ChIP-chip and ChIP-seq Protocols

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Formaldehyde Crosslinking and Chromatin Purification

Based on Toth, J. and Biggin, M.D. (2000) Nucleic Acids Res., 28. e4.

i. Formaldehyde Crosslinking of Drosophila Embryos

The flies are maintained in large population cages in a chamber under standard conditions. Prior to embryo collection, the old molasses/yeast plates are replaced with fresh new plates covered with yeast, which is repeated twice to clear away the aged embryos retained by the mother flies. For embryo collection, a molasses plate covered with yeast is placed in each cage for a certain time, usually one hour. Then, the plates are removed and set aside to let the embryos to age into desired developmental stage before being harvested and fixed with formaldehyde. The yield of embryos is about 0.5 g – 0.7 g per cage for *D. melanogaster* The yield for chromatin is dependent on the age of the embryos. For stage 4 to early 5 embryos, about 100 μg chromatin, enough for one ChIP, can be obtained from each 2g of embryos. More chromatin can be obtained for older embryos.

1. Prepare crosslinking solution by combining hexane with 10X PBS and 37% formaldehyde so that the final concentrations are 5% formaldehyde and 1X PBS. Shake vigorously and then mix with a stir bar for at least 30 minutes before use. Be sure to use only the top layer for crosslinking embryos.

10X PBS	/1L
1.37 M NaCl	80g NaCl
27 mM KCl	2g KCl
43 mM Na_2HPO_4	6.10g Na_2HPO_4
14 mM KH_2PO_4	2g KH_2PO_4

adjust pH to 7.3 and autoclave

5% Formaldehyde saturated hexanes
210.5 ml hexanes
37 ml 37% formaldehyde
27.5 ml 10X PBS

Prepare this mixture on the day of crosslinking. Aliquot the top (hexanes) layer to a separate container if small amounts are needed during the course of several hours.

* modification of Beta-galactosidase staining protocol from Christian Klambt

2. Collect properly staged embryos on double nitex mesh. Wash away yeast and dechorionate embryos for 2 minutes in 50 mL of 50% bleach per 5 g of embryos. Pour into small mesh and wash extensively.
3. Transfer embryos to a cup with mesh on the bottom. Completely immerse the embryos in a beaker of isopropanol and shake back and forth to disperse clumps. Remove cup and dry embryos as much as possible with paper towels beneath the mesh.
4. Transfer embryos to a 50ml Falcon tube containing 10 ml formaldehyde saturated hexanes per gram of embryos. Shake vigorously for 5 minutes at room temperature. Allow the embryos to settle and pour off as much of the hexanes as possible.
5. Resuspend embryos in the same volume of 1X PBS, 0.5% Triton X-100 and shake vigorously at room temp for 5 minutes. Pour onto nylon mesh and repeat with fresh buffer. Embryos should clump at first but then eventually become monodispersed. Dry embryos as much as possible and transfer them to plastic tubes. Flash freeze embryos in liquid nitrogen. If the yield of embryo is low, combine different batches of collection in the same tube so that the embryos in each tube will be enough for a CsCl gradient in the chromatin purification step.

ii <u>Formaldehyde Crosslinked Chromatin Purification</u>

Use autoclaved glassware and stir bars. Set up the required amounts of $CsCl_2$ gradient solutions so that they will be ready when needed

1. Thaw up the frozen embryos. To each embryo sample, which should contain enough embryos for one $CsCl_2$ gradient (check table at end of the protocol for amount of embryos according to their stages), add 35 mls of cold NIB buffer + DTT + PMSF (shake hard to break the clumps, and to decrease embryos sticking to tube wall).
2. Transfer embryos to a glass dounce tube, and dounce the embryos at 8,000 rpm for one stroke and two strokes at 7,000 rpm using a motor – drive homogenizer system (Glas-Col).
3. Transfer to a large hand held dounce and dounce five times using A size pestle.
4. Pour into an SS34 tube and add 0.5 ml 20% Triton X100 to a final concentration of 0.3% and put on rotating wheel for 10 minutes. Spin for 15 min at 4,000 rpm at 4˚C.
5. Pour off supernatant and add 5ml Nuclear Lysis Buffer (+ 1 mM PMSF) and loosen pellets by swirling gently.
6. Transfer to a small (B pestle) dounce and completely homogenize with several strokes.
7. Pour into a 15 ml falcon tube, sonicate 20" at setting 1.5 (this is for Branson sonifier 450, and may vary depending on type of sonicator) to partially fragment chromatin. This sonication step is not to reduce the chromatin fragment to sizes desired for ChIP, but rather to decrease viscosity of the sample in the following step with the addition of detergent.
8. Transfer to a clean SS-34 tube. Add 1.8 ml 20% SDS (to final 3%) and quickly vortex.
9. Add 1.2 ml 20% Sarkosyl, and 1.2 ml 20% Triton X-100. Incubate on rotator at room temperature for 10 minutes.

10. Spin at 4,000 for 10 minutes in an SS-34 rotor. There should be a small pellet of vitelline membranes and unlysed cells.
11. Set up $CsCl_2$ gradients (check table at end of protocol for volumes of $CsCl_2$ solutions to each gradient), and layer the lysed nuclei on top of them.
12. Stop the ultra spin after the appropriate length of time. Secure each SW28 tube in a clamp and locate the gelatinous grey band (which is the chromatin) about 3 cm from the bottom of the tube. Gently insert an 18 1/2 Ga needle attached to a 5 ml syringe 0.5 – 1 cm below this band. Slowly pull the plunger and remove this material, collect 2-3 ml for each gradient.
13. Transfer the material to Spec 2 dialysis bags and dialyze against 0.1 % Sarkosyl, 2mM EDTA, 20 mM Tris pH 7.9 + 1 mM PMSF at 4oC for 2 hrs, repeat 2 more times with fresh buffer. The chromatin appears as aggregates at this stage, but will be solublized after sonication.
14. Adjust the Sarkosyl concentration in the chromatin solution to 0.5%. Sonicate the chromatin to average of ~750 bp. Power and duration of sonication depends on the sonicator, and have to be determined empirically.
15. Store chromatin at -80^{o}C

Buffers and solutions for chromatin purification

NIB buffer	1L
0.3 M Sucrose ultrapure	205.4g
15 mM NaCl	0.88g
5 mM $MgCl_2$	1.02g
15 mM Tris pH 7.5	7.5 ml of 2M Tris pH 7.5
60 mM KCl	4.48g
0.1 mM EDTA	0.2 ml of 0.5M EDTA
0.1 mM EGTA	0.038g

Filter sterilize using a 0.2 µm filter and store at 4˚C. Add DTT to 0.5 mM and PMSF to 1 mM just before use.

Nuclei Lysis Buffer	1L
100 mM NaCl	20ml of 5M
10 mM Tris pH 7.9	5 ml of 2M
1 mM EDTA	2 ml of 0.5M
0.1% NP-40	1 ml of pure NP-40

Filter sterilize using a 0.2 µm filter and store at 4˚C. Add PMSF to 1 mM before use.

$CsCl_2$ gradient buffer	1L
2% Sarkosyl	20 g
1 mM EDTA	0.372 g

Parameters for chromatin purification:

Ultracentrifugation:		
Beckman rotor	SW41	SW28
Run speed/time (20˚C)	37K/~24hr	25K/~40hr
Embryos (grams) per gradient:		
4-5 hour (embryo age)	1-3 gr	5-8 g
5.5-7.5 hour		2.5-5 g
8-10 hour	0.3-1gr	1.5-2.5 g
0-12 hour		5 g
Embryo homogenization		
Nuclei Irradiation buffer	13 ml	35 ml
Nuclear Lysis		
Nuclear Lysis buffer	1.7 ml	5 ml
20 % SDS	0.6 ml	1.8 ml
20 % Sarkosyl	0.4 ml	1.2 ml
20 % Triton X 100	0.4 ml	1.2 ml
total volume	3.9 ml	11.8 ml
$CsCl_2$ steps:		
1.5 g/ml	2.6 ml	8.5 ml
1.4 g/ml	2.6 ml	8.5 ml
1.3 g/ml	2.6 ml	8.5 ml

$CsCl_2$ preparation (to make 400 ml of each solution or just enough for the experiment)

Density	gram CsCl	mls Buffer*
1.50 g/ml	266.8	333.2
1.40 g/ml	213.5	346.5
1.30 g/ml	160	360

* Buffer should contain final concentrations of 2% Sarkosyl, 1mM EDTA

Chromatin Immunoprecipitation Using Chromatin from Crosslinked Drosophila Embryos

Modified from Walter, J, Dever, C. A. and Biggin, M.D. (1994) Genes and Dev. 8, 1678-1692

Chromatin IP (ChIP) is carried out using chromatin that has been isolated from formaldehyde crosslinked embryos and sonicated to an average fragment size of 750 bp. Use 100 µg of chromatin for each ChIP reaction.

1. Just before use, spin the chromatin solution (in TE + 0.5% sarkosyl) in a microcentrifuge at full speed for 15 min at 4 °C. Transfer the chromatin solution to new Ependorf tubes.
2. Add 5x IP buffer to the chromatin solution at 1 : 4 ratio, to final concentration of 1x.
3. Add 7 µl of normal rabbit IgG (0.4 µg/µl, Santa Cruz BioTechnology)/100 µg chromatin. Incubate 30 min on ice.
4. Transfer the sample to an Ependorf tube containing protein-A sephacryl-1000 beads, use 15 µl (packed volume) of beads for each 100 µg of chromatin. Incubate 1 hr on a rotator.

   Preparation of protein-A sephacryl beads: wash beads twice with 1x IP buffer, and after finishing washing, remove buffer and leave just the beads in the tube.

5. Spin in a microcentrifuge at full speed for 15 mins., transfer the precleared chromatin solution to a 15 ml falcon tube.
6. Dilute chromatin to 100 µg/1.2 ml with 1x IP buffer, add 4 µl 50 mg/ml BSA for each one ml of diluted chromatin, and add PMSF to 1 mM. Save 24 µl of the chromatin solution as **Input** sample (representing 2% of total input DNA).
7. For each IP or control IP sample, use 1200 µl of the diluted chromatin (~100 µg). For IP samples, add 0.5 – 3 µg of purified polyclonal antibody, or appropriate amount of monoclonal antibody or antiserum.
   For control IPs, add an equivalent amount of normal IgG, or preimmune serum. Incubate the samples on ice for 3 hr, or overnight at 4°C.
8. Spin the samples in a microcentrifuge at full speed for 15 mins. at 4 °C, Transfer supernatant to tubes containing 100 µl of the protein A-sephacryl suspension.
   Incubate 30 mins. on a rotator.

   Prepare Protein A beads
   wash beads 2x with 1 ml 1xIP buffer + 200 µg/ml BSA. Resuspend beads in 1xIP buffer with BSA at a volume of 100 µl for each 10 µl beads.
   Use 100 µl of the protein A beads suspension for each IP reaction.
9. Pellet the beads by spinning the samples at 4K rpm for 1 min. in a microcentrifuge, discard supernatant.
10. Resuspend beads in 1. 4 ml 1x IP buffer, transfer to a new tube, rotate at room temp for 5 mins., pellet beads, discard supernatant – this is the 1st wash.

11. Carry out the following consecutive washes at room temperature similar to step 10, except do not change the tube:

    1x in IP buffer, 10 min
    2x in 0.5 M IP buffer, 10 min each*
    1x in LiCl buffer, 10 min*
    1x with TE, 10 min

    *Note: if the antibody binding reaction is incubated overnight, increase these washing steps to 15 - 20 min.

12. Resuspending the pellet in 1 ml TE, transfer to a new tube, and spin down the beads. Discard supernatant. Remove the remaining buffer in the beads with a 30G1/2 needle attached to a 1 ml syringe.

13. Add 150 µl elution buffer, put on a rotator for continuous mixing for 15 min at room temperature, spin at 4K rpm for 1 min, transfer supernatant to a new tube.
    Repeat the elution step, and combine the supernatant.

14. To each sample, add 30 µl of the following PK solution:

    15 µl 1 M Tris pH 7.5
    7 µl 0.5 M EDTA
    4 µl 5 M NaCl
    4 µl 10 mg/ml Proteinase K (Invitrogen)

    For input sample (saved from step 6), add 235 µl H2O, 15 µl 20% SDS, and 30 µl of the above PK solution

15. Incubate all the samples at 55^{o}C, overnight

16. Transfer the samples to 65°C, incubate for 6 hrs to reverse crosslinks

17. DNA purification.
    add 30 µl 3M NaOAc, and 20 µg glycogen to each sample.
    Extract once with phenol:chloroform
    Extract once with chloroform.
    Ethanol precipitate at -80°C for 4 hrs or more.
    Spin 15 mins.
    Wash with 75% ethanol twice. Remove as much ethanol as possible after the last wash.
    Dry the DNA pellet at room temperature ~10 min.

18. Resuspend in TE (10mM Tris, 0.1 mM EDTA): 20 µl for IP samples, and 40 µl for Input DNA samples (2% of total DNA). The samples are now ready to be analyzed by PCR, or processed for chip analysis.

Buffers and solutions

1x IP buffer
10 mM TrisCl pH 8.0
1 mM EDTA
150 mM NaCl
0.5% Triton-X100
0.1% Nadeoxycholate
0.5% Sarkosyl

add BSA and PMSF as indicated in procedure

5x IP buffer
50 mM TrisCl pH 8.0
5 mM EDTA
750 mM NaCl
2.5% Triton-X100
0.5% Nadeoxycholate
0.5% Sarkosyl

LiCl buffer
10 mM TrisCl pH 8
1mM EDTA
250 mM LiCl
1% NP40 (now called IGEPAL CA630)
1% Nadeoxylcholate

TE
10 mM Tris-Cl, pH8.0
0.1 mM EDTA

Elution buffer
0.1 M $NaHCO_3$ (pH 10)
1% SDS

Elution buffer should be made fresh each time from $NaHCO_3$ (2x) stock solution, which is stored in aliquot at –20°C. [a solution of 120 mM $NaHCO_3$+80 mM $NaCO_3$ has a pH around 10)

Other reagents
Chromatin: purified through $CsCl_2$ gradient, sonicated to average length of less than 1 kb.
Antibodies: affinity purified, Normal IgG as controls.
Protein A –agarose beads*
BSA
Glycogen

* We use protein A –sephacryl 1000 beads prepared in house. This seems to work better than commercial protein A-agarose beads for critical applications, see Appendix for protocol.

DNA Amplification and Hybridization to Genomic DNA Tiling Array for ChIP/chip

Based on Li et al. (2008) PloS Biol 6, e27.

i. DNA Amplification

This protocol is derived from an Affymetrix Protocol with following modifications: i) A modification to the original primer sequence is introduced to avoid primer dimer formation, ii) 15x higher primer A concentration accompanied with different purification procedures following random priming, iii) higher sequenase concentration, iv) more random priming cycles (4 vs. 2), v) some changes in PCR conditions, and some other adjustments. These modifications together improves the DNA amplification efficiency and consistency. With this protocol, highly reproducible amplification can be obtained even when as little as 0.5 ng Drosophila whole genomic DNA is used.

For a typical ChIP reaction that uses ~100 µg chromatin (corresponds to ~$1x10^8$ cells), resuspend the DNA samples after the final ethanol precipitation step in small volumes (10-20 µl). Normally, use at least 50% of the IPed material for amplification. But if the efficiency of ChIP is high (e.g. if more than 0.1% of target sequenced is IPed), a smaller portion of the IPed sample can be used. The amount of input DNA used can vary from 5-200ng without affecting the results. But, typically, it can be determined based on the ChIP efficiency of a known target sequence, e.g., if 0.2% of the target sequence is IPed based on a PCR analysis, use an mount corresponding to 0.2% (200 ng) of input DNA for each amplification reaction.

1. Set up first round reaction as follows:

__µl	DNA
__µl	H2O
4 µl	5x sequenase buffer
4 µl	200 µM primer A
18.5 µl total	

primer A (new version): GTTTCCCAGTCACGGTC(N)9

2. Carry out the reactions using the following cycle conditions (total 4 random priming cycles) using PE 9600 or 9700 PCR machine.
   i) 95 °C 4 min
      snap freeze sample (i.e. transfer sample to ice)
   ii) 10 °C 5 min, or longer if more time needed to finish adding NTP mix or sequenase
      once cycler temperature reaches 10 °C, place snap frozen samples back in cycler, and add 1.6 µl NTP mix (only the first of the four random priming cycles) and 1 µl of 6x diluted sequenase (USB Corp).
   iii) ramp to 37 °C over 9 -10 min (1 °C/20sec) [With 9600 PCR machine, the ramp time can be set directly to 9 min; with 9700 PCR machine, set ramping speed at 3%, for 60 µl rxn (even though the actual volume is less), it will take 10 min to reach 37°C]
   iv) 37 °C hold for 8 min
   Repeat (i-iv) 3 more times.

NTP mix (per reaction):

0.1 µl 20 mg/ml BSA
1 µl 0.1 M DTT
0.5 µl 25 mM dNTPs

6x diluted sequenase

dilute sequenase with sequenase dilution buffer

3. Purification of DNA samples from the 1st round: add 20 µl TE to each sample, pass the samples through two Amersham microspin S-300 HR columns sequentially by following manufacturer's instructions (e.g. 1st, remove buffer from column by spinning column at 3 krpm for 1 min in microcentrifuge; then load sample, and spin at 3 krpm for 2 min, save flow through). For the second column, after removing buffer in the column, re-equilibrate the column by adding 300 µl of 10 mM TrisCl, pH8.5, followed by a 1 min centrifugation, and then load the sample. The purified DNA is ready for PCR amplification

4. 2nd round PCR amplification: (It is worth checking amplification first with 7 ul of sample from 1st round in 20 ul reaction, if not enough amplification obtained, use more cycles).

35 µl of the DNA sample from the 1st round
10 µl 10X PCR buffer
3 µl 25 mM $MgCl_2$
1.5 µl 25 mM dNTPs
4 µl 100 µM Primer B (new version: GTTTCCCAGTCACGGTC)
44.5 µl H2O
2 µl 5U/µl Taq (Stratagene Taq2000)
Total reaction volume 100 ul

Cycle conditions (total 30 – 35 cycles):

15 cycles of
95 °C 30 sec
45 °C 30 sec
55 °C 30 sec
72 °C 1 min

15 -20 cycles of
95 °C 30 sec
45 °C 30 sec
55 °C 30 sec
72 °C 1 min + D5"

Hold 4 °C

5. Purify amplified DNA. Use Qiagen PCR purification kit. Elute DNA using 35 µl H2O. Check DNA concentration by measuring OD260. Up to 12 µg of DNA can be obtained for each sample.

ii. DNA Fragmentation

Since efficiency of DNase I digestion is sensitive to a number of conditions (for example purity of DNA, the vendor and lot of DNAse I), the exact amount of DNase I to be used needs to be determined empirically.

1. Dilute DNase I (1U/µl; Epicentre) 15x into 1X One-Phor-All buffer (Roche)
2. Set up reactions as follows:

2.5-3 µg	Amplified DNA
? µl	H2O
3.28 µl	10x One phor all buffer
2.2 µl	15X diluted DNase I
35 µl total	

3. Incubate for 5 min at 37^{o}C
4. Inactivate DNase I at 99^{o}C for 10 min, then place on ice.
5. Check 10% rxn on 1.5 % agarose gel, the bulk of DNA should be at 50-100 bp. If not, repeat the digest.

iii. TdT labeling

1. Set up reaction:

31.2 µl	DNase I treated DNA
10 µl	5x TdT buffer
5 µl	25 mM CoCl2
3.6 µl	1 mM biotin-ddATP
0.18 µl	TdTase (Roche)
50 µl total	

2. Incubate at 37^{o}C for 2 hr

iv. Hybridization to chips

1. Dilute 2x MES-triton to 1x, inject 200 µl to each chip
2. Prehybe ~ 1 hr in the Affymetrix hybridization oven at 45 rpm and 45 ^{o}C
3. Mix 200 µl hybridization cocktail with 50 µl of the sample from TdT reaction.
4. Boil sample for 10 min, transfer to a 45 ^{o}C TempBlock, incubate for 10 min
5. Spin in a microcentrifuge at maximum speed for 3 min
6. Inject 200 of the sample in to the chip.
7. Carry out hybridization in the hybridization oven at 45 rpm and 45 ^{o}C for 18 hrs
8. At end of hybridization, save the hybridization solution and store at -80oC so that rehybridization can be carried out if necessary; the chips are ready to be processed.

Hybridization cocktail each chip

		final
20.83 µl	12x MES	1x
150 µl	5 M TMAC	3 M
3 µl	3 nM B2	30-50 pM
2 µl	herring sperm DNA (10mg/ml)	100 µg/ml
5 µl	1% Triton	0.02%
18.67 µl	H2O	
200 µl total		

12X MES 1 liter
70.4 g MES free acid monohydrate
193.3 g MES-Na
800 ml water, mix and adjust to 1000 ml

pH should be between 6.5 and 6.7, do not adjust
filter as above

2x MES-triton 10 ml

1.66 ml	12x MES
3.44 ml	5 M NaCl
0.8 ml	0.5 M EDTA
0.2 ml	1% triton-x100
3.9 ml	H2O

Filer sterilize, store at 4°C, shield from light

v. Washing / staining

Use Affymetrix GeneChIP Fluidics Station to process the hybridized chips following the wash/stain - protocol EukGeWS2v4.

Materials and Solutions

10mg/ml n.goat IgG**:** resuspend 10 mg in 1 ml 150 mM NaCl, aliquot, store at 4^{o}C, and long term at -20^{o}C

0.5 mg/ml biotinylated anti-streptavidin: 0.5 mg resuspend in 1 ml H2O, aliquot, store at 4^{o}C, and long term at -20^{o}C

wash buffer A 1 liter

300 ml	20x SSPE
1 ml	10% Tween 20
700 ml	H2O

filter sterilize

Wash buffer B	500 ml
41.6 ml	12xMES
2.6 ml	5M NaCl
0.5 ml	10% Tween20
455.2 ml	H2O

filter sterilize and store at 4°C, shield from light

2x stain buffer	50 ml
8.35ml	12x MES
18.5ml	5 M NaCl
0.5ml	10% Tween20
22.65ml	H2O

Filter sterilize and store at 4°C

SAPE sol	1200 µl each chip
600µl	2x MES stain buffer
48 µl	50 mg/ml BSA
12 µl	1mg/ml SAPE
540 µl	DD H2O

Ab solution	600 µl each chip
300 µl	2x MES stain buffer
24 µl	50 mg/ml BSA
6 µl	10 mg/ml n. Goat IgG
3.6 µl	0.5 mg/ml biotinylated antibody
266.4 µl	H2O

For each array, need 1 tube of 600 µl of Ab solution and two tubes each containing 600 µl SAPE

vi. Scanning Chips

Scan chips using Affymetrix GeneChip scanner

ChIP-seq Sample Preparation Protocol

All the steps in this protocol are the same as the "illumina protocol for sample preparation for chip-seq", except that the PCR amplification step was carried out before instead of after size selection, see also Bradley et al. (2010) PloS Biol. 8, e1000343. A few notes are added

i. 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.

Consumables Illumina-Supplied
- T4 DNA ligase buffer with 10mM ATP
- dNTPs mix
- T4 DNA polymerase
- Klenow DNA polymerase
- T4 PNK
- Water

User-Supplied
- ChIP enriched, QPCR verified DNA (approx.10 ng in 30 µl water)
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

1. Dilute Klenow DNA polymerase 1:5 with water for a final Klenow concentration of 1U/µl.

2. Prepare the following reaction mix:

   - ChIP enriched DNA * (30 µl)
   - Water (10 µl)
   - T4 DNA ligase buffer with 10mM ATP (5 µl)
   - dNTP mix (2 µl)
   - T4 DNA polymerase (1 µl)
   - Klenow DNA polymerase (1 µl)
   - T4 PNK (1 µl)

   The total volume should be 50 µl.

3. Incubate in the thermal cycler for 30 minutes at 20ºC.

4. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 34 µl of EB.

* We include two control samples for each experiment: Input DNA control and a Mock IP control. For Input DNA sample, use 1 ng of the input DNA. The amount of DNA in the reaction in our Mock IP and IP reactions is quite low, and may be less than 0.5 ng.

ii. 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.

Consumables Illumina-Supplied
- Klenow buffer
- dATP
- Klenow fragment (3' to 5' exo minus)

User-Supplied
- MinElute PCR Purification Kit (QIAGEN, part # 28004)

1. Prepare the following reaction mix:
    - DNA sample (34 µl)
    - Klenow buffer (5 µl)
    - dATP (10 µl)
    - Klenow exo (3’ to 5’ exo minus) (1 µl)

    The total volume should be 50 µl.

2. Incubate for 30 minutes at 37°C.

3. Follow the instructions in the MinElute PCR Purification Kit to purify on one MinElute column, eluting in 10 µl of EB.

iii. 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.

Consumables Illumina-Supplied
- DNA ligase buffer
- Adapter oligo mix
- DNA ligase
- Ultra pure water

User-Supplied
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

1. Dilute the Adapter oligo mix 1:10 with water to adjust for the smaller quantity of DNA.

2. Prepare the following reaction mix:
    - DNA sample (10 µl)
    - DNA ligase buffer (15 µl)
    - Diluted adapter oligo mix (1 µl)
    - DNA ligase (4 µl)

    The total volume should be 30 µl.

3. Incubate for 15 minutes at room temperature.

4. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 34 µl of EB.

iv. Enrich the Adapter-Modified DNA Fragments by PCR

Note: this step would be carried out after size selection in standard Solexa protocol, but we found higher yield is obtained after fewer cycles of amplification if this step is carried out before the size selection step. This apparently is due to the significant loss of DNA during the size selection step in the standard protocol, which is not desirable

Consumables Illumina-Supplied
- Phusion* polymerase
- 5x Phusion* buffer
- dNTP mix
- PCR primer 1.1
- PCR primer 2.1

User-Supplied
- MinElute PCR Purification Kit (QIAGEN, part # 28004)

1. Prepare the following PCR reaction mix (Note: A 25 ul reaction is carried out instead of the 50 ul reaction as in the Solexa standard protocol, and only half of the ligation product is used):

- DNA (18 µl)
- 5x Phusion* buffer (5 µl)
- dNTP mix (0.75 µl)
- PCR primer 1.1 (0.5 µl)
- PCR primer 2.1 (0.5 µl)
- Phusion* polymerase (0.25 µl)

The total volume should be 25 µl.

2. Amplify using the following PCR protocol:

a. 30 seconds at 98°C
b. 12 cycles of: (See Note #)
10 seconds at 98°C
30 seconds at 65°C
45 seconds at 72°C (See Note ## below).
c. 5 minutes at 72°C
d. Hold at 4°C

Note # 12 cycle is enough to produce about 10 ul of 2-10 nM DNA from 0.5 ng or less starting material. More cycles will generate more DNA, but overamplification may be a concern. Perform a trial experiment to make sure you get enough DNA (after the gel selection step that follows) on your hand, otherwise increase the cycle number).

Note ##: the standard extension time is 30 seconds, which is fine for fragment less than 500 bp. We have being using longer extension time when our fragments are relatively large)

V. Size Select the Library

1. Prepare an agarose gel in TBE or TAE buffer. Add 5 ul of 10 mg/ml EtBr to 100 ml gel. Use a comb with the well wide enough so that the whole 25 ul sample can be loaded into one single lane.

2. Load 500 ng of 100 bp DNA ladder to one lane of the gel.

3. Add loading buffer to the PCR product.

4. Load the entire sample in another lane of the gel, leaving at least one empty lane between ladder and sample, and between samples. Load DNA ladder on both sides of the samples.

   Be careful to avoid samples escaping from the wells which can cause cross contamination, and minimize the number of samples to be purified in each gel (3 is the maximum for me)

5. Run gel at 120 V for 60 minutes.

6. To avoid being exposed to UV light, there are two options to excise the gel:

   i.) Cut out the DNA ladder lane part of the gel; place over a transilluminator, mark the location of the DNA band (e.g. 200 bp and 400 bp if the fragments ranging from 200 bp – 400 bp are to be selected) by cutting a small wedge of gel at that position. Then put the gel slice into the original gel tray, and excise the areas in the sample lanes between the pair of marked gel positions.

   ii) Use blue-light transilluminator. In this case run the gel without EtBr, and instead after finishing running the gel, stain gel with SYBR gold. The DNA ladder and the DNA smear in the sample lanes can be easily visualized with a blue – light transilluminator.

   Note: sometime a band corresponding to ~140 bp in size is seen from the PCR amplification. To avoid contamination of the sample by this band, the DNA excised from the gel should be > 175 bp.

7. Take a photograph the gel after the slice is excised.

8. Use a QIAGEN Gel Extraction Kit (QIAGEN, part # 28704) to purify the DNA from the agarose slices and elute DNA in 36 µl.

Quantification of DNA sample concentration prepared for Solexa by Q_PCR

One advantage of ChIP-seq is the small amount of DNA sample required – theoretically 1 nanogram of DNA with an average size of 250 bp can generate millions of reads, as compared to ChIP-chip, which requires microgram quantities. However, such a small amount of DNA can be difficult to quantify. We have found Q_PCR works quite well. For this method, a DNA standard with known concentration and size range will be required. This DNA standard can be any DNA that is generated using Solexa sequencing kit.

Reagents

Primers we use

ilu-F cCaAGCAGAAGACGGCATACGAGCTCTTCCGATC

ilu-r CAATGATACGGCGACCACCGAGATCTACACTCTTTCCCTAC

These primers correspond to sequences of the solexa linkers (but the C at 5' end of each is cloning artifact)

Q_PCR kit

ABI Power SYBR green PCR master mix, PN 4367659

DNA Standard with known concentration

Method

1. The amount of DNA for standard used in each reaction: 0.0001 – 0.01 ng .
   For DNA sample to be tested, make four different dilutions: 200x, 1000x, 5000x, 25000x. This will ensure at least one of the diluted sample will have the amount of DNA within the standard range (e.g. with starting concentration of 0.5 ng/ul to 250 ng/ul, at least one of the diluted sample will have a concentration of 0.002 – 0.01 ng/ul).

2. Q_PCR reaction contains:
   DNA: varying amount
   Primers: 100 nM final
   ABI power SYBR green PCR master mix

3. Q_PCR conditions*:
   1) 50°C 2 min
   2) 95°C 10 min
   3) 40 cycles of
      a. 95°C 0:15
      b. 68°C 0:30
      c. 72°C 0:45

* i) The primers used were designed on purpose for this Q_PCR application. They have high Tms, that's why these PCR conditions were "picked". These PCR parameters were not optimized either, by it just works perfectly well. ii) Sometimes, the DNA samples may be contaminated by adaptor fragments. To check this, run a dissociation curve analysis following Q_PCR. Without the contaminants, there will only be one major peak, but if there is significant contamination, a second peak with higher Tm will appear.

Appendix

Coupling of Protein A to Sephacryl S1000 Resin

Refernce: Hornsey VS, Prowse CV, Pepper DS. Reductive amination for solid-phase coupling of protein. A practical alternative to cyanogen bromide. Journal of Immunological Methods, 93 (1986): 83-88.

rProtein A is from Pierce. Reconstitute by resuspending 5 mg in 1 ml H2O. (even though it is said that after reconstitution protein A can be stored at 4^{o}C for up to 1 month, we found some degradation already occurred (10%?) following storage through weekend).

Protein A / beads ratio: 1.5 mg protein/ml beads.

i. Activation

1. Wash Sephacryl S-1000 (Amersham Pharmacia) 3-5 times with 0.1 M NaOAC pH4.7. Use 50 ml Falcon tube, and resuspend beads at 5 ml buffer/ml beads. Pellet the beads by spinning at 0.5 krpm for 30 seconds in a Beckman J6 centrifuge.

2. Resuspend beads in 3mM sodium periodate in 0.1 M NaOAC pH4.7 (5 ml/ml beads), mix end-over-end for 15 min at room temperature IN THE DARK.

3. Wash with H_2O (10ml/ml beads), and 3 times with 0.1 M trisodium citrate in 50 mM Sodium Carbonate pH10 (10 ml/ml beads).

   Oxidized gel can be stored in distilled water at 4^{o}C up to 4 mo. (NaN_3 may be added).

ii. Protein Coupling

4. Dilute Protein A into fresh pH10 buffer that contains 5mM ascorbic acid (For each ml beads, use 0.3 ml 5 mg/ml protein A, and 5 ml pH10 buffer + 0.25 ml 100 mM ascorbic acid). Add this solution is to the settled gel, and incubate for 20 hrs at room temperature in dark with mixing.

5. Let the gel to settle. Remove supernatant and resuspend beads in pH10 buffer 5 ml/ml beads), and transfer to a empty BioRad chromatography column. This and all wash steps below are carried out in the column.

iii. Blocking

6. Gel is resuspended with 1M Ethanolamine pH9 containing 5 mM ascorbic acid (5 ml/ml beads). Incubate with mixing for 1 hour at room temperature.

7. Gel washed successively with 5 ml /ml beads of each:

   a. Distilled water

   b. 0.1M glycine, pH3.0; incubate 30 min while mixing.

c. Distilled water

d. 1M Ethanolamine pH9

e. 5M Urea

f. 3M Potassium thiocyanate

8. A final wash with 10 ml 15 mM citrate, 150 mM NaCl pH7, +0.03% NaN3.

9. Store in 20% ethanol.

Solutions

1. 0.1M NaOAC pH4.7:
   1.23g NaOAC, dissolve in H_2O; adjust pH with acetic acid.
2. 3mM Sodium m-periodate:
   6.42mg, dissolve in 10ml 0.1M NaOAC (above).
3. 0.1M Trisodium citrate; 50mM Sodium carbonate, pH10:
   4.41g Trisodium citrate, 0.795g Sodium carbonate, dissolve in 150 ml H_2O.
4. 100mM Ascorbic acid:
   0.176g, dissolve in 10ml H_2O
5. 1M Ethanolamine pH9.0:
   6.1g, dissolve in 100ml H_2O
6. 0.1M Glycine pH3.0:
   0.376g, dissolve in 50ml H_2O
7. 5M Urea pH6.0:
   15g, dissolve in 50ml H_2O
8. 3M Potassium thiocyanate:
   14.6g, dissolve in 50ml H_2O
9. 15mM Citrate, 150mM Sodium chloride pH7.0:
   315g sodium citrate, and 0.87g sodium chloride, dissolve in 100ml H_2O.

All solutions were filtered sterilized with 0.22μ filters. The proper pH was achieved using HCl or NaOH.