

RRBS-SEQ
ICE
OS-SEQ
MAINE-SEQ
RIBO-SEQ
CHIP-SEQ

WORKSHOP 16: LIBRARY PREP FOR NGS

4-C
TC-SEQ
NET-SEQ
UMI
CAP-SEQ
FAIRE-SEQ
DUPLX-SEQ
DNASE-SEQ
PAR-CLIP-SEQ
BS-SEQ
MEDIP-SEQ
GRO-SEQ

MBDCAP-SEQ
PARE-SEQ/GMUT
HITS-CLIP
HI-C/3-C
TRAP-SEQ
RC-SEQ
FRAG-SEQ

DAY 2

Marco Morselli
Pellegrini Lab
QCBio Collaboratory Fellow

Apr 15th, 2020



YESTERDAY (day 1)

- **INTRO to SEQUENCING**
- **LIBRARY PREP 101**

TODAY (day 2)

- **SPECIALTY DNA PREPs**
- **RNA Library Preps**
 - **RNAseq**
 - **small RNA-seq**
 - **Low-input RNAseq**

TOMORROW: ONLINE LAB Experiments (day 3)

(Use the TapeStation for QC; Library Preparation; submit libraries for sequencing)

1. REPETITA IUVANT



1bis. SPECIALTY DNA-seq



2. RNA LIBRARY PREP – UNSTRANDED & STRANDED



3. RNA QC & INTEGRITY



4. low-input//scRNA-seq

5. small RNA-seq



6. BEST PRACTICES IN NGS



1. DNA

2. short (average 200-800 bp)

3. modified with adapters*

CONCENTRATION
& QC



CONCENTRATION
& QC

FINAL LIBRARIES

LAB

It's going to
be in the
final QUIZ

PCR
Primer
Dimers

A

B

C

1,500
1,000
700
500
400
300
200
100
50
25

Adapter
Dimers

Final
Libraries

REPETITA

IUVANT

GOAL: Less than 1%

Final Libraries: 200-700 bp

Adapter Dimers: 120-140 bp

Primer Dimers < 100 bp

SEQUENCING

REPETITA
IUVANT



SE50: ChIPseq, RNAseq, smallRNAseq

SE100: BS-seq

PE50: ATACseq, Hi-C

PE100 (or longer): RNAseq (splicing or *de novo* transcriptome), WGS, WES



**KEEP
CALM
IT'S
BREAK
TIME**



QUESTIONS
#8

LIBRARY PREPS

SPECIALTY PIZZAS

PEPPERONI LOVERS

Two layers of pepperoni

BBQ CHICKEN

BBQ chicken, green peppers, onions,
BBQ sauce

HAWAIIAN

Ham, pineapple

VEGETARIAN

Mushrooms, green peppers,
onions, tomatoes, black olives

PPP • Small • Medium • Large • Stuffed crust
\$475 \$975 \$1675 \$2000 \$2200 (Large only)

MEAT LOVERS

Pepperoni, beef, pork, Italian sausage,
ham, bacon

Small • Medium • Large • Stuffed crust
\$1200 \$2200 \$2850 \$3000 (Large only)

SUPREME

Cheese, pepperoni, mushrooms,
green peppers, onions, beef, pork

PPP • Small • Medium • Large • Stuffed crust
\$490 \$995 \$1950 \$2500 \$2700 (Large only)

SUPER SUPREME

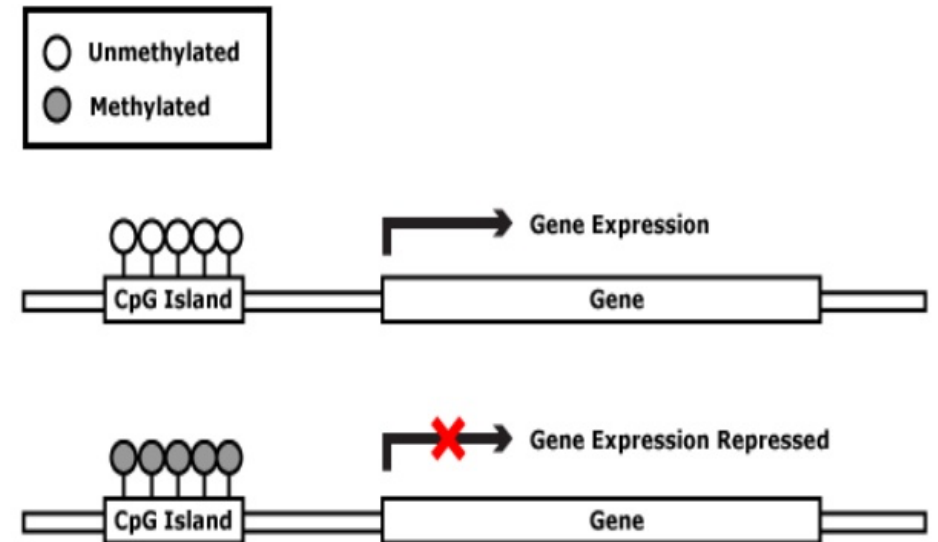
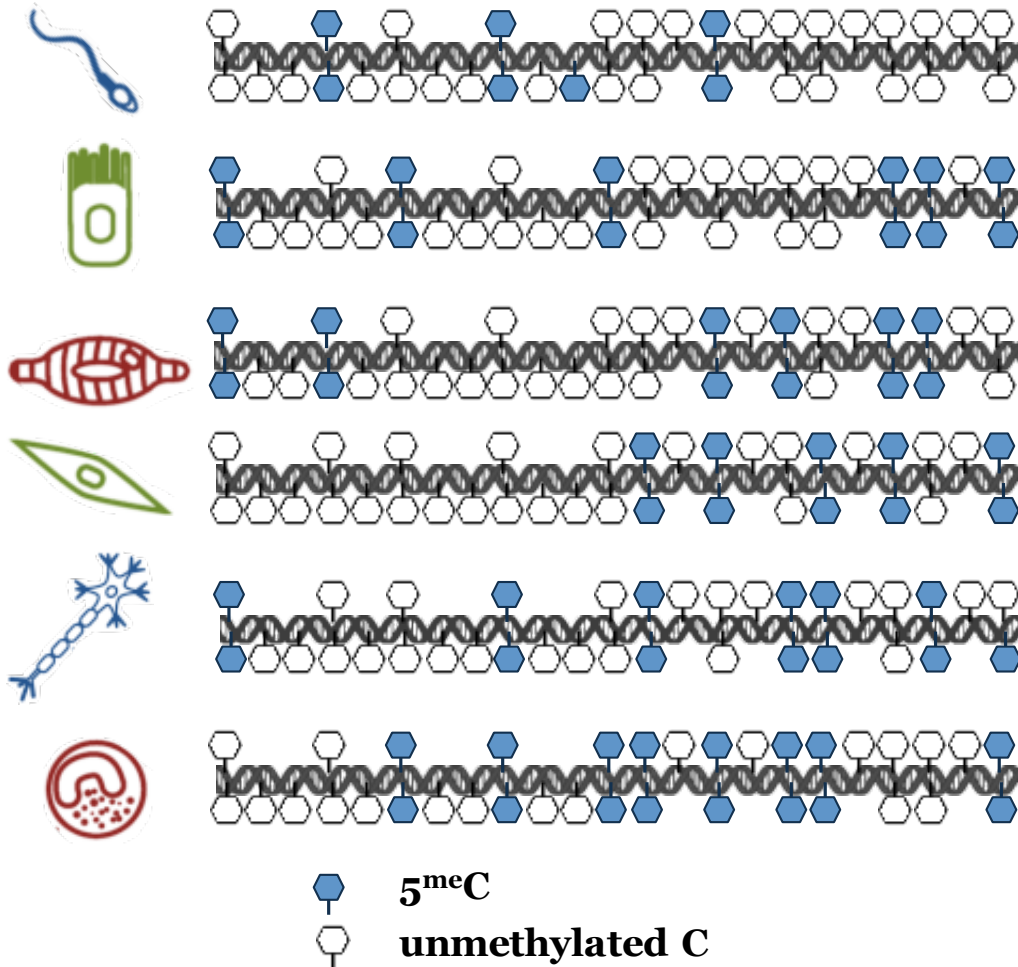
Cheese, pepperoni, ham, Italian sausage,
mushrooms, onions, black olives,
green peppers, beef, pork.

Small • Medium • Large • Stuffed crust
\$1250 \$2200 \$2950 \$3150 (Large only)

Visuals shown are representational

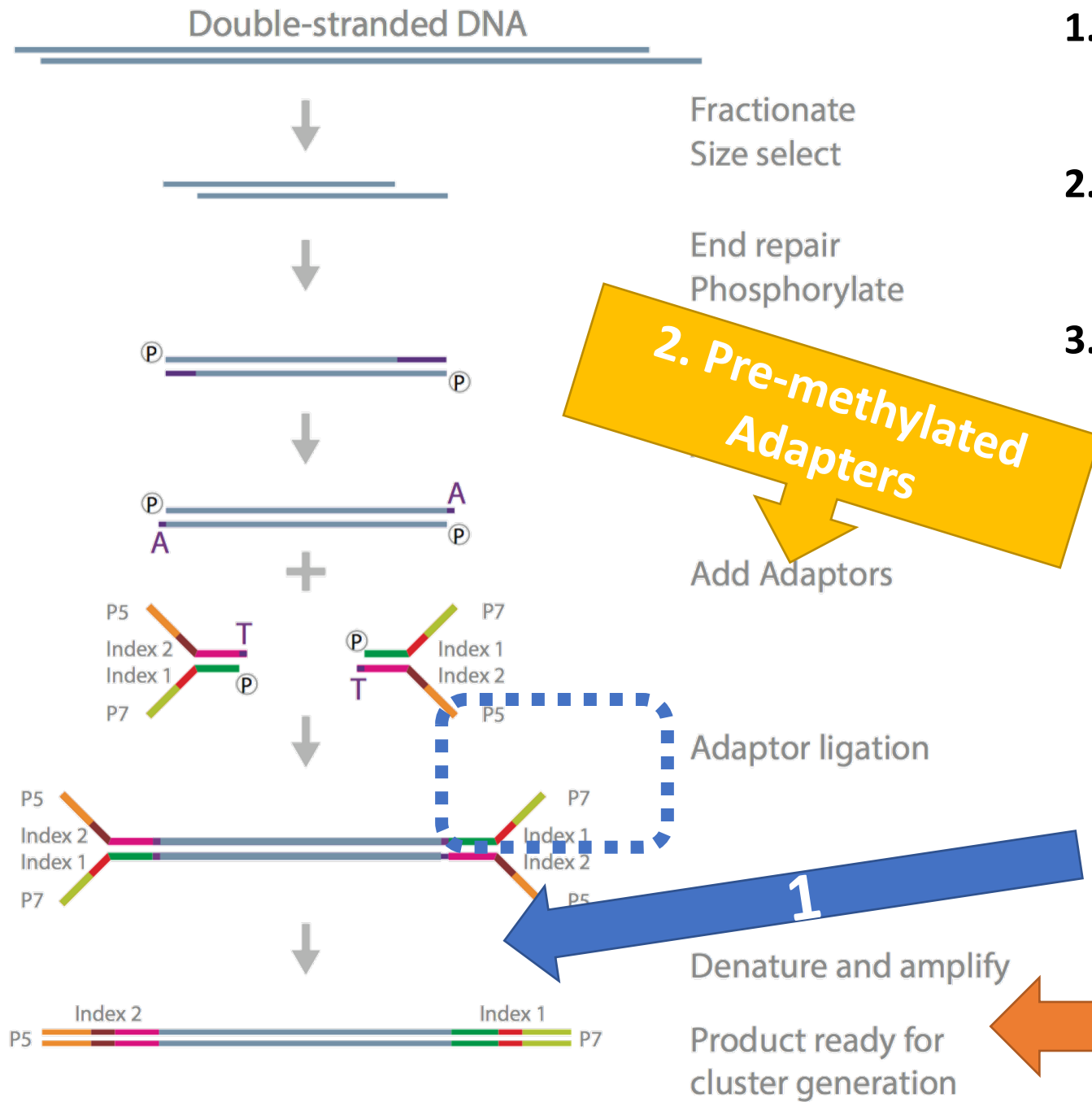


DNA METHYLATION



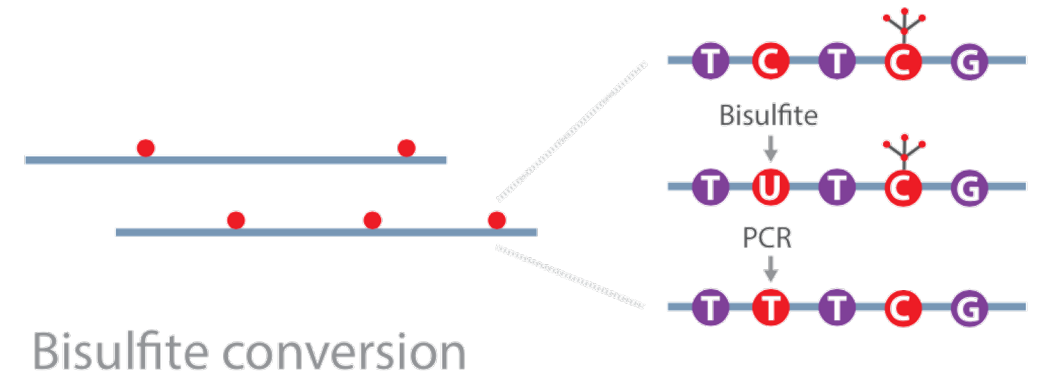
. Métyvier, R. et al. Cyclical DNA methylation of a transcriptionally active promoter. *Nature* **452**, 45–50 (2008).

Different cells have different DNA methylation patterns and can vary between individuals and in diseases.



1. PCR before bisulfite treatment will dilute the methylation signal in the original DNA
2. Bisulfite treatment modifies the cytosines on DNA: BOTH INSERT AND ADAPTERS
3. After BS-treatment → ssDNA and dU

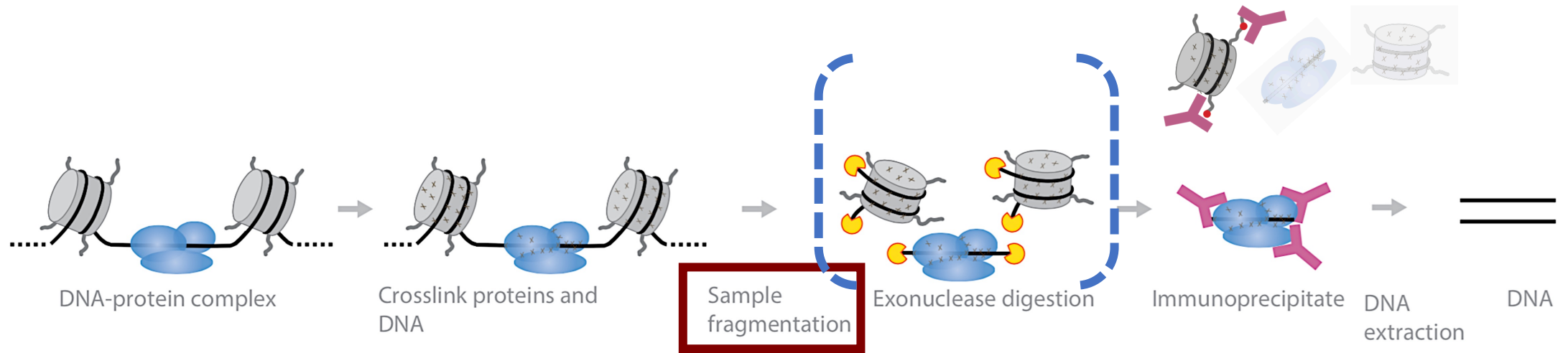
Higher input amount than regular libraries****



3. Not all DNA polymerases are compatible with BS-treated DNA (contains Uracil → Stranded RNA-seq)

There are variations of the protocol

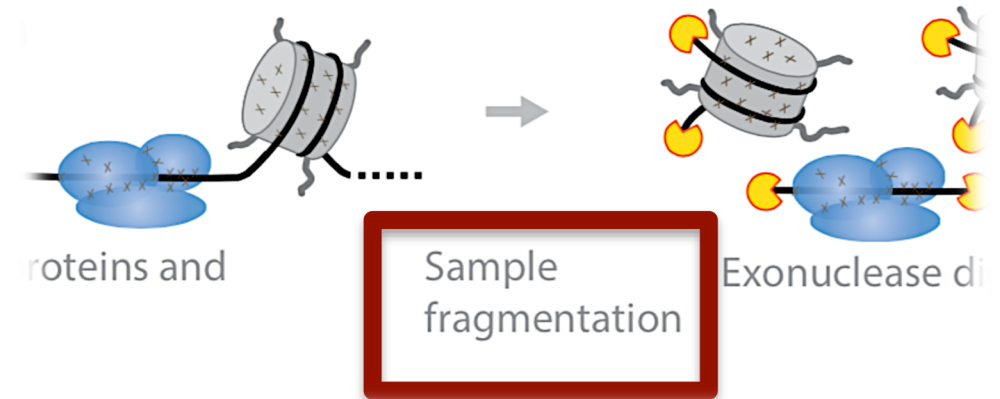
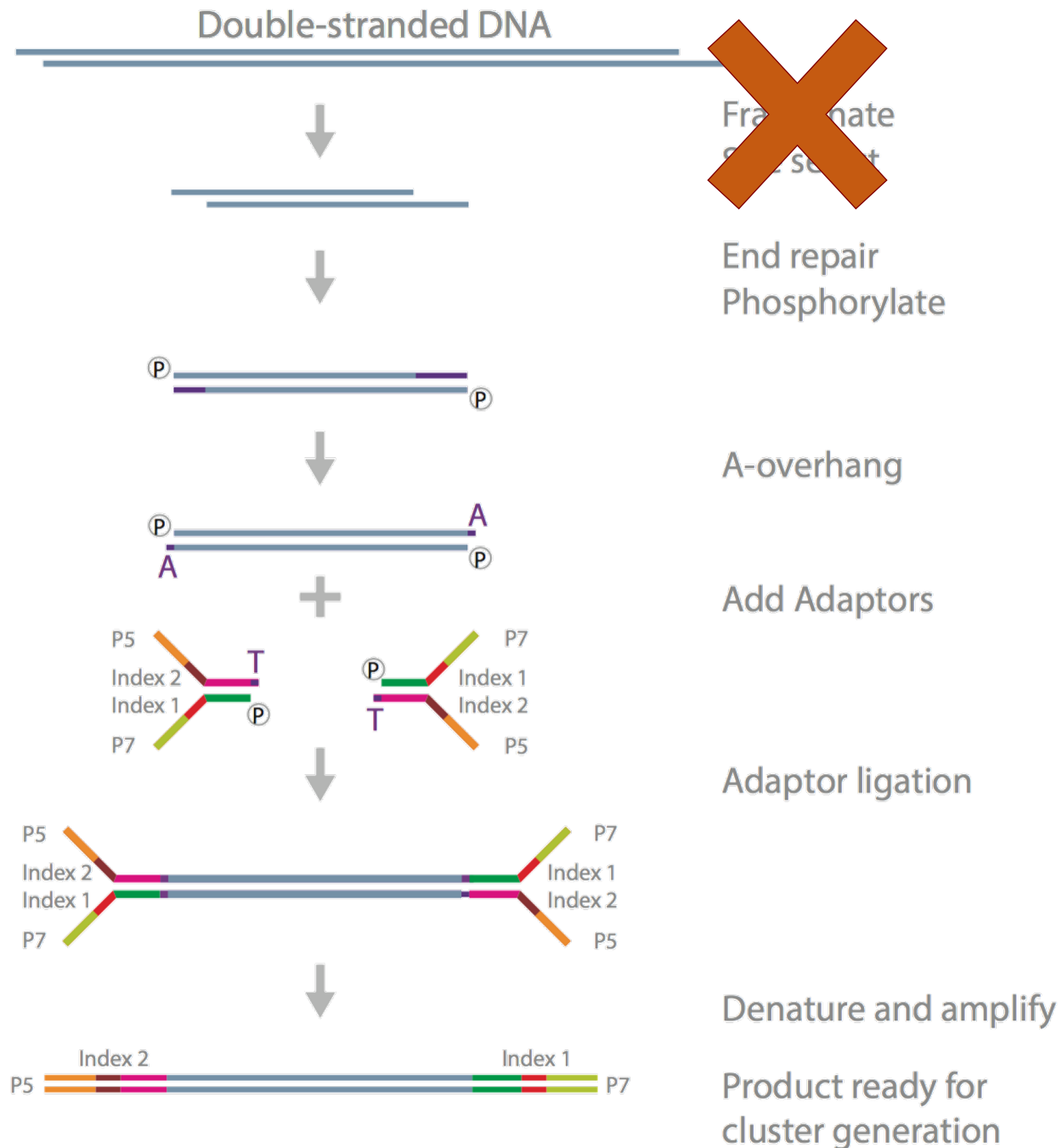
ChIP-seq



**Sonication,
endonucleases...**

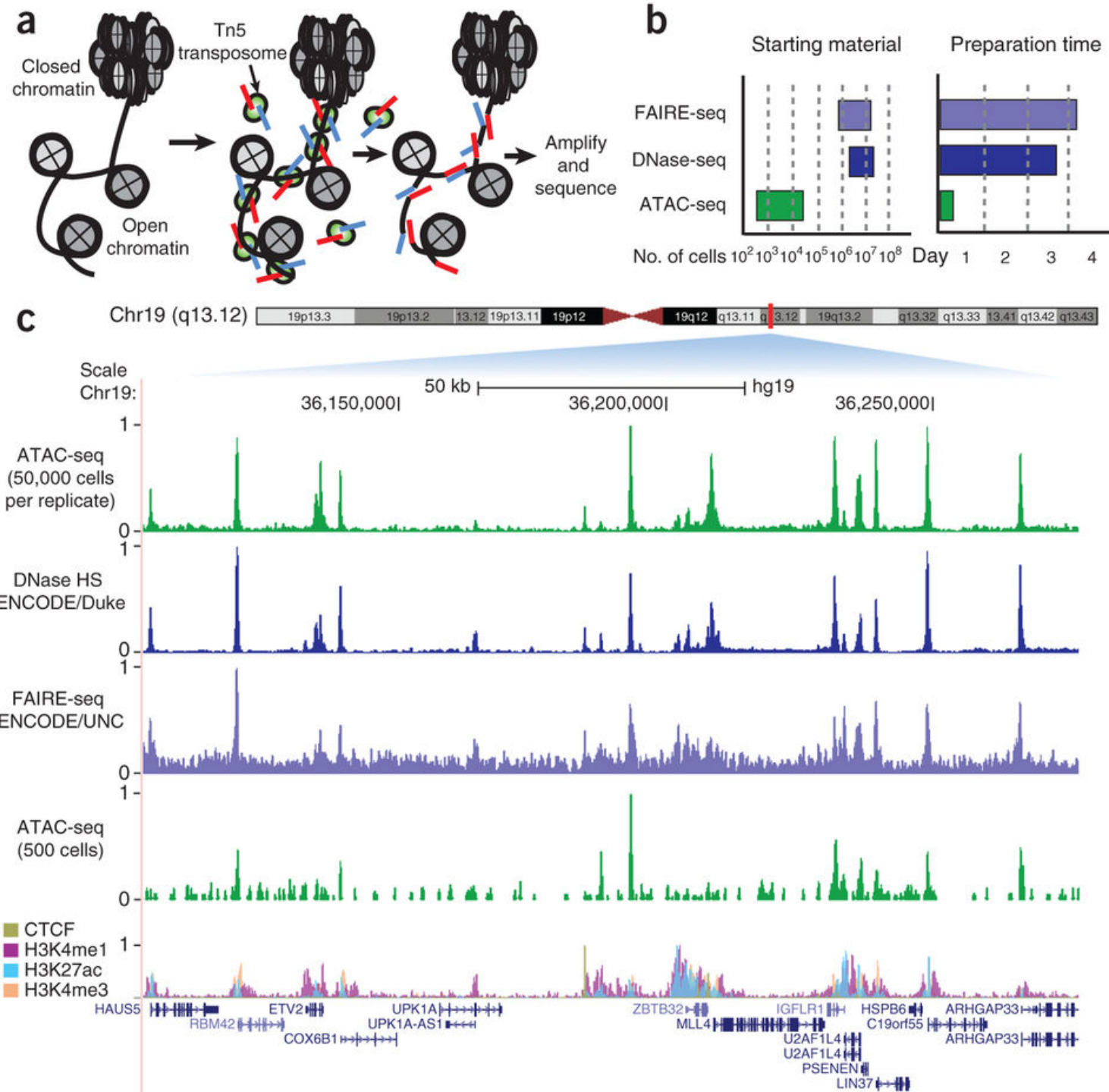
Chromatin immunoprecipitation sequencing (ChIP-Seq) is a well-established method to map specific protein-binding sites. In this method, DNA-protein complexes are crosslinked *in vivo*. Samples are then fragmented and protein-specific antibodies are used to immunoprecipitate the DNA-protein complex.

ChIP-seq

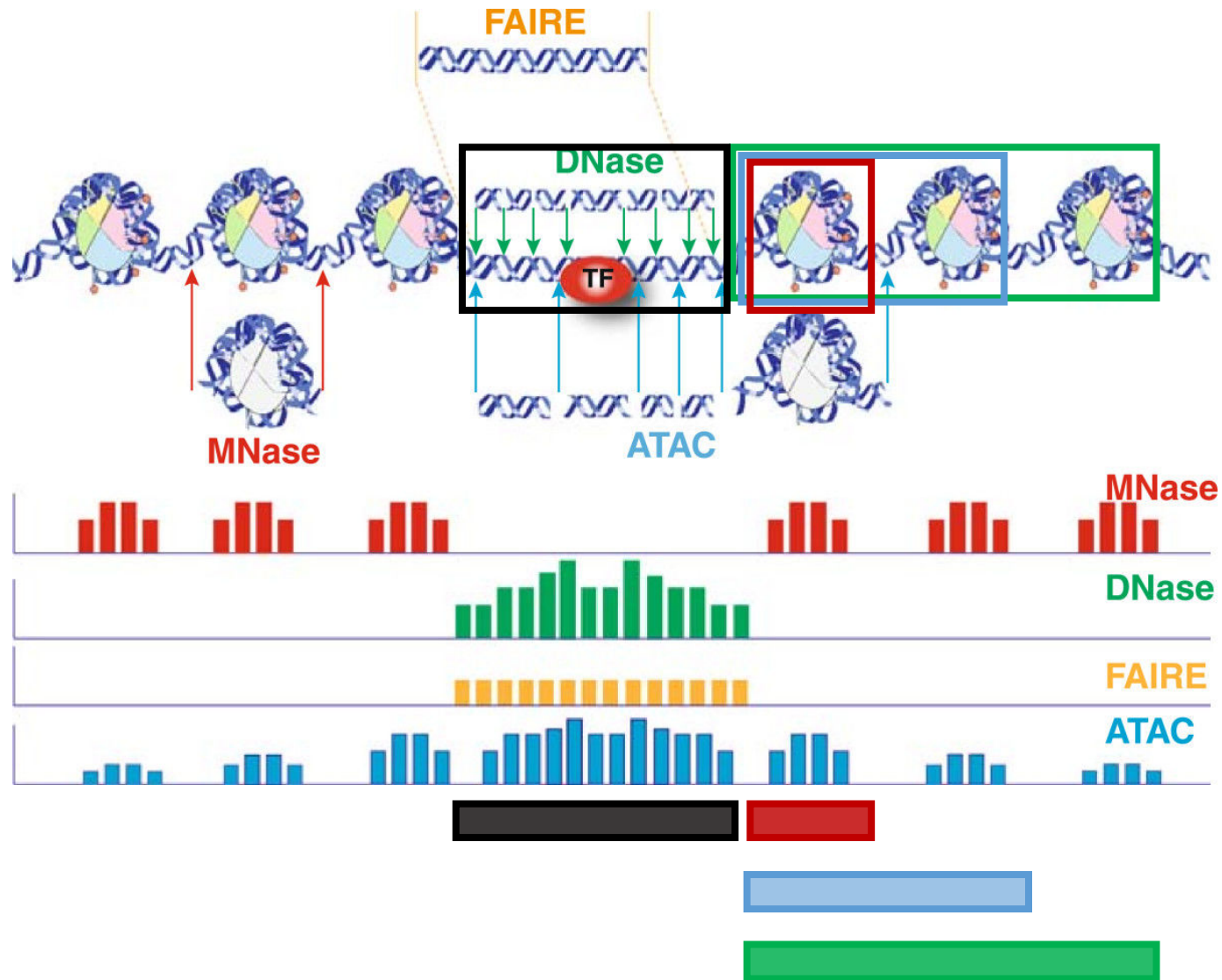


- 1. Sonication/fragmentation and Ab: EXTREMELY IMPORTANT**
- 2. Make sure your IP works: qPCR on known regions (enrichment of IP vs. INPUT – sonicated chromatin or IgG-IP)**
- 3. You can do qPCR on your final library – enrichment decrease 2-3 fold**

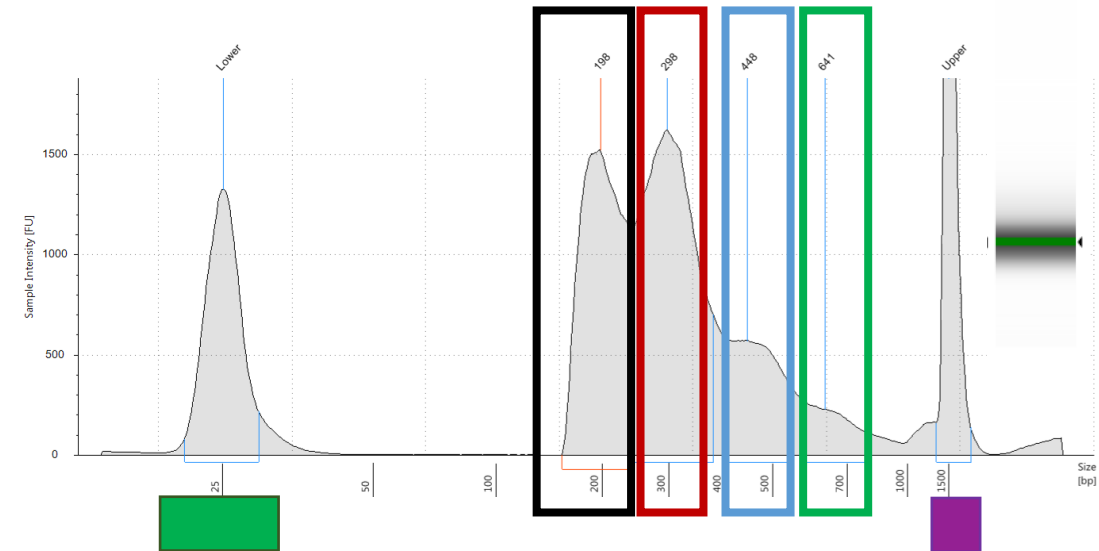
ATAC-seq



ATAC-seq



Trinucleosome
Dinucleosome
Mononucleosome
Sub-nucleosomal DNA



SIZE????

ATAC-seq

- Fast
 - Native Chromatin (no fixation, cross-linking)
 - Few cells (**now protocols down to single cell**)
-
- mtDNA: big contaminant (waste of reads!!!!)
 - Only fresh cells

Letter | Published: 17 June 2015

Single-cell chromatin accessibility reveals principles of regulatory variation

Jason D. Buenrostro, Beijing Wu, Ulrike M. Litzenburger, Dave Ruff, Michael L. Gonzales, Michael P. Snyder, Howard Y. Chang  & William J. Greenleaf 

Nature **523**, 486–490 (23 July 2015) | [Download Citation](#) 

Article | **OPEN** | Published: 17 December 2018

A rapid and robust method for single cell chromatin accessibility profiling

Xi Chen, Ricardo J. Miragaia, Kedar Nath Natarajan & Sarah A. Teichmann 

Nature Communications **9**, Article number: 5345 (2018) | [Download Citation](#) 

An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues **OMNI-ATAC**

M Ryan Corces, Alexandro E Trevino, Emily G Hamilton, Peyton G Greenside, Nicholas A Sinnott-Armstrong, Sam Vesuna, Ansuman T Satpathy, Adam J Rubin, Kathleen S Montine, Beijing Wu, Arwa Kathiria, Seung Woo Cho, Maxwell R Mumbach, Ava C Carter, Maya Kasowski, Lisa A Orloff, Viviana I Risca, Anshul Kundaje, Paul A Khavari, Thomas J Montine, William J Greenleaf  & Howard Y Chang 

Nature Methods **14**, 959–962 (2017) | [Download Citation](#) 

Article | **OPEN** | Published: 24 January 2019

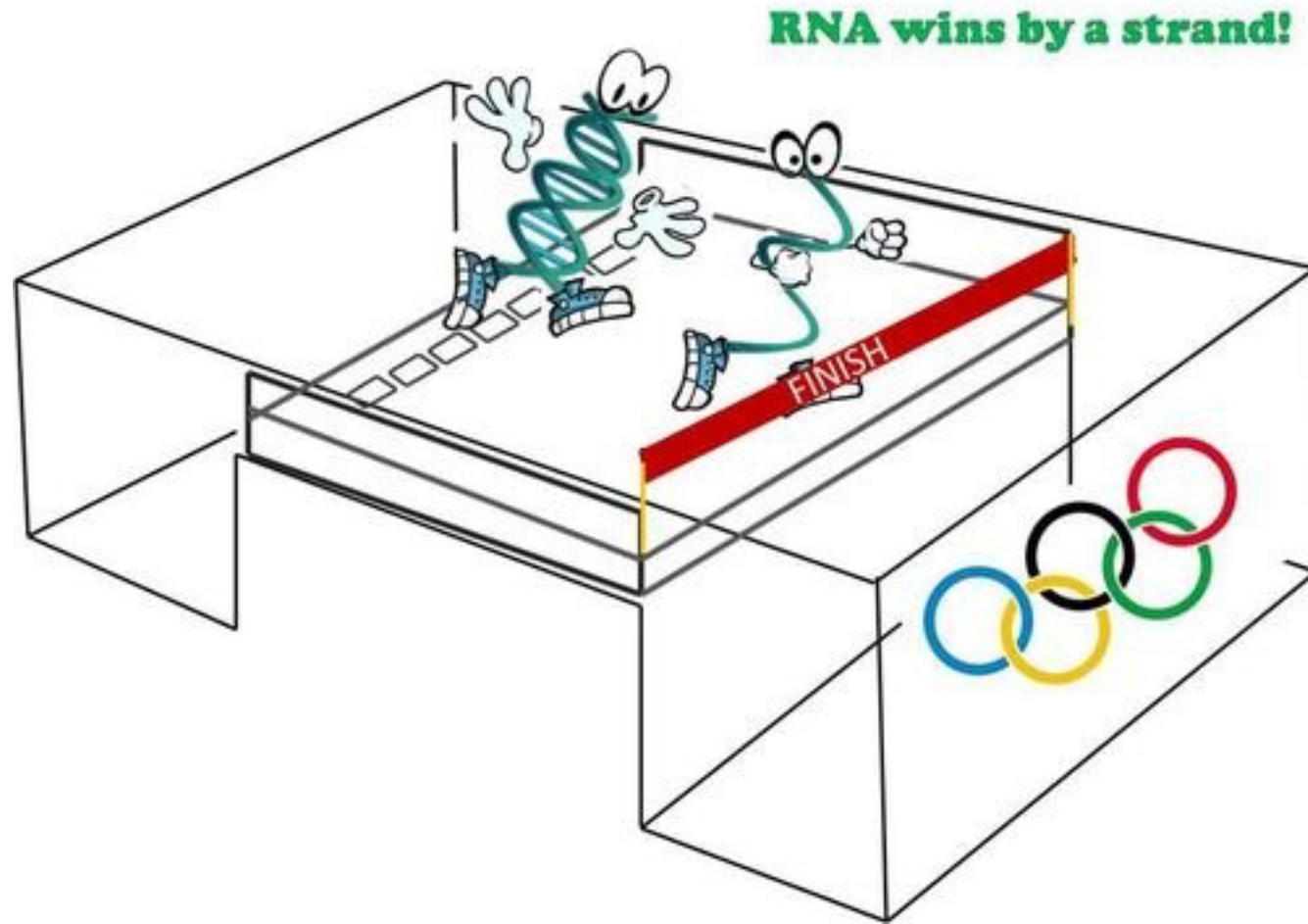
High Quality ATAC-Seq Data Recovered from Cryopreserved Breast Cell Lines and Tissue **OPTI-ATAC**

Saori Fujiwara, Songjoon Baek, Lyuba Varticovski, Sohyoung Kim & Gordon L. Hager 

Scientific Reports **9**, Article number: 516 (2019) | [Download Citation](#) 

**GOOGLE FORMs for
ATTENDANCE and mini-QUIZ
(DAY 1 + SPECIALTY PREPs)**

RNA SEQ



1. DNA

2. short (average 200-800 bp)

3. modified with adapters



POLL 2



1. DNA

2. short (average 200-800 bp)

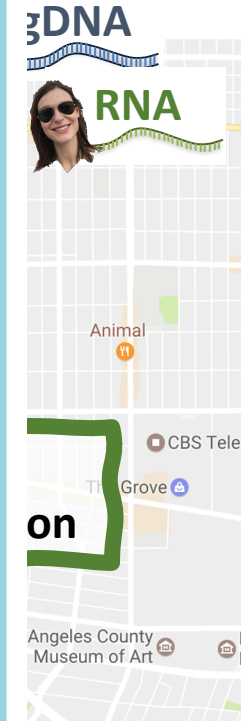
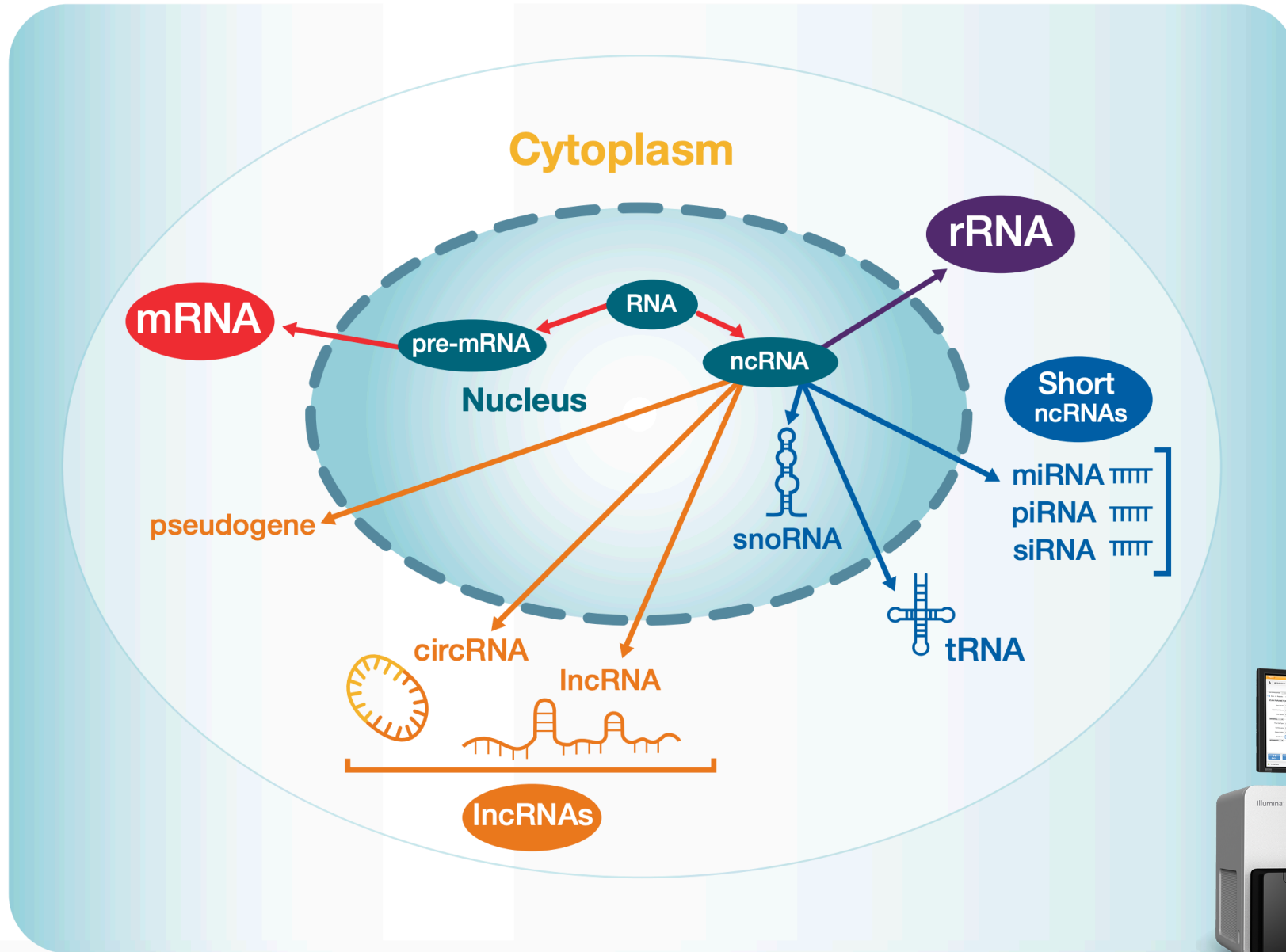
3. modified with adapters



1. DNA

2.

3.



1. DNA

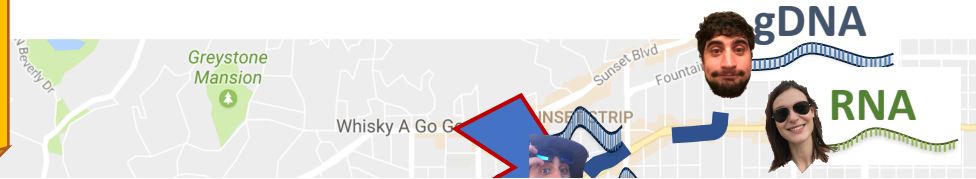
2. short (average 200-800 bp)

3. mod

RNA species	Biological function	Estimated percent of total RNA*
Ribosomal RNA (rRNA)	Protein synthesis	80–90
Transfer RNA (tRNA)	Protein synthesis	10–15
Messenger RNA (mRNA)	Translation	3–7
Long intergenic noncoding RNA (lincRNA)	Various (cell identity and differentiation)	<0.2
Small nucleolar RNA (snoRNA)	Post-transcriptional modification of RNA	0.04–0.2
Pseudogenes	Various (transcriptional regulation)	Variable**
MicroRNA (miRNA)	RNA silencing, post-transcriptional regulation	0.02

* Percentage by mass [7].

** Pseudogene transcripts can be counted in both coding and noncoding RNA.



3. modified with adapters



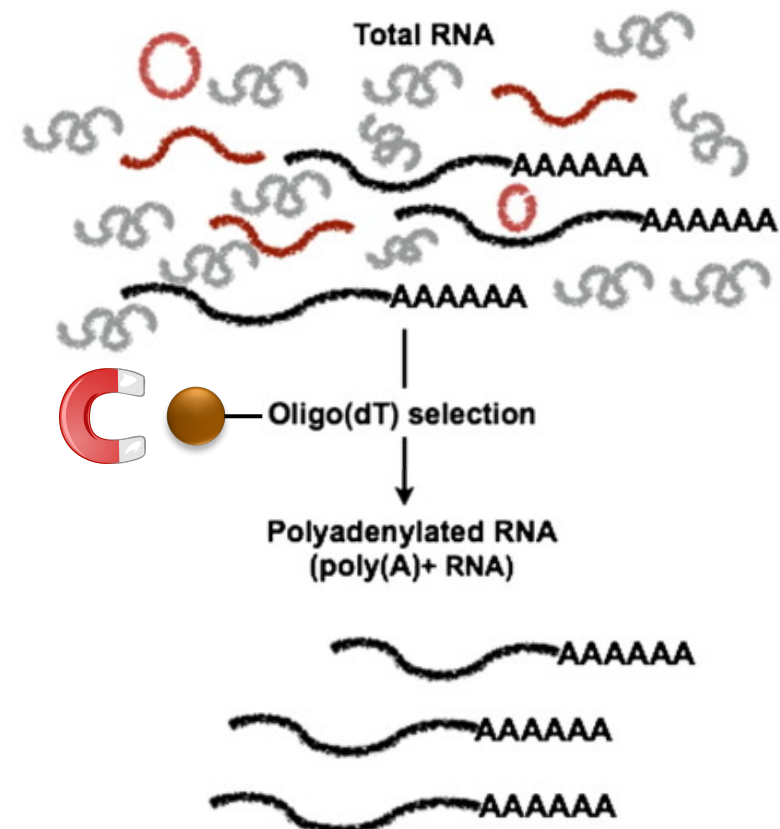
RNA species	Biological function	Estimated percent of total RNA*
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Pseudogenes	Various (transcriptional regulation)	Variable**
MicroRNA (miRNA)	RNA silencing, post-transcriptional regulation	0.02

* Percentage by mass [7].

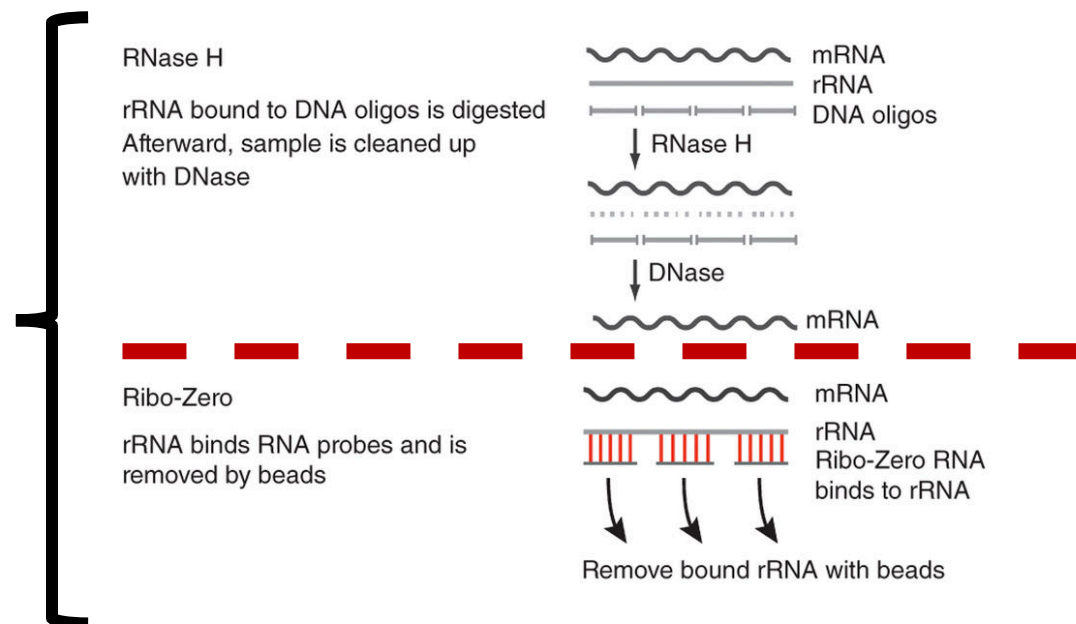
** Pseudogene transcripts can be counted in both coding and noncoding RNA.

RNAseq → enrichment

1. polyA Enrichment

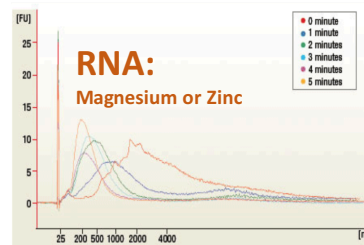
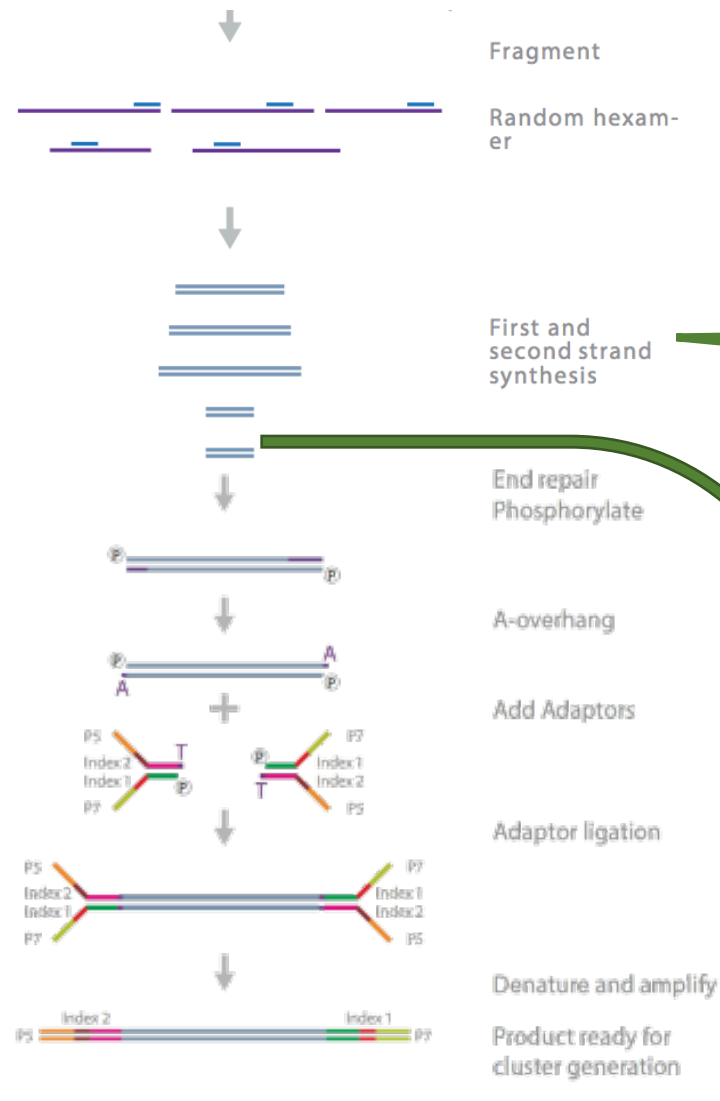


2. rRNA removal (+ other versions)



RNAseq → Library Prep

Enriched RNA



A. Reverse Transcriptase

**B. Second Strand Synthesis Enzymes:
DNA pol I, RNase H, DNA ligase (*E. coli*)**

Same as the DNA Prep

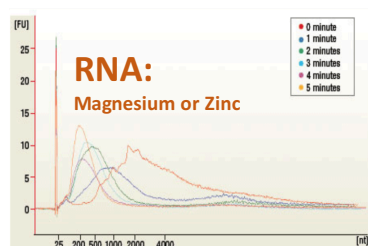
RNAseq → Library Prep

Enriched RNA



Fragment

Random hexamer



First and second strand synthesis

End repair
Phosphorylate

A-overhang

Add Adaptors

Adaptor ligation

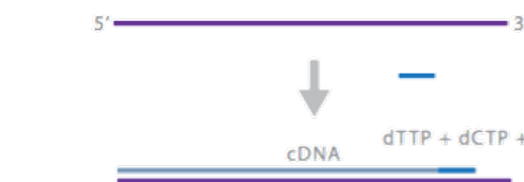
Denature and amplify

Product ready for cluster generation

A. Reverse Transcriptase

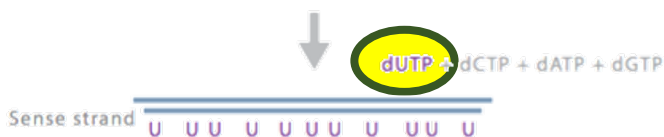
B. Second Strand Synthesis Enzymes:
DNA pol I, RNase H, DNA ligase

STRANDED RNA PROTOCOL



RNA
Random primer

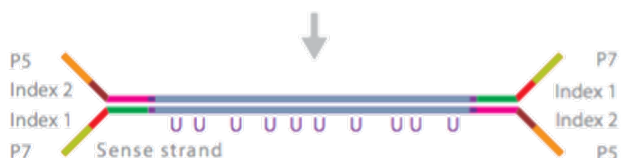
Create cDNA
Reverse Transcriptase



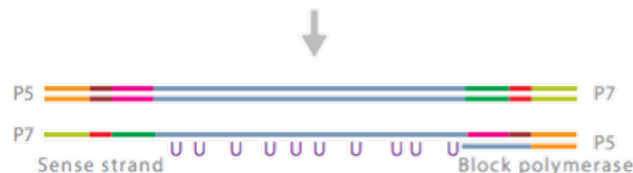
Create second
strand cDNA



End repair
Phosphorylate
A-overhang



Adaptor ligation



Denature and
amplify



Product ready for
cluster generation

***Sense strand: strand of the DNA that has the same sequence as the mRNA

**DRAW
STRANDED
PREP**

RNAseq → Library Prep

5'  3' Original RNA

vs.

3'  5' FINAL LIBRARY
Read 2 Read 1

REVERSE COMPLEMENT!!!!





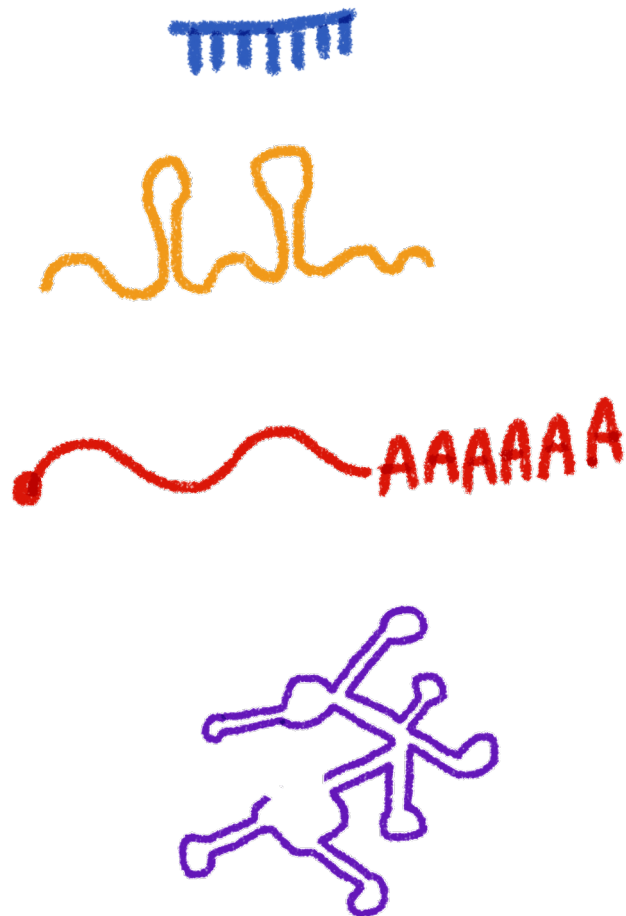
**KEEP
CALM
IT'S
BREAK
TIME**



QUESTIONS
#9

RNAseq → The Enrichment Strategy Depends also on the Initial Quality

Intact RNA



miRNA

lncRNAs

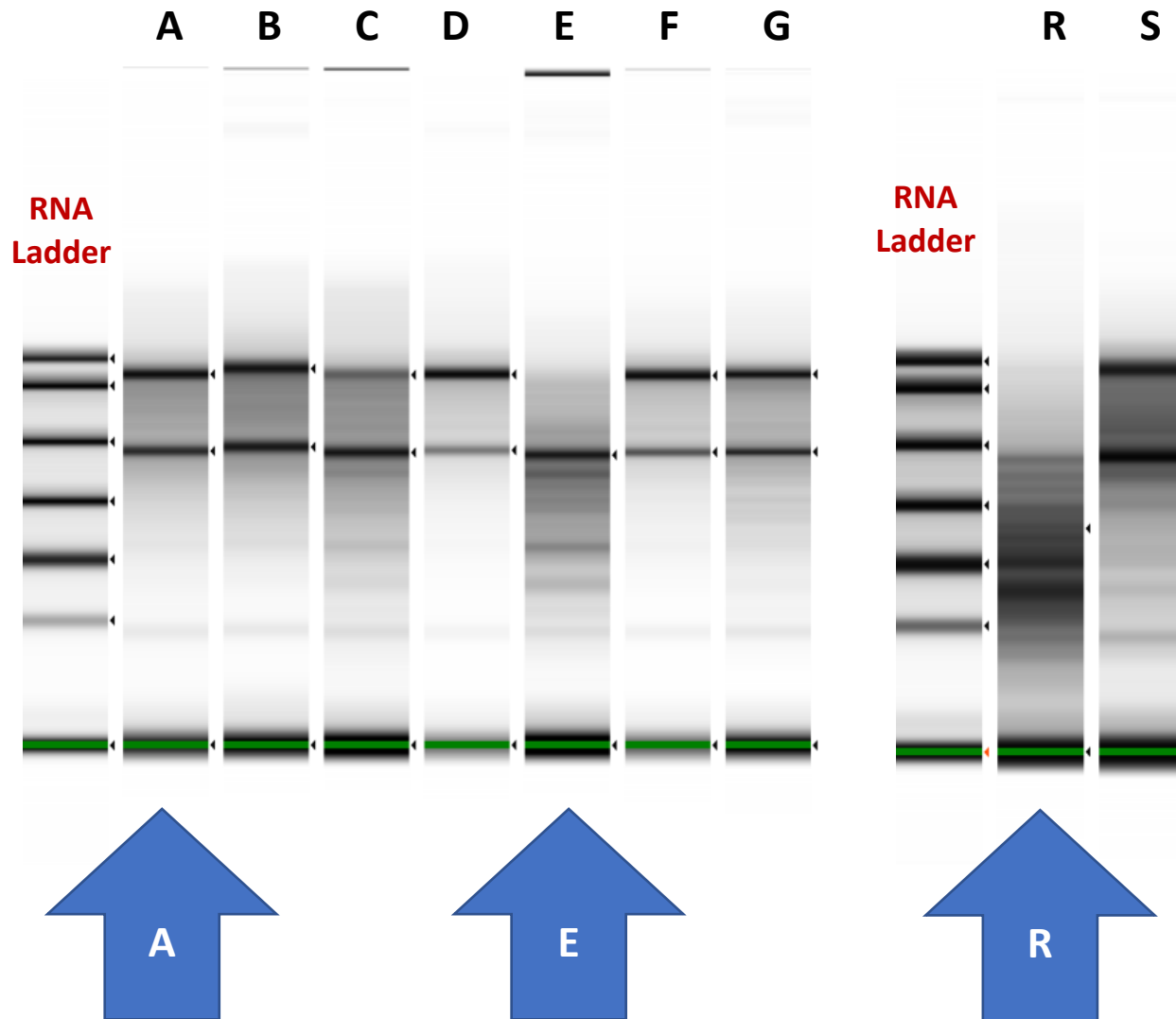
mRNA

rRNA

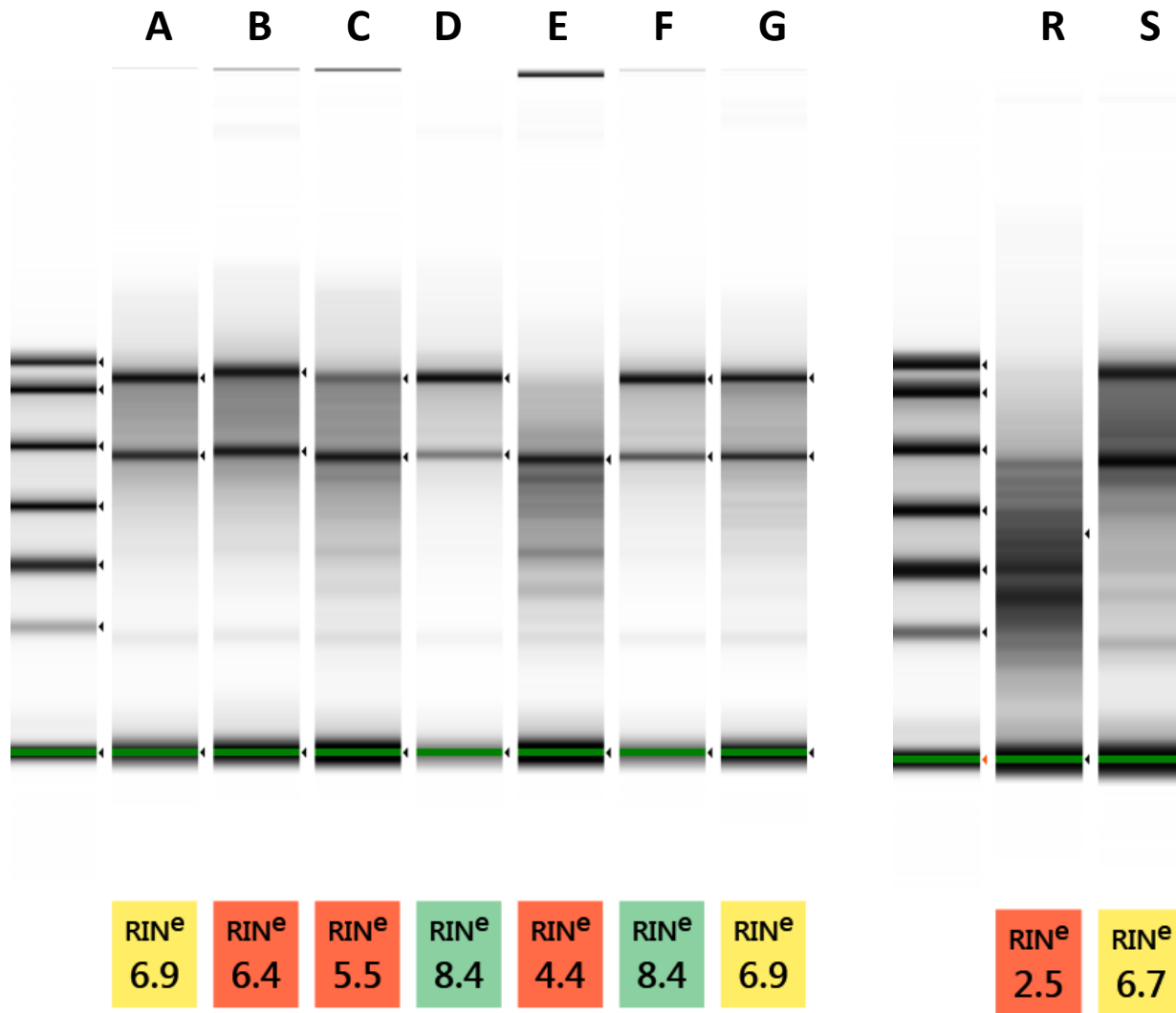
Fragmented RNA



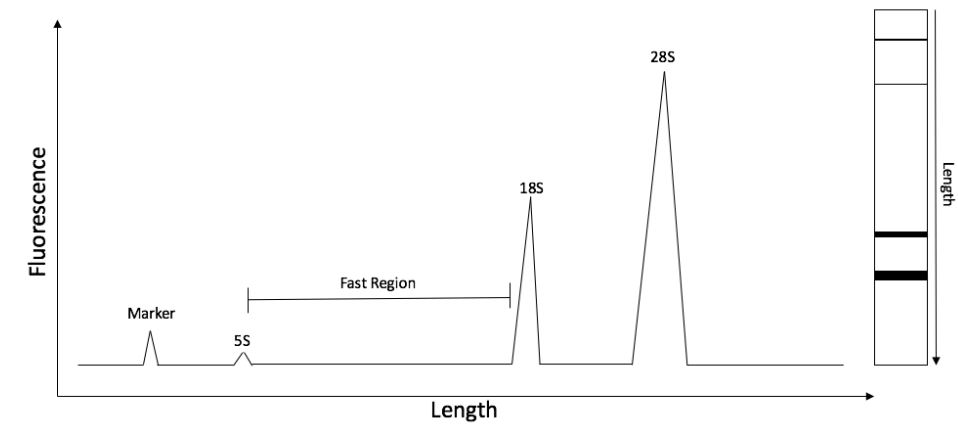
RNAseq → RNA Integrity



RNAseq → RNA Integrity

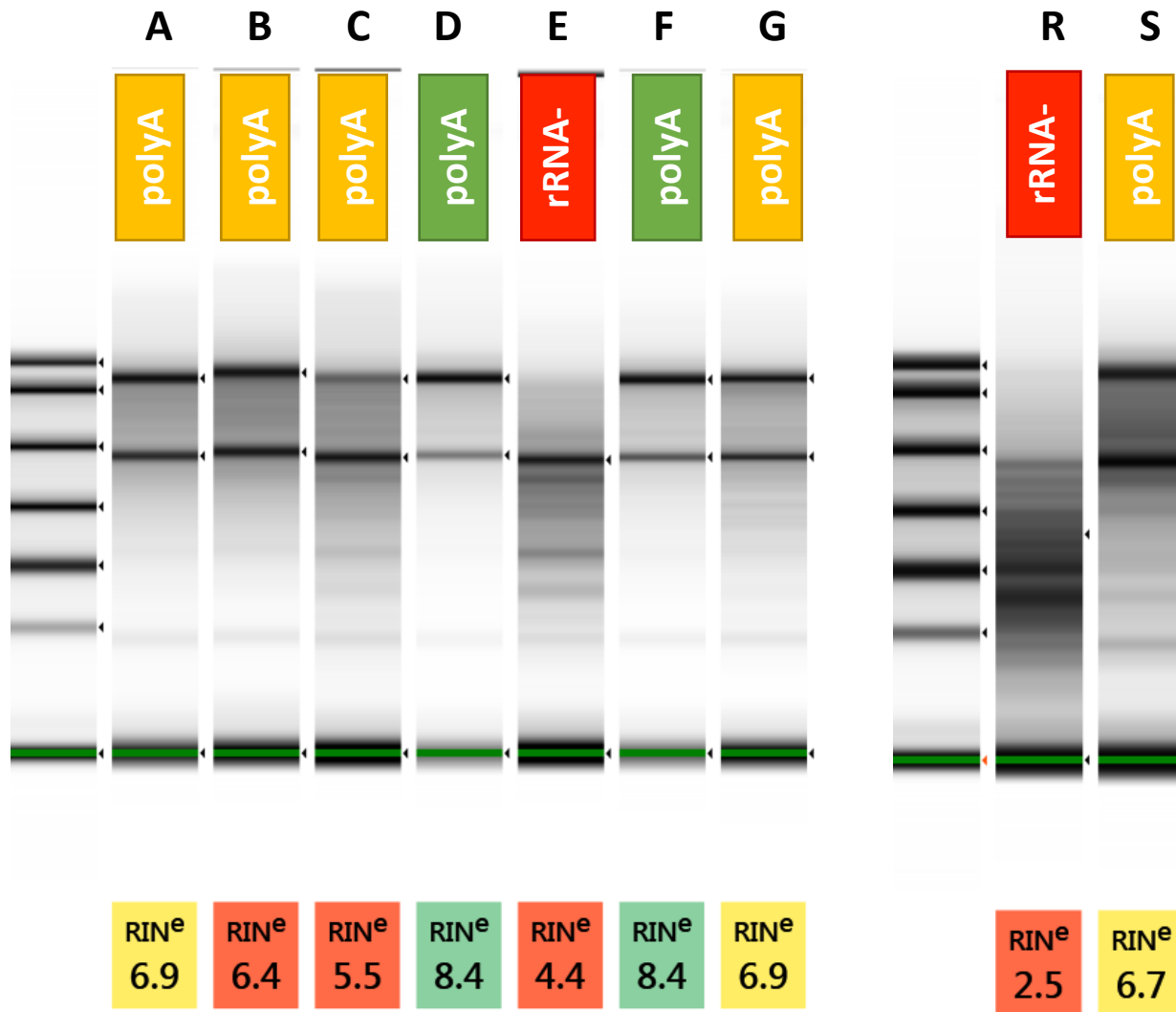


RIN Score: RNA Integrity Number
1 = Degraded
10 = Intact

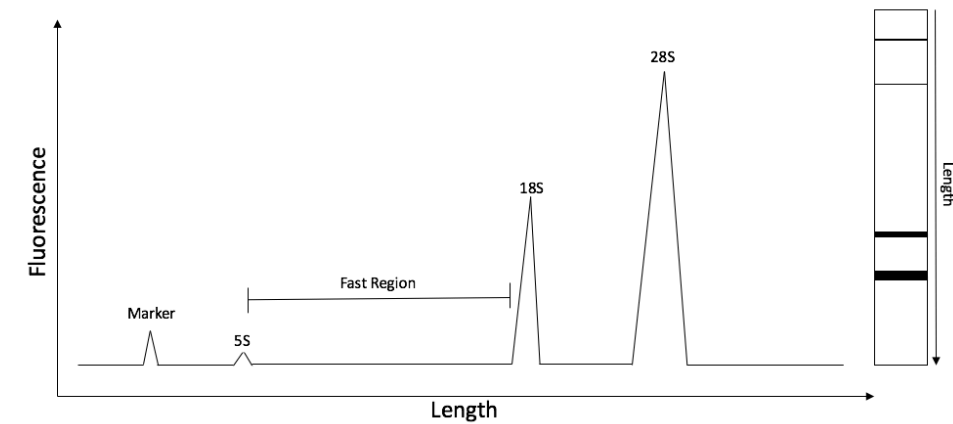


DV200 Method: for degraded samples, the RIN score is not accurate

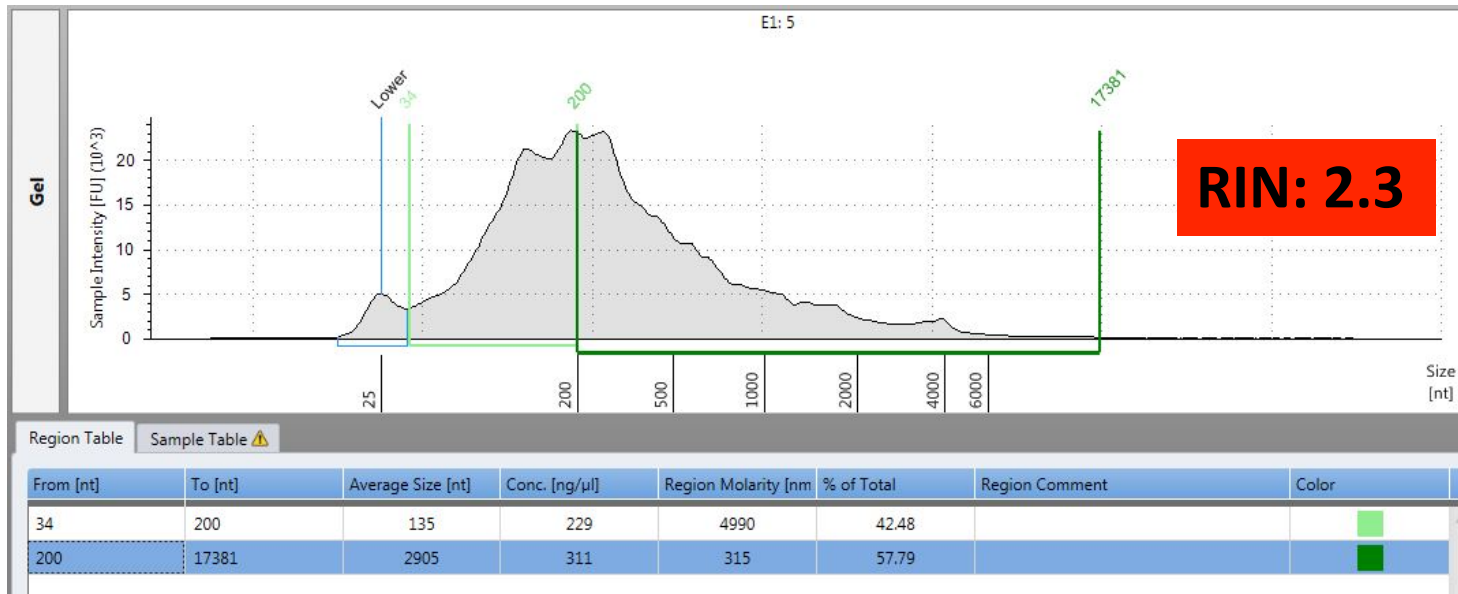
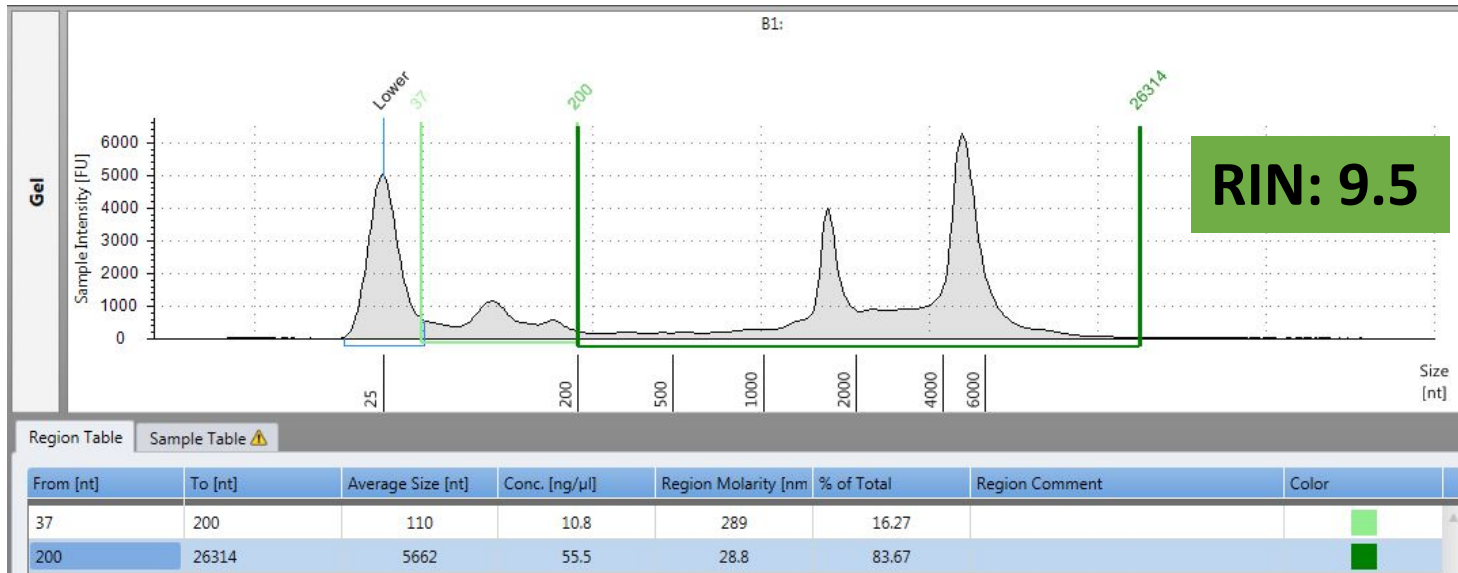
RNAseq → RNA Integrity



RIN Score: RNA Integrity Number
1 = Degraded
10 = Intact



DV200 Method: for degraded samples, the RIN score is not accurate



Parameter
Quality*†‡

DV₂₀₀

High

> 70%

Medium

50–70%

Low

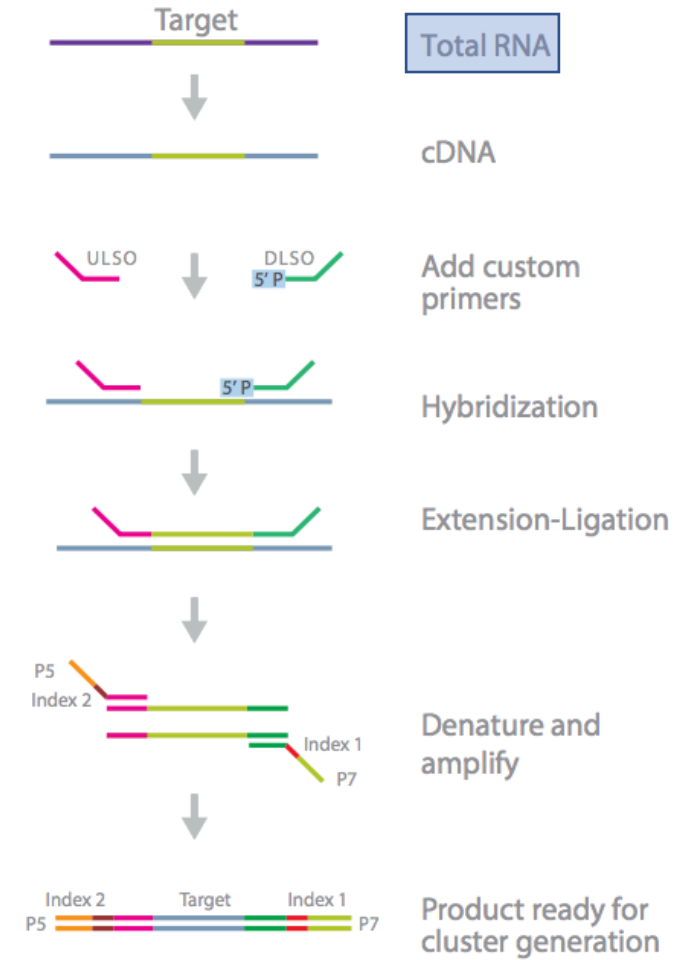
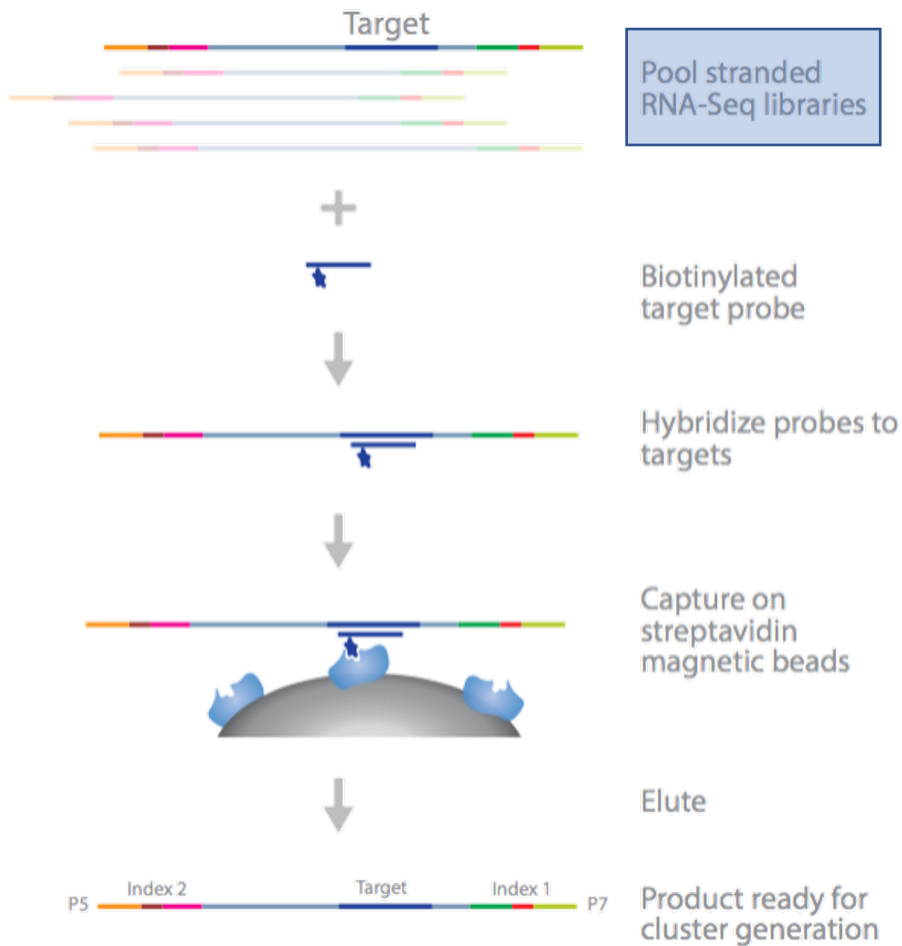
30–50%

Too Degraded

< 30%

DV200 Method: for degraded samples, the RIN score is not accurate

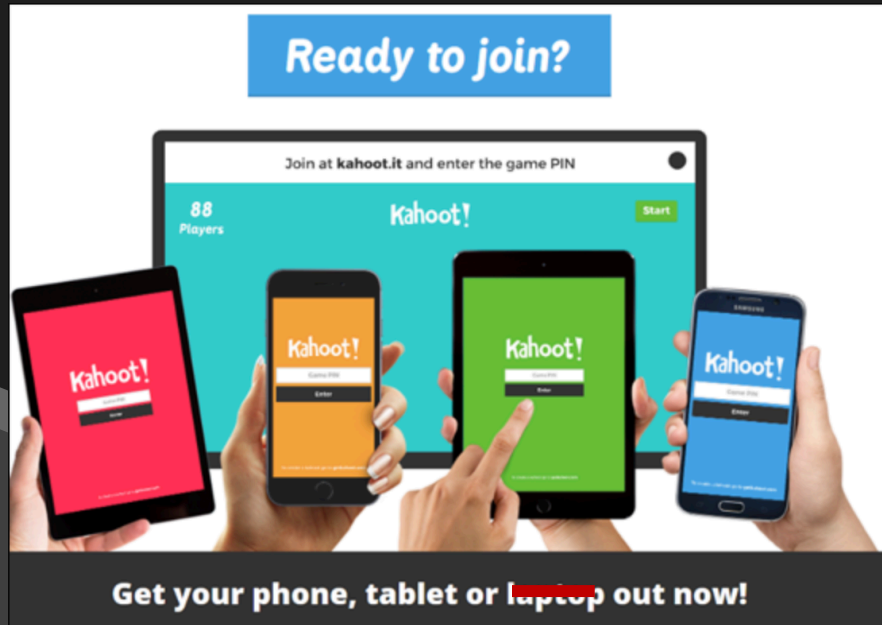
RNAseq → Targeted



Better exon coverage in low quality samples – it requires less coverage, but library prep more expensive

→ Go to Kahoot.it

then follow on-
screen instruction



QUIZ RNA

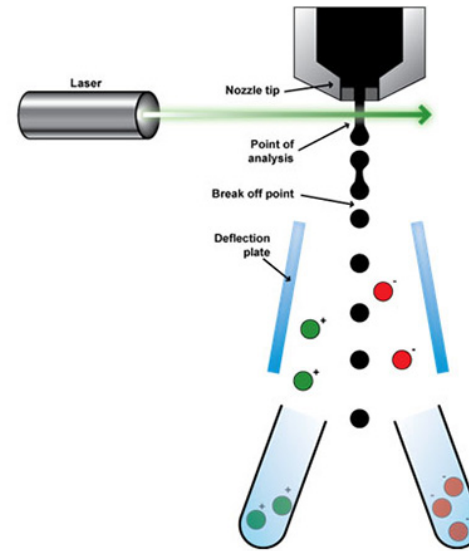
Kahoot!

Game PIN

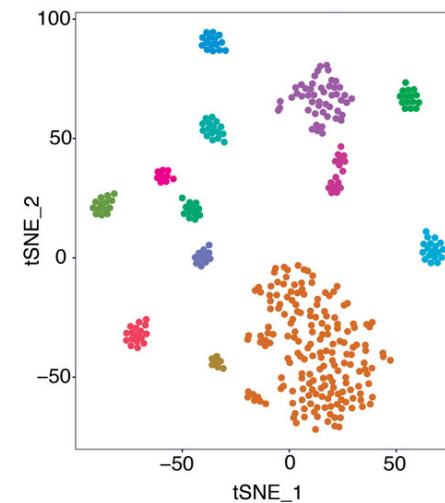
Enter

RNAseq → low-input

a. Sorted cell population
(low input)

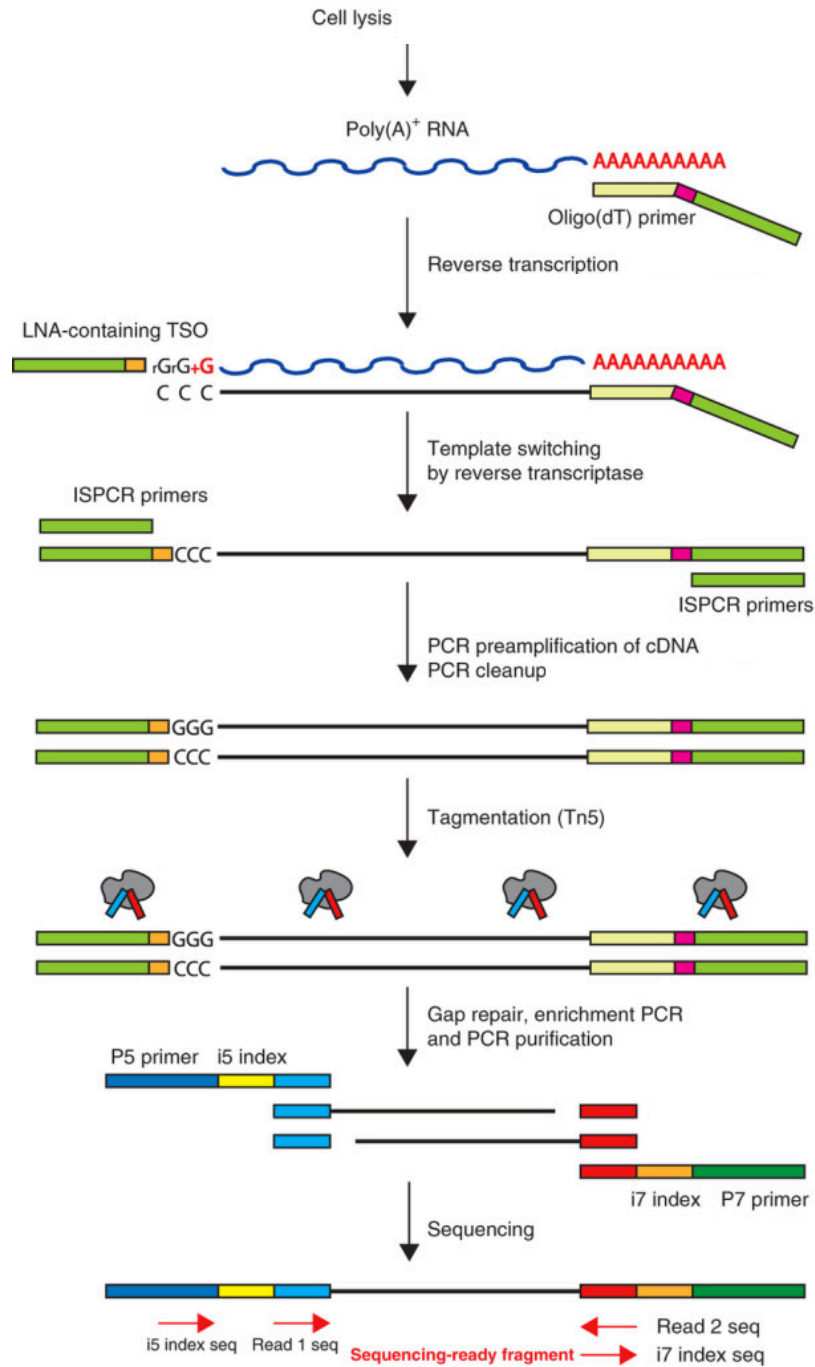


b. Single cells



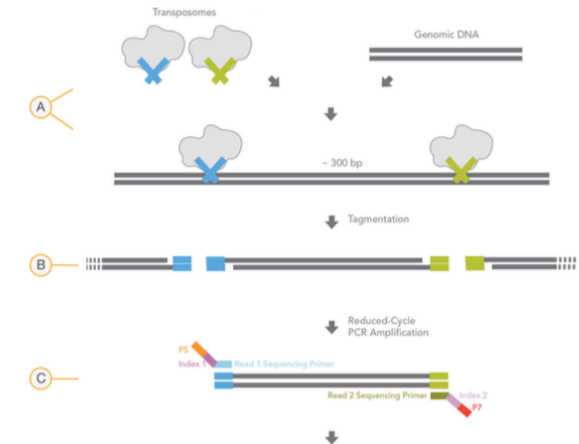
RNAseq → low-input

Commercial kits and home-brewed protocols
SMARTseq



Template switch → Property of RT enzymes

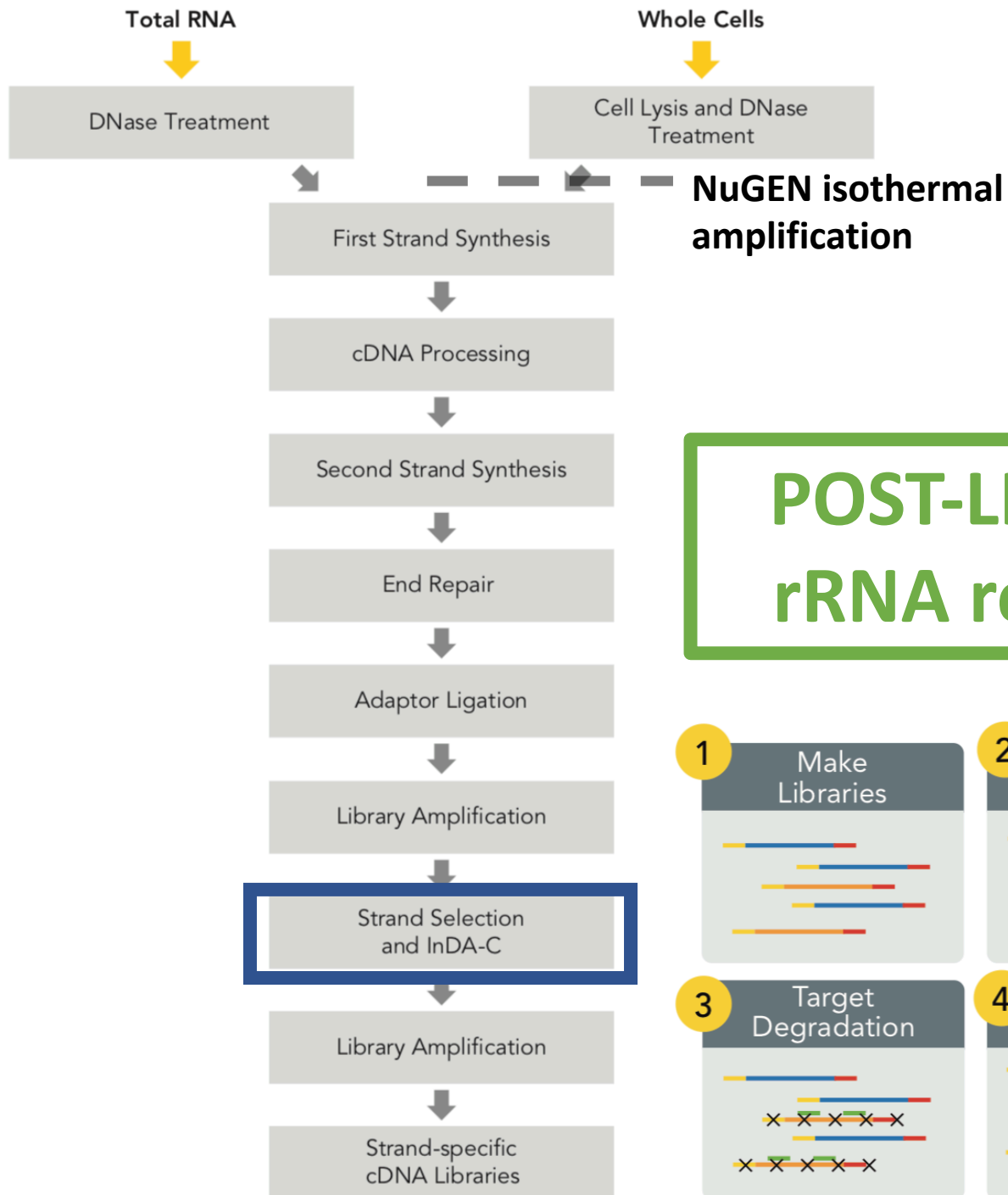
Tagmentation → Low input



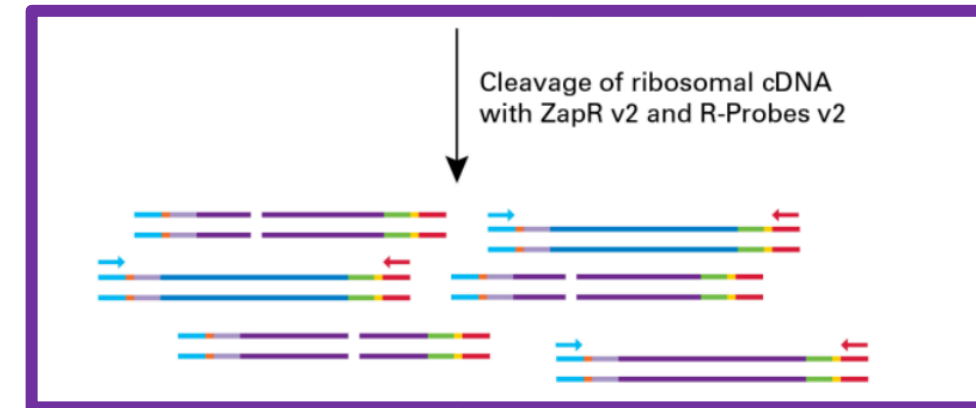
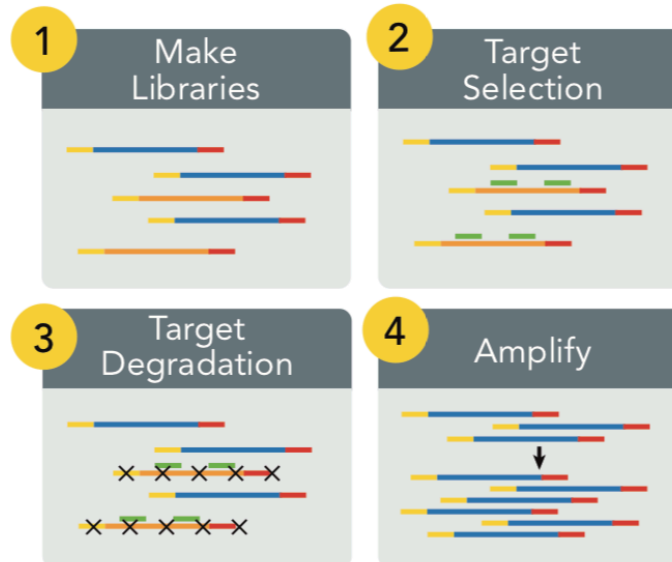
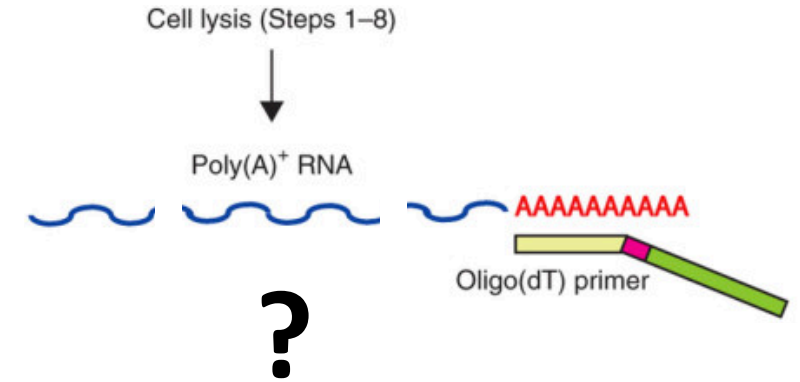
Single cells: 10-50pg of total RNA

→ mRNA is 1-5% of total RNA: 0.1 – 2.5 pg

RNAseq → low-input and degraded RNA #2

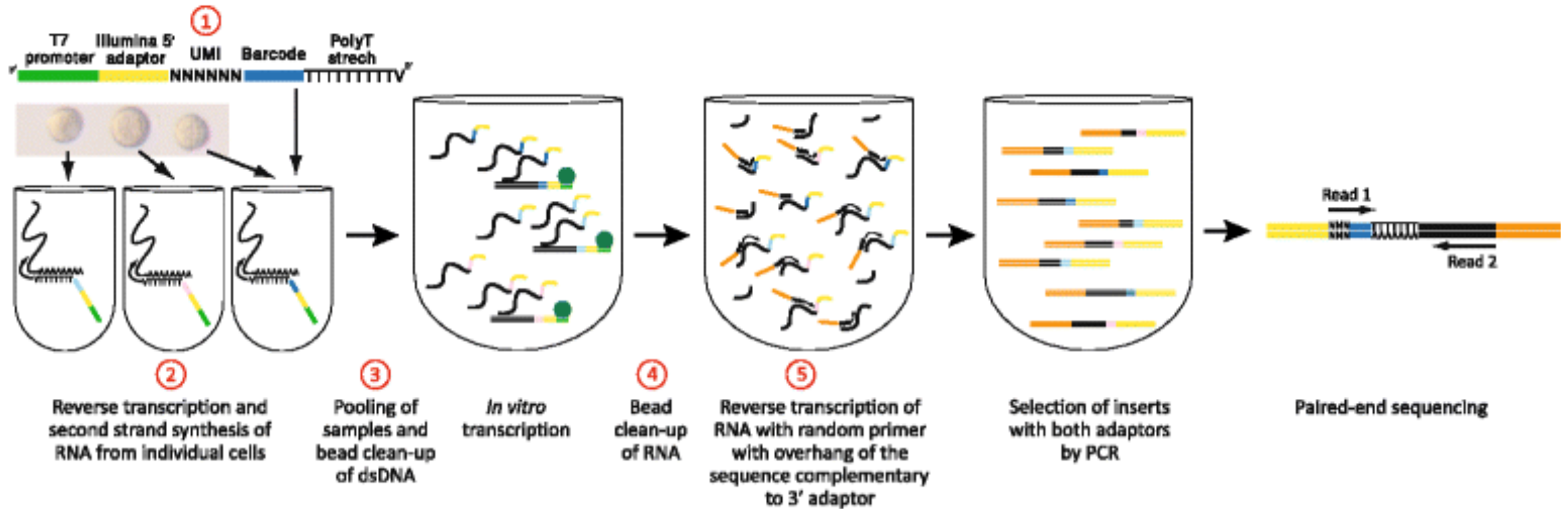


**POST-LIBRARY
rRNA removal**

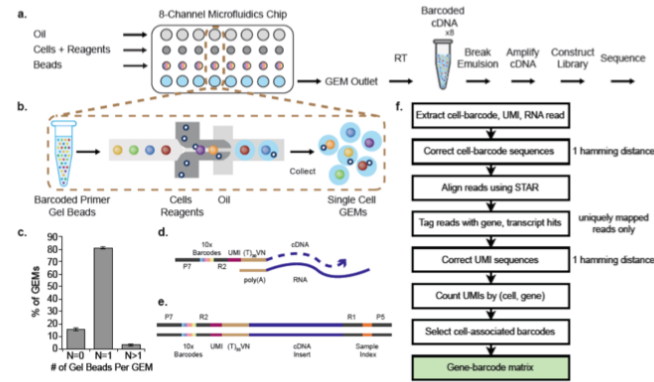
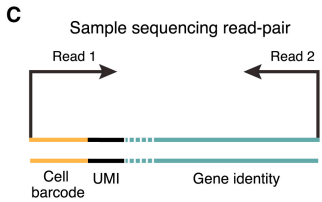
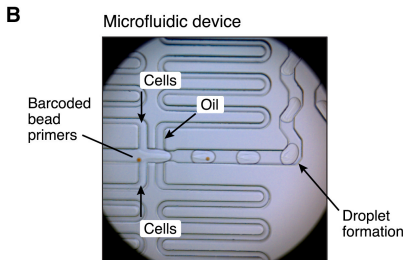
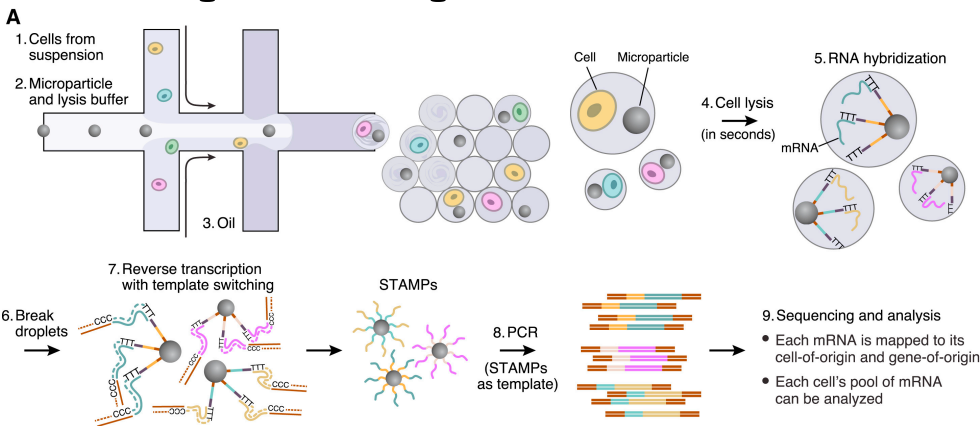


CELseq → *in vitro* transcription instead of PCR

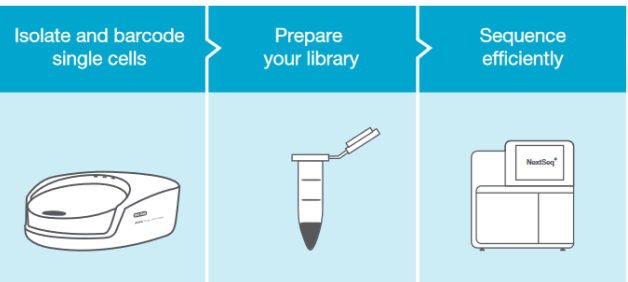
a



DropSeq & Co.



BD Targeted scRNAseq + Ab-seq



Fluidigm C1

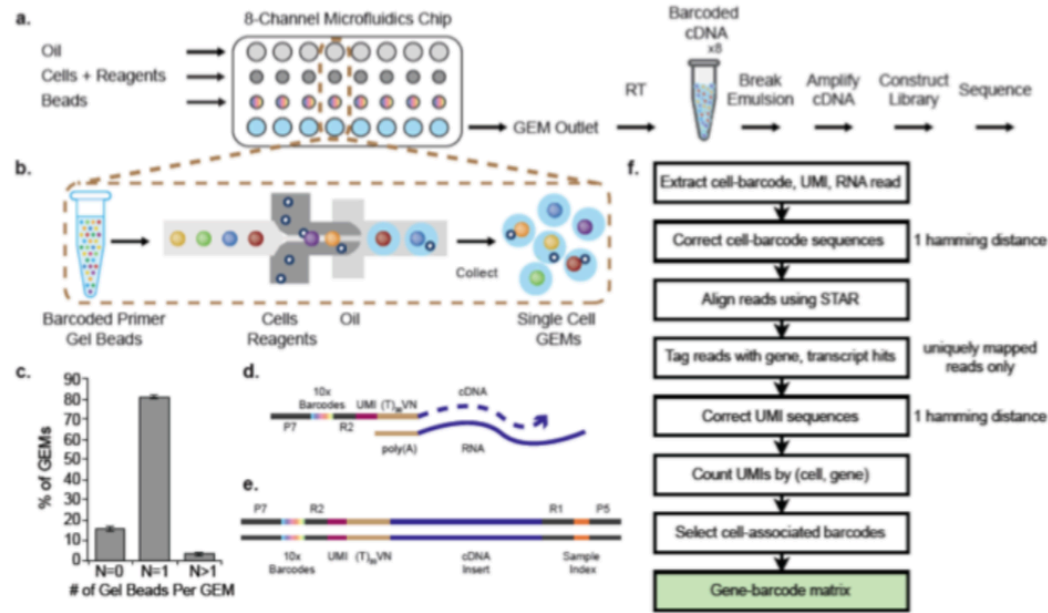


Fluidigm C1

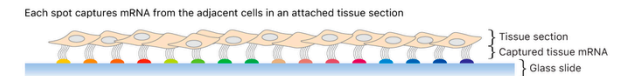
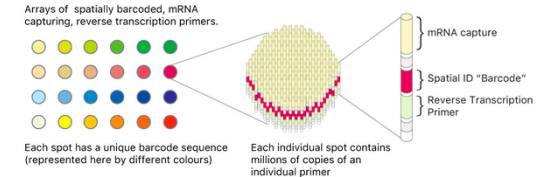


Illumina BioRad

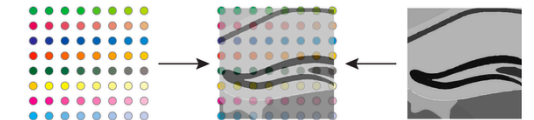
10x GENOMICS™



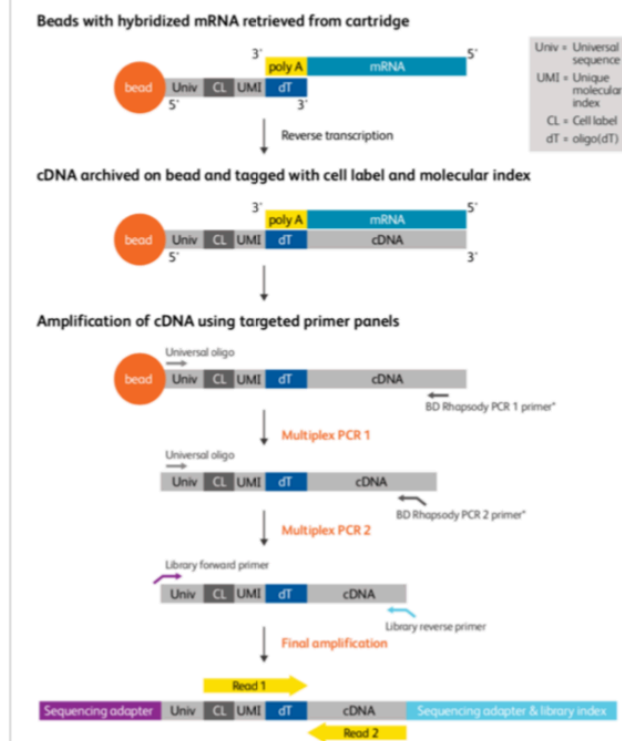
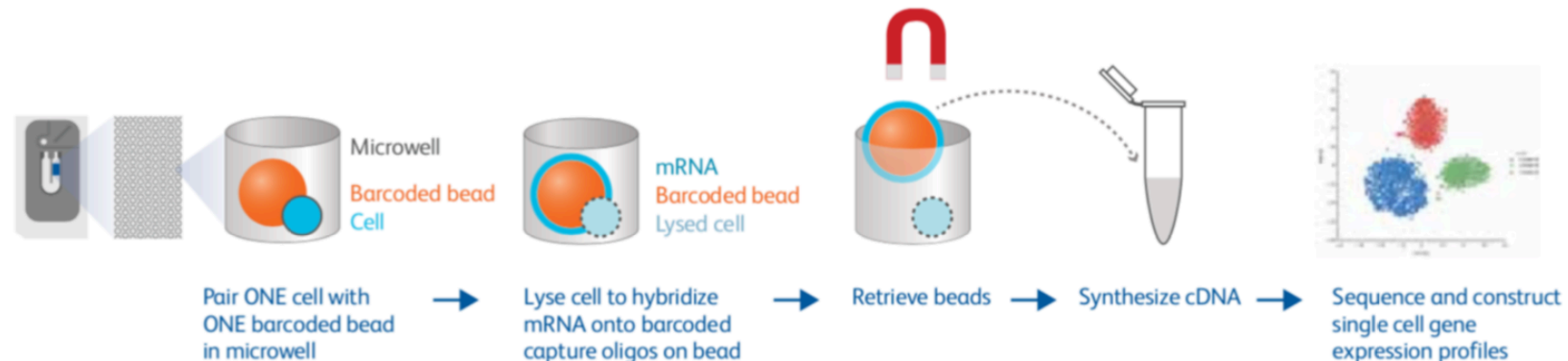
- Single cell DNA
- Single cell 3'-end
- V(D)J
- Single cell ATAC-seq
- Genome
- Exome
- *de novo* DNA seq



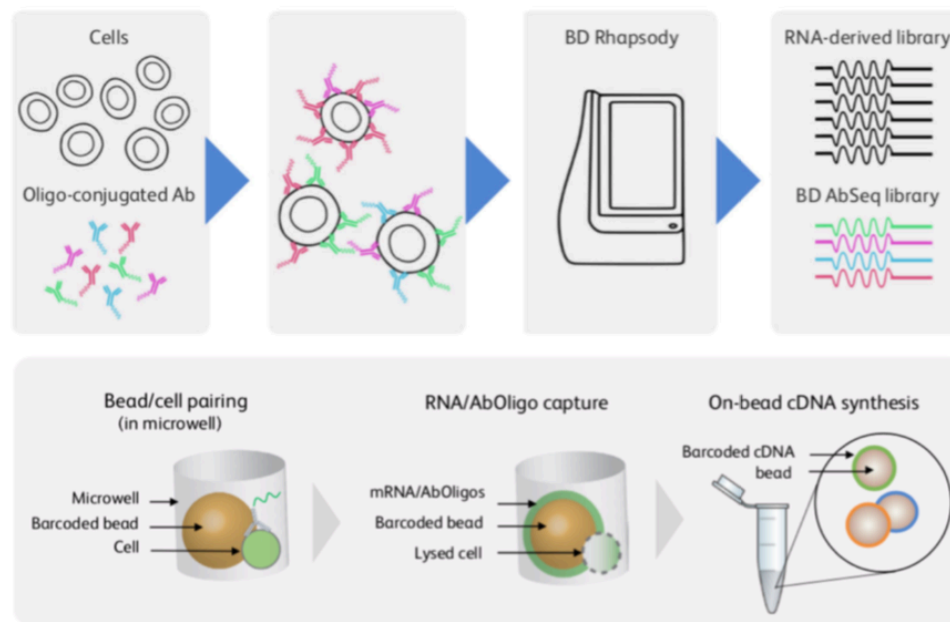
Sequencing data for each individual mRNA can be mapped back to its spot of origin on the array. Alignment of the array with a previously captured image of the tissue section then allows the spatial analysis of all tissue mRNAs.



+ Spatial Transcriptomics



BD Rhapsody targeted single-cell + Ab-seq

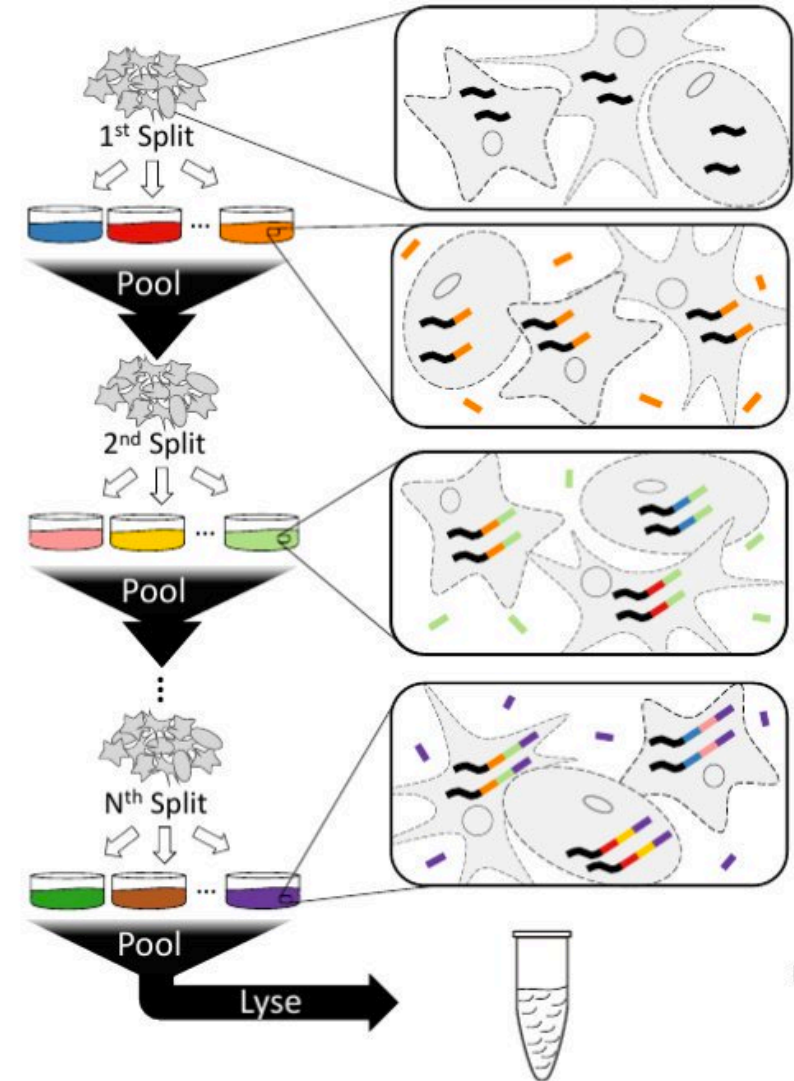


STANDARD SINGLE CELL

1. Physical isolation of single cells (droplet, well, ...)
2. Reverse Transcription with cell barcode
3. Pooling and PCR

VS.

Combinatorial Indexing

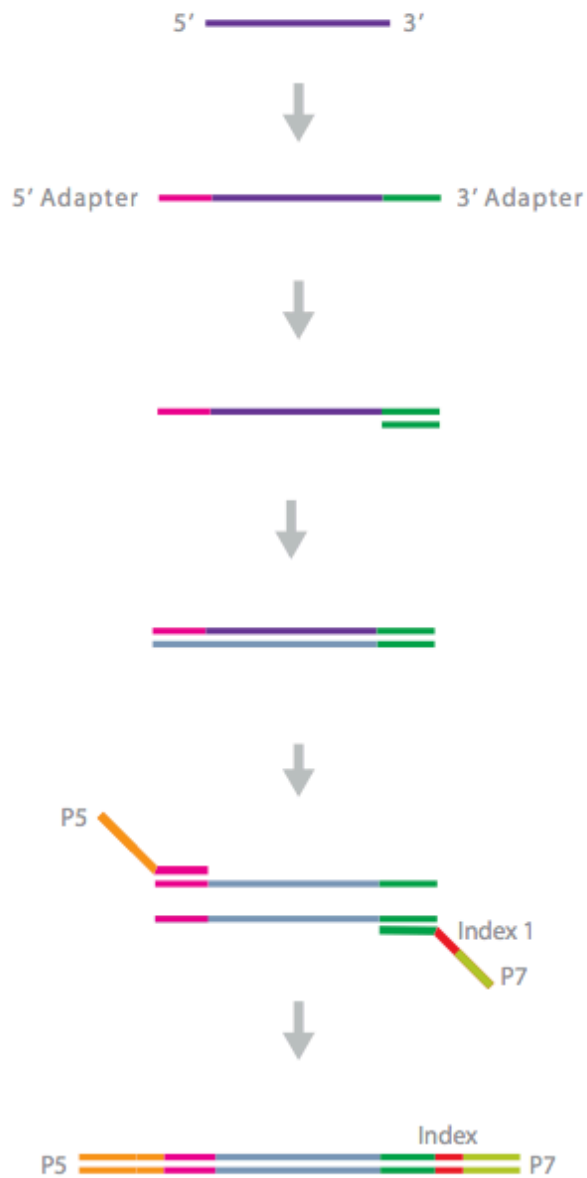


SplitBio

PRODUCTS ▼ TECHNOLOGY CAREERS COMPANY CONTACT

Scalable
Single Cell
Sequencing
Without the Instrument

RNAseq → smallRNA



Small RNA fragment

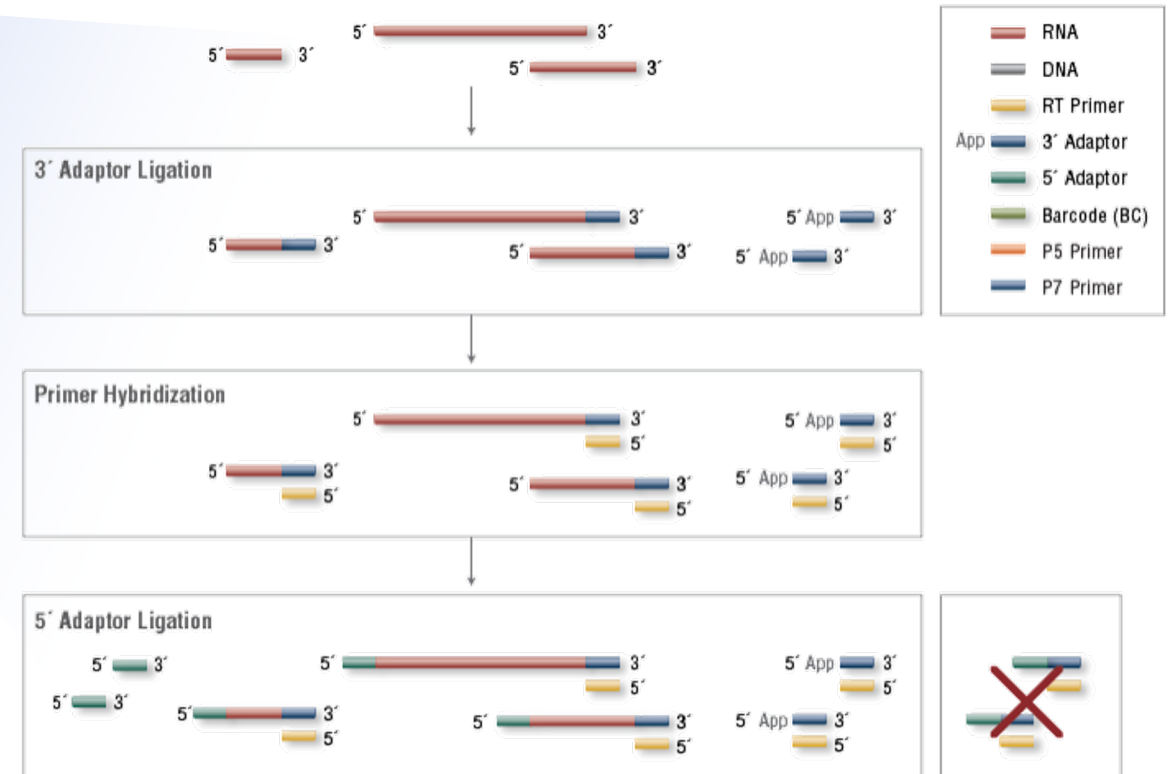
Ligate adaptors

Add primer

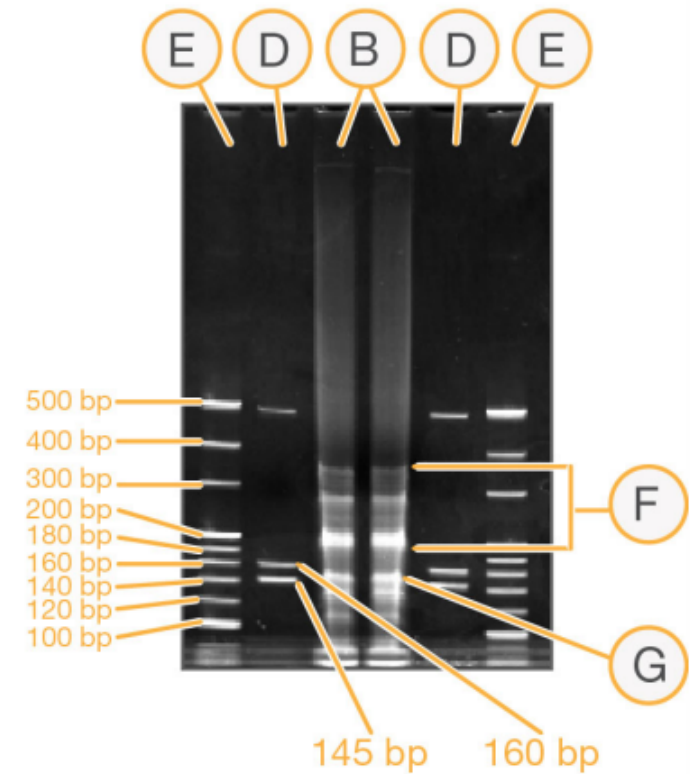
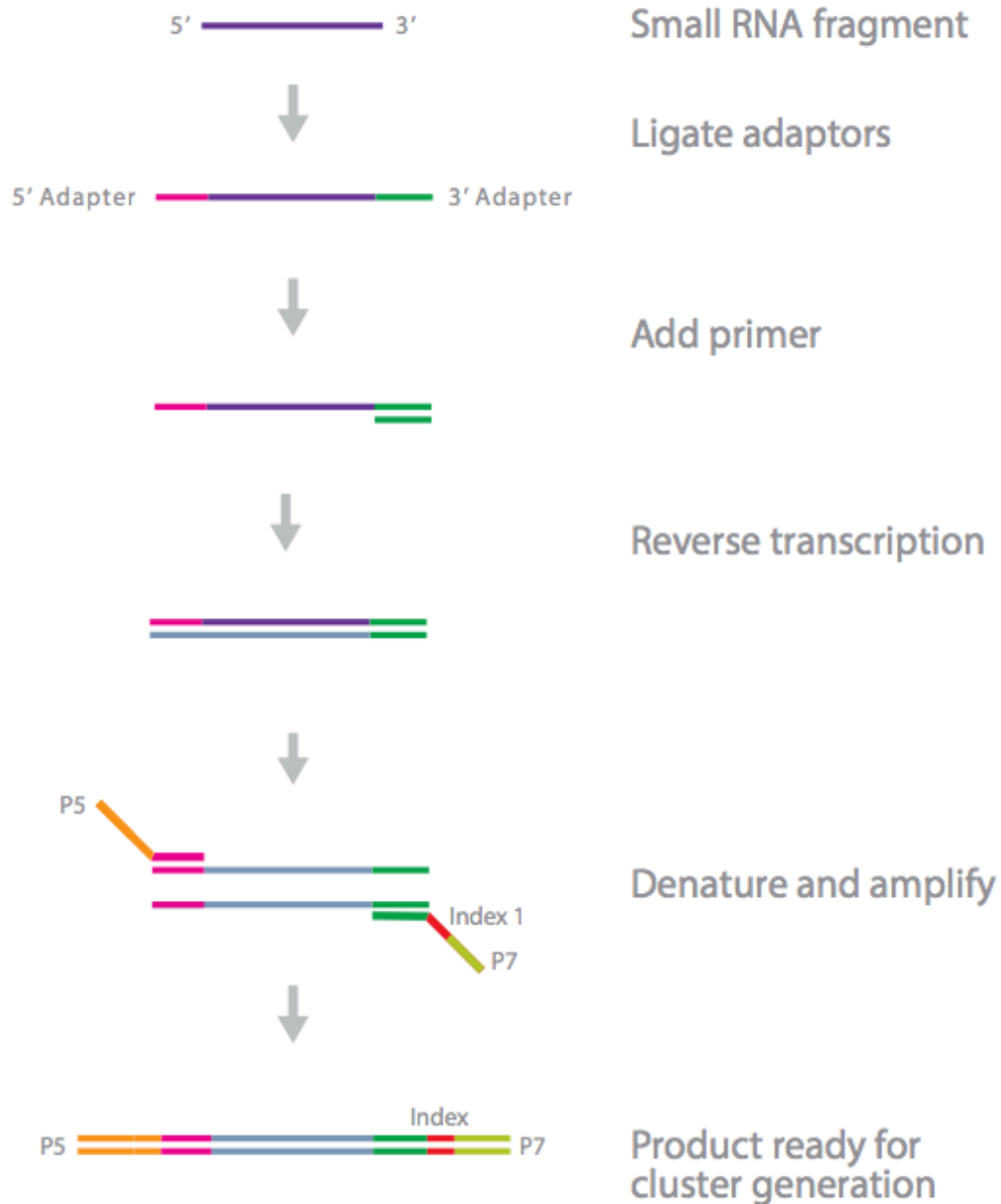
Reverse transcription

Denature and amplify

Product ready for cluster generation



RNAseq → smallRNA

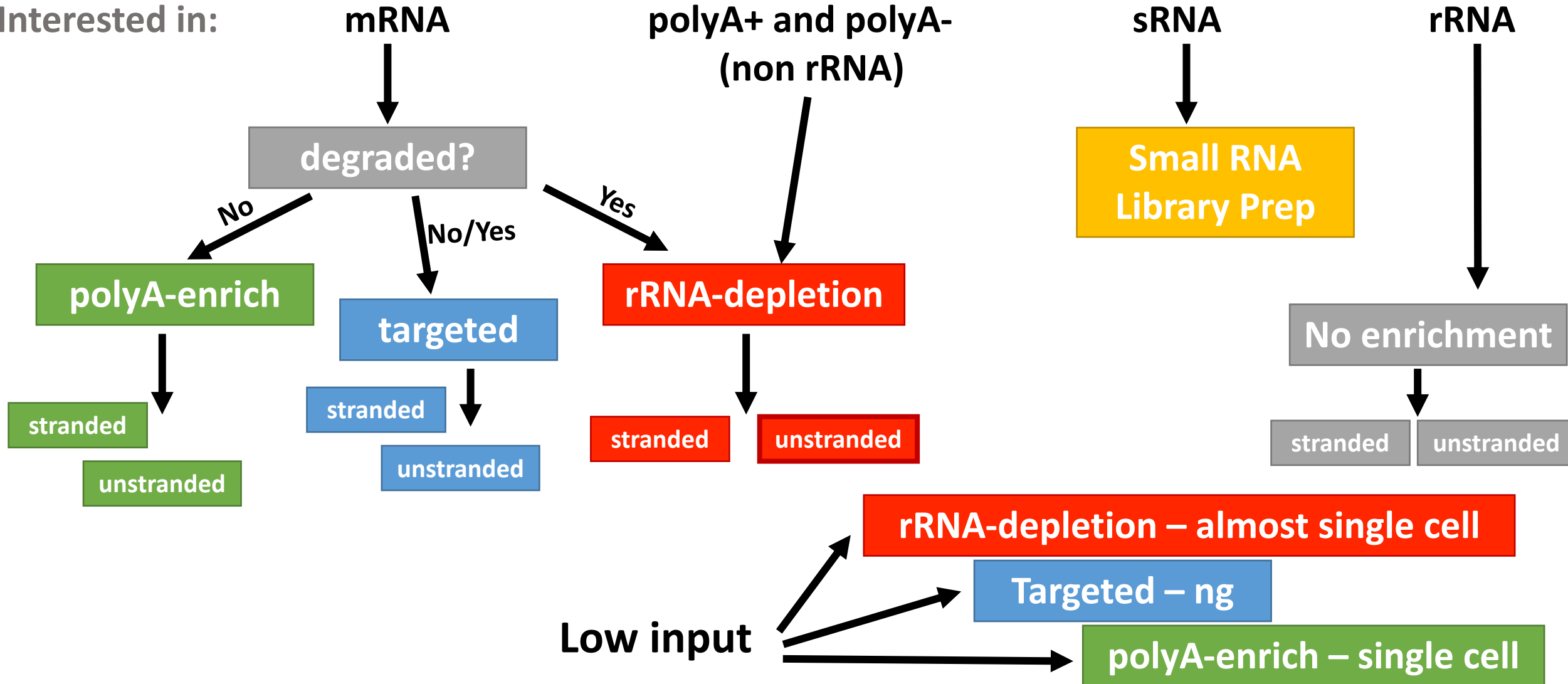


Size selection options:

1. At the very beginning, before library prep – denaturing polyacrylamide gel (10-15%)
2. After PCR – TBE PAGE 6%
3. both

RNAseq selector

Interested in:





**KEEP
CALM
IT'S
BREAK
TIME**



QUESTIONS
#10

OTHER RELATED WORKSHOPS

WGS

W8: Variant Discovery with GATK	May 5, 6, and 7	1:30 PM to 4:30 PM	Peter Scott, Ph.D.
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WGS or 16S-seq

W11: Metagenomics Analysis	May 12, 13, and 14	9:00 AM to 12:00 PM	TBD
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ChIP-seq

W7: ChIP-seq	May 12, 13, and 14	1:30 PM to 4:30 PM	Nathan Zemke, Ph.D.
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W24: MERFISH/seqFISH	May 19, 20, and 21	9:00 AM to 12:00 PM	Rob Foreman, Ph.D.
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BS-seq

W23: DNA Methylation Analysis	May 19, 20, and 21	1:30 PM to 4:30 PM	Anela Tosevska, Ph.D.
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RNA-seq

W5a: RNA-seq I Analysis	May 26, 27, and 28	9:00 AM to 12:00 PM	Karolina Kaczor-Urbanowicz, Ph.D. Nicolas Rochette, Ph.D.
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W4: Galaxy for NGS	May 26 and 27	1:30 PM to 3:30 PM	Weihong Yan, Ph.D.
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RNA-seq

W5b: RNA-seq II Analysis	June 2 and June 4	10:00 AM to 12:00PM	Karolina Kaczor-Urbanowicz, Ph.D. Nicolas Rochette, Ph.D.
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scRNA-seq

W20: Single-Cell RNA-Seq	June 2 and 3	1:30 PM to 3:30 PM	Igor Mandric, Ph.D. Ying Tang, Ph.D.
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BEST PRACTISES IN NGS

1. **SPIKE-IN CONTROLS** → Especially for Quantitative Applications, such as RNAseq, ChIPseq...
2. **UMIs (Unique Molecular Identifiers)** → In order to distinguish the True Biological Signal vs. PCR Duplicate Artifacts
3. **UDI (Unique Dual Indices)** → Important with the advent of patterned flow cells. Each barcode is not present in any other sample (different from Combinatorial Dual Indices)

Spike-in controls



Chen K, Hu Z, Xia Z, Zhao D, Li W, Tyler JK. 2016. The overlooked fact: fundamental need for spike-in control for virtually all genome-wide analyses. *Mol Cell Biol* 36:662–667. doi:[10.1128/MCB.00970-14](https://doi.org/10.1128/MCB.00970-14)

The Overlooked Fact: Fundamental Need for Spike-In Control for Virtually All Genome-Wide Analyses

Kaifu Chen,^{c,d,e} Zheng Hu,^{a,e} Zheng Xia,^b Dongyu Zhao,^{c,d,e} Wei Li,^b Jessica K. Tyler^{a,e}

Department of Epigenetics and Molecular Carcinogenesis, University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA^a; Dan L. Duncan Cancer Center and Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA^b; Institute for Academic Medicine, Houston, Texas, USA^c; Center for Cardiovascular Regeneration, Department of Cardiovascular Sciences, The Methodist Hospital Research Institute, Houston, Texas, USA^d; Weill Cornell Medicine, New York, New York, USA^e

Genome-wide analyses of changes in gene expression, transcription factor occupancy on DNA, histone modification patterns on chromatin, genomic copy number variation, and nucleosome positioning have become popular in many modern laboratories, yielding a wealth of information during health and disease states. However, most of these studies have overlooked an inherent normalization problem that must be corrected with spike-in controls. Here we describe the reason why spike-in controls are so important and explain how to appropriately design and use spike-in controls for normalization. We also suggest ways to retrospectively renormalize data sets that were wrongly interpreted due to omission of spike-in controls.

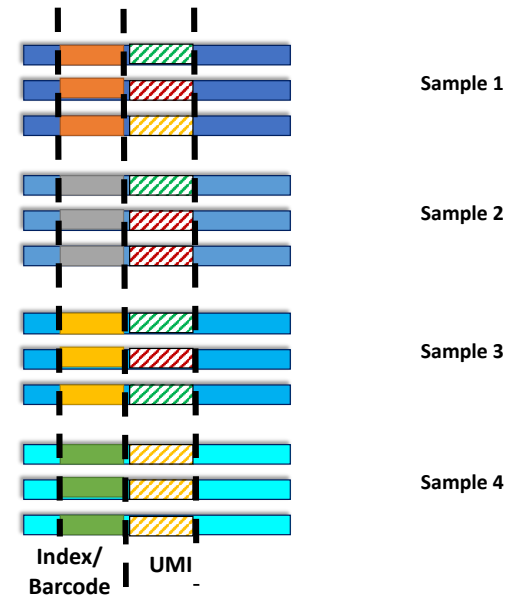
UMIs in NGS

Counting absolute numbers of molecules using unique molecular identifiers

Teemu Kivioja^{1-3,5}, Anna Vähärautio^{1,3,5},
Kasper Karlsson⁴, Martin Bonke¹, Martin Enge³,
Sten Linnarsson⁴ & Jussi Taipale³

Counting individual RNA or DNA molecules is difficult because they are hard to copy quantitatively for detection. To overcome this limitation, we applied unique molecular identifiers (UMIs), which make each molecule in a population distinct, to genome-scale human karyotyping and mRNA sequencing in *Drosophila melanogaster*. Use of this method can improve accuracy of almost any next-generation sequencing method, including chromatin immunoprecipitation–sequencing, genome assembly, diagnostics and manufacturing-process control and monitoring.

Counting absolute numbers of molecules using unique molecular identifiers. Kivioja T, Vähärautio A, Karlsson K, Bonke M, Enge M, Linnarsson S, Taipale J. Nat Methods. 2011 Nov 20;9(1):72-4. doi: 10.1038/nmeth.1778.



Incorporation of unique molecular identifiers in TruSeq adapters improves the accuracy of quantitative sequencing

Jungeui Hong[†] and David Gresham
Center for Genomics and Systems Biology, Department of Biology,
New York University, New York, NY

[†]Present address: Memorial Sloan Kettering Cancer Center, New York, NY

BioTechniques 63:221-226 (November 2017) doi 10.2144/000114608

Keywords: unique molecular identifier (UMI); RNA-Seq; TruSeq; PCR duplicates; TrUMIseq



Custom NGS Adapters

Adapter and indexing primer selections for DNA library prep

Choose from a variety of options that personalize the adapters to your NGS workflow. Order Custom NGS Adapters when you need to:

- Scale up your experiments—get the delivery format (volume, plate, single-use) and volume-pricing that best suit your NGS study.
- Expand your set of barcodes to increase sample throughput—augment limited sets of stocked adapters.
- Supplement commercial library kit components—create your own custom library prep workflow that fits your experimental design.

The Custom Adapter Configurator tool helps you design the adapters best suited to your experiments by walking you through these choices:

- Library prep method
- Configuration (SI, CDI, UDI, UDI-UMI)
- Attachment style
- Indexing strategy
- Number of indexes
- Size (reaction number or nmol)
- Delivery format (tubes, plates)
- Methylated/unmethylated
- TruGrade processing

Sample 1:

Sample 2:

Sample 3:

Index 1



Index 2



CDIs
Combinatorial

Sample 1:

Sample 2:

Sample 3:

Index 1

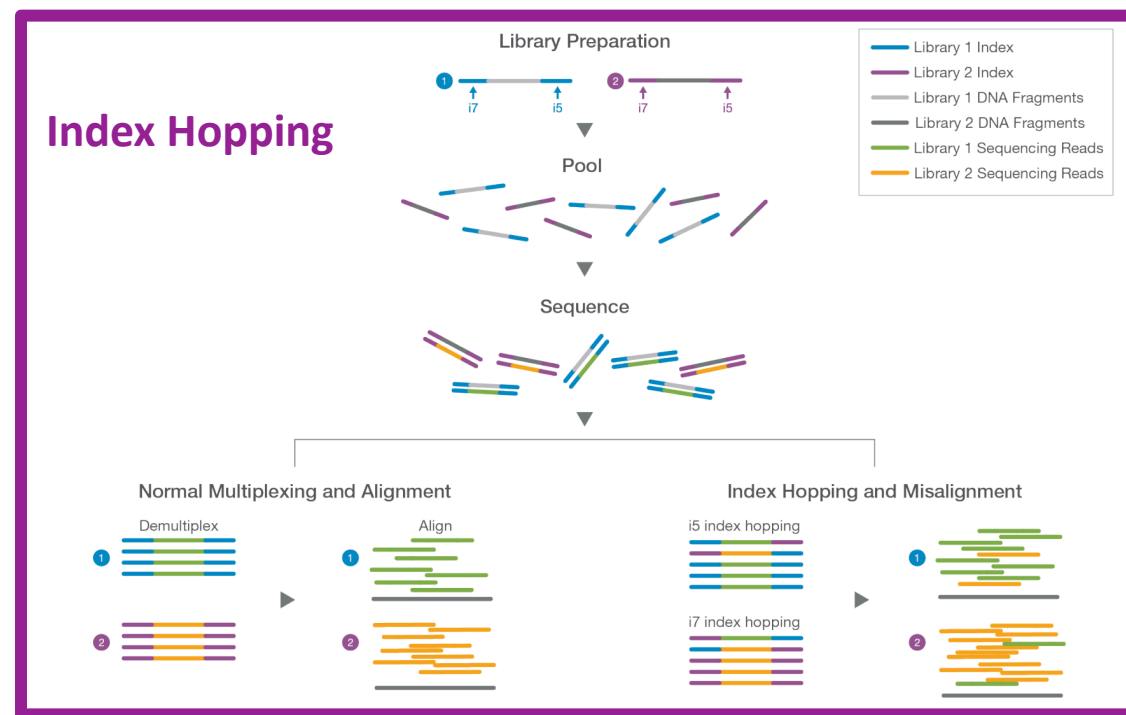


Index 2



UDIs
Unique

UDIs in NGS and Index Hopping





Custom NGS Adapters

Adapter and indexing primer selections for DNA library prep

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- Methylated/unmethylated
- TruGrade processing

Sample 1:

Sample 2:

Sample 3:

Index 1



Index 2



CDIs

Sample 1:

Sample 2:

Sample 3:

Index 1



Index 2



UDIs

IDT for NGS

Single index



Unique dual index



Dual index UMI



Flow cell binding sequence: Platform-specific sequences for library binding to instrument



Sequencing primer sites: Binding sites for general sequencing primers



Sample indexes: Short sequences specific to a given sample library



Molecular index/barcode: Short sequence used to uniquely tag each molecule in a given sample library



Insert: Target DNA or RNA fragment from a given sample library

**GOOGLE FORMs for
ATTENDANCE and
mini-QUIZ
(LIBRARY and SEQ)**

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QUESTIONS #11



Casalmaggiore, Italy