Clinical and Experimental Health Sciences

Optimizing qPCR Annealing Temperatures for Cancer-Related lncRNAs

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ABSTRACT

Objective: To determine the optimal annealing temperatures to detect copy number variation and expression levels of specific cancer-associated long non-coding RNAs (lncRNAs), to improve the accuracy of clinical tests in precision medicine.

Methods: Gradient qPCR analysis was performed to identify the optimal annealing temperatures for the detection of lncRNAs. These lncRNAs were H19, CCAT1, HOTAIR, NEAT1, MALAT1, PVT1, GAS5, BANCR, UCA1, HULC, and MEG3.

Results: Optimal qPCR annealing temperatures for the detection of lncRNA expression and copy number variations were determined as follows: 62.4°C for PVT1 and BANCR; 60°C for H19, CCAT1-b, HOTAIR, NEAT1, MALAT1; 58°C for GAS5 and HULC; 56.7°C for CCAT1-a, UCA1; and 56°C for MEG3.

Conclusion: In this study, we determined optimal annealing temperatures for some lncRNAs, which is crucial for the precision and accuracy of qPCR used to identify lncRNA expression and copy number changes. These results confirm the optimization of lncRNA analysis by determining optimal annealing temperatures, which is essential for the precision and accuracy of qPCR.

Keywords: cancer, lncRNA, qPCR, annealing temperature

1. INTRODUCTION

Cancer, one of the serious diseases that threaten human health, is caused by a combination of genetic and non-genetic environmental factors. In most cases, it is also characterized by mutations occurring in the non-coding region of the genome, beyond the protein-coding regions, which make up less than 2% of the genome (1,2).

Advances in genome sequencing technologies have revealed that more than 80% of the genome produces different RNA molecules, in contrast to the less than 2% that is transcribed to messenger RNA (mRNA) for protein synthesis. This means that a large part of the human genome that is transcribed are called non-coding RNAs (ncRNAs). Long ncRNAs (lncRNAs) are non-protein-coding RNA molecules longer than 200 nucleotides that are transcribed by RNA polymerase II (Pol II) (3).

Genome and transcriptome analyses have highlighted the involvement of lncRNAs in numerous epigenetic regulations, including chromatin remodeling, DNA methylation, and histone modification (4). In addition, lncRNAs have crucial post-translation roles, controlling the function of proteins by silencing genes through the production of endogenous siRNA or microRNA binding. Some lncRNAs have oncogenic roles or tumor suppressor functions, while others play dual roles in tumor suppression and oncogenesis in cancer (5).

H19, a paternally imprinted lncRNA first isolated in the 1980s, exhibits both oncogenic and tumor-suppressive functions. As a tumor suppressor, it inhibits cell proliferation in some cancers such as colorectal cancer, hepatocellular carcinoma, and osteosarcoma. It also has an oncogenic role in breast cancer, ovarian cancer, gastric cancer, and other

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cancers by promoting survival, metastasis, and epithelial-mesenchymal transition (6,7).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) gene, one of the most studied lncRNAs, produces various splicing variants and full-length MALAT1. It can exhibit both oncogenic and tumor-suppressive functions in several cancer types. Similarly BANCR, NEAT1, MEG3, and PVT1 (8) (9).

HOTAIR, a trans-regulator lncRNA that has been identified in fibroblasts, shows oncogenic effects by regulating HoxD cluster genes. It has an important role in proliferation, metastasis, apoptosis, invasion, and drug resistance in various cancers like breast, lung, ovarian, and gastric cancers(10). Other lncRNAs with reported oncogenic effects include CCAT1, HULC, and UCA1 (9).

GAS5 (Growth Arrest-Specific 5) serves as a tumor suppressor lncRNA which is frequently deactivated in various cancers, especially in breast cancer, hepatocellular carcinoma, and gastric cancer. GAS5 functions by suppressing proliferation, cell survival, metastasis, and therapy resistance. (11).

H19, CCAT1, HOTAIR, NEAT1, MALAT1, PVT1, GAS5, BANCR, UCA1, HULC, and MEG3 are the most commonly studied cancer-related lncRNAs. There are different primer sequences published for targeting these lncRNAs in the literature. However, limited publications shared both primer sequences and optimal annealing temperature information. Optimizing primer assay temperatures in qPCR is crucial for obtaining precise and reliable results.

In this study, we aimed to optimize the qPCR annealing temperature of the target-specific primers for these lncRNAs. The experimental design considered annealing temperatures identified from a comprehensive review of previous studies (Table 1).

2. METHODS

2.1. RNA Isolation and Reverse Transcription

5 mL of whole blood was collected from a healthy individual and aliquoted to 500 µL. Total RNA was extracted from the 500 µL whole blood sample using the QIAamp RNA Blood Mini Kit (Qiagen Cat. No. / ID: 52304) following the manufacturer's protocol. The concentration and purity of the extracted RNA were measured at 230 nm, 260 nm, and 280 nm using a NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific).

Total RNA was converted to cDNA using random hexamers with the SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA; Cat: 18080051) following the manufacturer's protocol.

2.2. Annealing Temperature Optimization

The annealing temperature optimization of BANCR, CCAT1-a, CCAT1-b, GAS5, H19, HOTAIR, HULC, MALAT1, MEG3, NEAT1, PVT1, and UCA1 was carried out with abm® Bright Green 2X qPCR Master Mix-No Dye (Cat. No. Master Mix-S; Lot No. 023.485.4746001, Canada) and the BIO-RAD® CFX96™ Touch Real-Time PCR Detection System. The gradient qPCR was performed as follows: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 15 seconds, and 30 seconds of annealing temperature adjusted between 56°C and 66°C.

The total reaction volume was 20 µL containing 5 µL of cDNA sample. Forward and reverse primers shown in Table 1 were synthesized and purified via HPLC (High-Performance Liquid Chromatography) by Oligomer (Turkey). Each primer was employed at a concentration of 500 nM in the reaction. Also RPPH1 F: 5'-GTCAGACTGGGCAGGAGATG-3' and R: 5'-TGGCCGTGAGTCTGTTCC-3' was used as a reference gene. All temperatures were assessed in duplicate. Bio-Rad CFX Maestro 1.1. Software was used to analyze the qPCR results.

Table 1. *Primer Sequences of lncRNAs*

lncRNA	Forward Primer Sequences	Reverse Primer Sequences	References
BANCR	5'-ACAGGACTCCATGGCAAACG-3'	5'-ATGAAGAAAGCCTGGTGCAGT-3'	(12)
CCAT1-a	5'-TTTATGCTTGAGCCTTGA-3'	5'-CTTGCCTGAAATACTTGC-3'	(13)
CCAT1-b	5'-AGAAACACTATCACCTACGC-3'	5'-CTTAACAGGGCATTGCTAATCT-3'	(14)
GAS5	5'-CTTCTGGGCTCAAGTGATCCT-3'	5'-TTGTGCCATGAGACTCCATCAG-3'	(15)
H19	5'-ACTCAGGAATCGGCTCTGGAA-3'	5-CTGCTGTTCCGATGGTGTCTT-3'	(16)
HOTAIR	5'-CAGGAGTGATTATGCAGTGGG – 3'	5'-ACCCCTTCTGTGTCTACATGC-3'	(17)
HULC	5'-TCATGATGGAATTGGAGCCTT-3'	5'-CTCTTCCTGGCTTGCAGATTG-3'	(18)
MALAT1	5-AGGCGTTGTGCGTAGAGGA-3'	5'-GGATTTTTACCAACCACTCGC-3'	(19)
MEG3	5'-ATCATCCGTCCACCTCCTTGTCTTC-3'	5'-GTATGAGCATAGCAAAGGTCAGGGC-3'	(20)
NEAT1	5-TGGCTAGCTCAGGGCTTCAG-3	5-TCTCCTTGCCAAGCTTCCTTC-3	(21)
PVT1	5'-GGGGAATAACGCTGGTGGAA-3'	5'-CCCATGGACATCCAAGCTGT-3'	(22)
UCA1	5'-ACGCTAACTGGCACCTTGTT-3'	5'-TGGGGATTACTGGGGTAGGG-3'	(23)

3. RESULTS

This study aims to optimize the annealing temperature for primer assays that target specific cancer-related lncRNAs. The experimental design includes annealing temperatures defined in the review of the literature. For the gradient qPCR analysis, the annealing temperature was adjusted between 56°C and 66°C.

Following gradient qPCR analysis, optimum annealing temperatures were determined for various lncRNAs expressions. For analyzing the expression of PVT1 and BANCR, the optimal annealing temperature was determined as 62.4°C. For H19, CCAT1-b, HOTAIR, NEAT1, and MALAT1, the annealing temperature was determined as 60°C, which revealed lower Cq level and gave a higher fluorescence signal compared to other temperatures tested. The negative control samples did not show any false positive findings.

The optimal annealing temperature for GAS5 and HULC expression was found to be 58°C among the temperatures analyzed. Similarly, the ideal annealing temperature for CCAT1-a, UCA1 expression analysis was determined as 56.7°C. Additionally, the optimal annealing temperature for MEG3 expression analysis was found to be 56°C (Table 2). For all assays, no false positive signals were observed in the negative control samples.

Table 2. *Cq Values from Gradient qPCR for Determining Optimum Annealing Temperatures*

Annealing Temperature °C	H19		MEG3		CCAT1-a		CCAT1-b		GAS5		HOTAIR	
	Cq	±	Cq	±	Cq	±	Cq	±	Cq	±	Cq	±
66	33.7	-	-	-	-	-	28.7	1.6	22.8	0.3	28.3	0.7
65.5	28.5	-	-	-	-	-	28.9	4.4	22.5	0.1	26.1	1.0
64.3	28.1	-	40.0	3.0	-	-	24.5	0.3	22.4	0.2	24.9	0.0
62.4	34.0	7.8	43.7	2.4	-	-	24.0	0.6	22.1	0.0	24.5	0.0
60	28.6	0.3	39.7	0.7	-	-	23.5	0.2	22.3	0.1	24.5	0.0
58	29.5	0.2	36.4	0.1	28.7	2.0	23.9	0.2	22.2	0.0	24.7	0.1
56.7	28.5	0.5	34.3	0.2	26.6	0.0	23.9	0.0	22.4	0.1	31.7	0.0
56	29.2	1.7	33.8	0.0	28.0	0.1	24.1	0.0	23.0	0.9	24.9	0.0
Annealing Temperature °C	NEAT1		UCA1		BANCR		MALAT1		PVT1		HULC	
	Cq	±	Cq	±	Cq	±	Cq	±	Cq	±	Cq	±
66	29.5	0.7	24.6	0.0	25.3	0.2	26.5	0.1	24.9	0.1	25.6	0.2
65.5	32.5	0.6	24.2	0.1	25.0	0.1	26.4	0.0	24.8	0.2	25.3	0.1
64.3	28.1	0.0	24.1	0.1	25.0	0.1	26.0	0.2	25.1	0.5	25.0	0.0
62.4	27.0	0.3	24.1	0.0	24.6	0.2	33.8	0.2	24.5	0.1	25.0	0.2
60	26.3	0.1	23.6	0.0	25.1	-	26.3	0.0	24.6	0.1	25.0	0.0
58	26.4	0.0	23.1	0.1	32.3	0.0	32.5	0.1	32.2	0.1	24.8	0.2
56.7	33.8	0.1	22.9	0.0	29.8	0.1	31.8	0.0	25.3	0.1	24.9	0.4
56	27.2	0.2	23.0	0.1	25.4	0.1	31.5	0.2	25.2	0.1	24.7	0.1

In this study, the annealing temperatures of primer assays were determined by using the TM calculator available at https://www.thermofisher.com/. The calculated TMs were compared with values obtained from gradient PCR. It was observed that the annealing temperature values of some lncRNAs were found to be 2°C higher than the calculated values with informatics tools (Table 3). This temperature difference might potentially affect the qPCR results (24). It requires accurate measurement of the expression level of lncRNAs to investigate their distinctive roles in carcinogenesis. It is required that the annealing temperature be optimized in qPCR experiments to quantify accurate and reliable lncRNA transcripts.

Table 3. *Calculated TM and Optimum Annealing Temperatures*

lncRNA	Optimum TM	Calculated TM	Differences
H19	60	61.9	-1.9
CCAT1-b	60	53.4	6.6
HOTAIR	60	59.9	0.1
NEAT1	60	60.6	-0.6
MALAT1	60	59.6	0.4
PVT1	62.4	59.3	3.1
GAS5	58	61.3	-3.3
BANCR	62.4	59.8	2.6
CCAT1-a	56.7	50.6	6.1
UCA1	56.7	58.2	-1.5
HULC	58	58.7	-0.7
MEG3	56	63.5	-7.5

4. DISCUSSION

While numerous primer assays have been developed to analyze lncRNA expression profiles in different cancers, experimental results tend to differ from predicted theories. The method most popularly employed to predict optimal annealing temperatures is computational prediction according to GC content, but optimal PCR efficiency is usually attained by optimizing variables including RNA secondary structures and minor primer design specifics (25).

In the literature, HOTAIR, ANRIL, CCAT1, H19, PVT-1, and MEG3 annealing temperatures were published as 55°C using different target-specific primer sequences (26). Another study for MALAT1 and UCA1 at 55°C, for HOTAIR and PVT1 at 57°C, and for CCAT1 at 60°C annealing temperature was published (27).

Moreover in the literature, for BANCR expression analysis 57°C (28), for HULC expression analysis 52°C (29), and for GAS5 expression analysis 62.5°C set as annealing temperature (30) have been reported.

Accurate determination of annealing temperatures for specific lncRNA targets is necessary to enhance the sensitivity and accuracy of assays in cancer research. We know that lower and non-optimized annealing temperatures could lead to non-target amplifications. Thus optimization of PCR conditions is essential for accurate analysis of gene expression and copy number changes. Validated methods not only improve the reliability of PCR-based assay data but also enhance our understanding of the roles of the target genes in cancer biology and their potential as therapeutic targets.

In this study, we performed annealing temperature optimization for several cancer-related lncRNAs. Optimization of the annealing temperatures for lncRNAs has the importance for improving the accuracy of biomarker detection and for developing the most suitable personalized treatment strategies tailored to individual patients.

5. CONCLUSION

As a conclusion, long non-coding RNAs (lncRNAs) play a crucial role in regulating oncogenic processes in cancer due to their involvement in several epigenetic mechanisms. Their importance as biomarkers and therapeutic targets has led to a significant increase in lncRNA research exponentially over the last several years.

Accurate measurement of lncRNA expression by qPCR, which is considerably dependent on optimization of experimental conditions such as annealing temperature, is crucial for obtaining precise and reliable results.

Our findings establish a foundation for future studies, contributing to the growing body of knowledge and guiding additional research in the field of lncRNA-targeted studies.

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