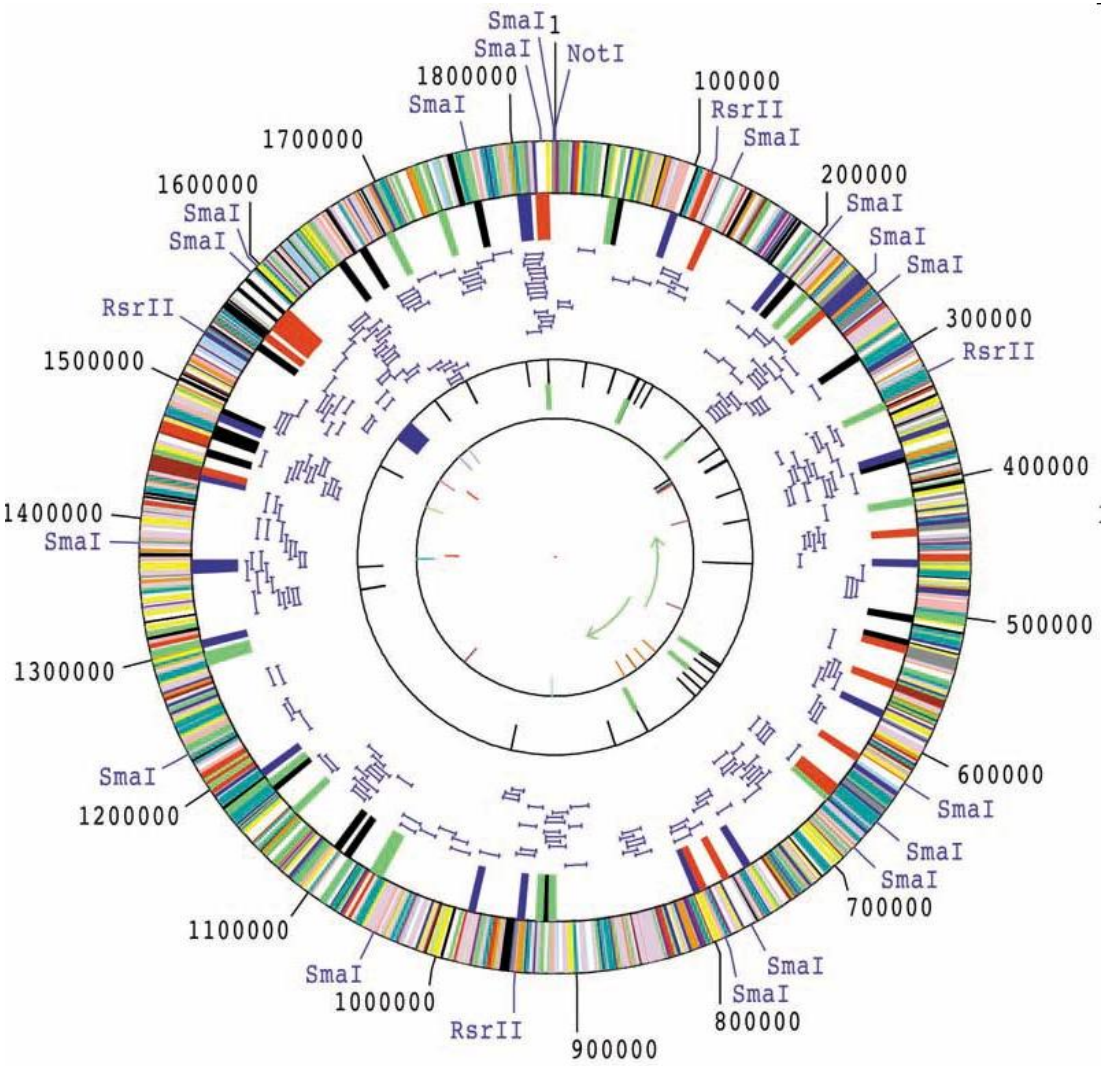
Next generation sequencing (ab 2005)

Vollautomatische Systeme, die Gesamt-DNA-Gemische zur Sequenzierung nutzen können.

Sequenzierungsstrategien

Shot-gun Sequenzierung von Zufallsfragmenten (Venter)
Zerlegen großer DNA-Fragmente in viele kleine Fragmente (partieller Verdau, mechanisch), Klonierung in pUC-Vektoren, Sequenzierung, Computer (coverage – 6-10mal, entstehen viele „Contigs“, trotzdem Lücken!!)



1995
Craig Venter, Hamilton Smith, Claire Fraser, and colleagues at TIGR elucidate the first complete genome sequence of a microorganism - *Haemophilus influenzae Rd*. 1.830.137 bp

Since that time, the genome sequencing was mainly done using this strategy. (**T**he **I**nstitute for **G**enome **R**esearch - TIGR)

2010
Die Sanger-Sequenzierung kann pro „Automat“ im Jahr maximal 350 Mio. bp Sequenz generieren, allerdings mit sehr guter Qualität. Die neuen Verfahren bringen das am Tag.

Different platforms

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- 454 Sequencing / Roche
  - GS Junior System
  - GS FLX+ System
- Illumina (Solexa)
  - HiSeq System
  - Genome analyzer IIx
  - MySeq
- Applied Biosystems - Life Technologies
  - SOLiD 5500 System
  - SOLiD 5500xl System
- Ion Torrent - Life Technologies
  - Personal Genome Machine (PGM)
  - Proton
- Helicos
  - Helicos Genetic Analysis System
- Pacific Biosciences
  - PacBio RS
- Oxford Nanopore Technologies
  - GridION System
  - MinION

Next Generation Sequencing
Amplified Single Molecule Sequencing

Third Generation Sequencing,
Next Next Generation Sequencing,
Single Molecule Sequencing

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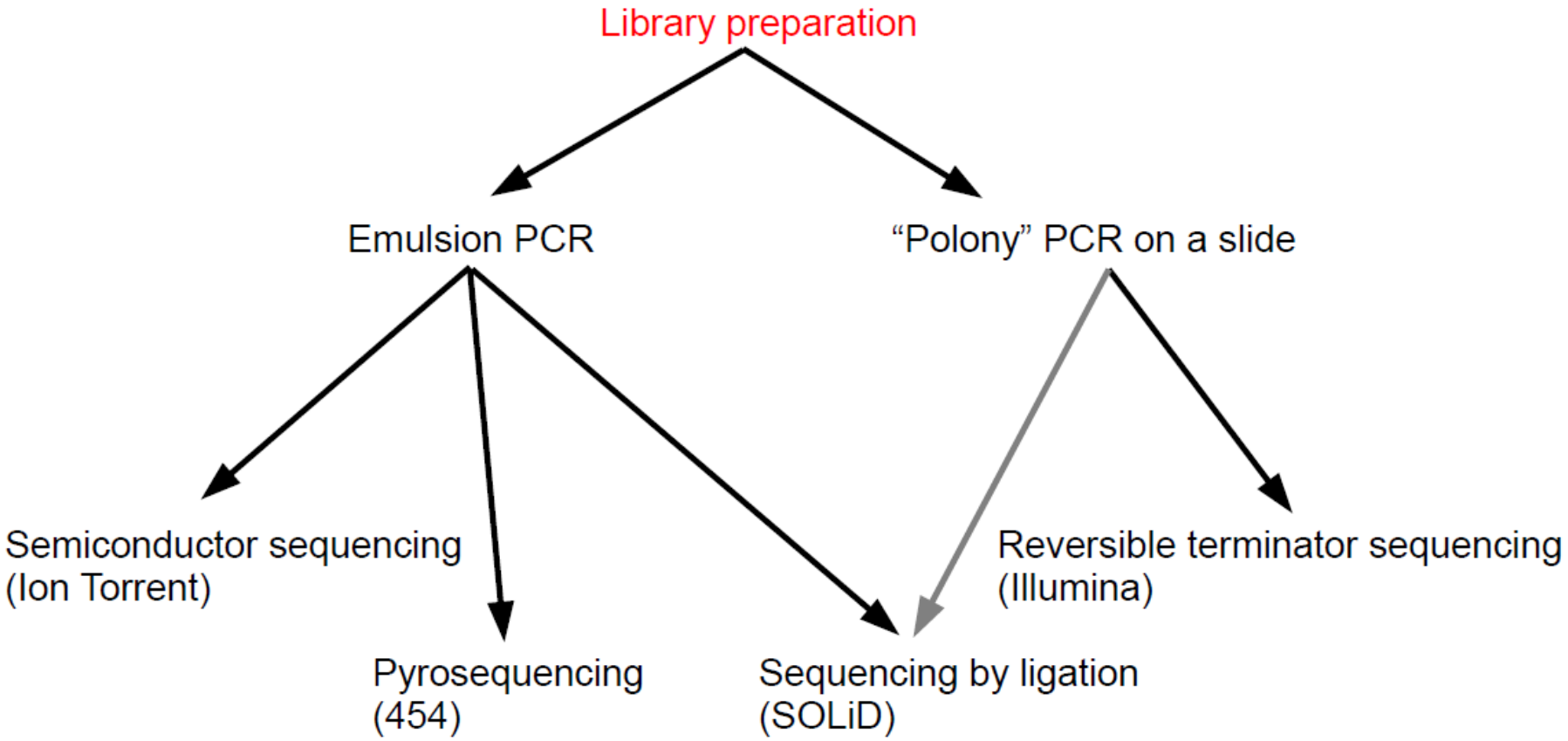
Next Generation Sequencing : Amplified Single Molecule Sequencing



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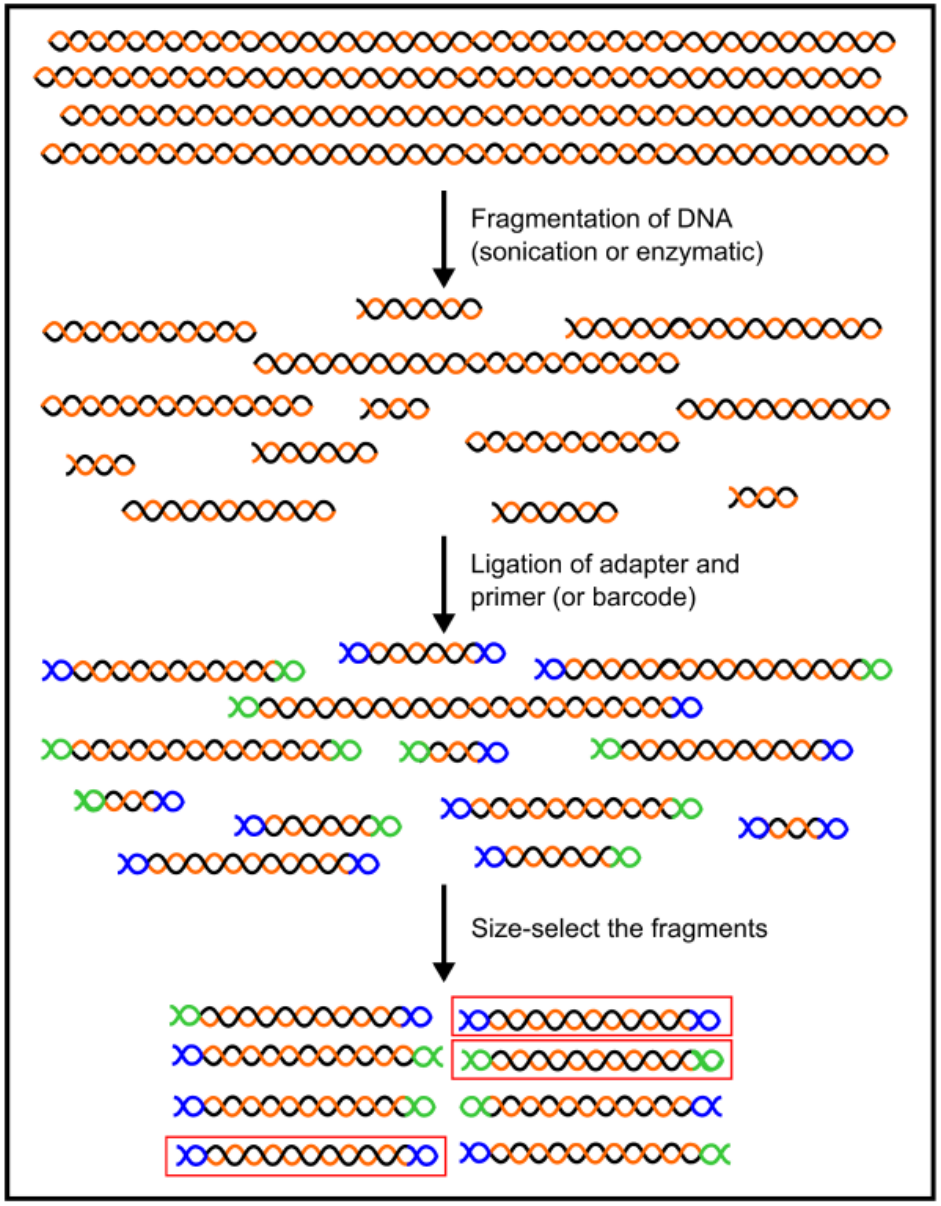
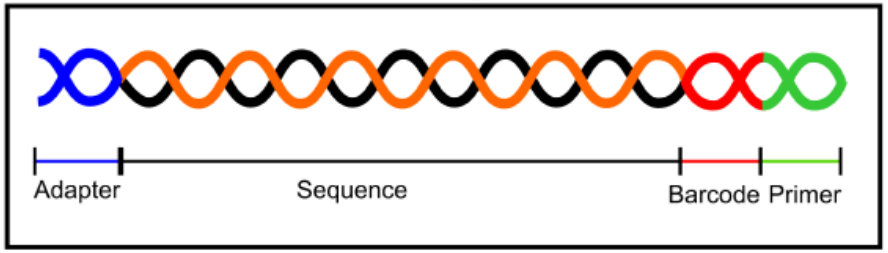
Next Generation Sequencing : Amplified Single Molecule Sequencing



Library preparation

Good fragments :



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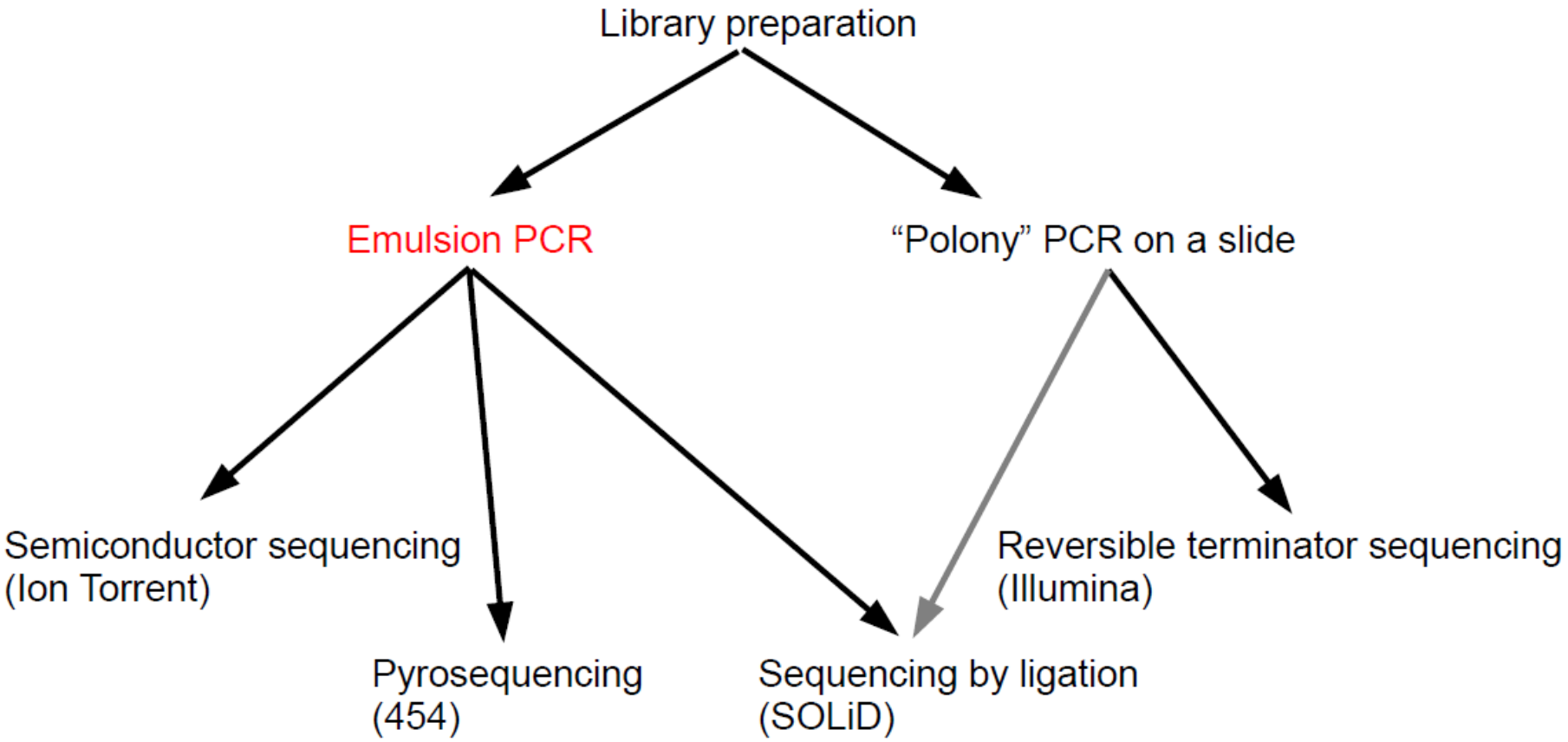
Next Generation Sequencing : Amplified Single Molecule Sequencing



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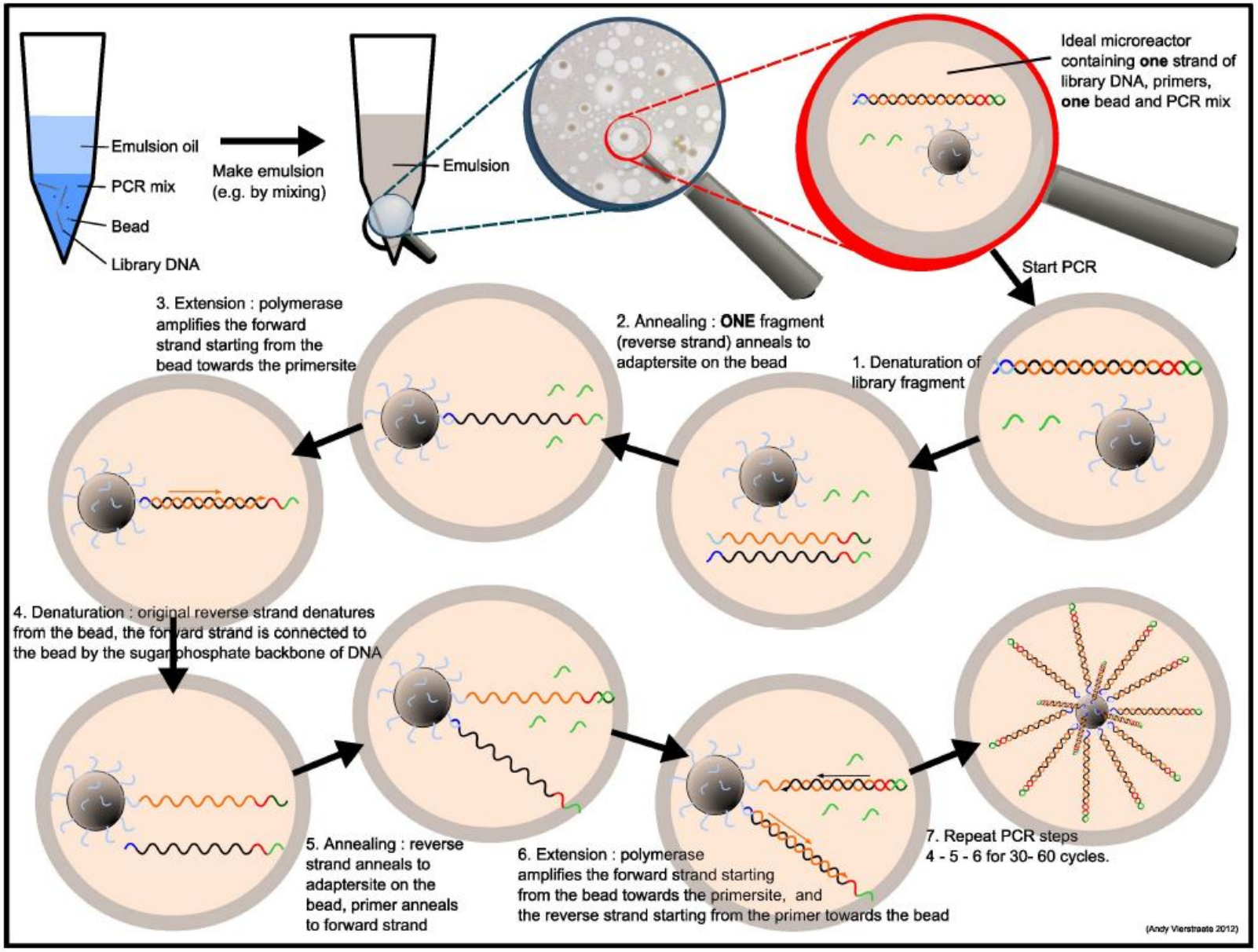
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR



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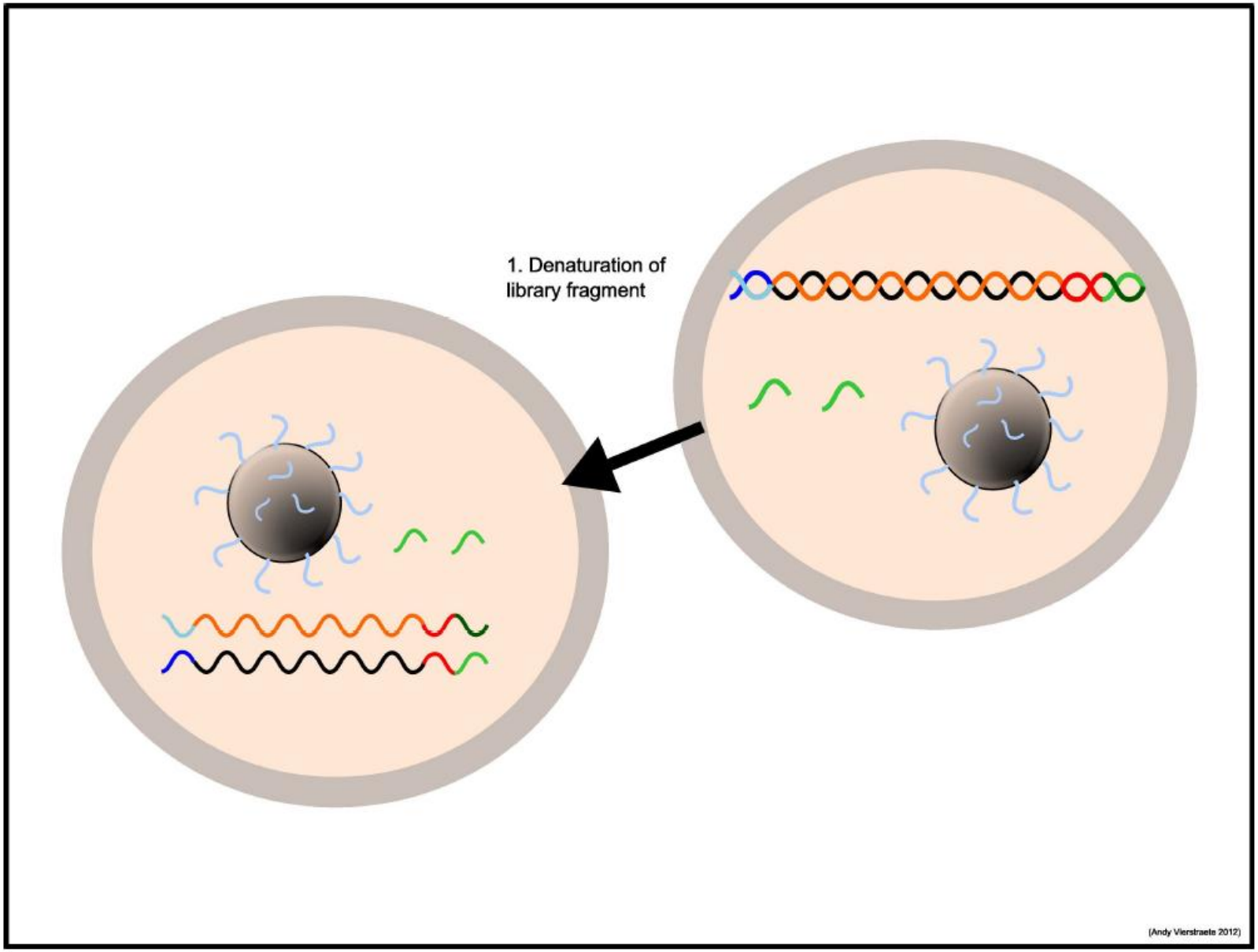
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR



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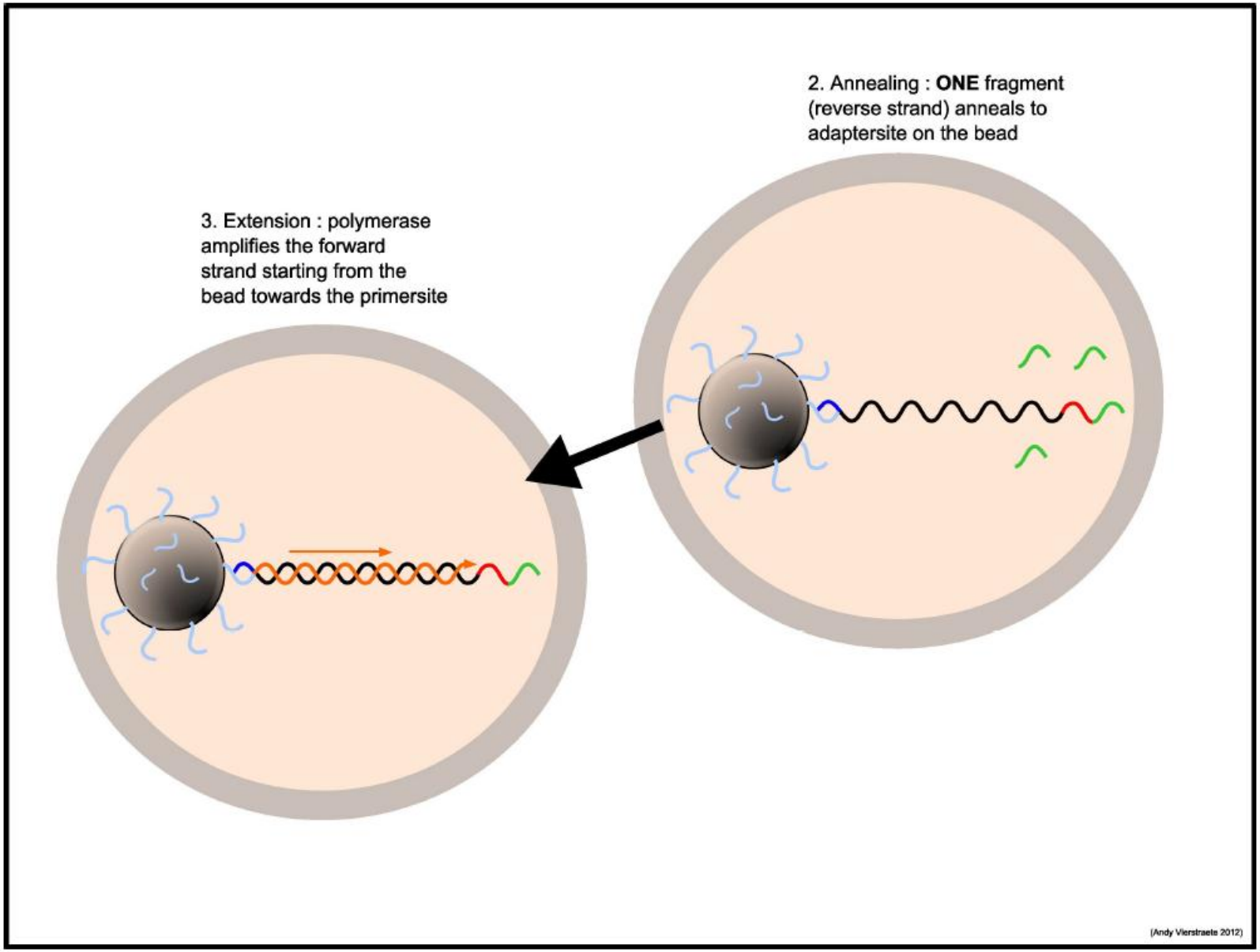
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR



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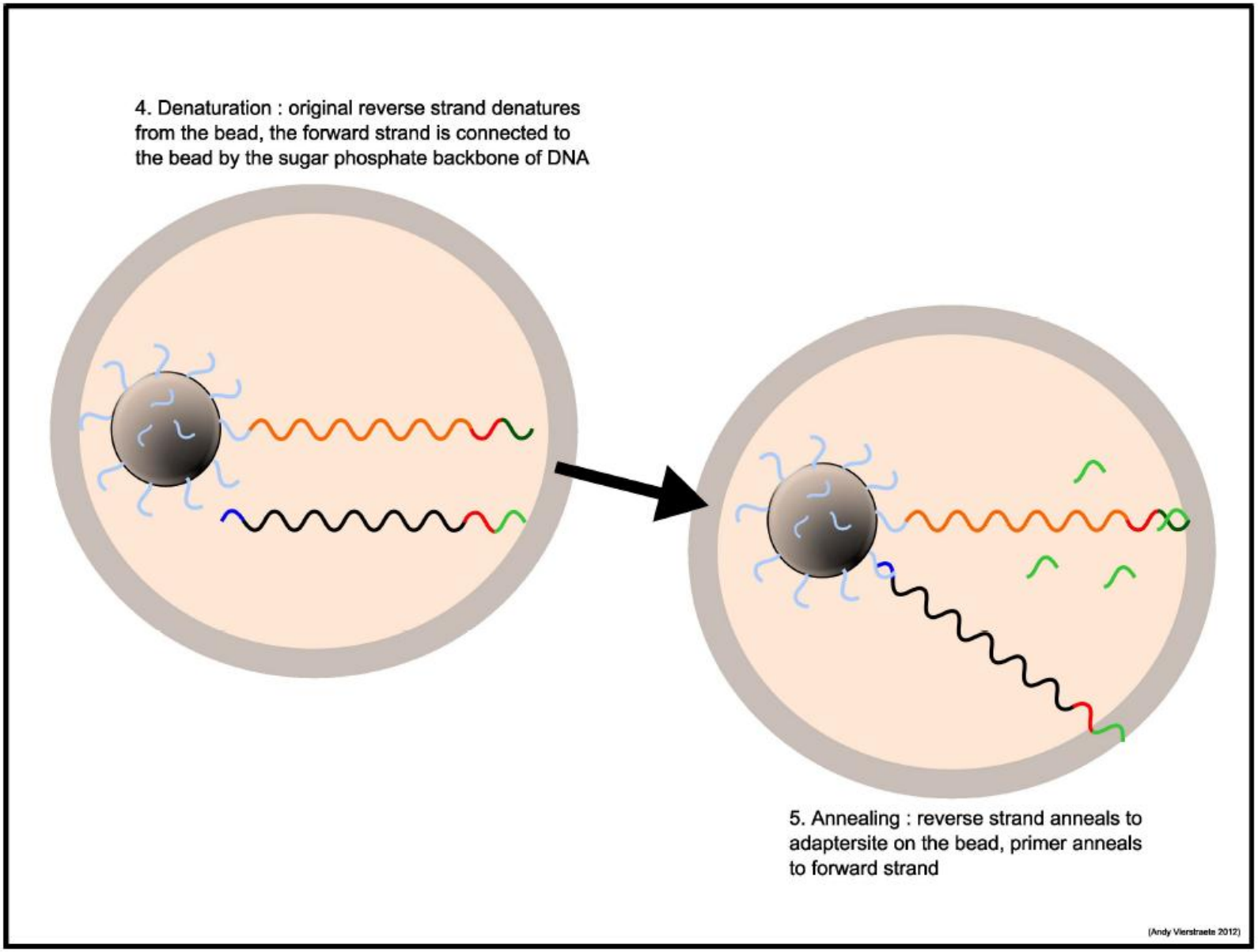
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR



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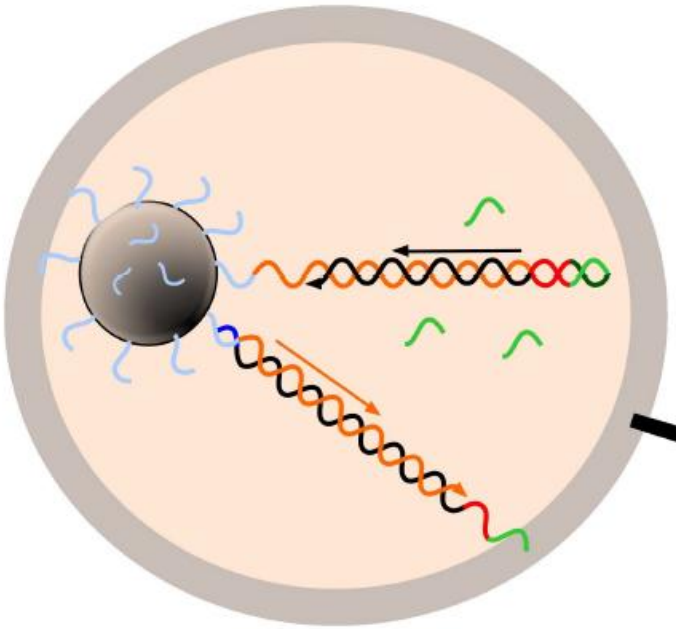
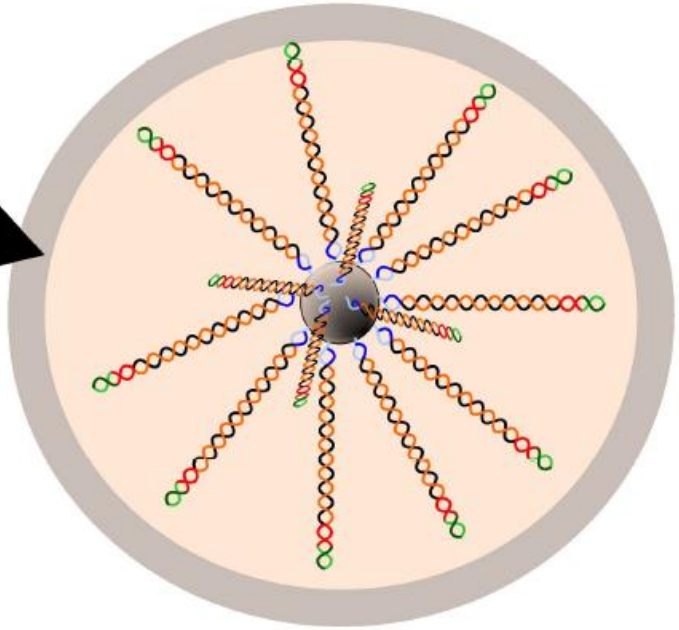
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR



6. Extension : polymerase amplifies the forward strand starting from the bead towards the primersite, and the reverse strand starting from the primer towards the bead



7. Repeat PCR steps
4 - 5 - 6 for 30- 60 cycles.

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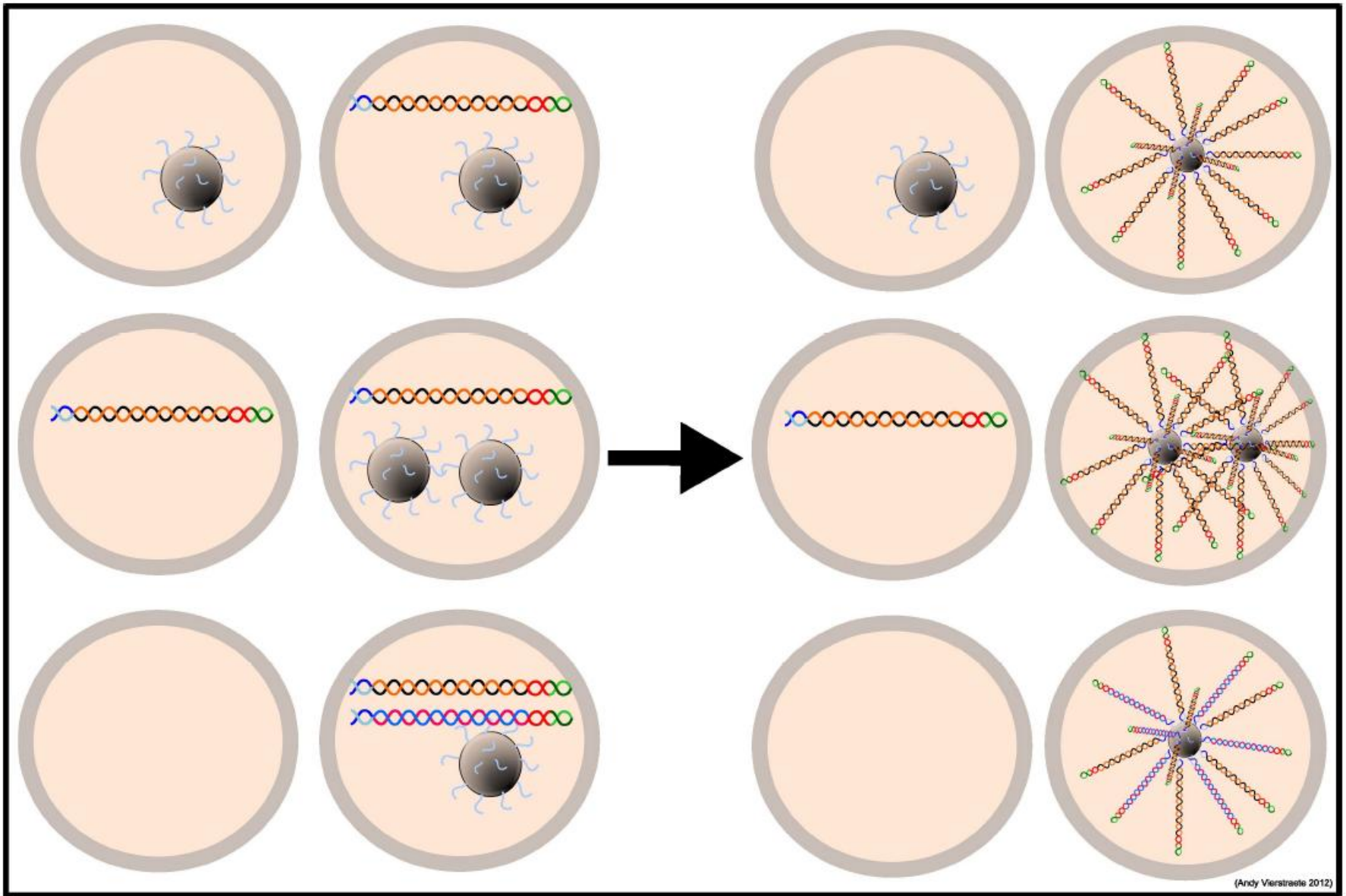
Next Generation Sequencing : Amplified Single Molecule Sequencing Emulsion PCR

different micro reactors : only 15 % are good ones



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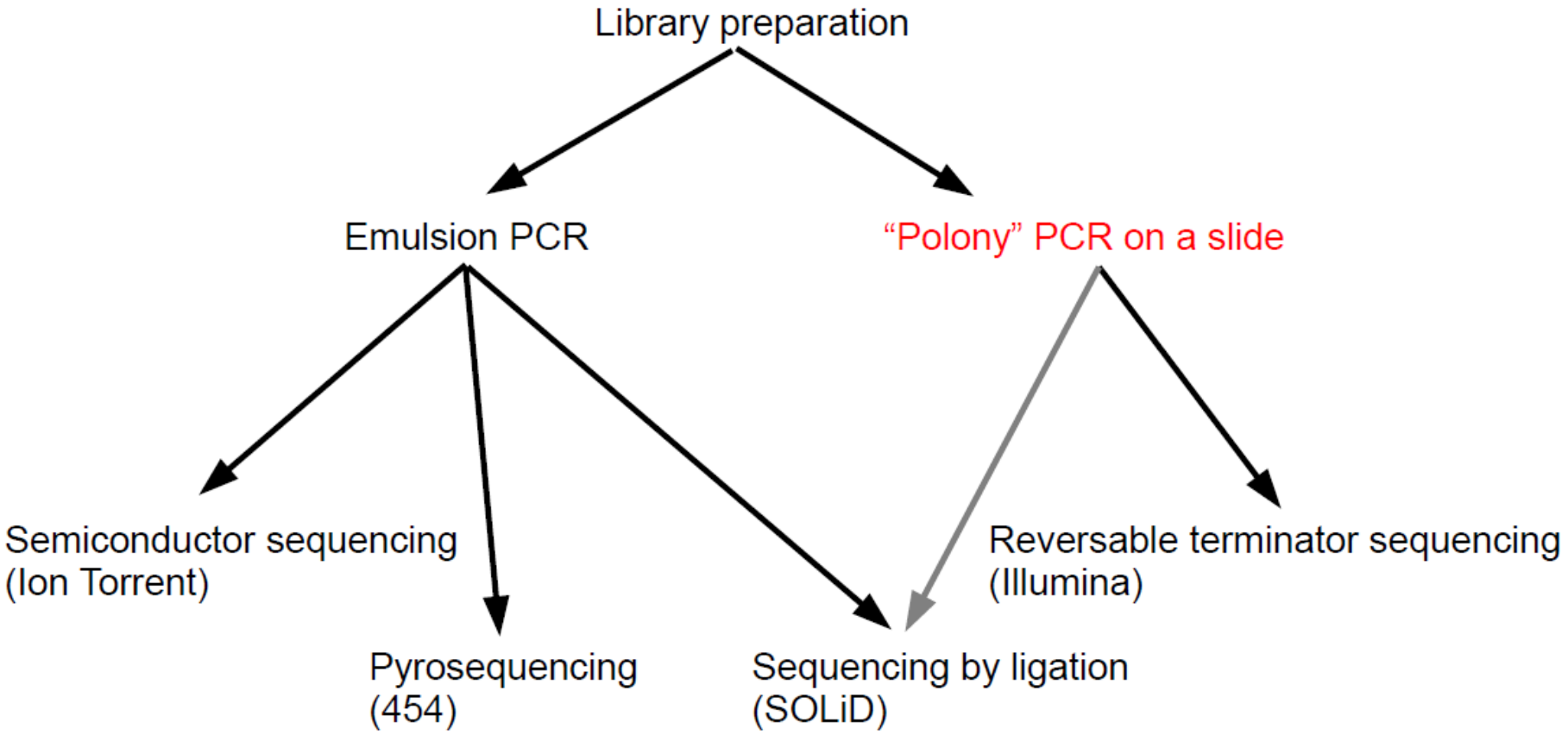
Next Generation Sequencing : Amplified Single Molecule Sequencing



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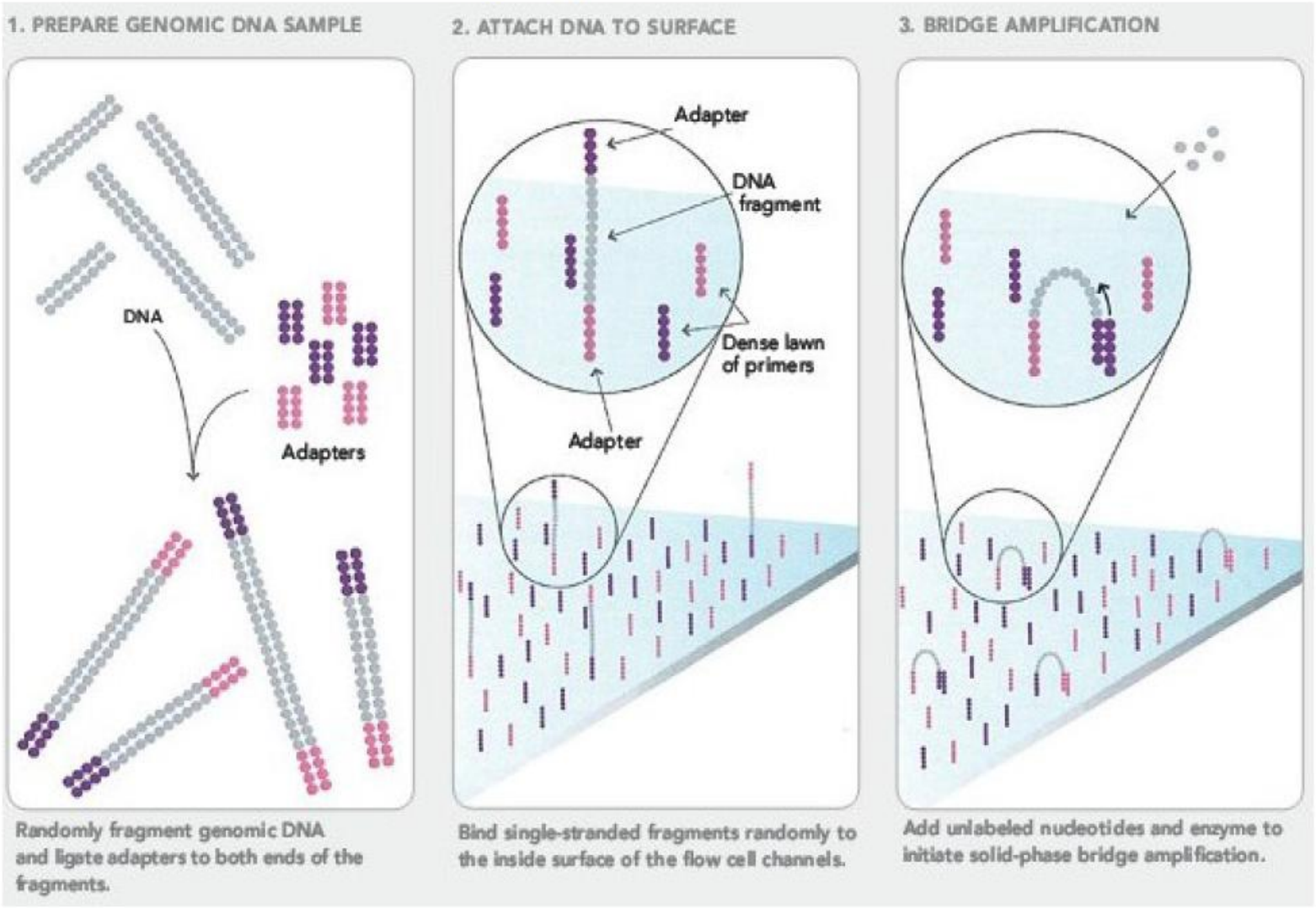
Next Generation Sequencing : Amplified Single Molecule Sequencing "Polony" PCR

Bridge amplification : Illumina



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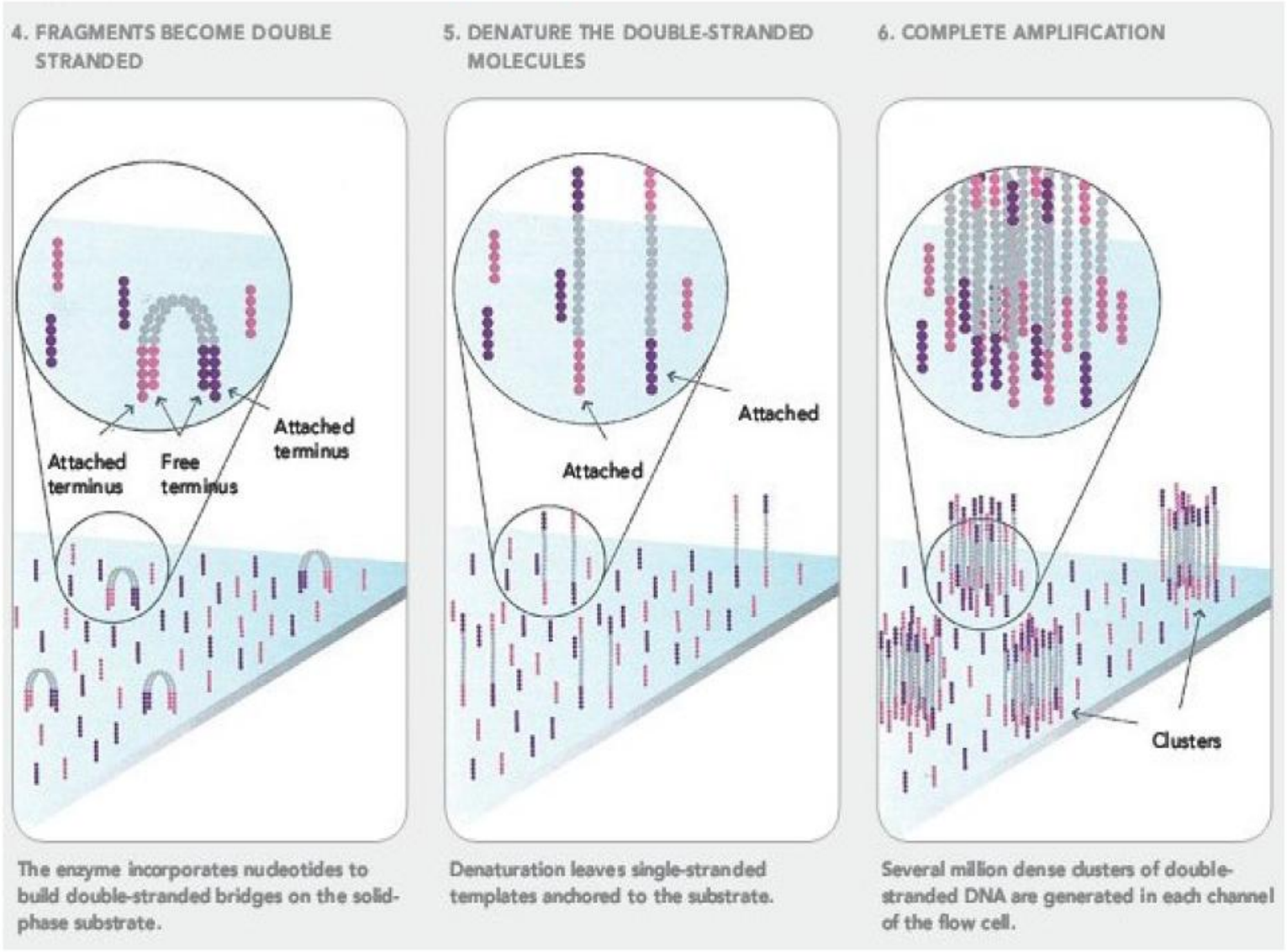
Next Generation Sequencing : Amplified Single Molecule Sequencing "Polony" PCR

Bridge amplification : Illumina



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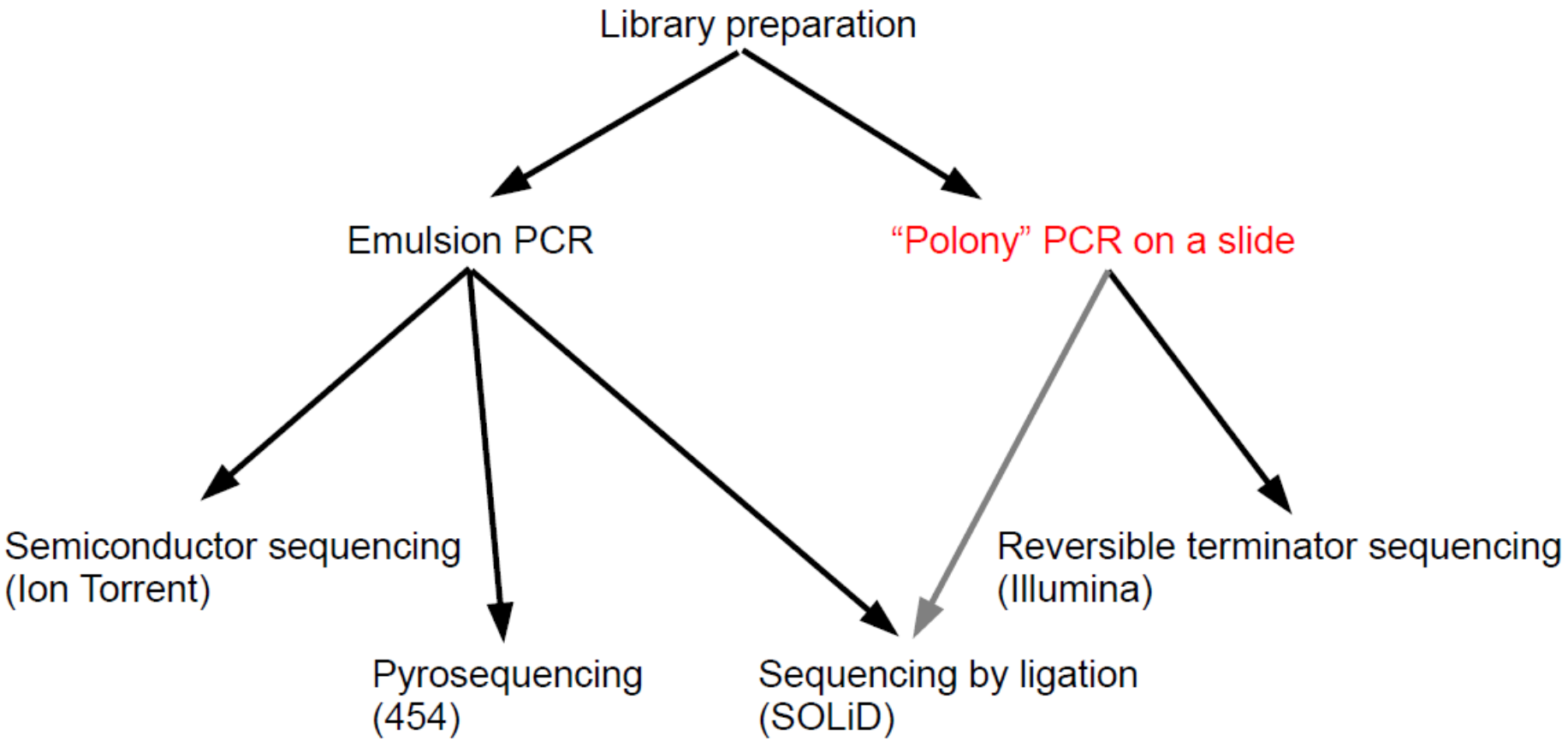
Next Generation Sequencing : Amplified Single Molecule Sequencing



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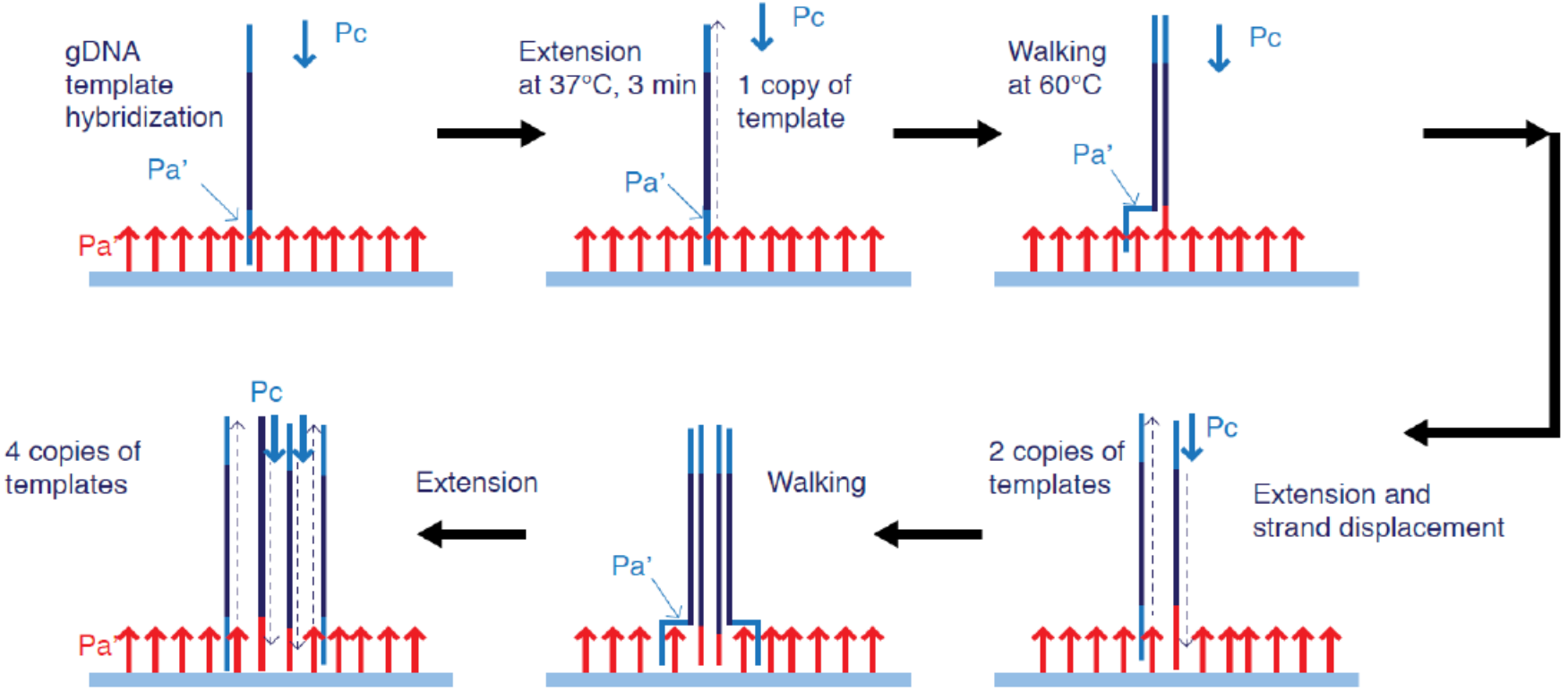
Next Generation Sequencing : Amplified Single Molecule Sequencing "Polony" PCR

Wildfire amplification : SOLiD



Wildfire chemistry schematic.

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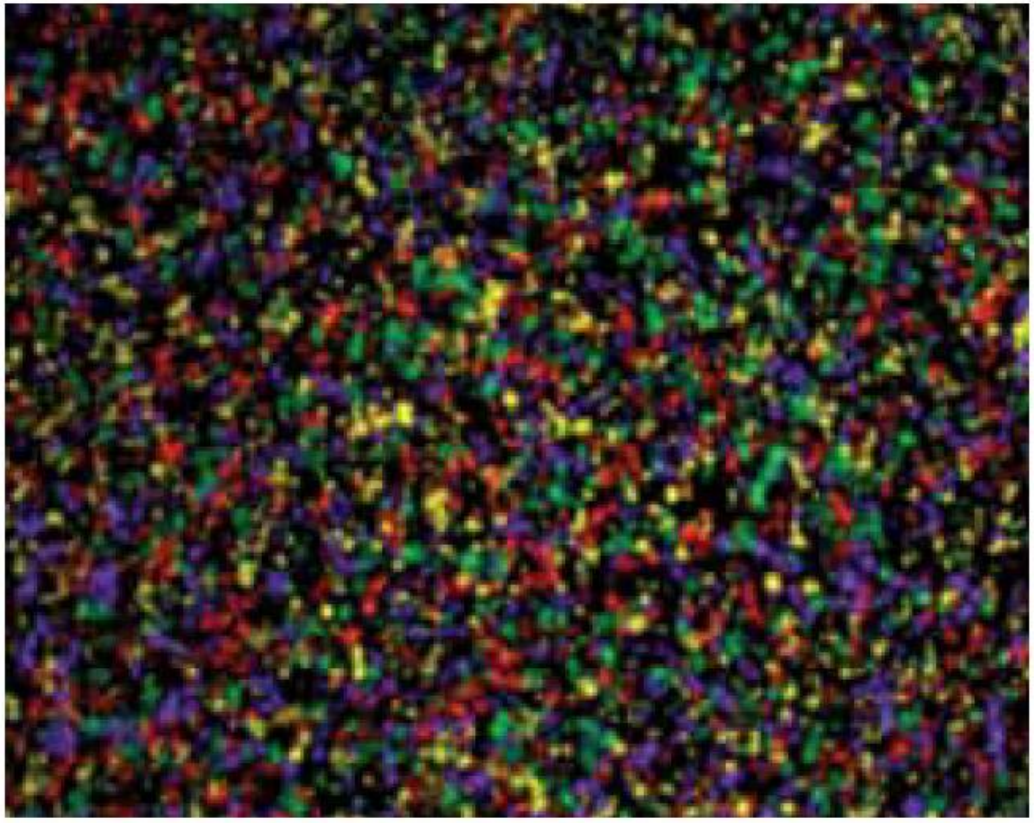
Next Generation Sequencing : Amplified Single Molecule Sequencing "Polony" PCR

Wildfire amplification : SOLiD



One million colonies/mm² per FlowChip surface.

Quality scores in sequencing :
Q17, Q20, Q30, ...

Quality score	Probability of incorrect bases	Base call accuracy
10	1 in 10	90 %
17	1 in 50	98 %
20	1 in 100	99 %
30	1 in 1000	99,9 %
40	1 in 10.000	99,99 %
50	1 in 100.000	99,999 %
60	1 in 1.000.000	99,9999%

1 Gb genome : 1 time coverage :
Q20 : 10.000.000 errors
Q30 : 1.000.000 errors
More coverage reduce the errors

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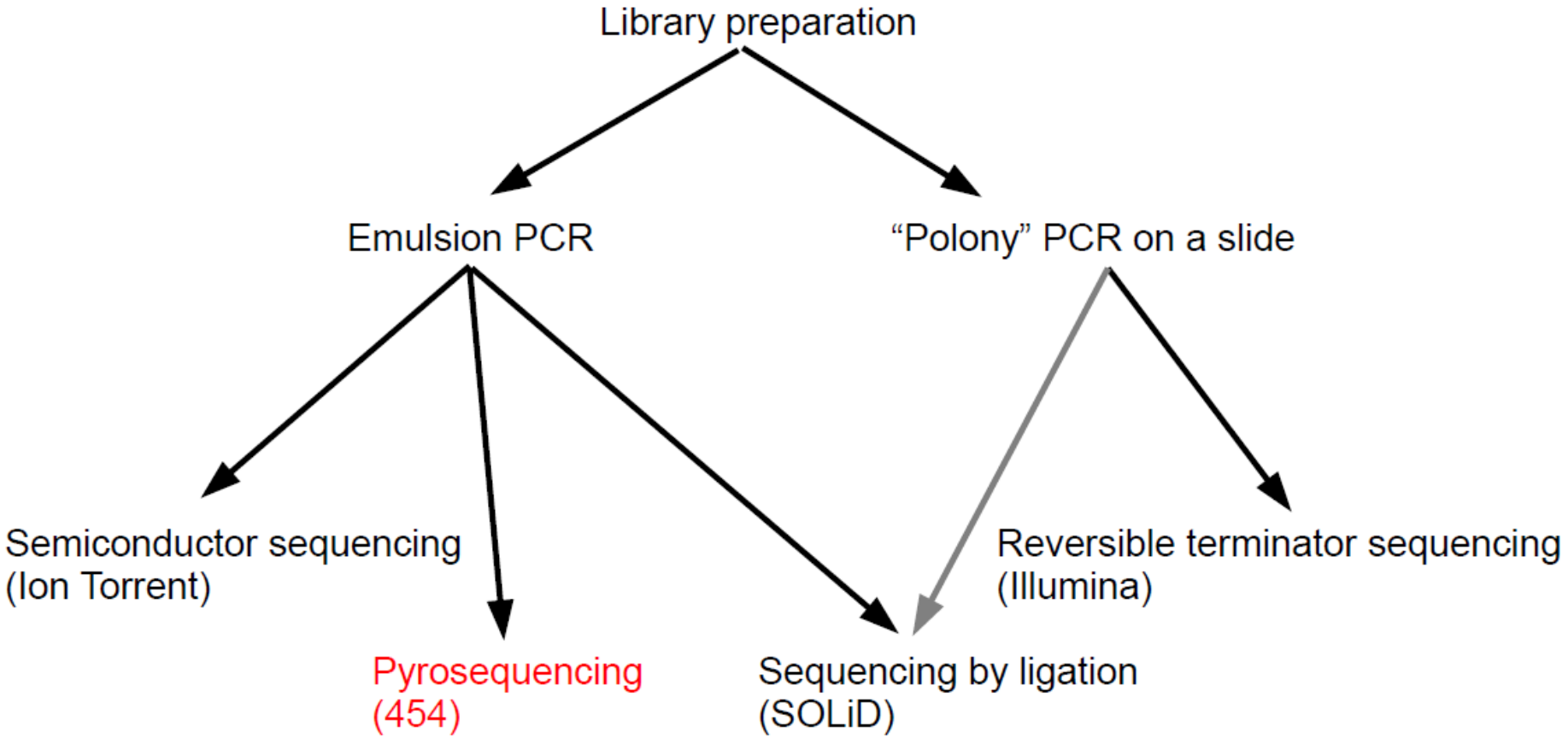
Next Generation Sequencing : Amplified Single Molecule Sequencing



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Next Generation Sequencing : Amplified Single Molecule Sequencing

454 Sequencing / Roche

	GS Junior	GS FLX Titanium XL+	GS FLX Titanium XLR70
Read Length	400 bp	700 bp	450 bp
Throughput	35 Mb	700 Mb	450 Mb
Reads per run	100,000	1,000,000	1,000,000
Accuracy	99 %	99,997 %	99,995 %
Run Time	10 hours	23 hours	10 hours

Workflow : Library preparation → Emulsion PCR → Pyrosequencing

GS Junior System

GS FLX+ System

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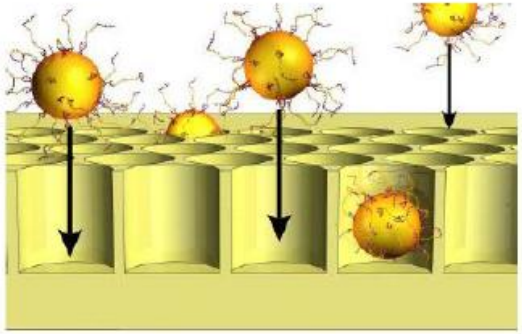
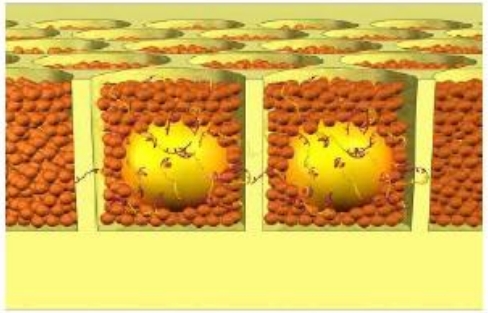
Next Generation Sequencing : Amplified Single Molecule Sequencing

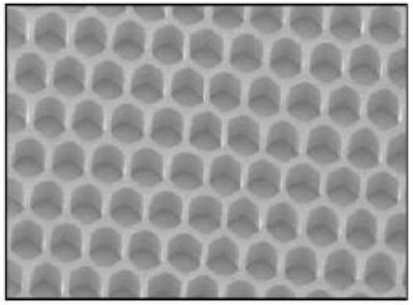
454 Sequencing / Roche Pyrosequencing

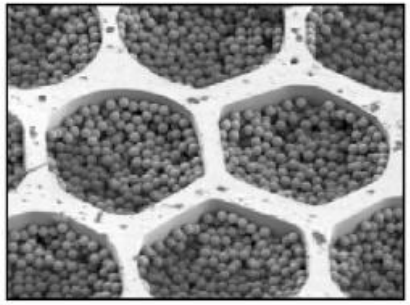


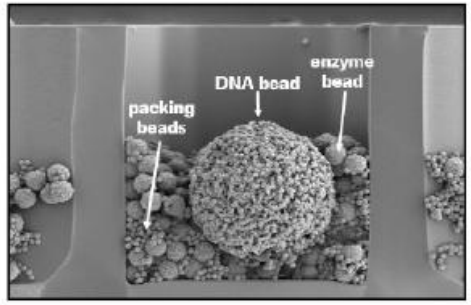
Load enzyme + packing beads



Single clonally amplified DNA beads are deposited in a PicoTiter™Plate (max 4 *10^6 beads)







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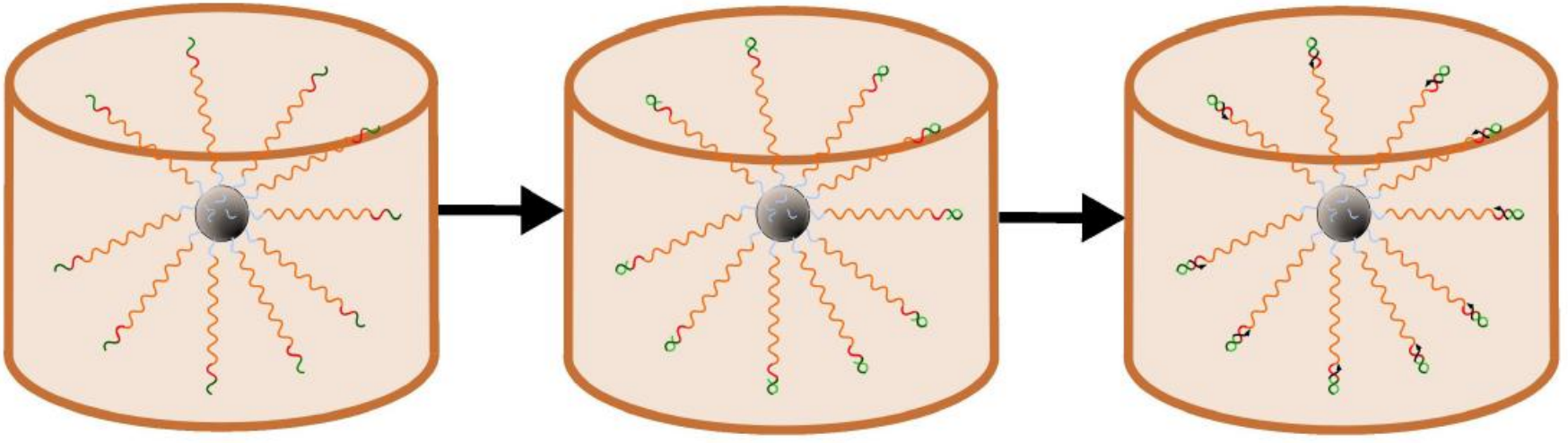
Next Generation Sequencing : Amplified Single Molecule Sequencing

454 Sequencing / Roche Pyrosequencing



1. Denaturation : the bead contains single stranded DNA. The complementary strand is washed away.

2. Annealing : the sequencingprimer anneals to the single strands.

3. Extension : polymerase amplifies the strand starting from the primer towards the bead. Nucleotides flow sequentially over the beads.

(Andy Vierstraete 2012)

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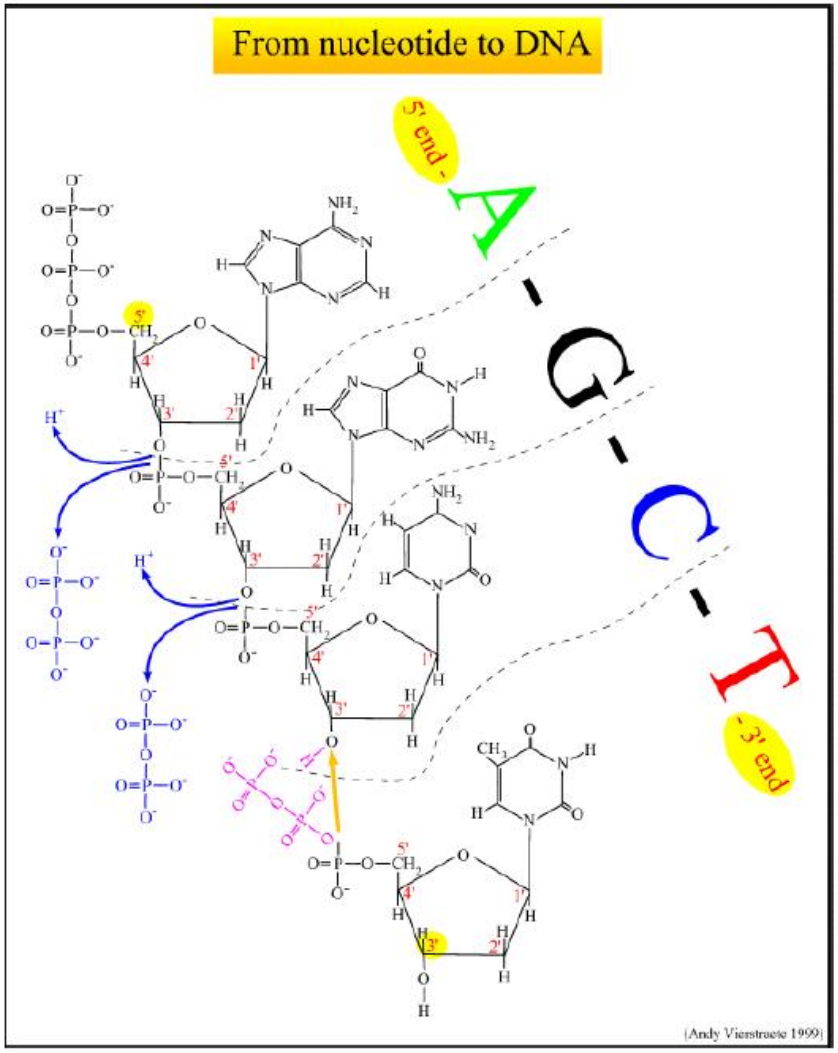
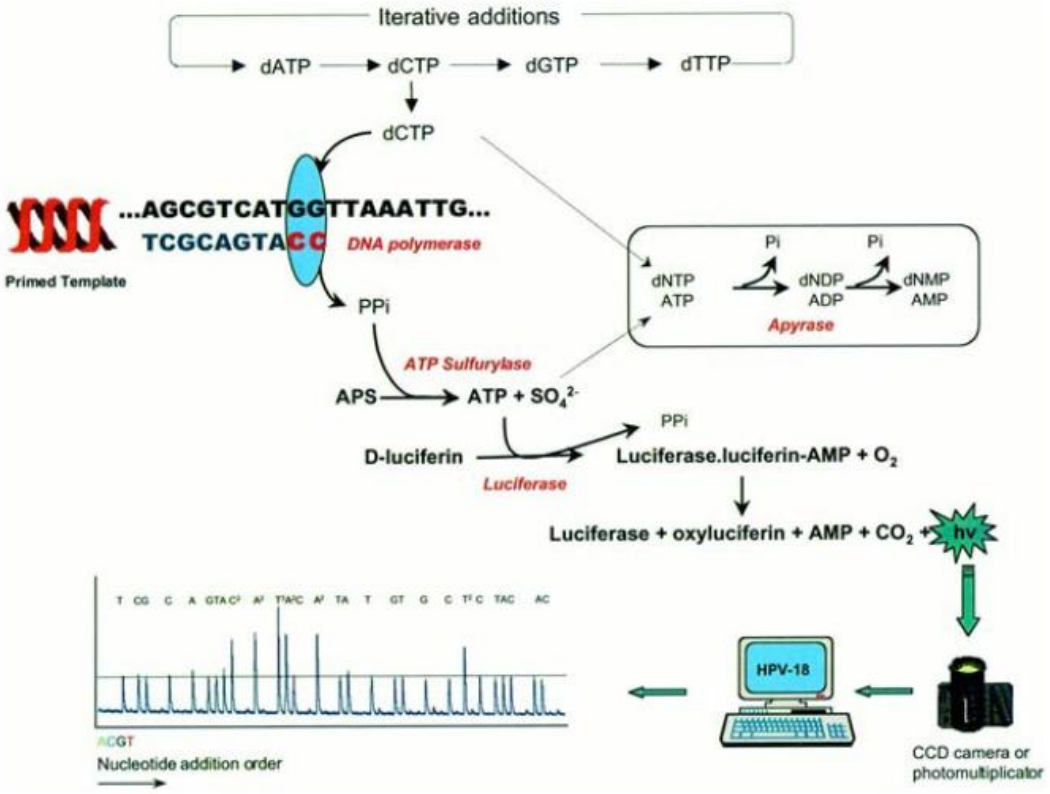
Next Generation Sequencing : Amplified Single Molecule Sequencing

454 Sequencing / Roche Pyrosequencing



4 nucleotides flow sequentially



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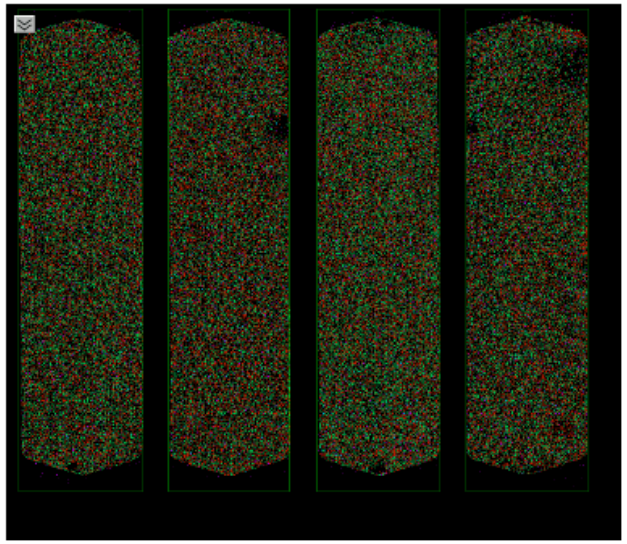
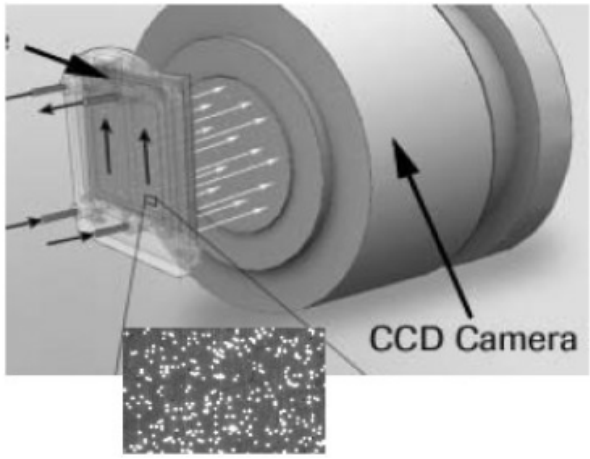
Next Generation Sequencing : Amplified Single Molecule Sequencing

454 Sequencing / Roche Pyrosequencing





The signal strength is proportional to the number of nucleotides incorporated

TCAG for signal calibration and normalization

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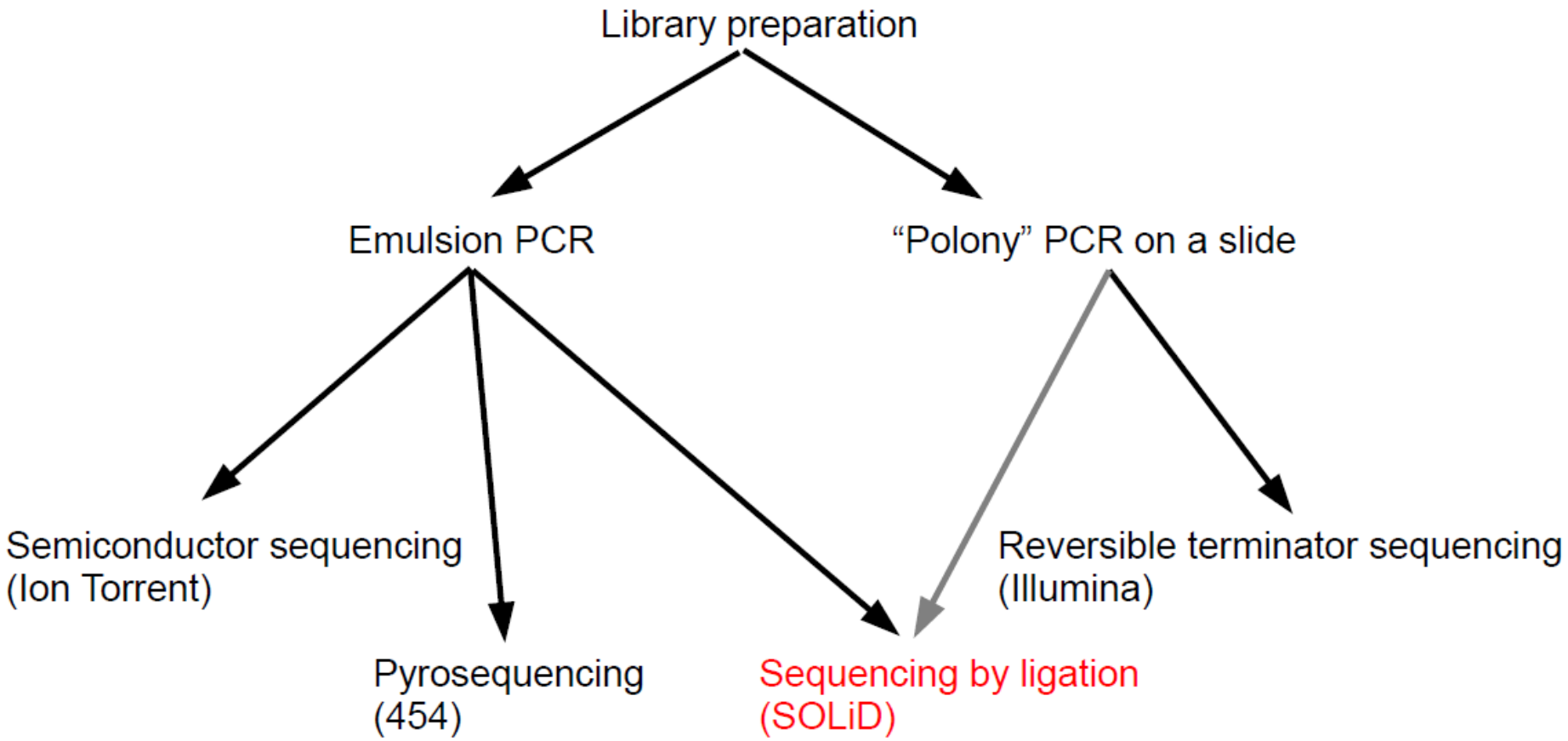
Next Generation Sequencing : Amplified Single Molecule Sequencing



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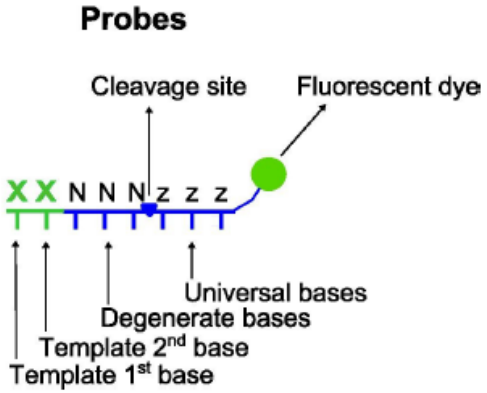
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD

	5500xl	5500	5500 W
Read Length	75 bp	75 bp	75 bp
Throughput	180 Gb	90 Gb	360 Gb ?
Reads per run	3,000,000	1,500,000	6,000,000 ?
Accuracy	99,99 %	99,99 %	99,99 %
Run Time	7-12 days	7-12 days	7-12 days

Workflow : Library preparation → Emulsion PCR → Sequencing by Ligation
Wildfire PCR



dual base encoding

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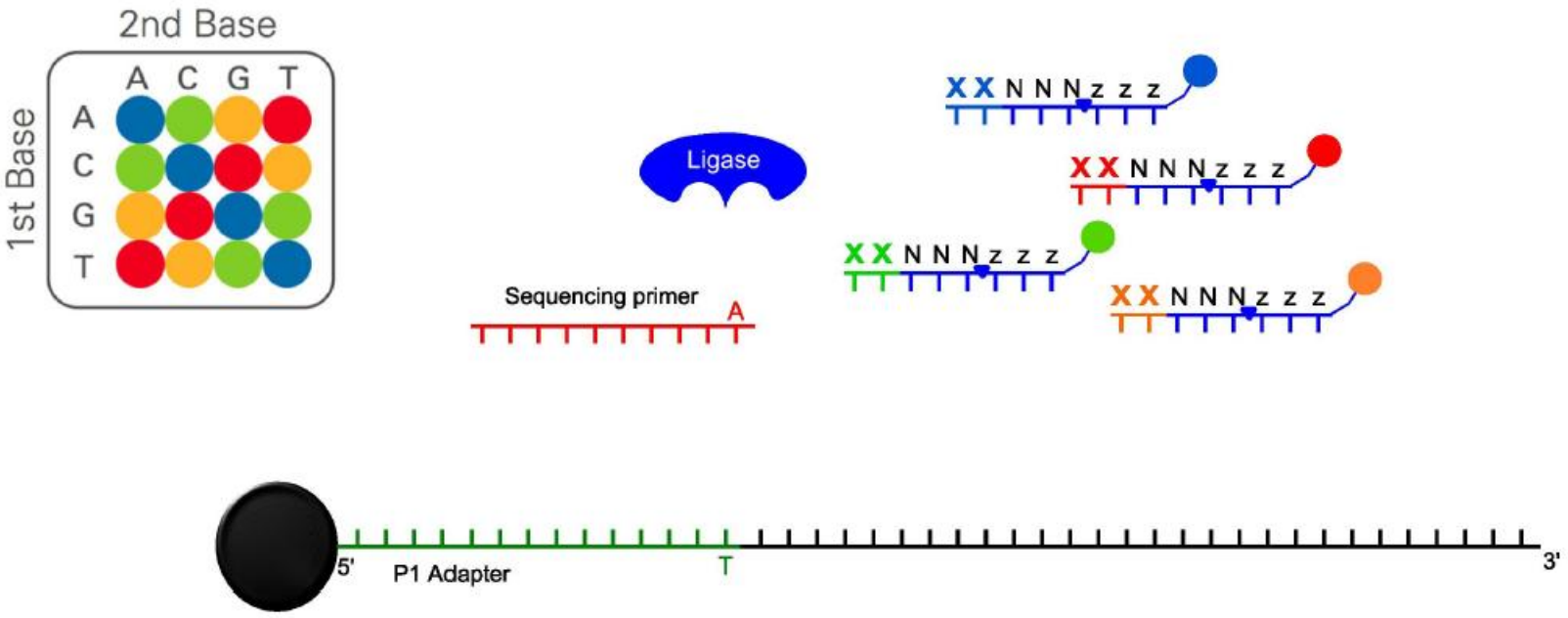
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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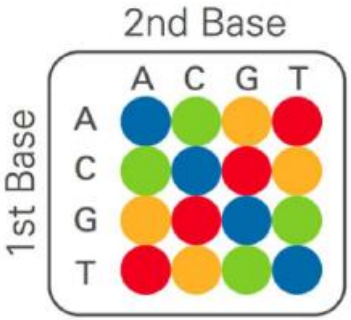
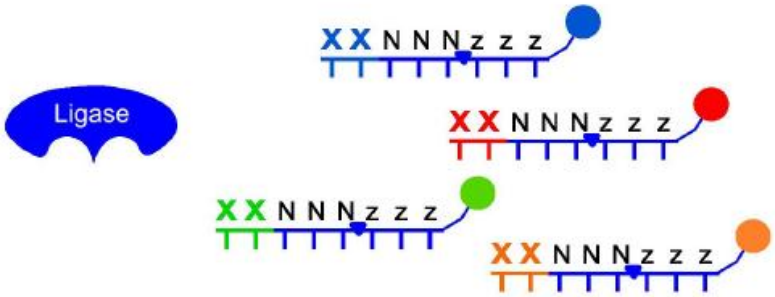
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD





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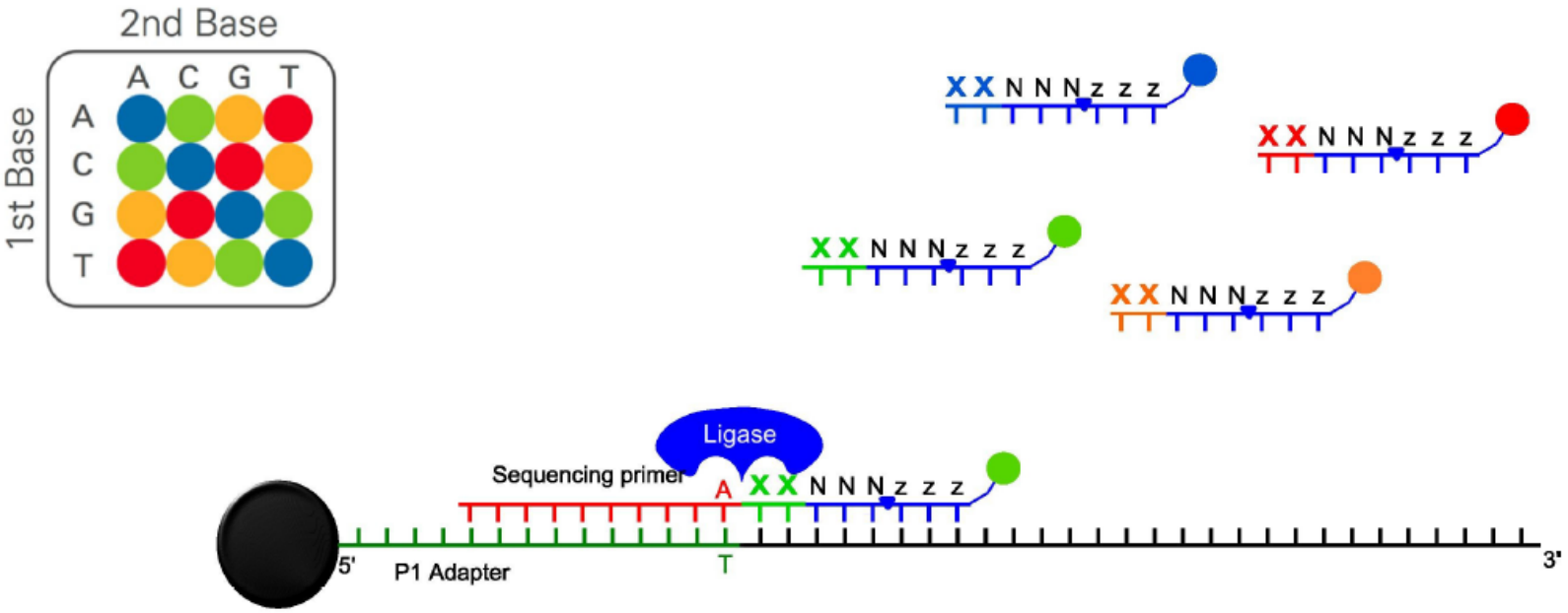
SOLiD



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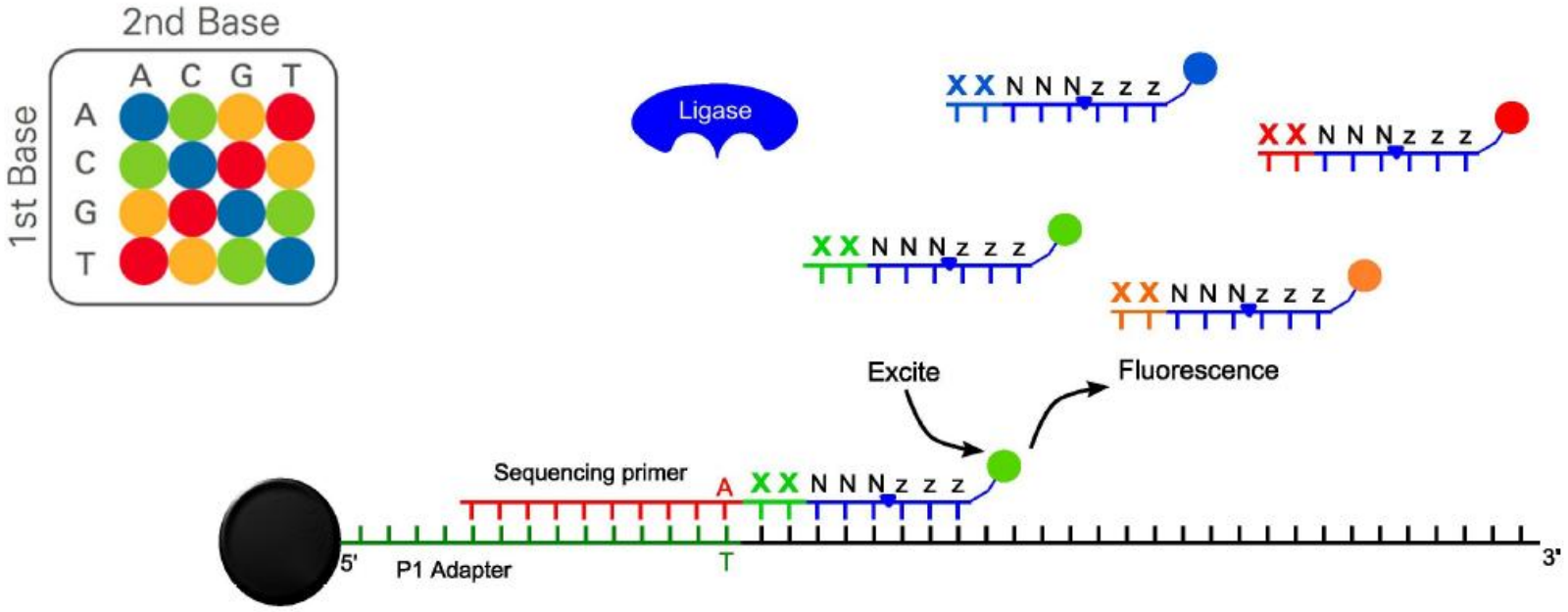
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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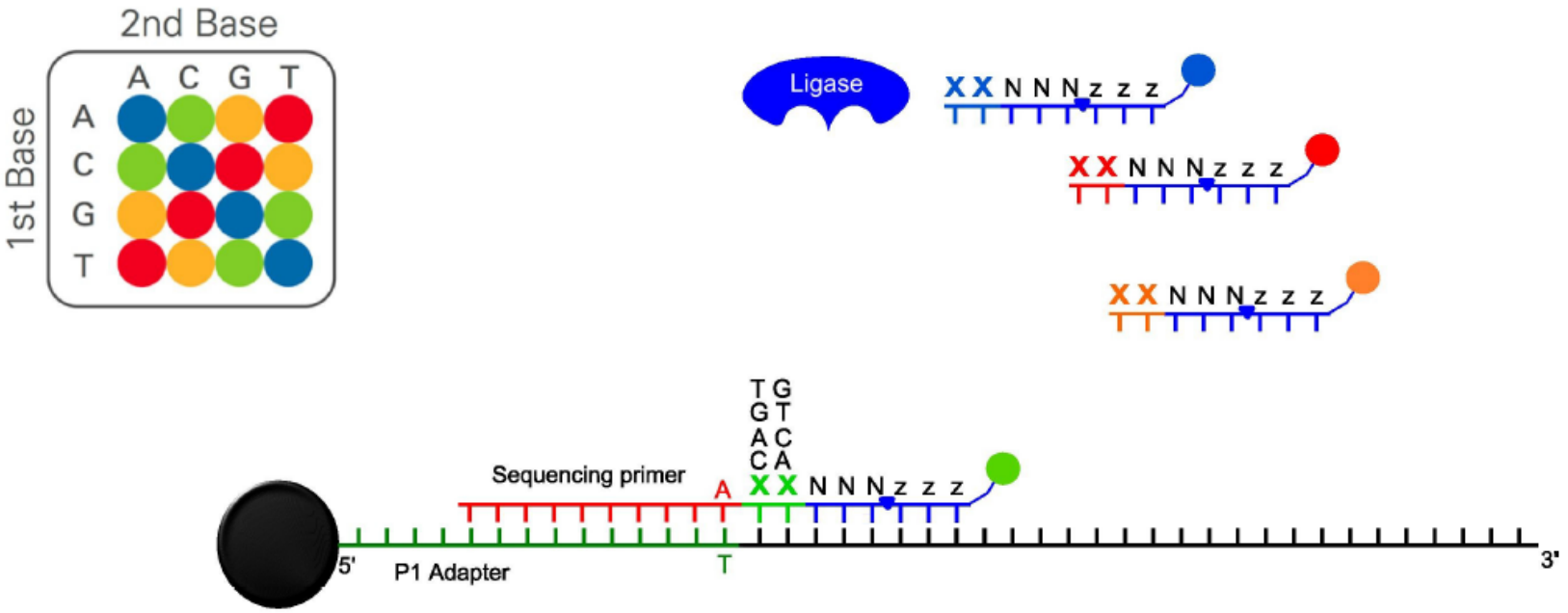
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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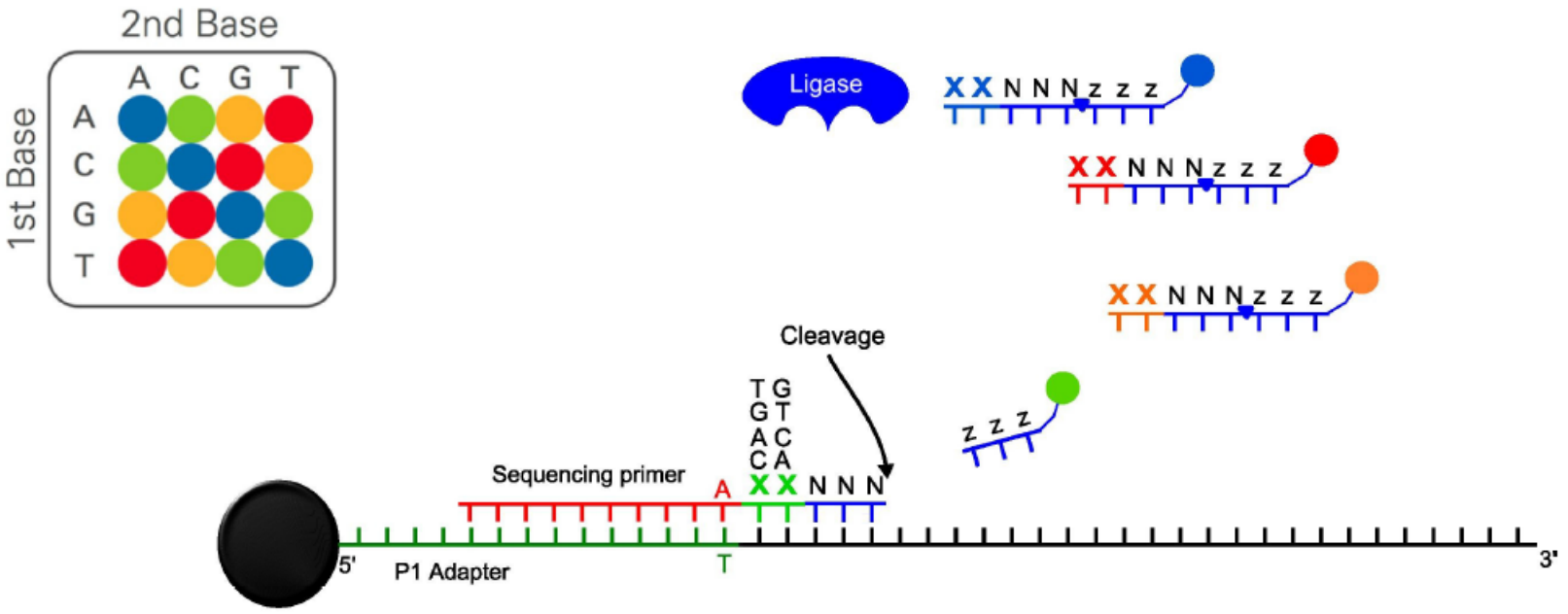
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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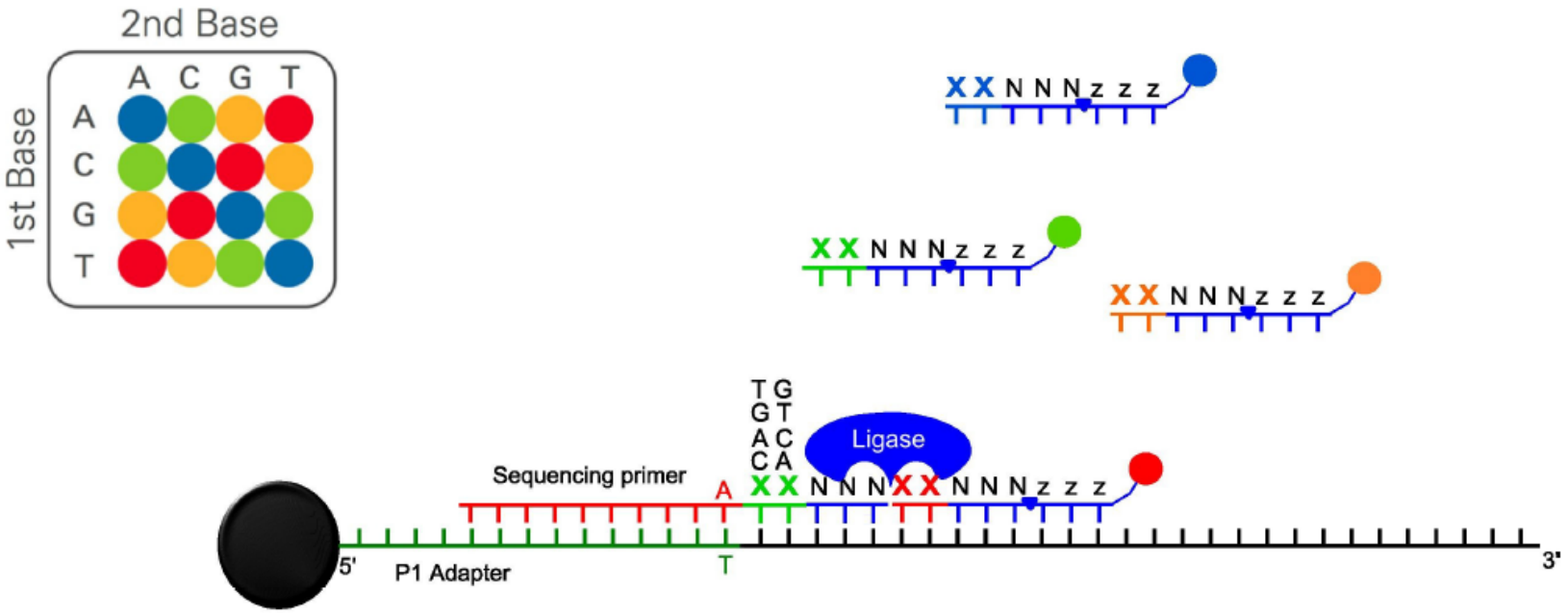
SOLiD



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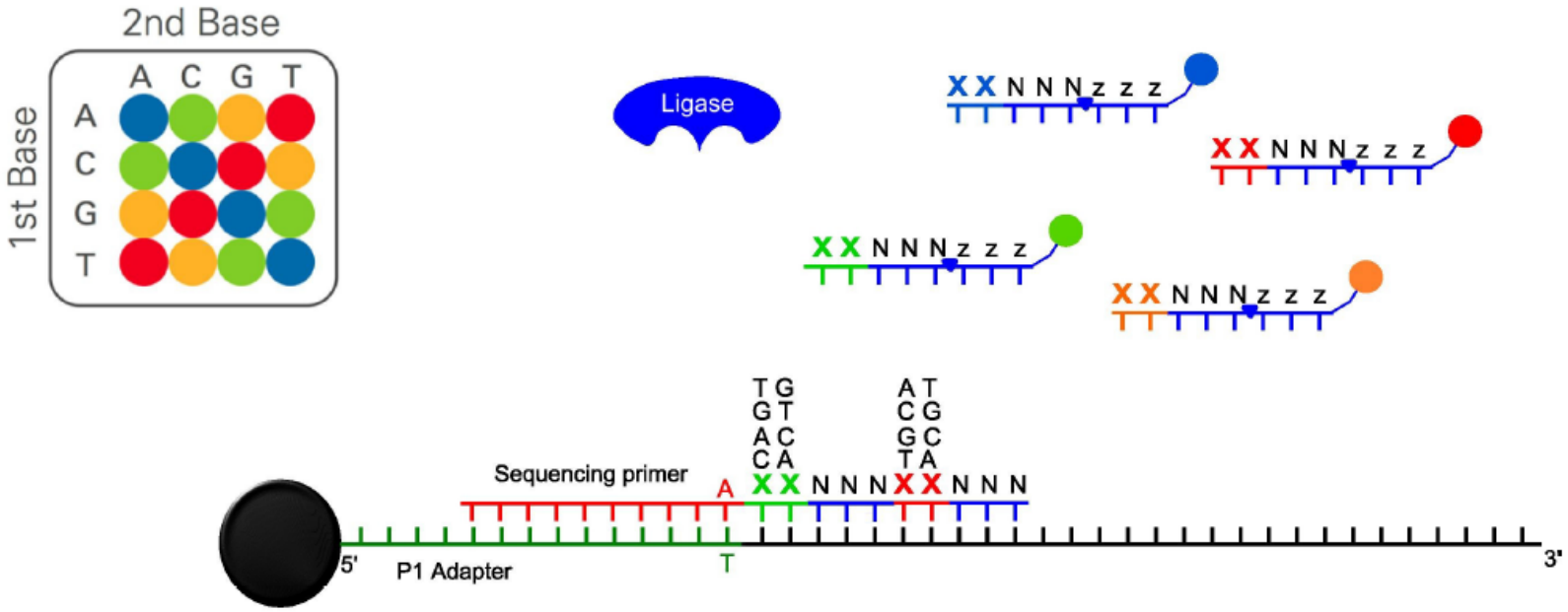

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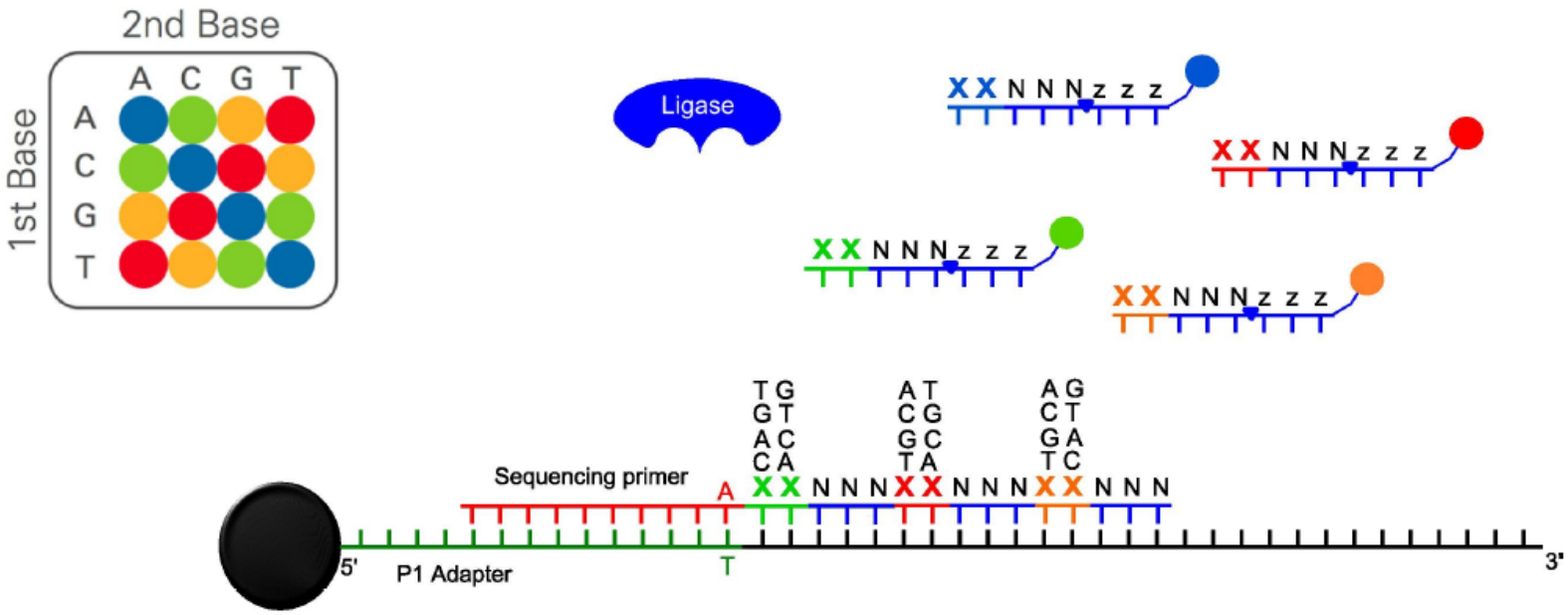
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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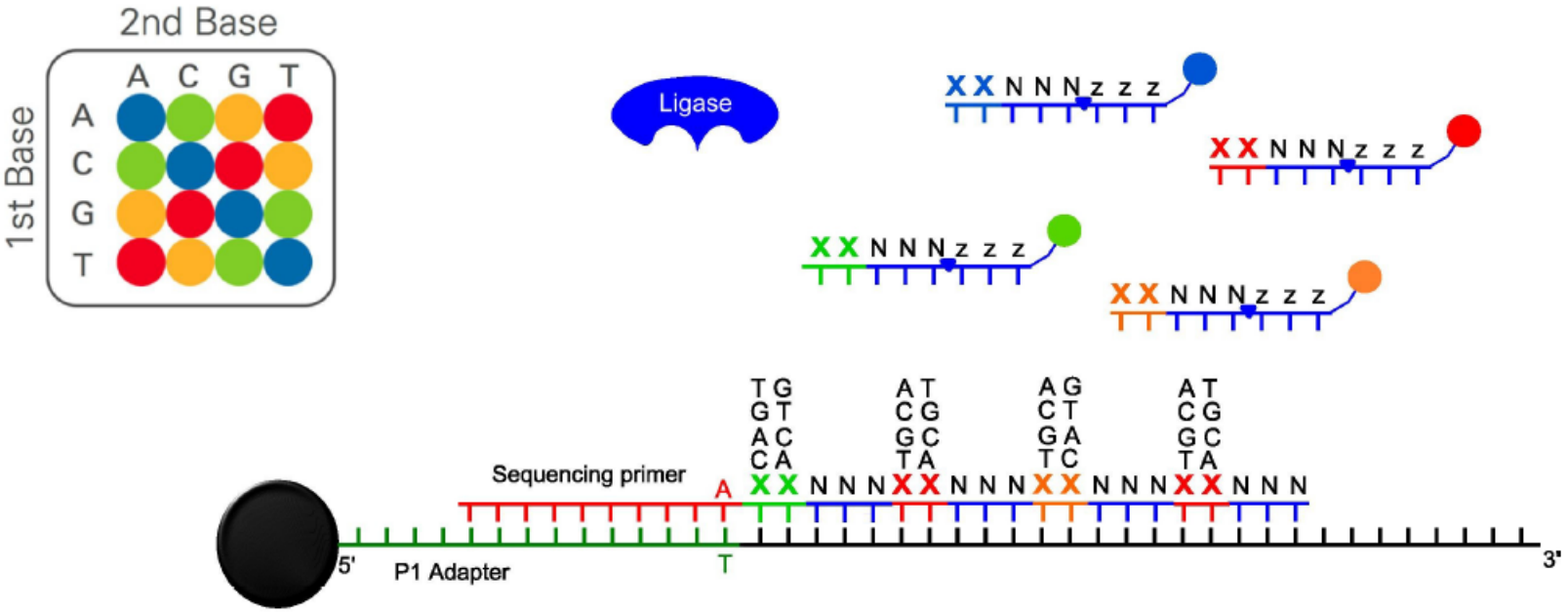
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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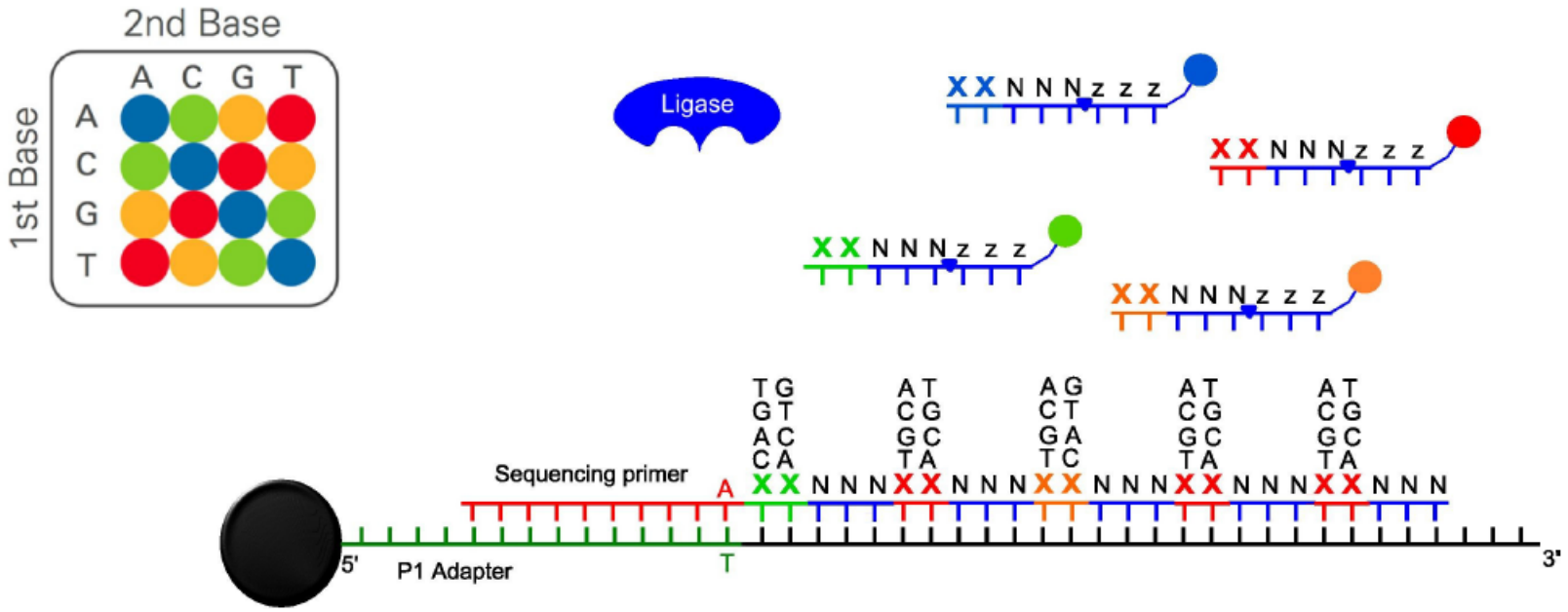
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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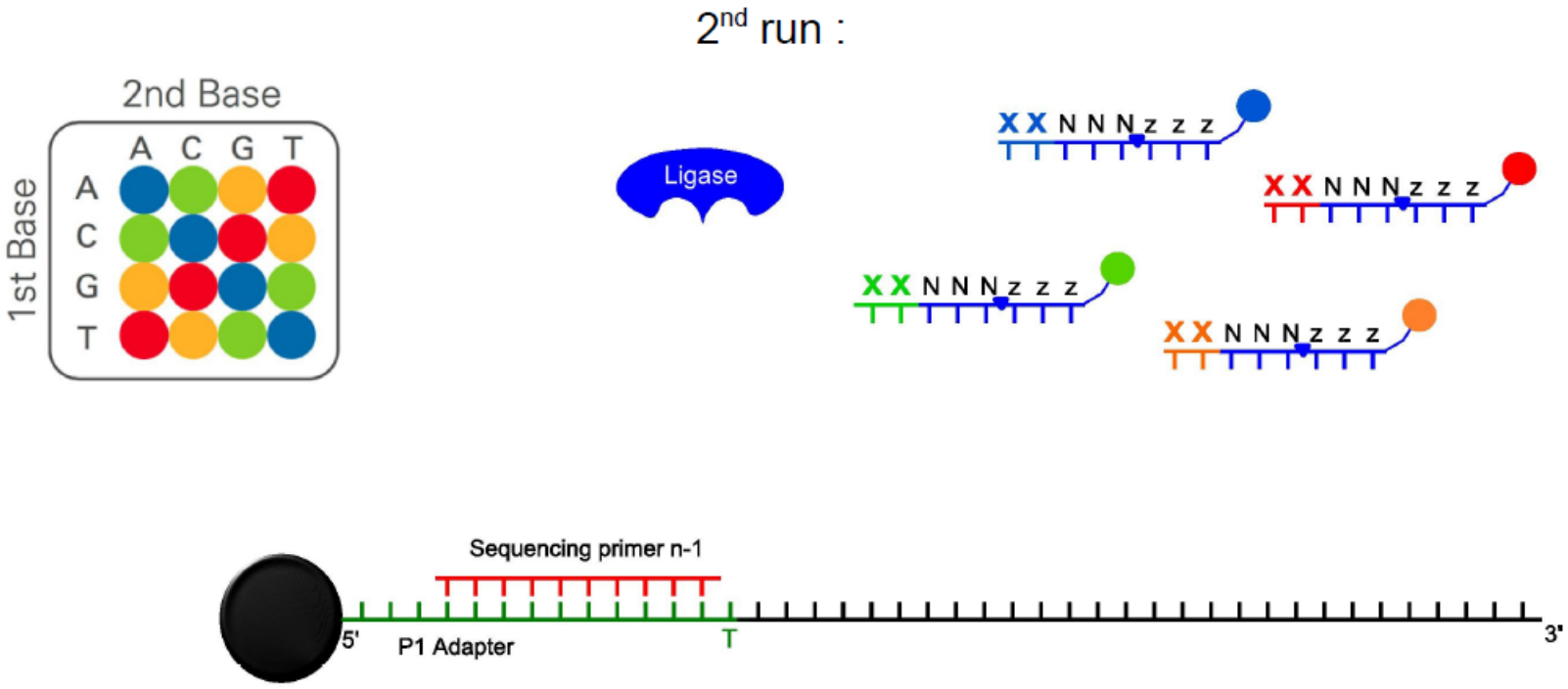
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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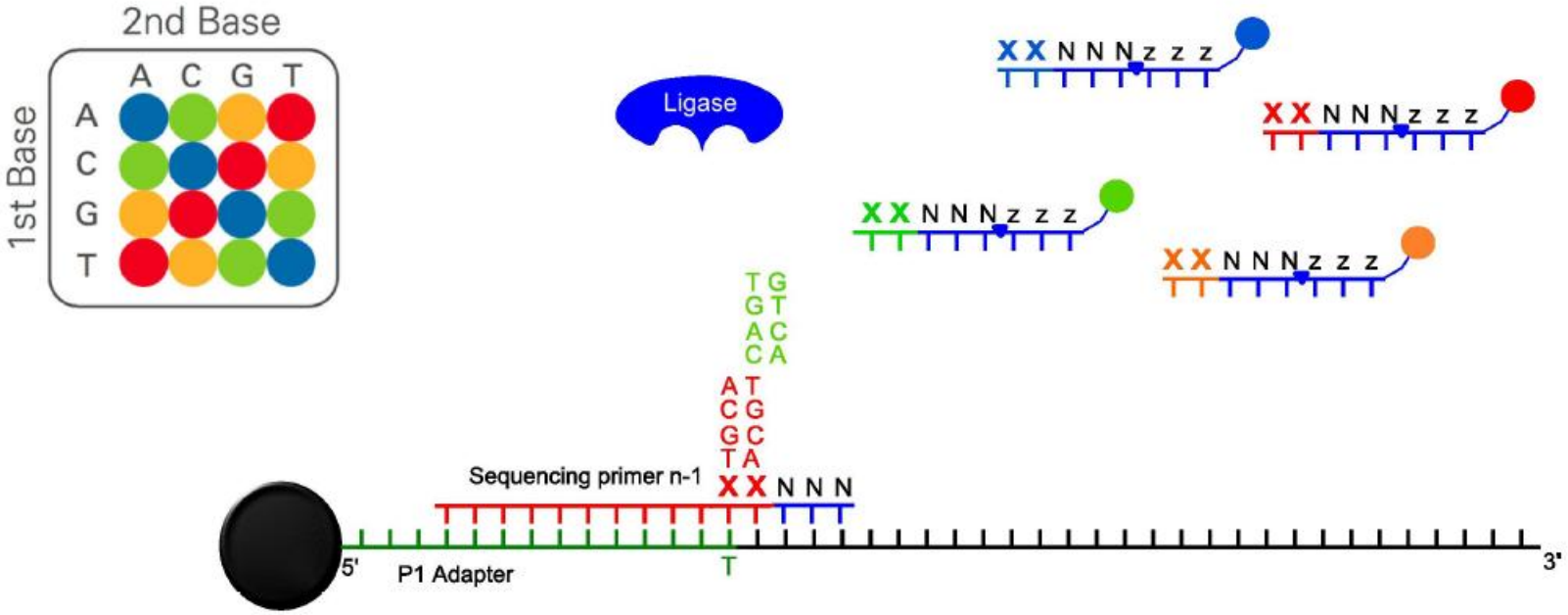
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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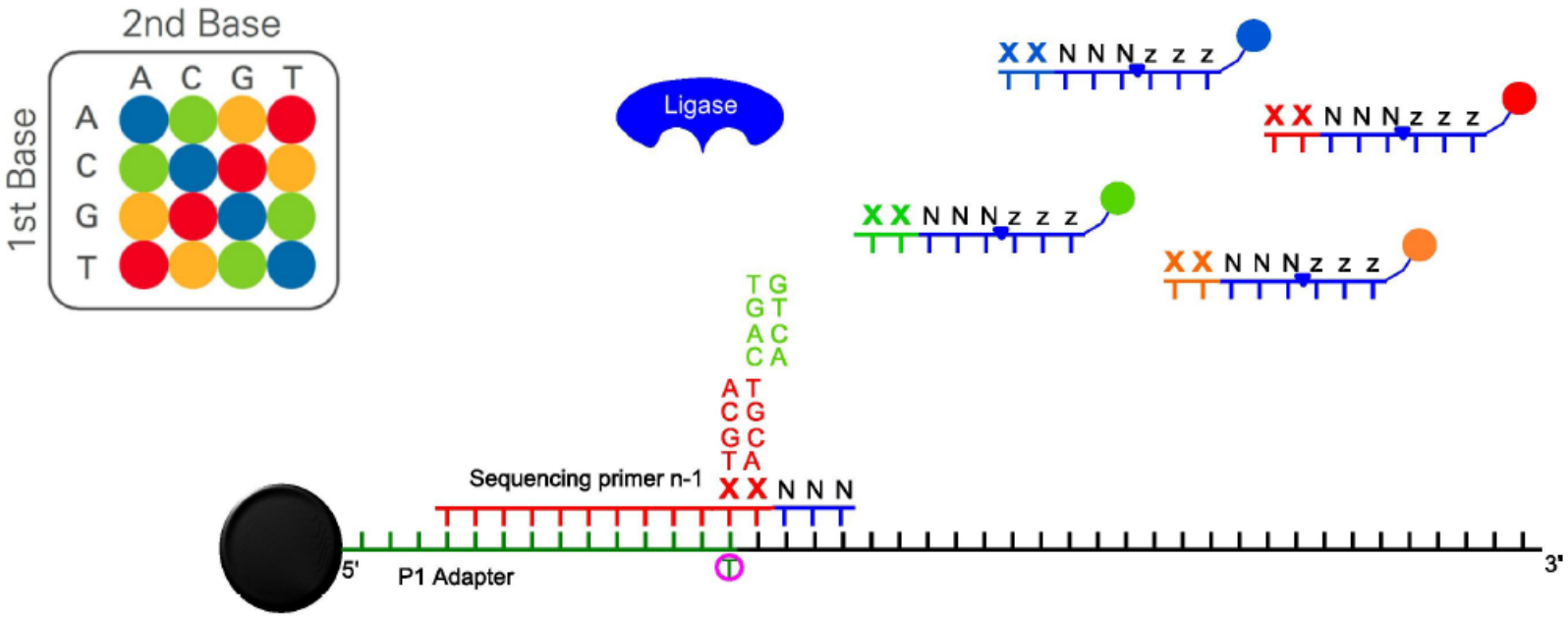
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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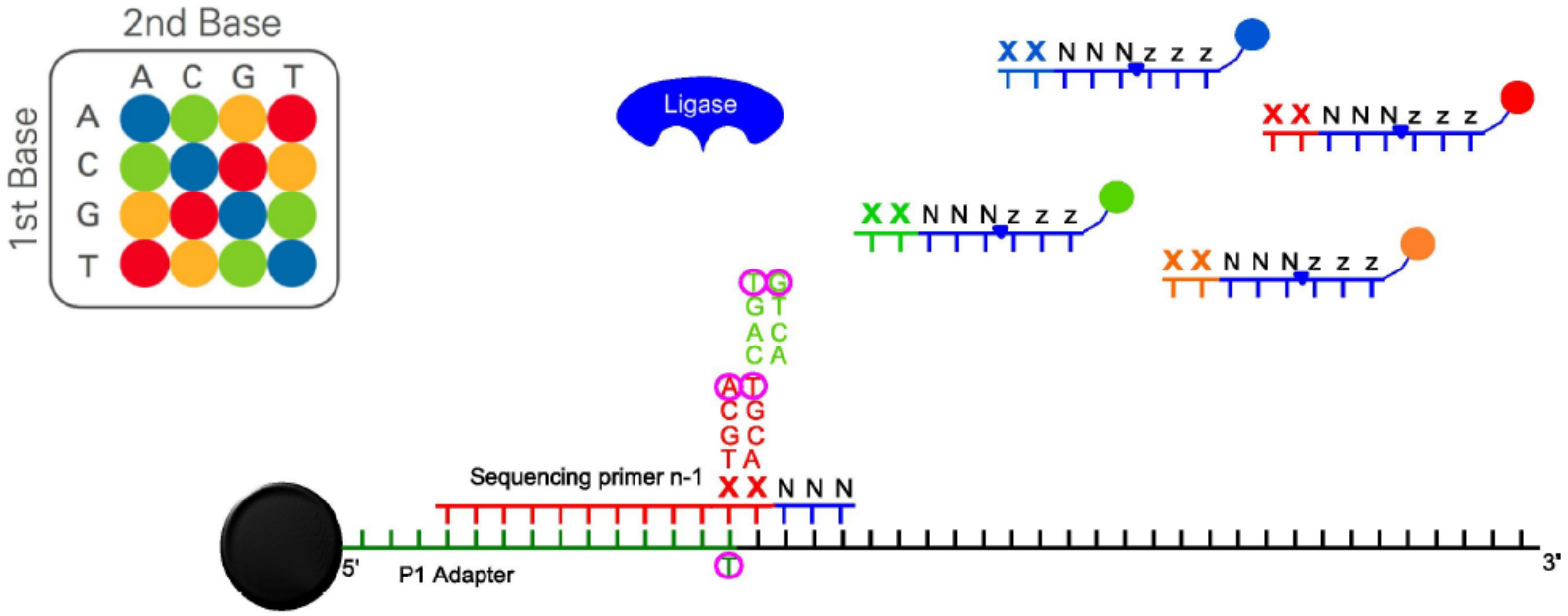
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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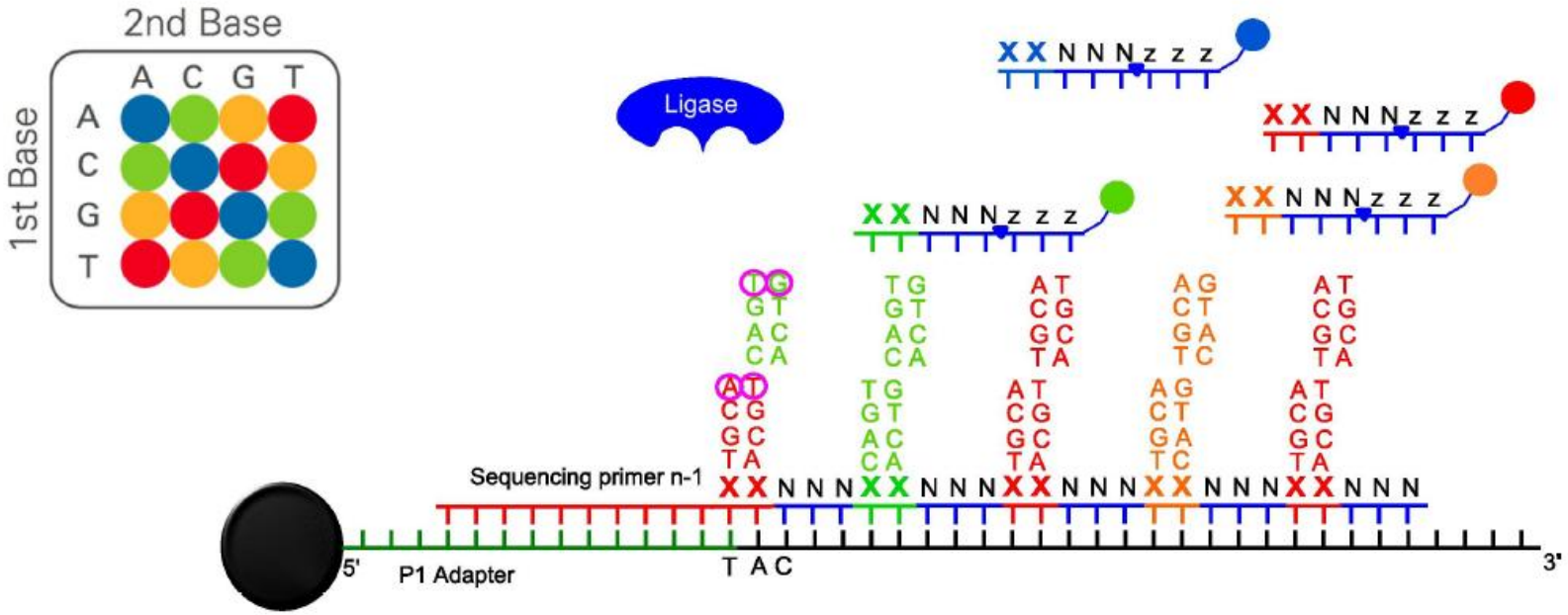
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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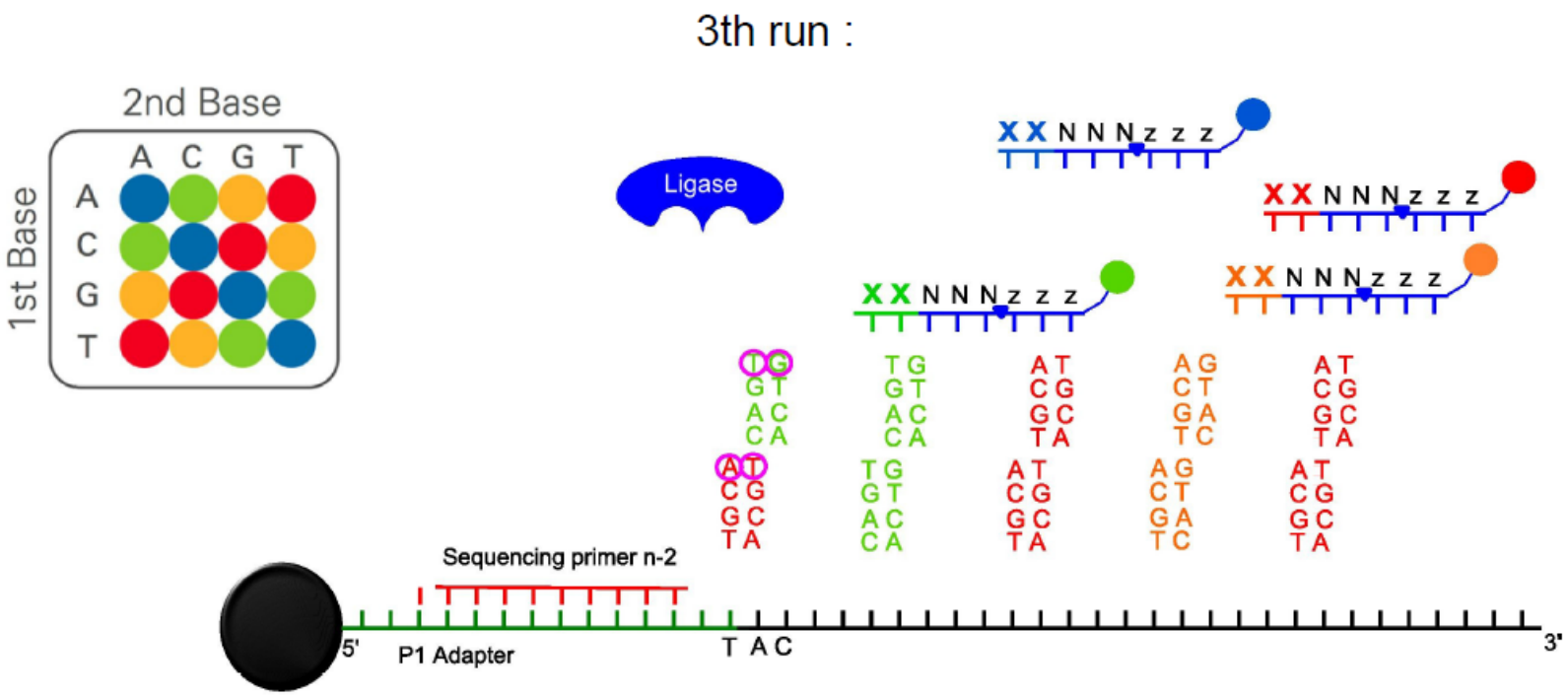
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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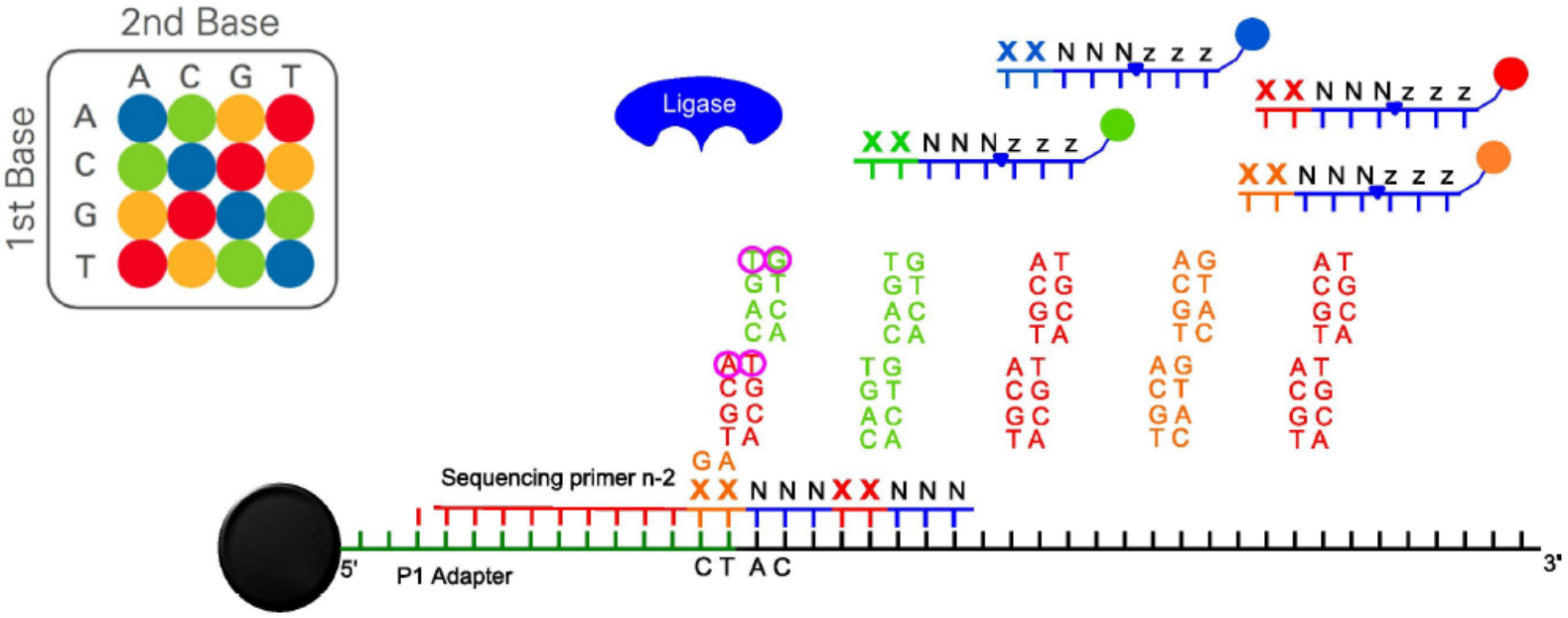
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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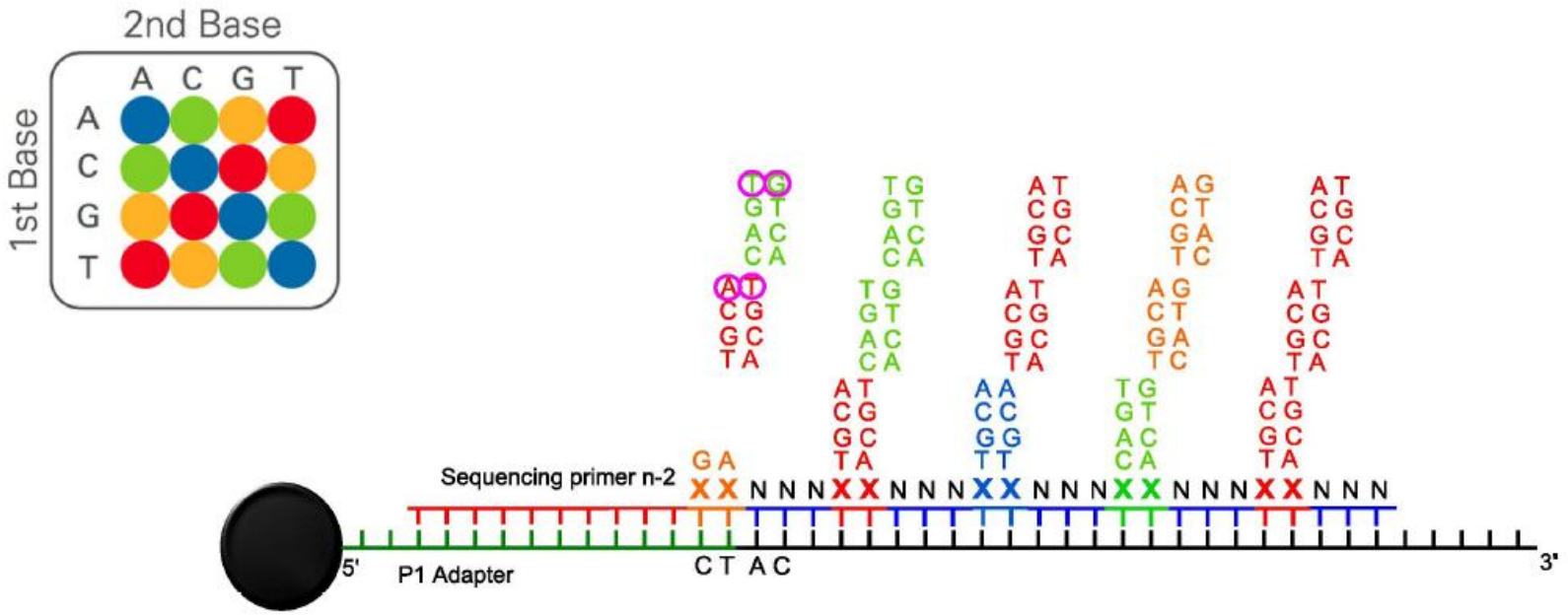
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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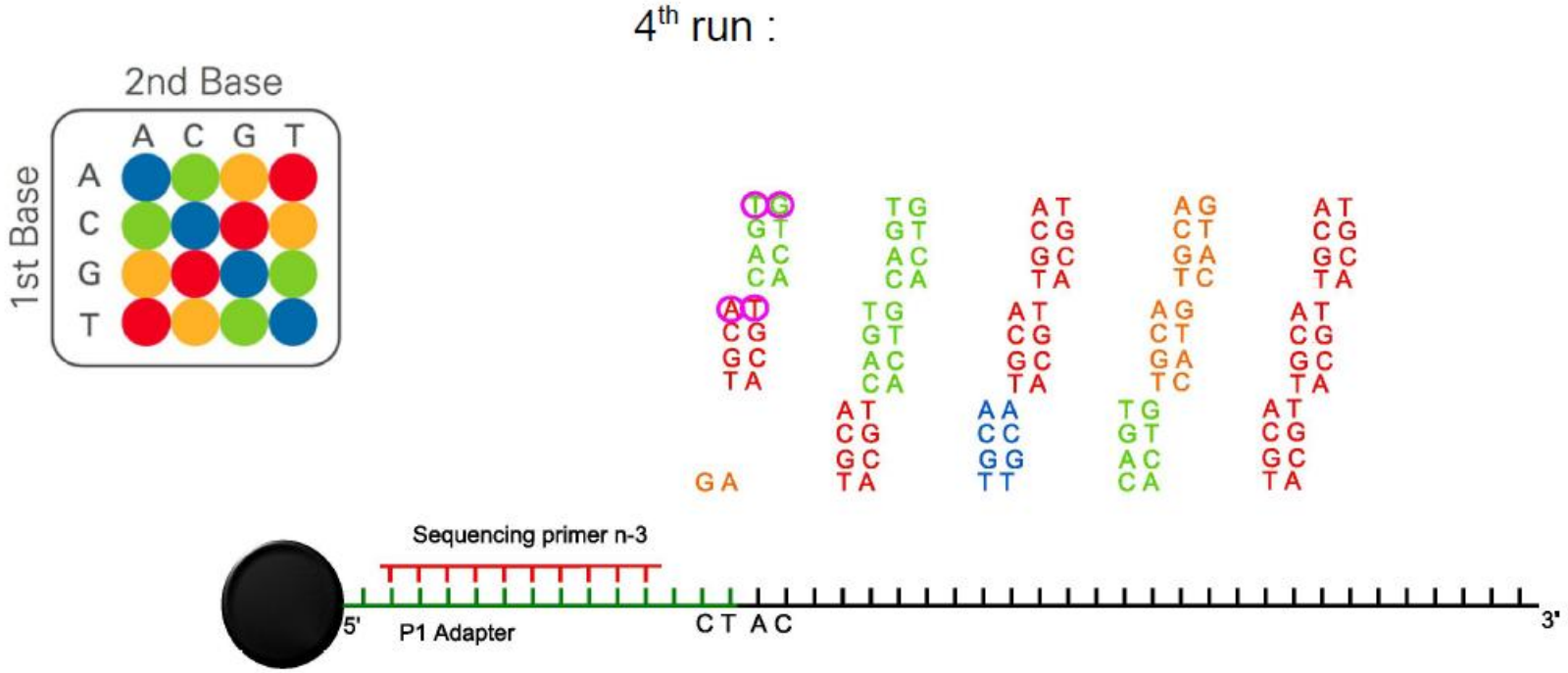
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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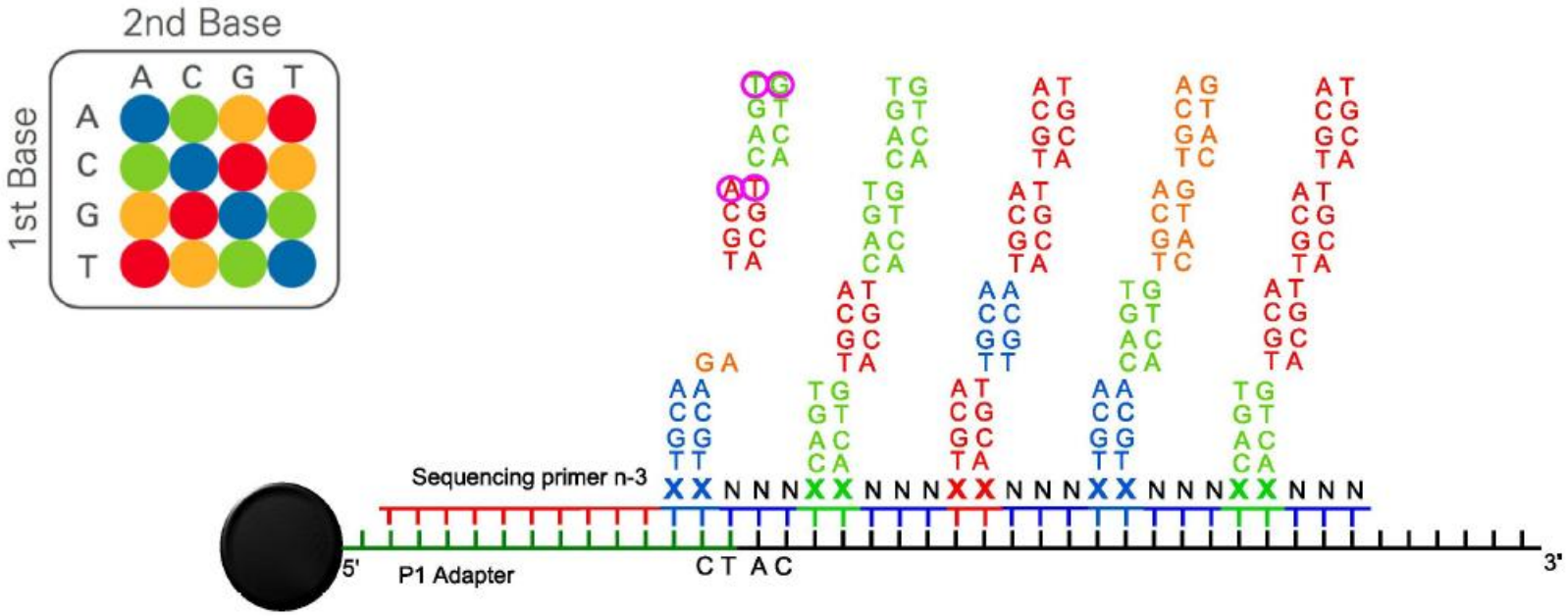
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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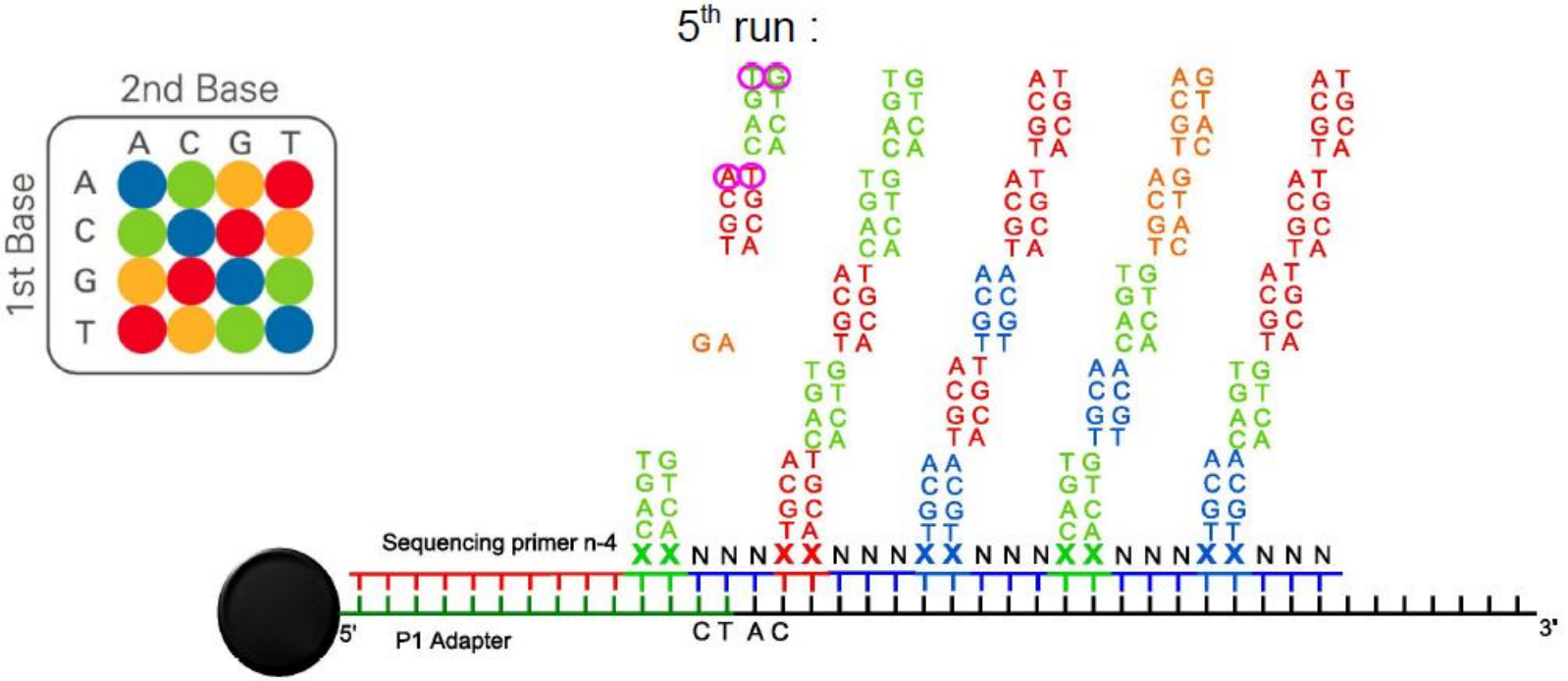
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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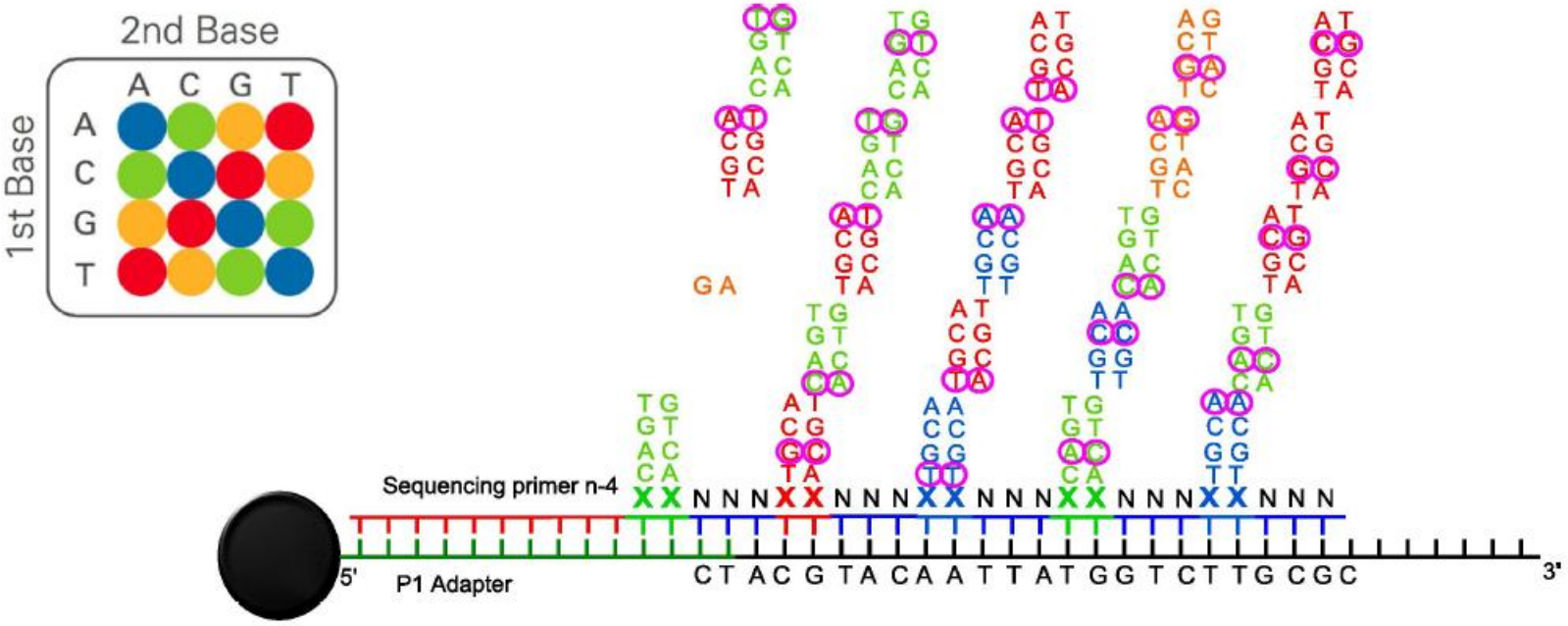
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD



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Next Generation Sequencing : Amplified Single Molecule Sequencing

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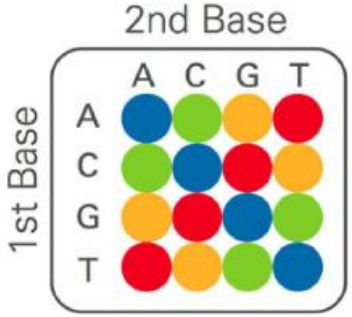
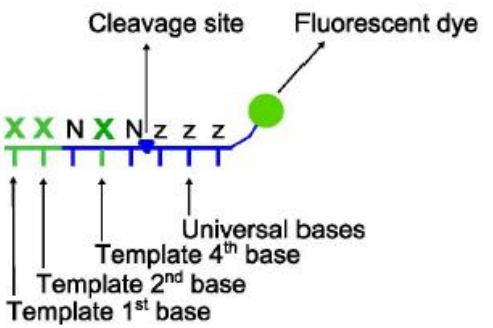
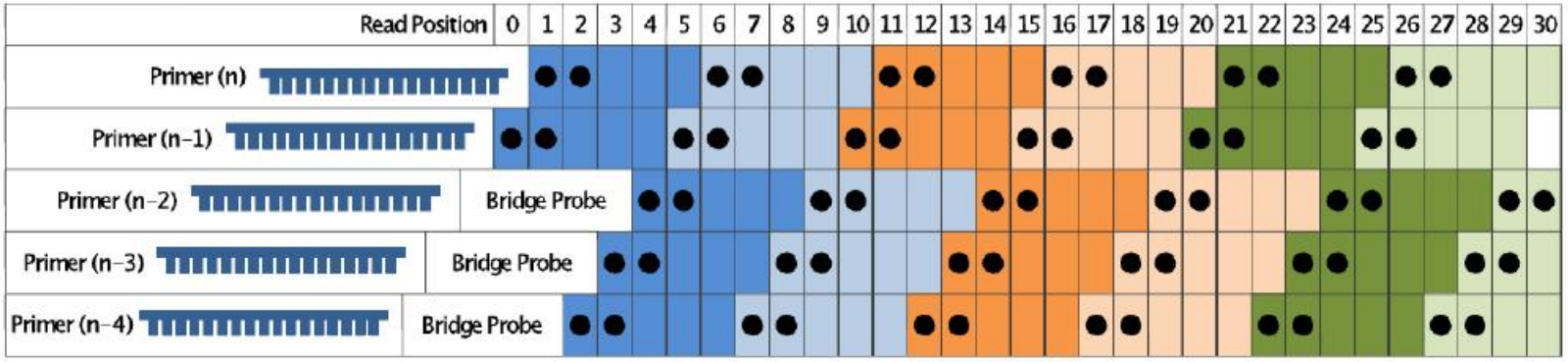
More accuracy : 3-base encoding probes



Probes set 2 : 3-base encoding

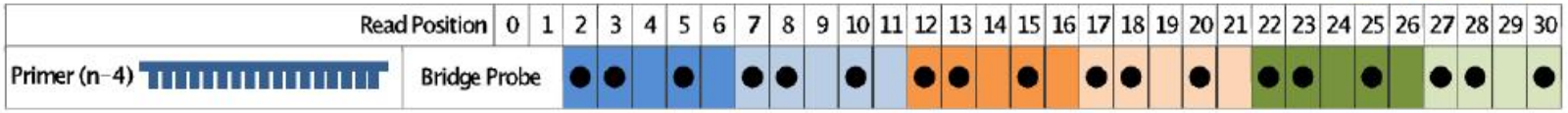


8. Repeat steps 1–7 with Primers n–1, n–2, n–3, and n–4 using Probe Set 1



9. Repeat steps 1–7 with Primer n–4 using Probe Set 2

● Interrogated Positions Ligation Cycle 1 2 3 4 5 6



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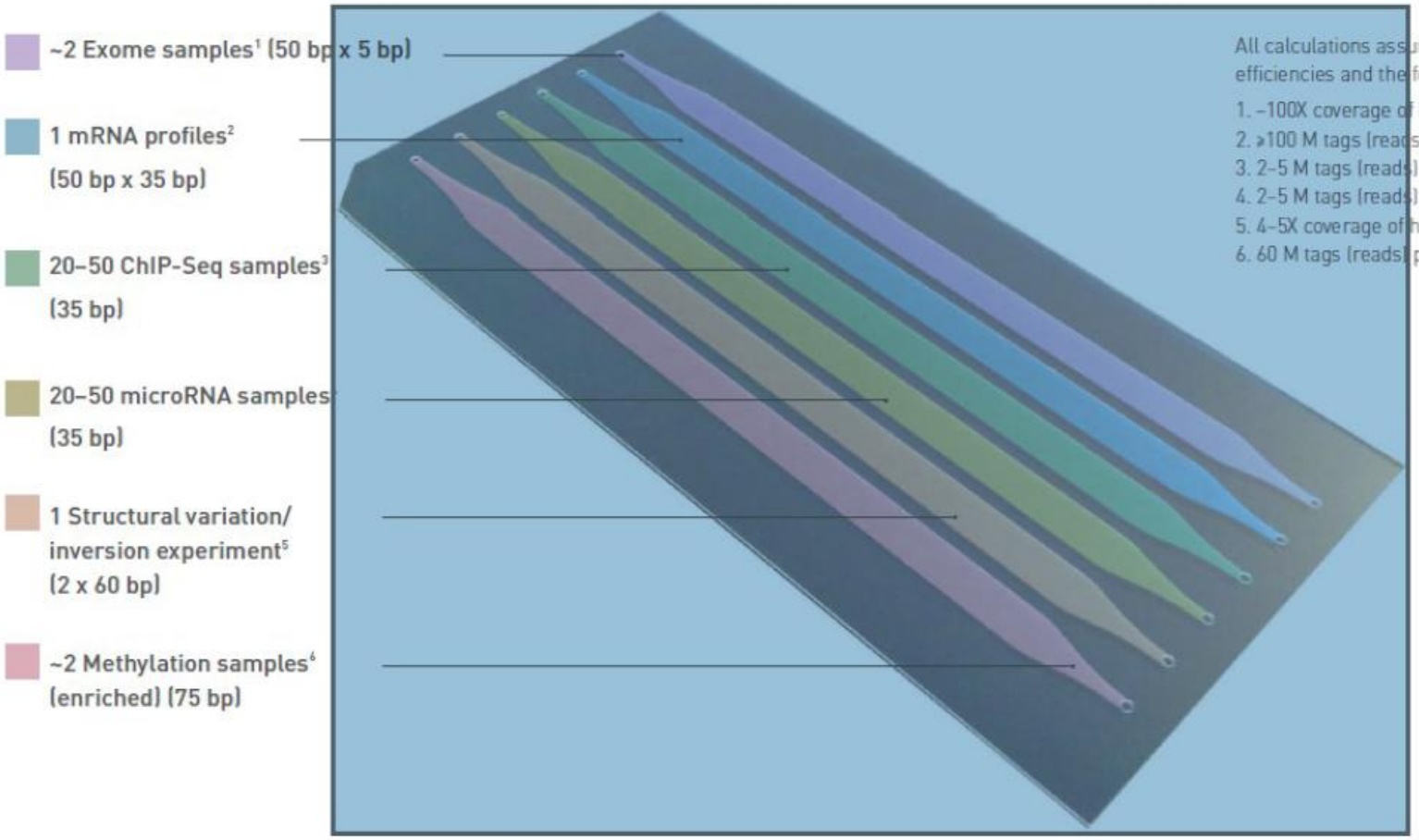
Next Generation Sequencing : Amplified Single Molecule Sequencing

SOLiD

Figure 1. Multiple applications on a single FlowChip with different read lengths and chemistries.



life technc

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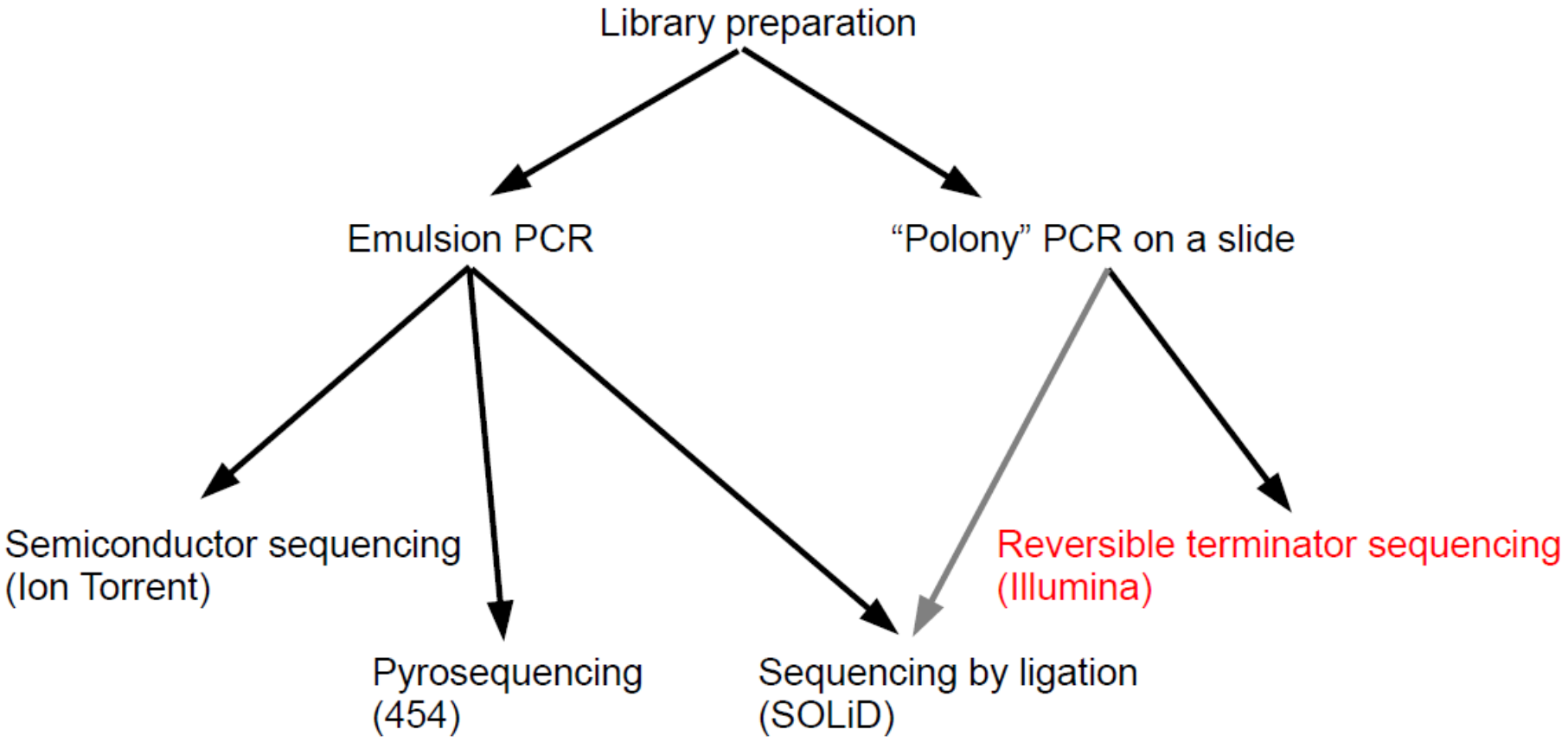
Next Generation Sequencing : Amplified Single Molecule Sequencing



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Next Generation Sequencing : Amplified Single Molecule Sequencing
Illumina

	HiSeq	HiScanSQ	Genome Analyzer IIx	MiSeq
Read Length	100 bp	100 bp	150 bp	250 bp
Throughput	600 Gb	150 Gb	95 Gb	6 Gb
Reads per run	3,000,000,000	750,000,000	320,000,000	12,000,000
Accuracy	99,9 %	99,9 %	99,9 %	99,9 %
Run Time	11 days	8 days	14 days	20-35 hours

Workflow : Library preparation → Bridge amplification → Reversible termination sequencing

Next Generation Sequencing : Amplified Single Molecule Sequencing
Illumina

HiSeq 2500 / 2000 / 1500 / 1000

HiScanSQ

Genome Analyzer IIx

MiSeq

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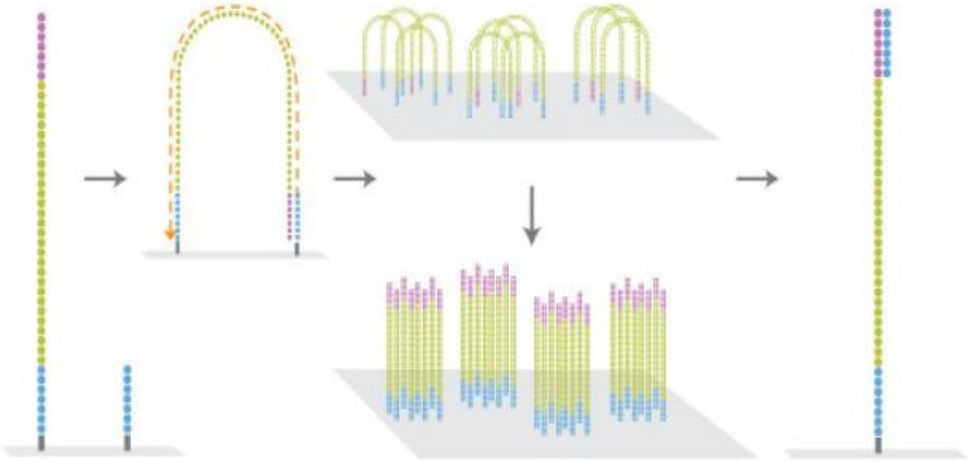
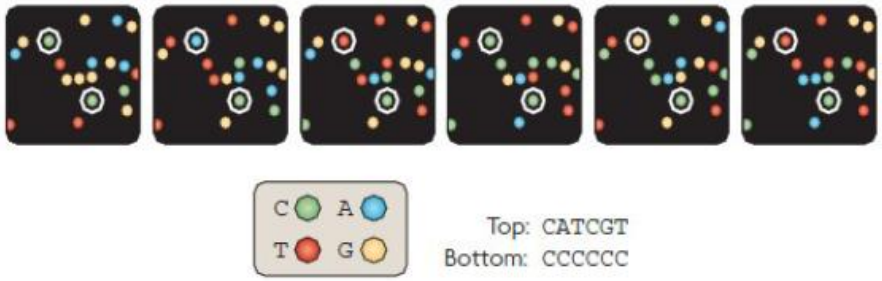
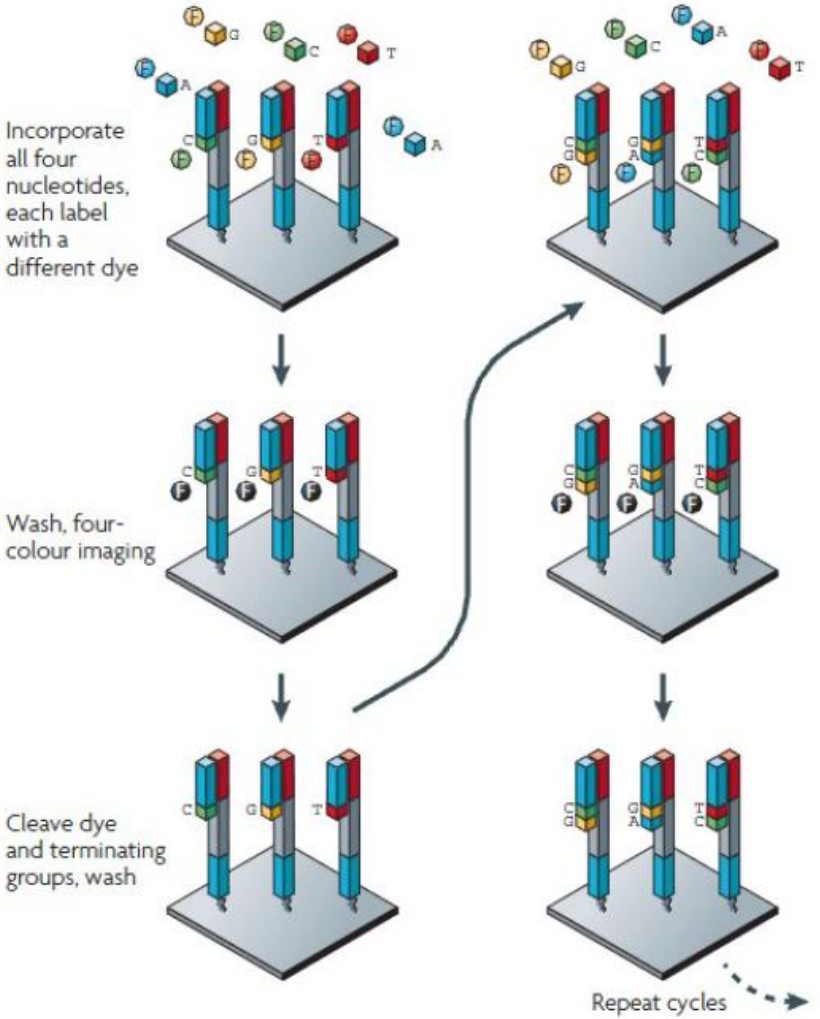
Next Generation Sequencing : Amplified Single Molecule Sequencing

Illumina



4 nucleotides with different dye flow simultaneous





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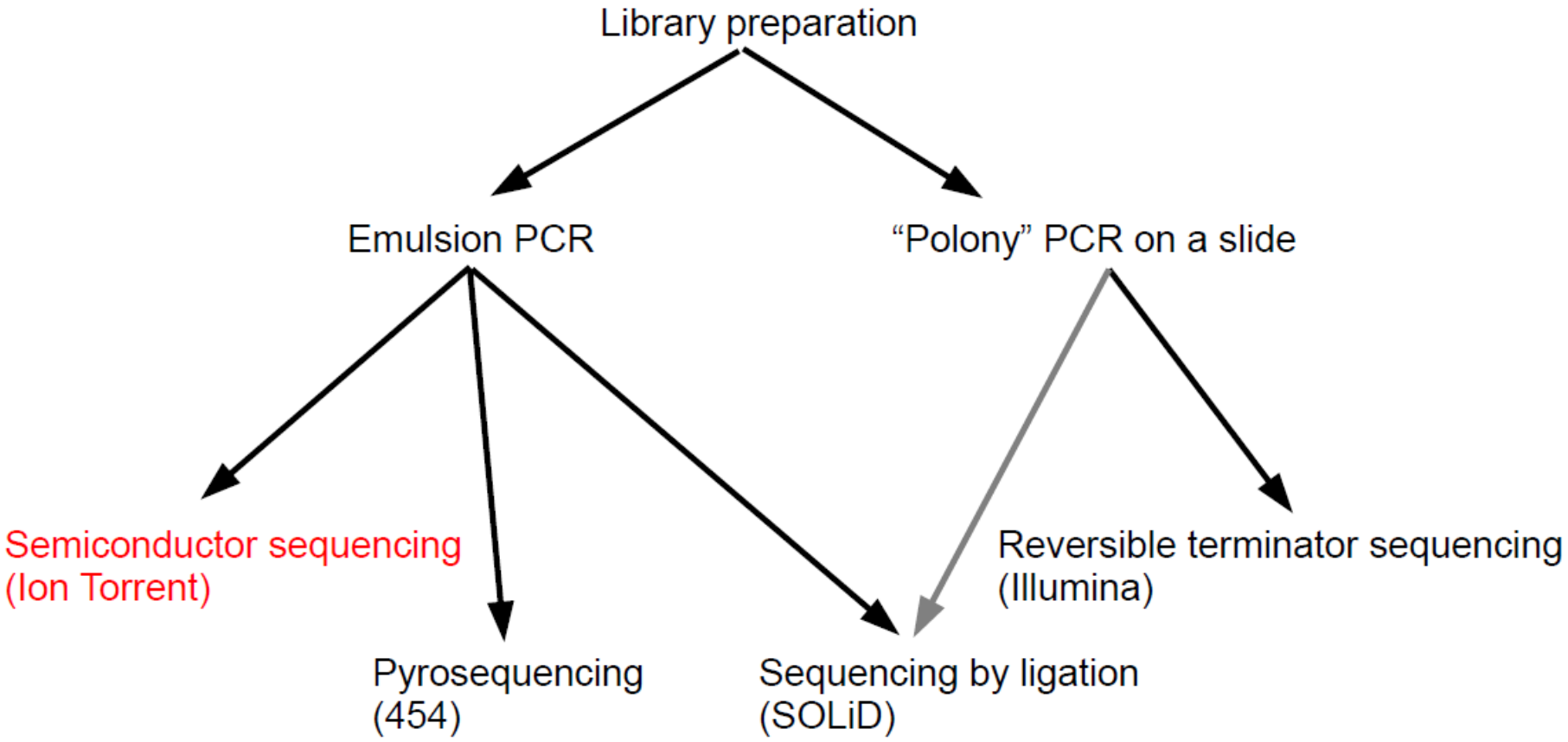
Next Generation Sequencing : Amplified Single Molecule Sequencing



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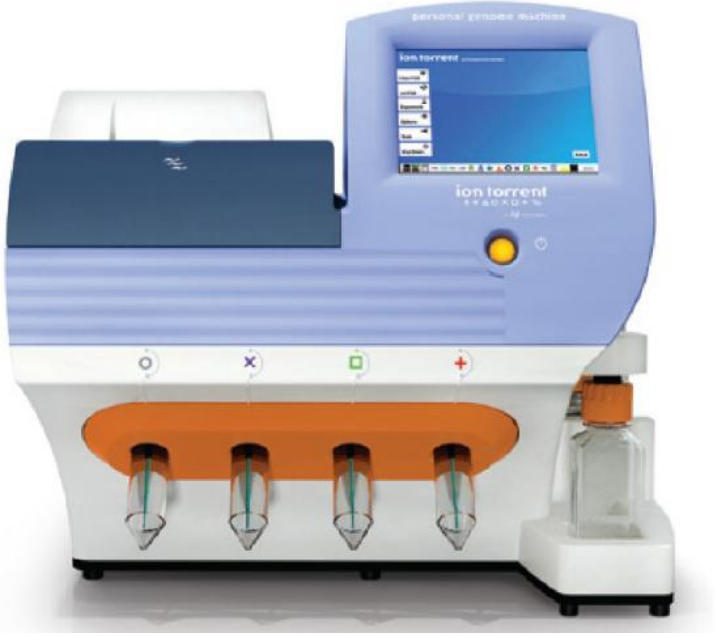
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent

	PGM	Proton
Read Length	200 bp	200 bp
Throughput	20 Mb - 1 Gb	10 -100 Gb
Reads per run	11,000,000	660,000,000
Accuracy	99 %	99 %
Run Time	4,5 hours	4,5 hours

PGM (Personal Genome Machine)



Proton

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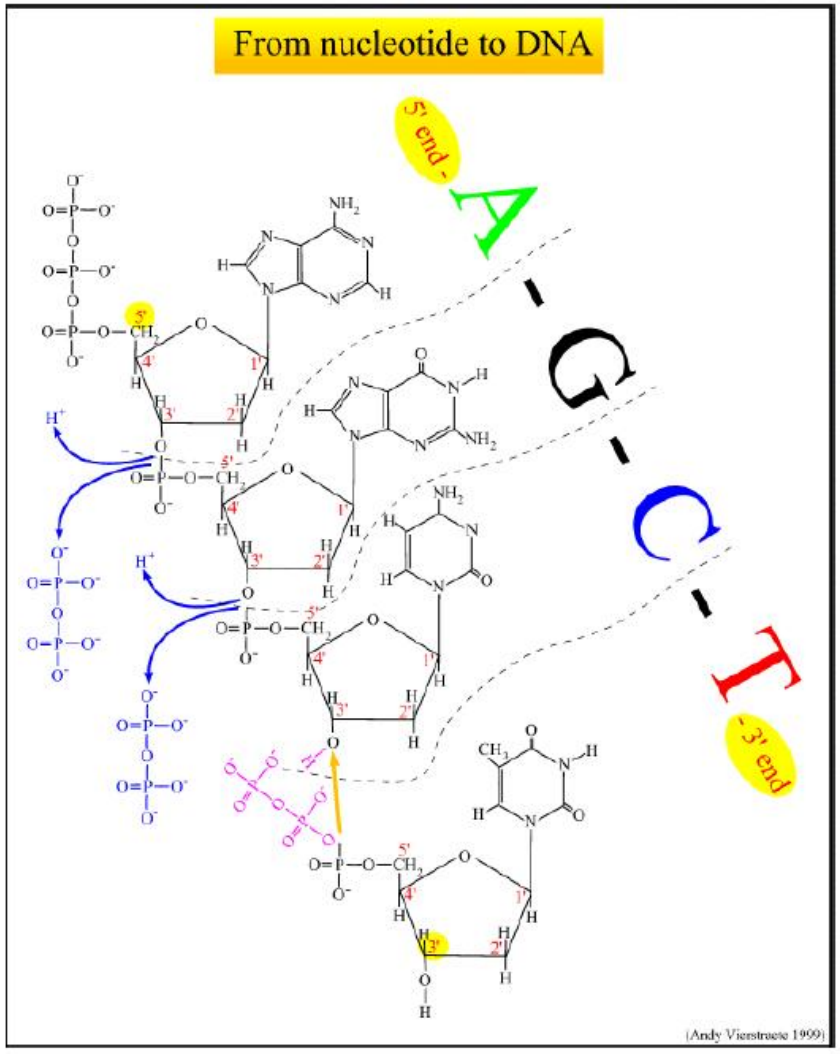
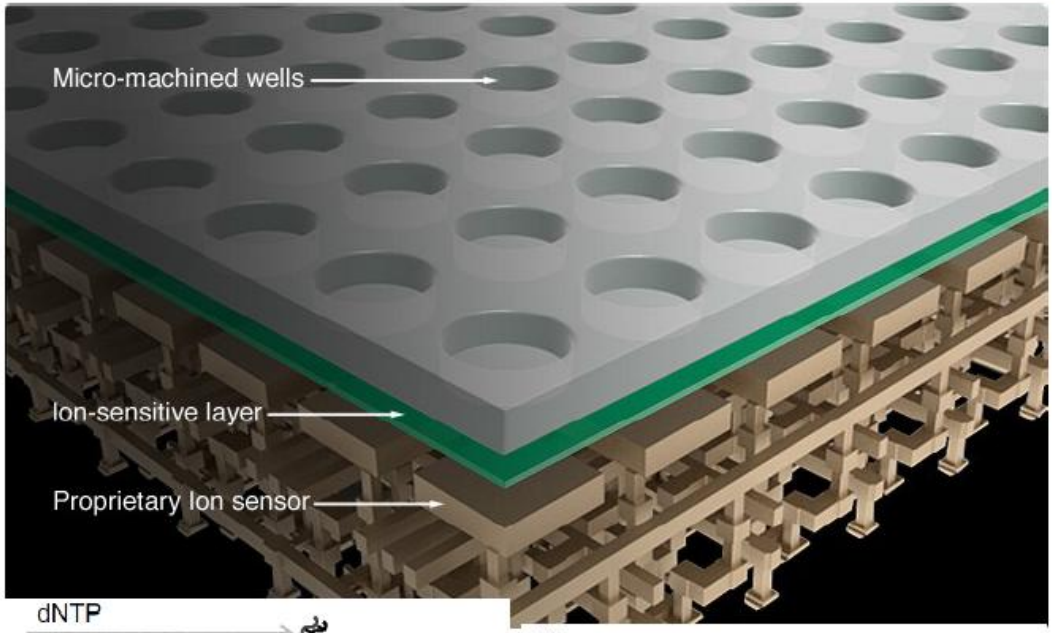
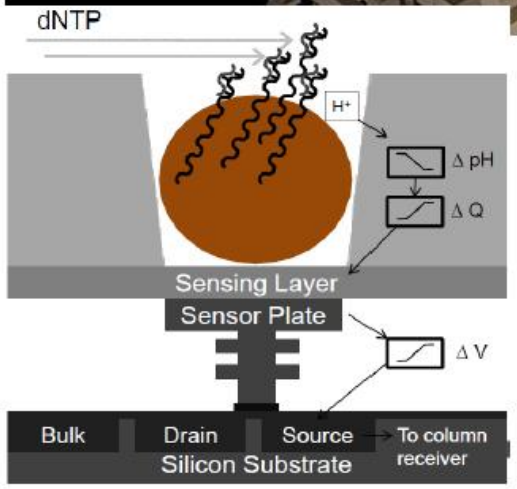
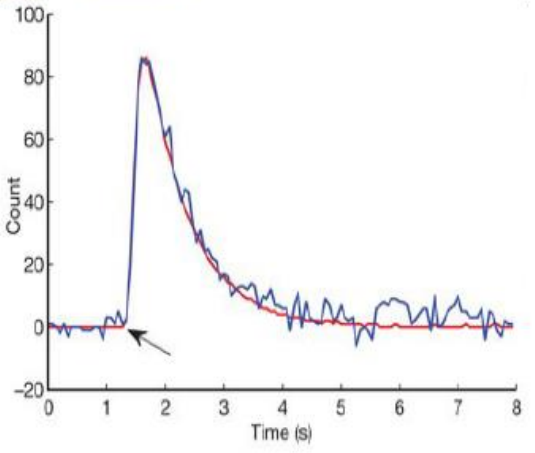
Next Generation Sequencing : Amplified Single Molecule Sequencing
Ion Torrent

Workflow : Library preparation → Emulsion PCR → Semiconductor Sequencing









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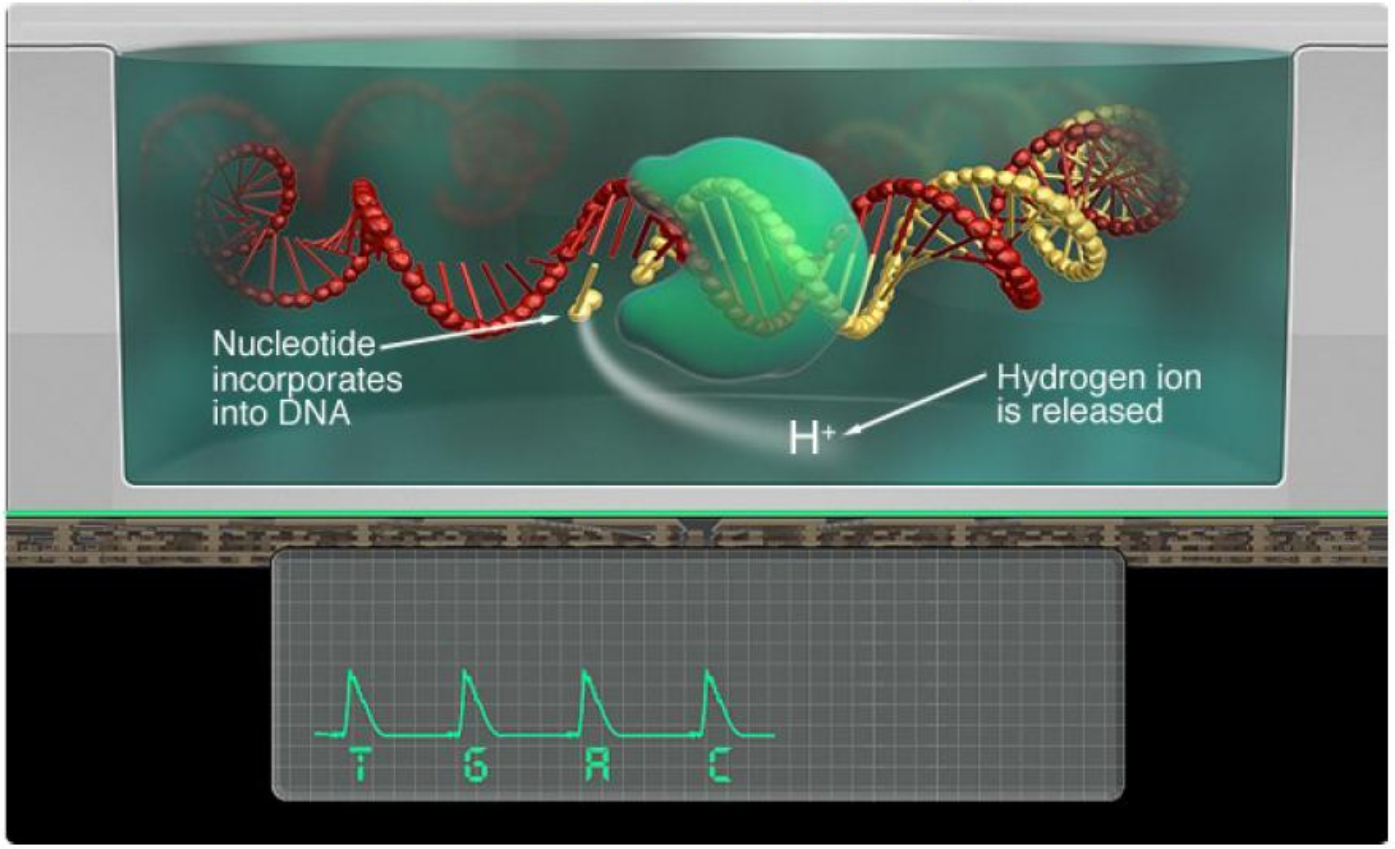
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent

4 nucleotides flow sequentially



No camera, just a pH sensor

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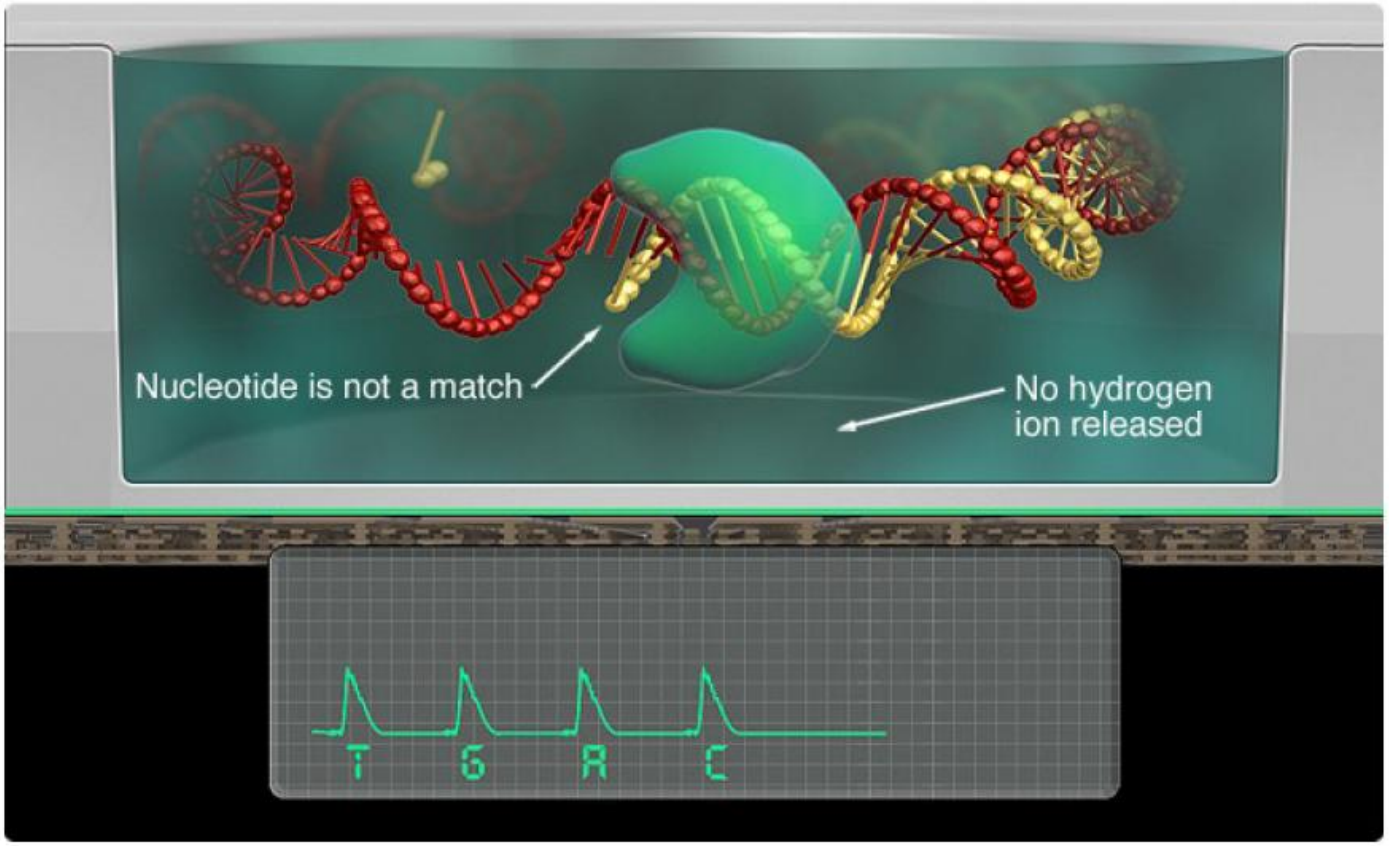
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent



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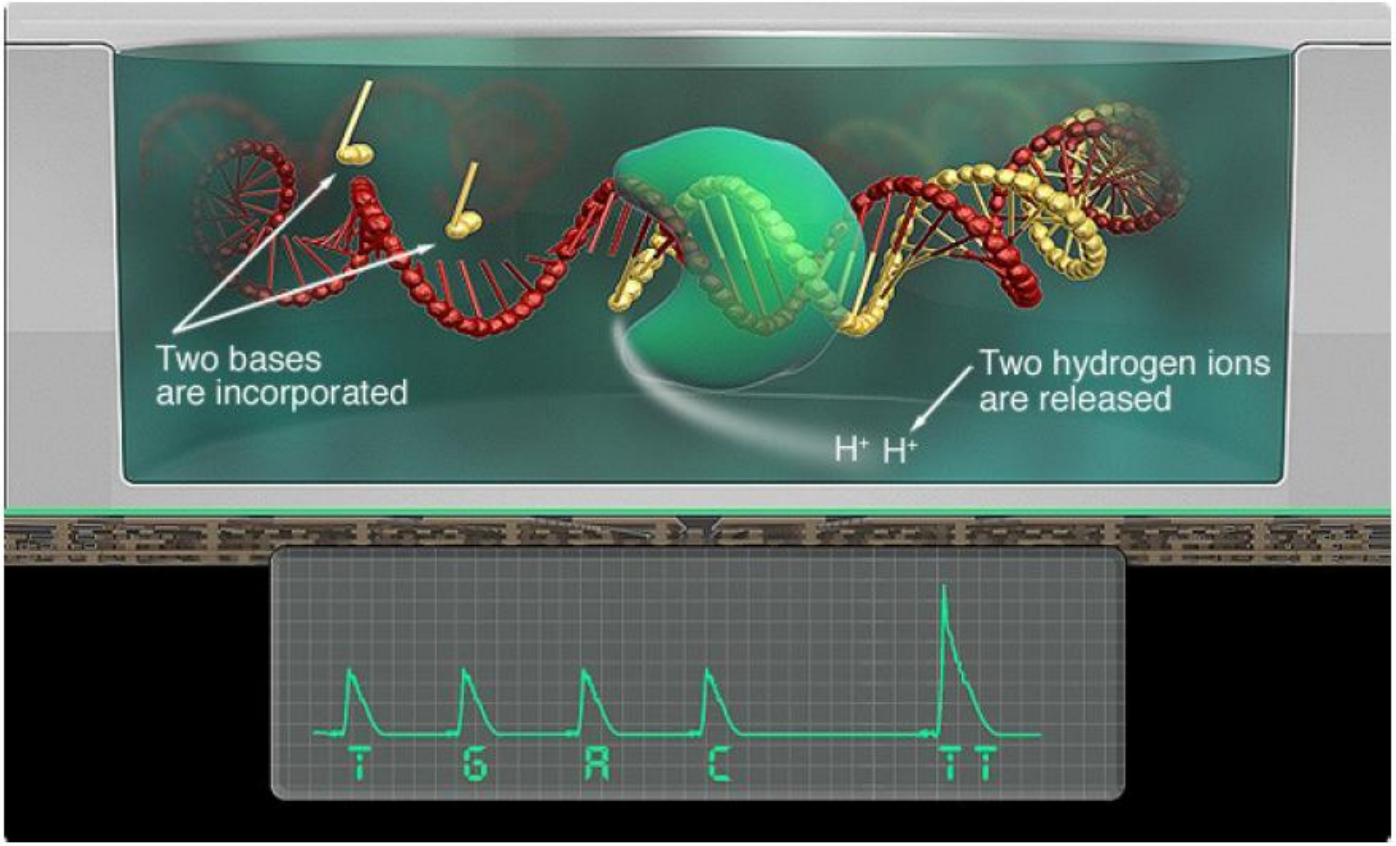
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent



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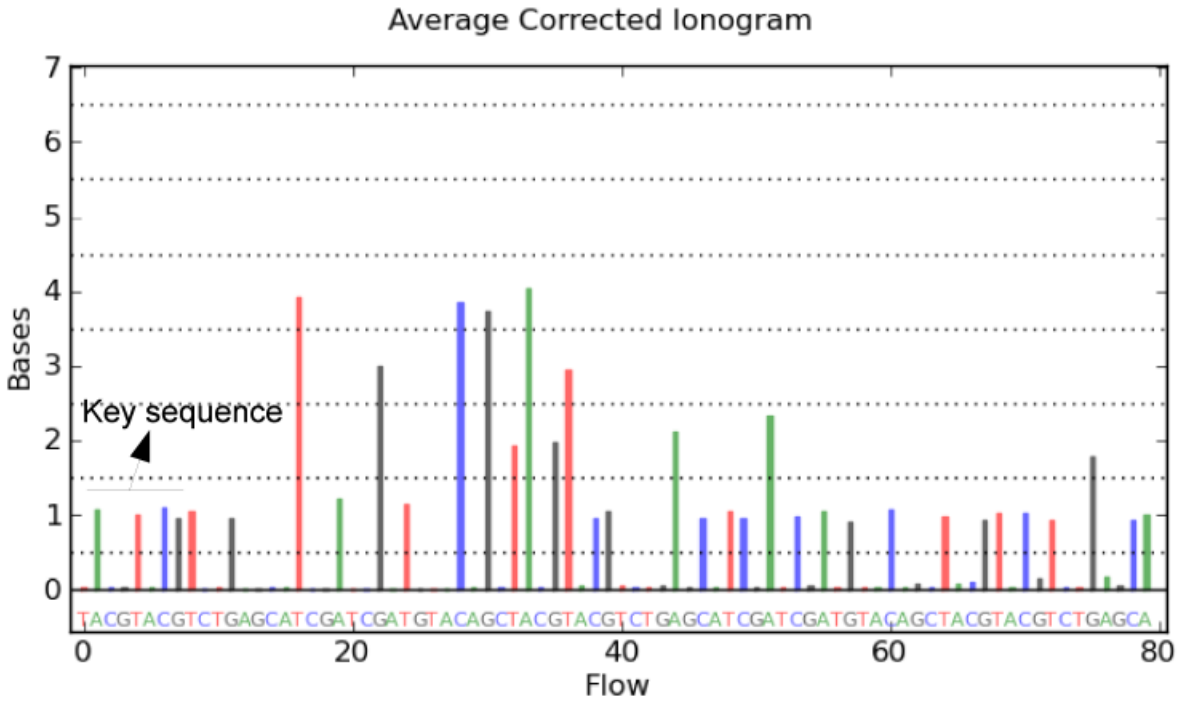
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent



ATCGTGTTTTAGGGTCCCCGGGGTT...

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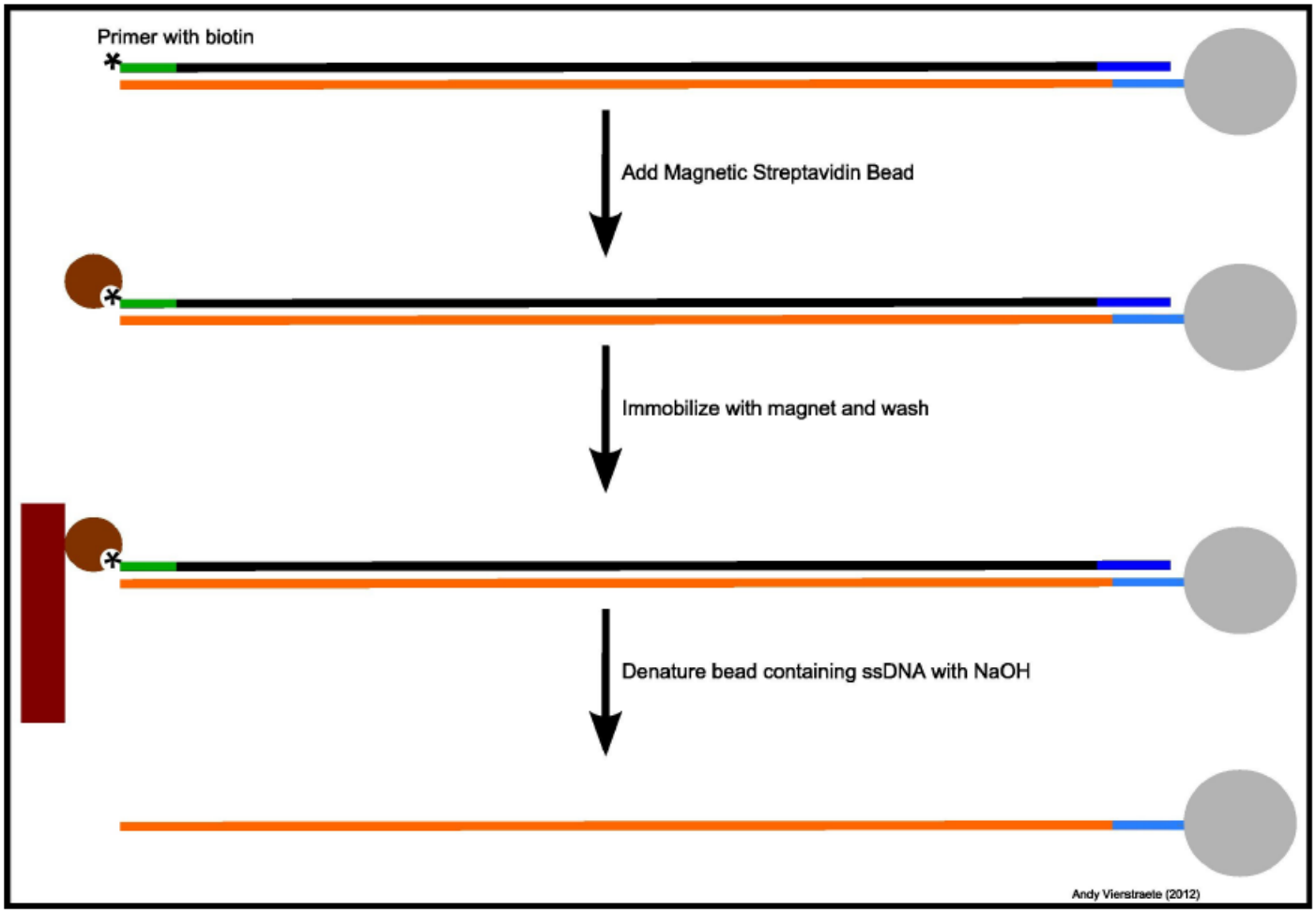
Next Generation Sequencing : Amplified Single Molecule Sequencing

Ion Torrent

Enrichment : select only the beads that contain DNA
-> maximizing sequencing yield



Different platforms

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- 454 Sequencing / Roche
  - GS Junior System
  - GS FLX+ System
- Illumina (Solexa)
  - HiSeq System
  - Genome analyzer IIx
  - MySeq
- Applied Biosystems - Life Technologies
  - SOLiD 5500 System
  - SOLiD 5500xl System
- Ion Torrent - Life Technologies
  - Personal Genome Machine (PGM)
  - Proton
- Helicos
  - Helicos Genetic Analysis System
- Pacific Biosciences
  - PacBio RS
- Oxford Nanopore Technologies
  - GridION System
  - MinION

Next Generation Sequencing
Amplified Single Molecule Sequencing

Third Generation Sequencing,
Next Next Generation Sequencing,
Single Molecule Sequencing

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Third Generation Sequencing : Single Molecule Sequencing

Helicos (BioSciences Corporation)

Helicos Genetic Analysis System

	Helicos
Read Length	35 bp
Throughput	35 Gb
Reads per run	600,000,000 - 1,000,000,000
Accuracy	97 %
Run Time	8 days

Workflow : Library preparation → Sequencing

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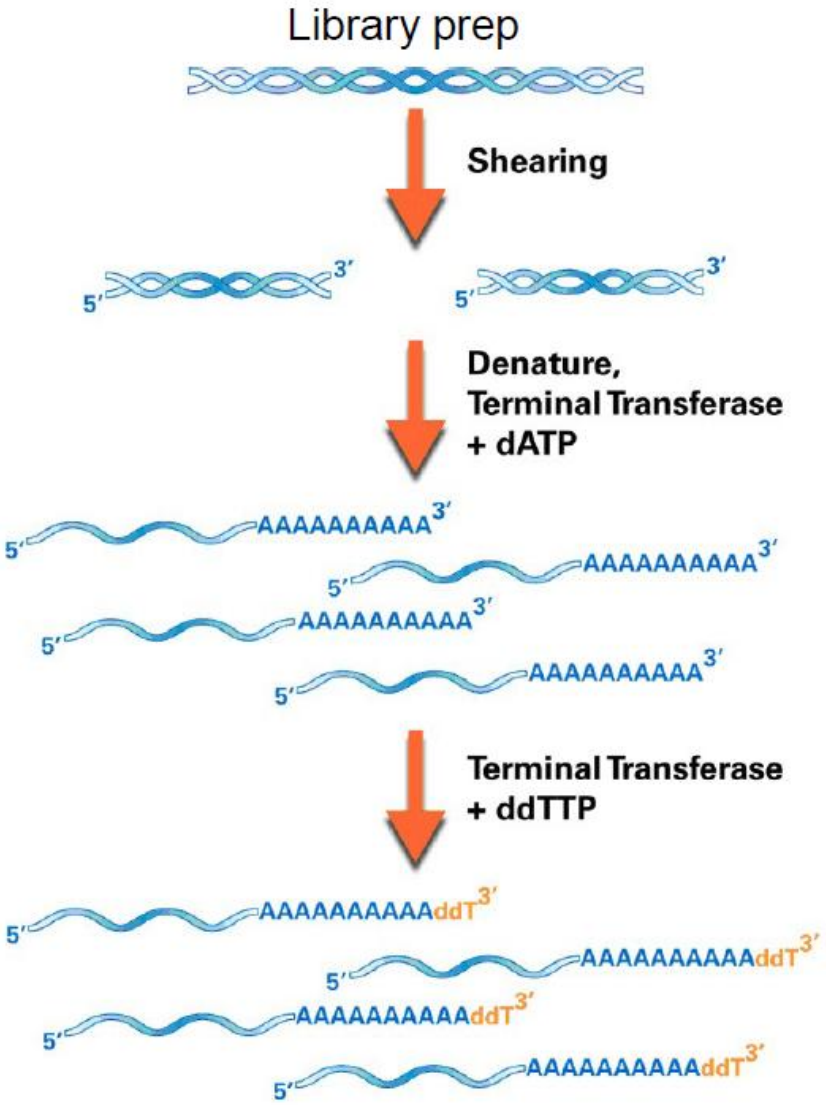
Third Generation Sequencing : Single Molecule Sequencing

Helicos



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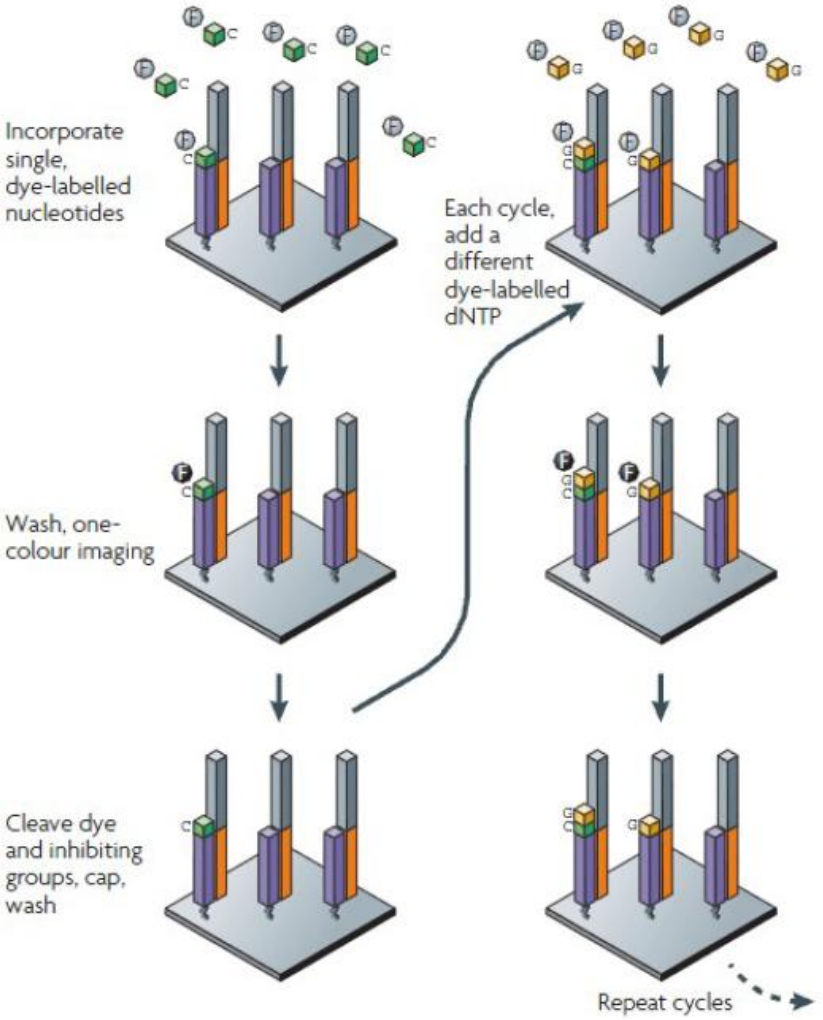
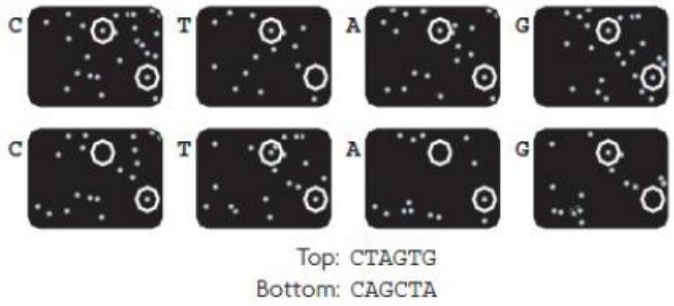
Third Generation Sequencing : Single Molecule Sequencing

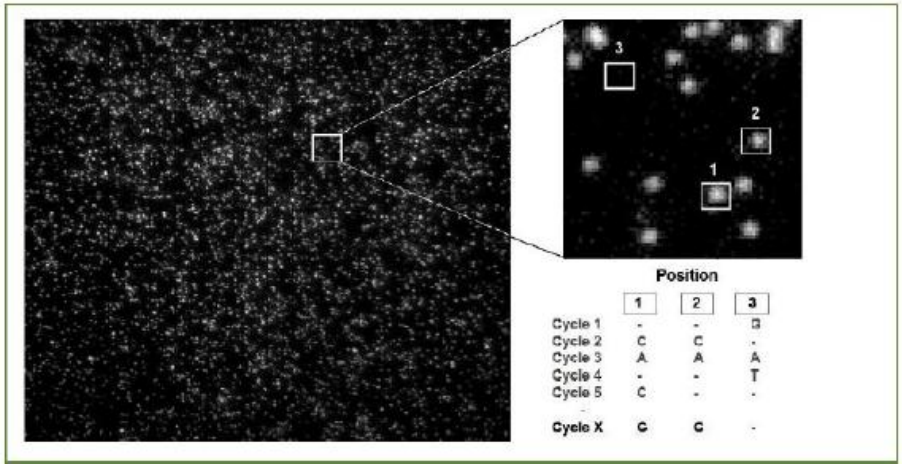
Helicos





Nucleotides flown sequentially

(Dark nucleotides : incorporation not detected)



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Third Generation Sequencing : Single Molecule Sequencing

Helicos

Advantages Single molecule sequencing :

- Less sample preparation (no PCR)
- No amplification
  - -> no PCR errors
  - -> fewer contamination issues
  - -> no GC-bias
  - -> analyze every sample (unPCRable / unclonable)
  - -> analyze low quality DNA (museum, archeological, forensics samples)
- Absolute quantification
- Sequence RNA directly

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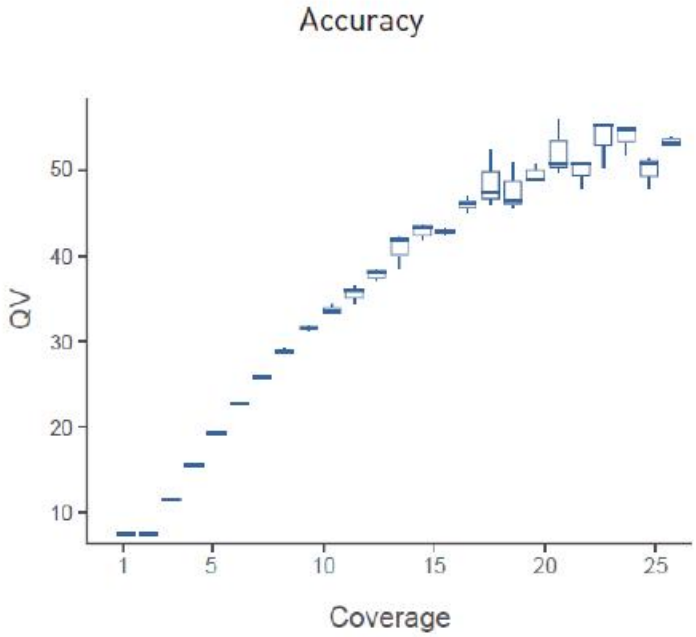
Third Generation Sequencing : Single Molecule Sequencing

Pacific Biosciences

Pacbio RS



	Pacbio RS
Read Length	3000 - 15,000 bp
Throughput	1 Gb
Reads per run	70,000
Accuracy	95 %
Run Time	30 minutes

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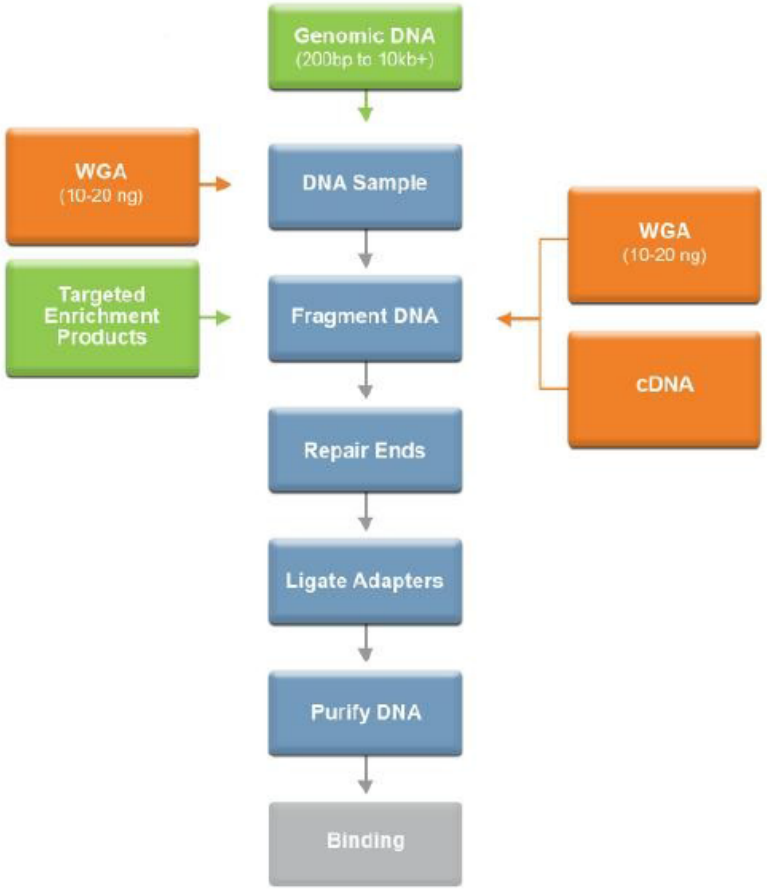
Third Generation Sequencing : Single Molecule Sequencing

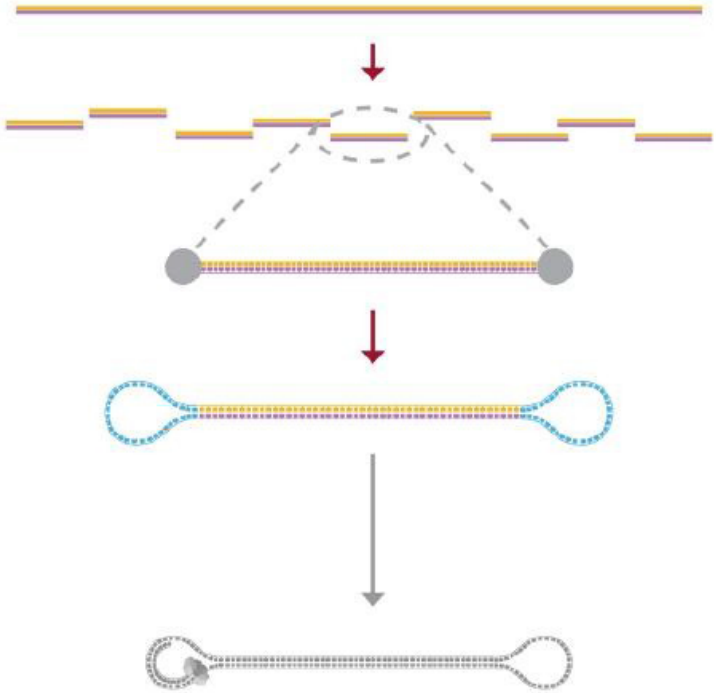
Pacific Biosciences

Workflow : Library preparation → Sequencing

Sample Preparation



Building of SMRTbell



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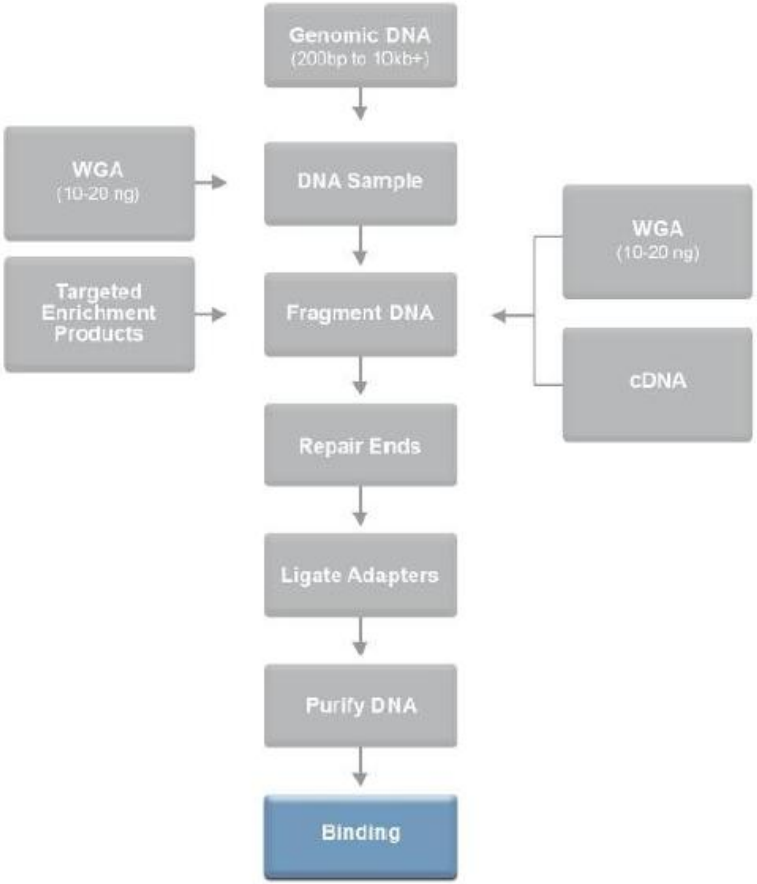
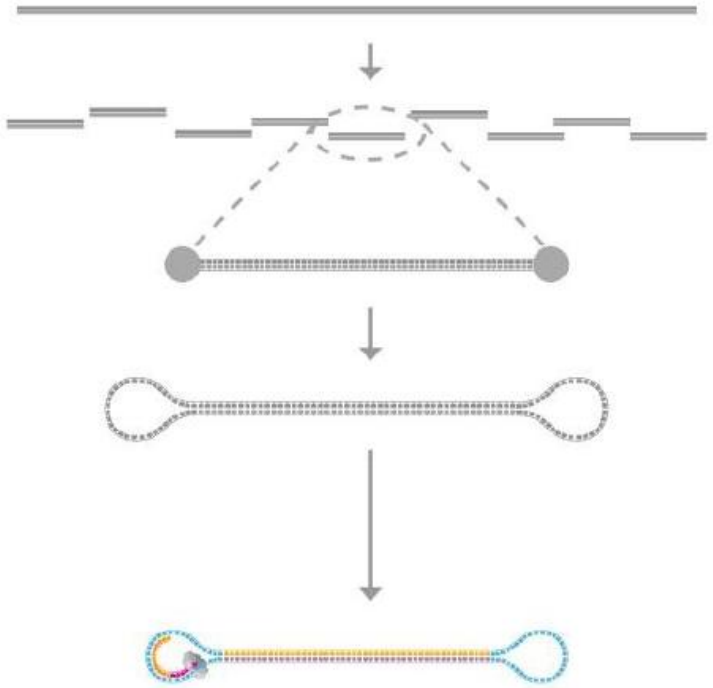
Third Generation Sequencing : Single Molecule Sequencing

Pacific Biosciences

Sample Preparation



Building of SMRTbell



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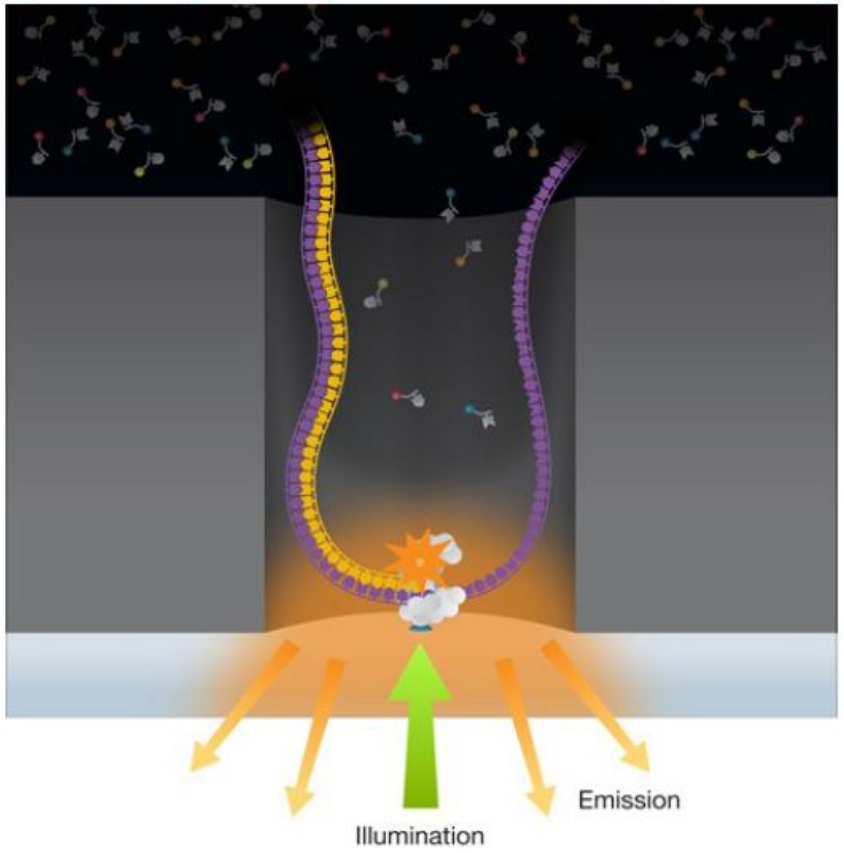
Third Generation Sequencing : Single Molecule Sequencing

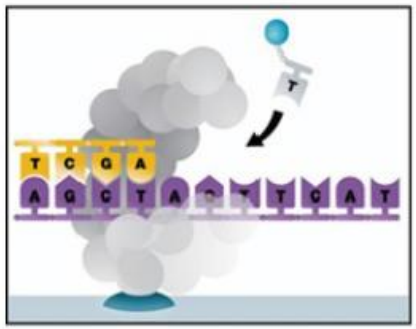
Pacific Biosciences

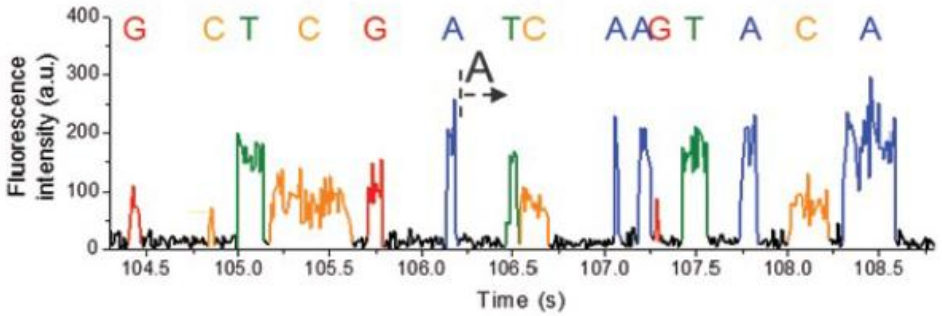


4 nucleotides with different fluorescent dye simultaneous present

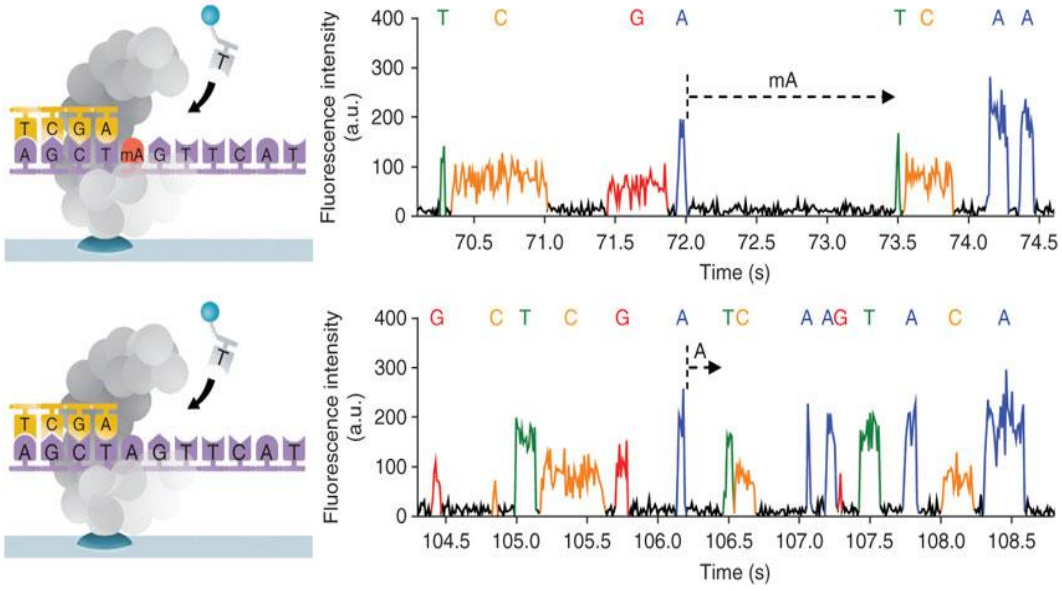
2-3 nucleotides/sec
2-3 Kb (up to 50) read length
6 TB data in 30 minutes

laser damages polymerase





Single molecule real time (SMRT) DNA sequencing allows evaluation of the methylome.



(Flußberg, 2010)

Fluorescence intensity shows incorporated base.

Time delay demonstrates Methylation.

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Ghent University. June 2012

Third Generation Sequencing : Single Molecule Sequencing

Pacific Biosciences

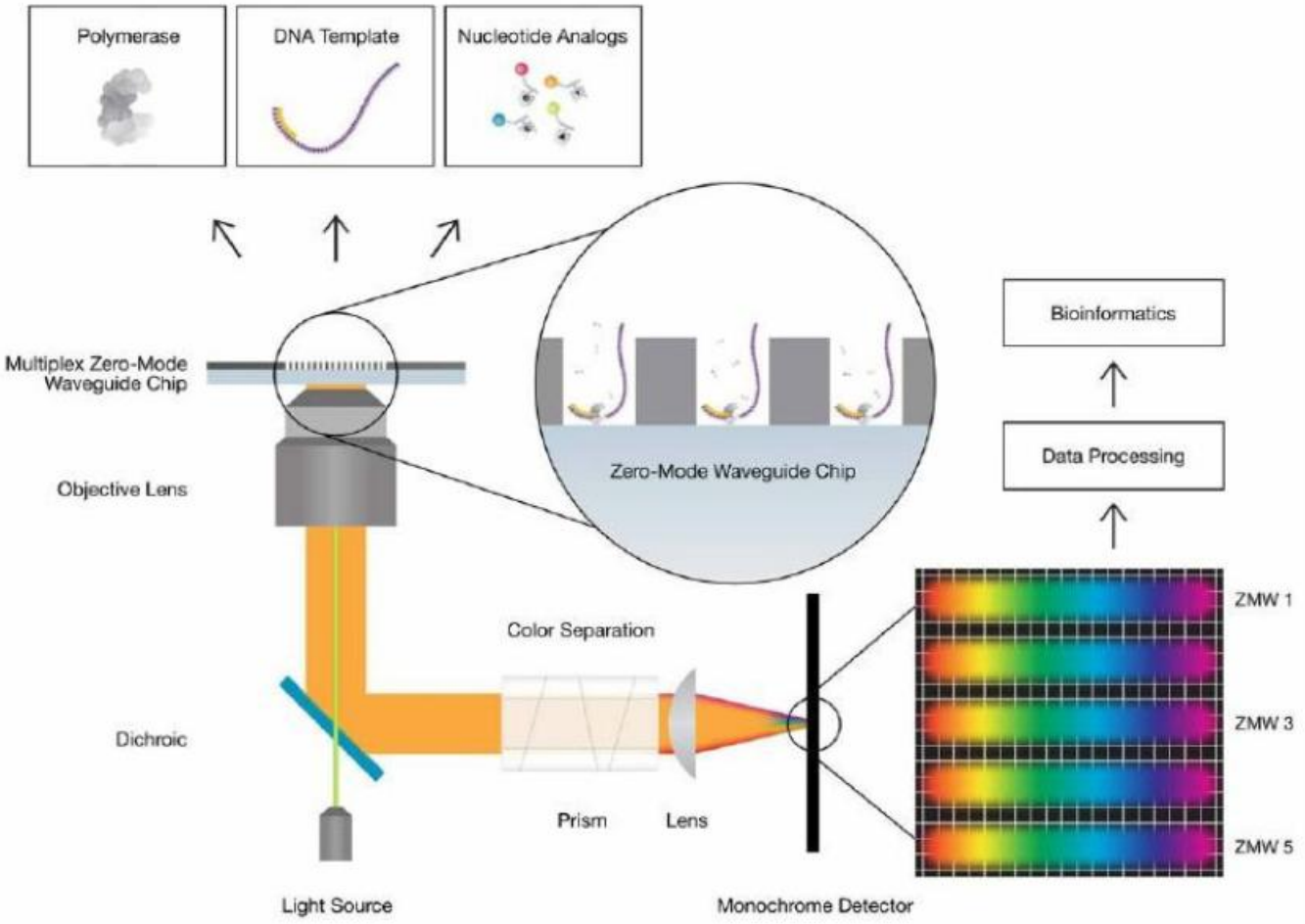

Figure 13. Highly parallel optics system.

The detected flash of light is separated into a spatial array, from which the identity of the incorporated base is determined.

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Third Generation Sequencing : Single Molecule Sequencing

Oxford Nanopore

Single use cartridge

GridION system

MinION

	Nanopore
Read Length	48 kb ?
Throughput	? Gb
Reads per run	2000
Accuracy	75 %
Run Time	? minutes

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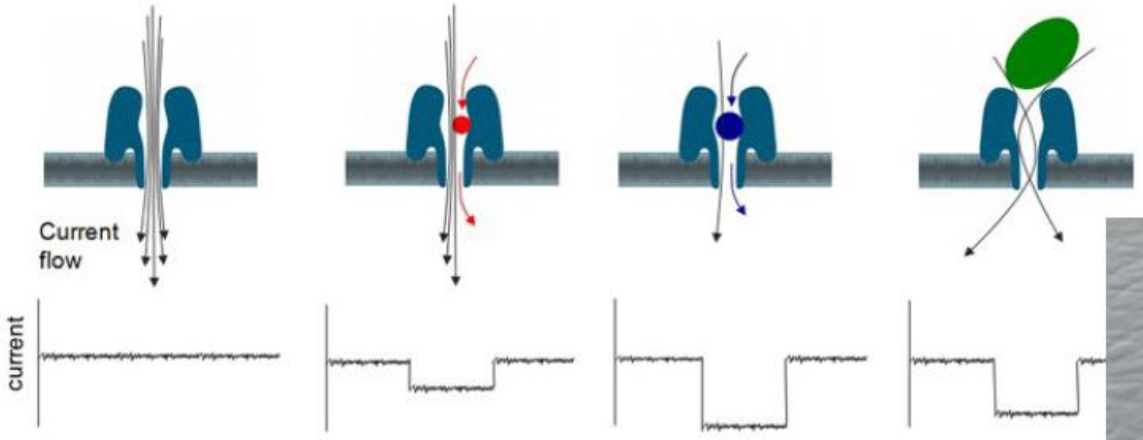
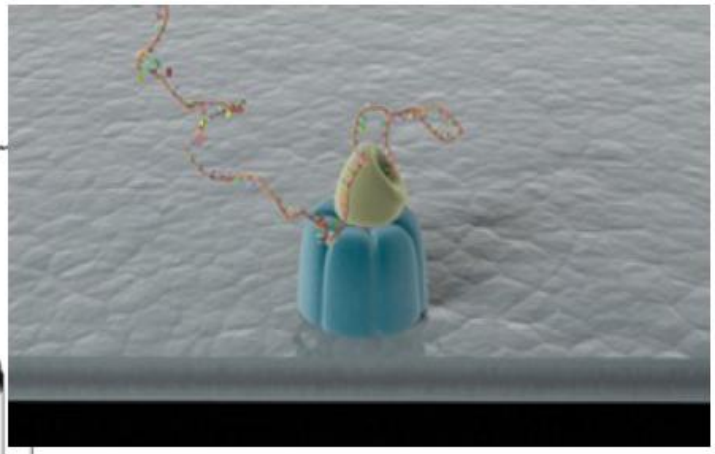
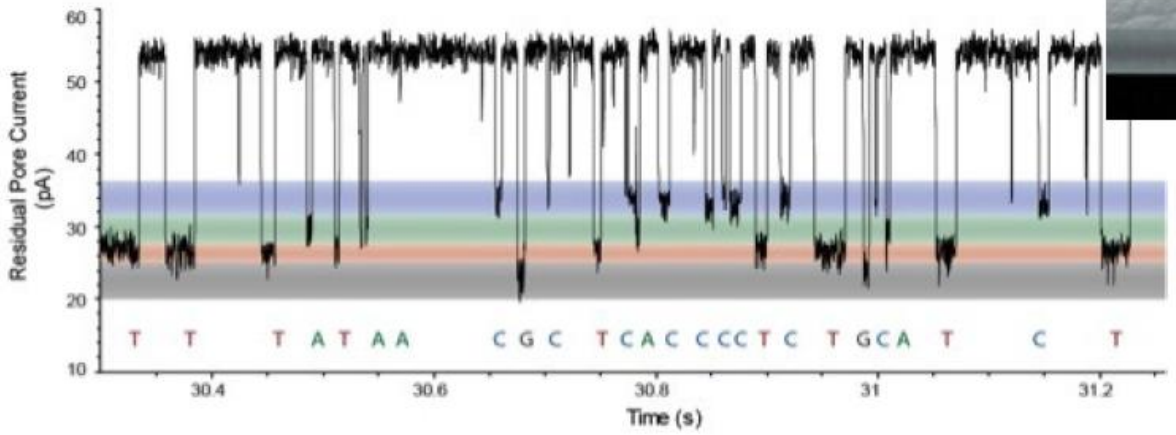
Third Generation Sequencing : Single Molecule Sequencing

Oxford Nanopore







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Department of Biology,
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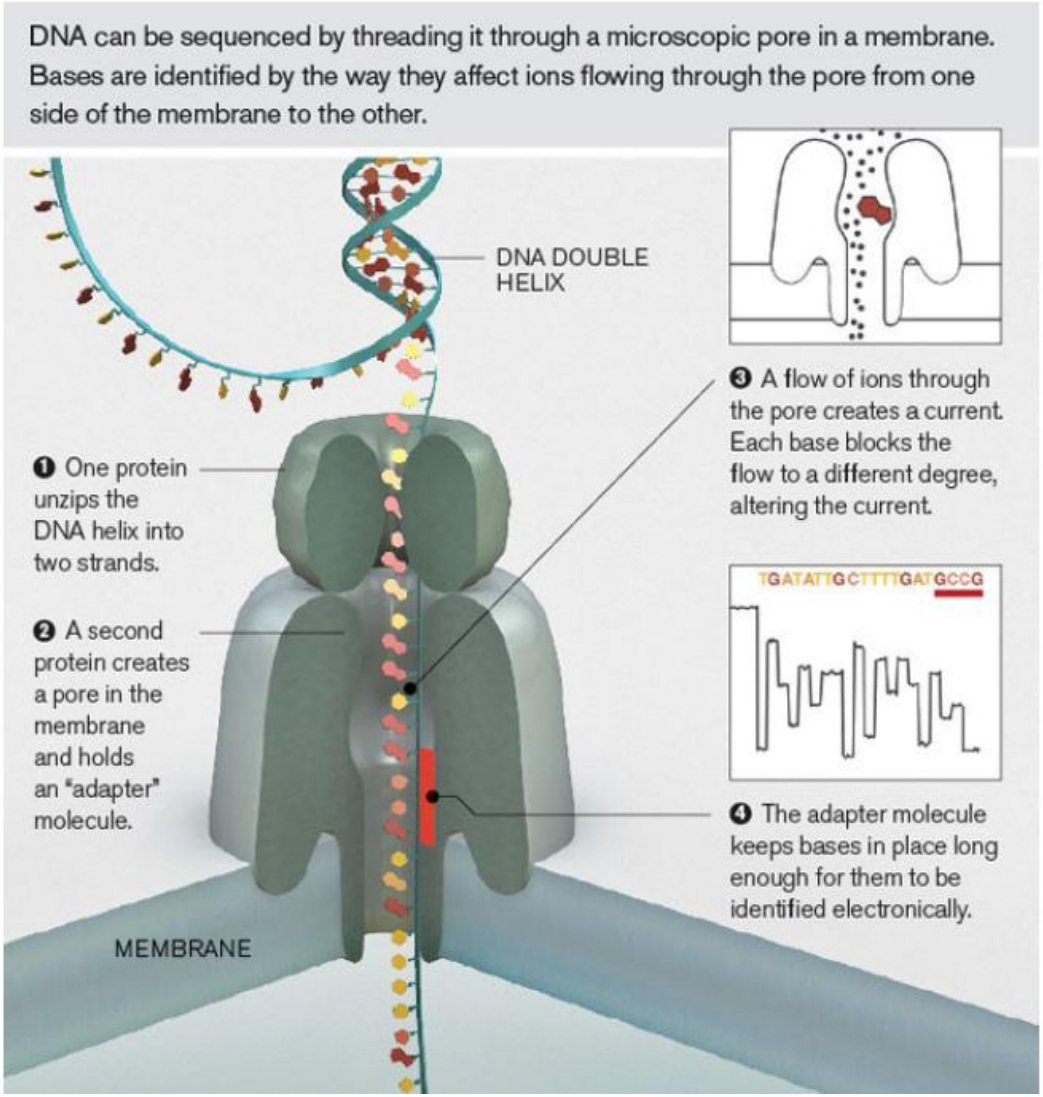
Third Generation Sequencing : Single Molecule Sequencing

Oxford Nanopore



Different platforms

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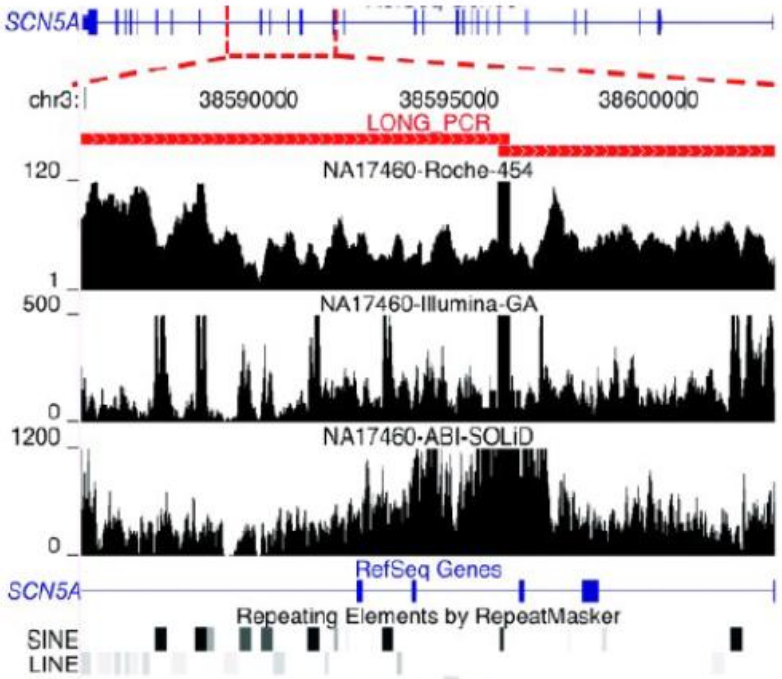
Which Next Generation Sequencer to choose for your project ?

	Capacity	Speed	Read Length	Homopolymers	Cost/run	Amplification
454 Roche	35-700 Mb	10-23 hours	400-700 bp	-	5.000 €	Yes
SOLiD	90-180 Gb	7-12 days	75 bp	+	5.000 €	Yes
Illumina	6-600 Gb	2-14 days	100-250 bp	+	10.000-20.000 €	Yes
Ion Torrent	20 Mb- 1Gb	4,5 hours	200 bp	-	1.000-2.000 €	Yes
Helicos	35 Gb	8 days	35 bp	+	20.000 €	No
PacBio	1Gb	30 minutes	3000 bp	+	600-800 €	No

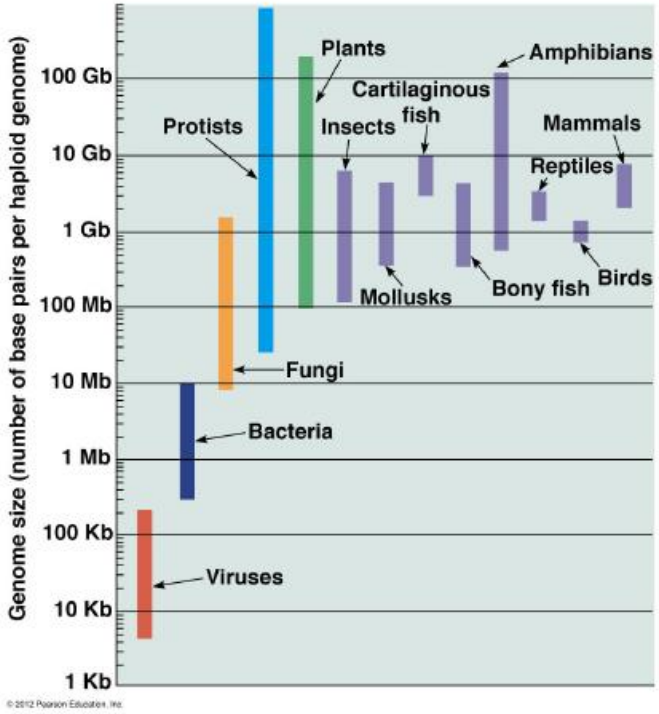
Roche →

Illumina →

SOLID →



Harismendy et al., Genome Biol. 2009



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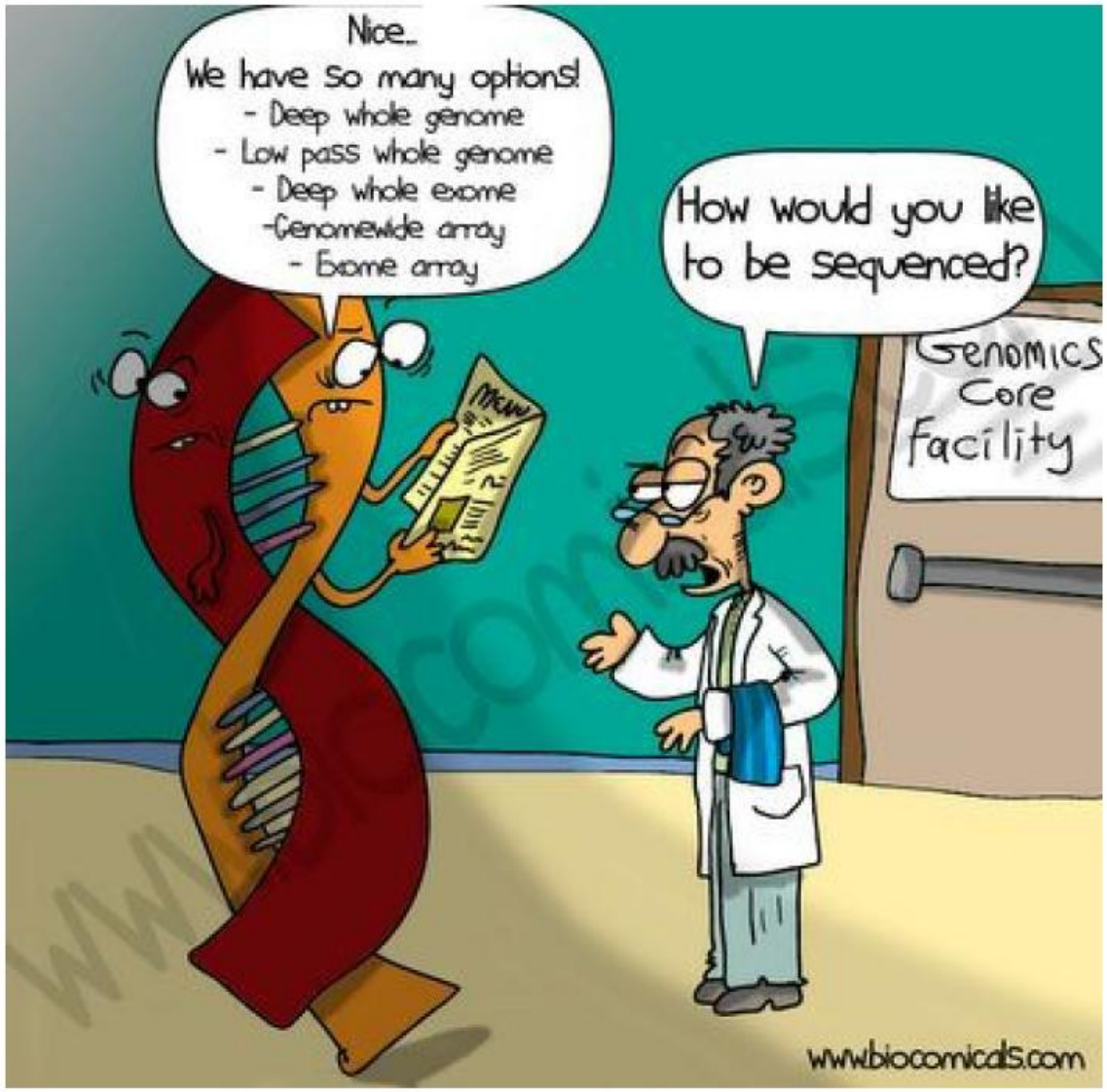

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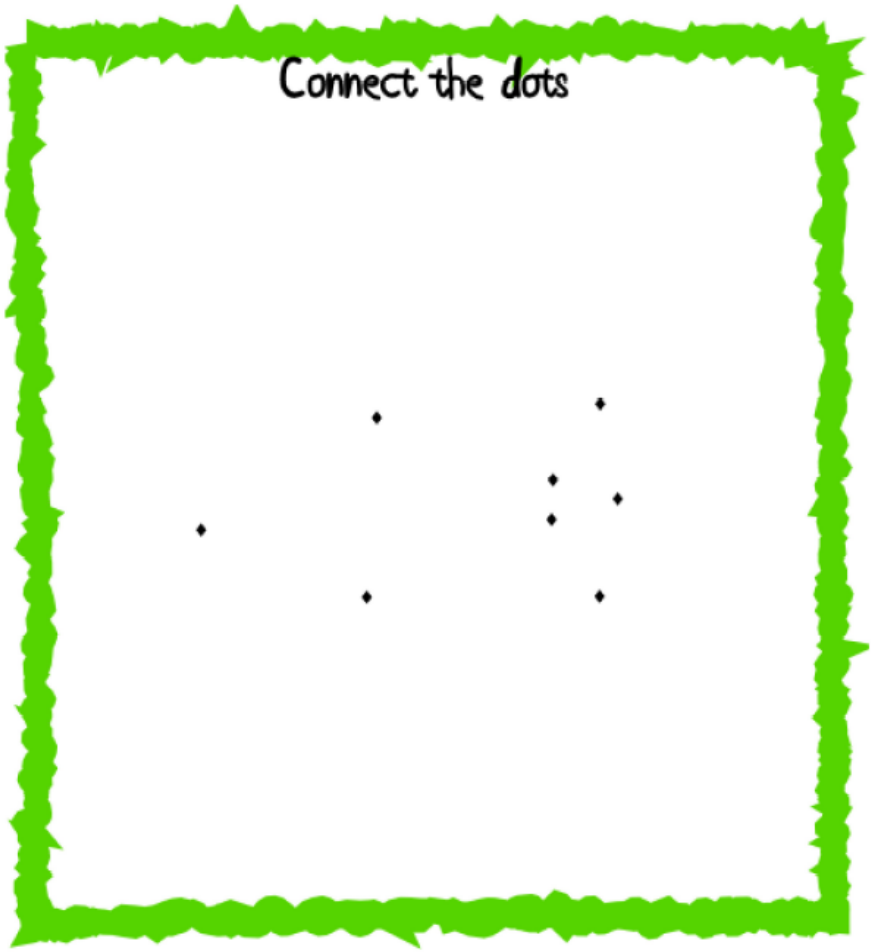
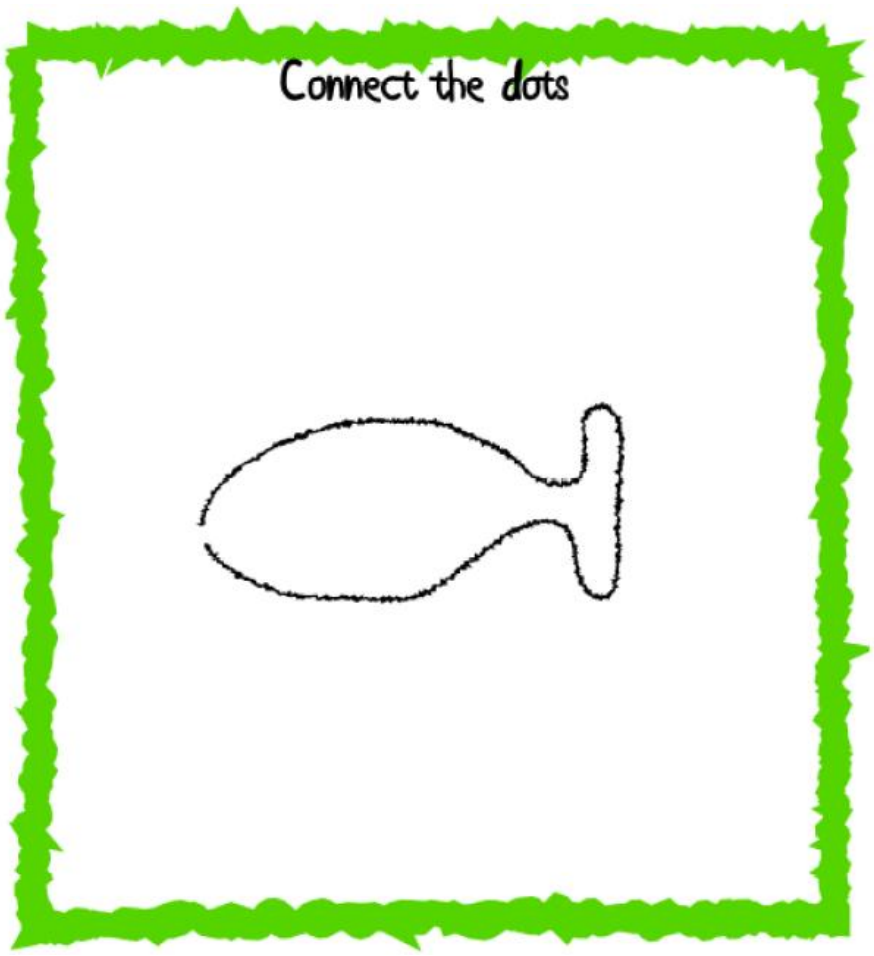
Sanger sequencing : simplified :





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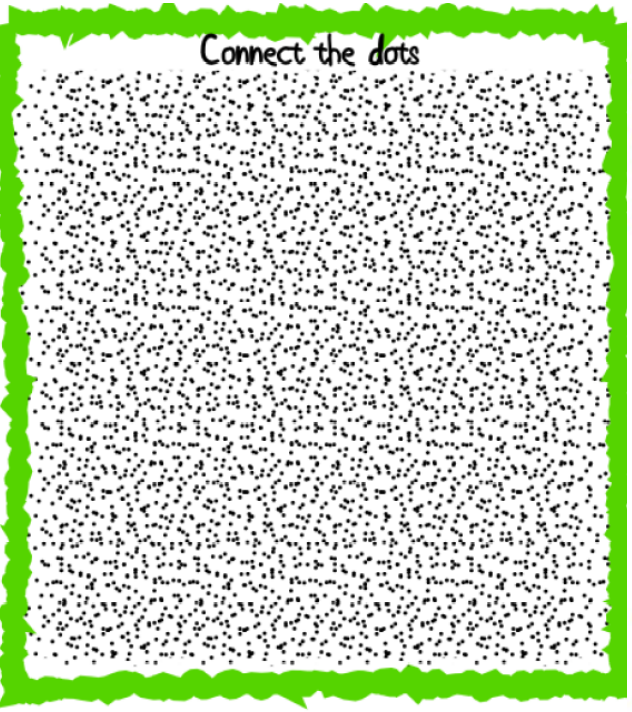
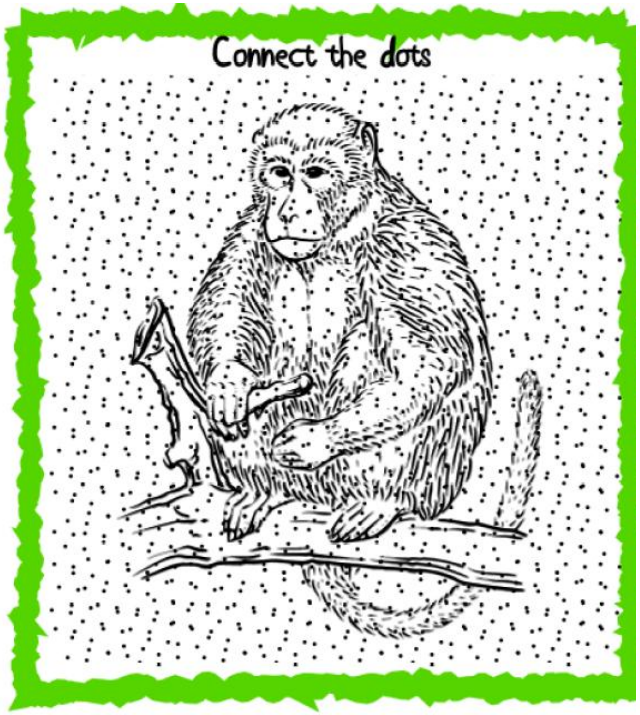
Next Generation sequencing : simplified :



Impossible to assemble manually



Same dataset, different parameters

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Department of Biology,
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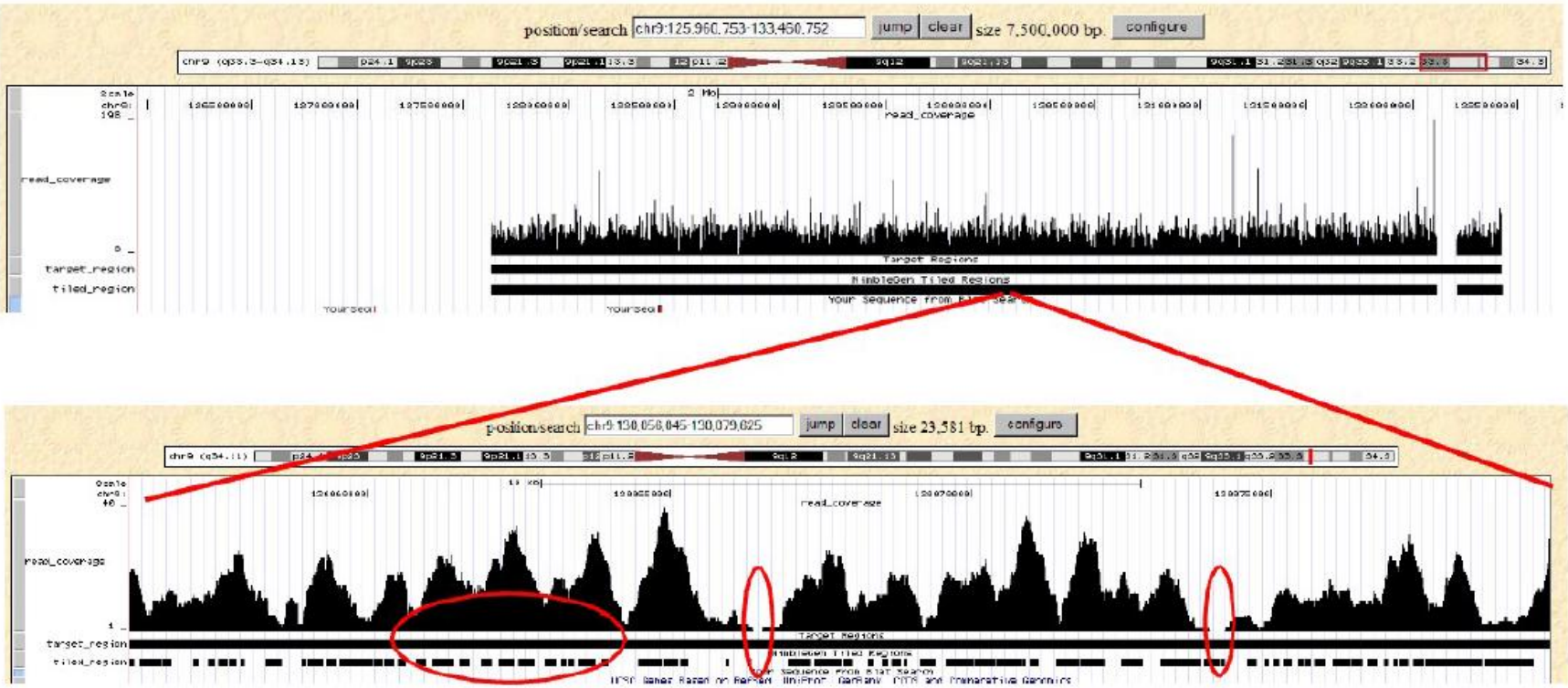
Next Generation sequencing : Impossible to check manually



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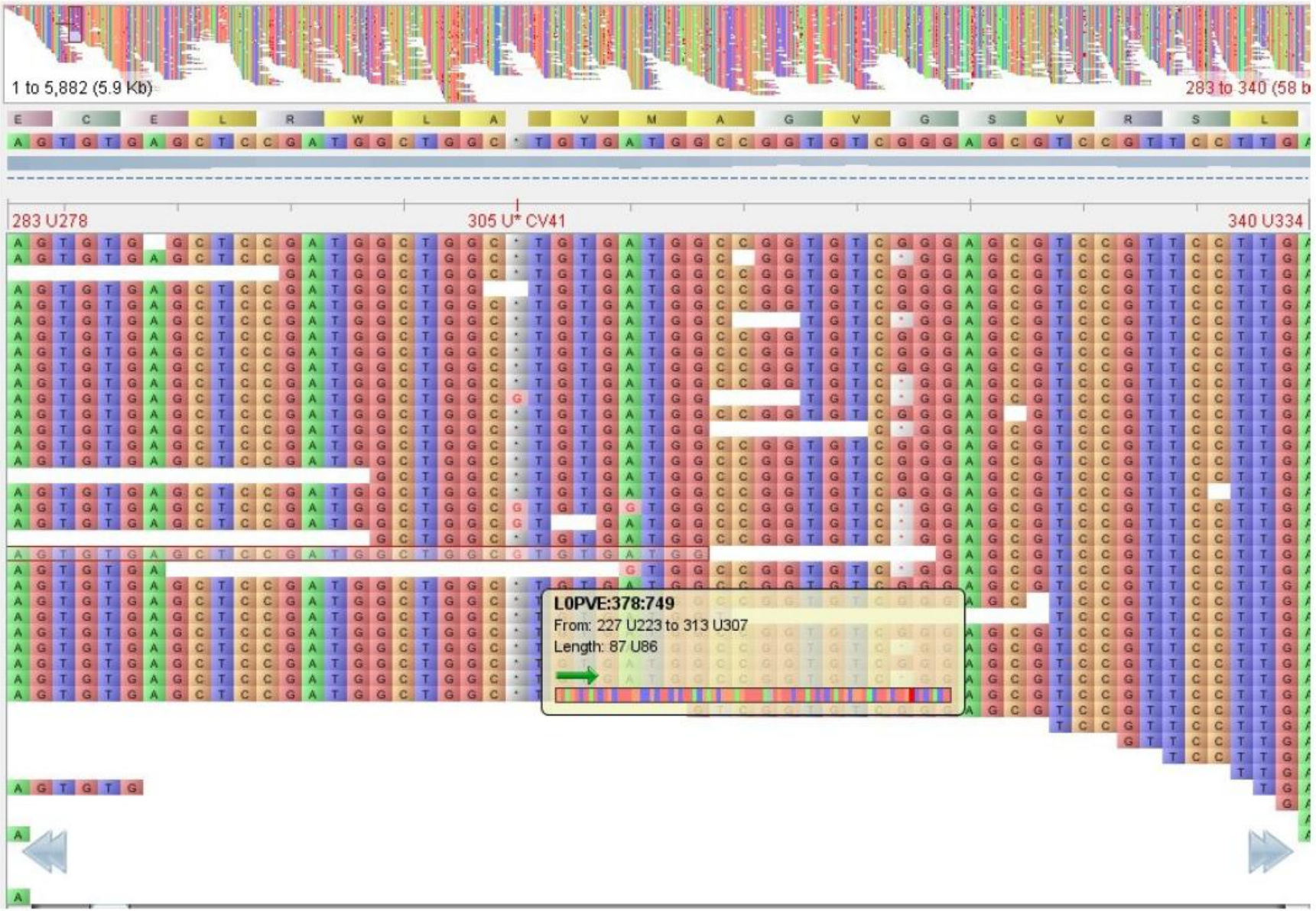
Next Generation sequencing : Assembly of the largest contig



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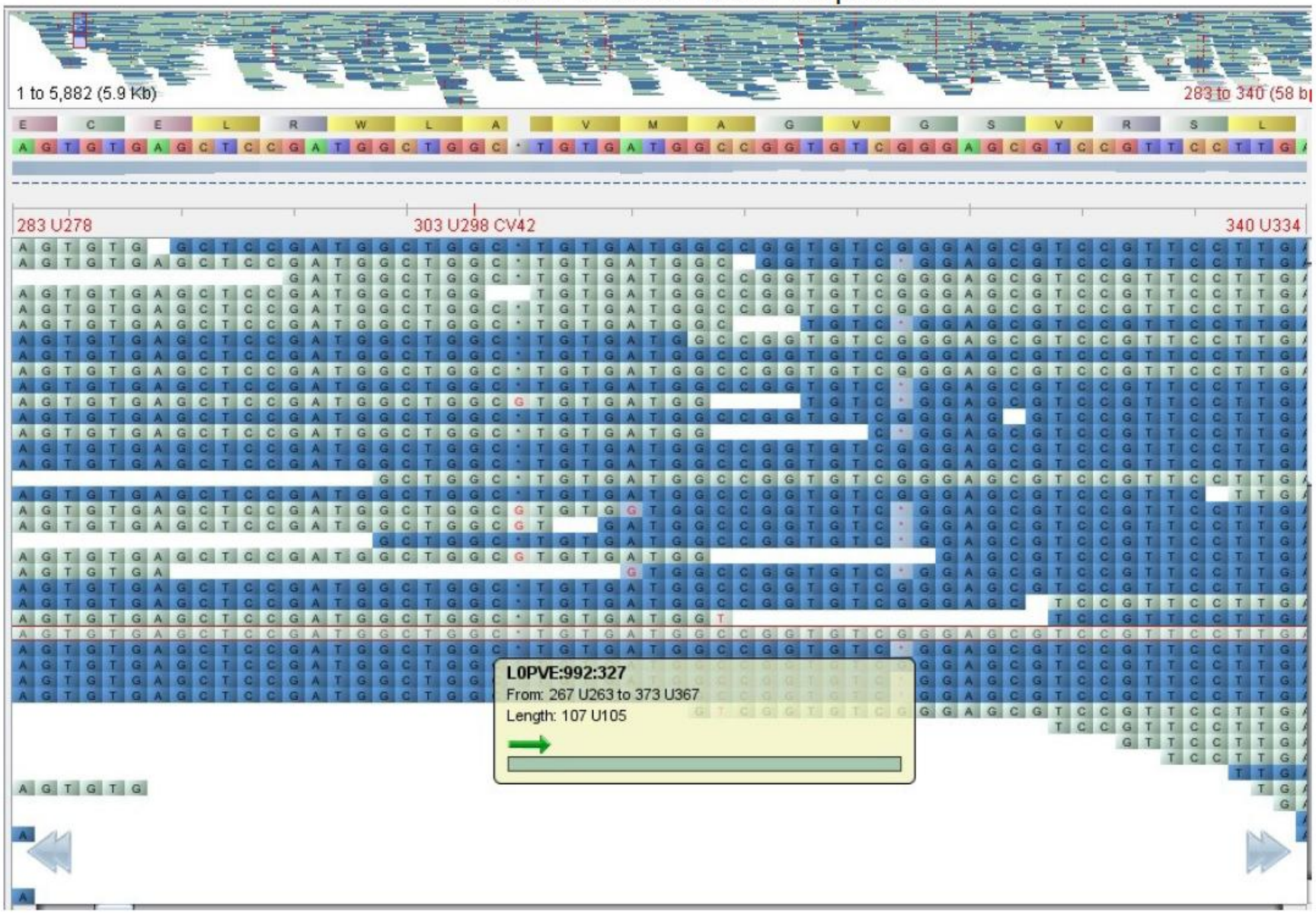
Next Generation sequencing :

Light blue : forward
dark blue : reverse sequence



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Department of Biology,
Ghent University. June 2012

Next Generation sequencing :

CV : coverage of a specific part

Data analyses

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Department of Biology,
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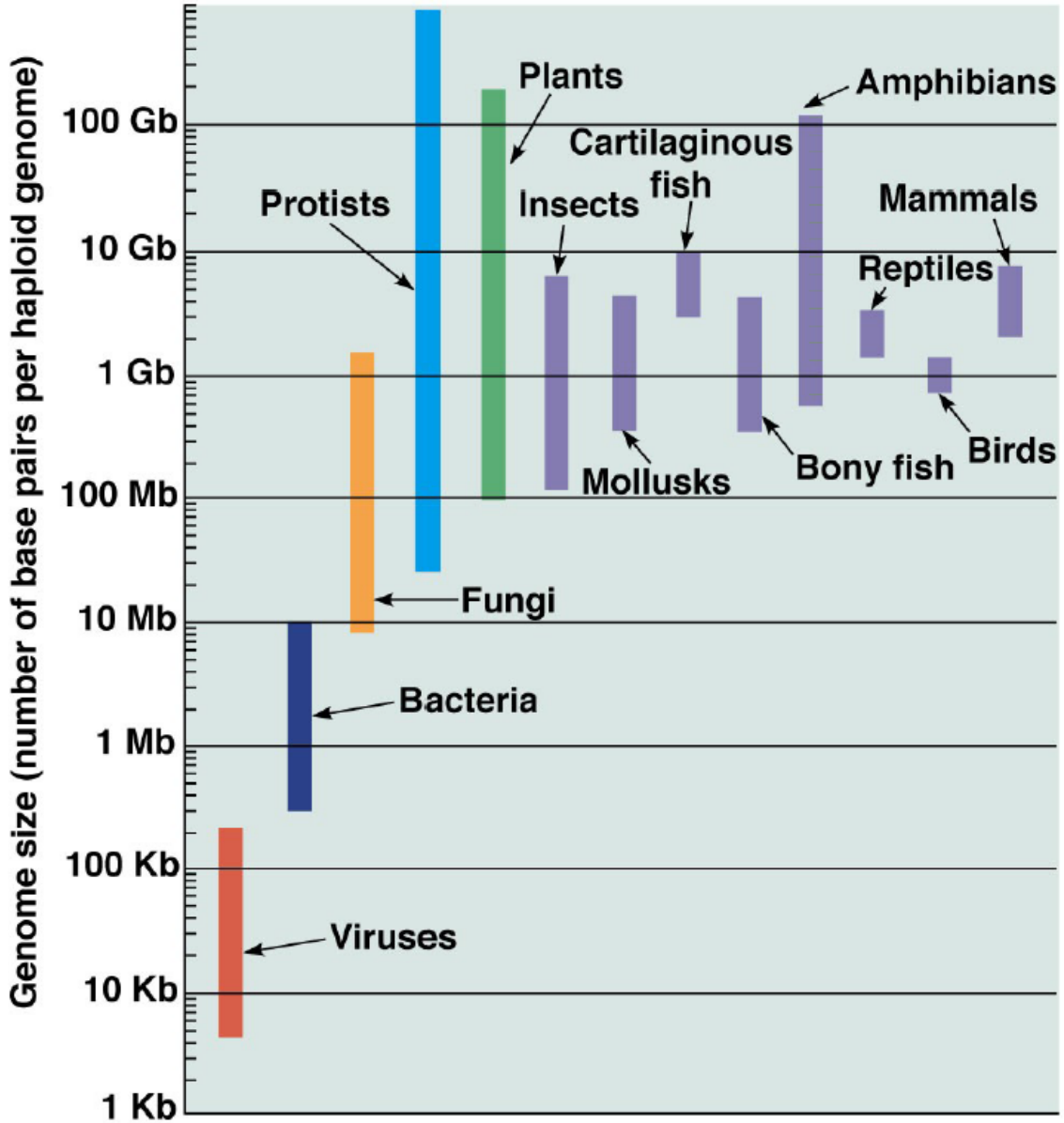

One SOLiD run : 90 Gb (gigabases)
-> 200 GB (gigabytes) of raw data
-> mapping to reference :
4 h on 250 cores server

1 Gb genome, 15 x coverage =
15 Gbases to assemble or
to map to a reference !

Total DNA sequencing :
1x gDNA
100x mDNA

Thanks for your interest !

http://users.ugent.be/~avierstr/

CTAGGTAGCTAGTCG
GCTLIFECISGATAG
C4-LETTERTWORDT
GCTATATCGTAGCTG

WWW.DNA.UGENT.BE