Supplementary information

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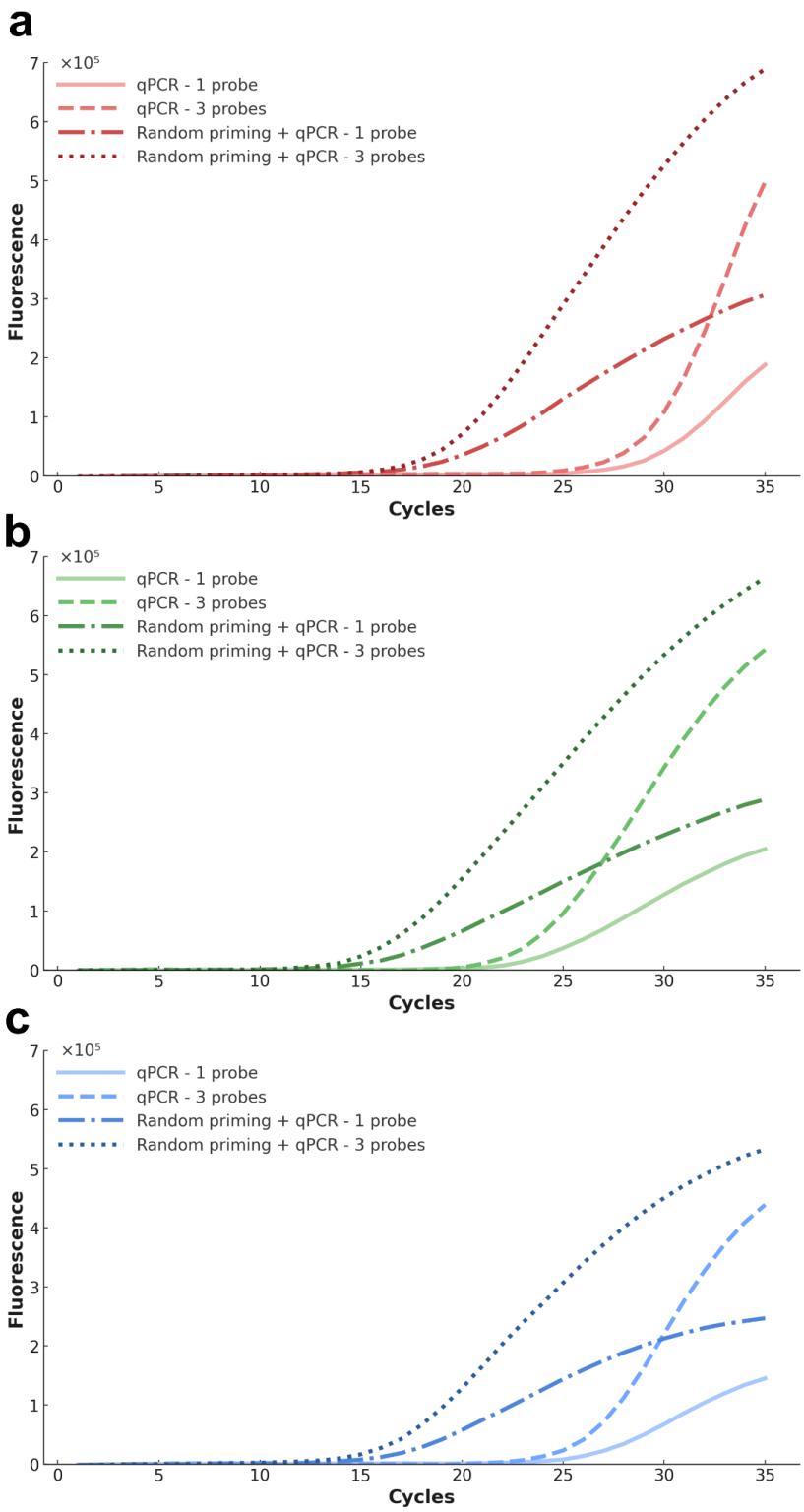
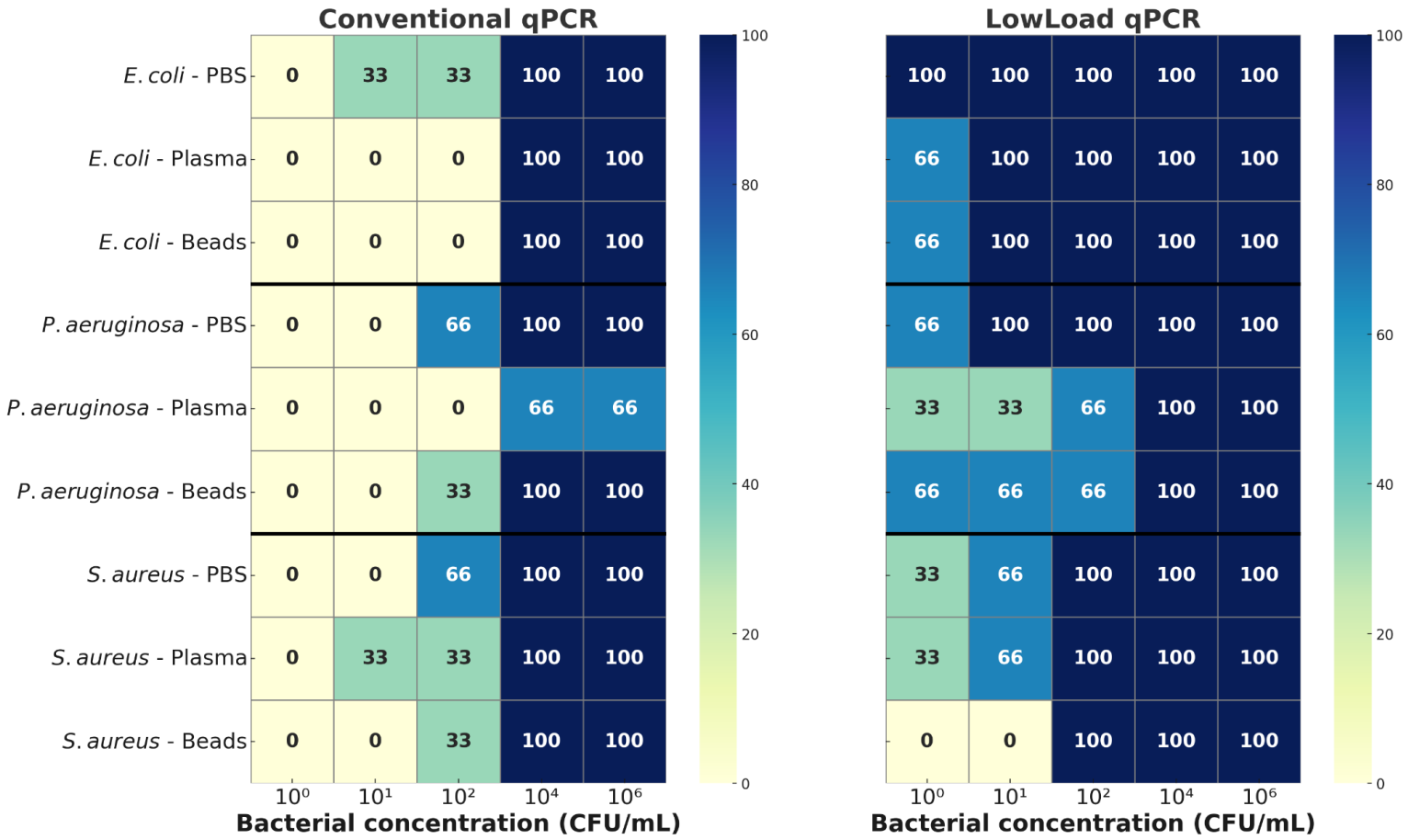
Supplementary Note 1 | Enhanced qPCR signal through random priming amplification and probe multiplexing

To evaluate strategies for enhancing qPCR fluorescence signal intensity under low bacterial load conditions, we compared four amplification workflows across *E. coli*, *P. aeruginosa* and *S. aureus*: conventional qPCR with a single hydrolysis probe, qPCR with three probes sharing the same fluorophore targeting the same gene, random priming followed by qPCR with one probe, and random priming followed by qPCR with three probes. The use of three probes substantially increased fluorescence signal intensity and consistently lowered Ct values compared to single-probe qPCR, reflecting improved detection sensitivity. Incorporating a random priming step prior to qPCR resulted in an even greater reduction in Ct values across all species, suggesting enhanced template accessibility and amplification efficiency. When combined, random priming and probe multiplexing yielded the highest signal intensities and earliest amplification onset, highlighting a synergistic effect that improves qPCR performance under limiting conditions (**Supplementary Fig. 1**).

To assess how these improvements impact detection performance, we conducted a broader comparison using clinical-like samples spiked with decreasing bacterial concentrations. Detection rates were measured for each species and sample matrix (PBS, plasma, beads), using both conventional qPCR and LowLoad-qPCR. As shown in the heatmap (**Supplementary Fig. 2**), LowLoad-qPCR consistently achieved higher detection rates across all concentrations. This improvement was especially notable at lower bacterial loads ($\leq 10^2$ CFU/mL), where conventional qPCR frequently failed. These results confirm that both probe multiplicity and random priming enhance sensitivity and robustness, particularly in complex or low-input samples.



Supplementary Fig. 1 | Fluorescence amplification curves of qPCR and random priming–qPCR with one and three probes across three bacterial species. Real-time qPCR fluorescence curves from (a) *E. coli*, (b) *P. aeruginosa,* and (c) *S. aureus* using four amplification conditions. Curves represent ΔRn fluorescence over 35 amplification cycles. All data are baseline-corrected (cycles 1–10). Y-axes are scaled equally (0–7 $\times 10^5$) to facilitate comparison across conditions and species.



Supplementary Fig. 2 | Detection performance across bacterial loads using conventional qPCR or LowLoad-qPCR. Heatmaps display the percentage of positive replicates (n = 3 per condition) for *E. coli*, *P. aeruginosa,* and *S. aureus* spiked into PBS, plasma or bead-treated blood, and tested at five concentrations (10^6, 10^4, 10^2, 10^1, and 10^0 CFU/mL). LowLoad-qPCR consistently improved detection, particularly in low-burden and complex matrices. Black lines separate bacterial species.

Supplementary Note 2 | Design and selection of the different genes for the detection of the three species

The selection of gene targets for species-specific detection was based on their high specificity, stable genomic presence, and widespread use in molecular diagnostics. Each target—*uidA* for *E. coli*, *gyrB* for *P. aeruginosa*, and *nuc* for *S. aureus*—was amplified using species-specific primers combined with **t**hree hydrolysis probes per gene, all labelled with the same fluorophore (FAM) to allow multiplex detection within a single channel.

Probe sequences were either newly designed or adapted from validated qPCR protocols (see references in **Supplementary Table 1**), and were selected to bind distinct, non-overlapping regions of the amplicon. This design aimed to improve detection sensitivity under limiting DNA conditions, while maintaining signal specificity.

All oligonucleotides were synthesized by Microsynth AG (Balgach, Switzerland) and prepared at a final working concentration of 10 µM. Primer and probe sequences are listed in **Supplementary Table 1**.

Supplementary Table 1 | Primers and probes used for qPCR detection of each bacterial species

Escherichia coli		
Gene	*uidA*	
Forward primer	5'- AGC GAA GAG GCA GTC AA-3'	This study
Reverse primer	5'-TGA GCG TCG CAG AAC ATT ACA-3'	(13)
Probe 1	5'-CGC GTC CGA TCA CCT GCG TC-3'	(13)
Probe 2	5'-ACC CGT CCG CAA GGT GCA CG-3'	This study
Probe 3	5'-CAG CAG GCG CAC TTA CAG GC-3'	This study
Pseudomonas aeruginosa		
Gene	*gyrB*	
Forward primer	5'-TGA CCA TCC GTC GCC ACA AC-3'	This study
Reverse primer	5'-ACG CCG GAG TTG AGG AAG GA-3'	This study
Probe 1	5'-TTC CGC AGT TCC CAC TGC G-3'	This study
Probe 2	5'-CGG CTT GAA GTG AAC TTC GGT-3'	This study
Probe 3	5'-CCA CTT CAG TTG GGA CAT CCT G-3'	This study
Staphylococcus aureus		
Gene	*nuc*	
Forward primer	5'-AGT GCT GGC ATA TGT ATG G-3'	This study
Reverse primer	5'-AGT CGC AGG TTC TTT ATG TA-3'	This study
Probe 1	5'-CGA AAG GGC AAT ACG CAA AGA GG-3'	This study
Probe 2	5'-CGG CGT AAA TAG AAG TGG TTC TGA-3'	This study
Probe 3	5'-AGT CTA AGT AGC TCA GCA AAT GC-3'	This study