Experiment time: 2025-06-21, 13:30 - 2025-06-21, 16:30

qRT-PCR (I)

I. Objective of the experiment

After reverse transcription of RNA from positive, low, medium and high drug-resistant cell lines T24-RC48 to obtain cDNA, the expression levels of specific genes (PDL1, VEGF, RBPJ, ICAM1, DSP, SOD2, CAT, GAPDH) were detected by real-time fluorescent quantitative PCR (SYBR Green method), and quantitative analysis was performed to determine whether there were significant differences in gene expression compared to the control group.

II. Experimental content

In this experiment, real-time fluorescence quantitative PCR (qPCR) was used to analyze the RNA of cDNA of T24 wild-type cell line and low/medium/high drug-resistant T24-RC48 cell line by reverse transcription and gene expression quantification. The specific contents included:

2.1 Experimental design

First, thaw the cryopreserved cDNA samples from the T24 wild-type (control) and the low-, medium-, and high-drug-resistant T24-RC48 cell lines in a -80°C freezer. After room temperature thawing, perform immediate centrifugation and store on ice. Next, slowly thaw ROX Reference Dye, RNase-free ddH_2O, and specific gene primers (PDL1, VEGF, etc.) from a -20°C freezer. Then, prepare qPCR reaction mix according to the preset protocol (15μl cDNA, 75μl ROX, 3μl each positive/counter-primers, 54μl ddH_2O per group) and transfer aliquots to labeled EP tubes labeled "control, low, medium, high". Following the pre-designed 96-well plate layout (red, orange, yellow, green corresponding to four concentration groups), add reaction mix to designated wells ensuring no bubbles and without touching well walls. Finally, load the 96-well plate into real-time fluorescence quantitative PCR instrument with the following program: denaturation (95 ° C 5s), annealing/extension (60 ° C 30s) for 40 cycles. Run SYBR Green detection mode to collect fluorescence signals and export data.

2.2 Sample types

T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line cDNA (obtained

from pre-reverse transcription of RNA).

2.3 Measurement principle

Quantitative Polymerase Chain Reaction (qPCR), also known as real-time quantitative PCR, is a technique that monitors DNA amplification through real-time fluorescence detection. The principle relies on fluorescence intensity changes during PCR amplification: When fluorescent dyes (e.g., SYBR Green) or specific probes (e.g., TaqMan probes) binding to double-stranded DNA are added to the reaction system, the fluorescence signal intensifies with each amplification cycle. The instrument generates an amplification curve by continuously monitoring fluorescence levels. The cycle number (Ct value) corresponding to the signal reaching a preset threshold is inversely proportional to the initial template quantity. Through standard curves or relative quantification methods, the original DNA template concentration can be precisely calculated. This technology finds extensive applications in gene expression analysis, pathogen detection, and gene copy number determination.

III. Materials and reagents

3.1 Materials

96-well optical reaction plate, optical sealing film (Pantley): both purchased from domestic companies

RNAase Free EP tubes, octet tubes and pipette tips: purchased from AxyGen

Ice box

3.2 Reagents

cDNA (synthesized by reverse transcription from previously extracted RNA)

ROX Reference Dye

Primers

Internal reference GAPDH

RNase-free ddH_2O

IV. Experimental instruments

Micropipettes, benchtop low temperature and low speed centrifuge: purchased from Eppendorf

Real-time quantitative PCR instrument: AB IQ Quant Studio 5 1.5ml

Centrifuge tubes: Purchased from Corning

Cubee centrifuge: Purchased from GeneReach

V. Experimental steps

This experiment requires strict adherence to the entire procedure and reagent protocols. The preparation of PCR reaction mixtures must be conducted on ice to ensure enzyme-free conditions. Real-time quantitative PCR (qPCR) instrument parameters—including primer configuration, sample names, temperature gradients for denaturation and annealing, and reaction time—must be precisely calibrated according to specified requirements. The detailed procedures are as follows:

5.1 Take the EP tubes of four kinds of cDNA (control, low, medium and high) frozen in the last time from the -80 degree refrigerator, tear off the sealing film, let it melt at room temperature for 5-10 minutes, invert the tubes up and down 5-10 times to mix thoroughly, then use centrifuge to snap the tubes to the bottom, and put them on ice for standby.

5.2 At the same time, ROX reagent, RNase-free ddH_2O and positive and negative primers of specific genes (PDL1, VEGF, RBPJ, ICAM1, DSP, SOD2, CAT, GAPDH) were taken out from the -20 degree refrigerator and slowly thawed on ice.

5.3 Set up a group in vivo control with three small holes of the primer gene, and calculate the amount of each reagent according to the qpcr system configuration instructions:

Table 1 qPCR Reaction Master Mix Components per Sample

	cDNA (μl)	**ROX Reference Dye (μl)**	**Forward primer (μl)**	**Reverse primer (μl)**	**RNase-free ddH_2O (μl)**
Wild type (Control)	15	75	3	3	54
Low Resistance	15	75	3	3	54
Medium Resistance	15	75	3	3	54
High Resistance	15	75	3	3	54

5.4 Use a pipette with a range of 10μl and 200μl to draw the above amount and add it to four new

EP tubes labeled "control", "low", "medium" and "high". Mix gently without producing bubbles during the process.

5.5 Edit the position relationship between the corresponding primer gene and the four concentrations ("control", "low", "medium", "high") on the 96-well plate in Excel (red, orange, yellow and green represent positive, low, medium and high concentrations respectively);

PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1	PDL1
VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF
RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ	RBPJ
ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1	ICAM1
DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP	DSP
IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B	IL1B
CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT	CAT
GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH

Figure 1 Layout of the 96-well Plate

5.6 Prepare 12-row eight-tube tubes and carefully add 40 μl of qPCR system solution to each well according to the positional relationship diagram. Note: Do not touch the wall of the well with the pipette tip during the addition process; change the pipette tip when switching samples.

5.7 After the sampling is completed, cover the lid and put the 12-row octaplex tube into the high-speed mini centrifuge for 1000rpm centrifugation for 1 minute, and the liquid is centrifuged to the bottom of the qPCR plate to ensure the accuracy of the experiment.

5.8 Remove the 96-well plate and its supporting tray, and put the 12-row eight-connected tubes into the tray in order. Pay attention not to touch the middle and bottom of the 96-well plate with your hands to avoid affecting the detection accuracy due to contamination.

5.9 Put the 96-well plate into the qPCR machine and set the reaction conditions as follows:

Table 2 Standard reaction conditions on the qPCR amplifier

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Reaction step	Temperature	Time	Recurring number	Remarks
Pre-transformation	95℃	30 seconds	1	Activate hot start DNA polymerase and eliminate cold start effect
Chains of amplification			40	Fluorescence signal acquisition point: at the end of each annealing/elongation cycle
denaturation	95℃	5 seconds		Double strand DNA unwinding
-Annealing/Extending	60℃	30 seconds		Primer binding and chain extension (annealing temperature adjusted according to primer Tm value)
Melting curve analysis	65℃→95℃	Continuous heating	1	Each temperature increase of 0.5℃ for 5 seconds, and the fluorescence signal was monitored (used to verify product specificity)

5.10 Start the device, select SYBRGreen program, mark the sample and primer, and wait for results.

5.11 Take out the 96-well plate in the instrument and copy the data.

5.12 Analyze and process the data to calculate ΔCT, ΔΔCT and $2^{-\Delta\Delta CT}$ values.

VI. Experimental results

The PCR experimental results revealed multiple melting curves with peaks. Some samples exhibited additional secondary peaks or shoulder peaks near the main peak, indicating the presence of non-uniform amplification products. Variations in melting temperature (Tm) values among different samples suggested inconsistent amplification outcomes. Furthermore, the fluorescence signals during the plateau phase of the amplification curve showed significant heterogeneity, with some samples displaying markedly higher or lower plateau signals compared to others, indicating substantial variations in amplification efficiency or initial template amounts.

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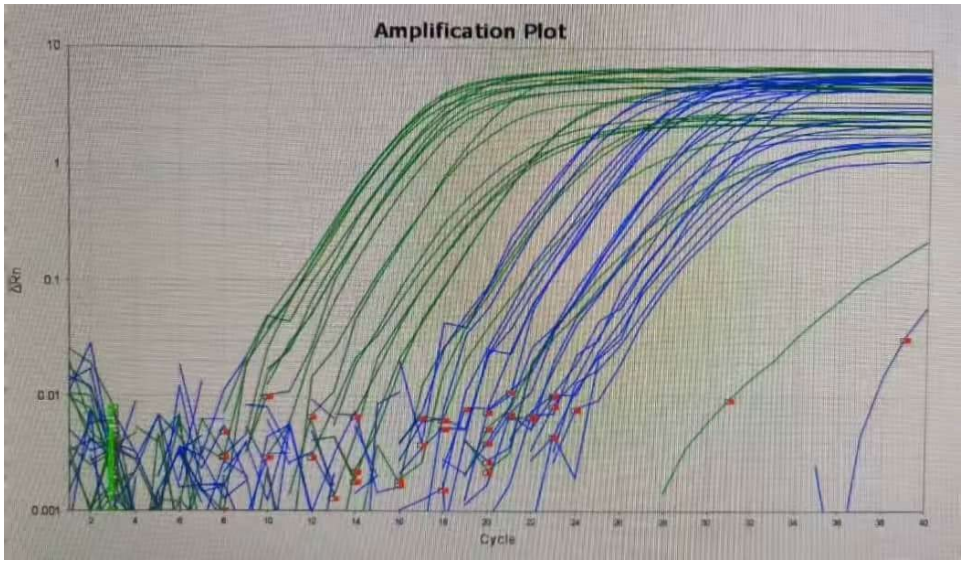

Figure 2 Partial sample amplification curve

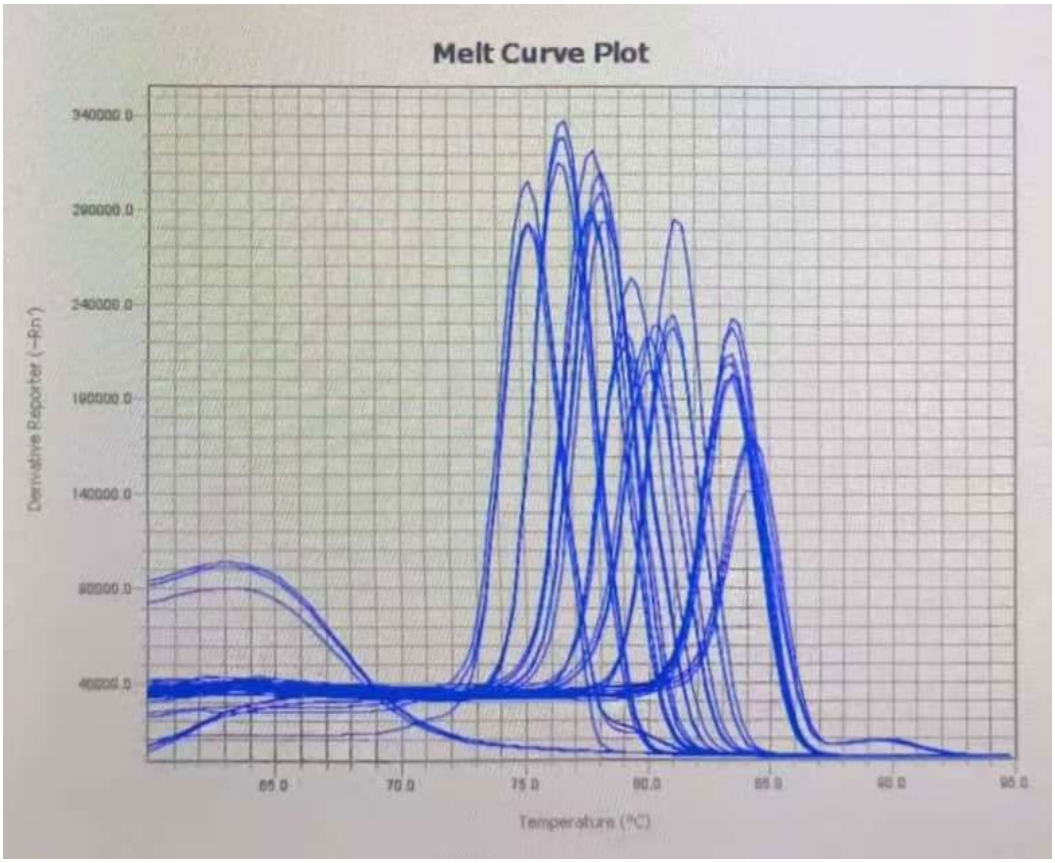

Figure 3 Partial sample melting curves

VII. Results analysis

The multi-peak phenomenon of melting curve suggests that the following problems may exist: (1) primer dimer formation; (2) nonspecific amplification (which needs to be verified by gel

electrophoresis of amplified products); (3) genomic DNA contamination (which can be avoided by designing inter-intron primers).

The significant difference in plateau of amplification curve may be due to: (1) template concentration determination error (re-measurement with fluorescence quantification instrument); (2) deviation in sample addition operation (use of pipette for sample addition); (3) residual PCR inhibitors (verified by 1:5 template dilution experiment).

To address potential issues, we implemented the following experimental adjustments: (1) Redesignated primer specificity verification using Primer-BLAST; (2) Reprocessed RNA plate preparation; (3) Gradient PCR optimization (55-65℃) annealing temperature; (4) DMSO (2-5%) supplementation for enhanced amplification of high GC templates; (5) 1:5 dilution of all cDNA templates to verify inhibition effects; (6) Triple-dilution sampling using calibrated pipettes; (7) Switching to TaqMan probe method for improved specificity if unresolved. These modifications are expected to significantly enhance qPCR result quality, providing reliable data for subsequent analysis of gene expression-drug resistance correlations.