Jan 2012

Preparing Samples for ChIP Sequencing of DNA

Contents

Component Information

A kit containing all reagents and oligos for library preparation can be purchased from Illumina:

ChIP Seq DNA Sample Prep Kit (Illumina IP-102-1001)

A kit containing all reagents and except the Adapter Oligo Mix and PCR Primers can be purchased from NEB. **One can make their own Adapter and Primers (page 2):**

NEBNext ChIP-Seq DNA Sample Prep Reagent Set 1 (NEB E6200S/L)
NEBNext ChIP-Seq DNA Sample Prep Master Mix Set 1 (NEB E6240S/L)

Individual Reagents, if you want to purchase each separately:

- 10X T4 DNA Ligase Buffer with 10 mM ATP (NEB B0202S)
- T4 DNA Polymerase (NEB M0203S)
- Klenow DNA Polymerase (NEB M0210S)
- T4 Polynucleotide Kinase (PNK) (NEB M0201S)
- 10 mM dNTPs Mix
- 1 mM dATP
- Klenow Fragment (3' to 5' exo minus) (NEB M0212S)
- Quick DNA Ligation Kit (NEB M2200S)
- Phusion HF DNA Polymerase (NEB M0530S/L) OR Phusion HF DNA PCR Kit (NEB E0553S/L) OR Phusion HF PCR Master Mix (NEB MO531S/L)

Gel Purification Components:

- 6X Loading Dye (NEB B7021S)
- Low Molecular Weight DNA Ladder (NEB N3233L)
- Certified Low Range Ultra Agarose (Bio Rad 161-3106)
- SYBR Gold Nucleic Acid Gel Stain (Invitrogen S-11494)
- Roche Restriction Enzyme Buffer H
- Gel x-tracta (USA Scientific 5454-2500)
- QIAquick Gel Extraction Kit (QIAGEN 28704)
- TaKara DNA-OFF (TaKara 9036)

DNA Purification Kits:

- QIAquick PCR Purification Kit (QIAGEN 28104)
- MinElute PCR Purification Kit (QIAGEN 28004)
- Agencourt AMPure XP Beads - 5 mL (Beckman Coulter A63880)

General Recommendations

- Make sure to have good sonication of your chromatin before doing the ChIP. If there is not a decent amount of gDNA in the 100-200bp range, then that could lead to low yield in the library prep. Troubleshoot multiple different conditions to find the right sonication protocol.

- Do not block your ChIP with Salmon Sperm or tRNA. You will sequence it. Use BSA.

- An Input sample should be run as a Positive Control, as this should work every time.

- To help ensure good results, start with at least 10µg of chromatin. However, all antibodies are different, so one should expect varying results between ChIPing for different histone modifications and transcriptional factors. For most transcriptional factors it can be difficult to obtain a good library, while for the majority of histone modifications it is very easy.

Preparing your own Multiplex Adapters and PCR Primers

One can order their own adapters and primers from Operon/IDT. Follow our helpful information below to get better results. Order the adapters PAGE purified. Order the PCR primers HPLC or PAGE purified. The * stands for phosphorothioate bond, which helps protect against endonuclease activity and have stronger bond. Adapter and Primer preparation are on page 3. All sequences are written 5' to 3'. The resulting library can be used for Multiplexed, Paired End, and Single Read sequencing.

Some sequencing experiments require the use of fewer than 12 index sequences in a lane with a high cluster density. In such cases, a careful selection of indexes is required to ensure optimum cluster discrimination by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

2 Samples: Indexes 6 and 12
3 Samples: Indexes 4, 6, and 12
4 Samples: Indexes 4, 6, 12, and 23
5 Samples: Indexes 4, 5, 6, 12, and 23
6 Samples: Indexes 2, 4, 5, 6, 7, and 12

Multiplexing Adapters:
MP_Adapt1: OP: [Phos]GATCGGAAGAGCACACGTC*T IDT: /5Phos/GATCGGAAGAGCACACGTC*T
MP_Adapt2: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Multiplexing PCR Primer 1:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Multiplexing PCR Primer 2: *Order one Index per sample.*

Primer Name	PCR Primer2 Sequence (each containing a different Index)	Index
PCR_Primer2_Index 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ATCACG
PCR_Primer2_Index 2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CGATGT
PCR_Primer2_Index 3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TTAGGC
PCR_Primer2_Index 4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TGACCA
PCR_Primer2_Index 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACAGTG
PCR_Primer2_Index 6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GCCAAT
PCR_Primer2_Index 7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CAGATC
PCR_Primer2_Index 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	ACTTGA
PCR_Primer2_Index 9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GATCAG
PCR_Primer2_Index 10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	TAGCTT
PCR_Primer2_Index 11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GGCTAC
PCR_Primer2_Index 12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	CTTGTA
PCR_Primer2_Index 23	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	GAGTGG

Preparing the Adapters

1. Dilute MP_Adapt1 and MP_Adapt2 to 100µM in EB.
2. Mix 20 µl of MP_Adapt1 and MP_Adapt2 with 10 µl of NEB Buffer 2 for a total volume of 50 µl in a PCR tube.
3. Incubate in a thermal cycler with the following program:
   - 95°C 5min
   - 80°C 1min
   - 70°C 1min
   - 60°C 1min
   - 50°C 1min
   - 40°C 1min
   - 30°C 1min
   - 25°C ∞
   - One can also Incubate at 95°C for 5min in a heat block and then remove the block and put on the bench for 2-3hrs.
4. The final molarity of the Multiplexing Adapter Mix is now approximately 40µM.
5. Dilute the Multiplexing Adapter Mix 1:20 in EB, for a final molarity of ~2µM. This is the molarity needed for ligation.

Preparing the Primers

1. Dilute all the PCR Primers to 25µM in EB. *Do not dilute below 25µM. If necessary, the primers can have a higher molarity of 50µm.*

Perform End Repair

This protocol converts the overhangs into phosphorylated blunt ends, using T4 DNA polymerase, E. coli DNA Pol I large fragment (Klenow polymerase), and T4 polynucleotide kinase (PNK). The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs. Use all 40µl of ChIP DNA. Dilute 10ng Input DNA in 40µl water.

Cleanup Method

- QIAquick PCR Purification Kit

Sample Prep Kit Components

- T4 DNA ligase buffer with 10mM ATP
- 10mM dNTPs mix
- T4 DNA polymerase
- Klenow DNA polymerase
- T4 Polynucleotide Kinase (PNK)

Master Mix (MM) Kit Components

- End Repair Reaction Buffer
- End Repair Enzyme Mix

Procedure

1. Prepare the following reaction mix:

	x1	x5
Water	0.8 µl	4 µl
T4 DNA ligase buffer	5 µl	25 µl
10mM dNTP mix	2 µl	10 µl
T4 DNA polymerase	1 µl	5 µl
T4 PNK	1 µl	5 µl
Klenow DNA polymerase	0.2 µl	1 µl
	10 µl	

2. Add 10 µl of reaction mix to 40 µl DNA
   OR
   MM: Add 5 µl of End Repair Reaction Buffer and 1µl End Repair Enzyme Mix to 44 µl DNA

3. Incubate for 30 minutes at 20ºC.

4. Purify using QIAquick PCR Purification Kit:
   - Add 250 µl Buffer PB and mix by pipetting
   - Apply to column and spin at max speed 30 sec
   - Pour flowthrough back on to column and spin at max speed 30 sec
   - Discard flowthrough
   - Add 750 µl Buffer PE and spin at max speed 45 sec
   - Discard flowthrough
   - Spin empty column at max speed 1 min
   - Transfer column to new 1.5 ml tube
   - Aspirate around clear ring inside column
   - Allow to air dry 1 min
   - Apply **36 µl Buffer EB** and let stand 2 min (accounts for loss of 2 µl volume during elution)
     - *MM*: Elute in 46 µl EB
   - Spin at max speed 1 min and discard column

Add ‘A’ Bases to the 3' End of the DNA Fragments

This protocol adds an ‘A’ base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single ‘T’ base overhang at their 3' end.

Cleanup Method

- MinElute PCR Purification Kit

Sample Prep Kit Components

- Klenow buffer (NEB buffer #2)
- 1mM dATP
- Klenow fragment (3' to 5' exo minus)

Master Mix (MM) Kit Components

- Klenow fragment (3' to 5' exo minus)
- dA-Tailing Reaction Buffer (10x)

Procedure

1. Prepare the following reaction mix:	x1	x5
▪ Klenow buffer (NEB buffer #2)	5 µl	25 µl
▪ 1mM dATP	10 µl	50 µl
▪ Klenow exo (3’ to 5’ exo minus)	1 µl	5 µl
	16 µl	

2. Add 16 µl of reaction mix to 34 µl DNA sample from previous step.
   OR
   MM: Add 5 µl of da-Tailing Reaction Buffer and 1 µl of Klenow exo to 44 µl DNA from previous step

3. Incubate for 30 minutes at 37ºC.

4. Remove the Ampure XP Beads from 4^{o}C and let sit at Room Temp.

5. Purify using MinElute PCR Purification Kit:
   - Add 250 µl Buffer PB and mix by pipetting
   - Apply to column and spin at max speed 30 sec
   - Pour flowthrough back on to column and spin at max speed 30 sec
   - Discard flowthrough
   - Add 750 µl Buffer PE and spin at max speed 45 sec
   - Discard flowthrough
   - Spin empty column at max speed 1 min
   - Transfer column to new 1.5 ml tube
   - Aspirate around purple ring inside column
   - Allow to air dry 1 min
   - Apply **15 µl Buffer EB** and let stand 2 min (accounts for loss of 2 µl volume during elution)
     - *MM:* Elute in 21 µl of EB
   - Spin at max speed 1 min and discard column

Ligate Adapters to DNA Fragments

This protocol ligates adapters to the ends of the DNA fragments, preparing them to be hybridized to a flow cell. Final Adapter mix molarity should be 1.5 - 2µM.

Cleanup Method

- Agencourt Ampure XP Beads (or Optional QIAquick PCR Purification Kit below)
- Freshly prepared 70-80% Ethanol

Sample Prep Kit Components

- 2X Quick DNA ligase buffer
- Adapter oligo mix (either Illumina's or Multiplexing Adapter Mix)
- Quick T4 DNA ligase

Master Mix (MM) Kit Components

- 5X Quick DNA ligase buffer
- Adapter oligo mix
- Quick T4 DNA ligase

Procedure

1. If you have not done so, dilute Illumina's Adapter 1:10 in EB OR Multiplexing Adapter 1:20.

2. Prepare the following reaction mix:

	x1	x5
▪ 2X Quick DNA ligase Buffer	15 µl	75 µl
▪ Diluted Adapter oligo mix	1 µl	5 µl
▪ Quick DNA ligase	1 µl	5 µl
	17 µl	

OR *MM:*

	x1	x5
▪ 5X Quick DNA ligase Buffer	6 µl	30 µl
▪ Diluted Adapter oligo mix	1 µl	5 µl
▪ Quick DNA ligase	4 µl	20 µl
	11 µl	

3. Add 17 µl of reaction mix to 13 µl DNA sample from previous step.
   OR
   MM: Add 11 µl of reaction mix to 19 µl of DNA sample from previous step.

4. Incubate for 15 minutes at 20ºC.

5a. Purify using Agencourt Ampure XP Beads: *Make sure beads came to Room Temp before proceeding*

- Vortex/mix the beads until they are well dispersed, and keep doing so every couple of samples
- Add 50 µl of mixed Ampure XP beads and mix by pipetting at least 10x
- Incubate the tubes at Room Temp for 10-15 mins
- Place tubes on magnetic stand and let sit for 2 mins or until the liquid appears clear
- Remove and discard 75 µl of the supernatant from each tube
- Add 200 µl 70-80% Ethanol and let it incubate on the stand for 1 min and then Discard Ethanol
- Repeat Ethanol wash one more time, making sure to completely remove all traces of Ethanol
- Let stand at Room Temp for 5-10 mins to dry and remove all Ethanol
- Remove from magnetic stand and resuspend the dried beads in **11 µl EB for 2% Agarose Gel**
  - **For Pippin Prep elute in 31 µl EB**
- Mix by pipetting 10x and Incubate at Room Temp for 5 mins.

Ligate Adapters to DNA Fragments (cont.)

- Place tubes on magnetic stand and let sit for 2 mins or until the liquid appears clear
- Transfer **10 µl** of the supernatant to a new tube for **2% Agarose Gel** procedure
    - Transfer **30 µl** of the supernatant to a new tube for **Pippin Prep** procedure

5b. Purify using QIAquick PCR Purification Kit: *Optional for Pippin Prep or Higher Elutions*

- Add 150 µl Buffer PB and mix by pipetting
- Apply to column and spin at max speed 30 sec
- Pour flowthrough back on to column and spin at max speed 30 sec
- Discard flowthrough
- Add 750 µl Buffer PE and spin at max speed 45 sec
- Discard flowthrough
- Spin empty column at max speed 1 min
- Transfer column to new 1.5 ml tube
- Aspirate around clear ring inside column
- Allow to air dry 1 min
- Apply **32 µl Buffer EB** and let stand 2 min (accounts for loss of 2 µl volume during elution)
- Spin at max speed 1 min and discard column

Size Select the Library

This protocol removes excess adaptors and selects a size range of templates to go on the Cluster Station. There are many ways to do size selection. Running a normal **2% Agarose Gel** and Sage Science's **Pippin Prep** are highlighted in this protocol. In addition, Invitrogen's E-Gel SizeSelect 2% Agarose gels or Caliper's Caliper XT can be used, but not mentioned in this protocol.

2% Agarose Gel Size Selection

Components

- Certified Low Range Ultra Agarose
- 50X TAE buffer
- Low Molecular Weight DNA ladder
- 6X loading dye
- 10X Roche Buffer H
- SYBR Gold
- QIAquick Gel Extraction Kit
- Gel x-tracta (USA Scientific 5454-2500)
- TaKara DNA-OFF (TaKara 9036)

Procedure

1. Make sure to have previously washed the gel tray, combs, cutting glass, etc. with DNA Off before proceeding.

2. Dilute 50X TAE Buffer to 1X TAE Buffer in 500 ml (10ml of 50X TAE into 490 ml of diH_2O).

3. Prepare a 50 ml, 2% agarose gel with 1X TAE buffer and Ultra Low Agarose. *Do not add EtBr or any DNA stain.*

4. Dilute SYBR Gold 1:1000 in water (add 0.5 µl to 500 µl water)

5. Prepare the DNA ladder mix:

	x1	x2
▪ Diluted SYBR Gold	10 µl	20 µl
▪ 6X loading dye	3.75 µl	7.5 µl
▪ 10X Roche Buffer H	1.25 µl	2.5 µl
▪ Low Mol. Weight ladder	2.5 µl	5 µl
	17.5 µl	35 µl

6. Prepare the loading dye mix:

	x1	x5
▪ 6X loading dye	3 µl	15 µl
▪ 10X Roche Buffer H	2 µl	10 µl
	5 µl	

7. Add 5 µl of loading dye mix to each DNA sample from previous step.

8. Load 17 µl of DNA ladder mix to outside wells.

9. Load entire sample (~15 µl) on gel leaving an empty lane between samples to avoid cross contamination.

10. Run gel at 100 V for 45 minutes.

11. View the gel on a **Dark Reader transilluminator**. *Do not expose gel to UV light.*

12. Using a Gel x-tracta or clean razor for each lane, excise a region of gel just above the 200bp band in the **220 ±25** bp range for **Genomic DNA Adapters** (or **200 ±25** bp range for **Multiplexed Adapters**). In addition, excise a region just above the first cut (**250 ±25** bp for Genomic DNA Adapters), to save as a backup for just in case there are PCR issues or one may get a better result with a higher insert. One can cut out as many bands as one wishes, just make sure they are small and not below 180bp. *DO NOT cut a wide range or a big piece. Photograph the gel after excisions for reference later.*

2% Agarose Gel Size Selection (cont.)

13. Purify DNA from gel slices using the QIAquick Gel Extraction Kit.
    - Weigh empty 1.5 ml tube to zero scale
    - Weigh each tube containing gel piece
    - To each tube add **6 volumes** of Buffer QG per volume of gel
        - ie. add 600 µl QG to a 100 mg gel piece
    - Incubate at Room Temp for 10 mins, vortexing regularly until gel piece is dissolved.
    - To each tube add **2 volumes** of isopropanol per volume of gel and mix by pipetting.
        - ie. add 200 µl isopropanol to a 100 mg gel piece
    - Apply to column and spin at max speed 30 sec
    - Pour flowthrough back on to column and spin at max speed 30 sec
    - Discard flowthrough
    - Repeat until entire volume has been passed through column
    - Add 500 µl Buffer QG and spin at max speed 30 sec
    - Discard flowthrough
    - Add 750 µl Buffer PE and spin at max speed 45 sec
    - Discard flowthrough
    - Spin empty column at max speed 1 min
    - Transfer column to new 1.5 ml tube
    - Aspirate around purple ring inside column
    - Allow to air dry 1 min
    - Apply **38 µl Buffer EB** and let stand 1 min (accounts for loss of 2 µl volume during elution)
    - Spin at max speed 1 min and discard column

Pippin Prep Size Selection

Using Sage Science's Pippin Prep helps standardize library size selection and most importantly vastly increases yield over the normal gel extraction technique. Turn on the machine by a button in the back, on the bottom right. The machine does not catch your mistakes, so carefully follow the directions and be aware of what you are doing. **CAUTION: some gels contain Ethidium Bromide, so wear gloves at all times.**

Components

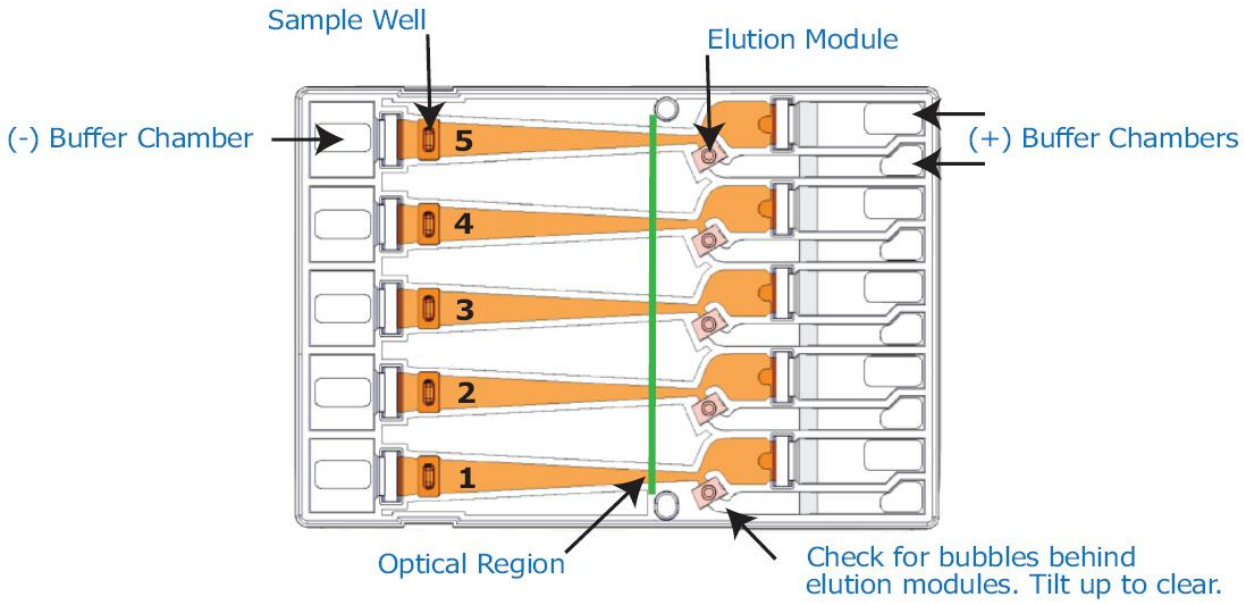
- 2% EF (Ethidium Free) Agarose Gel Cassette (Sage Science CEF-2010)
  OR
- 2% Agarose Gel Cassette (Sage Science CSD-2010)
- Loading Solution (in 4°C)
- Marker (ladder): 2% EF use Marker E, 2% use Marker B (in 4°C)
- Electrophoresis Buffer (Clear for 2% EF and Brown for 2%)

Procedure

1. Remove marker and loading solution from refrigerator let stand at Room Temp for 20 mins

2. Add 10 µl of loading solution to 30 µl DNA sample from the previous step. Mix by vortex and spin.

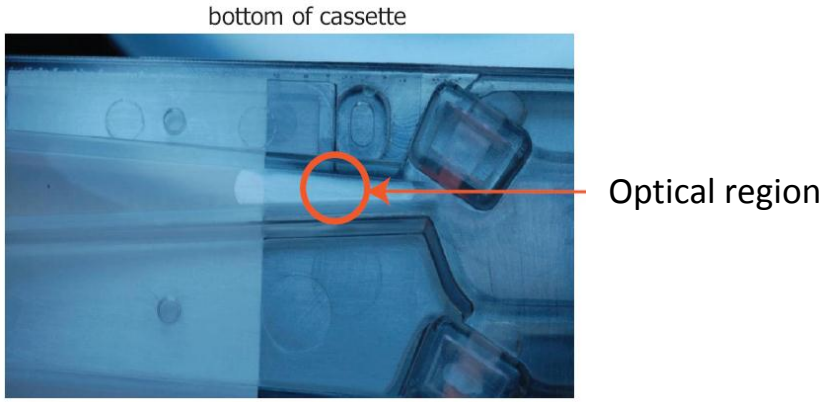
3. Remove gel cassette from foil pack and **Visually Inspect the Cassette**:



- ✓ **Inspect the gel columns.** Look for obvious breakage of the agarose column in each channel. If there is breakage, *do not use the lane at all*. Remaining lanes can be used

- ✓ **Inspect for bubbles in the optical detection region.** If bubbles are present, *do not use the lane as a reference (marker lane)*. It can be used for sample collection. However, the samples tend to run faster in those lanes, so add 10-20 bp to size range to account for this issue. Bubbles on the top surface of the gel will have no effect on optical detection or purification, so it can be used.

Pippin Prep Size Selection (cont.)



- ✓ **Inspect the levels of buffer in all buffer reservoirs.** Reservoirs should be nearly full, so if any buffer level appears less, fill with spare electrophoresis buffer after tape is removed.

4. Dislodge bubbles from behind the elution wells. Tilt the cassette samples well side down and tap the elution well side to release any trapped bubbles.

5. Place gel cassette into the optical nest. Keep the cassette slightly tilted down so that the bubbles by the elution well do not return.

6. Remove the white tabbed adhesive strips from the cassette and place them on the foil wrapper (one can reuse the tape to reseal the gel if there are any unused lanes). Grab the white tabs of the tape and pull the strips firmly and slowly toward the front of the Pippin Prep. *Do not remove the tape of the elution modules if there are individual seals over each elution well and skip to step 8 (and skip step 10).*

7. Remove the buffer from all the elution wells and replace with 40 µl of fresh electrophoresis buffer. Make sure the pipette tip is all the way at the bottom of the well and then dispense the buffer while pulling up with the pipette.

8. Check the buffer level in the sample wells. The wells should be completely filled to the top with buffer and if any wells are under-filled, and then top them up with additional electrophoresis buffer.

9. **Perform the continuity TEST**. Close the lid and press the "Test" button located in the lower right area of the Main screen. The test routine runs automatically and measures the current in each separation and elution channel. If continuity tester indicates "PASS" for each separation and elution channel, then click "RETURN" and proceed to the next step. If the tester indicates "FAIL", the lane and channel (separation or elution) that are out of range will be highlighted with an orange color and do one of the following steps:

- ✓ If a separation lane is out of specified range, try restarting the software and trying again. In addition, check the levels of the buffer in the sample wells and anode buffer chamber. If the lane fails again, do not use lane and notify Sage Science of this problem.

- ✓ If an elution lane is out of specified range, remove buffer from the elution well and replace with 40 µl of fresh electrophoresis buffer. Retest. If it fails again, try replacing all the elution wells with fresh electrophoresis buffer and then Retest. If the lane fails again, it maybe be used as a reference (marker) lane, but may not be used to collect a sample.

Pippin Prep Size Selection (cont.)

10. If there was a single white tabbed adhesive strip over the elution wells, then replace with a new piece that is next to the machine. Start the tape at the corner of the elution modules, just past the optical region, and make sure it covers the elution well, small hole in gel, and rectangle part of the cathode chamber. *No tape should be in the optical region.* One can score the tape between each elution well with a razor, to help prevent sample cross contamination from the wicking of droplets when peeling off the adhesive later.

11. Press the tape over the elution wells firmly with a blunt smooth object (finger or sharpie), to ensure a good seal.

12. Remove 40 µl of buffer from the sample wells. Take care not to pierce the agarose with the pipette tip. There is gel on all sides and bottom of the sample well. There should be some buffer (~30 µl) left in the well. Wait 30 secs and check to make sure no wells refill with buffer. If a lane does refill, take out an additional 40 µl of buffer. If the lane keeps refilling with buffer, then one can use this lane as the reference (marker) lane or Input sample lane, but *do not use the lane for a ChIP sample*. In step 14, load this well last by removing 40 µl of buffer and quickly adding the marker or Input sample.

13. **Set Up Size Selection Protocol:**
- Open up the **"Protocol Editor"** tab
- Click "Load" if using a previously saved protocol, if not continue to next step
- Select Cassette Type:
  - For 2% EF Agarose Gel Cassette select **2%EF Marker E V2**
  - For 2% Agarose Gel Cassette select **2% Marker B No Overflow Detection**
- Select Mode for each Lane:
  - **Ref** for the marker
  - **Range** for the samples
  - **Off** for the lanes not being run
- Enter Size Range:
  - For Genomic DNA Adapters; **BP Start:** 180, **BP End:** 270, **BP Pause:** 220
  - For Multiplexed Adapters; **BP Start:** 170, **BP End:** 260, **BP Pause:** 210
  - For TruSeq Adapters; **BP Start:** 230, **BP End:** 320, **BP Pause:** 270
- Enter Sample ID Template Names
- Make sure the BP Range Flag is Broad and the Pause Enabled is highlighted yellow
- Click "**Save As**" at the bottom right of the screen to save your protocol.
- Enter Protocol Name, whatever you like. It automatically stamps it with date and time.
- If you make any changes to the protocol, hit "**Save**" from now on.
- Click "**Main**" tab to get back to the main screen. Your protocol should appear there now.

14. **Load 40 µl of sample into their respective sample well**. Once again, take care not to pierce the agarose with the tip. Do not be concerned if the sample well slightly overfills. The density of the sample will allow it to sink before it can flow out of the well.

15. Press the "**Start**" button.

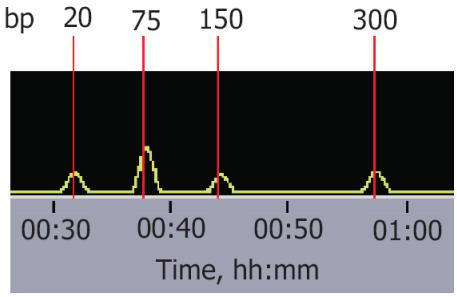
16. If any mistakes were made, like adding the wrong marker or selecting the wrong cassette type, one can catch the error just by checking on the progress of the run. Make sure the ladder peaks come up when they are supposed to (see markers below). The machine will continue to run and not PAUSE, unless it can detect the marker. Worst case, Stop the run and rerun it in Manual Mode to collect fractions.

Pippin Prep Size Selection (cont.)

✓ **2% EF Agarose Gel Cassette Marker E**

Size (bp)	Time to Detector (min.)	Time to Collect (min.)
20	34	NA
75	40	NA
150	46	51
300	60	65
600	88	96

run times vary based on temperature and other factors. This is an approximate guideline — actual values may vary up to ±5 min.

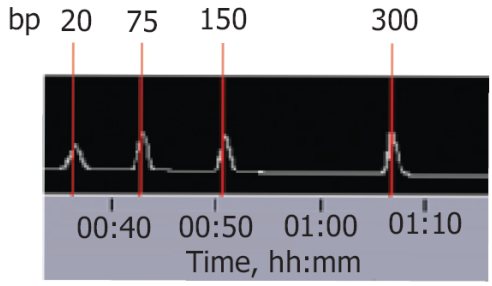


Reference DNA, 2EF% Gel
Marker E

✓ **2% Agarose Gel Cassette Marker B**

Size (bp)	Time to Detector (min.)	Time to Collect (min.)
20	37	NA
75	43	NA
150	50	57
300	65	73
600	94	111

run times vary based on temperature and other factors. This is an approximate guideline — actual values may vary up to ±5 min.



Reference DNA, 2% Gel
Marker B

17. After approx. 50 mins the run should be paused and the **"RESUME"** button will be available. If the run is still progressing and the **Elute** boxes are orange, that means it is still collecting the first fraction and just wait until it pauses. If the run is still progressing and the **Separate** boxes are green, that means it has not reached the collection BP Start size or there was a mistake.

18. Remove the adhesive over the elution wells and collect the entire volume (~40-60µl) and place into a new Eppendorf tube for each sample/lane.

19. Using a p10 pipette and thinner tip, Rinse each elution well with 10 µl of Elution Buffer and put into corresponding tube. Make sure to get into the corners to get every last drop.

20. Add 40 µl of fresh electrophoresis buffer to the elution well and Replace Adhesive.

21. Click the **"RESUME"** button.

22. After 10-15 mins return to machine and make sure the **Elute** boxes are not orange and the 300bp marker has appeared. Manually stop the run by clicking the **"STOP"** button.

23. Remove the adhesive over the elution wells and collect the entire volume (~40-60µl) and place into a fresh Eppendorf tube for each sample/lane. This is your "top band" equivalent, and is a failsafe measure. ***Throw out Gel, and do not leave it in the machine.***

Enrich the Adapter-Modified DNA Fragments by PCR

In this protocol you will perform PCR amplification using the size selected DNA. It is beneficial to use 0.5 ml PCR tube, so one can add 250 µl of PB for cleanup easily.

Cleanup Method

- Agencourt Ampure XP Beads (or Qiagen MinElute PCR Purification Kit below)
- Freshly prepared 70-80% Ethanol

Sample Prep Kit Components

- Phusion polymerase
- 5x Phusion HF buffer
- 10 mM dNTP mix
- PCR primer 1.1 or Multiplex PCR Primer 1
- PCR primer 2.1 or Multiplex PCR Primer 2 with Index

Master Mix (MM) Kit Components

- Phusion HF PCR Master Mix (Optional)
- PCR primer 1.1 or Multiplex PCR Primer 1
- PCR primer 2.1 or Multiplex PCR Primer 2 with Index

Procedure

1. Prepare the following PCR mix:	x1	x5
▪ 5x Phusion HF buffer	10 µl	50 µl
▪ dNTP mix	1.5 µl	7.5 µl
▪ PCR primer 1.1	1 µl	5 µl
▪ PCR primer 2.1	1 µl	5 µl
▪ Phusion polymerase	0.5 µl	2.5 µl
	14 µl	

2. **2% Agarose Size Selection:** Add 14 µl of PCR mix to 36 µl of DNA sample.
Pippin Prep Size Selection: Add H_2O until the DNA sample volume is 72 µl. Double the PCR Mix volumes above and Add 28 µl of PCR mix to 72 µl of DNA sample. Either run the PCR in 100 µl or split the PCR reaction into two 50 µl reaction volumes and recombine on the same column during purification.

OR

Pippin Prep Size Selection PCR Master Mix: Add 2 µl of each PCR Primer (4 µl total) and 50 µl of Phusion HF PCR Master Mix to 40-60 µl DNA sample. Either run the PCR in 100 µl or split the PCR reaction into two ~50 µl reaction volumes and recombine on the same column during purification.

3. Amplify using the following PCR protocol (solexa1):

a. 30 seconds at 98°C
b. 18 cycles of:
- 10 seconds at 98°C
- 30 seconds at 65°C
- 30 seconds at 72°C
c. 5 minutes at 72°C
d. Hold at 4°C

Enrich the Adapter-Modified DNA Fragments by PCR (cont.)

4a. Purify using Agencourt Ampure XP Beads: *Make sure beads came to Room Temp before proceeding*

- Vortex/mix the beads until they are well dispersed, and keep doing so every couple of samples
- Add 90 µl of Ampure XP beads for every 50 µl PCR volume and mix by pipetting at least 10x
- Incubate the tubes at Room Temp for 10-15 mins
- Place tubes on magnetic stand and let sit for 2 mins or until the liquid appears clear
- Remove and discard the majority of the supernatant from each tube
- Add 200 µl 70-80% Ethanol and let it incubate on the stand for 1 min and then Discard Ethanol
- Repeat Ethanol wash one more time, making sure to completely remove all traces of Ethanol
- Let stand at Room Temp for 5-10 mins to dry and remove all Ethanol
- Remove from magnetic stand and resuspend the dried beads in **16 µl EB**
- Mix by pipetting 10x and Incubate at Room Temp for 5 mins.
- Place tubes on magnetic stand and let sit for 2 mins or until the liquid appears clear
- Transfer **15 µl** of the supernatant to a new tube

4b. Purify using MinElute PCR Purification Kit: *Recommended if used the Pippin Prep*

- Add 250 µl Buffer PB and mix by pipetting
    - **Pippin Prep**: If Split the PCR reaction, then Recombine the sample reactions. Add 500 µl PB and 2 µl of pH Indicator. If orange or purple in color, Add 4 µl of 3 M sodium acetate (NaOAc pH5.5). If it's not bright yellow, Add NaAc in 1 µl increments until it turns yellow.
- Apply to column and spin at max speed 30 sec
- Pour flowthrough back on to column and spin at max speed 30 sec
- Discard flowthrough
- Add 750 µl Buffer PE and allow to stand 1 min
- Spin at max speed 45 sec and discard flowthrough
- Spin empty column at max speed 1 min
- Transfer column to new 1.5 ml tube
- Aspirate around purple ring inside column
- Allow to air dry 1 min
- Apply **15 µl Buffer EB** and let stand 2 min
- Spin at max speed 1 min and discard column

Validate the Library

The amount of starting material is very low, and after 18 cycles of PCR, the yield could still be too low to see on a regular gel, even though it is enough for cluster generation. Illumina recommends using an Agilent Technologies 2100 Bioanalyzer to check the size, purity, and concentration of the sample library. Generally 40nM and below has PCR artifacts and is not a good library. The Good Libraries below have high molarity and nice narrow peak. The Okay MP Library is broad and slightly higher in size than recommend, but can be sequenced. In addition, the Okay MP Library has a peak at ~80bp, which is the PCR primers, that will not cluster to the flowcell. Bad Library has adapter junk at 100bp (for Genomic Adapters, it will be 120-130bp for Multiplex and TruSeq Adapters) that will cluster and thus will cause less usable reads. In addition, Bad Library is broad, which could still be used, if not for the adapter junk, just it is better for the analysis if the peak is narrower. Okay Library has too low molarity and a broad peak, which usually means there will be PCR biasis and is not worth proceeding; however, there is a possibility it could have worked, but it will have to be sequenced to know. If followed this protocol, one should not get a Bad Library, but one could still get a low molarity library, as not every ChIP has the same yield and this is dependent on the starting amount and sonication.