2ND FACULTY OF MEDICINE

CHARLES UNIVERSITY IN PRAGUE

DEPARTMENT OF BIOLOGY AND MEDICAL GENETICS

MOLECULAR GENETIC DIAGNOSTICS OF CYSTIC FIBROSIS, HYPERHOMOCYSTEINEMIA-RELATED DISORDERS AND MALE INFERTILITY: VALIDATION AND APPLICATION OF HIGH RESOLUTION MELTING

PATRICIA NORAMBUENA BARAQUET

DOCTORAL DISSERTATION

PRAGUE, 2012.

Doctorate study program in Biomedicine

Charles University in Prague and the Academy of Sciences of the Czech Republic

Discipline:	Molecular and Cellular Biology, Genetics and Virology.
Chairman of the discipline board:	Prof. Ing. Zdeněk Sedláček, DrSc.
Study department:	Department of Biology and Medical Genetics, Charles University 2nd Faculty of Medicine and Faculty Hospital Motol.
Author:	Patricia Alejandra del Pilar Norambuena Baraquet.
Dissertation advisor:	Prof. Milan Macek Jr. M.D., DrSc.
Opponents:	

Dissertation work was sent on:	……..
Defense takes place on:	……..... at ….....hrs. Where: .....

This dissertation may be for available for revision at Dean's office on the 2nd Faculty of Medicine, Charles University - Prague.

ACKNOWLEDGEMENT

I am heartily thankful to my supervisor, Milan Macek Jr., whose guidance, enthusiasm and support during this time has been of great importance to me and this work. It has been an honor for me to be under his supervision.

I would like to thank Milan Macek, Sr. for encouragement and financial grant support of my PhD project. I also need to thank Alexandra Štambergová for listening and support my ideas and to Petra Křenková for her friendship, collaboration and enjoyable discussions.

I would also like to show my gratitude to all my colleagues from the Cystic Fibrosis Centre of the Faculty Hospital Motol for their teamwork and atmosphere.

Lastly, I would like to thank my beloved husband, my new family and my friend Marcia Villalba for they support during this work. And, to my family, especially to my mother, that even though the distance they have been always there.

SUMMARY

Diagnostic test results are crucial for treatment management and family planning of an individual. Considering that around 80% of medical decisions are based on diagnostic tests and that genotyping is usually concluded only once in a lifetime, it is of a great importance to assure highly accurate test results and provided under high quality standards.

Cystic fibrosis (CF) is one of the most common and life-threatening autosomal recessive genetic disease affecting mainly Caucasian populations. CF is caused by mutations in the *CFTR* gene and until this date, more than 1900 mutations have been detected, while only few of them have frequencies higher than 1% worldwide. Thus, to confirm the diagnosis of cystic fibrosis in patients where only one disease-causing mutation has been found, it is necessary to apply a sensitive test to search for uncommon *CFTR* gene mutations/variants. In this work, we have successfully used HRM for gene scanning of certain exons of the *CFTR* gene. We have confirmed the numerous advantages of the HRM method for gene scanning and also detect some limitations that must be considered through an implementation process in a DNA diagnostic laboratory.

Hyperhomocysteinemia has been proposed as a risk factor for several diseases such as recurrent pregnancy loss and inherited thrombophilia and might be caused from acquired or genetic factors. One of the genetic factors are the c. 677 C>T and c. 1298 A>C variants in the *MTHFR* gene which alter the enzymatic activity of the Methylenetetrahydrofolate reductase enzyme (MTHFR) and participate in the homocysteine metabolism. Hence, the genotyping of these variations has become of importance to establish the diagnosis of disorders related to hyperhomocysteinemia in affected patients. Using a variation of HRM reducing amplicon sizes we were able to successfully genotype the c. 677 C>T and c. 1298 A>C variations. For the implementation of this method in our laboratory we performed a full validation utilizing suggested validation parameters within a quality assurance framework.

Since the terminology involved in the validation/verification process does not provide detailed information it usually leads to confusion, Eurogentest project created a framework for validation and published a simple and detailed generic scheme for the validation and verification of molecular genetic tests for DNA diagnostics. To assure the quality of genetic tests results, diagnostic laboratories, should be accreditated and as a part of this process, validate/verificate every implemented test. The author of this dissertation was a member of the validation group.

Infertility affects 10 – 15% of couples trying to conceive in Western countries and in about 50 % of male infertility cases the original cause has not yet been identified. Thus, there have been many attempts to identify genetic factors associated with male infertility. A previous report made in Japanese population proposed that the rs6836703: G>A variation of the ADP-ribosyltransferase 3 (*ART3*) gene is related to non-obstructive azoospermia (NOA) with high significance. We have used the previously validated method HRM to assess the importance of this variation in sub-/infertile Czech men in a case/control study. Significant differences in allele/genotype distribution between fertile versus oligozoospermic men were found, while in azoospermic men, this difference was not observed. This study was the first replication effort to evaluate in a different population the implication of rs6836703: G>A in impaired spermatogenesis. We found that the "A" allele of the rs6836703: G>A variant of *ART3* is a genetic risk factor for oligozoospermia in Czech male population and reveals a protective effect of the "G" allele in

spermatogenesis. Further studies in related populations are required to confirm these results and to determine the clinical utility of this test for its potential implementation into clinical diagnostics.

The overall impact of this dissertation is the implementation and clinical validation of HRM in DNA diagnostics, including utilization of this method in cystic fibrosis, hyperhomocysteinemia-related disorders and male infertility. Validation of diagnostic techniques is topical and the work performed by the author of this dissertation has been cited since August 2009 fourteen times. We have provided a useful model for other techniques and/or diseases.

SOUHRN

Správné výsledky laboratorních diagnostických testů mají zásadní význam pro diagnostiku a léčbu u pacientů s genetickými onemocněními. Vzhledem k tomu, že a) přibližně 80% lékařských algoritmů je založeno na těchto vyšetřeních, b) genotypizace zárodečného genomu se obvykle provádí jednou za život, je velice důležité, aby výsledky testů byly přesné a prováděné v rámci systémů kvality.

Cystická fibróza (CF) je jednou z nejčastějších monogenních autozomálně recesivních genetických onemocněních vyskytující se převážně u evropských populací. CF je zapříčiněna mutacemi v genu *CFTR*. Do dnešní doby bylo odhaleno více jak 1900 mutací, ale pouze několik z nich má svou četnost vyšší než 1% z celosvětového hlediska. Pro potvrzení diagnózy tohoto onemocnění u pacientů, u kterých se detekuje standardním screeningovým vyšetřením pouze jedna patogenní mutace, je nutné následně použít citlivý test pro vyhledání méně častých mutací nebo variant genu *CFTR*. V této disertační práci jsme úspěšně použili screening mutací a variant ve vybraných exonech genu *CFTR* pomocí metody HRM. Potvrdili jsme četné výhody metody HRM pro mutační skanování a charakterizovali jsme některá důležitá omezení, která musí být brána v úvahu v rámci zavádění HRM do rutinní praxe v diagnostických laboratořích.

Hyperhomocysteinémie se považuje jako jeden z rizikových faktorů pro několik závažných onemocnění jako jsou například opakované potraty nebo vrozené trombofilní stavy. V tomto kontextu jsou nejčastěji vyšetřovány varianty c.677C>T a c.1298A>C genu *MTHFR* měnící enzymatickou aktivitu enzymu methylenetetrahydrofolát reduktázy (MTHFR), který se podílí na metabolismu homocysteinu. Genotypizace těchto variant je proto důležitou součástí stanovení diagnózy u hyperhomocysteinémie. Pomocí modifikované metody HRM, kde je zkrácena celková délka analyzovaných amplikonů (HRM of small amplicons), se podařilo úspěšně genotypizovat varianty c.677C>T a c.1298A>C u vybrané skupiny pacientů s těmito onemocněními. Při diagnostické implementaci této metody v naší laboratoři jsme provedli kompletní validaci modifikované formy HRM za použití doporučených validačních parametrů v rámci zavedeného mezinárodního systému kvality.

Vzhledem k tomu, že terminologie používaná při validacích a verifikacích obvykle vede k nejasnostem, výzkumné konsorcium Evropské Unie (Eurogentest) vytvořilo doporučení pro validace a verifikace molekulárně genetických testů pro rutinní diagnostické účely. Pro zajištění kvality výsledků genetických testů, diagnostické laboratoře by měly být akreditovány a v rámci tohoto procesu by měly být genetické testy validovány. Autorka této disertační práce byla členkou validační skupiny tohoto projektu.

Neplodnost postihuje 10 - 15% párů, přičemž asi u přibližně 50% případů mužské neplodnosti nebyla původní příčina doposud identifikována. Z tohoto důvodu bylo publikováno velké množství publikací analyzujících genetické příčiny mužské neplodnosti. Předchozí publikace zabývající se japonskou populací prokázala, že variant rs6836703:G>A v genu pro ADP-ribosyltransferázu (*ART3*) je signifikantně asociována s neobstrukční azoospermií (NOA). Tuto studii jsme replikovali pomocí dříve validované metody HRM k posouzení významu této variace u českých sub-/infertilních mužů oproti fertilním kontrolám. Nalezli jsme významné rozdíly v distribuci alel/genotypů mezi fertilními muži a pacienty s oligozoospermií, zatímco u mužů s azoospermií, nebyl tento rozdíl signifikantní. Tato studie byla první úspěšnou replikací patogenetického potenciálu varianty rs6836703: G> A u poruch spermatogeneze. Zjistili jsme, tato varianta je asociována s oligozoospermií u českých mužů, přičemž jsme odhalili ochranný účinek

alely "G" v spermatogenezi. Je však potřeba tento nález objektivizovat v dalších populacích a určit klinickou významnost tohoto vyšetření před jejím zavedením do diagnostické praxe.

V souhrnu tato práce je modelem pro diagnostickou validaci u ostatních genetických laboratorních vyšetření a validace HRM of small amplicons byla již 14x citována. Validovaná metodika byla použita pro studium patogenetického potenciálu variant v genu *ART3* u mužské neplodnosti.

ABBREVIATIONS LIST

ACCE:	Analytical validation, Clinical validation, Clinical utility and consideration of Ethical, legal and social implications
ACMG:	American College of Medical Genetics
AR:	Androgen Receptor
ART3:	ADP-ribosyltransferase 3
ASO:	Allele-specific oligonucleotide
AZF:	Azoospermia Factor
bp:	base pair
CAIS:	Complete androgen insensitivity
CBAVD:	Congenital bilateral absense of vas deference
CF:	Cystic fibrosis
CFTR:	Cystic fibrosis transmembrane conductance regulator
CPC:	Cumulative probabilities of conception
DNA:	Deoxyribonucleic acid
dsDNA:	double-stranded DNA
FSHR:	Follicle-Stimulating Hormone Receptor
Hcy:	Homocysteine
HRM:	High Resolution Melting
HWE:	Hardy-Weinberg Equilibrium
ICH:	International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use
IRT:	Immunoreactive Trypsinogen
ISO:	International Organization for Standardization
IVD:	In-vitro diagnostics
kb:	kilobase

KS:	Klinefelter syndrome
MAIS:	Mild androgen insensitivity
MS-HRM:	Methylation Sensitive High Resolution Melting
MTHFR:	Methylenetetrahydrofolate reductase
NGRL:	National Genetics Reference Laboratory
NOA:	Non-obstructive azoospermia
OECD:	Organization for Economic Co-operation and Development
OR:	Odds-Ratio
PCR:	Polymerase Chain Reaction
PD:	Potential Difference
POLG:	Polymerase (DNA directed), gamma gene
PRM1:	Protamine 1
PRM2:	Protamine 2
PRSS1:	Protease, serine, 1 (trypsin 1)
QFPCR:	Quantitative Fluorescent Polymerase Chain Reaction
RPRGL1:	Reccurent pregnancy loss
SA:	Spontaneous abortion
SD:	Standard deviation
SNP:	Single Nucleotide Polymorphism
SPINK:	Serine peptidase inhibitor, Kazal type 1
spz:	spermatozoa
ssDNA:	single-stranded DNA
STARD:	STAndards for the Reporting of Diagnostic accuracy studies
Tm:	Temperature of melting
TTP:	Time-to-pregnancy
WHO:	World Health Organization
WT:	wild-type

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1. INTRODUCTION

1.1 CFTR gene and Cystic Fibrosis

1.1.1 CF Description and epidemiology

Cystic fibrosis (CF; MIM# 219700), also known as mucoviscidosis, is one of the most common and life-threatening autosomal recessive genetic disease in Caucasians (Bobadilla *et al.,* 2002; Yankaskas *et al.,* 2004; Ratjen 2009). Formerly known as cystic fibrosis of the pancreas, CF is a multisystem disorder which impairs clearance of secretions due to thickness of mucosa in a variety of organs affecting mainly lungs, pancreas, sweat glands and reproductive organs. It is characterized by recurrent pulmonary infections which leads to pulmonary hypertension and respiratory failure, exocrine pancreatic function deficiency causing malabsorption and growth retardation, excessive electrolyte secretions by the sweat glands, and also may cause congenital bilateral absence of the vas deferens (CBAVD; MIM# 277180) an important cause of infertility in men (Phillipson *et al.,* 2000; Noone and Knowles, 2001; Yankaskas *et al.,* 2004).

The increased viscosity of bronchial mucus is caused by defects in the Cystic Fibrosis Transmembrane Conductance Regulator protein (CFTR; ATP binding cassette, subfamily C, member 7; MIM# 602421). CFTR protein has a length of 1480 amino acids (UniProt# P13569). It is a chloride channel located in the outer layer of epithelial cell membranes, its dysfunction causes accumulation of chloride (negative charged) and consequently, the flux of sodium producing salt (recognized by the salty sweat in patients) resulting in an impaired mucociliary clearance (Ballard *et al.,* 1999; Boucher, 2007).

The *CFTR* gene is located in the chromosome 7q31.2, contains 27 exons and spans 189 kb of DNA (Riordan *et al.,* 1989; Rommens *et al.,* 1989). Until this date, more than 1900 genetic variants for *CFTR* gene have been reported, but it is still important to elucidate the functional consequences of many of these mutations since the majority of

them are rare. Actually, only 5 mutations occur with a frequency more than 1% in worldwide estimates (www.genet.sickkids.on.ca; accesed by February, 2012). Cystic fibrosis phenotypes vary depending on the *CFTR* mutations carried by the patient. Some mutations such as p.F508del (ΔF508), the most common mutation for cystic fibrosis with an estimated frequency of 66% worldwide (http://www.genet.sickkids.on.ca), are associated with severe phenotypes, while others are associated with mild symptoms (Noone and Knowles, 2001; Rohlfs *et al.,* 2002; Ratjen and Döring, 2003). Also vary the frequency and distribution of *CFTR* variants depending on the population of origin. For example, p.F508del, ranges from as high as 100% in the Faroe Islands of Denmark to as low as 24.5% in Turkey (Bobadilla *et al.,* 2002). In Albania, Finland and Lithuania only 4 mutations occur at frequencies ≥ 0.5%, while in Belgium there are 27 mutations (Bobadilla *et al.,* 2002) and in the Czech Republic, 17 mutations with frequencies ≥ 0.5% (Balascakova, 2010). Moreover, some common mutations are specific to ethnic groups, such as pW1282X among Ashkenazi Jews and c.3120 + 1G>A among native Africans (Montgomery *et al.,* 2007). In the Czech Republic, the most prevalent deletion is the p.F508del with a frequency of 66.90% (Balascakova, 2010). See Table 1.

Despite intensive and long-term treatment, majority of CF patients experience recurrent illnesses and progressive disability. The average life expectancy for a CF patient in the United States is 32 years (Yankaskas *et al.,* 2004). In the United States, CF occurs with a frequency of 1 in 2500 - 3500 births to Caucasians; 1 in 4,000–10,000 Hispanic Americans; 1 in 15,000–20,000 African Americans; and 1 in 100,000 Asian Americans. (http://www.cff.org/), while in the Czech Republic the incidence established by newborn screening is of 1 in 4000 newborns (Balascakova *et al.,* 2009). In the United States, approximately 1 in 29 Caucasians is a carrier for CF, 1 in 46 Hispanic Americans; 1 in 65 African Americans; and 1 in 90 Asian Americans (http://www.cff.org/; accessed by February 2012). Due to the high frequency of carriers, it is of importance to examine for CF carrier mutations in couples planning to have or expecting a child especially in Caucasian populations.

Table 1. Most common mutations (frequencies higher than 1%) detected at the Cystic Fibrosis Center, University Hospital Motol, Prague, Czech Republic (*).

Mutation	N	%
F508del (c.1520_1522delTCT)	768	66.90
c.CFTRdele2.3/21kb/	67	5.84
G551D (c.1652G>A)	34	2.96
N1303K (c.3909C>G)	29	2.53
G542X (c.1624G>T)	23	2.00
3849+10kbC>T (c.3717+12191C>T)	20	1.74
1898+1G>A (c.1766+1G>A)	15	1.31

(*) Adapted from Balascakova, 2010.
Using both, the legacy and Human Genome Variation Society (HGVS) nomenclature.

1.1.2 CF Diagnostics

When a patient is suspected of having CF it is necessary to confirm the diagnosis by biochemical substantiation of the CFTR dysfunction. Laboratory tests performed to confirm CF are: 1/ sweat test; 2/ measurement of nasal potential difference (PD; an abnormal nasal PD measurement recorded on 2 separate days can be used as evidence of CFTR dysfunction); and/or 3/ genetic analysis of CF-causing mutations also used for prenatal diagnosis and carrier test (Rosenstein and Cutting, 1998; Yankaskas *et al.*, 2004; Dequeker *et al.*, 2009). In newborn screening programs, the concentration of immunoreactive trypsinogen (IRT) in newborns' blood is tested. The amount of IRT tends to be increased above arbitrary "threshold" in people with CF, but may be increased also

due to other reasons. Therefore, an examination test to confirm CF is needed (Balascakova *et al.,* 2009; http://www.cff.org/).

In adults, the quantitative pilocarpine iontophoresis sweat test is the initial diagnostic examination and with the highest utility for the diagnosis of CF. Within CF adult patients, sweat chloride concentration is abnormal in > 90% of patients, but there is still a number of CF patients with normal or borderline sweat chloride values. Therefore, this method could not be used as a single assay to diagnose CF due to false negativity in atypical CF patients (Yankaskas *et al.,* 2004).

Particularly in suspected patients with normal or borderline sweat chloride concentrations, a test for *CFTR* mutations ought to be performed. However, the detection of mutations in adult patients becomes more complicated since they likely carry a rare or an unidentified mutation not included in commercial mutation screening panels (Yankaskas *et al.,* 2004). Complete analysis of *CFTR* gene may be used in those patients when disease alleles are not identified by routine *CFTR* genotyping assays. Nonetheless, complete gene analysis is not generally recommended as variants of unknown clinical significance complicate genetic counseling. Moreover, sequencing of the entire *CFTR* gene remains expensive and laborious (Yankaskas *et al.,* 2004; Montgomery *et al.,* 2007; Polou *et al.,* 2012).

Alternatively, scanning techniques such as DGGE (denaturing gradient gel electrophoresis), DHPLC (denaturing high performance liquid chromatography) and SSCP (single strand conformation polymorphism) have been used to identify *CFTR* variants, however, despite their high degree of accuracy and relatively low cost, the labor-intensive nature of these techniques restricts their use to research and selected reference laboratories (Montgomery *et al.,* 2007; Dequeker *et al.,* 2009).

1.2 Hyperhomocysteinemia related to MTHFR

1.2.1 Homocysteine and MTHFR

Homocysteine (Hcy) is a sulfur amino acid derived from the metabolism of methionine, which after conversion to S-adenosylmethionine, is the most important methyl group donor in the body. Hcy is metabolized by either remethylation or transsulfuration pathways. The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) regulates the remethylation pathway of homocysteine catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine and the predominant circulating form of folate, see Figure 1 (Legnani *et al.,* 1997; Selhub, 1999; Brustolin *et al.,* 2010).

Hyperhomocysteinemia arises from disrupted homocysteine metabolism and might be due genetic and/or acquired factors. Acquired causes mainly include low vitamin intake of B6, B12 and folic acid vitamins leading to an increase in homocysteine levels. Genetic determinants include the cystathione β-synthetase deficit producing hyperhomocysteinemia with very high levels in homozygous carriers, and, variants on the methyltetrahydrofolate reductase (*MTHFR*) gene (Selhub, 1999; den Heijer, 2003; Slavik *et al.,* 2009; Brustolin *et al.,* 2010).

Common variants of the methylenetetrahydrofolate reductase (MTHFR) gene are c.677 C>T (rs1801133:C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p.E429A). These variants result into reduction of MTHFR enzymatic activity, in particular in the case of the c.677 C>T variant, since the MTHFR protein carrying the Ala222Val variation (rs1801133: C>T; c. 677 C>T; p.A222V), located in a highly conserved residue of the molecule, is thermolabile and has a reduced activity increasing homocysteine levels (Legnani *et al.,* 1997: Gemmati *et al.,* 1999). The c.1298 A>C variant also affects protein activity but it is not associated with high levels of Hcy and is not associated with low folate levels. Combined heterozygosity for both common MTHFR mutations (i.e. c.677 C>T and c.12989 A>C) results in similar characteristics to those observed in homozygotes for the c.677 C>T variation (van der Put *et al.,* 1998).

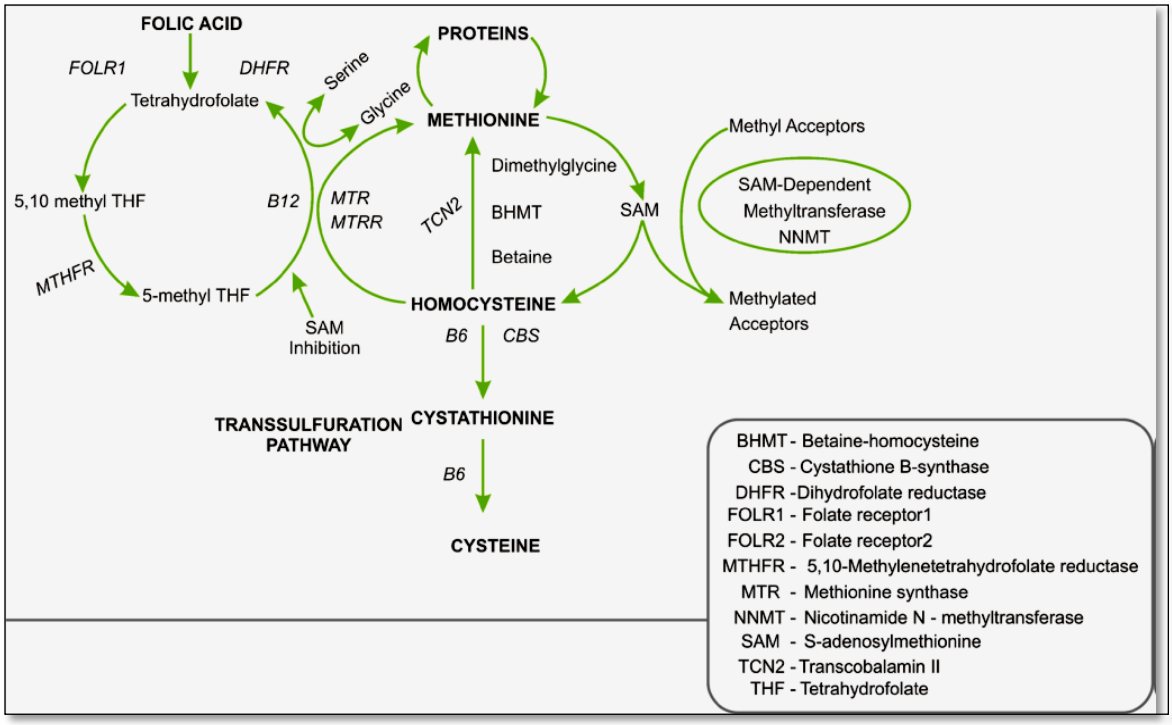

Figure 1. Homocysteine metabolism (Brustolin *et al.*, 2010).

The *MTHFR* gene (GeneID: 4524; MIM# 607093) is located in the chromosome 1p36.3, has 12 exons, spans 21 kb of DNA and encodes a protein of 656 amino acids (UniProt# P42898). Currently, the most common methods to genotype *MTHFR* c.677 C>T (rs1801133: C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p.E429A) variants include RFLP (Restriction Fragment Length Polymorphism) (Frosst *et al.*, 1995; Hanson *et al.*, 2001) and commercial kits (based on hybridization). Both techniques are a laborious and time-consuming.

1.2.2 Hyperhomocysteinemia and related disorders

High levels of homocysteinemia have been related to several disorders such as neural tube defects, diabetes, vascular dementia, Alzheimer's disease, spontaneous abortion, thrombophilia, and certain types of cancer (Schwahn & Rozen, 2001; den Heijer, 2003; Kono & Chen, 2005; Blom *et al.,* 2006; Bolufer *et al.,* 2006; Eldibany and Caprini, 2007; Brustolin *et al.,* 2010; La Merrill *et al*., 2012).

1.2.2.1 Inherited thrombophilia

Already, in the 19th century, it has been established that increase in blood coagulability (hypercoagulability) may lead to venous thrombosis. Therefore, thrombophilia is defined as "an inherited or acquired abnormality of hemostasis predisposing to thrombosis" (Middeldorp, 2011).

Patients affected with thrombophilic alterations present a wide variability of symptoms (Legnani *et al.,* 1997). Hyperhomocysteinemia is a risk factor of atherosclerosis and venous thrombosis. It is associated either with atherosclerosis or venous thrombosis but not with both (Eldibany and Caprini, 2007). The *MTHFR* c.677 C>T variation is an independent risk factor for arterial and venous thrombosis (Gemmati *et al.,* 1999; Salomon *et al.,* 1999).

Mild/moderate hyperhomocysteinemia is a cause for recurrence of venous thrombosis in patients with coexistent risk factors and represents 5–10 % of all clinical thrombotic episodes (Legnani *et al.,* 1997; Gemmati *et al.,* 1999; den Heijer, 2003; Eldibany and Caprini, 2007; Slavik *et al.,* 2009). Hyperhomocysteinemia is a surrogate for low levels of a group of B vitamins, which represent an objective risk for vascular disease (Eldibany and Caprini, 2007).

1.2.2.2 Recurrent Pregnancy loss

Recurrent pregnancy loss (RPRGL1; MIM: #614389), also called recurrent miscarriage or recurrent spontaneous abortion (SA) is the most common complication of pregnancy and is defined as the spontaneous fetal loss before 20 weeks of gestation (i.e. before the fetus has reached its viability). RPRGL1 is defined as three or more consecutive SAs. About 1% of couples are affected by RPRGL1 and around 5% of couples are affected by two or more SAs (Rai and Regan, 2006; Rodriguez-Guillen *et al.,* 2009).

Lifestyle, diet, and, maternal "genetic background" have been proposed as predisposition factors for SA. Mainly, maternal and paternal smoking, including maternal alcohol and coffee consumption during pregnancy have been associated with a higher risk of SA. Moreover, low folate levels and suboptimal folate metabolism, determined by hyperhomocysteinemia, have also been associated with RPRGL1 (Rodriguez-Guillen *et al.,* 2009).

MTHFR c.677 C>T and c.1298 A>C variants increase the risk of spontaneous abortion, regardless of dietary intake of B vitamins. (Rodriguez-Guillen *et al.,* 2009). Additionally, the c.1298 A>C variant may be an independent risk factor for the development of SA associated with fetal chromosomal aneuploidy (Kim *et al.,* 2011).

1.3 Male infertility

1.3.1 Definition, classification and idiopatic infertility

Subfertility and infertility definitions are particularly important for the proper care in patients with repeated reproductive failure. However, the terminology used in reproductive medicine usually leads to confusion (Habbema *et al.,* 2004). The term *subfertility* should be used to name any form of reduced fertility in couples unsuccessfully trying to conceive within a prolonged time (Jenkins *et al.,* 2004; Gnoth *et al.,* 2005). Time-to-pregnancy (TTP) estimations and cumulative probabilities of conception (CPC)

are crucial to determine the degree of subfertility in order to provide the appropriate care and avoid over or under-treatment of subfertile couples (Brosens *et al.,* 2004; Gnoth *et al.,* 2005).

Infertility is defined as one year of "unwanted non-conception" with unprotected intercourse within the fertile phase of menstrual cycles (Evers, 2002) and may be used in equivalence to sterility (Gnoth *et al.,* 2005). Timing and prevalence are described in Table 2.

Infertility affects 10 – 15% couples trying to conceive in Western countries (McClure 1986; de Kretser, 1997). It has been estimated that 50% are of female origin, 35% are due to male factor, and 15% are unexplained (Ayensu-Coker *et al.,* 2007).

Sperm disorders are the single most common cause of "male factor" infertility (Razvi *et al.,* 1999). Ejaculatory disorders and impotence also result in infertility but could be effectively treated (Ayensu-Coker *et al.,* 2007). Sperm disorders may be classified according to sperm counts into oligozoospermia and azoospermia. A*zoospermia* refers to no spermatozoa in the ejaculate (given as the limit of quantification for the utilized examination method). O*ligozoospermia* is defined as the total number (or concentration, depending on the outcome reported) of spermatozoa below the threshold of $39x10^6$ spermatozoa per ejaculate or $15x10^6$ spz/mL (WHO, 2010).

Approximately 50 % of male infertility cases have been classified as "idiopathic" since their original cause has not yet been identified (Shefi and Turek, 2006; Krausz and Giachini, 2007). Considering the complexity of spermatogenesis and the high number of genes involved, it is likely that genetic causes alone contribute to the development of unexplained cases of male infertility (Ferlin et al., 2006; Shefi and Turek, 2006; Krausz and Giachini, 2007).

Table 2. Definition and prevalence of subfertility and infertility (*).

Time	Prevalence/grading	Chances to conceive spontaneously in the future
After six unsuccessful cycles	About 20% at least slightly subfertile couples	50% of these couples will conceive spontaneously in the next six cycles, the remaining are moderately subfertile [Equivalent to slightly reduced fertility (Habbema *et al.*, 2004)]
After 12 unsuccessful cycles	About 10% at least moderately or seriously subfertile couples	50% of these couples will conceive spontaneously in the next 36 months, the remaining are nearly complete infertile [Equivalent to moderate/seriously reduced fertility (Habbema *et al.*, 2004)]
After 48 months	About 5% nearly complete infertile couples	Couples with only sporadic spontaneous conceptions [Equivalent to sterile couple (Habbema *et al.*, 2004)]

(*) *Gnoth et al., 2005.*

1.3.2 Male infertility – Genetic background and candidate factors

It has been proposed that around 15% of male infertility cases are due to chromosomal abnormalities and single gene defects (Ferlin *et al.*, 2006). Chromosomal abnormalities that significantly affect fertility comprise the Klinefelter syndrome (47, XXY), sex chromosome alterations such as 47, XYY and Y-chromosome microdeletions (AZF deletions) (Simoni *et al.*, 2004; Ferlin *et al.*, 2006; Martin, 2008). Klinefelter syndrome (KS) represents the most common forms of male hypogonadism and is the most frequent sex chromosome aneuploidy in males, with a prevalence of 0.1 – 0.2% in newborns. Its frequency among sub-/infertile men is further increased: 5% in severe oligozoospermic and 10% in azoospermic men (Ferlin *et al.*, 2006).

Klinefelter syndrome (KS) and AZoospermia Factor (AZF) microdeletions of the Y chromosome: AZFa, AZFb and AZFc, are the most common genetic cause of male infertility (Lanfranco *et al.,* 2004; Simoni *et al.,* 2004; Vogt *et al.,* 1996). The Y chromosome contains a high number of gene and gene families that are necessary for spermatogenesis. Many of these genes are localized in AZF regions that are replete with repetitive elements that may undergo complex deletions events (Repping *et al.,* 2002). The most frequent Y chromosome microdeletion is the deletion of the AZFc region which causes azoospermia or severe oligozoospermia. AZFc microdeletion has a prevalence ranging from 2% to 10% in infertile men and could even be higher in other populations (Simoni *et al.,* 2004).

Described single-gene causes of male sub-/infertility have been related to androgen receptor (*AR*) and *CFTR* genes. Androgens and a functional AR are essential for development and maintenance of the male phenotype and spermatogenesis. Approximately 90% of mutations in the *AR* gene have been reported in different types of defects known as a group as androgen insensitivity syndrome (AIS), ranging from mild (MAIS) to complete androgen insensitivity (CAIS) (Ferlin *et al.,* 2006; Rajender *et al.,* 2007).

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene lead to congenital bilateral absence of the vas deference (CBAVD), causing obstructive azoospermia in more than 95 % of males with CF. Conversely, 50 – 80% men with CBAVD (but no digestive or pulmonary symptoms suggestive for CF) will carry a CF-causing mutation (Dohle *et al.,* 2006; Ferlin *et al.,* 2006; Radpour *et al.,* 2008). *CFTR* mutations have also been related to non-obstructive azoospermia (NOA) or idiopathic infertility, but larger studies corroborating these results are missing (Schlegel, 2007).

There have been many attempts to identify other genetic factors associated with male infertility, but until this date only inconsistent results have been found thus far. This trait has been associated with a broad list of genes and variants: gr/gr deletions of the AZFc region on the Y chromosome, variants in genes involved in endocrine regulation of spermatogenesis, such as CAG repeats in the exon 1 of the androgen receptor gene (*AR*)

and follicle stimulating hormone receptor gene (*FSHR*), variants in genes of common cell functions as polymerase (DNA directed), gamma gene (*POLG*) and 5, 10-methylenetetrahydrofolate reductase gene (*MTHFR*). In addition, variants in genes involved in specific spermatogenic functions, such as deleted in azoospermia-like gene (*DAZL*), protamine 1 and 2 genes (*PRM1* and *PRM2*) and ubiquitine specific peptidase 26 gene (*USP26*) (Ferlin *et al.,* 2006; Rajender *et al.,* 2007; Tuttelmann *et al.,* 2007, Krausz and Giachini 2007; Giachini *et al,* 2008). These discordant results might be due to the heterogeneity of the studied populations, penetrance of these polymorphisms (which usually depends on the ethnical background), different selection criteria of cases and control cohorts (fertile, normospermic vs. azoospermic, oligozoospermic or infertile all together); influence of environmental and epigenetic factors, or statistic approaches used for analysis (Tuttelmann *et al.,* 2007, Aston and Carrell, 2009). Also the list of single studies reporting an association between a polymorphism and male infertility is increasing, but no replicate studies or meta-analyses has been published, so far (Aston and Carrell, 2009).

1.3.3 ADP-ribosyltransferase 3, rs6836703: G>A SNP

Outcomes of a previous genome-wide expression study suggested that the rs6836703: G>A single-nucleotide polymorphism (SNP) from the ADP-ribosyltransferase 3 (*ART3*) gene, located into the intron 10 (NCBI database, reference sequence NT016354.18, accessed by 05-25-2010), is related to NOA in the Japanese population (Okada *et al.,* 2008).

Mono-ADP-ribosyltransferases (ART) (EC 2.4.2.31) catalyzes the reversible post-translational protein modification, mono-ADP-ribosylation, which involves the transfer of the ADP-ribose moiety from NAD^+ to a specific amino acid in a target protein, while the nicotinamide moiety is released. This modification is presumed as a mechanism to regulate endogenous protein functions. Mono-ADP-ribosylation was originally discovered as the mechanism by which diphtheria toxin blocks protein synthesis (Honjo *et al.,* 1968). The best known ARTs are represented by bacterial products (e.g. cholera toxin, pertussis

toxin, *Escherichia coli* heat-labile enterotoxin and *Pseudomonas aeruginosa* exotoxin A). They produce profound changes in the cellular metabolism of the human host cells by interfering with signal transduction via attachment of ADP-ribose onto regulatory G-proteins (Koch-Nolte *et al,*. 1997; Glowacki *et al.,* 2002; Friedrich *et al.,* 2006a).

The *ART3* gene is a single-copy gene, member of the mono-ADP-ribosyltransefrase family genes (the family of human ART comprises four members: ART1, ART 3-5) (Koch-Nolte *et al,*. 1997; Glowacki *et al.,* 2002; Friedrich *et al.,* 2006a). *ART3* was mapped to the chromosome 4p15.1-p14 (Koch-Nolte *et al,*. 1997). This gene contains 11 exons and spans 33.13 kb of DNA (Glowacki *et al.,* 2002; Friedrich *et al.,* 2006b).

ART3 gene encodes a protein of 389 amino acids (isoform a). The ART3 protein has another two isoforms: isoforms b and c have a length of 378 and 367 amino acids, respectively (NCBI database, accessed by 02-22-2011). According to the protein sequence, ART3 contains two signal peptides. The hydrophobic N-terminal peptide exhibits features of an extracellular protein, while the C-terminal contains a long stretch of hydrophobic amino acids, a characteristic feature of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins (Friedrich *et al.,* 2006a; Friedrich *et al.,* 2006b).

The biological function of ART3 is still unknown since this protein lacks the active site motif (R-S-EXE) thus which is essential for catalytic activity of the arginine-specific transferases. ART3 under cell culture conditions is not able to catalyze the transfer of ADP-ribose to arginine, but also to other amino acids indicating that it had lost its enzyme activity and could have acquired a new protein binding function (Glowacki *et al.,* 2002; Friedrich *et al.,* 2006a; Friedrich *et al.,* 2006b). Interestingly, the ART3 protein is expressed in human testis, especially in spermatocytes. Temporary expression profile of ART3 suggests that it might play a key role in a tightly regulated specific stage of spermatogenesis (Friedrich, *et al.* 2006a; Friedrich, *et al.* 2006b).

1.4 High Resolution Melting (HRM)

1.4.1 Description and characteristics

Advances in DNA testing technologies have provide an impetus for the development of different mutation detection techniques. Direct DNA "Sanger" sequencing remains as the "gold standard". However, it is still expensive, laborious and time consuming. Several methods have been developed to simplify the detection and reduce the overload of sequencing, with the most common diagnostic techniques being based on restriction enzyme analysis (Botstein *et al.,* 1980), allele-specific amplification (Newton *et al*., 1989), ligation based assays (Baron *et al.,* 1997), single-base extension (Li *et al.,* 1999), fluorogenic ASO hybridization probes (Schaeffeler *et al.,* 2003; Murugesan *et al.,* 2006) and pyrosequencing (Ahmadian *et al.,* 2000). Nevertheless, despite automatization, most of them still are time consuming and their success is dependent on the analyst's experience.

A new alternative for mutation scanning was introduced by Carl Witter and his group in 2003 (Wittwer *et al.,* 2003). The high Resolution Melting (HRM) quickly emerged as a rapid, simple, economical and high-throughput mutation scanning method (Reed & Wittwer, 2004; Wittwer *et al.,* 2003; Reed *et al.,* 2007; Erali *et al.,* 2008). HRM is based on DNA thermodynamics of melting (Figure 2). With the utilization of fluorescence saturating dsDNA-binding dye and an instrument able to record fluorescence transitions in high resolution, it is feasible to monitor DNA melting transitions as gradually the temperature increases and as the dye is being released by measuring the proportional decrease of fluorescence. This way, it is possible to record more than 25 readings per 1 ºC. The fluorescence decreases as DNA intercalating dye is released from double-stranded DNA during the process of its dissociation (melting) into single strands. (Wittwer *et al.,* 2003; Reed & Wittwer, 2004; LightCycler® 480 Operator's Manual, Roche 2007).

Other advantage, particularly important for a routine diagnostic setting, is that PCR amplification and melting curve analysis are performed within the same tube or plate, without any post-PCR processing. HRM thus, has similar or higher sensitivity and

specificity than methods that require physical separation (Erali *et al.,* 2008). Furthermore, HRM is a highly sensitive and specific technique for mutation scanning that could be easily integrated into clinical diagnostic pre-screening strategies (NGRL, 2006; Erali *et al.,* 2008).

Instruments which merely perform melting are not sensitive enough for high resolution applications. Most of the platforms used for HRM use microtiter plates that allow the simultaneous analysis of 96 or 384 samples (Vossen *et al.,* 2009). Recently, a new platform called LightCycler® 1536 System from Roche Applied Science with the proprietary plate allows the analysis of 1536 samples at the same time. In most of these platforms using microtiter plates, a well-to-well difference in temperature is present thereby affecting HRM sensitivity. Seipp and colleagues (Seipp *et al.,* 2007) developed control temperature calibration probes that melt at low and high temperatures, while the software use such probes to adjust Tm values. These authors, where able to reduce Tm SD by 38% increasing the overall sensitivity of the method (Seipp *et al.,* 2007). It is important to notice that not all softwares for HRM are able to use respective calibration probes (Vossen *et al.,* 2009).

Additionally, the dsDNA-binding dye utilized must be "*saturating*". This feature allows recording the release of the dye as the DNA strands are being separated, while the redistribution along the DNA strand is minimal (Figure 3). HRM success also depends on PCR product purity. Therefore, PCR amplification must be specific and have an adequate yield. DNA quality plays an important role in order to achieve good quality melting profiles. Any extraction method could be used but it is important to use same buffers for dilutions (NGRL, 2006; Montgomery *et al.,* 2007; Fortini *et al.,* 2007).

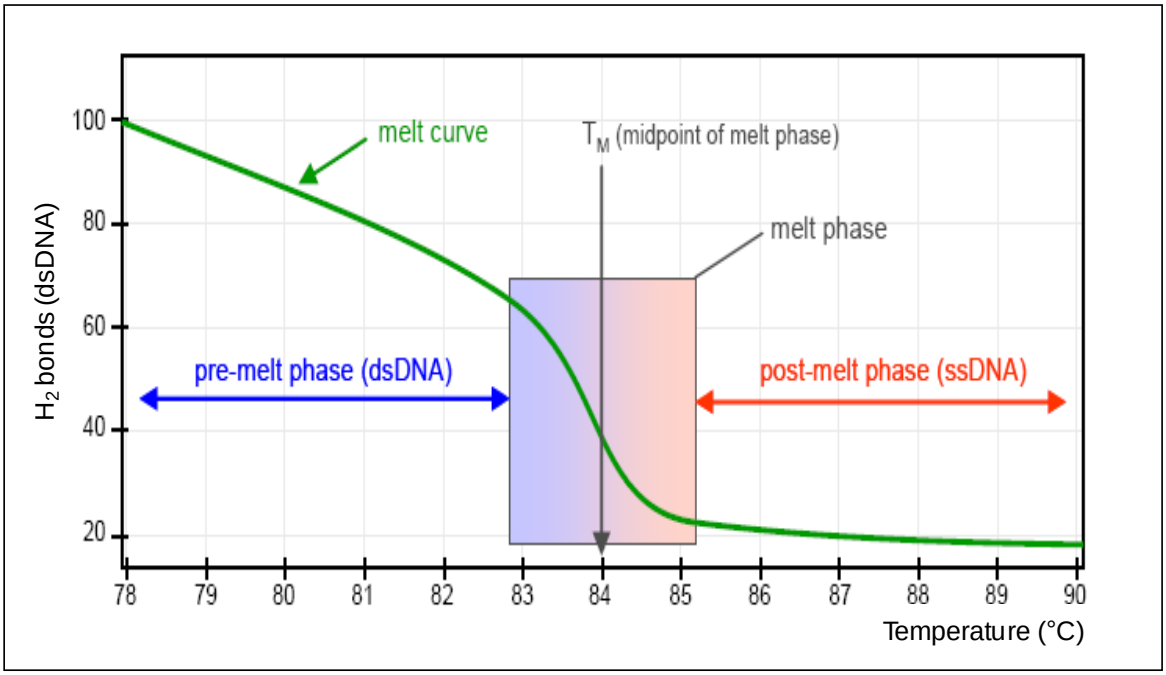

Figure 2. DNA Melting Curve Profile. The melt curve (green) plots the transition from high number of H_2 bonds of the initial pre-melt phase (high number of double-stranded DNA (dsDNA)) through the sharp loss of H_2 bonds of the melt phase due to an increase of the temperature causing the release of the H_2 bonds, to the complete loss of H_2 bonds, having only single-stranded DNA (ssDNA) at the post-melt phase. The midpoint of the melt phase defines the temperature of melting (Tm) of the particular DNA fragment under analysis. *Adapted from Corbett Research, 2006.*

With the development and understanding of the HRM technology, different applications have arisen such as genotyping with labeled/unlabeled probes, genotyping using small amplicons or snap back primers, detection of variable-number tandem repeats, detection of RNA editing, and methylation-sensitive high resolution melting (MS-HRM) (Pubmed search keywords: "high resolution melting", accessed by March 2011). In this thesis, we will be focusing on two of these applications: 1/ the original application of HRM which is mutation scanning and on 2/ HRM genotyping by small amplicons. These applications will be further discussed in the paragraphs below.

1.4.2 HRM for gene scanning

High Resolution Melting of PCR products can be used as a mutation scanning method to identify any heterozygous DNA between PCR primers. Differences in the melting curve shape allow identification of a heterozygous sample (i.e. via simple eye-inspection by the analyst) (Wittwer *et al.*, 2003; Graham *et al.*, 2005; Montgomery *et al.*, 2007). These differences can be displayed by superimposing normalized curves and plotting the fluorescence difference between wild-type and heterozygous samples (Erali *et al.*, 2008) (Figure 4).

Once a variant is found, sequencing of the specific amplicon usually detects the variant identified by HRM. Although with lower sensitivity, homozygous mutations may also be identified, while small homozygous deletions/insertions are usually missed. This disadvantage can be overcome by: 1/ 1:1 mixes of tested sample with wild-type samples (i.e. creating artificial heterozygotes) or by 2/ decreasing the length of the DNA fragment to be analyzed (Montgomery *et al.*, 2007).

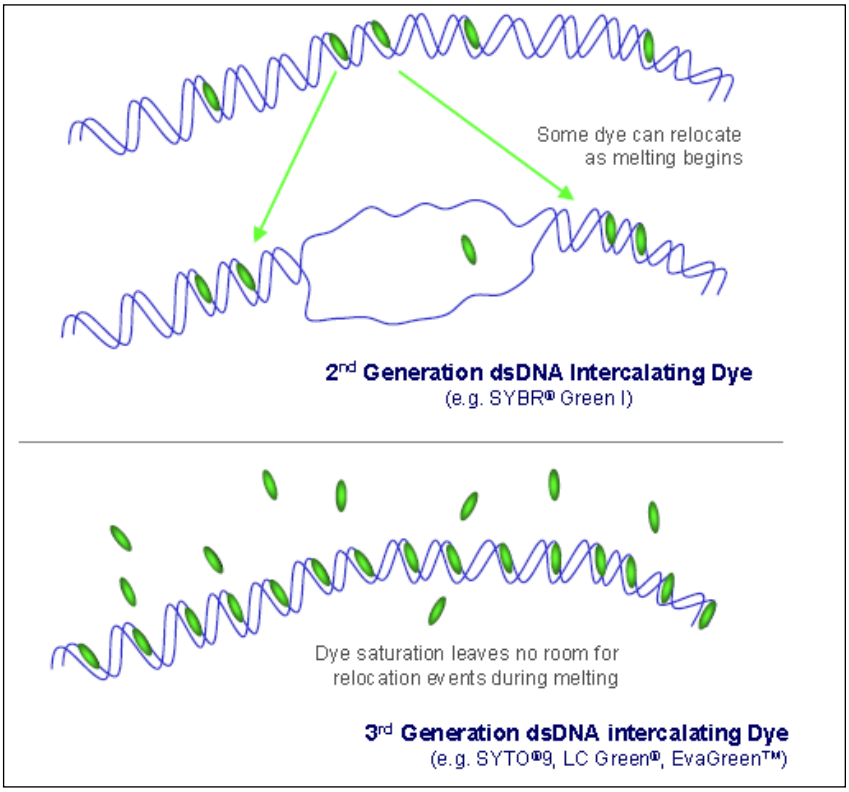

Figure 3. Redistribution of DNA intercalation dyes during DNA dissociation. The reduced reaction toxicity of 3rd generation dyes means that a higher concentration of dye can be used. Higher dye concentration increases the level to which the DNA becomes saturated with dye molecules. Saturation is believed to reduce dye redistribution effects during DNA dissociation (as illustrated) which increases the resolution of melt analysis (Corbett Research, 2006).

The capacity to detect heterozygous mutations by HRM is very good, but still one issue remains: whether HRM is able to distinguish between different heterozygotes in the same amplicon. Four classes of SNPs have been established based on the homo- and heteroduplexes that are formed after amplification of a heterozygous sample (see Table 3). Tm differences between homozygous samples are greater in the first two classes; with differences usually being between 0.8 – 1.4 °C (Liew *et al.,* 2004). These two classes comprise > 84% of human genome (Venter *et al.,* 2001). Difference in Tm in the classes 3 and 4 are usually < 0.4 °C, since the base pair (A::T or C::G) is just inverted and the difference is only due to the nearest-neighbor stability (Liew *et al.,* 2004). Since heteroduplexes mismatches from different SNP class are different, it should be possible to visualize such differences by their melting profiles. Moreover, it should be possible to detect heterozygotes within the same SNP class considering that nearest-neighbor stability parameters also determine their melting behavior (Liew *et al.,* 2004; Graham *et al.,* 2005). We addressed this question in a previous publication originating from our group and attached to this dissertation (Krenkova *et al.,* 2009).

Table 3. SNP classification according to the homo- and heteroduplexes formed after amplification of a heterozygous sample (*).

Class	SNP Transitions	Homoduplex matches	Heteroduplex matches
1	C/T or G/A	C::G and A::T	C::A and T::G
2	C/A or G/T	C::G and A::T	C::T and A::G
3	C/G	C::G	C::C and G::G
4	T/A	A::T	T::T and A::A

(*) *Adapted from Liew et al., 2004.*

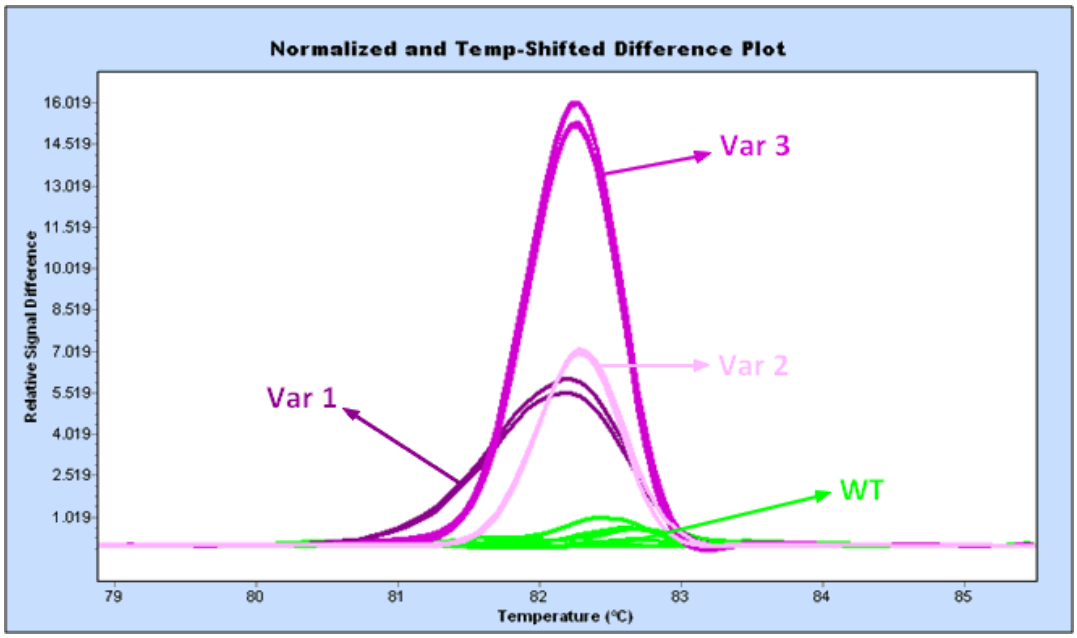

Figure 4. Heterozygote detection by HRM. Different DNA sequences have different melting profiles and this difference in the melting curve shape allows identification of heterozygous samples. In this normalized and temperature shifted difference plot we may easily recognize three different heterozygous variants (Var1: variant 1; Var 2: variant 2; Var3: variant 3) from the wild-type samples (WT) for the same DNA fragment amplified.

1.4.3 HRM for genotyping

High Resolution Melting is based on thermodynamic differences between DNA fragments. Discrimination between homozygous genotypes is difficult because the difference between homozygous sequence melting profiles is usually merely represented by a slight shift in the melting temperature (T_m), but not by a change of the melting curve profile. Moreover, some homozygous SNPs have melting curves impossible to differentiate from the wild-type profile, as predicted by nearest-neighbor thermodynamic models (Palais *et al.,* 2005). In order to solve this issue, laboratories usually prepare 1:1 sample mixes with a tested sample and a wild-type sample for the amplicon tested. This creates an artificial heterozygote. However, this procedure increases the time, workload and wild-type DNA sample needed for the test.

Reducing the amplicon size allows a better discrimination between homozygous samples. As amplicon size is reduced, the Tm differences between genotypes are accordingly increased facilitating the discrimination between genotypes and increasing the sensitivity of the technique (Liew *et al.,* 2004; Erali *et al.,* 2008) (Figure 5). Moreover, decreasing the amplicon size simplifies the primer design because these are chosen as close as possible from the SNP to be detected. Shortening in fragment size, also reduces the PCR cycling time since lower melting temperatures are used for the denaturation phase and no holds are needed on the extension phase (Erali *et al.,* 2008).

In the current literature there are many examples for the use of HRM for gene scanning or genotyping (Reed & Wittwer, 2004; Liew *et al.,* 2004; Reed *et al.,* 2007; Erali *et al.,* 2008; Nomoto *et al.,* 2006; Hung *et al.,* 2008; Bastien *et al.,* 2008; Audrezet *et al.,* 2008). Despite the rapid introduction of this technique into DNA diagnostics (Erali *et al.,* 2008) there is no publication on exhaustive validation as required for quality assurance purposes stipulated by the "OECD guidelines for quality assurance in genetic testing" (http://www.oecd.org/).

Therefore, our laboratory reported an example of validation strategies for SNP genotyping by HRM focused in a diagnostic setting using as a model the examination of common variants of the methylenetetrahydrofolate reductase (*MTHFR*) gene: c.677 C>T

(rs1801133: C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p.E429A) (Norambuena *et al.*, 2009). These variants have been proposed to be associated with different disorders such as neural tube defects, infertility, thrombosis or some types of cancer associated to impaired folate metabolism due to a measurable reduction of enzymatic activity of MTHFR, in particular for the c.677 C>T variant (Schwahn & Rozen, 2001; Blom *et al.*, 2006; den Heijer, 2003; Kono & Chen, 2005; Bolufer *et al.*, 2006).

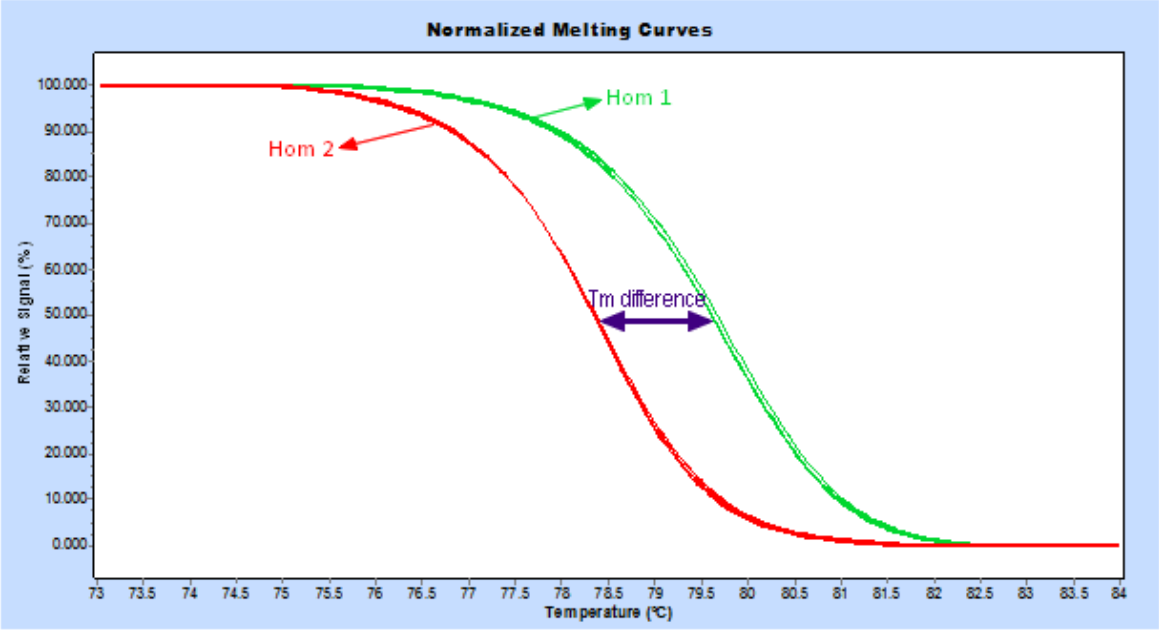

Figure 5. Homozygous DNA identification by HRM of small amplicons. Normalized melting curves plot of two different homozygous samples. The reduction in the size of the fragment amplified increases the difference in meting profiles of a DNA sample allowing an easier detection between homozygous samples.

1.5 Diagnostic genetic tests and method validation

1.5.1 Quality assurance in molecular genetic tests

The development and improvement of new technologies for molecular tests and the progress in understanding the molecular basis of diseases as in immunology, cancer, inherited diseases and infectious diseases, have fostered rapid introduction of molecular genetic tests for the diagnosis of a 1/ disease, 2/ the predisposition to a disease or to 3/ predict drug response of a patient (Jennings *et al.*, 2009).

Considering that genotyping is performed usually only once per lifetime, it is crucial to guarantee highly accurate test results since these affect the further treatment management and family planning of an individual (OECD guidelines, 2007).

Approximately 80% of medical decisions are made based on diagnostic tests, showing a high value and influence of laboratory test results (IVD manufacturers, 2004). Therefore, as Jennings states in their article, "it is encouraging to know that a laboratory's efforts assure patients and the public that we in the health care system are acting in their best interests" (Jennings *et al.*, 2009). Consequently, is an obligation for diagnostic laboratories to provide high quality tests results (OECD guidelines, 2007) and even though genetic tests are precise, there are always some limitations that must be considered before implementing a method within a diagnostic setting (Jennings *et al.*, 2009). Thus, in the frame of quality assurance, which is defined according to ISO9000 standards as "all those planned and systematic activities implemented within a quality system, and demonstrated as needed to provide adequate confidence that an entity will fulfill requirements for quality" (www.iso.org), each genetic laboratory working within a health care system should be accredited. For example, to reach accreditation a laboratory must, together with other requirements, have qualified personnel, adequate infrastructure, participate in external quality assurance schemes, validate or verify all implemented tests and document any deviation performance of those tests (OECD guidelines, 2007).

1.5.2 Validation and verification

Formal requirements for accreditation of laboratories according to ISO15189 and ISO17025 standards (www.iso.org) comprise validation and verification of implemented tests. Additionally, the ACCE framework stipulates that a laboratory implementing a new test must subject it to analytical validation, clinical validation, clinical utility and consideration also ethical, legal and social implications (Sanderson *et al.,* 2005). However, there is vagueness about terminology and the choice of most appropriate quality assurance parameters. Regulatory organizations do not accurately define the terminology related to test validation, and this is translated into confusing interpretation of these terms (OECD, 2007; Jennings *et al.*, 2009). In this respect, FP6 – FP7 Eurogentest projects (www.eurogentest.org) have created a working group focused on developing a framework for validation that is expected to be extensively implemented in laboratories to improve the quality of genetic services. This working group published a generic scheme for the validation and verification of molecular genetic tests for diagnostic use (Mattocks *et al.,* 2010). Previously, our laboratory suggested validation-related parameters as stipulated by ISO15189 (www.iso.org); QSOP 23 (www.evaluations-standards.org.uk); the American College of Medical Genetics (ACMG) (ACMG, 2006); the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 1994), including recommendations of the Czech Clinical Biochemistry and Medical Genetics societies (Brdička *et al.*), or as reported elsewhere (Prence, 1999). An example for diagnostic validation related to molecular genetic tests confined to qualitative test results was published by the author of this dissertation (Norambuena *et al.,* 2009).

A survey carried out by Eurogentest (www.eurogentest.org; 2005) where laboratories were asked to share their experiences with CE-marked in vitro diagnostic (IVD) assays within routine cystic fibrosis (CF) diagnostics, highlighted the necessity to strengthen the awareness of validation/verification usefulness in all types of genetic tests, even in the case of CE-marked commercial diagnostic assays. This is due to the fact that DNA diagnostic laboratories do not routinely verify CE-marked assays prior to their implementation in diagnostic settings. Likewise, almost 50 % of laboratories modified

manufacturer-recommended protocols of CE-marked IVD CF assays and, interestingly, many of them did not validate these changes (Camajova *et al.*, 2009).

In the USA, introduction of a new method for diagnostic use does not require FDA approval and as result of this, laboratories have developed the majority of their tests taking the obligation to fully validate all implemented tests (Jennings *et al.,* 2009). The implementation of new tests in a clinical setting is a complex process, as exemplified by a diagram on Figure 6.

When novel tests or technologies are being implemented in a laboratory and where there is no previous performance specification or reference material it is necessary to perform a full "*validation"*. In this case the test performance is measured in comparison of a reference test that is the best available method, or a combination of methods, in order to determine the status of a tested sample (Bossuyt *et al.,* 2003). Validation is performed to evaluate 1/ a technology (e.g. TaqMan technology for genotyping) or 2/ a specific test (e.g. TaqMan technology for genotyping FSHR variants). When it comes to a new technology it is crucial to perform the evaluation on a larger scale. Inter-laboratory studies or exchanges have higher chance to find deviations in the performance of a method.

When a previous performance specification of a method is available, it is required that this method meets specifications within the laboratory where is going to be implemented. This simplified process is called "*verification*" and it is a confirmation that the method is performed correctly. Verification is suitable for the implementation of a new test using an established method in the laboratory, a test where the performance specification has been done by another laboratory or for CE-marked IVD-compliant kits. In the case of a performance specification done by another laboratory it is prerequisite to collect as much information as possible. Furthermore, it is important to highlight that any modification of a CE-marked kit must be duly validated (Camajova *et al.*, 2009; Mattocks *et al.,* 2010).

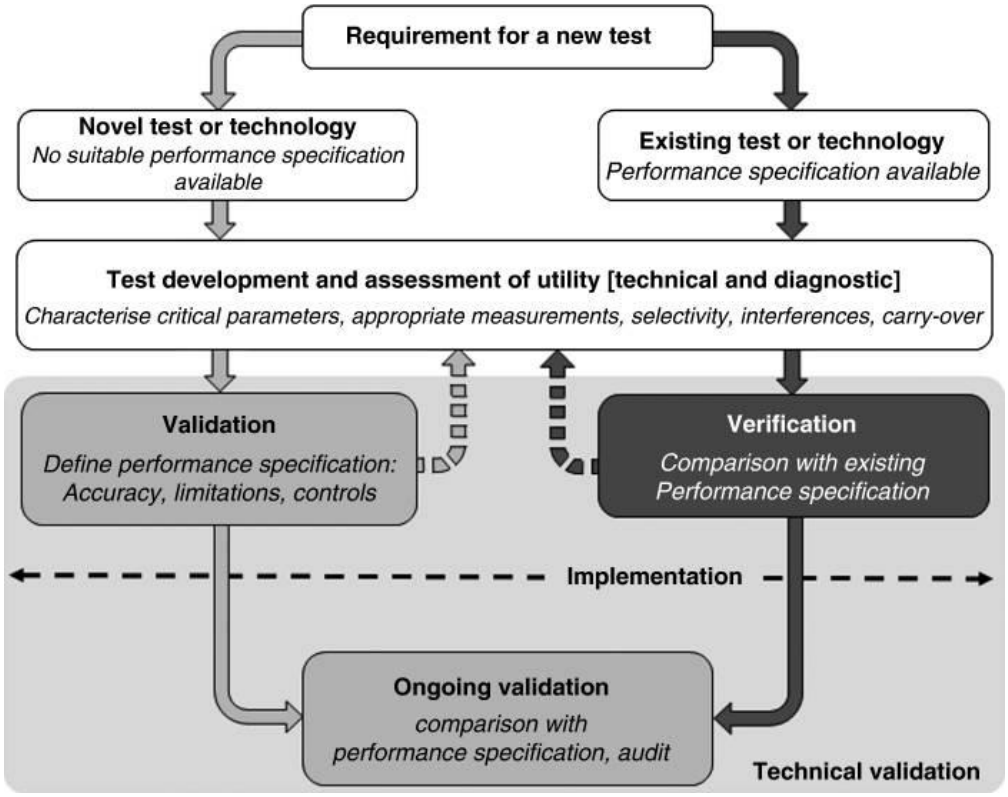

Figure 6. The process of implementing a molecular genetic test for diagnostic use. The shaded arrows represent the two general routes to implementation, depending on the availability of a suitable performance specifications: validation (lighter) and verification (darker). Broken arrows represent the situation in which validation or verification fails to meet the specified requirements (Mattocks, *et al.*, 2010).

In summary, validation determines that one is carrying out a correct test (i.e. is appropriate for its intended use), while verification confirms that we are performing this test correctly (i.e. confirmation that predetermined specifications are consistently met) (Jennings *et al.*, 2009).

1.5.3 Validation of qualitative molecular genetic tests

Molecular genetic tests are generally qualitative tests since genotyping has only two result categories: positive or negative. The assignment of results can be done by establishing a cut-off value or by simple qualitative observation of the analyst (Mattocks *et al.*, 2010). When it comes to accuracy, there are four results categories which include the "correct" or "incorrect" categorization in comparison with the reference method; this is illustrated in Table 4.

Table 4. Result categories for a qualitative validation study. *Adapted from Mattocks et al., 2010.*

	Reference method result	
Test result	+	-
+	True positive (TP)	False positive (FP)
-	False negative (FN)	True negative (TN)

Accuracy is the amount of agreement between a test result and the reference standard (Jennings *et al.*, 2009). *Diagnostic accuracy* is the ability of a test to correctly assign genotype irrespective of any clinical implication since the genotype not necessarily implies if a disease will develop (Mattocks *et al.*, 2010). In this case, accuracy is determined by correct or incorrect status of a result in comparison with the reference test. The STARD (STAndards for the Reporting of Diagnostic accuracy studies) committee defines reference standard as "the best available method for establishing the presence or absence of the condition of interest. The reference standard can be a single method, or a combination of methods, to establish the presence of the target condition. It can include laboratory test, imaging and pathology, but also dedicated clinical follow-up of participants" (Bossuyt *et al.*, 2003).

The diagnostic accuracy of a qualitative test can be determined by its specificity and sensitivity. *Sensitivity* is the probability of a positive test result in the presence of a risk allele (heterozygous and homozygous for risk allele samples) and concordant to the reference method, expressed as the ratio between true positivity (TP) and the sum of true positivity and false negativity (FN): **TP/(TP+FN)**, meanwhile *specificity* is the probability of a negative test result of the test in the absence of risk alleles (homozygous wild-type samples) and in conformity to the reference method, expressed as a ratio between true negativity (TN) and the sum of true negativity and false positivity (FP): **TN/(TN+FP)** (ICH, 1994; Prence, 1999; ACMG, 2006; Brdička *et al.*).

The *overall accuracy* can be determined by the total number of true results in comparison of a reference test represented by a proportion of total results: **(TP + TN)/(TP+FN+TN+FP)** (Mattocks *et al.*, 2010).

To exhaustively validate a new test we must determine its characteristics and limits. Additional recommended validation parameters as suggested by ISO15189 (www.iso.org), QSOP 23 (www.evaluations-standards.org.uk), the American College of Medical Genetics (ACMG) (ACMG, 2006), the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human

Use (ICH) (ICH, 1994), the Czech Clinical Biochemistry and Medical Genetics societies (Brdička *et al.*) or as published elsewhere (Prence, 1999; Jennings *et al.*, 2009) are:

- *Intra-run precision – Repeatability.* Comparison of results within a single series in parallel within a single day performed by one analyst in a test where one tends to receive consistent results from following a specific procedure.

- *Inter-run precision – Reproducibility.* Comparison of results between the series – on different days (day to day reproducibility).

- *Robustness.* Ability of a method to remain unaffected by minor deliberated modifications.

It is important to highlight that not always a method that is impeccable analytically could be implemented in a diagnostic setting. First, it is crucial to establish its clinical utility. In this matter, it is critical to have a physician consultant in every diagnostic laboratory to approve a test prior its implementation and likewise, to properly interpret its results to the patient (Jennings *et al.*, 2009; Lwoff, 2009).

2. AIMS OF THE DISSERTATION

Quality of laboratory genetic tests is of great importance for the diagnosis of inherited diseases. Therefore, below we have addressed three issues related to the application of HRM in clinical diagnostic practice: cystic fibrosis, hyperhomocysteinemia-related disorders and male infertility. We have validated and optimized the HRM technique for rapid and reliable DNA diagnostics.

1/ The first topic of this dissertation is aimed to evaluate the performance of HRM for gene scanning, focused in the detection of uncommon mutations/variations of the *CFTR* gene to confirm the diagnosis in patients clinically suspected of having CF. This evaluation belongs to the analytical validation of the *CFTR* screening test for its implementation in our laboratory.

2/ The second topic of this dissertation is the development of a new test for genotyping the most common variants of *MTHFR* for the diagnosis of inherited thrombophilia and recurrent pregnancy loss related to MTHFR deficiency. The method of choice is HRM of small amplicons due to its numerous advantages in a diagnostic laboratory setting.

With this regard, we validated High Resolution Melting (HRM) genotyping of small amplicons as is a crucial step for its use in routine diagnostics. We also elaborated the steps needed for implementation of a new technology or method in a genetic diagnostic laboratory under quality assurance standards and demonstrate the importance of method validation/verification in molecular genetic diagnostics.

3/ The last topic of this dissertation is aimed at the determination of the impact of the rs6836703: G>A variant in *ART3* on impaired spermatogenesis within the Czech male population in a case-control study using the previous validated method HRM in order to evaluate its clinical utility and establish medical indications targeted to the Czech infertile male population. Implementation of this test in reproductive medicine diagnostics and provision of evidence on its population distribution related to male infertility belongs to practical outcomes of this dissertation.

3. RESULTS AND DISCUSION OF ATTACHED PUBLICATIONS

3.1. Krenkova, et al. (2009) Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol (Praha). (IF: 1.140).

The identification and genotyping of disease-causing mutations has become important for the treatment and future management of patients carrying different genetic diseases as is the case of CF patients. The *CFTR* gene contains 27 exons and more than 1900 mutations (www.genet.sickkids.on.ca/cftr, accesed by January, 2012). In this respect, mutation detection in this type of large genes (or even larger) is an intricate, laborious and time-consuming process. HRM is a cost-effective, simple and precise technique useful for gene scanning purposes. It reduces the need for sequencing of large number of samples thereby decreasing costs and turnaround time used for a test (Provaznikova *et al.,* 2008). In our laboratory, HRM for gene scanning has been successfully implemented for pre-sequencing scanning analysis in CF patients in which common mutations have not been found.

In this study, 16 different mutations localized in 6 different exons were analyzed by HRM. All SNP classes (see Table 2) and 1-/3-base pair deletions were included. In conformity with the high mutation detection rate reported for HRM (Chou *et al.,* 2005; NGRL, 2006; Kennerson *et al.,* 2007, Montgomery *et al.,* 2007; Dobrowolski *et al.,* 2009; van der Stoep N *et al.,* 2009; Vossen *et al.,* 2009; Wittwer, 2009), all heterozygous samples from all SNP classes were successfully distinguished from wild-type samples. Furthermore, we were also able to confirm that the position of sequence variations within the fragment amplified (i.e. in the middle of the amplicon or next to primers) is not decisive for their better discrimination (Reed and Wittwer, 2004; van der Stoep N *et al.,* 2009). Thus allowed, the detection of variants regardless of their position within full-lenght amplified exons or fragments.

By using HRM we were also able to discriminate among different heterozygous samples within the same amplicon in accordance with other studies (Graham *et al.,* 2005;

Tindall *et al.,* 2009). Nevertheless, amplicon melting-based methods lack specificity necessary for genotyping to a certain degree (von Ahsen, 2005; Tindall *et al.,* 2009). Therefore, it was not possible to differentiate between p.G551D and p.R553X, and p.L1335F and p.L1335P mutations (all these transitions belong to SNP class 1). In such cases, transitions are very similar but since nearest-neighbor stability also influences melting behaviors (Graham *et al.,* 2005), a shortening on the PCR fragment surrounding the SNP area could improve the resulting resolution of the melting profiles (Norambuena *et al.,* 2009).

The p.F508del and p.I507del mutations in the exon 10 have very similar melting profiles and the peaks of the Tm from the normalized difference plots are very close to each other. This proximity may lead to future errors in differentiating these two mutations by the operator. This last issue might be overcome by adding DNA-unlabeled probes matching the "conflict" region to the PCR reaction, and thus analyze separately the melting profile of the probe area from the amplicons region (Zhou *et al.,* 2005).

Homozygous samples discrimination was not achieved in this study. This is a common disadvantage of the HRM gene scanning application. The fragment size is too long to detect such small changes within the sequence under study. By preparing 1:1 mixes with a WT sample and creating artificial heterozygous we were able to detect all homozygous samples (Montgomery *et al.,* 2007).

The rate of specificity reached in our evaluation could be improved by reducing the number of false positives samples. One approach could involve "cleaning" of DNA samples and resuspending them in a fresh common buffer with the same final concentration. Usually helps in the analysis of old samples that do not have "clean" profiles, by standardizing buffer conditions.

In this study, we have not only replicated the high precision of HRM, but also utilized a not so widely-used platform: the Rotor-Gene ™ 6000 (Corbett) compared to LigthScanner from Idaho Technologies or the LightCycler® 480 (Roche). The Rotor-Gene™ 6000 device does not use a well-plate, instead, uses a rotor with tubes. Therefore, samples reach a better uniformity in terms of temperature-based cycling.

In summary, we demonstrated that HRM for gene scanning is a useful technique for the detection of *CFTR* mutations and could be applied for the detection of unknown mutations/variations. This decreases the amount of sequencing for disease diagnostic purposes and increases diagnostic throughput.

3.2. Norambuena, et al. (2009) Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem. (IF: 1.926).

In this article, we have substantiated all the advantages of HRM. First of all, we were able to accurately genotype all homozygous samples, an advantage rendered by the reduction of amplicon size (Liew *et al.,* 2004; Grievink and Stowell, 2008; Erali *et al.,* 2008; Vossen *et al.,* 2009; Wittwer, 2009). We did not record any false calls from the software which attests that the sensitivity and specificity of this method is very high. In addition, given the high repeatability and reproducibility we could to use the same normalization settings through different days providing that the chemistry of the reaction was kept the same. In any case, the limitations due to physical and chemical variations of procedure are overcome by adding oligonucleotide calibrators thereby increasing the sensitivity of this method. With this optimized procedure, it is possible even to differentiate between homozygous samples that have identical nearest-neighbor stability predictions (Gundry *et al.,* 2008).

Following optimization of HRM, we were able to register the same calls made by the software. Due to the simplicity of this technique, there is no need of an expertise for its daily-laboratory use and it can be performed by any technician in a diagnostic laboratory.

Another advantage associated to this HRM variation is related to its cost effectiveness. Only two oligonucleotide primers, a common PCR reaction buffer and a dsDNA-saturating binding dye are used, thereby excluding the need of expensive labeled-oligonucleotide probes for genotyping. Furthermore, it uses small reaction volumes and utilization of 384 or 1536-well plates increases the number of samples which can be processed at the same time. In conclusion, as HRM is a very simple and fast method which decreases the hands-on time required. In this regard, personnel costs could be decreased.

Importantly, this article is the first study that performed a detailed method validation for diagnostic use of HRM of small amplicons in accordance to OECD and ISO

15189 guidelines (OECD guidelines, 2007; www.iso.org) and as recommended elsewhere (Prence, 1999; Jennings *et al.,* 2009; Mattocks *et al.,* 2010). This study represented a validation model for other laboratories that are implementing new qualitative genetic tests, i.e. HRM of small amplicons, into diagnostic routine settings.

The accuracy of HRM relies on appropriate instrumentation, proper operator manipulation, chemistry of the reaction, DNA quality, DNA saturating dyes and analysis software (Erali *et al.,* 2008; Wittwer, 2009); features that still need improvement.

High Resolution Melting is rapidly becoming the method of choice to screen patients for pathogenic variants. The high precision and robustness, and easy use of this method make HRM suitable for a diagnostic routine laboratory (Reed *et al.,* 2007; Erali *et al.,* 2008; Vossen *et al.,* 2009; Wittwer *et al.,* 2009). Additionally, HRM as a flexible and low cost method makes it suitable for a broader use in research laboratories.

Our experience confirms that some laboratories have difficulties to implement this technique due to the lack of detailed information in the literature and/or instructions in manufacturer manuals. The most complete guide for a successful HRM is the report from the original group that established HRM (Montgomery *et al.,* 2007). However, this report does not provide details which are at the end of technical significance. For example, pipetting plays a key role to get "clean" melting profiles as we proved by varying reaction/sample volumes. Moreover, we have seen differences after altering the order of reaction/sample addition to the well (data not shown). Since we are working on a high resolution environment, a small volume change affects the recorded fluorescence signal. Also it is important not to analyze samples with late amplification (as monitored by the real-time PCR) or with fluorescence less than 60% of the average melting profiles since these samples could generate unreliable melting profiles (Zhou *et al.,* 2010). These recommendations increase the practical utility of our article, which has been cited fourteen times since published.

The high standards reached by HRM genotyping of the most common variants in the *MTHFR* gene and its successful validation makes this test appropriate for its inclusion in the diagnosis of disorders related to hyperhomocysteinemia. Specifically, in the case of

our laboratory for the diagnosis of recurrent pregnancy loss and various forms of thrombophilia.

3.3. Mattocks, et al. (2010) A standardized framework for the validation and verification of clinical molecular genetic tests. Eur J Hum Genet. (IF: 3.564).

The implementation of a new genetic test into a clinical diagnostics laboratory involves according to the ACCE framework (http://www.cdc.gov/genomics/gtesting/ACCE/index.htm) the following phases: a/ analytical validation, b/ clinical validation, c/ clinical utility and d/ evaluation of legal and ethical implications of the test (Sanderson *et al.,* 2005). Moreover, the interpretation of terminology concerning quality assurance in molecular genetic diagnostics differs among laboratories and organizations, creating confusion at the moment for the implementation of a new diagnostic test thereby increasing complexity.

Therefore, this article collates and explains the confusing terminology of clinical molecular genetic tests in order to provide practical guidance and an easier procedure to introduce molecular tests into the clinical laboratory. It focuses on analytical validation/verification, the last ones being formal requirements for laboratory accreditation as stipulated by the International Organization for Standardization through the ISO 15189 and ISO 17025 standards (www.iso.org).

The main objective of the validation process is to define the intended use of a test and identify and/or quantify the possible sources of error as well as reasons for analytic and biological variation.

The planning and design phase is decisive for the validation procedure. First of all, is crucial to define the clinical utility and medical indications for the new test. Also, it is important to notice that a "better" test is not only better in test performance characteristics but also improved in factors such as turnaround time, cost and less invasive specimen collection (Jennings *et al.,* 2009). Another critical step is to choose the adequate number of samples, replicates and number of replication tests useful for a proper method validation/verification. Results obtained in the validation study must meet the requirements of quality and reach statistical significance. Usually lower number of replicates and samples leads to imprecise and unreliable results. However, we should

recognize that statistically significant data may not be available for every item of every validation study. Moreover, there are not enough biological samples or scientist/technologist time to support such laborious data collection. It is also important to consider the economical cost of the validation procedure within specific laboratory capacities. Then, the decision of when a test is ready to be introduced for clinical diagnostics is left to medical judgment considering in-laboratory and health system possibilities and characteristics.

Another matter to be aware of during a method implementation is the enrollment in an external quality assessment testing program (EQA) or identification of an alternate form of external quality control. Hence, internal and external quality controls are essential for evaluating the reliability and accuracy of a diagnostic laboratory. In addition, satisfactory performance in external quality assessment gives assurance both to patients and referring clinicians that the diagnostic laboratory results are reliable and accurate. EQA is recognized by international standards and accreditation bodies as a true measure of the quality of a laboratory's performance. In this regard, it is important to keep documented continuous training and certificate's records of personnel who will be involved with the new method in an accredited laboratory.

Laboratory accreditation demonstrates competence, impartiality, performance capability and international acceptance. Since there is a high number of diagnostic laboratories offering a wide number of genetic tests, accreditation of a laboratory and validation/verification of used tests is the prove of high quality standards that strengthens a laboratory vis-à-vis competition.

The published recommendations in this article are of utility to each genetic diagnostics laboratory serving as a practical guide to implement a new method and maintain an accreditated laboratory with high quality standards. This continuous process assures high performance and reliable test results, crucial for patient diagnosis and management.

The author of this dissertation was part of the validation group of the Eurogentest.org consortium.

3.4. Norambuena, et al. (2012) An ADP-ribosyltransferase 3 (ART3) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from a Japanese population. Neuro Endocrinol Lett. (IF: 1.05).

In this study we have corroborated the results found by a Japanese study, where they reported an association between the rs6836703: G>A genetic variant and impairment in spermatogenesis (Okada *et al.*, 2008), in an independent population.

Since the major aim of our study was to assess the clinical utility of the rs6836703: G>A variant genotyping in reproductive DNA diagnostics, we did not used sperm counts as the primary selection criterion in our control group. We assume that normozoospermia is crucial indicator of male fertility. However, sperm counts alone do not provide direct evidence about the ability of a sperm to fecund an oocyte and result in a live birth. Other important parameter to consider are i.e. semen quality, motility, morphology and vitality of sperm (WHO, 2010). In addition, the fertility status of our controls was "clinically" proven by a birth of their child-/children. Potential for non paternity-based bias was excluded by previous family-based marker/mutation segregation studies in our genetic diagnostic laboratory (data not shown).

The inclusion/exclusion criteria of our infertile male cohort were in accordance with previous studies: patients with known clinical or genetic causes of infertility were excluded (Hucklenbroich *et al.*, 2005; Wu *et al.*, 2007; Yang *et al.*, 2008). Sub-stratification of sub-/infertile males according to their sperm counts widely differs in association reports, to exclude any potential ascertainment bias we used the established classification by the World Health Organization (WHO, 2010).

Based on our previous studies, HRM of small amplicons was our method of choice for rs6836703: G>A SNP genotyping because of its multiple advantages, as discussed earlier (section 3.2).

Call assignments by the software were made in almost every sample in the first run. Control samples for each genotype (G/G, G/A and A/A) were run in duplicate while

tested samples were run only once due to the high accuracy of the method reported since there is no need to run additional replicates (Liew *et al.,* 2004; Grievink and Stowell, 2008; Erali *et al.,* 2008; Norambuena *et al.,* 2009; Vossen *et al.,* 2009; Wittwer, 2009). In samples where we were not able to perform assignments at the first instance, they were repeated only once when reaching "clean" melting profiles. We presume that the cause of previous failure was due to pipetting inaccuracies (Norambuena *et al.,* 2009).

We determined that the allele "A" was increased in sub-/infertile patients compared to the fertile cohort (p=0.007). This was reflected by the increase in the genotype distribution for G/A and A/A genotype frequencies (p = 0.008). Reduction of the allele "G" in cases suggests a "protective" effect of this allele on the spermatogenesis process. Furthermore, the low frequency of the A/A genotype in European population (HapMap-CEU: G/G=0.750; G/A=0.250; A/A=0.0; dbSNP accesed by 01-10-2012), further supports the impeding effect of the allele "A" in the spermatogenesis process and possible negative evolutionary consequences.

Nevertheless, in contrast with the first study to describe an association between rs6836703: G>A and male infertility (Okada *et al.,* 2008), our results differ from the Japanese study since we found an association particularly with oligozoospermic men (p = 0.002) and azoospermic patients showed no differences in allele and genotype distribution in comparison with fertile men cohort (p = 1 and p=0.939, respectively). These data suggest that the *ART3* variant: rs6836703: G>A likely cause a milder effect in spermatogenesis not disrupting male fertility within Czech men as seen in the Japanese population. Unfortunately, due to a different study design we do not count with more information of the status of this association in oligozoospermic Japanese men since they were not included in the case cohort under investigation.

This last feature reflects once again the importance of proper study design, consideration of the ethnic background and environmental or epigenetic factors in the determination of the penetrance of a genetic variation related to a specific phenotype (Tuttelmann *et al.,* 2007 and Aston and Carrell, 2009). This trait has been seen in different association studies, e.g. the partial deletion "gr/gr" of the AZFc region has been found in

association with male infertility in several studies in particular populations (Repping *et al.,* 2003; Ferlin *et al.,* 2005; Giachini *et al.,* 2005; de Llanos *et al.,* 2005; Lynch *et al.,* 2005; Navarro-Costa *et al.,* 2007; Giachini *et al.,* 2008; Yang *et al.,* 2008); while in many others, no association has been found (Machev *et al.,* 2004; Hucklenbroich *et al.,* 2005; Carvalho *et al.,* 2006; de Carvalho *et al.,* 2006; Fernando *et al.,* 2006; Ravel *et al.,* 2006; Zhang *et al.,* 2006; Imken *et al.,* 2007; Lardone *et al.,* 2007; Lin *et al.,* 2007; Wu *et al.,* 2007; Stouffs *et al.,* 2008). Another example comprises CAG repeats in the androgen receptor gene where an association between a moderate expansion in the CAG repeats and reduced spermatogenesis has been found in Asian populations, while in European studies this association has not been replicated (Ferlin *et al.,* 2006; Rajender *et al.,* 2007).

Therefore, evaluation of the impact of any genetic variant in a particular disease should be focused on the population where a new diagnostic test is planned to be implemented. Moreover, it is crucial to establish clinical utility of a test before its inclusion into diagnostic routine.

Since rs6836703: G>A variant is a risk factor for oligozoospermia in Czech male population, genetic diagnostics for this variation could be implemented as a routine testing in male infertility. However, it is still needed to determine the exact protein function and role of ART3 in spermatogenesis. The effect of allele "A" is likely related to the alteration of the expression/regulation of ART3. More studies in genetically related populations i.e. German, Hungarian (Lao *et al.,* 2008) are required to confirm our results. The finding of positive associations in unrelated populations is of importance to further support the role of *ART3* rs6836703: G>A in spermatogenesis and utilization of this test in diagnostic practice.

4. CONCLUSIONS

1/ We have substantiated advantages of gene scanning by HRM for its use in clinical DNA diagnostics. *CFTR* screening by HRM is of high utility in DNA diagnostics of cystic fibrosis as it has a very high rate of sequence variation detection. Moreover, HRM is a rapid, economical and simple technology. However, this form of HRM is not applicable for genotyping of CF-causing mutations as it lacks the required level of precision.

2/ Laboratory accreditation is a crucial process which assures patients and the public accuracy and quality of test results. Validation/verification of genetic tests is a critical phase in the implementation of a new test or technology in accordance to quality assurance purposes. In this dissertation, we have successfully validated High Resolution Melting (HRM) genotyping of small amplicons for the most common *MTHFR* variants for its inclusion into diagnostics of disorders associated to hyperhomocysteinemia due to MTHFR deficiency. This HRM validation study could be used as a model for diagnostic validations of other qualitative genetic techniques in routine DNA diagnostics.

3/ Since have demonstrated the utility and accuracy of HRM, we used this method to determine whether novel variants are risk factors for different diseases. We applied our experiences in the field of reproductive genetics. In this regard, we have found a significant association between rs6836703: G>A variant and a reduction in sperm counts within Czech male population, this was the first replication study different from the Japanese population suggesting the role of rs6836703: G>A in male infertility. Our results suggest that the "A" allele of the rs6836703: G>A variant in *ART3* is a risk factor for oligozoospermia and thus genetic diagnostics for this variation could eventually be applied. More studies are needed to corroborate these results in independent populations prior to its potential inclusion into routine clinical diagnostics in reproductive medicine.

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6. LIST OF PUBLICATIONS, POSTERS, TRAININGS AND PRESENTATIONS

6.1 Publications related to the topic of Ph.D. project

1. Norambuena PA, Diblík J, Křenková P, Paulasová P, Macek M Sr, .Macek M Jr (2012) ADP-ribosyltransferase 3 (ART3) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from a Japanese population. Neuro Endocrinol Lett 33(1): 48-52.

2. Mattocks CJ, Morris MA, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, Müller CR, Pratt V, Wallace A; EuroGentest Validation Group (2010) A standardized framework for the validation and verification of clinical molecular genetic tests. European Journal of Human Genetics 18(12):1276-1288.

   EuroGentest Validation Group: Zoccoli M, Camajova J, Krenková P, Norambuena P, Stambergova A, Macek M, Moix I, Bossuyt PM, Voorhoeve E, Bakker B, Berwouts S, Janssens T, Salden I, McDevitt T, Barton D, Amos-Wilson J, Mann I and Scheffer H.

3. Norambuena PA, Copeland JA, Krenková P, Stambergová A, Macek M Jr. (2009) Diagnostic method validation: High resolution melting (HRM) of small amplicons genotypingfor the most common variants in the MTHFR gene. Clinical Biochemistry 42(12):1308-1316.

4. Krenková P, Norambuena P, Stambergová A, Macek M Jr. (2009) Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biologica (Praha) 55(6):238-242.

6.2 Other publications

1. Colombres M, Garate JA, Lagos CF, Araya-Secchi R, Norambuena P, Quiroz S, Larrondo L, Pérez-Acle T and Eyzaguirre J. (2007) An eleven amino acid residue deletion expands

the substrate specificity of acetyl xylan esterase II (AXE II) from Penicillium purpurogenum. Journal of Computer-Aided Molecular Design 22 (1): 19-28.

2. Balascakova M, Piskackova T, Holubova A, Rausova E, Kazarova V, Krebsova A, Koudova M, Stambergova A, Camajova J, Norambuena P, Krenkova P, Votava F, Skalicka V, Vavrova V, Macek M st, Macek M ml. (2008) Současné metodické postupy a přehled preimplantační, prenatální a postnatální DNA diagnostiky cystické fibrózy v České republice. Časopis České a Slovenské Pediatrické Společnosti 63 (2): 62-75.

3. Macek M ml, Čamajová J, Křenková P, Norambuena P, Goetz P, Macek M st, Havlovicová M, Štambergová A. (2007) EuroGentest: evropský projekt zaměřený na harmonizaci a zvýšení úrovně genetických služeb. Časopis České a Slovenské Pediatrické Společnosti 62 (7-8): 444-446.

6.3 Oral Presentations

1. Norambuena P, Křenková P, Štambergová A, Macek M Jr., *Macek M Sr (speaker).* ADP-ribosyltransferase 3 (ART3) variant is associated with idiopathic oligospermia. 10. Česko-slovenská konference reprodukční gynekologie a 21. Sympózium asistované reprodukce. Brno, Czech Republic. November 2011.

2. Norambuena P, Piskáčková T, Balaščaková M, Koudová M, Štambergová A, Gromoll J and Macek M Sr. "AZFc region partial deletions on the Y chromosome in Czech fertile men". 3rd. Czech and International Congress of Andrology. Prague, Czech Republic. June 2008.

3. Norambuena P and Krenkova P. "Our experiences with HRM method evaluation for gene scanning and small amplicon genotyping". Seminar - "Roche Your Partner in Discovery". Prague, Czech Republic. June 2008.

4. Norambuena P, Copeland J, Krenkova P, Nestorovic A, Stambergova A and Macek M Jr. Genotyping of MTHFR 677 C>T and 1298 A>C polymorphisms by High Resolution Melting of Small Amplicons. 11th National DNA diagnostics Conference, Prague, Czech Republic. December 2007.

5. Štambergová A, Křenková P, Norambuena P and Macek M Jr. Zkušeností s analyzátorem křivek tání s vysokým rozlišením (HRMCA) pro detekci mutací/SNP genu CFTR a BRCA1. "HRM – High Resolution Melting" Workshop. Genetica. Prague, Czech Republic. May 2007.

6. Štambergová A, Křenková P, Norambuena P. První zkušenosti s analyzátorem křivek tání s vysokým rozlišením (HRMCA) pro detekci mutací/SNP CFTR a BRCA 1 genu. "Mutation Detection" Workshop and laboratory demonstration for LightScanner use. Organized by KRD. Prague, Czech Republic. February 2007.

6.4 Poster presentations

1. Norambuena P, Krenkova P, Stambergova A, Macek M Jr., Macek M Sr. "PARP-3 polymorphism of an ADP-ribosyltransferase 3 (ART3) is associated with reduced sperm count in Czech dysfertile men". The 12th International Congress of Human Genetics and the American Society of Human Genetics 61st Annual Meeting. Montreal, Canada. October, 2011.

2. Norambuena PA, Krenková P, Stambergová A, Macek M Sr and Macek M Jr. "An ADP-ribosyltransferase 3 (ART3) polymorphism is associated with reduced sperm count in Czech men". Scientific Conference of 2nd Faculty of Medicine, Charles University. April, 2011.

3. Macek M Jr, Křenková P, Norambuena P and Štambergová A. "Evaluation of high-resolution melting (HRM) for mutation scanning of the CFTR gene". 2008 North American CF Conference (NACFC). Orlando, Florida, USA. October 2008.

4. Křenková P, Štambergová A, Norambuena P and Macek M Jr. "Mutation scanning of the CFTR gene by high resolution melting analysis (HRM)". 31th European Cystic Fibrosis Conference. Prague, Czech Republic. June 2008.

5. Norambuena P, Copeland J, Křenková P, Macek M Jr and Štambergová A. "Genotyping of MTHFR 677C>T and 1298 A>C Variants by High Resolution Melting of Small Amplicons: an Example of Method Validation". European Society of Human Genetics Conference 2008. Barcelona, Spain. May-June 2008.

6. van der Stoep N, van Paridon CDM, Stambergova A, Norambuena P, Macek M Jr, Janssens T, Matthijs G and Bakker E. "Interlaboratory validation study of High Resolution Melting Curve Analysis for mutation scanning of BRCA1 using the LightScanner (IT)". European Society of Human Genetics Conference 2008. Barcelona, Spain. May-June 2008.

7. Macek M Sr, Kluckova H, Norambuena P, Piskackova T, Balascakova M, Koudova M, Stambergova A, Macek M Jr and Gromoll J. "N680S and -29 (G>A) FSH-R polymorphisms in Czech fertile men and female population". European Society of Human Genetics Conference 2008. Barcelona, Spain. May-June 2008.

8. Balaščaková M, Křenková P, Norambuena P, Fialová M, Štambergová A, Macek M Jr. "Selekční výhoda nosičství mutace F508del ve vztahu k laktózové intolerancí". Vědecká Konference 2008, 2. Lékařská Fakulta, Univezity Karlovy. Prague, Czech Republic. April 2008.

9. Norambuena P, Gromoll J, Müllerová M, Štambergová A,Vilímová Š, and Macek M Sr. "CAG Repeats Length in Androgen Receptor within Czech Fertile Male and Female Population". 6th Czech-Slovak Conference in Reproductive Gynecologie and 17th Simposium in Assisted Reproduction. Brno, Czech Republic, November 2007.

10. Norambuena P, Gromoll J, Müllerová M, Štambergová A, Vilímová Š, and Macek M Sr. Androgen Receptor CAG repeats in infertile Czech men. SLG Conference, Prague, Czech Republic, September 2007.

6.4 Participation in training courses

- Course in GeneMapper program, Applied Biosystems. Prague, Czech Republic. April 23, 2008.

- AutoGen Flex Star – Automated DNA Extraction. Eric Soter. Prague, Czech Republic. January 15 -17, 2008.

- Internship for training in TaqMan assays and Fragment Analysis. Institute of Reproductive Medicine, University of Muenster, Muenster, Germany. May 14 – June 8, 2007. At mentor Prof. Joerg Gromoll.

- 20th Medical Genetics Course. European School of Genetic Medicine. European Genetics Foundation. Bertinoro di Romagna, Italy. May 5-11, 2007.
- "Mutation Detection" workshop and laboratory demonstration for LightScanner (Idaho Technologies) use. Organized by KRD. Prague, Czech Republic, February 2006.

7. PUBLICATION ATTACHMENTS

Short Communication

Evaluation of High-Resolution Melting (HRM) for Mutation Scanning of Selected Exons of the *CFTR* Gene

(*CFTR* / high-resolution melting / HRM / mutation detection)

P. KŘENKOVÁ, P. NORAMBUENA, A. ŠTAMBERGOVÁ, M. MACEK JR.

Charles University in Prague, 2nd Faculty of Medicine and University Hospital Motol, Department of Biology and Medical Genetics, Cystic Fibrosis Centre, Prague, Czech Republic

Abstract. Hereby we present evaluation of high-resolution melting for mutation scanning applied to the cystic fibrosis transmembrane conductance regulator gene. High resolution melting was used for mutation scanning of selected samples derived from cystic fibrosis patients with a known cystic fibrosis transmembrane conductance regulator genotype. We tested 19 different disease-causing cystic fibrosis transmembrane conductance regulator mutant genotypes located within six exons of the cystic fibrosis transmembrane conductance regulator gene (4, 7, 10, 11, 14b and 22). Normalized melting curves of tested samples were compared to sequenced-verified wild-type samples. Determined mutations are as follows: p.F508del, p.I507del, p.G551D, p.R347P, c.1717-1G>A, c.621+1G>T, p.Y122X, p.I336K, p.R553X, c.2789+5G>A, c.574delA, c.1811+1G>C, p.L1335F, p.L1335P, p.L1324P and p.M470V and represent minimally 76.5 % of all cystic fibrosis alleles detected in the Czech cystic fibrosis population. All analysed samples with mutant genotypes were unambiguously distinguished from wild-type samples. High-resolution melting analysis enabled reliable detection of all single-nucleotide polymorphism classes and 1- or 3-base pair deletions. We examined the specificity, sensitivity and precision of this methodology. High-resolution melting analysis is an economical, sensitive and specific close-tube method and has a high utility for the detection of unknown mutations in cystic fibrosis DNA diagnostics.

Introduction

To date, more than 1,600 mutations have been identified in the cystic fibrosis transmembrane conductance regulator (*CFTR)* gene (http://www.genet.sickkids.on.ca/cftr). The *CFTR* gene consists of 27 exons (Riordan et al., 1989), thus making the detection of non-common mutations by sequencing laborious, expensive and time-consuming. To simplify the analysis of such a broad mutation spectrum, a rapid and reliable method is required. There are many available scanning methods, such as single-strand conformation polymorphism analysis (SSCP) (Orita et al., 1989), denaturing gradient gel electrophoresis (DGGE) (Lerman and Silverstein, 1987), temperature gradient capillary electrophoresis (TGCE) (Li et al., 2002), denaturing high-performance liquid chromatography (dHPLC) (Xiao and Oefner, 2001) or heteroduplex analysis (HA) (Highsmith et al., 1999) which are time-consuming and the sensitivity often depends on the experience of the operator. On the other hand, HRM presents a rapid, high-throughput, closed-tube method for mutation scanning and genotyping (Wittwer et al., 2003). The sample preparation consists of a standard PCR reaction with a dsDNA intercalation fluorescent dye and does not require any post-PCR handling. Products can be analysed directly after PCR amplification using specially designed instruments for high-resolution melting (HRM) analysis. The homozygous, heterozygous and wild-type samples are differentiated according to their melting profile, which is represented by plotting fluorescence over the temperature range. The heterozygous genotype is distinguished from a wild-type sample by different melting temperatures (T_m) and the shape of the melting curve, whereas homozygous genotypes are distinguished only by a change in T_m.

Received October 7, 2008. Accepted May 6, 2009.

This study was supported by VZFNM00064203, EuroGentest (FP6-512145) and NR-9448-3/2007.

Corresponding author: Petra Křenková, Charles University in Prague, 2nd Faculty of Medicine and University Hospital Motol, Department of Biology and Medical Genetics, Cystic Fibrosis Centre, V Úvalu 84, 150 06 Prague 5, Czech Republic. Phone: (+420) 224 433 548; Fax: (+420) 224 433 520; e-mail: petra.krenkova@email.cz

Abbreviations: CF – cystic fibrosis, CFTR – cystic fibrosis transmembrane conductance regulator, DGGE – denaturing gradient gel electrophoresis, dHPLC – denaturing high-performance liquid chromatography, HA – heteroduplex analysis, HRM – high-resolution melting, PCR – polymerase chain reaction, SSCP – single-strand conformation polymorphism analysis, SNP – single nucleotide polymorphism, TGCE – temperature gradient capillary electrophoresis, T_m – melting temperature.

Many publications have documented the successful use of HRM for mutation scanning and genotyping (Wittwer et al., 2003; Liew et al., 2004; Reed and Wittwer, 2004; Zhou et al., 2004, 2005; Chou et al., 2005; Graham et al., 2005; Krypuy et al., 2006, 2007; Montgomery et al., 2007; Audrezet et al., 2008, Nguyem-Dumont et al., 2009). HRM is a mutation detection and scanning technique that has high reliability. It has been reported to have near 100% sensitivity and specificity when the analysed PCR products were up to 400 bp in length (White and Potts, 2006).

To evaluate the reliability of HRM in our laboratory, we tested the specificity, sensitivity and repeatability in detecting 16 representative mutations (19 genotypes) within six exons of the *CFTR* gene. In comparison to previously published studies we focused on the utilization of Rotor-Gene™ 6000. This instrument has a specially tuned high-intensity optical channel and extreme thermal resolution (± 0.02 °C). Due to the unique rotary design there is the highest thermal uniformity between samples (± 0.01 °C), hence there is no need for temperature shifting. Compared to most block-based systems the light in our case is highly focused, while in the block-based systems it is most intense in its centre and becomes dispersed on the edges. This leads to optical variability and non-uniformity within the analysed plate. We also used the IdahoTechnology dye, which provides highest sensitivity compared to all subsequent proprietary dyes from other companies. All these properties make this technical platform unique and highly suitable for the diagnostic setting.

Material and Methods

DNA samples

We selected clinical samples derived from CF patients of our Institute with known genotypes previously determined by sequencing analysis. Genomic DNA was extracted from leukocytes of peripheral blood using a commercial PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions or by the "salting out" procedure. All DNA samples were diluted in the same buffer PUREGENE DNA Hydration Solution (Gentra Systems) at the concentration 15 ng/µl.

Polymerase chain reaction conditions

Human genomic DNA was amplified by polymerase chain reaction (PCR) using previously published primers (Macek et al., 1997) in the presence of the intercalating fluorescent dye LCGreen plus (Idaho Technology, Salt Lake City, UT). Amplification efficiency was monitored using real-time PCR. PCR reactions were performed in 10 µl reaction volume which consisted of 1× PCR buffer including 2 mM $MgCl_2$ (final concentration), 0.3 µM of each primer, 1 mM of dNTPs, 1× LCGreen plus, 1 U of FastStart Taq DNA Polymerase (Roche Diagnostics, Indianapolis, IN) and 30 ng of genomic DNA. To determine the precision, all PCR reactions were performed five times.

PCR cycling and HRM analysis were performed in Rotor-Gene™ 6000 (Corbett Life Science, Qiagen, San Francisco, CA). The amplicons were run according to the following conditions: one cycle of initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 s, annealing for 30 s, 72 °C for 40 s and one cycle of final extension at 72 °C for 2 min. The annealing temperature was 62 °C for amplicons 10, 11, 14b and 22, 67 °C for amplicon 4 and 58 °C for amplicon 7. Following amplification, PCR products were denatured at 95 °C for 1 min and rapidly cooled to 25 °C for 1 min to form heteroduplexes.

Melting conditions

Melting analysis was performed immediately after PCR in the same instrument. The fluorescence signal was acquired from 65 °C to 95 °C at a ramp rate 0.1 °C/s. Melting data were visualized and analysed using Rotor-Gene™ 6000 Series Software 1.7 (Corbett Life Science). Melting curves of examined samples were normalized and the difference temperature graphs were compared against wild-type control samples.

Results and Discussion

We scanned 19 different mutant genotypes (Table 1) located within six exons of the *CFTR* gene (4, 7, 10, 11, 14b and 22). Analysed mutations included all SNP classes (which divided SNPs into four groups according to the intensity of T_m change) and 1- or 3-base pair deletions and represent at least 76.5 % of all CF alleles detected in the Czech Republic.

Evaluated amplicons varied in size from 101 bp to 380 bp and had a GC content ranging from 33.7 % to 45.9 %. The tested samples were evaluated by initial visual inspection of melting curves and by software analysis. All examined samples with mutant heterozygous genotype were unambiguously distinguished from wild-type samples by a different shape of the melting curves (Figs 1A, 1B, 1C, 1D, 1E, 1F, 1G and 1H). Heterozygous profiles of p.G551D versus p.R553X and p.L1335F versus p.L1335P were not distinguishable (Figs. 1A and 1B).

A sample with homozygous genotype both for p.F508del and p.M470V mutations showed a similar melting pattern as the wild-type control samples and the T_m shift not easily distinguished (Fig. 1C). The presence of the homozygous mutations was confirmed by mixing (1 : 1) the PCR product with the wild-type PCR product, denaturing and reanalysing the melting. In that way, heterozygosity of both mutations was established and the detection of the homozygous mutations by melting analysis was obvious – the melting pattern was similar as the original sample with heterozygous genotype p.M470V/p.F508del (Fig. 1D).

We scanned nine samples for exon 4 (three heterozygous, six wild-type controls), eight samples for exon 7 (four heterozygous, four wild-type controls), nine samples for exon 10 (four heterozygous, one homozygous

Table 1. CFTR mutations analysed in the study

Exon	Genotype	Nucleotide Change	SNP class*
4	c.621+1G>T	G>T	2
	p.Y122X	T>A	4
	c.574delA	del	--
7	p.R347P	G>C	3
	p.I336K	T>A	4
10	p.F508del/p.M470V/p.M470V	del/A>G/A>G	1
	p.F508del/p.F508del/p.M470V/p.M470V	del/del/A>G/A>G	1
	p.F508del/p.M470V	del/A>G	1
	p.M470V	A>G	1
	p.I507del	del	--
11	c.1717-1G>A	G>A	1
	c.1811+1G>C	G>C	3
	c.1717-1G>A / c.1811+1G>C	G>A / G>C	1/3
	p.G551D	G>A	1
	p.R553X	C>T	1
14b	c.2789+5G>A	G>A	1
22	p.L1335F	C>T	1
	p.L1335P	T>C	1
	p.L1324P	T>C	1

* (Venter et al., 2001)
SNP class 1 represents C/T and G/A base changes with typical T_m melting curve shift larger than 0.5 °C. SNP class 2 represents C/A and G/T base changes, class 3 C/G base change with T_m melting curve shift between 0.5–0.2 °C. SNP class 4 comprises A/T base change, which caused T_m melting curve shift smaller than 0.2 °C.

and four wild-type controls), twelve samples for exon 11 (seven heterozygous, five wild-type controls), seven samples for exon 14b (two heterozygous, five wild-type controls) and seven samples for exon 22 (three heterozygous, four wild-type controls). To determine the precision, each sample was prepared five times. In total, we analysed 120 wild-type melting curves and 140 mutant melting curves with 100% sensitivity and 96% specificity.

Currently, there is discussion whether mutation scanning techniques still have a role in DNA diagnostics, since many argue that lowering costs for sequencing render these unnecessary. However, even in the case of sequencing, false positivity/negativity could occur if one does not sequence both strands. Usually, in a routine diagnostic setting, laboratories only sequence one strand – an approach associated with higher risk of error. We have confirmed this contentious issue (currently in press) within our participation in the Eurogentest (www.eurogentest.org) consortium. Moreover, next-generation sequencing techniques still do not have the desirable sensitivity and specificity, since enrichment strategies are not yet optimized for DNA diagnostics (Hert et al., 2008; Voelkerding et al., 2009).

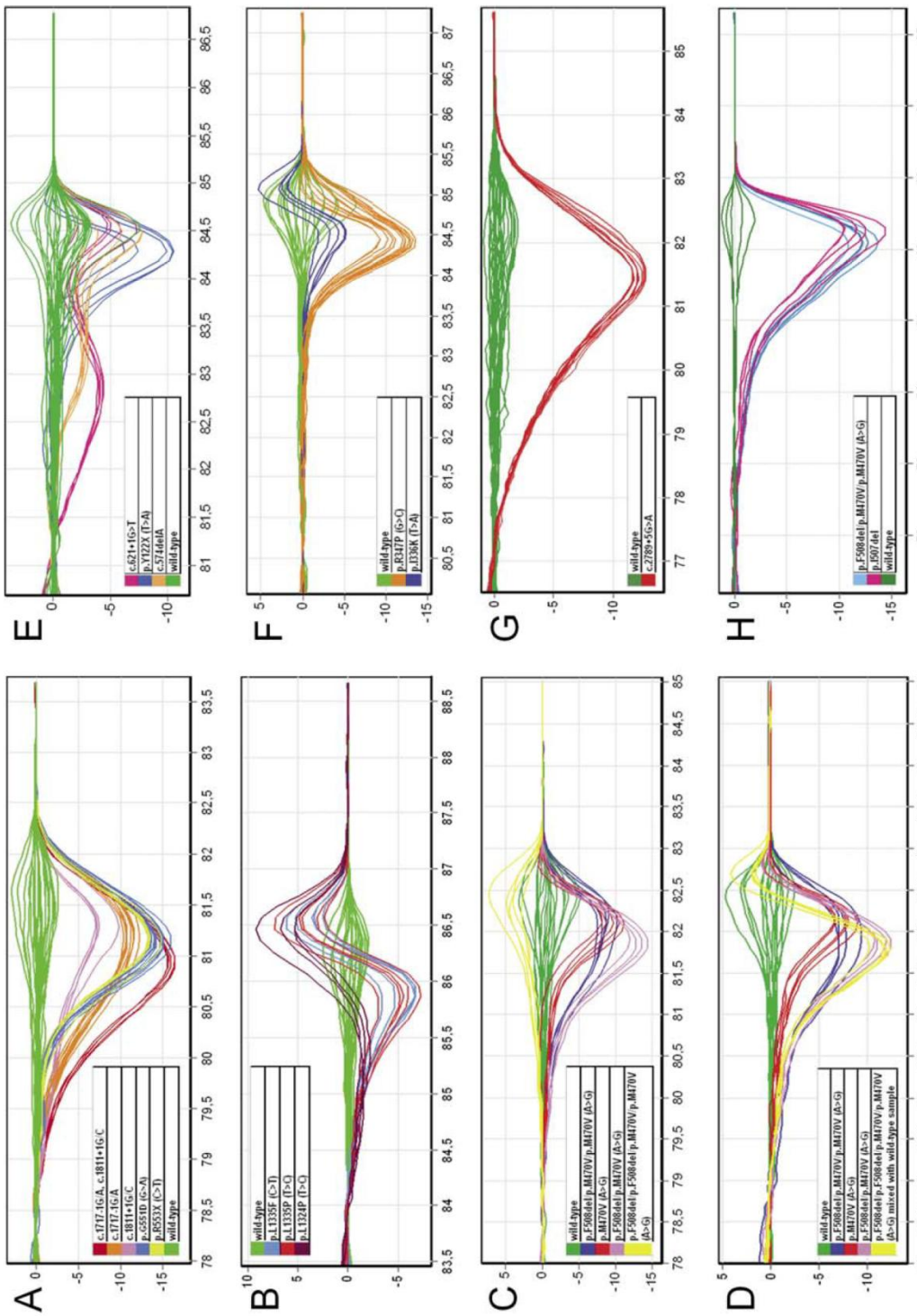
Therefore, there is still space for mutation pre-scanning prior to sequencing the "positives", as we have proved in the case of HRM. In this respect, we have studied a particular technical variant of HRM, based on the RotorGene™ 6000 device and the original HRM dye developed by IdahoTechnology. In this way we accounted for previously described disadvantages of alternative HRM platforms.

In our hands HRM allowed easy detection of all SNP classes as well as 1- and 3-base pair deletions. Heterozygous mutations belonging to the first SNP class cause the biggest change of T_m and should thus be the easiest mutations to detect. We detected this group very clearly, and we were also able to simply identify the most difficult fourth SNP class (Figs. 1E and 1F). The detection of homozygous mutations is complicated since generally the T_m difference is not high enough to allow proper discrimination. By generating "artificial" heterozygous samples from the 1 : 1 mixture, we were able to accurately detect homozygous sample.

All analysed samples with heterozygous mutant genotypes were unambiguously distinguished from wild-type samples. This method exhibits very high specificity and sensitivity, making it suitable for its use as a pre-screening method in diagnostics.

In some cases false positives appeared, hence we reached an almost 96 % of specificity. This could have been caused by analysing various old DNA samples, which were isolated by different techniques. Various DNA storage solution buffers can affect the melting behaviour in HRM, leading to broader dispersal of melting profiles, and thus could have contributed to false-positive "calls" by the software. It is also possible that the observed false-positive rates could be lowered following further optimization. Nevertheless, in diagnostic setting there is usually no time to perform such optimization, since it is expected that the technique should provide uniform results.

Figs. 1A–1H demonstrate the repeatability of the method; the melting curves of each sample were prepared in one day, by one analyst in the same instrument. Excellent repeatability is demonstrated by the overlapping curves of multiple samples for both the wild-type and the mutant samples.



Figs 1A-1H. Normalized difference graphs. Each genotype was melted and displayed in five replicates. A – exon 11. B – exon 22. C – exon 10. D – exon 10; homozygous sample (yellow colour) was mixed 1 : 1 with wild-type PCR product, denatured and reanalysed. E – exon 4. F – exon 7. G – exon 14b. H – exon 10.

In conclusion, HRM analysis is an economical, sensitive and specific close-tube method that can dramatically reduce the need for sequencing. Consequently, it has a high utility for the detection of unknown mutations in CF DNA diagnostics. The only caveat, which pertains to all other PCR-based techniques, is the quality of template DNA to which one needs to pay extra attention. We hope that our experience could be applicable to other HRM diagnostic applications.

Acknowledgements

We thank Andrea Tesoriero, Thomas Kaiser and Pavel Votýpka for their assistance with the manuscript.

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7.2. Norambuena, et al. (2009)

Available online at www.sciencedirect.com
ScienceDirect
Clinical Biochemistry 42 (2009) 1308–1316
CLINICAL BIOCHEMISTRY

Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the *MTHFR* gene

Patricia A. Norambuena, Joshua A. Copeland, Petra Křenková, Alexandra Štambergová, Milan Macek Jr.*

Department of Biology and Medical Genetics, Charles University 2nd Medical School and University Hospital Motol, V Úvalu 84, 150 06 Praha 5, Czech Republic

Received 15 December 2008; received in revised form 29 March 2009; accepted 24 April 2009
Available online 8 May 2009

Abstract

Objectives: According to OECD guidelines methods implemented in a diagnostic laboratory should be properly validated prior their implementation. For this purpose we selected genotyping by High Resolution Melting (HRM) of small amplicons using common variants in *MTHFR* as a model.

Design and methods: We selected previously typed samples on which selected analytical validation-related parameters relevant to DNA diagnostics — specificity, sensitivity, precision, robustness and ability to perform reliable calls were evaluated.

Results: Correct genotype was assigned in 375/381 (98.4%) for c.677 C>T (rs1801133: C>T; p.A222 V) and in 102/104 (98.1%) for c.1298 A>C (rs1801131: A>C; p.E429A) of all cases. Low analytical failure rate and very high specificity/sensitivity were achieved. Similarly, precision and robustness were consistent.

Conclusions: We have successfully validated HRM of small amplicons using common *MTHFR* variants as a model. We proved that this technique is highly reliable for routine diagnostics and our diagnostic validation strategy can serve as a model for other applications.

Keywords: DNA diagnostics; Genotyping; High resolution melting (HRM); Diagnostic validation; Methylenetetrahydrofolate reductase gene (*MTHFR*); Organization for Economic Cooperation and Development (OECD) guidelines; Small amplicons

Introduction

Although direct DNA sequencing is considered as a "gold standard" for genotyping of known or unknown mutations, it still remains relatively expensive, laborious and time consuming. Different methods have been developed to simplify the detection of novel mutations, with the most common techniques being based on restriction enzyme analysis [1], allele/specific amplification [2], ligation based assays [3], single-base extension [4], fluorogenic ASO hybridization probes [5,6] and pyrosequencing [7].

High Resolution Melting (HRM) is a simple, rapid and low-cost mutation scanning method [8–11]. Its advantage is the fact that PCR amplification and melting curve analysis are performed within the same tube or plate, without any post-PCR processing. This feature is particularly important for a routine diagnostic setting. HRM is based on computer analysis of DNA melting transitions, whereby it is possible to record more than 25 readings per 1 °C [12], via monitoring of the change in fluorescence that results from gradual temperature-dependent release of a saturating ds-DNA binding dye [8,9].

Since HRM is based on thermodynamic differences between DNA fragments, it has been used in particular for scanning of heterozygous sequences. However, in its original form, discrimination between homozygous genotypes is more difficult, because the difference between homozygous sequence melting profiles is usually merely represented by a slight shift in the melting temperature (T_m), but not by a change of the melting curve profile [13]. Therefore, HRM has been adapted for analysis of polymorphic SNPs via PCR amplification of small amplicons. Such a reduction of the amplicon length results in a broader divergence between melting profiles and increases the sensitivity of the technique, which then could be used not only

* Corresponding author. Fax: +420 2 2443 3520.
E-mail addresses: patynorambuena@gmail.com (P.A. Norambuena), milan.macek.jr@lfmotol.cuni.cz (M. Macek Jr.).

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doi:10.1016/j.clinbiochem.2009.04.015

for scanning, but also for accurate genotyping. Moreover, differences between homozygous wild type and homozygous mutant DNA fragments are thus more apparent [14].

Due to the advantageous features of the HRM of small amplicons this technique is currently being rapidly introduced into diagnostic laboratories for genotyping of disease-associated genes. According to the "OECD guidelines for Quality Assurance in Molecular Genetic Testing" [15], there is an obligation for diagnostic laboratories to provide high quality results. Therefore, all methods implemented within a routine setting must be duly validated prior to their diagnostic use.

In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters as stipulated by ISO15189 [16], QSOP 23 [17], the American College of Medical Genetics [18], the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [19], recommendations of the Czech Clinical Biochemistry and Medical Genetics societies [20] or as published elsewhere [21]. Parameters mostly relevant for the diagnostic setting comprise sensitivity, specificity, precision (reproducibility and repeatability) and robustness [18–21].

Although reviews and reports on the use of HRM for mutation scanning and genotyping were published previously [8,10,11,22–25], there is no report on diagnostic validation of this technique as required by OECD and/or ISO guidelines [15,16]. Furthermore, we feel that there is a particular need to provide examples of proper validation strategies for genotyping of SNPs by HRM of small amplicons due to its increasing use in diagnostics [11].

Therefore, the aim of this work was diagnostic validation of small amplicon genotyping by HRM using as model the examination of common variants of the *m*ethylene*t*etra*h*ydro *f*olate *r*eductase (*MTHFR*) gene: c.677 C>T (rs1801133: C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p. E429A). These variants are associated with a measurable reduction of enzymatic activity of *MTHFR*, in particular in the case of the c.677 C>T variant, and have been proposed to be in association with several disorders related to impaired folate metabolism such as neural tube defects, infertility, thrombosis or some types of cancer [26–30].

We hope that our study would serve as an example for diagnostic validations of other molecular genetic techniques applied in routine practice.

Materials and methods

DNA template preparation

Since the main objective of our study was to validate HRM genotyping we have randomly selected DNA aliquots from patients examined at our Department for inherited thrombophilia prior to assisted reproduction [31]. All patients or their legal representatives signed an informed consent approving general/anonymous research use of respective specimens and this study was approved by the Internal Ethics Committee of University Hospital Motol. Genomic DNA was extracted from blood using Puregene™ "Genomic DNA Purification Kit" (Gentra Systems, MN, U.S.A; currently distributed by Qiagen, Germany) and diluted to 10 ng/μL using the "DNA Hydratation Solution" provided by the manufacturer.

All samples used in this study were previously genotyped for respective *MTHFR* variants by an alternative technique and were selected retrospectively with the aim to have enough samples for each genotype. The rs1801133: C>T variant was genotyped utilizing the RHA Kit Thrombo™ (Labo Bio-medical Products, The Netherlands) and/or by RFLP-based (Restriction Fragment Length Polymorphism) typing [32], while the rs1801131: A>C variant, only RFLP-based analysis was used [33].

Primer design and annealing temperature

Primers were designed to amplify a small fragment surrounding the polymorphisms and avoid the presence of other sequence variations in the primer region (Table 1). Primer T_m and general suitability were calculated using FastPCR software, version 4.0.27 [34]. In order to select the optimal annealing temperature (T_a) for our assay, we performed a gradient PCR within the range of 10 °C using the median temperature from the T_a range proposed by the FastPCR software as the starting point. Gradient PCR was performed using the PTC-220 thermocycler (MJ Research, MA, U.S.A.) under the PCR cycling and HRM conditions described in the next section.

After the gradient PCR reaction and for the purpose of the optimization of T_a, we initially performed HRM on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany) followed by 4% agarose gel electrophoresis in order to detect spurious bands. The T_a with an optimal melting profile and associated with no unspecific amplification products, and which could be used for amplification of both SNPs, was selected.

PCR conditions and HRM acquisition

PCR amplification for rs1801133: C>T and rs1801131: A>C variants were performed under the same conditions in a 96-well plate in the LightCycler® 480 Real-Time PCR System. Reaction volume was 10 μL; 2 μL of genomic DNA (10 ng/μL) was added to 8 μL of reaction master mix consisting of 1× LightCycler® 480 High Resolution Melting Master (containing the proprietary ds-DNA saturating binding dye), with 2.5 mM

Table 1
Primer sequences and amplicon sizes used for rs1801133: C>T and rs1801131: A>C variants HRM small amplicon genotyping.

SNP	Primer sequences	Amplicon size (bp)
rs1801133: C>T	5′-GAAGGAGAAGGTGTCTGCG	45
	5′-AGCTGCGTGATGATGAAATC	
rs1801131: A>C	5′-GGGAGGAGCTGACCAGTGAA	50
	5′-GTAAAGAACGAAGACTTCAAAGACACT	

Table 2
Settings for the analysis of *MTHFR* variant genotyping.

SNP	Pre-melting normalization (°C)	Post-melting normalization (°C)	Temperature shift	Sensitivity
rs1801133: C>T	73.0–75.0	82.0–84.0	5	Auto-group: 0.7 In-run standards: 1.0
rs1801131: A>C	70.0–72.0	79.7–81.7	5	Auto-group: 0.7 In-run standards: 1.0

Legend: "Auto-group" calculation was used for the comparison of the control sample replicates for the three genotypes when reproducibility, repeatability and robustness parameters were evaluated. "In-run standards" calculation was used to analyze blinded samples using control samples for the three genotypes analyzed as melting standards.

$MgCl_2$ (Roche Diagnostics, Germany) and 0.5 μM of forward and reverse primers. For this study only one batch of the commercial master mix was used. The PCR program started with an initial denaturation of 10 min at 95 °C, continued with 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 10 s at 72 °C. This program also allows one step for heteroduplex formation by heating to 95 °C for 30 s and cooling down to 40 °C for 1 min. For HRM, the plate was heated from 65 °C to 95 °C performing 25 acquisitions per 1 °C.

HRM analysis

Melting curve analysis was performed using the Light-Cycler® 480 Gene Scanning software version 1.2 (Roche Diagnostics, Germany). All the samples with a late amplification, as monitored by real-time PCR or associated with fluorescence of less than the 60% of the maximum, were excluded from the analysis. According to manufacturer's recommendations these could generate unreliable melting profiles. The

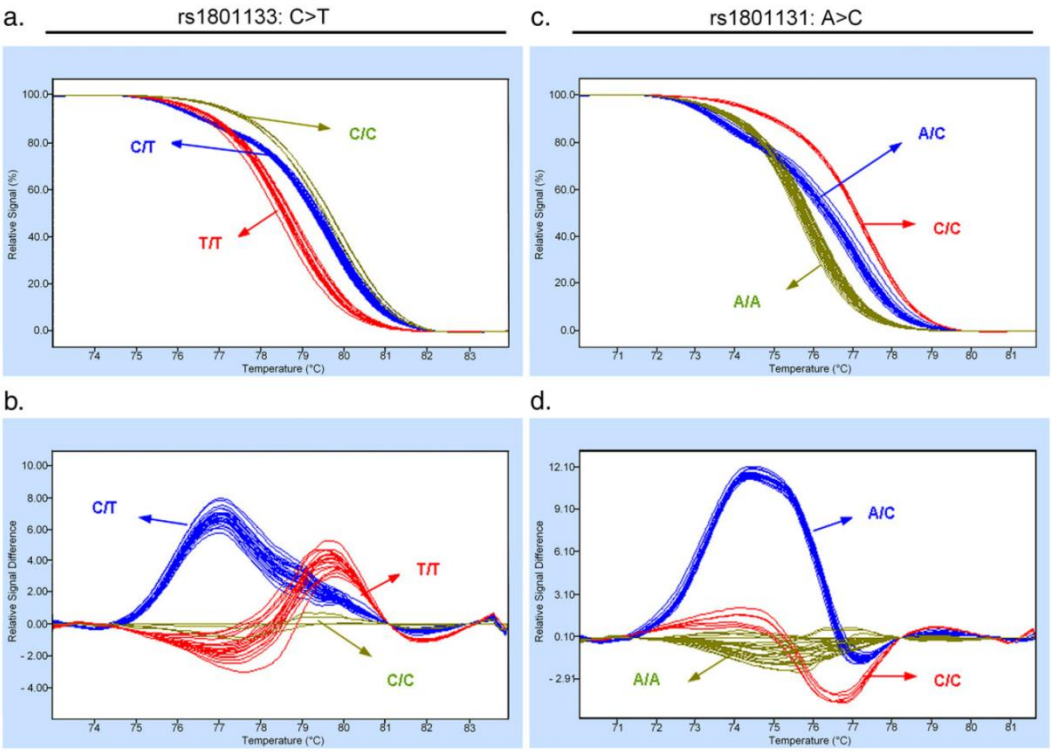

Fig. 1. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. In order to calculate sensitivity and specificity, samples were blinded. From the normalized melting curves for rs1801133: C>T and rs1801131: A>C (panels "a" and "c", respectively) it is possible to distinguish both homozygous groups by their T_m variation (C/C wild type homozygous and T/T homozygous for the variation for rs1801133: C>T and A/A wild type homozygous and C/C homozygous for the variation for rs1801131: A>C), heterozygous samples (C/T and A/C, respectively) have a different melting curve shape.

normalization settings were exactly the same for each experiment performed (Table 2).

Validation parameters

Sensitivity and specificity: sensitivity is the probability of a positive test result in the presence of a risk allele (heterozygous and homozygous for risk allele samples) expressed as the ratio between true positivity (TP) and the sum of true positivity and false negativity (FN): TP/(TP+FN), while specificity is the probability of a negative test result of the test in the absence of risk alleles (homozygous wild type samples), expressed as a ratio between true negativity (TN) and the sum of true negativity and false positivity (FP): TN/(TN+FP) [18–21]. To evaluate sensitivity and specificity we ran two replicates (i.e. same sample ran two times within the same run) of each genotype as melting control standards and one replicate of tested samples. Altogether we tested 381 samples for rs1801133: C>T and 104 samples for rs1801131: A>C, proportionate to their availability in our laboratory.

For the validation of HRM of small amplicons, these samples were organized into blinded groups. For specificity evaluation we examined 178 negative samples for rs1801133: C>T variant, while for rs1801131: A>C, 46 negative samples were analyzed. To determine sensitivity, we analyzed 203 positive samples for rs1801133: C>T variant and 58 positive samples for rs1801131: A>C.

Intra-run precision–repeatability: is the comparison of results within a single series in parallel within a single day performed by one analyst [20]. To test repeatability a single analyst ran 10 replicates of each genotype control sample within the same run.

Inter-run precision–reproducibility: is the comparison of results between the series — on different days (day to day reproducibility) [20]. For reproducibility (inter-run precision) parameter test, a single analyst prepared one sample from each genotype in triplicate, with that same analyst repeating the procedure on three different days.

Robustness: is the ability of a method to remain unaffected by minor modifications [18–21]. To evaluate robustness we ran one sample from each genotype in triplicate. The tested parameters were DNA template amount, annealing temperature, cycle number, analyst variation and pipetted volume variation. We added 10 ng, 20 ng, 50 ng and 100 ng of DNA into the reaction, respectively, for the evaluation of DNA template amount variation. Annealing temperature was modified within the range of ±1 °C. Cycle number variation was assessed adding or decreasing by two cycles in the PCR program. Three

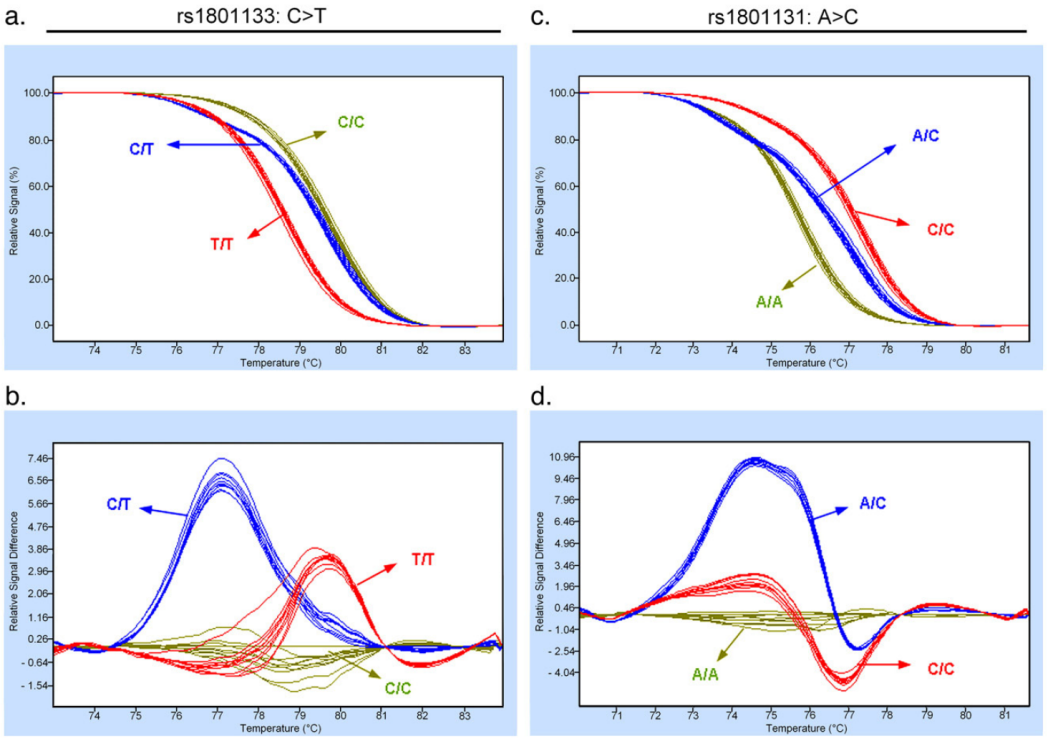

Fig. 2. Repeatability. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. The same control sample for a genotype was analyzed in 10 replicates. Panels "a" and "c" represent normalized melting curves, while panels "b" and "d" depict difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C, respectively.

different analysts repeated the same PCR and HRM procedure in order to assay analyst variation. For examining the influence of reaction master mix and DNA volume variation during pipetting, we modified respective volume in both instances by ±0.5 μL and 1.0 μL.

Results

HRM genotyping

The analytical failure rate was 6 out of 381 for rs1801133: C>T and in 102 out of 104 calls for rs1801131: A>C, while proper software-based genotype assignments ("calls") were obtained in the remaining cases. In aggregate, from the normalized melting curves and difference plots we were able to clearly distinguish three genotype melting profiles (Fig. 1). Individually, all calls corresponded to previous genotype assignments.

Sensitivity and specificity

Since we did not have any errors (no false calls) in genotyping assignments made by the software, we reached 100% sensitivity and specificity for both tested SNPs.

Intra-run precision–repeatability

All genotype replicates grouped together with regard to their melting pattern in both tested SNPs. Additionally, melting profiles were the same for each sample in all 10 replicates (Fig. 2).

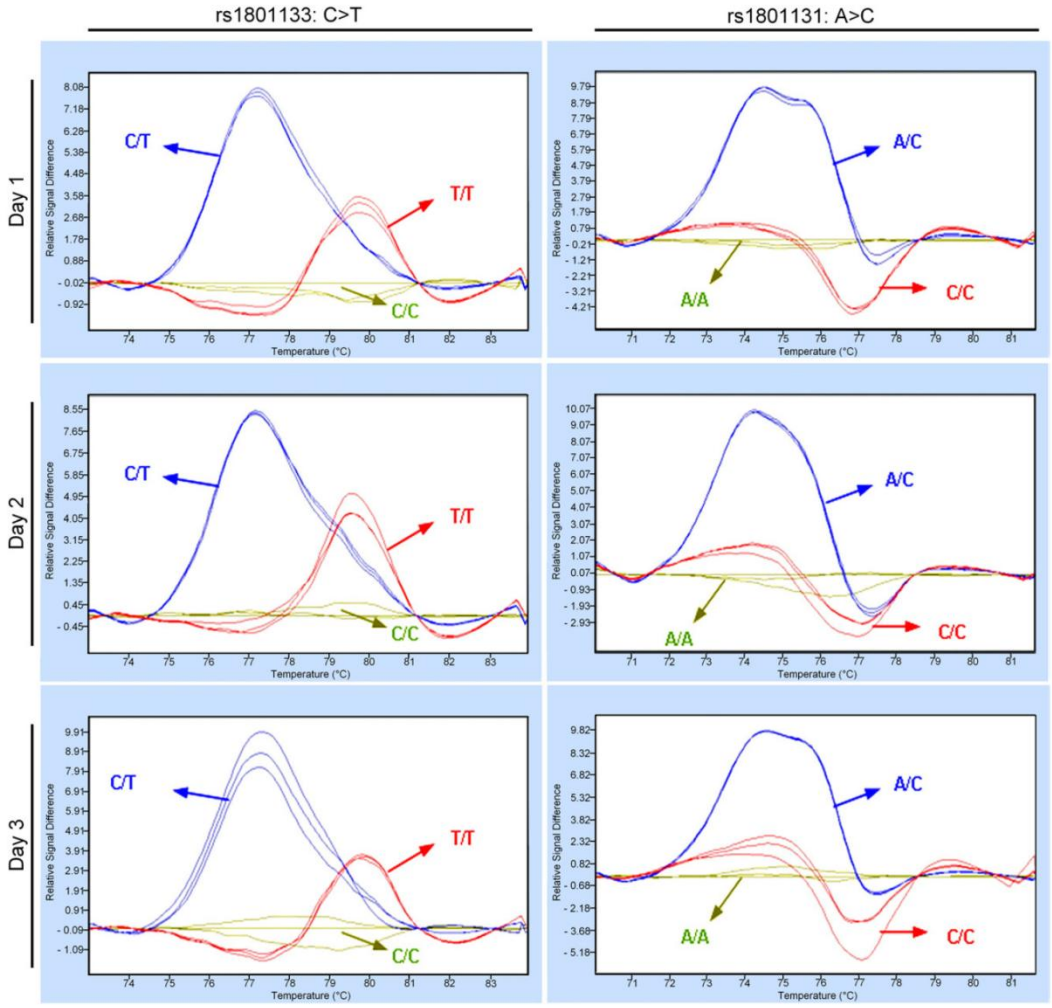

Fig. 3. Day-to-day reproducibility. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C performed on three different days by the same analyst are shown. Control samples for each genotype were run in triplicates. Each genotype group is indicated by arrows.

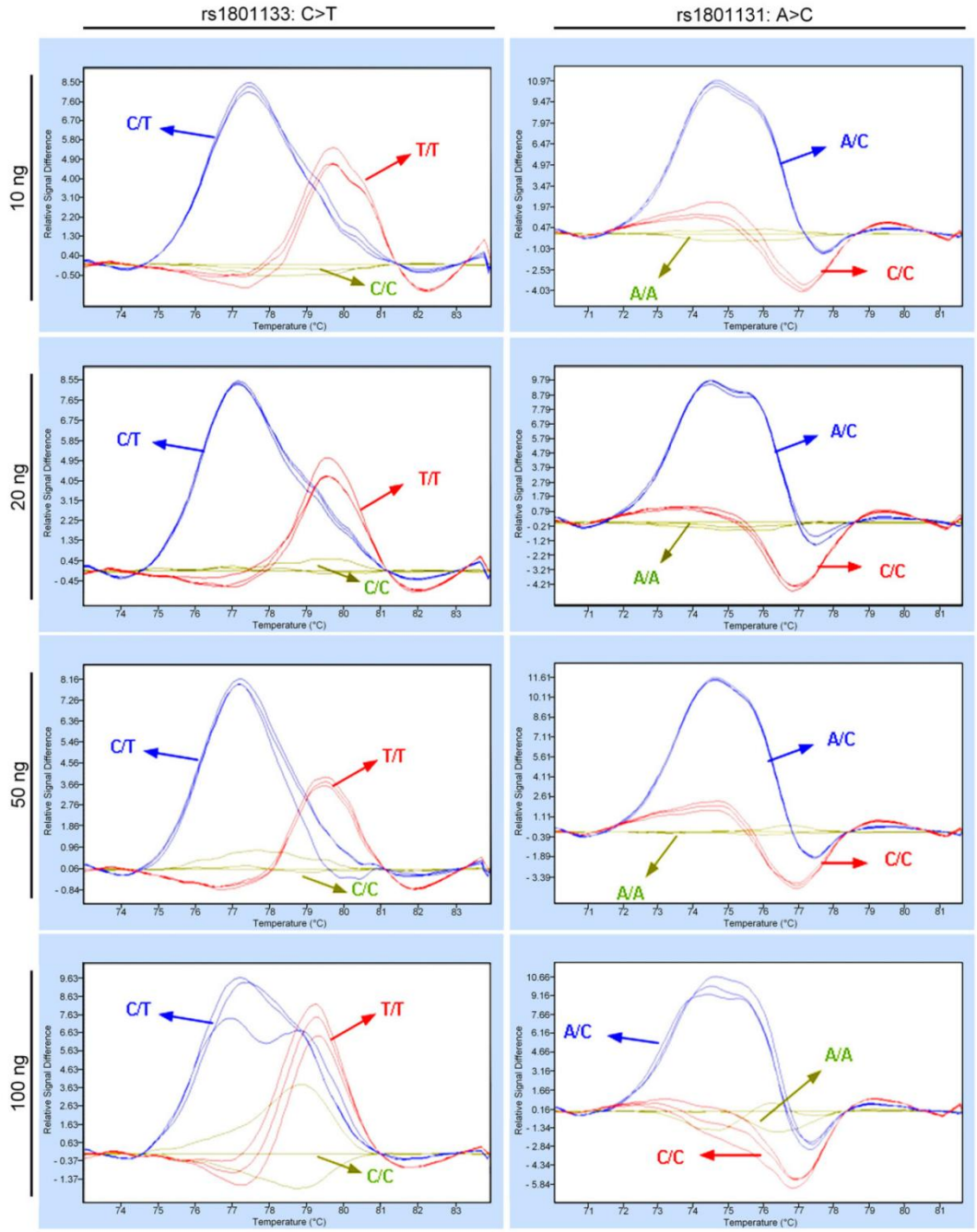

Fig. 4. DNA template amount variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C are shown. 10 ng, 20 ng, 50 ng and 100 ng of gDNA template per 10 μL of reaction were tested. Each genotype group is indicated by arrows.

Inter-run precision–reproducibility

There was no difference in the calls and difference plots when the same procedure was repeated on different days (Fig. 3).

Robustness

Template DNA amounts ranging from 10 ng to 50 ng provided correct grouping within the three different genotype groups (Fig. 4). Only when 100 ng of DNA was used in the reaction, melting profiles became unreliable. An annealing temperature variation of ±1 °C did not change the ability of the method to render correct grouping (data not shown). An increase in the cycle number, for both tested SNPs, did not modify the correct grouping nor did influence the melting profile. There was no detectable difference when we decreased the PCR program by two cycles for rs1801131: A>C, whereas a decrease in two cycles for rs1801133: C>T modified the melting profiles (Fig. 5).

Three different analysts repeated the same procedure in order to group same control samples (in three replicates) within the different genotype groups. With regard to the grouping ability, neither the software calls, nor the melting profiles varied (data not shown).

A difference in the melting profile was observed only when the same sample was analyzed by adding 7.0 μL of reaction master mix instead of 8.0 μL, and when template DNA volume added was 3.0 μL instead of 2.0 μL (data not shown).

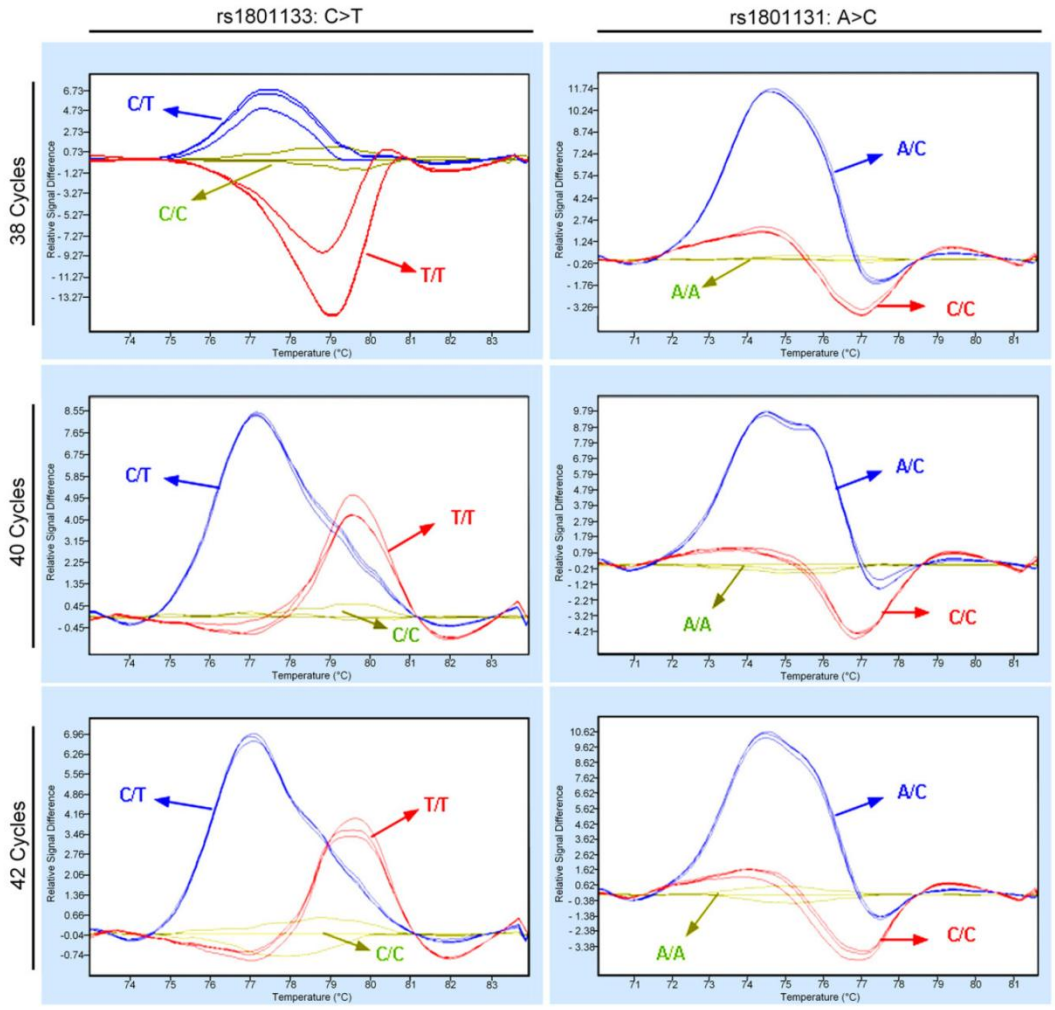

Fig. 5. Cycle number variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C with ±2 cycles variation from the normal PCR program (40 cycles) are shown. One sample from each genotype was run in triplicate; each genotype group is indicated by arrows.

Discussion

Genotyping by HRM of small amplicons is a technique associated with high sensitivity and specificity [14,35–37]. We were able to prove these observations by discriminating between homozygous wild type and homozygous mutant melting profiles on the model of two common *MTHFR* variants. Moreover, the advantage of this approach is that it is carried out in a closed tube environment. There is no need to add an extra oligonucleotide probe for genotyping as in the "original" form of HRM gene scanning [38,39] and even after a T_m shift we can still clearly distinguish both homozygous genotypes. We only used two flanking primers, a proprietary saturating DNA fluorescence dye and ready-to-use commercial master mix. Costs per analysis could be further decreased by preparing an "in house" master mix. These features make this technique very simple, customizable and fast, thus useful for a routine diagnostic setting [10,11,35].

Although previous studies analyzed all types of HRM from the point of view of sensitivity and specificity [8,10,11,22–25], none of them subjected HRM of small amplicons to diagnostic validation as required by OECD guidelines or ISO 151989 [15,16]. In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters which are particularly relevant in DNA diagnostics [18–21]. Assessment of sensitivity and specificity alone is not sufficient enough to cover all aspects of the diagnostic use of a given technique [40], since these do not account for possible variability of scenarios encountered at DNA diagnostic laboratories (i.e. uneven DNA template quality, imprecise DNA template concentration, change of personnel, different laboratory devices, variations in ambient temperature etc). In addition, due to the small master mix volume (10 μL) this method is particularly prone to pipetting inaccuracies. Therefore, it is necessary to include additional parameters, such as precision and robustness [18–21].

Monitoring of the progress of amplification by real-time PCR enabled us to exclude poor quality or insufficiently amplified template DNA samples. These quality measures contributed to high sensitivity and specificity [41] and our observations are in accordance with other studies [8,10,11,20–25].

Nevertheless, despite strictly applied quality measures the analytical failure rate was within the range of 1.6 to 1.9% for the tested variants. These samples cannot be assessed as "false negatives" since the assessor cannot infer any conclusions when the proprietary software discards unreliable data acquisitions due to its internal, quality-based algorithms, as is the case in LightCycler® 480 Real-Time PCR System. The only plausible technical explanation is that failure could likely result from inaccurate pipette handling of very small reaction master mix volumes, demonstrating that there is still a space for further improvements. When we repeated the analysis for the second time correct genotyping was achieved.

Since we were using the same reagents and DNA dilution conditions (i.e. same reaction chemistry), the precision (repeatability and reproducibility) of HRM was very high and we are able to use the same normalization settings between runs made on different days. This observation supports the high value of this technique for reliable genotyping in routine diagnostics.

Minor modifications of the technique do not generally affect its performance. Nevertheless, a shortening of the PCR by 2 cycles, as was the case in rs1801133: C>T, might produce different melting profiles in instances when PCR has not reached its plateau. This can be avoided by monitoring amplification in real-time and stopping the PCR after its plateau has been reached, as is possible when using the LightCycler 480 system. This step can be done manually, but we recommend to evaluate this issue at the optimization stage to set up the correct number of PCR cycles prior to future analyses. The use of larger template DNA amounts impedes HRM due to altering ds-DNA/amplicon/proprietary saturating dye ratio, thereby leading to less precise melting curves [42].

In conclusion, we have successfully performed diagnostic validation of High Resolution Melting of small amplicons for the genotyping of rs1801133: C>T and rs1801131: A>C *MTHFR* variants according to OECD and ISO guidelines [15,16] by using parameters and approaches pertinent to a diagnostic setting. In addition to accurate genotyping HRM of small amplicons, altered melting profiles could signal another mutation within the analyzed sequence. As is the case with all mutation scanning-based techniques direct sequencing will elucidate the reason for altered melting profiles. Shortening of amplicons in HRM further decreases the false negativity rate due to poor discrimination of homozygous sequences. This feature substantially decreases the necessity to implement sequencing in samples where the estimated clinical risk is highly discordant with a "negative" test result.

Finally, we believe that our approach could be of general use for diagnostic validations of other methods.

Acknowledgments

We thank I. Anguelová, M. Fialová, A. Nestorovic and E. Raušová for sample preparation and technical assistance. We acknowledge helpful consultations with Dr. N. van der Stoep (Leiden University Medical Centre) and Dr. M. Morris (Geneva University Hospital). Supported by VZFNM 00064203(6112) and IGA NR9448-3 from the Czech Ministry of Health and EuroGentest (FP6-512148).

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EJHG Open

European Journal of Human Genetics (2010), 1–13

www.nature.com/ejhg

ARTICLE

A standardized framework for the validation and verification of clinical molecular genetic tests

Christopher J Mattocks*[,1,7], Michael A Morris[2,7], Gert Matthijs[3,7], Elfriede Swinnen[3], Anniek Corveleyn[3], Els Dequeker[3], Clemens R Müller[4], Victoria Pratt[5] and Andrew Wallace[6], for the EuroGentest Validation Group[8]

The validation and verification of laboratory methods and procedures before their use in clinical testing is essential for providing a safe and useful service to clinicians and patients. This paper outlines the principles of validation and verification in the context of clinical human molecular genetic testing. We describe implementation processes, types of tests and their key validation components, and suggest some relevant statistical approaches that can be used by individual laboratories to ensure that tests are conducted to defined standards.
European Journal of Human Genetics advance online publication, 28 July 2010; doi:10.1038/ejhg.2010.101

Keywords: validation; verification; molecular genetics; statistics; diagnostic

INTRODUCTION

The process of implementing a molecular genetic test for diagnostic use is complex and involves many levels of assessment and validation. The key components of the process, as detailed by the ACCE framework, are analytical validation, clinical validation, clinical utility and consideration of the ethical, legal and social implications of the test.[1] After making a decision to set up a diagnostic test, the technology to be used must be chosen and built into a suitable laboratory process. The development stage involves assessment of both the diagnostic and technical use of the process to ensure that the measurements obtained are relevant to the diagnostic question(s) and that the analyte(s) can be unambiguously identified (ie, that there are no confounding factors). The final stage of the laboratory process is to determine whether the performance of the test, in terms of accuracy, meets the required diagnostic standards. Whether this is achieved by performing analytical validation or verification depends on the existence of a suitable performance specification that details the expected accuracy of the test under given conditions. The results of the analytical validation or verification determine whether, and how, the test will be implemented and set the requirements for performance monitoring (ongoing validation) of the test. A simplified process diagram illustrating these concepts is given in Figure 1.

The validation or verification of methods, as defined in Table 1, is a formal requirement for the accreditation of laboratories according to the two major international standards applicable to genetic testing laboratories, ISO 15189[2] and ISO 17025.[3] Although the general requirements are clearly stated (Table 1), the standards provide very little guidance about the detailed requirements or procedures.

To provide more detailed and specific guidance, Eurogentest[4] set up a working group comprising clinical and laboratory scientists and experts on quality assurance and statistics from both Europe and the United States. The aims were to develop a framework for validation that could be widely implemented in laboratories to improve the overall quality of genetic testing services while respecting the need for flexibility imposed, for example, by regional requirements and regulations, as well as practical constraints such as test volume and resources. In a recently generated parallel initiative, Jennings *et al*[5] have provided a thorough discussion of FDA regulation, together with a good review of validation procedures. However, specific interpretation of the standards and practical guidance for molecular genetic tests are still lacking. In this paper we propose a generic scheme for the validation and verification of molecular genetic tests for diagnostic use.

SCOPE

This paper is specifically focused on processes involved in analytical validation and verification of tests in human molecular genetics so as to provide working detail of the first component of the ACCE framework.[1] These processes seek to confirm that a particular laboratory process or test delivers reliability that is consistent with the intended diagnostic use. Analytical validation/verification relates only to laboratory processes, and makes no assessment of the manner in which the decision to set up a test is made, as well as the clinical validation, clinical utility or the ethical, legal and social implications of the test.[1] In particular, the clinical relevance of the test and the suitability of the chosen measurements with respect to diagnosing a particular genetic disorder are left to professional judgement.

There is much debate about the exact boundary between development and validation, and good cases can be made for different divisions. For the purpose of simplicity, we have defined a definitive boundary placing all concepts that relate to test utility in development

[1]National Genetics Reference Laboratory (Wessex), Salisbury District Hospital, Salisbury, UK; [2]Molecular Diagnostic Laboratory, Service of Genetic Medicine, CMU, Geneva, Switzerland; [3]Centre for Human Genetics (EuroGentest), campus Gasthuisberg, Leuven, Belgium; [4]Department of Human Genetics, University of Würzburg, Biozentrum, Würzburg, Germany; [5]Quest Diagnostics Nichols Institute, Chantilly, VA, USA; [6]National Genetics Reference Laboratory (Manchester), St Mary's Hospital, Manchester, UK
*Correspondence: Professor CJ Mattocks, National Genetics Reference Laboratory (Wessex), Salisbury District Hospital, Odstock Road, Salisbury SP2 8BJ, UK.
Tel: +44 1722 429016; Fax: +44 1722 338095; E-mail: chris.mattocks@salisbury.nhs.uk
[7]Chief editors.
[8]See the appendix for a list of members of the 'EuroGentest (Unit 1) working group'.
Received 11 February 2010; revised 26 May 2010; accepted 1 June 2010

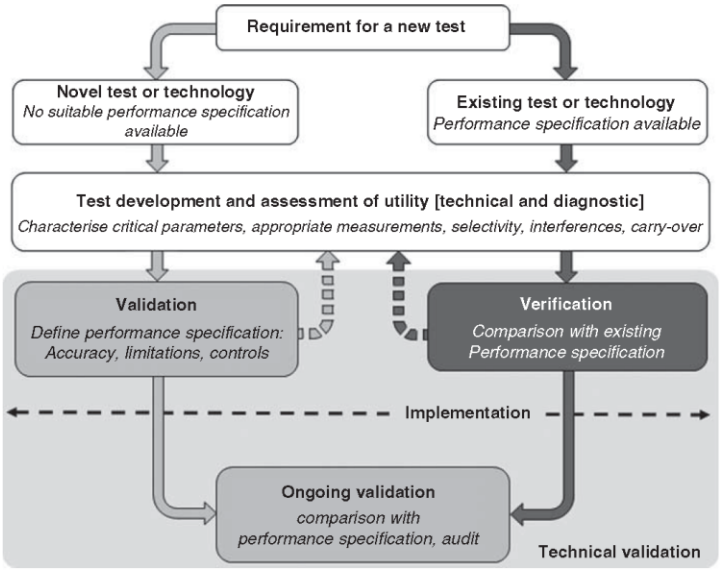

Figure 1 The process of implementing a molecular genetic test for diagnostic use. The shaded arrows represent the two general routes to implementation, depending on the availability of a suitable performance specification: validation (lighter) and verification (darker). Broken arrows represent the situation in which validation or verification fails to meet the specified requirements.

Table 1 Validation and verification

Definitions (from ISO 9000:2005) Also see the VIM[20]	*Verification*: 'Confirmation, through the provision of objective evidence, that specified requirements have been fulfilled' (doing test correctly) *Validation*: 'Confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled' (doing correct test)
Principle requirements of ISO 17025:2005[3]	5.4.2 'Laboratory-developed methods or methods adopted by the laboratory may also be used if they are appropriate for the intended use and if they are validated'. 5.4.5.2 'The laboratory shall validate non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for the intended use. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is fit for the intended use'. 5.4.5.3 'NOTE 1 Validation includes specification of the requirements, determination of the characteristics of the methods, a check that the requirements can be fulfilled by using the method, and a statement on the validity. NOTE 3 Validation is always a balance between costs, risks and technical possibilities. There are many cases in which the range and uncertainty of the values (eg accuracy, detection limit, selectivity, linearity, repeatability, reproducibility, robustness and cross-sensitivity) can only be given in a simplified way due to lack of information'.
Principle requirements of ISO 15189:2007[2]	5.5.1 '[...] If in-house procedures are used, they shall be appropriately validated for their intended use and fully documented'. 5.5.2 'The methods and procedures selected for use shall be evaluated and found to give satisfactory results before being used for medical examinations. A review of procedures by the laboratory director or designated person shall be undertaken initially and at defined intervals'. 5.6.2 'The laboratory shall determine the uncertainty of results, where relevant and possible'.

Definitions and summarized requirements of the major international standards for accreditation of genetic testing laboratories.

(ie, out of scope) and parameters relating to test accuracy in validation (ie, within scope).

These limitations of the scope should not be taken as assigning different levels of importance to the various processes; making clinically useful and appropriate measurements is clearly critical to setting up a valid diagnostic test. For this reason, we have included a brief section outlining the processes involved and important factors that should be considered at the development stage.

Although we are concerned with appropriate use of statistics and sample sizes, this paper is not intended to be a treatise on the subject, but a practical guide for diagnostic molecular geneticists to aid them in designing, performing and reporting suitable validation or verification for the tests they wish to implement. References have been provided in which more complex statistical concepts are involved, but it is recommended that the advice of a statistician be sought in case of doubt. Above all, we seek to promote a pragmatic approach; although validation and verification must be carefully considered, test implementation must also be achievable and not overburdening.

Although there is much literature addressing validation on a more general level,[6–8] we propose a first attempt to identify and organize the components required for validation/verification in the context of molecular genetic diagnostics, and have consequently included some measure of simplification of statistical principles and their interpretation. It is intended that this paper be a starting point for the ongoing development of validation/verification guidelines that will necessarily become more sophisticated as knowledge and experience in the area increase and scenarios that are not adequately covered by this paper are identified. Although these recommendations are aimed primarily at molecular genetic testing, we believe that the principles and concepts are also applicable in the context of cytogenetics.

To help guide the validation process and provide a format for recording validations, a standardized validation *pro forma* (template) has been provided in Supplementary data. An initial draft of this form was developed from an amalgamation of standard forms used in a range of small and large laboratories that undertake genetic testing. This prototype underwent a field trial to assess its use, as well as ease of use and appropriate amendments made. We recognize that a single format is unlikely to suit all laboratories; hence we recommend that this form should be used as a starting point for the development of a suitable format for local needs.

This paper can be used as detailed explanatory notes for the validation form.

THE IMPLEMENTATION PROCESS

Development

The purpose of development is to establish a testing procedure and broadly show that it is fit for the intended purpose, in terms of what is being tested and of the desired laboratory procedure. This involves defining the analyte(s) to be tested and designing an appropriate methodology, including any assay-specific reagents (eg, primers), controls and a testing workflow. The development process should be used to gain the necessary experience with the test, with the aim of identifying any critical parameters that may affect performance and any necessary control measures and limitations that need to be considered. Examples of critical parameters may include primer design, location of known polymorphisms, the G+C content of the region of interest, fragment length, type of mutations to be detected and location of mutations within fragments. Suitable control measures might include the use of positive, negative and no-template controls, running replicates of the test and a quality scoring system. It therefore follows that the amount of development required would depend on the novelty of the testing procedure, both on a general level (ie, in the literature) and within the laboratory setup of the test. For example, development of a sequencing test for a new gene in a laboratory with extensive experience in sequencing may simply be a case of primer design, whereas setting up an entirely new methodology would require much more extensive investigation.

Assessment of use

Before a test can be validated, it is necessary to establish (a) that the particular measurements are diagnostically useful and (b) that the correct analyte(s), and only the correct analyte(s), are measured. This could involve, for example, ensuring that primers do not overlay known polymorphisms in the primer-binding site and that they are specific to the target of interest. It should be noted that use of a CE-marked kit does not preclude assessment of use; care should still be taken to ensure that the test measures suitable analyte(s) for the intended purpose, as *in vitro* diagnostic device (IVDD) compliance relates only to technical performance and not to clinical or diagnostic validity. Three other critical concepts that should be considered at this stage are the following:

Selectivity. How good is the method at distinguishing the signal of the target from that of other components? For example, a PCR product run on a denaturing polyacrylamide gel to detect the presence of the CFTR p.Phe508del (p.F508del) mutation associated with cystic fibrosis will also detect the rarer p.Ile507del (p.I507del) mutation, without distinguishing between them. For most genetic tests, selectivity issues are best avoided by careful design (eg, BLAST[9] primers to avoid nonspecific amplification) or by applying adapted controls and/or limitations.

Interference. Are there any substances the presence of which in the test can affect the detection of the target sequence? If so, will this cause the reaction to fail or is there a possibility of an incorrect result? For most genetic tests, this is likely to relate to substances that cause a reaction to fail (eg, heparin or ethanol in a DNA sample as a result of the stabilization or extraction procedure). Although failures may not generate false results, there can be issues relating to the use and timeliness of tests if failure rates are too high. In situations in which interference could cause incorrect results, great care needs to be taken to avoid interfering substances, for example, by running a pretest quality check on samples or by including more controls.

Because of their complex nature, multiplex assays are particularly susceptible to interference, which could give rise to incorrect results. Validation and verification of this type of assay can be particularly demanding and is beyond the scope of this paper. The Clinical Laboratory Standards Institute (CLSI) has published a guideline that deals comprehensively with this specialist topic.[10]

Carryover (cross-contamination). This relates to residual products from previous or concurrent analyses that may be introduced into an assay (eg, through a contaminated pipette). Stringent procedural precautions should be used as a matter of routine to minimize the risk of such cross-contamination. In particular, physical separation of pre- and post-PCR areas for both reagents and laboratory equipment is critical. Other controls/precautions may include the use of no-template controls and uracil-*N*-glycosylase treatment.[11–13]

Performance specification

Once a suitable test procedure has been established and it is judged that there is sufficient local knowledge of critical parameters, it is necessary to show that

(a) test performs to a suitable level of accuracy for the intended purpose: that is, it produces results that can satisfactorily answer the clinical question allowing for uncertainty of measurement; and that
(b) this level of accuracy is routinely maintained.

The level of testing required is dependent on the availability of a suitable performance specification. This should define all the test conditions necessary to achieve a particular level of accuracy, together with measurable parameters that can be used to show that this is the case; specifically,

(a) an estimate of the test accuracy including measurement uncertainty (eg, confidence limits);
(b) control measures required to ensure routine maintenance of accuracy;
(c) limitations on critical parameters that will ensure the desired level of accuracy.

For validation of a specific test, limitations may include factors such as input DNA concentration or details on how DNA extraction needs to be performed. When a technology is being validated (as opposed to a specific test), there may also be limitations related to physical parameters such as PCR fragment length or G+C content. It should be stressed that a performance specification will only apply within particular limits of certain critical parameters; hence, care should be taken to ensure that the new test falls within these limits. For example, the performance specification for a hypothetical method for mutation scanning (>95% sensitivity for mutations in fragments <300 bp long and 25–60% G+C content) would not be applicable to a new test involving a 400-bp fragment or fragments with 70% G+C content.

Validation

Full validation is required when there is no suitable performance specification available, for example, with novel tests or technologies. This process involves assessing the performance of the test in comparison with a 'gold standard' or reference test that is capable of assigning the sample status without error (ie, a test that gives 'true' results). In simple terms, validation can be seen as a process to determine whether we are 'performing the correct test'. In the field of medical genetics, with the almost complete absence of reference tests or certified reference materials, the reference should be the most reliable diagnostic method available. It is worth noting that the gold standard does not have to comprise results from a single methodology; different techniques could be used for different samples and in some cases the true result may represent a combination of results from a portfolio of different tests. To avoid introducing bias, the method under validation must not, of course, be included in this portfolio.

Validation data can be used to assess the accuracy of either the technology (eg, sequencing for mutation detection) or the specific test (eg, sequencing for mutation detection in the *BRCA1* gene). Generally speaking, the generic validation of a novel technology should be performed on a larger scale, ideally in multiple laboratories (interlaboratory validation), and include a much more comprehensive investigation of the critical parameters relevant to the specific technology to provide the highest chance of detecting sources of variation and interference.

Verification

If a suitable performance specification is available, it is necessary to establish that the new test meets this specification within the laboratory; this process is called verification. In simple terms, verification can be seen as a process to determine that 'the test is being performed correctly'.

Verification should usually be appropriate for CE-marked IVDD-compliant kits, but care should be taken to ensure that the performance specification is sufficient for the intended use of the kit, particularly with kits that are self-certified. Most diagnostic genetic tests are classified by the IVD directive as 'low-risk' and can be self-certified by the manufacturer without assessment by a third party. Such tests can be identified by the absence of a number following the CE mark (Article 9: IVDD Directive 98/79/EC).[14,15] If, at any stage, the test procedure associated with the performance specification is modified (eg, if reaction volumes of a CE-marked kit are reduced), verification is not appropriate and validation is required.[16]

Other applications of verification may include a new test being implemented using a technology that is already well established in a laboratory (eg, a sequencing assay for a new gene), or a test for which a suitable performance specification is available from another laboratory in which the test has already been validated. In all cases, it is essential that laboratories obtain as much information as possible with regard to the validation that has been performed.

Reporting validation and verification

The plan, experimental approach, results and conclusions of the validation or verification should all be recorded in a validation file, along with any other relevant details (see the section 'Reporting the results'). In addition, the validation plan and outcome should be formally reviewed and approved. When reporting validations or verifications in peer-reviewed publications, it is strongly recommended that the STARD initiative (Standards for Reporting of Diagnostic Accuracy)[17] be followed as far as possible.

Performance monitoring (ongoing validation)

Once a test validation has been accepted (ie, the use and accuracy have been judged to be fit for the intended diagnostic purpose), it is ready for diagnostic implementation. However, this is not the end of performance evaluation. The performance specification derived from the validation should be used to assess the 'validity' of each test run and this information should be added to the validation file at appropriate intervals. In many cases, the accumulation of data over time is an important additional component of the initial validation, which can be used to continually improve the assessment of test accuracy and quality. The ongoing validation should include results of internal quality control, external quality assessment and nonconformities related to the test or technique as appropriate.

TYPES OF TEST

The core aim of validation is to show that the accuracy of a test meets the diagnostic requirements. Essentially, all tests are based on a quantitative signal, even if this measurement is not directly used for the analysis. Although measuring the proportion of a particular mitochondrial variant in a heteroplasmic sample is, for example, clearly quantitative, the presence of a band on a gel is commonly considered as a qualitative outcome. However, the visual appearance of the band is ultimately dependent on the number of DNA molecules that are present, even though a direct measurement of this quantity is rarely determined. These differences in the nature of a test affect how estimates of accuracy can be calculated and expressed.

For the purpose of this paper, we are concerned with two types of accuracy. Determining how close the fundamental quantitative measurement is to the true value is generally termed 'analytical accuracy'. However, it is often necessary to make an inference about the sample or the patient on the basis of the quantitative result. For example, if the presence of a band on a gel signifies the presence of a particular mutation, test results are categorized as either 'positive' or 'negative' for that mutation, on the basis of the visible presence of the band. Such results are inferred from the quantitative result, but are not

Table 2 Types of test

	Description	Examples	Sensitivity[a]	Specificity[b]	Accuracy[c]	Trueness	Precision[d]	Limits of detection	Probability[e]
A	**Quantitative** tests. The result can have any value between two limits (including decimals).	Determination of methylation load (%); characterization of a mosaic mutation; heteroplasmy of mitochondrial variants.				++	++	++	
B	**Categorical** tests where the quantitative signal is placed into an ordinal series to give the final result.	Sizing a PCR product; determination of triplet repeat size (FRAXA, Huntington disease, etc.)			+	++	++	++	+
C	**Categorical** tests where the quantitative signal is placed into one of a limited series of predefined categories to give the final result.	Determination of copy number using PCR or MLPA.: exon deletion / duplication in *BRCA1*; *PMP22* gene dosage in CMT and HNPP;			+	To establish correction factors and/or cut-offs			++
D	**Qualitative** tests where the true quantitative signal can have one of many possible values, but the required result can only have one of two possible values.	Mutation scanning for unknown mutations e.g. by sequencing or high resolution melt.	++	++	+	To establish correction factors and/or cut-offs		++[f]	
E	**Qualitative [binary]** tests where the true quantitative signal can only have one of two possible values	Genotyping for a specific mutation e.g. *CFTR* Phe508del in cystic fibrosis or *HFE* Cys282Tyr in hemochromatosis .	++	++	+	To establish correction factors and/or cut-offs		++[f]	+

Legend

- Metric used for implementation validation
- Metric used for implementation or ongoing validation
- Metric used for ongoing validation
- ++ Recommended parameter
- + Applicable parameter (less used)

Notes

a. Sensitivity = True Positive / (True Positive + False Negative)
b. Specificity = True Negative / (True Negative + False Positive)
c. Accuracy = True Result / (True Result + False Result)
d. Precision should be measured in terms of repeatability and intermediate precision (as well as reproducibility for inter-laboratory validations)
e. The term 'probability' is used to describe situations where a probability that the result is correct can be assigned – primarily in on-going validation (e.g. competitive hypothesis testing)
f. Should be used in tests where genotyping of low level variations is required for example mitochondrial DNA

NB: In addition to the parameters detailed above, appropriate robustness testing should be carried out for all types of test.

in themselves quantitative. Determination of how often such a test gives the correct result is termed 'diagnostic accuracy'. The term diagnostic accuracy is generally used to describe how good a test is at correctly determining a patient's disease status. However, genotype does not necessarily equate directly to disease status (phenotype) for various reasons, including incomplete penetrance/modifying factors or simply because the patient is presymptomatic. The purpose of these guidelines is to enable laboratories to establish how good their tests are at correctly determining genotype; clinical interpretation of the genotype is not considered in this context. Therefore, for the purpose of this paper, the term diagnostic accuracy will be taken to relate exclusively to the ability of a test to correctly assign genotype irrespective of any clinical implication.

We distinguish three broad test types (quantitative, categorical and qualitative) that can be subdivided into five groups according to the method for interpreting the raw quantitative value to yield a meaningful result.

The following sections discuss each of these test types in more detail and provide guidance on appropriate measurement parameters in each case. A summary of the characteristics of the different test types and examples is given in Table 2, together with recommendations for appropriate measurement parameters and timing of validation.

Type A quantitative tests

For a quantitative test, the result is a number that represents the amount of a particular analyte in a sample. This can be either a relative quantity, for example, determining the level of heteroplasmy for a particular mitochondrial allele, or an absolute quantity, for example, measuring gene expression. In either case, the result of a quantitative test can be described as continuous as it can be any number (between two limits), including decimal numbers.

Two components of analytical accuracy are required to characterize a quantitative test: trueness and precision.[18,19] Trueness expresses how close the test result is to the reference value. Typically, multiple measurements are made for each point and the test result is taken to be the mean of the replicate results (excluding outliers if necessary). As quantitative assays measure a continuous variable, mean results are often represented by a regression of data (a regression line is a linear average). Any deviation of this regression from the reference (ie, the line where reference result equals test result) indicates a systematic error, which is expressed as a bias (ie, a number indicating the size and direction of the deviation from the true result).

There are two general forms of bias. With constant bias, test results deviate from the reference value by the same amount, regardless of that value. With proportional bias, the deviation is proportional to the reference value. Both forms of bias can exist simultaneously (Figure 2).

Although measurement of bias is useful (Figure 3), it is only one component of the measurement uncertainty and gives no indication of how dispersed the replicate results are (ie, the degree to which separate measurements differ). This dispersal is called precision and provides an indication of how well a single test result is representative of a number of repeats. Precision is commonly expressed as the standard deviation of the replicate results, but it is often more informative to describe a confidence interval (CI) around the mean result. For example, a result for a test investigating mutation load in a tumour sample might be described as 7% (95% CI: 5–10%).

Precision is subdivided according to how replicate analyses are handled and evaluated. Here, there is some variability in the use of terminology; however, for practical purposes, we recommend the following scheme based on ISO 3534-1[20] and the International Vocabulary of Metrology:[21]

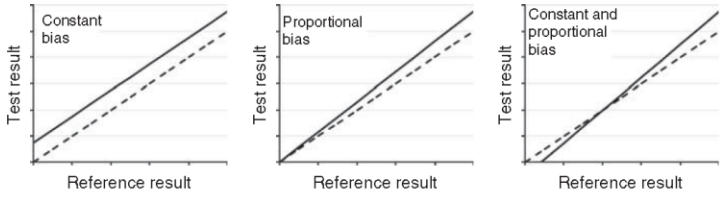

Figure 2 Types of bias. In each case, the broken line represents the perfect result in which all test results are equal to the reference.

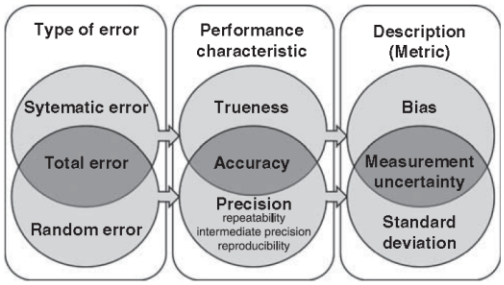

Figure 3 Performance characteristics, error types and measurement metrics used for quantitative tests (adapted from Menditto *et al*)[19].

Repeatability refers to the closeness of agreement between results of tests performed on the same test items, by the same analyst, on the same instrument, under the same conditions in the same location and repeated over a short period of time. Repeatability therefore represents 'within-run precision'.

Intermediate precision refers to closeness of agreement between results of tests performed on the same test items in a single laboratory but over an extended period of time, taking account of normal variation in laboratory conditions such as different operators, different equipment and different days. Intermediate precision therefore represents 'within-laboratory, between-run precision' and is therefore a useful measure for inclusion in ongoing validation.

Reproducibility refers to closeness of agreement between results of tests carried out on the same test items, taking into account the broadest range of variables encountered in real laboratory conditions, including different laboratories. Reproducibility therefore represents 'inter-laboratory precision'.[22]

In practical terms, internal laboratory validation will only be concerned with repeatability and intermediate precision and in many cases both can be investigated in a single series of well-designed experiments. Reduced precision indicates the presence of random error. The relationship between the components of analytical accuracy, types of error and the metrics used to describe them is illustrated in Figure 3.

Any validation should also consider robustness, which, in the context of a quantitative test, could be considered as a measure of precision. However, robustness expresses how well a test maintains precision when faced by a specific designed 'challenge', in the form of changes in preanalytic and analytic variables. Therefore, reduced precision does not represent random error. Typical variables in the laboratory include sample type (eg, EDTA blood, LiHep blood), sample handling (eg, transit time or conditions), sample quality, DNA concentration, instrument make and model, reagent lots and environmental conditions (eg, humidity, temperature). Appropriate variables should be considered and tested for each specific test. The principle of purposefully challenging tests is also applicable to both categorical and qualitative tests and should be considered in these validations as well. Robustness can be considered as a useful prediction of expected intermediate precision.

As trueness and precision represent two different forms of error, they need to be treated in different ways. In practice, systematic error or bias can often be resolved by using a correction factor; constant bias requires an additive correction factor, whereas proportional bias requires a multiplicative correction factor. For example, results from a test that has +5% bias can be multiplied by 100/105. Random error, in contrast, cannot be removed, but its effects can generally be reduced to acceptable levels by performing an appropriate number of replicate tests.

For the purpose of this paper, a basic understanding of the concepts described above is the main objective. However, it is worth outlining some of the complexities that can arise in estimating the analytical accuracy of quantitative tests. In molecular genetics, quantitative measurements are most often relative, that is, two measurements are taken and the result is expressed as a proportion (eg, the percentage of heteroplasmy of a mitochondrial mutation). In such cases, it is preferable to perform both measurements in a single assay to minimize the effects of proportional bias, as the assay conditions are likely to affect both the measurements in a similar way.

If the measurements must be taken in separate assays, each measurement is effectively an absolute measurement and must be quantified in comparison with a set of calibration standards run with each test batch. In this scenario, it is important to assess the variation in each test/standard pair, as even minor variation can dramatically affect the overall analytical accuracy. This is most effectively achieved by monitoring the efficiencies of the two reactions over time.[23]

For quantitative tests, particularly those requiring absolute quantification, it is most effective to estimate analytical accuracy on an ongoing basis by running a set of calibration standards (standard curve) with each batch or run. In this case, it is important that linearity be evaluated[24] and that the lower and upper standards are respectively below and above the expected range of the results as precision cannot be assessed on extrapolated results. Where possible, calibration standards should be traceable to absolute numbers or to recognized international units.

Other factors that may need to be evaluated include the limit of detection defined as the lowest quantity of analyte that can be reliably detected above background noise levels and the limits of quantification that define the extremities at which the measurement response to changes in the analyte remains linear.

A detailed description of the determination of these limits is given in CLSI document EP17-A.[25] In situations in which test results are likely to fall close to these extremities or there are significant clinically relevant boundaries within the linear range (eg, the intermediate

expansion/mutation boundary in Huntington's disease), it is useful for both implementation and ongoing validation to use controls on or close to the boundary.

It should be noted that limit of detection is sometimes referred to as 'sensitivity'; that is, how sensitive a methodology is to detecting low levels on a particular analyte in a large background. Use of the term 'sensitivity' in this context should be avoided, as it may be confused with sensitivity described in the section 'Qualitative tests' (ie, the proportion of positive results correctly identified by a test).

It can be seen that the analysis of all but the simplest quantitative assays can be complex and it is recommended that statistical advice be sought to determine those factors that need to be measured and the best way to achieve it.

Categorical tests

Categorical tests (sometimes referred to as semiquantitative[26]) are used in situations in which quantitative raw data, which could have any value including decimals, are grouped into categories to yield meaningful results. For example, fluorescent capillary analysis might be used to determine the size of PCR products (in base pairs) by analysing the position of the peaks relative to an internal size standard. The quantitative results from this analysis will include numbers with decimal fractions, but the length of the product must be a whole number of base pairs; a fragment cannot be 154.3 bp long. Therefore cutoffs must be used to assign quantitative results to meaningful categories. The parameters used to describe the estimates of analytical accuracy for a quantitative test (Figure 3) can be used to describe the performance of the categorical test in much the same way. However, there is an added level of complexity here, as the primary (quantitative) result is manipulated (ie, placed into a category). The categorized results for these tests retain a quantitative nature (although this is distinct from the quantitative primary data) and, in practice, trueness and precision can be determined at the category level, as well as at the level of the primary result. We divide categorical tests into two subgroups, depending on the number and type of categories and the degree of importance placed on knowing how accurate a result is (Figure 4).

Type B categorical tests. This group includes tests in which there are (essentially) unlimited categories, such as the sizing example cited above. In this case, each cutoff forms the upper boundary of one category and the lower boundary of the next, so that all results can be categorized (except for those that have failed). Generally, less-stringent levels of accuracy are acceptable with this type of test. In this case, estimation of precision can be performed before implementation (eg, ±1 bp), whereas trueness is dealt with by running a standard curve with each experiment (ie, a size standard).

Type C categorical tests. When the number of predefined categories is limited, for example, with allele copy number determination, accuracy tends to be critical and a more definitive approach is often required. The most informative way to express accuracy for this type of test is the probability that a particular (quantitative) result falls into a particular category. Here, cutoffs are defined at particular level(s) of probability, typically 95% CI, which means that each category has its own unique upper and lower boundaries with regions in between, where results would be classified as unreportable.

Results can be assigned to the appropriate categories by a process of competitive hypotheses testing. For example, a test to determine constitutional allele copy number has three expected results: normal (2n), deleted (n) and duplicated (3n). The odds ratios p(2n):p(n) and p(2n):p(3n) can be used to assign results (Figure 5). It should be noted that mosaic variants may give rise to intermediate values; detection of mosaics should be considered under quantitative tests. A good example of this methodology is described in the MLPA spreadsheet analysis instructions that are freely available from NGRL

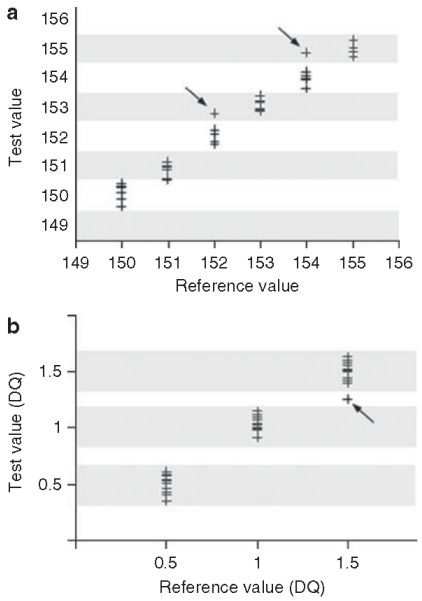

Figure 4 (**a**) A type-B categorical test to size PCR fragments. Each category (indicated by alternating shading) has an upper cutoff that is also the lower cutoff of the next category. Results marked with arrows are not precise but fall within the given accuracy for the test of ± 1 bp. (**b**) A type-C categorical test for allele quantification. Each category (shaded) has unique upper and lower cutoffs. Results falling between categories are classed as unreportable (marked with an arrow). A dosage quotient (DQ) of 0.5 represents a sample with a deleted allele, 1.0 represents normal and 1.5 represents a sample with a duplicated allele.

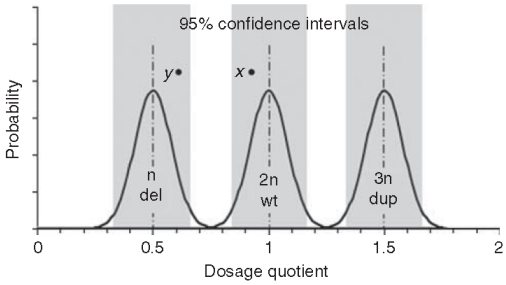

Figure 5 Multiplex ligation-dependent probe amplification to detect exon copy number (Categorical test type C). Dosage quotient (DQ)=relative height of test peak compared with control peaks. DQ=0.5 represents exon deletion, DQ=1.0 represents wild type and DQ=1.5 represents exon duplication. Population distributions of DQs are shown with 95% confidence intervals shaded. Results falling between categories are unreportable.

(Manchester).[27] In this case, the validation of accuracy is predominantly carried out on an ongoing basis by running replicate control assays during the actual test run to determine the extent of the random error observed within that particular run.

Qualitative tests

This is the extreme form of a categorical test, in which there are only two result categories, positive and negative. This binary categorization can be based either on a cutoff applied to a quantitative result, for example, peak height or a mathematical measure representing peak shape, or on direct qualitative observation by the analyst, for example, the presence or absence of a peak (in the latter case, as discussed in the section 'Types of test', the underlying data will generally be quantitative in nature, even though no formal quantification is performed). In terms of accuracy, categorization can be either correct or incorrect with respect to the 'true' (reference) result. A simple contingency table can be used to describe the four possible outcomes (Table 3).

The diagnostic accuracy of a qualitative test can be characterized by two components, both of which can be calculated from the figures in the contingency table:

(i) Sensitivity – the proportion of positive results correctly identified by the test=TP/(TP+FN);
(ii) Specificity – the proportion of negative results correctly identified by the test=TN/(TN+FP).

In addition, the overall accuracy can be characterized by the total number of true results as a proportion of the total results ((TP+TN)/(TP+TN+FP+FN)), although, in practice, this parameter is rarely used. For comparison with quantitative tests (Figure 3), the relationship between the components of accuracy is depicted in Figure 6.

There is an inverse relationship between sensitivity and specificity (Figure 7). As more stringent cutoffs are used to reduce the number of false positives (ie, increase specificity), the likelihood of false negatives increases. Therefore, the desirable characteristics of a test must be considered in the context of the required outcome and the diagnostic consequences. For example, laboratory procedures for mutation scanning tests often involve a primary screen to determine which fragments carry mutations, followed by a second confirmatory test by sequencing to characterize the mutations present. In the primary screen, sensitivity is much more critical than specificity, to avoid missing mutations that are present; the only consequence of poor specificity is increase in the workload for confirmatory sequencing. Obviously, there is a limit to the lack of specificity that can be tolerated, even if only on the grounds of cost and efficiency.

In situations in which sensitivity and specificity are both critical, it is desirable to use two cutoffs to minimize both false-positive and false-negative rates. In this case, results falling between the two cutoffs can either be classified as test failures or be passed for further analysis.

Table 3 Possible outcomes for a qualitative validation experiment

	Reference result	
	+	–
Test result		
+	True positive (TP)	False positive (FP)
–	False negative (FN)	True negative (TN)

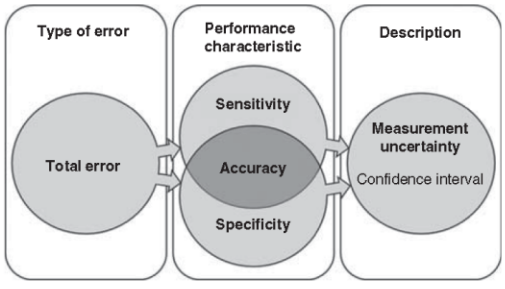

Figure 6 The relationship between performance characteristics, error and measurement uncertainty used for qualitative tests (adapted from Menditto *et al*)[19].

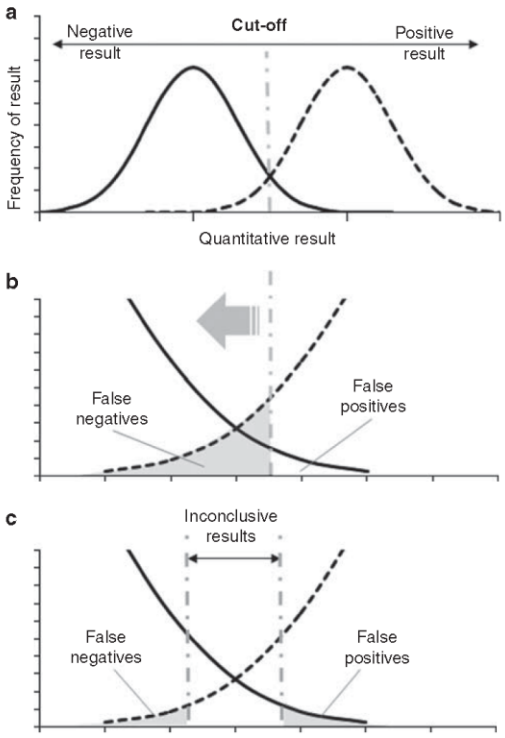

Figure 7 (**a**) The relationship between sensitivity and specificity. The figure shows frequency distributions of the primary quantitative results for a qualitative (binary) test. Solid line represents gold standard negatives (wild type), broken line represents gold standard positives (mutant). Using a single cutoff to categorize the results as either positive or negative gives rise to both false negatives and false positives. (**b**) Cutoff location. Positioning the cutoff to the right encompasses more of the negative distribution, giving a low false-positive rate but a high false-negative rate (shaded). As the cutoff is moved to the left, the false-negative rate is reduced but the false-positive rate increases. (**c**) Use of two cutoffs. It is possible to minimize both false-positive and false-negative rates by using two cutoffs. In this case, results falling between the two cutoffs can either be classified as test failures or be passed for further analysis.

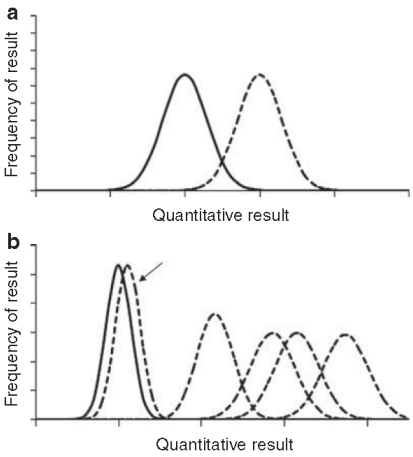

Figure 8 (**a**) Truly binary test. Only two states of the analyte can be measured: one wild type (solid line) and one mutant (broken line). Competitive hypothesis testing could be used to determine the relative probability (odds ratio) that a result is either wild type or mutant. (**b**) Arbitrary binary test. There are many different possible states of the analyte; one wild type (solid line) and five different mutants (broken lines) are shown. The mutant state indicated is essentially indistinguishable from the wild type. Hypothesis testing could be used to estimate the probability that a result is not negative, but not that it is not positive.

Type D qualitative tests. In many cases, particularly for mutation scanning methods, it is necessary to use a qualitative description to distinguish between a single normal state (negative result) and any number of mutated states (positive result). Although quantitative results for the normal state would be expected to be normally distributed, positive results would not, as they combine many (known or potential) different mutations, each with its own distribution (Figure 8b). Although it is still theoretically possible to use basic hypothesis testing to assign a probability that a result is not normal, competitive hypotheses cannot be used, as it is not possible to know the mean quantitative result for all possible mutations (unless they have all been tested). In this scenario, assessment of accuracy is therefore best performed in a preimplementation validation using a suitable number of positive (ie, known mutant) and negative (known normal) samples (see the section 'Study design').

Type E qualitative (binary) tests. In cases in which the test is designed to measure only two states of the analyte (eg, a specific SNP genotyping assay), the quantitative results for each state can be expected to be normally distributed (Figure 8a). In this case, results can be assigned to appropriate categories by competitive hypothesis testing, as described for type C categorical tests (see the section 'Type C categorical tests'). Again, this model can be used as an ongoing validation method, minimizing the need for implementation validation. Test accuracy can also be described in terms of sensitivity and specificity, given particular cutoffs. This method would require much more stringent validation before implementation (see the section 'Study design').

Sequencing

Direct sequencing (currently, fluorescent dideoxy-terminator sequencing by capillary electrophoresis) is the method of choice for a wide range of clinical genetic tests and is widely considered to be the 'gold standard' (reference) method for identifying and characterizing DNA variations. As such, it is often not possible to develop a suitable reference for comparative validation of new sequencing-based tests. In this situation, it is recommended that validation be treated as a verification that sequencing is being performed to the required standard, in the context of the new test. Factors to be considered should include confirmation that the new test specifically targets the region of interest (ie, BLAST primers, and check sequence), that both alleles are reliably amplified (ie, ensure that no SNPs are located in primer binding sites) and that the sequencing data generated are consistently of suitable quality across the whole region of interest (eg, monitoring PHRED scores across the region of interest). It is important to note that, as sequencing methodologies can vary, for example, by the cleanup method, thermal cycling regime, or whether single or bidirectional sequencing is used for analysis, the validation scheme should be carefully tailored to the application. This is of particular importance when a new sequencing test is being 'imported' from another laboratory, as most laboratories will have their own particular sequencing methodology and this is unlikely to be identical to the local method.

As with other tests, it is important to participate regularly in an external quality assurance (EQA) scheme where possible. In the case of sequencing, this may be dealt with at the technology level in addition to disease-specific schemes; for example, the MSCAN and SEQ DNA schemes run by the European Molecular Genetics Quality Network (EMQN).[28]

CONSIDERATIONS FOR EXPERIMENTAL DESIGN

Extrapolation of results (validation constraints)

The results of a validation can be applied beyond its immediate coverage; however, some rationale needs to be applied to such extrapolation. Let us consider the validation of a mutation-scanning technology that tested 100 different mutations in a particular gene (5000 bp) resulting in a sensitivity of '≥97% (95% CI)' (see the section 'Qualitative tests' for calculating and reporting sensitivities). What does this actually mean in practice?

Only a very small number of the possible mutations in the region of interest were actually covered; there are 15 000 possible single-base substitutions in 5000 bp and virtually limitless insertion/deletion mutations. If only substitutions were tested in the validation, the estimated sensitivity could only reasonably be considered to apply to these types of mutations. However, assuming that all different types of mutations were broadly covered by the validation (eg, all possible nucleotide substitutions, different lengths of insertion and deletion and so on), it would be reasonable to say that sensitivity of mutation scanning in this gene using this method had been shown to be ≥97% (95% CI).

It is often appropriate to examine particular categories separately on the basis of specific knowledge of a test system. For example, it is known that certain single-base insertions or deletions in homopolymer stretches can be refractory to detection by high-resolution melting. To gain a realistic understanding of how relevant this might be, particular attention might be paid to this group of variations by including a disproportionate number in the validation. The specific gene or disease should also be considered: if only amino-acid substitutions are expected, a reduced sensitivity to single-base insertions would be irrelevant.

Broadly speaking, the limits of extrapolation can be defined by coverage of the parameters considered to be critical to the successful outcome of the test. That is, if mutation type is considered as a critical factor in achieving a correct result with the given test, then as many

different types of mutations need to be included in the validation as possible. Equally, if the G+C content of the template is considered a critical factor, validation is only applicable to fragments within the G+C content range covered by the validation. This means that a validation of a technology could be applicable to a new gene even if the validation was carried out exclusively on another gene or genes, provided the test carried out on the new gene falls within the critical parameters of the validation (obviously, in this case, it is critical to ensure that the correct fragments are being amplified). In this case, the gene itself is not a critical factor.

Potentially critical factors should be identified and evaluated at the development stage, on the basis of previous experience and expertise with the technology being validated. However, with primary validation of new technology, attempts should be made to identify the key parameters by performing an evaluation covering as many different potential factors as possible (full or partial factorial). It is also recommended that interlaboratory reproducibility be evaluated (see the section 'Type A quantitative tests').

Sample selection

The limits of extrapolation of the validation results is ultimately defined by the choice of samples, which itself is generally limited by the availability of positive controls. For this reason, it is essential that the sample profile be clearly detailed in the validation report, together with an analysis of how this relates to the factors considered critical to the performance of the test.

Positive (mutant) samples should be chosen to represent as broad a range of results as possible, bearing in mind the desire or requirement for extrapolation of the results. This will depend on the purpose of the test under validation. For example, for validation of a method for mutation scanning, samples containing as many different mutations as possible should be included in the validation. In this context, it is not normally important that the mutations/variations chosen for the validation are actually pathogenic, as this is not normally relevant to whether they will be detectable. It should be noted that including multiple examples of the same mutation in the same amplicon will not increase the power of the study to determine sensitivity, as each repeat cannot be considered different with respect to sensitivity. It is also valuable to include examples in which potentially confounding variations exist (ie, is it possible to detect a mutation in a fragment containing a certain common polymorphism?).

In general, it is desirable to include samples containing mutations that represent the range of possible variation in parameters that are important to the technique under test. For example, key parameters for a technique that relies on heteroduplexing or melting would include the G+C content of the fragment, the position of the mutation in the fragment and the actual nucleotide change.

In some cases, particularly when validating a new technology, local limitations of sample availability may necessitate an interlaboratory collaboration to collect a suitable number of samples to attain the required power for diagnostic validation.

Sample size (numbers)

The number of samples used in a validation determines its statistical power, which is a measure of how much confidence can be placed on the results of the validation. Therefore, validation sample size is ultimately one of the most important factors in determining the analytical use of the test. Unfortunately, definitive guidelines defining specific sample sizes cannot be realistically given, as the requirement is so dependent on a wide range of factors, including the nature and performance of the test, the critical parameters, the way in which the test will be used in practice and the confidence level required for clinical use. A large number of tools for determining sample size, given certain input criteria (eg, confidence interval), are freely available on the internet (eg http://www.statpages.org/#Power, accessed May 2010).

The Clinical and Laboratory Standards Institute provides a number of evaluation protocols (prefixed EP) making reference to sample size requirements for a variety of situations.[29–33] Although these tools will give useful estimates of the numbers of samples required, the limiting factor is often the availability of suitable control samples,[34] even in the case of verification, which requires less-stringent analysis and therefore fewer samples. In this case, it is critical to understand the statistical relevance of using the given sample size and how the confidence level achievable with this sample size affects the utility of the test. It is recommended that statistical advice be sought and this is carefully reviewed in the context of clinical utility. As Jennings *et al*,[5] state: 'Although supporting evidence is essential to scientific understanding, it must be recognised that statistically significant data may not be available for every aspect of every validation study. There is simply not enough patient material, financial support, or scientist/technologist time to support such rigorous data collection. Therefore, medical judgement in the context of in-laboratory data and the larger healthcare system is essential to deciding when a test is ready to be introduced for patient care.'

Whatever the availability of samples or the outcome of the validation, it is important to accurately record all details in the validation file, including confidence levels and the basis of any decisions made.

Qualitative tests

Estimating power. In the case of qualitative tests, there is a useful rule of thumb that can be used to estimate the power of a study given a particular number of samples. This can be illustrated by the following two qualitative validations of a methodology for mutation scanning:

(a) Validation using 30 different mutations.
(b) Validation using 300 different mutations.

If all mutations were correctly identified in both validations, the measured sensitivity would be 100% in both cases. However, we are likely to be much more confident in the results of validation (b) because a wider range of different mutations has been tested. This difference relates to the confidence that certain mutations, which cannot be detected by the technique, have not been excluded from the validation by the random selection of samples. This confidence increases as more different mutations are tested. This problem is referred to in statistics as sampling error. For a qualitative test, the goal is to determine a sample size that will provide sufficient power to determine sensitivity and specificity to the desired level of confidence for the particular application.

Precise calculations can be complex, but for practical purposes the 'rule of 3' provides a sufficiently accurate estimate of power according to sample size.[35–37] This states that, at 95% confidence, the probability of an event that is not seen in validation of sample size n is 3/n. An illustration of the use of the 'rule of 3' using the examples above is given in Table 4.

With molecular genetic tests, technologies are often highly sensitive and the target of validation is often a sensitivity approaching 100%; a test that does not achieve a measured sensitivity of 100% is often not considered suitable for diagnostic purposes. Although it is likely that a false negative would be found given a big enough sample size, this expectation does mean that sample numbers calculated using the 'rule of 3' generally yield the required results.

Table 4 The effect of sample size on statistical power to determine sensitivity

Validation	*n*	*Experimental sensitivity (%)*	*3/n (probability of an FN)*	*Maximum sensitivity*
(a)	30	100	0.1 (or 10%)	≥90% (95% CI)
(b)	300	100	0.01 (or 1%)	≥99% (95% CI)

Abbreviation: FN, false negative.

In practical terms, the 'rule of 3' will give very accurate estimates for studies in which $n > 60$; below this, the estimates become overcautious, which is not a bad thing in diagnostics. This rule is valid for any proportion; therefore, it can be used for either sensitivity or specificity.

Study design. As we have seen, the power of validation data is related to sample size. The number of positive samples (mutant) will prescribe the power to estimate sensitivity, and the number of negative samples (normal) that of specificity. For most applications it is sufficient to include equal numbers of mutant and wild-type samples; this will yield equal power to estimate both sensitivity and specificity.

This has a practical implication: for the validation of a mutation scan of over 50 amplicons using 100 mutant samples, it is not useful to screen all samples for all amplicons (ie, total of $50 \times 100 = 5000$ tests). This equates to 100 analyses of mutant samples (power to estimate sensitivity=97% (1−3/100 by 'rule of 3')) but to 4900 analyses of normal samples (power to estimate specificity=99.94% (1−3/4900)). There is clearly a disproportionate power to estimate specificity, which in this case is likely to be the less important measure. It would be sufficient to perform 100 analyses of normal samples (total analyses 200), although it would be sensible to evenly distribute these analyses among the amplicons. In situations in which sensitivity or specificity is considered to be particularly important, it may be appropriate to weight the number of mutant and normal samples appropriately.

It is critical that validation be performed without any knowledge of the actual status of each sample (ie, blinded analysis), especially in the case of categorical and qualitative tests. To eliminate systematic errors or bias, consideration should also be given to sample order, which should be randomized as much as is practically possible. It may also be beneficial to introduce redundancy into the experiment (eg, by duplication) to ensure coverage of all the required results. Although this is not critical to the validation results *per se*, it can save time repeating failed analyses. In addition, these data can be used in the determination of precision (repeatability and/or reproducibility).

REPORTING THE RESULTS

Comprehensive and clear documentation of validation is extremely important, both during the preimplementation phase and during ongoing validation. When reporting the results of a validation experiment, it is important to include the derived estimates of diagnostic accuracy, including confidence intervals and all details that may affect the interpretation of these estimates, including the following:

Sample inclusion criteria
Nature of the samples
Details of reference method
Technical details
Handling of failures
Critical parameters tested
Equipment details.

Reporting estimates of accuracy

Quantitative and categorical tests. In all cases in which estimates of accuracy are reported, some measure of the confidence that is applicable to the estimate should also be given. The confidence applied to quantitative measures is essentially the precision (with the exception of measures of probability, which are measures of confidence in themselves). This can most usefully be expressed as a confidence interval around the mean of the replicate results. The following is a simple guide to calculating confidence intervals:

1. Calculate the mean of the replicates (M)
2. Calculate the standard deviation of the replicates (SD)
3. Calculate the standard error $s_M = s/\sqrt{N}$ (where N=number of replicates)
4. Calculate degrees of freedom, d.f.$=N-1$
5. Find t for this d.f. using a Student's t table
6. Lower confidence limit$=M-(t \times s_M)$
7. Upper confidence limit$=M+(t \times s_M)$.

For a comprehensive discussion on expression of uncertainty in relation to quantitative tests, refer to the European co-operation for Accreditation document EA-4/14.[38]

Qualitative tests. When reporting estimates of accuracy for a qualitative test, the measured sensitivity and specificity are not useful figures on their own, as they only relate to the specific samples tested in the validation (eg, the proportion of gold standard positives correctly identified). To apply the estimates to a wider population and to allow the validation results to be realistically compared with others, a confidence interval must be given. This is a function of the measured results and the sample size. Table 5 gives an example of the results of three experiments with different sample sizes but for which the measured sensitivities were identical. It is clear that the larger sample size of experiment C gives a much smaller confidence interval.

Such ambiguities are very common in the reporting of diagnostic accuracy.[39] At best, they can preclude any realistic comparison of different validation experiments; at worst, they can provide misleading diagnostic information with potentially serious consequences for patient care.

To improve this situation, estimates of accuracy should always be based on valid calculations and be given with appropriate confidence intervals; for example, the lower and upper limits between which there is 95% confidence that the sensitivity/specificity for the wider population falls.

In cases in which the measured sensitivity and/or specificity is 100% and the sample size is ≥60, the 'rule of 3' (as described in the section 'Estimating power') reference is sufficiently accurate to determine the confidence interval. Only the lower confidence limit need be stated, as the upper limit is 100%.

It is important to note that using the 'rule of 3' in this context is only valid if all tested mutations are detected. In situations in which the measured diagnostic accuracy is less than 100%, more complex statistics are required to calculate the confidence interval. It is recommended that the exact method based on the binomial distribution be used, as confidence intervals near 100% need to be skewed (ie, the interval above and below the measured result is not equal) to avoid upper confidence limits '>100%'. A detailed description of the method, together with instructions on performing the calculations in Microsoft Excel, is available on the NIST engineering statistics handbook website.[40] In all cases in which measured diagnostic accuracy is less than 100%, it is recommended to consult a competent statistician.

Table 5 Confidence intervals for experiments with apparently equivalent sensitivities

Experiment	Experimental result	Experimental sensitivity (%)	Confidence interval (95% confidence) (%)	Confidence range (%)
A	1 FN in 150	99.3	96.9–99.9	3.09
B	2 FN in 300	99.3	97.9–99.9	1.96
C	20 FN in 3000	99.3	99.0–99.6	0.53

Abbreviation: FN, false negative.

CONCLUSION

This paper has outlined the basic principles for including validation and verification in an implementation process for molecular genetic testing. We have described the different types of tests and the key components for validation, and suggested some relevant statistical approaches. The standardized validation *pro forma* provided in the Supplementary data can be used to guide and record validations and verifications for the purposes of quality management and accreditation. Any suggestions for additions or alterations should be addressed to the corresponding author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Patrick M Bossuyt (Department of Clinical Epidemiology, Biostatistics & Bioinformatics, Amsterdam, The Netherlands), Ian Mann (Swiss Accreditation Service) and Paul Strike (Salisbury Foundation Trust, UK) for their help and advice, and Milan Macek Jr (Charles University, Prague) for hosting the meetings. This study was funded by Eurogentest and by the UK Department of Health.

USEFUL WEB SITES AND DOCUMENTS

Statistics:

http://www.fao.org/docrep/W7295E/w7295e08.htm (accessed May 2010) – Basic statistics

http://davidmlane.com/hyperstat/index.html (accessed May 2010) – Comprehensive handbook of statistics

http://www.itl.nist.gov/div898/handbook/index.htm (accessed May 2010) – Comprehensive handbook of statistics

http://en.wikipedia.org/wiki/Statistics (accessed May 2010) – Useful descriptions of statistical tests

http://faculty.vassar.edu/lowry/vsmap2.html (accessed May 2010) – A useful resource for statistical tests (with calculators).

Validation procedures:

Clinical laboratory standards institute (CLSI) publish a range of protocols and standards that may be useful for diagnostic genetic applications. http://www.clsi.org/Source/Custom/Currentdocs.cfm?Section=Current_Versions_of_CLSI_Documents (accessed May 2010).

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APPENDIX

EuroGentest (Unit 1) working group: Mike Zoccoli (Celera, Alameda, USA), Jana Camajova, Petra Křenková, Patricia Norambuena, Alexandra Stambergova and Milan Macek (Charles University Prague, 2nd Faculty of Medicine and University Hospital Motol, Department of Biology and Medical Genetics; Prague, Czech Republic), Isabelle Moix (Molecular Diagnostic Laboratory, Service of Genetic Medicine, Geneva University Hospitals, Switzerland), Patrick M. Bossuyt (Department of Clinical Epidemiology, Biostatistics & Bioinformatics, Amsterdam, The Netherlands), Els Voorhoeve and Bert Bakker (Department of Human and Clinical Genetics, LUMC St Radboud, Leiden, The Netherlands), Sarah Berwouts, Tom Janssens and Ivo Salden (KU Leuven, Centre for Human Genetics, Leuven, Belgium), Trudi McDevitt and David Barton (National Centre for Medical Genetics, Dublin, Ireland), Jean Amos-Wilson (Sequenom, San Diego, USA), Ian Mann (Swiss Accreditation Service SAS, Lausanne, Switzerland), Hans Scheffer (University Medical Centre Nijmegen, Nijmegen, The Netherlands).

Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

7.4 Norambuena, et al. (2012)

Neuroendocrinology Letters Volume 33 No. 1 2012

ADP-ribosyltransferase 3 (*ART3*) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from a Japanese population

Patricia Alejandra NORAMBUENA, Jan DIBLÍK, Petra KŘENKOVÁ, Petra PAULASOVÁ, Milan MACEK Sr., Milan MACEK Jr.

Department of Biology and Medical Genetics – Center for Reproductive Medicine & Genetics, Charles University 2nd Medical Faculty and University Hospital Motol, Prague, Czech Republic

Correspondence to: Prof. Milan Macek Jr. MD., PhD.
Department of Biology and Medical Genetics, Charles University
2nd Medical Faculty and University Hospital Motol
V Úvalu 84, 150 06 Praha 5, Czech Republic.
TEL: +420 2 2443 3501; FAX. +420 2 2443 3520;
E-MAIL: milan.macek.jr@lfmotol.cuni.cz

Submitted: 2011-10-21 Accepted: 2011-11-15 Published online: 2012-00-00

Key words: **ADP-ribosyltransferase 3; High Resolution Melting; male infertility; male subfertility; small amplicons**

Neuroendocrinol Lett 2012; 33(1):101–105 **PMID:** ----- NEL330112AXX © 2012 Neuroendocrinology Letters • **www.nel.edu**

Abstract

OBJECTIVES: In about 50% of male infertility the underlying pathogenesis remains unknown. A recent Japanese study provided evidence that the rs6836703: G>A single-nucleotide polymorphism (SNP) from the ADP-ribosyltransferase 3 (*ART3*) gene is significantly associated with non-obstructive azoospermia. However, the functional significance of this association is unknown and replication studies in unrelated populations are thus necessary.
DESIGN: In this study, 257 fertile Czech controls of proven paternity and 98 sub-/infertile patients selected according to stringent exclusion / inclusion criteria were genotyped by High Resolution Melting (HRM) of small amplicons.
Setting: This study was performed at University Hospital Motol – Laboratory of reproductive genetics using routinely analyzed cases.
RESULTS: Significant differences in allele distribution between fertile and sub-/infertile men were found (OR=1.78, 95% CI: 1.17–2.70; p=0.007). Following sub-stratification of cases according to their sperm counts we found that observed differences in allele distributions were increased in oligozoospermic men with sperm counts of <15 million sperm/mL (OR=1.98, 95% CI: 1.28–3.07; p=0.002). This difference was also reflected in genotype distributions between fertile and sub-/infertile men (p=0.008), and fertile versus oligozoospermic men (p=0.004).
CONCLUSIONS: Our study serves as a first replication of the original Japanese report and opens new avenues of research. Compared to the Japanese patient cohort, where cases with AZF microdeletions were included, we provided evidence that the analyzed *ART3* variant is associated with quantitative impairment of spermatogenesis.

To cite this article: **Neuroendocrinol Lett** 2012; 33(1):101–105

ORIGINAL ARTICLE

Abbreviations:

ART3	- ADP-ribosyltransferase 3
ART	- Assisted Reproduction Treatment
ARTs	- mono-ADP-ribosyltransferases
AZF	- Azoospermia Factor
bp	- base pair
χ^2	- chi-square
DNA	- Deoxyribonucleic Acid
HapMap-CEU	- Haplotype Map of Utah residents with ancestry from northern and western Europe
HRM	- High Resolution Melting
HWE	- Hardy-Weinberg Equilibrium
NOA	- Non-Obstructive Azoospermia
OR	- Odds-Ratio
PCR	- Polymerase Chain Reaction
SNP	- Single-Nucleotide Polymorphism
WHO	- World Health Organization

INTRODUCTION

Infertility affects approximately 10–15% of couples in Western countries and in about 50% of male infertility cases the underlying pathogenesis has not been identified (Krausz & Giachini 2007; Ferlin *et al.* 2006). Considering the complexity of spermatogenesis and the increasing number of genes associated with this process, it is likely that molecular alterations in such genes are responsible, at least in part, for the "idiopathic" cases of male infertility (Aston & Carrell 2009). Thus far, there have been multiple attempts to identify associated genetic risk factors (Krausz & Giachini 2007; Ferlin *et al.* 2006; Aston & Carrell 2009).

Recently, a Japanese report provided evidence that the rs6836703: G>A single-nucleotide polymorphism (SNP) of the ADP-ribosyltransferase 3 (*ART3*) gene located into the intron 10 (NCBI database, reference sequence NT016354.18, accessed by 05-25-2010), is significantly associated with non-obstructive azoospermia (NOA; p=0.027) (Okada *et al.* 2008). The *ART3* is a single-copy gene located on chromosome 4p15.1-p14, contains 11 exons and spans 33.13 kb of DNA (Glowacki *et al.* 2002; Friedrich *et al.* 2006b). The ART3 protein is a member of the mono-ADP-ribosyltransferases (ARTs) family (EC 2.4.2.31); they catalyze the reversible post-translational protein modification mono-ADP-ribosylation that can be used as a mechanism to regulate endogenous protein functions (Koch-Nolte 1997; Glowacki *et al.* 2002; Friedrich *et al.* 2006a). Currently, the best characterized ARTs are bacterial toxins, e.g. related to cholera or pertussis, which interfere with signal transduction by attachment of ADP-ribose onto regulatory G-proteins (Koch-Nolte 1997; Glowacki *et al.* 2002; Friedrich *et al.* 2006a). However, the biological function of ART3 remains unclear since this protein lacks the active site motif (R-S-EXE) that is essential for the catalytic activity of arginine-specific transferases (Friedrich *et al.* 2006a; Friedrich *et al.* 2006b). In humans, the ART3 protein is expressed predominantly in spermatocytes, suggesting that it could play an important role in spermatogenesis (Friedrich *et al.* 2006a; Friedrich *et al.* 2006b; Okada *et al.* 2008).

The aim of this study is to replicate the Japanese observation in a representative cohort of Czech sub-/infertile males versus fertile controls in order to substantiate the association of the *ART3* rs6836703: G>A variant with impaired spermatogenesis and open new areas of research which may elucidate the role of *ART3* in spermatogenesis.

MATERIALS AND METHODS

A total of 257 males with proven paternity (i.e. fathered at least one child by natural conception) from a random cohort examined between 2003–2011 at our Department were genotyped for the rs6836703: G>A *ART3* variant. Their results were compared with corresponding data from 98 sub-/infertile men who underwent assisted reproduction treatment (ART) at our Center. Their clinical selection was in accordance with previously published exclusion criteria (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008). Patients with foreign origin and known causes of infertility were excluded (e.g. with varicocele, AZF microdeletions, orchitis, cryptorchidism, chromosomal aneuploidies associated with infertility such as aberrations of gonosomes – data not shown, obstructive azoospermia) (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008). The sub-/infertile group was further substratified into two groups according to the WHO classification (WHO 2010): a) azoospermic (n=18) and b) oligozoospermic (n=80, sperm counts <15 million sperm/mL). All cases and controls involved signed the informed consent that was approved by the Ethics Committee of the University Hospital Motol.

For genotyping we used the previously validated method High Resolution Melting (HRM) of small amplicons (Norambuena *et al.* 2009). Genomic DNA was extracted from leukocytes of peripheral blood using PUREGENE Genomic DNA Purification Kit (Gentra Systems, MN, USA) according to manufacturer's recommendations and stored at –20 °C. Before storage, all DNA samples were diluted to a concentration of 10 ng/µL using the PUREGENE™ DNA Hydratation Solution from Gentra Systems as dilution buffer, dilutions were kept at +4 °C. Primers were designed to amplify a 49 bp fragment around the SNP site and to avoid other sequence variations within the primer region (F: 5´-GTTGCTCTGGGTGGTGTTGAGC/R: 5´-ACAGTAGTGTCCCAGGCCTTCAC). PCR reaction was performed in a 10 µL reaction volume which consisted in 2 µL of genomic DNA (10 ng/µL) added to 8 µL of "reaction master mix" consisting of 1X LightCycler® 480 High Resolution Melting Master with 2.5 mM $MgCl_2$ (Roche Diagnostics, Germany) and 0.5 µM of forward and reverse primers. Rapid two-step PCR cycling, with an initial denaturation of 5 min at 95 °C continued by 35 cycles of 5 s at 95 °C and 25 s at 69 °C for annealing

and extension. Then, the program allowed one step for heteroduplex formation by heating to 95 °C for 10 s and cooling down to 40 °C for 5 s. For HRM, the plate was pre-heated at 72 °C for 10 s and afterward, heated from 72 °C to 95 °C performing 25 acquisitions per °C. The PCR reaction was performed on a 96-well plate in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany).

For melting curve analysis all samples with late amplification, monitored by real time PCR and/or with a fluorescence <60% of the maximum value, were excluded (Norambuena *et al.* 2009). Following amplification and HRM respective normalized and normalized temperature-shifted difference plots for the rs6836703: G>A variant were analyzed. We were able to perform software-based genotype calls and visually differentiate the three expected melting profiles for each genotype group: G/G, G/A and A/A (Figure 1). HRM of small amplicons was our method of choice for the examination of the rs6836703: G>A SNP since it is a rapid, accurate and cost effective method (Norambuena *et al.* 2009).

Fertile controls are in conformity with Hardy-Weinberg equilibrium (HWE; non-significant results from Pearson's chi-square test /χ^2/ calculated from rs1801133: C>T genotype/allele distributions; data available upon request) which marginalizes sampling bias. Association studies were analyzed by the odds-ratio (OR) and χ^2 (with Yates´s correction, where applicable), where *p*-values ≤0.05 were considered statistically significant.

RESULTS

We observed a marked overall difference in allelic distribution between fertile and sub-/infertile Czech cohorts, since the frequency of allele "A" was significantly increased in infertile males (p=0.007; Table 1). This observation was also reflected in the distribution of G/A and A/A genotypes in sub-/infertile men (p=0.008; Table 2). When breaking down our aggregate results according to sperm counts (as specified above), we observed that allele "A" was particularly "enriched" in oligozoospermic men, i.e. in group b), compared to fertile controls (p=0.002; Table 1).

DISCUSSION

In this study we have replicated the significant association between the *ART3* rs6836703: G>A variant originally detected in the Japanese population in an unrelated Czech cohort. The "A" allele was comparatively "enriched" in oligozoospermic men, while the corresponding reduction of the allele "G" in infertile cases indirectly supports the "protective effect" of this allele on spermatogenesis.

Our observation of the increase of allele "A" frequencies in cases versus controls is not skewed due to insufficient sample size and/or sampling bias as verified by non significant deviation from the Hardy-Weinberg equilibrium in control samples. Given the low allele frequency for the "A" allele in the *ART3* variant rs6836703: G>A, we calculated HWE equilibrium from rs1801133: C>T (MTHFR c.677C>T; p.A222V) genotype/allele distributions which has a higher frequency for the recessive allele (data available upon request) minimizing type I error which is the probability of rejecting a true null hypothesis leading to false exclusion of associated markers, usually disease-associated SNPs, in HWE calculations. Tests for HWE presume that genotypes are randomly collected from the general population. In most association studies controls are selected by their apparently health status, thus being relatively "over-represented" compared to the general population. This could be a compounding feature for common diseases but is not applicable to our case (Salanti *et al.* 2005; Li & Li 2008; Wang & Shete 2010). In addition, potential of false positivity in HRM of small amplicons is negligible (Liew *et al.* 2004; Norambuena *et al.* 2009; Wittwer 2009).

Absence of association with azoospermia (Tables 1 and 2) may indicate that the *ART3* variant rs6836703: G>A likely causes a milder, i.e. "quantitative", reduction in spermatogenesis within the Czech population. However, we did not find any homozygous for the "A" allele within the azoospermic cohort most likely due to the lower number of azoospermic patients (n = 18) who

Tab. 1. *ART3* rs6836703: G>A allele distribution.

	Allele		OR (95% CI)	*p*-value
	G	A		
Fertile	442/514 (0.860)	72/514 (0.140)		
Sub-/infertile men (all)	152/196 (0.776)	44/196 (0.224)	**1.78 (1.17-2.70)**	**0.007**
Azoospermic	31/36 (0.861)	5/36 (0.139)	0.99 (0.37-2.63)	1
Oligozoospermic	121/160 (0.756)	39/160 (0.244)	**1.98 (1.28-3.07)**	**0.002**

Tab. 2. *ART3* rs6836703: G>A genotype distribution.

	Genotype			
	G/G	G/A	A/A	*p*-value
Fertile	194/257 (0.755)	54/257 (0.210)	9/257 (0.035)	
Sub-/infertile men (all)	58/98 (0.592)	36/98 (0.367)	4/98 (0.041)	**0.008**
Azoospermic	13/18 (0.722)	5/18 (0.278)	0/18 (0.000)	0.939 (1)
Oligozoospermic	45/80 (0.563)	31/80 (0.388)	4/80 (0.050)	**0.004**

(1) Yate's correction

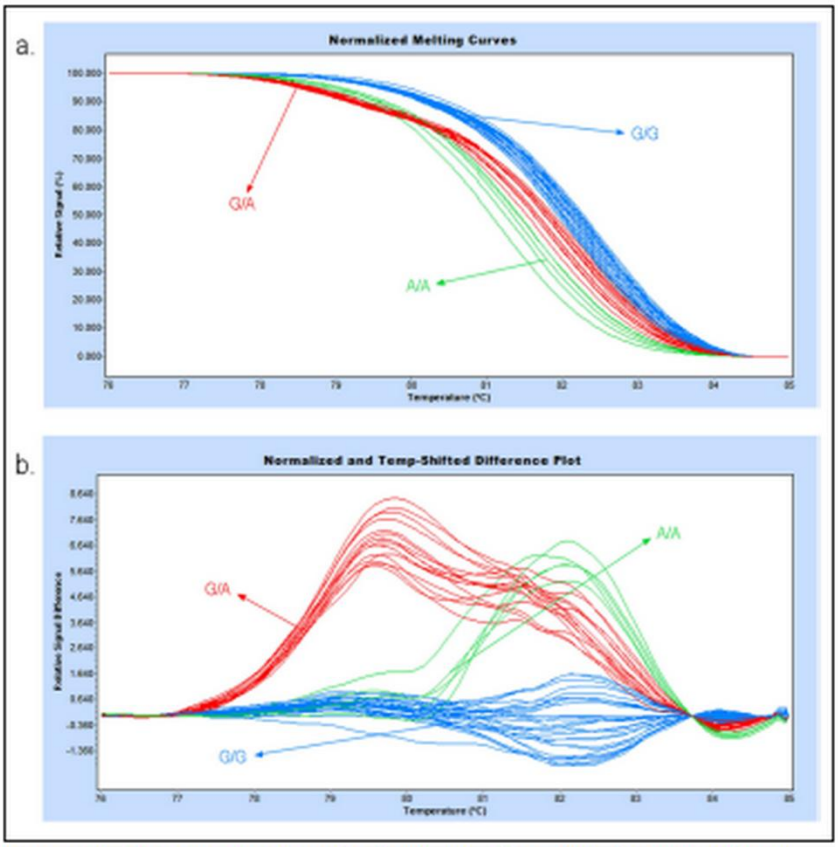

Fig. 1. Genotyping of rs6836703: G>A SNP by HRM of small amplicons. From the normalized melting curves (panel "a") it is possible to recognize both homozygous groups (G/G and A/A) by the difference in their melting temperature (Tm) and the heterozygous group (G/A) by the difference in the melting curve shape. Normalized temperature-shifted difference plot (panel "b") helps to the assignment by eye-inspection increasing the difference of the shape between melting profile groups. Control samples for each genotype (G/G, G/A and A/A) were run in duplicate. Each genotype group is indicated by arrows.

were selected according to stringent exclusion / inclusion criteria (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008) from a larger initial cohort of infertile cases. Moreover, there is a particularly low frequency of the "A/A" genotype in the European population (HapMap-CEU: G/G=0.750; G/A=0.250; A/A=0.0; dbSNP accesed by 09-19-2011) which further substantiates its potentially negative evolutionary selection.

It needs to be noted that the Japanese study included only patients with "non-obstructive azoospermia" without any further sub-stratification of cases according to their sperm counts by inclusion of sub-fertile patients (Okada *et al.* 2008). Moreover, the Japanese study group included patients with AZF microdeletions who could create, compared to the Czech patient cohort, a strong confounding variable. We presume that observed inter-population differences might also reflect alternative study designs (multiple variant versus single variant testing), differences in cohort sizes and/or the role of ethnic background / environmental factors (Tüttelmann *et al.* 2007; Aston & Carrell 2009).

Furthermore, the ethnic background of case-control studies needs to be taken into account. For instance, the previously reported association between a moderate expansion in the CAG repeats in the androgen receptor and reduced spermatogenesis in Asian populations has

not been replicated in European studies (Ferlin *et al.* 2006; Rajender *et al.* 2007). Thus, in this study replication of original Japanese findings in an unrelated Czech cohort markedly increases the likelihood of its "real" functional association.

To date there is no further information on the pathway that disrupts spermatogenesis which could involve the rs6836703: G>A and/or ART3 protein (Glowacki *et al.* 2002; Friedrich *et al.* 2006a; Friedrich *et al.* 2006b; Okada *et al.* 2008). No difference was observed in testicular ART3 protein expression among haplogroups containing the rs6836703: G>A variant (Okada *et al.* 2008). The rs6836703: G>A variation might affect the expression/regulation of ART3 and/or of another protein(-s). In this regard, possible DNA-protein binding-sites for TFII-I, E2F-1 and PEA3 transcription factors were found for the DNA sequence containing the observed variation in *ART3* (PROMO – online program; Messeguer *et al.* 2002; Farré *et al.* 2003). Such *in silico* predictions may provide leads into additional studies of function of the ART3 protein in spermatogenesis. In any case, further replication studies in unrelated European-derived populations (Lao *et al.* 2008) are necessary in order to corroborate this difference. Eventual positive associations detected in unrelated populations could provide additional evidence for utilization of the respective *ART3* variant in reproductive genetics diagnostics.

In summary, our study provided the first replication of the original Japanese report which suggested that *ART3* rs6836703: G>A variant is implicated in impaired spermatogenesis and thus opens new avenues of research.

ACKNOWLEDGEMENTS

We would like to thank to Drs. Tereza Piskáčková, Miroslava Balasčaková for providing patient data, and to Dr. Alexandra Štambergová for technical assistance. This study was supported by MZ0FNM2005 from the Czech Ministry of Health and CZ.2.16/3.1.00/24022OPPK to MM Jr.

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