荧光定量 PCR 实验报告

1 实验器材及试剂

1.1 实验器材及耗材

名称	厂家	货号
高速低温组织研磨仪	Wanwu	KZ-III-F
台式高速冷冻型微量离心机	DragonLab	D3024R
荧光定量 PCR 仪	ABI	Stepone plus
超净工作台	苏净安泰	SW-CJ-1FD
超微量分光光度计	Thermo	NanoDrop2000
标准试剂型纯水仪	青岛富勒姆科技有限公司	FBZ2001-up-p
离心管	Wanwu	
TIP 头	Wanwu	

1.2 主要实验试剂

试剂	厂家	货号
RNA 提取液	Wanwu	G3013
三氯甲烷	国药集团化学试剂有限公司	10006818
异丙醇	国药集团化学试剂有限公司	80109218
无水乙醇	国药集团化学试剂有限公司	10009218
HyPure ™Molecular Biology Grade Water	HyClone	SH30538.02
Wanwu®RT First Strand cDNA Synthesis Kit	Wanwu	G3330
2×SYBR Green qPCR Master Mix (High ROX)	Wanwu	G3322
引物	Wanwu	

2、荧光定量 PCR 实验步骤

2.1 总 RNA 抽提（*枪头和离心管均经过湿热灭菌，无 RNA 酶*）

2.1.1 样本前处理

2.1.1.1 组织：取匀浆管，加入 1ml 的 RNA 提取液，置冰上预冷。取 100mg 组织，加入到匀浆管中。匀浆仪充分研磨直至无可见组织块。

2.1.1.2 贴壁细胞：用移液器将培养瓶/板中培养液吸除干净，加入 1ml 4℃ 预冷的 PBS 溶液，轻摇洗涤。用移液器将 PBS 吸除干净。加入 1ml 的 RNA 提取液，轻缓振荡或用枪头吹打，破碎细胞。

2.1.1.3 全血：取 200μl 全血，加入 800μl 的 RNA 提取液，震荡混匀。

2.1.2 相分离

样本前处理之后，12000rpm 离心 10min 取上清。加入 250 μl 三氯甲烷，颠倒离心管 15s，充分混匀，静置 3min，4℃ 下 12000rpm 离心 10min，将 400 μl 上清转移到一新的离心管中。

2.1.3 沉淀 RNA

加入 0.8 倍体积的异丙醇，颠倒混匀。-20℃ 放置 15min。4℃下 12000rpm 离心 10min，管底的白色沉淀即为 RNA。

2.1.4 洗涤 RNA

吸除液体，加入 75%乙醇 1.5ml 洗涤沉淀。4℃下 12000rpm 离心 5min，将液体吸除干净。

2.1.5 溶解 RNA

将离心管置于超净台上吹 3min，加入 15μl 无 RNA 酶的水溶解 RNA，55℃ 孵育 5min。

2.1.6 RNA 浓度测定

用 Nanodrop 2000 检测 RNA 浓度及纯度：仪器空白调零后取 2.5μl 待测 RNA 溶液于检测基座上，放下样品臂，使用电脑上的软件开始吸光值检测。

2.2 反转录（*枪头和 PCR 均经过湿热灭菌，无 RNA 酶*）

2.2.1 逆转录反应体系配制（推荐 20 μL 反应体系，逆转录试剂盒货号：G3330）

Component	Volume
5 x Reaction Buffer	4 μL
Oligo $(dT)_{18}$ Primer (100 μM)	0.5 μL
And Random Hexamer primer (100 μM)	0.5 μL
Wanwu®RT Enzyme Mix	1 μL
Total RNA *	0.1 ng-5 μg / 10 pg-0.5 μg
RNase free water	Add to 20 μL

2.2.2 轻轻混匀并离心

2.2.3 逆转录程序设置*

Temperature	Time
25℃	5 min
42℃	30 min
85℃	5 sec

2.3 定量 PCR

2.3.1 取 0.2ml PCR 管，配制如下反应体系，每个反转录产物配制 3 管。

2× qPCR Mix	7.5μl
2.5μM 基因引物	1.5μl
反转录产物	2.0μl
ddH_2O	4.0μl

2.3.2 PCR 扩增

预变性	95℃，10min
循环（40 次）	95℃，15s→60℃，60s
熔解曲线	60℃→95℃，每 15s 升温 0.3℃

2.4 结果处理

ΔΔCT 法：

A=CT(目的基因，待测样本)- CT(内标基因，待测样本)

B=CT(目的基因，对照样本)- CT(内标基因，对照样本)

K=A-B

表达倍数=2^{-K}

Real Time PCR Report

1 Laboratory equipment and reagents

1.1 Laboratory equipment and consumable

Equipment	Manufacturers	Model
High-speed-microtherm Homogenizer	Wanwu	KZ-III-F
Centrifuge	DragonLab	D3024R
Real time PCR System	ABI	Stepone plus
Clean bench	Suzhou Antai Air Tech Co., Ltd	SW-CJ-1FD
Ultramicro spectrophotometer	Thermo	NanoDrop2000
Standard Reagent Type Lab Ultra pure Water Purifier	Qingdao Fulum Technology Co., Ltd	FBZ2001-up-p
Centrifuge tube	Wanwu	
TIP	Wanwu	

1.2 Reagents

Reagents	Manufacturers	Order
RNA Extraction	Wanwu	G3013
Trichloromethane	Sinopharm Group Chemical Reagent Co., Ltd.	10006818
iso-Propyl alcohol	Sinopharm Group Chemical Reagent Co., Ltd.	80109218

Ethanol	Sinopharm Group Chemical Reagent Co., Ltd.	10009218
HyPure ™Molecular Biology Grade Water	HyClone	SH30538.02
Wanwu®RT First Strand cDNA Synthesis Kit	Wanwu	G3330
2×SYBR Green qPCR Master Mix (High ROX)	Wanwu	G3322
Primer	Wanwu	

2 Protocol:

2.1 Isolate RNA

2.1.1 Homogenization

2.1.1.1 Tissues: Homogenize tissue samples in 1 ml of RNA Extraction per 100 mg of tissue using power homogenizer. The sample volume should not exceed 10% of the volume of RNA Extraction used for the homogenization.

2.1.1.2 Cells grown in Monolayer: Rinse cell monolayer with ice cold PBS once. Lyse cells directly in a culture dish by adding 1 ml of RNA Extraction per 3.5 cm diameter dish . Pass the cell lysate several times through a pipette. Vortex thoroughly. The amount of RNA Extraction added is based on the area of the culture dish (1 ml per 10 cm2) and not on the number of cells present. An insufficient amount of RNA Extraction may result in DNA contamination of the isolated RNA.

2.1.1.3 Cells Grown in suspension: Spin cells for 10 min at 3000rpm. Remove media and resuspend cells in ice cold PBS. Pellet cells by spinning at 3000rpm for 10 min. Lyse cells with RNA Extraction by repetitive pipetting . Use 1 ml of the reagent per 1X 10^6 of animal cells.

2.1.1.4 Blood: Mix 0.2 mL of blood and 0.8 mL RNA Extraction, vortex thoroughly.

2.1.2 PHASE SEPERATION

Centrifuge the samples at 12000rpm for 10 min at 4℃ to remove cell debris. Transfer the supernatant to a new tube. Add 0.25 ml of chloroform per 1 ml of RNA Extraction. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at 12,000rpm for 10 minutes at 4℃. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube.

2.1.3 RNA PRECIPITATION

Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of RNA Extraction used for the initial homogenization. Incubate samples at -20℃ for 15 minutes and centrifuge at 12,000rmp for 10 minutes at 4℃. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

2.1.4 RNA WASH

Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of RNA Extraction used for the initial homogenization. Mix the samples by vortexing and centrifuge at 12,000rmp for 10 minutes at 4 ℃. Remove all leftover ethanol.

2.1.5 REDISSOLVING RNA

Air-dry RNA pellet for 5-10 minutes. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in DEPC-treated water by passing solution a few times through a pipette tip.

2.1.6 SPECTROPHOTOMETRIC ANALYSIS

Dilute 1 µl of RNA with 39 µl of DEPC-treated water (1:40 dilution). Using 2.5 µl RNA, take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.8. Apply the convention that 1 OD at 260 equals 40 µg /ml RNA.

2.2 First Strand cDNA Synthesis

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

2.2.1 Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order: Template RNA 2µg , Primer Oligo (dT) 18 primer 0.5 µL and Random Hexamer primer 0.5 µL or gene-specific primer 1 µL , 5X Reaction Buffer 4 µL , Wanwu®RT Enzyme Mix[a] 1 µL, Water, nuclease-free to 20 µL , Total volume 20 µL

2.2.2 Optional. If the RNA template is GC-rich or contains secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.

2.2.3 Mix gently and centrifuge briefly.

2.2.4 Incubate for 5 min at 25°C ,30 min at 42°C.

Note. For GC-rich RNA templates the reaction temperature can be increased up to 50°C.

2.2.5 Terminate the reaction by heating at 85°C for 5 seconds.

2.3 Preparation of PCR Master Mix

2.3.1 For each 15µL reaction, prepare the following reation mix:

2×SYBR Green qPCR Master Mix (High ROX)	7.5µL
Primer(2.5µM)	1.5µL
cDNA template	2.0µL
ddH2O	4.0µL

2.3.2 PCR amplification

Pre-denaturation	95℃，10min
cycle（40 次）	95℃，15s→60℃，60s
Melt Curve	60℃→95℃，0.3 ℃/15s

2.4 the results of processing

ΔΔCT method:

A = CT (target gene, sample) - CT (internal standard gene, sample)

B = CT (target gene, control) - CT (internal standard gene, control)

K = A-B

RNA Expression = 2^{-K}