ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 4, 2016

Online - 2455-3891
Print - 0974-2441

Review Article

A REVIEW ON PRODUCTION OF PCDNA3.1-EGFP WITH CLONING PRINCIPLE METHOD

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Received: 10 May 2016, Revised and Accepted: 21 May 2016

ABSTRACT

pcDNA3.1-e green fluorescent protein (GFP) is an important compound that is already established and widely used as a marker in biomolecular works. Producing pcDNA3.1-eGFP is not very complicated. It can be inserted to *Escherichia coli* and replicate in millions. Due to the availability of *E. coli* is in Indonesia, this process should not be difficult at all. A description of the method of producing pcDNA3.1-eGFP will be covered so that many Indonesian and other researchers as well can use it and develop it in their own research. The production of pcDNA3.1-eGFP has been done by several researchers around the globe. In this review article, five relevant studies have been included. Their respective results were analyzed. The pcDNA3.1 can be expressed differently apart from the eGFP form. Therefore, it can serve different purposes, especially when the different cloning and sub-cloning processes are involved. It can be used to improve outcomes in various fields such as veterinary science, health, wellness, medicine, and even agriculture. Through the use of the cloning principle, mass production of pcDNA3.1-eGFP can be carried out easily.

Keywords: pcDNA3.1-Green fluorescent protein, Cloning, *Escherichia coli*.

INTRODUCTION

The GFP in pcDNA3.1-eGFP stands for green fluorescent protein. Deoxyribonucleic acids (DNAs) are typically made up of protein, so it makes sense for a special protein to make up a specific type of DNA add-gene such as the pcDNA3.1. The study of how pcDNA3.1-eGFP is obtained and reproduced, either naturally or by artificial means falls under the field of molecular biology. In this study, the author reviews the different methods how the pcDNA3.1-eGFP can be reproduced. Studies that focus on cloning as the primary means of creating pcDNA3.1-eGFP would be emphasized, although other means besides cloning would also be openly discussed. In this study, a detailed overview of how pcDNA-eGFP can be produced through cloning is done thanks to the use of secondary sources; a proper review of the pcDNA3.1-eGFP is first carried out to understand this compound. Afterward, the importance of a vector in the cloning process is looked at in detail and finally the cloning system is covered extensively.

UNDERSTANDINGTHE PCDNA3.1-EGFP

GFP proteins are generally composed of 238 amino acids with molecular masses of around 26.9 KD [1] and are typically used in the field of molecular biology as one of the many reporter genes. Basically, a reporter gene is a type of gene that researchers, especially in laboratory experiments, use to attach to a pre-specified sequence of the gene (oftentimes an experimental one) such as that of bacteria, plants, animals, and cell cultures. Some of the criteria that researchers use when selecting a reporter gene include, but may not be limited to, having easily identifiable and selectable markers and their ability to introduce changes that tend to be easily spotted under certain conditions. When conducting laboratory experiments in molecular biology, it would be important for researchers to know the different variables involved and the impact that they create on the experimental environment. Therefore, it makes sense to select reporter genes that possess these qualities such as the GFP [1]. In previously published studies involving the use of GFPs, including but not limited to pcDNA3.1, it has been common for researchers to introduce the GFP gene into cells using vector-based systems. In some cases, the researchers also used recombinant viruses (attaching the GFP to them). Being used as a reporter protein, the location of the target protein can be easily identified and expressed. However, in many of the laboratory experiments, the selection market of the GFPs used was not specific enough and there is often no selection market to normalize the transfection among other reactions, making it harder to reproduce results under similar conditions - an area which again falls on the reproducibility and therefore the reliability of the results. In a study carried out Izadi *et al*. [1], focus was on the creation of a GFP expression vector that contains a neo gene. A neo gene is a type of gene that often gets included in DNA plasmid creations because of their ability to create stable and replicable mammalian cell lines, which automatically makes them a good candidate for protein culturing and in some cases even cloning. This explains why many commercially available protein expressions being manufactured today, including the pcDNA3.1-eGFP in theory, contain neo as a selectable marker.

THE NEED OF VECTOR WHILE CLONING STEP

In the study Izadi *et al*. [1], a GFP gene was created by separating it from a peGFP-N1 vector and later on inserting it into the backbone of a pcDNA3.1/His/lacZ vector that also contained the neo gene. Based on the researchers' theoretical framework, the resulting gene should then contain the target GFP. Their results indicated that the resulting gene indeed contained the target GFP beside the neo gene that they apparently used as a reporter gene. Based on the study's discussion, the researchers were able to produce a new vector for GFP expression using their own framework of delivery (cloning) that contained a neomycin resistant gene. "In the absence of a selection marker, neomycin gene, in this case, the level of transfection using GFP expression vector would be different in various wells" and "by using this vector (GFP/Neo) transfection could be normalized" [1]. The total number of hours it took for the researchers to complete the transfection process was 48. In some iterations of their experiment, they also made use of the G418 antibiotic to confirm the level and specificity of antibiotic resistance – If the resulting gene was indeed resistant to the neomycin antibiotic, as well as to select only cells with the GFP expression that they are looking for and then, later on, remove cells that lack such quality. Theoretically, these methods were used to filter out the results and focus on what was needed for the study. According to the researchers of that study, this was done to come up with a higher rate of transfection normalization.

The scientists used colonies of *Escherichia coli* Top 10F strain cells that contained the peGFP-N1 vector and the pcDNA3.1/His/lacZ vector in the study. The use of *E. coli* bacterial colonies in the study was a good choice due to the fact that these colonies can be cultured easily, reproduced, and filtered, depending on the laboratory conditions created by the researchers.

First, the preparation of the plasmid DNA to be used was carried out. This was done by inoculating a single colony with a specific measurement (4.5 ml of LB solution that contains ampicillin at 100 µg/ml). The resulting suspension is then left growing at 37° C overnight. The DNA cultures were then prepared using a 4 ml culture using a tool, specifically the Accuprep Plasmid Extraction Kit manufactured by Bioneer from Korea. To systematize the results and prevent errors caused by mixing up of variables, the plasmids were analyzed using positive sampling and restriction digestion in all cloning experiments that they conducted using the said samples. This was done to confirm that the insertions of the insert DNA into the sample vectors was done correctly. This is a highly delicate procedure. Therefore, minor errors during the procedure cannot be ruled out. They recruited the help of the Genfanavaran Company, an Iranian molecular biology company, to do the sequencing. Afterward, they used the *E. coli* strain cell colony to do the delivery. The peGFP-N1 and pcDNA3.1 vector maps were used in guiding them. The PvuII and EcoRI enzymes were used for the digestion of these gene vectors, respectively. Based on their hypothesis, they were expected to produce two fragments after the digestion of the peGFP-N1 plasmids using the PvuII as the enzyme or catalyst. The two fragments, they were expecting to see were the 3.1 kb and 605 bp. On the other hand, what they were expecting after the digestion of the pcDNA3.1/His/lacZ plasmids using the ERORI as the enzyme or catalyst was the 8.7 KB band. So far, using their unique cloning process, the researchers were able to successfully verify their hypothesis.

Studies involving the use of bacteria as samples are often taken for granted by the public because of the apparent notion that they have little significance to the human life. Scientifically, the opposite of that perceived notion is true owing to the fact that there are certain molecular principles that are applicable to bacterial colonies and certain animals including mammals.

There are also studies that make use of mammals as samples. In another study Todoroki *et al*. [2], they examined the suppressive effects of DNA vaccines encoding heat shock protein on *Helicobacter pylori*-induced gastritis in mice. Mice are mammals and compared to bacterial colonies; they share far more qualities with humans. What makes this study significant is the fact that they used pylori heat shock protein A and B (pcDNA3.1-hspA and-hspB) injections as the independent variable. The goal of the study was to determine the effect of DNA vaccines encoded with the *H. pylori* heat shock protein A and B in the process of inducing immune responses on a sample population composed of six 5-week-old mice. Two sample groups were used: The intervention and the control group. The control group simply lacked the pcDNA3.1 vaccinations. The results suggested that 3 months after vaccination, a significant buildup of antibodies against the *H. pylori* were detected by the enzyme-linked immunosorbent assay microscope that the researchers used. This only shows that the DNA vaccination involving the use of the pcDNA3.1-hspA and - hspB led to the dramatic suppression of the target colonies of bacteria in the stomach of the vaccinated mice compared to the control mice. It is important to note that there was essentially no cloning done in this study. The goal of this study was not to create pcDNA3.1 but rather to test its effectiveness when used as a vaccine against the outgrowth of certain bacterial colonies typically found in mammalian digestive tracts. What makes this particular study significant, however, is the fact that it made use of vaccines that have the pcDNA3.1 vector map such as the -hspA and -hsp B vaccines. These were used to introduce immunity against certain bacteria in the mice [2].

In another study that was published in 2015 Fathpur *et al*. [3], the authors focused on the generation of pcDNA3.1 as a recombinant expression vector of ostrich growth hormone cDNA (GH cDNA) in *Saccharomyces cerevisiae*. The dependent variable used in the study was the GH. For starters, the GH is a hormone that is typically produced and released by the responsible endocrine glands in most of the mammalian and bird species as seen in this case of ostriches. It is responsible for the stimulation of growth (in terms of size and length) of tissues and organs, facilitation of carbohydrate metabolism, assimilation of protein, among other vital functions. In vertebrates (including ostriches), the GH is secreted by the pituitary gland located adjacent to the brain. The researchers generated the pcDNA3.1 by the first extracting ribose nucleic acid from the creatures' pituitary gland tissue from which the cDNA samples were extracted and synthesized. GHs from the ostrich samples were then extracted from the pituitary gland tissue. It was then cloned into a pCR8/GW/TOPO vector using the TA cloning technique [3].

Cloning system

Basically, the TA cloning technique is a sub-cloning procedure that annuls the use of restriction enzymes. Compared to other sub-cloning procedures, TA cloning is easier and is often dubbed as the more efficient compared to other traditional sub-cloning techniques because of this very fact. The process typically involves two important phases. The first phase is where the cloners create the insert. The insert is where the DNA that will be cloned (from another sample) would be inserted. The inserts are created by inducing a reaction called polymerase chain reaction (PCR).

Despite the obvious benefit of being the simpler and faster alternative – because of its linear qualities and the fact that it removes the complexity of introducing restriction enzymes, there are some drawbacks to the use of TA cloning. First and foremost, directional cloning would be impossible to simulate using the TA cloning. This means that the gene would have a 50% chance to be cloned in the reverse direction. For studies whose outcomes depend highly on the direction of the cloning procedure (whether the surrogate organism or a donor organism would be the one getting the qualities or characteristics they are targeting), TA cloning may not be a viable option. However, TA cloning was used in the study. It can only be presumed that the reason behind their use of TA instead of the traditional sub-cloning processes was because the direction of the cloning process that they simulated was not important.

In the study Fathpur *et al*. [3], after the GH from the ostrich samples were obtained and the PCR 8s were already produced, they were submitted to GenBank for verification. The GH cDNA was then sub-cloned (in another cloning procedure) but this time using the pcDNA3.1. They were cloned into *Saccharomyces cerevistae* and pcDNA3.1. Based on the results of their study, the researchers were able to successfully clone and sub-clone the ostrich GH into *S. cerevistae*.

The researchers were also able to generate the necessary pcDNA3.1 vector to clone it. It is important to note that the pcDNA3.1 generation was essentially a smaller part of this study. The generation of pcDNA3.1 was done in order to use it as a catalyst to clone the *S. cerevistae*. One major advantage of this study is that the pcDNA3.1 HG recombinant expression vector that was generated. This is because it can be useful in the expression of the ostrich GH in yeast cells as a simple and affordable way to produce the said hormone on a large scale – after all, the cloning procedure used was specifically designed to be faster, simpler, and cheaper alternative compared to the traditional cleaning methods. What this study failed to consider, however, was the fact that the cloning outcomes can be reversed simply because of their choice of cloning procedure. This is one of the drawbacks of TA cloning – it could go the other way around at any point in their experiment or cloning procedures. Therefore, the reliability of the outcomes may be questionable.

In another study Baghani *et al*. [4], they designed and constructed a eukaryotic expression vector containing *Mycobacterium tuberculosis* using the pcDNA 3.1 vector encoding map. This research is significant in the field of public health because *M. tuberculosis* is the pathogen responsible for the respiratory infection named after it. It has so far caused a lot of human mortality and morbidity. With the findings of this research, future researchers would be able to create more effective vaccines, including DNA vaccines as a way of preventing the spread of the disease. A PCR was used to amplify a genomic DNA of the pathogen after it was cultured in the Lowenstein-Jensen medium and extracted. After

this, the amplified sample was then ligated (essentially cloned) into the vector pcDNA 3.1. To verify the correctness of the cloning procedure, the researchers observed the colony's PCR stability, restriction enzyme digestion, and sequencing. After the confirmation, they proceeded with the analysis. Their results and analysis showed that "electrophoresis of the PCR product on gel showed a 303 basis point target fragment; colony PCR, restriction enzyme digestion, and sequencing methods confirmed the accuracy of the gene cloning; colony PCR and restriction enzyme digestion confirmed the cloning" [4]. Therefore, the cloning procedure was termed a success.

The researchers were able to create a pcDNA vector map that was ligated with a strain of *M. tuberculosis*. In this study, the generation of the pcDNA3.1 was used as a research milestone in order for the *M. tuberculosis* to have something to attach to in the cloning procedure. The main outcome indicator used in the study was the correctness of the insertion. Essentially, the success of the cloning procedure was verified using this process. Based on the results of the study, it can be stated that the pcDNA3.1 vector map is very useful in the field of health, medicine, and even veterinary science as in the case of the previous studies reviewed. The authors in the said study, however, did not dig deeper into the application of their findings. In this study Baghani *et al*. [4], the findings can be used to further the research on the development of a vaccine perhaps a DNA-based one that can prevent the spread of tuberculosis, which so far still remains as one of the deadly and most infectious diseases internationally.

Finally, in a study published in the Journal of Experimental and Clinical Cancer Research in 2010, the researchers investigated the *in-vivo* transfection of pcDNA3.1 in melanoma growth inhibition in mice through apoptosis induction and vascular endothelial growth factor down expression. This was essentially similar, in terms of research design, to the study about the GHs and ostriches that were discussed earlier. This time, however, the focus was on melanoma growth in mice, particularly its inhibition, and the cloning of pcDNA3.1. What they wanted to find out was whether the *in-vivo* transfection of pcDNA3.1 could have any significant effect on the inhibition of melanoma growth in their chosen samples mice.

CONCLUSION

To briefly summarize their methods, pcDNA3.1 was transfected into B16-F10 cells. The specific expression that they used was the insulin-like growth factor-binding protein 7 (IGFBP7). This was detected and confirmed by the real-time PCR detection tool and the western blot method. They used cell counting kit-8 and flow cytometry to verify the apoptosis and proliferation rates of the control cells (no interventions made, therefore, non-cloned or non-transfected) and the transfected cells (the ones that were based on the pcDNA3.1 expression that was used). The main outcome that can be considered here would be the tumor tissue growth rate and its size. This was assessed by immunohistochemistry. To provide for a more specific and measurable outcome, in this case, the researchers used the terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay. This is one of the most effective ways of detecting fragmentation in DNA, which is basically apparent in most if not all tumor growths.

The results of the said study showed that the resulting plasmid from the *in-vivo* transfection of pcDNA3.1 led to significant inhibition of proliferation of B16-F10 melanoma cells efficiently. It was also observed that *in-vivo* transfection of the pcDNA3.1 expression inhibited it significantly on the population of mice as measured by the TUNEL assay. It has been verified and confirmed that the inhibition of melanoma tumor growth can be attributed to the presence of the pcDNA3.1 [5].

This basically opens a lot of opportunities for further research. Melanoma is a type of malignant skin cancer wherein cancerous growths or tumors develop because of severe damages to the DNA of skin cells. This is often caused by genetic mutations and excessive exposure to ultraviolet radiation; in some cases, it can be caused by the lack of melanin, which protects the skin from ultraviolet rays among other forms of radiation – which is what causes the genetic mutations in the first place. Traditionally, malignant skin tumors are resected. This, however, proves to be not only costly but also unreliable in terms of being a cancer treatment. This is because it does not address the main problem on a molecular level. With the findings of this study, although it can be argued that inhibition of tumor growth, at least to some extent which is yet to be verified, it can be achieved using the procedure that the researchers just used – *in vivo* transfection of pcDNA3.1 using the IGFBP7 gene expression.

Cloning is a diverse process. It opens a lot of opportunities to improve the methods and mechanisms through which certain organisms and cells interact. This can easily be proven by the numerous studies reviewed in this study. What can be inferred from them collectively though, is that the pcDNA3.1 has a lot of possible expressions aside from being an eGFP. This means that it can be used in a multitude of ways, especially when the different cloning and sub-cloning processes are involved. It can be used to improve outcomes in various fields such as, but not limited to, veterinary science, health, wellness, medicine, and even agriculture. However, further research is recommended. Future researchers should focus on identifying the specific effects of using cloning or genetic modification in general on these proposed fields. The possibility of using cloning to modify the behavior of certain cells and organisms has so far been confirmed already by the very fact that researchers are discovering alternations in cancer cell growth, reproduction of GH in certain organisms through artificial means, among others. Before these discoveries can be significant for human development, however, further testing is needed. Tests, involving live human subjects and the introduction of certain pcDNA3.1 expressions may be possible or even be necessary in the future.

ACKNOWLEDGMENT

Special thanks to Dr. Fera Ibrahim and Dr. Budiman Bella that has allowed me to do there search in IHVCB. The research is supported by "Hibah Awal Year 2013" from University of Indonesia.

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