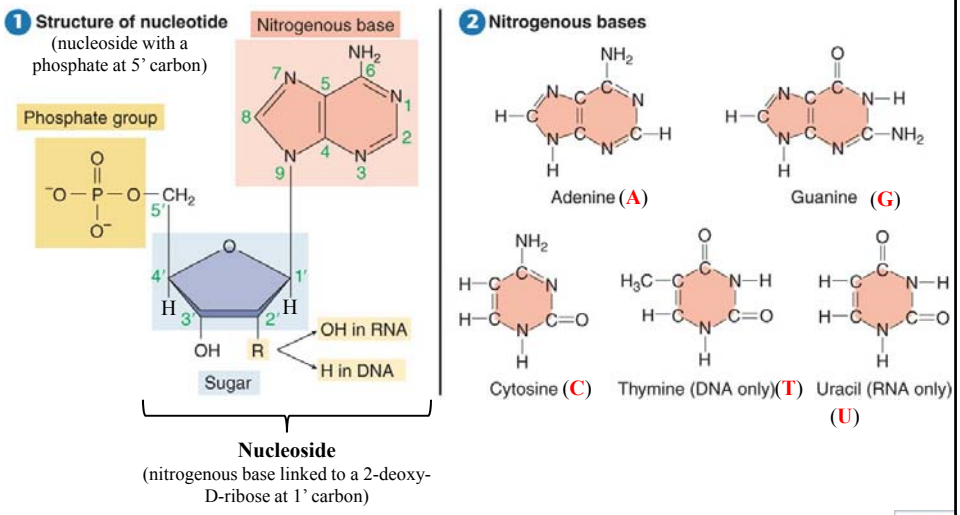
ГЕНОМИКА
4 апреля 2017, Вторник

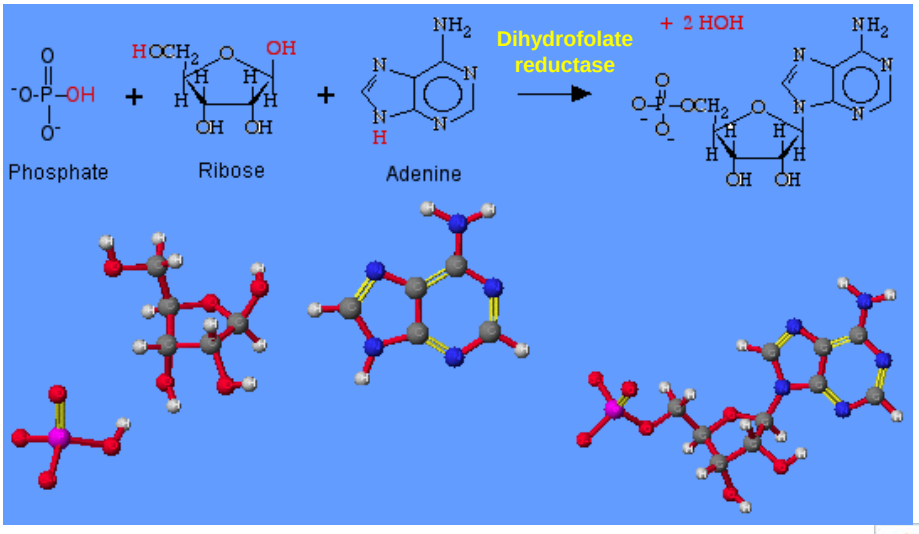
2. Технология секвенирования ДНК:

- по Sanger
- next-generation sequencing (NGS) technology
- полногеномное *de novo* (whole genome *de novo* sequencing)
- ресеквенирование (resequencing)
- целевое (target)
- метагеномное (community (metagenomics) sequencing)

Nitrogenous, Nucleoside and Nucleotide Bases

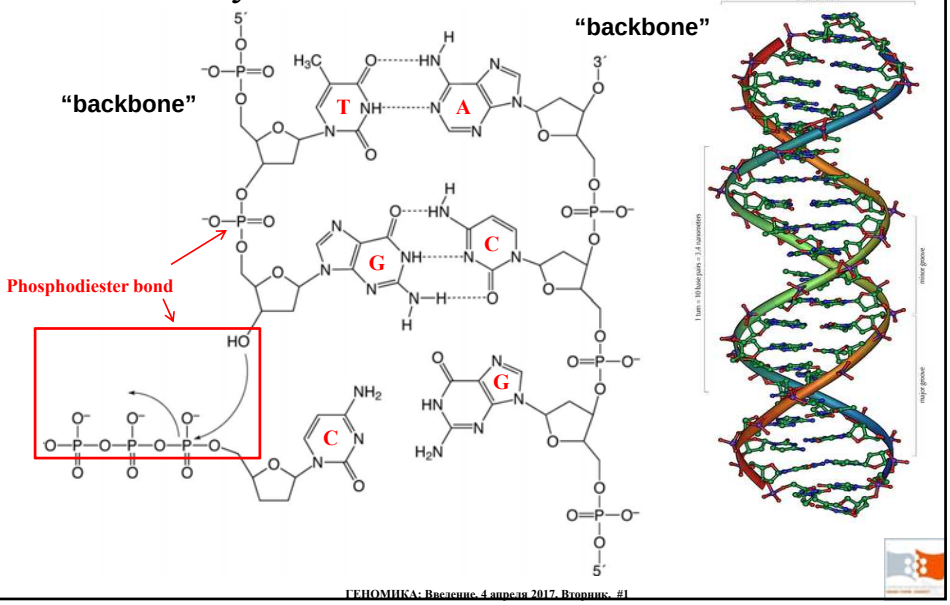


Synthesis of a Nucleotide: Adenosine monophosphate (AMP)



Nucleotide polymer

- DNA Polymerase



DNA Sequencing Methods

1nd generation sequencing methods:

- chemical degradation of nucleotides method (Allan Maxam and Walter Gilbert, 1977)
- chain-termination or dideoxy method (Frederick Sanger, 1977)

Next-Generation Sequencing (NGS) Methods

2nd generation high-throughput massively parallel shotgun sequencing methods:

- **sequencing-by-synthesis methods:**
  - **pyrosequencing** by Jonathan Rothberg (Marguilis et al. 2005) (originally **454 Corp.,** a subsidiary of **CuraGen Corp.**, then **454 Life Sciences**, a subsidiary of **Roche Diagnostics**) based on fixing fragmented (nebulized) and adapter-ligated DNA fragments to small DNA-capture beads PCR amplified in a water-in-oil emulsion in a PicoTiterPlate, and then sequenced using DNA polymerase, ATP sulfurylase, and luciferase (to generate light for detection of the individual nucleotides added to the template DNA) and adding sequentially 4 DNA nucleotides in a fixed order using the Genome Sequencer FLX Instrument (there is also a downscaled Junior version);
  - **based on reversible dye-terminators**, the DNA are extended one nucleotide at a time followed by image acquisition - the camera takes images of the fluorescently labeled nucleotides, then the dye along with the terminal 3' blocker is chemically removed from the DNA, allowing the next cycle (**Solexa**, now **Illumina Inc.**) using Illumina GAII, HiSeq and MiSeq instruments;
  - **semiconductor sequencing** developed by **Ion Torrent Systems Inc.** founded by Jonathan Rothberg (acquired by **Applied Biosystems Inc.** that became **Life Technologies Corp.** as a merger of **Invitrogen Corp.** and **Applied Biosystems Inc.** in 2008; now **Thermo Fisher Scientific Corp.** since 2014), based on the detection of hydrogen ions that are released during the DNA synthesis, as opposed to the optical methods used in other sequencing systems (Ion Torrent and Ion Proton Sequencers - Personal Genome Machines);
- **sequencing-by-ligation method** developed by **Applied Biosystems Inc.** (now **Thermo Fisher Scientific Corp.** since 2014), the DNA is amplified by emulsion PCR and then sequenced using the SOLiD Instrument.

DNA Sequencing Methods

3rd generation **single molecular** (**SM**) based sequencing methods:

- **sequencing-by-synthesis methods:**
  - **SM** method by **Helicos Biosciences (**Cambridge, MA) uses bright fluorophores and laser excitation to detect base addition events from individual DNA molecules fixed to a surface, eliminating the need for molecular amplification. First, DNA fragments with added poly-A tail adapters are attached to the flow cell surface. The next steps involve extension-based sequencing with cyclic washes of the flow cell with fluorescently labeled nucleotides (one nucleotide type at a time, as with the Sanger method). The reads were short, up to 100 bp per run, and on November 15, 2012, Helicos BioSciences filed for Chapter 11 bankruptcy (purchased recently by SeqLL, LLC; seqll.com);
  - **SM real time (SMRT)** sequencing by **Pacific Biosciences** is based on the sequencing by synthesis of the DNA in zero-mode wave-guides (ZMWs) - small well-like containers with the signal capturing tools located at the bottom of the well using DNA polymerase attached to the ZMW bottom and producing reads of up to 15-20 Kbp, with mean read lengths of 2.5-2.9 Kbp;

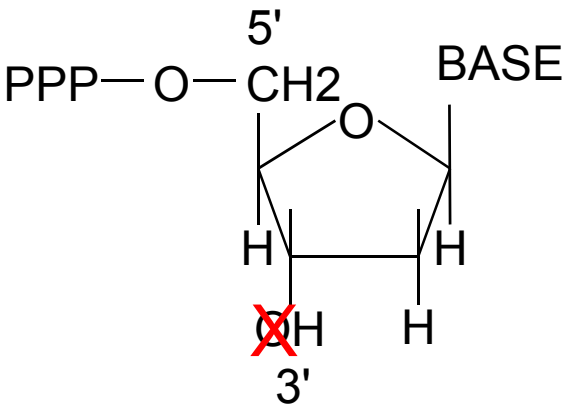
4th generation **single molecular** (**SM**) based sequencing methods:

Roche Nanopore Sequencing and **Oxford Nanopore Technologies** are based on the readout of electrical signals occurring at nucleotides passing through artificially manufactured pores in membranes, for example, in case of **Oxford Nanopore Technologies** the pores are created by the pore-forming protein α-hemolysin covalently bound with cyclodextrin within the nanopore that will bind transiently to the DNA molecule being detected. The DNA passing through the nanopore changes its ion current. Each type of the nucleotide blocks the ion flow through the pore for a different period of time (GridION and PromethION systems and miniaturised MinION instrument).

Dideoxy (Sanger) Method

Dideoxynucleotides without a hydroxyl group at 3'-end of ribose, which prevents strand extension, are used together with normal nucleotides in the sequencing reaction:

Frederick Sanger
1918 –2013



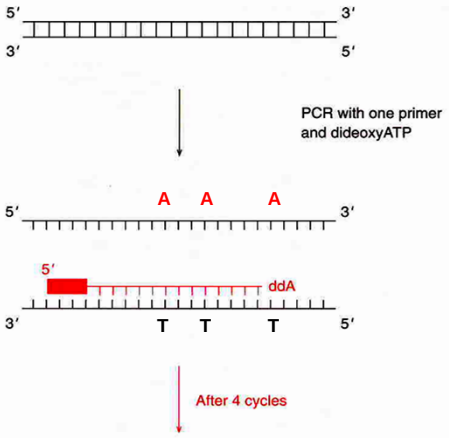
Dideoxy (Sanger) Method

As a result there is a mixture of fragments of different size in a sequencing reaction:

Sequencing cycle:

- 94°C: DNA denaturing
- 45°C: primer annealing
- 60-72°C: thermostable DNA polymerase primer extension

Repeat 25-35 times



ddATP in the reaction: a **ddA** will be occasionally added to the growing strand whenever there is a **T** in the template strand

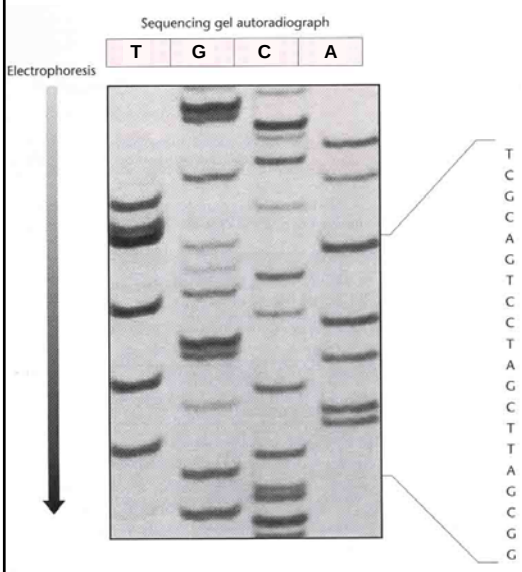
How to visualize DNA fragments?

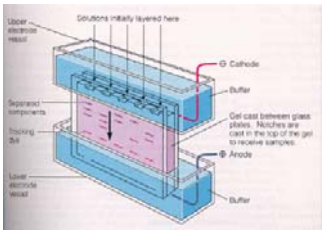
- **Radioactivity**
  - radioactive isotope labeled primers (kinase with ^{32}P)
  - radioactive isotope labeled dNTPs (gamma ^{35}S or ^{32}P)
- **Fluorescence**
  - ddNTPs chemically synthesized to contain fluorescent dye
  - each ddNTP with a different fluorescent dye emits a light at a different wavelength allowing its identification

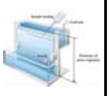
Then, fragments for both methods are separated in high resolution **polyacrylamide gel electrophoresis**

- slab gels
- capillary gels: require only a tiny amount of sample to be loaded, run much faster than slab gels, have higher resolutions, automated (best for high throughput sequencing)

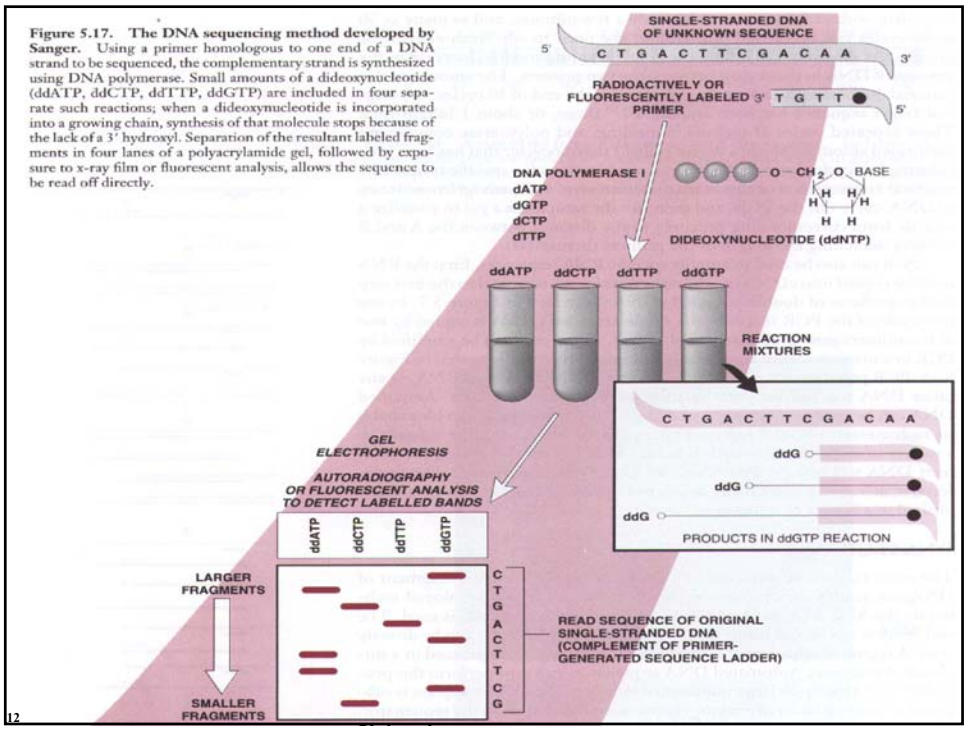
Sequencing gel electrophoresis of DNA fragments amplified using radioactive isotope labeled primers or ddNTP





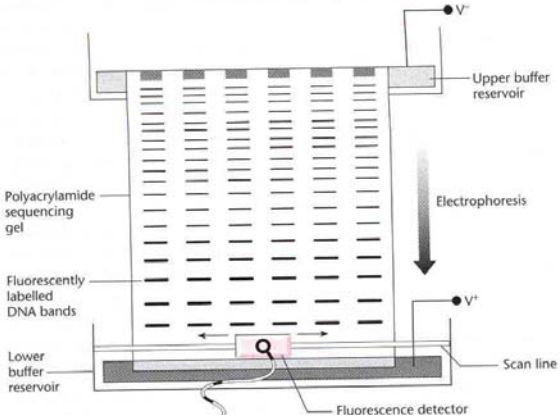


- Radioactively labelled primer or dNTPs in sequencing reaction
- 4 different ddNTPs used in 4 separate reactions, respectively
- Separation of sequencing products by slab gel electrophoresis
- Autoradiography

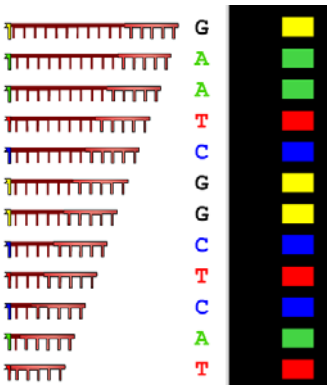


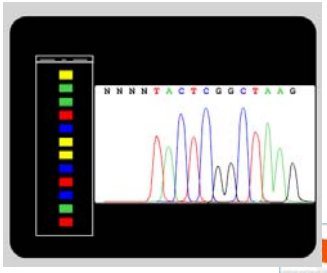
An automated sequencer using mixed ddNTPs labelled by 4 different fluorescent dyes

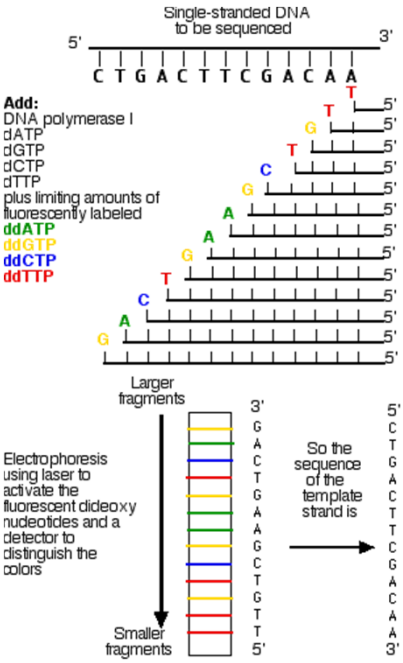
Slab or capillary gel electrophoresis:

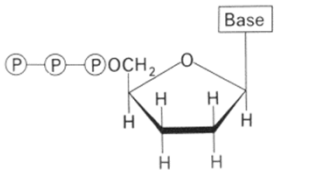


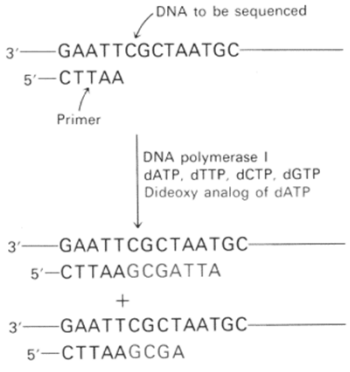
Single lane or capillary output:



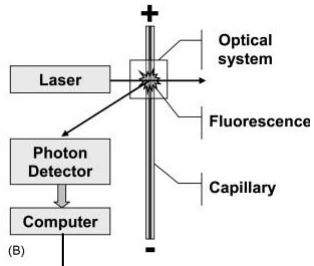




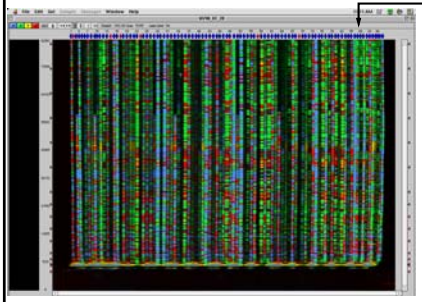


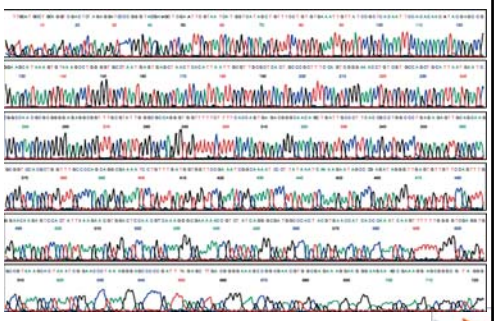


Automated Version of the Dideoxy Method



96 capillaries





Dideoxy (Sanger) Method

Advantages:

- relatively long fragments (500-750 bp)
- low frequency of sequencing errors ("gold standard")

Disadvantages:

- expensive
- laborious
- low productivity

Next Generation Sequencing (NGS)

- Overview of the **Next Generation Sequencing (NGS)** technologies: **second, third and fourth generations**
- **Sequencing-by-synthesis** using reversible nucleotide terminators and luciferase and luminescence signal detection - **Pyrosequencing** (**454 / Roche**)
- **Sequencing-by-synthesis** using reversible nucleotide terminators and fluorescent signal detection - **Illumina** sequencing technology
- Application of NGS for marker discovery and genotyping
  - whole genome *de novo* sequencing
  - whole genome resequencing
  - whole transcriptome sequencing
  - genotyping by sequencing (GBS)
  - genotyping using high density SNP genotyping assays (Infinium)
  - genomic complexity reduction and target resequencing

Available 2nd-Generation Sequencing Technologies

Company	Platform name	Method of sequencing	Method of detection	Read length	Advantages	Relative limitation
Roche/454	GS FLX Titanium	Pyrosequencing	Optical	500-800	Longest read lengths among second generation; high-throughput with respect to first generation (Sanger) sequencing	Challenging sample prep; difficulty reading through repetitive/ homopolymer regions; sequential reagent washing gives steady accumulation of errors; expensive instrument ($500K)
Illumina	HiSeq/(Miseq)	Reversible terminator Sequencing by Synthesis	Fluorescence/Optical	2x150 (HiSeq)/2x300 (MiSeq) (MiniSeq)	Very high-throughput (HiSeq)/Desktop Sequencer (MiSeq)	Expensive instrument (HiSeq); significant cost of data managing and analysis (HiSeq)
ABI/SOLiD **Thermo Fischer Scientific**	5500xl SOLiD System	Sequencing by Ligation	Fluorescence/Optical	25-35	Very high throughput; lowest reagent cost needed to reassemble a human genome among the widely accepted second generation platforms (Illumina, 454, SOLiD)	Long sequencing runs; short reads increase cost and difficulty of data analysis and genome assembly; high instrument cost (~$700K)
Helicos (SeqLL now)	Heliscope	Single-molecule sequencing by synthesis	Fluorescence/Optical	11-100	High throughput; single-molecule nature of technology unique among second-gen platforms	Short reads increasing the costs and reduce quality of genome assembly; very costly instrument (~$1M)

Niedringhaus et al. (2011) Landscape of Next-Generation Sequencing Technologies. *Anal. Chem.* 83: 4327–4341

In 2014, **Illumina** dominated the market ($ 3.3 bln) with 70% of the 2nd generation sequencers, while **ABI Life Technologies/Thermo Fischer Scientific** and **Roche** split nearly all of the remaining market at 14% each.

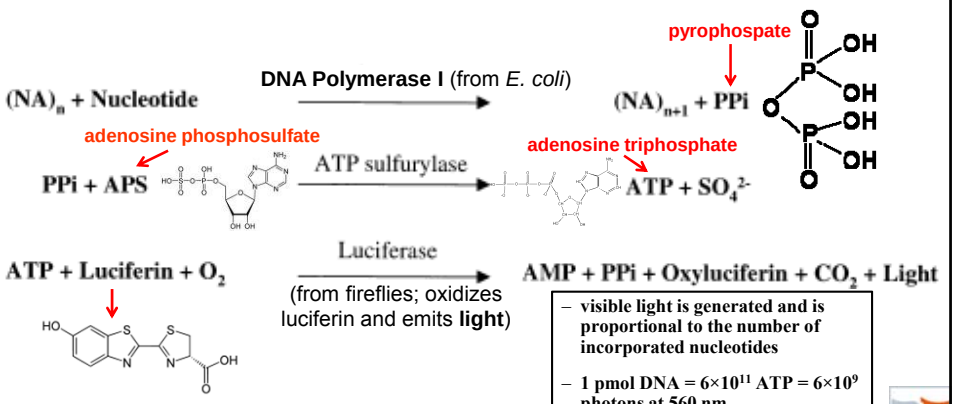
However, the 454 sequencers have been be phased out in 2016!

Available 2nd-Generation Sequencing Technologies

- **454/Roche (www.roche-applied-science.com): GS FLX Titanium**
- **Illumina (www.illumina.com): HiSeq1000/2000/2500/X, MiSeq, MiniSeq**
- **Thermo Fischer Scientific/Life/ABI (www.appliedbiosystems.com): Solid 5500xl**
- **Helicos BioSciences (http://www.helicosbio.com/): HeliScope**

2nd Generation Sequencing: Pyrosequencing method – Basic Principle

- Pyrosequencing is based on the generation of **light** signal through release of **pyrophosphate** (**PPi**) on nucleotide addition: **$(NA)_n$ + dNTP → $(NA)_{n+1}$ + PP_I**
- **PPi** is used to generate **ATP** from **adenosine phosphosulfate** (**APS**): **APS + PP_I → ATP**
- **ATP** and **luciferase** generate **light** by conversion of **luciferin** to **oxyluciferin**.



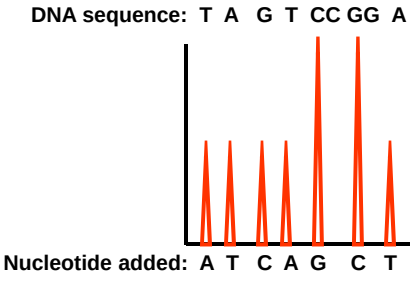
- visible light is generated and is proportional to the number of incorporated nucleotides
- 1 pmol DNA = 6×10^{11} ATP = 6×10^{9} photons at 560 nm

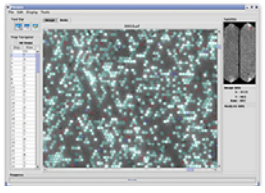
Pyrosequencing: Preparation of DNA library

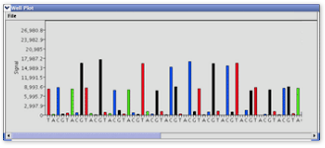
- shearing genomic DNA to small DNA fragments 500-800 bp long
- attaching single DNA fragments to very small plastic beads (one fragment per bead)
- emulsion-based clonal PCR amplification (emPCR) of the DNA on each bead to cover each bead with a cluster of identical fragments to enhance the light signal
- placing each bead in a separate well on a PicoTiterPlate, a fiber optic chip with up to 1.6 million wells (A mix of enzymes such as DNA polymerase, ATP sulfurylase, and luciferase are also packed into the well.)
- The PicoTiterPlate is then placed into the GS FLX System for sequencing.

Pyrosequencing – Basic Principle

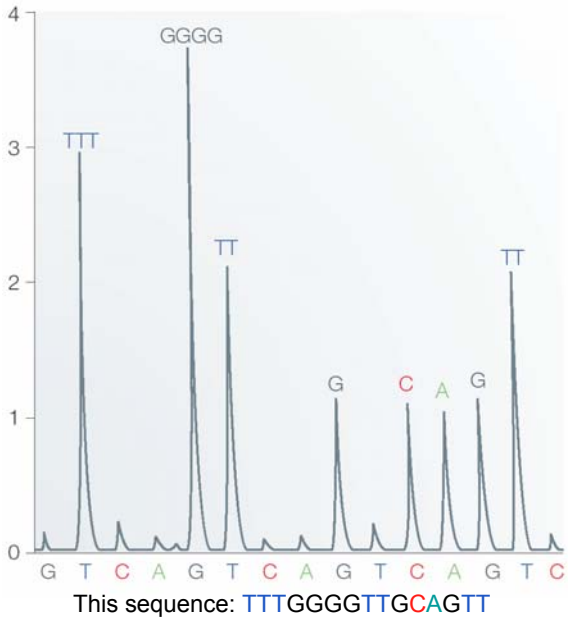
- Sequence-by-synthesis via DNA polymerase directed chain extension, one base at a time in the presence of a reporter (luciferase). Each nucleotide is added separately in a separate cycle.
- Only one of four will generate a light signal. Luciferase will emit a photon of light in response to the pyrophosphate (PPi) released upon nucleotide addition by DNA polymerase
- The remaining nucleotides are removed enzymatically.
- The light signal is recorded on a **pyrogram**:







Pyrosequencing Results:

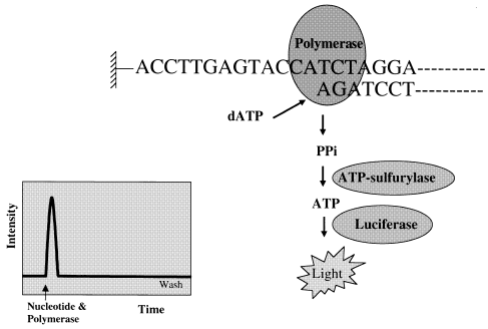


Height of peak indicates the number of dNTPs added

This sequence: TTTGGGGTTGCAGTT

Pyrosequencing

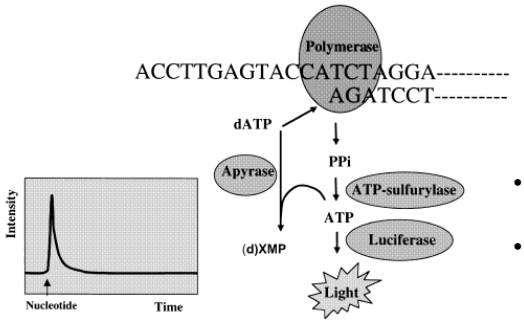
- 1st Method
  - Solid Phase
    - immobilized DNA
    - 3 enzymes
    - wash step to remove nucleotides after each addition



Pyrosequencing

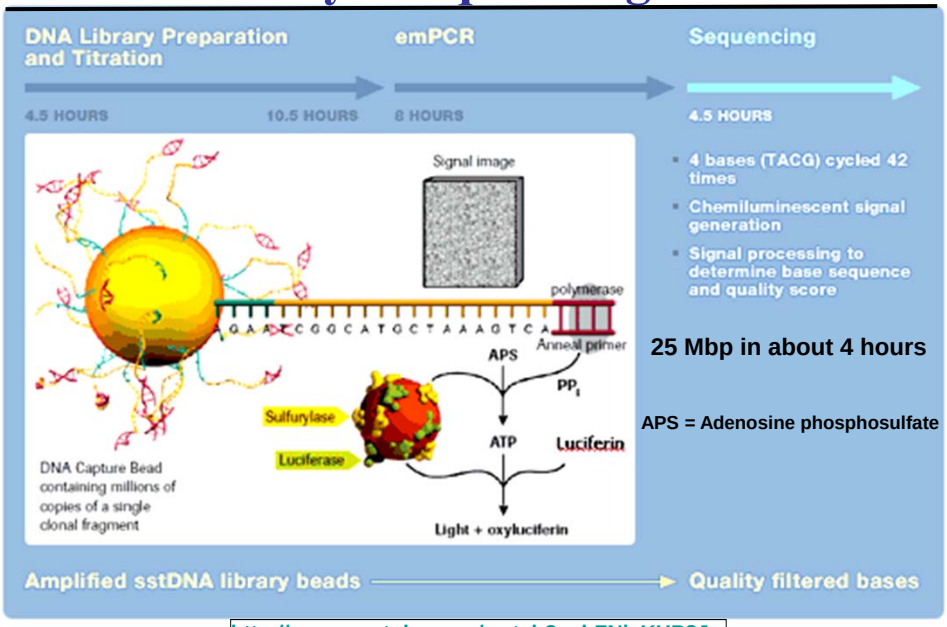
- 2nd Method
  - Liquid Phase
    - 3 enzymes + apyrase (nucleotide degradation enzyme)

      Eliminates need for washing step



- In the well of a microtiter plate:
  - primed DNA template
  - 4 enzymes
- Nucleotides are added stepwise
- Nucleotide-degrading enzyme degrade previous nucleotides

Pyrosequencing



http://www.youtube.com/watch?v=bFNjxKHP8Jc

Pyrosequencing

- Sequencing by synthesis
- **Advantages**:
  - accurate - low frequency of sequencing errors
  - relatively long fragments (500-750 bp)
  - parallel processing
  - automated
  - no need for labeled primers and nucleotides
  - no need for gel electrophoresis
- **Disadvantages**:
  - expensive
  - laborious
  - low productivity
  - nonlinear light response after more than 5-6 identical nucleotides

References

Applied Biosystems Automated DNA Sequence Chemistry Guide. (2000)

Garrett & Grisham. (2007) Biochemistry. Thomson and Brooks/Cole. 3rd ed. Pgs 337-340.

Maxam, A. & Gilbert, W. (1977) A new method for sequencing DNA. *Proc. Natl. Acad. Sci.* **74**, 560-564.

Ronaghi, M. (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Res.* **11**, 3-11.

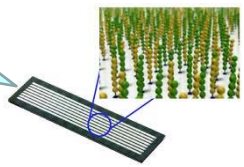
Sanger, F., Nicklen, S., & Coulson, A.R. (1977) DNA Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **94**, 5463-5467.

Shendure, J. & Ji, H. (2008) Next-generation DNA Sequencing. *Nature Biotech.* **26**, 1135-1145

Venter, C, et al. (2001) The sequence of the human genome. *Science*. **291**, 1304.

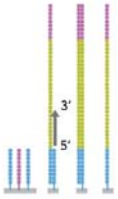
Illumina: Preparation a DNA library for sequencing - Cluster formation

Samples are loaded in a flowcell with 8 lanes and clusters attached to the cell surface are generated and prepared for sequencing using cBot instrument

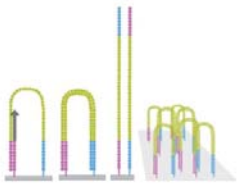


Flowcell with 8 separate lanes and primers complementary to adapters immobilized on the flowcell surface.

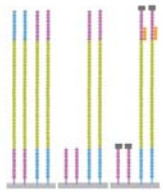
cBOT



Hybridization and second strand synthesis



Bridge PCR



Linearization and primer annealing

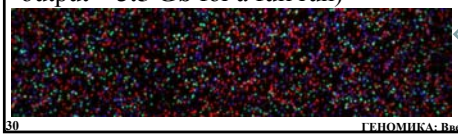
HiSeq2000

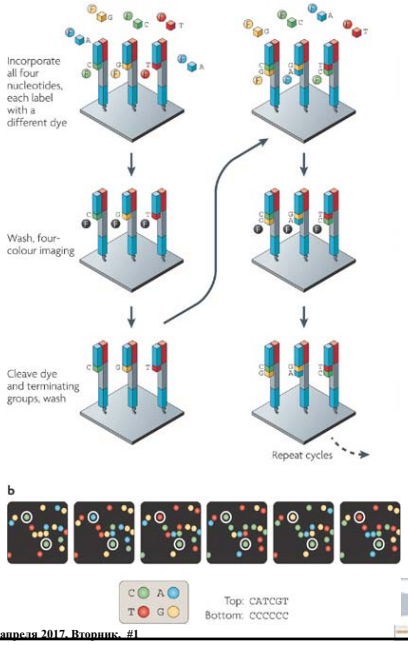
Illumina: Sequencing by Synthesis (SBS)

- Sequencing is performed by addition of labeled reversible terminators (1 base is added per cycle for all the fragments).
- The flowcell is then scanned
- The block and phluorophore is then removed, and another cycle starts up to the desired read length.

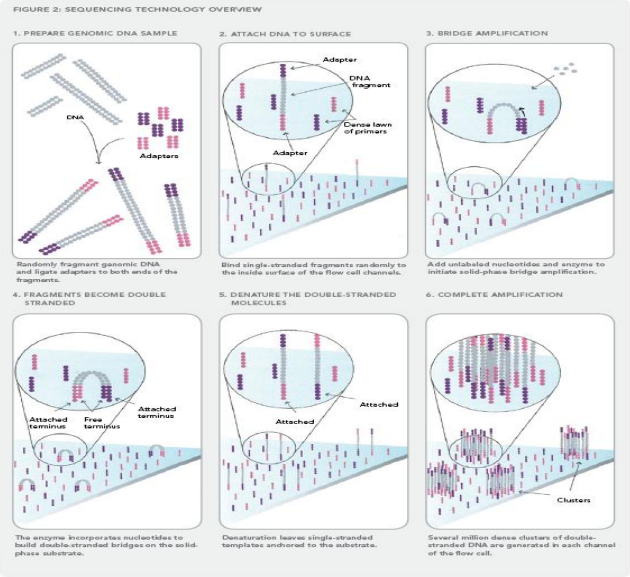
HiSeq X: **330-375 millions of clusters per lane** (8 lanes per flowcell) – **5.3-6 billions** for dual flowcell; total output up to **1.6-1.8 Tb** for a full run with 2 flowcells for paired-end sequences 150 bp × 2

MiSeq: 7 Millions of clusters per flowcell with a single lane; up to 250 bp × 2 (total output = **3.5 Gb** for a full run)





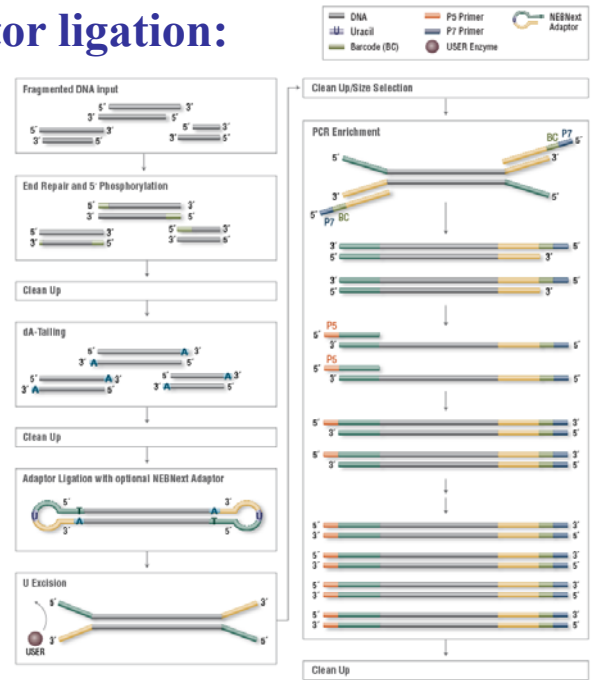
Illumina sequencing technology in 15 steps



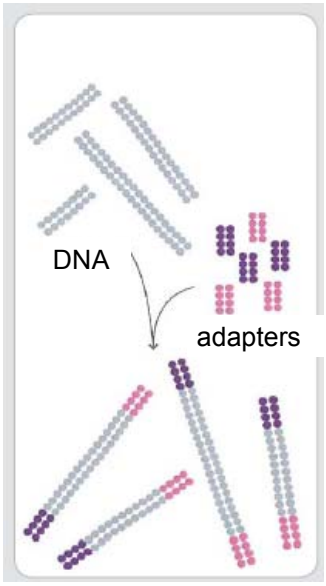
Important note:
Between 1. and 2. steps PCR is performed to selectively enrich those DNA fragments that have adapters on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR primer cocktail that anneals to the ends of the adapters.

http://www.illumina.com/downloads/SS_DNAsequencing.pdf

Adaptor ligation:



https://www.neb.com/products/e6040-nebnext-dna-library-prep-master-mix-set-for-illumina



1. Fragment genomic DNA and ligate adapters

2. Attach DNA to surface

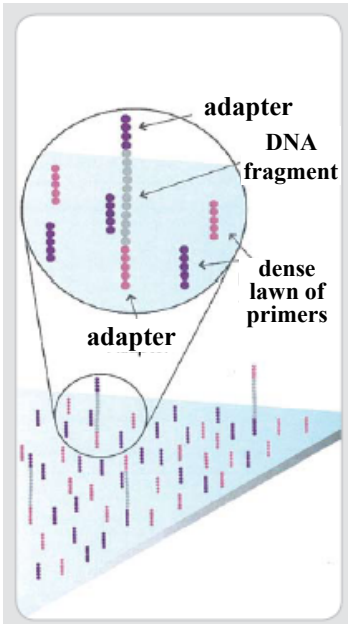
3. Bridge amplification

4. Fragments become double stranded

5. Denature the double-stranded molecules

6. Complete amplification

Randomly fragment genomic DNA and ligate adapters to both ends of the fragments



1. Fragment genomic DNA and ligate adapters

2. Attach DNA fragments to surface

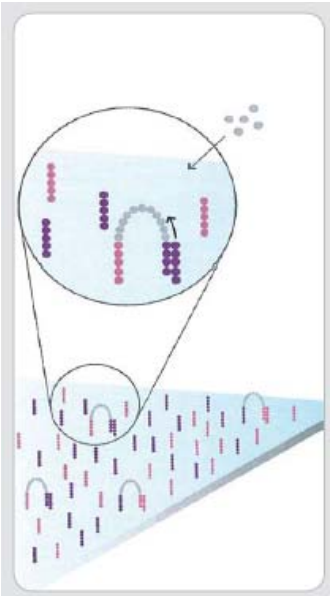
3. Bridge amplification

4. Fragments become double stranded

5. Denature the double-stranded molecules

6. Complete amplification

Bind single-stranded fragments randomly to the inside surface of the flow cell channels



1. Fragment genomic DNA and ligate adapters

2. Attach DNA fragments to surface

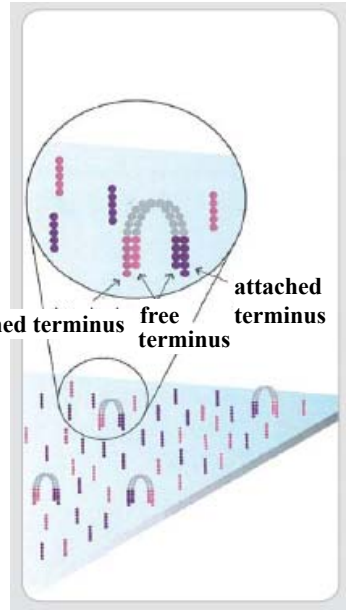
3. Bridge amplification

4. Fragments become double stranded

5. Denature the double-stranded molecules

6. Complete amplification

Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification



1. Fragment genomic DNA and ligate adapters

2. Attach DNA fragments to surface

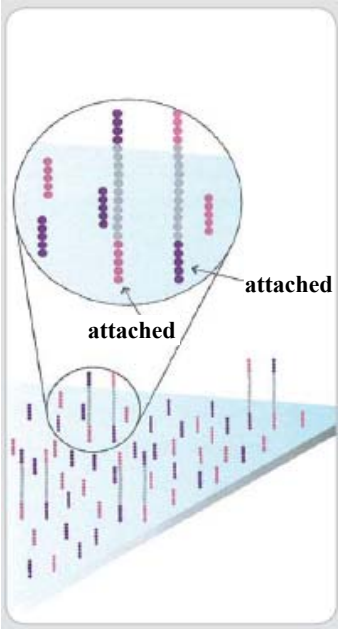
3. Bridge amplification

4. Fragments become double stranded

5. Denature the double- stranded molecules

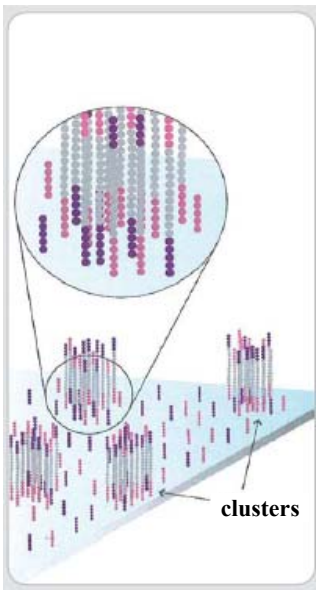
6. Complete amplification

The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate



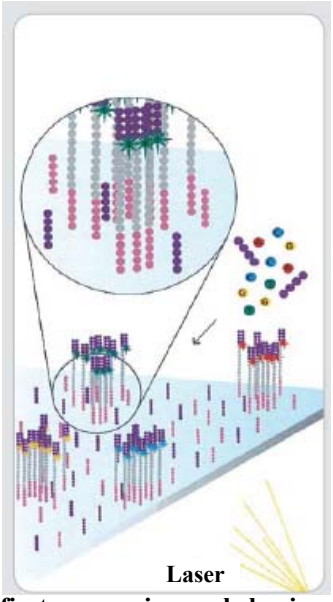
Denaturation leaves single-stranded templates anchored to the substrate

1. Fragment genomic DNA and ligate adapters
2. Attach DNA fragments to surface
3. Bridge amplification
4. Fragments become double stranded
5. **Denature the double-stranded molecules**
6. Complete amplification



Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell

1. Fragment genomic DNA and ligate adapters
2. Attach DNA fragments to surface
3. Bridge amplification
4. Fragments become double stranded
5. Denature the double- stranded molecules
6. **Complete amplification**
7. **Reversed complement strands are cleaved and washed away**



The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase

8. First nucleotide base adding and the first nucleotide base synthesis

9. Image first base

10. The block and phluorophore are then removed

11. Second nucleotide base adding and the second nucleotide base synthesis

12. Image second base

13. The block and phluorophore are then removed

14. Sequencing over multiple chemistry cycles

15. Process sequence reads, etc.

After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified

8. First nucleotide base adding and the first nucleotide base synthesis

9. Image first base

10. The block and phluorophore are then removed

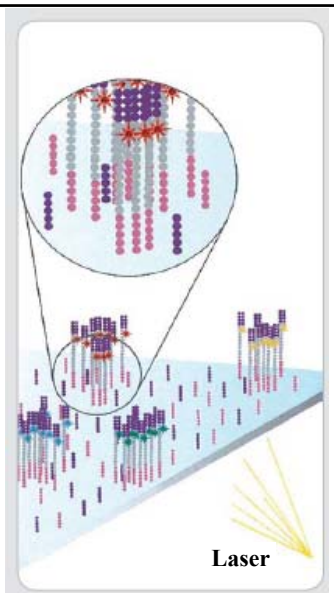
11. Second nucleotide base adding and the second nucleotide base synthesis

12. Image second base

13. The block and phluorophore are then removed

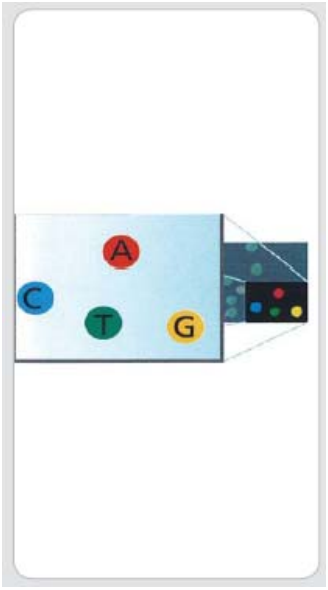
14. Sequencing over multiple chemistry cycles

15. Process sequence reads, generate contigs, map (align) reads or contigs to the reference sequence, if it is available



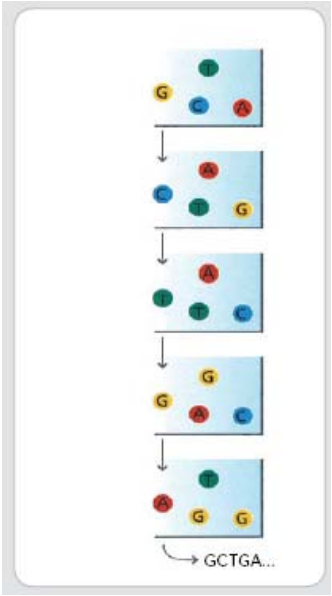
The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase

8. First nucleotide base adding and the first nucleotide base synthesis
9. Image first base
10. The block and phluorophore are then removed
11. **Second nucleotide base adding and the second nucleotide base synthesis**
12. Image second base
13. The block and phluorophore are then removed
14. Sequencing over multiple chemistry cycles
15. Process sequence reads, generate contigs, map (align) reads or contigs to the reference sequence, if it is available



After laser excitation the image is captured as before, and the identity of the second base is recorded.

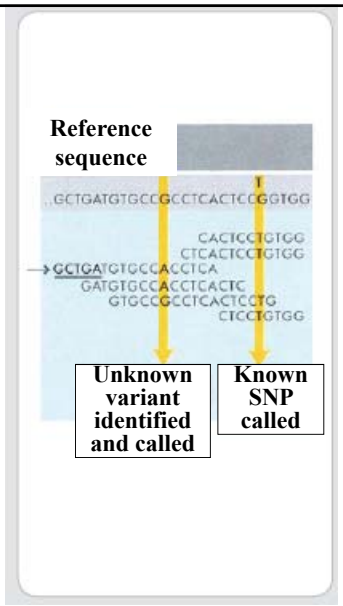
8. First nucleotide base adding and the first nucleotide base synthesis
9. Image first base
10. The block and phluorophore are then removed
11. Second nucleotide base adding and the second nucleotide base synthesis
12. **Image second base**
13. The block and phluorophore are then removed
14. Sequencing over multiple chemistry cycles
15. Process sequence reads, generate contigs, map (align) reads or contigs to the reference sequence, if it is available



The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

https://www.youtube.com/watch?v=fCd6B5HRaZ8

8. First nucleotide base adding and the first nucleotide base synthesis
9. Image first base
10. The block and phluorophore are then removed
11. Second nucleotide base adding and the second nucleotide base synthesis
12. Image second base
13. The block and phluorophore are then removed
14. **Sequencing over multiple chemistry cycles**
15. Process sequence reads, generate contigs, map (align) reads or contigs to the reference sequence, if it is available



The data are aligned and compared to a reference, and sequencing differences are identified.

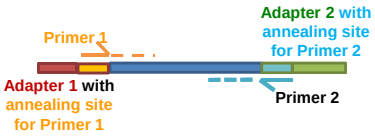
8. First nucleotide base adding and the first nucleotide base synthesis
9. Image first base
10. The block and phluorophore are then removed
11. Second nucleotide base adding and the second nucleotide base synthesis
12. Image second base
13. The block and phluorophore are then removed
14. Sequencing over multiple chemistry cycles
15. Process sequence reads, generate contigs, **map (align)** reads or contigs **to the reference sequence**, if it is available

Single, paired-end and multiplexed sequencing

Single read sequencing:

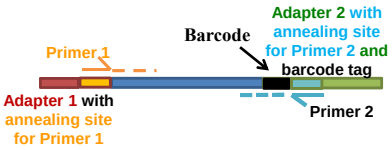
- resequencing
- expression quantification

Pair-end sequencing:



- *de novo* sequencing for better assembly
- allow to resolve better transcript isoforms
- can be used to detect gene fusion

Multiplexed sequencing:



- The sequencing of a 6-nucleotides barcode on one of the adapters allows to identify samples pooled together and sequenced in a single lane.
- Up to 96 samples per lane.

Illumina Sequencers and Genotypers

From Genome-Wide Discovery to Targeting Validation and Screening

	Sequencing					Arrays	
Instrument	HiSeq X™ Ten*	HiSeq® 2500	NextSeq™ 500	MiSeqDx™	MiSeq®	HiScan®	iScan
Technologies	Sequencing by Synthesis (SBS) Powered by TruSeq Chemistry					BeadArray, Infinium, GoldenGate, DASL	
Applications	Population-Scale Whole Human Genome Sequencing	Production-Scale Genome, Exome, Transcriptome Sequencing and More	Exome, Transcriptome, Whole-Genome, Sequencing and More	FDA-Cleared *in vitro* Diagnostic System Cystic Fibrosis Screening and User-Defined Assays	Small Genome, Amplicons, Targeted Gene Panel Sequencing	SNP and Whole-Genome Genotyping, CNV Analysis, Gene Regulation and Epigenetic Analysis, Gene Expression Analysis, Cytogenetic Analysis	

* The HiSeq X Ten consists of 10 sequencing systems.

Illumina Sequencers

	 MiniSeq System	 MiSeq Series	 NextSeq Series	 HiSeq Series	 HiSeq X Series*
Key Methods	Amplicon, targeted RNA, small RNA, and targeted gene panel sequencing.	Small genome, amplicon, and targeted gene panel sequencing.	Everyday exome, transcriptome, and targeted resequencing.	Production-scale genome, exome, transcriptome sequencing, and more.	Population- and production-scale whole-genome sequencing.
Maximum Output	7.5 Gb	15 Gb	120 Gb	1500 Gb	1800 Gb
Maximum Reads per Run	25 million	25 million†	400 million	5 billion	6 billion
Maximum Read Length	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp	2 × 150 bp
Run Time	4–24 hours	4–55 hours	12–30 hours	<1–3.5 days (HiSeq 3000/HiSeq 4000) 7 hours–6 days (HiSeq 2500)	<3 days
Benchtop Sequencer	Yes	Yes	Yes	No	No
System Versions	• MiniSeq System for low-throughput targeted DNA and RNA sequencing	• MiSeq System for targeted and small genome sequencing • MiSeq FGx System for forensic genomics • MiSeqDx System for molecular diagnostics	• NextSeq 500 System for everyday genomics • NextSeq 550 System for both sequencing and cytogenomic arrays	• HiSeq 3000/HiSeq 4000 Systems for production-scale genomics • HiSeq 2500 Systems for large-scale genomics	• HiSeq X Five System for production-scale whole-genome sequencing • HiSeq X Ten System for population-scale whole-genome sequencing

http://www.illumina.com/systems/sequencing.html

Illumina HiSeq 2500 or X

Read length	HiSeq 2500 Dual Flow Cell	HiSeq 2500 Single Flow Cell	Dual Flow Cell Run Time
1×36	128-144 Gb	64-72 Gb	29 hrs
2×50	360-400 Gb	180-200 Gb	2.5 days
2×100	720-800 Gb	360-400 Gb	5 days
2×125	900-1 Tb	450-500 Gb	6 days
Reads Passing Filter	Up to 4 billion single read or 8 billion paired-end reads	Up to 2 billion single read or 4 billion paired-end reads	
Quality	Greater than 85% of bases above Q30 at 2×50 bp Greater than 80% of bases above Q30 at 2×100 bp Greater than 80% of bases above Q30 at 2×125 bp		

Table 1: HiSeq X Ten Performance Parameters*

	Dual Flow Cell	Single Flow Cell
Output/Run	1.6–1.8 Tb	800–900 Gb
Reads Passing Filter†	5.3–6 billion	2.6–3 billion
Supported Read Length	2 × 150	
Run Time	< 3 days	
Quality	≥ 75% of bases above Q30 at 2 × 150 bp	

Illumina MiSeq & MiniSeq Personal Sequencers

MISEQ REAGENT KIT V2

READ LENGTH	TOTAL TIME*	OUTPUT
1 × 36 bp	~4 hrs	540-610 Mb
2 × 25 bp	~5.5 hrs	750-850 Mb
2 × 150 bp	~24 hrs	4.5-5.1 Gb
2 × 250 bp	~39 hrs	7.5-8.5 Gb

MISEQ REAGENT KIT V3

READ LENGTH	TOTAL TIME*	OUTPUT
2 × 75 bp	~20 hrs	3.3-3.8 Gb
2 × 300 bp	~55 hrs	13.2-15 Gb

MiniSeq:

Read length	Output
2 x 150 bp	7.5 Gb (25 mln reads)

Third-Fourth Generation Sequencing Technologies

- **Pacific Biosciences (http://www.pacificbiosciences.com/): PacBioRS**
- **Complete Genomics (BGI-Shenzhen) (http://www.completegenomics.com/) (only for human genome)**
- **Ion Systems (Life/ABI/Thermo Scientific Inc.) (http://www.iontorrent.com http://www.lifetechnologies.com): Ion Torrent, Ion Proton, Ion S5, Ion Personal Genome Machine (PGM™) sequencers**

Fourth generation:

- **Oxford Nanopore (http://www.nanoporetech.com/): GridION, PromethION and miniaturised MinION**
- **Roche Nanopore Sequencing (http://sequencing.roche.com/)**

PacBio Single Molecule Real Time (SMRT) Sequencing

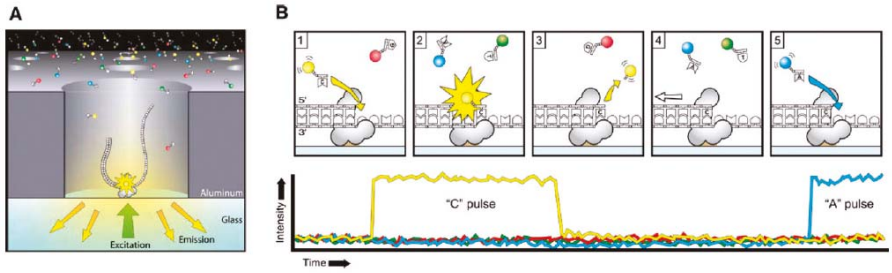


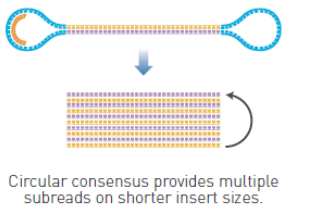
Figure 2. Schematic of PacBio's real-time single molecule sequencing. (A) The side view of a single ZMW nanostructure containing a single DNA polymerase (Φ29) bound to the bottom glass surface. The ZMW and the confocal imaging system allow fluorescence detection only at the bottom surface of each ZMW. (B) Representation of fluorescently labeled nucleotide substrate incorporation on to a sequencing template. The corresponding temporal fluorescence detection with respect to each of the five incorporation steps is shown below. Reprinted with permission from ref 39. Copyright 2009 American Association for the Advancement of Science.

ZMW = Zero Mode Waveguide

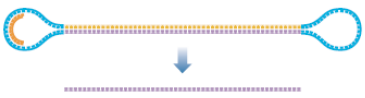
http://www.youtube.com/watch?v=v8p4ph2MAvI

PacBio Sample prep

Insert Size (bp)	Input DNA required per library prep (ng)
250	250
500	250
1,000	500
2,000	500
5,000	2,000
10,000	5,000

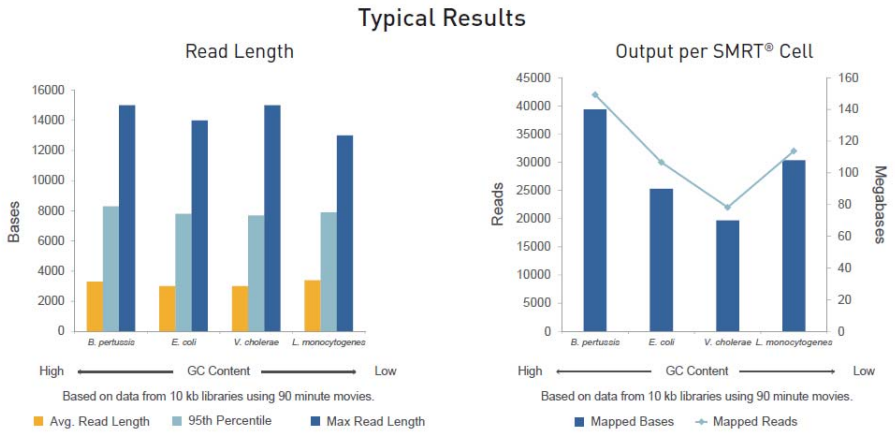


Circular consensus provides multiple subreads on shorter insert sizes.

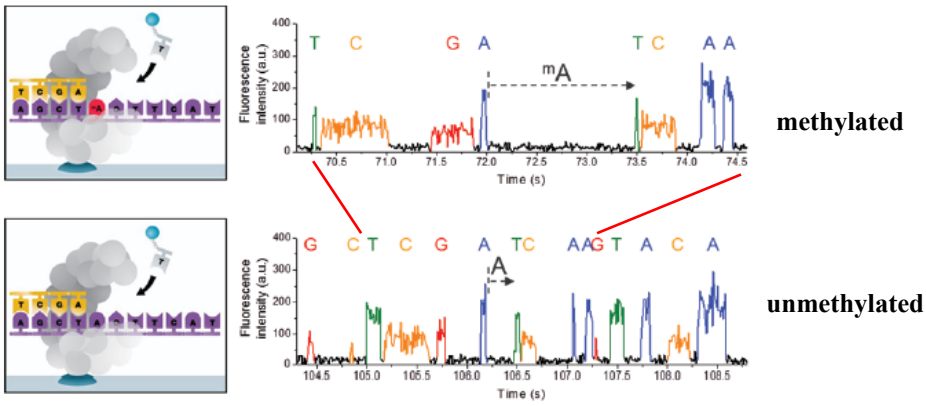


Standard sequencing provides a single pass read on longer insert sizes.

PacBio SMRT Sequencing



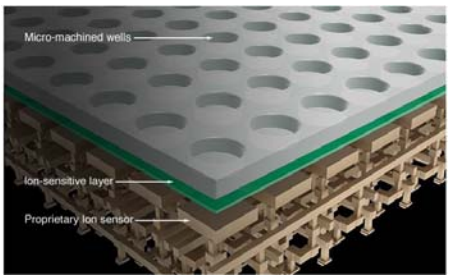
PacBio Base Modification Detection (Application in development)

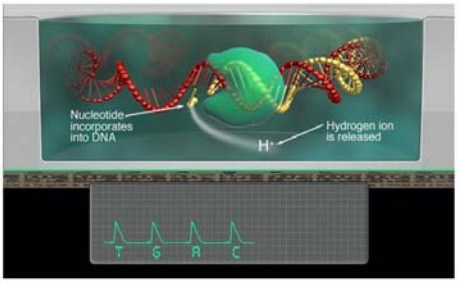


A methylated adenine in the template (top) slows the incorporation of a thymine in the replicating strand of DNA. The rate of incorporation can be compared to an unmodified version of the same template (bottom) which has a much faster thymine addition. Differences between the modified and unmodified incorporation rates indicate potential sites of modified bases. These differences often span multiple bases, creating a distinctive signature.

Flusberg et al. (2010) Nature Methods 7: 461-465

Ion Torrent & Proton: Personal Genome Machine (PGM)





When a nucleotide is added to a DNA template and is then incorporated into a strand of DNA, a hydrogen ion is released. The charge from that ion changes the pH of the solution, which can be detected by a ion sensor.

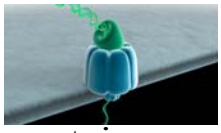
- **reads 200 or 400 bp long (S5, Torrent, Proton, PGM)**
- **from 0.6 to 15 Gb per run (different chips)**
- **max 3-80 M reads (2.5-16.5 hours)**
- **useful for Amplicon-seq, smallRNA-seq, small genomes sequencing (i.e. bacterial, virus)**

(https://ioncommunity.thermofisher.com/welcome)

http://www.youtube.com/watch?v=yVf2295JqUg

Oxford Nanopore: The GridION system

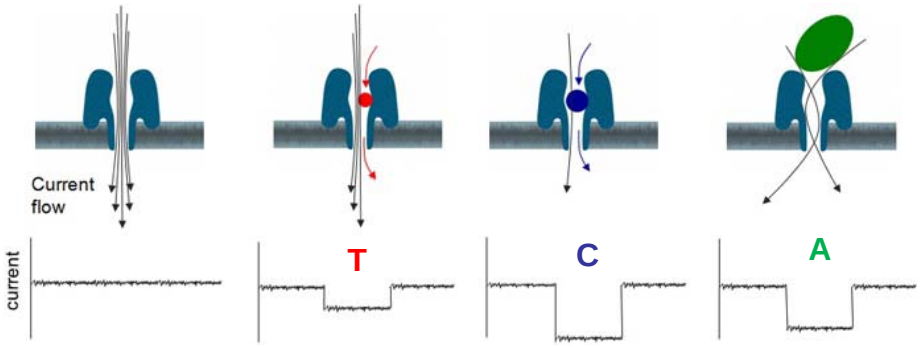
- Electrical single-molecule sequencing
- Protein nanopores used as biosensor



- Exonuclease sequencing: combining a protein nanopore and processive enzyme for the sequential identification of DNA bases as they pass through the pore

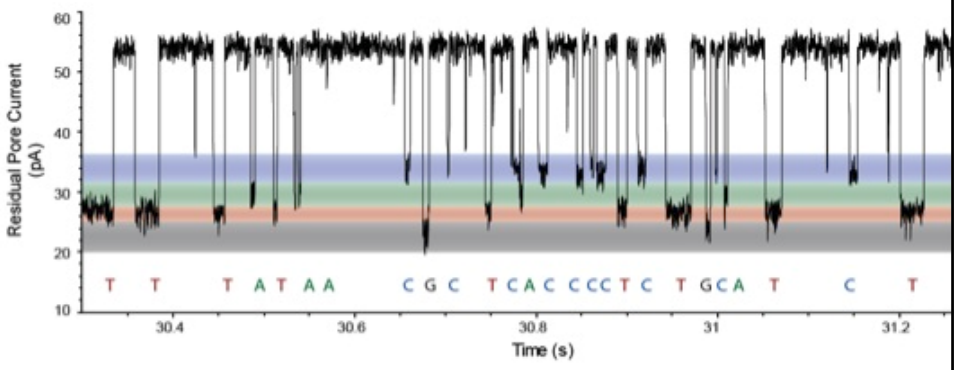
- Oxford Nanopore signed a commercialisation agreement with Illumina for this technology

The GridION system: Nanopore sensing



Exonuclease sequencing: combining a protein nanopore and processive enzyme (e.g., a exonuclease) for the sequential identification of DNA bases as they pass through the pore.

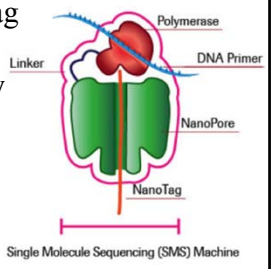
The GridION system: Electrical sequencing trace



- The system is designed to give ultra-high read length (tens of Kb)
- First generation commercial system is designed to achieve tens of Gb per day

Roche Nanopore Sequencing: Genia Technologies

- electrical single-molecule sequencing
- biological nanopores used as biosensor (a protein pore embedded in a lipid bilayer membrane)
- based on a proprietary integrated circuit and NanoTag chemistry from Genia Technologies developed in collaboration with Columbia and Harvard University
- uses a DNA replication enzyme to sequence a template strand with single base precision as base-specific engineered NanoTags are captured by the nanopore
- as the phosphate tagged nucleotides (NanoTags) enter the pore, they attenuate the current flow through the pore in an identity-dependent manner



http://sequencing.roche.com/research---development/nanopore-sequencing.html

Third-Forth Generation Sequencing Technologies

Company	Platform name	Method of sequencing	Method of detection	Read length	Advantages	Relative limitation
Pacific Biosciences	PacBio RS	Real-time, singlemolecule DNA sequencing	Fluorescence/Optical	variable: 200 bp to few thousand Kbp	Long average read lengths; decreased sequencing time compared to secgen platforms; no amplification of sequencing fragments; longest individual reads approach 3,000 bases	Inefficient loading of DNA polymerase in ZMWs; low single-pass accuracy (81-83%); degradation of the polymerase in ZMWs; overall, high cost per base (expensive instrument)
Complete Genomics / BGI	In-house lab-built instrumentation	Combinatorial probe anchor hybridization and ligation (cPAL)	Fluorescence/Optical	35 bp paired-end reads	Highest (claimed) throughput of thirdgen platforms; lowest reagent cost for reassembling a human genome of all sequencing technologies; each sequencing step is independent, minimizing accumulation of errors	Short read lengths; template preparation prevents sequencing through long repetitive regions; labor intensive sample preparation; no commercially available instrument
Ion Torrent/Life Technologies	Personal Genome Machine (PGM) sequencer	Sequencing by synthesis	Change in pH detected by Ion-Sensitive Field Effect Transistors (ISFETs)	175, 200 or 400 bp	Direct measurement of nucleobase incorporation events; DNA synthesis reaction operates under natural conditions (no need for modified DNA bases)	Sequential washing steps can lead to accumulation of errors; potential difficulties in reading through highly repetitive or homopolymer regions of the genome
Oxford Nanopore	gridION	Nanopore exonuclease sequencing	Current	9-10 Kb	Potential for long read lengths; low cost of RHLnanopore production; no fluorescent labeling or optics necessary	Cleaved nucleotides may be read in the wrong order; difficult to fabricate a device with multiple parallel pores

2016 NGS Field Guide: Overview

http://www.molecularecologist.com/next-gen-fieldguide-2016/

NGS Platform statistics



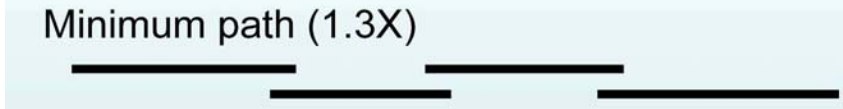

Instrument	Amplification	Run time	M of Read / run	Bases / read	Reagent Cost / run, $	Reagent Cost / Gb, $	Reagent Cost /M reads, $	Gbp / run	cost / Gb, $
Illumina HiSeq X (2 flow cells)	BridgePCR	3 days	6000	300	12750	7	2	1800	7
Illumina HiSeq 2500 - high output v4	BridgePCR	6 days	2000	250	14950	30	7	500	30
Life Technologies SOLiD – 5500xl	emPCR	8 days	1410	110	10503	68	7	155.1	68
Illumina NextSeq 500	BridgePCR	30 hrs.	400	300	4000	33	10	120	33
Oxford Nanopore GridION 8000	None - SMS	varies	10	10000	1000	10	100	100	10
Ion Torrent - Proton III	emPCR	6 hrs.	500	175	1000	11	2	87.5	11
Illumina MiSeq v3	bridgePCR	55 hrs.	22	600	1442	109	66	13.2	109
Ion Torrent – PGM 318 chip	emPCR	7.3 hrs.	4.75	400	874	460	184	1.9	460
Ion Torrent – PGM 316 chip	emPCR	4.9 hrs.	2.5	400	674	674	270	1	674
Oxford Nanopore MinION	None - SMS	≤6 hrs.	0.1	9000	900	1000	9000	0.9	1000
454 FLX+	emPCR	20 hrs.	1	650	6200	9538	6200	0.65	9538
Ion Torrent – PGM 314 chip	emPCR	3.7 hrs.	0.475	400	474	2495	998	0.19	2495
Pacific Biosciences RS II	None - SMS	2 hrs.	0.03	3000	100	1111	3333	0.09	1111

'Benchtop' NGS technology: will substitute the capillary electrophoresis (CE) sequencers for common experiments, such as Illumina libraries verification, amplicon sequencing and small genome sequencing.

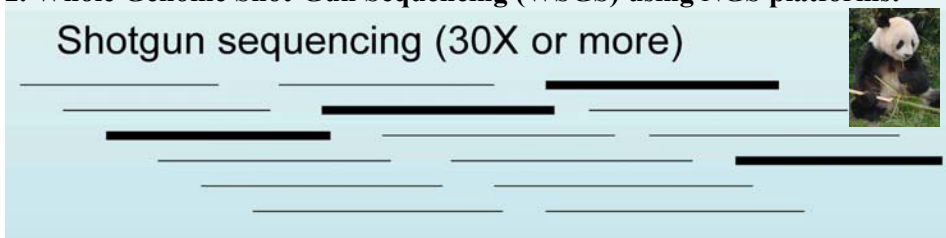
Major approaches for *de novo* complete genome sequencing

1. Traditional based on BAC library – very costly for large genomes:

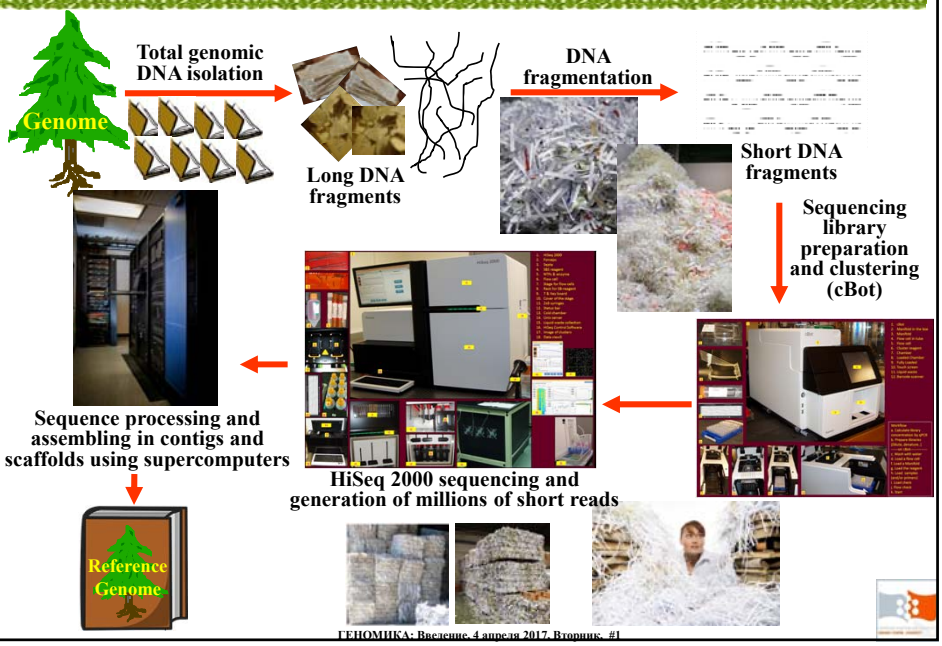
- 1) developing BAC library (arranged and microplatted) (costly for large genomes, for instance, loblolly pine (*Pinus taeda*) library with ~1.8M BACs (~8X genome coverage) costs ~$1.8M (http://www.pine.msstate.edu/bac.htm);
- 2) fingerprinting each clone - ~$ 5 mln;
- 3) selection of unique overlapping BACs, building a "**minimum tiling path**" and sequencing



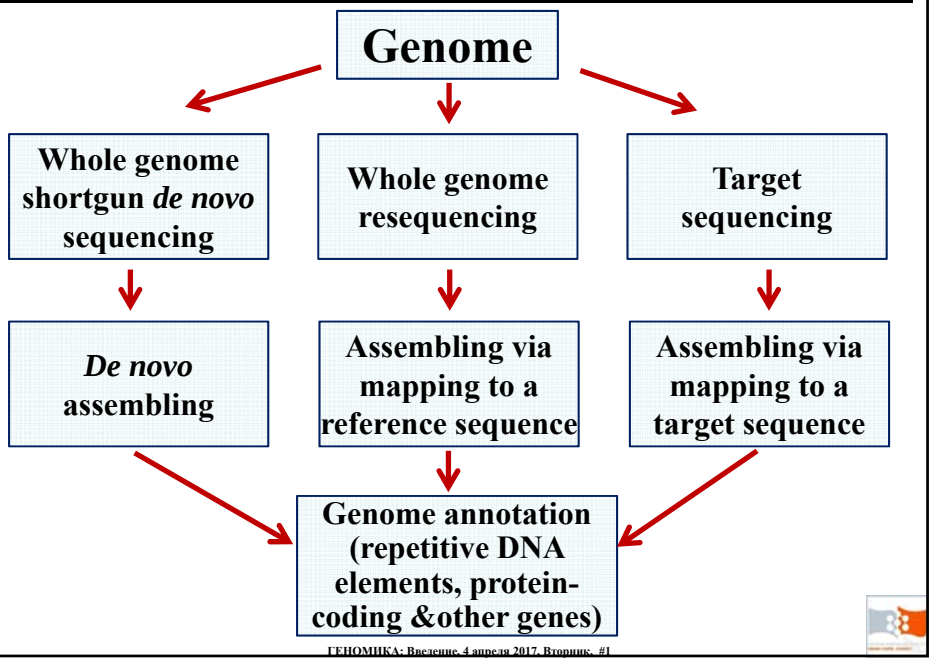
2. Whole Genome Shot-Gun Sequencing (WSGS) using NGS platforms:



Whole genome *de novo* sequencing and assembling using NGS short reads and supercomputers



Overview of genome sequencing analysis



Applications of Genome Sequencing

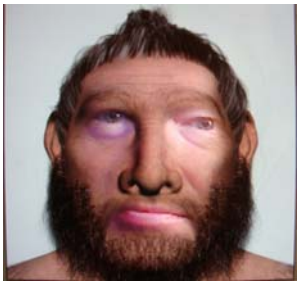
Purpose	Template	Example
De novo sequencing	genome sequencing	**The Genome 10K project** (sequencing 10,000 vertebrate species genomes, approximately one for every vertebrate genus); **1K Plant Genomes Project, 10K fungi genomes**
	ancient DNA	Extinct Neanderthal genome
	metagenomics	Human guts
Resequencing	whole genomes	Sequencing **1000 individual human genomes** project
	genomic regions	Assessment of genomic rearrangements or disease-associated regions
	somatic mutations	Sequencing mutations in cancer
Transcriptome	full-length transcripts	Defining regulated messenger RNA transcripts
	Noncoding RNAs	Identifying and quantifying microRNAs in samples
Epigenetics	Methylation changes	Measuring methylation changes in cancer

Table 13.15 in Bioinformatics and Functional Genomics by J. Pevsner (2nd ed., Wiley-Blackwell, 2009) p. 538

Ancient genome DNA sequencing projects

Special challenges:

- fragmented & degraded by nucleases
- deamination of cytosine to uracil
- the majority of DNA from unrelated organisms such as bacteria that invaded after death
- The majority of DNA in samples is contaminated by human DNA
- Determination of authenticity requires special controls, and analysis of multiple independent extracts



Green, R. E. *et al.* (2010) A draft sequence of the Neandertal genome. Science 328, 710–722

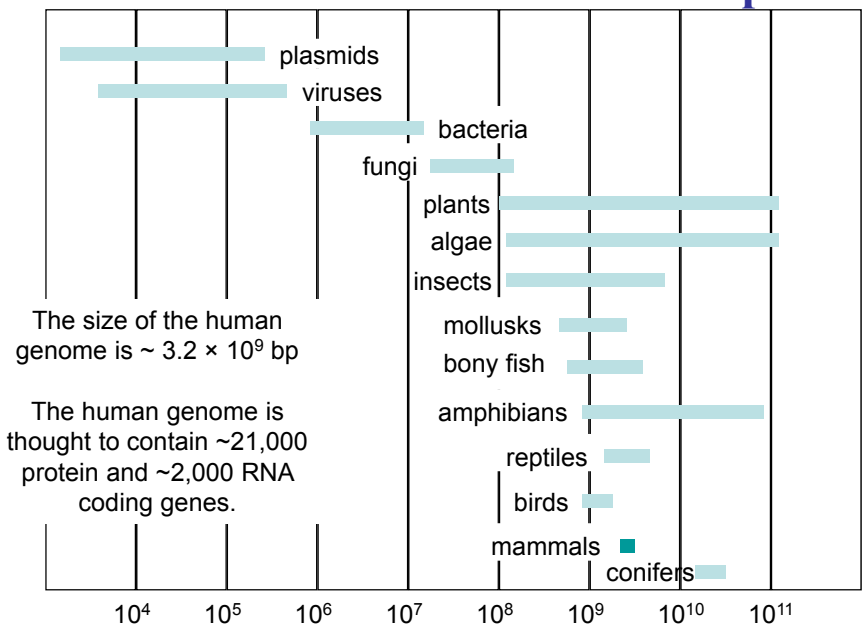
Microbial Community Sequencing (Metagenomics) Projects

Two broad areas:

- Environmental (ecological)
  e.g. hot spring, ocean, sludge, soil

- Organismal
  e.g. human gut, feces, lung

Genome sizes in nucleotide base pairs



http://www3.kumc.edu/jcalvet/PowerPoint/bioc801b.ppt

Eukaryotic completed genome projects > 2 Gb

Genus, species	Subgroup	Size (Mb)	#chr	Common name
Pinus taeda	Land Plants	20150	12	Loblolly pine
Picea abies	Land Plants	20000	12	Norway spruce
Triticum urartu	Land Plants	4940	7	wheat A-genome progenitor
Aegilops tauschii	Land Plants	4360	7	Tausch's goatgrass
Macropus eugenii	Mammals	3800	8	tammar wallaby
Oryctolagus cuniculus	Mammals	3500	22	rabbit
Capsicum annuum	Land Plants	3480	12	pepper
Cavia porcellus	Mammals	3400	31	guinea pig
Homo sapiens	Mammals	3200	23	human
Pan troglodytes	Mammals	3100	24	chimpanzee
Bos taurus	Mammals	3000	30	cow
Dasypus novemcinctus	Mammals	3000	32	nine-banded armadillo
Loxodonta africana	Mammals	3000	28	African savanna elephant
Sorex araneus	Mammals	3000	20	European shrew
Rattus norvegicus	Mammals	2750	21	rat
Nicotiana sylvestris	Land Plants	2636	12	tobacco
Canis familiaris	Mammals	2400	39	dog
Zea mays	Land Plants	2365	10	corn

GC content varies across genomes

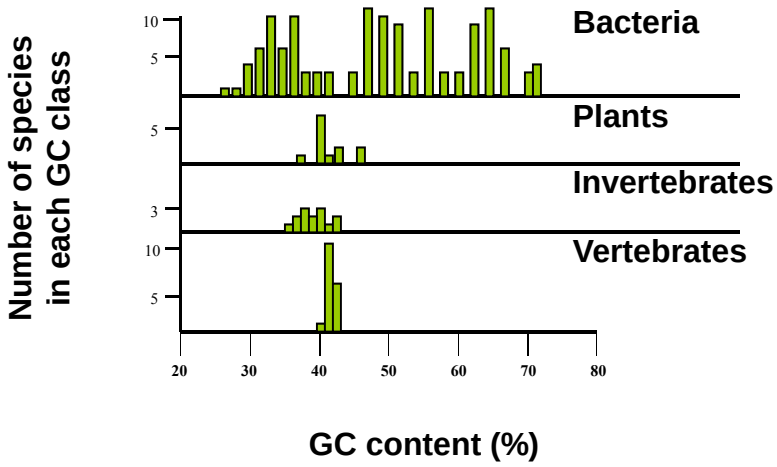
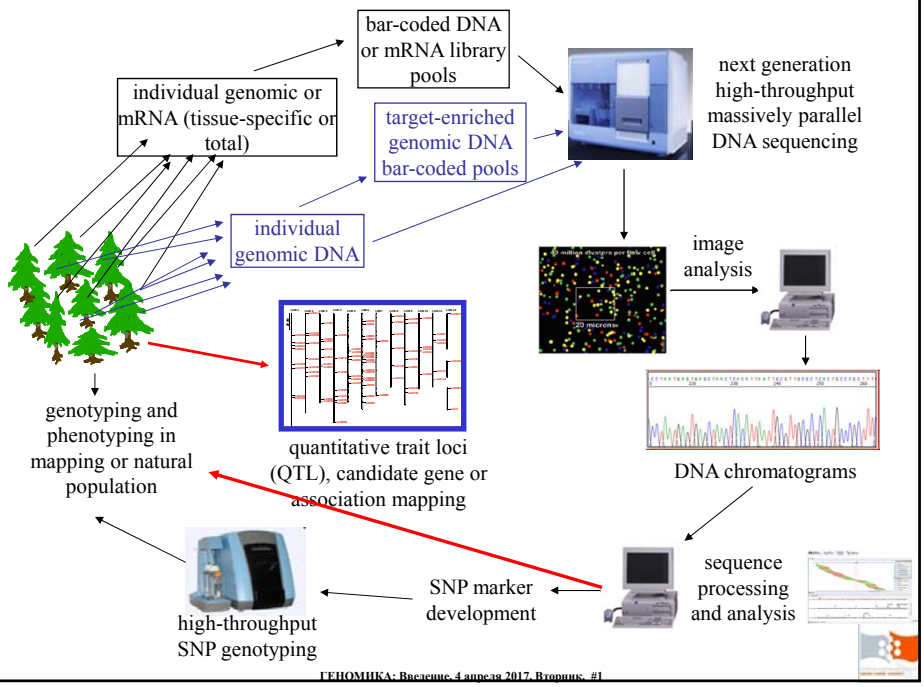


Fig. 13.15 in Bioinformatics and Functional Genomics by J. Pevsner (2nd ed., Wiley-Blackwell, 2009) p. 556

Genomic markers development and genotyping using next generation sequencing



Genomic DNA target enrichment for high-throughput massively parallel sequencing using the Agilent's SureSelect Target Enrichment System

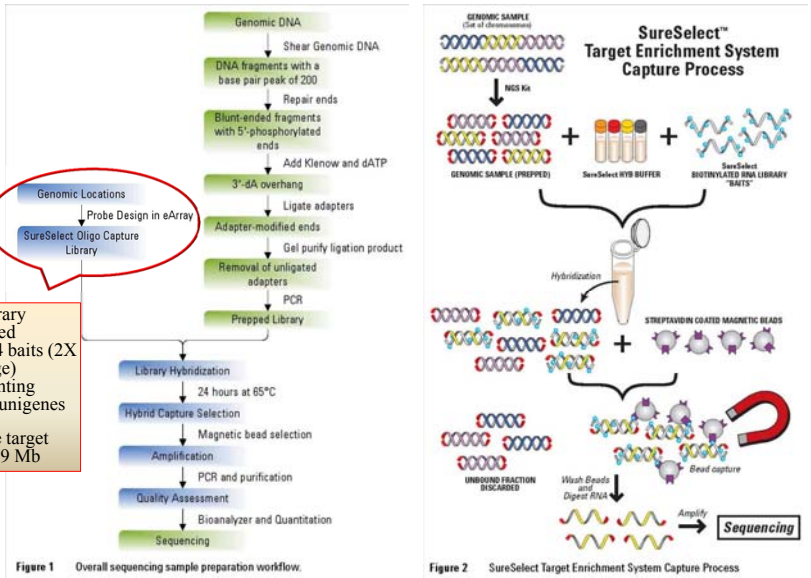


Figure 1 Overall sequencing sample preparation workflow.

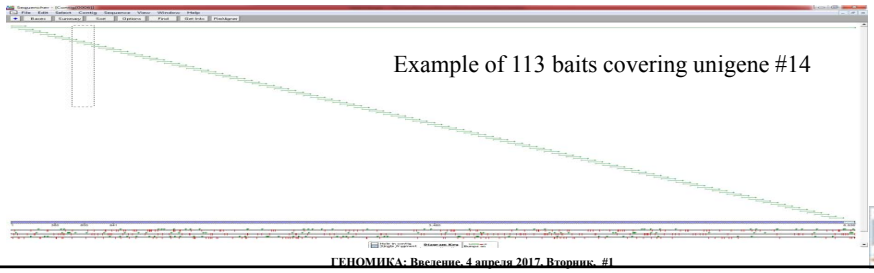
Figure 2 SureSelect Target Enrichment System Capture Process

- Our library contained 647,634 baits (2X coverage) representing 35,550 unigenes
- Capture target size ≈ 39 Mb

http://www.opengenomics.com/SureSelect_Target_Enrichment_System

Genomic DNA enrichment for 35,550 genes in loblolly pine for high-throughput massively parallel sequencing using bar-coding and the Agilent's SureSelect Target Enrichment System

647,634 oligonucleotide hybridization 120 bp long probes (baits) based on 35,550 unigenes build by Dr. Chun Liang (Miami University) were designed to target 39 Mb of gene space using Agilent Genomic Workbench software to gene enrich DNA libraries for sequencing



Example of 113 baits covering unigene #14