

METHODS

Evaluation and Use of a Synthetic Quality Control Material, Included in the European External Quality Assessment Scheme for Cystic Fibrosis

Sarah Berwouts,¹ Joan T. Gordon,² Clark A. Rundell,² David E. Barton,³ and Elisabeth Dequeker^{1*}

¹Centre for Human Genetics, University of Leuven, Leuven, Belgium; ²Maine Molecular Quality Controls, Inc., Scarborough, Maine; ³National Centre for Medical Genetics, Our Lady's Children's Hospital, Dublin, Ireland

Communicated by Garry R. Cutting

Assuring high quality within the field of genetic testing is fundamental, as the results can have considerable impact on the patient and his or her family. The use of appropriate quality control (QC) samples is therefore essential. Diagnostic laboratories mainly use patient samples as QC material, which of course include a maximum of two mutations per sample. Bearing in mind that some assays (such as for cystic fibrosis [CF] testing) can test for more than 100 mutations, multiplex QC materials including more than two mutations could save valuable time and reagents. Based on this need, synthetic multiplex controls have been developed by Maine Molecular Quality Controls, Inc. (MMQCI) for CF. A synthetic control, containing six homozygous mutations and one polymorphism for CF transmembrane conductance regulator (CFTR), was evaluated by distributing it through the CF external quality assessment (EQA) scheme, along with the EQA samples in 2005. A total of 197 participants returned results of the yearly EQA scheme and 133 laboratories participated in the evaluation of the synthetic sample. Respectively, 76% and 73% of the participants were assigned as successful. This evaluation study revealed that the multiplex QC material performed well in the majority of assays and could be useful in method validation, as a tool to challenge interpretation skills, and as potential proficiency testing (PT) material. *Hum Mutat* 29(8), 1063–1070, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: cystic fibrosis; CF; CFTR; external quality assessment; proficiency testing; synthetic control material; quality assurance

INTRODUCTION

The decoding of the human genome and the identification of mutations associated with human diseases have led to a continuous expansion of molecular genetic testing. Testing is moving gradually from research laboratories identifying disease-related genes, into diagnosing rare or monogenetic diseases. In contrast to other diagnostic testing, genetic tests are usually carried out only once in the life of an individual; therefore, a single result can have a considerable impact on the patient and his or her relatives. Misinterpretation of the result could have serious consequences affecting more than one individual. It is therefore essential that the quality of a genetic test is assured, encompassing the whole process from sample collection to the interpretation and reporting of the genetic results. Increased attention to ethical, legal, and social aspects is also necessary.

However, laboratory regulation and accreditation measures have not kept pace with the growing integration of genetic tests into clinical practice and rapid expansion of technology development. Efforts to assess and improve the level of quality within genetic testing laboratories have been initiated by diverse organizations and networks in Europe and the United States. The European Science and Technology Observatory Network (ESTO) [Ibarreta et al., 2004], the Organisation for Economic Co-operation and Development (OECD) [McGovern et al., 2007], and the Centers for Disease Control and Prevention (CDC) [Chen et al., 2005] conducted studies to document and compare the status of quality

assurance and quality control (QC) practices in genetic testing laboratories. The Cystic Fibrosis (CF) Network [Dequeker and Cassiman, 1998] and European Molecular Quality Network [Muller, 2001] are among the largest external quality assessment (EQA) providers in Europe for molecular testing. EQA is also known as proficiency testing (PT). Despite the various activities mentioned above, there continues to be a need to improve the availability of positive quality control samples (QCS) [Williams et al., 2003]. Positive control samples are of fundamental importance for verifying the accuracy of existing tests, validating new assays, and test development. They can also serve as a resource for EQA schemes, so the availability of these materials is therefore a key in assuring quality of molecular genetic testing. Diagnostic laboratories can obtain some of the necessary controls

Received 17 October 2007; accepted revised manuscript 22 January 2008.

*Correspondence to: Prof. Dr. Els Dequeker, University of Leuven, Centre for Human Genetics, Herestraat 49, box 602, 3000, Leuven, Belgium. E-mail: els.dequeker@med.kuleuven.be

Grant sponsor: European CF Network; Grant number: QLK3-CT99-00241; Grant sponsor: EU project EuroGentest; Grant number: FP6-512148.

DOI 10.1002/humu.20764

Published online 9 May 2008 in Wiley InterScience (www.interscience.wiley.com).

from research laboratories, colleagues, EQA schemes, or cell lines and DNA samples available in repositories such as the Coriell Institute for Medical Research. However, positive control materials for some mutations are difficult to obtain, especially those for rare mutations and diseases. In addition, informed consent procedures, privacy issues, and the fact that clinicians are not aware of this need in genetic laboratories, all contribute to the difficulty in obtaining the necessary samples for positive controls. As a result, initiatives were taken to develop novel methods of collecting or producing control materials for molecular genetic testing. The European Commission funded two projects in this context. The CRMGEN project (fifth framework, G6RD-CT-2001-00581) [Barton et al., 2004] and the EuroGentest Network of Excellence (sixth framework, FP6-512148) [Cassiman, 2005; Hayhurst and Cassiman, 2006], both focus (partly) on the production of various types of certified reference materials (CRM) for a range of molecular genetic tests and assist in making them available to end users [Barton and Kalman, 2007]. In the United States, the CDC funded projects to promote the development and distribution of control materials [Grody, 2003; Bernacki et al., 2003; Jarvis et al., 2005] and was involved in the Genetic Testing Reference Material Coordination Program [Chen et al., 2005] (GeT-RM; www.phppo.cdc.gov/dls/genetics/qcmaterials/default.aspx). Three commercial entities in the United States, Molecular Controls [Christensen et al., 2007], AcroMetrix [Huang and Pan, 2007], and Maine Molecular Quality Controls, Inc. (MMQCI) [Johnson et al., 2007], have developed synthetic reference materials (RM) for molecular testing. These synthetic RM were developed in different ways and optimized to work on diverse testing platforms. Synthetic controls are common in other clinical laboratory disciplines. For example, synthetic cholesterol derivatives have been successfully used as a substitute for biological cholesterol for several years in quality controls and in proficiency samples in clinical biochemistry [Klein et al., 1974; Proksch and Bonderman, 1978]. Until recently, molecular testing has chiefly employed biological materials, such as cell lines and previously-tested patient DNA, for control materials. However, biological materials are somewhat problematic, because they contain at most two mutations per sample and rare mutations are often not available. Consequently, the usefulness of recently developed synthetic RMs for molecular testing was investigated. To evaluate synthetic RM on a large scale, the European CF Network agreed to enclose one of the controls encompassed in MMQCI's CF Panel I Control as an additional sample (MMQCI-CF-PI) in the EQA scheme of 2005. This study is the first time synthetic nucleic acid RM have been tested in such a wide variety of extraction and mutation detection methods. The quality assurance challenges faced by laboratories testing for CF transmembrane conductance regulator (CFTR; MIM# 602421) mutations support the rationale for using CF testing as a synthetic control test model. CF (MIM# 219700) is the most common lethal autosomal recessive disorder with an incidence of 1 in 2,500 in Caucasians. More than 1,000 mutations are identified in the CF gene (Cystic Fibrosis Mutation Database; www.genet.sickkids.on.ca/cftr/StatisticsPage.html). In addition, the American College of Medical Genetics (ACMG) and American College of Obstetricians and Gynecologists (ACOG) have compiled a recommended standard screening panel of 23 CFTR mutations for CF [Grody et al., 2001a; Watson et al., 2004]. Most laboratories test for these and other mutations cited in current literature in multiplex formats. To meet quality assurance requirements for these complex tests, it is recommended by the U.S. Clinical Laboratory Improvement Amendments of 1988 (CLIA) that laboratories include controls for all these mutations

during testing. However, the mutant genotype samples are difficult to find and testing of many control samples per run is not feasible. Synthetic controls have the advantage of being able to contain multiple mutations in single samples and can be manufactured to contain any desired combination of mutations. CLIA also requires that the entire testing process be monitored. The control samples of MMQCI comply with these needs as they mimic blood and can therefore be extracted as routine patient samples. Furthermore, these samples were recently cleared for marketing as genetic testing controls by the U.S. Food and Drug Administration (FDA). Additionally, synthetic controls can be configured to mimic genotypes that may challenge interpretation skills and therefore serve as an educational tool. For example, true homozygous I507del (c.1519_1521delATC, p.Ile507del) must be distinguished from heterozygous I507del (c.1519_1521delATC, p.Ile507del) with F508C (c.1522T>G, p.Phe508Cys) in *trans*. Including such genotypes in proficiency samples provides the laboratories with valuable practice, which may assure accurate patient results. The preceding points and the fact that it has been shown that participation in EQA schemes can improve laboratory performance [Dequeker et al., 2001; Dequeker and Cassiman, 2000], shows the importance of investigating the use of multiplex RMs as potential PT materials.

MATERIALS AND METHODS

CF EQA Samples

In 2005, the European CF Network distributed a panel of six DNA samples, along with clinical information, for the EQA scheme, to genetic testing laboratories routinely screening for CF. These samples, derived from CF patients, CF mutation carriers, and noncarriers, represented the mutations: 1717-1G>A (c.1585-1G>A), 3272-26A>G (c.3140-26A>G), F508del (c.1521_1523delCTT, p.Phe508del), and R117H (c.350G>A, p.Arg117His). Each participating laboratory was asked to indicate the mutations tested for, the methods used, and the genotypes detected. Moreover, reports and raw data were requested in order to conduct a proper evaluation of the interpretation given by the participant.

CF Synthetic Control Sample

Additionally, the participants were optionally able to take part in an evaluation study of the synthetic QC sample, MMQCI-CF-P1 from MMQCI. To obtain the desired mutations in one sample, DNA control sequences were first amplified and cloned. Second, desired mutations were created *in vitro* and incorporated into the construct, which was then bidirectionally sequenced and analyzed using Phred-based quality score analysis (Phred; <http://www.phrap.com/phred>). Finally the MMQCI-CF-P1 construct was added to a proprietary stabilizing matrix that allows for DNA extraction by common methods. The control material was thoroughly evaluated by MMQCI beforehand according to good manufacturing practice (GMP) by testing internally at MMQCI and externally in multiple extraction and CFTR mutation detection methods for accuracy, reproducibility, stability, homogeneity, and robustness.

Preliminary to the large scale evaluation study, a small pilot with seven laboratories was executed. They all used different but common extraction and detection methods. Comments from the pilot laboratories were used to improve the clarity of the final instruction letter. The results of the pilot are not included in this work, as they are similar to those from the main evaluation study.

The MMQCI-CF-P1 control distributed to EQA participants contained six homozygous mutations and one polymorphism:

R553X (c.1657C>T, p.Arg553X), I507del (c.1519_1521delATC, p.Ile507del), R117H (c.350G>A, p.Arg117His), 394delTT (c.262_263delTT, p.Leu88fs), 2183AA>G (c.2051_2052delAAinsG, p.Lys684fs), R347H (c.1040G>A, p.Arg347His), and 5T (c.1210-12T[5]). The laboratories that accepted the challenge to test this extra sample were not informed as to the number or identity of the mutations present, and were requested to submit the genotype and raw data to the organizers via an electronic datasheet. Additionally, they agreed to report on their CF test menu, the extraction and detection methods used, and to give feedback on the usefulness of this type of control material.

Evaluation

There is no standardization as to which detection methods should be used to identify mutations and polymorphisms in the *CFTR* gene, with the consequence that different assays used by the participating centers were not all able to detect the entire mutation panel included in the EQA and QC samples. Therefore, all evaluated items reported with the correct result were assigned as correct, and no assignment was made (neither correct nor incorrect) for a mutation in the sample for which a laboratory did not screen. When a laboratory reported an incorrect zygosity (heterozygous instead of homozygous), this was considered a genotype error. Since MMQCI-CF-P1 is not fully compatible with the ElucigeneTM (Tepnel Molecular Diagnostics, Abingdon, United Kingdom) CF kits due to insufficient intronic DNA sequence being included in the MMQCI's synthetic constructs of exon 11, a missed R553X (c.1657C>T, p.Arg553X) mutation was not counted as an error by users of this method.

For the EQA scheme, laboratories were designated as successful only if they provided a written report including the correct genotypes and a correct and sufficient interpretation of the clinical information. Laboratories that made a genotype error in combination with an interpretation error were included in the group that made a genotype error.

In contrast, in order to be classified as completely correct in the QCS study, laboratories were only required to report correctly all of the mutations (plus correct zygosity) included in their assay which were present in the QCS. Difficulties with the analytical process were counted as errors; however, additional mutations not present in the QCS but reported or commented upon were not counted as errors. The reviewers felt that, except for occasional cross-reactivity issues, faint signals were reported as additional mutations solely due to the synthetic nature of the sample and did not truly reflect a laboratory's ability to correctly test and report on genomic samples.

Further, although reflex 5T (c.1210-12T[5]) testing is required clinically when R117H (c.350G4A, p.Arg117His) is detected in carriers [Grody et al., 2001b], not all laboratories performed or reported a reflex test for the polymorphic tract in intron 8 (polyT), again probably due to the synthetic nature of the sample. Therefore, lack of a polyT report was not counted as an error. All of the 64 laboratories that did report polyT, were correct.

Finally, not all raw data, especially from sequencing analysis, were sent to the assessors, which made it more difficult in some situations to evaluate and elucidate the reason for a particular problem.

Mutation Nomenclature

The mutation nomenclature complies with the guidelines from the Human Genome Variation Society (www.hgvs.org). These state that nucleotide number 1 should correspond to the A of the

ATG translation initiation start site. The traditional nomenclature is also included, using nucleotide position 133 as translational start site. The description of all variants is preceded by a letter indicating the type of reference sequence used; "c." relates to a coding DNA sequence and "p." a protein sequence. The GenBank reference sequence for *CFTR* is NM_000492.3.

RESULTS

In total, 31 countries joined the EQA scheme, of which 23 took part in the QCS evaluation (Fig. 1). Fig. 1 also compares in general the performance of the participants in both schemes. In the EQA scheme, laboratories had to provide a written report including a correct genotype and interpretation, in contrast to the QCS study, in which only a correct genotype was required.

A total of 197 participants returned results for the EQA samples. The error types ranged from incorrect genotyping (10 participants) to absent, incorrect, or insufficient interpretation (33 participants) regardless of genotype. A total of five participants failed because they did not provide the written report required for a successful EQA evaluation (Table 1). Two of them are companies and the other three laboratories are from Italy, the Netherlands, and the United States. A few laboratories from Italy, Spain, the Netherlands, and the United States reported a correct genotype for the EQA samples, but provided insufficient interpretation of one or more clinical cases. In general, difficulty with the requirement for correct interpretation of the results in EQA is the reason these countries scored worse for the EQA sample than for the QC sample (Fig. 1). It is important to know that genetic testing in some countries is increasingly being performed in clinical chemistry laboratories, where, so far, insufficient attention is paid to specific interpretation related to the genetic result (E.D., personal communication). A growing number of clinical chemistry laboratories participate in the CF EQA scheme, but most fail due to the lack of sufficient interpretation in the report. In total, 149 (76%) of the laboratories that returned data on the EQA samples were assigned as successful.

A total of 133 laboratories of the 197 that participated in the EQA scheme also took part in the evaluation of the synthetic QC. Of these, 97 laboratories (73%) found the correct genotype. Apart from 24 (18%) laboratories that did not detect all mutations or reported an incorrect zygosity, 12 (9%) laboratories reported difficulties during the analytical process (extraction or detection phase): four stated the test was invalid due to lack of control bands, which ordinarily indicates a failed test; three reported an unclear result after detection; and five laboratories mentioned extraction failure (Table 1).

A total of 78 laboratories participated successfully in both schemes. Three laboratories made a genotype error in both trials. A total of 19 laboratories correctly identified the QCS genotypes, but failed in the EQA scheme due to incorrect interpretation (14), incorrect genotype (three), or not providing written reports (two). Within the category of the laboratories that made a genotype error for the QC sample, 17 laboratories participated successfully in the EQA scheme, but four laboratories were not able to provide sufficient interpretation in the EQA scheme (Table 1).

The 12 laboratories that encountered difficulties in the analytical process did not succeed due to a combination of inexperience, method incompatibility with the QCS, or failure to read the instructions accompanied with the sample.

First, the instruction letter explains that the sample lacks the internal controls amplified by ElucigeneTM CF29 and CF30. The seven laboratories that reported a lack of control bands or an

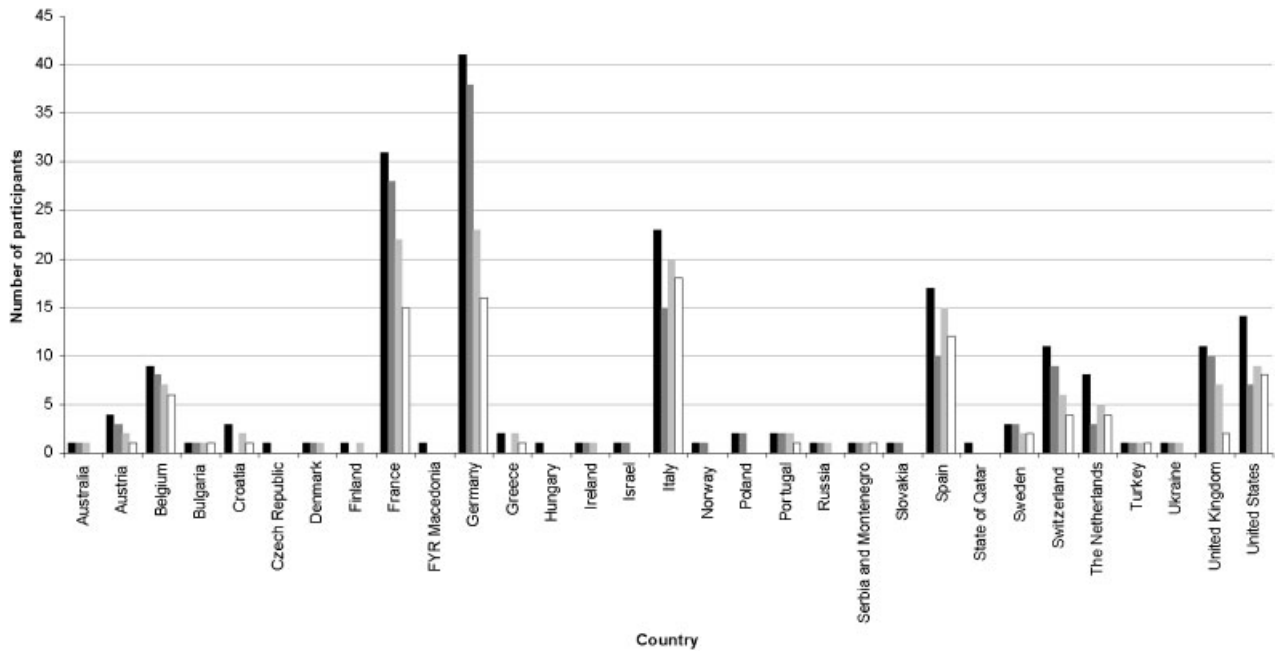


FIGURE 1. EQA scheme (197 laboratories) and QC trial (133 laboratories) participation per country and general overview of performance in both schemes. Black bars, number of laboratories participating in the EQA scheme; dark-gray bars, number of laboratories that reported a correct genotype and submitted a report including the correct interpretation in the EQA scheme; light-gray bars, number of laboratories participating in the QCS trial; white bars, number of laboratories that reported a correct genotype and encountered no difficulties in the analytical process in the QCS trial.

TABLE 1. EQA Scheme and QC Trial Participation, Common Errors*

		Number of participants in QC trial (133)		
		Genotype correct	Genotype error	Difficulties in analytical process
Number of participants in EQA scheme	197 (100%)	97 (72.9%)	24 (18.0%)	12 (9%)
Correct genotype + report including correct interpretation	149 (75.6%)	78 (58.6%)	17 (12.8%)	9 (6.8%)
Genotype error	10 (5.1%)	3 (2.3%)	3 (2.3%)	0
No, insufficient or wrong interpretation	33 (16.8%)	14 (10.5%)	4 (3.0%)	3 (2.3%)
No written reports	5 (2.5%)	2 (1.5%)	0	0

*Bold text indicates only the laboratories that participated in both schemes (133).

unclear result all used the amplification refractory mutation system assay (ARMS), either in-house developed (two laboratories) or as Elucigene™ CF29 (five laboratories). On the other hand, the instruction sheet did not notify the user of any cautions against using in-house ARMS.

Second, although the instruction sheet stated that the synthetic DNA sample was not quantifiable by usual spectrophotometric methods, comments by the five laboratories that reported an extraction failure indicated that their attempts to quantify the sample led them to believe the extraction failed so they did not continue to test. Synthetic DNA samples, unlike genomic DNA samples, do not contain DNA of sufficient quantity to be measured by ordinary clinical methods. In laboratories whose standard protocol is always to measure the DNA yield from each extracted sample, the DNA extraction from synthetic controls will appear to have failed. The solution for such laboratories is to have an alternative protocol, which does not include determination of the DNA yield or concentration, specifically for the extraction of DNA from synthetic control materials. This issue should be addressed more thoroughly in future instruction sheets. A total of 21 different extraction methods were used, with QIAamp DNA

Blood Kit (Qiagen, Hilden, Germany), salting out, and MagNA Pure Kit (Roche Diagnostics, Basel, Switzerland) being the most common (Table 2). There was no relation observed between the extraction problems reported and the extraction method used.

The observations among the 121 laboratories that did not have analytical difficulties, are presented in Table 3 (general) and Table 4 (detail). The tables are organized as follows: 1) genotype error only, in which the QCS mutations and zygosity were detectable by the method used; 2) no genotype error but one or more additional mutations reported that were not present in the QCS; and 3) genotype error and one or more additional mutations reported. For evaluation, the laboratories in this study are split into two categories: a category for laboratories that used a single detection assay and another for those that used more than one assay (combination). Table 4 represents the detailed results of the laboratories (75 laboratories) that used only one mutation detection assay. The most common supplemental detection methods were sequencing and heteroduplex analysis. The laboratories in the “miscellaneous” group of Table 3 used more than one assay, including a combination of non-commercial assays such as polymerase chain reaction (PCR),

restriction fragment length polymorphism (RFLP), sequencing, and restriction enzyme analysis (REA).

As shown in Table 3, the assays most frequently used to detect the six mutations are the commercially available oligonucleotide ligation assay (46), reverse dot blot (44), and ARMS method (17). Among the group that did not encounter problems during the analytical process, nine laboratories made a genotype error, 11 laboratories reported one or more additional mutations not included in the QCS, and 15 laboratories found extra mutations and also reported an incorrect genotype.

The observed causes of reporting genotype errors or additional mutations are as follows: 1) cross-reactivity of probes for similar alleles; 2) lack of internal controls amplified by ARMS-Elucigene™ CF29 and CF30, which makes it more difficult to interpret results; 3) near neighbor interferences (loss of signal of one allele due to a mutation at a nearby allele); 4) nonspecific

signal possibly due to DNA overload; and 5) misinterpretation, nonspecific binding.

Mutations 2183AA>G (c.2051_2052delAAinsG, p.Lys684fs) and R347H (c.1040G>A, p.Arg347His) cross-react with 2184delA (c.2052delA, p.Lys684fs) and R347P (c.1040G>C, p.Arg347Pro), respectively, in many CFTR detection methods. This explains why seven laboratories made genotype errors (missing 2183AA>G (c.2051_2052delAAinsG, p.Lys684fs) and R347H (c.1040G>A, p.Arg347His)) and/or reported mutations not present in the QCS (2184delA (c.2052delA, p.Lys684fs) and R347P (c.1040G>C, p.Arg347Pro)); they encountered difficulties in interpreting typical cross-reaction patterns that are explained in the manual of the assays. Five of them used the ARMS-Elucigene™ method, which requires control bands not amplifiable from the synthetic QCS as a guideline for interpretation. The remaining two laboratories analyzed the sample with the OLA-CF v3.0 from Abbott Molecular (Des Plaines, IL). The raw data of the laboratory that used the U.S. version (does not contain 2183AA>G (c.2051_2052delAAinsG, p.Lys684fs)) clearly showed that the software (Genotyper® 3.7, Applied Biosystems, Foster City, CA) called mutation 2184delA (c.2052delA, p.Lys684fs) homozygous, although this was a very small peak. No raw data was provided by the laboratory using the European Union (EU) version.

Furthermore, additional mutations, 1717-1G>A (c.1585-1G>A) and 3849+10kbC>T (c.3717+10kbC>T), were found by ARMS-Elucigene™, caused by challenging patterns in the raw data; or it might be that the laboratory did not dilute the extract as instructed in the letter.

A total of three laboratories, using INNO-LiPA assay (In-nogenetics NV, Gent, Belgium), reported a very weak signal for wild-type R553X (c.1657C>T, p.Arg553X) apart from a signal for mutant R553X (c.1657C>T, p.Arg553X), which indicates a heterozygous R553X (c.1657C>T, p.Arg553X) mutation. Two of the laboratories that saw this weak signal for wild-type R553X (c.1657C>T, p.Arg553X) also reported weak mutant signals for Q552X (c.1654C>T, p.Gln552X) or G542X (c.1624G>T, p.Gly542X), possibly indicating DNA overload. The Q552X (c.1654C>T, p.Gln552X) wild-type and mutant signal should disappear when R553X (c.1657C>T, p.Arg553X) homozygous is present, using INNO-LiPA. This type of near neighbor interaction is explained in the manual of the assay; therefore, the laboratory should have recognized this pattern. Two other laboratories

TABLE 2. Extraction Methods Used When Analyzing the QCS*

Extraction method	Number of laboratories	Extraction problem reported
QIAamp DNA Blood Kit (Qiagen)	41	1
Salting Out	14	2
MagNA Pure Kit (Roche)	10	
EZ1 DNA Blood Kit (Qiagen)	9	
PUREGENE DNA Purification Kit (Gentra)	8	1
Nucleon DNA Extraction Kit (GE Healthcare, Amersham Biosciences)	7	
Phenol/chloroform extraction	6	
Wizard System (Promega)	6	
NucleoSpin (Macherey-Nagel)	4	
Chemagic DNA Blood Kit (Chemagen)	3	
High Pure Kit (Roche)	3	
OLA-CF V.3.0 ASR sample preparation (Abbott Molecular)	3	
Chelex extraction	2	
FlexiGene DNA Kit (Qiagen)	2	
GFX Genomic Blood DNA Purification Kit (GE Healthcare, Amersham Biosciences)	2	
Miscellaneous*	6	
Not specified method	7	1
Total	133	5

*DNA Extraction Kit (Fermentas, Burlington, Ontario), Generation DNA Purification System (Gentra, Minneapolis, MN), Genisol Maxi-Prep Kit (ABgene, Epsom, United Kingdom), GenoPrep Cartridge B (GenoVision, Philadelphia, PA), MagAttract DNA Blood Kit (Qiagen), UltraClean Kit (MO BIO Laboratories, Inc., Carlsbad, CA).

TABLE 3. Observations for the QCS Per Mutation Detection Assay Among the Laboratories That Did Not Have Analytical Difficulties: Genotype Error and/or Additional Mutations Reported That Were Not Present in the Sample

Detection assay ^a	Number of laboratories	Genotype error	Additional mutation(s) reported, not present in the QCS	Genotype error + additional mutation(s) reported, not present in the QCS
OLA-CF ASR v3.0 EU	25	0	0	1
OLA-CF ASR v3.0 US	5	0	1	0
OLA-CF ASR v2.0	1	1	0	0
OLA-CF ASR v3.0 EU combination	15	1	0	0
INNO-LiPA CFTR36	27	2	3	1
INNO-LiPA CFTR36 and Italian regional	3	0	0	1
INNO-LiPA CFTR36 combination	14	0	1	2
ARMS-Elucigene™ CF29	4	0	3	1
ARMS-Elucigene™ CF29 and CF-HT	1	0	1	0
ARMS-Elucigene™ CF29 combination	1	0	0	1
ARMS-Elucigene™ CF30 combination	6	1	0	4
DNA sequencing	6	3	1	0
ASPE-CFTR 40+4 Tag-It	3	0	1	0
Miscellaneous	10	0	0	5
Total	121	9	11	15

^aCombination: commercial assay used in combination with one or more other detection assays.

TABLE 4. Error Types for the QCS in More Detail, for the Laboratories That Used Only One Detection Assay*

Genotype error				
Detection assay	Number of labs	Genotype		Comment
		Expected	Reported	
OLA-CFASR v2.0	1	R117 H hom	–	Correct on raw data
INNO-LiPA CFTR36	1	R117 H hom	R117 H het	No signal for wt R117 H visible on copy of the raw data, could be very weak on original raw data
INNO-LiPA CFTR36	1	R553X hom	R553X het	No signal for wt R553X visible on copy of the raw data, could be very weak on original raw data
Sequencing	2	1507del hom	1507del/F508del	No complete raw data received
Sequencing	1	R347 H hom	–	No raw data received
Sequencing	1	1507del hom	–	No raw data received

Additional mutation(s) reported			
Detection assay	Number of labs	Additional mutation(s)	Comment
OLA-CFASR v3.0 US	1	2184delA ^a hom	Software called it
INNO-LiPA CFTR36	3	A455E het (3labs), F508del (1lab)	No signal for mut A455E visible on copy of the raw data, could be very weak on original raw data
ARMS-Elucigene TM CF29	3	2184delA ^a (3labs), R347P (3labs), 1717-1G>A (3labs), 3849+10kbC>T (2labs)	Cross reaction with 2183AA>G ^b and R347 H and no full compatibility of MMQCI-CF-P1 and ARMS method: no control bands visible
ARMS-Elucigene TM CF29 +CF-HT	1	2184delA ^a , R347P	Cross reaction with 2183AA>G ^b and R347H
Sequencing	1	W1282X het, N1303 K het	No raw data received
ASPE-CFTR 40+4 Tag-It	1	711+1G>T het	No raw data received

Genotype error + additional mutation(s) reported						
Detection assay	Number of labs	Genotype		Comment	Additional mutation(s)	Comment
		Expected	Reported			
OLA-CFASR v3.0 EU	1	R117 H hom 394deTT ^c hom 2183AA>G ^b hom	– 394deTT ^c het –	No raw data received; probably 2183AA>G ^b missed, but 2184delA ^a reported due to cross reaction	2184delA ^a hom	No raw data received, probably due to cross-reaction with 2183AA>G ^b
INNO-LiPA CFTR36	1	R553X hom 1507del hom	R553X het 1507del/ F508del	No signal for wt R553X visible on copy of the raw data, could be very weak on original raw data	G542X het A455E het	No signal for mut G542X and mut A455E visible on copy of the raw data, could be very weak on original raw data
INNO-LiPA CFTR36 + Italian regional	1	R553X hom	R553X het	No signal for wt R553X visible on copy of the raw data, could be very weak on original raw data	Q552X het	Misinterpretation: wt and mut signal for Q552X not visible, but this is a normal reaction pattern when R553X is hom present; the lab reported R553X het
ARMS-Elucigene TM CF29	1	1507del hom 2183AA>G ^b hom	– –	No full compatibility of MMQCI-CF-P1 and ARMS method: no control bands	R347P	Cross-reaction with R347H

*If the zygosity is not mentioned in the table, the laboratory did not report it.

^ac.2052delA.

^bc.2051_2052delAAinsG.

^cc.262_263deTT.

lab, laboratory; hom, homozygous; het, heterozygous; wt, wild type; mut, mutant.

using INNO-LiPA in combination with an extra detection assay (real-time PCR or REA) made the same error (not shown in Table 4).

Similarly, one laboratory reported a weak signal for wild-type R117H (c.350G>A, p.Arg117His) and normal signal for mutant R117H (c.350G>A, p.Arg117His) instead of mutant R117H (c.350G>A, p.Arg117His) homozygote.

Finally, some laboratories using the INNO-LiPA assay obtained a very faint hybridization signal for the A445E (c.1364C>A, p.Ala445Glu) mutant probe, but a normal signal for the wild-type probe.

These very faint signals for wild-type R553X (c.1657C>T, p.Arg553X), wild-type R117H (c.350G>A, p.Arg117His), mutant G542X (c.1624G>T, p.Gly542X), and mutant A455E (c.1364C>A, p.Ala445Glu) signal were often not visible to the assessors on the copies of the raw data. Based on the accompanying comments given by the laboratories, for example, “signal would not pass quality control,” addition of a question mark (?) after a weak signal or listing the mutation only in the comments section, not in datasheet itself, it was clear that most of them took very weak signals into account only because they did not know exactly what to expect for a multiplex synthetic sample containing more than two unknown mutations.

Moreover, these patterns could occur with overload of genomic DNA, and in that situation laboratories should not report weak signals unless they are confirmed.

Two laboratories were not able to confirm if the QCS included I507del (c.1519_1521delATC, p.Ile507del) homozygous or F508del/I507del (c.1521_1523delCTT, p.Phe508del/c.1519_1521delATC, p.Ile507del). However, the INNO-LiPA assay is able to distinguish these genotypes. Therefore, these laboratories did not interpret the results correctly; this is worrying, as it implies that laboratories could also misinterpret such a result for patient samples.

DISCUSSION

In discussing the utility of the QCS it is important to note that a disadvantage of synthetic RMs is that they may not contain all the sequence necessary for all test methods. Laboratories using ARMS-ElucigeneTM products encountered interpretation difficulty chiefly because the QCS did not contain the internal control DNA required by the assay. Interpretation of the QCS is possible but somewhat challenging without the presence of the control bands as a guide. Further, this version of the QCS did not contain sufficient sequence for the ElucigeneTM CF29 to detect R553X (c.1657C>T, p.Arg553X), but the revised ElucigeneTM CF29 v2 can now detect R553X (c.1657C>T, p.Arg553X) in the QCS. Finally, a synthetic sample only mimics a blood sample, but could be complementary where appropriate patient samples are not available. Since synthetic materials can be manufactured reproducibly, and the study results show that amplifiable DNA was recovered from the QCS and successfully analyzed in the majority of laboratories, it seems reasonable to conclude that changes in test result outputs of a synthetic control may indicate system changes. In this manner, synthetic controls can potentially serve as a monitor for the entire test system. Currently, the most commonly used controls consist of purified genomic DNAs from cell lines or from previously-tested patients. Neither of these provides any information about the quality of DNA extraction at the time of testing.

Overall, the laboratories did consider the control useful (85 reactions), as this synthetic sample can contain rare mutations, which are hard to obtain. Moreover the sample can enclose multiple mutations, in homozygous or heterozygous forms, which reduces the number of positive controls necessary in a test. The QCS is safe to handle and can be produced in large amounts. Some laboratories see the advantage of using synthetic QC samples as a component of method validation. However, others were more critical (18 reactions), mentioning the fact that the control can never include all genotypes and is not totally comparable with human or tissue samples. In addition, the assessors saw a potential benefit of using multiplex synthetic samples as an interesting tool to challenge the interpretation skills of the laboratories. A synthetic sample carrying rare genotypes and mutations with potential cross-reactions can be used, in addition to patient material, as PT material to educate laboratories.

Despite the fact that this was the first time the majority of the laboratories tested control material of a nonbiological nature, most of them were able to extract the sample and detect the correct genotype without making serious interpretation errors. The assessors came across three types of problems, starting from difficulties during the analytical process, to genotype errors and detecting additional mutations not present in the QCS. The putative cause of these problems can be divided into several categories: 1) insufficient understanding of method interferences such as cross-reactivity and near neighbor interferences, some of

which are described in the instructions of the detection assay; 2) insufficient reading of the QCS instruction letter; 3) incompatibility with the ARMS method; and 4) misinterpretation due to inexperience with synthetic controls.

In the first category, several participants were not able to recognize typical cross-reaction patterns despite the fact that the patterns are explained in the manual of the assays. The lack of understanding is significant because these patterns are likely to occur in multiplex tests and can also occur with DNA overload of genomic samples.

Second, several laboratories reported that they stopped the analysis when no DNA was obtained after extraction or no internal control bands were detected during analysis. Both comments indicate that laboratories did not read the instructions of the QCS carefully as both these issues were explained in detail.

However, the lack of control bands for ARMS and the inability of ElucigeneTM CF29 to detect R553X (c.1657C>T, p.Arg553X) in the QCS are due to an incompatibility of the control with the detection methods. It is understandable that laboratories using these methods would have difficulty with the QCS analysis. Both MMQCI and Tepnel Molecular Diagnostics have made product modifications that improve the compatibility of MMQCI's CF controls with ElucigeneTM CFTR test methods.

Finally, it is possible that a few laboratories reported mutations they would not normally report for a patient sample, but did so in the framework of an evaluation study. For example, reporting the weak bands for wild-type R553X (c.1657C>T, p.Arg553X) and R117H (c.350G>A, p.Arg117His), mutant G542X (c.1624G>T, p.Gly542X) and A455E (c.1364C>A, p.Ala455Glu) seen by some of the laboratories using the INNO-LiPA assay could be explained in this respect.

Overall, the synthetic sample appears to be robust for a wide variety of extraction and detection assays. In addition to the positive reactions of the participants regarding the sample's usefulness in method validation and application as RM, the assessors view this type of sample as a valuable means of challenging laboratories' interpretative skills. This might be interesting especially when rare or homozygous mutations are not available in genomic material. Finally, the EuroGentest project aims to improve the quality provided by genetic testing laboratories. Within this scope, multiplex samples, which can be designed to include rare alleles and multiple common mutations, have potential to educate laboratories on the use of control materials and help them to improve the accuracy of genotype detection when circulated as PT material.

ACKNOWLEDGMENTS

This study was performed within the framework of the EU project EuroGentest (FP6-512148) and supported by the European CF Network in the distribution of the samples and collecting the data. We are grateful to all laboratories that took part in the evaluation scheme, especially to those that participated in the pilot testing: Dr. David E. Barton (Dublin, Ireland), Dr. Emmanuelle Girodon (Créteil, France), Prof. Dr. Gert Matthijs (Leuven, Belgium), Dr. Michael A. Morris (Geneva, Switzerland), Prof. Pier Franco Pignatti (Verona, Italy), Dr. Martin J. Schwarz (Manchester, United Kingdom) and Prof. Dr. Manfred Stuhmann-Spangenberg (Hannover, Germany). We thank Nick Nagels and Dr. Anniek Corveleyn for their valuable contributions in developing the electronic datasheet. We appreciate the collaboration and input from Abbott Molecular, Innogenetics, and Tepnel Molecular Diagnostics.

REFERENCES

- Barton DE, Klein C, Stacey GN. 2004. Certified reference materials for genetic testing. In: Fuchs J, Podda M, editors. *Encyclopedia of Medical Genomics and Proteomics*. New York: Dekker Encyclopedias p 226–231.
- Barton DE, Kalman LV. 2007. Reliable genetic testing. *BioWorld Eur* 1:18–21.
- Bernacki SH, Stankovic AK, Williams LO, Beck JC, Herndon JE, Snow-Bailey K, Prior TW, Matteson KJ, Wasserman LM, Cole EC, Stenzel TT. 2003. Establishment of stably EBV-transformed cell lines from residual clinical blood samples for use in performance evaluation and quality assurance in molecular genetic testing. *J Mol Diagn* 5:227–230.
- Cassiman JJ. 2005. Research network: EuroGentest—a European Network of Excellence aimed at harmonizing genetic testing services. *Eur J Hum Genet* 13:1103–1105.
- Chen B, O'Connell CD, Boone DJ, Amos JA, Beck JC, Chan MM, Farkas DH, Lebo RV, Richards CS, Roa BB, Silverman LM, Barton DE, Bejjani BA, Belloni DR, Bernacki SH, Caggana M, Charache P, Dequeker E, Ferreira-Gonzalez A, Friedman KJ, Greene CL, Grody WW, Highsmith WE, Jr, Hinkel CS, Kalman LV, Lubin IM, Lyon E, Payne DA, Pratt VM, Rohlf's E, Rundell CA, Schneider E, Willey AM, Williams LO, Willey JC, Winn-Deen ES, Wolff DJ. 2005. Developing a sustainable process to provide quality control materials for genetic testing. *Genet Med* 7:534–549.
- Christensen TM, Jama M, Ponck V, Lyon E, Wilson JA, Hoffmann ML, Bejjani BA. 2007. Design, development, validation, and use of synthetic nucleic acid controls for diagnostic purposes and application to cystic fibrosis testing. *J Mol Diagn* 9:315–319.
- Dequeker E, Cassiman JJ. 1998. Evaluation of CFTR gene mutation testing methods in 136 diagnostic laboratories: report of a large European external quality assessment. *Eur J Hum Genet* 6:165–175.
- Dequeker E, Cassiman JJ. 2000. Genetic testing and quality control in diagnostic laboratories. *Nat Genet* 25:259–260.
- Dequeker E, Ramsden S, Grody WW, Stenzel TT, Barton DE. 2001. Quality control in molecular genetic testing. *Nat Rev Genet* 2:717–723.
- Grody WW, Cutting GR, Klinger KW, Richards CS, Watson MS, Desnick RJ. 2001a. Laboratory standards and guidelines for population-based cystic fibrosis carrier screening. *Genet Med* 3:149–154.
- Grody WW, Cutting GR, Klinger KW, Richards CS, Watson MS, Desnick RJ. 2001b. Laboratory standards and guidelines for population-based cystic fibrosis carrier screening. *Genet Med* 3:149–154.
- Grody WW. 2003. Quest for controls in molecular genetics. *J Mol Diagn* 5:209–211.
- Hayhurst R, Cassiman JJ. 2006. EuroGentest standing up to scrutiny—first year demonstrates good progress harmonizing community approaches. *J Appl Genet* 47:5–7.
- Huang CK, Pan Q. 2007. Validation of cystic fibrosis mutation analysis using ABI 3130XL genetic analyzer. *Diagn Mol Pathol* 16:57–59.
- Ibarreta D, Elles R, Cassiman JJ, Rodriguez-Cerezo E, Dequeker E. 2004. Towards quality assurance and harmonization of genetic testing services in the European Union. *Nat Biotechnol* 22:1230–1235.
- Jarvis M, Iyer RK, Williams LO, Noll WW, Thomas K, Telatar M, Grody WW. 2005. A novel method for creating artificial mutant samples for performance evaluation and quality control in clinical molecular genetics. *J Mol Diagn* 7:247–251.
- Johnson MA, Yoshitomi MJ, Richards CS. 2007. A comparative study of five technologically diverse CFTR testing platforms. *J Mol Diagn* 9:401–407.
- Klein B, Kleinman NB, Foreman JA. 1974. Preparation and evaluation of a water-soluble cholesterol standard. *Clin Chem* 20:482–485.
- McGovern MM, Elles R, Beretta I, Somerville MJ, Hoefler G, Keinänen M, Barton D, Carson N, Dequeker E, Brdicka R, Blazkova A, Ayme S, Schnieders B, Muller CR, Dalen V, Martinez AA, Kristoffersson U, Ozguc M, Mueller H, Boone J, Lubin IM, Sequeiros J, Taruscio D, Williamson B, Mainland L, Yoshikura H, Ronchi E. 2007. Report of an international survey of molecular genetic testing laboratories. *Community Genet* 10:123–131.
- Muller CR. 2001. Quality control in mutation analysis: the European Molecular Genetics Quality Network (EMQN). *Eur J Pediatr* 160:464–467.
- Proksch GJ, Bonderman DP. 1978. A water-soluble cholesterol derivative for use in augmenting serum control materials. *Clin Chem* 24:1924–1926.
- Watson MS, Cutting GR, Desnick RJ, Driscoll DA, Klinger K, Mennuti M, Palomaki GE, Popovich BW, Pratt VM, Rohlf's EM, Strom CM, Richards CS, Witt DR, Grody WW. 2004. Cystic fibrosis population carrier screening: 2004 revision of American College of Medical Genetics mutation panel. *Genet Med* 6:387–391.
- Williams LO, Cole EC, Lubin IM, Iglesias NI, Jordan RL, Elliott LE. 2003. Quality assurance in human molecular genetics testing: status and recommendations. *Arch Pathol Lab Med* 127:1353–1358.