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RESEARCH PAPER

Establishment and characterization of a genomic DNA reference material for Group B Streptococcus detection

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Abstract

Group B Streptococcus (GBS) is a leading cause of maternal and neonatal morbidity and mortality. While molecular assays, like nucleic acid amplification tests, are widely used for GBS detection, their accuracy depends on reliable reference standards. We developed a DNA reference material (RM) for GBS from genomic DNA extracted from reference strain ATCC® 13813™. This RM was quantified using droplet digital PCR and exhibited excellent homogeneity and stability. The certified value of the RM, determined through a multi-center collaborative study, was established as $(7.53 \pm 0.48) \times 10^3$ copies/μL, where the uncertainty represents the expanded uncertainty. We further applied the RM to assess the analytical performance of three commercial GBS detection kits. None of the kits consistently achieved their claimed limits of detection, a discrepancy that could lead to false-negative results and subsequent misdiagnosis or inappropriate treatment. Clinical validation confirmed the stability and suitability of the GBS DNA RM for clinical application. The establishment of this GBS DNA RM provides a reliable tool for validating commercial diagnostic kits and platforms, thereby improving the accuracy and reliability of GBS nucleic acid detection.

Keywords GBS · Genomic DNA · Reference material · Digital PCR · Nucleic acid detection

Introduction

Group B Streptococcus (GBS), or *Streptococcus agalactiae*, is a β-hemolytic, Gram-positive bacterium that often colonizes the gastrointestinal and genitourinary tracts asymptomatically [1, 2]. It remains a leading cause of high-risk populations, especially pregnant women and neonates [3, 4]. Maternal colonization elevates the risk of preterm birth, stillbirth, and postpartum sepsis, with approximately half of colonized mothers transmitting GBS to their newborns [5]. In the absence of intrapartum prophylaxis, 1–2% of exposed infants develop early-onset disease, which can present as pneumonia, sepsis, or meningitis [6]. Globally, GBS is responsible for 409,000 severe maternal, fetal, and neonatal infections annually, leading to approximately 147,000 stillbirths and neonatal deaths; survivors often experience long-term sequelae such as neurodevelopmental impairment or sensory loss [7, 8].

Universal screening combined with intrapartum antibiotic prophylaxis represents the standard strategy for preventing vertical GBS transmission and has significantly reduced the incidence of early-onset disease [9]. The current gold standard for prenatal GBS screening involves vaginal–rectal swab culture at 35–37 weeks of gestation [10]. However, culture-based methods require up to 3 days of enrichment, delaying result reporting [11]. Its accuracy can also be affected by competitive inhibition from other microorganisms, undetected non-hemolytic GBS strains that yield false-negative results, and cross-reactivity with *Streptococcus porcinus* causing false positives [12]. Although antigen-based assays and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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have been explored as alternatives, their clinical application for GBS detection remains limited—antigen tests often exhibited suboptimal sensitivity and cross-reactivity with other *streptococci*, while mass spectrometry faces challenges in differentiating polymicrobial specimens and depends on expensive instrumentation [13].

Nucleic acid amplification tests, particularly quantitative real-time polymerase chain reaction (qPCR), provide rapid results with high sensitivity and specificity [14]. These characteristics make qPCR especially useful when prenatal screening results are unavailable or ambiguous, and for neonates requiring prompt diagnosis [15]. Growing evidence supports the use of qPCR for both prenatal screening and intrapartum testing [16]. Nevertheless, qPCR-based GBS detection often exhibits considerable inter-laboratory variability due to differences in protocols, reagents, and operator skill [17, 18]. Standardized GBS DNA reference material (RM) is therefore essential to improve consistency and ensure comparability across laboratories.

To date, no GBS DNA RM has been reported in China. In this study, we developed a GBS DNA RM using genomic DNA (gDNA) extracted from the reference strain ATCC® 13813™, which was also employed in our earlier external quality assessment (EQA) study [17]. The RM was quantified via droplet digital PCR (dPCR), a method that enables absolute nucleic acid quantification with high precision [19, 20]. This assigned value allows the GBS DNA RM to be applied for validating current detection methods and commercial kits, thereby enhancing the diagnostic accuracy and reliability of GBS nucleic acid testing.

Materials and methods

GBS strain culture and DNA extraction

A *Streptococcus agalactiae* strain (ATCC® 13813™) was inoculated into sterile brain-heart infusion medium (3.85%) and at 37 °C with shaking at 220 rpm for 36 h. After centrifugation of a 20 mL culture aliquot at 3220 × g for 5 min, the pellet was washed twice with 1 × PBS to remove residual medium. The pellet was subsequently resuspended in 1.8 mL lysozyme solution (20 mg/mL) and 200 µL proteinase K solution, followed by incubation at 50 °C for 40 min. gDNA was extracted using the Tianlong Bacterial Genomic DNA Extraction Kit (Tianlong Biotechnology, Xi'an, China) according to the manufacturer's instructions. The quality of the gDNA was assessed with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, USA) to ensure that the GBS DNA met the requirements for subsequent RM preparation.

Table 1 Sequences of primers and probes for dPCR

Gene	Type	Sequence (5′–3′)
cfb	Fw 1	GTGGCTGGTGCATTGTTATTT
	Rv 1	CCATTTGCTGGGCTTGATTATT
	Probe 1	FAM-TGCTGATCAAGTGACAACTCCACA AGT-BHQ1
	Fw 2	GGTTAATGAGGCTATTACTAGCGTTGA
	Rv 2	ATCACATCTGTTAAGGCTTCTACACG
	Probe 2	FAM-TGCGTGCCAACCCTGAGACAGTTT ATG-BHQ1
	Fw 3	AAAATTAAAGACTTCATTGCG
	Rv 3	GTTAAGGCTTCTACACGAC
	Probe 3	FAM-ATAAACTGTCTCAGGGTTGGC-BHQ1

Fw forward primer, *Rv* reverse primer

Primer-probe design and synthesis

The *cfb* gene, which encodes the CAMP factor, was selected for GBS DNA quantification due to its unique and highly conserved nature within *Streptococcus agalactiae* [21–24]. Thus, the *cfb* locus is widely used in molecular diagnostic assays for GBS detection and is recommended by the US Centers for Disease Control and Prevention as a standard target for qPCR-based identification [25, 26]. To verify the target region sequence, conventional PCR primers were initially designed according to the reference sequence (NZ_CP012480) and synthesized by Proprobes Biotechnology Co., Ltd. (Kunshan, China). The forward primer was 5′-GTG GCTGGTGCATTGTTATTT-3′, and the reverse primer was 5′-CCATTTGCTGGGCTTGATTATT-3′. Primers and probes for dPCR targeting the same *cfb* region were then designed using Oligo 7 (Molecular Biology Insights, Inc., CO, USA) and Primer Premier 5 (Premier Biosoft International, CA, USA). Three primer-probe sets were synthesized by Proprobes Biotechnology Co., Ltd., to evaluate analytical performance, and the optimal set was selected. The sequences are listed in Table 1.

GBS DNA quality assessment and RM preparation

PCR amplification was performed in a 20 µL reaction mixture containing 10 µL of 2 × Q5® Hot Start High-Fidelity Master Mix (Applied Biosystems, Foster, USA), 2 µL of each primer (10 µM), 6 µL of nuclease-free water, and 2 µL of GBS DNA. The thermal cycling protocols began with an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 50 s, and conducted with a final extension at 72 °C for 10 min. The PCR products were analyzed via agarose gel electrophoresis; the

corresponding bands were then purified and sent for Sanger sequencing to Shanghai Baoteng Medical Laboratory Co., Ltd. (Shanghai, China) to confirm successful amplification of the *cfb* gene. The resulting GBS DNA RM was adjusted to the appropriate concentration using 10 mM Tris-buffer pH 7.4 containing 1 mM EDTA and 5 mg/mL trehalose, aliquoted into sterile 2 mL microtubes (100 µL per tube), and stored at −20 °C until use.

dPCR method establishment

The dPCR assay was established using the ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Each 20 µL reaction mixture contained 1.8 µL of forward and reverse primer (final concentration 900 nM), 0.5 µL of probe (final concentration 250 nM), 10 µL of ddPCR™ Supermix, 2 µL of GBS DNA RM, and nuclease-free water to volume. The primers and probe concentrations followed the manufacturer's optimized protocol, eliminating the need for further condition adjustment. Reactions were performed on a QX ONE system (Bio-Rad Laboratories, Inc, CA, USA) under the following cycling conditions: an initial hold at 25 °C for 3 min, enzyme activation at 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 30 s and annealing/extension at 60 °C for 1 min, enzyme deactivation at 98 °C for 10 min, and a final hold at 25 °C for 1 min. A ramp rate of 2 °C/s was used to maintain droplet stability during amplification. This configuration provided consistent partitioning and amplification efficiency, allowing the study to focus on quantifying and validating the GBS DNA RM without additional assay optimization.

Homogeneity and stability assessment

The homogeneity and stability of the GBS DNA RM were evaluated in accordance with ISO 33405: 2024. To the homogeneity assessment, 15 randomly selected tubes of the RM were quantified by dPCR, with each tube analyzed in triplicate. Homogeneity was evaluated using the Shapiro-Wilk test for normality and the *F*-test for variance analysis. Short-term stability was evaluated by quantifying two tubes in duplicate after storage at 4 °C for 1, 2, 4, and 7 days; at 25 °C for 4, 8, 24, and 48 h; and at 37 °C for 2, 4, 8, and 24 h. Long-term stability was evaluated by storing the RM at −20 °C and testing two tubes in duplicate after 1, 2, 4, and 6 months. A linear model was estimated using Ordinary Least Squares regression, expressed as follows:

$$Y = \beta_0 + \beta_1 X \tag{1}$$

Stability was analyzed using linear regression analysis and *t*-test. To evaluate freeze-thaw stability, three randomly selected vials of the prepared RM underwent repeated freezing and thawing cycles. Each vial was thawed at ambient temperature (approximately 25 °C) and then refrozen at −20 °C, constituting one complete cycle. This procedure was repeated once to twice daily, with 10 µL aliquots withdrawn after the first, third, and fifth cycles for subsequent analysis. Three additional vials stored continuously at −20 °C served as controls and were analyzed once. The data were statistically evaluated using an independent samples *t*-test with IBM SPSS Statistics 27.

Assessment of assigned value

Following ISO 33405: 2024, the assigned value of the GBS DNA RM was determined from 27 units distributed across nine laboratories (#1 Shanghai Center for Clinical Laboratory (Shanghai, China), #2 Shanghai Institute of Measurement and Testing Technology Co., Ltd. (Shanghai, China), #3 Guangzhou BDS Biological Technology Co., Ltd. (Guangzhou, China), #4 Ningbo Health Gene Technologies Co., Ltd. (Ningbo, China), #5 Suzhou Sniper Medical Technology Co., Ltd. (Suzhou, China), #6 Shanghai Little Turtle Biomedical Technology Co., Ltd. (Shanghai, China), #7 Suzhou LDT Bioscience Co., Ltd. (Suzhou, China), #8 TargetingOne Technology (Beijing) Corporation (Beijing, China), #9 Zhenzhun Bio-Tech (Shanghai) Co., Ltd. (Shanghai, China)), with three units per laboratory [27]. Each unit was measured in duplicate. Outliers were identified and removed using Dixon's *Q* test and Grubbs' test. The arithmetic mean of the valid results was taken as the final assigned value.

Uncertainty assessment

The uncertainty of the assigned value mainly incorporated contributions from Type A components of characterization: homogeneity (u_1), long-term stability (u_2), and value assignment (u_3). The combined standard uncertainty (u_c) was calculated using Eq. (2), and the expanded uncertainty (U) was derived at a 95% confidence level with a coverage factor of $k = 2$, as shown in Eq. (3).

$$u_c^2 = u_1^2 + u_2^2 + u_3^2 \tag{2}$$

$$U = k \times u_c \tag{3}$$

Commercial qPCR kit validation using the GBS DNA RM

To evaluate the practical utility of the GBS DNA RM, three commercial qPCR kits (Assay I, Assay II, and Assay III) were selected based on market share, manufacturer

reputation, and technical support availability. All qPCR analyses were performed on the QuantStudio 5 Real-Time PCR platform (Thermo Fisher Scientific, CA, USA). The limit of detection (LOD) acceptance criterion required a detection rate of ≥95% (i.e., at least 19 positive results out of 20 replicates) at the claimed concentration. For LOD verification, the GBS DNA RM was serially diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) to span the concentration range specified by each manufacturer. Each dilution was prepared fresh under sterile conditions to ensure accuracy and minimize the risk of contamination. These diluted RM samples were tested in 20 replicates per concentration to evaluate assay performance. Detection results were recorded as positive ("detected") or negative ("undetected") to determine whether the RM could reliably evaluate performance across different commercial assays. This approach provided a standardized and transparent means of assessing assay performance, consistent with common clinical diagnostic practices.

Clinical laboratory validation

Three accredited clinical laboratories in Shanghai—the Department of Laboratory Medicine of Shanghai First Maternity and Infant Hospital, the Department of Laboratory Medicine of Shanghai Tongren Hospital, and the Department of Laboratory Medicine of Wusong Central Hospital of Baoshan District—perform the clinical evaluation experiments. Prepared vials of GBS DNA RM (100 µL each) were shipped to these sites under refrigerated conditions (2–8 °C). Each laboratory received three vials and a trial instruction report. Qualified personnel performed quantitative analysis using their routine commercial qPCR kits. Testing was completed in three independent batches, with three replicate measurements per batch. All procedures were carried out strictly following the manufacturer's instructions.

Results

GBS DNA quality assessment

The extracted GBS DNA was of high quality, exhibiting a concentration of 300.15 ng/µL, an A260/A280 ratio of 1.83, and an A260/A230 ratio of 2.02, confirming its suitability for subsequent experiments (Fig. S1). Agarose gel electrophoresis showed a PCR amplification product slightly larger than 100 bp, aligning with the expected 112 bp fragment (Fig. 1a). Sanger sequencing of the forward and reverse strands of the *cfb* gene revealed 100% identity with the reference sequence (Fig. 1b). These results—high DNA quality, clear electrophoretic band, and accurate sequencing—collectively confirm that the GBS DNA is suitable for developing the RM.

Establishment of dPCR method for GBS DNA RM detection

PCR amplification using different primer-probe sets yielded clear separation between positive and negative droplets, with few intermediate signals (Fig. 2). Despite variations in annealing temperatures and amplification efficiencies among the three prime-probe combinations, an *F*-test performed with IBM SPSS Statistics 27 showed no statistically significant differences in DNA copy numbers across the datasets ($F = 0.1 < F_{0.05}$ (2, 6) = 5.14; Table S1). Because primer-probe set 1 exhibited the most consistent and efficient performance, it was selected as the optimized assay for subsequent dPCR amplification.

The dPCR assay was established using the manufacturer's optimized ddPCR™ Supermix system, which eliminated the need for further optimization of primer, probe, or thermal cycling conditions. Preliminary testing confirmed that the selected parameters provided stable droplet generation, efficient amplification, and clear positive/negative partitioning (Fig. 2). Therefore, further analysis focused on quantifying

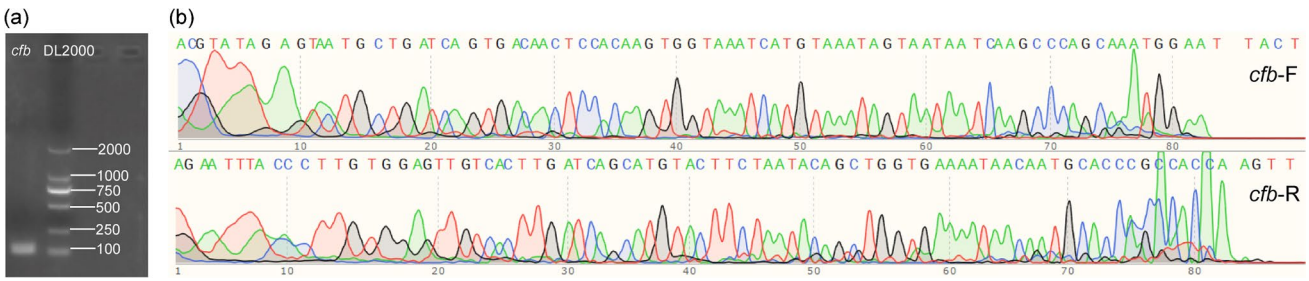

Fig. 1 Agarose gel electrophoresis and Sanger sequencing of the *cfb* gene. **a** PCR amplification of GBS gDNA using *cfb*-specific primers. **b** Sanger sequencing chromatogram of the amplified *cfb* fragment

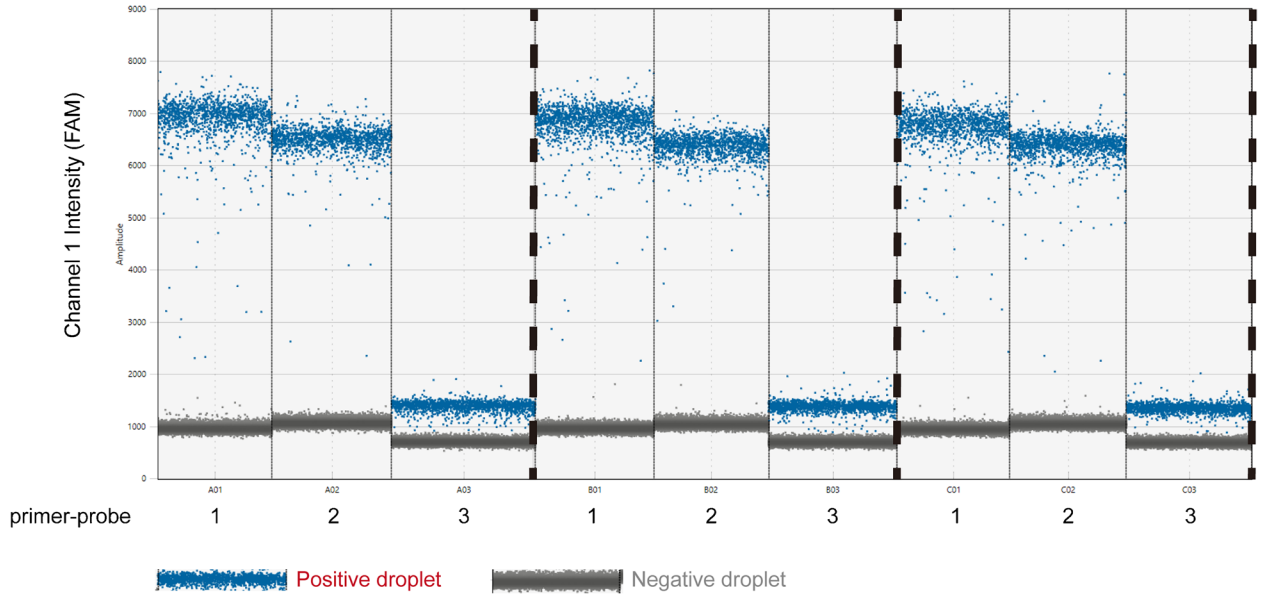

Fig. 2 dPCR analysis evaluated three different primer-probe sets targeting the GBS *cfb* gene. The droplet fluorescence separation and amplification patterns obtained from these three assays using GBS DNA are compared

the GBS DNA RM and evaluating its suitability for method validation, rather than on refining assay conditions.

Homogeneity and stability for GBS DNA RM

Normality of the data was first verified using the Shapiro-Wilk test, which yielded a statistic of 0.962 ($p = 0.146 > 0.05$), confirming a normality distribution. An F-test showed no statistically significant difference between the between-unit and within-unit variance ($F = 1.955 < F_{0.05}$ (14, 30) = 2.04, $p > 0.05$), confirming the homogeneity of the RM (Table 2).

Short-term stability was evaluated at three temperatures (4 °C, 25 °C, and 37 °C) over specified time intervals. The slope parameter (β_1) met the criterion $|\beta_1| < t_{0.95,n-2} \times SE\ (\beta_1)$, indicating no temporal trend (Eq. (1)). A t-test analysis confirmed that the material remained stable for up to 7 days at 4 °C, 48 h at 25 °C, and 24 h at 37 °C (Fig. 3; Table S2). Long-term stability analysis further demonstrated that the GBS DNA RM was stable for at least 6 months when stored at −20 °C (Fig. 3; Table S2).

The freeze-thaw stability results are summarized in Table 3, with comprehensive statistical data in Table S3. Levene's test for equality of variances yielded a p-value of 0.577 (> 0.05) for the one-cycle group, confirming variance homogeneity between the test and control samples. An independent samples t-test produced a two-tailed p-value of 0.392 (> 0.05), indicating no statistically significant difference between their means. These results demonstrate that the prepared RM tolerates at least one freeze-thaw cycle without measurable degradation. Likewise, comparisons of the three- and five-cycle groups with the initial material showed no significant copy number variations, verifying that the GBS DNA RM maintains stability through five freeze-thaw cycles.

Table 2 Assessment of homogeneity for GBS DNA RMs (copies/μL)

Tube	Number of tests			Relative standard deviation (%)
	1	2	3	
#1	7627	8019	7486	3.58
#2	7587	7128	8060	6.14
#3	7155	8005	7564	5.61
#4	7148	7624	7987	5.55
#5	6925	7334	7210	2.93
#6	7469	7476	7726	1.94
#7	7706	7518	7319	2.58
#8	7328	6954	7578	4.31
#9	7612	7768	7703	1.02
#10	7189	6938	6975	1.93
#11	7328	7499	7462	1.21
#12	7615	7429	7533	1.24
#13	7074	7108	7413	2.59
#14	6944	7137	7488	3.84
#15	7998	7383	7632	4.03

Calculation of assigned value for GBS DNA RM

The value assignment for the GBS DNA RM was conducted through a multi-center collaboration involving nine

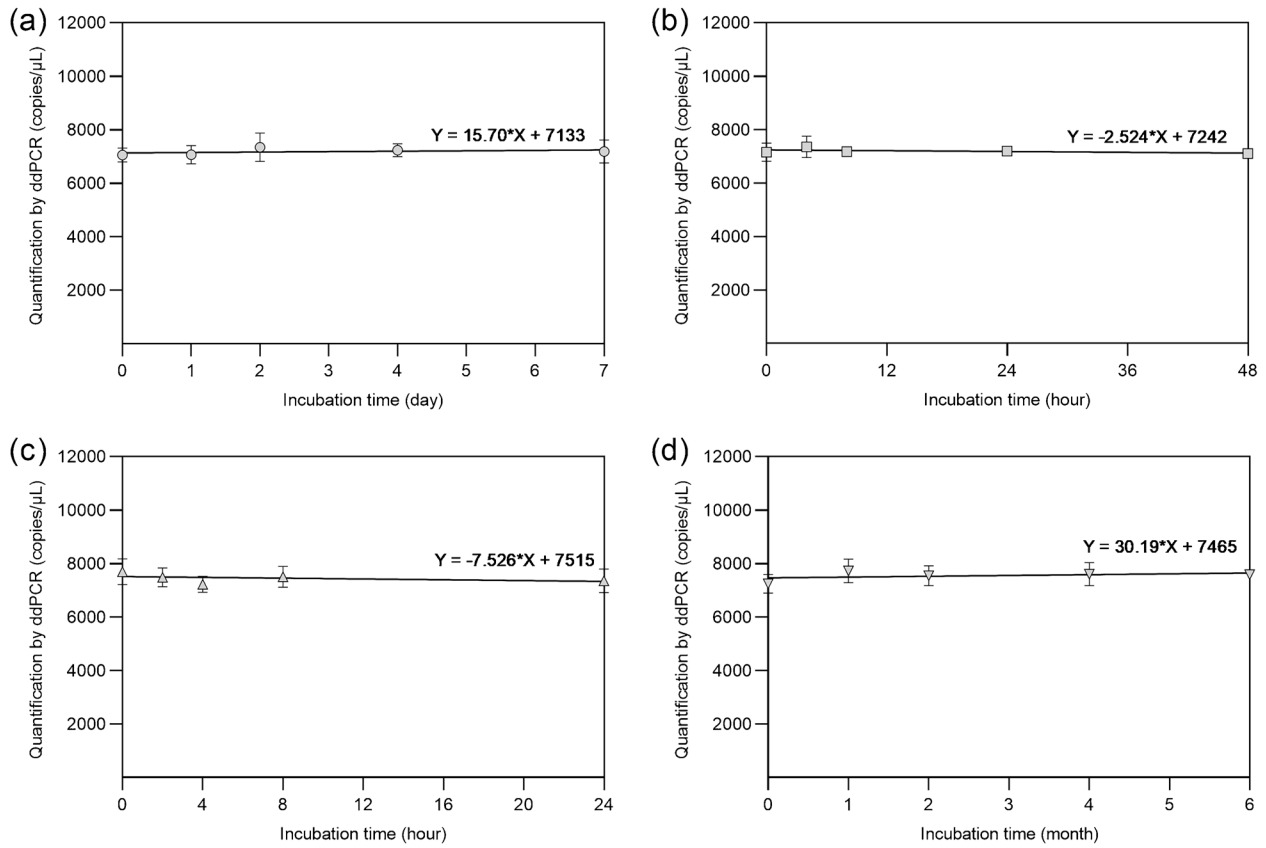

Fig. 3 The stability of the GBS DNA RM was evaluated under different temperature conditions. The RM was stored at 4 °C (**a**), 25 °C (**b**), 37 °C (**c**), and −20 °C (**d**) and analyzed by dPCR at defined time intervals

Table 3 Results of the repeated freeze-thaw stability test (copies/μL)

Freeze-thaw cycles	RM#1	RM#2	RM#3
0	7136	7719	7125
1	7903	7841	7139
3	7515	6928	8101
5	7706	6994	6995

independent laboratories, each utilizing one of seven common dPCR platforms (QX ONE, QX200, Snip DQ24, BioDigital, Rainsure SG2000, TD-1, and AccuONE-R200PB) (Table 4). Twenty-seven units were distributed among the laboratories, and each unit was analyzed in duplicate; the resulting assigned values are summarized in Tables 4 and S4. Dixon's Q test was applied to assess intra-laboratory consistency and identify potential outliers (Table S4). All calculated $Q_{\min}$ and $Q_{\max}$ values fell below the critical thresholds (for $n=6$, $Q_{0.05,\,6}=0.560$ and $Q_{0.01,\,6}=0.698$), indicating no outliers within the laboratory datasets. For inter-laboratory distribution, a Shapiro-Wilk test (appropriate for sample size under 50) gave a statistic of 0.957 ($p=0.316>0.05$), supporting a normal distribution. This conclusion reinforced by a normal Q-Q plot of the combined values (Fig. S2). Given the normal distribution, a Grubbs' test was used to screen for outliers. The Grubbs' statistic was calculated as follows:

$$G=\frac{max\left|x_i-\bar{x}\right|}{s} \tag{4}$$

where $\bar{x}$ is the overall mean and s is the standard deviation. With a mean of 7532.44 and a standard deviation of 247.67, the maximum deviation was $\left|x_i-\bar{x}\right|=464.44$, yielding $G=1.88$. For $n=27$ and $\alpha=0.05$ (two-tailed), the critical value was $G_{\text{crit}}=2.69$. Since $G<G_{crit}$, no outliers were detected. The arithmetic mean of the quantitative results was therefore adopted as the certified value, giving 7532.44 copies/μL for the GBS DNA RM.

Evaluation of uncertainty for GBS DNA RM

The uncertainty attributable to homogeneity was derived from the mean squares between- and within-bottle. Given that $MS_b > MS_w$, the relative standard uncertainty was calculated using the following equation:

Table 4 Average assigned values obtained from nine independent laboratories (copies/μL)

No. of laboratory	dPCR platform	GBS DNA			Mean assigned value
		RM#1	RM#2	RM#3	
#1	QX ONE[a]	7812	7904	7327	7681
#2	QX200[b]	7160	7387	7068	7205
#3	QX200[b]	7715	7550	7530	7598
#4	Snip DQ24 PLUS[c]	7225	7814	7616	7552
#5	Snip DQ24[c]	7883	7684	7843	7803
#6	BioDigital[d]	7530	7574	7748	7617
#7	Rainsure SG2000[e]	7187	7650	7441	7426
#8	TD-1[f]	7577	7423	7481	7494
#9	AccuONE-R200PB[g]	7799	7089	7359	7416

[a]QX ONE (Bio-Rad Laboratories, Inc., CA, USA)

[b]QX200 (Bio-Rad Laboratories, Inc., CA, USA)

[c]Snip DQ24/DQ24 PLUS (Suzhou Sniper Medical Technology Co., Ltd., Suzhou, China)

[d]BioDigital (Shanghai Little Turtle Biomedical Technology Co., Ltd., Shanghai, China)

[e]Rainsure SG2000 (RainSure Scientific Co., Ltd., Suzhou, China)

[f]TD-1 (TargetingOne Technology (Beijing) Corporation, Beijing, China)

[g]AccuONE-R200PB (ZhenZhun Bio-Tech Co., Ltd., Shanghai, China)

$$u_1 = \sqrt{\frac{MS_b - MS_w}{n}} \tag{5}$$

where $n = 3$, $MS_b = 143,450.64$, and $MS_w = 73,372.47$. This gave $u_1 = 152.84$. The uncertainty associated with stability was estimated from an isochronous study in which the RM was stored at −20 °C for 6 months. The uncertainty was calculated according to the following expression:

$$u_2 = |\beta_1| \times T \tag{6}$$

where $\beta_1 = 30.19$ and $T = 6$ months, yielding $u_2 = 181.14$. The uncertainty from value assignment characterization was determined as follows:

$$u_3 = \frac{s}{\sqrt{n}} \tag{7}$$

where $s = 247.67$ and $n = 27$, resulting in $u_3 = 47.66$.

In summary, the relative uncertainties arising from homogeneity, long-term stability, and value assignment characterization were $u_1 = 152.84$, $u_2 = 181.14$, and $u_3 = 47.66$, respectively. Substituting these values into Eq. (2) produced a combined relative uncertainty of $u_c = 241.75$. The certified value of the RM is expressed as the assigned value ± expanded uncertainty (U), where $U = 483.50$ (Eq. (3)). Thus, the certified value for the GBS DNA RM was established as $(7.53 \pm 0.48) \times 10^3$ copies$/\mu L$.

Validation of qPCR kit performance using GBS DNA RM

Three commercial qPCR kits targeting the *cfb* gene, approved by the National Medical Products Administration and intended for prenatal and perinatal GBS screening, were evaluated using the GBS DNA RM. The specifications of the kits are summarized in Table S5. Standard curves were constructed by quantifying serial dilutions of the RM for each kit; all curves showed excellent linearity, with a correlation coefficient (R^2) above 0.9950 (Fig. 4). Amplification efficiency (E) was derived from the following equation:

$$\mathrm{E} = -1 + 10\text{^}\left(\frac{-1}{\text{slope}}\right) \tag{8}$$

The calculated efficiencies for the three kits ranged from 90.29 to 95.77%. Additionally, the kits' claimed LODs were verified using the GBS DNA RM. Contrary to the manufacturers' claims, the observed detection rates at the stated LOD concentrations (20 replicates each) were only 35% (7/20), 55% (11/20), and 50% (10/20), respectively.

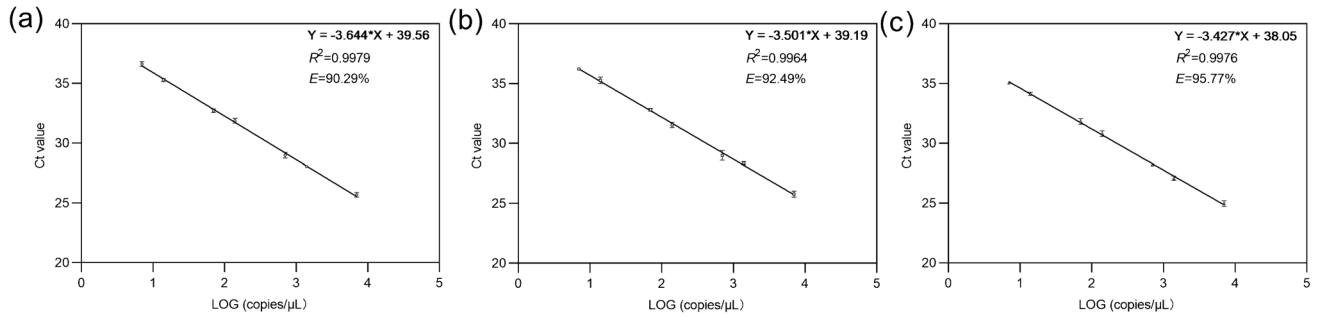

Fig. 4 The performance of three commercial qPCR kits was evaluated using the GBS DNA RM. Serial dilutions of the RM were analyzed with Assay I (**a**), Assay II (**b**), and Assay III (**c**), and standard curves were generated for comparison

Clinical validation of GBS DNA RM

Clinical validation of the GBS DNA RM was conducted in three International Organization for Standardization 15,189-accredited clinical laboratories in Shanghai. Each site performed qualitative testing with its own routinely used commercial qPCR kit, such as those from TIB and Biochain, through three independent batches, each comprising three replicates. As shown in Table S6, all participating laboratories reported consistent and reproducible results, with each evaluation outcome designated as "+". This confirms that the GBS DNA RM satisfies the necessary quality control criteria for clinical GBS detection. These collective data underscore the stability, reliability, and applicability of the prepared RM in routine clinical molecular testing.

Discussion

GBS remains a leading cause of neonatal sepsis and continues to pose significant risks to pregnant and immunocompromised individuals [28]. Despite molecular diagnostics having advanced in speed and accuracy, the lack of standardized RMs has impeded assay comparability and long-term quality assurance [3]. The GBS DNA RM developed here addresses this gap by providing a reliable benchmark for assay calibration, quality control, and inter-laboratory comparison. Beyond clinical diagnostics, this RM also supports epidemiological surveillance, thereby strengthening the overall robustness of GBS monitoring systems [29].

Value assignment for the RM was performed through a multi-center collaboration involving nine independent laboratories and seven distinct dPCR platforms. The high concordance observed across these laboratories and platforms confirms the robustness of the developed GBS DNA RM and underscores its broad applicability as a cross-platform standard. This multi-platform validation further demonstrates its suitability as a common benchmark for diverse dPCR systems, improving the comparability of quantitative results across different laboratory settings. Moreover, confirmation of homogeneity and stability according to ISO requirements affirms the reliability of the material under both long-term storage and routine transportation conditions. Taken together, these features ensure the GBS DNA RM can be effectively applied to assay calibration, routine quality control, and the performance evaluation of emerging molecular detection technologies.

We further evaluated the applicability of the GBS DNA RM using three commercial qPCR kits. Standard curves were successfully generated, and the kits' claimed LODs were evaluated; however, inconsistencies among the kits were evident. These discrepancies are likely attributable to assay-specific variables, such as differences in primer-probe design, amplification efficiencies, and threshold settings, rather than from deficiencies in the RM itself, since the GBS DNA RM consists of purified bacterial DNA without interfering substance. These findings underscore the necessity of understanding assay-specific variables prior to clinical implementation [30]. Insufficient analytical sensitivity can lead to false-negative results, potentially causing misdiagnosis or inappropriate treatment [31]. Our data show that the observed detection rates (35–55%) at the manufacturers' claimed LODs were substantially lower than the claimed threshold, emphasizing the necessity of independent verification of assay performance using certified RMs to ensure reliable detection result [32]. The consistent results obtained across multiple laboratories and different qPCR kits confirm the reliability and broad applicability of the GBS DNA RM for routine clinical molecular testing.

Several limitations of this study should be acknowledged. First, validation was performed under controlled laboratory conditions using purified DNA and only three commercial qPCR assays; thus, it does not account for pre-analytical variability such as sampling collection, transport, or matrix effects present in clinical specimens. Moreover, the developed RM consists of purified genomic DNA material and lacks an internal control sequence, which is a critical component in most clinical assays for monitoring sample extraction and inhibition. Future studies involving routine clinical samples and multi-center participation in EQA are needed to verify robustness under real-world conditions. Second, the current GBS DNA RM represents a single-locus, single-strain material and therefore does not encompass the genomic diversity of circulating GBS, including variation in capsular serotypes and sequence/genotype backgrounds, which can affect assay performance through primer-probe mismatches or target dropout. Although this strain has been applied successfully in EQA studies, demonstrating its general utility [17], expanding toward a panelized RM set covering representative serotypes/genotypes or other targeted loci (e.g., *sip*, *hylB*, *atr*, and *scpB*) would further broaden its applicability. Despite these limitations, the GBS DNA RM described here provides a metrologically assigned, transferable standard that facilitates calibration, lot-to-lot quality control, and inter-laboratory comparability, thereby promoting standardization and normalization of GBS molecular testing across platforms and laboratories.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-025-06242-y.

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Data availability The datasets generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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