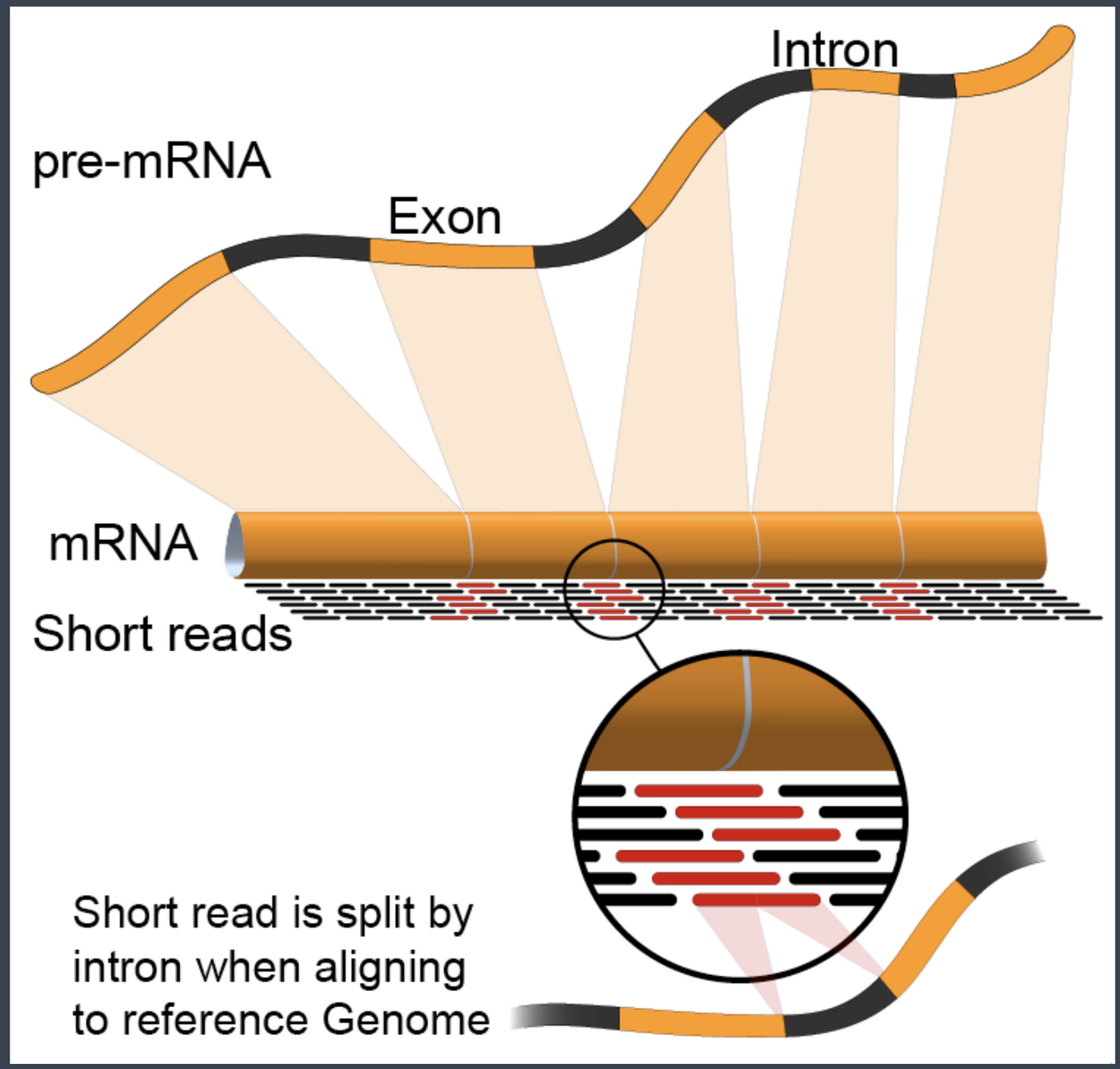
# RNA-seq workflow




http://upload.wikimedia.org/wikipedia/commons/0/01/RNA-Seq-alignment.png

# 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

# Outline

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

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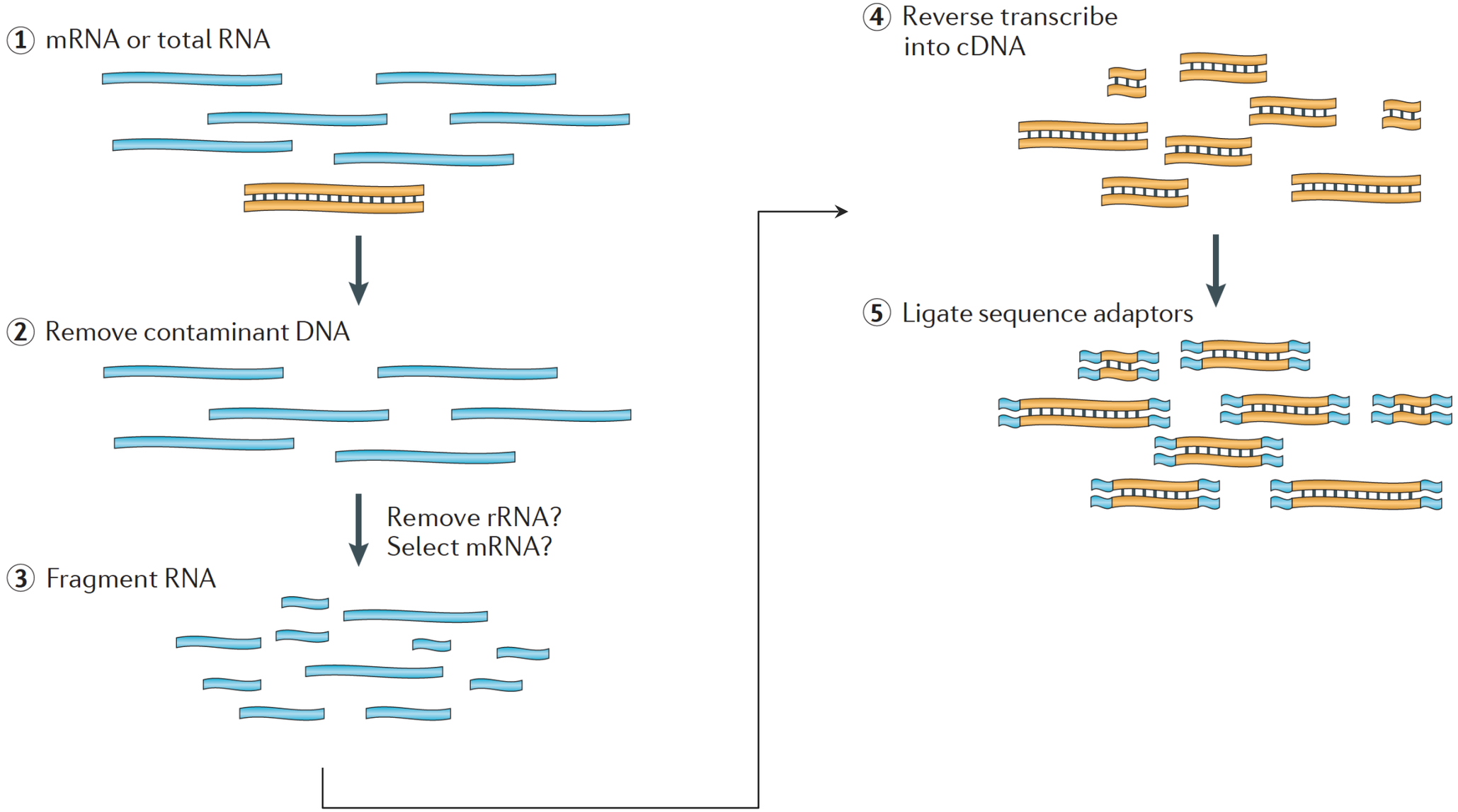
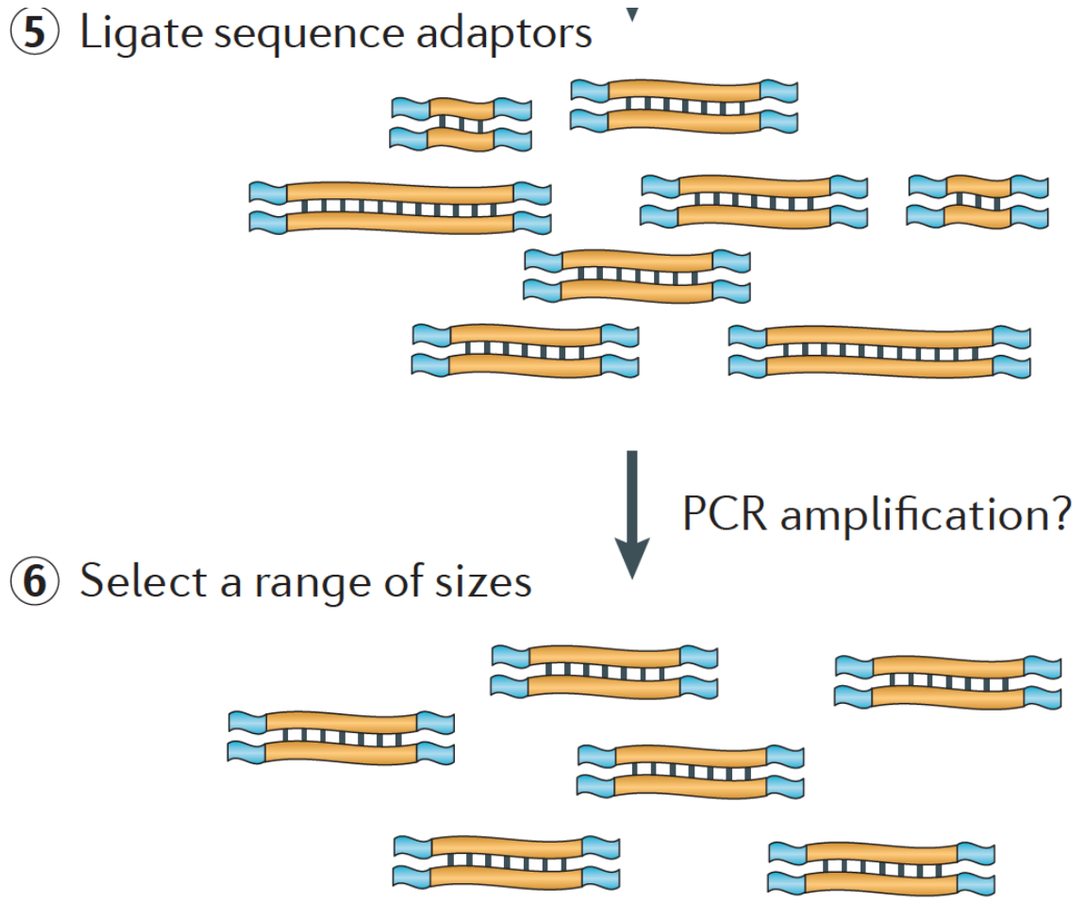



# RNA-Seq library prep

http://rnaseq.uoregon.edu/#rna-prep

Martin J.A. and Wang Z., Nat. Rev. Genet. (2011) 12:671–682

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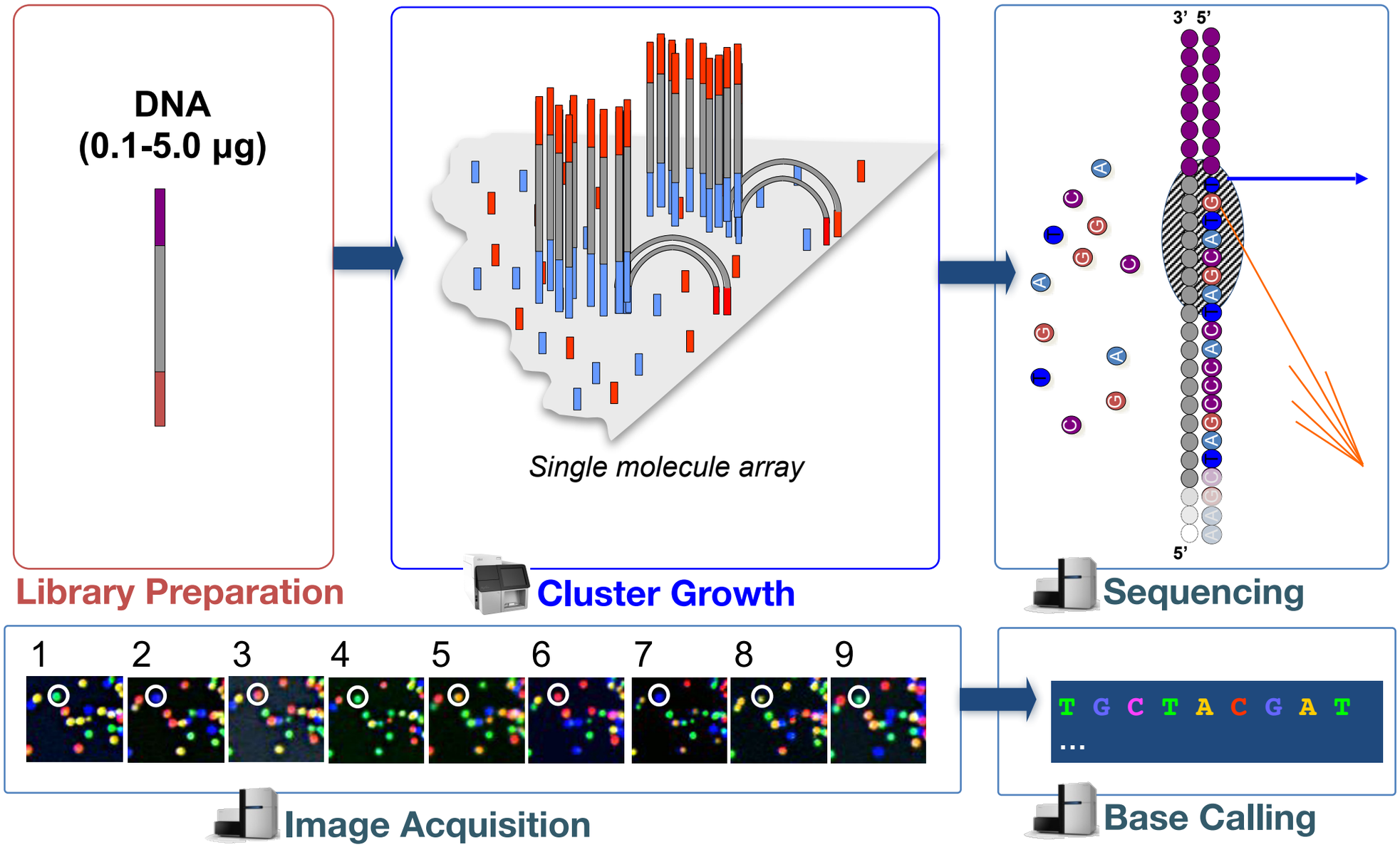


https://www.youtube.com/watch?v=fCd6B5HRaZ8&t=3s

# Illumina: Sequencing by Synthesis

Number of clusters ~= Number of reads

Number of sequencing cycles ~= 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

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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

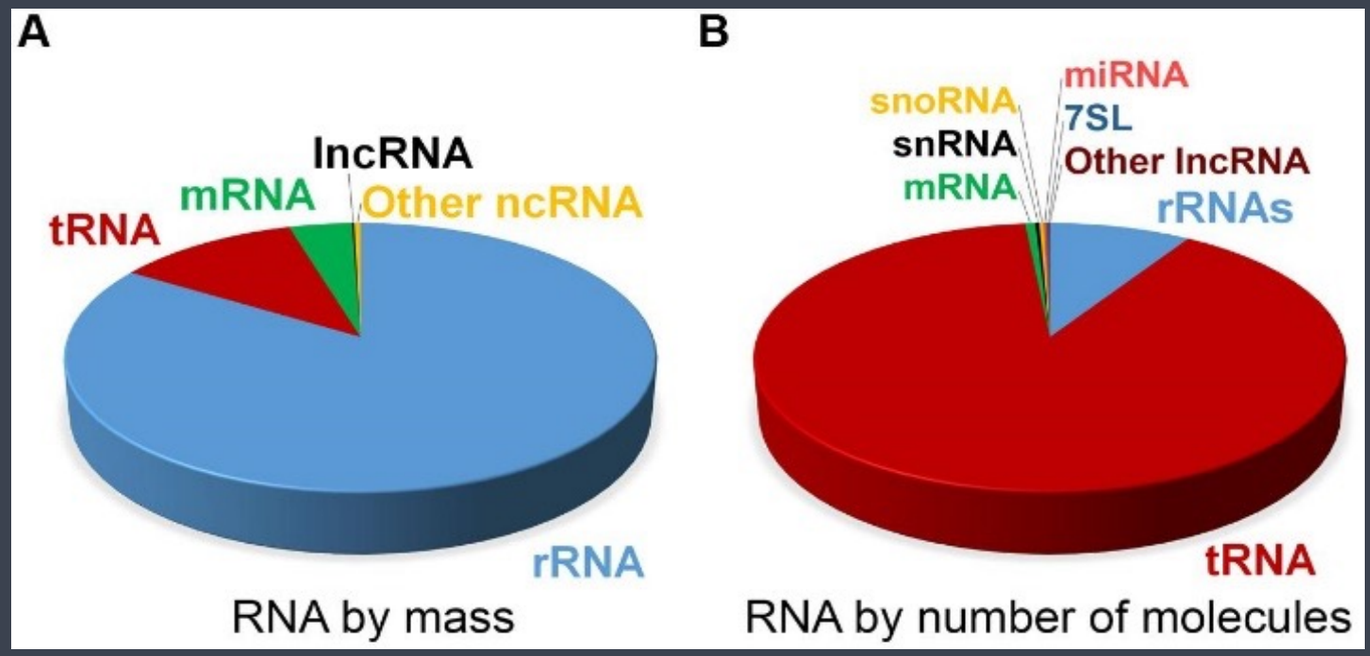
# Experimental and Practical considerations

## 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?



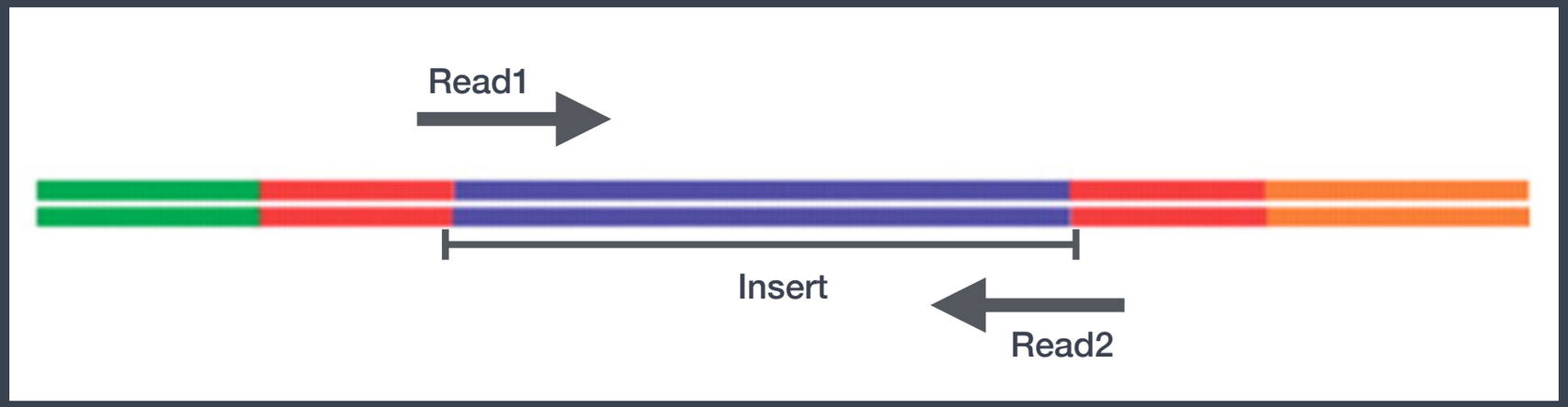

Depends on which RNA entities you are interested in…

- 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.

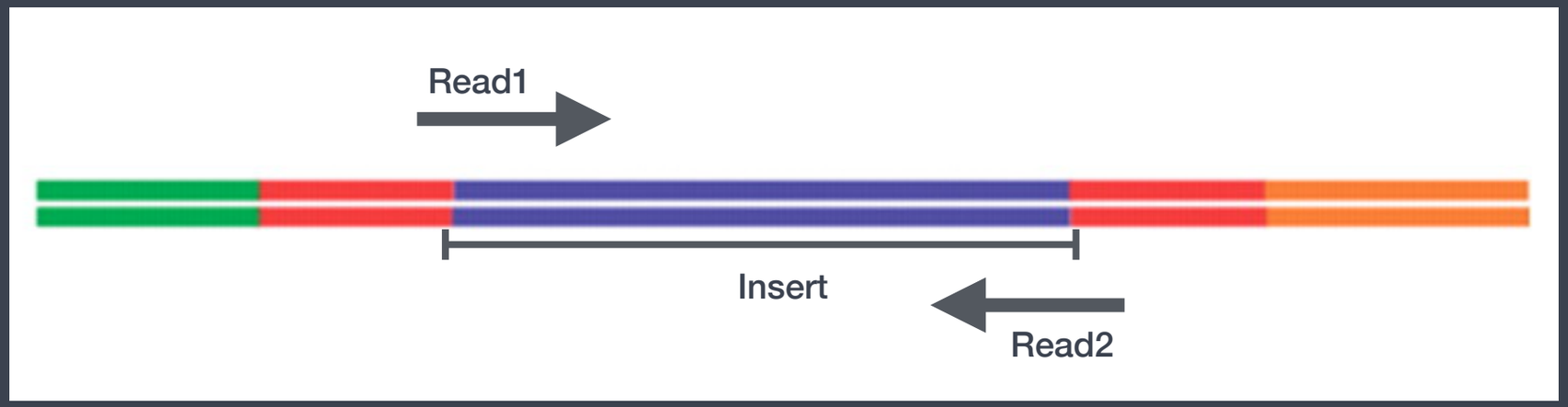



- ✓ SE - Single end dataset => Only Read1
- ✓ PE - Paired-end dataset => Read1 + Read2

  - can be 2 separate FASTQ files or just one with interleaved pairs

# Options for sequencing

http://tucf-genomics.tufts.edu/home/faq




- ✓ 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

http://tucf-genomics.tufts.edu/home/faq

# 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:

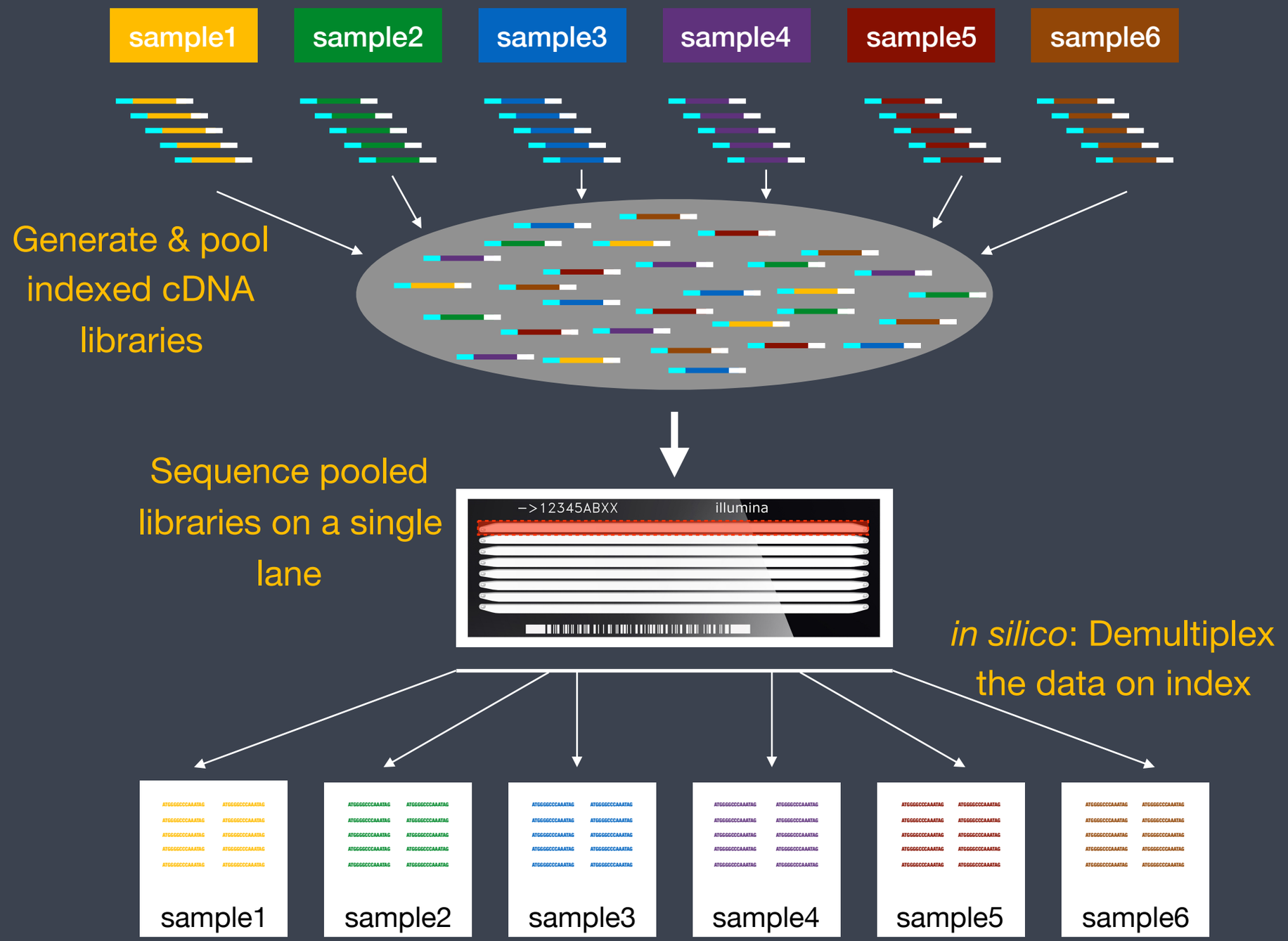
  More replicates >> More reads (for standard differential expression analysis)

# 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)



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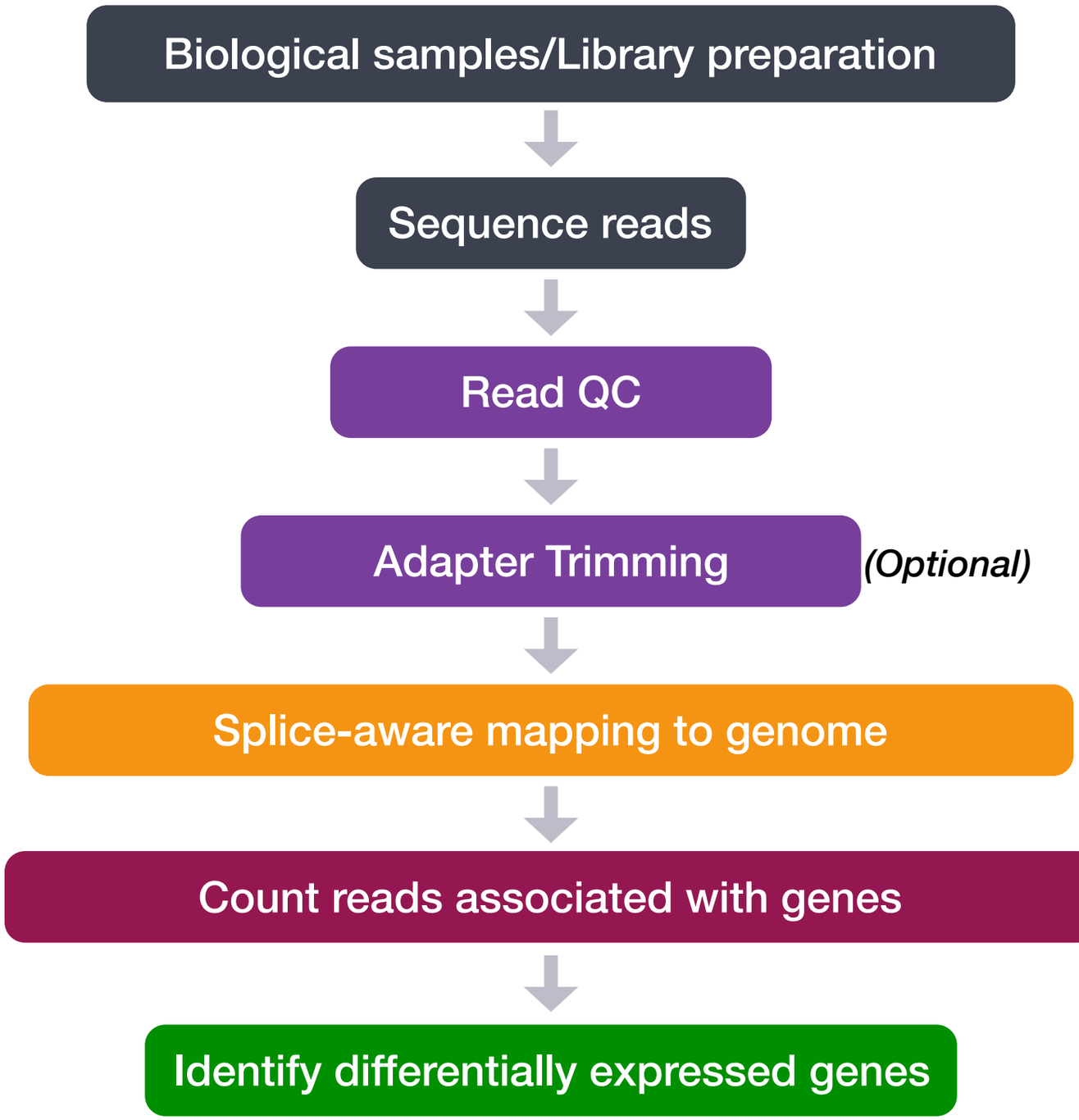
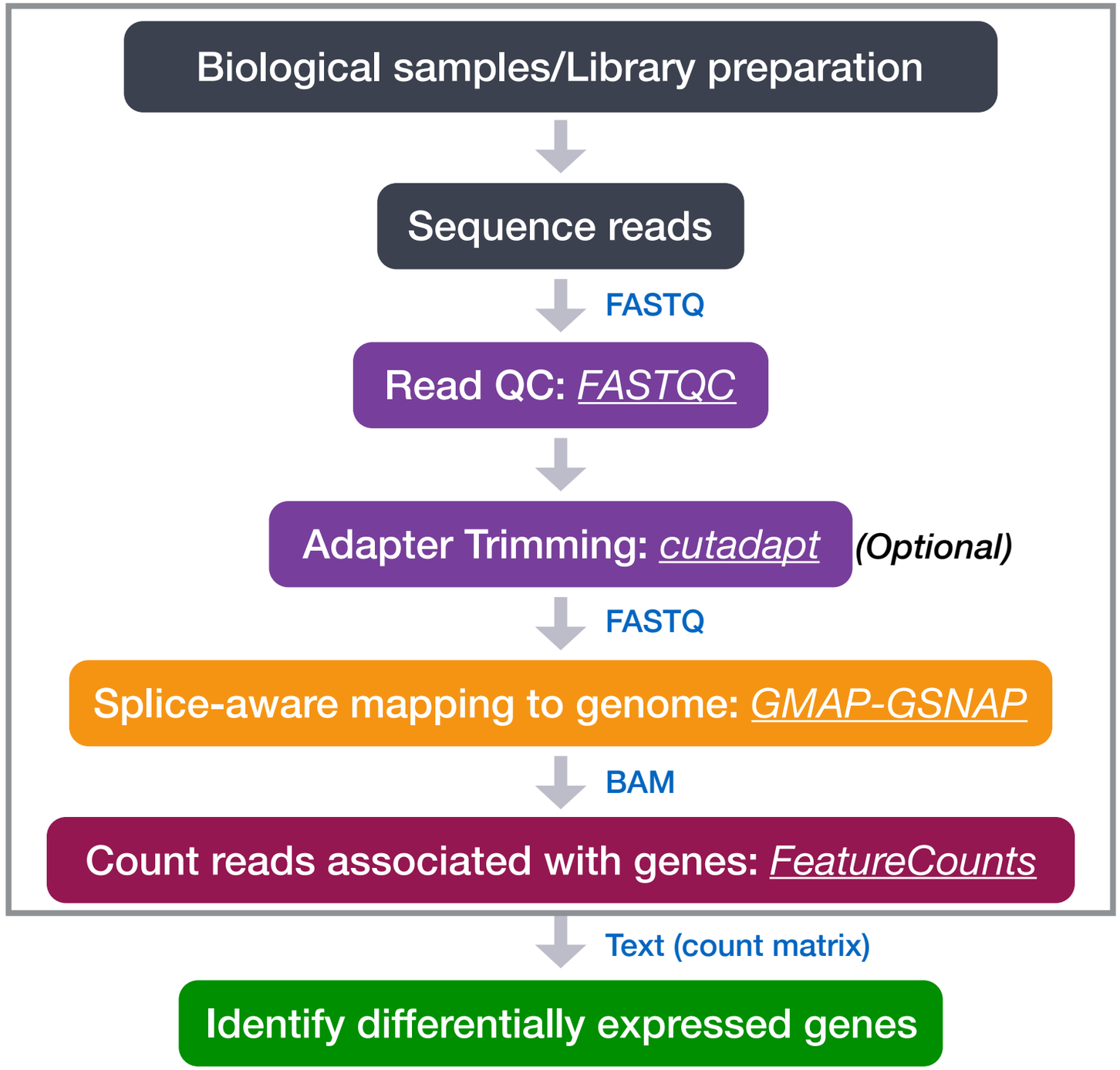



# Analysis Workflow




# Analysis Workflow