How do we sequence DNA

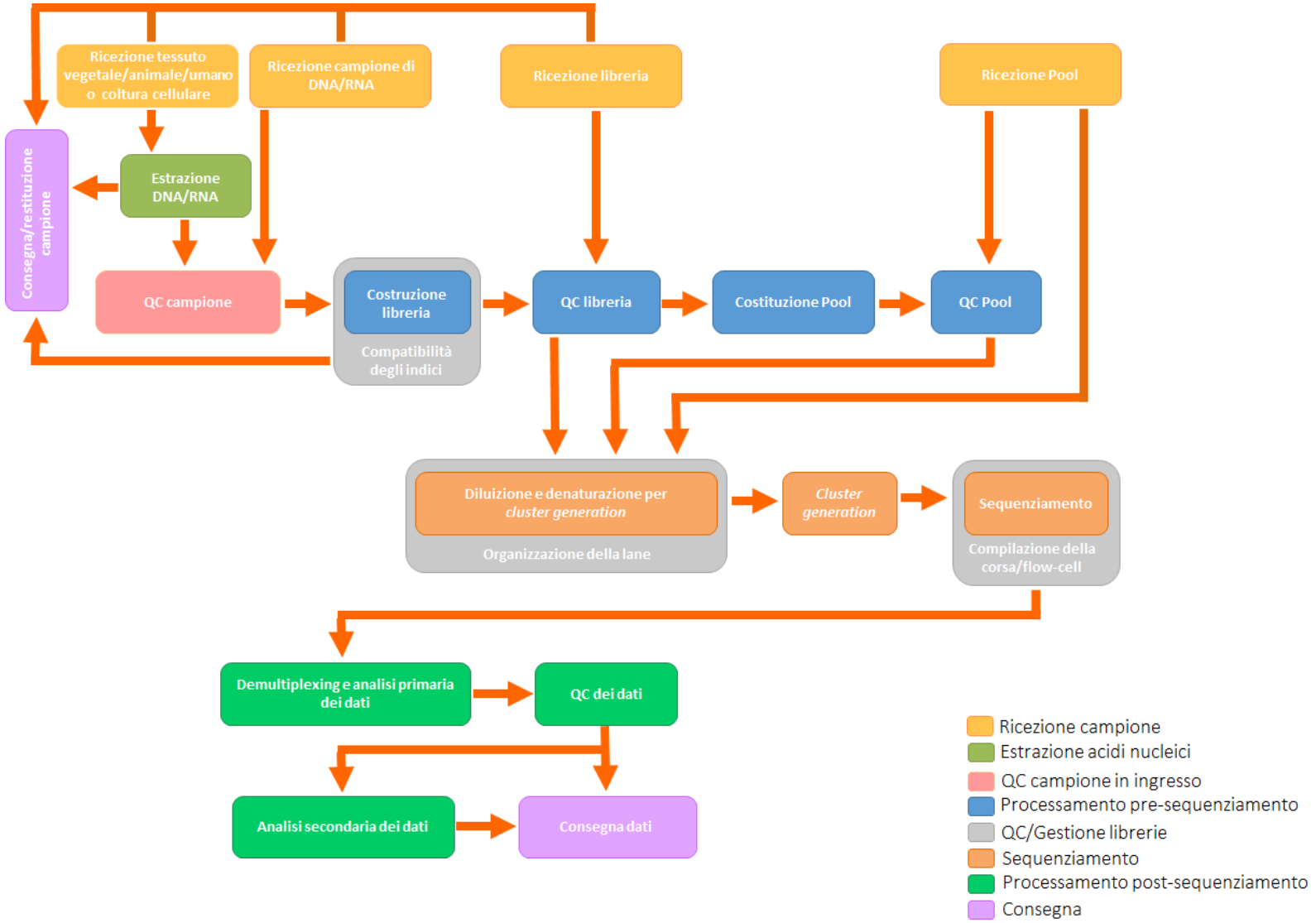
September 2018

This manual was developed on the occasion of the visit of the high-school students to our laboratories.

The activity of a sequencing centre is very complex and based on a teamwork.
We hope that by reading this document you will find some useful information and hopefully we have rised your curiosity.

Special thanks to all the IGA Team for conceiving this document with expertize and ethusiasm.

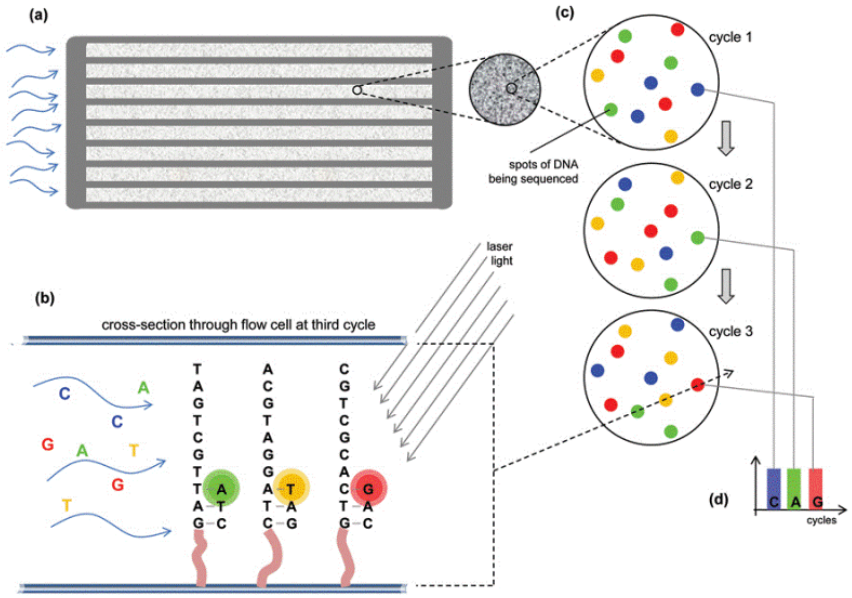
NGS Workflow



Flow Cell

illumina

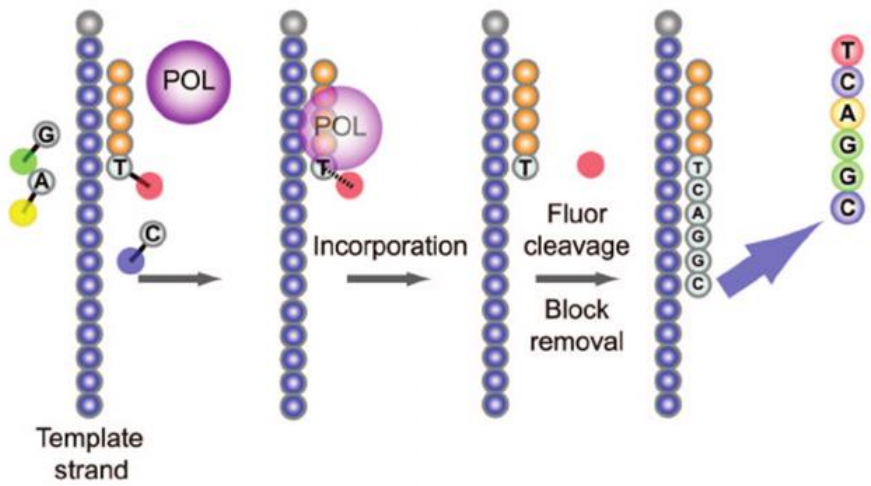
Sequencing templates are immobilized on a proprietary flow cell surface designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surfacebound template and low non-specific binding of fluorescently labeled nucleotides.



Sequencing By Reversible Dye Terminators

illumina

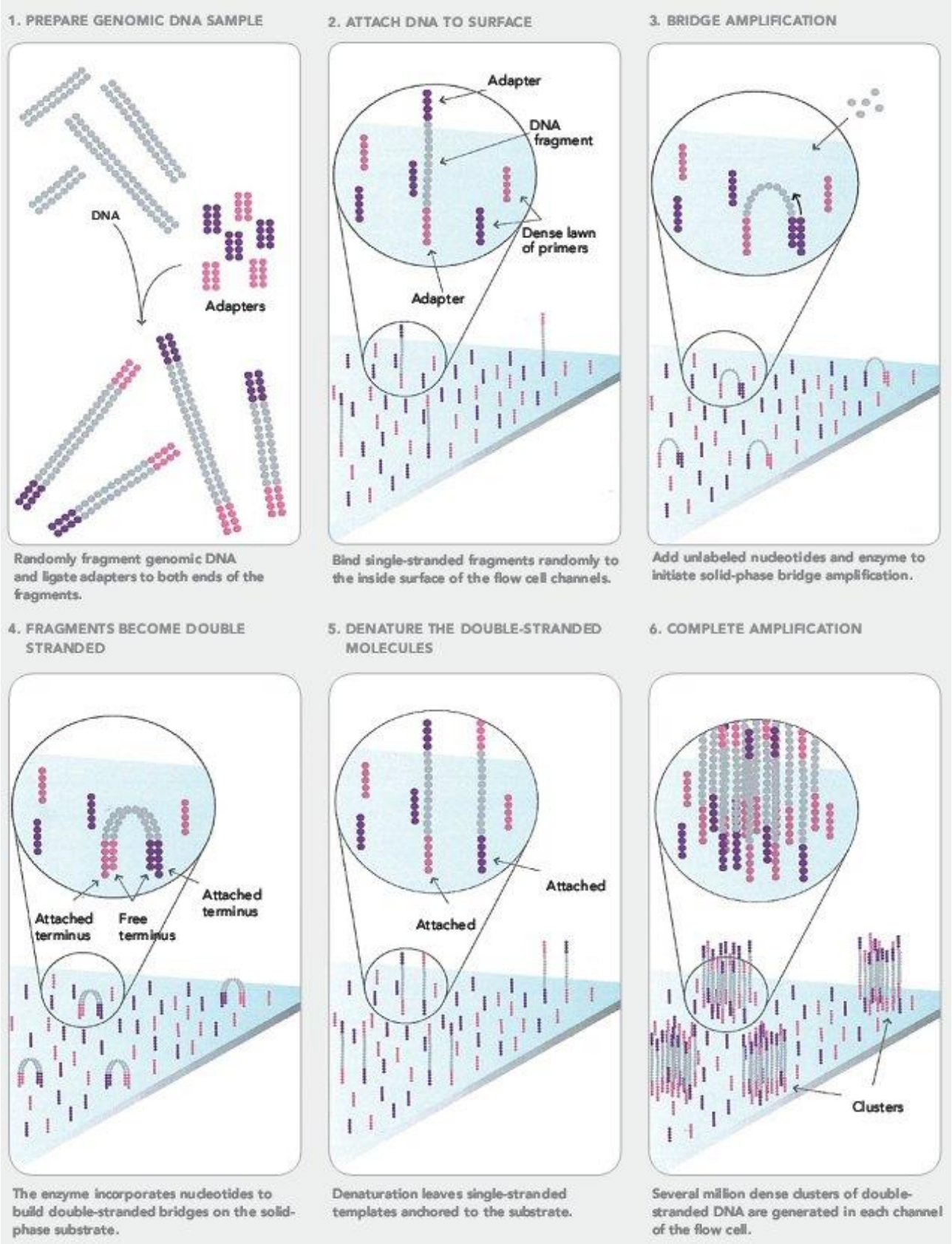
Sequencing by synthesis (SBS) technology uses four fluorescentlylabeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labeled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Base calls are made directly from signal intensity measurements during each cycle.



Cluster Generation

illumina

Solid-phase amplification creates up to 1,000 identical copies of each single template molecule in close proximity (diameter of one micron or less).



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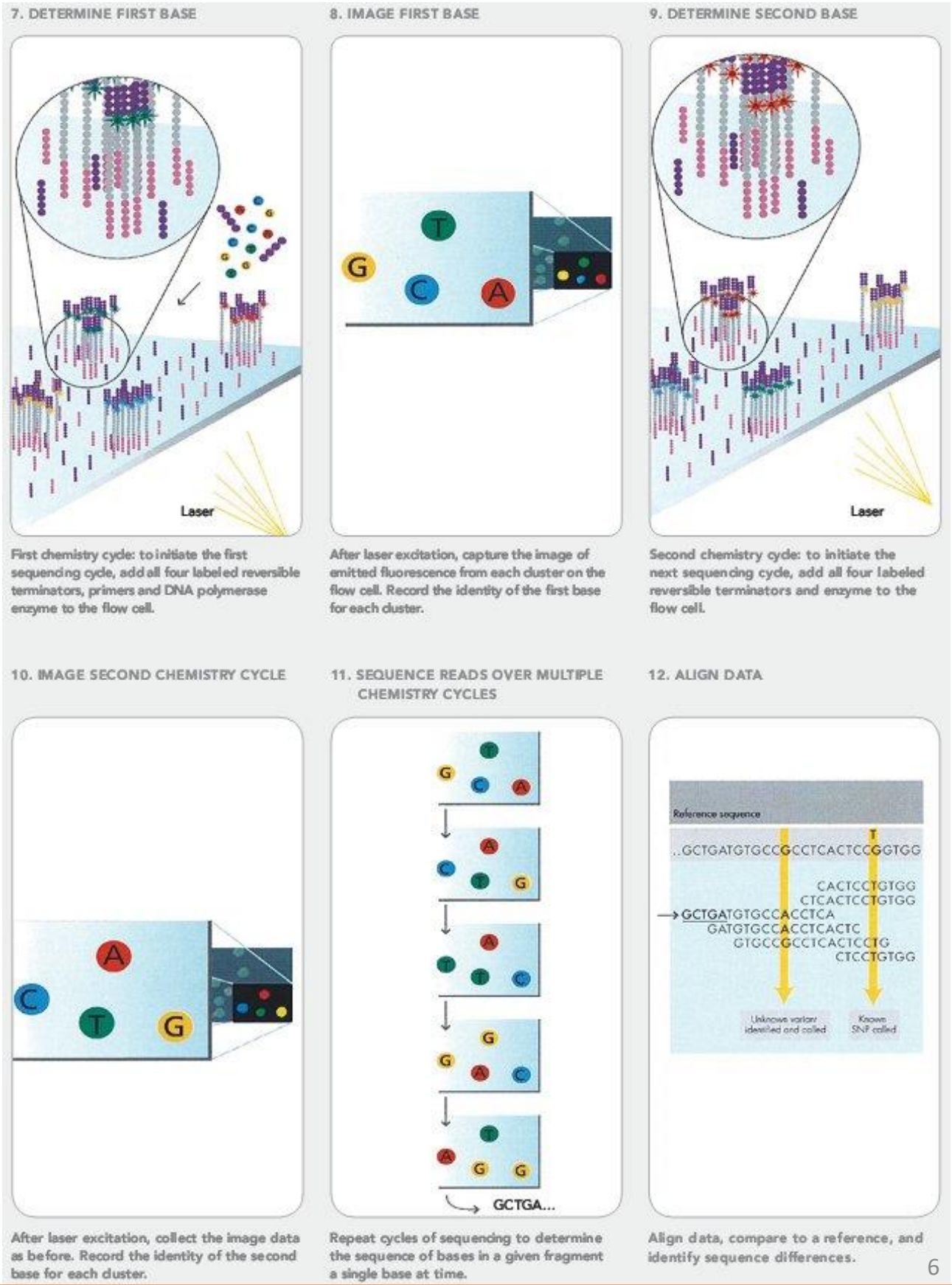
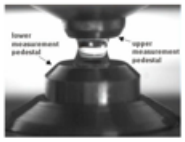


IGATech EUROPEAN BIOTECH WEEK IGA

NanoDrop 1000 Spectrophotometer

Instrument for sample/library QC → quantitative QC

https://www.thermofisher.com/it/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-nucleic-acid-quantification.html



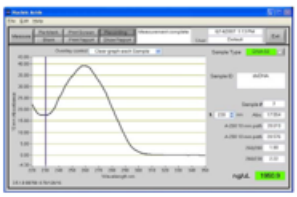
Instrument description

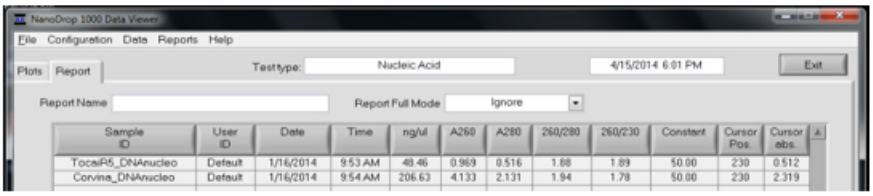
The Thermo Scientific NanoDrop 1000 Spectrophotometer measures 1 uL samples with high accuracy and reproducibility.
The full spectrum (220-750nm) spectrophotometer utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. This eliminates the need for cumbersome cuvettes and other sample containment devices and allows for clean up in seconds.

Applications

Nucleic Acid – concentration and purity of nucleic acid
MicroArray – dye incorporation concentration and purity of nucleic acid
UV-Vis – general UV-Vis measurements
Cell Cultures – absorbance (light scattering) measurement of suspended microbial cells
Protein A280 – concentration and purity of purified protein
Protein & Labels – concentration of dye-labeled proteins, conjugates and metalloproteins
Protein BCA – protein concentratrion using the BCA assay
Protein Bradford – protein concentration using the Bradford assay
Protein Lowry – protein concentration using the Modified Lowry assay

Example of Assay → DNA





Qubit 2.0 Fluorometer

Instrument for sample/library QC → quantitative QC

https://www.thermofisher.com/it/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html?gclid=EAIaIQobChMIkqaEyu221gIVSjwbCh1MOQQbEAAYASAAEgLpFPD_BwE&s_kwcid=AL!3652!3!222404684081!b!!g!!qubit%20dna&ef_id=VwJTAQAAAXpGBz08:20170921175916:s

Instrument description

The Qubit 2.0 Fluorometer is a benchtop fluorometer for the quantitation of DNA, RNA and protein, using the highly sensitive and accurate fluorescence-based Qubit quantitation assays.
Use of the state-of-art dyes selective for dsDNA, RNA and protein minimizes the effects of contaminants in your sample that affect the quantitation. Further, the very latest illumination and detection technologies used in the Qubit 2.0 Fluorometer for attaining the highest sensitivity allow you to use as little as 1 uL of sample and still achieve high levels of accuracy, even with very dilute samples.

Assays

DNA Qubit ssDNA Assay 1-200 ng
Qubit dsDNA BR Assay 2-1000 ng
Qubit dsDNA HS Assay 0.2-100 ng

RNA Qubit RNA BR Assay 20-1000 ng
Qubit RNA Assay 5-100 ng

Protein Qubit Protein Assay 0.25-5 ug

Example of Assay

Name	Date/Time	Assay Conc.	Units	Stock Conc.	Units	Assay Type	Sample Vol (µL)	Dilution Factor
Sample_A	2/4/2014 16:27	172	ng/ml	34.4	ng/µL	dsDNA HS	1	200
Sample_B	2/4/2014 16:27	329	ng/ml	65.8	ng/µL	dsDNA HS	1	200
Sample_C	2/4/2014 16:28	144	ng/ml	28.8	ng/µL	dsDNA HS	1	200
Sample_D	2/4/2014 16:28	204	ng/ml	40.8	ng/µL	dsDNA HS	1	200

GloMax

Instrument for sample/library QC → quantitative QC

https://ita.promega.com/products/fluorometers-luminometers-multimode-readers/multimode-readers/glomax-explorer-system/?catNum=GM3500

Instrument description

The GloMax® Explorer is a high-performance multimode detection instrument that allows you to get up and running quickly, generating the data you need from your experiments. Simply unpack it, plug it in, and begin your experiments. You can also interpret your results using integrated data analysis software. Developed with Promega reagents to provide a simple means of detecting advanced chemistries, the GloMax® Explorer measures luminescence, fluorescence intensity and visible absorbance. The GloMax® Explorer can be used as a standalone instrument or integrated into your high-throughput automated workflow.

Example of Assay

Raw fluorescence data

	1	2	3	4	5	6
A	1.60E+03	1.26E+03	1.75E+03	2.10E+03	1.47E+03	1.60E+03
B	1.21E+03	7.56E+02	1.28E+03	1.61E+03	1.25E+03	1.81E+03
C	1.53E+03	8.15E+02	1.50E+03	1.03E+03	1.36E+03	2.52E+03
D	1.08E+03	1.44E+03	1.33E+03	8.63E+02	1.33E+03	1.90E+03
E	1.98E+03	1.27E+03	1.90E+03	5.25E+02	1.79E+03	1.52E+03
F	2.23E+03	1.10E+03	1.60E+03	8.57E+02	4.28E+02	7.80E+02
G	9.99E+02	7.09E+02	1.41E+03	6.64E+02	8.87E+02	1.48E+03
H	1.46E+03	1.29E+03	1.31E+03	5.94E+02	2.02E+03	1.15E+03

Blank corrected fluorescence data

	1	2	3	4	5	6
A	1584.0	1242.5	1733.4	2087.7	1454.1	1589.4
B	1195.6	742.1	1269.0	1591.6	1238.3	1798.8
C	1515.6	800.5	1481.9	1011.5	1342.2	2506.9
D	1062.2	1420.8	1312.2	849.1	1319.5	1887.3
E	1966.9	1251.6	1286.6	510.1	1772.3	1110.3
F	2214.9	1089.2	1587.7	842.5	415.5	765.9
G	984.6	694.7	1394.4	649.9	872.1	1446.4
H	1443.0	1273.1	1296.3	519.6	2006.5	1139.5

gDNA concentrations (ng/µL)

	1	2	3	4	5	6
A	22.4	17.6	24.5	29.6	20.6	22.5
B	16.9	10.5	18.0	22.5	17.5	25.5
C	21.5	11.9	21.0	14.3	19.0	35.5
D	15.0	20.1	18.6	12.0	18.7	26.7
E	27.9	17.7	18.2	7.2	25.1	15.7
F	31.4	15.4	22.5	11.9	5.9	10.8
G	13.9	9.8	19.7	9.2	9.5	20.5
H	20.4	18.0	18.4	7.4	28.4	16.1

Bioanalyzer 2100

Instrument for sample/library QC → quantitative and qualitative QC

http://www.genomics.agilent.com/en/Bioanalyzer-System/2100-Bioanalyzer-Instruments/?cid=AG-PT-106

Instrument description

The Agilent 2100 Bioanalyzer is a microfluidics-based platform for sizing, quantification and quality control of DNA, RNA, proteins and cells. Miniaturization of analytical instrumentation has many advantages over conventional techniques: improved data precision and reproducibility, short analysis times, minimal sample consumption, improved automation and integration of complex workflows.
Results are delivered within 30-40 minutes in automated, high quality digital data.

Assays

DNA DNA 1000 Series II
DNA 7500 Series II
DNA 12000 Series II
High Sensitivity DNA

RNA Eukariote Total RNA Nano Series II
Eukariote Total RNA Pico Series II
mRNA Nano Series II
mRNA Pico Series II
small RNA Series II
Plant RNA Nano Series II
Plant RNA Pico Series II
Prokaryote Total RNA Nano Series II
Prokaryote Total RNA Pico Series II

Protein Protein 230 Series II
Protein 80 Series II
High Sensitivity Protein Series II

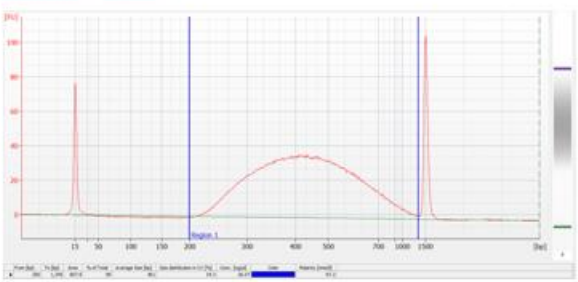
Biochemical analysis

DNA quantification
sizing

RNA integrity
quantification
sizing

Protein quantification
sizing
purity

Example of Assay



LabChip GX

Caliper LifeSciences

Instrument for sample/library QC → quantitative and qualitative QC

http://www.perkinelmer.com/category/microfluidic-capillary-electrophoresis-systems

Instrument description

The LabChip GX assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip format dramatically reduces separation time and provides automated sizing and quantitation information in a digital format.
In addition to the instrument the complete system also includes the LabChip GX software for data analysis. This adavanced data management suite allows users to visualize results via an electropherogram or virtual gel view. Additionally it provides data in tabular form, which can then be analyzed or easily exported into a spreadsheet format.
With sample acquisition time less than a minute, the instrument can throughly analyze 96 samples in less than an hour, virtually eliminating throughput bottlenecks and improving efficiency.

Assays

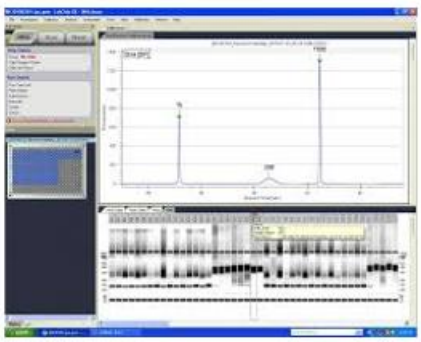
HTRNA
HTDNA 5K
HTDNA 12K
HTDNA 1K

Applications

DNA PCR Analysis & QC
Genotyping
Restriction digest analysis
RLFP
DNA Diagnostics

RNA Quantitation and Quality Assessment of Total RNA
Smear Analysis of mRNA and cRNA

Example of Assay



Mx3000P qPCR System

Agilent Technologies

Instrument for sample/library QC → quantitative QC

https://www.genomics.agilent.com/en/Mx3005P-Mx3000P-qPCR-Instruments-Software/Mx3000P-qPCR-System/?cid=AG-PT-199&tabId=AG-PR-1126

Instrument description

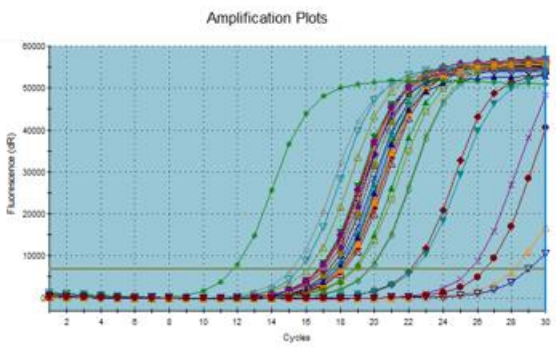
The Mx3000P™ real-time PCR system is a fully integrated real-time PCR detection system. The system includes a state-of-the-art thermal cycler, a quartz-tungsten halogen lamp to excite fluorescence, a photomultiplier tube for high-sensitivity detection, and real-time quantitative detection and analysis software.
The Mx3000P system has an open format that allows closed-tube real-time PCR detection with many detection chemistries including SYBR® Green dye and fluorogenic probe systems.
The software features a variety of specific experiment types with customized plate setup, thermal profile setup and analysis screens that streamline the process of collecting and analyzing data for specific applications.

Assays

Quantitative PCR
Comparative Quantitation
SYBR Green (with Dissociation Curve)
Allele Discrimination/SNP's Real-Time
Molecular Beacon Melting Curve

Example of Assay

Replicate	Well Name	Well Com	Threshold	Ct (dR)	Quantity (	RSq (dR)	Slope (dR)
1	8A_TruSeq01_Libr20140307	1:1000000	6714.914	16.9	6.80E-02	1	-3.478
2	8B_TruSeq02_Libr20140307	1:1000000	6714.914	18.09	3.11E-02	1	-3.478
3	8C_TruSeq03_Libr20140307	1:1000000	6714.914	18.74	2.01E-02	1	-3.478
4	8D_TruSeq04_Libr20140307	1:1000000	6714.914	17.95	3.40E-02	1	-3.478
5	8E_TruSeq05_Libr20140307	1:1000000	6714.914	19.7	1.07E-02	1	-3.478
6	8F_TruSeq06_Libr20140307	1:1000000	6714.914	19.76	1.03E-02	1	-3.478
7	8G_TruSeq07_Libr20140307	1:1000000	6714.914	17.38	4.97E-02	1	-3.478



Bioruptor® Sonication System

Instrument for library preparation

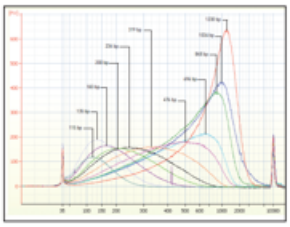
https://www.diagenode.com/en/categories/bioruptor-shearing-device

Instrument description

Some of the key design features of the Bioruptor® are the laboratory friendly format, ability to use many sample tube types in a water bath-based rotor, and flexible power controls. The walls of the waterbath reflect the ultrasound waves in a random but reproducible pattern. The samples in the adaptor are rotated through the ultrasound field to expose each sample to the same level and intensity of energy. This novel technology enables a wide range of applications for superior yields and quality.

The effect of ultrasound on biological samples

The Bioruptor® sonication system uses ultrasound to create focused mechanical stress to shear DNA. Ultrasound waves pass through the sample expanding and contracting the liquid. During expansion, negative pressures pull the molecules away from one another to form a cavity or bubble. This process is called cavitation. The bubble continues to absorb energy until it can no longer sustain itself and implodes. This produces intense focused shearing forces, that disperse and break biomolecules. The fragmentation of DNA takes place as a consequence of this mechanical stress or shear.
With the Bioruptor®, the entire volume of water present in the water bath is exposed to ultrasound, allowing all the samples to be efficiently sonicated in parallel.



DNA size distributions of sheared genomic DNA

Figure shows different DNA size distributions of sheared genomic DNA produced by varying the duration of sonication using power setting high (H). The different curves depict a specific Bioruptor® NGS run, optimized to produce specific mean sizes and size ranges for next-generation sequencing.
All samples were analysed on Bioanalyzer 2100 using DNA High Sensitivity chip.

Biomek FX

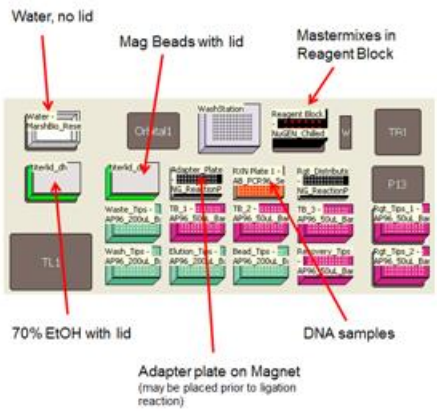
Instrument for library preparation

http://liquidhandlersbybeckman.com/?pi_ad_id=65645902942

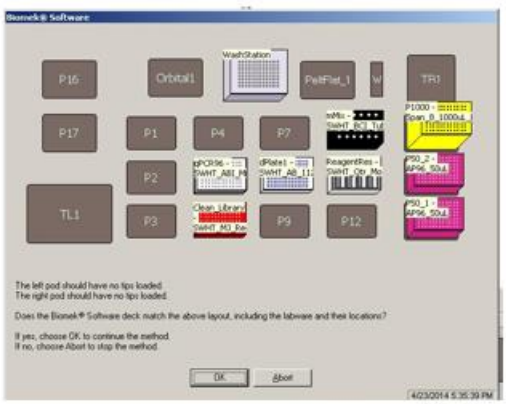
Instrument description

The Biomek FX^P is the latest entry in the Biomek line.
With an up-to-date hardware design for greater positional accuracy and increased robustness, it can meet the needs of just about any application. It can be configured with either one or two pipetting pods. With its large deck capacity, the Biomek FX^P sets the standard for flexible laboratory solutions to meet your changing needs. It puts every aspect of liquid handling – including pipetting, dilution, dispensing and integration – into a single, automated system

Example of Deck Setup for Library construction



Example of Deck Setup for qPCR Reaction Setup



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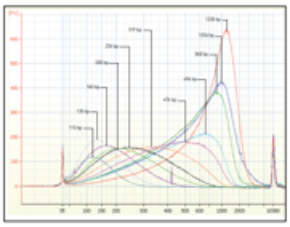

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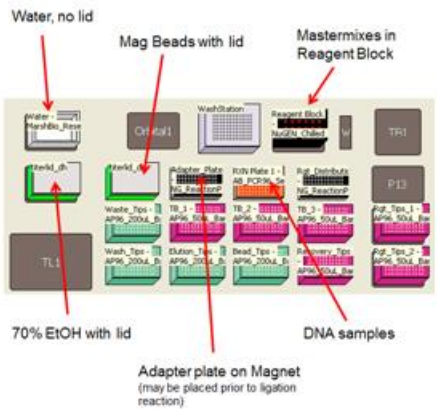

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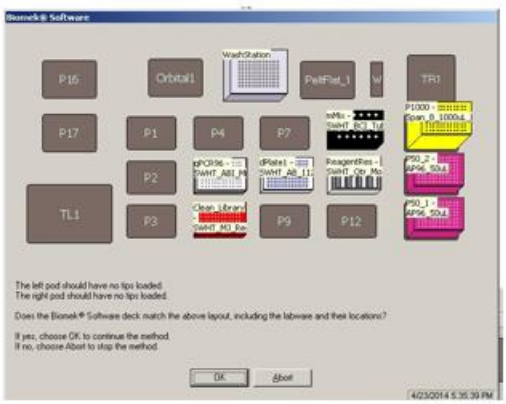


cBot

Instrument for sequencing

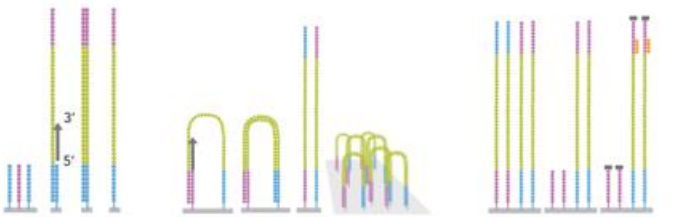
https://www.illumina.com/products/by-type/accessory-products/cbot.html

Instrument description

The Illumina cBot Cluster Generation System provides complete automation of a complex process.
cBot is a revolutionary automated system that creates clonal clusters from single molecule DNA templates, preparing them for sequencing by synthesis on the HiSeq, Genome Analyzer, HiScanSQ, and HiSeq X Ten sequencers.
With very little hands-on time and no reagent preparation, the cBot uses bridge amplification to create over 100 million DNA templates simultaneously in less than five hours. The cBot dispenses reagents from a pre-aliquoted 96-well plate, and controls reaction times, flow rates, and temperatures. The run is set up using the cBot software interface, which simplifies operation and provides a visual report of run status. An on-instrument barcode reader records the reagents and flow cell used for each experiment.

cBot Cluster Generation Process

cBot isothermally amplifies cDNA fragments that have been captured by complementary adapter oligonucleotides covalently bound to the surface of Illumina flow cells.
Flow cells facilitate access of bound DNA to enzymes while ensuring high stability of surface-bound template and low non-specific binding of fluorescently-labeled nucleotides. Attached DNA fragments are extended and bridge amplified to create hundreds of millions of clusters, each of which contains ~1,000 identical copies of a single template molecule.

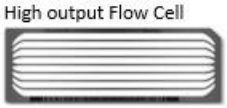


HiSeq2500

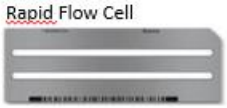
Instrument for sequencing

https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html

High output Flow Cell



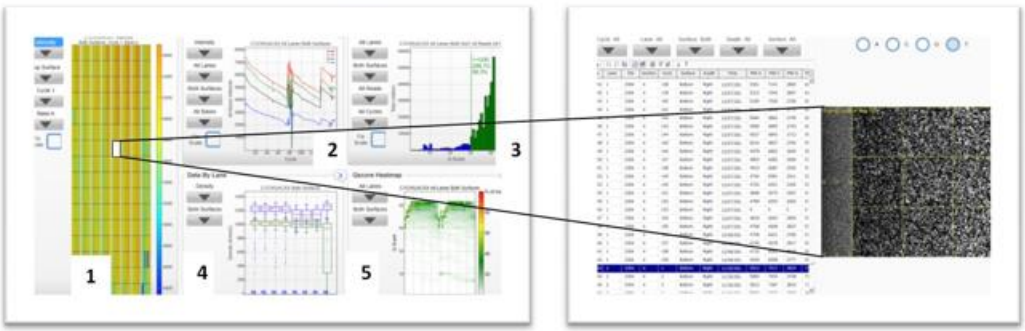
Rapid Flow Cell



Instrument description

Based on the same core architecture as the groundbreaking HiSeq 2000, and with advancements in cycle time reduction and onboard cluster generation originally developed for the MiSeq system, the HiSeq 2500 can be switched between a high output run mode and a rapid turnaround run mode.
Just as the HiSeq 2000 was a breakthroough evolution for next-generation sequencing, HiSeq 2500 has revolutionized the speed at which whole human fenome scale sequencing can be performed. Rapid turnaround time combined with superior data quality is critical for situations where quick answers are needed

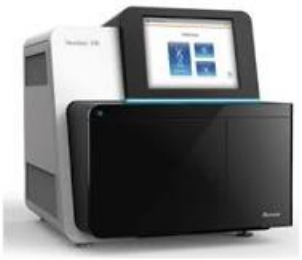
Real-time metrics accessed through HiSeq Control Software



NextSeq500

Instrument for sequencing

https://www.illumina.com/systems/sequencing-platforms/nextseq.html



Instrument description

The NextSeq 500 System Whole-Genome Sequencing (WGS) Solution enables researchers and clinicians to explore the entire genome of any species cost-effectively for a deeper understanding of biology. It leverages industry-standard Illumina next-generation sequencing (NGS) technology responsible for most global WGS, delivering the best data quality and highest coverage to identify variants in coding and noncoding regions of the genome. High-quality library preparation kits are optimized for low-input, unbiased coverage, and rapid workflow. With push-button sequencing, simple data analysis, and minimal hands-on time, the NextSeq 500 System WGS Solution enables researchers to interrogate simple prokaryotic and complex eukaryotic genomes quickly and efficientrly.

Real-time metrics accessed through HiSeq Control Software

MiSeq

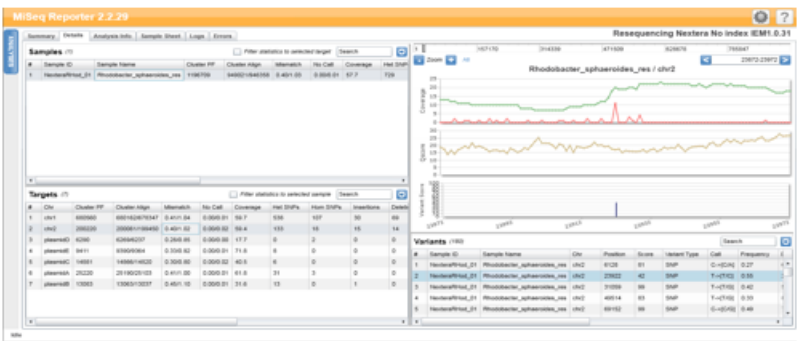
Instrument for sequencing

https://www.illumina.com/systems/sequencing-platforms/miseq.html

Instrument description

The MiSeq system uses TruSeq chemistry, the same proven reversible-terminator sequencing by synthesis technology used by all Illumina sequencing platforms.
The MiSeq system offers the first end-to-end sequencing solution, integrating cluster generation, amplification, sequencing, and data analysis into a single instrument.
MiSeq is the only desktop sequencer that can produce 2 x 300 paired-end reads in a single run. This allows small genome sequencing and assembly, and enables detection of target variants with unmatched accuracy, especially within homopolymer regions. Now, even more samples can be processed in less time while generating more reads per run than any previous versions. All of this can be achieved using the targeted gene and small genome sequencer with the shortest sample-to-data workflow.

Real-time metrics accessed through HiSeq Control Software



Applications

Target Resequencing Amplicon Sequencing
Hybrid Capture
16S Metagenomics
Clone Checking

Small-Genome Sequencing *De novo*
Resequencing
Plasmids

RNA Sequencing small RNA Sequencing
RNA-Seq (microbial)

Quality Control Library QC

Regulation ChIP- Seq

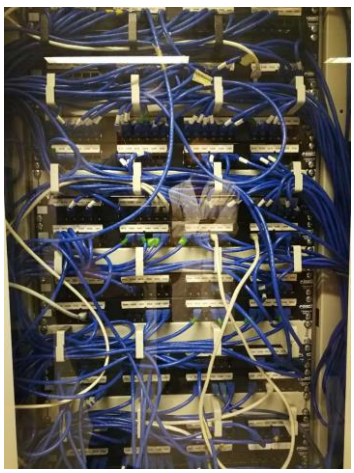
Data Elaboration Center

Instrument for data processing and IT system

RAM totale: 2 Terabyte

Storage: 300 Terabyte

PEv4 Run 0.5 Terabyte



Topic: terapia genica
Area: salute umana
Content: la FDA approva il Kymriah (Università della Pennsylvania), trattamento che modifica geneticamente le cellule del paziente, rendendole i grado di contrastare il cancro. Test condotti su 63 bambini e giovani adulti gravemente malati a causa della LLA. La malattia è andata in remissione entro tre mesi nell'83 per cento dei casi.
Source: http://www.ilpost.it/2017/08/31/kymriah-terapia-genica-leucemia/

Topic: medicina personalizzata FVG
Area: salute umana , IT
Content: sposalizio "made in friuli" tra realtà che trattano Information technology, genomica, clinica con l'obiettivo comune di mettere a punto una piattaforma per la medicina molecolare e personalizzata per integrare i dati classici clinici e le informazioni provenienti dalla genetica
Source: https://issuu.com/friulinnovazione/docs/pubblicazione_finale_d.namica

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Topic: diagnosi precoce tumore al seno e all'ovaio
Area: salute umana
Content: Le donne portatrici (come l'attrice Angelina Jolie) hanno un elevato rischio di sviluppare tumori al seno e all'ovaio. Pro e contro di tutte le opzioni terapeutiche e di prevenzione al momento disponibili. Il test per chi ha familiari con una mutazione già accertata oppure due/tre parenti di primo grado che hanno avuto un tumore al seno o all'ovaio, in convenzione con il sistema sanitario nazionale costa un ticket inferiore a 70 euro.
Riflessioni:
Farmacoprevenzione?
Controlli più frequenti?
Mastectomia e ovariectomia?
Source: https://www.fondazioneveronesi.it/magazine/i-blog-della-fondazione/il-blog-di-airicerca/la-scelta-di-angelina-jolie-cosa-significa-avere-mutazioni-nei-geni-brca-12

Topic: viti resistenti alle malattie

Area: agrigenomica

Content: Cinque vitigni a bacca bianca e cinque a bacca rossa. i primi vitigni resistenti alle malattie costituiti in Italia dai ricercatori dell'Università di Udine e dell'Istituto di Genomica applicata. Frutto di 15 anni di lavoro, questi nuovi vitigni non sono OGM e consentiranno di abbattere notevolmente i costi delle viticoltura, grazie al risparmio sui trattamenti.

E' da notare che la coltivazione della vite, pur occupando soltanto il 3,3% della superficie agricola, utilizza ben il 65% di tutti i funghicidi impiegati in agricoltura.

Source: https://qui.uniud.it/notizieEventi/ricerca-e-innovazione/ecco-i-magnifici-dieci-primi-vitigni-resistenti-alle-malattie-prodotti-in-italia

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Topic: dove si può trovare il DNA?

Area: agrigenomica , alimentazione , ambiente ed ecologia

Content: I materiali grezzi da cui partire possono essere I più vari ed impensabili: dal succo di frutta, alla mozzarella, alla farina, l'acqua, la terra, un frutto, il legno, le radici, i semi, le foglie, gli escrementi, il sangue….

Source: IGATECH

Topic: piante più forti col gene editing

Area: agrigenomica

Content: Il controllo delle malattie in agricoltura attraverso la tecnica del gene editing (CRISPR/Cas9) sembra l'approccio più fattibile ed economico, I risultati sul brusone (Il brusone è la più dannosa malattia fungina del riso a livello mondiale ed è causata da un fungo) sono incoraggianti!

Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4846023/

Topic: un gioco on line per migliorare il gene editing

Area: scienza democratica

Content: scienziati di Stanford coinvolgono la web community con un gioco on line per farsi aiutare a sviluppare un sistema "interruttore" per il sistema CRISPR. Il gioco prevede diversi livelli di difficoltà, ed i risultati migliori potrebbero essere applicati direttamente in laboratorio, con la possibilità degli utenti di poter interagire direttamente con i ricercatori.

Source: https://www.wired.it/scienza/lab/2017/09/01/gioco-online-crispr/

Topic: i batteri di CSI

Area: forense

Content: come riuscire a determinare correttamente quando da quanto tempo è deceduta una persona? Grazie alla metagenomica c'è la possibilità di svelare il ruolo dei microbi nella decomposizione di un cadavere, con conseguente applicazione in campo forense per determinare il Post Mortem Interval

Source: https://www.nature.com/articles/srep24197

Topic: collaborazione vincente!

Area: agrigenomica

Content: la pianta e alcuni batteri benefici che collaborano per intrappolare il patogeno! Il sequenziamento "entra in campo" per aiutare a comprendere i meccanismi sinergici pianta-batterio.

Source: https://www.nature.com/articles/nmicrobiol2016167

Topic: miglioramento genetico accellerato

Area: agrigenomica

Content: le tecniche di sequenziamento consentono di accellerare notevolmente le tempistiche che servono ad individuare piante resistenti alle malattie, con l'obiettivo di ridurre i costi dei trattamenti fitosanitari e di conseguenza l'impatto ambientale

Source: http://www.nature.com/nbt/journal/v34/n6/full/nbt.3540.html

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