

SPECIAL ARTICLE

Diagnostic DHPLC Quality Assurance (DDQA): A Collaborative Approach to the Generation of Validated and Standardized Methods for DHPLC-Based Mutation Screening in Clinical Genetics Laboratories

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Genetic testing in a clinical diagnostic environment must be subject to rigorous quality control procedures, in order to ensure consistency and accuracy of results. Denaturing high performance liquid chromatography (DHPLC) has become a standard prescreening tool for mutation detection, offering very high efficiency and sensitivity of detection. Despite the relatively simple software-assisted assay setup, DHPLC is a complex assay, and quality control is reliant on ensuring optimal instrument performance, excellent assay design and validation, and sufficient user training and proficiency to interpret results. We describe here a unique collaborative effort by a group of diagnostic clinical genetics laboratories with DHPLC expertise who, together with the manufacturer of one of the most widely used DHPLC platforms, have generated standard operating procedures (SOPs) for instrument operation and maintenance, and for mutation detection by DHPLC. We also describe the validation of a disease-specific SOP for DHPLC assisted mutation screening of the *MECP2* gene associated with Rett syndrome. The proposed SOP was validated, and used independently in two laboratories to introduce *MECP2* testing. In addition, we provide empirically derived normal ranges for the WAVE[®] System Mutation Standards, which are essential for optimal instrument performance. This effort was initiated to try to standardize DHPLC-based mutation screening procedures across laboratories, and so increase the overall quality of this testing method. This endeavor will thus save each laboratory from having to generate SOPs on their own, which is a lengthy and laborious task. In this respect, we define “generic” SOPs as procedures that are easily adaptable to the individual laboratories’ quality systems. *Hum Mutat* 25:583–592, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: DHPLC; standard operating procedure; SOP; *MECP2*; Rett syndrome; quality control; mutation detection

INTRODUCTION

The success of the Human Genome Project and a universal demand for significant breakthroughs in the diagnosis, prevention, and treatment of the world’s major genetic disorders have resulted in the discovery of a plethora of human genes, the number of which appears to increase daily. As the number of these genes rises, so does the need for new methods and technologies to deliver accurate, efficient, and cost effective genetic diagnosis. As a direct result, the demand for genetic testing from clinical diagnostic laboratories is increasing at a rate of over 20% per year [McGovern et al., 1999], making this the fastest growing discipline in diagnostics.

Quality control and harmonization of genetic testing procedures has therefore become ever more important. In a relatively complex and often technically demanding field, it is essential to ensure that all laboratories who offer a genetic testing service meet minimum

levels of expertise, follow standardized protocols, and participate in internationally recognized external quality assessment schemes (also known as proficiency testing) to continuously monitor and improve the quality of results produced [Dequeker and Cassiman, 2000; Dequeker et al., 2001; Ibarreta et al., 2004].

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In stark contrast to simple “home-brew” and “kit-based” protocols for the analysis of common single gene mutations, molecular genetic analysis for many diseases now demands a higher level of expertise to both perform the analysis and interpret the results. For some diseases, hundreds of mutations within a single gene have been found, and can often be unique to an individual. Molecular diagnostics therefore frequently necessitates complete gene scanning rather than simple mutation calling. The technologies currently available for novel mutation detection are usually semiautomated, complex assays, requiring not only a relatively high level of user expertise in setting up and analyzing the results, but also the continual monitoring of instrument performance to ensure sensitivity and reproducibility of results.

Denaturing high performance liquid chromatography (DHPLC) (Fig. 1) has been widely adopted as the method of choice for disease gene screening for novel mutations, as it offers a very high efficiency of detection in a relatively cost-effective and semiautomated format [Ellis et al., 2000; Eng et al., 2001; Holinski-Feder et al., 2001; Le Marechal et al., 2001; Sevilla et al., 2002; Xiao and Oefner, 2001]. The molecular analysis is usually carried out using DHPLC analysis of PCR products followed by direct sequencing of abnormal fragments. Although DHPLC assay design is software-assisted, methods for each fragment (amplicon) to be analyzed must be thoroughly validated by the laboratory to ensure the set detection level and maximize the sensitivity for mutation detection along the entire length of that fragment [for example, see Oldenburg et al., 2001]. Test and instrument variables include PCR yield and quality, oven calibration, column quality, and the composition and stability of the buffers. Once optimized, the accuracy of a method can only be assured if the test is performed according to a robust standard operating procedure (SOP), documented and validated in each individual laboratory.

For those wishing to adopt this technology, this can be a daunting prospect. For existing users, validation of individual protocols in individual laboratories is time-consuming, laborious, and often limited by the number of amplicons with known variants available in that laboratory.

In an unprecedented effort, we have established the Diagnostic DHPLC Quality Assurance (DDQA) collaborative project whose specific aims are to develop standardized and validated SOPs for mutation detection by DHPLC. These SOPs should be universally applicable to all laboratories, and prevent constant reduplication of effort. Three different SOPs were generated: one for the operation and maintenance of the WAVE[®] System (Transgenomic, Omaha, NE; and Transgenomic Limited, Elancourt, France), one for mutation detection by DHPLC analysis as a whole, and one for specific applications to disease genes. In particular, we describe here the generation and validation of a SOP for *MECP2* gene (MIM# 312750) screening in the context of Rett syndrome (RS or RTT; MIM# 312750), as the first milestone of the DDQA project. The *MECP2* gene is relatively small, so it was chosen as the first gene for the DDQA group, to act as a model for subsequent SOPs. During the optimization of the different procedures, the experiments also included the fibrillin (*FBN1*; MIM# 134797) gene (Marfan syndrome; MIM# 154700) and the breast cancer genes *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600195) (familial breast cancer; MIM# 114480).

The initial intention was to make all of these SOPs publicly available in a published format. The DDQA collaborative group is not exclusive, and participation from other laboratories with DHPLC expertise has already been solicited.

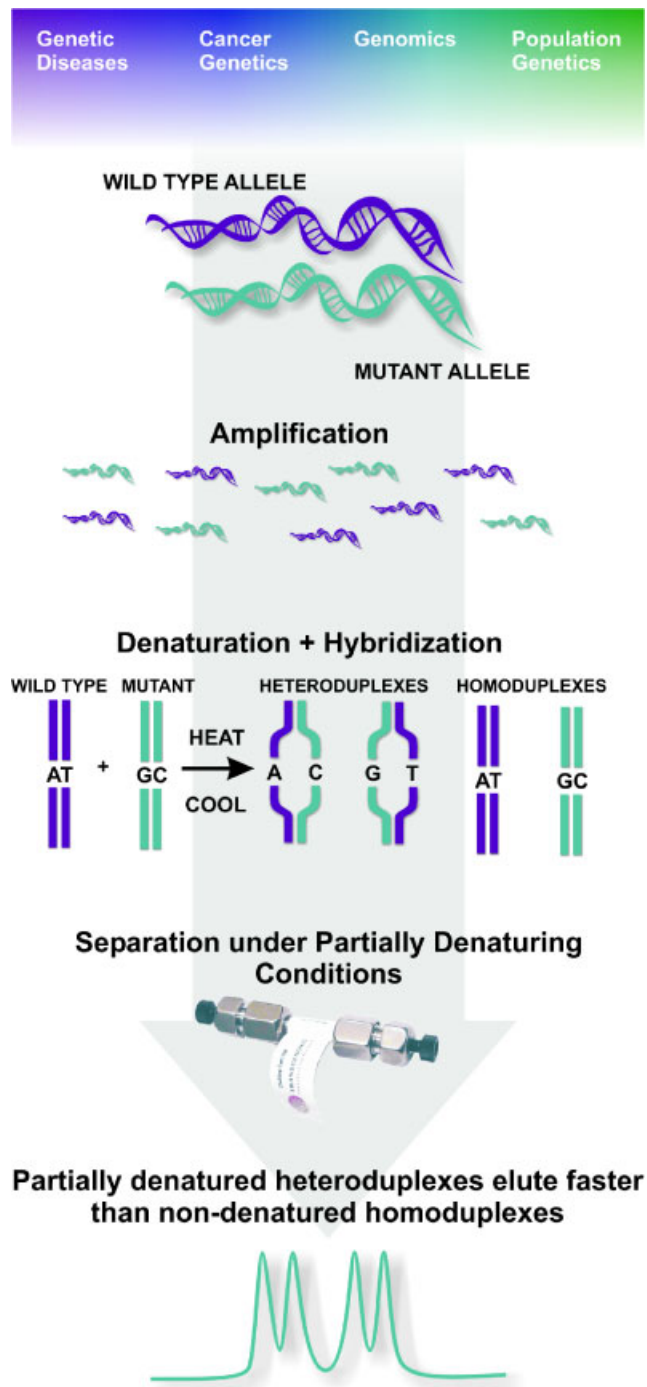


FIGURE 1. Principle of mutation detection by DHPLC analysis: after amplification of a specific allele from a DNA sample with a heterozygous variation for that locus, heteroduplexes are formed by denaturation and subsequent hybridization. Heteroduplexes have a lower denaturing temperature than homoduplexes and can be separated from the homoduplexes on a DNASep column using partial denaturing conditions.

MATERIALS AND METHODS

Diagnostic DHPLC Quality Assurance (DDQA)

DDQA is a nonexclusive group of diagnostic laboratories with DHPLC expertise working in close collaboration with Transgenomic, the manufacturer of the most commonly used platform for DHPLC analysis, the WAVE System. The aim of the group effort

is the quality assurance of molecular diagnostic tests performed with this system. The initial group consisted of coworkers from five partners: Center for Human Genetics, Leuven, Belgium; National Genetic Reference Laboratory, Wessex, UK; National Centre for Medical Genetics, Our Lady's Hospital for Sick Children, Dublin, Ireland; Department of Clinical Genetics and Human Genetics, VU University Medical Center, Amsterdam, the Netherlands; and Transgenomic Ltd., Elancourt, France. The group also included an expert in quality assurance with knowledge of International Organization for Standardization (ISO; www.iso.org/welcome.html) regulations and quality management systems. The Department of Clinical Genetics, Copenhagen, Denmark and the Division de Génétique Médicale, Hôpitaux Universitaires de Genève, Geneva, Switzerland were involved in the validation of the *MECP2* SOP.

DHPLC Systems

The DHPLC analysis described in this work was mainly performed on a WAVE 3500 HT System with a L7300+ oven and WAVEMAKER™ software 4.1.44 (Transgenomic) unless otherwise indicated. The experiments on heteroduplex stability of the *FBN1* fragments were performed on a DNASep® Cartridge (Transgenomic) and a normal run (7 min, with flow rate of 0.9 ml/min). The optimization of the *MECP2* mutation screening and analysis of the *BRCA2* fragments were performed on a DNASep HT Cartridge (Transgenomic) with a rapid run (3 min, with flow rate 1.5 ml/min).

The different PCR conditions used are described below, in the relevant sections. The equipment used for optimization was regularly maintained and calibrated against international standards according to the local quality system.

Heteroduplex Stability

Exons 26, 37, and 63 of the fibrillin (*FBN1*) gene were amplified using Platinum Taq (Invitrogen, Breda, The Netherlands) and standard PCR conditions using 100 ng of genomic DNA. The samples included normal control sample and patient samples heterozygous for mutations c.3299C>T (p.G1100V), c.3299G>A (p.G1100D), c.3302A>G (p.Y1101C) (exon 26), c.4588C>T (p.R1530C), c.4589G>T (p.R1530L), c.4605T>A (p.Y1535X) (exon 37), c.8002G>T (p.G2668C), and c.8003G>A (p.G2668D) (exon 63). Mutations in all control samples were confirmed by sequencing. Heteroduplexes were obtained by denaturation for 5 min at 95°C, followed by cooling to 26°C in 25 min, and further cooling to 4°C using a GeneAmp® PCR System 9600 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Heteroduplexes obtained from identical amplicons were stored at 4 and -20°C for 14 days prior to DHPLC analysis. The DHPLC

analyses were done at the appropriate temperatures. Details on primer sequences, PCR conditions, and DHPLC methods (gradient and analysis temperature) may be obtained upon request.

Heteroduplex Formation for Two Deletions in Exon 11 of *BRCA2*

Exon 11 of the *BRCA2* gene was amplified from DNA of different affected breast cancer patients heterozygous for two frameshift mutations (c.6497_6498delTA and c.6502_6503delTT, confirmed by direct sequencing) in a PCR fragment of 503 bp. Details on primer sequences and PCR conditions can be obtained upon request. Aliquots of the mutated (heterozygous) PCR samples and control samples were submitted to different conditions for heteroduplex formation prior to DHPLC analysis. These conditions were: 1) denaturation (95°C, 5 min); rapid cooling (-4°C/sec) to 56°C followed by an incubation of 45 min at 56°C; 2) denaturation (95°C, 5 min) followed by slow cooling at -0.03°C/sec to 45°C and 30 min incubation at 45°C; and 3) denaturation (95°C, 5 min) followed by cooling at -0.02°C/sec to 65°C [Wagner et al., 1999]. All three performed on a Biometra Uno-II thermocycler (Westburg, Leusden, The Netherlands). The samples were analyzed at optimal DHPLC conditions (conditions available on request) in a single run. In parallel, aliquots of the same samples were also submitted to heteroduplex analysis by electrophoresis on a 6% polyacrylamide gel.

Normal Range Values for the Low Range and High Range Mutation Standards for Calibration and Optimization of the WAVE System

Elution profiles of the WAVE Low Range and High Range Mutation Standards (Transgenomic) were obtained for 22 different WAVE System Model 3500 and WAVE System Model 3500A (with accelerator) (DNASep, 0.9 ml/min flow rate) and 29 Model 3500 HT (DNASep HT, 1.5 ml/min flow rate) running under Navigator® software across two independent studies. On each system, two injections of 5–8 µl of the Low Range and High Range Mutation Standard were analyzed at 56 and 70°C, respectively, using the conditions described in the WAVE System Operation and Maintenance SOP (SOP-O&M, Supplementary Appendix S1; available online at <http://www.interscience.wiley.com/jpages/1059-7794/suppmat>). All peak profiles with optimal “full” separation (four peaks) and a minimal peak intensity of 2 mV were included in determining a mean and estimating a standard deviation (SD) using a moving range. The retention time (RT) was measured for the first heteroduplex peak (RT-Het1) and the last homoduplex peak (RT-Hom2) (Fig. 2A). Delta-Het (Δ -Het) is the difference in

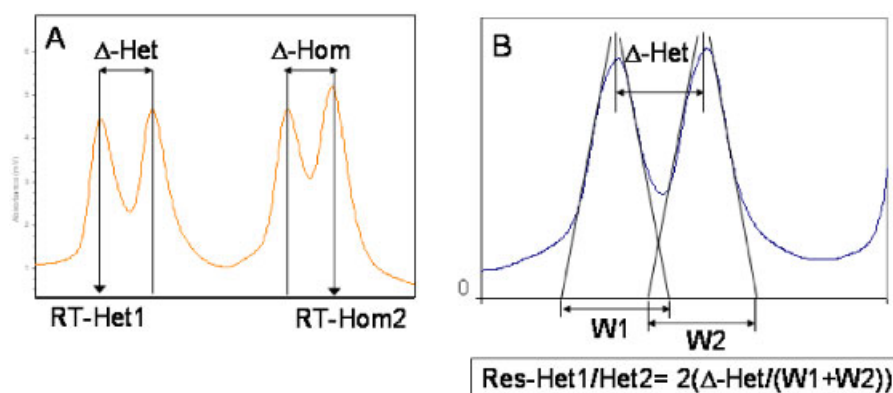


FIGURE 2. Graphical representation of the critical parameters for evaluation of the WAVE Low Range mutation standards, run and analyzed under Navigator. **A:** Retention times of the first heteroduplex peak (RT-Het1) and the last homoduplex peak (RT-Hom2) and the difference in retention time between the heteroduplex peaks (Δ -Het) and the homoduplex peaks. **B:** The resolution of the peaks determined as the difference in retention time between adjacent peaks (e.g., Δ -Het) divided by the average of the full width of the peaks (represented by $W1$ and $W2$).

RT between the two heteroduplex peaks; delta-Hom (Δ -Hom) is the difference in RT between the two homoduplex peaks. Within Navigator, the resolution of the peaks (Res-Het1/Het2, Res-Het2/Hom1, and Res-Hom1/Hom2) is determined as the difference in retention time between adjacent peaks divided by the average of the full width of the peaks. For example, the resolution of the heteroduplex peaks (Res-Het1/Het2) is defined as $(2 * (\Delta\text{-Het}) / (W1 + W2))$ (Fig. 2B). W1 and W2 are determined by drawing tangents to each side of the adjacent peaks, then calculating the distance between the two points where these tangents intercept the baseline.

The lower and upper control limit of each parameter are determined using standard Statistical Process Control protocol as the mean \pm 3SD, where the SD is estimated using a moving range ($n=2$) of the data points ($R\text{-bar}/1.128$) [Moen et al., 1999]. The consistency of the buffers (WAVE Optimized buffers; Transgenomic) used in these studies were tighter than the full range of variation allowed in their manufacturing specification. This was factored into the estimate of the SD. This additional variance adjustment is based on known effects of buffer variation on retention times of mutation standards (Transgenomic internal study of effects of WAVE Optimized buffer variation, unpublished data).

Optimization of MECP2 DHPLC Screening

A complete new set of primers was designed for amplification of the different genomic fragments of the MECP2 gene with Optimase™ DNA polymerase (Transgenomic) in one single PCR program using the prediction program available at the website www.MutationDiscovery.com. Primers were based on the MECP2 gene sequence (NT_025965.11), taking into account the SNPs available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=4204) and on www.MutationDiscovery.com. The coding exons (exon 2–4) and a minimum of 30-bp flanking intronic region were amplified in 1, 2, and 5 overlapping fragments (Ex2, Ex3Fa-b, and Ex4Fa-e, respectively). Amplification conditions were optimized on a GeneAmp PCR System 9700 (Applied Biosystems), using 100 ng gDNA, 1 μ l Optimase Polymerase (2.5 U/ μ l) and Optimase PCR buffer with Mg^{2+} (Transgenomic). Details on primer sequences, PCR conditions and heteroduplex formation are given in the SOP-MECP2 (see Supplementary Appendix S3).

The evaluation of the theoretical DHPLC analysis temperatures and gradients, as predicted by WAVEMAKER 4.1.44, was performed with the following control mutations, previously confirmed by direct sequencing: c.28G>C (p.E10Q (exon 3 fragment a, Ex3Fa); c.316C>T (p.R106W) (exon 3 fragment b, Ex3Fb); c.397C>T (p.R133C), c.455C>G (p.P152R), c.473C>T (p.T158M), c.502C>T (p.R168X) (exon 4 fragment a, Ex4Fa); c.674C>G (p.P225R), c.763C>T (p.R255X), c.808C>T (p.R270X) (exon 4 fragment b, Ex4Fb); c.880C>T (p.R294X), c.916C>T (p.R306C) (exon 4 fragment c, Ex4Fc); c.1125 C>T (p.P376S), c.1151_1191del45 (exon 4 fragment d, Ex4Fd); and c.1339G>A (p.A447T) (exon 4 fragment e, Ex4Fe). A volume of 5–10 μ l of the PCR product was injected on a DNASep HT column in a L7300+ oven and analyzed using gradients as calculated by WAVEMAKER 4.1.44. Premade 1 \times WAVE Optimized buffers were used. Analysis temperatures were optimized to achieve an optimal resolution of the homoduplex and heteroduplex peaks; gradients were adopted with time shifts to obtain homoduplex peaks between 1.5 and 2.5 minutes.

Validation of the SOP for DHPLC Screening of MECP2

The conditions given in the SOP-MECP2 were validated according to international guidelines ISO/IEC17025[1999] section 5.4 and EN-ISO 15189[2003] section 5.5. The reproducibility and repeatability of PCR and DHPLC conditions (analysis temperature, gradient, injection volume) have been assured by

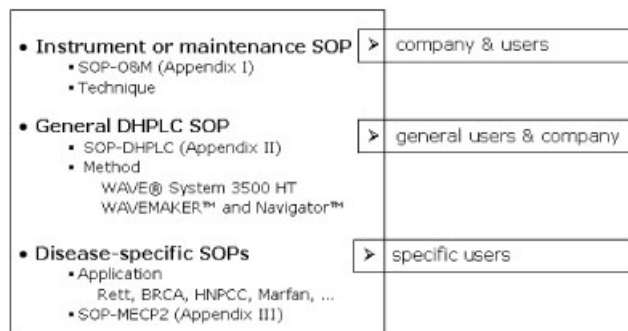


FIGURE 3. Schematic representation of the collaboration between users and the manufacturers in the generation of the three (proto)types of SOPs.

repeating the amplification and DHPLC analysis of each fragment three times with known control samples, including at least one normal control sample, the positive control described above and a negative (no DNA) control. Before each run, column and buffer conditions were assessed as described in SOP-O&M (Supplementary Appendix S1). The amplification accuracy and yield were assessed by DHPLC screening.

The influence of DNA quality and concentration on the amplification quality and yield has not been extensively tested. Poor DNA quality or low DNA concentration will result in peak signals below 2mV and, hence, the results will be rejected (see SOP-DHPLC; Supplementary Appendix S2).

The final draft of the MECP2 SOP was sent to Dr. M. Schwartz at the Department of Clinical Genetics, Copenhagen and to Dr. M. Morris at the Division de Génétique Médicale, Geneva. The laboratory in Copenhagen adopted the SOP successfully. They tested and identified clearly the variations p.R106W (Ex3Fb), c.378–109A>G, p.R168X (Ex4Fa), c.582C>T (p.S194S), and p.R255X (Ex4Fb). The laboratory in Geneva found clear and reproducible results for the mutations p.E10Q (Ex2), p.S134C (c.401C>G), p.R167P (c.502G>C), p.R168X (Ex4Fa), p.R255X, p.225R (Ex4Fb), p.R294X (Ex4Fc), p.P376S, p.E397K (c.1189G>A), and A447T (Ex4Fd). Although exactly the same protocol was used, amplification of fragment Ex4Fd failed. Different batches of primers or DNA samples did not solve the problem.

Mutation Nomenclature and Reference Sequences

For nomenclature of mutations and variations, the recommendations of the Human Genome Variation Society (HGVS; latest update November 7, 2004; www.hgvs.org/mutnomen/index.html) have been followed. DNA mutation numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon. An overview of the used reference sequences is given in Table 1.

RESULTS

Rationale and Strategy

A detailed investigation of the process of mutation screening of a disease gene by DHPLC (Fig. 1) revealed that there are

TABLE 1. GenBank Accession Numbers of the Used Reference Sequences

Gene	Gene seq	mRNA	Protein
MECP2	NT_025965.11	NM_004992.2	NP_004983.1
FBN1	NT_010194.16	NM_00138.2	NP_00129.2
BRCA2	NT_024524.13	NM_000059.1	NP_000050.1

numerous parameters and variables that need to be critically considered, especially those that specifically relate to quality assurance, i.e., the reliability, reproducibility, and accuracy of the procedure. That is why from the outset of the study, DDQA had to consider two main issues.

First, as generally applied in quality systems, a modular system of SOPs was defined. The overall procedure was split into three (proto)types of SOPs. The “operation and maintenance” SOP (SOP-O&M) describes in detail the requirements necessary for optimal use and performance of the WAVE System. The “general methodology” SOP (SOP-DHPLC) describes DHPLC as a method, applicable to mutation detection. Several “disease-specific” SOPs describe the laboratory’s approach towards mutation analysis by DHPLC of a specific gene or disease. The major advantage of this modular system is the add-on option, i.e., once a laboratory has implemented and validated the operation and maintenance SOP and general methodology SOP, individual disease-specific SOPs can easily be introduced to the framework (Fig. 3).

Second, to write these SOPs on a collaborative basis, the working group faced practical challenges. Because of the extensive experience of the diagnostic users in the group, it was unanimously agreed that the SOPs should definitely reflect and promote “best practice” in the field. In the context of the aims of DDQA, it was then possible to gather and include previous attempts from the partners to write up SOPs, and produce procedures specifically focusing on issues of quality assurance. In addition, it was important that the SOPs should also be of value to the larger diagnostic community. This is why DDQA defined and put forward the concept of generic SOPs: these SOPs give a description of the basic requirements for a platform or method and are easily adaptable to individual laboratories’ quality systems. Once these issues were addressed, it was relatively easy to chalk up the topics for inclusion in the different SOPs.

An SOP for DHPLC Instrument Operation and Maintenance

WAVE System Operation and Maintenance, SOP-O&M, Supplementary Appendix S1. The WAVE System basically consists of a pump, a buffer mixer, an autosampler, an oven, a detector, an interface, and software. Buffers and columns can be looked upon as consumables and were handled as such.

In gathering information from the DDQA working group, it turned out that, although all laboratories use the WAVE System, there is no “standard” system. First, different laboratories use different ovens. Both the L7300+ and L7300 ovens with 0.1 and 1°C accuracy, respectively, are currently in use. Also both the normal (0.9 l/min flow rate) and high throughput (HT) (1.5 l/min flow rate) systems are commonly used. Second, a number of different versions of WAVEMAKER and Navigator software (Transgenomic) with WAVEMAKER 4.1.44 build 19 and Navigator 1.5.4 being the latest versions currently in use. The fact that different systems and software are in use is not a problem per se. However, because there is no standard system, a full and detailed description of the WAVE System employed is essential for publications (articles, SOPs, validation reports, etc.) to allow experimental work to be repeated and validated by others. For this project, it was chosen to focus on the WAVE 3500 Systems with an L7300+ oven, since these systems are most widely used. In the SOP-O&M and SOP-DHPLC, notes pinpoint the most important differences for the other systems.

Most aspects of the SOP were extracted from the WAVE System user manual and the maintenance guidelines from Transgenomic; however, this SOP-O&M is unique because it is written from a specific user’s point of view, i.e., a diagnostic clinical genetic laboratory with particular emphasis on quality assurance.

A first aspect to deal with is the preventative maintenance of the system. The prescribed prerun, weekly, and quarterly maintenance are generally applicable and acceptable as good laboratory practice and were transcribed into the SOP. A regular (minimally yearly) preventative maintenance procedure, performed by a Transgenomic service representative (or a user trained by Transgenomic) is indispensable for the company’s guarantee. Quality assurance of the system without a company’s guarantee is much more elaborate and less efficient.

For the second aspect, the day-to-day WAVE System performance monitoring and quality assessment of the associated consumables (column and buffers), most WAVE customers rely on the analysis of the Low and High Range Mutation Standards (hereafter called mutation standards). However, to date, assessing the quality of the elution profiles of the mutation standards has depended entirely on the operator’s eye and estimation, a purely subjective determination. The DDQA Collaborative Group judiciously recognized that specific objective criteria, i.e., empirically-derived normal range values for mutation standards are the missing link for quality assurance of mutation detection by DHPLC.

Transgenomic agreed with the importance of these critical data and offered to generate values applicable for both users and manufacturer. The critical parameters for the Low Range Mutation Standards are the retention times of the first heteroduplex peak (RT-Het1) and the last homoduplex peak (RT-Hom2), the difference in retention time between the two heteroduplex peaks (Δ -Het) and the two homoduplex peaks (Δ -Hom) and the resolution of the different peaks (Res-Het1/Het2, Res-Het2/Hom1, and Res-Hom1/Hom2) (Fig. 2A and B). Resolution is used to characterize the quality of separation of adjacent peaks in a chromatogram and is, within Navigator, automatically calculated in the analysis page. For the High Range Mutation Standards, only the retention times of the first heteroduplex peak (RT-Het1) and the last homoduplex peak (RT-Hom2) and the difference in retention time for the heteroduplex peaks (Δ -Het) and homoduplex peaks (Δ -Hom) are taken into account. If the seven parameters of the Low Range Mutation Standard and the retention times of the High Range Mutation Standard are within the specified limits, the resolution of the peaks for the High Range Mutation Standard is optimal and is therefore not included in the minimal set of critical parameters (Transgenomic, unpublished data).

Normal ranges (mean \pm 3SD) for mutation control standards (Table 2) were calculated based on quantitative data from system capability studies by Transgenomic with the SD estimated from moving range determinations. Data were obtained for both the WAVE System Model 3500(A) and Model 3500HT, running under Navigator Software. Optimal performance of the DHPLC System can be assured if, for each parameter, the average of two injections of each mutation standard, injected back-to-back, is within the specified limits. The back-to-back injections are recommended to check the consistency of the analysis and the short-term stability.

Some users might prefer to reduce costs by replacing the commercial mutation standards with their own in house validated controls. Ideally, in such a control the four duplexes are perfectly separated within a narrow temperature range (2–4°C). The complete validation of all variables of the DHPLC system with such a standards might be less comprehensive because it does not

TABLE 2. Normal Ranges for the Critical Parameters of the Mutation Standards

	WAVE System Model 3500 and 3500A DNASep [®] Cartridge 0.9 mL/min flow rate		WAVE System Model 3500 HT DNASep [®] HT Cartridge 1.5 mL/min flow rate	
	Low range mutation standard	High range mutation standard	Low range mutation standard	High range mutation standard
RT-Het1 (min.)	3.52–4.52	3.70–5.01	1.33–1.75	1.48–2.00
RT-Hom2 (min.)	4.27–5.58	4.49–5.76	1.70–2.20	1.84–2.35
Δ-Het (min.)	0.05–0.17	0.05–0.13	0.05–0.12	0.03–0.08
Δ-Hom (min.)	0.05–0.19	0.05–0.16	0.05–0.15	0.03–0.10
Peak Intensity (mV)	2–12	2–12	2–12	2–12
Res-Het1/Het2	0.65–1.39	–	0.50–1.42	–
Res-Het2/Hom1	1.63–3.21	–	1.92–3.78	–
Res-Hom1/Hom2	0.66–1.36	–	0.85–1.53	–

build on the extensive internal quality assurance of the manufacturer and its use for the calibration of the oven. A practical option may be to use the commercial standards for the oven calibration, trouble shooting, and the weekly and monthly maintenance procedure and replace them with in-house controls only for the daily maintenance and evaluation of the individual runs. For the validation of the in-house controls for this purpose only, it could be sufficient to compare the elution profiles with the commercial mutation standards at several (at least three, covering a window of 3°C) temperatures, column conditions, etc., on a regular basis.

A logbook or some permanent form of documentation is indispensable to continuously record the performance of the mutation standards as a system assay control and to trace back the effect of changes of reagents or other consumables on previous runs.

A final aspect that is often neglected is the adequate training of the users of the equipment. No standard operating procedure can adequately deal with this issue, unless a properly operating quality system is in place.

An SOP for DHPLC as a General Method for Mutation Analysis

DHPLC mutation detection on WAVE 3500HT with WAVEMAKER 4.144 and HSM 3.0–2.1 build 2 or Navigator 1.54, SOP-DHPLC, Supplementary Appendix S2. DDQA has listed the basic variables that apply to DHPLC, irrespective of the genomic or cDNA fragment or gene under scrutiny in the general methodology SOP.

These include PCR related aspects, such as PCR composition, amount of template, and yield and quality of the amplicon, but also heteroduplex formation, project setup, and interpretation of the chromatograms. At first glance, this SOP largely rehearses the Clinical Molecular Genetics Society (CMGS, UK) best practice guidelines for the use of the WAVE System, issued in 2001 and revised in 2002 and 2003 (www.cmgs.org/BPG/Guidelines/2002/dhplc.htm). However, the nature of a SOP is to document detailed instructions in order to work reproducibly and accurately, independent of the performing technician, rather than to be a set of helpful suggestions for the user.

The basis of DHPLC—the differences in thermodynamic properties between homoduplex DNA and heteroduplex DNA—defines two critical aspects of the technique. First, it is generally assumed that formation of heteroduplexes is a simple process, only requiring a denaturation step followed by cooling. Hence, all kinds of procedures have been published. However,

we previously encountered problems with two different 2-bp deletion mutations in exon 11 of BRCA2 (G. Michils, unpublished results). Although the amplicons were proven to be heterozygous for the respective mutations by direct sequencing, the DHPLC results were inconsistent and not reproducible, resulting in false negative results in a quarter of the samples. Surprisingly, there was a perfect correlation between the DHPLC results and the results obtained after heteroduplex analysis on 6% PAGE, i.e., between the presence/absence of heteroduplexes and positive/negative detection by DHPLC. After changing the protocol for heteroduplex formation from a fast cooling (−4°C/cycle) and incubation at annealing temperature to a slow cooling procedure (−0.02 or −0.03°C/cycle) followed by incubation at 45 or 65°C, the DHPLC profiles were reproducible and displayed the variant (data not shown). Also, it is generally assumed that heteroduplexes are very stable, but some anecdotal evidence suggested that this might not necessarily be so. Therefore to investigate, we tested the stability of heteroduplexes for eight different missense mutations in the fibrillin gene (*FBN1*). Heteroduplexes were formed immediately after PCR amplification and aliquots were directly analyzed or kept for 14 days at 4 or −20°C before DHPLC screening. No significant difference was observed (data not shown). Still, the physicochemical properties of fragments and the influence of mutations might differ significantly, hence the importance of positive (mutation) controls in each single run.

The second element, the accurate prediction of the melting characteristics of the amplicons, is the key requisite in obtaining maximal sensitivity. In the case of a small gene with a range of typical mutations like *MECP2*, validation of the selected temperatures is fairly easy. For larger genes, however, control mutations are often not available. In this case the temperature selection is mainly based on mathematical predictions, which might change slightly with the software [Rudolph et al., 2002].

Different sources give also different guidelines, defining the optimal analysis temperatures as those temperatures resulting in a percentage helicity between 30 and 99% (www.MutationDiscovery.com) or between 50 and 98% (WAVE System manual, Transgenomic). This discrepancy reflects the essential problem: perfect algorithms, predicting 100% sensitivity, do not exist. For most fragments, the software is very accurate. However, several diagnostic laboratories with expertise in using the WAVE System have shown that the software often fails to detect known mutations in GC-rich fragments or complex melting profiles. Empirical determination of melting profiles can be an option (see SOP-DHPLC).

A Disease-Specific SOP: *MECP2* as a Key Example

DHPLC screening of *MECP2*, SOP-*MECP2*, Supplementary Appendix S3. DDQA has considered several disease genes, which are currently tested widely. They include *MECP2* (the gene mutated in Rett syndrome), *MSH2* and *MLH1* (the two major mismatch repair genes, involved in hereditary nonpolyposis colon cancer [HNPCC]), *BRCA1* and *BRCA2* (the two major genes involved in hereditary breast and ovarian cancer), *CFTR* (the cystic fibrosis gene), and *FBN1* (the fibrillin gene, responsible for Marfan syndrome). The list is evidently nonexhaustive.

From a practical standpoint, it was obvious that the first disease-specific SOP should be drafted for a small gene. The *MECP2* gene was a good choice because 1) the coding region is relatively short and only three exons are commonly screened, 2) a plethora of mutations in this gene is available, and 3) there is an important clinical interest in the diagnostic service for Rett syndrome, so it is foreseeable that more laboratories will offer this test in the future.

Of the two laboratories in the DDQA Collaborative Group that had already introduced *MECP2* screening, both had based their primer design on existing published methods, basically [Amir et al., 1999; Buyse et al., 2000]. Interestingly, in the gradual divergence of practice, no single primer set matched between the two laboratories after they had “optimized” the published primer sequences themselves. Although essentially this kind of diversification does not impair the molecular genetic diagnosis of Rett syndrome, standardization of laboratory methods does simplify the comparison of patient reports, particularly when no mutation is found.

We developed a new protocol for the DHPLC screening of *MECP2* in the context of Rett syndrome, complying with two specific criteria: a robust amplification of the coding region of the *MECP2* gene with one single PCR program and a clear detection of the eight recurrent *MECP2* missense mutations (p.R106W, p.R133C, p.T158M, p.R168X, p.R255X, p.R270X, p.R294X, and p.R306C) and small deletions in the C-terminal region [Miltenberger-Miltenyi and Laccone, 2003]. The initial division of exon 3 and exon 4, in two and five fragments, respectively, as described by Buyse et al. [2000] was not changed. Some users might prefer to use larger amplicons and to screen the gene in fewer fragments. This is of course acceptable, as it would also reduce the cost of the testing.

PCR conditions have been optimized with Optimase Polymerase. The accurate proofreading activity of this enzyme assures single, symmetric peaks on DHPLC and minimizes the chance of false positives. Other enzymes can be used, but it must be noted that some other enzymes with a poor PCR fidelity can produce asymmetric peaks (making interpretation more difficult) and that components of the buffers may affect the performance and lifetime of the cartridge (see Transgenomic Application Notes 118 and 119e at www.transgenomic.com or Section 4.4 in SOP-DHPLC).

Validation of DHPLC Screening

The validation of the DHPLC screening described here is based on the definition of validation defined by ISO/IEC 17025 [1999] section 5.4 or EN ISO 15189 [2003] section 5.5-5.6 as “the confirmation by examination and the provision of objective evidence that the particular requirements of a specific intended use are fulfilled.” This guideline is written for all kind of medical

laboratories and is therefore not very specific, but should be looked upon as a framework for the in-house validation by each single user, according to the local criteria.

Validation of a method includes the quality control of critical consumables, which means for DHPLC, the buffers and the column. Transgenomic manufacturing facilities are ISO9000 compliant with the scope of ‘the design and production of DNA standards and consumables for use with Transgenomic WAVE DHPLC Systems. This does not, however, imply that the buffers and columns themselves are ISO9000 compliant. New batches of buffers or columns have to be validated in-house before use, even in spite of the QC document provided by the manufacturer with each column. Transgenomic indicates an expected shelf life for unopened bottles of its WAVE Optimized buffers for at least a year when stored at normal room temperatures. Practical experience suggests that storage of more than 1 year would not be a concern. Laboratories can still choose to use self-made buffers on the condition that their consistency is confirmed using the mutation standards as described earlier (or SOP-O&M).

To assure the quality of each single run we propose the following criteria: 1) Low and High Range Mutation Standards should be run and evaluated against known normal range values at the beginning and end of each project (WAVEMAKER) or plate (Navigator). For longer runs, it is even recommended to include mutation standards every 100–150 injections. 2) Optimally, confirmed sequence variants should be included and evaluated for each single fragment. For large genes with a limited number of known mutations, this is very hard to achieve. However, it is recommended to include at least some positive controls per project/plate to evaluate other critical steps in the DHPLC process, such as sufficient heteroduplex formation. 3) Elution profiles of each single injection should be minimally 2 mV and represent more than 30% of the average peak height of that fragment in the given run.

We propose individual and separate validation reports for each amplicon, as a useful format for validation reports of screening methods of (large) genes. A typical validation report includes all necessary information (sequence, melting profiles, analysis temperatures, buffer gradients, and elution profiles of the validated mutations) for each amplicon (Supplementary Appendix S4). When necessary, a redesign and subsequent reevaluation of an amplicon can then be inserted easily in the quality assurance (QA) files. The validation framework suggested in this report was evaluated by validating the DHPLC screening of *MECP2* in the context of Rett syndrome. The *MECP2* screening protocol described here (SOP-*MECP2*) was validated in Leuven for the eight recurrent missense mutations and a C-terminal deletion on a WAVE System 3500HT. Two other independent laboratories that were familiar with DHPLC but did not perform *MECP2* screening before evaluated the robustness of the procedure. Although exactly the same protocol was assessed, amplification of one of the eight fragments (Ex4Fd) failed in one of the laboratories, while reproducible results were obtained in both Leuven and the second independent laboratory. The only difference was the use of a gold-plated silver sample block module in the GeneAmp PCR System 9700, which has slightly different heating/cooling features compared with the aluminum sample blocks. Primers for this fragment will be redesigned and further validated, while the scope will also include exon 1, which was recently published to contain an alternative translation initiation signal [Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004]. This stresses the importance of validation of new procedures in each single laboratory to ensure

optimal performance. In total 19 different control variations (p.E10Q, p.R106W, c.378-109A>G, p.R133C, p.S134C, .P152R, p.T158M, p.R167P, p.R168X, p.S194S, p.P225R, p.R255X, p.R270X, p.R294X, p.R306C, p.P376S, p.E397K, c.1151_1191-del45, and p.A447T) have been detected repeatedly and clearly using the validated conditions, confirming the sensitivity of the screening conditions (Fig. 4).

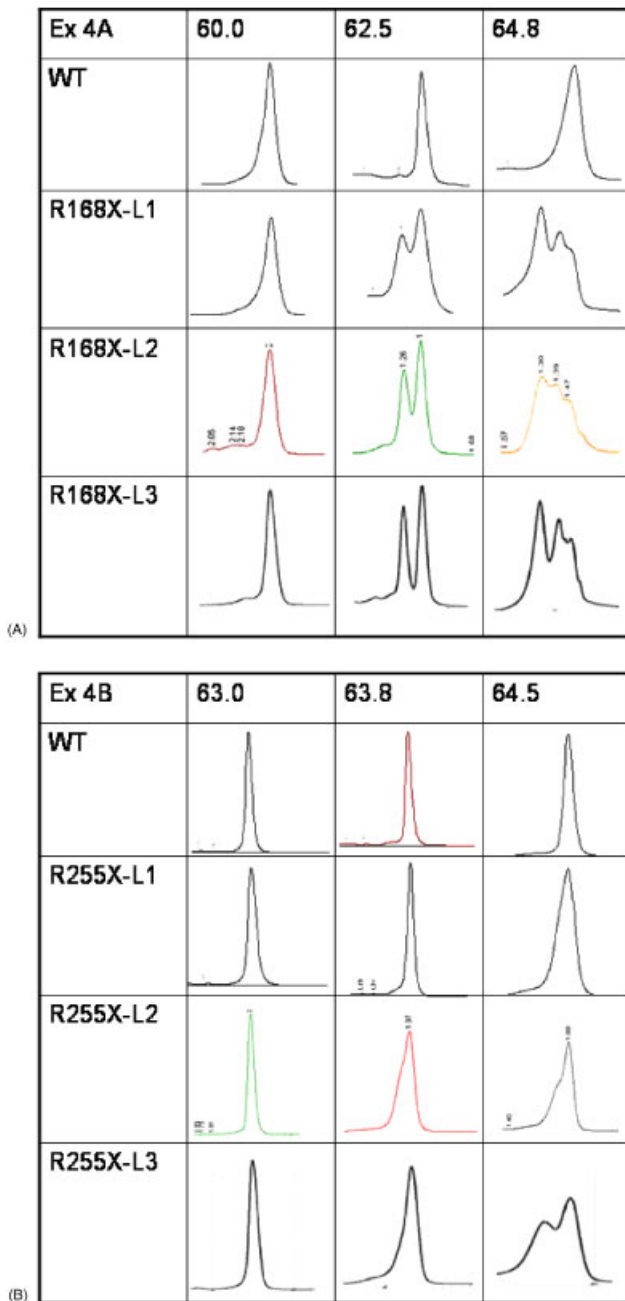


FIGURE 4. Comparison of the elution profiles obtained by the three test laboratories (L1=Leuven; L2=Geneva; L3=Copenhagen) for amplicons of fragments Ex4Fa (A) and Ex4Fb (B) from a wild-type sample (WT) and patients heterozygous for the mutation p.R168X (Ex4Fa) or p.R255X (Ex4Fb). p.R168X is clearly visible at 62.5 and 64.8°C. p.R255X is very sensitive to slight temperature changes and is often only visible as a shoulder at 63.8 and 64.5°C.

DISCUSSION

The growing lists of publications for mutation screening by DHPLC witnesses the breakthrough of this platform in molecular genetics. The technique is widely adopted because of its high sensitivity, relative ease-of-use, and its automation. However, the generally stated high sensitivity of the method should be considered with caution.

First, this high sensitivity can only be achieved after thorough test design, optimization, and validation. Second, quality control and continual validation of the system and the testing procedures are essential to maintain this sensitivity. Third, the expertise of the user should not be underestimated. Fourth, even in an ideal setting, a 100% sensitivity can not be guaranteed for every amplicon. Finally, though not directly related to the method, but rather to the fact that it is performed on PCR products of small amplicons: DHPLC analysis only allows the detection of single base changes and small deletions or insertions [Cremonesi et al., 2003]. Large deletions or insertions will not be detected.

The apparent ease-of-use of the system is in contrast with its technical complexity and the intricacy of most of the diagnostic tests performed by DHPLC. It is much easier to validate a restriction digestion! Quality assurance for complex tests is a major challenge. The DDQA Collaborative Group dealt with this challenge by providing generic SOPs for instrument operation and maintenance, for DHPLC as a general method for mutation screening, and by offering an exemplar disease-specific SOP. These generic SOPs include all the essential requirements necessary to comply with the various quality systems but should of course be revised and further completed with local references (e.g., numbering and indexing, location of specific reagents, etc.) and local requirements, before implementation and in-house validation.

With respect to validation, three main issues came to prominence.

1. A fundamental issue is the lack of calibration of the system, including instrument oven, column, and buffer, to international standards or other stated references. Elution profiles of the mutation standards can be accepted for the latter one, on the condition that well-defined acceptance/rejection criteria based on statistical calculation are defined. In close collaboration with the manufacturer, we have delineated these criteria for the Low and High Range Mutation Standards on both the normal and HT System running under Navigator software. Instruments running with the parameters falling outside of these limits are not operating within the limits of what the manufacturer, Transgenomic, considers normal. The instrument may still provide adequate test results, depending on the particular fragment and mutations under investigation, but the normal detection sensitivity cannot be assured under these conditions and a higher chance of false negatives should be considered. The use of in-house validated controls instead of or in combination with the commercial mutation standards is still an option but has not been further explored within the DDQA workgroup.

2. Although numerous publications report a 100% detection rate, in most cases the predicted screening conditions have to be adapted to reach this sensitivity [for example, see Oldenburg et al., 2001]. Previously, a lot of effort has gone into the optimization and evaluation of the melting profile prediction programs [Rudolph et al., 2002]. In practice, it comes down to evaluating the runs with known variations or comparing the theoretical predictions with empirical melting profiles. In this context it is important to note that a particular diagnostic test is

only validated for the mutations that have been successfully detected by the given conditions. However, as validation is a continuous process, variations not included in the initial validation can be added as testing progresses. Hence the importance of programs like Certified Reference Materials for molecular GENetic testing, CRMGEN (www.crmgen.org) or the reference development by NGRL (Wessex; www.ngrl.org.uk/Wessex/controls.htm), which strive to make known mutation samples for specific genes available to the diagnostic community.

3. In terms of heteroduplexing, we have shown that the heteroduplexes of eight different missense mutations of the *FBN1* gene are stable for at least 14 days at 4 and -20°C . Also, it was reassuring to find that a careful heteroduplex formation, with a slow cooling procedure, gave repeatable good results for a difficult *BRCA2* fragment. Although problems with heteroduplex formation or stability may be only anecdotal, heteroduplexing is a critical parameter of the analysis and should be monitored in each single run.

At least two beneficial aspects of collaboration became clear.

First, the preparation of SOPs and the validations of the instruments and methods are ideally done in a collaborative effort between the manufacturers and the users. As shown in Figure 3, the contribution of different partners is required for the different aspects of such a project. The collaboration accelerated the information flow on product details, maintenance procedures etc. and was indispensable in the description of the QA criteria for the mutation standards. We claim that this approach is more widely applicable to other existing and emerging methods and platforms. We call upon the manufacturers to try to offer uniform systems. It is very important for the manufacturers to realize that any update or change to the system (including software) will require revalidation and thus results in extra costs both in terms of time and labor and consumables on the diagnostic laboratories.

Second, in contrast to the limited material and method information presented in published research papers, the benefits of a disease-specific SOP are enormous. Two independent diagnostic laboratories have proved that this specifically written, optimized, and validated SOP was highly beneficial in enabling both laboratories to successfully set up the mutation screening of the *MECP2* gene by DHPLC within in 1 month of receiving the SOP. The benefits in terms of time, labor, and consumables saving are self-evident. The lack of amplification of one fragment in one of the test laboratories does not affect the validation of the procedure in the two other labs. In contrast, it supports the importance of the final validation by each laboratory individually. Also, there is still room for further optimization, for example by reducing the number of overlapping amplicons to cover exon 3 and exon 4. During the time of this collaboration, a previously unidentified *MECP2* open reading frame, including exon 1 has been shown to be involved in Rett syndrome [Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004]. Using the suggested framework with individual validation reports for each amplicon, screening of this exon could easily be added to the current validation report, thus enhancing the sensitivity of this diagnostic assay with the minimum of effort.

Although the given SOPs and the examples of the validation procedures do not provide an alibi for a blind start, we hope to have contributed significantly to allowing laboratories to take a swift start when introducing these methods, and to have provided the quality control procedures necessary to achieve

and maintain the high sensitivity of this mutation screening platform.

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