Time: 2024.05.28-2024.05.29

1. **Experiment:** Enrichment and screening of dominant sequences
2. **Time:** 2024.05.28-2024.05.29
3. **Members:** Xinyu Zhu, Hanyue Liu, Xiaoyuan Chen
4. **Materials:** 75% ethanol (prepared with DEPC water), reverse transcription system (RNA, Enzyme Mix, 5×All-in-one qRT SuperMix, RNase Free dH$_2$O), quantitative PCR reaction system (2×ChamQ Universal SYBR qPCR Master, Primer F (10 μM), Primer R (10 μM), Template DNA/cDNA, ddH$_2$O).
5. **Method:**

(1) Cell Culture

Cultivate the cells sorted in experiment record 7 in a 37°C, 5% CO_2 incubator for 2 days.

(2) RT-qPCR

①RNA 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 seconds, incubate at room temperature for 3 minutes, then centrifuge at 12,000 rpm for 15 minutes 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 minutes.
- Centrifuge at 12,000 rpm for 30 minutes 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 minutes at 4°C.
- Allow the RNA pellet to air-dry at room temperature for 5 minutes. Add 32 μL of RNase-free water to dissolve the RNA and then measure the RNA concentration and purity.

②cDNA Synthesis

Reverse transcription system:

Reagent	Amount (μL)
RNA	≤ 1 μg
Enzyme Mix	1
5×All-in-one qRT SuperMix	4
RNase Free dH2O	Up to 20

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

③Real-Time PCR

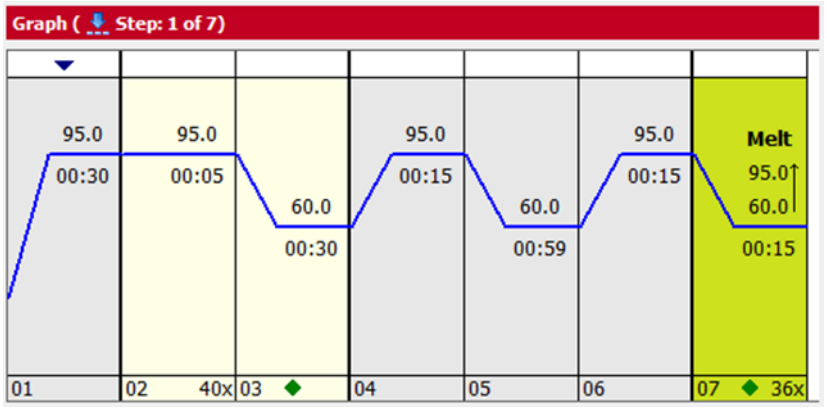
- Primer Design

Design primers flanking the barcode region 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
ddH2O	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

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

②Classify 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).

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

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

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

6. Result:

For the PCR analysis of the barcode, create a schematic diagram based on the results from Experimental Record 7. In this diagram, green is used to represent monoclonal, yellow is used for polyclonal, gray is used for non-amplified, and red is used for unsuccessful detection.

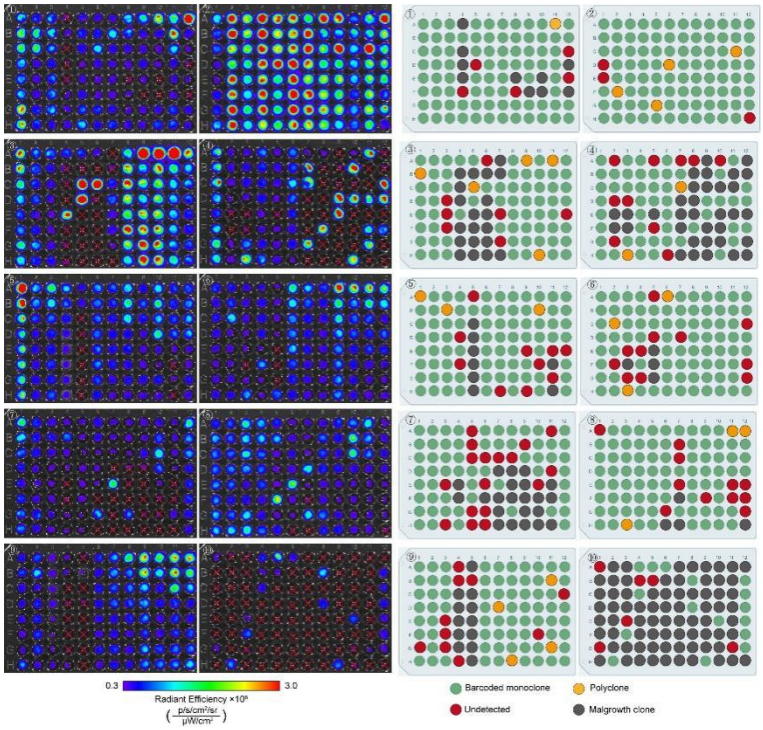


Fig.1 Dish A amplification sorting plot

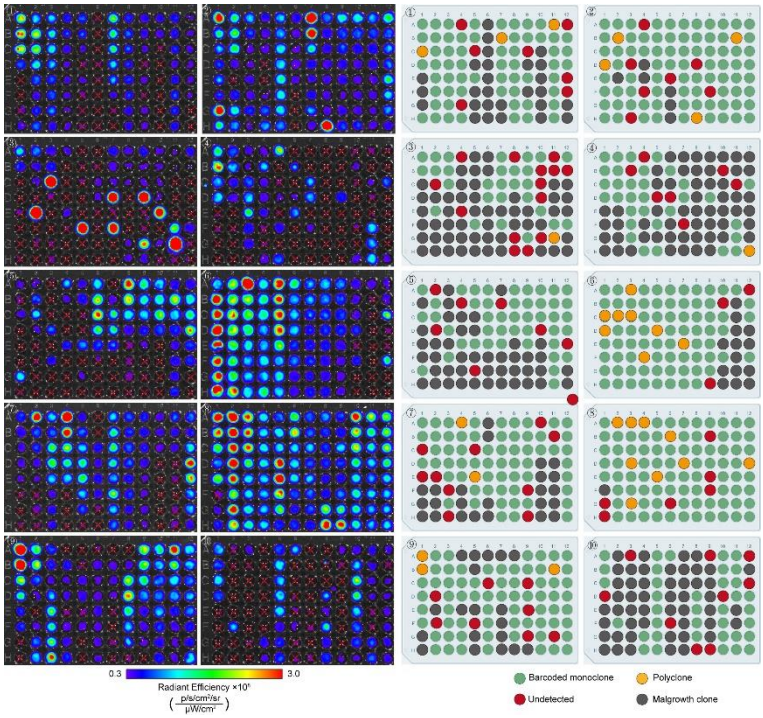


Fig.2 Dish B amplification sorting plot

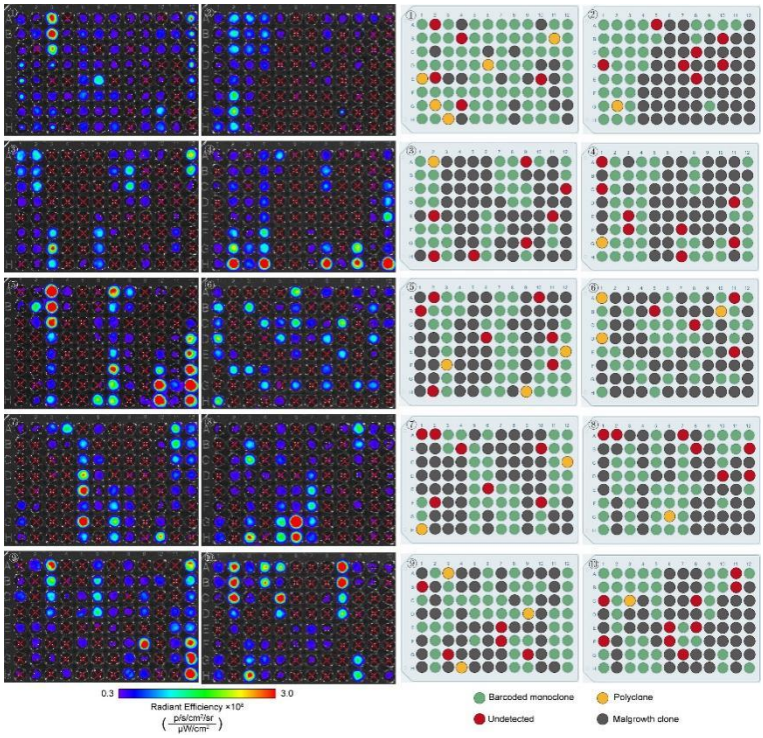


Fig.3 Dish C amplification sorting plot