HLA-Typing Strategies

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Human MHC

[Diagram showing HLA class I, II, and III region loci with specific gene names and abbreviations like TAPBP, DPB2, DOA, DQA, DMB, FSB9 (LMP2), PSMB8 (LMP1), DOB, DOA1, DRB1, DRB2, DRB3, DRA, P450, C21B, C2, HSPA1B, HSPA1A, HSPA1L, LTB, TNF-α, LTA, MICA, HLA-B, HLA-C, HLA-E, HLA-A, HLA-G, HLA-F, HFE]
Annual increase of known HLA-Alleles

- Class I Alleles
- Class II Alleles

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Current Number of recognized HLA-Alleles

<table>
<thead>
<tr>
<th>HLA</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>3.830 (733)</td>
</tr>
<tr>
<td>HLA-B</td>
<td>4.647 (1.115)</td>
</tr>
<tr>
<td>HLA-Cw</td>
<td>3.382 (392)</td>
</tr>
<tr>
<td>HLA-DRB</td>
<td>2.252 (697)</td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>1.054 (95)</td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>740 (132)</td>
</tr>
</tbody>
</table>

A total of **16,251** HLA Alleles as of Apr. 2017
HLA alleles possess a “patchwork” pattern of polymorphism
Inheritance of HLA

Mother

| HLA-A: | 01:01 29:01 |
| HLA-B: | 08:01 35:03 |
| HLA-Cw: | 07:01 04:01 |
| HLA-DRB1*: | 07:01 03:01 |
| HLA-DQA1*: | 02:01 05:01 |
| HLA-DQB1*: | 03:03 02:01 |
| HLA-DPB1*: | 09:01 04:01 |

Father

| HLA-A: | 02:05 03:01 |
| HLA-B: | 08:01 40:01 |
| HLA-Cw: | 03:03 07:02 |
| HLA-DRB1*: | 13:01 08:01 |
| HLA-DQA1*: | 01:03 04:01 |
| HLA-DQB1*: | 06:03 04:02 |
| HLA-DPB1*: | 04:01 15:01 |

Child 1

| HLA-A: | 29:01 03:01 |
| HLA-B: | 35:03 40:01 |
| HLA-Cw: | 04:01 07:02 |
| HLA-DRB1*: | 03:01 08:01 |
| HLA-DQA1*: | 05:01 04:01 |
| HLA-DQB1*: | 02:01 04:02 |
| HLA-DPB1*: | 04:01 15:01 |

Child 2

| HLA-A: | 01:01 03:01 |
| HLA-B: | 08:01 40:01 |
| HLA-Cw: | 07:01 07:02 |
| HLA-DRB1*: | 07:01 08:01 |
| HLA-DQA1*: | 02:01 04:01 |
| HLA-DQB1*: | 03:03 04:02 |
| HLA-DPB1*: | 09:01 15:01 |

Child 3

| HLA-A: | 29:01 03:01 |
| HLA-B: | 35:03 40:01 |
| HLA-Cw: | 04:01 07:02 |
| HLA-DRB1*: | 03:01 08:01 |
| HLA-DQA1*: | 05:01 04:01 |
| HLA-DQB1*: | 02:01 04:02 |
| HLA-DPB1*: | 04:01 15:01 |

Child 4

| HLA-A: | 01:01 02:05 |
| HLA-B: | 08:01 08:01 |
| HLA-Cw: | 07:01 03:03 |
| HLA-DRB1*: | 07:01 13:01 |
| HLA-DQA1*: | 02:01 01:03 |
| HLA-DQB1*: | 03:03 06:03 |
| HLA-DPB1*: | 09:01 04:01 |
Why do we type for HLA

- Transplantation
  - Solid Organs
    - Kidney
    - Pancreas
    - Heart
    - Cornea
  - Bone Marrow + HSC
  - HLA-A, B for platelet transfusions
- Disease association
  - B27 with AS
  - DR4 with RA
  - DR3, 4 & DQ2, 8 with Diabetes and coeliac disease
- Recurrent foetal loss
- Paternity testing
Methods for HLA-Typing

• Resolution degree
  – Allelic Resolution = > 2 fields: e.g. A*02:01:01:01
  – High Resolution with G-Codes: e.g. A*02:01:01G
  – High Resolution = 2 fields: e.g. A*02:01
  – Low Resolution = 1 field: e.g. A*02

• Method
  – Serological
  – Molecular
  – Cellular
  – Biochemical
HLA Typing for Donor Registries
Considerations

• Typing Quality – Errors make the Registry unattractive
• Typing Resolution – „Two fields“ currently sufficient
• Number of Loci – The more the better
• Expenses – The cheaper the better
• Data Handling – The lesser the better
• Only 1% of the donors typed will be needed
Classical Methods

- **Serological Typing (CDC)**
  - NIH-Test, LCT, Micro-lympho-cytotoxicity test

- **Cellular Tests**
  - MLC (Mixed Lymphocyte Culture)
  - PLT (Primed Lymphocyte Typing)

- **Biochemical Typing**
  - One-dimensional, isoelectric focussing
  - Two-dimensional, isoelectric focussing
Serology

• Pros:
  – Low implementation costs (no expensive equipment)
  – Cheap and quick

• Cons:
  – Low Resolution
  – Fresh material required!
  – Insufficient quality for HLA-class II Typing
### Microlymphocytotoxicity Test

<table>
<thead>
<tr>
<th></th>
<th>+ve Reaction</th>
<th>-ve Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLA antiserum (Ab) + lymphocyte suspension (Ag)</strong>&lt;br&gt;(Ag - Ab reaction)</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
</tr>
<tr>
<td><strong>Complement dependent cell lysis</strong></td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
</tr>
<tr>
<td><strong>Staining with AO/EB/Hb</strong>&lt;br&gt;(as seen through microscope)</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
</tr>
</tbody>
</table>
## SCORING

<table>
<thead>
<tr>
<th>Percent dead cells</th>
<th>Score</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 -10%</td>
<td>1</td>
<td>negative</td>
</tr>
<tr>
<td>11 - 20%</td>
<td>2</td>
<td>possibly negative</td>
</tr>
<tr>
<td>21 - 50%</td>
<td>4</td>
<td>weakly positive</td>
</tr>
<tr>
<td>51 - 80%</td>
<td>6</td>
<td>positive</td>
</tr>
<tr>
<td>81 - 100%</td>
<td>8</td>
<td>strongly positive</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>not interpretable</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Kev.</th>
<th>R.</th>
<th>sp1sp2sp3sp4sp5sp6sp7sp8</th>
</tr>
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<tbody>
<tr>
<td>A: 1</td>
<td>1</td>
<td>+ + + + + + + + + + + +</td>
</tr>
<tr>
<td>B: 1</td>
<td>1</td>
<td>+ + + + + + + + + + + +</td>
</tr>
<tr>
<td>C: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
<tr>
<td>D: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
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<tr>
<td>E: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
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<tr>
<td>F: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
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<tr>
<td>G: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
<tr>
<td>H: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
<tr>
<td>I: 1</td>
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<td>A A A A A A A A A A A A</td>
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<tr>
<td>J: 1</td>
<td>8</td>
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</tr>
<tr>
<td>K: 1</td>
<td>8</td>
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<tr>
<td>L: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
<tr>
<td>M: 1</td>
<td>8</td>
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<tr>
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<td>O: 1</td>
<td>8</td>
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<tr>
<td>P: 1</td>
<td>8</td>
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<td>Q: 1</td>
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<tr>
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<td>S: 1</td>
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<tr>
<td>U: 1</td>
<td>8</td>
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<tr>
<td>V: 1</td>
<td>8</td>
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</tr>
<tr>
<td>W: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
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<td>X: 1</td>
<td>8</td>
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<tr>
<td>Y: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
<tr>
<td>Z: 1</td>
<td>8</td>
<td>A A A A A A A A A A A A</td>
</tr>
</tbody>
</table>

**Notes:**
- HLA-A: 1
- HLA-B: 3
- HLA-C: 6
Molecular Methods for HLA-Typing

- PCR-SSP
- PCR-SSO – Luminex

- SBT – Sanger
- Next Generation Sequencing (NGS)
  - Amplicon Based
  - Whole Gene
PCR-SSO, SBT, NGS

PCR-SSP, SBT
PCR-SSP

Sequence Specific Primer Design

Primer 1

Primer 2

Primer 3

Primer 4

Primer 5

Primer 6

Primer 1

Primer 7

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PCR-SSP - Workflow

DNA-Isolation

→

PCR with sequence-specific Primer-combinations

→

Electrophoresis

→

Reading and Interpretation
Example of a simple PCR-SSP-Typing

Mix Nr. 1 2 3 4 5 6 7 8

DQB1* 05:01 06 05/06 02 0301 03:02 03:03 03/04

Typing Result: **DQB1*03:02, 05:01**
PCR-SSP

• Pros:
  – Low implementation costs (no expensive equipment)
  – Straight forward interpretation of results
  – Quickest molecular method (<3 hours)

• Cons:
  – High consumption of DNA (in particular for high res)
  – Limited resolution in view of the increas. number of alleles
  – Constant need for primer updating – too cumbersome
  – Only low throughput possible
PCR-SSO
Sequence Specific Oligonucleotides Design

Primer 1

Oligo 1          Oligo 2     Oligo 3
Oligo 4
Oligo 5    Oligo 6

Oligo 7          Oligo 8     Oligo 3
Oligo 4
Oligo 5    Oligo 9

Oligo 1          Oligo 2     Oligo 10
Oligo 4
Oligo 5    Oligo 6

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Example of an allele specific hybridization reaction:
Specific bead set is coupled with a specific oligonucleotide to the surface.

To develop the biological reaction an orange fluorescent labeled PCR product is used
Beads pass through laser in a single file.
Sample will be taken up by the instrument and will pass the lasers.
PCR-SSO (Luminex)

• Pros:
  – Simultaneous Hybridisation of one PCR product with >100 oligonucleotides in one single tube (liquid chip)
  – High throughput with minimal staff requirements
  – Very few DNA material required

• Cons:
  – Reagent costs
  – Constant need for probe updating – too cumbersome
  – Limited resolution in view of the increases number of alleles

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SBT  
Sequence Based Typing
Why Sequencing Approaches

A*01:001: AACGCCGATCCGTTACGCTAG

SSO: ???GCC???????????????????TAG

SSP: ???GCC????????????????????????TAG

SBT: AACGCCGATCCGTTACGCTAG

Some years later....

A*01:040: AACGCCGAACAGGTTACGCTAG
SBT Steps
1. DNA-Isolation
2. Locus +/- Allele-group specific Amplification (PCR)
3. PCR Control
4. Purification of Amplification Product
5. Cycle Sequencing
6. Purification of the „Cycle Seq“ Products
7. Separation of the „Cycle Seq“ Fragments in an automatic Sequencer
8. Interpretation and Evaluation of the Sequences

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5. SBT – Cycle Seq Step
7. SBT – Separation of the Cycle Seq fragments
SBT

• Pros:
  – High Resolution approach
  – Direct reading of sequence information
  – Very few DNA material required

• Cons:
  – Costs for reagents and equipment
  – Rather time consuming and complex if more than exons 2 and 3 need to be considered
  – Limited resolution in view of the steadily increasing number of alleles (no cis-trans-separation -> Ambiguities)
Problem: Ambiguities

Allele 1: \[\text{Acg} \text{TTA} \text{AggTagcgcATcTgA} \text{cccAATCTT}\]

Allele 2: \[\text{Acg} \text{CTTA} \text{AggTagcgcATcTgA} \text{gggTTACTT}\]

Sequencing-Result: \[\text{Acg} \text{YTA} \text{AggTagcgcATcTgA} \text{SSSWWWCTT}\]

Allele 3: \[\text{Acg} \text{TTA} \text{AggTagcgcATcTgA} \text{gggAATCTT}\]

Allele 4: \[\text{Acg} \text{CTTA} \text{AggTagcgcATcTgA} \text{cccTTACTT}\]

Sequencing-Result: \[\text{Acg} \text{YTA} \text{AggTagcgcATcTgA} \text{SSSWWWCTT}\]

Final Result: \[\text{Allele 1} + \text{Allele 2} \text{ or } \text{Allele 3} + \text{Allele 4}\] = Ambiguities

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NGS

• Principle:
  – Massive Parallel Sequencing
  – Different Plattforms and Technologies
    (Illumina, PGM, PacBio, Nanopore etc)

• Resolution: High, Maximum
Why NGS?

• Reduction of Expenses due to the massive parallel testing feasible by the use Multiplex Identifier Sequences (MID)

• Higher Resolution and avoidance of Ambiguities.

• Higher Throughput

• More Information (more Loci) per Test Run
  – HLA-Loci
  – Other Genes (ABO, KIR, CCR5 etc)
NGS Workflow Overview

1. DNA-Isolation
2. Locus specific Amplification (PCR)
3. PCR Control – Quantification steps
4. Library Preparation (differs dramatically between platforms)
5. Quantification - Purification
6. Pooling
7. Sequencing
7. Interpretation and Evaluation of the Sequences
Current NGS Platforms

- ThermoFisher – Ion Torrent (PGM)
- Illumina – Miseq
- Pacific Biosciences RS
- Oxford Nanopore - Minion
NGS Typing Approaches

Exon-based typing (selected exonic/intronic sequences)
= Amplicon based

cDNA-based typing (selected exons can be included)
= „Shot gun“ based

Full genomic typing
= „Shot gun“ based

Full length Amplification (5’UTR to 3’UTR) of:
- HLA-A, HLA-B, HLA-C (~3kb)
- HLA-DQA1 (~7kb)
- HLA-DQB1 (~7kb)

Partial Amplification of:
- HLA-DRB1 amplicon ~5 kb (gene ~15kb)
- HLA-DPB1 amplicon ~7kb (gene ~12kb)
NGS – Important Molecules
(for Whole Gene and Amplicon Based Approaches)

- Adapter
- Reverse Adapter
- MID (Multiplex Identifier = Molecular Barcode
- Target Specific Primer (= Locus specific)
- Individual Sequence (Targeted Region)
NGS – Library Preparation

= Ligation of double-stranded DNA adaptors to the ends of the DNA template, that are used throughout the process to amplify, capture, and sequence the DNA.

Two strategies for library preparation depending on the size of the DNA region to be sequenced

Whole Gene (Long range PCR)
- for targeted regions >500 bases in length
- the starting DNA template (genomic DNA or large PCR amplicons) must be fragmented prior to adaptor ligation.

Amplicon based (Short PCR)
- for targeted regions <500 bases in length
- does not require fragmentation prior to adapter ligation.
NGS – Amplicon Based Workflow

Pool and end-repair amplicons

Ligate adapters and nick-repair

Barcoded Library

Purification, Quantification and dilution

Pool

Exons 1 2 3 4 5 6 7

5'UTR 3'UTR

NGS – Amplicon Based Workflow

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Shotgun Genome Sequencing

Complete genome copies

-> Fragmented genome chunks
Shotgun Genome Sequencing

NOT REALLY DONE BY DUCK HUNTERS
Hydroshearing, sonication, enzymatic shearing
Assembly

Consensus:
TAATGCAGACCTCGATGCGCGGCGAAGCATTGTTCACCACAGACCCTGTTTTCCGACCCTGAATGGCTCC

6x coverage
100% identity

Coverage: # of reads underlying the consensus

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Assembly

Consensus:

TAATGCAGACCTCGATGCCCCGCGAAGCATTGTTCCCACAGACCGTGTTTTCCGACCACCAGAAATGGCTCC

AGCTCGATGCCGCCGGCGAAG

TTGTCCCACAGACCGTGTTTTCCGACCACCAGAAATGGCTCC

ATGCCCGGCGAAGCATTG

ACAGACCCGGTTTTCCCCG

TAATGCAGACCTCGATGCC

AAGCATTGTTCCCACAG

TGTTTTCCGACCACCAGAAAT

CCGACCACCAGAAATGGCTCC

5x coverage

80% identity

Coverage: # of reads underlying the consensus
Assembly

Consensus:
TAATGCACCTCGATGCGCAGCGACATTGTTCACAGACCGTGTTTTTCCGACCGAAATGGCTCC

2x coverage
50% identity

Coverage: # of reads underlying the consensus
Assembly

Consensus:
TAATCGGACCTCGATGCCGGGCGAAGCATTGTTCCCACAGACCGTGTTTTTCCGACCGAAATGGCTCC

Coverage: # of reads underlying the consensus

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Assembly
NGS – Whole Gene Workflow - Library

1. **Purification + Quantification**
2. **Fragmentation**
3. **Ligate adapters and nick-repair**
4. **Size-select**
5. **Purification, Quantification and dilution**
6. **Barcoded Library**

This process involves the following steps:

1. **Purification + Quantification**
2. **Fragmentation**
3. **Ligate adapters and nick-repair**
4. **Size-select**
5. **Purification, Quantification and dilution**
6. **Barcoded Library**

Additionally, there is a mention of "NGS – Whole Gene Workflow - Library" which includes the following:

- **Exons**: 1, 2, 3, 4, 5, 6, 7
- **5’UTR**
- **3’UTR**
- **Exons 1, 2, 3, 4, 5, 6, 7**
- **Barcode Adapters**
- **P1**

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Current NGS Platforms

- ThermoFisher – Ion Torrent (PGM)
- Illumina – Miseq
- Pacific Biosciences RS
- Oxford Nanopore - Minion

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NGS – Sequence Analysis
NGS
Workflow, Automation and Data Processing

PRÄ-PCR Setup

Post-PCR, Cleanup, MID-PCR, Quantification, Pooling

Analysis

Sequencing
NGS – Critical Points

• Fragment length of products to be sequenced – *Platform depended*
• Sequence „Depth“ – *Number of reads per position*
• Sequence „Coverage“ – *reliable sequences per gene area*
• Error Rate of Sequence Reading – *Platform depended*
• Complexity of Library Preparation - *Automation*
• Turn Around Time - *Platform and Approach depended*
• Throughput – *Approach and platform depended*
• Expenses – *Approach depended*
• Data Overload
NGS

• Pros:
  – Maximum Resolution possible
  – Cis-Trans Information provided (less ambiguities)
  – Very few DNA material required
  – Parallel Testing of large number of Material (= low cost)

• Cons:
  – Costs for reagents and equipment
  – Complex Procedures – Automation mandatory
  – Time consuming – TAT significantly longer
  – Large data volumes – Data storage and processing logistics required
HLA Typing for Donor Registries
Considerations

• Typing Quality – Errors make the Registry unattractive
• Typing Resolution – „Two fields“ currently sufficient
• Number of Loci – The more the better
• Expenses – The cheaper the better
• Data Handling – The lesser the better
• Only 1% of the donors typed will be needed
HLA Typing for Donor Registries
Recommendation from today‘s point of view

- NGS – exon based for typing at registration
- ABCRDQDP at „two field“ level

In order to keep costs reasonably low
And have a reasonably high level of quality and information in an acceptable time frame
HLA-Typising in Future?
Nanopore Technology