MOLECULAR (GENO)TYPING IN IMMUNOHAEMATOLOGY

Primož Rožman
Blood Transfusion Centre of Slovenia
2010
Chromosomal localization

Rh

HNA

HLA

AB0

Duffy

FcγRIIIb

GPIa

GPIV

GPIIa

GPIIIa

GPIc'

Kell

Kidd

HPA

1p34-36

5q23.3

7q11

17p12

1q22

1q22

17q21-32

17q12

18q11-q12

18p12

18q11-12
MOLECULAR EVENTS LEADING TO BLOOD GROUP ANTIGENS AND PHENOTYPES

- gene conversion or recombination
- duplication of an exon
- deletion of a gene, exon, or nucleotide(s)
- insertion of a nucleotide(s)
- single-nucleotide substitutions (most blood group systems)
Molecular genotyping of blood cell antigens

- red blood cells (blood groups)
- platelet antigens (HPA)
- leukocytes (HLA)
- granulocyte antigens (HNA)
The rationale for genotyping in immunohaematology

- If no sera exist for phenotyping
- If no samples available
- Serological questions
- Inheritance – zygosity
- Mixed samples - hymeras
Clinical applications of molecular typing

- patient genotyping
- donor genotyping
- prenatal diagnostics
Clinical applications of genotyping - 1

- recently transfused or multitransfused patients
- patients with DAT+
- resolving of serological problems (weak D, partial D, acquired phenotypes)
- detection of weakly expressed Ag (Fy^b-Fy^x)
- Ab is not available or is too weak (Do^a, Do^b, Go^a etc.)
- to distinguish allo- from autoantibody
Clinical applications of genotyping -2

• post-transfusion thrombocytopenia
• refractoriness to platelet transfusion – HLA - HPA
• neonatal alloimmune thrombocytopenia (NAIT)
• neonatal alloimmune neutropenia (NAN)
• transfusion related acute lung injury (TRALI)
METHODS
Methods

- PCR-RFLP
- PCR-SSP
- PCR-SSO
- hybridization
- real-time PCR – TaqMan
- sequencing
- microarrays
- high throughput methods
The specificity of PCR-SSP relies on the relative inability of Taq-Polymerase to start from 3 mismatched primers...
1. DNA is denatured. Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand.

2. Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.

3. Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.

4. Another round: DNA is denatured, primers are attached, and the number of DNA strands are doubled.

5. Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.
PCR-SSP - 2
Real Time PCR
Real time PCR – TaqMan - 1

**TAQMAN® PROBE-BASED ASSAY CHEMISTRY**

1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.

   ![Diagram of polymerization](http://www.appliedbiosystems.com/etc/medialib/appliedbio-media-library/images/application-and-technology/real-time-pcr/taqman-sybr-green-chemistry/data-images.Par.6739.Image.634.541.1.gif.Figure_1.gif)

2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.

   ![Diagram of strand displacement](http://www.appliedbiosystems.com/etc/medialib/appliedbio-media-library/images/application-and-technology/real-time-pcr/taqman-sybr-green-chemistry/data-images.Par.6739.Image.634.541.1.gif.Figure_1.gif)

3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.

   ![Diagram of cleavage](http://www.appliedbiosystems.com/etc/medialib/appliedbio-media-library/images/application-and-technology/real-time-pcr/taqman-sybr-green-chemistry/data-images.Par.6739.Image.634.541.1.gif.Figure_1.gif)

4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

   ![Diagram of polymerization completed](http://www.appliedbiosystems.com/etc/medialib/appliedbio-media-library/images/application-and-technology/real-time-pcr/taqman-sybr-green-chemistry/data-images.Par.6739.Image.634.541.1.gif.Figure_1.gif)
PCR v reálnem čase – TaqMan - 2

Model of real-time quantitative PCR plot


Choice of primers/probes
Very low copy number detection
6700 Instrument (door open)

- Robotic arm
- Plate heat sealer
- Output plates
- Vacuum purification station
- Purification reagents
- Dilutions, controls & master mixes
Reagents are commercially available

Universal PCR Master Mix (gold-Taq polymerase, nucleotides, buffer, Mg salts, stabilizer, control dye)

Detection System (TaqMan®, MGB®)

SYBR® green
Testing of meat: is there beef in the schnitzel?

- beef
- other
- Mixed grill
MOLECULAR TYPING OF RED CELL ANTIGENS

Fig. 2. Models of the structure of the major blood group-active proteins. Adapted from Anstee & Mallinson (1994).
(□) glycocalyx; (—) O-glycans; (→) site of blood group antigens; (↑) N-glycan; (GPA,B,C,D) glycoporphins A,B,C,D
Chromosomal localization of....
ABO blood group system

9. chromosome

19. chromosome

Hh
Sese

Modif. po Danielsu
Organization of AB0 locus 9q34

Exons [bp] 28 70 57 48 36 135 688
Exons [kbp] 1 2 3 4 5 6 7
Introns [kbp] ~12 0.9 1.7 1.5 0.4 1.0

ABO locus to scale: exons (blue) and introns (yellow)
* Genetically heterologous blood groups
ABO Glycosyltransferase Enzymes

*H. Clausen

\[ \text{Colgi Lumen} \]

\[ \begin{align*}
A_1 \quad & \quad \text{A}_2 : 1059 \\
& \quad \text{B: 703, 796, 802} \\
& \quad \text{O}_1 : 261 \\
& \quad \text{O}_2 : 802 \\
\end{align*} \]
ABO-Glycosyltransferase
Gene, Exons and Introns

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Razlike med ABO aleli na nivoju nukleotidov

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DEL

Pro Arg Phe Gly Leu INS Gly Gly Asp Arg DEL

Leu Gly Iso Ser Met Arg Ala Asn Trp
## Primers for AB0-PCR-SSP

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<th>Allele</th>
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<th>Sequence of primers</th>
<th>Rct. No / PCR product (bp)</th>
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Interpretation of PCR-SSP for ABO Glycosyltransferases

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Why ABO-PCR-SSP?

ABO-typing - no red cells needed!

- prenatal diagnosis
- polytransfused patients
- ABO serological problems
- post-transplantational ABO typing
- acquired B*
- forensic investigations (paternity testing)
Serological problems – acquired B

phenotype: A1 B

Anti-B antibodies present!

genotype: $A^1 O^1$
Detection of weakly expressed Ag (Fy^b with the Fy^x phenotype)

Fya  Fya/b  FynullFy^x

phenotype: Fy(a+b+?)
genotype: FYA/FYX
Donor typing

- resolving serological problems (weak D, partial D, Del)
- selections of rare donors (antigen-negative)
- Ab is not available or is too weak (Do^a, Do^b, Go^a etc.)
- high-throughput donor typing (donors registry)
- preparing reagent RBCs (Ab screening, identification panels)
- population genetic study
Resolving serological problems (weak D, partial D) PCR-SSP

D2, D3, D4, D5, D6, D7, D9, D10

C

weak D type 1

1 2 3 4 5 DHMi

RhD^{VI-2}
Multitransfused patient – 1a

Original blood group: B; D-C+c+ E-e+; K-k+; Jk(a-b+); Fy(a+b+)
0²/B; Rh(d)/Rh(d); RhCe/Rhce; k/k; Jk^b/Jk^b; Fya/Fyb; M/N; S/s

- after 2. tr. event
received 22 blood units
- nonidentical units
  - 20x D+
  - 10x C-
  - 4x c-
  - 10x E+
  - 1x e-
  - 3x K+
  - 17x Jk(a+)
  - 7x Jk(b-)
  - 8x Fy(a-)
  - 5x Fy(b-)

before transfusion

after transfusion
Multitransfused patients – 1b

Original blood group: B; D-C+c+ E-e+; K-k+; Jk(a-b+); Fy(a+b+)
0^2/B; Rh(d)/Rh(d); RhCe/Rhce; k/k; Jk^b/Jk^b; Fya/Fyb; M/N;
S/s

- after 2. tr. event received 22 blood units
- nonidentical units:
  - 20x D+
  - 10x C-
  - 4x c-
  - 10x E+
  - 1x e-
  - 3x K+
  - 17x Jk(a+)
  - 7x Jk(b-)
  - 8x Fy(a-)
  - 5x Fy(b-)

before transfusion

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control product
434 bp

after transfusion

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</tbody>
</table>

specific product
bp

M/N
FY (AB) typing - (TaqMan – end point detection)
FY (AB, X) typing (TaqMan)
HPA-1 donor typing (TaqMan)
MOLECULAR METHODS IN PRENATAL DIAGNOSTICS

- fetal genotyping in women with an immune antibody
  - to identify a fetus at risk of HDN (RhD, K)
  - to identify a fetus at risk of FMAIT (HPA-1, HPA-5)

- determination of \(RHD\) zygosity (RhD-pos partner)

- screening of RhD-neg women to avoid antenatal prophylaxis
Fetal genotyping in women with an immune antibody (PCR-SSP)

Determination of fetal blood group from:
• amniocytes
• chorionic villi
• cordocentesis

invasive procedures

KEL2/KEL2

RHe/RHe
Screening of RhD-neg women for carrying D-pos fetus (to avoid unnecessary antenatal prophylaxis)

- isolation of fetal cell-free DNA from maternal plasma in 28th week
- the prediction of fetal \textit{RHD} genotype using real-time PCR
- confirmation of the fetal cell-free DNA in maternal plasma
Prenatal diagnostics of HDN

• Amniocentesis
• Chordocentesis
• Biopsy of chorion villi

⇒ 1-2% casualties

Lo et al. 1997: free DNA of the fetus in the peripheral blood of pregnant women
48% RhD-neg pregnant women with RhD-neg fetus receive IgG anti-D
1. DNA from the fetal cells
2. Free fetal DNA
   – In the peripheral blood of the mother
   – Quantity rises with gestational age
   – Represents 3.6% → 6.2% of total free DNA
   – ↓ in immunized pregnant women
   – Post partem dissapears in few hours!
Algorythm

Isolation of free DNA from mothers plasma sample (week 28).

Real time PCR – RHD and SRY genes

- $D$-pos SRY-pos
- $D$-pos SRY-neg
- $D$-neg SRY-pos
- $D$-neg SRY-neg (or fetal DNA missing)
- $D$-unclear SRY-pos or neg

Testing of mother’s bi-allelic polymorphisms

Testing of free DNA for alleles not present in mother

Pos result - any

Neg result for all alleles

Fetal DNA not confirmed <1%!

New sample

$D$-neg
RHD-pos fetus
(detection of the RHD gene - exon 7)

Real-time PCR amplification plots
Genotype SRY (TaqMan) - female

Real-time amplification plots
**RHD-pos female fetus**

*(RHD gene - intron 4, exon 7, exon 10 and SRY)*

Real-time PCR amplification plots
PLATELETS

- refractoriness to platelet transfusion
- neonatal alloimmune TR-cytopenia (NAIT)
- post-transfusion thrombocytopenia
PLATELET SPECIFIC GP COMPLEXES

Glanzmann's thrombasthenia
Bernard-Soulier syndrome
vWillebrand's disease - platelet form
HPA polymorphism

LEGEND:
- Glanzmann's thrombasthenia
- vWillebrand's disease - platelet form
- Cleavage of soluble glycocalicin
- Bernard-Soulier syndrome
- HPA polymorphism
Suspected NAIT – HPA genotyping
HPA-1, 2, 3, 5, 4 and 15

mother

HPA-1 B/B
HPA-2 A/A
HPA-3 A/A
HPA-5 A/A
HPA-4 A/A
HPA-15 A/B

ewborn

genotype:

HPA-1 A/B
HPA-2 A/A
HPA-3 A/A
HPA-5 A/B
HPA-4 A/A
HPA-15 A/B
Genotyping of donors - HPA (PCR-SSP)
GRANULOCYTES

- neonatal alloimmune neutropenia (NAN)
- transfusion related acute lung injury (TRALI)
Genotyping of HNA-1
HIGH THROUGHPUT METHODS
High-throughput donor typing (microarrays)
Mikromreže - 1

Gene fragments on the array

SNP
Oligo
Spacer
Glass

Spot 1
"No fit"

Spot 2
"Fit"

http://liebel.lab.fzk.de/liebelwiki/images/5/57/Agilent.jpg
Mikromreže - 2
High throughput genotyping of donors - microarrays (mikromreže - BloodChip)
BLOODBCHIP

- English BT service & University of West England (Dr. G. Daniels, Dr. N. Avent & Dr. M. Scott)
- University of Lund (Dr. M. Olsson and Dr. J. Storry)
- Czech Blood Service in Prague (Dr. M. Pisacka)
- Barcelone Transfusion and Tissue Centre (Dr. E. Muniz-Diaz & Dr. N. Nogues)
- DRK/University of Ulm (Dr. W. Flegel)
- Sanquin (Dr. M. de Haas & Dr. E. van der Schoot)
BloodChip

**STEP 1 - AMPLIFICATION (PCR)**
45 fragments, which contain the main allelic variants of red blood cells groups and human platelets antigens, are amplified.

**STEP 2 - FRAGMENTATION AND LABELLING**
PCR products are fragmented and labeled with two different fluorophores (Cy3 and Cy5) to allow specific detection of RHD and RHCE genes.

**STEP 3 - HYBRIDIZATION**
The labeled PCR products are hybridized to a DNA array printed with allele-specific oligonucleotides probes.

**STEP 4 - DATA ANALYSIS**
Hybridized slides are scanned on a confocal scanner. The data is analyzed by proprietary software and a report is generated.
BLOODCHIP – procedure

isolation DNA

multiplex PCR

Blood sample

hybridisation washing

result output (genotype – phenotype)
<table>
<thead>
<tr>
<th>BLOOD GROUP</th>
<th>PHENOTYPES</th>
<th>GENOTYPES DETECTED BY BLOODchip®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABO (ABO/001)</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>RhD (RH/004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RhCcEe (RH/004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kell (KEL/006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidd (JK/009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Duffy (FY/008)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MNS (MNS/002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diego (DI/010)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dombrock (DO/014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colton (CO/015)</td>
<td></td>
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<tr>
<td></td>
<td>PLATELETS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPA</td>
<td>24</td>
</tr>
</tbody>
</table>

- ABO (ABO/001): O, A, B, AB and Weak A
- RhD (RH/004): RhD+, RhD-, Del, Partial D, Weak D
- RhCcEe (RH/004): C, c, Cw, Cx, E, e, VS, r’s
- Kell (KEL/006): K, k, Kp, Kp, Kmod, Js, Js
- Kidd (JK/009): Jk, Jk
- Duffy (FY/008): Fy, Fy
- MNS (MNS/002): M, N, S, s, U, Mia
- Diego (DI/010): Di, Di
- Dombrock (DO/014): Do, Do
- Colton (CO/015): Co, Co
- HPA: HPA1a, HPA1b, HPA2a, HPA2b, HPA3a, HPA3b, HPA4a, HPA4b, HPA5a, HPA5b, HPA6a, HPA6b, HPA7a, HPA7b, HPA8a, HPA8b, HPA9a, HPA9b, HPA10a, HPA10b, HPA11a, HPA11b, HPA15a, HPA15b
additional blood groups:

- Lutheran: Lua/Lub
- Scianna: Sc1/Sc2
- Diego: Wra/Wrb
- Landsteiner-Wiener: Lwa/Lwb (N Europe, Baltic states)
- Dombrock: Hy, Joa
- Cromer: Cra
# BLOODCHIP - results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Predicted Phenotype</th>
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<tbody>
<tr>
<td>ABO A101v</td>
<td>A</td>
</tr>
<tr>
<td>RHD (&quot;Apparently non-negative&quot;)</td>
<td>RHD +</td>
</tr>
<tr>
<td>RHCE Cc CC</td>
<td>C+c-</td>
</tr>
<tr>
<td>RHCE Ee ee</td>
<td>E-e+</td>
</tr>
<tr>
<td>RHCE Cx NO Cx</td>
<td>Cx-</td>
</tr>
<tr>
<td>RHCE Cw NO Cw</td>
<td>Cw-</td>
</tr>
<tr>
<td>RHCE r's no r's</td>
<td>no r's</td>
</tr>
<tr>
<td>RHCE VS no 712 ce AR/EK/BL no 733 VS</td>
<td>VS-</td>
</tr>
<tr>
<td>KELL Kk kk</td>
<td>K-k+</td>
</tr>
<tr>
<td>KELL KpA/KpB KPb/KpB</td>
<td>Kpa- Kpb+</td>
</tr>
<tr>
<td>KELL KpC NO KpC</td>
<td>Kpc-</td>
</tr>
<tr>
<td>KELL Kmod NO Kmod-1</td>
<td>Kmod-1-</td>
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<tr>
<td>KELL JsA/JsB JSB/JSB</td>
<td>Jsa- Jsb+</td>
</tr>
<tr>
<td>KIDD JkA/JkB JKB/JKB</td>
<td>Jka- Jkb+</td>
</tr>
<tr>
<td>KIDD Jknull no Jknull</td>
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<tr>
<td>Duffy FyA/FyB FYA/FYB</td>
<td>Fya+ Fyb+</td>
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<tr>
<td>Duffy FyGATA/Fyx NO FyGATA, NO Fyx</td>
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</tr>
<tr>
<td>MN MN</td>
<td>M+N+</td>
</tr>
<tr>
<td>Ss ss</td>
<td>S-s+</td>
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<tr>
<td>U</td>
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<tr>
<td>GpMur NO Gp.Mur</td>
<td>Mia-</td>
</tr>
<tr>
<td>Diego DiA/DiB DIB/DIB</td>
<td>Dia- Dib+</td>
</tr>
<tr>
<td>Dombrock DoA/DoB DOA/DOB</td>
<td>Doa+ Dob+</td>
</tr>
<tr>
<td>Colton CoA/CoB COA/COA</td>
<td>Coa+ Cob-</td>
</tr>
</tbody>
</table>
Luminex

1. Amplification
   - Target DNA
   - primers + primers
   - 99 °C
   - biotin

2. Denaturation
   - Denatured DNA

3. Hybridization
   - Hybridized DNA

4. Labeling
   - Labeling reaction

5. Acquisition
   - Acquisition equipment

6. Analysis
   - Analysis data
BeadChipTM DNA Microarray  
(BioArray Solutions, Warren, NJ)

Bead assembly protocol on silicon wafer, array imaging system, and decoding and assay images, of HEA analysis charts generated automatically by BeadChipTM analysis software using web-based software (wHEA; BioArray Solutions, Warren, NJ), showing 28 antigen states derived from DNA analysis, linked to a carrier-ID and sample-ID, stored in a database.
<table>
<thead>
<tr>
<th>Blood factor</th>
<th>Analyte</th>
<th>Polymorphism</th>
</tr>
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<tbody>
<tr>
<td>Rh</td>
<td>Cc</td>
<td>203A&gt;G, Int 2</td>
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<tr>
<td></td>
<td>Ee</td>
<td>676G&gt;C</td>
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<tr>
<td>Kell</td>
<td>K/k</td>
<td>698T&gt;C</td>
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<tr>
<td>Kidd</td>
<td>Jk^a/Jk^b</td>
<td>838G&gt;A</td>
</tr>
<tr>
<td>Duffy</td>
<td>Fy^a/Fy^b</td>
<td>FY125G&gt;A</td>
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<td>Duffy-GATA</td>
<td>Silencing FY</td>
<td>FY-33T&gt;C</td>
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<td>Fy^x (Fy[b+]^x )</td>
<td>265C&gt;T</td>
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<tr>
<td>MNS</td>
<td>GYP A (M/N)</td>
<td>60C&gt;T</td>
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<tr>
<td></td>
<td>GYP B (S/s)</td>
<td>143T&gt;C</td>
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<tr>
<td></td>
<td>GPB Silencing</td>
<td>230C&gt;T, +5 g&gt;t</td>
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<td>Lutheran</td>
<td>Lu^a/Lu^b</td>
<td>230A&gt;G</td>
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<td>Diego</td>
<td>Di^a/Di^b</td>
<td>DI2561T&gt;C</td>
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<td>Co^a/Co^b</td>
<td>CO134C&gt;T</td>
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<td>Do^a/Do^b</td>
<td>DO793A&gt;G</td>
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<td>Jo(a+)/Jo(a−)</td>
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<td>Hy+/Hy−</td>
<td>DO323G&gt;T</td>
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<td>LW308A&gt;G</td>
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<td>Sc1/Sc2</td>
<td>SC169G&gt;A</td>
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<td>Hemoglobin S</td>
<td>HbS</td>
<td>173A&gt;T</td>
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</table>
Genotyping in immunohaematology – what should be considered - 1

- Several areas knowledge are needed
  - molecular techniques
  - serological techniques
  - gene structure and molecular bases of blood groups
  - expression of blood groups Ags
  - factors that may affect the interpretation of genotype (natural chimerism)

- Patients after HSC transplantation
Genotyping in immunohaematology – what else should be considered - - 2

- genotype does not always reflect the phenotype
  - incorrect genotyping strategy
  - discovery of an unknown allele

- serological methods will remain a normal procedure
  - ABO, RhD (genotyping more expensive, time consuming etc.)
  - Ab detection
Future - mass scale genotyping for blood donors

- better matching of donor blood to patient
- implementation of electronic cross-match
- repeat testing is unnecessary
- needs to be:
  - high throughput
  - automated (equipment)
  - accurate
  - positive sample identification (from the beginning to the end of the process)
  - cost effective