counts and basic urine analysis. All were asymptomatic and underwent a complete examination by a cardiologist, including a visit, standard 12-lead electrocardiogram, Doppler echocardiographic examination, and a bicycle stress test (when older than 50 years), which excluded silent heart disease. The median ACCESS and TRIAGE BNP concentrations were 10.5 ng/L (range, 1–53 ng/L; 97.5th percentile, 45 ng/L) and 8.9 ng/L (range <5 to 52 ng/L; 97.5th percentile, 35 ng/L), respectively. The distribution of BNP values measured by the 2 methods in the healthy group is shown in Fig. 1 of the Data Supplement that accompanies this Technical Brief at http://www.clinchem.org/content/vol51/issue7/.

To evaluate the diagnostic accuracy of the 2 methods, we measured only samples from the 193 patients with a clinically ascertained diagnosis of heart failure. The clinical characteristics of these patients are reported in Table 1. Cardiac morphology and function were assessed by 2-dimensional echocardiography, or cardiac catheterization when needed. The median (range) ACCESS and TRIAGE BNP concentrations measured were 171 (2–4833) ng/L and 118 (<5 to 4930) ng/L, respectively. The distributions of BNP values measured by the 2 methods in these patients grouped according to the severity of heart failure [New York Heart Association (NYHA) functional classes I to IV] are shown in Figs. 1 and 2 of the online Data Supplement. However, the results obtained with both the TRIAGE and ACCESS indicated a significant difference between the mean BNP values in healthy persons vs heart failure patients ($P < 0.0001$ by Scheffè test after ANOVA).

We used ROC curve analysis to evaluate the diagnostic accuracy of the 2 methods in differentiating between healthy persons and patients with heart failure. We found no difference in diagnostic accuracy between the 2 methods for differentiating healthy persons from patients with mild (NYHA class I and II; n = 122; $P = 0.196$) or severe (NYHA class III and IV; n = 71; $P = 0.697$) heart failure. For the TRIAGE, the areas under the curves were 0.840 (SE, 0.027; 95% CI, 0.788–0.893) for patients with mild disease and 0.998 (SE, 0.002; 95% CI, 0.995–1.000) for patients with severe heart failure, whereas for the ACCESS system, the areas under the curves were 0.870 (SE, 0.023; 95% CI, 0.825–0.916) for patients with mild disease and 0.997 (SE, 0.002; 95% CI, 0.993–1.000) for patients with severe heart failure. The BNP values corresponding to a sensitivity of 95% in differentiating healthy persons from patients with mild heart failure were 7.5 ng/L (corresponding specificity, 40%) for the ACCESS and 5.1 ng/L for the TRIAGE (corresponding specificity, 29%). The BNP values corresponding to a specificity of 95% were 41 ng/L (corresponding sensitivity, 65%) for the ACCESS and 29 ng/L (corresponding sensitivity, 63%) for the TRIAGE (Fig. 3 of the online Data Supplement). However, it is important to emphasize that the 95% sensitivity with the TRIAGE was obtained at a cutoff near the assay detection limit. The decision cutoff values from the current study are strictly related to our specific clinical setting, comparing healthy persons and patients with clinically ascertained heart failure. In routine clinical practice, several groups of individuals/patients suspected of having a specific disease are usually compared.

The present data confirm and extend previous results suggesting that BNP results are method dependent and that a single predefined common cutoff value cannot be used *(5, 6)*. Furthermore, we demonstrated that immunoassays that use the same antibodies and calibration materials do not automatically give the same results. However, the cutoff and reference values of these 2 methods are similar.

References

1. Clerico A, Emdin M. Diagnostic accuracy and prognostic relevance of the measurement of the cardiac natriuretic peptides: a review [Review]. Clin Chem 2004;50:33–50.
2. Galvani M, Ferrini D, Ottani T. Natriuretic peptides for risk stratification of patients with acute coronary syndromes [Review]. Eur J Heart Fail 2004;6:327–33.
3. Doust JA, Glasziou PP, Pietrzak E, Dobson AJ. A systematic review of the diagnostic accuracy of natriuretic peptides for heart failure. Arch Intern Med 2004;164:1978–84.
4. Clerico A, Del Ry S, Giannessi D. Measurement of cardiac natriuretic hormones (atrial natriuretic peptide, brain natriuretic peptide, and related peptides) in clinical practice: the need for a new generation of immunoassay methods [Review]. Clin Chem 2000;46:1529–34.
5. Clerico A, Prontera C, Emdin M, Passino C, Storti S, Poletti R, et al. Analytical performance and diagnostic accuracy of immunometric assays for the measurement of plasma B-type natriuretic peptide (BNP) and N-terminal proBNP. Clin Chem 2005;51:445–7.
6. Rawlins ML, Owen WE, Roberts WL. Performance characteristics of four automated natriuretic peptide assays. Am J Clin Pathol 2005;123:439–45.
7. Apple FS, Panteghini M, Ravkilde J, Mair J, Wu AHB, Tate J, et al., on behalf of the Committee on Standardization of Markers of Cardiac Damage of the IFCC. Quality specifications for B-type natriuretic peptide assays. Clin Chem 2005;51:486–93.
8. Storti S, Prontera C, Emdin M, Passino C, Prati P, Fontani G, et al. Analytical performance and clinical results of a fully automated MEIA system for BNP assay: comparison with a POCT method. Clin Chem Lab Med 2004;42:1178–85.
9. National Committee for Clinical Laboratory Standards. Evaluation of precision performances of clinical chemistry devices: approved guideline. NCCLS Document EP5-A, Vol. 19, No 2. Wayne, PA: NCCLS, February 1999:1–43.
10. Bland JM, Altman DG. Applying the right statistics: analysis of measurement studies. Ultrasound Obstet Gynecol 2003;22:85–93.

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Invasive Trophoblast Antigen (Hyperglycosylated Human Chorionic Gonadotropin) as a First-Trimester Serum Marker for Down Syndrome, *Martin J.N. Weinans,[1*] Ulrich Sancken,[2] Raj Pandian,[3] Jody M.W. van de Ouweland,[4] Henk W.A. de Bruijn,[4] Jozien P. Holm,[1] and Albert Mantingh[1]* ([1] Antenatal Diagnosis Unit, Department of Obstetrics and Gynaecology, and [4] Department of Pathology and Laboratory Medicine, University Hospital, Groningen, The Netherlands; [2] Institut für Humangenetik der Universität Göttingen, Göttingen, Germany; [3] Quest Diagnostics Nichols Institute, San Juan Capistrano, CA; * address correspondence to this author at: Department of Obstetrics and Gynaecology, University Hospital, PO Box 30.001, 9700 RB Groningen, The Netherlands; fax 31503611806, e-mail martinweinans@planet.nl)

Hyperglycosylated human chorionic gonadotropin (hCG) is a variant of hCG with more asparagine (N)-linked

Table 1. Median MoM values, means, and SDs of ITA, free β-subunit, and PAPP-A in unaffected (control) and Down syndrome-affected first-trimester pregnancies.

		Down syndrome-affected pregnancies	Controls
ITA	Median MoM	2.56	1.03
	Mean, log MoM	0.822	0.00
	SD, log MoM	0.489	0.701
Free β-subunit	Median MoM	2.19	0.99
	Mean, log MoM	0.82	0.00
	SD, log MoM	0.553	0.628
PAPP-A	Median MoM	0.35	1.02
	Mean, log MoM	−1.053	0.00
	SD, log MoM	0.572	0.673

triantennary carbohydrates and a serine (O)-linked tetrasaccharide core structure in the β-subunit of hCG *(1)*. Whereas nonhyperglycosylated hCG is secreted by differentiated syncytiotrophoblast cells, hyperglycosylated hCG is secreted solely by invasive cytotrophoblast cells and is therefore also called invasive trophoblast antigen (ITA) *(2, 3)*. Before the sixth week of gestation, ITA appears to be the predominant form of hCG *(2–4)*. In Down syndrome pregnancies, differentiation of the cytotrophoblast into a syncytiotrophoblast may be delayed, leading to increased production of ITA *(5)*.

Urinary ITA is a promising candidate for use as a biochemical marker in Down syndrome screening. In one study conducted during the second trimester of pregnancy, ITA alone detected 78% of the Down syndrome cases at a 5% false-positive rate *(6, 7)*. In a setting that simulated routine use, urinary ITA was reported to be the best single marker in the second trimester *(8)*. In the first trimester of pregnancy, however, the performance of urinary ITA is lower, with a reported 63% Down syndrome detection rate at a 10% false-positive rate *(9)*.

ITA is detectable in serum as well as in urine *(10)*. Studies have not been performed with serum ITA because of concerns about the stability of ITA in serum, possible loss of ITA when blood is collected in gel barrier tubes, and possible aggregation. A recently developed automated immunochemiluminometric assay measures ITA in various sample types, including serum *(11)*. In the present study, we investigated the Down syndrome screening performance of serum ITA before 12 weeks of gestation and compared it with the performance of pregnancy-associated plasma protein A (PAPP-A) and free β-subunit in the same sample set.

Sera from 24 women with Down syndrome-affected pregnancies and 320 unaffected pregnant women were used in this retrospective study. Samples were collected from women at 9 weeks and 5 days (9 + 5) of gestation to 11 weeks and 4 days (11 + 4). These samples were collected between 1999 and 2002, with permission, before chorionic villus sampling. The primary indication for chorionic villus sampling was advanced maternal age (≥36 years). All samples were collected at the Antenatal Diagnosis Unit of the University Hospital Groningen, The Netherlands, into non–gel-barrier Vacutainer Tubes. An Institutional Review Board-approved protocol was followed. Control samples were matched for gestational age, maternal age, and length of storage. The mean gestational age was 76.4 days for cases and 76.0 days for controls. The mean (SD) maternal age was 38.8 (2.36) years for cases and 37.2 (3.04) years for controls. The median duration of sample storage was 2 years and 1 month for cases and 2 years and 2 months for controls. Blood samples were allowed to clot for 1–3 h at room temperature and were centrifuged at 2500*g* and 10 °C for 10 min. Serum fractions were frozen immediately and stored at −20 °C and never thawed at room temperature except before being assayed. Serum samples were then thawed overnight at 4 °C and analyzed within 4 h.

ITA was measured by an immunochemiluminometric assay on the Nichols Advantage® platform (Nichols Institute Diagnostics) with an acridinium-ester–labeled, anti-hCGβ monoclonal antibody (B207) and a biotinylated ITA-specific monoclonal capture antibody (B152) *(11)*. The assay has a calibration range up to 300 μg/L, with automatic dilution at higher concentrations. The assay has <1% cross-reactivity with recombinant hCG *(11)*. The reported intra- and interassay variations (as CV) are <8% and 12%, respectively.

PAPP-A and free β-subunit were both measured by a fluoroimmunoassay (AutoDELFIA® PAPP-A and Free hCGβ reagent sets; Perkin-Elmer). The detection limits of the assays are 5 mIU/L for PAPP-A and 0.2 μg/L for free β-subunit. The intra- and interassay variations (CVs) for PAPP-A were <2% and 4%, respectively, at a concentration of 1500 mIU/L. For free β-subunit, the CVs were <4% and <5% at 40 μg/L.

We used STATISTICA for Windows, Ver. 6 (StatSoft Inc). Multivariate discriminant analysis was performed to calculate the risks. The multiples of the median (MoM) were derived from regressed medians, with gestational days used as the independent variable. All parametric statistical procedures were based on the natural logarithms of the concentrations or MoM values. A Monte Carlo model was applied to adapt detection and false-positive rates to the present age-standardized population of The Netherlands. Each patient was assigned the mean of 10 randomized maternal age-related risks where the randomization was based on the proportion of maternal age rates according to the present birth frequencies in The Netherlands.

Means, SD values based on logarithmic MoM values, and median MoM values for all 3 markers are summarized in Table 1.

The squared Mahalanobis distance was 1.42 for ITA, 1.73 for free β-subunit, and 2.50 for PAPP-A.

The predicted detection rates for Down syndrome for the combination of ITA and PAPP-A at 3%, 5%, and 10% false-positive rates were 62%, 71%, and 83%, respectively. For the combination of free β-subunit and PAPP-A, the predicted detection rates were 58%, 75%, and 79%, respectively.

The associations among ITA, PAPP-A, and free β-subunit concentrations were calculated in both affected and control pregnancies. In controls, there was a significant correlation between ITA and free β-subunit. The Pearson correlation coefficients (*r*) between the log-transformed MoM values were as follows: ITA/free β-subunit, 0.63; ITA/PAPP-A, 0.15; and PAPP-A/free β-subunit, 0.27.

The ROC curves for both ITA and free β-subunit in combination with PAPP-A, including maternal age after modeling against the age-standardized population of The Netherlands in 2002 *(12)*, are shown in Fig. 1. From these curves, the predicted detection rate for a given screen-positive rate can be determined.

We have shown that serum ITA is a useful first-trimester marker for Down syndrome screening. In the present study the combination of PAPP-A and ITA detected 71% of the Down syndrome cases at a 5% false-positive rate. The predicted detection rate for the combination PAPP-A/ITA, including maternal age after modeling against the age-standardized population of The Netherlands, was 63% at a 5% false-positive rate (Fig. 1).

PAPP-A was the most powerful biochemical marker in this study, as evidenced by the highest Mahalanobis distance. Although the median MoM value in Down syndrome cases was slightly higher for ITA (2.6 MoM) than for free β-subunit (2.2 MoM), ITA was not a better marker than free β-subunit, perhaps because there was less variation in free β-subunit concentrations in the control population (see the SDs in Table 1). The higher Mahalanobis distance for free β-subunit (1.7) compared with ITA (1.4) indicates that free β-subunit was a slightly better marker in this study.

The ROC curves (Fig. 1) indicate that at false-positive rates of 2%–7.5%, the PAPP-A/free β-subunit combination outperforms the PAPP-A/ITA combination, whereas at false-positive rates <2% and between 7.5% and 10%, the PAPP-A/ITA combination performed better.

Because the gestational age window in the present study was only 2 weeks (9 + 5 to 11 + 4) and the sample size was small, we do not know whether ITA is a better marker than free β-subunit during the very early first trimester (~9 weeks of gestation). In late first trimester (12–14 weeks of gestation), serum ITA might be a better marker than free β-subunit because urinary ITA concentrations in affected pregnancies have been shown to be very high at those gestational ages *(13)*. Moreover, because the correlation between ITA and PAPP-A is less than that of free β-subunit and ITA (i.e., ITA is more independent of PAPP-A), an ITA/PAPP-A combination may be a more effective screen than the free β-subunit/PAPP-A combination.

ITA should not be added as a third marker (i.e., added to free β-subunit and PAPP-A) because of the high correlation between ITA and free β-subunit in unaffected pregnancies.

The results of this study are comparable to the early first-trimester (10–11 weeks of gestation) study of ITA in maternal urine samples *(9)*. Because urinary ITA studies during the second trimester of pregnancy show a greater discriminatory power (i.e., 78% detection at 5% false-positive rate) *(6, 7)*, it is expected that serum ITA will also have a higher Down syndrome detection rate in the second trimester. Currently, studies are in progress to establish the role of ITA in the second trimester of pregnancy as a serum marker for Down syndrome.

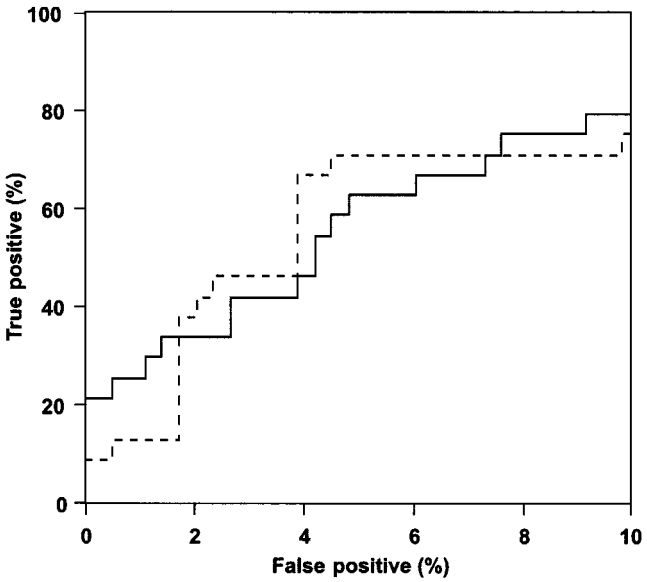

Fig. 1. ROC curves showing the utility of PAPP-A/ITA (*solid line*) and PAPP-A/free β-subunit (*dashed line*) combinations after modeling against the age-standardized population of The Netherlands (2002).

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References

1. Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the α- and β-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine 1997;7:15–32.
2. Kovalevskaya G, Genbacev O, Fisher SJ, Caceres E, O'Connor JF. Trophoblast origin of hCG isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG. Mol Cell Endocrinol 2002;194:147–55.
3. Cole LA, Khanlian SA, Sutton JM, Davies S, Stephens ND. Hyperglycosylated hCG (invasive trophoblast antigen, ITA) a key antigen for early pregnancy detection. Clin Biochem 2003;36:647–55.
4. Kovalevskaya G, Birken S, Kakuma T, Ozaki N, Sauer M, Lindheim S, et al. Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of hyperglycosylated hCG epitope. J Endocrinol 2002;172:497–506.
5. Frendo JL, Vidaud M, Guibourdenche J, Luton D, Muller F, Bellet D, et al. Defect of villous cytotrophoblast differentiation into syncytiotrophoblast in Down's syndrome. J Clin Endocrinol Metab 2000;85:3700–7.
6. Cole LA, Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ. Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: a new basis for gestational Down syndrome screening. Clin Chem 1999;45:2109–19.
7. Cole LA, Shahabi S, Oz UA, Rinne KM, Omrani A, Bahado-Singh RO, et al. Urinary screening tests for fetal Down syndrome: II. Hyperglycosylated hCG. Prenat Diagn 1999;19:351–9.
8. Palomaki GE, Knight GJ, Roberson MM, Cunningham GC, Lee JC, Strom CM, et al. Invasive trophoblast antigen (hyperglycosylated human chorionic gonadotropin) in second-trimester maternal urine as a marker for Down syndrome: preliminary results of an observational study on fresh samples. Clin Chem 2004;50:182–9.
9. Weinans MJN, Butler SA, Mantingh A, Cole LA. Urinary hyperglycosylated

hCG in first trimester screening for chromosomal abnormalities. Prenat Diagn 2000;20:976–8.
10. Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ, Omrani A, Baumgarten A, et al. Serum hyperglycosylated hCG: a potential screening test for fetal Down syndrome. Prenat Diagn 1999;19:488–9.
11. Pandian R, Lu J, Ossolinska-Plewnia J. Fully automated chemiluminometric assay for hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen). Clin Chem 2003;49:808–10.
12. Central Bureau of Statistics. Voorburg/Heerlen, The Netherlands: CBS, 2004.
13. Cole LA, Omrani A, Cermik D, Bahado-Singh RO, Mahoney MJ. Hyperglycosylated hCG, a potential alternative to hCG in Down syndrome screening. Prenat Diagn 1998;18:926–33.

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Validating a Rapid Method for Detecting Common Polymorphisms in the *APOA5* Gene by Melting Curve Analysis Using LightTyper, *Francesc Francés,* Dolores Corella, José Vicente Sorlí, Marisa Guillén, José I. González, and Olga Portolés* (Genetic and Molecular Epidemiology Unit, Department of Preventive Medicine, School of Medicine, University of Valencia, Valencia, Spain; * address correspondence to this author at: Department of Preventive Medicine, School of Medicine, Avda. Blasco Ibañez, 15, 46010 Valencia, Spain; fax 34-963864166, e-mail francesc.frances@uv.es)

The recently identified apolipoprotein A-V gene (*APOA5*) has been shown to play an important role in hypertriglyceridemia *(1)*. Genetic variation in *APOA5* has been consistently associated with plasma triglyceride concentrations in several studies *(2–4)*. Moreover, some studies have demonstrated additional associations with lipoprotein subclasses, remnant-like particles, and cardiovascular disease risk *(4–6)*. Several single-nucleotide polymorphisms (SNPs) in the human *APOA5* gene have been detected with differing frequencies depending on the population analyzed *(7, 8)*, and Klos et al. *(7)* have also suggested context-dependent associations in different populations. Overall, 5 common SNPs, −1131T>C, −3A>G, 56C>G, IVS3 + 476G>A, and 1259T>C, have been widely reported. Apart from the 56C>G SNP, the other SNPs are in strong linkage dysequilibrium, producing 3 haplotypes (11111, 22122, and 11211) representing ~98% of the population in Caucasians *(5, 9)*; therefore, 2 independent *APOA5* SNPs (56C>G and −1131T>C) can be analyzed in association studies as indicators of the corresponding haplotypes. The former consists of a nonsynonymous substitution, changing codon 19 from serine to tryptophan (S19W), whereas the latter is a T-to-C substitution 1131 nucleotides upstream of the initiation codon. To date, in most published reports, these SNPs have been genotyped by PCR with restriction fragment length polymorphism (RFLP) analysis *(3)*.

A system for high-throughput genotyping using fluorescence melting curve analysis, the LightTyper™ (Roche), has recently become commercially available. This instrument *(10)* offers higher throughput than the original LightCycler™ *(11)*. The LightTyper is designed explicitly for melting curve analysis to perform rapid, straightforward, reliable allelic discrimination. The system, which uses 384-well plates, provides postamplification genotyping within 10–15 min and performs genotyping automatically. A variety of probe chemistries are compatible for genotyping, including single-labeled probes and fluorescence resonance energy transfer probes *(10)*. SimpleProbes™ are designed to specifically hybridize to a target sequence that contains the SNP of interest *(12)*. Once hybridized, the SimpleProbe emits a larger fluorescent signal than when it is not hybridized to its target sequence. SimpleProbes are more cost-effective than fluorescence resonance energy transfer probes and represent a major advance in decreasing the cost and complexity of SNP analysis *(12)*. We report here the validation of an assay based on the LightTyper system and SimpleProbes to rapidly screen for the 56C>G and −1131T>C SNPs in the *APOA5* gene.

In the validation study we genotyped 825 randomly selected individuals (age range, 18–75 years) from the Spanish Mediterranean population. All participants gave informed consent, and the study protocol was approved by the Ethics Committee of the School of Medicine of the University of Valencia.

DNA, isolated from blood, was first genotyped for the 56C>G SNP in the *APOA5* gene by melting curve analysis with the LightTyper; the results were then compared with those obtained with the classic RFLP method. For the melting curve analysis, a 136-bp fragment containing the 56C>G SNP was amplified with primers 5′-AGAGCCCAGGCCCTGATTA-3′ and 5′-CATCTTCTGCTGATGGATCTGCT-3′ (TIB MOLBIOL) together with the SimpleProbe Flq-TCTCCACAGCGTTTTCGGCC-p (TIB MOLBIOL), where Flq represents a fluorescence quencher. PCR was carried out in 384-well plates with a total volume of 10 μL per well in a Thermocycler (Mastercycler-ep380®; Eppendorf). The reaction mixture used in the PCR consisted of 40 ng of genomic DNA, 0.2 μL of each primer (10 μM), 1 μL of the SimpleProbe (1.6 μM), 5 U of Fast Start Taq Polymerase (Roche Diagnostics GmbH), 1.2 μL of 25 mM $MgCl_2$, and 200 μM each deoxynucleotide triphosphate (Roche Diagnostics). After an initial denaturation at 94 °C, 34 PCR cycles were performed with 30 s of denaturation at 94 °C, 45 s of annealing at 55 °C, and 45 s of extension at 72 °C; final extension was at 72 °C for 10 min. A final melting cycle was performed on the LightTyper by heating to 85 °C and cooling to 40 °C at a ramping rate of 0.2 °C/s, with fluorescence data collected continuously. The assay exploits the thermal properties of DNA, i.e., the melting temperature (T_m). We designed the probe to match the wild-type DNA (56C allele), so that the wild-type DNA is 100% complementary to the fluorescent probe, making this complex more stable and thus giving it a higher T_m (65.5 °C). The presence of the mutation gives a lower T_m (56 °C). This difference in T_m allows genotypes to be assigned. The fluorescence signal (F) is plotted in real time against the temperature (T) to generate melting curves for