Time: 2024.06.29-2024.06.30

1. **Experiment:** qRT-PCR detect the scfv on the NK cells' surface
2. **Time:** 2024.06.29-2024.06.30
3. **Member:** Song Zhang, Yaqi Gao, Xudong Tang, Hanyue Liu
4. **Material:** Chloroform, Trizol, isopropanol, freezer, centrifuge, PCR machine, 75% ethanol (prepared with DEPC water), reverse transcription system (RNA, Enzyme Mix, 5×All-in-one qRT SuperMix, RNase Free dH_2O), quantitative PCR reaction system (2×ChamQ Universal SYBR qPCR Master, Primer F (10 μM), Primer R (10 μM), Template DNA/cDNA, ddH2O)
5. **Method:**

(1) Cell culture

Cultivate the cells sorted in experiment record 11 in a 37°C, 5% CO_2 incubator for 2 days.

(2) RT-qPCR

① RNA Extraction

- Aspirate the culture medium, add 1 mL of Trizol to each well of a six-well plate, shake 3-5 times, then pipette up and down several times to ensure complete lysis. Transfer the mixture to a 1.5 mL centrifuge tube.
- Add 0.2 mL of chloroform to each well, vortex vigorously for 15 secs, incubate at room temperature for 3 mins, then centrifuge at 12,000 rpm for 15 mins at 4°C.
- Transfer the upper aqueous phase to a new tube and precipitate the RNA with isopropanol. Add 0.5 mL of isopropanol to each tube, invert several times to mix, and place in a -20°C freezer for 30 mins.
- Centrifuge at 12,000 rpm for 30 mins at 4°C. RNA precipitation should be visible at the bottom of the tube.
- Discard the supernatant and wash the RNA pellet with 75% ethanol (prepared with DEPC water). Add 1 mL of 75% ethanol to each tube. Centrifuge at 7,500 rpm for 5 mins at 4°C.
- Allow the RNA pellet to air-dry at room temperature for 5 mins. Add 32 μL of RNase-free water to dissolve the RNA and then measure the RNA concentration and purity.

② cDNA Synthesis

- Reverse transcription system:

Reagent	Amount (μL)
RNA	≤ 1 μg
Enzyme Mix	1
5×All-in-one qRT SuperMix	4
RNase Free dH_2O	Up to 20

- Perform reverse transcription in a PCR machine at 50°C for 15 mins, then deactivate the RT enzyme by heating at 85°C for 5 secs. The resulting cDNA product can be stored at -80°C for later use.

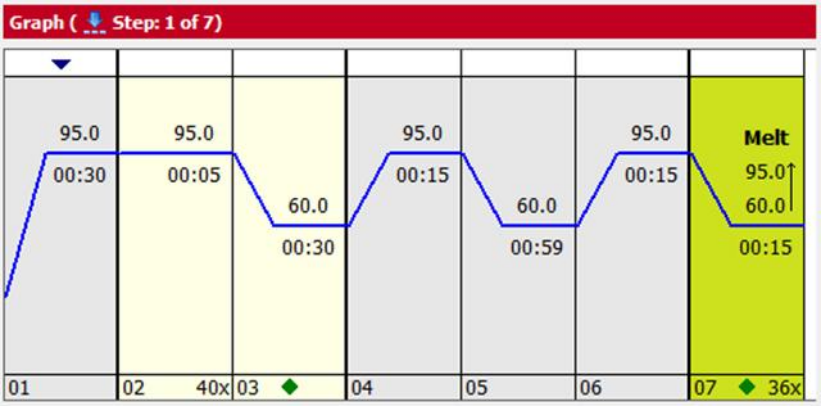
③ Real-Time PCR

- Primer Design
- Design primers flanking the KIR or the KAR using a primer design tool, ensuring the forward primer binds upstream and the reverse primer binds downstream of the barcode. Primers should be 18-25 nucleotides in length, have a GC content of 40-60%, and a melting temperature (Tm) of 55-65°C. Verify primer specificity, avoiding secondary structures such as primer-dimers or hairpins. Validate the primer sequences to ensure no off-target binding occurs, and adjust primer concentrations or annealing temperatures as needed for optimal amplification efficiency.

- Quantitative PCR Reaction System:

Reagent	Amount (μL)
2×ChamQ Universal SYBR qPCR Master	10
Primer F (10 μM)	0.5
Primer R (10 μM)	0.5
Template DNA/cDNA	1
ddH_2O	Up to 20

- Prepare the mix using the above system and add to qPCR tubes, ensuring no air bubbles are present.
- Perform real-time PCR using a two-step method with the following reaction program:



(3) Data Processing

① After completing the RT-qPCR, analyze the data by first exporting the raw fluorescence data and determining the baseline and threshold to exclude background noise.

② Classify each well based on the Ct values and amplification curves into four categories: monoclonal (a single, specific amplification curve indicating one barcode sequence), polyclonal (multiple amplification curves indicating mixed barcode sequences), nonspecific (amplification curves that do not match the expected pattern, possibly due to primer-dimers or nonspecific amplification), and undetected (Ct values beyond the detection limit or no amplification observed).

③ Create a schematic representation matching the layout of the experimental plate, using colors or symbols to indicate the categories.

④ Perform statistical analysis to quantify the distribution of each category and validate the data for accuracy by checking melt curves and other parameters.

⑤ Finally, visualize the results to interpret the biological significance of the different sample groups.

6. Result:

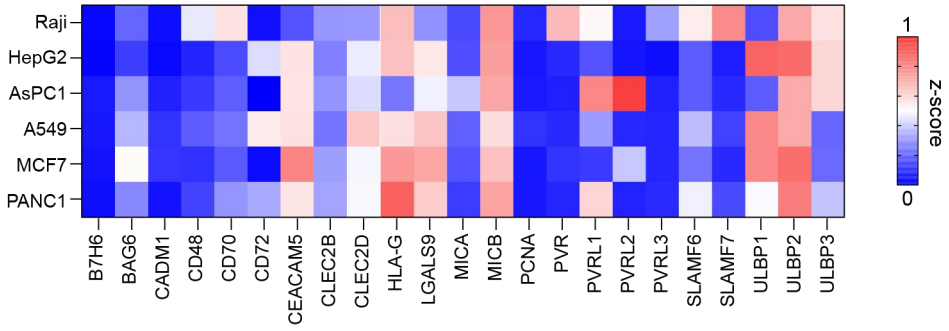


Fig.1 The expression levels of tumor ligands by qPCR in heatmap

Fig.2 The expression levels of NKRs by qPCR