

Application of ddPCR in Bovine Tuberculosis Diagnosis

WOAH Asia-Pacific Technical Training

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**WOAH Regional Hands-on Training on
Zoonotic TB Diagnosis**

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First-generation PCR / endpoint PCR

Three steps:

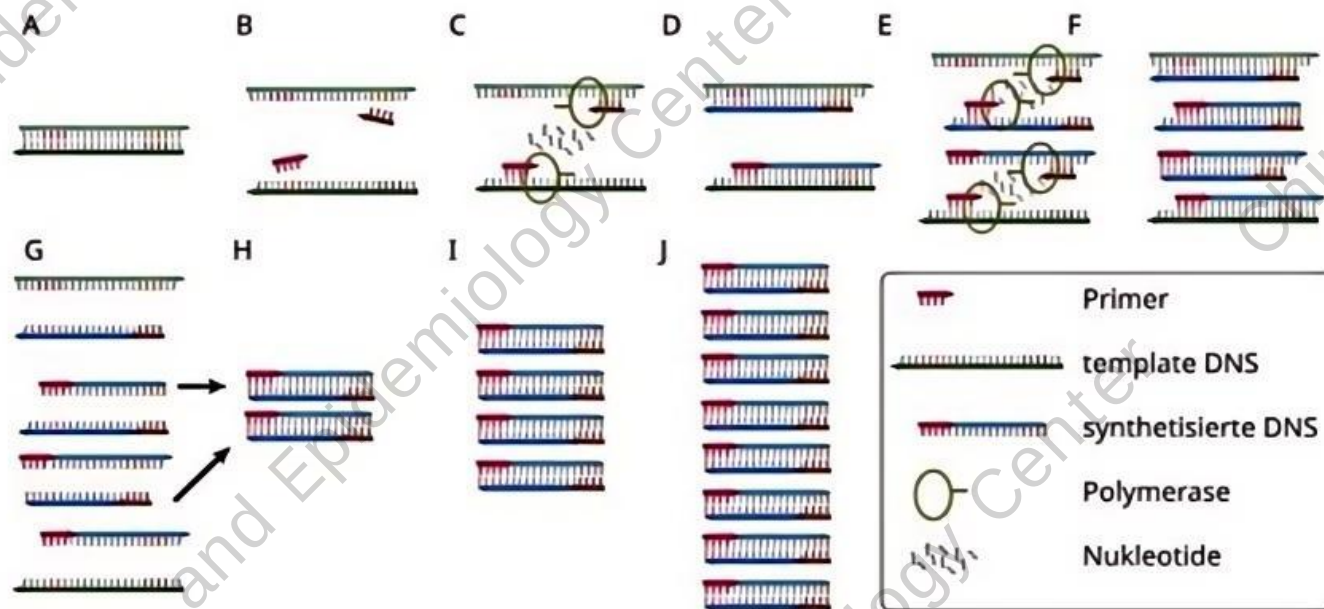
- Denaturation: $\sim 95^{\circ}\text{C}$
- Annealing: $\sim 55^{\circ}\text{C}$
- Extension: Taq DNA polymerase 30–40 cycles
million-fold amplification

Detection:

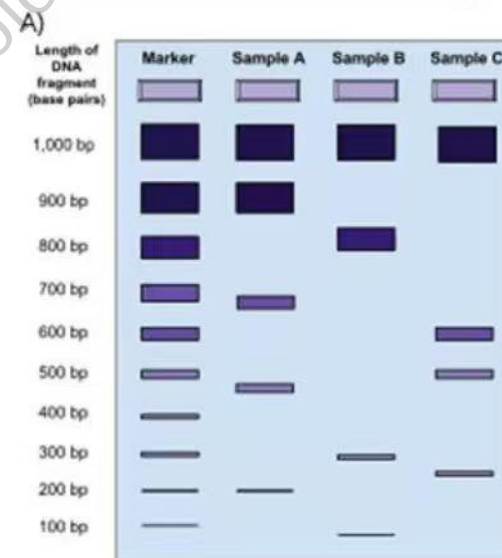
- agarose gel electrophoresis
- Band at expected position = positive
- No band = negative

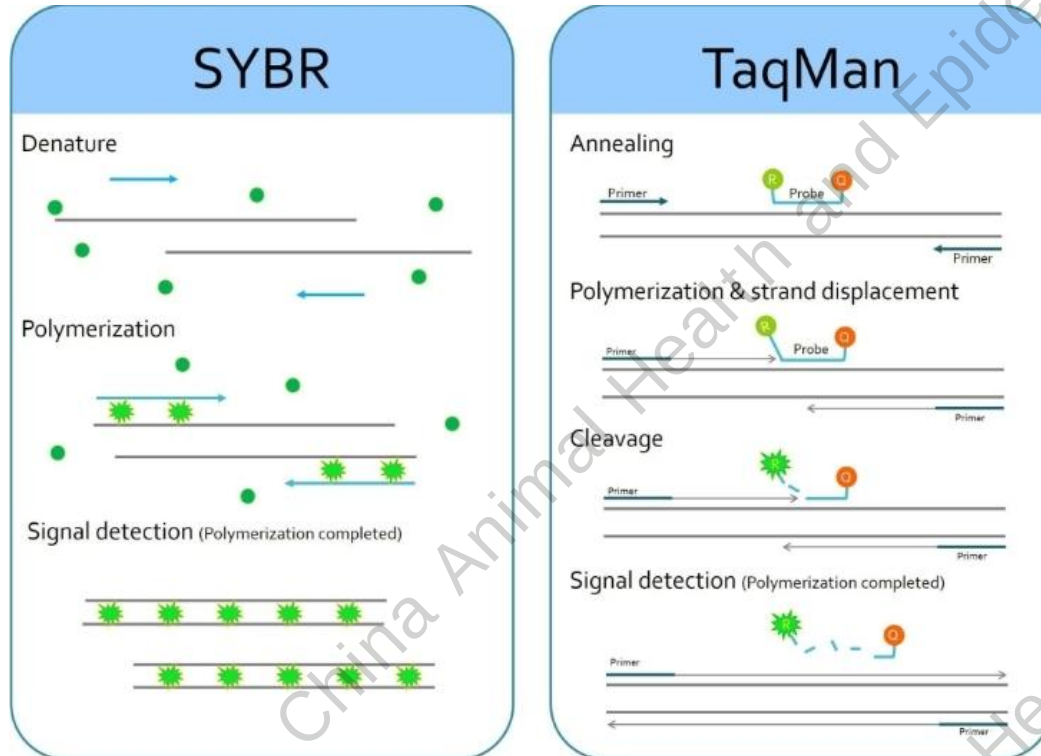
Limitations:

- Qualitative only
- Gel-based, labor-intensive
- Low sensitivity for low-load samples



(A) Double-stranded DNA template.
(B) Thermal denaturation separates the template into single strands. (C) Sequence-specific primers anneal to complementary regions, and DNA polymerase binds to the primer-template complex.
(D) During extension, DNA polymerase synthesizes new DNA strands from the primer 3' ends using nucleotides.
(E–F) The three steps—denaturation, annealing, and extension—are repeated in successive cycles
(G–J) Exponential amplification generates increasing amounts of the target amplicon.





Left: **SYBR Green** binds newly synthesized dsDNA and emits fluorescence.

Right: **TaqMan probe** is cleaved by Taq polymerase, separating the reporter from the quencher and generating fluorescence.

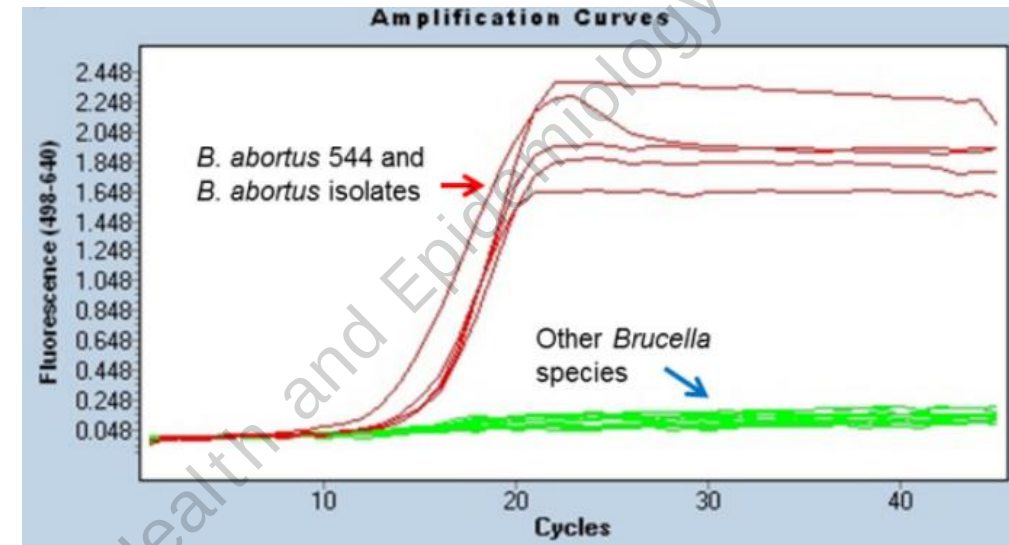
Legend: green star, reporter; orange circle, quencher.

Second-generation PCR: real-time quantitative PCR

Fluorescence monitored during amplification

Common chemistries: **SYBR Green**, **TaqMan probes**

- Ct value = cycle threshold
- More template → lower Ct
- Less template → higher Ct



Amplification curves in *B. abortus* 544 reference strain and Korean *B. abortus* isolates.



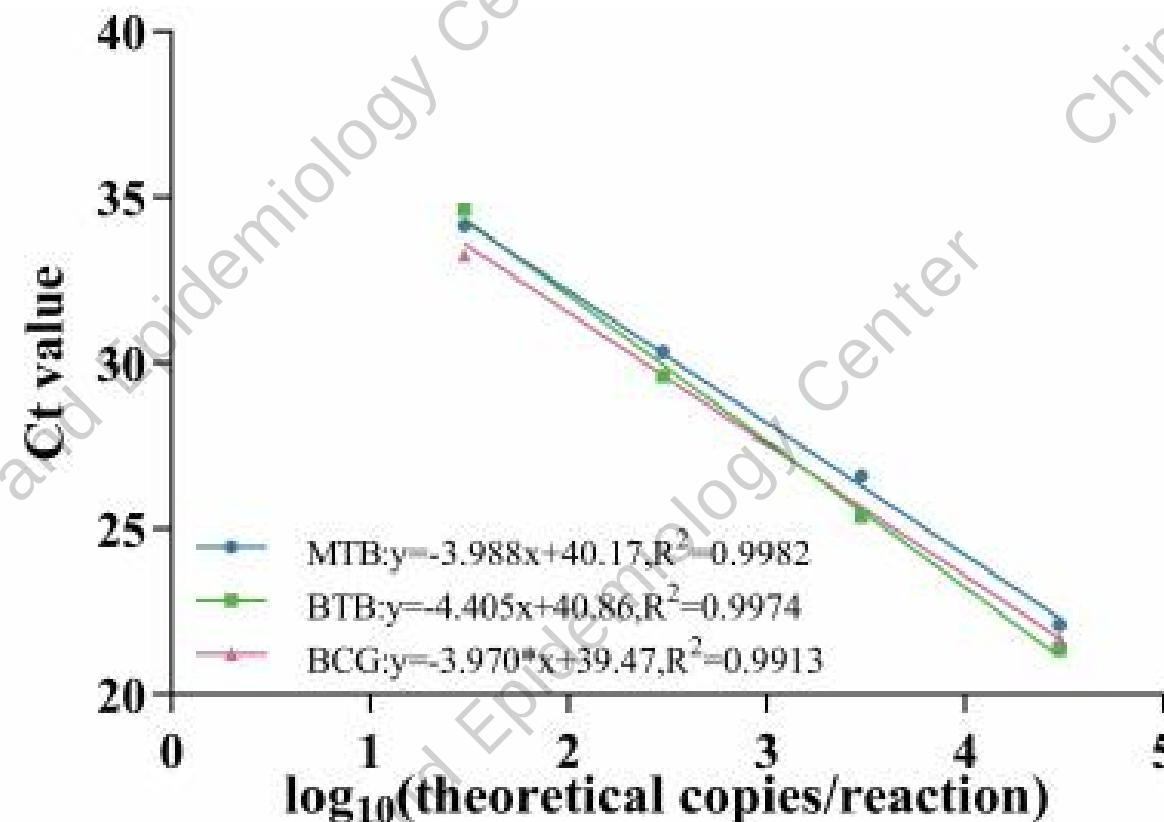
Absolute quantification requires a standard curve

Advantages:

- Quantitative
- Higher sensitivity
- No gel electrophoresis

Limitations:

- Standard curve dependent
- Poor reproducibility at low concentration
- Sensitive to PCR inhibitors



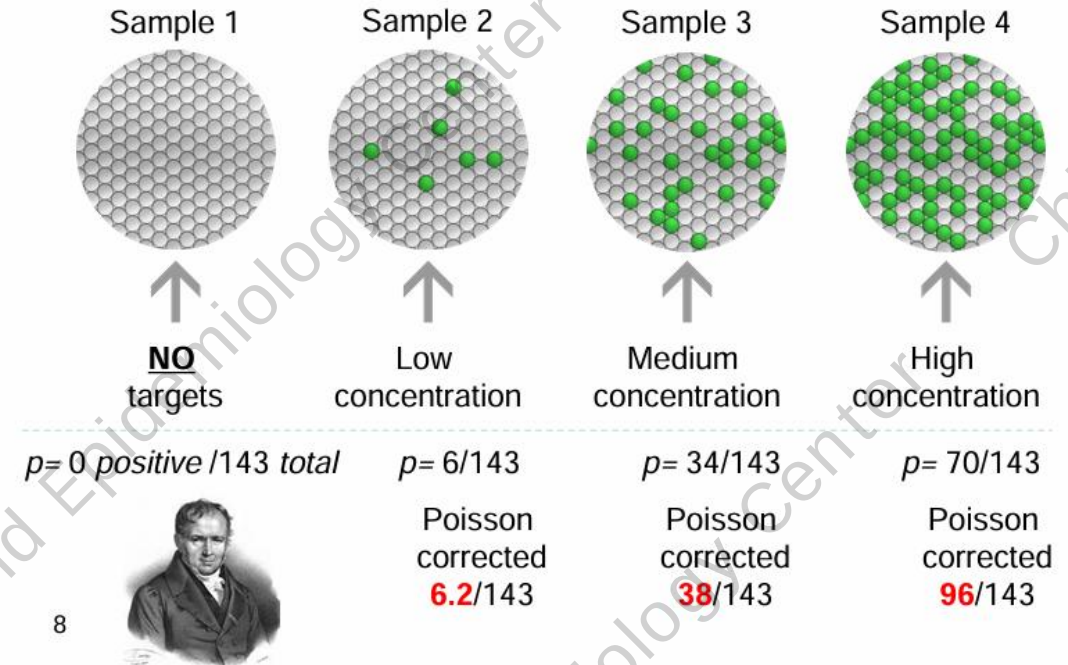
Triplex qPCR standard curve of *M. tuberculosis*, *M. bovis* and **BCG**

Key concepts:

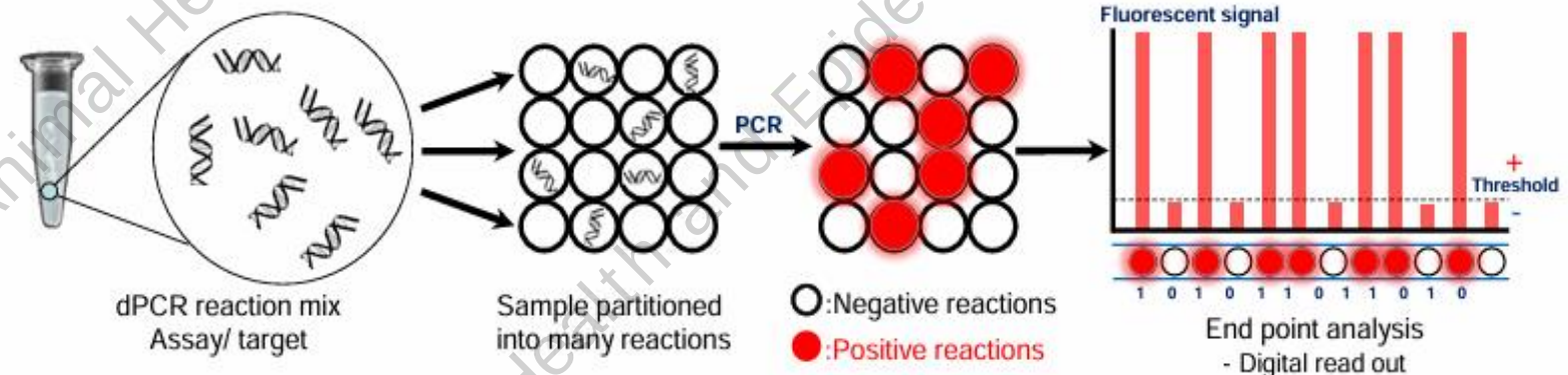
- Dropletization
- Endpoint amplification
- Poisson distribution
- Reaction divided into tens of thousands of nanoliter droplets
- Each droplet: 0 or 1 target molecule
- Positive droplet = 1; negative droplet = 0
- Absolute quantification without standard curve
- Unit: copies/ μ L

Advantages:

- Single-copy sensitivity
- Absolute quantification
- Strong inhibitor tolerance
- High reproducibility



Principle of ddPCR absolute quantification



Characteristics and Advantages of Droplet Digital PCR

Paper: **Development and evaluation of a triplex droplet digital PCR method for differentiation of *M. tuberculosis*, *M. bovis* and BCG**

Frontiers in Microbiology, 2024

Differentiates:

M. tuberculosis, *M. bovis*, BCG

Targets:

- *M. tuberculosis*: RD1 + RD4
- *M. bovis*: RD1
- BCG: Δ RD1

The optimal probe concentrations (FIGURE 1)

- RD1: 150 nM
- RD4: 500 nM
- Δ RD1: 250 nM

TABLE 1 Primers and probes sequences were chosen for three targets

Target gene	RD region	Fragment length	Design sequence 5'–3'
CFP-10 to ESAT-6	RD1	623	F: CCTCGCAAATGGGCTTCT
			R: GACGTGACATTCCCTGGATT
			P: FAM-AGTGGAAATTCGCGGGTATCGAGG-DHQ1
Rv0222	RD4	789	F: TATGCGATAGCCATGGAGTTG
			R: CCATTGGCGGTGATCTTCT
			P: VIC-TCGATGCTGCGATCGCGTTG-DHQ1
Rv3871 and Rv3879c	Δ RD1	996	F: GGATTGACGTGCTGCTTCT
			R: CGATCTGGCGGTTTGGG
			P: CY5-ATCCAGCATCTGTCTGGCATAGCT-DHQ2

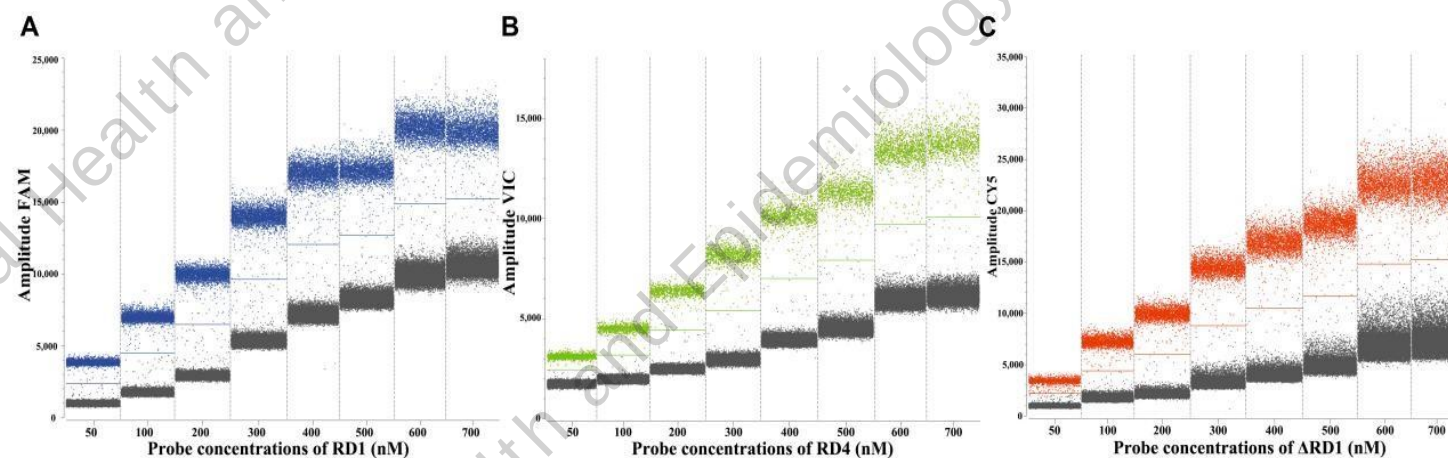


FIGURE 1: Determination of the optimal probe concentrations of the ddPCR of RD1 (A), RD4 (B), Δ RD1 (C).

The optimal ddPCR annealing temperature (FIGURE 2): 60°C

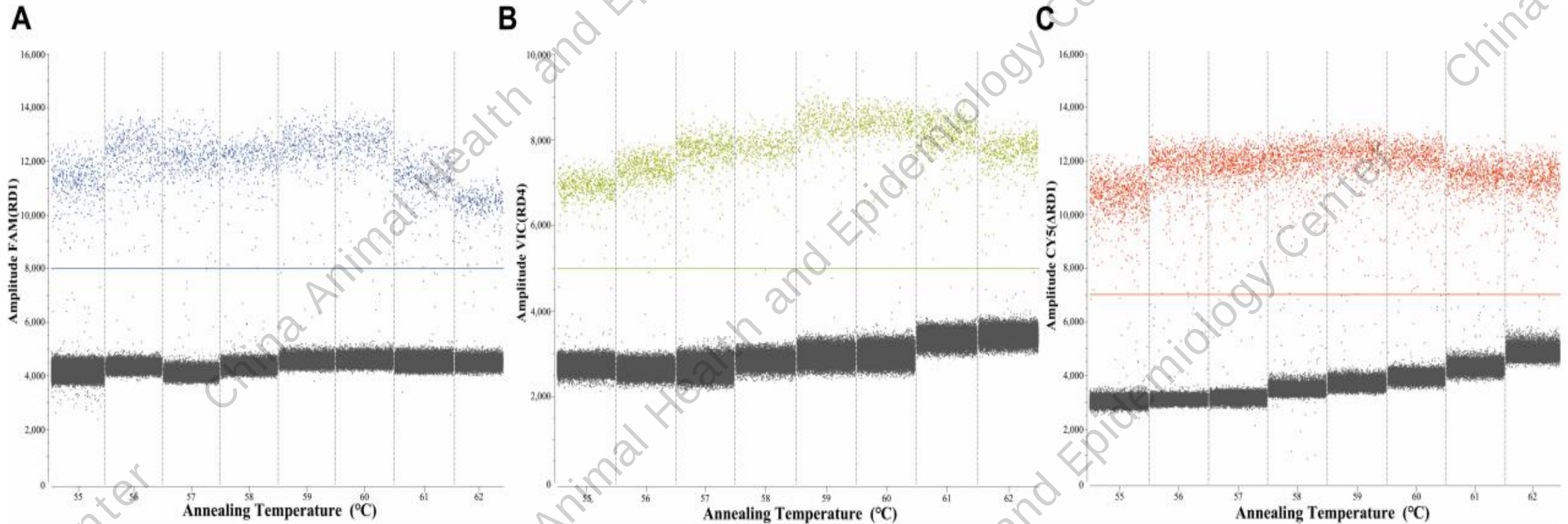


FIGURE 2: Screening the optimum annealing temperature from 55 to 62°C of RD1 (A), RD4 (B), 1 RD1 (C)

Three fluorescence channels (FIGURE 3)

(A–C) Detection results of the target *M. tuberculosis*.

(D–F) Detection results of the target *M. bovis*.

(G–I) Detection results of the target BCG

LOD:

- *M. tuberculosis*: 3.08 copies/reaction
- *M. bovis*: 4.47 copies/reaction
- BCG: 3.59 copies/reaction

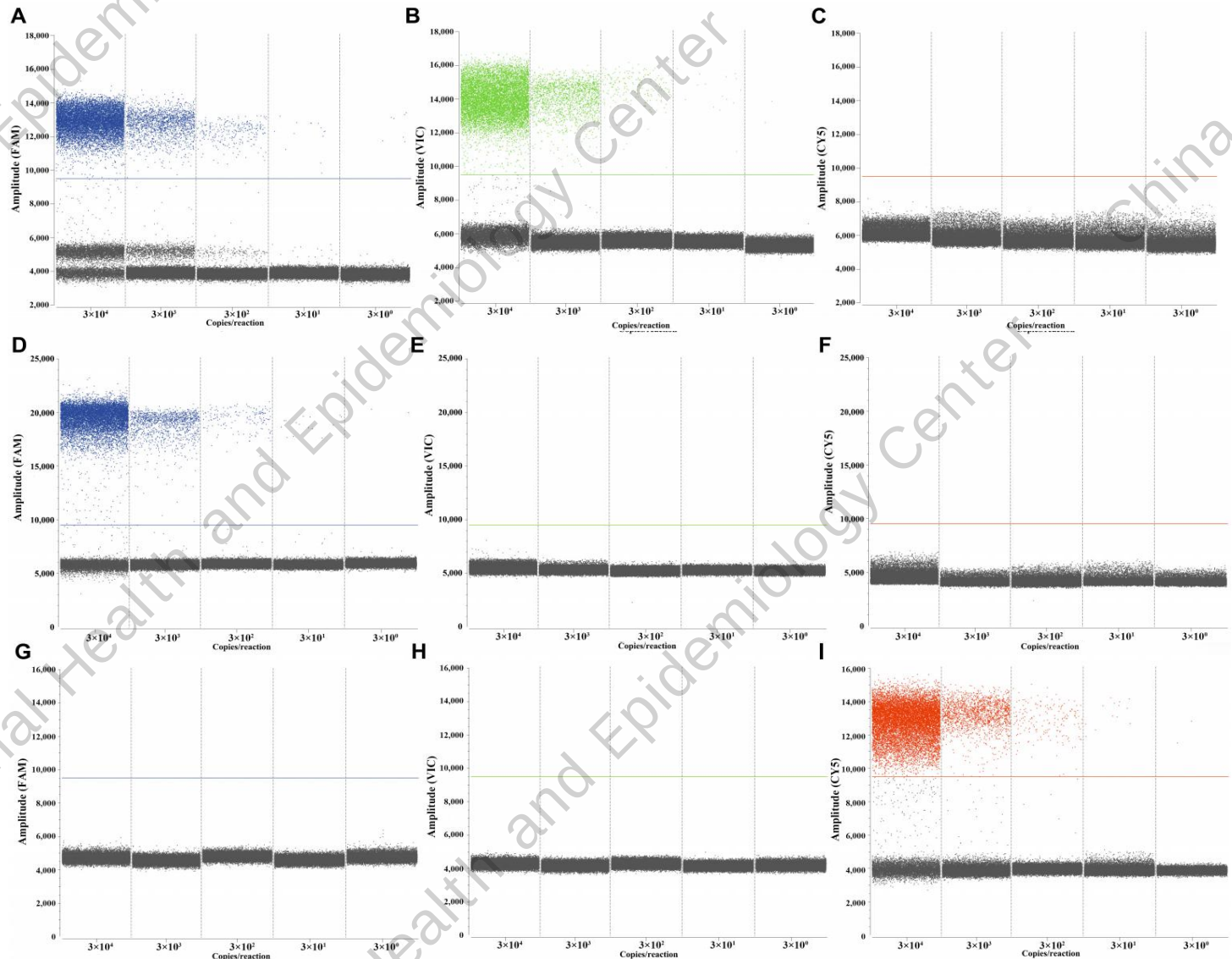


FIGURE 3: Performances of the triplex ddPCR assay by target DNA from 3×10^4 to 3×10^0 copies/ μ L.

Specificity: No cross-reaction with common non-target bacteria

repeatability: CV < 10% (TABLE 2)

TABLE 2 Analysis of the repeatability of the triplex ddPCR.

DNA	Final concentration (copies/ μ L)	Repeatability assay		
		SD	AVG	CV%
<i>M. tuberculosis</i>	3×10^4	283.18	14646.28	1.93%
	3×10^3	74.68	1574.18	4.74%
	3×10^2	6.17	175.95	3.50%
<i>M. bovis</i>	3×10^4	178.50	19320.18	0.90%
	3×10^3	29.31	1338.84	2.19%
	3×10^2	10.76	117.62	9.15%
BCG	3×10^4	769.98	24532.97	3.14%
	3×10^3	98.09	2365.36	4.15%
	3×10^2	22.10	238.48	9.27%

The 60 clinical DNA samples were tested using the triplex ddPCR and the triplex qPCR

Milk LOD: 5×10^3 CFU/mL; 10 $\times$ more sensitive than triplex qPCR

Clinical samples: including milk, tissue, and swabs

- ddPCR positive rate: 11.67%
- qPCR positive rate: 8.33%

Agreement: 100%, 96.7%, 100%

TABLE 3 Clinical results for triplex ddPCR

Detection method	Detection results (positive samples/total samples)		
	<i>M. tuberculosis</i>	<i>M. bovis</i>	BCG
ddPCR	1/60	6/60	0/60
qPCR	1/60	4/60	0/60
Coincidence rates	100%	96.7%	100%



ddPCR: Concept Map of Advantages and Limitations

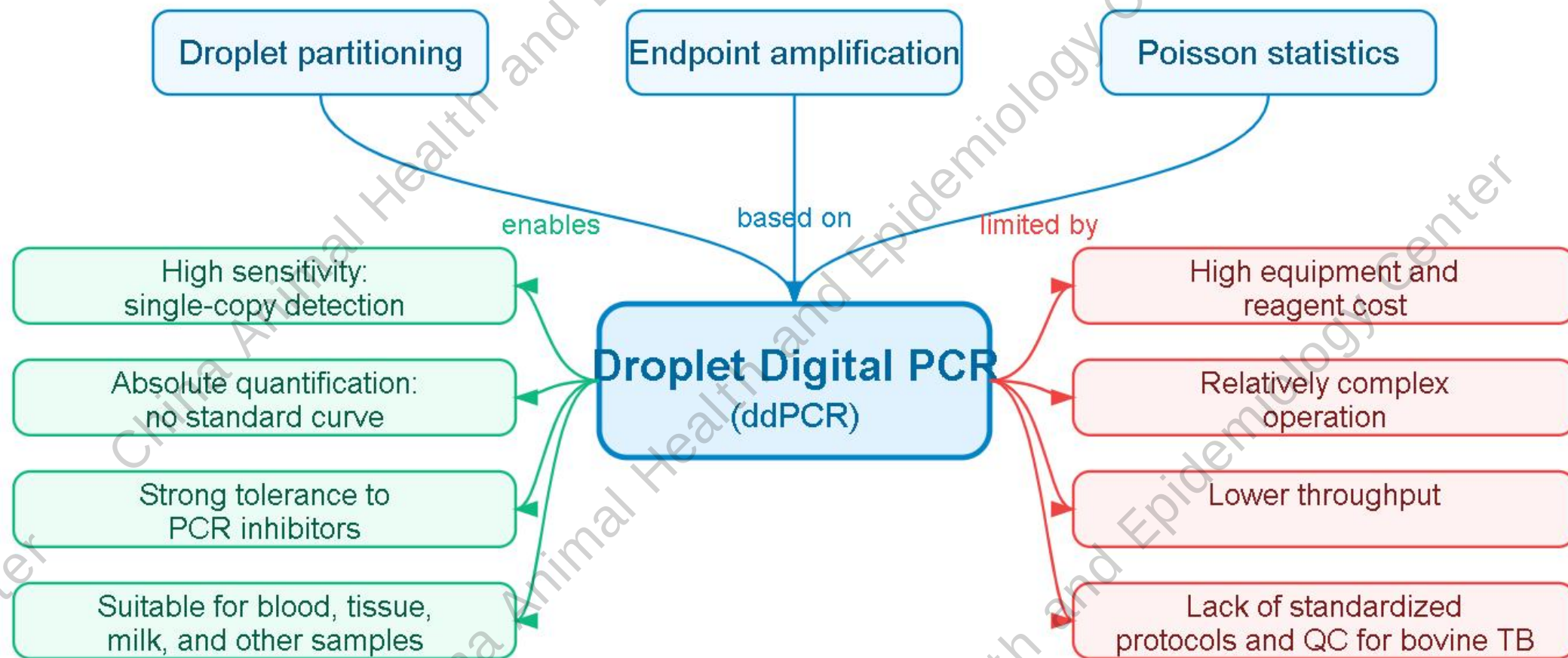


Figure 4 Concept map of ddPCR advantages and limitations. Advantages arise from the core ddPCR principles; limitations are mainly practical and infrastructural.

"Two Diseases": bovine tuberculosis and brucellosis

Precise diagnosis is central to eradication and disease-free zone construction

Application 1: TB nucleic acid reference materials

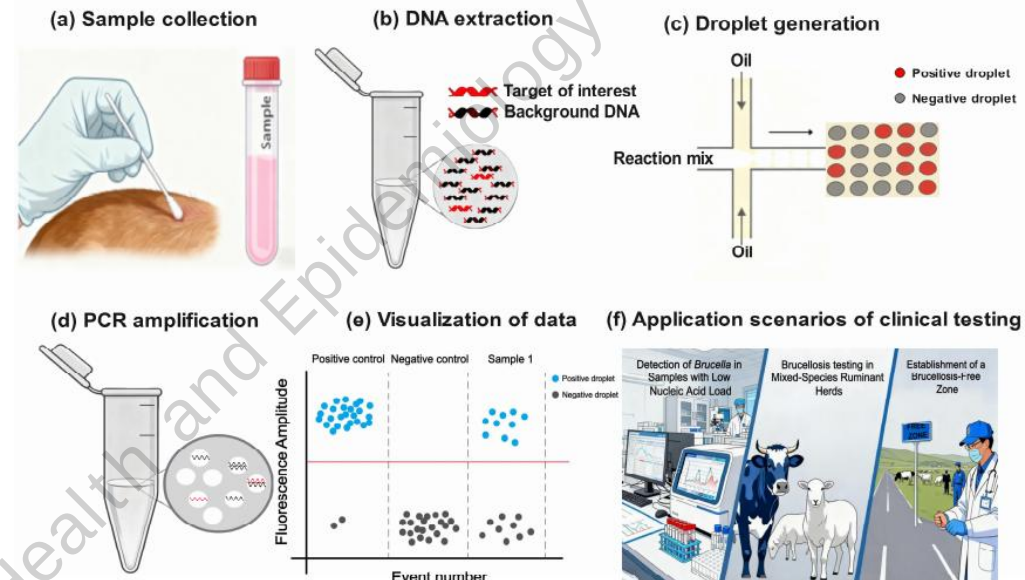
- Laboratory has obtained multiple TB reference materials
- Supports diagnostic standardization, traceability, and quality control

Application 2: Eradication and disease-free zones

- Precise detection and removal of positive animals
- Samples: vaginal swabs and milk
- Milk is excellent for detecting active TB
- Collection is easy and non-invasive
- ddPCR is suitable for low-load, complex-matrix samples

Remaining needs:

Cost control; Standardization



ddPCR is a powerful third-generation PCR tool

Key strengths:

- High sensitivity
- Absolute quantification
- Inhibitor tolerance
- Valuable for bovine TB diagnosis
- Supports China's "Two Diseases" control and disease-free zone development

Cooperation welcome in the Asia-Pacific region



Thank you!

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