



Associazione Italiana di Immunogenetica e Biologia dei trapianti

SUMMER SCHOOL AIBT

Ercolano (Na), 13 – 15 Giugno 2019

La gestione della qualità in NGS

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Dipartimento di Oncematologia e Terapia Cellulare e Genica
IRCCS Ospedale Pediatrico Bambino Gesù



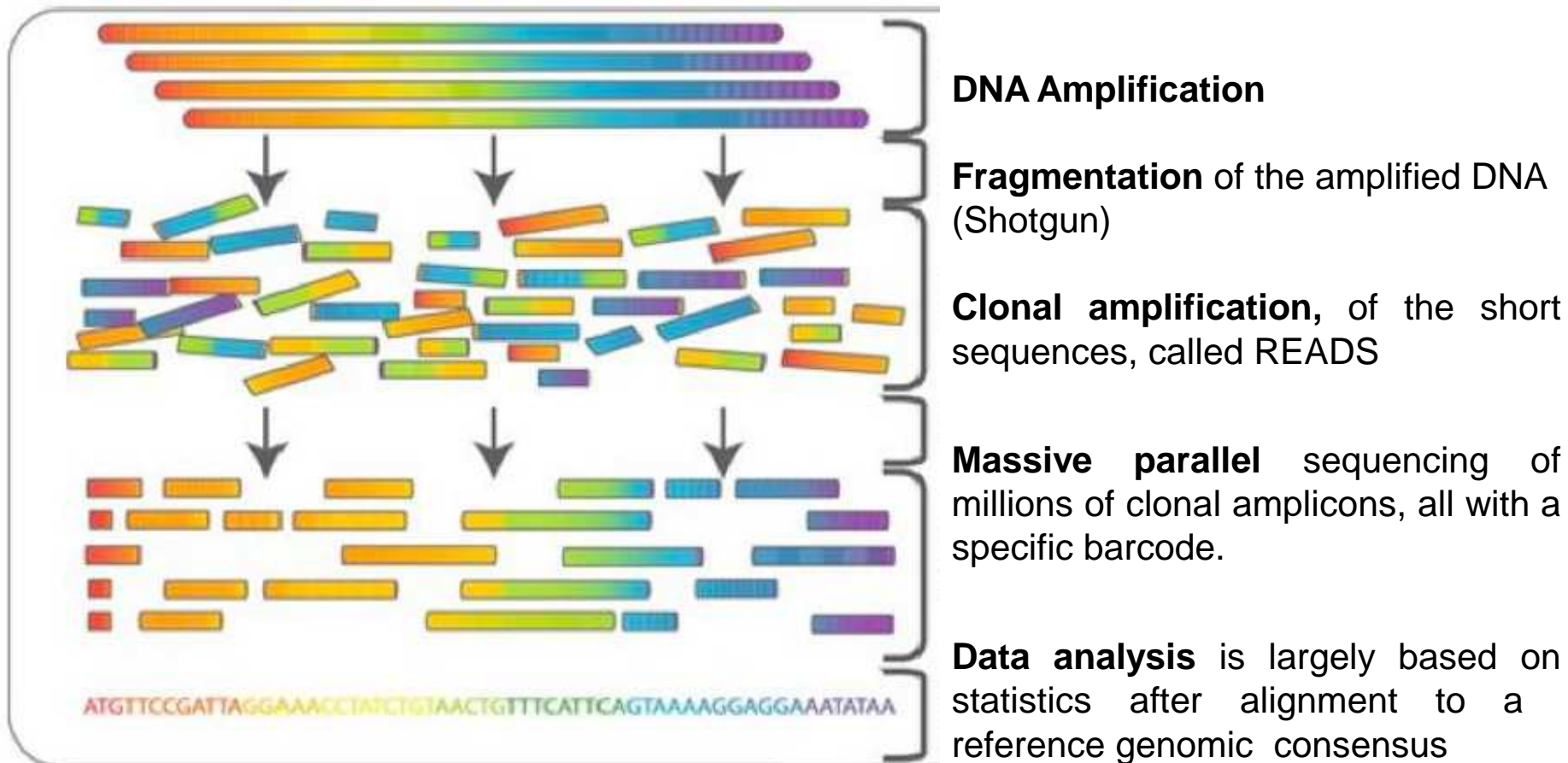
Comune di Roma



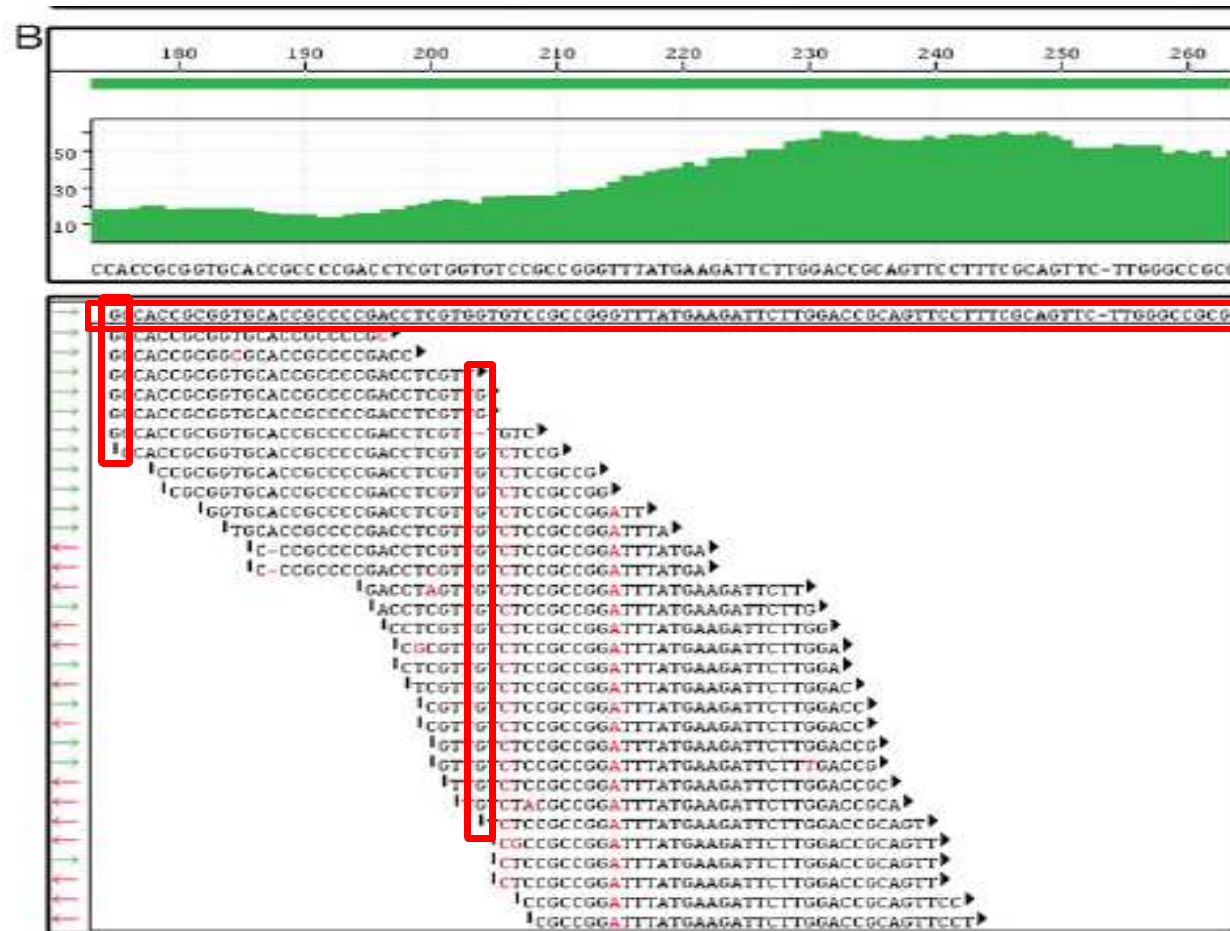
- **Overview on NGS technology**
- **EFL standards and NGS**
- **Validation of the method before the introduction in routine**

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- EFL standards and NGS
- Validation of the method before the introduction in routine

How NGS works



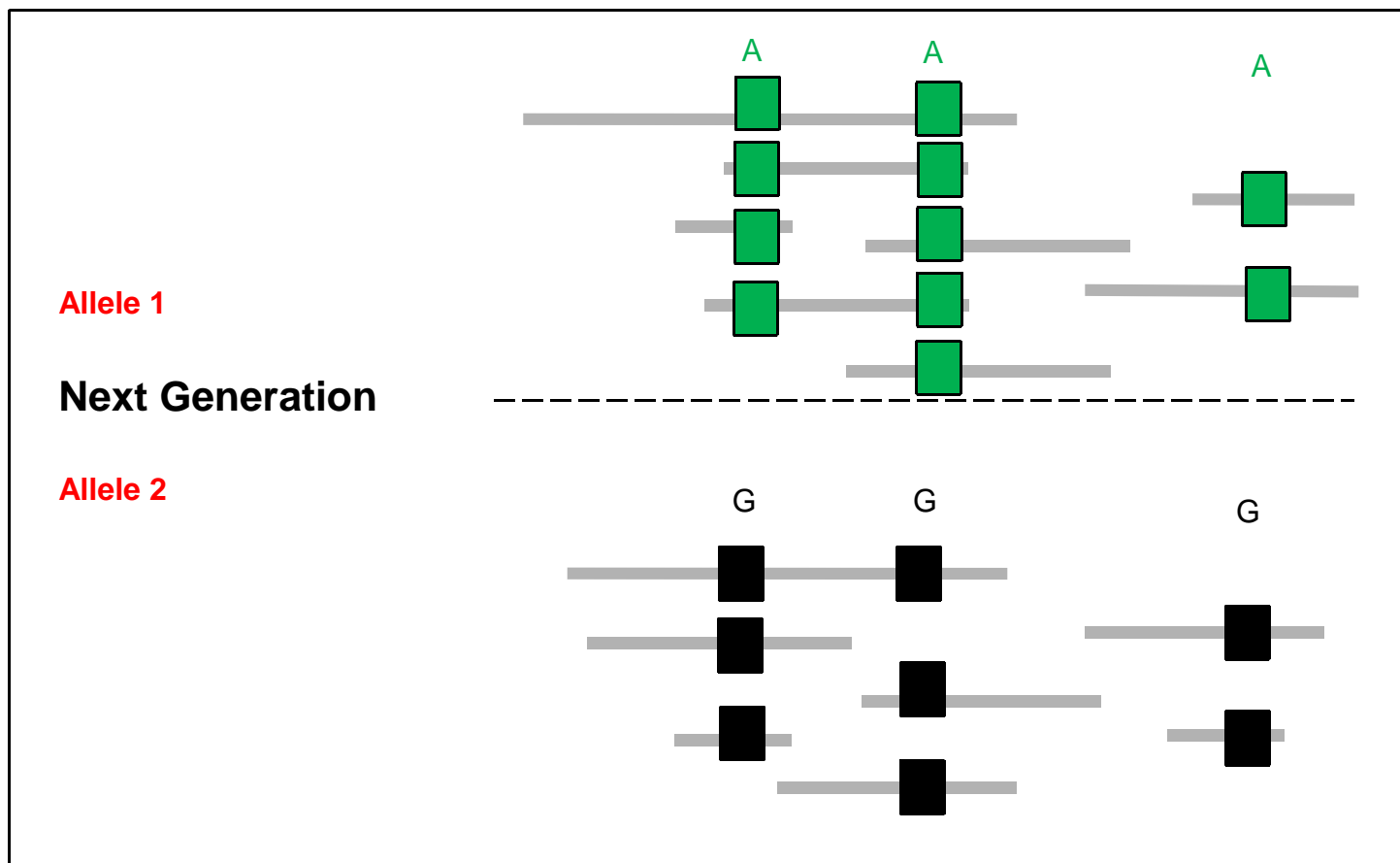
COVERAGE DEPTH



How NGS works

1 - Solve ambiguities

in phase



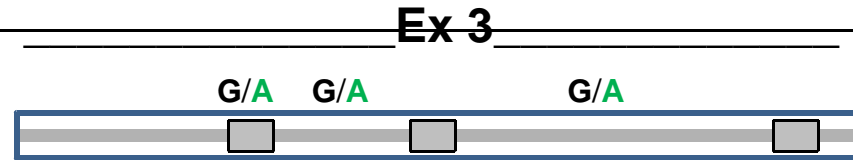
Sequencing by Sanger method (SBT)

First PCR

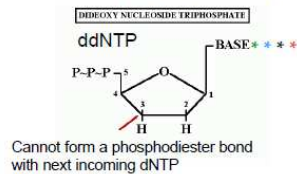
HLA Class I Ex2 and Ex3 (Ex4)



Sanger Allele 1&2 EX 3
...some ambiguities...



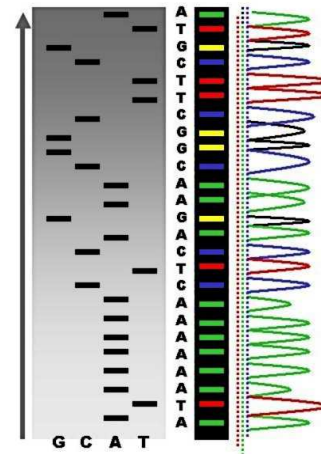
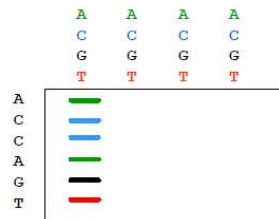
1. Dideoxy chain termination



2. Electrophoretic separation of the extension fragments

cttggacttACTGGTCTA

T*
 TG*
 TGA*
 TGAC*
 TGACCA*

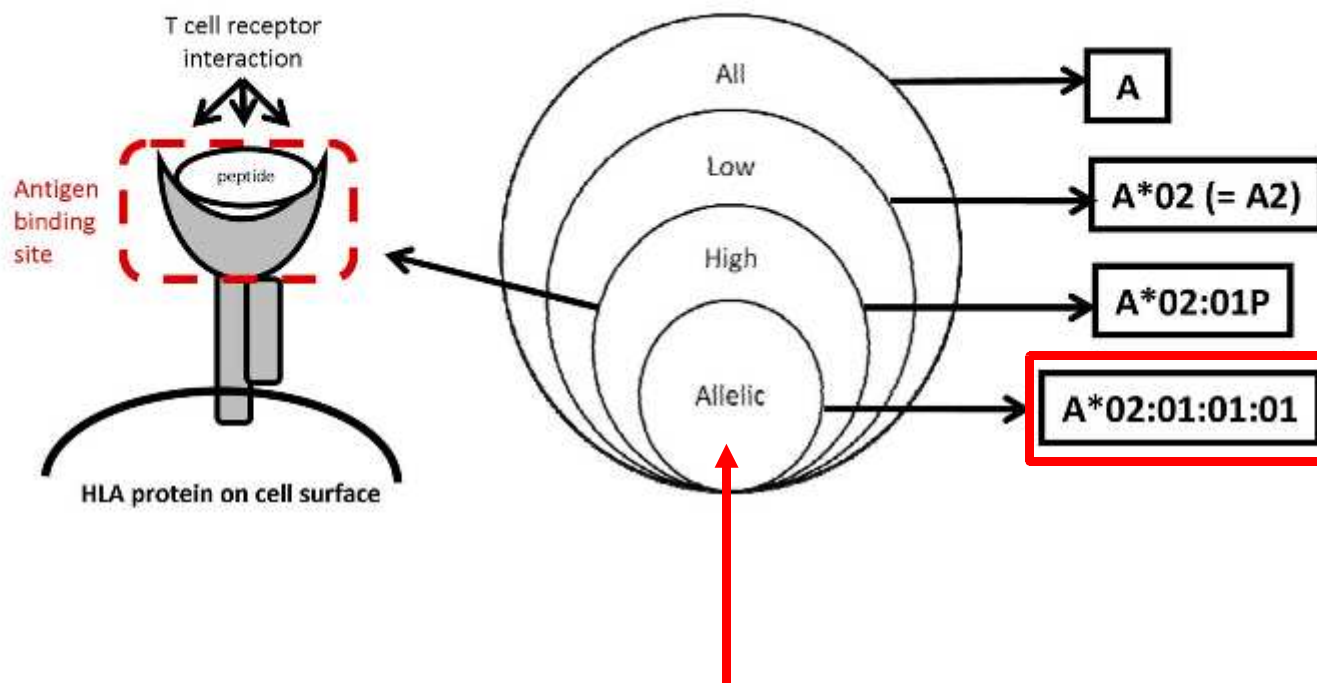


Combination of different techniques to solve allelic or cis/trans ambiguities

How NGS works

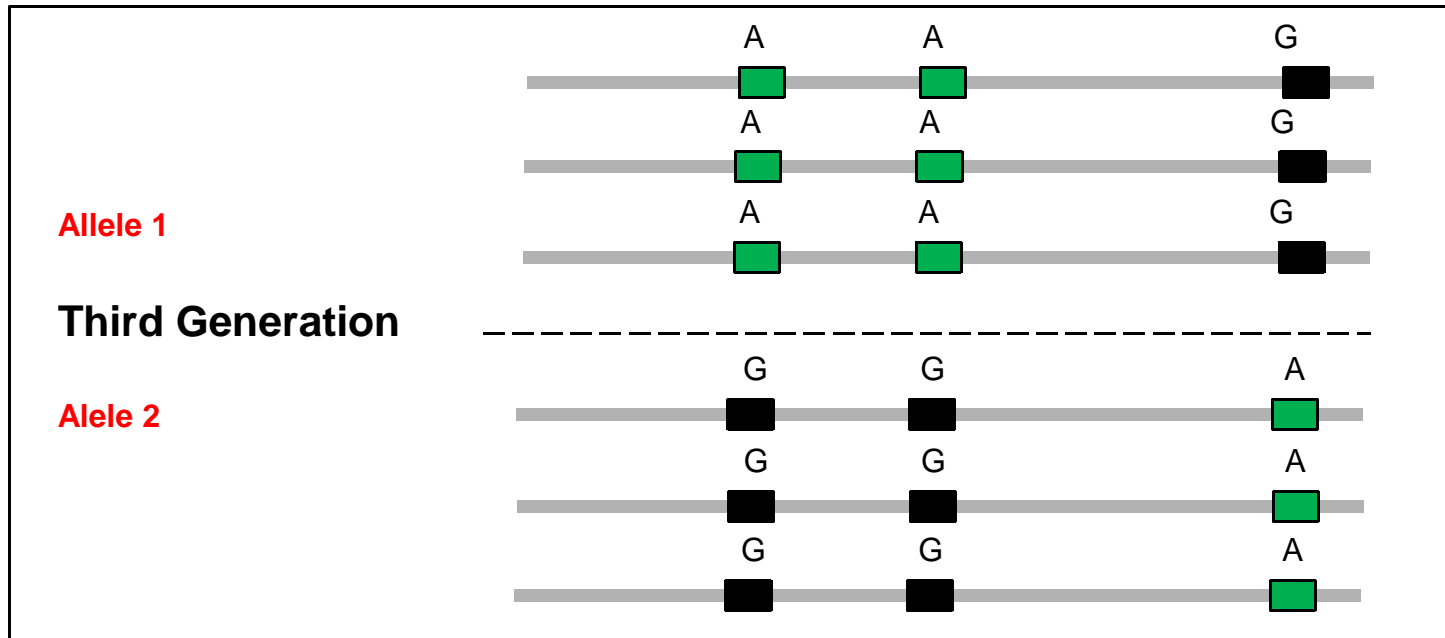
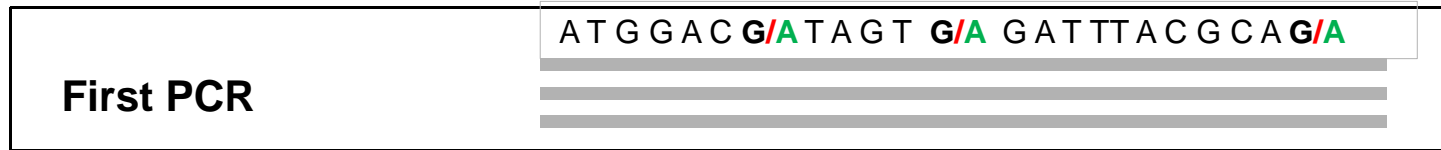
BLOOD, 1 DECEMBER 2011 • VOLUME 118, NUMBER 23

DEFINITIONS OF HISTOCOMPATIBILITY TYPING TERMS e181



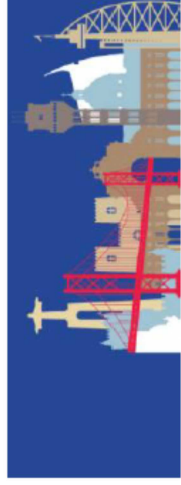
2 - Allelic resolution

How third generation sequencing works



in phase

Long reads sequencing (Nanopore, Biopacific)



**33rd EUROPEAN IMMUNOGENETICS AND
HISTOCOMPATIBILITY CONFERENCE**
FUNCTIONAL IMMUNOGENETICS: THE HISTORICAL CHALLENGE

MAY 8-11, 2019
CENTRO CULTURAL DE BELEM
LISBON PORTUGAL

- Overview on NGS technology
- **EFL standards and NGS**
- Validation of the method before
the introduction in routine

EFI standards: version 7.0

E4.10 Next Generation Sequencing

....and many others "common standards"

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

Fragmentation and end-preparation
Barcoding
Purification
Size selection
Second Amplification
Pooling

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Sequencing

Different options:
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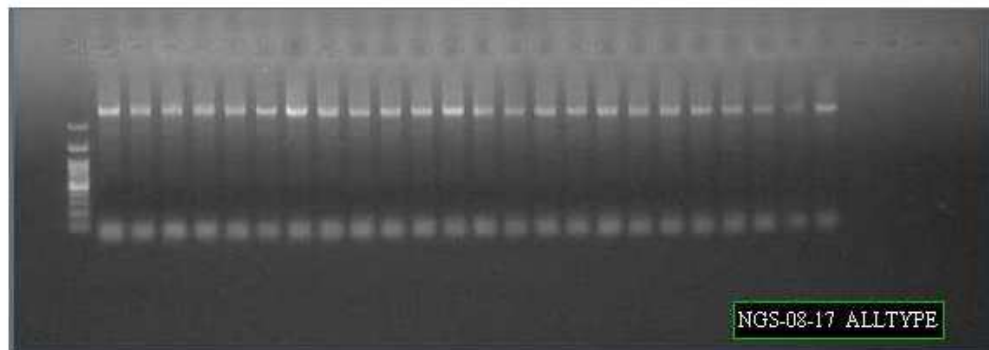
Data analysis

Locus assignment,
Generating consensus sequence

Template generation

First Amplification of HLA loci

Analyze first PCR products, running on 1% agarose gel



PURIFICATION WITH MAGNETIC BEADS



E4.10.1.1 Must have sufficient purity, specificity, quantity and quality to provide interpretable sequencing data

E4.10.3 Steps must be taken to prevent creation of PCR artefacts

E4.10.1.2 Should be purified after amplification to eliminate the presence of dNTPs, Taq polymerase and amplification primers

E4.10.2 The following must be documented:

E4.10.2.2 Purification



HLA typing with NGS

Template generation

First Amplification of HLA loci

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Barcoding
Size selection
Second Amplification
Pooling

Clonal amplification

Fragment cluster generation:
- Bridge Amplification (Illumina)
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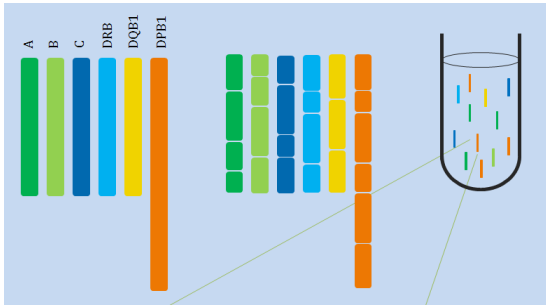
Sequencing

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

Data analysis

Locus assignment,
Generating consensus sequence

Library preparation



E4.10.6 If shotgun sequencing is used

E4.10.6.1 Method of fragmentation must be specified

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 |
| | | Ed. 3 – Rev. 0 |
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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. Spinare l'ENZYME MIX II e metterlo subito in ghiaccio
7. Scongellare a temperatura ambiente il 10x Reaction Buffer

Library preparation

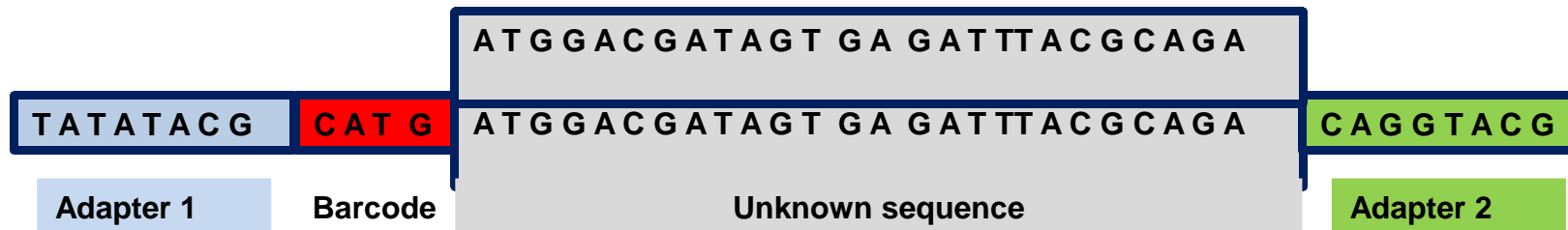
Fragmentation and end-preparation

Barcoding

Size selection

Second Amplification

Pooling



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

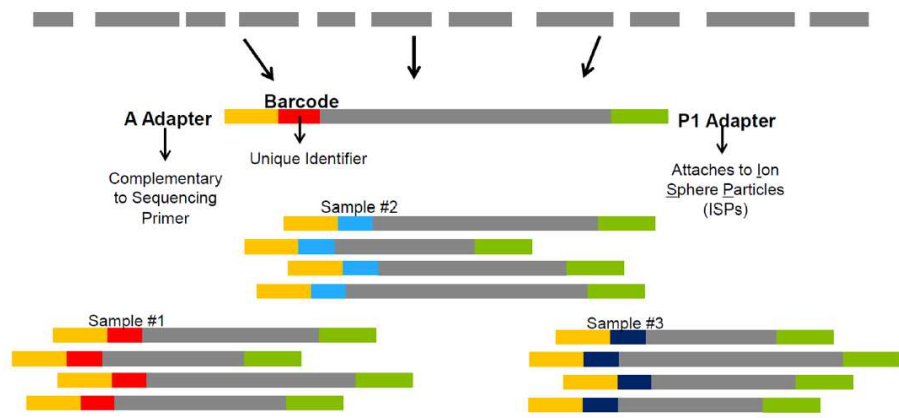
Barcoding

Size selection

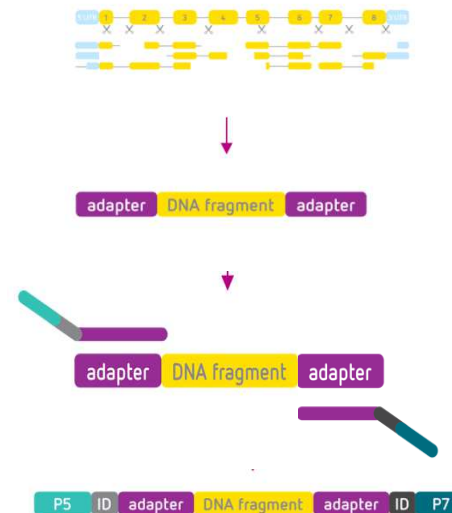
Second Amplification

Pooling

Ion Torrent



Illumina



E4.10.2

E4.10.2.1

The following must be documented:

Sample tagging

Library preparation

Fragmentation and end-preparation
Barcoding
 Size selection
 Second Amplification
 Pooling

| | | |
|---|--|---------------------|
|  | TIPIZZAZIONE HLA MEDIANTE NGS | Cod.: ISTR 01 PO 20 |
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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^a amplificazione (Platinum PCR Supermix High Fidelity e la Library Amplification Primer mix, mentre il LOW TE viene messo da parte a T.A.)

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

✓ION XPRESS BARCODE ADAPTERS KIT

- o ION XPRESS P1 ADAPTER (tappo viola)
- o ION XPRESS BARCODES (tappo bianco, si consiglia di aliquotarlo in strip da 8 well, per ogni singola 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 μ L.
3. Prendere il ION PLUS FRAGMENT LIBRARY KIT e il ION XPRESS 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

E4.10.5

Controls and procedures must be established to ensure sample tracking during pooling and barcoding

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 |
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Double control from a second operator

E4.10.5.1

Periodic barcode rotation is recommended to detect contamination. If contamination is detected Standard E4.5.4 must be followed

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

| | | |
|--|--|--|
|  | TIPIZZAZIONE HLA MEDIANTE NGS | Cod.: ISTR 01 PO 20 Data: 15/09/2018 Ed. 3 – Rev. 0 Pagina 19 di 34 |
|--|--|--|

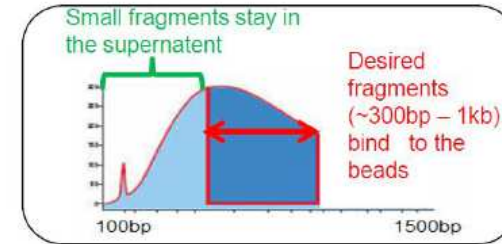
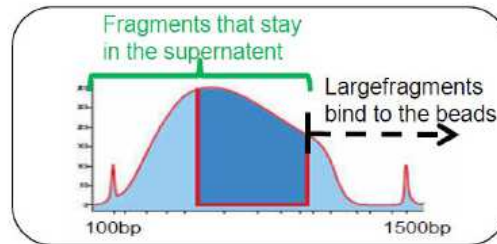
4.8 SIZE-SELECTION (Tempo ~ 1h)

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*)



E4.10.6.2

For each run the size of fragments must be documented and the selection must be specified

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

TAPE station or subsequent software analysis

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

Second amplification of selected fragments



| | |
|-----------|--|
| E4.10.4 | PCR artefacts must be documented |
| E4.10.4.1 | The information must be used in the routine interpretation of data following established policies. (i.e. PCR cross-over and/or artefact) |

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

NORMALIZATION: to obtain the same concentration of the amplified DNA in each single



Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

Double control from a second operator

| Barcode | Plate Position | DNA ID | | |
|---------|----------------|--------|--|--|
| 65 | 1 | 605-19 | | |
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E4.10.2

E4.10.2.3

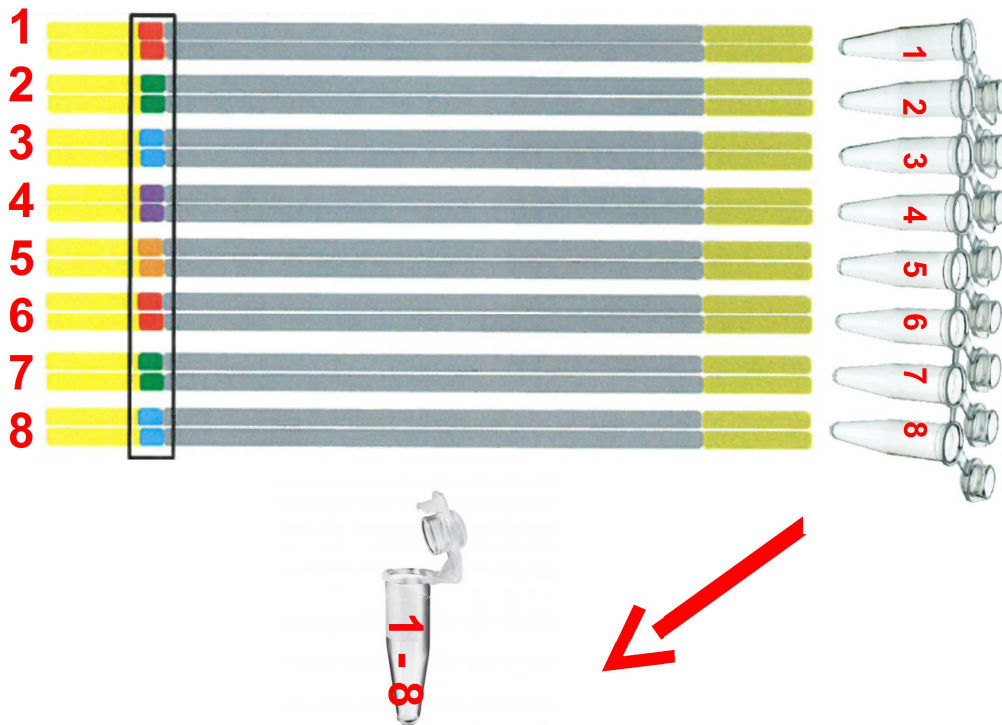
The following must be documented:

Normalization

Library preparation

Fragmentation and end-preparation
Barcoding
Size selection
Second Amplification
Pooling

Reached the same concentration in each sample with the specific bare code, they are pooled in a single tube



LIBRARY

E4.10.2

The following must be documented:

E4.10.2.4

Pooling methods

HLA typing with NGS

Template generation

First Amplification of HLA loci

Library preparation

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Barcoding
Size selection
Second Amplification
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Clonal amplification

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

Sequencing

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

Data analysis

Locus assignment,
Generating consensus sequence

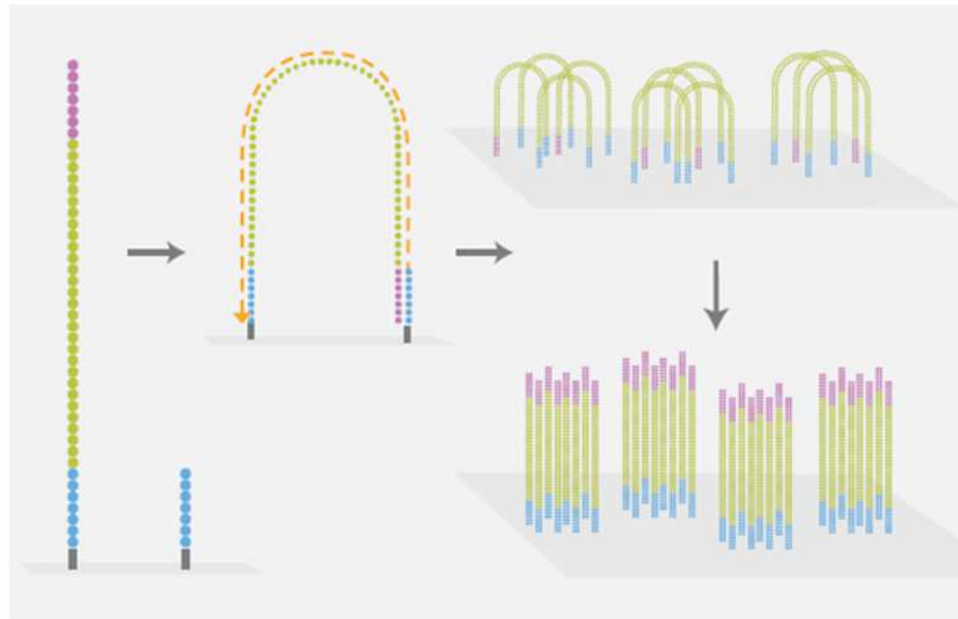
Clonal amplification

Illumina

Fragment cluster generation:

- Bridge Amplification (Illumina)
- Isothermal Amplification (Ion Torrent)

The library is flowed over the flow cell and DNA fragments will anneal to specific sites



E4.10.6.3

Methods for enrichment strategies of multi gene panels must be defined.

Clonal amplification

Fragment cluster generation:

- Bridge Amplification (Illumina)

- **Isothermal Amplification (Ion Torrent)**

Ion Torrent

Clonal Amplification :

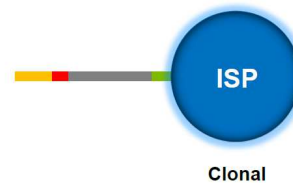
1 Ion Sphere™ Particle (ISP) + 1 Barcoded Library Fragment

1 Ion Sphere™ Particle (ISP) + Clonally Amplified Barcoded Library Fragments



Ideal

Attachment of one fragment to one Ion Sphere Particle



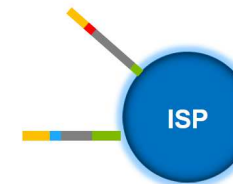
Non-Ideal



Fragments ONLY



ISPs ONLY



Polyclonal

E4.10.6.3

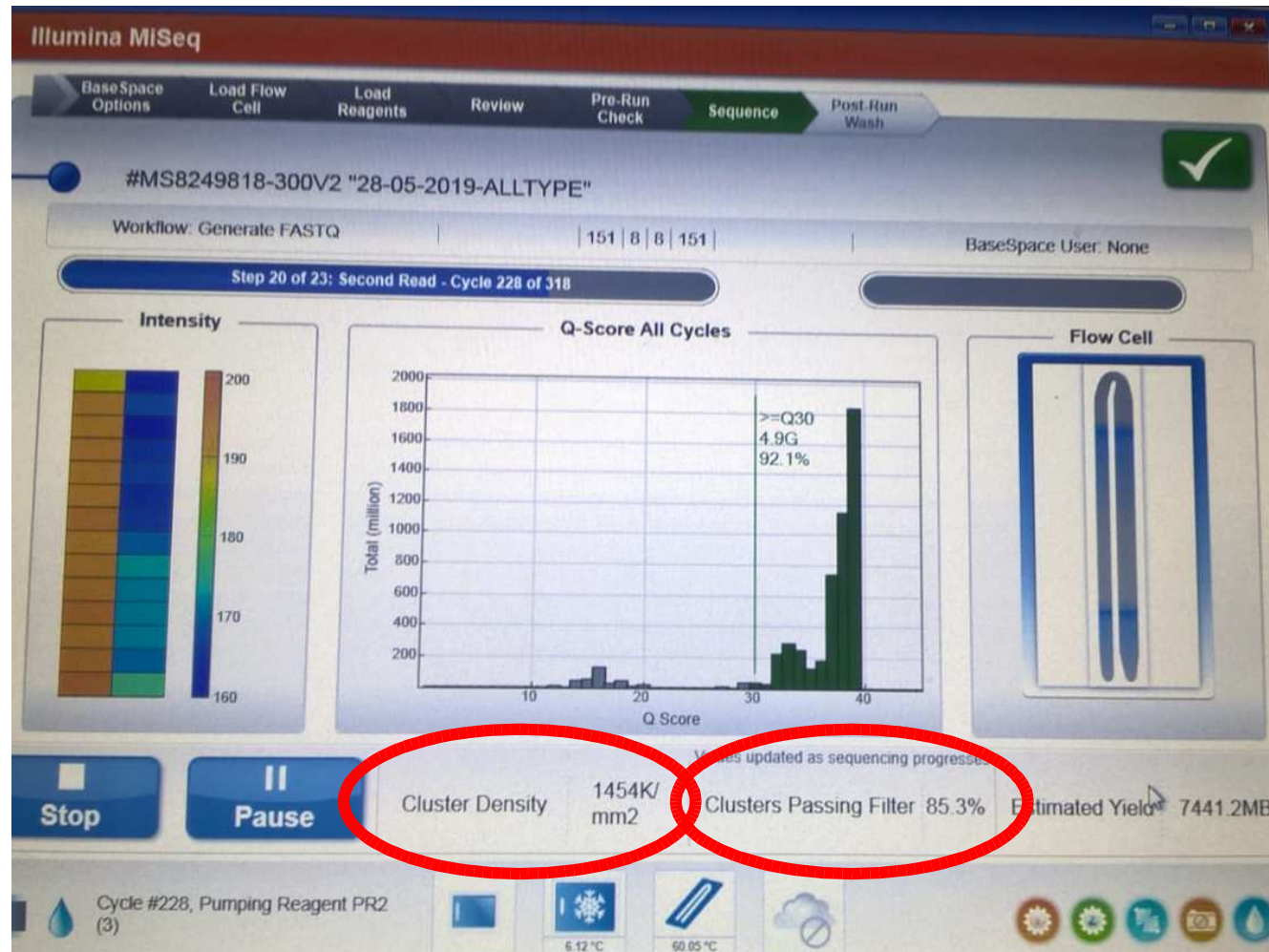
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Clonal amplification

Fragment cluster generation:

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Illumina



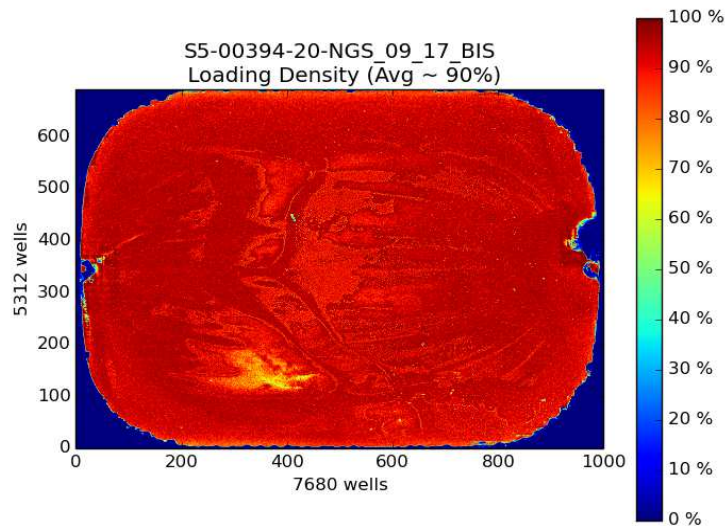
Clonal amplification

Fragment cluster generation:

- Bridge Amplification (Illumina)
- **Isothermal Amplification (Ion Torrent)**

Chip of the new
run by NGS

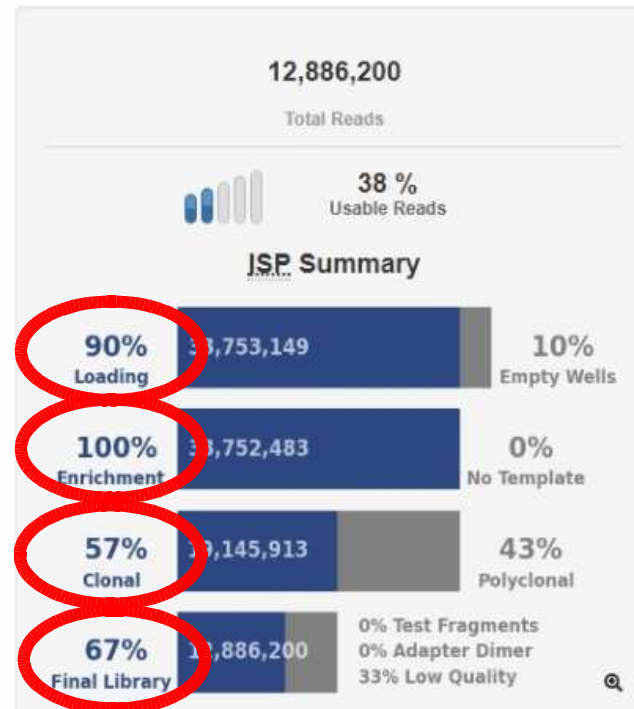
Ion Torrent



First observation of the data

Optimal colors: **RED, ORANGE**

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



E4.10.6.3

Methods for enrichment strategies of multi gene panels must be defined.

Automation of the production process

Validation of automation

HLA typing with NGS

Template generation

First Amplification of HLA loci

Library preparation

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Barcoding
Size selection
Second Amplification
Pooling

Clonal amplification

Fragment cluster generation:
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Sequencing

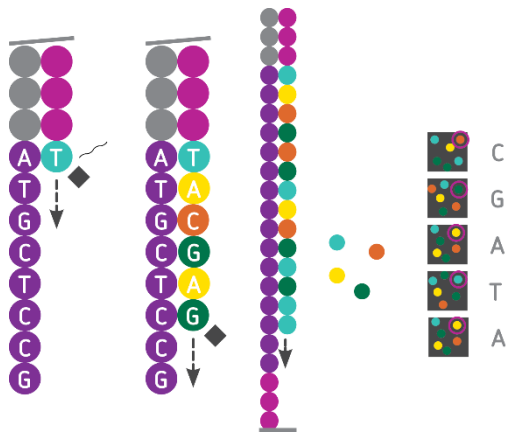
Different options:
- Sequencing by Synthesis
- Semiconductor Sequencing
- Single Molecule Real Time PCR

Data analysis

Locus assignment,
Generating consensus sequence

Sequencing

Sequencing-by-synthesis (SBS) Semiconductor sequencing



Mix of 4 fluorescent dNTPs, which are incorporated **one-by-one** during each cycle of the sequencing

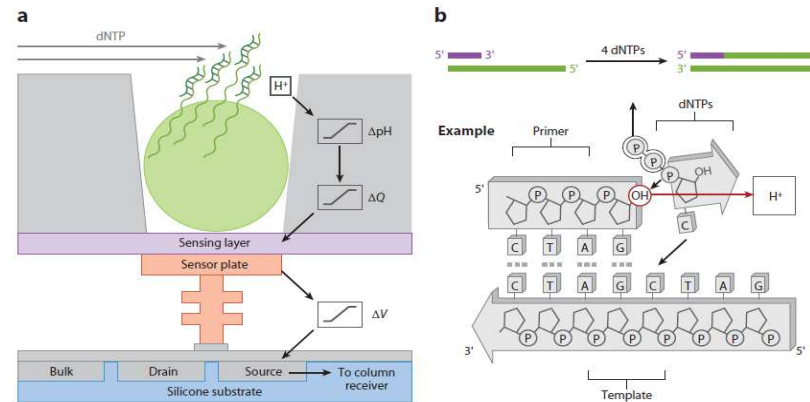


Illumina

Different options:

- Sequencing by Synthesis
- Semiconductor Sequencing
- Single Molecule Real Time PCR

Sequencing reaction Semiconductor sequencing



When a nucleotide is incorporated into a strand DNA, a **hydrogen ion** will be released

Massive parallel sequencing of millions of clonal amplicons, all with a specific barcode



Ion Torrent

HLA typing with NGS

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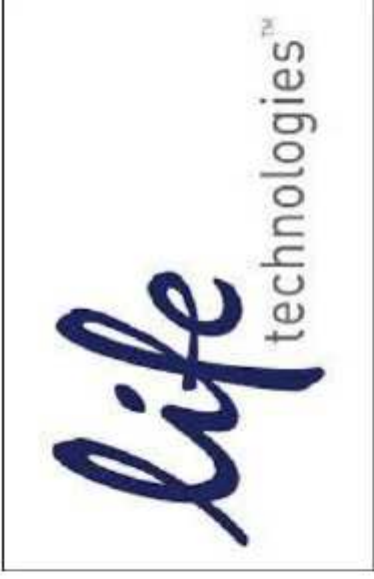
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Generating consensus sequence



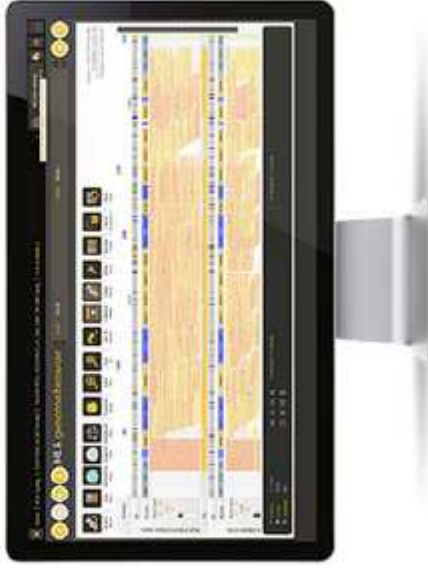
Immucor MIA FORA



Ion Torrent HLA Plug In



Illumina Conexo



Omixon HLA Twin



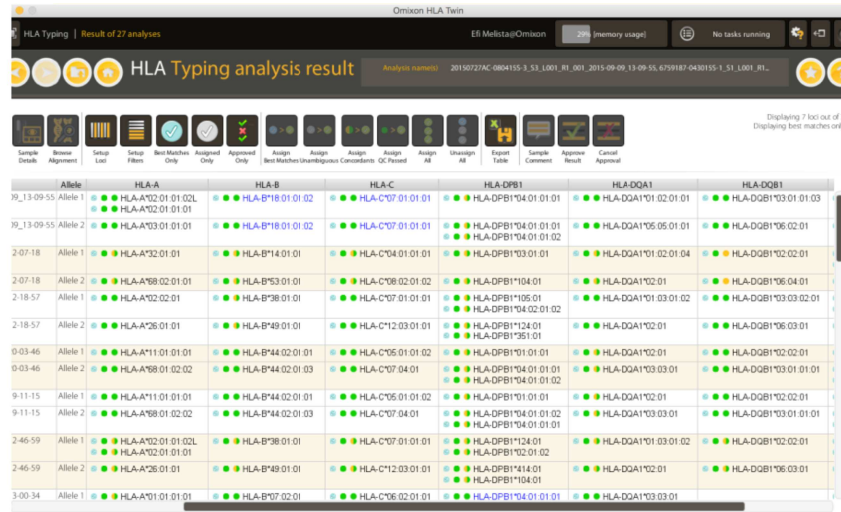
GENDX NGSengine



R.O.S.E. GenTec Ltd.

Data analysis

Locus assignment,
Generating consensus sequence



SOFTWARE

Overview Statistics Reports

| Resolution: | All fields | Quality metrics: | None | Status | Actions |
|-------------|-------------------|------------------|------------------|--------|--|
| P 133-19 | | | | | |
| HLA-A | 87249/88847 (98%) | 137 [1-151] | 7559 [3995-9323] | 2 | [C][C] A*01:01:01:01, A*24:02:01:01 |
| HLA-B | 64940/66079 (98%) | 136 [1-151] | 5223 [2078-6612] | 3 | [C][C] B*35:01:01:02, B*35:08:01:01 |
| HLA-C | 92131/93744 (98%) | 139 [1-151] | 7623 [3606-8809] | 4 | [C][C] C*04:01:01:01, C*04:01:01:06 |
| DRB1 | 14796/18178 (81%) | 137 [1-151] | 919 [92-1587] | 9 | [C][C] DRB1*03:01:01:01, DRB1*14:04:01:01 DRB3 |
| DQB1 | 63244/64614 (97%) | 140 [1-151] | 4752 [1781-5895] | 2 | [C][C] DQB1*02:01:01, DQB1*05:03:01:01 |
| DPB1 | 11556/12113 (95%) | 139 [1-151] | 529 [185-758] | 6 | [C][C] DPB1*01:01:01:01, DPB1*13:01:01:01 |
| P 2166-18 | | | | | |
| HLA-A | 8769/8986 (97%) | 141 [1-151] | 762 [551-1103] | 1 | [C][C] A*02:05:01:01, A*30:01:01:01 |
| HLA-B | 18746/19062 (98%) | 139 [1-151] | 1590 [830-1965] | 2 | [C][C] B*53:01:01, B*58:01:01:01 |
| HLA-C | 30665/31092 (98%) | 142 [1-151] | 2636 [891-3213] | 4 | [C][C] C*04:01:01:01, C*07:18 |
| DRB1 | 28311/32788 (86%) | 142 [1-151] | 2376 [925-2878] | 10 | [C][C] DRB1*07:01:01:01, DRB1*07:01:01:01 DRB4 |
| DRB4 | 0/0 (0%) | 0 [0-0] | 0 [0-0] | | Detected but not analyzed |

EN Ingles

Data

Sample

Patient Info

Profile

Utilities

About

Exit

Session: WGS-11-17

Analysis Date: 6/1/2017

Status: Completed

Locus

☒ Show All

☐ A☐ B☐ C☐ DRB1☐ DRB345☐ DQB1☐ DPB1☐ DQA1☐ DPA1

Health Number Missed

☒ 0

☐ 1

☐ 2

☐ 3

☒ 4

| | Barcode | Sample ID | Patient ID | Total Read Count | Avg Read Length | Anal |
|--|---------|-----------|------------|------------------|-----------------|------|
| | 016 | 828-14 | | 300000 | 246 | Bar |

| | Locus | Allele 1 | [K/N/I] 1 | Notes 1 | Allele 2 | [K/N/I] 2 |
|--|--------|------------------|-----------|--------------|-------------------|-----------|
| | A | A*11 [+] | [0/0/0] | Low Coverage | A*26:01:01:01 | [0/0/0] |
| | B | B*35:08:01 | [0/0/0] | | B*38:01:01 | [0/0/2] |
| | C | C*06:127:01 | [0/0/1] | NEW | C*12:03:01:01 | [0/0/0] |
| | DRB1 | DRB1*04:02:01 | [0/0/0] | | DRB1*11:01:01 | [0/0/4] |
| | DRB345 | DRB3*02:02:01:02 | [0/0/0] | | DRB4*01:03:01 [+] | [0/0/0] |
| | DQB1 | DQB1*03:01:01:03 | [0/0/0] | | DQB1*03:02:01:01 | [0/0/0] |

| | | |
|---|--|------------------|
|  | PROCEDURA OPERATIVA Tipizzazione HLA mediante NGS | Cod.: PO 20 LIT |
| | | Data: 15/09/2018 |
| | | Ed. 3 Rev. 0 |
| | | Pagina 8 di 11 |

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 **Typestream** 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. È 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:

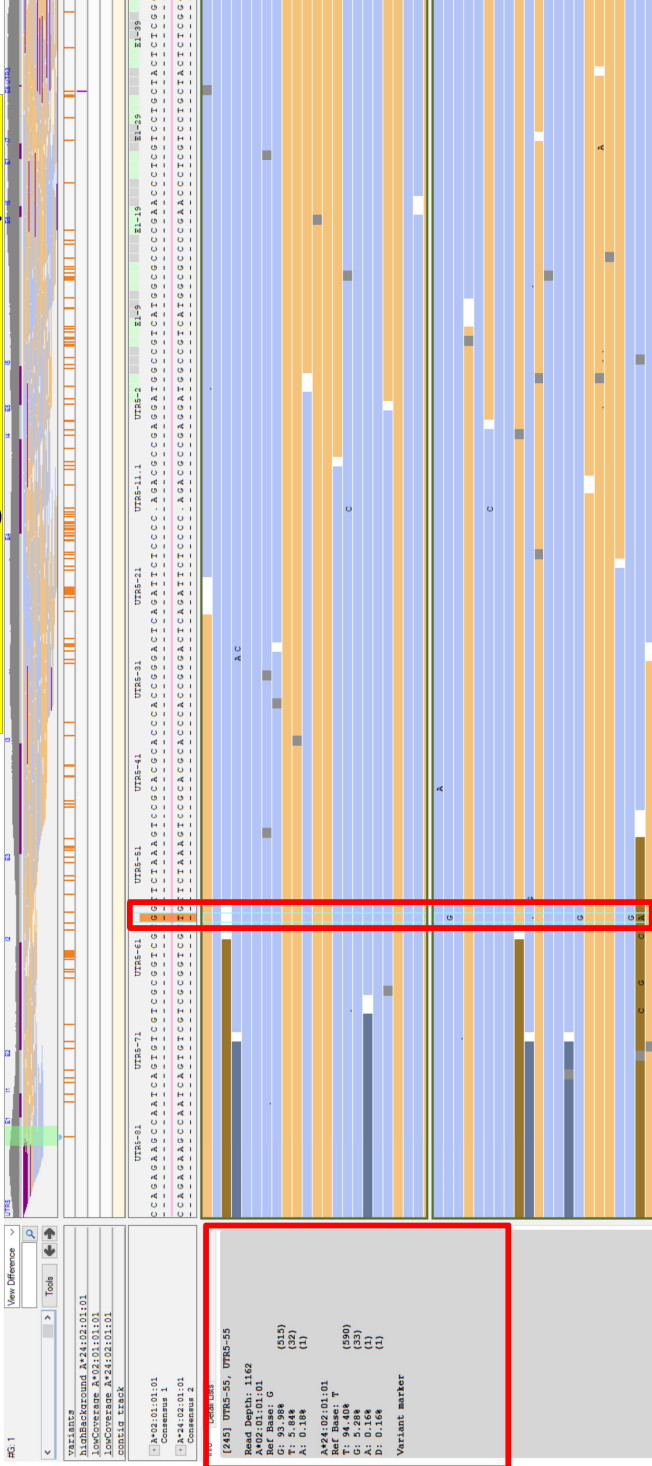
| Analysis Parameter | Value | Analysis Parameter | Value |
|----------------------|-------|------------------------|---------|
| Min Read Length: | 100 | Min Valid Reads: | 500 |
| Max Insertion: | 3 | Cut off Value: | 20 |
| Max Deletion: | 3 | Min Hetero Allele Bal: | 10 |
| Max Mismatch Bases: | 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 |
| | |

Data analysis

Locus assignment,
Generating consensus sequence



E4.10.7

E4.10.7.1

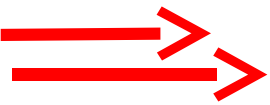
The signal to noise ratio

must be sufficient to

ensure reliable nucleotide

assignments

| 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 |



```
[245] UTR5-55, UTR5-55
```

```
Read Depth: 1162
```

```
A*02:01:01:01
```

```
Ref Base: G
```

```
G: 93.98% (515)
```

```
T: 5.84% (32)
```

```
A: 0.18% (1)
```

```
A*24:02:01:01
```

```
Ref Base: T
```

```
T: 94.40% (590)
```

```
G: 5.28% (33)
```

```
A: 0.16% (1)
```

```
D: 0.16% (1)
```

```
Variant marker
```

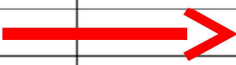
E4.10.7

Nucleotide Assignment

E4.10.7.1

The signal to noise ratio must be sufficient to ensure reliable nucleotide assignments

| 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 |
| | |

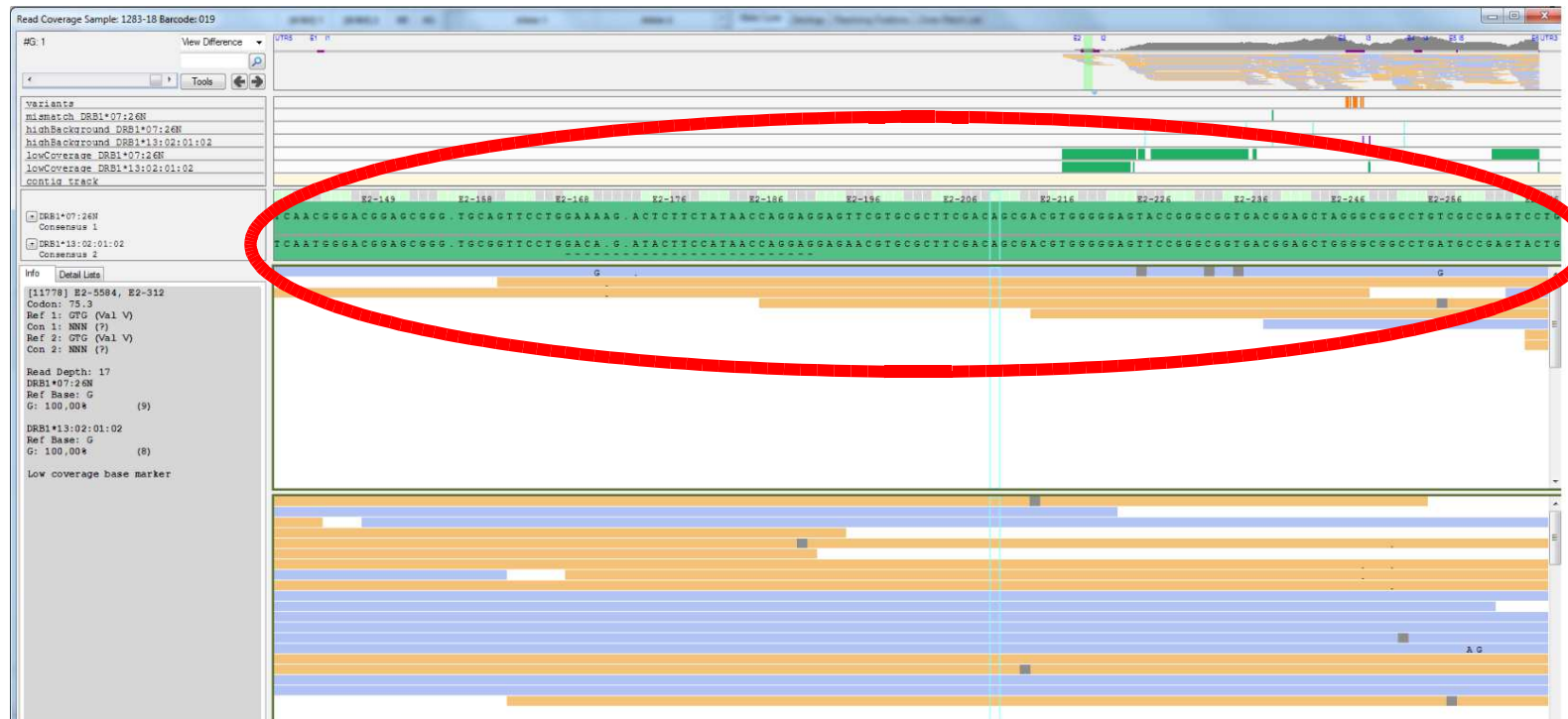


Data analysis

Locus assignment,
Generating consensus sequence

Ion Torrent

E4.10.7.2 A scientific and technically sound method must be established for interpretation, acceptance and/or rejection of sequences from regions which are difficult to resolve



E4.10.7.3

Established sequence-specific characteristics should be
do

| | | |
|---|--|------------------|
|  | PROCEDURA OPERATIVA Tipizzazione HLA mediante NGS | Cod.: PO 20 LIT |
| | | Data: 15/09/2018 |
| | | Ed. 3 Rev. 0 |
| | | Pagina 8 di 11 |

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

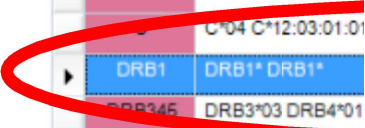
Successivamente vanno osservati parametri come il **Loading**, che deve essere superiore al 90%, **enrichment** che deve avvicinarsi al 100%, la **clonalità**, che deve essere superiore al 55% e la percentuale di libreria, intorno al 70%. Deve essere controllata inoltre la lunghezza dei frammenti (**read 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 **Typestream 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. È 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

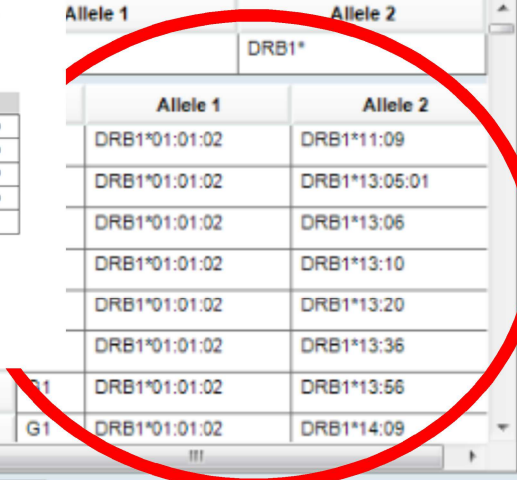
Prima di accettare i risultati di una tipizzazione in NGS, è necessario analizzare i parametri impostati per ogni singolo campione e visibili nel software di interpretazione come da seguente tabella:

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 | | |




| Analyze Data Reports Data | |
|---------------------------|---------------------|
| << Summary < 128 | |
| Set Config Analysis Par | |
| User Comments : | |
| Locus | |
| A | A*02:01:01:01 A*68 |
| B | B* B*44 |
| C | C*04 C*12:03:01:01 |
| DRB1 | DRB1* DRB1* |
| DRB345 | DRB3*03 DRB4*01 |
| DQA1 | DQA1*01 DQA1*01 |
| DQB1 | DQB1*02 DQB1*02 |
| DPA1 | DPA1*01 DPA1*01 |
| DPB1 | DPB1*02:01:02 DPB1* |



| System Comments : Conf | |
|------------------------|---------------|
| Allele 1 | Allele 2 |
| DRB1* | DRB1* |
| Allele 1 | Allele 2 |
| DRB1*01:01:02 | DRB1*11:09 |
| DRB1*01:01:02 | DRB1*13:05:01 |
| DRB1*01:01:02 | DRB1*13:06 |
| DRB1*01:01:02 | DRB1*13:10 |
| DRB1*01:01:02 | DRB1*13:20 |
| DRB1*01:01:02 | DRB1*13:36 |
| DRB1*01:01:02 | DRB1*13:56 |
| DRB1*01:01:02 | DRB1*14:09 |

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 x |
| 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 |

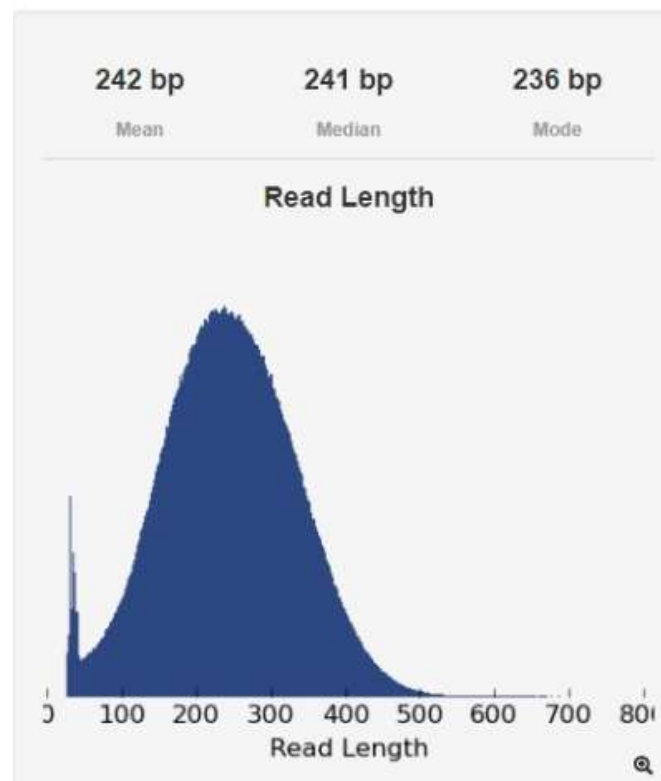
monitor and share written information within the group

Data analysis

Locus assignment,
Generating consensus sequence

Nucleotide Assignment

| 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 |
| | |



E4.10.7.4

Percentage of low quality reads and passed filter reads must be specified and in defined range

Data analysis

Locus assignment,
Generating consensus sequence

Allele assignment

E4.10.8.1

Methods must ensure that sequences contributed by amplification primers are not considered in the assignment of alleles

The reads containing the primers are trimmed to remove the primer sequence from analysis.

The catalog file must guide the software

Data analysis

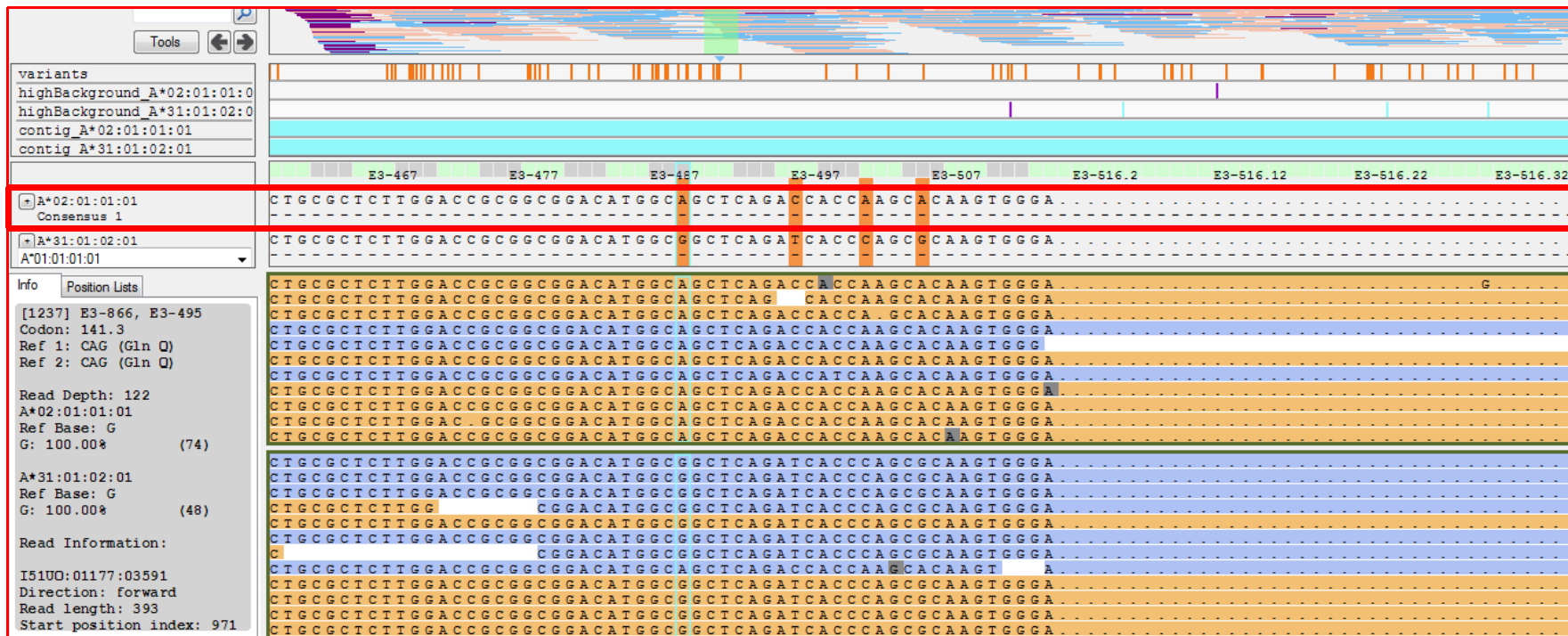
Locus assignment,
Generating consensus sequence

Allele assignment

E4.10.8.2

Criteria for allele assignment must be established

The consensus must be compared to the known updated alleles library (IMG)



Data analysis

Locus assignment, Generating consensus sequence

E4.10.8.3

Adequate depth of coverage threshold necessary to make accurate allele calls must be established and documented empirically during the validation phase

E4.10.8.4

Overlap of sequences must be sufficient to determine the phase of alleles for the methods where phasing is possible

```
[1237] E3-866, E3-495
Codon: 141.3
Ref 1: CAG (Gln Q)
Ref 2: CAG (Gln Q)

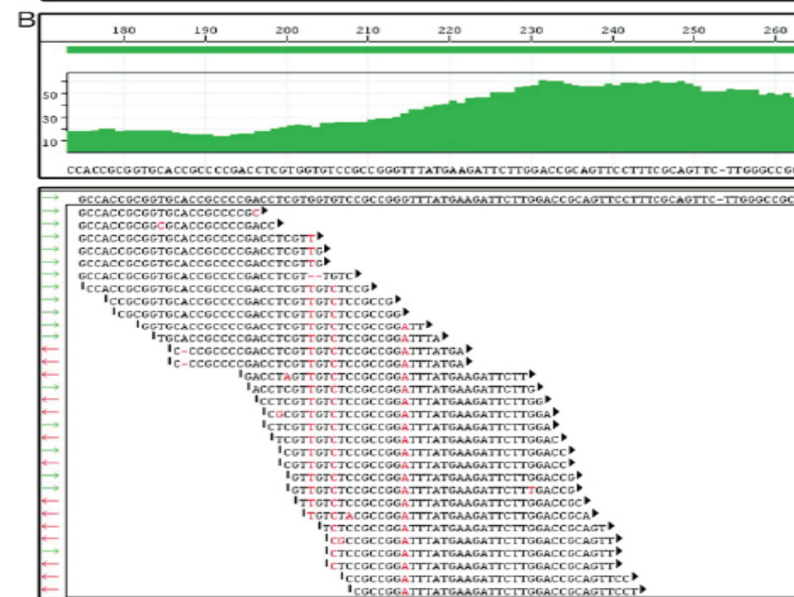
Read Depth: 122
A*02:01:01:01
Ref Base: G
G: 100.00% (74)

A*31:01:02:01
Ref Base: G
G: 100.00% (48)

Read Information:

I51UO:01177:03591
Direction: forward
Read length: 393
Start position index: 971
```

SOP and Validation



| 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 |

Data analysis

Locus assignment,
Generating consensus sequence

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 |

E4.10.9 Bioinformatics

E4.10.9.1 Sequencing metrics and QC parameters for optimal performance must be documented, specified and in range

E4.10.9.2 Each deviation from the standard operation procedure must be documented

Data relative to each single allele analyzed



Data analysis





Locus assignment,
Generating consensus sequence

 **Overview** > 133-19 HLA-A






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**

Data analysis

Locus assignment,
Generating consensus sequence

| | |
|------------------|--|
| E4.10.9.3 | Detailed documentation and validation of the bioinformatics process supporting the analysis, interpretation and reporting results must be established |
|------------------|--|

| | |
|------------------|---|
| E4.10.9.4 | Revalidation of bioinformatics processes must be performed after upgrading or changes of any affected components |
|------------------|---|

Specific SOP and/or Instructions for validation

Data analysis

Locus assignment,
Generating consensus sequence

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



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



Transferring the raw data



The real back-up

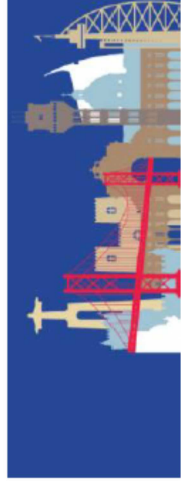
Data analysis

Locus assignment,
Generating consensus sequence

| | |
|------------------|---|
| E4.10.9.6 | The version of the bioinformatics process must be traceable for each sample analyzed |
|------------------|---|

| | |
|------------------|--|
| E4.10.9.7 | Algorithms for modification of raw sequence reads must be described in detail and validated (i.e. sequence trimming, quality filtering) |
|------------------|--|

| | |
|------------------|--|
| E4.10.9.8 | Each sample processed must be traceable through the whole process including data analysis and reporting |
|------------------|--|



**33rd EUROPEAN IMMUNOGENETICS AND
HISTOCOMPATIBILITY CONFERENCE**
FUNCTIONAL IMMUNOGENETICS: THE HISTORICAL CHALLENGE

MAY 8-11, 2019
CENTRO CULTURAL DE BELEM
LISBON PORTUGAL

- Overview on NGS technology
- EFI standards and NGS
- **Validation of the method before
the introduction in routine**

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 | *07.01:01 | 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 | *11.01 | 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 | *11.01 | 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

Suggestions for NGS validation

KIT

NGS PRODUCTS FOR ION TORRENT

| | |
|---------------------|--|
| 1st PCR | ALLTYPE NGS 11 ALL-11LX |
| LIBRARY PREPARETION | AMPURE XP BEADS |
| | ION XPRESS PLUS FRAGMENT (20 test) |
| | ION XPRESS BARCODE ADAPTERS 1-16 |
| | ION 520&530 EXT KIT-CHEF (reagenti+soluzioni S5) 4 run |
| | QUBIT ASSAY TUBES |
| | QUBIT DSDNA HS ASSAY KIT |
| NGS AMPLIFICATION | ION 520&530 EXT KIT-CHEF (48x4 CHIP) 4 run |

Test a known sample in each new NGS run



**33rd EUROPEAN IMMUNOGENETICS AND
HISTOCOMPATIBILITY CONFERENCE**
FUNCTIONAL IMMUNOGENETICS: THE HISTORICAL CHALLENGE

MAY 8-11, 2019
CENTRO CULTURAL DE BELEM
LISBON PORTUGAL

Inspectors' Workshop

Tuesday 7 May 2019

Many thanks for your attention

Laboratorio d'Immunogenetica dei Trapianti - Polo di Ricerca di San Paolo
Dipartimento di Oncematologia e Terapia Cellulare e Genica
IRCCS Ospedale Pediatrico Bambino Gesù



Comune di Roma





Tiziana Galluccio
Mangione

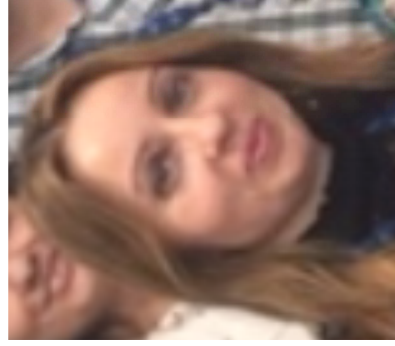
Annalisa Guagnano Martina



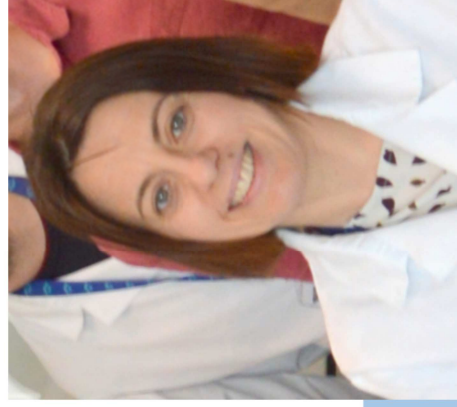
Mariarosa Battarra Giuseppe Testa



Andrea Di Luzio



Paola Giustiniani



Maria Troiano





Tiziana Galluccio
Mangione

Annalisa Guagnano

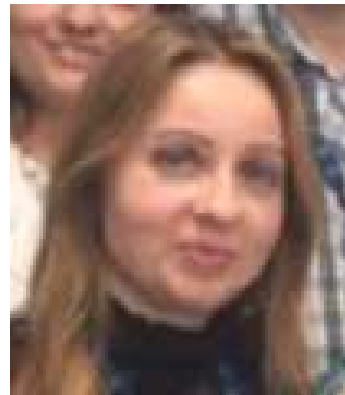
Martina



Mariarosa Battarra Giuseppe Testa



Andrea Di Luzio



Paola Giustiniani



Maria Troiano