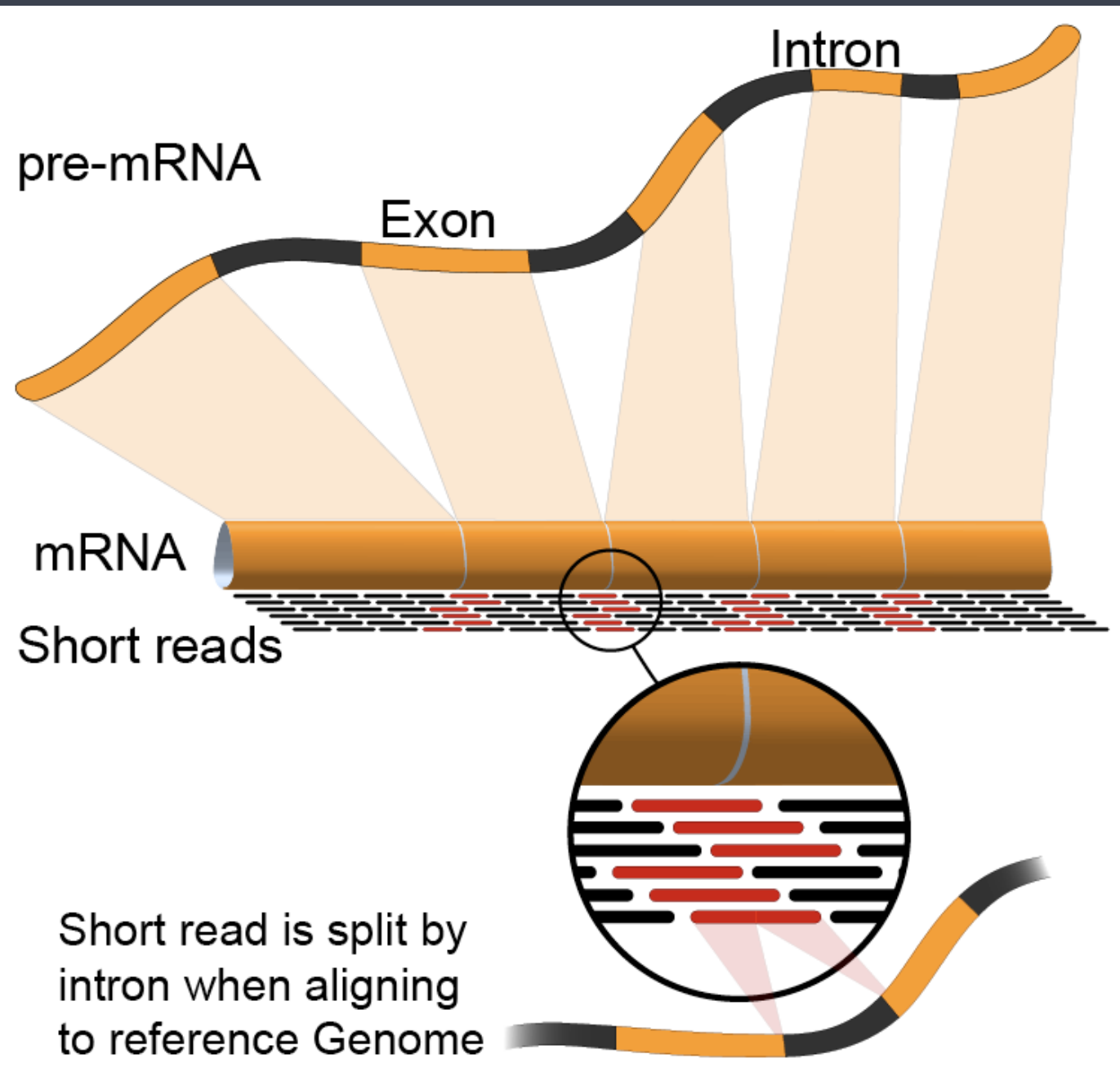


RNA-seq workflow



Transcriptomics (RNA-Seq)

- The process of sequencing the “transcriptome”
- Uses include –
 - Differential Gene Expression
Quantitative evaluation and comparison of transcript levels
 - Transcriptome assembly
Building the profile of transcribed regions of the genome, a qualitative evaluation.
 - Can be used to help build better gene models, and verify them using the assembly
 - Metatranscriptomics or community transcriptome analysis

Biological samples/Library preparation



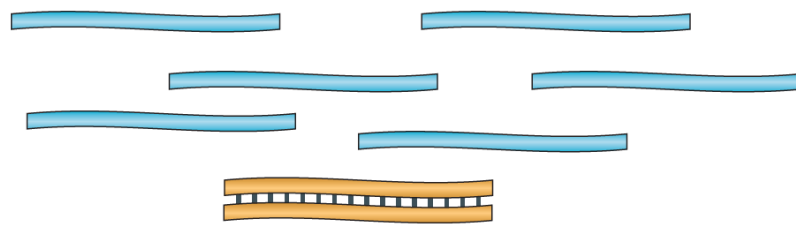
Sequence reads

Analysis Workflow

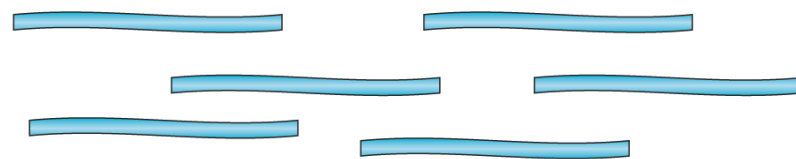
Outline

- Library preparation and sequencing with Illumina
- Experimental and Practical Considerations
- Analysis workflow

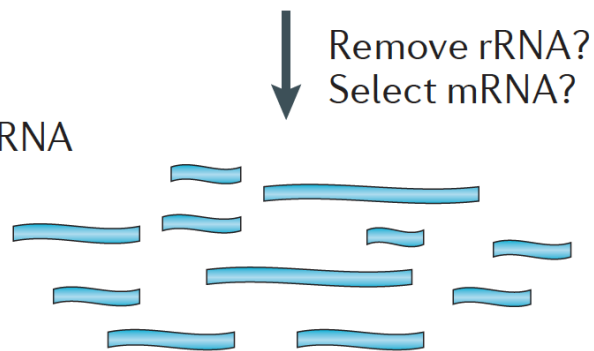
① mRNA or total RNA



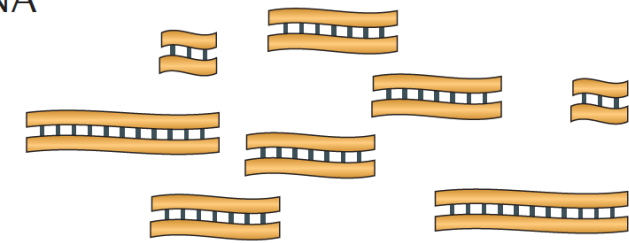
② Remove contaminant DNA



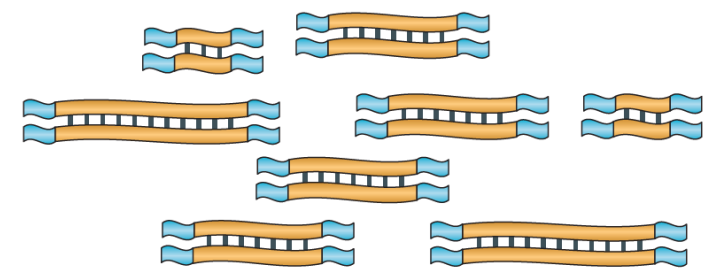
③ Fragment RNA



④ Reverse transcribe into cDNA

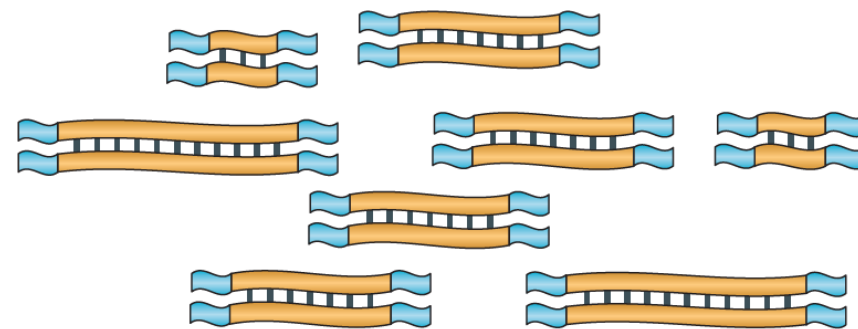


⑤ Ligate sequence adaptors



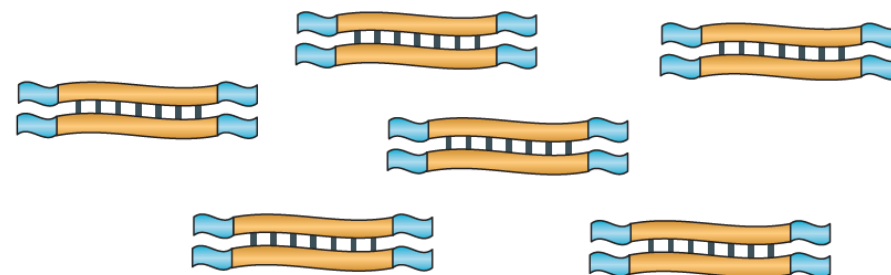
RNA-Seq library prep

⑤ Ligate sequence adaptors ▼

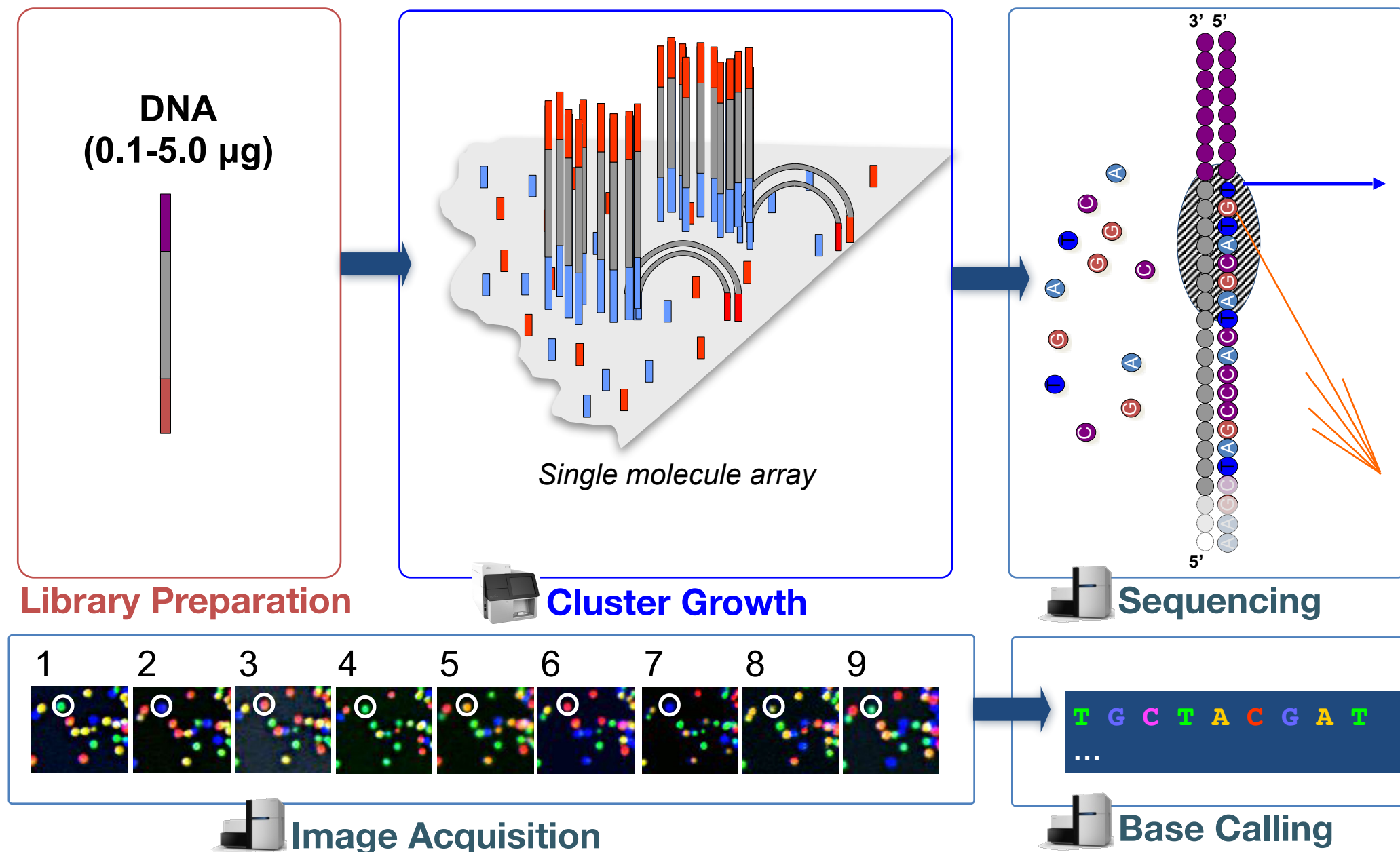


PCR amplification?

⑥ Select a range of sizes



RNA-Seq library prep



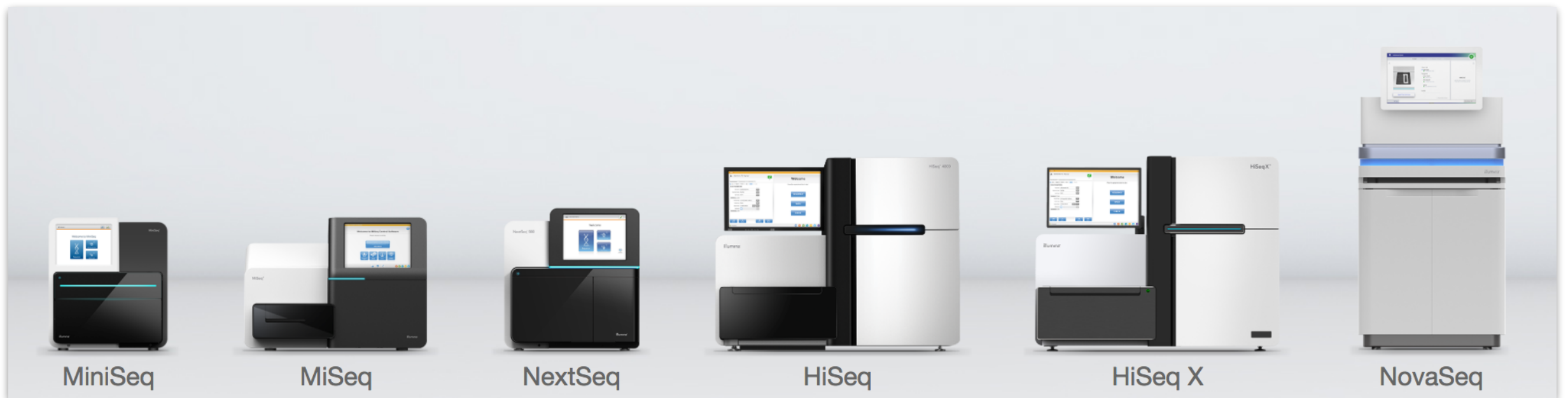
<https://www.youtube.com/watch?v=fCd6B5HRaZ8&t=3s>

Illumina: Sequencing by Synthesis

Number of clusters \sim Number of reads

Number of sequencing cycles \sim Length of reads

Illumina: Sequencing by Synthesis



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

Illumina: Sequencing Platforms

Oxford Nanopore (MinION): <https://nanoporetech.com/>

Pacific Biosciences: <http://www.pacb.com/>

Other Sequencing Platforms

Outline

- Library preparation and sequencing with Illumina
- Experimental and Practical Considerations
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Experimental and Practical considerations

1. Experimental Design
2. Poly(A) enrichment or ribosomal RNA depletion?
3. Single-end or Paired-end data?
4. Stranded libraries?
5. How much sequencing data to collect?
6. Multiplexing

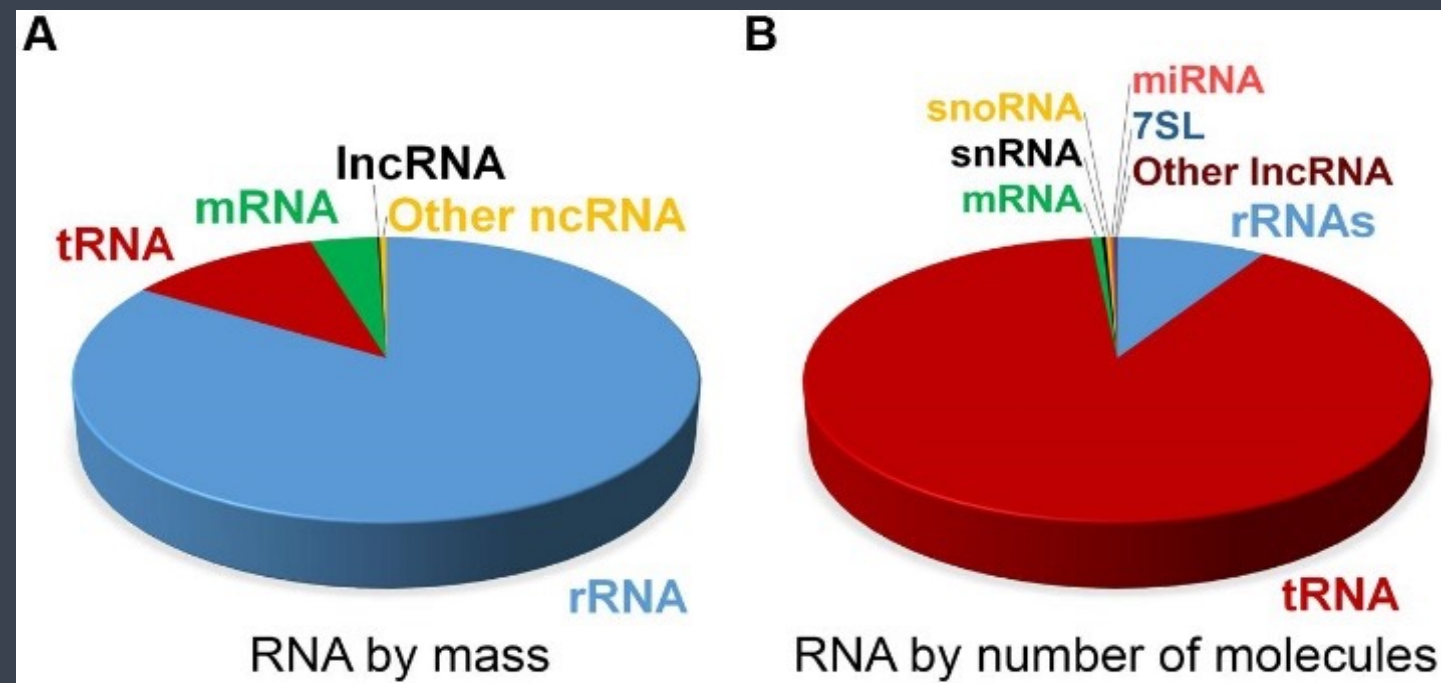
Experimental and Practical considerations

1. Experimental design

- ✦ **Technical replicates**: Illumina has low technical variation unlike microarrays, hence technical replicates are unnecessary.
- ✦ **Biological replicates**, are absolutely essential. Have at least 3!
- ✦ **Batch effects** are still a problem. Be consistent!
- ✦ For differential gene expression, **pooling** RNA from multiple biological replicates can be tricky; do so only if you have multiple pools from each experimental condition.

Experimental and Practical considerations

2. Poly(A) enrichment or ribosomal RNA depletion?



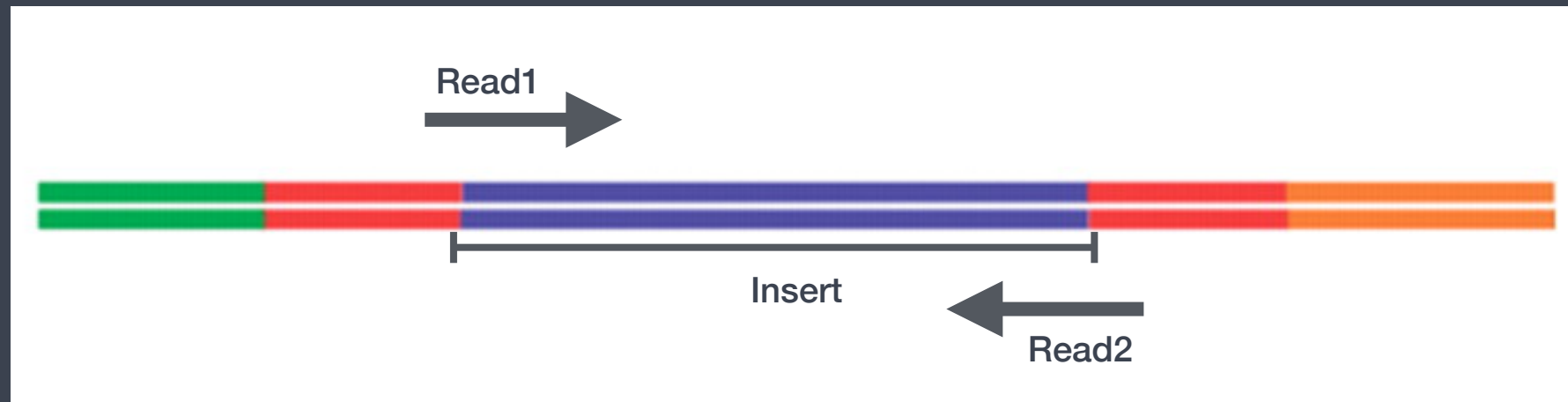
Depends on which RNA entities you are interested in...

- ✦ For differential gene expression, it is best to enrich for Poly(A)+
 - EXCEPTION – If you are aiming to obtain information about long non-coding RNAs, then do a ribosomal RNA depletion.

Experimental and Practical considerations

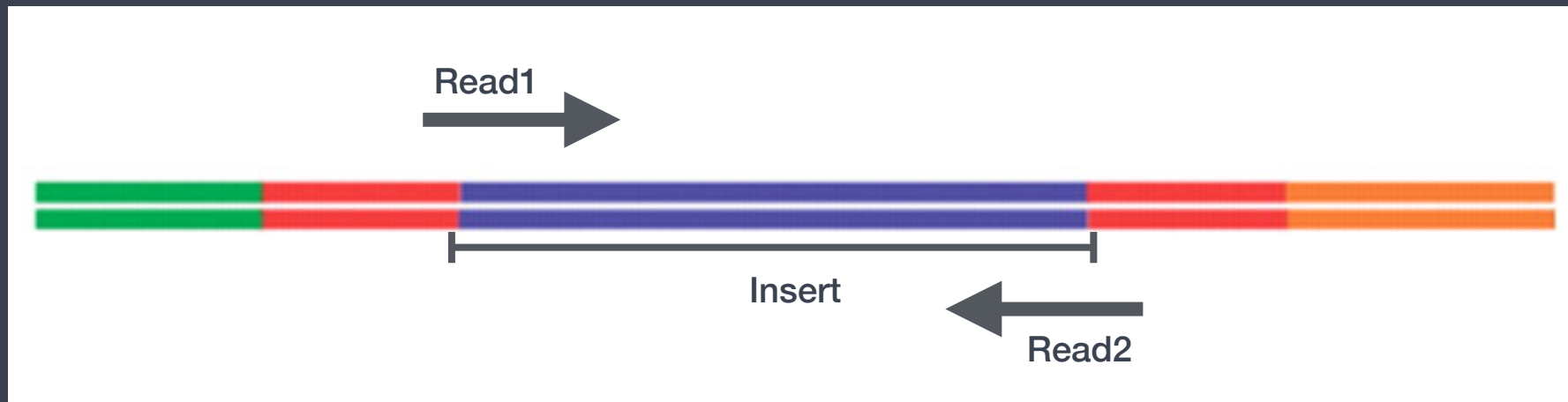
3. Single-end or Paired-end data?

Depends on your goals, paired-end reads are better for reads that map to multiple locations, for assemblies and for splice isoform differentiation.



- ✓ SE - Single end dataset => Only Read1
- ✓ PE - Paired-end dataset => Read1 + Read2
 - can be 2 separate FASTQ files or just one with interleaved pairs

Options for sequencing



- ✓ SE - Single end dataset => Only Read1
- ✓ PE - Paired-end dataset => Read1 + Read2
 - can be 2 separate FASTQ files or just one with interleaved pairs
- ✓ Fragment length: ~300-500bp
- ✓ Read length: 50bp - 300bp, depends on the sequencer (HiSeq2500, MiSeq, NextSeq)

Options for sequencing

Experimental and Practical considerations

3. Single-end or Paired-end data?

Depends on your goals, paired-end reads are better for reads that map to multiple locations, for assemblies, and for splice isoform differentiation.

- ✦ For differential gene expression, which one you pick depends on-
 - If you are specifically interested in **isoform-level differences**
 - The abundance of **paralogous genes** in your system of interest
 - Your **budget**, paired-end data is usually 2x more expensive

Experimental and Practical considerations

4. Stranded libraries?

Stranded libraries are now standard with Illumina's TruSeq stranded RNA-Seq kits. This means that with a great amount of certainty you can identify which strand of DNA the RNA was transcribed from.

3 types of libraries –

- ✦ Reverse (firststrand)– reads resemble the complementary sequence (TruSeq)
- ✦ Unstranded
- ✦ Forward (secondstrand) – reads resemble the gene sequence

Experimental and Practical considerations

5. How much sequencing data to collect?

- ✦ Only ~2% of the human genome transcribes protein-coding RNA
- ✦ Some mRNAs will be much more abundant than others
- ✦ Some genes are much longer than others

Recommendations:

- ✦ For human samples ~30-50 million reads/sample (ENCODE guidelines)
- ✦ Modify that number based on the size of your transcriptome (crude estimate)
- ✦ If working with a tight budget:

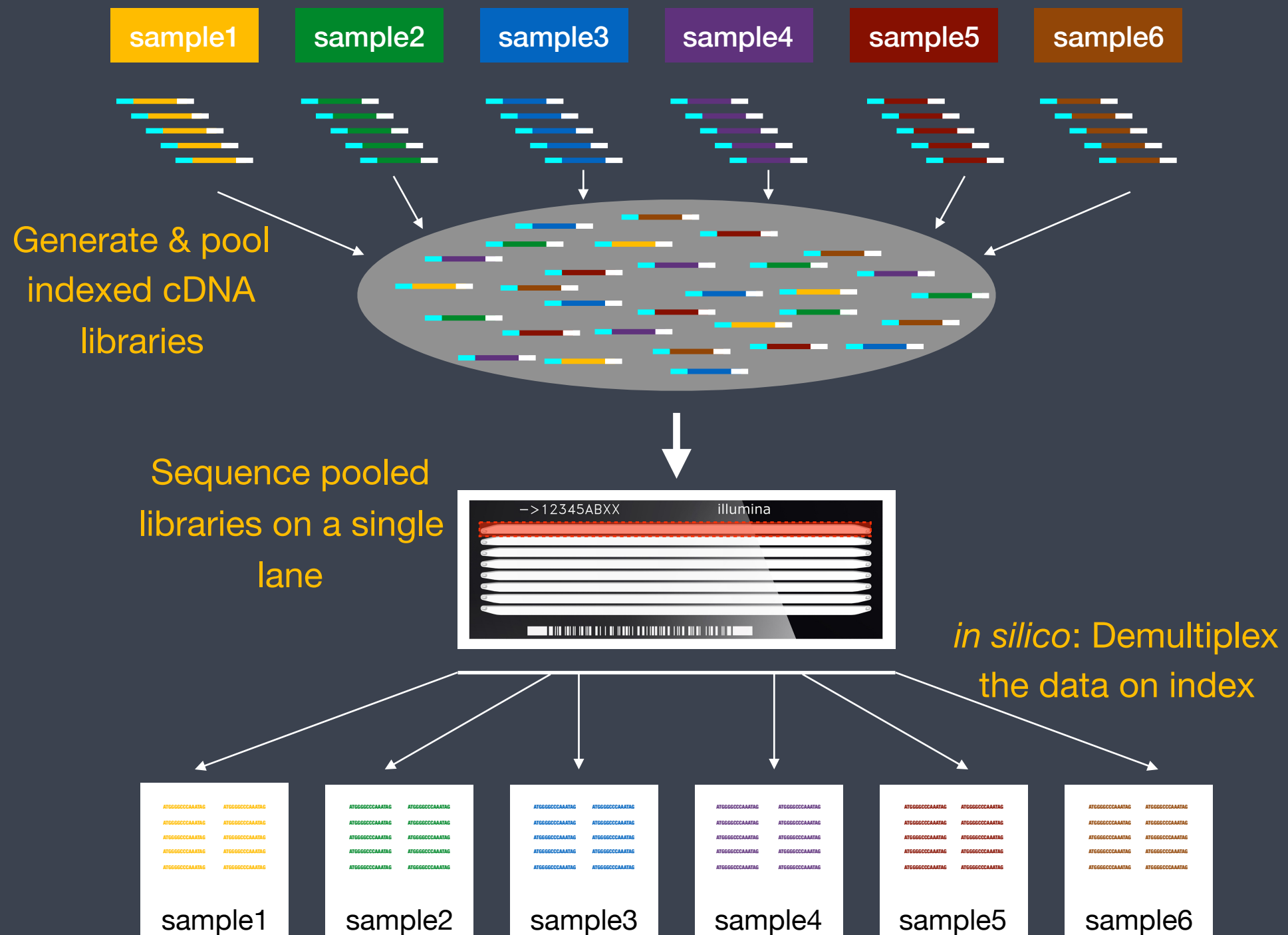
More replicates >> More reads (for standard differential expression analysis)

Experimental and Practical considerations

6. Multiplexing (with barcodes and indices)

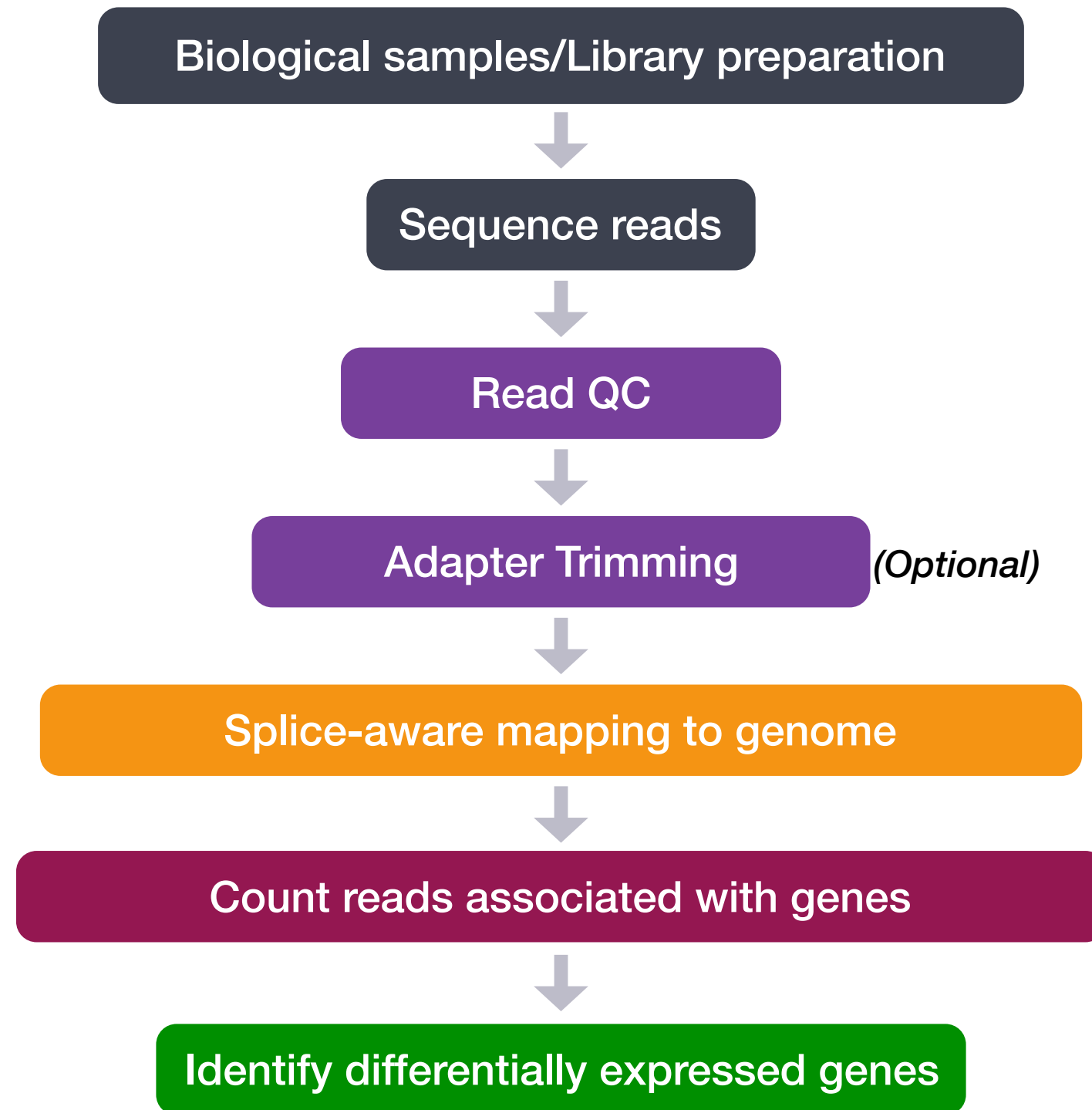
- ✦ Charges for sequencing are usually per lane of the flow cell
- ✦ Each lane generates ~150 million reads
- ✦ For RNA-Seq, the required data per sample is much lower than that
- ✦ Sequencing of multiple samples per lane possible with addition of indices (within the Illumina adapter) or special barcodes (outside the Illumina adapter).

6. Multiplexing (with barcodes and indices)

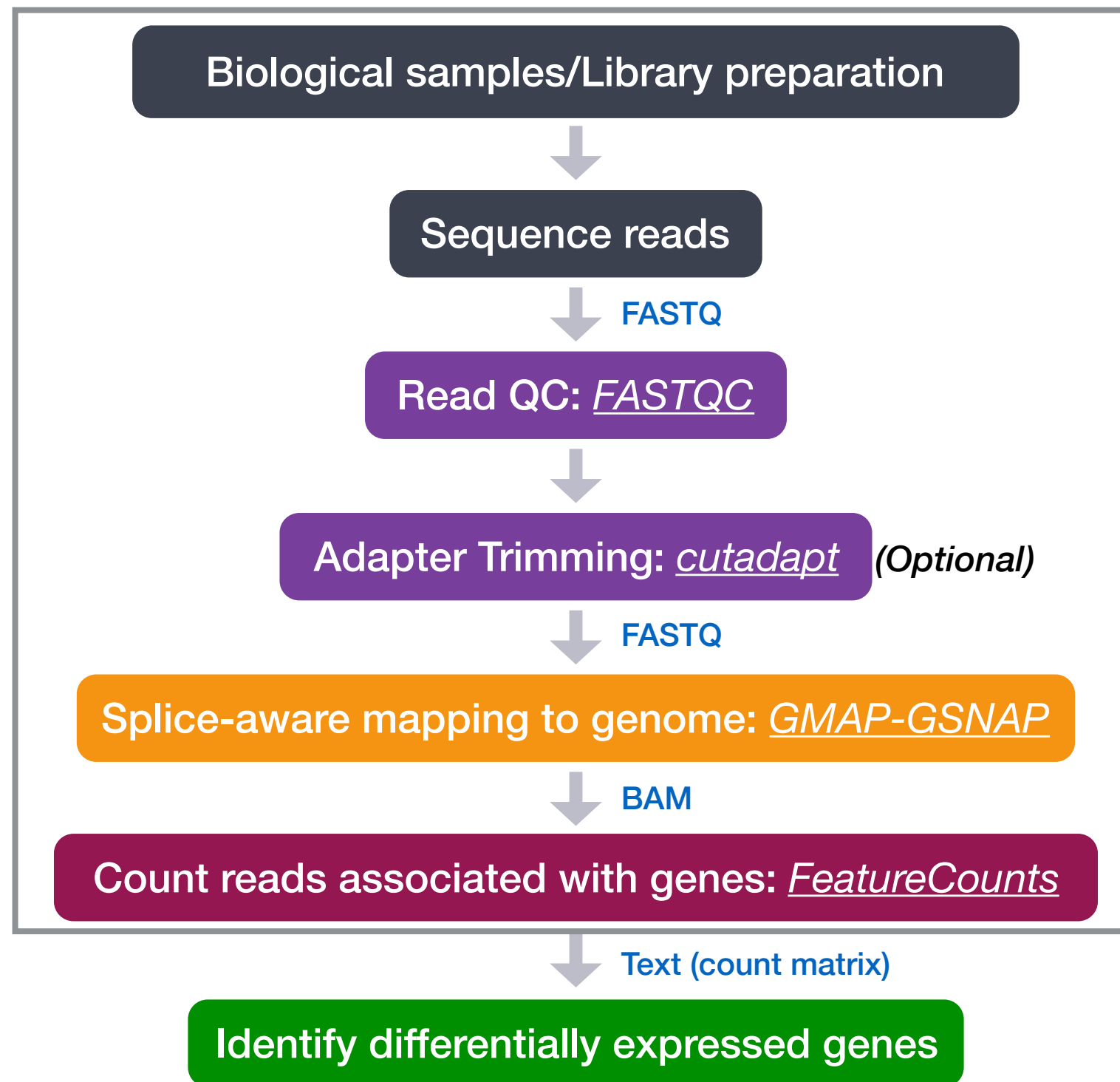


Outline

- Library preparation and sequencing with Illumina
- Experimental and Practical Considerations
- Analysis workflow



Analysis Workflow



Analysis Workflow

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