



Associazione Italiana di Immunogenetica e Biologia dei trapianti

NGS NEL LABORATORIO DI ISTOCOMPATIBILITA'

Ospedale Pediatrico Bambino Gesù
Auditorium Nobili - Polo di ricerca San Paolo, Viale F. Baldelli 38
Roma, 19 novembre 2019

Come introdurre e validare la metodica NGS nel laboratorio HLA

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IRCCS Ospedale Pediatrico Bambino Gesù



Bambino Gesù
OSPEDALE PEDIATRICO



Come introdurre e validare la metodica NGS nel laboratorio HLA

Validazione della metodica
Validazione dei risultati ottenuti

Suggerimenti pratici:

- Dottoressa Mariarosà Battarra
- Dottoressa Maria Troiano

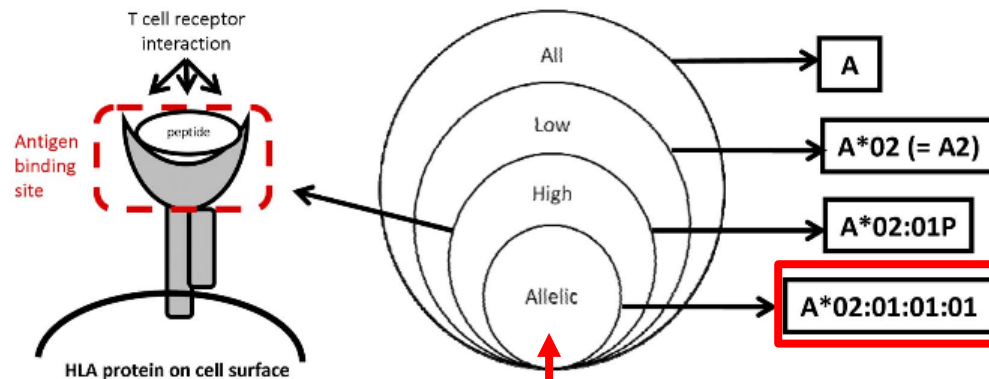


NGS in HLA typing for HSCT: short background

1 - Solve ambiguities

BLOOD, 1 DECEMBER 2011 • VOLUME 118, NUMBER 23

DEFINITIONS OF HISTOCOMPATIBILITY TYPING TERMS e181



2 - Allelic resolution



NGS in HLA typing for HSCT: short background

First PCR

ATGGAC **G/A** TAGT **G/A** GATTACGCAG **G/A**

Shotgun sequencing

in
phase

Next Generation

Allele 1

Allele 2

A

A

G/A??

G

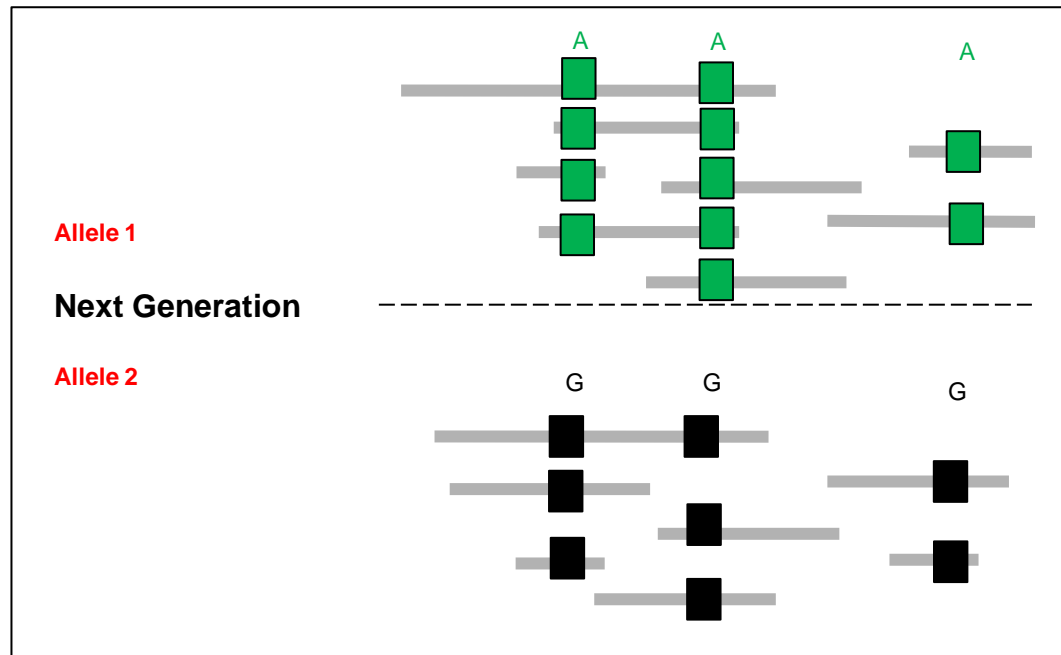
G

G/A??



NGS in HLA typing for HSCT: short background

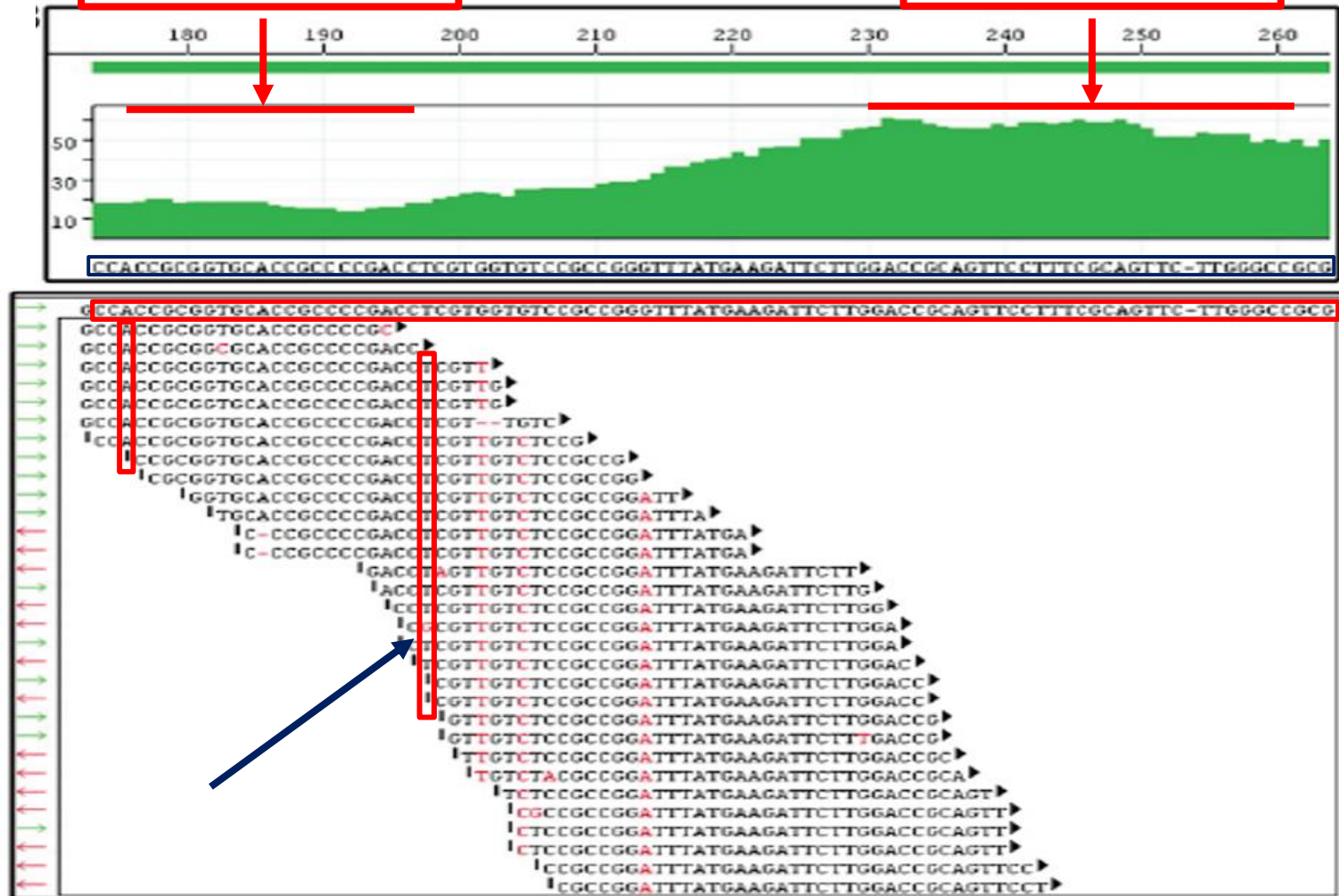
in
phase



NGS in HLA typing for HSCT: short background

Lower coverage

Higher coverage



COVERAGE DEPTH



Qualità dei dati influenzata da:

- Bilanciamento allelico nell'amplificazione
- Lunghezza delle reads
- Coverage
- Phasing
- Background



Come introdurre e validare la metodica NGS nel laboratorio HLA

Validazione della metodica

Validazione dei risultati ottenuti



Suggestions for NGS validation

Comparison with known LR and HR results

Comparison with known allelic results

Comparison with rare alleles results

Reproducibility of the method



Suggestions for NGS validation

Comparison with previous LR and HR results

Work flow		
Platform	Illumina, Thermo Fisher etc.	
Kit used	Commercial or Local	
Number of samples to test	30 – 60 up to 100	
Number of alleles to test		
Analyze HLA-A, -B, -C, -DRB1, -DQB1		150 – 300 up to 500 test
If used in routine, also analyze HLA-DRB3/DRB4/DRB5, -DQA1, -DPB1		300 – 600 up to 1000 test



Suggestions for NGS validation

Comparison with previous LR and HR results

CONFRONTO RISULTATI ATTESI																
N°	TEST	DATA		SAMPLE	A1	A2	B1	B2	C1	C2	DR1	DR2	DR345-1	DR345-2	DQA1-1	DQA1-2
19	P5	12/12/2016	S5 LIBT	1105-13	*24.02.01:01	*33 NEW	*14.02.01:01	*44.05.01	*02.02.02:01	*08.02.01:01	*01.02.01	*15.01.01:03/04	5*01.01:01 NEW			
19	rianalisi P5	04/05/2017	S5 LIBT	1105-13	*24.02.01:01	*33	*14.02.01:01	*44.05.01	*02.02.02:01	*08.02.01:01	*01.02.01	*15.01.01:03/04	5*01.01:01			
20	LIBT		LIBT	149-12T	*02:140	*23.01P	*44.03	*51.01	*04.01P	*14.02	*07.01		4*01.01	4*01.03	*02.01	
20	P5	12/12/2016	S5 LIBT	149-12T	*02:140	*23.01:01	*44.03:01:01	*51.01:01 NEW	*04.01:01:01	*14.02:01	*07.01:01:01/02		4*01.01:01:01	4*01.03:01 NEW		
20	rianalisi P5	04/05/2017	S5 LIBT	149-12T	*02:140	*23.01:01:01	*44.03:01:01	*51.01:01:03/12	*04.01:01	*14.02:01:01	*07.01:01:01/02		4*01.01:01:01	4*01.03:01:01/03		
20	P11	16/06/2017	S5 LIBT	149-12T	*02:140	*23.01:01:01	*44.03:01:01	*51.01:01:03/12	*04.01:01:01	*14.02:01:01	*07.01:01:01/02		4*01.01:01:01	4*01.03:01		
21	LIBT		LIBT	987-15	*66.01	*68:24	*07.05P	*35.03	*12.03	*15.05	*01.01	*04.05	4*01.03		*01.01P	*03.01P
21	P5	12/12/2016	S5 LIBT	987-15	*66.01:01	*68:24	*07.06	*35.03:01	*12.03:01:01	*15.05:02	*01.01:01	*04.05:01	4*01.03:01:01/03			
21	rianalisi P5	04/05/2017	S5 LIBT	987-15	*66.01:01:01	*68:24	*07.06:01	*35.03:01:01	*12.03:01:01	*15.05:02	*01.01:01	*04.05:01	4*01.03:01:01/03			
22	LIBT		LIBT	1602-11T	*01.01	*02.01	*08.01	*27:12	*02.02	*07.01	*03.01		3*01.01	3*02.02	*05.01	*05.05
22	P5	12/12/2016	S5 LIBT	1602-11T	*01.01:01:01	*02.01:01:01	*08.01:01	*27:12	*02.02.02:01	*07.01:01:01	*03.01:01:01	*11.01:01	3*01.01:02:01	3*02.02:01 NEW		
22	rianalisi P5	04/05/2017	S5 LIBT	1602-11T	*01.01:01:01	*02.01:01:01/16	*08.01:01:01	*27:12	*02.02.02:01	*07.01:01	*03.01:01:01	*11.01:01:01	3*01.01:02:01	3*02.02:01:02		
23	LIBT		LIBT	1111-09T	*23.01	*29.02	*15:71	*44.03	*03.03	*16.01	*07.01		3*02.02	4*01.01	*02.01	*05.05
23	P5	12/12/2016	S5 LIBT	1111-09T	*23.01:01	*29.02:01:01	*15:71	*44.03:01:01	*03.03:01	*16.01:01:01	*07.01:01:01/02	*11.01:01	3*02.02:01NEW	4*01.01:01:01		
23	rianalisi P5	04/05/2017	S5 LIBT	1111-09T	*23.01:01:01	*29.02:01:01	*15:71	*44.03:01:01/02/03	*03.03:01:01	*16.01:01:01	*07.01:01:01/02	*11.01:01:01	3*02.02:01:02	4*01.01:01:01		
24	LIBT		LIBT	570-09T	*02.01		*18:07:01	*57.01	*06.02	*12.03	*11.01	*11.04	3*02.02		*05.05	
24	P5	12/12/2016	S5 LIBT	570-09T	*02.01:01:01	*02.327	*18:07:01	*57.01:01	*06.02:01:02	*12.03:01:01	*11.01:01	*11.04:01	3*02.02:01 NEW			
24	rianalisi P5	04/05/2017	S5 LIBT	570-09T	*02.01:01:01/16		*18:07:01	*57.01:01	*06.02:01:02	*12.03:01:01	*11.01:01:01	*11.04:01	3*02.02.01			
24	P11	16/06/2017	S5 LIBT	570-09T	*02.01:01:01/16		*18:07:01	*57.01:01	*06.02:01:01	*12.03:01:01	*11.01:01:01	*11.04:01	3*02.02.01:02	3*02.02.01		
25	LIBT		LIBT	2293-16	*02.01P	*11.01	*07.02	*44.02	*05.01P	*07.02P	*14.01P	*16.01P	3*02.02	5*02.02	*01.01P	*01.02P
25	P6	09/01/2017	S5 LIBT	2293-16	*02.01:01:01	*11.01:01:01	*07.02:01	*44.02:01:01	*05.01:01:02	*07.02:01:03	*14.54:01 NEW	*16.01:01	3*02.02:01 NEW	5*02.02		
25	rianalisi P6	04/05/2017	S5 LIBT	2293-16	*02.01:01:01/16	*11.01:01:01	*07.02:01:01/03	*44.02:01:01	*05.01:01:02	*07.02:01:03	*14.54:01	*16.01:01	3*02.02.01	5*02.02		
26	LIBT		LIBT	786-14	*03.01	*24.02	*14.02	*18.01	*08.33	*12.03	*11.04	*16.01	3*02.02	5*02.02	*01.02P	*05.01P
26	P6	09/01/2017	S5 LIBT	786-14	*03.01:01:01	*24.02:01:01	*14.02:01:01	*18.01:01:02	*08.33:01	*12.03:01:01	*11.04:01	*16.01:01	3*02.02:01 NEW	5*02.02		
26	rianalisi P6	04/05/2017	S5 LIBT	786-14	*03.01:01:01	*24.02:01:01	*14.02:01:01	*18.01:01:02/05	*08.33:01	*12.03:01:01	*11.04:01	*16.01:01	3*02.02:01:02	5*02.02		
26	P11	16/06/2017	S5 LIBT	786-14	*03.01:01:01	*24.02:01:01	*14.02:01:01	*18.01:01:02/05	*08.33:01	*12.03:01:01	*11.04:01	*16.01:01	3*02.02:01:02	5*02.02		
27	LIBT		LIBT	828-14	*11.01	*26.01	*35:08	*38.01	*06.02	*12:02:08	*04.02	*11.01P	3*02.02	4*01.03	*03.01P	*05.01P
27	P6	09/01/2017	S5 LIBT	828-14	*11.01:01:01	*26.01:01:01	*35:08.01	*38.01:01	*06:127:01	*12:03:01:01	*04.02:01	*11.01:01 NEW	3*02.02:01 NEW	4*01.03:01 NEW		
27	rianalisi P6	04/05/2017	S5 LIBT	828-14	*11.01:01:01	*26.01:01	*35:08.01	*38.01:01	*06:127:01	*12:03:01:01	*04.02:01	*11.01:01	3*02.02:01:02	4*01.03:01:01/03		
27	P11	16/06/2017	S5 LIBT	828-14	*11.01:01:01	*26.01:01:01	*35:08.01	*38.01:01	*06:127:01:01	*12:03:01:01	*04.02:01	*11.01:01:01	3*02.02:01:02	4*01.03:01		
28	LIBT		LIBT	2032-11T	*02.01	*24.02	*51.01	*52.01	*12:10:02	*14.02	*03.01	*15.02	3*01.01	5*01.02	*01.03	*05.01
28	P6	09/01/2017	S5 LIBT	2032-11T	*02.01:01:01	*24.02:01:04	*51.01:01 NEW	*52.01:08	*12:10.02	*14.02:01	*03.01:01:01	*15.02:01 NEW	3*01.01:02:01	5*01.02		
28	rianalisi P6	04/05/2017	S5 LIBT	2032-11T	*02.01:01:01/16	*24.02:01:04	*51.01:01:03/12	*52.01:01:02	*12:10.02	*14.02:01:01	*03.01:01:01	*15.02:01:01/*15:140	3*01.01:02:01	5*01.02		
28	P11	16/06/2017	S5 LIBT	2032-11T	*02.01:01:01/16	*24.02:01:04	*51.01:01:03/12	*52.01:01:02	*12:10.02	*14.02:01:01	*03.01:01:01	*15.02:01/*15:140	3*01.01:02:01	5*01.02		



Suggestions for NGS validation

Comparison with previous allelic results

Work flow	
Platform	Illumina, Thermo Fisher etc.
Kit used	Commercial or Local
Number of samples	10 up to 20
Number of alleles	

Analyze HLA-A, -B, -C, -DRB1, -DQB1	50 up to 100 test
If used in routine, also analyze HLA-DRB3/DRB4/DRB5, -DQA1, -DPB1	100 up to 200 test



Suggestions for NGS validation

Comparison with previous rare alleles results

DNA - ID	Alleles carachteristic	Standard HLA typing	NGS HLA typing
725-08T	allele new	B*07:69	B*07:69
647-10T	allele new	A*31:48	A*31:48
1180-10T	allele new	C*06:58	C*06:58
1413-10T	allele new	C*16:07:02	C*16:07:02
1087-10T	allele new	C*06:47	C*06:47
146-11	allele new	C*07:195	C*07:195
54-12T	allele new	A*03:143	A*03:143
956-10T	allele new	B*35:240	B*35:240
413-13	allele new	A*24:02:65	A*24:02:65
1734-13	allele new	C*07:02:60	C*07:02:60
1778-14	allele new	C*02:106	C*02:106
2140-15	rare	A*23:18	A*23:18
2072-15	rare	A*02:17:02	A*02:17:02
149-12T	rare	A*02:140	A*02:140
570-09T	rare	B*18:07:01	B*18:07:01
457-12T	rare	B*15:01:06	B*15:01:06
786-14	rare	C*08:33	C*08:33:01



Suggestions for NGS validation

Reproducibility of the method

Repeat Investigation from 2 up to 5 times

Analyze HLA-A, -B, -C, -DRB1, -DQB1

If used in routine, also analyze HLA-DRB3/DRB4/DRB5, -DQA1, -DPB1



Come introdurre e validare la metodica NGS nel laboratorio HLA

Validazione della metodica

Validazione dei risultati ottenuti



HLA typing with NGS

Template generation

First Amplification of HLA loci

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

Clonal amplification

Fragment cluster generation:
- Bridge Amplification (Illumina)
- Isothermal Amplification (Ion Torrent)

Sequencing

Different options:
- Sequencing by Synthesis
- Semiconductor Sequencing
- Single Molecule Real Time PCR

Data analysis

Locus assignment,
Generating consensus sequence



HLA typing with NGS

Template generation

First Amplification of HLA loci

Library preparation

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Clonal amplification

Fragment cluster generation:
- Bridge Amplification (Illumina)
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Sequencing

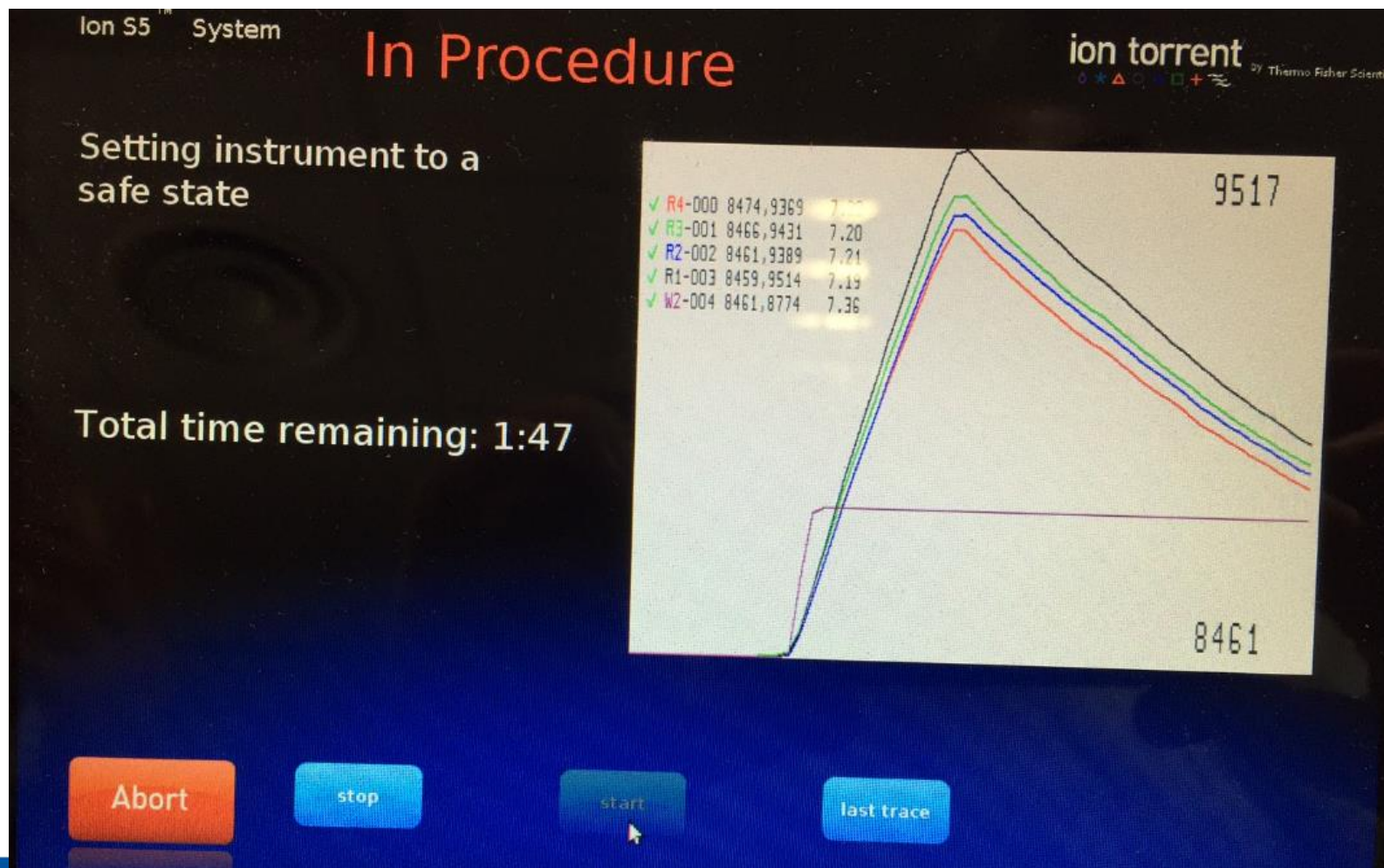
Different options:
- Sequencing by Synthesis
- Semiconductor Sequencing
- Single Molecule Real Time PCR

Data analysis

Locus assignment,
Generating consensus sequence

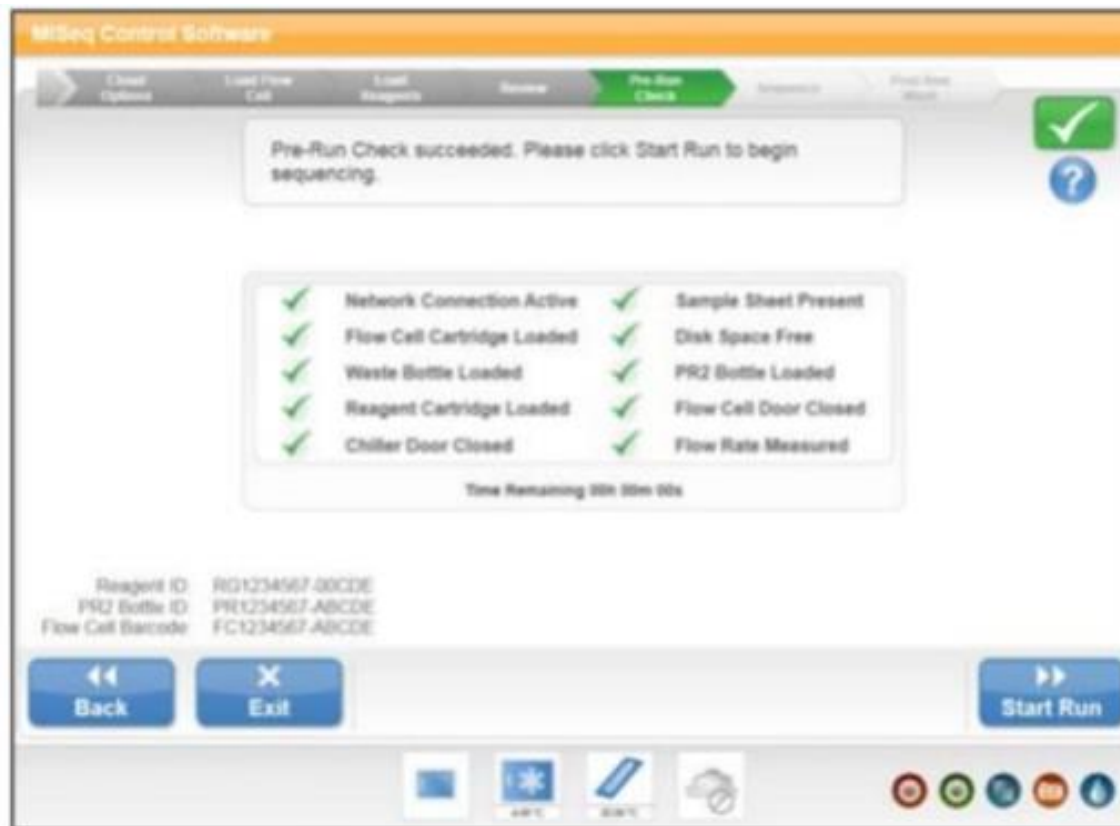


In base alla piattaforma



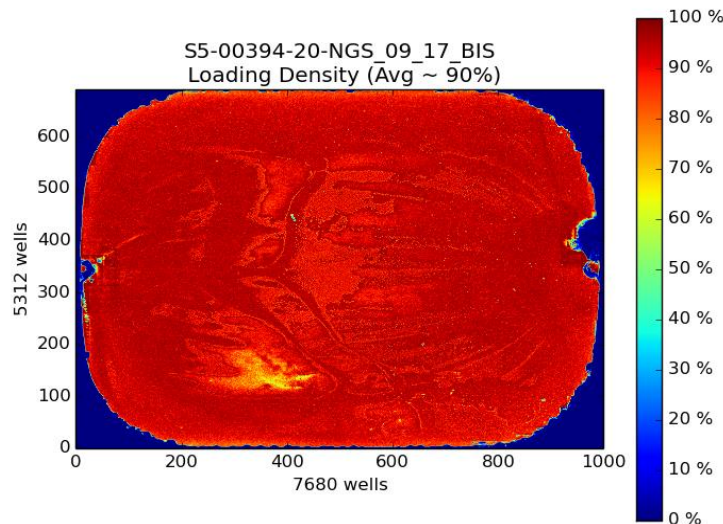
Ion Torrent

In base alla piattaforma



In base alla piattaforma

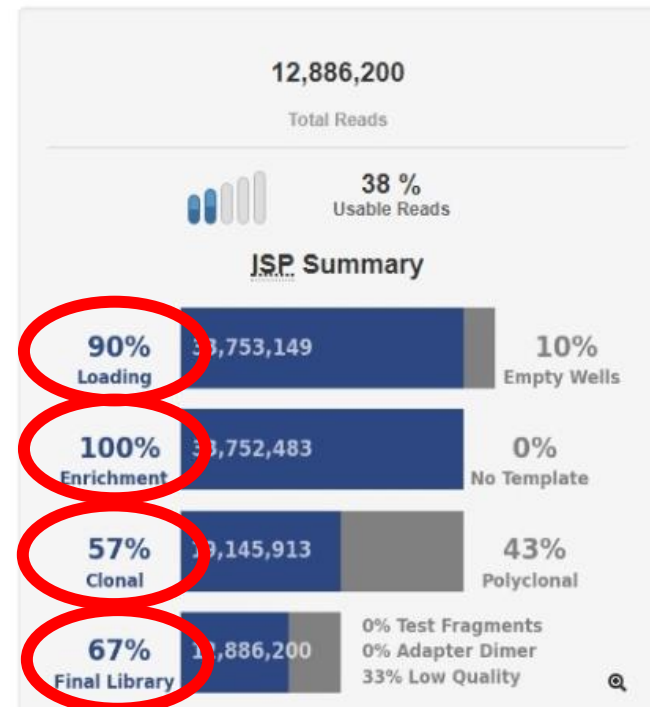
Chip of the new run by NGS



First observation of the data

Optimal colors: **RED, ORANGE**

NOT Optimal colors: **YELLOW, GREEN and BLUE**

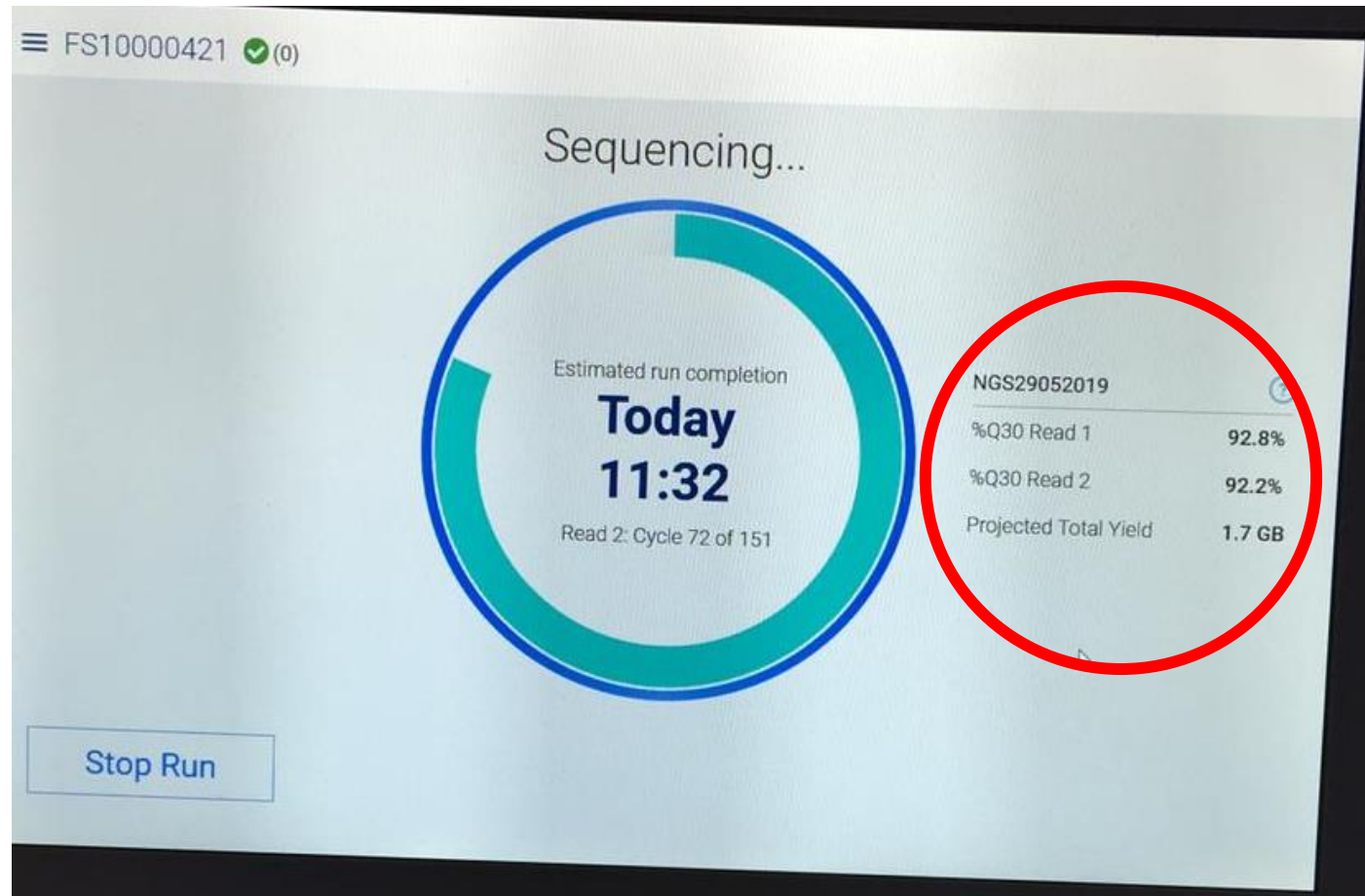


Ion Torrent



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In base alla piattaforma

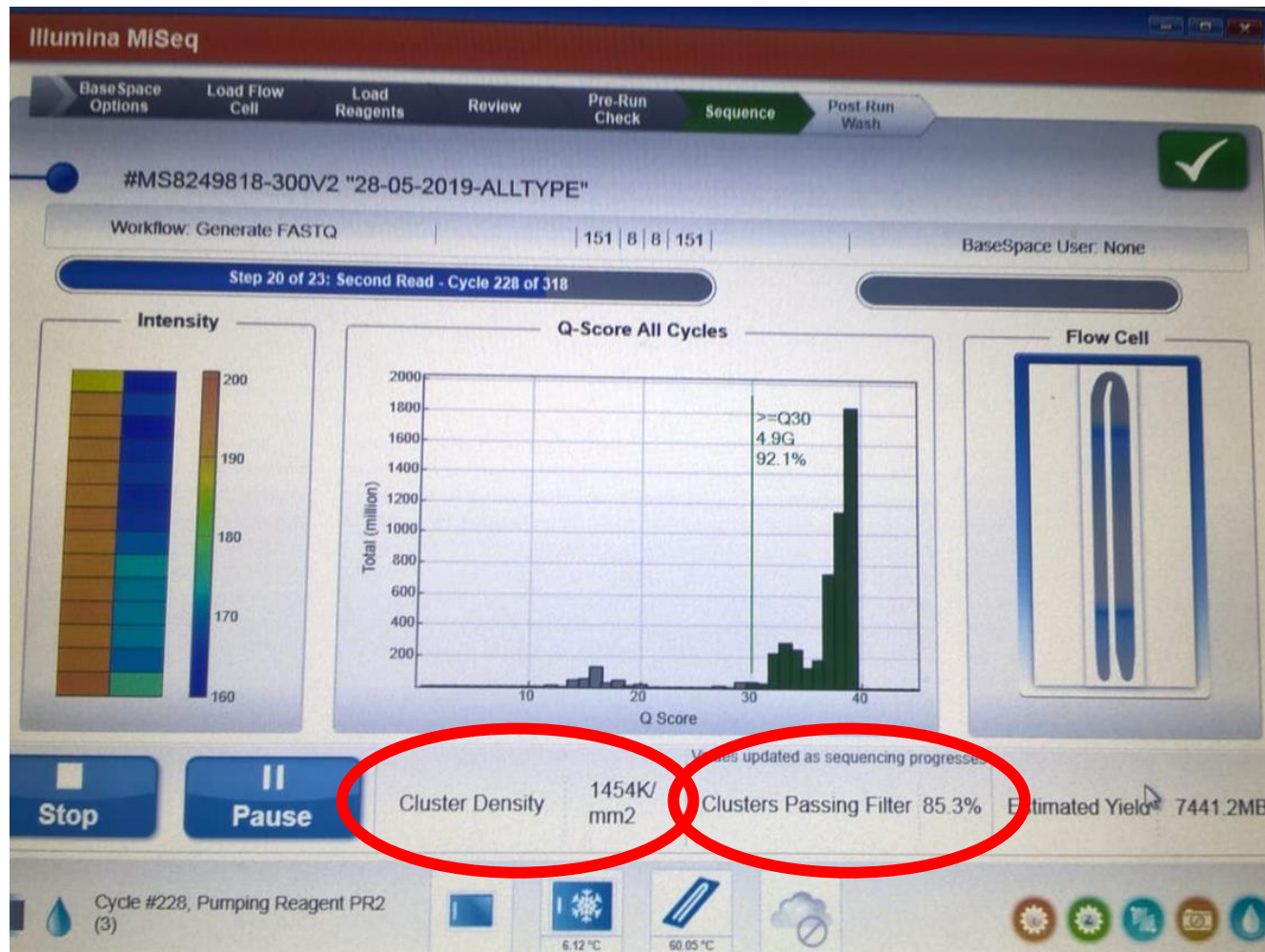


Illumina



Bambino Gesù
OSPEDALE PEDIATRICO

In base alla piattaforma



Illumina



Bambino Gesù
OSPEDALE PEDIATRICO

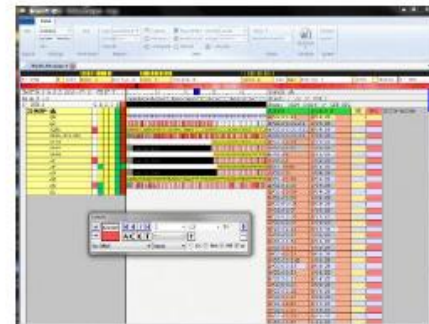
SOFTWARE



Immucor MIA FORA



Ion Torrent HLA Plug In



Illumina Conexio



Omixon HLA Twin

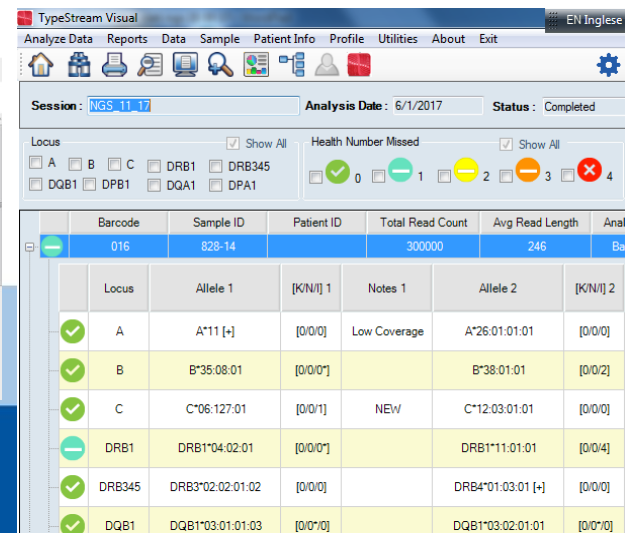
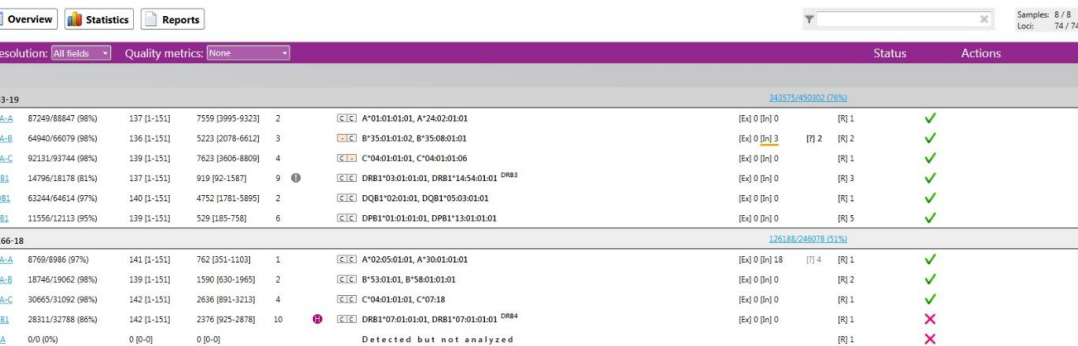


GENDX NGSengine



R.O.S.E. GenTec Ltd.

SOFTWARE



Parametri di qualità



Qualità dei dati influenzata da:

- Bilanciamento allelico nell'amplificazione
- Lunghezza delle reads
- Coverage
- Phasing
- Background



Validazione del risultato

4.4.6 Analisi dei risultati ottenuti

La prima osservazione dei dati va eseguita sui colori che l'immagine del chip riporta sono:

COLORI OTTIMALI: ROSSO E ARANCIONE

COLORI NON OTTIMALI: GIALLO, VERDE E BLU

Successivamente vanno osservati parametri come il **Loading**, che deve essere superiore al 90%, **enrichment** che deve avvicinarsi al 100%, la **clonalità**, che deve essere superiore al 55% e la percentuale di libreria, intorno al 70%. Deve essere controllata inoltre la lunghezza dei frammenti (**read length**), che deve essere compresa in una curva gaussiana tra 100 bp e 450 bp, con una media che deve aggirarsi intorno a 250bp. L'acquisizione dei dati viene eseguita attraverso l'utilizzo del software **Typstream Visual**, secondo le istruzioni ISTR 01 PO 20 che assegna la tipizzazione e il numero dei **mismatches** all'interno degli esoni (MM) per ogni determinata combinazione allelica. L'operatore esegue una prima analisi del primo risultato valutando la presenza di eventuali **mismatches** segnalati dal software. E' molto importante tener presente che il software esclude dall'analisi le zone di DNA complementari ai **primers** utilizzati per l'amplificazione.

4.5 Validazione assegnazione sequenza nucleotidica e alleli

Prima di accettare i risultati di una tipizzazione in NGS, è necessario analizzare i parametri impostati per ogni singolo campione e visibili nel software di interpretazione come da seguente tabella:

Analysis Config:

Analysis Parameter	Value	Analysis Parameter	Value
Min Read Length:	100	Min Valid Reads:	500
Max Insertion:	3	Cut off Value:	20
Max Deletion:	3	Min Hetero Allele Bal:	10
Max Mismatch Bases:	5	Max Read for Typing:	300,000
Min Base Read Depth:	20		

Quality Parameters

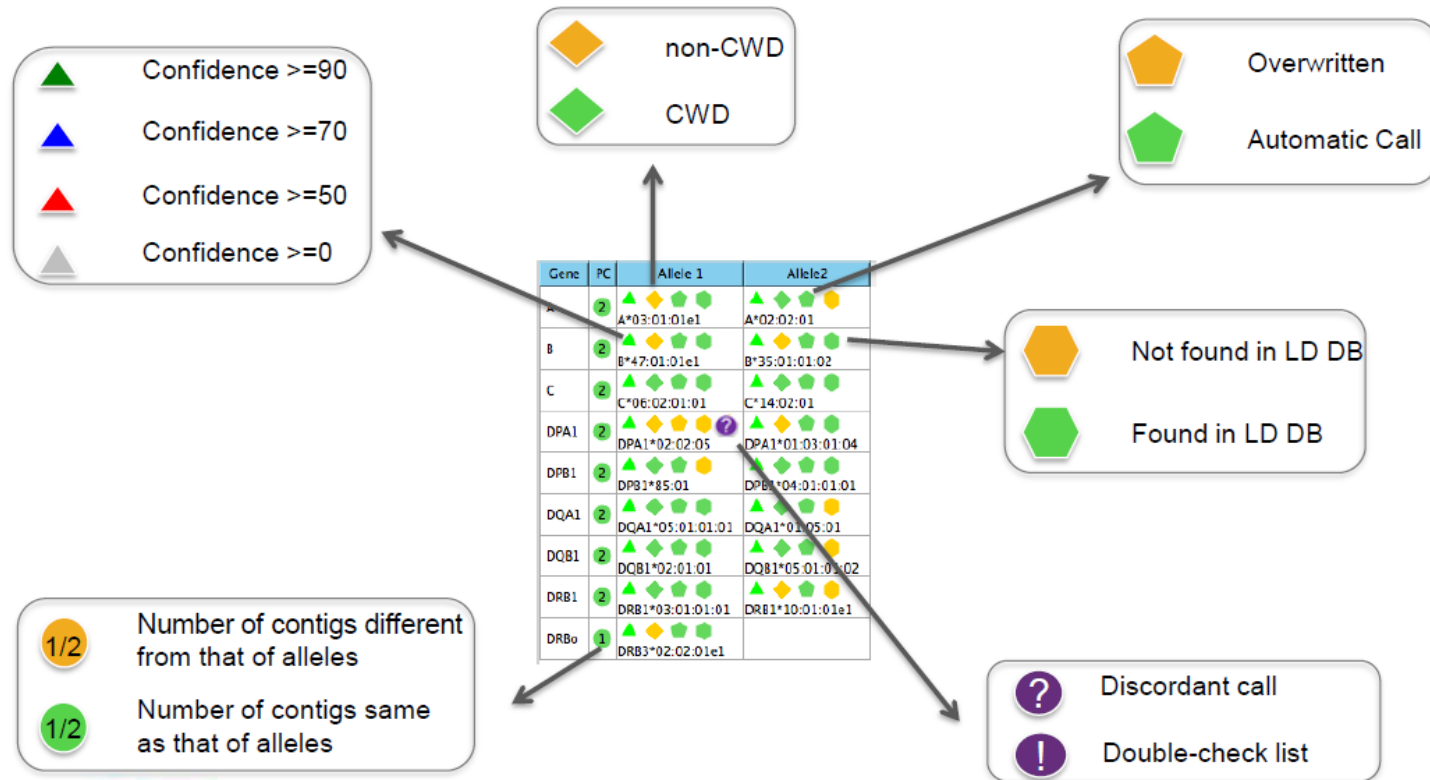
Analysis Parameter	Value
Min Read Length:	100
Max Insertion:	3
Max Deletion:	3
Max Mismatch Bases:	5
Min Base Read Depth:	20
Min Valid Reads:	500
Cut off Value:	20
Min Hetero Allele Bal:	10
Max Read for Typing:	300,000

Immucor



Validazione del risultato

Smart Flag System e codici colori



Il sistema Smart Flag permette di capire immediatamente la qualità del risultato, anche prima dell'analisi manuale, basandosi sul confidence score, sulla qualità delle read e i diversi siti polimorfici individuati.



Validazione del risultato

Call:

- Alleles selected by software
- Green check mark-no warning for selected alleles
- Yellow check mark-warning associated with selected alleles (e.g. CWD, MME, Homozygous)

Sequencing Library QC Metrics

MME: Number of mismatches in exon
MMI: Number of mismatches in intron

Additional mapping parameters in Detail Window

Alele	Call Cmt	MME	MMI	cRead	Cov	Cen	eRead	Cov	Cen	gRead	Cov	Cen	xRead	Cov	Cen
DPB1*04:01:01:01	✓	0	8	8423	329	86	1606	329	86	53375	417	8	52360	655	145
DPB1*04:01:01:02		0	16	8423	329	86	1606	329	86	51431	-21	0	50416	-21	0
DPB1*02:01:02	✓	0	8	8223	319	82	1406	319	82	52831	388	2	51969	547	145
DPB1*01:01i1		1	8	7717	114	11	900	114	11	52321	121	1	51807	547	145
DPB1*01:01		1	999	898	114	11	898	114	11						
DPB1*33:01		1	999	5354	111	5	939	111	5						
DPB1*33:01i1		1	8	7758	111	5	941	111	5	52611	110	3	52024	629	145
DPB1*23:01:01i1		1	8	7736	106	12	919	106	12	52654	119	8	52139	655	145
DPB1*23:01:01		1	999	5332	106	12	917	106	12						
DPB1*105:01		1	999	7748	103	15	931	103	15						
DPB1*165:01i1		1	8	7748	103	15	931	103	15	52341	115	2	51803	547	145

cRead: Number of mapped read against cDNA reference

eRead: Number of mapped read against exons 2&3

gRead: Number of mapped read against genomic reference

xRead: Number of mapped read against all sequence except exons 2&3

Cov: Minimum overall coverage (guideline = >20)

Cen: Minimum central coverage (guideline = >10)



One Lamda



Validazione del risultato

TypeStream™ Visual (IVD in EU only, USA and Canada: For Research Use Only. Not for use in diagnostic procedures.)

Analyze Data Reports Data Sample Patient Info Profile Utilities About Exit

Session TSV_S5_172.16.92.13_173 Analysis Date 25/09/2019 Status Finished Samples 32 Catalog ALL-11LX_012_01 IMGT Ver 3.35.0.0 TypeStream Ver 1.3.0.27232 Engine Ver V1.3.0.37 Analysis Parameters Comment:

Locus ☐ A ☐ B ☐ C ☐ DRB1 ☐ DRB345 ☐ DRB1 ☐ DPB1 ☐ DQA1 ☐ DPA1 ☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4

Health Metric ☐ Key Exon Coverage ☐ Uniformity ☐ Possible Null ☐ Break In Phase ☐ Linkage ☐ High Bgnd Exon ☐ Match in Exon ☐ Allele Balance ☐ Homozygous ☐ Intron Match

Barcode ☐ Show All

Barcode	Sample ID	Patient ID	More Test	Total Read Count	Avg Read Length	Analysis Status	System Comments	User Comments
001	1951-2019		<input type="checkbox"/>	300000	260	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
002	1967-2019		<input type="checkbox"/>	300000	256	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
003	2008-2019		<input type="checkbox"/>	300000	267	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
004	2013-2019		<input type="checkbox"/>	300000	257	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
005	2017-2019		<input type="checkbox"/>	300000	254	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
006	2018-2019		<input type="checkbox"/>	300000	256	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
007	2019-2019		<input type="checkbox"/>	300000	254	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
008	2020-2019		<input type="checkbox"/>	300000	256	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
009	2022-2019		<input type="checkbox"/>	208788	268	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
010	2026-2019		<input type="checkbox"/>	300000	255	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
011	2027-2019		<input type="checkbox"/>	300000	268	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
012	2028-2019		<input type="checkbox"/>	88626	261	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
013	2029-2019		<input type="checkbox"/>	53722	259	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
014	2030-2019		<input type="checkbox"/>	300000	269	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
015	2031-2019		<input type="checkbox"/>	300000	267	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
016	2032-2019		<input type="checkbox"/>	300000	272	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
017	2033-2019		<input type="checkbox"/>	300000	265	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
018	2034-2019		<input type="checkbox"/>	300000	266	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
019	2035-2019		<input type="checkbox"/>	300000	266	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
020	2036-2019		<input type="checkbox"/>	300000	264	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
021	2037-2019		<input type="checkbox"/>	300000	251	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
022	2038-2019		<input type="checkbox"/>	300000	261	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
023	2039-2019		<input type="checkbox"/>	300000	272	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
024	2040-2019		<input type="checkbox"/>	221262	268	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
025	2041-2019		<input type="checkbox"/>	300000	273	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
026	2042-2019		<input type="checkbox"/>	300000	263	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
027	2043-2019		<input type="checkbox"/>	261387	279	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
028	2044-2019		<input type="checkbox"/>	300000	258	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
029	2045-2019		<input type="checkbox"/>	300000	254	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
030	2046-2019		<input type="checkbox"/>	300000	258	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	
031	2047-2019		<input type="checkbox"/>	300000	255	Confirmed	Confirmed Locus/Confirmed By: Altroiano.Bltroiano.Ctro.	

☐ Collapse All ☐ Select All

Replace XX Code Retype Current Barcode Reanalysis Assign All XML Report Genotype Summary (pdf) Genotype Summary (csv) Genotype Summary Health (csv) Close

User Name: 1 Server Name: 172.16.32.23 Database Name: TSV Ver: 1.3.0 SID: TSV S5 172.16.92.13 173



GenDx



Validazione del risultato

Data analysis: NGSengine®

File Users Aligner View Help

GENDX

User: [First reviewer] loes

Library: IMGT 3.18.0-Core

Project: 20150203CR N\ANGS\projects\

Data folders: N\ANGS\data\2015_MiSeq_GenDx\data

Analyze

Pause Resume

Cancel Reset

Overview Statistics Reports

385 / 385 samples

Analysis										Status	Actions	
[P] C1-201-i509-i713_S201										440296/475488 (92%)	✓ X	Reanalyze
HLA-A	91543/100494 (91%)	147 [35-151]	4370 [1235-5225]	1	A*02:01:01:01, A*80:01:01:02	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
HLA-B	104158/113712 (91%)	147 [35-151]	4502 [2602-5192]	1	B*14:01:01, B*44:03:01	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
HLA-C	106113/115190 (92%)	147 [35-151]	4601 [714-5835]	1	C*04:01:01:01, C*08:02:01:02	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
DRB1	26362/31204 (84%)	146 [35-151]	861 [275-1622]	2	DRB1*03:04:01, DRB1*07:01:01:01	[Ex] 0 [In] 0	[R] 2	✓ X	Reanalyze			
DQB1	72371/79696 (90%)	146 [35-151]	2370 [944-3955]	1	DQB1*02:01:01, DQB1*02:02:01	[Ex] 0 [In] 0	[R] 3	✓ X	Reanalyze			
[P] C1-201-i510-i715_S234										338888/368224 (92%)	✓ X	Reanalyze
HLA-A	68450/75760 (90%)	147 [35-151]	3300 [958-3779]	1	A*02:01:01:01, A*80:01:01:02	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
HLA-B	78918/86876 (90%)	147 [35-151]	3423 [1807-4047]	1	B*14:01:01, B*44:03:01	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
HLA-C	79670/87134 (91%)	147 [35-151]	3471 [557-4406]	1	C*04:01:01:01, C*08:02:01:02	[Ex] 0 [In] 0	[R] 1	✓ X	Reanalyze			
DRB1	20665/24892 (83%)	146 [35-151]	662 [254-1254]	2	DRB1*03:04:01, DRB1*07:01:01:01	[Ex] 0 [In] 0	[R] 2	✓ X	Reanalyze			
DQB1	57605/64226 (89%)	146 [35-151]	1909 [716-3084]	1	DQB1*02:01:01, DQB1*02:02:01	[Ex] 0 [In] 0	[R] 3	✓ X	Reanalyze			

Mappability

Read length

Read depth

possible genotypes and typing result

Mismatches

Phasing regions

GENDX, ROW: RUO



Omixon



HLA Typing sample result

Sample: 0517165-DLP-AB6298-4_S4_L001_R1_001
Application build id: 5cbfb97c9ec98a3c24a2e5561a506b057212925c

Browse Alignment

Browse Allele 1

Browse Allele 2

Genotype Details

Show Mismatches

Show Novelties

Setup Loci

Setup Filters

Best Matches Only

Assignment State

Genotype Precision

Assignment

Comments

Send For Approval

In Progress

Approve Result

Cancel Approval

Export Result

Turn LD on/off

Show LD details

State	Allele	HLA-A	HLA-B	HLA-C	HLA-DPA1	HLA-DPB1
✓ In Progress	Allele 1	HLA-A*03:01:01:01	HLA-B*07:02:01	HLA-C*04:01:01:01	HLA-DPA1*01:03:01:04	HLA-DPB1*04:01:01:01 HLA-DPB1*105:01
✓ In Progress	Allele 2	HLA-A*26:01:01:01	HLA-B*44:03:01:01	HLA-C*07:02:01:03	HLA-DPA1*01:03:01:05	HLA-DPB1*126:01 HLA-DPB1*04:02:01:02

HLA-A

HLA-B

HLA-C

HLA-DPA1

HLA-DPB1

HLA-DQA1

HLA-DQB1

HLA-DRB1

HLA-DRB3

HLA-DRB4

HLA-DRB5

Genotype	Quality control	Data statistics
Measure		
Overall	HLA-A PASSED	HLA-B INFO
Primary QCs for Interpretation		
Read count	3412	3416
Noise ratio	0%	0.29%
Key exon spot noise ratio	0%	0%
Consensus coverage key exon minimum depth	78	49
Key exon allele imbalance	0.54 : 0.46	0.63 : 0.37
Genotype available	Yes	Yes
Secondary QCs for Interpretation		
Fragment size	362	359
Read quality	36.24	36.14
Other exon spot noise ratio	0%	0%
PCR crossover artifact ratio	2.81%	1.96%
Key exon mismatch count	0	0
Warnings for Troubleshooting		
Read length	231	233



Parametri di qualità

**Sequencing Library
QC Metrics**



Validazione del risultato

Sequencing Library QC Metrics

Assignments Coverage Stats **Health Stats** Variant

Health Metric	Standard	Value
Full Key Exon Coverage	= 100%	= 100.00%
Uniformity	< 1	= 0.28
Allele Balance	> 0.3	= 0.90
Mismatch in Exon	= 0	= 0.00

Mapped Read Metrics	Value
Reads for Typing	14145
Forward Read Count	7171
Averaged Forward Read Length	289
Reverse Read Count	6974
Averaged Reverse Read Length	289

Data relative to each single allele analyzed



Validazione del risultato



Overview

> 133-19 HLA-A

Sequencing Library QC Metrics

Alignment Statistics Genotype ranking XML Report SNP Calling Approval Quality metrics

Data quality metrics

Mappability perct. [accepted / total reads]	98% [87249 / 88847]
? Read length (median)	151
? Insert size (median)	546

	Core+	Exon+	Amplicon
? Read depth			
Median	7546	7510	7559
Minimum	4500	4097	3995
? Coverage	100 %	100 %	100 %
? QV (median)	37	37	37
? Noise			
Median	0.2 %	0.2 %	0.2 %
Maximum	5.0 %	5.0 %	5.0 %

Analysis quality metrics

? Analyzed	100 %	100 %	100 %
Ignored positions count	0	0	0
Heterozygous positions count	32	46	86
? Delta signal to noise			
Median	36.4 %	36.4 %	35.8 %
Minimum	36.4 %	36.4 %	35.8 %
? Second allele			
Median	45.3 %	44.5 %	43.9 %
Minimum	41.4 %	41.4 %	40.8 %
? Phased regions	-	-	1
Mismatches	0	0	0
? Question mark positions	-	-	-

Sequencing Library QC Metrics



Validazione del risultato

Qualità dei risultati dei singoli campioni

Sample: Barcode_D01 [Ctrl] ▼

Locus: A ▼

Last visited: 2017-06-14T11:14:16

Sample QC

✓ Approve ✓ Confirm

PC	Allele 1	Allele 2
2	A*24:02:01:01	A*03:01:01:01
2	B*55:01:01	B*35:03:01e1
2	C*03:03:01e1	C*04:01:01:01
2	DRB1*14:54:01	DRB1*12:01:01:03
1	DRB3*02:02:01:01	
2	DQA1*01:04:01:03	DQA1*05:05:01:05
2	DQB1*05:03:01:01	DQB1*03:01:01:01
1	DPA1*01:03:01:01	DPA1*01:03:01:05
2	DPB1*02:01:02	DPB1*04:02:01:02

MIA FORA

QC Metrics Sample: Ctrl 3 (Barcode_D01)

Context	Key	Value
Barcode_D01	RawReadCount	828857
Barcode_D01	MappedReadCount	474896

Context	FragLength (min, avg, max)	ReadLength (min, avg, max)
Project	132 ,475, 716	142 ,142, 142
Barcode_D01	144 ,451, 680	142 ,142, 142

Minimum and maximum fragment lengths are 5th and 95th percentile fragment lengths, respectively.

Context	A	B	C	DPA1	DPB1	DQA1	DQB1	DRB1	DRB3
Project Total Mapped Reads	655616	641054	815064	402072	1.07877e+06	753241	1.13952e+06	1.00958e+06	N/A
Project Normalized Reads	30.4137	23.9856	28.7959	12.3787	31.6794	19.4489	26.6435	22.1332	N/A
Barcode_D01 Average Coverage	907	648	555	718	1330	1173	1442	2013	4235
Barcode_D01 Mapped Reads	36134	38156	49191	21921	63568	59522	71461	62849	N/A


Average coverage depth minimum for automated call: 40

Per ogni singolo campione è disponibile l'analisi qualitativa del risultato basato su:

- 1 - Il numero di read Q30 utilizzate per l'analisi
- 2 - La lunghezza minima, media e massima delle read
- 3 - Il numero di read utilizzate per l'analisi dei singoli loci

Data analysis

Locus assignment,
Generating consensus sequence

 Bambino Gesù OSPEDALE PEDIATRICO		Monitoraggio Qualità NGS					PO 20 MD 02 01/08/2018		Pag. 2		
FILE: \\srvopbgfs4\Dip-FS4\Dip010-DOEMT\10786-HLA\Archivio\AAA - CARTELLA CONDIVISI\Qualita\Procedure\1 - In uso\2 - Allegati in uso							PO di riferimento: PO 20				
Amp	KIT	Lotto	scadenza	Ditta	data	#DNA	Cod Anomalia	Natura dell'anomalia	Note		
15-18	ALLTYPE	008	01/2019	onelambda	09/11/2018	2030-18	4	low coverage ex2 B	confermare B in SBT/confermato		
15-18	ALLTYPE	008	01/2019	onelambda	09/11/2018	2056-18	4	low coverage ex2 B	confermare B in SBT/confermato		
15-18	ALLTYPE	008	01/2019	onelambda	09/11/2018	2058-18	4	low coverage ex2 B	confermare B in SBT/confermato		
18-18	ALLTYPE	008	01/2019	onelambda	09/11/2018	2201-18	2		Ripetere/ripetuto ok		
01-19/59	ALLTYPE	009	01/2019	onelambda	04/01/2019	24-19	2		Ripetere/ripetuto ok		
01-19/59	ALLTYPE	009	01/2019	onelambda	04/01/2019	25-19	2		Ripetere/ripetuto ok		
01-19/59	ALLTYPE	009	09/2019	onelambda	04/01/2019	17-19	4	numero di reads lette sotto il limite di	Confermare/confermato		
05-19	ALLTYPE	009	09/2019	onelambda	23/01/2019	133-19	3	MM EX1	CONFERMATO DA SBT		
06-19/71	ALLTYPE	009	09/2019	onelambda	24/01/2019	168-19	2		Ripetuto 2 volte NV e fatto xr		
06-19/71	ALLTYPE	009	09/2019	onelambda	24/01/2019	170-19	2		Ripetere/ripetuto ok		
09-19/77	ALLTYPE	009	09/2019	onelambda	06/02/2019	302-19	3	MM DQA1 EX2	Confermare/confermato		
15-19	ALLTYPE	009	09/2019	onelambda	01/03/2019	454-19	4	allele raro DPB1	Confermare/confermato		
15-19	ALLTYPE	009	09/2019	onelambda	01/03/2019	488-19	3	MM C EX5	Confermare/confermato		
16-19	ALLTYPE	011	09/2019	onelambda	08/03/2019	538-19	1	Sbilanciamento DQB1	Tipiz. Confermata da studio familiare		
17-19	ALLTYPE	011	09/2019	onelambda	15/03/2019	561-19	4	reads basse	MM ex3 DPB1 escluso allele new ripetuto 18/19		
19-19	ALLTYPE	011	09/2019	onelambda	22/03/2019	606-19	3	MM ex7 B forse allele new ripetere	CONFERMATO MM EX7		
20-19/99	ALLTYPE	011	09/2019	onelambda	29/03/2019	704-19	½	drop out DRB1/DQA1 e DPB1 NV	Ripetere/ripetuto ok		

monitor and share written information within the group



Validazione dei reagenti



HLA typing with NGS

Template generation

First Amplification of HLA loci

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

Clonal amplification

Fragment cluster generation:
- Bridge Amplification (Illumina)
- Isothermal Amplification (Ion Torrent)

Sequencing

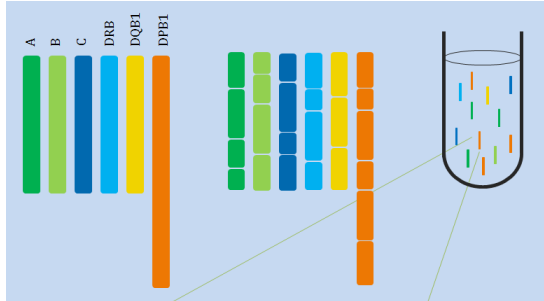
Different options:
- Sequencing by Synthesis
- Semiconductor Sequencing
- Single Molecule Real Time PCR

Data analysis

Locus assignment,
Generating consensus sequence



Library preparation



The first main step in preparing nucleic acid for NGS is **fragmentation** DNA in millions of short reads included between 300 and 1400 bp.

Fragmentation and end-preparation

Barcoding
Size selection
Second Amplification
Pooling

Shotgun sequencing

Physical Fragmentation

- Acoustic shearing
- Sonication
- Hydrodynamic shear

Enzymatic Methods

- **DNase I or other restriction endonuclease, non-specific nuclease**
- Transposase

Chemical Fragmentation

- Heat and divalent metal cation

	TIPIZZAZIONE HLA MEDIANTE NGS		Cod.: ISTR_01 PO 20
			Data: 15/09/2018
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			Pagina 14 di 34

4.6 FRAGMENTATION (Tempo ~ 30 min)

Reagenti

- ION SHEAR PLUS REAGENTS KITS (parte del ION XPRESS PLUS FRAGMENT LIBRARY KIT, -20°C)
- ION SHEAR PLUS ENZYME MIX II (tappo trasparente, 2 provette da unire)
- ION SHEAR PLUS 10x REACTION BUFFER (tappo trasparente, 2 provette da unire)
- Buttare tutto il resto del kit che non serve (STOP BUFFER)

Procedura

1. Accendere il termociclatore.
2. Avviare il programma "Fragmentation PCR Program":

Fragmentation PCR Program			
Step	Temp	Time	# of Cycles
Step 1	37°C	6 min	1
Step 2	70°C	10 min	1
Step 3	4°C	∞	1

3. Settare il volume a 49 µL.
4. Mettere la piastra con gli amplificati a 100 ng in ghiaccio
5. Prendere il numero sufficiente di kit ION SHEAR PLUS REAGENTS KIT, considerando che ogni kit è sufficiente per 20 campioni
6. Spinnare l'ENZYME MIX II e metterlo subito in ghiaccio
7. Scongellare a temperatura ambiente il 10x Reaction Buffer

Library preparation

Fragmentation and end-preparation

Barcoding

Size selection

Second Amplification

Pooling

ATGGACGATAGT GA GATTACGCAGA

TATATACG

CAT G

ATGGACGATAGT GA GATTACGCAGA

CAGGTACG

Adapter 1

Barcode

Unknown sequence

Adapter 2

TATATACG

T GCA

ATGGACGATGGACATAGTTACGC

CAGGTACG

TATATACG

T GCA

GGACGATGGACATAGTTA

CAGGTACG

TATATACG

T GCA

TACGCCGATGGACATAGT

CAGGTACG

TATATACG

CAT G

GATGGACATGT GA GATTACGCACGGCA

CAGGTACG

Adapter 1: is identical across all samples

Adapter 2: is identical across all samples

Barcode (index): is unique to each sample



Library preparation

Fragmentation and end-preparation
Barcoding
 Size selection
 Second Amplification
 Pooling

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4.7 BARCODE LIGATION & NICK-REPAIR (Tempo ~ 1h)

Reagenti

✓ ION PLUS FRAGMENT LIBRARY KIT (parte del ION XPRESS PLUS FRAGMENT LIBRARY KIT), di cui 3 reagenti saranno utilizzati nella 2° amplificazione (Platinum PCR Supermix High Fidelity e la Library Amplification Primer mix, mentre il LOW TE viene messo da parte a T.A.)

- 10x LIGASE BUFFER (tappo giallo)
- DNA LIGASE (tappo celeste)
- NICK REPAIR POLYMERASE (tappo trasparente)
- dNTP MIX (tappo viola)

✓ ION XPRESS BARCODE ADAPTERS KIT

- ION XPRESS P1 ADAPTER (tappo viola)
- ION XPRESS BARCODES (tappo bianco, si consiglia di aliquotarlo in strip da 8 well, per ogni singolo barcode possiamo fare 10 test)

✓ Buttare tutto quello che non serve del kit (provette tappo Rosso, Arancione, Verde)

Procedura

1. Accendere il termociclatore e impostare il programma "Ligate and Nick-Repair Program"
2. Assicurarsi che il volume impostato sia 100 µL.
3. Prendere il ION PLUS FRAGMENT LIBRARY KIT e il ION EXPRESS BARCODES and ADAPTERS P1
4. Spinnare il DNA LIGASE e il NICK REPAIR POLYMERASE, mettere tutto in ghiaccio

Barcode	Plate Position	DNA ID
49	1	542-19
50	2	543-19
51	3	547-19
52	4	561-19
53	5	562-19
54	6	563-19
55	7	567-19
56	8	569-19
57	9	580-19
58	10	581-19
59	11	585-19
60	12	586-19
61	13	587-19
62	14	588-19
63	15	599-19
64	16	600-19

Double control from a second operator

Library preparation

Barcode	Plate Position	DNA ID
65	1	605-19
66	2	606-19
67	3	607-19
68	4	635-19
69	5	640-19
70	6	642-19
71	7	646-19
72	8	647-19
73	9	651-19
74	10	652-19
75	11	653-19
76	12	654-19
77	13	655-19
78	14	656-19
79	15	657-19
80	16	658-19
81	17	659-19
82	18	660-19
83	19	661-19
84	20	662-19
85	21	663-19
86	22	664-19
87	23	668-19
88	24	669-19

Fragmentation and end-preparation

Barcoding

Size selection

Second Amplification

Pooling

Barcode	Plate Position	DNA ID
49	1	542-19
50	2	543-19
51	3	547-19
52	4	561-19
53	5	562-19
54	6	563-19
55	7	567-19
56	8	569-19
57	9	580-19
58	10	581-19
59	11	585-19
60	12	586-19
61	13	587-19
62	14	588-19
63	15	599-19
64	16	600-19

Double control from a second operator



Library preparation

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		Data: 15/09/2018
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Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

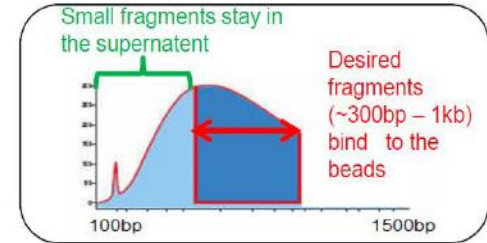
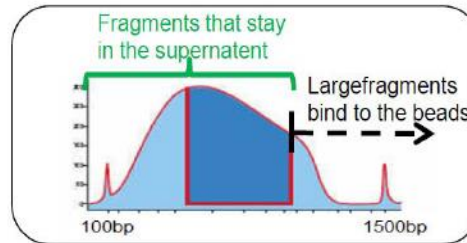
4.8 SIZE-SELECTION (Tempo ~ 1h)

Reagenti

- AGENTCOURT AMPURE XP BEADS
- LOW TE BUFFER
- ETHANOL
- NUCLEASE-FREE WATER

Procedura

1. Prendere le biglie dal frigo +4°C e portarle a temperatura ambiente
2. Prendere due piastre Round-Bottom e nominarle come Piastra 1 e Piastra 2
3. Preparare una piastra PCR per la size-selection.
4. Vortexare le biglie a velocità media per 30''
5. Calcolare il giusto volume delle biglie usando 63,1 uL di biglie per campione con un eccesso del 15% (per esempio: 16 campioni: 1262 uL; 24 campioni: 1742 uL; 48 campioni: 3483 uL)
6. Versare il volume delle biglie in un Reservoir.
7. Nella piastra 1 (Round-Bottom), trasferire 48,5 uL di biglie in ogni pozzetto con una multicanale (*selezione di frammenti di piccola taglia*)
8. Trasferire 97 uL dei campioni ligati nei corrispettivi pozzetti della piastra 1
9. Spipettare per 5-7 volte con gli stessi puntali usati nel trasferimento
10. Incubare a temperatura ambiente per 5 minuti
11. Durante l'incubazione, risospendere e trasferire 14,6 uL di biglie dal Reservoir alla piastra 2 (*selezione di frammenti di grande taglia*)



Short and too long fragments must be eliminated to select correct size fragments

TAPE station or subsequent software analysis

Double control from a second operator



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Library preparation

Fragmentation and end-preparation
 Barcoding
 Size selection
Second Amplification
 Pooling

Barcode	Plate Position	DNA ID	Co
65	1	605-19	
66	2	606-19	
67	3	607-19	
68	4	635-19	
69	5	640-19	
70	6	642-19	
71	7	646-19	
72	8	647-19	
73	9	651-19	
74	10	652-19	
75	11	653-19	
76	12	654-19	
77	13	655-19	
78	14	656-19	
79	15	657-19	
80	16	658-19	
81	17	659-19	
82	18	660-19	
83	19	661-19	
84	20	662-19	
85	21	663-19	
86	22	664-19	
87	23	668-19	
88	24	669-19	

Double control from a second operator

Suggestions for NGS validation

KIT

NGS PRODUCTS	
1st PCR	PRIMERS (COMMERCIAL OR HOME MADE)
LIBRARY PREPARATION	BARCODE
	FRAGMENTS PREPARATION
	ADAPTERS
	QUBIT REAGENTS
	PRODUCTS FOR AUTOMATION
NGS AMPLIFICATION	OTHERS
	REAGENTI SPECIFICI PER PIATTAFORMA: CHIPS OR CELL FLOW

Test a known sample in each new NGS run



Attenzione al back-up dei dati



Attenzione al back-up dei dati

E4.10.9.5 Storage and back-up of data (input, raw data, intermediate and final data) must be defined in accordance with the national laws



HiSeq2000/2500



Ion PGM



Ion Proton



Pacbio RS II



NextSeq500

Analysis and storage of the row data
Insufficient to maintain all the data



Transferring the row data



The real back-up



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Roma, 19 novembre 2019

Many thanks for your attention

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