Introduction to NGS data

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Why do sequencing?

Microarrays vs sequencing

- Probe design issues with microarrays
  - *'Dorian Gray effect'* http://www.biomedcentral.com/1471-2105/5/111 (http://www.biomedcentral.com/1471-2105/5/111)
  - '…mappings are frozen, as a Dorian Gray-like syndrome: the apparent eternal youth of the mapping does not reflect that somewhere the 'picture of it' decays'
- Sequencing data are 'future proof'
  - if a new genome version comes along, just re-align the data!
  - can grab published-data from public repositories and re-align to **your** own choice of genome / transcripts and aligner
- Limited number of novel findings from microarays
  - can't find what you're not looking for!
- Genome coverage
  - some areas of genome are problematic to design probes for
- Maturity of analysis techniques
  - on the other hand, analysis methods and workflows for microarrays are well-established
  - until recently…

The cost of sequencing

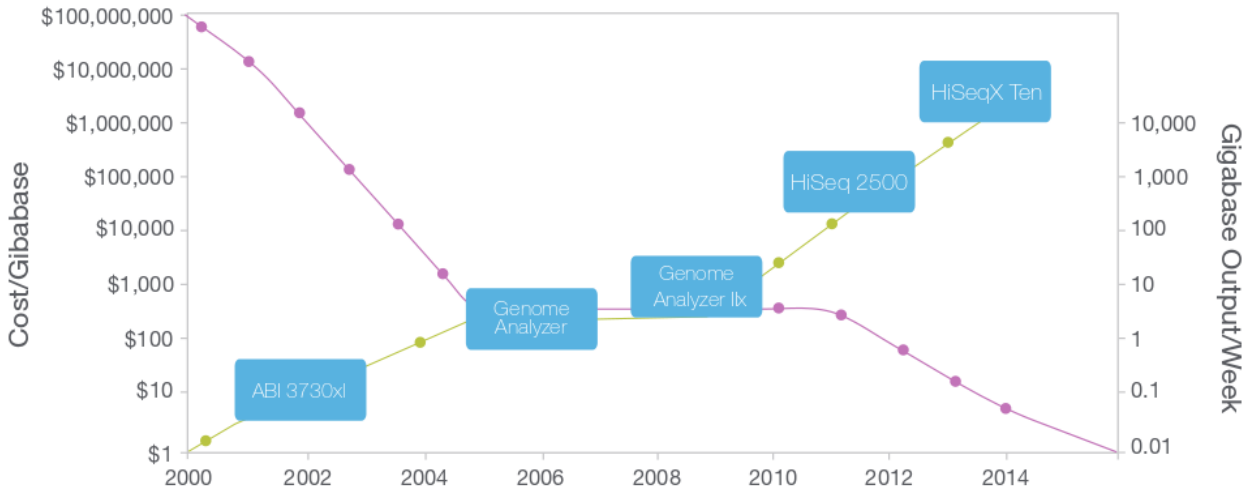

Figure 1: Sequencing Cost and Data Output Since 2000—The dramatic rise of data output and concurrent falling cost of sequencing since 2000. The Y-axes on both sides of the graph are logarithmic.

Reports of the death of microarrays

nature International weekly journal of science

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Published online 15 October 2008 | *Nature* **455**, 847 (2008) | doi:10.1038/455847a

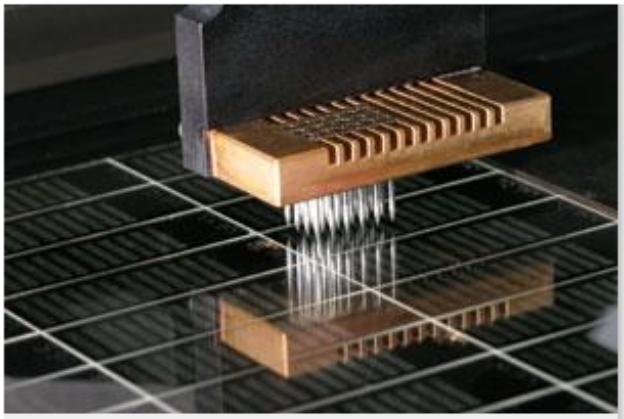
News

The death of microarrays?

High-throughput gene sequencing seems to be stealing a march on microarrays. Heidi Ledford looks at a genome technology facing intense competition.

Heidi Ledford

Faster, cheaper DNA sequencing technology is revolutionizing the burgeoning field of personal genomics. But it is having another, more subtle effect. Scientists are using the sequencers to tackle a wide range of research applications, including monitoring gene expression, mapping where proteins bind to the



DNA is deposited on a glass slide to make a microarray — but is the technology losing its allure?

P. DUMAS/EURELIOS/SPL

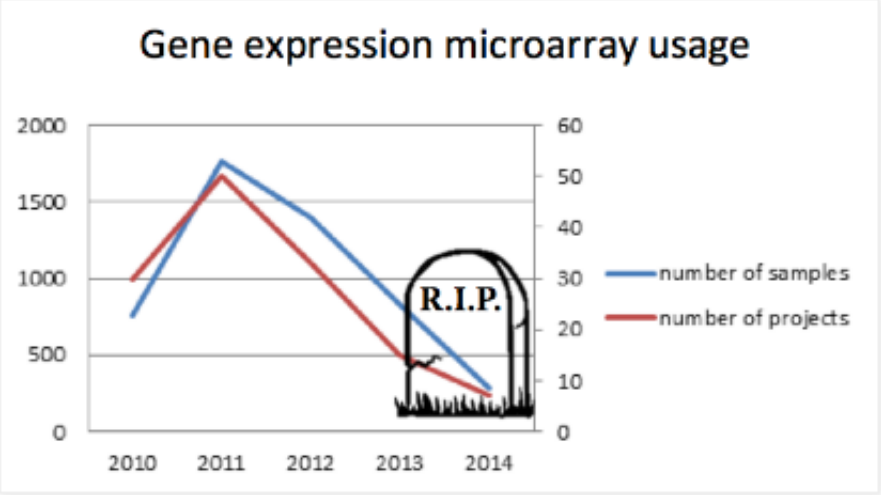
Reports of the death of microarrays. Greatly exagerated?

http://core-genomics.blogspot.co.uk/2014/08/seqc-kills-microarrays-not-quite.html (http://core-genomics.blogspot.co.uk/2014/08/seqc-kills-microarrays-not-quite.html)

Thursday, 28 August 2014

SEQC kills microarrays: not quite

I've been working with microarrays since 2000 and ever since RNA-seq came on the scene the writing has been on the wall. RNA-seq has so many advantages over arrays that we've been recommending them as the best way to generate differential gene expression data for a number of years. However the cost, and lack of maturity in analysis meant we still ran over 1000 arrays in 2013, but it looks like 2014 might be the end of the line. RIP: microarrays.



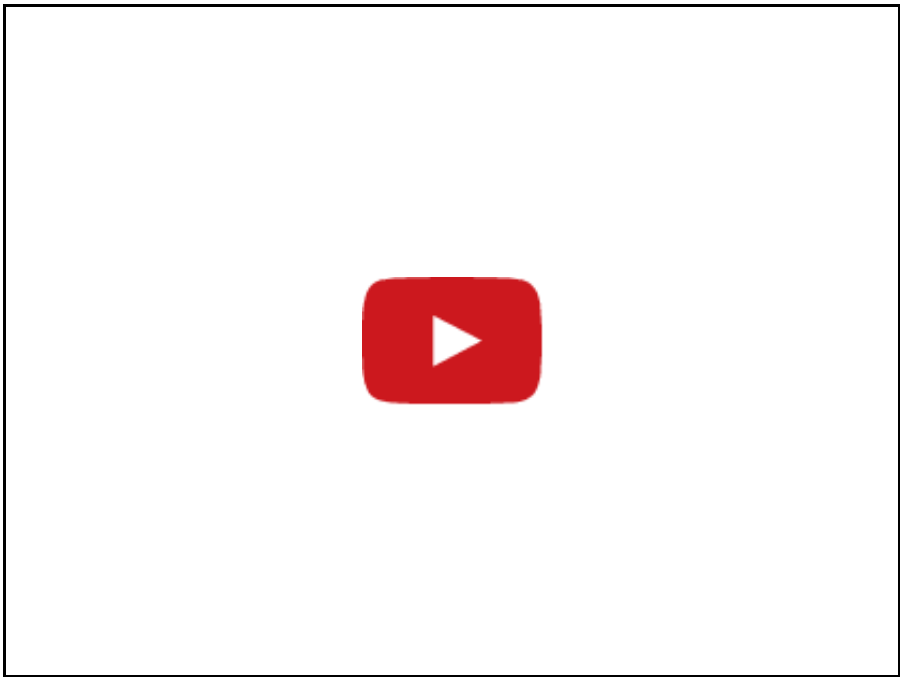
What are NGS data?

Different terminologies for same thing

- *N*ext *G*eneration *S*equencing
- *H*igh-*T*hroughput *S*equencing
- 2nd Generation Sequencing
- Massively Parallel Sequencing
- Also different library preparation
  - RNA-seq *
  - ChIP-seq *
  - Exome-seq
  - DNA-seq
  - Methyl-seq
  - …..

Illumina sequencing *

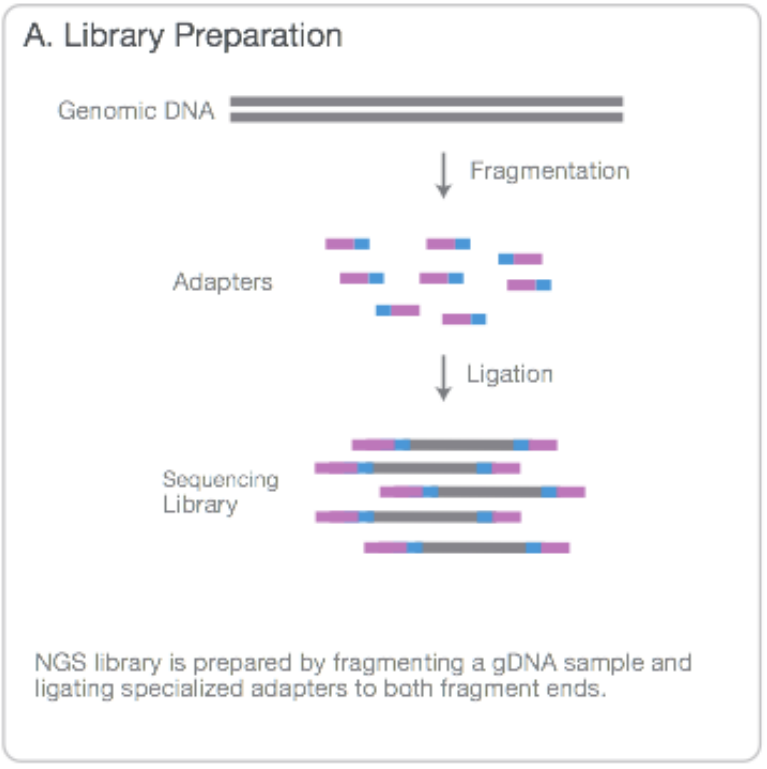
- Employs a *'sequencing-by-synthesis'* approach



http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf (http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf)

* Other sequencing technologies are available

Illumina sequencing



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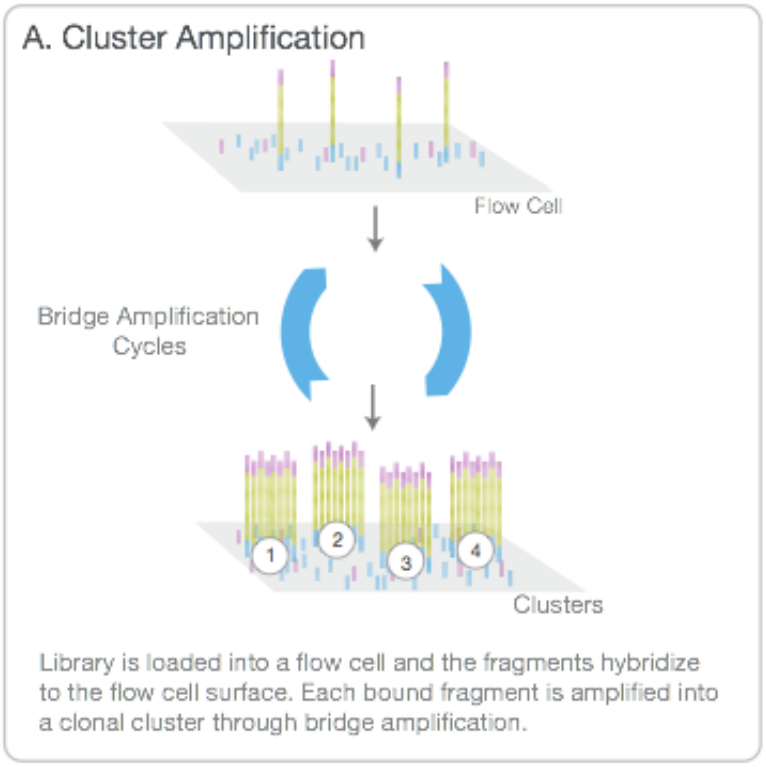
Illumina sequencing



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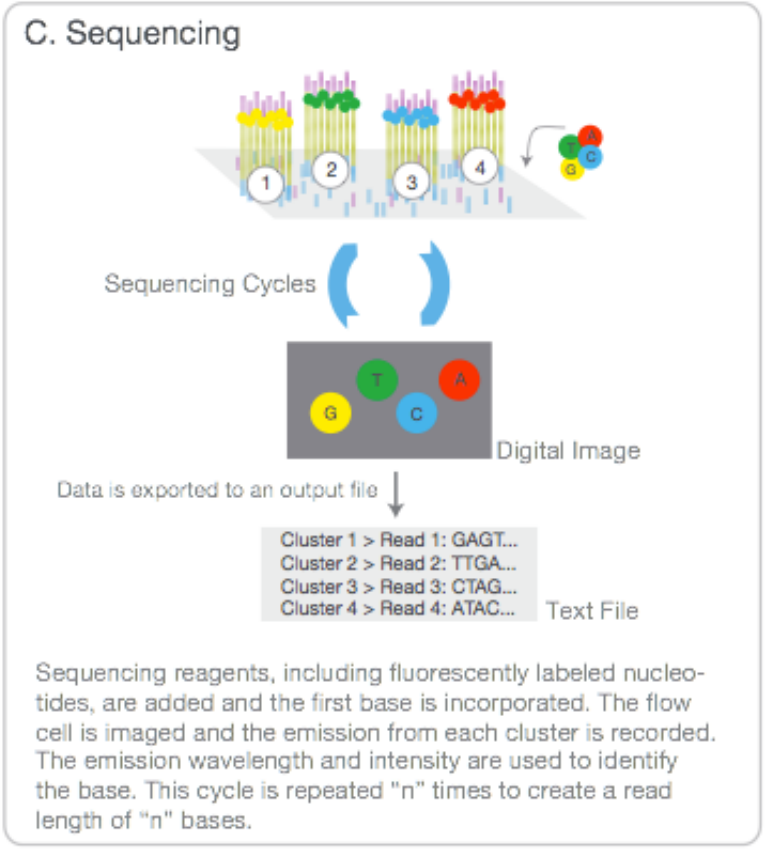
Illumina sequencing



http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf (http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf)

Paired-end

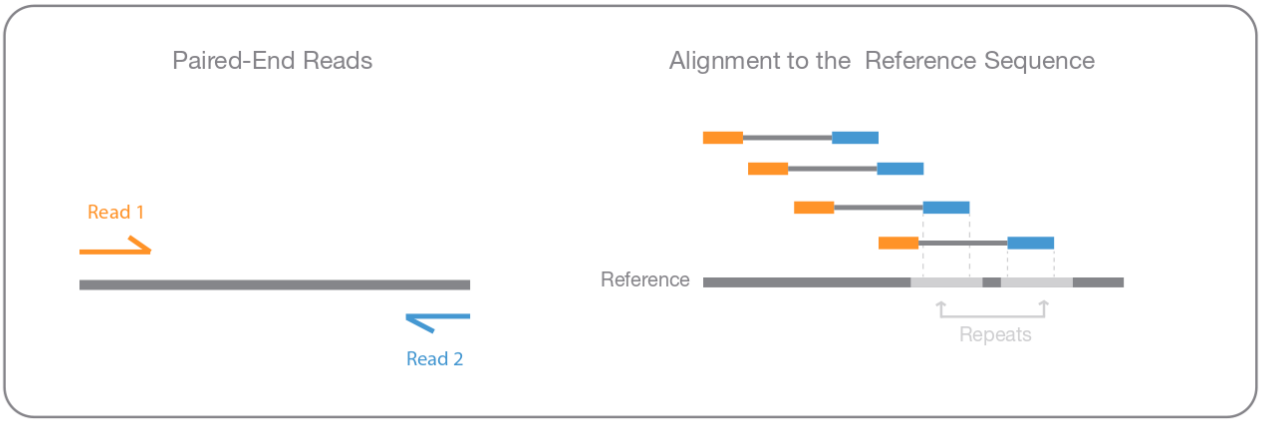


Figure 4: Paired-End Sequencing and Alignment—Paired-end sequencing enables both ends of the DNA fragment to be sequenced. Because the distance between each paired read is known, alignment algorithms can use this information to map the reads over repetitive regions more precisely. This results in much better alignment of the reads, especially across difficult-to-sequence, repetitive regions of the genome.

Multiplexing

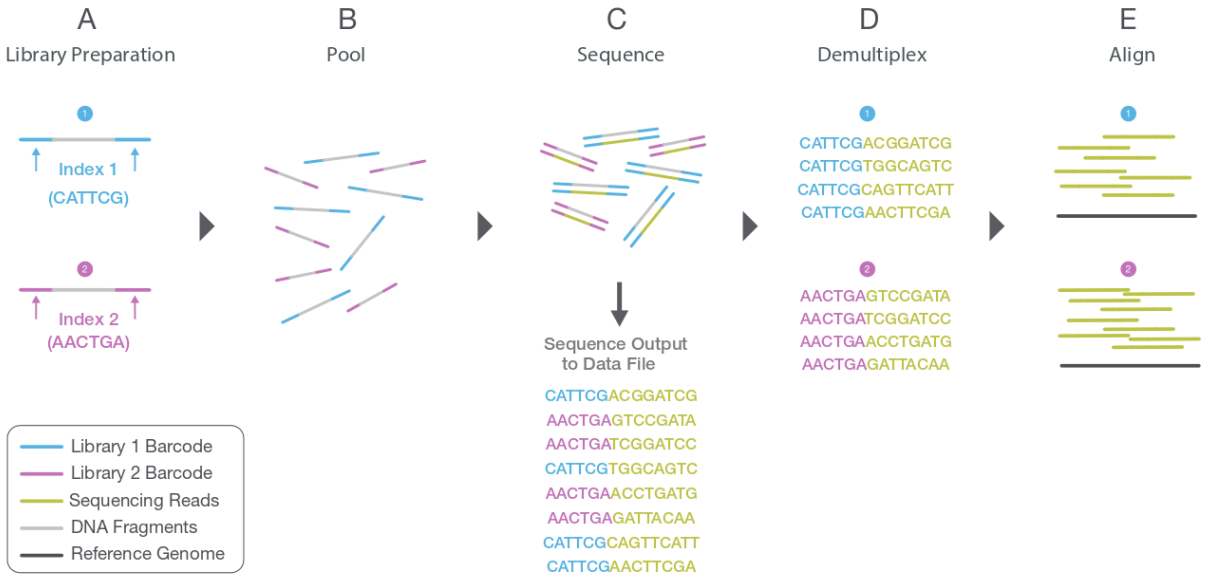


Image processing

- Sequencing produces high-resolution TIFF images; not unlike microarray data
- 100 tiles per lane, 8 lanes per flow cell, 100 cycles
- 4 images (A,G,C,T) per tile per cycle = 320,000 images
- Each *TIFF* image ~ 7Mb = 2,240,000 Mb of data (**2.24TB**)

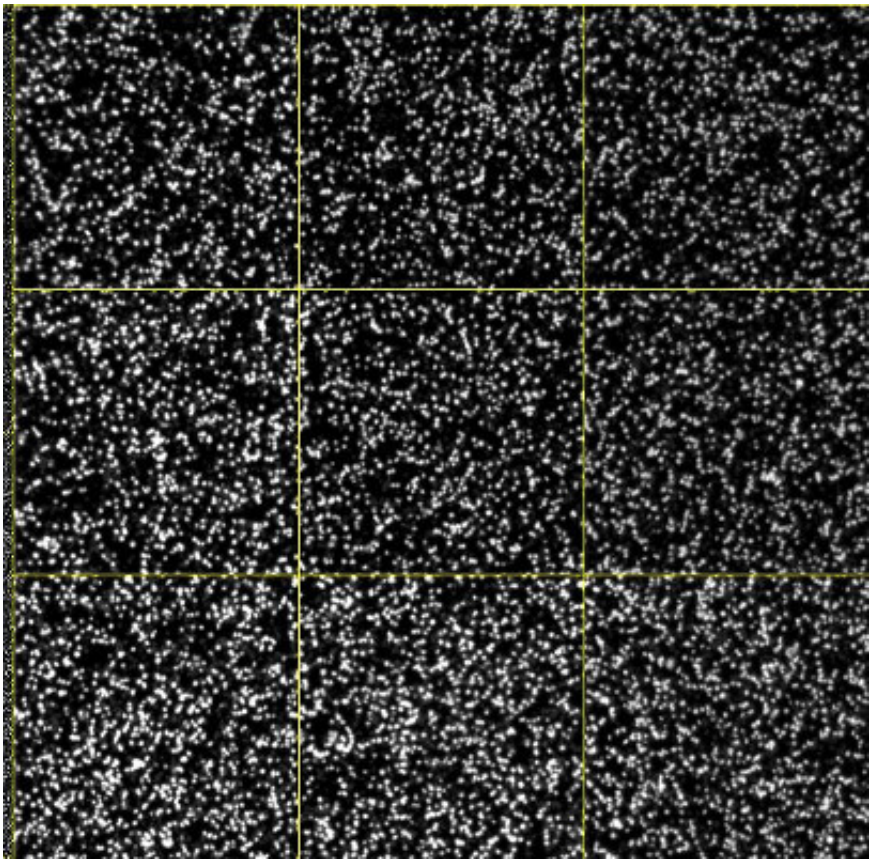


Image processing

- Firecrest

- *“Uses the raw TIF files to locate clusters on the image, and outputs the cluster intensity, X,Y positions, and an estimate of the noise for each cluster. The output from image analysis provides the input for base calling.”*
    - http://openwetware.org/wiki/BioMicroCenter:IlluminaDataPipeline (http://openwetware.org/wiki/BioMicroCenter:IlluminaDataPipeline)
- **You will never have to do this**
    - In fact, the TIFF images are deleted by the instrument

Base-calling

- Bustard

- *“Uses cluster intensities and noise estimate to output the sequence of bases read from each cluster, along with a confidence level for each base.”*
    - http://openwetware.org/wiki/BioMicroCenter:IlluminaDataPipeline (http://openwetware.org/wiki/BioMicroCenter:IlluminaDataPipeline)
- **You will never have to do this**

Alignment

- Locating where each generated sequence came from in the genome
- Outside the scope of this course
- *Usually* perfomed automatically by a sequencing service
- For most of what follows in the course, we will assume alignment has been performed and we are dealing with aligned data
    - Popular aligners
    - bwa http://bio-bwa.sourceforge.net/ (http://bio-bwa.sourceforge.net/)
    - bowtie http://bowtie-bio.sourceforge.net/index.shtml (http://bowtie-bio.sourceforge.net/index.shtml)
    - novoalign http://www.novocraft.com/products/novoalign/ (http://www.novocraft.com/products/novoalign/)
    - stampy http://www.well.ox.ac.uk/project-stampy (http://www.well.ox.ac.uk/project-stampy)
    - many, many more…..
- ***Demo to follow after this talk***

Post-processing of aligned files

- Marking of PCR duplicates
    - PCR amplification errors can cause some sequences to be over-represented
    - Chances of any two sequences aligning to the same position are *unlikely*
    - Caveat: obviously this depends on amount of the genome you are capturing
    - Such reads are *marked* but not usually removed from the data
    - Most downstream methods will ignore such reads
    - Typically, ***picard*** (http://broadinstitute.github.io/picard/) is used
- Sorting
    - Reads can be sorted according to genomic position
        - ***samtools*** (http://www.htslib.org/)
- Indexing

- Allow efficient access
  - ***samtools*** (http://www.htslib.org/)

Data formats

Raw reads - fastq

- The most basic file type you will see is *fastq*
  - Data in public-repositories (e.g. Short Read Archive, GEO) tend to be in this format
- This represents all sequences created after imaging process
- Each sequence is described over 4 lines
- No standard file extension. *.fq*, *.fastq*, *.sequence.txt*
- Essentially they are text files
  - Can be manipulated with standard unix tools; e.g. *cat*, *head*, *grep*, *more*, *less*
- They can be compressed and appear as *.fq.gz*
- Same format regardless of sequencing protocol (i.e. RNA-seq, ChIP-seq, DNA-seq etc)

```
@SEQ_ID
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65
```

~ 250 Million reads (sequences) per Hi-Seq lane

Fastq sequence names

```
@HWUSI-EAS100R:6:73:941:1973#0/1
```

- The name of the sequencer (HWUSI-EAS100R)
- The flow cell lane (6)
- Tile number with the lane (73)
- x co-ordinate within the tile (941)
- y co-ordinate within the tile (1973)
- #0 index number for a multiplexed sample
- /1; the member of a pair, /1 or /2 (paired-end or mate-pair reads only)

Fastq quality scores

```
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65
```

- Quality scores

$$Q = -10log_{10}p$$

  - Q = 30, p=0.001
  - Q = 20, p=0.01
  - Q = 10, p=0.1

- These numeric quanties are *encoded* as **ASCII** code
  - Sometimes an offset is used before encoding

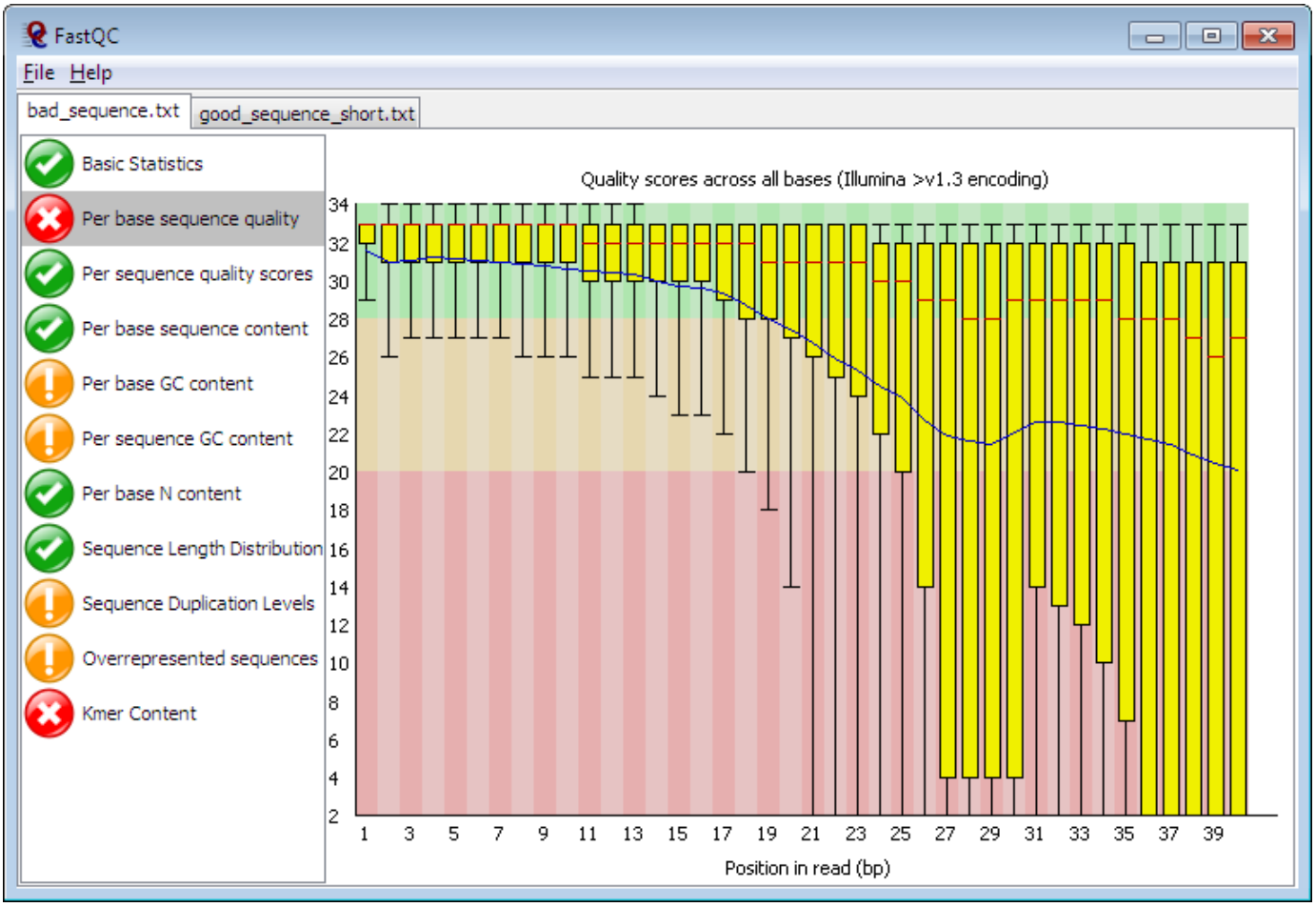
Fastq quality scores

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
 ..............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
 ..............................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                         |    |        |                              |                     |
33                        59   64       73                            104                   126
 0........................26...31.......40
                          -5....0........9.............................40
                               0........9.............................40
                                  3.....9.............................40
 0.2......................26...31........41

S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
    with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
    (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

Useful for quality control

- FastQC, from Babraham Bioinformatics Core; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
  - ***we will look at this in detail later***



- Based on these plots we may want to *trim* our data
  - A popular choice is ***trimmomatic*** http://www.usadellab.org/cms/index.php?page=trimmomatic (http://www.usadellab.org/cms/index.php?page=trimmomatic)
  - or ***Trim Galore!*** from the makers of FastQC

Aligned reads - sam

- **S**equence **A**lignment **M**atrix (sam) http://samtools.github.io/hts-specs/SAMv1.pdf (http://samtools.github.io/hts-specs/SAMv1.pdf)
- *Header* lines followed by tab-delimited lines
  - Header gives information about the alignment and references sequences used

```
@HD     VN:1.0  SO:coordinate
@SQ     SN:chr1 LN:249250621
@SQ     SN:chr10        LN:135534747
@SQ     SN:chr11        LN:135006516
```

```
HWI-ST1001:137:C12FPACXX:7:1115:14131:66670     0       chr1    12805   1
 42M4I5M *
0       0       TTGGATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTCACCAATATG
CCCFFFFFHHGHHJJJJJHJJJJJJJJJJJJJJJIJJJJJJJJJJJJIJJ
AS:i:-28        XN:i:0  XM:i:2  XO:i:1XG:i:4   NM:i:6  MD:Z:2C41C2     YT:Z:UU
NH:i:3
CC:Z:chr15      CP:i:102518319  XS:A:+  HI:i:0
```

- http://homer.salk.edu/homer/basicTutorial/samfiles.html (http://homer.salk.edu/homer/basicTutorial/samfiles.html)
- Large size on disk; ~100s of Gb
  - Can be manipulated with standard unix tools; e.g. *cat*, *head*, *grep*, *more*, *less*

Sam format - key columns

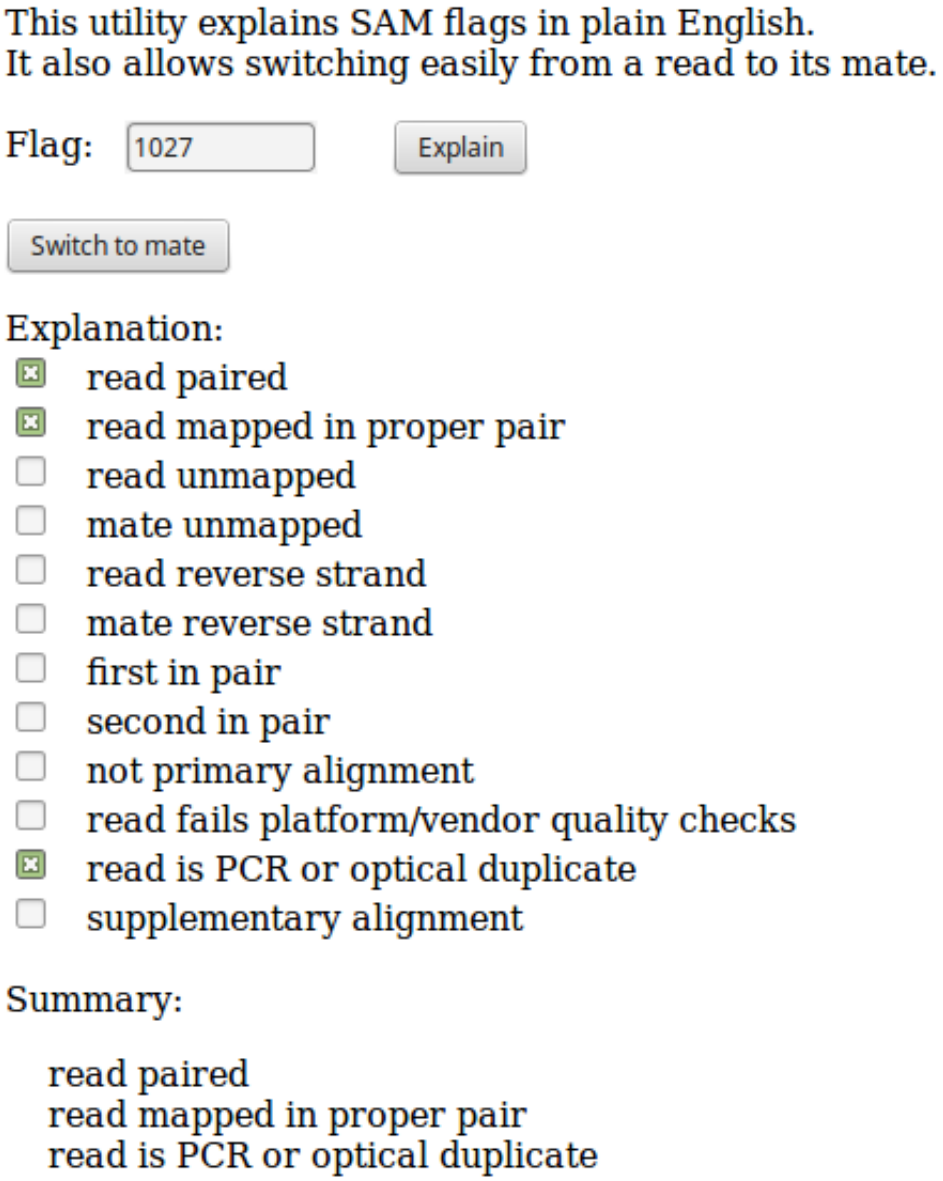
```
HWI-ST1001:137:C12FPACXX:7:1115:14131:66670     0       chr1    12805   1
 42M4I5M *
0       0       TTGGATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTCACCAATATG
CCCFFFFFHHGHHJJJJJHJJJJJJJJJJJJJJJIJJJJJJJJJJJJIJJ
AS:i:-28        XN:i:0  XM:i:2  XO:i:1XG:i:4   NM:i:6  MD:Z:2C41C2     YT:Z:UU
NH:i:3
CC:Z:chr15      CP:i:102518319  XS:A:+  HI:i:0
```

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	`[!-?A-~]{1,255}`	Query template NAME
2	FLAG	Int	$[0,2^{16}-1]$	bitwise FLAG
3	RNAME	String	`\*|[!-()+-<>-~][!-~]*`	Reference sequence NAME
4	POS	Int	$[0,2^{31}-1]$	1-based leftmost mapping POSition
5	MAPQ	Int	$[0,2^{8}-1]$	MAPping Quality
6	CIGAR	String	`\*|([0-9]+[MIDNSHPX=])+`	CIGAR string
7	RNEXT	String	`\*|=|[!-()+-<>-~][!-~]*`	Ref. name of the mate/next read
8	PNEXT	Int	$[0,2^{31}-1]$	Position of the mate/next read
9	TLEN	Int	$[-2^{31}+1,2^{31}-1]$	observed Template LENgth
10	SEQ	String	`\*|[A-Za-z=.]+`	segment SEQuence
11	QUAL	String	`[!-~]+`	ASCII of Phred-scaled base QUALity+33

- http://samtools.github.io/hts-specs/SAMv1.pdf (http://samtools.github.io/hts-specs/SAMv1.pdf)
  - Read name
  - Chromosome
  - Position
  - Mapping quality
  - etc…

Sam file flags

- Represent useful QC information
  - Read is unmapped
  - Read is paired / unpaired
  - Read failed QC
  - Read is a PCR duplicate
- https://broadinstitute.github.io/picard/explain-flags.html (https://broadinstitute.github.io/picard/explain-flags.html)



Aligned reads - bam

- *Exactly* the same information as a sam file
- ..except that it is *binary* version of sam
- compressed around x4
- Attempting to read will print garbage to the screen
- bam files can be indexed
    - Produces an index file with the same name as the bam file, but with **.bai** extension

```
samtools view mysequences.bam | head
```

- N.B The sequences can be extracted by various tools to give *fastq*

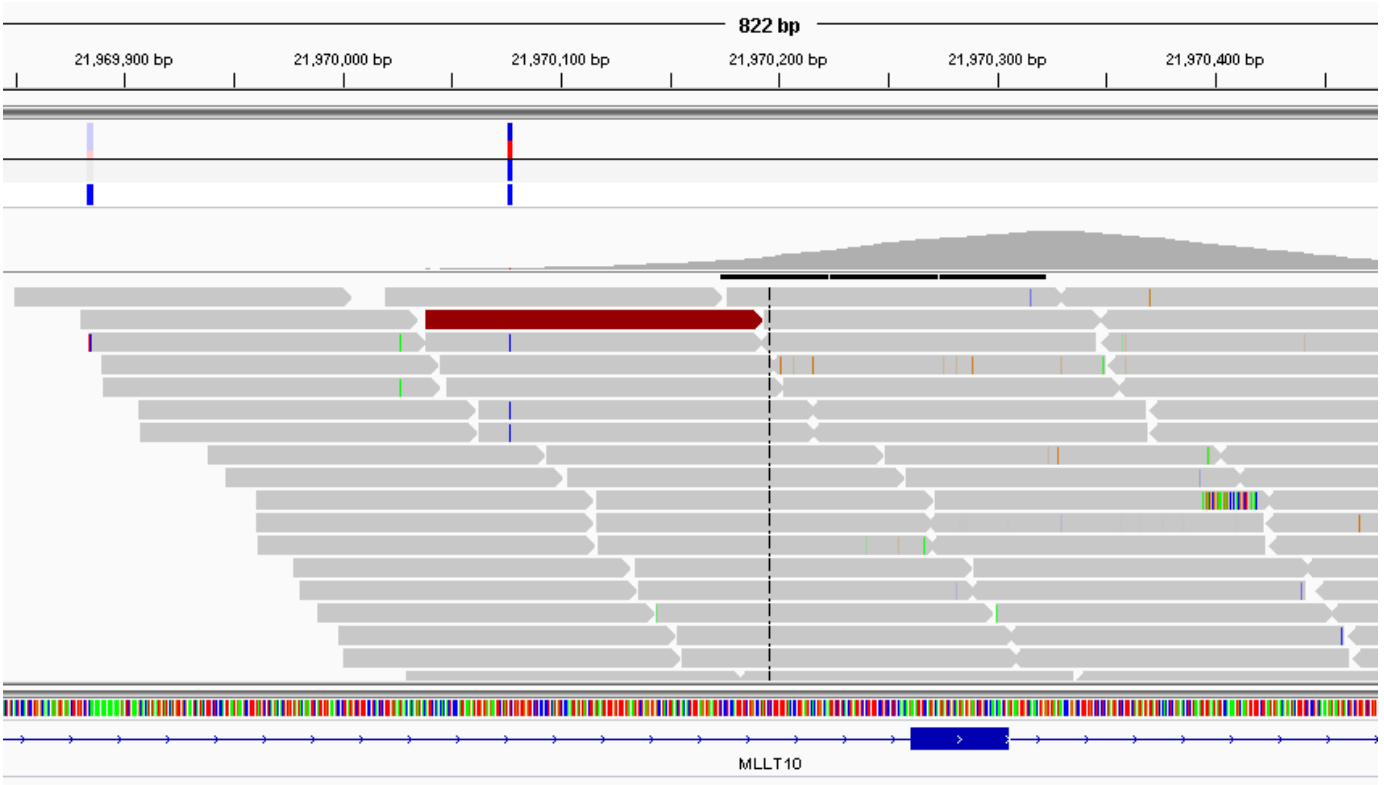
samtools flagstat

- Useful *command-line* tool as part of **samtools**

```
$ samtools flagstat NA19914.chr22.bam
2109857 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 secondary
0 + 0 supplimentary
40096 + 0 duplicates
2064356 + 0 mapped (97.84%:-nan%)
2011540 + 0 paired in sequencing
1005911 + 0 read1
1005629 + 0 read2
1903650 + 0 properly paired (94.64%:-nan%)
1920538 + 0 with itself and mate mapped
45501 + 0 singletons (2.26%:-nan%)
5134 + 0 with mate mapped to a different chr
4794 + 0 with mate mapped to a different chr (mapQ>=5)
```

Aligned files in IGV

- Once our bam files have been *indexed* we can view them in IGV
- This is **highly recommended**



Other misc. format

Often said that Bioinformaticians love coming up with new file formats

- Useful link : http://www.genome.ucsc.edu/FAQ/FAQformat.html (http://www.genome.ucsc.edu/FAQ/FAQformat.html)
- bed ; only first three columns are required

```
track name=pairedReads description="Clone Paired Reads" useScore=1
chr22 1000 5000 cloneA 960 + 1000 5000 0 2 567,488, 0,3512
chr22 2000 6000 cloneB 900 - 2000 6000 0 2 433,399, 0,3601
```

- gff; (gene feature format)

```
track name=regulatory description="TeleGene(tm) Regulatory Regions"
visibility=2`
chr22  TeleGene enhancer  10000000  10001000  500 +  .  touch1
chr22  TeleGene promoter  10010000  10010100  900 +  .  touch1
chr22  TeleGene promoter  10020000  10025000  800 -  .  touch2
```

- wig;

```
variableStep chrom=chr2
300701 12.5
300702 12.5
300703 12.5
300704 12.5
300705 12.5
```

What happens next?

- Hands-on examples of NGS data
  - Alignment (Shamith)
  - Quality assessment of NGS data (Ines)