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	Wanwu	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	Wanwu	MC-700
涡旋混匀仪	Wanwu	MV-100
高速低温组织研磨仪	Wanwu	KZ-III-F

1.2 主要实验试剂及耗材

试剂	厂家	货号
血液/细胞/组织基因组 DNA 提取试剂盒	Tiangen Biotech(Beijing) CO.,LTD	DP304
Methylation-Gold Kit	ZYMO	D5005S(10)
琼脂糖凝胶 DNA 回收试剂盒	Tiangen Biotech(Beijing) CO.,	DP209
TaKaRa Taq Hot Start DNA 聚合酶	LTDTAKARA Bioiotechnology (Dalian)	DR007A
T-Vector PMD19	Co. ,LTD	K1901AA
2×Fast Pfus PCR Master Mix	TAKARA	G3305
琼脂糖	Wanwu	G5056
无水乙醇	Wanwu	10009218
异丙醇	国药集团化学试剂有限公司	80109218
MarkerI DNA ladder	国药集团化学试剂有限公司	WGM1100
1.5ml 离心管（无酶）	Wanwu	EP-150-M

网址：http://www.wanwusw.com
细胞网址：http://www.hfwanwu.com
地址：合肥市蜀山区长江西路248号11层
电话： 400-1016-218
邮箱：2028438226@qq.com

Gold view	Wanwu	G1066
蛋白酶 K	Wanwu	G1205
红细胞裂解液	Wanwu	G2015
50*TAE	Wanwu	G3001
10×TE（pH 8.0）	Wanwu	G3003
6× DNA Loading Buffer	Wanwu	G3011
EB 染液	Wanwu	G3010
LB 液体培养基（干粉）	Wanwu	G3102

2 实验步骤

2.1 组织基因组 DNA 提取

2.1.1 将组织处理为细胞悬液，10000 g 离心 1min，用移液器吸取上清液，丢弃上清液，向样本管中加 300μl 缓冲液 GA，振荡 15 秒，室温放置 5 分钟

2.1.2 加入 30 μl 蛋白酶 K 溶液，混匀，放置于 55 度干式恒温器中，放置 3h 以上。

2.1.3 加入 300 μl 缓冲液 GB，充分颠倒混匀，70℃放置 10 分钟，简短离心以去除管盖内壁的水珠。

2.1.4 加入 300 μl 无水乙醇，充分振荡混匀 15 秒，简短离心以去除管盖内壁的水珠。

2.1.5 将上一步所得溶液和絮状沉淀都加入一个吸附柱 CB3 中（吸附柱放入收集管中），12000 g 离心 30 秒，倒掉废液，将吸附柱 CB3 放回收集管中。

2.1.6 向吸附柱 CB3 中加入 500 μl 缓冲液 GD（已加入无水乙醇），12000 g 离心 30 秒，倒掉废液，将吸附柱 CB3 放回收集管中。

2.1.7 向吸附柱 CB3 中加入 700 μl 漂洗液 PW（已加入无水乙醇），12000 g 离心 30 秒，倒掉废液，将吸附柱 CB3 放回收集管中。

2.1.8 向吸附柱 CB3 中加入 500 μl 漂洗液 PW，12000 g 离心 30 秒，倒掉废液。

2.1.9 将吸附柱 CB3 放回收集管中，12000 g 离心 2 分钟，倒掉废液，室温放置 10 分钟，以彻底晾干吸附材料中残余的漂洗液。

2.1.10 将吸附柱 CB3 转入一个干净的离心管中，向吸附膜的中间部位悬空滴加 50 μl TE 洗脱缓冲液，室温放置 5 分钟，12000 g 离心 2 分钟，将溶液收集到离心管中。

2.1.11 将离心得到的溶液再加入吸附柱 CB3 中，室温放置 2 分钟，12000 g 离心 2 分钟，将溶液收集到离心管中。

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。

10×PCR Buffer	5.0µl
2.5mM dNTPs	5.0µl
10µM 基因引物（F+R）	2.0µl+ 2.0µl
甲基化回收产物	1.5µl
5U/µl Taq HS	0.2µl
ddH_2O	34.3µl

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

2×Solution	6ul
T-Vector PMD19	1.0µl
胶回收产物	5.0µl

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 100ml LB 培养基中，37℃振荡培养 3h。

2.7.2 将培养液于冰上放置 10min，使其冷却。

2.7.3 将培养液转移至预冷的 50ml 离心管中。

2.7.4 4℃下 4000rpm 离心 10min 收集菌体。

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

2.7.6 4℃下 4000rpm 离心 10min 收集菌体。

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

2.7.8 按每管 200μl 的量分装到 1.5ml 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 30min。

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 800μl　LB 培养基，于 37℃振荡培养 45min。

2.7.13 吸取 200℃培养液，涂布于含 50μg/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 1.5ml 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 0.2ml PCR 管，配制如下反应体系，

2×Fast Pfus PCR Master Mix	25ul
10μM 基因引物（F+R）	1.5ul+1.5ul
菌液	2ul
ddH_2O	20ul

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	Wanwu	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	Wanwu	MC-700
Vortex oscillator	Wanwu	MV-100
High-speed-microtherm Homogenizer	Wanwu	KZ-III-F

1.2 Reagents

Reagents	Manufacturers	Order
TIANamp Genomic DNA Kit	Tiangen Biotech(Beijing) CO.,LTD	DP304
Methylation-Gold Kit	ZYMO	D5005S(10)
TIANgel Midi Purification Kit	Tiangen Biotech(Beijing) CO.,	DP209
TaKaRa Taq Hot Start DNA Polymerase	LTDTAKARA Bioiotechnology (Dalian)	DR007A
T-Vector PMD19	Co. ,LTD	K1901AA
2×Fast Pfus PCR Master Mix	TAKARA	G3305
Agarose	Wanwu	G5056

Anhydrous ethanol	Sinopharm Group Chemical Reagent Co., Ltd.	10009218
Isopropyl alcohol	Sinopharm Group Chemical Reagent Co., Ltd.	80109218
MarkerI DNA ladder	Wanwu	WGM1100
Centrifuge tube(1.5 mL)	Wanwu	EP-150-M
TIP	Wanwu	
Gold view	Wanwu	G1066
Proteinase K	Wanwu	G1205
Red Blood Cell Lysis Buffer	Wanwu	G2015
50*TAE	Wanwu	G3001
10×TE（pH 8.0）	Wanwu	G3003
6× DNA Loading Buffer	Wanwu	G3011
Ethidium bromide	Wanwu	G3010
Luria-Bertani Liquid Medium（Powder）	Wanwu	G3102

Tube and TIP are moist heat sterilization 40min, dry。

2 Experimental steps

2.1 Genomic DNA Extraction Experimental steps

2.1.1 Samples preparation

2.1.1.1 For blood, please use 200 µl fresh, frozen or anticoagulantadding blood. If less than 200 µl, please make up with buffer GA to 200 µl.

2.1.1.2 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.

2.1.2.3 The adherent cells should be treated to cell suspension first, then centrifuge the cells for 1 min at 10,000 rpm (~11,200 × g), then discard the flow-through and re-suspend cell pellet in 200 µl buffer GA.

2.1.2.4 Animal tissue (spleen＜10mg) should be treated to cell suspension first, then centrifuge the cells for 1 min at 10,000 rpm (~11,200 × g), then discard the flow-through and re-suspend cell pellet in 200 µl buffer GA.

2.1.2 Add 20 µl Proteinase K, mix thoroughly by vortex. If the sample is tissue: incubate at 56°C until the tissue is completely lysed.

2.1.3 Add 200 µl Buffer GB to the sample, mix thoroughly by vortex, 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.

2.1.4 Add 200 µl ethanol (96-100%) 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.

2.1.5 Pipet the mixture from step 4 into the Spin Column CB3 (in a 2 ml collection tube) and centrifuge at 12,000 rpm (~13,400 × g) for 30 s. Discard flow-through and place the spin column into the collection tube.

2.1.6 Add 500 µl Buffer GD (Ensure ethanol (96-100%) has been added) to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g) for 30 s, then discard the flow-through and place the spin column into the collection tube.

2.1.7 Add 600 µl Buffer PW (Ensure ethanol (96-100%) has been added) to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g) for 30 s. Discard the flow-through and place the spin column into the collection tube.

2.1.8 Repeat Step 7.

2.1.9 Centrifuge at 12,000 rpm (~13,400 × g) for 2 min to dry the membrane completely. Note: The residual ethanol of buffer PW may have some affection in downstream application

2.1.10 Place the Spin Column CB3 in a new clean 1.5 ml microcentrifuge tube, and pipet 50-200 µl Buffer TE 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 12,000 rpm (~13,400 × g).

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.

10×PCR Buffer	5.0µl
2.5mM dNTPs	5.0µl
10µM primers(F+R)	2.0µl+ 2.0µl
DNA	1.5µl
5U/µl Taq HS	0.2µl
ddH_2O	34.3µl

2.3.2 PCR amplification

Pre-denaturation	95°C，5min	
Denatured	95°C，30s	40×cycle
Annealing	55°C，30s	
Extended	72°C，30s	
End extension	72°C，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 Spin Column CA2 (put Spin Column CA2 into a collection tube). Centrifuge for 1 min at 12,000 rpm (~13,400 × g) in a table-top microcentrifuge. Discard the flow-through, and put Spin Column CA2 back into the collection tube.

2.5.2 Cut the DNA fragment from agarose gel with a clean, sharp scalpel. Weigh the gel slice in a clean tube.

2.5.3 Add equivalent volume of Buffer PN to the gel (If the gel is 0.1 g, it is defaulted to be 100 µl, then add 100 µl Buffer PN). Incubate at 50°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 PN 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 Spin Column CA2 (put Spin Column CA2 into a collection tube). Let the column stand for 2 min at room temperature (15-25°C), then centrifuge for 30-60 s at 12,000 rpm (~13,400 × g) in a table-top microcentrifuge. Discard the flow-through; place the Spin Column CA2 back into the collection tube again.

2.5.5 Wash the Spin Column CA2 with 600 µl Buffer PW (ensure that ethanol (96-100%) has been added) and centrifuge for 30-60 s at 12,000 rpm (~13,400 × g). Discard the flow-through and place the Spin Column CA2 back into the collection tube.

2.5.6 Repeat Step 5.

2.5.7 Place the Spin Column CA2 back to the collection tube and centrifuge at 12,000 rpm (~13,400 × g) 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 Spin Column CA2 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 12,000 rpm (~13,400 × g) for 2 min.

2.6 Recommended DNA insert volume

2×solution	6.0µl
T-Vector PMD19	1.0µl
purify DNA	5.0µl

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	25ul
10μM primers(F+R)	1.5μl+1.5μl
Bacteria liquid	2.0μl
ddH2O	20.0μl

2.8.2 PCR amplification

Pre-denaturation	98℃，2min	
Denatured	98℃，20s	30×cycle
Annealing	55℃，20s	
Extended	72℃，10s	
End extension	72℃，5min	

2.9 DNA sequencing

DNA sequencing by Wuhan GeneCreate Biological Engineering Co., Ltd.