**CR裂核版**

Gaolab-CM

**1、5% Digitonin（65mg粉末+1.3mL DMSO 70℃）**

To reconstitute enough digitonin for an experiment, weigh out the powder in a 2 ml microcentrifuge tube, boil water in a small beaker in a microwave oven, and pipette in and out to warm the 1000 μL pipette tip. Pipette the hot water into the tube with the digitonin powder to make 5% (w/v), close the cap and quickly vortex on full until the digitonin is completely dissolved. If refrigerated, this stock can be used within a week, but will need reheating as the digitonin slowly precipitates.

**2、Binding Buffer**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积 |
| HEPES-KOH pH 7.9 | 1 M | 20 mM | 200 μL |
| KCl | 1 M | 10 mM | 100 μL |
| CaCl2 | 1 M | 1 mM | 10 μL |
| MnCl2 | 1 M | 1 mM | 10 μL |
| ddH2O |  |  | 9.68 mL |
| Total |  |  | 10 mL |

Store the buffer at 4℃ for 6 months.

**3、Wash Buffer**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积（样品数 = 6） |
| HEPES pH 7.5 | 1 M | 20 mM | 1 mL |
| NaCl | 5 M | 150 mM | 1.5 mL |
| Spermidine | 2 M | 0.5 mM | 12.5 μL |
| BSA | 10 % | 0.1 % | 500 μL |
| PIC | 50 X | 1 X | 1片（EDAT-free） |
| ddH2O |  |  | 47 ml |
| Total |  |  | 50 ml |

Store the buffer at at 4℃ for up to 1 week.

**4、Blocking Buffer = Wash Buffer + EDTA + Dig**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积 (N=样品数+1) |
| EDTA（250X） | 500 mM | 2 mM | 28 μL |
| Digitonin（50X） | 5 % | 0.1 % | 140 μL |
| Wash Buffer |  |  | 7 mL |

现用现配。

**5、Dig-wash Buffer = Wash Buffer + Dig**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | Volume 2 |
| Digitonin（50X） | 5 % | 0.1 % | 200 μL |
| Wash Buffer |  |  | 10 mL |

现用现配。

**6、2XSTOP（Low Salt）- 普通用（每个样品准备150 μL体系）**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积（样品数 = 6） |
| NaCl | 5 M | 200 mM | 40 ul |
| EDTA | 0.5 M | 20 mM | 40 ul |
| EGTA | 0.2 M | 4 mM | 20 ul |
| Digitonin | 5 % | 0.02 % | 4 ul |
| RNase A | 10 mg/mL | 50ug/ml | 5 ul |
| glycogen | 2 mg/ml | 40ug/ml | 20 ul |
| spike-in DNA | 10pg/ul | 5 pg/ml | 0.5 ul |
| ddH2O |  |  | 870.5 ul |
| Total |  |  | 1 ml |

Store the buffer at 4℃ for up to 1 week.

**7、2XSTOP（High Salt）- 裂核用（每个样品准备50 μL体系）**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积（样品数 ≤ 20） |
| NaCl | 5 M | 4 M | 800 ul |
| EDTA | 0.5 M | 20 mM | 40 ul |
| EGTA | 0.2 M | 4 mM | 20 ul |
| Digitonin | 5 % | 0.02 % | 4 ul |
| glycogen | 2 mg/ml | 40 ug/ml | 20 ul |
| spike-in DNA | 10 pg/ul | 5 pg/ml | 0.5 ul |
| ddH2O |  |  | 115.5 ul |
| Total |  |  | 1 ml |

Store the buffer at 4℃ for up to 1 week.

**8、67 μg/mL RNase A - 裂核用（每个样品准备X μL体系）**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock | Final | 推荐体积（样品数 ≤ 20） |
| RNase A | 10 mg/mL | 67 ug/ml | 20 ul |
| ddH2O |  |  | 2.98 ml |
| Total |  |  | 3 ml |

***1，Binding cells to beads***

**TIMING 30 min**

**CRITICAL STEP:** All steps prior to the addition of antibody are performed at room temperature to minimize stress on the cells. Because it is crucial that DNA breakage is minimized throughout the protocol, we recommend that cavitation during resuspension and vigorous vortexing be avoided.

1）Harvest fresh culture(s) at room temperature and count cells. The same protocol can be used for 100 to 250,000 mammalian cells per sample and/or digestion time point.

PAUSE POINT: If necessary, cells can be cryopreserved in 10% DMSO using a Mr. Frosty isopropyl alcohol chamber. We do not recommend flash freezing, as this can cause background DNA breakage that may impact final data quality.

2) Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

3) Wash cells twice with 1 ml room temperature Wash buffer and centrifuge 3 min 600 x g at room temperature and withdraw liquid.

4) Resuspend in 600ul room temperature Wash buffer by gently pipetting.

(这一步数细胞的话就用少量wash buffer重悬，在wash buffer里数，另取一个低吸附1.5ml管子加入600ul wash buffer，数好的细胞吹进去)

5）10ul/reaction beads were transferred to a microfuge tube containing 3X volume cold Binding buffer

6）Beads were washed twice in 1mL cold Binding buffer and resuspended in 300μL binding buffer.

7）beads were added to cells with gentle vortexing and incubated for 10 minutes at room temperature.

（整个实验过程都不要用枪用力吹打，尽量加入buffer后颠倒混匀，以免细胞从beads上掉下来）

**CRITICAL STEP:** To evaluate success of the procedure without requiring library preparation, include in parallel a positive control antibody (*e.g.* α-H3K27me3) and a negative control antibody (*e.g.* α-rabbit). Do not include a no-antibody control, as the lack of tethering may allow any unbound pA-MN to act as a “time-bomb” and digest accessible DNA, resulting in a background of DNA-accessible sites.

***2，Bind (primary) antibodies***

**TIMING 15 min–overnight, with longer incubations providing higher yields**

7) Place on the magnet stand to clear and pull off the liquid.

8）Bead-bound nuclei were blocked with 1mL cold Blocking buffer，incubated for 5 minutes at room temperature.

9）The supernatant was discarded and nuclei/beads were washed in 1mL cold Dig-Wash Buffer and resuspended in 250μL cold Dig-Wash Buffer

10）1ug primary antibody was added with gentle vortexing of bead-bound cell in 250μL cold Dig-Wash buffer.

11) Samples were incubated with rotation at 4°C for 2 hours or overnight.

12) The supernatant was discarded and samples were washed twice in 1mL cold Dig-Wash buffer. The supernatant was discarded and samples were resuspended in 250μL cold Dig-Wash buffer.

***3，Bind secondary antibody (as required)（目前的抗体不需要这一步）***

**TIMING 15 min-1.5 hr**

**CRITICAL STEP:** The binding efficiency of Protein A to the primary antibody depends on host species and IgG isotype. For example, Protein A binds well to rabbit and guinea pig IgG but poorly to mouse and goat IgG, and so for these latter antibodies a secondary antibody, such as rabbit α-mouse is recommended.

13) Secondary antibody was added with gentle vortexing of the nuclei in 250μL cold Dig-Wash buffer to a final concentration of 1:100.

14) Samples were incubated with rotation at 4°C for 1 hour.

15)The supernatant was discarded and samples were washed twice in 1mL cold Dig-Wash buffer. The supernatant was discarded and samples were resuspended in 250μL cold Dig-Wash buffer.

***4，Bind Protein A-MNase fusion protein***

**TIMING 15 min–1.5 hr**

16) pA-MN was added with gentle vortexing of the cells in 250μL cold Dig-Wash buffer to a final concentration of 1:500.

17) Samples were incubated with rotation at 4°C for 1 hour.

18)The supernatant was discarded and samples were washed twice in 1mL cold Dig-Wash buffer. The supernatant was discarded and samples were resuspended in 150μL（裂核时体系改为50μL）cold Dig-Wash buffer.

***5，Targeted digestion***

**TIMING 45 min**

19) Samples were equilibrated to 0°C on ice water for 5-10 minutes

20)To initiate cleavage, 3μL（裂核时改为1μL）100mM CalC2 was added during gentle vortexing, samples were flicked quickly to mix and returned to ice water.

21) After 5 minutes of digestion, reactions were stopped with addition of 150μL 2XSTOP buffer

***5（裂核版），Targeted digestion***

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***6.Target chromatin release***

**TIMING 20 min**

22) Samples were incubated at 37°C for 20 minutes to digest RNA and release DNA fragments

23) Samples were centrifuged at 16,000g for 5 minutes and supernatants were transferred to a new microfuge tube while pellets and beads were discarded.

***7，DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)***

**TIMING 1.5 hr**

48) To each sample add 3 μL 10% SDS (to 0.1%), and 2.5 μL Proteinase K (20 mg/ml). Mix by inversion and incubate 10 min 70 oC.

49) Add 300 μL PCI and mix by full-speed vortexing ~2 s.

50) Transfer to a phase-lock tube, and centrifuge 5 min room temperature at 16,000 x g.

51) Add 300 μL chloroform and invert ~10x to mix.

52) Remove liquid by pipetting to a fresh tube containing 0.5 μL glycoblue.

53) Add 300 μL isopropanol and mix by vortexing or tube inversion.

54) Put in -80 oC for 20min or overnight

55) centrifuge 15 min at 4 °C 16,000 x g.

56) Wash the pellet twice with 1 ml 80% ethanol and incubate in room temperature for 5 min, then centrifuge 5 min at 4 °C 16,000 x g.

57) Carefully pour off the liquid and drain on a paper towel. Air dry.

58) When the pellet is dry, dissolve in 17μL EB buffer.

利用KAPA试剂盒建库，最后PCR的过程建议尝试常规方法和快速扩增两种

快速扩增：循环中没有专门的延伸时间

98℃ 45s

98℃ 15s

60℃ 10s，go to step 2 for 14-16 cycle

72℃ 1min

4℃ hold

建议尝试抗体 OCT4(100cells,500cells,30Kcells)，H3K4me3(20cells,100cells)

每个做两个重复，扩增时选择不同的扩增方法（常规和快速）