

- Overview of molecular diagnostics (qPCR) in TB detection
- On-site RAA(Recombinase Aided Amplification) rapid testing

Xiangxiang Sun





- Quantitative Real-time PCR (9:15-9:23)
- Recombinase Aided Amplification (9:23-9:30)
- Practical on the processing of qPCR (9:30-10:20)
- Operation Demonstration of RAA (10:30-11:30)



Quantitative Real-time PCR

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Quantitative Real-time PCR

Quantitative Real-time PCR (qPCR) involves adding fluorescent markers to the PCR reaction. By continuously **monitoring** the sequence and intensity changes of **the fluorescent signals**, the initial quantity of the target gene can be analyzed in real time.

This technology marked a leap from **qualitative** to **quantitative** PCR.



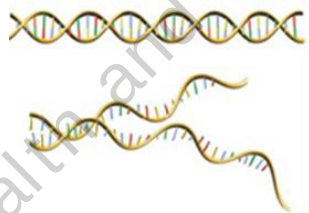
Applied Biosystems, USA



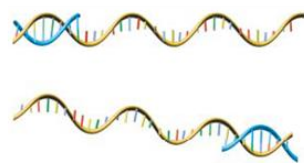
Basic Process of quantitative Real-time PCR

4

PCR Step 1:
Template Denaturation



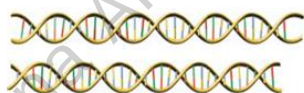
PCR Step 2:
Primer Annealing



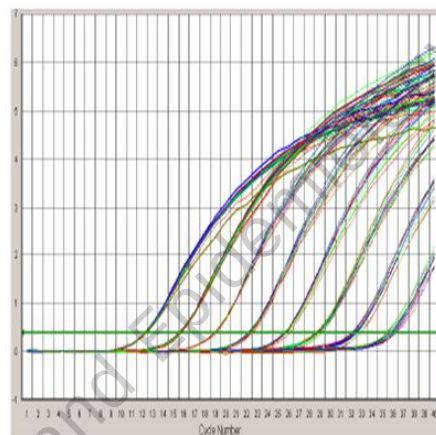
PCR Step 3:
Polymerase Extension



PCR Step 4:
Exponential Amplification



Linear Plot



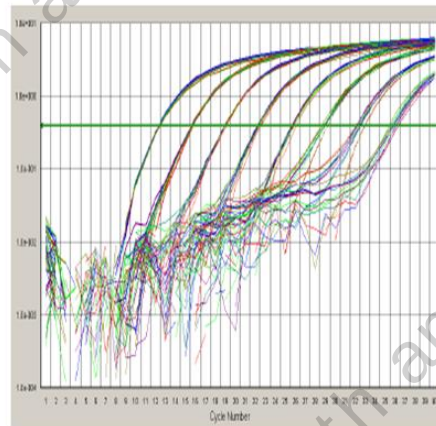
Plateau
Phase

Linear Growth Phase

Exponential Growth Phase

Baseline Phase

Logarithmic Plot



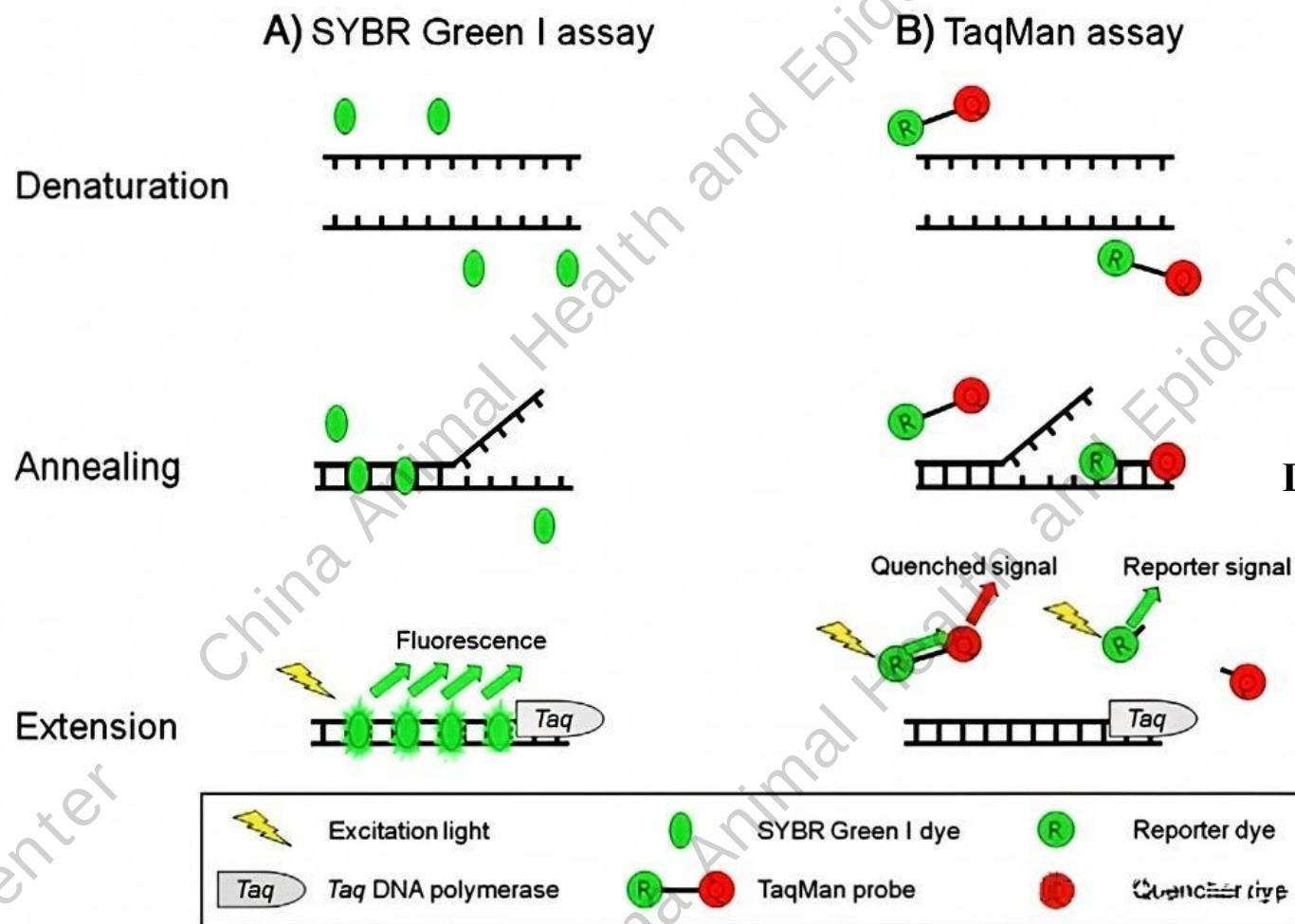
Plateau Phase

Linear Growth Phase

Exponential
Growth Phase

Baseline Phase



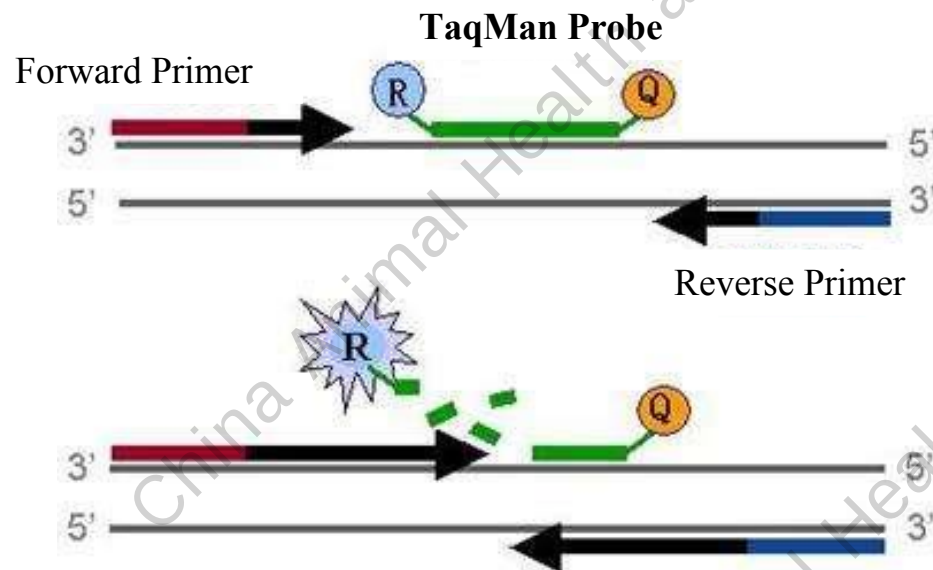


SYBR Green Advantages:

- Easy to use
- Binds to any PCR product
- Cost-effective

Disadvantages:

- Cannot distinguish between different double-stranded DNA
- Primer dimers may affect detection sensitivity
- Non-specific products may impact result accuracy



Advantages:

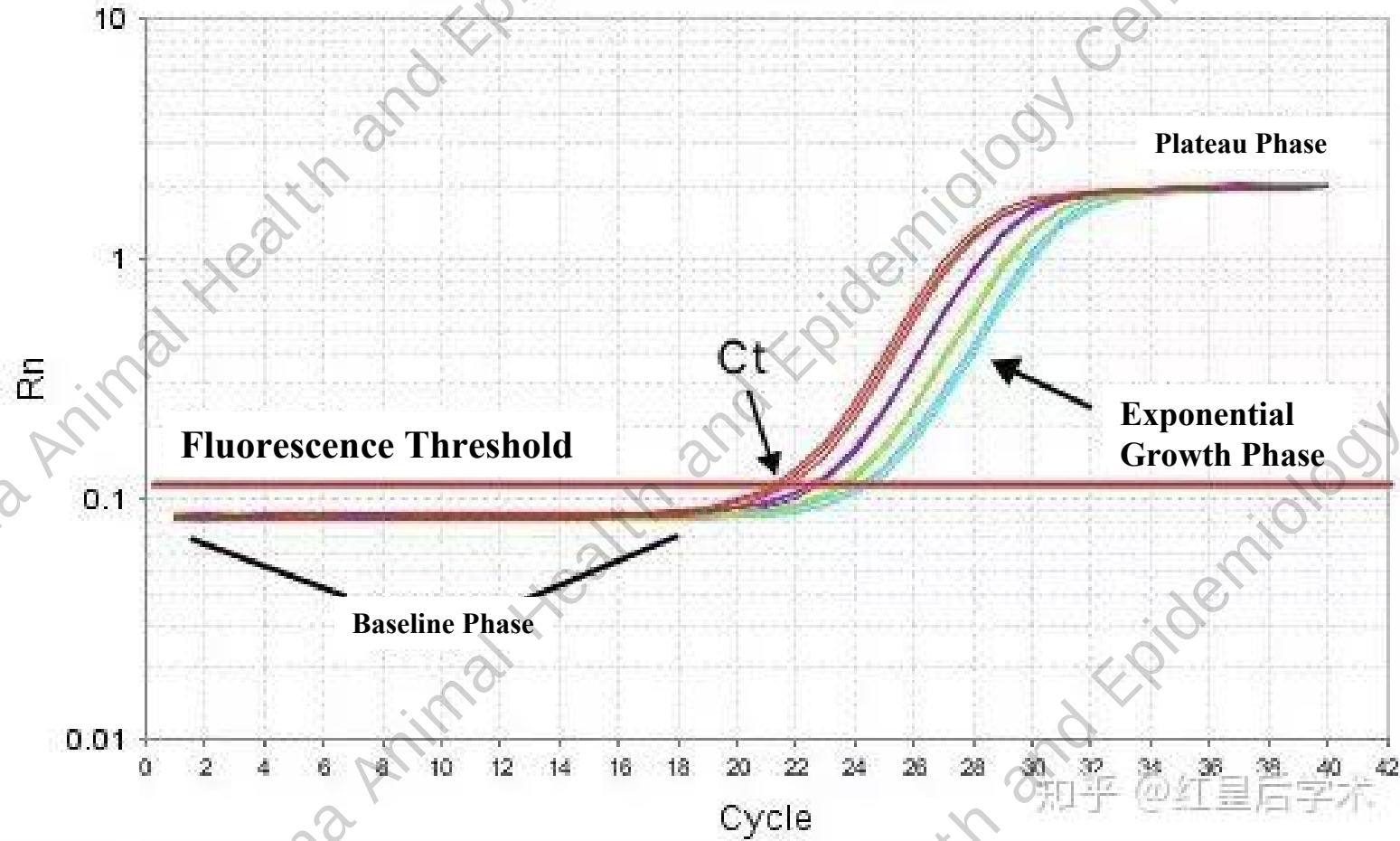
- Low fluorescent background, high sensitivity
- High hybridization stability
- Good fluorescence spectral resolution
- High specificity

Disadvantages:

- High cost
- Complex design
- Only suitable for detecting products shorter than 150 bp

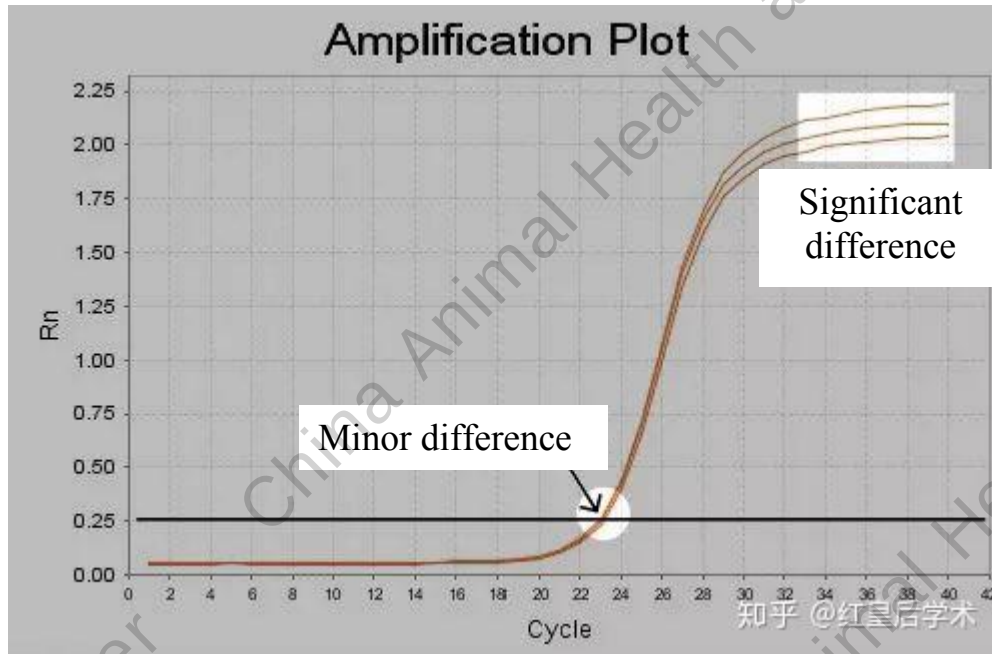


Amplification Plot





Cycle Threshold



The optimal Ct value ranges between 18 and 30 cycles. Values too high or too low will compromise experimental accuracy.

$$X_n = X_0 \times 2^n$$

$$X_n = X_0 \times (1+E\%)^n$$

$$X_{Ct} = X_0 \times (1+E\%)^{Ct}$$

$$\lg X_0 = -\lg(1+E\%)^{Ct} + \lg X_{Ct}$$

Advantages of Quantitative Real-time PCR

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High Specificity

Uses specific probes for accurate target identification, with dual control by primers and probes, reducing false positives.

High Sensitivity

Combines PCR, fluorescence labeling, laser, and digital imaging technologies for exceptional detection sensitivity.

High Accuracy and Broad Detection Range

Fluorescence signals correlate linearly with amplification products, enabling direct quantification (0–10¹⁰ copies/mL).

Simple, Safe, and Pollution-Free

Amplification and detection occur in a closed tube, eliminating contamination risks and post-processing steps.

Fast and High-Throughput

Quantitative analysis of 96 samples can be completed in 2–3 hours.



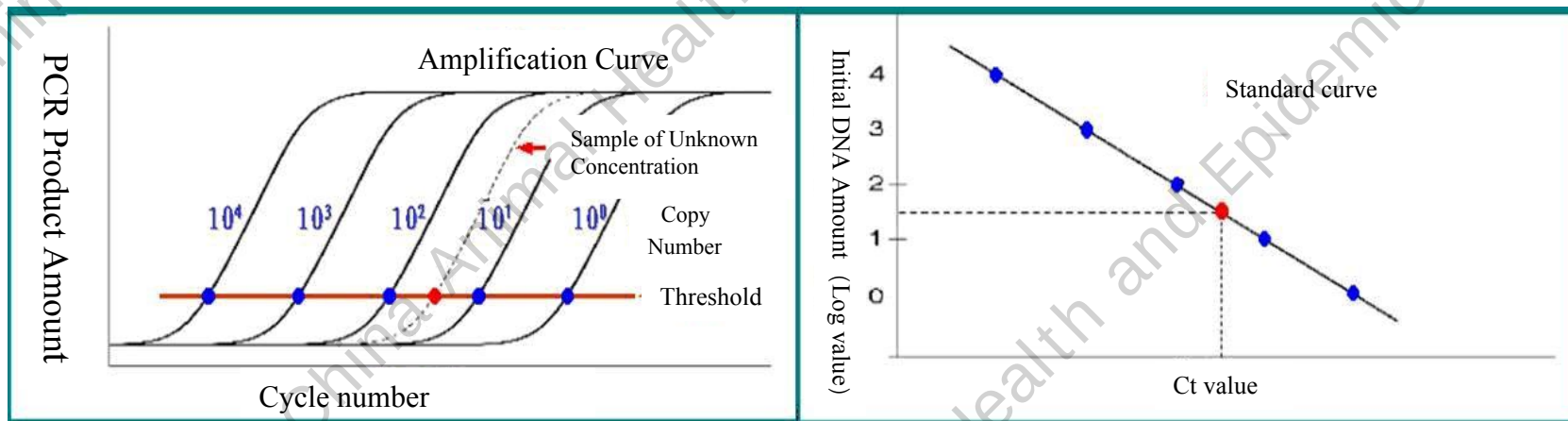
Key Elements of Real-time PCR Experiments

- **Target Gene:** Unknown sample
- **Standard Curve:** Standards of known gradient concentration
- **Ensuring System Validity:** Positive controls
- **Monitoring Contamination:** Negative controls
- **Calibrating Biological Errors:** Housekeeping genes
- **Reducing Other Errors:** Replicate experiments
- **Reaction Conditions**

Standard Curve

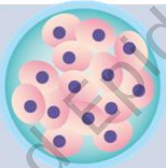
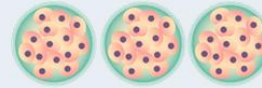
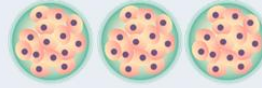
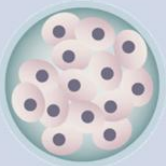
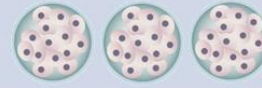
Purpose: Establish a mathematical relationship between Ct values and concentration.

- ❑ Requires at least 5 gradient points of the same DNA.
- ❑ Known concentrations.
- ❑ Consistent PCR efficiency, ideally close to 100%.



Replicate Experiments

- Typically, 3 replicates are required.
- Inter-replicate variation should be <0.5 Ct.
- Includes biological and technical replicates.

	No Treatment	Low dosage 	High dosage 
Cell A 	 A-NT1,A-NT2,A-NT3	 A-L1,A-L2,A-L3	 A-H1,A-H2,A-H3
Cell B 	 B-NT1,B-NT2,B-NT3	 B-L1,B-L2,B-L3	 B-H1,B-H2,B-H3
Cell C 	 C-NT1,C-NT2,C-NT3	 C-L1,C-L2,C-L3	 C-H1,C-H2,C-H3



Main Sources of Contamination:

- ❑ Cross-contamination between samples.
- ❑ Laboratory equipment contamination.
- ❑ Residual contamination from previous PCR products or primers.

Preventive Measures:

- ❑ Strict laboratory zoning and protocols.
- ❑ Chemical decontamination (e.g., 10% sodium hypochlorite followed by 70% ethanol or water).
- ❑ UV irradiation (5–20 minutes) before experiments to eliminate residual contamination.
- ❑ Use of uracil-DNA glycosylase (UNG or UDG) to prevent contamination.



What is RAA?

Recombinase Aided Amplification

- Key Point 1: Works at a steady temperature (37-42°C, like human body)
→ No heating/cooling cycles (different from PCR)
- Key Point 2: Fast (10-30 minutes) + sensitive (finds 1-10 target molecules)
- Key Point 3: No expensive machine (uses simple water bath/heating block)
→ Good for on-site testing (clinics, farms, remote areas)



How RAA Works (3 Core Steps)

Step 1: Recombinase + Primers → Find Target

Recombinase (protein) binds to primers

Scans DNA, finds target, makes "D-loop"

Step 2: Polymerase → Makes New DNA

Polymerase (protein) extends primers

Builds new DNA strand

Step 3: Cycle → More Copies

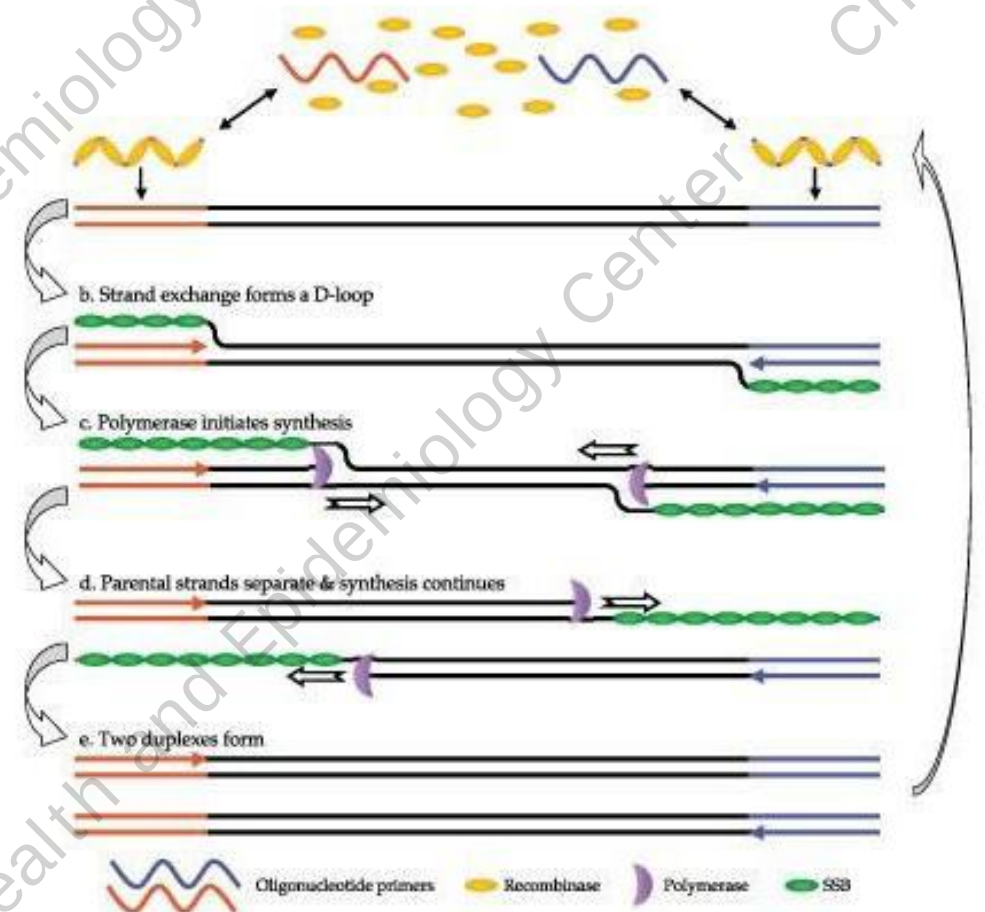
Displaced DNA becomes new template

Process repeats → Millions of copies

The RPA Cycle

All steps operate at low constant temperature (optimum 37°C)

a. Recombinase/oligonucleotide primer complexes form and target homologous DNA





What RAA Needs (6 Key Parts)

Component	Function
Recombinase	Helps primers find target DNA
Strand-Displacing Polymerase	Builds new DNA strands
Single-Stranded Binding Protein (SSB)	Keeps single-stranded DNA from sticking back
Primers	"Starting lines" for making new DNA
dNTPs	"Building blocks" of DNA (A/T/C/G)
Buffer	Good environment for proteins to work



Amplification Process

1. Start (0-5 min):

- Recombinase binds to primers, forming active complexes.

2. Attach to Target (5-10 min):

- Complexes scan dsDNA, bind target sequences, and form D-loops.

3. Primer Extension (10-20 min):

- Polymerase extends primers, building new dsDNA and displacing one strand.

4. Exponential Amplification (20-30 min):

- Displaced ssDNA acts as a new template; SSB stabilizes ssDNA.
- Cycle repeats, leading to $\sim 10^6$ - 10^9 target copies.



Technical Advantages

- Steady Temperature: No expensive machine
- Very Fast: 10-30 minutes for results
- Finds Small Amounts: Detects 1 target copy
- Works for DNA/RNA: Add 1 protein for RNA (RT-RAA)
- Easy to Carry: Reagents don't need fridge (freeze-dried)



Typical Applications

1. Clinical Diagnostics:

- Rapid detection of pathogens (e.g., COVID-19, influenza, malaria, and antibiotic-resistant bacteria).
- Prenatal testing (e.g., Down syndrome screening via fetal DNA).

2. Food Safety:

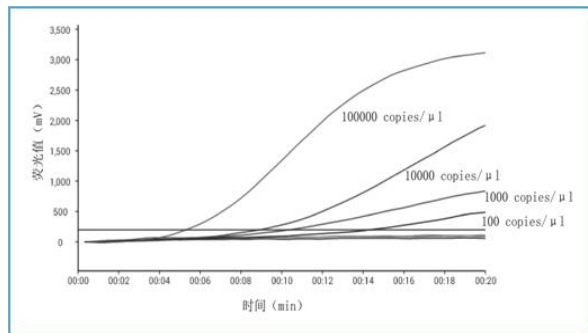
- Detection of foodborne pathogens (e.g., Salmonella, E. coli O157:H7) in meat, vegetables, and water.

3. Environmental Monitoring:

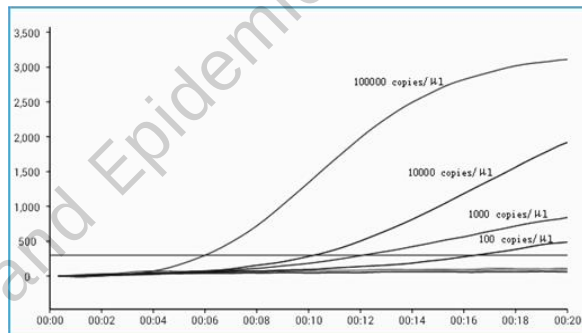
- Identification of harmful algae or pollutant-degrading microorganisms in water/soil.

4. Forensic Science:

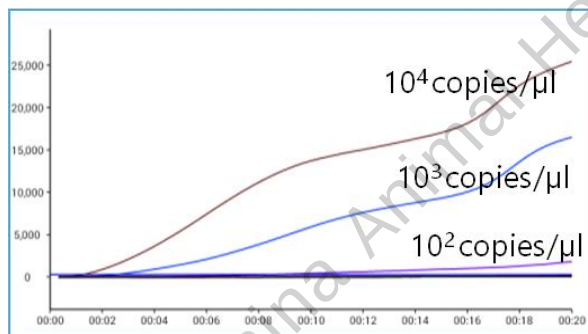
- Amplification of trace DNA from crime scenes (e.g., hair, blood stains).



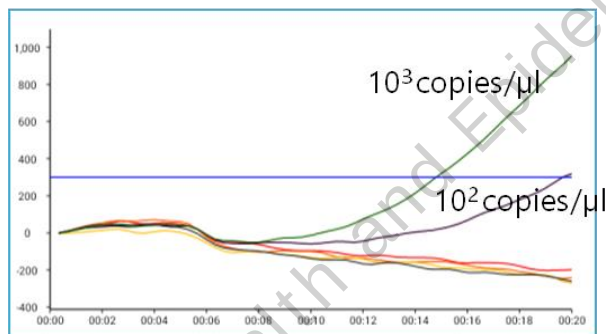
Brucellosis



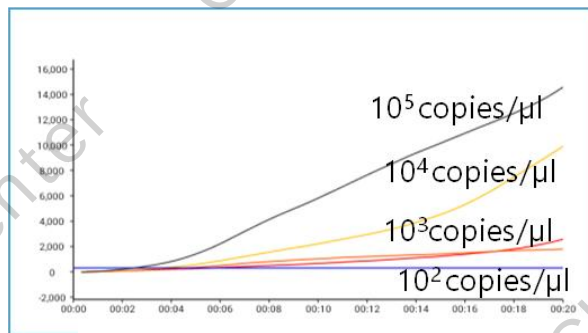
Tuberculosis



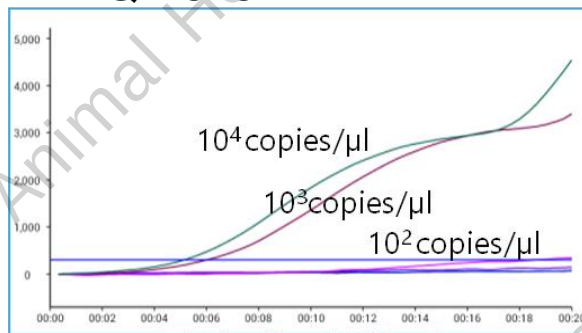
Paratuberculosis



Q Fever



Chlamydia



Tularemia

ICS 11.220
CCS B 41

中华人民共和国国家标准

GB/T 18646—2025
代替 GB/T 18646—2018

动物布鲁氏菌病诊断技术

Diagnostic techniques for animal brucellosis

2025-05-30 发布

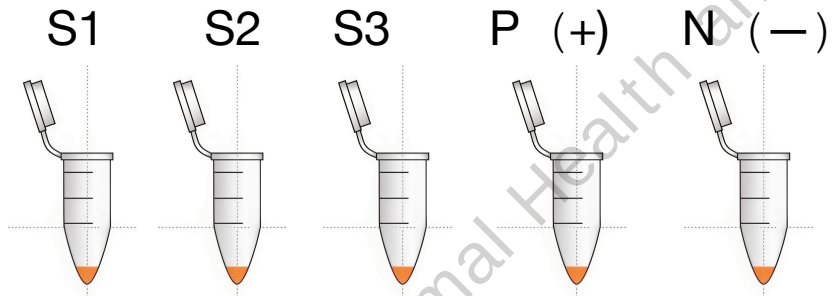
2025-12-01 实施

国家市场监督管理总局 发布
国家标准化管理委员会



Practical on the processing of qPCR

DNA



Reagent

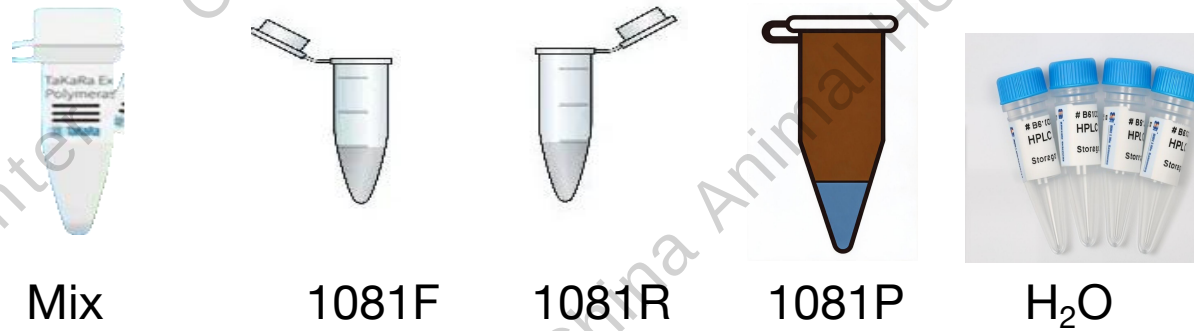


Table RT-PCR Reaction System

Reagent	Volume (μL)	Volume (μL) 6
2 × Taq PCR Master Mix	10	60
Forward Primer	0.8	4.8
Reverse Primer	0.8	4.8
Probe	0.4	2.4
ddH ₂ O	6	36
DNA Template	2	12
Total Volume	20	120



Practical on the processing of qPCR

Basic test information		
Test Date	2026.9.17	
Tester	Xiangxiang Sun	
Instrument ID	2-C-001	
Ambient Temperature		
Ambient Humidity		
Sample ID	Test Result	Remarks
S1		
S2		
S3		
P		
N		

WOAH Regional Hands-on Training on Zoonotic TB Diagnosis
Qingdao, China P.R., September 2026

Basic Information			Experiment Execution Information			
Task No.	Tester	Test Time	Signature			
—	Xiangxiang Sun	10:30	Sun Xiangxiang			
Refrigerator Temperature		4 °C	Ambient Temperature		Ambient Humidity	
Pipettor ID			2-E-011 2-E-012 2-E-013			
PCR Instrument ID			2-C-001			
Reagent Supplier			Takara	Batch No.		
DNA Extraction			Completed (√)	-		
Prepare n+1 reaction mixes, mix thoroughly, brief centrifugation, aliquot into tubes to collect all components at the bottom, refer to Table 2 for operation			Completed ()	ROOM1		
Add DNA template			Completed ()	ROOM2-1		
Mix, brief centrifugation			Completed ()	ROOM2-1		
Load to PCR instrument, set programme and run			Completed ()	ROOM2-3		
Post-PCR result analysis			Completed ()			
Result interpretation, data saving, end experiment			Completed ()			



Thank you!

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Qingdao, China P.R., September 2026

