Experimental of qRT-PCR

1 Materials and Methods

1.1 Sample

Six human granulocyte samples were cryopreserved at -80°C.

1.2 Reagents

Animal Total RNA lsolation Kit, item No. RE-03014, specification 200 times, Ltd.; PrimeScript RT reagent Kit, lot number RR047A, size 100 Preps, produced by Bao Ri Doctor Biotechnology Co. Ltd.; anhydrous ethanol (AR grade), lot 220718 1, 2500 mL/bottle, produced by Sichuan Xilong Science Co.

1.3 Instruments and Consumables

Real-time fluorescence quantitative (RT-PCR) instrument, model QuantStudio TM3, produced by ThermoFisher Instruments Co., Ltd; low temperature centrifuge, model C2500, produced by Hunan Xiang Yi Experimental Instrument Factory; thermal cycler, model TCA0096, produced by ThermoFisher Instruments Co. Model UPH-II-10T, produced by Chengdu Ultrapure Technology Co., Ltd; Vortex Mixer, Model XH-B, produced by Jiangsu Kangjian Medical Supplies Co. Gun, specifications 1000μL, 200μL, 20μL, 10μL, 2μL, produced by Dalong Xingchuang Experimental Instruments (Beijing) Co., Ltd; Gun head, specifications 1000μL, 200μL, 20μL, produced by Axygen, USA; EP tube, specifications 1.5mL, 0.2mL, 100μL, produced by Axygen, USA.

1.4 Experimental Method

RT-PCR is a technique that combines reverse transcription (RT) of RNA and polymerase chain amplification (PCR) of cDNA. The cDNA is first synthesized from RNA by the action of reverse transcriptase, and then the cDNA is used as a template to amplify and synthesize the target fragment.

1.5 Experimental Steps

1.5.1 Total RNA extraction from samples

1.5.1.1

Add anhydrous ethanol to Buffer RL2 and Buffer RW2 before use, refer to the label on the bottle for the volume to be added.

1.5.1.2

Add an appropriate amount of Buffer RL1 to the EP tube containing the sample and mix with repeated blowing with a pipette for digestion and lysis. (Add 0.5 mL Buffer RL1 for 2-5×106 cells)

1.5.1.3

Transfer the mix to a DNA-Cleaning Column and centrifuge at 12,000 rpm for 2 min. Remove the DNA-Cleaning Column and retain the supernatant in the collection tube.

1.5.1.4

Add 1.6 times the volume of Buffer RL2 to the above supernatant and mix gently. Then transfer 0.7mL of the mixture into the RNA-only Column, centrifuge at 12000rpm for 1min, and discard the waste solution in the collection tube.

1.5.1.5

Put the purification column back into the collection tube, add all the remaining mixture into the purification column, centrifuge at 12000 rpm for 1 min, and discard the waste solution in the collection tube.

1.5.1.6

Add 0.5 mL of Buffer RW1 to the purification column, centrifuge at 12000 rpm for 1 min, and discard the waste solution in the collection tube.

1.5.1.7

Add 0.7 mL of Buffer RW2 to the purification column, centrifuge at 12000 rpm for 1 min, and discard the waste solution in the collection tube.

1.5.1.8

Add 0.7mL Buffer RW2 to the purification column, centrifuge at 12000rpm for 1min and discard the waste solution from the collection tube.

1.5.1.9

The purification column was placed back into the collection tube and the empty tube was centrifuged at 12,000 rpm for 2 min to remove the residual Buffer RW2.

1.5.1.10

Transfer the purification column to a new centrifuge tube, add 50-200 ul of RNase-Free ddH2O pre-warmed at 65°C dropwise to the centre of the purification column and leave at room temperature for 2 min. centrifuge at 12000 rpm for 1 min to collect the RNA solution for testing or store at -80°C.

1.5.2 Removal Reactions of Genomic DNA (Table 1)

Table 1 Genomic DNA removal reaction system

Reagents	Volume (μL)
5×gDNA Eraser Buffer	2
gDNA Eraser	1
Total RNA	2
RNase Free dH_2O	5
Total	10

Note: After adding each reagent in turn, 42°C, 2min

1.5.3 Reverse Transcription Reaction (Table 2)

Table2 Reverse transcription reaction system

Reagents	Volume (μL)
5×PrimeScript Buffer 2	4
PrimeScript RT Enzyme Mix I	1
RT Primer Mix	1
RNA	10
RNase Free dH_2O	4
Total	20

Note: Add each reagent in turn and set up the PCR instrument for the reaction.

1.5.4 Real-time Fluorescence Quantitative Polymerase Chain Reaction (real-time PCR)

1.5.4.1 Primer design

The full sequences of the genes were searched from the National Center for Biotechnology Information (NCBI) database and primers specific to each gene were designed and screened using Primer Premier primer design software. All primers were designed and synthesized by Shanghai Bioengineering Bioengineering Technology Service Co Ltd and purified by ULTRAPAGE (Table 3).

Table 3 Primers and base sequences

Primer name	Upstream Downstream	Upstream Downstream
GAPDH	tgacttcaacagcgacaccca	caccctgttgctgtagccaaa
DLAT	gggttattgcacagcgattaat	gaagaatttgcttcgggaactt
NUDT16	tcccaatttcctcttctcccaaagc	gatcacgatcccaactccaccaag

1.5.4.2 Real-time Fluorescent Quantitative PCR Reactions

Table 4 PCR Reaction System

Reagents	Volume (μL)
2×Real PCR Easy^{TM} Mix-SYBR	10.0
Forward Primer(10μM)	0.8
Reverse Primer(10μM)	0.8
Template（DNA）	2.0
ddH_2O	6.4
Total	20.0

Table 5 PCR Reaction System

Reaction temperature	Time	Remarks
95℃	30s	Pre-mutability
95℃	5s	Denaturation
55℃	30s	Annealing
72℃	30s	Fully extended, collecting fluorescence

Note: Remarks: 45 cycles

2 Results

CT (Threshold cycle) values for each assay sample of the PCR process were analysed using Thermo Scientific PikoReal software (Thermo). In our laboratory, X relative mRNA expression levels were calculated by $2^{-\Delta\Delta CT}$ at :

$\Delta CT = CT_{\text{target gene}} - Ct_{\text{internal reference}}$

$\Delta\Delta CT = \Delta CT_{PCOS} - \Delta CT_{\text{control}}$

XmRNA expression difference ploidy is expressed as $2^{-\Delta\Delta CT}$.

The analysis of real-time fluorescence quantitative PCR results is shown in Tables 6~7, and the statistical results are shown in Tables 8~9; the lysis curve and amplification curve are shown in Annex 1, and the corresponding amplification curve for each sample is shown in Annex 2~4.

2.1Data Analysis and Results

Table 6 Analysis of DLAT Gene Expression Value Data

	Gene Name		△CT= CT $_{\text{target gene}}$-Ct $_{\text{internal reference}}$		ΔΔCT = ΔCT$_{PCOS}$ – ΔCT $_{\text{control}}$	Amplification ploidy = 2 - ΔΔCT (gene expression value)
Sample	GAPDH	DLAT				
C1	18.874	28.663	9.79		-0.05	1.04
C2	19.140	28.869	9.73		-0.11	1.08
C3	18.896	28.895	10.00	9.84	0.16	0.89
P1	19.113	29.517	10.40		0.56	0.68
P2	18.694	29.761	11.07		1.23	0.43
P3	18.960	29.304	10.34		0.50	0.70

Table 7 Analysis of NUDT16 Gene Expression Value Data

	Gene Name		△CT= CT $_{\text{target gene}}$-Ct $_{\text{internal reference}}$		ΔΔCT = ΔCT$_{PCOS}$ – ΔCT $_{\text{control}}$	Amplification ploidy = 2 - ΔΔCT (gene expression value)
Sample	GAPDH	DLAT				Sample
C1	18.874	29.754	10.88		-0.20	1.15
C2	19.140	30.259	11.12		0.04	0.97
C3	18.896	30.141	11.25	11.08	0.16	0.89
P1	19.113	30.696	11.58		0.50	0.71
P2	18.694	30.878	12.18		1.10	0.47
P3	18.960	31.099	12.14		1.06	0.48

2.2 Statistical Results

Table 8 Changes in DLAT mRNA Expression in Human Granulosa Cells（$\bar{x}$ ±SD）

Group	Number of samples	$2^{-\Delta\Delta CT}$（gene expression value）
Control	3	1.003±0.100
PCOS	3	0.603±0.150 #

Note: Compared to Group Control, # p <0.05，## p <0.01

The statistical results in Table 8 show that DLAT mRNA expression was reduced in human granulosa cells in group D compared with group E. The difference was significant（p <0.05）。

Table 9 Changes in NUDT16 mRNA Expression in Human Granulosa Cells ($\bar{x}$ ±SD）

Group	Number of samples	$2^{-\Delta\Delta CT}$（gene expression value）
Control	3	1.003±0.133
PCOS	3	0.553±0.136 #

Note: Compared to Group Control, # p <0.05，## p <0.01

The statistical results in Table 9 show that NUDT16 mRNA expression was reduced in human granulosa cells in group D compared to group E. The difference was significant (p < 0.05).

Annex: Summary of original images

Annex1: Dissolution and Amplification Curve Plots

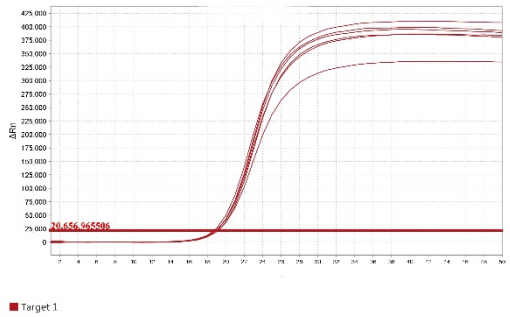

Figure 1-1 GAPDH Amplification Curve

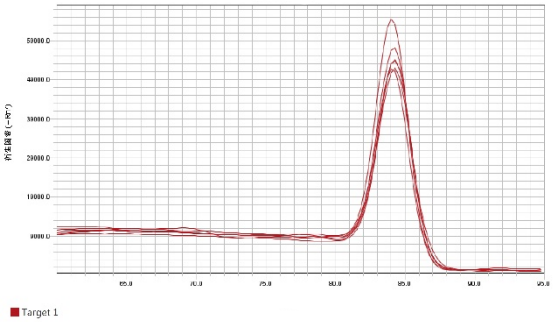

Figure 1-2 GAPDH Solubility Curve Graph

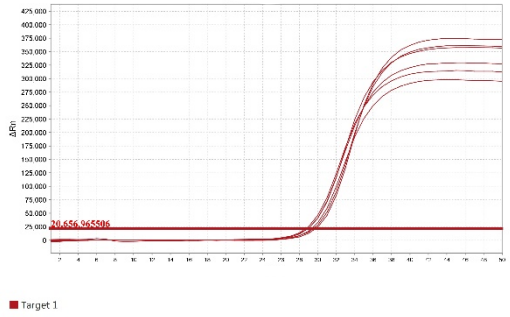

Figure 1-3 DLAT Amplification Curve

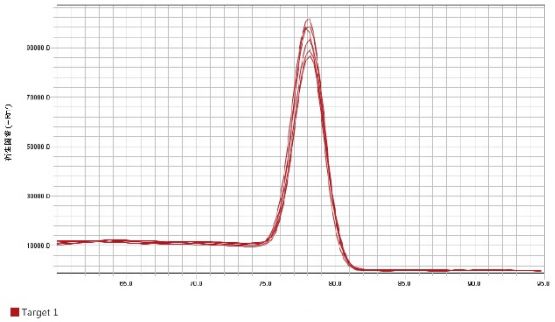

Figure 1-4 DLAT Dissolution Curve Graph

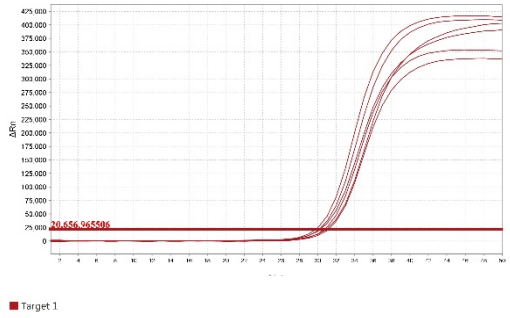

Figure 1-5 NUDT16 Amplification Curve

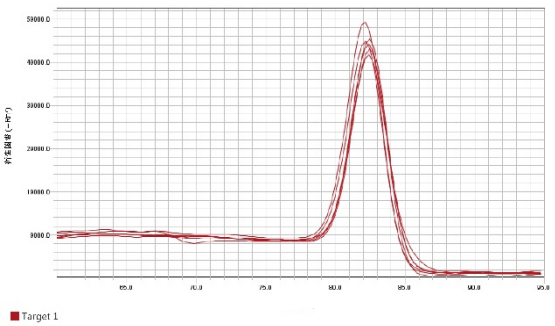

Figure 1-6 Dissolution Profile of NUDT16

Annex2: Plot of GAPDH Amplification Curves for Individual Samples.

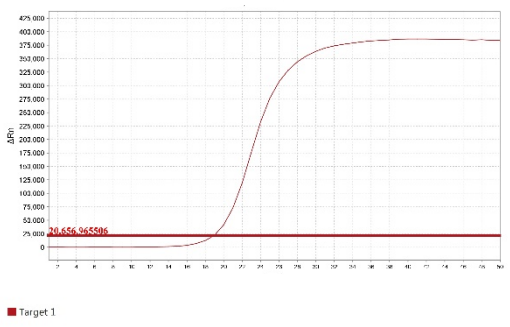

Figure 2-1 C1 Amplification Curve

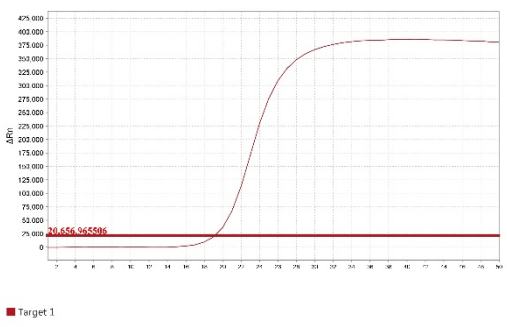

Figure 2-2 Plot of C2 Amplification Curve

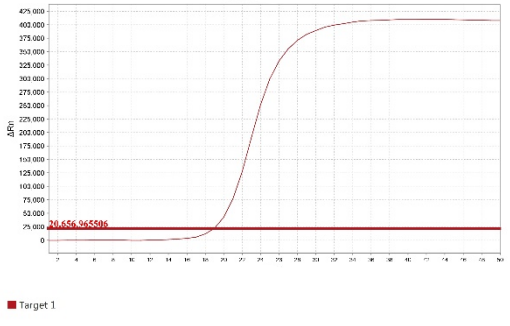

Figure 2-3 Plot of C3 Amplification Curve

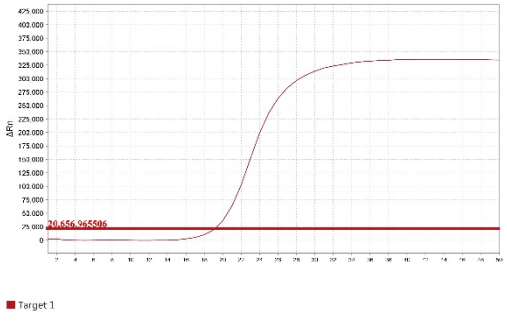

Figure 2-4 Plot of P1 Amplification Curve

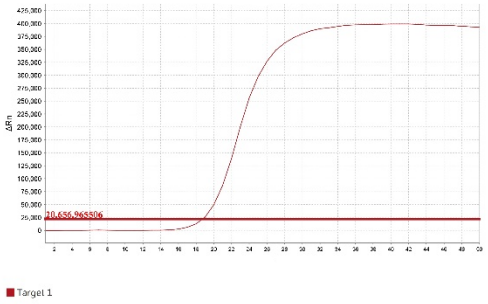

Figure 2-5 Plot of P2 Amplification Curve

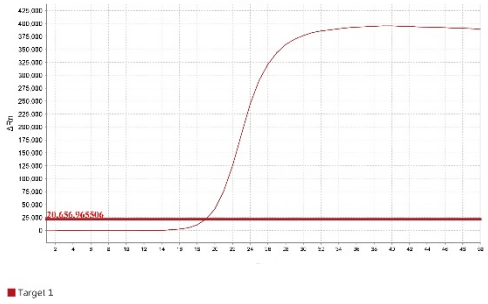

Figure 2-6 Plot of P3 Amplification Curve

Annex 3: Plot of DLAT Amplification Curves for Individual Samples

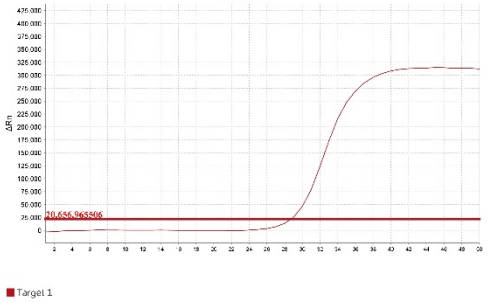

Figure 3-1 C1 Amplification Curve

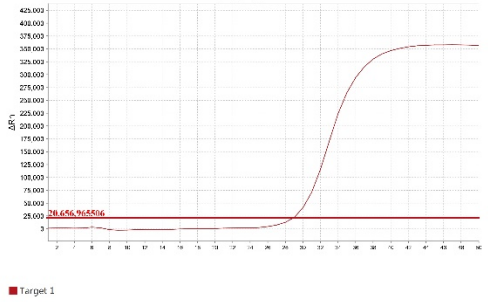

Figure 3-2 C2 Amplification Curve

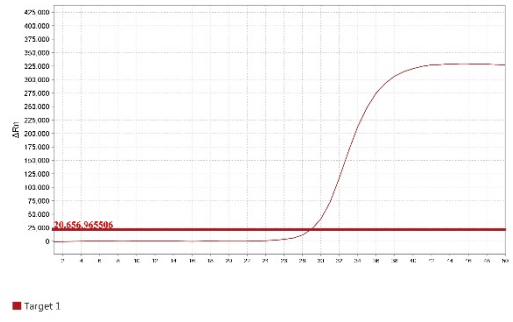

Figure 3-3 C3 Amplification Curve

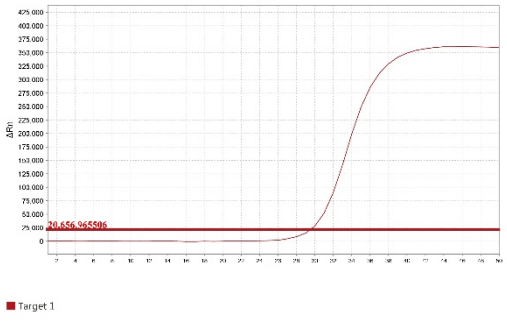

Figure 3-4 P1 Amplification Curve

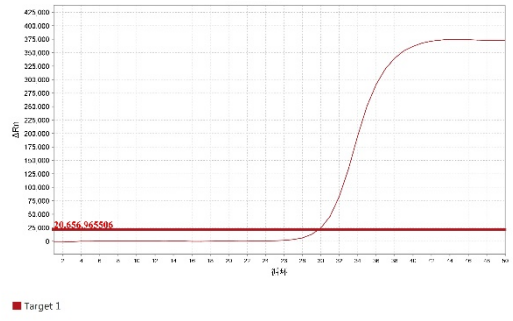

Figure 3-5 P2 Amplification Curve

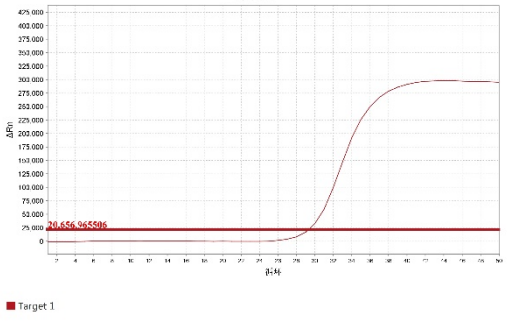

Figure 3-6 P3 Amplification Curve

Annex 4: Plot of NUDT16 Amplification Curves for Individual Samples.

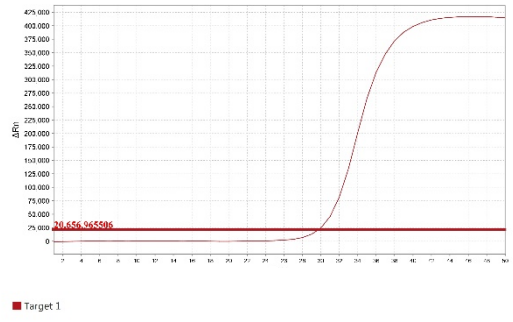

Figure 4-1 C1 Amplification Curve

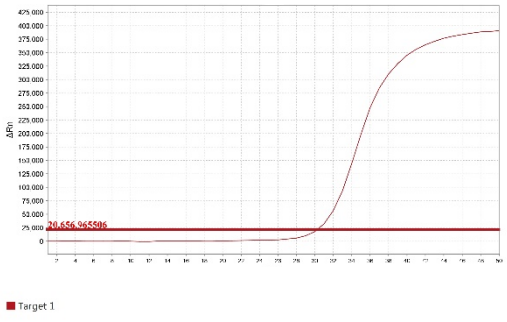

Figure 4-2 C2 Amplification Curve

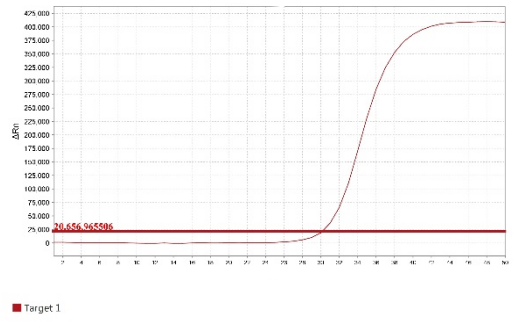

Figure 4-3 C3 Amplification Curve

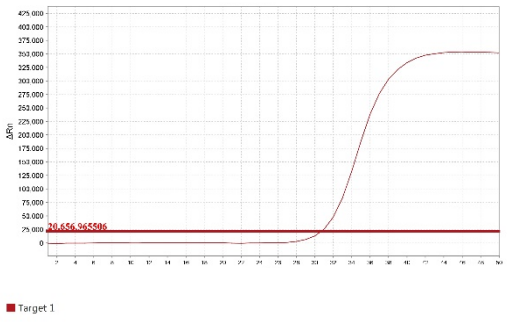

Figure 4-4 P1 Amplification Curve

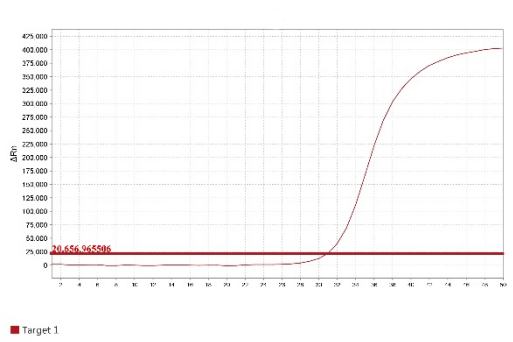

Figure 4-5 P2 Amplification Curve

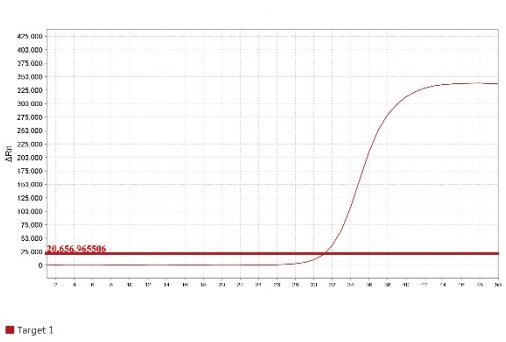

Figure 4-6 P3 Amplification Curve