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FLASH v2.0 V.2

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We use this protocol and it's working

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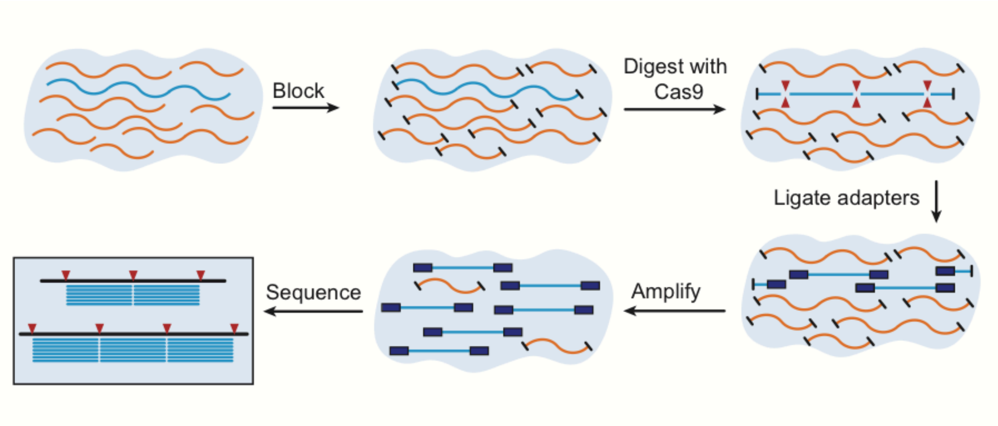
Abstract



FLASH workflow

FLASH is a crispr-cas9 technology that enriches for targeted sequences in sequencing libraries. The initial DNA sample undergoes a blocking step that removes of the 5′ phosphoryl groups of the DNA fragments, resulting in a product that is not amendable for downstream adaptor ligation or amplification via standard Illumina-based library preparation. The subsequent incorporation of targeted CRISPR-cas9 library exposes the desired regions of interest, allowing them to be processed into a library. For more information on methods and results, please see the **FLASH paper**. For FLASH guide RNA design help, please see our **github**.

Guidelines

1. This protocol has been used most successfully with starting inputs of DNA ranging from 10 pg – 100 ng. Limited success may be achieved with as little as 100 fg DNA.
2. Keep all enzymes on a chilled enzyme block. Immediately before use, allow them to come to room temperature for 5 minutes and then vortex briefly.
3. The NEBNext® dA-Tailing Module includes the Klenow fragment and the dA-Tailing buffer. The Klenow fragment allows for the 5′ ends to remain dephosphorylated after the initial blocking step. Do NOT use the NEBNext Ultra II End Prep kit for this step.
4. AmpureXP beads or other magnetic beads may be used instead of SPRI beads. Adjust the ratios of beads:sample accordingly to ensure proper removal of unwanted products. Different beads yield varying size selection cut-offs. Refer to this table to adjust ratios according to desired effect.

	SPRI Beads : sample ratio	**Step(s)**	**Desired cut-off/effect**
	1.7 : 1	Post-FLASH cleanup	No size selection; cleanup of unwanted buffers and deactivated cas9 protein from reaction
	1 : 1	Post-adaptor ligation clean up	Removal of buffers AND Stringent removal of all adaptor dimers
	1: 0.9	post-indexing Q5 PCR cleanup	Removal of buffers AND Stringent removal of all primer dimers
	1: 1	post-KAPA amplification cleanup	Removal of buffers AND Stringent removal of all primer dimers

SPRI bead cut-offs

Materials

MATERIALS

- NEBNext dA-Tailing Module - 100 rxns **New England Biolabs Catalog #**E6053L
- Sodium Orthovanadate (Vanadate) - 1 ml **New England Biolabs Catalog #**P0758S
- USER Enzyme - 250 units **New England Biolabs Catalog #**M5505L
- NEBNext Adaptor for Illumina **New England Biolabs Catalog #**E7337 in Kits E7335, E7500, E771
- NEBNext Ultra II Ligation Module - 96 rxns **New England Biolabs Catalog #**E7595L
- NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs Catalog #**M0544L
- Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #**Q32854
- Nuclease-free water **Ambion Catalog #**AM9932
- Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**
- PCR Thermocycler
- TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina) **New England Biolabs Catalog #**E7500L
- Proteinase K **New England Biolabs Catalog #**P8107S
- SPRI beads (homemade) or Ampure XP beads
- Kapa HiFi Real-Time Amplification Kit **Kapa Biosystems Catalog #**KK2702
- Magnetic rack for PCR strips
- rAPID alkaline phosphatase enzyme and buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4898133001
- cas9 4µM or higher concentration
- Dual guide RNAs (4µM - targeted to genes or regions to be depleted - crisprRNA and tracr RNA - quantified by RNA Qubit)
- Illumina P5 and P7 primers 5uM combined; P5: 5′ AATGATACGGCGACCACCGAGATCT P7: 5′ CAAGCAGAAGACGGCATACGAGAT
- 10X Cas9 Activity Buffer (500mM Tris pH 8.0 - 1M NaCl - 100mM MgCL2 - 10mM TCEP)

STEP MATERIALS

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- Dual guide RNAs (4µM - targeted to genes or regions to be depleted - crisprRNA and tracr RNA - quantified by RNA Qubit)
- Proteinase K **New England Biolabs Catalog #**P8107S
- SPRI beads (homemade) or Ampure XP beads

- 80% Ethanol
- TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina) **New England Biolabs** **Catalog** #E7500L
- SPRI beads (homemade) or Ampure XP beads
- 80% Ethanol
- NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs** **Catalog** #M0544L
- USER Enzyme - 250 units **New England Biolabs** **Catalog** #M5505L
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- SPRI beads (homemade) or Ampure XP beads
- 80% Ethanol
- Qubit 1X dsDNA High Sensitivity Assay Kit **Thermo Fisher Scientific** **Catalog** #Q33230
- Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**
- Sodium Orthovanadate (Vanadate) - 1 ml **New England Biolabs** **Catalog** #P0758S
- Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs** **Catalog** #M0212L
- NEBNext Adaptor for Illumina **New England Biolabs**
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- Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs Catalog #**M0212L
- NEBNext Ultra II Ligation Module - 96 rxns **New England Biolabs Catalog #**E7595L
- NEBNext Adaptor for Illumina **New England Biolabs**

- 80% Ethanol
- TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina) **New England Biolabs** **Catalog** #E7500L
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- Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**
- Sodium Orthovanadate (Vanadate) - 1 ml **New England Biolabs** **Catalog** #P0758S

Troubleshooting

Before start

Ensure that you are working in a PCR hood in a pre-PCR space if you are working with metagenomic samples or in a PCR hood if you are working with isolate samples. FLASH is very sensitive to environmental contamination.

Please refer to the guidelines section in this protocol if you are using Ampure beads or other SPRI beads to ensure you use the correct cut-offs.

For mixing of sample prior to PCR, avoid vortexing to keep DNA intact, and instead mix by pipetting and tapping sides of tube.

Dephosphorylation

1 Normalize your cDNA or gDNA to anywhere between 10pg-100ng. For most samples, we recommend 5-10ng input.

2 Prepare a reaction for each cDNA or gDNA sample and mix well. Add the components in the order specified below. You can make a master mix (MM) of the rapid alkaline phosphatase (RAP) buffer and enzyme. If you are using the RAP MM, mix 3 µL of MM with each sample. Mix thoroughly with a pipette or by tapping to avoid shearing.

rAPID alkaline phosphatase enzyme and buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4898133001

	Component	**1X**
	DNA, 10pg - 100ng	x µL
	rAPid Alkaline Phosphatase Buffer	2 µL
	rAPid Alkaline Phosphatase	1 µL
	H2O	up to 20 µL

RAP MM.

3 Incubate at 37 °C for 00:30:00 with heated lid OFF.

4 Add 1 µL sodium orthovanadate (competitive inhibitor of phosphatases) to the quench the reaction and mix well with a pipette or by tapping.

Sodium Orthovanadate (Vanadate) - 1 ml **New England Biolabs Catalog #**P0758S

Cas9 Treatment

5 Prepare dual-guide RNAs by annealing crRNA and tracrRNA at an equimolar amount at 95 °C for 00:00:30 then allowing the mixture to cool to room temperature on the bench. (Note. If crRNA and tracrRNA have been previously annealed and stored, it is recommended to re-anneal the dual-guide RNAs under the above conditions)

Dual guide RNAs (4µM - targeted to genes or regions to be depleted - crisprRNA and tracr RNA - quantified by RNA Qubit)

6 If your starting stock of Cas9 is more than 4µM, you must dilute your stock of Cas9 to 4µM by using 1X Cas9 activity buffer.

cas9 4µM or higher concentration

10X Cas9 Activity Buffer (500mM Tris pH 8.0 - 1M NaCl - 100mM MgCL2 - 10mM TCEP)

7 Make a Cas9 master mix as described below. **Add the components in the order specified to prevent precipitation.**

	Component	**1X**	___ X
	10x Cas9 Activity buffer	3 µL	___ µL
	Cas9 4µM*	2.5 µL	___ µL
	dgRNAs 4µM**	3 µL	___ µL
	H2O	0.5 µL	___ µL

	Total	*9 µL*	___ *µL*

Cas9 MM

**Remember to dilute your Cas9 stock to 4uM if not already at 4uM. Use 1x Cas9 activity buffer to dilute your Cas9 enzyme if you do need to dilute your stock.*
***Remember to dilute your dgRNAs to 4uM using water if needed.*

Note: Most experiments for the 2018 FLASH paper were performed at this Cas9 concentration. However, we have demonstrated that lower concentrations work equally well on bacterial isolate DNA. Consult the manuscript for more details.

8 Add 9 µL of the master mix to each of your 21 µL blocked DNA samples.

9 Mix well by pipetting or tapping the PCR tubes and incubate the reaction at 37 °C for 02:00:00 .

10 Deactivate the Cas9 by adding 1 µL of Proteinase K to each of your sample tubes, mixing again by pipetting or tapping. Incubate at 37 °C for 00:15:00 .

Proteinase K **New England Biolabs Catalog #**P8107S

SPRI Clean-up at 1.7X

11 Equilibrate clean SPRI beads to room temperature and vortex well to mix.

SPRI beads (homemade) or Ampure XP beads

12 Add beads equivalent to 1.7X the sample volume to each sample tube (for 31 µL of sample, add 53 µL beads).

13 Mix well by pipetting or tapping the tubes. Pulse-spin in a picofuge for no more than 2 seconds.

14 Incubate for 00:05:00 at room temperature, then put the tubes on the magnetic rack. Allow beads to separate on the magnet for 3-5 minutes, or until the supernatant is clear.

15 Keeping the tubes on the magnet, carefully remove and discard the supernatant.

16 Add 200 µL [M] 80 % volume ethanol (prepared fresh). Incubate beads for 00:01:00 and then remove the ethanol

80% Ethanol

17 Repeat the above ethanol wash step.

18 Allow the beads to air dry for 00:05:00 . Do not overdry. Dry beads should appear matte (rather than glossy), but should not have a cracked appearance. Overdried beads, as indicated by a cracked appearance, may not resuspend or elute well.

19 Remove tubes from magnet and resuspend in 53 µL nuclease-free H2O by pipetting up and down.

20 Continue to resuspend by tapping the tubes and then spin down briefly in a picofuge.

21 Allow 00:02:00 for DNA to elute from beads, then transfer tubes back to magnet

22 Allow the beads to separate for at least 2 minutes.

23 Collect 50.4 µL of supernatant to clean PCR tubes.

dA tailing

24 Prepare the following mixture for each sample. You can prepare the dA-tailing buffer and Klenow fragment ahead of time as a MM. If you prepared dA-tailing MM, add 9.6uL of MM to each sample.

	Component	**1X**
	FLASHed sample	50.4 µL
	dA-Tailing buffer	6 µL
	Klenow fragment	3.6 µL
	Total	*60 µL*

Klenow Fragment (3'-5' exo-) - 1,000 units **New England Biolabs Catalog** #M0212L

25 Mix well by pipetting up and down several times with a P200 set to 40uL.

26 Incubate all tubes at 37 °C for 00:30:00 with heated lid OFF

27 Cool all tubes to 4 °C **and proceed with the next part as soon as possible**.

Adaptor Ligation

28

Note

! The reagents used in this step are very viscous and must be mixed well before using.

! Do NOT make a master mix for this step, although the Ligation Master Mix and Ligation Enhancer may be mixed up to 4 hours before and kept at 4°C.

29 Prepare the following mixture for each sample. Alternatively, if you prepared an adaptor MM with Ligation MM and Ligation Enhancer, add 31uL of MM to each sample and then add 2.5uL of adaptor to each sample.

	Component	**1X**
	dA-tailed sample from part IV	60 µL
	NEB Ultra II Ligation Master Mix	30 µL
	NEBNext Ligation Enhancer	1 µL
	NEBNext Adaptor 1:100 or 1:300 dilution*	2.5 µL
	Total	*93.5 µL*

Note: Adaptor dilution of 1:100 works for an initial DNA input up to 100 ng. A 1:300 dilution is recommended for an initial DNA input of under 10 ng. The subsequent cleanup step should remove all extra adaptor and adaptor dimers.

NEBNext Adaptor for Illumina **New England Biolabs**

NEBNext Ultra II Ligation Module - 96 rxns **New England Biolabs** **Catalog #**E7595L

30 Prepare the above mixture and mix well by pipetting up and down several times with a P200 set to 50 µL .

31 Incubate at 20 °C for 00:15:00 in a thermocycler with the heated lid OFF.

SPRI Clean Up 1X + Addition of TruSeq Indexing Primers

32 Thaw a TruSeq i5/i7 barcode plate or other TruSeq primers, and choose barcodes for each sample. Take note of plate color/barcodes to be used. **DO NOT** use the same barcode for more than one sample in a sequencing run. Dual unique TruSeq barcodes are preferable.

TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina) **New England Biolabs Catalog** #E7500L

33 Equilibrate clean SPRI beads to room temperature and vortex well to mix.

SPRI beads (homemade) or Ampure XP beads

34 Add beads equivalent to 1X the sample volume to each sample tube (for 93.5 µL of sample, add 93.5 µL beads).

35 Mix well by pipetting or tapping the tubes. Pulse-spin in a picofuge for no more than 2 seconds.

36 Incubate for 00:15:00 at room temperature, then put the tubes on the magnetic rack. Allow beads to separate on the magnet for 3-5 minutes, or until the supernatant is clear.

Note

Longer incubation time during SPRI addtion is because it may be helpful in removing unwanted fragments/adapter dimers

37 Keeping the tubes on the magnet, carefully remove and discard the supernatant.

38 Add 200 µL [M] 80 % volume ethanol (prepared fresh). Incubate beads for 00:01:00 and then remove the ethanol

80% Ethanol

39 Repeat the above ethanol wash step.

40 Allow the beads to air dry for 00:05:00 . Do not overdry. Dry beads should appear matte (rather than glossy), but should not have a cracked appearance. Overdried beads may not resuspend or elute well. Ensure the beads are fully dry before eluting.

41 Remove tubes from magnet and resuspend in 17 µL nuclease-free H2O.

42 Resuspend well by tapping the tubes and spin down briefly in a picofuge.

43 Allow 00:02:00 for DNA to elute from beads, then transfer tubes back to magnet

44 Allow the beads to separate for at least 2 minutes.

45 Collect 15 µL of elution.

46 Mix 10 µL of the elution with the appropriate TruSeq indexing primer barcodes as planned.

Note

STOPPING POINT: If necessary, samples may be stored at -20C.

USER Enzyme + Indexing PCR

47 Spin down plate or tube strips briefly to collect liquid to bottom of the well.

48 Prepare the following mixture for each sample. You can make a master mix of USER enzyme and Q5. If doing this, add 28µL of MM to your 25µL sample.

NEBNext Ultra II Q5 Master Mix - 250 rxns **New England Biolabs** **Catalog** #M0544L

USER Enzyme - 250 units **New England Biolabs** **Catalog** #M5505L

	Component	**1X**
	Ligated sample + TruSeq indexing primers from part VI	25 µL
	NEBNext Ultra II 2X Q5 PCR Master Mix	25 µL
	NEB USER Enzyme	3 uL
	Total	*53 µL*

User/Q5

49 Set up the following cycling conditions for USER enzyme cutting and indexing PCR **in a post-PCR room**:

	Temperature	**Time**	**Cycles**

	37°C	15mins	1
	98°C	30 sec	1
	98°C	10 sec	12 cycles
	65°C	75 sec	
	65°C	5 min	1
	4°C	-	-

Set the lid heat to **ON** at 105°C.

SPRI clean-up 0.9X

50 Equilibrate clean SPRI beads to room temperature and vortex well to mix.

SPRI beads (homemade) or Ampure XP beads

51 Add beads equivalent to 1:0.9x the sample volume to each sample tube (for 53 µL of sample, add 47.7 µL beads).

52 Mix well by pipetting or tapping the tubes. Pulse-spin in a picofuge for no more than 2 seconds.

53 Incubate for 00:05:00 at room temperature, then put the tubes on the magnetic rack. Allow beads to separate on the magnet for 3-5 minutes, or until the supernatant is clear.

54 Keeping the tubes on the magnet, carefully remove and discard the supernatant.

55 Add 200 µL [M] 80 % volume ethanol (prepared fresh). Incubate beads for 00:01:00 and then remove the ethanol

80% Ethanol

56 Repeat the above ethanol wash step.

57 Allow the beads to air dry for 00:05:00 . Do not overdry. Dry beads should appear matte (rather than glossy), but should not have a cracked appearance. Overdried beads may not resuspend or elute well.

58 Remove tubes from magnet and resuspend in 53 µL nuclease-free H2O.

59 Resuspend well by tapping the tubes and spin down briefly in a picofuge.

60 Allow 00:02:00 for DNA to elute from beads, then transfer tubes back to magnet

61 Allow the beads to separate for at least 2 minutes.

62 Collect 23 µL of supernatant to clean PCR tubes.

KAPA Fluorescence-guided Amplification

63 Using optical PCR strip tubes which are separated from each other so that they can be removed from the thermocycler one at a time, add KAPA amplification MM and Illumina P7 and P5 (5sol-20 and 5sol-21) primers at 5µM to your samples as below. You can make a master mix of KAPA master mix and primers.

Kapa HiFi Real-Time Amplification Kit **Kapa Biosystems** **Catalog** #KK2702

Illumina P5 and P7 primers 5uM combined; P5: 5′ AATGATACGGCGACCACCGAGATCT P7: 5′ CAAGCAGAAGACGGCATACGAGAT

Component	1X
Amplified and indexed DNA	23 µL
Kapa amplification master mix	25 µL
Illumina P5 and P7 5uM primers	2 µL
Total	*50 µL*

KAPA amplification

64 Add 50 µL of STD 2 to a clean optical PCR tube.

65 Cap all tubes with optical caps. Do not write on the caps.

66 Place your labeled samples in the RT-PCR thermocycler.

67 Set up the following PCR conditions:

Temperature	Time	Cycles
98°C	45 sec	1
98°C	15 sec	20
60°C	30 sec	
72°C	1 min 30 sec	
Plate read		
72°C	30 sec	

Thermocycling Conditions for KAPA Amplification

Run the program, and watch until your sample either:
1. Crosses the standard (STD) 2 threshold
2. Starts to plataeu

Then pull your sample out **DURING THE 72C 30sec INCUBATION** after the plate read. It is critical to pull it out during this step, and not when it is denaturing or annealing.

If your sample still has not reached the STD 2 by 20 cycles, let the program finish.

Alternatively, if you have many samples, you can simply apply 10 cycles to all, and evaluate if you need further amplification later.

You may want to take note of the unique number of cycles each sample needed to reach the STD 2 threshold.

SPRI Clean-up 1X

68 Equilibrate clean SPRI beads to room temperature and vortex well to mix.

SPRI beads (homemade) or Ampure XP beads

69 Add beads equivalent to 1X the sample volume to each sample tube (for 50 µL of sample, add 50 µL beads).

70 Mix well by pipetting or tapping the tubes. Pulse-spin in a picofuge for no more than 2 seconds.

71 Incubate for 00:05:00 at room temperature, then put the tubes on the magnetic rack. Allow beads to separate on the magnet for 3-5 minutes, or until the supernatant is clear.

72 Keeping the tubes on the magnet, carefully remove and discard the supernatant.

73 Add 200 µL [M] 80 % volume ethanol (prepared fresh). Incubate beads for 00:01:00 and then remove the ethanol

80% Ethanol

74 Repeat the above ethanol wash step.

75 Allow the beads to air dry for 00:05:00 . Do not overdry. Dry beads should appear matte (rather than glossy) but should not have a cracked appearance. Overdried beads may not resuspend or elute well.

76 Remove tubes from magnet and resuspend in 27 µL nuclease-free H2O.

77 Resuspend well by tapping the tubes and spin down briefly in a picofuge.

78 Allow 00:02:00 for DNA to elute from beads, then transfer tubes back to magnet

79 Allow the beads to separate for at least 2 minutes.

80 Collect 25 µL of supernatant to clean PCR tubes.

Library Analysis

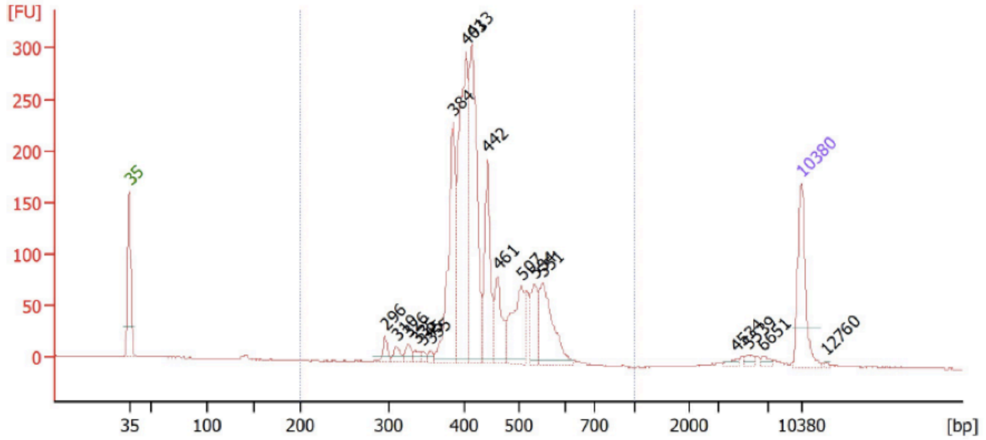
81 Quantify by HS DNA Qubit

Qubit 1X dsDNA High Sensitivity Assay Kit **Thermo Fisher Scientific Catalog** #Q33230

82 Run a fragment analysis, such as with the HS DNA Bioanalyzer. You are expecting a fragment trace that is characterized by sharp peaks in the 250-650 range. A successful FLASHed sample trace looks approximately like this: (lower marker in green, upper marker in purple)

Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**

Expected result



FLASH Bioanalyzer Trace

83 If there is a large spike at ~138 bp, this is indicative of adaptor dimer in the sample. We recommend: 1) Additional SPRI clean-ups with a sample:bead volume ratio of 1X (as many as necessary for removal of dimers), or 2) size selection to 250-650bp with the BluePippin on a 2% gel.

84 If pooling multiple samples, use the concentration of DNA between 250-650bp for normalization.

85 When satisfied with the quality of your pooled/individual library, proceed with quantitative PCR and sequencing.