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Novel Wide-Range Quantitative Nested Real-Time PCR Assay for *Mycobacterium tuberculosis* DNA: Development and Methodology▽

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Previously, we designed an internally controlled quantitative nested real-time (QNRT) PCR assay for *Mycobacterium tuberculosis* DNA in order to rapidly diagnose tuberculous meningitis. This technique combined the high sensitivity of nested PCR with the accurate quantification of real-time PCR. In this study, we attempted to improve the original QNRT-PCR assay and newly developed the wide-range QNRT-PCR (WR-QNRT-PCR) assay, which is more accurate and has a wider detection range. For use as an internal-control "calibrator" to measure the copy number of *M. tuberculosis* DNA, an original new-mutation plasmid (NM-plasmid) was developed. It had artificial random nucleotides in five regions annealing specific primers and probes. The NM-plasmid demonstrated statistically uniform amplifications ($F = 1.086$, $P = 0.774$) against a range (1 to 10^5) of copy numbers of mimic *M. tuberculosis* DNA and was regarded as appropriate for use as a new internal control in the WR-QNRT-PSR assay. In addition, by the optimization of assay conditions in WR-QNRT-PCR, two-step amplification of target DNA was completely consistent with the standard curve of this assay. Due to the development of the NM-plasmid as the new internal control, significantly improved quantitative accuracy and a wider detection range were realized with the WR-QNRT-PCR assay. In the next study, we will try to use this novel assay method with actual clinical samples and examine its clinical usefulness.

Tuberculous meningitis (TBM) is the severest form of infection of *Mycobacterium tuberculosis*, causing death or severe neurological defects in more than half of those affected in spite of antituberculosis treatment (25). The diagnosis of TBM remains a complex issue because the most widely used conventional bacteriological detection methods, such as direct smear for acid-fast bacilli and culture for *M. tuberculosis*, are unable to rapidly detect *M. tuberculosis* with sufficient sensitivity in the acute phase of TBM (7, 8, 11, 12, 18, 19, 21, 22, 23, 25). At present, the detection of *M. tuberculosis* DNA in cerebrospinal fluid (CSF) by use of PCR is widely used as a more rapid, sensitive, and specific diagnostic method (1, 7, 8, 10, 11, 12, 15, 17, 18, 19, 21, 22, 23, 26). Recently, we designed a novel internally controlled quantitative nested real-time PCR (QNRT-PCR) assay based on TaqMan PCR (Applied Biosystems) (22). This novel assay technique combines the high sensitivity of nested PCR with the accurate quantification of real-time PCR (22, 23). However, this original QNRT-PCR (OR-QNRT-PCR) assay is still unstable and has many points that should be improved (22, 23).

In this study, to reliably detect *M. tuberculosis* DNA in CSF samples with a wider detection range, we attempted to improve on the OR-QNRT-PCR technique; therefore, a new internal control for use as a "calibrator" was prepared. We named this improved assay technique wide-range QNRT-PCR (WR-QNRT-PCR) and examined its ability to quantitatively detect *M. tuberculosis* DNA in samples. In this paper, the development and methodology of the WR-QNRT-PCR assay are stated.

MATERIALS AND METHODS

This study was approved by the Nihon University Institutional Review Board.

Preparation of the new internal control (plasmid) for use as a calibrator. For the WR-QNRT-PCR assay, two types of the original plasmid, wild plasmids (W-plasmids) and new-mutation plasmids (NM-plasmids), were prepared for a quantitative detection of *M. tuberculosis* DNA, and this was done as well for the OR-QNRT-PCR assay (22, 23).

W-plasmid, which was inserted into a 239-bp DNA fragment of the gene sequence encoding the MPB64 protein of *M. tuberculosis* (MPT64; GenBank accession no. NC_000962) (22, 23) into pCR 2.1 vector (Invitrogen Corp., San Diego, CA) was constructed for use as the standard template by the previously reported procedure (22, 23).

NM-plasmid was developed based on the previously reported M-plasmid (22, 23) for use as a new internal-control "calibrator" in the WR-QNRT-PCR assay. In NM-plasmid, a total of four regions, where two pairs of (outer and inner) forward and reverse primers annealed, were replaced with the artificial random nucleotides added to the TaqMan probe annealing region in the M-plasmid (Fig. 1). The sequences of the artificial random nucleotides were set to have the same nucleotide composition as MPT64 of wild *M. tuberculosis*. Replacing procedures

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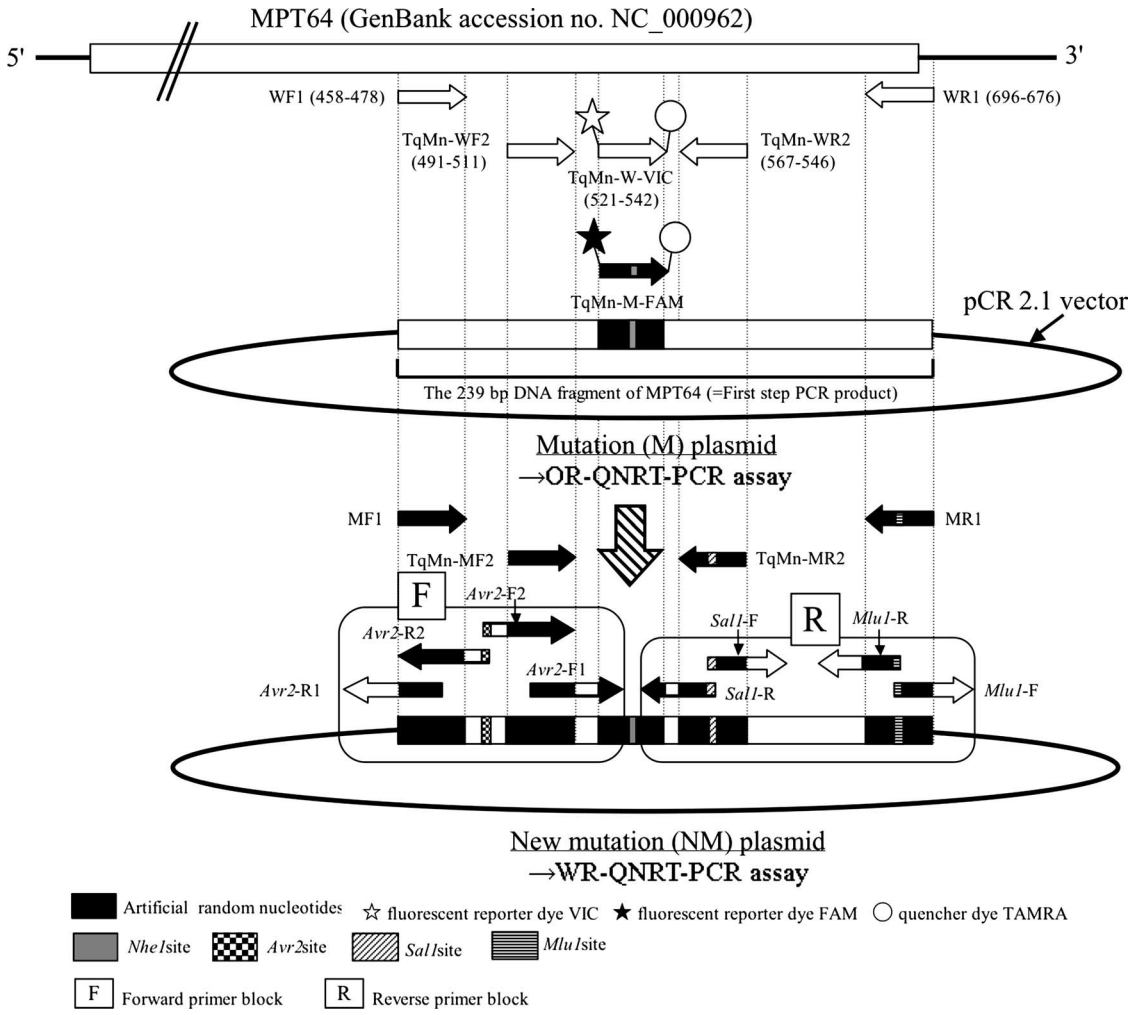


FIG. 1. Position of primers and probes in the MPB64 protein encoding gene (MPT64), and the procedure for the development of the NM-plasmid for use as a new internal control. TAMRA, 6-carboxytetramethylrhodamine.

were gradually performed by two steps (forward and reverse primer blocks) using the following four pairs of primers: *Avr2*-F1 and *Avr2*-R1, *Avr2*-F2 and *Avr2*-R2, *Mlu1*-F and *Sal1*-R, and *Sal1*-F and *Mlu1*-R (Fig. 1). The sequences and positions of these primers are shown in Table 1 and Fig. 1. These four pairs of primers were also specific for MPT64 and contained additional artificial random nucleotides including one restriction enzyme site at the 5′ end. Since each restriction enzyme site (*Avr2*, *Mlu1*, and *Sal1*) in these primers was not contained within pCR2.1 vector, it was possible to accurately digest both ends of each PCR product. In respective replacing steps, each PCR product was digested by restriction enzymes and then ligated (Fig. 1). The final ligation product, i.e., NM-plasmid, was cloned using a TA cloning kit (Invitrogen Corp.) (Fig. 1). The 10^3 copies of NM-plasmid were adopted as a new internal-control "calibrator." This copy number was determined by the preliminary experiments (described below).

(The NM-plasmid is available from us through the laboratory at the High-Tech Research Center, Nihon University School of Medicine, Tokyo, Japan: please e-mail corresponding author Teruyuki Takahashi to request.)

Primers and probes for WR-QNRT-PCR. For use in the WR-QNRT-PCR assay, four pairs of new specific primers and two types of specific (TaqMan) probes were prepared. The sequences and positions of these new primers and probes are shown in Table 1 and Fig. 1. In the first step of WR-QNRT-PCR assay, two pairs of outer forward and reverse primers, WF1 and WR1, as well as MF1 and MR1, were used. WF1 and WR1 were specific for MRT64 of wild *M. tuberculosis* or W-plasmid, whereas MF1 and MR1 were specific for the artificial random nucleotides in the NM-plasmid for use as a new internal-control "calibrator." In the second step, two pairs of inner forward and reverse primers, TqMn-WF2 and TqMn-WR2, as well as TqMn-MF2 and TqMn-MR2, were used. TqMn-WF2 and TqMn-WR2 were specific for wild MPT64. TqMn-MF2 and TqMn-MR2 were specific for the artificial random nucleotides in the NM-plasmid. In addition, two types of probes, TqMn-W-VIC and TqMn-M-FAM, were used. TqMn-W-VIC was labeled with fluorescent reporter dye VIC and specifically annealed to wild MPT64. While TqMn-M-FAM was labeled with fluorescent reporter dye 6-carboxyfluorescein (FAM) and specifically annealed to the artificial random nucleotides in the NM-plasmid. These primers and probes were set to have the same nucleotide composition but a different and random sequence (Table 1). Therefore, the annealing efficiencies of these primers and probes to wild MPT64 or NM-plasmid as a template can be regarded as the same.

In the OR-QNRT-PCR assay, two consecutive PCR amplification steps were performed by using the common two pairs of primers WF1 and WR1 at the first step and TqMn-WF2 and TqMn-WR2 at the second step for both *M. tuberculosis* DNA and M-plasmid as the old internal control (Table 1 and Fig. 1). Two types of probes, TqMn-W-VIC and TqMn-M-FAM, were also used to specifically detect each *M. tuberculosis* DNA or M-plasmid (Table 1 and Fig. 1).

Extraction and purification of DNA from CSF samples. A 500-μl aliquot of original lysis buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.8% (vol/vol) sodium dodecyl sulfate, and 0.5 mg of proteinase K was prepared. This lysis buffer was added to 500 μl of the CSF sample, followed by incubation in a water bath at 65°C overnight. After incubation, the total 1,000-μl suspension was divided into two 500-μl aliquots for use in the WR-QNRT-PCR assay.

In advance, the 10^3 copies of NM-plasmid as a new internal-control "calibra-

TABLE 1. Sequences of primers and TaqMan probes for PCR assays

Objective	First- or second-step PCR	Target	PCR product size (bp)	Type	Sequence[a]
Developing NM-plasmid		Forward primer block (NM-plasmid)	4146	*Avr2*-F1: *Avr2* random forward primer-1	5′-GGATAGCCAGCACGGAGACAACAGGATGGGACGGC-3′
				Avr2-R1: *Avr2* random reverse primer-1	5′-GGTGGGACAGAATCGAAAGCCGAATTCCAGCACACT-3′
			4178	*Avr2*-F2: *Avr2* random forward primer-2	5′-ATCCTAGGAGAGATCGGATAGCCAGCACGGAGACA-3′
				Avr2-R2: *Avr2* random reverse primer-2	5′-CTCCTAGGATAGACGGCGGTGGGACAGAATCGAAAGC-3′
		Reverse primer block (NM-plasmid)	4048	*Mlu1*-F: *Mlu1* random forward primer:	5′-CTACGCGTCGAGTCTAAGCCGAATTCTGCAGATAT-3′
				Sal1-R: *Sal1* random reverse primer	5′-GCGTCGACATATTCTAAAGGACGGATTGCTAGCCGT-3′
			136	*Sal1*-F: *Sal1* random forward primer	5′-ATGTCGACGCAGCGCATTCGCAGTCACGAACGACGG-3′
				Mlu1-R: *Mlu1* random reverse primer	5′-CGACGCGTAGTCCTCGCGAGTCGATCGCGGAACGTG-3′
WR-QNRT-PCR assay	First-step PCR	Wild *M. tuberculosis* DNA (MPT64) or W-plasmid	239	WF1: outer wild forward primer	5′-ATCCGCTGCCAGTCGTCTTCC-3′; total of 21 nucleotides, A:2, T:6, G:4, C:9 (G+C, 62%)
				WR1: outer wild reverse primer	5′-CTCGCGAGTCTAGGCCAGCAT-3′; total of 21 nucleotides, A:4, T:4, G:6, C:7 (G+C, 62%)
		New internal control (NM-plasmid)		MF1: outer mutation forward primer	5′-TCGATTCTGTCCCACCGCCGT-3′; total of 21 nucleotides, A:2, T:6, G:4, C:9 (G+C, 62%)
				MR1: outer mutation reverse primer	5′-AGACTCGACGCGTAGTCCTCG-3′; total of 21 nucleotides, A:4, T:4, G:6, C:7 (G+C, 62%)
	Second-step PCR	Wild *M. tuberculosis* DNA (MPT64) or W-plasmid	77	TqMn-WF2: TaqMan inner wild forward primer	5′-GTGAACTGAGCAAGCAGACCG-3′; total of 21 nucleotides, A:7, T:2, G:7, C:5 (G+C, 57%)
				TqMn-WR2: TaqMan inner wild reverse primer	5′-GTTCTGATAATTCACCGGGTCC-3′; total of 22 nucleotides, A:4, T:7, G:5, C:6 (G+C, 50%)
		New internal control (NM-plasmid)		TqMn-MF2: TaqMan inner mutation forward primer	5′-AGATCGGATAGCCAGCACGGA-3′; total of 21 nucleotides, A:7, T:2, G:7, C:5 (G+C, 57%)
				TqMn-MR2: TaqMan inner mutation reverse primer	5′-TGCGCTGCGTCGACATATTCTA-3′; total of 22 nucleotides, A:4, T:7, G:5, C:6 (G+C, 50%)
		Wild *M. tuberculosis* DNA (MPT64) or W-plasmid		TqMn-W-VIC: TaqMan probe-wild-VIC	5′-VIC-TATCGATAGCGCCGAATGCCGG-TAMRA-3′; total of 22 nucleotides, A:5, T:4, G:7, C:6 (G+C, 59%)
		New internal control (NM-plasmid)		TqMn-M-FAM: TaqMan probe-mutation-FAM	5′-FAM-ATGGGACGGCTAGCAATCCGTC-TAMRA-3′; total of 22 nucleotides, A:5, T:4, G:7, C:6 (G+C, 59%)
OR-QNRT-PCR assay	First-step PCR	Wild *M. tuberculosis* DNA (MPT64) and old internal control (M-plasmid)	239	WF1	5′-ATCCGCTGCCAGTCGTCTTCC-3′
				WR1	5′-CTCGCGAGTCTAGGCCAGCAT-3′
	Second-step PCR	Wild *M. tuberculosis* DNA (MPT64) and old internal control (M-plasmid)	77	TqMn-WF2	5′-GTGAACTGAGCAAGCAGACCG-3′
				TqMn-WR2	5′-GTTCTGATAATTCACCGGGTCC-3′
		Wild *M. tuberculosis* DNA (MPT64)		TqMn-W-VIC	5′-VIC-TATCGATAGCGCCGAATGCCGG-TAMRA-3′
		Old internal control (M-plasmid)		TqMn-M-FAM	5′-FAM-ATGGGACGGCTAGCAATCCGTC-TAMRA-3′

[a] Underlining indicates artificial sequence; double underlining indicates restriction site. TAMRA, 6-carboxytetramethylrhodamine.

TABLE 2. PCR assay conditions

Step	Parameter at indicated step for:	
	WR-QNRT-PCR	OR-QNRT-PCR
First-step PCR		
Initial denaturing	96.0°C; 3 min	96.0°C; 3 min
Amplification	25 cycles[a]	35 cycles
Denaturing	95.0°C; 30 s	95.0°C; 30 s
Annealing	60.0°C; 30 s	60.0°C; 30 s
Extension	72.0°C; 1 min	72.0°C; 1 min
Final extension	72.0°C; 10 min	72.0°C; 10 min
Second-step PCR		
Incubation	50.0°C; 2 min	50.0°C; 2 min
Initial denaturing	95.0°C; 10 min	95.0°C; 10 min
Amplification	40 cycles	40 cycles
Denaturing	95.0°C; 15 s	95.0°C; 15 s
Annealing-extension	60.0°C; 1 min	60.0°C; 1 min

[a] Improved amplification cycle number.

tor" were added to one of the 500-μl aliquot containing 250 μl each of CSF and lysis buffer. The DNA specimens including *M. tuberculosis* DNA and NM-plasmid were extracted and purified from these 500-μl aliquots by a previously reported conventional phenol-chloroform method and ethanol precipitation (7, 8, 22). To efficiently extract a small amount of DNA, a high-molecular-weight carrier, Ethachinmate (Nippon Gene, Tokyo, Japan), was used as a coprecipitating agent for the nucleotides in the ethanol precipitation. After complete vacuum desiccation, the extracted DNA specimen was resuspended in 20 μl of pure water and then stored at −20°C until use.

Assay condition of WR-QNRT-PCR. Both WR and OR-QNRT-PCR assays consist of two consecutive PCR amplification steps, which were conventional PCR at the first step and real-time (TaqMan) PCR at the second step. *M. tuberculosis* DNA and NM-plasmid were amplified and detected in separate tubes and wells. However, the entire procedure was performed simultaneously under the same assay conditions.

In the first-step PCR, 18-μl conventional PCR solution mixtures containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 400 μM of each deoxynucleoside triphosphate mix, 20 pM each of outer primers WF1 and WR1 or MF1 and MR2, and 2.5 U of *Taq* DNA polymerase were prepared. As a template, 2 μl of the extracted DNA specimen, including *M. tuberculosis* DNA and the new internal control (NM-plasmid), was added to the PCR solution mixture (each total reaction volume was 20 μl). This preparation was subjected to the protocol shown in Table 2 at 25 amplification cycles, using the GeneAmp PCR system 9700 (Perkin Elmer, Norwalk, CT). The assay protocol of OR-QNRT-PCR is additionally described in Table 2. The first-step PCR in the OR-QNRT-PCR assay was set at 35 amplification cycles (22).

In the second-step PCR, 23-μl PCR solution mixtures containing 12.5 μl of TaqMan universal PCR master mix, 0.9 μM each of inner primers TqMn-WF2 and TqMn-WR2 or TqMn-MF2 and TqMn-MR2, and 0.2 μM TaqMan probe TqMn-W-VIC or TqMn-M-FAM were prepared. As a template, 2 μl of the first-step PCR product was added to this PCR solution mixture (each total reaction volume was 25 μl). This preparation was subjected to the protocol shown in Table 2, using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA).

Quantitative detection of *M. tuberculosis* DNA. In the WR-QNRT-PCR assay, the procedures of extraction, amplification, and detection for both *M. tuberculosis* DNA and the new internal control were performed simultaneously by using two pairs of primers and two probes that had annealing efficiencies equivalent to those of the templates. Therefore, the initial copy number of *M. tuberculosis* DNA in CSF samples was able to be calculated based on the amplification ratio against the new internal control (10^3 copies of NM-plasmid) as a "calibrator." Therefore, we adopted equation 1 as well as the OR-QNRT-PCR assay (22, 23).

$$X : W = C : M \therefore X = W \times C/M \quad (1)$$

where X is the initial copy number of *M. tuberculosis* DNA per 1 ml of CSF sample, C is the initial copy number of the new internal control (i.e., "calibrator" [10^3 copies of NM-plasmid]), and W and M are the copy numbers of *M. tuberculosis* DNA and NM-plasmid, respectively, after passing through extraction and PCR amplification procedures. In *M. tuberculosis*, it was universally acceptable that a single copy of the MPT64 gene represented one bacterial cell (7, 8). Therefore, we considered that the copy numbers calculated by the WR-QNRT-PCR assay corresponded to the bacterial cell numbers of *M. tuberculosis* in CSF samples.

Statistical analysis. The statistical analysis was calculated using data analysis software program SPSS 13.0 for Windows. A P value of <0.05 was considered statistically significant.

RESULTS

Precision of the standard curves. For the WR-QNRT-PCR assay, two specific standard curves for the quantitative detection of *M. tuberculosis* DNA and the new internal control are needed, and this is also the case for OR-QNRT-PCR assay (22). Therefore, the precision of these two specific standard curves was statistically evaluated using the standard templates in the previously reported preliminary experimental protocols (22). The two specific standard curves are shown in Fig. 2A and B. In simple regression analysis, both of these two standard curves demonstrated a significant linear relationship ($R^2 = >0.99$) between the threshold cycle numbers (C_T values) (y axis) and log of the starting copy numbers for each standard template (x axis). In both standard curves, no significant differences were found among the plots in each preliminary experiment ($F = 1.007$, $P = 0.65$ and $F = 1.015$, $P = 0.53$) by two-way analysis of variance (ANOVA). The PCR efficiency (PCR-Eff) of real-time PCR can be calculated by the slope of the standard curve by the following equation: PCR-Eff = $10^{(-1/\text{slope})} - 1$ (14). In the WR-QNRT-PCR assay, the PCR-Eff values calculated by this equation based on the slopes (−3.33 and −3.28) of two standard curves were 99.7 and 101.8%, respectively.

Optimization of WR-QNRT-PCR assay conditions. For the WR-QNRT-PCR assay, two important parameters may affect assay conditions: the amplification cycle number for the first-step PCR and the copy number of the new internal control. These two parameters were determined by previously reported preliminary experimental protocols (22) using five serial sets of 10-fold-diluted W-plasmids (1 to 10^5 copies) as the templates instead of actual *M. tuberculosis* DNA. The C_T value data collected under various assay conditions were statistically analyzed.

(i) Optimal amplification cycle number for first-step PCR. To determine the optimal amplification cycle number for the first-step PCR, cycle numbers were set at 5-cycle intervals in the range from 20 to 35 cycles. When the first-step PCR was set at 25 cycles, the most constantly isolated amplification curves were demonstrated in all starting copy numbers of W-plasmids (Fig. 2C). The C_T value data (means ± standard deviations) at 25 cycles in first-step PCR are shown in Table 3. Reflecting Fig. 2C, a significant linear relationship ($R^2 = 0.996$) was demonstrated between C_T values (y axis) and the log of the starting copy numbers of W-plasmids (x axis) by simple regression analysis (Fig. 2D). The slope of this linear regression curve (−3.33) was completely consistent with that of the standard curve shown in Fig. 2A. Therefore, 25 cycles was adopted as the optimal cycle number in the first-step PCR. Whereas, when the first-step PCR was set at the previously reported 35 cycles in the OR-QNRT-PCR assay (22), a significant linear relationship ($R^2 = 0.991$) was also demonstrated by simple regression analysis for the C_T value data (Table 3 and Fig. 2E). However,

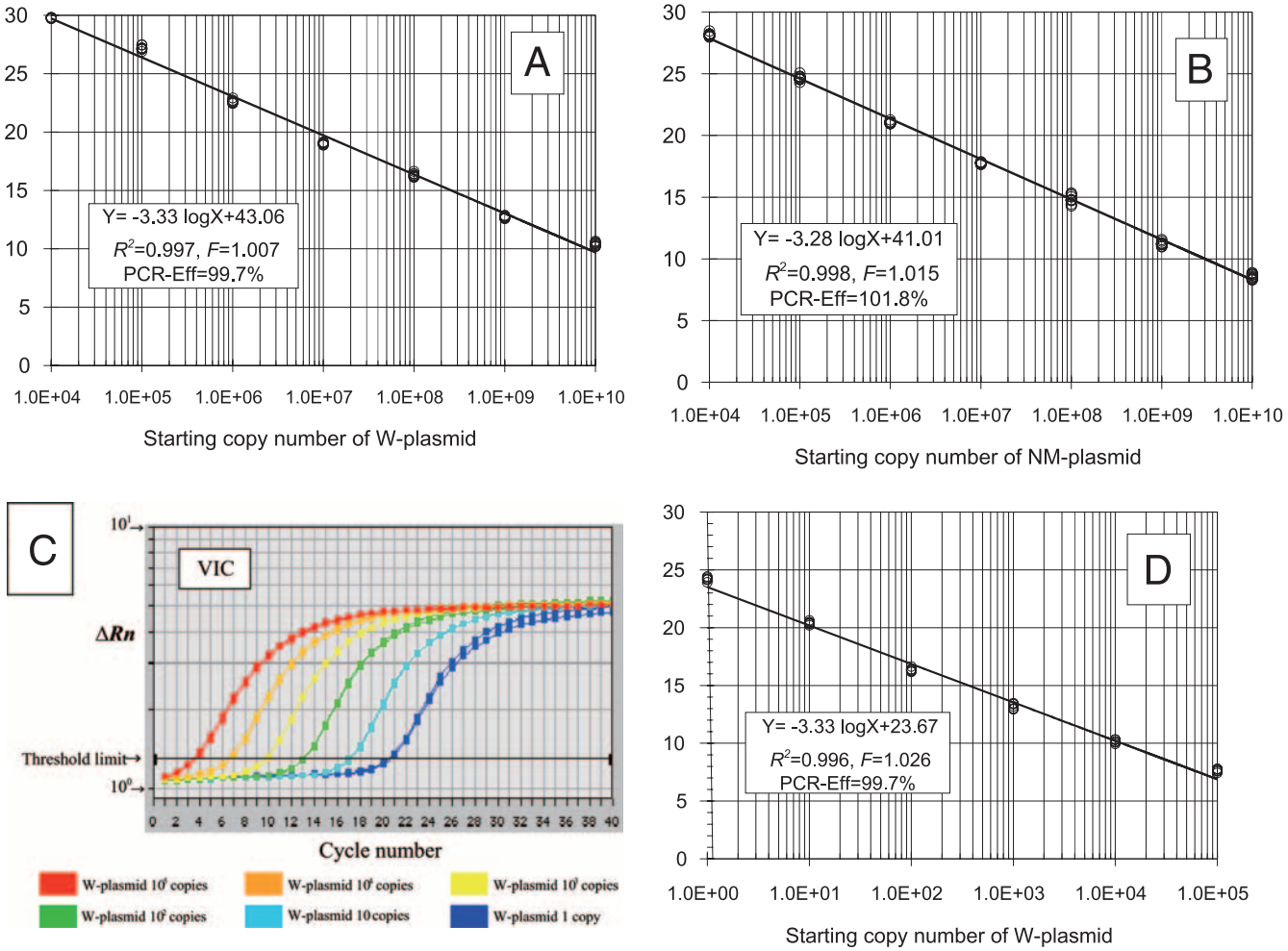


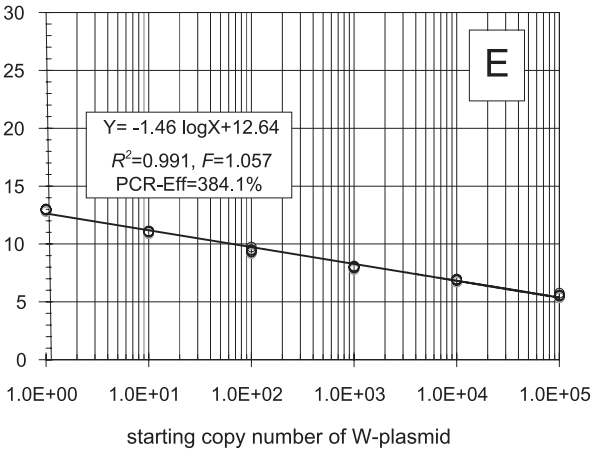
FIG. 2. Statistical evaluation of C_T value data in preliminary experiments. (A) Specific standard curve for use in the quantitative detection of *M. tuberculosis* DNA or W-plasmid. VIC (TqMn-W-VIC) was used for analysis. (B) Specific standard curve for use in the quantitative detection of the NM-plasmid as a new internal control. FAM (TqMn-M-FAM) was used for analysis. (C) Amplification curves for W-plasmids after first-step PCR at 25 cycles. (D) Result of simple regression analysis between C_T values (y axis) and the log of the starting copy numbers of W-plasmids (x axis) in setting first-step PCR at 25 cycles. (E) Result of simple regression analysis between C_T values (y axis) and the log of the starting copy numbers of W-plasmids (x axis) in setting first-step PCR at 35 cycles (OR-QNRT-PCR assay). (F) Amplification curves for 10^3 copies of NM-plasmids as the new internal control. (G) Comparative results of one-way ANOVA against C_T values for 10^3 copies of NM-plasmid (WR-QNRT-PCR assay) or M-plasmid (OR-QNRT-PCR assay) as an internal control.

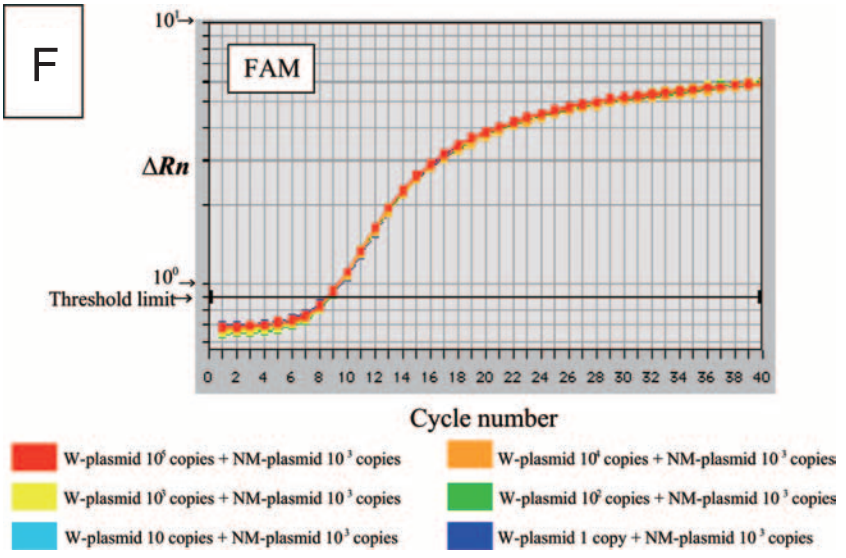
the slope of this linear regression curve (−1.46) indicated overamplification (PCR-Eff = 384.1%).

(ii) Optimal copy number of new internal control. To determine the optimal copy number of the new internal control, 10^3, 10^4, and 10^5 copies of NM-plasmids were examined. When 10^3 copies of NM-plasmid were set as the new internal control, the amplification curves of NM-plasmids revealed extremely uniform patterns in all starting copy numbers of W-plasmids (Table 3 and Fig. 2F). Reflecting Fig. 2F, the C_T values for 10^3 copies of the NM-plasmid also revealed significantly uniform variance between all starting copy numbers of W-plasmids ($F = 1.086$, $P = 0.774$) by one-way ANOVA (Fig. 2G). Therefore, 10^3 copies of NM-plasmid were adopted as the sufficient and optimal copy number of the new internal control for use as a "calibrator." However, when 10^3 copies of M-plasmid were set as the old internal control in the OR-QNRT-PCR assay (22), the C_T values for M-plasmid revealed unbalanced patterns (Table 3 and Fig. 2G). In particular, a significant difference was demonstrated for 10^3 to 10^5 copies of W-plasmid (Fig. 2G). In addition, when the first-step PCR was set at 25 cycles, as same as the WR-QNRT-PCR assay, the C_T values for the old M-plasmid were inconstant, and there was no difference in the C_T values for the 1 to 10^2 copies of W-plasmid (Table 3). These results indicated that M-plasmid as the old internal control was incomplete and needed more improvement.

DISCUSSION

We have developed an improved WR-QNRT-PCR assay technique for the accurate quantitative detection of *M. tuberculosis* DNA in CSF samples collected from patients with clinically suspected TBM. In the WR-QNRT-PCR assay, the initial copy number of *M. tuberculosis* DNA in CSF samples was





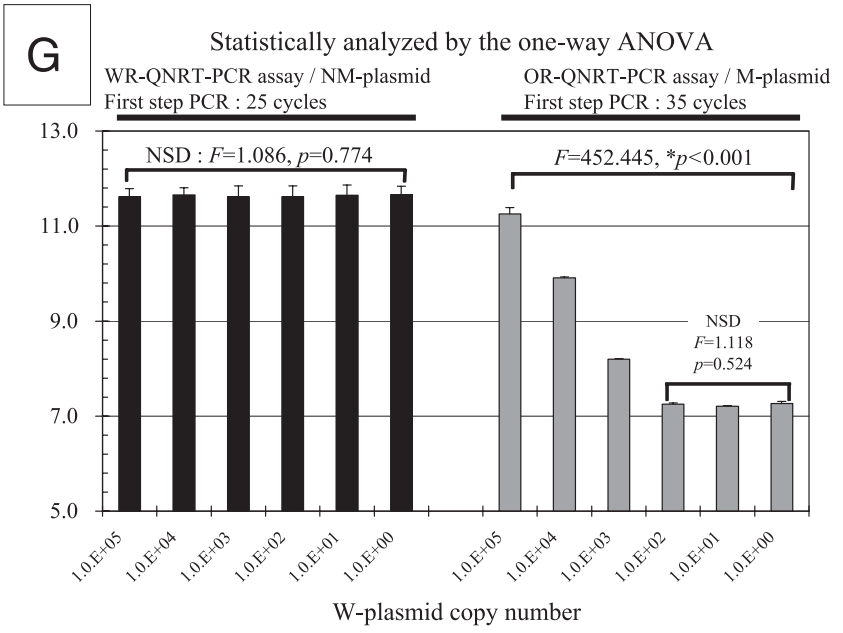


FIG. 2—*Continued*.

TABLE 3. C_T value data collected under different assay conditions in the WR- and OR-QNRT-PCR assays

W-plasmid starting copy no.	C_T value for[a]:					
	WR-QNRT-PCR assay (internal control, NM-plasmid [10^3 copies]); 25 cycles		OR-QNRT-PCR assay (internal control, M-plasmid [10^3 copies])			
			35 Cycles		25 Cycles	
	VIC	FAM	VIC	FAM	VIC	FAM
10^5	7.64 ± 0.17	11.62 ± 0.20	5.79 ± 0.01	25.27 ± 0.95	8.80 ± 0.03	40.00 (NA)[b]
10^4	10.16 ± 0.18	11.66 ± 0.18	6.99 ± 0.05	16.78 ± 0.37	12.85 ± 0.3	28.67 ± 0.05
10^3	13.20 ± 0.26	11.62 ± 0.27	10.19 ± 0.05	14.03 ± 0.08	16.87 ± 0.09	22.80 ± 0.13
10^2	16.37 ± 0.20	11.62 ± 0.26	11.29 ± 0.07	13.39 ± 0.06	19.61 ± 0.09	19.36 ± 0.21
10	20.48 ± 0.18	11.65 ± 0.25	12.71 ± 0.09	13.37 ± 0.06	19.99 ± 0.04	16.64 ± 011
1	24.20 ± 0.22	11.66 ± 0.20	14.25 ± 0.05	13.32 ± 0.04	20.04 ± 0.24	16.45 ± 0.33

[a] C_T value data represent the means ± standard deviations in duplicate for five independent experiments. The numbers of cycles are for first-step PCR. Reporter dyes VIC and FAM are indicated.
[b] NA, no amplification.

calculated from the amplification ratio of the specific new internal control used as a "calibrator," as was the case for the OR-QNRT-PCR assay. For use as the specific new internal control, the original NM-plasmid was designed to have equivalent amplification and detection efficiency against actual *M. tuberculosis* DNA. Based on a similar concept, specific primers and probes were prepared. Consequently, we were able to formulate equation 1, which can be used to determine initial copy number.

For the accurate quantitative detection of a small amount of *M. tuberculosis* DNA in CSF samples by WR-QNRT-PCR assay, it is extremely important that both *M. tuberculosis* DNA and the new internal control are amplified with sufficient balance. Therefore, the precision of the two specific standard curves was strictly examined by statistical evaluation in a series of preliminary experiments. Previously, many investigators have reported that the precision of the standard curve is the principal factor for quantitative detection in real-time (TaqMan) PCR assays (1–6, 9, 10, 13, 15–17, 20, 24, 26, 27). In this study, the two specific standard curves demonstrated statistically significant precision ($R^2 > 0.99$, $F = 1.007$ or 1.015) (Fig. 2A and B). Therefore, we consider that any overall errors relating to the dilution procedure or within each experiment can be disregarded. In addition, the PCR-Eff values of two standard curves calculated by the slopes (−3.33 and −3.28) were 99.7 and 101.8%, respectively. These results indicated that the efficiency of amplification and detection for both *M. tuberculosis* DNA and the new internal control was almost equivalent in the WR-QNRT-PCR assay. Therefore, our hypothesis was proved experimentally by these results.

The optimal assay conditions were examined in detail by statistical analysis for C_T value data collected from the preliminary experiments. In setting 25 cycles as the optimal cycle number for the first-step PCR, the primary concentration gradient for all starting copy numbers of W-plasmid was completely preserved (Fig. 2C and D). Moreover, in setting 10^3 copies of NM-plasmid as the optimal copy number of the new internal control, extremely uniform amplifications were demonstrated for all starting copy numbers of W-plasmids (Fig. 2F and G). These results indicate that there is no interference between *M. tuberculosis* DNA and the new internal control in the entire PCR amplification procedure. Therefore, the new internal control could be regarded as appropriate for use as a "calibrator" in the WR-QNRT-PSR assay.

In the OR-QNRT-PCR assay, both *M. tuberculosis* DNA and the old internal control (M-plasmid) were simultaneously amplified using two pairs of common primers (22). This system is the most serious weak point of the OR-QNRT-PCR assay because the amplification for a small copy number of template (*M. tuberculosis* DNA or M-plasmid) was poor owing to the interference by a high copy number of template. In order to obtain sufficient amplification for a small amount of *M. tuberculosis* DNA (<100 copies), the first-step PCR cycle number need to be set at a large number (35 cycles) in the OR-QNRT-PCR assay (22). This led to the problems of overamplification (Fig. 2E) and the instability of the M-plasmid for a large copy number (>1,000) of *M. tuberculosis* DNA (Fig. 2G). Therefore, the OR-QNRT-PCR assay was limited necessarily to being within a narrow detection range (22). Due to the development of NM-plasmid as the new internal control, the stable and accurate quantitative detection of *M. tuberculosis* DNA was possible in a detection range wider than that for the OR-QNRT-PCR assay.

In this study, we attempted to improve the OR-QNRT-PCR assay and developed NM-plasmid for use as a new internal control. Due to the development of NM-plasmid, significantly improved quantitative accuracy and a wider detection range were realized with the WR-QNRT-PCR assay. In the clinical application of the WR-QNRT-PCR assay, the advantages of this method would be powerful tool for rapid and accurate diagnosis in the difficult cases in which it is impossible to detect *M. tuberculosis* by conventional assay methods. In our next study, we plan to examine and evaluate the clinical usefulness of the WR-QNRT-PCR assay for the rapid and accurate diagnosis of TBM and for assessing the clinical course of TBM.

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