荧光定量 PCR 实验报告

一、实验原理

实时荧光定量 PCR（Quantitative Real-time PCR）是一种在 DNA 扩增反应中，以荧光化学物质测每次聚合酶链式反应（PCR）循环后产物总量的方法。通过内参或者外参法对待测样品中的特定 DNA 序列进行定量分析的方法。

二、实验器材及试剂

1、实验器材

名称	厂家	型号
匀浆仪	Servicebio	KZ-II
台式高速冷冻离心机	海尔	LX-165T2R
荧光定量 PCR 仪	ABI	7500
超净工作台	BIOBASE	BBS-DDC
超微量分光光度计	可帮基因	CANHELP-100
标准试剂型纯水仪	芷昂	Clever-S

2、实验试剂

试剂	厂家	货号
总 RNA 提取剂	杭州浩克生物技术有限公司	HKR022
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
All-in-One First-Strand cDNA Synthesis SuperMix for qPCR	TRAN	AE341
2X SYBR Green qPCR Master Mix	APExBIO	K1070

三、实验方法

1. 总 RNA 抽提（枪头和离心管均经过湿热灭菌，无 RNA 酶）

1) 取匀浆管，加入 1mL 的 Trizol Reagent，置冰上预冷。
2) 取 100mg 组织，加入到匀浆管中。
3) 匀浆仪充分研磨直至无可见组织块。
4) 12000rpm 离心 10min 取上清。
5) 加入 250 μL 三氯甲烷，颠倒离心管 15s，充分混匀，静置 3min。
6) 4℃ 下 13000rpm 离心 15min。
7) 将上清转移到纯化管中，加入 1.2 倍体积的无水乙醇，混匀。
8) 4℃下 12000rpm 离心 5min，纯化管滤膜上的白色沉淀即为 RNA。
9) 弃去纯化管下部液体，加入 75%乙醇 650μL 4℃下 12000rpm 离心 5min 洗涤。
10) 重复洗涤 2-3 次。
11) 4℃下 12000rpm 离心 5min，弃去残留液体，将离心管置于超净台上吹 3-5min。
12) 加入 50μL 无 RNA 酶的水溶解 RNA。
13) 使用超微量分光光度计检测 RNA 浓度及纯度：仪器空白调零后取 2μL 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。
14) 将浓度过高的 RNA 进行适当比例的稀释，使其终浓度为 100-500 ng/μL.

2. 反转录（枪头和 PCR 均经过湿热灭菌，无 RNA 酶）

1) 取一 PCR 管，加入含 1μg RNA 的溶液。
2) 加入 1μL dsDNASE 和 1μL 10*dsDNase Buffer 。
3) 用无核糖核酸酶的去离子水补足至 10μL，混匀。
4) 于 PCR 仪上 37℃温育 2min，以去除基因组 DNA 污染。65℃温育 2min，使 dsDNAase 失活。
5) 加入 4μL All-in-One First-Strand Synthesis MasterMix。
6) 用无核糖核酸酶的去离子水补足至 20μL，混匀。
7) 于 PCR 仪上 50℃孵育 15min，结束后 85℃孵育 5min 灭活反转录酶。

3. 定量 PCR

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。

Reagent	Volume
cDNA	1 μL
Forward Primer (10 μM)	0.4 μL
Reverse Primer (10 μM)	0.4 μL
Taq SYBR Green qPCR Premix	10 μL
ddH_2O	To 20 μL

2）PCR 扩增

Stage1 预变性	Stage2（50 个循环）	Stage3（熔解曲线）
94℃，2min	94℃，5s→58℃，10s→72℃，15s	60℃→95℃，每 10s 升温 0.5℃

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

Experimental principle

Quantitative Real-time PCR is a method in which a fluorescent chemical is used to measure the total amount of product after each PCR cycle in DNA amplification. A method of quantitative analysis of a specific DNA sequence in a test sample by internal or external parameters.

Laboratory equipment and reagents

Laboratory equipment

Equipment	Manufacturers	Model
Tissue Grinder	Servicebio	KZ-II
High speed refrigerated centrifuge	Haier	LX-165T2R
Real time PCR System	ABI	7500
Clean bench	BIOBASE	BBS-DDC
Ultramicro spectrophotometer	Canhelp Genomics Co., Ltd	CANHELP-100
Laboratory pure water system	Zhiang Instrument (Shanghai) Co., Ltd	Clever-S

Laboratory reagents

Reagents	Manufacturers	Catlog
RNA Extraction	Hangzhou Haoke Biotechnology Co., Ltd	HKR022
trichloromethane	Sinopharm Chemical Reagent co., Ltd.	10006818

Isopropyl alcohol	Sinopharm Chemical Reagent co., Ltd.	80109218
Anhydrous ethanol	Sinopharm Chemical Reagent co., Ltd.	10009218
All-in-One First-Strand cDNA Synthesis SuperMix for qPCR	TRAN	AE341
2X SYBR Green qPCR Master Mix	APExBIO	K1070

Experimental method

Isolate RNA

1) Take homogenate tube, add 1mL of Trizol Reagent, place on ice for pre-cooling.

2) Take 100mg tissue and add it to the homogenate tube.

3) The homogenizer fully grinds until no tissue blocks are visible.

4) Centrifuge at 12000rpm for 10min to take the supernatant.

5) Add 250μL trichloromethane, reverse the centrifuge tube for 15s, mix thoroughly, and let stand for 3min.

6) Centrifuge at 13000rpm at 4℃ for 15min.

7) Transfer the supernatant to the purification tube, add 1.2 times the volume of anhydrous ethanol, and mix well.

8) Centrifuge at 12000rpm at 4℃ for 5min, and purify the white precipitate on the tube filter membrane as RNA.

9) Discard the liquid in the lower part of the purification tube, add 75% ethanol 650μL at 4℃, centrifuge at 12000rpm for 5min and wash.

10) Repeat washing 2-3 times.

11) Centrifuge at 12000rpm at 4℃ for 5min, discard the residual liquid, place the centrifuge tube on a super-clean table and blow for 3-5min.

12) Add 50μL of RNA-free water to dissolve RNA.

13) Use an ultramicrospectrophotometer to detect RNA concentration and purity: After blank zero adjustment of the instrument, take 2μL RNA solution to be tested and put it on the detection base, lower the sample arm, and use the software on the computer to start the absorption value detection.

14) The RNA with excessive concentration is diluted in an appropriate proportion so that its final concentration is 100-500 ng/μL.

First Strand cDNA Synthesis

1) Take a PCR tube and add a solution containing 1μg RNA.

2) Add 1μL dsDNASE and 1μL 10*dsDNase Buffer.

3) Fill with ribonuclease free deionized water to 10μL and mix well.

4) Incubate at 37℃ for 2min on PCR apparatus to remove genomic DNA contamination. dsDNAase was inactivated after incubation at 65℃ for 2min.

5) Add 4μL All-in-One First-Strand Synthesis MasterMix.

6) Fill with ribonuclease free deionized water to 20μL and mix well.

7) Incubation at 50℃ for 15min on the PCR apparatus, and then incubation at 85℃ for 5min to inactivate reverse transcriptase.

Preparation of PCR Master Mix

1）Take 0.2mL PCR tubes and prepare the reaction system as follows, with 3 tubes for each retroproduct.

Reagent	Volume
cDNA	1 μL
Forward Primer (10 μM)	0.4 μL
Reverse Primer (10 μM)	0.4 μL
Taq SYBR Green qPCR Premix	10 μL

ddH_2O	To 20 μL

2）PCR amplification

Stage1 predegeneration	Stage2（50cycles）	Stage3（Melt Curve）
94℃，2min	94℃，5s→58℃，10s→72℃，15s	60℃→95℃

Data analysis

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 $=2^{-K}$