

AIBT Winter School
Ravascletto (UD) 3-5 Dicembre 2015

NGS: vantaggi, ambiti e ricadute.

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HLA Typing Methods

- Serologic assays
 - Microlymphocytotoxicity test
- Cellular assays
 - Mixed lymphocyte culture
- Molecular assays
 - Sequence-specific primer (SSP)
 - Sequence-specific oligonucleotide probe (SSOP)
 - Sequence-based testing (SBT)



T/A 2.24 2009-01-16 ..221.....23.....241.....251.....261.....271.....281.....

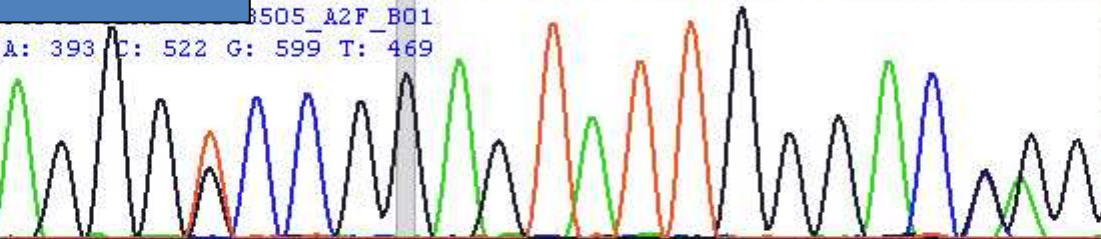
e 172 (244) RCBGTRGATRGAGCRGRAGRRKSYDGMGTATTRGGAYVNGVAVACRSGGMAHRTRARGDBCCVSTCACA

n 2 171 C A 2 1 R

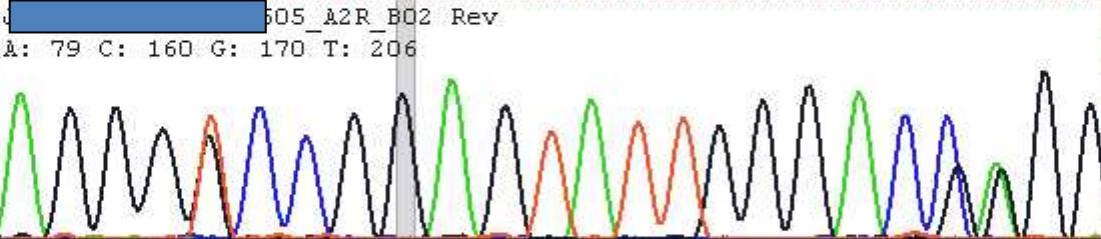
Start: 2 (74) Exon 2 1
 Stop: 823 (895) Exon 4 276

GCCGTGGATAGAGCAGGAGGGKCCGGAGTATTGGGACSRGGAGACACGGAAWRTGAAGGCCCACTCACA
 505 B
 505 Cw
 505 DQF
 505 DRI

Allele 1	Allele 2	MM
A*01010101	A*02010101	0
A*01010101	A*02010102L	0
A*01010101	A*02010103	0
A*01010102N	A*02010101	0
A*01010102N	A*02010102L	0
A*01010102N	A*02010103	0
A*0114	A*9201	0
A*0236	A*3604	0



GCCGTGGATAGAGCAGGAGGGKCCGGAGTATTGGGACSRGGAGACACGGAAWRTGAAGGCCCACTCACA
 505_A2R_B02 Rev



A*01010101	A*020103	1
A*01010101	A*020104	1
A*01010101	A*020105	1
A*01010101	A*020106	1
A*01010101	A*020107	1
A*01010101	A*020108	1
A*01010101	A*020109	1
A*01010101	A*020110	1
A*01010101	A*020111	1
A*01010101	A*020112	1
A*01010101	A*020113	1
A*01010101	A*020114	1
A*01010101	A*020115	1
A*01010101	A*020118	1
A*01010101	A*020119	1
A*01010101	A*020121	1
A*01010101	A*020122	1
A*01010101	A*0204	1
A*01010101	A*0207	1
A*01010101	A*0209	1
A*01010101	A*022001	1
A*01010101	A*022002	1
A*01010101	A*0222	1

8505

* A C M [Navigation] 89 [Next]

G R S V Master [Dropdown] [Next]

T W Y H No Offset [Dropdown] [Next]

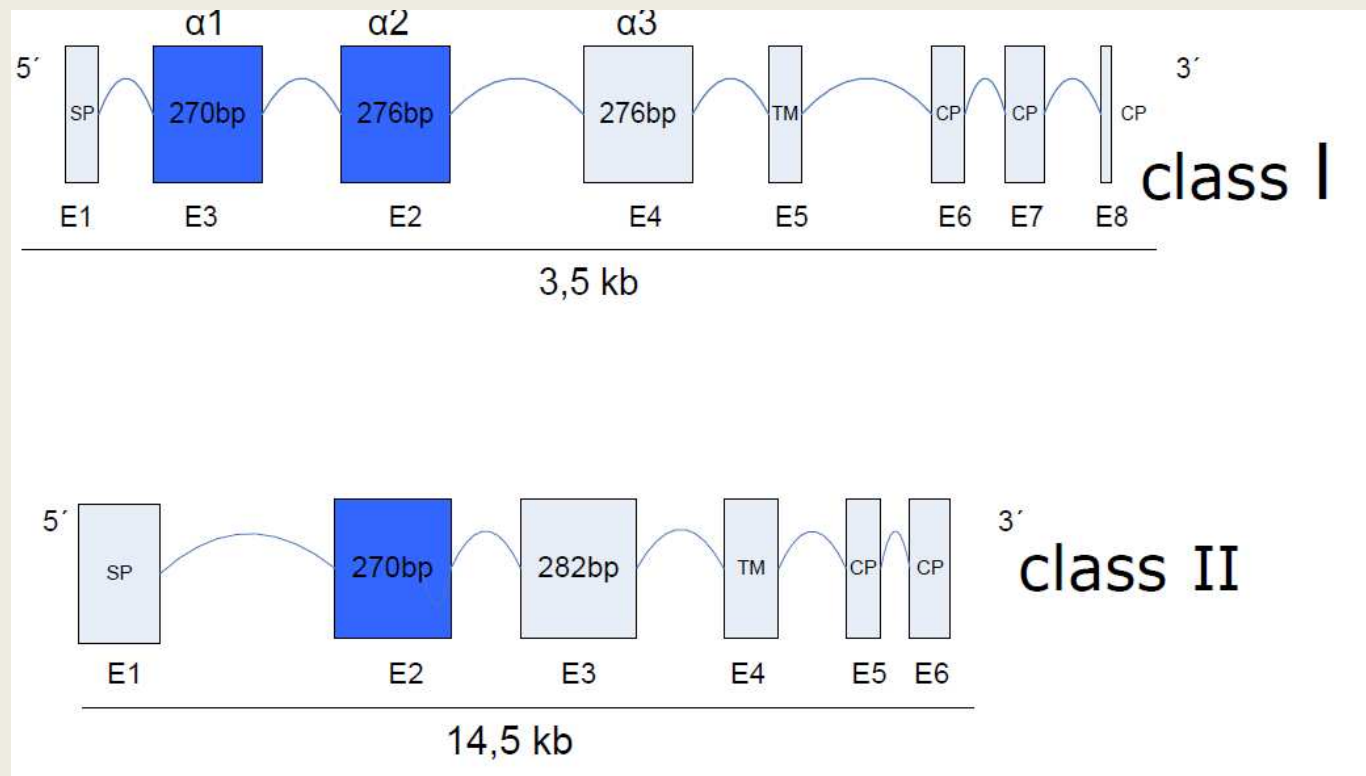
K D B N 82.1 [Dropdown] 244 [Dropdown] [Next]

+ - [Close]

A*01:01, 02:01

Sequenziamento secondo Sanger dei loci HLA

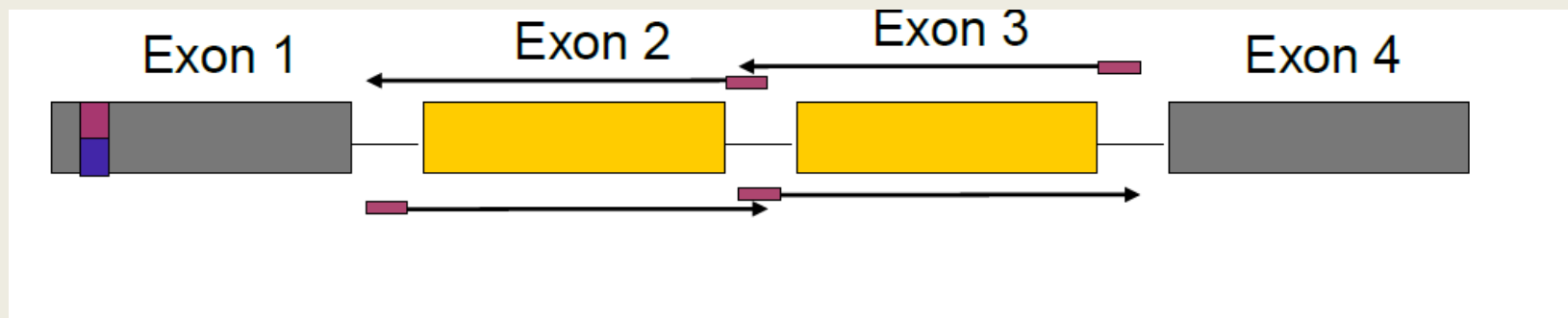
A causa dei costi e della laboriosità del metodo, l'analisi dei loci HLA è solo raramente condotta sull'intera regione codificante.



Allele ambiguity

outlier mutations: allele ambiguity

Si verifica quando un polimorfismo che distingue un allele cade fuori dalla regione esaminata



Polymorphic positions



Core heterozygous sequence data

example: HLA-B

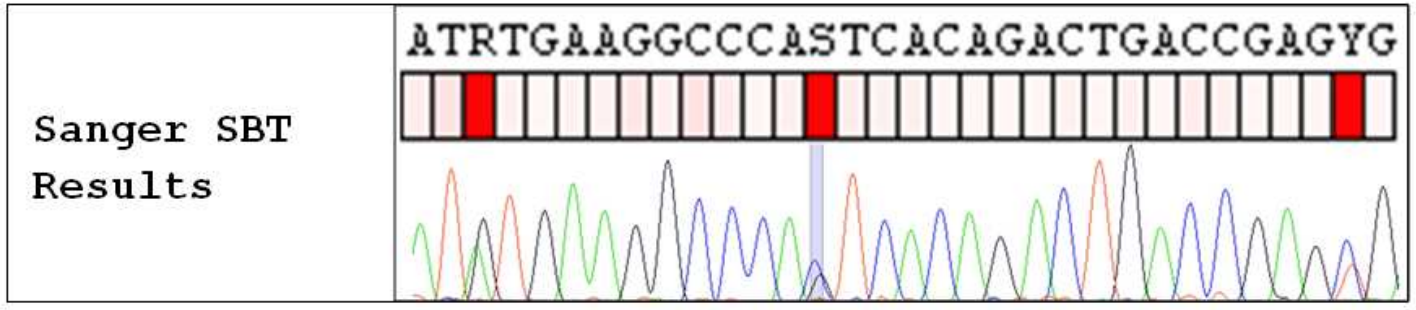
B*07:02, 44:02

B*07:02, 44:19N

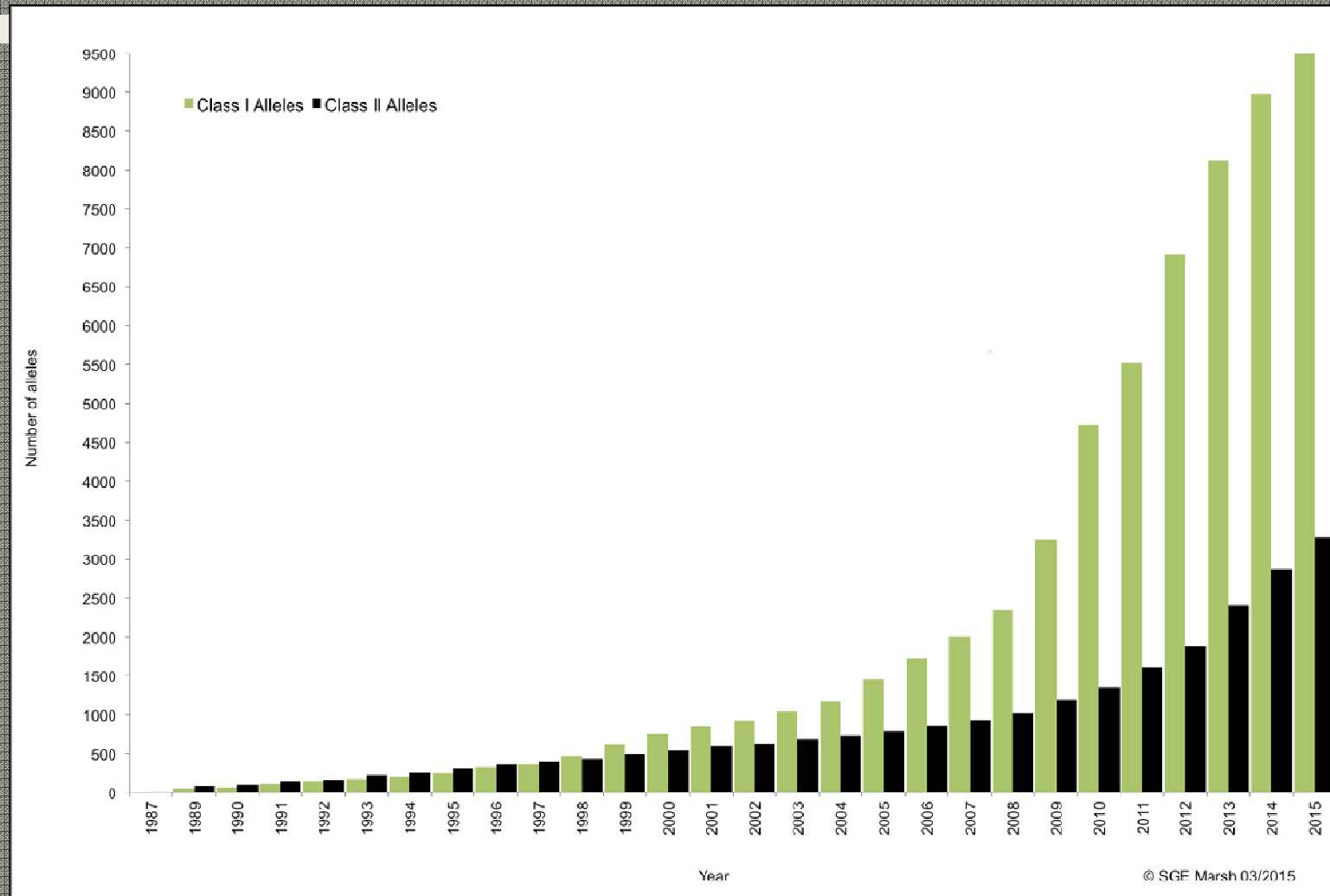
**Ambiguità generate dal sequenziamento dovute a
 mancanza di determinazione della fase gametica.**

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

cDNA	270	280	290	300
A*01010101	AT ATGAAGGCC	ACTCACAGAC	TGACCGAGCG	
A*0117	---	-G-	-----	-----
A*110101	-- G-	-----	-G-	-----T-
A*1119	-- G-	-----	-----	-----T-



Increased number of HLA alleles = more ambiguities



The new Gold Standard for genetic test

The new Gold Standard for genetic test

La Next Generation Sequencing (NGS) è un metodo di sequenziamento del DNA che ha la capacità di processare milioni di frammenti (reads) in parallelo a un costo estremamente ridotto.

Attraverso la NGS un genoma complesso come quello umano può essere sequenziato in meno di 7 giorni a un costo di \$ 700-1000.

La NGS è attualmente il metodo di analisi del DNA col più basso tasso di errore per base.

Problemi? A volte il troppo guasta!!

Problemi? A volte il troppo guasta!!

Piattaforme di Next Generation Sequencing (NGS)

- Illumina (Solexa)
 - HiSeq System
 - Genome analyzer Iix
 - MySeq
- Ion Torrent - Life Technologies
 - Personal Genome Machine (PGM)
 - Proton

Next Generation Sequencing
Amplified Single Molecule Sequencing

- Pacific Biosciences
 - PacBio RS
- Oxford Nanopore Technologies
 - GridION System
 - MinION

Third Generation Sequencing,
Next Next Generation Sequencing,
Single Molecule Sequencing

Benchtop genome sequencers

MiSeq



PGM (Personal Genome Machine)



	MiSeq	PGM
lunghezza reads (paia di basi)	300	200
throughput (giga basi G)	15	1
numero di reads per run (milioni)	25	11
Accuratezza (%)	99,90	99,00
Tempo di run (h)	65	4,5

Illumina MiSeq vs Ion Torrent PGM

INTERNATIONAL JOURNAL OF
IMMUNOGENETICS

doi: 10.1111/iji.12213

Towards allele-level human leucocyte antigens genotyping – assessing two next-generation sequencing platforms: Ion Torrent Personal Genome Machine and Illumina MiSeq

J. L. Duke*, C. Lind*, K. Mackiewicz*, D. Ferriola*, A. Papazoglou*, O. Derbenevat,
D. Wallace†‡ & D. S. Monos*‡

International Journal of Immunogenetics, 2015, 42, 346–358

Apparecchiature associate alla NGS

AmpliGen 2100 D

Pip
Target
for N
(90b



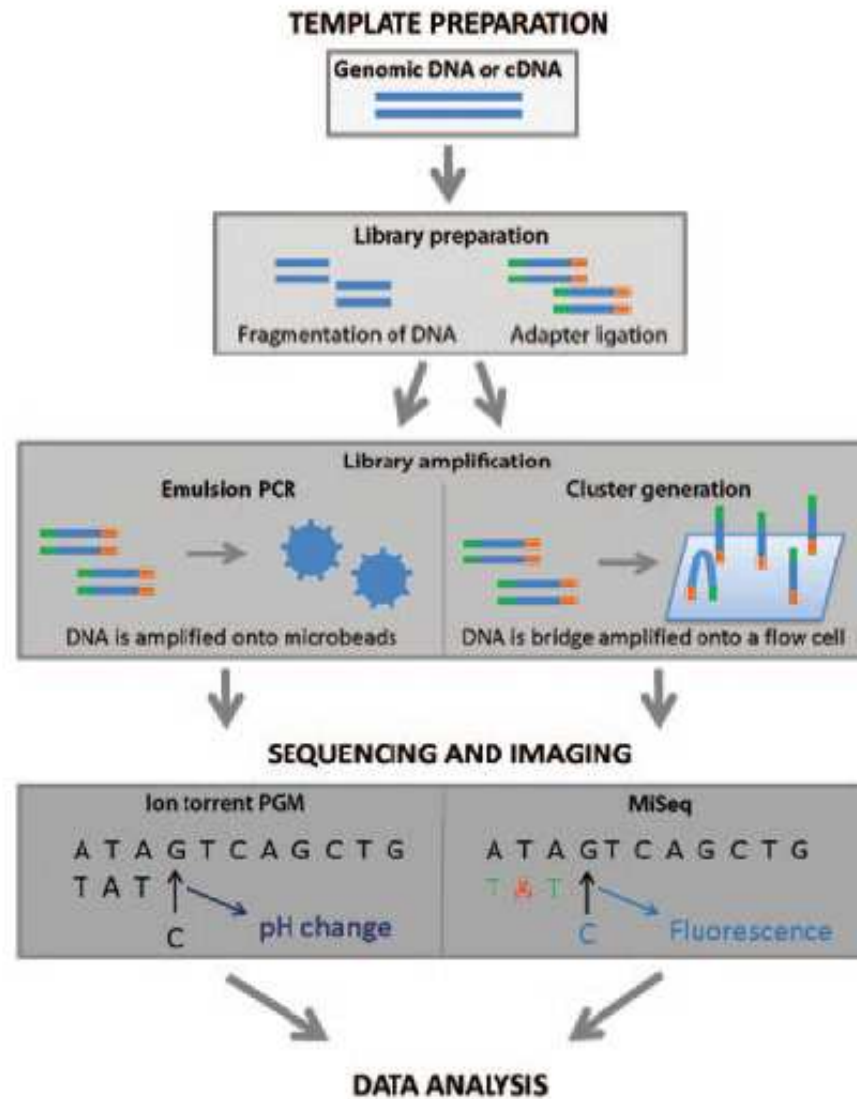
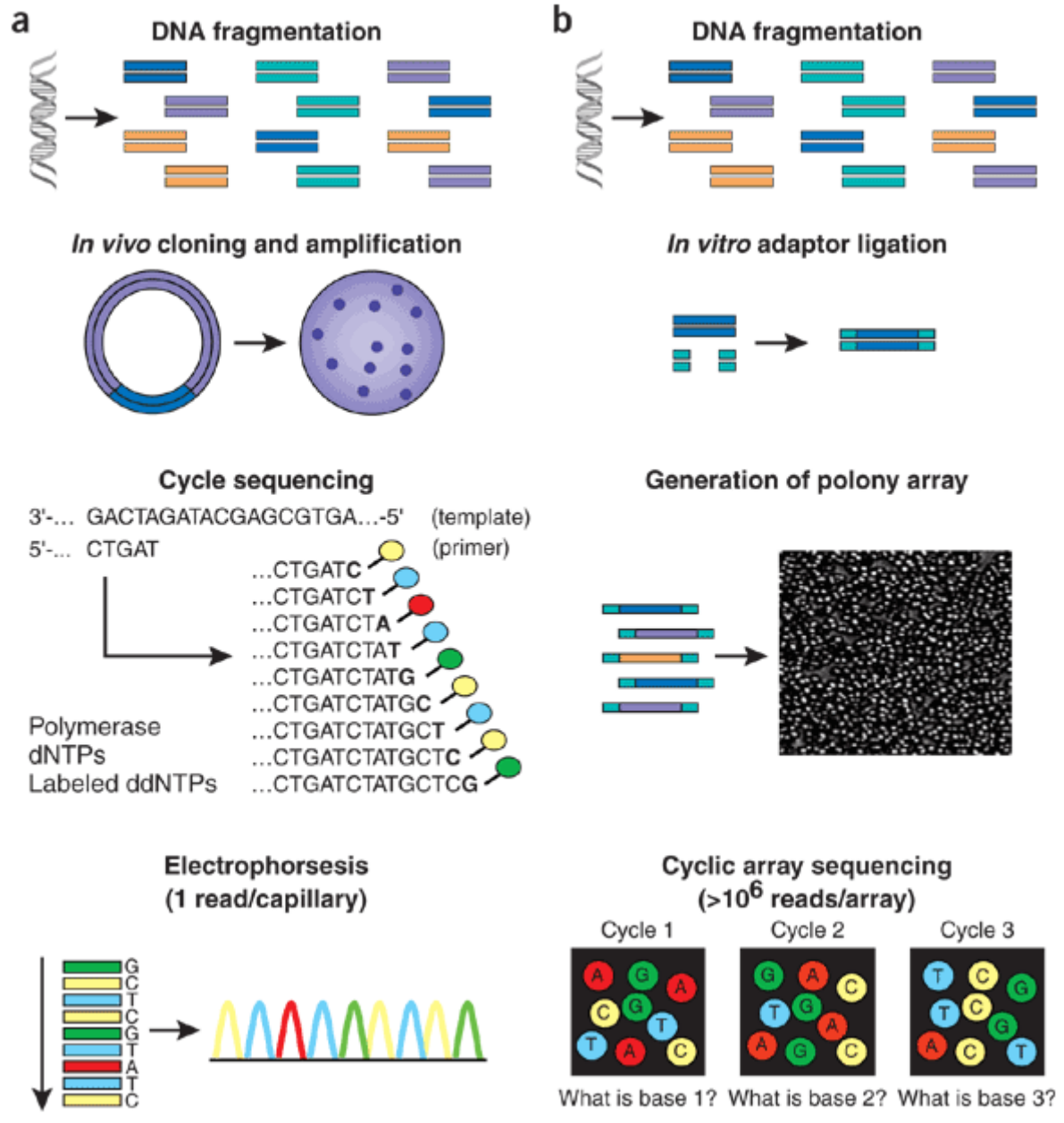


Figure 1. Next-generation sequencing methodology.



SOME DEFINITIONS

Reference GCTCCCACTCCATGAGGTATTTCTTCACATCCGTGTCCC...
Frag[0001] AGGTATTTCTTCACATCCGTGTCCC...
Frag[0004] TTCTTCACATCCGTGTCCC...
Frag[0002] ATTTCTTCACATCCGTGTCCC...
Frag[0003] CATCCGTGTCCC...
Frag[0005] GTGTCCC...
Frag[0006] GGAGCCCCGCTTCATCGCC...
Frag[0007] CCGCTTCATCGCC...
Frag[0010] CCGTGTCCC...
Frag[0008] CATCGCC...
Frag[0011] GCTTCATCGCC...
Frag[0012] CATCGCC...
Frag[0009] ACGACACGCAGTT...
Frag[0013] GGAGCCCCGCTTCATCGCC...

Fragment base #126 is selected. Base 62 of 62

Read: sequenza ottenuta da un singolo cluster originato a partire da un frammento di DNA o cDNA.

Coverage o Read Depth: numero medio di volte che una base viene analizzata, corrisponde al numero di reads che mappano sulla base.

mappano sulla base.

HLA & NGS

HLA Typing:

Risoluzione allelica a livello del 6-8 digit

Alta processività

Basso costo

La quasi totalità delle tipizzazioni HLA per i registri di donatori è prodotta attualmente con metodi NGS

HLA & NGS

HLA e MALATTIE

Esistono oltre 100 malattie HLA associate

HLA e MALATTIE

Esistono oltre 100 malattie HLA associate

FARMACOGENOMICA

FARMACOGENOMICA

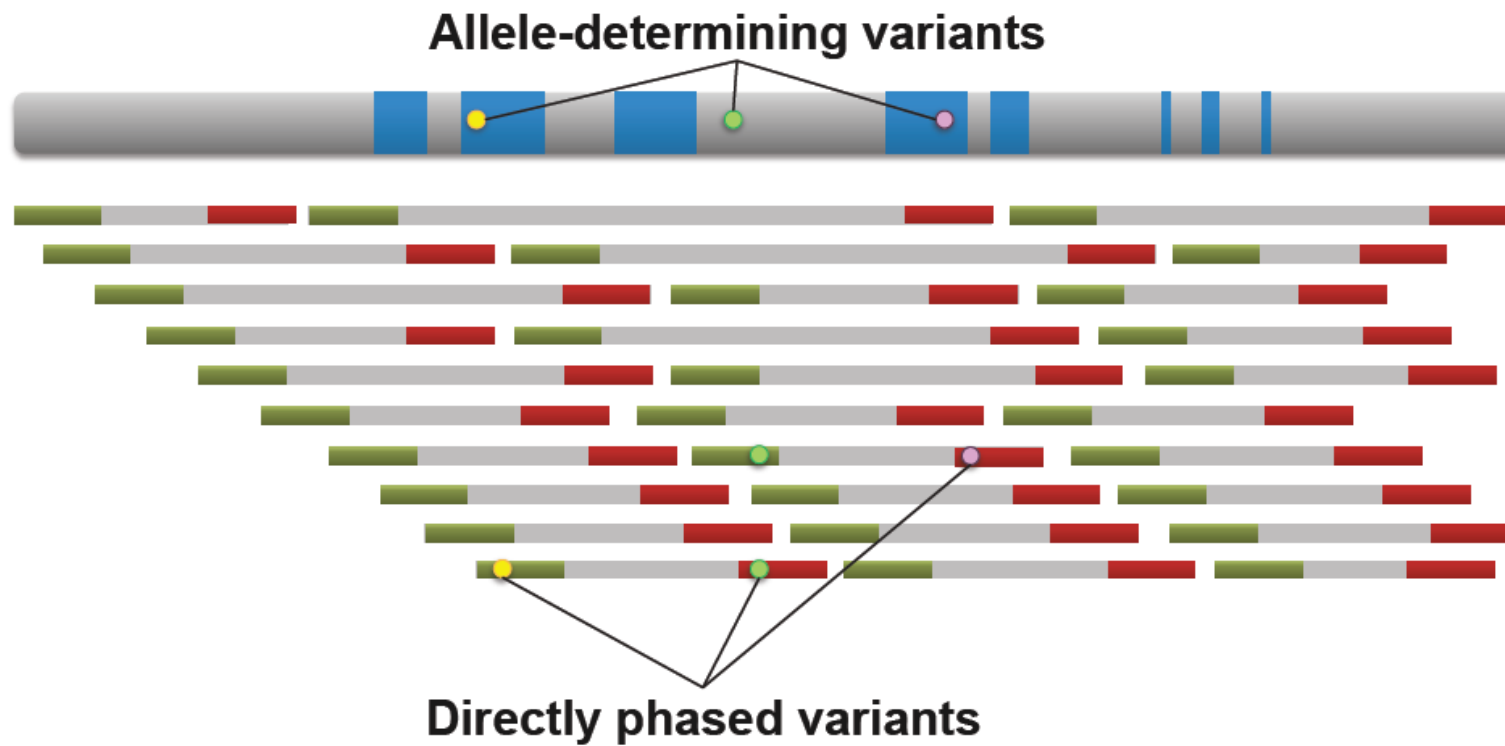
E' crescente l'individuazione di risposte avverse ai farmaci mediate
dal specifici alleli HLA.

dal specifici alleli HLA.

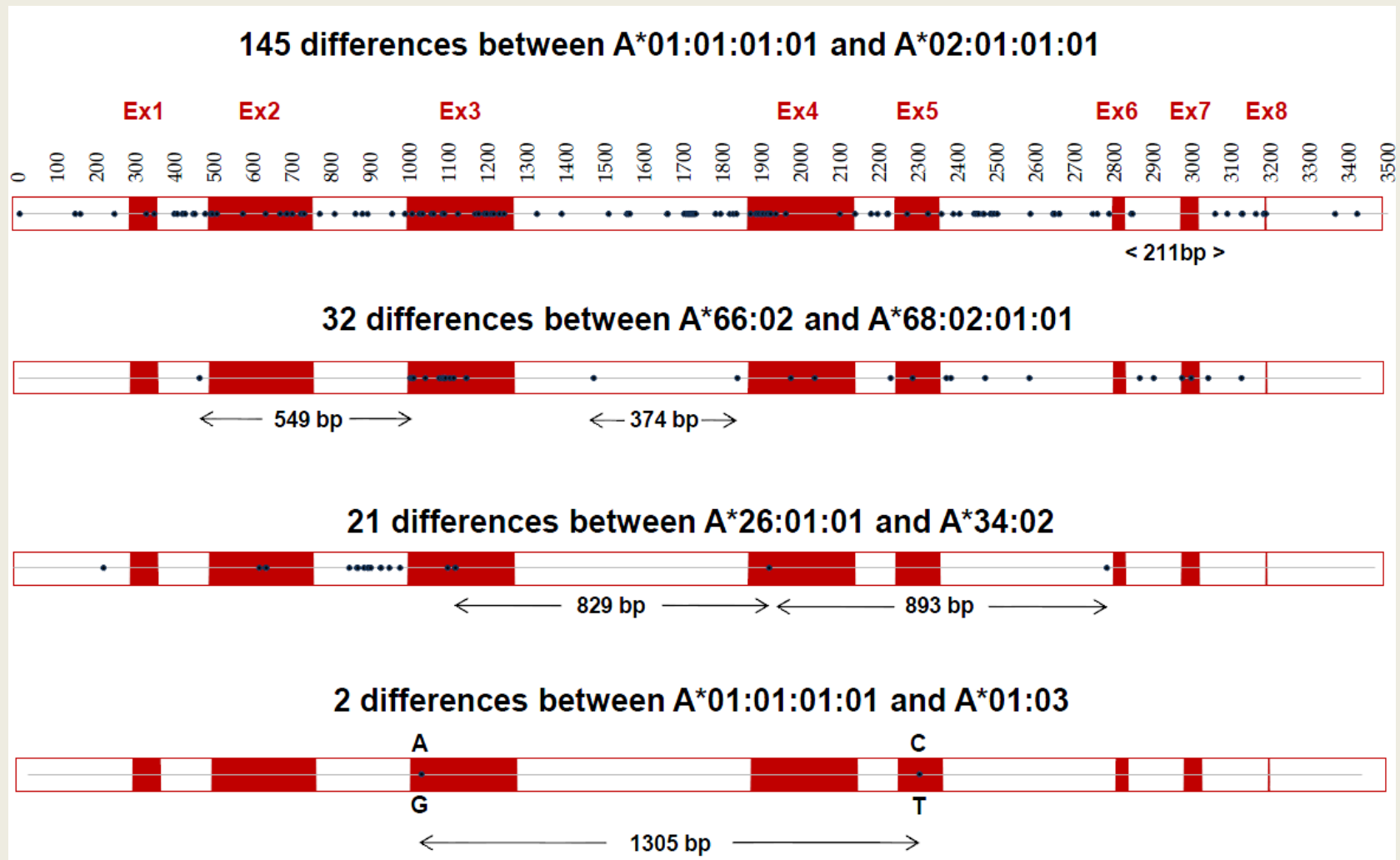
Con l' NGS sarà possibile indagare sui meccanismi attraverso cui i
geni HLA sono modulati nella loro espressione.

geni HLA sono modulati nella loro espressione.

TruSight HLA Phase Determination



The Significance of Read Length for HLA Typing by NGS



Applicazione di un metodo di high-throughput basato sulla Next Generation Sequencing per la tipizzazione dei loci HLA

Per la validazione del metodo sono stati identificati 96 campioni di DNA, precedentemente tipizzati mediante SBT ad alta risoluzione.

Kit commerciali (32 Campioni con GENDX NGSgo; 20 con OMIXON Holotype HLA)

288 con un sistema Home-made individual tagging sequencing method



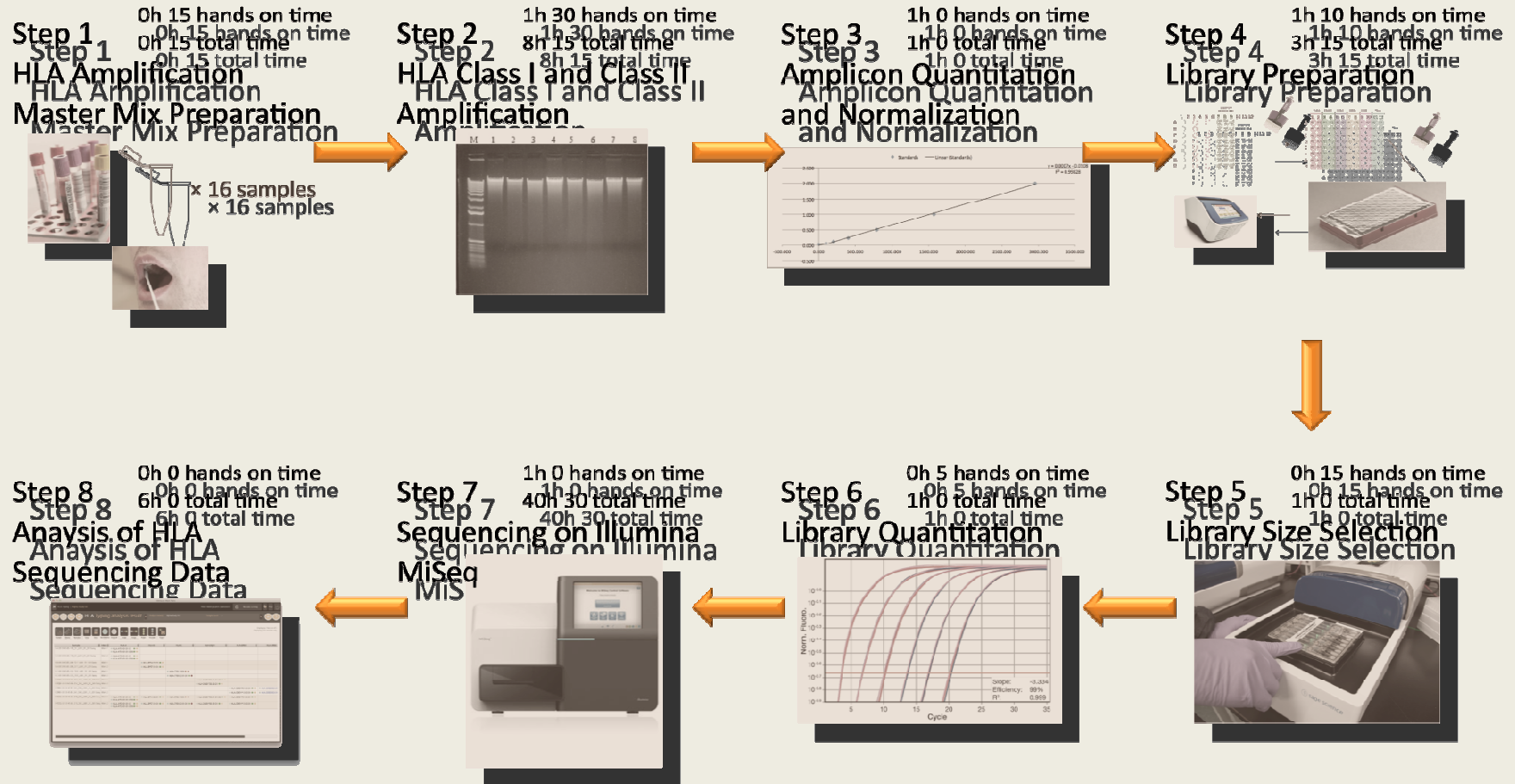
Nel protocollo di validazione abbiamo incluso i kit commerciali

GENDX



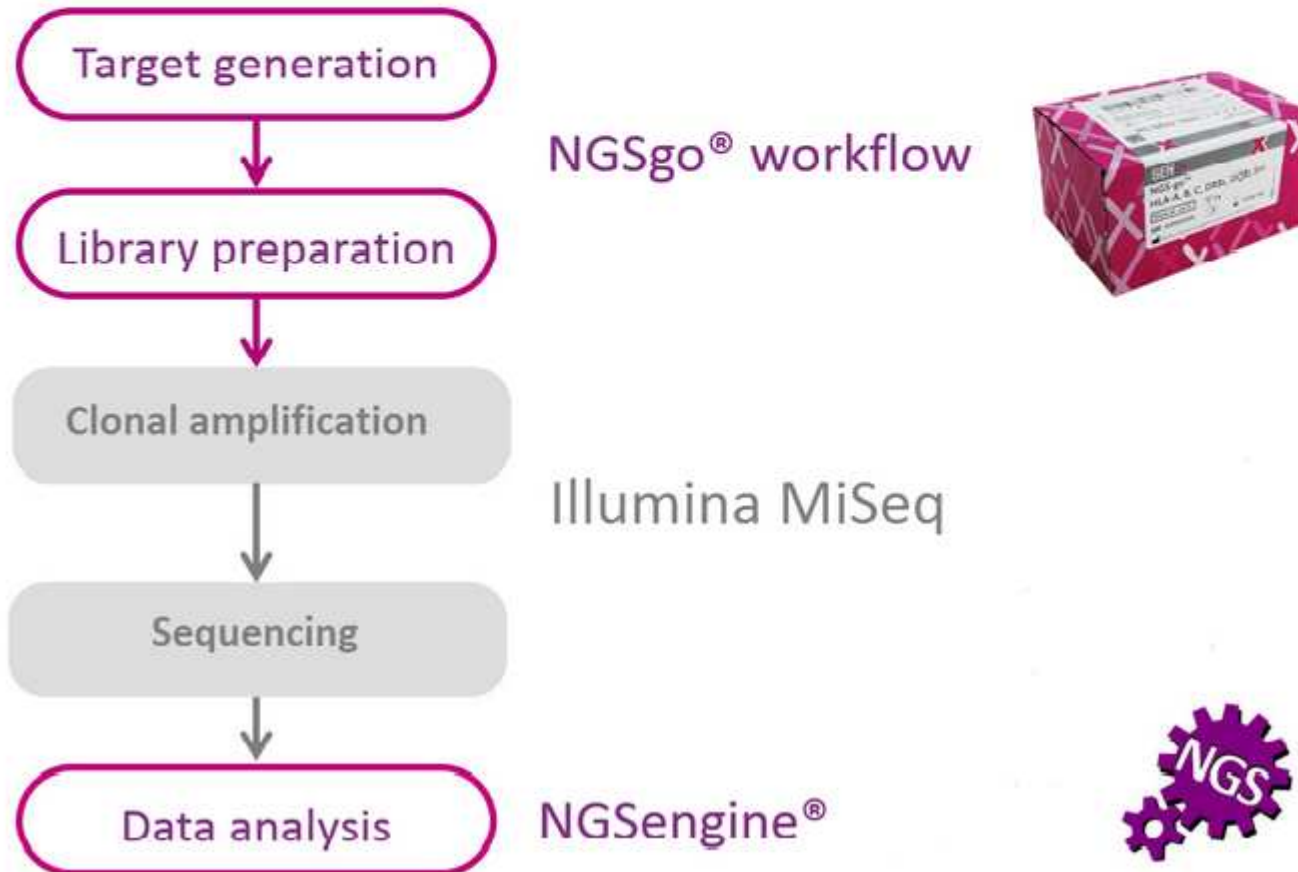
Steps in Holotype HLA

5 hours hands-on time



Overview Illumina Workflow

GenDx offers with NGSgo[®] a full workflow for the Illumina MiSeq platform, from amplification, library preparation to data analysis. We made it easy for you to make a headstart by integrating reagents, software and our know-how in this fully integrated workflow. Together we enter this next level of NGS-HLA typing.



Our Method

Long Range PCR specifica per i loci HLA-A, B, C, DRB1, DQB1, DPB1, basata su piattaforma Illumina Nextera XT e sequenziamento su Illumina MiSeq

Sviluppo di un protocollo di liquid handling semi-automatico, su MTP 96

Sviluppo di un supporto informatico Home Made.

Perché un metodo Home-made?

Perché un metodo Home-made?

La NGS è un metodo complesso con contenuti importanti di genomica e bioinformatica.

Interpretazione dei dati finali più confidente, soprattutto nel casi in cui il software produce un risultato dubbio.

Permette di ottenere un *know how* utile a sviluppare altri sistemi di analisi genetica (loci KIR)

Permette di trovare soluzioni specifiche a problemi specifici.

METHODOLOGY ARTICLE

Open Access

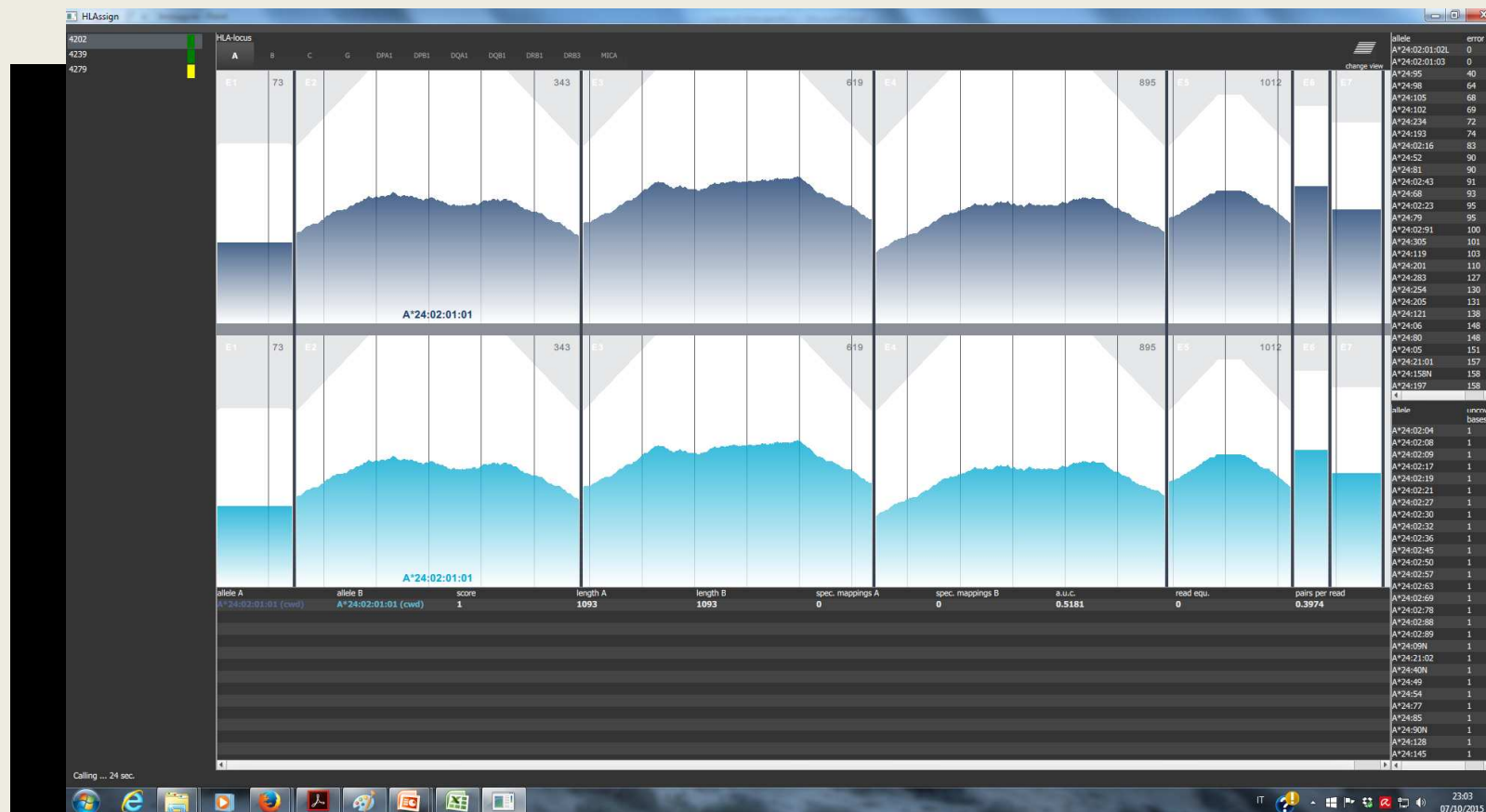
Phase-defined complete sequencing of the HLA genes by next-generation sequencing

Kazuyoshi Hosomichi¹, Timothy A Jinam¹, Shigeki Mitsunaga², Hirofumi Nakaoka¹ and Ituro Inoue^{1*}

- 7 Prodotti di LongRange PCR dal 5' al 3' UTR per i loci: A, B, C, DRB1, DPB1, DQB1, G per un target di ~ 50Kb
- Preparazione delle librerie con kit Nextera XT modificato e individual tag
- Selezione dei frammenti mediante doppio taglio con AmpureXP Beads
- Normalizzazione delle librerie con Nextera XT normalization module
- Sequenziamento su Illumina MiSeq con Flow Cell v3 300+300 cicli

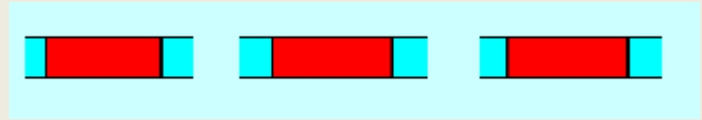
Development of a high-resolution NGS-based HLA-typing and analysis pipeline

Michael Wittig^{1,*}, Jarl A. Anmarkrud^{2,3,4}, Jan C. Kässens⁵, Simon Koch⁶, Michael Forster¹,
Eva Ellinghaus¹, Johannes R. Hov^{2,3,4,7}, Sascha Sauer⁸, Manfred Schimpler⁵,
Malte Ziemann⁹, Siegfried Görg⁹, Frank Jacob⁶, Tom H. Karlsen^{2,3,4,7,*} and Andre Franke^{1,*}

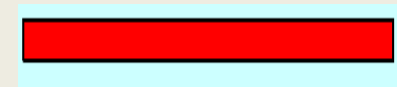


Strategies for HLA Typing by NGS

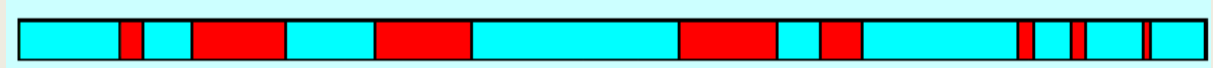
Exon-based typing (selected exonic/intronic sequences)



cDNA-based typing (selected exons can be included)



Full genomic typing



Full length Amplification (5'UTR to 3'UTR) of : HLA-A, HLA-B, HLA-C (~3kb)

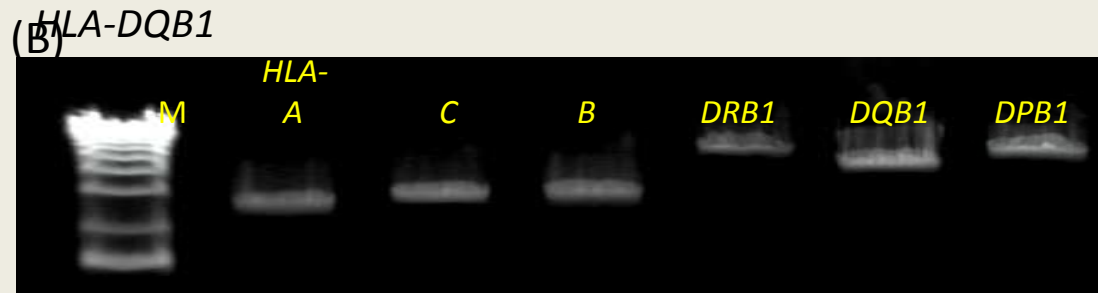
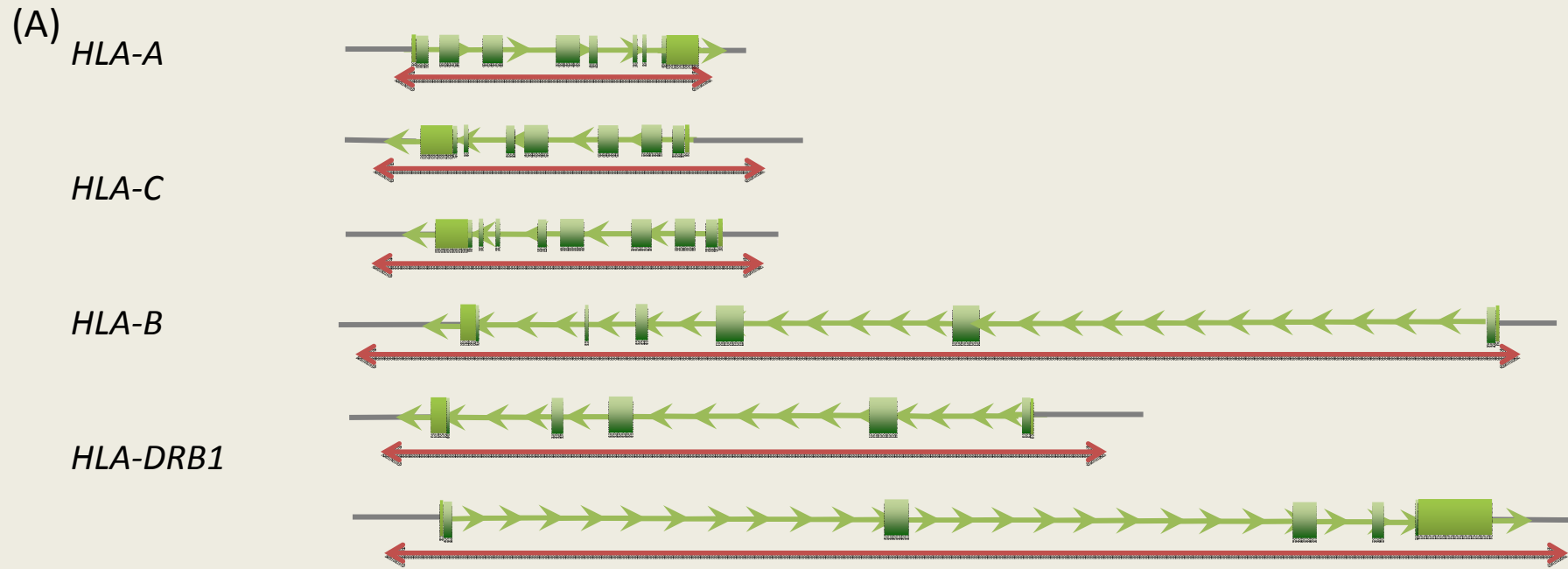
HLA-DQA1 (~7kb)

HLA-DQB1 (~7kb)

Partial Amplification of:

HLA-DRB1 amplicon ~5 kb (gene ~15kb)

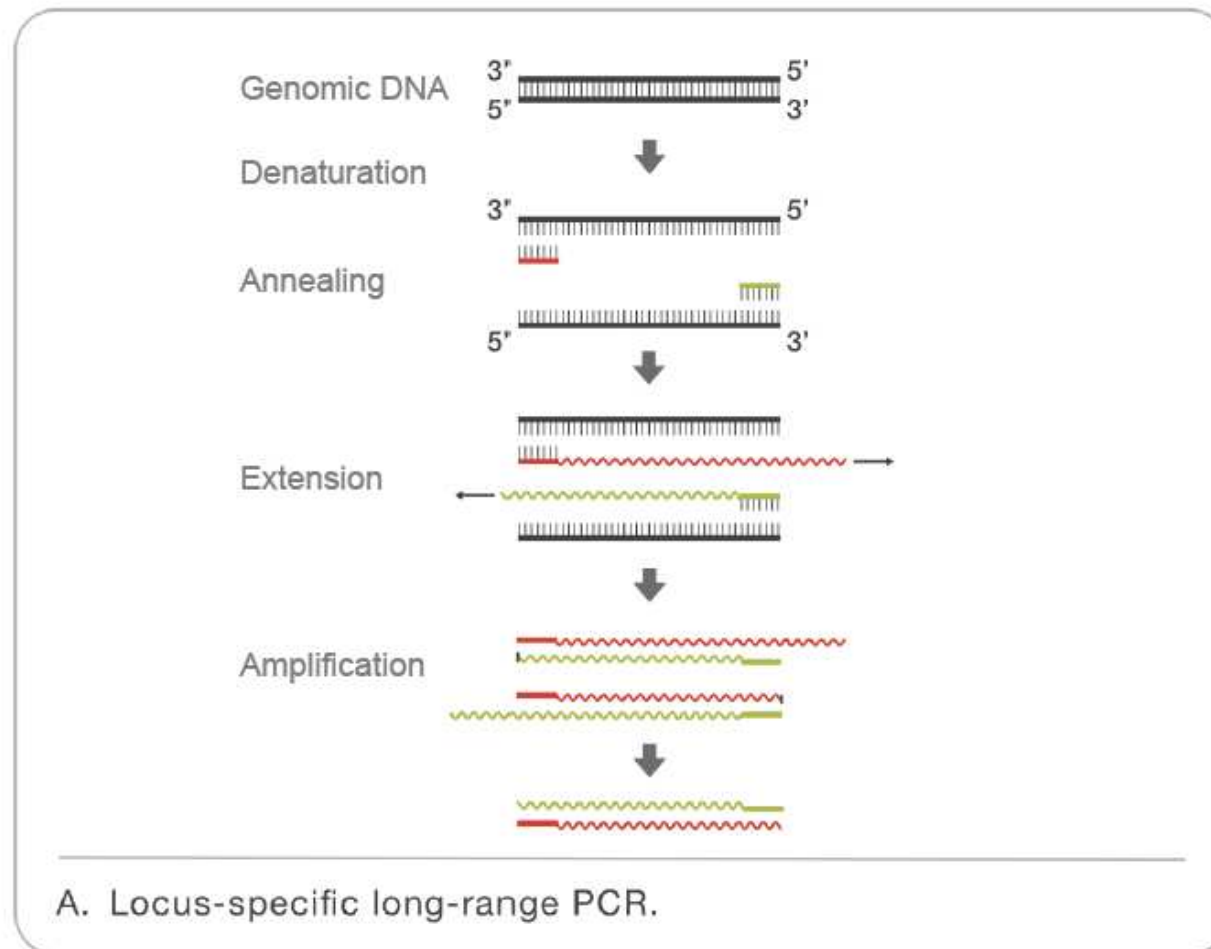
HLA-DPB1 amplicon ~7kb (gene ~12kb)



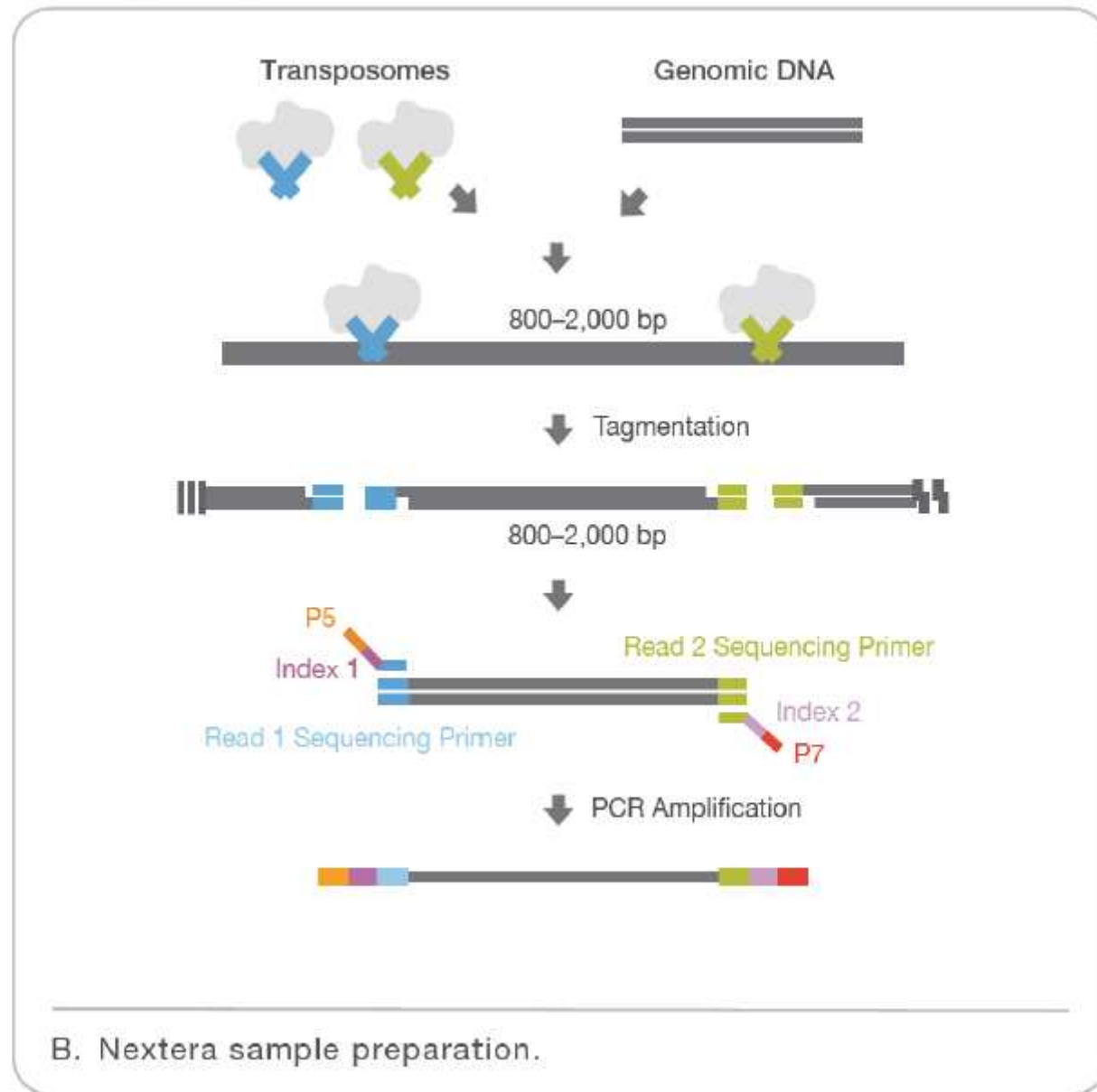
Locus	length (bp)
HLA-A	3,398
HLA-C	4,296
HLA-B	4,440
HLA-DRB1	11,899
HLA-DQB1	7,118
HLA-DPB1	13,605

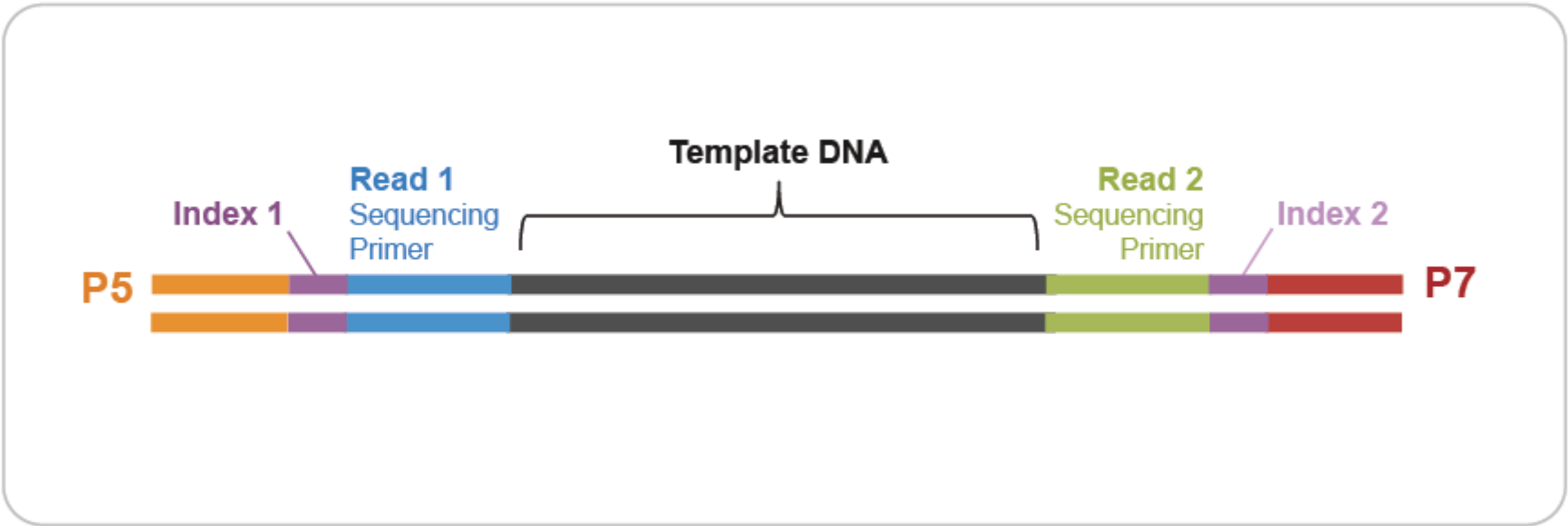
Figure S1: PCR amplification of the six HLA genes. (A) Amplified region of the six loci, where dark green boxes represent exons. Red arrows indicate the amplified region. (B) Agarose gel electrophoresis of PCR products and sizes of each amplicon.

Step 1: Amplification

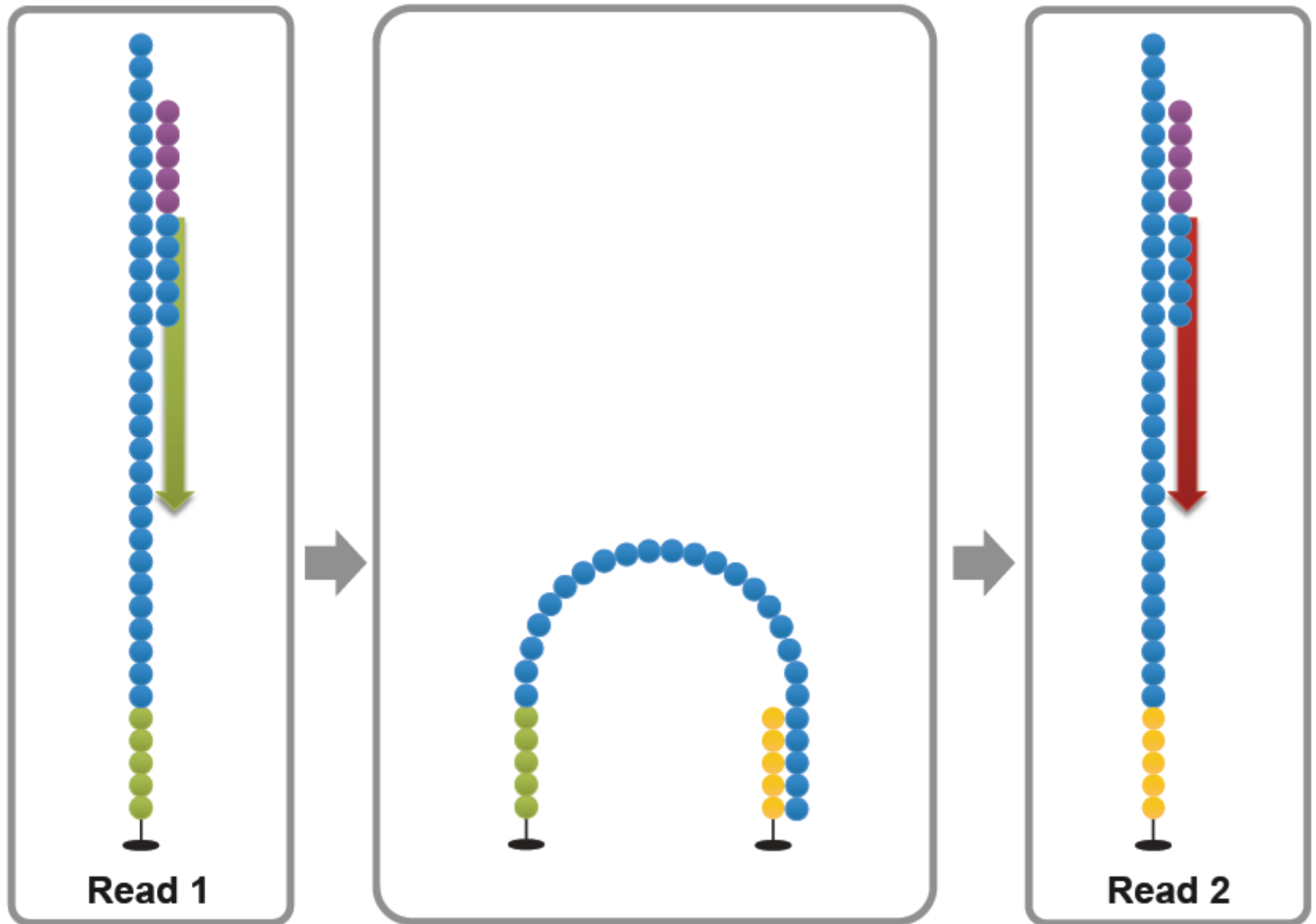


Step 2: Nextera Library Prep

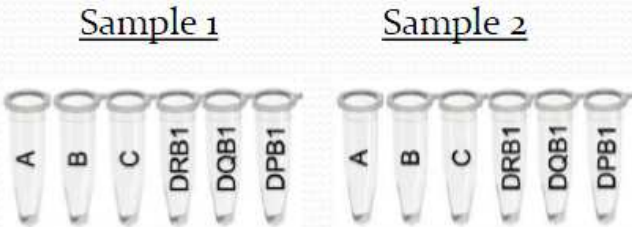




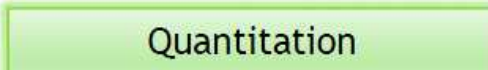
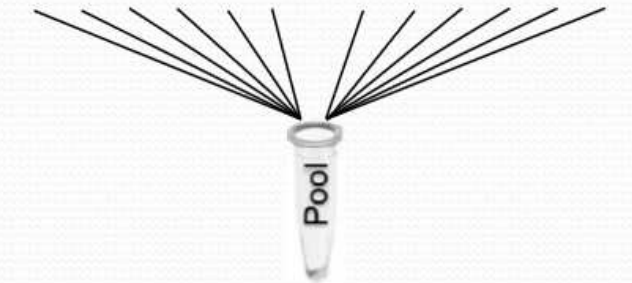
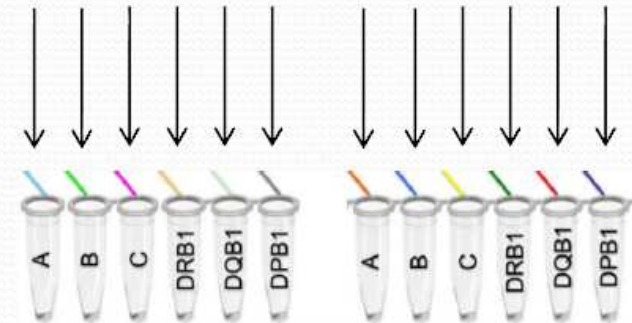
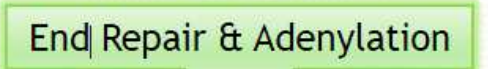
Illumina Paired-End Sequencing



Day 1
(4-6 hrs)



Day 2
(6-8 hrs)



Day 3,4



Day 5



Workflow

(tempi riferiti a una plate da 96 campioni)

1. Amplificazione O/N dei loci d'interesse

1° giorno

2. Dosaggio dei frammenti e produzione di un pool di prodotti equimolari

3. Dosaggio fluorimetrico e diluizione intermedia dei pool

2° giorno

4. Dosaggio fluorimetrico e diluizione di lavoro dei pool

5. Tagmentazione e purificazione

6. Indexing PCR

7. Doppia purificazione per la selezione dei frammenti

8. Normalizzazione delle librerie

9. Produzione del pool di librerie equimolari

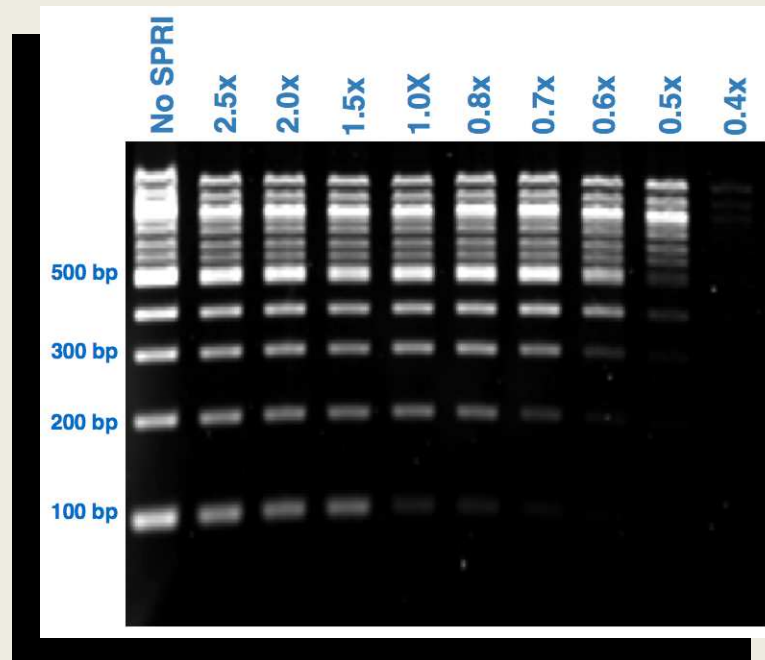
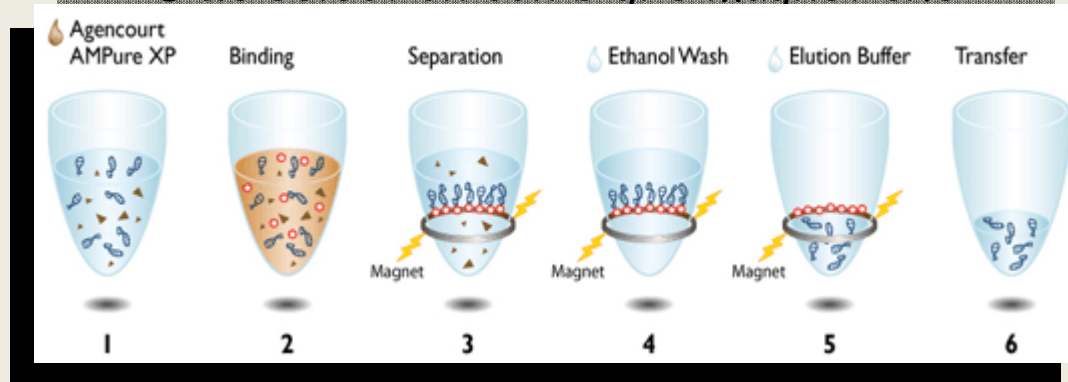
10. Sequenziamento su Illumina MiSeq con Flow Cell v3 301+301 cicli

56h

11. Analisi dei dati

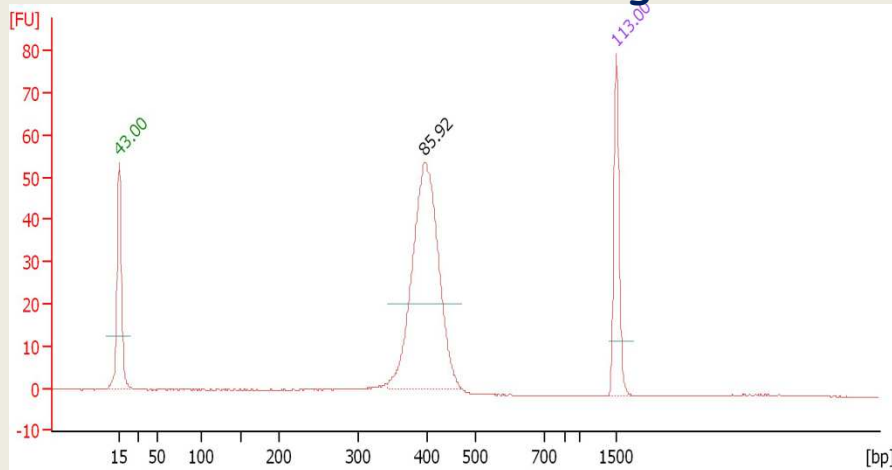
La preparazione delle librerie

Size selection con biglie magnetiche



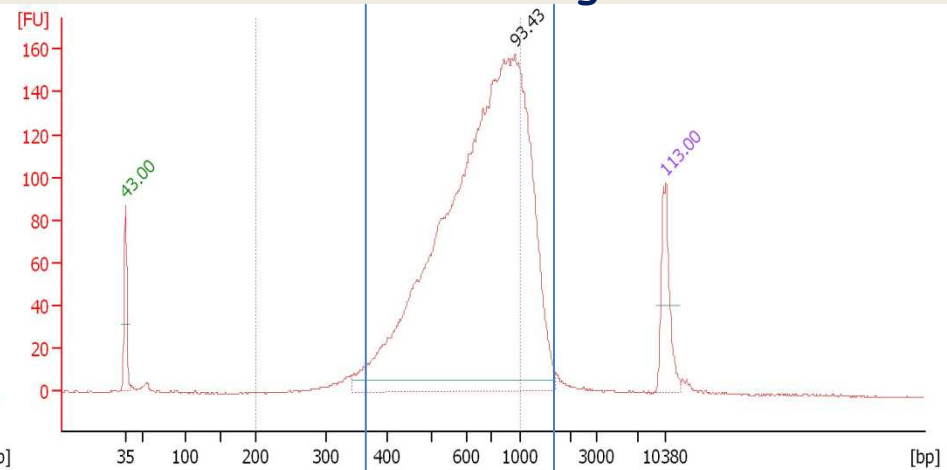
La preparazione delle librerie

Selezione da gel



Peak	Size [bp]	Conc. [ng/μl]	Molarity [nmol/l]
1	15	4.20	424.2
2	396	8.88	34.0
3	1,500	2.10	2.1

Selezione con biglie



Observations	Peak	Size [bp]	Conc. [pg/μl]	Molarity [pmol/l]	Observations
Lower Marker	1	35	125.00	5,411.3	Lower Marker
	2	940	1,790.38	2,886.1	
Upper Marker	3	10,380	75.00	10.9	Upper Marker

2° step, beads 0,6X
lega frammenti più piccoli



Discard supernatant
Keep beads

1° step, beads 0,5X
lega frammenti grandi



Keep supernatant
Discard beads

NGSEngine GenDX

Overview		Statistics		Reports					
Analysis							Progress	Actions	
P A1-B-C-DR1-DP-DQ-G_S2 181186/200000 (90%)							Analyzed	Reanalyze	
HLA-A	36801/39380 (93%)	271 [33-301]	(2225, 2798)	2	A*02:01:01:01, A*26:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
HLA-B	18018/20304 (88%)	276 [34-301]	(1037, 1298)	1	B*35:01:01:02, B*51:01:01	[Ex] 0 [In] 0	[R] 2	Analyzed	Reanalyze
HLA-C	12891/14076 (91%)	274 [35-301]	(723, 886)	1	C*01:02:01, C*16:02:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
HLA-G	26512/28712 (92%)	274 [35-301]	(1793, 2140)	1	G*01:01:01:01, G*01:01:02:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DRB1	9529/10522 (90%)	276 [35-301]	(150, 269)	1	DRB1*11:01:01, DRB1*11:04:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DQB1	25287/26358 (95%)	278 [35-301]	(713, 940)	4	DQB1*03:01:01:02, DQB1*03:01:01:02	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DPB1	39157/41834 (93%)	277 [36-301]	(654, 1010)	2	DPB1*104:01, DPB1*13:01:01	[Ex] 0 [In] 0	[R] 7	Analyzed	Reanalyze
P A1-B-C-DR1-DP-DQ-G_S1 185414/200000 (92%)							Analyzed	Reanalyze	
HLA-A	53904/59966 (89%)	271 [33-301]	(3399, 4077)	1	A*26:01:01, A*32:01:01	[Ex] 0 [In] 0	[R] 3	Analyzed	Reanalyze
HLA-B	20343/23808 (85%)	276 [35-301]	(1149, 1534)	1	B*40:02:01, B*51:01:01	[Ex] 0 [In] 3	[R] 1	Analyzed	Reanalyze
HLA-C	11049/12486 (88%)	277 [37-301]	(630, 807)	1	C*01:02:01, C*02:02:02:01	[Ex] 0 [In] 0	[R] 2	Analyzed	Reanalyze
HLA-G	20011/22396 (89%)	279 [37-301]	(1425, 1729)	1	G*01:01:01:01, G*01:01:02:01	[Ex] 0 [In] 0	[R] 3	Analyzed	Reanalyze
DRB1	10567/11608 (91%)	279 [32-301]	(172, 347)	1	DRB1*11:01:01, DRB1*11:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DQB1	16297/17328 (94%)	280 [38-301]	(475, 614)	4	DQB1*03:01:01:02, DQB1*03:01:01:02	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DPB1	34915/37822 (92%)	278 [37-301]	(606, 860)	5	DPB1*04:01:01:01, DPB1*13:01:01	[Ex] 0 [In] 0	[R] 2	Analyzed	Reanalyze
P A1-B-C-DR1-DP-DQ-G_S3 182608/200000 (91%)							Analyzed	Reanalyze	
HLA-A	32193/35168 (91%)	270 [33-301]	(1907, 2621)	2	A*02:01:01:01, A*68:01:01:02	[Ex] 0 [In] 0	[R] 3	Analyzed	Reanalyze
HLA-B	20971/23964 (87%)	274 [34-301]	(1172, 1352)	1	B*14:02:01, B*35:01:01:02	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
HLA-C	11876/13032 (91%)	273 [34-301]	(654, 782)	1	C*04:01:01:01, C*08:02:01:01	[Ex] 0 [In] 0	[R] 2	Analyzed	Reanalyze
HLA-G	26250/28790 (91%)	278 [34-301]	(1842, 2152)	1	G*01:01:01:01, G*01:01:02:01	[Ex] 0 [In] 0	[R] 2	Analyzed	Reanalyze
DRB1	18272/19506 (93%)	281 [38-301]	(370, 488)	1	DRB1*01:01:01, DRB1*01:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DQB1	24031/25112 (95%)	280 [35-301]	(718, 903)	1	DQB1*05:01:01:02, DQB1*05:01:01:02	[Ex] 0 [In] 0	[R] 1	Analyzed	Reanalyze
DPB1	34074/37036 (92%)	281 [33-301]	(576, 962)	1	DPB1*10:01, DPB1*104:01	[Ex] 0 [In] 0	[R] 3	Analyzed	Reanalyze

HLA Twin Omixon

Omixon HLA Twin

HLA Typing | Result of 1 analysis

sandro.orrù@medical.sciences 37% [memory usage] No tasks running

HLA Typing analysis result

Analysis name(s) BM1615_S3_L001_R1_001_2015-10-07_14-30-37

Displaying 7 loci out of 7. Displaying best matches only.

Sample Details Alignment Setup Load Setup Filters Best Matches Only Assigned Only Approved Only Assign Best Matches Unambiguous Concordants Assign All Unassign All Export Table Sample Comment Approve Result Cancel Approval

Approval	Sample	Allele	HLA-A	HLA-B	HLA-C	HLA-DPB1	HLA-DQA1	HLA-DQB1	HLA-DRB1
Not approved	BM1615_S3_L001_R1_001_2015-10-07_14-30-37	Allele 1	HLA-A*02:05:01	HLA-B*41:01:01	HLA-C*07:01:02	HLA-DPB1*04:01:01:01	HLA-DQA1*01:02:02	HLA-DQB1*02:01:01	HLA-DRB1*03:01:01:01
Not approved	BM1615_S3_L001_R1_001_2015-10-07_14-30-37	Allele 2	HLA-A*23:01:01	HLA-B*58:01:01:01	HLA-C*07:18	HLA-DPB1*104:01	HLA-DQA1*05:01:01:01	HLA-DQB1*05:02:01	HLA-DRB1*16:01:01

Omixon HLA Twin

HLA Typing | Result of 1 analysis | BM1606_S2_L001_R1_001_2015-10-07_14-21-11

sandro.orrù@medical.sciences 44% [memory usage] No tasks running

HLA Typing sample result

Sample: BM1606_S2_L001_R1_001_2015-10-07_14-21-11 Application build id: 02c2833e4ac47cd3879d30a1703165039bc64c6f Application version: 1.1.1

HLA database version: 3.21.0

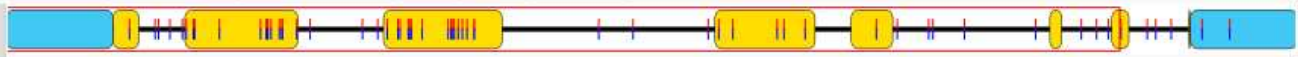
Displaying 7 loci out of 7. Displaying best matches only.

Browse Alignment Browse Allele 1 Browse Allele 2 Genotype Details Show Mismatches Show Novelles Setup Load Setup Filters Best Matches Only Assigned Only Genotype Precision Assign Best Matches Unambiguous Concordants Assign All Unassign All Export Result Sample Comment Locus Comment

Genotype	Quality control	Data statistics	HLA-A	HLA-B	HLA-C	HLA-DPB1	HLA-DQA1	HLA-DQB1	HLA-DRB1
Overall			INFO	INFO	INFO	INFO	INFO	PASSED	FAILED
Fragment size			310	305	299	318	320	300	290
Read length			213	208	209	217	220	215	213
Crossmapping (intergenic ambiguity)			23.26%	30.09%	32.607%	0.029%	0.057%	0.117%	22.621%
Ambiguous layout (intra-genic ambiguity)			26.665%	18.42%	26.347%	13.27%	7.205%	12.973%	19.152%
Read quality			35.292	35.058	35.063	36.209	36.473	36.399	36.373
Read count			3319	3355	3340	6948	6967	6845	6725
Noise ratio			0%	0.298%	0.03%	0.144%	0.344%	0%	1.502%
PCR crossover artifact ratio			0.482%	0%	0.599%	1.713%	0.459%	0.409%	0.416%
Continuous consensus			1	1	1	1	1	1	1
Fully phased consensus			1	1	1	0	0	1	0
Consensus coverage exon minimum depth			33	98	34	61	131	59	7
Consensus coverage non-exon minimum depth			34	29	36	20	20	20	0
Allele imbalance			64.312%:35.688%	51.343%:48.657%	58.149%:41.851%	52.905%:47.095%	55.505%:44.495%	55.06%:44.94%	59.259%:40.741%
Genotype available			1	1	1	1	1	1	1
Exon mismatch count			0	0	0	0	0	0	0
Non-exon mismatch count			0	0	0	0	0	0	1



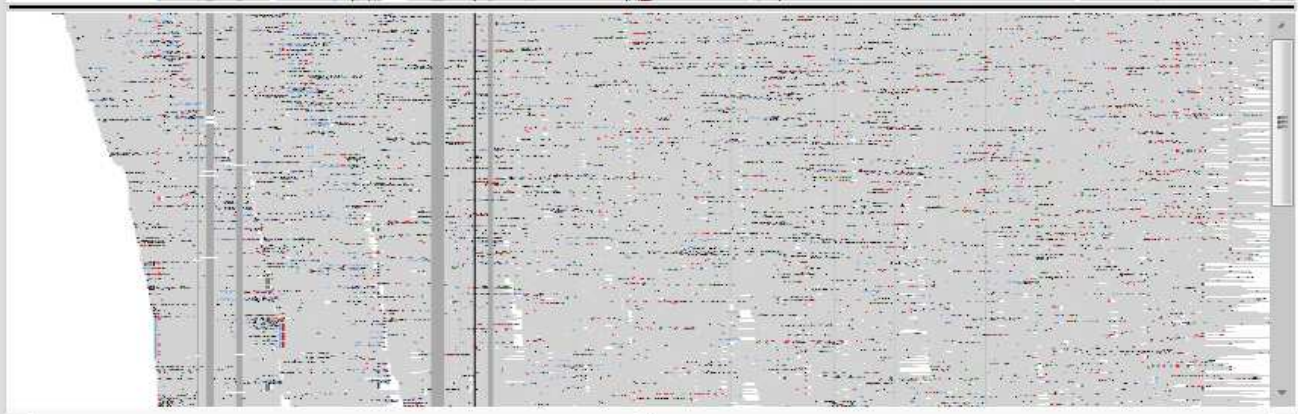
Alignment Statistics SNP Calling Allele ranking Genotype ranking XML Report Approval



-201 -101 -2 99 199 271 351 450 550 649 749 848:1908 990 1090 1190 1285 1385 1488 1588 1663 1781 1861 1961 2061 2159 2259 2359 2459 2559 2659 2759 2859 2959 3058 311



A*02:01:01:01
A*26:01:01



gDNA Position: 931 A: 5, C: 995, G: 887, T: 6, -: 0

Rejections Separate alleles

Show library

Show reads



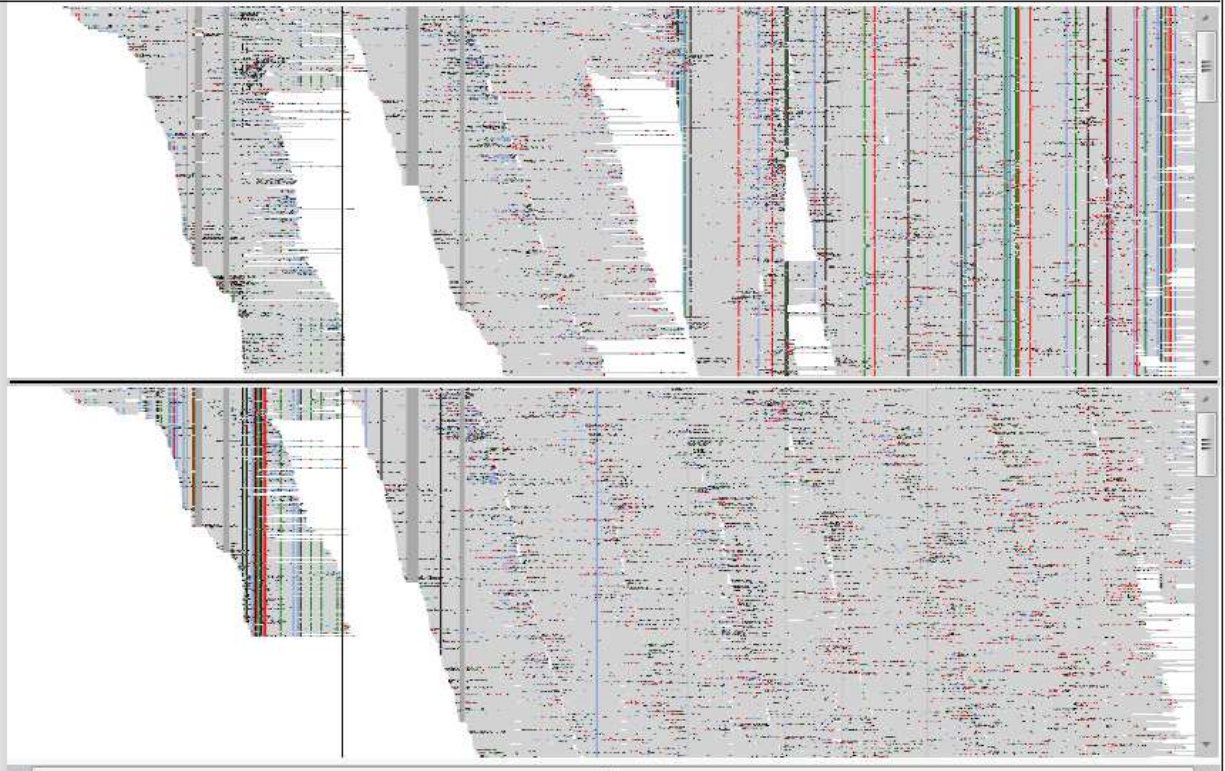
Analysis										Progress	Actions
P P978-IndTag_S2							144542/200000 (72%)	Analyzed			Reanalyze
HLA-A	40345/45226 (89%)	285 [32-301]	(2473, 6976)	2	A*02:05:01, A*02:81	[Ex] 33 [In] 55	[R] 3	Analyzed		Reanalyze	
HLA-B	22541/26424 (85%)	285 [35-301]	(1379, 3080)	1	B*49:01:01, B*58:01:01	[Ex] 0 [In] 4	[R] 14	Analyzed		Reanalyze	
HLA-C	13124/14816 (88%)	286 [35-301]	(705, 1937)	1	C*07:01:01:01, C*07:18	[Ex] 0 [In] 0	[R] 3	Analyzed		Reanalyze	
DRB1	11583/14494 (79%)	286 [33-301]	(247, 439)	1	DRB1*16:01:01, DRB1*16:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed		Reanalyze	
DQB1	28022/29516 (94%)	287 [38-301]	(958, 1484)	2	DQB1*05:02:01, DQB1*05:02:03	[Ex] 11 [In] 285	[R] 1	Analyzed		Reanalyze	
DPB1	12939/14066 (91%)	290 [34-301]	(256, 543)	1	DPB1*02:01:02, DPB1*02:01:02	[Ex] 0 [In] 0	[R] 1	Analyzed		Reanalyze	
P P1020-IndTag_S26							186222/200000 (93%)	Analyzed			Reanalyze
HLA-A	47711/56706 (84%)	265 [32-301]	(2512, 6196)	6	A*30:12, A*32:37	[Ex] 67 [In] 200	[R] 3	Analyzed		Reanalyze	
HLA-B	12714/15274 (83%)	266 [33-301]	(685, 1166)	3	B*44:03:01, B*56:21	[Ex] 23 [In] 28	[R] 20	Analyzed		Reanalyze	
HLA-C	14515/16814 (86%)	275 [35-301]	(986, 1750)	2	C*04:01:01:01, C*15:02:01	[Ex] 0 [In] 4	[R] 11	Analyzed		Reanalyze	
DRB1	22138/24680 (89%)	273 [35-301]	(348, 682)	1	DRB1*01:01:01, DRB1*01:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed		Reanalyze	
DQB1	52361/55886 (93%)	263 [33-301]	(1246, 2452)	1	DQB1*03:02:01, DQB1*05:01:01:02	[Ex] 0 [In] 20	[R] 5	Analyzed		Reanalyze	
DPB1	15338/16862 (90%)	278 [34-301]	(235, 659)	5	DPB1*04:01:01:01, DPB1*04:02:01:02	[Ex] 0 [In] 0	[R] 3	Analyzed		Reanalyze	
P P1042-IndTag_S37							186430/200000 (93%)	Analyzed			Reanalyze
HLA-A	34129/40992 (83%)	270 [33-301]	(1885, 4791)	2	A*01:81, A*11:50Q	[Ex] 34 [In] 120	[R] 9	Analyzed		Reanalyze	
HLA-B	16683/19662 (84%)	274 [32-301]	(805, 2208)	1	B*18:01:01:02, B*44:97	[Ex] 10 [In] 21	[R] 17	Analyzed		Reanalyze	
HLA-C	14078/16152 (87%)	279 [32-301]	(871, 1639)	2	C*05:01:01:02, C*12:03:01:01	[Ex] 0 [In] 0	[R] 9	Analyzed		Reanalyze	
DRB1	15534/17938 (86%)	280 [34-301]	(301, 446)	>10	DRB1*15:01:01:01, DRB1*15:01:01:01	[Ex] 0 [In] 0	[R] 1	Analyzed		Reanalyze	
DQB1	64841/68916 (94%)	274 [32-301]	(1754, 3285)	1	DQB1*02:02:01, DQB1*05:02:01	[Ex] 0 [In] 46	[R] 4	Analyzed		Reanalyze	
DPB1	20739/22770 (91%)	284 [36-301]	(335, 899)	2	DPB1*02:01:02, DPB1*04:01:01:01	[Ex] 0 [In] 0	[R] 2	Analyzed		Reanalyze	
P P1138-IndTag_S13							184792/200000 (92%)	Analyzed			Reanalyze
HLA-A	42279/48876 (86%)	269 [33-301]	(2294, 6014)	2	A*30:03, A*74:13	[Ex] 57 [In] 214	[R] 3	Analyzed		Reanalyze	
HLA-B	14514/17202 (84%)	273 [32-301]	(775, 1435)	1	B*13:02:01, B*35:254	[Ex] 13 [In] 24	[R] 20	Analyzed		Reanalyze	



-201 -101 -2 99 199 271 351 450 550 649 749 848:1908 990 1090 1190 1285 1385 1488 1588 1683 1781 1861 1961 2061 2159 2259 2359 2459 2559 2659 2759 2859 2959 3058 31

CGGCG GAGGAG TAGC G
AAGCA AAGGGA GAA AG
GG T CT G CG AT G GG AAT CAG G CG CA
GA G T C C AA GC A ABA GGG GCC G TG GC

- A*02:05:01
- A*02:81

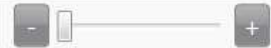


gDNA Position: 656 A: 143, C: 1, G: 280, T: 1, -: 2

Rejections Separate alleles

Show library

Show reads



Identificazione di nuovi alleli by Omixon Twin HLA typing

HLA Typing analysis result Analysis name(s) 4239-LMC-39_577_L001_R1_001_2015-10-07_21-29-55

Displaying 7 loci out of 7
Displaying best matches only

Sample Details Alignment Setup Loci Setup Filters Best Matches Only Assigned Only Approved Only Assign Best Matches Unambiguous Assign QC Passed Assign All Unassign All Export Table Sample Comment Approve Result Cancel Approval

Approval	Sample	Allele	HLA-A	HLA-B	HLA-C	HLA-DPB1	HLA-DQB1	HLA-DRB1	HLA-G
Not approved	4239-LMC-39_577_L001_R1_001_2015-10-07_21-29-55	Allele 1	HLA-A*02:01:01:02L HLA-A*02:01:01:01	HLA-B*18:01:01:02	HLA-C*07:01:01:01#1	HLA-DPB1*02:01:02	HLA-DQB1*05:01:01:02	HLA-DRB1*01:01:01	HLA-G*01:01:01:05
Not approved	4239-LMC-39_577_L001_R1_001_2015-10-07_21-29-55	Allele 2	HLA-A*30:02:01:01	HLA-B*78:01:01	HLA-C*16:01:01	HLA-DPB1*04:01:01:01 HLA-DPB1*04:01:01:02	HLA-DQB1*05:01:01:03	HLA-DRB1*10:01:01	HLA-G*01:01:01:06

Omixon HLA Twin

HLA Typing | Result of 1 analysis | 4239-LMC-39_577_L001_R1_001_2015-10-07_21-29-55 | HLA-C/Pair 1 sandro orru@medicalsciences 60% (memory usage) No tasks running

HLA Typing result novelties Locus HLA-C Pair no. 1

Jump to Novelty Setup Filters Best Matches Only Assigned Only Displayed Allelic Export Results

Allele	Chromosome	Region	Position	Type	Reference	Consensus
HLA-C*07:01:01:01#1	6	ex7	39,340,000	SNP	A	G

HLA-Locus	Fragment size	Read length	Crossmapping (intergenic ambiguity)	Ambiguous layout (intra-genic ambiguity)	Read quality	Read count	Noise ratio	PCR crossover artifact ratio	Continuous consensus	Fully phased consensus	Consensus coverage exon minimum depth	Consensus coverage non-exon minimum depth	Allele imbalance	Genotype available	Exon mismatch count	Non-exon mismatch count
HLA-C	310	213	23.26%	26.665%	35.292	3319	0%	0.482%	1	1	33	34	64.312%; 35.688%	1	0	0
HLA-DPB1	305	208	30.09%	18.42%	35.058	3355	0%	0%	1	1	98	29	51.343%; 48.657%	1	0	0
HLA-DQA1	299	209	32.607%	26.347%	35.063	3340	0.03%	0.599%	1	0	34	36	58.149%; 41.851%	1	0	0
HLA-DQB1	318	217	0.029%	13.27%	36.209	6948	0.144%	1.713%	1	0	61	20	52.905%; 47.095%	1	0	0
HLA-DRB1	320	220	0.057%	7.205%	36.473	6967	0.344%	0.459%	1	0	131	20	55.505%; 44.495%	1	0	0
Passed	300	215	0.117%	12.973%	36.399	6845	0%	0.409%	1	1	59	20	55.06%; 44.94%	1	0	1
Failed	290	213	22.621%	19.152%	36.373	6725	1.502%	0.416%	0	0	7	0	59.259%; 40.741%	0	0	0

Conclusioni

La NGS può essere usata per lo sviluppo di sistemi di genotipizzazione dei geni HLA producendo molti vantaggi rispetto ai sistemi correnti.

Diventa indispensabile quando è necessario un livello High-throughput come nel caso della tipizzazione per i registri di donatori di midollo osseo o cellule cordonali.

D' altra parte è necessario almeno inizialmente, una attenta programmazione del numero di campioni massimo e minimo da analizzare per flow cell, utilizzo della tagmentazione, size selection dei frammenti, coverage stimato ecc.



abbiamo osservato:

Che il protocollo da noi utilizzato ha mostrato una concordanza con metodi alternativi prossima al 100%.

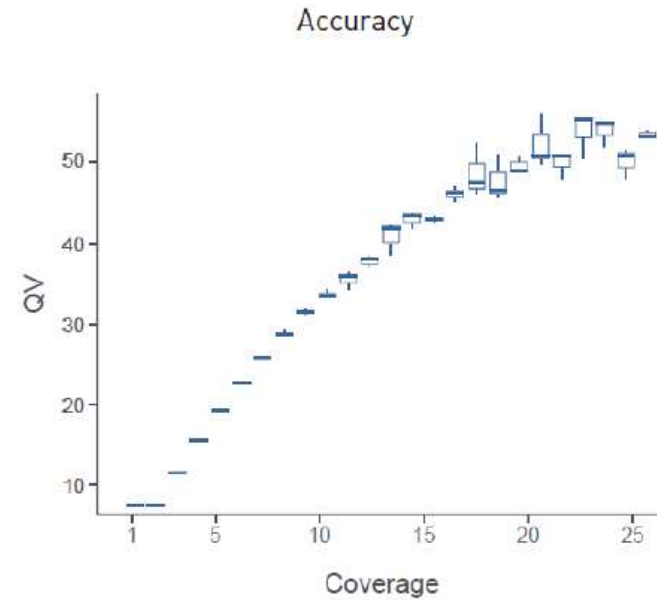
Che l'identificazione di primers specifici che riducano il rischio di sbilanciamento allelico o perdita allelica, è un elemento fondamentale nella riuscita della tipizzazione.

Che fino a 192 campioni indipendenti possono essere analizzati nella stessa flow cell da 18 Gb con un coverage sufficiente.

In queste condizioni sperimentali il costo di una tipizzazione HR per 7 loci HLA è circa 70 euro.

Third Generation Sequencing : Single Molecule Sequencing

Pacbio RS



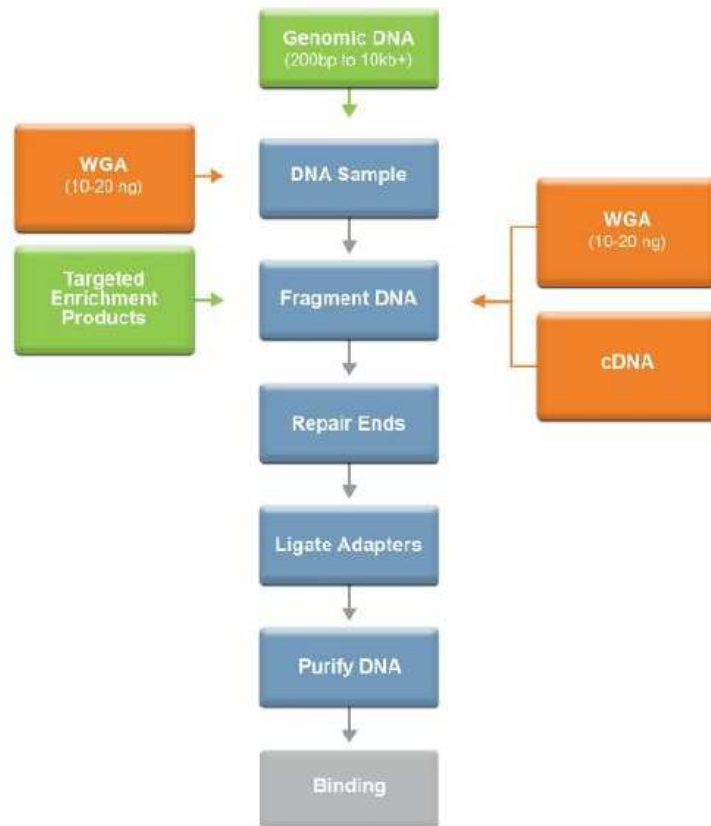
	Pacbio RS
Read Length	3000 - 15,000 bp
Throughput	1 Gb
Reads per run	70,000
Accuracy	95 %
Run Time	30 minutes

Third Generation Sequencing : Single Molecule Sequencing

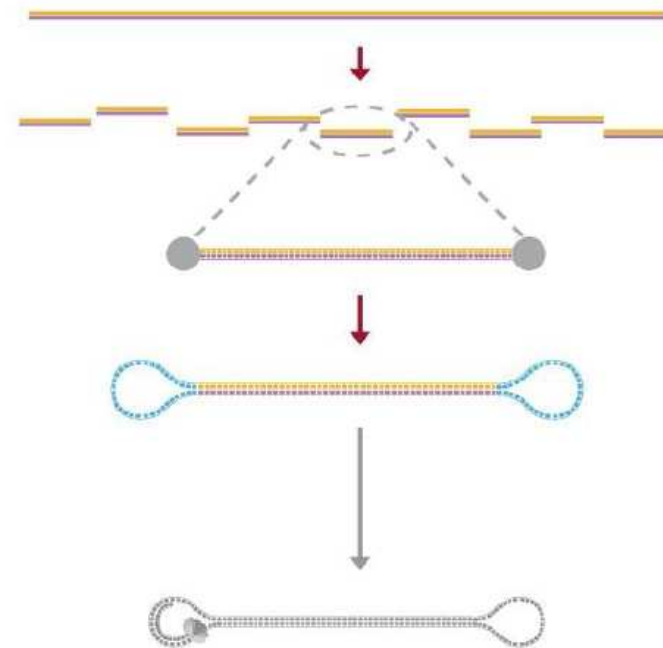
Pacific Biosciences

Workflow : Library preparation \longrightarrow Sequencing

Sample Preparation

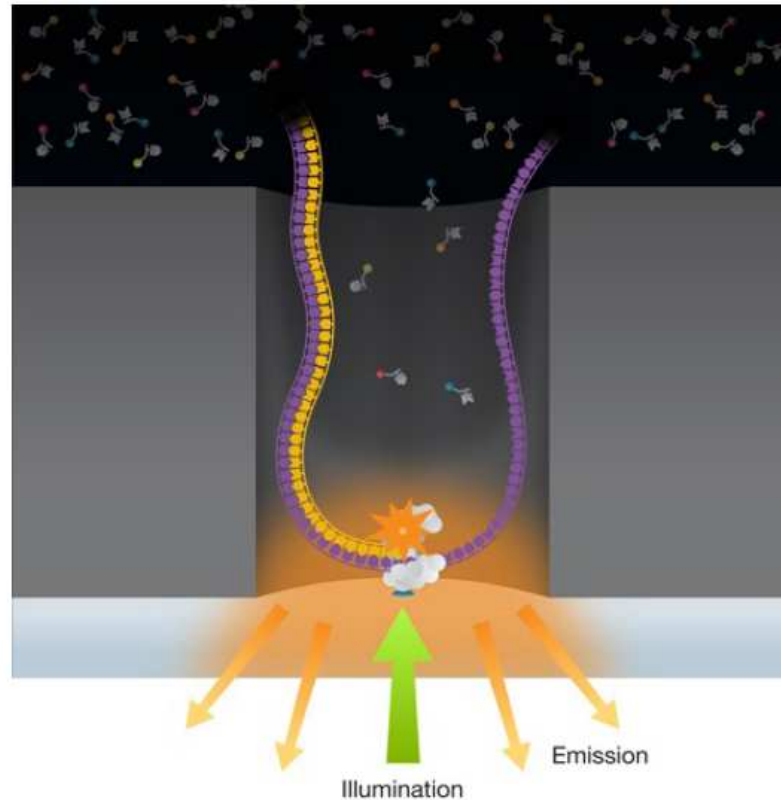


Building of SMRTbell



Third Generation Sequencing : Single Molecule Sequencing

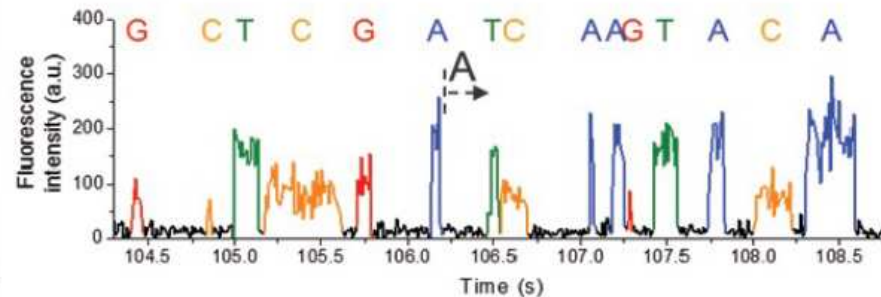
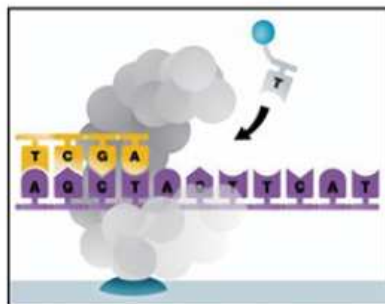
Pacific Biosciences



4 nucleotides with different fluorescent dye simultaneous present

2-3 nucleotides/sec
2-3 Kb (up to 50) read length
6 TB data in 30 minutes

laser damages polymerase



Third Generation Sequencing : Single Molecule Sequencing

Oxford Nanopore

Single use cartridge



GridION system



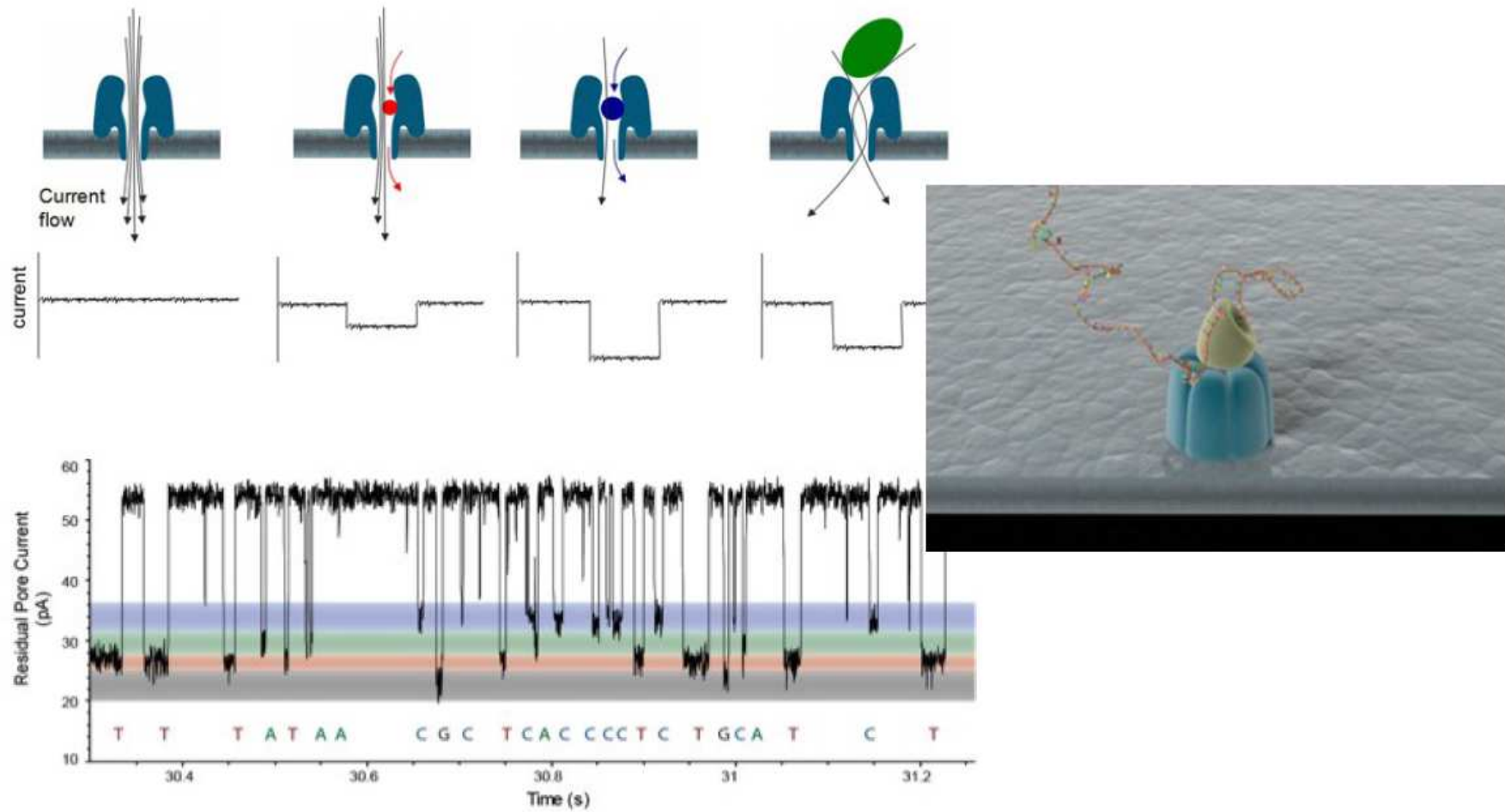
MinION



	Nanopore
Read Length	48 kb ?
Throughput	? Gb
Reads per run	2000
Accuracy	75 %
Run Time	? minutes

Third Generation Sequencing : Single Molecule Sequencing

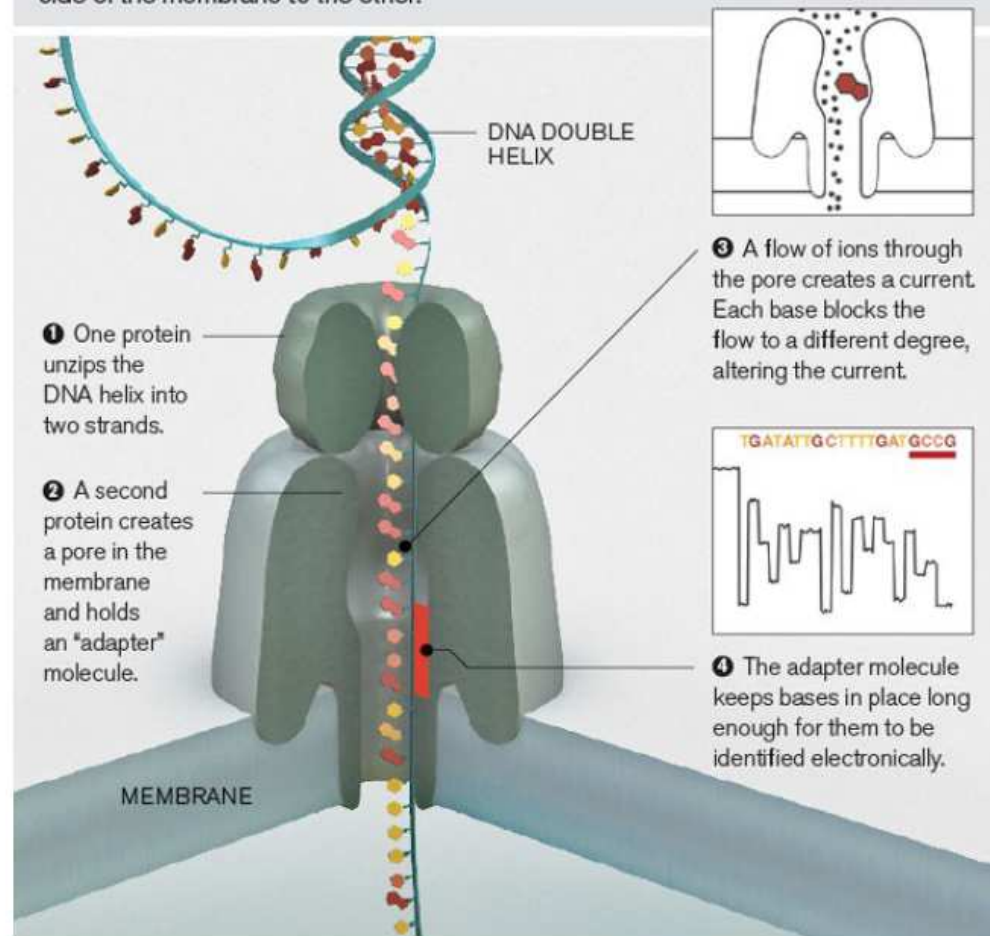
Oxford Nanopore



Third Generation Sequencing : Single Molecule Sequencing

Oxford Nanopore

DNA can be sequenced by threading it through a microscopic pore in a membrane. Bases are identified by the way they affect ions flowing through the pore from one side of the membrane to the other.



Ringraziamenti

Ringraziamenti

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