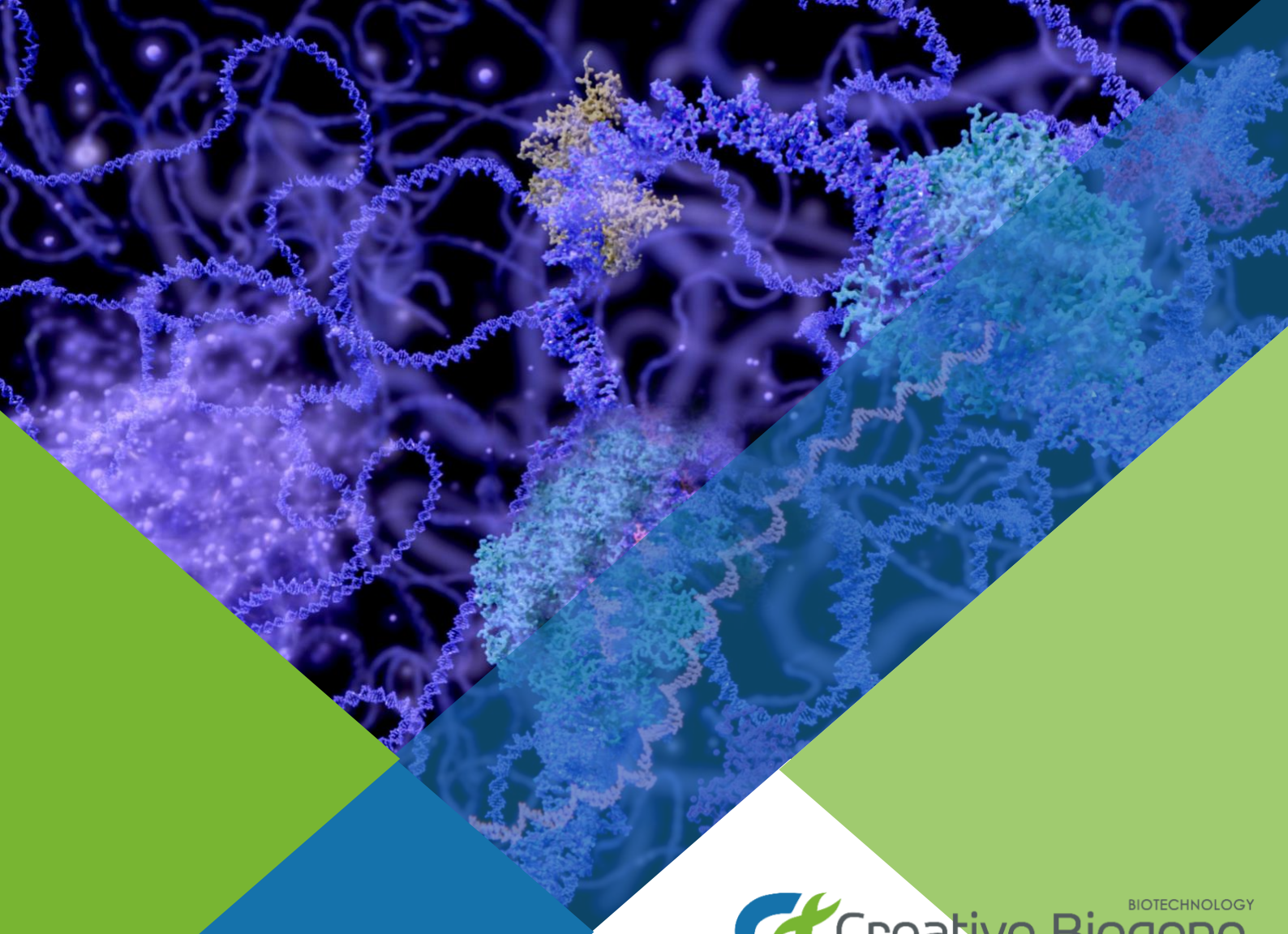

Creative Biogene BIOTECHNOLOGY

FINAL REPORT

miRNA Luciferase Reporter Assay

This report is provided by:
Creative Biogene
SUITE 115, 17 Ramsey Road,
Shirley, NY 11967, USA

Order# XXXXXXXXXX

Date: Jan 14, 2022

Submitted by:
Lily Tsai
Technical Support

This study was conducted according to the procedures described in this report. All data presented are authentic, accurate and correct to the best of our knowledge. Information and materials provided by this investigation are for research use only.

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1. Project Summary

This report describes that testing the binding and activity of miR-XXX-5p on the 3'UTR of human XXXX by luciferase reporter gene assay. It is based on the expression of Luciferase as a reporter gene fused to the XXXX 3'UTR sequence in the presence of miR-XXX-5p mimic to test in an in vitro cell culture assay.

2. Experimental Method

2.1 Construction of miRNA luciferase reporter vector

There are a number of available luciferase reporter vectors, including recently introduced ones that are designed for testing miRNA-mediated gene silencing. In this project, we use pmirGLO (Promega), one of the most commonly used luciferase reporter vectors. pmirGLO luciferase reporter constructs are created by cloning the specific miRNA binding sequence (wild-type/mutants) into the XhoI/XbaI site located at 3'UTR of pmirGLO-Control vector.

a. Digestion of pmirGLO vector: linearize the pmirGLO vector with XhoI/XbaI restriction enzymes.

b. WT/mutant human XXXX 3'UTR synthesis and amplification

>WT human XXXX 3'UTR sequence (miRNA binding site)

GCCAGTCTGCTGTCCTGAAACCCAGAAGTGATGGAGAGAAACCAACAAGAGATCTCGAACCCTGTCTAGAAGGAAT
GTATTTGTTGCTAAATTTCGTAGCACTGTTTACAGTTTTCCTCCATGTTATTTATGAATTTTATATTCCGTGAATGTATATT
GTCTTGTAATGTTGCATAATGTTCACT

>mutant human XXXX 3'UTR sequence

GCCAGTCTGCTGTCCTGAAACCCAGAAGTGATGGAGAGAAACCAACAAGAGATCTCGAACCCTGTCTAGAAGGAAT
GTATTTGTTGCTAAATTTCGTAGCACGCACAGTAGTTTTCCTCCATGTTATTTATGAATTTTATATTCCGTGAATGTATAT
TGTCTTGTAATGTTGCATAATGTTCACT

c. Ligate the WT/mutant human XXXX 3'UTR fragment into linearized pmirGLO by recombination reaction.

d. Transform the ligation product into DH5 alpha competent cells and plate cells on LB plates containing Amp selection overnight at 37°C.

e. Identify positive clones by colony PCR and sanger sequencing.

2.2 Design and synthesis of miRNA/NC mimic

hsa-miR-XXX-5p mimic sequence: UGUAAACAUCCCCGACUGGAAG

Negative control mimic: UCACAACCUCCUAGAAAGAGUAGA

Purification: HPLC

2.3 Co-transfection of luciferase reporter constructs with miRNA/NC mimic

Plate 1 x 10^4 cells per well in 50 µl culture medium from a sub-confluent HEK293T cell suspension (cell density < 1.3 x 105 cells/cm2). Plate 3 wells for each condition to produce triplicates.

Transfect cells after 5-16 h in culture. The following is transfection condition for 1 well:

a. Dilute 150 ng of pmirGLO-UTR reporter plasmid in combination with miRNA/NC Mimic at a final concentration of 20 nM in 25 μl of pure DMEM.

b. Dilute 0.5 μl Lipofectamine in 25 μl of pure DMEM.

c. Incubate both diluted solutions 5 min at room temperature.

d. Combine diluted nucleic acids with diluted Lipofectamine (50 μl final), mix gently and incubate complexes 20 min at room temperature.

e. Add the transfection complexes (50 μl) to the well containing plated cells in 50 μl medium.

Incubate transfected cells at 37 °C in a 5% CO2 incubator for 48 h without medium change.

Experimental group:

pmirGLO+mimic NC

pmirGLO+miR-XXX-5p mimic

XXXX 3'UTR WT+mimic NC

XXXX 3'UTR WT+miR-XXX-5p mimic

XXXX 3'UTR MUT+mimic NC

XXXX 3'UTR MUT+miR-XXX-5p mimic

2.4 Luciferase activity assay

Measure luciferase using the Dual-Glo Luciferase assay system as recommended by the manufacturer.

a. Prepare the stop solution, according to the number of wells, by diluting Dual-Glo® Stop &Glo® Substrate (1:100) in Dual-Glo® Stop &Glo® Buffer.

b. Due to evaporation of the medium with time, the final volume per well is less than 100μl after 48 h in culture. Measure the volume of the medium left in one well by pipetting and discard the appropriate amount of medium to reduce the volume to 50 μl from the well by pipetting out.

c. Add 50 μl of Dual-Glo® Luciferase Reagent to each well (equal volumes). Avoid depositing Reagent solution along the walls of the well. Mix gently by tapping the plate. Because the plate is not shaken, when a drop of reagent is on the wall of the well, the volume of reagent in contact with the culture medium is reduced and this has an impact on the measurement of the luciferase.

d. Incubate 10 min in the dark at room temperature without shaking.

e. Measure firefly luciferase activity using the luminometer.

f. Remove the plate from the luminometer and add 50 μl of Dual-Glo® Stop&Glo® Reagent (stop solution prepared in a.) per well. Mix gently

g. Incubate 10 min in the dark at room temperature.

h. Measure Renilla luciferase activity using the luminometer.

Note: Dual-Glo® Luciferase and Dual-Glo® Stop&Glo® Reagents are stable for 2 h.

Calculate the ratio of luminescence from the experimental reporter (firefly) to luminescence from the control reporter (Renilla). Calculate the mean ratio for each triplicate and normalize this ratio to the ratio of control wells.

3. Experimental Results

3.1 Amplification results of WT/mutant human XXXX 3'UTR

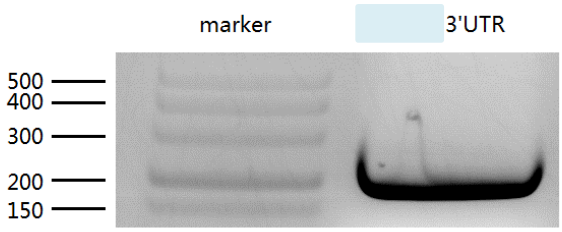


Figure1. Agarose gel electrophoresis of XXXX 3'UTR amplification

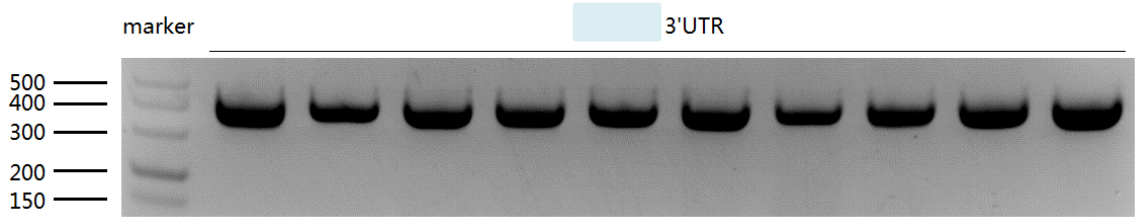
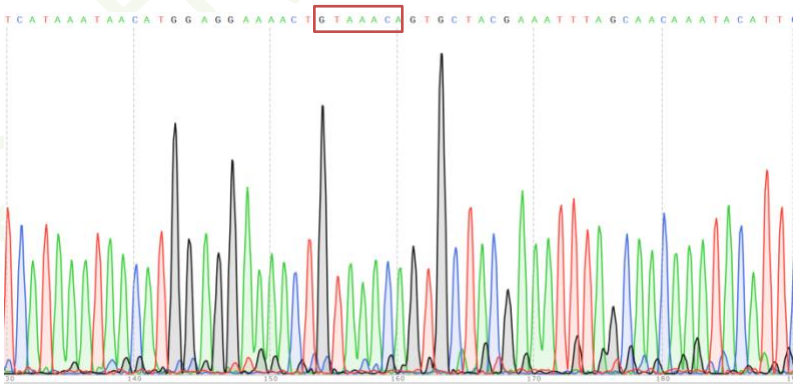


Figure 2. Identification of positive clone

Primer: P3799-GCGAGATTCTCATTAAGGCCAAGAAG

P3800-GTTATGCTAGTTATTGCTCAGCGG

3.2 Sequencing results of pmirGLO-XXXX 3'UTR



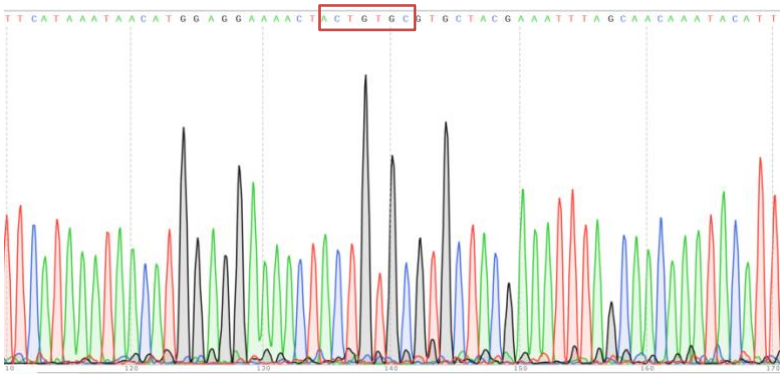


Figure 3. Sanger sequencing results of pmirGLO-XXXX 3'UTR

The Raw data of this part is shown in attachment.

3.3 Luciferase reporter assay results for miR-XXX-5p effect on XXXX 3'UTR

Table 1. Luciferase reporter assay data

Target gene	Experiment Group	Luciferase	Luciferase Activity Value		
			Data 1	Data 2	Data 3
XXXX	HEK293 control	firefly	51	46	45
	pmirGLO+mimic NC	firefly	65470000	56090000	59080000
	pmirGLO+miR-XXX-5p mimic	firefly	60950000	50230000	58900000
	XXXX 3'UTR WT+mimic NC	firefly	75610000	74090000	79630000
	XXXX 3'UTR WT+miR-XXX-5p mimic	firefly	69380000	70880000	68120000
	XXXX 3'UTR MUT+mimic NC	firefly	81260000	86850000	87180000
	XXXX 3'UTR MUT+miR-XXX-5p mimic	firefly	81690000	77740000	81470000
	HEK293 control	Renilla	82	78	54
	pmirGLO+mimic NC	Renilla	2147000	1974000	2064000
	pmirGLO+miR-XXX-5p mimic	Renilla	2022000	1816000	1926000
	XXXX 3'UTR WT+mimic NC	Renilla	1905000	2027000	2288000
	XXXX 3'UTR WT+miR-XXX-5p mimic	Renilla	2400000	2360000	2286000
	XXXX 3'UTR MUT+mimic NC	Renilla	2916000	3399000	3111000
	XXXX 3'UTR MUT+miR-XXX-5p mimic	Renilla	2775000	3041000	3337000

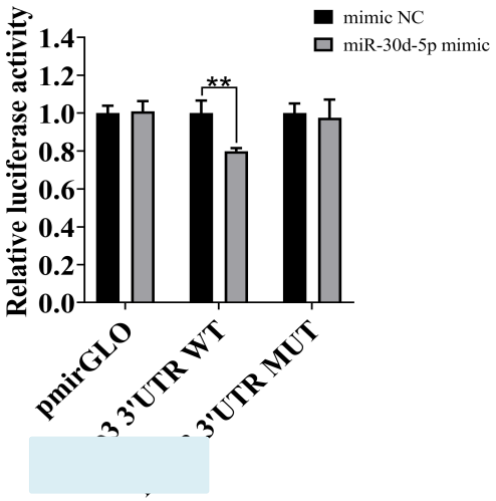


Figure 4. Luciferase reporter assay using miR-XXX-5p and XXXX 3'UTR

4. Conclusion

The result of luciferase reporter assay suggests that miR-XXX-5p inhibiting XXXX gene expression via binding to the 3'UTR of human XXXX gene.