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Thirteen Years of an International External Quality Assessment Scheme for Genotyping: Results and Recommendations

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BACKGROUND: Suboptimal laboratory procedures resulting in genotyping errors, misdiagnosis, or incorrect reporting bear greatly on a patient's health management, therapeutic decisions made on their behalf, and ultimate outcome. Participation in external quality assessment (EQA) is a key element of quality assurance in molecular genetic diagnostics. Therefore, the Reference Institute for Bioanalytics has tried for 13 years to improve the quality of genetic testing by offering an EQA for different clinically relevant sequence variations.

METHODS: Within each of the biannual EQA schemes offered, up to 18 samples of lyophilized human genomic DNA were provided for up to 50 different molecular genetic tests. Laboratories were asked to use their routine procedures for genotyping. At least 2 expert peer assessors reviewed the final returns. Data from 2002 to 2014 were evaluated.

RESULTS: In total, 82 462 reported results from 812 characterized samples were evaluated. Globally, the number of participants increased each year along with the number of sequence variations offered. The error rate decreased significantly over the years with an overall error rate of 1.44%. Additionally, a decreased error rate for samples repeated over time was noted. Interestingly, the error rate showed a high difference depending on the locus analyzed and the method used.

CONCLUSIONS: Based on the evaluation of this long-term EQA scheme, various recommendations can be given to improve the quality of molecular genetic testing, such as the use of 2 different methods for genotyping. Furthermore, some methods are inappropriate for analysis of certain sequence variations.

QC in a laboratory setting requires the establishment of both internal procedures including standard operating procedures, internal QC and validation of test results, and external quality assessment *(1–7)*. External quality assessment (EQA),[5] also known as proficiency testing (PT), is a key strategy for comparing analytical test performance among different laboratories *(1)*. This concept is firmly established in medical laboratory diagnostics and has led to major standardization and harmonization of test methods, laboratory procedures, and test results. Furthermore, participation in EQA schemes is a requirement for accreditation to the International Organization for Standardization (ISO) 15 189 and 17 025 standards and is mandatory for performing genetic testing in some countries depending on country-specific laws, guidelines, and recommendations *(8–12)*.

Molecular genetic diagnostics has some unique characteristics, commonly referred to as genetic exceptionalism, that need to be considered *(13, 14)*. These characteristics include the high proportion of laboratory-developed tests being used for genotyping, which results in lower comparability of laboratory test results. Furthermore, a genetic test differs from other diagnostic tests in respect to its far-reaching consequences in the lives of patients and their relatives. Compared to other tests, molecular genetic testing is often only performed once in a patient's lifetime, as constitutional genetic information is stable throughout life *(15, 16)*. Accordingly, accurate results are of special importance for genetic testing, and this

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[5] Nonstandard abbreviations: EQA, external quality assessment; PT, proficiency testing; ISO, International Organization for Standardization; NGS, next generation sequencing; RfB, Reference Institute for Bioanalytics; gDNA, genomic DNA; FRET, fluorescence resonance energy transfer; ASA, allele-specific amplification PCR; CAP, College of American Pathologists.

stresses the need for EQA schemes for inherited disorders and conditions. This demand was first met in the 1990s *(17)*. Since then, different providers have been offering EQA schemes for a number of genetic tests, although they only cover a small proportion of the molecular genetic tests being performed worldwide *(18, 19)*. However, since the decoding of the human genome in 2003, our knowledge about the origin of diseases, the influence of genetics, and environmental factors such as lifestyle habits has changed dramatically. One important consequence is the increasing number of genetic tests that have been introduced to clinics. This development is speeding up as more human genetics laboratories are using next generation sequencing (NGS)-based approaches for analysis. Walkaway automation is becoming increasingly popular, and new genotyping methods are entering the molecular diagnostic market.

To investigate influences on DNA amplification and genotype determination as the most frequently performed techniques in molecular diagnostics, the Reference Institute for Bioanalytics (RfB) established a regular EQA scheme focusing on clinically relevant sequence variations in 1998. Since then, its scope has been extended continuously, establishing the RfB as one of the main EQA providers in molecular genetic diagnostics. Here, we present the results of the last 13 years, and provide recommendations on how to establish a genotyping assay. We advise on which methods are preferable.

Materials and Methods

EQA DESIGN

Each EQA scheme is announced to the clinical molecular genetics community through the RfB program, its website (www.dgkl-rfb.de), and mailings. The EQA scheme is framed in a biannual cycle, with up to 9 panels of lyophilized human genomic DNA. Each of the panels consists of 2 samples selected by the scheme organizers. The EQA samples are aliquoted and distributed by the RfB at environmental temperature. Each sample dispatch is accompanied by a cover letter giving basic instructions and a reporting sheet. Participants are asked to use their routine procedures for genotyping. Participants are requested to report results within 6 weeks. After the closing date, all reports are assessed by at least 2 of the scheme organizers. For each scheme, a general report summarizing the statistics, any problems, and upcoming changes is sent to all participating laboratories, together with a certificate for each molecular genetic test they passed. Additionally, the final report is made available via the RfB website. For this EQA scheme, an accreditation according to DIN EN ISO/IEC 17043:2010 exists.

EQA SAMPLE PREPARATION

Sufficient DNA is supplied to perform all necessary analyses. The EQA samples are prepared and approved by the RfB according to standard operating procedures. DNA for the scheme is obtained either from anonymous leftover blood of healthy blood donors or from patients who have given informed consent. Genomic DNA (gDNA) from cell lines is isolated using the Wizard® genomic DNA purification kit (Promega) according to the manufacturer's instructions, and gDNA from buffy coats is isolated via red blood lysis and subsequent salt precipitation according to standard operating procedures *(20)*. Genetic variations are generally checked by more than 1 analytical method.

The scheme organizers select samples for each scheme, which are precharacterized and validated. After gDNA isolation, the quality of gDNA is checked by spectrophotometric analysis (NanoDrop 1000, ThermoFisher) and amplification of a 1.5 kb glyceraldehyde-3-phosphate dehydrogenase (*GADPH*)[6] fragment (GAPDH_forward: TGAA TGGGCAGCCGTTAGGAAAGC, GAPDH_reverse: ATCCTAGTTGCCTCCCCAAA, Sigma-Aldrich) and subsequent gel analysis. The concentration of the gDNA is determined by fluorometric quantification using the Qubit™ dsDNA assay kit (ThermoFisher) according to the manufacturer's instructions. The isolated gDNA is aliquoted and the samples are stabilized by lyophilization and then stored at 4 °C until shipment. Representative lyophilized gDNA samples are checked with respect to their purity and quality by amplifying a 902-bp runt related transcription factor 1 (*RUNX1*) (RunX1_for: CCGGGCTGGGTTATAACTTT, RunX1_rev: ACC TCTGCAAGCAAGATTCG, Sigma-Aldrich) fragment and subsequent analysis by gel electrophoresis.

[6] Human genes: *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *RUNX1,* runt related transcription factor 1; *TPMT,* thiopurine S-methyltransferase; *UGT1A1,* UDP glucuronosyltransferase 1 family member A1; *ATP7B,* ATPase, copper transporting beta; *ITGA2,* integrin subunit alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor); *BCHE,* butyrylcholinesterase; *CYP2C8,* cytochrome P450 family 2 subfamily C member 8; *NOD2,* nucleotide binding oligomerization domain containing 2; *CYP2C19,* cytochrome P450 family 2 subfamily C member 19; *CYP2C9,* cytochrome P450 family 2 subfamily C member 9; *HFE,* hemochromatosis; *F13A1,* coagulation factor XIII A chain; *APOB,* apolipoprotein B; *MCM6,* minichromosome maintenance complex component 6; *LCT,* lactase; *F2,* coagulation factor II, thrombin; *APOE,* apolipoprotein E; *BRAF,* B-Raf proto-oncogene, serine/threonine kinase; *ACE,* angiotensin I converting enzyme; *ALDOB,* aldolase, fructose-bisphosphate B; *CETP,* cholesteryl ester transfer protein; *COL1A1,* collagen type I alpha 1; *DPYD,* dihydropyrimidine dehydrogenase; *F12,* coagulation factor XII (Hageman factor); *F5,* coagulation factor V (proaccelerin, labile factor); *FGB,* fibrinogen beta chain; *HABP2,* hyaluronan binding protein 2; *HLA-B*27,* major histocompatibility complex, class I, B, variant 27; *ITGB3,* integrin subunit beta 3 (platelet glycoprotein IIIa, antigen CD61); *KRAS,* KRAS proto-oncogene, GTPase; *MTHFR,* methylenetetrahydrofolate reductase (NAD(P)H); *SERPINA1,* serpin family A member 1 (alpha-1 antiproteinase, antitrypsin); *SERPINE1,* serpin family E member 1 (nexin, plasminogen activator inhibitor type 1); *VDR,* vitamin D (1,25- dihydroxyvitamin D3) receptor; *VKORC1,* vitamin K epoxide reductase complex subunit 1.

COMPUTATIONAL AND STATISTICAL ANALYSIS

EQA data from 2002–2014 derived from up to 36 EQA samples per year were analyzed. Data analysis and graph plotting were performed using Microsoft Excel for Mac 2011 Ver. 14.4.8 (Microsoft). The means and SDs were calculated for the overall error rate in each EQA scheme offered. A Mann–Whitney *U*-test with a nonparametric approach (SAS™ software, Ver. 8.2, SAS Institute) was performed to test statistical differences in the overall error rate of the different EQA schemes. *P* values <0.05 were considered significant.

Results

SCOPE OF EXTERNAL QC

Since 2002, participation in the EQA scheme has increased steadily from 161 laboratories in 20 different countries to 401 laboratories in 35 countries in 2014. The majority of the participating laboratories are from central Europe: 131 from Germany, 46 from France, 45 from the Benelux countries, and the remainder from 30 different countries. The number of genetic tests offered within the scheme has increased to 49 (Table 1). The mean number of loci analyzed per laboratory increased from 10.43 to 15.23.

Since 2002, the number of genetic variations analyzed by participating laboratories rose constantly from 3180 reported results in 2002 to 11 923 in 2014. In total, 82 462 results were generated in 26 offered EQA schemes. The genetic variation rs6025 (NM_000130.4:c.1601G>A), commonly referred to as Factor V Leiden, was the most frequently determined locus at 13.25%. Table 1 details the number of results reported per locus. For further evaluation, a genetic variation was called frequently determined if the mean number of determinations in each EQA exceeded 40; if fewer, it was defined as a rarely analyzed genetic variation.

Interestingly, the number of determinations of pharmacogenetic relevant sequence variations increased. For instance, for the sequence variations rs1800460, rs1800462, and rs1142345 of the thiopurine S-methyltransferase (*TPMT*) gene (NM_000367.2:c.238G>C; NM_000367.2:c.460G>A; NM_000367.2:c.719A>G), a 20-fold overall increase in participating laboratories was demonstrated when comparing 2002 with 2014. Additionally, comparing 2005 and 2014, the genetic variant rs3481510 of UDP glucuronosyltransferase 1 family member A1 (*UGT1A1*) (NM_000463.2:c.-54_-53insTA) showed the strongest increase in results reported (3.17-fold). On the other hand, no decrease in determinations could be noted within the last 6 years for any of the genetic tests offered (see Supplemental Table 1 that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue8).

METHODS USED FOR GENOTYPE DETECTION

For evaluation of the methods used for genotype detection, as well as for analysis of the method-specific error rate, only data from 2009 or 2010, respectively, to 2014 could be evaluated because participants did not provide this information before 2009. Within the last 5 years, the participating laboratories have reported the methods used for analyses on sequence variations in 73.5% (35 986/48 795) of tests carried out. The 5 methodological approaches most frequently reported were: hybridization probe techniques (30%), hydrolysis probe assays (11.9%), RFLP (10.6%), direct DNA sequencing (including Sanger sequencing and NGS) (10.4%), and reverse dot blot (10.3%). By hybridization probe techniques, we mean the use of 2 adjacent hybridization probes, 1 labeled with a donor and 1 with an acceptor fluorochrome. In this case, the fluorescence resonance energy transfer (FRET) occurs after hybridization by quenching of the donor and sensitization of the acceptor fluorescence *(21, 22)*. In the case of hydrolysis probes, a quencher molecule quenches the fluorescence emitted using a reporter fluorochrome via FRET. As soon as the quencher molecule is cleaved by the 5′–3′ exonuclease activity of *Taq* polymerase, the light emitted is detected *(23, 24)*. In addition to these 5 methods, others less commonly used (0.2–9.4%) included allele-specific PCR (ASA), pyrosequencing, microchips, molecular beacon *(25–27)*, laboratory-developed tests, and others (Table 2). Tests that could not be assigned to one of the methods mentioned were categorized as laboratory-developed if developed by the laboratory or as other in the case of a commercially available test. A detailed overview of the different methodological approaches used for analyses on loci in each EQA can be found in online Supplemental Table 2.

Compared to 2009, the number of laboratories using RFLP and hybridization probes (14%/35% in 2009 compared to 8%/25% in 2014) decreased, whereas, for DNA sequencing and the usage of hydrolysis probes (7%/9% in 2009 compared to 14%/13% in 2014), an increase was noted (see online Supplemental Table 2).

A more detailed overview of the methods used depending on the locus analyzed is displayed in online Supplemental Table 3.

Interestingly, there was a clear preference for methods used with respect to certain sequence variations. For instance, for analysis of the sequence variants rs76151636 (NM_000053.3:c.3207C>A) of ATPase, copper transporting beta (*ATP7B*) and rs1126643 (NM_002203.3:c.759C>T) of integrin subunit alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) (*ITGA2*), approaches based on hybridization probes were used in more than 50% of cases. In contrast, the sequence variations rs1799807 (NM_000055.2:c.293A>G) and rs1803274 (NM_000055.2:c.1699G>A) of butyryl-

Table 1. Summary of genetic variations, nomenclature, percentage of determinations, and locus-specific error rate.

Gene	Genetic variation[a]	rs-number	% of all determinations (n = 82 462)[b]	Locus-specific mean error rate, %[c]
ACE	NM_000789.3:c.2306–119_2306–118ins50	rs1799752	2.11	1.49
ALDOB	NM_000035.3:c.448G>C	rs1800546	0.54	0.00
ALDOB	NM_000035.3:c.524C>A	rs76917243	0.54	0.21
ALDOB	NM_000035.3:c.1005C>G	rs78340951	0.54	0.18
APOB	NM_000384.2:c.10580G>A	rs5742904	3.10	0.90
APOE	NM_000041.2:c.388T>C; NM_000041.2: c.526C>T	rs429358, rs7412	5.24	2.37
ATP7B	NM_000053.3:c.3207C>A	rs76151636	0.39	0.35
BCHE	NM_000055.2:c.1699G>A	rs1803274	0.23	0.63
BCHE	NM_000055.2:c.293A>G	rs1799807	0.21	0.14
BRAF	NM_004333.4:c.1799T>A	rs113488022	0.43	0.36
CETP	NM_000078.2:c.118 + 279G>A	rs708272	0.49	2.64
COL1A1	NM_000088.3:c.104–441G>T	rs1800012	0.13	0.99
CYP2C19	NM_000769.1:c.681G>A; NM_000769.1: c.-806C>T	rs4244285, rs12248560	1.04	3.49
CYP2C8	NM_000770.3:c.1196A>G	rs10509681	0.20	0.56
CYP2C9	NM_000771.3:c.430C>T; NM_000771.3: c.1075A>C	rs1799853, rs1057910	1.58	1.86
DPYD	NM_000110.3:c.1905 + 1G>A	rs3918290	1.33	0.11
F12	NM_000505.3:c.-4T>C	rs1801020	0.29	1.01
F13A1	NM_000129.3:c.103G>T	rs5985	1.34	0.80
F2	NM_000506.3:c.*97G>A	rs1799963	13.07	0.88
F5	NM_000130.4:c.1601G>A	rs6025	13.25	0.98
F5	NM_000130.4:c.3980A>G	rs1800595	0.53	1.29
FGB	NM_005141.4:c.-463G>A	rs1800790	0.72	0.16
HABP2	NM_004132.3:c.1601G>A	rs7080536	0.14	0.96
HFE	NM_000410.3:c.845G>A	rs1800562	7.41	0.85
HFE	NM_000410.3:c.187C>G	rs1799945	7.49	2.32
HFE	NM_000410.3:c.193A>T	rs1800730	4.04	1.09
*HLA-B*27*			0.30	0.15
ITGA2	NM_002203.3:c.759C>T	rs1126643)	0.18	0.38
ITGB3	NM_000212.2:c.176T>C	rs5918	1.09	0.96
KRAS	NM_033360.2:c.34G>A/C/T; NM_033360.2:c.35G>A/C/T	rs121913530, rs121913529	0.65	0.87
KRAS	NM_033360.2:c.37G>A/C/T; NM_033360.2:c.38G>A/C/T	rs121913535, rs112445441	0.59	1.20
KRAS	NM_033360.2:c.181C>A/G; NM_033360.2:c.182A>C/G/T; NM_033360.2:c.183A>C/T	rs121913238, rs121913240, rs17851045	0.42	0.62
LCT	NM_002299.2:c.-13910C>T	rs4988235	3.03	2.29
MTHFR	NM_005957.4:c.1286A>C	rs1801131	4.50	0.50
MTHFR	NM_005957.4:c.665C>T	rs1801133	8.79	0.83
NOD2	NM_022162.1:c.2722G>C	rs2066845	0.35	0.17
NOD2	NM_022162.1:c.3017_3018insC	rs2066847	0.35	0.52

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Table 1. Summary of genetic variations, nomenclature, percentage of determinations, and locus-specific error rate. (*Continued from page 1087*)

Gene	Genetic variation[a]	rs-number	% of all determinations (n = 82 462)[b]	Locus-specific mean error rate, %[c]
NOD2	NM_022162.1:c.2104C>T; NM_001002235.2:c.710T>C; NM_001002235.2:c.863A>T	rs2066844, rs6647, rs17580	0.35	2.64
SERPINA1	NM_001002235.2:c.1096G>A	rs28929474	2.92	2.77
SERPINE1	NM_000602.4:c.-817_-816insG	rs1799889	3.20	1.59
TPMT	NM_000367.2:c.238G>C; NM_000367.2: c.460G>A; NM_000367.2:c.719A>G	rs1800460, rs1800462, rs1142345	2.47	1.17
UGT1A1	NM_000463.2:c.-54_-53insTA	rs34815109	2.22	1.81
VDR	NM_000376.2:c.1025-49G>T	rs7975232	0.04	0.00
VDR	NM_000376.2:c.1024 + 283G>A	rs1544410	0.16	0.62
VDR	NM_000376.2:c.1056T>C	rs731236	0.07	0.96
VKORC1	NM_024006.4:c.174–136C>T	rs9934438	0.78	0.19
VKORC1	NM_024006.4:c.-1639G>A	rs9923231	1.21	0.79

[a] The correct HGVS (Human Genome Variation Society) coding of principal genotypes is given in *(39)*.
[b] The percentage of determinations for each locus reported between 2002 and 2014 is shown.
[c] The locus-specific error rate is calculated by the absolute number of false determination per locus divided by the absolute number of results reported per locus between 2002 and 2014.

cholinesterase; BRAF, B-Raf proto-oncogene, serine/threonine kinase (*BCHE*) as well as *CYP2C8*3* [CYP2C8, cytochrome P450 family 2 subfamily C member 8 (NM_000770.3:c.1196A>G)] were most frequently determined by direct DNA sequencing (57%, 54%, respectively).

Table 2. Methods used for analysis of genetic variations by participating laboratories.

Method used for genotyping[a]	% of all determinations (n = 35986)
Hybridization probe	30.0
RFLP	10.6
Reverse dot blot	10.4
Hydrolysis probe	11.9
ASA	9.4
DNA sequencing	10.4
Other	8.1
Pyrosequencing	4.9
Laboratory-developed	2.7
Microchips	1.4
Molecular beacons	0.2

[a] All of the different categories (with the exception of laboratory-developed and other) included commercially available tests as well as laboratory-developed tests.

ERROR RATE

The overall error rate based on 82 462 results reported within the last 13 years for the different genetic tests offered was 1.44% (n = 1191; range 0.62%–3.62%). The error rates for the different genetic tests offered in each EQA scheme are displayed in Table 3. Furthermore, Table 3 shows the *P* values determined by comparing the mean error rate for the genetic tests offered in each scheme with the error rates for the same tests across schemes. The mean error rate decreased significantly since 2010.

The locus-specific error rate based on data from 2010 to 2014 is shown in Table 1. An increased error rate was noted for rarely analyzed sequence variations: 4.83% for the sequence variant rs2066844 (NM_022162.1: c.2104C>T) in nucleotide binding oligomerization domain containing 2 (*NOD2*) and 4.55% for rs1800012 (NM_000088.3:c.104–441G>T). With respect to frequently determined genetic variations, the highest number of misidentifications could again be demonstrated for pharmacogenetic-relevant sequence variations. For instance, error rates of 8.52% for the sequence variations *CYP2C19*2* and *CYP2C19*17* (cytochrome P450 family 2 subfamily C member 19 NM_000769.1:c.681G>A and NM_000769.1:c.-806C>T) and 3.76% for the genetic variations *CYP2C9*2* and *CYP2C9*3* (cytochrome P450 family 2 subfamily C member 9 NM_000771.3: c.430C>T and NM_000771.3:c.1075A>C) were observed.

Table 3. Statistics for error rate in each EQA scheme.

EQA[a]	n[b]	Minimum	Median	Mean	Maximum	SD	*P value*[c]
1 (2002)	9	0.00	2.63	3.00	8.85	3.32	0.4527
2 (2002)	11	0.00	0.77	1.32	5.05	1.74	0.177
1 (2003)	11	0.00	1.35	1.67	5.71	1.55	0.1763
2 (2003)	12	0.00	2.61	3.62	20.0	5.42	0.133
1 (2004)	13	0.00	2.10	1.93	4.95	1.95	0.9385
2 (2004)	14	0.00	0.00	0.62	2.42	0.84	0.0257
1 (2005)	16	0.00	0.00	1.36	8.54	2.53	0.0196
2 (2005)	16	0.00	1.08	1.97	11.11	2.91	0.5204
1 (2006)	16	0.00	1.54	1.27	4.17	1.15	0.3852
2 (2006)	17	0.00	0.00	1.10	5.32	1.55	0.0792
1 (2007)	19	0.00	0.00	1.48	12.24	2.87	0.0467
2 (2007)	24	0.00	0.00	0.83	5.1	1.40	0.0009
1 (2008)	24	0.00	1.46	1.98	7.84	2.35	0.5902
2 (2008)	28	0.00	0.49	2.16	8.7	2.79	0.2484
1 (2009)	32	0.00	0.55	1.83	12.5	2.92	0.0865
2 (2009)	32	0.00	0.81	2.04	21.43	4.11	0.0767
1 (2010)	37	0.00	0.52	2.55	25.00	4.98	0.0459
2 (2010)	37	0.00	0.00	1.29	11.54	2.75	<0.0001
1 (2011)	41	0.00	0.00	2.00	50.00	7.90	<0.0001
2 (2011)	41	0.00	0.37	1.45	9.38	2.24	0.0172
1 (2012)	46	0.00	0.00	1.81	25.00	4.06	0.0049
2 (2012)	46	0.00	0.00	1.24	8.33	2.03	0.0005
1 (2013)	48	0.00	0.00	1.08	18.00	3.43	<0.0001
2 (2013)	49	0.00	0.71	2.59	22.73	4.55	0.0744
1 (2014)	49	0.00	0.17	1.50	10.64	2.46	0.0084
2 (2014)	49	0.00	0.00	1.10	8.33	1.79	0.0005

[a] Numbers of the EQA scheme and the corresponding year (indicated in brackets) are shown.
[b] Number of genetic tests offered in each EQA scheme.
[c] The level of significance for the error rates of tests in each scheme compared to the error rates for the same tests across schemes is calculated using the Mann–Whitney *U*-test with a nonparametric approach. *P* values < 0.05 are considered significant.

Furthermore, within the last 13 years, different EQA samples have been provided more than once. In most of the cases, fewer laboratories failed when the same sample was analyzed the second time. For instance, for analysis of the sequence variations rs17999445, rs1800730, and rs1800562 (NM_000410.3:c.845G>A; NM_000410.3:c.187C>G; NM_000410.3:c.193A>T) in the hemochromatosis (*HFE*) gene, error rates of 8.33%, 6.31%, and 2.17%, respectively, for the first scheme in 2010 compared to error rates of 1.4%, 2.79%, and 0.7%, respectively, for the first scheme in 2012 were registered. Other examples include the testing of the sequence variant rs5985 (NM_000129.3:c.103G>T) in coagulation factor XIII A chain; F2, coagulation factor II, thrombin (*F13A1*) with an error rate of 2.9% (1/2012) compared to 0% (1/2014), or the analysis of the sequence variations rs1799853 and rs1057910 (NM_000771.3:c.430C>T; NM_000771.3:c.1075A>C) in *CYP2C9* with error rates of 15.2% (1/2010) and 3.85% (1/2012), respectively.

Depending on the method used, the highest error rate over the last 5 years was observed for microchips at 4%, followed by RFLP-based methods and laboratory-developed tests at 2.4% and 2.1%, respectively (Fig. 1).

Surprisingly, certain methods seemed to be inappropriate for analysis of certain sequence variations (Table 4). To determine a cutoff, we defined those methods for analysis of a certain locus as inappropriate if the error rate reported over the last years was twice as high as the percentage of laboratories that used that method for analysis. In this case, the resulting factor was larger than 2. To give an example, for analysis of the sequence variation

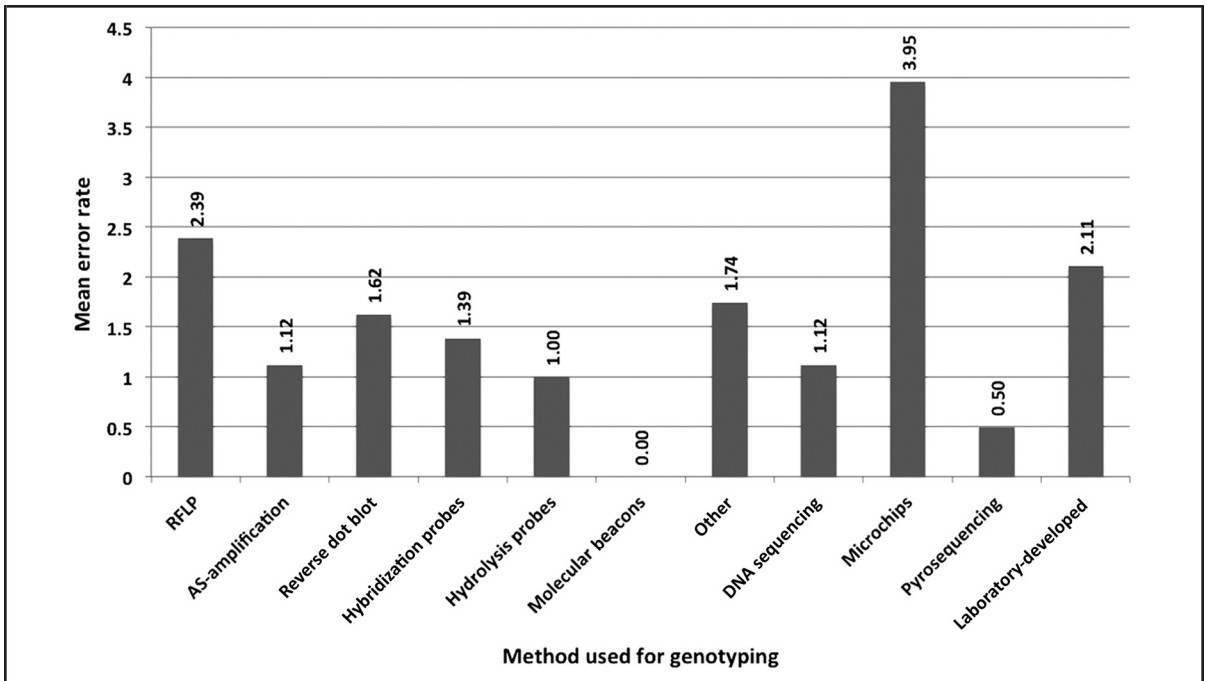

Fig. 1. Mean error rate per method used.

Data presented are based on methods reported for loci analyses between 2010 and 2014. In total, 48 795 results are considered. Displayed is the mean error rate per method. Other indicates all commercially available tests that cannot be assigned to one of the other methods; Microchips, microchip analysis.

rs5742904 (NM_000053.3:c.3207C>A) in apolipoprotein B (*APOB*), 17% of laboratories have used RFLP-based approaches within the last 5 years, whereas 100% of reported errors have been made by laboratories using RFLP for analysis of this sequence variation. Thus, the resulting factor (17% divided by 100%) was 6.05. This means that the percentage of an error made by laboratories using RFLP-based approaches for analysis of rs5742904 (NM_000053.3:c.3207C>A) in *APOB* was 6 times higher than the percentage of laboratories using this method for genetic testing. According to our cutoff given above, RFLP-based approaches are inappropriate for analysis of rs5742904 (NM_000053.3:c.3207C>A) in *APOB*. Other examples are highlighted in Table 4. A more detailed overview including a locus-specific error rate and the percentage of laboratories using each method for analysis of the different sequence variations can be found in online Supplemental Table 4.

ERROR TYPES

In the course of EQA assessments, several errors were identified that could involve technical or analytical processes *(16)*. These could be divided into technical and analytical errors. Technical errors included mislabeling of samples, errors due to cross-contamination, or incorrect assignment of the genetic sequence variation identified to the correct phenotype. The latter case was often found in genetic testing for lactose intolerance due to the sequence variation rs4988235 (NM_002299.2:c.-13910C>T) in the minichromosome maintenance complex component 6 (*MCM6*) gene that influences the lactase (*LCT*) gene. Here, the homozygous constellation NM_002299.2:c.[-13910C>T];[-13910C>T] is associated with lactase persistence and, though defined as mutant, is often falsely assigned to a hypolactasia phenotype by participating laboratories *(28, 29)*.

Analytical errors can be caused either by rare sequence variations or by inadequate assignment of the genetic variation (haplotype) identified to the correct genotype. The second case is often found in the genetic testing of sequence variations in cytochrome P450 enzymes like *CYP2C19* or *CYP2C9*, as these have their own nomenclature system *(30–32)*.

With respect to rare sequence variations, no methodological approach seemed to be especially prone to error. Retrospectively, we could demonstrate misidentifications due to rare sequence variations on almost every basis of molecular genetic testing. For instance, during the second scheme in 2011, 4 laboratories using TaqMan® assays from the same commercial supplier failed

Table 4. Locus-specific error rate depending on method used.

Gene	Genetic variation	RFLP[a]	ASA[a]	Reverse dot blot[a]	Hybridization probe[a]	Hydrolysis probe[a]	Molecular beacons[a]	Other[a]	DNA sequencing[a]	Microchips[a]	Pyrosequencing[a]	Laboratory-developed[a]
ACE	NM_000789.3:c.2306-119_2306-118ins50	3.49	0.00	3.56	0.00	0.00	0.00	0.59	0.00	0.00	0.00	0.00
ALDOB	NM_000035.3:c.448G>C; NM_000035.3:c.524C>A; NM_000035.3:c.1005C>G	0.00	0.00	0.00	0.00	0.00	0.00	5.75	0.00	41.29	0.00	0.00
APOB	NM_000384.2:c.10580G>A	6.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
APOE	NM_000041.2:c.388T>C; NM_000041.2:c.526C>T	0.77	0.00	0.48	1.38	0.72	0.00	4.6	0.47	23.81	0.00	0.00
ATP7B	NM_000053.3:c.3207C>A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BCHE	NM_000055.2:c.1699G>A; NM_000055.2:c.293A>G	0.00	0.00	0.00	0.00	2.86	0.00	6.2	0.88	0.00	0.00	0.00
BRAF	NM_004333.4:c.1799T>A	0.00	4.83	0.00	3.27	1.25	0.00	0.00	1.19	0.00	0.00	0.00
CETP	NM_000078.2:c.118 + 279G>A	1.13	0.00	0.00	0.00	0.00	0.00	1.05	0.00	6.89	0.00	0.00
COL1A1	NM_000088.3:c.104-441G>T	0.74	0.00	1.36	0.00	4.83	0.00	0.00	0.00	0.00	0.00	0.00
CYP2C19	NM_000769.1:c.681G>A; NM_000769.1:c.-806C>T	2.53	0.00	0.99	1.52	0.65	0.00	0.00	0.00	1.4	0.84	1.24
CYP2C8	NM_000770.3:c.1196A>G	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.86	0.00	0.00	0.00
CYP2C9	NM_000771.3:c.430C>T; NM_000771.3:c.1075A>C	2.83	0.00	3.02	0.97	0.54	0.00	0.00	0.65	0.00	0.00	0.00
DPYD	NM_000110.3:c.1905 + 1G>A	0.00	0.00	0.00	2.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F12	NM_000505.3:c.-4T>C	0.00	0.00	0.00	5.56	1.03	0.00	0.00	0.00	0.00	0.00	0.00
F13A1	NM_000129.3:c.103G>T	2.63	2.09	3.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F2	NM_000506.3:c.*97G>A	3.1	0.8	0.83	1.08	0.00	0.00	0.00	0.00	0.00	0.00	5.98
F5	NM_000130.4:c.1601G>A	2.57	0.88	1.86	1.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F5	NM_000130.4:c.3980A>G	0.00	2.36	1.26	1.07	0.00	0.00	5.04	0.00	0.00	0.00	0.00
FGB	NM_005141.4:c.-463G>A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HABP2	NM_004132.3:c.1601G>A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HFE	NM_000410.3:c.187C>G; NM_000410.3:c.193A>T; NM_000410.3:c.845G>A	0.72	0.38	0.69	1.45	1.04	0.00	0.78	1.23	6.53	0.00	0.78
*HLA-B*27*		0.00	1.25	2.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.38

Continued on page 1092

Table 4. Locus-specific error rate depending on method used. (*Continued from page 1091*)

Gene	Genetic variation	RFLP[a]	ASA[a]	Reverse dot blot[a]	Hybridization probe[a]	Hydrolysis probe[a]	Molecular beacons[a]	Other[a]	DNA sequencing[a]	Microchips[a]	Pyrosequencing[a]	Laboratory-developed[a]
ITGA2	NM_002203.3:c.759C>T	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ITGB3	NM_000212.2:c.176T>C	3.63	1.47	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00
KRAS	NM_033360.2:c.34G>A/C/T; NM_033360.2:c.35G>A/C/T; NM_033360.2:c.37G>A/C/T; NM_033360.2:c.38G>A/C/T; NM_033360.2:c.181C>A/G; NM_033360.2:c.182A>C/G/T; NM_033360.2:c.183A>C/T	1.44	0.58	1.91	0.00	0.00	0.00	1.96	0.83	5.07	0.68	0.00
LCT	NM_002299.2:c.-13910C>T	1.6	1.73	0.6	0.76	1.41	0.00	1.74	0.00	0.00	0.00	4.77
MTHFR	NM_005957.4:c.1286A>C; NM_005957.4:c.665C>T	1.84	0.45	0.84	1.35	0.36	0.00	0.74	0.00	11.96	0.00	0.00
NOD2	NM_022162.1:c.2722G>C; NM_022162.1:c.3017_3018insC; NM_022162.1:c.2104C>T	0.00	0.00	0.00	2.93	0.00	0.00	0.00	1.5	0.00	0.00	0.00
SERPINA1	NM_001002235.2:c.710T>C; NM_001002235.2:c.863A>T; NM_001002235.2:c.1096G>A	2.23	1.64	0.82	0.85	1.4	0.00	0.00	0.85	0.00	0.00	0.00
SERPINE1	NM_000602.4:c.-817_-816insG	2.67	1.46	1.57	0.48	0.00	0.00	0.00	0.00	18.32	0.00	0.00
TPMT	NM_000367.2:c.238G>C; NM_000367.2:c.460G>A; NM_000367.2:c.719A>G	1.48	1.36	7.41	0.62	1.44	0.00	1.77	0.00	0.00	0.00	0.00
UGT1A1	NM_000463.2:c.-54_-53insTA	0.00	0.00	0.00	0.86	0.00	0.00	1.6	1.07	0.00	0.66	0.37
VDR	NM_000376.2:c.1025-49G>T	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
VDR	NM_000376.2:c.1024 + 283G>A	0.99	0.00	1.53	0.00	0.00	0.00	0.00	3.81	0.00	1.41	0.00
VDR	NM_000376.2:c.1056T>C	0.00	0.00	0.00	0.00	0.00	0.00	3.3	0.00	0.00	0.00	0.00
VKORC1	NM_024006.4:c.174-136C>T; NM_024006.4:c.-1639G>A	4.13	0.00	0.00	0.44	2.06	0.00	2.56	0.00	0.00	0.00	0.00

[a] Data are based on methods reported and errors for each locus from 2010 to 2014. For each of the methods used for analysis of the different sequence variations, a factor is displayed. This factor is calculated by dividing the error rate by the percentage of laboratories using that method for genotyping of the corresponding sequence variation. If the resulting factor is larger than 2, the method is defined as inappropriate for the corresponding locus and is highlighted in gray.

to identify the *17 sequence variant rs12248560 (NM_000769.1:c.-806C>T) in *CYP2C19* due to an additional sequence variation 41 bp downstream of the loci investigated.

Discussion

In general, QC in a laboratory setting includes the establishment of effective training and standard operating procedures, internal QC, validation of tests and participation in EQA schemes. EQA is educational, allowing long-term, retrospective analysis of laboratory performance. This permits participating laboratories to compare results with peers, which can reveal intermethod variability *(33)*. To identify the appropriate EQA, a few databases exist providing information on EQA providers and their available schemes. To the best of our knowledge, the listings on the IFCC and the Eurogentest *(34)* websites cover all European and globally active providers for molecular genetic diagnostics *(18, 19)*.

One of these providers is the RfB, for which we describe here the results of this long-term EQA scheme between 2002 and 2014.

Over a 13-year period, participation in the scheme more than doubled, which reflected an increasing number of laboratories performing molecular genetic tests worldwide and an increasing awareness of the importance of participation in EQA schemes, as well as the implementation of gene-diagnostic laws in some countries by which the participation in EQA schemes has become mandatory *(12)*. In particular, the assay frequency of pharmacogenetic-relevant sequence variations increased. This might be explained by the US Food and Drug Administration recommendations for genetic testing before administration of certain drugs to avoid adverse events or optimize drug dose. Giving an example, testing of the *TPMT* gene is recommended before administration of azathioprine *(35)*.

Additionally, the number of genetic tests performed per laboratory nearly doubled, which mirrored the increasing range of genetic tests being performed in routine diagnostics. This development stresses the need for EQA schemes covering the different tests. To meet this demand, the number of genetic tests offered by the RfB quintupled and is still increasing.

Regarding the methods used for genetic testing, one development is obvious: the number of laboratories using hydrolysis probes and sequencing-based approaches for analysis of sequence variations has increased. This is most likely because of the fact that these approaches in particular allow a higher throughput and broader range of tests to be performed at the same time, thereby reducing time and costs for analysis. The observation that hybridization probes are more commonly used for frequently determined variations, whereas, for rarely analyzed loci, DNA sequencing is the most commonly used method, is likely because of the fact that commercially available kits are limited to frequently determined variations because of monetary reasons.

In total, 82 462 results reported from 812 characterized samples were evaluated, revealing an overall error rate of 1.44%. This means that every 70th sequence variation reported was incorrect. These results are in accordance with the data from other EQA providers. For instance, CAP stated an overall error rate of 1.5% for the cystic fibrosis mutation analysis scheme *(36)*. The error rate within each of the different schemes offered decreased significantly over the years, demonstrating that the performance of laboratories increased. This indicates the importance of quality assurance and the improvement that can be achieved by participation in EQA schemes. Furthermore, the ability of EQA schemes to improve the quality of diagnostics can be proven by the decreased error rate of samples provided twice. Explanations include the increased awareness of the participants for rare sequence variations causative for assay-specific or method-specific problems and the importance of the correct usage of different nomenclature systems.

With regard to the methods used for genetic testing, it is important to note that some methods seem to be inappropriate for analysis of certain sequence variations because they are particularly error-prone for genetic tests on particular loci. For example, the usage of hybridization probe assays is error-prone in respect to the analysis of the sequence variation rs4988235 (NM_002299.2:c-13910C>T) in *MCM6* or the sequence variations rs1799945 and rs1800730 (NM_000410.3:c.187C>G, NM_000410.3:c.193A>T) in *HFE*. This can be explained by the close vicinity of the 2 variations in the case of *HFE* or of another rare nonrelated variant in the case of *LCT (37)*. This demonstrates the importance of choosing a suitable and accurate method depending on the locus analyzed. To give advice on which methods should not be used for genotyping on particular loci, we have highlighted these methods in Table 4 and have provided the loci- and method-specific error rates in online Supplemental Table 4.

With respect to the error types, analytical errors are often a result of inadequate assignment of the genetic variation identified to the correct genotype. This is especially the case for pharmacogenetic-relevant sequence variations, but also explains the high error rate for genotyping of *APOE* because the 2 sequence variations rs429358 and rs7412 (NM_000041.2:c.388T>C; NM_000041.2:c.526C>T) define the 3 major alleles of apolipoprotein E (*APOE*), *APOE2*, *APOE3,* and *APOE4*. Rare sequence variations often affect the ability of a test to detect certain sequence variations, leading to false-positive or false-negative results *(38)*. By providing such samples within the last 13 years, 2 results were ob-

tained. On the one hand, the quality of commercial kits could be assessed, which allowed notification of laboratories and suppliers of kit-specific problems. On the other hand, the results from such samples demonstrate that none of the methods seem to be prone to be affected by rare sequence variations. For that reason, and taking into account the far-reaching consequences of a false-positive result, we recommend the use of 2 different methods or at least 2 different sets of primers and probes for molecular genetic testing. In fact, more and more laboratories are mentioning this need *(15)*.

In conclusion, the following recommendations can be made. Molecular genetic testing should be performed in accordance to ISO 15 189 and 17 025 standards and to the OECD (Organisation for Economic Co-operation and Development) guidelines. In particular, attention should be paid to the following 5 points. First, after primer design, sequences should be blasted to ensure the specificity of the primers. Additionally, for given sequence variations, the site under the primer or probe binding must be searched. The same applies for commercially available kits. Second, laboratory-developed methods should be validated with appropriate samples and numbers (e.g., 20) by at least 2 different methods. Furthermore, cross-validation with another laboratory or, if possible, with a reference institute should be performed. The same applies to in vitro diagnostic kits. Third, when establishing a new assay, the appropriate method should be chosen. The data provided in Table 4 give more detailed recommendations. Those methods highlighted in grey should not be used for genotyping of the corresponding loci because they are particularly error-prone. Fourth, laboratory and medical staff should be trained in how to use the HUGO (Human Genome Organization), HGVS (Human Genome Variation Society), and star allele nomenclature systems. Fifth, 2 different methods should be used for analysis of sequence variations.

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