Gene Regulation with O-GlcNAc Glycosylation by dCas9-OGT/OGA Fusion Proteins

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Abstract

O-linked β -N-acetylglucosamine (O-GlcNAc) is a post-translational modification that plays important role in cellular network/diseases such as autophagy, transcriptional activity, and protein stability, phosphorylation modification competition, cancers, neurodegenerative disorders, etc. However, there are still lots of unknown roles in gene regulation especially while there is the existence of more than 1000 transcription factors and many other coactivators/repressors which might have an influence on expression level with or without O-GlcNAc modification.

In this project, we use CRISPR technology with catalytically inactive Cas9 from *Streptococcus pyogenes* fused with the only writer and eraser of O-GlcNAc to selectively target the DNA sequence of interest by guide RNA to see what the result of additional modification or cleavage of O-GlcNAc on proximal proteins in gene regulation is.

A cost and time-efficient way for guide RNA construction is developed in the project with one-piece PCR and Gibson assembly for 2 different backbones of guide RNA: 11 guide RNA for each. Other different cloning methods have also been used for future work. For future work, a second reporter will be introduced to normalize the luciferase signal. In addition, a new metabolic O-GlcNAc reporter and Y289L GalT/UDP-GalNAz could be used to find out proteins that might be modified by dCas9-OGT/OGA with click reaction. Furthermore, a split OGA/Turbo ID system could also be used to reduce the background and find out proteins/modification sites that might

be important to the gene regulation for DNA-protein or protein-protein interaction.

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Chapter 1: Introduction of O-GlcNAc and CRISPR

1.1 Introduction and importance of O-GlcNAc modification

O-GlcNAc (*O*-linked β -*N*-acetylglucosamine) is a post-translational modification that is found on serine and threonine which plays an important role in many cellular networks such as transcription, protein stability, phosphorylation competition, etc. Dysregulation of *O*-GlcNAc has been found in many pathologies: including neurodegenerative disorders, and cancers, and is implicated in increasing the phenomenon of autophagy. Contrary to its diverse role in the cellular network, the dynamic modification of O-GlcNAc is only controlled by one eraser and one writer, OGA and OGT, which is relatively simple than phosphorylation dynamics.^{1,2,3,4}

Post-translational modification is a process the side chain of an amino acid is added or cleaved with modification after biosynthesis. Readers, writers, and erasers involve in the process and result in changes in protein structure, protein function, protein-protein interactions, and protein-DNA/RNA interaction. There are many modifications such as methylation, acetylation, phosphorylation, and ubiquitination found important in various biological processes. However, the knowledge of O-GlcNAc modification in the role of gene expression is still not well-known.

In this research, we are interested in the role and impact of O-GlcNAc modification. Here, we propose to construct the CRISPR (clustered, regularly interspaced, short palindromic repeats) technology with the only eraser and the writer for further discovery. The hypothesis is made that by selectively target to the sequence,

the CRISPR system fused with OGT/OGA will modify the proteins proximal to the DNA region and gene regulation will change because of the O-GlcNAc role which we are interested in for further discovery.

1.2 CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats): introduction and variants

There are two types of CRISPR systems: Class I and Class II. Class I system works with protein complexes that are not suitable for usage in gene editing or gene regulation approach. Hence, the CRISPR system we use in our project is one of Class II; SpCas9 which is from *Streptococcus pyogenes* and works by only a single protein with multi-subunit domains. Other Class II Cas9 proteins include SaCas9, NmeCas9, CjCas9, StCas9, and CdCas9 and they are from *Staphylococcus aureus*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Streptococcus thermophilus*, and *Corynebacterium diphtheriae* separately. Cas9 proteins from different strains have different Cas9 protein structures (sequence), sequence of scaffold RNA, and the protospacer adjacent motif (PAM site) that is recognized by Cas9 protein and is directly 2-6 nucleotide downstream of the sequence targeted by guide RNA.^{5,6}

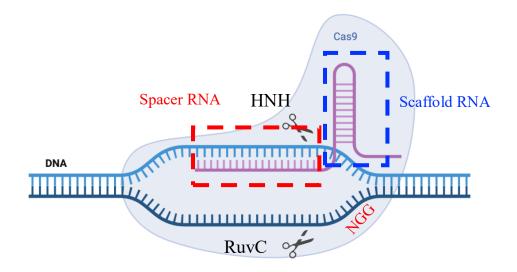


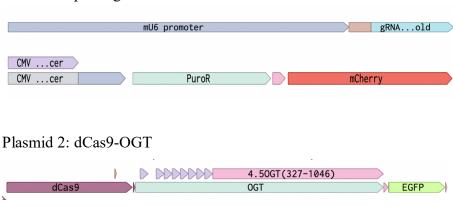
Figure 1. CRISPR: SpCas9 components

Native Cas9 protein and mutant Cas9 protein have been developed for different applications. In the beginning, native SpCas9 was found to make a blunt end doublestrand break of DNA targeted sequence by the HNH nuclease domain and RuvC nuclease domain of Cas9. HNH nuclease cleaves the DNA strand complementary to the guide RNA and RuvC nuclease cleaves the opposite strand of DNA. The most popular mutants made for catalytic amino acids of the HNH domain are H840A and D10A for RuvC.

Nickase Cas9 (nCas9) is made by one of two mutants and dead Cas9 (dCas9) is made by double mutants. So far, nCas9 has been used for base editings such as A to G, C to T, and C to G, prime editing, enhanced selectivity of gene editing by homologydirected repair, etc.^{7,8} However, dCas9 has been used in a different application even though it loses its nuclease ability while sustaining the ability to bring the Cas9 system to the target sequence. Catalytic inactive Cas protein is fused with many kinds of proteins such as transcription activator, transcription repressor, methyltransferase, fluorescence reporter, DNA polymerase, and kinases to regulate the cellular system, label genomic locus, or find out the role of different kinds of modifications on protein, DNA or RNA in the cellular network.

1.3 Experimental design

In the project, we use catalytic inactive Cas9 protein from *Streptococcus pyogenes* to fuse with OGT/OGA and target the sequence next to NGG to further discover the O-GlcNAc modification roles in gene regulation. We will transiently transfect HEK293T with three plasmids including guide RNA, dCas9-OGT/OGA, and luciferase reporter which the protein expression is driven by SV40 promoter. Several guide RNAs will be constructed to target the SV40 promoter and regulate the luciferase protein expression. T2A is installed between PuroR/mCherry and dCas9-OGT/EGFP.



Plasmid 1: pU6-sgGAL4-4

Plasmid 3: pMLS-SV40-firefly luciferase



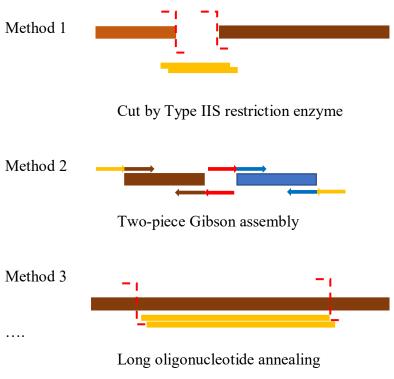
Figure 2. Experiment designs of 3 plasmids

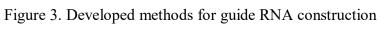
Chapter 2: Results and Experiments

2.1 gRNA plasmid construction

Since the development of CRISPR technology, different kinds of gRNA plasmid construction have been developed to full fill the need to target genome loci with gRNA libraries. In the system of SpCas9, spacer RNA is usually composed of 18-24 nucleotides of RNA which is complementary to the DNA that the Cas9 system targets. Two-piece Gibson assembly, long oligonucleotide annealing, and application of Type IIS restriction enzyme have been developed to reach out to the need. Among those methods, double recognition and cuts by Type IIS restriction enzyme is the way which is used most broadly. However, there is no double Type IIS in the backbone of the plasmid used in our project. To construct gRNA with the method, small oligonucleotide annealing inserted with double Type IIS recognition of AarI could be used to construct the backbone. On the other hand, two-piece Gibson assembly and long oligonucleotide annealing seem to be relatively not cost-efficient or time-efficient methods to construct gRNA.^{9,10,11}

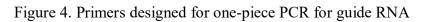
Hence, the possibility of a one-piece PCR/one-piece Gibson assembly has been developed in the project, and the reaction is optimized and applied to two gRNA plasmid backbones, one is for native gRNA of SpCas9, and another is for scaffold RNA which is inserted with double MS2 stem-loop. With the optimization, only one forward primer is needed for tons of different kinds of spacer, and we believe this kind of design logic could be applied to different kinds of Cas proteins that have different scaffold RNA.

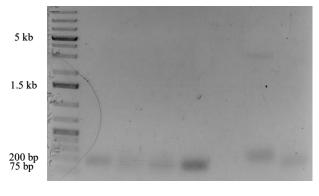




Firstly, assemble junction is set up between forward and reverse primer over 30 nt. However, there is no PCR product according to the design even though several ways have been used such as adding DMSO into the PCR reaction, using more DNA polymerase, or diluting the concentration of the primers. There is still a faint band under the gel which is around 200 bp. The reason each PCR reaction doesn't work might be because forward and reverse primer bind together and interrupt their binding to the backbone. To avoid the situation, the junction is moved to the spacer RNA region, but there is a new problem which is a non-specific product derives from the design. The junction is then moved to the scaffold RNA region that expected size band is shown after diluting the concentration of the primers.

				tctcgatctttatcgttc		
	mU6 promoter	X	Guide RNA B		gRNA scaffold	20
2,900	2,920	2,940	2,960	2,980	3,000	3,020
tgtgttttgagac	tataagtatcccttggag	and an and a superstant and the second	ccccaggetecccagegtttt			atcaacttgaaaaagtggcaccgag
						tagttgaactttttcaccgtggctc
	agggaaccto	ttggtggaacaacttca	ggggtccgaggggtcgcaaaa	atctcgatcttta		

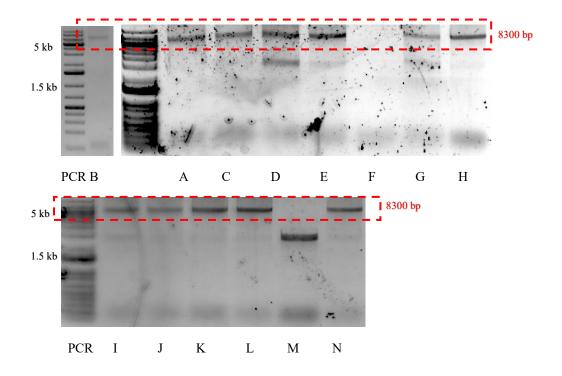


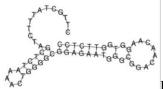




a: more Q5 polymerase

b: primer concentration dilution





F reverse primer: Free Energy (delta G) = -17.20 kcal/mol Figure 5. PCR results for each set of primers

However, there is no PCR band shown on gRNA F, and a non-specific PCR shown gRNA M. The reason for the failure of gRNA F might be the reverse F primer will form a very stable hairpin that is not good for the PCR reaction. And the non-specific band from gRNA M might be solved by moving the junction 4 nucleotide left so the annealing affinity part to the target site is larger than the non-specific binding site.

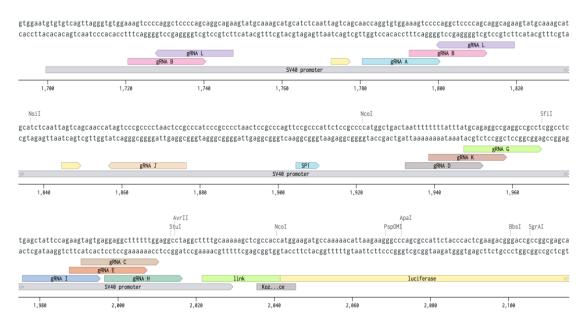


Figure 6. Region of SV40 promoter targeted by 11 different guide RNA

gRNAs are designed to target the SV40 promoter which drives luciferase protein expression. Each spacer RNA is 20 nucleotides in length and NGG is downstream of the region targeted by guide RNA.

The second problem comes after transformation/miniprep, there is almost no target sequence that the spacer RNA designed but most of them are templates with some undesired bands. The reason might result from the low efficiency of the assembly and there is still a template plasmid in the assembly reaction before the transformation. To purify the PCR reaction which might benefit the efficiency of Gibson Assembly reaction or the possibility to get the newly constructed spacer RNA. DpnI (37 °C, 1-4 h) is added to degrade the template plasmid which is amplified by TOP10 or Turbo strain of bacteria (both are Dam⁺), and the spin column is used to purify the PCR solution by excluding protein that might interrupt the efficiency of Gibson Assembly. However, none of them works. Finally, gel extraction is performed which almost excludes the chance of getting a template but there is still some issue about the salt which might have an impact on Gibson assembly and transformation. While doing electroporation with assembly solution, arcing happens much of the time and there is still some undesired band after picking colonies/miniprep. The phenomenon is much excluded and optimized by increasing the ratio of deionized water and the time of assembly reaction from 15 minutes 60 °C to an hour 60 °C.

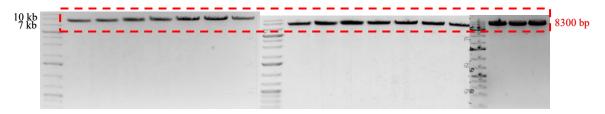
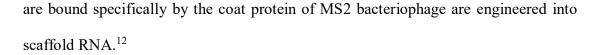


Figure 7. Miniprep results after condition optimization

After transformation and miniprep, the plasmid is digested with BamHI with one cut on the plasmid. There is no undesired band and 1 template after analytic gel or sequencing result.

The optimized primer designs and the procedure is used directly on another backbone for gRNA construction which is used in synergistic activation mediator (SAM). The gRNA difference from the native one is that two MS2 stem-loops which



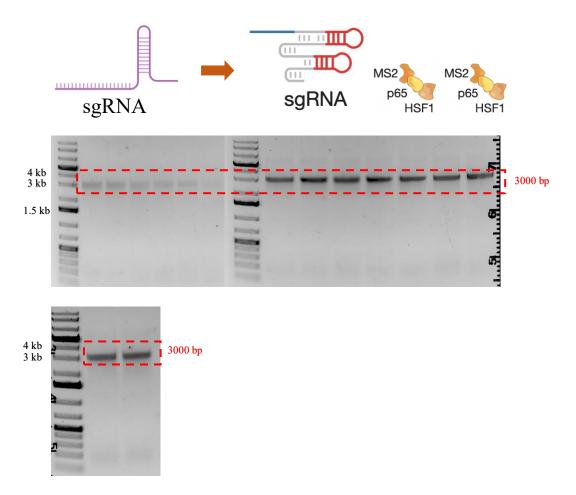


Figure 8. Engineered sgRNA with 2 MS2 stem-loop and PCR results

PCR product is shown above which includes all newly constructed spacers from the first backbone. There is no non-specific product shown in the gel but still with some faint primer dimer product around 75-200 bp. An interesting phenomenon is found that the band at the bottom is relatively faint compared to PCR the first backbone. The reason might be because the junction for Gibson assembly is moved 4 nucleotides left and the reverse primer could anneal much more than the original design.

After Gibson assembly and transformation, miniprep is performed and digested with one cut. There is no undesired band and there is almost no template. Correct sequence results are obtained which only need to pick 2 colonies for each different spacer RNA.

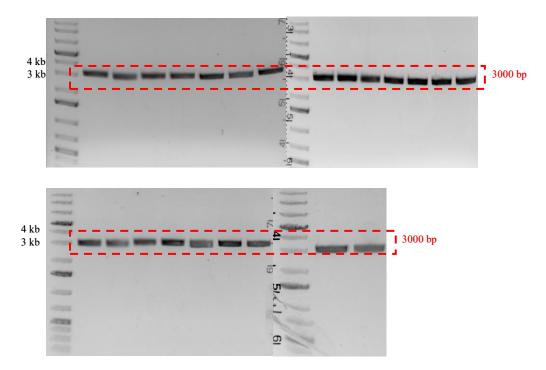


Figure 9. Miniprep results with the optimized condition

2.2 Other cloning methods: two-piece Gibson, ligation, and oligonucleotide annealing

2.2.1 Plasmid construction with ligation

Ligation is performed as a practice for the following oligonucleotide annealing. To construct plasmid with ligation, recognition site of restriction enzyme which causes sticky end is chosen and inserted in the 5'-end of forward and reverse primer with 5-7 nt added upstream of the recognition site. After PCR reaction and double restriction enzyme digestion for each piece, gel extraction (spin column is not good) is performed to exclude the rest of the primers and restriction enzymes which might interrupt the ligation reaction. Ligation reaction is conducted under 4 °C 24 hours and heat inactivation is performed before electroporation. Transformation done in TOP10 has more colonies than Turbo in my case. However, because of further consideration about

if there is a frameshift, especially for translation fusion and the low reaction efficiency, a two-piece PCR combination is performed mainly by a two-piece Gibson Assembly.

2.2.2 Plasmid construction with two-piece Gibson Assembly

Two-piece Gibson Assembly is performed for a replacement of fluorescence reporter, chemiluminescent reporter (codon-optimized), and promoter (sgRNA target to the different promoter) for future work. Before assembly reaction, gel extraction which is performed to increase assembly efficiency is still needed. (46919_pMLS-SV40-EGFP to 46919_pMLS-SV40-mCherry, 46919_pMLS-SV40-EGFP to 46919_pMLS-SV40-luciferase to 46919_pMLS-SV40-luciferase to 46919_pMLS-SV40-luciferase codon-optimized).

2.2.3 Plasmid construction with oligonucleotide annealing

Oligonucleotide annealing is tried to insert one AarI recognition site into the backbone as a practice to see if the protocol and experiment design work. In my case, Type IIS restriction enzyme (BbsI, BsaI) could not be used because they will cut the regions outside the guide RNA. Hence, two pairs of oligonucleotides one of which contains an AarI recognition site are designed and successfully inserted into the backbone. Three pairs of oligonucleotides are designed to target the region targeted by gRNA F and M but finally, the results are not expected. The reason might because the ligation reaction is not efficient enough for 3 pairs of oligo ligation to work in only 15 minutes. Hence the following ligation with the vector doesn't work either. Vector is prepared by digestion with BstXI and BamHI separately in different buffers and is purified by gel extraction. Each pair of small oligonucleotides is annealed and

phosphorylated in different Eppendorf tubes. After 5'-end phosphorylation, two or three pairs of nucleotides are ligated together and then ligated with the vector.

The sequencing result shows the newly constructed backbone with the AarI recognition site is inserted and the design is shown as follows.

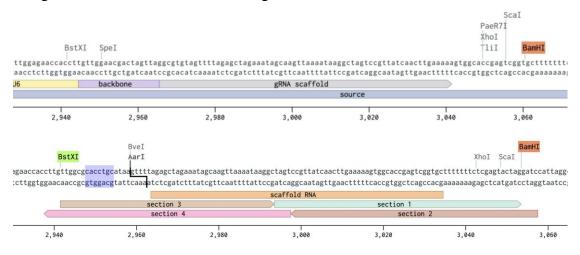


Figure 10. Backbone inserted with sequence cut by type IIS restriction enzyme

2.3 Mammalian Cell Culture and experiments

2.3.1 Troubleshooting of cell culture issue

There is a cell culture issue that happened in the lab which is after cell passages cells couldn't attach to the dish and die. The reason might be that we didn't add FBS in our DMEM, glutaMax medium. Even though glutaxMax supplement is already added in the commercially available medium which could improve cell viability and growth, attachment form cells such as the cell line used in the project, HEK293T, still need growth factors, hormones, etc, which are included in FBS. Without FBS, cells couldn't grow more than 3 passages and will die or lose their ability to attach to the dish in starvation conditions based on my observation.

A Series of troubleshooting has been made to fix or find out the problem which is shown in the table

Entry	Improvement	Reason
1	Disperse cells in the middle of dish	Avoid clamp while dispending cell
2	Disperse cells evenly in dish	Avoid clamp while dispending cell
3	Add Penicillin-Streptomycin	Anti-bacteria, negative and positive gram
4	Pipette smoothly	Pipette too strong will kill the cell
5	Pipette strongly	Suspend cell into single cell, not clamp
6	Finish cell passage in 5 minutes	Avoid death of cell from external pressure, temperature, % of CO ₂ , possible contamination
7	Trypsin time longer	Make cell into single cell
8	Trypsin time shorter	Over-trypsinization kill cell or cell can't attach
9	Lab coat contamination	Specific lab coat for cell culture room
10	Medium contamination	Open a new bottle of medium
11	Technical issues	Not crossing over dishes, not possible contact contamination of medium, trypsin or PBS.

Table 1. Troubleshooting of cell culture issue

2.3.2 Transient transfection

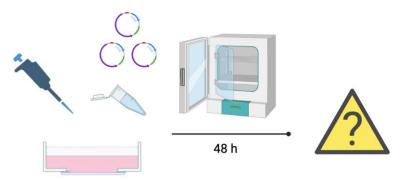


Figure 11. Steps after gRNA prepared: 3 plasmids transient transfection

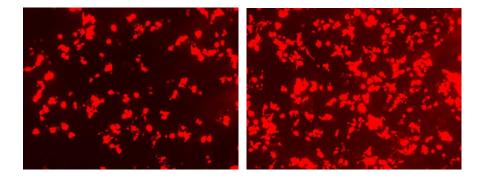
 $5 \ \mu g$ of each plasmid is transiently transfected into HEK293T cell. The ratio of plasmid/PEI (1 mg/mL) is tested at 1:3, 1:4, or 1:5 during the incubation. Less Opti-MEM for incubation is found to have higher transfection efficiency which might result

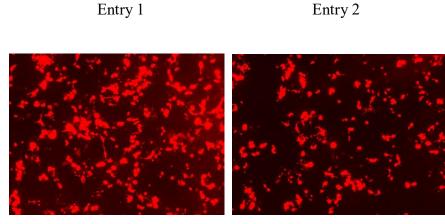
from a higher interaction rate of PEI and plasmid so more plasmids could be bound with PEI with a positive charge and enter the cell with endocytose pathway. The difference in transfection efficiency is found between the cell just revived and the cell already passaged 25 times. Furthermore, transfection efficiency is found to be higher when the experiment is performed while the cell is at 60-70% confluency than at 80% confluency or above.

To further increase transfection efficiency which might be needed in the future, more amount of plasmid could be transfected into a 10-cm dish from 5 μ g to 10 or more and the ratio of transfection reagent and plasmid should be tried further. Different kinds of methods could be used such as lipid-based transfection reagent, lipofectamine 2000, or lipofectamine 3000. Bright-field should be presented overlapping with fluorescence reporter imaging, but the data can't be read for no reason.

Entry	Amount of plasmid µg	Volume of	Opti-MEM (µL)	Incubation time
		1 mg/mL		(min)
		PEI (µL)		
1	5	15	125	15
2	5	20	125	15
3	5	25	125	15
4	5	25	200	15

Table 2. Transient transfection condition optimization





Entry 3 Entry 4 Figure 12. pU6-sgGAL4-4 transfection condition optimization

Condition of entry 2 is applied to another plasmid with eGFP and the image is presented as follows. The transient transfection of eGFP plasmid is replicated and the result of transfection efficiency seems to be reliable and fine.

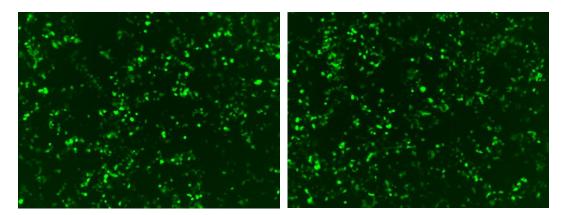


Figure 13. dCas9-OGT transfection efficiency

2.3.3 Western blot practice for future work for anti-O-GlcNAc with RL2 or CTD110.6.

To perform western blot, the cell medium is removed, and the cell is washed with PBS (could be +/+ Ca²⁺/ Mg²⁺) 2-3 times to make sure there is no rest of the medium with a little pink color which might interrupt the western blot result. The cell is then collected with 2 mL PBS in a 15 mL tube and incubated in ice for 15-20 minutes before

ultrasonication for protein extract. This step is to lower the overall temperature so cavitation bubbles from ultrasonication couldn't reach out to high temperatures and cook the proteins in lysates.

The cell is ultrasonicated until there are no pale white particles (cell) precipitate at the bottom of the tube then the protein extract could be used directly for the following western blot procedure or storage at -20 °C or -80 °C temperature. Repeat freezing and thawing should be avoided so 2 mL (or a little bit more because of the cell) could be aliquot into 40 (less or more) 0.5 mL tubes with 50 μ L protein extract for each depending on the expected repeat time of western blot or storage capacity.

15 μ L protein extract is mixed with a 5 μ L blue ladder (4X to 1X) and incubated under a condition of 90 °C for 5 minutes. Stacking gel (4%) solution is added to the stack and then add about 1 mL of isopropanol on the top evenly to make the end of the curve straight. After 30 minutes of polymerization, isopropanol is removed. Resolving gel (12%) is added to the specific height and the cap is inserted. After 30 minutes of polymerization, the cap is carefully removed, and samples are loaded into each well that the tip should be deep enough to make sure samples do not disperse/diffuse into the running buffer solution but do not destroy the gel. The protein ladder is loaded aside.

In the beginning, 120 V 1 hour is performed to separate the protein, but it does not work well. Several trials have been made to troubleshoot the problem if there is an error made in buffer preparation or gel preparation. Finally, the condition is changed to 180V for 1 hour and it works well which means it is not any problem with preparation in advance.

After protein separation, the gel is carefully removed from the stack and is cut into a shorter piece that can fit better into the sandwich for protein transfer to the PVDF membrane. 10% of methanol contained in transfer buffer is found to have better transfer ability. PVDF membrane is incubated into methanol for 15 sec for activation and is

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found to have better transfer ability too. The first time, 20 mA 2 hours is performed for the transfer condition, however, it doesn't work well for the rest of the imaging detection or even the protein ladder transfer. Hence, 150 mA 1 hour is performed (sometimes need 10-15 minutes more) to transfer the protein.

Membrane blocking is performed at room temperature for 4 hours to overnight but finally found 1 hour is enough and over blocking under room temperature conditions is not good. The membrane is then washed with wash buffer and the following primary antibody incubation; secondary antibody incubation is performed. After each time of incubation, the membrane is washed with wash buffer 3 times, 5 minutes for each. Secondary antibody incubation should be less than 2 hours. Horseradish peroxidase is conjugated with a secondary antibody so ClarityTM Western ECL substrate, 200 mL is added to the membrane for detection. 1 mL of each luminol reagent and peroxide reagent is added to the membrane with an area of around 10 cm².

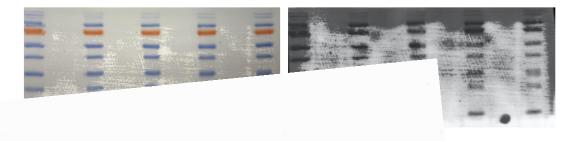


Figure 14. Western blot of GAPDH

While performing primary antibody incubation, 10 mL solution is found out that it couldn't cover all the membrane in the smallest tips covered for incubation procedure by the rocker. The middle of the membrane is found sometimes expose to the air while rocking. This could be fixed by using more primary antibody solution or a shaking mode of incubation.

Buffer preparation, gel preparation, and the procedure of membrane transfer seem not to be an issue in the picture shown below. The picture is filmed after membrane transfer and before blocking/primary antibody incubation. There is no uneven distribution of bands between each well ranging from small to large proteins and the

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line of each well of the protein ladder is straight.

Figure 15. PVDF membrane image is shown to exclude some possible errors made

2.3.4 luciferase assay

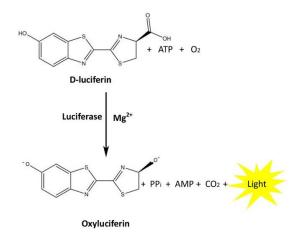
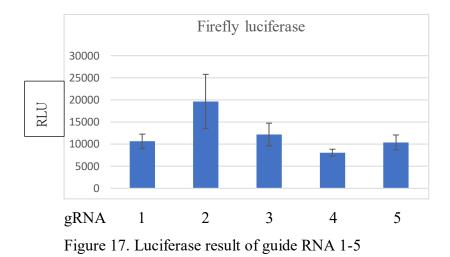


Figure 16. Brief mechanism of luciferase chemiluminescence

Luciferase, guide RNA, and the dCas9-OGT plasmid are transfected into HEK293T with PEI and the signal is detected after 48 hours of transfection. Before cell lysis and the following procedure to do the luciferase assay, the medium is removed, and the cell is washed with PBS 2-3 times until there is no pink color in the solution (medium). To make the assay result more reliable, the cell must be cultured with FBS because once the cell is cultured in starvation conditions, the cell is extremely easy to come off by PBS which is very hard to avoid losing some cells. The deviation of some part of the signal is huge and might also come from each transient transfection

efficiency or after cell lysis with the kit procedure, cell lysates from some of the sample aggregate easily even with smooth/harsh pipetting.



Those five guide RNA are constructed by our collaborator with a long oligonucleotide annealing method to construct the gRNA. Each of the oligo pairs is over 100 bp. According to the result, different gRNA designed to target the SV40 promoter results in the different protein expression levels of firefly luciferase, and the rest of the gRNA constructed by one-piece Gibson assembly would be tested and tried to find out the role of O-GlcNAc among the signaling pathway.

To improve the reliability of the assay result, further improvements to the experiment designs should be applied and written in future work.

Chapter 3: Conclusions and Discussion

3.1 Summary of work

One-piece PCR and Gibson Assembly are developed for gRNA plasmid construction which avoids using Type IIS restriction enzyme and are more feasible to construct plasmid with less limitation. In addition, it is also a relatively cost and timeefficient gRNA plasmid construction developed by optimized primer designs and the following procedure to exclude the template, and undesired bands and increase the assembly efficiency. To construct gRNA, only one forward primer is needed for different reverse primers and is confirmed to work on two backbones. The table below shows the Type IIS restriction enzyme cut site in some elements while designing plasmids.

NEB/ThermoFisher	Cut Site
BbsI/BpiI	WPRE, mCherry, VSV-G, NmCas9, BGH polyA, luciferase*,
BsaI/Eco31I	Puro, Amp, 5'-LTR, VSV-G, hGH polyA,
AlWI/BspPI	Amp, 5'-LTR, WPRE, NeoR/KanR,
BfuAI/BveI	SaCas9, PP7CP, chimeric intron, HIV-pol, nucleoplasmin NLS,
	NmCas9, luciferase*, IRES, NeoR/KanR, SaABE8e,
	SpCas9,
PaqCI/AarI	HIV-pol, chimeric intron, NmCas9, luciferase*, IRES,
BsmBI/Esp3I	Puro, gentamicin resistance, hGH polyA,

Table 3. Plasmid elements cut by different types of IIS restriction enzymes

Other cloning methods have been used to construct different plasmids for different needs including ligation, two-piece Gibson, and oligonucleotide annealing. All of them are successfully performed so there are several alternative ways to construct recombinant DNA if one has an issue. Experiments such as western blot, luciferase, transient transfection, and cell culture issues have been performed and the reasonable/possible improvement or the issue which is already confirmed is pointed out.

3.2 Experiment designs improvement

To develop a reliable luciferase assay, cells should be cultured with FBS, and experiment designs should include a second bioluminescence reporter for normalization. The second reporter should not be constructed on the same plasmid as the first luciferase or use self-cleavage peptides to construct them together. Luciferase of pMLS-SV40-Luc is replaced with luciferase PLR1. CMV-Puro-T2A-mCherry construct with gRNA is cloned into EF1 alpha-luciferase PLG3-T2A-mCherry by two-piece Gibson Assembly.¹³

In addition, experiments would be performed with a 96-well plate with control PLR1/PLG3 only transfected. After 48 hours of transfection, the cell is lysed and both substrates are added to each well at the same time, and data is collected by the plate reader.^{14,15}

	Name	Substrate	RLU wavelength
Luciferase 1	PLR1	LH2	635 ± 16 nm
Luciferase 2	PLG3	BtLH2	516 ± 10 nm

Table 4. Dual-luciferase system used for experiment design improvement

The cell line used in the project is HEK293T but not HEK293 which might be more complicated to discuss because of a non-natural protein in a mammalian cell.

3.3 Future Work

Once all gRNAs are tested triplicate in 96-well with a second reporter, the candidates with the most and least signal from PLR1 after normalization would be further tested with one or some of the following methods to try to find out which proteins are modified or less modified by O-GlcNAc.

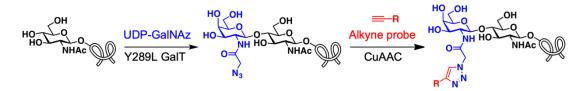


Figure 18. O-GlcNAc Chemoenzymatic labeling

3.3.1 After 48 hours of transfection with three plasmids, candidates are then added with expressed and purified Y289L GalT/ UDP-GalNAz at the end of O-GlcNAc and N-glycosidase F (PNGase F) is treated to remove GlcNAc-containing N-linked glycans. CuAAc (Copper-catalyzed azide-alkyne cycloaddition) reaction is performed to click the azide and Biotin/TAMRA conjugated alkyne for following in-gel fluorescence or biotin/streptavidin pull-down for protein identification.⁴ However, there should be more other proteins O-GlcNAcylated by dCas9-OGT after biosynthesis but are not related to the gene regulation of luciferase.

3.3.2 To lower the signal-to-noise ratio, split turbo ID and split OGA could be used to specifically find out which proteins are further modified.¹⁶ However, the scaffold RNA should be designed to insert two different stem-loop such as MS2 and PP7 which their corresponding coat proteins bound specifically. And for example, plasmid encodes PCP-Turbo(N) and MCP-Turbo(C) should be transfected at the same time. Split-OGA system developed by Woo's lab could also be used in the project to deregulate

GlcNAcylation of the proteins proximal to the genome locus.¹⁷ Selective metabolic O-GlcNAc reporter (Ac₃6AzGlcNAc) could also be used in the project for easier discovery of proteins that are O-GlcNAcylated or not.¹⁸

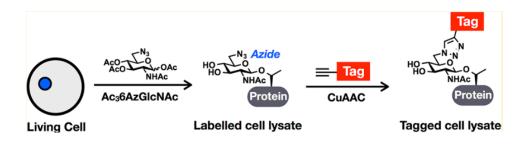
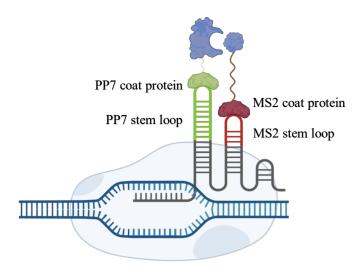


Figure 19. Newest O-GlcNAc metabolic reporter



PCP is fused with one part of turbo ID or OGA.

MCP is fused with another part of turbo ID or OGA.

Dead SpCas9 is still needed to localize genome loci.

Scaffold RNA is engineered with one PP7 stem-loop and an MS2 stem-loop.¹⁹

Figure 20. Future work: split OGA/Turbo ID system with sgRNA inserted with 2

different stem-loops

Chapter 4: Material and Methods

Gels and western blot were imaged with the BioRad ChemiDoc imaging system. Absorbances were taken using the Thermo Fisher Scientific NanoDrop One Micro-UV/Vis Spectrophotometer. Cells were monitored for viability with EVOS XL Core imaging system from Thermo Fisher Scientific and imaged with Zeiss Axio Observer A1 X-Cite Series 120 Q combination microscope.

To clone the plasmids of interest using Gibson assembly, the backbones and inserts were amplified with PCR. A 50 µL reaction of 1X Q5 reaction buffer (NEB), 200 µM dNTPs (NEB), 0.25-0.5 µM forward primer (Genewiz), 0.25-0.5 µM reverse primer (Genewiz), and 0.02 U/µL Q5 polymerase (NEB) was subjected to the thermal cycler with the following program: $[98 \degree C \text{ for } 30 \text{ s}] + [98 \degree C \text{ for } 15 \text{ s} + \text{ annealing temperature}$ for 30 s + 72 °C for (# k length + 20-50 s depends on target size)] * 30 + [72 °C for 2]min] + [4 °C hold]. The amplified product was purified by PCR purification using the QIAGEN QIAquick PCR purification kit according to the manufacturer's protocol, and the resulting DNA template concentration was quantified by UV spectroscopy at Abs₂₆₀. DNA integrity was analyzed by agarose gel electrophoresis with a 1% agarose gel, alongside the GeneRuler 1 kb Plus DNA ladder (ThermoFisher Scientific) in 1X TAE buffer at 120 V for 45 minutes stained with 0.33 μ g/mL EtBr solution. Gel extraction is performed based on the protocol of the QlAquick Gel Extraction Kit. Once the PCR products were purified and quantified, an assembly reaction comprising 1X Gibson assembly master mix (NEB), 0.03 pmol vector, and 0.06 pmol insert(s) was diluted to 10 µL with nuclease-free water. The reaction was subjected to the thermal cycler with the following program: [50 °C for 15 min] + [4 °C hold]. For one-piece Gibson assembly for sgRNA, the solution is diluted, and time is increased from 15 min to 1 h.

After the assembly reaction, 1 μ L of the product was transformed into NEB Turbo or other strain of electrocompetent *E. coli* cells using a benchtop electroporator, and the transformed cells were incubated in 1 mL SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 20 mM glucose) for recovery at 37 °C for 1 hour. After recovery, 100 μ L recovered cells were cultured on Luria broth agar plates containing 100 μ g/mL ampicillin and incubated at 37 °C for 12-16 hours for Turbo, 18-24 hours for TOP10. The following day, several single colonies were inoculated in Luria broth supplemented with 100 μ g/mL ampicillin and grown at 37 °C for 12-14 hours for Turbo, 16-24 for TOP10 at 250 rpm. The overnight cultures were then isolated and purified using the GeneJET Plasmid Miniprep kit (ThermoFisher Scientific), and the resulting plasmid concentration was quantified by UV spectroscopy at Abs₂₆₀. To confirm that the isolated plasmid was correct, restriction enzyme digests were performed following NEB's standardized protocols, and the samples were sent out for Sanger sequencing (Azenta/Genewiz) to confirm the DNA sequence.

HEK293T cells were treated in 100-mm culture dishes (Corning). Cells were transfected at approximately 70% confluency with 1 μ g/ μ L or 1 mg/mL PEI (Sigma Aldrich) and 5 μ g of the purified plasmid of interest. The cell viability was measured every 24 hours post-transfection using the bright-field microscope, and fluorescence was visualized with the fluorescent microscope after 48 hours to visualize mCherry, eGFP, and gene expression levels. HEK293T was seeded in a 10 mm dish and transfected with luciferase. Luciferase assay is detected with the protocol Pierce Firefly Luciferase Glow Assay Kit under BioTek synergy Neo-2, Gen5 Image Prime 3.10. Stacking gel (4%): 1.98 mL 30% Acrylamide/bis, 3.78 mL 0.5M Tris-HCl, pH 6.8, 9 mL diH₂O, 150 μ L 10% SDS, 15 μ L TEMED, 75 μ L 10% APS. % of the Resolving gel is adjusted based on the size of the protein of interest. Resolving gel (12%): 6.0 mL 30% Acrylamide/bis, 3.75 mL 1.5M Tris-HCl, pH 8.8. 5.0 mL diH₂O, 150 μ L 10%

SDS, 7.5 μL TEMED, 75 μL 10% APS. SDS-PAGE running buffer: 1g SDS, 3 g Trisbase, 14.4 g glycine. Transfer buffer: 3 g Tris-base, 14.4 g glycine, 900 mL diH₂O, 100 mL methanol (% of methanol in the solution could be adjusted). Transfer membrane PVDF. Blocking buffer: 1 g blotting grade blocker non-fat dry milk, 200 μL Tween 20, 50 mL 1X PBS. Wash buffer: 50 mL 1XPBS, 200 μL Tween 20.

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Chapter 6: Sequencing Results

Template										
FWD pYS1_Guide	ctaactgtaaa	gtaattgtgtgttttga	gactataagtatccct	ggagaaccaccttgttga	accaggtgtggaaagto	cccgttttagagctag	aaatagcaagttaaaat	aaggctagtccgttatca	acttgaaaaagtggcac	cgagtcggtgc
RNA A check 🖍		2,900	2,920	2,940	2,96	0	2,980	3,000	3,020	3,0
		2,900	2,920	2,940	2,96		2,980	3,000	3,020	3,0
	6 promoter		promoter	<u> </u>	Guide RNA A	linkir		gRNA scaffold		
FWD A- × sequenceprimerforopr	CTAACTGTAAA			GGAGAACCACCTTGTTG						
oject_R (1) 🖍	80	2,900	2,920	2,940 240	2,96	260	2,980	3,000	3,020	3,0
Template		200	220	240		200	200	300	320	
FWD pYS 2_Guide RNA B check 🖌	aaacagcacaaaag	zgaaactcaccctaactgta 2,880		actataagtatcccttggaga	accaccttgttgaagtccc 2,940	caggctccccagcgtttta 2,960				
	,860	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040
		2,000	2,500		BB			3,000	3,020	5,040
	mU	6		mU6 promot		ie RNA B	linkpart	gRNA scaffold		
					R	everse B (DNA)	F	1 (DNA)		
FWD B- ×	AAACAGCACAAAAG	GGAAACTCACCCTAACTGTA	AAGTAATTGTGTGTTTTGAG	ACTATAAGTATCCCTTGGAGA	ACCACCTTGTTGAAGTCCC	CAGGCTCCCCAGCGTTTTA	GAGCTAGAAATAGCAAGTTA	AAATAAGGCTAGTCCGTTAT	CAACTTGAAAAAGTGGCACCC	GAGTCGGTGCTTT
sequenceprimerforopr oject_R 🖌	,860	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040
	160	180	200	220	240	260	280	300	320	340
Template										
FWD pYS 3_Guide RNA C check 🖍	cacaaaaggaaact	tcaccctaactgtaaagtaa	ttgtgtgttttgagactata 1,540	agtatcccttggagaaccacc	ttgttgtagtgaggaggct	tttttgggttttagagcta	gaaatagcaagttaaaataa 1,620	aggctagtccgttatcaactt	gaaaaagtggcaccgagtcgg 1,660	gtgctttttttct
	1,500	1,520	1,540	1,560	1,580	1,600	1,620	1,640	1,660	
	1,500	1,520	1,540	В				1,640	1,660	
	mU6			mU6 promoter	Guide RNA	C		RNA scaffold		
FWD C- × sequenceprimerforo				AGTATCCCTTGGAGAACCACC						STGCTTTTTTC
project_R.ab1 (928 hn)	1,500	1,520	1,540 200	1,560 220	1,580 240	1,600 260	1,620 280	1,640 300	1,660 320	340
Template FWD pYS 4_Guide	cagcacaaaagga	aactcaccctaactgtaaa	taattgtgtgttttgagact	ataagtatcccttggagaacc	accttgttgaattttttt	atttatgcaggttttagag	ctagaaatagcaagttaaaa	taaggctagtccgttatcaac	ttgaaaaagtggcaccgagt	cggtgcttttt
RNA D check 🖌	0	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040
		2,880	2,900	2,920	2,940 BB	2,960	2,980	3,000	3,020	3,040
	mU6			mU6 promoter	Guid		nkpart	gRNA scaffold		
PWD D-				ATAAGTATCCCTTGGAGAACC						
sequenceprimerforopr oject_R 🖌	0	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040
Template FWD pYS 5_Guide	aacagcacaaaag	gaaactcaccctaactgtaa	agtaattgtgtgttttgag	actataagtatcccttggagaa	accaccttgttgaagtagt	gaggaggctttttgtttta	gagctagaaatagcaagtta	aaataaggctagtccgttatc	aacttgaaaaagtggcaccg	agtcggtgcttt
RNA E check 🖌	860	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040
	860	2,880	2,900	2,920	2,940 BB	2,960	2,980	3,000	3,020	3,040
	BUm			mU6 promot	Gu	ide E	linkpart	gRNA scaffold		
FWD E- × sequenceprimerforopr	AACAGCACAAAAG		AGTAATTGTGTGTTTTTGAG		ACCACCTTGTTGAAGTAGT			AAATAAGGCTAGTCCGTTATC		
oject (1) 🖍	160	2,880	2,900	2,920	2,940	2,960 260	2,980	3,000	3,020	3,040
Template				tggagaaccaccttgttgtgca						
FWD pYS 7_Guide		2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040	3
	2,880					2,980	3,000	3,020	3,040	3,
FWD pYS 7_Guide	2,880	2,900	2,920 mU6	2,940 BB promoter	2,960	linkpart				
FWD pYS 7_Guide RNA G check 🖌	2,880		mU6	BB	guide g	linkpart	gRNA scaffc		CACCGAGTCCGTCCTTTTTT	CTCGAGTACTAC
FWD pYS 7_Guide RNA G check 🖌	2,880		mU6	BB promoter TGGAGAACCACCTTGTTGTGCA	guide g	linkpart			CACCGAGTCGGTGCTTTTTT	
FWD pYS 7_Guide RNA G check 🖍	2,880 × ACTCACCCTAAC	CTGTAAAGTAATTGTGTGTT	mU6 TTGAGACTATAAGTATCCCT	BB	guide g	linkpart	AGTTAAAATAAGGCTAGTCCI	GTTATCAACTTGAAAAAGTGG		
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FWD pYS 7_Guide RNA G check ✓ FWD G1- sequenceprimetror projectab10049 bpj (I lears chouseishu Template	2,880 × ACTCACCCTAAC 2,880 180	статалаатна тагаатат 2,990 200	mU6	BB promoter TGGAGAACCACCTTGTTGTGC/ 2,940 240	guide g AGAGGCCGAGGCCGCCTGT 2,960 260	linkpart	AGTTAAAATAAGGCTAGTCCI 3,000 300	5TTATCAACTTGAAAAAGTGG 3,020 320	3,040	3,
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FWD pYS 7_Guide RNA G check / Sequenceprimeforo projectabl (1045 pp) (I lears choruxichu Template FWD pYS 8_Guide RNA H check /	× ACTCACCTANG 2,880 2,880 2,880 2,880 100 100 0 0 0	200 200 200 200 200 200 200 200 200 200	mUG TTGAGACTATAGTATCCCT 2,920 228 gtaattgfgtgttttgagac 2,990 2,990	BB prosocter TGGAGAACCACCTTGTTGTGC 2,940 240 240 240 240 240 240 240 2	guide g RAAGGCCCAGCCCCCTGT 2,960 260 260 2,940 2,940 BB guide	linkpart ITTAGAGCTAGAATAGCA 2,980 280 Ittggaggc-tgttttaga 2,960 2,960 2,960	AGTTAMATAAGGCTAGTCCC 3,000 300 gctagnaatagcaagttaaa 2,980 2,980 2,980 1mkpart	GTATCAACTIGAAAAAGTGG 3,220 320 ataaggetagtcogttatcaa 3,600 3,600 gRNA scaffold	3,040 340 hcttgaaaagtggcaccgag 3,020 3,020	3, ttcggtgctttttt 3,040 3,040
 FWD GI- sequenceprimerforo projectab1 (049 pp) (1 liters charavielu) Template FWD pYS 8_Guide 	× ACTCACCTANG 2,880 2,880 2,880 2,880 100 100 0 0 0	200 200 200 200 200 200 200 200 200 200	mUG TTGAGACTATAGTATCCCT 2,920 228 gtaattgfgtgttttgagac 2,990 2,990	BB promoter 2,940 240 240 240 240	guide g RAAGGCCCAGCCCCCTGT 2,960 260 260 2,940 2,940 BB guide	linkpart ITTAGAGCTAGAATAGCA 2,980 280 Ittggaggc-tgttttaga 2,960 2,960 2,960	AGTTAMATAAGGCTAGTCCC 3,000 300 gctagnaatagcaagttaaa 2,980 2,980 2,980 1mkpart	GTATCAACTIGAAAAAGTGG 3,220 320 ataaggetagtcogttatcaa 3,600 3,600 gRNA scaffold	3,040 340 hcttgaaaagtggcaccgag 3,020 3,020	3, ttcggtgctttttt 3,040 3,040

46916_pU6-sgGAL4-4 Guide RNA: A, B, C, D, E, G, H, I, J, K, L

FWD pYS 9_Guide	aaaaggaaactcaccctaactgt	aaagtaattgtgtgttttga	gactataagtatcccttgga	agaaccaccttgttggagc	tattccagaagtagtggtt	ttagagctagaaatagcaagtta	aaataaggctagtccgtt	atcaacttgaaaaagtggc	accgagtcggtgctttt	tttctcga
IA I check 🖍	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040	
	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040	
				BB						
	mU6		mU6 pro	moter	guide I	linkpart	gRNA scaffold			
VD 16- ×	AAAAGGAAACTCACCCTAACTGT	AAAGTAATTGTGTGTTTTGA	GACTATAAGTATCCCTTGGA	AGAACCACCTTGTTGGAGC	TATTCCAGAAGTAGTGGTT	TTAGAGCTAGAAATAGCAAGTTA	AAATAAGGCTAGTCCGTT	ATCAACTTGAAAAAGTGGC	ACCGAGTCGGTGCTTTT	TTTCTCG
quenceprimerforo bject.ab1 (959 bp)	2,880	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040	
leare chouvushu	180	200	220	240	260	280	300	320	346	
mplate										
WD pYS 10_Guide	ctcaccctaactgtaaagtaat	tgtgtgttttgagactataa	gtatcccttggagaaccacc	ttgttgcggagttagggg	cgggactagttttagagcta	agaaatagcaagttaaaataagg	ctagtccgttatcaactt	gaaaaagtggcaccgagtc	ggtgctttttttctcga	gtactag
IA J check 🖌	2,880 2	,900 2	,920 2	940	2,960	2,980 3	, 000	3,020	3,040	З,
	2,880 2,	,900 2	,920 2	,940	2,960	2,980 3	, 000	3,020	3,040	3,
			mU6 promoter	8	14-14	part				
			mus promoter	guide			scaffold			
WD J1-		TGTGTGTTTTGAGACTATAA	GTATCCCTTGGAGAACCACC	TTGTTGCGGAGTTAGGGG	CGGGACTAGTTTTAGAGCT	AGAAATAGCAAGTTAAAATAAGG	CTAGTCCGTTATCAACTT	GAAAAAGTGGCACCGAGTC	GGTGCTTTTTTTCTCGA	GTACTAC
quenceprimerforo	2,880 2	,900 2	,920 2	2,940	2,960	2,980 3	, eeo	3,020	3,040	З,
oject (1).ab1 (1147	180 20	20 2	20 2	40	260	280 3	00	320	340	36
FWD pYS 11_Guide	ctgtaaagtaattgtgtgtttt 2,900	tgagactataagtatccctt 2,920	ggagaaccaccttgttgttt 2,940	tatttatgcagaggccgg 2,960	ttttagagctagaaatagca 2,980	aagttaaaataaggctagtccgt 3,000	tatcaacttgaaaaagtg 3,020	gcaccgagtcggtgctttt 3,040	tttctcgagtactagga 3,06	
FWD pYS 11_Guide										0
FWD pYS 11_Guide	2,900	2,920	2,940 2,940 BB	2,960	2,980	3,000	3,020	3,040	3,06	0
FWD pYS 11_Guide	2,900	2,920	2,940	2,960	2,980	3,000	3,020	3,040	3,06	0
FWD pYS 11_Guide	2,900	2,920 2,920 mUG p	2,940 2,940 BB promoter	2,960 2,960 guide K	2,980 2,980 linkpart	3,000 3,000 gRNA scaffol	3,020 3,020	3,040	3,06	0
WD pYS 11_Guide NA K check 🖍	2,900	2,920 2,920 mUG p	2,940 2,940 BB promoter	2,960 2,960 guide K	2,980 2,980 linkpart	3,000 3,000 gRNA scaffol	3,020 3,020	3,040	3,06	0 0 TCCATT/
FWD K4- equenceprimerforo roject.abf (1026 bp)	2,900 2,900 2,900	2,920 2,920 mUG p	2,940 2,940 BB promoter GGAGAACCACCTIGTIGTITT	2,960 2,960 guide K	2,980 2,980 linkpart	3,000 3,000 gRNA scaffol	d TATCAACTTGAAAAAGTG	3,040 3,040 GCACCGAGTCGGTGCTTTTT	3,06	0 0 TCCATT
FWD pYS 11_Guide RNA K check FWD K4- equenceprimerforo roject.ab1 (1026 bp) Users chouwishu	2,900 2,900 × CTGTAAAGTAATTGTGTGTTT 2,900	2,920 2,920 mUG p TGAGACTATAAGTATCCCTT 2,920	2,940 2,940 BB promoter GGAGAACCACCTTGTTGTTT 2,940	2,960 2,960 guide K TATTTATGCAGAGGCCGG 2,960	2,980 2,980 linkpart TTTTAGAGCTAGAAATAGC/ 2,980	3,000 3,000 <u>gRNA scaffol</u> AGTTAAAATAAGGCTAGTCCGT 3,000	3,020 3,020 d TATCAACTTGAAAAAGTG 3,020	3,040 3,040 GCACCGAGTCGGTGCTTTTT 3,040	3, 66 3, 86 TTTCTCGAGTACTAGGA 3, 86	0 0 TCCATT/
EWD bYS 11_Guide INA K check EWD K4- equenceprimerforo rojectabi (1026 bp) Users chousebu emplate	2,900 2,900 2,900 × CTGTAAGTAATTGTGTGTTTT 2,900 200	2,920 2,920 mU5 p TGAGACTATAAGTATCCCTT 2,920 220	2,940 2,940 BB promoter 2,940 2,940 2,940 240	2,960 2,960 guide K TATTTATGCAGAGGCCGG 2,960 250	2,980 2,980 linkpart TTTTAGAGCTAGAAATAGC/ 2,980 280	3,000 3,000 <u>gRNA scaffol</u> AGTTAAAATAAGGCTAGTCCGT 3,000	3, 020 3, 020 d TATCAACTTGAAAAAGTG 3, 020 3, 020 3, 020	3, 040 3, 040 GCACCGAGTCGGTGCTTTT 3, 040 340	3,00 3,00 TTTCTCGAGTACTAGGA 3,00 360	0 0 TCCATT/ 0
FWD pYS 11_Guide NNA K check ✓ FWD K4- equenceprimefroro project.ab (1026 bp) Libers, choruserbu utemplate	2,900 2,900 2,900 × CTGTAAGTAATTGTGTGTTTT 2,900 200	2,920 2,920 mU5 p TGAGACTATAAGTATCCCTT 2,920 220	2,940 2,940 BB promoter 2,940 2,940 2,940 240	2,960 2,960 guide K TATTTATGCAGAGGCCGG 2,960 250	2,980 2,980 linkpart TTTTAGAGCTAGAAATAGC/ 2,980 280	3,000 3,000 gRNA scaffol gRNA scaffol 3,000 300	3, 020 3, 020 d TATCAACTTGAAAAAGTG 3, 020 3, 020 3, 020	3, 040 3, 040 GCACCGAGTCGGTGCTTTT 3, 040 340	3,00 3,00 TTTCTCGAGTACTAGGA 3,00 360	0 0 TCCATTA 0
FWD pYS 11_Guide RNA K check ✓ FWD K4- Requenceprimefror projectabl (1026 bp) 1 Isers: chouserbui Isers: chouserbui Isers: chouserbui Isers: chouserbui	2,900 2,900 2,900 2,900 2,900 200 200 200 200 2,800 2,800 2,800	2,520 2,520 mUG p rGAGACTATAAGTATCCCTT 2,520 220 aattgtgtgttttgagacta 2,900	2, 949 2, 949 88 900000000000000000000000000000000	2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,940	2,980 2,980 11nkpart 11TTAGAGCTAGAATAGC 2,980 280 280	3,000 3,000 gRNA scaffol gRNA scaffol 3,000 300 gctagaaatagcaagttaaaata 2,980	3,020 3,020 TATCAACTTGAAAAAGTG 3,020 320 320 320 3,020 3,020 3,020 3,020	3, 040 3, 040 3, 040 340 340 340 3, 040 340	3,00 3,00 1TTCCCGAGTACTAGGA 3,00 360 101 101 3,040	0 0 TCCATTA 0
FWD pYS 11_Guide RNA K check ✓ FWD K4- Bacquenceprimerforo rojectabi (1026 bp) Itsers: characuthu Template FWD pYS 12_Guide	2,900 2,900 × CTGTAAAGTAATTGTGTGTTTT 2,900 200 aaactcaccctaactgtaaagt	2,920 2,920 2,920 1046 p 1046 p 1046 p 1046 p 1046 p 2,920 2,920 2,920 2,920 2,920	2,940 2,940 BB promoter 2,940 2,940 2,940 2,940 2,940	2,960 2,960 2,960 TATTATGCAGAGGCCGG 2,960 260	2,980 2,980 1inkpart TTTTAGAGCTAGAATAGC/ 2,980 280 280	3,000 3,000 BNA scaffol MGTTAAATAAGCTAGTCCGT 3,000 300 300	3,020 3,020 TATCAACTTGAAAAAGTG 3,020 320 320	3,040 3,040 3,040 3,040 3,040 340	3,00 3,00 TTTCTCGAGTACTAGGA 3,00 360	0 0 TCCATTA 0
FWD pYS 11_Guide NNA K check ✓ FWD K4- equenceprimefroro project.abt (1026 bp) Libers, choruserbu utemplate FWD pYS 12_Guide	2,900 2,900 2,900 2,900 2,900 200 200 200 200 2,800 2,800 2,800	2,520 2,520 mUG p rGAGACTATAAGTATCCCTT 2,520 220 aattgtgtgttttgagacta 2,900	2,940 2,940 BB GGAGAACCACCTIGITI 2,940 240 taagtatcccttggagaac 2,920	2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,960 2,940 2,940 88 6	2,580 2,580 11TTLAGACTAGAATAGC/ 2,580 280 280 2,560 2,560	3,000 3,000 gRNA scaffol MGTTAAAATAAGCCTAGTCGGT 3,000 300 gctagaaatagcaagttaaaata 2,980 2,980 2,980	3,028 3,028 d TATCAACTTGAAAAGTG 3,028 320 320 3,020 3,020 3,020 3,020 3,020 3,020 3,020	3, 040 3, 040 3, 040 340 340 340 3, 040 340	3,00 3,00 1TTCCCGAGTACTAGGA 3,00 360 101 101 3,040	0 0 TCCATTA 0
FWD p/S 11_Guide RNA K check FWD K4- Lequenceprimerforo vojectab1 (026 bp) Target Provincerbur Template RNA L check	2,900 2,900 2,900 2,900 2,900 200 200 200 200 2,800 2,800 2,800	2,920 2,920 mU6 p TGAGACTATAAGTATCCCTT 2,920 220 220 2,920 2,920 2,920 2,920 2,920 2,920	2,940 2,940 2,940 BB GGGAACCACCTIGITIGITI 2,940 240 240 2,94	2,960 2,960 2,960 2,960 2,960 260 260 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,95	2,980 2,980 11nkpart 11TTACAGCTAGAATAGCC 2,980 280 2,960 2,960 2,960	3,000 3,000 3,000 300 300 300 300 300 30	3,020 3,020 TATCAACTTGAAAAGTG 3,020 320 3,020 3,020 3,020 3,020 3,020 3,020 3,020	3, 040 3, 040 6CACCGAGTCGGTGCTTTTT 3, 040 340 1000 3, 020 3, 020	3,00 3,00 3,00 360 100 3,040 3,040	0 0 TCCATTA 0
FWD K4- sequenceprimerforo project.ab1 (1026 bp) I lisers chomolishu Template FWD pYS 12_Guide RNA L check 🖍	2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,900 2,800 2,880 2,880 2,880	2,920 2,920 mU6 p TGAGACTATAAGTATCCCTT 2,920 220 220 2,920 2,920 2,920 2,920 2,920 2,920	2,940 2,940 2,940 BB GGGAACCACCTIGITIGITI 2,940 240 240 2,94	2,960 2,960 2,960 2,960 2,960 260 260 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,940 2,95	2,980 2,980 11nkpart 11TTACAGCTAGAATAGCC 2,980 280 2,960 2,960 2,960	3,000 3,000 3,000 300 300 300 300 300 30	3,020 3,020 TATCAACTTGAAAAGTG 3,020 320 3,020 3,020 3,020 3,020 3,020 3,020 3,020	3, 040 3, 040 6CACCGAGTCGGTGCTTTTT 3, 040 340 1000 3, 020 3, 020	3,00 3,00 3,00 360 100 3,040 3,040	0 0 TCCATTA 0

46916_pU6-sgGAL4-4 Guide RNA (1 AarI inserted)

Template FWD Aarl checked	actataagtatcccttgg	agaaccaccttgttggcgcac	ctgcataagttttagagctag	gaaatagcaagttaaaata	aggctagtccgttatcaactt	gaaaaagtggcaccgagtcgg	gtgetttttttetegagtae	taggatccattaggcggccg	cgtggataaccgtattaccgc	catgcat
/	2,920	2,940	2,960	2,980	3,000	3,020	3,040	3,060	3,080	3
	2,920	2,940	2,960	2,980	3,000	3,020	3,040	3,060	3,080	3
					scaffold RNA					
			section	4	K K	section	2			
		BB	section 3	3		section 1				
▼ FWD Aa1- ×	ACTATAAGTATCCCTTGG	AGAACCACCTTGTTGGCGCAC	CTGCATAAGTTTTAGAGCTAG	GAAATAGCAAGTTAAAATA	AGGCTAGTCCGTTATCAACTT	GAAAAAGTGGCACCGAGTCG	STGCTTTTTTTCTCGAGTAC	TAGGATCCATTAGGCGGCCG	CGTGGATAACCGTATTACCGC	CATGCAT
sequenceprimerforo	2,920	2,940	2,960	2,980	3,000	3,020	3,040	3,060	3,080	3
project.ab1 (935 bp)	2,520	2,510	_,	2,200	2,500	-,-20	-,	1,100	-,	
(Users chouvushu	220	240	260	280	300	320	340	360	380	

gRNA sgRNA (MS2): A, B, C, D, E, G, H, I, J, K, L

•	Template										
	FWD gRNA A	ttgtggaaaggacga	acaccaccaggtgtggaaag	tccccgttttagagctag	gccaacatgaggatcacccat	gtctgcagggcctagcaag	ttaaaataaggctagtccgtt	atcaacttggccaacatgag	gatcacccatgtctgcagg	gccaagtggcaccgagtcgg	tgcttttttgaat
	sgRNA(MS2) ck 🖋	240	260	280	300	320	340	360	380	400	420
		240	260	280	300	320	340	360	380	400	420
					MS2 sloop	>					
			gRNA A	linkart	source			MS2 :	sloop		
_		TTCTCCAAACCACCA		TOCOCCTTTTACACCTAC	GCCAACATGAGGATCACCCAT	CTCTCCACCCCCTACCAAC	TTAAAATAACCCTACTCCCT	ATCAACTTCCCCAACATCAC	CATCACCOATCTCCACC	CCCANCTOCOCOCOCOCOC	TCCTTTTTTCAAL
	FWD A1-	Transadancara	hencencenderererer	rececutititianacina						occano roocaccono reou	
	MSFYSsequencing	240	260	280	300	320	340	360	380	400	420
	(1).ab1 (1060 bp)	200	220	240	260	280	300	320	340	360	380
	Template	cttgtggaaggacg	aacaccaagtocccaggoto	cccarcettttagageta	receased rarrat cacces	tetctecagegectageaa	gttaaataaggctagtccgt	tatcaacitggccaacatg	aggatcacccatgtctgcag	gccaagtggcaccgagtog	gtgcttttttgaa
•	Template FWD gRNA B				ggccaacatgaggatcaccca						
•	Template	cttgtggaaaggacg 240	aaacaccaagtccccaggctc 260	cccagcgttttagagcta 280	ggccaacatgaggatcaccca 300	tgtctgcagggcctagcaa	gttaaaataaggetagteegt 340	tatcaacttggccaacatga 360	aggatcacccatgtctgcag 380	ggccaagtggcaccgagtcg 400	gtgcttttttgaa 426
•	Template FWD gRNA B										
•	Template FWD gRNA B	240	260 260	280	300 300 MS2 sloop	320	340	360	380	400	420
•	Template FWD gRNA B	240	260	280	300 300 MS2 sloop	320	340	360	380	400	420
•	Template FWD gRNA B sgRNA(MS2) ck 🖌	240	260 260 gRNA B	280 280 linkar	300 300 MS2 sloop t source	320	340 340	360 360 MS2	380 380 5loop	400	420
•	Template FWD gRNA B sgRNA(MS2) ck 🖌	240	260 260 gRNA B	280 280 linkar	300 300 MS2 sloop	320	340 340	360 360 MS2	380 380 5loop	400	420
•	Template FWD gRNA B sgRNA(MS2) ck 🖌	240	260 260 gRNA B	280 280 linkar	300 300 MS2 sloop t source	320	340 340	360 360 MS2	380 380 5loop	400	420
•	Template FWD gRNA B sgRNA(MS2) ck /	240 240 CTTGTGGAAAGGACG	260 260 gRNA B	280 280 linkar	300 300 MS2 sloop t source	320 320 TGTCTGCAGGGCCTAGCAA	340 340	360 360 MS2	380 380 5loop	400 400 GGCCAAGTGGCACCGAGTCG	42¢ 42¢

FWD gRNA C	atcttgtggaaaggad	gaaacacctagtgaggaggc	ttttttgggttttagagctag	ggccaacatgaggatcaccca	tgtctgcagggcctage	caagttaaaataaggctagtccs	ttatcaacttggccaacatg	aggatcacccatgtcts	cagggccaagtggcaccgagtcgg	gtgcttttt
sgRNA(MS2) ck 🖌	240	260	280	300	320	340	360	380	400	
	240	260	280	300	320	340	360	380	400	
		gRNA (linkart	MS2 sloop			MS2	2 sloop		
FWD C1-	× ATCTTGTGGAAAGGAG	GAAACACCTAGTGAGGAGGC	TTTTTTGGGTTTTAGAGCTAG	SOUFCE SGCCAACATGAGGATCACCCA	TGTCTGCAGGGCCTAGC	CAAGTTAAAATAAGGCTAGTCCC	GTTATCAACTTGGCCAACATG	GAGGATCACCCATGTCTC	CAGGGCCAAGTGGCACCGAGTCG	GTGCTTTT
MSFYSsequencing.a	240	260	280	300	320	340	360	380	400	
b1 (1090 bp) (Users chouvushu	200	220	240	260	280	300	320	340	360	
			nd Mismatches < 🗴					Add Comment	Sort By • Export	· 6
SFYSsequencing.at	Rename Reali		na mismatches (, 			, ,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		Soft By C Export	
FWD gRNA D	ttgtggaaaggacgaaa	caccaattttttttatttat	caggttttagagctaggcca	acatgaggatcacccatgtc	tgcagggcctagcaagt				ggccaagtggcaccgagtcggtgc	ctttttt
sgRNA(MS2) ck 🖍	240	260	280	300	320	340	360	380	400	
	240	260	280	300	320	340	360	380	400	
		gRNA D	linkpart	MS2 sloop			MS2 s.	loop		
FWD D1-	× TTGTGGAAAGGACGAAA	CACCAATTTTTTTTTTTTTTTTT	CAGGTTTTAGAGCTAGGCCA		TGCAGGGCCTAGCAAGT	TTAAAATAAGGCTAGTCCGTTA	TCAACTTGGCCAACATGAGG/	ATCACCCATGTCTGCAG	GGCCAAGTGGCACCGAGTCGGTGC	сттттттт
MSFYSsequencing.a b1 (1079 bp)	240	260	280	300	320	340	360	380	400	
Users chouvushu		220 2	40 2	60 2	80	300	320	340	360	380
MSFYSsequencing.at	Rename Rea	lign In Sync 😗 F	ind Mismatches <	>			4	Add Comment	Sort By • Export	• \$
Template										
				ccaacatgaggatcacccatg	CCCRCaRREccraRcas	aktraaaaraakkcrakteekt	rarcaactiggccaacaigag	ggateacceatgretge	agggccaagtggcaccgagtcggt	gettett
FWD gRNA E sgRNA(MS2) ck				300	320	340	360	380	400	
	240	260	280	300	320	340	360	380	400	
	240	260 260	280				360	380		
	240	260 260 gRNA E	280 280 linkart	300 MS2 sloop source	320	340	360 MS2 :	380 sloop	400	
sgRNA(MS2) ck 🖌	248 248 × TCTTGTGGAAAAGGACG/	260 260 gRNA E	280 280 linkart	300 MS2 sloop source	320	340	360 MS2 :	380 sloop	400	GCTTTTTT
sgRNA(MS2) ck ✔ FWD E1- MSFYSsequencing.a b1 (1060 bp)	240	260 260 gRNA E 04ACACCAAGTACTCAGGAGG 260	280 280 linkart	300 MS2 sloop source cccacatGagGATCACCCATC 300	320 STCTGCAGGGCCTAGCA/ 320	340 AGTTAAAATAAGGCTAGTCCGT 340	360 MS2 : TATCAACTTGGCCAACATGAC 360	380 Sloop SGATCACCCATGTCTGCA 380	400 AGGGCCAAGTGGCACCGAGTCGGT 400	_
sgRNA(MS2) ck FWD EI- MSFYSsequencing.a b1 (1060 bp) (Lisers, chourushu	240 240 240 × TCTTGTGGAAAGGACGA 240	260 260 gRNA E 260 260 220	280 280 1 linkart CTTTTGTTTTAGAGCTAGGG 280 240	300 M52 sloop source CCAACATEAGGATCACCCATO 300 260	320	340	TATCAACTTGGCCAACATGAC 360 320	388 sloop GGATCACCCATGTCTGC/ 388 348		380
SgRNA(MS2) ck / FWD EI- MSFYSsequencing.a b1 (1060 bp) (11sers choroushu ASFYSsequencing (1)	248 248 × TCTTGTGGAAAAGGACG/	260 260 gRNA E 260 260 220	280 280 linkart	300 M52 sloop source CCAACATEAGGATCACCCATO 300 260	320 STCTGCAGGGCCTAGCA/ 320	340 AGTTAAAATAAGGCTAGTCCGT 340	TATCAACTTGGCCAACATGAC 360 320	380 Sloop SGATCACCCATGTCTGCA 380	400 AGGGCCAAGTGGCACCGAGTCGGT 400	380
sgRNA(MS2) ck / FWD E1- MSFYSsequencing,a b1 (1060 bp) (11kers choiseshi) 4SFYSsequencing (1) Template FWD gRNA G	× TCTTGTGGAAAGGACGA 240 Rename Real	260 260 8RNA E 8RNA E 260 260 220 gn In Sync • Fi	280 280 11nkart CTITIGTITIAGAGCTAGGG 280 240 nd Mismatches < 2	380 MS2 sloop source ccAACATGAGGATCACCCATG 380 260	320 320 320 320 280	340 MGTTAMATAAGGCTAGTCCGT 340 300	360 MS2 : 1ATCAACTTGGCCAACATGAC 360 320	380 sloop 36ATCACCCATGTCTGC. 380 340 Add Comment		380
sgRNA(MS2) ck / FWD E1- MSFYSsequencing,a b1 (1060 bp) (11kers choiseshi) 4SFYSsequencing (1) Template FWD gRNA G	× TCTTGTGGAAAGGACGA 240 Rename Real	260 260 8RNA E 8RNA E 260 260 220 gn In Sync • Fi	280 280 11nkart CTITIGTITIAGAGCTAGGG 280 240 nd Mismatches < 2	380 MS2 sloop source ccAACATGAGGATCACCCATG 380 260	320 320 320 320 280	340 MGTTAMATAAGGCTAGTCCGT 340 300	360 MS2 : 1ATCAACTTGGCCAACATGAC 360 320	380 sloop 36ATCACCCATGTCTGC. 380 340 Add Comment	400 AGGECCAAGTGGCACCGAGTCGGT 400 360 Sort By • Export	380
sgRNA(MS2) ck / FWD E1- MSFYSsequencing,a b1 (1060 bp) (11kers choiseshi) 4SFYSsequencing (1) Template FWD gRNA G	249 249 * TCTTGTGGAAAGGACG 249 Rename Reali	260 260 260 260 260 220 20 10 10 5ym 0 Fi	280 280 11nkart 280 240 nd Mismatches < 1 gcctgttttagagctaggecc	300 HSZ sloop Source CCAACATGAGGATCACCCATG 300 200 300 300	320 TICTGCAGGGCCTAGCAN 320 280 280	340 MGTTAMATAMGGCTAGTCCGT 340 300	360 MS2 TATCAACTIGGCCAACATGAC 360 320 atcaacttggccaacatgag	380 sloop 380 340 Add Comment gatcaccatgtctgca	400 AGGECCAAGTGGEACCGAGTCGGT 400 360 Sort By • Export gggeccaagtggcaccgagtcggt	380 t • t
sgRNA(MS2) ck / FWD E1- MSFYSsequencing,a b1 (1060 bp) (11kers choiseshi) 4SFYSsequencing (1) Template FWD gRNA G	248 248 x TCTTGGGAMGGACG/ 248 Rename Reall cttgtggaaaggaacgaa	260 260 9RNA E 260 260 220 9n In Symc 0 Fi acacet geogaggeo gaggeo 250	280 280 11nkart 280 240 dd Mismatches < 2 240 geotgttttagagetaggeo 280	300 HS2 5100p Source CCAACATGAGGATCACCCATG 300 300 HS2 5100p	320 320 320 280 280 280 320 320	340 MGTTAMATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtccgtt 340	360 MS2 1 360 320 320 360 320 360 360 360 360	380 SEATCACCCATGTCTGC. 380 340 Add Comment gatcacccatgtctgca 380	400 KGGCCAAGTGGCACCGAGTCGGT 400 360 Sort By • Export 8888ccaagtgcaccgagtcggt 400	380
sgRNA(MS2) ck FWD EI- MSFYSsequencing a bt (1060 bp) () lans, charanshin ISFYSsequencing () Template FWD gRNA G sgRNA(MS2) ck	x TCTTGTGGAAAGACGA 248 x TCTTGTGGAAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGTGGAAGGACGA 248 x TCTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG	260 260 260 220 220 220 220 220 10 10 250 250 250 250 250 250	280 280 280 280 240 240 addismatches < 2 280 280 280 280 280 280 280 280 280	300 HSZ 5loop Source 260 260 260 300 300 300 300 MS2 5loop Source	320 STCTGCAGGGCCTAGCAA 320 2280 Ctgcagggcctagcaag 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 gttaaaataaggctagtccgtt 340 340	360 HS2 TATCAACTTGCCAACATGAC 360 320 atcaacttggccaacatgaag 360 360 HS2 s	380 Sloop SGATCACCCATGTCTGC 380 340 Add Comment gatcacccatgtctgca 380 380 380 380 380 380 380 380	400 KGGCCAAGTGGCACGCAGTGGGT 400 360 Sort By • Export 8888ccaagtggcacgagtggcggtg 400	380 t - 4 gctttttt
syRNA(MS2) ck / FWD EI- MSFYSsequencing a b1 (1060 ba) (Likers, chonaishu USFYSsequencing () FWD gRNA G ggRNA(MS2) ck / FWD GI- ()	x TCTTGTGGAAAGACGA 248 x TCTTGTGGAAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGGAAGGACGA 248 x TCTTGTGTGTGGAAGGACGA 248 x TCTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG	260 260 260 220 220 220 220 220 10 10 250 250 250 250 250 250	280 280 280 280 240 240 addismatches < 2 280 280 280 280 280 280 280 280 280	300 HSZ 5loop Source 260 260 260 300 300 300 300 MS2 5loop Source	320 STCTGCAGGGCCTAGCAA 320 2280 Ctgcagggcctagcaag 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 gttaaaataaggctagtccgtt 340 340	360 HS2 TATCAACTTGCCAACATGAC 360 320 atcaacttggccaacatgaag 360 360 HS2 s	380 Sloop SGATCACCCATGTCTGC 380 340 Add Comment gatcacccatgtctgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCGAGTCGGTC 400 350 Sort By - Export 400 400 400	380 t - 4 gctttttt
sgRNA(MS2) ck /	248 248 248 248 x TCTTGTGGAAGGACGACGACGACGACGACGACGACGACGACGACGA	260 260 260 220 220 220 220 220 220 220	280 280 280 280 280 280 280 280 280 280	300 HSZ 5loop Source 260 260 260 300 300 MSZ 5loop Source	320 STCTGCAGGGCCTAGCAM 320 280 Ctgcagggcctagcaag 320 Ctgcagggcctagcaag	340 AGTTAAAATAAGGCTAGTCCGT 340 300 gttaaaataaaggctagtcogtt 340 340 340	360 HS2 TATCAACTTGCCAACATGAC 360 320 320 360 HS2 s 40 40 40 40 40 40 40 40 40 40	380 sloop SGATCACCCATGTCTGC 380 340 Add Comment 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCGAGTCGGT 400 350 Sort By • Export 400 400 400	380 t - t
sgRNA(MS2) ck / FWD EI- MSFVSsequencing a bt (1060 bp) (1 liters choronichu ISFVSsequencing () Template FWD Gfi- MSFVSsequencing () abd 1084 bp) 1 liters choronichu	240 240 240 240 240 240 240 240 Cttgtggaaggaggaggaggaggaggaggaggaggaggagg	260 260 280 220 200 200 200 200 200 200 200 20	280 280 280 280 280 280 240 240 240 240 240 240 240 240 240 24	300 HS2 sloop Source CCAACATGAGGATCACCCATG 300 220 220 300 300 HS2 sloop Source 300 300 500 500 500 500 500 500	320 320 320 280 ctgcagggcctagcagg 320 320 320 280 ctgcagggcctagcagg 320 320 280 280 280 280 280 280 280 2	340 AGTTAAAATAAGGCTAGTCCGT 340 300 gttaaaataaggctagtccgtt 340 340 340 340 340 340	360 HS2 : TATCAACTTGGCCAACATGAC 360 320 320 360 360 HS2 : HS2 : 4C3ACTTGGCCAACATGAG 360 320 4C3ACTTGGCCAACATGAG 360 320	380 Sloop 380 340 Add Comment gatcaccatgtctgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCGAGTCGGTT 400 360 Sort By - Export 400 400 400 400 400 400 400 40	380 t • 1 gcttttt GCTTTTT
sgRNA(MS2) ck FWD EI- MSFVSsequencing,a bf (1060 bp) (1 lkers: chousishu SFYSsequencing () Template FWD G1- MSFYSsequencing () Jab (1084 bp) 1 lkers: chousishu 1 femplate	240 240	260 260 260 220 220 20 20 20 20 20 20 20 20 20 20	280 280 280 240 nd Mismatches < 2 260 240 240 240 240 240 240 240 240 240 24	300 HS2 5100p Source 200 200 200 300 300 MS2 5100p Source 300 300 200 200 200 200 200 200	320 320 320 320 320 320 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtccgtt 340 340 340 517AAAATAAGGCTAGTCCGTT. 340	360 HS2 1 TATCAACTTGGCCAACATGAG 360 320 AtCaaCt tggccaacatgagg 360 HS2 s ATCAACTTGGCCAACATGAG 360 HS2 s	380 Sloop GATCACCCATGTCTGC 380 340 Add Comment GatCacccatgtCtgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCGAGTGGGT 400 350 Sort By - Export 400 400 400 400	380 t - 1 gctttttt GCTTTTTT 380
sgRNA(MS2) ck FWD EI- MSFVSsequencing a b1 (1060 bp) (1 likers chownshu FWD gRNA G gRNA(MS2) ck FWD G1- mathematical sectors and a MSFVSsequencing (ab1 (1084 bp) 1 likers chownshu	240 240	260 260 260 220 220 20 20 20 20 20 20 20 20 20 20	280 280 280 240 nd Mismatches < 2 260 240 240 240 240 240 240 240 240 240 24	300 HS2 5100p Source 200 200 200 300 300 MS2 5100p Source 300 300 200 200 200 200 200 200	320 320 320 320 320 320 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtccgtt 340 340 340 517AAAATAAGGCTAGTCCGTT. 340	360 HS2 1 TATCAACTTGGCCAACATGAG 360 320 AtCaaCt tggccaacatgagg 360 HS2 s ATCAACTTGGCCAACATGAG 360 HS2 s	380 Sloop GATCACCCATGTCTGC 380 340 Add Comment GatCacccatgtCtgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCCAGTCGGTC 400 Sort By - Export 400 400 400 400 400 400 400 40	380 t - 1 gctttttt GCTTTTTT 380
SyRNA(MS2) ck / FWD EI- MSFYSsequencing a b1 (1060 bp) (Liser, chorunschu KSFYSsequencing (1) Template FWD GI- MSFYSsequencing 1, Jahr (1084 bp) Lisers, chorunschu Template FWD GI- KSFYSsequencing 1, Jahr (1084 bp) Lisers, chorunschu	240 240	260 260 260 220 220 20 20 20 20 20 20 20 20 20 20	280 280 280 280 280 280 280 280 280 280	300 HSZ 5loop Source 200 200 300 400 300 MSZ 5loop 300 MSZ 5loop 300 MSZ 5loop 300 200 200 200 200 200 200 200 200 200	320 TICTGCAGGGCCTAGCAA 320 280 CTgCAGgggCCTAGCAAG 320 320 320 320 320 320 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtcogtt 340 340 311AAAATAAGGCTAGTCCGTT. 340 300	360 HS2 1 TATCAACTTGCCAACATGAC 360 320 atCaacttggccaacatgag 360 360 HS2 s ATCAACTTGGCCAACATGAG 360 HS2 s	380 Sloop GATCACCCATGTCTGC 380 340 Add Comment gatcacccatgtctgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCCAGTCGGTC 400 Sort By - Export 400 400 400 400 400 400 400 40	380 t - 4 gctttttt GCTTTTTT 380
SgRNA(MS2) ck / FWD EI- MSFVSsequencing a b1 (0600 bp) (1 likers chorusichi FWD gRNA G gRNA(MS2) ck / FWD GFI- FWD GFI- FWD GRNA(MS2) ck / FWD GRNA(MS2) ck	248 248 248 248 249 249 249 249 249 249 249 249 240	260 260 260 220 220 20 20 20 20 20 20 20 20 20 20	280 280 280 240 nd Mamatches < 2 280 240 240 240 240 240 240 240 240 240 24	300 HS2 5100p Source 200 200 200 400 300 400 400 500 400 500 400 500 5	320 STCTGCAGGGCCTAGCAA 320 280 CTGCAGGGCCTAGCAAG 320 320 320 320 320 320 320 320	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtcogtt 340 340 340 340 340 340	360 HS2 1 TATCAACTTGGCCAACATGAG 360 320 Atcaacttggccaacatgag 360 MS2 3 ATCAACTTGGCCAACATGAG 360 MS2 3 40 ATCAACTTGGCCAACATGAG 360 MS2 3 360 MS2 3 360 360 360 360 360 360 360 36	380 Sloop GATCACCCATGTCTGC 380 340 Add Comment gatcacccatgtctgca 380 380 380 380 380 380 380 380	400 AGGGCCAAGTGGCACCCAGTCGGTC 400 500 Export 400 400 400 400 400 400 400 40	380 t - 1 gcttttt GCTTTTTT 380
sgRNA(MS2) ck / FWD EI- MSFVSsequencing, a b1(1060 bp) (Here: chousehu SFVSsequencing () FWD G1- WSFVSsequencing () ab1004 bp) (Lears: chousehu Template FWD G1- () ab1004 bp) Lears: chousehu Template FWD G1- () ab1004 bp) Lears: chousehu SgRNA(MS2) ck /	240 240	260 260 260 220 220 220 220 220 220 260 26	280 280 280 240 nd Mamatches < 2 240 240 240 240 240 240 240 240 240 24	300 HS2 sloop source 200 200 200 200 300 300 500 500 500 500 500 5	320 320 320 320 280 320 320 320 320 320 320 320 32	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtcagtt 340 340 317AAAATAAGGCTAGTCCGTT. 340 300 agttaaaataaggctagtccgt 340 340	360 HS2 1 TATCAACTTGGCCAACATGAG 360 320 Atcaacttggccaacatgag 360 HS2 s 40 360 130 140 140 360 130 140 140 140 140 140 140 140 14	380 Sloop GATCACCCATGTCTGC 380 Add Comment gatcaccatgtctgc 380 340 Sloop GATCACCCATGTCTGCA 380 340 Sloop	400 AGGECCAAGTGGCACCCAGTCGGT 400 500 Sort By - Export 400 400 400 400 400 400 400 40	380 C C C C C C C C C C C C C C C C C C C
sgRNA(MS2) ck / FWD EI- MSFVSsequencing,a bt (1060 bp) (Liear, chousehu ASPVSsequencing (t) Template FWD GI- MSFVSsequencing (t) (Liear, chousehu (Liear, chousehu (Liear, chousehu (Liear, chousehu)	240 240	260 260 260 220 220 220 220 220 220 260 26	280 280 280 240 nd Mamatches < 2 240 240 240 240 240 240 240 240 240 24	300 HS2 sloop source 200 200 200 200 300 300 500 500 500 500 500 5	320 320 320 320 280 320 320 320 320 320 320 320 32	340 AGTTAAAATAAGGCTAGTCCGT 340 300 tttaaaataaggctagtcagtt 340 340 317AAAATAAGGCTAGTCCGTT. 340 300 agttaaaataaggctagtccgt 340 340	360 HS2 1 TATCAACTTGGCCAACATGAG 360 320 Atcaacttggccaacatgag 360 HS2 s 40 360 130 140 140 360 130 140 140 140 140 140 140 140 14	380 Sloop GATCACCCATGTCTGC 380 Add Comment gatcaccatgtctgc 380 340 Sloop GATCACCCATGTCTGCA 380 340 Sloop	400 AGGGCCAAGTGGCACCCAGTCGGTC 400 500 Export 400 400 400 400 400 400 400 40	380 C C C C C C C C C C C C C C C C C C C

I1-MSFYSsequencing (1).	Rename Rea	lign In Sync 😧 F	ind Mismatches <	>			A	dd Comment	Sort By * Export	• \$ •
- Template										
FWD gRNA I	tcttgtggaaaggacga	aaacaccgagctattccagaa	gtagtggttttagagctagg	ccaacatgaggatcacccat	gtctgcagggcctagcaagt	taaaataaggctagtccgtta	atcaacttggccaacatgag	gatcacccatgtctgcagg	gccaagtggcaccgagtcgg	lgcttttttg
sgRNA(MS2) ck 🖍	240	260	280	300	320	340	360	380	400	4
	240	260	280	300	320	340	360	380	400	4
				MS2 sloop						
					missing					
				1			MS2 s	loop		
		gRNA I	linkart					1		
				source						
FWD II-	× TCTTGTGGAAAGGACGA	AACACCGAGCTATTCCAGAA	GTAGTGGTTTTAGAGCTAGG	CCAACATGAGGATCACCCAT	GTCTGCAGGGCCTAGCAAGT	TAAAATAAGGCTAGTCCGTT/	ATCAACTTGGCCAACATGAG	GATCACCCATGTCTGCAGG	GCCAAGTGGCACCGAGTCGG	IGCTTTTTTTG.
MSFYSsequencing	240	260	280	300	320	340	360	380	400	4
(1).ab1 (1090 bp)	240	200	200		520	540		500	100	
(Users chouvushu	200	220	240	260	280	300	320	340	360	380

J2-MSFYSsequencing.al	Rename Re	align In Sync 😧	Find Mismatches <	>				Add Comment	Sort By • Expo	rt • • •
Template FWD gRNA J	cttgtggaaaggacga	aacacccggagttaggggcg	ggactagttttagagctag	gccaacatgaggatcaccca	atgtctgcagggcctagcaag	ttaaaataaggctagtccgtt	atcaacttggccaacatgag	gatcacccatgtctgcagg	gccaagtggcaccgagtcgg	tgcttttttgaa
sgRNA(MS2) ck 🖋	240	260	280	300	320	340	360	380	400	42€
	240	260	280	300	320	340	360	380	400	42€
		gRNA J	linkart	MS2 sloop			MS2 s	sloop		
				source						
▼ FWD J2- ×	CTTGTGGAAAGGACG	AAACACCCGGAGTTAGGGGCG	GGACTAGTTTTAGAGCTAG	GCCAACATGAGGATCACCCA	TGTCTGCAGGGCCTAGCAAG	TTAAAATAAGGCTAGTCCGTT	ATCAACTTGGCCAACATGAG	GATCACCCATGTCTGCAGG	GCCAAGTGGCACCGAGTCGG	TGCTTTTTTTGAA
MSFYSsequencing.a b1 (1070 bp)	240	260	280	300	320	340	360	380	400	420
(Users chouvushu	200	220	240	260	280	300	320	340	360	380

K1-MSFYSsequencing.at	Rename R	ealign In Sync 🕄	Find Mismatches <	>				Add Comment	Sort By * Expe	ort • 🔹 •
Template FWD gRNA K	ttgtggaaaggacga	aaacaccttttatttatgcaga	ggccggttttagagctagg	ccaacatgaggatcacccatg	tctgcagggcctagcaag	ttaaaataaggctagtccgtt	atcaacttggccaacatgag	gatcacccatgtctgcag	ggccaagtggcaccgagtcgg	tgctttttttgaa
sgRNA(MS2) ck 🖍	240	260	280	300	320	340	360	380	400	420
	240	260	280	300	320	340	360	380	400	420
		gRNA K	linkart	MS2 sloop	>		MS2 :	sloop		
				source						
• FWD K1-	× TTGTGGAAAGGACGA	AAACACCTTTTATTTATGCAGA	GCCGGTTTTAGAGCTAGG	GCCAACATGAGGATCACCCATG	TCTGCAGGGCCTAGCAAG	TTAAAATAAGGCTAGTCCGTT	ATCAACTTGGCCAACATGAG	GATCACCCATGTCTGCAG	GGCCAAGTGGCACCGAGTCGG	TGCTTTTTTTGAA
MSFYSsequencing.a	240	260	280	300	320	340	360	380	400	420
b1 (1069 bp)										
b1 (1069 bp) (Users chouvushu	00	220	240	260	280	300	320	340	360	380
	Rename R	ealign In Sync 🕢	Find Mismatches <	>				Add Comment	Sort By • Expo	ort • • •
(Users chouvushu L1-MSFYSsequencing.ab	Rename R		Find Mismatches <	>				Add Comment	Sort By • Expo	ort • • •
Users chouvushu L1-MSFYSsequencing.ac Template	Rename R	ealign In Sync 🕢	Find Mismatches <	>				Add Comment	Sort By • Expo	ort • • •
LI-MSFYSsequencing.ab Template FWD gRNA L	Rename R	ealign In Sync 🕢	Find Mismatches <	> aggccaacatgaggatcaccca 300 300	tgtctgcagggcctagca	agttaaaataaggctagtccg	ttatcaacttggccaacatg	Add Comment	Sort By • Expo	ort ▼ ♥ ▼ ggtgcttttttga 42
LI-MSFYSsequencing.ab Template FWD gRNA L	Rename R tcttgtggaaagga 240	ealign In Sync Q cgaaacacctctgcctgctggg 260 260	Find Mismatches c gagcctggttttagagcta 280 280	> ggccaacatgaggatcaccca 300 MS2 sloop	tgtctgcagggcctagca 320	agttaaaataaggctagtccg 340	ttatcaacttggccaacatg 360 360	Add Comment aggatcaccatgtctgca 380 380	Sort By Expo	ggtgcttttttga
LI-MSFYSsequencing.ab Template FWD gRNA L	Rename R tcttgtggaaagga 240	ealign In Sync 🕢	Find Mismatches	> ggccaacatgaggatcaccca 300 MS2 sloop	tgtctgcagggcctagca 320	agttaaaataaggctagtccg 340	ttatcaacttggccaacatg 360 360	Add Comment	Sort By Expo	ggtgcttttttga
(Kers: charanshi LI-MSFYSsequencing.at. Template FWD gRNA L sgRNA(MS2) ck /	Rename R tcttgtggaaagga 240 240	ealign In Sync Q cgaaacacctctgcctgctggg 260 260	Find Mismatches < gagcctggttttagagcta 280 280 linkar	> 300 452 sloop	tgtctgcagggcctagca 320 	agttaaaataaggctagtccg 340 340	ttatcaacttggccaacatg 360 360 MS2	Add Comment aggatcaccatgtctgca 380 380 : sloop	Sort By • Expo agggccaagtggccacgagtcy 400 400	sgtgcttttttga 42
(Kers: choronshu LI-MSFVSsequencing.at. Template FWD gRNA L sgRNA(MS2) ck /	Rename R tcttgtggaaagga 240 240	ealign In Sync Q cgaaacacctctgcctgctggg 260 260 gRNA L	Find Mismatches < gagcctggttttagagcta 280 280 linkar	> 300 452 sloop	tgtctgcagggcctagca 320 	agttaaaataaggctagtccg 340 340	ttatcaacttggccaacatg 360 360 MS2	Add Comment aggatcaccatgtctgca 380 380 : sloop	Sort By • Expo agggccaagtggccacgagtcy 400 400	ggtgcttttttge 42

46919_pMLS-SV40-FLuc->46919_pMLS-CMV-FLuc

SV40-FLuc change to CMV 🖌	660		1,680		1,700		1,720		1,740		1,760	
	660	1,670	1,680	1,690	1,700	1,710	1,720	1,730	1,740	1,750	1,760	1,770
											CMV enhancer	
				pYS22 (DNA)								
				pYS22 (DNA)	pYS20	(DNA)						
REV cluc1-	TTTTT	CAATTCGCC/	AGCACAGTGG				CTAGTTATTA	ATAGTAATCA	ATTACGGGGT	CATTAGTTCA	TAGCCCATATA	ATGGAGT
REV cluc1- × Seqpromoterluc.ab1 (1021 bp)	TTTTTT 660	CAATTCGCC/	AGCACAGTGG				CTAGTTATTA	ATAGTAATCA	ATTACGGGGT	CATTAGTTCA	TAGCCCATATA 1,760	ATGGAGT

 Template 											
FWD 46919_pMLS-	GTAACAACTCCC	GCCCCATTGAC	GCAAATGGGC	GGTAGGCGTG	TACGGTGGGA	GTCTATATA	AGCAGAGCTA	AGCTCGCCAC	CATGGAAGAT	GCCAAAAACA	TTAAGAAG
SV40-FLuc change to CMV 🖌	2,220		2,240		2,260		2,280		2,300		2,320
	2,220	2,230	2,240	2,250	2,260	2,270	2,280	2,290	2,300	2,310	2,320
				С	MV promoter			Koza	.nce		
								pYS23 (DN	A)		
							pYS21				
• REV cluc1-	× GTAACAACTCCC	GCCCCATTGAC	GCAAATGGGC	GGTAGGCGTG	TACGGTGGGA	GTCTATATA	AGCAGAGCTA	AGCTCGCCAC	CA <mark>N</mark> GGAAGAT	GCCNNNNNN	NNNNNNN
Seqpromoterluc.ab1 (1021 bp)	2,220		2,240		2,260		2,280		2,300		2,320
(_Users_chouyushu_	910	920	930	940	950	960	970	980	990	1.000	1.010

46919_pMLS-SV40-FLuc to 46919_pMLS-SV40-FLuc codon-optimized

 Template FWD 46919_pMLS- 	AGGCTTTTG	CAAAAAGCTC	GCCACCATGG	AAGACGCCAA	AAACATAAAG	AAAGGCCCG	GCGCCATTCTA	TCCGCTGGAA	GATGGAACCO	GCTGGAGAGC	AACTGCATAA	GCT/
SV40-FLuc codon optimzed 🖌	2,020		2,040		2,060		2,080		2,100		2,120	
	2,020	2,030	2,040	2,050	2,060	2,070	2,080	2,090	2,100	2,110	2,120	2
								ok 1	uciferase 46.76	X		
* FWD op1-SV40-		erse back (DNA)		se F (DNA)			GCGCCATTCTA	CCCCTCC A A	CATCOALCO	CTCCACACC		CCTA
F.ab1 (1087 bp)	2,020		2,040	AAGACGCCNA	2,060	JAAAGGEEEGG	2,080	I CCGC I GGAA	2,100	GCTGGAGAGC/	2,120	36017
Downloads_op1-		20	30	40	50	60	70	80	90	100	110	120
 Template FWD 46919_pMLS- SV40-FLuc codon optimzed 	2,200	2,250	2,300	2,350	2,400	2,450	2,500	2,550	2,600	2,650	2,700	
			luci	ferase 46.76%								

FWD 46919_pMLS-	ACAACCGCGAAAA	AGTTGCGCGGAGG	AGTTGTGTTTC	STGGACGAAGTAC	CGAAAGGTCTTACCGGAA		ATCAGAGAGATCCTCAT	AAAGGCCAAGAAGGGC	GAAAGATCGCCGTGTA	ATAATGAATTAATTAAG	GAATTATCAAGCTTATCGA	TAAAATAAAAGATTI
SV40-FLuc codon	560	3,580		3,600	3,620	3,640	3,6	60	3,680	3,700	3,720	3,740
	560	3,580		3,600	3,620	3,640	3,6	60	3,680	3,700	3,720 anothe	3,740 r part
									lucife	Forward ba	ack (DNA)	
FWD op1-4luc-F.ab1	× ACAACCGCGAAAA	AGTTGCGCGGAGG	AGTTGTGTTT	GTGGACGAAGTAC	CGAAAGGTCTTACCGGAA	AACTCGACGCAAGAAAA	ATCAGAGAGATCCTCAT	AAAGGCCAAGANGGGC	M GGAAAGATCGCCGTGTA/	ATAATGAATTAATTAAG	GAATTATCAAGCTTATCGA	TAAAATAAAAGATTI
1058 bp) _Users_chouyushu_ Downloads_on1-	560	3,580	780	3,600	3,620	3,640	3,6	60 860	3,680	3,700	3,720	3,740
WD 46919_pMLS- V40-FLuc codon	CGAGTCG	ТСТТААТ	GTATAG	ATTTGAA	GAAGAGCTG	TTTCTGAGG	AGCCTTCAGG	ATTACAAGA	TTCAAAGTG	CGCTGCTGG	GTGCCAACCC	TATTCTCC
ptimzed 🖍			2,840			2,860		2,880		2,900		2,92
		2,830	2,840		2,850	2,860	2,870	2,880 and	2,890 other part	2,900	2,910	2,92
					ok luciferase 46.	. 76%						
VD op1-4luc-F.ab1	* <mark>N</mark> GAGTCG	TCTT <mark>-</mark> AT		ATTTGAA	GAAGAGCTG		AGCCTTCAGG		TTCAAAGTG		GTGCCAACCC	
			2,840			2,860		2,880		2,900		2,9
ownloads_op1-	/ILS-S		EGF	^E P ->		_pMLS		-mChe	rry	98	100	
919_pN mplate WD ReC pMLS-		V40-	EGF	⁵ P ->	46919_	_pMLS	-SV40	-mChe	rry		GGTGCACATGG	
919_pN mplate WD ReC pMLS-	TTGCAAAA	V40-	EGF CTTGCC	SP ->	46919_ CATGGTGAGC	_pMLS	-SV40-	-mChe	AAGGAGTTCAT	IGCGCTTCAAC	GGTGCACATGG 2,120	AGGGCTCCC
919_pN mplate WD ReC pMLS-		V40-	EGF	⁵ P ->	46919_	_pMLS	-SV40	-mChe	rry	TGCGCTTCAA(2,110	GGTGCACATGG 2,120 2,120	
919_pN mplate WD ReC pMLS-	TTGCAAAA	V40-	2,040	5P -> GGTGCCAC 2,050	46919_ CATGGTGAGC	_pMLS	-SV40-	-mChe	AAGGAGTTCAT	IGCGCTTCAAC	GGTGCACATGG 2,120 2,120	AGGGCTCCC
919_pN mplate WD ReC pMLS-	TTGCAAAA	V40-	EGF CTTGCC	GGTGCCAC 2,050	46919_ CATGGTGAGC	_pMLS	-SV40-	-mChe	AAGGAGTTCAT	TGCGCTTCAAC 2,110 mChe	GGTGCACATGG 2,120 2,120	AGGGCTCCC
919_pN	TTGCAAAA	V40-	38 EGF CTTGCC 2,040 2,040	GGTGCCAC 2,050	46919_ CATGGTGAGC	_pMLS	-SV40-	-mChe	AAGGAGTTCAT	TGCGCTTCAAC 2,110 mChe	GGTGCACATGG 2,120 2,120	AGGGCTCCC
wwioads_opt- 9919_pN mplate WD RC pMLS- /40-mchery ×	TTGCAAAA	V40-	2,040 2,040 2,040	2, 050	46919_ ::catggtgagc, 2,660	_pMLS AAGGGCGAGG/ 2,070		-mChe	11111111111111111111111111111111111111	2,110 80000000000000000000000000000000000	GGTGCACATGG 2,120 2,120	AGGGCTCCC 2,130
wwiloads_opt- 6919_pN wplate WD ReC pMLS- /40-mcherry / WD YS1F1ab1 V40-brr) wbsrs_chousyushu_	TTGCAAAA	V40- AGCTCAAG	2,040 2,040 2,040	2, 050	46919_ ::catggtgagc, 2,660	_pMLS AAGGGCGAGG/ 2,070		-mChe	11111111111111111111111111111111111111	Z,110 x,110x	GGTGCACATGG 2,128 2,128 2,128	AGGGCTCCC 2,130
Users_cholyusthu_ cownloads_opt- 5919_pt seplate WD_RC_PMLS- V40-mchery / Users_cholyusthu_ cownloads_V51- Template	TTGCAAAA 2,93 222 (DNA)	NG V40- AGCTCAAG	38 EGF CTTGCCC 2,040 2,040 22 2,040 22 2,040 22	GGTGCCAC	46919_ 2,660 2,660 2,660 2,660 2,660	_pMLS AAGGGCGAGG, 2,070 222 AAGGGCGAGG,	AGGATAACATC 2, 880 2, 880 AGGATAACATC 2, 880	-mChe	44GGAGTTCA1 2,100 2,100 2,100	IGCGCTTCAAG 2, 110 eChe 11 IGCGCTTCAAG	GGTGCACATGG 2, 120 2, 120 2, 120 11 GGTGCACATGG 2, 120 110	AGGGCTCCC 2, 130 AGGGCTCCC
WD YSI-FLab1 004 bp) Users_chouryush_ VWD YSI-FLab1 004 bp) Users_chouryush_ VWD YSI-FLab1 Users_chouryushu_ VWD YSI-FLab1 Users_chouryushu_ VWD YSI-FLab1 Users_chouryushu_	2,81 222 (0NA)	NG V40- AGCTCAAG	38 EGF CTTGCCC 2,040 2,040 22 2,040 22 2,040 22	GGTGCCAC	46919_ 2,660 2,660 2,660 2,660 2,660	AAGGGCGAGGA	AGGATAACATC 2, 880 2, 880 AGGATAACATC 2, 880	source	44GGAGTTCA1 2,100 2,100 2,100	rgcgcttcaad 2,118 Che 11 rgcgcttcaad	GGTGCACATGG 2,128 2,128 GGTGCACATGG 2,128	AGGGCTCCC
WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 Users_choyushu_ wmloads_YSI- Template FWD RcC pMLS-	2,81 2,22 (0NA) 222 (0NA) ATCGTGG, 2,788	AGCTCAAG	2,040 2,040 2,040 2,040 2,040 2,040 2,040 2,040 30 30 30 30 30 30 30 30 30 30 30 30 30	FP -> GGTGCCAC 2, eso New 2 GGTGCCAC 40 SCCGAGGG 2, 720	46919_ ccatggtgagc, 2,660 2,650 ccatggtgagc, 2,650 50 ccgccactcca	AAGGGCGAGG, 2,070 222 AAGGGCGAGG, 60 .CCGGCGGCAT 2,740	AGGATAACATC 2,880 2,880 AGGATAACATC 2,880 70 GGACGAGCTG1	source	AGGAGTTCAT 2,100 2,100 AGGAGTTCAT 2,100 90 ATCCATCGATA	IGCGCTTCAAG 2, 110 «Che 11 IGCGCTTCAAG 100 100 100 2, 780	GGTGCACATGG 2,120 2,120 crry 11 GGTGCACATGG 2,120 110 110	AGGGCTCCC 2, 130 AGGGCTCCC 120 TCTCCAGAA 2, 80
WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 Users_choyushu_ wmloads_YSI- Template FWD RcC pMLS-	2,81 222 (0NA)	NG V40- AGCTCAAG	38 EGF 2,640 2,640 2,640 2,640 2,640 2,640 30 6 GAACGCC	GGTGCCAC	46919_ 2,660 2,660 2,660 2,660 2,660	AAGGGCGAGGA	AGGATAACATC 2, 880 2, 880 AGGATAACATC 2, 880	source	44GGAGTTCA1 2,100 2,100 2,100	rgcgcttcaad 2,118 Che 11 rgcgcttcaad	GGTGCACATGG 2, 120 2, 120 2, 120 11 GGTGCACATGG 2, 120 110	AGGGCTCCC 2,130 AGGGCTCCC 120 TCTCCAGAA
WD YSI-FI.ab1 204 pmplate WD YSI-FI.ab1 204 pb) Users_cholyushu_ ownloads_YSI- Template FMO ReC pMLS-	2,81 2,22 (0NA) 222 (0NA) ATCGTGG, 2,788	AGCTCAAG	38 EGF 2,640 2,640 2,640 2,640 2,640 2,640 30 6 GAACGCC	FP -> GGTGCCAC 2, eso New 2 GGTGCCAC 40 SCCGAGGG 2, 720	46919_ ccatggtgagc, 2,660 2,650 ccatggtgagc, 2,650 50 ccgccactcca	AAGGGCGAGG, 2,070 222 AAGGGCGAGG, 60 .CCGGCGGCAT 2,740	AGGATAACATC 2,880 2,880 AGGATAACATC 2,880 70 GGACGAGCTG1	source	AGGAGTTCAT 2,100 2,100 AGGAGTTCAT 2,100 90 ATCCATCGATA	IGCGCTTCAAG 2, 110 «Che 11 IGCGCTTCAAG 100 100 100 2, 780	GGTGCACATGG 2,120 2,120 crry 11 GGTGCACATGG 2,120 110 110	AGGGCTCCC 2, 130 AGGGCTCCC 120 TCTCCAGAA 2, 80
WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 WD YSI-P1.ab1 Users_choyushu_ wmloads_YSI- Template FWD RcC pMLS-	2,81 2,22 (0NA) 222 (0NA) ATCGTGG, 2,788	AGCTCAAG	38 EGF 2,640 2,640 2,640 2,640 2,640 2,640 30 6 GAACGCC	FP -> GGTGCCAC 2, eso New 2 GGTGCCAC 40 SCCGAGGG 2, 720	46919_ ccatggtgagc, 2,660 2,650 ccatggtgagc, 2,650 50 ccgccactcca	AAGGGCGAGG, 2,070 222 AAGGGCGAGG, 60 .CCGGCGGCAT 2,740	AGGATAACATC 2,080 2,080 AGGATAACATC 2,080 70 GGACGAGCTG1 2,750	source	AGGAGTTCAT 2,100 2,100 AGGAGTTCAT 2,100 90 ATCCATCGATA 2,770	IGCGCTTCAA(2,110 mcheh1 11 IGCGCTTCAA(100 AAAATAAAAG/ 2,780 2,780	GGTGCACATGG 2,120 2,120 2,120 GGTGCACATGG 2,120 110 ATTTTATTTAG 2,790	AGGGCTCCC 2, 130 AGGGCTCCC 120 TCTCCAGAA 2, 80
wwioads_opt- 5919_pX mplate WD PKC-pKLS- 40-mcherry ✓ WD VS1-F1.ab1 04-bp) Users_chouyushu_ writoads_v51- Template	2,01 222 (DNA) 222 (DNA) 2,700 2,700	AGCTCAAG AGCTCAAG Ie AACAGTACI	а EGF СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТТБССС СТБС СТБСС	EP → GGTGCCAC 2, 950 New 2 GGTGCCAC 40 GGTGCCAC 40 GCCGAGGGC 2, 720 2, 720	46919_ 2,650 2,650 2,650 2,650 50 50 50 50 2,730	AAGGGCCGAGG, 2,070 222 AAGGGCCGAGG, 60 	AGGATAACATC 2,880 2,880 AGGATAACATC 2,880 70 GGACGAGCTG1 2,750 222	-mChe SGCCATCATC/ 2,090 SOURCE SGCCATCATC/ 80 SGCCATCATC/ 100 SGCCATCATCATCATCATC/ SGCCATCATCATCATCATCATCATCATCATCATCATCATCAT	AGGAGTTCAT 2,100 2,100 AGGAGTTCAT 2,100 90 ATCCATCGATA 2,770	IGCGCTTCAAG 2,118 Che 11 IGCGCTTCAAG 180 AAAATAAAAG 2,789 2,789 2,789	GGTGCACATGG 2,120 2,120 2,120 GGTGCACATGG 2,120 110 ATTTTATTTAG 2,790	AGGGCTCCC 2,139 AGGGCTCCC 120 TCTCCAGAA 2,899 2,899 2,899

Target	Forward Primer	Reverse Primer
gRNA A		CTTGCTATTTCTAGCTCTAAAACGGGGGACTTTCCACACCTGGTCAACAAGGTGGTTCTCC
gRNA B		CTTGCTATTTCTAGCTCTAAAACGCTGGGGAGCCTGGGGACTTCAACAAGGTGGTTCTCC
gRNA C		CTTGCTATTTCTAGCTCTAAAAACCCAAAAAAGCCTCCTCACTACAACAAGGTGGTTCTCC
gRNA D	AGAGCTAGAAATA	CTTGCTATTTCTAGCTCTAAAAACCTGCATAAATAAAAAAAA
gRNA E	GCAAGTTAAAAT	CTTGCTATTTCTAGCTCTAAAAAAAAAAAGCCTCCTCACTACTTCAACAAGGTGGTTCTCC
gRNA G	AAGGCTAGTCCGT	CTTGCTATTTCTAGCTCTAAAACAGGCGGCCTCGGCCTCTGCACAACAAGGTGGTTCTCC
gRNA H	TATCAACTTGAAA	CTTGCTATTTCTAGCTCTAAAACAGGCCTCCAAAAAAGCCTCCCAACAAGGTGGTTCTCC
gRNA I		CTTGCTATTTCTAGCTCTAAAACCACTACTTCTGGAATAGCTCCAACAAGGTGGTTCTCC
gRNA J		CTTGCTATTTCTAGCTCTAAAACTAGTCCCGCCCCTAACTCCGCAACAAGGTGGTTCTCC
gRNA K		CTTGCTATTTCTAGCTCTAAAACCGGCCTCTGCATAAATAA
gRNA L		CTTGCTATTTCTAGCTCTAAAACCAGGCTCCCCAGCAGGCAG

Table 5. Primer Sets for SpCas9 Scaffold RNA: 46916_pU6-sgGAL4-4 backbone

Target	Forward Primer	Reverse Primer
gRNA A		TTGGCCTAGCTCTAAAACGGGGGACTTTCCACACCTGGTGGTGTTTCGTCCTTTCC
gRNA B		TTGGCCTAGCTCTAAAACGCTGGGGAGCCTGGGGACTTGGTGTTTCGTCCTTTCC
gRNA C		TTGGCCTAGCTCTAAAAACCCAAAAAAGCCTCCTCACTAGGTGTTTCGTCCTTTCC
gRNA D	GTTTTAGAGCTAG	TTGGCCTAGCTCTAAAACCTGCATAAATAAAAAAAATTGGTGTTTCGTCCTTTCC
gRNA E	GCCAACATGAGGA	TTGGCCTAGCTCTAAAAAAAAAGCCTCCTCACTACTTGGTGTTTCGTCCTTTCC
gRNA G	TCACCCATGTCTG	TTGGCCTAGCTCTAAAACAGGCGGCCTCGGCCTCTGCAGGTGTTTCGTCCTTTCC
gRNA H	CAGGGCCTAGC	TTGGCCTAGCTCTAAAACAGGCCTCCAAAAAAGCCTCCGGTGTTTCGTCCTTTCC
gRNA I		TTGGCCTAGCTCTAAAACCACTACTTCTGGAATAGCTCGGTGTTTCGTCCTTTCC
gRNA J		TTGGCCTAGCTCTAAAACTAGTCCCGCCCCTAACTCCGGGTGTTTCGTCCTTTCC
gRNA K		TTGGCCTAGCTCTAAAACCGGCCTCTGCATAAATAAAAGGTGTTTCGTCCTTTCC
gRNA L		TTGGCCTAGCTCTAAAACCAGGCTCCCCAGCAGGGCAGAGGTGTTTCGTCCTTTCC

Table 6. Primer sets for SpCas9 Scaffold with Two MS2 Stem Loop: sgRNA (MS2) cloning backbone