

La tipizzazione HLA con metodo NGS

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Giornata di formazione AIBT
NGS nel laboratorio di Istocompatibilità
Roma, Ospedale Pediatrico Bambin Gesù, 19.11.2019

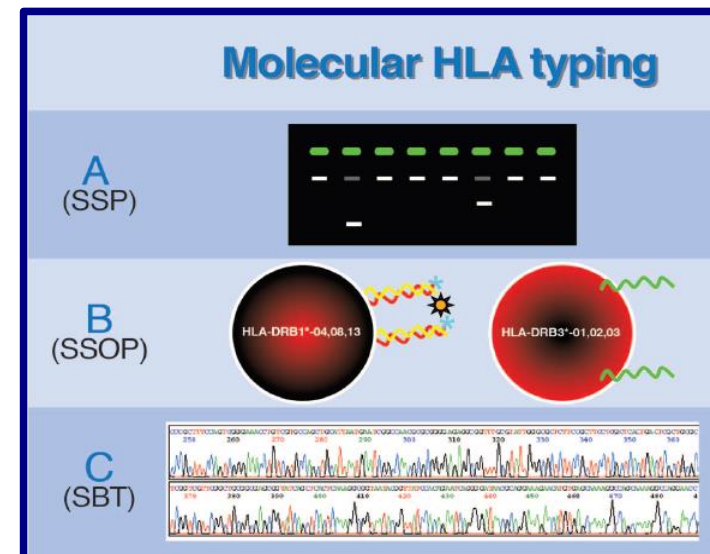


Implementare la tipizzazione HLA mediante NGS....

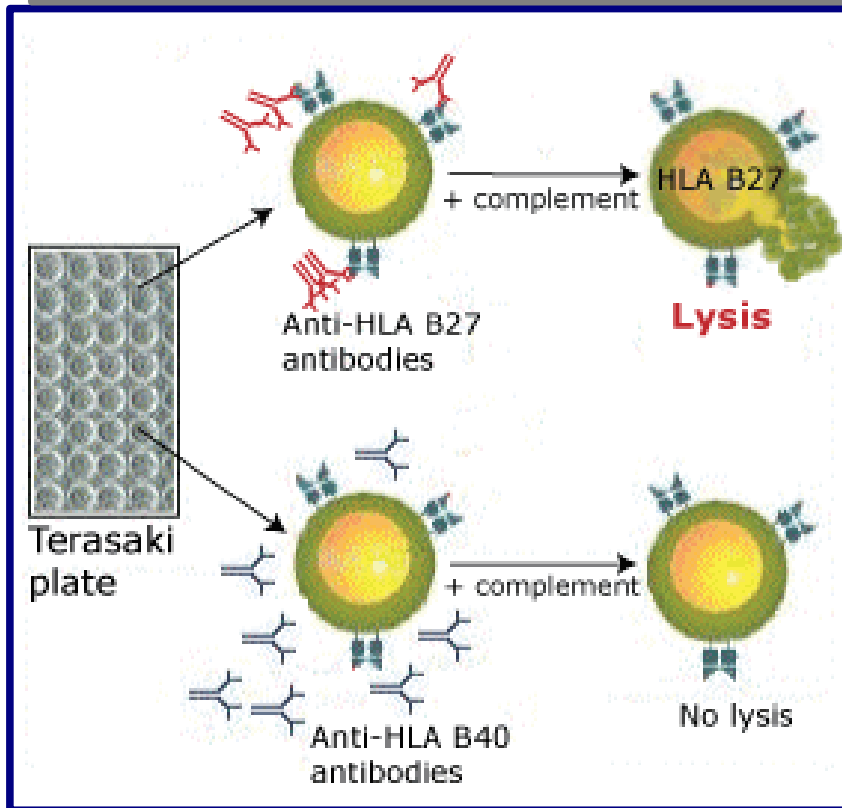
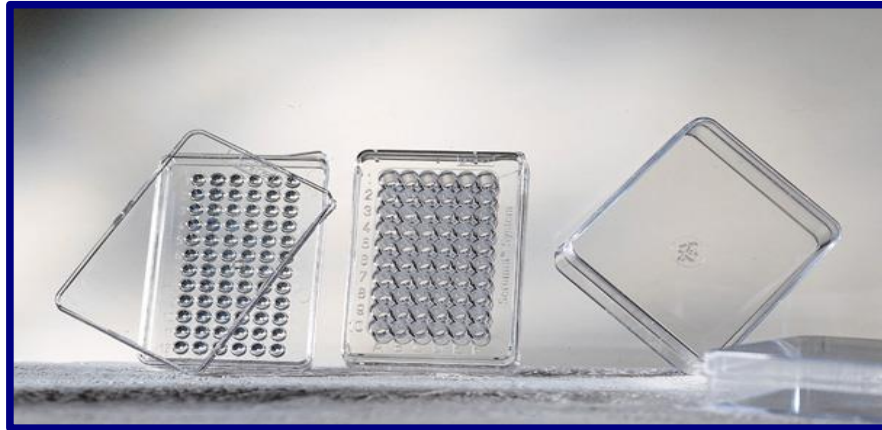
- Perché?
- Per quali campioni?
- In quale laboratorio?

Tipizzazione HLA

- **Metodi:**
 - Sierologia:
 - Test mediante citotossicità complemento dipendente (CDC)
 - Molecolare:
 - PCR-SSO (sequence-specific oligonucleotides)
 - PCR-SSP (sequence-specific primers)
 - Sequenziamento (sequencing-based typing, SBT)
- **Livello di risoluzione:**
 - Bassa: 2 digits, 1 campo
 - Alta: 4 digits, 2 campi
 - Allelica: tutti i campi



Tipizzazione Sierologica

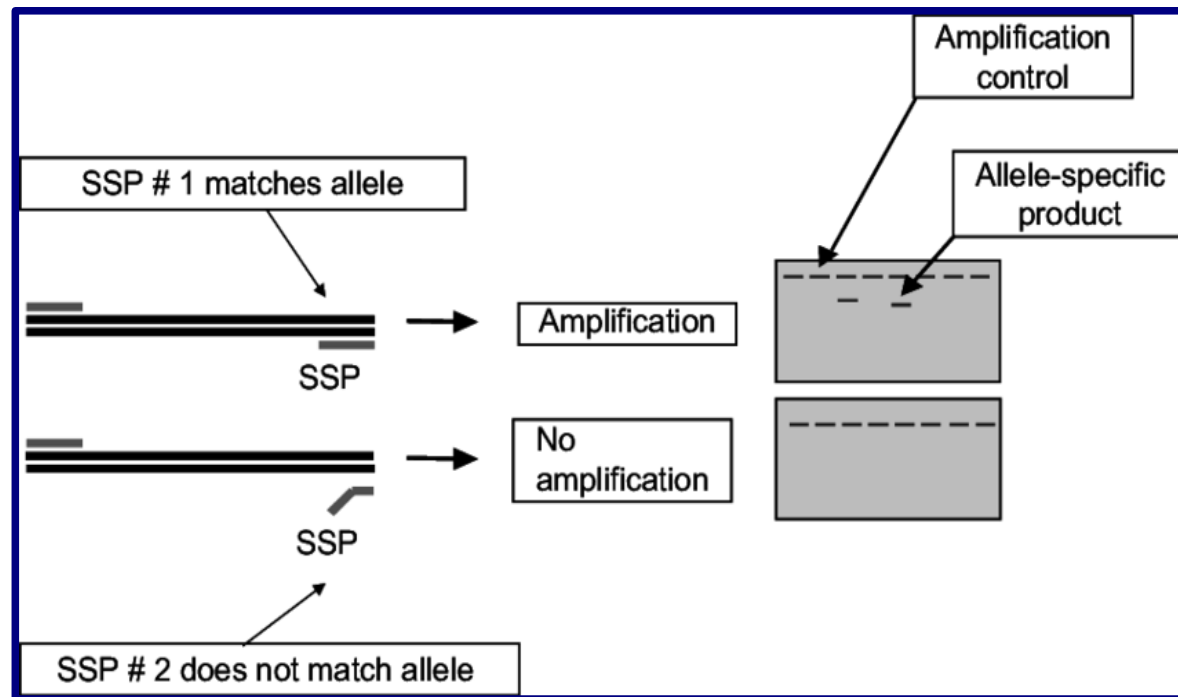


HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	HLA-DP
A1	B5	Cw1	DR1, DR103	DQ1	DPw1
A2, A203, A210	B51(5), B5102, B5103	Cw2	DR2	DQ5(1)	DPw2
A3	B52(5)	Cw3	DR15(2)	DQ6(1)	DPw3
A9	B7, B703	Cw9(w3)	DR16(2)	DQ2	DPw4
A23(9)	B8	Cw10(w3)	DR3	DQ3	DPw5
A24(9), A2403	B12	Cw4	DR17(3)	DQ7(3)	DPw6
A10	B44(12)	Cw5	DR18(3)	DQ8(3)	
A25(10)	B45(12)	Cw6	DR4	DQ9(3)	
A26(10)	B13	Cw7	DR5	DQ4	
A34(10)	B14	Cw8	DR11(5)		
A66(10)	B64(14)		DR12(5)		
A11	B65(14)		DR6		
A19	B15		DR13(6)		
A74(19)	B62(15)		DR14(6), DR1403, DR1404		
	B63(15)		DR7		
	B75(15)		DR8		
A29(19)	B76(15)		DR9		

Antigeni HLA					
A	B	C	DR	DQ	DP
28	60	10	21+3	9	6

Tipizzazione Molecolare

PCR-SSP



1 test per ogni locus

1 test per ogni allele

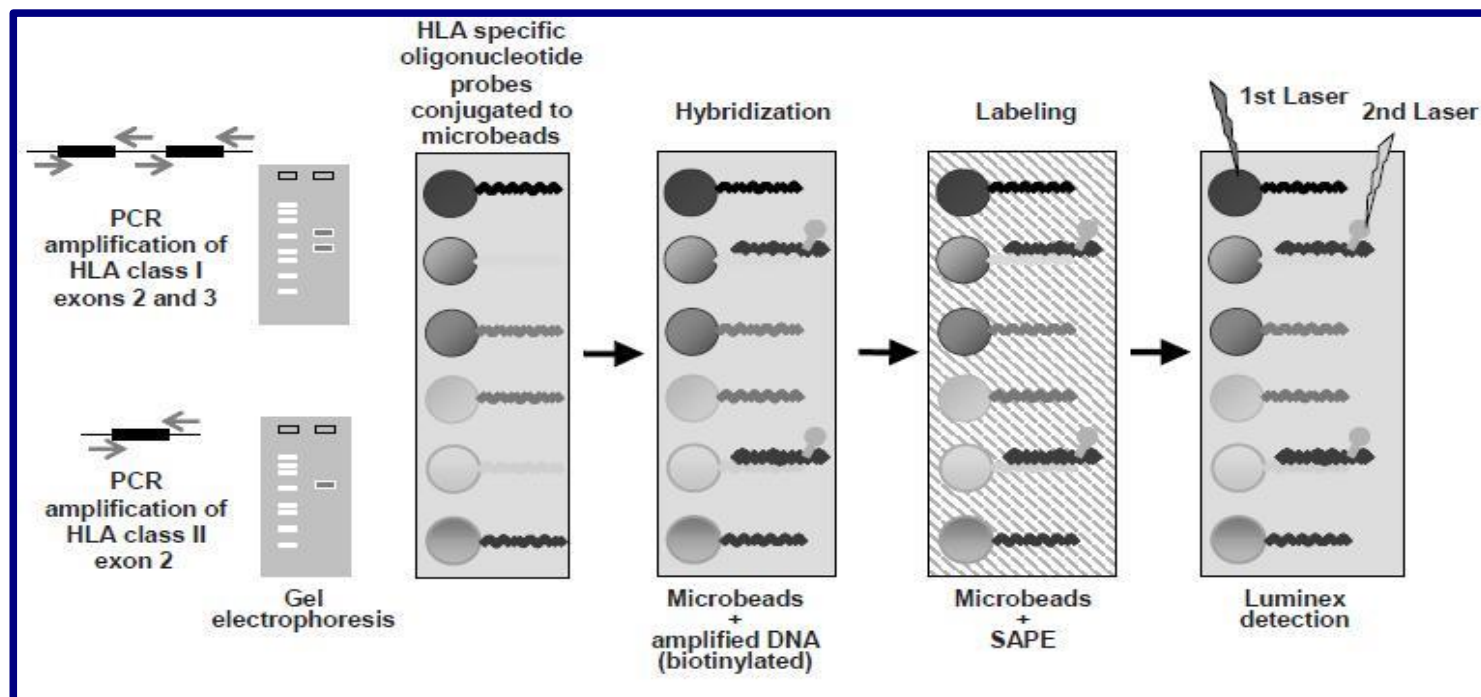
Difficile aggiornamento dei primers

Risoluzione???



Tipizzazione Molecolare

PCR-SSO



Risoluzione?

Oggi presenti sul mercato kit SSO/SSOP per alta risoluzione con amplificazione di + esoni rispetto ai test standard.

Limitata sempre al target generato con l'amplificazione e all'esistenza di probes che legano le posizioni polimorfiche.



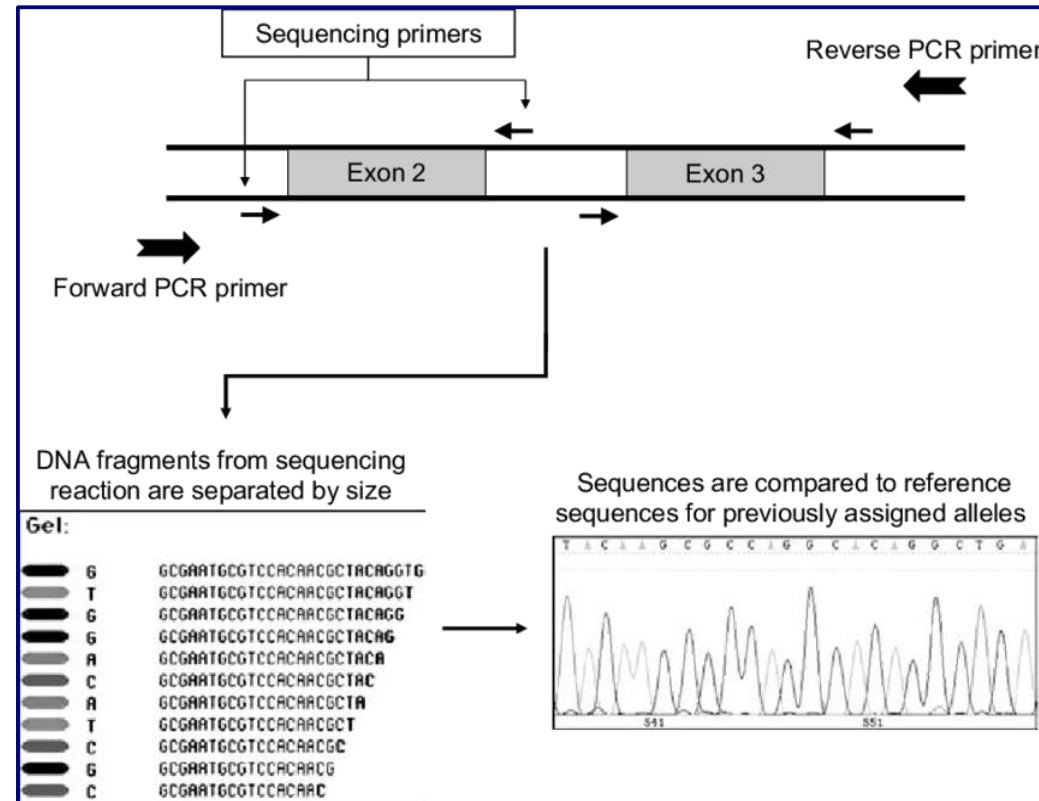
shutterstock.com • 1140473522

Patient	Sample	Exon2	Exon3	Exon4&5	Code Definition	Assigned Allele Pairs
305319	375692	4043	3814	3599	BRTSF:=:02:05/02:791N/02:807N BSCDK:=:03:01/03:289/03:290/03:291/03:292/03:293/03:300/03:301/03:302/03:310/03:312/03:313/03:315/03:325/03:329N/03:331	A*02:05:01:01 A*03:01:01:01
305320	375693	3446	2279	2346	BPVPA:=:02:01/02:570/02:704/02:706/02:707/02:716/02:719/02:720/02:722/02:724/02:726/02:729/02:730/02:733/02:735/02:739/02:740/02:742/02:744/02:745/02:752/02:753/02:755/02:756/02:761/02:762/02:763/02:765/02:769/02:776/02:779/02:786/02:788N/02:789N/02:792N/02:793N/02:794/02:795/02:796N/02:803N/02:806N/02:810/02:812/02:816/02:819/02:823/02:824/02:825 BPVPB:=:24:02/24:353/24:385/24:388N/24:389N/24:391/24:393/24:396N/24:398/24:400/24:401/24:402/24:407/24:412/24:416/24:417/24:418/24:419/24:420/24:422/24:423/24:426N/24:428N/24:429N/24:431/24:432/24:433N	A*02:01:01:01 A*24:02:01:01
305363	375732	3102	2505	2185	1988 XX1:=:02:02/02:26/02:69/02:70/02:86/02:88/02:91/02:100/02:101/02:102/02:103/02:105N/02:112/02:118/02:119/02:122/02:127/02:128/02:134/02:136/02:138/02:139/02:145/02:147/02:149/02:151/02:154/02:155/02:162/02:163/02:165N XX2:=:16:01/16:02/16:25/16:89N/16:94/16:99/16:107/16:108/16:121/16:123N	C*02:02:01 C*16:02:01
305364	375733	3140	2453	2090	2758 BRUPB:=:02:31/51/72/80/81/83/85/94/95/100/102/104 XX1:=:01:02/15/83/94/95/97/105/106/112/118/125/126/127/130/131/134/135/137/138/142/146	C*14:02:01:01 C*16:01:01:01
305365	375734	3723	3041	2510	3118 BRBPE:=:01:02/01:40/01:44/01:85/01:91/01:117N/01:126/01:127/01:130/01:133/01:138/01:139/01:142/01:150/01:151/01:155/01:158/01:159/01:162/01:163/01:164/01:165/01:167/01:171N/01:172/01:174/01:175/01:176 BRBPG:=:07:01/07:78/07:103/07:153/07:166/07:408/07:417/07:418/07:419/07:422/07:424/07:435/07:442/07:443/07:448/07:453/07:458/07:461/07:462/07:463/07:469/07:470/07:471/07:479/07:491/07:493/07:502/07:506/07:508/07:526/07:545/07:547/07:554/07:555/07:561/07:570/07:588/07:591/07:603N/07:610/07:617/07:619/07:623/07:624/07:627/07:633N/07:635/07:654/07:657/07:663Q/07:669/07:682/07:685/07:690N/07:694/07:696/07:699/07:708/07:712/07:713	C*01:02:01:01 C*07:01:01:01

KEEP
CALM
AND
EMBRACE
AMBIGUITY

Tipizzazione Molecolare

SBT - Sequenziamento



Risoluzione?

Target generato con l'amplificazione

Possibilità di risolvere ambiguità CIS/TRANS

Possibilità di separare i due alleli



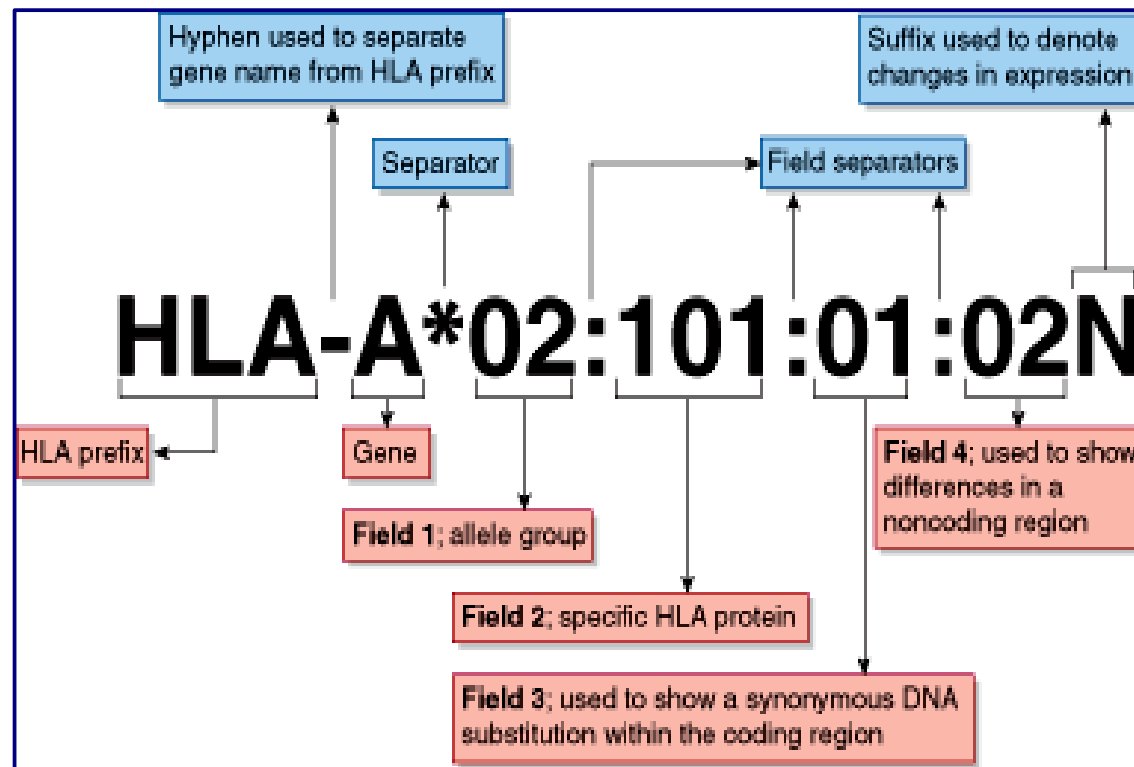
Alta risoluzione

Arrivare ad una tipizzazione a 2 campi senza
AMBIGUITA'

in zone codificanti per la peptide binding groove

.....

escludendo tutti i possibili alleli Null

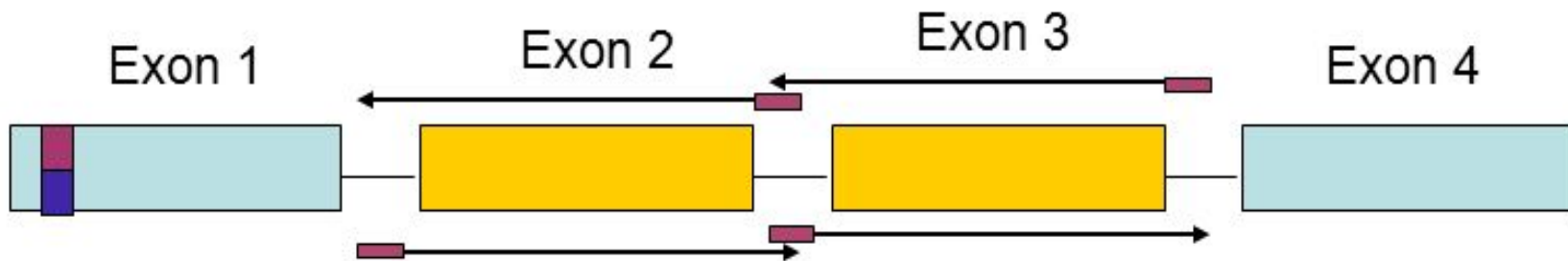


Alta risoluzione

Ambiguità?

- Polimorfismi che si trovano al di fuori delle regioni amplificate e studiate
- Sequenze depositate in modo incompleto
- Ambiguità cis-trans

Ambiguità



Polymorphic positions



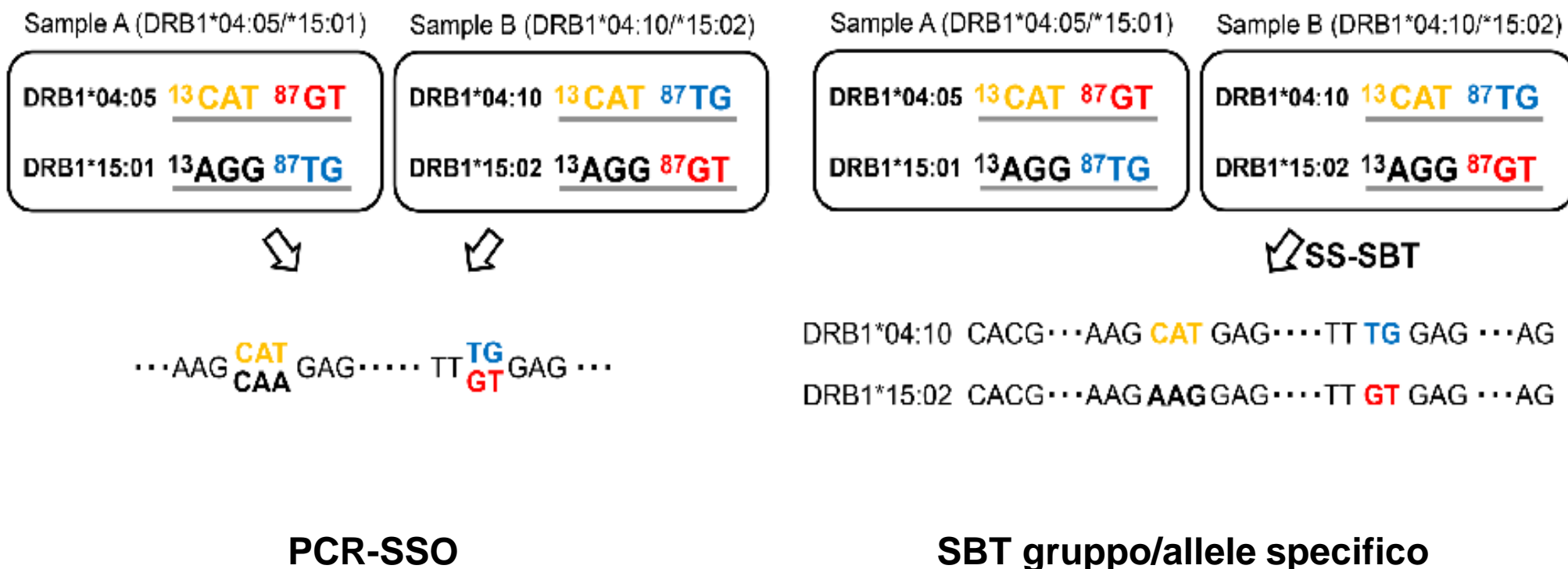
Core heterozygous sequence data

example: HLA-B

B*0702, 4402

B*0702, 4419N

Ambiguità

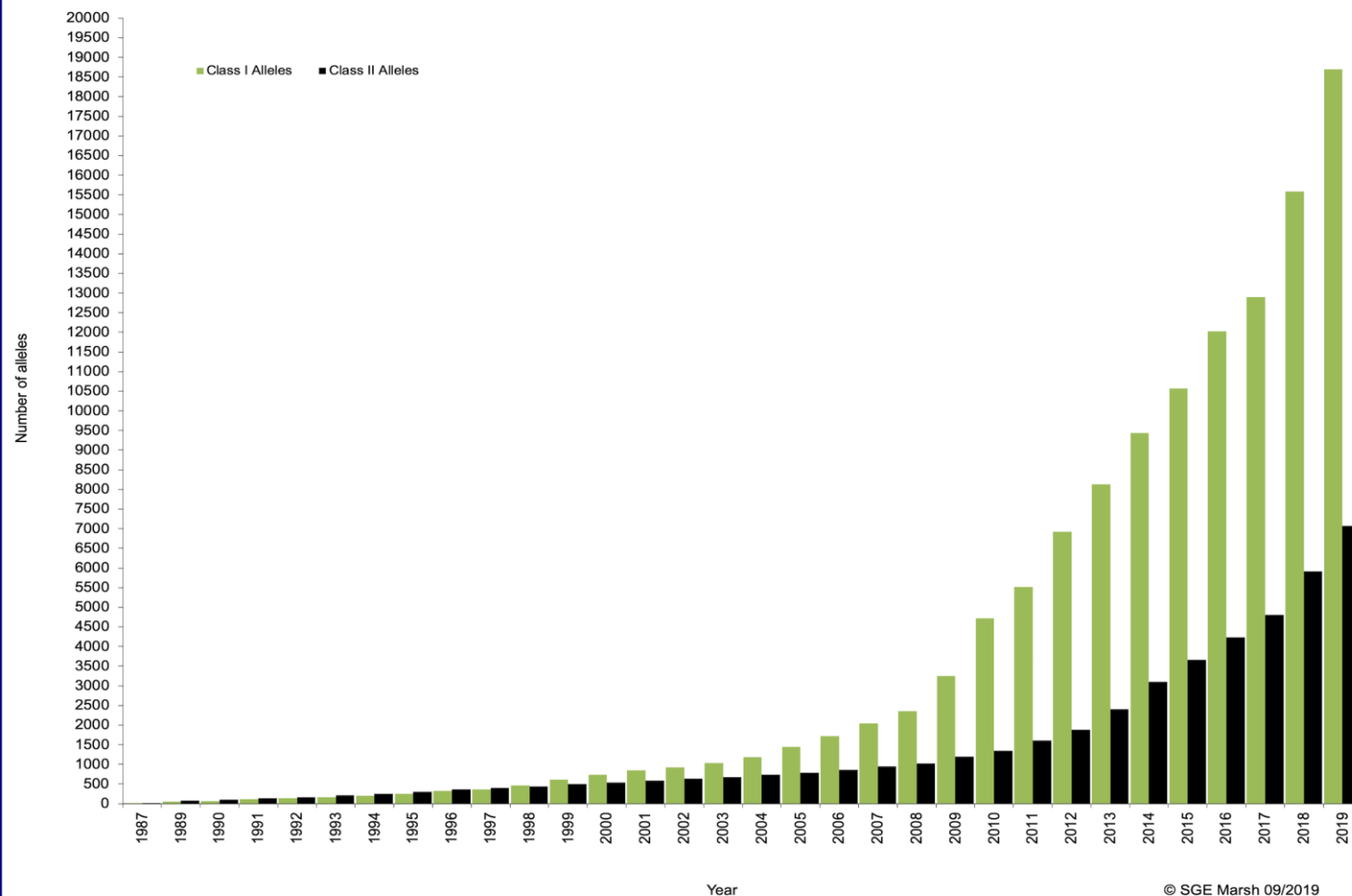


DataBase IMGT-HLA

1.0	1998-12	964	1,853
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2.11	2005-10	2,280	5,510
2.12	2006-01	2,337	5,585
2.13	2006-04	2,429	5,598
2.14	2006-07	2,533	5,753
2.15	2006-10	2,607	5,849
2.16	2007-01	2,683	5,957
2.17	2007-04	2,816	6,139
2.18	2007-07	2,901	6,246
2.19	2007-10	3,043	6,430
2.20	2008-01	3,095	6,506

3.33	2018-07	18,955	36,161
3.34	2018-10	20,272	38,209
3.35	2019-01	21,683	40,451
3.36	2019-04	22,528	41,623
3.37	2019-07	24,093	45,307
3.38	2019-10	25,958	48,804



HLA Class I				HLA Class II					
Gene	A	B	C	Gene	DRB	DQA1	DQB1	DPA1	DPB1
Alleles	5,735	7,053	5,653	Alleles	3,296	216	1,771	161	1,519
Proteins	3,629	4,572	3,447	Proteins	3,158	90	1,179	62	993
Nulls	300	241	240	Nulls	138	6	77	2	78

Come l'NGS ci può aiutare?



Template Preparation

Genomic DNA or cDNA



Library preparation



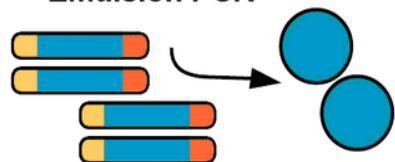
Fragmentation of DNA



Adapter ligation

Library amplification

Emulsion PCR



DNA is amplified onto microbeads

Cluster generation



DNA is bridge amplified onto flow cell

Sequencing and imaging

Ion Torrent PGM

ATAGTACTTACTA
TAT↑
C

pH change

MiSeq

ATAGTACTTACTA
TAT↑
C

Fluorescence

Data Analysis

Amplification

Library Preparation

HLA Class I & II

Amplicon Purification

Amplicon Quantitation

Amplicon Normalization & Fragmentation

Barcode Ligation

Size Selection

Secondary Amplification

Purification

Quantitation

Equimolar Pooling

Template Preparation & Sequencing

Data Analysis

Sample Sheet Preparation

Review/Report

Sequencing Preparation, Denature and Dilute Library Pool

Cluster Generation

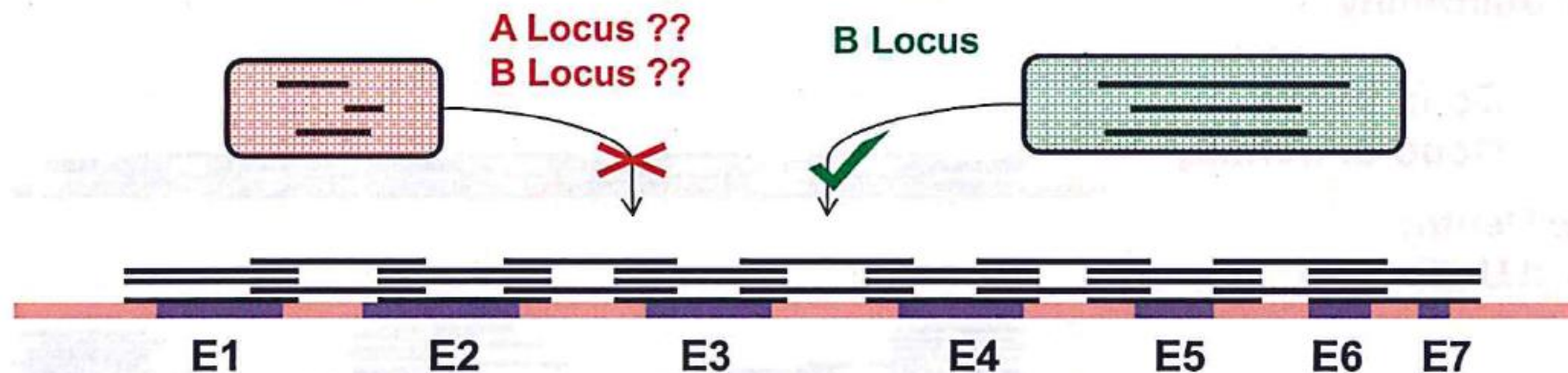
Sequencing

Clean MiSeq Instrument

Read Length

Each run typically produces millions of individual reads. Each read is of a differing **read length**. Longer reads lead to (1) higher specificity, (2) higher read depth, and (3) better phasing ability.

1. Higher Specificity:



Read Depth & Uniformity

Due to the massively parallel nature of NGS, multiple reads (different library fragments) will have overlapping sequence. **Read Depth** at a position is defined by the number of reads covering that position. Higher read depth = greater accuracy. Read **Uniformity**, describes how uniform the read depth is across the targeted region.



NGS - Algoritmi informatici

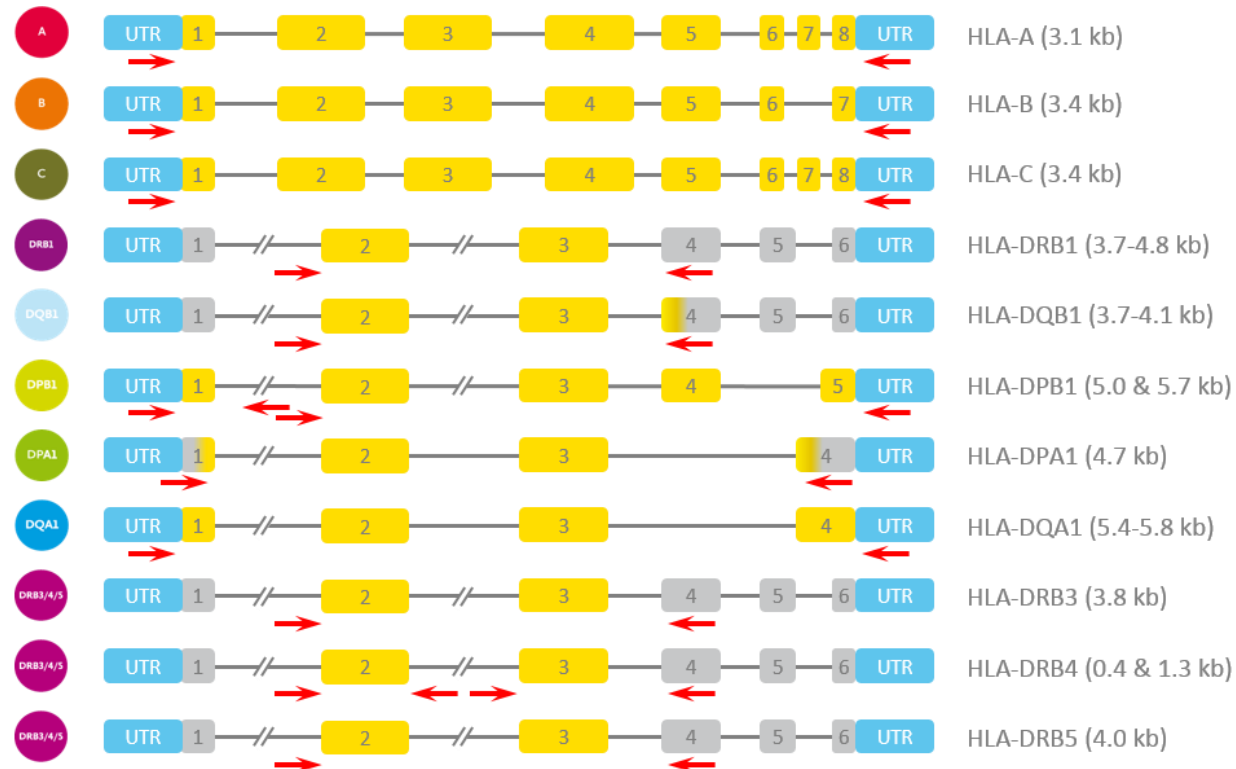
L'analisi dei frammenti ottenuti viene eseguita da software che utilizzano uno o più algoritmi informatici.

Due grandi categorie:

- Compongono una sequenza lineare a partire dai diversi frammenti (Contig) e la appaiano alla sequenza più simile trovata nel database IMGT-HLA
- Allineano i diversi frammenti al database IMGT-HLA fino a trovare l'allele a cui appartengono

Frequenze??

Target amplificato NGS



→ NGSgo-AmpX HLA
amplification primer

Amplified exon

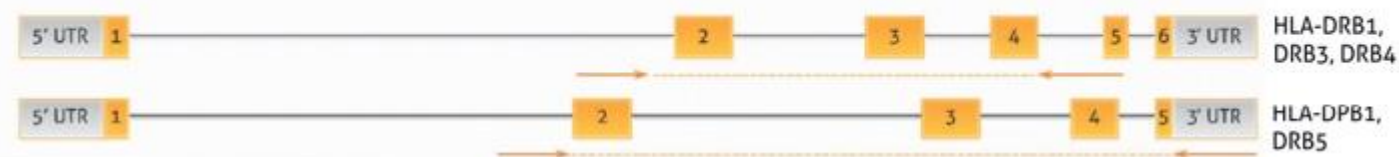
Exon not amplified

Target amplification NGS

Whole Gene Characterization



Key Region Characterization



Target amplificato NGS

A Locus



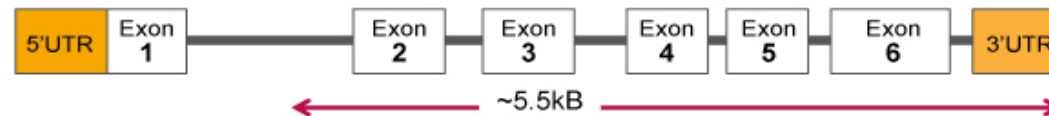
B Locus



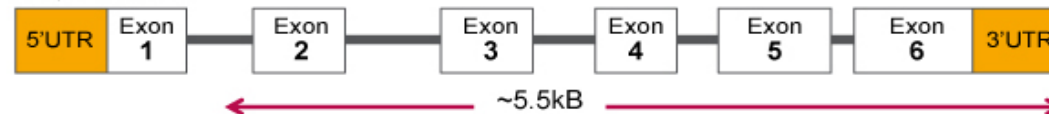
C Locus



DRB1/3/4/5 Loci



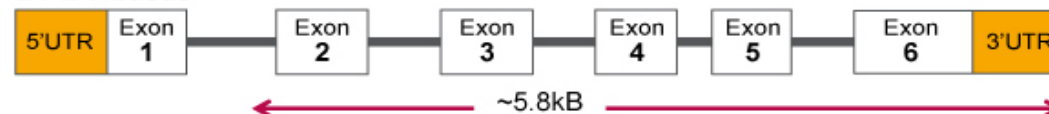
DQB1 Locus



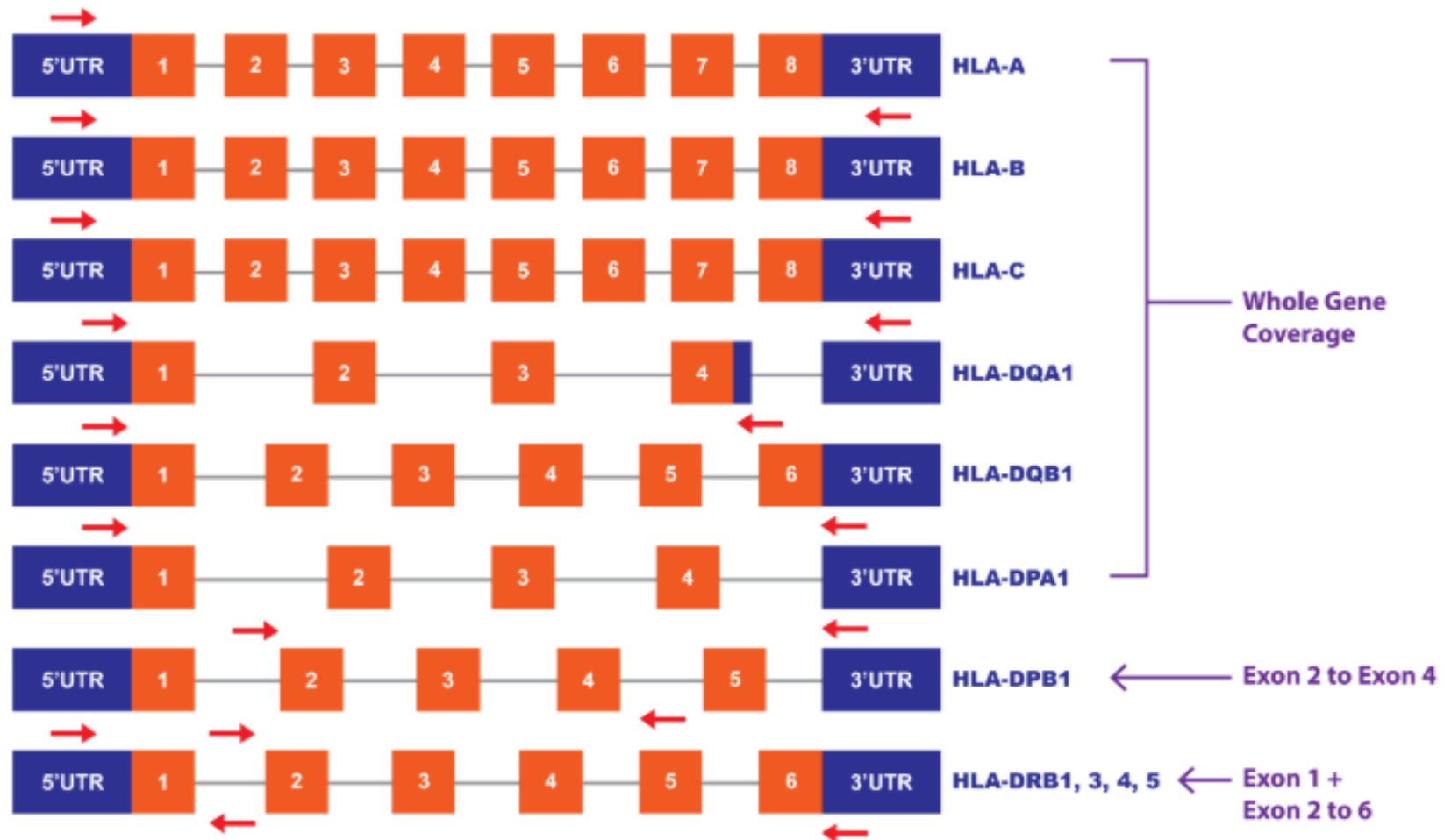
DQA1 Locus



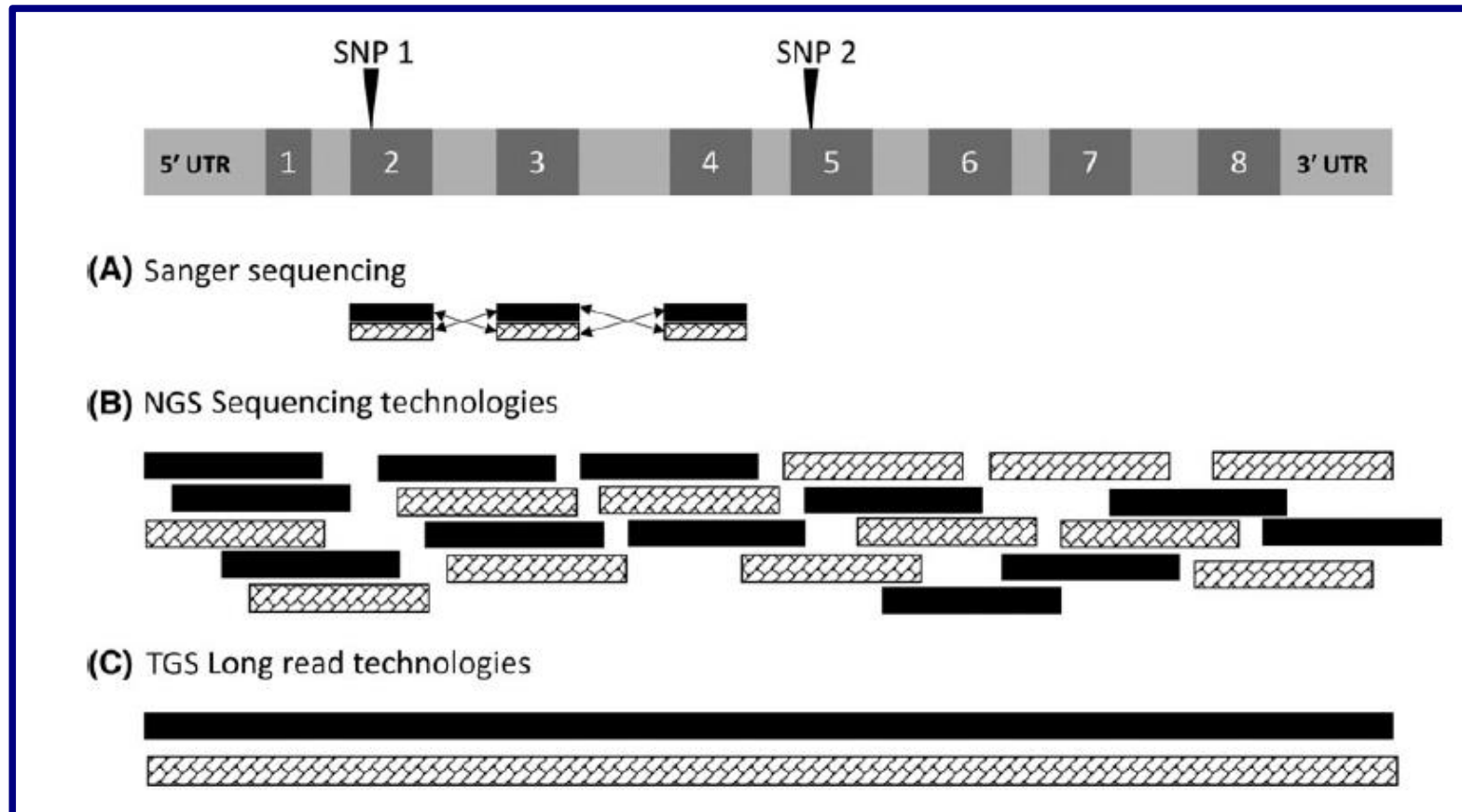
DPB1 Locus



Target amplificato NGS



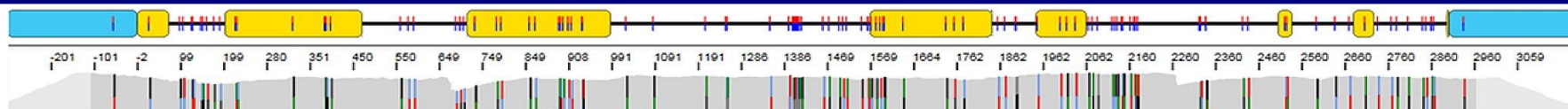
Risoluzione di ambiguità



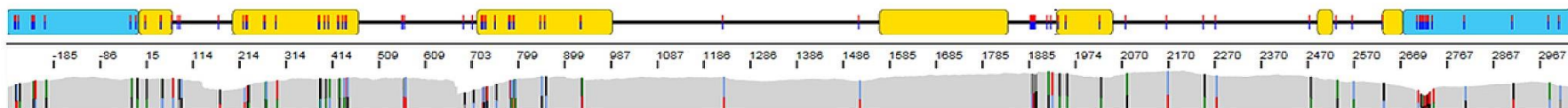
Sequenziamento di polimorfismi non visibili con altre metodiche

Possibilità di eliminare ambiguità CIS-TRANS

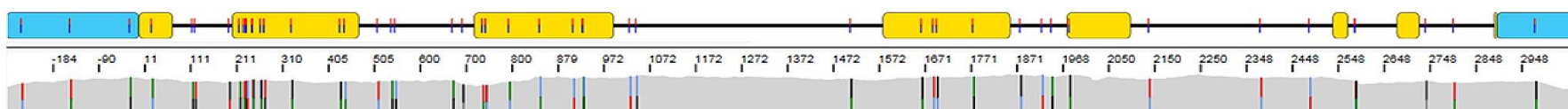
HLA-A



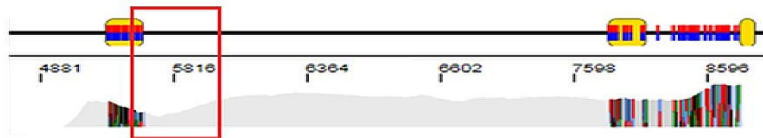
HLA-B



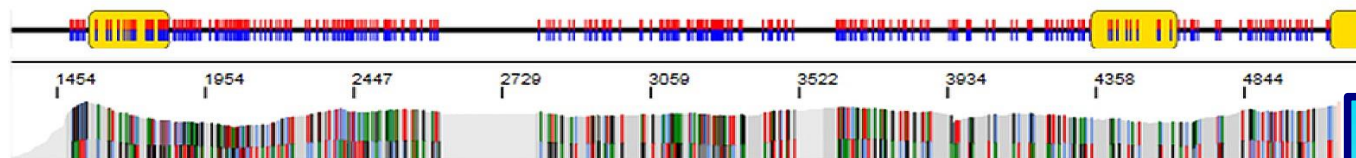
HLA-C



HLA-DRB1



HLA-DQB1



NGS...tutto semplice?

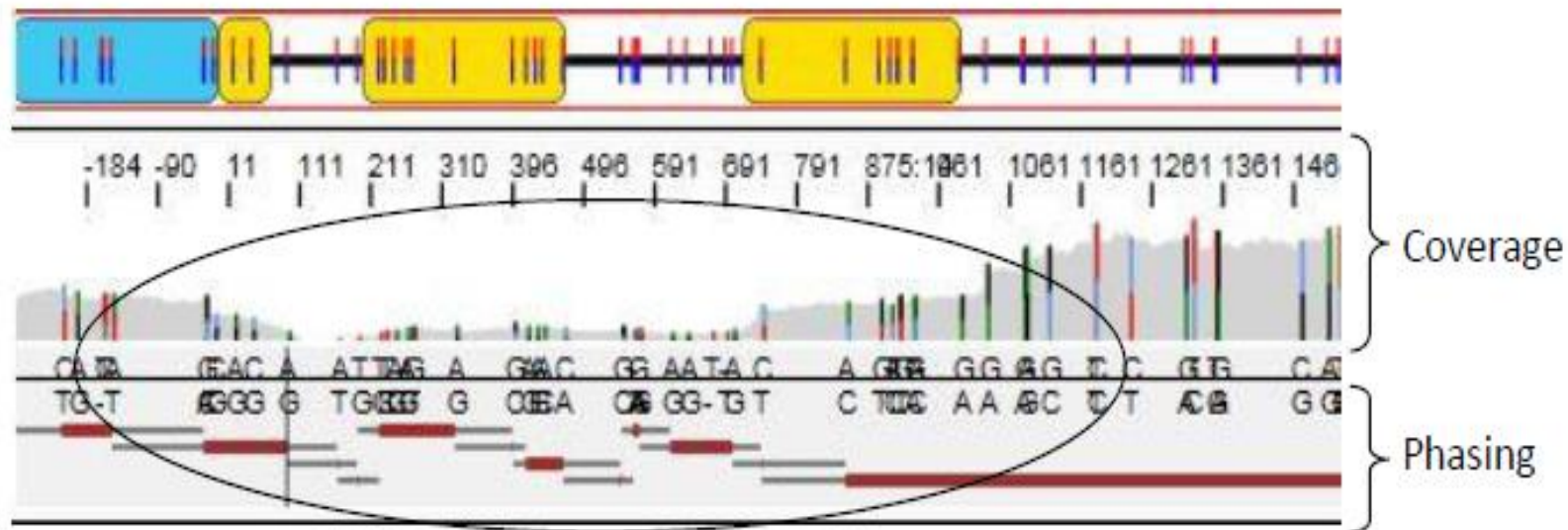
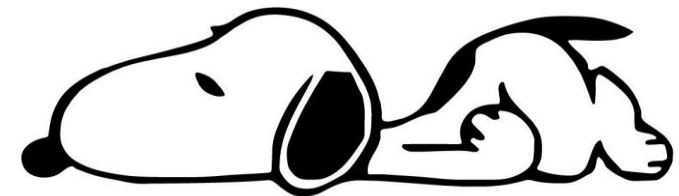


Figure 5.6 An example of uneven coverage and the resulting poor phasing across the gene. Uneven coverage observed in a sample from sequencing run #RPT2. Phased regions are indicated by the solid red line, with grey lines in between representing regions with incomplete phasing, particularly noticeable in regions of low coverage.



**Interruzione di fase
Low Coverage**



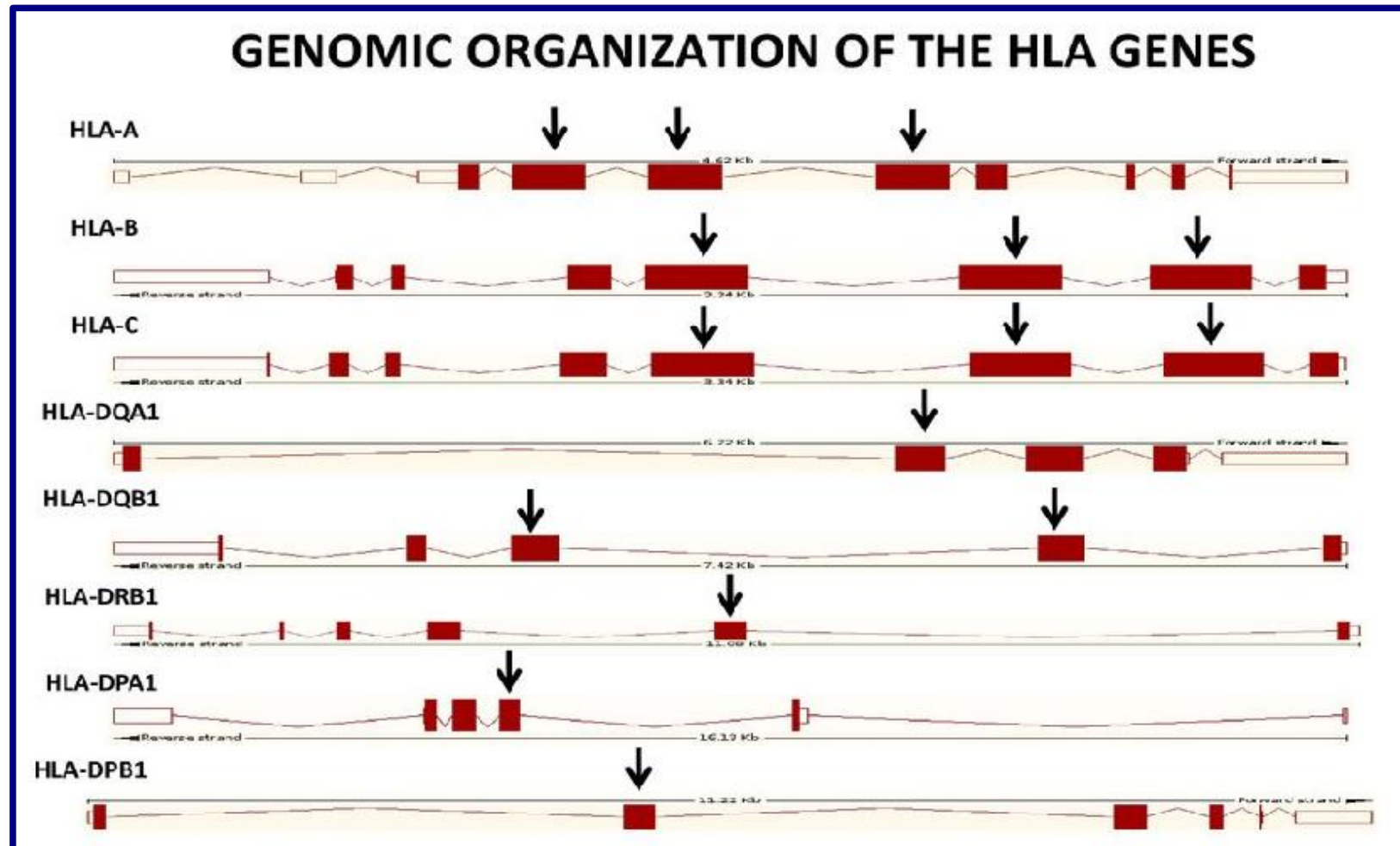
NGS...tutto semplice?

Si ottiene sempre una tipizzazione a 4 campi?

NO!

- Nella classe II ci sono zone difficili da sequenziare
- Le sequenze introniche sono spesso di difficile analisi
- Se ottengo più di una fase, posso avere ancora ambiguità CIS-TRANS
- Database IMGT-HLA incompleto per alleli depositati parzialmente
- L'elevato polimorfismo HLA con alleli diversi anche solo per una base mette a dura prova gli algoritmi di analisi
- Co-amplificazione di molecole non-HLA con alta omologia di sequenza può generare alto background

NGS – Classe II...difficoltà?



Classe II

- ✓ Introni molto lunghi
- ✓ Zone ricche in GC
- ✓ Zone molto ripetute

Alcuni alleli come il DRB1*04 ed il DQA1 richiedono particolare attenzione!
Ambiguità DPB1 difficili da risolvere

Sequenze incomplete in IMGT-HLA

SVERCHKOVA ET AL.

HLA
Immune Response Genetics

WILEY 3

FIGURE 1 Bar plot representing the number of available exons and introns per locus in the IPD-IMGT/HLA database (release 3.36.0; 17 April 2019). Only a small portion of all alleles is fully sequenced (darkest green)

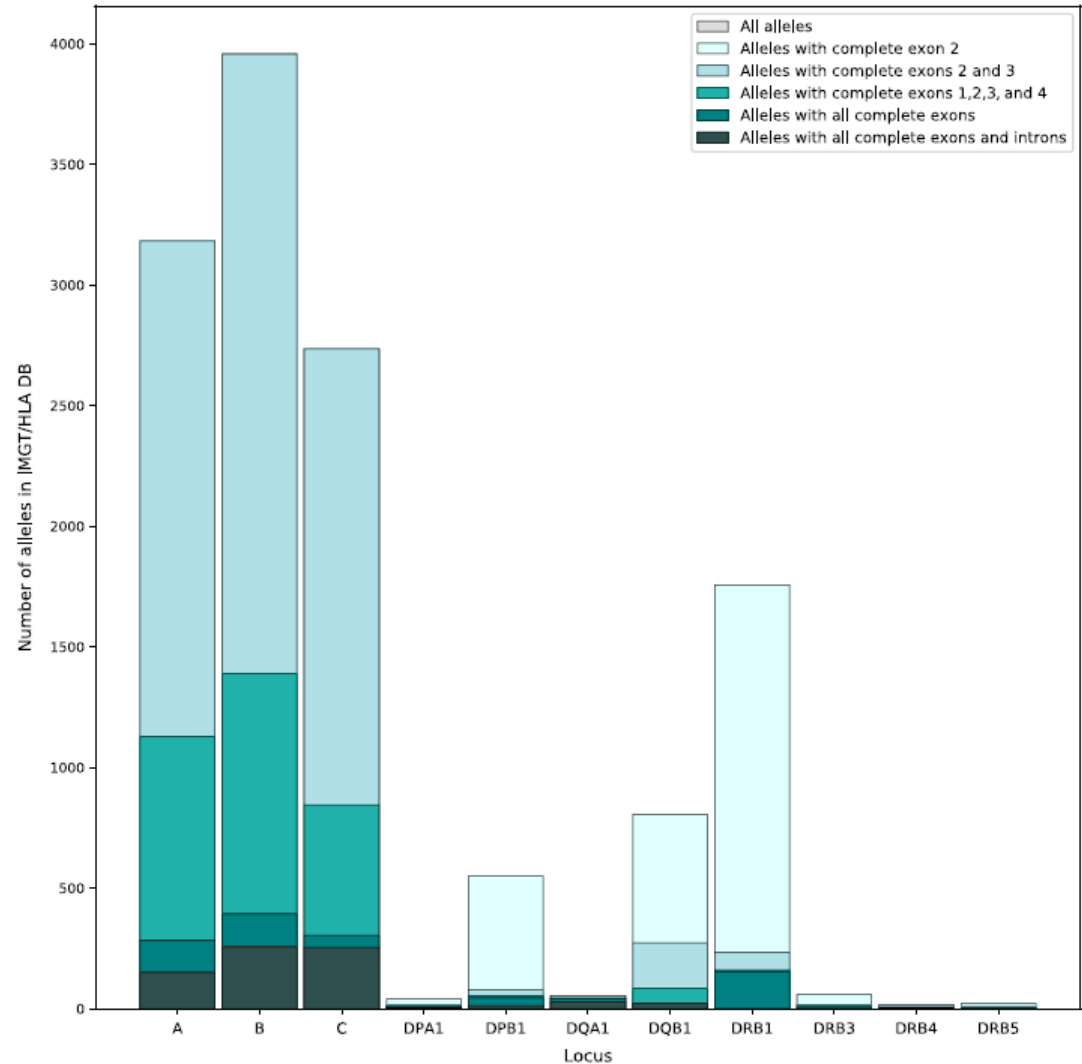


Figure 1.3 Cartoon showing a typical HLA class I gene and the structures each exon encodes. Exons 2 and 3 encode the alpha 1 and 2 domains, which form the peptide binding groove. The alpha 3 domain is encoded by exon 4, with the transmembrane region and cytoplasmic tail encoded by exons 5 and 6/7, respectively.

Di molti alleli conosciamo solo gli esoni che codificano per la Peptide Binding groove (2-3 per classe I e 2 per classe II).

Per Quali campioni?

	LOCI TIPIZZATI	RISOL.	METODICA	N. / ANNO
PAZIENTI IN LISTA	A,B,DRB1,DQB1	Low Res	Luminex	985
PAZ e DON PER TX DA VIVENTE	A,B,DRB1,DQB1	Low Res	Luminex	380
DONATORI DI ORGANI	A,B,C,DRB1,DRB,DQ,DP	Low Res	Real Time	740
CORD BLOOD	A,B,C,DRB1,DRB,DQ,DP	High Res	Luminex/SBT	230
HLA E MALATTIE	A seconda della patologia	Low Res	Luminex	400
PAZIENTI EMATOLOGICI	A,B,C,DRB1,DQB1	High Res	SBT	220
DONATORI MUD e FAMILIARI	A,B,C,DRB1,DQB1	High Res	SBT	385
DONATORI DA REGISTRO	A,B,C,DRB1	High Res	Luminex	3800

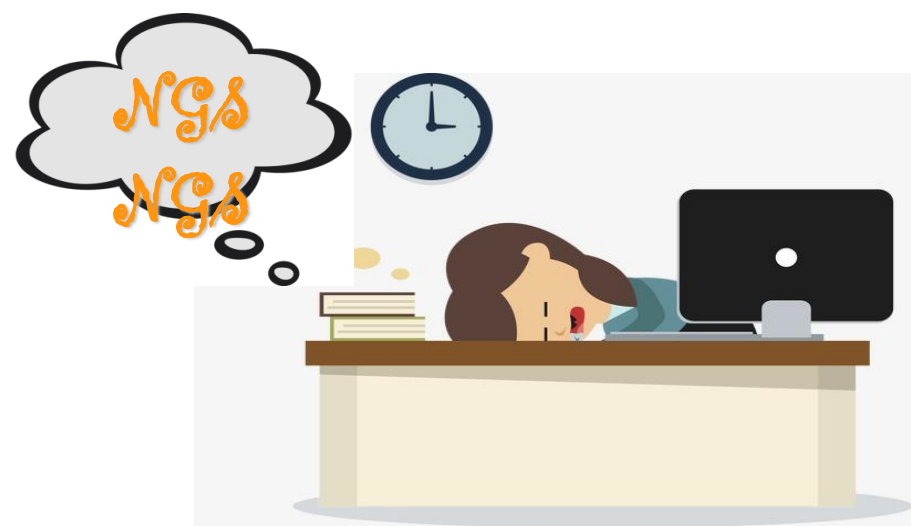
	LOCI TIPIZZATI	RISOL.	METODICA	N. / ANNO
PAZIENTI IN LISTA	A,B,DRB1,DQB1	Low Res	Luminex	985
PAZ e DON PER TX DA VIVENTE	A,B,DRB1,DQB1	Low Res	Luminex	380
DONATORI DI ORGANI	A,B,C,DRB1,DRB,DQ,DP	Low Res	Real Time	740
CORD BLOOD	A,B,C,DRB1,DRB,DQ,DP	High Res	Luminex/SBT	230

DONATORI DA REGISTRO	A,B,C,DRB1	High Res	Luminex	3600
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DONATORI DA	A,B,C,DRB1	High Res	Luminex	3600
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PAZIENTI EMATOLOGICI	A,B,C,DRB1,DQB1	High Res	SBT	220
DONATORI MUD e FAMIGLIARI	A,B,C,DRB1,DQB1	High Res	SBT	385

CORD BLOOD	A,B,C,DRB1,DRB,DQ,DP	High Res	Luminex/SBT	230
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NGS e trapianto di CSE

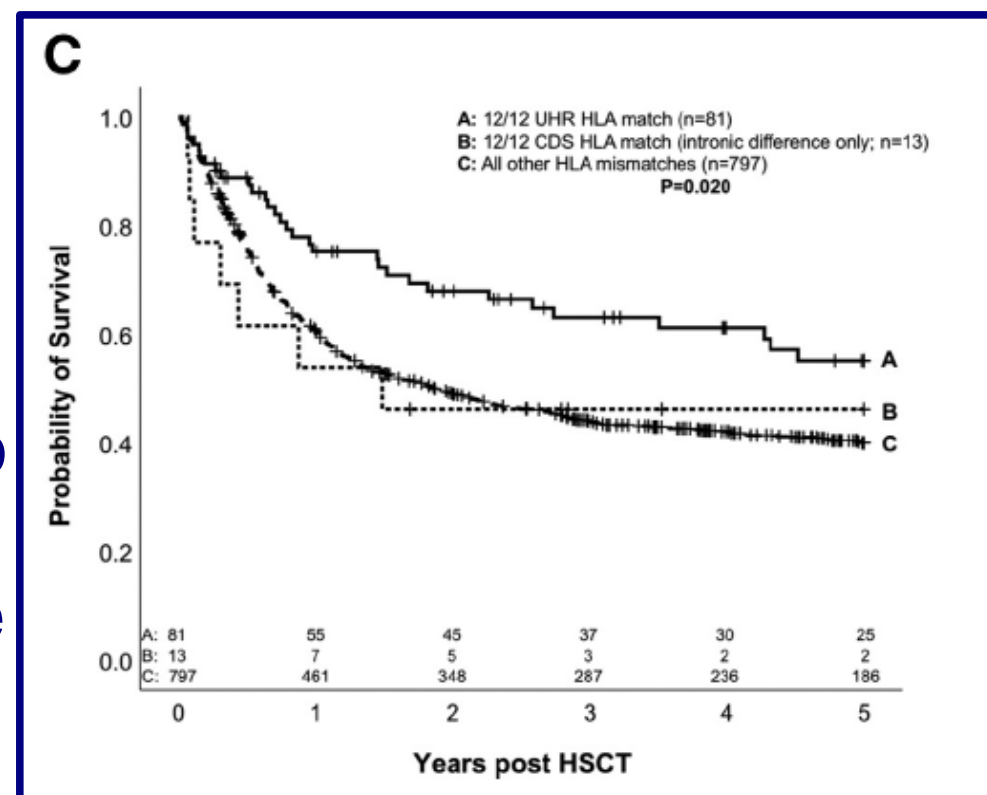
Recipients Receiving Better HLA-Matched Hematopoietic Cell Transplantation Grafts, Uncovered by a Novel HLA Typing Method, Have Superior Survival: A Retrospective Study



Neema P. Mayor^{1,2}, James D. Hayhurst¹, Thomas R. Turner^{1,2}, Richard M. Szydlo^{1,3}, Bronwen E. Shaw⁴, Will P. Bultitude^{1,2}, Jex-Ray Sayno¹, Franco Tavarozzi¹, Katy Latham¹, Chloe Anthias^{1,5}, James Robinson^{1,2}, Henny Braund¹, Robert Danby^{1,6}, Julia Perry⁷, Marie C. Wilson⁷, Adrian J. Bloor⁸, I. Grant McQuaker⁹, Stephen MacKinnon¹⁰, David I. Marks¹¹, Antonio Pagliuca¹², Michael N. Potter⁵, Victoria T. Potter¹², Nigel H. Russell¹³, Kirsty J. Thomson¹⁰, J. Alejandro Madrigal^{1,2}, Steven G.E. Marsh^{1,2,*}

N.P. Mayor et al. / Biol Blood Marrow Transplant 25 (2019) 443–450

Il match 12/12 considerando anche le sequenze introniche sembra favorire la sopravvivenza



NGS e trapianto di organi?

AJBT

Received: 1 November 2018

Revised: 18 December 2018

Accepted: 3 January 2019

DOI: 10.1111/ajt.15258

ORIGINAL ARTICLE

AJT

Assessing the utilization of high-resolution 2-field HLA typing in solid organ transplantation

Yanping Huang¹ | Anh Dinh¹ | Steven Heron¹ | Allison Gasiewski¹ | Carolina Kneib¹ | Hilary Mehler¹ | Michael T. Mignogno¹ | Ryan Morlen¹ | Larissa Slavich¹ | Ethan Kentzel¹ | Edward C. Frackelton¹ | Jamie L. Duke¹ | Deborah Ferriola¹ | Timothy Mosbruger¹ | Olga A. Timofeeva³ | Steven S. Geier³ | Dimitri Monos^{1,2}

Tipizzazione di 11 loci HLA in NGS aiuta a definire meglio il profilo anticorpale del paziente?

Received: 5 February 2019

Revised: 9 May 2019

Accepted: 18 June 2019


DOI: 10.1111/tan.13619

ORIGINAL ARTICLE

HLA
Immune Response Genetics

WILEY

Comparison of sequence-specific oligonucleotide probe vs next generation sequencing for HLA-A, B, C, DRB1, DRB3/B4/B5, DQA1, DQB1, DPA1, and DPB1 typing: Toward single-pass high-resolution HLA typing in support of solid organ and hematopoietic cell transplant programs

Anajane G. Smith¹ | Shalini Pereira² | Andrés Jaramillo³  | Scott T. Stoll³ | Faisal M. Khan⁴ | Nouredine Berka⁴ | Ahmed A. Mostafa⁴ | Marcelo J. Pando⁵ | Crystal Y. Usenko⁵ | Maria P. Bettinotti⁶ | Chul-Woo Pyo¹ | Wyatt C. Nelson¹ | Amanda Willis⁷ | Medhat Askar⁷ | Daniel E. Geraghty¹

Per quale laboratorio?

- Per il laboratorio che tipizza per il trapianto di CSE ma anche per quello che tipizza per il trapianto di organi
- Per il laboratorio che ha grandi numeri, ma anche per quello un po' più piccolo
- Per il laboratorio che ha un sequenziatore, ma anche per quello che può condividere uno strumento con un altro laboratorio
- Per chi ha un'esperienza pregressa con SBT, ma anche no...
- Per il laboratorio che vuole investire nella tipizzazione ad alta risoluzione

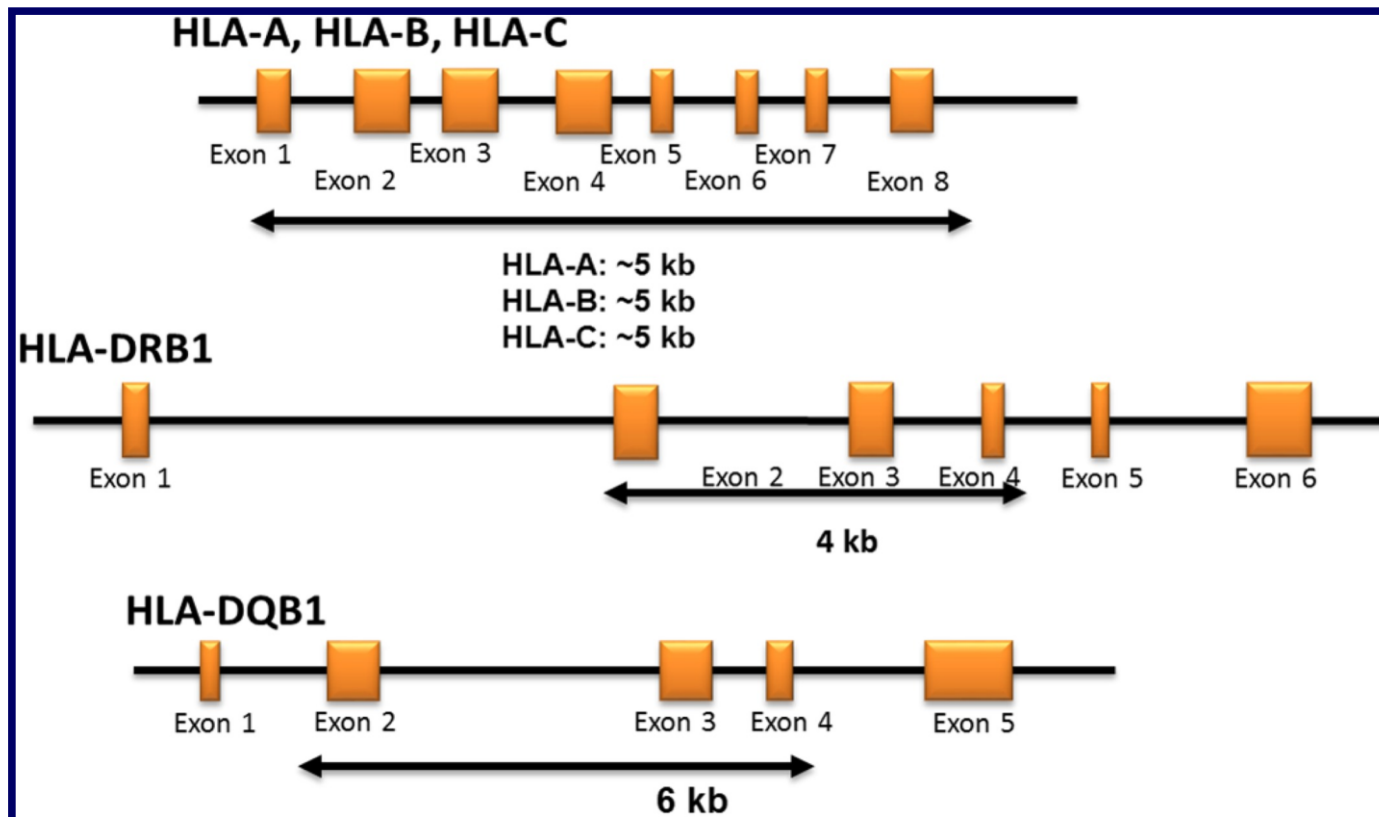
quindi....

...





NGS – Classe II problematica?



Classe II

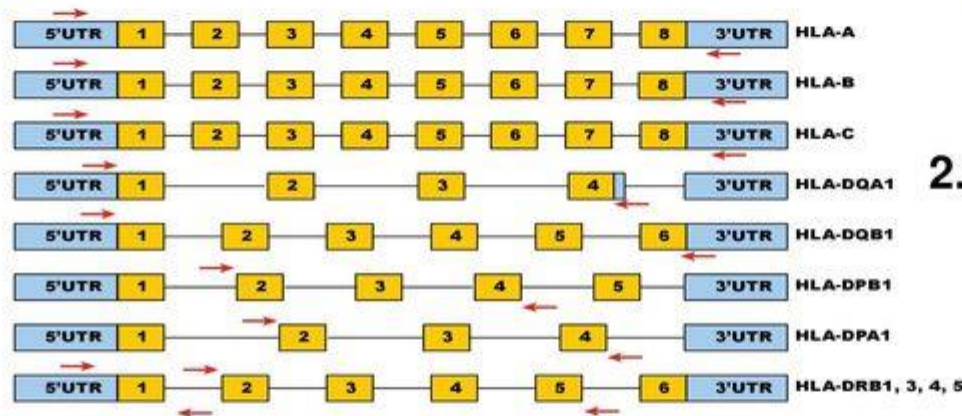
- ✓ Introni molto lunghi
- ✓ Zone ricche in GC
- ✓ Zone molto ripetute

Alcuni alleli come il DRB1*04 ed il DQA1 richiedono particolare attenzione!

NGS HLA TYPING SYSTEMS

7. Data analysis

1. Sample Collection



2. Long-Range PCR

6. Sequencing

5. Library preparation
& Pooling

3. Quantification
& Pooling

4. Fragmentation

Ambiguità

Results from an inability to establish **phase** between closely linked polymorphisms identified by the typing system

Allele A — ACGT — GAACT — TACC —

Allele B — CCAG — TAATA — CGGC —

Allele A+B — MCRK — KAAYW — YRSC —

Allele D — ACGT — GAACT — CGGC —

Allele E — CCAG — TAATA — TACC —

$A+B=D+E$

example: HLA-B

B*0702, 4402

B*0720, 4416

B*0724, 4421

Il Martedì è un Lunedì
che cerca di fare il
simpatico.

Buongiorno

