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A Stepwise Workflow for Optimizing Multiplex RT-qPCR-Based Gene Expression Analysis: From Primer Concentrations to RNA Extraction Method Selection

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A Stepwise Workflow for Multiplex RT-qPCR Optimization

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Daniil Kornilov is the first author.

Ekaterina Voroshilina and Danila Zornikov share senior authorship.

ABSTRACT

Multiplex reverse transcription quantitative PCR (RT-qPCR) is a cornerstone of molecular diagnostics, yet its reliability depends on systematic optimization. Here, we present a stepwise workflow for optimizing a multiplex RT-qPCR assay for *MTOR* (mechanistic target of rapamycin) gene expression analysis, normalized to *RPLP0* (ribosomal protein lateral stalk subunit P0) and *TBP* (TATA-box binding protein). The workflow comprised four sequential stages: amplification protocol refinement, primer concentration and probe:primer ratio titration, comparative assessment of three RNA extraction methods (phenol-chloroform extraction, isopropanol precipitation, and silica-membrane spin-columns) across blood, cell cultures, and tumor tissue, and evaluation of DNase treatment necessity. Optimal conditions yielded amplification efficiencies of 97% for *MTOR*, 92% for *RPLP0*, and 84.3% for *TBP* with high linearity ($R^2 \geq 0.97$). Phenol-chloroform extraction provided the highest RNA purity (A260/A280 1.8–2.0), completely eliminated genomic DNA from blood and cell culture samples, and ensured the best reproducibility for blood (interquartile range, IQR = 13.2%). Spin-columns offered the best balance for cell cultures (IQR = 22.5%). Isopropanol precipitation produced the highest RNA yield for both blood and cell culture samples but caused significant DNA contamination and poor linearity for blood samples ($R^2 = 0.55–0.58$). DNase treatment was dispensable when using intron-overlapping primers, as genomic DNA contribution did not exceed technical variability for phenol-chloroform and spin-column methods. Sanger sequencing confirmed 100% specificity of all amplification products. The proposed stepwise workflow provides a practical framework for the development of reliable multiplex RT-qPCR assays.

Keywords: RT-qPCR, multiplex PCR, *MTOR*, gene expression, RNA extraction, primer optimization

INTRODUCTION

Quantitative assessment of gene expression represents one of the fundamental methodologies in modern molecular biology, enabling investigation of cellular regulatory mechanisms, identification of pathological biomarkers, and development of diagnostic approaches for personalized medicine [1]. Among available methods for quantitative gene expression analysis, reverse transcription quantitative PCR (RT-qPCR) occupies a unique

position due to its combination of high sensitivity, specificity, broad dynamic range, and relative procedural simplicity [2].

Accurate quantification of gene expression by RT-qPCR requires normalization to reference genes with stable expression across cell types and experimental conditions. Commonly used reference genes include *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *ACTB* (beta-actin), *B2M* (beta-2-microglobulin), *TBP* (TATA-box binding protein), and *RPLP0* (ribosomal protein lateral stalk subunit P0) [3, 4]. Among these, *TBP* and *RPLP0* are recognized as two of the most stably expressed housekeeping genes across human tissues and are widely used for normalization in gene expression studies [5].

We previously developed and validated a multiplex RT-qPCR assay for relative quantitative analysis of *MTOR* (mechanistic target of rapamycin) expression with normalization to *RPLP0* and *TBP* reference genes [6]. However, the initial study revealed suboptimal amplification efficiency for all target genes, along with relatively high variability in quantitative expression estimates. Although these metrics were acceptable at the preclinical validation stage, the considerable data dispersion raises concerns about the practical applicability of the developed tool and underscores the need for further optimization of both amplification conditions and sample preparation protocols.

The need for optimizing PCR conditions arises because even with correctly designed primers and standardized reagents, multiplex amplification efficiency can vary substantially depending on multiple factors. Suboptimal reaction conditions compromise amplification efficiency, promote nonspecific product formation and primer-dimer artifacts, and ultimately distort quantitative readouts. This becomes particularly critical in multiplex formats, where competitive interaction of multiple primer and probe pairs within a single reaction mixture creates additional risks that are absent in monoplex systems [7].

Multiplex RT-qPCR optimization must address several key aspects. First, optimization of temperature-time parameters is critical for reaction specificity. Without such optimization, nonspecific products and primer-dimers accumulate, competing with and attenuating the specific amplification signal [8, 9]. To enhance specificity and quantitative accuracy, several approaches have been proposed. These include touch-down PCR (gradual annealing temperature reduction), which minimizes non-specific primer binding [10, 11], and baseline correction normalization procedures, which eliminate background signal variation between samples [12].

Second, primer concentrations and probe:primer ratios must be carefully optimized. Insufficient primer concentration limits amplification efficiency and compromises sensitivity [13], whereas excessive concentration increases the risk of nonspecific products and primer-dimers [8, 9].

Finally, choice of RNA extraction method is equally important, as it allows adaptation of the test to specific biological material types, since RNA quality and purity directly determine reverse transcription efficiency and subsequent quantitative PCR detection [14, 15]. In the present work, three methods were used for total RNA isolation from peripheral blood and cell culture: phenol-chloroform extraction, isopropanol precipitation (hereafter referred to as precipitation), and affinity sorption on spin-columns with silica membrane. Phenol-chloroform

extraction is considered the classical method, providing effective protein denaturation and endogenous RNase inactivation [16, 17], but it requires stringent purity control or additional purification steps to mitigate the risk of phenol contamination [17, 18]. The precipitation method is relative, simple, cost-effective, with numerous commercially available kits that are certified for commercial usage. Furthermore, this method enables nucleic acid isolation from a wide range of biological samples, including those containing significant quantities of potential PCR inhibitors [19, 20]. Spin-columns are simple, standardized, and amenable to automation. They provide high amplification efficiency even with low nucleic acid yields [17], yielding RNA suitable for RT-qPCR [15].

Each of these stages requires a systematic approach and empirical parameter adjustment, as their effects on the final result are interdependent and cannot be assessed in isolation. Stepwise optimization of all three factors will not only improve the efficiency and reproducibility of the developed test for *MTOR* expression analysis but also establish a universal workflow applicable to the optimization of other multiplex PCR assays.

The aim of this study was to optimize the previously developed multiplex PCR test for *MTOR* expression analysis and derive a systematic stepwise workflow applicable to the optimization of analogous multiplex RT-qPCR assays.

RESULTS

Amplification Protocol Optimization

The initial amplification protocol (reverse transcription at 45°C for 30 min, pre-denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 5 s, 68°C for 11 s) was modified by adding a touch-down step with gradual annealing temperature reduction from 75°C at 1°C per cycle over 10 cycles (touch-down step), and data normalization with automatic baseline correction.

Optimal Primer Concentration Selection

Exclusion of Non-optimal Primer Concentrations

Analysis was performed both with and without baseline normalization, allowing for assessment of normalization procedure influence on final quality indicators. Without normalization, all primer concentrations except the minimum (0.06 μM) demonstrated 100% validity regardless of probe:primer ratio. At 0.06 μM, critical validity reduction was observed: for 1:1 ratio validity was 0%, for 1:2 ratio — 73.3%.

Normalization markedly changed the results. Normalization revealed quality differences that had not been apparent from the raw data alone: at 0.12 μM with 1:2 probe:primer ratio, validity decreased to 40%. At 0.06 μM, validity dropped to 0% for both ratios after normalization. Thus, the optimal primer concentrations that maintained 100% validity were 0.3 μM, 0.24 μM, and 0.18 μM. The 0.06 μM concentration was considered invalid and excluded from further analysis.

Amplification Efficiency

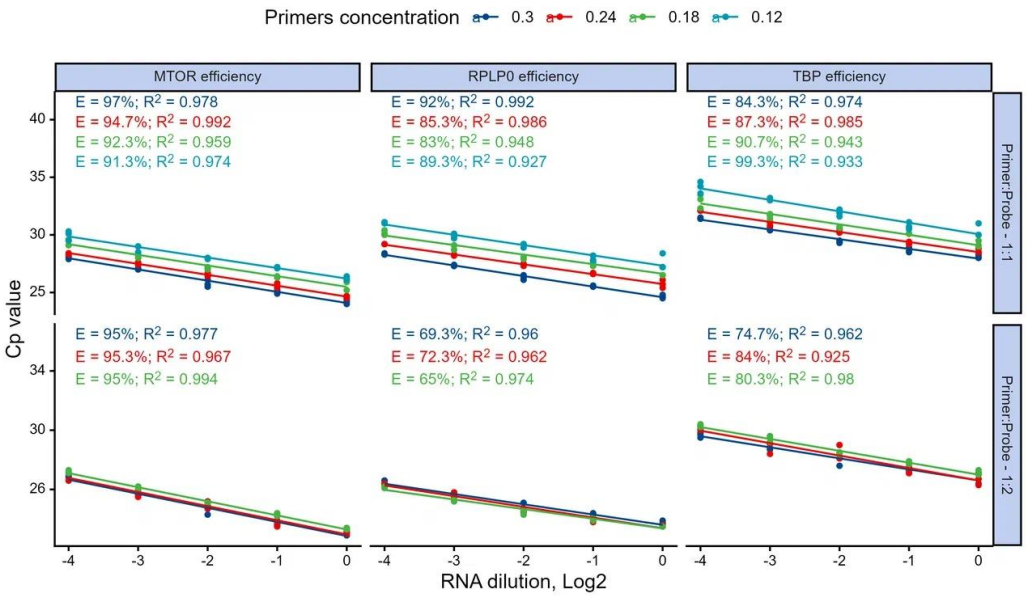


Figure 1. Dependence of amplification efficiency on primer concentration and probe:primer ratio in multiplex RT-qPCR for tissue samples using phenol-chloroform extraction. *MTOR* amplification efficiency remained high (91–97%) across all reactions. Reference gene efficiency showed significant variation with parameter changes: 1:2 ratio reduced *RPLP0* (65–72%) and *TBP* (75%) efficiency.
Lane designations: Primers concentration, μM — primer concentration in micromolars; *MTOR* efficiency — amplification efficiency of the target gene *MTOR*; *RPLP0* efficiency — amplification efficiency of the reference gene *RPLP0*; *TBP* efficiency — amplification efficiency of the reference gene *TBP*; Cp value — Crossing Point; Probe:Primer-1:1 — probe to primer ratio 1:1; Probe:Primer-1:2 — probe to primer ratio 1:2; RNA dilution, Log_2 — logarithmic transformation of RNA template dilution; E — amplification efficiency.

PCR efficiency was estimated from the slope of the standard curve (Cp vs. log_2 cell number), with efficiency expressed as the slope magnitude relative to the theoretical optimum of −1 (i.e., E = |slope| × 100%) (Figure 1). For *MTOR*, amplification efficiency remained stably high (91.3–97%) at all tested primer concentrations and both probe:primer ratios, with $R^2 \geq 0.95$ for all tested concentrations. For reference genes at 1:1 probe:primer ratio, efficiency also remained high (*RPLP0*: 83–92%, *TBP*: 84.3–99.3%) across the entire concentration range; R^2 for *RPLP0* and *TBP* was at least 0.97 at 0.3 and 0.24 μM. However, at 1:2 ratio, efficiency decreased: for *RPLP0* values were 65–72.3%, for *TBP* — 74.7–84%. It should be noted that high formal efficiency did not always accompany acceptable linearity: at 0.12 μM primer concentration and 1:1 ratio, *TBP* efficiency reached 99.3%, yet R^2 was only 0.93, indicating amplification nonlinearity.

Optimal balance of efficiency and linearity for all three genes simultaneously was achieved at 0.3 μM primer concentration and 1:1 ratio, where efficiency was 97% for *MTOR* (R^2 = 0.98), 92% for *RPLP0* (R^2 = 0.99), and 84.3% for *TBP* (R^2 = 0.97).

Result Reproducibility

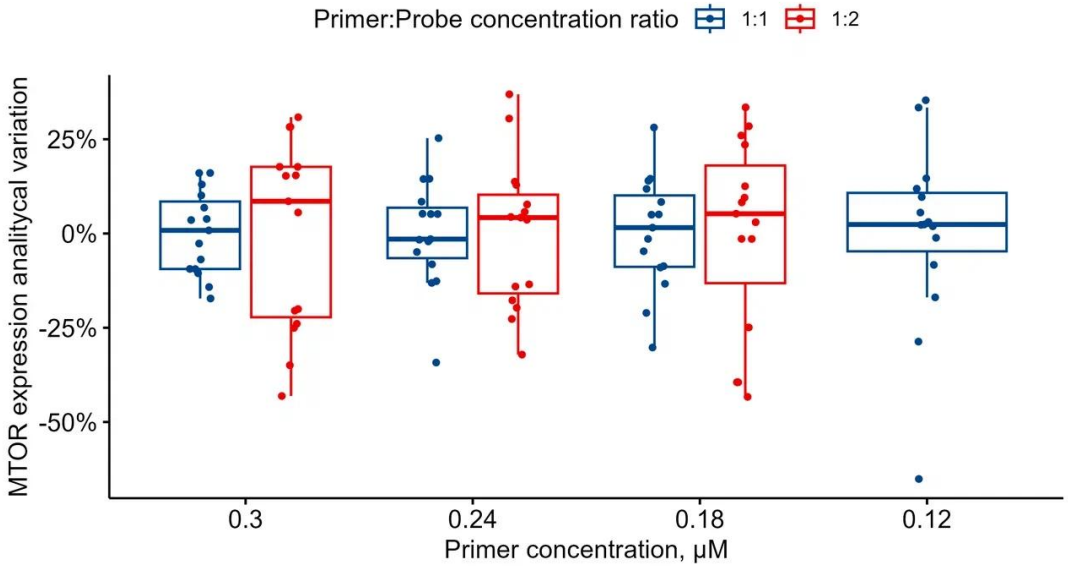


Figure 2. Fold Change (expression) variability depending on primer concentration and probe:primer ratio. 1:1 probe:primer ratio provides narrower Fold Change dispersion (median values –1.5%–2.4%; IQR 13.4–19%) than 1:2 (median values 4.2–8.5%; IQR 26.2–39.9%). **Lane designations:** Primer:Probe concentration ratio — ratio of primer to probe concentrations; *MTOR* expression analytical variation — analytical variation of *MTOR* expression; Primer concentration, μM — primer concentration in micromolars.

Reproducibility of *MTOR* expression quantification was assessed by Fold Change (FC) value variability depending on primer concentration and probe:primer ratio. For each condition combination, the median deviation of FC from the mean was calculated. The IQR was used as a measure of data dispersion (**Figure 2**). At 1:1 probe:primer ratio, IQR was substantially narrower (13.4–19%) compared to 1:2 ratio (26.2–39.9%). The lowest value dispersion was observed at 0.24 μM primer concentration and 1:1 ratio, where IQR was 13.4% (median –1.5%, Q1–Q3: –6.6% – 6.8%), and at 0.12 μM (IQR = 15.5%, median 2.4%, Q1–Q3: –4.7% – 10.8%). Concentration 0.3 μM also demonstrated high reproducibility (IQR = 17.9%, median 0.8%, Q1–Q3: –9.4% – 8.5%). For subsequent experiments, 0.3 μM concentration and 1:1 probe:primer ratio were used.

Optimal RNA Extraction Kit Selection

Three commercial RNA extraction kits with different extraction principles (phenol-chloroform, spin-columns, and precipitation) were compared across blood and cell culture samples; tissue samples were extracted by phenol-chloroform only. For each biomaterial-method combination, RNA yield and purity, matrix integrity, PCR efficiency, and quantitative analysis result reproducibility were assessed.

RNA Yield and Purity

RNA and DNA concentrations in isolated preparations were determined by fluorimetry, and purity was assessed spectrophotometrically by A260/A280 ratio (**Table 1**).

Table 1. Comparison of RNA yield and purity from three extraction methods across biomaterial types.

Biomaterial	Parameter	Phenol-chloroform	Precipitation	Spin-columns
Blood	RNA, µg/ml	4.6 (3.5-5.2)	19.4 (18.4-20.2)	17.2 (16.9-20.8)
	DNA, µg/ml	0 (0-0)	7.2 (6.1-7.4)	4.8 (4.3-5.9)
	RNA/DNA ratio	only RNA	2.7 (2.5-3.5)	3.5 (3.5-4)
	Purity A260/A280	1.8 (1.7-1.9)	2 (1.9-2)	3 (3-3.2)
Cells	RNA, µg/ml	36.3 (35.5-37.2)	150 (147-154)	112 (99.4-116)
	DNA, µg/ml	0 (0-0)	15.1 (14.6-17.5)	6.1 (5.3-6.9)
	RNA/DNA ratio	only RNA	9.5 (8.5-10.3)	18.4 (17-18.7)
	Purity A260/A280	2 (1.9-2.1)	2 (2-2.1)	2.2 (2.2-2.2)
Tissue	Purity A260/A280	2 (2-2)	-	-

Note to Table 1: Data are presented as median (Q1–Q3) from three independent isolation replicates for each biomaterial–method combination. Lowest RNA yield was determined with phenol-chloroform extraction (for all biomaterial types); however, this method showed complete absence of DNA in samples (except tissue — minimal DNA content). Maximum RNA yield was observed with precipitation with relatively high DNA content in samples (RNA/DNA ratio 2.7 for blood; 9.5 for cells). Spin-column RNA isolation showed intermediate results compared to the other two methods. Acceptable RNA purity results (in the 1.8–2.2 range) were observed for all methods and biomaterials, except spin-columns (blood).

Phenol-chloroform extraction was the only method providing complete absence of genomic DNA in blood and cell culture samples, and minimal contamination in tissue (3.6 µg/ml, Q1–Q3: 2.4–4.5) with RNA/DNA ratio 7.1 (Q1–Q3: 5.9–8.8). However, this method gave the lowest RNA yield: blood — 4.6 µg/ml (Q1–Q3: 3.5–5.2), cells — 36.3 µg/ml (Q1–Q3: 35.5–37.2), tissue — 25 µg/ml (Q1–Q3: 16.9–31.3). A260/A280 values for phenol-chloroform extraction were 1.8 (Q1–Q3: 1.7-1.9) for blood, 2 (Q1–Q3: 1.9-2.1) for cells, and 2 (Q1–Q3: 2.02-2) for tissue.

Precipitation provided maximum RNA yield for blood (19.4 µg/ml, Q1–Q3: 18.4–20.2) and cell culture (150 µg/ml, Q1–Q3: 147–154), but was accompanied by significant genomic DNA contamination (7.2 µg/ml, Q1–Q3: 6.1–7.4 for blood; 15.1 µg/ml, Q1–Q3: 14.6–17.5 for cells). RNA/DNA ratio for precipitation was lowest among all methods (2.7 — Q1–Q3: 2.5–3.5 for blood; 9.5 — Q1–Q3: 8.5–10.3 for cells), indicating a significant DNA fraction in preparations. A260/A280 values were 2 (Q1–Q3: 1.9-2) for blood and 2 (Q1–Q3: 2-2.1) for cells.

Spin-columns demonstrated intermediate results: RNA yield lower than precipitation (17.2 µg/ml, Q1–Q3: 16.9–20.8 for blood; 112 µg/ml, Q1–Q3: 99.4–116 for cells) but higher than phenol-chloroform extraction. DNA contamination was 4.8 µg/ml (Q1–Q3: 4.3–5.9) for blood and 6.1 µg/ml (Q1–Q3: 5.3–6.9) for cells, less than precipitation but greater than phenol-

chloroform. A260/A280 values for spin-columns were 3 (Q1–Q3: 3–3.2) for blood and 2.2 (Q1–Q3: 2.2-2.2) for cells.

RNA Integrity

RNA integrity was assessed by agarose gel electrophoresis (**Figure 3**). For cell culture and tissue samples, well-defined 28S and 18S rRNA bands with an approximately 2:1 intensity ratio were observed, confirming RNA integrity and the absence of substantial degradation. A different pattern was observed for blood samples: phenol-chloroform extraction yielded no detectable large ribosomal RNA bands, whereas both precipitation-based and spin-column methods produced a single, intense band at approximately 4000 bp.

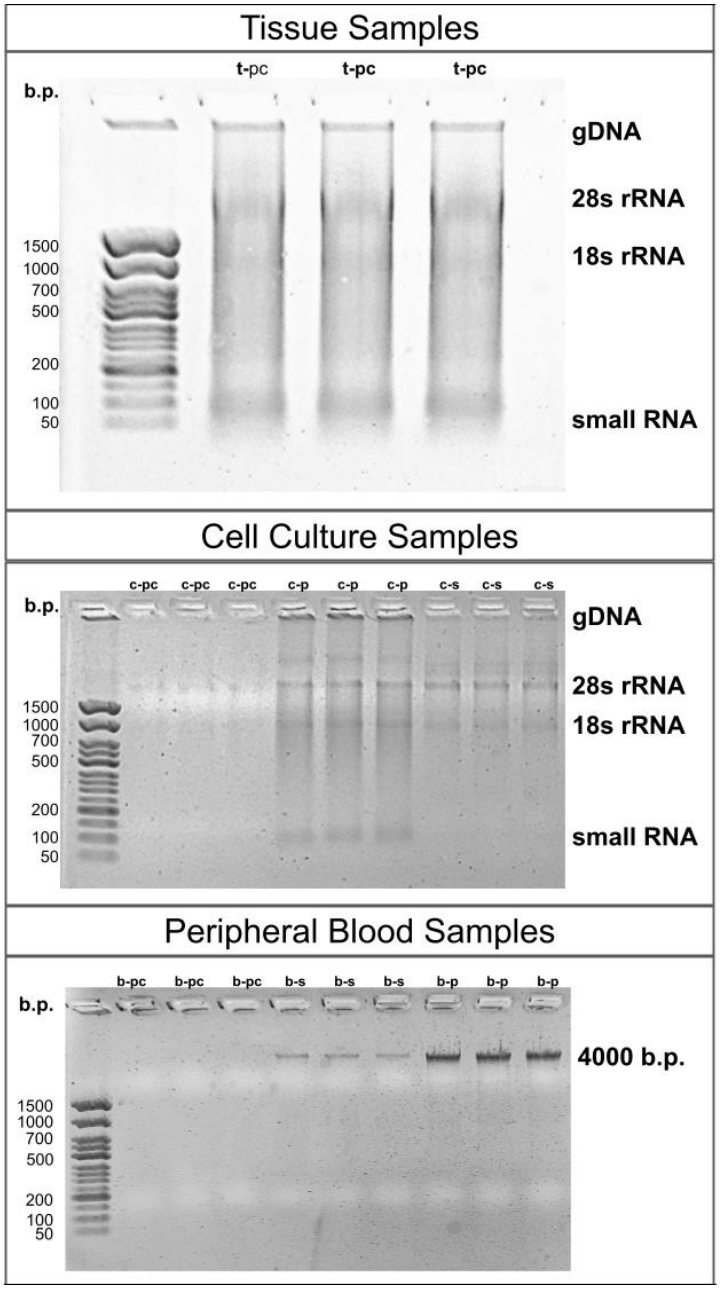


Figure 3. Integrity of isolated RNA from different biomaterial types using three extraction methods. Distinct 28S and 18S ribosomal RNA bands were observed for cell and tissue samples across all three methods. For blood RNA samples, ribosomal bands corresponding to 28S and 18S rRNA were not detected.

Lane designations: t-pc — tissue (breast tumor tissue), phenol-chloroform extraction; c-pc — cell culture (SCP-1 line), phenol-chloroform extraction; c-p — cell culture, precipitation; c-s — cell culture, silica membrane spin-columns; b-pc — peripheral blood, phenol-chloroform extraction; b-s — peripheral blood, spin-columns; b-p — peripheral blood, precipitation.

Amplification Curve Validity

Validity (presence of a clear sigmoidal curve) was 100% for all samples, regardless of biomaterial type or extraction method. Specifically, all three extraction methods — phenol-chloroform, precipitation, and spin-column — performed equally well for both blood and cell culture samples.

PCR Efficiency

Amplification efficiency was assessed for each biomaterial–extraction method combination by calibration curve slope (**Figure 4**), as it was done previously during the PCR efficiency analysis for different primer concentrations. Based on the results of the primer concentration optimization stage, all subsequent experiments were performed at the optimal primer concentration of 0.3 μM and a probe:primer ratio of 1:1.

For cell culture, phenol-chloroform extraction gave the best perfomabce: *MTOR* efficiency 96.3% (R^2 = 0.99), *TBP* — 93% (R^2 = 0.97); for *RPLP0*, efficiency was 119.3% with relatively low R^2 = 0.74, indicating amplification nonlinearity for this gene. Both spin-column and precipitation methods yielded comparable *MTOR* efficiencies (87.7% vs. 87%) with good R^2 values (0.98 and 0.94). However, *RPLP0* efficiency was markedly lower with spin-columns, falling to 67.7% (R^2 = 0.81).

For blood samples, spin-columns gave the best *MTOR* amplification efficiency (93.3%, R^2 = 0.98), exceeding that of phenol-chloroform extraction (90.7%, R^2 = 0.95) and precipitation (88.7%, R^2 = 0.55). For *RPLP0* in blood samples, efficiency was comparable with phenol-chloroform extraction and precipitation (90.3% and 92.7% respectively), however R^2 for precipitation was critically low (0.58 versus 0.86). For *TBP* in blood samples, best linearity was achieved with spin-columns (R^2 = 0.95, efficiency 78%), while phenol-chloroform extraction and precipitation demonstrated low R^2 (0.84 and 0.57 respectively). Precipitation for blood samples systematically showed extremely low calibration curve linearity (R^2 = 0.55–0.58) for all three genes, making this method insufficiently reliable for blood quantitative analysis, despite formally acceptable efficiency values.

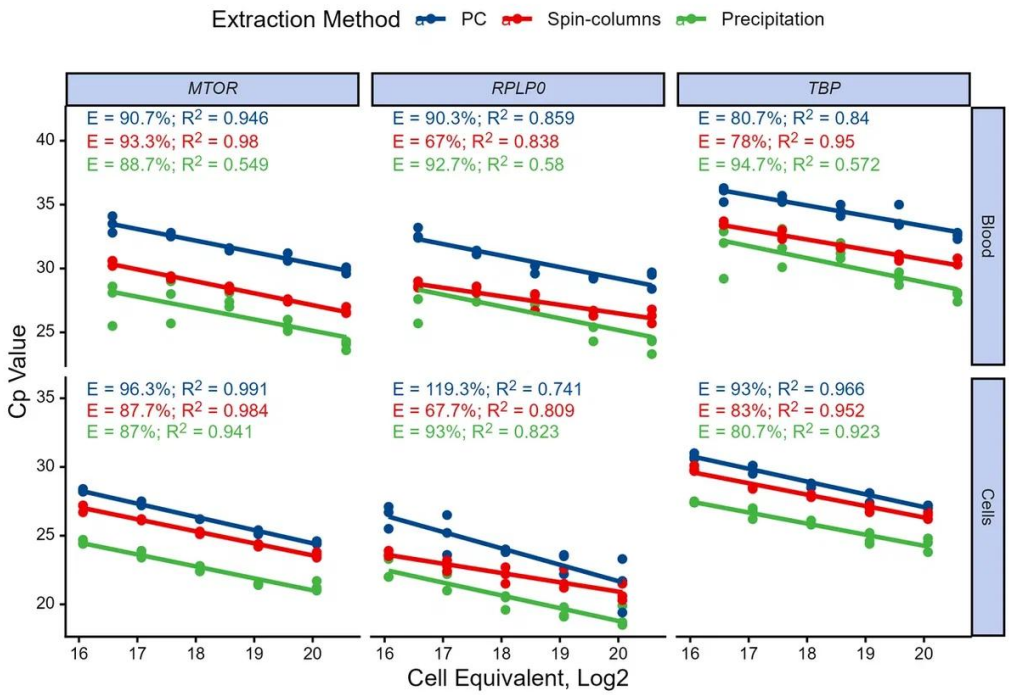


Figure 4. Comparison of three RNA extraction methods (phenol-chloroform, spin-columns and precipitation) on amplification efficiency of each gene in blood and cell culture samples. To assess amplification efficiency, calibration curves of Cp versus $\log_2$ RNA template dilution were constructed with calculation of coefficient of determination R^2. For blood samples, best *MTOR* performance was with spin-columns (93.3%, R^2 = 0.98); precipitation showed low linearity (R^2 = 0.55–0.58) for all three genes. For cells, phenol-chloroform extraction gave best results (efficiency 93-119.3% with high R^2 values). Precipitation and spin-columns showed lower efficiency results while maintaining high R^2 values.
Lane designation: PC extraction — phenol-chloroform extraction; Cp value — Crossing Point; E — amplification efficiency; Blood — peripheral blood samples; Cells — cell culture samples (SCP-1 line); Calculated number of cells, Log_2 — logarithmic transformation of the calculated cell number equivalent.

Result Reproducibility

Reproducibility of *MTOR* expression quantification was evaluated by determining the IQR of FC deviations across each biomaterial and extraction method combination. FC deviation for an individual reaction was calculated as the difference between FC value in the reaction and mean FC for the group.

For blood samples, greatest variability was with spin-columns (IQR = 29.8%; median 3.5%; Q1–Q3: –11.8 – 18.0%). Precipitation exhibited a slightly narrower spread (IQR = 29.4%; median 6.3%; Q1–Q3: –9.4 – 20.0%). Phenol-chloroform extraction showed lowest variability (IQR = 13.2%; median 5.7%; Q1–Q3: –6.1 – 7.1%).

For cell culture, best reproducibility was with spin-columns (IQR = 22.5%; median 7.7%; Q1–Q3: –6.0 – 16.5%). Precipitation showed higher spread (IQR = 28.2%; median 3.6%; Q1–Q3: –12.2 – 16.0%), while phenol-chloroform extraction showed maximum variability (IQR = 37.3%; median 10.0%; Q1–Q3: –19.0 – 18.3%).

For tissue samples, reproducibility was assessed under selected conditions: IQR = 17.9% (median 0.8%; Q1–Q3: –9.4 – 8.5%).

Genomic DNA Influence on Quantitative Analysis Results

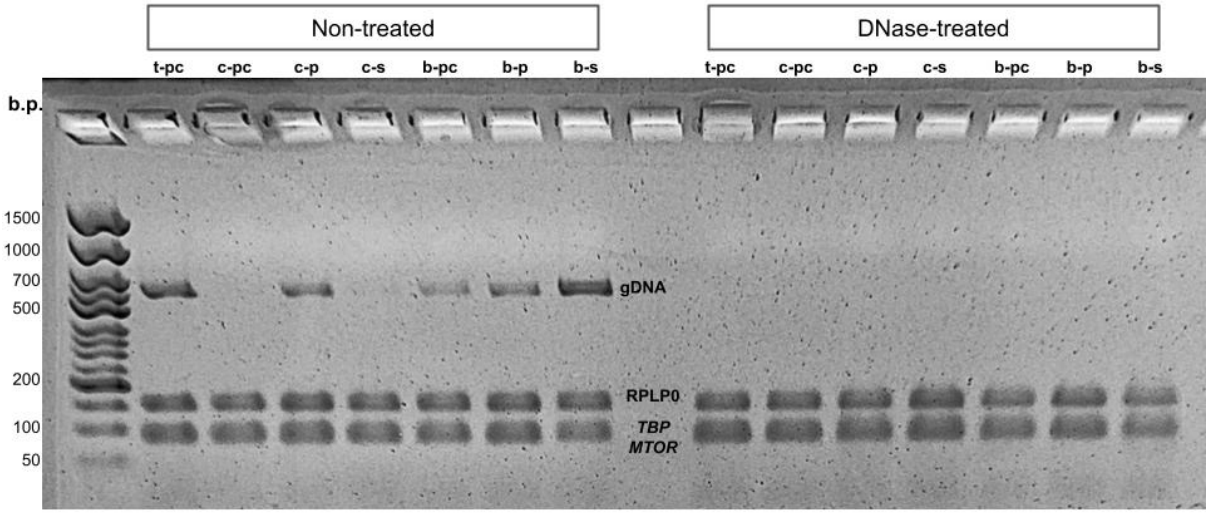


Figure 5. Electrophoregram of amplification products with and without thermolabile DNase pretreatment of RNA matrix. The most intense band corresponding to genomic DNA fragment was observed for precipitation method (blood, cells) and phenol-chloroform extraction from tissue; minimal intensity — for phenol-chloroform extraction (blood, cells) and spin-columns (cells). In all DNase-treated samples, genomic DNA was not detected. **Lane designations:** t-pc — tissue (breast tumor tissue), phenol-chloroform extraction; c-pc — cell culture (SCP-1 line), phenol-chloroform extraction; c-p — cell culture, precipitation; c-s — cell culture, silica membrane spin-columns; b-pc — peripheral blood, phenol-chloroform extraction; b-s — peripheral blood, spin-columns; b-p — peripheral blood, precipitation.

The genomic fragment band (~600 bp) intensity varied considerably depending on the isolation method and biomaterial (**Figure 5**). The most intense ~600 bp band was observed with precipitation RNA isolation from both cell culture and blood. With spin-columns for cell culture, genomic fragment band was absent or barely visible, while for blood its intensity ranged from moderate to intense. With phenol-chloroform extraction, genomic fragment band was absent in cell culture samples, barely visible or moderately intense in blood samples, and most intense in tissue samples.

Minimal genomic DNA contribution was thus observed with phenol-chloroform extraction and spin-column RNA isolation from cell culture, and phenol-chloroform extraction from blood; maximal — with precipitation from any biomaterial and phenol-chloroform extraction from tissue. Following DNase treatment, no genomic DNA fragments were detectable in any of the samples.

Sequencing verified amplification specificity for all five investigated products. For the ~600 bp band obtained from DNase-untreated matrix, an *MTOR* intron fragment (chromosome 1, NC_000001.11) was identified, confirming its genomic origin. For the ~150 bp and ~90 bp bands obtained from both untreated and DNase-treated matrix, cDNA fragments of *RPLP0* (NM_001002.4), *MTOR* (NM_004958.4), and *TBP* (NM_003194.5) were respectively identified. In the case of the ~90 bp band, sequencing using both primer sets (*MTOR* and

TBP) confirmed the presence of both target products in a single band, due to the proximity of amplicon molecular weights (84 and 89 bp respectively). All obtained nucleotide sequences showed 100% identity to reference sequences. Thus, sequencing confirmed that all detectable signals in multiplex amplification correspond to target genes, and the ~600 bp band represents an *MTOR* genomic DNA fragment.

Amplification of all target genes proceeded successfully in every sample under both DNase-treated and untreated conditions, irrespective of varying degrees of genomic DNA contamination. However, detailed ΔCp and ΔFC analysis revealed dependence of genomic DNA contribution magnitude on isolation method and biomaterial type (**Table 2**).

Table 2. Genomic DNA influence on quantitative analysis results assessed by ΔCp and ΔFC (differences in Crossing Point and Fold Change between DNase-treated and untreated samples).

Biomaterial	Extraction Kit	Gene	ΔCp (Q1-Q3)	IQR ΔCp	ΔFC (Q1-Q3), %	IQR ΔFC
Blood	Phenol-chloroform	*MTOR*	0.2 (0.2-0.7)	0.5	-14 (-20.25-3.4)	23.6
		RPLP0	0.6 (0.4-1.4)	1.0		
		TBP	0.2 (-0.1-0.5)	0.6		
	Precipitation	*MTOR*	2.4 (1.8-2.5)	0.8	39.1 (13.45-91.95)	78.5
		RPLP0	0.7 (0.5-2)	1.5		
		TBP	1.6 (1.2-2)	0.8		
	Spin-columns	*MTOR*	1 (0.8-1.4)	0.6	24.9 (17.35-44.05)	26.7
		RPLP0	0.7 (0.4-1.5)	1.0		
		TBP	0.4 (0.4-0.7)	0.2		
Cells	Phenol-chloroform	*MTOR*	0.3 (-0.1-0.6)	0.7	-8.4 (-9.3-3.2)	12.5
		RPLP0	0.1 (-0.2-0.3)	0.6		
		TBP	0.2 (0.1-0.9)	0.8		
	Precipitation	*MTOR*	0.6 (0.3-0.6)	0.3	-36.3 (-36.3--10.9)	25.4
		RPLP0	0.6 (0.5-0.8)	0.2		
		TBP	0.7 (0.5-1.3)	0.8		
	Spin-columns	*MTOR*	0.1 (0-0.9)	0.9	-17.7 (-18.95--12.25)	6.7
		RPLP0	0.2 (0.1-1.6)	1.5		

		TBP	0.4 (0.3-0.6)	0.3		
		MTOR	0.9 (0.9-0.9)	0.0		
Tissue	Phenol-chloroform	*RPLP0*	0.5 (0.4-0.8)	0.4	36.1 (14.5-48.75)	34.2
		TBP	0.4 (0.2-0.6)	0.4		

Note to Table 2: ΔCp analysis revealed minimal genomic DNA contribution with phenol-chloroform extraction for blood and cells, and with spin-columns for cells. Maximum sample contamination with genomic DNA was observed for tissue with phenol-chloroform extraction. ΔFC analysis revealed minimal values for phenol-chloroform extraction from blood and cells, maximal values — for precipitation method (blood and cells) and spin-columns (cells).

Genomic DNA contribution by ΔCp data. The median ΔCp values between DNase-treated and untreated samples did not exceed 1 cycle for most combinations of biomaterial, extraction method, and gene (**Table 2**). Minimal genomic DNA contribution (ΔCp < 0.5) was observed for all three genes with phenol-chloroform extraction from blood (*MTOR*: 0.2; *RPLP0*: 0.6; *TBP*: 0.2) and cells (*MTOR*: 0.3; *RPLP0*: 0.1; *TBP*: 0.2), and spin-columns from cells (*MTOR*: 0.1; *RPLP0*: 0.2; *TBP*: 0.4). Somewhat higher ΔCp values (0.7–2.4) were recorded with precipitation from blood (*MTOR*: 2.4; *RPLP0*: 0.7; *TBP*: 1.6), correlating with residual genomic DNA in preparations; however, these values remained within one cycle in most cases (except *MTOR*, where ΔCp reached 2.4). For tissue isolated by phenol-chloroform method, ΔCp values were 0.9 for *MTOR*, 0.5 for *RPLP0*, and 0.4 for *TBP*. Thus, even in samples with maximal genomic DNA contamination (tissue, phenol-chloroform), ΔCp for most genes did not exceed 1 cycle, with the only exception being ΔCp 2.4 for *MTOR* in blood with precipitation.

Genomic DNA contribution by ΔFC data. Differences in FC between DNase-treated and untreated samples varied and depended on biomaterial and extraction method combination. Minimal genomic DNA influence on FC was observed for cell culture with phenol-chloroform extraction (median ΔFC = –8.4%, Q1–Q3: –9.3% – 3.2%). For blood with phenol-chloroform extraction, median ΔFC was –14% (Q1–Q3: –20.3% – 3.4%), also indicating negligible genomic DNA influence. Moderate influence was observed for blood with spin-columns (median ΔFC = 24.9%, Q1–Q3: 17.4 – 44.1%) and tissue with phenol-chloroform extraction (median ΔFC = 36.1%, Q1–Q3:14.5 – 48.8%). Greatest genomic DNA contribution to FC variability was recorded for blood with precipitation (median ΔFC = 39.1%, Q1–Q3: 13.5 – 92%) and cell culture with precipitation (median ΔFC = –36.3%, Q1–Q3: –36.3% – –10.9%) and spin-columns (median ΔFC = –17.7%, Q1–Q3: –19% – –12.3%).

Comparison of ΔFC with Technical Variability. To assess genomic DNA influence on quantitative results, FC change with DNase treatment (ΔFC) was compared with technical variability. For each combination, the position of median ΔFC relative to technical variability IQR (TV-IQR) and the magnitude of exceedance (as percentage of TV-IQR width) were calculated.

For blood samples with phenol-chloroform extraction, median ΔFC was –14.0% (Q1–Q3: –20.3 – 3.4%) with TV-IQR = 13.2% (Q1–Q3: –6.1 – 7.1%). Median ΔFC fell 7.9% below the lower technical variability boundary (60% of IQR width). For blood with spin-columns,

median ΔFC = 24.9% (Q1–Q3: 17.4 – 44.1%) with TV-IQR = 29.8% (Q1–Q3: –11.8 – 18.0%); exceeded upper boundary by 6.9% (23% of IQR). For blood with precipitation, median ΔFC = 39.1% (Q1–Q3: 13.5 – 92.0%) with TV-IQR = 29.4% (Q1–Q3: –9.4 – 20.0%); exceeded upper boundary by 19.1% (65% of IQR).

For cell culture with phenol-chloroform extraction, median ΔFC = –8.4% (Q1–Q3: –9.3 – 3.2%) with TV-IQR = 37.3% (Q1–Q3: –19.0 – 18.3%); median value was within technical variability IQR. For cell culture with precipitation, median ΔFC = –36.3% (Q1–Q3: –36.3 – – 10.9%) with TV-IQR = 28.2% (Q1–Q3: –12.2 – 16.0%); fell below lower boundary by 24.1% (85% of IQR). For cell culture with spin-columns, median ΔFC = –17.7% (Q1–Q3: –19.0 – – 12.3%) with TV-IQR = 22.5% (Q1–Q3: –6.0 – 16.5%); fell below lower boundary by 11.7% (52% of IQR).

For tissue with phenol-chloroform extraction, median ΔFC = 36.1% (Q1–Q3: 14.5 – 48.8%) with TV-IQR = 17.9% (Q1–Q3: –9.4 – 8.5%); exceeded upper boundary by 27.6% (154% of IQR).

Analytical Sensitivity

Analytical sensitivity was assessed based on data from two experiments. In the PCR efficiency experiment for blood samples and cell culture, RNA matrix from six samples was analyzed in five dilutions (undiluted, 1:2, 1:4, 1:8, 1:16). In the primer optimization experiment for tissue, the same dilution series was used. Initial biomaterial amounts added at the isolation stage were: 1,560,000 cells for blood, 1,100,000 cells for cell suspension, and 100 mg for tissue. Target gene detection was maintained to 1:16 dilution inclusive, allowing calculation of minimum detectable quantity. Minimum detectable RNA matrix in terms of original sample was: 97,500 cells for blood samples, 68,750 cells for cell culture, and 6.25 mg tissue. For blood samples and cell culture, values were identical for all three extraction methods.

DISCUSSION

In this study, we developed a stepwise workflow for multiplex RT-qPCR assay optimization for quantitative *MTOR* expression analysis, encompassing sequential adjustment of amplification protocol, primer concentrations, and RNA extraction method. While individual optimization steps have been described in the literature, they are typically presented either as isolated recommendations addressing a single parameter or as comprehensive, resource-intensive protocols designed primarily for industrial diagnostic kit development. In contrast, the integrated scheme described herein offers a practical, reproducible, and methodologically sound template that can be readily adopted by researchers who need to rapidly establish a reliable multiplex qPCR assay without unnecessary procedural complexity.

Amplification Protocol Optimization

The protocol modifications (adding of touch-down step) introduced in this study enabled the use of higher primer concentrations (0.3 μM instead of 0.12 μM) without compromising specificity, while also minimizing the effect of background signal on real-time detection [6].

Primer Concentration Optimization: From Suboptimal to High-Efficiency Amplification

The key finding of the first step was the identification of optimal primer concentration (0.3 μM) and probe:primer ratio (1:1), achieving amplification efficiencies of 97% for MTOR, 92% for RPLP0, and 84.3% for TBP with high linearity ($R^2 \geq 0.97$). These values substantially exceed those from our previous study (73–81%). This improvement is attributed to two key protocol modifications: the introduction of touch-down PCR, which allowed the use of higher primer concentrations (0.3 μM vs. 0.12 μM) without compromising specificity, and data normalization with baseline correction, which minimized the influence of background signal on Cp determination [6]. These optimization steps align with current standards for quantitative PCR method validation [21, 22], and their efficacy is supported by studies demonstrating enhanced specificity and reliability with modified amplification protocols [23].

Notably, during concentration optimization, we observed cases where high amplification efficiency did not correspond to acceptable linearity. For example, at 0.12 μM primer concentration and a 1:1 probe:primer ratio, TBP exhibited 99.3% efficiency but an R^2 of only 0.93. This discrepancy led us to propose $R^2 \geq 0.95$ as a mandatory criterion for quantitative analysis, underscoring the need to assess both efficiency and linearity [24].

RNA Extraction Method Selection: Trade-off Between Yield, Purity, and Reproducibility

Comparison of three RNA extraction methods revealed clear patterns enabling method recommendations based on biomaterial type and research objectives.

Phenol-chloroform extraction was the only method that completely eliminated genomic DNA from blood and cell culture samples, consistent with fluorimetry data and literature [17, 25]. However, it yielded the lowest RNA concentrations: for blood — 4.6 μg/ml, four-fold lower than precipitation. Nevertheless, phenol-chloroform extraction provided the best reproducibility for blood samples (IQR = 13.2%), making it the preferred choice for blood analysis where low variability is critical.

Precipitation, conversely, provided maximum RNA yield (19.4 μg/ml for blood and 150 μg/ml for cells), but with significant genomic DNA contamination (7.2 and 15.1 μg/ml respectively) and low regression plot linearity ($R^2 = 0.55–0.58$) for all three genes in blood analysis. This makes precipitation unacceptable for blood quantitative PCR analysis, despite formally acceptable efficiency (88.7–92.7% and 80.7-93% respectively). This observation is consistent with reports of PCR inhibitor co-precipitation, which can disproportionately suppress amplification across dilutions and disrupt linearity [15]. However, DNase treatment effectively eliminates genomic DNA from precipitated preparations (Figure 3), potentially allowing the use of precipitation for samples with extremely low cell numbers near the analytical sensitivity threshold, where maximizing RNA yield is critical.

Spin-columns occupied an intermediate position: offering good RNA yield (17.2 μg/ml for blood, 112 μg/ml for cells), moderate DNA contamination (4.8 and 6.1 μg/ml), and high calibration curve linearity ($R^2 = 0.95–0.98$). This method showed the best *MTOR* efficiency from blood samples (93.3%) and good reproducibility for cell culture (IQR = 22.5%). However, for blood, reproducibility with spin-columns was somewhat lower (IQR = 29.8%) than with phenol-chloroform extraction (IQR = 13.2%), possibly due to variability in RNA binding to silica membrane in the presence of hemoglobin, lipids, and other co-extracted components that can compete with RNA for binding or alter binding efficiency, leading to

yield variability and consequently reduced quantitative result reproducibility [26]. For cell cultures, spin-columns demonstrated the best overall balance among all tested methods: high reproducibility (IQR = 22.5%) combined with good amplification efficiency (87.7% for *MTOR*) and calibration curve linearity (R^2 = 0.94–0.98). Thus, spin-columns are the method of choice for RNA isolation from cell cultures in quantitative PCR analysis.

In this study, RNA extraction methods were compared by RNA concentration (µg/ml) rather than total RNA yield (µg). This choice was deliberate. Total yield depends on both RNA recovery and elution volume, whereas equal volumes of RNA matrix are used in downstream RT-qPCR. Therefore, it is the concentration, not the total yield, that determines the amount of template entering the reaction and directly affects analytical sensitivity. When elution volumes differ between methods, total yield can be misleading, whereas RNA concentration provides a more relevant basis for comparing extraction methods intended for quantitative RT-qPCR.

In summary, comparative evaluation of the three RNA extraction methods enabled the determination of an optimal strategy for each biomaterial type. Phenol-chloroform extraction is recommended for peripheral blood and tissue samples due to minimal genomic DNA contamination and best quantitative reproducibility. Spin-columns are preferred for cell cultures, offering an optimal balance of yield, purity, amplification efficiency, and reproducibility with procedural simplicity. Precipitation, despite high RNA yield, is characterized by significant DNA contamination and low linearity; its use is only justified with mandatory DNase treatment and in cases where maximum RNA yield is essential.

RNA Integrity

Electrophoretic analysis of RNA revealed unexpected features. For samples from cell culture and tissue, classical 28S and 18S rRNA bands were observed, indicating matrix preservation. However, for blood samples, the pattern differed: with phenol-chloroform extraction, large ribosomal bands were not detected; with precipitation and spin-columns, a single intense band was observed in the ~4000 bp region, approximately 1000 bp larger than expected human 28S rRNA. Notably, this unexpected band disappeared after DNase treatment with subsequent thermal inactivation at 65°C, indicating its DNA nature.

We could not find a direct explanation for this phenomenon in the literature; however, several hypotheses can be proposed. The ~4000 bp band may represent genomic DNA fragments released from damaged nucleated blood cells during Ficoll centrifugation [27]. Additionally, extracellular DNA fragments up to several thousand base pairs are known to circulate in blood plasma [28] and may co-precipitate with RNA during isolation. Finally, it cannot be excluded that electrophoresis under non-denaturing conditions may lead to anomalous migration of ribonucleoprotein complexes that dissociate after DNase treatment and heating. This phenomenon requires further investigation.

Importantly, the presence of this anomalous band did not correlate with PCR efficiency and result reproducibility — blood samples with atypical electrophoretic patterns demonstrated quite acceptable amplification performance. This is consistent with reports indicating that RT-qPCR is more tolerant to partial matrix degradation than methods requiring full-length transcripts [29]. Thus, we do not recommend using electrophoretic assessment of blood

RNA as a universal predictor of RT-qPCR success, at least for samples isolated by Ficoll gradient.

DNase Treatment: When Is It Really Necessary?

One of the most important and unexpected results of this work was the demonstration that DNase treatment is not mandatory for all biomaterial types and extraction methods. ΔCp analysis showed that for most combinations of biomaterial, extraction method, and gene, median ΔCp values between DNase-treated and untreated samples did not exceed 1 cycle. The only exception was *MTOR* in blood samples with precipitation, where ΔCp reached 2.4 cycles.

However, the most informative metric was the comparison of ΔFC with technical assay variability. Critical genomic DNA influence (median ΔFC exceeding technical variability IQR) was observed only for precipitation (both blood and cell culture samples). For phenol-chloroform extraction and spin-columns, genomic DNA influence did not exceed technical variability, making DNase treatment unnecessary, even when a genomic fragment band was visible on the electrophoregram.

This finding carries important practical implications. DNase treatment not only increases the cost and time of analysis but may also introduce additional variability due to incomplete enzyme inactivation [30]. Furthermore, when using intron-overlapping primers (as in our test system), genomic DNA amplification, even if it occurs, should not affect quantitative expression assessment, since cDNA and genomic DNA amplicons differ in length and consequently in detection timing [31]. In practice, we observed that the 574 bp band appeared on electrophoregrams only at high genomic DNA content in the sample, but its contribution to Cp value was minimal. We therefore propose reconsidering the routine practice of mandatory DNase treatment for all RNA samples and recommend it only when the extraction method is known to introduce substantial genomic DNA contamination.

Analytical Sensitivity

In our previous study, analytical sensitivity was 125,000 cells for blood and cell culture samples [6]; tissue sensitivity had not been determined. In the present work, the minimum detectable amount was 97,500 cells for blood samples and 68,750 cells for cell culture samples. For tissue, sensitivity was established for the first time at 6.25 mg. It should be noted that the observed differences for blood and cell culture fall within a single two-fold dilution step; therefore, these values should be interpreted as comparable rather than as a true improvement in sensitivity. Nevertheless, the established detection limits expand the potential clinical utility of the test for samples with limited biomaterial availability.

A Stepwise Workflow for Optimizing Multiplex RT-qPCR-Based Gene Expression Analysis

Synthesizing the results of the sequential optimization steps, we propose the following stepwise algorithm (Figure 6).

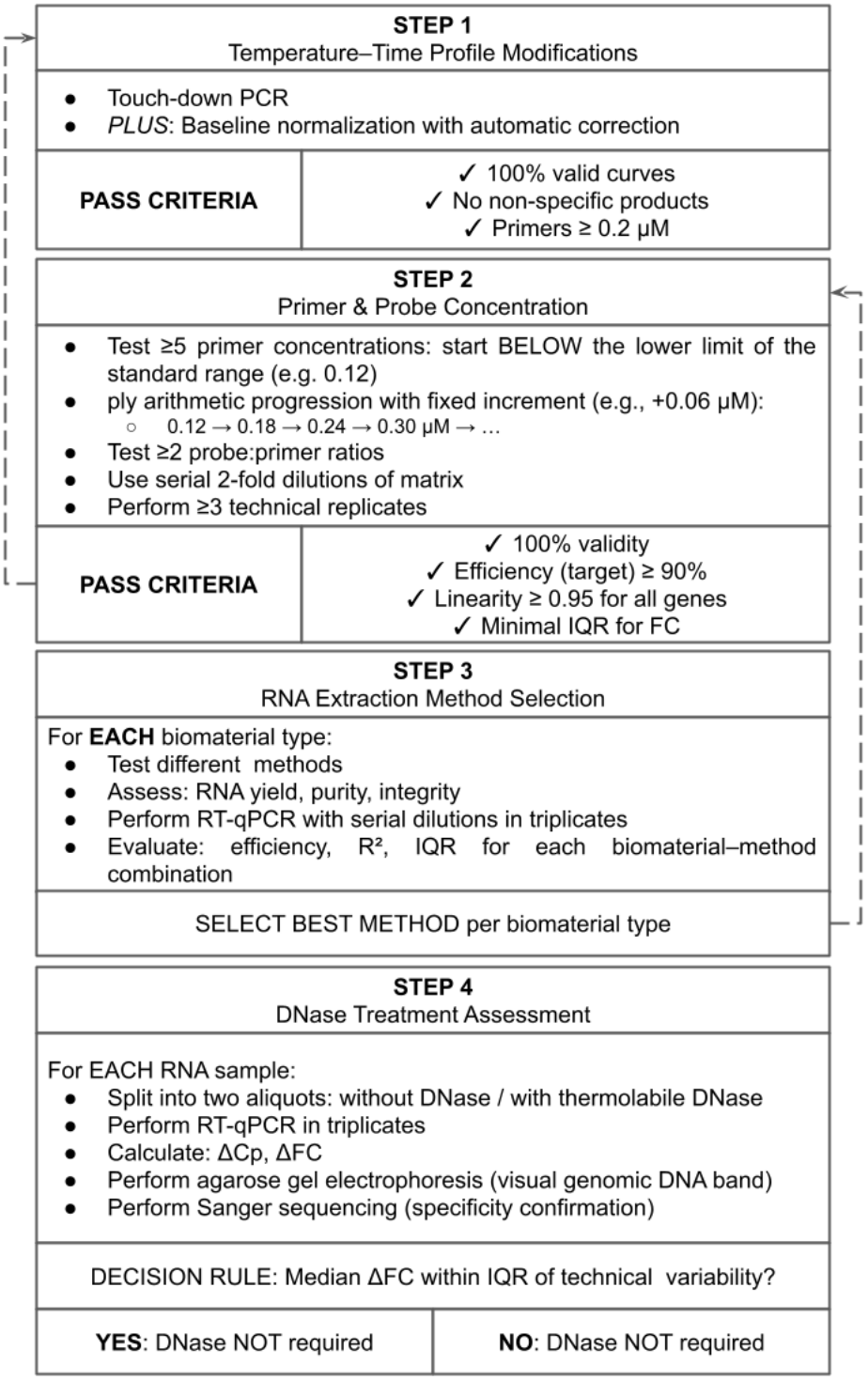


Figure 6. Stepwise workflow for multiplex RT-qPCR optimization. The algorithm comprises four sequential steps: (1) amplification protocol optimization via temperature–time profile modification and baseline normalization; (2) primer concentration and probe:primer ratio titration using serial RNA dilutions with simultaneous criteria (100% validity, efficiency ≥ 90%, results linearity - $R^2 \geq 0.95$, minimal IQR for FC); (3) RNA extraction method selection based on yield, purity, integrity, efficiency, R^2, and IQR for each biomaterial type; (4) DNase treatment necessity assessment by ΔCp and ΔFC comparison with technical variability, confirmed by gel electrophoresis and Sanger sequencing. Dashed return arrows indicate iterative adjustments between steps when criteria are not met.

At the first step, we recommend optimizing the amplification protocol by modifying the temperature–time profile. In the present study, we employed the touch-down PCR (TD-PCR) method; however, alternative strategies described in the literature may also be considered, including gradient PCR, step-down PCR, touch-up PCR, two-step protocols, extended initial denaturation, shortened annealing time, extended elongation time, and multiple annealing steps [32-38]. Additionally, we recommend including data normalization with automatic baseline correction as an integral component of the protocol. The criteria for proceeding to the second stage is the attainment of 100% valid amplification curves, the absence of non-specific products, and the feasibility of using primer concentrations of at least 0.2 μM [22]. If these conditions are not met, an iterative return to the selection of an alternative modification or combination thereof is required.

At the second step, we propose optimizing primer concentrations and the probe:primer ratio by testing at least five primer concentrations and at least two ratios using serial dilutions of the RNA matrix. Primer titration should start below the lower limit of the range generally recommended for probe-based qPCR assays (0.3-0.9 μM) [22] and proceed upward in fixed increments (e.g., +0.06 μM) until amplification efficiency and curve linearity. This approach identifies the minimum sufficient primer concentration, reducing reagent consumption and avoiding unnecessary testing across the entire recommended range .The optimal combination can be selected when the following criteria are simultaneously fulfilled: 100% validity, amplification efficiency for the target gene ≥ 90%, results linearity -R^2 ≥ 0.95 for all genes, and minimal IQR for FC. Failure to meet any of these criteria necessitates a return to the first step for adjustment of the temperature profile.

At the third step, we recommend selecting the most appropriate RNA extraction method: for each biomaterial type, several methods should be tested (e.g., phenol-chloroform extraction, precipitation, spin-columns, etc.). To select the optimal kit, RNA yield, purity (A260/A280 ratio), and integrity (agarose gel electrophoresis) should be assessed, followed by RT-qPCR with serial dilutions to evaluate efficiency, results linearity, and IQR for each biomaterial–method combination. The method should be chosen based on the best combined performance across all parameters for the specific biomaterial type; if all methods yield unsatisfactory results, a return to the second step for primer concentration revision is recommended.

At the fourth step, we recommend assessing the necessity of DNase treatment. To this end, each RNA sample should be divided into two aliquots (untreated and treated with thermolabile DNase); following RT-qPCR, ΔCp and ΔFC should be calculated, agarose gel electrophoresis of amplicons should be performed for visual assessment of the genomic DNA fragments, and Sanger sequencing conducted to confirm specificity. If the median ΔFC value does not exceed the IQR of technical variability, DNase treatment is not required; if it exceeds this threshold, DNase treatment is recommended [30].

Study Limitations

This study has several limitations that should be considered when interpreting the results.

All experiments were performed using a limited number of samples: one blood sample, one cell line (SCP-1) sample and one tumor tissue sample (luminal subtype A breast cancer). This design does not allow assessment of interindividual variability and influence of clinical factors (e.g., comorbidities, patient age) on amplification efficiency and reproducibility. However, this study was focused on technical method optimization rather than clinical validation. Subsequent validation on representative cohorts of breast cancer patients of various molecular-biological subtypes will be the next necessary step.

A single sample analysis did not allow statistical comparison of ΔFC values between different extraction methods within the same sample type, nor comparison of these differences with technical variability for each combination. Consequently, within the framework of this study, we cannot definitively state that quantitative results obtained for one biomaterial type using different RNA extraction methods are comparable. Assessment of inter-method compatibility requires further studies on representative samples and remains a promising task for future work.

For tissue samples, we used only phenol-chloroform extraction, limiting the ability to compare with other methods for this biomaterial type. For example, phenol-chloroform extraction could be modified using various standard column-based kits modifications or precipitation-based kits with enzymatic pretreatment (e.g., proteinase K) [39, 40]. Nevertheless, future studies should conduct comparative analysis of such approaches for tissue samples under optimized protocol conditions.

This study is limited to comparison of three RNA extraction methods; alternative approaches such as magnetic particle-based isolation or silica-based solid-phase extraction were not considered. This decision was made because phenol-chloroform extraction and spin-columns represent the "gold standard" for tissue, blood, and cell culture samples in most molecular diagnostics laboratories, while precipitation was included as a widely available alternative [16, 41]. Magnetic particle-based methods typically require specialized equipment and additional protocol optimization [42], which is beyond the scope of this work focused on standard approaches available for routine application. Silica-based solid-phase extraction methods, in turn, are characterized by less standardization and variable efficiency, limiting their use in clinical diagnostics [43]. Nevertheless, we acknowledge that comparative evaluation including these alternative methods could complement the present study, and their investigation represents interest for future work; however, the chosen design fully corresponds to the task of test optimization using the most reproducible and standardized approaches.

All samples used in this study were cryopreserved and subjected to thawing before RNA isolation. Although cryopreservation is standard practice for nucleic acid integrity preservation, the freeze-thaw process may affect RNA quality and yield, which should be considered when interpreting results [44].

RNA integrity and amplicon assessment by agarose gel electrophoresis was performed exclusively visually, without densitometric analysis or microchip electrophoresis. Although visual assessment is widespread practice in laboratory research, it does not allow objective quantitative measurements [45]. Nevertheless, in the context of this work, where the primary task was technical PCR protocol optimization, visual control was deemed sufficient.

For RNA purity control, we used only the A260/A280 optical density ratio, which reflects protein contamination degree. However, we did not analyze A260/A230 ratio, sensitive to residual guanidine thiocyanate, phenol, salts, and other PCR inhibitors that may be present in preparations, especially with phenol-chloroform extraction and precipitation [46]. This limitation does not allow complete exclusion of inhibitors that reduce amplification efficiency, which could explain low linearity for some methods.

In this study, we used relative quantification (2^(–ΔCp) method) without applying external calibration curves with known standard concentrations. This approach, widely used in gene expression studies, has several limitations. As shown in the review by Chen et al., the 2^(–ΔCp) method assumes that PCR efficiency for target and reference genes is 100% or close to 100% (ideally in the 90–110% range), which is not always achieved in practice [47]. In our study, amplification efficiency was 84–97%, close to ideal values; however, even small efficiency deviations can accumulate exponentially and lead to systematic errors [22]. Moreover, relative quantification does not allow direct comparison of absolute expression levels between different studies, since results are expressed as fold change relative to control sample [48]. Nevertheless, for clinical purposes where relative expression level between samples is important (e.g., comparison of tumor and normal tissue), this approach is standard and widely accepted [47].

We did not evaluate the influence of different treatment regimens (chemotherapy, targeted therapy) on RNA quality and PCR efficiency, which may be relevant when analyzing clinical samples from patients receiving therapy.

Finally, we did not evaluate long-term RNA stability after isolation by different methods, which may be important for clinical laboratories working with accumulated biomaterial.

Clinical Significance and Perspectives

The developed stepwise workflow for multiplex RT-qPCR optimization has significance extending beyond the specific *MTOR* test. The proposed methodology — sequential adjustment of amplification protocol, primer concentrations, RNA extraction method, and reconsideration of DNase treatment necessity — can be applied to optimize any multiplex PCR assays intended for quantitative gene expression analysis.

At the same time, the test developed in this study is oriented toward assessing *MTOR* expression — a key component of the PI3K/AKT/MTOR signaling pathway, whose dysregulation plays a central role in tumor cell proliferation in breast cancer [49], making it a promising tool for diagnosis and monitoring of this disease. The optimized protocol provides high analytical sensitivity and reproducibility across different biomaterial types, expanding clinical application possibilities: from tumor tissue analysis at primary diagnosis to monitoring MTOR signaling pathway activity in peripheral blood and cell cultures during experimental studies.

In conclusion, the developed stepwise workflow enabled the creation of a reliable, highly sensitive, and reproducible test for quantitative *MTOR* expression assessment, suitable for analyzing different biomaterial types. The key finding is the demonstration that DNase treatment is not mandatory when using properly designed intron-overlapping primers, and

that RNA extraction method choice should be determined by biomaterial type: phenol-chloroform extraction is preferred for blood samples, spin-columns for cell culture samples, while precipitation, despite high yield, is not recommended for quantitative PCR analysis due to low linearity and high DNA contamination.

CONCLUSION

In this study, we successfully performed comprehensive optimization of the previously developed multiplex RT-qPCR assay for quantitative *MTOR* expression analysis. Through sequential adjustment of the amplification protocol (introduction of TD-PCR and baseline normalization), empirical optimization of primer concentrations and probe:primer ratios, and comparative evaluation of three RNA extraction methods, we achieved amplification efficiencies of 97% for *MTOR*, 92% for *RPLP0*, and 84.3% for *TBP* with high calibration curve linearity ($R^2 \geq 0.97$), substantially exceeding the performance of our previous study. Optimal conditions were determined as 0.3 μM primer concentration with 1:1 probe:primer ratio. For peripheral blood samples, phenol-chloroform extraction is recommended, providing minimal genomic DNA contamination and best reproducibility (FC IQR = 13.2%); for cell cultures, silica membrane spin-columns are optimal (RNA yield 112 μg/ml, FC IQR = 22.5%). Precipitation, despite maximum RNA yield, is characterized by low calibration curve linearity ($R^2 = 0.55–0.58$) and significant DNA contamination; its use is justified only for samples with extremely low cell numbers (near the analytical sensitivity threshold), where achieving sufficient matrix concentration becomes critical. Electrophoretic analysis of amplicons revealed an additional 574 bp band corresponding to an *MTOR* intron fragment in DNase-untreated samples. This band was absent in all DNase-treated preparations. Sanger sequencing confirmed multiplex amplification specificity: the ~600 bp band was identified as an *MTOR* genomic DNA fragment (chromosome 1, NC_000001.11), while ~150 bp and ~90 bp products were identified as cDNA fragments of *RPLP0* (NM_001002.4), *MTOR* (NM_004958.4), and *TBP* (NM_003194.5), fully confirming correct performance of the intron-overlapping primers. DNase treatment of RNA is not mandatory when using phenol-chloroform extraction and spin-columns, as genomic DNA contribution to quantitative assessment does not exceed technical assay variability. DNase treatment is recommended only for precipitation. Analytical sensitivity was for 97,500 blood sample cells and 68,750 cells for cell culture; for tissue, the detection threshold was established for the first time at 6.25 mg. Through this work, we developed a universal stepwise workflow for multiplex RT-qPCR assay optimization, encompassing amplification protocol adjustment, oligonucleotide concentration titration, RNA extraction method selection based on biomaterial type, and assessment of DNase treatment necessity. The developed workflow can be recommended as a methodological framework for multiplex RT-qPCR assay optimization in research applications.

MATERIALS AND METHODS

The study comprised four sequential stages. First, the amplification protocol was optimized by adding a touch-down step and data normalization with baseline correction. Second, optimal primer concentrations (5 concentrations) and probe:primer ratios (2 variants) were determined using breast tumor tissue material; for each variant, 5 serial dilutions of RNA template were analyzed in three technical replicates (150 PCR reactions total), assessing amplification curve validity, PCR efficiency, and reproducibility of *MTOR* expression quantification. Third, three commercial RNA extraction kits (phenol-chloroform extraction

with column purification, precipitation, and affinity sorption on spin-columns) were compared across three biological materials (SCP-1 cell culture, peripheral blood, and tumor tissue); for each material-method combination, RNA yield and purity, matrix integrity, PCR efficiency, and reproducibility were evaluated (90 PCR reactions). Fourth, in 7 RNA samples (three from blood, three from cell culture, one from tissue) isolated by each method, PCR was performed with and without thermolabile DNase pretreatment in three technical replicates (42 PCR reactions total), comparing ΔCp and FC values and analyzing amplicon electrophoretic profiles to determine the necessity of enzymatic genomic DNA removal when using the optimized protocol with intron-overlapping primers.

Amplification Protocol Optimization

One-step multiplex RT-qPCR was performed on a DTprime amplifier (DNA-Technology LLC, Russia) using manufacturer's software. Fluorescence signal detection was performed in FAM (*MTOR*), HEX (*RPLP0*), and ROX (*TBP*) channels with Crossing Point (Cp) registration. The initial amplification protocol included reverse transcription at 45°C for 30 min, pre-denaturation at 95°C for 5 min, followed by 35 cycles: 95°C for 5 s, 68°C for 11 s (elongation step was excluded to minimize nonspecific genomic DNA amplification, under the condition that final primer and probe concentrations did not exceed 0.12 μM) [50]. To enable use of higher primer and probe concentrations, a touch-down step was added: in initial cycles, annealing temperature exceeded primer melting temperature (Tm) with subsequent gradual 1°C decrease per cycle to the optimal value (negative temperature increment from 75°C over 10 cycles) [51]. Additionally, data normalization with automatic baseline correction was performed to minimize background signal differences between samples and improve Cp determination accuracy [12].

Primer and Probe Concentration Optimization

Study Material

The material for this study was an operative fragment of tumor tissue obtained from breast resection from a patient diagnosed with luminal subtype A breast cancer at the Sverdlovsk Regional Oncology Dispensary, Yekaterinburg, Russia. The study was approved by the Local Ethics Committee of Ural State Medical University (Protocol No. 6 dated 18.10.2024), and informed consent was obtained from the participant. Selection of this material for the initial optimization stage was based on tissue containing high genomic DNA concentrations, pronounced cellular heterogeneity, and PCR inhibitors. Thus, amplification conditions optimized on tissue would subsequently be applicable to blood and cell culture analysis without additional protocol modification.

Sample transport to the laboratory was performed in disposable sterile tubes using Hanks' Balanced Salt Solution (HBSS) without Ca and Mg ions (ServiceBio, China) at +2–+8°C in a medical thermocontainer with cooling elements within 3 hours of biomaterial collection. Upon arrival, the tissue fragment was divided into equal 100 mg portions. For long-term storage and nucleic acid integrity preservation, fragments were immersed in cryomedium (ServiceBio, China) and cryopreserved (cryostorage temperature not exceeding –196°C). Biomaterial was stored until RNA extraction.

Experimental Design

Total RNA from tissue was isolated using the "Lira" reagent kit (Biolabmix LLC, Russia), which combines sequential homogenization, phenol-chloroform extraction, and spin-column purification as integral steps of the manufacturer's protocol. This method was selected as most appropriate for tissue samples due to effective removal of PCR inhibitors and genomic DNA characteristic of this biomaterial type, as well as its ability to handle samples with high protein content without requiring elevated temperatures, thereby preserving the integrity of the RNA matrix. Isolation was performed strictly according to manufacturer's instructions; homogenization was performed using a manual homogenizer (Miulab, China). One-step multiplex RT-qPCR was performed using the BioMaster RT-qPCR kit (Biolabmix LLC, Russia). Primers and probes were taken from our previous study [6] and synthesized by DNA-Synthesis LLC (Russia); their full characteristics, including sequences, amplicon lengths, melting temperatures, and fluorescent labels, are summarized in Supplementary Table 1 [52]. According to the manufacturer's protocol, reaction mixtures were prepared from 12.5 µl Reaction Buffer (containing dNTP mixture and Mg ions), 1 µl Reaction Mix (containing genetically modified Moloney murine leukemia virus reverse transcriptase and recombinant Taq DNA polymerase inactivated by specific monoclonal antibodies), and 5 µl of probe/primer mix at various concentrations.

To evaluate amplification efficiency and quantitative analysis reproducibility, RNA matrix isolated from tissue was serially two-fold diluted in physiological saline to obtain the following template concentrations: undiluted, and 1:2, 1:4, 1:8, and 1:16 dilutions (N = 5). Tested primer concentrations were: 0.3, 0.24, 0.18, 0.12, and 0.06 µM (N = 5). For each concentration, probe:primer ratios of 1:1 and 1:2 were tested (N = 2). Each combination was analyzed in three technical replicates. Total reaction number was 150 (5 RNA template dilutions × 5 primer concentrations × 2 probe:primer ratios × 3 technical replicates). Additionally, three negative controls were included in each run. One-step multiplex RT-qPCR was performed according to the protocol and equipment described above.

For each variant, detection was performed with and without baseline normalization to evaluate nonspecific background fluorescence levels for correct quantitative expression assessment of target genes.

Evaluation Criteria

Quality assessment for each combination of primer concentration and probe:primer ratio was performed sequentially according to the following criteria.

Amplification validity was defined as the proportion of reactions with clear sigmoidal fluorescence accumulation curves characterized by a distinct exponential phase. Curves lacking exponential growth, showing "stepwise" profiles, or exhibiting early plateau were considered invalid.

Amplification efficiency for *MTOR* and reference genes was assessed using linear regression of Cp values against $\log_2$ of RNA template dilution. PCR efficiency was expressed as the slope magnitude relative to the theoretical optimum of −1 (E = |slope| × 100%). PCR results linearity was evaluated by R^2 values. Results with $R^2 < 0.95$ were considered unacceptable for quantitative analysis, as low linearity indicates presence of inhibitors or amplification instability.

Regression analysis yielded the following equation for each gene:

$$y = a - b \cdot x,$$

where y is the predicted Cp value, a is the Y-intercept, b is the regression slope, and x is $\log_2(N)$, where N is the estimated number of cells in a tested sample.

Reproducibility of *MTOR* expression quantification was assessed by variability of *MTOR* expression values. Expression values were determined based on Cp values using the FC formula described by Livak and Schmittgen [53]:

$$FC = 2^{-\Delta Cp}, \Delta Cp = Cp_{target} - Cp_{reference},$$

where Cp_{target} is the Cp value of the target gene and $Cp_{reference}$ is the geometric mean of Cp values of both normalizers.

For each condition combination (primer concentration × probe:primer ratio), the median FC and IQR were determined across all technical replicates pooled across the entire series of RNA template dilutions. Reproducibility was assessed by IQR magnitude: narrower IQR indicated higher reproducibility. Optimal priment concentration and probe:primer ratio selection was based on minimal IQRwhile maintaining 100% validity and high amplification efficiency.
The primary data of RT-qPCR obtained during primer concentration and probe:primer ratio optimization are presented in **Supplementary Dataset 1** [54].

Extraction Kit Comparison

Study Materials

This stage utilized three biological material types: cell culture, venous blood, and tumor tissue.

Cell culture. The SCP-1 human bone marrow stromal mesenchymal stem cell line, obtained from the Cell Culture Collection of Ural State Medical University (Yekaterinburg, Russia), was used. Cell cultivation and passaging were performed according to previously described protocols [55]. Cell suspension at 1 million cells/mL concentration was prepared for the experiment; counting was performed in a Goryaev chamber using trypan blue exclusion method:

$$X = A \times 10^4$$

where X is cell concentration in 1 mL suspension, A is total counted cells in 25 large squares, and 10^4 is the conversion factor per 1 mL volume.

Cell viability was at least 95%. After obtaining suspension, cells were resuspended in 100 µl RNA Stabilizer (Biolabmix LLC, Russia) and incubated at +4°C for 24 h, as immediate RNA isolation was not organizationally possible. After incubation, samples were stored at –80°C.

Venous blood. A venous blood sample was obtained from a healthy female volunteer at the City Clinical Hospital №40, Yekaterinburg, Russia. The study was approved by the Local Ethics Committee of Ural State Medical University (Protocol No. 6 dated 18.10.2024), and informed consent was obtained. Sample transport to the laboratory was performed in K_2-EDTA vacuum tubes (Hebei Xinle Sci&Tech Co., China) at +2–+8°C in a medical thermocontainer with cooling elements within 3 hours of collection. Upon arrival, nuclear fraction was isolated. The blood sample was diluted 2-fold with phosphate-buffered saline (PBS, ServiceBio, China) and layered onto a Ficoll solution (PanEco, Russia) at density 1.077 g/mL in 3:2 ratio respectively. Centrifugation was performed at 400 g for 30 min, and the meniscus at the Ficoll-plasma interface was collected. Cells were resuspended in 500 µl PBS and centrifuged at 1500 g for 10 min. Supernatant was removed and cells were resuspended in 500 µl PBS. Cells were counted in a Goryaev chamber using the formula:

$$X = \frac{A \times N \times 4000}{V}$$

where X is cell concentration in 1 µl suspension, A is total counted cells, N is cell suspension dilution factor, V is number of small squares counted, and 4000 is the conversion factor per 1 µl volume.

Cell suspension was adjusted to 1.56 million cells/mL. Cells were centrifuged at 400 g for 5 min, supernatant removed, and resuspended in 100 µl RNA Stabilizer (Biolabmix LLC, Russia), then incubated at +4°C for 24 hours. After the incubation, samples were stored at –80°C.

Tumor tissue. The same breast tumor tissue sample (luminal subtype A) as in the primer concentration optimization section was used. Sample preparation, including collection, transport, and cryopreservation, was performed according to the protocol described above.

Total RNA Isolation Methods

Total RNA isolation was performed using three commercial reagent kits implementing different nucleic acid extraction principles: phenol-chloroform extraction with subsequent column purification using "Lira" kit (Biolabmix LLC, Russia), precipitation using "Proba NK" kit (DNA-Technology LLC, Russia), and affinity sorption on spin-columns using "RUplus-50" kit (Biolabmix LLC, Russia). Method selection was determined by the need for comparative evaluation of their effectiveness for different biomaterial types: cell culture, peripheral blood, and tumor tissue.

For blood and cell culture samples, RNA isolation was performed by all three methods. For tissue, isolation was performed only by phenol-chloroform extraction with spin-column purification ("Lira", Biolabmix LLC, Russia), since it is well established that methods not involving homogenization, guanidine thiocyanate treatment, and phenol-chloroform purification cannot isolate nucleic acids of sufficient quality for quantitative analysis without additional processing [56]. RNA isolation from all sample types was performed according to manufacturer's instructions, with the exception of adding PolyA RNA reagent (Biolabmix LLC, Russia) at 5 mg/mL before isolation with "RUplus-50".

According to the manufacturer's instructions, the final elution volumes were 50 µl for the "Lira" kit and 200 µl for both the "Proba NK" and "RUplus-50" kits. These volumes were kept constant within each method throughout the study, ensuring that RNA concentrations could be directly compared across extraction methods.

PCR Efficiency and Reproducibility Comparison

To assess PCR efficiency and expression measurement reproducibility, RNA matrix obtained from each of 6 individual samples (three from a single blood sample and three from a single cell culture sample, each processed by the three different extraction methods) (N = 6) was serially two-fold diluted in physiological saline: undiluted, and 1:2, 1:4, 1:8, and 1:16 dilutions (N = 5). Amplification was then performed in three technical replicates. Total reaction number was 90 (6 samples × 5 template dilutions × 3 replicates). Negative controls were included in all runs. For the tissue sample, efficiency and reproducibility comparison was not performed in this experiment as it had been conducted previously.

Assessment of Genomic DNA Influence on RT-qPCR Results and Quantitative Expression Measurement

To evaluate genomic DNA influence on PCR results and quantitative expression measurement, PCR was performed with and without thermolabile DNase pretreatment (Biolabmix LLC, Russia).

For each of seven RNA samples, mixtures were prepared. These seven samples comprised aliquots of a single blood sample and a single cell culture sample processed by the three different extraction methods (three RNA samples each), plus one RNA sample isolated from tumor tissue. Each RNA sample was divided into two equal aliquots — one for DNase treatment and one untreated control. For DNase-treated tubes, 10 µl RNA matrix, 1 µl DNase (2 U/µl), 2 µl 10× reaction buffer (Biolabmix LLC, Russia), and 7 µl PCR-grade water (Biolabmix LLC, Russia) were added. For untreated samples, DNase was replaced with equivalent PCR-grade water. All samples were incubated for 30 min at 37°C and 15 min at 65°C. Each sample (with and without DNase) was analyzed in three technical replicates. Total reaction number was 42 (7 samples × 2 treatment variants × 3 replicates). Negative controls were included in all runs.

Amplification Conditions

RT-qPCR was performed using the same reagents as described in the Primer and Probe Concentration Optimization section. Final primer and probe concentration was 0.3 µM, probe:primer ratio 1:1. Amplification protocol corresponded to the optimized protocol described in the Amplification Protocol Optimization section.

The primary data of RT-qPCR obtained after protocol optimization and RNA extraction method selection are provided in **Supplementary Dataset 2** [57].

Amplicon Agarose Gel Electrophoresis

To assess DNase effectiveness, confirm the presence of specific amplification products and verify the absence of nonspecific amplification products, amplicon electrophoresis was performed in 2% agarose gel. Gel was prepared by dissolving 2 g agarose (Helicon, Russia) in 100 ml 1× TBE buffer (Biolabmix LLC, Russia) with ethidium bromide addition (Servicebio, China) at final concentration 0.3 mg/L. Electrophoresis was performed in a horizontal

chamber WIX-midDNA (WIX, China) using 1 L fresh 1× TBE buffer (100 ml 10× TBE buffer diluted in 900 ml distilled water), to which 30 µl ethidium bromide (final concentration 0.3 mg/L) was added. Electrophoresis was performed at 100 V (5 V/cm electrode distance) for 50 min. Results were visualized on UV transilluminator (Vilber, France) with orange filter using Step50 plus DNA ladder (Biolabmix LLC, Russia). Amplicons (2 µl) mixed with 4 µl bromophenol blue (Biolabmix LLC, Russia) were loaded into wells. Images were captured digitally and processed in Adobe Photoshop CC 26.11 (Adobe Inc., USA).

For quantitative assessment of genomic DNA influence on analysis results, ΔCp and ΔFC were calculated for DNase-treated versus untreated samples for each sample, followed by comparison with technical assay variability.

Amplicon Sequencing

To confirm amplification specificity and identify PCR products corresponding to target genes, Sanger sequencing of amplicons obtained from multiplex amplification of RNA matrix isolated from breast tissue was performed. Analysis was performed for both DNase-treated and untreated samples, allowing assessment of genomic DNA contribution to amplification signal formation.

Five bands corresponding to amplification products were excised from agarose gel: ~600 bp band (DNase-untreated matrix, *MTOR* primers), ~150 bp band (untreated matrix, *RPLP0* primers), ~90 bp band (untreated matrix, *MTOR* and *TBP* primers), ~150 bp band (DNase-treated matrix, *RPLP0* primers), and ~90 bp band (DNase-treated matrix, *MTOR* and *TBP* primers). DNA extraction from gel and purification were performed using a column-based agarose gel extraction kit «Cleanup Standard» (Evrogen, Russia) according to the manufacturer's instructions. Quality control and concentration determination of purified samples were performed on a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA).

Termination reactions were performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a Veriti thermal cycler (Applied Biosystems, USA). Sequencing was performed on a 3130 DNA Analyzer genetic analyzer (Applied Biosystems, USA) using forward and reverse primers for each gene at 3.2 pM each.

Obtained chromatograms were analyzed in SnapGene software (GSL Biotech LLC, USA). Nucleotide sequence identification was performed using the BLAST algorithm (NCBI) by comparison with reference sequences: for cDNA amplification products — *MTOR* (NM_004958.4), *RPLP0* (NM_001002.4), and *TBP* (NM_003194.5); for genomic DNA amplification product — chromosome 1 (NC_000001.11) containing the *MTOR* gene locus.

The nucleotide sequences of amplicons obtained by Sanger sequencing have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1551020 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1551020).

DNA and RNA Yield Determination by Fluorimetry

To compare effectiveness of different isolation methods and identify possible genomic DNA contamination, RNA and DNA concentrations obtained from three different extraction kits were compared using fluorimetry on Qubit 2.0 (Invitrogen, USA). RNA concentration was

measured using the QuDye RNA Assay Kit (Vazyme, China); DNA quantification was performed using the QuDye dsDNA HS Assay Kit (Lumiprobe, Russia). All measurements were performed according to the manufacturer's instructions.

For each sample, a working dye solution was prepared by diluting concentrate in the corresponding buffer at 1:200 ratio. Measurement tubes were loaded with 190 µl working solution and 10 µl matrix sample. Incubation was performed at room temperature for 2 minutes in the dark. Fluorescence measurement was performed according to standard Qubit 2.0 protocol. Instrument calibration was performed before each measurement using standard solutions included in kits. Each sample from three technical isolation replicates was measured once. RNA and DNA concentrations were expressed in µg/ml.

Purity Assessment of Isolated RNA by Spectrophotometry (A260/A280)

Matrix purity was assessed using spectrophotometry on NanoDrop 2000 (Thermo Fisher Scientific, USA). Standard solutions (Lumiprobe, Russia; Nanodrop Standards, USA) were used for instrument calibration and quality control. Before each sample measurement, instrument "blanking" was performed using the same buffer or water in which the sample was dissolved (eluent), allowing solvent background absorption to be nullified.

Spectrophotometry was performed in the 220–350 nm wavelength range with optical density recording at 260 nm (A260) and 280 nm (A280). A260/A280 ratio served as an indicator of protein contamination removal: values in the 1.8–2.2 range were considered acceptable for RNA used in RT-qPCR. Each sample from three technical isolation replicates was measured once.

RNA Integrity Assessment by Agarose Gel Electrophoresis

To prevent RNA degradation during electrophoresis, equipment was pretreated with 70% ethanol with 3% hydrogen peroxide to remove RNases. Electrophoresis was performed in 1.2% agarose gel. Gel preparation, electrophoresis buffer, visualization, and image processing were performed similarly to the protocol described above (in section *Amplicon Agarose Gel Electrophoresis*). Electrophoresis at this stage was performed at 200 V (10 V/cm electrode distance) for 20 min. Higher voltage and shorter electrophoresis time were chosen to prevent thermal RNA degradation and preserve ribosomal band clarity, as well as due to larger target sequence sizes. RNA quality was assessed by presence and ratio of 28S and 18S ribosomal RNA bands: preparations with clear 28S and 18S bands at approximately 2:1 ratio were considered intact.

Statistical Analysis

Statistical processing and data visualization were performed in R version 4.6.1 (R Foundation for Statistical Computing; Vienna, Austria). The complete R script project used for statistical analysis and data visualization are available in **Supplementary Script 1** [58].

The group Cp values, FC values, ΔCp values, and ΔFC values are presented as median with 1st and 3rd quartile values. All analyses were descriptive; no inferential statistical testing was performed.

Amplification efficiency was assessed using linear regression of Cp values against $\log_2$ of cell equivalent (RNA matrix dilution). PCR efficiency was expressed as the slope magnitude relative to the theoretical optimum of −1 (i.e., E = |slope| × 100%). PCR results linearity was assessed by the value of R^2.

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Figures and Tables and Legends

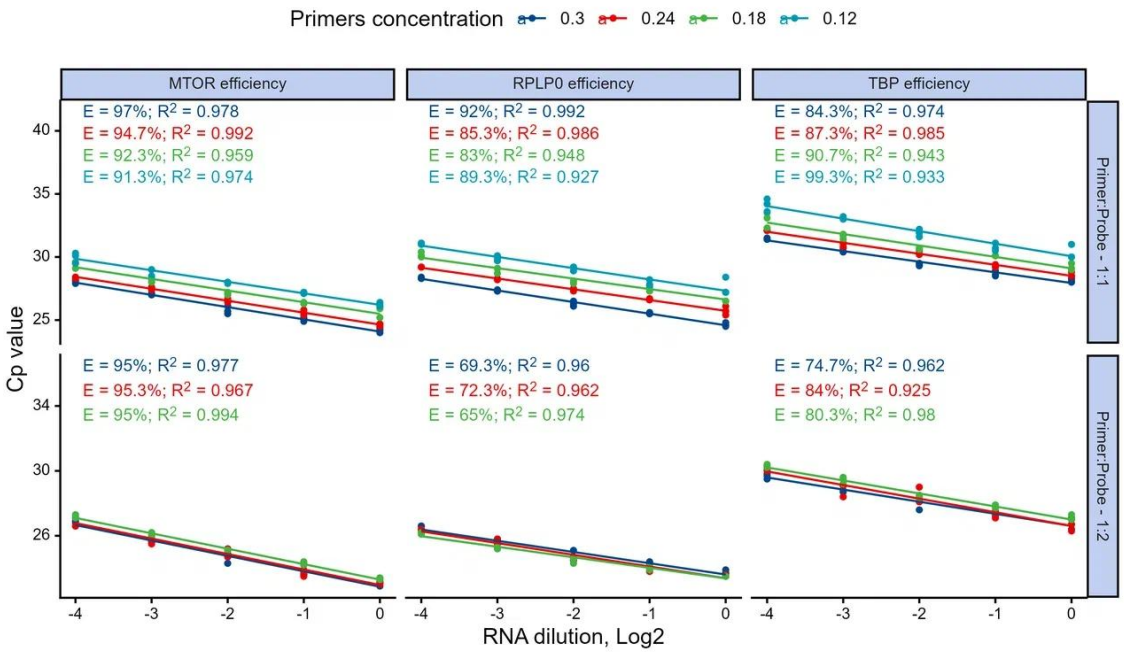


Figure 1. Dependence of amplification efficiency on primer concentration and probe:primer ratio in multiplex RT-qPCR for tissue samples using phenol-chloroform extraction. *MTOR* amplification efficiency remained high (91–97%) across all reactions. Reference gene efficiency showed significant variation with parameter changes: 1:2 ratio reduced *RPLP0* (65–72%) and *TBP* (75%) efficiency.
Lane designations: Primers concentration, μM — primer concentration in micromolars; *MTOR* efficiency — amplification efficiency of the target gene *MTOR*; *RPLP0* efficiency — amplification efficiency of the reference gene *RPLP0*; *TBP* efficiency — amplification efficiency of the reference gene *TBP*; Cp value — Crossing Point (threshold cycle); Probe:Primer-1:1 — probe to primer ratio 1:1; Probe:Primer-1:2 — probe to primer ratio 1:2; RNA dilution, Log_2 — logarithmic transformation of RNA template dilution; E — amplification efficiency.

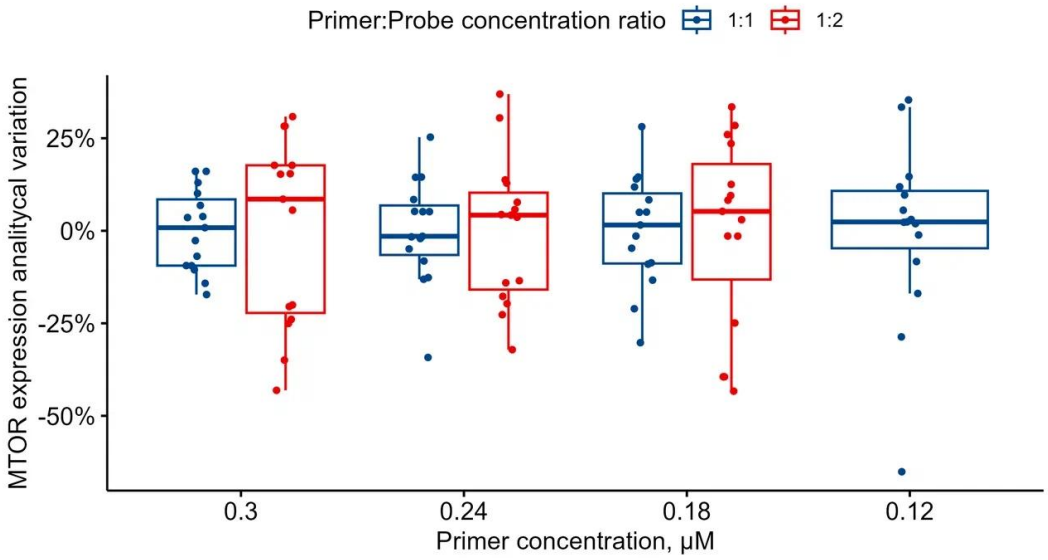


Figure 2. Fold Change (expression) variability depending on primer concentration and probe:primer ratio. 1:1 probe:primer ratio provides narrower Fold Change dispersion (median values –1.5%–2.4%; IQR 13.4–19%) than 1:2 (median values 4.2–8.5%; IQR 26.2–39.9%). **Lane designations:** Primer:Probe concentration ratio — ratio of primer to probe concentrations; *MTOR* expression analytical variation — analytical variation of *MTOR* expression; Primer concentration, μM — primer concentration in micromolars.

Table 1. Comparison of RNA yield and purity from three extraction methods across biomaterial types.

Biomaterial	Parameter	Phenol-chloroform	Precipitation	Spin-columns
Blood	RNA, μg/ml	4.6 (3.5-5.2)	19.4 (18.4-20.2)	17.2 (16.9-20.8)
	DNA, μg/ml	0 (0-0)	7.2 (6.1-7.4)	4.8 (4.3-5.9)
	RNA/DNA ratio	only RNA	2.7 (2.5-3.5)	3.5 (3.5-4)
	Purity A260/A280	1.8 (1.7-1.9)	2 (1.9-2)	3 (3-3.2)
Cells	RNA, μg/ml	36.3 (35.5-37.2)	150 (147-154)	112 (99.4-116)
	DNA, μg/ml	0 (0-0)	15.1 (14.6-17.5)	6.1 (5.3-6.9)
	RNA/DNA ratio	only RNA	9.5 (8.5-10.3)	18.4 (17-18.7)
	Purity A260/A280	2 (1.9-2.1)	2 (2-2.1)	2.2 (2.2-2.2)
Tissue	Purity A260/A280	2 (2-2)	-	-

Note to Table 1: Lowest RNA yield was determined with phenol-chloroform extraction (for all biomaterial types); however, this method showed complete absence of DNA in samples (except tissue — minimal DNA content). Maximum RNA yield was observed with precipitation with relatively high DNA content in samples (RNA/DNA ratio 2.7 for blood; 9.5 for cells). Spin-column RNA isolation showed intermediate results compared to the other two methods. Acceptable RNA purity results (in the 1.8–2.2 range) were observed for all methods and biomaterials, except spin-columns (blood).

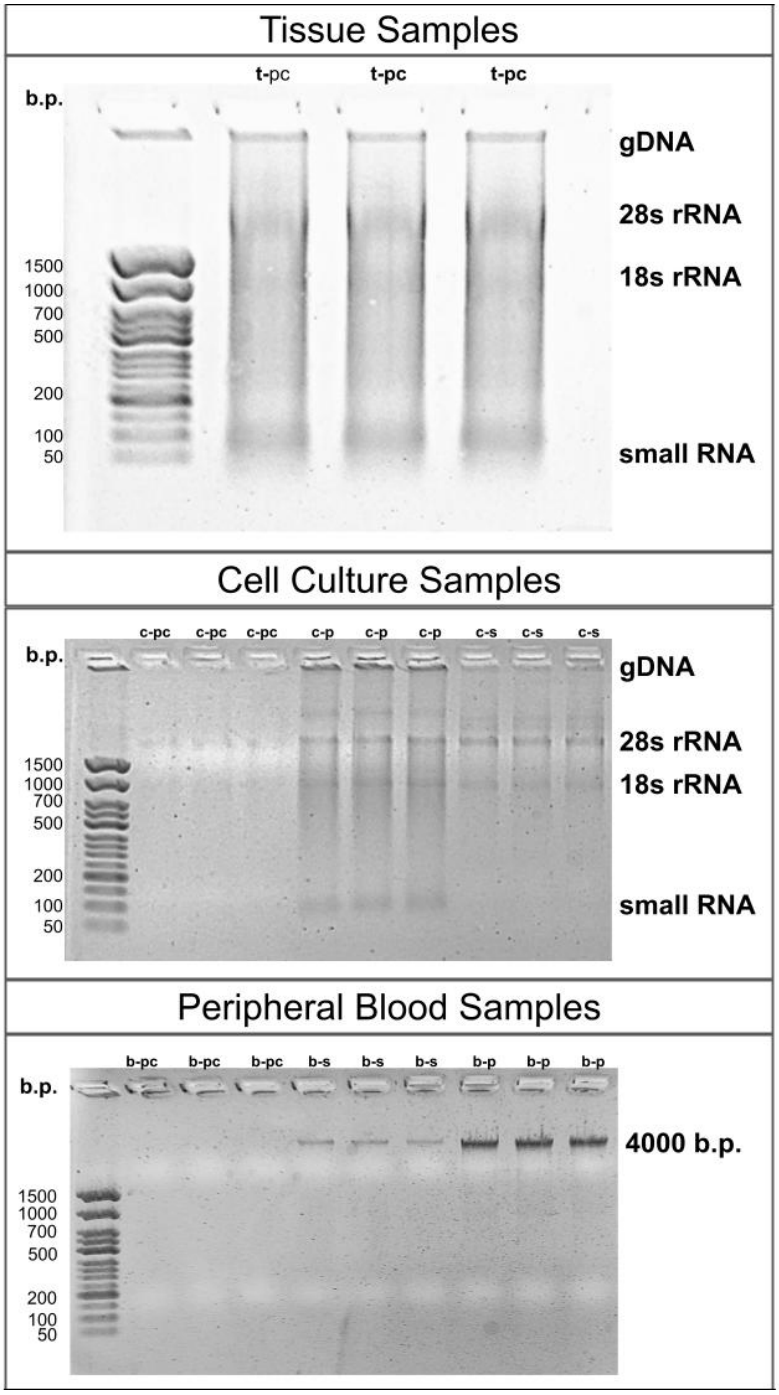


Figure 3. Integrity of isolated RNA from different biomaterial types using three extraction methods. Clear 28S and 18S ribosomal RNA bands were observed for cell and tissue samples across all three methods. For blood RNA samples, ribosomal bands corresponding to 28S and 18S rRNA were not detected.

Lane designations: t-pc — tissue (breast tumor tissue), phenol-chloroform extraction; c-pc — cell culture (SCP-1 line), phenol-chloroform extraction; c-p — cell culture, precipitation; c-s — cell culture, silica membrane spin-columns; b-pc — peripheral blood, phenol-chloroform extraction; b-s — peripheral blood, spin-columns; b-p — peripheral blood, precipitation.

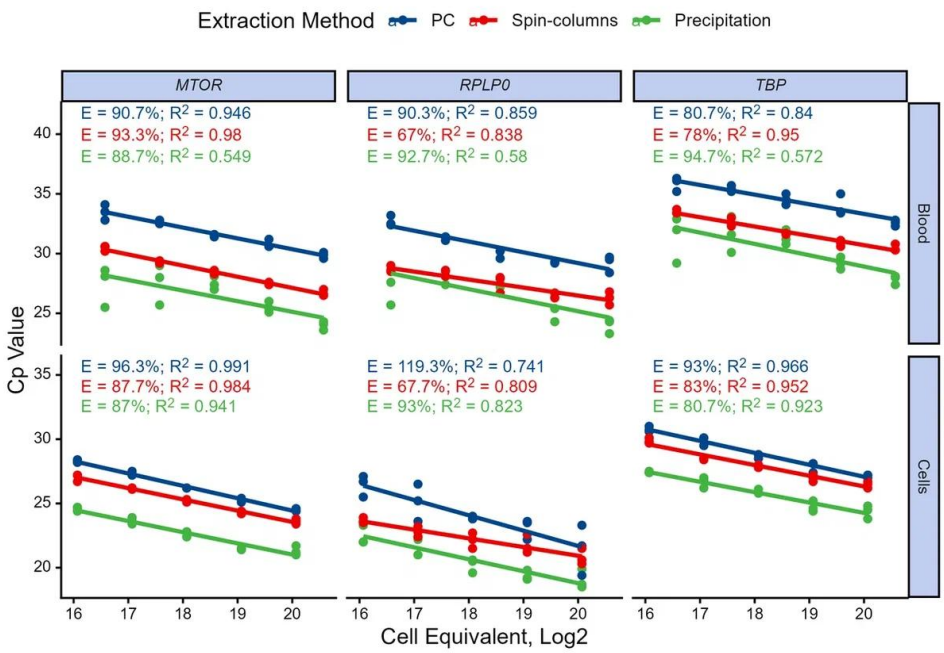


Figure 4. Comparison of three RNA extraction methods (phenol-chloroform, spin-columns and precipitation) on amplification efficiency of each gene in blood and cell culture samples. To assess amplification efficiency, calibration curves of Cp versus $\log_2$ RNA template dilution were constructed with calculation of coefficient of determination R^2. For blood samples, best *MTOR* performance was with spin-columns (93.3%, $R^2 = 0.98$); precipitation showed low linearity ($R^2 = 0.55–0.58$) for all three genes. For cells, phenol-chloroform extraction gave best results (efficiency 93-119.3% with high R^2 values). Precipitation and spin-columns showed lower efficiency results while maintaining high R^2 values.
Lane designation: PC extraction — phenol-chloroform extraction; Cp value — Crossing Point; E — amplification efficiency; Blood — peripheral blood samples; Cells — cell culture samples (SCP-1 line); Calculated number of cells, Log_2 — logarithmic transformation of the calculated cell number equivalent.

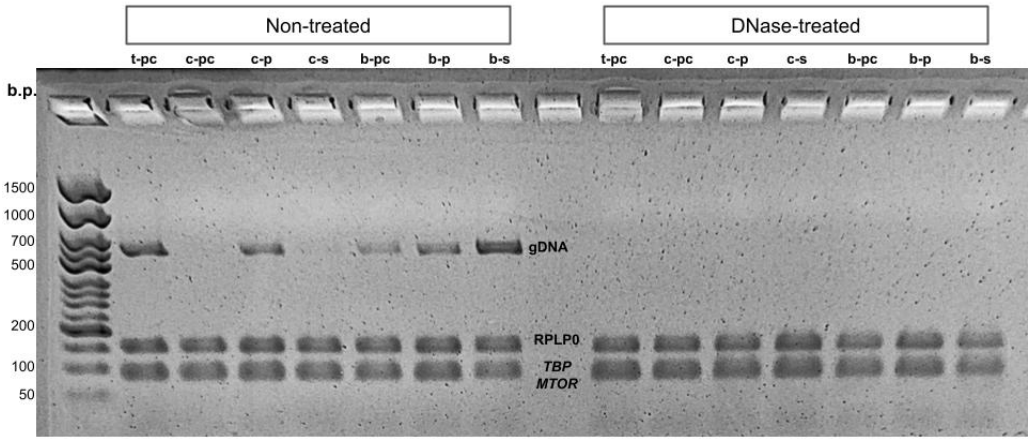


Figure 5. Electrophoregram of RNA matrix with and without thermolabile DNase pretreatment. The most intense band corresponding to genomic DNA fragment was observed for precipitation method (blood, cells) and phenol-chloroform extraction from tissue; minimal intensity — for phenol-chloroform extraction (blood, cells) and spin-columns (cells). In all DNase-treated samples, genomic DNA was not detected.

Lane designations: t-pc — tissue (breast tumor tissue), phenol-chloroform extraction; c-pc — cell culture (SCP-1 line), phenol-chloroform extraction; c-p — cell culture, precipitation; c-s — cell culture, silica membrane spin-columns; b-pc — peripheral blood, phenol-chloroform extraction; b-s — peripheral blood, spin-columns; b-p — peripheral blood, precipitation.

Table 2. Assessment of genomic DNA influence on quantitative analysis results.

Biomaterial	Extraction Kit	Gene	ΔCp (Q1-Q3)	IQR ΔCp	ΔFC (Q1-Q3)	IQR ΔFC
Blood	Phenol-chloroform	*MTOR*	0.2 (0.2-0.7)	0.5	-14 (-20.25-3.4)	23.6
		RPLP0	0.6 (0.4-1.4)	1.0		
		TBP	0.2 (-0.1-0.5)	0.6		
	Precipitation	*MTOR*	2.4 (1.8-2.5)	0.8	39.1 (13.45-91.95)	78.5
		RPLP0	0.7 (0.5-2)	1.5		
		TBP	1.6 (1.2-2)	0.8		
	Spin-columns	*MTOR*	1 (0.8-1.4)	0.6	24.9 (17.35-44.05)	26.7
		RPLP0	0.7 (0.4-1.5)	1.0		
		TBP	0.4 (0.4-0.7)	0.2		
Cells	Phenol-chloroform	*MTOR*	0.3 (-0.1-0.6)	0.7	-8.4 (-9.3-3.2)	12.5
		RPLP0	0.1 (-0.2-0.3)	0.6		
		TBP	0.2 (0.1-0.9)	0.8		
	Precipitation	*MTOR*	0.6 (0.3-0.6)	0.3	-36.3 (-36.3--10.9)	25.4
		RPLP0	0.6 (0.5-0.8)	0.2		
		TBP	0.7 (0.5-1.3)	0.8		
	Spin-columns	*MTOR*	0.1 (0-0.9)	0.9	-17.7 (-18.95--12.25)	6.7
		RPLP0	0.2 (0.1-1.6)	1.5		
		TBP	0.4 (0.3-0.6)	0.3		
Tissue	Phenol-chloroform	*MTOR*	0.9 (0.9-0.9)	0.0	36.1 (14.5-48.75)	34.2
		RPLP0	0.5 (0.4-0.8)	0.4		
		TBP	0.4 (0.2-0.6)	0.4		

Note to Table 2: ΔCp analysis revealed minimal genomic DNA contribution with phenol-chloroform extraction for blood and cells, and with spin-columns for cells. Maximum sample

contamination with genomic DNA was observed for tissue with phenol-chloroform extraction. ΔFC analysis revealed minimal values for phenol-chloroform extraction from blood and cells, maximal values — for precipitation method (blood and cells) and spin-columns (cells).

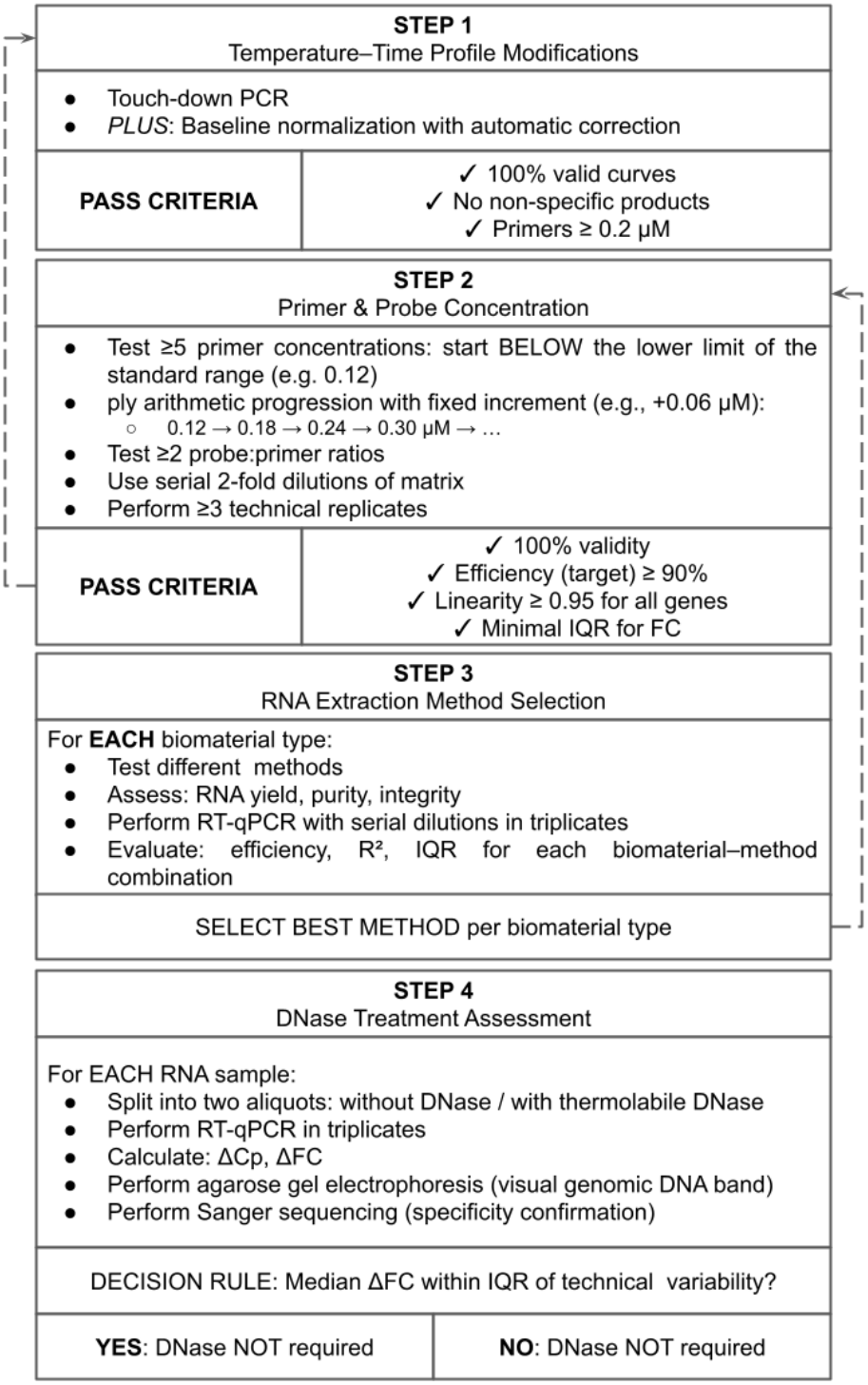


Figure 6. Stepwise workflow for multiplex RT-qPCR optimization. The algorithm comprises four sequential steps: (1) amplification protocol optimization via temperature–time profile modification and baseline normalization; (2) primer concentration and probe:primer ratio titration using serial RNA dilutions with simultaneous criteria (100% validity, efficiency ≥ 90%, results linearity - $R^2 \geq 0.95$, minimal IQR for FC); (3) RNA extraction method selection based on yield, purity, integrity, efficiency, R^2, and IQR for each biomaterial type; (4) DNase

treatment necessity assessment by ΔCp and ΔFC comparison with technical variability, confirmed by gel electrophoresis and Sanger sequencing. Dashed return arrows indicate iterative adjustments between steps when criteria are not met.

Supplementary Material

Supplementary Table 1: Primers and probes characteristics. (File: Table_1_Primers.xlsx)
Supplementary Dataset 1: Complete dataset of Cp values from primer concentration and probe:primer ratio optimization experiment. (File: Dataset_1_Cp_Values.xlsx)
Supplementary Dataset 2: Complete dataset of Cp values from RNA extraction method comparison experiment. (File: Dataset_2_Cp_Values.xlsx)
Supplementary Script 1: Complete R script project for statistical analysis and figure generation. (File: Script_1_R_Analysis_Primer_Optimization.rar)

Ethical and Legal Declarations

Research ethics

The study was approved by the Local Ethical Committee of the Federal State Budgetary Educational Institution of Higher Education "Ural State Medical University" (USMU) of the Ministry of Health of Russia, Yekaterinburg, Russia (Protocol No. 6, dated October 18, 2024).

Informed consent

Written informed consent was obtained from all participants included in the study. All participants were informed of the study's purpose, procedures, potential risks, and benefits, and their right to withdraw at any time without penalty. For the patient tissue samples used in the study, additional informed consent for publication of de-identified data was obtained. The study was conducted in accordance with the principles of the Declaration of Helsinki. All identifying information has been anonymized to protect participant privacy. Documentation of informed consent is retained by the authors and is available upon request.

Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Daniil O.Kornilov: Conceptualization, Methodology, Writing — Original Draft Preparation.
Alexey A.Bekhterev: Investigation, Methodology (optimization of PCR protocols performed electrophoresis experiments), Writing — Original Draft Preparation.
Veronika M.Simarzina: Investigation, Methodology, Visualization, Writing — Original Draft Preparation.
Valeria A. Utemova: Investigation, Methodology (RNA extraction and PCR), Visualization, Writing — Original Draft Preparation.
Tarek Itani: Investigation, Design of methodology (sequencing facility organization and management).
Narine L. Adamyan: Investigation, Methodology (performed the sequencing experiments).
Natalia V. Kazantseva: Sample collection.
Evgenii E. Tarasov: Sample collection.
Georgiy P. Maslakov: Investigation, Software, Validation.
Ekaterina S. Voroshilina: Supervision, Project Administration, Writing — Review & Editing.
Danila L. Zornikov: Supervision, Visualization, Formal Analysis, Writing — Review & Editing.

Use of Large Language Models, AI and Machine Learning Tools

The authors utilized DeepSeek (Hangzhou DeepSeek Artificial Intelligence Co., Ltd.) for language polishing, grammar checking, and manuscript text refinement during the preparation of this work. The AI was accessed between May 2026 and August 2026. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the entire content of the published work.

Conflict of interest

The authors state no conflict of interest.

Research funding

None declared.

Data availability

The authors confirm that the data supporting the findings of this study are available in its supplementary materials and public repositories. The primers and probes with their full characteristics, including sequences, amplicon lengths, melting temperatures, and fluorescent labels, are provided in Supplementary Table 1. The complete datasets of Cp values used for the calculation of amplification efficiency, gene expression stability, and technical variability from both the primer optimization and RNA extraction method comparison experiments are provided as Supplementary Datasets 1 and 2, respectively. The nucleotide sequences of amplicons obtained by Sanger sequencing have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1551020 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1551020). The complete R scripts containing all statistical analyses and graph generation for the primer optimization and RNA extraction method comparison experiments are provided as Supplementary Script 1. All supplementary materials are available with the online version of this article.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Script1RAnalysisPrimerOptimization.rar
- Dataset1CpValues.xlsx
- Dataset2CpValues.xlsx
- Table1Primers.docx