

Myers Lab ChIP-seq Protocol, v042211.1 and v042211.2

Modified April 22, 2011

Contact information:

Dr. Florencia Pauli
HudsonAlpha Institute for Biotechnology
601 Genome Way
Huntsville, AL 35806
Telephone: 256-327-5220
Email: fpauli@hudsonalpha.org

Dr. Richard Myers
HudsonAlpha Institute for Biotechnology
601 Genome Way
Huntsville, AL 35806
Telephone: 256-327-0431
Email: rmyers@hudsonalpha.org

Introductory information

This set of protocols are currently in use by the Myers lab at HudsonAlpha Institute for Biotechnology for performing ChIP-seq, a method for identifying on a genome-wide level the sites of occupancy of DNA binding proteins in the nuclei of living cells (see Johnson et al., 2007, Science 316: 1497-1502). We have used this method in our work as part of the ENCODE Project, where our group is analyzing the occupancy of dozens of sequence-specific transcription factors, as well as RNA Polymerase and several other general factors, in a variety of human cell lines. We have also applied these methods to other human and mouse projects.

This set of protocols describes the experimental details of how we perform the steps of ChIP-seq in human cells. This includes growing cultured cells and cross-linking the chromatin, shearing the chromatin, immunoprecipitating the desired complexes by ChIP, and preparing the library for sequencing on Illumina GAIIx sequencers.

For each ChIP-seq experiment, we typically perform one ChIP reaction on chromatin prepared from about 2×10^7 human cells, then use the ChIP'd DNAs to construct libraries for the Illumina sequencing platform.

It is critical to perform a Control experiment in every cell line for which ChIP-seq is done. Control chromatin can be either i) sonicated reverse-cross-linked crosslinked chromatin (which has not been subjected to any antibody treatment), or ii) immunoprecipitation with a control IgG antibody that does not recognize any DNA binding protein (a "mock IP"). At HudsonAlpha, we use the reverse-crosslinking method for our Control experiments, as we have found that the mock IP approach generates very little DNA and does not provide a good baseline for a peak calling control. The reason a Control experiment is so important is that some regions of the genome, particularly those at open chromatin regions, are sheared more readily than others, and these small fragments can be over-

represented in the sequencing step, giving the false conclusion that these regions are sites of occupancy. By performing the reverse-crosslink Control separately from the antibody ChIP experiments, these false signals can be subtracted on a region-by-region basis. Indeed, the peak-calling algorithms for ChIP-seq take this issue into account.

In the following pages, we describe each of the steps for preparing samples for sequencing in ChIP-seq. This protocol differs from our previous protocols in that the incubation times for ChIP have been reduced from overnight to 2 hours, a Bioruptor Twin (Diagenode) was used for chromatin sonication and fragment size selection for sequencing library construction was done using Agencourt Ampure XP beads.

I. Growth of cells

Note: ChIP works best when cells are healthy and in “log phase” growth. Do not grow suspension cells to maximum density or to high acidity, and, for adherent cells, do not grow cells to confluency.

A. Suspension cells (for example, K562, Jurkat, GM06990)

1. Grow cells to log phase growth (10^6 cells/ml maximum density).
2. Count cells and measure viability by staining an aliquot of the cells with trypan blue.

If viability, as measured by exclusion of the blue dye, is >90%, proceed to the next steps. If viability is lower, discard the experiment and start over with new cells.

B. Adherent cells (for example, HepG2, HCT-116)

It is easier to process adherent cells growing on tissue culture plates rather than in flasks.

1. Prepare an additional plate for counting. Grow cells to a final cell density of $2-5 \times 10^7$ per 150-mm dish.
2. Trypsinize one tissue culture plate. Count and test for viability by removing a small aliquot and staining with trypan blue.

If viability, as measured by exclusion of the blue dye, is >90%, proceed to the next steps. If viability is lower, discard the experiment and start over with new cells.

II. Cross-linking of chromatin, harvest, and storage

Note: Suspension and adherent cells are cross-linked and collected differently. For ease of experimentation, we recommend harvesting in aliquots of 2×10^7 cells.

Reagents:

All reagents are at room temperature unless otherwise noted.

20% formaldehyde [36.5% Formaldehyde for molecular biology Sigma F87750]

2.5 M glycine

1X PBS 4°C

Farnham Lysis buffer 4°C 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40 (adherent cells) + Roche Protease Inhibitor Cocktail Tablet (Complete 11836145001 for 50 ml or mini tablets 11836153001 for 10 ml)
[Add protease inhibitor tablet just before use]

A. Suspension cells

1. Transfer cells and media from the tissue culture flasks into either 50-ml conical tubes or centrifuge bottles.
2. Add formaldehyde to a final concentration of 1%, mix gently, and incubate at room temperature for 10 minutes.
3. To stop the cross-linking reaction, add glycine to a final concentration of 0.125 M and mix gently.
4. Pellet cells at 2,000 rpm for 5 minutes at 4°C.
5. Place cells on ice and remove supernatant. Add equal volume cold (4°C) 1X PBS and gently resuspend cells, then centrifuge again.
6. Carefully remove supernatant and either proceed to sonication step or snap-freeze in liquid nitrogen and store at -80°C or in liquid nitrogen.

Note: Frozen cross-linked cells appear to be stable indefinitely. Therefore, this is a convenient step in the protocol to collect many samples for testing multiple antibodies, biological replicates, controls, etc.

B. Adherent cells

1. To adherent cells growing on tissue culture plates, add formaldehyde directly to the media to a final concentration of 1%, swirl gently, and incubate at room temperature for 10 minutes.
2. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M and swirl gently to mix.
3. Remove media from plates and wash cells with equal volume cold (4°C) 1X PBS.
4. Aspirate PBS and add 5-8 ml cold (4°C) Farnham lysis buffer + protease inhibitors.
3. Scrape the cells off the plate with a cell scraper and transfer into 15-ml conical tubes on ice.
4. Pellet cells at 2,000 rpm for 5 minutes at 4°C.
5. Place cells on ice. Carefully remove supernatant and either proceed to sonication step or snap-freeze in liquid nitrogen and store at -80°C or in liquid nitrogen.

Note: Frozen cross-linked cells appear to be stable indefinitely. Therefore, this is a convenient step in the protocol to collect many samples for testing multiple antibodies, biological replicates, controls, etc.

III. Sonication

Note: The Myers lab has used three methods for sonicating chromatin. All of our experiments until Fall 2009 used a Sonics VibraCell sonicator, a relatively inexpensive approach that we fine-tuned to fragment the chromatin to a specific size range. After that time, we began using a Bioruptor sonicator, model XL, which is much easier (multiple samples can be sonicated at the same time) and cleaner (the samples are closed during the sonication treatment). Beginning in October of 2010, we began using a Bioruptor Twin (Diagenode) sonicator. All of the ChIP-seq datasets submitted to the ENCODE project and labeled as protocol v042211.1 or v042211.2 were done with the Bioruptor Twin. The reagents used are the same, but the methods differ. All methods are described below.

Reagents:

Farnham Lysis buffer: 5 mM PIPES pH 8.0 / 85 mM KCl / 0.5% NP-40 (filtered 0.2 - 0.45 micron filter unit) + Roche Protease Inhibitor Cocktail (complete) 4°C
[Add protease inhibitor tablet just before use]

RIPA Buffer: 1XPBS / 1% NP-40 / 0.5% sodium deoxycholate / 0.1% SDS (filtered 0.2 - 0.45 micron filter unit) + Roche Protease Inhibitor Cocktail 4°C
[Add protease inhibitor tablet just before use]

Sonics VibraCell Sonicator:

1. Resuspend each fresh or frozen pellet (containing 2×10^7 cells) on ice in 1 ml Farnham Lysis Buffer and mix gently by flicking the test tube.

Note: This treatment breaks the cells while keeping the nuclei mostly intact. The Farnham lab includes a douncing step here to ensure that the cells are completely broken. We have found that douncing has not been necessary, but it is possible that it will increase yields and/or purity with some cell types.

2. Collect the crude nuclear prep by centrifuging at 2,000 rpm at 4°C for 5 minutes.

3. Resuspend pellet to 1 ml with RIPA Buffer.

Do not vortex the tubes and try to avoid bubbles. Bubbles will cause popping and loss of samples during sonication.

4. Sonicate each 1.0 ml ChIP sample on ice, in a cold room, at Power Output 5 watts 6 times for 30 seconds each, with at least 30 second cooling on ice between each 30-second sonication.

Note: For ChIPSeq, it is critical that the average length of the sheared chromatin is about 250 bp or less, with a range of about 100-600 bp. If the average size range is

much higher, there will be very little DNA in the portion of the gel from which the 100-250 bp fragments will be extracted.

Important note: *Our lab uses a Sonics VibraCell sonicator. Different sonicator models and even different individual sonicators of the same model may vary in the settings and conditions needed for fragmenting DNA to a particular size range. We strongly recommend that each group do a titration experiment with cross-linked chromatin to determine conditions for shearing to an average of 250 bp before attempting a ChIP experiment. See detailed discussion of sonication below.*

5. Spin the sonicated mixture at 14,000 rpm in a microfuge for 15 minutes at 4°C and collect the supernatant.
6. Snap-freeze the sample in liquid nitrogen and store at -80°C, or do not freeze and continue with the immunoprecipitation steps below.

Bioruptor (Diagenode, model XL) Sonicator:

1. Resuspend each fresh or frozen pellet (containing 2×10^7 cells) on ice in 1 ml Farnham Lysis Buffer and mix gently by flicking the test tube.

Note: This treatment breaks the cells while keeping the nuclei mostly intact. The Farnham lab includes a douncing step here to ensure that the cells are completely broken. We have found that douncing has not been necessary, but it is possible that it will increase yields and/or purity with some cell types.

2. Collect the crude nuclear prep by centrifuging at 2,000 rpm at 4°C for 5 minutes.
3. Resuspend pellet to 300 μ l with RIPA Buffer.
4. Process samples in Bioruptor at high setting for a total time of 15 minutes, 30 seconds ON, 30 seconds OFF. The Bioruptor is connected to a re-circulating water chiller that maintains the water bath temperature at 4°C.

Important note: *Our lab uses a Diagenode XL Bioruptor. Different sonicator models and even different individual sonicators of the same model may vary in the settings and conditions needed for fragmenting DNA to a particular size range. We strongly recommend that each group do a titration experiment with cross-linked chromatin to determine conditions for shearing to an average of 250 bp before attempting a ChIP experiment.*

5. Spin the sonicated mixture at 14,000 rpm in a microfuge for 15 minutes at 4°C and collect the supernatant.
6. Bring the volume of the sonicated sample to 1 μ l with RIPA buffer.

7. Snap-freeze the sample in liquid nitrogen and store at -80°C , or do not freeze and continue with the immunoprecipitation steps below.

Bioruptor (Diagenode, model Twin) Sonicator:

1. Resuspend each fresh or frozen pellet (containing 2×10^7 cells) on ice in 1 ml Farnham Lysis Buffer and mix gently by flicking the test tube.

Note: This treatment breaks the cells while keeping the nuclei mostly intact. Although we had found that a douncing step was not necessary when using the Vibracell or Bioruptor XL sonicators, we found that passing the lysate through a 20G needle 20 times increased our yield when using the Bioruptor Twin. This step is optional and should be tested for each cell line.

2. Collect the crude nuclear prep by centrifuging at 2,000 rpm at 4°C for 5 minutes.

3. Resuspend pellet to 300 μl with RIPA Buffer.

4. Process samples in Bioruptor Twin with circulating water bath according to the manufacturer's instructions for temperature (less than 4°C) and speed of circulation (<500 mL per minute) at high setting. We perform a minimum of two 10-minute rounds of 30 seconds ON, 30 seconds OFF, but these conditions should be determined empirically for each cell type.

Important note: *Our lab uses a Diagenode Twin Bioruptor. Different sonicator models and even different individual sonicators of the same model may vary in the settings and conditions needed for fragmenting DNA to a particular size range. We strongly recommend that each group do a titration experiment with cross-linked chromatin to determine conditions for shearing to an average of 250 bp before attempting a ChIP experiment.*

5. Spin the sonicated mixture at 14,000 rpm in a microfuge for 15 minutes at 4°C and collect the supernatant.

6. Bring the volume of the sonicated sample to 1 μl with RIPA buffer.

7. Snap-freeze the sample in liquid nitrogen and store at -80°C , or do not freeze and continue with the immunoprecipitation steps below.

IV. Immunoprecipitation

Reagents:

All reagents are at room temperature unless otherwise noted.

Magnetic beads:	Dynal / Invitrogen Dynalbeads (sheep anti-rabbit or anti-mouse IgG, or protein G)
PBS/BSA:	1XPBS + 5 mg/ml BSA (fraction V) (freshly-prepared and filtered 0.2 - 0.45 micron filter unit) 4°C + Roche Protease Inhibitor Cocktail [Add protease inhibitor tablet just before use]
LiCl IP Wash Buffer:	100 mM Tris pH 7.5 / 500 mM LiCl / 1% NP-40 / 1% sodium deoxycholate (filtered 0.2 - 0.45 micron filter unit) 4°C
TE:	1X TE (10 mM Tris-HCl pH 7.5 + 0.1 mM Na ₂ EDTA) 4°C
IP Elution Buffer:	1% SDS / 0.1 M NaHCO ₃ (filtered through 0.2 - 0.45 micron filter unit)

1. Perform all steps in an ice bucket or in the cold room at 4°C. Couple the primary antibody for each transcription factor or chromatin protein to magnetic beads as follows:
 - a. Add 200 µl re-suspended magnetic bead slurry to a 1.5 ml microfuge tube on ice containing 1 ml PBS/BSA. Vortex briefly to mix well.
 - b. Place the microfuge tubes on the magnet and remove supernatants.
 - c. Resuspend the beads in 1 ml PBS/BSA.
 - d. Repeat Steps b and c 3 times.
 - e. Add 1 ml PBS/BSA to beads.
 - f. Add 5 µg primary antibody (25 µl 200 µg/ml from Santa Cruz).
Do not vortex beads with bound antibody.
 - g. Gently mix on a rotator platform for at least 2 hours at 4°C.
 - h. Wash beads 3 times (steps b-c), resuspending the beads by inverting the tubes during each wash.
 - i. Resuspend in 100 µl PBS/BSA, and proceed to Step 2.

2. Add 100 μ l of antibody-coupled beads (from step 1.i above) to each 1 ml chromatin preparation (from Sonication protocol) and incubate on a rotator for one hour at room temperature, followed by one hour at 4°C.
3. Perform all steps in the cold room at 4°C. Collect beads containing immuno-bound chromatin by placing the microfuge tube on the magnet.
4. Remove and discard supernatant.
5. Wash beads 5 times with LiCl Wash Buffer, mixing 3 minutes for each wash on a rotator.
6. Wash bead pellet with 1 ml TE Buffer. Mix 1 minute on rotator and place tubes on magnet.
7. Discard supernatant. Resuspend the bead pellet in 200 μ l IP Elution Buffer (at room temperature). Vortex to mix.

V. Reverse Cross-linking and recover DNA ChIP and Control DNA

1. Incubate in a 65°C water bath for 1 hour, shake or vortex every 15 minutes, to elute the immuno-bound chromatin from the beads.
2. Spin at 14,000 rpm in a microfuge at room temperature for 3 minutes. Collect the supernatant, which contains the ChIP'd DNA. The tubes can be placed on the magnet to facilitate supernatant recovery.
3. Incubate the supernatant containing the ChIP'd DNA in a 65°C water bath overnight to complete the reversal of the formaldehyde cross-links.
4. To prepare a Reverse Cross-link Total Chromatin library, thaw a 1-ml chromatin tube, divide into two 500 μ l aliquots and incubate in a 65°C water bath overnight to reverse the cross-links in that sample.
5. Add 5 volumes Qiagen Buffer PB (PCR cleanup kit) to one volume of ChIP'd DNA or Reverse Cross-link sample. Upon addition of Buffer PB, the sample should be yellow, indicating the correct pH. If the sample is not yellow, the pH should be adjusted with 3M sodium acetate as recommended by the manufacturer (Qiagen).
6. Add about half (~600 μ l) of the solution to a Qiagen PCR Cleanup column, centrifuge, and then repeat with other half to bind the 1.2 ml sample on a Qiagen column.
7. Wash the column with 750 μ l Qiagen Buffer PE, centrifuge, empty, and centrifuge the column, which contains the DNA, to dry.

8. Elute the DNA from the column with two 30 μ l aliquots of warmed ($\sim 55^{\circ}\text{C}$) Qiagen Buffer EB.

9. Measure DNA concentration by using a Qubit (Invitrogen) fluorometer.

DNA recovery varies by antibody used for ChIP and ranges from 5-50+ ng from a 1-ml chromatin prep of 2×10^7 cells. Total chromatin recovery is about 20 μ g. Reserve 25 μ l for qPCR. Store at -80°C .

DNA is ready for Illumina library construction as per our protocol in Section IX.

VI. Checking size of sonicated ChIP'd fragments.

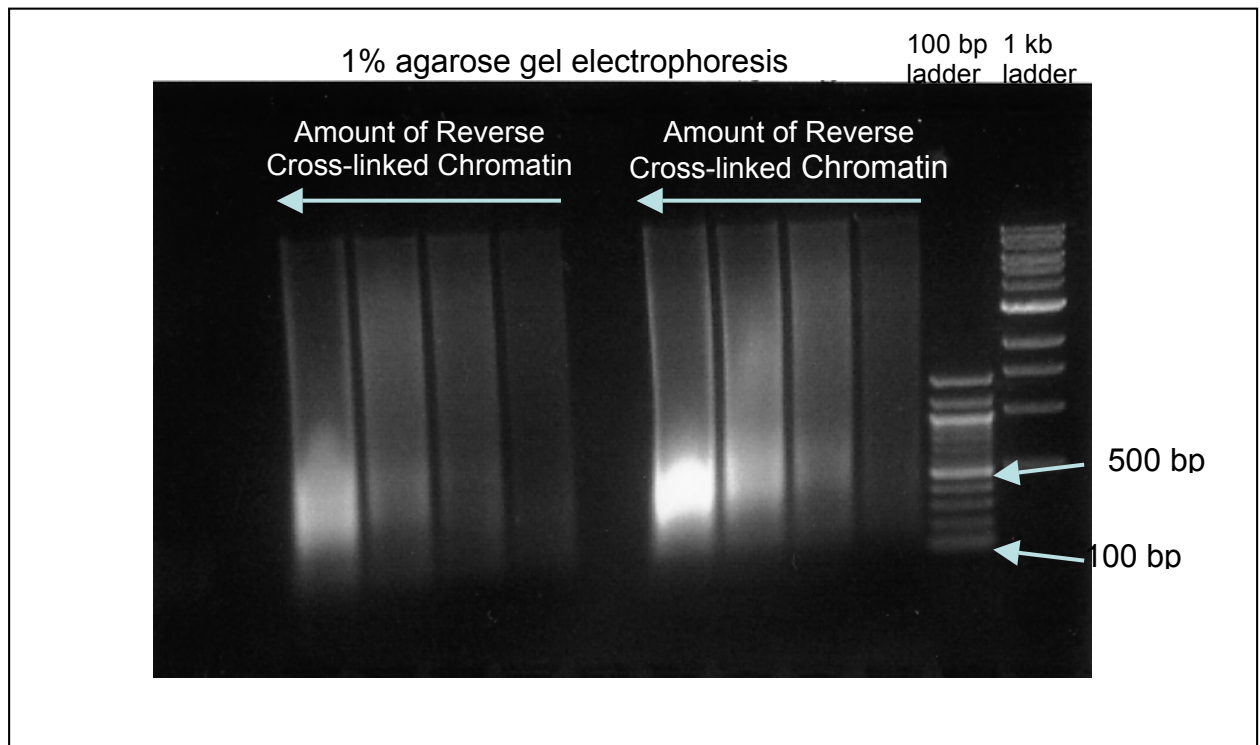
We perform this step only on the total chromatin DNA, as both are prepared from the same sonicated sample. This test should be done on each experiment prior to constructing the Illumina sequencing library.

1. Load ~1 μ l, 2 μ l, 4 μ l and 8 μ l aliquots of reverse cross-linked DNA (from samples described in Step 4 in Section V: Immunoprecipitation Protocol) in lanes of a 1% agarose gel.

Note: We run this wide range of amounts to be sure we can assess the size range accurately; due to variation in the yield and range of fragment sizes varies, you may learn that your sonication protocol requires a different range of amounts for this test.

2. After electrophoresis, examine the gel and compare the midpoint of the sample's size distribution to 100 bp ladder size markers (NEB).

3. Ideally, the DNA should be in a relatively tight distribution between about 100 bp and 500 bp, such as in the gel below.



VIII. Sonication details for VibraCell Sonicator.

We use the stepped microtip #630-0422 (the distal tip of this microtip is a 3-mm diameter cylinder). For the first use and when the microtip is changed, it is important to recalibrate the sonication conditions. Calibrate as follows:

1. Prepare 4 samples of cross-linked chromatin as in Section III (1-34) above.
2. Sonicate for 30 seconds, continuously, on amplitude setting 5 or 6 as follows:
 - a. Sample 1: 2 x 30 seconds
 - b. Sample 2: 4 x 30 seconds
 - c. Sample 3: 6 x 30 seconds
 - d. Sample 4: 8 x 30 seconds

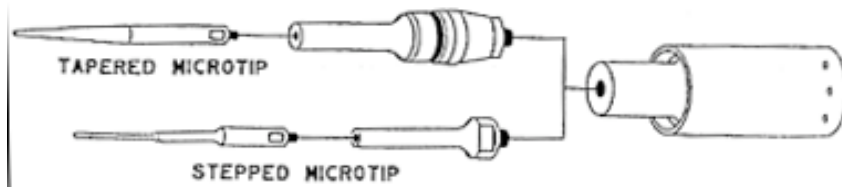
Notes: Dial up slowly to amplitude 5, and start counting to 30 seconds after the sonicator reaches the 5 setting. Watch the dial the entire time to be sure that it does not go below 5; if it does, dial up slowly to 5 again.

The sonicator tip should be submerged about halfway to the bottom of the liquid in the microfuge tube. These conditions should not result in bubbling of the liquid.

Important: Leave microfuge tube on ice for 30 seconds between each 30-second sonication.

3. After sonication, reverse the cross-links as in Section V, steps 4-12.
4. Load 1, 2, 4 and 8 ml of each sample from each sonication condition onto a 1% agarose gel as in Protocol VI above.
5. Determine which sonication condition gives the appropriate size range for ChIPSeq (100-500 bp, with the center of the distribution at 250 bp or lower).

Below is a drawing of the microtips of the Sonics VibraCell sonicator. The stepped microtip on the bottom is the one we use.



IX. Illumina Sequencing Library Construction

DNA fragments recovered from ChIPs or reverse cross-linked chromatin are repaired, ligated to adapters, size selected and PCR-amplified to make the library for sequencing.

Illumina DNA Library Construction Kit reagents are substituted in this protocol with reagents from NEB and Finnzymes, except for the adapter oligo mix and the PCR primers, which can be ordered from Illumina. We currently use paired-end adapters for library construction, even though we sequence our ChIP libraries with a single-end sequencing run.

NEB reagents:

- NEB N0447 – dNTP Deoxynucleotide Solution Mix
- NEB M0203 – T4 DNA Polymerase 3,000 units/mL
- NEB M0201 – T4 Polynucleotide Kinase 10,000 units/mL
- NEB M0210 – Klenow DNA Polymerase 5,000 units/mL
- NEB M0212 – Klenow Fragment (3' to 5' exo-) 5,000 units/mL
 - Supplied with 10X NEBuffer2 used for dA addition step
- NEB M0202 – T4 DNA Ligase
 - Supplied with 10X DNA ligase buffer. This buffer is used for the end-repair and the adapter ligation steps of the protocol.

Finnzymes:

- F531 – Phusion High-Fidelity PCR Master Mix

DNA in EB can be stored in the freezer at any step.

DNA in agarose can be stored overnight at 4°C.

1. End Repair

Be sure to use Klenow (E. coli DNA polymerase large fragment) and not the Klenow exo-polymerase for this step.

- a. Mix in a PCR tube on ice:
 - 5.0 µl 10X T4 DNA ligase buffer (with 10mM ATP)
 - 0.5 µl 10 mM dNTP mix (each @ 10 mM)
 - 41.5 µl recovered DNA fragments
 - 1.0 µl T4 DNA polymerase
 - 1.0 µl T4 Polynucleotide Kinase
 - 1.0 µl Klenow DNA polymerase
- Total volume = 50 µl

Note: when processing more than one sample, a master mix that includes all reagents except for the recovered DNA fragments can be prepared.

- b. Spin down briefly and incubate at 20°C in a PCR machine for 30 minutes.

- c. Purify on one QIAquick PCR cleanup column and elute with 32 μ l EB warmed to 55°C. Allow EB to sit on the filter in the column for 1 minute before spinning for 1 minute.

2. dA Addition

Be sure to use correct Klenow enzyme (3' to 5' exo-)

- a. Mix in a PCR tube on ice:
 - 32 μ l end-repaired DNA fragments
 - 10 μ l 1mM dATP
 - 5 μ l 10X NEBuffer2
 - 3 μ l Klenow fragment (3' to 5' exo-)Total volume = 50 μ l
- b. Spin down briefly and incubate at 37°C in a PCR machine for 30 minutes.
- c. Purify on one QIAquick PCR cleanup column and elute with 42 μ l EB warmed to 55°C. Allow EB to sit on the filter in the column for 1 minute before spinning for 1 minute.

3. Adapter Ligation

- a. Mix in a PCR tube on ice:
 - 42 μ l DNA recovered from dA addition (Step 2)
 - 5 μ l T4 DNA Ligase buffer (NEB)
 - 0.5 μ l Adapter oligo mix
 - 0.5 μ l ddH₂O
 - 2 μ l T4 DNA ligase (NEB)Total volume = 50 μ l
- b. Spin down briefly and incubate at 20°C in a PCR machine for 15 minutes.
- c. **Protocol version v042211.1:** Purify DNA fragments with Agencourt Ampure XP beads (Beckman-Coulter), following manufacturer's recommendations. Elute with 32 μ l EB, then proceed to step 5 (Library Amplification by PCR).

Agencourt Ampure beads use solid phase reversible immobilization (SPRI) technology to recover dsDNA greater than 100bp. This step is carried out instead of gel fragment size selection since it serves to exclude adapters that were not ligated to DNA (mimicking the lower threshold of gel size selection in step 4). An extension time of 30 seconds during PCR amplification ensures that the fragments sequenced are not too long (mimicking the upper threshold of gel size selection in step 4).

Protocol version v042211.2: Purify on one QIAquick PCR cleanup column and elute with 50 μ l EB warmed to 55°C. Allow EB to sit on the filter in the column for 1 minute before spinning for 1 minute. Alternatively, skip this purification step if you are proceeding to step 4 immediately and can load the sample directly to the gel.

4. Gel Purification and Size Selection (only for protocol v042211.2)

Note: Gel purification removes the ~50 bp sequencing adapters and isolates the 150-300 bp fragments, allowing for higher density of productive clusters on the flow cell lanes.

- a. Pour and run gel in the cold room. Pour a 2% low-melting agarose gel (SeaPlaque) in 1X TAE with EtBr (final concentration in gel is 0.4 μ g/ml). Use NEB PCR Marker (N3234L) as DNA ladder.
- b. Load PCR products on gel and run gel at ~115 V until the loading dye has migrated 6 cm (~2 hours).
- c. Excise the gel region from 150 bp to 300 bp for each sample. Due to the low concentration of library DNA fragments at this step, they will not be visible on the gel. The adapters, however, are visible and should be carefully excluded from the extracted gel fragment. Image the gel before and after excision of the library, if desired.
- d. Use QIAquick Gel Extraction Kit with columns to extract DNA from the gel fragments. Follow the Qiagen protocol, with the exception of warming the gel and Buffer QG to 55°C to melt the gel. Instead, this should be done at room temperature by vortexing every 2-3 minutes until the gel is dissolved. Include the optional step of washing the column with 0.5 ml of Buffer QG before adding Buffer PE. Elute with 32 μ l 50°C EB. Allow EB to sit on the filter in the column for 1 minute before spinning for 1 minute.

5. Library Amplification by PCR

- a. Mix in a PCR tube on ice:
 - 32 μ l DNA fragments
 - 32 μ l Phusion DNA Polymerase Mix
 - 0.5 μ l PCR primer 1.1 (Illumina)
 - 0.5 μ l PCR primer 2.1 (Illumina)Total volume = 65 μ l
- b. Spin down briefly.

- c. Amplify in thermal cycler using the following protocol:
 - 98°C for 30 sec
 - 15 cycles of: 98°C for 10 sec
65°C for 30 sec
72°C for 30 sec
 - 72°C for 5 min
 - 4°C hold
- d. Clean up using Agencourt Ampure XP beads, following manufacturer's recommendations. Elute in 32 μ l EB.
- e. Quantify using a Qubit fluorometer with a high-sensitivity (HS) kit. The library is now ready for sequencing on the Illumina GAIIIX platform.