วารสาร
เทคนิคการแพทย์และกายภาพบำบัด
JOURNAL OF MEDICAL TECHNOLOGY AND PHYSICAL THERAPY

นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

ปัจจัยที่มีผลต่อความไวในการตรวจวิเคราะห์ Dual Luciferase Reporter Assay เพื่อตรวจหาการควบคุมการแสดงออกของยีนในเซลล์ 293T

วิภาพร วงค์เฟียง[1,2] กรรณิการ์ กัวหา[3] อมรรัตน์ จำเนียรทรง[2,3] และ ชาญวิทย์ ลีลายุวัฒน์[2,3]*

Received: *February 6, 2018*
Revised: *March 8, 2018*
Accepted: *March 11, 2018*

บทคัดย่อ

Luciferase reporter เป็นหนึ่งในวิธีการที่ได้รับความนิยมมากที่สุดสำหรับการศึกษาการควบคุมการแสดงออกของยีน เนื่องจากวิธีการตรวจวัดง่ายสะดวกและนำไปประยุกต์ใช้ได้อย่างกว้างขวาง อย่างไรก็ตามข้อจำกัดของวิธีนี้คือความแปรปรวนของการตรวจวัดที่มีผลต่อความคงที่และความไวทำให้ส่งผลต่อค่านัยสำคัญทางสถิติ ดังนั้นในการศึกษานี้จึงได้ตรวจสอบปัจจัยที่ส่งผลต่อการตรวจหาการควบคุมที่ขั้นตอนหลังการถอดรหัสด้วยวิธี dual luciferase reporter เพื่อให้ได้ความไวและความคงที่ที่ดีที่สุด เริ่มศึกษาโดยการสร้างพลาสมิด luciferase reporter ที่มี 3'UTR แบบ wild type และสร้าง 3'UTR แบบ mutants 2 ชนิด ที่มีการกลายพันธุ์ ณ ตำแหน่งการจับของไมโครอาร์เอ็นเอ พลาสมิดแต่ละชนิดถูกเจือจางให้ได้ความเข้มข้น 500 ng 50 ng และ 25 ng และถูกถ่ายโอนเข้าสู่เซลล์ 293T พร้อมกับพลาสมิด renilla reporter ด้วยอัตราส่วนระหว่าง firefly/renilla เป็น 10:1 และตรวจวัด luciferase activity นอกจากนี้ยังศึกษาเปรียบเทียบจำนวนตำแหน่งที่ mutants เพื่อประเมินว่ามีผลต่อความไวของ luciferase หรือไม่ พบว่าความเข้มข้นของพลาสมิด luciferase reporter ที่ 50 ng ให้ความไวสูงสุดและสอดคล้องกับการประเมินการแสดงออกของ luciferase ในการควบคุมผ่าน 3'UTR ที่มีค่านัยสำคัญทางสถิติสูงสุดเมื่อเปรียบเทียบระหว่าง wild type และ mutants นอกจากนี้ยังพบว่าจำนวนของตำแหน่งการกลายพันธุ์มีผลต่อความไวของการตรวจวิเคราะห์ด้วย ดังนั้นการหาความเข้มข้นที่เหมาะสมของพลาสมิดดีเอ็นเอจึงเป็นสิ่งสำคัญ และจำเป็นอย่างยิ่งในการเพิ่มความไวของการตรวจวิเคราะห์ ข้อมูลนี้จะเป็นประโยชน์สำหรับนักวิจัยและนักวิทยาศาสตร์ที่ใช้ dual luciferase reporter ในการตรวจสอบการควบคุมการแสดงออกของยีนที่ขั้นตอนหลังการถอดรหัสดีเอ็นเอ

คำสำคัญ: การควบคุม 3' untranslated region, การแสดงออกของยีน, ระบบ Luciferase reporter

[1] นักศึกษาปริญญาเอก สาขาวิชาชีวเวชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยขอนแก่น
[2] ศูนย์วิจัยและพัฒนาการตรวจวินิจฉัยทางห้องปฏิบัติการทางการแพทย์ (ศ.ว.ป.) คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น
[3] กลุ่มวิชาภูมิคุ้มกันวิทยาคลินิกและวิทยาศาสตร์การบริการโลหิต คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น
*ผู้รับผิดชอบบทความ

วารสาร
เทคนิคการแพทย์และกายภาพบำบัด
JOURNAL OF MEDICAL TECHNOLOGY AND PHYSICAL THERAPY

นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

Factors affecting sensitivity of dual luciferase reporter assay for detection of gene regulation in 293T cells

Wipaporn Wongfieng [1,2], Kunnika Kuaha[3], Amonrat Jumnainsong[2,3] and Chanvit Leelayuwat [2,3]*

Abstract

The Luciferase reporter gene is one of the most popular methods for studying gene expression and regulation because of its convenience, simple and the broad dynamic range of applications. However, a limitation of this method is a high variability of measurement affecting consistency and sensitivity leading to varied statistical significances. Here, we investigated the factors affecting the sensitivity and consistency of detection of posttranscriptional regulatory sequences in the dual luciferase reporter system. We generated firefly luciferase reporter plasmids containing 3'untranslated region (UTR) of a gene. One construct contained a wild type and 2 constructs contained mutant binding sites for miRNAs. Each plasmid construct was diluted to the concentration of 500 ng, 50 ng and 25 ng and was co-transfected with the renilla reporter plasmid to 293T cells using the ratio between firefly/renilla plasmids of 10:1. Then cells were measured for dual luciferase activities. We also compared luciferase activities regarding the number of mutation sites to assess whether they affected the detection of luciferase sensitivity. Our results indicated that at the 50 ng concentration of the reporter plasmid gave the highest sensitivity and consistency for evaluation of luciferase expression via 3'UTR regulatory region with the highest statistical significance when compared to those of wild type and mutant sequences. Moreover, the numbers of mutation sites also had the effect on sensitivity of the luciferase reporter assay. Thus, optimization of plasmid DNA concentration is essential to improve the sensitivity. This information would be useful for researchers and scientists who employ the dual luciferase reporter system in investigating the posttranscriptional regulation of sequences.

Keywords: 3' Untranslated region regulation, Gene expression, Dual luciferase reporter system

---

[1]Ph.D candidate of Biomedical Sciences Program, Graduates School of Khon Kaen University

[2]The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University

[3]Department of Clinical Immunology and Transfusion Sciences, Faculty of Associated Medical Sciences, Khon Kaen University

*Corresponding author: (e-mail: chanvit@kku.ac.th)

Introduction

Regulation of gene expression is a process of cellular function to control the amount and manner of protein expression which can be up-regulated or down-regulated[1]. Regulation of gene expression assays were widely used in identification of gene expression/regulation mechanisms both in a cell and tissue. Recently several techniques have been used to detect the gene expression regulation such as to investigate transcriptional rates and regulatory sequences using the nuclear run-off transcription assays[2, 3], to characterize DNA-protein interactions by DNase I foot printing analysis and mobility shift assays[4]. In addition, to evaluate the role of specific regulatory regions of DNA sequences or investigate factors and mechanisms affecting gene expression often required the reporter gene assay[5].

The reporter gene assay has several systems based on the detectable reporter genes. Chloramphenicol acetyltransferase (CAT) is the first gene reporter used to investigate regulation at transcriptional level in mammalian cells[6]. However this assay is limited due to its dependence on the use of radioisotopes[7]. The alkaline phosphatase (AP) reporter gene system consists of AP which requires optimal activity at alkaline pH. Thus, this system is limited because AP enzyme is expressed practically in all cell types[8]. β-galactosidase (β-gal) is a reporter gene assay that was designed to particularly evaluate β-galactosidase activity of bacteria, thus, it is not optimal for the eukaryotic system[9]. The green fluorescence protein (GFP) reporter system is commonly used to evaluate gene regulation including the study pattern of protein localization and intra-cellular trafficking[10]. It is not optimal for a quantitative detection. The luciferase reporter assay now is one of the most commonly used gene reporter system that emits light generated by chemical reaction with oxygen and a substrate and can be quantitative. The most common luciferase enzymes in the eukaryotic system are firefly luciferase and renilla (sea pansy) luciferase. Differences between these luciferases are that renilla luciferase does not require ATP and uses a different substrate to generate chemical reactions[11, 12]. The dual luciferase reporter assay is one of the luciferase reporter assay systems which is performed by sequentially measuring the firefly and *renilla* luciferase activities of the same sample based on the different properties of both enzymes. The results expressed as the ratio of firefly to *renilla* luciferase activities[13]. Generally, one luciferase acts as an experimental reporter to indicate the biological expression while the other is an internal control used to normalize the data. This is recommended as a good choice for investigating a regulation of protein expression via miRNA binding at the regulatory regions. Because of the binding of a candidate miRNA to its regulatory sequence on the mRNA target will suppress the production of reporter protein. Thus, its activity or expression is reduced which can be measured via instrument and compared to a negative control. Although luciferase reporter system is the most commonly used to study regulation of gene expression because of its convenience, simple and the broad dynamic range of applications[14], a limitation of this method is a high variability of measurement affecting consistency and sensitivity leading to varied statistical significances[15]. Thus, it is essential to optimize the reporter plasmid conditions for every cell type

because each cell type is different resulting in different efficiency of transfection[16].

In this report, we investigated the factors affecting the dual luciferase reporter system regarding sensitivity by optimization of DNA concentration of luciferase reporter. Firstly, the regulatory sequence of a gene of interest was inserted into down -stream of the luciferase gene in a reporter construct. Then, different concentrations of these vectors were transfected into 293T and luciferase activities were measured. We also compared luciferase activities regarding the varied number of mutations on regulatory sequences to assess whether the differences had any effect on luciferase sensitivity. Our result indicated that both concentration and number of mutations had effect on sensitivity and consistency of the dual luciferase reporter assay.

Materials and Method

Cell line

293T cells (human embryonic kidney) were cultured in Dulbecco's Modified Eagle Medium (DMEM)(Invitrogen, Carlsbad, CA, USA) supplemented with 1%penicillin-streptomycin (Sigma, St Louis, MO, USA) and 10% fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA) and incubated at 37°C in 5% CO_2.

Plasmid construction

Luciferase reporter vectors, the firefly luciferase vector (pcDNA3.1-Zeo(+)Pp) and the renilla luciferase vector (pRL-SV40) were kindly provided by Dr Yong Sun Lee, the University of Texas Medical Branch, USA. The 1,250 bp fragment of the wild-type 3´UTR of the MICB gene was amplified from genomic DNA and was inserted into downstream of the luciferase gene in the reporter vector (pcDNA3.1-Zeo(+)Pp)using restriction enzyme, BamH I and Not I[17]. Site directed mutagenesis was used to generate specific mutation miRNA binding sites, using the designed primers purchased from Bio basic Inc., Markham, Canada. Both the 3 and 6 specific mutation sites were generated, pMICB_3 mut and pMICB_6 mut, respectively[17]. Further, the inserts and their proper orientations were confirmed by DNA sequencing (Macrogen, Seoul, Republic of Korea).

Luciferase reporter transfection

The firefly luciferase reporter used in this study was pcDNA3.1-Zeo(+)Pp (Promega, Madison, WI, USA) which contained regulatory sequences of 3'UTR of MICB (pMICB_3U), 3'UTR with 3 mutation sites (pMICB_3 mut) and 6 mutation sites (p3UTR_6 mut) as well as the luciferase reporter vector alone (empty vector). Their concentrations were varied at 500 ng, 50 ng and 25 ng. The renilla luciferase vector derived from pRL-SV40 (Promega) was used for normalization. To make the ratio between firefly luciferase and renilla luciferase stable as 10:1 according to the manufacturer's instruction, the concentration of renilla luciferase vector was varied at 50 ng, 5 ng and 2.5 ng, accordingly. These plasmids were co-transfected into the 293T cells. After 24 hrs transfection, each transfection with varied concentrations was measured for firefly and renilla luciferase activities, accordingly.

Luciferase reporter analysis

The emitted light of firefly and renilla luciferases was measured by the dual-luciferase reporter assay kit (Promega) with the GloMax® 20/20 luminometer machine (Promega). Briefly, cells were collected from a 24-well plate, lysed

by passive lysis buffer provided in the kit. Then, the cell lysates were transferred to 1.5 ml tubes and the substrate solution (Luciferase Assay Reagent II (LAR II)) was added before measuring the firefly luciferase activities. Finally, stop the reaction of firefly by adding the stop reagent (Stop & Glo® Reagent) and then, measured renilla luciferase activities in the same sample. The percentages of luciferase inhibitions were calculated via several normalizations. Firstly, calculations of relative luciferase (RL) by firefly luciferase activities from wild-type, mutants or empty plasmid were normalized with renilla luciferase activity. Secondly, calculations of relative luciferase ratio (RLR) by relative luciferases (RL) of wild-type or mutants were normalized with relative luciferases (RL) of the empty plasmid. Finally, calculations of percent inhibition of luciferase by the formula: (1- RLR) x 100 were performed.

Statistical analysis

The data were tested for normal distributions by Shapiro-Wilk (SPSS inc., Chicago, IL, USA). Then, significant differences between the wild-type and the mutant groups were analyzed by Student's t-test via the GraphPad Pro. Prism 5.0 (GraphPad, San Diego, CA, USA). The data were shown as means±S.E.M. The *p*–values less than 0.05 were considered as statistical significance. All data were represented of at least 3 independent experiments.

The receiver-operating characteristic (ROC) curves were generated to estimate the sensitivity for detection of luciferase reporter assay by using relative luciferase ratio of each condition compared with the negative control. The cut-off value for the luciferase expression was determined by the score of specificity, sensitivity, positive likelihood ratio (+LR), negative likelihood ratio (-LR) and Youden's index (YI) for each condition. The data were presented as means ±S.D.

Results

1. Effect of different concentrations of reporter plasmids containing 3'-UTR on luciferase expression in the 293T cells.

To investigate the optimal reporter plasmid concentrations on the effect of 3'-UTR in the luciferase reporter system, each varied concentration was co-transfected with renilla reporter plasmid into 293T cells with the ratio of 10:1. After complete transfection, cells were measured for dual luciferase activities by the dual luciferase reporter assay kit. The law data of luciferase expressions were shown as Relative Light Units (RLU). Expectedly, the highest dual luciferase expression levels (10^9 RLU) were obtained from the concentration of 500 ng of luciferase reporter vector and followed by 50 ng (10^6 RLU) and 25 ng (10^4 RLU), respectively (Figure 1A, B and C). However, the highest luciferase expression levels (500 ng) had higher variability of both firefly and renilla luciferase activities **(Figure 1A).** In contrast, the other groups (50 ng and 25 ng) had lower variabilities **(Figure 1B and C)**.

To evaluate the effect of different mutated miRNA binding sites containing 3'-UTR on luciferase expression in the 293T cells, we compared luciferase activity of wild type with 3 or 6 specific mutation sites. The wild-type reporters should have activity less than those of the mutated reporters. Our data showed that at the concentration of 500 ng of reporter plasmid, there was no significant difference of percentages

of inhibition of luciferase activities between the vector containing the wild-type 3'-UTR (p3UTR_WT) (42 %) and vector containing the 3 (p3UTR_3mut) (30 %) or 6 (p3UTR_6mut) (23 %) specific mutation sites **(Figure 1D)**. However, the significance was found at the 50 ng concentration of wild-type 3'-UTR (p3UTR_WT) (60%). Interestingly, luciferase activities were recovered when they were 3 (p3UTR_3 mut) and 6 (p3UTR_6 mut) mutated specific binding sites. Inhibition percentages of luciferase expressions were reduced to 30% and 5%, respectively and were differently significant when compared with the vector containing wild-type 3'-UTR (p3UTR_WT) (black bar graph of **Figure 1D**). At 25 ng, luciferase activities were repressed in vector containing wild-type 3'-UTR (p3UTR_WT) (40%) but not significantly different from the 3 mutation binding sites (35%) (p3UTR_3 mut). However, significant differences of percent inhibition of luciferase expressions between the wild-type 3'-UTR (p3UTR_WT) and the 6 mutation binding sites (p3UTR_6 mut) was observed (slant bar graph of **Figure 1D**). These results indicated that co-transfection between 50 ng of firefly reporter plasmid with 5 ng of renilla reporter plasmid was the best concentration for evaluating effect of 3'-UTR of MICB on luciferase expression in 293T cells.

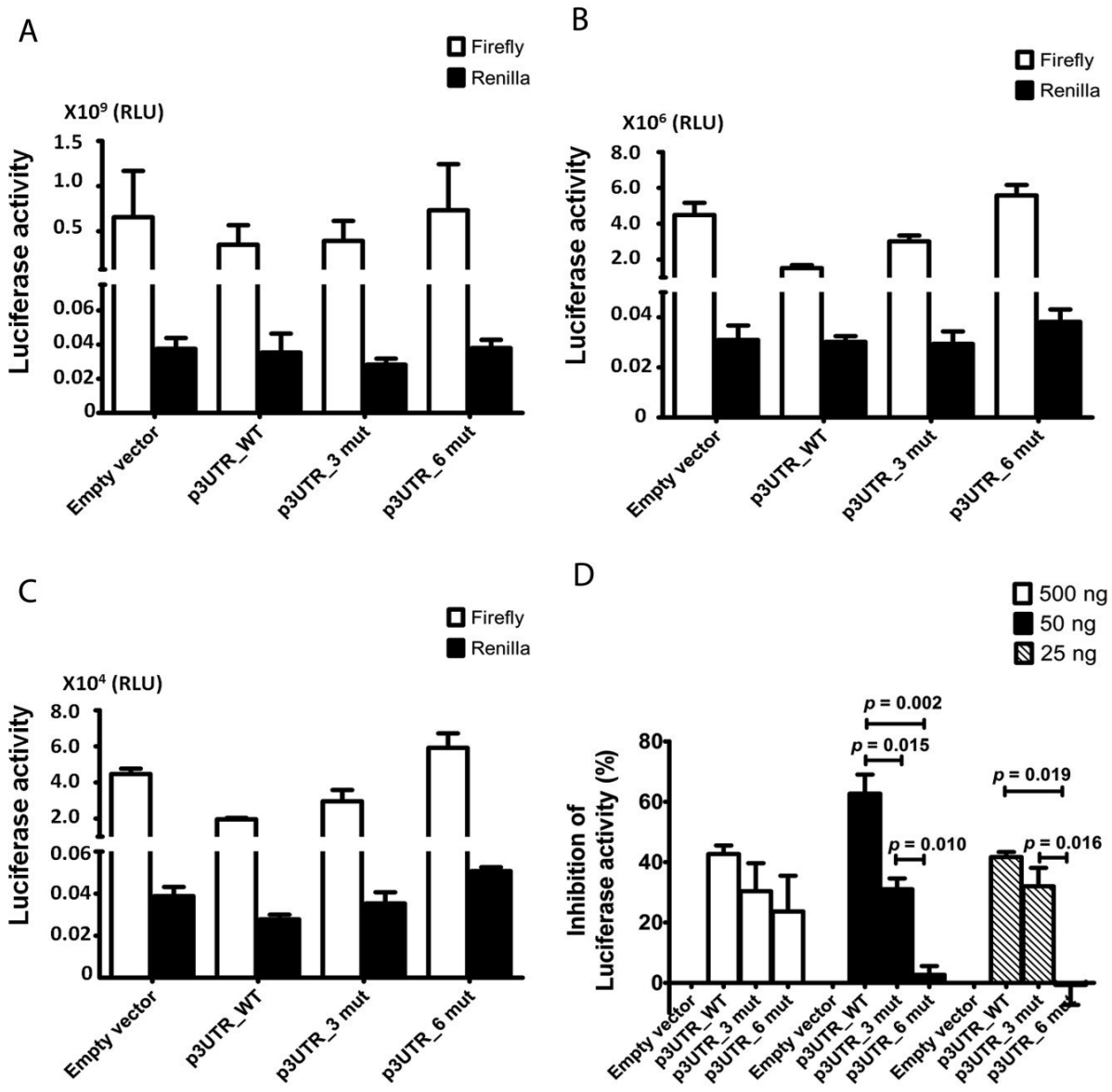

Figure 1. Effect of different concentration of reporter plasmid containing 3'-UTR on dual luciferase expressions.

Relative Light Units (RLU) of firefly and renilla luciferase activities were shown from the transfections of 500 ng luciferase reporter plasmid (A) 50 ng (B) and 25 ng (C). Inhibition percentages of luciferase activities were shown in (D). Firefly luciferase activity was divided by renilla luciferase activity to give relative luciferase activity (RL) and relative luciferase activity (RL) was divided by the relative luciferase activity of control reporter to give relative luciferase ratio (RLR). % of luciferase inhibition = (1- RLR) x 100. Results are shown as mean ± SEM, n=3 experiments.

2. ROC analysis of luciferase reporter assay

To confirm the previous results, the ROC curve analyses were performed. ROC analysis of each concentration was analyzed by SPSS 17.0 (SPSS inc., Chicago, IL, USA). The analysis results were presented in **Table 1** and ROC curves were presented in figure 2. Observing in the area under the ROC curve of the 3 mutant binding sites, 50 ng of reporter plasmid gave the highest score of 0.975 and followed by 500 ng with the score of 0.784, and 25 ng of 0.716 **(Figure 2A)**. When we considered on the 6 mutation binding sites, again 50 ng had the highest score of 1.000 and followed by 25 ng of 0.914 while 500 ng had the lowest score of 0.895 **(Figure 2B)**. Based on the results of ROC curve analyses, the concentration of 50 ng was confirmed as the optimal condition for detection of the 3'-UTR regulation in 293T cells.

Table 1. Area under the ROC curve analysis of luciferase expressions in 293T cells

Plasmid Conc.(ng)	AUC (95 % CI)		SE		*p*-value	
	3 mut	6 mut	3 mut	6 mut	3 mut	6 mut
500	0.784 (0.534, 1.014)	0.895 (0.741, 1.048)	0.116	0.078	0.042	0.005
50	0.975 (0.913, 1.037)	1.000 (0.913, 1.037)	0.031	0.031	<0.001	<0.001
25	0.716 (0.465, 0.967)	0.914 (0.748, 1.079)	0.128	0..084	0.122	0.003

Conc: concentration, AUC: area under the ROC curve, SE: standard error, 3 mut: p3UTR_3 mut and 6 mut: p3UTR_6 mut

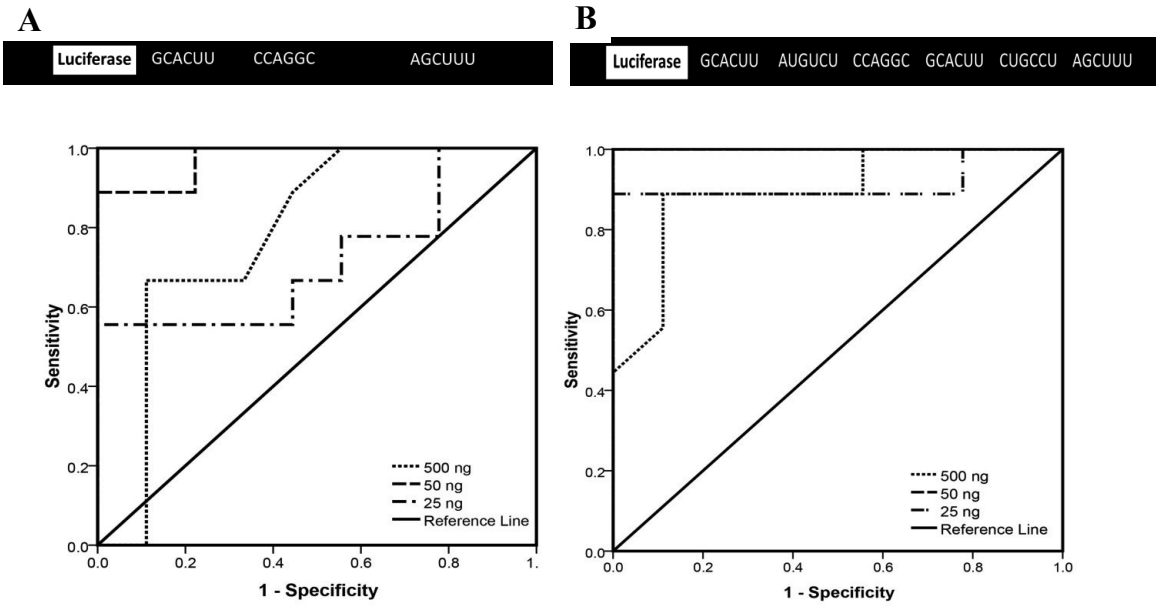

Figure 2. ROC analyses of luciferase expressions of 500 ng, 50 ng and 25 ng.
ROC analyses of 3 different concentrations of reporters with 3 and 6 mutation binding sites were shown. (A) A schematic showed a construct containing 3 specific binding sites of miRNAs and the relative luciferase ratio (RLR) of the 3 specific mutation binding sites of each concentration regarding the sensitivity 1-specificity and (B) for 6 specific binding sites of miRNAs.

Further assessment was demonstrated by the best score of the sensitivity, specificity and Youden's index. The positive likelihood ratio (+LR) was used to indicate the probability for a correct analysis of method. Usually, a number greater than one or a number that near 10 is indicated as an excellently appropriate method. In contrast, the negative likelihood ratio (-LR) was used to indicate the possibility of incorrect analysis of method. The lower value indicated optimal, usually less than one. The study demonstrated that, in all of the three conditions, the concentration of 50 ng of both 3 and 6 mutation binding sites had the best sensitivity, specificity, LRs and YI **(Table 2)**. These results supported the above data indicated that the 50 ng concentration of firefly reporter plasmid with 5 ng of renilla reporter plasmid was the best concentration for evaluating effect of 3'-UTR on luciferase expressions in 293T cells.

Discussions and Conclusions

To study the gene regulation, the luciferase reporter gene is commonly and popularly used to investigate the function of promoter or untranslated region (UTR) because the luciferase reporter assay is convenient, simple and can be used at the broad dynamic range approaches. However, limitation of luciferase reporter is rather high variability data resulting in reduce significance values of data analysis. Several studies try to optimize luciferase reporter assay by using differently approaches. In 2003, Burn et al[18] try to increase the level of gene expression in neuronal and glial cells by using several different posttranscriptional regulatory elements in a plasmid that contained a luciferase gene in lentiviral vectors. In 2013, Etten et al.[19] designed and optimized approach of luciferase reporter assays to investigate the regulatory RNA sequences that had effect on protein and RNA expressions by transfected the luciferase reporter into cells

Table 2. Assessment index of the optimal concentrations of plasmids to transfect into 293T cells based on ROC analysis results

Plasmid	Sensitivity		Specificity		+LR		-LR		YI	
Conc.(ng)	3 mut	6 mut	3 mut	6 mut	3 mut	6 mut	3 mut	6 mut	3 mut	6 mut
500	0.667	0.778	0.667	0.889	2.000	7.000	0.499	0.249	0.334	0.667
50	0.889	1.000	0.889	0.889	8.100	9.000	0.124	0.000	0.778	0.889
25	0.778	0.889	0.677	0.889	2.366	8.100	0.327	0.124	0.455	0.778

Conc: concentration, +LR: positive likelihood ratio, -LR: negative likelihood ratio , YI: Youden's index, 3 mut: p3UTR_3 mut and 6 mut: p3UTR_6 mut

and measured protein expression levels with activity of luciferase expression, and measured mRNA levels of luciferase by qRT-PCR. Here we aimed to investigate the best sensitivity of a luciferase reporter gene assay by optimizing concentration of luciferase reporter vectors. We also compared luciferase activity between the numbers of mutation sites on regulatory sequences to assess whether their different mutations had effect on luciferase sensitivity. We found that at 50 ng, the reporter plasmid gave low variability and greater significance values of data analysis when compared between the wild-type 3'-UTR (p3UTR_WT) and the 3 specific mutation binding sites (p3UTR_3 mut), *p*-value of 0.015 or compared between the wild-type 3'-UTR (p3UTR_WT) and the 6 specific mutation binding site (p3UTR_6 mut), *p*-value of 0.002. On the other hand, at 500 ng, the reporter plasmid gave the highest luciferase activities (10^9 RLU) but also gave higher variability data leading to non-significant value of data analysis. The high activities of luciferases maybe out of linear dynamic range of the GloMax®-20/20 machine which over 8 logs according to the manufacturer's instructions[20]. Thus, this concentration gave higher variability data within the same group leading to statistical non-significance. Too high activities also affect the sensitivity of the test because small changes would not be demonstrated. The low concentration (25 ng of reporter plasmid) gave low variability data but too low signal would also affect the statistical significance. This was demonstrated when comparing between the wild-type 3'-UTR (p3UTR_WT) with the 3 specific mutation binding sites (p3UTR_3 mut). Thus, the low concentration of reporter plasmid maybe insufficient to express luciferase activities especially, renilla luciferases which were very low (10^2 RUL) **(Figure 1C)** that maybe out of the limit of machine detection.

To identify the efficacy of each concentration, we used several statistics to estimate such as ROC analysis method[21] and Youden's index (YI)[22]. Based on ROC curve analysis, the index of sensitivity (the concentration that showed the most different relative luciferase ratio between wild type and mutant plasmids) correlated with the potentiality of experimental method to identify the best method. The concentration of 50 ng reporter plasmid showed the highest sensitivity of 0.889 and 1.000, close to optimal. Youden's index (YI) is a statistic used to estimate the efficacy of the analysis test which is calculated from "sensitivity + specificity – 1"[22]. Of all the three studied concentrations, 50 ng also gave the better of YI, +LR and -LR scores. This study demonstrated that at 50 ng concentration was an appropriate condition for detection of 3'UTR regulation in 293T cell. In addition, the number of mutation sites also affected the luciferase activities leading to different sensitivity, specificity, YI, +LR and -LR scores.

In conclusion, we have optimized the reporter concentration for the dual luciferase reporter system in 293T cells. Thus, the optimization of reporter plasmid concentration is essential for reliable and valid experiments because each cell type is different and required optimization. This approach can also be applied to investigate the regulation on 5'UTR. This information will be useful for researchers and scientists who employ the luciferase reporter system in investigating the regulation at posttranscriptional regulation.

Acknowledgements

We thank Dr. Yong Sun Lee and members of laboratory in the University of Texas Medical Branch for providing luciferase reporter constructs.

Funding

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Center of Excellence in Specific Health Problems in Greater Mekong Sub-region Cluster (SHeP-GMS), Khon Kaen University (KKU) the Centre for Research and Development of Medical Diagnostic Laboratories (CMDL) and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University. WW is holding a scholarship supported by the Royal Golden Jubilee Ph.D. program (RGJ: PHD/0269/2551), Thailand Research Fund (TRF).

References

1. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. Bioessays. 2009;31(1):51-59.
2. Srivastava RA, Schonfeld G. Measurements of rate of transcription in isolated nuclei by nuclear "run-off" assay. Methods Mol Biol. 1998;86:201-207.
3. Hu ZW, Hoffman BB. Nuclear run-on assays for measurement of adrenergic receptor transcription rate. Methods Mol Biol. 2000;126:169-180.
4. Woo AJ, Dods JS, Susanto E, Ulgiati D, Abraham LJ. A proteomics approach for the identification of DNA binding activities observed in the electrophoretic mobility shift assay. Mol Cell Proteomics. 2002;1(6):472-478.
5. Jiang T, Xing B, Rao J. Recent developments of biological reporter technology for detecting gene expression. Biotechnol Genet Eng Rev. 2008;25:41-75.
6. Gorman CM ML, Howard BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. molecular and cellular biology. 1982;2(9):1044-1051.
7. Li L, Higaki K, Ninomiya H, Luan Z, Iida M, Ogawa S, et al. Chemical chaperone therapy: luciferase assay for screening of beta-galactosidase mutations. Mol Genet Metab. 2010; 101(4):364-369.
8. Martin CS, Wight PA, Dobretsova A, Bronstein I. Dual luminescence-based reporter gene assay for luciferase and beta-galactosidase. Biotechniques. 1996;21(3):520-524.
9. Smale ST. Beta-galactosidase assay. Cold Spring Harb Protoc. 2010;2010(5):pdb prot 5423.
10. Kitsera N, Khobta A, Epe B. Destabilized green fluorescent protein detects rapid removal of transcription blocks after genotoxic exposure. Biotechniques. 2007;43(2):222-227.
11. Khuc T, Hsu CW, Sakamuru S, Xia M. Using beta-Lactamase and NanoLuc Luciferase Reporter Gene Assays to Identify Inhibitors of the HIF-1 Signaling Pathway. Methods Mol Biol. 2016;1473:23-31.
12. Hill SJ, Baker JG, Rees S. Reporter-gene systems for the study of G-protein-coupled receptors. Curr Opin Pharmacol. 2001;1(5): 526-532.
13. Erika Hawkins MS, Michael Beck, M.S., Braeden Butler, B.S., and Keith Wood. Dual-luciferase reporter assay: an advanced co-reporter technology integrating firefly and Renilla luciferase assays. Promega Notes 1996;57:2-8.

14. Bronstein I, Martin CS, Fortin JJ, Olesen CE, Voyta JC. Chemiluminescence: sensitive detection technology for reporter gene assays. Clin Chem. 1996;42(9):1542-1546.
15. Strehler BL, Totter JR. Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. Arch Biochem Biophys. 1952;40(1):28-41.
16. Lundin A. Optimization of the firefly luciferase reaction for analytical purposes. Adv Biochem Eng Biotechnol. 2014;145:31-62.
17. Wongfieng W, Jumnainsong A, Chamgramol Y, Sripa B, Leelayuwat C. 5'-UTR and 3'-UTR Regulation of MICB Expression in Human Cancer Cells by Novel microRNAs. Genes (Basel). 2017; 8(9).
18. Brun S, Faucon-Biguet N, Mallet J. Optimization of transgene expression at the posttranscriptional level in neural cells: implications for gene therapy. Mol Ther. 2003;7(6):782-789.
19. Van Etten J, Schagat TL, Goldstrohm AC. A guide to design and optimization of reporter assays for 3' untranslated region mediated regulation of mammalian messenger RNAs. Methods. 2013;63(2):110-118.
20. Promega. Technical manual of glomax 20/20 luminometer. Available online.https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/glomax-20-20-luminometer-protocol.pdf.
21. Demler OV, Pencina MJ, D'Agostino RB, Sr. Equivalence of improvement in area under ROC curve and linear discriminant analysis coefficient under assumption of normality. Stat Med. 2011; 30(12):1410-1418.
22. Schisterman EF, Perkins NJ, Liu A, Bondell H. Optimal cut-point and its corresponding Youden Index to discriminate individuals using pooled blood samples. Epidemiology. 2005;16(1):73-81.