CHP_10v4

Pombe ChIP Dynabeads protocol w/ column DNA clean up

(modified from CHP_10v2 by **MF** and **SB;** updated by **PG** 22/05/2016 and **SB** 30/08/2016))

Day 1: Set up pre-cultures. Growth for 1 day at 30ºC.

Day 2: Set up main 100 ml main cultures w/ YES media. Depending on individual strain growth, use a dilution of 1:100 – 1:500 of post-log phase pre-culture (main culture should have a starting OD of 0.01-0.05). Grow 14-16 hrs at 30ºC.
Alternatively: main cultures can also be i/o directly from plate w/ starting OD of 0.02-0.04 OD.

Day 3: Growth, formaldehyde (FA) crosslink (X-link), and harvest

- Grow cultures to OD = 0.4-0.6
- Add FA (37%, Roth p.a.) to f.c. = 1% and **X-link 10 min** at RT with occasional mixing
  (i.e. for 100 ml culture -> 2.7 ml FA, for 400 ml -> 10.8 ml, for 500 ml -> 13.5 ml)
  Optional: If exact cross-linking efficiency is critical, use **fresh** FA (25 mL bottles/Fisher Scientific)
- Quench remaining FA by adding 2.5 M glycine to f.c. = 125 mM and incubate for 10 min
  (i.e. for 100 ml -> 5 ml glycine, for 400 ml -> 20 ml, for 500 ml -> 25 ml)
- Collect cells by centrifugation in 2x 50 ml Falcon tubes for 5 min at 700g at 4ºC.
- Decant and re-suspend pellets in **25 ml ice-cold PBS** and pool aliquots in one tube. Pellet, decant and repeat wash 1 more time, then re-suspend pellet in **1ml ice-cold PBS**.
- Transfer cell suspension to a **2 ml screw-cap tube**, shortspin for 12 s at max. speed, decant, and freeze pellets in liquid nitrogen.
  If larger cell cultures have been grown, split into several aliquots (e.g. 40-60 OD for each aliquot).
- Store at -80ºC or proceed to step 1 of Day 4 (skip freezing).

Day 4: Lysis, sonication, and IP

(estimated time for lysis + sonication + IP preparation: 2-3 hr;
antibody incubation 1.5-3 hr; magnetic beads incubation O/N or > 4 hr)

- Thaw cell pellets on ice and re-suspend pellets in **500 µl ice-cold lysis buffer w/ protease inhibitors** (for a harvest volume and OD of 100 mL and 0.5, respectively, this corresponds approx. to 0.1 OD/µl). Add protease inhibitors prior to use at the following final concentrations (indicated volumes for 10 ml working solution)
  1 mM **AEBSF** (100 µl of 100X stock, 100 mM),
  100 µg/ml **Leupeptin** (10 µl 1000X stock, 100 mg/ml),
  Roche complete protease inhibitor cocktail (400 µl from 25X stock = 1 tablet in 2 ml PBS);
- Add 2 scoops (i.e. a 250 µl PCR tube ~ 250 µl vol equivalent) of zirconia beads.
- Lyse cells 4X for 20 sec (max) in *Precyllis* cell disruptor (*PeqLab*) w/ intervals on ice.
  important: use only screw-cap tubes! (otherwise leakage!)
- Optional: Check cell lysis under the microscope and proceed if more than 80% of the cells are lysed (otherwise do one more lysis cycle).
- Invert tubes and puncture bottoms with a **hot** (use gas burner) **22-gauge (grey) needle** and place tubes in 2.0 ml tubes, centrifuge for 3 min at 700 xg.
- Transfer samples to **polystyrene sonication tubes** (*Active Motif*, #53071 or #53072)
- Shear chromatin of samples by sonicating in a water bath sonicator (***Q800R*****,** *QSonica*) **for 30 min** with cycles of 30”ON/30”OFF (total incubation time: 60 min)
- Transfer sonicated material into 1.5 ml tubes and pellet cell debris for 10 min at 16,000g at 4ºC; transfer supernatant into a fresh tube
- Repeat pelleting and transfer to reduce variability in results due to remaining debries in later steps (“lysate“ or supernatant “SN”).

- Optional: Input samples can be stored at -20ºC, lysates at -80ºC.
- For each **IP**, use crude lysate corresponding to **5-30* ODs** and add lysis buffer (w/ protease inhibitors) to a final volume of 540 µl (e.g. for a concentration of crude lysate of 0.1 OD/µl, this corresponds to 125-250 µl lysate). **For H3 and H3K36me3 use 5-10 ODs, for H3K9me2 use 10-15 ODs, for FLAG IP use 30 ODs*
- Add **2-4 µl** of antibody per sample and incubate 4 hrs or o/n on a nutator; alternatively, place eppendorf tubes in a Falcon tube on a rolling mixer at 4ºC. Final volume is 600 µl.
  (e.g. for H3K9me2 use 2 µl of Abcam ab1220; for FLAG use 2 µl of Sigma F3165; however, it is recommended to dilute the antibody prior addition w/ lysis buffer and pipette larger volumes to the samples to minimize pipetting errors; e.g. for anti-H3K9me2 prepare a total 1:50 dilution and add 100 µl to each sample.)
- **INPUT**: Remove **40 µl of lysate** and add **160 µl of TE/1% SDS** solution. This is the "Input DNA" sample. The Input DNA will be processed along with the IP'd chromatin on Day 5 at step 6.
- Prepare ProtG/Dynabeads (Invitrogen). Per sample calculate **25 µl of magnetic beads:**
  - Wash the magnetic beads 2x with PBS/0,1%Tween (incubation is not required, beads for several IP samples can be processed together, e.g. for 100 µl beads use 1ml PBS/T)
  - Wash once with 1 mL lysis buffer to reduce residual detergent.
  - Resuspend the beads in lysis buffer in a total volume corresponding to 100 µl (or 50 µl) per sample. Add 100 (50) µl to each sample and incubate on a nutator (or in a Falcon tube on a rolling mixer) at 4ºC for >**4 hours or O/N**. (Final volume is 700 µl)

Day 5: IP wash; reverse X-link (estimated time: wash 1 hr; reverse cross-link 4 hrs)

- Put samples onto the magnetic stand, incubate for 1min. Remove SN with a pipette or vacuum bottle and pipette tipped holder (exchange tip after each step).
- **Wash** beads as follows (at RT with cold buffer):
  - 2X with 0.5 ml **Lysis buffer** (buffer 1)
  - 2X with 0.5 ml **High salt lysis buffer** (buffer 2)
  - 2X with 0.5 ml **Wash buffer** (buffer 3)
  - 1X with 150 µl TE and transfer all into a **fresh tube**, keep IPs with TE on ice when not used
    optional: spin down samples for 10s at 0.1g to remove buffer from the lids before TE addition and transfer.
  - Put samples onto the magnetic stand and completely remove the SN
    (This step is important, as proteins and DNA bind non-specifically to the tube wall).
- Add **200 µl Elution buffer 3** (TE, 0.8% SDS)
- **Reverse X-link**: incubate IP samples together with INPUT samples **10 min at 95ºC and max speed** on thermomixer, shortspin to remove condensation from the lid and transfer the supernatant to a new tube **3 hr at 65ºC**
  (reverse X-link of eluate together with beads before transfer into fresh tube is possible and does not affect the efficiency of the rev. X-link; the extended incubation may actually increase the elution efficiency)

Day 6: Proteinase K treatment; DNA clean-up; qPCR
(estimated time for Proteinase K: 2 hrs; clean up: 0.5 hr; qPCR setup1-2 hr; qPCR run 1.5 hr)

- Prepare **fresh** Proteinase K (4mg/ml), add 10 µl to each sample and incubate for 2x 1hr at 55ºC, vortex and briefly spin after the first hour to keep the suspension homogeneous.
- Clean-up the DNA with *Zymo Research*, ChIP DNA Clean & Concentrator™, #D5201:
  - Add **5 volumes** of **DNA-binding buffer t**o samples
    (1x 1 ml Binding Buffer + 200 µl sample)
  - Add the mix onto the column in two subsequent steps (each ~600 µl) and spin for 30''
  - Add 200 µl Wwash Buffer and 30" spin (use the wash buffer that contains EtOH!)
  - Add 200 µl Wash Buffer and 30" spin (use the wash buffer that contains EtOH!)

- Do empty spin for 2',
- Transfer columns into new tubes and add Elution Buffer: **50 µl** for input, **15-25** µl for IP.
- Elute samples by a 1' spin

- **Optional:** Treat INPUT samples with **RNaseA** (f.c. 10 ug/ml) for 30 min at 37ºC prior to ProtK (This allows the analysis of mono-nucleosome preparation by agarose gel electrophoresis: load 25-50% of INPUT material on 1.8-2% agarose gel **w/out** EtBr; run at 50-75 V for 45-60 min; stain with EtBr for 2-3', destain with running buffer for 1' with agitating)

- for qPCR use **5 µl** sample (**1:20** dilutions for **ChIP, 1:100** dilutions for **large tiling arrays** (test on small scale before) and **INPUT**)

  2.5 µl Primer For/Rev mix (1.5 uM)

  7.5 µl PowerUp! SYBR Green Mastermix

  Use one of the input samples for normalization (standard curve). Prepare 6 standard samples (1/10 serial dilution), starting with a 1/20 dilution of the non-diluted input sample (use non-tag or WT if possible)

  Use **96-well MicroAmp® Optical 96-Well Reaction Plate** and **qPCR Seal**
- qPCR program stored on Dell™ Notebook of 7500 Fast Real-Time PCR System machines as PowerUp w/o melting curve (use w/ for untested primers)

  50ºC: 2 min, 95ºC: 2 min,

  [95ºC: 20 s, 50ºC: 20 s, 72ºC: 40 s, 73ºC: 1 s, read] X40 cycles

  record melting curve 70ºC to 90ºC; cool down to 10ºC.

Buffers and Solutions:

Formaldehyd (fresh - stock: 37%)
2.5 M glycine (MW 75.07): 93.75 g in 500 ml (sterile filt.)
Zirconia beads

Tris/HCl 1 M, pH 8
TBS (20mM Tris/HCl pH 7.5, 150mM NaCl) (autoclaved)
TE (10 mM Tris/HCl pH 7.6, 1 mM EDTA) (autoclaved)
0.5 M HEPES/KOH, PH 7.5 (sterile filtered)

5 M NaCl (autoclaved)
0.5 M EDTA (autoclaved)
10% SDS (autoclaved)

10% Triton X-100 (H2O)
10% Na-Deoxycholate (H2O)
10% Tween 20 (H2O)

Buffers:	final conc (mM or %)	stock solution	stock conc (mM or %)	100 ml	500 ml
Lysis buffer	50	HEPES/KOH pH 7.5	500	10	50
	140	NaCl	5000	2.8	14
	1	EDTA	500	0.2	1
	1	Triton X-100	10	10	50
	0.1	Na-DeoXycholate	10	1	5
Lysis buffer high salt	50	HEPES/KOH pH 7.5	500	10	50
	500	NaCl	5000	10	50
	1	EDTA	500	0.2	1
	1	Triton X-100	10	10	50
	0.1	Na-DeoXycholate	10	1	5
Wash buffer	10	Tris/HCl, pH 8	1000	1	5
	250	LiCl	4000	6.25	31.25
	1	EDTA	500	0.2	1
	0.5	NP-40	10	5	25
	0.5	Na-DeoXycholate	10	5	25
PBS-Tween	0,1%	**PBS-Tween**	100x	10	50
Elution buffer 3	50	Tris/HCl, pH 8	1000	5	
	10	EDTA	500	2	
	0.8	SDS	10	8	
TE + 1% SDS	10	Tris/HCl, pH 8	1000	1	
	1	EDTA	500	0.2	
	1	SDS	10	10	

Antibodies used for ChIP:

anti-H3:	polyclonal (rabbit), box B	Abcam ab1791	3-4 µl
anti-dimeK9H3:	monoclonal (mouse), box G	Abcam ab1220	3 µl
anti-trimeK9H3:	polyclonal (rabibit), box C	Upstate 07-523	2 µl
anti-trimeK4H3:	polyclonal (rabbit), box B	Abcam ab8580	3 µl
anti-Flag:	monoclonal (mouse)	Sigma F3165	2 µl

CHP_10v3: Quick protocol guide

Growth, crosslink, and harvest

- At OD = 0.4-0.6, add FA (f.c. 1%) and **x-link 10'** at RT, (i.e. 2.7 ml for 100 ml culture)
- Quench with 2.5 M glycine (f.c. 125 mM) for 10 min (i.e. 5 ml for 100 ml culture)
- Pellet at 700x g at 4ºC for 5', wash 2x w/ **25 ml ice-cold PBS,** re-suspend in **2ml ice-cold PBS**,
- Transfer cell suspension to a **2 ml screw-cap tube**, pellet, decant, and freeze pellets in liquid nitrogen.

Lysis, sonication, and IP

- Add **500 µl ice-cold lysis buffer** w/ protease inhibitors + 2 scoops of **zirconia beads**;
  for 10 ml working solution prepare 100 µl **AEBSF,** 10 µl **Leupeptin,** 400 µl **Roche complete**
- Lyse cells 4X for 20 sec (max) in Precyllis (PeqLab) w/ the intervals on ice.
  important: use only screw-cap tubes! (otherwise leakage!)
- Puncture tubes with a **22-gauge needle** and transfer samples to **polystyrene sonication tubes**
- Sonicate w/ **Q800R (*QSonica*) for 30 min** with cycles of 30"ON/30"OFF
- Pellet cell debris for 2x 10 min at 16,000Xg at 4ºC and transfer supernatant to a fresh tube.
- For each IP, use **5-30 ODs** of lysate and add lysis buffer to 540 µl
- For input, remove **40 µl of lysate** and add **160 µl of TE/1% SDS** solution
- Add **2-4 µl** of antibody and incubate 4 hrs or o/n on a nutator at 4ºC. Final volume = 600 µl
- ProtG/Dynabeads —per sample **25 µl beads** (2x washed with PBS/0,1%Tween). **2-4 hours.**

IP wash; reverse crosslink

- Put the samples in the magnet stand, wash beads as follows (at RT with cold buffer):
  - 2X with 0.5 ml **Lysis buffer** (buffer 1)
  - 2X with 0.5 ml **High salt lysis buffer** (buffer 2)
  - 2X with 0.5 ml **Wash buffer** (buffer 3)
  - 1X with 150 µl TE and transfer all into a **fresh tube**
- Add **200 µl Elution buffer 3** (0.8% SDS
- **reverse X-ink**: incubate IP samples with beads and "input DNA" samples for 10 min at 95º and max. rpm, and SN only **3 hrs at 65ºC**.

Proteinase K treatment; DNA clean up; qPCR

- Prepare **fresh Prot K** (4mg/ml), add 10 µl to each sample and incubate for **2x 1hr at 55ºC**.
- **Clean-up** the DNA with *Zymo Research*, ChIP DNA Clean & Concentrator™
  - Add **5 volumes** of DNA-binding buffer (2x 600 µl) ->load -> 30" spin
  - Wash w/ **200 µl** Wash Buffer -> 30" spin
  - Empty spin 2' -> transfer into new tube -> add 50 µl (input) or 15-25 µl (IP) -> 2' spin
  - Elute samples by 2' spin
- **qPCR mix** **5 µl** sample (**1:25** dilutions for **ChIP**, **1:100** dilutions for **INPUT**)
  2.5 µl Primer For/Rev mix (1.5 µM)
  7.5 µl FAST SYBR Green Mastermix
  Use input samples as standard: 8 standard points (1/5 serial dilution), first dilution 1:20
  Use **96-well MicroAmp® Optical 96-Well Reaction Plate** and **qPCR Seal**
- **qPCR** program: 50ºC: 2 min, 95ºC: 2 min
  [95ºC: 20 s, 50ºC: 20 s, 72ºC: 40 s, 73ºC: 1 s, read] X40 cycles
  optional: melting curve 70ºC to 90ºC; cool down to 10º.