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APPLICATION NOTE No. 515

Rapid 1-Step Multiplex Reverse Transcription PCR from Whole Blood: Assessing Direct and Extracted RNA Workflows

Abstract

Reliable and rapid gene expression analysis from human whole blood is critical for clinical diagnostics, biomarker discovery, and translational research. However, it is often challenged by complex sample matrices and variable RNA quality. This Application Note demonstrates the performance of 1-step multiplex reverse transcription PCR (RT-PCR) for the detection of five reference genes in human whole blood, comparing workflows with and without RNA extraction. Using the SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load, robust amplification of intracellular transcripts was achieved from purified RNA, while a cell lysis without mRNA extraction enabled detection of most but not all targets. The study highlights how RNA preparation strategy and transcript origin critically impact multiplex RT-PCR results from whole blood.

Key words

Human whole blood samples, 1-step Multiplex RT-PCR, Multiplex Reverse Transcription PCR, whole blood gen expression analysis, reference gene validation, housekeeping genes, crude blood lysate

Introduction

One-step reverse transcription PCR (1-step RT-PCR) combines reverse transcription and PCR amplification in a single reaction, simplifying RNA workflows while maintaining high sensitivity for transcript detection [1]. The SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load (Solis BioDyne) supports multiplex analysis of RNA targets, while the inclusion of uracil-N-glycosylase (UNG) provides carryover contamination control and improves reliability in assays that rely on consistent detection of housekeeping genes for normalization.

A more detailed overview of the workflow and kit performance is provided in application note 494 [2]. However, no single reference gene is universally stable across all biological samples. Expression stability depends on tissue type, cellular composition, physiological state, and RNA preparation method [3,4]. In whole-blood experiments, transcript origin becomes especially relevant because blood contains both intracellular leukocyte RNA and extracellular RNA derived from plasma, platelets, and circulating vesicles [5].

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In this study, five commonly used reference genes were evaluated in whole-blood RT-PCR workflows: β-actin (ACTB; 443 bp), Phosphoglycerate Kinase 1 (PGK1; 206 bp), β-glucuronidase (GUSB; 159 bp), Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0; 109 bp) and Albumin (ALB; 72 bp). These genes are frequently applied as internal controls across multiple tissues and sample types [4], yet their suitability in whole-blood assays may depend strongly on RNA extraction strategy [6]. In this application note we compared two common preparation approaches: (1) A column-based RNA extraction using the RiboPure-Blood kit and (2) a treatment using cell lysis without mRNA extraction.

Materials and Methods

RNA extraction from human whole blood

Human whole blood was purchased from the Belgian Red Cross. RNA was isolated using a combination of stabilization, chemical extraction, and column purification following the manufacturer's protocol (RiboPure-Blood Kit, Invitrogen, AM1928). Briefly, whole blood was first stabilized in RNAlater solution, and cells were pelleted and lysed using Lysis Solution and Sodium Acetate Solution. Initial RNA purification was achieved via Acid-Phenol: Chloroform extraction was conducted under a chemical hood. Following recovery of the aqueous phase and precipitation with ethanol, the RNA was passed through a Filter Cartridge for final purification and washing with proprietary Wash Solutions. The final RNA elution was performed using preheated Elution Solution, followed by enzymatic treatment with DNase I to eliminate genomic DNA contamination. RNA concentration was measured using the Nanodrop at 260 nm. RNA was stored at -20°C.

Human whole blood treatment for RT-PCR without RNA extraction

Human whole blood was treated following the protocol of Shi & Liu, 1995 [7] with minor modifications. In brief, a small volume of fresh human whole blood (20 µL) was diluted 10 times with deionized water treated with diethyl pyrocarbonate (DEPC-Water, Invitrogen, AM9906). The solution was cooled on ice for 5 min to destroy the erythrocytes, then centrifuged at 3300 ×g for 10 min. The supernatant was discarded. The pellet was resuspended in 20 µl of RNAsecure (Invitrogen, AM7006) diluted in water (1:25) and cooled on ice for 20 min to destroy leukocytes and release RNA. RNA concentration was measured using the Nanodrop at 260 nm. This suspension was directly used as the total RNA stock solution in the plate for the RT-PCR or stored at -20°C.

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 One-step RT-PCR Kit with UNG, Ready to Load (#04-54-00250). The primer mix contains specific primers designed to amplify the following 5 reference genes: β-actin, Phosphoglycerate Kinase, Ribosomal Protein Lateral Stalk Subunit P0, β-glucuronidase, and Albumin. The RNA template was diluted to the desired concentrations in TE buffer 1X (20, 10, 3.2, 1, 0.32, 0.1 ng/reaction). All RT-PCR reactions were conducted in Eppendorf twin.tec® PCR Plates 96 (skirted, 150 µL, #0030128680) sealed with Eppendorf Heat Sealing Film (0030127838) on an Eppendorf HeatSealer S200 (#5392000005). The RNA was reverse transcribed by using Eppendorf Mastercycler® X50s (#6311000010). The RT-PCR settings applied are described in Table 1. Each RNA concentration was run in technical triplicate.

Eppendorf Header Settings	Lid Energy Saving Mode Temperature Mode	105°C On Standard	
Reverse Transcription		50°C	15 min
Initial Denaturation		95°C	10 min
Denaturation	35 cycles	96°C	10 s
Annealing		58°C	30 s
Elongation		72°C	30 s
Post-Cycle Elongation		72°C	4 min extension
Storage		10°C	Hold

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

DNA amplicons analysis

Each individual RT-PCR amplified product was analyzed using either the D1000 ScreenTape (Agilent, 5067-5582) and DNA Reagent D1000 (Agilent, 5067-5583) or the high-sensitivity D1000 Screentape (Agilent, 5067-5584) and high sensitivity D1000 reagents (Agilent, 5067-5585) in the Agilent 4150 TapeStation System (Agilent, G2992AA).

Results and Discussion

Amplification of all targets in human RNA extracted from human whole blood

When using human RNA extracted from whole blood via the column-based approach, all five amplicons were detected at 20 and 10 ng/reaction (Figure 1). At lower mRNA concentrations the two unspecific bands below 50 bp indicate the presence of unused primers and primer dimers upon the PCR run and thus an incomplete amplification of the expected fragments. β-actin, PGK1, RPLP0 and GUSB are intracellular leukocyte transcripts [10] that exhibited robust amplification, consistent with effective stabilization and isolation of intact leukocyte RNA [11]. In contrast, ALB exhibited a comparable weak amplification following column-based extraction. This is expected, as albumin transcripts detected in blood largely originate from hepatocytes and circulate as extracellular RNA rather than being transcribed in leukocytes [12,13]. Because plasma is removed during RiboPure-Blood extraction, extracellular RNA species are poorly retained. Consequently, reduced ALB signal reflects enrichment of intracellular leukocyte RNA rather than assay failure. When excluding albumin as a target detection range of the other four targets is 1.0 ng/reaction.

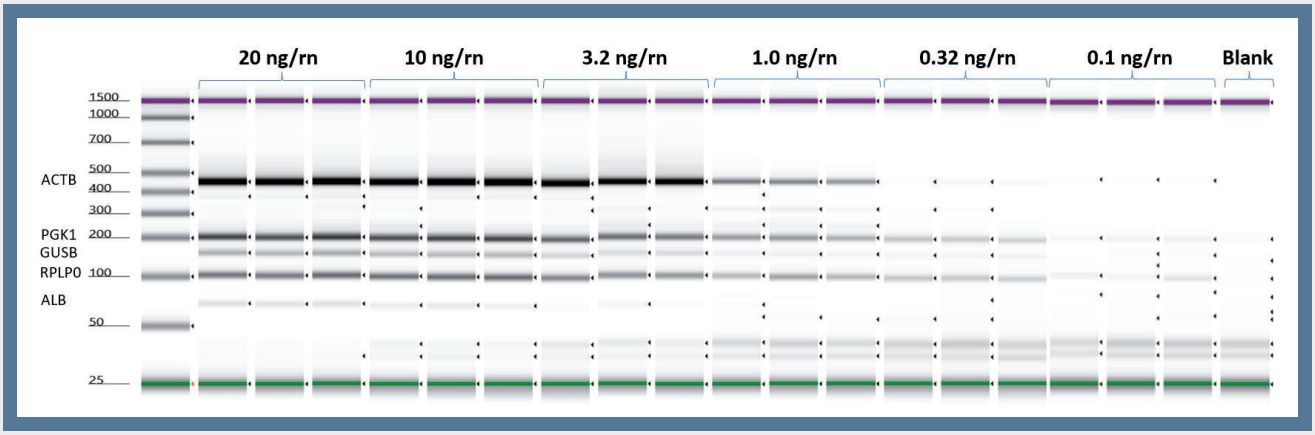

Figure 1: 1-step multiplex RT-PCR of RNA extracted from human whole blood. The multiplex RT-PCR was conducted with SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load in Eppendorf twin.tec® PCR Plate 96 on the Mastercycler X50s. The mRNA extracted from human whole blood was diluted to the desired concentrations of either 20, 10, 3.2, 1 or 0.1 ng per 20 µl RT-PCR reaction. The presence of expected amplicons at 72, 109, 159, 206 and 443 bp were analyzed in 3 technical replicates with D1000 ScreenTape assay. The lower and upper detection limit is indicated by the green and purple line.

4 of 5 target genes could be amplified directly in treated whole blood

In treated whole blood, β-actin, PGK1, RPLP0 and ALB were detected at 20 and 10 ng/reaction (Figure 2A). At lower mRNA concentrations, the two non-specific bands below 50 bp indicate the presence of unused primers and primer dimers in the PCR, underlining incomplete amplification of the expected fragments. GUSB could not be detected also when using a high-sensitivity D1000 Screentape assay (Figure 2B).

This is most plausibly explained by partial RNA degradation and RT-PCR inhibition. Whole blood contains substantial RNase activity, and hypotonic lysis may permit partial RNA degradation before complete nuclease inactivation [10]. Fragmentation disproportionately affects medium-length amplicons such as the 159 bp GUSB target, reducing reverse transcription efficiency. Additionally, crude lysates may contain heme, proteins, and other PCR inhibitors that selectively impair amplification of less abundant or structurally complex targets [14].

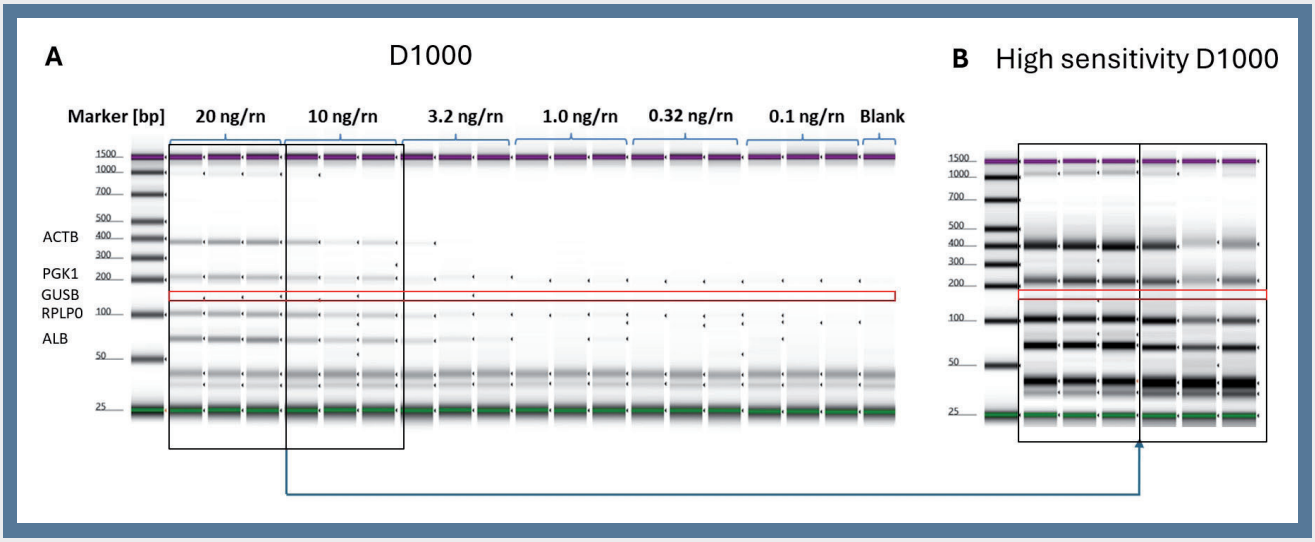

Figure 2: 1-step multiplex RT-PCR of RNA present in treated human whole blood. The multiplex RT-PCR was conducted with SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load in Eppendorf twin.tec® PCR Plate 96 on the Mastercycler X50s. Either treated human whole blood was diluted to the desired concentrations of either 20, 10, 3.2, 1 or 0.1 ng RNA per 20 µl RT-PCR reaction. 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. (A) All RT-PCR amplified products were analyzed with D1000 ScreenTape assay. (B) In addition, high-sensitivity D1000 Screentape assay was used for samples with starting material of 20 ng and 10 ng mRNA per reaction.

The results demonstrate that reference gene performance in whole blood is strongly influenced by RNA source and extraction chemistry. Importantly, whole blood contains multiple RNA pools, including intracellular leukocyte RNA, platelet-associated RNA, extracellular vesicle (EV) RNA, and circulating cell-free RNA [8]. Different preparation methods enrich or exclude these RNA populations, thereby affecting detectable transcript profiles [9].

Conclusion

Transcript detectability in whole blood is primarily determined by RNA compartment origin and extraction chemistry rather than enzyme performance alone. Overall, the multiplex SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load and the Mastercycler® X50s provided a robust amplification across multiple targets with minimal non-specific amplification in mRNA extracted from human whole blood.

Acknowledgement

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Ordering information

Description	Manufacturer	Order no.
SolisFAST® 1-step RT-PCR Kit with UNG Ready to Load	Solis BioDyne	04-54-00200
twin.tec® PCR Plates 96 (skirted, 150 µL)	Eppendorf	0030128680
Heat Sealing Film	Eppendorf	0030127838
HeatSealer S200	Eppendorf	5392000005
Mastercycler® X50s	Eppendorf	6311000010

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