specificity, normalization to select suitable samples and analysis range, producing difference plots according to reference genotypes and auto-calling the genotypes with user-set confidence values as integrity check for auto-called results, have been applied for the three commercial EvaGreen® based HRM mixes for their efficiency in application of genotype auto-calling function of Rotor-Gene 6000™ platform. This platform with suitable ready to use HRM kits, can characterize samples using the auto-calling function of the program clustering similar curve shapes automatically into groups repre-senting different genotypes with high accuracy.

MATERIALS AND METHODS

This work has been conducted as part of a thesis work in Fatih University Biology Department Laboratories. A comparative assessment and validation assay design work has been the subject to adopt HRM in prescreening for clinically significant NAT2 SNP (rs1801280 341T>C), as part of thesis titled ―Optimizations in SNP Detection Using Real-Time PCR High Resolution Melting Analysis‖.

Subjects and controls

The subjects used in the work are 21 (12 females and 9 males) healthy Turkish volunteers with their signed informed consents. Purification of genomic DNA from human whole blood samples was done using Magnesia 16 Genomic DNA Whole Blood Kit (Cat. No. MGB400-01), and via Magnesia 16 automatic machine of Anatolia Inc. The controls used in the work have been selected among the 21 subjects. A prescreen with KASP v.4 (KASP® DIAGNOSTIC and GTTT Metabolic-Dx®) showed 3 samples number 11,14 and 16 to have the genotypes T/T, C/C and C/T respectively; and a further confirmation of the genotypes were done by direct sequencing of the purified (Purification of the PCR products was done by using QIAquick PCR Purification Kit (50) (Cat. No. 28104 QIAGEN) PCR samples on an automated sequence analyzer (ABI-3730XL Capillary system, USA). The sequencing results were viewed in the version 1.4 of Finch TV DNA sequence viewer (Geospiza, USA). All products are prepared and used according to the manufacturer's instructions unless otherwise mentioned. The confirmed samples were used as controls of each genotype for sequence matching in HRM analysis to auto-call the genotypes of the unknown samples.

Gradient PCR and real-time PCR

A SNP encompassing primer set was designed on the DNA sequence (NCBI Reference Sequence: NC_000008.10), using primer 3 online software, according to Corbett research amplicon design protocol (CorProtocol™ 6000-1 July, 2006). It is critical to decide for the optimum annealing temperature (AT) when results depend on the PCR melting profile, so gradient thermal cycler (TECHNE TC512) was used to obtain the best annealing temperature for each commercial HRM real-time PCR kit (5 × HOT FIREPol® EvaGreen® HRM Mix (Cat. no. 08-25-00001 Solis BioDyne), SsoFastTMEvaGreenSupermix (Cat. no. 172-5200; Bio-Rad), Type-it® HRM™ PCR Kit (Cat. no. 206544; Qiagen) to have discriminating results in HRM analysis. Gradient facility of TECHNE TC512 has been used for each kit on separate runs with the plate design given in Figure 1. All the control samples were amplified and analyzed simultaneously in duplicates/triplicates as positive control for T/T, C/C and T/C. The PCR components and the conditions as adopted in this study according to each HRM kits' instructions are given in Tables 3 and 4. The amplified samples were analyzed with HRM of Rotor-Gene 6000™ real-time PCR instrument and Rotor-Gene Q Series Software 1.7 (Build 94) to auto-call the genotypes that match the controls with the highest confidence percentage.

High-resolution melting and data analysis

HRM analysis allows for sequence matching and auto-calling of genotypes for unknown samples with appropriate references. Results can be viewed as a normalized melt plot, difference plots and auto-called genotypes with confidence values (%). Normalization provides the selection of suitable samples and analysis range with the basic representation of the different genotypes based on curve shifting (for homozygotes) and curve shape change (for heterozygotes). Difference plots are an aid to visual interpretation.

HRM was performed in two platforms: preamplification with HRM or postamplification (amplification was done in TECHNE TC512 gradient PCR) and HRM analysis on the Rotor-Gene 6000™ real-time instrument with EvaGreen™ based three commercial kits. HRM curves were normalized and genotypes were assigned both manually and automatically according to HRM curve shapes by the Rotor-Gene 6000™ software with the user-set confidence threshold. A confidence value is calculated as an integrity check of auto-called results. The threshold value, above which auto-calls are made, can be edited. Samples that fall below the set threshold will be flagged as a variation for closer investigation with sequencing.

RESULTS

Genomic DNAs from controls: wild-type homozygote T/T, heterozygote T/C and risky homozygote C/C were amplified in duplicates/triplicates for optimization of the AT to provide the best resolution of genotypes of

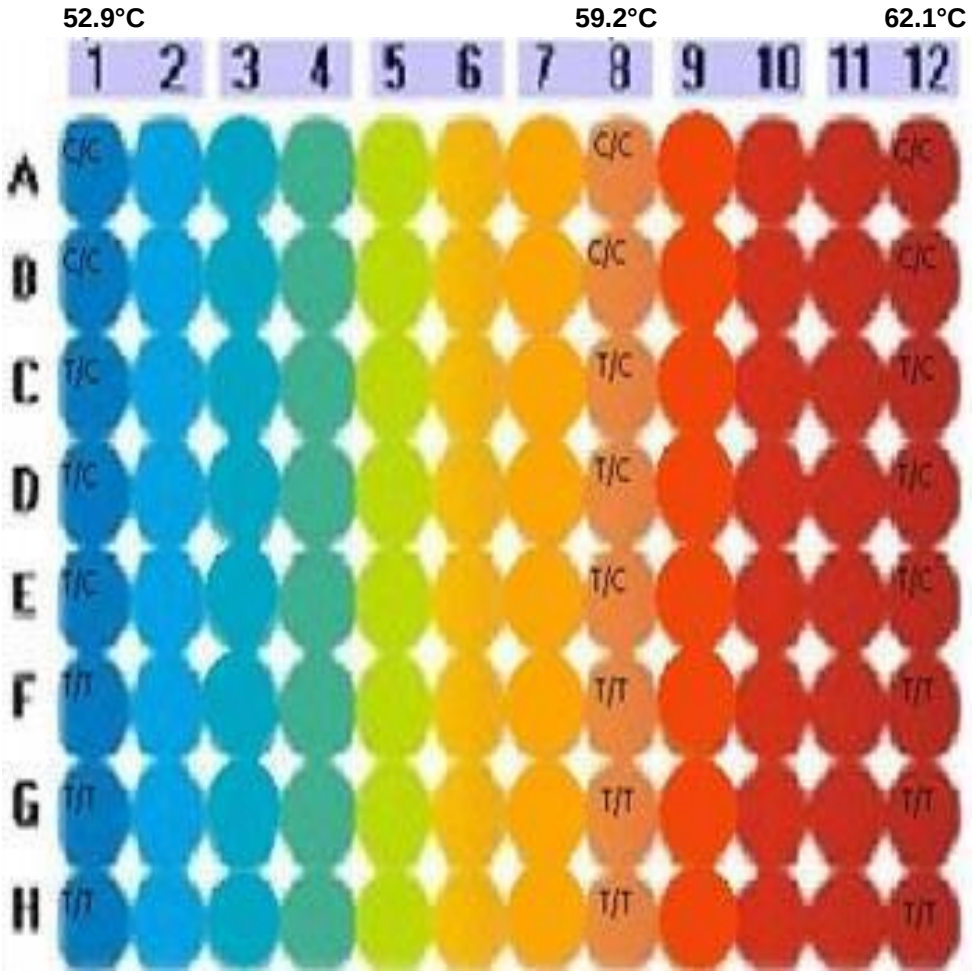

Figure 1. Gradient PCR plate design for control samples of known genotypes (C/C, T/C, T/T) in duplicates and triplicates for each HRM kit prior to HRM analysis to obtain the optimum AT for genotype discriminative amplification. Gradient 11°C, distributed evenly, starting from 52.9 to 62.1°C.

Table 3. PCR components for three commercial HRM kits as used in this study according to manufacturer's instructions and on Rotor-Gene 6000™ real-time instrument.

Components	BioRadSsoFast (2×) Vol (final concentration)	Qiagen Type-it (2×) Vol (final concentration)	Solis BioDyne (5×) Vol (final concentration)
Master mix	10 µl (1×)	12.5 µl (1×)	4 µl (1×)
Forward primer (12.5 µM)	0.8 µl (500 nM)	1.4 µl (0.7 µM)	0.4 µl (250 nM)
Reverse primer (12.5 µM)	0.8 µl (500 nM)	1.4 µl (0.7 µM)	0.4 µl (250 nM)
Template DNA	1 - 5 µl (1 - 50 ng/µl)	1-5 µl (1 - 50 ng/µl)	1 - 5 µl (1 - 50 ng/ µl)
ddH_2O	up to 20 µl	up to 25 µl	up to 20 µl
Total volume	20 µl	25 µl	20 µl

rs1801280 (Figure 2A, B and C) using the sequencing and KASP confirmed controls (sample numbers 11, 16 and 14). Then, the controls were co-amplified with unknown samples (Figure 3A and B) using 5 × HOT FIREPol® EvaGreen® HRM Mix, SsoFast^{TM} EvaGreenSupermix, Type-it® HRM™ PCR Mix and NAT2 rs1801280 specific forward and reverse primers F: 5'-TACAGCACTGGCATGGTTCACC-3' and primer R: 5'-

Table 4. Thermal cycling and HRM conditions for three commercial HRM kits as used according to manufacturer's instructions on Rotor-Gene 6000™ real-time instrument.

Cycling steps	BioRadSsoFast	Qiagen Type-it	Solis BioDyne
Enzyme activation	98°C – 2 min	95°C – 5 min	95°C – 15 min
Denaturation	98°C – 5 s	95°C – 10 s	95°C – 10s
Annealing Extension	59.2°C – 30 s	59.2°C – 30 s 72°C – 10 s	59.2°C – 20s 72°C – 20 s
Cycle #	40	40	40
HRM	75 – 95°C 0.1°C / 2 s	75–95°C 0.1°C / 2 s	75 – 95°C 0.1°C / 2 s

GAGCTTCCAGACCCAGCATCG-3' designed in this work to obtain auto-called genotypes with the highest confidence value (%) for each kit.

An 83-bp amplicon encompassing the rs1801280 SNP of NAT2 was amplified using a gradient thermal cycler followed by HRM analysis to optimize AT and usage of compatible kits in the Rotor-Gene 6000™ real-time PCR instrument Rotor-Gene Q Series Software 1.7 (Build 94). Three HRM kits with their specific PCR components (Table 3) and amplification/HRM protocols (Table 4) were compared for their accuracy in detection and discrimination of the three genotypes for rs1801280 SNP by visual inspection and genotype auto-calling function of the software with results shown as difference plots in Figure 2 A, B and C and selected auto-called genotypes in Table 5A, B, C and D with the highest confidence values for each HRM kit. Only the results for AT 59.2°C are shown in Figure 2A, B and C, and Table 5A, B and C according to the configuration in Figure 1. The most convenient temperatures for each commercial kit used for the genotype discrimination efficiency (evaluated as difference plots and auto-calling of genotypes with high enough confidence values in Table 5A, B and C), have been evaluated. For Solis BioDyne and Bio-Rad kits, the discrimination can efficiently be done at different annealing temperatures (refer to Table 5 D with different AT). Whereas for Qiagen's kit at 59.2°C for all genotypes, a highly discriminative difference plot and confident genotype auto-calling has been achieved.

The thermal gradient function of the conventional PCR instrument (TECHNE TC512) enabled determination of the optimum annealing temperature for the amplification of the 83 bp amplicon applicable with each HRM kit. Using the pre-determined annealing temperature HRM with pre-amplification has been performed in Rotor-Gene 6000™ real-time PCR instrument for the screening of all subjects (21) for each genotype with reference to samples 11,14 and 16 as controls. The best resolution of the three genotypes for each of the three different commercial HRM kits with the third generation EvaGreen™ dye was obtained at different optimum annealing temperatures (Figure 2A, B, C and Table 5A, B, C). Type-it among the three produced the most accurate sample clustering, genotype calling and discriminating curve shapes (Figure 2C) in difference plots and auto-called genotype confidence values (%) (Table 5D). Still, it is possible to make further optimizations with the kits' components and PCR conditions to obtain a better resolution for each kit or to work out with a real-time PCR instrument having a thermal gradient facility in itself.

In the last part of the work, 21 subjects were screened for sequence matching melt curve profiles of the amplicons to that of the controls (samples 11, 14 and 16) with genotype auto-calling function of the program with confidence values set higher than 88% to obtain the best visual separation in difference plot with reference to any of the three genotypes (T/T in our case) and normalized melt curves (Figure 3A and B, and Table 6). As these SNP screening results are a part of the optimizations for a novel application of HRM analysis with sequence matching function that is intended as a prescreening tool to reduce the workload of sequencing, a further confirmation of the genotype with sequencing has been done for the sample that has the lowest accepted confidence value 88.71% (sample with auto-called C/T genotype in Table 6). The samples having confidence values above 88.71% do not need further sequencing, but the ones below this threshold may contain other nearby SNPs to be detected by sequencing. These samples have also aberrant plots that do not fit any of the automatically clustered curve shapes.

DISCUSSION

It is very important to optimize all factors in the HRM workflow of SNP genotyping— from the amplicon design to the use of well characterized controls as references for

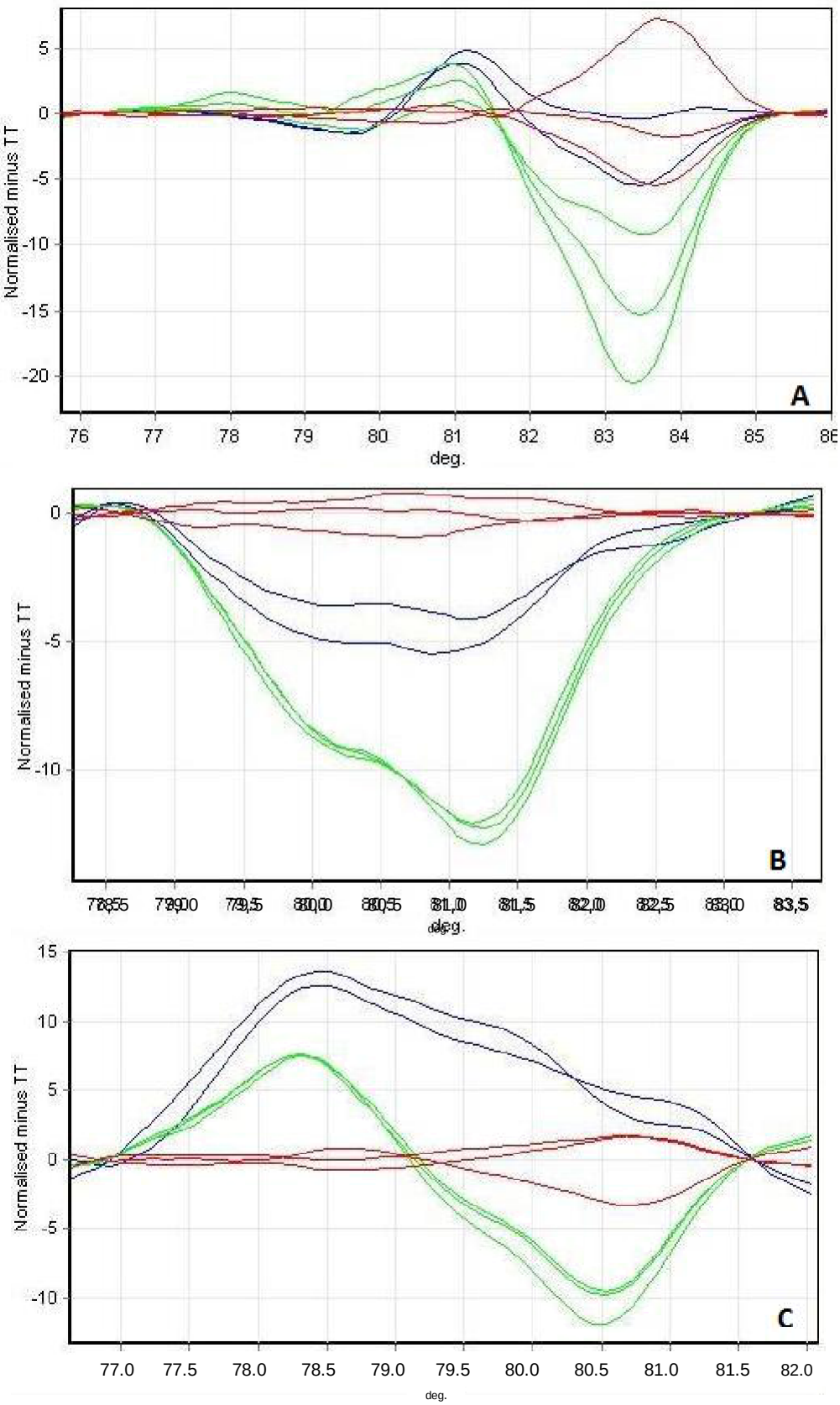

Figure 2. Difference plots to show the best resolution of the genotypes C/C (blue), C/T (green) and T/T (red) by using different kits at annealing temperature 59.2°C. Reference samples 14 in duplicates, 16 and 11 with triplicates.

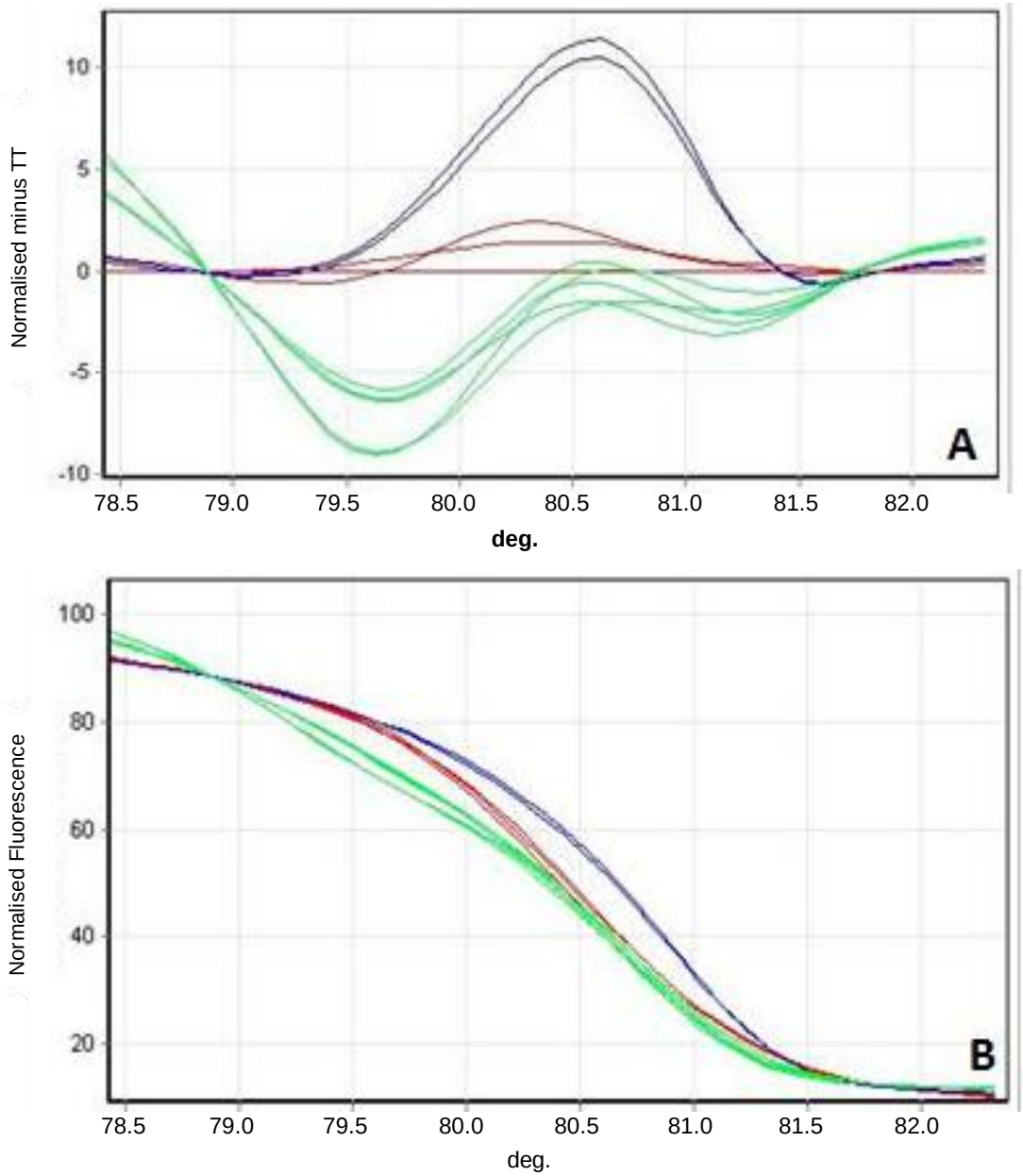

Figure 3. Co-amplification results of the unknown samples with controls. Difference plot (A) and normalized plot (B) for 10 samples filtered after prescreening and auto-calling genotypes above 88% confidence value.

auto-calling genotypes and to standardization of buffers and reagents as mentioned in previous works done with HRM (Liew et al., 2004; Herrmann et al., 2006; Reed et al., 2007; Wittwer 2009; Taylor et al., 2010). Results vary considerably depending upon DNA template quality, the particular sequences analyzed, chemistries used (fluorescent dyes and PCR components), HRM and gradient temperature availability of the real-time PCR instruments and the HRM compatible softwares and the way analysis is done.

In this work, a novel application for the optimization of NAT2 rs1801280 SNP suitable HRM assay design has been done successfully with aforementioned criteria to prescreen and genotype for samples to be used in downstream applications like pharmacogenetics, toxicogenetics and cancer researches.

Sequencing seems to be the gold standard for NAT2 SNP genotyping, also for rs1801280 where the work load can be reduced with our novel strategy in determining genotypes of some of the subjects with HRM sequence matching to the confirmed references. Although the Tm of a PCR product is a convenient metric, it is only one point

Table 5. Auto-called genotypes AT 59.2°C with the highest confidence value for each HRM kit selected from part D results.

Samples		Genotype	Confidence (%)
Color code	Names		
(A) HOT FIREPol Solis BioDyne			
	14 at 59.2°C	CC	100.00
	14 at 59.2°C	CC	66.87
	16 at 59.2°C	CT	98.90
	16 at 59.2°C	CT	68.46
	16 at 59.2°C	CT	65.56
	11 at 59.2°C	TT	67.93
	11 at 59.2°C	TT	96.21
	11 at 59.2°C	TT	53.31
(B) Sso Bio-Rad			
	14 at 59.2°C	CC	100.00
	14 at 59.2°C	CC	96.02
	16 at 59.2°C	CT	99.63
	16 at 59.2°C	CT	99.91
	16 at 59.2°C	CT	99.69
	11 at 59.2°C	TT	98.85
	11 at 59.2°C	TT	99.89
	11 at 59.2°C	TT	99.04
(C) Type-it Qiagen			
	14 at 59.2°C	CC	100.00
	14 at 59.2°C	CC	91.10
	16 at 59.2°C	CT	97.31
	16 at 59.2°C	CT	98.87
	16 at 59.2°C	CT	99.59
	11 at 59.2°C	TT	90.10
	11 at 59.2°C	TT	96.80
	11 at 59.2°C	TT	97.20

on the melting curve and in our case it is nearly impossible to discriminate the genotypes with only Tm values. More information is contained in the complete melting curve than in the Tm, so is used extensively in sequence matching and scanning in our case for more complex genotypes due to mighty neighbouring SNPs (Table 2), at least 10 less common genetic variants exist in our 83 bp region amplified.

To overcome challenges intrinsic in the nature of our work we have conducted experiments to optimize factors in the HRM workflow in the following way: from the amplicon design to the use of positive controls and, to standardization of buffers, reagents and thermal cycling instruments used and identifying the ideal number of

Table 5D. Continuation of Table 5.

Samples		HOT FIREPol Solis BioDyne		Sso Bio-Rad		Type-it Qiagen	
Color codes	Names	Genotype	Confidence (%)	Genotype	Confidence (%)	Genotype	Confidence (%)
	14 at 52.9°C	TT	72.75	CT	87.49	CC	55.94
	14 at 52.9°C	CC	94.22	CC	83.16	CC	89.55
	14 at 59.2°C	CC	100.00	CC	100.00	CC	100.00
	14 at 59.2°C	CC	66.87	CC	96.02	CC	91.10
	14 at 62.1°C	CT	41.29	CC	98.79	CC	78.86
	14 at 62.1°C	CC	90.31	CC	96.76	CC	84.23
	16 at 52.9°C	CT	72.91	CT	71.48	CT	83.25
	16 at 52.9°C	CT	48.26	CT	70.97	CT	93.34
	16 at 52.9°C	CT	76.11	CT	43.36	CT	96.98
	16 at 59.2°C	CT	98.90	CT	99.63	CT	97.31
	16 at 59.2°C	CT	68.46	CT	99.91	CT	98.87
	16 at 59.2°C	CT	65.56	CT	99.69	CT	99.59
	16 at 62.1°C	CT	72.28	CT	97.48	TT	60.48
	16 at 62.1°C	CT	0.00	CT	91.58	TT	70.22
	16 at 62.1°C	TT	68.69	CT	9.72	CT	55.21
	11 at 52.9°C	CT	65.26	CT	90.57	CT	43.99
	11 at 52.9°C	CT	24.90	CT	91.79	CT	43.85
	11 at 52.9°C	TT	75.46	CC	84.08	TT	75.83
	11 at 59.2°C	CT	67.93	TT	98.85	TT	90.10
	11 at 59.2°C	TT	96.21	TT	99.89	TT	96.80
	11 at 59.2°C	TT	53.31	TT	99.04	TT	97.20
	11 at 62.1°C	TT	70.44	TT	78.73	TT	62.22
	11 at 62.1°C	TT	59.25	TT	45.88	TT	46.55
	11 at 62.1°C	TT	37.05	TT	24.59	TT	46.24

Table 6. Auto-called genotypes for subjects above 88% confidence value.

Samples	Qiagen Type-it	
Color codes	**Genotype**	**Confidence %**
	CC	100.00
	CC	99.15
	CT	100.00
	CT	96.68
	CT	98.88
	CT	90.83
	CT	88.71
	TT	100.00
	TT	96.05
	TT	98.09

samples in each experiment have been optimized for rs1801280. 10 samples per experiment has proven ideal for our case (Figure 3), samples exhibiting similar curves are prone to be the same genetic variants, a phenomenon inherent in our technique based on melting of amplicons so samples with aberrant melting curves and auto-called genotypes with less than 88% confidence should be sequenced to confirm the genetic variants.

In conclusion, this study focused on how to use HRM analysis efficiently in genotyping and scanning of a clinically important SNP (rs1801280) of *NAT2* gene. HRM analysis is the simplest and the most cost-effective among its equivalents used as a prescreening tool for sequencing. For prescreening of samples prior to sequencing, it can be the method of choice using Rotor-Gene 6000™ HRM platform especially with genotype auto-calling function of the Rotor-Gene 6000™ software.

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