Micro-C XL Protocol

I. First Crosslinking

1. Culture 100 mL of yeast to the midlog stage, OD=0.55 o/n.
2. Add 37% formaldehyde directly to the culture to 3% of a final concentration.
3. Shake the culture at 210 rpm for 15 min at 30˚C (FA only) or 10 min at 30˚C (Dual crosslinking).
4. Quench the crosslinking by adding 10 mL of 2.5M Glycine.
5. Incubate for 5 min at room temperature.
6. Centrifuge the cells at 4000 rpm for 5 min at 4˚C.
7. Pour off the medium and wash the cells in 50 mL of sterile water by vortexing.
8. Centrifuge the cells at 4000 rpm for 5 min at 4˚C.
9. Pour off the water.

II. Permeabilize the cell wall

1. Resuspend the cell pellet in 10 mL of Buffer Z and add 7 µL of 2-Mercaptoethanol (final 10mM).
2. Add 250 µL Zymolyase solution (final 250 µg/mL).
3. Shake the tube at 210 rpm for 40 min at 30˚C.
4. Centrifuge the cells at 4000 rpm for 10 min at 4˚C.
5. Aspirate the supernatant with a vacuum suction.
6. Rinse the permeabilized cells by 5mL cold 1× PBS.
7. Centrifuge the cells at 4000 rpm for 2 min at 4˚C.
8. Aspirate the supernatant with a vacuum suction.

III. Second Crosslinking

1. Freshly prepare the long crosslinker stock and working solution as below:

Crosslinkers	MW	Spacer (Å)	Stock	Working
DSG	326.26	7.7	0.3M in DMSO	3mM in PBS
EGS	456.36	16.1	0.3M in DMSO	3mM in PBS

2. Resuspend the cells homogenously by 5 mL of working solution.
3. Rotate the tube for 40 min at 30˚C.
4. Quench the crosslinking by adding 1 mL of 2.5M Glycine.
5. Centrifuge the cells at 4000 rpm for 10 min at 4˚C.
6. Aspirate the supernatant with a vacuum suction.
7. Rinse the permeabilized cells by 5 mL cold 1× PBS.
8. Centrifuge the cells at 4000 rpm for 2 min at 4˚C.
9. Aspirate the supernatant with a vacuum suction.
10. The crosslinked pellet can be store at -80˚C for few months.

IV. Chromatin fragmentation

1. Resuspend the cell pellet in 200 µL of MBuffer#1 (freshly complete).
2. Add the appropriate amount of MNase to digest the chromatin to > 95% mononucleosomes.
3. Incubate the tube for 20 min at 37˚C.
4. Add 2 mM EGTA and incubate the tube for 10 min at 65˚C to stop the MNase activity.
5. Here, you can continue processing the sample in multiple ways prior to Micro-C sequencing library generation, depending on the desired experimental design. Micro-C has been successfully carried out using three chromatin preps: 1) Total chromatin. 2) Supernatant. 3)

Pellet. Any of these fractions can be subjected to the following Micro-C protocol, although we note that optimal signal-to-noise is achieved using relatively insoluble (Pellet) chromatin.

V. Chromatin cleaning and concentration

1) Total chromatin:

1. Transfer the whole sample into the 0.5 mL Amicon 10K spin column.
2. Concentrate the sample at 16000xg for at 4˚C until the volume goes down to ~ 50 µL.
3. Wash / pipette the sample by 450 µL MBuffer#2.
4. Repeat wash step 2 - 3.
5. Concentrate the sample at 16000xg for at 4˚C until the volume goes down to ~ 30 µL.
6. Add BSA to 1× final concentration.

2) Supernatant:

1. Centrifuge the tube at 16000xg for 5 min at 4˚C.
2. Collect the supernatant.
3. Concentrate the sample by the 0.5 mL Amicon 10K spin column at 16000xg for at 4˚C until the volume goes down to ~ 50 µL.
4. Wash / pipette the sample by 450 µL MBuffer#2.
5. Repeat wash step 2 - 3.
6. Concentrate the sample at 16000xg for at 4˚C until the volume goes down to ~ 30 µL.
7. Add BSA to 1× final concentration.

3) Pellet:

1. Centrifuge the tube at 16000xg for 5 min at 4˚C.
2. Collect the pellet.
3. Resuspend the pellet in 1 mL MBuffer#2.
4. Centrifuge the tube at 16000xg for 5 min at 4˚C.
5. Aspirate the buffer with a vacuum suction.
6. Repeat wash steps 3 - 5.
7. Resuspend the pellet to 30 µL of MBuffer#2 + final 1× BSA (or NEBuffer 2.1).

VI. Repair and label the end of chromatin fragments

1) De-phosphorylation

Total	**32.5µL**	**Final condition**
Chromatin sample	30µL	50mM NaCl, 10mM Tris, 10mM MgCl2, 1X BSA
1U/µL r-Shrimp alkaline phosphatase	2.5	2.5U

→ Incubate for 45min at 37°C.
→ Inactivate for 5min at 65°C.

2) End-Chewing

Total	**42.7µL**	**Final condition**
Chromatin sample from VI.1	32.5µL	-
10X NEBuffer#2	3	70mM NaCl, 14mM Tris, 14mM MgCl2
100mM ATP	0.5	1mM ATP
200X BSA	0.2	1X
0.1M DTT	1	3mM
3U/µL T4 DNA polymerase	2.5	7.5U
10U/µL T4 PNK	3	30U

→ Incubate at 37°C for 7min.

3) End-labeling

Total	**100µL**	**Final condition**
Chromatin sample from VI.2	42.7µL	-

0.4mM Biotin-dATP	25	100μM
0.4mM Biotin-dCTP	25	100μM
10mM dTTP + dGTP	1	100μM
10X T4 DNA ligase buffer	6	30mM NaCl, 35mM Tris, 12mM MgCl2, 7mM DTT
200× BSA	0.3	1× BSA

→ PCR machine: Incubate for 25min at 25°C → 15min at 12°C → 4°C.

→ Add EDTA (final 30mM) and heat inactivation for 20 min at 65°C.

VII. Proximity ligation and Remove unligated ends

1) Ligation

Although in our test Micro-C in "pellet" can be scaled down to a 1 mL ligation reaction, we suggest using at least 2.5 mL for routine experiments.

Total	**2.5mL** **(pellet)**	**10mL** **(Total & Sup)**	**Final condition**
Chromatin sample from VI.	100μL	100μL	-
Water	2122	8809	-
10× T4 DNA ligase buffer *w/ ATP*	250	1000	1×
200× BSA	12.5	50	1×
1M MgCl2	3	3	Equal to the moles of EDTA from previous part.
400U/μL T4DNA ligase	12.5	38	1.5 – 2U/μL

→ Incubate for 60min at room temperature.

→ **Pellet:** Centrifuge the pellet by 16000xg for 10min at 4°C.

→ **Total/Sup**: 15mL Amicon 30k concentrates sample by spin at 4000g for 40min at 4°C.

2) Remove the biotin-dNTP at unligated ends

Total	**100μL** **(pellet)**	**280μL** **(Total & Sup)**	**Final**
Chromatin sample	-	250	-
10× NEBuffer#1	10	28	1×
Water	89	-	-
100U/μL Exonuclease III	1	1	100U

→ Incubate for 5 min at 37°C.

3) Reverse crosslinking

→ Add 20X proteinase K to 1× final concentration.

→ Incubate for overnight at 55°C.

VIII. Dinucleosomal DNA purification

1. Phenol:Chloroform:Isoamyl Alcohol extraction twice → spin at 19800xg for 10min.
2. Ethanol precipitation: 0.1x volume of sodium acetate and 2.5× volume of 100% ethanol → -80°C for > 1hr → spin at 19800xg for 15min at 4°C → wash pellet by 75% ethanol → spin at 19800xg for 5 min at 4°C → Air dry pellet for 10min.
3. Resuspend pellet in 50μL of TE buffer (+ 1× RNase solution) and incubate for 30min at 37°C.
4. ZymoClean to purify DNA.
5. Run DNA samples on 3% Nusieve agarose DNA gel.
6. Size selection of the band between 250 – 350 bp.
7. ZymoGel purification and dissolve final product in 17μL of elution buffer.
8. Quantify the input DNA by Qubit.

IX. Library construction by "with-bead" method

1) End-it

Total	25µL
DNA	17
10× End-it buffer	2.5
10× ATP	2.5
10× dNTP	2.5
End-it enzyme mix	0.5

→ Incubate for 45 min at room temperature.
→ 2× Ampure XP purification.

2) A-tailing

Total	25µL
DNA	16
10X Exo- Klenow buffer	2.5
1mM dATP	5
Exo- Klenow fragment enzyme	1.5

→ Incubate for 30min at 37°C.
→ 2× Ampure XP purification. (PEG/NaCl solution: 20% PEG, 2.5M NaCl)

3) Adapter ligation

Total	15µL
In-line / Indexing Adapter	Ratio of Adapter:Input DNA = 10:1 – 50:1
Water	to total 15µL
10× Fast-link DNA ligase buffer	1.5
10× ATP	1.5
Fast-link DNA ligase	1

→ Incubate for > 2 hr at room temperature.
→ Add 10µL of EB to total volume 25 µL.
→ 2× Ampure XP purification and elute DNA in 150 µL of water.

4) Streptavidin beads purification

1. Wash 2.5 µL of beads per sample (100mL culture) by 1× TBW twice.
2. Resuspend the washed beads in 150 µL 2× BW.
3. Mix with 150 µL of adapter-ligated DNA sample.
4. Rotate for 15 min at room temperature.
5. Wash by 500 µL of 1× TBW twice.
6. Rinse by 200 µL of MBuffer#2.
7. Resuspend in 15 – 20 µL of EB buffer.

5) On-beads PCR

Total	**10µL** **(scalable)**

Streptavidin-Biotin-DNA sample	1
Water	3.5
2× KAPA HiFi Hot Start Mix	5
10μM PE1 primer	0.25
10μM PE2 primer	0.25

Denaturation	98°C	45sec
8-12 cycles	98°C	15sec
	60°C	30sec
	72°C	30sec
Extension	72°C	1min
	4°C	Hold

→ Check the size and quantity of library by DNA gel or Fragment analyzer.
→ Size-selection of dimer size library by 3% Nusieve agarose DNA gel or 1:1 Ampure XP beads purification.

X. Deep sequencing by Illumina PE-50

Solutions and Enzymes

- **YPD**: yeast extract/peptone/dextrose
- **37% Formaldehyde** (Sigma Aldrich # 252549)
- **DSG (disuccinimidyl glutarate)** (ThermoFisher #20593)
- **EGS (ethylene glycol bis(succinimidyl succinate))** (ThermoFisher #21565)
- **2.5M Glycine** (Sigma Aldrich #G7126)
- **Buffer Z:** 1M sorbitol, 50mM Tris pH 7.4
- **14.3M 2-Mercaptoethanol** (Sigma Aldrich # M6250)
- **Zymolyase solution:** 10mg/ml in Buffer Z; lasts up to 2 weeks at 4°C (Sunrise Science #N0766555)
- **MBuffer#1:** 50mM NaCl, 10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1mM $CaCl_2$, and **freshly** complete with 0.5mM spermidine, 1mM β-ME, and NP-40 (percentage is determined by titrating the ratio of pellet / sup).
- **Micrococcal Nuclease** (Worthington Biochem): resuspended from lyophilized powder at 20 U/μl in Tris pH 7.4. Aliquot into tubes upon first use and freeze at –80°C.

- **0.5M EDTA** (Life technology # AM9261)
- **MBuffer#2 (NEBuffer#2):** 50mM NaCl, 10mM Tris-HCl pH 7.5, 10mM MgCl2
- **Shrimp Alkaline Phosphatase** (**rSAP**) (New England Biolabs # M0371)
- **T4 DNA Polymerase** (New England Biolabs # M0203)
- **T4 Polynucleotide Kinase** (New England Biolabs # M0201)
- **Biotin-14-dCTP** (Life Technologies # 19518018)
- **Biotin-14-dATP** (Life Technologies # 19524016)
- **T4 DNA Ligase** (New England Biolabs # M0202)
- **Exonuclease III (*E. coli*)** (New England Biolabs # M0206)
- **20× Proteinase K solution:** TE with 20 mg/ml proteinase K and 50% glycerol. Store in -20°C.
- **Elution buffer (EB):** 10 mM Tris-HCl pH 7.5
- **TE buffer:** 10 mM Tris-HCl pH 8.0, 1 mM EDTA
- **End-It DNA End-Repair Kit** (EpiCentre BioTechnologies # ER81050)
- **Exo-Minus Klenow DNA Polymerase** (EpiCentre BioTechnologies # KL111)
- **Fast Link DNA Ligation Kit** (EpiCentre BioTechnologies # lk6201)
- **Dynabeads® MyOne Streptavidin C1** (Life Technologies # 65001)
- **KAPA HiFi HotStart ReadyMix** (KAPA Biosystems # KK2601)