Sample Preparation

Module 1: Overview

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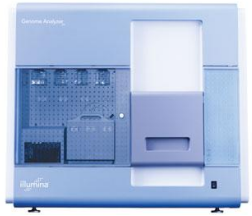
Sequencing Workflow

Sample Preparation

Cluster Generation



Sequencing

Data Analysis

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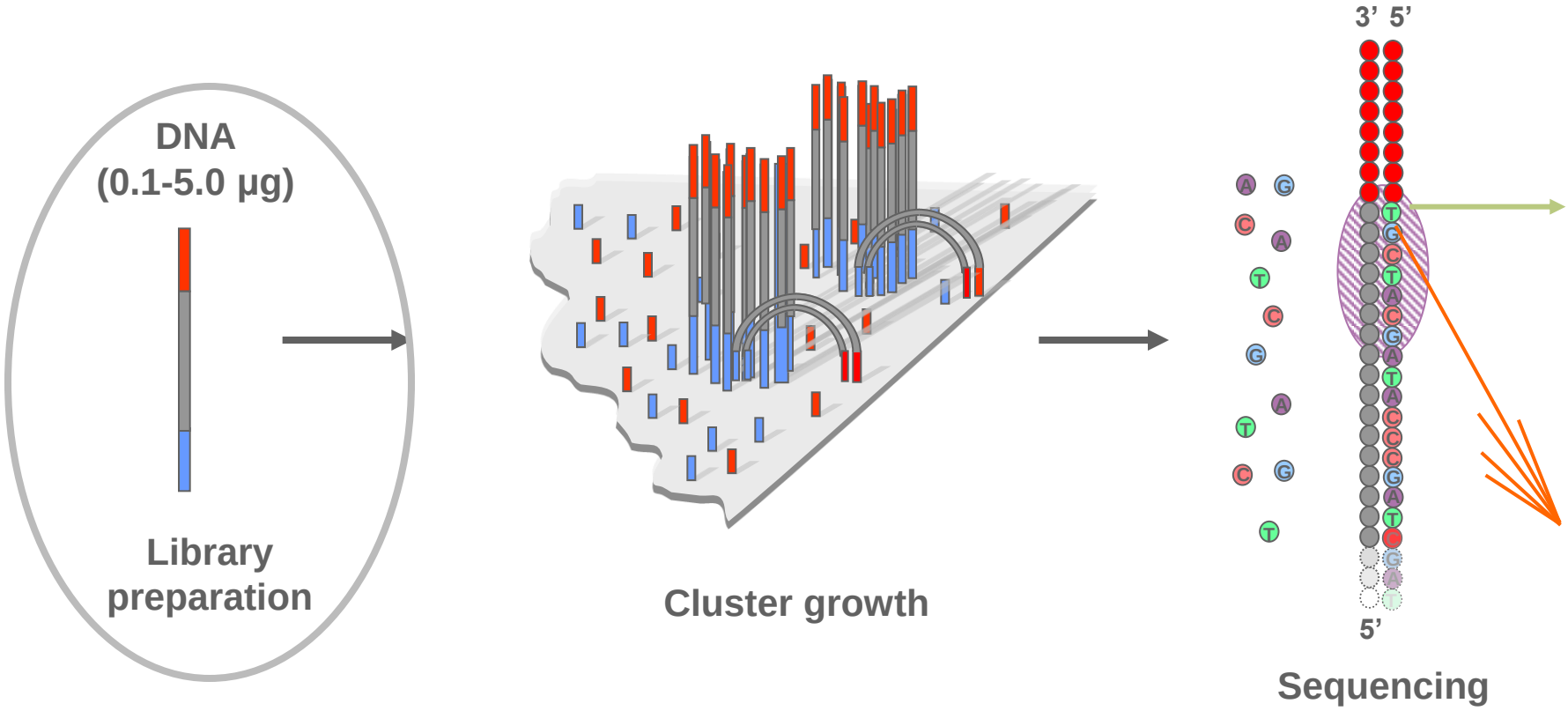
Library Preparation



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Library Preparation

Sample Preparation

- Prepares sample nucleic acid for sequencing
  - Fragmenting
  - Generates double-stranded DNA (if necessary)
  - Flanks with Illumina adapters
- All preparation ends with the same general template structure
  - Double-stranded DNA flanked by adapters
  - Variables include
    - Insert Size
    - Adaptor type
    - Index

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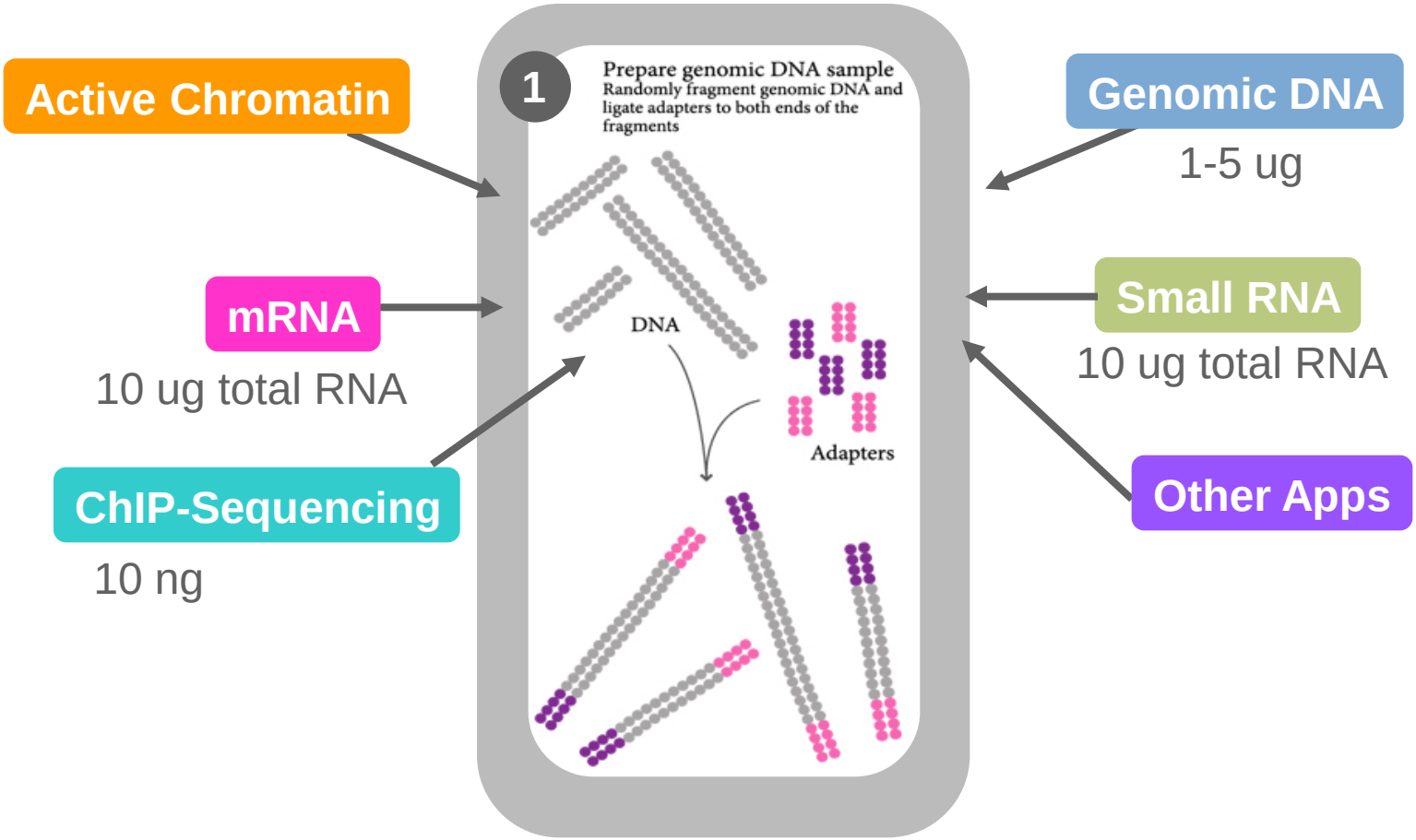
Library Preparation



Library preparation:
Attach adapters to both ends of fragmented nucleic acid

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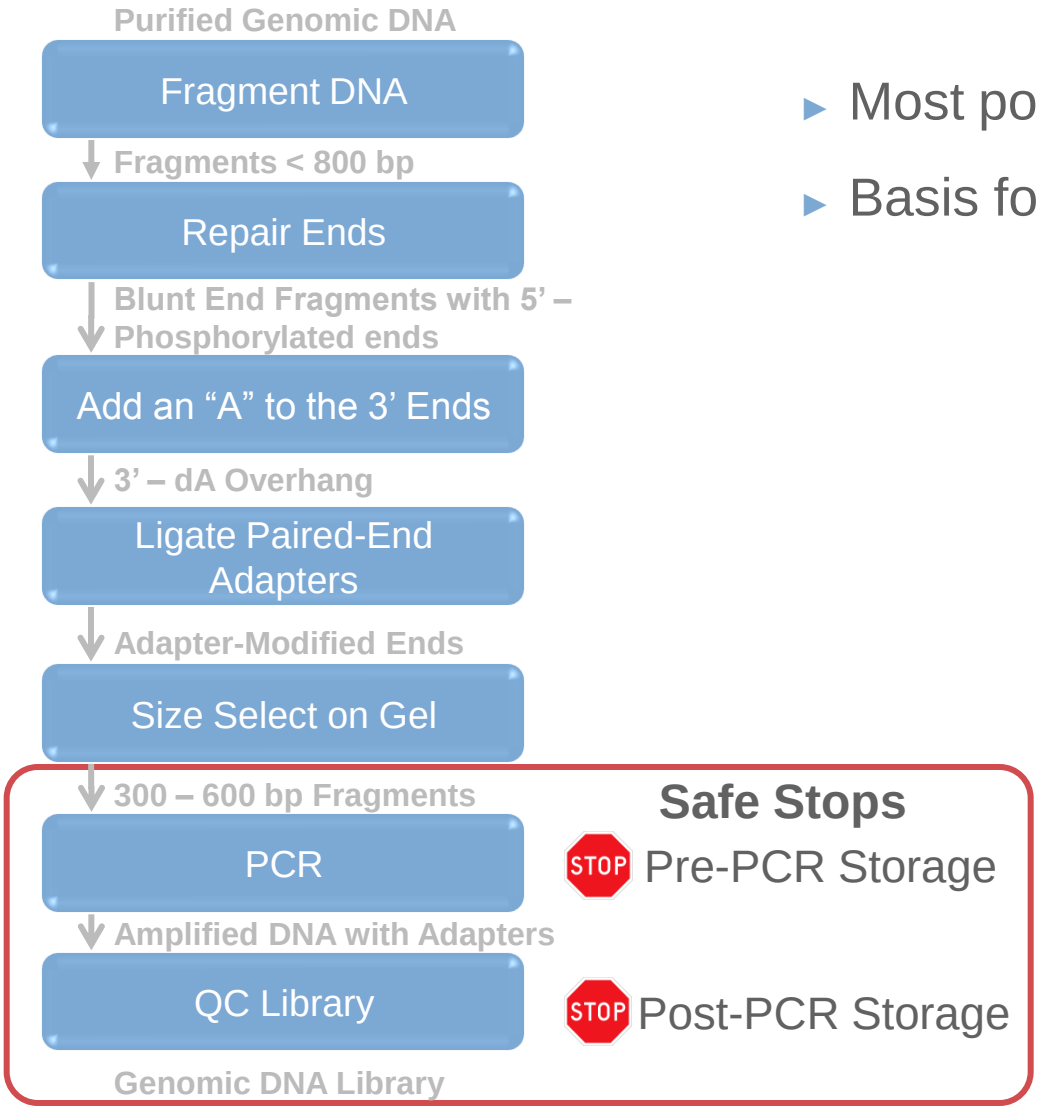
Genomic DNA Protocol Overview



- Most popular protocol
- Basis for all other protocols

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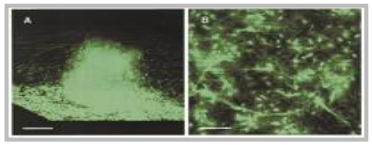
Starting Material Input

- Starting Materials
  - Quantify
  - Quality Control
- Methods for Quantification:
- 




  NanoDrop* PicoGreen® * Qubit™

- Methods for Quality Control
  - Visualization on gel
  - Agilent Bioanalyzer* trace especially useful for RNA (RIN)

***Methods utilized at the Broad**

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Step 1: Fragment DNA

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

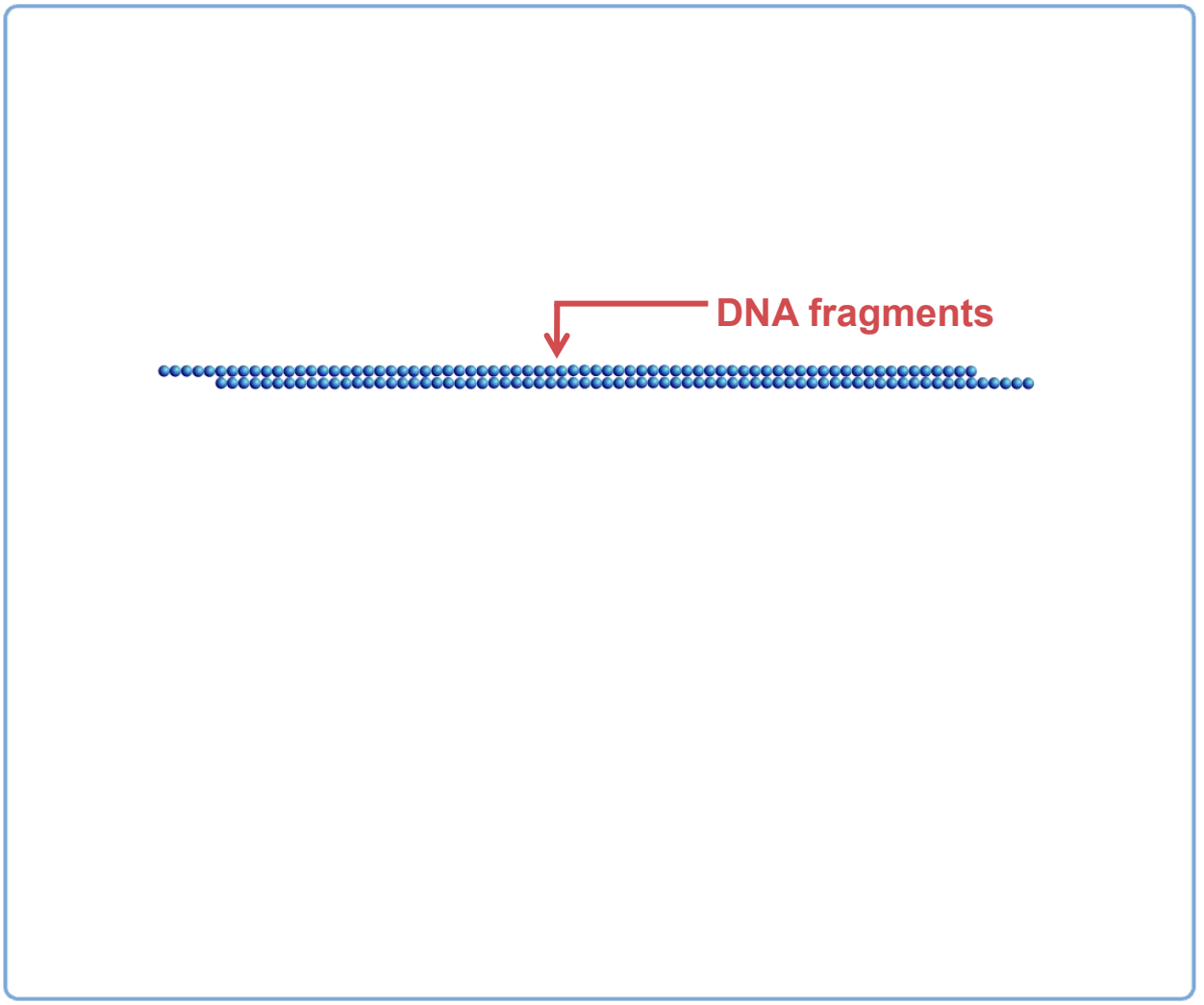
300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library



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Step 1: Fragment DNA

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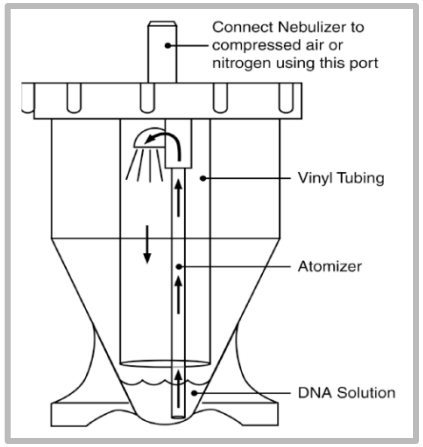

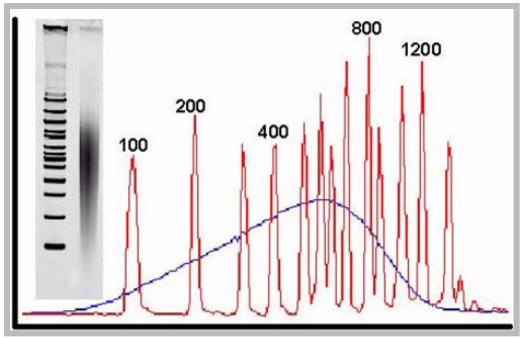

- Nebulizer
  - Very inexpensive
  - Works well for 1-5 µg starting input
- Other methods available
  - Covaris™
  - Sonication
  - HydroShear®
  - Enzymatic
  - Others

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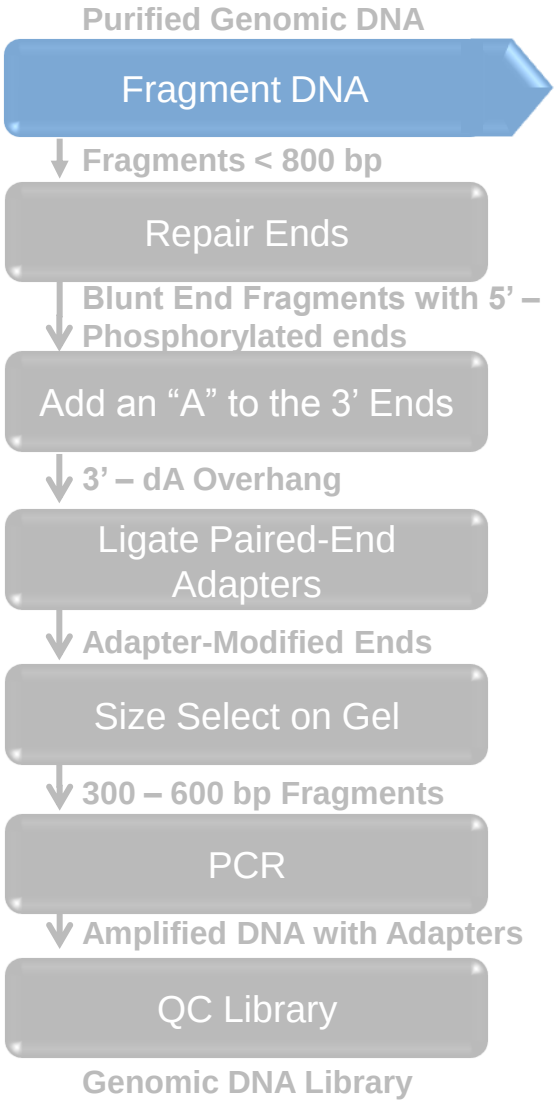
Step 1: Fragment DNA



	Nebulizer	Covaris™	HydroShear®	Sonication	Chemical	Enzymatic
DNA	✓	✓	✓	✓		
mRNA					✓	
DGE						✓
small RNA						
CHIP-Seq		✓		✓		
Mate Pair	✓		✓			

- Fragmentation methods have benefits and limitations
- Can be application-specific
- Depends on desired end-product

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Step 2: Repair Ends

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

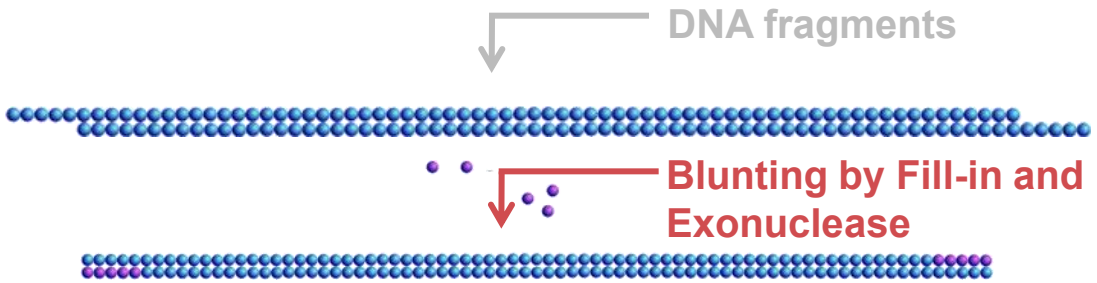
300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library



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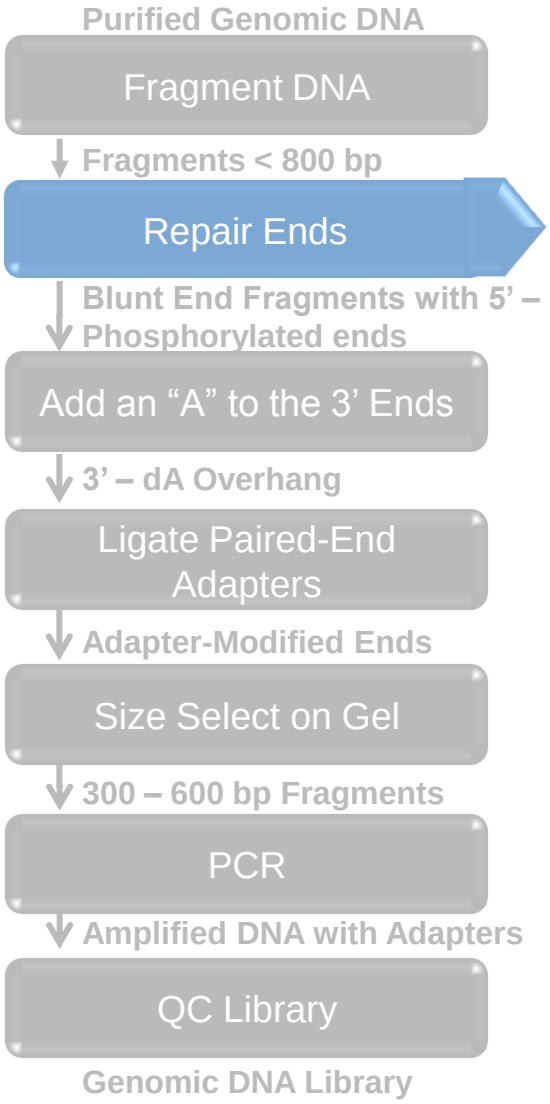
Step 2: Repair Ends



- Converts overhangs
  - Blunt ends
  - Phosphorylates 5' End
- Reagents:
  - dNTP, T4 DNA pol, Klenow
  - Kinase/ATP (T4 PNK)
- Simple enzymatic reaction
- 30 minutes incubation

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Step 3: Add “A” Overhang

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5’ – Phosphorylated ends

Add an “A” to the 3’ Ends

3’ – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

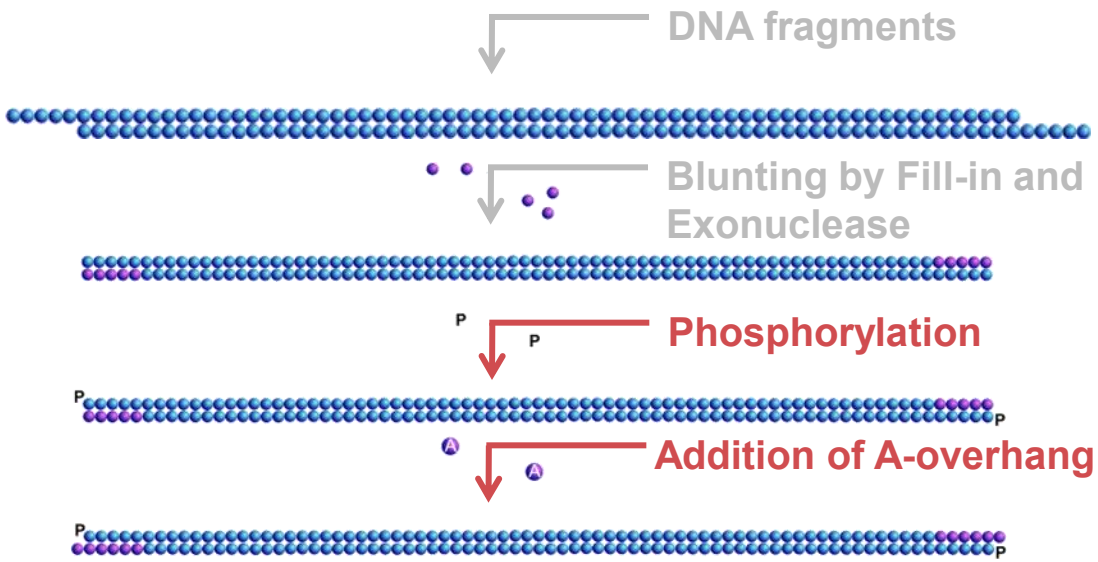
300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library



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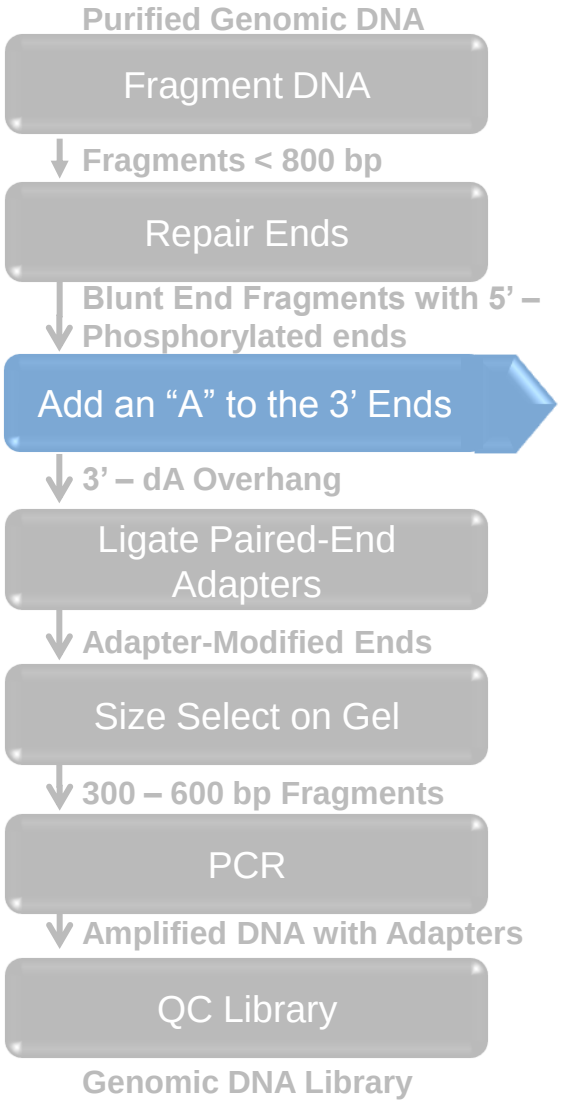
Step 3: Add “A” Overhang



- Adds
  - ‘A’ base to the 3' end of the blunt phosphorylated DNA fragments
- Prevents
  - Formation of adapters dimers
  - Concatemers
- Reagents
  - 1 mM dATP, Klenow exo (3' to 5' exo minus)

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Step 4: Ligate Adapters

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

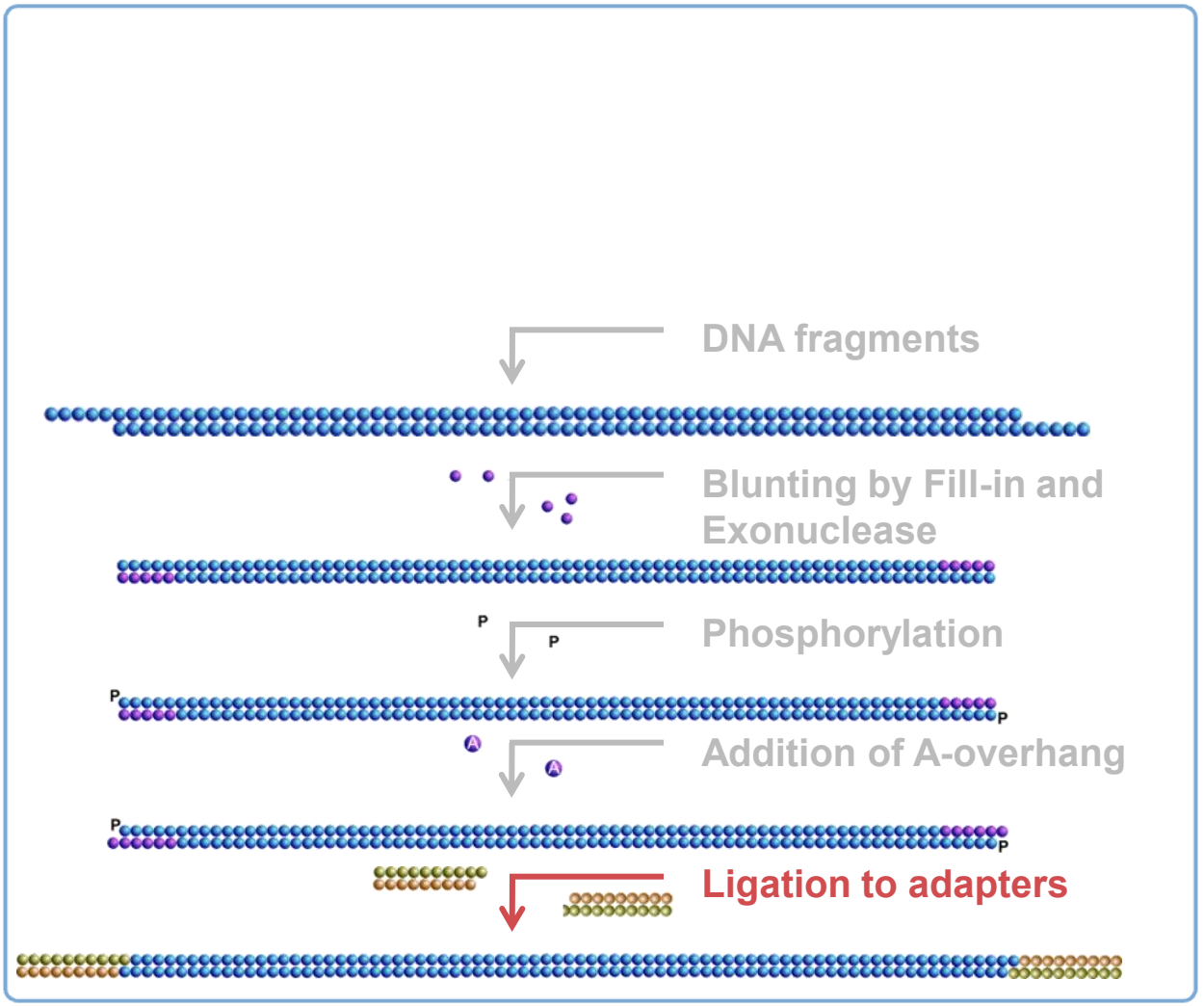
300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library



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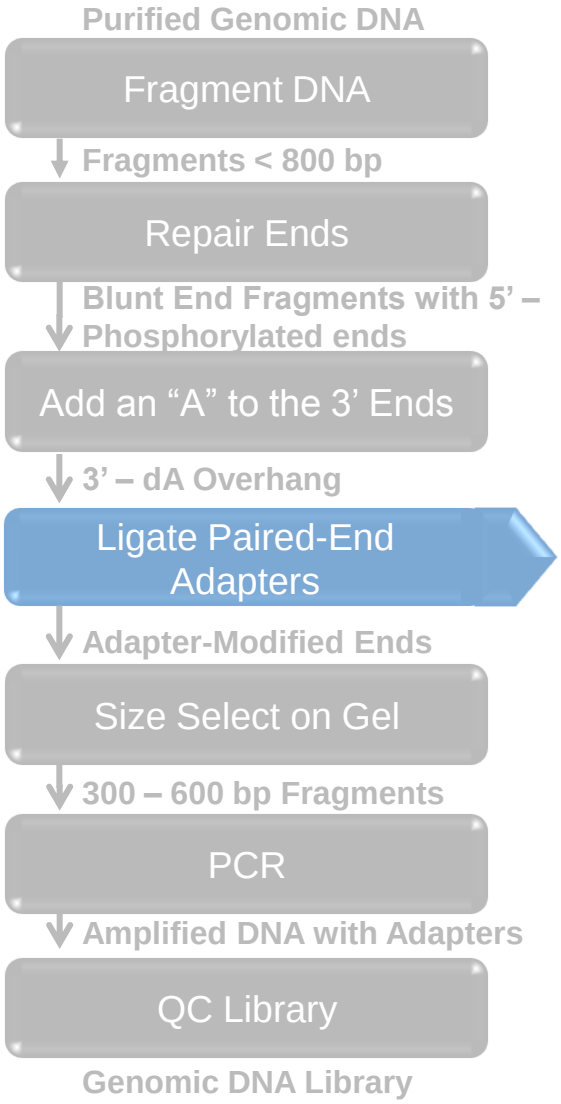
Step 4: Ligate Adapters



- Same library structure regardless of Application
  - gDNA
  - RNA
  - miRNA
  - ChIP-seq
  - Exon Pull-down
  - etc.

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Step 4: Ligate Adapters

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

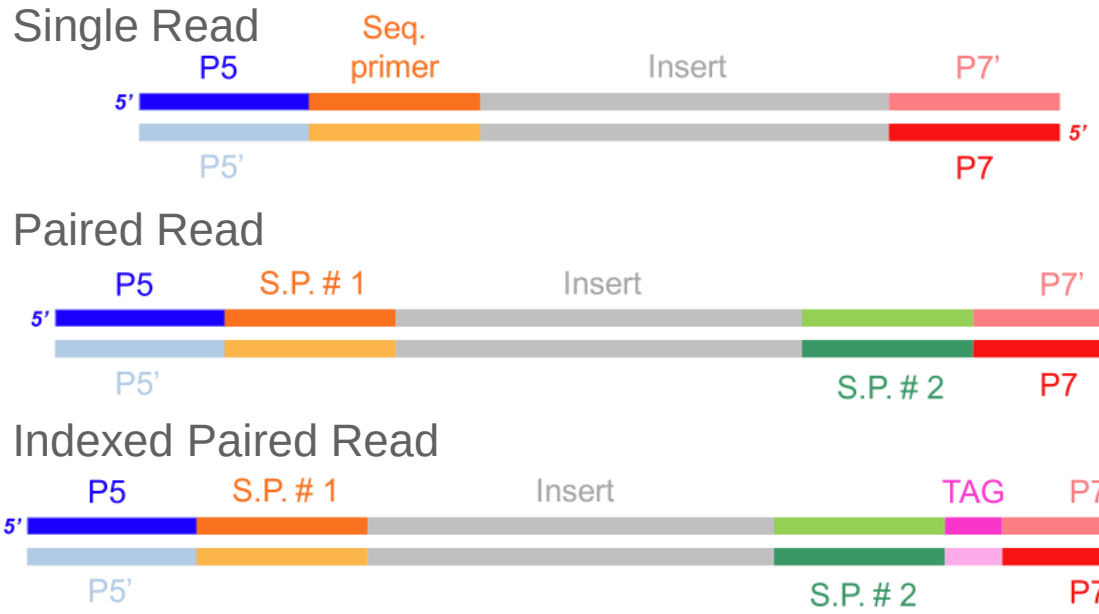
300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library



- 10:1 molar ratio of adapter to genomic DNA insert
  - Assumes 5 µg initial input
  - Titrate for different insert amounts

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Choosing a Library Type

- Single read libraries:
  - Unidirectional Sequencing
  - Single Read Flowcells ONLY
  - Counting applications: ChIP or low coverage resequencing projects

- Paired end libraries:
  - Uni- OR Bi-directional (paired reads)
  - Paired End Flowcells; Single: Unidirectional only
  - Most applications, #1 whole genome shotgun assembly
  - Tailor insert size and distribution per project:
    - Tight size distribution – Assembly, structural rearrangement detection
    - Wide distribution libraries - Resequencing, high coverage

- Multiplex Paired End (aka Indexing or Barcoding)
  - Uni- OR Bi-directional
  - Allows multiple libraries per lane
  - 12 Index tags available x 8 lanes = 96 libraries per flowcell

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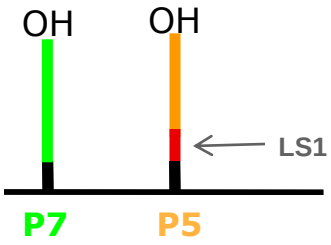
Flowcell Compatibility

Single Read Flowcell

Periodate Linearization:

- Cleaves off most of the P5 oligo



5' P-GATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

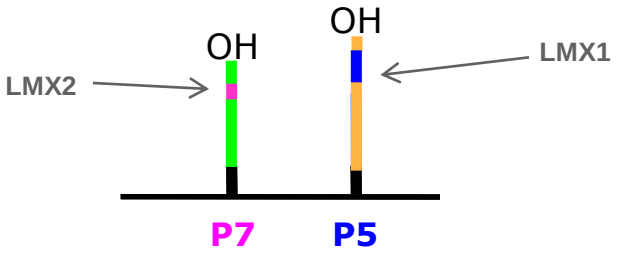
SR Adaptors

Paired End Flowcell

Linearization 1 Enzyme:

-Leaves part of P5 oligo intact for Read 2 resynthesis

Linearization 2 Enzyme



5' P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT

PE Adaptors

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Step 5: Gel Purify Ligation Products

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' –Phosphorylated Ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

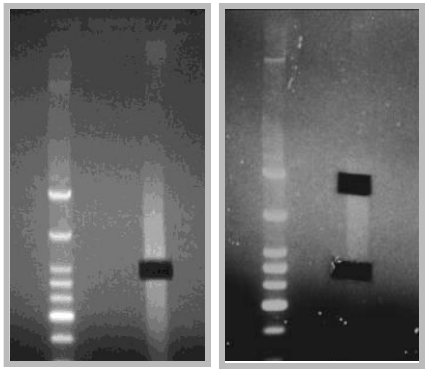
300 – 600 bp Fragments

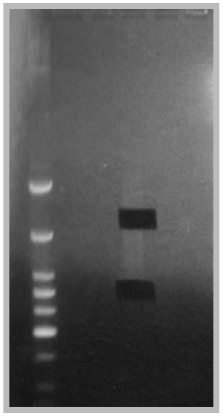
PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library





- 1µg of DNA Starting Material
  - 300/600bp area excised
- 5µg of DNA Starting Material
  - 300/600bp area excised

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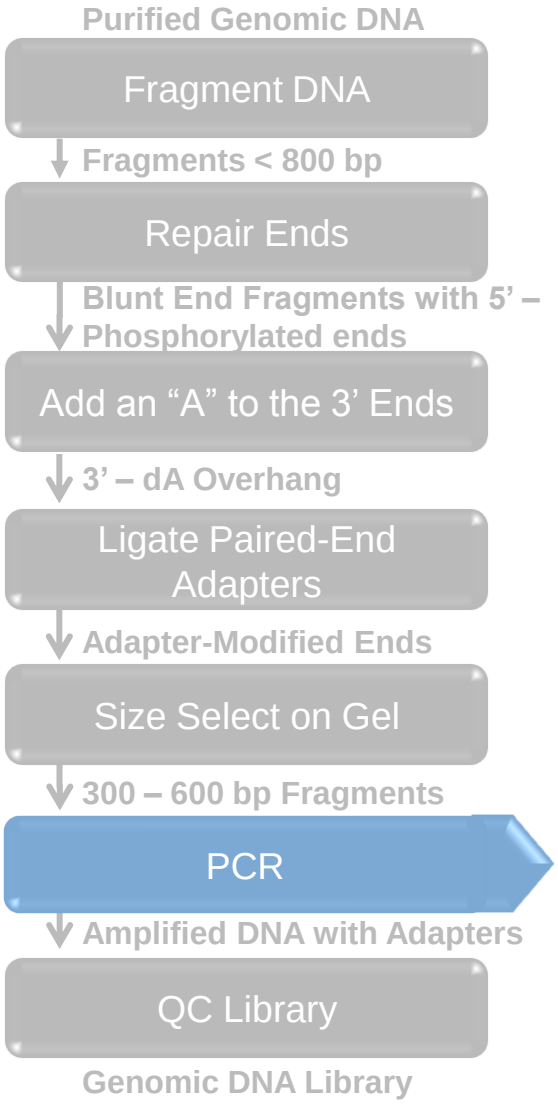
Step 6: PCR Enrich Ligation Products



- Selectively enrich DNA fragments with adapter molecules on both ends
- Adds additional sequences to the end of the adapters for hybridization
- Amplifies the amount of DNA in the library
- Reagents:
  - Adapter Specific Primers
  - High Fidelity Enzyme (Phusion)

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Step 7: QC Library

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

300 – 600 bp Fragments

PCR

Amplified DNA with Adapters

QC Library

Genomic DNA Library

- Quantitate by NanoDrop or Qubit
  - NanoDrop can overestimate concentration
  - Qubit or PicoGreen gives more accurate measurement
- QC by Agilent Bioanalyzer®
  - Gives size confirmation
  - Visualizes unwanted products

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Step 7: QC Library

Purified Genomic DNA

Fragment DNA

Fragments < 800 bp

Repair Ends

Blunt End Fragments with 5' – Phosphorylated ends

Add an "A" to the 3' Ends

3' – dA Overhang

Ligate Paired-End Adapters

Adapter-Modified Ends

Size Select on Gel

300 – 600 bp Fragments

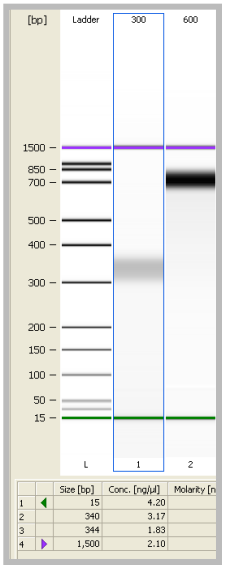
PCR

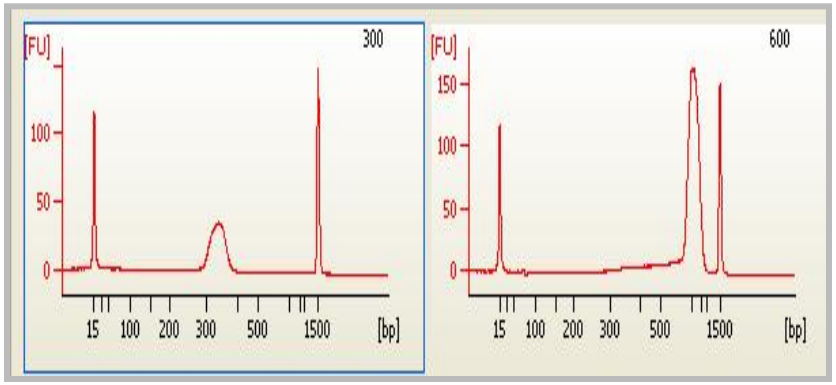
Amplified DNA with Adapters

QC Library

Genomic DNA Library

- Agilent Bioanalyzer Results
  - Lane 1: 300 bp library
  - Lane 2: 600 bp library





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Other DNA Applications

- Multiplexing (aka Indexing/Barcoding)
- Large Insert Mate Pair

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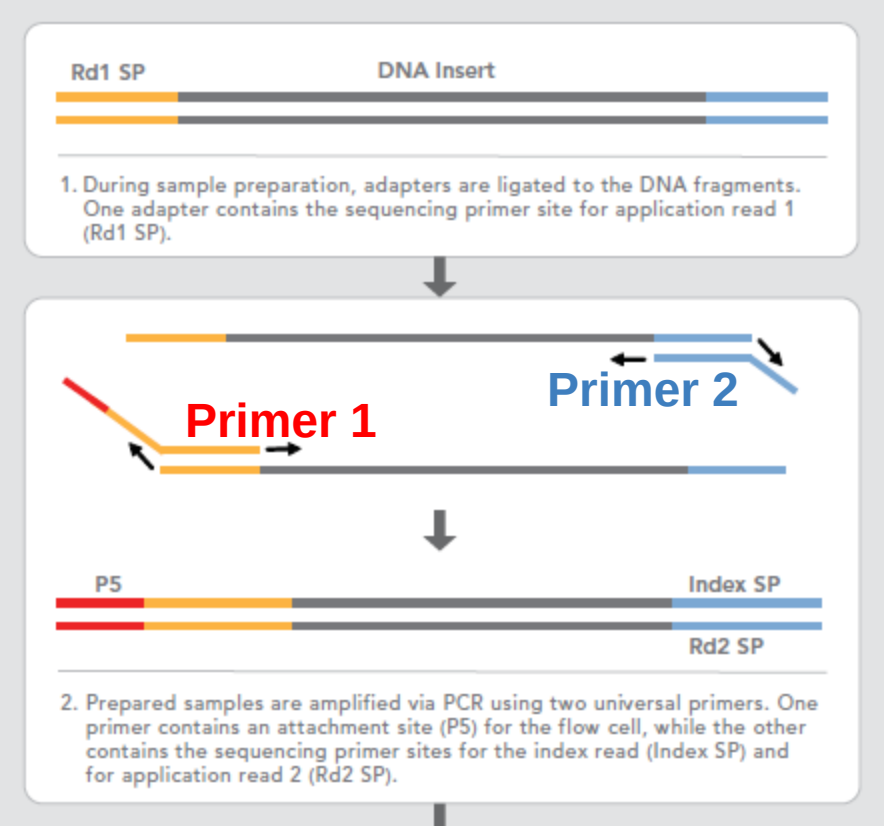
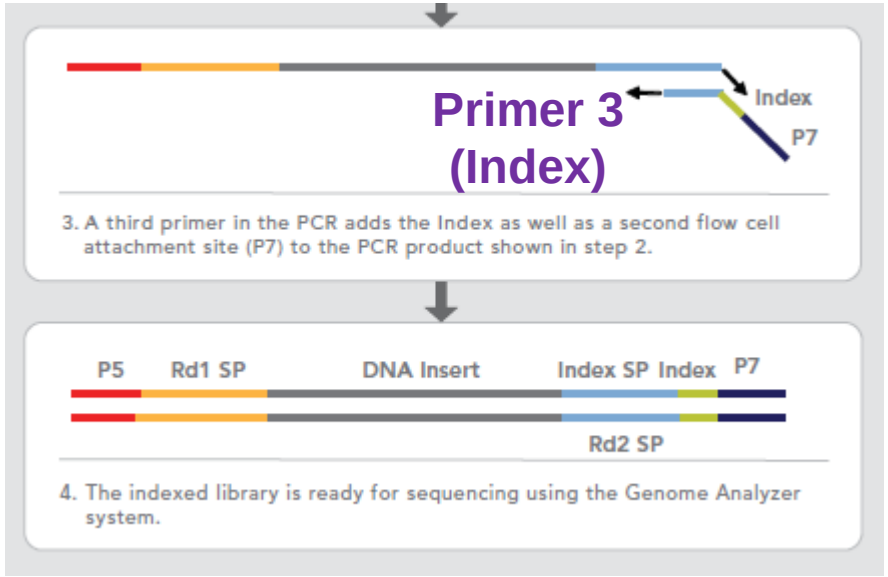
Multiplex Paired End Libraries

Index Tag is incorporated during Sample Prep PCR, using a 3-primer system





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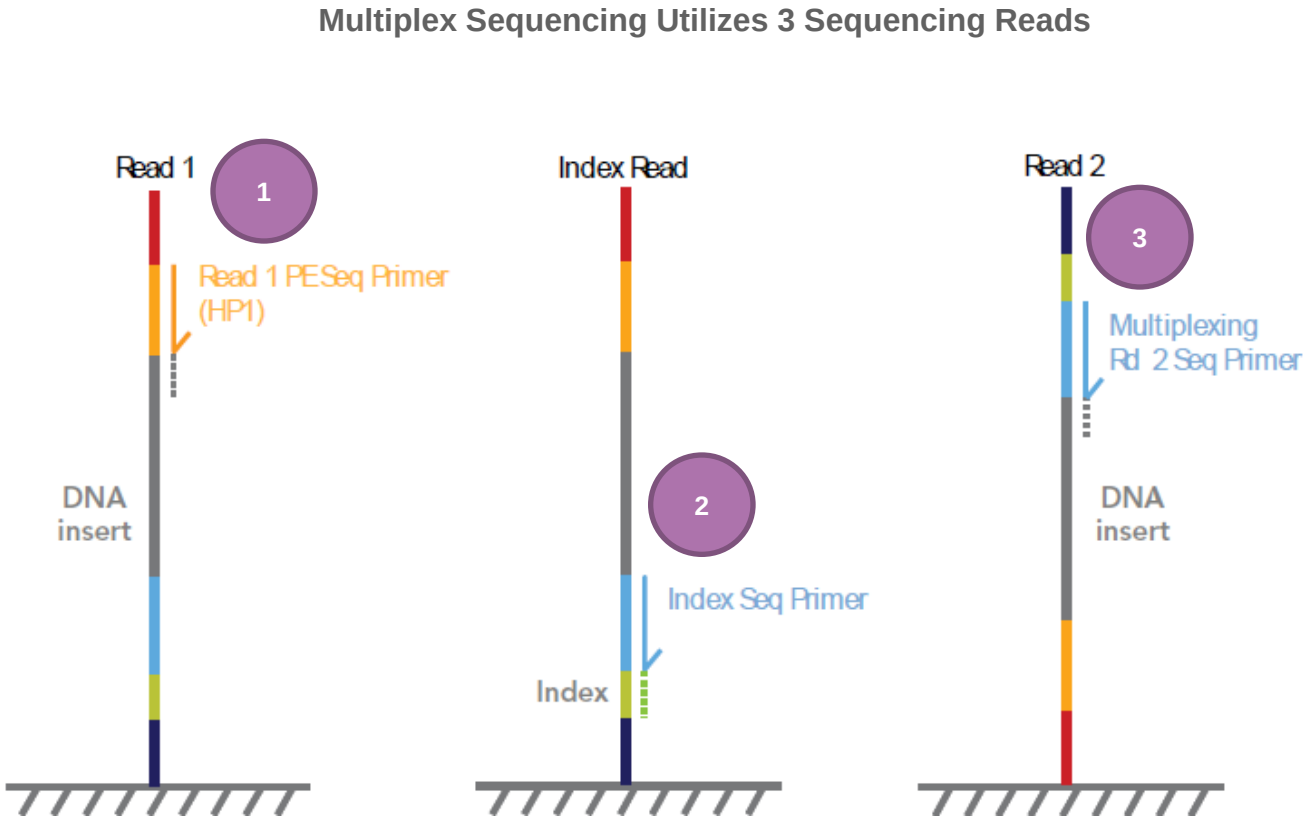
Multiplex Paired End Libraries



Long Insert Mate Pair

- Allows gap sizes of 2-5kb
- Useful for whole genome shotgun assembly; connecting contigs
- Detection of structural/chromosomal rearrangements

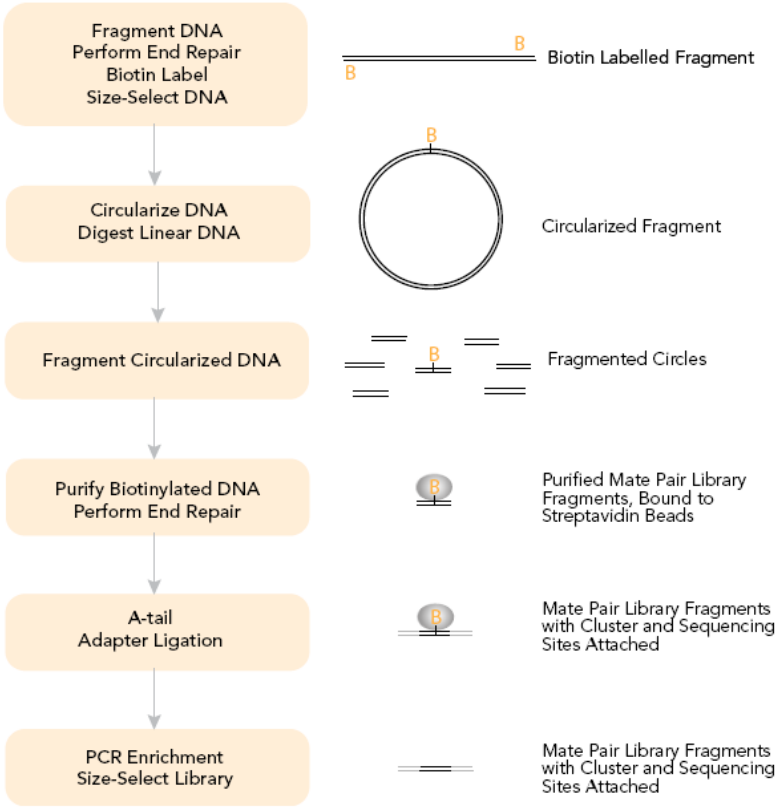


Figure 1 *Mate Pair Library Preparation Overview*

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RNA Applications Sample Prep

- mRNA-Seq
- Small RNA Seq

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mRNA-Seq Sample Prep

- Isolate poly-A containing mRNA
  - Magnetic oligo dT beads
- Randomly fragment RNA
- Random prime mRNA → cDNA
- Make 2nd strand cDNA
- Ligate sequencing adapters
- Size select on gel
- Enrich with 15 cycles of PCR

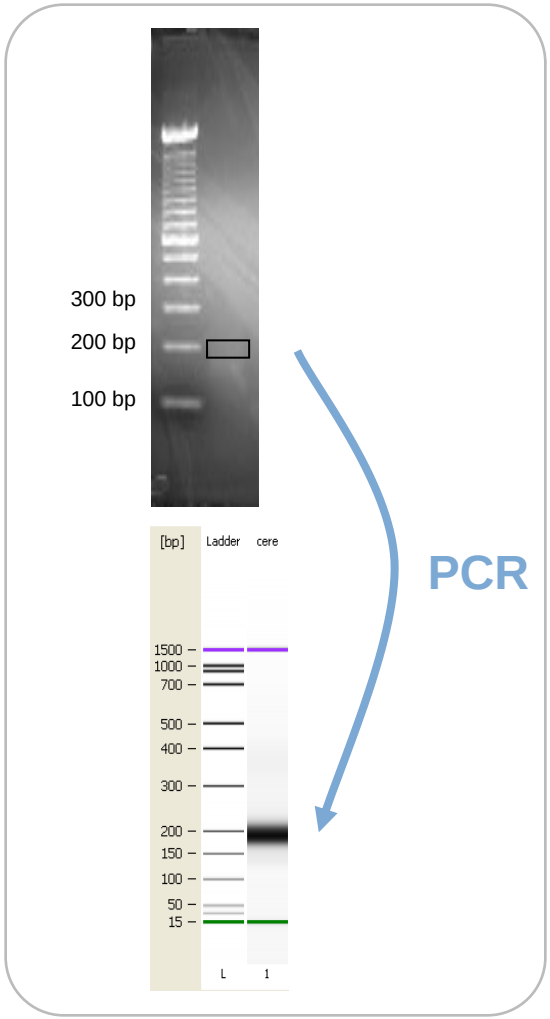

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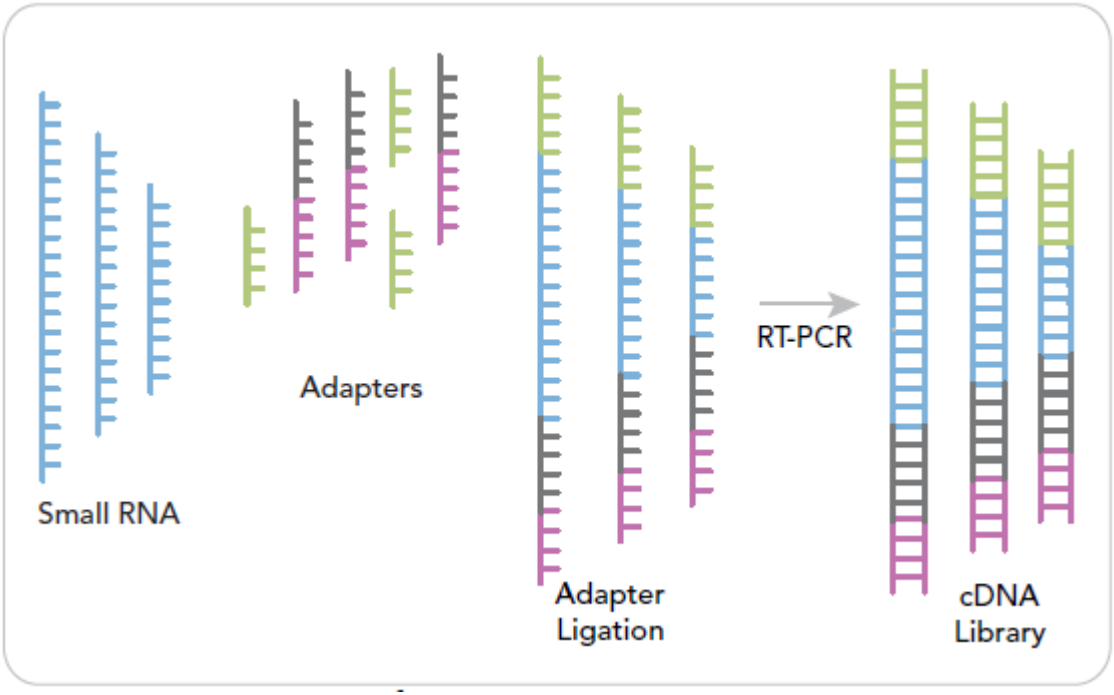
Diagram of Ligation Reaction

- Total RNA
- 3’ adapter ligation
- 5’ adapter ligation
- RT-PCR
- Sequencing



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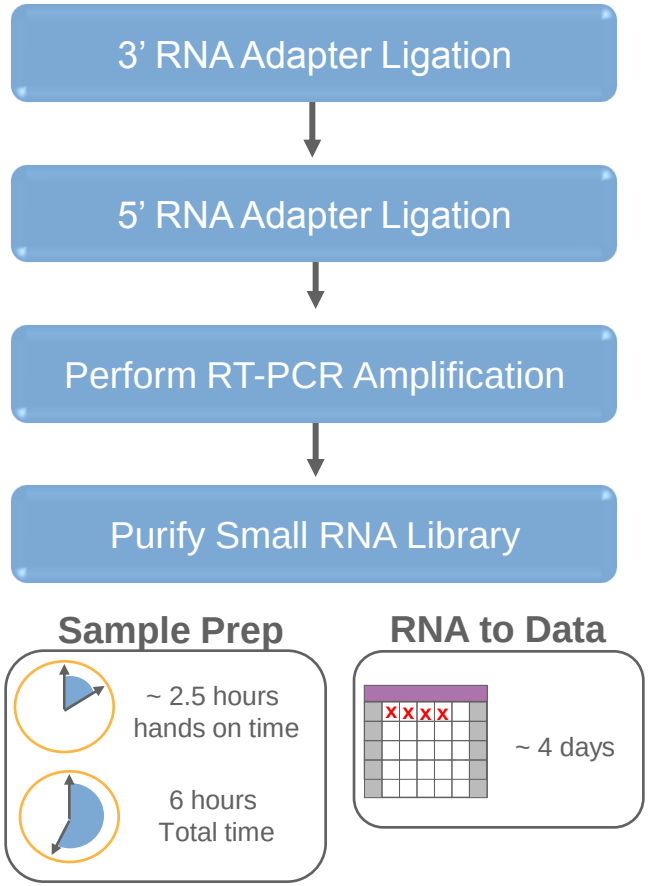
Small RNA V1.5 Sample Prep

- 6 hour library preparation
  - Shortest time from library to sequence
  - 2.5 hrs hands on time (6 hr total)
  - Single gel excision
- NO need for small RNA purification!
  - Starts directly from total RNA
- Reduced RNA input requirements
  - 1.0 µg total RNA input
    - Titrated to 200 ng total
- Strand specific information
  - Discover and profile microRNA
  - Sequence other non-coding RNAs
  - Can be used with stranded mRNA-Seq



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Sample Preparation Best Practices

Sample Preparation

- Be familiar with the protocol before starting it
  - Have all user-supplied reagents and equipment
- Use high-quality starting material
  - QC and quantify material before starting protocol
  - Aim for middle to high end of starting material requirements if possible
  - Very important for RNA applications
- Use proper material handling techniques
  - Prevent contamination and degradation
  - Particularly important for RNA applications

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Sample Preparation Best Practices

Sample Preparation

- Use positive controls if necessary for troubleshooting
- Quantitate and QC final library
  - This is essential for cluster lawn quality and sequencing success

Sample Preparation

Best Practices from The Broad Institute

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Module Objectives – Sample Preparation

- **Overview of Broad's sample preparation workflow**
- **How we track samples through the lab**
- **Broad developed modifications to sample preparation and their application in our protocols:**
  - Shearing methods
  - Enzymatic reaction clean up methods
  - Size selection methods
    - Both agarose gel and gel-free methods
  - PCR enrichment protocols
- **Quality control methodology for sample preparation**
  - Overview of sequencing metrics that can be used to assess sample preparation quality

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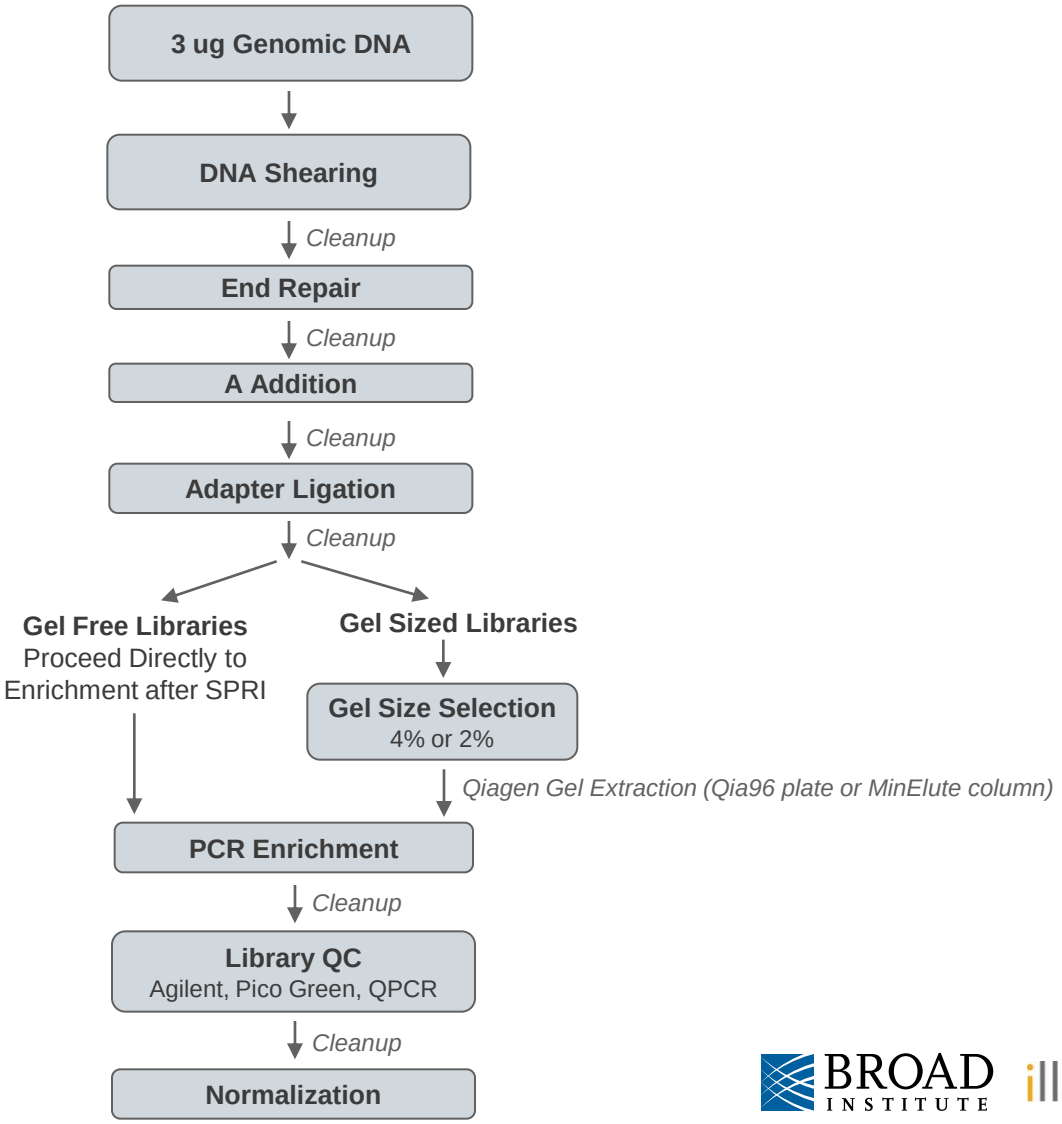
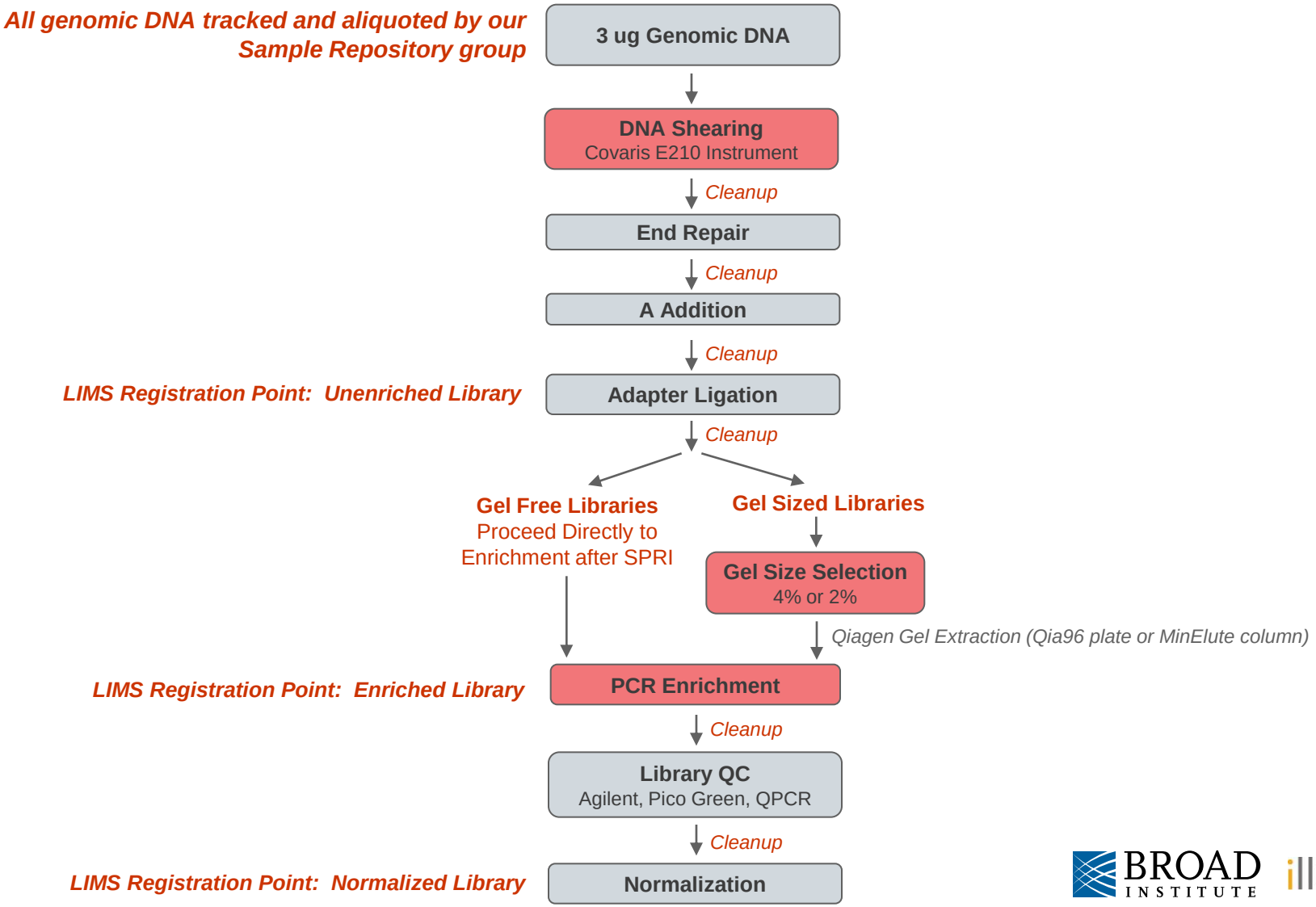
Sample Prep Workflow at the Broad

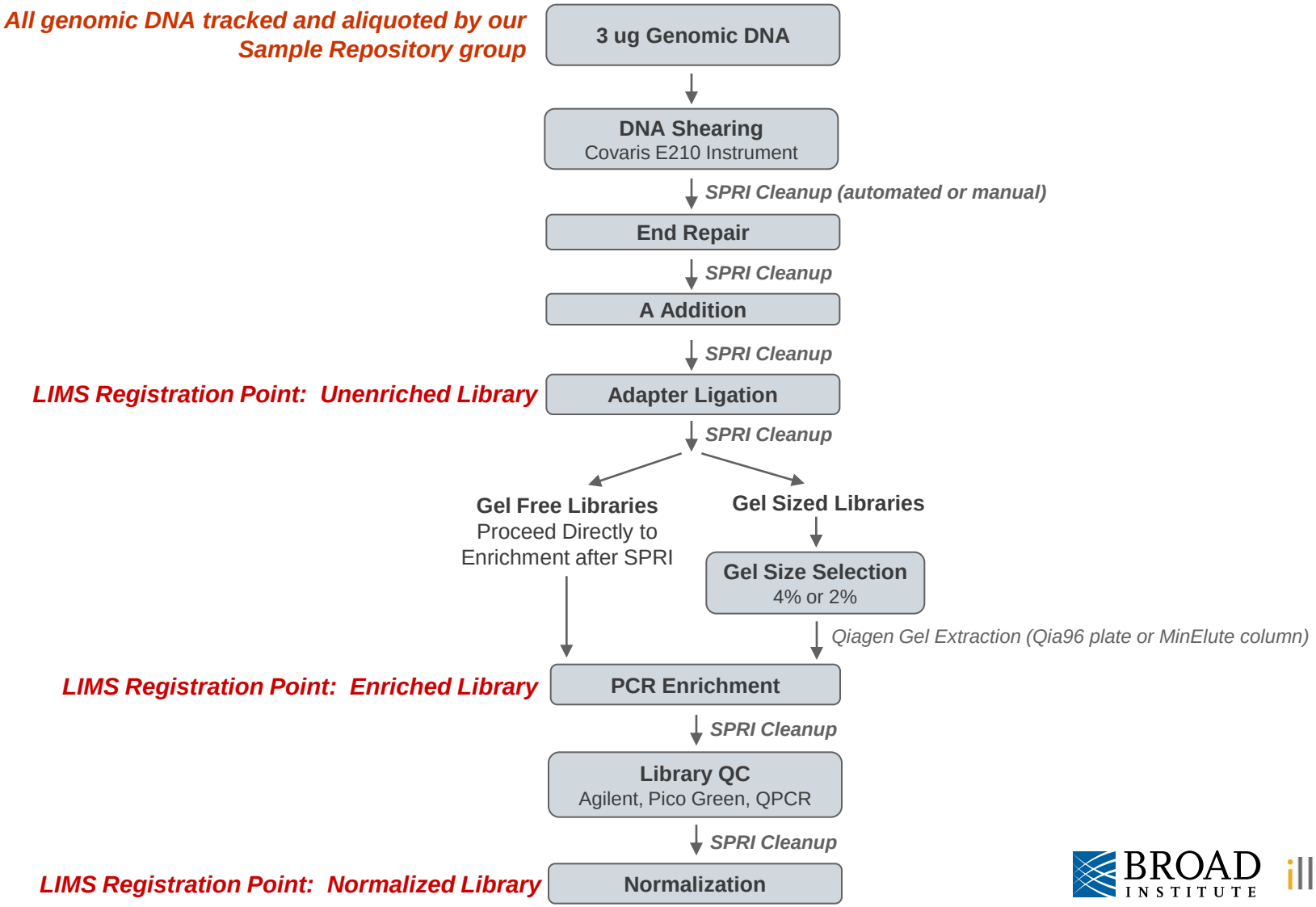


Sample Prep Workflow at the Broad



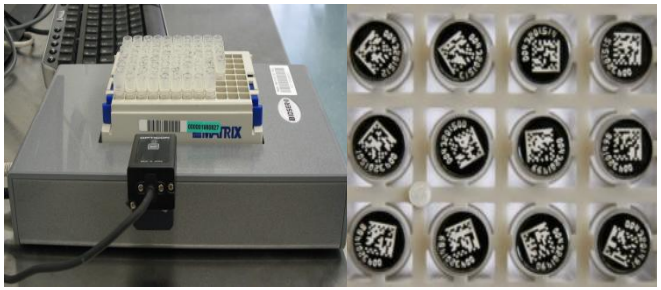
Sample Tracking

Sample Tracking

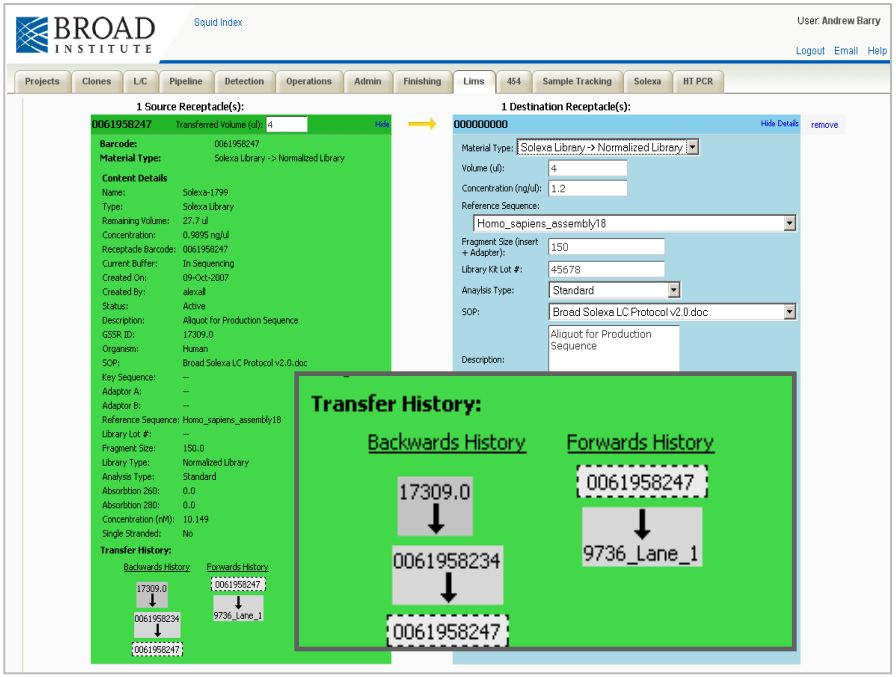


Sample Tracking in a High Throughput World

- DNA stored in 2D barcoded tubes
  - Source Genomic DNA
  - Unenriched libraries
  - Enriched libraries
  - Normalized libraries



- All transfers and details are tracked via web-based LIMS



- Tube picking system error-proofs selecting libraries

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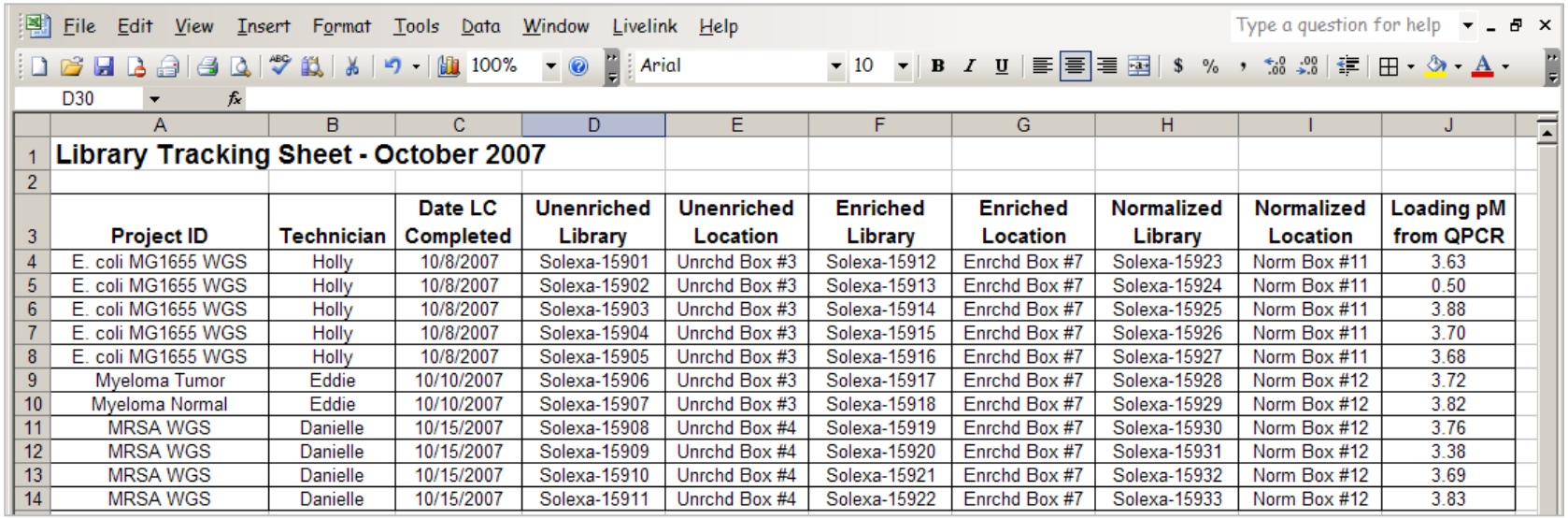
Sample Tracking at Lower Throughputs



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D30

Library Tracking Sheet - October 2007

Project ID	Technician	Date LC Completed	Unenriched Library	Unenriched Location	Enriched Library	Enriched Location	Normalized Library	Normalized Location	Loading pM from QPCR
E. coli MG1655 WGS	Holly	10/8/2007	Solexa-15901	Unrchd Box #3	Solexa-15912	Enrchd Box #7	Solexa-15923	Norm Box #11	3.63
E. coli MG1655 WGS	Holly	10/8/2007	Solexa-15902	Unrchd Box #3	Solexa-15913	Enrchd Box #7	Solexa-15924	Norm Box #11	0.50
E. coli MG1655 WGS	Holly	10/8/2007	Solexa-15903	Unrchd Box #3	Solexa-15914	Enrchd Box #7	Solexa-15925	Norm Box #11	3.88
E. coli MG1655 WGS	Holly	10/8/2007	Solexa-15904	Unrchd Box #3	Solexa-15915	Enrchd Box #7	Solexa-15926	Norm Box #11	3.70
E. coli MG1655 WGS	Holly	10/8/2007	Solexa-15905	Unrchd Box #3	Solexa-15916	Enrchd Box #7	Solexa-15927	Norm Box #11	3.68
Myeloma Tumor	Eddie	10/10/2007	Solexa-15906	Unrchd Box #3	Solexa-15917	Enrchd Box #7	Solexa-15928	Norm Box #12	3.72
Myeloma Normal	Eddie	10/10/2007	Solexa-15907	Unrchd Box #3	Solexa-15918	Enrchd Box #7	Solexa-15929	Norm Box #12	3.82
MRSA WGS	Danielle	10/15/2007	Solexa-15908	Unrchd Box #4	Solexa-15919	Enrchd Box #7	Solexa-15930	Norm Box #12	3.76
MRSA WGS	Danielle	10/15/2007	Solexa-15909	Unrchd Box #4	Solexa-15920	Enrchd Box #7	Solexa-15931	Norm Box #12	3.38
MRSA WGS	Danielle	10/15/2007	Solexa-15910	Unrchd Box #4	Solexa-15921	Enrchd Box #7	Solexa-15932	Norm Box #12	3.69
MRSA WGS	Danielle	10/15/2007	Solexa-15911	Unrchd Box #4	Solexa-15922	Enrchd Box #7	Solexa-15933	Norm Box #12	3.83

- Implementing a library numbering system allows for easy tracking, even without barcodes
- Keeping Excel tracking sheets can associate libraries to each other and to project, assisting in downstream troubleshooting
  - Records who made the libraries, prep date, lot #s, etc.
  - Identifies where in the freezer the tubes are stored

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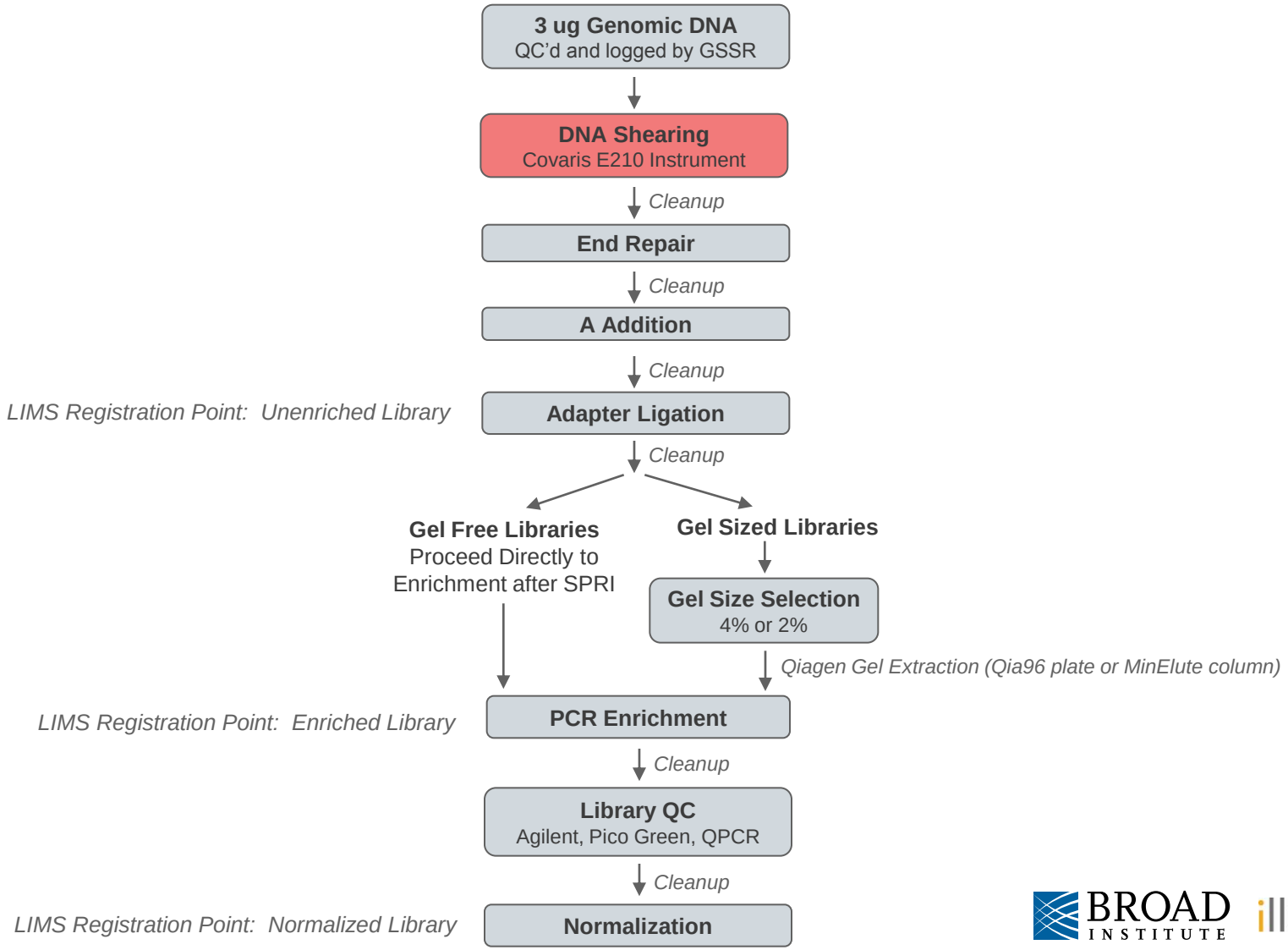
Shearing of Genomic DNA

Shearing of Genomic DNA



DNA Shearing with Covaris

- Founded in 1999, Covaris, Inc. developed a line of instruments capable of providing controlled delivery of acoustic energy to closed vessels.
- Originally, Covaris targeted the chemical industry in order to facilitate mixing, homogenizing, or pulverization of chemical compounds.
- We were the first to work with Covaris to develop methods to apply their “adaptive focused acoustics” technology to the process of shearing DNA for next generation sequencing applications.
- Main reason for moving to Covaris was the throughput:
  - Up to 96 samples can be loaded into the machine at a time in the E210 plate based model.

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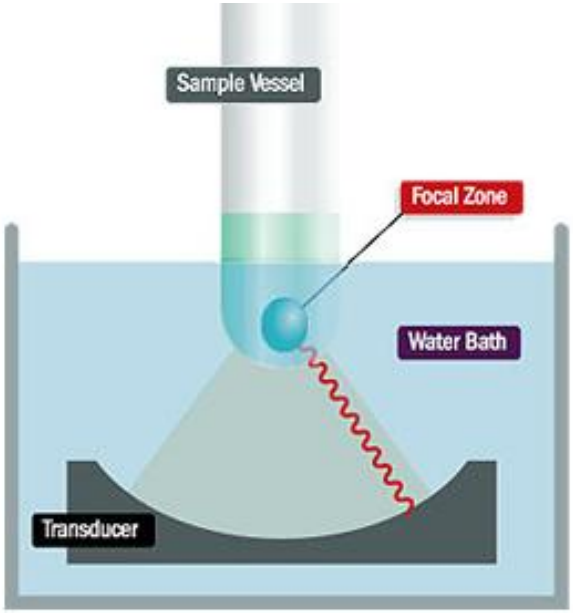
DNA Shearing with Covaris

- How it works:
  - Acoustic ultrasonic waves aimed at glass shearing vessels containing DNA cause “cavitation” within the sample.
  - This extremely rapid creation and high-energy collapsing of tiny bubbles leads to the mechanical shearing of DNA.
  - We will see a demo in the Lab Section.



E210 Plate Based Model

S2 Single Tube Based Model

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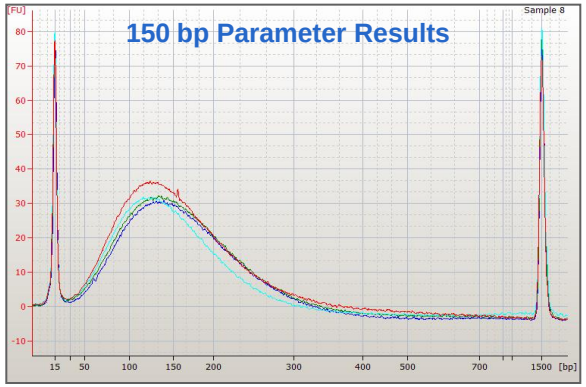
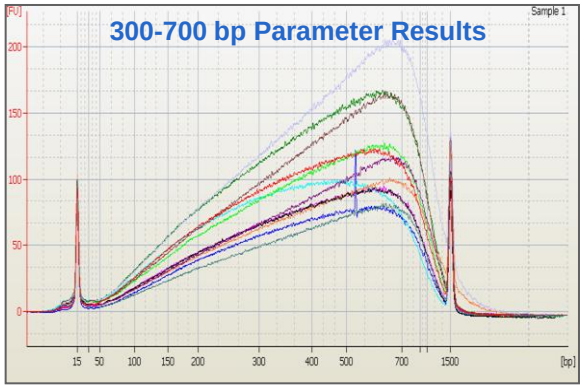
DNA Shearing with Covaris

- We have performed a multitude of experiments to understand how the parameters can be manipulated to produce DNA in various sizes:

Desired Fragment Size	Volume	Time (T)	Duty Cycle	Intensity	Cycles Per Burst (CPB)	Z Axis (mm)
150bp	100	300 sec	20%	5	200	0mm
200bp	100	90 sec	20%	5	200	0mm
200bp	50	360 sec	5%	5	200	0mm
300-700bp	100	30 sec	20%	5	200	0mm
300-700bp	50	300 sec	1%	5	200	0mm





- *You will see more examples of these data in the lab section.*

Choosing a DNA Shearing Method

	DNA in Desired Range	Reproducible	Customizable Settings	No Additional Materials Cost	No Additional Instrument Required	Multiple Samples at a Time	Compatible with automation
Nebulizer	✓	✓	*Limited - can vary time and pressure*	✓	✓		
Covaris S2 Single Tube	✓	✓	✓				
Covaris E210 Plate Based	✓	✓	✓			✓	✓

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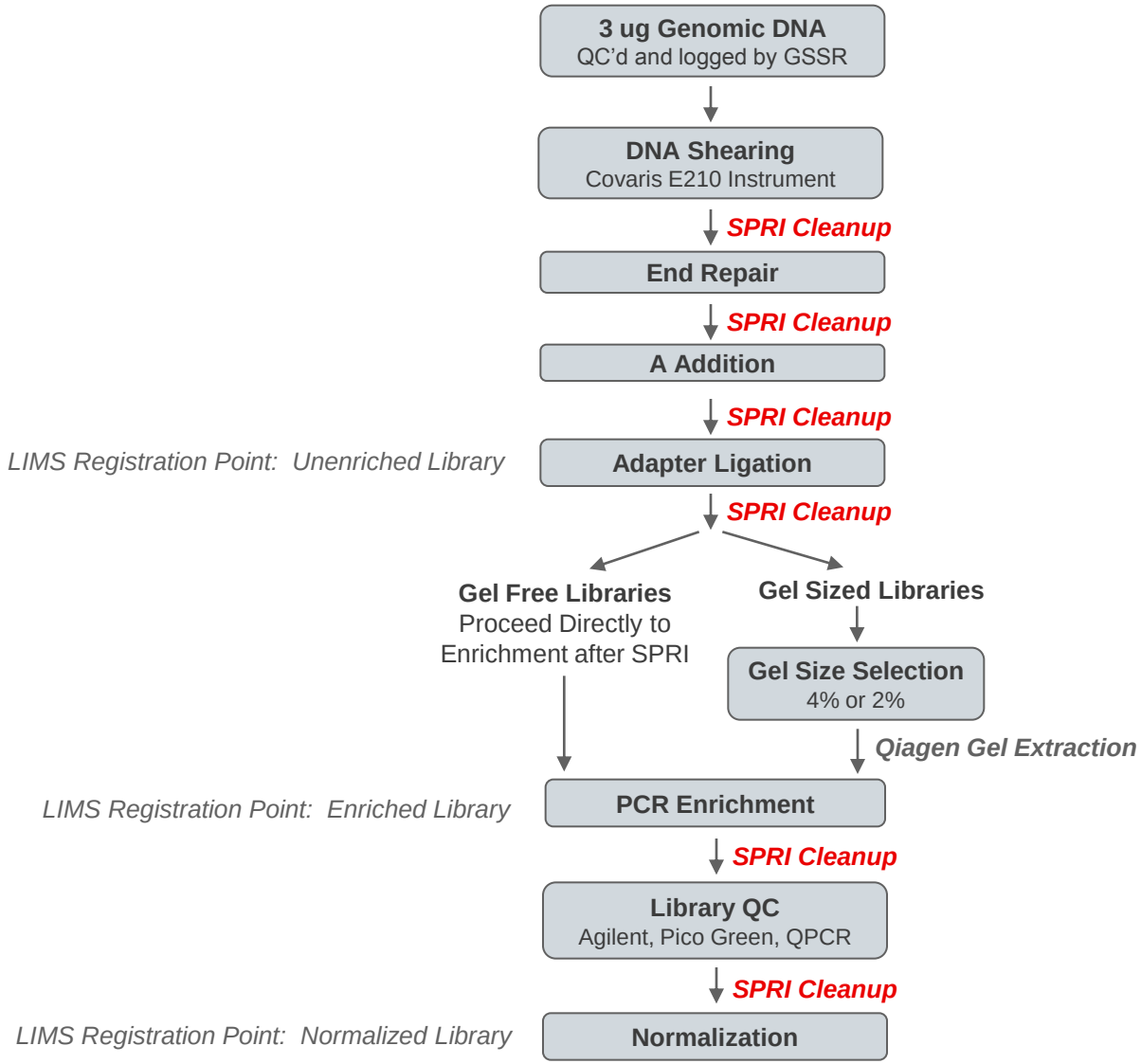
Reaction Cleanups

Reaction Cleanups



Reaction Cleanup Methods

- Column based cleanups with Qiagen MinElute columns
  - Reliable and reproducible, ~80% recovery
  - Easy to perform
  - Manual - ideal for lower throughput
    - Not compatible with automation: reasonable set size <12
    - Tubes more prone to sample swapping than samples in a plate
- Magnetic bead based cleanup methods
  - Reliable and reproducible, 80-90% recovery
  - Easy to perform
  - Can be done manual or automated
    - Manual: small sets of 8-12 can be reasonably done by hand in tubes
    - Automated: up to 96 can be done at a time using a liquid handler
- **Broad has moved away from column cleanups to magnetic bead based cleanups to accommodate higher throughput**

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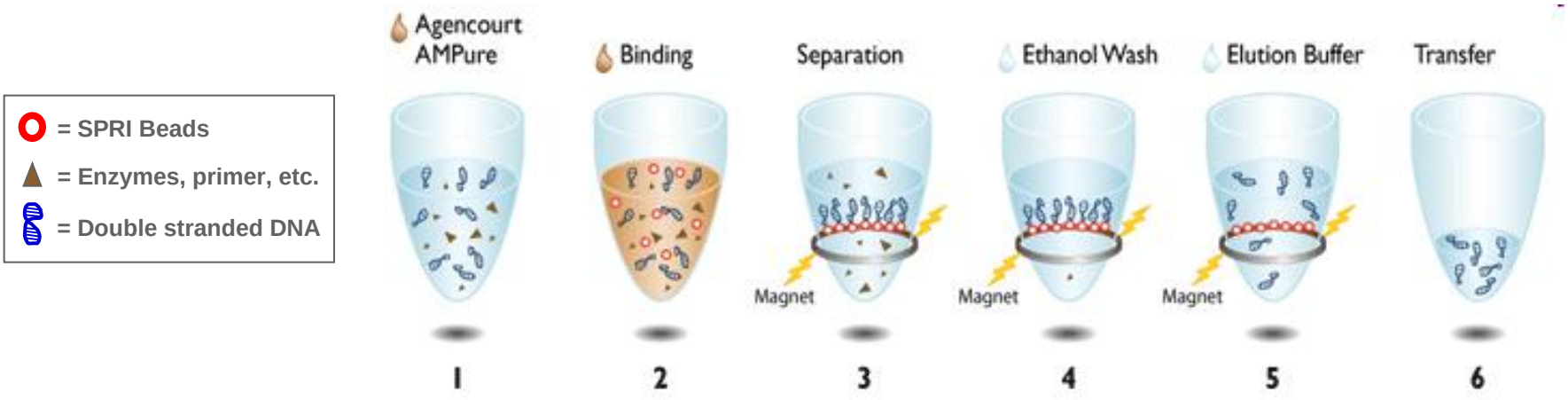
SPRI: An Alternative to Column Cleanups

- SPRI: **S**olid **P**hase **R**eversible **I**mmobilization (*AMPure by Agencourt*)
  - SPRI technology is a magnetic bead based DNA clean up method
  - Beads are specific for double stranded DNA
  - Enzymes, excess primer, etc. are washed away & DNA is then eluted
  - Reference: Hawkins TL et al. Nucleic Acids Res. 1994 Oct 25;22(21):4543-4.
- How the SPRI chemistry works:



Courtesy of Agencourt

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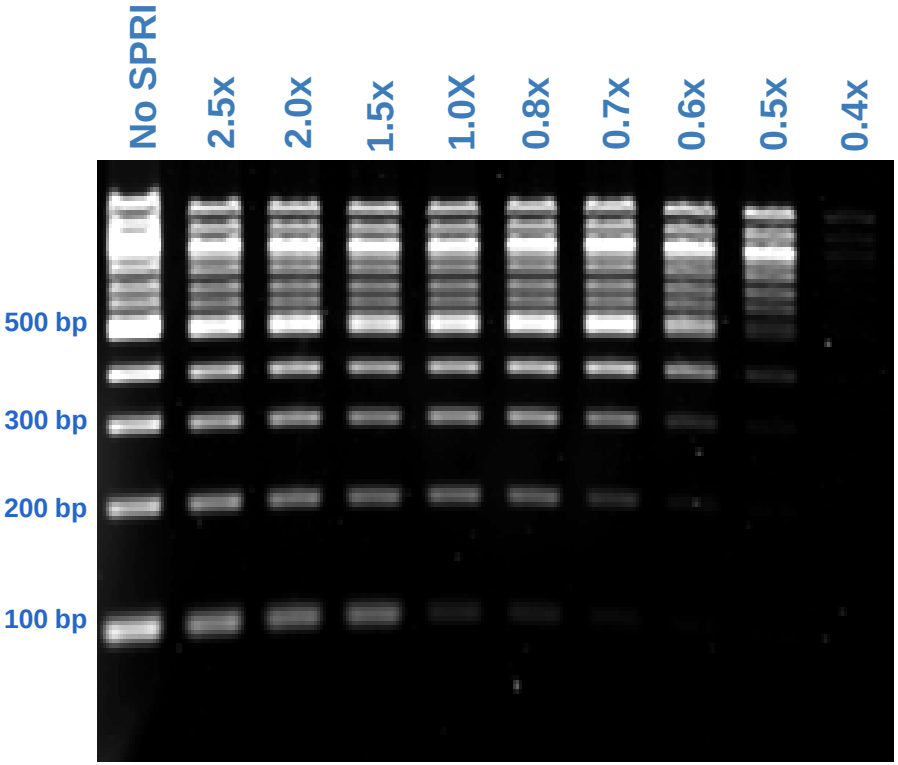
SPRI: An Alternative to Column Cleanups

- The length of fragments that SPRI binds can be controlled by varying the amount that is added to the reaction
- The “X” of SPRI refers to the volume to volume ratio of SPRI to DNA
  - 1X SPRI is a 1:1 vol of SPRI:DNA
- The **lower** the X of SPRI, the **higher** the size cut off becomes
  - See titration at right
  - 0.7X SPRI removes fragments below 150 bp such as adapter dimer
- As we will see in the size selection section, this property can be used to perform gel free library sizing



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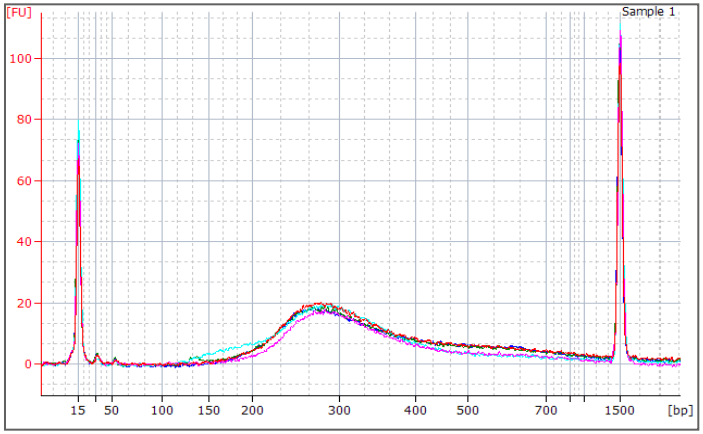
Application of SPRI - High Throughput Libraries

- To fill our sequencing capacity, we needed to be able to do the following:
  - Make 96 libraries or more per week
  - Start with 3 ug of DNA
  - Average final fragment size ~300 bp but tight sizing NOT required

- Implementing Covaris shearing and SPRI cleanups made this possible:
  - Covaris shear of 96 samples
  - Automated SPRI cleanups on the Velocity 11 Bravo liquid handler
  - No size selection method
    - 150 bp Covaris shearing
    - 0.7X SPRI to remove dimer after adapter ligation

Completed Library Agilent QC



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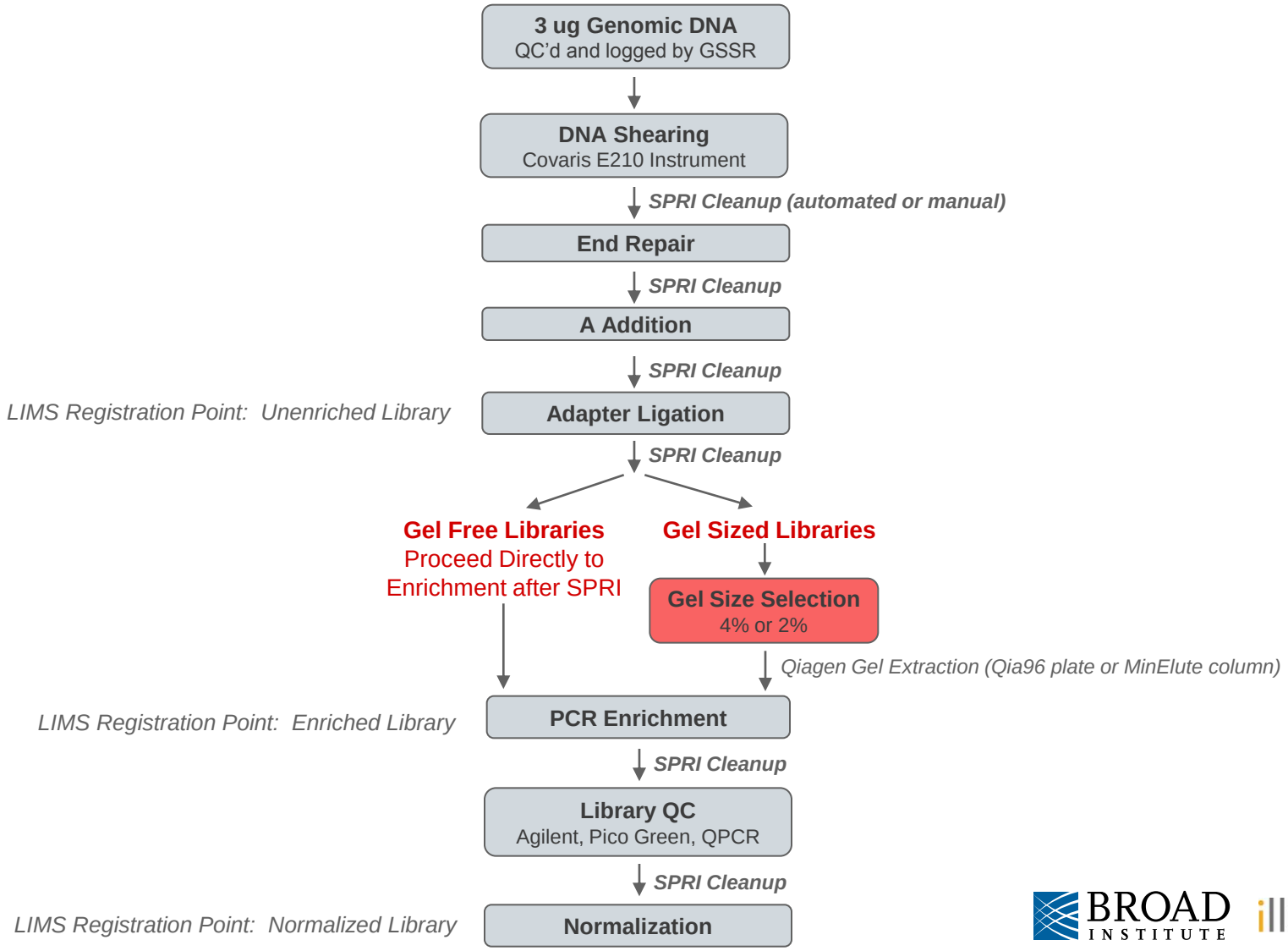
Size Selection Methods

Size Selection



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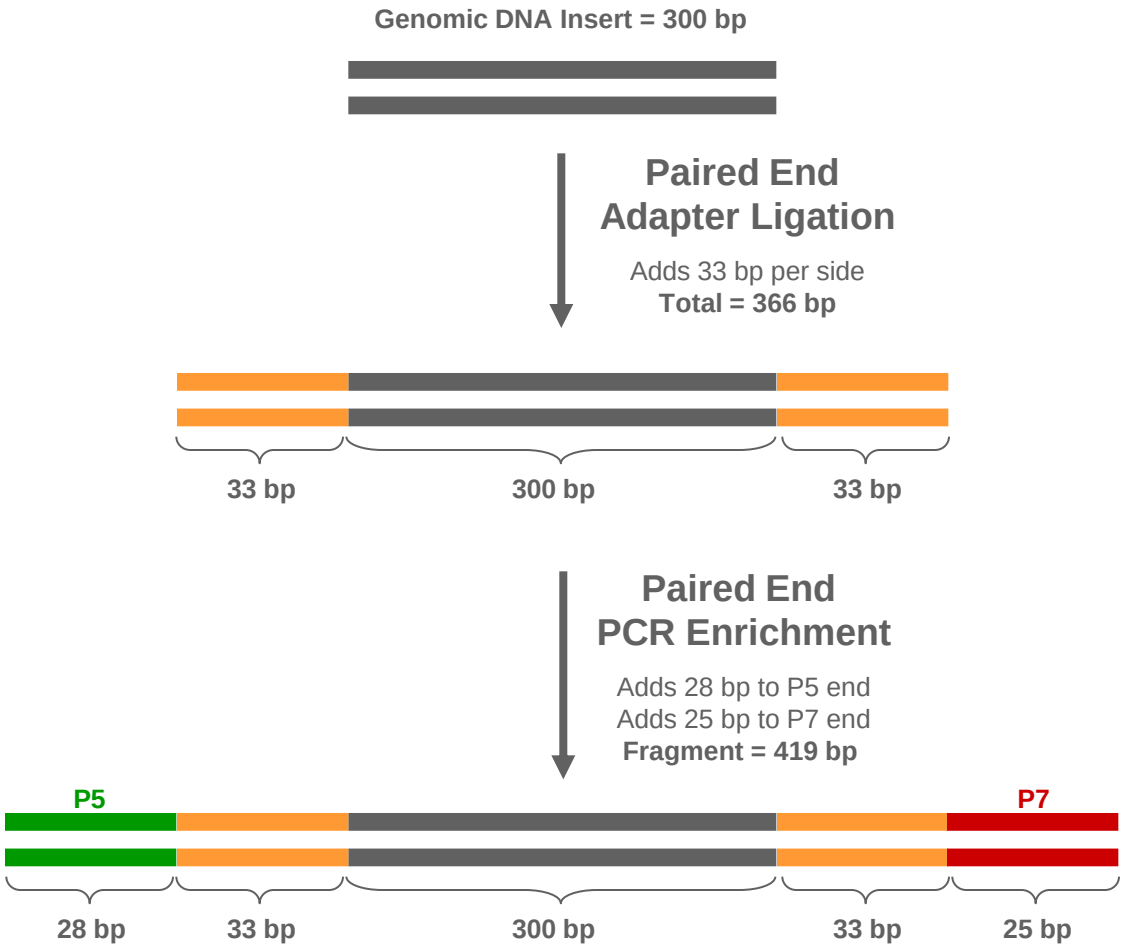
Insert Size vs. Library Fragment Size



Choosing a Library Fragment Size

- A library fragment size of ~400 bp is ideal for optimal cluster density as well as for sequencer optics and cluster-finding algorithms
- However, the sequencer can tolerate fragment sizes up to 800 bp
  - Fragment size can be tailored to suit the needs of a given project
- Desired read length determines the minimum insert size
  - For paired end, the targeted insert size should usually be at least twice the read length to avoid reading into the adapter or sequencing the same stretch
    - For 36 base paired sequencing: at least 72 bases
    - For 76 base paired sequencing: at least 152 bases
- **Larger fragments → Larger clusters → Lower max density**
- **Wider range → Wider variety of cluster sizes → Lower max density**

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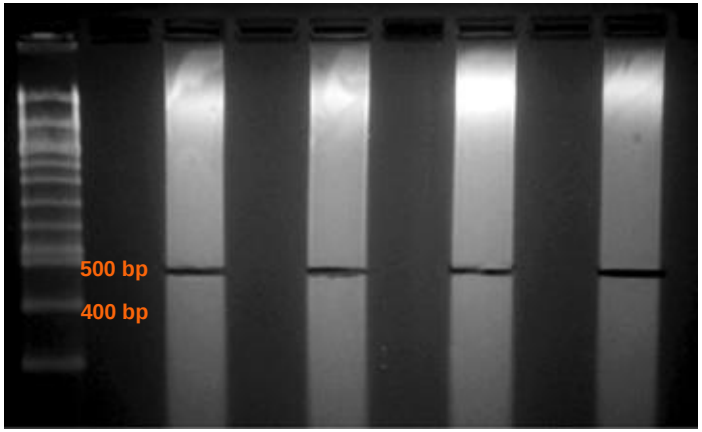
Choosing a Size Selection Method

- Option 1: Agarose gels
- Option 2: Double-sided SPRI
- Option 3: No size selection

Option 1: Agarose Gels

- Gel size selection is necessary to achieve size deviations of < 30%.
- Broad's recommendations for gel conditions:
  - **For fragments <400 bp: 4% NuSieve 3:1 TAE gel, 85 volts, 3 hours, room temp**
  - **For fragments >400 bp: 2% SeaPlaque TAE gel, 110 volts, 3 hours, 4°C**
- By taking ~2 mm gel slices at desired size range, a size distribution of +/- 10% or less can be achieved:

We will see a lab demo of a gel cutting tool we have developed to make these tight cuts easier.



Example of a tight cut library targeting fragment size of 490 bp.

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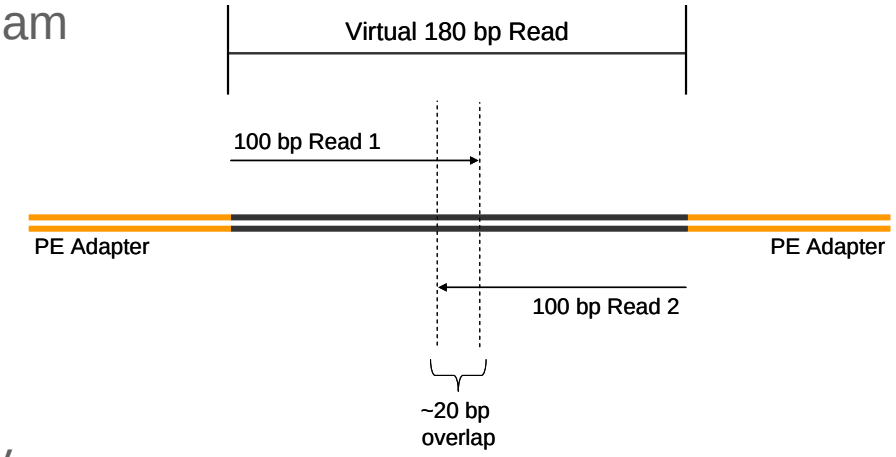
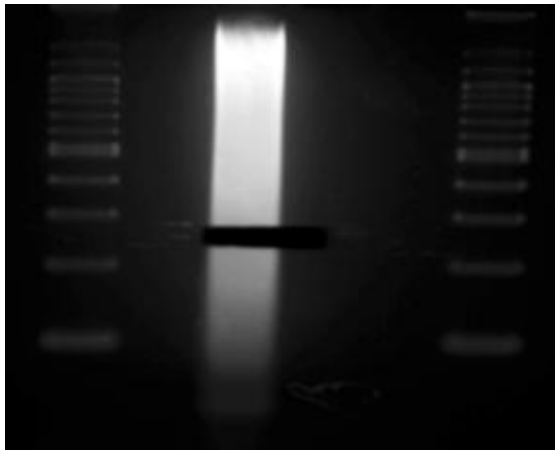
Application of Tight Cuts - 180 bp Libraries

- We were requested by our Assembly team to make libraries with inserts that were EXACTLY 180 bp with less than a 10% deviation
  - 2x101 run = virtual 180 bp read
  - 180 bp virtual read more robust to assemble than 100 bp alone
- To accomplish this, we take 3 extremely tight gel slices at 246 bp after adapter ligation (180 bp insert + 66 bp adapters)
  - Use a gel cutting tool that holds 4 razor blades - Will see a demo in lab section
  - Perform “high diversity” PCR protocol
  - QC size & distribution by Agilent chip
    - After PCR, should be 299 bp
- Select library closest to 299 bp with smallest standard deviation for sequencing





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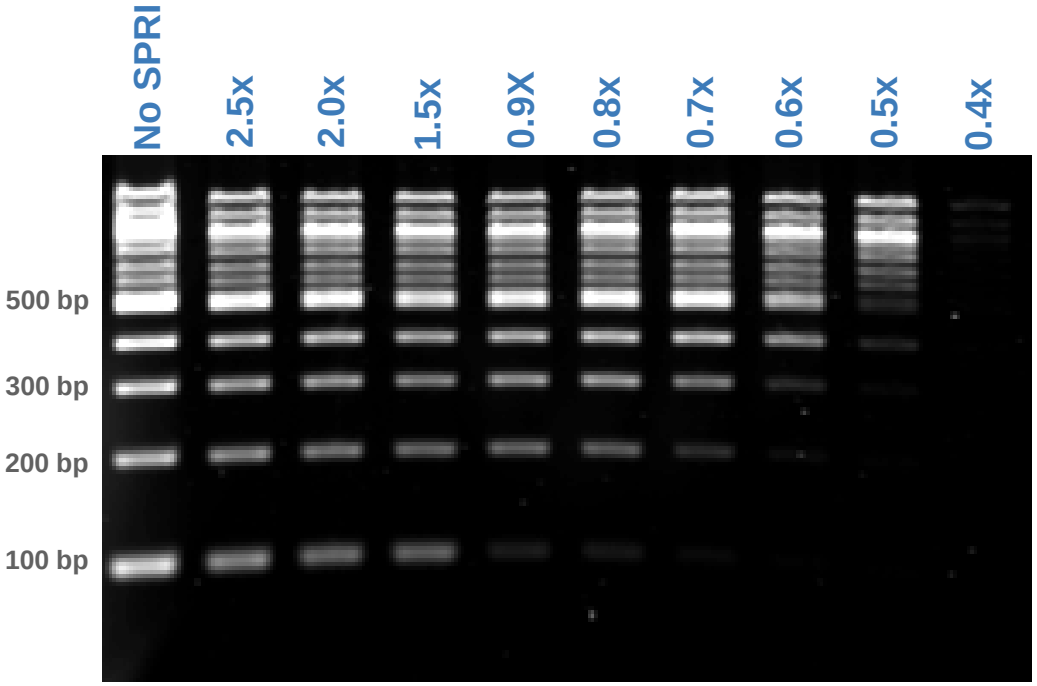
Option 2: Double-Sided SPRI

- As discussed previously, the size of fragments that SPRI binds can be varied by varying the “X” fo SPRI that is added to the reaction containing the DNA:



- We can utilize this property to perform a gel-free and automated size selection that we have termed “Double-sided SPRI”

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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

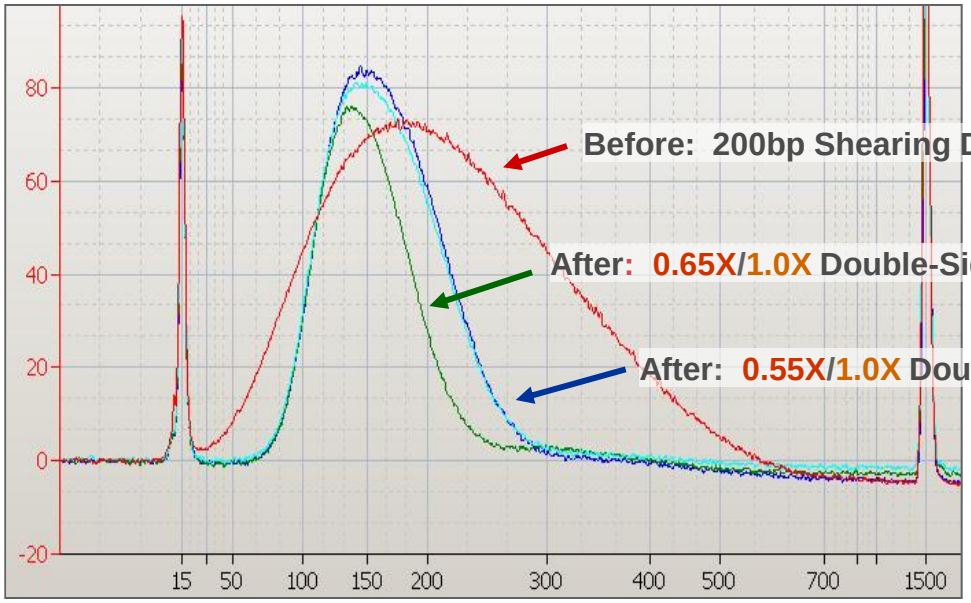
Fragments >100 bp on beads
Fragments <100 bp in supernatant

KEEP BEADS
100-250 bp

→

DISCARD SUPERNATANT

Results:



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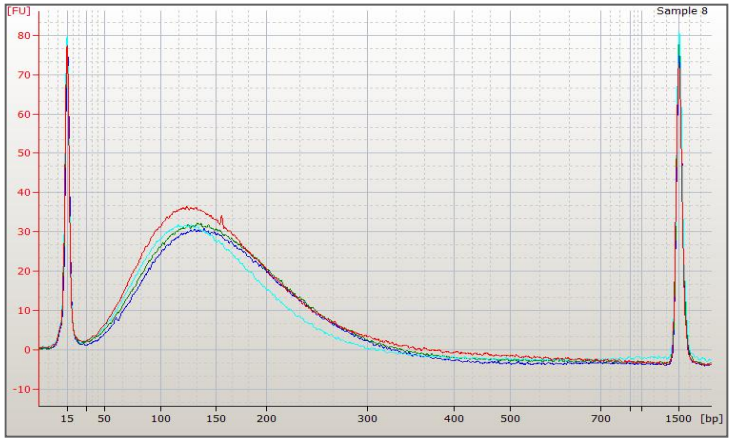
Option 3: No Size Selection At All

- If tight sizing not required for a project, size selection may be unnecessary.
- Combining a tight 150 bp shear with a 0.7X SPRI clean-up after adapter ligation to remove excess adapter dimer may be suitable.

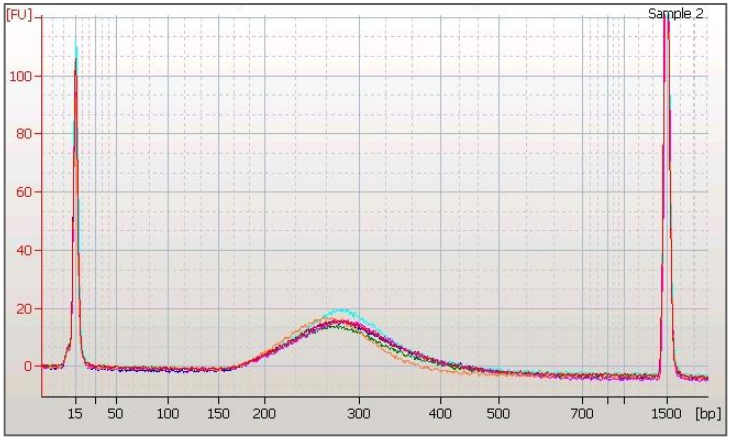
POST SHEARING (150 bp protocol)



End Repair
A Addition
Adapter Lig
NO GEL
O.7X SPRI

PCR Enrichment
1 ul DNA
10 cycles

POST PCR ENRICHMENT & 0.7X SPRI



- This works because of the tightness of our 150 bp shear. If shearing isn't optimized or if a larger size is needed, than double-sided SPRI is required.

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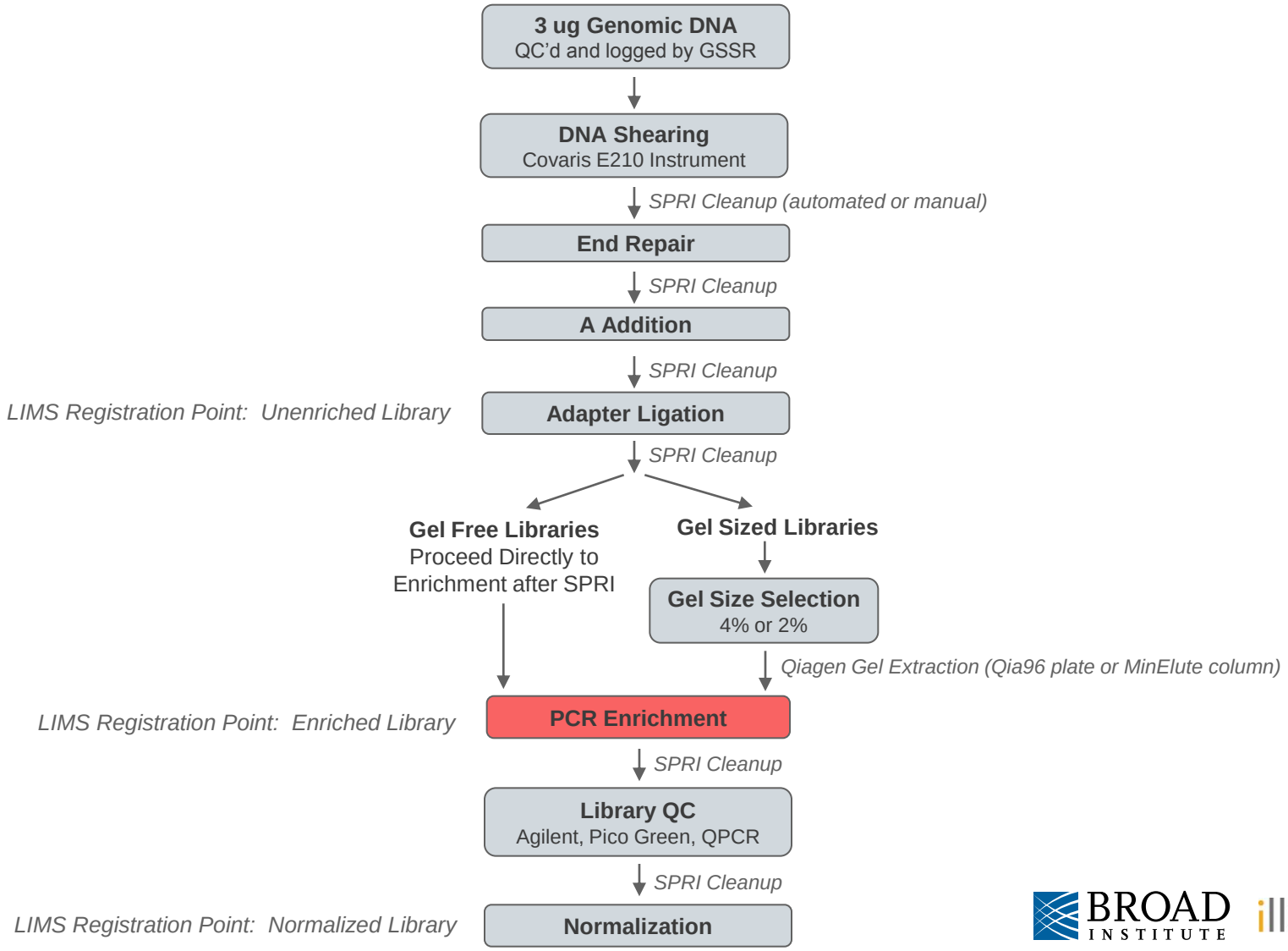
PCR Enrichment

PCR Enrichment



Library Complexity

- Library complexity refers to the number of unique fragments present in a given library
- Complexity is affected by:
  - Amount of starting material
  - Amount of DNA lost during cleanups and size selection
  - Amount of duplication introduced via PCR
- For most libraries that only need to be run across a few lanes, the standard protocol provides libraries with ample complexity
- However, certain projects require very deep coverage from a single sample - i.e. SNP discovery, mammalian assembly, cancer resequencing
- When dozens of lanes are required, library complexity becomes very important

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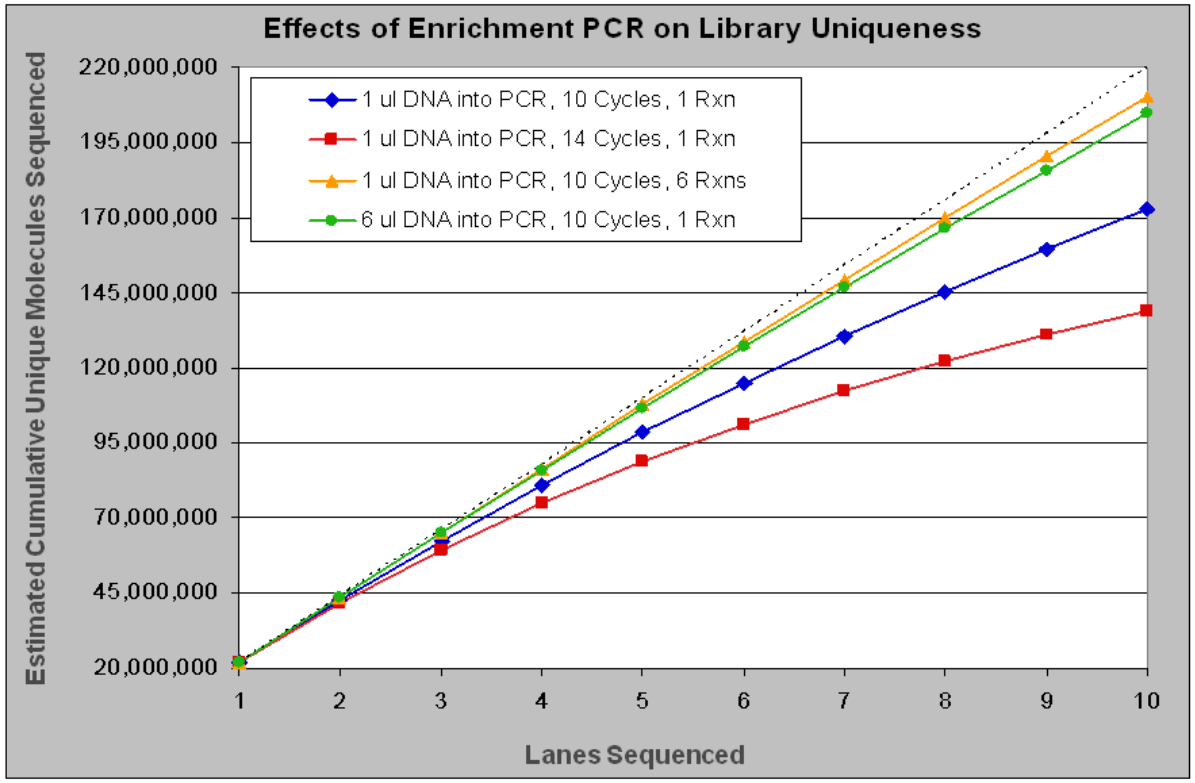
Relationship Between PCR and Complexity

Experiment:

- Varied input volume, PCR cycle number, & number of reactions pooled
- Sequenced to determine % duplication & estimated library size
- Based on data, number of new molecules seen after each successive lane of sequencing was estimated

Results:

- Decreasing PCR cycles decreases % duplication
- Increasing DNA input and pooling multiple PCR reactions decreases % duplication



Starting DNA	Cleanup Method	DNA Input to PCR (ul)	PCR Cycle #	# of PCR Rxn Pooled	% Duplication	Estimated Library Size
3 ug	MinElute	1	10	1	2.79%	247,441,814
3 ug	MinElute	1	14	1	5.51%	195,858,361
3 ug	MinElute	6	10	1	0.83%	1,255,845,288
3 ug	MinElute	1	10	6	0.48%	1,390,926,598

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Application - “High Diversity” Sample Preparation

- Cancer resequencing projects required 30X unique coverage from tumors and their matched normals
- We needed a way to reach this goal while minimizing both the amount of genomic DNA used and the amount of sequencing lanes needed
  - Highest complexity, lowest % duplication
  - Maintain a very tight size distribution of about 500 bp +/- 10%
- To accomplish this, we developed a high diversity protocol:
  - Take **two** extremely tight gel cuts from each sample: 500bp & 520bp
    - Creates two libraries for each 3 ug input
    - Double the amount of fragments from the same starting input
  - Enrich **ENTIRE** sample from both cuts to maximize diversity
    - 16 x 50 ul PCR reactions with 10 cycles enrichment
    - Pool 16 reactions for each cut & clean up in same column or SPRI

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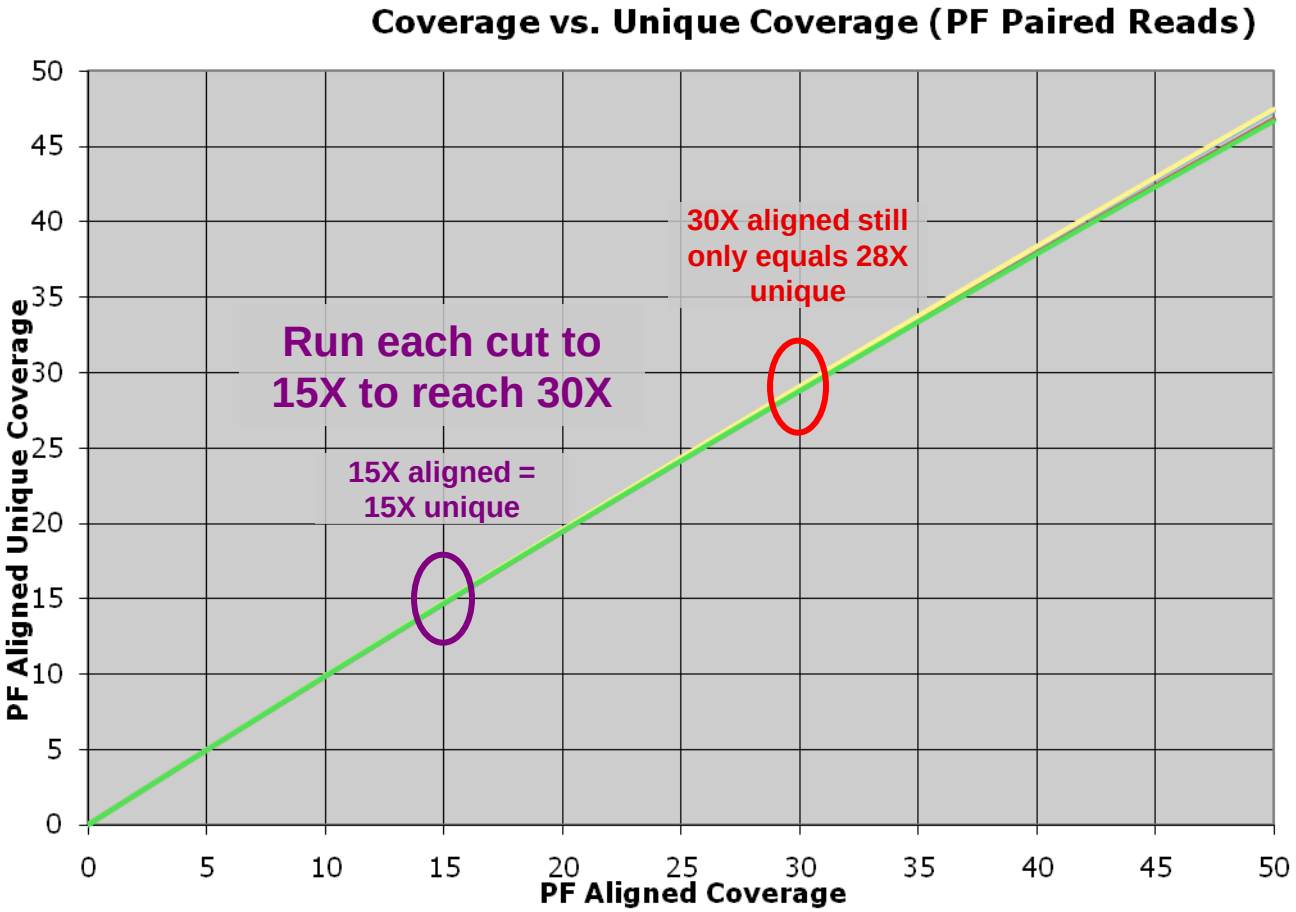
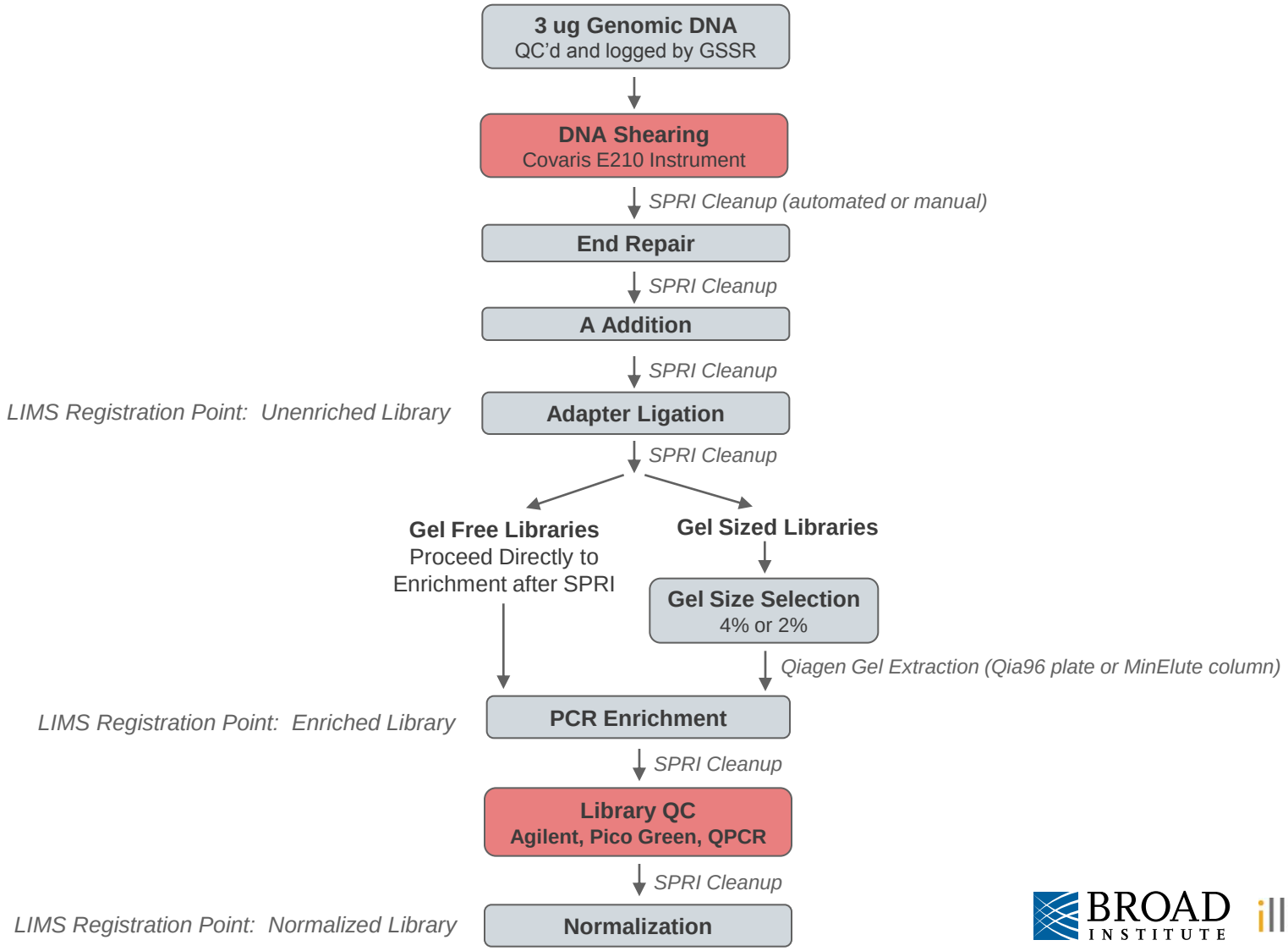
Application - “High Diversity” Sample Preparation



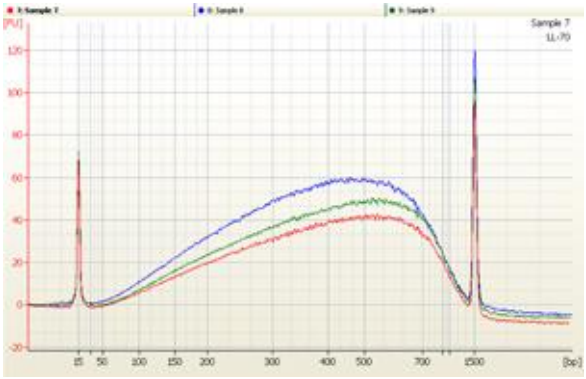
Library Quality Control

Library Quality Control



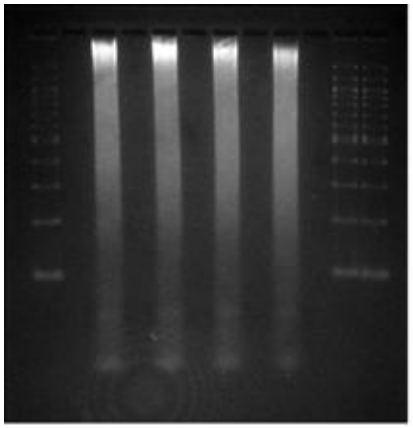
Library Quality Control

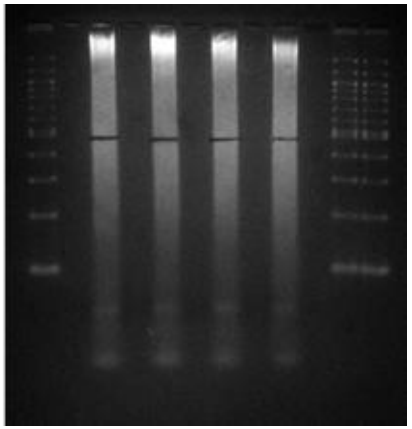
- **Agilent BioAnalyzer 2100**
  - Ensure proper size distribution after both shearing & PCR
  - Failing samples enter rework loop: re-sheared or re-enriched



- **Pico Quantification Post-Enrichment**
  - Samples <20nM are re-enriched with more input and/or cycles

- **Gel Images**
  - Look for excessive adapter, DNA in unexpected size range, etc.
  - Pictures taken before and after cutting are saved for downstream troubleshooting





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Library Quality Sequencing Metrics

- **Always check the sequencing metrics for a run before looking at the LC metrics**

Metric	Definition	How to Interpret…
% Aligned	% of passing filter (PF) reads that aligned to reference sequence.	Low % aligned may indicate sample contamination or swap.
% Adapter	% of PF reads whose first 16 bases match any part of the Illumina adapter sequence.	>1% adapter indicates inefficient removal of adapter dimer in size selection.
% Chimerism	% PF of reads that have 2 ends over 100kb apart or on 2 chromosomes.	>1% chimerism indicative of problem in adapter ligation or with genomic DNA prep itself.
% Duplication	% of PF aligned reads originating from duplicate fragments (i.e. multiple reads with exact same R1 and R2 start sites).	High % duplication indicates a low complexity library, possibly due to low amount of starting material and/or excessive PCR cycles.
Estimated Library Size	Estimated number of unique molecules in library, calculated using % duplication and reference genome.	Library size depends on starting material. Human WGS libraries should have a size of 1-3 billion.

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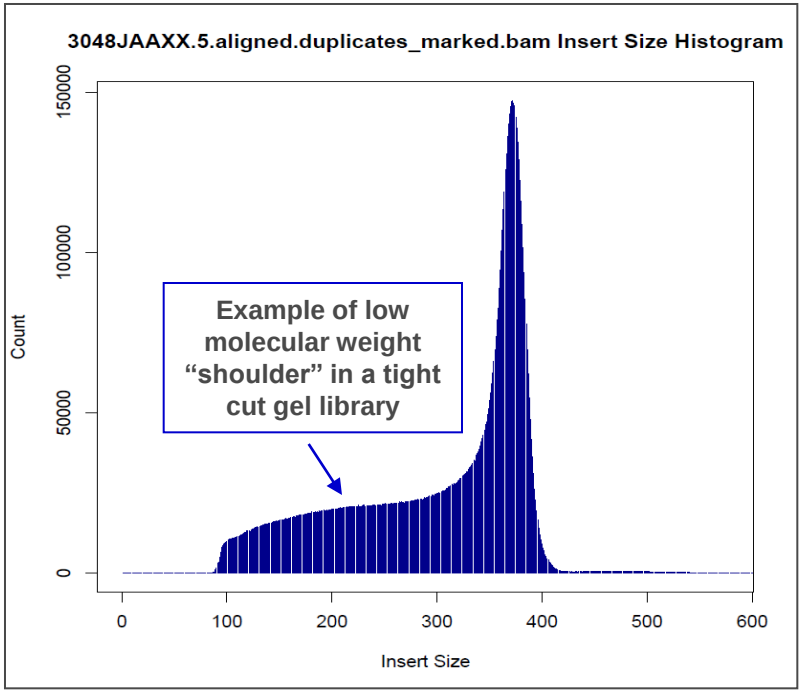
Library Quality Sequencing Metrics

Metric	Definition	How to Use Interpret…
Concordance	Concordance of sequencing results with previous genotyping/SNP data.	Can detect sample swap or contamination events.
Insert Size Plots	Show the size distribution of sequenced fragments.	“Shoulders” or spurious peaks indicate problem in size selection.



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Module Summary - Sample Preparation

- Sample preparation is the most customizable area of the Illumina process - Can adapt the basic protocol to meet the goals of any project
- Sample tracking is important for any size laboratory to ensure sample integrity
  - High throughput: barcoded libraries, plates, and LIMS tracking
  - Low throughput: Excel sheets or databases to record project details & QC information
- Broad has developed many modifications to the standard protocol to increase throughput and reproducibility, as well as to meet specific project needs:
  - Optimized shearing with Covaris
  - Implementation of AMPure SPRI for clean-ups and gel-free size selection
  - Optimized gel cutting conditions for libraries with <10% size deviation
  - Development of high diversity protocols for deep coverage libraries
- Library quality control monitoring and sample tracking are essential to ensure that only high quality libraries make it to the sequencer

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What You Will See in the Lab Sections:

- In lab sample tracking methods and tools
- Covaris E210 demo
- Agarose gel cutting tips & tricks
- Library QC methods and review of example data
- SPRI clean-up hands-on demo & automation overview

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