

Table 1: Lifecodes LSA Class I Results

| Bead  | MFI undiluted | MFI dilution 1:10 |
|-------|---------------|-------------------|
| A0205 | 891           | 14.876            |
| A0201 | 2.719         | 14.195            |
| A6802 | 801           | 13.788            |
| A0203 | 1.656         | 13.144            |
| A6901 | 6.260         | 12.740            |
| A6801 | 684           | 12.091            |
| A0202 | 2.707         | 10.800            |

(Cutoff: 1500)

Table 2: HLA Genotyping

|          | HLA-type             |
|----------|----------------------|
| mother   | A*01, *03; B*35, *39 |
| father   | A*02, *68; B*44, *51 |
| child I  | A*01, *68; B*35, *44 |
| child II | A*02, *03; B*39, *51 |

**Conclusions:** This unusual case of NAIT in siblings shows that HLA antibodies obviously can cause NAIT. It is remarkable that each twin had inherited an alternate HLA haplotype from the father, and the mother was producing strong antibodies against the cross-reactive antigens A2 and A28 (68,69).

## P-0836

#### SET UP AND VALIDATION OF REAL-TIME PCR TECHNOLOGY FOR MOLECULAR RHD TYPING OF CELL FREE FOETAL DNA IN MATERNAL PLASMA: THE EXPERIENCE OF PAVIA

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One of the most important application of real-time PCR is the determination of RHD genotype on cell free foetal DNA (cff-DNA) from maternal plasma to assess whether the foetus is or not at risk from haemolytic disease (HDFN). The incidence of HDFN has declined sensibly since the introduction of anti-D immunoglobulin prophylaxis, however some D-negative women still make anti-D, thus resulting in foetal morbidity and mortality. The knowledge of foetal RHD genotype might state if the pregnancy should be strictly monitored or if unnecessary intervention could be avoided. In this setting, amniocentesis and chorionic villous sampling can be used for obtaining foetal DNA, but both procedures are invasive and associated with an increased risk of transplacental haemorrhage and spontaneous abortion. Recently, it has been demonstrated that, starting from the 16th week of pregnancy, in peripheral mother's circulation cff-DNA is present in amount sufficient to perform real-time PCR tests to determine if the foetus is RHD positive. To provide this specific diagnostic test in our Transfusion Service a collaboration with IBGRL of Bristol (Prof. G. Daniels, Dr. P. Martin) was

established. The IBGRL protocol was tailored to carry out the experiments with our reagents (Roche labelled probes) and instrument (Roche Lightcycler<sup>®</sup> 480). The procedure consist of amplification of exons 4, 5 and 10 of RHD gene with probes specifically designed to prevent the amplification of the homologous region of the RHCE gene. Moreover, the reaction of exon 10 allows to establish the eventual presence of confounding pseudogene RHD<sup>Y</sup> and hybrid gene RHCE-D-CE<sup>5</sup> that are peculiar to Africans. Reactions for detecting the Y-linked gene SRY are also included in the test providing an internal control if the foetus is of male gender. Setting up our protocol we performed real-time PCR to determine RHD foetal genotype in 20 blind mother's plasma samples provided by IBGRL. In all cases our results were in agreement with IBGRL reports (data not shown). To validate our technique we processed five more samples of plasma and genomic DNA from amniocentesis of D-negative mothers at 16th week of gestation who referred to our Hospital for prenatal diagnosis of chromosomal pathologies. The assay was carried out in double blind using real-time PCR on cff-DNA extracted from plasma and PCR-SSP for genomic foetal DNA testing. Also in this comparison the results were in agreement as reported in Table 1. Finally, to test the performance of our reagents and instruments we took part to the Collaborative Study establishing the 1st sensitivity standard for non-invasive prenatal determination of foetal RHD genotype, organized by NIBSC (Dr. P. Metcalfe). Our outcomes demonstrate that accurate procedures for determining foetal RhD genotype, also in HDFN high risk ethnicities, are feasible in our Lab, thus allowing the diagnosis of at risk pregnancies and the correct management of HDFN. This avoids unnecessary treatments, and prevents wastage of anti-D immunoglobulins. We trust that RHD typing on cff-DNA obtained from plasma of any D-negative pregnant women will become a standard practice in many Laboratories monitored through international quality controls.

Table 1

| SAMPLE | REAL-TIME RESULTS |          |         |       |       |              | PCR-SSP RESULTS |                      |
|--------|-------------------|----------|---------|-------|-------|--------------|-----------------|----------------------|
|        | Ct ex 4           | Ct ex 10 | Ct ex 5 | SRY   | COR5  | GENOTYPE     | SEX             | SEX (from KARYOTYPE) |
| PV1    | 43,51             | 39,01    | 37,89   | 41,07 | 35,21 | RHD positive | male            |                      |
|        | 43,86             | 37,5     | 39,06   | 42,04 | 35,42 |              |                 |                      |
|        | 41,2              | 38,31    | 39      | 41,87 |       |              |                 |                      |
|        | 41,1              | 38,23    | 41,42   | 42,16 |       |              |                 |                      |
| PV2    | 43,42             | nd       | 40,19   | 41,82 | 37,65 | RHD positive | female          |                      |
|        | 43,53             | 39,89    | 39,26   | nd    | 36,89 |              |                 |                      |
|        | nd                | 36,92    | 40,8    | nd    |       |              |                 |                      |
|        | 43,87             | 38,83    | 39,45   | nd    |       |              |                 |                      |
| PV3    | nd                | nd       | nd      | nd    | 36,58 | RHD negative | female          |                      |
|        | nd                | nd       | nd      | nd    | 36,41 |              |                 |                      |
|        | nd                | nd       | nd      | nd    |       |              |                 |                      |
|        | nd                | nd       | nd      | nd    |       |              |                 |                      |
| PV4    | 42,27             | 39,89    | 39,16   | 39,85 | 37,14 | RHD positive | male            |                      |
|        | 42,42             | 39,53    | 39,69   | nd    | 37,32 |              |                 |                      |
|        | 42,11             | 39,84    | 38,6    | 40,08 |       |              |                 |                      |
|        | 42,08             | 40,21    | 40,45   | 40,88 |       |              |                 |                      |
| PV5    | 43,35             | 38,92    | 40,25   | nd    | 36,26 | RHD positive | male            |                      |
|        | 42,03             | 39,47    | 39,84   | 41,2  | 36,29 |              |                 |                      |
|        | 43,11             | 40,22    | 40,09   | 41,38 |       |              |                 |                      |
|        | nd                | 40,33    | 40,41   | nd    |       |              |                 |                      |

## P-0837

#### AUTOMATED QUANTIFICATION OF ANTI-D USING THE AUTOMATIC COOMBS TEST (ACT) ANALYSER

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**Background:** The level of an antibody's concentration in the serum of a pregnant woman can indicate the severity of Hemolytic Disease of Fetus and Newborn (HDFN). Monitoring the changes of the concentration helps identifying the fetuses threatened by HDNF. Values below 5 IU/ml of anti-D indicate the unlikelihood, greater than 15 IU/ml the high risk of HDFN, whereas between 5 and 15 IU/ml moderate HDFN can be expected. Under standard circumstances the quantity of the anti-D correlates to the strength of the antigen-antibody reaction. The recommended method for the quantification of anti-D is the Indirect Antiglobulin Test (IAT). ACT ana-

was tailored to carry out the experiments (labeled probes) and instrument (Roche) consist of amplification of exons 4, 5 specifically designed to prevent the region of the RHCE gene. Moreover, the establishment of the eventual presence of and hybrid gene RHCE-D-CE<sup>s</sup> that are or detecting the Y-linked gene SRY are an internal control if the foetus is of the foetus we performed real-time PCR to in 20 blind mother's plasma samples our results were in agreement with IBGRL validate our technique we processed five genomic DNA from amniocentesis of D-f gestation who referred to our Hospital somal pathologies. The assay was carried-time PCR on cf-DNA extracted from mic foetal DNA testing. Also in this agreement as reported in Table 1. Finally, reagents and instruments we took part to ing the 1st sensitivity standard for non- of foetal RHD genotype, organized by comes demonstrate that accurate proced- d genotype, also in HDFN high risk b, thus allowing the diagnosis of at risk agement of HDFN. This avoids unnec- vantage of anti-D immunoglobulins. We DNA obtained from plasma of any D- become a standard practice in many international quality controls.

| PHENOTYPE  | SEX    | PCR-SSP RESULTS |                           |
|------------|--------|-----------------|---------------------------|
|            |        | GENOTYPE        | SEX (from RFLP/PHENOTYPE) |
| D positive | male   | CcDdee          | male                      |
| D positive | female | CcDdee          | female                    |
| D negative | female | CcDdee          | female                    |
| D positive | male   | CcDdee          | male                      |
| D positive | male   | CcDdee          | male                      |

#### USE OF ANTI-D USING THE (ACT) ANALYSER

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body's concentration in the serum of a severity of Hemolytic Disease of Fetus the changes of the concentration helps by HDNF. Values below 5 IU/ml of anti- than 15 IU/ml the high risk of HDFN, moderate HDFN can be expected. Under ity of the anti-D correlates to the strength n. The recommended method for the direct Antiglobulin Test (IAT). ACT ana-

lyser is the only instrument capable to ensure standard conditions for routine blood group serological methods performed in tube. The measuring principle of the ACT is the analysis of the size of the agglutinated particles produced in saline medium (photo-sedimentometry). The strength of the reaction is characterized by the sedimentation curve. As in the ACT analyser every step of the IAT is automatically standardized, it is a sensitive and reliable instrument for antibody quantification, too.

**Aim:** The factors influencing the reproducibility of agglutination reactions had to be excluded. Firstly the strength of reaction was determined to which the sedimentation curve belonged that showed most sensitively the concentration of the antibody, then based on it the optimal conditions of the quantitative measurement was established.

**Methods:** It was found that the type of sedimentation curve belonging to the weak positive reaction ("+" ) was the most suitable to follow the effects of the changing parameters. The varied parameters were: the medium, the incubation time, use of enhancement solutions, the phenotype and the age of the red blood cells, and the concentration of the cell suspensions. The test results were compared with known standards in International Units (IU/ml).

**Results:** Using various commercially available anti-D-s of known concentration, it was proven that the reaction strength and the corresponding sedimentation curve of "+" reaction equals to 0.1 IU/ml anti-D. As the critical interval of anti-D is 5-15 IU/ml, measuring two dilutions of the test sera (50 and 150 times), a fair estimation can be reached to the severity of HDFN. (Both positive reactions mean high risk, both negative mean no risk.) The results got by 15 min incubation time while using enhancement medium were not significantly different from those where the incubation time was 45 min and the medium was Normal Ionic Strength Solution (NISS). The enhancement medium that had the strongest influence on sensitivity was Polyethylene Glycol (PEG). The most influential factor was the concentration of the red cell suspensions.

**Conclusions:** In the laboratories where ACT analysers are used, it is possible to measure quantitatively the level of the antibodies and to monitor the changes without the regular use of the international standards. In case of anti-D, if the test serum is diluted (N-times) until it gives a "+", then the anti-D concentration of the serum is 0,1N IU/ml. Any questions will be answered: transzfuzio@erg.bme.hu

#### P-0838

**A 42-YEARS OLD PREGNANT AFRICAN WOMAN WITH ANTI-U**  
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**Background:** The U high frequency antigen is present on red cells of all Caucasians and in 99% of Africans. Anti-U are immune antibodies stimulated by transfusion or pregnancy, generally non-complement binding IgG antibodies. They may be involved in delayed hemolytic transfusion reactions and in hemolytic disease of the newborn (HDN).

**Case report:** A specimen collected from a 42-year old African woman at 25th week gestation was sent to Immunohematology Reference Laboratory (IRL) from Trieste Hospital in order to identify an antibody to high incidence antigen. The patient had never been transfused but had a history of five previous abortions at 2nd trimester for cervical incompetence. The antibody screening after the last abortion (1 year previously) was negative. The serum sample referred to our attention reacted 3+ to 4+ with all cells tested in the screening and identification panel in microcolumn method (BioVue System, Ortho-Clinical Diagnostics, Raritan, USA). The direct antiglobulin test (DAT) was negative. Her group was B Rh(D)+ and she was negative for E, Cw, K, Jkb, Fya, Fyb, S, s and U antigens. An antibody to anti-Fy3 or anti-U high incidence antigens was suspected since her husband was Caucasian, A Rh(D)+ negative E, Cw, K, Fyb and S. The compatibility with Fy(a-b-), U+ and Fy(a-b-), U- red blood cells (RBCs) was performed using microcolumn and negative results were obtained only with Fy(a-b-) U- RBCs. To rule out additional clinically significant al-

loantibodies, alloabsorption with PEG (Immucor Inc) ficin-treated B Rh(D)+ RBCs, negative for E, K, Fya, performed. Non reactivity with a selected panel w alloabsorbed serum. The eluate from RBCs used firmed the anti-U specificity. The titre of anti-U in 1:8 and the score 1:28 in antiglobulin test-IgG. Cr from a O Rh(D)+, U(-) blood donor of whom two fr in our inventory was negative. The donor was donation for transfusion support at delivery. The n program of predeposit autologous blood donation no siblings in Italy. The patient delivered at the 30th preterm newborn's DAT was negative and present of HDN. No transfusion support was required by y **Conclusions:** The role of IRL in identifying rare al also in view of the increasing number of mixe adequate counselling and the availability in these phenotype units will help to provide a safe trans racial societies.

#### P-0839

**NON-INVASIVE PRENATAL DIAGNOSIS OF AUSTRALIAN OBSTETRIC POPULATION D 2.1% RATE OF MOLECULAR VARIANTS IN WOMEN**

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**Background:** Non-invasive prenatal diagnosis (NIPD) assist in the clinical management of Rh(D) negative women have Rh(D) antibodies as a result of isoimmunisation. Tests cell-free fetal (cff) DNA in maternal plasma and maternal cell-free DNA. The interpretation is only RhD negative phenotype arises from the deletion of the RHD gene. The RHD gene is located on chromosome 1. Validating NIPD in an Australian obstetric population of women who had been typed as Rh(D) negative molecular variants.

**Aim:** To characterize RHD variants detected in a women.

**Methods:** Venous blood samples from 376 Rh(D) negative women, including 125 isoimmunised, were investigated by the Australian Red Cross Blood Service. Fetal RHD status was determined with an interpretation algorithm based on replicates for exons 4, 5 and 10. Samples with either inconsistent rates of exon amplification were retested and investigated by analysis of maternal genomic DNA to determine maternal RHD status. This included comprehensive BLOODchip TM v2.0 array in Bilbao, Spain. Additional derived cfDNA was restricted to RT PCR tests for RHD variants. **Results:** For Ten (10) of 376 subjects the outcome of the analysis was "indeterminant". In eight cases, analysis of maternal variants comprising four partial RHD types: RHD VI types II and one RHD DBT II; one weak D (one K409K and one IVS3+1G>A); and one non-D (RHD-CE(3-8)-D). The weak D and DBT variants were of Asian origin, the remainder were of Caucasian origin. In cases the variant D genes were associated with either the D gene. These were initially identified as Rh(D) negative frequency Ce or cE rather than ce phenotypes. In the variant RhD allele in the cell free DNA was paternally. Both were silent RHD genes, one an RHD gene and one an RHD gene lacking exons 4, 5 and 7 but both cases Rh D negative babies were delivered.