National Aeronautics and Space Administration

Short Read Sequencing Overview

GL4U: Introduction 2024
Lecture 4 of 4

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Contractor: KBR

Biological & **P**hysical **S**ciences

Sequencing Through the Ages

1977

1990

Now

∞

1st Generation Sanger Sequencing

Assembly required

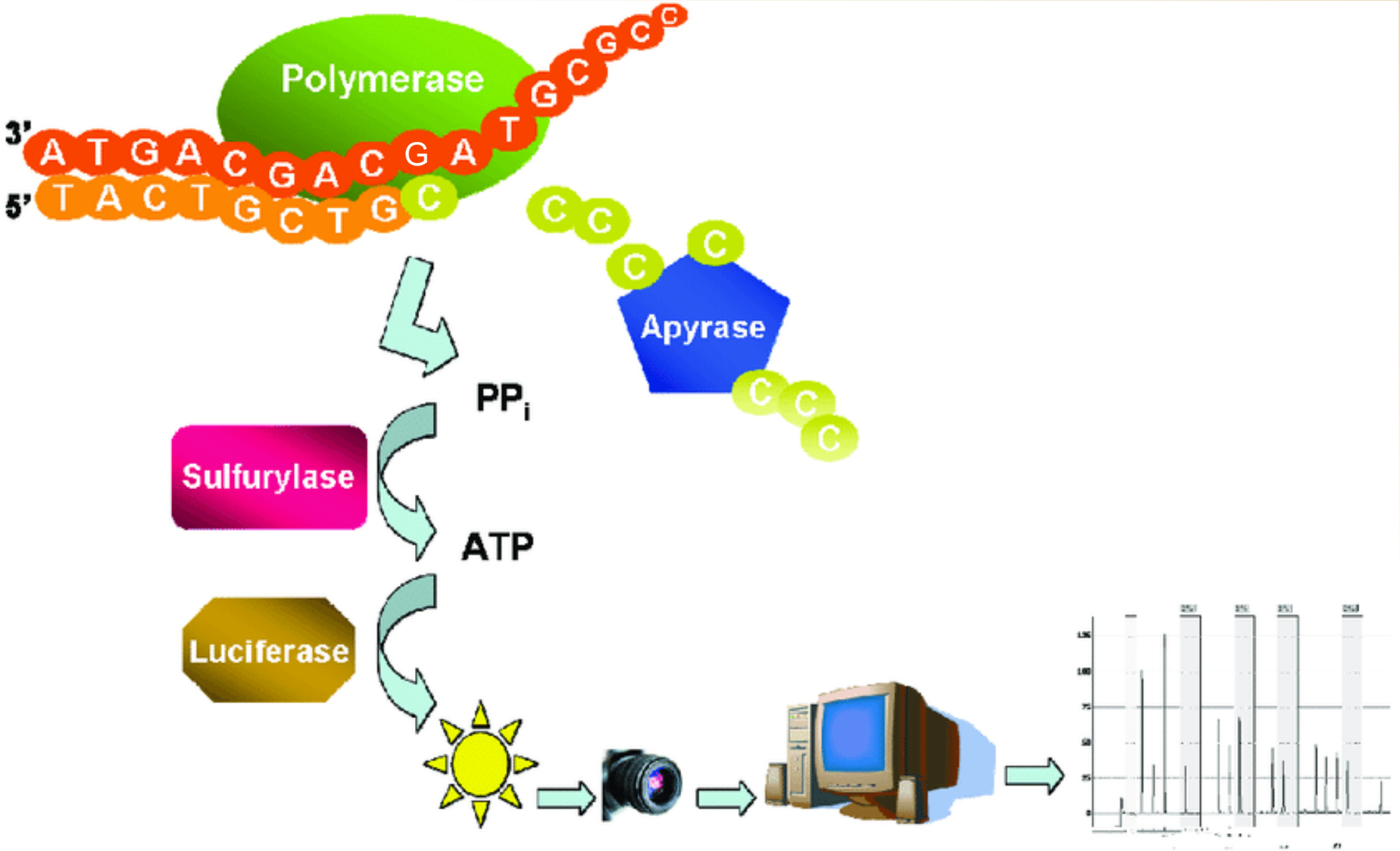
2nd Generation "NextGen" Pyrosequencing

Assembly required

3rd Generation Single Molecule Nanopores

Not much assembly required

Pyrosequencing aka Sequencing by Synthesis



https://www.youtube.com/watch?v=wY8to-_zAEo

https://www.dissertations.se/dissertation/e7acf1968c/

Illumina Sequencing Workflow

- Illumina is a very successful biotech company specializing in next generation technology that uses the pyrosequencing method
- ~90% of all sequencing worldwide is performed on an Illumina instrument (including GeneLab)
- The Illumina sequencing workflow has the following 3 steps:

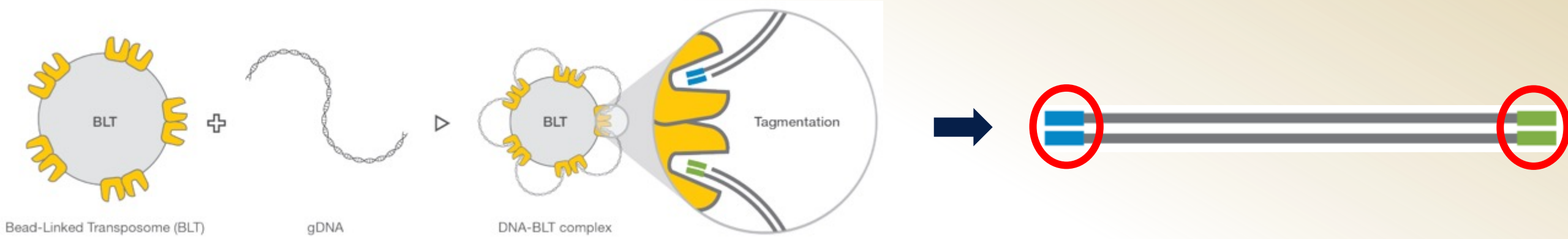
  - Library Construction
  - Cluster Formation
  - Sequencing

Library Preparation
DNA Sequencing

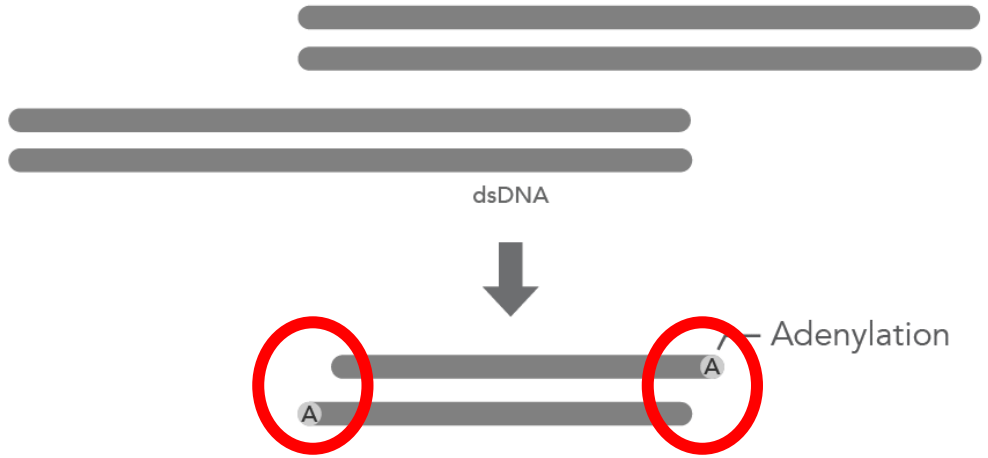
DNAseq: Library Preparation

Step 1: Create DNA fragments (with a means to attach adapters) from the extracted sample DNA

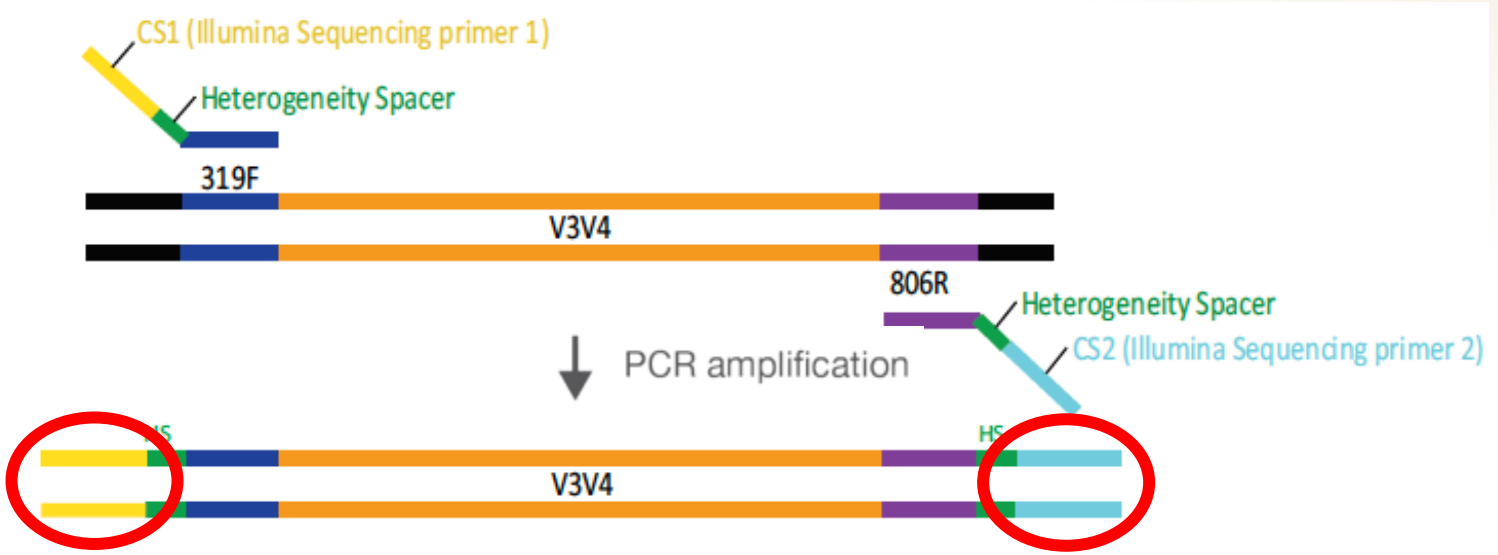
Tagmentation: Illumina DNA Prep (formerly Nextera Flex)



Fragmentation/A-tailing: IDT



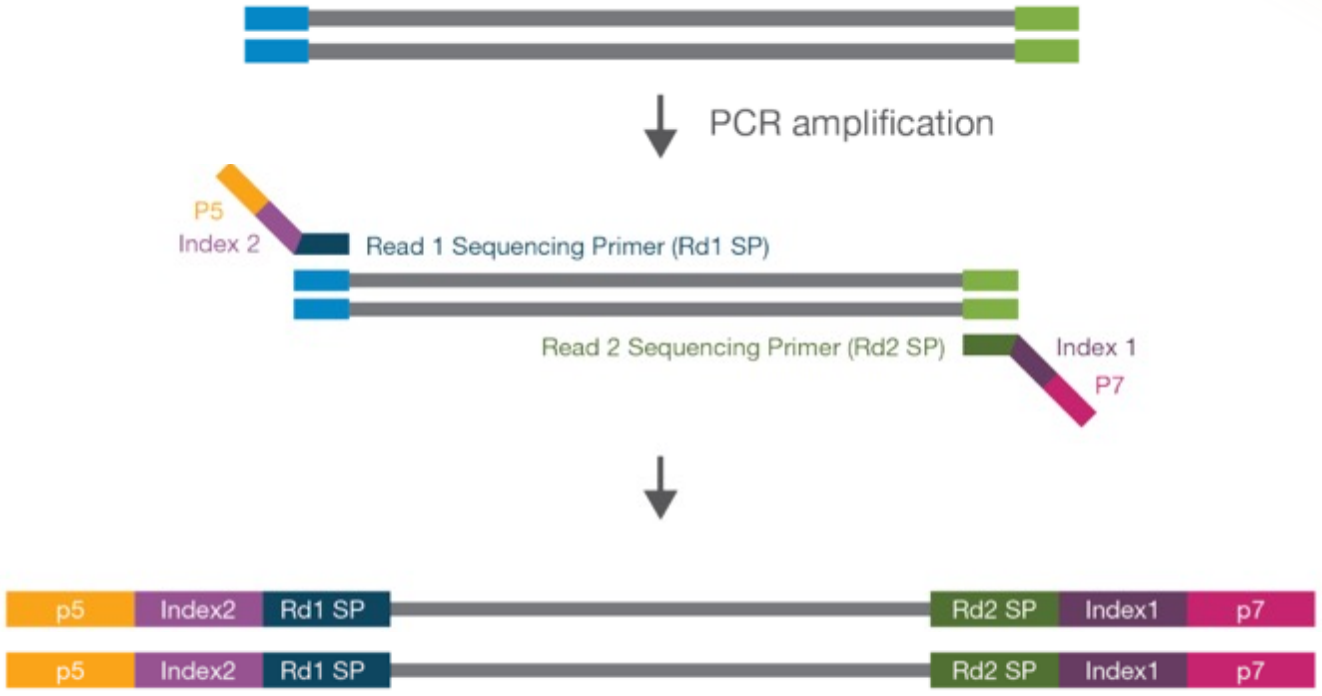
Target PCR: Illumina Amplicon



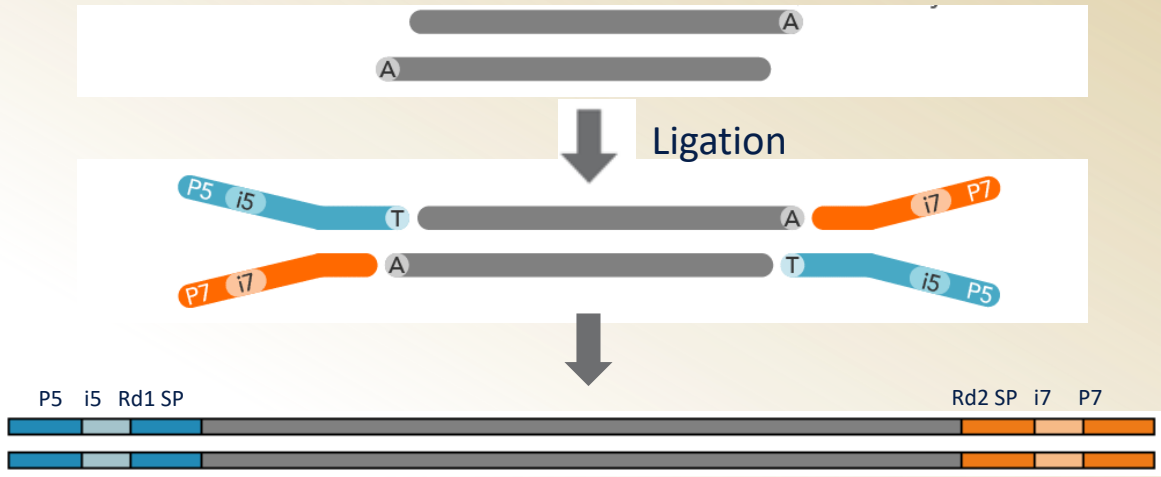
DNAseq: Library Preparation

Step 2: Attach adapters

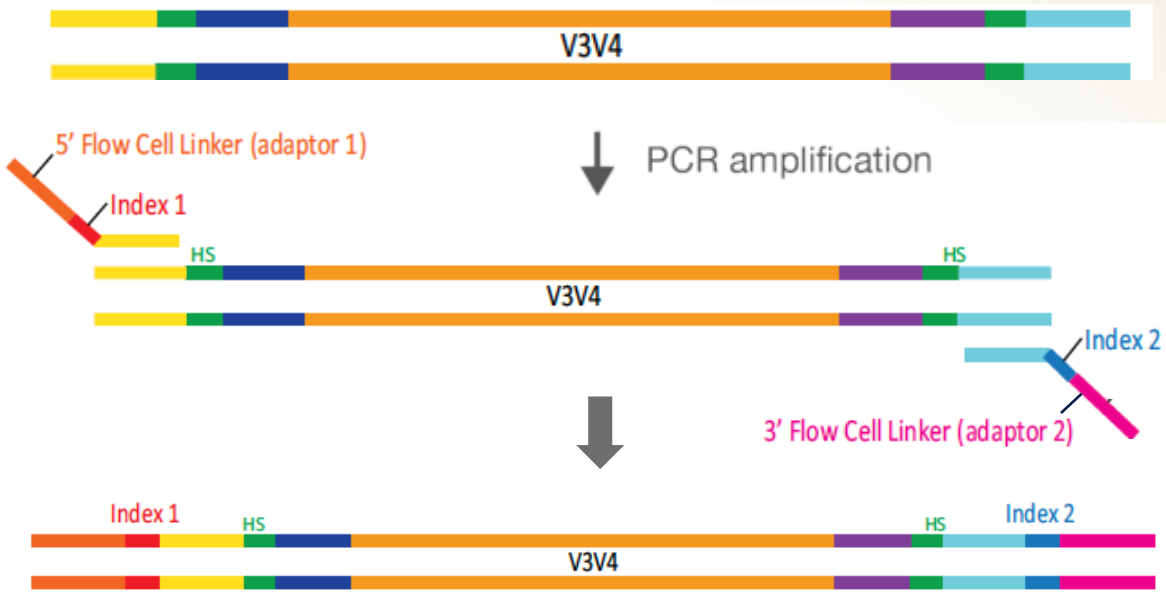
Tagmentation: Illumina DNA Prep



Fragmentation/A-tailing: IDT



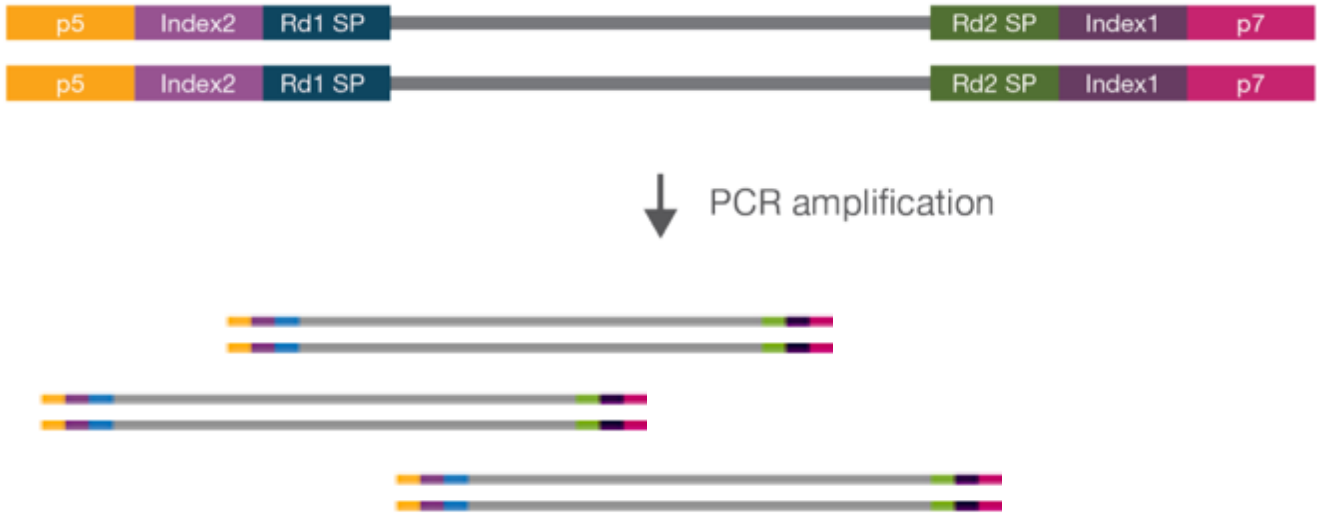
Target PCR: Illumina Amplicon



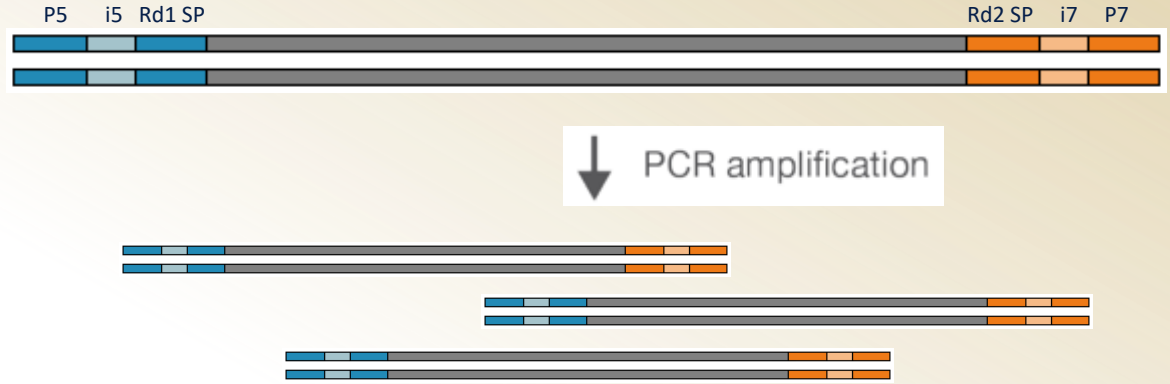
DNAseq: Library Preparation

Step 3: Amplify libraries

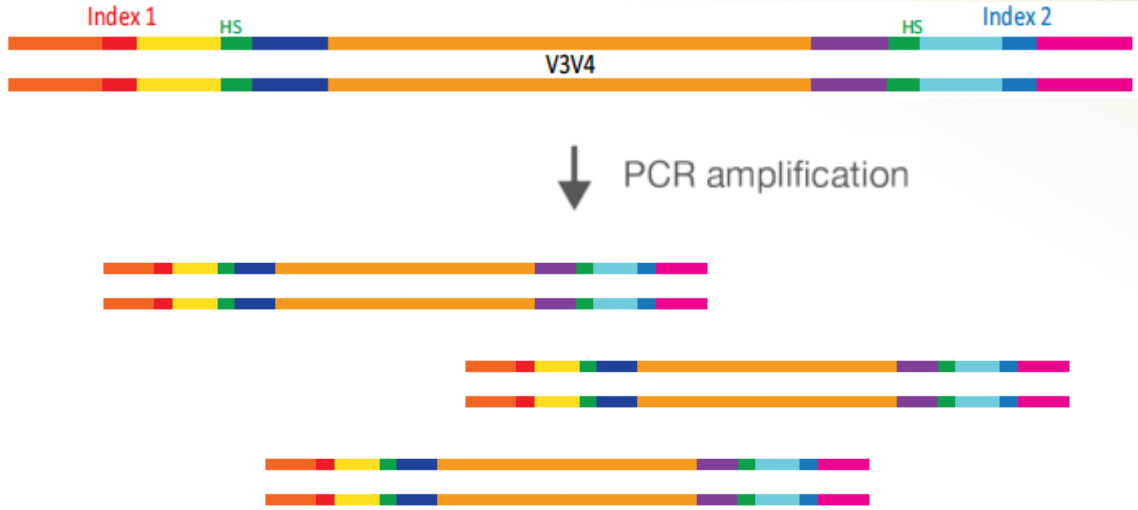
Tagmentation: Illumina DNA Prep



Fragmentation/A-tailing: IDT

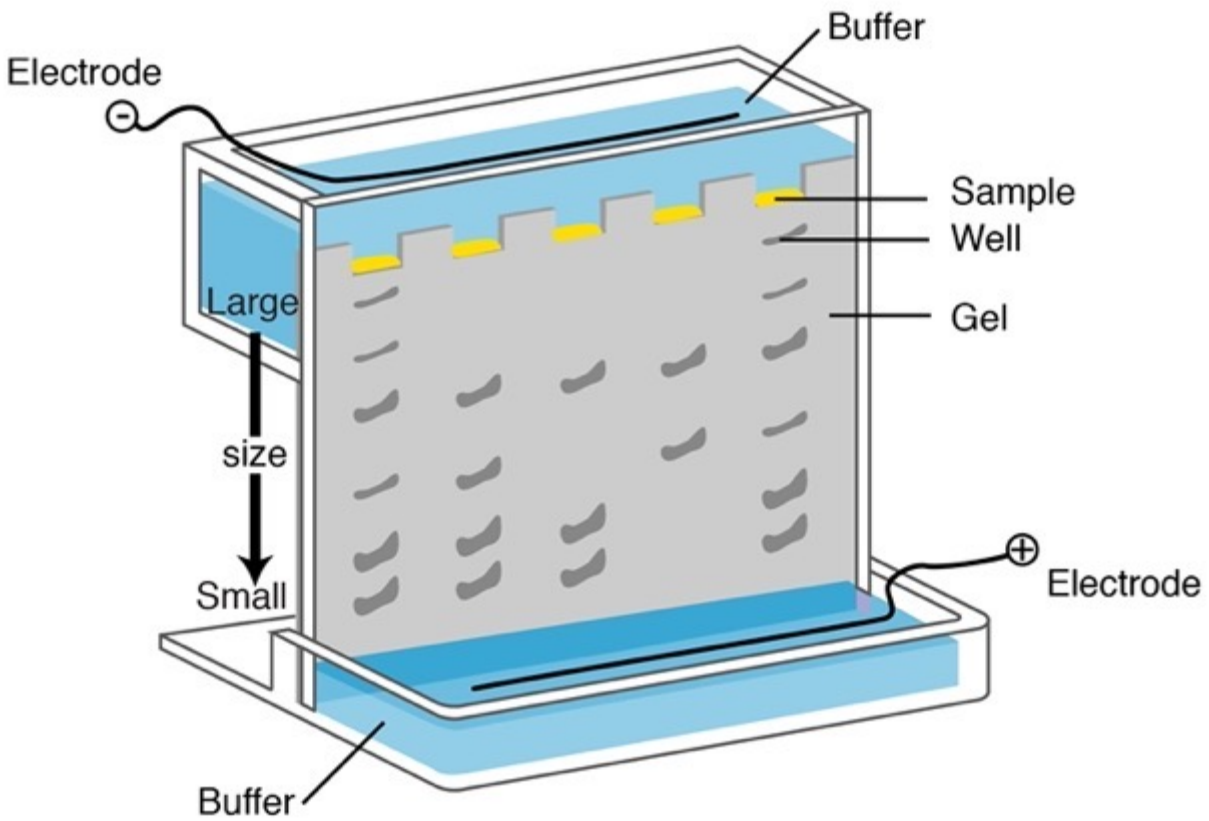


Target PCR: Illumina Amplicon

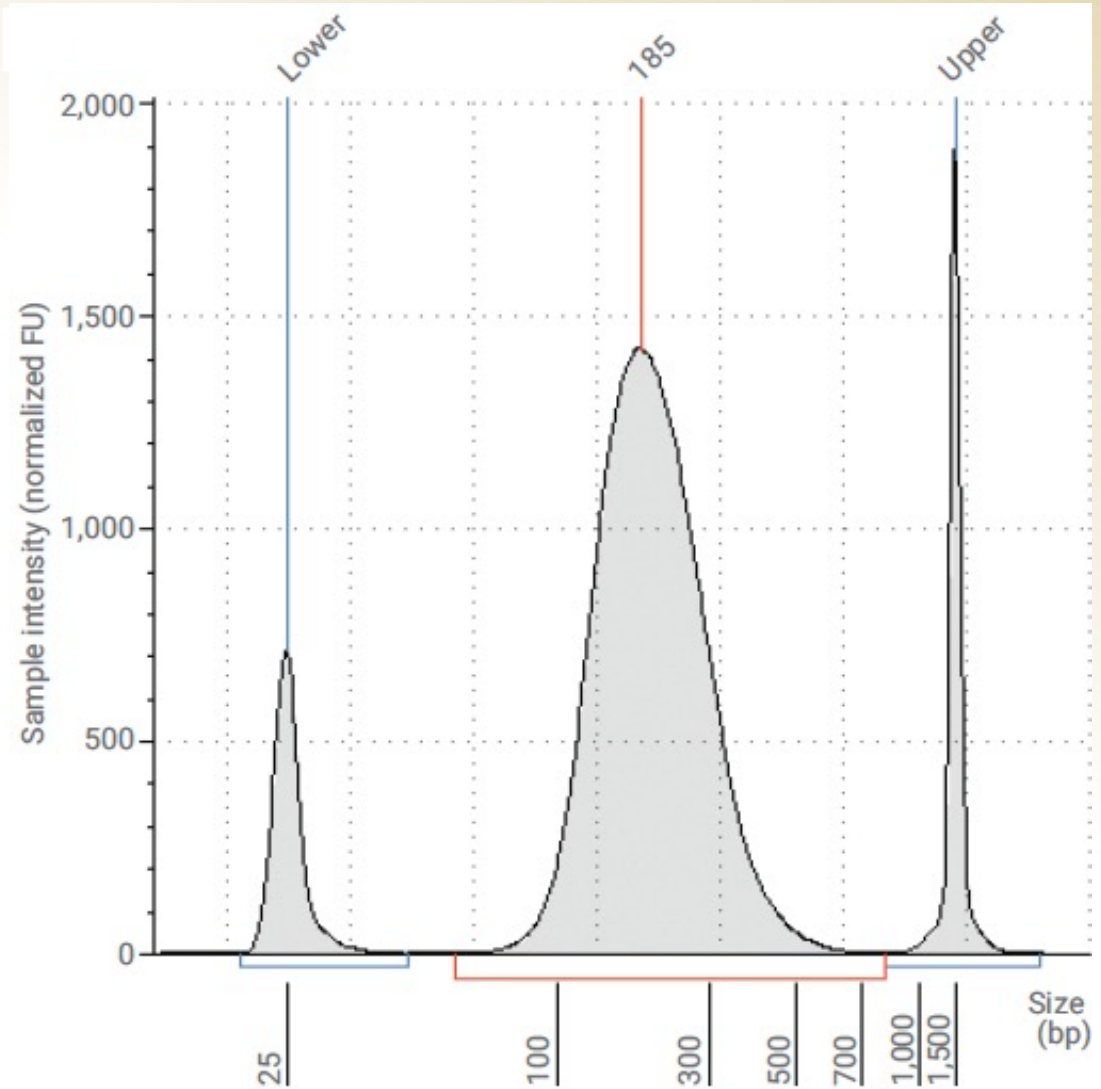


DNA Library QC: Electrophoresis and Electropherogram

Electrophoresis



Electropherogram



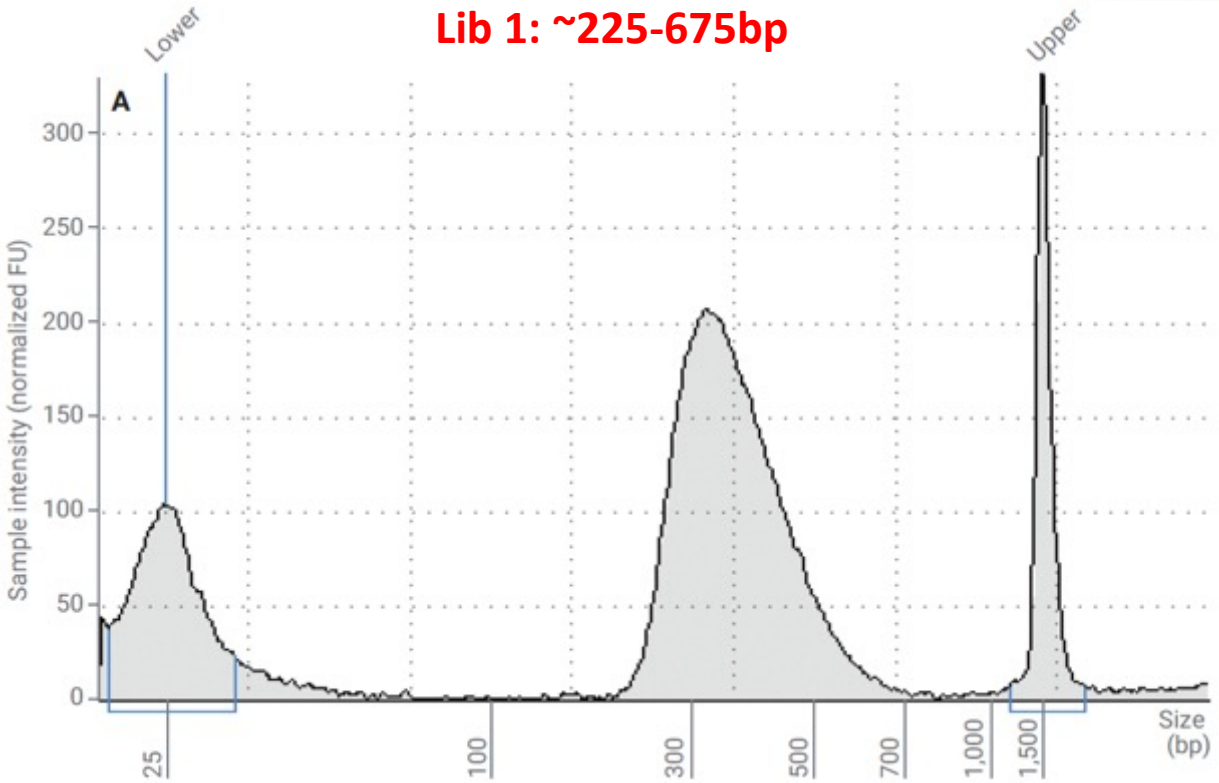
https://www.agilent.com/cs/library/applications/application-ngs-electrophoresis-samplequalitycontrol-tapestation-5994-0127en-agilent.pdf

DNAseq: Library QC

- Libraries are evaluated using a bioanalyzer or a tape station to create an electropherogram to assess quality

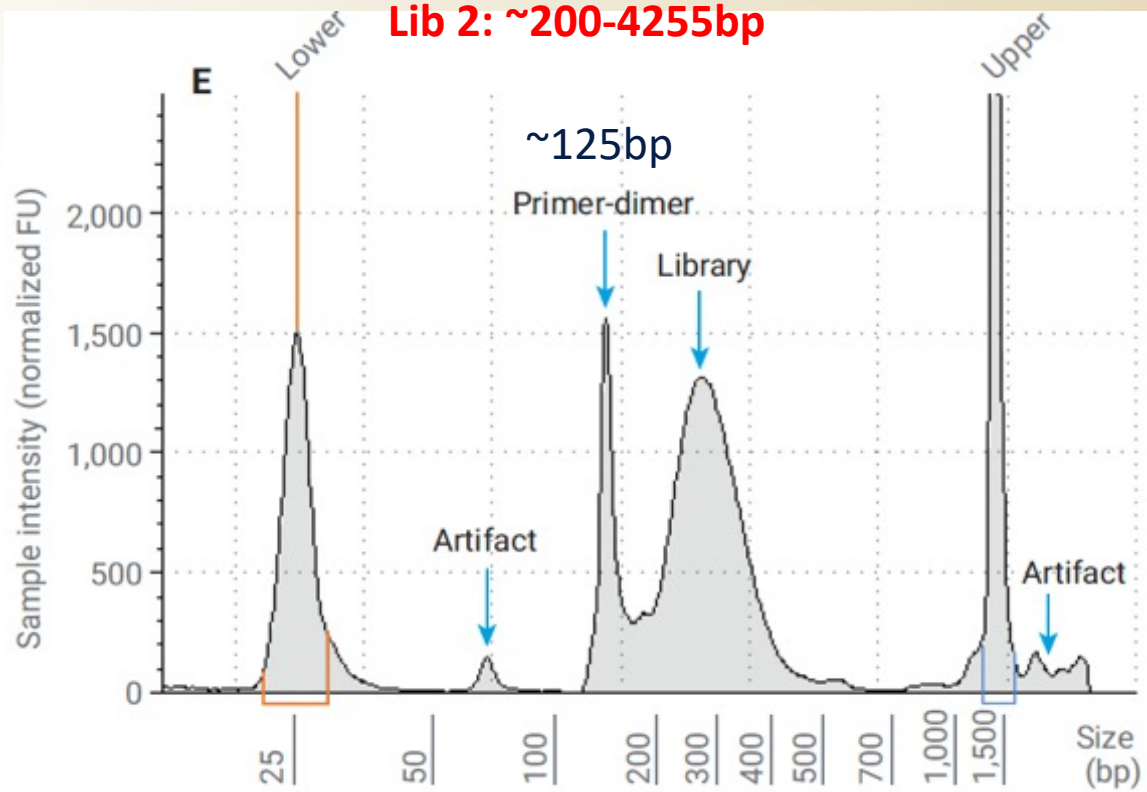
Good Library - 1

Lib 1: ~225-675bp



Bad Library - 2

Lib 2: ~200-4255bp



When assessing library quality look for the following:

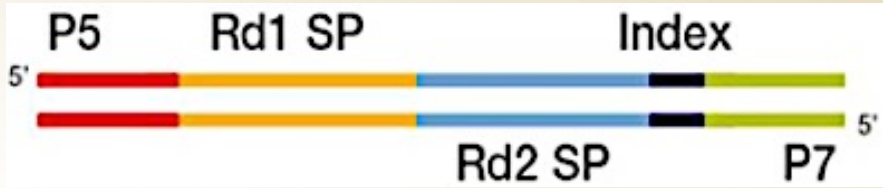
- NO adapter dimers! – Why?
- Library size is consistent with the number of desired sequencing cycles
  - If you're sequencing at PE 250, what is a good library size? (hint: library size = insert length + adapter length)

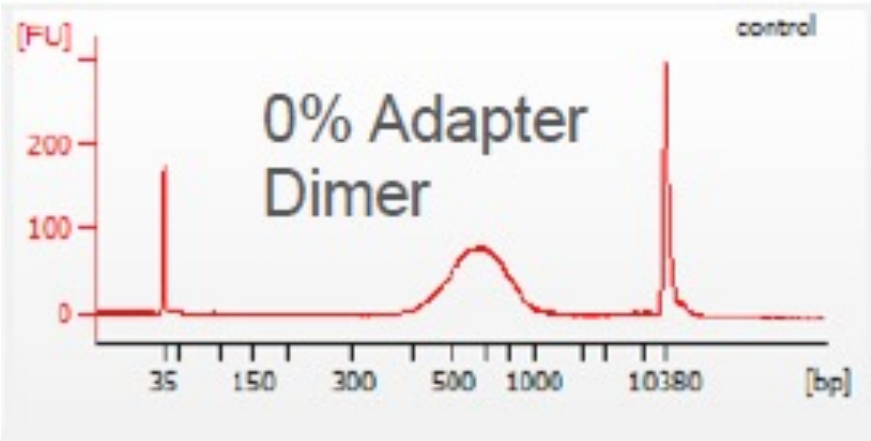
Assuming adapters are ~65bp each (130bp)
~600bp that would give an insert length of ~470,
allowing a ~30bp overlap between R1 and R2

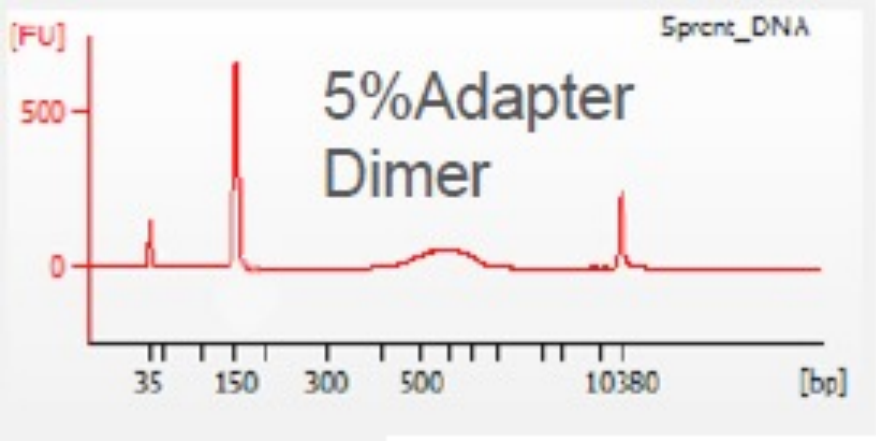
https://www.agilent.com/cs/library/applications/application-ngs-electrophoresis-samplequalitycontrol-tapestation-5994-0127en-agilent.pdf

DNAseq: Library QC

- Assess library quality (bioanalyzer, TapeStation)
  - Adapter dimers
  - Fragment size
- Determine library quantity (Qubit, qPCR)







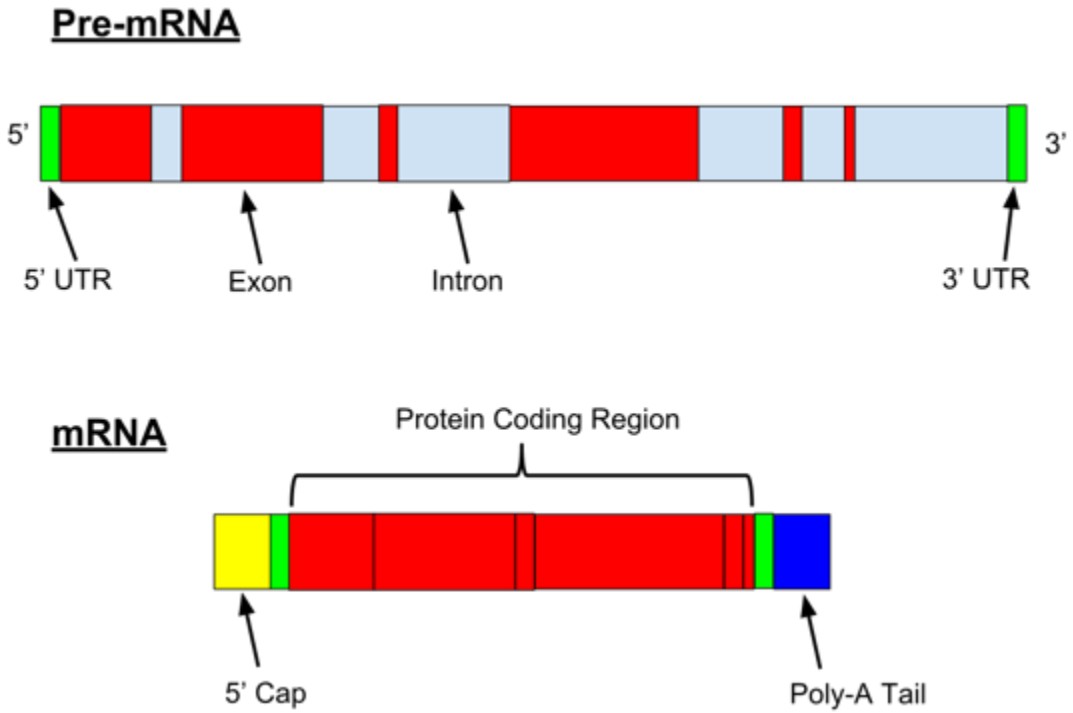
% AD	% PF	% AD Reads
Control	69.54	0.24
10%	10.87	84.25
5%	21.39	60.44
1%	51.88	6.46

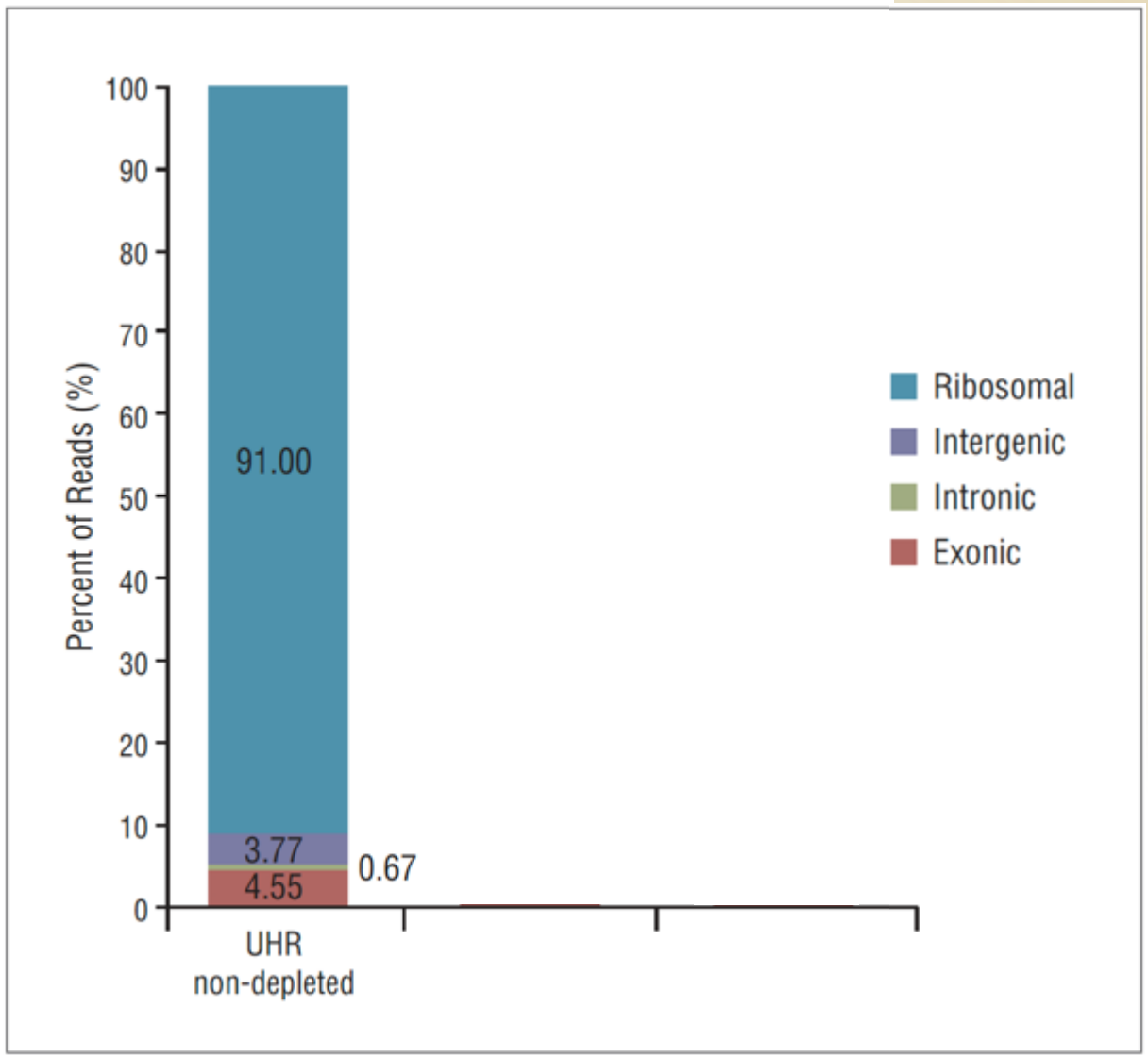
https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseq-stranded-total-rna-workflow/truseq-stranded-total-rna-workflow-reference-1000000040499-00.pdf

Library Preparation
RNA Sequencing

rRNA Contamination

mRNA makes up only ~2-5% of a total RNA sample





https://www.neb-online.de/wp-content/uploads/2015/04/NEB_selectivedepletionofabundantrnas_d.pdf

RNAseq: Library Preparation

Figure 1 Ribo-Zero Depleting and Fragmenting RNA

Figure 2 Synthesizing First Strand cDNA

Figure 3 Synthesizing Second Strand cDNA

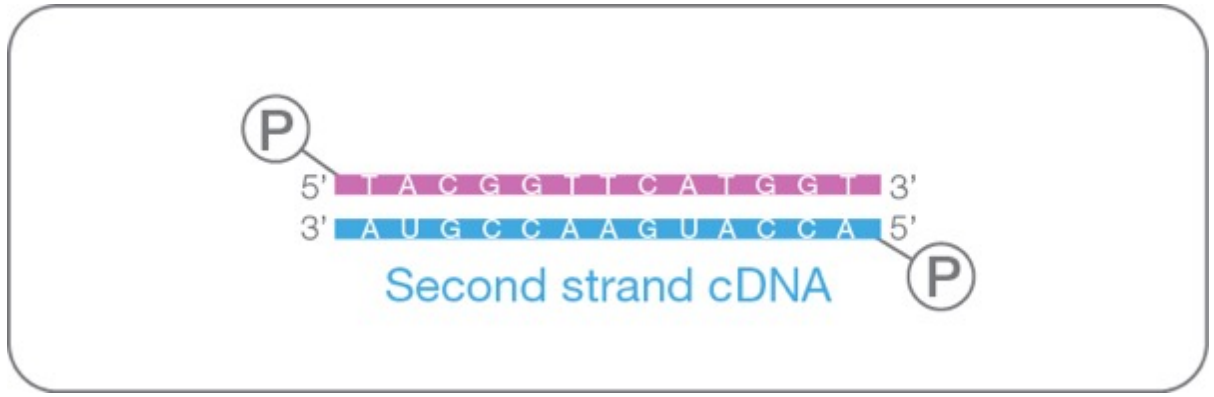
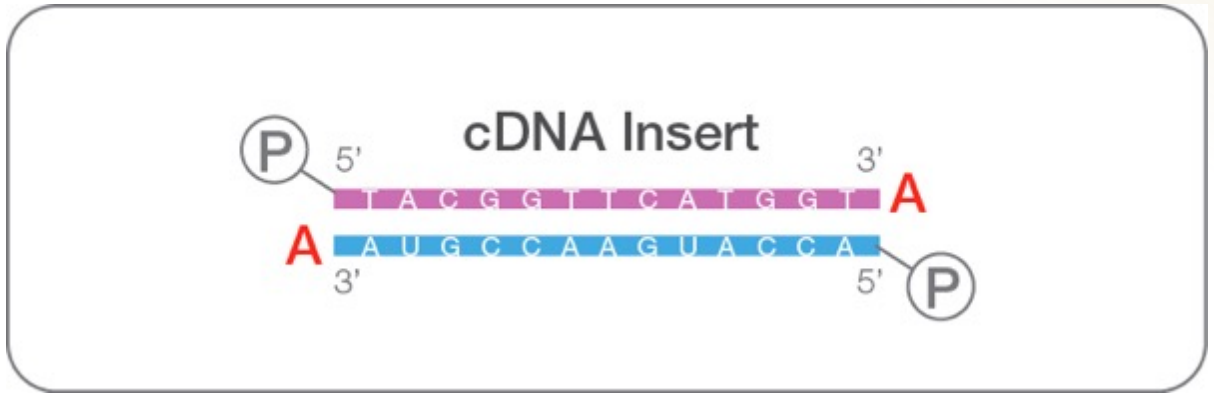
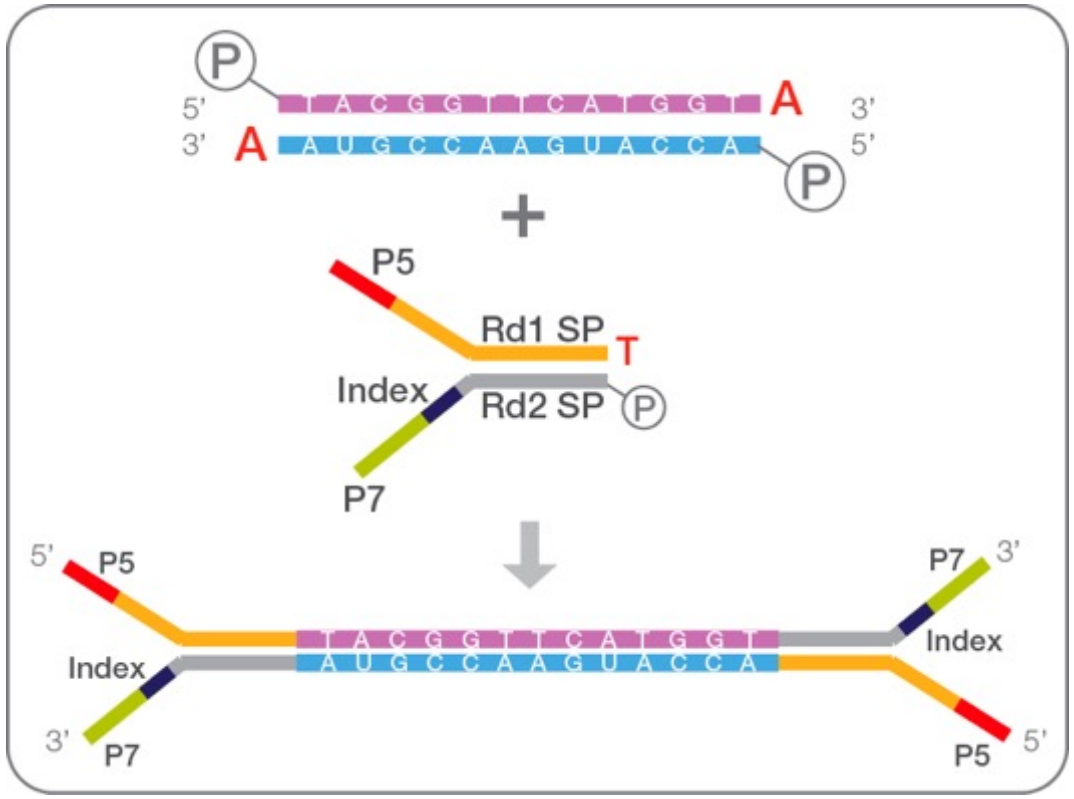


Figure 4 Adenylating 3' Ends*



RNAseq: Library Preparation

Figure 5 Ligating Adapters



Illumina TruSeq Stranded Total RNA Reference Guide

https://www.idtdna.com/pages/products/next-generation-sequencing/adapters

RNAseq: Library Preparation

Figure 6 Enriching DNA Fragments*

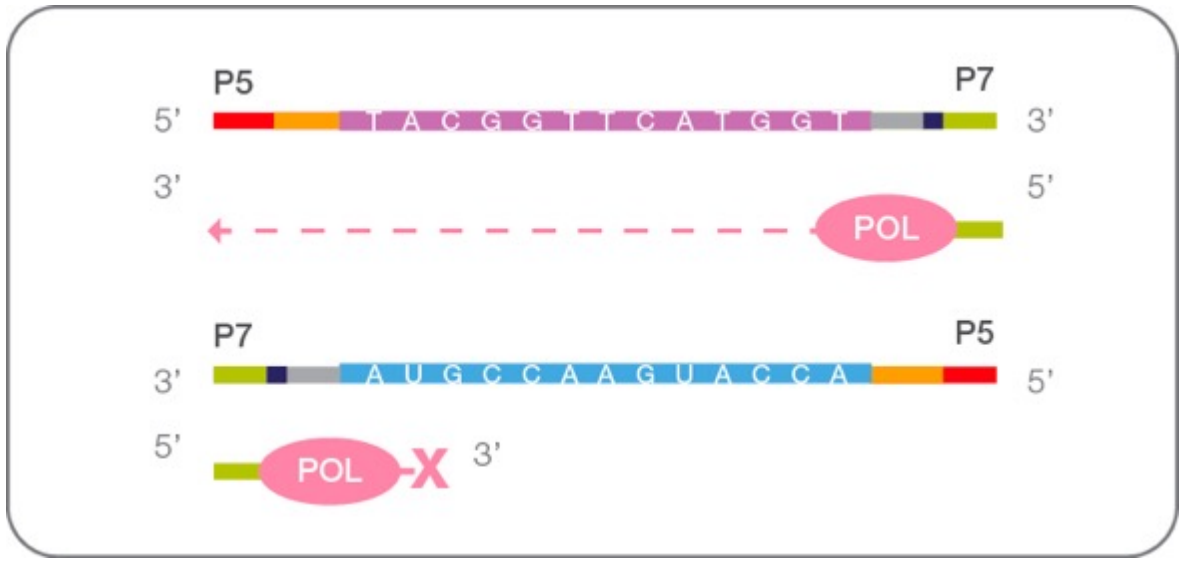
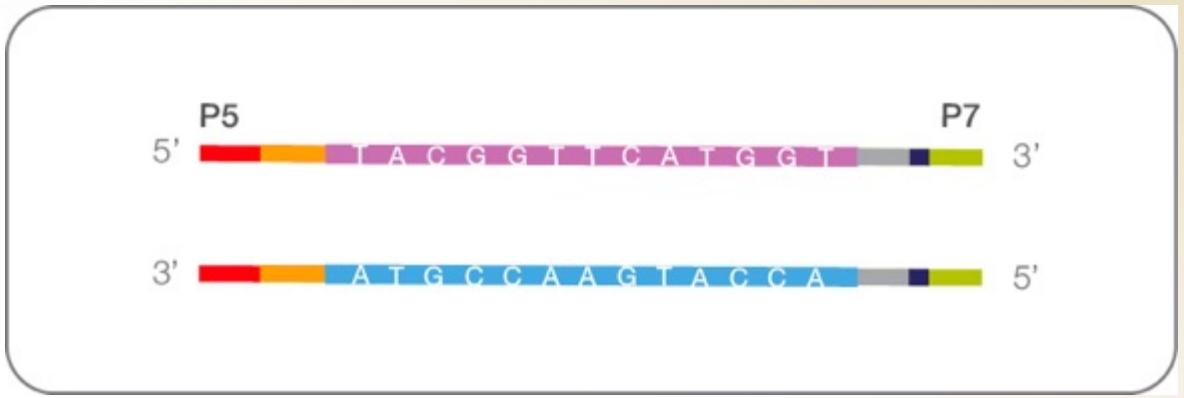
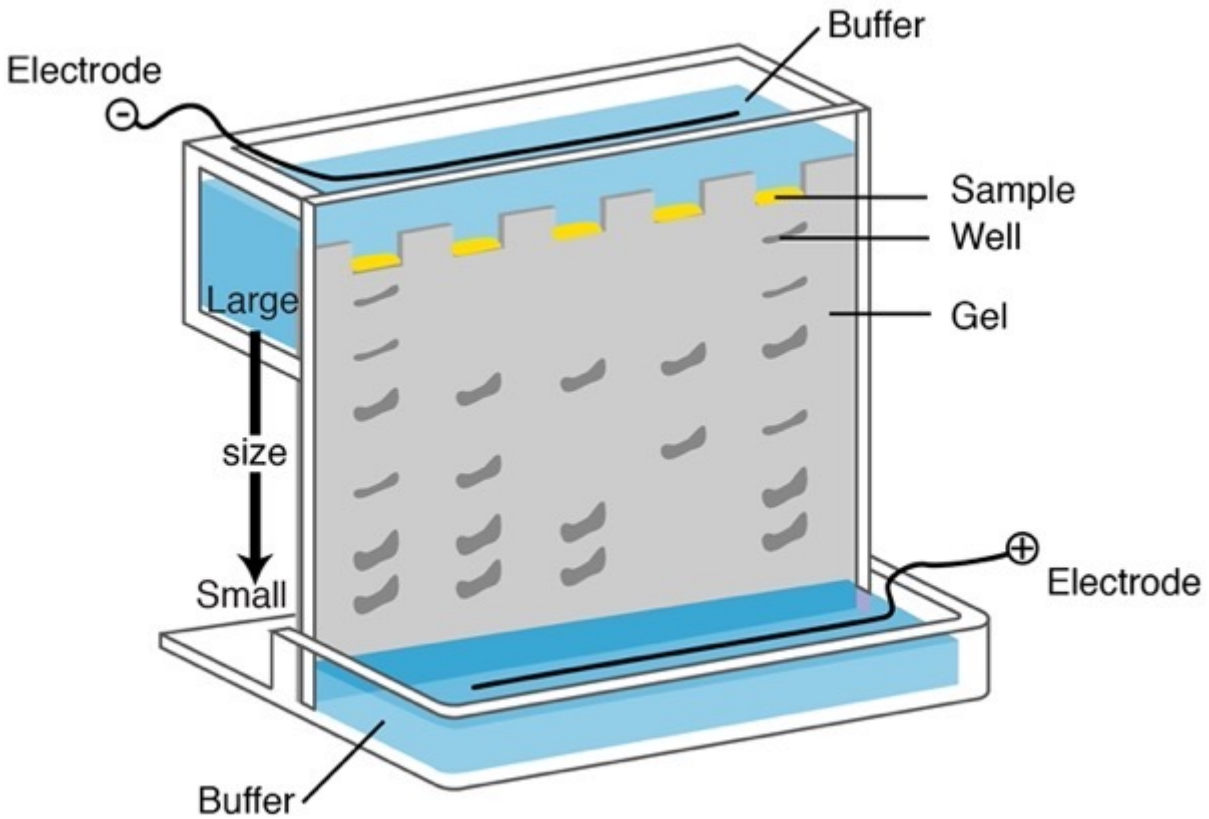


Figure 7 LS Final Library

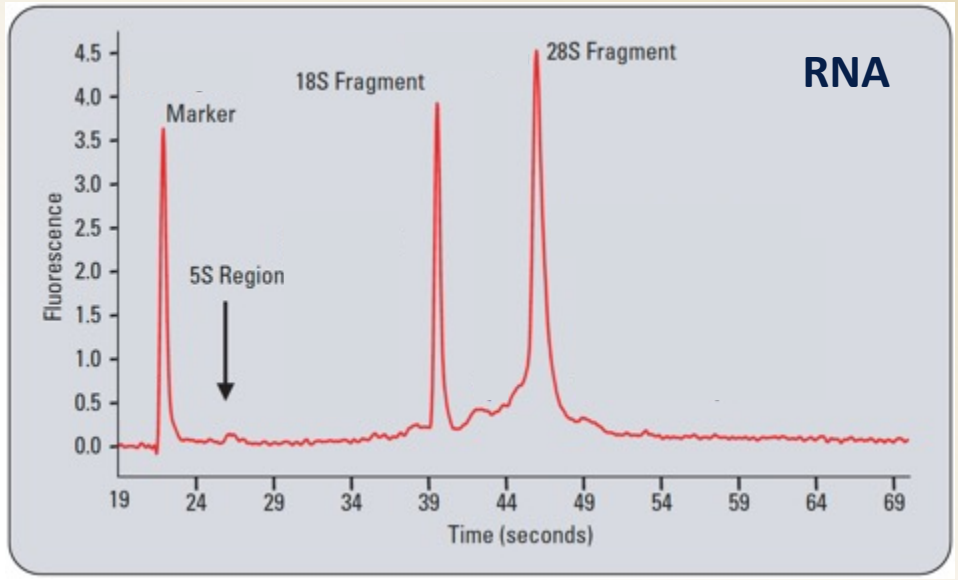


RNA Library QC: Electrophoresis and Electropherogram

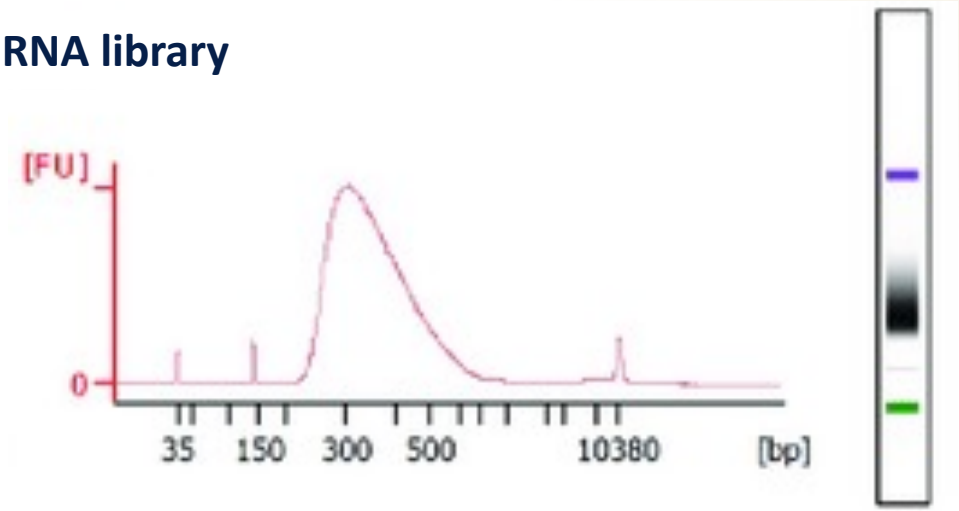
Electrophoresis



Electropherogram



RNA library

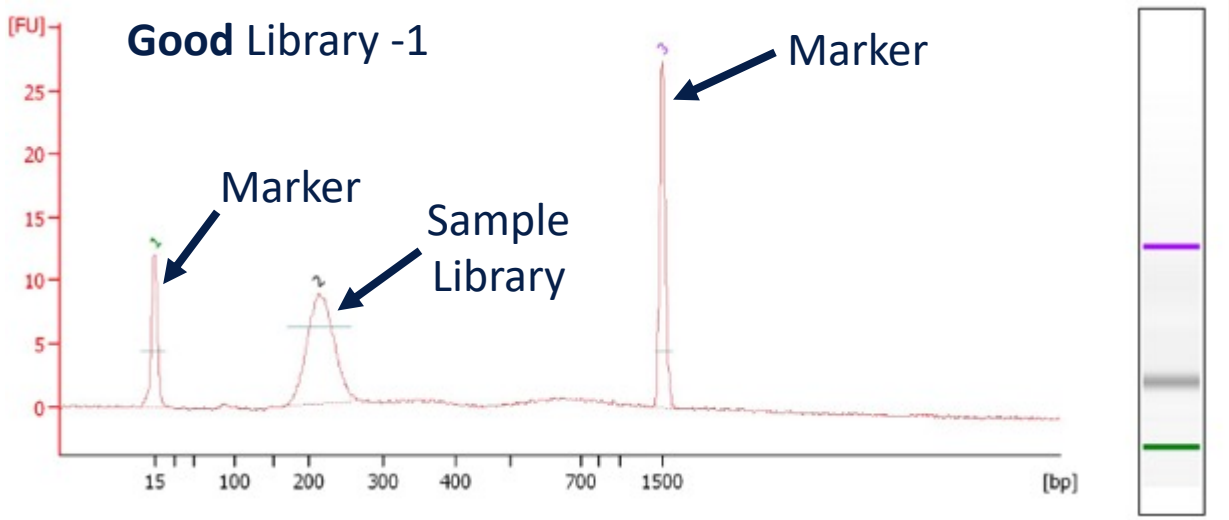


https://www.agilent.com/cs/library/applications/5989-1165EN.pdf

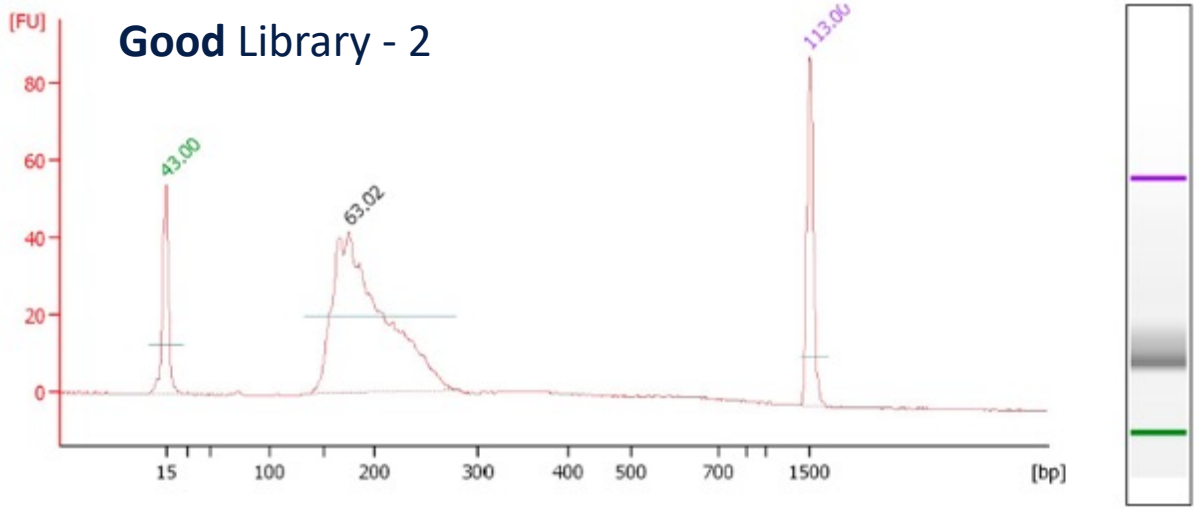
RNAseq: Library QC

- Libraries are evaluated using a bioanalyzer or a tape station to create an electropherogram to assess quality

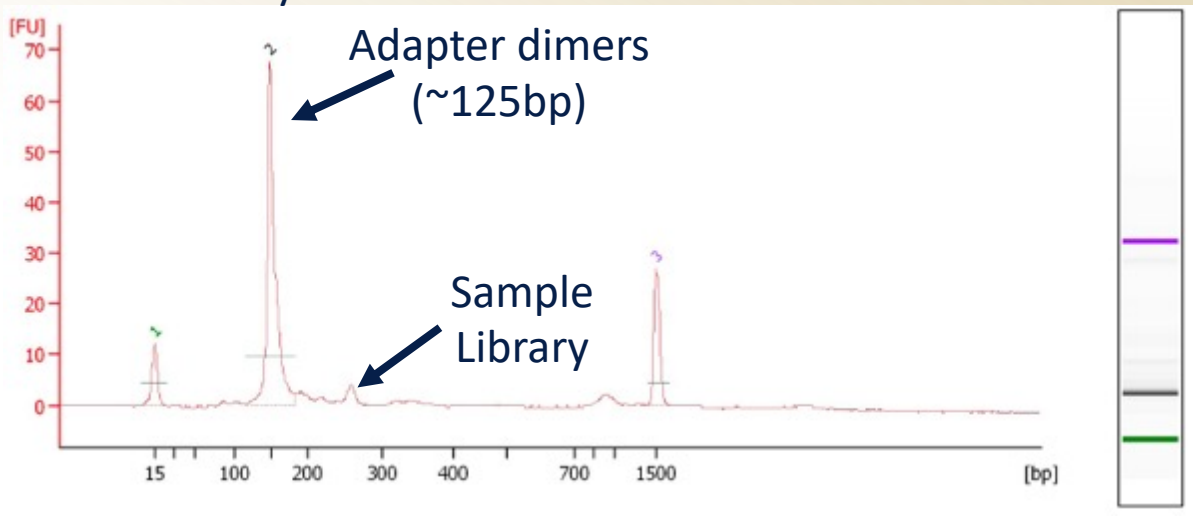
Good Library -1



Good Library - 2



Bad Library - 3



When assessing library quality look for the following:

- NO adapter dimers! – Why?
- Library size is consistent with the number of desired sequencing cycles
  - If you're sequencing at PE 100, what is a good library size? (hint: library size = insert length + adapter length)

Assuming adapters are ~65bp each (130bp): ~300bp that would give an insert length of ~170, allowing a ~30bp overlap between R1 and R2

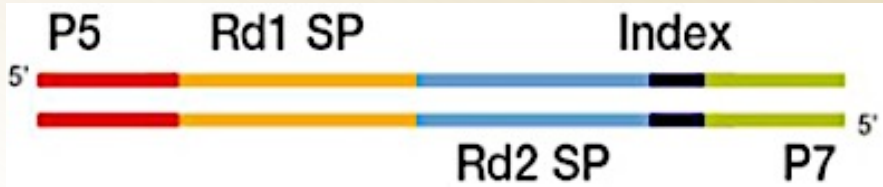
- What is the size range of library 1? Library 2?

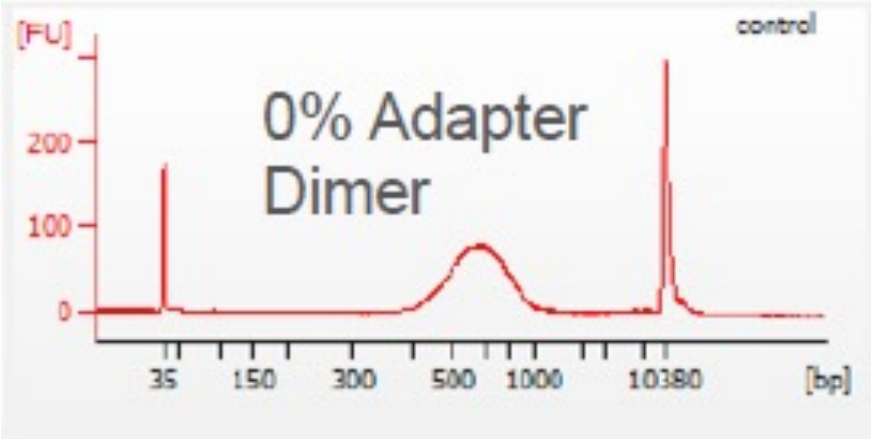
~175-250bp; ~150-275bp

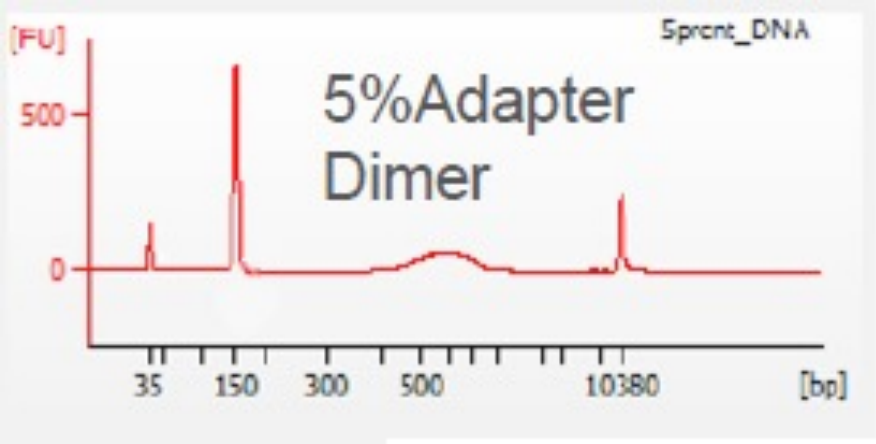
Modified from UC Davis Bioinformatics RNAseq Training

RNAseq: Library QC

- Assess library quality (bioanalyzer, TapeStation)
  - Adapter dimers
  - Fragment size
- Determine library quantity (Qubit, qPCR)







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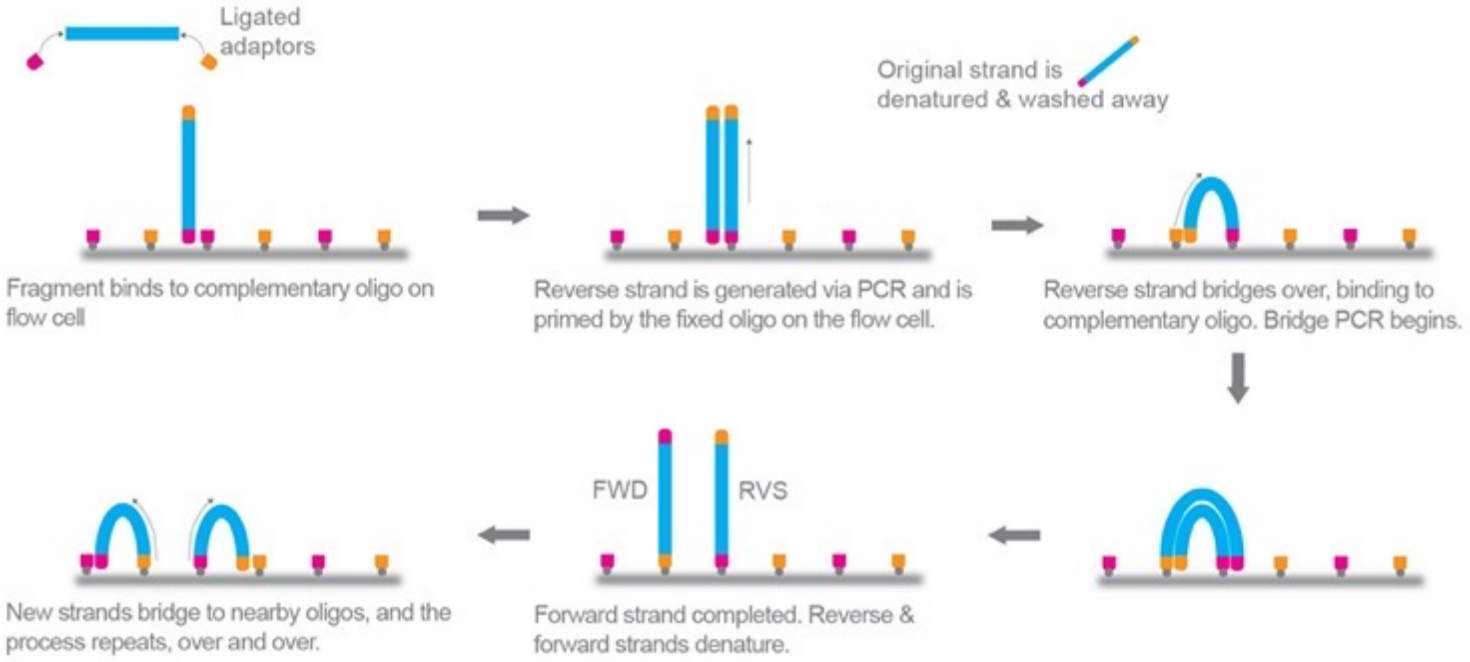
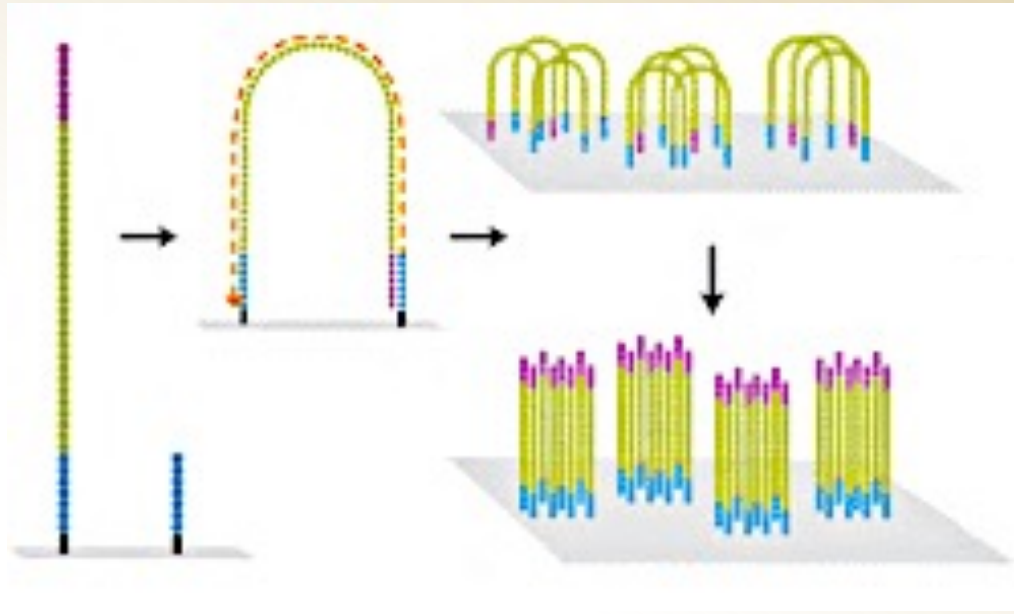
https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseq-stranded-total-rna-workflow/truseq-stranded-total-rna-workflow-reference-1000000040499-00.pdf

Cluster Formation and Sequencing by Synthesis

Cluster Formation





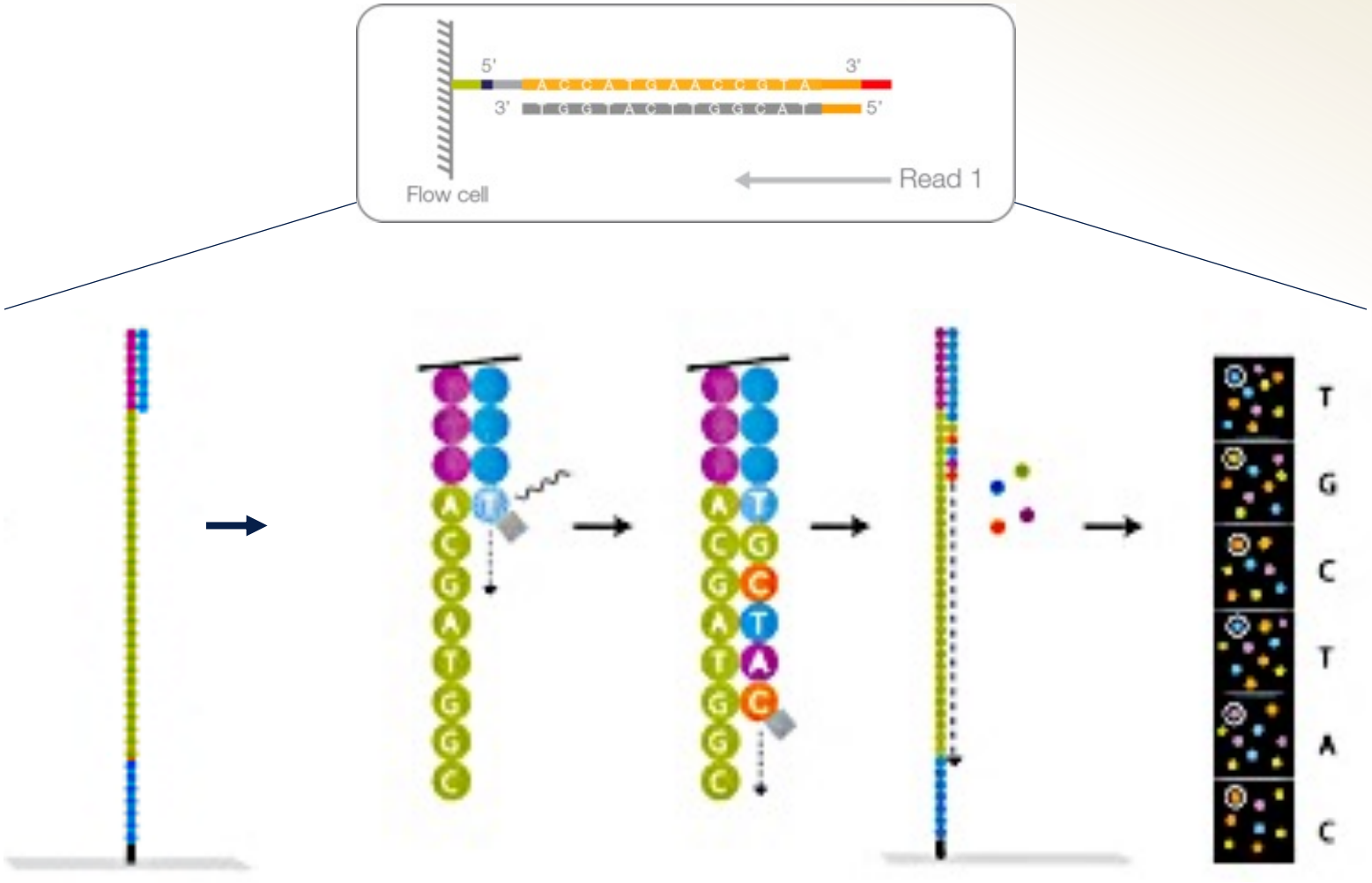
Attach DNA to flow cell → Bridge Amplification → Generate Clusters

Figure 4. Bridge PCR - a PCR method used to amplify samples for sequencing.

https://www.goldbio.com/articles/article/Types-of-PCR-used-for-Genetic-Research

Illumina's High Throughput Sequencing by Synthesis

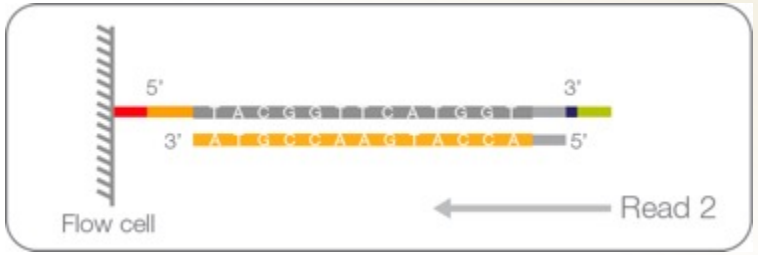
Figure 8 Read 1 Sequencing



Anneal Sequencing Primer → Extend first base, read, and deblock → Repeat previous step to extend strand → Generate base calls

- For paired-end (PE) sequencing, after read 1 is sequenced, forward strand reagents are washed
- The index read(s) are sequenced next
- Sequencing of the reverse read (read 2) is initiated after the index read(s)

Figure 9 Read 2 Sequencing



Sequencing Parameters

	Read length	Sequencing depth	Paired-end (PE) or Single-end (SE)
DNAseq	➢ Longer reads enable greater confidence in taxonomic classifications and functional annotations ➢ "Assembly" is often performed with short reads to facilitate this ➢ GL standard is 2x250bp	➢ Greater depth increases the likelihood of sequencing low-abundance organisms (if metagenomics) and detecting things like single-nucleotide variants and genetic rearrangements with greater confidence ➢ GL standards: ➢ Single organism or tissue: ➢ Re-sequencing (reference available): 10X minimum ➢ De novo sequencing (no reference available): 50X minimum ➢ Metagenomics (mixed community): 10M per sample, minimum	➢ PE is generally preferred
RNAseq	➢ Longer reads increase gene ID confidence ➢ GL standard is 2x150bp for bulk RNAseq	➢ Greater depth increases the likelihood of sequencing low-abundant transcripts, detecting novel transcripts, and quantifying isoforms ➢ Greater depth is necessary for ribo-depleted samples (vs. poly-A enriched samples) – for RNAseq ➢ GL RNAseq standard for mammalian samples prepared with ripo-depletion is ~60M reads/sample and ~40M reads/sample for samples prepared with polyA-selection. ➢ More replicates is usually preferred over greater depth	➢ PE is preferred

Illumina's High Throughput Sequencing by Synthesis

https://www.youtube.com/watch?v=fCd6B5HRaZ8

END
GL4U: Introduction
Lecture 4 of 4