Data S1

Detailed Protocol and Quality Control Results of RDD Library Construction

Part I Prepare RNA enriched chromatin

Day-1

1. Cell lysis and nuclei lysis

Around 1× 10^9 cells for S2-WT (non-treatment) and S2-HS (heat-shock treatment)

1.1. Thaw 2 tubes of frozen dual-crosslinking S2 cells on wet-ice for 1 hr.

0.8~1 × 10^9 cells are for one library generation.

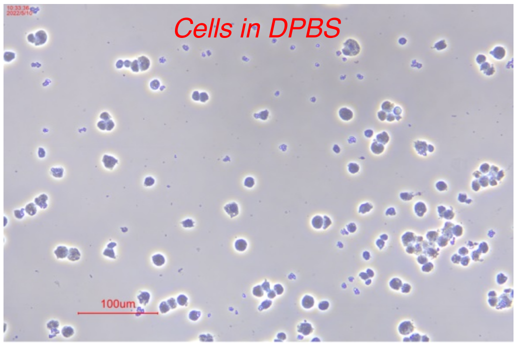
Preparation: *clean the bench and instrument with 75% ethanol and RNasezip. Use new reagents and pipettes. All the RNA-related buffers should be freshly set up.*

1.2. Prepare **4 tubes** of 50 ml of **0.1% FA lysis buffer without Triton-X100** at room temperature (RT).

0.1% FA lysis buffer without Triton X100	Volume (50 ml)	Stock conc.	Catalog	Company
50 mM Tris-HCl pH 7	2.5 ml	1 M	AM9851	Thermo Fisher
150 mM NaCl	1.5 ml	5 M	AM9759	Thermo Fisher
1 mM EDTA	0.1 ml	0.5 M	AM9261	Thermo Fisher
0.1% SDS	0.5 ml	10%	AM9822	Thermo Fisher
ddH_2O	45.4 ml	-	AM9932	Thermo Fisher

- *Do it during step 1.1*
- *Use RNase free tube.*
- *Mix the solution after adding each reagents*
- *Do not put lysis buffer on ice.*
- *Do not add PI (Proteinase inhibitor) and RI (RNAse inhibitor) at this step.*

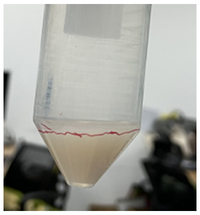
In this case: take one tube with 50 ml buffer and add 5 tablets of PI (Roche, 11836170001).



1.3. **Wash cells** with 50 ml **1 × DPBS** (Thermo Fisher Scientific, 14190250) by incubating on the Intelli-Mixer (Program F1, 20 rpm) for 5 min at room temperature. Spin down at 2,000 rpm for 5 min at room temperature.

- *Combine the same treatment cells into one new RNase-free 50ml-tube.*

- *In this case: each tube adds 1 ml of DPBS, combine all the cells in one 50 ml-tube, wash with 50 ml DPBS without PI or RI* (Thermo Fisher Scientific, AM2696).



1.4. Spin down at 2,000 rpm for 5 min at room temperature and discard the supernatant by pouring.

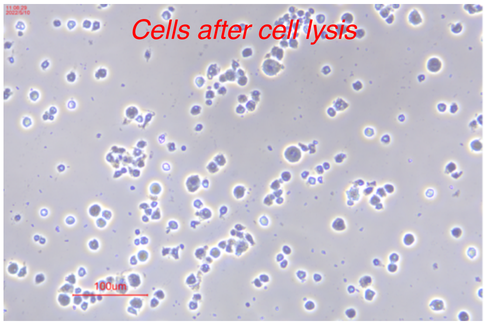
Firstly use 5 ml-pipette to remove the bubbles and then pool the supernatant, mark the size of pellets

1.5. Perform **cell lysis** using ***20*** ml of **0.1% FA lysis buffer without Triton-X100** freshly supplemented with PI, by incubating on the Intelli-Mixer (Program F1, 11 rpm) for 15 min at room temperature.

- *Add PI freshly before use at step 1.4.*
- *Do not put lysis buffer on ice.*
- *Check cells under microscope.*

In this case: firstly add 5 ml of buffer (contain PI) and use 1 ml-pipette to suspend the pellet, and then add another 5 ml buffer, finally add the remaining 10 ml buffer.

After 15 min incubation, check the cell under microscope.

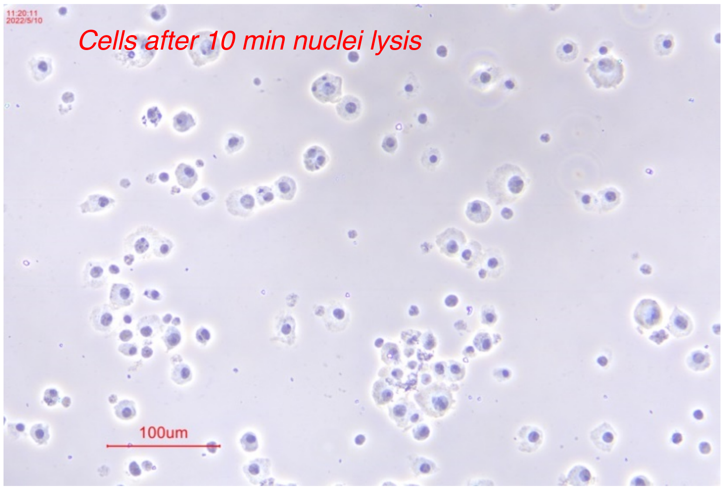


1.6. Perform **nuclei lysis** by adding ***1.8*** ml of 10% **SDS** to a final concentration of **1%**, mix and incubate on the Intelli-Mixer (Program F1, 30 rpm) for 10 min at 37 °C.

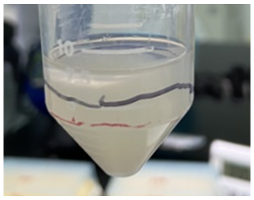
- *Do not put lysis buffer on ice.*
- *Check cells under microscope, the nuclei should be darker than step 1.5.*

Take out 5 µl samples to check the cells under microscope.

If the nuclear shows up a clear profiling, continue the nuclei wash step, if not, repeat the nuclei lysis.



1.7. Spin down at 3,890 rpm for 20 min at room temperature and remove the supernatant using the 5 ml-tip.

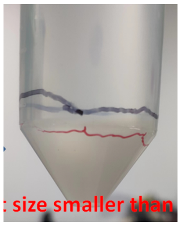


After spin down, the cell pellet tends to flow up, keep some remaining buffer in the pellet when remove the supernatant.

2. Nuclei wash

2.1. **Wash nuclei** with ***20*** ml of **0.1% FA lysis buffer without Triton-X100** freshly by incubating on the Intelli-Mixer (Program F1, 20 rpm) for 5 min at room temperature.

Do not put lysis buffer on ice.



2.2. Spin down at 3,990 rpm for 20 min at room temperature and remove the supernatant by ***5 ml-pipette***.

The size of nuclei pellet after incubating with 0.1% SDS would be smaller than 1% SDS at step 1.7.

Add the sonication buffer to the nuclei pellets and put on ice until sonication.

3. Sonication

3.1. Prepare 50 ml of **0.1% FA lysis buffer with Triton-X100**.

0.1% FA lysis buffer with Triton-X100	**Volume (50 ml)**	**Stock conc.**	**Catalog**	**Company**
50 mM Tris-HCl pH 7	2.5 ml	1 M	AM9851	Thermo Fisher
150 mM NaCl	1.5 ml	5 M	AM9759	Thermo Fisher
1% Triton-X100	2.5 ml	20%	327371000-100ml	Acros Organics
1 mM EDTA	0.1 ml	0.5 M	AM9261	Thermo Fisher
0.1% SDS	0.5 ml	10%	AM9823	Thermo Fisher
ddH$_2$O	42.9 ml	-	AM9932	Thermo Fisher

- *Do it during or before step 1.2.*
- *Use RNase free tube.*
- *Mix the solution after adding each reagent.*
- *Do not put lysis buffer on ice.*
- *Do not add PI and RI at this step.*

In this case: after preparing 50 ml sonication buffer, keep it at 4 ℃

3.2. **Clean the probe** of the sonication machine with 3~4 ml ddH$_2$O in 14 ml- RNase-free tube and setting the program (38%, 20 sec on/ 30 sec off, 4.5 min).

Put the tube containing ddH$_2$O to the tip of sonicator, and press "start" button to run 20 sec and stop, then keep the tip in ddH$_2$O until use.

3.3. Prepare 30 ml of **0.1% FA lysis buffer with Triton-X100** freshly supplemented with PI

15 ml for one tube of the nuclei pellet

In this case: add PI freshly in buffer before use

3.4. Resuspend nuclei pellet with 15 ml of **0.1% FA lysis buffer with Triton-X100** using 5 ml-pipette tip.

In this case: we only have two 50 ml-tubes of sample, the volume of the pellet is about 5 ml, add 5 ml buffer and suspend it, then add another 5 ml buffer, resuspended. Place it on ice until use.

3.5. **Aliquot** 1.5 ml into 14 ml-Falcon round bottom tube on ice.

Do put samples on ice.

In this case: for each treatment, aliquot 1.5 ml sonication sample to 10 tubes (14 ml-tube).

3.6. **Shearing** the nuclei by **sonication**.

- *Take one of the tubes and then perform sonication, which takes* about ***9*** *min.*
- *It may take half a day for sonication procedure.*

3.7. Combine all tubes into ***two*** 14 ml-Falcon round bottom tubes and spin down at ***3,890 rpm for 20 min*** at room temperature.

The pellet should be very small after sonication

3.8. Transfer the supernatant into ***two*** 14 ml-RNase-free tube (*~14 ml per tube*).

3.9. *Prepare C1 beads* (Thermo Fisher Scientific, 65001).

Take 200 µl of C1 beads to 1.5 ml-tube, place at magnetic rack for 1 min, remove the supernatant, wash with 200 µl of 0.1% FA lysis buffer with Triton-X100, twice. Then suspend the beads and place it on rack for 1 min, and remove the supernatant, finally, keep beads in 100 µl of buffer.

4. Preclear chromatin with C1 beads

4.1. Add 50 µl of *pre-washed* C1 beads to the two tubes containing ~14 ml of chromatin. Incubate on the Intelli-Mixer (Program F1, 20 rpm) for 20 min at room temperature.

4.2. Transfer the chromatin to two new tubes.

In this case: two 50 ml-tubes, each tube contains ~13.6 ml chromatin, keep at least 10 µl sample for QC by adding SDS with final concentration at 1%, and Proteinase K (Thermo Fisher Scientific, AM2546)*, then incubate at 65 ℃ with shaking overnight for decrosslinking.*

5. Hybridization overnight

5.1. Prepare two tubes of **35** ml **hybridization buffer**.

Hybridization buffer	**Volume** ***(35 ml)***	**Stock conc.**	**Catalog**	**Company**
750 mM NaCl	*5.25 ml*	5 M	AM9759	Thermo Fisher
50 mM Tris.HCl pH 7	*1.75 ml*	1 M	AM9851	Thermo Fisher
1 mM EDTA	*70 µl*	0.5 M	AM9261	Thermo Fisher
1% SDS	*3.5 ml*	10%	AM9823	Thermo Fisher
15% Formamide	*5.25 ml*		AM9342	Thermo Fisher
ddH$_2$O	*19.18 ml*	-		Thermo Fisher

5.2. Add 2 volume **(*26 ml*)** of **freshly prepared hybridization buffer** into the ***13 ml*** of pre-cleared chromatin. Mix well and split them into 3 of 14 ml-RNase-free tube (*13 ml per tube, 3 tubes for S2-WT, 3 tubes for S2-HS, ~13 ml reaction solution per tube*).

5.3. Rotated at RT for 30 min

5.4. Add **biotinylated probes** into each tube. Each treatment adds ***6 μl rox2 probes (2 μl per 14 ml tube)***. Seal the cap with film.

5.5. Incubate on the Intelli-Mixer (Program F1, 11 rpm) overnight at 37 °C.

Day-2

5.6. **Block** C1 beads with iBlock buffer.

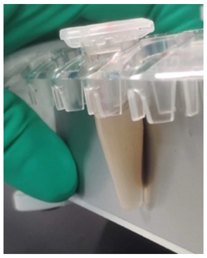
5.6.1. Take ***800* μl** C1 beads to a 1.5 ml-tube.

5.6.2. Place it on magnetic rack and remove the supernatant.

5.6.3. Wash the beads three times with 800 μl (per tube) of **0.1% FA lysis buffer without Triton-X100**.

- *In this case: after adding wash buffer, mix beads by flick and invert the tubes (may introduce some bubbles), spin briefly and place the tube on magnetic rack, keep tube on rack for 1 min before removed the supernatant.*
- *Wash step: do not need rotation.*

5.6.4. Add 800 μl iBlock (Thermo Fisher Scientific, T2015), incubate on the Intelli-Mixer (Program *F8, 20 rpm*) for 30 min at room temperature. Then place the tube on magnetic rack for 7~10 min.



5.6.5. Wash beads three times with 800 µl (per tube) of **0.1% FA lysis buffer without Triton-X100**.

Wash step: do not need rotation, just invert tube to mix beads.

5.6.6. Resuspend beads to original volume with corresponding hybridization reaction buffer.

In this case, resuspend beads in 600 µl of Hybridization buffer (for 6 tubes)

5.7. **Immobilize** the probe-chromatin to C1 beads. Add iBlock treated C1 beads to the corresponding hybridization chromatin. 300 µl C1 beads for S2-WT and 300 µl for S2-HS (100 µl per 14 ml-tube). Incubate on the Intelli-Mixer (Program F1, 11 rpm) for 2.5 hrs at room temperature.

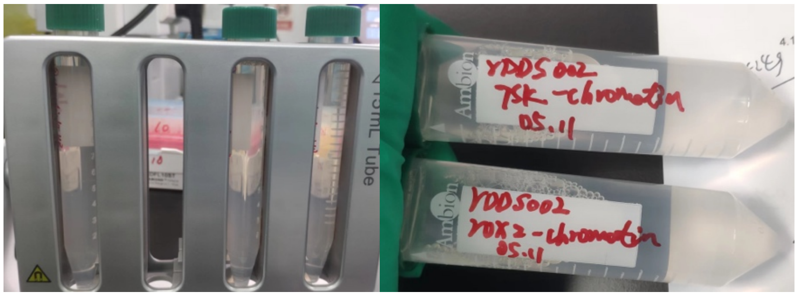
In this case, take 100 ul of iBlock treated C1 beads to each 14 ml-tube of corresponding hybridization chromatin.

5.8. Prepare 50 ml of **Wash buffer** and pre-warm at 37 °C.

Wash buffer	Volume (50 ml)	Stock conc.	Catalog	Company
2 × SSC	5 ml	20%	AM9770	Thermo Fisher
1% SDS	2.5 ml	10%	AM9823	Thermo Fisher
ddH$_2$O	42.5 ml	-	AM9932	Thermo Fisher

5.9. Transfer C1 beads bounded chromatin from the 14 ml-tube to 1.5 ml-tubes using magnetic rack (two 1.5 ml-tubes, one for S2-WT, one for S2-HS).

In this case: Place the 14 ml-tube on magnetic rack for 5 min, remove the supernatant. Take 1 ml of Wash buffer to suspend the beads and transfer to a new 1.5 ml-tube. Repeat and combine other beads from 14 ml-tube of same treatment to the 1.5 ml-tube, and then wash the 14 ml-tube with Wash buffer, collect them into the 1.5 ml-tube.

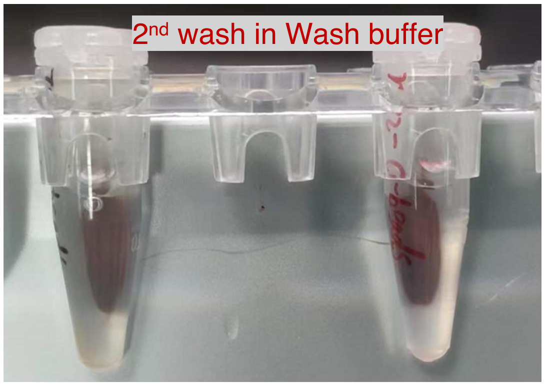


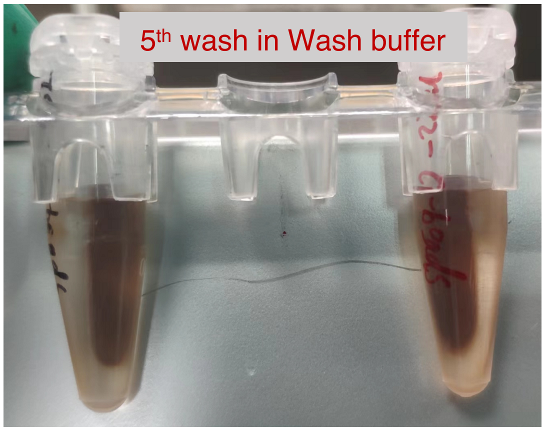
- *Keep the supernatant into to RNA-free 50 ml-tubes in case for trouble shooting.*
- *When transferring, perform tube by tube, place one tube on magnetic rack, remove the supernatant, repeat.*

5.10. Take 10 ml **Wash buffer** and 10 ml **TE buffer** (Thermo Fisher Scientific, AM9849) that supplemented with PI

- *In this case: Wash buffer do not need add PI or RI (because they contained 1% SDS), TE buffer need add PI before use.*
- *This period should start to prepare the IPB (weight IPB).*

5.11. **Wash beads** with 800 µl of **freshly prepared Wash buffer** for 5 times *(rotation for 5 min)*, then wash with 800 µl **TE buffer** for 3 times. At the last wash step, keep the tube at room temperature in TE buffer (1000 µl).





- *During the wash step of TE buffer, beads tend to be scatter and sticky, place the tube on the rack for 2 min before remove supernatant.*
- *Attention: 30 min before completed the wash step, we need start to prepare IPB solution and β-ME solution.*
- *For each wash step with Wash buffer, just flick then spin down briefly.*

Part II Proximity ligation of chromatin DNA

6. Block beads with denatured IPB

6.1. Prepare the denatured IPB during beads wash at step 5.10.

6.1.1. Weight 4.8 mg of IPB (Thermo Fisher Scientific, 11821334) and dissolve them into 400 µl of **IPB wash buffer**, incubate on the Intelli-Mixer (Program F1, 11 rpm) for 15 min at room temperature. Avoid of light using foil.

- *In this case, take 7.8 mg IPB, and dissolve them into 650 ul wash buffer.*
- *Make sure the IPB was dissolved before rotation.*

IPB wash buffer	Volume (10 ml)	Stock conc.	Catalog	Company
50 mM Tris-HCl pH 8	0.5 ml	1 M	AM9851	Thermo Fisher
50 mM NaCl	0.1 ml	5 M	AM9759	Thermo Fisher
1 mM EDTA	0.02 ml	0.5 M	AM9261	Thermo Fisher
ddH$_2$O	9.38 ml	-		Thermo Fisher

6.1.2. At the same time, take 1 µl of β-ME (Sigma, M6250) to 312 µl of H$_2$O. Incubate on the Intelli-Mixer (Program F1, 11 rpm) for 15 min at room temperature. Vortex briefly for 10 times before rotation.

Add the β-ME (stored at 4 °C) at chemical fume hood to avoid of the foul smell.

6.1.3. Take 44 µl β-ME solution into 400 µl IPB solution, mix and incubate on the Intelli-Mixer (Program F1, 11 rpm) for 15 min at room temperature to denature the IPB.

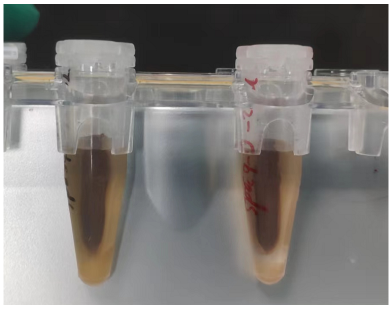
Attention: IPB should pre-warm at room temperature at least 1 hr before weighting. Both IPB and β-ME should be avoid of light.

6.2. Add 220 µl of the **denatured IPB** to the 1000 µl beads in TE. Incubate on the Intelli-Mixer (Program F1, 10 rpm) for 15 min at room temperature.

Attention: Keep the tubes to avoid light during rotation by packaging them with foil.

6.3. Rinse the beads once and wash beads with **TE (added PI freshly)** for five times *(rotation for 5 min at RT with F8, 10 rpm)*

Attention: the beads are sticky, should rinse the tips before using it to remove the supernatant. Some beads may float up during the wash step with TE buffer. Keep the beads in TE buffer for next step.



7. On beads end-repair, A-tailing and proximity ligation

7.1. Put buffer and dNTP on ice for end-repair.

7.2. Prepare 50 ml ChIA-PET wash buffer. Take out ***10*** ml and add ***25*** µl RI ***and 1 tablet of PI***.

ChIA-PET wash buffer	Volume (50 ml)	Stock conc.	Catalog	Company
10 mM Tris.HCl pH 7	0.25 ml	1 M	AM9851	Thermo Fisher
10 mM Tris.HCl pH 8	0.25 ml	1 M	AM9856	Thermo Fisher
1 mM EDTA	100 µl	0.5 M	AM9261	Thermo Fisher
150 mM NaCl	1.5 ml	5 M	AM9759	Thermo Fisher
ddH_2O	47.9 ml	-	AM9937	Thermo Fisher

This buffer should be stored at 4 ℃.

7.3. Prepare two times of volume of End-Blunt Mix ***on ice***. When adding dNTPs and RI, then flick, vortex, and spin down briefly each time to mix well.

End-Blunt Mix	1 ×	2.2 ×	Catalog	Company
Nuclease-free water	613 µl	1348.6 µl	AM9937	Thermo Fisher
NEB 10 X Buffer 2.1	70 µl	154 µl		*NEB*
10 mM dNTPs	7 µl	15.4 µl	*N0447S*	*NEB*
0.1 U/µl RI	3 µl	6.6 µl	AM2696	Invitrogen

Notice: Prepare all solution (2.2×) in one 2 ml-tube and then use 693 µl volume for one reaction.

7.4. Place tube at magnetic rack. Remove the supernatant in step *7.3*, and add 693 µl End-Blunt Mix into the beads. Flick it to mix.

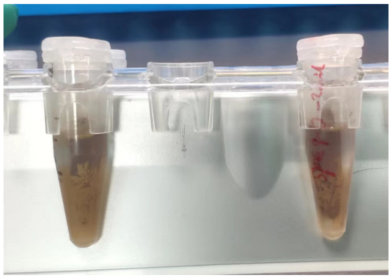
7.5. Add 7 µl of T4 DNA Polymerase (NEB, M0203S). Flick it to mix.

In this case: incubate the reaction at 12 ℃ on Thermomixer for 30 min with 800 rpm shaking, and then transfer to the 16 ℃- incubator for 15 min with rotation (F8, 10 rpm).

7.6. Thaw buffer and dATP for A-tailing on ice.

7.7. Take out the End-repair chromatin and spin down briefly. Then put on the magnetic rack and discard the supernatant. Wash beads with 800 µl ice-cold ChIA-PET buffer *(supplemented with PI and RI)* three times.

In this case: the wash steps do not need rotation, just by flick to mix beads, then place tube on magnetic rack for 2 min, rinse the pipette before removed the supernatant (The beads are very sticky).



7.8. Add 800 µl TE buffer, flick to mix, spin down briefly, put it at RT.

7.9. Prepare two times of volume of A-tailing Mix. When adding dATPs and RI, flick, vortex and spin down briefly each time to mix.

A-tailing Mix	1 ×	2.2 ×	Catalog	Company
Nuclease-free water	613 µl	1348.6 µl	AM9937	Thermo Fisher
10 X NEB Buffer 2	70 µl	154 µl	B7002S	NEB
10 mM dATPs	7 µl	15.4 µl	*18252015*	*Thermo Fisher*
0.1 U/µl RI	3 µl	6.6 µl	AM2696	Invitrogen

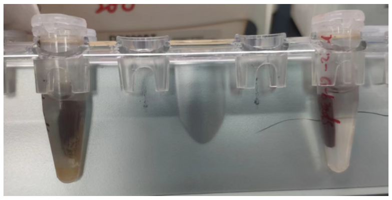
Notice: Prepare all solution (2.2×) in one 2 ml-tube and then use 693 µl volume for one reaction.

7.10. Place tube on magnetic rack, remove supernatant, add 693 µl A-tailing mix, flick to mix and spin down briefly.

7.11. Add 7 µl of Klenow fragment (3'-5' exo-) (NEB, M0212L). Flick to mix. Incubate at 37 °C for 50 min with rotation (F1, 11 rpm).

7.12. Put linker and buffer for ligation on ice.

7.13. Spin down the A-tailing chromatin briefly. Then put on the magnetic rack and discard the supernatant. Wash beads with 800 µl ice-cold ChIA-PET wash buffer three times *(do not need rotation)*.



7.14. Add 800 µl **EB** buffer (Qiagen, 19086), flick to mix, spin down briefly, then keep it at RT.

7.15. Prepare the Proximity Ligation Mix. When adding linker, flick, and upside down to mix. When adding buffer and RI, flick, vortex to mix each time.

Proximity Ligation Mix	1 ×	Catalog	Company
Nuclease-free water	*1243 µl*	AM9937	Thermo Fisher
Bridge Linker (*133 ng*/µl) (In-house)	*6 µl*	-	IDT
10 X T4 DNA Ligase Buffer	*140 µl*	*M0202s*	*NEB*
0.1 U/µl RI	5 µl	AM2696	Thermo Fisher

Notice: Here prepare one tube (1.394 ml) and then add all of them into the reaction tube. Rotated the tube at RT with rotation (F8, 10 rpm).

7.16. Place tube at magnetic rack, remove supernatant, add 1394 µl of Proximity Ligation Mix, Flick to mix and spin down briefly.

7.17. Put tubes on shaker (F8, 30 rpm).

7.18. Take out one tube from the shaker and add ***10*** µl T4 DNA ligase (NEB, M0202S), flick, up-side down and put it on shaker again. Then add enzyme to another tube. ***Incubate at RT for 50 min with rotation (F1, 20 rpm).*** After that seal the tube with film.

7.19. Then incubate overnight at 16 °C with rotation (F1, 20 rpm).

Day-3

8. Elution of proximity ligation DNA

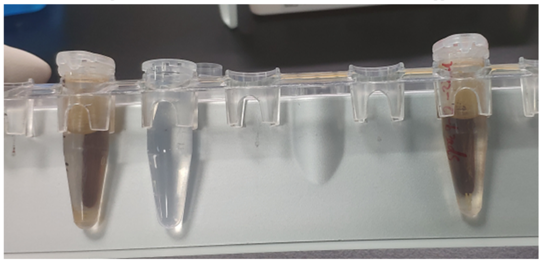
8.1. Prepare 10 ml **low salt concentration (LC) ChIP elution buffer**. Do not place them on ice.

LC ChIP Elution Buffer	10 ml	50 ml	Stock conc.	Catalog	Company
50 mM Tris.HCl pH 7	0.25 ml	1.25 ml	1 M	AM9851	Thermo Fisher
50 mM Tris.HCl pH 8	0.25 ml	1.25 ml	1 M	AM9856	Thermo Fisher
0.5 % SDS	0.5 ml	2.5 ml	10%	AM9823	Thermo Fisher
ddH$_2$O	9 ml	45 ml	-	AM9932	Thermo Fisher

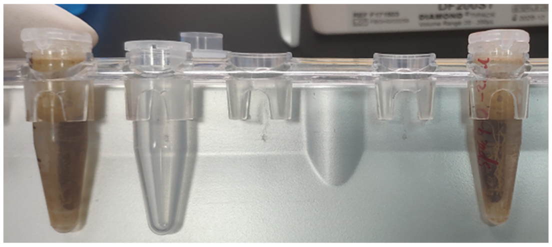
8.2. Wash beads with 800 µl of ChIA-PET wash buffer three times, and TE buffer twice.

Notice: Flick the tube once and upside down to mix in the rest procedures. Upside down for mixing, spin down briefly and keep on magnetic rack for removing the supernatant. When upside down for mixing, the beads were sticky, and may remain something on the beads.

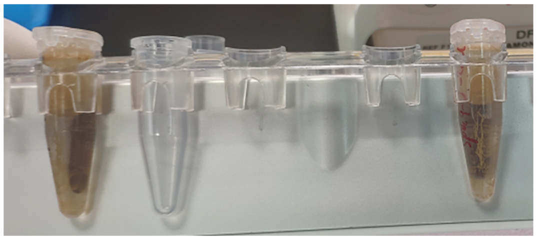
After 2nd Wash in ChIA-PET wash buffer



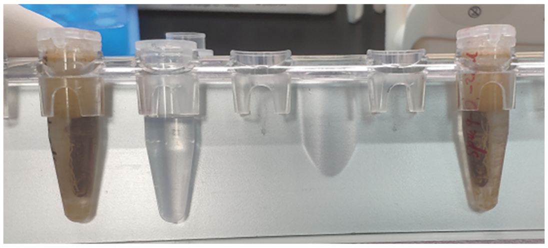
After 3rd Wash in ChIA-PET wash buffer



After 1st Wash in TE buffer



After 2nd Wash in TE buffer



- *In this case: Place ChIA-PET wash buffer on ice.*
- *Wash step: rotated at RT with program (F8, 10 rpm) for 5 min during each wash step. Beads are easy spreading on the magnetic rack. The beads are sticky to the tips, and some beads may bind at the tube, hard to wash both in ChIA-PET wash buffer and TE buffer.*

8.3. Add 480 µl LC ChIP elution buffer into the beads bounded with ligated DNA. Mix and add 20 µl of 20 mg/ml Proteinase K (Thermo Fisher Scientific, AM2546).

Notice: Just upside down to mix in case of introducing bubbles (elution buffer contains SDS, beads in bubbles may not be fully eluted). Working concentrations of the proteinase K is from 50 to 250 µg/ml.

8.4. Incubate at 65 °C on Thermomixer (Eppendorf) with 950 rpm overnight.

Notice: Do not put the tube to the Thermomixer until the temperature reach to 65 °C.

Day-4

9. DNA purification (Part A)

9.1. Put the ligated products with beads on magnetic rack.

Notice: The products include: 10 µl input DNA; 20 µl beads after Hybridization for rox2 or 7sk respectively; 800 µl beads after ligation for rox2 or 7sk respectively.

9.2. Centrifuge the MaXtract High Density Tube (Qiagen, 129046) at 12,000 rpm for 2 min at RT.

9.3. Add the de-crosslinked products (500 µl) into the MaXtract tube.

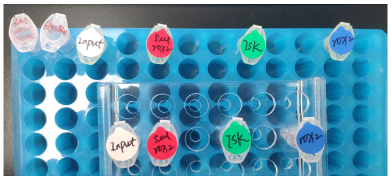
9.4. Then transfer 500 µl Phenol:Chloroform:IAA (Solarbio, P1012) to the MaXtract tube using a 5 ml-pipette tip in fume hood. Mix gently by hand.

9.5. Spin down at 12,000 rpm for 6 min at RT.

9.6. During step 9.5, prepare the reagents: add 52 µl of 3 M Sodium Acetate pH 5.5 (Ambion, AM9740) to the bottom of a 1.5 ml-tube, and add 1 µl of GlycoBlue (ThermoFisher, AM9516) to the cap of tube. Do not mix. Do not close the cap. Transfer the supernatant from the MaXtract tube into the 1.5 ml-tube quickly.

9.7. Add 520 µl ice-cold isopropanol (Sigma, I9516) to the1.5 ml-tube. Mix gently. Put it at -80 °C overnight.

In this case: first we add 1.5 µl GlycoBlue to the cap of tube, secondly add 50 µl of 3 M Sodium Acetate pH 5.5 (Ambion, cat. AM9740) to the bottom, and then transfer the supernatant of MaXtract tube by pouring, finally added the 500 µl of isopropanol (RT) into the tube. Mix by inverting the tubes and put it at -80 °C overnight.



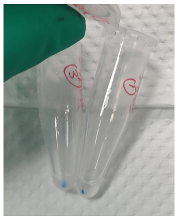
Day-5

10. DNA purification (Part B)

10.1. Take out the mixture from -80 °C and thaw at RT *(about 10 min)*, then spin down at 12,000 rpm for about 1 hr at 4 °C to precipitate DNA.

In this case: we centrifuge for 1 hr.

There will have a blue pellet:



10.2. Wash DNA pellet with 75% ethanol: carefully remove the supernatant. Add 800 µl of 70% ice-cold ethanol (Sigma, E7023), spin down at 12,000 rpm for 5 min at 4 °C, remove the supernatant. Repeat it once.

Notice:

a. Once after the centrifuge, remove the supernatant quickly by tips (first use 1 ml-tips following by 100 µl-tips).

b. Do it just nearby the centrifuge, do not take all of them out. If the temperature recovers, the blue pellet may not stick to the tube wall.

In this case: first we pour out the supernatant quickly, and then add 1 ml of ice-cold 75% ethanol to rinse the pellet and pour out the supernatant quickly. After 1st rinse, add 1 ml of ice-cold 75% ethanol, and flick the tube to make the pellet float up. Then spin down at 12,000 rpm for 10 min at 4 °C, remove the supernatant. Repeat it once.

10.3. Spin down the tube at 12,000 rpm for ***10*** min at 4 °C to collect the extra ethanol at the bottom of the tube. Then remove the rest ethanol.

10.4. Dry the DNA pellet by Vacuum (from 1 min 30 sec to 2 min generally).

10.5. Resuspend DNA pellet in 10 µl Qiagen elution buffer for 1~2 hrs at RT.

In this case: S2-WT and S2-HS samples were dissolved in 30 µl of EB buffer, input DNA was dissolved in 100 µl of EB buffer. Place the tube at RT for 2 hrs, flick the tube several times during the dissolved step.

10.6. Calculate the concentration using Qubit.

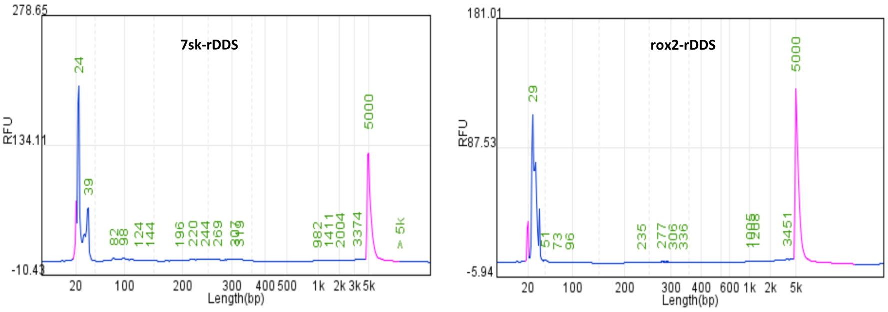
Sample ID	Factor	Elution volume (µl)	Concentration (ng/µl)	Elution DNA Amount (ng)
rox2-original tube	rox2	30	12.8	384
7sk-original tube	7sk	30	12	360
input DNA-original tube		100	86.8	8680

10.7. Put the DNA on ice when perform Qubit. Then store them at -20 °C for later library construction.

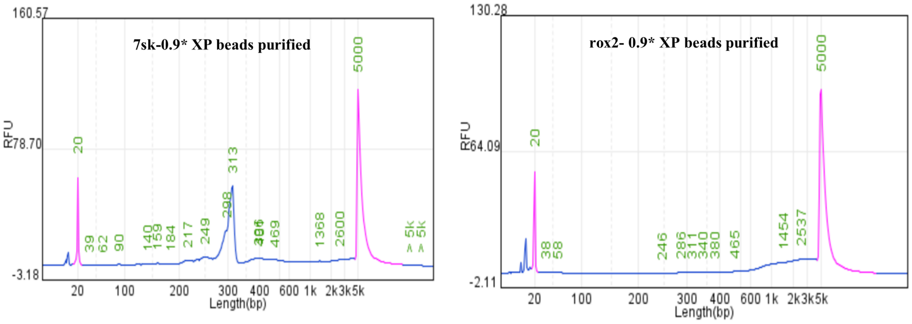
In this case, the samples are aliquot for Tn5 testing and scale-up tagmentation directly.

10.8. Check DNA size and profiling using Qsep.

rDDS002 Qsep results



After 0.9* Ampure XP beads purification



DNA amount after 0.9× Ampure XP beads purification:

NO.	Sample ID	Elution volume (µl)	Concentration (ng/µl)	Elution DNA Amount (ng)	Remaining Volume (µl)	Remaining DNA Amount (ng)
1	**rox2-rDD**	20	1.16	23.2	16.9	**19.6**
2	**7sk-rDD**	20	1.97	39.4	17.4	**34.28**

11. Tn5 tagmentation testing and scale-up

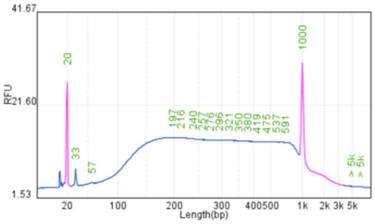
11.1. Prepare the reaction in a PCR tube on ice as below, add water in advance, followed by DNA sample, 4× THS TD buffer, then mix 10-20 times by pipetting with setting at 30 µl, briefly spin down, finally add Tn5, mix 10-20 times by pipetting with setting at 30 µl, spin down briefly, and make sure there is no bubble.

11.2. Then incubate at 55 °C for 10 min, and keep at 4 °C in PCR instrument.

PCR instrument setting:

Temperature	**Time**
70 °C	Heat the lid
55 °C	10 min
4 °C	Hold

This is quality control, and the majority of DNA fragments should fall into the range of 200-bp to 1-kb (shown as below).



11.3. According to Tn5 testing result, the rest products were scale-up with the same condition.

11.4. Then release Tn5 from DNA template: take out the sample from PCR instrument and place on ice. Spin down briefly, put at RT for 2 min, then add 5.5 µl 10% SDS to the sample (finally concentration is 1%), mix 10-20 times by pipetting with setting at 30 µl, avoid bubbles and spin down briefly, incubate in PCR instrument at 37 °C for 15 min at least.

12. Prepare M280 beads for immobilization of ligated products

12.1. Prepare M280 beads (Thermo Fisher Scientific, 11205D): take 40 µl of M280 (for 2 samples), wash with 200 µl of 1× B&W buffer twice.

Wash step: suspend the M280 beads with pipette, spin down briefly, then put on the tube on magnetic rack, rinse the tip in the supernatant for 5 times, keep the tube staying on magnetic rack for 30 sec, then remove the supernatant

1× B&W buffer (10 ml)

Component (Stock)	**Final concentration**	**Amount for 10ml**
1M Tris-HCl pH 7.0	5 mM	0.025
1M Tris-HCl pH 8.0	5 mM	0.025
5M NaCl	1 M	2
0.5M EDTA	0.5mM	0.01
Water (Molecular Grade)		7.94

12.2. Resuspend beads in 200 µl of iBlock Buffer, mix and incubate at RT from 45 min on rotating Intelli-Mixer (F1,12).

12.3. Briefly spin down and keep the tube on magnetic rack for about 15 min, remove the iBlock buffer, then wash beads with 500 µl of 2× B&W buffer twice.

2× B&W buffer (10 ml)

Component (Stock)	**Final concentration**	**Amount for 10ml**
1M Tris-HCl pH 7.0	5 mM	0.05
1M Tris-HCl pH 8.0	5 mM	0.05
5M NaCl	1 M	4
0.5M EDTA	0.5mM	0.02
Water (Molecular Grade)		5.88

12.4. Add 100 µl 2x B&W buffer to suspend the M280 beads, then add 100 µl of genomic DNA mixture (around 1,000 ng), mix well with the iBlocked beads, then incubate on Intelli-Mixer with rotation for at least 30 min (UU 50rpm) at RT for at least 30 min.

12.5. Briefly spin down the tube and place tube on magnetic rack for 15 min, discard iBlock buffer, and then wash beads with 200 µl of 1× B&W buffer twice.

Wash step: add 200 µl of 1× B&W, mix by pipette, and then rotate at RT with program (uu 70 rpm) for 2~5min, then place on magnetic rack, discard the supernatant.

12.6. Add 40 µl of 1× B&W buffer and stay on RT, aliquot to 2 tubes for 2 samples (20 µl per tube).

13. Immobilization of DNA to streptavidin dynabeads

13.1. Remove the remaining buffer in M280 beads, add 50 µl of 2× B&W buffer, transfer all the 50 µl of fragmented DNA library product from PCR tube to the M280 beads, then rinse the PCR tube with 55 µl 1× B&W buffer, and collect them to M280 beads. Mix well and incubate on Intelli-Mixer at RT for 45min (UU, 50 rpm).

13.2. Briefly spin down the tube, place the tube on magnetic rack, discard supernatant, wash beads with 500 µl of 0.5% SDS/ 2× SSC buffer at 37 °C for five times.

Wash step: rotating the tube at 37℃ for 5 min with program (F8 30 rpm, uu 20 rpm, UU 30 rpm), Buffer was pre-warmed at 37℃.

0.5% SDS/ 2× SSC

Wash buffer	Volume (10 ml)	Stock conc.	Catalog	Company
2 × SSC	1 ml	20%	AM9770	Thermo Fisher
0.5% SDS	0.5 ml	10%	AM9823	Thermo Fisher
ddH$_2$O	8.5 ml	-	AM9932	Thermo Fisher

13.3. Add 500 µl of 1× B&W buffer, rotate at 37℃ for 5 min (F8, 30 rpm, uu 20, UU 30) and discard the supernatant. Then add 200 µl of 1× B&W buffer, rotate at 37 °C for 5 min (UU50). Place the tube on the magnetic rack for 1 min, then use 200 µl-pipet to mix the buffer on the bottom of tube to allow all the beads attaching to the wall of tubes. Then discard the supernatant. Add 30 µl of EB buffer to the beads and store at -20℃.

14. PCR amplification of M280 enriched DNA

14.1. Prepare PCR reaction (according to the manual of Vazyme TD501 kit)

14.2. Thaw the samples and reagents on ice. All the preparation steps for PCR should be on ice.

14.3. Set up PCR reaction mixture as follow

Solution Name	Component (Stock)	×1 (µl)
PCR mix	High-Fidelity 2X PCR Master Mix	25.00
	PPM (dilute 5 times)	1
	N5XX (dilute 5 times)	1
	N7XX (dilute 5 times)	1
	M280 beads (1/3)	10
	H2O	12
	Total	50.00

14.4. Transfer the PCR tubes to PCR machine and set up the program.

Temperature	Time	Cycles
105 ℃	Heat the lid	
72 ℃	3 min	
98 ℃	30 sec	
98 ℃	15 sec	x
60 ℃	30 sec	
72 ℃	2 min	
72 ℃	5 min	
4 ℃	Hold	

The PCR amplification cycle is important; the more PCR cycles used; the lower complexity of the library obtained. Thus, the optimal number of PCR amplification cycles needs to be determined empirically. Generally, do not perform more than 15 cycles.

15. Purify PCR product using AMPure beads (Beckman Coulter, A63881)

15.1. Transfer the PCR product to a new 1.5 ml-tube. Add 20 µl EB buffer to rinse the PCR tube, then collect the EB buffer to the 1.5 ml-tube and place tube on magnetic rack.

15.2. Transfer the supernatant (about 100 µl) to a new 1.5ml-tube. Add 100 µl (1× volume) of AMPure XP beads (pre-warmed at RT) to the 1.5 ml-tube (details are described as the manual)

15.3. Finally, vacuum samples about 1 min. Then elute the DNA from beads with 12 µl EB buffer.

15.4. DNA quantification by Qubit.

15.5. According to the concentration of purified DNA, the optimal PCR cycle is determined, and the rest sample is amplified for size selection.

Sample	PCR index	PCR index	Tn5 input	1/3 PCR cycle	1/3 Amount (ng)	1/3 Remained (ng)	2/3 PCR cycle	Estimated 2/3PCR product (ng)	Estimated all PCR product (ng)
7sk	N505	N703	34.2ng	15	14.3	11.44	14	14.3	25
rox2	N505	N704	19.6ng	15	7.2	5.76	15	14.4	20

16. Size selection for sequencing (double size selection with AMPure beads)

16.1. Use Ampure XP beads for size selection. Generally, DNA size range of 250~600 bp is suitable for Illumina sequencing.

16.2. Take out Ampure XP beads and keep at RT for at least 30 min. Thaw the PCR samples (~50 µl per tube, 2 tubes) and purified DNA (1/3 of M280-bounded DNA for testing, ~8 µl) on ice. Spin down the samples briefly.

16.3. Combine the PCR product (2 tubes) and purified DNA in a 1.5 ml-tube. Place the tube on magnetic rack for 1 min. Use a new 50 µl-tip to transfer the 100 µl of supernatant to a new 1.5 ml-tube and evaluate the volume (in this case, 120 µl in total).

16.4. Add 73.2 µl (0.61 ×) Ampure XP beads to the above tubes. Vortex 10 times softly. Rotate at RT for 10 min with rotation (F8, 12rpm). Spin down briefly, and place on magnetic rack for 5min. Transfer 193.2 µl of supernatant to a new tube.

16.5. Then add 22.8 µl (0.8-0.61×) Ampure beads to the 193.2 µl of supernatant, vortex 10 times softly. Rotate at RT for 10 min with rotation (F8, 12rpm). Spin down briefly, and place on magnetic rack for 5 min. Wash the beads with 80% ethanol twice, dry beads by vacuum. Elute DNA from beads with 12 µl EB buffer for 10 min with rotation (UU 70 rpm), and then obtain 8 µl of supernatant and transfer to a new tube.

16.6. Take 1 µl for the concentration test by Qubit. And take samples to run Qsep for size distribution analysis (details are refer to the manual of Qsep).

Samples	**Conc. (ng/µl)**			**Amount (ng)**				**Remained Amount (ng)**		
	Large-(ng/µl)	Small-(ng/µl)	Double-(ng/µl)	Large	Small	Double	Remained double-size volume (µl)	Large	Small	Double
7sk	0.7	0.77	0.61	7	7.7	6.1	8	5.2	5.7	4.88
rox2	0.42	0.54	0.46	4.2	5.4	4.6	8	3.1	4	3.68

**The Qsep profile of the size distribution should be at length of 250~ 600 bp. In addition, 10~30 ng of library would be obtained.*

16.7. The double size selected library is ready for Illumina Novaseq sequencing with length of 2× 150 bp.

Attach: Time schedule for RDD experiments

Time Schedule		In Solution RDD Experiments	Time course
DAY 1	08:50-12:00	Thaw cells on ice	60 min
		Wash cells in DPBS	15 min
		Cell lysis	15 min
		Nucleus lysis	25 min
		Repeat nuclei lysis	25 min
		Wash nuclei	20 min
	13:00-18:30	Aliquot nuclei to Falcon tube	10 min
		Sonication	250 min
		Preclear chromatin with C1 beads	20 min
		Prepare for Hybridization	20 min
		Hybridization overnight	O/N
DAY 2	09:00-11:00	Block C1 beads with iBlock buffer	60 min
		Immobilize chromatin to C1 beads	150 min
	13:30-20:30	Transfer C1 beads bounded chromatin to 1.5 ml-tube	40 min
		Wash C1 beads	40 min
		Prepare denatured IPB	40 min
		Block C1 beads with denatured IPB	20 min
		Wash C1 beads with TE buffer	40 min
		On beads end repair	60 min
		Wash beads	15 min
		A-tailing	60 min
		Prepare reaction for proximity ligation	30 min
		Ligation overnight	O/N
DAY 3	14:30-16:00	Wash beads	60 min
		Decrosslinking overnight	O/N
DAY 4	14:30-15:30	DNA purification (Part A)	40 min
DAY 5	10:00-16:00	DNA purification (Part B)	120 min
		Qsep test	30 min
		Qubit test	30 min
DAY 6	09:00-11:30	Tn5 testing	120 min
		Proximity ligation product tagmentation	30 min
	13:30-21:00	Prepare streptavidin dynabeads M280	120 min
		Immobilization of DNA to M280	60 min
		Wash M280 beads	120 min
		PCR cycle test (use 1/3 M280 bounded DNA)	60 min
		Purify PCR product using AMPure beads	60 min
		DNA quantification by Qubit	30 min
DAY 7	09:30-11:00	PCR amplification (2/3 M280 bounded DNA)	90 min
	14:00-17:00	Double size selection	180 min