Preparing Samples for ChIP Sequencing of DNA

Contents

Component Information

A kit containing all reagents for library preparation can be purchased from Illumina:
ChIP Seq DNA sample prep kit is: IP-102-1001

Except for Adapter Oligo Mix and PCR Primers which should be acquired from Illumina, most other reagents can be purchased individually:

NEBNext™ DNA Sample Prep Reagent Set 1 (NEB E6000L or E6000S)
- this kit contains all reagents except for the Adapter Oligo Mix and PCR Primers

Individual Components:

- 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)
- 10mM dNTPs Mix
- 1 mM dATP
- Klenow Fragment (3' to 5' exo minus) (NEB M0212S)
- Quick DNA Ligation Kit (NEB M2200S)
- Phusion Polymerase (NEB F-530S)
- 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
- Oligo Only Kit (Illumina FC-102-1003)
    - Adapter Oligo Mix
    - PCR Primer 1.1
    - PCR Primer 2.1
- QIAquick PCR Purification Kit (QIAGEN 28104)
- MinElute PCR Purification Kit (QIAGEN 28004)
- QIAquick Gel Extraction Kit (QIAGEN 28704)

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.

Components

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

Procedure

1. Prepare the following reaction mix:

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

2. Add 10 ul of reaction mix to 40 ul ChIP DNA
   or
   Add 10 ul of reaction mix to 10ng Input DNA diluted to 40 ul with water

3. Incubate for 30 minutes at 20ºC.

4. Purify using QIAquick PCR Purification Kit:
   - Add 250 uL 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 ul 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 **36 ul Buffer EB** and let stand 1 min
     - accounts for loss of 2 ul volume during elution
   - 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.

Components

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

Procedure

1. Prepare the following reaction mix:

	x1	x5
▪ Klenow buffer (NEB buffer #2)	5 µl	25 ul
▪ 1mM dATP	10 µl	50 ul
▪ Klenow exo (3’ to 5’ exo minus)	1 µl	5 ul
	16 ul	

2. Add 16 ul of reaction mix to 34 ul DNA sample from previous step.

3. Incubate for 30 minutes at 37ºC.

4. Purify using MinElute PCR Purification Kit:
   - Add 250 uL 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 ul 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 **12 ul Buffer EB** and let stand 1 min
     - accounts for loss of 2 ul volume during elution
   - 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.

Components

- 2X Quick DNA ligase buffer
- Adapter oligo mix
- Quick DNA ligase
- MinElute PCR Purification Kit

Procedure

1. Dilute Adaptor oligo mix 1:10 in water

2. Prepare the following reaction mix:

	x1	x5
▪ 2X Quick DNA ligase Buffer	15 µl	75 ul
▪ Diluted adaptor oligo mix	1 µl	5 ul
▪ Quick DNA ligase	1 µl	5 ul
▪ Water	3 ul	15 ul
	20 ul	

2. Add 20 ul of reaction mix to 10 ul DNA sample from previous step.

3. Incubate for 15 minutes at 20ºC.

4. Purify using MinElute PCR Purification Kit:
   - Add 150 uL 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 ul 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 **12 ul Buffer EB** and let stand 1 min
     - accounts for loss of 2 ul 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.

Components

- 2% agarose gel
- 50X TAE buffer
- Low Molecular Weight DNA ladder
- 6X loading dye
- 10X Roche Buffer H
- SYBR Gold
- QIAquick Gel Extraction Kit

Procedure

1. Prepare a 50 ml, 2% agarose gel with TAE buffer. Final concentration of TAE should be 1X. **Do not add EtBr or any DNA stain.**

2. Dilute SYBR Gold 1:1000 in water (add 0.5 ul to 500 ul water)

3. Prepare the DNA ladder mix:

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

4. Prepare the loading dye mix:

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

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

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

7. Load 15 ul of DNA ladder mix to outside wells.

8. Run gel at 100 V for 45 minutes.

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

10. Using a clean razor for each lane, excise a region of gel containing the material in the 200 ±25 bp range. **Photograph the gel after the slice is excised.**

Size Select the Library (cont.)

11. 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 ul QG to a 100 mg gel piece)
    - Incubate at room temp. for 10 min, 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 ul 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 ul Buffer QG and spin at max speed 30 sec
    - Discard flowthrough
    - Add 750 ul 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 ul Buffer EB** and let stand 1 min
      - accounts for loss of 2 ul volume during elution
    - Spin at max speed 1 min and discard column

Enrich the Adapter-Modified DNA Fragments by PCR

In this protocol you will perform PCR amplification using the gel-extracted DNA.

Components

- Phusion polymerase
- 5x Phusion HF buffer
- 10 mM dNTP mix
- PCR primer 1.1
- PCR primer 2.1
- MinElute PCR Purification Kit

Procedure

1. Prepare the following PCR mix:

	x1	x5
5x Phusion HF buffer	10 ul	50 ul
dNTP mix	1.5 ul	7.5 ul
PCR primer 1.1	1 ul	5 ul
PCR primer 2.1	1 ul	5 ul
Phusion polymerase	0.5 ul	2.5 ul
	14 ul	

2. Add 14 ul of PCR mix to 36 ul of DNA sample from previous step.

3. Amplify using the following PCR protocol:
   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

4. Purify using MinElute PCR Purification Kit:
   - Add 250 uL 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 ul 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 ul Buffer EB** and let stand 1 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.