The Effective Small Interfering RNA of PRNP gene is Screened Out by Real-time Quantitative PCR Based on MIQE Guidelines

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Abstract

MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines are the gold standards for the experimental operation of real-time fluorescent quantitative PCR (qPCR). In this study, through qPCR based on MIQE guidelines we would select the effective sequence of small interfering RNA (siRNA) of PRNP (prion protein) gene. The integrity of RNA extracted from the cells via referring to MIQE was high, and the qPCR primer designed was practicable, which led to that the melting curve of PRNP gene was single- not multi-peak, and that the slope of the standard curve of exogenous reference gene luciferase was close to the theoretical value. Moreover, the use of exogenous reference gene reduced the differences between samples and enabled us to screen out the most effective sequence of PRNP siRNA. Finally, we showed that the siPRNP1259 significantly decreased its expression and reduced the ability of osteosarcoma cell colony formation. Taken together, our results indicated that MIQE guidelines could facilitate a more effectively screening of the small interference fragments of genes by qPCR.

Keywords

MIQE, qPCR, PRNP, siRNA, External reference gene.

1. Introduction

In the past, a lot of literatures about real-time quantitative fluorescent reverse transcriptase PCR (qPCR or RT-qPCR) assay lacked sufficient experimental details, which seriously hindered the readers from evaluating the quality and repeatability of the experimental data[1]. In 2009, MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines was reported for the first time by Bustin SA et al., which offered the minimum information necessary for evaluating qPCR experiments and then considered as the gold standard for qPCR practice[5]. In cellular biology, a very meaningful application of MIQE guidelines is to guide the screening of effective sequence of small interfering RNA targeting the specific gene, which will allow qPCR results more reliable and accurate[2,3,4].

MIQE guidelines are divided into experimental design, sample preparation, nucleic acid extraction, reverse transcription, oligonucleotides and target gene information for qPCR purpose, qPCR protocol, qPCR verification and data analysis[5]. To put it simply, in terms of experimental design, MIQE requires that each experimental group and control group must have three independent biological replications, and each experimental sample with biological replications must have more than three technical replications[6]. In nucleic acid extraction process, in order to ensure the completeness of total RNA extraction, each sample should be taken to agarose gel electrophoresis. Before the fluorescence quantitative experiments, the sample should undergo genomic DNA contamination removal processing[7,8]. The standard curve was made by cDNA gradient dilution to detect whether

the amplification efficiency of the target primers, internal reference gene or external reference gene was between 90-110%, whether the fusion curve was multi-peak, and the single peak of the dissolution curve was usually the performance of primers' specific amplification[9,10,11]. In most cases, the reference gene is the housekeping gene. However, it is not accurate to use the housekeping gene as a reference gene without proving that the target gene has no relationship with the housekeping gene, so the use of external reference genes for qPCR has become an increasingly reliable experimental method[12,13].

Prions are divided into PRNP and PRND, which are mainly involved in neurodegenerative spongiform diseases [14], have been recently confirmed to be involved in anti-apoptosis and closely related to tumorigenicity, proliferation and metastasis[15,16]. The expression level of PRNP (the gene encoding PrPc) may have prognostic value for colorectal cancer. In addition, it has been recently found that this gene is overexpressed in osteosarcoma, which may play a role in promoting the growth and proliferation of osteosarcoma cells[14]. Overexpression of PrPc (PRNP) is a prognostic marker for disease recurrence and may become a new target for anticancer therapy[17]. Another prion protein, PRND, found mainly in the testes, is a membrane glycosylphosphatidylinositol immobilized glycoprotein. PRND gene encodes about 20 kbp bases downstream of the prion gene on chromosome 20, similar to prion proteins in biochemistry and structure[18].

In this study, MIQE standards were used to guide the screening of effective siRNA of PRNP gene by qPCR and we identified that siPRNP 1259 significantly decreased the expression of PRNP.

2. Materials and Methods

2.1 Cell culture and siRNA transfection

Cells were cultured in humidified chambers at 5% CO2 and 37°C. U2-OS (ATCC) cell lines were grown in McCoy's 5A Modified Media Formulation (Sigma, USA) supplemented with 10% fetal bovine serum(Gibco, USA), the PRNP siRNA (GenePharma, Su Zhou, China) were transfected into cells with Lipofectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The sequences of PRNA siRNA were shown as below:

PRNP781 sence : 5'CAUCAUACAUUUCGGCAGUTT 3'

PRNP781 antisence: 5'ACUGCCGAAAUGUAUGAUGTT 3'

PRNP 902 sence: 5'GACUGCGUCAAUAUCACAATT 3'

PRNP902 antisence: 5'UUGUGAUAUUGACGCAGUCTT 3'

PRNP1259 sence: 5'CAAUACCCUUGGCACUGAUTT 3'

PRNP1259 antisence: 5'AUCAGUGCCAAGGGUAUUGTT 3'

PCR primers for external reference genes:

luciferase forward:

5'AAATTAATACGACTCACTATAGGGATGGAAGACGCCAAAAACATAAAG 3'

luciferase reverse:

5'TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTCGAGTTTGGACTTTCCGC 3'

PCR system:

Component	Amount
Template plasmid	200 ng
Primer forward	1 μL
Primer reverse	1 μL
Primer Master Mix (2x)	25 μL
ddH_2O	Add to 50 μL

And then, PCR reactions were following procedure:

Temperature	Time	Cycle
95℃	5 min	1
95℃	30 sec	35
60℃	30 sec	
72℃	30 sec	
72℃	5 min	1

2.2 Gradient dilution of cDNA template to make standard curve

After removing genome contamination, the RNA samples were reversely transcribed into cDNA, and the reverse transcriptional system was 20 μL. Then we took four 0.5 mL EP tubes, marked them as 0.1, 0.01, 0.001, 0.0001 respectively and added 18 μL ddH2O into them. Next, we transferred 2 μL undiluted cDNA sample to 0.1 tube for 10 fold dilution, took 2 μL 0.1-fold cDNA sample to 0.01 EP tube for another 10 fold dilution, successively diluted the undiluted cDNA sample into 5 gradients of 1, 0.1, 0.01, 0.001 and 0.0001. The CT values of target gene and reference gene in the samples with these 5 gradients were detected by qPCR, and the standard curves were made. The standard amplification efficiency of qPCR was 100%, and the slope of the standard curve was -3.4.

2.3 qPCR

Total RNA was isolated from the above cells using TRIzol® reagent (TransGen, Bei Jing, China). RNA integrity was detected using agarose gel electrostatin. DNaseI was used to remove genomic contamination prior to reverse transcription. Complementary DNA was prepared with 1 μg of total RNA for mRNA quantification using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, China). RNA expression was measured by Stepone plus (ABI, USA) and SYBR® Green (TOYOBO, Japan). The primers were purchased from TSINGKE Company (Guangzhou, China).

The qPCR primer sequences of this study were as follows:

PRNP forward: 5’CGAGTAAGCCAAAAACCAACAT 3’

PRNP reverse: 5’TAGTACACTTGGTTGGGGTAAC 3’

GADPH forward: 5’GACAGTCAGCCGCATCTTCT 3’

GADPH reverse: 5’GCGCCCAATACGACCAAATC 3’

ACTB forward: 5’CACCATTGGCAATGAGCGGTTC 3’

ACTB reverse: 5’ AGGTCTTTGCGGATGTCCACGT 3’

luciferase forward: 5’GCAGGTCTTCCCGACGATGA 3’

luciferase reverse: 5’TCCTCCGCGCAACTTTTTCG 3’

PCR system:

Component	Amount
Template cDNA	2 μL
Primer forward	1 μL
Primer reverse	1 μL
SYBR Green Mix (2x)	10 μL
ddH_2O (RNase Free)	Add to 20 μL

Step One plus Program module:

Temperature	Time	Cycle
95℃ 95℃ 60℃ 72℃	1 min 15 sec 15 sec 45 sec	1 40 40 40
55℃ 95℃	The dissolution curve section is drawn from 55℃, and each gradient increases by 0.3℃ until it is completed at 95℃.	

2.4 Colony formation assay

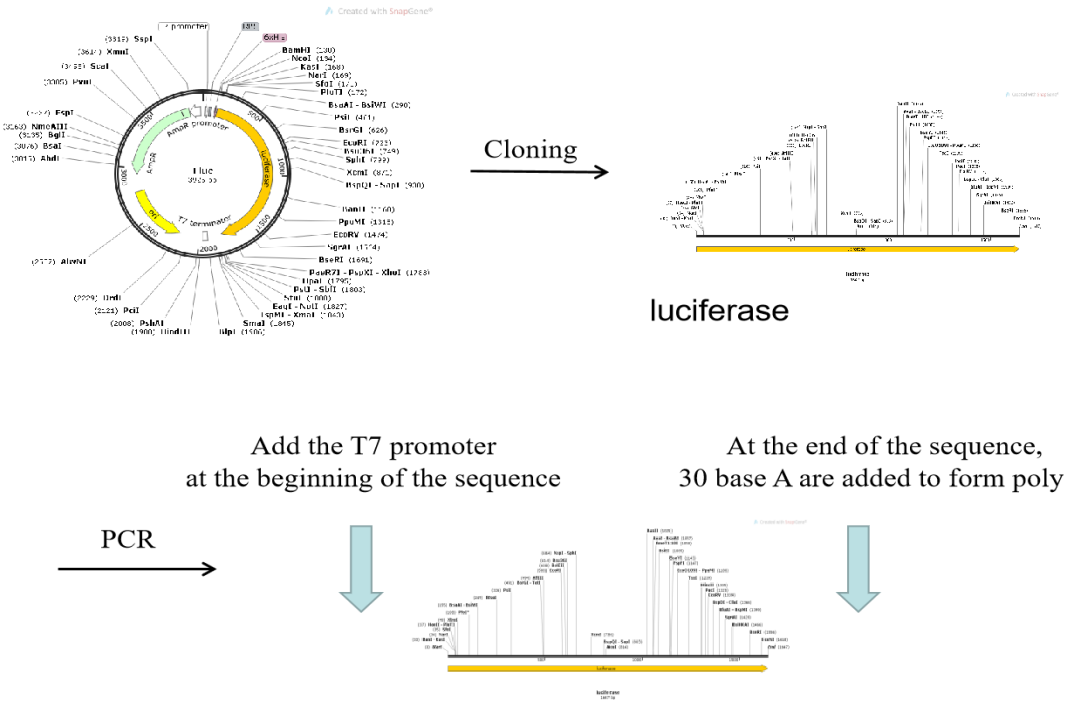
Cells were transfected with PRNP siRNA. After 24 hours, cells were digested with trypsin and replanted at a density of 2500 cells/10 cm^2. After 2 weeks of incubation, cells were fixed with methanol and then stained with 0.1% crystal violet. This experiment was repeated in triple.

3. Results

3.1 Preparation of luciferase, the external reference gene of qPCR

In order to improve the accuracy and reliability of qPCR experimental data, the use of exogenous reference gene, not expressed in human body, was introduced. Here, the cDNA of luciferase was supposed to be the external reference, which was amplified by PCR from a plasmid containing luciferase gene. The primers used for PCR to clone luciferase were added with T7 promoter sequence at the 5 'end and 30 A bases at the 3' end to recognize Oligo DT for reverse transcription. After a large number of luciferase cDNA were amplified by PCR, the samples were separated by DNA agarose gel electrophoresis. Then agarose gel was harvested for purification and recovery, and the luciferase cDNA were transcribed into RNA by *in vitro* transcription kit. The results of agarose gel electrophoresis showed that the linear foreign reference gene luciferase was successfully constructed (Figure 1).

A



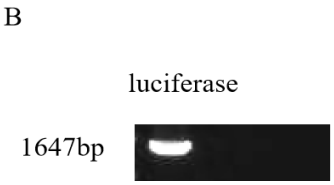


Figure 1 Preparation of exogenous reference gene luciferase

A. Flow chart of construction of exogenous reference gene luciferase. B. Electrophoretic result of successful construction of luciferase.

3.2 The RNA samples extraction based on MIQE had high integrity

According to the MIQE, the A260/A280 ratio must be measured in a buffer with neutral pH, but if the nucleic acid is to be used for quantitative analysis, such measurement is not sufficient[5]. Therefore, it is necessary to detect the integrity of RNA samples by agarose gel electrophoresi. In order to select effective siRNA of PRNP gene, three siRNA of PRNP and their negative control were transfected into the tumor cells respectively. 48 hours later, cells were scraped from the culture dish and counted. Appropriate amount of exogenous reference RNA was added according to the proportion of 1 ng external reference RNA per 1 million cells. The total RNA was extracted by TRIzol® reagent and the quality of RNA was detected by agarose gel electrophoresis. As shown in Figure 2, there were three bands composed of 28sRNA, 18sRNA and 5sRNA/5.8sRNA in the RNA sample, and the ratio of 28sRNA to 18sRNA was 2:1. The results showed the high integrity of RNA sample extraction and no degradation.

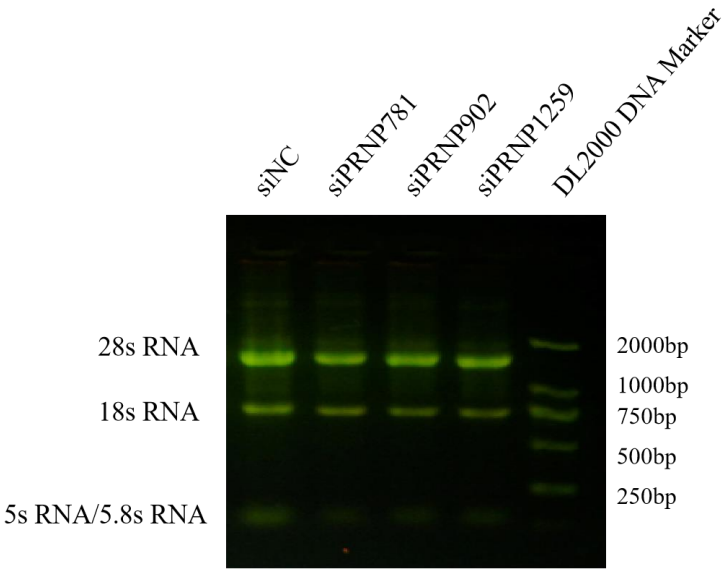


Figure 2 The identification of the quality of total RNA by agarose gel electrophoresis

3.3 High specificity of qPCR primers identified according to MIQE

In the MIQE guidelines, qPCR primer design based on one or at most two exons of mRNA is not enough to detect the expression level of specific genes. Therefore, the sequence information for primers must be provided together with the specific evaluation of SNPs (single-nucleotide polymorphism) locations recorded in known splice variants and SNPs databases[5]. Collectively, the design of qPCR primers should span introns as more as possible and the qPCR primers of the target gene and reference gene must be verified first by qPCR experiment. As shown in Figure 3A, the melting curve of the primer of PRNP was single-peak, which suggested that the amplification product of the primer was single and had good specificity. According to MIQE, gradient dilution cDNA template to make standard curve is an important way to check the amplification efficiency of primers.

The result in Figure 3B showed that the R^2 of standard curve is larger than 0.95, amplification efficiency is within 90% ~ 110%, and slope is close to the theoretical value of 3.4, which fully illustrated that the primer design of exogenous reference gene luciferase guided by MIQE was reliable and that the amplification product has high specificity (Figure 3).

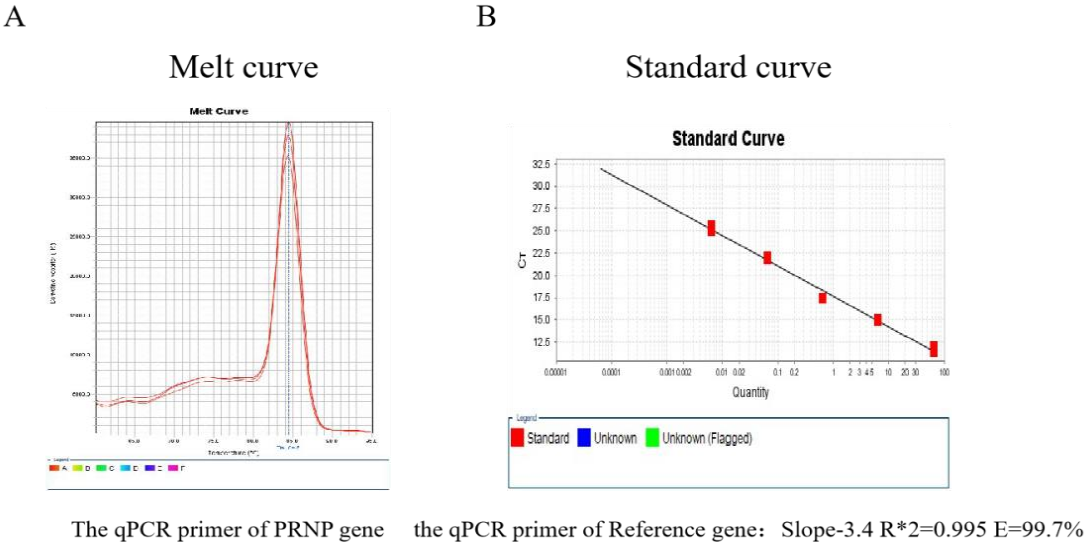


Figure 3 The identification of specificity of primers of PRNP and luciferase

A. The melt curve of PRNP primer. B. The standard curve after cDNA amplification by gradient of the luciferase (exogenous reference gene) primers.

3.4 The standard deviation of reference gene of different samples under the MIQE standard was small

Samples of PRNP siRNA-transfected cells and the control cells were treated according to MIQE guidelines, and then the exogenous reference gene luciferase was added to the samples according to the proportion of 1 ng luciferase RNA per 1 million cells. In the traditional qPCR experiment, housekeping genes (GADPH, ACTB, 5.8sRNA, etc) were used as the reference genes for quantitative analysis, but without determining whether the target gene (PRNP) is related to the housekeping genes, the results of this method are possibly inaccurate. As shown in Table 1, by comparing the CT values of internal and external reference genes in the control group (siNC) and knockdown groups (siPRNP781, siPRNP902, siPRNP1259), it is found that under the same experimental conditions, the standard deviation of CT value of exogenous reference gene luciferase detected according to MIQE is much smaller, suggesting that the qPCR data under the guidance of MIQE is more accurate.

Table 1 The standard deviation of CT value of reference gene between different samples

Samples	Traditional qPCR experiment (CT value of Actin)	The standard deviation of CT value of ACTB	The qPCR experiment base on MIQE (CT value of luciferase)	The standard deviation of CT value of luciferase
siNC	22.63 22.68 22.50	0.092	16.94 16.90 16.89	0.022
siPRNP781	18.97 18.80 19.51	0.371	16.78 16.83 16.92	0.058
siPRNP902	16.50 16.34 16.52	0.096	16.90 16.69 16.78	0.086
siPRNP1259	22.86 22.52 21.55	0.676	16.92 16.89 16.92	0.014

3.5 Effective siRNA of PRNP gene was screened out under the MIQE standard

Currently, PRNP is mainly reported to regulate the proliferation of osteosarcoma cells[14], but there are few reports about its other functions in cells and its effective siRNA. Above, we used the melt

curve and standard curve to determine the specificity of the qPCR primers of PRNP gene. Then we designed three siRNA targeting PRNP and their targeting sequences on PRNP was shown in materials and methods, The PRNP siRNA and its control siRNA were transiently transfected into the U2-OS cells (osteosarcoma cells). After 48 hours of expression, the cells were harvested for counting. According to the above proportion, an appropriate amount of RNA of luciferase gene was added to the cells, and then the total RNA was extracted. Based on the requirements of MIQE, we subsequently remove the genome contamination in the samples using DNase before reverse transcription. After RNA reverse transcription, qPCR assay was carried out. The experiments included three independent biological repeats, and each contained three same samples. The result in Figure 4 showed that siPRNP1259 effectively knocked down the expression of PRNP gene in U2-OS cells.

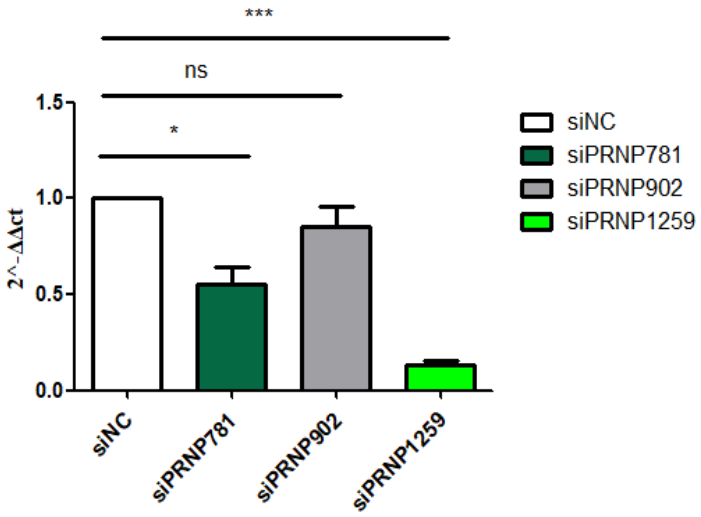


Figure 4 The effect of three siRNAs of PRNP on its expression

3.6 siPRNP1259 decreased the ability of cells to form colony through reducing the expression of PRNP

It has been reported that PRNP gene is overexpressed in osteosarcoma and may promote the growth and proliferation of osteosarcoma[14]. Therefore, we tested whether knockdown of PRNP with siPRNP1259 would affect the colony formation of osteosarcoma cells. To this end, siPRNP1259 was transfected into osteosarcoma U2-OS cells to observe its effect on the number of colony formation. Two weeks later, the results showed that the number of colony decreased after PRNP expression was knocked down (Figure 5), which suggested that PRNP knockdown inhibited the ability of colony formation of osteosarcoma cells, and also suggested that it was feasible and reliable to guide the screening of effective siRNA according to MIQE guidelines.

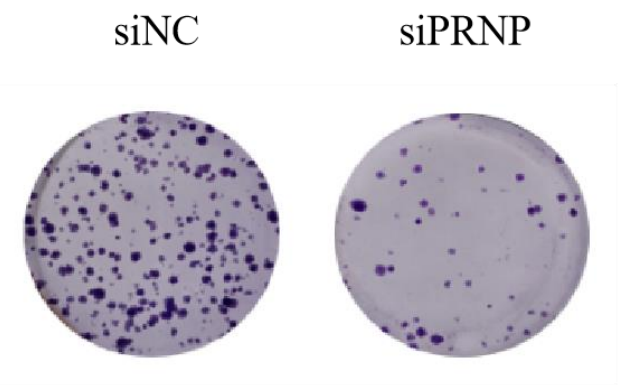


Figure 5 Knockdown of PRNP inhibited the ability of colony formation of U2-OS cells

4. Conclusion

In many published literatures, due to the lack of detailed information of qPCR experiments, there are many uncertainties and low repeatability in the experimental results of qPCR. For example, the storage and preparation of samples, if the quality of nucleic acid is not qualified, the experimental results will be unstable, the improper selection of several pairs of primers involved in qPCR will lead to low detection efficiency, and inappropriate data and statistical analysis will mislead the experimental results[19,20], so the emergence of MIQE guidelines has practical scientific significance. It is precisely because of the standardized experimental operation and experimental environment required by the guidelines that the repeatability and accuracy of qPCR results are greatly improved [21].

In this study, MIQE standard was used to guide qPCR assay to screen valid sequence of siRNA of PRNP genes. From the success of construction and *in vitro* reverse transcription of exogenous reference gene, to the successful application of exogenous reference gene in qPCR experiments, as well as the small standard deviation of CT value of exogenous reference gene among different samples, it showed that MIQE guidelines ensured the reliability of qPCR experiments. It was reported that PRNP gene was over expressed in osteosarcoma[14], suggesting that PRNP gene might promote the growth of osteosarcoma. In agreement with this, our result showed that PRNP gene knockdown inhibited the colony formation ability of osteosarcoma cells, indicating that PRNP played a role in promoting cell growth. This also proved the effectiveness of siRNA screened under the guidance of MIQE standards. In our previous experiments, we screened siPRNP902 from three siRNAs by traditional qPCR, but it had no effect on the ability of osteosarcoma cell colony formation. Therefore, under the guidance of MIQE standards, we could better screen out the effective siRNA.

Acknowleagements

This work was supported by the Natural Science Foundation of Guangdong Province (2018A030313544, 2019A1515010182), and the Guangzhou Science and Technology Project (201707010263).

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