甲基化 MSP 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
琼脂糖	TAKARA	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
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

Methylation-specific PCR(MSP) Lab Report

1 Laboratory equipment and reagents

1.1 Laboratory equipment

Equipment	Manufacturers	Model
Centrifuge	DragonLab	D3024R
PCR instrument	Beijing Dongsheng Innovation Biotechnology Co., Ltd.	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 pure water meter	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)
TaKaRa Taq Hot Start DNA Polymerase	TAKARA Bioiotechnology(Dalian) Co. ,LTD	DR007A
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

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℃，5min	
Denatured	95℃，30s	40×cycle
Annealing	55℃，30s	
Extended	72℃，30s	
End extension	72℃，5min	

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