A RELIABLE AND REPRODUCIBLE MULTIPLEX RT-QPCR ASSAY FOR *mTOR* GENE EXPRESSION ANALYSIS

Kornilov DO[1] ✉, Simarzina VM[1], Bekhter AA[1], Maslakov GP[2], Nechaeva DM[1], Kariakina AE[1], Fadeev FA[1,3], Voroshilina ES[1,4], Zornikov DL[1]

[1] Ural State Medical University, Yekaterinburg, Russia

[2] Institute of Cytology of the Russian Academy of Sciences, Saint Petersburg, Russia

[3] State Autonomous Health Institution of the Sverdlovsk Region "Center for Specialized Types of Medical Care 'Institute of Medical Cellular Technologies'", Yekaterinburg, Russia

[4] Medical Center "Garmoniya", Yekaterinburg, Russia

The PI3K/AKT/mTOR signaling pathway is a key regulator of cell growth, and its dysregulation is involved in oncogenesis. Existing methods for assessing *mTOR* activity have design flaws. The aim of this work was to develop and validate a novel multiplex RT-qPCR assay for relative quantification of *mTOR* gene expression normalized to *RPLP0* and *TBP*. Primers and probes were designed *in silico*. Validation was performed using the human SCP-1 cell line. Specificity was assessed in 10 separate and 10 multiplex runs. Analytical sensitivity and efficiency were determined from 27 technical replicates using a protocol without an elongation step. Specificity of amplification was assessed by agarose gel electrophoresis, and quantitative analysis was performed in real-time PCR using FAM (*mTOR*), HEX (*RPLP0*), and ROX (*TBP*) fluorescence channels. The assay showed 100% specificity. Stable detection was achieved at 125,000 cells/mL. Amplification efficiencies were 73–81%. The variation of *mTOR* expression normalized to *RPLP0* ranged from –21.5% to 26.4%, and normalized to *TBP* from –14.3% to 19.2%. Normalization to the geometric mean of both reference genes provided the best reproducibility, with an interquartile range from –9% to 23.4%. The developed assay demonstrates high specificity, sensitivity, and reproducibility, making it a reliable tool for subsequent clinical research.

Keywords: gene expression profiling, molecular diagnostics, multiplex polymerase chain reaction, reproducibility of results, reverse transcriptase polymerase chain reaction, TOR serine-threonine kinases

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✉ **Correspondence should be addressed:** Daniil O. Kornilov
Klyuchevskaya, 17, Yekaterinburg, 620109, Russia; danilovkornil@gmail.com

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НАДЕЖНАЯ И ВОСПРОИЗВОДИМАЯ МУЛЬТИПЛЕКСНАЯ ОТ-ПЦР-РВ ТЕСТ-СИСТЕМА ДЛЯ КОЛИЧЕСТВЕННОЙ ОЦЕНКИ ЭКСПРЕССИИ ГЕНА *mTOR*

Д. О. Корнилов[1] ✉, В. М. Симарзина[1], А. А. Бехтер[1], Г. П. Маслаков[2], Д. М. Нечаева[1], А. Е. Карякина[1], Ф. А. Фадеев[1,3], Е. С. Ворошилина[1,4], Д. Л. Зорников[1]

[1] Уральский государственный медицинский университет, Екатеринбург, Россия

[2] Институт цитологии Российской академии наук, Санкт-Петербург, Россия

[3] Центр специализированных видов медицинской помощи «Институт медицинских клеточных технологий, Екатеринбург, Россия

[4] Медицинский центр «Гармония», Екатеринбург, Россия

Сигнальный путь PI3K/AKT/mTOR — ключевой регулятор роста клеток, его дисрегуляция вовлечена в онкогенез. Существующие способы оценки активности *mTOR* имеют недостатки дизайна. Целью работы было разработать и провести валидацию новой мультиплексной ОТ-ПЦР-РВ-тест-системы для относительного количественного анализа экспрессии гена *mTOR* с нормализацией на гены *RPLP0* и *TBP*. Праймеры и зонды сконструированы *in silico*. Валидацию проводили на клеточной линии стромальных клеток костного мозга человека SCP-1. Специфичность оценивали в 10 сепарированных и 10 мультиплексных постановках. Аналитическую чувствительность и эффективность определяли по 27 техническим повторам с использованием протокола без элонгации. Специфичность амплификации оценивали с помощью электрофореза в агарозном геле, а количественный анализ выполняли в режиме реального времени по каналам флуоресценции FAM (*mTOR*), HEX (*RPLP0*) и ROX (*TBP*). Тест показал 100%-ю специфичность. Стабильная детекция достигалась при 125 тыс. клеток/мл. Эффективность амплификации составила 73–81%. Вариация экспрессии *mTOR*, нормализованной на *RPLP0,* составила от –21,5 до 26,4%, по *TBP* от –14,3 до 19%. Нормализация на среднее геометрическое двух генов обеспечила лучшую воспроизводимость от –9 до 23,4%. Разработанный тест отличается высокой специфичностью, чувствительностью и воспроизводимостью, что делает его надежным инструментом для последующих клинических исследований.

Ключевые слова: профилирование экспрессии генов, молекулярная диагностика, мультиплексная полимеразная цепная реакция, воспроизводимость результатов, полимеразная цепная реакция с обратной транскрипцией, TOR-серин-треониновые киназы

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✉ **Для корреспонденции:** Даниил Олегович Корнилов
ул. Ключевская, д. 17, г. Екатеринбург, 620109; danilovkornil@gmail.com

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The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) signaling pathway is a crucial regulator of cellular processes like proliferation and metabolism, with mTOR kinase acting as its central hub [1, 2]. Its dysregulation is implicated in a wide spectrum of pathologies, ranging from progeria and tuberous sclerosis to neurological disorders and cancer, including breast cancer [1, 3–5]. Consequently, the study of mTOR signaling attracts significant interest from diverse scientific fields, including oncology, aging research, and neurobiology [6–8].

This central role makes mTOR a high-priority target for novel therapeutics to restore pathway homeostasis. The broader scientific community actively pursues this direction [9]. In our previous work, miR-162a suppressed osteosarcoma cell proliferation and viability, suggesting potential *mTOR* inhibition [10]. The lack of a reliable *mTOR* expression assay prevented direct confirmation of this mechanism, motivating the development of the assay presented.

The high interest in studying mTOR regulation and its functions drives the demand for reliable methods to assess its activity. While established methods like Western blot [11] and luminescence microscopy [12] are reliable, they suffer from high cost, complexity, and operator-dependence.

An alternative method for evaluating protein activity is to assess the level of gene expression (mRNA quantity) using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) with relevant reference genes, typically housekeeping genes which are stably expressed in most cells under normal and pathological conditions, for example Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Beta-Actin (*ACTB*), Beta-2-Microglobulin (*B2M*), as well as some of the most stable ones — *TATA-binding protein* (*TBP*) and *Ribosomal protein P0* (*RPLP0*) [13–14].

A multiplex PCR assay for mTOR expression analysis was previously developed by Quidville et al. [15], utilizing the reference genes *TBP* and *RPLP0*, which are among the most stable known to date [14]. However, a fundamental flaw in its design — specifically, a probe-primer overlap for the *TBP* gene — renders this system unreliable and highlights the critical need for a properly constructed alternative [13, 15, 16].

The aim of this study was to develop and validate a new multiplex RT-qPCR assay for the relative quantitative analysis of *mTOR* expression normalized to the reference genes *TBP* and *RPLP0*.

METHODS

In silico development and analysis of the PCR assay

Target gene mRNA sequences (FASTA) were retrieved from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) [Accessed 2024 Sep 23]. Transcripts were annotated using the Assemble by CDS tool in Geneious Prime 2019.2.1 software (Biomatters Ltd., USA). Primers and probes were designed using the PrimerQuest Tool web service (Integrated DNA Technologies, Inc., USA; https://www.idtdna.com/pages/tools/primerquest) [Accessed 2024 Sep 23]. The following selection criteria were used for primer design [17]:

– Specificity — primers complementary to a single region.
– Primer length of approximately 20 nucleotides.
– Guanine-cytosine (GC) content of approximately 50%.
– At least one guanine-cytosine base pair at the primer ends.
– No complementary regions between primers.
– Primer melting temperature (Tm) between 55 °C and 65 °C.

The following requirements were applied to the probes:

– Length of approximately 20 nucleotides.
– GC content of approximately 50%.
– No more than four consecutive guanine or cytosine repeats.
– The probe must be located as close as possible to the primers but must not overlap them.
– The probe Tm must be 8–10 °C higher than the primer Tm.

Analysis of primer specificity, flanking, and spanning was performed in Geneious Prime 2019.2.1 software using the BLAST algorithm. Primer dimer formation was checked using the OligoAnalyzer Tool web service (Integrated DNA Technologies, Inc., USA; https://www.idtdna.com/pages/tools/oligoanalyzer) [Accessed 2024 Sep 23] [17].

In vitro validation of the PCR assay

Cell line

The study used the human bone marrow stromal cell line SCP-1, which was obtained from the Cell Culture Collection of the Ural State Medical University (Yekaterinburg, Russia). Cells were cultured in T-25 flasks (Sarstedt, Germany) with an adhesive coating in a medium containing 94% LoSera basal medium (HiMedia, India), 5% Fetal Bovine Serum (FBS) (HiMedia, India), 0.01% L-glutamine (Servicebio, China), and 0.01% (1 : 1 : 1) solution of penicillin, streptomycin, and amphotericin B (Servicebio, China), in a CO_2 incubator (Panasonic (Sanyo) MCO-15A, Japan) at 5% CO_2 and 37 °C [10]. Upon reaching 90–100% confluency, cells were passaged by removing the spent medium, washing twice with Hanks' Balanced Salt Solution (HBSS) without Ca and Mg ions (Servicebio, China), and then adding 2.5 mL of 0.25% trypsin-EDTA solution (Servicebio, China). The solution was removed after 10 seconds of exposure. The flask was placed in the CO_2 incubator for 1 minute 30 seconds. After adding fresh medium with serum, detached cells were collected, centrifuged at 350g for 3 minutes, resuspended, and distributed to new flasks at a 1 : 3 ratio [18]. For this study, a cell suspension was prepared as described, and cells were counted in a Goryaev chamber using the trypan blue exclusion method.

Nucleic acid extraction

For total RNA extraction, containing the mRNA fraction, the cell suspension (1 million cells/mL) was placed in a 1.5 mL Eppendorf tube and titrated with twofold dilutions in normal saline to obtain samples with concentrations of 1 million, 500 thousand, 250 thousand, 125 thousand and 62,5 thousand cells/mL. RNA was extracted from all dilutions to calculate the analytical parameters of the PCR assay.

RNA extraction from cell culture samples was performed using the «Proba-NK» reagent kit («DNA-Technology» LLC, Russia) according to the manufacturer's protocol. After the RNA extraction, total RNA from the 1 million cells/mL suspension was serially twofold diluted in normal saline to obtain samples with the matrix concentrations of 1, 1 : 2, 1 : 4, 1 : 8 and 1 : 16. All RNA matrix concentration variants were used for amplification to validate the PCR assay and calculate PCR efficiency.

Amplification

To analyze the specificity of the amplification product, 10 separate RT-qPCR technical repeats for each gene were performed using the BioMaster RT-qPCR SYBR Blue reagent kits («Biolabmix» LLC, Russia). Undiluted RNA matrix

from a suspension of 1 million cells/mL was used. Primers, according to the designed sequences, were synthesized by «DNK-sintez» LLC, Russia. According to the manufacturer's protocol, reaction mixtures were prepared from 12.5 µl of “Reaction Buffer” (containing dNTP mix, Mg ions and SYBR Green), 1 µl of “Master Mix” (containing genetically modified Moloney Murine Leukemia Virus reverse transcriptase and recombinant Taq DNA polymerase inactivated by specific monoclonal antibodies), 5 µl of a mixture with probe, forward and reverse primers (each at 0.12 µmol/L, 3 pmol/reaction), and 6.5 µl of RNA matrix. The final reaction volume was 25 µl. Additionally, 10 multiplex technical repeats (amplification of the target gene and normalizers in one tube) were performed using the BioMaster RT-qPCR reagent kits («Biolabmix» LLC, Russia). Reaction mixtures were prepared as above, except that the reaction buffer lacked an intercalating dye. Detection was performed by endpoint analysis using horizontal agarose gel electrophoresis.

The assay's analytical performance was assessed using the comprehensive dilution scheme as described in the section Nucleic Acid Extraction. This scheme incorporated both pre-analytical (cell concentration) and analytical (RNA dilution) variables, enabling simultaneous evaluation of amplification efficiency, analytical sensitivity, and technical reproducibility. All resulting RNA samples — from the five different cell concentrations and the four serial dilutions — were analyzed in triplicate using the multiplex RT-qPCR protocol described (Fig. 1). Three negative control replicates were included. This design resulted in a total of 27 individual reactions (9 unique sample types × 3 replicates). The results from these 27 technical repeats formed the primary dataset for all subsequent calculations of PCR efficiency, reference gene stability, and technical variability.

The amplification protocol consisted of reverse transcription for 30 minutes at 45 °C, initial denaturation for 5 minutes at 95 °C, followed by 45 cycles of denaturation (15 seconds at 95 °C) and primer annealing (30 seconds at 68 °C). The elongation step was excluded from the amplification protocol to limit DNA amplification [19]. For real-time detection of *mTOR*, *RPLP0*, and *TBP* amplification products, the fluorescent channels FAM, HEX, and ROX were selected, respectively.

Real-time detection was performed, and the Crossing Point (*Cp*) value was determined by the instrument's software as the cycle number at the maximum of the second derivative of the fluorescence growth curve [20]. As this study focused on the relative quantification of gene expression using the comparative method, no external calibration curves were run alongside the experimental samples. Results were considered valid if the *Cp* value was detected before the 31st amplification cycle. This threshold was established experimentally: when using a protocol that included an elongation step, additional amplification products appeared starting from cycle 31, which upon electrophoretic analysis corresponded in length to a fragment amplified from genomic DNA (574 bp, the size of the intron). Exclusion of the elongation step from the protocol eliminated this nonspecific amplification, and no signals were detected after the 31st cycle. Thus, the 31-cycle threshold was set as the limit beyond which no contribution from genomic DNA amplification is guaranteed to occur.

All runs used the «DTprime» thermal cycler («DNA-Technology» LLC, Russia) with the manufacturer's software.

Agarose gel electrophoresis

A 2,5% agarose gel was prepared using 2,5 g agarose powder (Helicon, Russia) in 100 mL 1x TBE buffer («Biolabmix» LLC, Russia) with the addition of 3 µL ethidium bromide (Servicebio, China). Electrophoresis ran at 100 V/A in a Sub-Cell GT Cell horizontal electrophoresis chamber (Bio-Rad, USA) for 40 minutes. Results were analyzed on a UV table transilluminator (Vilber, France) using a 10–25 DNA marker (Servicebio, China) [25, 26]. Images were captured digitally and processed in Adobe Photoshop CC 26.11 software (Adobe Inc., USA).

Statistical analysis

The expression value was determined based on the *Cp* value using the "Fold Change" (*FC*) formula presented by Livak and Schmittgen [22]. The formula was as follows:

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

where Cp_{target} is *Cp* value of the target gene and $Cp_{reference}$ is *Cp* value of the normalizer.

For the analysis, the Δ*Cp* value was calculated using each normalizer individually (*RPLP0* or *TBP*) and, additionally,

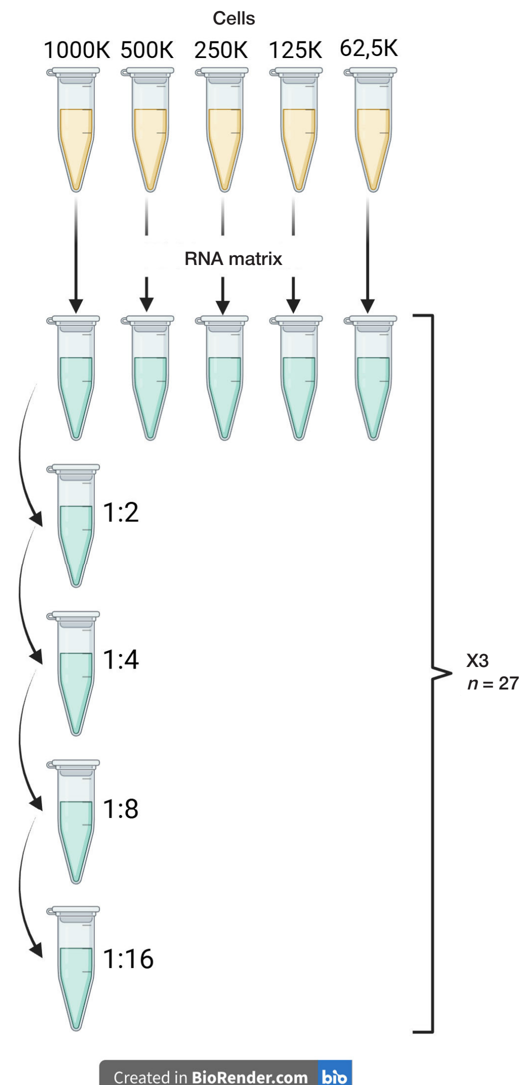

Fig. 1. Experimental design for multiplex RT-qPCR assay validation. The scheme illustrates the two independent dilution series used to assess assay performance. The pre-analytical series (*yellow*) involved total RNA isolation from SCP-1 cells at five different starting concentrations. The analytical series (*blue*) was created by twofold serial dilution of the RNA isolated from the highest cell concentration. Each resulting RNA sample (*n* = 9) was analyzed in triplicate, totaling 27 individual reactions for the evaluation of PCR efficiency, analytical sensitivity, and technical reproducibility

Table. Oligonucleotide characteristics for the multiplex RT-qPCR assay

Type	Gene name	Sequence, 5' → 3'	Amplicon length, bp	Melting T, °C	GC%	Detection
F-primer	*mTOR*	GCCCAGGCCGCATTGTCTCTAT	84	68	59.1	FAM
R-primer		GCAGTAAATGCAGGTAGTCATCCAGGTT		73	60.0	
Probe		TGGCTGCAATCCAGCTGTTTGGCGC		68	46.4	
F-primer	*RPLP0*	GGCGACCTGGAAGTCCAACTA	149	66	57.1	HEX
R-primer		CCATCAGCACCACAGCCTTC		65	60.0	
Probe		ATCTGCTGCATCTGCTTGGAGCCCA		72	56.0	
F-primer	*TBP*	CACGAACCACGGCACTGATT	89	65	55.0	ROX
R-primer		TTTTCTTGCTGCCAGTCTGGAC		65	50.0	
Probe		TGTGCACAGGAGCCAAGAGTGAAGA		69	52.0	

Note: F — forward; R — reverse. Gene symbols refer to mammalian target of rapamycin (*mTOR*), ribosomal protein lateral stalk subunit P0 (*RPLP0*), and TATA-box binding protein (*TBP*). All primers and probes were designed to meet standard criteria for multiplex real-time PCR, including amplicon size (<150 bp), matched melting temperatures within each set, optimal GC-content, and distinct, non-overlapping fluorescent detection channels

using the geometric mean of both normalizers *Cp* values as a combined reference value.

PCR efficiency for the *mTOR* gene and reference genes was assessed using linear regression of the *Cp* value dependence on the $\log_2$ of Cell Equivalent (*CE*). This metric, expressed in cell equivalents, represents the theoretical number of cells from which the RNA in the reaction was derived, accounting for both cell suspension dilution and RNA dilution. The correlation between the *CE* in the sample and the *Cp* value was assessed using Spearman's correlation coefficient (ρ, considered statistically significant at $p < 0.05$). The amplification efficiency (E) for each gene was calculated based on the slope (*b*) of the linear regression line plotted for the dependence of the *Cp* value on the $\log_2$ of the *CE*. 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 slope of the regression line, and *x* is $\log_2(N)$, with *N* being the calculated *CE*.

The *CE* was calculated as:

$$CE = \text{initial cell concentration} \div \text{cell dilution} \div \text{matrix dilution}.$$

The stability of the reference genes was assessed by analyzing the distribution of the *mTOR* expression value normalized to each reference gene (*FC*) and described by the median with the 0.25 and 0.75 percentiles.

Technical variability of the *mTOR FC* for a corresponding reference gene(s) was represented as median with the 0.25 and 0.75 percentiles of variability value, which had been calculated according to the formula:

$$\text{variability value} = 1 - (\text{mTOR FC sample value} / \text{mean mTOR FC value}).$$

mTOR FC values were calculated for the corresponding normalization strategy (normalized to *RPLP0*, *TBP*, or the geometric mean of both).

All analyses and graphs were performed in the R environment, version 4.5.2.

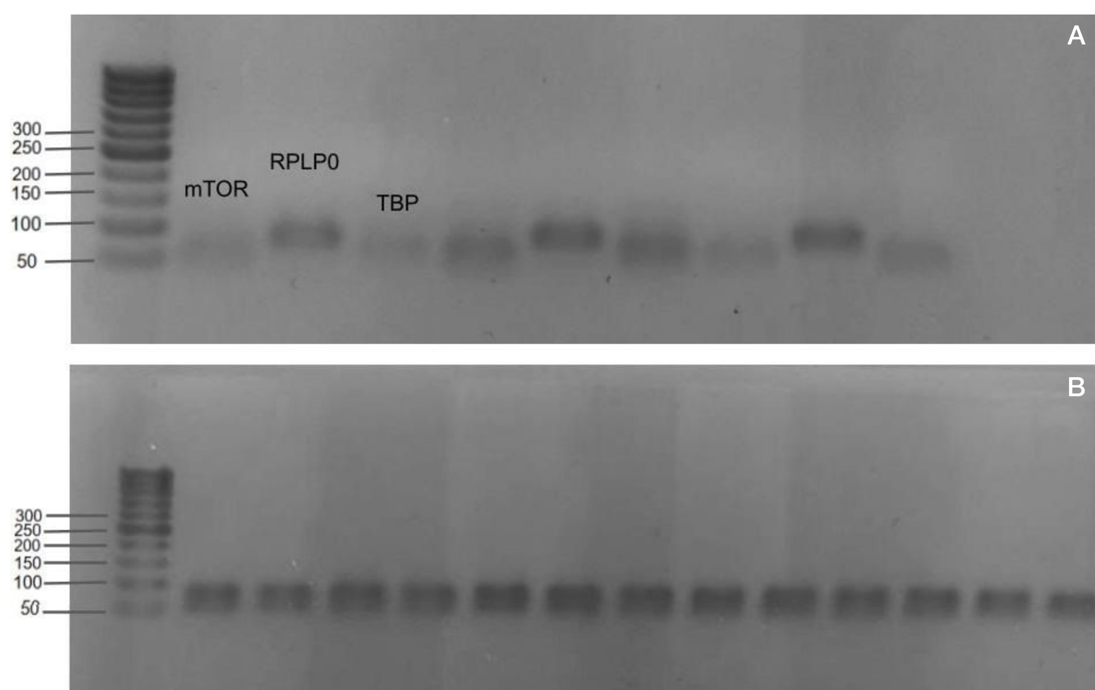

Fig. 2. Results of amplification product specificity analysis by agarose gel electrophoresis. **A**. Separate RT-qPCR technical repeats for each target gene. **B**. Multiplex RT-qPCR technical repeats amplifying all three targets simultaneously. Lanes for each gene show a single amplicon corresponding to the expected size, confirming high assay specificity

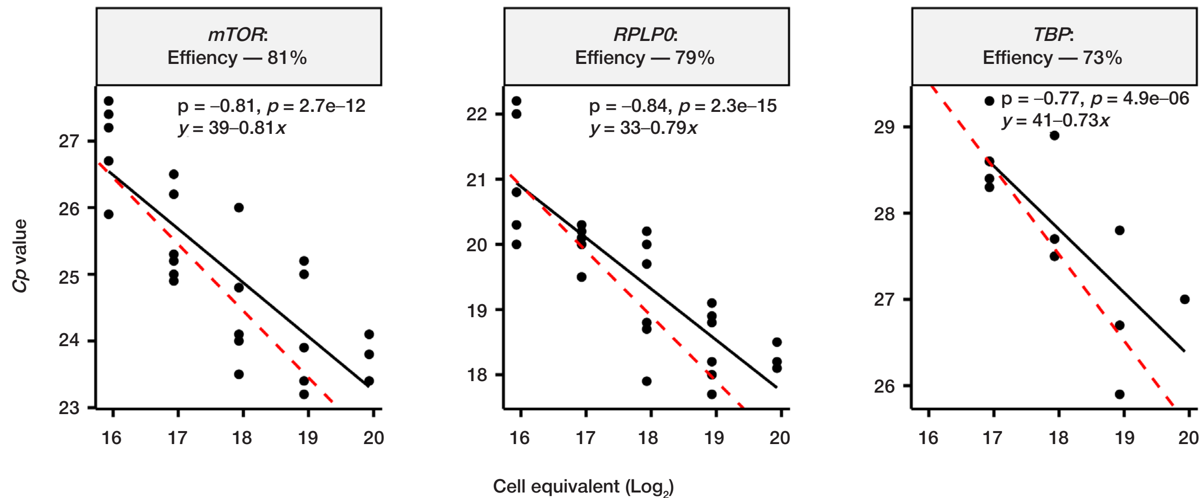

Fig. 3. Amplification efficiency of the target gene *mTOR* and reference genes *RPLP0* and *TBP*. Amplification efficiency analysis for *mTOR*, *RPLP0*, and *TBP* genes showing linear regression plots of *Cp* values versus log_2 of the Cell Equivalent. Experimental amplification efficiencies were 81% for *mTOR* (regression equation: $y = 39 – 0.81x$), 79% for *RPLP0* ($y = 33 – 0.79x$), and 73% for *TBP* ($y = 41 – 0.73x$). The dashed *red line* represents theoretical 100% amplification efficiency (slope = –1), when *Cp* value decreases by one for each twofold increase of cell concentration, while *solid black lines* show actual regression lines, and *black dots* indicate individual data points from multiplex RT-qPCR technical repeats

RESULTS

In silico development and analysis of the PCR assay

Available methods to exclude genomic DNA amplification include "spanning" — positioning one of the primers or the probe to span two exons — and "flanking" — positioning primers on exons separated by a large intron.

To subsequently check the primers for meeting this condition, sequences (FASTA) of the *mTOR* (NM_004958.4), *RPLP0* (NM_001002.4), and *TBP* (NM_003194.5) genes were retrieved. Data from human chromosomes 1 (NC_000001.11), 12 (NC_000012.12), and 6 (NC_000006.12), where the target genes are located, respectively, were used as reference sequences, which were then annotated to highlight introns and exons.

Using obtained exons sequences of the genes, the primer pairs and probes for the *mTOR* and *RPLP0* genes, described previously [16], were verified and their effectiveness was confirmed. 10 primer-probe sets for *TBP* were designed, and the most specific and suitable combination was selected (Table). Primer and probe sequences and parameters are presented in Table.

The proposed normalization strategies and oligonucleotide designs were verified in silico. For the *mTOR* and *RPLP0* genes, primer flanking of exon-exon junctions was confirmed (exons 21–22 and 2–3, respectively, flanking strategy), and for the *TBP* gene, probe spanning of the intron between exons 5 and 6 was confirmed (spanning strategy), which guarantees the absence of genomic DNA amplification. According to NCBI reference sequences (NM_004958.4, NM_001002.4, NM_003194.5), the primers and probes are localized on the CDS as follows: for *mTOR* — forward primer 3381–3402, probe 3411–3435, reverse primer 3437–3464; for *RPLP0* — forward primer 95–115, probe 181–205, reverse primer 224–243; for *TBP* — forward primer 863–882, probe 902–926, reverse primer 930–951.

The primers for *mTOR*, *RPLP0*, and *TBP* did not form stable dimers, indicating a low risk of nonspecific amplification products.

In vitro validation of the PCR assay

When assessing the specificity of amplification products in all separate ($n = 10$) and all multiplex ($n = 10$) technical repeats, a single clear amplification product was obtained for each of the target genes — mTOR, RPLP0, and TBP. All amplicon lengths matched the calculated values (Table). Side or nonspecific bands were not observed when analyzing amplification products by gel electrophoresis (Fig. 2). The analytical specificity of the PCR assay was 100%. To assess analytical sensitivity, various cell dilutions were used; stable detection of all three genes was maintained at a minimum concentration of 125 thousand cells/mL.

A linear regression plot between *Cp* and the *CE* (log_2) was constructed for all three genes (Fig. 3).

Spearman correlation coefficients for the relationship between *Cp* and the *CE* (log_2) were –0.81, –0.84, and –0.77 for *mTOR*, *RPLP0*, and *TBP*, respectively ($p < 0.05$ in all cases), indicating a strong and statistically significant inverse correlation between the number of investigated cells and the *Cp* value for each target.

Amplification efficiency was calculated for each gene from the slope of the linear regression. For the *mTOR* gene, the efficiency was 81%, for *RPLP0* — 79%, for *TBP* — 73%. All efficiency values were obtained based on the deviation of the slope of the experimental regression from the theoretical slope corresponding to 100% efficiency (slope = –1). The obtained values indicate high amplification efficiency of the investigated primers, with the slopes of the experimental lines close to –1 ($y = 39 – 0.81x$ for *mTOR*, $y = 33 – 0.79x$ for *RPLP0*, $y = 41 – 0.73x$ for *TBP*).

The stability of the reference genes was assessed in 27 multiplex technical repeats by analyzing *FC* of mTOR normalized to RPLP0 and TBP. The median *FC* value of *mTOR* normalized to *RPLP0* was 0.02 (0.02 – 0.03), indicating a consistently higher expression level of the *RPLP0* gene compared to *mTOR* in the studied samples. When normalized to *TBP*, the median *FC* of *mTOR* was 9.85 (8.57 – 12.13), indicating a consistently lower expression level of *TBP* relative to mTOR (Fig. 4).

To assess variability between technical replicates, the *mTOR FC* deviation from the mean value was calculated for each sample. The median variability value for *mTOR FC* when normalized to *RPLP0* was –2.3% (–21.5 – 26.4%). The median variability value for *mTOR FC* when normalized to *TBP* was 7.2% (–14.3 – 19.2%). When normalizing *mTOR FC* to both reference genes, the median *mTOR FC* value was 4.9% (–9 – 23.4%) (Fig. 5). In this case, the narrower interquartile range indicates higher reproducibility of results when using the geometric mean of the two reference genes for normalization.

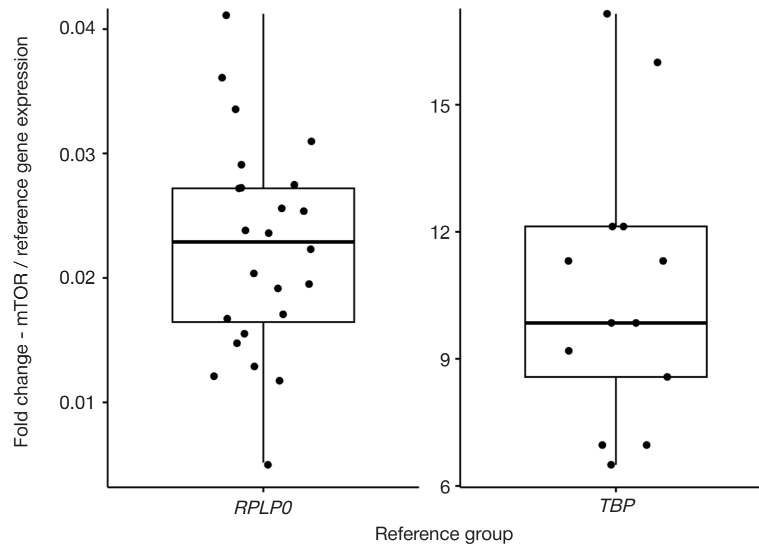

Fig. 4. Analysis of *mTOR* expression relative to reference genes *RPLP0* and *TBP*. The expression of *mTOR* was consistently lower than *RPLP0* and higher than *TBP* in the SCP-1 cell line

DISCUSSION

In this study, we successfully developed and validated a multiplex PCR assay for determining the expression level of the *mTOR* gene normalized to the reference genes *TBP* and *RPLP0*. The in silico analysis stage involved careful selection and verification of primers and probes based on specificity, thermodynamic parameters, and the absence of stable dimer formation. A crucial achievement was the elimination of a drawback of the previously published assay [15] — the overlap between the probe and primer sequences for the *TBP* gene, which had called into question the reliability of the results obtained with it. Our proposed assay completely solves this problem.

Preclinical validation of the PCR assay confirmed high analytical specificity: 100% analytical specificity was achieved with the detection of a single, clearly defined amplification product for each target gene in the complete absence of nonspecific products. Furthermore, the developed PCR assay demonstrated reliable analytical sensitivity, providing stable detection at a minimum concentration of 125 thousand cells/mL.

There was stability of the expression ratios of both reference genes *RPLP0* and *TBP*, regardless of cell concentration or matrix RNA concentration, combined with low median variability between technical replicates. This indicates the high reliability and reproducibility of the developed multiplex PCR assay for analyzing *mTOR* expression levels.

The amplification efficiency for all three genes ranged from 73 to 81%. This range is attributable to two key methodological choices made to ensure the assay's clinical relevance. First, we validated the protocol using a widely adopted RNA extraction kit to reflect real-world diagnostic conditions; the resulting RNA purity, while representative, can modestly impact efficiency [23]. Second, to mitigate the risk of amplifying contaminating genomic DNA — a residue not fully removed by even advanced kits — we omitted the elongation step from the amplification protocol [19]. This precaution is particularly relevant given the high processivity of modern Taq polymerases [24]. Despite these necessary trade-offs, assay specificity remained uncompromised [22]. Future work may focus on fine-tuning primer concentrations to further improve efficiency.

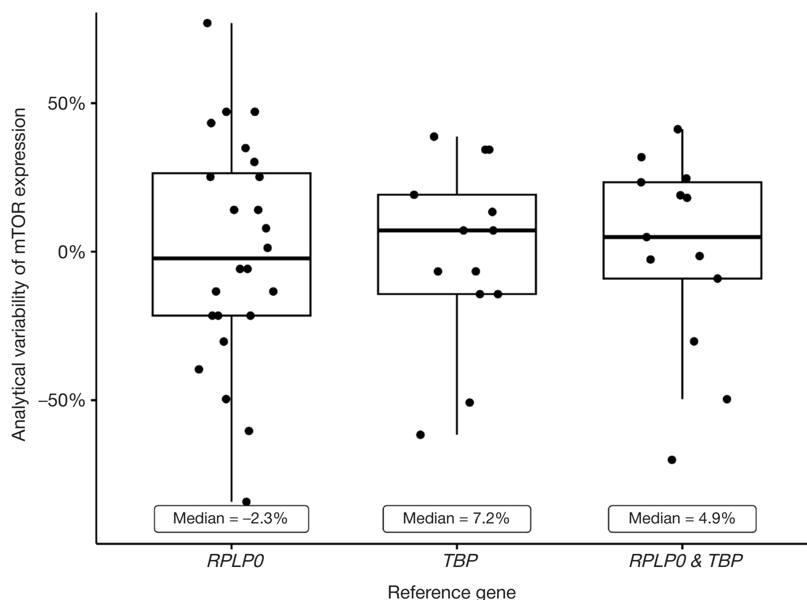

Fig. 5. Assessment of analytical reproducibility using different normalization strategies. The percentage deviation of *FC* values for each sample from the overall median. The reproducibility of results when *mTOR* expression normalized to *RPLP0*, *TBP*, and their geometric mean (*RPLP0* and *TBP*). The reduced variability (narrower interquartile range) achieved by using two reference genes simultaneously

Despite good median values, the interquartile ranges remain relatively wide. This is explained by the use of the Fold Change metric, based on an exponential formula, which amplifies the influence of even small changes in expression levels on the magnitude of percentage variability, which is an expected result for relative quantitative PCR analysis. The original *Cp* differences varied within a one-cycle range.

Such ranges of analytical *FC* variation fully correspond to the data from most published studies on the development of relative quantitative PCR kits without the use of absolute standards and are consistent with the results of fundamental works [22, 25]. In the study by Gentle et al. [26], the authors report technical *FC* variation values from 23 to 52%, depending on the number of technical replicates [26].

A key advantage of this study is the performance of rigorous analytical validation on a standardized cell line. The use of SCP-1 cells provided a homogeneous and controlled system, free from the pre-analytical variables inherent in clinical samples (such as tissue heterogeneity or variable RNA quality). This allowed us to accurately determine the intrinsic analytical parameters of the PCR assay — specificity, sensitivity, and reproducibility — without the influence of confounding biological factors. The demonstrated robustness of this PCR assay is a prerequisite for its successful application in the analysis of more complex biomaterial types, such as human tumor tissues, which will be the next step in our research.

Our multiplex RT-qPCR assay provides a direct and reproducible tool for assessing *mTOR* expression, capturing the integrated activity of the PI3K/AKT/mTOR pathway [27] and representing a promising objective alternative to operator-dependent methods like immunohistochemistry [28]. Unlike detecting mutations in genes like *BRCA1/2* or *PIK3CA* — which, despite their clinical utility, are limited to specific patient subgroups and provide information on predisposition or disease aggressiveness rather than serving as true diagnostic tools — our method quantifies pathway output directly. Given the role of mTOR overexpression in breast cancer [1, 6, 28], this assay holds significant potential for integration into future diagnostic and research strategies.

A limitation of this study is the use of a standardized cell line (SCP-1), which does not reflect the full heterogeneity of clinical tumor samples and does not account for the influence of pre-analytical variables [30]. Furthermore, assay performance is inextricably linked to the specific reagents and protocols employed in this work. Therefore, the clinical applicability of the developed test system for assessing mTOR expression in breast cancer diagnosis and stratification must be established through future studies on representative tissue cohorts. Such studies will determine its diagnostic accuracy (sensitivity, specificity, AUC) and evaluate the potential for implementation into routine clinical practice. It should also be noted that increased mTOR expression is observed not only in oncological but also in other diseases (e.g., rheumatic pathologies); therefore, the interpretation of results will depend on the type of biological material studied and the clinical study design. Depending on the specific task, future applications may require either the establishment of diagnostic thresholds for particular sample types (e.g., tumor biopsies) or the introduction of additional markers (e.g., RILP) to differentiate conditions with similar mTOR expression profiles. Despite the need for further research, the developed PCR assay represents a promising foundational tool for advancing molecular diagnostics in breast cancer.

CONCLUSIONS

In this study, we designed and validated a novel multiplex RT-qPCR assay for the relative quantification of *mTOR* gene expression normalized to *RPLP0* and *TBP*. A critical flaw of the the previously described design, which consisted of overlapping sequences of the probe and primer for the *TBP* gene was eliminated in the developed assay through the correct design of oligonucleotides, verification of the absence of primer-dimer interactions, and the use of optimal strategies to prevent genomic DNA amplification. The assay demonstrated 100% analytical specificity and a sensitivity of 125 thousand cells/mL. The amplification efficiency was 73% for *TBP*, 79% for *RPLP0*, and 81% for *mTOR*. In the SCP-1 cell line, the expression of *mTOR* was substantially higher than that of *TBP* but lower than *RPLP0*. Normalization to the geometric mean of both reference genes yielded the highest reproducibility, with a median Fold Change deviation of 4.9% between technical replicates. The developed PCR assay allows to overcome a critical design flaw of a previously published assay and, given the role of *mTOR* overexpression in carcinogenesis, is a promising tool for advancing molecular diagnosis in breast cancer.

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