Cost-effective and high-throughput genotyping assays will enable the routine detection of single nucleotide polymorphisms (SNPs) in iron-related genes in the Philippines, where anemia due to iron deficiency remains a public health concern throughout the years. This paper presents the development of high-resolution melting (HRM) assays that were able to identify SNPs in \textit{HFE} or homeostatic iron regulator gene (rs1800562, rs1799945); \textit{TF} or transferrin gene (rs3811647, rs1799852); and \textit{TMPRSS6} or transmembrane serine protease 6 gene (rs4820268) in the Nutritional Genomics Laboratory of the Department of Science and Technology – Food and Nutrition Research Institute (DOST-FNRI NuGen Lab). Genomic DNA was extracted from 112 peripheral blood samples and SNP-genotyped using the Precision Melt Analysis™ Software of Bio-Rad CFX96™ Real-Time PCR (polymerase chain reaction) Detection System. Real-time amplification reactions were evaluated in terms of varying conditions in annealing temperature (Ta) and number of amplification cycles. HRM assays were then compared against that of the gold-standard sequencing technique using the validation parameters: sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The ideal parameters of HRM assays that can genotype the target SNPs were determined. Genotype discrimination resulted in three genotypes of \textit{TF} rs3811647, \textit{TF} rs1799852, and \textit{TMPRSS6} rs4820268 with varying frequencies among samples used. Two genotypes were detected for \textit{HFE} rs1799945, while only one genotype was obtained for \textit{HFE} rs1800562. The confidence of genotype clusterings were at least 95%. All assays had 100% concordance with the sequencing results except for the \textit{TMPRSS6} rs4820268 assay, which achieved a sensitivity of 97%. Therefore, in line with the establishment of the DOST-FNRI NuGen Lab, HRM assays can be considered a reliable and affordable alternative to sequencing. Identifying genetically high-risk Filipinos in the diagnosis of anemia due to iron deficiency may be facilitated in the future.

Keywords: HFE, high-resolution melting assay, iron, single nucleotide polymorphisms, TF, TMPRSS6

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INTRODUCTION

HRM is a post-amplification technique that allows for the genotyping and screening of genetic variants based on the characteristic melting points and profiles of DNA amplicons (Reed et al. 2007). Such melting profiles depend mainly on amplicon length, sequence covering the target loci and its guanine-cytosine (GC) content, and heterozygosity of a particular genetic marker locus. In essence, HRM analysis works by monitoring in real-time the fluorescence levels of a saturating DNA-binding dye (i.e., SYBR® Green Dye) during amplification followed by the generation of melting curves of fluorescence data against time (Erali et al. 2008). Compared to other polymerase chain reaction-based genotyping techniques such as restriction fragment length polymorphism, HRM is considered advantageous because it is simple, rapid, cost-effective, efficient, and can be high-throughput depending on the number of samples to be analyzed (Wittwer 2009). Current applications of HRM analysis among others include the genotyping of SNPs (i.e., variations in single base pairs that are being linked to disease phenotypes, personalized drug responses, etc.); assessment of DNA methylation (i.e., attachment of methyl groups to DNA bases that turns genes on or off and alters gene expression); and analysis of copy number variants or CNVs (i.e., varying number of copies of a particular gene among individuals) (Reed and Wittwer 2004, Wojdacz and Dobrovic 2007, Bruder et al. 2008).

One of the most significant challenges posed in establishing a molecular diagnostic laboratory in the Philippines, such as the DOST-FNRI NuGen Lab, is the development of assays and protocols that can be used efficiently and effectively in detecting SNPs and other biomarkers. Under the field of nutritional genomics (particularly in nutrigenetics, the science that studies genetic variants with respect to the interplay between diet and disease), the identification of SNPs associated with diet-related chronic diseases and even undernutrition cases among Filipinos will lead to more innovative strategies that can ultimately optimize human health by personalizing dietary recommendations based on an individual’s genetic make-up (Ordoñas and Corella 2004). Previously published papers of the DOST-FNRI NuGen Lab team revealed that certain SNPs were potentially associated with hypertension (Zumaranaga et al. 2015); iron deficiency, anemia, and iron deficiency anemia (Timoteo et al. 2018); and satiety and obesity (Nacis et al. 2018) among Filipinos. In order to verify if these SNPs will provide a true association with complex and non-communicable diseases, SNP detection assays that can be applied and utilized in large association studies (in addition to the routine detection of SNPs in the laboratory) must first and foremost be evaluated. Thus, this study aimed to develop and achieve the ideal conditions of PCR-based HRM assays that can detect and genotype SNPs in iron-related genes, namely: (1) HFE rs1800562, (2) HFE rs1799945, (3) TF rs3811647, (4) TF rs1799852, and (5) TMPRSS6 rs4820268.

Table 1 presents the genes and SNPs that were selected and investigated in this study, as well as the dysfunctions corresponding to these polymorphisms. The genes HFE or homeostatic iron regulator (RefSeq: NG_008720.2; O’Leary et al. 2016), TF or transferrin (RefSeq: NG_013080.2), and TMPRSS6 or transmembrane serine protease 6 (RefSeq: NG_012856.2) code for proteins that have distinct and essential roles in iron homeostasis (Hentze et al. 2010). These are the hereditary hemochromatosis protein, serotransferrin, and transmembrane protease serine 6, respectively. The SNPs found in these genes were then reported to be associated with abnormalities in the levels of iron status measures and prevalence of iron deficiency and anemia in the general population, which has been replicated across ethnicities (Soranzo et al. 2009, Kamatani et al. 2010, Lo et al. 2011, Gichohi-Wainaina et al. 2015).

In pregnant Filipino women, SNPs in TF and TMPRSS6 were found common (i.e., minor allele frequency or MAF > 5%) and copies of the risk alleles were observed to influence the levels of transferrin saturation and total and unsaturated iron-binding capacity (Timoteo et al. 2018). A’s compared to the reports from other ethnic populations, the MAFs of SNPs in TF and TMPRSS6 were relatively higher among the pregnant women who participated in this study. For example, in the South Asian population of the 1000 Genomes Project Phase 3, the minor alleles of TF rs3811647 (A), TF rs1799852 (T), and TMPRSS6 rs4820268 (G) were at 41%, 19%, and 43%, respectively (Zerbino et al. 2017). In the former, the frequencies of the risk alleles were reported at 44%, 38%, and 56%, respectively. The G allele of HFE rs1799945 is a rare variant in this study population (MAF = 0.9%).

Given the number of advantages that the HRM technique offers, the routine screening and detection of these SNPs in the DOST-FNRI NuGen Lab may be facilitated. To the authors’ knowledge, this paper is the first to provide the ideal conditions and validation of HRM assays that were specifically designed for genotyping selected SNPs of iron-related genes among Filipinos.

MATERIALS AND METHODS

Ethical Considerations

The study protocol was reviewed and given clearance by the DOST-FNRI Institutional Ethics Review Committee with Registry No. 2012-09-28-0010-2. A total of 112 pregnant
women residing in selected municipalities of Quezon, Palawan, Philippines, voluntarily participated in the study and provided duly signed informed consent forms. All information obtained from the participants were treated with the utmost confidentiality. Blood samples or any derivatives were disposed of properly upon study completion.

**Extraction and Quality Assessment of DNA Templates**

Genomic DNA (gDNA) was isolated from 200 µL EDTA-treated whole blood samples (N = 112) using the QIAamp® DNA Mini Kit (QIA GEN, Germany) following the manufacturer’s recommendation. The concentration and purity (i.e., measurement of A260/A280 ratio) of gDNAs were measured in triplicates using Epoch™ Micro-Volume Spectrophotometer System (BioTek Instruments Inc., USA). Samples were run in 1% agarose gel stained with GelRed™ (Biotium, USA) for 30 min at 100 V to visualize the integrity of starting templates for amplification. A TrackIt™ 1 Kb Plus DNA ladder (Invitrogen™, USA) - which spans 100–12,000 bp - was used as a molecular-weight size marker. The negative control consisted of 1X TBE buffer (Invitrogen™, USA) and nuclease-free water (Ambion®, USA). Extracted gDNAs were stored in a −40 °C laboratory freezer until analyzed and diluted with nuclease-free water to make a standard working concentration of 5 ng/µL before use in real-time amplification.

**Table 1. Genes and SNPs related to iron status, iron deficiency, and anemia that were investigated in the study.**

<table>
<thead>
<tr>
<th>Gene description</th>
<th>SNP</th>
<th>Position*</th>
<th>Minor allele</th>
<th>Reference allele</th>
<th>Consequence and clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE: homeostatic iron regulator; also known as HLA-H; codes for MHC class I-like protein that has a signal sequence, peptide-binding regions, transmembrane region, and small cytoplasmic portion</td>
<td>rs1800562</td>
<td>chr6:26092913</td>
<td>A</td>
<td>G</td>
<td>Missense; C282Y; results in hereditary (type 1) hemochromatosis or iron overload disorder (Feder et al. 1996) and was reported to affect serum transferrin levels (Feder et al. 1998, Benyamin et al. 2009, Blanco-Rojo et al. 2011)</td>
</tr>
<tr>
<td>TF: transferrin; codes for transferrin, which is a serum protein that is responsible in delivering iron to the cells</td>
<td>rs1799945</td>
<td>chr6:26090951</td>
<td>G</td>
<td>C</td>
<td>Missense; H63D; results in a mild form of hereditary hemochromatosis (Butler 1997)</td>
</tr>
<tr>
<td>TMRRSS6 transmembrane protease, serine 6; also known as matriptase 2; codes for matriptase-2, which is a serine protease that represses the expression of hormone hepcidin</td>
<td>rs3811647</td>
<td>chr3:133765185</td>
<td>A</td>
<td>G</td>
<td>Intron variant; results in antrastaffinemia (i.e., characterized by microcytic anemia and iron overloading) (Butler et al. 2000) and was shown to affect serum transferrin levels (Benyamin et al. 2009, Blanco-Rojo et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>rs1799852</td>
<td>chr3:133756878</td>
<td>T</td>
<td>C</td>
<td>Synonymous variant; L247L; also affects serum transferrin levels but was found protective against iron deficiency anemia (Benyamin et al. 2009, Blanco-Rojo et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>rs4820268</td>
<td>chr22:37073551</td>
<td>G</td>
<td>A</td>
<td>Missense; D521D; increases risk of iron deficiency and iron deficiency anemia (An et al. 2012) and was shown to be associated with hemoglobin and hematocrit levels (Chambers et al. 2009, Tanaka et al. 2010, Gichohi-Wainaina et al. 2014)</td>
</tr>
</tbody>
</table>

*Position according to the human reference genome GRCh38 assembly indicated in the Database of Single Nucleotide Polymorphisms (Sherry et al. 2001)


Design of Primers for Real-time Amplification and SNP Genotyping

Pairs of forward and reverse primers were designed to detect the target SNPs using Primer3Plus (Untergasser et al. 2012). The following parameters were chosen: length of 20–25 bases, optimal at 23 bases; GC content of 40–60%, optimal at 50%; melting temperature (Tm) of 55–65 °C, optimal at 60 °C and with a maximum difference of 5 units; and amplicon size of 100–250 bp. Reference sequences containing the target SNPs were obtained from the dbSNP database (Sherry et al. 2001), wherein the Genome Reference Consortium Human genome build 38 (GRCh38) was used as the standard reference assembly sequence. Primer-BLAST (Ye et al. 2012) was used to confirm individual Tm and GC% values per 5’→3’ primer sequence, as well as amplicon size per primer pair. Resulting primer sequences were compared with that of GenBank® through the Basic Local Alignment Search Tool or BLAST (TBLASTN) and BLASTN (Madden 2002) and predicted melting secondary structures (i.e.,
Hairpins and loops were examined using DINA MeLt web server (Markham and Zuker 2005). Alignment of primer sequences against FASTA sequences was performed using MultAlin (Corpet 1988). Primers were synthesized by AITbiotech Singapore Pte Ltd.

**Real-time Amplification of Target Regions**

Amplification of target loci in all 112 gDNA samples was carried out using the 96-well CFX 96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Singapore Pte Ltd) of the DOST-FNRI NuGen Lab. Ta of primer pairs at standard speed was initially calculated using the Ta calculator of CFX Manager™ Software (Bio-Rad Laboratories, Inc., Singapore Pte Ltd). Optimal Ta was then determined using a thermal gradient PCR technique, with the incorporation of a melt curve that was generated at 65–95 °C in 0.5 °C / 5 s increments. Ta optimization reaction mixture has a total volume of 10 μL and consisted of 4 μL of template DNA (20 ng), 1 μL of 100 nM forward and reverse primers, and 5 μL of 2X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc., Singapore Pte Ltd). In each temperature in the gradient, PCR reactions were in triplicate, and control reactions without template (i.e., NTC) were included in each assay. The specificity of each primer pair and PCR yield was visualized in 2% agarose gel electrophoresis using EZ Load™ 100 bp Molecular Ruler (Bio-Rad Laboratories, Inc., USA) as DNA ladder.

**Optimization of HRM Assays**

For each HRM assay, the cycling conditions included: (1) enzyme activation at 98 °C for 2–3 min; (2) cycles of denaturation and annealing at 98 °C for 5 s and using the optimal Ta of corresponding primer pair for 30 s, respectively; (3) another round of denaturation at 95 °C for 30 s; (4) cooling at 60 °C for 1 min; and (5) generation of melt curve at 65–95 °C in 0.2 °C / 10 s increments. A total reaction volume of 20 μL consisted of 4.4 μL template DNA (22 ng), 1 μL each of 100 nM forward and reverse primers, 11 μL of 2X SsoFast™ EvaGreen® Supermix, and 3.3 μL nuclease-free water. All PCRs were run in duplicate, which included two PCR reactions per gDNA sample, positive controls for all genotypes, and NTCs. Positive controls were chosen from samples that contain the SNP genotypes, as previously confirmed by capillary sequencing.

During HRM genotyping analyses, samples were blind tested because no prior information about the amplicon sequences nor genotypes was available to the analyst. Preliminary inspection of the characteristic melt curves was performed using Bio-Rad CFX Manger™ Software. Genotype discrimination of target SNP per HRM assay was then carried out using Precision Melt Analysis™ Software (Bio-Rad Laboratories, Inc., Singapore Pte Ltd). Different melting profiles were obtained for the target SNPs. Melting temperature shifts and curve shapes and differences were also generated to better discriminate the SNP genotypes (homozygous wild-type vs. homozygous minor vs. heterozygote). A melt study file was further created to compare the melt curve data generated from all HRM runs for each SNP investigated. Representative number of amplicons from the genotyping of HFE rs1800562, HFE rs1799945, TF rs3811647, TF rs1799852, and TMPRSS6 rs4820268 were visualized on 2% agarose gel electrophoresis.

**Post-HRM Analyses and Validation of SNP Genotype Results**

In order to confirm if the regions containing the target SNPs were correctly amplified, a representative number of amplicons from distinct samples (>50) per HRM assay was sent out for sequencing. It was ensured that at least 10% of the total number of samples per SNP genotype were validated.

Amplicons were submitted to AITbiotech Singapore Pte Ltd for capillary electrophoresis sequencing on ABI 3730XL DNA Analyzer using BigDye Terminator chemistry. A total of 30 μL of amplicons were purified using the GF-1 AmbiClean Kit (Gel & PCR) (Vivantis Malaysia) following the recommended protocol for PCR clean-up of DNA bands ranging from 100 bp to 20 kb. Resulting sequences were validated through BLAST using genomic databases for humans and aligned against the FASTA sequence spanning the target SNPs using MultAlin. SNP genotypes were visually identified from the bitmap electropherograms of sequences.

Finally, the SNP genotype results obtained from the HRM assays were compared against the results of the gold-standard sequencing technique. The following validation parameters were calculated for each HRM assay using blind samples: (1) sensitivity, (2) specificity, (3) PPV, and (4) NPV. Norambuena and colleagues (2009) described assay sensitivity and specificity as the probability of getting a positive test result in the presence of a risk allele (i.e., heterozygous and homozygous risk samples) and the probability of getting a negative test result in the absence of the risk alleles (i.e., homozygous wild-type samples), respectively. Sensitivity, specificity, PPV, and NPV are mathematically expressed as:

\[
\text{Sensitivity} = \frac{\text{number of true positive (TP)}}{\text{number of TP} + \text{number of false negative (FN)}}
\]

and

\[
\text{Specificity} = \frac{\text{number of true negative (TN)}}{\text{number of TN} + \text{number of false positive (FP)}}
\]

Where

- TP = true positive
- TN = true negative
- FP = false positive
- FN = false negative
RESULTS AND DISCUSSION

Quality of Genomic DNA Templates and Primer Pairs

The quality of starting DNA templates for amplification and primer design are crucial factors prior to the optimization and conduct of an HRM analysis. Taylor and co-authors (2010) emphasized that to ensure quality HRM data, DNA templates should display purity and good integrity. The adequacy of starting DNA templates will also lead to efficient amplification cycles (Wahyuningsih et al. 2017). Primers, on the other hand, should yield short DNA amplicons. While larger amplicons can still yield detectable variations in melting behavior, an estimated amplicon size smaller than 200 bp is generally recommended. Analyzing smaller amplicons was shown to result in more profound melting profile differences between SNP genotypes during an HRM analysis (Fisher et al. 2010, Wahyuningsih et al. 2017).

A total of 112 extracted gDNAs had concentrations ranging from 14.91–49.53 ng/µL and A_{260}/A_{280} readings within 1.7–1.9, which indicated that samples were of high purity with respect to contaminating proteins. Based on gel electrophoresis runs, gDNAs were intact (i.e., appearance of a single array of bands); not degraded (i.e., absence of smears); and contained high molecular weight DNA fragments (i.e., all bands appeared above the 12 kb marker). Table 2 presents the set of primers designed for the amplification of target regions and SNP genotyping. Primer pairs were considered ideal as the resulting amplicon sizes ranged 107–245 bp and Tm were at 60.3–63.3 °C.

Assessment of Optimal Annealing Temperatures and Amplification Conditions

In this study, Ta optimization of primer pairs was facilitated through thermal gradient PCR. Eight Ta values in decreasing order were evaluated in a single two-step (i.e., denaturation-annealing) amplification run for each primer pair. In Figure 1A, amplification of the target regions was observed to generally occur before 40 PCR cycles except with the primers designed for TMPRSS6 rs4820268 HRM assay (i.e., quantitation cycle or Cq > 30). The optimal Ta of each primer pair was then identified as having the highest fluorescence units as measured in relative fluorescence unit (RFU) and peak intensity from the resulting amplification and melt peak charts, respectively (Figure 1A–B). This was then validated with the observed distinct band intensities and absence of non-specific products in the gel electrophoretic results (Figure 1C). A amplification using the primer pairs designed for TF rs3811647 and TMPRSS6 rs4820268 HRM assays resulted in double peaks of melting curves and non-specific amplicon bands with lower Ta values. Therefore, the most ideal Ta values selected for the primer pairs were as follows: (1) 59.0 °C for HFE rs1800562, (2) 59.4 °C for HFE rs1799945, (3) 57.5 °C for TF rs3811647, (4) 59.4 °C for TF rs1799852, and (5) 56.8 °C for TMPRSS6 rs4820268. All reactions under the identified optimal Ta values displayed tight amplification and single melt peak, implicating that a single amplicon was produced (Wahyuningsih et al. 2017).

### Table 2. Primers used for real-time amplification and HRM genotyping.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Primer sequence (5’→3’)</th>
<th>Tm (°C)(^a)</th>
<th>GC% (%)(^a)</th>
<th>Amplicon size (bp)(^a)</th>
<th>Average Tm (°C)(^b)</th>
<th>Calculated Ta (°C)(^b)</th>
</tr>
</thead>
</table>
| HFE    | rs1800562 | F: AAT GGG GAT GGG ACC TAC CAG  
R: CTC TCA TCA GTC ACA TAC CCC AG | 60.98 | 57.14 | 136 | 62.9 | 58.9 |
|        | rs1799945 | F: ACA TGG TTA AGG CCT GTC TGT GC  
R: ATG TGA TCC CAC CCT TTC AG | 58.74 | 50.00 | 245 | 60.3 | 56.3 |
| TF     | rs3811647 | F: ACA CAA GTG GAC CCT AAG CTG  
R: ATG TGA TCC CAC CCT TTC AG | 59.93 | 52.38 | 122 | 61.5 | 57.5 |
|        | rs1799852 | F: ACA CAA GTG GAC CCT AAG CTG  
R: ATG TGA TCC CAC CCT TTC AG | 59.96 | 50.00 | 122 | 61.5 | 57.5 |
| TMPRSS6| rs4820268 | F: GAA GCA TGT AGC AGG CCT AGA  
R: CTG ATT GTC TCA ACG GCA GC | 60.20 | 57.14 | 137 | 63.3 | 59.3 |

\(^a\)Evaluated using Primer-BLAST  
\(^b\)Obtained from Ta calculator of Bio-Rad CFX Manager™ Software  
Figure 1. Evaluation of optimal Ta using thermal gradient PCR: (A) amplification chart shows RFU at every cycle for 40 PCR cycles, (B) melt peak chart presents specificity of primers within a specified range of Ta values, and (C) corresponding agarose gel corroborates primer specificity at each Ta. Each line trace in charts represents data from SYBR® Green fluorophore in one well. Abbreviations: rs – reference SNP, RFU – relative fluorescence unit, NTC – no template control.
In running an HRM assay, selecting an optimal Ta for a primer pair is considered as the most important factor as it directly influences the amplification of target loci (Huynh et al. 2017, Taylor et al. 2010, Wahyuningsih et al. 2017). Higher Ta values will result in lower amplicon yield due to insufficient primer-template hybridization, while lower Ta may cause mismatching of base pairs leading to the presence of non-specific products. All Ta values generated by Bio-Rad CFX Manager Ta calculator were shown as the ideal Ta of the primer pairs except for the primers used to amplify HFE rs1799945. Instead of the calculated and suggested Ta at 56.3 °C, Ta at 59.4 °C was selected because it gave the highest RFUs and the peak intensity in the HFE rs1799945 assay. As mentioned by Taylor and co-authors (2010), an optimal Ta identified using a thermal gradient PCR will produce highly specific amplicons and early amplification based on Cq values (i.e., < 30). These were both observed from the Ta optimization results.

In the case of TMPRSS6 rs4820268 HRM assay, the protocol was modified by increasing the number of PCR cycles to 50 due to the previously observed late amplification of the target region from the thermal gradient PCR run. The same approach was done by Wahyuningsih and colleagues (2017). In addition, a three-step run protocol (i.e., extension step at 72 °C for 30 s was included during the annealing/extension step) was adapted in genotyping HFE rs1799945 since the estimated amplicon size was > 200 bp. These modifications yielded better melt curve results (i.e., samples showed identical PCR efficiencies and plateau fluorescence); significant melting profiles (i.e., amplification curve increased after 30 PCR cycle); and improved PCR performance as evidenced by the HRM assay results.

**HRM Assay Results**

In the HRM analyses, Bio-Rad Precision Manager analysis™ Software was used to generate the melt profiles of SNP genotypes by collecting data over 65–95 °C in 0.2 °C increments and a hold time of 10 s. Genotype discrimination started from the identification of stable areas of pre- and post-melt fluorescence intensity (green and red bars, respectively, in Figure 2) and melt range region, which was automatically set by the software. This was followed by the normalization of curves from 0 to 100% (i.e., 0 and 1.0, respectively) within the melt range region that eliminated the background noise in fluorescence and thereby highlighted the subtle differences in the melt profiles of SNPs genotypes (Figure 2A). The difference curve plots complemented the melt curve plots, magnifying the differences between the melting profiles of the SNP genotype clusters (Figure 2B). The chosen reference cluster then resulted in the most distinct visual difference among the genotypes. Finally, the temperature-shifted difference curve plots (Figure 2C) – which were achieved by applying a temperature shift to each normalized fluorescence curve along the temperature (x) axis – resulted in better discrimination of the SNP genotypes through enhanced visualization of the homoduplexes (i.e., superimposed curves for homozygote wild-type and homozygote minor) and heteroduplexes.

From the resulting pre-melt, post-melt, and melt range regions of the HRM profiles, the ranges of temperatures that produced the genotype curves were as follows: (1) 81.3–81.8 °C and 86.0–86.5 °C in HFE rs1800562 assay, (2) 81.7–82.2 °C and 85.5–86.3 °C in HFE rs1799945 assay, (3) 76.5–77.0 °C and 81.8–82.3 °C in TF rs3811647 assay, (4) 80.5–81.0 °C and 85.6–86.1 °C in TF rs1799852 assay, and (5) 80.5–81.0 °C and 86.6–87.1 °C in TMPRSS6 rs4820268 assay. A utomatic calling of genotype clusters in the HRM analyses yielded percent confidence ≥ 95%.

With the use of HRM technique, the genotyping of TF rs3811647, TF rs1799852, and TMPRSS6 rs4820268 resulted in three distinct clusters of genotypes (i.e., homozygous wild-type, homozygous minor, and heterozygote). Two genotypes (i.e., homozygous wild-type and heterozygote) were produced using the HFE rs1799945 HRM assay, while only one resulting genotype (i.e., homozygous wild-type) was observed in HFE rs1800562 HRM assay. With reference to reports of the 1000 Genomes Project, HFE rs1800562 was observed to be common only among the American and Caucasian populations. The genotype results implicate that rs1800562 may be absent in the Filipino population (as what is also observed in other Asian populations). This demonstrates the differences in genomic profile across ancestries (i.e., population stratification), which can also be observed even between populations of common ancestry with different descent groups.

A melt profile generated from a particular HRM assay is said to be specific and sensitive enough for SNP detection because the melt curves are generally attributed to the minute alterations found in the nucleic acid sequences under investigation (Reed et al. 2007, Erali et al. 2008, Wittwer 2009, Vossen et al. 2009). In this study, robust and reliable melt curves that can discriminate SNP genotypes were produced in all HRM assays except probably for TMPRSS6 rs4820268. The presence of a nearby or neighboring SNP from TMPRSS6 rs4820268 such as rs387907018 or rs137853120 (identified from the Database of Single Nucleotide Polymorphisms; Sherry et al. 2001) masked the melting and caused the slight changes in the observed melt curve profiles of the genotypes. Such neighboring SNPs and DNA sequence heterozygosity were reported to reduce the performance of genotyping assays in general (Slomka et al. 2017). In a previously published paper (Timoteo et al. 2018), another common SNP in TMPRSS6 (rs855791) was detected and
Figure 2. Discrimination of SNP genotypes using melt curve charts of positive controls per HRM assay: (A) normalized melt curve chart shows melting curves with normalized RFUs plotted against temperatures, (B) difference curve chart presents clustering of SNP genotypes based on characteristic melting curve profiles, and (C) temperature-shifted difference curve chart offers easier detection of heterozygous from superimposed homozygous wild-type and minor genotypes. Each line represents a single replicate. Abbreviations: rs – reference SNP, RFU – relative fluorescence unit, A – adenine, C – cytosine, G – guanine, T – thymine.

reported to have high frequency among pregnant Filipino women. TMPRSS6 rs855791 has the strongest association with iron status measures in the general population to date (Gichohi-Wainaina et al. 2015); thus, there was also an attempt to develop an HRM assay for this SNP. However, the high linkage disequilibrium between the two SNPs in TMPRSS6 and the presence of nearby SNPs led to the assessment and successful genotypic discrimination of rs4820268 alone by HRM assay. Given this circumstance, further optimization of TMPRSS6 rs4820268 HRM assay and design for TMPRSS6 rs855791 HRM assay are highly warranted for the routine detection and assessment of these SNPs in the DOST-FNRI NuGen Lab. For instance, the use of a Ta > 56.8 °C during amplification or a redesign of the primer pairs targeting TMPRSS6 rs4820268 may be considered in order to further improve the performance efficiency of its HRM assay.

Three of the genetic variants investigated (rs1800562 and rs1799945 in HFE and rs4820268 in TMPRSS6) are missense mutations, which lead to the production
of altered protein products and thus affecting its protein function. For example, a change from ‘A’ to ‘G’ in rs1800562 of HFE leads to a cysteine-to-tyrosine substitution at position 282 of the homeostatic iron regulator protein. This causally leads to a hereditary type of hemochromatosis or iron overload disorder in the body (Feder et al. 1996) and affects the serum transferrin levels of the general population (Feder et al. 1998, Benyamin et al. 2009, Blanco-Rojo et al. 2011). On the other hand, TF rs3811647 is an intrinsic variant that – although situated within a non-coding region – may be functional and can influence the expression of its gene (Cooper 2010). Thus, rs3811647 was reported to cause ataferrinemia (Bueler et al. 2000). Lastly, TF rs1799852 is a synonymous variant that does not change the translated amino acid sequence (i.e., leucine-to-leucine change at position 247) but can significantly impact the expression levels, conformation, and function of its protein product (Ward et al. 2012). A synonymous mutation can have effects on either transcription, splicing, mRNA transport, or translation, which can ultimately alter a phenotype. Consequently, TF rs1799852 can protect against iron deficiency anemia by modifying the levels of blood iron concentration parameters such as serum transferrin (Benyamin et al. 2009, Blanco-Rojo et al. 2011).

This study further present two classes of SNPs: HFE rs1800562 (G > A), TF rs3811647 (G > A), TF rs1799852 (C > T), and TMPRSS6 rs4820268 (A > G) are all class I SNPs; while HFE rs1799945 (C > G) is a class III SNP. Liew and colleagues (2004) described the first class of SNPs as transition point mutations, wherein a purine base interchanges with another purine (A ↔ G) or, similarly, a pyrimidine base interchanges with another pyrimidine (C ↔ T). Class II SNPs are transversions or interchanges between purines and pyrimidines (i.e., C/A and G/T). CG homoduplex (i.e., complementary base pairs) and CC and GG heteroduplexes (i.e., noncomplementary base pairs) can be observed in class III SNPs, while AT homoduplex and AA and AT heteroduplex pairs are produced in class IV SNPs. As mentioned by Taylor and colleagues (2010), the use of HRM in genotyping becomes more advantageous in the identification of class IV SNP. Class IV SNP genotypes are the most difficult to identify because they generate melt curves that have the smallest temperature shifts because they only differ by a single hydrogen bond. Conversely, class I and II SNPs can be easily distinguished by melting analysis (Liew et al. 2004).

Validation of HRM Assay Results
Presented in Figure 3 are the sequence electropherograms of SNP genotypes wherein a single peak was displayed for both homozygous wild-type and homozygous minor genotypes, while distinct overlapping peaks of different colors were observed for heterozygous genotypes.

Clear, single amplicon bands that matched the estimated amplicon size desired in each HRM assay were observed. Non-specific PCR products such as primer-dimers (i.e., hybridization of primers due to base complementarity) were not detected in all assays.

In comparing the genotype results between HRM assays and capillary sequencing, Table 3 presents the following: (1) total number of samples falling per SNP genotype that were detected via HRM, (2) number of amplicon samples submitted for sequencing for validation, (3) sensitivity and specificity per assay, and (4) PPV and NPV per assay. All assays achieved 100% sensitivity, specificity, PPV, and NPV, except for TMPRSS6 rs4820268 (i.e., 97% sensitivity). Similar to other studies (Garritano et al. 2009, Norambuena et al. 2009, Zumaraga et al. 2015, Huynh et al. 2017, Faris et al. 2018), high concordance between HRM and capillary sequencing results were observed.

In summary, this paper presents the development of HRM assays that can successfully detect and distinguish between genotypes of selected SNPs in iron-related genes. This serves as an initiative in routinely detecting SNPs during diagnosis and easily identifying genetically at-risk Filipinos for anemia due to iron deficiency. A limitation of the study, however, was the failure to evaluate PCR efficiency and linearity of all HRM runs. Thus, determination of PCR efficiencies and comparison with other methods as suggested by Faris and co-authors (2018) will further validate the accuracy of the HRM technique. Evaluation of other analytical validation parameters such as method accuracy, inter- and intra-day precision, and instrument detection limits may also be considered (Norambuena et al. 2009, Zumaraga et al. 2015). A multiplex SNP genotyping via HRM or the simultaneous detection of multiple target SNPs in a single-tube reaction may also be done as this is more powerful and efficient in both diagnostic and research settings (Helmsmoertel et al. 2016). The use of probes designed to have different sets of Tm range (for instance, 75–80 °C and 90–95 °C) and a more extensive assay optimization should be considered, however, when adapting a multiplex HRM assay that will discriminate the SNP genotypes of iron-related genes in the future (Garritano et al. 2009).

In conclusion, the observed high call rates from HRM genotyping – as well as the high concordance between results of HRM assays and sequencing – all substantiate the acceptability and reliability of the developed HRM assays in the routine detection of SNPs in HFE, TF, and TMPRSS6 in the DOST-FNRI NuGen Lab. In the near future, through nutritional genomics, SNPs in HFE, TF, and TMPRSS6 can be used when providing personal dietary recommendations to iron-deficient and anemic Filipinos or in preventing the onset of nutritional anemia for genetically at-risk individuals.
Figure 3. Validation of HRM assay results: (A) difference curve chart presents clustering of SNP genotypes based on characteristic melting curve profiles, (B) sequencing electropherograms show corresponding SNP genotypes, and (C) representative 2% agarose gels confirm high specificity of assays resulting in single bands of correct amplicon size. Abbreviations: rs – reference SNP, RFU – relative fluorescence unit, A – adenine, C – cytosine, G – guanine, T – thymine, NTC – no template control.
Table 3. Validation parameters used to compare HRM genotyping assays from capillary sequencing.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Number of samples detected by HRM</th>
<th>Number of samples validated by sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TP</td>
<td>FN</td>
</tr>
<tr>
<td>HFE rs1800562 (G &gt; A)</td>
<td>GG</td>
<td>112</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>110</td>
<td>51</td>
</tr>
<tr>
<td>HFE rs1799945 (C &gt; G)</td>
<td>GG</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>110</td>
<td>51</td>
</tr>
<tr>
<td>TFF rs3811647 (G &gt; A)</td>
<td>GG</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>110</td>
<td>51</td>
</tr>
<tr>
<td>TFF rs1799852 (C &gt; T)</td>
<td>CC</td>
<td>41</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>56</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>TMPRSS6 rs4820268 (A &gt; G)</td>
<td>AA</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>40</td>
<td>16</td>
</tr>
</tbody>
</table>

Abbreviations: TP – true positive, FN – false negative, TN – true negative, FP – false positive, PPV – positive predictive value, NPV – negative predictive value, NA – not applicable, A – adenine, C – cytosine, G – guanine, T – thymine

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