Reference Measurement Systems in Genetic Testing: Development of DNA-based Reference Materials

Summary of PhD thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Klein CL, **Márki-Zay J**, Corbisier P, Gancberg D, Cooper S, Gemmati D, Halbmayer WM, Kitchen S, Melegh B, Neumaier M, Oldenburg J, Oppliger Leibundgut E, Reitsma PH, Rieger S, Schimmel HG, Spannagl HG, Tordai A, Tosetto A, Visvikis S, Zadro R, Mannhalter C. Reference Materials (RMs) for the analysis of the human factor II (prothrombin) gene G20210A mutation. Clin Chem Lab Med 43:862-68. (2005) IF: 1.685

II. Gancberg D, **Márki-Zay J**, Corbisier P, Klein C, Schimmel H, Emons H. Certification of a Reference Material consisting of purified plasmid DNA containing a fragment from the human prothrombin gene (wildtype) – Certified Reference Material IRMM/IFCC-490. Report EUR 22169 EN (2006)

III. Gancberg D, **Márki-Zay J**, Corbisier P, Klein C, Schimmel H, Emons H. Certification of a Reference Material consisting of purified plasmid DNA containing a fragment from the human prothrombin gene (G20210A mutant) – Certified Reference Material IRMM/IFCC-491. Report EUR 22170 EN (2006)

IV. Gancberg D, **Márki-Zay J**, Corbisier P, Klein C, Schimmel H, Emons H. Certification of a Reference Material consisting of purified plasmid DNA containing a fragment from the human prothrombin gene (heterozygous G20210 wildtype/G20210A mutant) – Certified Reference Material IRMM/IFCC-492. Report EUR 22167 EN (2006)

V. Gancberg D, Corbisier P, Meeus N, **Márki-Zay J**, Mannhalter C, Schimmel H. Certification of reference materials for detection of the human prothrombin gene G20210A sequence variant. Clin Chem Lab Med 46:463-9. (2008) IF: 1.888

VI. **Márki-Zay J**, Klein CL, Gancberg D, Schimmel HG, Dux L. European External Quality Control Study on the Competence of Laboratories to Recognize Rare Sequence Variants Resulting in Unusual Genotyping Results. Clin Chem 55:739-47. (2009) IF: 6.263

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PRESENTATIONS DIRECTLY RELATED TO THE THESIS

VII. **Márki-Zay J**, Klein CL, Corbisier P, Gancberg D, Schimmel H, Mannhalter C. How to prepare stable reference materials for genetic testing. European Human Genetics Conference 2004, 12-15. June 2004, Munich, Germany; Abstract: Eur J Hum Genet 12;Suppl 1:235-6[P697]

VIII. Klein CL, Rieger S, Gancberg D, **Márki-Zay J**, Corbisier P, Schimmel H, Mannhalter C. Production of Certified Reference Materials (CRMs) for the analysis of the human factor II (prothrombin) gene G20210A mutation. European Human Genetics Conference 2004, 12-15. June 2004, Munich, Germany; Abstract: Eur J Hum Genet 12;Suppl 1:236[P698]

IX. Barton D, Donohoe EM, **Márki-Zay J**, Klein CL. Preparation of certified reference materials for hereditary haemochromatosis. European Human Genetics Conference 2004, 12-15. June 2004, Munich, Germany; Abstract: Eur J Hum Genet 12;Suppl 1:257[P803]

X. Barton DE, Duke S, Donohoe E, **Márki-Zay J**, Klein CL and the CRMGEN Consortium. The CRMGEN Project: Preparation of Certified Reference Materials for Hereditary Haemochromatosis Testing. 10th Annual Meeting of the Association for Molecular Pathology, 10-13. Nov 2004, Los Angeles, USA; Abstract: J Mol Diagn 6;4:413[G35]

XI. Gancberg D, Corbisier P, Klein C, Mannhalter C, **Márki-Zay J**, Schimmel H. Production of Certified Reference Materials (CRMs) for the analysis of the human Factor II (prothrombin) gene G20210A mutation. European Human Genetics Conference 6-9 May 2006, Amsterdam, The Netherlands; Abstract: Eur J Hum Genet 14;Suppl 1:375[P1256]

XII. **Márki-Zay J**, Gancberg D, Klein C, Schimmel H, Dux L. European proficiency testing study on the competence of laboratories to recognise rare mutations resulting in unexpected genotyping results. European Human Genetics Conference 6-9 May 2006, Amsterdam, The Netherlands; Abstract: Eur J Hum Genet 14;Suppl 1:379[P1276]

1. INTRODUCTION

Recent scientific and technological advances, such as the invention of the polymerase chain reaction (PCR) technique and the deciphering of the human genome, revealed the genetic background of medical conditions and led to an enormous progress in our knowledge on the association between specific sequence variants and particular disease entities. On the other hand, many of the molecular biology techniques developed and applied originally for scientific purposes achieved the robustness and performance characteristics required for routine diagnostic application. Such molecular diagnostic assays are able to detect genetic alterations resulting in inheritable disorders, higher risk to develop diseases or altered drug metabolism and play an increasingly important role in the diagnosis, in risk stratification and even more also in the choice of the right treatment for the patients. These tests are highly specific and thousands of different tests are already in routine use. DNA test results can be predictive for future onset of disorders prior to the manifestation of clinical symptoms, and thus have significant implications for the individual patient as well as for his/her relatives.

Although public perception holds that genetic tests are highly accurate and represent a state-of-the-art technology, the development of systems to ensure the quality of methods and testing services has lagged behind the spread of application of DNA-based tests in medicine. Concerns over the accuracy of genetic test results were further supported by several studies and publications of international organisations reporting high error rates and lack of standardisation and available certified reference materials (CRMs) for clinical genetic testing. Quality issues in genetic testing are given added importance because individuals are usually only tested once in their lifetime, and an incorrect genotyping result may remain hidden for many years with dramatic consequences for the tested person. These facts combine to make standardisation and improvement of quality in molecular genetic testing a matter of utmost importance.

In order to achieve and ensure the highest level of accuracy in the laboratory medicine, a reference measurement system (RMS) has to be established. Reference measurement systems are needed for producing useful and reliable measurement results in the routine diagnostic services as

5. MAIN SCIENTIFIC ACHIEVEMENTS OF THE STUDY

The most important achievements of the present study can be outlined as follows.

1) Several types and presentation formats of DNA-based RMs were processed and investigated for their suitability as clinical genetic (C)RMs.

2) Different possibilities for the preservation of purified DNA-based RMs were examined and optimised to ensure a reasonable stability.

3) For the assessment of the homogeneity and stability of genetic RMs, analytical procedures were improved and new methods were developed.

4) A model system for certification of DNA-based RMs was established and protocols of the isochronous stability and homogeneity studies for certification of genetic RMs were optimised and described according to ISO Guide 34 and the BCR guidelines.

5) These protocols and the methodology for development and production of genetic (C)RMs can be utilised in DNA-based tests of all kinds and might facilitate the development of further nucleic acid based (C)RMs.

6) Three plasmidic CRMs for the detection of the G20210A variant in the human coagulation Factor II (prothrombin) gene, namely the IRMM/IFCC-490, -491 and -492, were developed, produced and certified according to the ISO Guides 30–35.

7) Commutability studies were designed, organised and carried out to demonstrate the fitness for purpose of the candidate Factor II CRMs.

8) These CRMs are available from the IRMM for the quality assurance of PCR-based methods and have been the first clinical genetic CRMs introduced worldwide.

9) Utilizing the Factor II reference plasmids and the site-directed mutagenesis technique, QCMs for the FII G20210A variant and neighbouring rare SNPs were developed, produced and successfully applied in a European proficiency testing study to identify weaknesses of molecular genetic testing laboratories and to challenge their competence to recognize and adequately report interfering sequence variants.

From another point of view, the reporting of unusual genotyping results in a collaborative study is of prime importance also for RM producers. The low rate of such feedbacks (e.g. only two laboratories from 14 participants using the affected LightCycler assay system reported the Tm shift in the collaborative study on the NIBSC-WHO Reference Panel for Prothrombin Mutation G20210A) stresses the need for a more careful selection of participants for field studies, which cannot rely on the accreditation of the laboratories alone, but this competence might be challenged in proficiency testing exercises.

In conclusion, different types and formats of DNA-based RMs for genetic testing were processed and characterised for their suitability for the QA of molecular diagnostic methods. Genomic DNA has proven to be a suitable type of RMs for various genotyping techniques; however, its certification according to the ISO Guides 30−35 has not yet been achieved. A set of plasmidic CRMs for the analysis of the G20210A mutation in the Factor II gene had been developed and produced, which is now available from the IRMM and have been the first clinical genetic CRMs introduced worldwide. In addition, using these reference plasmids and the site-directed mutagenesis technique, QCMs for rare sequence variants were prepared and employed in a European proficiency testing study. It could be shown that ISO compliant plasmid-type RMs containing the desired SNPs can be produced and successfully used for quality assurance in genetic testing. Because DNA testing uses the same techniques wherever it is applied, the results of these projects can serve as guide and pilot for the production and use of (C)RMs in genetic testing.

well as in science and technology, which are comparable among laboratories and ultimately traceable to measurement standards of the highest metrological level.

The three elements of RMSs are internationally accepted and commonly agreed reference measurement procedures (RMPs), certified reference materials (CRMs) and reference laboratories possibly collaborating in a network. Successful implementation of such systems can be further enhanced by External Quality Assurance (EQA) programmes. At present, the lack of RMSs in molecular genetic testing means that there are limitations in benchmarking against which an IVD manufacturer or a laboratory can judge the performance of its assays.

Reference materials (RMs) and certified reference materials (CRMs) are key elements of such RMSs and are recognised as an excellent tool for checking analytical accuracy and are valuable in creating crucial reference points in the development of comprehensive measurement systems. Although the use of appropriate RMs to validate test equipment or testing methods is an important part of the quality assurance (QA) of any analytical testing system, currently only a few RMs for clinical genetic testing are available. Therefore, the European Commission, Joint Research Centre, Institute of Reference Materials and Measurements (IRMM) launched several projects to develop and produce (certified) reference materials for molecular genetic testing and to elaborate the methodology to produce (C)RMs for *any* molecular genetic test.

The first major initiative, the Certified Reference Materials for Molecular Genetic Testing (CRMGEN) project focused on the development of four basic types of prototype RMs for inherited diseases and genetic risk factors, such as PCR product RMs for hereditary haemochromatosis, genomic DNA based RMs for the fragile X syndrome, cell line RMs for cystic fibrosis, and recombinant (i.e. plasmid) DNA RMs for the Factor V Leiden (G1691A) mutation. These prototype RMs were evaluated for their suitability and different possibilities for the preservation and packaging of purified DNA were investigated to ensure a reasonable stability of DNA-based RMs.

In line with the CRMGEN project, the Scientific Committee of Molecular Biology Techniques in Clinical Chemistry (C-MBT) of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has

initiated a joint project with the IRMM to develop and produce a set of plasmidic CRMs for the analysis of the human coagulation Factor II (FII, prothrombin) gene G20210A variant.

Furthermore, the IRMM and the Department of Biochemistry of the University of Szeged agreed on a proficiency testing exercise in cooperation with 3 European EQA organisations. In the frame of this study, new mutations were introduced into the wild-type FII reference plasmid using the site-directed mutagenesis technique and the quality control materials (QCMs) containing the modified and/or reference plasmids were distributed to clinical laboratories to assess their competence to recognize rare sequence variants resulting in unusual genotyping results.

2. AIMS AND SCOPE

The ultimate goals of this thesis were the development and production of DNA-based (C)RMs for clinical molecular genetic testing and to contribute to the quality improvement in genetic testing services. In order to achieve these general objectives, the main aims of the present study were:

1) To process different types and presentation formats of DNA-based RMs, and to investigate their suitability for clinical genetic (C)RMs.

2) To optimize the preservation of DNA-, in particular plasmid-based RMs.

3) We aimed to elaborate the know-how for the development and production of (C)RMs for any genetic tests, including the optimisation of protocols of the stability and homogeneity studies on genetic RMs as well as to establish a model system for certification of DNA-based RMs.

4) We aimed to develop and produce a set of plasmidic CRMs for the quality assurance of PCR-based methods used for the detection of the G20210A variant in the human coagulation Factor II gene.

5) We intended to design, organize and carry out commutability studies on these candidate CRMs to demonstrate their fitness for purpose.

6) Utilizing the Factor II reference plasmids and the site-directed mutagenesis technique, we pursued to develop QCMs for rare mutations.

7) We aimed to carry out a European proficiency testing exercise using the newly developed Factor II QCMs to identify the error sources in SNP genotyping as well as to assess the competence of clinical laboratories to recognize rare sequence variants resulting in unusual genotyping results.

skills and suggested that training may be an efficient tool to improve the quality of genotyping services [VI].

In the case of the unusual results, although a majority (54.6%) of laboratories did not report when they experienced unusual results on samples A and B, other participants recognised the presence of the sequence variants (24.3%) or attributed the unusual behaviour of the assay to technical issues (21.1%), including poor quality or low concentration of the DNA. However, the atypical melting peak on the [20175G; 20179_20180delAC] mutation was reported by only 25.4% of the participants probably because of the small deviation from the expected values (ca. 1.7 °C lower Tm than expected for the wild-type allele). Approximately half of these laboratories noted that such unusual melting peaks could indicate a variant in the probe region.

Incidence of rare sequence variants interfering with some genotyping techniques is relatively low; however, they can be easily misinterpreted and lead to an even higher error rate. Although the G20210A mutation is a well characterised risk factor for venous thromboembolism, the consequences on the analysis results of these adjacent variants were rarely investigated. Such rare polymorphisms should be reported as variants of unknown clinical significance clearly distinct from the wild-type or the G20210A mutation alleles. In previous studies, competence of testing laboratories to recognize and correctly report such sequence variants had not been challenged. Our ring trial revealed that where additional sequence variants resulted in unusual genotyping data and were therefore in principle detectable, only a fraction of the laboratories recognised and adequately reported unexpected SNPs.

These observations called the attention of the participants as well as of the broader community to the proper assessment of unusual genotyping results. For instance, as a consequence of the results published, Roche Diagnostics recalled and modified the Factor II (Prothrombin) G20210A Kit for the LC 2.0 instruments, because its macro component erroneously identified the C20209T mutation as wild-type genotype instead of an unknown variant. Furthermore, the Reference Institute for Bioanalytics started to challenge the recognition of rare SNPs in their EQA schemes.

based on the wild-type reference plasmid pIRMM-0001, a SNP (i.e. C20209T) or a complex variation (i.e. [T20175G;20179_20180delAC]) were introduced into the human FII gene fragment allowing the design of QCMs with rare genotypes/mutations [VI].

4.5.2. The proficiency testing study

After removing of duplications resulting from the overlaps of the EQA schemes, 189 laboratories from 21 countries participated to the study. These participants in the survey applied a large variety of genotyping techniques representing 50 different analytical procedures with sample intakes ranging 0.5–10 µL. All of the assays could amplify the target sequence from the plasmids proving that the samples were suitable for the interlaboratory comparison exercise.

Genotyping results could be split into usual and unusual results after analysis: Usual results are results of methods, which were not influenced by the mutations in samples A and B. In 67 and 85 laboratories for samples A and B respectively the mutations affected the methods and unusual genotyping results were obtained which should have been recognised and reported accordingly.

Among the usual results, the overall error rate was 5.1%. Interestingly, the error rates were higher on the samples containing the G20210A mutant allele (6.4% and 6.3%, respectively) than on samples B and D without the G20210A mutation (2.9% and 4.3%, respectively); however, this difference was not statistically significant. These rates of genotyping failures corresponded to the latest data in the literature and may result in thousands of misclassifications yearly in the participant laboratories, which emphasizes the need for more detailed scrutiny to identify the sources of errors and to eliminate the weaknesses. Such an evaluation of data could be carried out using the method specifications and the raw genotyping results, which are rarely requested from the participants in the EQA schemes.

This analysis elucidated that the majority of the false results arose from the inadvertence of laboratory personnel and not from technical failure of the method as such. However, the allele-specific PCR assays proved to be less robust than other techniques and the elevated error rate of certain LDTs indicates that they, as a group, have to be more carefully validated. These observations underlined the pivotal importance of the laboratory personnel

3. MATERIALS AND METHODS

3.1. Origin and Production of the RMs

The development of the CRMGEN prototype RMs and the isolation of DNS fragments for the candidate CRMs for the FII G20210A variant were carried out in clinical genetic centres according to the local, national and European regulations. Source materials were collected under informed consent from the patients. Lymphocytes or genomic DNA (gDNA) extracted from venous blood were anonymised. The CRMGEN prototype RMs specified below were designed and processed in close cooperation with the IRMM.

3.1.1. Origin and production of the CRMGEN prototype RMs

CRMGEN-HH PCR product RMs for the hereditary haemochromatosis were developed in the National Centre for Medical Genetics, Our Lady's Hospital for Sick Children (Crumlin, Ireland) using a long range nested PCR technique with Pfu DNA polymerase. The 2878 bp RM sequence (GenBank accession number Z92910, nt 4333 to 7211) encompasses both the C282Y (G6722A) and the H63D (C4762G) mutations of the human HFE gene. Wild-type and compound heterozygous prototype RMs were processed at 100 µl/vial without or with 100 µg/ml sonicated salmon sperm DNA (SS DNA) as carrier and were freeze-dried (FD) or not (NFD).

CRMGEN-CF cell line RMs for CF established in the Center for Human Genetics, Katholieke Universiteit Leuven (Leuven, Belgium) in cooperation with the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Epstein-Barr virus (EBV) transformed lymphocytes were generated from blood that was obtained from individuals carrying mutations in the CFTR gene (ΔF508/ΔF508; N1303K/ΔF508; ΔF508/G542X; S1251N/ΔF508; G551D/ΔF508; ΔI507/wt). After characterisation and validation, master and working cell lines were sent to the IRMM and stored in liquid nitrogen.

CRMGEN-FraX genomic DNA (gDNA) prototype RMs for fragile X syndrome were produced from an EBV immortalised cell line of a normal heterozygous female (Allele 1: $(CGG)_{10}$, AGG, $(CGG)_{11;}$ Allele 2: $(CGG)_{10}$, AGG, $(CGG)_9$, AGG, $(CGG)_{10}$; except of the repeat region, FMR1 gene sequences correspond to the GenBank reference sequence M67468. This cell line was established in the National Genetics Reference Laboratory

Manchester (Manchester, UK) and transferred to the NIBSC for large scale culturing, testing and characterisation. Whole genomic DNA was extracted from the stable lymphoblastoid cell line using four different extraction methods, such as two different phenol/chloroform extraction protocols, sodium perchlorate/chloroform, and the Puregene 'salting out' methodology. Extracted gDNA was homogenised in TE buffer and aliquoted at ca. 5 µg DNA in a volume of 33 µl. Half of the vials processed from each CRMGEN-FraX prototype RM were lyophilised at the IRMM.

CRMGEN-FV plasmidic RMs for the Leiden (G1691A) mutation in the coagulation factor V (proaccelerin, FV) were generated at the Department.of Human and Clinical Genetics, Leiden University Medical Center, (LUMC; Leiden, The Netherlands). DNA fragments containing the G1691A "Leiden" mutation or the wild-type (G1691) sequence were cloned into pUC19 vector. Except of the "Leiden" mutation, the 585 bp insert corresponds to the exon 10 sequence of Factor V gene (GenBank accession number L09137, nt 38296-38880; "Leiden" mutation: 38592 G>A). The first lot of CRMGEN-FV prototype RMs were dissolved in TE buffer at pH 7.5 containing $2\times5\times10^6$ copies/µl in 50 µl/vial without additive or with different additives (10 % sucrose / 50% glycerol / SS DNA crosslinked with a Psoralen/UVA treatment). The 2nd batch of CRMGEN-FV prototype RMs were prepared in 10×TE buffer at pH 8.0 containing $2\times5\times10^6$ plasmids/µl in 50 µl/vial. This second series consisted of 3 prototype RMs: without additive FD / not FD and with 10% of sucrose FD.

3.1.2. Origin of the NIBSC-WHO candidate Reference Panel samples

NIBSC-WHO Reference Panel for the Factor V Leiden Mutation and NIBSC-WHO Reference Panel for the Prothrombin Mutation G20210A samples were received from the NIBSC in the frame of the collaborative studies *CS246* and *CS262*, respectively.

3.1.3. Preparation of prototype RMs for a feasibility study to optimise DNA preservation

The insufficient stability of the 1st batch of the candidate plasmidic FII CRMs triggered an 'exploratory research project' at the IRMM on the preservation of DNA- and particularly plasmid-based RMs. In the frame of this systematic study, several prototype RMs were prepared using the wild-type FII reference plasmids at 1 ng/50 µL with different commercially

analysis, the assessment of their homogeneity and stability on the long-term have to be confirmed by quantitative techniques [V].

Despite some apparent shortcomings of the plasmidic RMs discussed above, these data and achievements have proven that the strategy applied for the development and production of DNA-based CRMs containing defined sequence variants seems well suited and plasmid-type RMs can be successfully used in the field of genetic testing for the analysis of SNPs.

4.5. Proficiency testing study using QCMs with rare sequence variants

The introduction of new mutations into the pIRMM-0001 (wild-type) FII reference plasmid, the production of the QCMs as well as the organisation and evaluation of the proficiency testing study has been published in details earlier [VI].

4.5.1. Preparation of the samples for the proficiency testing exercise

Following the site-directed mutagenesis procedure, screening of plasmid preparations detected two new variants displaying unusual melting peaks at ca. 54 °C and at 58 °C. Sequencing of these plasmids identified the newly introduced mutations identified as the C20209T and the [20175T>G; 20179_20180delAC] mutations, respectively. Using these plasmids and the FII reference plasmids four QCMs were produced containing 10 pg/µL plasmid DNA in 30 µL TE-buffer (10 mM : 1 mM; pH 8.0; autoclaved):

Sample A: 20210A / [20175G; 20179_20180delAC] – heterozygous for the G20210A mutation

Sample B: G20210 / 20209T – homozygous wild-type

Sample C: 20210A / 20210A – homozygous for the G20210A mutation

Sample D: G20210 / G20210 – homozygous wild-type for the G20210A mutation

The paucity of the mutation-positive clinical samples for rare sequence variants, haplotypes or complex genotypes and the ethical issues related to their isolation from human sources pose a significant barrier to the development of RMs for some analytically challenging genotypes and contribute to the high error rates in the field of genetic testing. However, plasmidic RMs containing a reference sequence of a human gene can be modified using the site-directed mutagenesis technique, which allows the generation of a defined mutation at the designated nucleotide position. The applicability of this approach had been successfully demonstrated, when

been indicated in the certification as additional information on the CRMs, but it is not a certified value. The sample intake recommended is 0.5 to 5 µL. The stability of the CRMs is being followed in the post-certification monitoring and the certification is valid for one year after purchase of the unit. Following the careful assessment of their stability, homogeneity and fitness for purpose in a field trial, these RMs were certified and are now available from the IRMM (http://www.irmm.jrc.be) [II,III,IV,V].

The CRMs IRMM/IFCC-490 (G20210/G20210 wild-type), IRMM/IFCC-491 (20210A/20210A homozygous mutant) and IRMM/IFCC-492 (G20210/20210A heterozygous mutant) have been the first clinical genetic CRMs introduced worldwide, which can be used for validation and harmonisation of PCR-based methods used for the detection of the G20210A sequence variant in the human prothrombin gene. Due to the lack of established reference methods in genetic testing, such CRMs play a crucial role to fulfil the traceability requirement of the IVD directive. In addition, any laboratories can use these CRMs as QC samples, which is particularly important for the rare homozygous mutant 20210A genotype.

Although the commutability of plasmid type RMs is in principle restricted due to the limited length of the gene insert, this issue could be tackled by the appropriate selection of the reference sequence. All of the genotyping techniques applied in the field studies or in the proficiency testing exercise – representing more than 50 different analytical procedures – were able to amplify the target sequence and to detect of the mutation of interest from the plasmids, proving that these CRMs are fit for purpose [I,V,VI]. Furthermore, none of the laboratories reported atypical results (e.g. shifts in the melting temperature or allele discrimination plot, etc.) on the G20210 wild-type and 20210A mutant reference plasmids [I,VI].

On the other hand, short DNA fragment based RMs can be described and quantified more precisely, which was a key advantage during the characterisation studies. For example, although two other sets of QCMs are also available to assess the performance of test systems for the analysis of the prothrombin G20210A variant [V], their homogeneity and long term stability have not been demonstrated clearly as required by the relevant ISO standards for RMs. Even though these RMs are to be used for qualitative

available additives dissolved in TE buffer at pH 8.0 and freeze-dried or not. Prototype RMs tested: TE-buffer or 10×TE buffer without additive; 1 mg/mL bovine serum albumin (BSA); 5%, 10%, 20% of sucrose; 0.5%, 5%, 10%, 20% of D(+)trehalose; 50% glycerol; 5% D-fructose; 5% D-mannitol; 5% D-sorbitol; 1% Tween 20; 1% PEG 8000; 1% Na-L(+)ascorbate; 0.01% sodium dodecyl sulphate (SDS); 200 µmol/L diethylene triamine pentaacetic acid; 200 µmol/L desferrioxamine. These series of prototype FII RMs were tested after 12 weeks of storage.

3.1.4. Production of the candidate CRMs IRMM/IFCC-490, -491 and -492 and the QCMs for the analysis of the FII G20210A variant

The prothrombin gene fragment was selected in the Molecular Biology Division, Department of Clinical Chemistry and Laboratory Medicine, Medical University Vienna (Vienna, Austria). The FII gene fragment (GenBank accession number M17262, nt 26302 to 26910) cloned into the pUC18 vector spans all primer annealing sites published until today. The C20209T mutation was introduced into the wild-type sequence using the site-directed mutagenesis technique. The first batches of the candidate CRMs were processed as lyophilised material containing 5×10^4 copies of plasmids in 100 µl solution and 100 µg/ml BSA as additive. The second batches of the IRMM/IFCC-490, -491 and -492 and the QCMs were prepared in TE buffer (10 mmol/L : 1 mmol/L, pH 8.0) at a concentration of approximately 20 and 10 pg/µL DNA, respectively.

3.2. Assessment of stability and homogeneity of RMs

Both the prototype and the candidate RMs were subjected to stability studies following an isochronous scheme developed at the IRMM according to the ISO Guide 35. The protocol consisted of a short- and long-term isochronous study for every RM. Samples for the short-time stability studies (STSS) were stored for 2, 4 and 8 weeks at 40 °C, 18 °C, 4 °C and -20 °C; however, the CRMGEN-HH prototype RMs were stored at 60 °C instead of 40 °C. Long-term stability of RMs were investigated by storing the RMs at 18 °C, 4 °C and -20 °C for 4, 8, and 12 months. However, long-term stability the IRMM/IFCC candidate FII CRMs was tested in 6, 12 and 24-month stability studies. In both type of stability studies, the safety factor was 2 and the reference temperature was set at -70 °C, corresponding to

time point 0. Three units per time point were tested in 4 replicates using 4 dilutions (i.e. 1, 1:10, 1:100, and 1:1000) under repeatability conditions.
Homogeneity of RMs could be deduced from the results on samples stored at -70 °C and at -20 °C (i.e. the usual storage temperature of the genetic RMs tested, n=18) in the short-term stability study.
Freeze-thaw tolerance: Liquid plasmidic prototype RMs prepared for the feasibility study to optimize DNA preservation were tested for any degradation due to 10 freeze-thaw cycles within 6 days. Samples stored at +4 °C served as control.

3.3. Measurement of DNA concentration and genotyping techniques

DNA concentrations were determined using the PicoGreen double-stranded DNA quantitation kit.
CRMGEN-HH prototype RMs were analyzed using a qualitative multiplex PCR–RFLP method and a quantitative real-time LightCycler (LC) assay.
CRMGEN-FraX samples were tested in Manchester using PCR/PAGE Genescan analysis followed by Southern blot to determine the methylation status of region surrounding the CGG repeat.
The Factor V G1691A "Leiden" mutation was genotyped using The LC Factor V Leiden Mutation Detection Kit with a modified protocol to enable the relative quantitation of the samples analyzed.
Techniques applied for the analysis of the *Factor II gene G20210A* mutation and for the characterisation of the candidate CRMs have been described in details previously in the certification reports on the CRMs IRMM/IFCC-490, -491, and -492 [II,III,IV] and other publications [I,V,VI].

3.4. Field trials

Field trials on the CRMGEN prototype RMs were carried out within the European Molecular Genetics Quality Network (EMQN).
Design, organisation and evaluation of field trials on the candidate CRMs IRMM/IFCC-490, -491, and -492 have been reported elsewhere [I,V] as well as the proficiency testing exercise using the plasmidic QCMs for the analysis of the G20210A variant in the prothrombin gene [VI].

Homogeneity: A plot of the Cp values of the PCR products versus sample number did not reveal a trend in the filling sequence of the vials. The slope of the regression line was not statistically different from zero (*t*-test at 95% confidence interval) and the data followed a normal and unimodal distribution, which indicated that the candidate CRMs were homogeneous. The homogeneity-related uncertainty on the DNA quantity per vial throughout the batches was inferior to 10% for all three materials. Every sample and its dilutions at least down to 1:10000 showed the expected melting curves corresponding to the genotype of the candidate CRM samples. This proved that the candidate CRMs are sufficiently homogeneous and that the minimal sample intake can be as low as 0.5 µL.
Stability: Condition of every sample was good, loss of volume or other changes could not be observed. Every melting curve corresponded to the expected genotype and no Tm-shift was detected. These results and the Cp values indicated a sufficient short-term (8 weeks at -20 °C, 4 °C and 18 °C) and long-term (6, 12 and 24 months at -20 °C and 4 °C) stability of these candidate CRMs at the tested temperatures [II,III,IV,V]. However, instead of an increase of the Cp values due to a degradation of the target DNA sequences, lower Cp values were recorded on samples stored at higher temperatures and this trend was found to be statistically significant. In addition, less dilution steps gave appropriate results for the samples stored at -20 °C or at the reference temperature, than for samples stored at higher temperatures. These observations could be explained by better accessibility or lower adsorption rate of target sequences in samples stored at higher temperatures.
Field study: In order to test the performance of the 3 candidate CRMs, they were sent to 7 diagnostic laboratories that used routinely different genotyping methods for the detection of the prothrombin G20210A variant. All DNA-containing samples were genotyped correctly and unambiguously demonstrating that the materials are fit for their intended purpose.
Certification: The identity of the gene fragments inserted in the plasmids was assessed by internal and external bi-directional sequencing with a probability of misreadings estimated to be $p<(1/609)^2$. This estimation is based on the agreement between forward and reverse sequencing of the 609-bp fragments inserted in the pUC18 vectors. DNA concentration has

presence in the original patient sample means that it was not introduced during the production. Although the A20054G variant did not cause any problems during the development and processing of the plasmid-type RMs or in the field study, difficulties may arise when a primer is used that binds with its 3' end to this sequence. Thus, this mutation is reported in the documentation of the plasmidic CRMs. The cloned wild-type fragment corresponded to 100% to the reported prothrombin gene sequence [I,II,III,IV,V].

4.4.1. Evaluation of the 1st batch of the candidate FII CRMs

In the absence of any reference method for genetic testing, CRMs have to be evaluated by a consensus of recognised reference laboratories. Test compatibility and commutability of such plasmids as candidate reference materials was demonstrated in a study involving 13 expert laboratories using 9 different PCR-based methods [I]. The study showed that plasmid-type RMs containing defined point mutations can be produced and successfully used in tests for detection of SNPs in a typical QA scheme. Feedbacks from the participants however suggested a higher concentration of the target sequences as well as to set the minimal sample intake to ≤1 µl.

Stability: Analysis of the STSS samples produced no or weak signals using the LightCycler platform and kit for the detection of the FII G20210A mutation. This applied as well for samples stored at -70 °C and -20 °C. These results indicated that stability of the candidate CRMs was an issue and the presentation format had to be re-investigated towards the processing of a new lot of the CRMs IRMM/IFCC-490, -491 and -492.

4.4.2. Characterisation of the new lot of the Factor II CRMs

Processing, characterisation and certification of the new batch of the FII CRMs have been published previously [V] and described in details in the certification reports [II,III,IV].

Utilizing the results of the feasibility study on the preservation of plasmidic DNA, the new batch of these RMs were produced as liquid frozen materials containing approximately 1 ng plasmid DNA in a volume of 50 µL TE solution (10 mM Tris : 1 mM EDTA, pH 8.0) in polypropylene vial. This batch was characterised according to the requirements of the ISO Guides 34 and 35.

4. RESULTS AND DISCUSSION

4.1. Results on the CRMGEN prototype RMs

4.1.1. CRMGEN-HH prototype RMs

Although prototype RMs containing SS DNA gave clearer results (i.e. sharper bands on the gels), all prototype RMs were homogenous and stable at +18 ºC or at lower temperatures and no significant differences were shown between the homogeneity and short-term stability of the different presentation formats (lyophilised or liquid, with or without salmon sperm DNA as additive). Lyophilised samples proved to be stable even at 60 ºC for 8 weeks, but all the liquid samples lost volume at this temperature and the detection of target sequences were ambiguous after this period. In case of the STSS samples stored at 60 °C, target sequences could not be detected at higher dilutions from the NFD prototype RMs containing SS DNA.

These observations confirmed that freeze drying can assure the stability of DNA-based RMs even at higher temperatures. On the other hand, the use of carrier SS DNA gave conflicting results (i.e. clearer bands on the gels, but no signals on higher dilutions) in the STSS indicating the need for a more reliable assay to compare the stability study samples, which enables a relative quantification of the sequences of interest in the different CRMGEN-HH prototype RMs. Therefore, one-year long-term stability study (LTSS) samples were analyzed applying a quantitative LightCycler assay. Using this real-time PCR method, no differences were found between the lyophilised and liquid RMs; however, Cp values on the prototype RMs with SS DNA were significantly higher than the Cp values on prototype RMs without SS DNA (18.66 vs. 17.52 $p<0.05$ for the homozygous wild-type and 19.43 vs. 17.73 $p<0.005$ for the compound heterozygous RMs). This data display a significantly lower concentration of target sequences in the liquid prototype RMs containing SS DNA, which indicates an impaired stability of such RMs. These data clearly disproved the speculation that SS DNA can improve the stability of small DNA molecules. Moreover, the field study on the CRMGEN-HH prototype RMs showed that SS DNA contains some amplifiable sequences under certain conditions, which might interfere with some techniques: 9 of 33 labs (27%) reported spurious results on the blank samples containing only SS DNA without human HFE sequences. (In the case of CRMGEN-FV prototype

RMs, this issue was avoided by crosslinking the SS DNA.) Unfortunately, despite the promising results on the CRMGEN-HH prototype RMs, the development of candidate CRMs for the analysis of the C282Y and H63D variants had been arrested by intellectual property right (IPR) issues.

4.1.2. CRMGEN-CF – Cell line based prototype RMs

At the IRMM, one of the vials containing these EBV transformed cells and stored in liquid N_2 was exploded in an incident and the production of cell pellet based materials were cancelled due to insurmountable regulatory barriers identified in handling, storage and transportation of such materials, which all require authorisation according to 'Biosafety Level 2'. Although the cell pellet based CRMGEN-CF RMs proved unfeasible due to the strict biohazard compliance and regulatory criteria applied to EBV transformed cells, such cell lines however could provide excellent starting material for the preparation of gDNA based (C)RMs. In addition, genotyped and well characterised cell lines established without using EBV or similar infectious agents (e.g. malignant cell lines) could be used as RMs for common sequence variants.

4.1.3. CRMGEN-FraX – Genomic DNA based prototype RMs

All short- and long-term stability study samples tested by PCR/PAGE Genescan produced acceptable results regardless of extraction method, freeze drying or liquid medium and temperature/time stored. Southern blot testing showed that samples were stable at both -20 °C and 4 °C; however, samples stored at 18 °C and 40 °C showed poor quality genotype test results in both series of Southern blot tests, so stability is compromised at these temperatures. In addition, treatment at 65 ºC for inactivation of nucleases resulted in DNA degradation in some phenol extracted samples. All prototype RMs performed well in the field trial.

Consequently, gDNA has proven to be a suitable type of RMs for various genotyping techniques, such as PCR and Southern blot. Among the DNA extraction techniques tested, phenol/chloroform extraction yielded in a slightly better stability and the Puregene method in a superior purity with comparable stability of extracted DNA. Therefore, this latter technique was chosen later on for the production of the 1st WHO international genetic reference panel for Fragile X syndrome already brought through the WHO certification route. Such a RM made of gDNA is of pivotal importance for

>>1%) prototype RMs containing sucrose showed a significant Tm-shift on the wild-type FII sequence (59.33±0.20 °C vs. 60.20±0.06 °C) after 12 weeks storage. This latter observation not only emphasizes the pivotal importance of a proper lyophilisation protocol, but might be a possible explanation for the Tm shift on the WHO-NIBSC reference panel samples as well.

On the other hand, our results showed that an acceptable shelf life of several years can be achieved even without freeze-drying in frozen aqueous solution at $>10^8$ copies of plasmid DNA using alkaline conditions (pH 8.0−8.5) in Tris-EDTA (TE) buffer. Freeze-thaw tolerance of plasmids was also sufficient even without additives. In the case of PCR product or plasmid type RMs, this presentation format can reduce also the contamination risk, which is significantly higher in FD format.

4.4. Production and certification of the CRMs IRMM/IFCC-490, -491 and -492

Whilst the studies discussed above aimed to investigate and compare the suitability of different types of genetic RMs and to optimize the preservation of nucleic acid based RMs, the ultimate goal of the projects was the development and certification of CRMs for molecular genetic tests as well as to demonstrate their fitness for purpose and applicability in the quality assurance of clinical genetic testing.

In co-operation with the IFCC C-MBT, two plasmids containing a 609 bp fragment of either the wild-type 20210G (named pIRMM-0001) or the 20210A (named pIRMM-0002) sequence variant of the human FII gene were used to generate a set of 3 plasmid-type RMs corresponding to the wild-type, mutant, and heterozygous genotypes of the G20210A variant. Construction, sequencing and characterisation of the reference plasmids pIRMM-0001 and pIRMM-0002 have been described in details previously [I,II,III,IV,V].

Sequence analysis of the selected fragments confirmed that the isolated 609 bp length sequences were identical to the GenBank reference sequence (accession number M17262, nt 26302 to 26910) except for the G20210A mutation (corresponding to the nt 26784 in the reference sequence) and an A→G point mutation at position 20054 (nt 26628 in the GenBank reference sequence). This second mutation was linked to the mutated sequence and its

Subsequent experiments showed that (i) testing of these gDNA-based RMs for other polymorphisms, the extent of the Tm shift was similar; (ii) the Tm-shift is linked to the quantity of RM engaged in the PCR reaction (could be abolished by 1:10 dilution of the samples) and (iii) cannot be attributed to the trehalose, which was used as lyoprotectant in these RMs.

This Tm shift was lower than the ±2.5 °C value specified in the pack insert as possible difference between samples and the control provided in the kit, but very much exceeds the total precision of the kit (median CV=0.36%) and highlighted a serious issue: Several methods, such as the LightCycler and the Taqman allelic discrimination techniques, distinguish the different alleles on the basis of their characteristic melting temperatures with the hybridisation/Taqman probes. Sequence variants resulting in a mismatch between the probe and the target sequence lead to a decrease of ca. 2–10 °C in the Tm. The shift of the melting peaks on the WHO-NIBSC RMs was very close or even reached this range; however, the typical separation between the melting peaks on the heterozygous samples was maintained. Such a pronounced shift in the Tm may affect also the sensitivity, specificity and efficiency of every PCR reaction, which might lead to biased or false genotyping results (e.g. allele-specific PCR, melting analysis) or quantification. Therefore, in order to avoid similar shortcomings, additives and presentation formats should be carefully selected and investigated for their effect on the test results.

4.3. Feasibility study on the preservation of plasmidic DNA

In the frame of the present study many lyo- and/or cryoprotectants as well as other preservatives were screened for possible interferences with the molecular diagnostic techniques. These experiments showed that some of the additives widely used to enhance the stability of biologic materials might interfere with such assays. For instance, glycerol led to a very significant decrease in the Tm and consequently to seriously impaired amplification efficiency at the concentration needed for effective cryoprotection, while the ionic detergent SDS completely inhibited the amplification already at 0.01%. Interestingly, addition of 1% Na-ascorbate to the prototype RM resulted in a significant decrease of the Cp values (Cp=13.46±0.21 vs. Cp=14.37±0.25; $p<0.05$) without affecting the Tm values. In addition, NFD or inadequately lyophilised (moisture content

the fragile X syndrome as Southern blot is the reference/confirmation method used for the diagnosis.

4.1.4. CRMGEN-FV – Plasmidic prototype RMs

Analysis of the 1st batch of CRMGEN-FV prototype RMs prior to the lyophilisation revealed a significant degradation of target sequences during the transport and 4 months storage at +4°C. Prototypes without additive or with 10% of sucrose gave relative low signals, but still could be genotyped. On the other hand, the prototype RMs prepared using 50% of glycerol or crosslinked SS DNA as additives could not be reliably genotyped due to the degradation of target sequences. In addition, melting peaks on the prototypes containing sucrose or glycerol were shifted towards lower temperature. Therefore, the new batch CRMGEN-FV prototype RMs were processed only in 3 formats. This 2nd batch however was dissolved in a 10×TE buffer (10 mM Tris : 1 mM EDTA) at pH 8.0, which performed better in the feasibility study.

Homogeneity testing of the 2nd batch of CRMGEN-FV prototype RMs resulted in significantly higher Cp values on the prototypes without additives either FD (34.33±1.80) or NFD (31.24±1.45) than the Cp value on the prototype containing 10% of sucrose as additive (26.15±0.49) indicating a serious loss of quantifiable target sequences during the processing of the prototype RMs without additives. Comparison of these data to the results on the raw plasmid preparations (Cp=24.63±0.30) revealed that aliquotation of the material into small vials lead already to a dramatic increase of the Cp values.

Target sequences could not be reliably amplified from the STSS samples of the 2nd lot CRMGEN-FV prototype RMs without additives stored at +40 °C. Prototype RM with 10% sucrose showed always the expected heterozygous genotype and the Cp value exhibited a smaller standard deviation as well. Following an initial increase in the Cp values, the 2nd batch of the CRMGEN-FV prototype RMs were stable at -20 °C and the prototype RM with 10% of sucrose also at +4 °C and no shift in the melting temperature (Tm) values was observed.

These data revealed a serious loss of the target sequences during the lyophilisation and/or reconstitution steps, which can be largely abolished applying an appropriate additive such as the sucrose used in this case.

Additional tests performed to investigate the reason of these weak signals revealed that using such low DNA concentrations, a large proportion of plasmids had been lost already due to the attachment of target sequences to the inner wall of the polypropylene vials used. Lyophilisation with an appropriate additive (e.g. 10% sucrose) was found to reduce also this phenomenon. Despite the successful development of the CRMGEN-FV RMs, these plasmidic RMs could not be processed as candidate CRMs due to a patent on the gene.

In summary, results of the FP5 CRMGEN project clearly demonstrated that it should be possible to create a (C)RM for any genetic test where the sequence variant is well characterised. On the other hand, the suitability of a certain type of genetic RM strongly depends on the kind of the sequence variant of interest. For example, PCR-based approach is not suited for triplet repeat diseases due to the instability of the triplet repeats during amplification. The advantages of short DNA fragment (i.e. PCR product or plasmid) type RMs include easy and economical production in large quantities, high reproducibility, versatility, and applicability for qualitative analysis. From another point of view, these genetic RMs cannot be used to assess the quality of DNA extraction procedures and contain only one defined specific sequence, which restricts the commutability limiting the wider use of such RMs. Assays for defined point mutations, such as the FV 'Leiden' mutation, usually amplify short DNA fragments, and thus the problem can be tackled by appropriate selection of the reference sequence. However, the lack of other genomic sequences still limits the applicability of such RMs in multiplex techniques, including assays with internal amplification controls and might prevent the recognition of possible interferences arising from similar sequences in the genome. In addition, such RMs based on isolated gene sequences are particularly affected by gene patents, while cell lines or gDNA-based RMs might bypass this issue.

Clearly, a very significant barrier of standardisation appears if a private company is able to obtain a patent on a particular gene. Such patents have strong impact on the molecular genetic diagnostics as well as on the standardisation of the genetic testing services.

In addition to an appropriate type and the overall suitability of the RMs, homogeneity and stability are by definition fundamental requirements for

any RMs, which have to be guaranteed by the producer. Although the homogeneity of RMs for qualitative molecular genetic tests was not a critical problem, to ensure a reasonable shelf life became a crucial issue. Beside enzymatic breakdown of the DNA − which was prevented by proteinase digestion and/or heat denaturation of nucleases − several mechanisms of physical/chemical degradation have been described, such as shearing during freezing, hydrolytic/oxidative cleavage, its enhanced form in the presence of metal ions, and depurination. The rate of these processes depends mainly on the storage conditions of which optimisation for long-term DNA preservation required comprehensive studies.

Lyophilisation turned out to be a practical and effective tool for preservation of nucleic acid RMs. However, solid content and components of the material determine its behaviour during the freeze-drying process. If the solid content is small (≤2%), the structure of the product is mechanically unstable and particles of the material might not adhere to the matrix. In such cases, water vapour can move these loose particles during the sublimation phase and deposit them somewhere in the vial or even transport them to the vacuum chamber resulting in loss of the compound of interest and dramatically increasing the contamination risk. Therefore, it is preferable to add substances that protect the DNA and form a stable structure, particularly in the field of clinical genetic RMs, where avoiding of any contamination is crucial. Furthermore, sugars (e.g. trehalose, sucrose) might serve as lyoprotectants and aid preservation forming a 'glass' structure, thus protecting the DNA from the breakdown of its helical structure and from oxidation during the freeze-drying process.

4.2. Analysis of the NIBSC-WHO candidate Reference Panel samples

Genotyping of the candidate Reference Panel samples for the respective sequence variant gave the expected genotypes from each vials; however, a significant shift in the melting temperature (Tm) towards lower temperatures was detected in both set of NIBSC-WHO samples using the LC Factor V Leiden Mutation Detection Kit and different lots of the LC Prothrombin G20210A Mutation Detection Kit with the LightCycler and an unusual Taqman allelic discrimination plot could be observed using the Taqman allelic discrimination assay on the ABI Prism 7700 instrument.