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Research Article

Evaluation/Investigation of Cleavage Efficiency of sgRNA Targeting TFIIAɣ5 Gene *in vitro*

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Abstract

The CRISPR/cas9 becomes a popular editing system in a wide variety of organisms. Potential candidates of sgRNA targeted single genomic site can be predicted by accessible web-based tools However, designed sgRNA displays different efficiencies in term of guiding Cas9 to introduce double-stranded breaks (DSB) at a specific site of target DNA. Thus, one critical step is to test whether the designed sgRNA could successfully edit the gene of interest. Ribonucleoprotein (RNP) in CRISPR format (Cas9-crRNA-tracrRNA), has been found to be an efficient and rapid system to minimise a likely mistake of CRISPR-mediated gene editing. In this study, designed crRNA has been used via the system to cleave TFIIAɣ5 gene *in vitro*. The system has successfully cleaved the gene *in vitro* into two fragments with approximately~195bp and ~125bp, of which the total fragment size is expected to be~320bp (the original size of TFIIAɣ5 gene). The results revealed that a high efficiency of the CRISPR cleavage system to pre-validate crRNA designed for the TFIIAɣ5 gene. Overall, one step *in vitro* cleavage of target DNA by CRISPR/Cas9 ribonucleoprotein complex can be potentially utilised for pre-validating designed crRNAs to determine its functionality and relative efficiency prior to utilising them in gene editing study via CRISPR system.

Keywords: *CRISPR-Cas9, in vitro transcription, in vitro cleavage sgRNA, ribonucleoprotein (RNP).*

Introduction

Zinc Finger Nuclease (ZFN) and Transcription Activator–Like Effector Nuclease (TALEN) are site-specific endonucleases developed in recent years for targeted genome modifications (Briggs. *et al*., 2012; Zhang. *et al*., 2010; Zhang. *et al*., 2011). These technologies involve the designing of complex proteins which is complicated and time-consuming, and moreover the assembly of these proteins are needed for each target (Carroll. *et al*., 2006; Sanjana. *et al*., 2012). Hence a specific and effective tool/system has been developed recently called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system associated with the Cas9 endonuclease (CRISPR/Cas9) (Zafar. *et al*., 2020). Compared to ZFNs and TALENs, CRISPR/cas9 is a simpler and inexpensive technology of which introduction of a double-strand break (DSB) is achieved just by the expression of Cas9 protein and a sgRNA (additional sgRNAs for multiplex genome editing) (Pennisi, 2013; Cong. *et al*., 2013; Mali. *et al*., 2013). CRISPR/Cas9 system consists of a *Streptococcus pyogenes* derived Cas9 nuclease and a RNA duplex or single-guide RNA (sgRNA) which is formed by fusion between a CRISPR RNA (crRNA), a linker loop and a trans-activating crRNA (tracrRNA) (Deltcheva. *et al*., 2011). How the system work is based on the base-pairing between short DNA sequence adjacent to an obligate Protospacer Adjacent Motif (PAM) NGG and a short complimentary RNA sequence prior to cleave by the Cas9 protein (Mali. *et al*., 2013; Baker, 2014; Jore. *et al*., 2011) to form Double Strand Breaks (DSBs). Induced DSB then can be repaired by either Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR) to produce new form of the gene by

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replacing or deletion (Lin *et al*., 2014). Thus, efficient targeting of sequences with a PAM site to be edited is ensured by Cas9 protein and exchangeable crRNA: tracRNA duplexes.

Eventhough CRISPR/Cas system has becoming a popular research tool nowdays for genome editing, there are still parameters affecting its efficiency and accuracy. For instance, the efficiency of cleavage at the target site is possibily affected by the accessibility of the targeted DNA sequence and difficulty of chromatin structure to allow binding of the Cas9: sgRNA complex (Ann Ran. *et al*., 2013). Furthermore, if there are similarities between the target sequence and sequences in the genome, it could lead to a so-called off-target cleavage. It is also important in making sure that no random mutations are being introduced (Naeem. *et al*., 2020). For that reason, bioinformatical tools were developed to predict off-target sites co-currently when designing potential crRNA. CRISPRdirect is one of the web tools for sgRNA prediction whereby this tool efficiently predicts possible sgRNA sequences by scoring according to their off-targeting potential (Naito. *et al*., 2015). Despite these *in silico* predictions, not every sgRNA can facilitate Cas9-mediated cleavage to exhibit equivalent cleavage efficiency. Therefore, after designing possible sgRNA sequences via web-based tools, it is critical to assess their cleavage efficiencies at the target locus and identify the most efficient gRNAs *in vitro* before employing binary plasmids for genome editing *in planta* (Cong. *et al*., 2013). Hence, the aim of this study was to test the efficiency of designed sgRNA to cleave TFIIAɣ5 gene via CRISPR-Cas9 system *in vitro*. This approach could help to screen suitability of designed sgRNA prior to be used for gene editing in plant.

CRISPR/Cas9 system can be applied *in vitro* (Karvelis. *et al*., 2013). In the traditional way, crRNA and tracrRNA were artificially made in pieces, where they were joined with a linker to form as a single guide RNA (sgRNA). Recently, a RNP complex of CRISPR known as Innovative Alt-R CRISPR- Cas9 system was designed with an optimum length of crRNAs and tracrRNAs, 36 and 67 nucleotides, respectively. This new format of CRISPR/Cas9 complex has been proved as the most rapid, efficient and convenient for gene editing study (Hendel. *et al*., 2015). In conjunction to this, nowadays, there is an increasing potential for genome editing study following dozens of commercial kits developed by research companies which could save time and reduce cell toxicity upon *in vivo* application (Sung. *et al*., 2014; Aida. *et al*., 2015; Burger. *et al*., 2016).

Materials and Methods

Target Selection

The 20-nt target site on TFIIAɣ5 gene was selected using online CRISPRdirect tools programme (https://crispr.dbcls.jp). Specific rules were made in the programme to make sure the selected target sites are unique, highly specific and have fewer off-target hits.

In vitro Assembly of a Cas9 Ribonucleoprotein (RNP) Complex

Commercial kit Guide-it™ sgRNA *in vitro* transcription and screening systems (Takara Bio. USA) was used to assemble Cas9 RNP complex *in vitro*. The first step was PCR amplification by using a unique forward primer consisting of the sequences of a T7 promoter (17 bp), target region (20bp) and scaffold template (15 bp) to create the *in vitro* transcription template for generating sgRNA. PrimeSTAR max premix (2X) (12.5 µl), Guide-it scaffold template (plus reverse primer of scaffold) (1 µl), forward primer (10 µM) (0.5 µl) and RNase free water (to 25 µl) were added in a PCR tube prior to placing in a thermal cycler and PCR cycling conditions at 98°C, 10 sec and 68°C, 10sec for 33 cycles. About 5 µl PCR product/ sgRNA was electroforesed on 2% agarose to analyse prior to use as a template for *in vitro* transcription (IVT) reaction to transcribe sgRNA. For IVT reaction, the template (5 µl) was combined together with Guide-it *In vitro* transcription buffer (7 µl), Guide-It T7 polymerase mix (3 µl), and RNase free water (added up to 20 µl) prior to incubating at 37°C for 4 hours. The incubation was continued for another 15 min after adding 2 µl of recombinant DNase 1 to

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remove excess DNA in the reaction. Transcribed sgRNA generated in this step was purified using Guide-it IVT RNA Clean-Up kit (TAKARA Bio. USA). Prior to use in Cas9 cleavage assay, purified sgRNA (50ng) was assembled using Guide-it recombinant Cas9 nuclease (250 ng) by incubation at 37°C for 5 min to form RNP complex.

In vitro DNA Cleavage by sgRNA-Cas9 RNP Complex Assembly

Cleavage activity of sgRNA-Cas9 RNP complex was assayed on the TFIIAɣ5 gene. The gene contains the target site (crRNA) and the PAM sequence that for recognition by RNP.All components used for cleavage reaction which are 1 µl of purified TFIIAɣ5 gene (250 ng/µl), 1 µl of Cas9 reaction buffer (15X), 1 µl of BSA (15X), 1.5 ul of RNP complex and RNase free water (to a final volume of 15 µl) were pooled in PCR tube. The mixture was then incubated at 37°C for 1 hr followed by 80°C for 5 min. Control reaction was included by using control sgRNA together with control fragment as provided by the kit. The cleavage reaction was subsequently assessed by electrophores the product on 2% agarose gel. The cleaving results of the TFIIAɣ5 gene were compared with the untreated TFIIAɣ5 gene and control DNA (provided by the kit) and the efficiency of designed crRNA was investigated.

Results

crRNA Selection and Designing Unique Primers To Create sgRNA Template

CRISPRdirect is user-friendly software to select rational crRNA on target gene by searching against existing sequences in database in order to minimise off-target sites. The selection of potential target sequence (crRNA) by this software was based on two-fold target sequence requirement. Location of crRNA sequence must be adjacent to the 50 -NGG protospacer adjacent motif (PAM) and the sequence compulsory to be specific within the entire genome to avoid off-target editing. In this study, to test the cleavage efficiency of the crRNA, the sgRNA (crRNA + tracrRNA) needs to be created by PCR and used as a template to transcribe *in vitro* under the control of T7 promoter. Thus, unique forward primer was designed which contained crRNA, T7 promoter and Scaffold Template annealing sequences in order to amplify the sgRNA (Fig. 1).

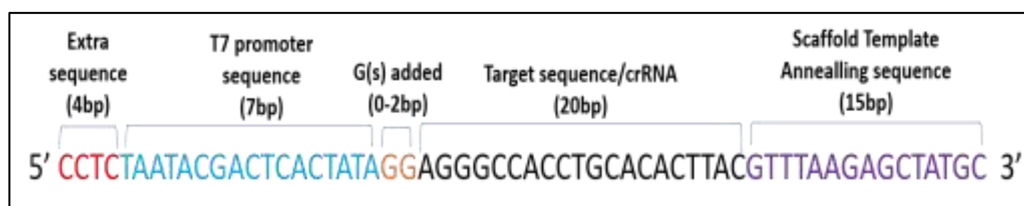

Figure 1: Sequence of forward primer for generating sgRNA *in vitro*. T7 promoter sequence was added before the crRNA to initiate the transcription reaction during the sgRNA synthesis process.

Transcription of sgRNA *in vitro* for Cas9 Cleavage Assay

Transcription template of sgRNA containing T7 promoter, crRNA and tracrRNA was generated by PCR which the components involved were unique forward primer (fig.1), scaffold reverse primer and scaffold template. An intact single PCR product with the size about ~130 bp was observed on 2% agarose gel (Fig. 2). This was the strategy used to transcribe sgRNA *in vitro* by taking linear DNA sequence (sgRNA) as a template and utilising T7 RNA polymerase to synthesise sgRNA (Fig. 2).

Figure 2: Gel electrophoresis image of sgRNA transcription template in lane 1 and the transcribed sgRNA in *vitro* in lane 3.

sgRNA-Cas9 RNP Complex Assembled *in vitro* for DNA Cleavage Assay

The experiment showed the efficiency and specificity of the resulting sgRNA-Cas9 RNP complex *in vitro*. The agarose gel electrophoresis demonstrated that cleaved TFIIAɣ5 gene fragments were at the target positions, leading to full cleaved into the two fragments with the sizes of 195 bp and 125 bp, respectively (Fig. 3). The result indicated that crRNA designed for the TFIIAɣ5 gene was active and efficient to guide Cas9 to produce DSB at the target site.

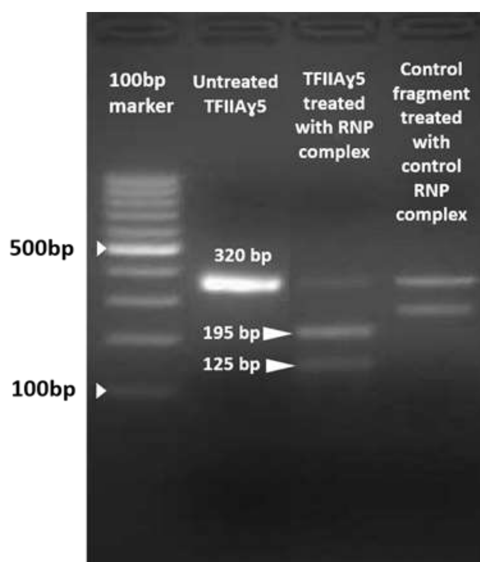

Figure 3: *In vitro* DNA cleavage by Cas9 nuclease and crRNA (CRISPR RNP) assembled *in vitro*. Lane 1: 100bp DNA ladder, Lane 2: untreated TFIIAɣ5 gene, Lane 3: TFIIAɣ5 gene treated with RNP complex, Lane 4: Control fragment treated with control RNP complex (provided by the kit).

Discussion

Testing on the specificity and efficiency of designed sgRNA are among those important steps to be performed for a successful CRISPR-Cas9 mediated gene editing study. *In vivo* application of CRISPR/Cas9 RNP complex via cell lines is normally used to investigate the efficiency of designed sgRNA of which the approach involved many complicated steps (Mehrayar. *et al.*, 2019). With CRISPR RNP complex, this system can be easily utilised *in vitro*. This is because sgRNA can be synthetically synthesised and assembled with Cas9 protein prior to

performing the Cas9 cleavage assay. The technology was able to produce a very high quality synthetic sgRNA and gave editing efficiency as high as 90%. Thus, it is unnecessary to pre-validate CRISPR/Cas9 RNP complex in respective cell lines but rather just to perform an *in vitro* cleavage assay. For instance, a previous plasmid-based approach for CRISPR-mediated genome editing, as illustrated by (Mehravar. *et al*., 2019), which was tested in the NIH3T3 cell lines, was found to be time-consuming. This was due to a series of experimental procedures were required in that system, such as preparation of cell culture, transfection, selection, DNA isolation, PCR and T7 endonuclease 1 assay for the cleavage analysis.

In order to test the functionality and relative efficiency of the CRISPR RNP system, *in vitro* cleavage of DNA was carried out in a single step by CRISPR/cas9 ribonucleoprotein complex. The coding target sequence of the TFIIAɣ5 gene that was complementary to the 5′ end of the crRNAs was designed based on the rules mentioned in the materials and methods section.

In this current study, we found *in vitro* digestion of targeted genes by CRISPR/Cas9 gene editing in RNP format provides a simple and rapid method for pre-validation/screening of crRNA/gRNA prior to delivering them into the cells. However, the shelf life of the chemically synthetic crRNA and tracrRNA of the RNP complex is considerably short (~6 months), whereby the efficiency reduces after this period. Progressive advancement on this system has managed to improve this setback where a longer shelf life of crRNA and tracrRNA of RNP complex has been achieved in some of the commercial kits, providing higher efficiency and more user-friendly system.

Conclusion

In conclusion, one-step *in vitro* cleavage of DNA by CRISPR/Cas9 ribonucleoprotein (RNP) complex is potential to be utilised for pre-validating the functionality and relative efficiency of designed crRNA, prior to its application in CRISPR/Cas9 system gene editing study. Nowadays, the CRISPR/cas9 RNP complex can be easily generated based on our requirement by using a commercial kit that can be found in the market. In this study, we used Guide-it™ sgRNA *in vitro* transcription and screening systems (TAKARA Bio. USA), and the results indicated that the kit is an ideal method for screening effective crRNA that to be used for editing the TFIIAɣ5 gene.

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