Target Resequencing of Selected Genomic Regions on the Illumina Platform

Illumina Seminars
23rd of June 2010

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IGA Technology Services Srl
What is target resequencing?

- Also referred to as genome partitioning or DNA capture
- Captures genomic material of interest for next generation sequencers
- Most of the remaining genomic material discarded
- ENRICHMENT
NGS Applications

Figure 41: Percent of Application Representing Next Generation Sequencing in the Laboratory over the Next 2 Years

- mRNA expression profiling: 15.4%
- Biomarker discovery: 13.8%
- Whole genome re-sequencing: 12.0%
- Diagnostics: 10.7%
- Targeted re-sequencing: 10.4%
- ChIP-sequencing: 9.0%
- De Novo sequencing: 6.8%
- Epigenomics: 5.7%
- Exome sequencing: 5.4%
- High throughput genotyping: 3.7%
- Metagenomics: 2.6%
- Protein analysis: 2.0%
- Digital karyotyping: 1.7%
- Forensics: 0.8%
- Other: 0.0%

From Next Gen Sequencing Survey, JP Morgan, May 2010
DNA shearing by nebulization or sonication

New generation sequencing (Illumina Genome Analyzer IIx)

Alignment to reference sequence

Reference genome

Genomic DNA

DNA-seq
Why Targeted Re-Sequencing

Whole Genome Resequencing

• Too expensive (60-100,000 Euro per sample)
• Too slow (>1 month per sample)

Targeted Resequencing

Genes of interest: up to 6.6 Mb of ‘custom’ genomic targets or human exons (38 Mb)

• Greater Depth of Coverage
• Reduced Cost
• Higher Sample Throughput
• Reduced Data Analysis

Human genome (3 Gbp)
PCR of the entire human 'exome'?  

- Sensitive  
- Specific  
- Easy to Use  
- Reproducible  
- 30+ years of validation  

Human exome = 38 Mbp  

47500 individual PCR (800bp long)  
19000 individual Long PCR (2000 bp long)  

Traditional PCR is expensive  

Traditional PCR is sensitive and specific but logistically unrealistic
Capture technologies

• On-Array methods (i.e. Nimblegen, Agilent, Febit)

• Agilent In-Solution Genome Partitioning (i.e. Agilent SureSelect, Illumina?)

• Selective Genomic Amplification Using Droplet-Based Microfluidics (Rain Dance Technologies)
On array capture

I. Genomic DNA preparation
   and hybrid selection

1. Randomly fragment high-
   molecular-weight DNA by
   by sonication or nebulization.

2. Repair, blunt and phosphorylate ends.

3. Ligate linkers, denature
   strands and capture with
   385k arrayed probes.

4. Recover selected fragments
   by thermal elution followed by
   lyophilization and PCR
   enrichment of ligated strands.

II. 1G sequencing

5. Blunt asymmetric capture linkers.
   Phosphorylate and adenylate ends.
   Ligate Illumina 1G-compatible adaptors.
   Gel purify and PCR enrich.

6. Denatured strands are injected
   into eight-lane flow cell. Clusters are
   generated from single molecules
   by in situ amplification.

7. Sequencing-by-synthesis primer is
   hybridized and cluster images are
   scanned with each successive
   round of fluorescent nucleotide
   incorporation.

8. Images are processed with
   Illumina base-calling software
   and aligned to reference.

From Emily Hodges et al.,
SureSelect™ Target Enrichment System: Design and Order Process

1. Design & Order
   - Select custom genome partitioning set using eArray
   - or, select catalog oligo set

2. Kit Production
   - 55K unique 120 mer oligos synthesized on one wafer
   - Oligos released
   - Oligo IVT to RNA-biotin

3. Single Tube
   - Kit Delivery
     - Agilent genome partitioning kit shipped to customer

Kit Includes
1. Biotinylated-cRNA
2. Buffers
3. Protocol
SureSelect™ Target Enrichment System Capture Process

- Standard NGS library prep
- Genomic Sample (Set of chromosomes)
- NGS Kit
- Genomic Sample (Prepped)
- SureSelect HYR bufffer
- SureSelect RIOTINATED RNA LIBRARY "BAITS"
- Hybridization
- Streptavidin coated magnetic beads
- Unbound fraction discarded
- Wash beads and digest RNA
- Bead capture
- Amplify
- Sequencing
# In-Solution vs On-Array

<table>
<thead>
<tr>
<th></th>
<th>In-solution method</th>
<th>On-Array method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Throughput</strong></td>
<td>HIGH</td>
<td>LOW</td>
</tr>
<tr>
<td><strong>Study Sizes</strong></td>
<td>Medium to Large</td>
<td>Small</td>
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<tr>
<td><strong>DNA input</strong></td>
<td>3 µg</td>
<td>20 µg</td>
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<tr>
<td><strong>Capture of target DNA</strong></td>
<td>5-30 Mb</td>
<td>1 Mb</td>
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</table>
Illumina indexing with multiplex custom SureSelect

Indexes inserted during PCR enrichment

INDEX_1_ATCACG
INDEX_2_CGATGT
INDEX_3_TTAGGC
INDEX_4_TGACCA
INDEX_5_ACAGTG
INDEX_6_GCCAAT
INDEX_7_CAGATC
INDEX_8_ACTTGA
INDEX_9_GATCAG
INDEX_10_TAGCTT
INDEX_11_GGCTAC
INDEX_12_CTTGTA
Primer Libraries in Droplets

Step 1. Design primers for loci of interest
No constraints on primer design associated with traditional multiplex PCR

Step 2. Synthesize primer pairs
Standard oligo synthesis

Step 3. Reformat primer pairs as droplets
Only one primer pair present in each droplet

Step 4. Pool emulsions as Primer Library
Droplet stability prevents cross-contamination of primer pairs

RainDance Technology (From CSHL NGS Sequencing Course, July 2008)
Droplet-Based PCR Process

- **Primer Library**
- **gDNA, Taq, dNTPs**
- **Waste**
- **Electrodes**
- **Collect in 96 well PCR plate**
- **100-200 µL per sample**
- **Less than 1 hour per sample**

**Droplet Rate = 3000 Hz**
**Combine Efficiency = 95%**

**PCR Amplification**

**Instrument**

RainDance Technology (From CSHL NGS Sequencing Course, July 2008)
Post-Amplification Workflow

1. Add equal volume Droplet Release Reagent to each well of PCR plate
2. Seal plate and vortex 30 seconds
3. Centrifuge for 5 minutes
4. Transfer (pipette) upper aqueous phase into new tube
5. Purify using PCR clean-up kit

RainStorm™ droplet chemistry enables simple and efficient recovery of amplified DNA following PCR

RainDance Technology (From CSHL NGS Sequencing Course, July 2008)
Monogenic Versus Polygenic Disease

Monogenic: One base change = disease. Relatively easy to detect and analyze.

Polygenic/complex trait: A set of base changes affect the probability of disease. Subtle – hard to detect and analyze
Single base changes are the most common mutations involved in human diseases

Number of entries in HGMD by type

<table>
<thead>
<tr>
<th>Data type</th>
<th>Number of entries (public release for academic/non-profits only)</th>
<th>Number of entries (HGMD Professional release 2010.1)</th>
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<td>TOTAL (public release) 72414</td>
<td>TOTAL (HGMD Professional 2010.1) 100329</td>
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<td>Missense/nonsense</td>
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<td>56457 (details)</td>
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<tr>
<td>Splicing</td>
<td>7011</td>
<td>9600 (details)</td>
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<tr>
<td>Regulatory</td>
<td>1051</td>
<td>1766 (details)</td>
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<tr>
<td>Small deletions</td>
<td>11745</td>
<td>15805 (details)</td>
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<td>1065</td>
<td>1473</td>
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<tr>
<td>Repeat variations</td>
<td>178</td>
<td>307</td>
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<tr>
<td>Gross insertions/duplications</td>
<td>744</td>
<td>1260</td>
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<td>Complex rearrangements</td>
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<tr>
<td>Gross deletions</td>
<td>4106</td>
<td>6201</td>
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<tr>
<td>Gene/sequence data</td>
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<td></td>
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<tr>
<td>Genes</td>
<td>2689</td>
<td>3739</td>
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<tr>
<td>cDNA reference sequences</td>
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</table>

Source: Human Gene Mutation Database (HGMD)
Human disease example: Hypertrophic Cardiomyopathy (HCM)

Dr Maria Iascone, Ospedali Riuniti di Bergamo

Normal heart  Hypertrophic heart. Left and/or right ventricular hypertrophy, usually asymmetric and involving the inter-ventricular septum

- Most well-known as a leading cause of sudden cardiac death in young athletes
- Hereditability
  - Autosomal dominant
  - Rarely X-linked or recessive
- Variable and age-dependent penetrance and expressiveness
- Hypertrophy, myocardial disarray, interstitial fibrosis
- Most frequent cardiovascular genetic disease (prevalence of 1:500)

Maron BJ, JAMA, 2002; 287:1308-1320.
Human Gene Mutation Database
Mutation/genes distribution

MYH7+MYBPC3
409 mutations
72%

Dr Maria Iascone, Ospedali Riuniti di Bergamo
Targeted resequencing of Genetic Loci

Multiple Genes  ↔  Multiple Mutations

Complex Genotype

Overlapping Clinical Phenotype

Hypertrophic Cardiomyopathy

Variable Outcome

Dr Maria Iascone, Ospedali Riuniti di Bergamo
The samples

3 HCM-patients

MYH7, MYBPC3, TNNT2 sequencing negative

+ 

5 HCM-patients, work currently ongoing in multiplex (Illumina index)

The target

- 36 genes with at least one mutation described in HGMD
- Distributed on 17 chromosomes (about 2 Mb repeat-masked regions)
Upload the genomic intervals of your targets and allow eArray’s algorithm to design baits for these targets.
Resequencing of the target

Genomic DNA

- NGS library construction
- SureSelect Hybr

Capture up to 6.6 Mb of 'custom' genomic targets or human exons (38 Mb)

New generation sequencing (Illumina Genome Analyzer)

Alignment to reference sequence

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...

AGCTGCTAGCTAGCTTGAGATCGATCGTTCGATCGATCGCATTTATTCGGATGATGCATCGTACTATCGAT...
Coverage level distribution (excl. zero coverage regions)

Sample T994
14 M reads (single-read 36bp)
(212x mean coverage)

Sample BG141
16 M reads (single-read 36bp)
(220x mean coverage)

Sample BG228
20 M reads (single-read 36bp)
(280x mean coverage)
Key Issues with Targeted Resequencing

✓ Completeness (Sensitivity): The percent of the targeted loci that are represented in the sequencing results.

✓ Specificity: The percent of sequencing reads that map to the targeted loci.

✓ Uniformity (Bias): The relative abundances of targeted loci in your sequencing results.

✓ Reproducibility

✓ Workflow & Cost: Does the method scale to enable large studies?

✓ Level of Multiplex: the number of selection events performed in a single assay. Flexibility to do tens to thousands of target regions.
Specificity = % of enrichment

i.e. percentage of sequencing reads that map to the targeted loci

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<thead>
<tr>
<th>Sample</th>
<th>Count</th>
<th>Total bases</th>
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<td>14518170</td>
<td>508135950</td>
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<td></td>
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<td></td>
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<td>1922607</td>
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<tr>
<td>70%</td>
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<td>66%</td>
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<td>BG228</td>
<td>20205635</td>
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<td></td>
<td>36</td>
<td>1922607</td>
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<tr>
<td>68%</td>
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Uniformity (Bias): i.e. the relative abundances of targeted loci in sequencing results. The coverage was distributed unevenly along the target sequence apparently reflecting the efficiency of hybridization of baits with their targets.

Reproducibility: good

Coverage level distribution along human chromosomes in 3 samples (2Mbp=36 genes, target resequenced region)
Small DIPs calling

chr1 AATTTGCACAGTGCTTTACATACTGCTAAAAGATTGAG

Consensus AATTTGCACRNGTSWTACAGAYGTGCTAAAAGVTGGMG

Coverage

NA-C3C24B:1:101:1115:1347#0/1 AAATTTGCACAGTGCTTTACAC--GTGCTAA
JMINA-C3C24B:1:8:679:1100#0/1 AAATTTGCACAGTGCTTTACAC--GTGCTAA
MINA-C3C24B:1:81:1221:439#0/1 AAATTTGCACAGTGCTTTACACATGTGCTA
INA-C3C24B:1:46:1388:1468#0/1 AAATTTGCACAGTGCTTTACACATGTGCTA
MINA-C3C24B:1:7:1759:1368#0/1 AAATTTGCACAGTGCTTTACACATGTGCTA
MINA-C3C24B:1:57:416:1070#0/1 AAATTTGCACAGTGCTTTACACATGTGCTA
MINA-C3C24B:1:74:559:1801#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:56:907:1121#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
JMINA-C3C24B:1:53:827:232#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
JMINA-C3C24B:1:39:1671:60#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:40:282:1411#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:10:87:1552#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
INA-C3C24B:1:83:1205:1114#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:74:1747:884#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:70:1040:1083#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:119:1419:29#0/1 AAATTTGCACAGTGCTTTACACATGTGCTAA
MINA-C3C24B:1:47:1327:568#0/1 AAATTTGCACAGTGCTTTACAC--GTGCT AAAGAT
JMINA-C3C24B:1:20:120:750#0/1 AAATTTGCACAGTGCTTTACAC--GTGCT AAAGAT
LUMINA-C3C24B:1:18:70:897#0/1 AAATTTGCACAGTGCTTTACAC--GTGCT AAAGAT
INA-C3C24B:1:100:839:1290#0/1 AAATTTGCACAGTGCTTTACAC--GTGCT AAAGATT
JMINA-C3C24B:1:92:466:630#0/1 AAATTTGCACAGTGCTTTACAC--GTGCT AAAGATT
### SNPs and DIPs number at different coverage level

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<th>Homozygous SNP</th>
<th>Total SNPs</th>
<th>DIPs</th>
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<td>T994 (4x)</td>
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<td>1125</td>
<td>1620</td>
<td>286</td>
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<tr>
<td>T994 (8x)</td>
<td>364</td>
<td>666</td>
<td>1030</td>
<td>82</td>
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<tr>
<td>T994 (10x)</td>
<td>313</td>
<td>502</td>
<td>815</td>
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<td>T994 (15x)</td>
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<td>BG141 (4x)</td>
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<td>1373</td>
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<td>770</td>
<td>1163</td>
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<td>BG228 (15x)</td>
<td>248</td>
<td>470</td>
<td>718</td>
<td>64</td>
</tr>
</tbody>
</table>

**SNP frequency in human genome about 1/300bp**

**About 1/1000 bp in coding regions**
Resequencing all ‘exome’

Protein-coding regions constitute about 1% of the human genome or about 30 megabases (Mb), split across 180,000 exons.

By examining only 1% of the genome you can get about 90-98% of the information about positions that cause changes in traits.

We used the Agilent SureSelect Human All Exon Kit designed to target all human exome totaling approximately 38 Mb.

The kit covers 1.22% of human genomic regions corresponding to the CCDS exons (the NCBI Consensus Coding Sequence database).
Read coverage distribution along human chromosomes for 3 samples (human exome captured regions)

200 Kb windows
Read coverage distribution along human chr 1 for 3 samples (exome captured regions)
Preliminary analysis (1 sample)

Specificity
alignment on human RefSeqGene NCBI db

<table>
<thead>
<tr>
<th></th>
<th>Reads</th>
<th>Matched</th>
<th>Not matched</th>
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<tr>
<td>Number</td>
<td>69,165,998</td>
<td>59,364,882</td>
<td>9,801,116</td>
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<tr>
<td>Total</td>
<td>2,489,975,928</td>
<td>2,137,135,752</td>
<td>352,840,176</td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td>85%</td>
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Alignment on HG18 human genome assembly

About 50,000 SNPs and 2,000 Short INDELs detected
BIOINFORMATIC ANALYSIS

1-Aligning reads to the reference
2-SNP and INDELs calling
3-SNP annotation:
   • NCBI dbSNP comparison
   • New SNP: Synonymous or non-synonymous?
   • Unknown SNP: prediction of functional effect of human nsSNPs

4-Validation (CE Sanger sequencing)
Target resequencing in plants?

• Whole genome resequencing is feasible at low price for small genomes (i.e. grape and peach genome)

• Target enrichment strategies associated with bar coded systems could be useful to survey nucleotide variations in plant genomes (need a reference)

• Useful in complex genome variation analysis (i.e. using full length cDNAs to design the baits if the genome sequence is not available, i.e. in polyploid genomes as wheat)
SUMMARY

• Current cost of whole genomes sequencing remains high for routine analysis of large populations, especially for complex genomes (i.e. humans, animals and big plant genomes)

• Techniques that allow targeted sequencing of defined genomic regions are valuable tools to facilitate the search for causative mutations in genetic disease and to study nucleotide variation in animals and plants

• Human exome sequencing probably will be important for the next 2-3 years and then moving to WGS if the cost falls low enough

• *In solution* technologies coupled with Illumina sequencing represent a good solution to analyze individual genome targets in order to find SNPs and small INDELs

• Continuing increases in throughput and multiplexing capabilities will further decrease the cost of targeted resequencing
IGA Projects

- **VIGNA/VIGNE.** Grape genome sequencing project (MiPAF with Genoscope 2006-2008)
- **Grape.** Development of new grapevine varieties (FVG Region, 2007-)
- **Grape.** Genotyping grapevine varieties and clones (FVG winemakers, 2008-)
- **Coffee.** Quality-controlling genes (Illy Co, 2008)
- **Barley.** BAC end sequencing (IPK, Gatersleben, 2008-)
- **Wheat.** Marker-assisted breeding (Barilla SpA, 2008-)
- **Wheat.** Physical map of 4 chromosomes (EU Triticeae, 2008-)
- **Wheat.** Physical map of chromosome 5 (MiPAF, 2009-)
- **Poplar.** Energy poplar: gene mining (EU Energy Poplar, 2008-)
- **Peach.** Peach genome sequencing project (MiPAF-Drupomics with JGI-USA, 2009-)
- **Citrus.** Clementine genome sequencing (MiPAF Citromics with JGI-USA, 2009-)
- ... other projects on grape, apple, olive, kiwifruit under negotiation ...
ULTRA HIGH-throughput SEQUENCING

• Illumina Genome Analyzer IIx (GAIllx)
• Acquired in July 2008
• Routinely ongoing: single reads and pair-end
• 36, 75, 100 bp reads
• More than 70 runs performed until now
IGA Technology Services S.r.l.
NGS facilities

Illumina Genome Analyzer IIx (April 2010):
50 Gbp/run (pair-end 2x100bp)

HiSeq2000 (by December 2010):
200 Gbp/run (pair-end 2x100bp)

Certification requested
Services

• DNA/RNA extraction and production of genomic libraries
• BAC physical maps
• Individual genotyping
• DNA and RNA sequencing by Sanger (i.e. BES)
• DNA and RNA sequencing by Illumina (DNA-seq, target resequencing, RNA-seq, smallRNA-seq)
• DNA sequence alignment and high-throughput queries on public and proprietary databases (i.e. transposable elements)
• WWW Blast server for HT sequences
• Metagenomics (by Illumina)
• Genome characterization by sample sequencing
• Hosting and set-up of GBrowse
• Data storage, backup and remote access to data
• Remote management of computing facilities

Customers

• Universities and public/private Research Institutes all around the world
• Hospitals
• Private Companies (seed firms, agro-industries…)

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Nicoletta Felice, Università di Udine e IGA
Simone Scalabrin, IGA
Luca Beretta, Illumina
Maria Iascone, Ospedali Riuniti di Bergamo
Laura Pezzoli, Ospedali Riuniti di Bergamo
Silvana Penco, Ospedale Niguarda Milano
Lorena Mosca, Ospedale Niguarda Milano