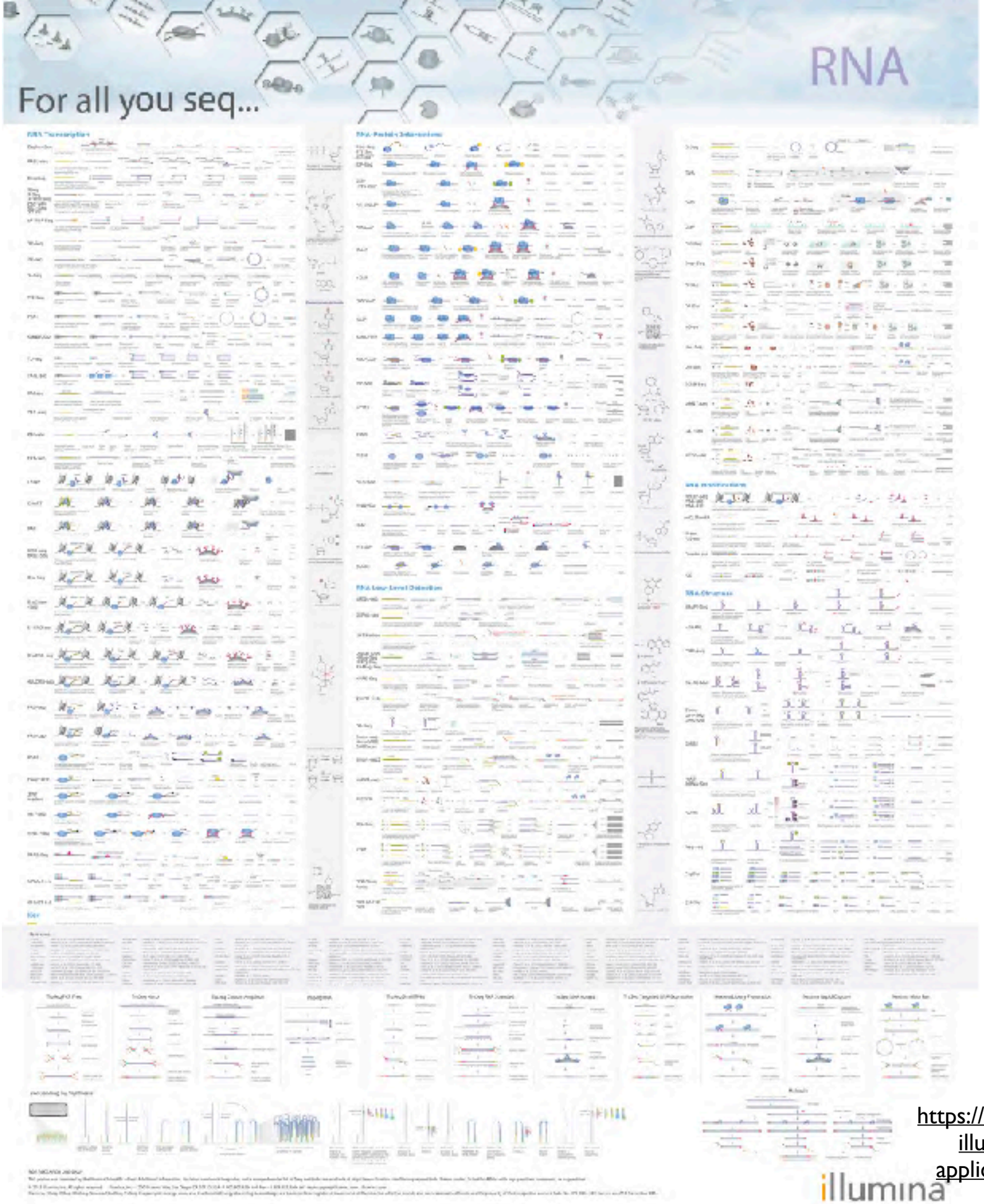
RNA-Seq Library Preparation

Josh Granek

- Ethernet
- Lanes

Rna-Seq Applications

- Transcriptome: "Which genes are expressed in this sample?"
  - Differential Expression
  - Genome Annotation
- SNPs
- Gene Fusions



https://www.illumina.com/content/dam/illumina-marketing/documents/applications/ngs-library-prep/for-all-you-seq-rna.pdf

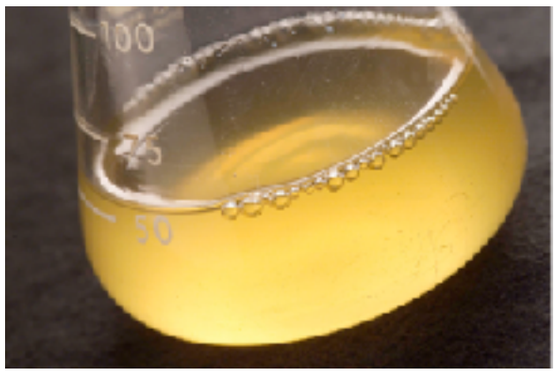
RNA-Seq

- Bulk RNA-Seq
- Single-Cell RNA-Seq (scRNA-Seq)

Overview

- From Cells to Library

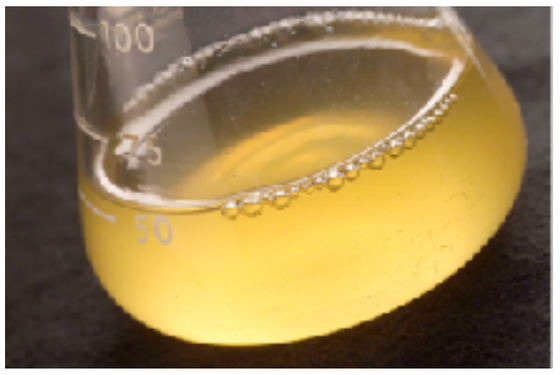
RNA-Seq:
Major Components



1. Sample Collection
2. RNA Extraction
3. Library Preparation

http://cse.ksu.edu/REU/S11/bvosburg/FLASK.jpg

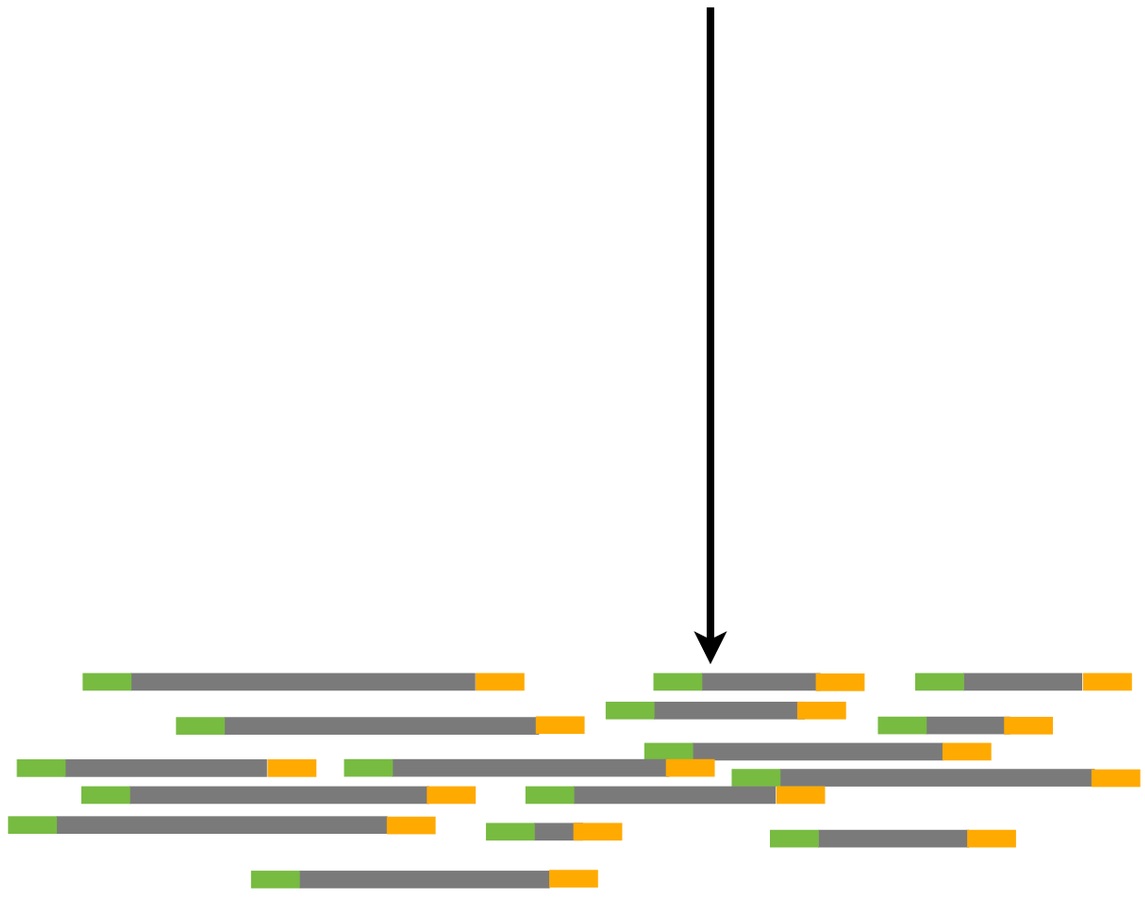
RNA-Seq:
Major Components



1. Sample Collection

2. RNA Extraction

3. Library Preparation



http://cse.ksu.edu/REU/S11/bvosburg/FLASK.jpg

Growth and Sample Collection

- Avoid Confounding Factors!
  - System Specific
  - Experiment Specific
  - Avoid RNA response to sample collection!

Sample Collection Options

- Flash freeze
- RNA stabilizers
    - RNA protect
    - RNAlater
- Phenol (hot acid phenol, trizol, etc)

RNA Extraction: Why?

- Have cells, need RNA

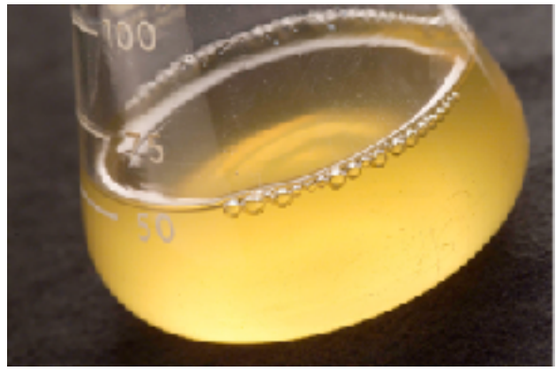
RNA Extraction Options

- Kits
  - Qiagen RNeasy Mini Kit
  - Etc
- Phenol (hot acid phenol, trizol, etc)

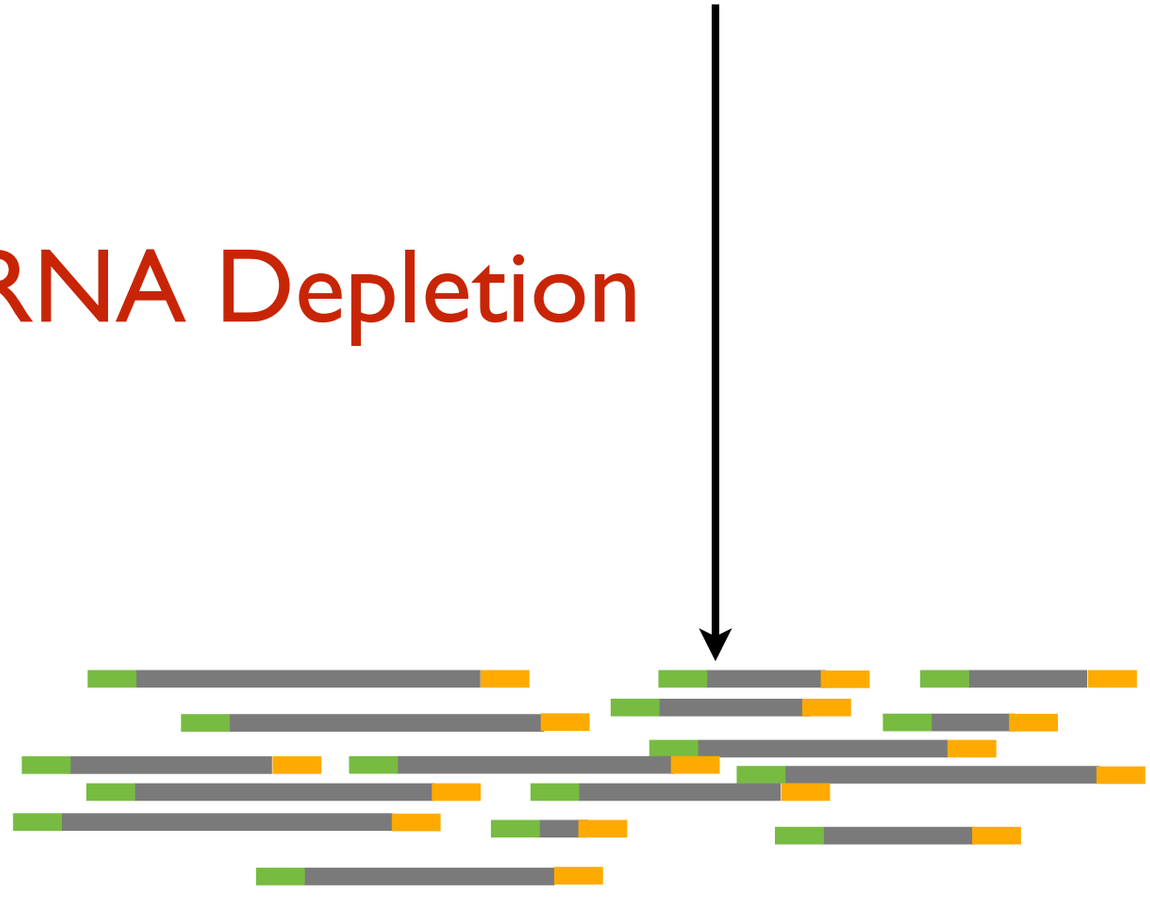
Our Samples

1. Collect cells (centrifuge liquid culture)
2. Flash freeze
3. Lyophilize overnight
4. Bead beating to break open cells
5. Qiagen RNEasy: "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi"

RNA-Seq:
Major Components



1. Sample Collection
2. RNA Extraction
3. mRNA Enrichment/rRNA Depletion
4. Library Preparation



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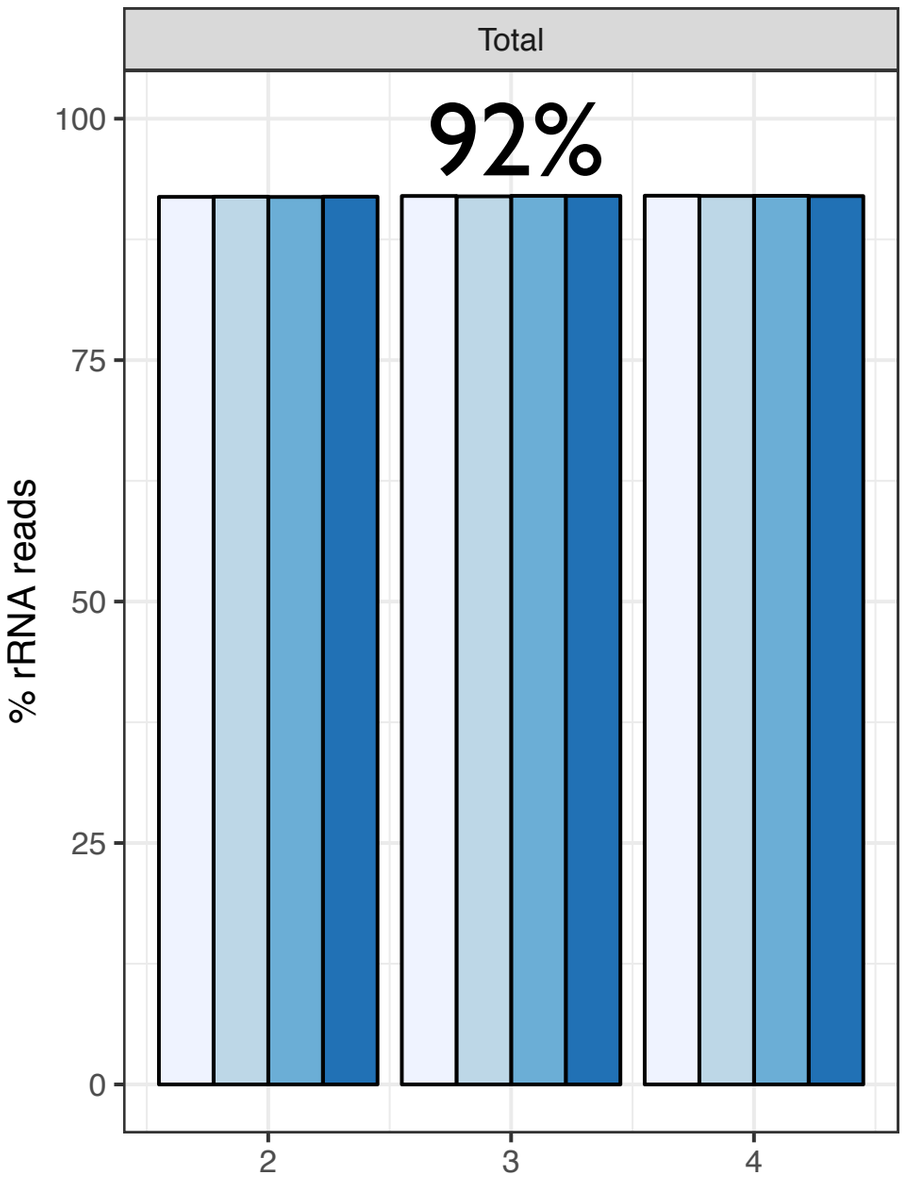
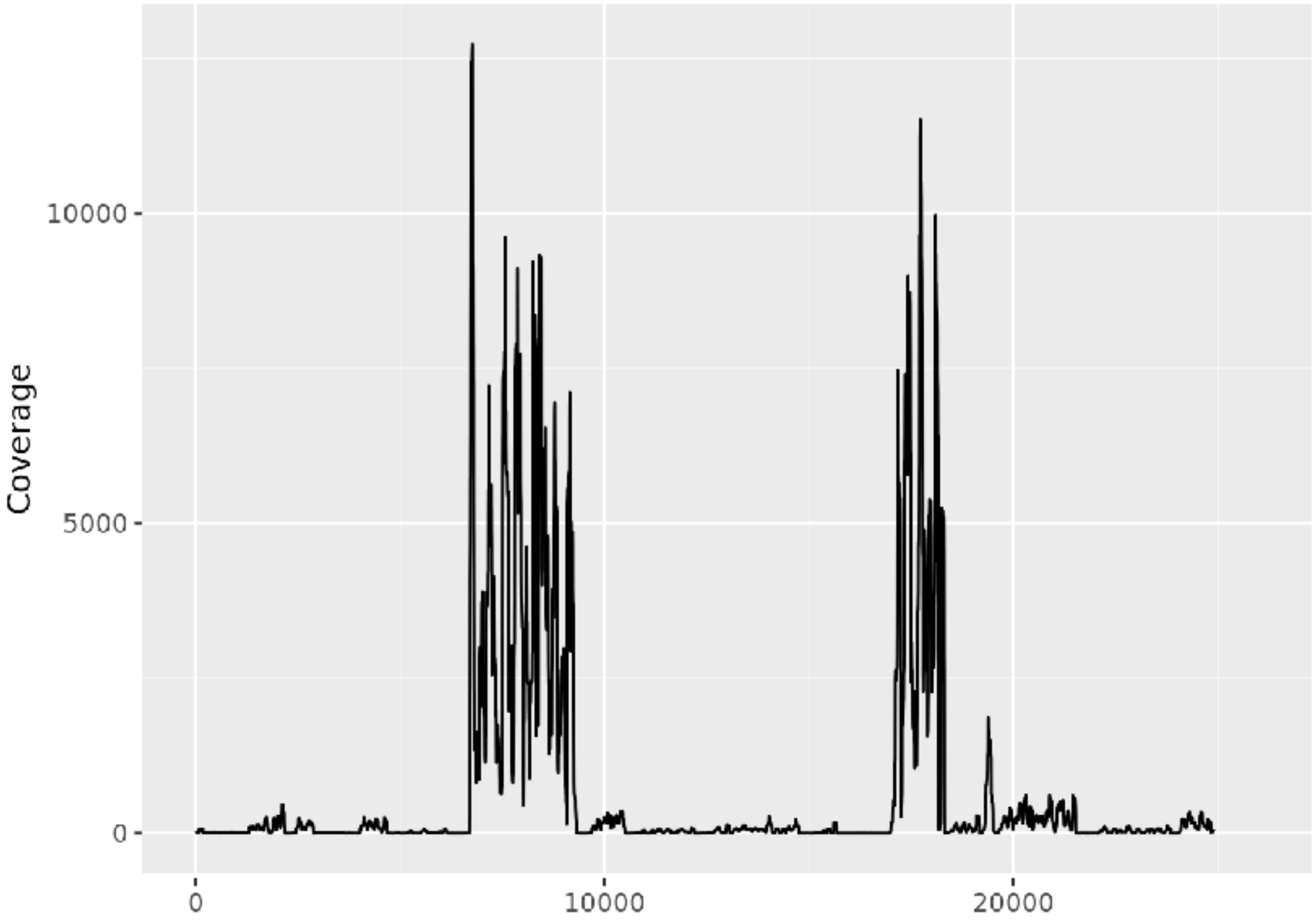
rRNA Depletion: Why?



rRNA Depletion: Why?



rRNA Depletion: How?

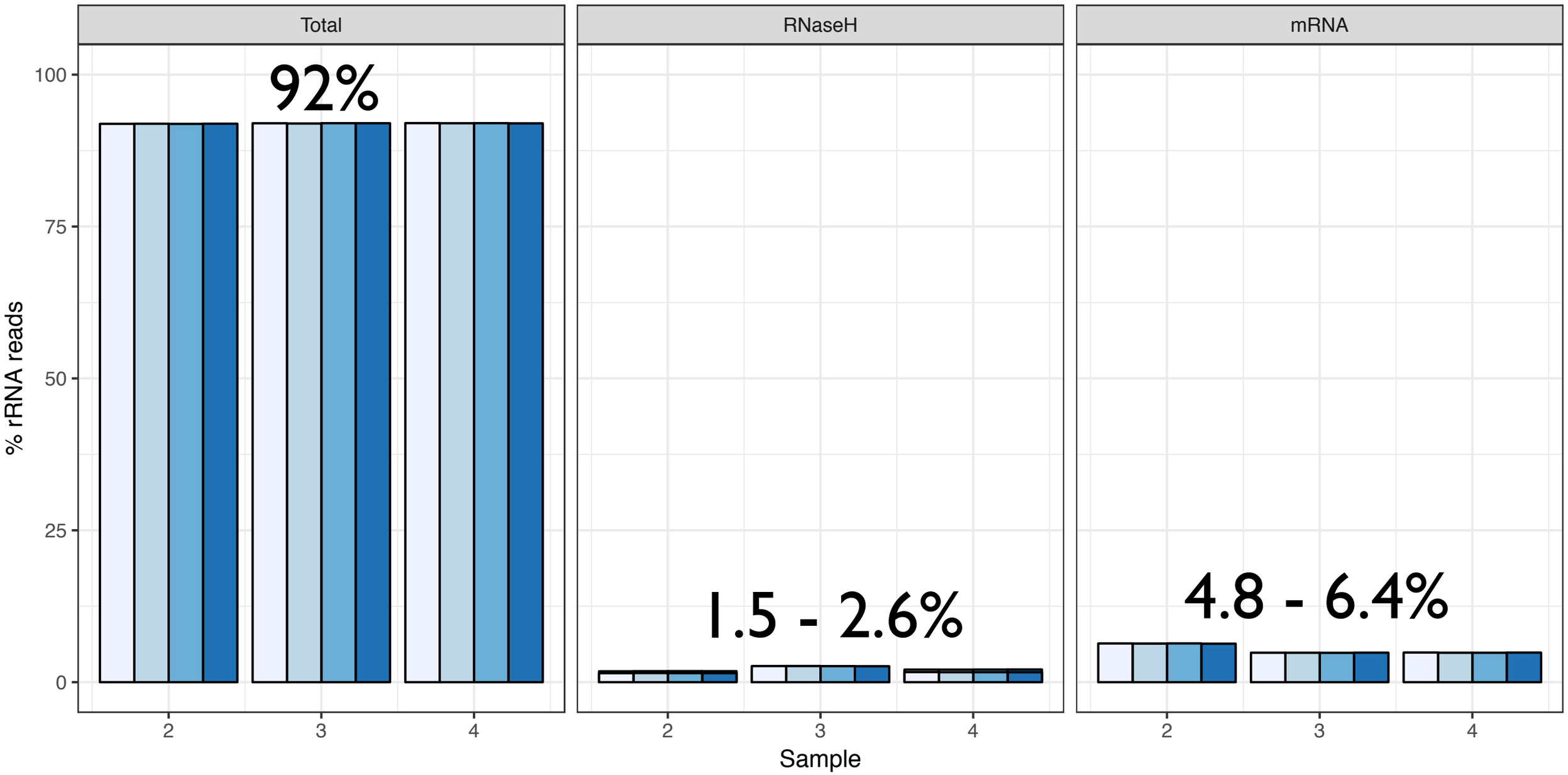
- Selection for desired RNA

*** • poly(A) mRNA enrichment

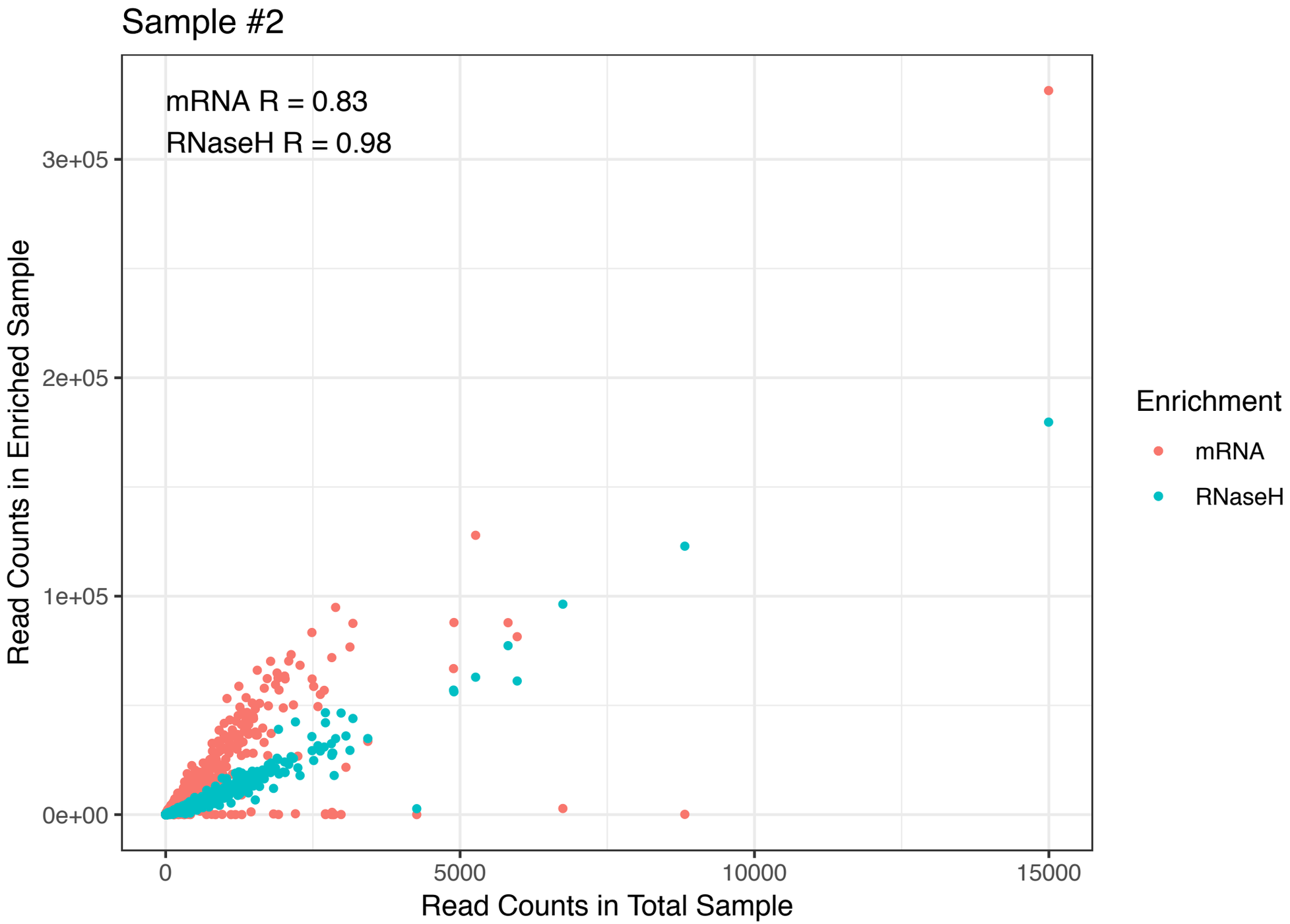
- Selective polyadenylation of mRNAs
- Antibody capture of RNAs that interact with a specific protein
- Non-random priming

- Selection against non-desired RNA
  - DNA targeted RNaseH degradation of rRNA
  - Ribosomal RNA capture
  - Duplex-specific nuclease (DSN) normalization
  - Degradation of processed RNA

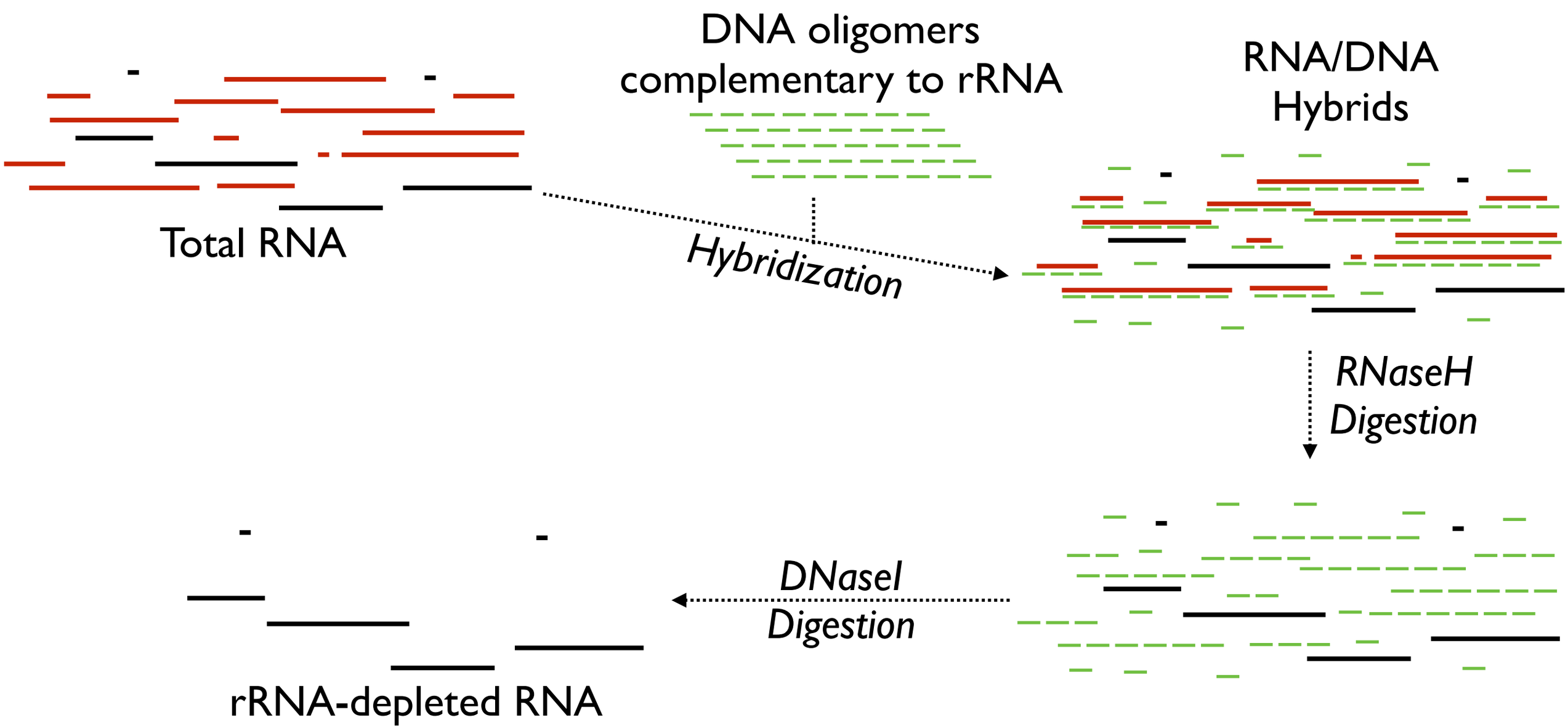
rRNA Depletion: How?



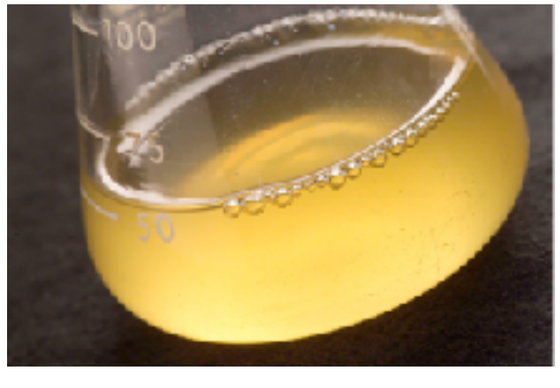
rRNA Depletion: How?



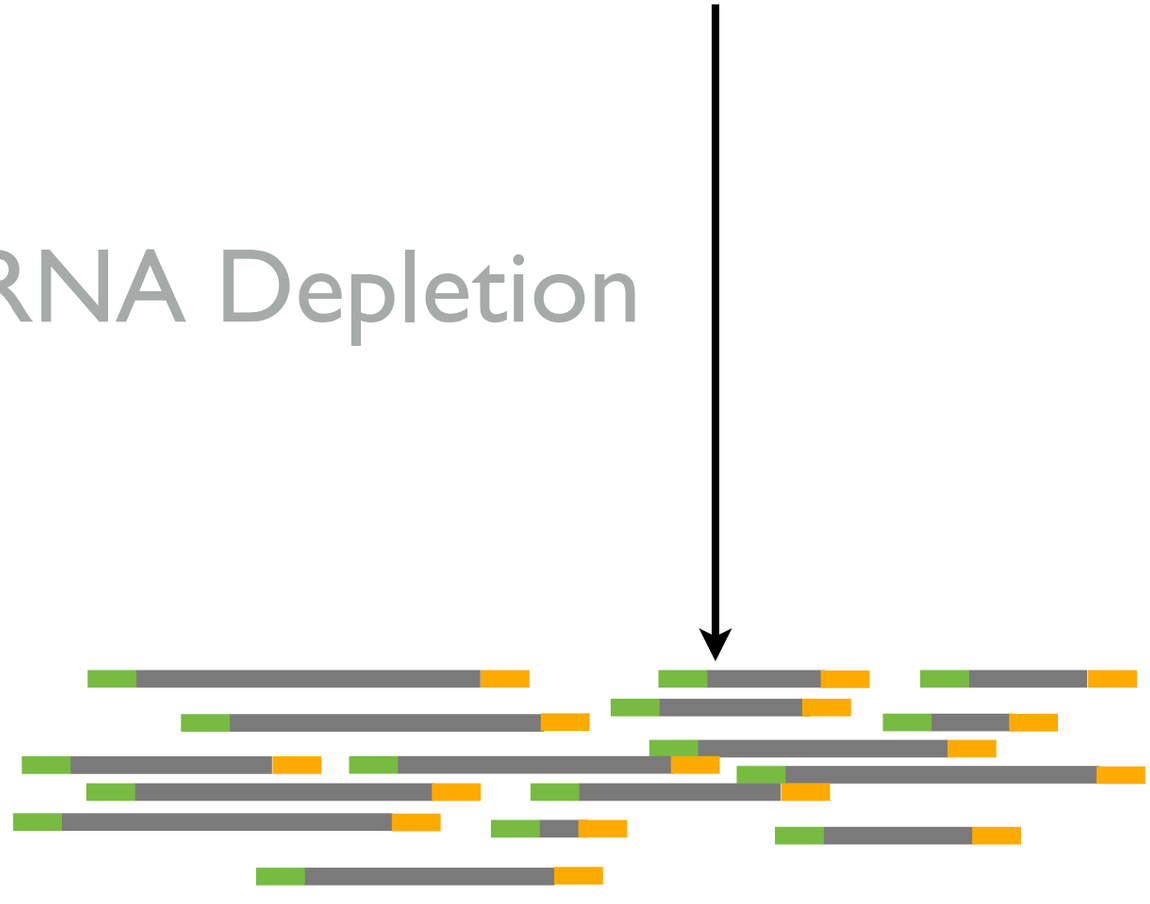
DNA Targeted RNaseH Degradation of rRNA



RNA-Seq:
Major Components



1. Sample Collection
2. RNA Extraction
3. mRNA Enrichment/rRNA Depletion
4. **Library Preparation**



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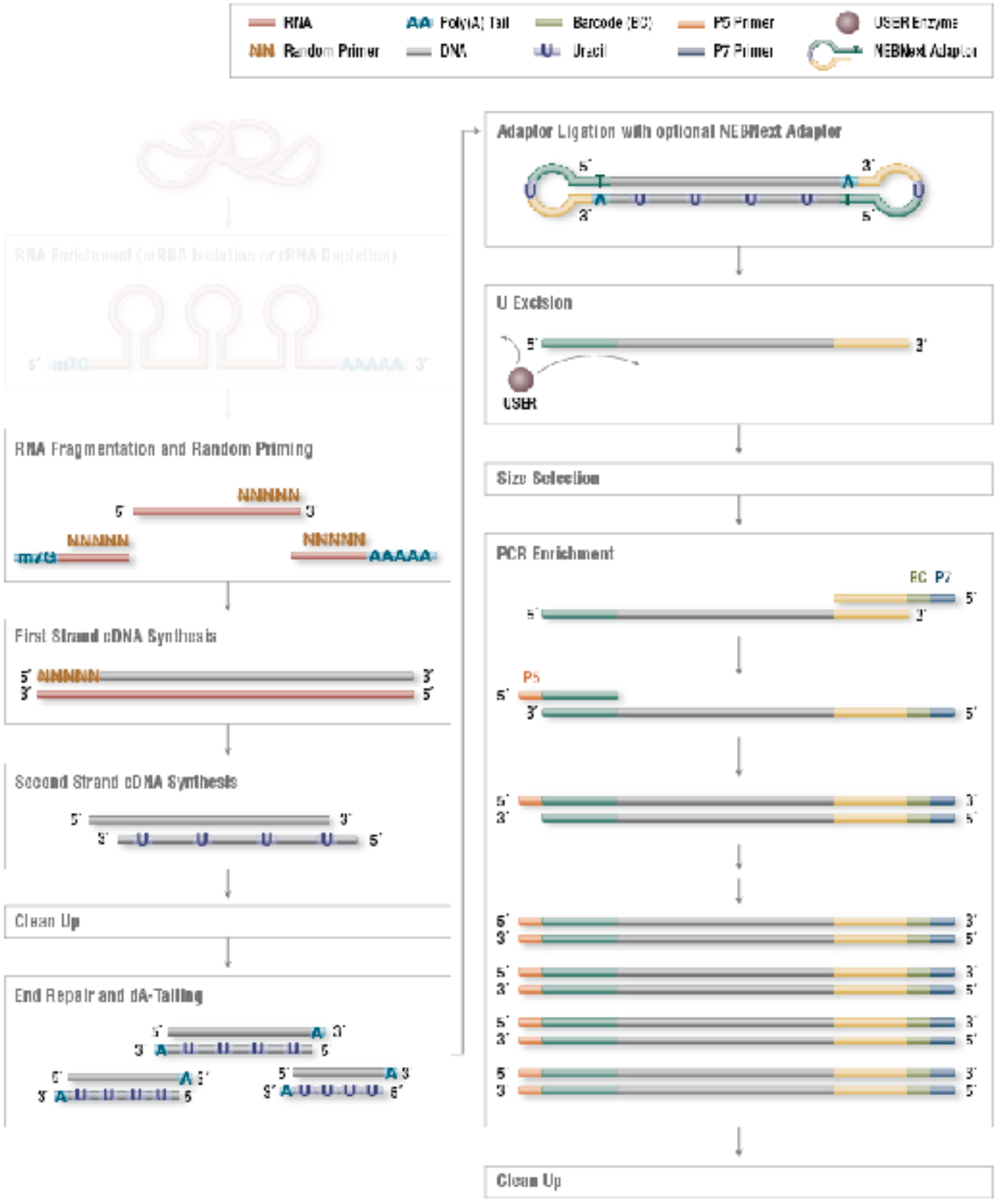
Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
3. Adapter Ligation
4. Size Selection
5. PCR Enrichment

Library Prep Workflow



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Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
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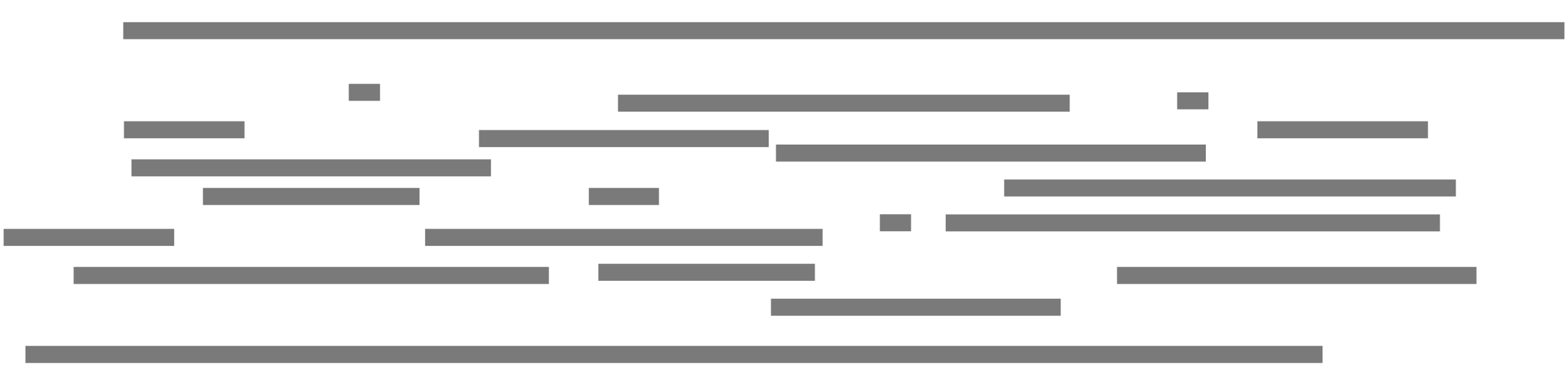
Fragmentation

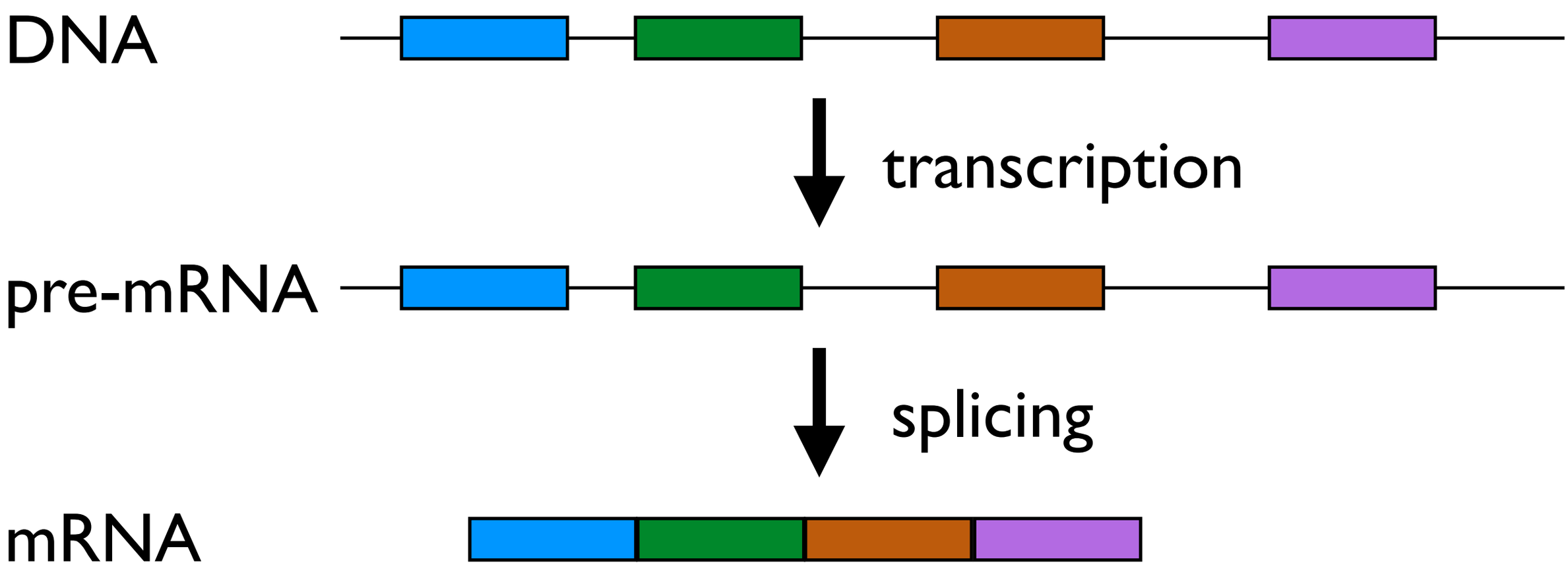


Fragmentation: Why?

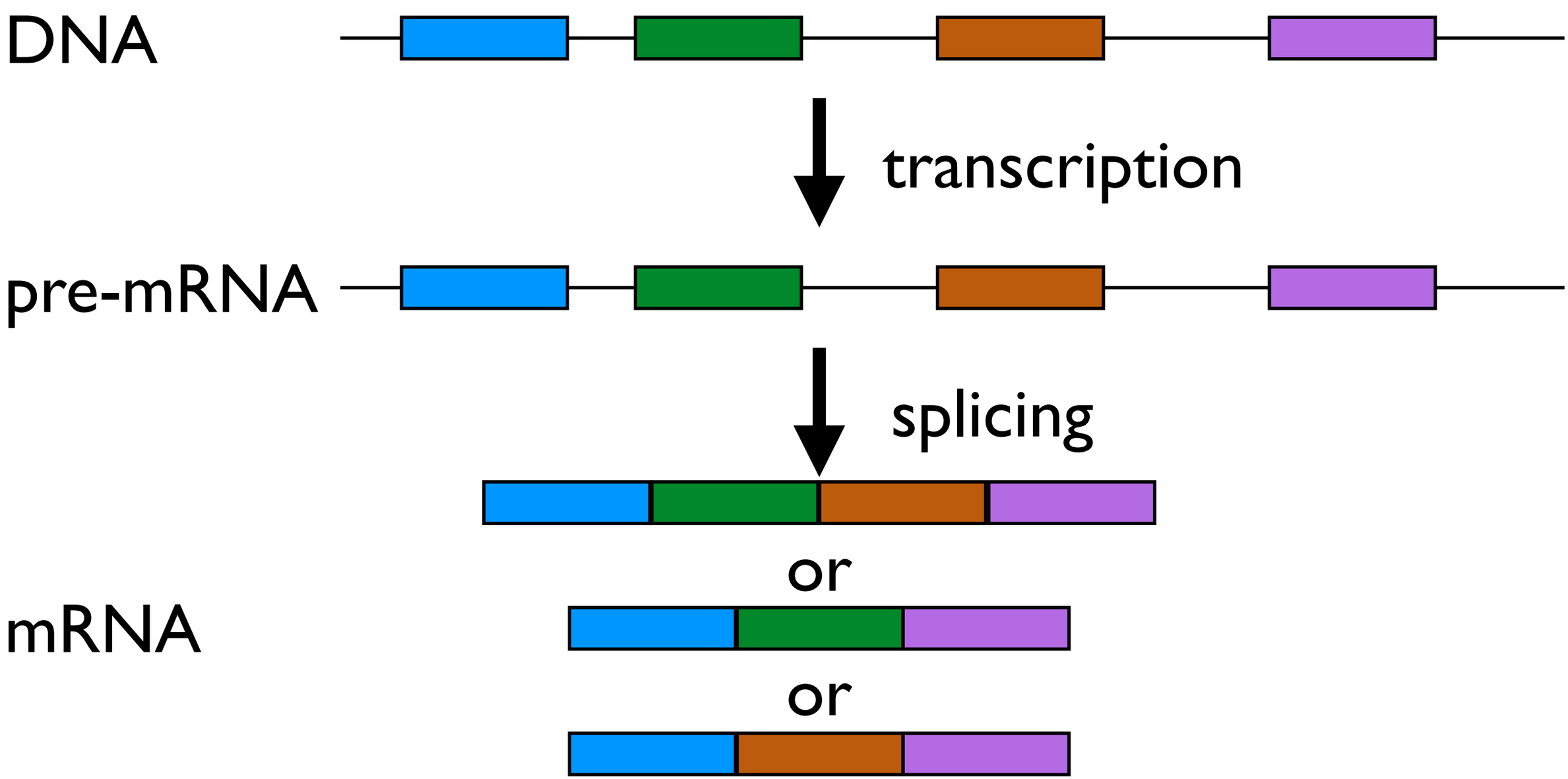
- Efficient cluster generation and sequencing
- Distribution of reads across mRNA

Differential Splicing

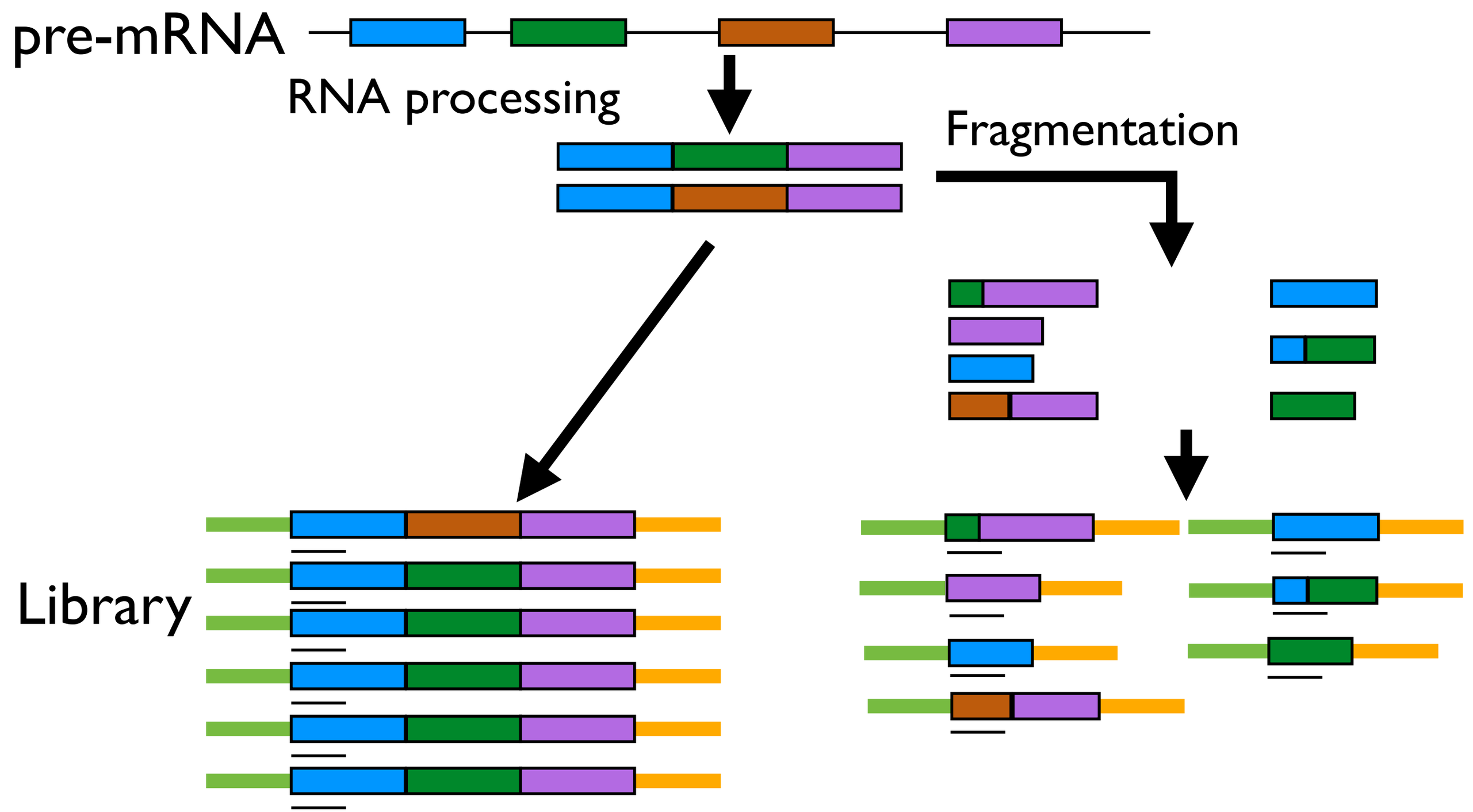
Splicing



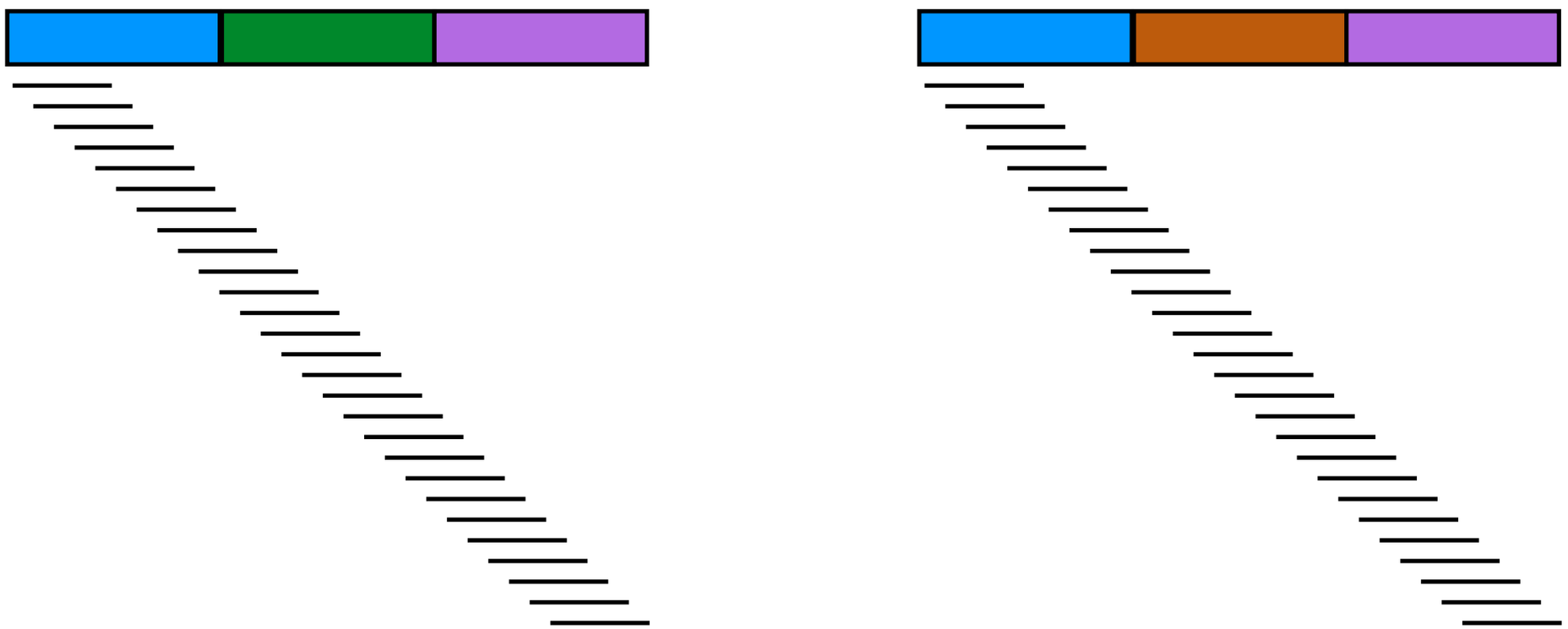
Differential Splicing



Library Prep Fragmentation: Why?

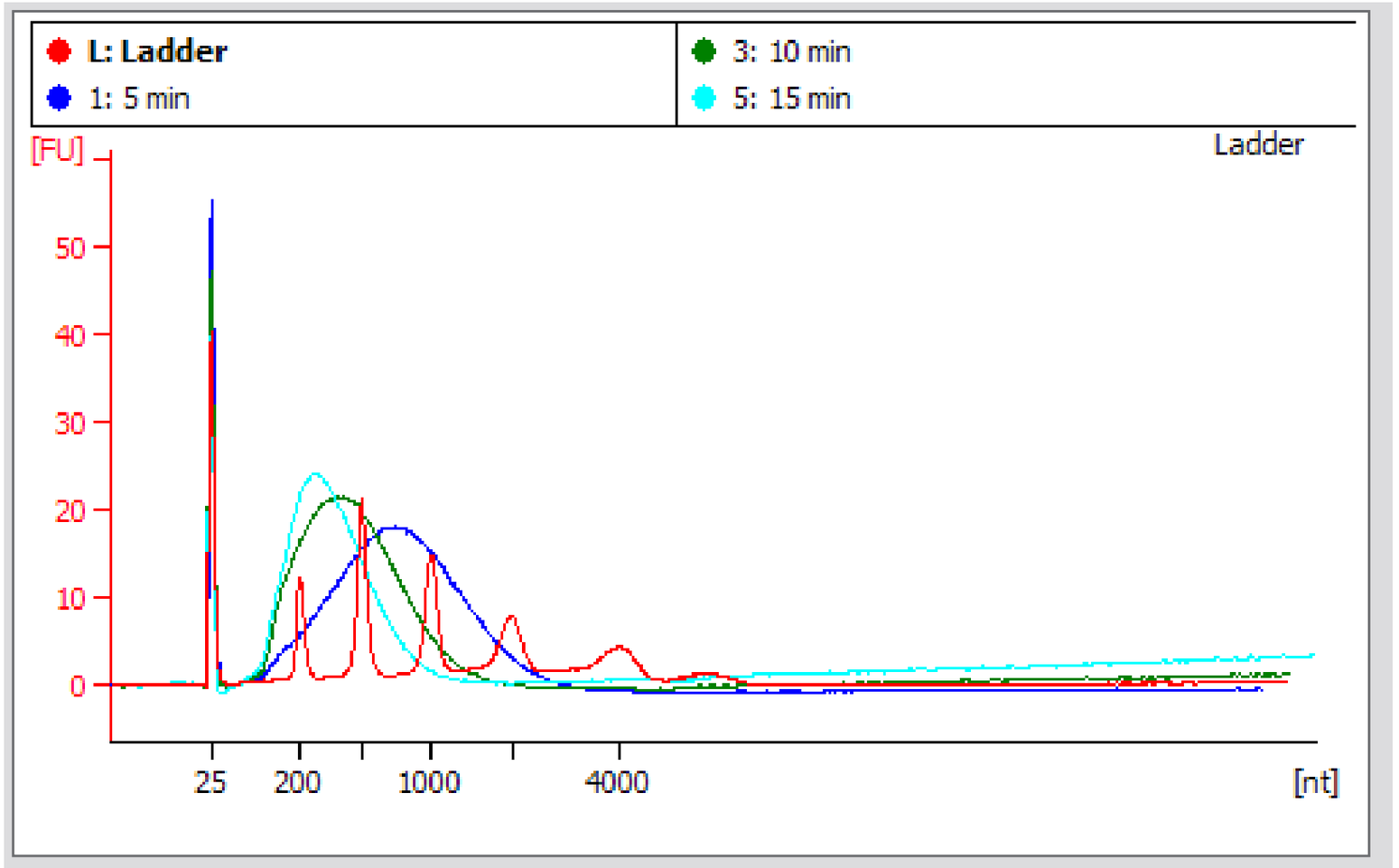


Library Prep Fragmentation: Why?



Fragmentation: How?

- Heat with divalent metal cation (Chemical)



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Library Prep Fragmentation: Alternatives?

- Degraded RNA
- Small RNAs
- DNA Fragmentation uses Physical or Enzymatic methods
- Needs to be Random!!!

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Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
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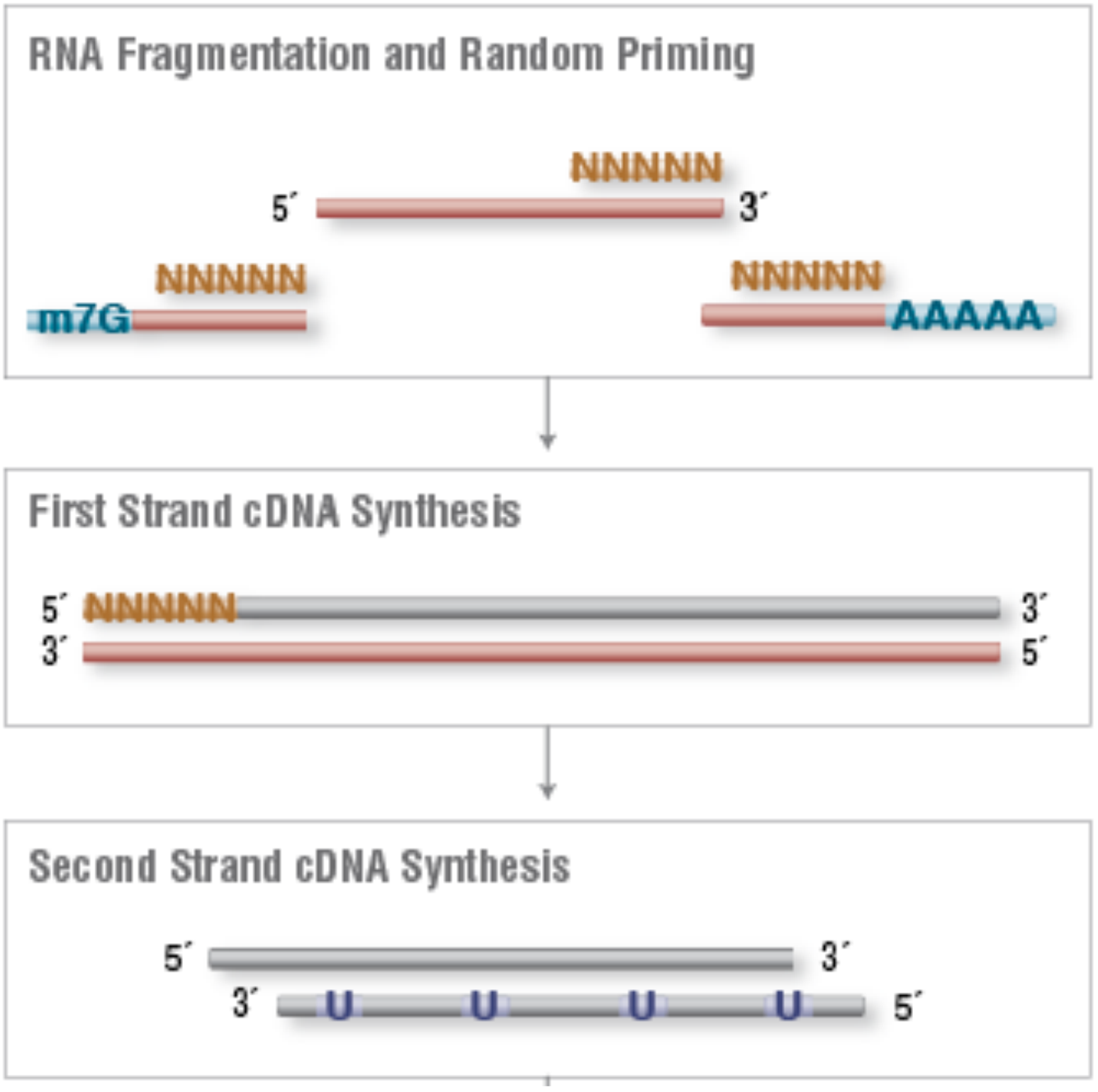
4. Size Selection
5. PCR Enrichment

cDNA Synthesis: Why?

- Have RNA, need DNA

cDNA Synthesis



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cDNA Synthesis: How?

- First Strand:
  - Reverse Transcriptase
  - Random Primers
  - dNTPs
- Second Strand:
  - RNaseH: generate RNA primers
  - DNA polymerase I: DNA synthesis
  - DNA ligase: ligate fragments
  - dNTPs

Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
3. Adapter Ligation
4. Size Selection
5. PCR Enrichment

Library Preparation

DNA Fragment

200-1000 bp

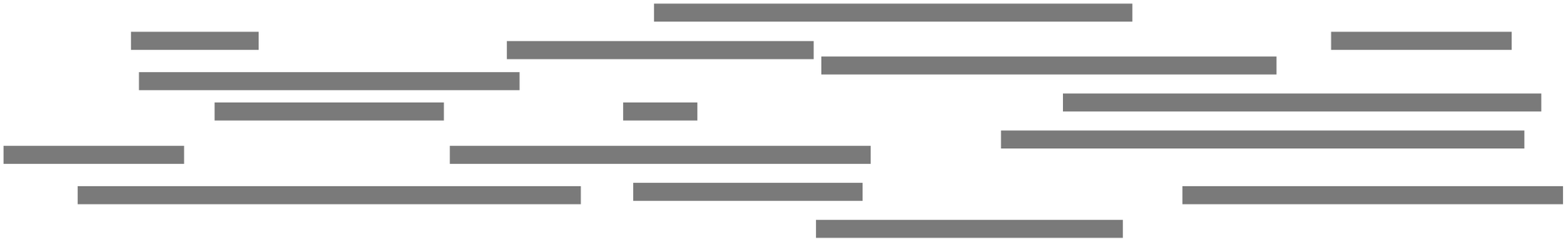
+

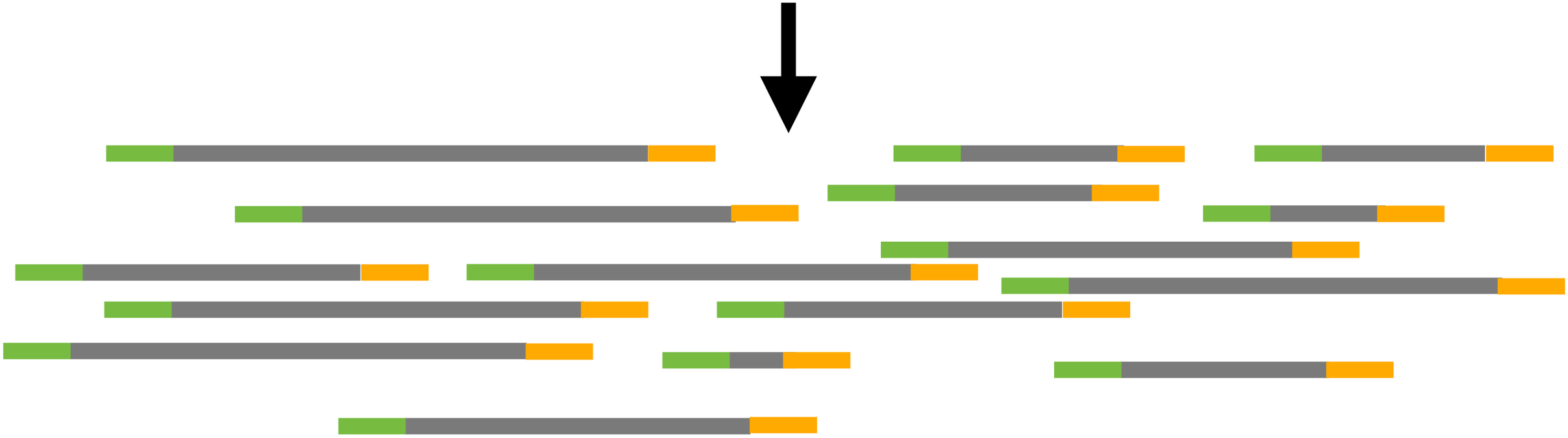
Adapters

=

Sequencing "Library"

Adapter Ligation





Library Preparation

1. RNA Fragmentation
2. cDNA Synthesis
3. Adapter Ligation
    1. End-Repair and dA-Tailing
    2. Adapter Ligation
    3. U Excision
4. Size Selection
5. PCR Enrichment

End-Repair and dA-Tailing

Prepare fragments for adapter ligation:

- Generate blunt ends
- Then generate 3' A overhang

DNA Ligation

DNA Ligation: What

Join two or more fragments of DNA into a single continuous strand

*Do not confuse with hybridization

DNA Ligation: How

- Requirements:
  - two or more DNA fragments
  - DNA Ligase
  - Phosphate/Energy

DNA Ligation: Basics

```
5′-CTGATCTGACTGA-3'
3′-GACTAGACTGACT-5′
          +
5′-TGCGTATGCTAGT-3'
3′-ACGCATACGATCA-5′
          +

   Ligase + ATP

          =

5′-CTGATCTGACTGATGCGTATGCTAGT-3'
3′-GACTAGACTGACTACGCATACGATCA-5′
```

Derived from:
https://en.wikipedia.org/wiki/Sticky_and_blunt_ends

Blunt End Ligation

```
5′-CTGATCTGACTGA-3'
3′-GACTAGACTGACT-5′
          +
5′-TGCGTATGCTAGT-3'
3′-ACGCATACGATCA-5′
          +
   Ligase + ATP
          =
5′-CTGATCTGACTGATGCGTATGCTAGT-3'
3′-GACTAGACTGACTACGCATACGATCA-5′
```

Derived from:
https://en.wikipedia.org/wiki/Sticky_and_blunt_ends

Sticky End Ligation

```
5′-CTGATCTGACT     -3'
3′-GACTAGACTGACTAC-5′
           +
5′-GATGCGTATGCTAGT-3'
3′-    GCATACGATCA-5′
           +
```

```
Ligase + ATP
```

```
=
```

```
5′-CTGATCTGACTGATGCGTATGCTAGT-3'
3′-GACTAGACTGACTACGCATACGATCA-5′
```

Derived from:
https://en.wikipedia.org/wiki/Sticky_and_blunt_ends

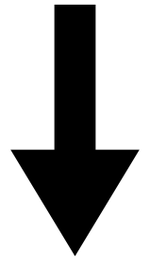
End Repair

End Repair: What

Fix overhanging ends so they are double-stranded

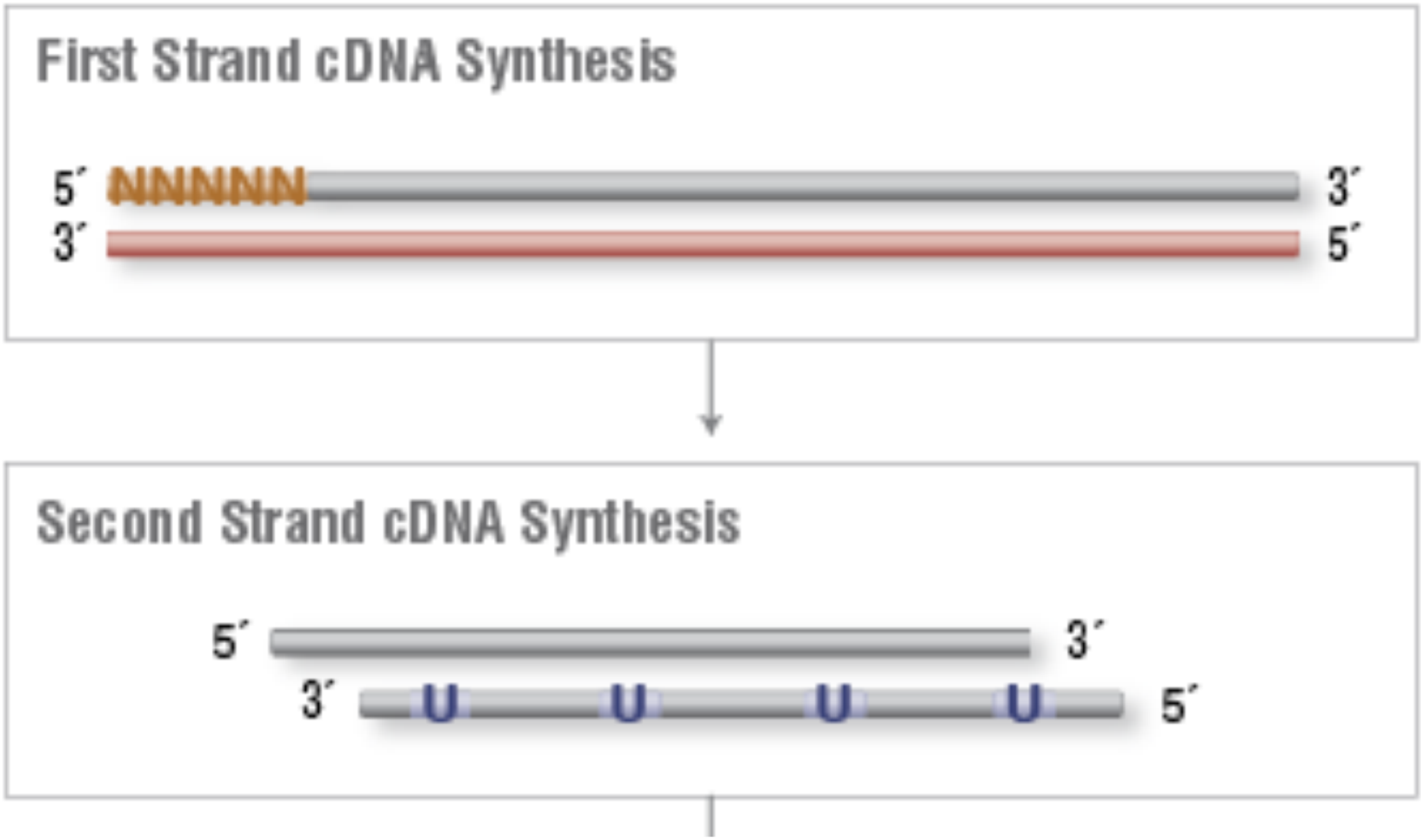
End Repair: What

```
5′-CTGATCTGACT     -3'
3′-GACTAGACTGACTAC-5′
```



```
5′-CTGATCTGACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

Why are ends NOT blunt?



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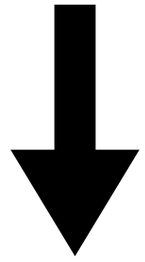
End Repair: What

```
5′-CTGATCTGACT     -3'
3′-GACTAGACTGACTAC-5′
```



```
5′-CTGATCTGACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

End Repair: Why

- Allow blunt end ligation

End Repair: How

```
5′-CTGATCTGACT     -3'
3′-GACTAGACTGACTAC-5′
```

+

?

=

```
5′-CTGATCTGACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

End Repair: How

```
5′-CTGATCTGACT     -3'
3′-GACTAGACTGACTAC-5′
```

+

DNA Polymerase

=

```
5′-CTGATCTGACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

End Repair: How

- Requirements:
  - DNA with overhanging end
  - DNA Polymerase
  - dNTPs

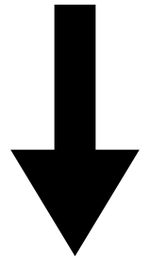
dA-Tailing

dA-Tailing: What

- Add a 3' "A" to blunt end fragments

dA-Tailing: What

```
5′-CTGATCTGACTGATG-3'
3′-GACTAGACTGACTAC-5′
```



```
5′- CTGATCTGACTGATGA-3'
3′-AGACTAGACTGACTAC -5′
```

dA-Tailing: Why

?

dA-Tailing: Why

Allow sticky-end ligation to a “universal fragment”

dA-Tailing: Why

```
5′- GATGATTGCTGAAGA-3'
3′-ACTACTAACGACTTC -5′
```

```
5′- AGTACTGTTCTTTATA-3'
3′-ATCATGACAAGAAATA -5′
```

+

```
5′- CCATG-3'
3′-TGGTAC-5′
```

=

```
5′- GATGATTGCTGAAGACCATG-3'
3′-ACTACTAACGACTTCTGGTAC-5′
```

```
5′- AGTACTGTTCTTTATACCATG-3'
3′-ATCATGACAAGAAATATGGTAC-5′
```

dA-Tailing: Why?

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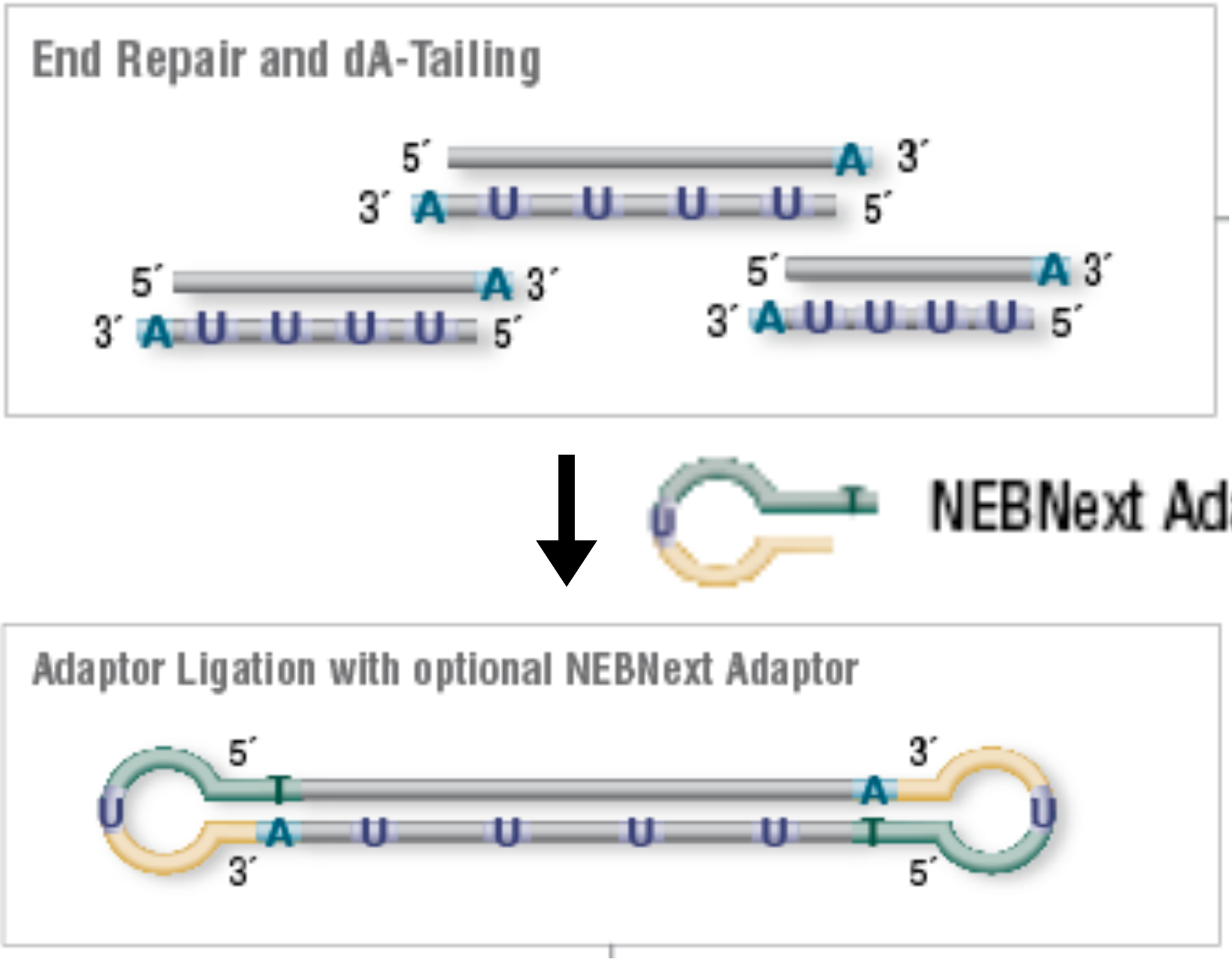
dA-Tailing: How

- Requirements:
  - Blunt-end DNA Fragment
  - Taq DNA Polymerase
  - ATP

Adapter Ligation



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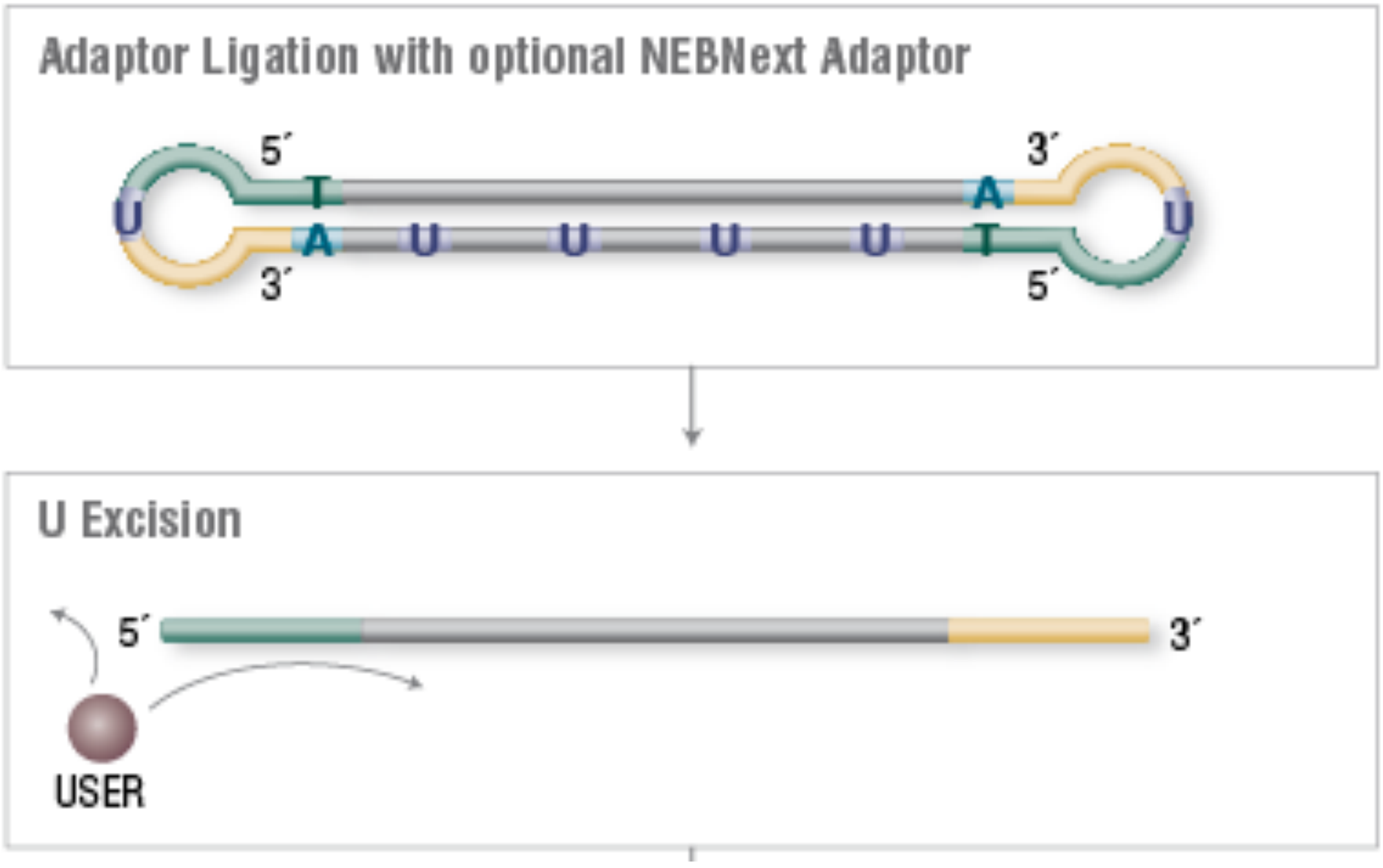
U Excision



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U Excision

Why?

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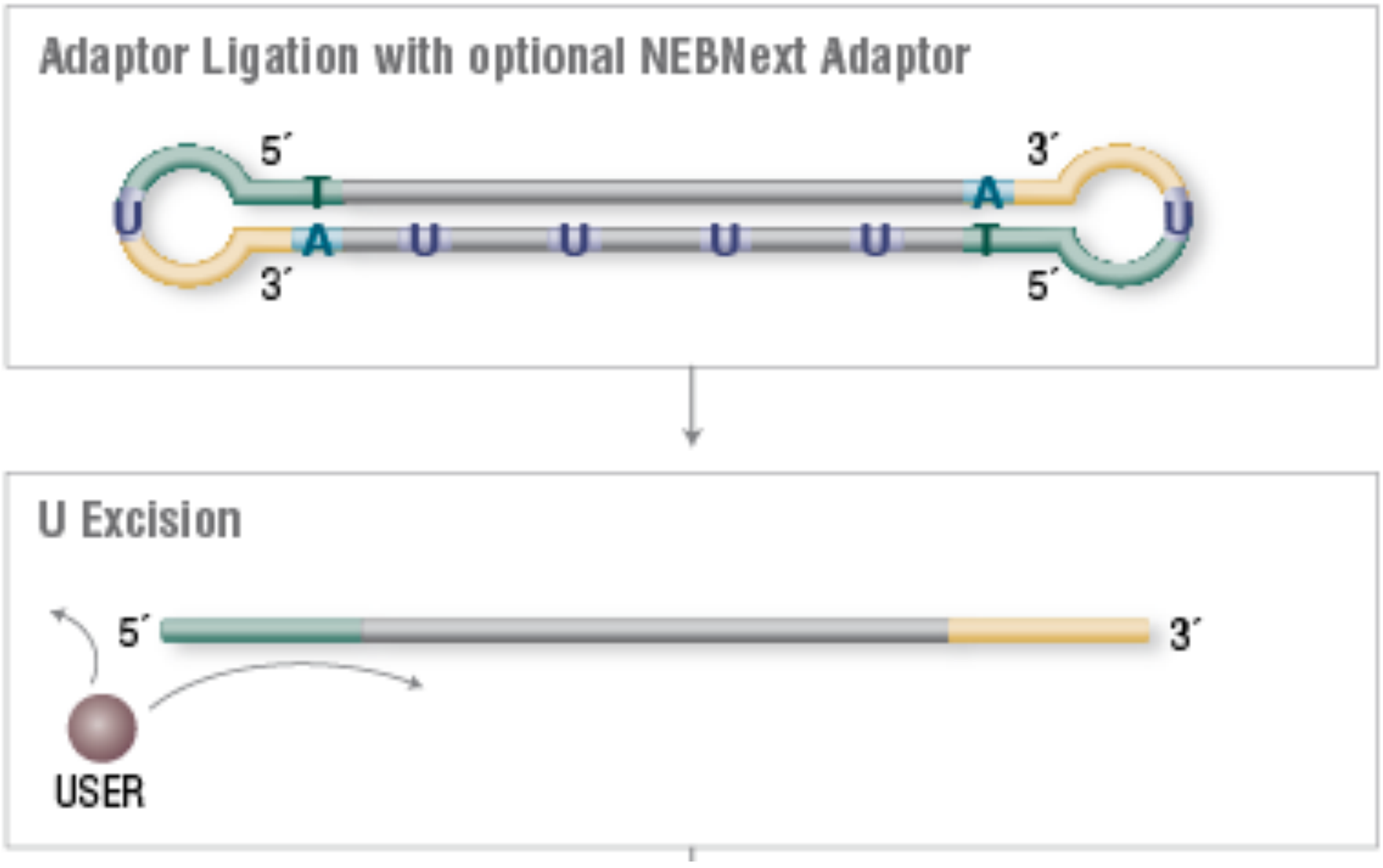
U Excision



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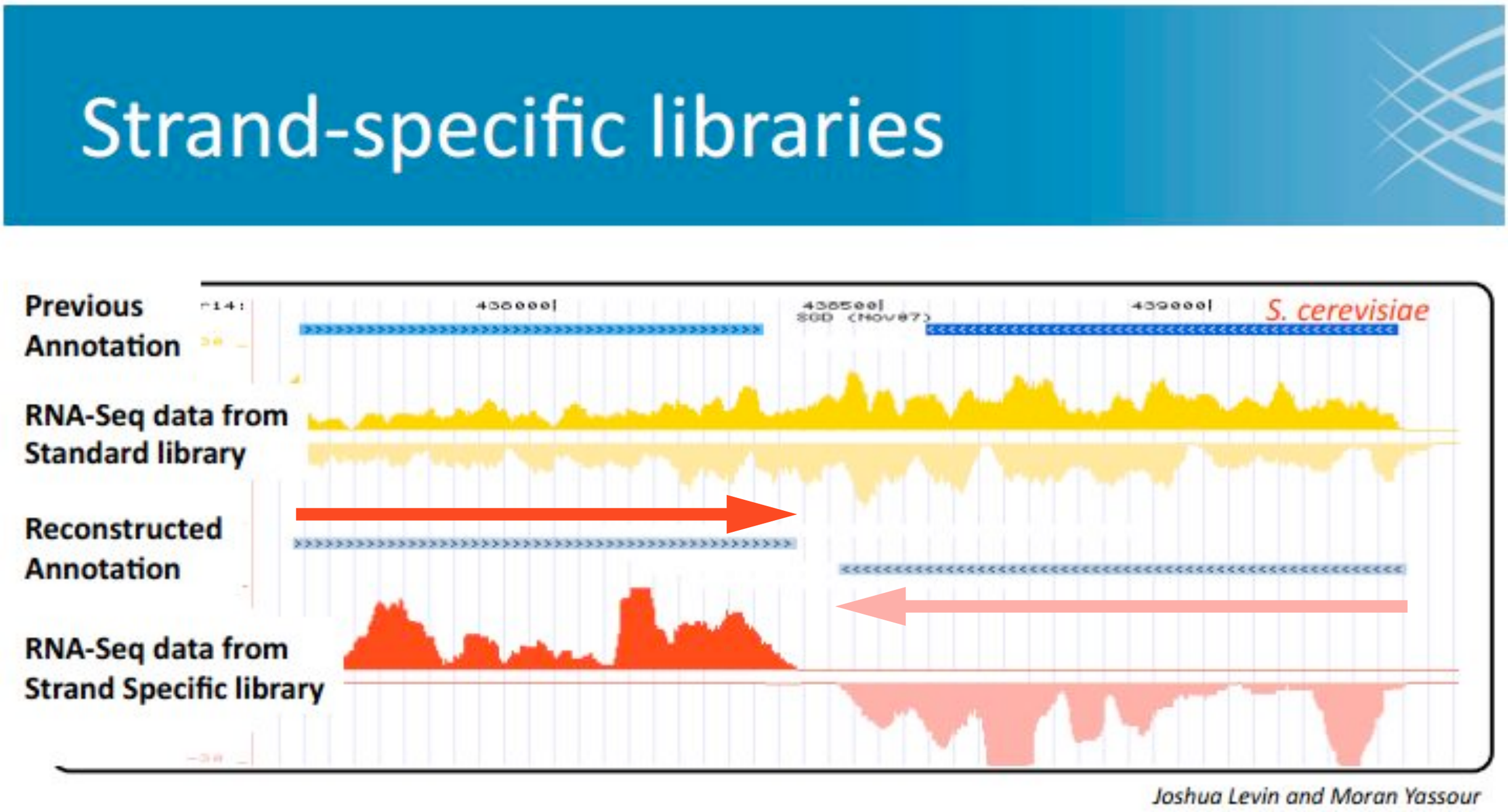
Strand-Specific Library

- Why Bother?

Strand-Specific Library



http://evomicsorg.wpengine.netdna-cdn.com/wp-content/uploads/2014/01/SSlib.jpg

Strand Specific Prep

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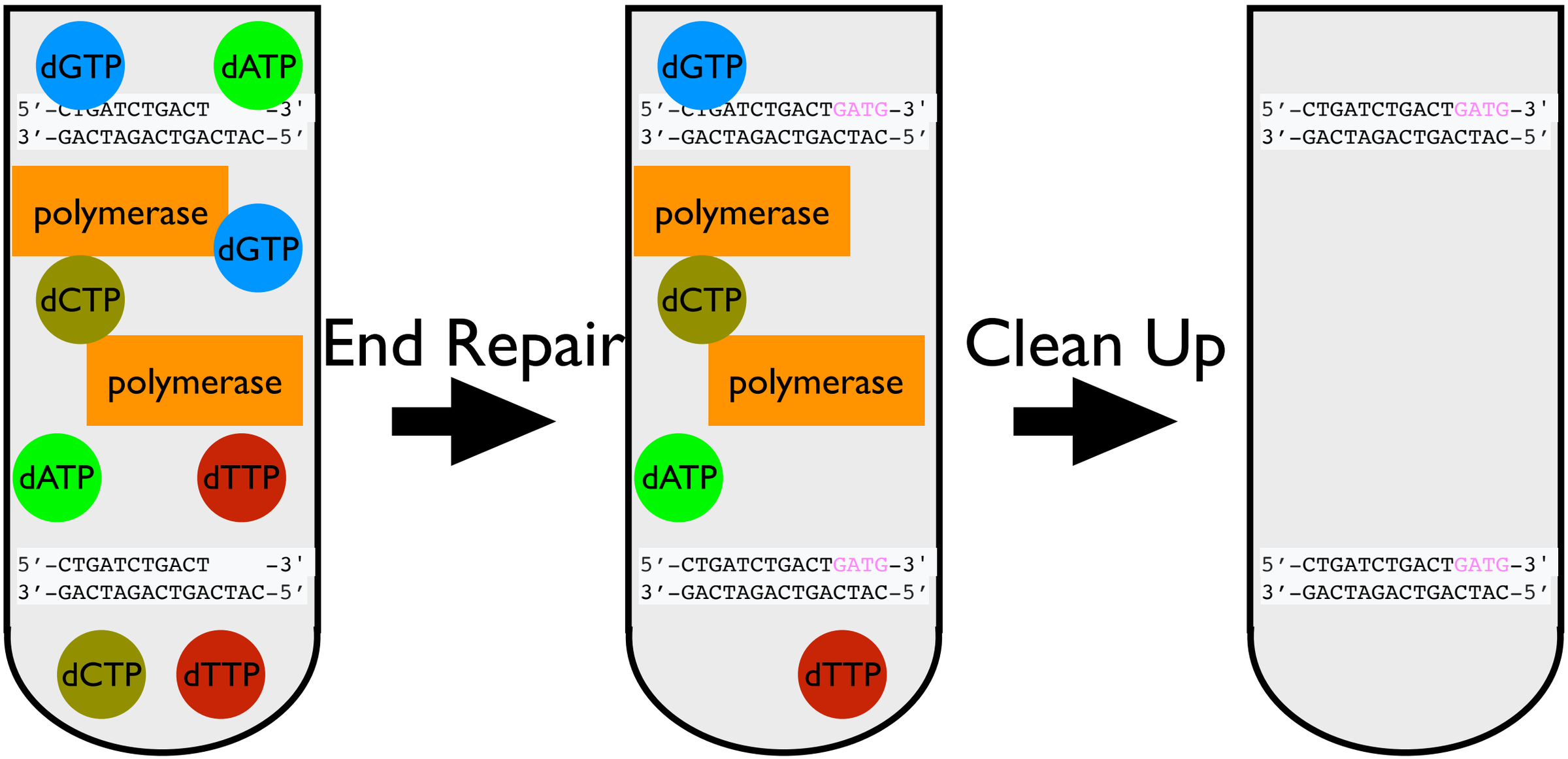
Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
3. Adapter Ligation
4. Size Selection
5. PCR Enrichment

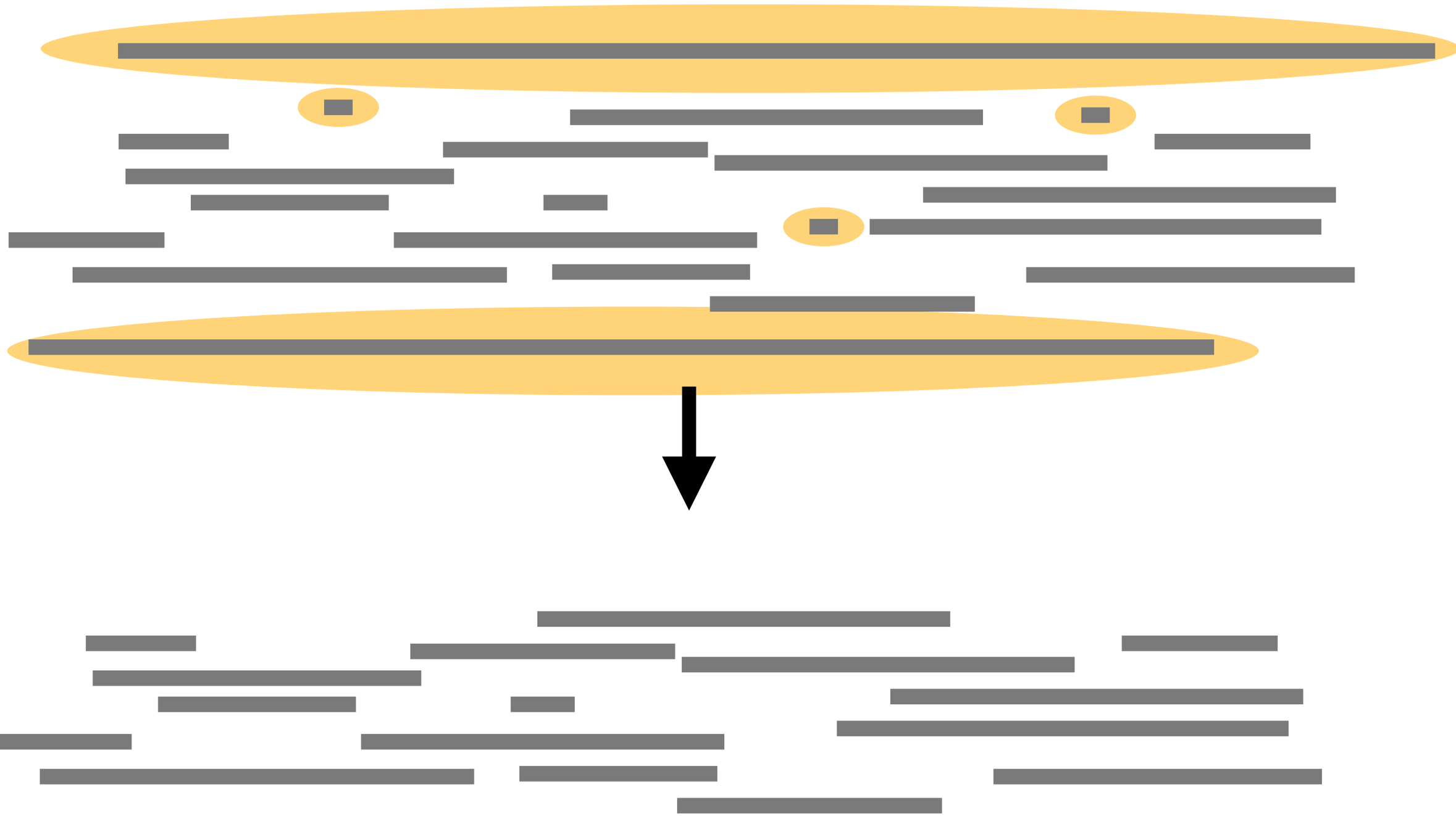
Clean Up and Size Selection: Why?

- Remove regents from previous step
- Eliminate unwanted fragments
  - Unligated adapter
  - adapter dimers
  - fragments without adapter
- Efficient cluster generation and sequencing

Sample Clean Up

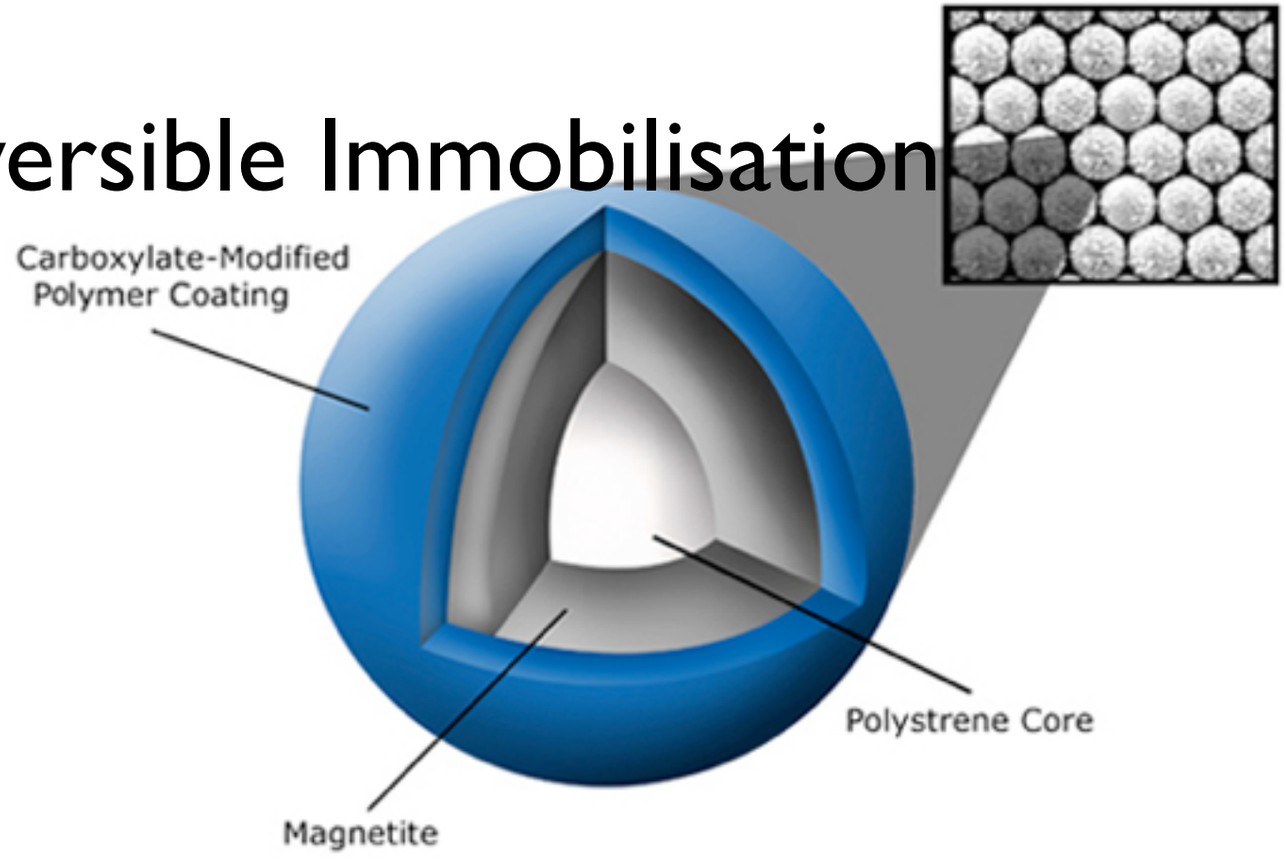


Size Selection



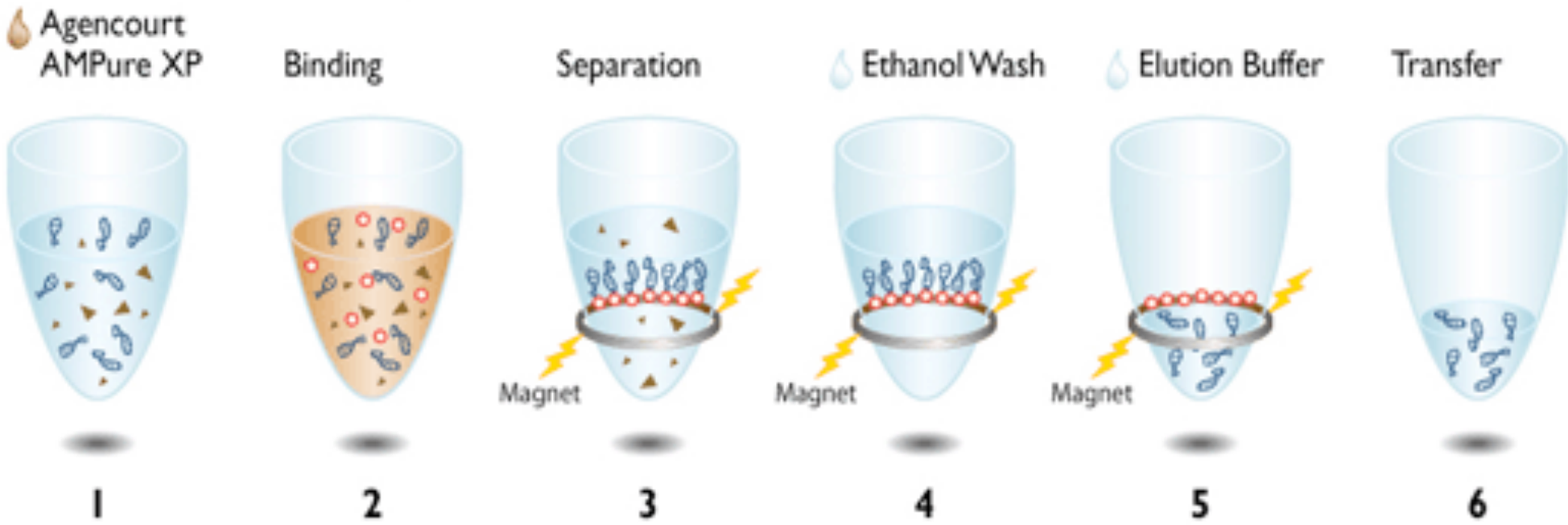
Clean Up and Size Selection: How?

- Solid Phase Reversible Immobilisation (SPRI) beads



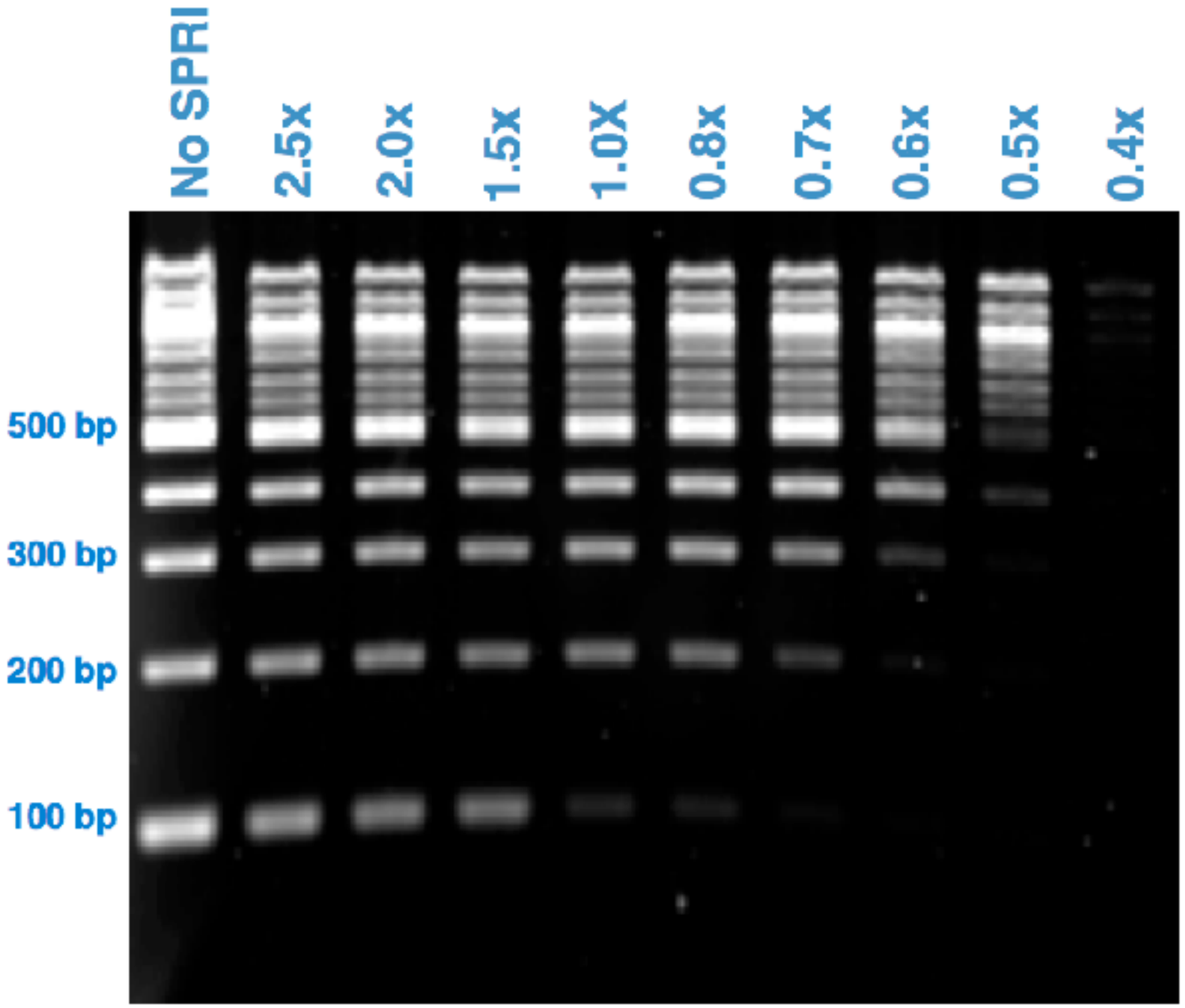
http://core-genomics.blogspot.com/2012/04/how-do-spri-beads-work.html

Clean Up and Size Selection: How?



https://www.beckmancoulter.com/ucm/idc/groups/public/documents/webasset/glb_bci_003489.gif

Size Selection: How?



http://core-genomics.blogspot.com/2012/04/how-do-spri-beads-work.html

Size Selection: How?

Option 2: Double-Sided SPRI

- By implementing a combination of good shearing with SPRI and "reverse" SPRI, one can select a fairly tight size range *with no gel*:

0.65X SPRI
Fragments >250 bp on beads
Fragments <250 bp in supernatant

KEEP SUPERNATANT
0-250 bp

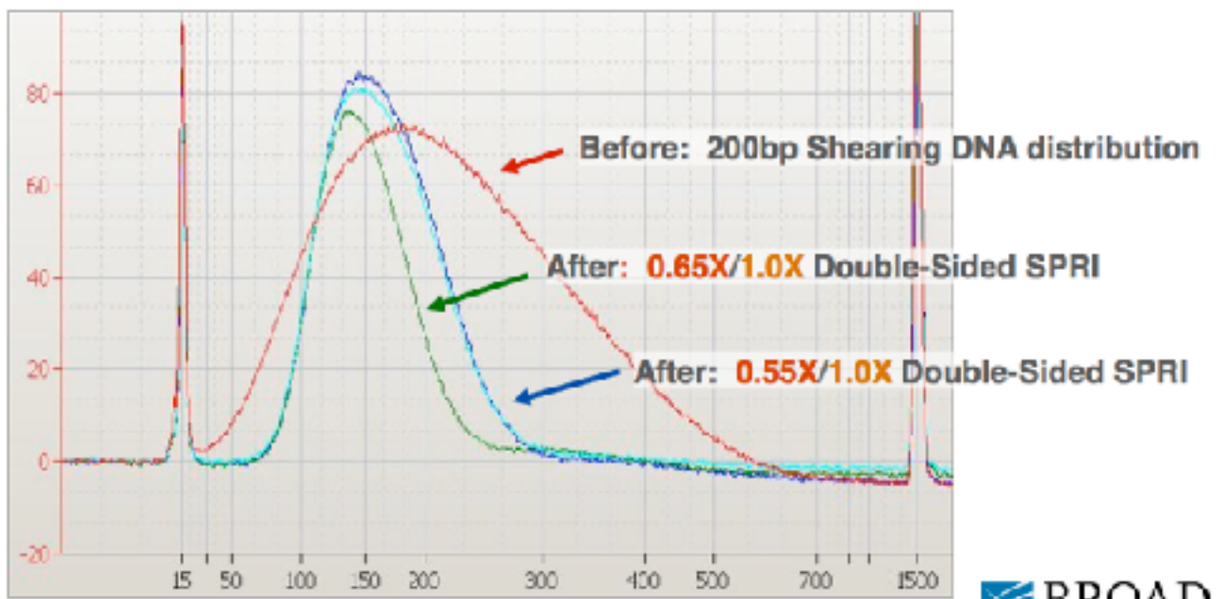
DISCARD BEADS
"Reverse SPRI"

1.0X SPRI
Fragments >100 bp on beads
Fragments <100 bp in supernatant

KEEP BEADS
100-250 bp

DISCARD SUPERNATANT

Results:



BROAD INSTITUTE illumina

http://core-genomics.blogspot.com/2012/04/how-do-spri-beads-work.html

Clean Up and Size Selection: Alternatives

- Spin Columns
- Gel Purification
- DIY SPRI

Library Preparation: Key Steps

1. RNA Fragmentation
2. cDNA Synthesis
3. Adapter Ligation
4. Size Selection
5. PCR Enrichment

PCR Enrichment: Why?

1. Extend adapter to full length
   A. add barcodes
   B. add priming sites
2. Amplify library
   A. Make more of the good fragments
   B. Leave the garbage in the dust

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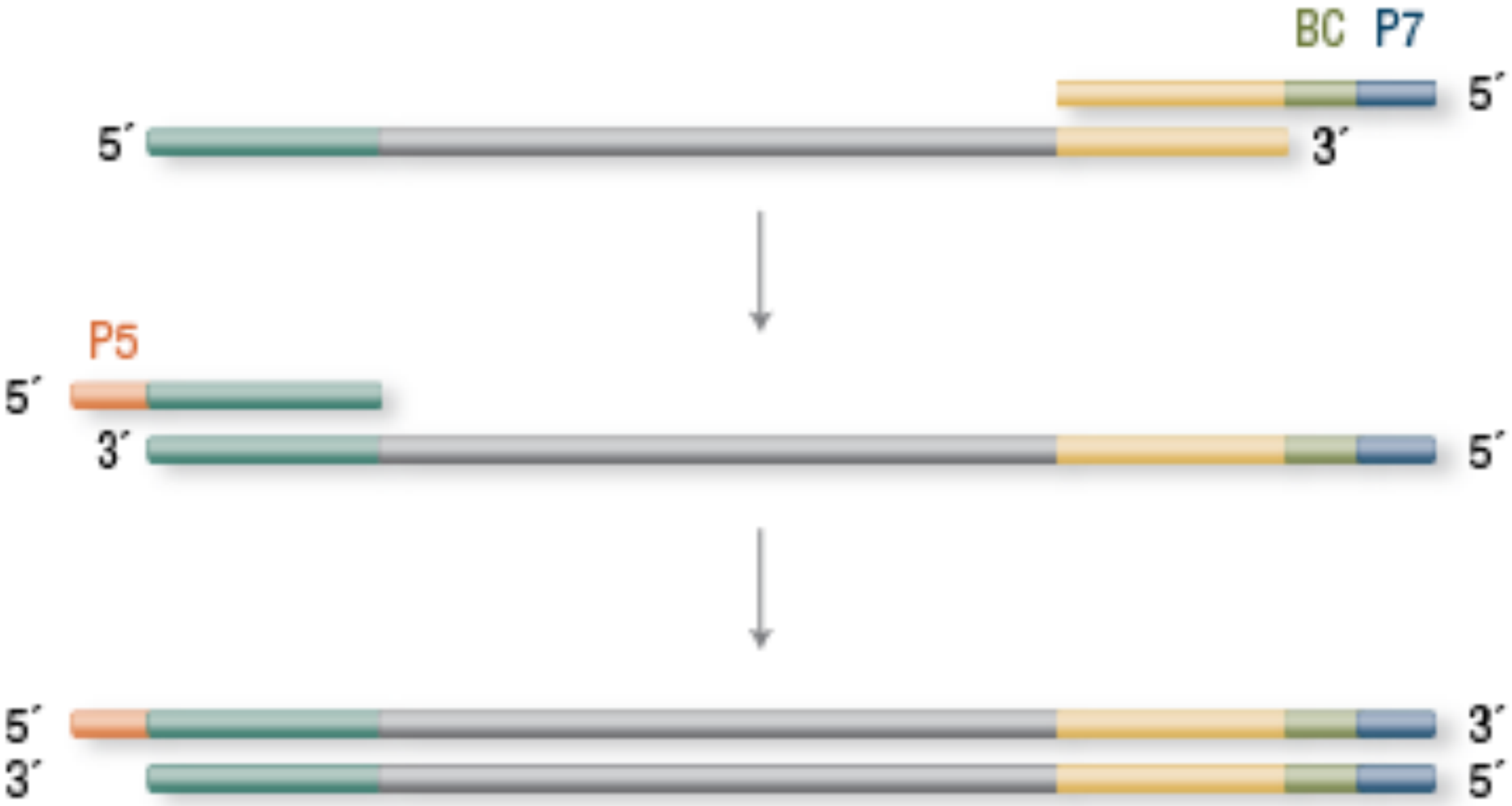
Extend Adapters



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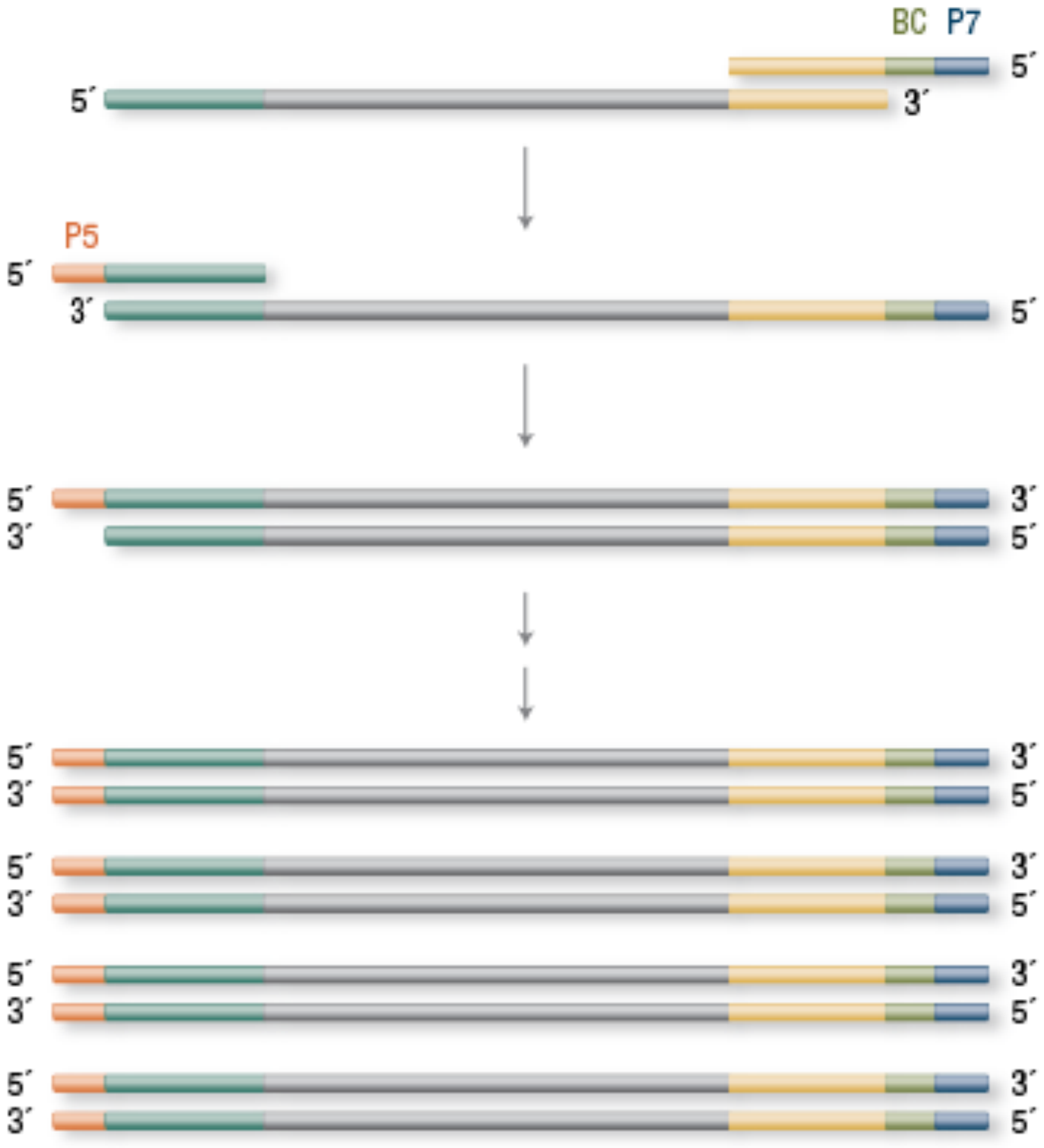
PCR Enrichment



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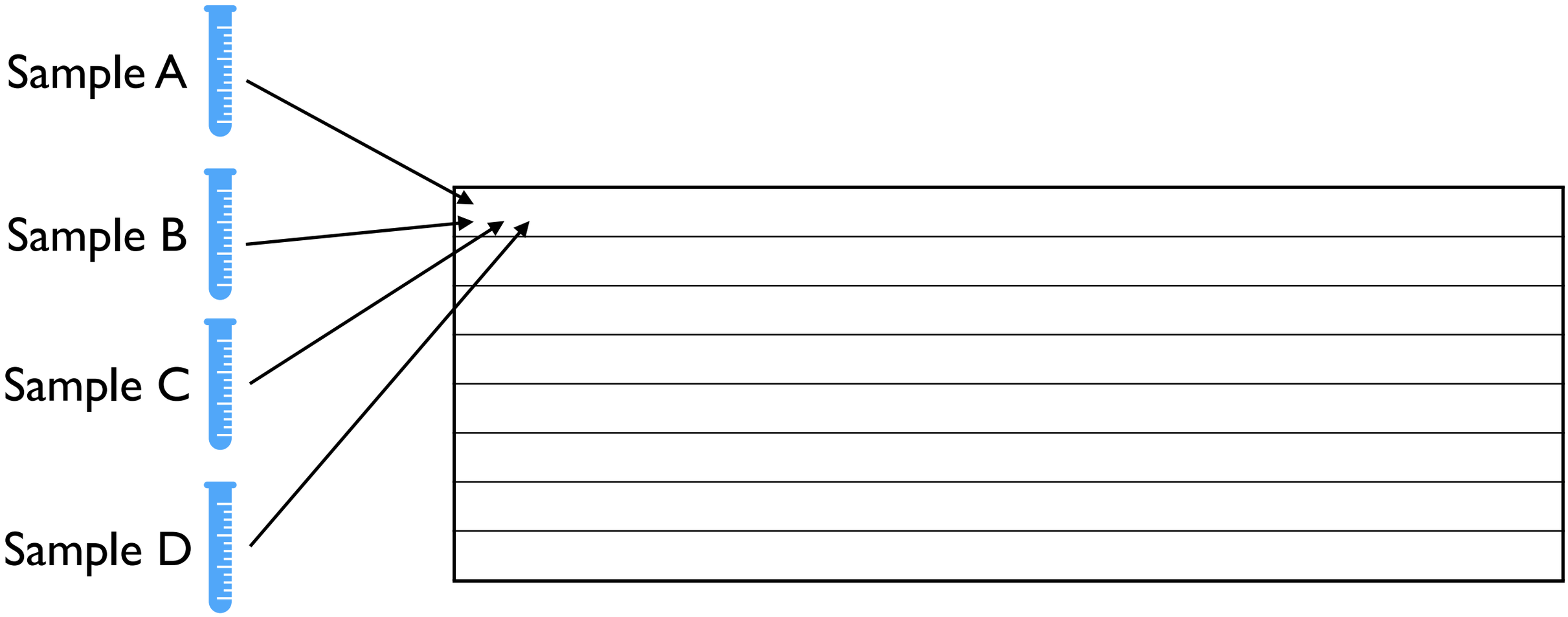
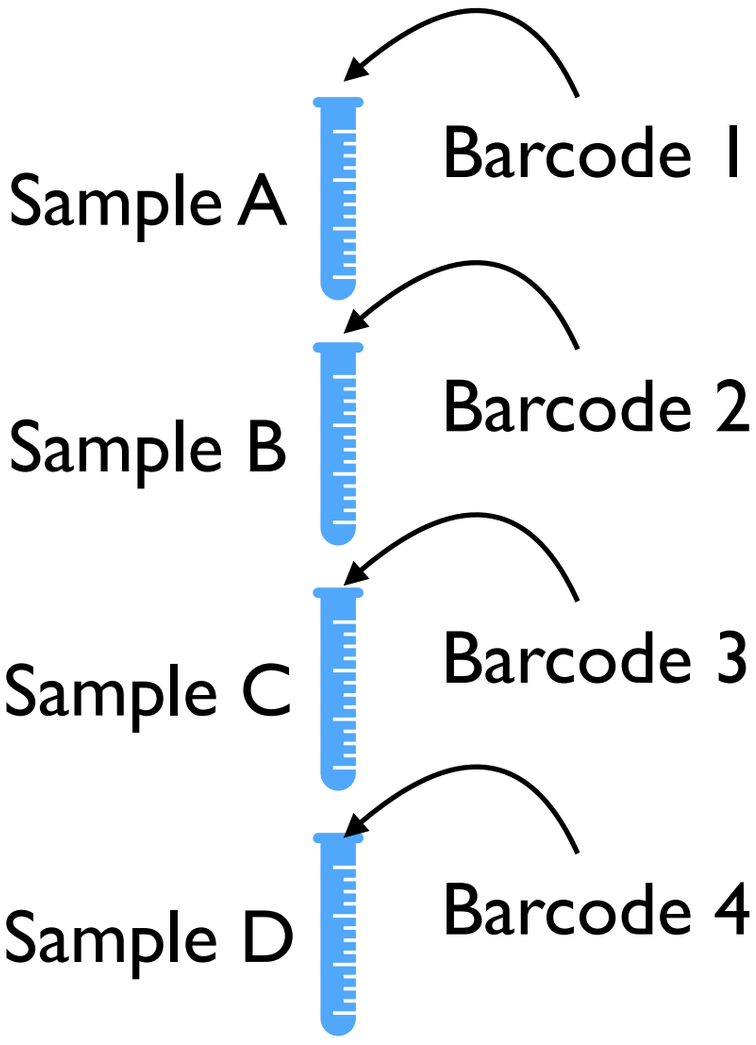
Barcodes: Why?

Multiplexing: Combine multiple samples in a lane



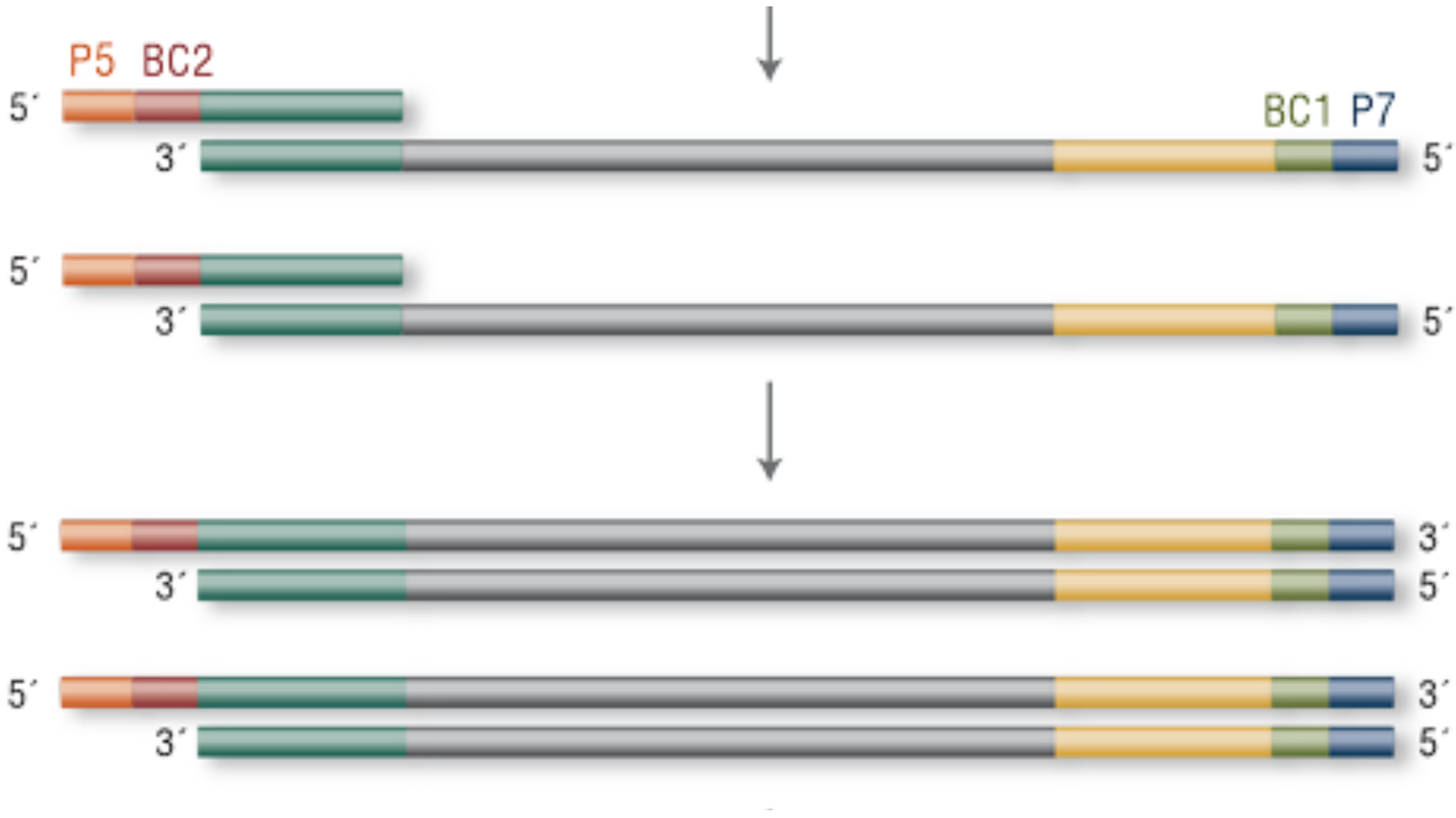
Barcodes


Sample A
Barcode 1
Sample B
Barcode 2
Sample C
Barcode 3
Sample D
Barcode 4

Barcodes

Sample_Name	I7_Index_ID	index
1_A	P49-E1	AAGACCGT
2_A	P50-E2	TTGCGAGA
3_A	P51-E3	GCAATTCC
4_A	P52-E4	GAATCCGT
5_A	P53-E5	CCGCTTAA
6_A	P54-E6	TACCTGCA
7_B	P55-E7	GTCGATTG
8_B	P56-E8	TATGGCAC
9_B	P57-E9	CTCGAACA
10_B	P58-E10	CAACTCCA

Barcodes: Dual Index



https://www.neb.com/-/media/nebus/page-images/products/library-preparation-for-next-generation-sequencing/dna_illumina_loopadapte6440.png'?la=en&device=modal

Barcodes: Dual Index

	BC1: A	BC1: B	BC1: C
BC2: W	Sample 1 A,W	Sample 2 B,W	Sample 3 C,W
BC2: X	Sample 4 A,X	Sample 5 B,X	Sample 6 C,X
BC2: Y	Sample 7 A,Y	Sample 8 B,Y	Sample 9 C,Y
BC2: Z	Sample 10 A,Z	Sample 11 B,Z	Sample 12 C,Z

Nasty Stuff

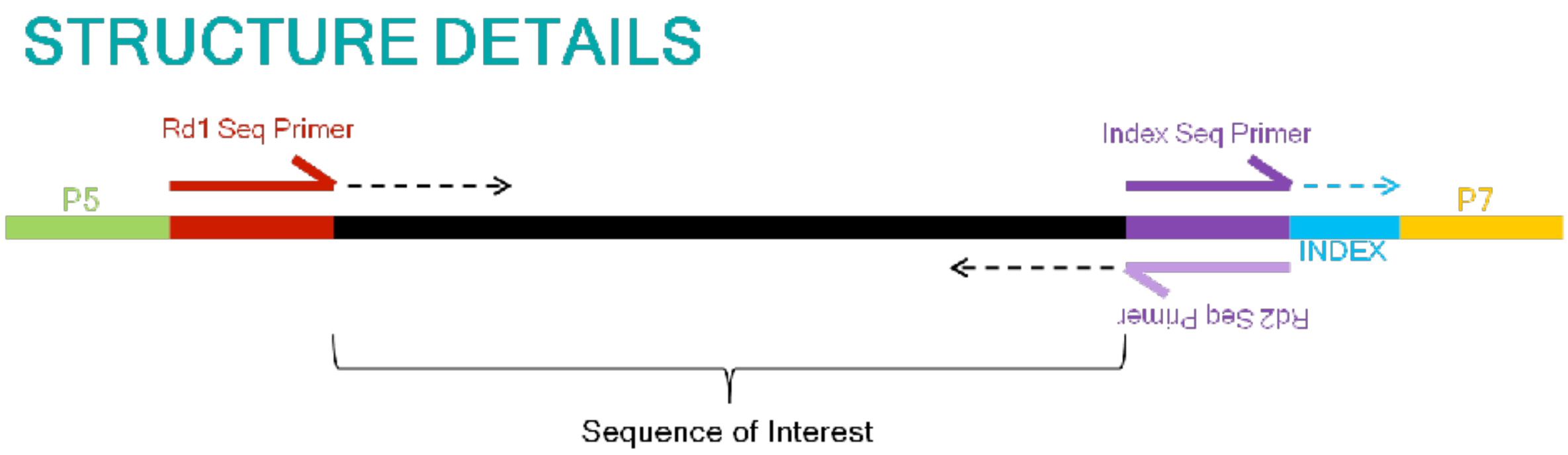
- Sodium Azide
- Actinomycin D

Library Preparation: Alternatives

1. Illumina Kits
2. Other Kits
3. DIY

Extra Stuff

Multiplexing (Barcodes)



http://nextgen.mgh.harvard.edu/IlluminaChemistry.html

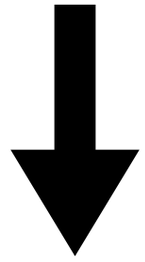
Uracil DNA glycosylase and DNA lyase

Uracil DNA glycosylase: What

- Remove Uracil base from DNA

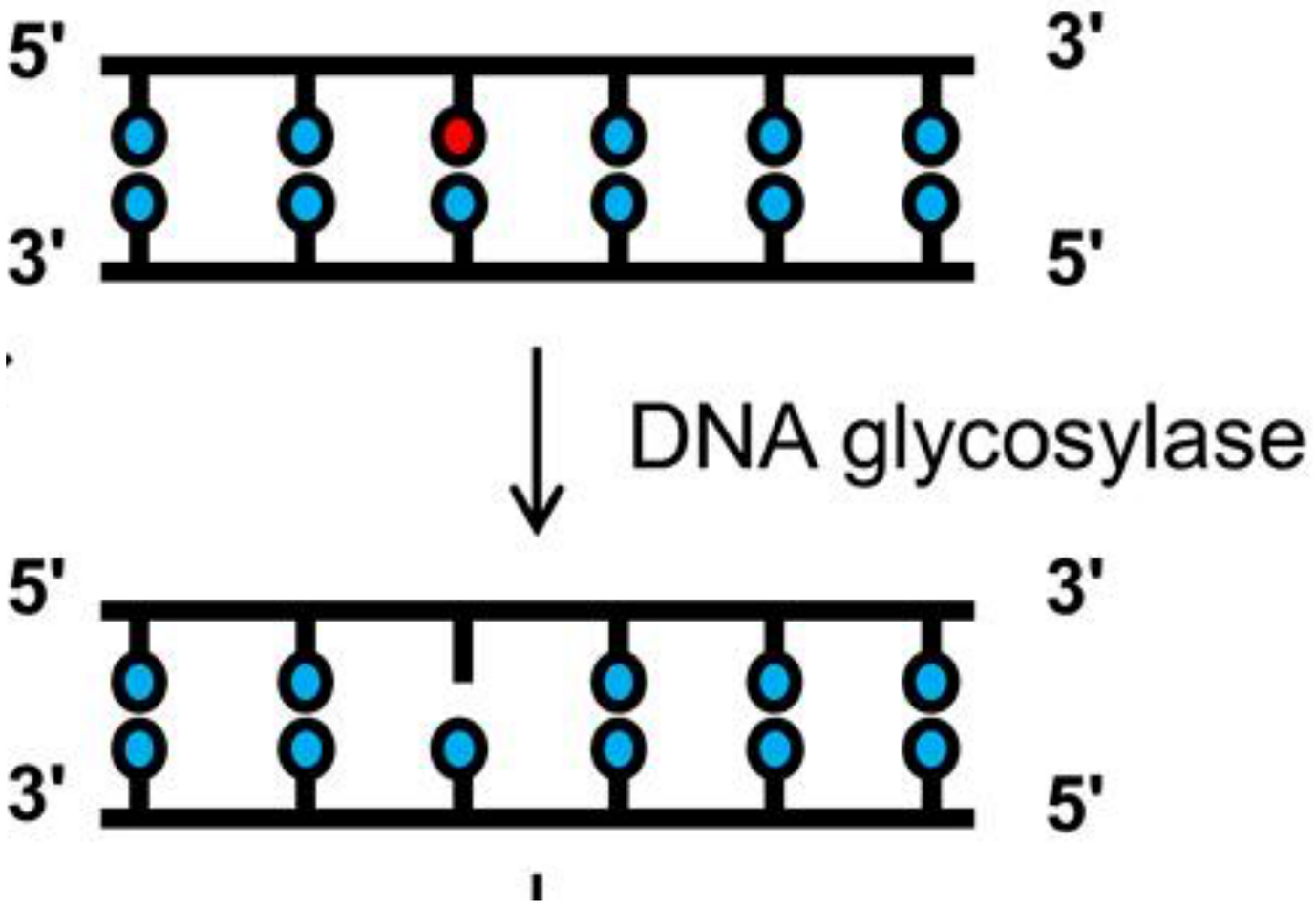
Uracil DNA glycosylase: What

```
5′-CTGATCUGACTGATG-3'
3′-GACTAGACTGACTAC-5′
```



```
5′-CTGATC-GACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

Uracil DNA glycosylase: What



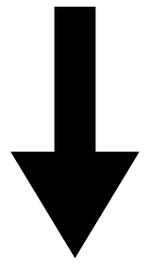
http://www.frontiersin.org/files/Articles/98174/fgene-05-00232-r2/image_m/fgene-05-00232-g001.jpg

DNA Lyase: What

- Cleave DNA backbone at abasic site

DNA Lyase: What

```
5′-CTGATC-GACTGATG-3′
3′-GACTAGACTGACTAC-5′
```



```
5′-CTGATC GACTGATG-3′
3′-GACTAGACTGACTAC-5′
```

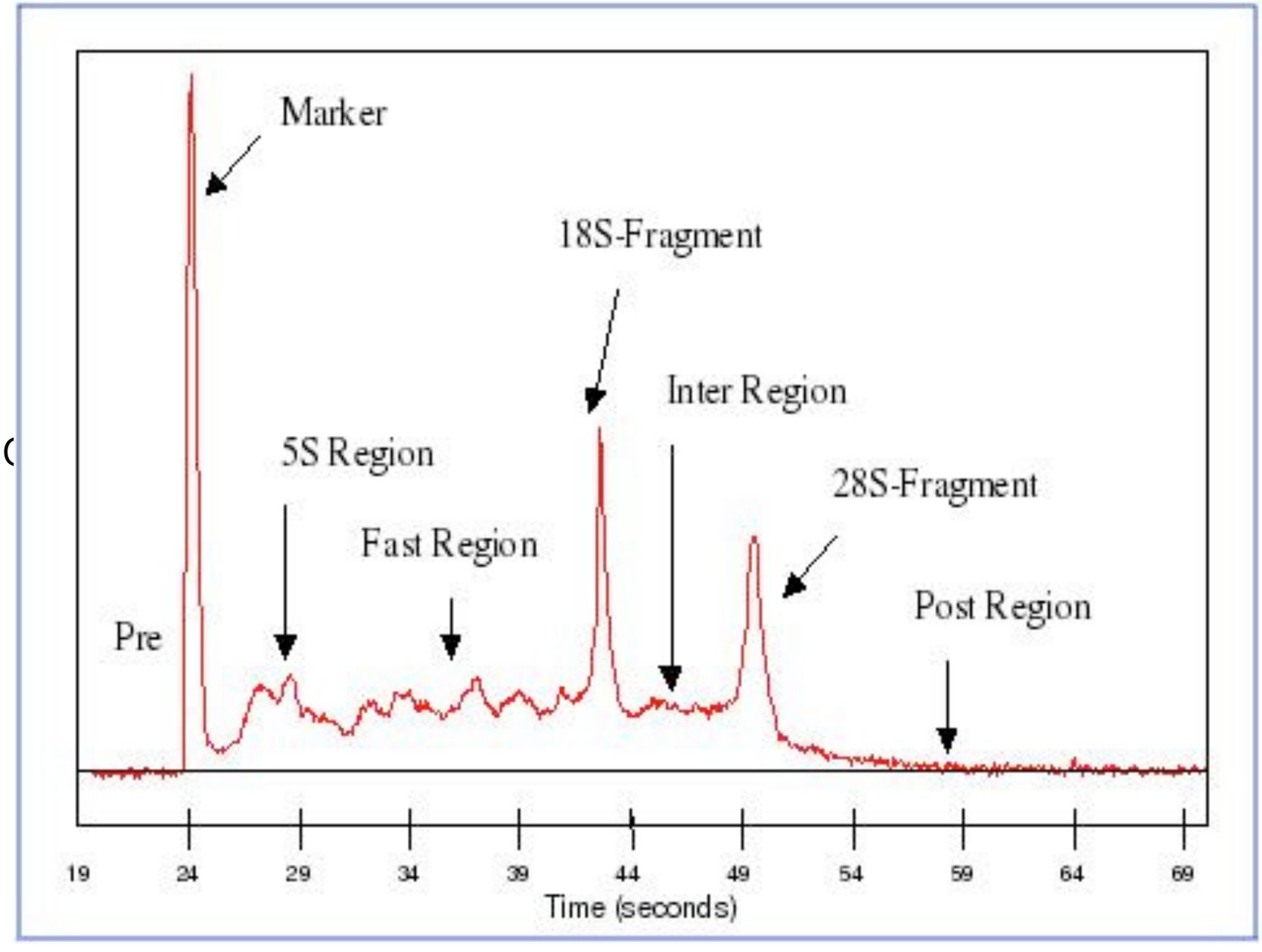
RNA Quality?

- RIN: RNA Integrity Number

http://cartoon-yeah.blogspot.com/2011/03/ren-and-stimpy-picture-4.html

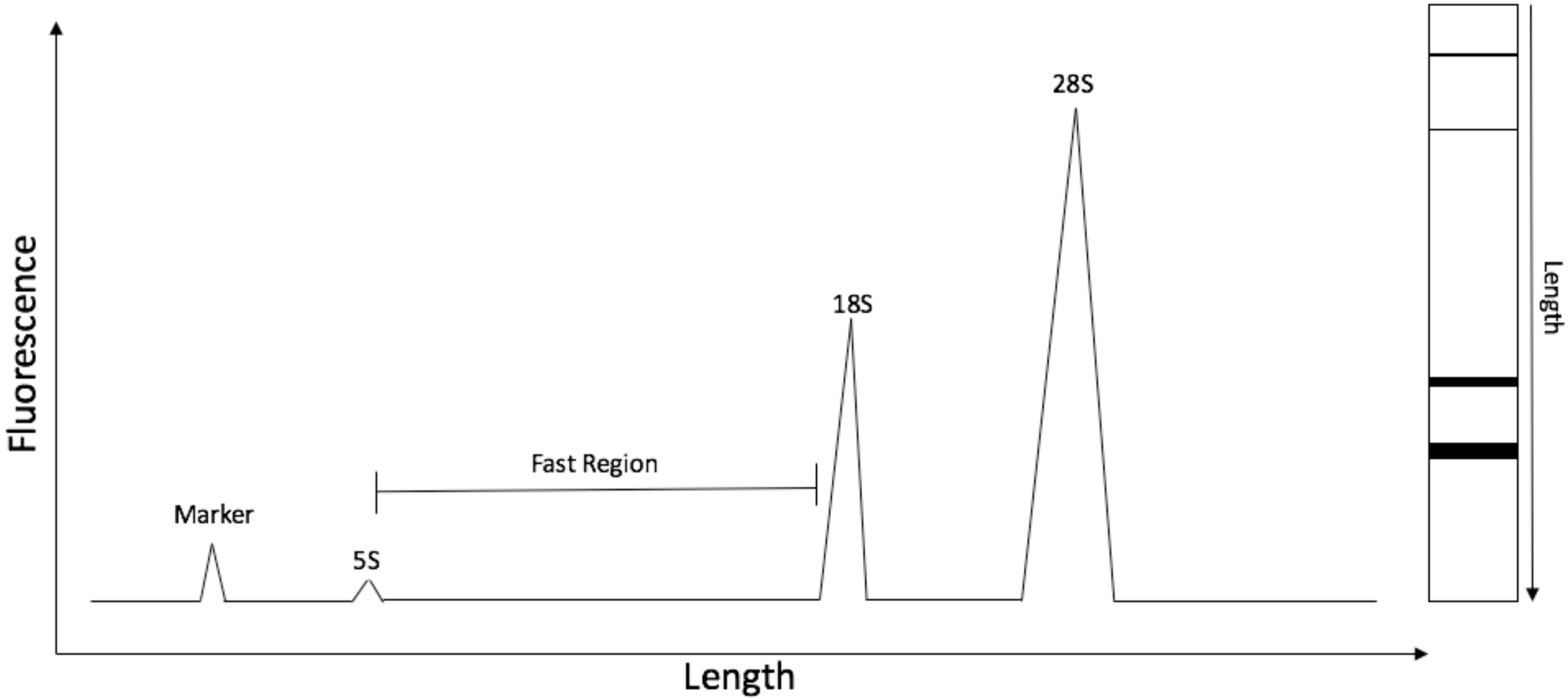
Ratio of 28S to18S ribosomal RNA

- Ratio o

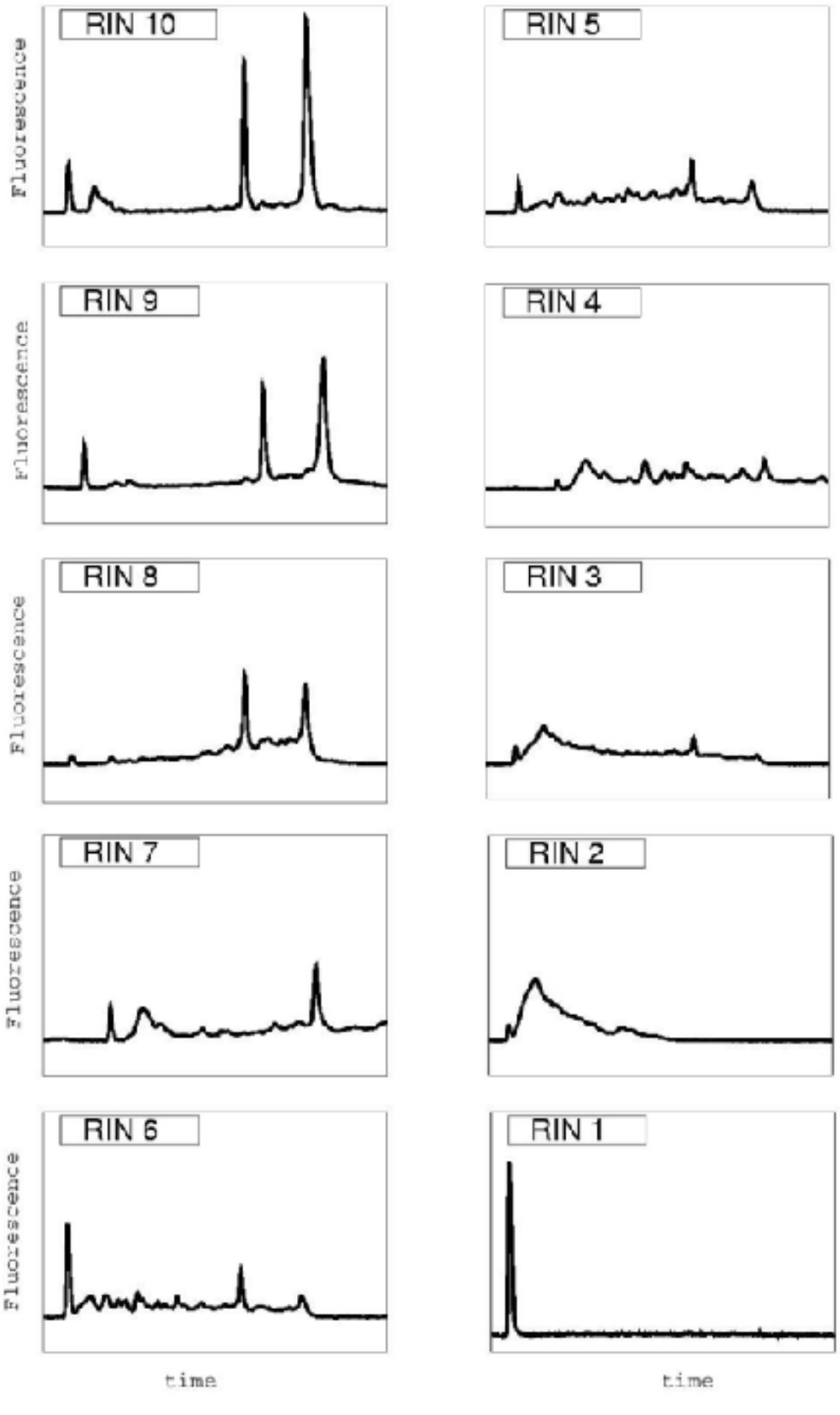


http://dx.doi.org/10.1186/1471-2199-7-3

Electropherogram

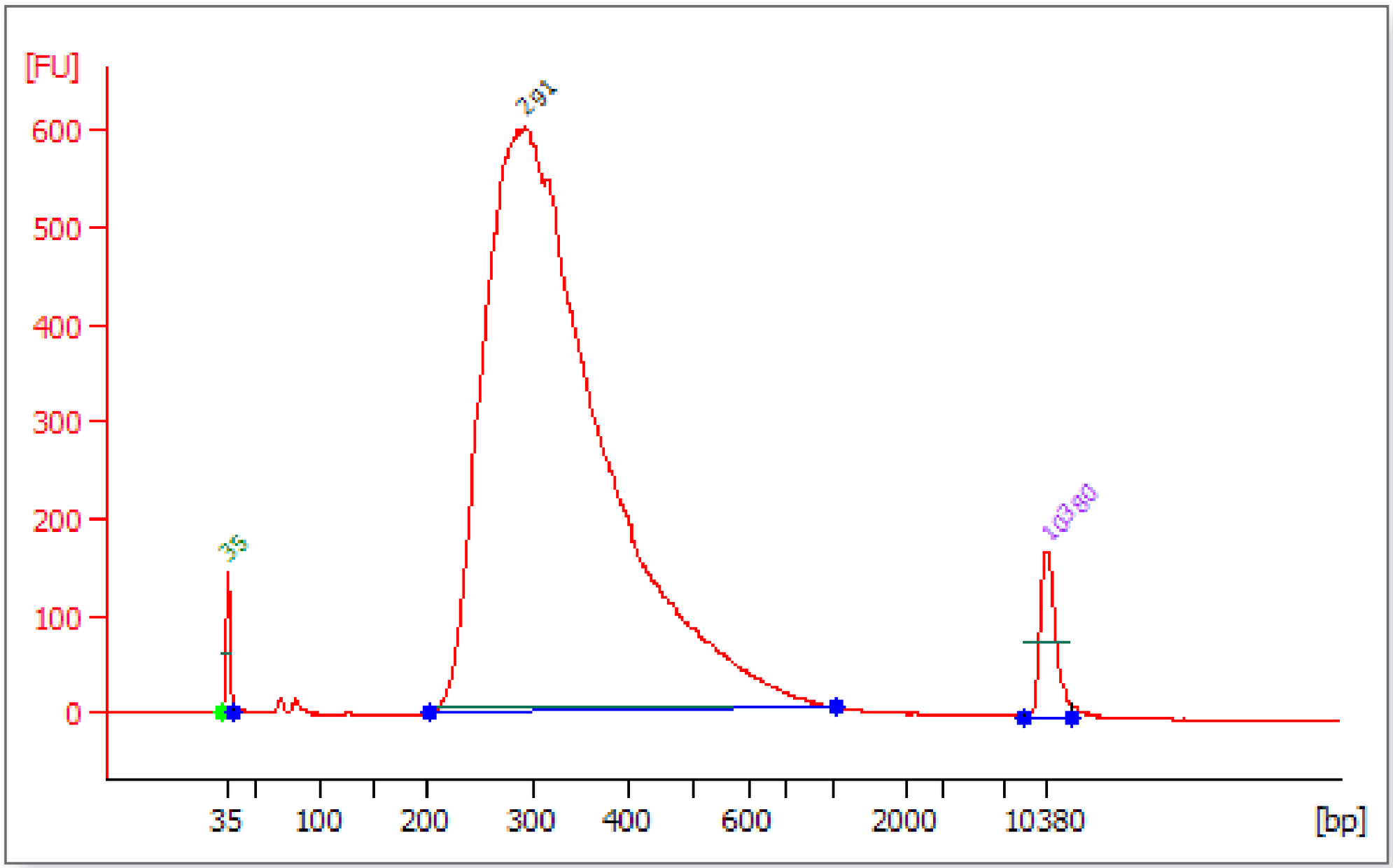


https://upload.wikimedia.org/wikipedia/commons/a/a2/Sample_RNA_electropherogram.png



http://dx.doi.org/10.1186/1471-2199-7-3

RNA Library Size Distribution



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Assessment of RNA/DNA Quantity and Quality

- Advanced Analytical: Fragment Analyzer
- PerkinElmer: LabChip GX Touch
- Agilent: Bioanalyzer
- Agilent: TapeStation

Barcode Combinations

- Excitation Frequency
  - Red: A and C
  - Green: G and T
- Need both frequencies in each cycle for image registration

Barcode Combinations

GOOD

PRIMER	INDEX SEQUENCE	PRIMER	INDEX SEQUENCE
P1-A1	T T A C C G A C	P41-D5	G A C G T C A T
P2-A2	A G T G A C C T	P42-D6	C T T A C A G C
P3-A3	T C G G A T T C	P43-D7	T C C A T T G C
P4-A4	C A A G G T A C	P44-D8	A G C G A G A T
	✔ ✔ ✔ ✔ ✔ ✔ ✔ ✔		✔ ✔ ✔ ✔ ✔ ✔ ✔ ✔

BAD

PRIMER	INDEX SEQUENCE	PRIMER	INDEX SEQUENCE
P9-A9	C G C A A C T A	P56-E8	T A T G G C A C
P10-A10	C G T A T C T C	P57-E9	C T C G A A C A
P11-A11	G T A C A C C T	P58-E10	C A A C T C C A
P12-A12	C G G C A T T A	P59-E11	G T C A T C G T
	✔ ✖ ✔ ✖ ✔ ✔ ✔ ✔		✔ ✔ ✔ ✔ ✔ ✖ ✔ ✔

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