Illumina Sample Prep – Best Practices and Troubleshooting

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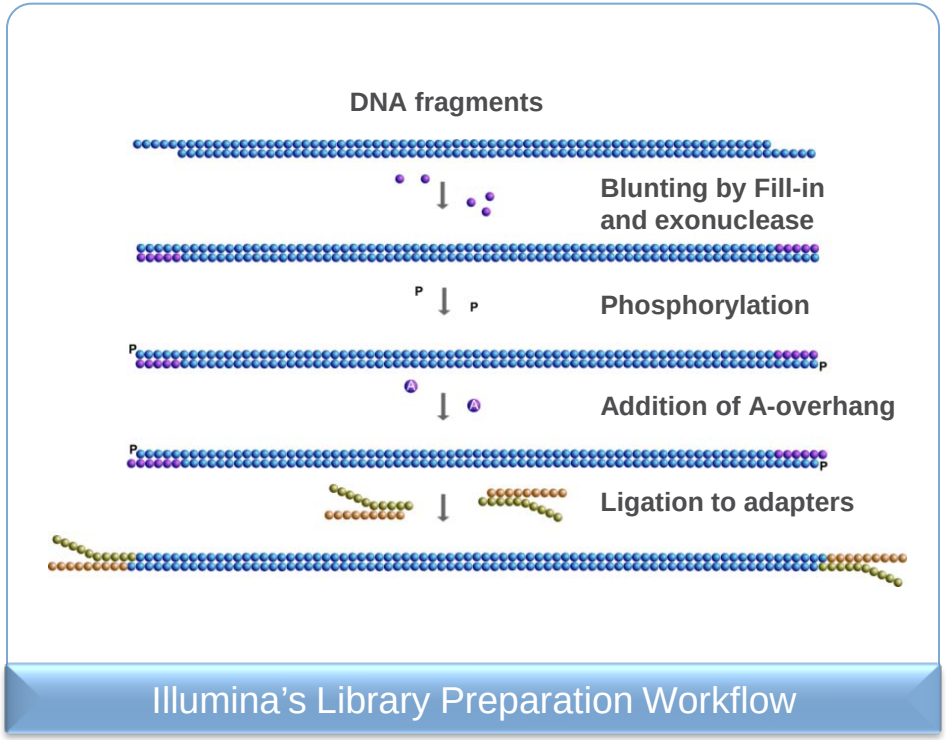
Outline

- TruSEQ Best practices
  - Starting material
  - DNA fragmentation
  - Reagent Handling
  - Agarose gel size selection
- Bioanalyzer Trace as a Troubleshooting tool
  - Commonly seen problems
- TruSEQ Small RNA Troubleshooting
- Nextera

Library Preparation

- Prepares sample nucleic acid for sequencing
  - Fragmenting
  - Generates double-stranded DNA (if necessary)
  - Flanks with Illumina adapters
- All preparation ends with the same product
  - Double-stranded DNA with insert to be sequenced flanked by adapters
- Same protocols all both platforms

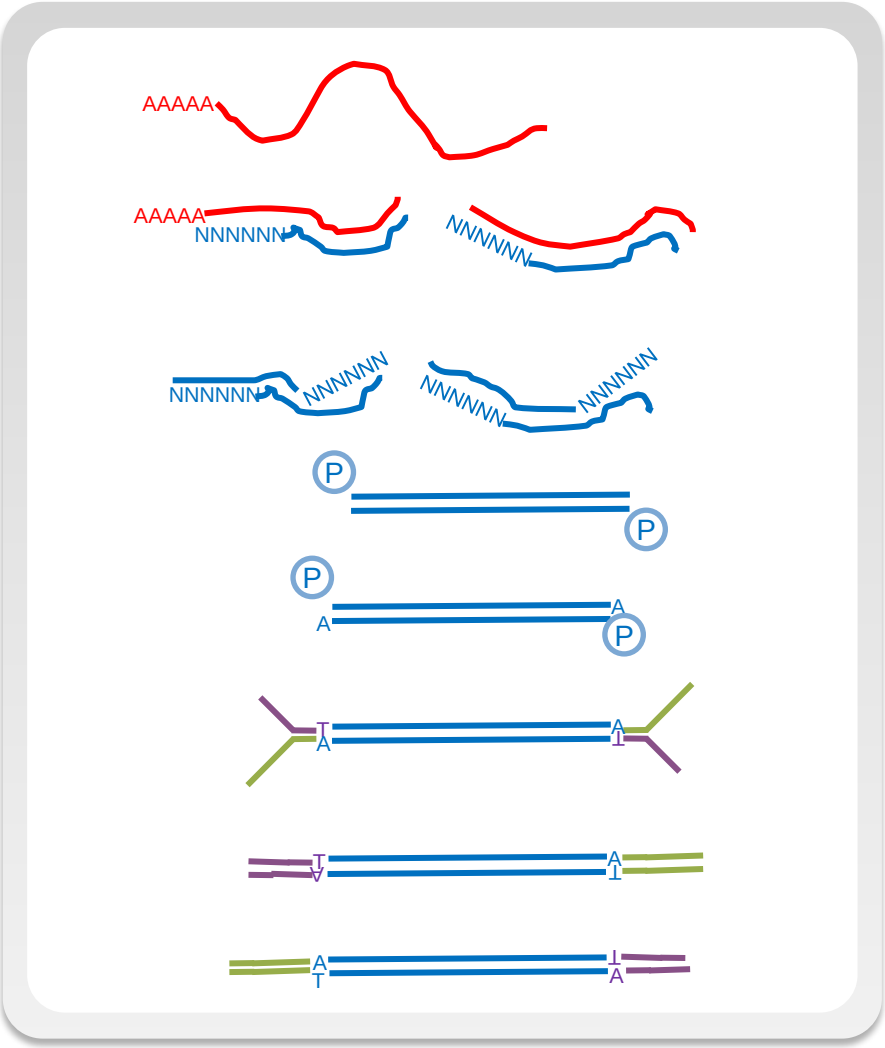


Illumina's Library Preparation Workflow

Overview of TruSeq RNA Workflow

Master-mixed reagents, optimized adapters, and a flexible workflow for simple method to prepare RNA for NGS

- Isolate mRNA from Total RNA with poly-A selection
- Randomly Fragment RNA
- Random Prime mRNA → cDNA
- Make 2nd Strand cDNA
- End repair, phosphorylate, A-tailing
- Ligate sequencing adapters (w/ indexes)
- Gel free size selection
- Enrich with 15 cycles of PCR
- Grow Clusters and Sequence



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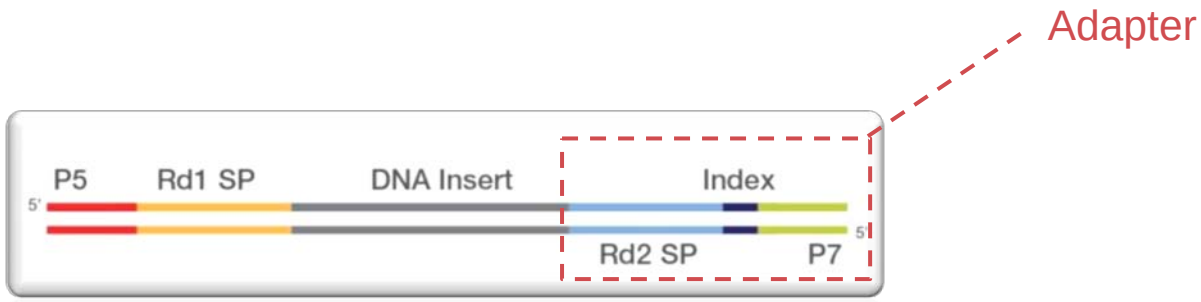
TruSeq DNA & RNA v2 Sample Prep

Additional 12 indexes available for a total of 24!

- Each TruSeq DNA and RNA v2 kits:
  - No changes to workflow or price, increase in index capabilities
  - Fill volumes and new consumables to support automation
  - Each kit contains 12 of 24 unique indexes
  - Each index reaction sufficient for 8 individual samples



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TruSeq Exome and Custom Enrichment

Simple, integrated and cost-effective

- Simple and scalable workflow
  - Plate-based processing and master-mixed reagents
  - Gel-free; integrated with TruSeq DNA Sample Prep
  - 1ug DNA input
  - 3 days total time with less than 4hr hands-on
- Pre-enrichment sample pooling
  - Dramatically reduces FTE time and increases throughput

	Exome	Custom
Region size	62Mb	500kb-25Mb
Pre-enrichment pooling	6 spls	12 spls
Read length	2 x 100	2 x 50-75
Min order	48 spls	48 spls
Demo/Test kit available	Exome	Cancer panel

Best Practices:
Starting Material

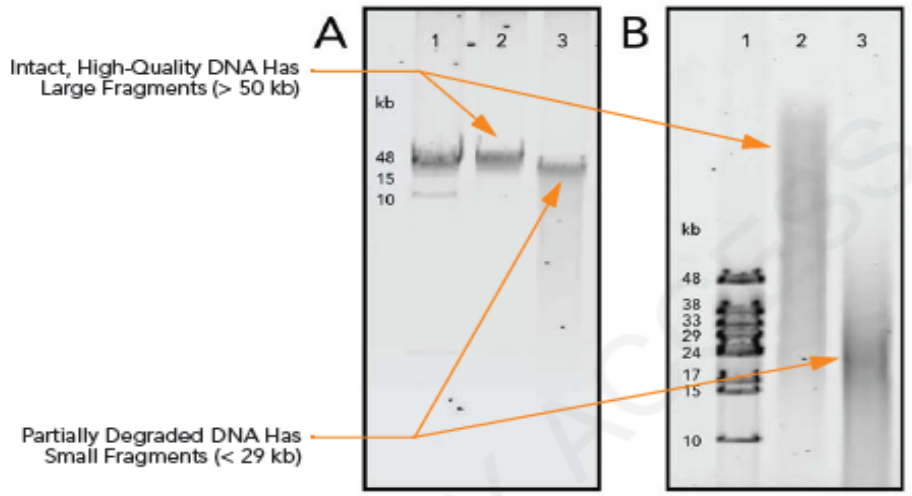
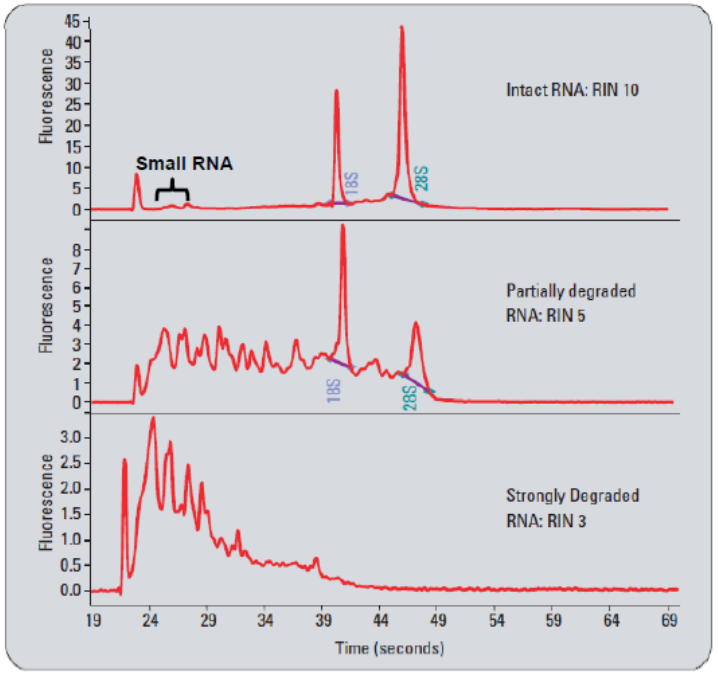


Figure 8 *Analysis of Genomic DNA Sample Integrity*

- Use recommended input amounts
- Quantify with Qubit, Picogreen, or Ribogreen
- Assess sample quality
  - 260/280 ratio and gel electrophoresis for DNA
  - RIN score for RNA (Bioanalyzer)
- Determine sample source for RNA protocols
  - Total RNA, purified mRNA
  - Origin species: no poly-A tail, dicer like processing



Best Practices:
Input Recommendations

	Recommended		Recommended
gDNA (SR;PE; Indexed) Nextera	500ng - 5 µg* 50ng	TruSeq DNA	1ug genomic
Targeted Resequencing	1 - 5µg	Enrichment	500ng DNA library
Mate Pair gDNA	10 - 20µg		
ChIP-Seq	10 - 50 ng		
mRNA	0.1 - 4 µg total RNA	TruSeq RNA	1ug total RNA
Small RNA	1 µg total RNA	TruSeq small RNA	1ug total RNA

*100 ng has been shown to work

Best Practices:
Starting Material, Enrichment

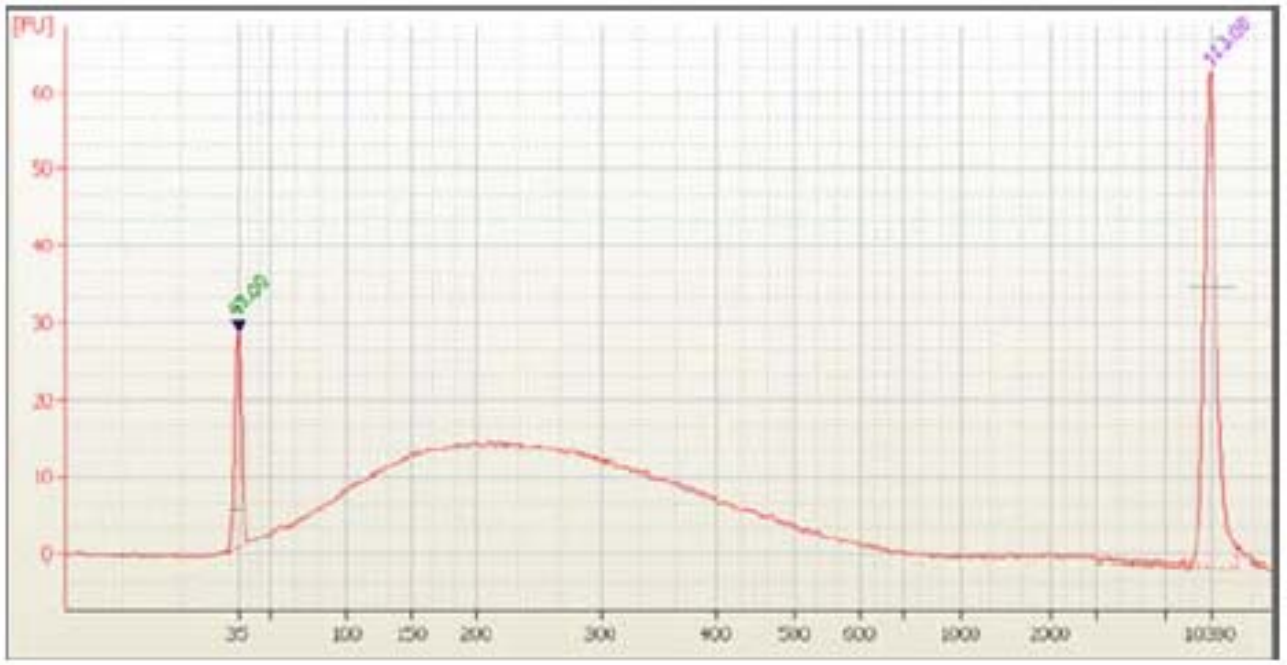
- Assess library quality and size
  - Run agarose gel
  - Run Bioanalyzer
- Determine library quantity
  - Fluorometric assay, like Qubit dsDNA Assay
  - Use 2ul DNA library in 200ul Qubit buffer
- Use 500ng of purified DNA library in Enrichment prep

Best Practices:
Fragmentation

- Fragmentation method: Covaris, nebulizer, sonicator
  - Were adjustments made for sample loss
  - Recommended to run a bioanalyzer trace, especially the first couple of times using a new fragmentation protocol
- Covaris fragmentation can be impaired by excessive air bubbles or airgap
  - Spin tube down before fragmentation to minimize risk
- Can sonicate up to 10ug depending on protocol
  - Ensure that only 1ug goes into TruSeq protocol (Follow recommendations on protocol input material

Best Practices:
Fragmentation

- Example of good Covaris fragmentation



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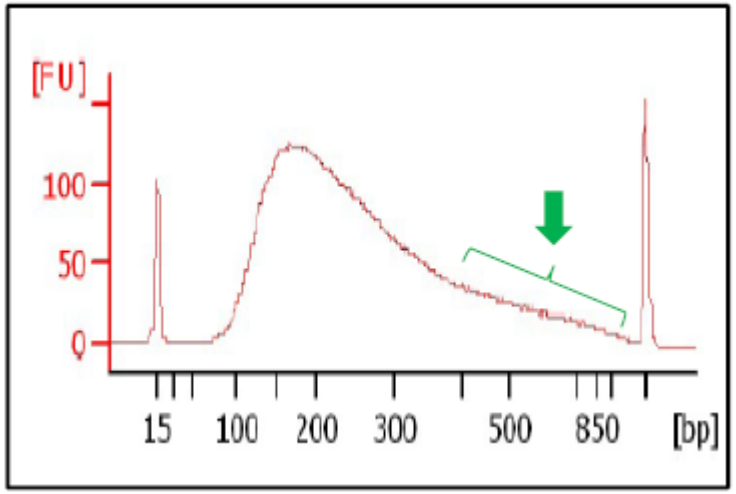
Fragmentation Problems

DNA Tailing

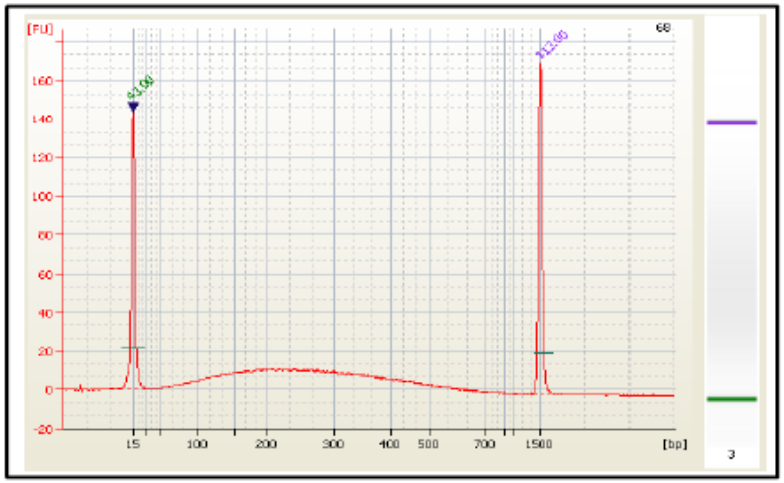
- Incomplete shearing caused by too much DNA input



- Adhere to recommended starting amounts

Increased size range

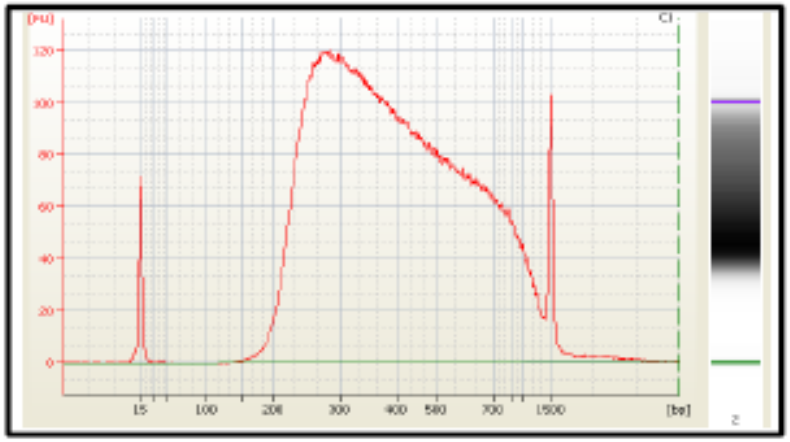
- Covaris problem

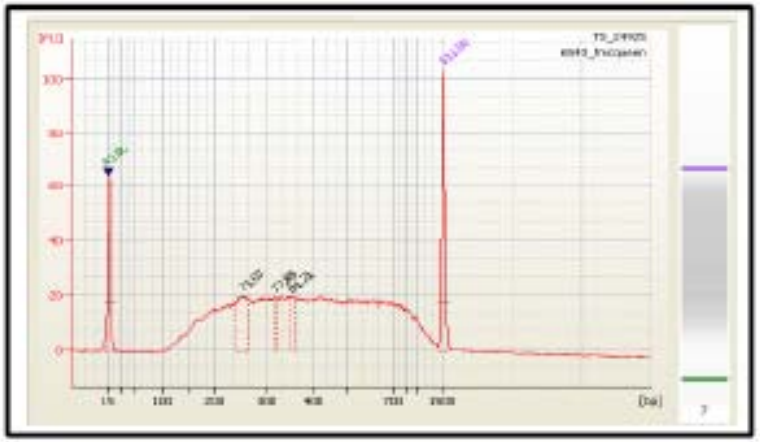


- Check Covaris with DNA of known quality (like lambda)

Fragmentation Problems

- Uneven shearing
- Poor DNA quality
- DNA eluted in wrong buffer (not TE)
- Covaris issues
  - Volume too low. Allows air gap and causes inconsistent fragmentation
  - Water level is too high or low
  - Temperature of water bath not between 6-8’C
  - Insufficient degassing





Best Practices:
Master Mix Reagent Handling

- Minimize freeze-thaw cycles
  - Dispense the reagent into aliquot
  - Beware: more aliquot -> reduced excessive reaction
- Reagents must be added in ORDER indicated in the TruSeq Sample Prep Guide
- A-Tailing Mix (ATL) and Ligation Mix (LIG) are viscous

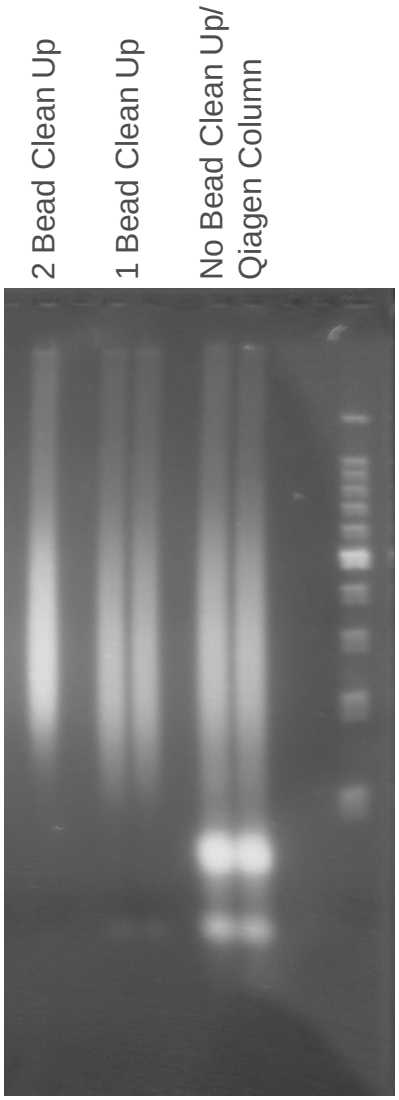
Best Practices:
AMPure XP Beads

- Clean up steps have only been verified using a 96-well PCR or MIDI plate. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats
- Ensure beads are well suspended, not settled
- Carefully pipet up and down to mix to minimize potential bead loss
- Utilize freshly prepared 80% EtOH for the washes
- While drying, allow the plate to remain on the magnetic stand to prevent potential bead loss due to electrostatic forces

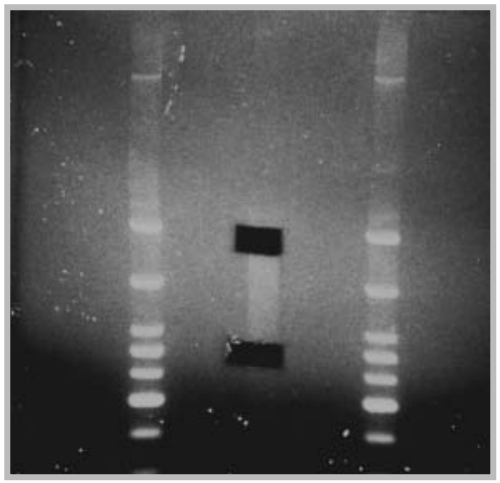
Common Questions
AMPure XP Beads

- Can I use columns in place of AMPure beads?
  - Not supported in the TruSeq protocol, but can work
  - Columns will not remove smaller DNA species, while the SPRI beads will remove everything below 150bp
  - Gel will not run as clean due to retention of the adapters

- Do the SPRI steps have to be done before the gel?
  - Gel size selection step will remove the adapters, but it is still recommended to do a SPRI cleanup before running the gel
  - This is because excess adapter dimers may cause the gel to run less reproducibly



Agarose Gel for Size Selection



- Familiar
- Cheap
- Very tight selection
- Can take several slices
- Not readily automatable

Tips:

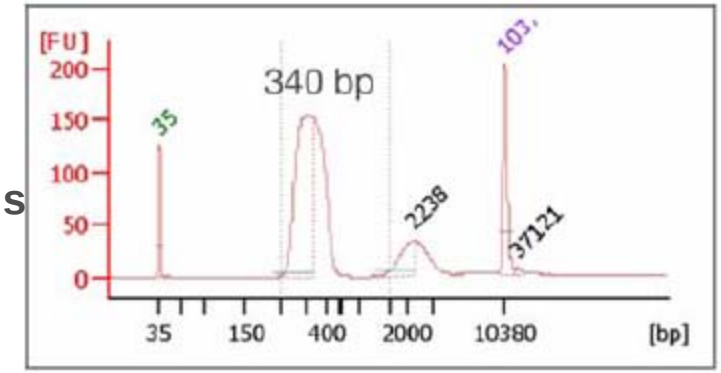
- Take care loading the sample – to avoid losses and cross contamination
- Don’t overload (keep to less than 3ug/lane)

DNA Migration Through Gels

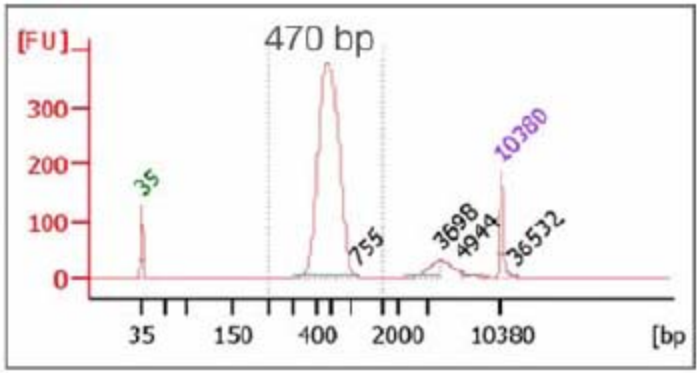
- To ensure accurate size selection, the protocol should be run exactly as listed in the user guide
- To minimize contamination, separate samples on the gel or run one sample per gel
- Ladders
  - We recommend a Promega ladder because it produces nice, sharp bands
    - BenchTop 100 bp DNA ladder, Promega, part # G829B
  - Do not overload your DNA ladder as this can lead to skewed migration patterns
- Pre- Vs. Post Gel Staining
  - We recommend a SyBr Gold Nucleic acid gel stain from Invitrogen
    - part # S11494

Pre and Post Staining (SyBr Gold at 1x)

Pre-electrophoresis Staining

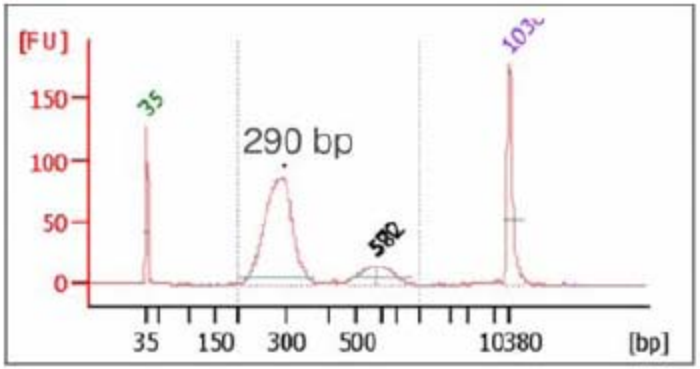


Selected 300 – 400 bp

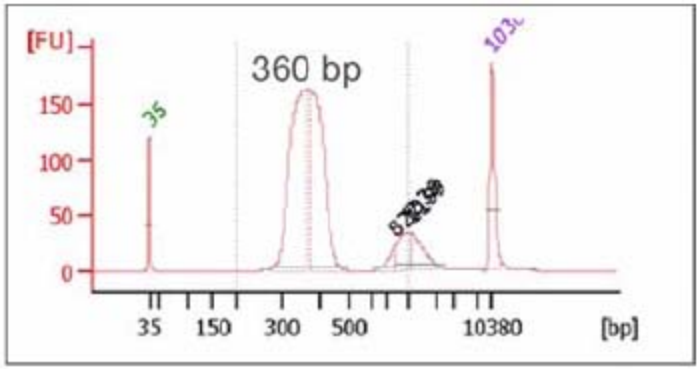


Selected 400 – 500 bp

Post-electrophoresis Staining



Selected 300 – 400 bp

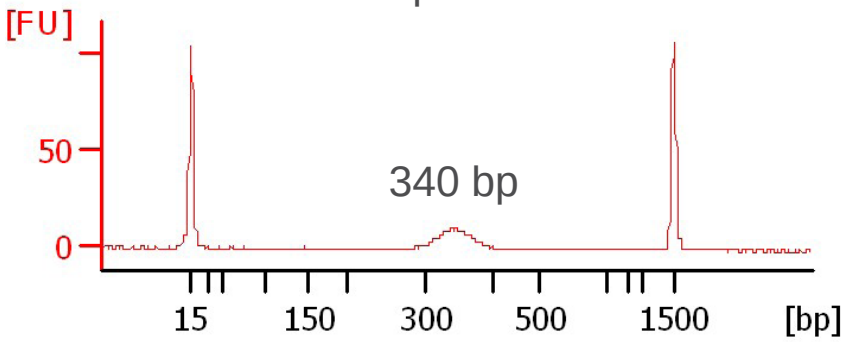


Selected 400 – 500 bp

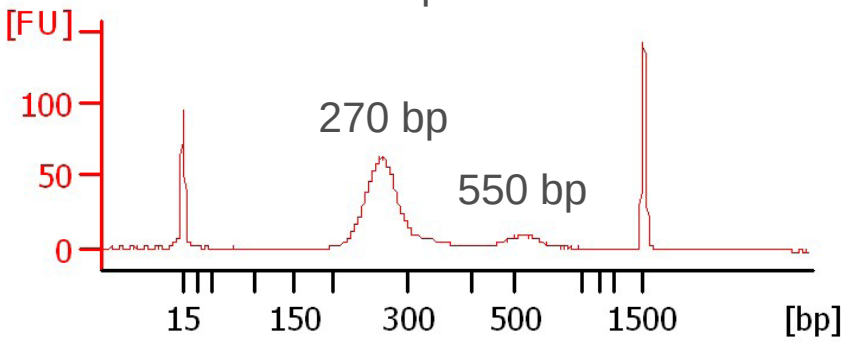
TruSeq DNA gel size selection

- Invitrogen E-gels have been shown to give inaccurate size selection
  - The selected product appears to be the right size after the gel but before amplification
  - After amplification, the product has a smaller apparent size and often shows a second larger band

Post-gel, 300-400 bp selected
Pre-amplification



Post-amplification

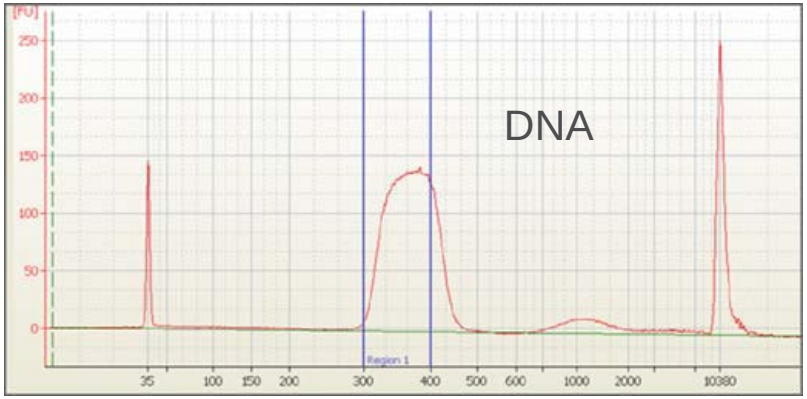


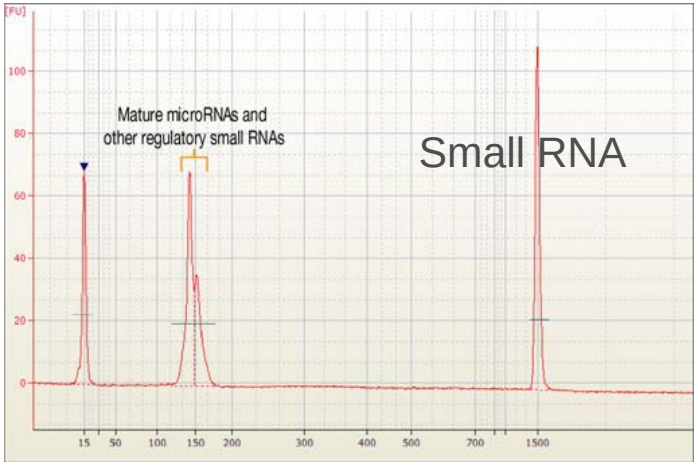
Troubleshooting:
Size Selection Step

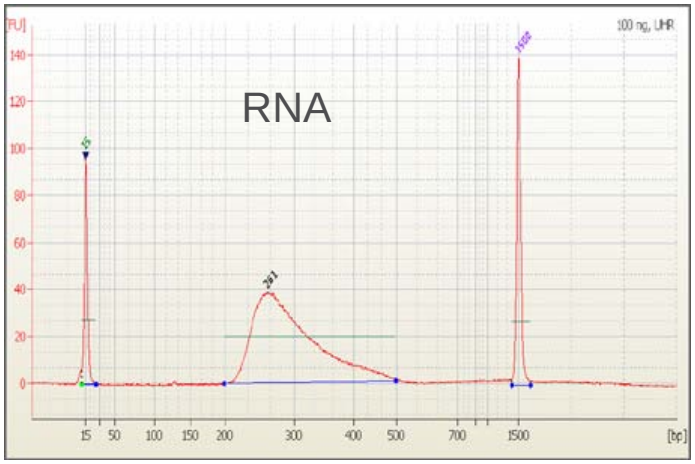
- Gather gel images before and after gel cutting from customer
- Ensure size range cut is what was intended for project
  - Make sure customer is running the correct ladder
- Determine size, type and staining method of gel
  - Gel should be 150ml, low-range ultra agarose prestained with SYBR gold
- Ensure that customer did not overload gel if starting with more input material then recommended

Bioanalyzer trace as a troubleshooting tool

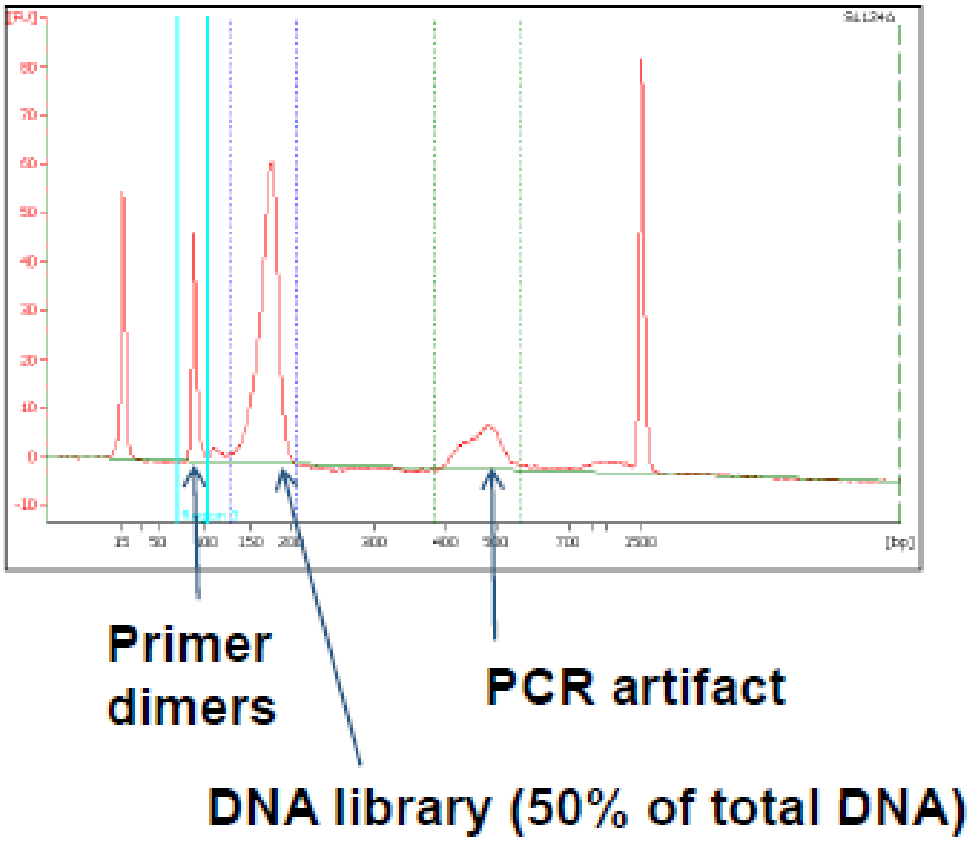
Expected Bioanalyzer Traces







Presence of peak <125bp:
Adapter dimers



From: Agilent's Bioanalyzer Applications for Next-Gen Sequencing: Updates and Tips March 1, 2011

Presence of peak <125bp:
Adapter dimers

Cause

- Error or omission of DNA gel cut
  - Proper cut should remove <150 bp
- Improper AMPure bead ratios
  - Proper ratios should remove <150bp
- Inefficient Ligation
  - Too much input DNA
  - Too little input DNA

Effect

- Dimers will form clusters and be sequenced. If ratio is low compared to library, may not be a problem but some reads will be dimers.
- Cause low diversity sample with improper matrix/phasing info and low clusters passing filter if clustered at higher densities.

- Primers <100bp cannot cluster or be sequenced, but can bind to flowcell

Libraries of Unexpected Size

Cause

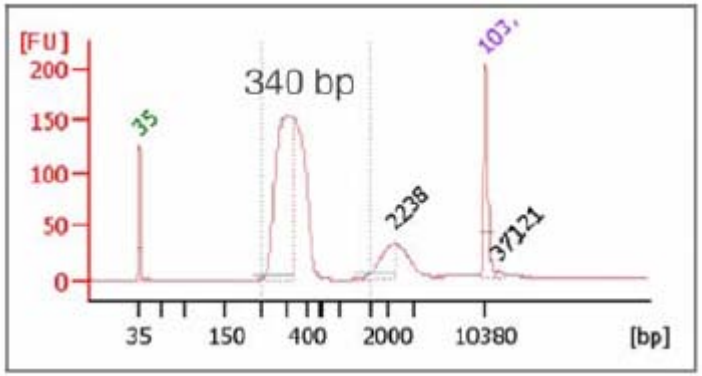
- Recommended gel conditions not used
- Check before and after gel purification pictures, if available

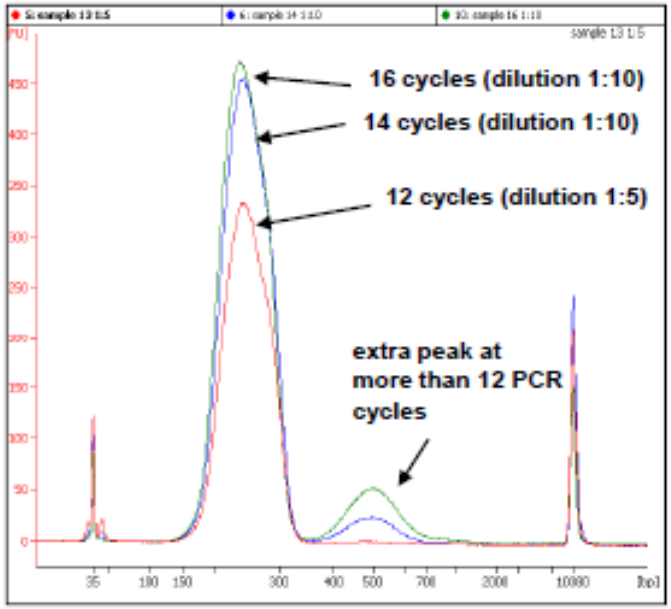
Effect

- Other types of gels and staining methods can shift size ranges leading to incorrect band excision

Libraries 2x larger than expected:
PCR artifact

- Possible single stranded species
- Overamplification

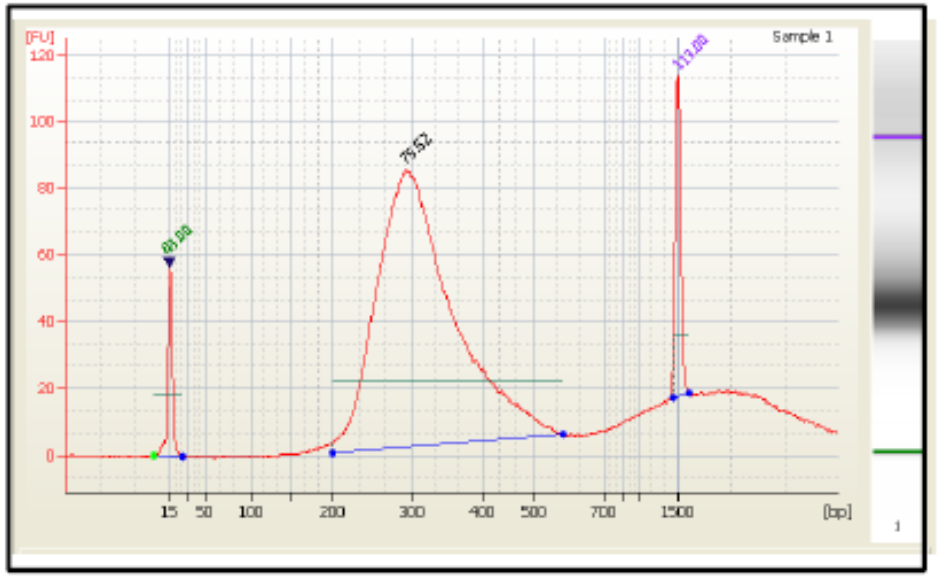




From: Agilent's Bioanalyzer Applications for Next-Gen Sequencing: Updates and Tips March 1, 2011

Additional Peaks:
SPRI bead carryover

- SPRI bead carry over following PCR Clean Up step

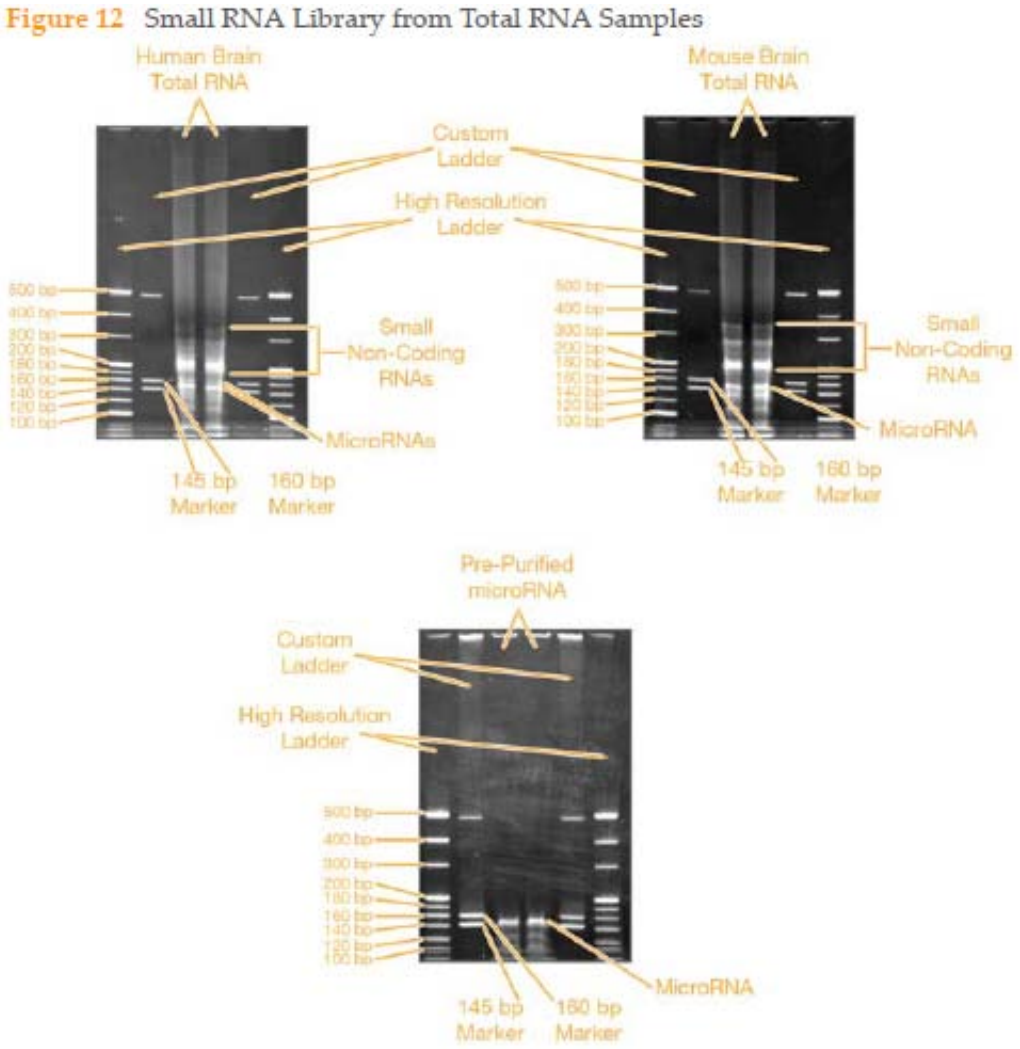


- Use a strong magnet for bead separation
- Pipet carefully during elution to avoid disturbing bead pellet

TruSeq Small RNA Troubleshooting

TruSeq Small RNA Prep:
Appropriate bands

- miRNA libraries 145-160bp
- 18bp insert = 136bp library
- 25bp insert = 143bp library
- All consistently run about 7bp higher on gel and Bioanalyzer



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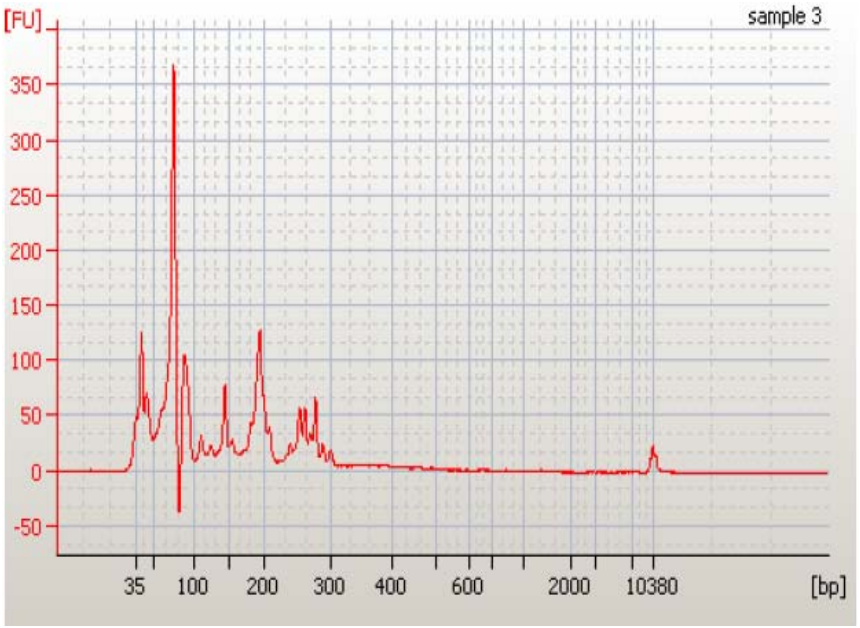
TruSeq Small RNA Prep: Inappropriate bands

- <100bp: Normal, Primers or primer dimers
- 120-125bp: Amplified adapter dimer
  - Low input increases dimer
- 130-138bp: Artifact band, Adapter concatamer
  - Reduced by increasing temp of RT
  - Seen at 44-47’, gone at 50’
  - Make sure ice->cycler->ice
- 144-150bp: PRODUCT. Cut band
- 150-155bp: pre-miRNA, piRNA
- 200bp smear: tRNA + adapters



TruSeq Small RNA Prep
Gel best practices

- Gels and gel running buffer should be fresh
  - Commercially available gels are recommended
- Don’t run gel so fast that it warms up
  - Warmed up gels will warp
  - May need to optimize running conditions on individual apparatus
- Premix stain to proper concentration before adding to gel
  - Adding concentrated stain directly can cause blobs of stain that cant be washed away
- Don’t overload gel
  - Load 25ul of post-PCR volume
  - More or less causes the band to shift up or down
- Don’t mix by vortexing
  - Vortexing causes fuzzier bands and lower yields

TruSeq Small RNA Prep
Loss of yield

- No band between 145-160bp
  1. Mostly caused by formation of secondary structure in adapter oligos that prevents ligation
- Solution: Verify 70’C incubation of adapters was performed

- Band present, and excised, at 145-160bp but lost in subsequent steps
  1. Gel disruption or elution
  2. Ethanol precipitation (if done)
- Solutions:
  1. Practice precipitation with 25bp DNA ladder
  2. Use freshly diluted ethanol and fresh sodium acetate
  3. Final pellet doesn’t adhere well to tube wall – do NOT lose during washes!

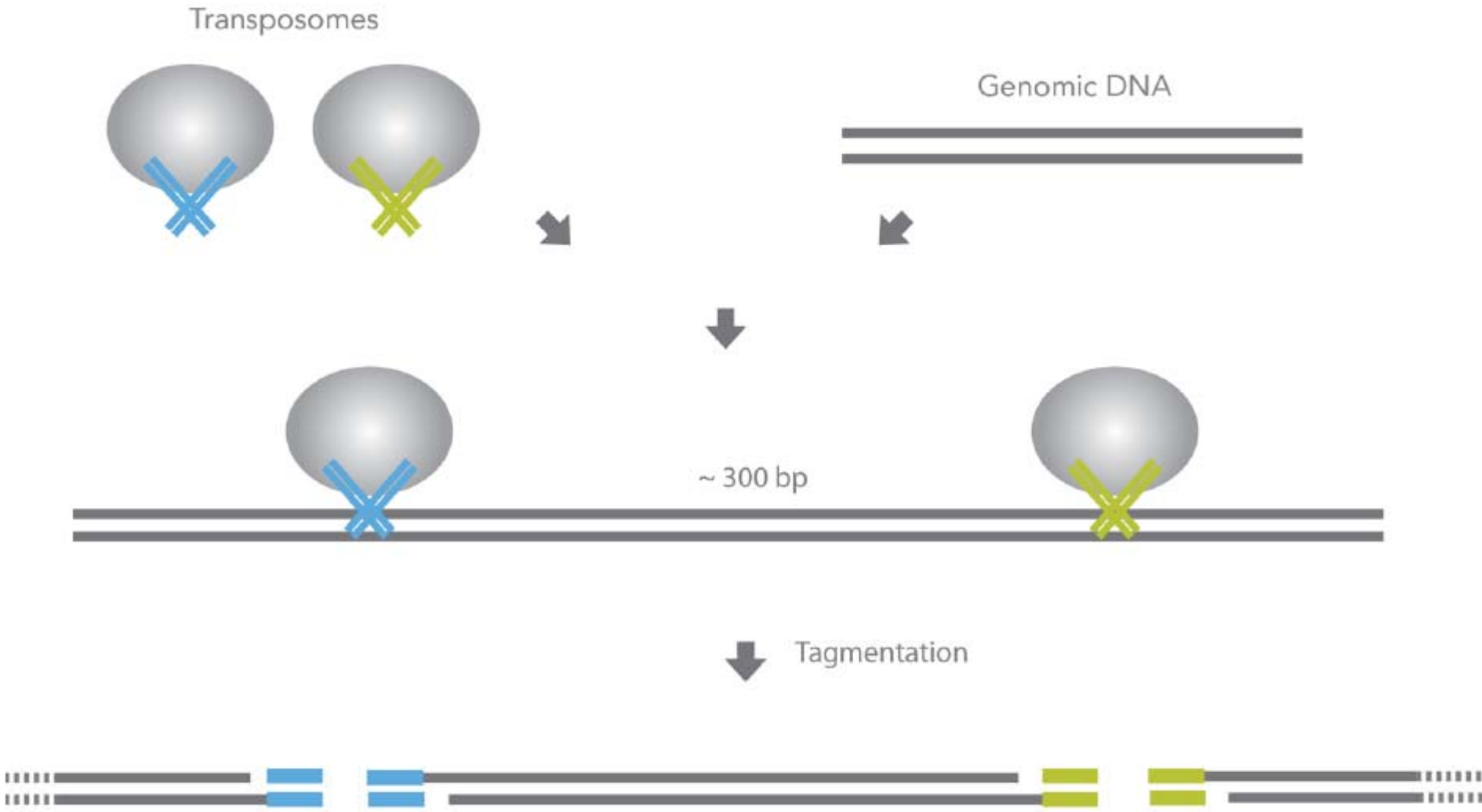
Nextera

Nextera DNA Sample Prep

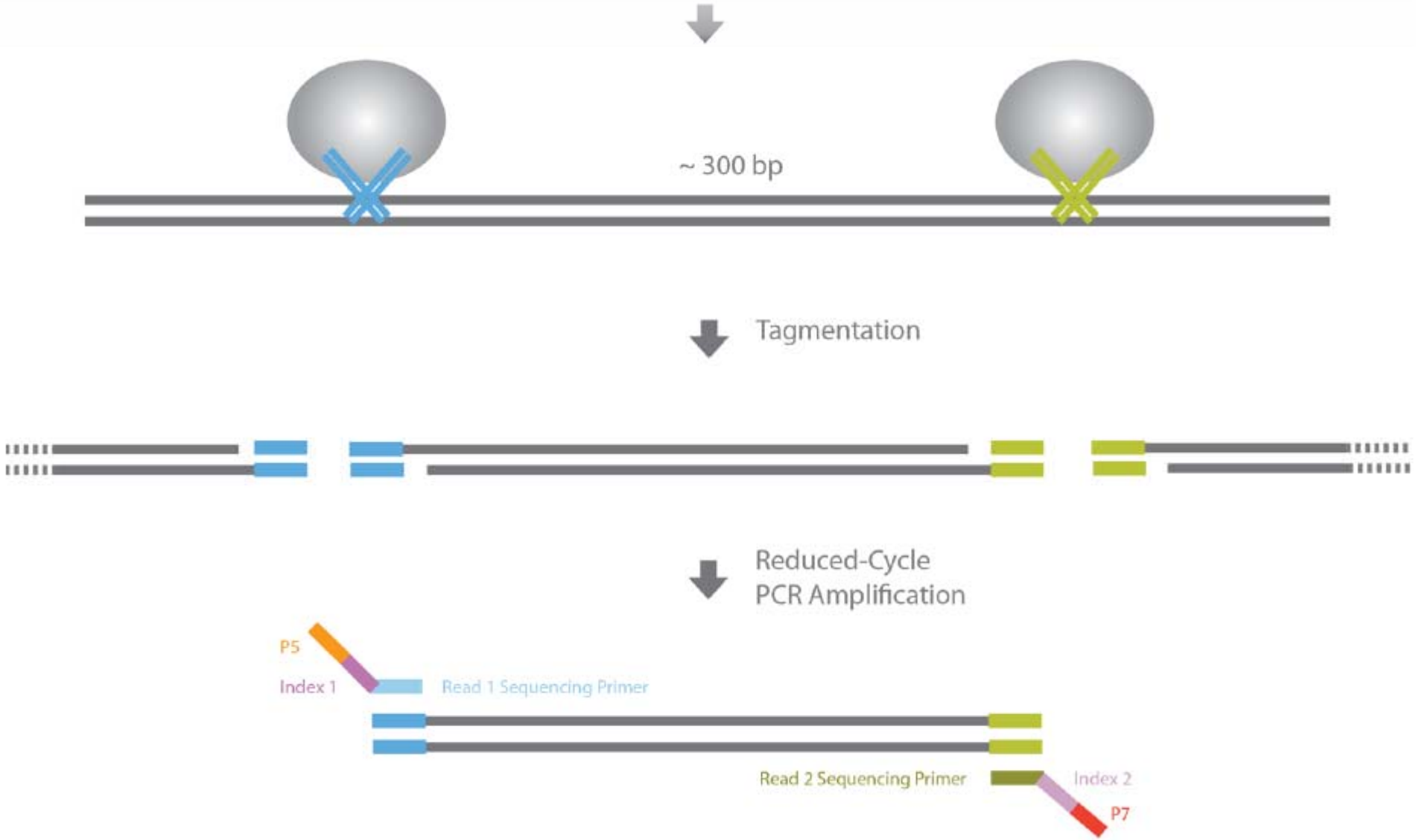
Sequencing's fastest and easiest sample prep

- Rapid and streamlined workflow
  - Complete protocol in < 90 min
  - Single well enzymatic reaction both fragments and adds adapter
  - no mechanical fragmentation/shearing required
  - Easy four step protocol
- Ultra-Low DNA input of only 50 ng
- High Throughput, High Indexing
  - Prepare up to 96 samples per batch
  - Master-mixed reagents and automation-friendly configurations
  - 96 indices supported on all Illumina sequencers

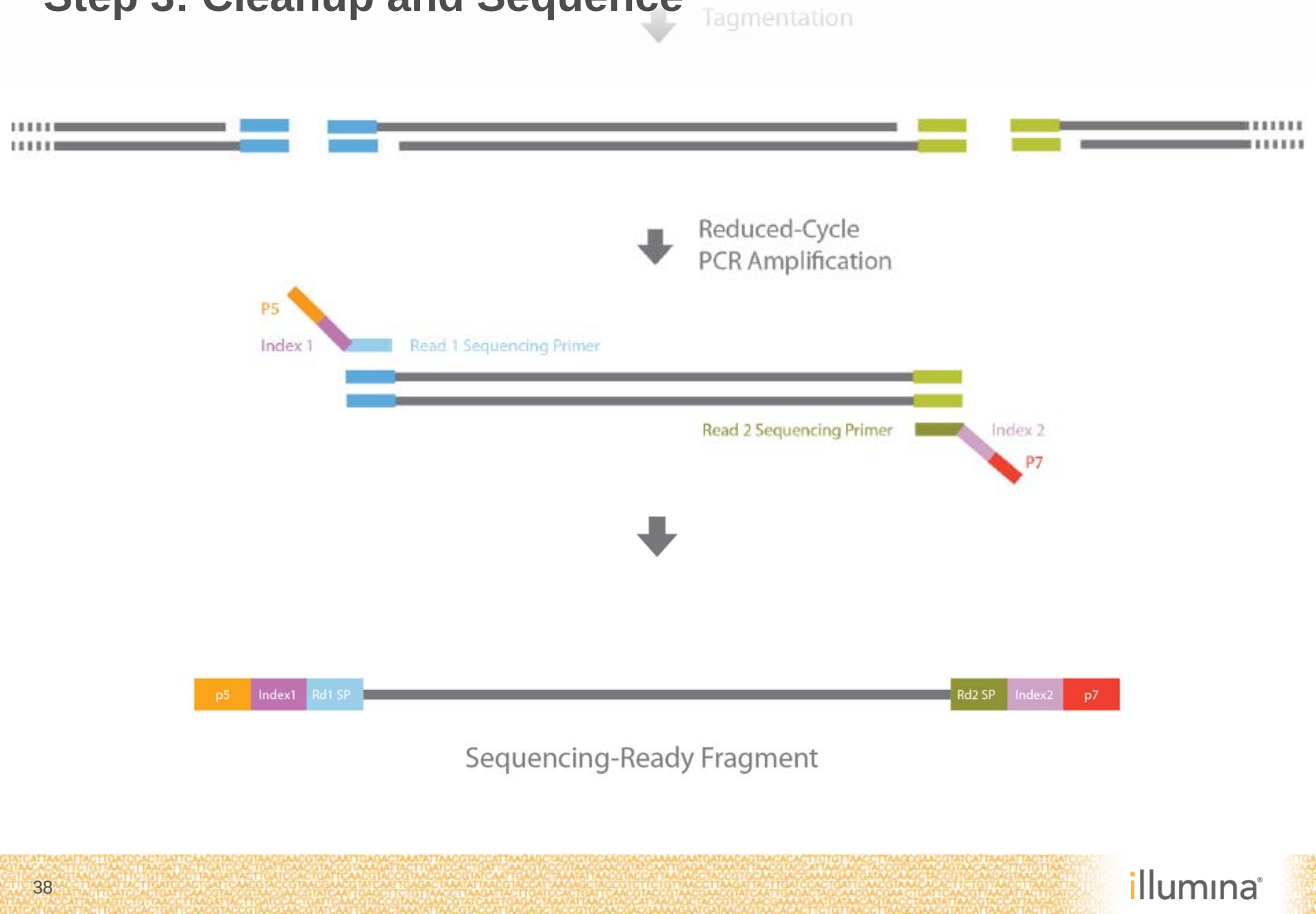
Step 1: Tagmentation of template DNA



Step 2: PCR to add adapters and indices



Step 3: Cleanup and Sequence

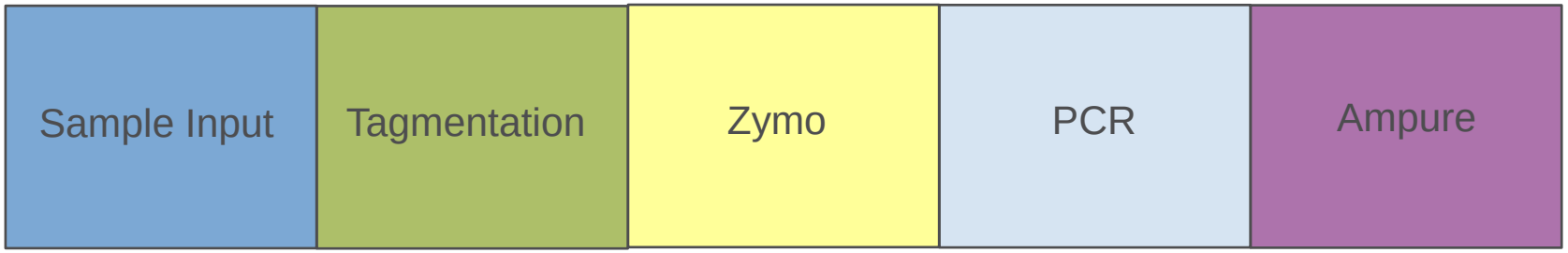


Improvements to Illumina's Nextera DNA Preparation Kit compared to legacy kit from Epicentre

- Optimized PCR
  - Reagents now included in the kit
  - Less cycles
  - New polymerase
  - Reduced GC bias and error rates
- 96 indices supported on all ILMN sequencers
- Improved master mixes – easier protocol with less tubes
- Optimized tagmentation reaction
  - shift insert size distribution to support longer 2x150 reads on MiSeq
- Plate-based protocol to support larger batch sizes

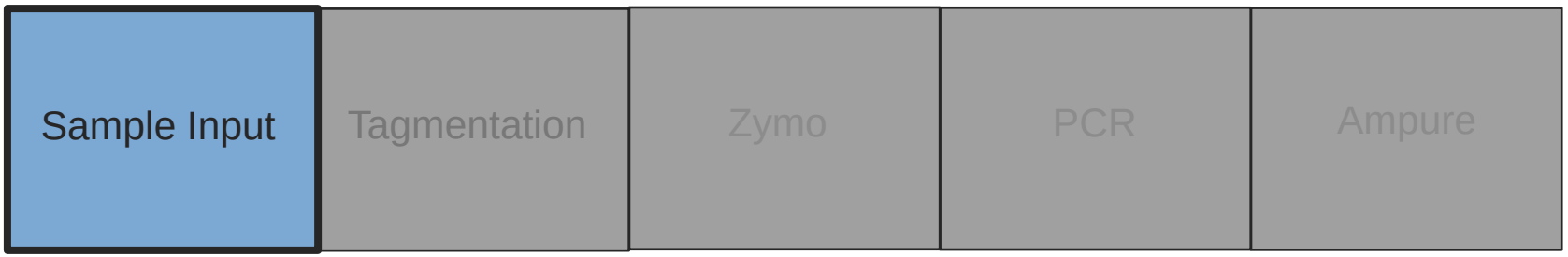
General Troubleshooting Strategy

- Typical complaint is about sample ‘loss’
- Is the sample completely gone or are there unexpected peaks in final trace?
- If the sample is gone, likely cleanup problem
- If there are unexpected peaks, likely tagmentation or PCR problem
- Confirm exactly 50ng of high quality genomic DNA was used
- Confirm which step in the protocol is the problem, run diagnostic traces after each step



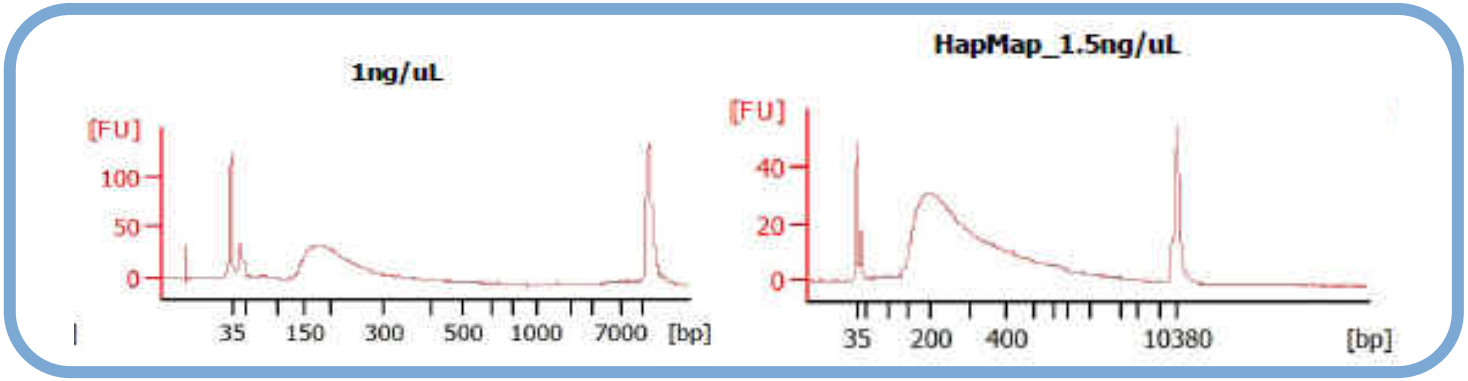
Sample Input

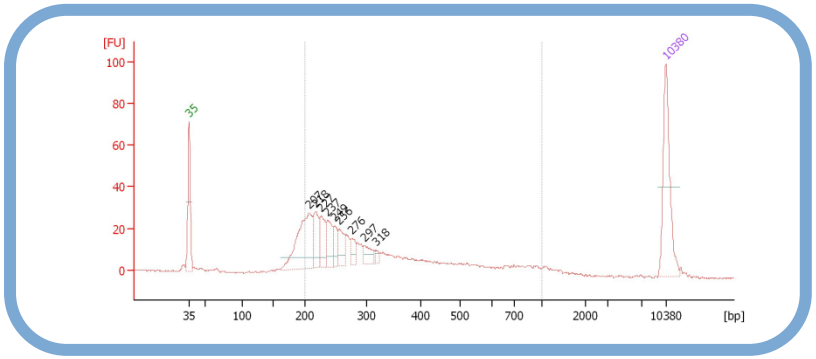
- General Comments:
  - We require 50ng of high quality genomic DNA of at least 300bp
  - Sample concentration can be measured with Qubit and quality can be assessed on a gel or trace
- Failure Modes:
  - over fragmentation --- too little sample
  - under fragmentation --- too much sample
  - poor performance in PCR --- degraded sample
  - loss in coverage within 50bp of the ends of the template --- template too short



Sample Input

- Failure Mode: over fragmentation --- too little sample

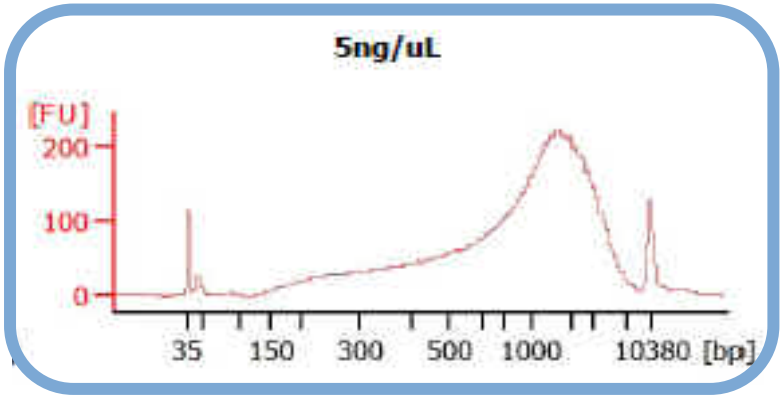


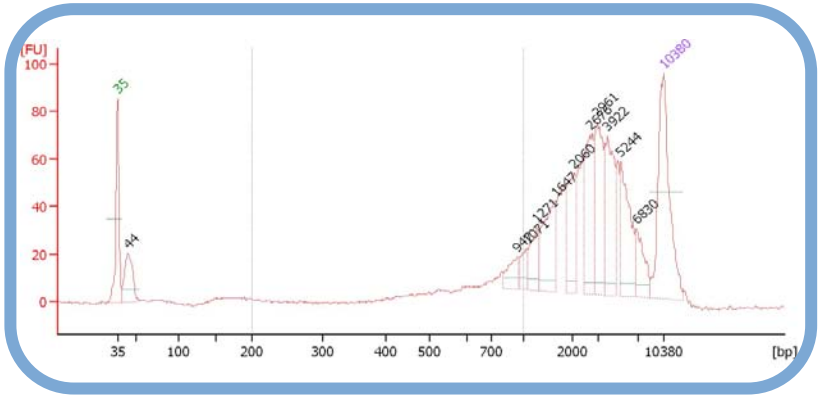


Sample Input	Tagmentation	Zymo	PCR	Ampure

Sample Input

- Failure Mode: under fragmentation --- too much sample

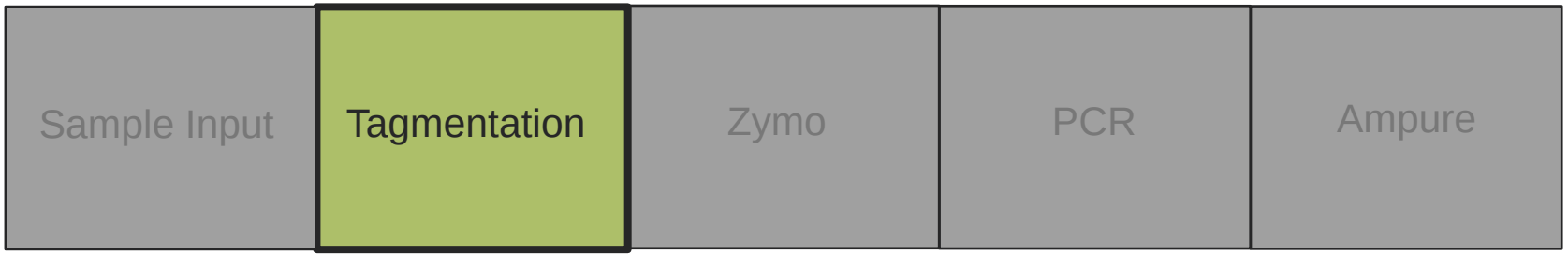




Sample Input	Tagmentation	Zymo	PCR	Ampure

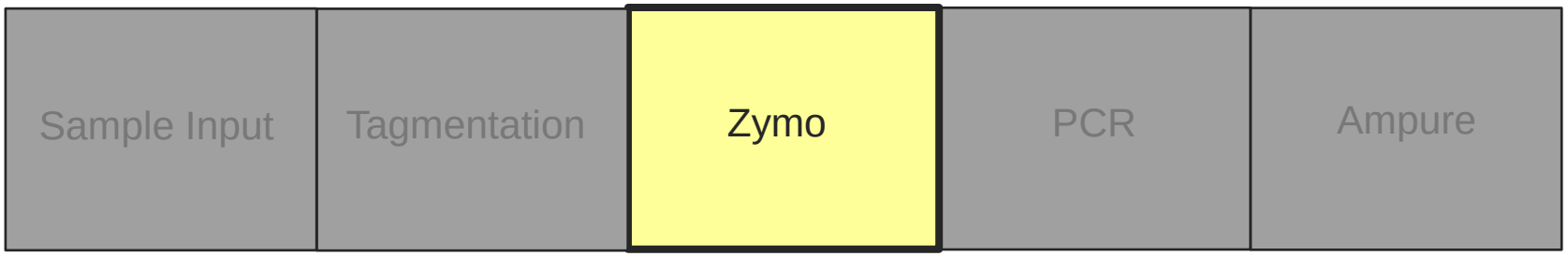
Tagmentation

- General Comments:
  - This is a 5 min. incubation at 55C
  - The protocol lists a hold at 10C . . . This means bring the sample to 10C and proceed **immediately** to Zymo cleanup. The transposomes are active at 10C and will continue fragmenting the sample.
- Failure modes:
  - inhibition of transposase (under fragmentation) --- presence of ethanol in sample
  - overfragmentation --- incorrectly calibrated thermal cycler: too hot
  - overfragmentation --- incorrect incubation time: too long



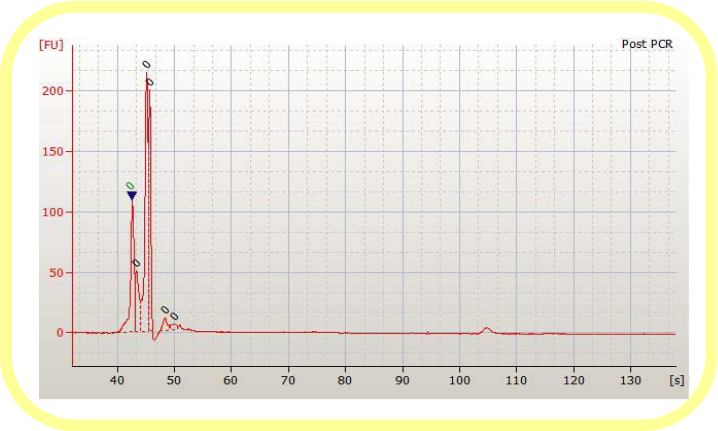
Zymo

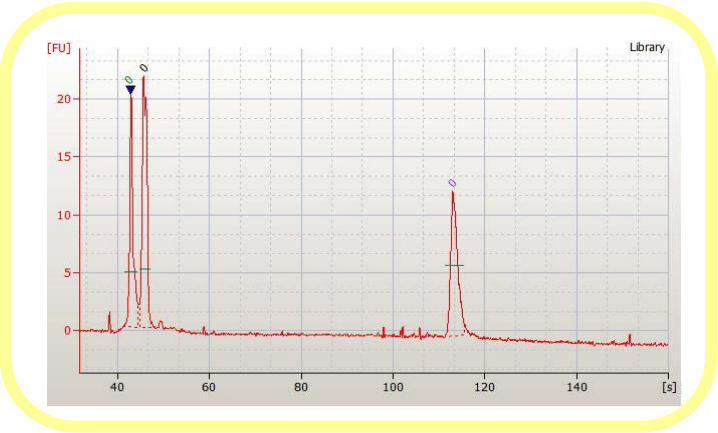
- General Comments:
  - This is a simple column purification.
  - A bioanalyzer trace of 1uL undiluted eluate should show a size range between 150bp and 1kb
- Failure modes:
  - low yield from PCR --- ethanol not added to wash buffer: transposomes may remain on DNA and inhibit PCR reaction
  - low yield from clean- up --- spinning at wrong speed and/or using incorrect volumes



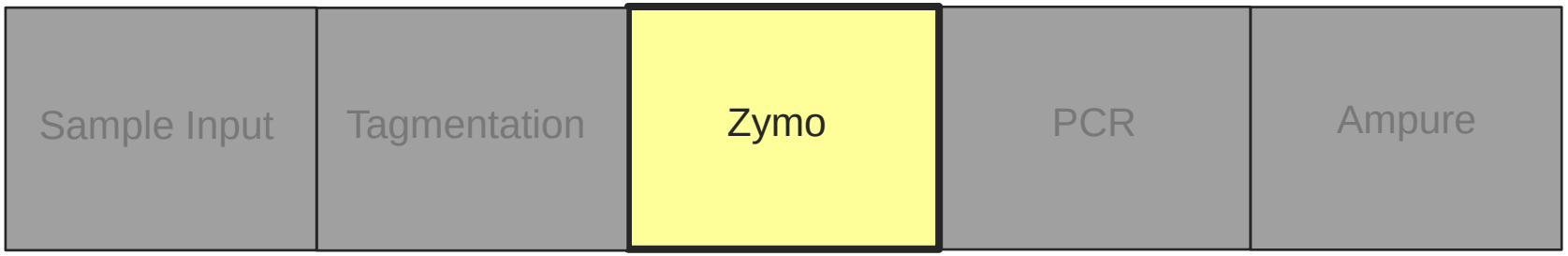
Zymo

- Failure Mode: transposome inhibition of PCR



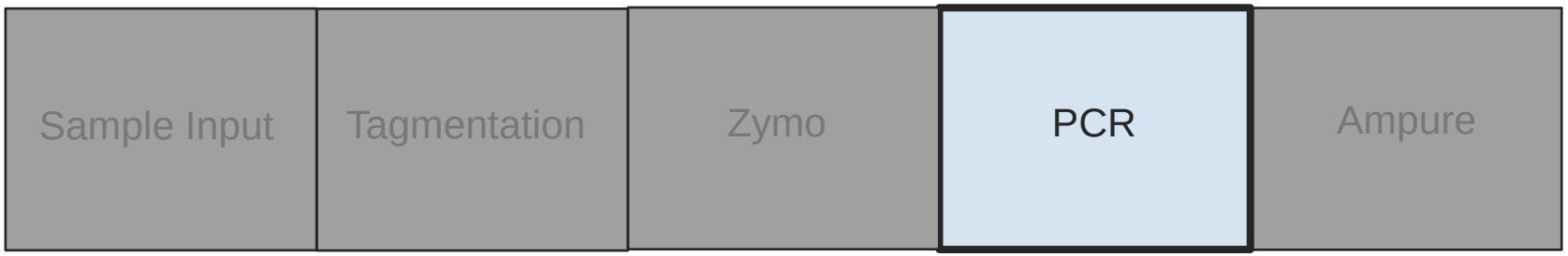


- The transposomes and both sets of PCR primers will add ~135bp to the template
- It is unlikely you will ever see full adapter+primer dimers
- PCR primer dimers will likely run at ~55-60bp



PCR

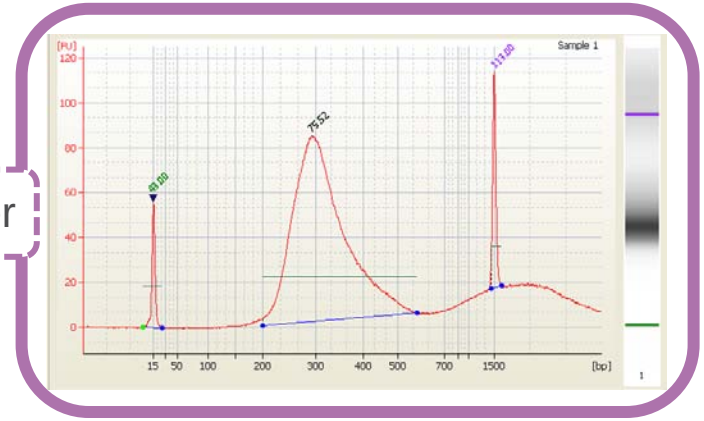
- General Comments:
  - This is the most complicated stage of the protocol: a 4 primer, 5 cycle PCR reaction
  - A very common failure mode is forgetting to add the two index primers
    - Two primers are in the PPC (PCR Primer Cocktail), the other two are in the index kit (purchased separately)
- Failure modes:
  - low yield, contamination --- pipetting inaccuracies
  - low yield --- forgetting to spin before PCR
  - low yield, dimer formation (55-60bp) --- wrong PCR setting on thermal cycler
  - low yield, dimer formation (55-60bp) --- miscalibration of thermal cycler
  - low yield, evaporation of sample --- not heating lid on thermal cycler



Ampure

- Important to use freshly prepared 80% ethanol, old ethanol will decrease wash efficiency
- As always, it is very important to let the supernatant clear before moving on
- Incorrect usage of magnetic stand can lead to sample loss and bead carryover
- Incorrect bead handling can cause contamination and/ or sample loss

Example of bead carryover



Sample Input	Tagmentation	Zymo	PCR	Ampure

Questions?