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Research article

Method validation of SYBR Green RT-PCR Assay for Identification of Strains of Pentavalent Rotavirus Vaccine

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Abstract

Rotavirus is a leading cause of severe gastroenteritis in infants and young children, contributing to significant global morbidity and mortality. Vaccination with rotavirus vaccines, particularly the pentavalent vaccine targeting G1P7, G2P7, G3P7, G4P7, and G6P[8] strains, provides broad protection against multiple serotypes responsible for severe disease. Accurate identification of these strains is essential for monitoring vaccine efficacy and safety. The World Health Organization (WHO) has established strict quality control guidelines to ensure the safety, efficacy, and consistency of rotavirus vaccines. This study aims to validate a SYBR Green-based reverse transcription polymerase chain reaction (SYBR Green-based RT-PCR) assay for the precise identification of strains included in the pentavalent rotavirus vaccine. The validated assay showed high specificity and reproducibility. Positive identification was confirmed for all target strains with cycle threshold (Ct) values <40.00. The assay yielded Ct values of 27.76±0.48 (G1P7), 27.76±0.74 (G2P7), 28.45±1.43 (G3P7), 28.35±0.42 (G4P7) and 30.19±1.30 (G6P[8]). Melting curve analysis confirmed specificity with single peaks at 77.03±0.03°C (G1P7), 74.50±0.07°C (G2P7), 76.42±0.21°C (G3P7), 75.58±0.17°C (G4P7), and 77.29±0.29°C (G6P[8]). Intra-assay repeatability showed %CV values of 0.14-11.47 (Ct) and 0.01-0.38 (Tm), while inter-assay reproducibility ranged from 0.68-9.51 (Ct) and 0.06-0.26 (Tm). Ruggedness/robustness testing across analysts yielded %CV 2.17-11.47 (Ct) and 0.04-0.32 (Tm). These results confirm the method's consistency and effectively identified rotavirus strains in vaccines, supporting reliable vaccine quality control and surveillance efforts.

Keywords: Validation, Identification, Rotavirus Vaccine, SYBR Green RT-PCR

Introduction

Rotavirus (RV) was first identified in the 1950s from rectal swab samples collected from monkeys[1]. In 1973, Bishop and colleagues reported the first report of rotavirus in children suffering from gastroenteritis[2]. Globally, it is estimated that approximately 258 million infants and young children under five contract rotaviruses annually. This infection frequently results in severe diarrhea and dehydration due to gastroenteritis,

often accompanied by electrolyte imbalances[3]. Recent estimates show that rotavirus remains a leading cause of diarrheal mortality, accounting for ~19% of all diarrhoea-related deaths in 2019 (≈ 235,331 deaths across all ages), with a substantial share occurring in children <5 years; globally, there were ~1.76 million rotavirus hospitalizations in 2019[4]. Rotavirus is a member of the Reoviridae family. It is a non-enveloped virus with a diameter ranging from 50 to 76 nanometers, containing 11 segments of double-stranded RNA. These segments encode six structural viral proteins (VP1-VP4, VP6, and VP7) and five nonstructural proteins (NSP1-NSP5)[5]. The viral capsid proteins (VPs) determine rotavirus subgroups and serotypes, while the nonstructural proteins are involved in viral replication and pathogenesis. Variations in the VP7 and VP4 proteins have led to the classification of rotaviruses into 36 glycoprotein (G) genotypes and 51 protease-sensitive (P) genotypes[6].

In 2009, the WHO recommended the inclusion of rotavirus vaccines in national immunization programs worldwide. Following this recommendation, Thailand's Department of Disease Control initiated a pilot program in 2011 to incorporate rotavirus vaccines into its national immunization schedule. Currently, four types of rotavirus vaccines are registered and available in Thailand: Rotarix™, RotaTeq™, Rotavac®, and Rotasiil®[7]. Clinical studies have consistently demonstrated the efficacy and safety of these vaccines. However, identifying the appropriate serotypes for multivalent vaccine production remains essential to mitigate the risk of the emergence of new strains via reassortment in live-attenuated vaccines.

Ensuring the safety, efficacy, and consistency of vaccines is paramount in the global fight against infectious diseases. The WHO's quality control guidelines emphasize the importance of precise strain identification, genetic stability testing, and comprehensive safety assessments. Although the genetic sequencing is technically demanding and costly, it remains essential for ensuring vaccine identity and potency. WHO guidelines stipulate the use of genetic sequences from the VP7 gene segment and quantitative PCR techniques for serotype identification and quality control[8]. Techniques such as rotavirus multivalent SYBR Green polymerase chain reaction (SYBR Green RT-PCR assay) have been developed to verify the identity and stability of multivalent rotavirus vaccine serotypes, providing rapid, sensitive, and specific results for both imported vaccines and potential future domestic production. Adherence to these guidelines ensures that manufacturers and health authorities produce high-quality vaccines, thereby enhancing public confidence in immunization programs and contributing to global efforts to reduce the burden of rotavirus-related illnesses. Continuous monitoring and validation by the WHO uphold the integrity of rotavirus vaccines, safeguarding public health on a global scale.

The SYBR Green RT-PCR assay offers a promising alternative for vaccine quality control, providing real-time quantification and high sensitivity in detecting viral RNA. This study focuses on improving and validating the SYBR Green RT-PCR assay for the precise identification of rotavirus strains in the pentavalent rotavirus vaccine. By optimizing assay parameters and validating its performance, the study aims to develop a robust and reliable method to enhance the monitoring and evaluation of rotavirus vaccines, ultimately contributing to better public health outcomes.

Thus, the purpose of this study is to provide an overview of the method validation of a SYBR Green RT-PCR assay for the detection of rotavirus strains in the pentavalent rotavirus vaccine. Through the use of advanced molecular techniques, this assay aims to standardize the identification of rotavirus strains for quality control purposes, contributing to the improved reliability and safety of rotavirus vaccines.

Method

This experimental research study was conducted between October 2023 and September 2024. The study focuses on developing optimal conditions and validating a method for identifying the strains of the pentavalent rotavirus vaccine using the SYBR Green RT-PCR assay.

1) Materials

In this study, we used a single production batch of the pentavalent rotavirus vaccine (RotaTeq®,

batch X008547). Live-attenuated RNA virus vaccines were included as negative controls, specifically one batch each of a tetravalent dengue vaccine (Qdenga®, batch 546543) and one batch of a yellow fever vaccine (Stamaril®, batch W3A371V), both of which were imported and commercially available in Thailand.

2) RNA Extraction and Purification

Total viral RNA was extracted from pentavalent rotavirus vaccine using phenol:chloroform:isoamyl Alcohol. Briefly, 500 µL of pentavalent Rotavirus Vaccine and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Invitrogen, cat no. 15593031) were added into a 1.5 mL microtube and homogenized for 5 minutes by vortexing. Then, the samples were centrifuged at 15,000 rpm for 15 minutes, and the upper aqueous phase was carefully transferred to a new 1.5 mL tube. Additionally, RNA in the aqueous phase was purified using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, cat. 12280050), applying explicitly defined protocol modifications to ensure clarity and reproducibility. The manufacturer's initial lysis step for biological specimens was omitted and processing commenced at the binding step because the phenol: chloroform aqueous phase is already denatured. The extracted RNA was stored immediately at −20°C until further processing. The total RNA concentration, quality and purity of RNA were assessed by measuring absorbance at 260/280 nm using the Nanodrop spectrophotometer.

3) Qualitative analysis of strains of pentavalent rotavirus RNA based on SYBR green RT-PCR

This protocol outlines the steps for conducting RT-PCR experiments to amplify and confirm the identity of strains in pentavalent rotavirus vaccine samples using SYBR Green as the detection method. The primers targeting the VP7 gene segment (G1P7, G2P7, G3P7, G4P7 and G6P[8] of rotavirus) were derived from published sequences[9] and are listed in Table 1

Table 1 Primers used in this protocol[9].

Reassortant	Primer	Sequence
G1P7	Forward	5′-TGTCTGTATTATCCAACTGAAGCAAGT-3′
	Reverse	5′-CCCTTTGTAAGAAAACATTTGCGA-3′
G2P7	Forward	5′-GATGAATGGGAAAATACTCTATCACAATT-3′
	Reverse	5′-TCATGGAAAATGTAGTAATGTCATTGTAGT-3′
G3P7	Forward	5′-ACTCCTTAATGCACAAAATTATGGAA-3′
	Reverse	5′-TCCTCTCGCGTTGAGTTCG-3′
G4P7	Forward	5′-AAAGATACACTATCTCAACTGTTTTTAACCA-3′
	Reverse	5′-TCGATGGAAAATTCTAAAACGTTT-3′
G6P[8]	Forward	5′-TGGAAAGATACCTTATCACAACTGTTCTTA-3′
	Reverse	5′-GAAAAGGCCGCTATATCAGCA-3′

SYBR Green–based RT-PCR was performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA) using the SensiFAST™ SYBR Lo-ROX One-Step Kit (Bioline, cat. no. BIO-74001). Each 20 µL reaction contained 10 µL of 2×SensiFAST SYBR Lo-ROX One-Step Mix, 0.2 µL reverse transcriptase, 0.4 µL RiboSafe RNase Inhibitor, 0.4 µM of each primer, and 5 ng of total RNA from the pentavalent rotavirus vaccine (1 µL at 5 ng/µL).

This assay is a qualitative identification procedure. In accordance with ICH Q2(R1), validation focused on analytical specificity, repeatability, and ruggedness; a formal limit-of-detection (LOD) study was not performed. To ensure robust detection, each reaction contained 5 ng total RNA per 20 µL, with templates prepared from 1 or 10 ng/µL working stocks. Serotype calls were based on exponential amplification together with a single melt peak whose Tm matched the serotype-specific reference window.

In this experiment, all PCR samples were run using RT-PCR cycling conditions adapted from the method previously reported by Ranheim et al.[9] The thermal profile included the following steps: reverse transcription at 45°C for 30 minutes to synthesize cDNA, initial denaturation at 95°C for 10 minutes to activate the Taq polymerase and denature double-stranded DNA, followed by 40 amplification cycles consisting of denaturation at 95°C for 20 seconds, annealing at 55°C for 1 minute, and extension at 55 °C for 15 seconds. To determine the melting temperatures to confirm the specificity of PCR products, the temperature was raised between 60-95°C and a pre-melting step at 60°C for 1 minute, followed by a temperature increase of 0.05°C/s to 95°C. From each reaction, the threshold cycle value (Ct) was established as the cycle number at which fluorescence was detectable over the threshold value calculated by the QuantStudio software.

4) Method Validation

The parameters for the validation of the analytical method using real-time PCR are based on the Guidelines for Validation of Qualitative Real-time PCR Methods[10] and Requirements for evaluating the performance of quantification methods for nucleic acid target sequences qPCR and dPCR (ISO 20395:2019(en)[11]

4.1) Specificity

Consistent with ICH Q2(R1)[12], specificity is the principal validation characteristic for an identification procedure. Our validation covered intra- and inter-serotype specificity across rotavirus vaccine serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8], and heterologous specificity using dengue (DENV) and yellow fever live-attenuated RNA vaccines as negative controls[12]. The SYBR Green RT-qPCR assay was used to determine rotavirus serotype identity in vaccine samples, while DENV and yellow fever vaccines served as negative specificity controls. For each rotavirus serotype and for DENV and yellow fever controls, 5 ng total RNA per 20 µL reaction (1 µL at 5 ng/µL) was used with SensiFAST™ SYBR Lo-ROX One-Step on a QuantStudio 6 Flex system. At least three independent replicated tests were performed, and coefficients of variation were calculated (acceptance criterion CV < 25%)[13]. To evaluate analytical specificity under matrix-matched conditions, DENV and yellow fever vaccines (Flaviviridae; +ssRNA) were used as heterologous negatives, taxonomically/genomically unrelated to rotavirus (Reoviridae; segmented dsRNA). Across all runs, negatives showed no exponential amplification (Ct = UND). Occasional low-amplitude melt deflections were observed in negatives but fell outside the predefined serotype-specific Tm windows established in Figure 2 and were therefore classified as UND for both Ct and serotype-specific Tm. Predefined acceptance criteria for negatives were the absence of Ct and the absence of a serotype-specific Tm within the Figure 2 windows (reported as UND).

4.2) Precision

Because the assay is qualitative, statistics for Ct and Tm (mean±SD; %CV) are reported as parameter precision (supportive) to document assay stability and are not interpreted as quantitative precision of analyte amount. Qualitative result reproducibility is presented as identity-call agreement (positive/negative) across technical replicates, days, and analysts. The precision assessment includes the following sections:

- Intra-assay or Repeatability Testing: This test involves determining the identity of the rotavirus serotype in the vaccine sample within the same day and time. For each rotavirus serotype strain, 1 µL of RNA (1 and 10 ng/µL) was mixed with SensiFAST™ SYBR Lo-ROX One-Step and was performed by QuantStudio 6 Flex system. Intra-assay precision was evaluated by assessing the consistency of detection and quantification of each rotavirus serotype within a single run (n=3 replicates). The resulting values

are used to calculate the coefficient of variation, which must be less than 25%[13].

- Inter-assay or Reproducibility Testing: This test assesses the amounts of the identity of the rotavirus serotype in the vaccine sample on different days and times. For each serotype strain of rotavirus, 1 µL of RNA (1 and 10 ng/µL) was mixed with SensiFAST™ SYBR Lo-ROX One-Step and was performed by QuantStudio 6 Flex system. Inter-assay precision was assessed by determining the reproducibility of detection and quantification of rotavirus serotypes across three independent runs performed on different days. Repeatability (intra-assay) and intermediate precision (inter-assay) assessed the consistency and reproducibility of measured Ct/Tm across serotypes, with an acceptance criterion of %CV <35%[13].

4.3) Ruggedness/Robustness

This test involves determining the amounts of the identity of the rotavirus serotype in the vaccine sample by two different analysts, with each performing three technical replicates. For each serotype strain of rotavirus, 1µL of RNA (1ng/µL) was mixed with SensiFAST™ SYBR Lo-ROX One-Step and was performed by QuantStudio 6 Flex system. The resulting values are used to calculate the coefficient of variation, which must be less than 35%[13].

Results

Specificity

We developed and applied a SYBR Green–based RT-PCR assay to detect strains present in the pentavalent rotavirus vaccine (G1P7, G2P7, G3P7, G4P7, and G6P[8]). During RNA extraction using phenol: chloroform: isoamyl alcohol and purified with spin column, for validation of the identification test, the sample was considered positive for VP7 gene segment from the G1P7, G2P7, G3P7, G4P7 and G6P[8] reassortants matched when the cycle threshold amplification curve for the target genes crossed the threshold line within 40 cycles (Ct<40.00) and was showed by a single serotype-specific melt peak. Figure 1 shows the distribution of cycle threshold (Ct) values by RT-PCR, a SYBR Green RT-PCR amplification plot showing the target genes of the VP7 gene segment from the G1P7, G2P7, G3P7, G4P7, and G6P[8] reassortants of rotavirus. Figure 1 illustrates one representative run (duplicate wells). All Ct values summarized in the text and tables represent mean ± SD across three independent runs; therefore, the representative traces in Figure 1 are not expected to match the pooled means exactly. The fluorescence signals yielded Ct values of 27.76 ± 0.48, 27.76 ± 0.74, 28.45 ± 1.43, 28.35 ± 0.42, and 30.19 ± 1.30 for G1P7, G2P7, G3P7, G4P7, and G6P[8], respectively (Table 2)

For clarity, Figures 2 and 3 present the same representative dataset from the same experiment. Figure 2 emphasizes identification of serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8], whereas Figure 3 demonstrates analytical specificity alongside negative controls (reported as UND for serotype-specific Tm).

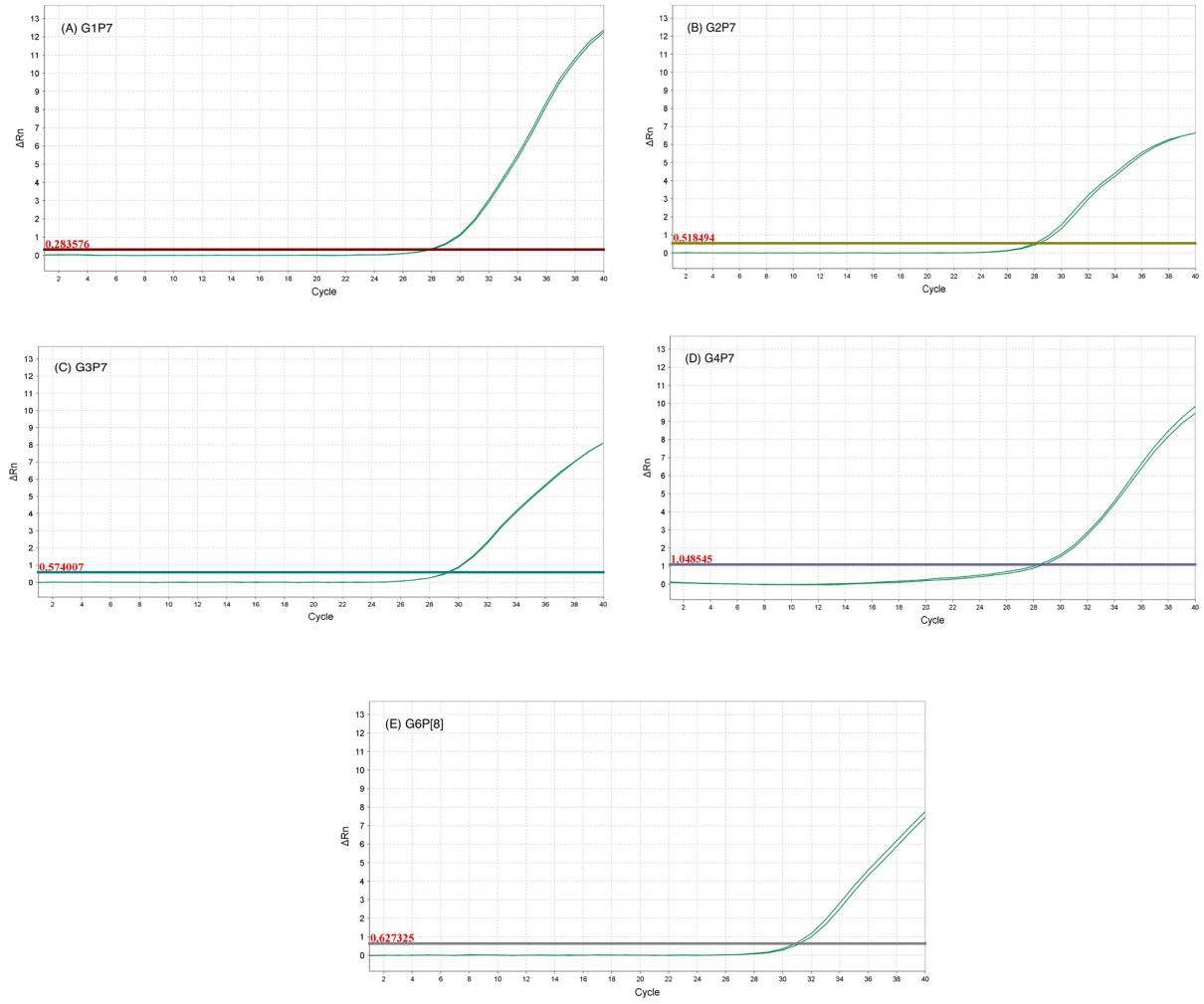

Figure 1 Representative amplification plots (one run, duplicate wells). Panels A–E: G1P7, G2P7, G3P7, G4P7, G6P[8]. Calls: Ct < 40 cycles. Note: Text/table Ct = mean ± SD across three independent runs, so traces from this single run will not numerically equal pooled means.

As shown in Figure 2A-E, the results of this representative experiment show that the melting curves displayed a single melting Tm for identified rotavirus strains. Table 4 shows that the Tm values at 1 ng were 77.03±0.03°C, 74.50±0.07°C, 76.42±0.21°C, 75.58±0.17°C and 77.29±0.29°C and the melting Tm for identified rotavirus strains at 10 ng were 77.12±0.01°C, 74.38±0.14°C, 76.59±0.09°C, 75.69±0.07°C and 77.47±0.09°C for the rotavirus strains G1P7, G2P7, G3P7, G4P7, and G6P[8] reassortants, respectively. In addition, all five SYBR Green RT-PCR assays gave a unique melting peak for each serotype with different Tm values.

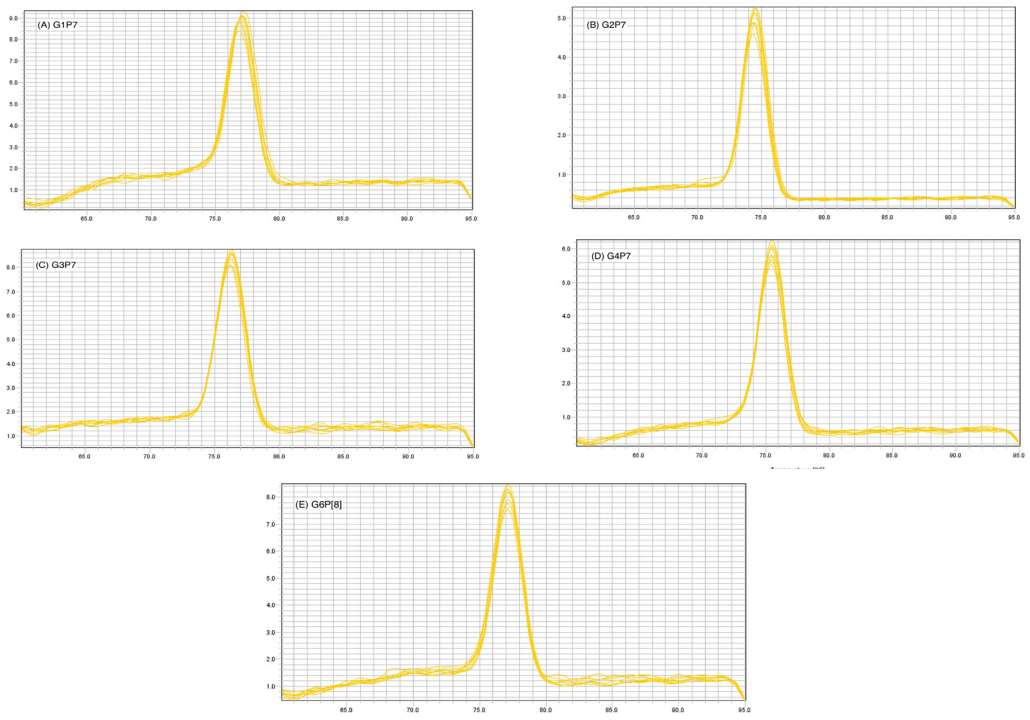

Figure 2 Melt-curve analysis showing single, serotype-specific peaks for G1P7, G2P7, G3P7, G4P7, and G6P[8] (Panels A–E). Serotype-specific Tm values are reported in the Results (Identification). RNA input: 5 ng total per 20 µL reaction (1 µL at 5 ng/µL). Note: Figures 2 and 3 present the same representative dataset from the same experiment.

The nucleotide sequences of primers specific to various rotavirus serotypes, including G1P7, G2P7, G3P7, G4P7, and G6P[8], were analyzed using the Primer BLAST function (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The results indicated that the designed primers exhibited specificity solely for rotavirus, without any cross-reactivity or mismatches with other rotavirus serotypes or DNA from other organisms. The specificity of the test was determined using the SYBR Green RT-PCR method with an RNA input of 5 ng total per 20 µL reaction (1 µL at 5 ng/µL). The rotavirus, dengue, and yellow fever vaccines served as negative controls for this test, and genetic material was amplified with a real-time PCR machine. The Cycle threshold (Ct) values were determined for rotavirus serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8], with three independent runs on separate days, each with n = 3 technical replicates per serotype. The mean values for each vaccine were calculated. The results confirmed that the designed primers were specific to rotavirus only, without any cross-reactivity with other rotavirus serotypes, as shown in Table 2. The one-step SYBR Green RT-PCR did not amplify the genomes of other viruses including dengue and yellow fever viruses. The melting curve analysis showed Tm values at 1 ng were 77.03±0.03°C, 74.50±0.07°C, 76.42±0.21°C, 75.58±0.17°C and 77.29±0.29°C for G1P7, G2P7, G3P7, G4P7, and G6P[8], respectively.

Moreover, Figure 3A-E shows melt-curve analysis from the same representative experiment as Figure 2. Rotavirus serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8] yielded single, serotype-specific melting peaks with Tm values of 77.03±0.03°C, 74.50±0.07°C, 76.42±0.21°C, 75.58±0.17°C, and 77.29±0.29°C, respectively (as reported in Figure 2). By contrast, dengue and yellow fever vaccines showed no exponential amplification (Ct = UND). Low-amplitude melt deflections occasionally reported by the instrument due to SYBR Green detecting nonspecific duplexes (e.g., primer-dimers) fell outside the serotype-specific Tm ranges defined in Figure 3 and, per our calling criteria, were reported as UND for both Ct and serotype-specific Tm. The clear separation between rotavirus Tm values and any nonspecific deflections supports the high analytical specificity of our primers and the absence of cross-reactivity with dengue or yellow fever RNA[14,15].

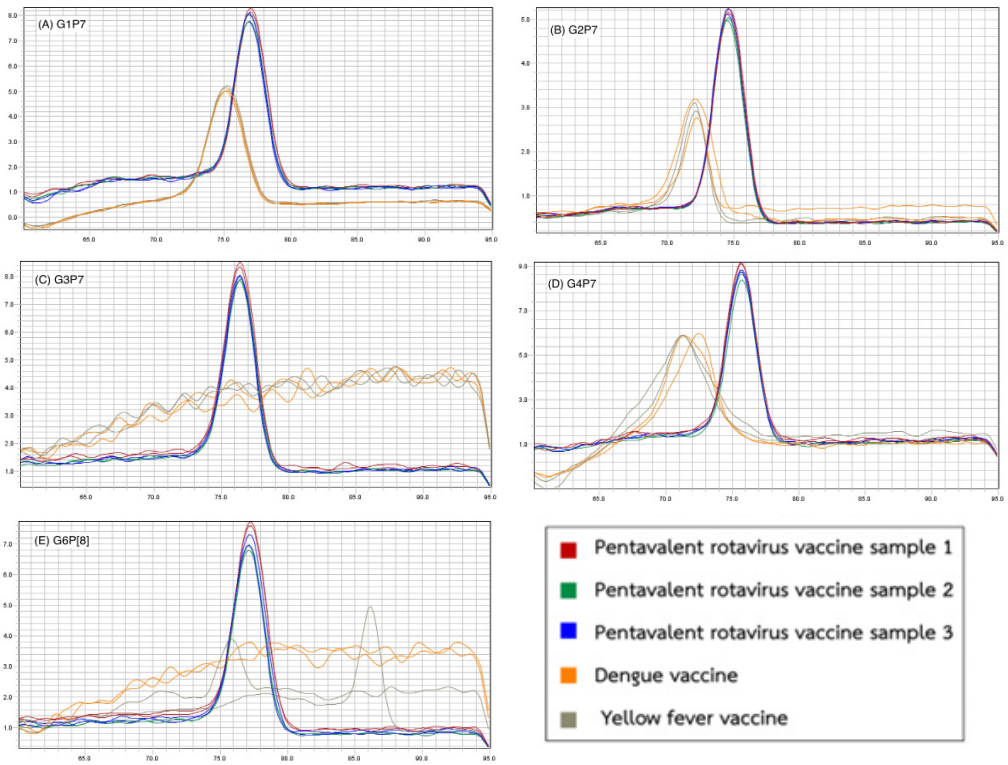

Figure 3 Panel 3A-E Analytical specificity testing using dengue and yellow fever vaccines as negative controls. Panels A–E show rotavirus melting curves for G1P7, G2P7, G3P7, G4P7, and G6P[8], respectively and comparison (from the same representative dataset as Figure 2) alongside the negative controls. Non-target vaccines (dengue, yellow fever) yielded no Ct and no rotavirus-range Tm, thus reported as UND for serotype-specific Tm. RNA input: 5 ng total per 20 µL reaction (1 µL at 5 ng/µL).

Table 2 The specificity testing of primers using the in vitro RT-PCR method, conducted in three technical replicates for rotavirus vaccine serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8].

Serotype	Rotavirus vaccine	Dengue vaccine	Yellow fever vaccine
	Mean±SD Ct value (N=3)	Mean±SD Ct value (N=3)	Mean±SD Ct value (N=3)
G1P7	27.76 ± 0.48	UND	UND
G2P7	27.76 ± 0.74	UND	UND
G3P7	28.45 ± 1.43	UND	UND
G4P7	28.35 ± 0.42	UND	UND
G6P[8]	30.19 ± 1.30	UND	UND

Remark; UND = Undetermined

Precision

Tables 3 and 4 show the results of intra-assay or repeatability testing of the amounts of identification tests of the vaccine sample on the same day and time involving at least three independent replicates. The analysis of the identity amounts for all three repeats shows a %CV value between 0.14-11.47 of Ct and of 0.01-0.38 of Tm values in G1P7, G2P7, G3P7, G4P7 and G6P[8] reassortants of rotavirus, which meets the specified criteria.

Table 3 Repeatability of the Ct values evaluated SYBR green RT-PCR assay for the VP7 segment of rotavirus. The repeatability test was performed with three technical replicates on the same days.

Strain of rotavirus	RNA per reaction (ng)	Repeatability on Ct	
		Ct mean±SD	%CV
G1P7	10 ng	27.80±0.17	0.62
	1 ng	33.06±1.07	3.23
G2P7	10 ng	31.70±0.23	0.72
	1 ng	34.09±0.74	2.17
G3P7	10 ng	24.50±0.03	0.14
	1 ng	30.88±1.96	6.35
G4P7	10 ng	30.76±3.18	10.33
	1 ng	32.90±1.72	5.22
G6P[8]	10 ng	22.43±0.29	1.29
	1 ng	28.10±3.22	11.47

Table 4 Repeatability of the Tm values evaluated SYBR Green RT-PCR assay for the VP7 segment of rotavirus. The repeatability test was performed with three technical replicates on the same days.

Strain of rotavirus	RNA per reaction (ng)	Repeatability on Tm	
		Ct mean±SD	%CV
G1P7	10 ng	77.12±0.01	0.01
	1 ng	77.03±0.03	0.04
G2P7	10 ng	74.38±0.14	0.19
	1 ng	74.50±0.07	0.09
G3P7	10 ng	76.59±0.09	0.12
	1 ng	76.42±0.21	0.27
G4P7	10 ng	75.69±0.07	0.10
	1 ng	75.58±0.17	0.23
G6P[8]	10 ng	77.47±0.09	0.12
	1 ng	77.29±0.29	0.38

The results of inter-assay or reproducibility testing of the identity of virus strains in the vaccine sample conducted on different days and at different times (separate experiments) were obtained from at least three technical replicates (Supplementary Table S1-S2). The Ct values for each strain were within the 27 to 35 cycle range in the amplification process at RNA concentration 1 ng/µL, while higher RNA concentrations as 10 ng/µL appeared within 22 to 30 cycles as shown in Table 5. As seen in Table 6, all plots displayed a single melting domain, typically between 74-77°C, and melting peaks over three different days show a %CV value of 0.06-0.26.

Table 5 Reproducibility of the Ct values evaluated SYBR Green RT-PCR assay for the VP7 segment of rotavirus. The reproducibility test was conducted as three technical replicates on different days (three days).

Strain of rotavirus	RNA per reaction (ng)	Repeatability on Ct	
		Ct mean±SD	%CV
G1P7	10 ng	27.73±0.19	0.68
	1 ng	33.06±0.68	2.06
G2P7	10 ng	30.82±0.94	3.06
	1 ng	34.42±0.56	1.62
G3P7	10 ng	25.00±0.71	2.84
	1 ng	30.51±1.55	5.07
G4P7	10 ng	27.64±2.54	9.19
	1 ng	35.54±1.43	4.03
G6P[8]	10 ng	22.79±0.83	3.64
	1 ng	27.59±2.62	9.51

Table 6 Reproducibility of the Tm values evaluated SYBR green RT-PCR assay for the VP7 segment of rotavirus. The reproducibility test was conducted as three technical replicates on different days (three days).

Strain of rotavirus	RNA per reaction (ng)	Repeatability on Tm	
		Ct mean±SD	%CV
G1P7	10 ng	77.04±0.08	0.11
	1 ng	76.96±0.12	0.16
G2P7	10 ng	74.48±0.08	0.11
	1 ng	74.44±0.19	0.26
G3P7	10 ng	76.54±0.03	0.04
	1 ng	76.42±0.17	0.22
G4P7	10 ng	75.71±0.05	0.06
	1 ng	75.61±0.16	0.21
G6P[8]	10 ng	77.47±0.10	0.12
	1 ng	77.36±0.19	0.25

Ruggedness/Robustness

For the ruggedness/robustness, the %CV was calculated for each serotype of rotavirus on the Ct and the Tm values. For each SYBR Green RT-PCR assay, this %CV was between 2.17 to 11.47% for the Ct values and between 0.04 to 0.32% for the Tm values. The %CV values of the developed SYBR Green RT-PCR comply with the acceptance limits are shown in Table 7.

Table 7 Ruggedness/robustness of the SYBR Green RT-PCR assay for five rotavirus serotypes: inter-analyst comparison of Ct and Tm. Ruggedness was assessed across analysts; each analyst performed three technical replicates per serotype (n=3 per analyst).

		Ruggedness/Robustness test of the five serotypes of rotavirus detected by SYBR Green RT-PCR assay				
		G1P7	**G2P7**	**G3P7**	**G4P7**	**G6P[8]**
Ct	**Analyst 1**	33.82	34.61	29.50	31.69	25.82
	Analyst 2	32.30	33.56	32.27	34.12	30.37
	%CV	3.25	2.17	6.35	5.22	11.47
Tm	**Analyst 1**	77.05	74.76	76.56	75.71	77.46
	Analyst 2	77.00	74.42	76.28	75.46	77.11
	%CV	0.04	0.32	0.27	0.23	0.32

Discussion

The strain-specific VP7 primers originally described by Ranheim et al.[9] Although these primers have been widely adopted in rotavirus research, primarily within cell-based quantitative RT-qPCR assay (C-QPA) in which vaccine virus is first propagated in culture and then quantified by RT-qPCR[16] their application outside this context has been limited. In contrast, prior RT-qPCR applications detecting vaccine components have focused on stool matrices rather than the vaccine[17]. In this study, we extracted viral RNA from the pentavalent vaccine using phenol:chloroform:isoamyl alcohol method and purified nucleic acids with a spin-column kit (PureLink™ Viral RNA/DNA Mini Kit), enabling direct RT-PCR detection of the component strains without prior cell culture, yielding results concordant with culture-based identification (Supplementary Table S3-S4, Figure S1-S2). Our findings show that a SYBR Green RT-PCR workflow targeting VP7 provides high analytical specificity, repeatable Ct/Tm parameters, and inter-analyst ruggedness sufficient to support qualitative identity calls for the five vaccine serotypes at a standardized input (5 ng per 20 µL reaction). In this role, the assay functions as a cost-effective, time-efficient first-line identity check that can triage samples for confirmatory sequencing when comprehensive genomic characterization is required.

The study demonstrated that the SYBR Green RT-PCR assay exhibited high specificity in detecting pentavalent rotavirus strains. Fluorescence signals yielded Ct values ranging from 27.76±0.48 to 30.19±1.30, and melting-curve analysis showed a single, serotype-specific Tm for each reassortant with tight dispersion: 77.03±0.03°C (G1P7), 74.50±0.07°C (G2P7), 76.42±0.21°C (G3P7), 75.58±0.17°C (G4P7), and 77.29±0.29°C (G6P[8]), supporting robust qualitative identification across serotypes. The single melting peak for each serotype further validated the specificity of the assay, ensuring no cross-reactivity with non-target sequences. These findings are consistent with previous research emphasizing the utility of qPCR assays for virus detection and vaccine monitoring[9]. Repeatability and reproducibility testing confirmed the assay's robustness, with %CV values for Ct and Tm well within acceptable limits. Intra-assay repeatability testing showed %CV values ranging from 0.14% to 11.47% for Ct and 0.01% to 0.38% for Tm, indicating consistent results within the same day. Similarly, inter-assay reproducibility testing across different days showed %CV values of 0.68% to 9.51% for Ct and 0.06% to 0.26% for Tm, affirming the assay's reliability in different experimental conditions. These results align with established guidelines for analytical method validation[13]. Additionally, the ruggedness/robustness analysis confirmed that the assay performed consistently across different analysts, with %CV values between 2.17% and 11.47% for Ct and between 0.04% and 0.32% for Tm. This underscores the reproducibility of the method across various laboratory settings, an essential criterion for routine application in vaccine quality control.

For routine vaccine quality control, SYBR Green RT-PCR provides a rapid, lower-cost alternative for strain identification, with high throughput and faster turnaround than conventional genotyping workflows that rely on RT-PCR plus Sanger sequencing. Reports in rotavirus and other viral systems show significantly faster turnaround and reduced labor for (multiplex) RT-qPCR while maintaining high agreement with sequencing. At the reagent level, SYBR-based qPCR is frequently the lowest-cost quantitative format, whereas probe-based assays and sequencing incur higher per-test and infrastructure costs[18]. Importantly, clinical NGS requires specialized personnel and validation frameworks; thus, while sequencing remains the definitive method for comprehensive genomic characterization, RT-qPCR is well-suited for routine lot-release/identity testing[19-21]. The use of SYBR Green RT-PCR, as demonstrated in this study, allows for rapid, high-throughput screening of vaccine strains with high specificity and minimal resource requirements. The findings reinforce previous reports advocating for the adoption of real-time PCR techniques in viral diagnostics and vaccine monitoring[3]. Rotavirus' segmented genome permits reassortment among live-attenuated vaccine strains; in RotaTeq recipients, human–bovine double-reassortant viruses such as G1[8] arising from G1P[5] and G6P[8] have been documented in symptomatic children. Moreover, vaccine-derived reassortants have been linked to household transmission and, if they circulate more broadly, could plausibly seed outbreaks; vaccine viruses may also reassort with co-circulating human wild-type strains (e.g., acquisition of vaccine NSP2 by G1P[8]). Genotype-specific real-time RT-PCR surveillance further demonstrates heterogeneous shedding patterns of the five vaccine genotypes across infants, underscoring the need to distinguish wild-type, vaccine, and vaccine-reassortant strains in post-licensure monitoring[22,23]. The WHO emphasizes the importance of strict quality control measures to ensure vaccine consistency and effectiveness. The findings from this study support the integration of SYBR Green RT-PCR into current WHO recommended quality control protocols to enhance rotavirus vaccine surveillance and improve public health outcomes[7]

The validated SYBR Green RT-PCR assay provides a rapid, sensitive, and reliable method for identifying and characterizing rotavirus strains in the pentavalent vaccine. The method demonstrated high specificity, with single melting peaks confirming accurate strain detection. Furthermore, intra- and inter-assay evaluations showed minimal variation, confirming the assay's precision and reproducibility. A formal LOD study was not part of this validation because the procedure is qualitative and aimed at identity confirmation (ICH Q2(R1)). Future work may include a quantified RNA LOD dataset to complement the current specificity-focused validation. By offering a streamlined workflow and cost-effective alternative to sequencing, the assay is well-suited for routine vaccine quality control applications. Its robustness and reproducibility make it a valuable tool for ensuring vaccine safety and efficacy, thereby supporting global immunization programs aimed at reducing rotavirus-related morbidity and mortality in children. Future studies should focus on expanding the validation of this assay across a broader range of vaccine formulations and laboratory settings. Additionally, comparative studies with alternative qPCR approaches and digital PCR could further refine detection capabilities and improve vaccine strain monitoring.

Conclusion

This assay is intended for health authorities and QC/research laboratories; this SYBR Green RT-PCR assay supports routine strain-identity confirmation and serotype-stability monitoring for multivalent rotavirus vaccines. By testing vaccine-derived RNA directly, without co-culture with cell lines, it delivers shorter turnaround and lower per-test costs than sequencing-based genotyping, while sequencing remains available for in-depth characterization. This manuscript outlined the successful validation of a SYBR Green RT-PCR assay for the Identification of strains of the pentavalent rotavirus vaccine. The performance of the assay was validated according to current literature guidelines, focusing on the evaluation of key parameters: specificity, repeatability, and reproducibility. This technique ensures their quality and effectiveness. This advancement plays a crucial

role in public health efforts to mitigate the burden of rotavirus infections globally.

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Reference

1. ADAMS WR, KRAFT LM. Epizootic diarrhea of infant mice: indentification of the etiologic agent. *Science.* 1963;141(3578):359-360. doi:10.1126/science.141.3578.359.
2. Bishop RF, Davidson GP, Holmes IH, Ruck BJ. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet.* 1973;2(7841):1281-1283. doi:10.1016/s0140-6736(73)92867-5
3. Omatola CA, Olaniran AO. Rotaviruses: From Pathogenesis to Disease Control-A Critical Review. *Viruses.* 2022;14(5):875. Published 2022 Apr 22. doi:10.3390/v14050875
4. Du Y, Chen C, Zhang X, et al. Global burden and trends of rotavirus infection-associated deaths from 1990 to 2019: an observational trend study. *Virol J.* 2022;19(1):166. Published 2022 Oct 20. doi:10.1186/s12985-022-01898-9
5. Gómez-Rial J, Rivero-Calle I, Salas A, et al. Rotavirus and autoimmunity. *J Infect.* 2020;81(2):183-189. doi:10.1016/j.jinf.2020.04.041.
6. Surendran S. Rotavirus infection: molecular changes and pathophysiology. *EXCLI J.* 2008;7:154. Doi: 0.17877/DE290R-14555
7. Meeting of the Strategic Advisory Group of Experts on immunization, October 2009: conclusions and recommendations. *Wkly Epidemiol Rec.* 2009;84(50):517-532.
8. World Health Organization. *Recommendations to Assure the Quality, Safety and Efficacy of Rotavirus Vaccines* (replacement of Annex 3 of WHO Technical Report Series, No. 941). TRS. 2007.
9. Ranheim T, Mathis PK, Joelsson DB, et al. Development and application of a quantitative RT-PCR potency assay for a pentavalent rotavirus vaccine (RotaTeq). *J Virol Methods.* 2006;131(2):193-201. doi:10.1016/j.jviromet.2005.08.013
10. Broeders S, Huber I, Grohmann L, et al. Guidelines for validation of qualitative real-time PCR methods. *Trends Food Sci Technol.* 2014;37(2):115-126. doi:10.1016/j.tifs.2014.03.008
11. International Organization for Standardization. *ISO 20395:2019—Biotechnology: Requirements for Evaluating the Performance of Quantification Methods for Nucleic Acid Target Sequences—qPCR and dPCR.* Geneva, Switzerland: ISO; 2019.
12. Borman P, Elder D. Q2(R1) validation of analytical procedures: text and methodology. In: Teasdale A, Elder D, Nims RW, eds. *ICH Quality Guidelines.* 1st ed. Hoboken, NJ: Wiley; 2017:127-166. doi:10.1002/9781118971147.ch5.
13. European Commission; Joint Research Centre; Institute for Health and Consumer Protection. *Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing: European Network of GMO Laboratories (ENGL).* Luxembourg: Publications Office of the European Union; 2008. doi:10.2788/65827.
14. Kubista M, Andrade JM, Bengtsson M, et al. The real-time polymerase chain reaction. *Mol Aspects Med.* 2006;27(2-3):95-125. doi:10.1016/j.mam.2005.12.007
15. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. *Nucleic Acids Res.* 2002;30(6):1292-1305. doi:10.1093/nar/30.6.1292
16. Wang Y, Liu Y, Bao H, et al. Application of the cell-based RT-qPCR assay (C-QPA) for potency detection of the novel trivalent rotavirus vaccine in China. *J Clin Lab Anal.* 2023;37(23-24):e24989. doi:10.1002/jcla.24989

17. Gautam R, Esona MD, Mijatovic-Rustempasic S, Tam KI, Gentsch JR, Bowen MD. Real-time RT-PCR assays to differentiate wild-type group A rotavirus strains from Rotarix® and RotaTeq® vaccine strains in stool samples. *Hum Vaccin Immunother.* 2014;10(3):767-777. doi:10.4161/hv.27388.
18. Tajadini M, Panjehpour M, Javanmard SH. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Adv Biomed Res.* 2014;3:85. Published 2014 Feb 28. doi:10.4103/2277-9175.127998
19. Rehm HL, Bale SJ, Bayrak-Toydemir P, et al. ACMG clinical laboratory standards for next-generation sequencing. *Genet Med.* 2013;15(9):733-747. doi:10.1038/gim.2013.92
20. Purcell RV, Pearson J, Frizelle FA, Keenan JI. Comparison of standard, quantitative and digital PCR in the detection of enterotoxigenic Bacteroides fragilis. *Sci Rep.* 2016;6:34554. Published 2016 Sep 30. doi:10.1038/srep34554.
21. Carossino M, Barrandeguy ME, Erol E, Li Y, Balasuriya UBR. Development and evaluation of a one-step multiplex real-time TaqMan® RT-qPCR assay for the detection and genotyping of equine G3 and G14 rotaviruses in fecal samples. *Virol J.* 2019;16(1):49. Published 2019 Apr 25. doi:10.1186/s12985-019-1149-1
22. Hemming M, Vesikari T. Detection of rotateq vaccine-derived, double-reassortant rotavirus in a 7-year-old child with acute gastroenteritis. *Pediatr Infect Dis J.* 2014;33(6):655-656. doi:10.1097/INF.0000000000000221
23. Higashimoto Y, Ihira M, Miyazaki Y, et al. Monitoring Shedding of Five Genotypes of RotaTeq Vaccine Viruses by Genotype-Specific Real-Time Reverse Transcription-PCR Assays. *J Clin Microbiol.* 2018;56(6):e00035-18. Published 2018 May 25. doi:10.1128/JCM.00035-18

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Online Access

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Table S1 Reproducibility of the Ct values evaluated SYBR Green RT-PCR assay for the VP7 segment of rotavirus. The reproducibility test was conducted as three technical replicates on different days (three days). (each run in duplicate).

Strain of rotavirus	RNA per reaction (ng)	Day 1	Day 2	Day 3	Ct mean	SD	CV (%)
		Ct (Mean of duplicate)					
G1P7	10ng	27.64	27.60	27.95	27.73	0.19	0.68
	1ng	33.30	33.70	32.38	33.12	0.68	2.06
G2P7	10ng	30.42	30.14	31.89	30.82	0.94	3.06
	1ng	34.77	34.73	33.78	34.42	0.56	1.62
G3P7	10ng	25.25	24.19	25.55	25.00	0.71	2.84
	1ng	29.77	29.48	32.29	30.51	1.55	5.07
G4P7	10ng	26.40	25.97	30.57	27.64	2.54	9.19
	1ng	32.06	31.40	34.15	32.54	1.43	4.41
G6P[8]	10 ng	22.47	22.18	23.74	22.79	0.83	3.64
	1ng	26.28	25.87	30.61	27.59	2.62	9.51

Table S2 Reproducibility of the Tm values evaluated SYBR green RT-PCR assay for the VP7 segment of rotavirus. The reproducibility test was conducted as three technical replicates on different days (three days). (each run in duplicate).

Strain of rotavirus	RNA per reaction (ng)	Day 1	Day 2	Day 3	Ct mean	SD	CV (%)
		Tm (Mean of duplicate)					
G1P7	10ng	77.01	77.14	76.98	77.04	0.08	0.11
	1ng	76.95	77.08	76.84	76.96	0.12	0.16
G2P7	10ng	74.49	74.56	74.40	74.48	0.08	0.11
	1ng	74.47	74.53	74.33	74.44	0.10	0.14
G3P7	10ng	76.56	76.56	76.50	76.54	0.03	0.04
	1ng	76.50	76.53	76.23	76.42	0.17	0.22
G4P7	10ng	75.75	75.72	75.66	75.71	0.05	0.06
	1ng	75.66	75.73	75.43	75.61	0.16	0.21
G6P[8]	10 ng	77.56	77.49	77.37	77.47	0.10	0.12
	1ng	77.47	77.47	77.13	77.36	0.19	0.25

Identification of Rotavirus Serotypes in Cell Culture Using SYBR Green RT-qPCR

Triplicate assays were performed to evaluate the detection of rotavirus serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8] in cell culture using SYBR Green real-time PCR. At a viral concentration of log3 CCID50, samples were analyzed at 24, 48, and 72 hours post-inoculation, and the results are presented in Tables S1-S2

Table S3 Cycle threshold (Ct) values for rotavirus serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8] at 24, 48, and 72 hours post-inoculation (SYBR Green RT-qPCR). Values are mean ± SD with %CV across triplicate runs (each run in duplicate).

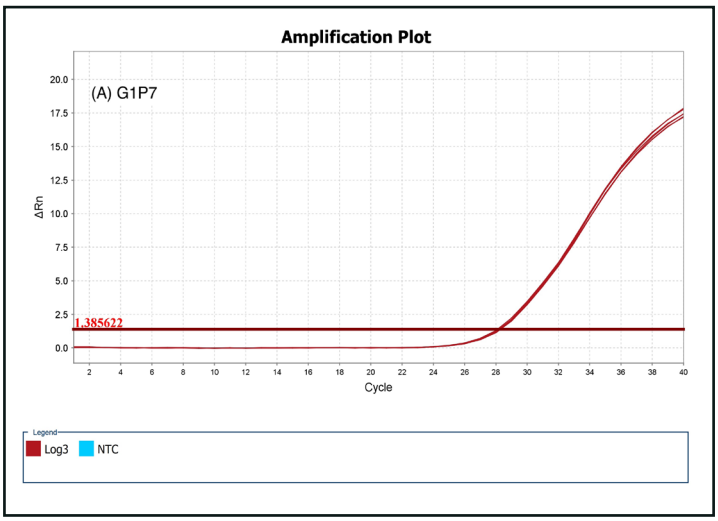
Rotavirus serotype	Time (h.)	Run1		Run2		Run3		Mean ±SD	%CV
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2		
G1P7	24	30.28	29.81	29.89	29.75	29.90	29.54	29.86±0.24	0.82
	48	26.56	26.46	26.59	26.95	26.48	26.44	26.58±0.19	0.72
	72	25.89	26.30	26.44	26.39	26.36	26.33	26.28±0.20	0.76
G2P7	24	25.73	25.69	25.78	25.49	25.82	25.76	25.71±0.12	0.46
	48	21.59	21.13	20.65	21.05	21.21	19.77	20.90±0.63	3.02
	72	20.78	20.33	20.42	20.30	20.24	20.70	20.46±0.22	1.09
G3P7	24	26.98	26.91	26.92	26.86	26.81	26.84	26.89±0.06	0.22
	48	22.78	22.76	22.71	22.58	22.51	22.68	22.67±0.11	0.47
	72	22.03	22.71	22.16	22.16	21.90	22.20	22.19±0.28	1.24
G4P7	24	27.31	27.35	27.65	28.19	27.42	27.39	27.55±0.33	1.21
	48	23.69	24.00	23.83	23.43	23.71	24.95	23.94±0.53	2.22
	72	24.33	24.08	25.03	23.57	23.45	23.80	24.04±0.58	2.42
G6P[8]	24	29.39	29.41	29.08	27.71	28.64	29.26	28.92±0.66	2.27
	48	27.39	26.03	26.46	26.46	26.98	26.20	26.59±0.51	1.91
	72	26.63	26.78	26.67	26.80	26.77	27.07	26.79±0.16	0.58

Table S4 Melting temperatures (Tm, °C) for rotavirus serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8] at 24, 48, and 72 hours (SYBR Green RT-qPCR). Values are mean ± SD with %CV across triplicate runs (each run in duplicate)

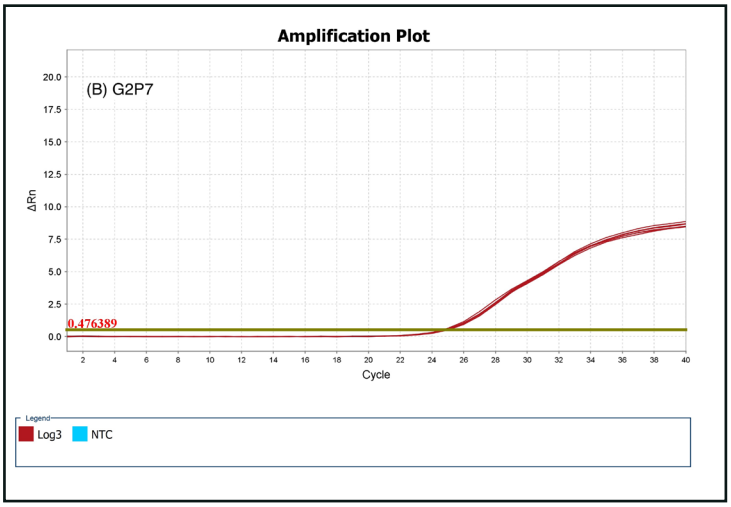
Rotavirus serotype	Time (h.)	Run1		Run2		Run3		Mean±SD	%CV
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2		
G1P7	24	77.11	77.01	77.11	77.20	77.49	77.59	77.25±0.23	0.30
	48	77.59	77.49	77.40	77.11	77.11	77.20	77.32±0.21	0.27
	72	77.49	77.49	77.59	77.49	77.49	77.49	77.51±0.04	0.05
G2P7	24	74.70	74.70	74.70	74.89	74.98	74.89	74.81±0.13	0.17
	48	74.89	74.98	74.98	74.89	74.79	74.79	74.89±0.09	0.12
	72	74.98	74.98	74.98	74.89	74.98	74.98	74.97±0.04	0.05
G3P7	24	76.43	76.53	76.62	76.62	76.62	76.72	76.59±0.10	0.13
	48	76.72	76.53	76.53	76.53	76.53	76.53	76.56±0.08	0.10
	72	76.62	76.62	76.53	76.62	76.53	76.53	76.58±0.05	0.07
G4P7	24	75.66	75.66	75.66	75.76	75.76	75.76	75.71±0.05	0.07
	48	75.76	75.76	75.76	75.66	75.66	75.66	75.71±0.05	0.07
	72	75.76	75.85	75.85	75.85	75.76	75.76	75.80±0.05	0.07
G6P[8]	24	77.11	77.11	77.01	77.20	77.11	77.11	77.11±0.06	0.08
	48	77.40	77.40	77.40	77.30	77.49	77.40	77.40±0.06	0.08
	72	77.30	77.30	77.40	77.30	77.30	77.30	77.32±0.04	0.05

Figure S1 Representative amplification plots and Ct distributions of cell-based SYBR Green RT-qPCR assay targeting the VP7 gene in rotavirus vaccine serotypes G1P7, G2P7, G3P7, G4P7, and G6P[8]. Panels A–E correspond to each serotype.

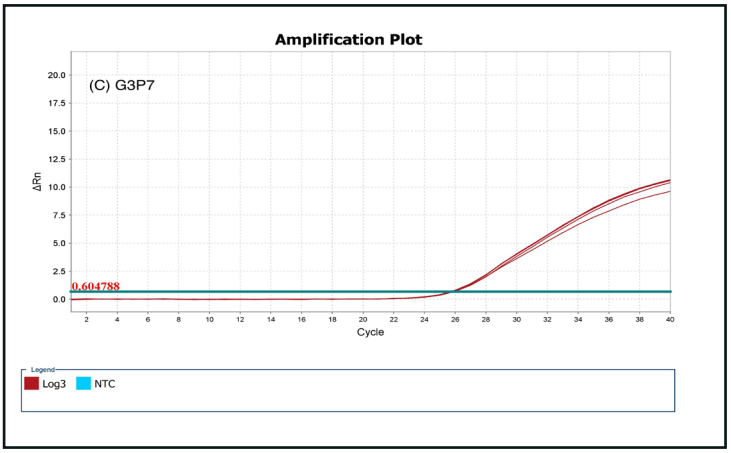
A. Rotavirus serotype G1P7



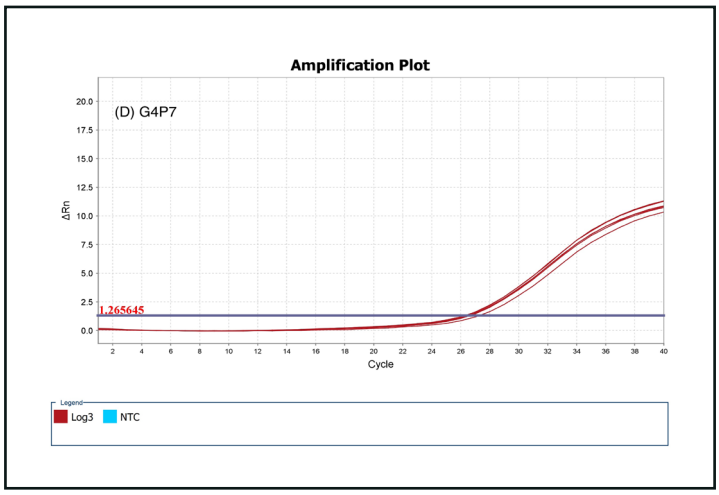
B. Rotavirus serotype G2P7



C. Rotavirus serotype G3P7



D. Rotavirus serotype G4P7



E. Rotavirus serotype G6P[8]

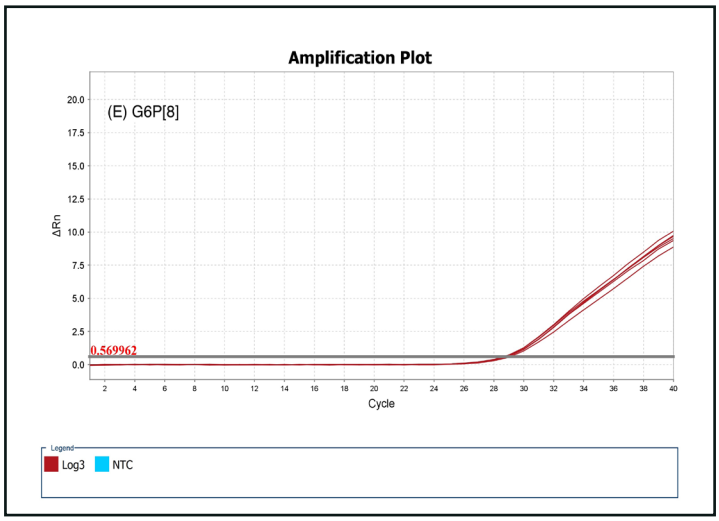
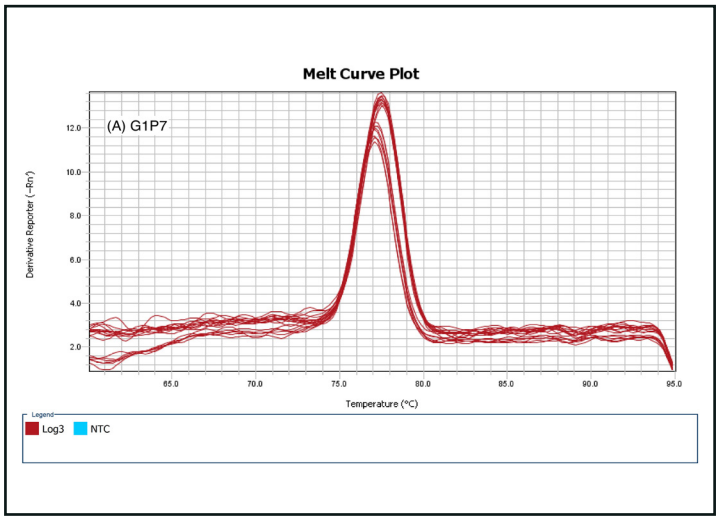
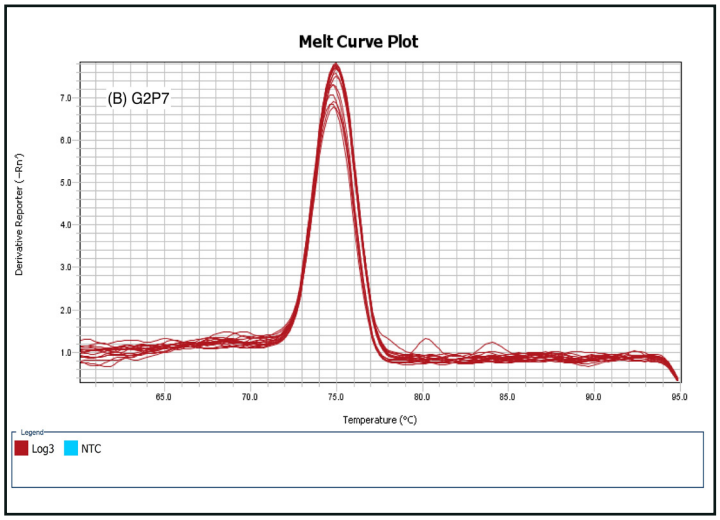


Figure S2 The melting curve analysis of rotavirus serotype G1P7, G2P7, G3P7, G4P7, and G6P[8], as determined by cell-based SYBR Green RT-qPCR assay. Panels A–E correspond to each serotype.

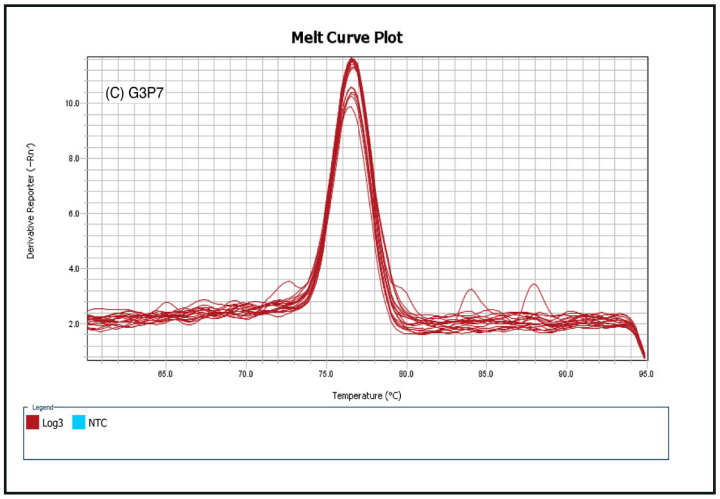
A. Rotavirus serotype G1P7



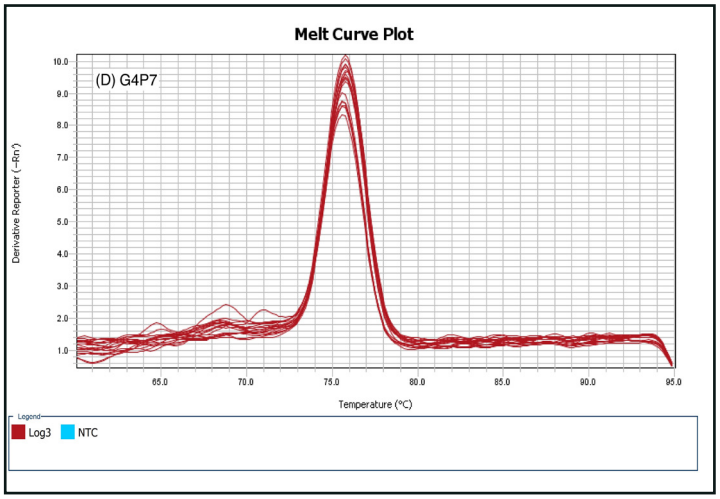
B. Rotavirus serotype G2P7



C. Rotavirus serotype G3P7



D. Rotavirus serotype G4P7



E. Rotavirus serotype G6P[8]