Native chromatin immunoprecipitation (N-ChIP) of *Crassostrea gigas* (16/03/2017) version 1

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This protocol is based on the protocol of David Umlauf.

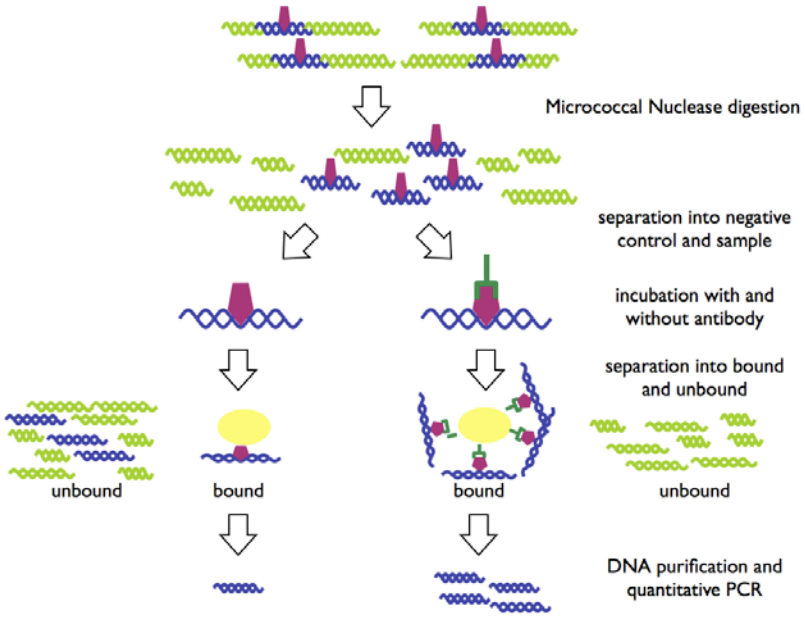

Figure 1: General scheme

Before everything begins: prepare solutions...

- 1 M KCl, autoclave
- 5 M NaCl, filter and autoclave
- 1 M MgCl, autoclave
- 1 M Tris/Cl pH 7.4 - 7.6, autoclave
- 0.5 M EDTA, autoclave
- 1 M $CaCl_2$, 10 ml, sterile filter
- 100 mM DTT, 1ml (store at -20°C)
- Roche CompleteProtease Inhibitor (ref: 11 697 498 001)
- 2.5 M Sodium butyrate (Sigma B5887 1g) (store at 4°C)
- 25 mM PMSF in isopropanol, 10 ml (store at -20°C)
- 15 U/µl Micrococccal nuclease (MNase) (USB 70196Y) in sterile 50% glycerol, aliquot to ~10µl and store at -20°C
- Protein A - sepharose CL-4B (Sigma P3391 250mg) (store at 4°C)
- agarose gel loading buffer
- 20% SDS, sterile filter
- 20 g/l glycogen solution (store at -20°C)
- 2% NaN_2 in water (store at 4°C), NaN_2 is very toxic!

- micro-dialysis units (Slide-a-Lyzer 3500 D cut-off, Pierce 69550). Note: You can also make your own (much cheaper) micro-dialysis units. For details see here (external link, not tested).

preparation of protein A - sepharose

1. weight 250 mg protein A - sepharose in 15 ml falcon tube
2. wash with 10 ml sterile water
3. centrifuge 10 min at 4000 rpm
4. remove supernatant
5. repeat step 3 - 4 four times
6. add sterile water to 5 ml

250 mg Protein A - sepharose swells to approx. 1 ml gel and binds approx. 20 mg human IgG. You will need 50 µl of the protein A sepharose homogeneously mixed in its 5 ml water volume per ChIP. Only if prolonged storage is anticipated (several month) add NaN_2 to 0.02 % and label tube appropriately.

optional if micro dialysis units are not available: preparation of dialysis tubing

1. cut tubing (e.g. VWR international dialysis tube 0.5 mm) into pieces of 10 - 20 cm length
2. boil for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA
3. rinse the tubing thoroughly with distilled water
4. boil for 10 min in 1 mM EDTA
5. cool and store in this solution at 4°C
6. before use, wash tubing inside and outside with distilled water

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day 1...

- reserve centrifuge and cool down to 4°C
- prepare a 2% 0.5x TBE agarose gel with 20 µl slots
- preheat a water bath to exactly 37°C
- prepare the following solutions with autoclaved distilled water

2x base buffer		
6 ml	1 M KCl	60 mM final 1x
0.3 ml	5 M NaCl	15 mM
0.5 ml	1 M MgCl2	5 mM
20 µl	500 mM EDTA	0.1 mM

1.5 ml	1 M Tris/Cl	15 mM
to 50 ml	water	
2	Roche protease inhibitor tabletts	

buffer1 (0.3 M sucrose)	
2.58 g	sucrose
12.5 ml	2x base buffer
50 µl	sodium butyrate
100 µl	PMSF
125 µl	DTT
to 25 ml	water
buffer 2	
10 ml	buffer 1 (0.3 M sucrose)
put on 37°C to allow NP40 to be pipetted into the buffer	
80 µl	NP40 (cut pipette tip)
put on 37°C to fully dissolve NP40 and put on ice	
buffer 3 (1.2 M sucrose) for 3 cell samples	
20.55 g	sucrose
25 ml	2x base buffer
100 µl	sodium butyrate
200 µl	PMSF
250 µl	DTT
to 50 ml	water
MNase digestion buffer	
1.1 g	sucrose
0.5 ml	Tris/Cl
80 µl	PMSF
40 µl	$MgCl_2$
20 µl	sodium butyrate
10 µl	$CaCl_2$ (essential for the enzyme)
to 10 ml	water
put at 37°C	
Dialysis buffer	
1mM Tris/Cl, 200 µM EDTA, 200 µM PMSF, 5 mM sodium butyrate	
50 µl	Tris/Cl
20 µl	EDTA

400 µl	PMSF
100 µl	sodium butyrate
to 50 ml	water

- put all buffer solutions on ice (except MNase buffer)
- ***cell lysis***
  - aliquote tissues of mantel or gills from adults or whole larvae (stored at -80°C or in liquid nitrogen)
  - adults tissues and larvae:
    - remove excess liquid (if any) and resuspend in 1 ml buffer 1, add 1 ml buffer 2 (lysis buffer) and transfer to Dounce
    - homogenize for 3 min with Dounce (pestle A) on ice
    - put on ice 7 min
  - fill 8 ml buffer 3 into a 50 ml corex centrifugation tube
  - overlay the 8 ml buffer 3 with 2 ml cell suspension so that the tubes are ready for centrifugation
  - disturb a little bit the interface
  - mark tubes at the exterior side (to know where to look for the nuclei)
  - centrifuge 8500 rpm 20 min 4°C
  - carefully remove supernatant completely (by pipetting with 10 ml pipette and then, pipetting with micropipettes 1000µl>100µl>10µl)
- ***MNase digestion***
  - resuspend pellet in 1 ml MNase digestion buffer (2 ml for 10 000 miracidia or cercariae)
  - aliquot 500 µl of this suspension in 1.5 ml Eppendorf tubes
  - add 1 µl MNase (15 U) and incubate **4 min at 37°C**
  - to stop the reaction add 20 µl 0.5 M EDTA to each 500 µl MNase digest and put the tube on ice
  - centrifuge 13000 g 10 min 4°C
  - transfer the supernatant to a new tube (**S1**) and keep the pellet (**P1**)
  - store S1 at -20°C
  - Quantify chromatin in S1 with the Qubit® 2.0 Fluorometer (HS DNA assay) following manufacturer instructions.
- **Dialysis of P1**
  - humidify Slide-a-Lyzer with 50µl dialysis buffer
  - resuspend the pellet P1 in 100 µl dialysis buffer and dialyze overnight at 4°C against 50 ml dialysis buffer with gentle stirring

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day 2...

  - the next day, transfer dialysed sample to Eppendorf tubes and...
    - centrifuge 13000 g 10 min 4°C
    - transfer the supernatant to a new tube and repeat the centrifugation 2 times
    - supernatant is fraction **S2**
  - yesterdays supernatant S1...

        - in parallel with the dialyses sample, centrifuge 13000 g 10 min 4°C
        - transfer the supernatant into a new tube and repeat this centrifugation 2 times
    - these triple centrifugations are IMPORTANT! They reduce the unspecific background!
    - use 50 µl of S1 and S2 for phenol/chlorofrom extraction, centrifuge and load 20 µl of supernatant on 2% 0.5x TBE gel (100V, 25 min)
- ***incubation with antibody***
    - Ideally, the antibody should be in excess over the protein you want to precipitate. The antigen/antibody ration must be determined experimentally for each antibody . Prepare a dilution series of your chromatin in MNase buffer starting with 20 - 40 µg DNA for histone ChIP.
    - Add appropriate amounts of stock solutions to generate the

antibody incubation buffer	
NaCl	150 mM
Tris/Cl	20 mM
sodium butyrate	20 mM
EDTA	5 mM
PMSF	100 µM

    - you can download an Excel worksheet for calculation here (v0.1) or here (v1.0)
    - dissolve chromatin from S1 (and S2 if you have dialyzed) in 1 ml buffer
    - add about 2 µg antibody
    - incubate overnight at 4°C on a rotating wheel

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day 3...

- ***precipitation***
    - prepare 50 µl of protein A - sepharose for each tube
    - wash the beads with milliQ water in 1.5ml Eppendorf: short spin, remove supernatant and replace with equal volume of sterile water
    - add 50 µl of protein A - sepharose to each tube
    - incubate at least 4 h at 4°C on a rotating wheel
    - prepare washing buffers (10 ml / tube) and cool down to 4°C:

washing buffers A B C			50 ml	100 ml	200 ml	300 ml
	Tris/Cl	50 mM	2.5 ml	5 ml	10 ml	15 ml
	EDTA	10 mM	1 ml	2 ml	4 ml	6 ml
	sodium butyrate	5 mM	100 µl	200 µl	400 µl	600 µl

washing buffer A	NaCl	75 mM	750 µl	1.5 ml	3 ml	4.5 ml
washing buffer B	NaCl	125 mM	1.25 ml	2.5 ml	5 ml	7.5 ml
washing buffer C	NaCl	175 mM	1.75 ml	3.5 ml	7 ml	10.5 ml

- centrifuge chromatin/antibody mixture 10 min 4°C 11600 g
- keep the supernatant in a 2 ml tube. **This is the unbound fraction UB**.
- resuspend the pellet in approx. 1 ml washing buffer A and transfer into a 15 ml Falcon tube containing 9 ml washing buffer A
- mix for 10 min on a rotating wheel at 4°C (speed 6)
- centrifuge 10 min 3400 g 4°C and pour off supernatant (by inversion)
- add 10 ml washing buffer B, mix for 10 min on a rotating wheel at 4°C and centrifuge 10 min 4000 rpm 4°C
- pour off supernatant (by inversion)
- add 10 ml washing buffer C, mix for 10 min on a rotating wheel at 4°C and centrifuge 10 min 4000 rpm 4°C
- pour off supernatant (by inversion)
- centrifuge 10 min 4000 rpm 4°C
- remove remaining supernatant completely (not by inversion but by pipetting with micropipettes 1000 µl, then 100 µl, then 10µl)
- resuspend pellet in 500 µl elution buffer

elution buffer		10 ml	20 ml
SDS (20% stock)	1 %	500 µl	1ml
Tris/Cl	20 mM	200 µl	400 µl
NaCl	50 mM	100 µl	200 µl
EDTA	5 mM	100 µl	200 µl
sodium butyrate	20 mM	80 µl	160µl
PMSF	100 µM	40 µl	80 µl
water		to 10 ml	to 20 ml

- transfer suspension to a 1.5 ml Eppendorf tube
- incubate 15 min at RT on a rotating wheel
- centrifuge 10 min 11600 g **18°C**
- transfer supernatant to a 1.5 ml Eppendorf tube
- **This is the bound fraction B.**

- ***DNA extraction***
  - DNA purification with QIAquick PCR Purification Kit (Quiagen, Cat. no. 28104), following manufacturer instructions. Step 3 and 4: Time of centrifugation : 60sec.
  - Step 5: Centrifuge the column in a 2 ml collection tube for 1 min, without the cap.
  - Step 7: Before the centrifugation, let the column stand for 2 minutes at room temperature. Elution volume: 50 µl EB or sterile water.

- In order to obtain more DNA, it is possible to proceed to a second elution in 30 µl
- OR: use a single elution in 65 µl EB or sterile water
- Quantify purified DNA with the Qubit® 2.0 Fluorometer (HS DNA assay) following manufacturer instructions.
- use 1 µl of this DNA for PCR in 25 µl reactions (quantitative real-time PCR) or 10 µl (PCR)

Ref.:

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