Sequence Based Typing (SBT) and Next Generation Sequencing (NGS) HLA Typing

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Objectives

- Basics of Sequence Based Typing (SBT)
- Next generation sequencing and HLA vs. SBT
- overview of Illumina NGS technology

Methods for HLA Typing

Serology Based Typing	DNA Molecular Based Typing
- Complement Dependent Cytotoxicity (CDC)	- Sequence Specific Primers(SSP) - Sequence Specific Oligonucleotide (SSO) - Sequence Based Typing (SBT) – Sanger - Next Generation Sequencing (NGS)

The Challenge of HLA Genotyping

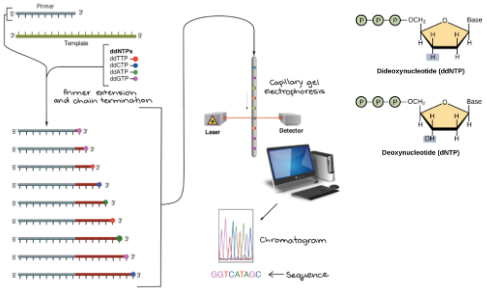
- Most polymorphic region of the genome
- Contains many repeated structures & pseudogenes
- Gene-level phasing of heterozygotes is difficult with current technology (e.g. SSP, SSO and SBT "Sanger")

→ → → Highly ambiguous results

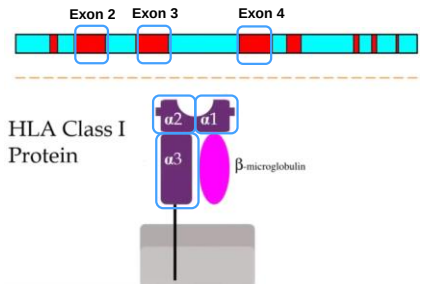
Current HLA Typing Methods

	Coverage*	Allele Ambiguity	Resolution
SSP	Exon	Yes	Low to Medium
SSO	Exon	Yes	Medium
SBT (Sanger)	Exon	Yes	High
Whole Gene NGS	Whole Gene	Few to None	Highest

Sequence Based Typing (SBT)
Chain Terminating (Sanger) Sequencing



Targets of Current HLA Typing Method: Class I Gene



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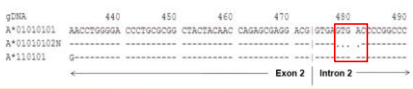
Ambiguities Generated Due to Uncharacterized Genomic Regions — Limitations of Existing Methods

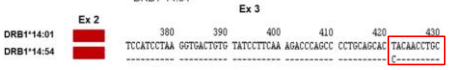
Example#1

Allele 1	Allele 2
A*01010101	A*110101
A*01010102N	A*110101



Example#2

Allele 1	Allele 2
DRB1*14:01	
DRB1*14:54	any other DRB1 allele



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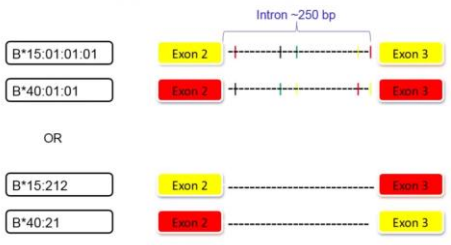
Ambiguities Generated Due to Uncharacterized Genomic Regions — Limitations of Existing Methods



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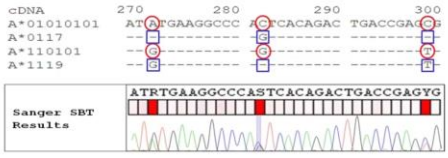
Ambiguities Generated by Sanger Sequencing Due to Lack of Phase — Limitations of Existing Methods

Allele 1	Allele 2
A*01010101	A*110101
A*0117	A*1119



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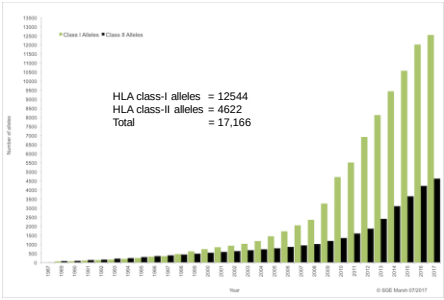
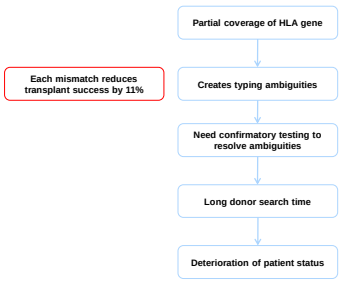
Number of HLA alleles 1987-2017

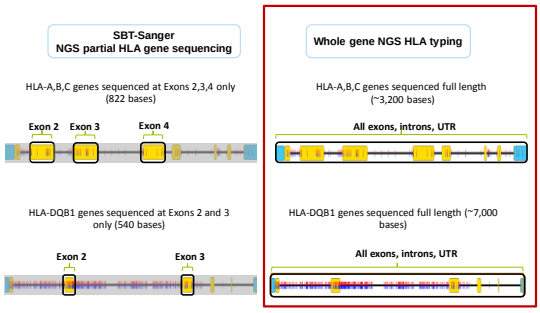


Increased Number of HLA Alleles = More Ambiguities

Problems with the Current HLA Typing Methods



Solution to Current Problems with HLA typing



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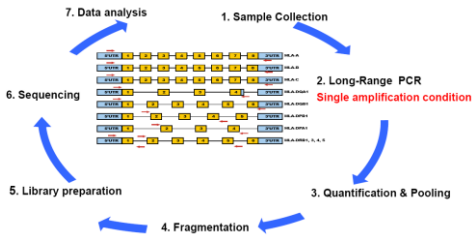
NGS HLA Typing Method



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Step 0 – gDNA preparation

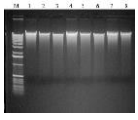
- DNA extraction
- DNA quantification (5 µL gDNA per amplification)
- Concentration adjustment (Final conc. = 20-30 ng/µl)

Step 1 – Master Mix Preparation for Long Rang PCR

Reagent	Volume/Sample	Volume/24 samples
Primer mix	2 ul	51 ul
Long Range PCR Buffer	2.5 ul	63.8 ul
dNTP mix	1.25 ul	31.9 ul
Molecular grad H2O	13.85 ul	353.2 ul
Total Volume	**19.6 ul**	**499.9 ul**

Step 2 – HLA Amplification- Long Range PCR

- Amplification
- Agarose gel electrophoresis



Step 3 – Amplicon Quantitation

- Quantify the amplicons
- Normalize the amplicons to the same level before pooling
- Quantifluor dsDNA system and plate fluorometer
- Per Sample Pooling (5 or 7 or 11 Loci)
- ExoSap Purification

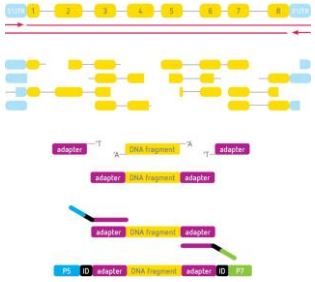
OR

Fluorometer

Real time PCR

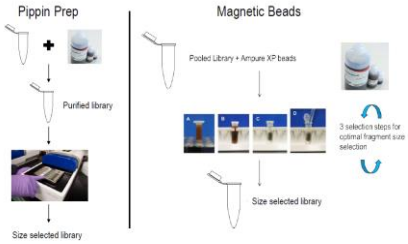
Step 4 – Library preparation

- Fragmentation- ideal size DNA fragment for sequencing on the Illumina Miseq
- End-repair-repaired into blunt ends and are adenylated to facilitate the adapter ligation
- Adaptor-ligation
- Reduced cycle amplification



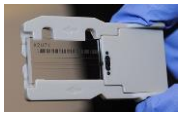
Step 5 – Size Selection

- 650 – 1300 bp collection range

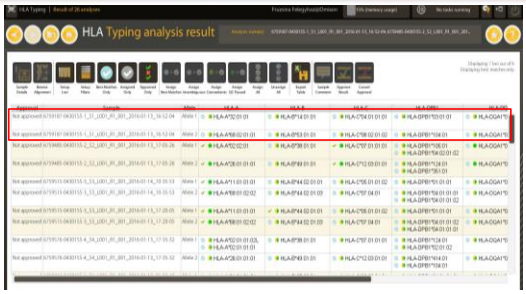


Step 6 – Library Quantification

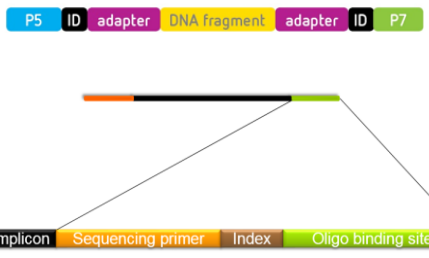
Step 7 – Sequencing on the MiSeq



Step 8 – Analysis of HLA Sequencing Data

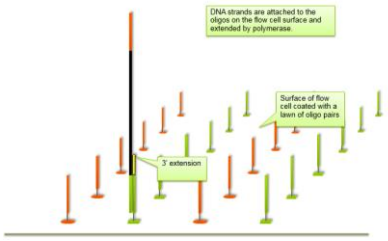


Adapter scheme



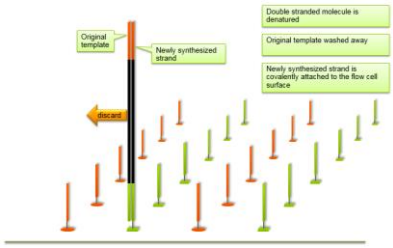
Libor Kolesar

Fragments hybridize and extend



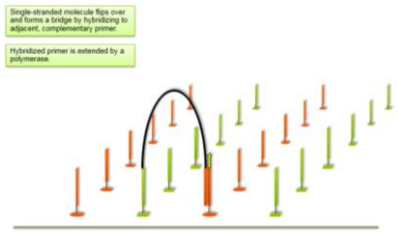
Libor Kolesar

Denature double stranded DNA



Libor Kolesar

Bridge amplification

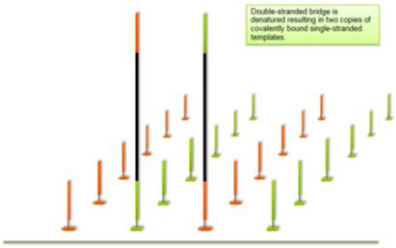


Libor Kolesar

Double-stranded bridge

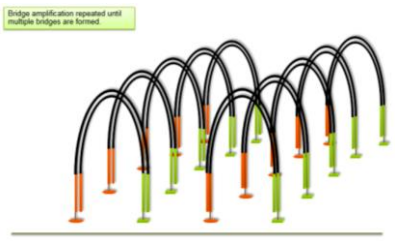
Libor Kolesar

Denature double-stranded bridge



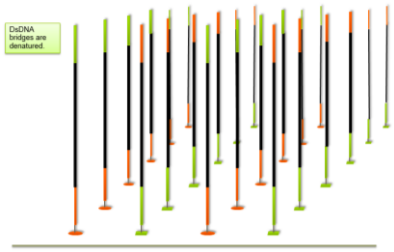
Libor Kolesar

Bridge amplification for cluster generation



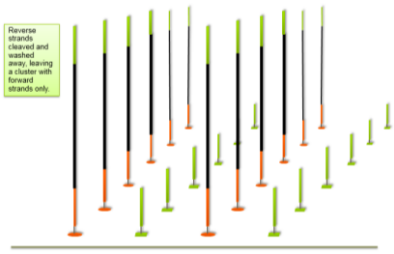
Libor Kolesar

Linearization



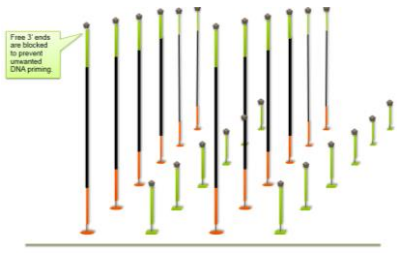
Libor Kolesar

Reverse strand cleavage



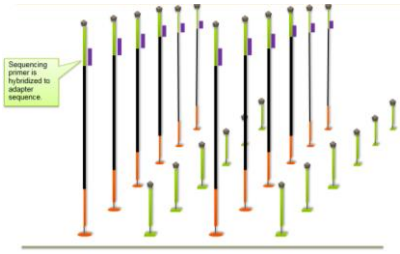
Libor Kolesar

Blocking



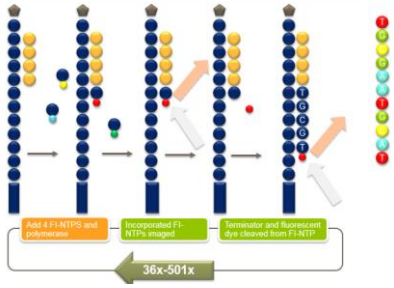
Libor Kolesar

Read 1 primer hybridization



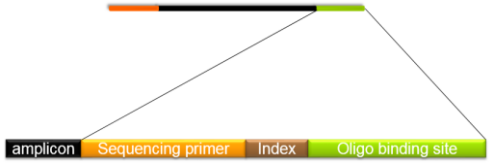
Libor Kolesar

Sequencing by synthesis



Libor Kolesar

Adapter scheme



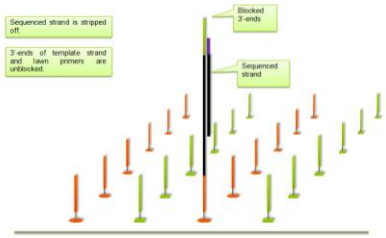
Paired-End Sequencing

Single-End Reads

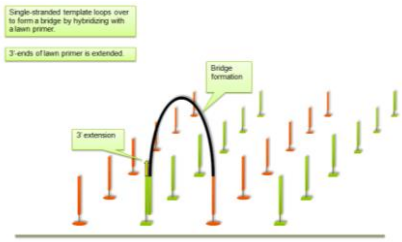
Paired-End Reads

- Paired reads are always phased with one another, because they come from the same template

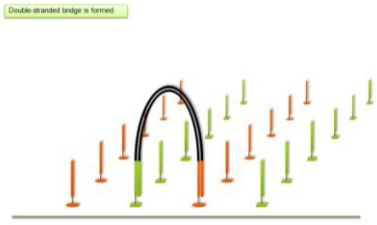
Paired-end sequencing



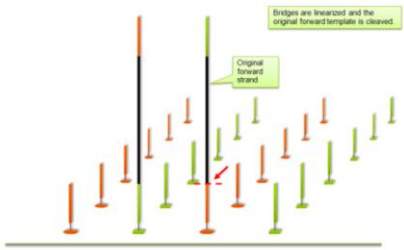
Paired-end sequencing



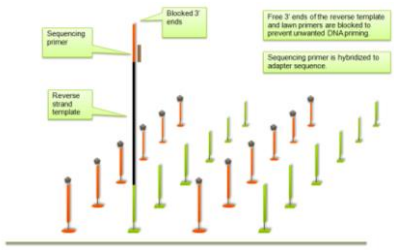
Double-stranded bridge



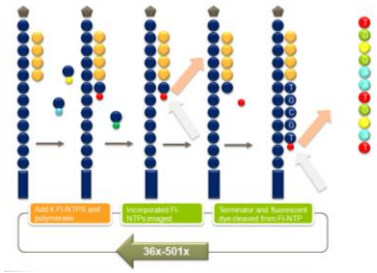
Denature double-stranded bridge



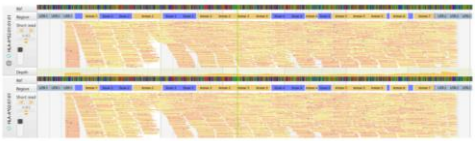
Sequencing of reverse strand



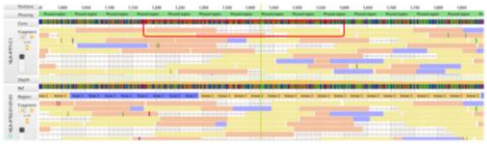
Sequencing by synthesis – Read 2



Piled reads



Paired-end reads



Benefits of NGS for HLA typing

- Clonal template amplification *in vitro* to eliminate problem of sequencing heterozygous DNA (minimize allele drop-out)
- Sufficiently long read length to cover entire exon or more (secure phase)
- Increased sequence coverage of HLA genes (whole gene sequencing)
- Capability to multiplex patient samples (24,48,96, 144, 196, etc.)
- Potential to complete run and data analysis within three days

Advantages of NGS

- **Detecting Null Alleles is Clinically Important**
  - Current methods assume that most alleles are expressed even when only partial gene region are tested
- In bone marrow transplantation, assuming that a donor carries an expressed allele when in fact the allele is not expressed results in a mismatch in the graft-versus host direction
  - **Patient:** A*02:01, 24:02 / ~~24:09N~~
  - **Donor:** A*02:01, ~~24:02~~ / 24:09N
- The NGS whole gene approach can detect null alleles routinely in single pass

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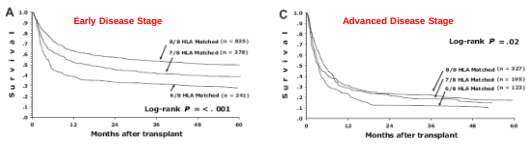
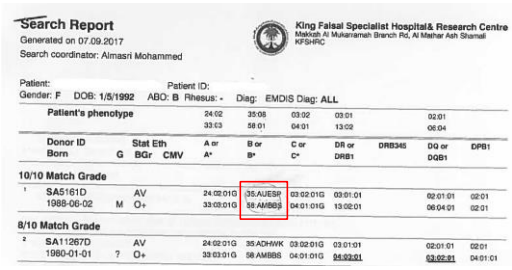
Application of NGS for Optimal Donor Selection (Bone Marrow Registry)

- All loci typed at full or extended coverage
- Immediate identification of eligible donors or indication that no donor is available
- Shortened donor search process
- Early decisions about treatment options and alternative therapies



Lee et al, Blood

Application of NGS for Optimal Donor Selection (Bone Marrow Registry)



Search Report

Generated on 07.09.2017

Search coordinator: Almasri Mohammed

King Faisal Specialist Hospital& Research Centre
Makkah Al Mukarramah Branch Rd, Al Mathar Ash Shamali
KFSHRC

Patient: Patient ID:
Gender: F DOB: 1/5/1992 ABC: B Rhesus: - Diag: EMDIS Diag: ALL

	Donor ID / Born	G	Stat Eth / BGr	CMV	A or A*	B or B*	C or C*	DR or DRB1	DRB345	DQ or DQB1	DPB1
	Patient's phenotype				24:02 33:03	35:08 58:01	03:02 04:01	03:01 13:02		02:01 06:04	
	10/10 Match Grade										
1	SA5161D		AV		24:02:01G	35:AUESR	03:02:01G	03:01:01		02:01:01	02:01
	1988-06-02	M	O+		33:03:01G	58:AMBBS	04:01:01G	13:02:01		06:04:01	02:01
	8/10 Match Grade										
2	SA11267D		AV		24:02:01G	35:ADHWK	03:02:01G	03:01:01		02:01:01	02:01
	1980-01-01	?	O+		33:03:01G	58:AMBBS	04:01:01G	04:02:01		03:02:01	04:01:01

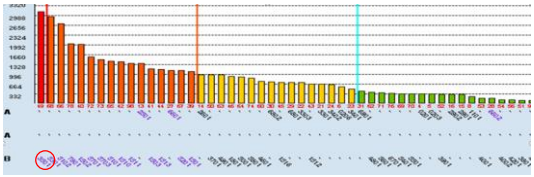
Application of NGS for Optimal Donor Selection (Solid Organ Transplant)

Anti-HLA: B35 (B*35:01), B53,B51, B15, B57

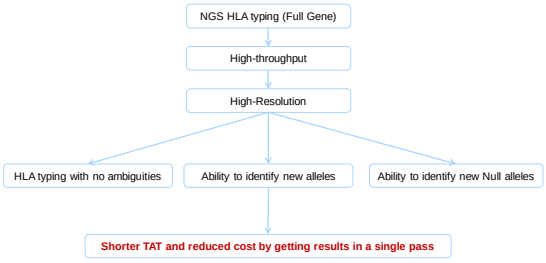
Donor HLA typing:

Low Resolution	A*02	A*03	B*27	B*35	C*05	C*07
High Resolution	A*02:03	A*03:01	B*27:01	B*35:02	C*05:01	C*07:04

DSA: anti-HLA-B35?!



Solution to Current Problems with HLA typing



Thank You!