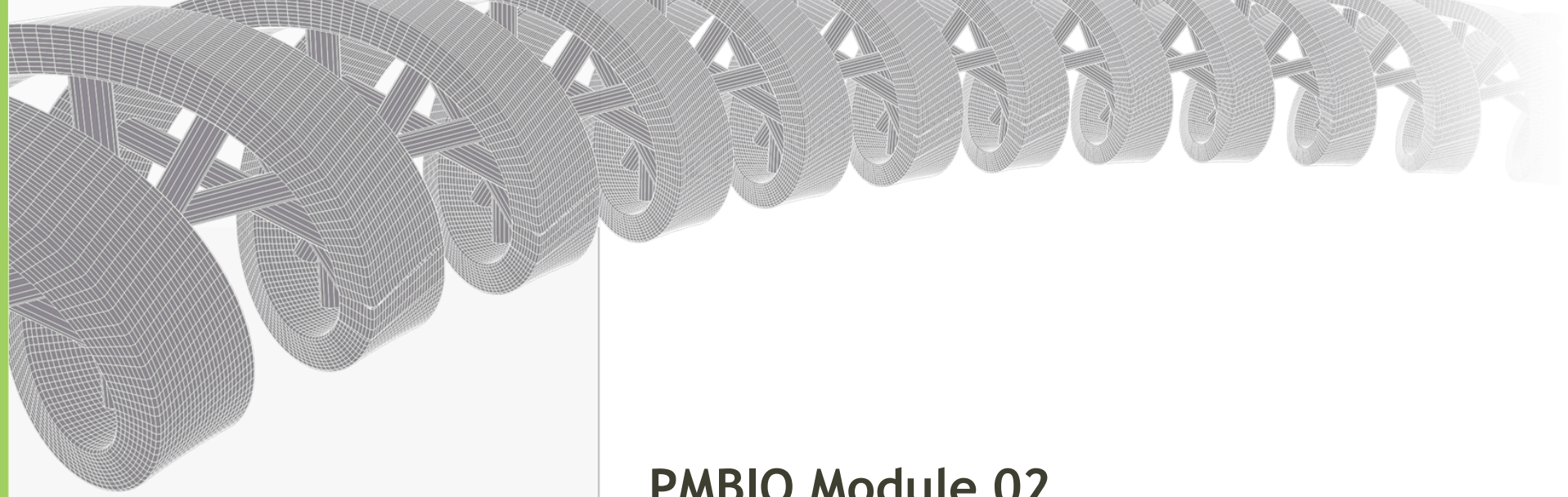

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School of Medicine

PMBIO Module 02

Inputs. References, Annotations, and Raw Data

Malachi Griffith, Obi Griffith, Zachary Skidmore, Huiming Xia
Introduction to bioinformatics for DNA and RNA sequence analysis (IBDR01)
29 October – 2 November, 2018
Glasgow

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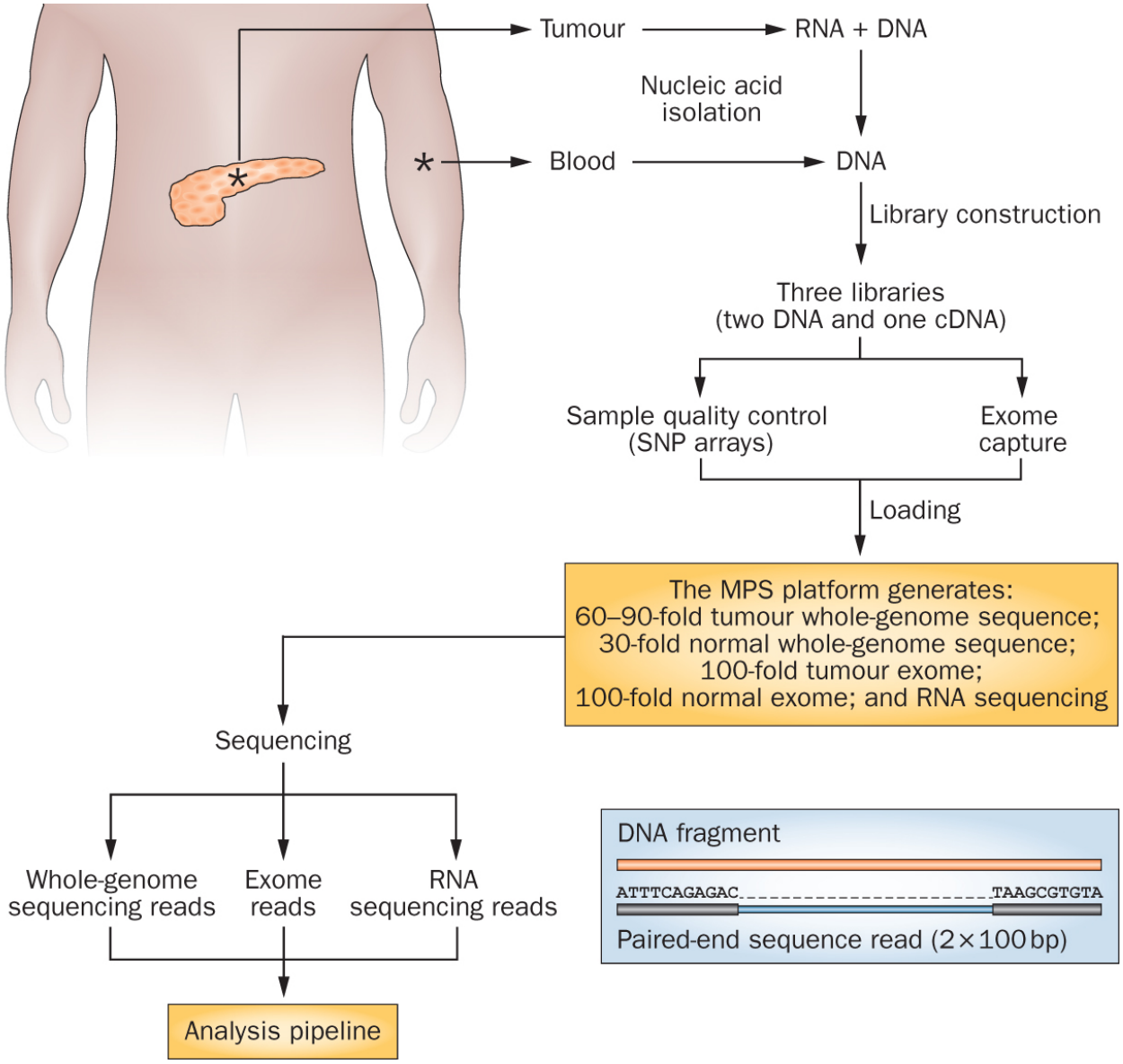
Learning objectives of module 02: Inputs

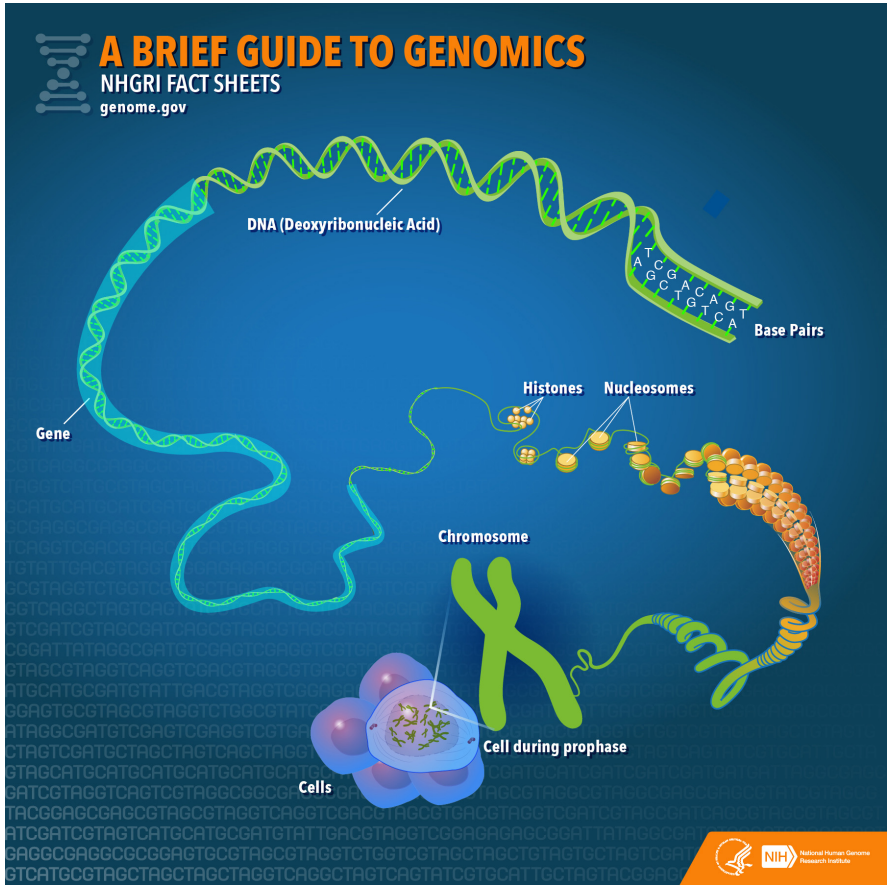
- **Key concepts**: Central dogma (chromosomes, genes, transcripts, proteins), reference genome assemblies, reference genome versions, FASTA file format, gene/transcript annotation pipelines (Refseq, Ensembl, UCSC, Gencode), GTF file format, sequence data generation, NGS reads, FASTQ file format, raw data QC.

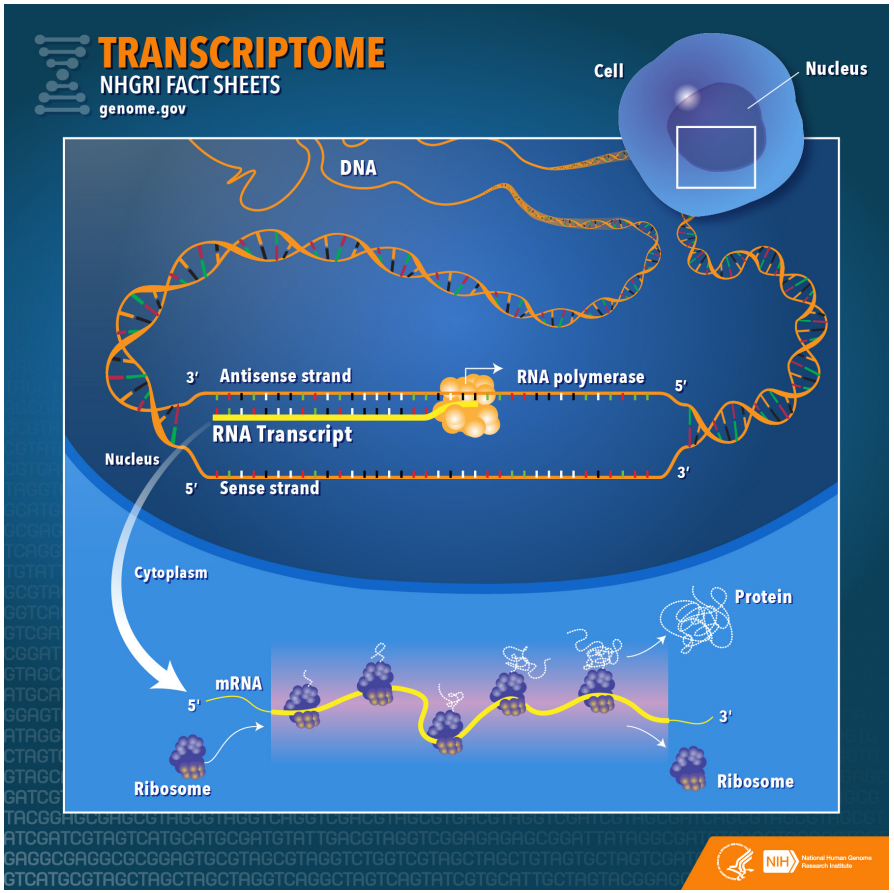
- Obtain reference genome and annotation files and understand the standard formats used to represent them
- Index large files for more efficient access/analysis
- Download and explore raw data files
- Review experimental details for a proof-of-principle personalized genomics exercise
- Perform a raw data quality assessment and discuss any data quality issues that are observed. What are their implications for interpretation of the results?

Cancer genomics data has exploded with rapid advances in sequencing technologies

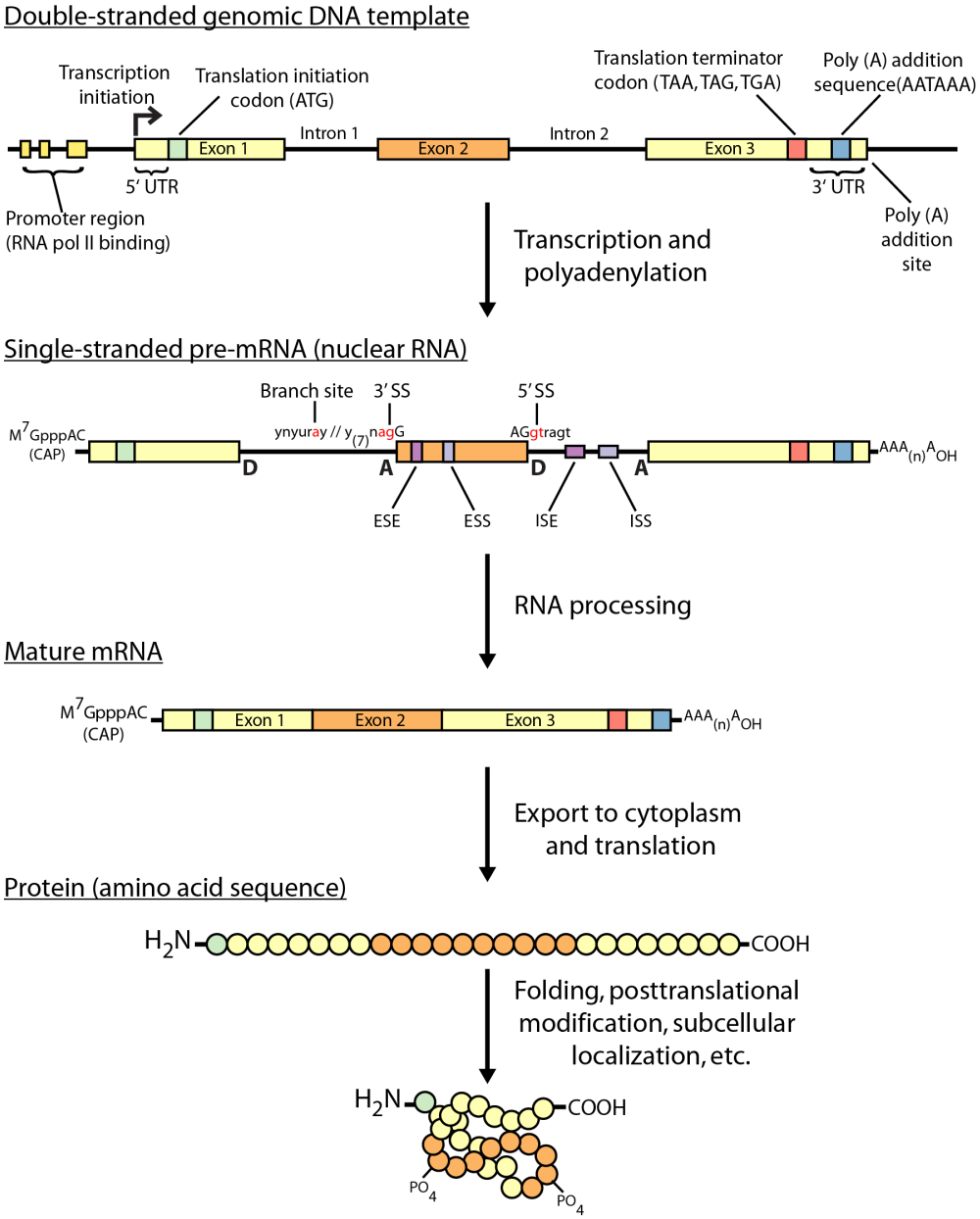


Genomes and transcriptomes

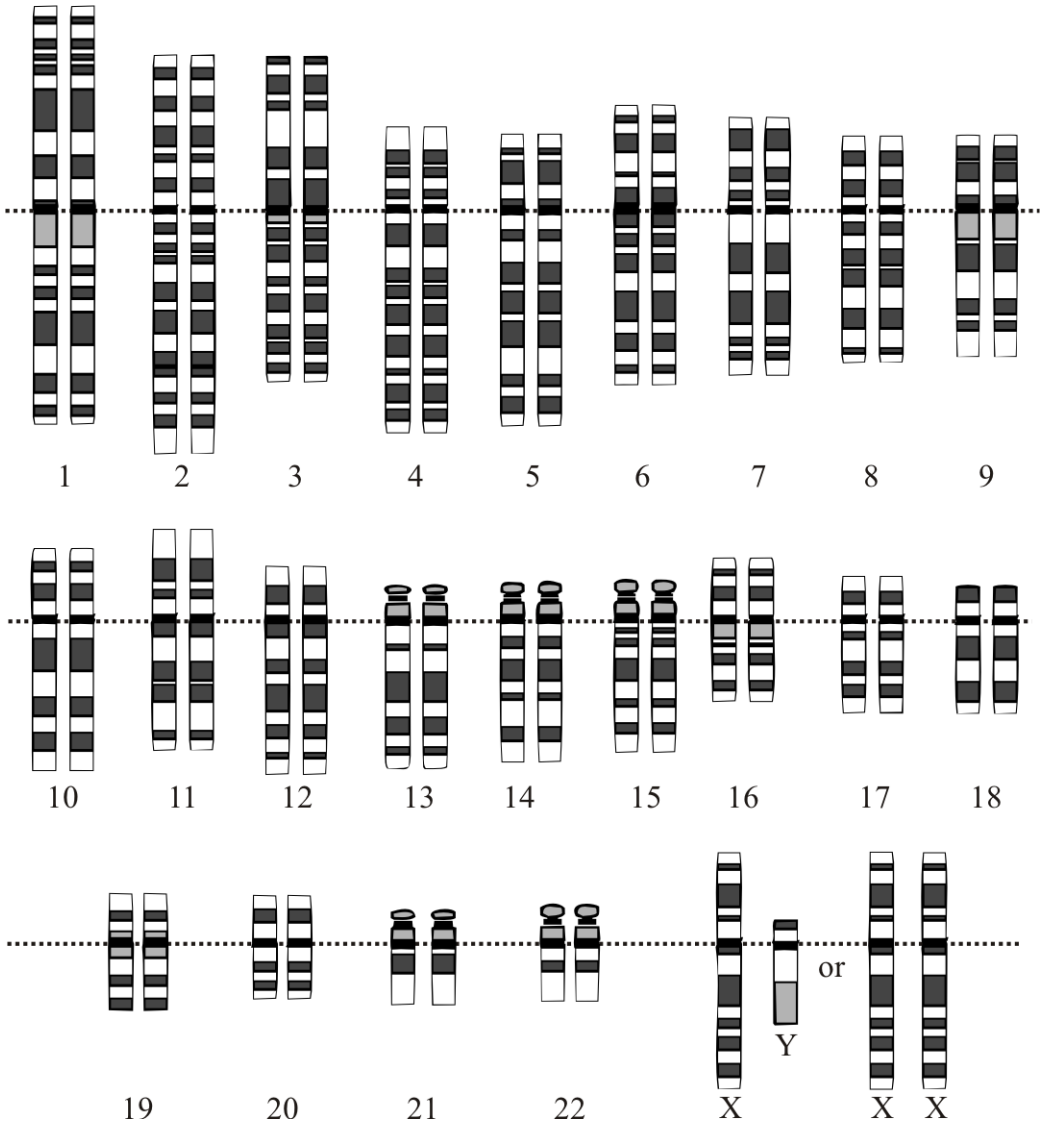




The Central Dogma

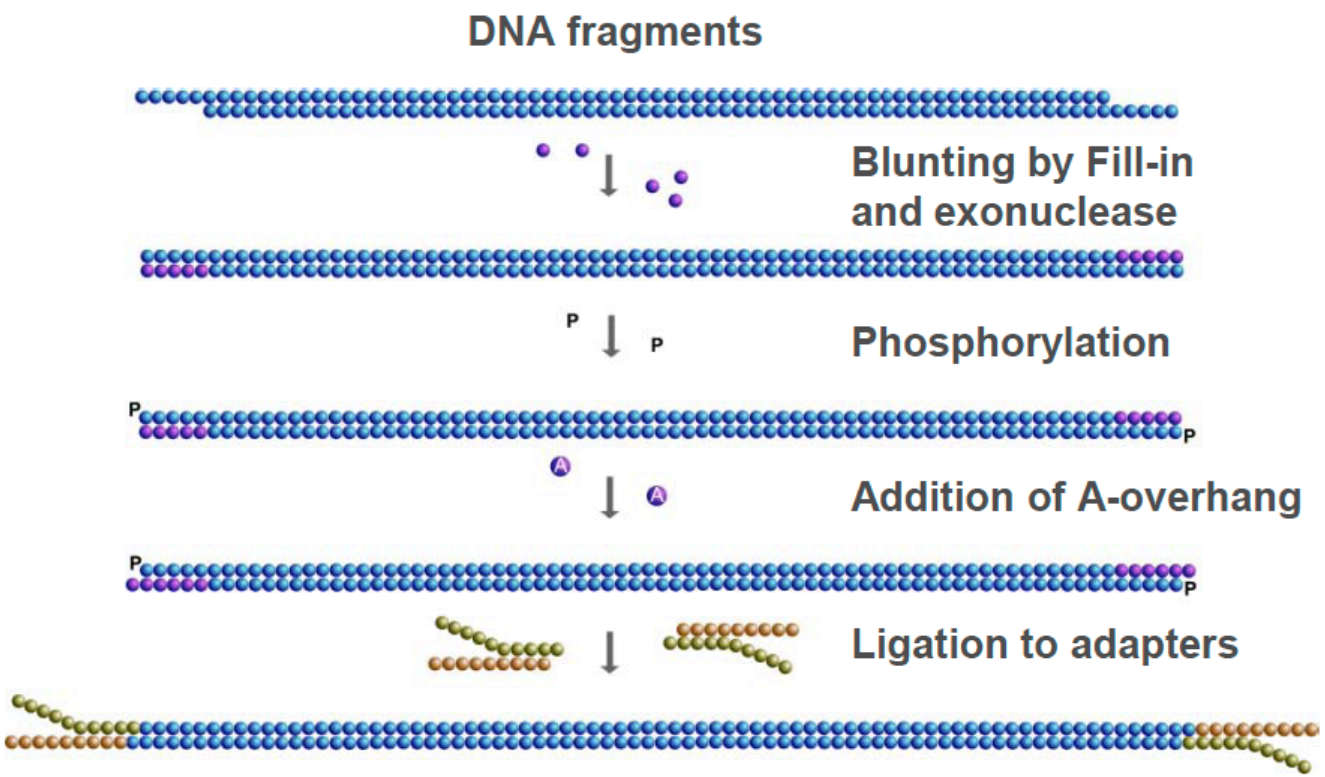


Human chromosomes (karyotype)



Data Generation

Library Construction for MPS



- Shear high molecular weight DNA with sonication
- Enzymatic treatments to blunt ends
- Ligate synthetic DNA adapters (each with a DNA barcode), PCR amplify
- Quantitate library
- Proceed to WGS, or perform exome or specific gene hybrid capture

Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

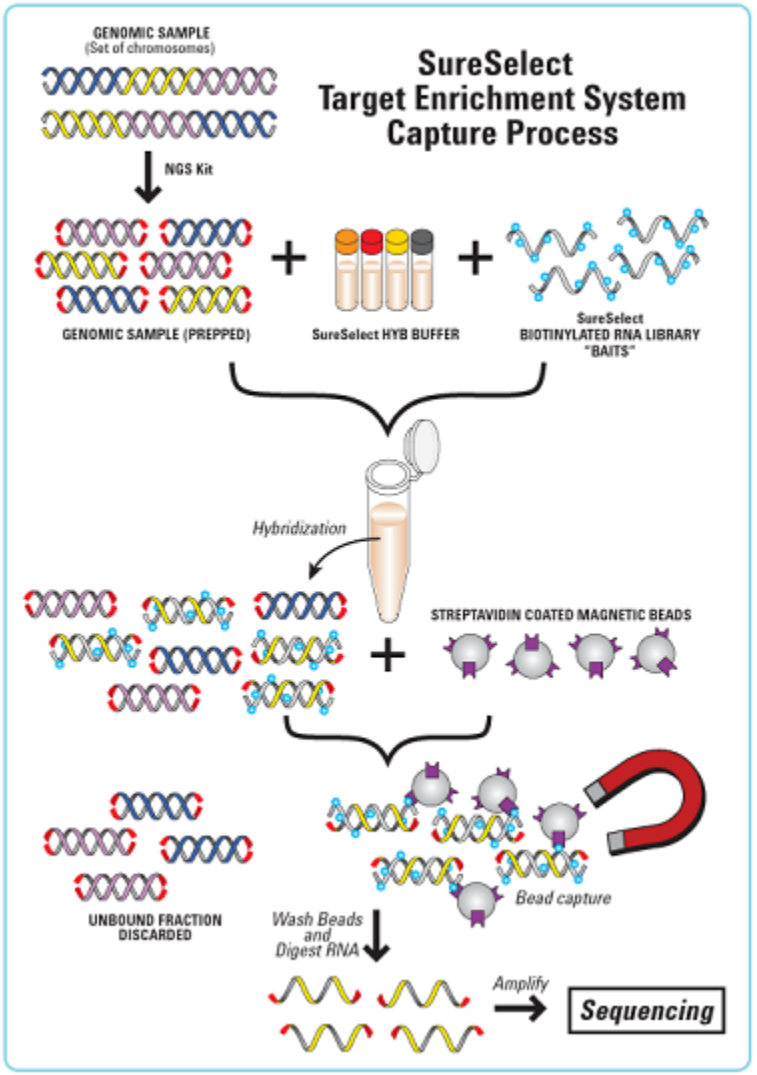
PCR-related Problems in MPS

- PCR is an effective vehicle for amplifying DNA, however…
- In MPS library construction, PCR can introduce preferential amplification ("jackpotting") of certain fragments
  - Duplicate reads with exact start/stop alignments
  - Need to "de-duplicate" after alignment and keep only one pair
  - Low input DNA amounts favor jackpotting due to lack of complexity in the fragment population
- PCR also introduces false positive artifacts due to substitution errors by the polymerase
  - If substitution occurs in early PCR cycles, error appears as a true variant
  - If substitution occurs in later cycles, error typically is drowned out by correctly copied fragments in the cluster
- Cluster formation is a type of PCR ("bridge amplification")
  - Introduces bias in amplifying high and low G+C fragments
  - Reduced coverage at these loci is a result

Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

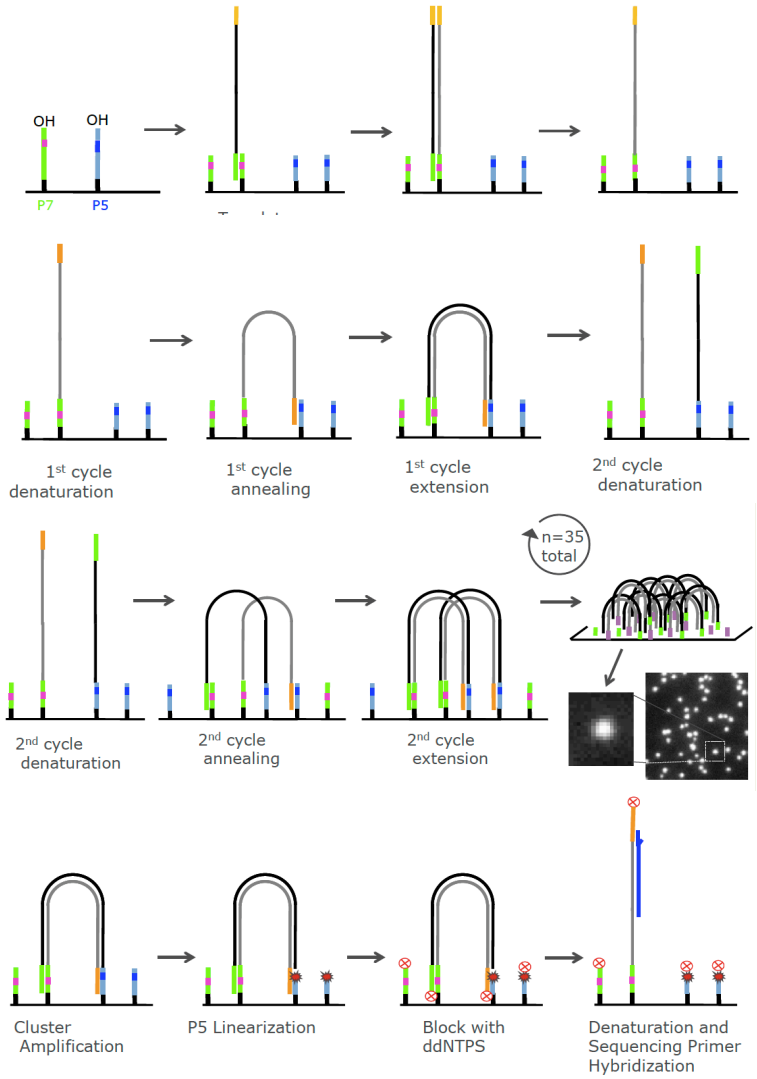
Hybrid Capture

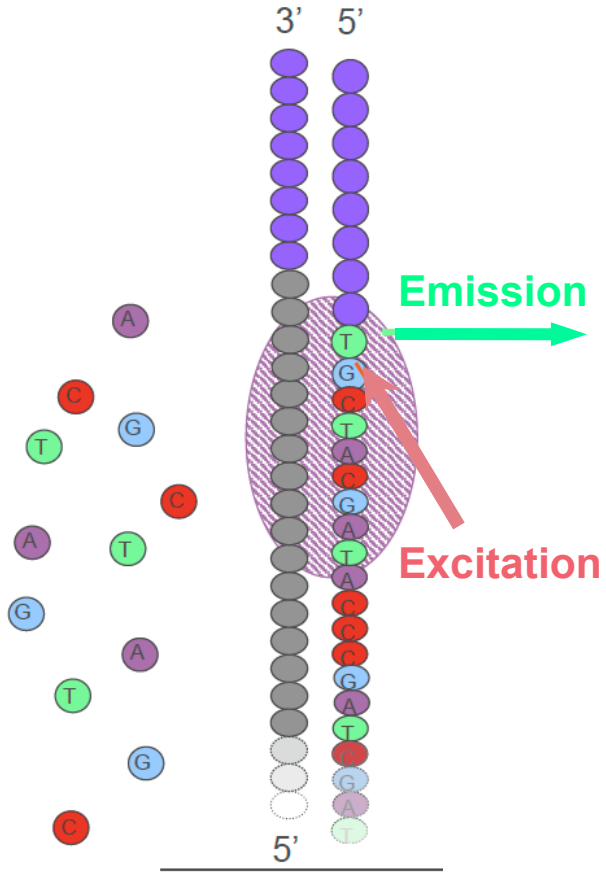
- **Hybrid capture** - fragments from a whole genome library are selected by combining with probes that correspond to most (not all) human exons or gene targets.
- The probe DNAs are biotinylated, making selection from solution with streptavidin magnetic beads an effective means of purification.
- An "**exome**" by definition, is the exons of all genes annotated in the reference genome.
- **Custom capture reagents** can be synthesized to target specific loci that may be of clinical interest.

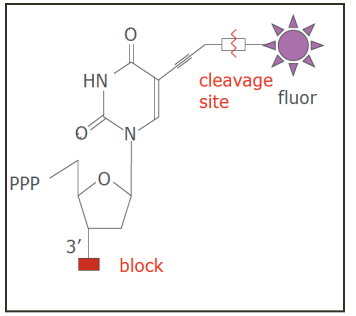


Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

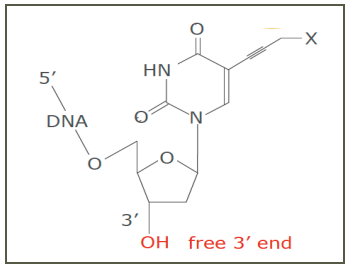
Massively Parallel Sequencing by Synthesis





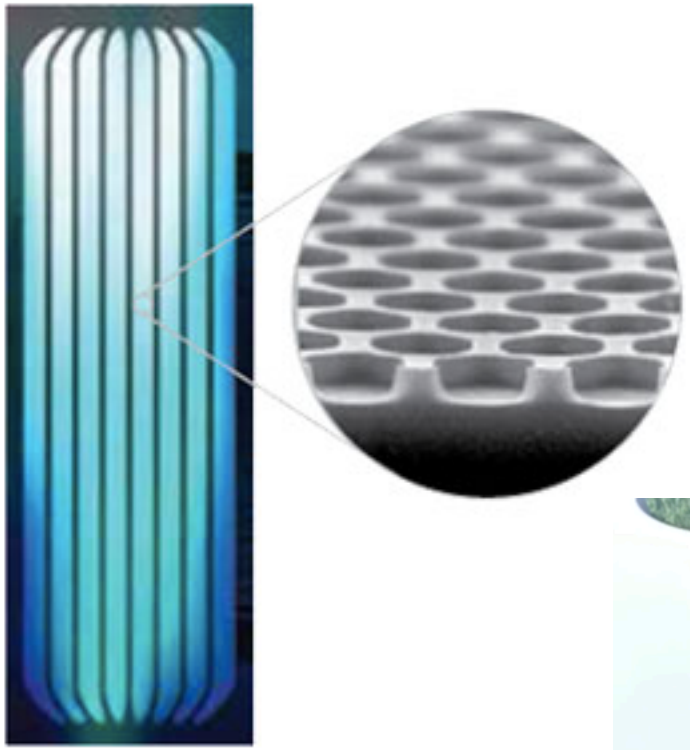


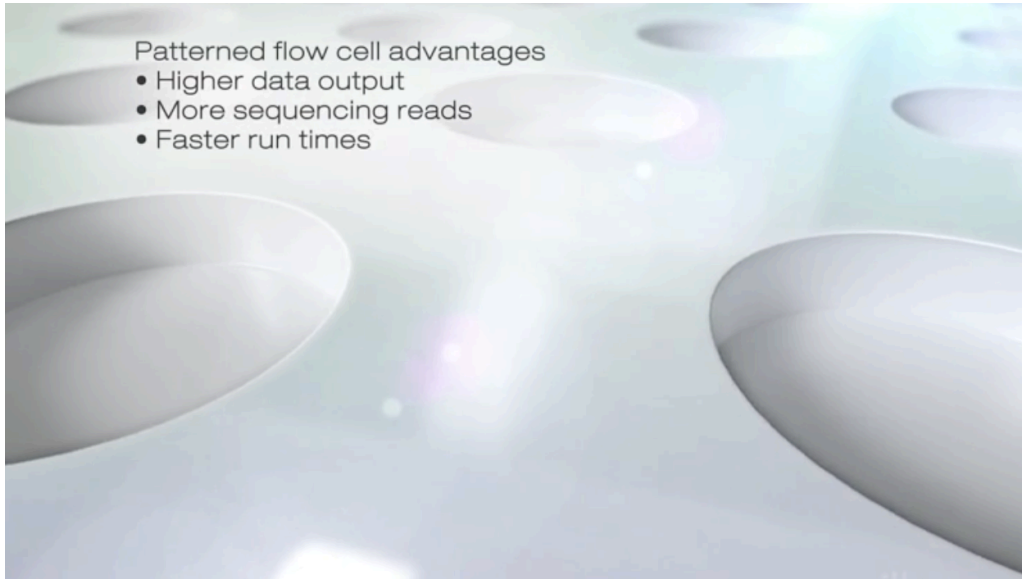
Incorporate
Detect
De-block
Cleave fluor

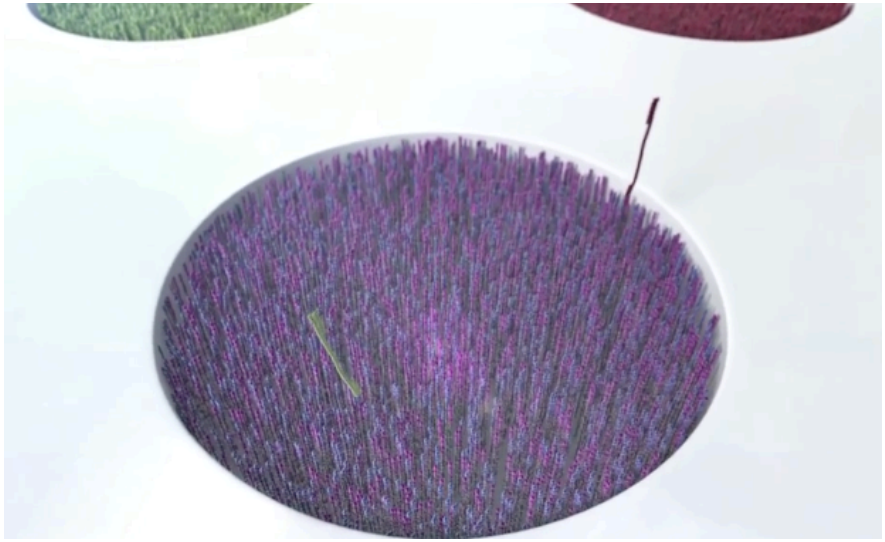


Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

Illumina Patterned Flow Cell







Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

Platforms: Illumina

	 NextSeq*	 HiSeq 4000*	 NovaSeq 6000†† *	 HiSeq X Ten†
Output Range	20-120 Gb	125-1500 Gb	167-6000 Gb	900-1800 Gb
Run Time	11-29 hr	<1-3.5 days	19-40 hr	< 3 days
Reads per Run	130-400 million	2.5-5 billion	1.4-20 billion	3-6 billion
Maximum Read Length	2 x 150 bp	2 x 150 bp	2 x 150 bp	2 x 150 bp
Samples per Run†	1	6-12	4-48	8-16
Relative Price per Sample†	Lower Cost	Lower Cost	Lower Cost	Lower Cost
Relative Instrument Price†	Higher Cost	Higher Cost	Higher Cost	Higher Cost

- High accuracy, range of capacity and throughput
- Longer read lengths on some platforms (MiSeq)
- Improved kits, improved software pipeline and capabilities, cloud computing in BaseSpace

Slide courtesy of Elaine Mardis (Nationwide Children's Hospital)

Our inputs

Common genomic data file formats

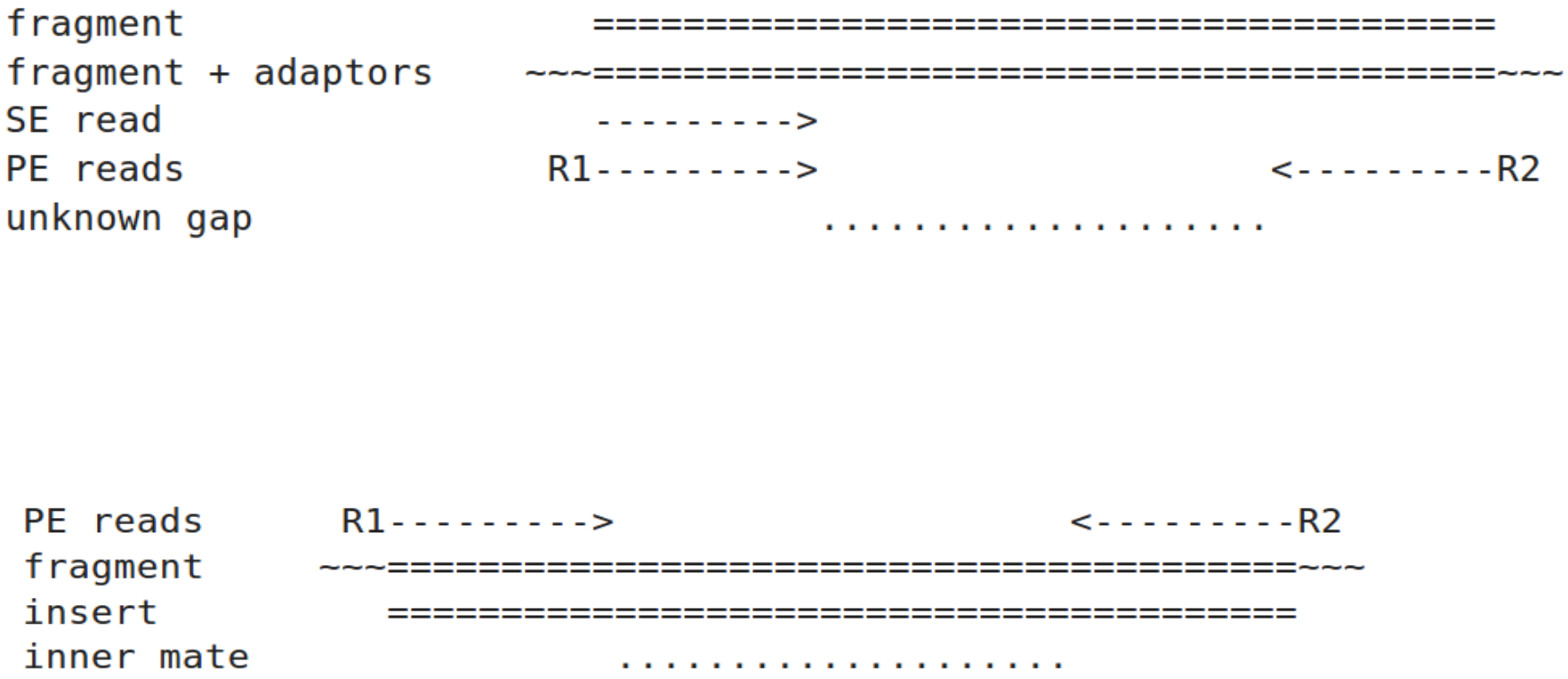
- FASTA – sequences (DNA, RNA, protein)
- FASTQ – raw sequence data (with qualities)
- SAM/BAM/CRAM – aligned sequence data
- GTF – gene/transcript annotations
- BED – other genome features
- VCF – variant calls (individual, multi-individual)
- MAF – aggregated variant information (project, population)

- Many, many custom data formats output by specialized tools … often in TSV format

Our reference genome

- All reference files were obtained from the 1000 genomes project
  - The GRCh38 build of the human genome is used
  - This is the latest version of the human reference
- For the tutorial, two chromosomes are used (chr. 6 and chr. 17)
  - The reason for this is to reduce run time for the tutorial
  - Performing this analysis on the complete genome reference would require only minor modification of the commands
    - Would also require more storage, compute resources, and time

Insert size terminology explained



http://thegenomefactory.blogspot.ca/2013/08/paired-end-read-confusion-library.html

FASTA file format example

```
>18 ENST00000603290 chr6+ 319926-320364,335114-335163,345854-345912
ATAGCAGGGATCGAAAACTGTCTAGTAATCGCTCTTTCTTGAAGCAAGCTGTATCTAGGT
TTCTCGTGTGGCAGTCGGGTGTGTAAGAGCGGCCCCTCCACTGGCTGGTCCTGCTACCAC
AGACTCACTTGCAAATGGAGCCCTAGAACTCCTGCATGGCTGGACTCAGAGGGCTTGAGA
TCTTCCAGTACTTAACTGAGTCTAGGGAGGACATGGTTTTTTAAACTTCTAAATCAAAGA
ACTCTTTTGGCCTGGCTTCCTAGAGGTACCCTGTCCTTCAGCTGGGGACCTTCTCTCTAG
GGCTCGTGCTCACTGACCAGCCTCTCCTTACAACTGAAGACTTTTGCAGGACACTCTTCC
TAGATGTTGCCTTCATGTTGAGTCCCGGTGTCTGAGCATTTCCTCCTGACGGCTGTACTT
GAGCAGTCACCTGGCACAGGGAGTTAAATACCTGTGCATCCCAGCAGCGGATTCACCATC
TCAAAACCTGACAAGACATTTCAAAGAAAGTATTAAATTCATTCACGAGTGCCGGCTCCG
CGGTGAGA
>19 ENST00000605391 chr6+ 319952-320364,345854-345928,348103-348161
AATCGCTCTTTCTTGAAGCAAGCTGTATCTAGGTTTCTCGTGTGGCAGTCGGGTGTGTAA
GAGCGGCCCCTCCACTGGCTGGTCCTGCTACCACAGACTCACTTGCAAATGGAGCCCTAG
AACTCCTGCATGGCTGGACTCAGAGGGCTTGAGATCTTCCAGTACTTAACTGAGTCTAGG
GAGGACATGGTTTTTTAAACTTCTAAATCAAAGAACTCTTTTGGCCTGGCTTCCTAGAGG
TACCCTGTCCTTCAGCTGGGGACCTTCTCTCTAGGGCTCGTGCTCACTGACCAGCCTCTC
CTTACAACTGAAGACTTTTGCAGGACACTCTTCCTAGATGTTGCCTTCATGTTGAGTCCC
GGTGTCTGAGCATTTCCTCCTGACGGCTGTACTTGAGCAGTCACCTGGCACAGGACAAGA
CATTTCAAAGAAAGTATTAAATTCATTCACGAGTGCCGGCTCCGCGGTGAGAGCTGCCTT
GTACACTGCCTGGCCGGGGTCTCCAGGAGCGTGACACTGGTGATCGCATACATCATGACC
GTCACTG
```

Raw read data

- For purposes of the tutorial, the test data has been pre-filtered
  - Identified reads that appear to match transcripts on the selected chromosomes
- The test data corresponds to a single hypothetical human breast cancer patient (cell lines)
  - HCC1395 and HCC1395/BL
  - Genomic DNA and RNA were isolated
  - The RNA samples also included one of two ERCC RNA "spike-in" mixes (Mix1 or Mix2)
- The input data is provided in 'fastq' format:
  - http://en.wikipedia.org/wiki/FASTQ_format

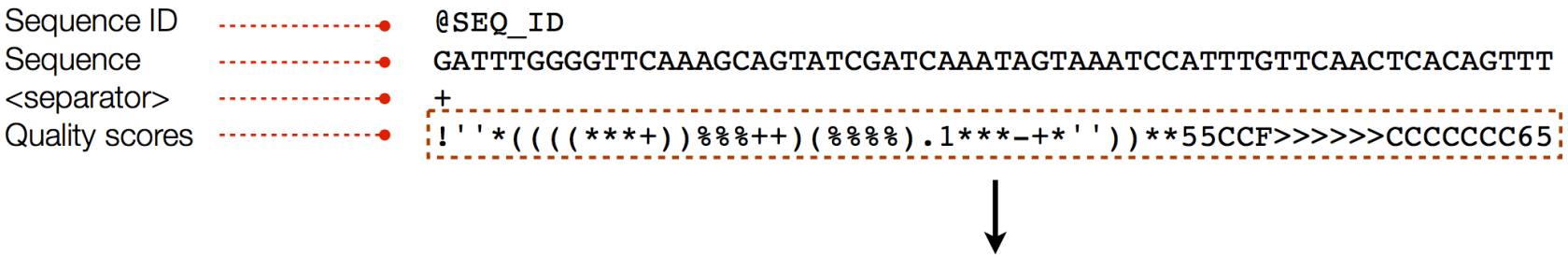
FASTQ file format example

Fastq files represent raw sequence – base calls and qualities

```
@HWI-ST718_146963544:6:1213:8996:10047/1
CTTTTTTATTTTTGTCTGACTGGGTTGATTCAAAA
+
CCCFFFFFHHHHGJHIIJHIHIIIFHIJJJJIJJGIBBFGE

@HWI-ST718_146963544:5:2303:11793:37095/1
ATGAATTATAGGGCTGTATTTTAATTTTGCATTTTAA
+
@@??BDDFFF<FHEGFFGGIEBGHIIIIIBEHIIGIH<FHE
```

Fastq record format



Qualities are based on the Phred scale and are *encoded*

$$Q = -10 * \log_{10}(P_{err})$$

Q scores are derived differently for each technology.

Slide courtesy of Andrew Farrell (University of Utah)

Phred scores and ASCII glyphs

Phred Q	Probability (P) of Wrong Base	Base Call Accuracy	Sanger "Q + 33" Shift	Sanger "Q + 33" Shift ASCII glyph
0	1	0	33	!
1	0.794	0.206	34	"
2	0.631	0.369	35	#
10	0.1	0.90	43	+
20	0.01	0.99	53	5
30	0.001	0.999	63	?

Encoding History:

- Sanger Format (shown above): Q of 0 to 93 using ASCII 33 to 126
  - Sanger data, SAM format, Illumina 1.8+
- Solexa/Illumina 1.0: Q of -5 to 62 using ASCII 59 to 126
- Illumina 1.3 to 1.8: Q of 0 to 62 using ASCII 64 to 126
- Illumina 1.5 to 1.8: Phred scores 0 to 2 have a slightly different meaning

Known transcript annotations

- All annotation files are obtained from Ensembl
  - http://useast.ensembl.org/info/data/ftp/index.html
  - There are many other ways to obtain gene annotation files. For example:
  - UCSC Genome Browser, Ensembl API, BioMart, Entrez, Galaxy, etc. could also be used
- You will download GTF files describing human transcripts (exon coordinates, gene ids, gene symbols, etc.)
- Descriptions of the GTF file format can be found here:
  - http://genome.ucsc.edu/FAQ/FAQformat.html#format4

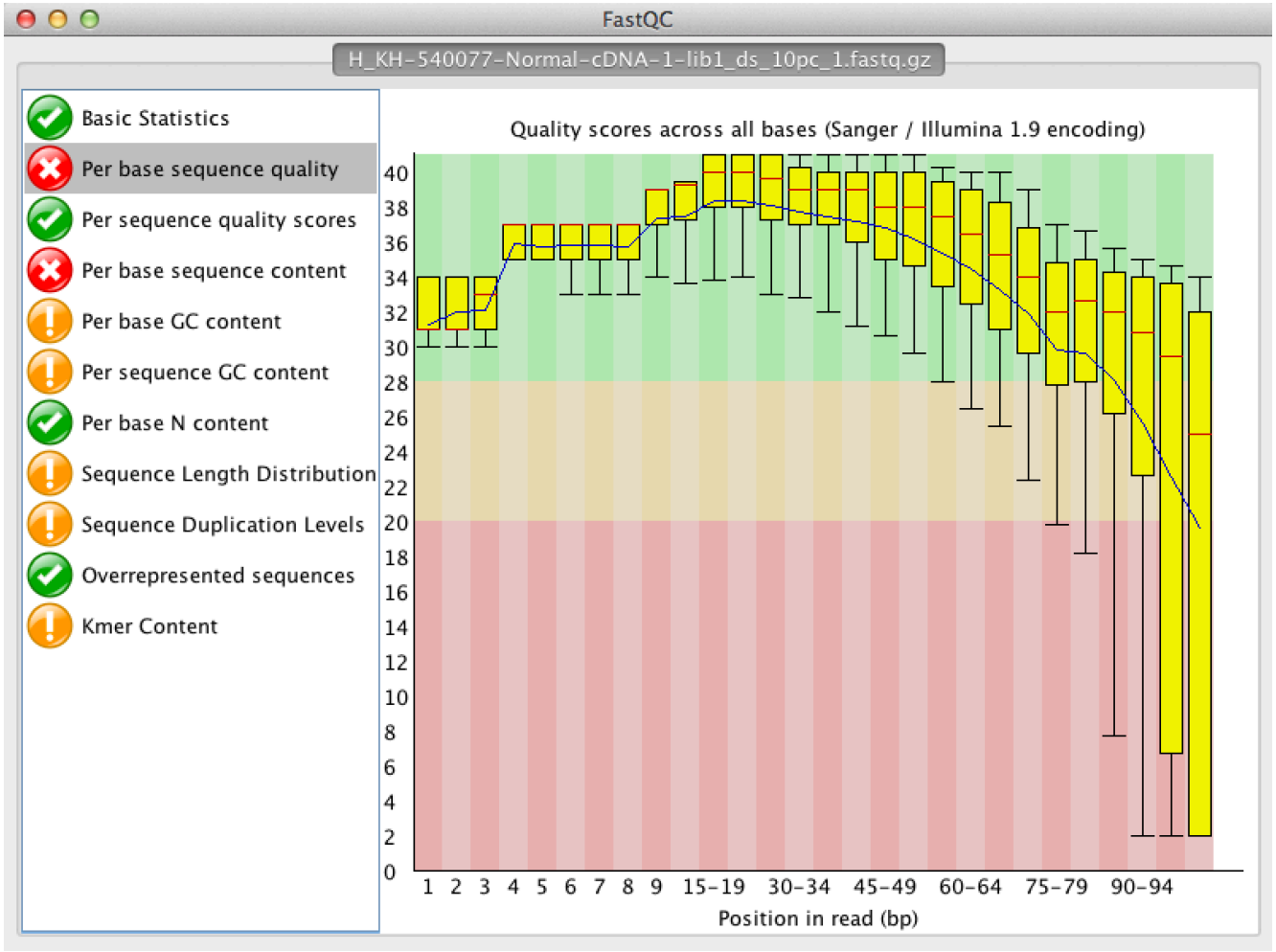
GTF file format example

```
chr6    havana  gene    167607  170631  .       -       .       gene_id "ENSG00000218577"; gene_ver
chr6    havana  transcript      167607  170631  .       -       .       gene_id "ENSG00000218577";
chr6    havana  exon    170352  170631  .       -       .       gene_id "ENSG00000218577"; gene_ver
chr6    havana  exon    169545  169753  .       -       .       gene_id "ENSG00000218577"; gene_ver
chr6    havana  exon    167607  167826  .       -       .       gene_id "ENSG00000218577"; gene_ver
chr6    havana  gene    181466  205484  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  transcript      181466  205484  .       -       .       gene_id "ENSG00000263667";
chr6    havana  exon    204887  205484  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    198165  198509  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    189476  189649  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    187736  187906  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    186356  186561  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    184593  184809  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    182366  182474  .       -       .       gene_id "ENSG00000263667"; gene_ver
chr6    havana  exon    181466  181695  .       -       .       gene_id "ENSG00000263667"; gene_ver
```

Indexing reference genomes

- Before sequences can be mapped to the genome, the reference genome files must be ‘indexed’ in a way that is compatible with the aligner being used
  - Each alignment algorithm generally requires a custom index to be built for that purpose
  - Do not use an index created for another aligner
- In general we will encounter the concept of “indexing” many times throughout the course
  - Reference genomes (FASTA files), reference transcriptomes (GTF files), alignments (BAM files), variants (VCF files), etc.

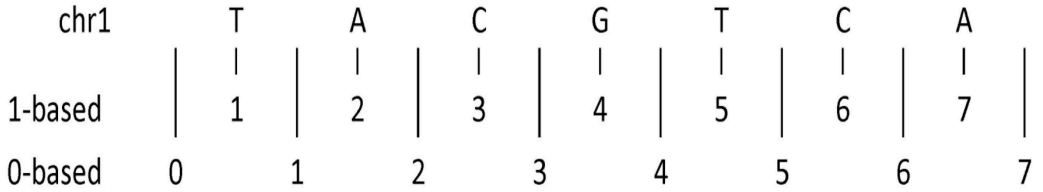
Pre-Alignment QC

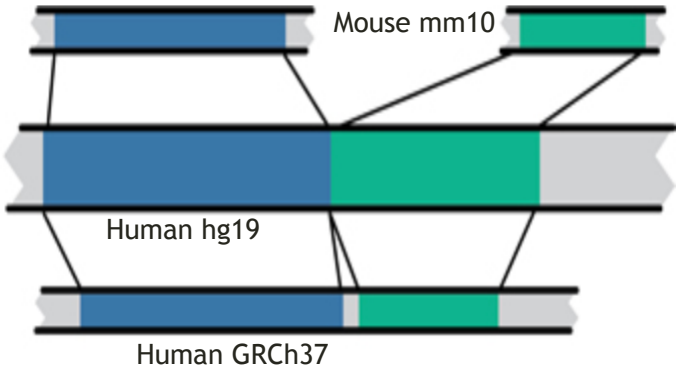


Common sources of confusion

Overview

- Genomic coordinate systems
  - 1-based vs. 0-based
- Genome builds
  - And annotation builds
  - “Liftover” tools
- Left-shifted vs right-shifted





REF	CAC	6	CAC	C	Not left aligned but parsimonious
ALT	C				
REF	GCACA	3	GCACA	GCA	Not right trimmed
ALT	GCA				
REF	GGCA	2	GGCA	GG	Not left trimmed
ALT	GG				
REF	GCA	3	GCA	G	Normalized (left aligned & parsimonious)
ALT	G				

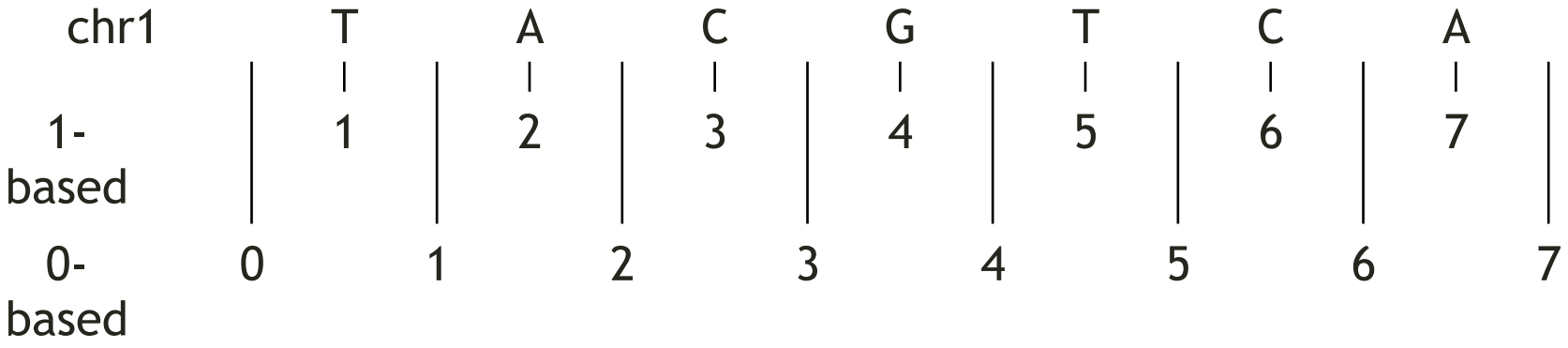
Reference genome assemblies/versions/builds

- NOTE!: Probably ~50% of all bioinformatics results problems where something unexpected is happening involve genome coordinate build incompatibility
  - e.g. reads were aligned to build 37 but you are using transcript annotations based on build 38 coordinates
- Learn to use ‘lift-over’ tools
  - https://www.biostars.org/p/65558/

1- versus 0-based coordinates

- NOTE!: The other ~50% relate to 1 versus 0 based coordinates or conceptually similar data parsing issues
- Learn the basics of these two coordinate systems that are both used ubiquitously in genomics
  - https://www.biostars.org/p/84686/

0-based vs 1-based method to indicate a single nucleotide or variant



	1-based	0-based
Indicate a single nucleotide	chr1:4-4 G	chr1:3-4 G
Indicate a range of nucleotides	chr1:2-4 ACG	chr1:1-4 ACG
Indicate a single nucleotide variant	chr1:5-5 T/A	chr1:4-5 T/A

- 1-based coordinate system
  - Single nucleotides, variant positions, or ranges are specified directly by their corresponding nucleotide numbers
  - GFF, SAM, VCF, Ensembl browser, …
- 0-based coordinate system
  - Single nucleotides, variant positions, or ranges are specified by the coordinates that flank them
  - BED, BAM, UCSC browser, …