Comparison of the new Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument to the 7500 Fast System by genotyping \textit{RHD} in cell-free fetal DNA from maternal plasma

**Introduction**

The new Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument is available for \textit{in vitro} diagnostic use in certain European countries. Prof. Dr. Dieter Schwartz and Dr. Irina Korschineck of the Clinic for Blood Group Serology and Transfusion Medicine at the Medical University of Vienna, and ingenetix GmbH, respectively, compared genotyping data from this new instrument to data obtained using the 7500 Fast Real-Time PCR Instrument in their research, which includes development of a method for noninvasive prenatal investigation of fetal markers.

In spite of widespread immunoprophylaxis, hemolytic disease of the fetus and the newborn (HDFN) still contributes to perinatal morbidity and mortality. Rh antigens are the main cause of HDFN. There are at least 45 antigens of the Rh blood group system, which are located on two proteins encoded by two homologous genes, \textit{RHD} and \textit{RHCE} [1]. The Rh blood group system is second to the ABO blood group system as the most clinically significant in transfusion medicine [2]. The most immunogenic of the Rh antigens, RhD, is encoded by \textit{RHD}. Because of its strong immunogenicity, RhD is the most important antigen of the polymorphic Rh system. Currently, a major problem in the management of pregnant RhD-negative women is avoiding invasive procedures that can cause transplacental hemorrhage and fetal loss.

Approximately 15% of Caucasians are RhD-negative, usually with a homozygous deletion of \textit{RHD}; those who are RhD-positive have either one or two copies of \textit{RHD} [3]. Tests for predicting RhD phenotype from DNA involve the amplification of one or more regions of \textit{RHD} to determine whether the gene is present. Numerous variants of \textit{RHD} exist: in some, all or part of \textit{RHD} is present, but no RhD antigen is expressed; in others, part of \textit{RHD} is absent, but a variant RhD antigen is expressed. \textit{RHD} variants are relatively rare in Caucasians, but an inactive \textit{RHD} gene, \textit{RHDΨ}, is present in 66% of RhD-negative black
Africans. RHDΨ contains a 37 bp duplication and a nonsense mutation, both of which must be taken into account when any method of RHD genotyping is being performed.

Prenatal determination of fetal RHD genotype can be useful in the management of pregnancies. When the father is heterozygous at the RHD locus, the fetus has a 50% chance of being Rhd-negative, hence, unaffected. The need for further invasive procedures is reduced in an immunized pregnant woman whose fetus is Rhd-negative. Therefore, transplacental hemorrhage and its associated complications, which include fetal loss related to these procedures, is reduced. Furthermore, additional investigations and administration of anti-D immune globulin can be avoided in nonimmunized pregnant women.

The fetal RHD genotyping protocol is based on one from the SAFE network, which is a European Union Framework Programme 6–funded Network of Excellence established to implement routine, cost-effective, noninvasive prenatal diagnosis and neonatal screening. This article compares the data obtained using the new Applied Biosystems® 7500 Fast Dx Real-Time PCR System with data obtained using the established 7500 Fast Real-Time PCR System.

This customer research workflow demonstrates concordance of RHD genotyping results from the Applied Biosystems® 7500 Fast and new 7500 Fast Dx Real-Time PCR instruments.

**Fetal RHD genotyping workflow**

**Samples and DNA extraction**

Blood samples from 50 Caucasoid pregnant women carrying RhD-negative and RhD-positive fetuses were used to establish and validate the real-time PCR assays. The donors were enrolled in this blinded study after their informed consent was obtained.

Fetal DNA was extracted from 2 x 500 µL fresh or frozen maternal plasma using the QiaAmp DSP Virus Kit and the QiaVac 24 Plus Vacuum System [4,6], and each extraction was eluted in a 40 µL volume. To minimize the risk of contamination, DNA was isolated in laminar airflow and aerosol-resistant tips were used. The extracted DNA was used for real-time PCR immediately or stored at −20°C until testing was performed.

**Real-time PCR to detect the presence of RHD**

Real-time PCRs were set up using 10 µL of extracted DNA in a 30 µL reaction containing TaqMan® Gene Expression Master Mix, which produces robust results for multiplex PCR. Each multiplex reaction contained primers and TaqMan® MGB probes to detect RHD exons 7 (FAM™ label), 5 (VIC® label), and 10 (NED™ label). Three exons were tested to allow maximum confidence for detecting rare genotypes from different ethnic groups and to minimize false-negative results. At least 2 exons would be amplified in rare genotypes. Primer and probe concentrations were optimized to determine the minimum concentrations for maximum efficiency. Universal TaqMan® PCR conditions were used. At least 3 replicate reactions were performed for each sample.

For each experimental plate, 2 controls were analyzed in triplicate: a negative control (extraction water) and a positive control consisting of synthetically derived plasmids for all 3 target exons in the multiplex reactions. Additionally, to account for occasional nonuniform amplification of cell-free fetal DNA, amplification curves from 3 control DNA samples were used to determine the best baseline and threshold settings for more reliable analysis (see Table 1 for details on controls). Data were analyzed by two scientists independently, using SDS software v1.4 with Manual Analysis parameters. The same protocol was performed on the 7500 Fast Real-Time PCR System and the new 7500 Fast Dx Real-Time PCR Instrument to demonstrate the reproducibility of the results. All results were analyzed using the same analysis parameters.

After internal validation studies, the detection cutoff was defined as a C, value of 41. Samples were considered positive for the presence of RHD if at least 7 of the 9 amplification curves, representing the three exons in triplicate, were above the detection cutoff. Negative samples were reanalyzed to assure the confidence of the results.

**The New 7500 Fast Dx Real-Time PCR Instrument delivers reproducible results comparable to the 7500 Fast Real-Time PCR Instrument**

The optimized real-time PCR workflow for detection of fetal RHD described in this application note was developed by the Laboratory of Transfusion Medicine, University of Vienna, and is based on the Safe Network protocol. The workflow was verified with 50 maternal blood samples analyzed on the Applied Biosystems® 7500 Fast and 7500 Fast Dx Real-Time PCR Instruments. Data obtained from the two instruments were compared.

Representative amplification plots generated by the two instruments are shown in Figure 1. The data show that similar amplification curves were obtained for the positive and negative samples as well as the control DNA. The results from the 7500 Fast Dx and 7500 Fast Instruments, as determined by two scientists independently, did not differ; comparable, reproducible results were obtained from the two instruments (Table 2), demonstrating the reproducibility between the two systems and the overall utility of the new 7500 Fast Dx Real-Time PCR Instrument for this application. SD values for the control

Table 1. Control DNA used in the analyses.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument positive control</td>
<td>Plasmid expressing different exons of RHD</td>
</tr>
<tr>
<td>Instrument negative control</td>
<td>Extraction from non-pregnant women</td>
</tr>
<tr>
<td>Baseline and threshold control 1</td>
<td>1 ng/well human genomic DNA</td>
</tr>
<tr>
<td>Baseline and threshold control 2</td>
<td>100 pg/well human genomic DNA</td>
</tr>
<tr>
<td>Baseline and threshold control 3</td>
<td>20 pg/well 1–5 copies/well human genomic DNA</td>
</tr>
</tbody>
</table>

**Benefits of TaqMan® Gene Expression Master Mix**

- Sensitive detection for reliable quantification of abundant and rare transcripts
- Duplex PCR for co-amplifying two targets in a single reaction
- Specificity for differentiating between gene family members
- Stable mix for high-throughput analysis
- Validated with TaqMan® Gene Expression Assays for exceptional performance
- Use with TaqMan® PreAmp Master Mix for limited amounts of sample
DNA samples were lower than those for the test samples because of the greater robustness of the control DNA samples, which are genomic DNA and not rare fetal DNA in a background of maternal cells (see sidebar, Tips for Successful Amplification of Rare Targets).

**Conclusion**
The data generated on the new 7500 Fast Dx Real-Time PCR Instrument were comparable to those from the 7500 Fast System. The sensitivity of the protocol and the limited starting material underlie the need to use procedures similar to those used in a forensic laboratory.

Dr. Korschineck noted that the 7500 Fast Dx Real-Time PCR Instrument, its maintenance procedures, and its security, auditing, and eSignature features are perfect tools for prenatal diagnostic laboratories. She says, “The security of the certified, standardized IQ/OQ installation and service procedure gives me confidence in the operation of my laboratory.” Moreover, the audit trail software features make it possible to track any changes in the analysis and subsequent results. These are all fundamental requirements for laboratories working under strict quality control procedures. The 7500 Fast Dx Real-Time PCR Instrument is available for in vitro diagnostic use in certain European countries.*

The open system software provides full flexibility in assay setup and data analysis options, making this versatile real-time PCR instrument adaptable to the unique needs of any clinical laboratory.

* The customer is responsible for any validation of assays, and compliance with any regulatory requirements that pertain to their procedures and instrument use.

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**Table 2. No significant difference between data generated by the 7500 Fast Dx and 7500 Fast Real-Time PCR Instruments.**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Overall Mean SD* of Replicate C&lt;sub&gt;t&lt;/sub&gt; Values</th>
<th>Mean SD of Replicate Control C&lt;sub&gt;t&lt;/sub&gt; Values†</th>
</tr>
</thead>
<tbody>
<tr>
<td>7500 Fast Dx Real-Time PCR Instrument</td>
<td>0.868</td>
<td>0.577</td>
</tr>
<tr>
<td>7500 Fast Real-Time PCR Instrument</td>
<td>0.803</td>
<td>0.511</td>
</tr>
</tbody>
</table>

* SD = standard deviation
† SD values for control DNA are lower due to the more robust nature of these samples.

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**Figure 1. Similar amplification plots are generated by the 7500 Fast Dx and 7500 Fast Real-Time PCR Instruments.**

A. Representative samples analyzed on the 7500 Fast Dx Real-Time PCR Instrument.  
B. Representative samples analyzed on the 7500 Fast Real-Time PCR Instrument.  
C. Control DNA samples analyzed on the 7500 Fast Dx Real-Time PCR Instrument.  
D. Control DNA samples analyzed on the 7500 Fast Real-Time PCR Instrument.  

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**Figure 2. Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument.**

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**Features of the 7500 Fast Dx Real-Time PCR Instrument**

- Available for in vitro diagnostic use in certain European countries*  
- Easy-to-use software  
  - User-friendly software includes plate setup wizards, multi-plate data viewing capabilities, user-defined templates, and powerful data analysis and reporting tools.  
  - Open software easily accommodates your assays.  
- Specialized Applied Biosystems® Assurance Service Plan for Diagnostics  
- Flexible formats—96-well block format accommodates tube strips and 96-well plates for easy plate setup without automation  
- Multiplex-capable, 5-color variable excitation and emission optics—detect multiple targets from one sample  
- Customizable security settings—optimize the levels of security features required for your environment  
- Run both standard and Fast cycling protocols—get results in <40 min with Fast run mode

* The customer is responsible for any validation of assays, and compliance with any regulatory requirements that pertain to their procedures and instrument use.
Acknowledgments
We acknowledge Thomas Halama, Life Technologies Senior Field Application
Scientist, Molecular Biology, for his generous contribution to this collaboration.

References
typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin
in RhD negative pregnant women: prospective feasibility study. BMJ
2. Avent ND, Reid ME [2000] The Rh
blood group system: a review. Blood
95:375–387.
et al. (1991) Genetic basis of the RhD-
positive and RhD-negative blood group
polymorphism as determined by Souther
Risk free simultaneous prenatal
identification of fetal Rhesus D status and
sex by multiplex real-time PCR using cell
free fetal DNA in maternal plasma. Swiss
5. Daniels G, Finning K, Martin P, Massey
E [2009] Noninvasive prenatal diagnosis
of fetal blood group phenotypes: current
practice and future prospects. Prenat
High levels of fetal erythroblasts and fetal
extracellular DNA in the peripheral blood
of a pregnant woman with idiopathic
polyhydramnios: case report. Prenat
Diagn 20: 838–841.

Tips for Successful Amplification of Rare Targets
The most important guidelines, which were also used for this fetal RHD genotyping research, are
listed:
• Control and limit access to the laboratory to authorized personnel.
• Conduct techniques performed before PCR amplification and PCR setup at separate times or
in separate locations.
• Follow written procedures for cleaning and decontaminating facilities and equipment.
• Use validated methods for DNA analyses.
• Use reagents that are suitable for the methods employed.
• Have written procedures for documenting commercial reagents and for formulating in-house
reagents.
• Follow written guidelines for data interpretation.
• Verify that all control results meet the laboratory’s interpretation.
• Monitor analytical procedures using the following controls and standards:
  — Positive and negative amplification controls associated with samples being analyzed, which
    are amplified concurrently for all loci.
  — Reagent blank controls associated with each extraction, analyzed in triplicate for each run.
• Run a minimum of triplicates for each test sample.
• Repeat negative results to ensure they are true negatives.