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Original Article

Improving Gene Expression Analysis Efficacy from Formalin-Fixed Paraffin Embedded Tissues

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Abstract

Introduction: Improving RNA isolation and cDNA synthesis techniques has emerged due to advancements in the knowledge of molecular basis of most diseases. This in turn increased the need of higher quantity and quality of the extracted genetic material to be used for a variety of diagnostic tests and experiments.

Aim: The aim of the study was to compare three modified methods for RNA extraction from formalin-fixed paraffin embedded (FFPE) biopsied tissue and different cDNA synthesis strategies to facilitate study of gene expression.

Materials and methods: Compared RNA extraction methods were: lysis buffer, phenol-based extraction, and combination of both with concomitant use of silica-based spin columns. RNA quantity and purity were estimated spectrophotometrically. Different priming strategies for cDNA synthesis were applied: oligo dT, combination of oligo dT and random hexamer, and gene specific primer. Two-step RT-qPCR of ribosomal protein L37A on preamplified and non-preamplified cDNA templates was performed.

Results: The combination of lysis buffer with phenol based extraction gave higher RNA yield. By doing cDNA preamplification, the confidence of detection by qPCR was raised, and efficiency was improved. The preamplified template increased the sensitivity of analysis.

Conclusions: Together, the combination of approaches improved substantially the reproducibility and validity of quantitative gene expression analyses from FFPE tissues.

INTRODUCTION

With the development of molecular biology techniques for research and diagnostics, there is a growing interest to use the vast archives of formalin-fixed paraffin-embedded (FFPE) tissue samples in these applications also.[1] The latter gave rise to the demand to optimize isolation techniques of genetic material to reach the highest yields possible, due to the limited amount of fixed tissue for the ever increasing possibilities in molecular biology, such as qPCR.[2]

The most important issue when manipulating genetic material from FFPE is nucleic acid fragmentation and chemical modifications, especially formation of nucleoprotein complexes during formaldehyde fixation. The methods

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to overcome these issues are amplification of short sequences[3,4], incubating deparaffinised tissue with an alkaline saline buffer[5,6], and incorporating silica based columns for purification.

The limitation of oligo dT cDNA synthesis priming strategy for mRNA is the preferential transcription of the 3' region, often lost in degraded samples, and the low number of intact transcripts may lead to inaccurate gene expression results.[7] Fragmentation and low yield of RNA extracted from FFPE tissue may fail to reach the necessary threshold for qPCR and often requires a preliminary mRNA amplification.[8]

AIM

The purpose of this study was improvement of qualitative gene expression analysis using FFPE tissue samples by combining strategies aiming at: 1) increasing the RNA yield; 2) more efficient cDNA synthesis, and 3) improving the detection limit in qPCR reaction.

MATERIALS AND METHODS

This retrospective study approved by the University Research Ethics Committee (P55/16.06.2016) was conducted at the Medical University of Varna in collaboration with St Marina University Hospital. Written informed consents were obtained in compliance with the Helsinki Declaration.

Tissue fixation

Tissue samples were collected during colonoscopy analysis as part of routine investigation for colorectal cancer at St Marina University Hospital, Varna, Bulgaria. Samples were immersed in 10% buffered neutral formalin solution for 24 hours before embedding them in paraffin according to a standard university hospital protocol (St Marina University Hospital, Varna, Bulgaria) and were stored at room temperature (RT).

RNA extraction methods

The experimental design is presented in **Fig. 1**. From a single FFPE tissue block, 3 sections of 5 μm each were cut using a microtome (LEICA RM2235, Germany) and placed in a microcentrifuge tube. The procedure was repeated until nine identical tissue samples were prepared from the same block. Three RNA extraction methods were tested (method A, B, and C) and each method was performed in triplicate.

Deparaffinisation of samples was performed using the following procedure: incubation of each sample with 1 mL xylene, followed by brief vortex and centrifugation at 14 000 rpm/2 min at RT. The procedure was repeated and after xylene removal the sample was washed with 1 mL absolute ethanol. Samples were centrifuged at 14 000 rpm/2 min at

RT and ethanol was removed. The procedure was repeated. After ethanol removal, samples were left to air-dry. At this stage, samples were divided in three groups for methods A, B, and C, respectively, each group containing three samples.

Method A: lysis buffer

Tissue samples were initially treated with 100 μL of Quickextract FFPE RNA extraction Lysis buffer (Epicentre, Illumina, USA). Samples were incubated at 56°C for 30 min and further heated at 80°C for 10 min. Then purification was performed using the RNA Clean and Concentrator 5 spin columns (Zymo Research, USA) according to the manufacturer's protocol. Elution of RNA was performed with 15 μL of DNAse/RNAse free water and stored at −70°C.

Method B: lysis buffer and phenol based extraction

Samples were treated with lysis buffer as described in method A. Immediately after the final incubation period (80°C for 10 min), standard phenol based extraction was performed using Accuzol (Bioneer, USA) and isolated samples were transferred to Clean and Concentrator 5 (Zymo Research, USA) silica spin columns for purification and concentration. Phenol based extraction and column purification steps were performed according to the respective manufacturer's protocols. RNA was further eluted, as described in method A.

Method C: phenol-based extraction

Accuzol (Bioneer, USA) 1 mL was added directly to the deparaffinised tissue samples. The steps followed further were as described in method B.

The concentration and purity of isolated RNA was estimated spectrophotometrically (Synergy 2, Biotek).

DNase treatment

For the removal of contaminating gDNA, a DNase reaction was performed adding 2 μL of DNase buffer and 2 μL of DNase I (1 U/μL) (Epicentre, Illumina, USA) to each sample following the manufacturer's protocol.

cDNA synthesis

For the synthesis of cDNA, 500 ng total RNA template was used. For all of the three replicates from methods A and B, three types of reverse transcription reactions were performed: 1) with oligo dT primer; 2) with oligo dT and random hexamer primer; 3) with gene specific reverse primer (RPL37A: Forward 5' ATTGAAATCAGCCAGCACGC 3' and Reverse 5' AGGAACCACAGTGCCAGATCC 3'). Samples were transcribed using RevertAid cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's protocol.

cDNA preamplification

Preamplification was performed in 50 µL volume PCR reaction for each sample containing: 5 µL template cDNA; 5 µL of Taq DNA polymerase (2U) (New England Biolabs, USA) buffer containing $MgCl_2$; 2 µL dNTPs (2.5 mM); forward and reverse gene specific primers (RPL37A, see cDNA synthesis) (Sigma-Aldrich, Germany) to a final concentration of 50 nM each; and PCR grade water (Sigma-Aldrich, Germany) up to 50 µL. Samples were amplified in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, USA). Initial denaturation was performed at 95℃ for 5 min, followed by 95℃ for 15 s and 60℃ for 4 min for 5 cycles. Samples were finally cooled down to 4℃ and stored at −20℃. The samples were placed on ice during all preparations. Each DNA-se treated sample provided a single preamplified sample.

Quantitative Real-Time PCR (qPCR)

Results were validated by qPCR using standard SYBR Green qPCR Master Mix (Thermo Scientific, USA). Re-actions in total volume of 10 µL were performed for each sample (preamplified and non-preamplified) as follows: 5 µL Master Mix with ROX dye; gene specific primers (RPL37A, see cDNA synthesis) (Sigma-Aldrich, Germany) to a final concentration of 0.25 µM each and 4 µL 10× diluted preamplified or non-preamplified cDNA. Reaction conditions were as follows: initial denaturation at 95℃ for 10 min, followed by 95℃ for 15 s and 63℃ for 1 min for 40 cycles. Melting curve was added at the end of each qPCR analysis. Reactions were performed in triplicate.

Standard curve

Absolute quantification method was performed and standard curve was created to assess preamplification efficacy of gene specific primed cDNA strategy for RNA isolation methods A and B. A serial decimal dilutions of the non-preamplified cDNA (25 ng/µL, 2.5 ng/µL, 0.25 ng/µL, 0.025 ng/µL, 0.0025 ng/µL) were used as standard. The change in Ct value resulting from preamplification was analysed by calculating the initial concentration of the template before preamplification (C=2.5 ng/µL) and running qPCR with
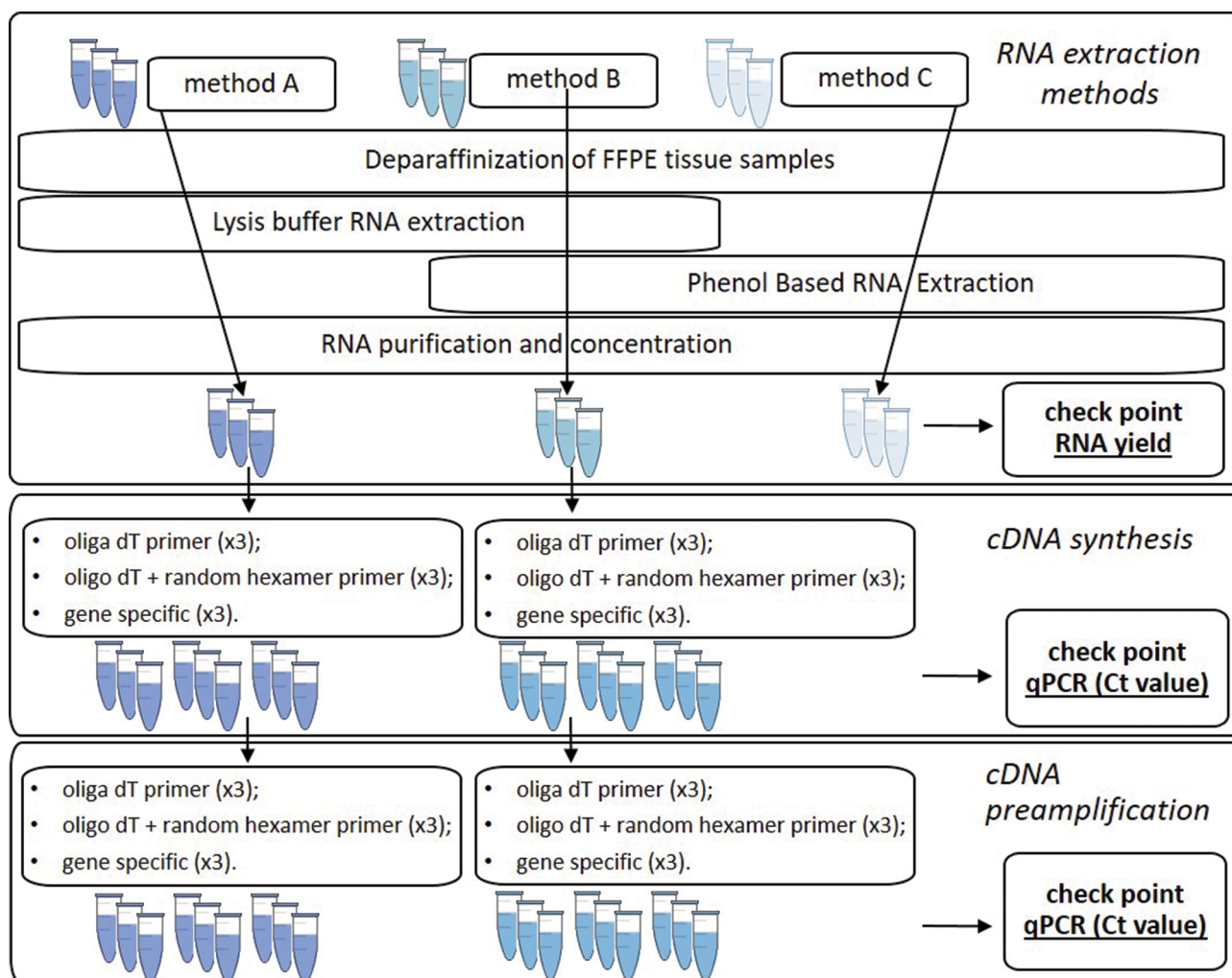
Figure 1. Experimental design and workflow, including number of samples for each step followed in the present study.

the same template volume of preamplified product, using SYBR Green qPCR Master Mix (Thermo Scientific, USA) according manufacturer's protocol. RPL37A gene primer set (see cDNA synthesis) was used in performing of qPCR.

Statistical analysis

Data were analysed using GraphPad Prism V6 software. For the estimation of statistical significance, single-way and two-way ANOVA statistical analyses were performed. *P* values <0.05 were considered as statistically significant.

RESULTS

RNA yield and purity

Analysis of concentration of RNAs obtained by the three different methods showed that combination of lysis buffer extraction with phenol-chloroform extraction (method B) outperformed the other methods (A and C) **(Table 1)**.

Quantitative PCR

Average Ct values of qPCR reactions of preamplified and non-preamplified samples, obtained after RNA extraction with methods A and B are presented in **Table 2**.

Gene specific primed cDNA synthesis gave significantly lower Ct values (*p*<0.001) for both RNA extraction methods (A and B) compared to oligo dT and to combination of oligo dT and random hexamer primers, for both non-pre-amplified and preamplified cDNA samples **(Table 2)**. For non-preamplified cDNA samples of both RNA extraction methods, application of gene specific priming strategy resulted in significantly lower Ct (*p*<0.001) also compared to combined oligo dT and random hexamer priming strategy. Melting curve analysis resulted in clear peaks for all repetitions for both methods A and B for the case of gene specific primer strategy.

Application of oligo dT priming strategy resulted in a statistically significant lower Ct value in RNA extraction method A than in method B, on non-preamplified (*p*<0.01) and on preamplified cDNA templates (*p*<0.01). This could be attributed to the ability of lysis buffer extraction, solely,

Table 1. Comparison of RNA quantity and purity isolated using three different extraction methods

Method	RNA Concentration [ng/μL]	RNA yield [ng]	$A_{260/280\,nm}$
A	163.36±17.96 ***	2450.40±269.43	2.03±0.07
B	259.87±38.60 ***, #	3898.00±579.02	2.02±0.02
C	15.51±2.06	232.60±30.90	1.83±0.21

Data are presented as mean±SD of three identical FFPE tissue samples. ***p<0.001 vs. C, #p<0.05 vs. A. Legend: **A**. Lysis buffer RNA extraction; **B**. Lysis buffer and phenol-based RNA extraction; **C**. Phenol-based RNA extraction. Since method C appeared to result in the lowest RNA yield, it was excluded from further analyses at this stage.

Table 2. Comparison of Ct values obtained through qPCR of non-preamplified and preamplified cDNA samples obtained after RNA extraction with methods A and B

Non-preamplified		
Priming strategy	**Ct $_A$**	**Ct $_B$**
Oligo dT primer	27.93±0.69**	31.50±0.46
Oligo dT+random hexamer primer	26.40±0.62##	27.26±0.60###
gene specific primer	20.20±0.53###, $$$	20.89±0.46###, $$$
Preamplified		
Priming strategy	**Ct $_A$**	**Ct $_B$**
Oligo dT primer	26.14±0.39**	28.54±0.49
Oligo dT+random hexamer primer	18.89±2.09###	19.29±1.49###
gene specific primer	17.17±0.62###	16.99±0.46###, $

Data are presented as mean ±SD of FFPE tissue samples for both methods utilizing three different reverse transcription priming strategies (oligo dT, oligo dT+random hexamer primer and gene specific primers), of non-preamplified and preamplified cDNA templates. **p<0.01 vs. Ct$_B$; ##p<0.01, ###p<0.001 vs. oligo dT primer of the respective non-preamplified/preamplified cDNA sample; $$p$<0.05, $$$p$<0.001 vs. oligo dT+random hexamer primer of the respective non-preamplified/preamplified cDNA sample. Legend: **A**. Lysis buffer RNA extraction; **B**. Lysis buffer and phenol-based RNA extraction.

to result in higher ratio of intact to fragmented, short RNAs, compared to concomitant phenol-based extraction.[9]

Comparing Ct values between non-preamplified and preamplified cDNA templates for all three priming strategies to assess the efficacy of preamplification, we noticed statistically significant improvement for both RNA extraction methods A ($p<0.01$) and B ($p<0.001$) **(Fig. 2)**. The Ct values of method A for oligo dT, combined oligo dT and random hexamer and gene specific priming strategies were lowered respectively by 1.78 ($p<0.05$), 7.52 ($p<0.01$), 3.03 ($p<0.01$) when cDNA preamplification was applied. According to method B, the preamplification lowered Ct values by 2.96 ($p<0.01$) for oligo dT, by 7.97 ($p<0.001$) for combined oligo dT and random hexamer, and by 3.90 ($p<0.001$) for gene specific priming strategies. The average of methods A and B decrease of Ct values for oligo dT and for gene specific primed cDNA were 2.37±0.82 and 3.46±0.61, respectively. The oligo dT+random hexamer primed cDNA gave an average decrease of Ct value by 7.74±0.32, which corresponded to a higher efficiency than expected, thus indicating the need of additional validation of the analysis by performing absolute quantification to assess the preamplification efficiency.

Preamplification efficacy assessment

By performing an absolute quantification, to assess preamplification efficacy of gene specific primed cDNA strategy for RNA isolation methods A **(Fig. 3A)** and B **(Fig. 3B)**, we found that the Ct value for preamplified template was 5.5
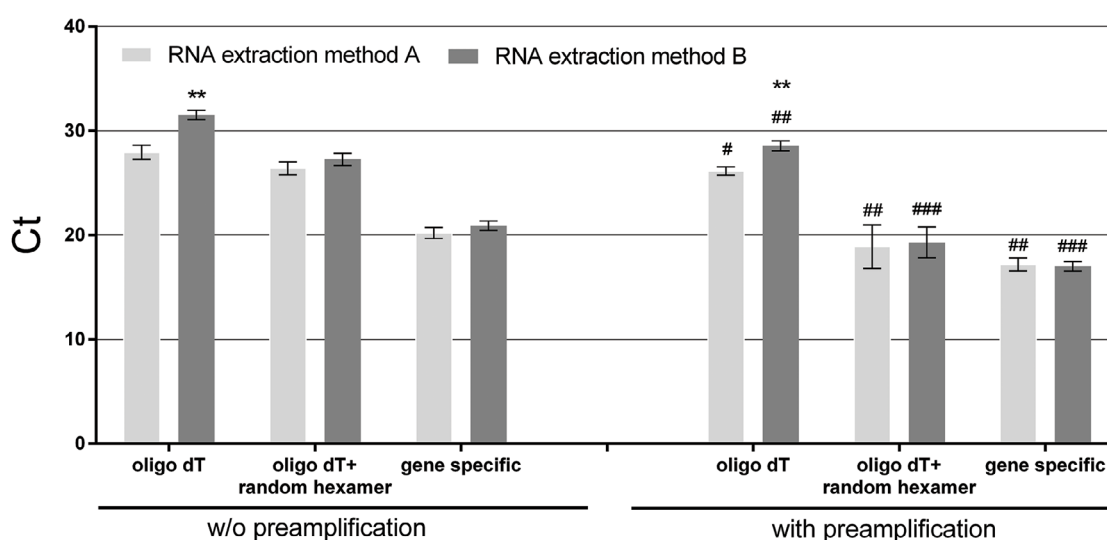
Figure 2. Comparison of Ct values obtained through two-step qPCR of non-preamplified and preamplified cDNA samples. Data are presented as mean ±SD of FFPE tissue samples for both RNA extraction methods utilizing three different reverse transcription priming strategies (oligo dT; oligo dT+random hexamer; gene specific reverse primer). **p<0.01 vs. RNA extraction method A; #p<0.05, ##p<0.01, ###p<0.001 vs. respective priming strategy and RNA extraction method w/o preamplification. **A**. Lysis buffer RNA extraction (grey); **B**. lysis buffer and phenol-based RNA extraction (dark grey).
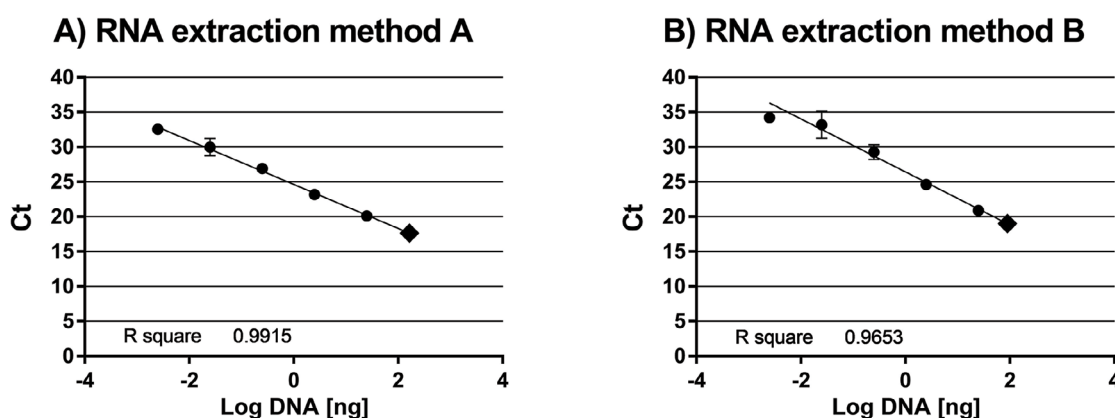
Figure 3. Relative quantification for construction of standard curve of gene specific primed cDNA for calculation of the preamplification efficiency, by taking r^2 (R square) value. Used standard dilutions of non-preamplified cDNA were 25 ng/µL, 2.5 ng/µL, 0.25 ng/µL, 0.025 ng/µL, 0.0025 ng/µL. Preamplified unknown cDNA sample volume was corresponding to the volume of 2.5 ng/µL standard dilution. **A**) RNA extraction method A; **B**) RNA extraction method B. Legend: standard dilutions (●); unknown sample (♦).

cycles lower for method A as well as for B, corresponding to 5.5 Ct of preamplification, although the preamplification run was performed for 5 cycles. Evaluation of quantification efficiency was confirmed by high r^2 score for RNA isolation methods A and B where r^2=0.9915 and r^2=0.9653, respectively.

DISCUSSION

The commonly used formalin fixation of tissue samples limits most molecular techniques by causing nucleic acid degradation[10,11] and protein-RNA cross links[12]. Therefore, it was interesting to modify standard protocols to achieve maximum quality and quantity of extracted RNA.

Three different RNA extraction methods were compared implementing basic techniques, involving a tissue lysis buffer and a phenol-based extraction, as well as a combination of both. By replacing traditional ethanol precipitation with the more advantageous silica based spin columns, we managed to provide maximum efficiency and purity of the extracted RNA. The combination produced the highest yield and purity of RNA, as expected. The phenol-based extraction failed to achieve high yield to an acceptable level and was excluded from further analysis.

As mentioned before, RNA degradation and modification compromise the reverse transcription reaction and directly affects the produced cDNA. Different priming strategies were compared including modified protocols using oligo dT, combination of oligo dT and random hexamer primers, and gene specific primed cDNA for a set of genes. The gene specific primed strategy outperformed the rest priming strategies for both RNA extraction techniques **(Table 2)**.

To increase the sensitivity of the analysis, we evaluated the effect of targeted preamplification of cDNAs. By comparing Ct values obtained through qPCR on preamplified and non-preamplified templates of all cDNA primed strategies for both extraction methods (A and B), we observed that preamplification can be safely used on gene specific primed cDNA templates, considering the preamplification conditions. More specifically, the 5-μl cDNA volume corresponding to 125 ng of transcribed total RNA along with a low number of amplification cycles (n=5) was chosen to avoid fluctuations due to Poisson noise. Low primer final concentration, 50 nM, and annealing-extension temperature of 60°C decreased nonspecific amplification. To compensate for the low primer concentration, annealing time was increased to ≥3 min.[13]

Quantification of the preamplified template by standard curve verified the consistency of preamplification efficiency, evaluating and correcting any variations. A higher Ct value of 5.5 was estimated, compared to the 5 run cycles. Confirmation through standard curve for the consistency of change in Ct values is highly recommended.

CONCLUSIONS

In conclusion, the combination of lysis buffer with phenol-based extraction giving the highest RNA yield, along with gene specific primed cDNA synthesis, is of great superiority. Increasing the target gene template by preamplification decreases Ct value and achieves higher accuracy of the results.

Acknowledgements

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Повышение эффективности анализа экспрессии генов из фиксированных формалином парафиновых тканей

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Резюме

Введение: Улучшение методов выделения РНК и синтеза кДНК появилось благодаря прогрессу в изучении молекулярных основ большинства заболеваний. Это, в свою очередь, увеличило потребность в более высоком количестве и качестве извлечённого генетического материала для использования в различных диагностических тестах и экспериментах.

Цель: Цель исследования состояла в том, чтобы сравнить три модифицированных метода экстракции РНК из фиксированной формалином ткани, залитой в парафин (FFPE) биопсийной ткани, и различные стратегии синтеза кДНК для облегчения изучения экспрессии генов.

Материалы и методы: Сравниваемые методы выделения РНК: лизирующий буфер, экстракция на основе фенола и их комбинация с одновременным использованием спин-колонок на основе диоксида кремния. Количество и чистоту РНК оценивали спектрофотометрически. Были применены различные стратегии праймирования для синтеза кДНК: праймер oligodT, комбинация oligodT и случайного гексамера, а также ген-специфический праймер. Была проведена двухэтапная RT-qPCR рибосомного белка L37A на предварительно амплифицированных и непредварительно амплифицированных матрицах кДНК.

Результаты: Комбинация буфера для лизиса с экстракцией на основе фенола дала более высокий выход РНК. Выполнение преамплификации кДНК повысило достоверность обнаружения с помощью qPCR и повысило эффективность. Предварительно амплифицированный шаблон повышал чувствительность анализа.

Заключение: Совокупность комбинаций подходов существенно улучшила воспроизводимость и достоверность количественного анализа экспрессии генов в тканях FFPE.

Ключевые слова

биопсия, кДНК, FFPE, qPCR, РНК-зонд

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