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Inclusion of Additional Reference Reagents in the Existing Collection of WHO International Reference Reagents for Blood Group Genotyping

Report of the international collaborative study to evaluate eighteen additional candidates for addition to the existing collection of four WHO International Reference Reagents for blood group genotyping

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **27 September 2019** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

An international collaborative study coordinated by CBER/FDA was conducted to assess the suitability of an additional new set of reagents consisting of 18 genomic DNA (gDNA) lyophilized samples generated from genotyped and phenotyped blood donors. These samples were characterized by 28 participating laboratories for detection of 40 red cell antigen polymorphisms (single nucleotide polymorphisms, insertions and deletions) associated with 17 blood group systems (Table 1). Some assays are designed to predict phenotypes rather than detect genotypes because some phenotypes are predicted based on more than a single polymorphism. Thus, this set of reference reagents was validated for detection of 38 genotypes or predicted phenotypes (Table 2). The red cell antigenic phenotypes of the donors are summarized in Appendix 2, and genotypes represented in this set of reference reagents are summarized in Table 1.

Introduction

Nearly 390 antigens organized in 36 blood group systems are recognized by the International Society of Blood Transfusion (ISBT), and the genetic background for most of these blood group antigens is known [1]. As a result, blood group genotyping methods for donor/recipient matching are becoming widely used to overcome serological typing limitations encountered by immunohematology laboratories [2-4].

Red blood cell (RBC) antigen matching between donors and recipients by means of DNA-based typing is an effective strategy to identify antigen negative donor units for prevention of alloimmunization and for transfusion into chronically transfused, alloimmunized patients such as those with sickle cell disease and thalassemia [5-9]. Molecular typing also predicts the correct RBC antigen of patients with a positive direct antiglobulin test [10], identifies a fetus at risk of hemolytic disease of the newborn by the prediction of fetal RhD phenotype and other blood group antigens (i.e. Rhc, RhC, RhE and K) in fetal DNA that circulates in maternal blood [11-16], resolves serological typing discrepancies when informative serological reagents are unavailable and aids identification of Rh variants [3,17]. Large scale blood group genotyping of blood donor populations allows identification of individuals with both rare and multiple antigen-negative phenotypes [18,19], assuring better transfusion outcomes for alloimmunized patients [17].

Molecular methods used for blood group genotyping vary from conventional PCR (Polymerase Chain Reaction) – either with Sequence-Specific Primers (PCR-SSP) or PCR-restriction fragment length polymorphism (RFLP) – to mass-scale genotyping. Commercially available blood group genotyping platforms differ in format, allele content, processing steps, result interpretation algorithms, cost and regulatory status. The preferred method of a laboratory is influenced by cost, throughput capabilities, turnaround time and coverage of specific genotypes.

The clinical use of any genotyping assay requires validation of test methods, which can be achieved by using reference reagents. However, there is a paucity of availability of validated reference reagents for blood group antigen genotypes. Genotyping kits manufacturers, genotyping laboratories, and proficiency schemes often resort to use of diverse clinical materials as reference[20]. Reference reagents are therefore limited in volume, not widely available and sometimes poorly characterized, potentially compromising the quality of results and patient care. World Health Organization (WHO) International Reference Reagents (IRR) for blood group genotyping have already been established in 2011 by the National Institute for Biological Standards and Control of the U.K. (NIBSC), and consist of four (4) reference reagents with limited coverage of blood group alleles in six blood group systems [21,22]. The CBER set of reference reagents for RBC blood group genotyping was developed and validated for institutional use and consists of 18 reference reagents covering 17 blood group systems. This set of reagents was produced using protocols similar to those used to produce the existing WHO RBC genotyping reference reagents and validated in an international collaborative study. This set of reagents was designed to contain the least number of samples with the greatest number of genotypes that were recommended by Consortium for Blood Group Genes (CBGG) for use as controls for prediction of certain RBC antigens [23]. The CBER set of 18 reference reagents has some overlap in allele coverage with the current collection of WHO IRR; however, the overlapping alleles have been extensively characterized in this study using a variety of advanced techniques not available at the time when the existing WHO RBC genotyping IRR collection was developed. The new set of 18 reference reagents is proposed to be added to the existing collection of 4 WHO IRR for blood group genotyping, expanding the existing collection to 22 reagents. These 22 reagents are available to assay manufacturers and researchers for development and validation of assay kits and to genotyping laboratories for qualitative test evaluation and monitoring of assay performance.

Materials and Methods for 18 Additional Reference Reagents

Preparation of Bulk Materials

Blood units were collected from consenting donors based on their historical RBC antigen profiles determined by serological screening (shown in Appendix 2) and by genotyping of donor leukocytes performed at Blood Center of Wisconsin (BCW, Milwaukee, WI). The blood units were screened for bloodborne pathogens and found negative for evidence of donor infection with HIV 1/2, HCV, HBV, HTLV-1/2, *T. pallidum*, *T. cruzi*, and WNV according to current U.S. regulations. Samples were anonymized and sent to the OBRR/CBER/FDA laboratory. Whole blood was separated into plasma, erythrocytes and leukocytes by centrifugation and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Fisher, Pittsburgh, PA). Aliquots of each component were cryopreserved: PBMC were preserved in Fetal Calf Serum (Hyclone, Logan, UT) containing 20% DMSO (Corning Cellgro, Manassas, VA), and RBC in 40% w/v glycerol (Fenwal, Lake Zurich, IL); gDNA was extracted from buffy coat using FlexiGene DNA Kit (QIAGEN, Germantown, MD).

Each gDNA sample was characterized for 41 polymorphisms associated with 17 blood group systems by Sanger sequencing, real-time qPCR assays, PCR-SSP, and/or PCR-RFLP. Fifteen predesigned and 23 custom designed TaqMan genotyping assays (Applied Biosystems, Foster City, CA) were run for each sample on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Forty SNPs/indels of interest were characterized by Sanger sequencing, and amplification and sequencing primers are listed in Table 3. Presence of *RHD* deletion and its zygosity were determined by a PCR-SSP assay and a PCR-RFLP assay[24]. The complete genotyping profiles of each reference reagent of this set are presented in Table 1.

PBMC were subjected to EBV transformation to generate immortalized B-lymphoblastoid cell lines (B-LCLs). Multiple vials of B-LCLs were cryopreserved, which will allow replacement of

each of the reagents upon depletion of the current stocks.

The B-LCLs were further expanded and used to extract gDNA in bulk followed by spectrophotometric analysis. The bulk DNA was diluted to the final concentration of 1 µg/ml in 0.40 mM Tris, 40 µM EDTA containing 3 mg/ml trehalose, and 1 ml aliquots were dispensed into 3 ml glass vials followed by the assessment of the coefficients of variation (CV) of the fills. Vials were lyophilized and sealed with rubber stoppers and aluminum caps at the ISO 17025 certified testing facility of the Office of Compliance and Biologics Quality/CBER/FDA using a VirTis Benchmark Lyophilizer (SP Scientific). Residual moisture was determined by a methanol extraction Karl Fischer coulometric method using non-pyridine reagent and a Mettler-Toledo Coulometric Titrator (Mettler-Toledo, Columbus, OH). Accuracy of the residual moisture determination may be impacted by the weights of lyophilizates that were lower than the quantities routinely analyzed in the facility. Fill details are summarized in Table 4.

Following freeze-drying, materials were stored at −20°C. To assess DNA integrity after freeze-drying we randomly selected 4 of the 18 reagents and compared their test performance to that of the same materials pre-lyophilization. The suitability of the reconstituted DNA for blood group genotyping was evaluated using real-time qPCR and PCR assays - the same genotyping methods and assays which were used for the sample characterization.

Stability Studies

Assessment of all 18 reagents in multiple tests and at multiple time points would be extremely time- and resource-consuming. Since all reference reagents were produced using the same established and verified protocols for cell line preparation, gDNA extraction, stocks preparation, filling and lyophilization, we arbitrarily selected three of the 18 reference reagents to serve as representative samples for assessment of their stability under accelerated degradation conditions and under recommended storage conditions over longer periods of time.

For the accelerated degradation study, on the day of lyophilization, 16 vials of each of these three selected reagents were placed at −80°C (n=4), room temperature (RT) (n=4), +37°C (n=4), and +45°C (n=4). One additional vial of each of them was used to perform tests on day 0. After initial testing, reconstituted materials in day 0 vials were kept at +4°C and tested along with other samples at each time point to establish open-vial stability. The accelerated degradation study included the following temperatures and time points: RT, +37°C and +45°C at 1 week, 2 weeks, 4 weeks, and 8 weeks.

For the long-term stability study, on the day of lyophilization vials with the same three reagents were stored at −20°C, +4°C, and RT and tested at time points of 3 months, 6 months, 1 year and biannually thereafter. Also, reagents stored at −80°C and open vials stored at +4°C were evaluated for up to 1 year.

For each time point, DNA materials in the tested vials were resuspended in 40 µl of water resulting in a gDNA concentration of 25 ng/µl in 10 mM Tris, 1 mM EDTA, and 75 mg/ml trehalose, and the following tests were performed:

- DNA quantification using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).
- Agarose gel electrophoresis using 1% agarose gel to detect visible degradation of genomic DNA.
- Real-time qPCR assay of a 432bp fragment of Human Growth Hormone (HGH) gene on chromosome 17 to quantify the percentage loss in the amount of DNA using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and following thermocycling conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min. The primers

sequences were as follows: HGH-F: TGCCTTCCCAACCATTCCCTTA, HGH-R: CCACTCACGGATTTCTGTTGTGTTTC[25].

Collaborative Study

A total of 41 laboratories from 19 countries were invited to participate in the study and 31 agreed. A total of 28 laboratories in 13 countries from North and South America, Europe, Asia, Middle East, and Australia participated in the study (Appendix 1) providing 30 sets of data that were analyzed.

Each collaborator received three vials of each of the 18 lyophilized members of the set and was asked to test them in 3 independent runs, preferably on different days, utilizing their routine blood genotyping assays. The collaborative study protocol is provided in Appendix 4. Participants were asked to report the genotyping results using a specifically designed worksheet with predefined genotypes (Appendix 4, Table 2), to avoid inconsistent terminology. We also requested the original reports (raw data) produced by their genotyping platform(s). Upon study completion, all participating laboratories received a study report with preliminary results, which allowed every collaborator to investigate possible reasons for discrepant results and correct previously overlooked discrepancies.

Data Analysis

Data reported back by each of the participants was analyzed and compared to the expected results. Discordant results were classified as: 1) “no calls” when the result was not determined; 2) reporting or clerical errors when mistakes and typos were made when transcribing original reports (raw data) to the electronic worksheet; 3) other discrepancies, for which the root causes could not be determined. Additionally, there were cases where the correct genotypes had not been determined due to inherent limitations of the assay used. Each reagent was analyzed for agreement rates for each polymorphism/phenotype, test method/platform and testing laboratory/site. The number of “no calls”, discrepant results, and in some cases reporting errors was subtracted from the total number of genotyping results/phenotype predictions, and the resulting value was divided by the total and presented as percentage.

Results

Collaborative Study

Results of the panel validation study were reported by 28 participants from 13 countries. Laboratories #4 and #25 each provided 2 sets of results produced by different methods, and these sets were considered separately (as laboratories 4a/4b, and 25a/25b); thus, there were 30 data sets included in the analysis.

Collaborators used a wide variety of genotyping techniques, including laboratory-developed tests and commercial methods. Data sets were most frequently obtained by PCR-SSP, either single- or multiplex, the HEA (human erythrocyte antigen), RHD, and RHCE BeadChip arrays from Immucor, Sanger sequencing, PCR-RFLP, and real-time PCR based assays. Less common methods included ID-CORE XT and BLOODchip REFERENCE by Progenika, MALDI-TOF-based assays such as Hemo ID from Agena Bioscience, Next Generation Sequencing, RBC-Ready Gene and RBC-FluoGene by Inno-train Diagnostik GmbH, droplet digital PCR, HI-FI Blood by AXO Science, SNaPshot, and high-resolution melting analysis (HRMA). For 15 data sets, a single genotyping method was used to analyze the panel, and for another 15 data sets, 2 - 4 methods were employed, which were generally used in a complementary fashion. The specific assay methods used by participants have been masked in this report since identification of the methods would likely reveal the identities of the participants and would violate the signed agreement of

participation in the studies, requested by some collaborators. Furthermore, due to disparities in methods' performance and the fact that the project was designed and conducted by the U.S. FDA, a regulatory body, it was necessary to avoid any appearance of endorsing any method or laboratory.

The number of polymorphisms tested per laboratory ranged from 6 to 39 polymorphisms. Participants performed their tests in triplicate (42.9%), duplicate (14.3%), and single (42.9%) runs. No differences were observed between the results produced by these 3 groups, and there was only 1 report of inconsistencies between replicates from 1 collaborator. Participants collectively performed 743 genotyping tests producing 13,374 results. Of these, 35 (0.26%) genotyping results were "no call"/undetermined, 71 (0.53%) were errors in reporting, 97 (0.73%) were discrepant, and 70 (0.52%) were produced by assays limited in their ability to distinguish homozygous from heterozygous variants for some targets.

To predict the phenotype of RhD, RhC, Rhc and Fy antigens, more than 1 polymorphism is often evaluated. Taken separately, genotyping results for each polymorphism were in many cases inaccurate or inconclusive; however, when analyzed together, they predicted correct phenotypes. Consequently, the agreement with expected results in these 3 cases was analyzed based on predicted phenotypes rather than individual genotypes, which decreased the number of discordant results to 34 for "no call", 64 for reporting errors and 85 for discrepant results (Table 2). Additionally, 72 results (0.54%) were not reported by a collaborator in the predesigned worksheet but were present and correct in the original report produced by their genotyping platform. Some collaborators have also performed analyses of the alleles that were beyond the scope of this study, such as in-depth testing of polymorphisms in RH, Scianna, VEL and other blood group systems.

Considering "no call", reporting errors, and discrepant results, agreement of 100% was obtained for 10 SNPs: IN c.137G>C, KEL c.841C>T, KEL c.1790T>C, KN c.4768A>G, KN c.4801A>G, LW c.299A>G, MNS c.143C>T, RHCE c.106G>A, RHCE c.122A>G, and SC c.169G>A (Table 2). For 24 polymorphisms or predicted phenotypes, the agreement ranged from 95% to 99.8%; for the polymorphisms RHD/D- deleted, KN c.4681G>A, CROM c.679G>C and OK c.274G>A the agreement rates were 94.2%, 91.1%, 85.7%, and 66.7%, respectively (Table 2).

When only "no call" and discrepant results were considered (reporting errors were ignored for the purposes of this analysis), 100% agreement was identified for 15 polymorphisms and 97% to 99.9% agreement for 19 polymorphisms or predicted phenotypes. The agreement for KN c.4681G>A and CROM c.679G>C remained at 91% and 85.7%, respectively, and for the OK c.274G>A polymorphism the agreement increased to 98% from 66.7%.

In addition, the results provided for *KN* c.4681G>A SNP by one collaborator were discrepant for 16 out of 18 panel members. Since discrepant results were found only for samples with homozygous genotypes and raw data were not provided by this laboratory, which may be caused by erroneous interpretation of wild type and mutated alleles. The agreement rate for *OK* c.274G>A polymorphism was increased from 66.7% to 98% when reporting errors were not considered in the analysis. The results for this SNP from laboratory #17 were initially considered incorrect in our analysis and later reclassified as reporting errors for 17 samples and as a "no call" for one sample after the collaborator reanalyzed the data using the correct chromosome location of the reference SNP. Additionally, the agreement of 85.7% for *CROM* c.679G>C resulted from "no call" results for all 18 samples reported by one collaborator. In contrast, the genotypes for this allele for all samples were correctly determined by other laboratories using both similar and different methodologies. Therefore, the low agreement rate obtained for these polymorphisms is not caused by the materials' intrinsic qualities, but rather by limitations in assay performance.

Discrimination between RhD-positive and RhD-negative phenotypes was performed by 21 laboratories; of these 14 performed an *RHD* zygosity test by assays that target the upstream and downstream Rhesus boxes surrounding the *RHD* gene, and 6 collaborators had differing outcomes when analyzing DNA panel members 10044, 10047, 10048, 10056 and 10058. It is well known

that some *RH* variant alleles carry genetic alterations in Rhesus boxes regions[26-28], which might have affected the outcomes of the assays[26,27,29,30] and caused discrepant *RHD* zygosity results for these DNA panel members. Therefore, we advise caution when using the aforementioned panel members as standards in the *RHD* zygosity assays targeting upstream and downstream Rhesus boxes.

Interference of an *RHD* variant with an assay outcome was observed in the determination of the *RHCE* c.733G>C SNP in panel member 10044 by a real-time allelic discrimination PCR assay. To interrogate this SNP a collaborator used *RHCE* nt.697C to specifically amplify *RHCE* allele. However, the *RHD*DAU-5* allele[31] present in the panel member 10044 also has C at that position. Presumably, the assay was detecting *RHD* c.733**G** with the *RHD*DAU-5* sequence "masquerading" as *RHCE*.

RHDΨ was tested by 16 laboratories by a variety of methodologies; erratic results for 10 (10007, 10012,10020, 10029, 10030, 10044, 10048, 10050, 10052, 10058) out of 18 DNA panel members were observed by one collaborator. Since these samples are not representative of the *RHDΨ* allele they should not be used as reference reagents in *RHDΨ* assays. Removal of this polymorphism from the initial list of 41 SNPs and indels resulted in the final list containing 40 genotypes. This genotype is covered by the existing collection of WHO IRR for blood group genotyping[21].

To calculate agreement with the expected results by laboratory and method used, reporting errors were not subtracted from the total number of tests performed. Three out of 13 methods provided 100% agreement of results and two of these three methods were used to only analyze 18 DNA panel members for one polymorphism, which precludes reaching meaningful conclusions regarding these methods' actual performance. For the remaining 10 methods used, agreement ranged from 92.22% to 99.97% (Table 5). Fifteen out of 30 laboratories had 100% agreement, 7 laboratories had 99.0 ─ 99.9%, 4 laboratories had 97.1 – 98.9%, and 4 less than 97.0% agreement (Table 6).

Stability Assessment

Results of the accelerated degradation study confirmed the stability of the reagents stored at temperatures of up to +45°C for 8 weeks (data not shown). The DNA materials stored at different temperatures for the long-term stability study have already been tested for up to three years showing no meaningful degradation as verified by real-time qPCR assay (Figure 1) and agarose gel electrophoreses (Figure 2). The concentration and quality of DNA remained acceptable. Concentrations were higher than 25 ng/µl for materials kept both at recommended storage temperature (─20°C) and at elevated temperatures (+4°C and +25°C) (Figure 3). Ct values obtained by real-time qPCR were within acceptable ranges at all time points, which is consistent with experiment-to-experiment variation. Additionally, we evaluated lyophilized materials stored at ─80°C and open vials stored at +4°C for up to one year with no recognizable differences observed. TaqMan allelic discrimination assays for DO c.793 and DO c.323 SNPs were performed for the three reagents at three-year time point using the same reagents pre-lyophilization as reference, and they performed similarly.

Instructions for Use

The draft Instructions for Use to accompany this reference material are provided in Appendix 3.

Recommendation

It is proposed that the 18 reference reagents candidates, namely 10006, 10007, 10012, 10014, 10018, 10020, 10029, 10030, 10035, 10038, 10044, 10047, 10048, 10050, 10052, 10053, 10056, and 10058 should be added to the existing 4 WHO International Reference Reagents to further expand those for validation and standardization of genotyping for 40 blood group polymorphisms based on the results of the international collaborative study. The allelic genotypes and phenotypes

associated with the reagents are summarized in Table 1 and Appendix 2. There are around 300 vials containing lyophilized DNA of each reference reagent available to the WHO, and the custodian laboratory is the Center for Biologics Evaluation and Research/U.S. FDA.

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Table 1. Genotypes of the 18 DNA panel members determined as described in the Methods section.

Gene	Polymorphism*	DNA panel member ¶																	
		10006	10007	10012	10014	10018	10020	10029	10030	10035	10038	10044	10047	10048	10050	10052	10053	10056	10058
ABO	c.1061delC	C/C	C/C	C/delC	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/delC	C/C	C/C	C/C	C/C	C/C
	c.526C>G	C/C	C/C	C/C	C/C	C/C	C/G	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/G	C/C	C/C	C/C	C/C
	c.703G>A	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G
	c.796C>A	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/C	C/C
	c.803G>C	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G	G/G
	c.261delG	G/delG	G/delG	G/G	delG/delG	delG/delG	G/delG	delG/delG	G/delG	G/delG	delG/delG	delG/delG	delG/delG	G/G	G/delG	delG/delG	delG/delG	G/G	delG/delG
GYPA	c.59C>T	C/C	C/C	C/C	C/C	C/T	C/C	C/T	C/T	C/T	T/T	T/T	C/T	T/T	T/T	T/T	C/C	T/T	C/T
GYPB	c.143C>T	C/C	C/C	T/T	C/C	C/C	C/C	T/T	C/C	C/T	C/C	C/C	C/T	C/C	C/T	C/C	C/C	C/C	C/C
	c.230C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.270+5G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
RHD	RHDdel†	+/—	+/—	+/+	—/—	+/+	+/+	+/+	+/+	+/—	+/+	+/+	—/—‡	+/+‡	+/+	+/+	+/+	—/—‡	+/+‡
RHCE	109bp intron 2 ins§	—/—	+/—	+/—	—/—	+/—	+/+	+/—	+/—	—/—	—/—	—/—	—/—	—/—	+/—	+/—	—/—	—/—	—/—
	c.307C>T	C/C	T/C	T/C	C/C	T/C	T/T	T/C	T/C	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C	C/C
	c.676G>C	C/G	G/G	C/G	G/G	G/G	G/G	C/G	G/G	C/G	C/G	G/G	G/G	G/G	C/G	C/G	G/G	G/G	C/G
	c.122A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
	c.106G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
	c.733C>G	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	G/G	G/C	G/C
	c.1006G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
BCAM	c.230G>A	G/G	G/G	A/A	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
KEL	c.578C>T	C/C	C/C	C/C	T/T	T/T	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.841C>T	C/C	T/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C
	c.1790T>C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/C	C/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T
ACKR1	c.125G>A	A/A	A/G	A/A	A/A	A/G	A/A	A/G	G/G	A/G	A/A	A/A	A/A	A/A	A/G	A/G	A/A	A/A	A/A
	c.265C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.—67T>C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	C/C	C/C	C/C	C/C	T/T	T/T	C/C	C/C	T/C
SLC14A1	c.838G>A	A/A	A/A	A/A	A/G	A/G	A/A	A/A	A/A	A/G	G/G	G/G	G/G	G/G	A/G	A/G	G/G	G/G	G/G
SLC4A1	c.2561C>T	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
ACHE	c.1057C>A	C/C	C/C	C/C	C/C	C/C	A/A	C/C	C/C	C/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
ERMAP	c.169G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G
ART4	c.793A>G	A/G	G/G	A/G	A/G	A/G	A/G	A/G	A/A	G/G	A/G	G/G	A/G	A/A	A/G	A/G	G/G	A/G	A/A
	c.323G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/T	G/G	G/G	G/G	G/G	G/G	G/G
	c.350C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	T/T	C/C	C/C	C/C	C/C	C/T
AQP1	c.134C>T	T/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C
ICAM4	c.299A>G	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
CD55	c.679G>C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G
CR1	c.4681G>A	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G
	c.4768A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/G	A/G	A/G	A/A	A/A	A/A	A/A	A/A
	c.4801A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/G	G/G	G/G	A/A	A/A	A/G	A/G	G/G
CD44	c.137G>C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
BSG	c.274G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G

*Nucleotide position refers to the position within the cDNA sequence (see reference sequence in Table 3). † +/+ indicates no *RHD* deletion, +/— indicates deletion of one *RHD* allele, —/— indicates deletion of both *RHD* alleles. ‡ Caution in the interpretation of results should be exercised if an assay targeting upstream and downstream Rhesus boxes is used for determination of this genotype. §+/+ indicates the presence of 109bp intron 2 insertion in both *RHCE* alleles, +/— indicates the presence of 109bp intron 2 insertion in one *RHCE* allele, —/— indicates absence of 109bp intron 2 insertion.

Table 2. Agreement of allele identification by study participants calculated for each blood group allele.

Blood Group System	Polymorphism*	# of Labs Testing	Results Reported	Reporting Errors	No Call	Discrepant Results	Agreement Rate (%)
ABO	c.1061delC	11	198	1	1		98.99
ABO	c.526C>G	6	108			2	98.15
ABO	c.703G>A	8	144		1	2	97.92
ABO	c.796C>A	7	126			2	98.41
ABO	c.803G>C	12	216			2	99.07
ABO	c.261delG	12	216		1	2	98.61
MNS	c.59C>T	24	432		3	1	99.07
MNS	c.143C>T	27	486				100
MNS	c.230C>T	21	378	16			95.77
MNS	c.270+5G>T	23	414	16			96.14
RH	*RHD+/RHD─*[†]	21	378	1	4	17	94.18
RH	37bp dup exon 4	15	270			10	96.3
RH	RhC/Rhc[‡]	26	468	1		6	98.5
RH	c.676G>C	28	504	2		1	99.4
RH	c.122A>G	18	324				100
RH	c.106G>A	11	198				100
RH	c.733C>G	21	378	1		2	99.21
RH	c.1006G>T	20	360	4			98.89
LU	c.230G>A	25	450	1	3	1	98.89
KEL	c.578C>T	28	504			4	99.21
KEL	c.841C>T	25	450				100
KEL	c.1790T>C	21	378				100
FY	[§]	28	504			6	98.81
JK	c.838G>A	27	486		1	3	99.18
DI	c.2561C>T	25	450	1			99.78
YT	c.1057C>A	17	306			2	99.35
SC	c.169G>A	16	288				100
DO	c.793A>G	27	486			3	99.38
DO	c.323G>T	22	396	1			99.75
DO	c.350C>T	22	396	2		2	98.99
CO	c.134C>T	24	432		1	1	99.54
LW	c.299A>G	15	270				100
CROM	c.679G>C	7	126		18		85.71
KN	c.4681G>A	10	180			16	91.11
KN	c.4768A>G	7	126				100
KN	c.4801A>G	6	108				100
IN	c.137G>C	5	90				100
OK	c.274G>A	3	54	17	1		66.67
Total			12078	64	34	85	98.48

*Nucleotide position refers to the position within the cDNA sequence. [†]Predicted by determining *RHD* deletion or *RHD* zygosity; [‡] Predicted by testing SNP c.307T>C and 109bp insertion within intron 2 of *RHCE*; [§] Predicted by testing SNPs c.125G>A, c.265C>T, c.─67T>C of *ACKR1* gene. The total number of results reflects reported results for RhD, RhCc and Fy predicted phenotypes.

Table 3. Primers used for characterization of the samples.

Primer Name	Sequence (5' – 3')
CO-PCR-F	TCAGAGGGAATTGAGCACCC
CO-PCR-R	GACACCCCACTCACGTCAT
CO-Seq-F	AAGAAGCTCTTCTGGAGGGCA
CO-Seq-R	AAGCGAGTTCCCAGTCAGGGA
CR-PCR-F	TGCATTCAAAGCTCCCACAT
CR_PCR-R	TCATTGAATGACTGCAACCC
CR-Seq-F1	GGCATTTATAAGCATCTCTTGTTGGT
CR-Seq-R1	CATATAGACCTGAGGGTAAGAAT
DI-PCR-F1	AGATGTGCCCTACGTCAAGC
DI-PCR-R1	TTAGGGGTCCAGCTCACTCA
DI-Seq-F1	GGTACAGGACCCTTTTCTGGCT
DI-Seq-R1	TGTCTGCCTCTTGACGCTCT
DO-PCR-F1	CTGCAACCACATTCACCATCTG
DO-PCR-R1	TCTGTGATCCTGAGTGGCCT
DO-Seq-F1	AGTACCAAGGCTGTAGCAAACAG
DO-Seq-R1	AATACCTTTTAGCAGCTGACAGTT
Duffy-PCR-F	CTGAGTGTAGTCCCAACCAGCCAA
Duffy-PCR-R	AGAGCTGCGAGTGCTACCTAGC
Duffy Seq-F1	CAAGGCCAGTGACCCCCATA
Duffy Seq-R1	GGCAAACAGCACGGGAAATGAG
Duffy Seq-F2	GATGGCCTCCTCTGGGTATGTCCT
Duffy Seq-R2	GCACCACAATGCTGAAGAGG
Kpab-PCR-F1	GCCCCTCAACCCTTAATCCC
KPab-PCR-R1	TAGCAGCATCTCCTCCACCA
Kpab-Seq-F1	TTGTGTCCATCTCCCAAGGC
Kpab-Seq-R1	AGGGACTGAGAAGGGCTCAG
KEL578PCR-F	TTTAGTCCTCACTCCCATGCTTCC
KEL578PCR-R	TATCACACAGGTGTCCTCTCTTCC
KEL578SeqF	CCTAGAGGAATCGAAGGGCG
KEL578SeqR	CCAACTGTGTCTTCGCCAGT
JS-PCR-F2	TTCCACCCTGGCTATCCCAG
JS-PCR-R1	AGAAGGGCCATCAGGCTCTA
JS-Seq-F1	TGCTGAGAGATTCTGGGGGT
JS-Seq-R1	ATGGCCCTTGCTCACTGGTT
JK-PCR-F2	CTATACCAGTGGGAGTTGGTCAGA
JK-PCR-R1	GGGGAGCTTTGAGAAGGCAT
JK-Seq-F1	ATTTTCCTGGGAGCCATCCT
JK-Seq-R1	ACAGCAAGTGGGCTCAAGCC
KN-PCR-F1	ACCATACTCTTCCTTCTCTCAGTCA
KN-PCR-R1	CCCTCACACCCAGCAAAGTC

KN-Seq-F1	GCAACAATAGAATAGAACATCTTTTCAC
KN-Seq-R1	CACTCACCCCTGGAGCAGTGT
LU-PCR-F3	AAAGAGGCAGAGCCAGGAGCTGCA
LU-PCR-R3	TCACCACGCACACGTAGTCTCG
LU-Seq-F3	GGGACACCCGGAGCTGAGAG
LU-Seq-R3	TCACCACGCACACGTAGTCTCG
LW-PCR-F2	CTTATCTCTAGAGCCGGCCCT
LW-PCR-R2	AAGGGGGAGCCTCTGAGTGA
LW-Seq-F1	TCTGTTCCCTCTGTCGCTGCT
LW-Seq-R1	GCTCCCTATAGAGCGACTGTCA
IN-PCR-F1	AGGTTCATGCCATTCTCCTG
IN-PCR-R1	CCAGGAGAGCTCTGTGGAAG
IN-Seq-F1	GGAGTCTGTCCTAAACTGAACTT
IN-Seq-R1	GTCGGGTGCTGGTCTCTTACC
IN-Seq-F2	TGTTAACCAGGCTGGTCTTGAG
IN-Seq-R2	GGAAAGGAGCCTTCCAGTTC
OK-PCR-2-4F	GAGGTGTGGGGTTCATCAGT
OK-PCR-2-4R	TCCCCCTCGTTGATGTGTTC
OK-Seq-F	CCAGAGGTGTGGGGTTCATC
OK-SeqR	TCAGGCCAACAACTTCTCCCGACA
SC-PCR-F1	GGCGTCCCCAGAATAAGGAA
SC-PCR-R1	GACACTTCCCTCTTGGGCAT
SC-Seq-F1	TGGCAATCCTTTCCAGAGTCCTT
SC-Seq-R1	ATTCCGGCATCAGAGATCTTCA
YT-PCR-F2	CAGGAGAACGTGGCAGCCTT
YT-PCR-R	GGAGGACTTCTGGGACTTCTG
YT-Seq-F	ACCAGCGCAGGTCCTGGTGAA
YT-Seq-R	AATGGGCCTGGAGAAGCCCTCAT
CwCx-PCR-F4	CCTCTTCTGAGCTTCAGTTTCCTTATTT
CwCx-PCR-R4	GCTTCTAAAGGAAAGCTTACATTGTTGA
CwCx-Seq-F2	CTCCATAGACAGGCCAGCACAG
CwCx-Seq-R2	GAAGATGGGGGAATCTTTTCCTC
VVS-EePCR-F5	CTGGGCAACAGAGCAAGAGTCC
VVS-Ee-PCR-R5	AGAAACGGGGTTCAAACTC
VVS-EeSeq-F	CCATCACAGAGCAGGTTCAGG
VVS-Ee-SeqR	GGGGTGGGGAGGGGCATAAA
VVS-Fprimer 6	AACAAACTCCCCATTGATGTGAGTA
VVS-Rprimer 6	GCTCTGTGTTTGTGGGGTCACAG
VVS- F primer-3	CCTTAGTGCCCATCCCCATTT
VVS-SeqR2	ATCCAAGGTAGGGGCTGGACAT
RHC109-PCRF3	GTGCCACTTGACTTGGGACT
RHC109-PCRR3	GTGGACCCAATGCCTCTG

109ins-Seq-3F	GGTACAATCATAGCTCATTGCTATAGC
109ins-Seq3R	CTATGATTGTACCACTGGGAAGTGAC
RHc307PCR-F3	GCTTCCCCCTCCTCCTTCTCAC
RHc307PCR-R1	TGAGAGGCCTTGAGAGGTCC
Rhc307SeqF1-S	TGGGCTTCCTCACCTCAAAT
RHc307PCR-F4	GTGCGAAAACAGTTGGTGATTATTGATAAG
RHc307PCR-R4	GGCAATATCCCAGATCTTCTGGAACC
RHc307SeqF4	CAGTTGAGAACATTGAGGCTCA
RHc307SeqR4	TTTCGGGGTCCATTCCCTCT
MN-PCR-F2	GCTTTATCTGTAAACCTCTGCTATGC
MN-PCR-R3	TGAGGTGACTGCGTGGACATAG
MN-Seq-F1	GAGGGAATTTGTCTTTTGCAAT
MN-Seq-R2	GGGTCTGAGCTGAACTCAGTTT
Ss-PCR-F3	TAAGGCAACCATACTATCAATTGCTA
Ss-PCR-R3	AGGCTTGGCCTCCCAAAATTATA
Ss Seq-F2	TGGCATCTCTGTGGAGTAATGGC
Ss-Seq-R2	GTTAACAACATATGCTCTTCTGTTTTAAG
SSI-PCR-F5	TGGGTCTGGAATCAGAAGCC
SSI-PCR-R5	GACTTCTATGTGTCCAGTTGAAAAAG
SSI-Seq-F2	CACATAATAGTATGTTAACTGTACTTTG
SSI-Seq-R4	GAATTTTATGCAGTTCTGTTTCTCTTC
ABO-PCR-F6	GGGCTGGGAATGATTTG
ABO-PCR-R6	GGTGTCCCCCTCCTGCTATC
ABO-PCR-F1	GGCAGAAGCTGAGTGGAGTT
ABO-O-SeqR2	GCTCAGTAAGATGCTGC
ABO-PCR-F7	CCCCGTCCGCCTGCCTTGCAG
ABO-PCR-R7	GGGCCTAGGCTTCAGTTACTC
ABO-B1-SeqF2	CTACTATGTCTTCACCGAC
ABO-PCR-R1	ACTCACAACAGGACGGACAA
DS4-S [32]	GCCGACACTCACTGCTCTTAC
DS4-AS [32]	TGAACCTGCTCTGTGAAGTGC
DS4-Seq[32]	GGGAGATTTTTTCAGCCAG
RhD-Intron4-F[33]	TAAGCACTTCACAGAGCAGG
Rh-I5R[33]	TATGTGTGCTAGTCCTGTTAGAC
DS6-S[32]	CAGGGTTGCCTTGTTCCCA
DS6-AS[32]	CTTCAGCCAAAGCAGAGGAGG

F, S: forward (sense) primer; R, AS: reverse (antisense) primer.

Table 4. Summary of fill details.

Dispensation	
Number of vials tested	12
Mean weight of the dispensed solution, g	1.0009
Standard deviation	0.0191
Coefficient of variation	0.0191
Residual moisture analysis	
Number of measurements	16
Mean residual moisture, %	7.72
Standard deviation	3.58

Table 5. Agreement calculated for each method

Method	# of Labs Using	Results Reported	No Call	Discrepant Results	Agreement Rate (%)
Method A	1	468	0	0	100.00%
Method B	1	18	0	0	100.00%
Method C	1	18	0	0	100.00%
Method D	8	3060	1	0	99.97%
Method E	3	1296	0	1	99.92%
Method F	3	1386	0	3	99.78%
Method G	2	1350	2	1	99.78%
Method H	1	306	0	1	99.67%
Method I	6	2038	2	21	98.87%
Method J	8	2434	4	32	98.52%
Method K	5	666	1	9	98.50%
Method L	6	958	20	10	96.87%
Method M	1	270	8	13	92.22%

Table 6. Agreement calculated for each laboratory.

# of Tests Performed by a Laboratory	Genotyping Results		Agreement Rate (%)
	Concordant	Reported	
37	663	666	99.55
36	630	648	97.22
35	607	630	96.35
34	612	612	100.00
31	548	558	98.21
30	540	540	100.00
28	502	504	99.60
28	500	504	99.21
28	500	504	99.21
26	460	468	98.29
25	450	450	100.00
23	414	414	100.00
22	382	396	96.46
22	395	396	99.75
22	396	396	100.00
21	377	378	99.74
21	374	378	98.94
21	378	378	100.00
21	378	378	100.00
21	378	378	100.00
21	377	378	99.74
21	378	378	100.00
21	378	378	100.00
18	324	324	100.00
18	324	324	100.00
13	213	234	91.03
10	180	180	100.00
9	162	162	100.00
4	72	72	100.00
4	67	72	93.06
671	11,959	12,078	98.88

Figure 1. Cycle threshold (Ct) values from real-time qPCR over a period of three years for lyophilized samples stored at -20°C, +4°C, and RT.

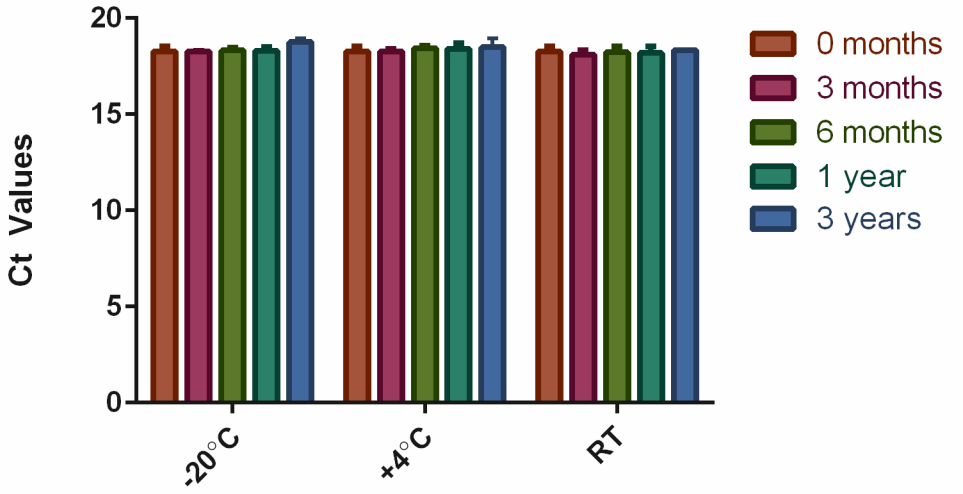


Figure 2. Agarose gel electrophoresis of three representative samples of the lyophilized DNA material stored at -20°C, +4°C, and RT for three years. 2-Log DNA ladder by New England Biolabs was used as DNA marker.

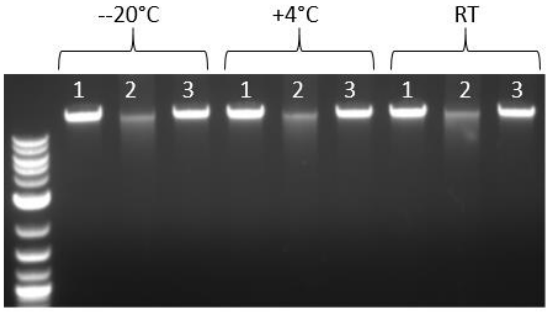
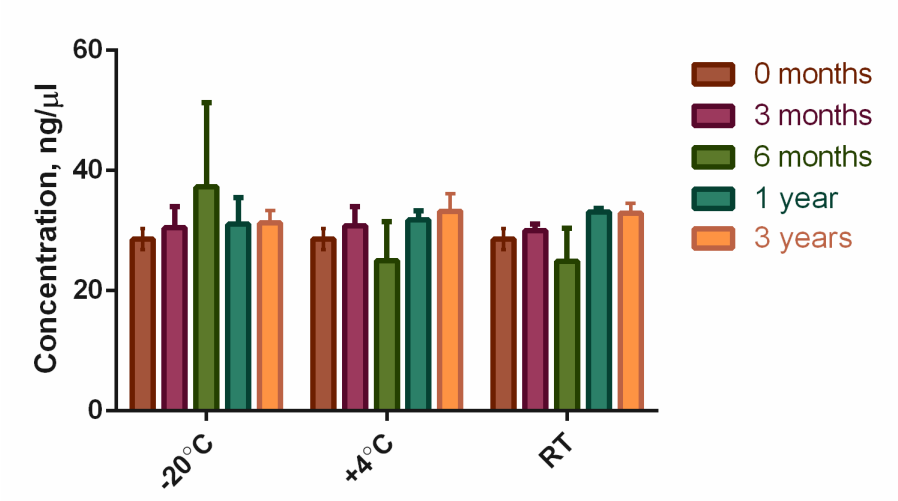


Figure 3. DNA concentrations of the lyophilized DNA material stored at -20°C, +4°C, and RT over a period of three years.



Appendix 1. Participants and their affiliations.

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Michael Wittig Andre Franke	Institute of Clinical Molecular Biology, Kiel University, Kiel, Germany
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Appendix 2. Phenotypes of the 18 panel members determined by established immunohematological techniques.

Panel member	ABO	MNS					RH						KEL						FY		JK		DI	YT*	DO*	CO*	
		M	N	S	s	U	D	C	c	E	e	C^w	K	k	kp^a	kp^b	Js^a	Js^b	Fy^a	Fy^b	Jk^a	Jk^b	Di^a	Yt^a	Do^b	Co^a	Co^b
10006	A	+	0	0	+	+	+	0	+	+	+	NA	0	+	NA	NA	NA	+	0	+	0	+	NA	NA	NA	0	NA
10007	A	+	0	0	+	NA	+	+	+	0	+	NA	0	+	+	0	NA	NA	+	+	0	NA	NA	NA	NA	NA	NA
10012	A	+	0	+	+	NA	+	+	+	+	+	NA	0	+	NA	NA	NA	+	0	+	0	+	NA	NA	NA	NA	NA
10014	O	+	+	0	+	NA	0	0	+	0	+	NA	+	0	0	NA	NA	NA	0	+	+	+	NA	NA	NA	NA	0
10018	O	+	+	0	+	NA	+	+	+	0	+	NA	+	0	0	NA	NA	+	+	+	+	+	NA	NA	NA	NA	0
10020	B	+	0	0	+	NA	+	+	0	0	+	NA	0	+	NA	NA	NA	NA	0	+	0	+	NA	0	NA	NA	NA
10029	O	+	+	+	0	NA	+	+	+	+	+	0	+	+	NA	+	NA	NA	+	+	0	+	+	+	NA	NA	NA
10030	A	+	+	0	+	NA	+	+	+	0	+	+	0	+	NA	NA	NA	NA	+	0	0	+	NA	NA	NA	NA	NA
10035	A	+	+	+	+	NA	+	0	+	+	+	NA	0	+	NA	NA	NA	NA	+	W	+	+	NA	NA	NA	NA	NA
10038	O	0	+	0	+	+	+	0	+	+	+	NA	0	NA	0	+	+	NA	0	0	+	0	NA	NA	NA	NA	NA
10044	O	0	+	0	+	+	+	0	+	0	+	NA	0	NA	NA	+	NA	0	0	0	+	0	NA	+	NA	+	NA
10047	O	+	+	+	+	+	0	0	+	0	+	NA	0	+	0	+	NA	+	0	0	+	0	NA	NA	+	NA	0
10048	A	0	+	0	+	+	+	0	NA	0	NA	NA	0	NA	NA	NA	0	+	0	0	+	0	NA	NA	NA	NA	NA
10050	B	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA	+	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
10052	O	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA	NA	+	NA	NA	NA	NA	NA	NA	NA	+	NA	+	NA
10053	O	+	0	0	+	NA	+	0	NA	0	+	NA	0	NA	NA	+	NA	+	0	0	+	0	NA	NA	NA	NA	NA
10056	A	0	+	0	+	+	0	0	NA	NA	+	NA	0	NA	NA	+	NA	+	0	0	+	0	NA	NA	NA	NA	NA
10058	O	+	+	0	+	NA	+	0	NA	+	+	NA	0	NA	NA	+	NA	+	+	NA	NA	0	NA	+	NA	+	NA

NA: not available; W: weak expression; *: no commercial reagents were available for these and the following antigens: Di^b, Yt^b, Do^a, VS, V.

Note: no serology data were available for the following blood group systems: CROM, IN, KN, LU, LW, OK, SC.

Appendix 3. Draft Instructions for Use for DNA Reference Reagents for genotyping of blood group antigens.

U.S. Food and Drug Administration
Center for Biologics Evaluation and Research
(CBER/FDA)

A WHO Collaborating Center
for Biological Standardization

Additional WHO International Reference Reagents for Blood Group Genotyping

CBER/FDA codes: 10006, 10007, 10012, 10014, 10018, 10020, 10029, 10030, 10035, 10038, 10044, 10047, 10048, 10050, 10052, 10053, 10056, 10058

Instructions for use

(Version 1.0, March 2019)

1. INTENDED USE

To standardize genotyping of 40 blood group alleles, present in different ethnic groups. The reagents are intended to be used either individually or together as a panel containing one vial of each of the 18 reagents. Additional WHO International blood group genotyping reference reagents are available from NIBSC (http://www.nibsc.org/science_and_research/advanced_therapies/genomic_reference_materials/red_blood_cell_genotyping_(who).aspx).

2. CAUTION

This preparation is not for administration to humans.

The material is of human origin. The DNA was extracted from B-lymphoblastoid cell lines generated from consenting blood donors that were tested and found negative for evidence of bloodborne pathogens HIV 1/2, HCV, HBV, HTLV-1/2, *T. pallidum*, *T. cruzi*, and WNV according to current U.S. regulations. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

N/A.

4. CONTENTS

Each vial contains 1 µg DNA and stabilizing agents. After reconstitution with 40 µl of nuclease-free water the final concentration of DNA will be 25 ng/µl in 10 mM Tris, 1 mM EDTA, and 75 mg/ml trehalose.

5. STORAGE

Store at –20°C.

Please note: because of the inherent stability of lyophilized material, these materials may be shipped at ambient temperature.

6. DIRECTIONS FOR OPENING

Vials have a “flip-up” circular cap. Either on the cap or the collar of the vial, there is an indication of the point at which to lever off the cap. This exposes an area of the rubber stopper through which the preparation can be reconstituted using a hypodermic needle and syringe. If use of a pipette is preferred, then fully remove the metal collar using, for example, forceps, taking care to avoid cuts by wearing appropriate gloves. Remove the stopper for access. Care should be taken to prevent loss of material during stopper removal.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.

Each vial should be reconstituted with 40 µl of nuclease-free water. Once reconstituted, gently agitate the vial over the period of 20 minutes before use. All 18 reagents have been validated for blood group genotyping in an international collaborative study. The genotypes are shown in Table 1.

8. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid at the assigned potency and status until withdrawn or amended. The reference materials are held at CBER/FDA in assured, temperature-controlled storage facilities. Reference Materials should be stored on the receipt as indicated on the label.

Once diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting deterioration in the characteristics of any reference preparation are encouraged to contact CBER/FDA.

9. REFERENCES

Volkova E, Sippert E, Liu M, Mercado T, Denomme GA, Illoh O, Liu Z, Rios M, and the Collaborative Study Group, Validated Reference Panel from renewable source of genomic DNA available for standardization of blood group genotyping, The Journal of Molecular Diagnostics (2019), doi: https: //doi.org/10.1016/j.jmoldx.2019.02.003.

Boyle J, Thorpe SJ, Hawkins JR, Lockie C, Fox B, Matejtschuk P, Halls C, Metcalfe P, Rigsby P, Armstrong-Fisher S, Varzi AM, Urbaniak S, Daniels G: International reference reagents to standardise blood group genotyping: evaluation of candidate preparations in an international collaborative study. Vox Sang 2013, 104:144-152.

World Health Organization. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). WHO Technical ReportSeries 2006. 932, 73-131.

10. ACKNOWLEDGEMENTS

We thank all of our collaborators for timely evaluation of reagents and providing invaluable feedback. We thank Simleen Kaur and members of Laboratory of Microbiology, In-Vivo Testing and Standards (DBSQC, FDA) for their assistance with filling and lyophilization of the DNA.

11. FURTHER INFORMATION
Further information can be obtained as follows:
This material: Maria.Rios@fda.hhs.gov
WHO Biological Standards:
http://www.who.int/biologicals/en/

12. CUSTOMER FEEDBACK
Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service.
Please send any comments to Maria.Rios@fda.hhs.gov

13. CITATION
In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the CBER/FDA code number, and the name and address of CBER/FDA are cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance:	Lyophilized powder
Corrosive:	No
Stable:	Yes
Oxidizing:	No
Hygroscopic:	No
Irritant:	No
Flammable:	No
Handling:	See caution, Section 2
Other (specify):	Contains human DNA
Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS
Information provided by CBER/FDA is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by CBER/FDA to the Recipient (“the Goods”) for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

CBER/FDA shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of CBER/FDA in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.
If any of the Goods supplied by the CBER/FDA should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to CBER/FDA together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), CBER/FDA shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of CBER/FDA’s liability under this Condition.

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Table 1. Genotypes of the 18 DNA panel members.

Gene	Polymorphism*	DNA panel member																	
		10006	10007	10012	10014	10018	10020	10029	10030	10035	10038	10044	10047	10048	10050	10052	10053	10056	10058
ABO	c.1061delC	C/C	C/C	C/delC	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/delC	C/C	C/C	C/C	C/C	C/C
	c.526C>G	C/C	C/C	C/C	C/C	C/C	C/G	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/G	C/C	C/C	C/C	C/C
	c.703G>A	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G
	c.796C>A	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/C	C/C
	c.803G>C	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G	G/G	G/G	G/G
	c.261delG	G/delG	G/delG	G/G	delG/delG	delG/delG	G/delG	delG/delG	G/delG	G/delG	delG/delG	delG/delG	delG/delG	G/G	G/delG	delG/delG	delG/delG	G/G	delG/delG
GYPA	c.59C>T	C/C	C/C	C/C	C/C	C/T	C/C	C/T	C/T	C/T	T/T	T/T	C/T	T/T	T/T	T/T	C/C	T/T	C/T
GYPB	c.143C>T	C/C	C/C	T/T	C/C	C/C	C/C	T/T	C/C	C/T	C/C	C/C	C/T	C/C	C/T	C/C	C/C	C/C	C/C
	c.230C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.270+5G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
RHD	RHDdel†	+/–	+/–	+/+	–/–	+/+	+/+	+/+	+/+	+/–	+/+	+/+	–/–‡	+/+‡	+/+	+/+	+/+	–/–‡	+/+‡
RHCE	109bp intron 2 ins§	–/–	+/–	+/–	–/–	+/–	+/+	+/–	+/–	–/–	–/–	–/–	–/–	–/–	+/–	+/–	–/–	–/–	–/–
	c.307C>T	C/C	T/C	T/C	C/C	T/C	T/T	T/C	T/C	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C	C/C
	c.676G>C	C/G	G/G	C/G	G/G	G/G	G/G	C/G	G/G	C/G	C/G	G/G	G/G	G/G	C/G	C/G	G/G	G/G	C/G
	c.122A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
	c.106G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
	c.733C>G	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	G/G	G/C	G/C
	c.1006G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
BCAM	c.230G>A	G/G	G/G	A/A	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
KEL	c.578C>T	C/C	C/C	C/C	T/T	T/T	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.841C>T	C/C	T/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C
	c.1790T>C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/C	C/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T
ACKR1	c.125G>A	A/A	A/G	A/A	A/A	A/G	A/A	A/G	G/G	A/G	A/A	A/A	A/A	A/A	A/G	A/G	A/A	A/A	A/A
	c.265C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
	c.–67T>C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	C/C	C/C	C/C	C/C	T/T	T/T	C/C	C/C	T/C
SLC14A1	c.838G>A	A/A	A/A	A/A	A/G	A/G	A/A	A/A	A/A	A/G	G/G	G/G	G/G	G/G	A/G	A/G	G/G	G/G	G/G
SLC4A1	c.2561C>T	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
ACHE	c.1057C>A	C/C	C/C	C/C	C/C	C/C	A/A	C/C	C/C	C/A	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
ERMAP	c.169G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G
ART4	c.793A>G	A/G	G/G	A/G	A/G	A/G	A/G	A/G	A/A	G/G	A/G	G/G	A/G	A/A	A/G	A/G	G/G	A/G	A/A
	c.323G>T	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/T	G/G	G/G	G/G	G/G	G/G	G/G
	c.350C>T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	T/T	C/C	C/C	C/C	C/C	C/T
AQP1	c.134C>T	T/T	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C
ICAM4	c.299A>G	A/A	A/A	A/A	A/A	A/G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
CD55	c.679G>C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/C	G/G
CR1	c.4681G>A	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G
	c.4768A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/G	A/G	A/G	A/A	A/A	A/A	A/A	A/A
	c.4801A>G	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/G	A/G	G/G	G/G	A/A	A/A	A/G	A/G	G/G
CD44	c.137G>C	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G
BSG	c.274G>A	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G

*Nucleotide position refers to the position within the cDNA sequence. † +/+ indicates no *RHD* deletion, +/– indicates deletion of one *RHD* allele, –/– indicates deletion of both *RHD* alleles. ‡ Caution in the interpretation of results should be exercised if an assay targeting upstream and downstream Rhesus boxes is used for determination of this genotype. § +/+ indicates the presence of 109bp intron 2 insertion in both *RHCE* alleles, +/– indicates the presence of 109bp intron 2 insertion in one *RHCE* allele, –/– indicates absence of 109bp intron 2 insertion.

Appendix 4. Collaborative study protocol.

COLLABORATIVE STUDY TO EVALUATE LYOPHILISED DNA PREPARATIONS FOR THEIR SUITABILITY TO SERVE AS REFERENCE REAGENTS FOR BLOOD GROUP GENOTYPING

Collaborative Study Protocol

Materials Provided

Three sets of 18 vials of lyophilized genomic DNA extracted from cell pellets of immortalized cell lines, 1 µg per vial.

Instructions

Each vial should be reconstituted in 40 µl distilled or deionized nuclease-free water and gently agitated over the period of 20 minutes to completely dissolve the contents before use. This will result in a DNA concentration of approximately 25 ng/µl in 10mM Tris, 1mM EDTA, and 75mg/ml trehalose.

Perform blood group genotyping using your usual methods; if feasible, perform three independent runs on different days.

Reporting

Report details of each run, methods used, and results using the provided forms (Table 1). Please refer to Alleles Database + Methods file (Table 2) for test numbers and information on alleles and corresponding DNA bases. Note that for each cell in Results file, values have been predefined in a drop-down menu.

Please return the completed forms to evgeniya.volkova@fda.hhs.gov for analysis of the data. If possible, also include the original report as outputted by your genotyping platform(s).

If you encounter discrepancies or the assay you are using gives ambiguous results, please report it to us and use an alternate method (if any are available to you or via a reference laboratory) for the allele(s) in question.

Please note that the performance of the panel in some genotyping assays (such as NGS, MALDI-TOF MS and others) has not been validated.

Table 1. Alleles Database + Methods file distributed with the panel.

1. Please indicate which allele variants you can test for by checking the box next to the variant (column 1).
2. Choose method(s) you use for each variant. If you use only one method for the majority of your genotyping, please indicate that method here --->
and fill out column 2 only for alleles that you test with different methods.

Test #	Blood group	Nt #	Change	AA #	Change	Allele	Phenotype	ISBT Allele Name	1 Test	2 Method(s) - Indicate all that apply
1	ABO	803	G	268	Gly	*ABO*A*	A		☐	1. High Resolution Melting Analysis
	ABO	803	C	268	Ala	*ABO*B*	B	*ABO*B.01.803C*		2. PCR with sequence-specific primers (PCR-SSP)
2	ABO	796	C	266	Leu	*ABO*A*	A		☐	3. PCR-restriction fragment length polymorphism (PCR-RFLP)
	ABO	796	A	266	Met	*ABO*B*	B	*ABO*B.01.796A*		4. Multiplex PCR-SSP
3	ABO	703	G	235	Gly	*ABO*A*	A		☐	5. Mass spectrometry
	ABO	703	A	235	Ser	*ABO*B*	B	*ABO*B.01.703A*		6. Real-time PCR allelic discrimination
4	ABO	526	C	176	Arg	*ABO*A*	A		☐	7. Multiplex PCR-based microarray (e.g. BeadChip)
	ABO	526	G	176	Gly	*ABO*B*	B	*ABO*B.01.526G*		8. Luminex-based microarray (e.g. BLOODchip)
5	ABO	261	G			*ABO*A1*	A1		☐	9. Other (please specify)
	ABO	261	ΔG	87	fs	*ABO*O*	O	*ABO*O.01.01*		
6	ABO	1061	C			*ABO*A*	A1		☐	
	ABO	1061	ΔC	354	fs	*ABO*A2*	A2	*ABO*A2*		
7	YT	1057	C	353	His	*YT*A*	Yt(a+)	*YT*01*	☐	
	YT	1057	A	353	Asn	*YT*B*	Yt(b+)	*YT*02*		
8	CO	134	C	45	Ala	*CO*A*	Co(a+)	*CO*01.01*	☐	
	CO	134	T	45	Val	*CO*B*	Co(b+)	*CO*02*		
9	CROM	679	G	193	Ala	*CR*A*	Cr(a+)	*CROM*01*	☐	
	CROM	679	C	193	Pro		Cr(a-)	*CROM*-01*		
10	DI	2561	T	854	Leu	*DI*A*	Di(a+)	*DI*01*	☐	
	DI	2561	C	854	Pro	*DI*B*	Di(b+)	*DI*02*		
11	DO	793	A	265	Asn	*DO*A*	Do(a+)	*DO*01*	☐	
	DO	793	G	265	Asp	*DO*B*	Do(b+)	*DO*02*		
12	DO	323	G	108	Gly	*HY*	Hy+		☐	
	DO	323	T	108	Val		Hy-	*DO*02.-04*		
13	DO	350	C	117	Thr	*JO*	Jo(a+)		☐	
	DO	350	T	117	Ile		Jo(a-)	*DO*01.-05*		
14	FY	-67	T						☐	
	FY	-67	C				Fy(a-b-)	*FY*01N.01 or FY*02N.01*		
15	FY	125	A	42	Asp	*FY*B*	Fy(b+)	*FY*02*	☐	
	FY	125	G	42	Gly	*FY*A*	Fy(a+)	*FY*01*		
16	FY	265	C	89	Arg	*FY*B*			☐	
	FY	265	T	89	Cys	*FY*265T*	Fy(x)	*FY*01W.01*		
17	IN	137	G	46	Arg	*IN*B*	In(b+)	*IN*02*	☐	
	IN	137	C	46	Pro	*IN*A*	In(a+)	*IN*01*		
18	KEL	841	C	281	Arg	*KEL*04*	Kp(b+)		☐	
	KEL	841	T	281	Trp	*KEL*03*	Kp(a+)	*KEL*02.03*		
19	KEL	578	C	193	Thr	*KEL*02*	k+	*KEL*02*	☐	
	KEL	578	T	193	Met	*KEL*01*	K+	*KEL*01.01*		
20	KEL	1790	T	597	Leu	*KEL*07*	Js(b+)		☐	
	KEL	1790	C	597	Pro	*KEL*06*	Js(a+)	*KEL*02.06*		
21	JK	838	A	280	Asn	*JK*B*	Jk(b+)	*JK*02*	☐	
	JK	838	G	280	Asp	*JK*A*	Jk(a+)	*JK*01*		
22	KN	4801	A	1601	Arg	*KN*04*	Sl(a+)		☐	
	KN	4801	G	1601	Gly	*KN*07*	Sl(a-) Vil+	*KN*01.07*		
23	KN	4768	A	1590	Lys	*KN*03*	McC(a+)		☐	
	KN	4768	G	1590	Glu	*KN*06*	McC(b+)	*KN*01.06*		
24	KN	4681	G	1561	Val	*KN*A*	Kn(a+)	*KN*01*	☐	
	KN	4681	A	1561	Met	*KN*B*	Kn(b+)	*KN*02*		
25	LW	299	A	100	Gln	*LW*A*	LW(a+)	*LW*05*	☐	
	LW	299	G	100	Arg	*LW*B*	LW(b+)	*LW*07*		
26	LU	230	G	77	Arg	*LU*B*	Lu(b+)	*LU*02*	☐	
	LU	230	A	77	His	*LU*A*	Lu(a+)	*LU*01*		
27	MNS	59	C	20	Ser	*GYPA*M*	M+	*GYPA*01*	☐	
	MNS	59	T	20	Leu	*GYPA*N*	N+	*GYPA*02*		
28	MNS	230	C	77		*GYPB*s*	s+		☐	
	MNS	230	T	77	Silent	*GYPB*NY*	S- U+^{w}	*GYPB*03N.01*		
29	MNS	intron 5 +5	g			*GYPB*s*	s+		☐	
	MNS	intron 5 +5	t			*GYPB*P2*	S- U+^{w}	*GYPB*03N.03*		
30	MNS	143	C	48	Thr	*GYPB*s*	s+	*GYPB*04*	☐	
	MNS	143	T	48	Met	*GYPB*S*	S+	*GYPB*03*		
31	OK	274	G	92	Glu	*OK*A*	Ok(a+)	*OK*01.01*	☐	
	OK	274	A	92	Lys		Ok(a-)	*OK*01.-01*		
32	RH	D				*RHD*	D+	*RHD*01.01*	☐	
	RH	D deletion				*RHd*	D-	*RHD*01N.01*		
33	RH	D				*RHD*	D+		☐	
	RH	exon 4 37bp insertion				*RHDψ*	D-	*RHD*04N.01*		
34	RH	733	G	245	Val		VS+	*RHCE*ce733G*	☐	
	RH	733	C	245	Leu		VS-	*RHCE*ce733C*		
35	RH	676	C	226	Pro	*RHCE*E*	E+	*RHCE*03*	☐	
	RH	676	G	226	Ala	*RHCE*e*	e+	*RHCE*01*		
36	RH	307	T	103	Ser	*RHCE*C*	C+	*RHCE*02*	☐	
	RH	307	C	103	Pro	*RHCE*c*	c+	*RHCE*01*		
37	RH	122	A	41	Gln		C(w-)	*RHCE*Ce122A*	☐	
	RH	122	G	41	Arg		C(w+)	*RHCE*Ce122G*		
38	RH	106	G	36	Ala		C(x-)	*RHCE*Ce106G*	☐	
	RH	106	A	36	Thr		C(x+)	*RHCE*Ce106A*		
39	RH	1006	G	336	Gly			*RHCE*ce1006G*	☐	
	RH	1006	T	336	Cys		VS+V-	*RHCE*ce1006T*		
40	RH	CE				*RHCE*c*	c+		☐	
	RH	intron 2 109bp insertion				*RHCE*C*	C+	*RHCE*intr2ins*		
41	SC	169	G	57	Gly	*SC*01*	Sc1	*SC*01*	☐	
	SC	169	A	57	Arg	*SC*02*	Sc2	*SC*02*		

Table 2. Results Reporting Table

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Sample # / Test #	10006	10007	10012	10014	10018	10020	10029	10030	10035	10038	10044	10047	10048	10050	10052	10053	10056	10058
2	1																		
3	2																		
4	3																		
5	4																		
6	5																		
7	6																		
8	7																		
9	8																		
10	9																		
11	10																		
12	11																		
13	12																		
14	13													323G/323G					
15	14													323G/323T					
16	15													323T/323T					
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41	40																		
42	41																		