甲基化 BSP 检测实验报告

1 实验器材及试剂

1.1 实验器材

名称	厂家	型号
台式高速冷冻型微量离心机	DragonLab	D3024R
PCR 仪	北京东胜创新生物科技有限公司	东胜龙 ETC811
全温震荡培养箱	Labotery	ZQPW-70
隔水式恒温培养箱	慧泰仪器	GHP-9050 型
超净工作台	苏净安泰	SW-CJ-1FD
电泳仪	Servicebio	FW-600
凝胶成像系统	上海天能科技有限公司	Tanon-1600R
标准试剂型纯水仪	青岛富勒姆科技有限公司	FBZ2001-up-p
干式恒温器	杭州奥盛仪器有限公司	K20
制冰机	SIMAG	SPR80
超微量分光光度计	Thermo	NanoDrop2000
掌上离心机	Servicebio	MC-700
涡旋混匀仪	Servicebio	MV-100
高速低温组织研磨仪	Servicebio	KZ-III-F

1.2 主要实验试剂及耗材

试剂	厂家	货号
组织/细胞/血液基因组 DNA 提取试剂盒	Servicebio	G3633
通用型 DNA 纯化回收试剂盒	Servicebio	G3631
pSWE-Topo Zero Cloning Kit	Servicebio	G3022
2×Taq PCR Master Mix	Servicebio	G3302
2×Fast Pfus PCR Master Mix	Servicebio	G3305
高纯度低电渗琼脂糖（Agarose）	Servicebio	G5056
GN100bp DNA Ladder II	Servicebio	G3366
1.5mL 离心管（无菌无酶）	Servicebio	EP-150-J
SerRed 核酸染料	Servicebio	G3606
Recombinant Proteinase K	Servicebio	G1234

红细胞裂解液	Servicebio	G2015
50*TAE	Servicebio	G3001
10×TE（pH 8.0）	Servicebio	G3003
6× DNA Loading Buffer	Servicebio	G3011
LB 液体培养基（干粉）	Servicebio	G3102
Water Nuclease-Free	Servicebio	G4700
Methylation-Gold Kit	ZYMO	D5005
无水乙醇	国药集团化学试剂有限公司	10009218
异丙醇	国药集团化学试剂有限公司	80109218

2 实验步骤

2.1 组织基因组 DNA 提取

2.1. 1 处理材料

a) 取 50-100 μL 无核红细胞全血（含抗凝剂），置于 1.5 mL 离心管中，补加溶液 **Buffer GA** 至 **200 μL**，使用涡旋振荡器涡旋混匀，接着进行第 3 步**（注意：如果处理更大体积血液，在样品中加入 3 倍体积红细胞裂解液（客户自备，目录号：G2015）颠倒混匀，室温放置 5 min，期间再颠倒混匀几次，1,000 rpm 离心 5 min，使用移液器轻轻吸弃上清，留下白细胞沉淀，加入 200 μL 溶液 Buffer GA，使用涡旋振荡器涡旋混匀，接着进行第 3 步）**；

b) 取 5-20 μL 有核红细胞全血（含抗凝剂），置于 1.5 mL 离心管中，补加溶液 Buffer GA 至 200 μL，使用涡旋振荡器涡旋混匀，接着进行第 3 步；

c) 取 10^6-10^7 细胞悬液，置于 1.5 mL 离心管中，1,000 rpm 离心 5 min，使用移液器轻轻吸弃上清；

d) 取 2-30 mg 的动物组织（切勿使用超过最大起始量的组织），置于 1.5 mL 离心管中，用剪刀尽可能地剪成碎块，对于一些质地坚硬的组织也可以进行液氮研磨；

2.1.2. 向离心管中加入 **200 μL 溶液 Buffer GA**，使用涡旋振荡器重悬样品；

2.1.3. 向离心管中加入 **20 μL 溶液 Proteinase K Stock Solution**，56℃孵育 30-60 min（每 10 min 涡旋混匀），较难裂解的材料可以适当延长裂解时间（**注意：**如果有未彻底裂解的样品，会导致提取量和纯度偏低）；

2.1.4. 向离心管中加入 **200 μL 溶液 Buffer GB**，充分上下颠倒混匀 6-8 次，70℃放置 10 min，溶液应变清亮（**注意：**加入缓冲液 GB 时可能会产生白色沉淀，一般 70℃放置时会消失，不会影响后续实验。如溶液未变清亮，说明细胞裂解不彻底，可能导致提取 DNA 量少和提取出的 DNA 不纯）；

2.1.5. 向离心管中加入 **200 μL 无水乙醇**，涡旋振荡混匀 15 sec，若是溶液中有沉淀，则 10,000 g 离心 5 min，收集的上清液转移至新的离心管中，注意尽量不要吸取沉淀；若是没有沉淀，则直接进行下一步；

2.1.6. 如果需要去除 RNA，可选**步骤 a）或 b）**

a) 向离心管中加入 **50μL RNase A（客户自备，目录号：G3405）**，吹打均匀后常温放置 5 min；

b) 第 12 步结束后的洗脱产物，向离心管中加入 **1μL RNase A（客户自备，目录号：G3405）**，吹打均匀后常温或 37℃放置 5-30 min 即可；

2.1.7. 将吸附柱（Bind DNA Mini Columns）置入收集管（Collection Tubes）中，向吸附柱中加入上一步所得产物，10,000 g 离心 30 sec，倒掉废液，将吸附柱放回收集管中；

2.1.8. 向吸附柱中加入 **500 μL 溶液 Buffer PD**，10,000 g 离心 1 min，倒掉收集管中的废液，将吸附柱放入收集管中；

2.1.9. 向吸附柱中加入 **600 μL 溶液 Buffer PW**（**使用前请先检查是否已加入无水乙醇**），10,000 g 离心 1 min，倒掉收集管中的废液，将吸附柱放入收集管中（**注意：**如果回收的 DNA 是用于盐敏感的实验，例如酶切或 PCR 等，建议 Buffer PW 加入后静置 2-5 min 再离心）；

2.1.10. 重复操作步骤 9；

2.1.11. 将吸附柱放入收集管中，10,000 g 离心 2 min，尽量除去残留的液体；将吸附柱置于室温放置 5 min 或者 37℃培养箱放置 3 min，彻底晾干（**注意：**漂洗液中乙醇的残留会影响后续的酶切、PCR 等实验）；

2.1.12. 将吸附柱放入一个干净离心管中，向吸附中的膜中间位置**悬空滴加 50-100μL 溶液 Elution Buffer 或 ddH_2O**（60-65℃预热 Elution Buffer 或 ddH_2O 后再使用效果更佳），室温放置 2 min，10,000 g 离心 2 min，收集 DNA 溶液（**注意：**洗脱液的体积不应少于 50 μL，体积过少会影响回收的效率。为了提高 DNA 的回收量，可将离心得到的溶液重新加回离心吸附柱中，室温放置 2 min，10,000 g 离心 2 min，将 DNA 溶液收集到离心管中。洗脱液的 pH 值对于洗脱效率有较大影响，其 pH 值在 7.0-8.5 范围内，pH 值低于 7.0 会降低洗脱效率；

DNA 产物应保存在-20℃，以防 DNA 降解）。

2.2 重亚硫酸盐处理 DNA

2.2.1 取 20 μL 基因组 DNA（500 pg—1μg）样品于 PCR 管中，加入 130 μL CT 转换试剂，振荡混匀。

2.2.2 将 PCR 管放入 PCR 仪中，98℃，10 min；64℃，2.5 h；4℃，放置 20 h。

2.2.3 加入 600 μL M-Binding 缓冲液到 Zymo-Spin 吸附柱中，将吸附柱放回收集管中。

2.2.4 将 2）中的样品加到 3）中的 Zymo-Spin 吸附柱里，颠倒混匀。

2.2.5 12000 g 离心 30 秒，倒掉废液，将吸附柱放回收集管中。

2.2.6 向吸附柱中加入 100 μL M-Wash 缓冲液（已加入无水乙醇），12000 g 离心 30 秒，倒掉废液，将吸附柱放回收集管中。

2.2.7 向吸附柱中加入 200 μL M-Desulphonation 缓冲液，室温静置 15-20 min，12000 g 离心 30 秒，倒掉废液，将吸附柱放回收集管中。

2.2.8 向吸附柱中加入 200 μL M-Wash 缓冲液，12000 g 离心 30 秒，倒掉废液。重复一次。

2.2.9 将吸附柱转入一个干净的离心管中，向吸附膜的中间部位悬空滴加 20 μL M-Elution 缓冲液，室温放置 5 分钟，12000 g 离心 30 秒，将溶液收集到离心管中。

2.3 PCR 扩增

2.3.1 取 0.2mL PCR 管，配制如下反应体系。

2×Taq PCR Master Mix	25 μL
Forward Primer (10 μM)	1 μL
Reverse Primer (10 μM)	1 μL
甲基化回收产物	1.5 μL
Water Nuclease-Free	Add to 50 μL

2.3.2 PCR 扩增

预变性	95℃，5min	
变性	95℃，30s	⟵┐
退火	55℃，30s	├ 40×循环
延伸	72℃，30s	⟵┘
末段延伸	72℃，5min	
降温	16℃，2min	

2.4 电泳：1×TAE，2.0% agarose，4V/cm

2.5 胶回收

2.6 连接

2.6.1 建立如下连接体系

pSWE-Topo Zero Vector (50 ng/μL)	1.0 μL
胶回收产物	4.0 μL

2.6.2 16℃过夜连接。

2.7 感受态制备及转化

于超净台上，按《分子克隆实验指南》（第三版）操作。

Joseph Sambrook and David W. Russell. 分子克隆实验指南。第三版。黄培堂等译。北京：科学出版社，2002 年 8 月第三版：96-99

2.7.1 从 37℃培养 16h 的平板上挑取单菌落，接种至 100 mL LB 培养基中，37℃振荡培养 3h。

2.7.2 将培养液于冰上放置 10 min，使其冷却。

2.7.3 将培养液转移至预冷的 50 mL 离心管中。

2.7.4 4℃下 4000 rpm 离心 10 min 收集菌体。

2.7.5 将废液倒干净，每 50 mL 培养液用 30 mL 预冷的 0.1M $CaCl_2$ 重悬菌体。

2.7.6 4℃下 4000 rpm 离心 10 min 收集菌体。

2.7.7 将废液倒干净，每 50 mL 培养液用 2 预冷的 0.1M $CaCl_2$ 重悬菌体。

2.7.8 按每管 200μL 的量分装到 1.5 mL EP 管中。

2.7.9 将连接产物加入到感受态细胞中，轻弹混匀，于冰上放置 30 min。

2.7.10 将管放入 42℃水浴锅中，静置 90s。

2.7.11 快速将管转移到冰浴中，冷却 3min。

2.7.12 加入 800 μL LB 培养基，于 37℃振荡培养 45min。

2.7.13 吸取 200℃培养液，涂布于含 50 μg/mL 卡那霉素的 LB 平板上，于恒温培养箱中 37℃下培养过夜。

2.8 菌液 PCR 鉴定（超净台进行）

2.8.1 取灭菌 1.5 mL 离心管，加入含千分之一抗生素的 LB 液体培养基。

2.8.2 醮取平板上的阳性菌落，接种到液体培养基中，37℃振荡培养。

2.8.3 以菌液为模板，进行 PCR 鉴定，步骤如下：

2.8.3.1 取 0.2 mL PCR 管，配制如下反应体系，

2×Fast Pfus PCR Master Mix	25 μL
Forward Primer (10 μM)	1 μL
Reverse Primer (10 μM)	1 μL
菌液	2 μL
Water Nuclease-Free	Add to 50 μL

2.8.3.2 PCR 扩增

预变性	98℃，2min	
变性	98℃，20s	←┐
退火	55℃，20s	├ 30×循环
延伸	72℃，10s	←┘
末段延伸	72℃，5min	
降温	16℃，2min	

2.9 测序

Bisulfite Sequencing PCR (BSP) Lab Report

1 Laboratory Equipment and Reagents

1.1 Laboratory equipment

Equipment	Manufacturers	Model
Centrifuge	DragonLab	D3024R
PCR instrument	EASTWIN	ETC811
Constant temperature oscillation shaker	Changzhou Aohua Instrument Co ., Ltd	SHZ-82A
Clean bench	Suzhou Antai Air Tech Co., Ltd	SW-CJ-1FD
Electrophoresis	Servicebio	FW-600
Gel imaging system	Tanon Science & Technology Co., Ltd.	Tanon-1600R
Standard Reagent Type Lab Ultra pure Water Purifier	Qingdao Fulum Technology Co., Ltd	FBZ2001-up-p
Dry thermostat	Hangzhou Allsheng Instruments Co.,Ltd	K20
Ice maker	Changshou City shircore Electrical Appliance Co. Ltd.	IMS-20
Ultramicro spectrophotometer	Thermo	NanoDrop2000
Mini-centrifuge	Servicebio	MC-700
Vortex oscillator	Servicebio	MV-100
High-speed-microtherm Homogenizer	Servicebio	KZ-III-F

1.2 Reagents

Reagents	Manufacturers	Order
Servicebio Genomic DNA Kit	Servicebio	G3633
DNA Purification Kit	Servicebio	G3631
2×Taq PCR Master Mix	Servicebio	G3302
2×Fast Pfus PCR Master Mix	Servicebio	G3305
pSWE-Topo Zero Cloning Kit	Servicebio	G3022
2×Fast Pfus PCR Master Mix	Servicebio	G3305

Agarose	Servicebio	G5056
GN100bp DNA Ladder II	Servicebio	G3366
Centrifuge tube(1.5 mL)	Servicebio	EP-150-J
TIP	Servicebio	
Recombinant Proteinase K	Servicebio	G1234
Red Blood Cell Lysis Buffer	Servicebio	G2015
50*TAE	Servicebio	G3001
10×TE（pH 8.0）	Servicebio	G3003
6× DNA Loading Buffer	Servicebio	G3011
SerRed	Servicebio	G3606
Luria-Bertani Liquid Medium（Powder）	Servicebio	G3102
Water Nuclease-Free	Servicebio	G4700
Methylation-Gold Kit	ZYMO	D5005
Ethanol absolute	Sinopharm Group Chemical Reagent Co., Ltd.	10009218
Isopropyl alcohol	Sinopharm Group Chemical Reagent Co., Ltd.	80109218

2 Experimental Steps

2.1 Genomic DNA Extraction Experimental Steps

2.1 Samples preparation

2.1.1 For blood, please use 50-100 μL fresh, frozen or anticoagulantadding blood. If less than 200 μL, please make up with **buffer GA** to 200 μL.

2.1.2 If the sample is blood from poultry, birds, amphibians, of which red blood cells have nucleolus, the amount should be reduced to 5-20 μL and adjust the volume to 200 μL with **buffer GA**.

2.1.3 The adherent cells should be treated to cell suspension first, then centrifuge the cells for 5 min at 1,000 rpm, then discard the flow-through and re-suspend cell pellet in 200 μL **buffer GA**.

2.1.4 Animal tissue (2-30mg) should be treated to cell suspension first, then centrifuge the cells for 1 min at 12,000 rpm, then discard the flow-through and re-suspend cell pellet in 200 μL **buffer GA**.

2.2 Add 20 μL **Proteinase K Stock Solution**, mix thoroughly by vortex. If the sample is tissue: incubate at 56°C for 30-60 min until the tissue is completely lysed(mix thoroughly by vortex per 10 min).

2.3 Add 200 μL **Buffer GB** to the sample, mix thoroughly for 6-8 times, and incubate at 70°C for 10 min to yield a homogeneous solution. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

2.4 Add 200 μL ethanol to the sample, and mix thoroughly by vortex for 15 s. A white precipitate may form on addition of ethanol. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.

2.5 Pipet the mixture from step 4 into the Bind DNA Mini Columns (in a 2 mL collection tube) and centrifuge at 10000g for 30 s. Discard flow-through and place the spin column into the collection tube.

2.6 Add **500 μL Buffer PD** to Bind DNA Mini Columns, and centrifuge at 10000g for 1 min , then discard the flow-through and place the spin column into the collection tube.

2.7 Add **600 μL Buffer PW** (Ensure ethanol has been added) to Bind DNA Mini Columns, and centrifuge at 10000g for 1 min. Discard the flow-through and place the spin column into the collection tube.

2.8 Repeat Step 7.

2.9 Centrifuge at 10000g for 2 min to dry the membrane completely. Note: The residual ethanol of buffer PW may have some effection in downstream application.

2.10. Place the Bind DNA Mini Column in a new clean 1.5 mL microcentrifuge tube, and pipet 50-100 μL **Elution Buffer or ddH_2O** directly to the center of the membrane. Incubate at room temperature (15-25°C) for 2-5 min, and then centrifuge for 2 min at 10000g.

2.2 Bisulfite Treatment of DNA

2.2.1 Add 130 µL of the CT Conversion Reagent to 20 µL of your DNA sample in a PCR tube. If the volume of the DNA sample is less than 20 µL, make up the difference with water. Mix the sample by flicking the tube or pipetting the sample up and down, then centrifuge the liquid to the bottom of the tube.

2.2.2 Place the sample tube in a thermal cycler and perform the following steps*:

1. 98°C for 10 minutes
2. 64°C for 2.5 hours
3. 4°C storage up to 20 hours.

2.2.3 Add 600 µL of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided Collection Tube.

2.2.4 Load the sample (from Step 2) into the Zymo-Spin™ IC Column containing the MBinding Buffer. Close the cap and mix by inverting the column several times.

2.2.5 Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.

2.2.6 Add 100 µL of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.

2.2.7 Add 200 µL of M-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

2.2.8 Add 200 µL of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µL of M-Wash Buffer and centrifuge for an additional 30 seconds.

2.2.9 Place the column into a 1.5 mL microcentrifuge tube. Add 10 µL of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

2.3 PCR Amplification

2.3.1 0.2mL PCR tube, 20ul PCR amplification system.

2×Taq PCR Master Mix	25 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
Template	1.5 µL
Water Nuclease-Free	Add to 50 µL

2.3.2 PCR amplification

Pre-denaturation	95℃，5min	
Denaturation	95℃，30s	40×cycle
Anealing	55℃，30s	
Extension	72℃，30s	
End extension	72℃，5min	

2.4 Electrophoresis： 1×TAE，2.0% agarose，4V/cm

2.5 Purify DNA from Agarose Gel

2.5.1 Column equilibration: add 500 μL Buffer BL to the Bind DNA Mini Columns (put Bind DNA Mini Columns into a collection tube). Centrifuge for 1 min at 10000g in a table-top microcentrifuge. Discard the flow-through, and put Bind DNA Mini Columns back into the collection tube.

2.5.2 Cut the DNA fragment from agarose gel. Weigh the gel slice in a clean tube.

2.5.3 Add equivalent volume of Buffer GL to the gel (If the gel is 0.1 g, it is defaulted to be 100 μL, then add 100 μL Buffer GL). Incubate at 60°C by inverting up and down the tube until the agarose gel dissolves completely. If the agarose gel does not dissolve completely, incubate for longer period or add additional Buffer GL until all the agarose gel dissolved completely.

2.5.4 When the gel dissolved completely and the solution temperature turns to room temperature (15-25°C), transfer the mixture to the Bind DNA Mini Columns (put Bind DNA Mini Columns into a collection tube). Let the column stand for 2 min at room temperature (15-25°C), then centrifuge for 30-60 s at 10000g in a table-top microcentrifuge. Discard the flow-through; place the Bind DNA Mini Columns back into the collection tube again.

2.5.5 Wash the Bind DNA Mini Columns with 700 μL Buffer PW (ensure that ethanol (96-100%) has been added) and centrifuge for 30-60 s at 10000g. Discard the flow-through and place the Bind DNA Mini Columns back into the collection tube.

2.5.6 Repeat Step 5.

2.5.7 Place the Bind DNA Mini Columns back to the collection tube and centrifuge at 10000g for 2 min to remove residual wash buffer. Discard the flow-through, and place column with the cap open for several minutes to air dry the membrane.

2.5.8 Transfer the Bind DNA Mini Columns to a clean 1.5 mL microcentrifuge tube. Add appropriate volume of Buffer EB to the center of the membrane, incubate at room temperature (15-25°C) for 2 min, then centrifuge at 10000g for 2 min.

2.6 Recommended DNA Insert Volume

pSWE-Topo Zero Vector (50 ng/μL)	1.0 μL
DNA	4.0 μL

16℃ connection overnight.

2.7 Transformation Experiment Using DH5α

2.7.1 Thaw competent cells on wet icefor 20 minutes.

2.7.2 For DNA from ligation reactions. Add 1 μL of the dilution to the cells , moving the pipette through the cells while dispensing. Gently tap tubes to mix.

2.7.3 Incubate cells on ice for 20 minutes.

2.7.4 Heat-shock cells 90 seconds in a 42°C water bath; do not shake.

2.7.5 Place on ice for 2 minutes.

2.7.6 Add 0.8 mL of room temperature LB Medium.

2.7.7 Shake at 220 rpm (37°C) for 45 minutes.

2.8 PCR Amplification Identification

The positive colonies on the plate were inoculated into the liquid medium and shaken at 37 ° C. pcr amplification for Positive clones.

2.8.1 0.2mL PCR tube, 20ul pcr amplification system.

2×Fast Pfus PCR Master Mix	25 μL
Forward Primer (10 μM)	1 μL
Reverse Primer (10 μM)	1 μL
Bacteria liquid	2 μL
Water Nuclease-Free	Add to 50 μL

2.8.2 PCR amplification

Pre-denaturation	98℃，2min	
Denaturation	98℃，20s	30×cycle
Anealing	55℃，20s	
Extension	72℃，10s	
End extension	72℃，5min	

2.9 DNA Sequencing