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Enhancing Reverse Transcriptase (RT)-PCR Efficiency: Optimizing Multiplex RT-PCR

Vincent Dufey[1], Steffen Riethmüller[2], Oliver Kahre[3], Sebastian Maurer[2] and Francoise Delongueville[1]

[1]Eppendorf Application Technologies S.A., Namur, Belgium

[2]Eppendorf SE, Hamburg, Germany

[3]Solis BioDyne OÜ, Tartu, Estonia

Abstract

Reverse transcription-polymerase chain reaction (RT-PCR) is a widely used technique e.g., for the detection and analysis of RNA pathogens. In a 1-step RT-PCR, reverse transcription and cDNA amplification occur in the same tube. This offers faster workflows, fewer pipetting steps (reducing errors and contamination), and less variation between reactions compared to 2-step approaches. Especially multiplexing RT-PCR is a great tool in different diagnostic settings.

However, due to differences in ramp rates and temperature profiles, the choice of a PCR cycler affects the, results along with the choice of consumables. Here, we show how optimized results can be achieved with the Mastercyler® X40, X50a, and X50s from Eppendorf with the SolisFAST® 1-step RT-PCR Kit by optimizing both, PCR protocol and the choice of consumables.

Key Takeaways

1. **Optimization for Different Consumables:** The initial RT-PCR program was suitable for fast PCR consumables but required optimization for standard consumables like the Eppendorf twin.tec® PCR Plate 96 to ensure reliable amplification of all target amplicons.
2. **Improved Specificity and Sensitivity:** By adjusting the denaturation temperature and extending the denaturation time, the optimized RT-PCR program improved specificity and sensitivity, eliminating non-specific bands, and ensuring complete primer utilization.
3. **Compatibility Across Thermal Cyclers:** The optimized program enabled consistent detection of all expected amplicons on different Mastercyclers, including the X50a and X50s, even at low RNA concentrations (0.1 ng/reaction), making it a robust solution for various laboratory setups.
4. **Efficient Run Times:** Despite the optimizations, the enhanced RT-PCR program maintained an efficient run time, only four minutes longer than the original, demonstrating that performance improvements do not significantly impact workflow efficiency.

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Introduction

1-step reverse transcription-quantitative PCR (RT-qPCR) is a popular choice for identifying and analyzing RNA pathogens and there is a wide selection of commercial kits available for researchers to choose from. However, end-point PCR is still often used in diagnostics, pathogen detection, and other areas when working with RNA sample material. The reason is that end-point analysis can often be simpler to perform and a cheaper alternative with equivalent performance when comparing to RT-qPCR [1]. Thus, the availability of robust 1-step RT-PCR kits for end-point analysis is equally relevant.

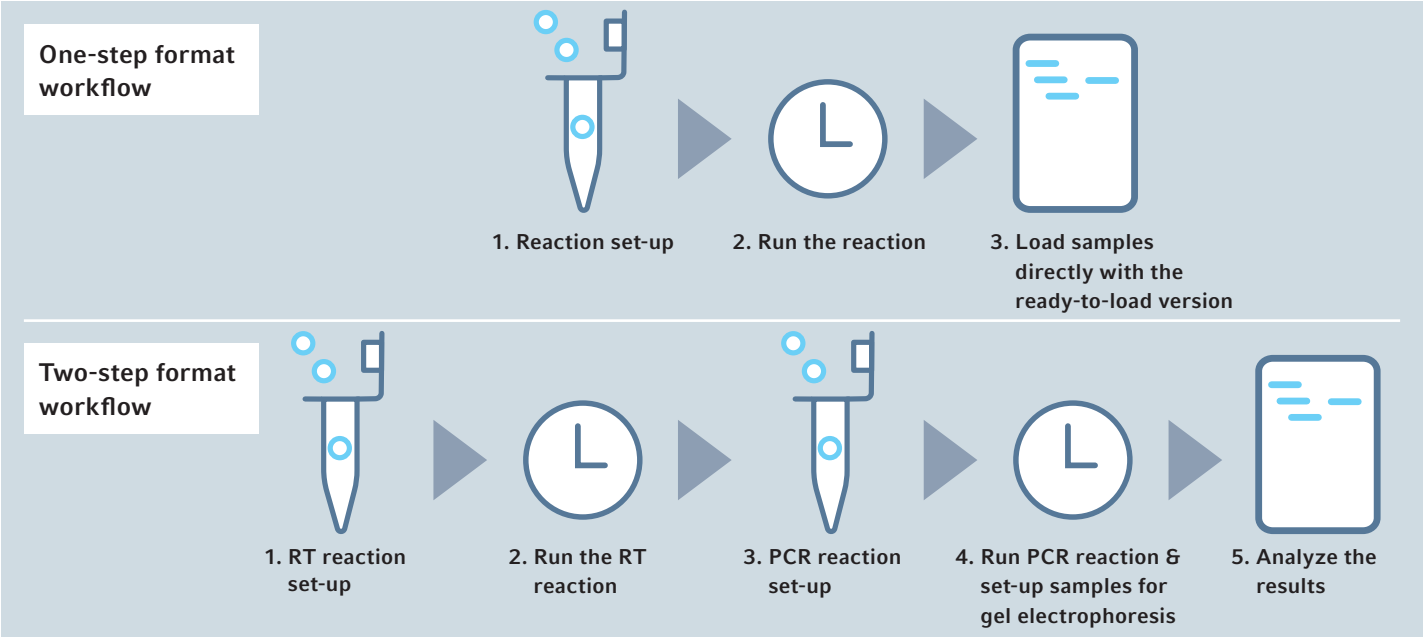


Figure 1: Comparison of one-step and two-step RT-PCR workflows. In 1-step RT-PCR, the reverse transcription (RT) of RNA and amplification of the resulting cDNA (PCR) are both performed in the same reaction tube. Depending on the application, a 1-step approach can offer many benefits over the common 2-step approach. It enables faster and simpler workflows, reduced hands-on and pipetting steps that can lead to errors or contamination, and reduces overall reaction-to-reaction variation.

Solis BioDyne has developed the SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load. This kit is designed for complex 1-step multiplex reactions, combining the exceptionally fast SolisFAST® polymerase and robust SOLIScript® and FIREScript® reverse transcriptases to offer a new way of doing multiplex 1-step RT-PCR reactions. The SolisFAST® 1-step RT-PCR Kit with UNG Ready To Load enables fast reaction times and robust multiplexing capabilities that allows convenient analysis of many targets at once. Even though multiplexing is a great option for researchers and in diagnostic settings, allowing to save time and resources, it can be a bit tricky at times and require optimization when different consumables and thermal cyclers with different heating and cooling ramp rates are used. Stable expression of a housekeeping gene is critical for any RT-PCR reaction, as it plays a critical role in interpretation of the results. However, a single ideal reference gene does not exist, and the choice depends on the species, tissue, cell type and condition of interest [2]. In this application note a selection of housekeeping genes is used. These include Phosphoglycerate Kinase, β-actin, Ribosomal Protein Lateral Stalk Subunit P0, β-glucuronidase and Albumin, that are commonly used in various sample types [3].

The multiplex RT-PCR program using SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load was initially established for the usage of fast PCR consumables like the Eppendorf Fast PCR Tube Strips. The Eppendorf Fast PCR Tube Strips made from polyethylene offer even faster temperature transfer than the Eppendorf twin.tec PCR Plate 96 (as its wells are made from polypropylene) and allow PCR users to speed up time-to-result [4]. Here, we showed that the PCR consumable can have an influence on the results and that a successful switch of fast to standard PCR consumables sometimes requires optimization of the PCR program. The optimized multiplex RT-PCR program (Table 1) is suitable for both fast PCR consumables and standard consumables, using the Mastercycler X40 as well as Mastercycler X50a and X50s.

Materials and Methods

Reverse transcription PCR

Each RT-PCR reaction was carried out in a total volume of 20 µL. The mix was prepared as described in the manual of the Solis BioDyne SolisFAST® 1-step RT-PCR Kit with UNG Ready To Load (#004-54-00200). The primer mix contained specific primers designed to amplify the following five reference genes: β-actin (443 bp), phosphoglycerate kinase (206 bp), ribosomal protein lateral stalk subunit P0 (109 bp), β-glucuronidase (159 bp) and albumin (72 bp). The RNA template (Agilent™ Human Reference RNA (#750500) was diluted to the intended concentrations in 0.1x TE buffer.

DNA amplicons analysis

Each individual RT-PCR amplified product was analyzed using the D1000 ScreenTape (# 5067-5582) and DNA Reagent D1000 (# 5067-5583) with the Agilent™ 4150 TapeStation.

All RT-PCR reactions were conducted either in Eppendorf Fast PCR Tube Strips (#0030124928) or Eppendorf twin.tec PCR Plate 96 (skirted, 150 µL, #0030128680) sealed with Eppendorf Heat Sealing Film (0030127838) on a HeatSealer S200 (#5392000005). The RNA was reverse transcribed by using three Mastercycler models from Eppendorf: the X50s (#6311000010), the X50a (#6313000018) and the X40 (#6381000018). The initial RT-PCR settings applied are described in the Table 1. Each RNA concentration was run in technical triplicates.

Table 1: Multiplex RT-PCR programs using the SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load.

Run settings	Lid	105 °C	
	Energy-saving mode	ON	
	Temperature mode	Standard	
Reverse transcriptase		50 °C/15 min	
Initial denaturation		95 °C/10 min	
35 Cycles	Denaturation	95 °C/5 s	optimized 96 °C/10 s
	Annealing	58 °C/30 s	
	Elongation	72 °C/30 s	
Post-Cycle Elongation		72 °C/1 min	
Storage	Hold	4 °C	

Result and Discussion

Multiplex reverse transcriptase PCR (RT-PCR) program suitable for Fast PCR consumables but not for standard consumables

In initial experiments using the SolisFAST 1-step RT-PCR Kit with UNG Ready to Load on Mastercycler X40, X50a, or X50s with Eppendorf Fast PCR Tube Strips (Figure 2A) all five expected amplicons were detected at 20 ng/reaction (rx) and 3.2 ng/rx htRNA. However, the band at 443 bp was faint. Furthermore, unspecific bands were observed at approximately ~60 bp and below 50 bp. The two unspecific bands below 50 bp indicate the presence of unused primer and primer dimers upon the PCR run and thus an incomplete amplification of the expected fragments.

When using Eppendorf twin.tec PCR Plate 96 with a starting material of 20 ng/rx htRNA, all five amplicons were detected (Figure 2B) as well. However, the 443 bp band was only observed when using 3.2 ng/rx starting material on the Mastercycler X40 but not on the Mastercycler X50a and Mastercycler X50s. Additionally, more intense unspecific bands below 50 bp were present suggesting incomplete primer utilization when Mastercycler X50a and Mastercycler X50s were used.

The Mastercycler X40, with a heating (and cooling) ramp rate of 3.3°C/s (and 1.5°C/s), completed the RT-PCR run in 1 hour and 41 minutes. In contrast, the Mastercycler X50a and X50s have higher heating (and cooling) ramp rates of up to 5°C/s (2.3°C/s) and 10°C/s (5°C/s) [5], respectively. The higher ramp rates significantly reduced the run times to 1 hour and 29 minutes for the X50a and 1 hour and 20 minutes for the X50s, as illustrated in Figure 2A/B.

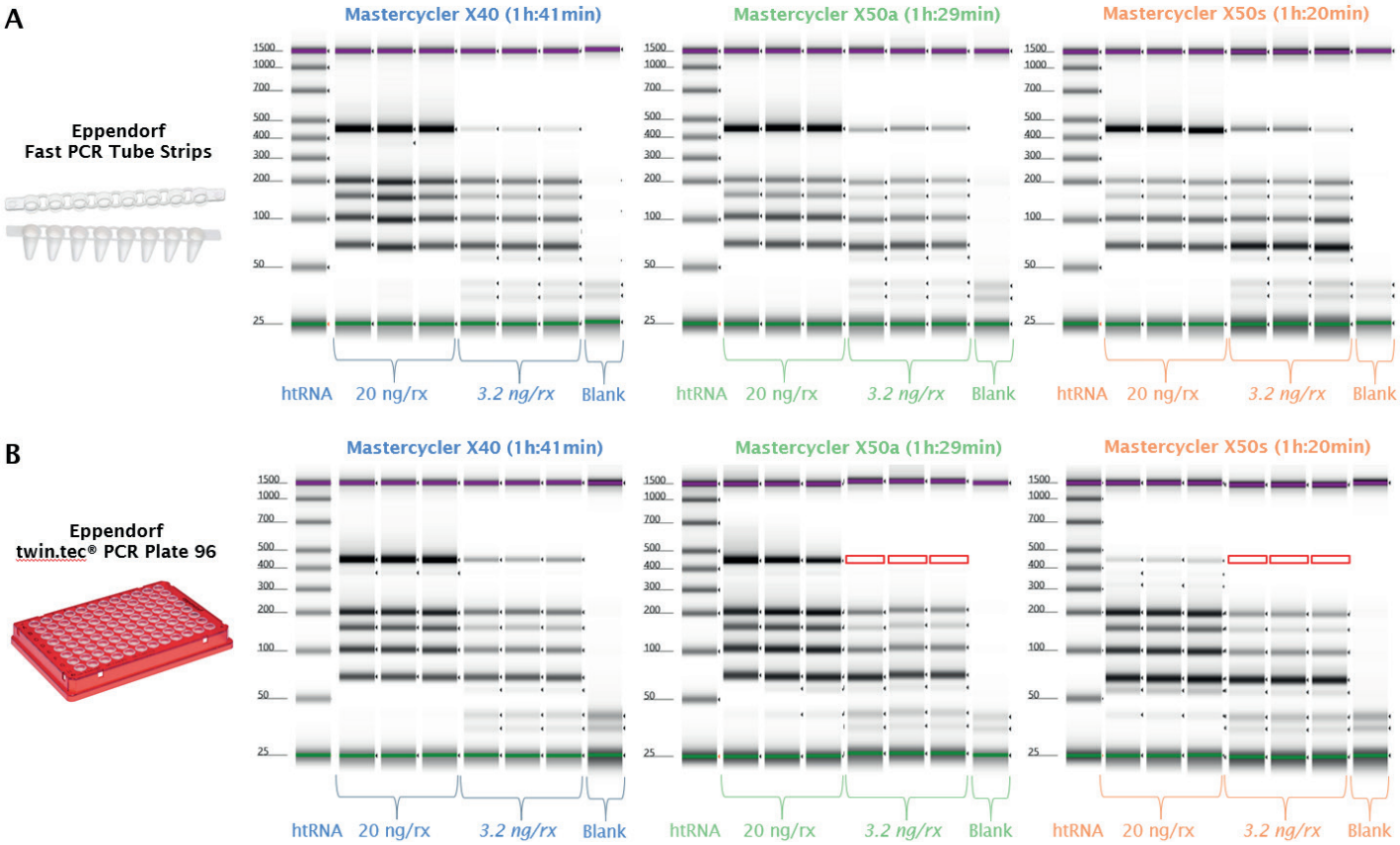


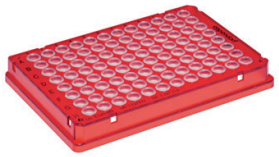
Figure 2: Choice of consumables affects RT-PCR results. Multiplex RT-PCR was conducted with SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load. Experiments were performed on the Mastercycler X40, Mastercycler X50a or Mastercycler X50s (runtime is displayed in brackets). Either 20 ng or 3.2 ng htRNA per 20 µl reaction were used as a starting material. The presence of expected amplicons at 72, 109, 159, 206, 443 bp were analyzed in 3 technical replicates. The experiment was performed in (A) Eppendorf Fast PCR Tube Strips and (B) Eppendorf twin.tec® PCR Plate 96. Red boxes indicate the absence of an expected band. The lower and upper detection limit is indicated by the green and purple lines.

Optimized multiplex RT-PCR program for Fast PCR and standard PCR consumables

The multiplex RT-PCR program required optimization for use with the Eppendorf twin.tec PCR Plate 96 on the rapid Mastercycler X50a and X50s as the largest fragment at 443 bp failed to amplify (depicted in Figure 2). The denaturation temperature was optimized to 96°C by using a 1D-gradient function (data not shown). Additionally, a prolongation of the denaturation step from 5 to 10 seconds further improved the results (data not shown).

This new program (Table 1) enabled the detection of all amplicons when using standard consumables like the Eppendorf twin.tec PCR Plate 96 on the Mastercycler X50s (Figure 3). The detection of all expected amplicons was still successful using 0.1 ng/rx htRNA. In addition, no unspecific band at ~60 bp and no bands of unused primers were observed at 3.2 ng/rx htRNA. The runtime of the optimized PCR program (1h:24min) was only four minutes longer than the original program.

Eppendorf twin.tec® PCR Plate 96



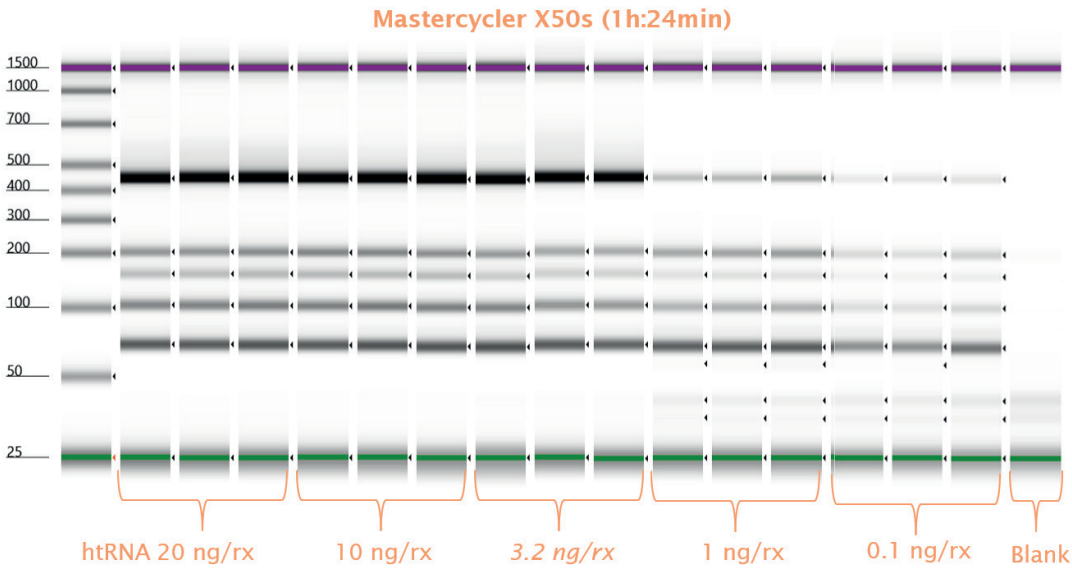


Figure 3: The optimized PCR program improved specificity and sensitivity. Optimized multiplex RT-PCR was conducted with SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load in Eppendorf twin.tec® PCR Plate 96 on the Mastercycler X50s (runtime is displayed in brackets). Either 20, 10, 3.2, 1, 0.1 ng htRNA per 20 µl reaction were used as a starting material. The presence of expected amplicons at 72, 109,159, 206 and 443 bp were analyzed in 3 technical replicates. Red boxes indicate the absence of an expected band. The lower and upper detection limit is indicated by the green and purple lines.

Conclusion

The optimized multiplex RT-PCR program demonstrates improved specificity and sensitivity and is suitable for standard consumables like Eppendorf twin.tec PCR plates 96. It offers a robust solution for detecting all expected amplicons with high efficiency and minimal non-specific amplification also at very low template concentrations. This Application note serves as a guide for researchers seeking to optimize their multiplex RT-PCR programs for enhanced performance across different PCR consumables.

Literature

[1] Cruz-Rangel A, Gómez-Romero L, Cisneros-Villanueva M et al. End-point RT-PCR based on a conservation landscape for SARS-COV-2 detection. Sci Rep 12, 4759 (2022). https://doi.org/10.1038/s41598-022-07756-6.
[2] Ho KH & Patrizi A. Assessment of common housekeeping genes as reference for gene expression studies using RT-qPCR in mouse choroid plexus. Sci Rep 11, 3278 (2021). https://doi.org/10.1038/s41598-021-82800-5.
[3] Kozera B & Rapacz M. Reference genes in real-time PCR. J Appl Genet 54, 4 (2013). doi:10.1007/s13353-013-0173-x.
[4] Isermann K & Phang A. Reduced PCR runtimes and increased yields using Eppendorf Fast PCR Consumables. Eppendorf Application Note 400.
[5] Gerke N & Phang A. Comparative run time evaluations of PCR thermal cyclers. Eppendorf SE, Application Note 274.

Ordering information

Description	Manufacturer	Order no.
SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load	Solis BioDyne	04-54-00200
Mastercycler® X40	Eppendorf	6381000018
Mastercycler® X50a	Eppendorf	6313000018
Mastercycler® X50s	Eppendorf	6311000010
twin.tec® PCR plate 96	Eppendorf	0030128680
Fast PCR Tube Strips	Eppendorf	0030124928
Heat Sealing Film	Eppendorf	0030127838
HeatSealer S200	Eppendorf	5392000005

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Your local distributor: www.eppendorf.com/contact
Eppendorf SE · 22339 Hamburg · Germany
eppendorf@eppendorf.com · www.eppendorf.com

Solis BioDyne OÜ: Teaduspargi 9, 50411 Tartu, Estonia
info@solisbiodyne.com, www.solisbiodyne.com

www.eppendorf.com