Construction and amplification of NPC1-luciferase fusion protein expression vector

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Summary

Mammalian cell expression vectors containing the NPC1 gene are difficult to construct and amplify using *E. coli,* resulting in mutations and partial deletions of the gene. In this application note, we report an example overcoming these difficulties with the OriCiro® Cell-free Cloning System.

1. Research Background

Niemann-Pick disease type C is an intractable and rare genetic disease. This disease is a neurodegenerative disorder caused mainly by mutations in the NPC1 gene, which encodes NPC intracellular cholesterol transporter 1 (NPC1), a membrane protein essential for intracellular cholesterol transport.

NPC1 is a 13-pass transmembrane protein that localizes to lysosomes and late endosomes to transport cholesterol from extracellular sources to other organelles. Mutation of NPC1 causes cholesterol accumulation in lysosomes and late endosomes. More than 100 mutations in NPC1 have been identified to date, and most of them are found to be unstable and defective in folding (the process of forming three-dimensional structures after translation) of the NPC1 protein (Yamamoto et al., 2004; Gelsthorpe et al. 2008; Shammas et al. 2019). We have been searching for small-molecule compounds, called "pharmacological chaperones", that can ameliorate the instability and folding abnormalities of NPC1 mutants. To evaluate such activity, we utilized imaging of a NPC1 mutant fused with green fluorescent protein (GFP) (NPC1^{I1061T}-GFP) in cultured cells (Ohgane et al., 2013; Shioi et al., 2020). In this study, in order to improve the throughput and sensitivity of activity-evaluation, we replaced the GFP with a luciferase by employing the OriCiro® Cell-free Cloning System.

2. Why did we use cell-free cloning?

In general, plasmids used for gene transfer into cultured cells are prepared using *E. coli.* Genetic engineering and amplification of plasmids with *E. coli* involves the following steps: introducing the plasmid into *E. coli*, culturing the *E. coli* transformed with the plasmid, and isolating the plasmid. Shortly after we started our research on NPC1, we encountered a problem in the preparation of plasmid vectors for mammalian expression of NPC1-GFP. Following standard methods, we introduced the pCMV-NPC1-GFP expression vector (10.4 kb) into **E. coli,** purified the plasmid from the growing *E. coli,* and found that the original plasmid could hardly be recovered and only small plasmids with partial deletions were recovered. Although the plasmid was relatively large, there was no problem in amplifying the plasmid containing NPC1-like 1 (NPC1L1) gene, an NPC1 homolog of approximately the same size, suggesting that leaky expression of NPC1 protein was toxic to *E. coli* or some unknown features in the NPC1 sequence might be responsible for the failure (Lewin et al., 2005). We also experienced similar problems with an ABC transporter, and similar observations for sodium ion channels have been previously reported (Feldmann & Lossin, 2014).

Consistent with the hypothesis that NPC1 expressed in *E. coli* from the leaky CMV promoter is toxic, *E. coli* carrying the NPC1-GFP expression vector in its original state exhibited slow growth rate and the resulting colonies on the agar plate were quite small. From relatively large colonies, we could only isolate smaller plasmids of various sizes, which contained deletions in the NPC1 gene. By picking up small colonies, culturing them in liquid medium at a slightly lower temperature, and purifying the plasmids, we were able to improve the chance to obtain plasmids without deletions. However, this required picking up more than 10 colonies for plasmid purification, screening of the plasmid size with agarose gel electrophoresis, and finally sequencing was essential to select plasmids without small deletions. This whole process was costly and time-consuming. In addition, the amount

of plasmid obtained was less than one-tenth of that of a normal plasmids, making it difficult to secure sufficient amount of plasmid and therefore complicating modification of the expression vector. Since the OriCiro® Cell-free cloning system does not use *E. coli* and is likely to solve the above problems, we chose this kit to modify GFP to luciferase, which we have wanted to do for a long time but have not been able to do.

3. Experiment and Result

Construction of NPC1^{I1061T}-ELuc expression vector

To replace GFP in the previous expression vector (pCMV-NPC1^{I1061T}-GFP) with the luciferase ELuc, we planned to assemble the following three fragments; *oriC* fragment PCR-amplified using *oriC* casette provided in the kit as a template, NPC1 fragment (including the ampicillin resistance gene and CMV promoter) PCR-amplified using pCMV-NPC1^{I1061T}-GFP as a template, and ELuc fragment (including the neomycin resistance gene necessary for creating stably expressing cells) PCR-amplified using pCMV-HaloTag-ELuc as a template (Figure 1).

PCR amplification of each fragment with a high fidelity polymerase KOD-plus-, followed by purification of the fragments from agarose gel gave the required fragments. Since the PCR primers were designed so that both ends of these three fragments have overlapping sequences of about 40 base pairs, the three fragments can be assembled in the intended order through complemetary base-pairing. The assembly reaction was performed using the standard protocol of the kit. Each fragment in equimolar ratio (2.5 µL in total) was mixed with 2.5 µL of 2X RA mix and reacted on a thermal cycler at 42°C for 30 minutes and 65°C for 2 minutes. 1 µL of the mixture was mixed with 9 µL of amplification reaction solution and allowed to react at 33°C overnight. The reaction solution was diluted 2-fold with the attached buffer III and treated at the same temperature for another 30 minutes.

The resulting solution and its BamHI digest were analyzed by agarose gel electrophoresis (Figure 2). A supercoiled band was observed for the reaction solution. The target plasmid has two BamHI cleavage sites within the NPC1 and *oriC* sequences, and the plasmid is expected to give 8.36 and 2.65 kb fragments upon BamHI digestion. Consistently, two bands of expected size were observed for the BamHI digest, supporting the successful assembly of the fragment. After purification of the plasmid, Sanger sequencing of the plasmid confirmed successful assembly of the fragments.

Passage amplification of NPC1^{I1061T}-Eluc expression vector

In order to prepare the required amount of the constructed plasmid, the amplification reaction was performed again using the assembled plasmid as a template at 6-fold scale of the standard protocol (passage amplification). The amplification reaction was carried out using 6 µL of a 10^6-fold dilution of the obtained plasmid solution, for a total of 60 µL. This reaction also proceeded without any problems, and the same band pattern as before was observed with agarose gel electrophoresis.

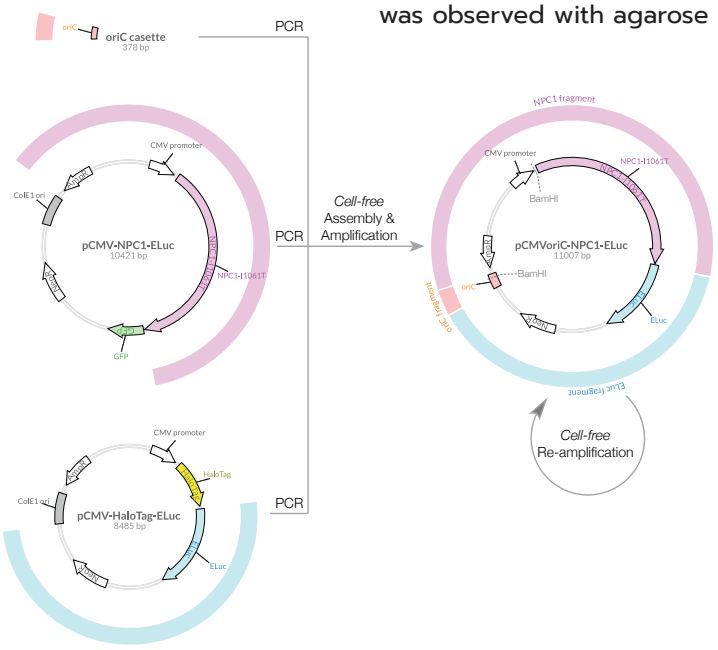

Figure 1: Flow of creating the target plasmid. Three fragments were prepared by PCR, and they were assembled and amplified. The obtained plasmids were re-amplified by passage amplification.

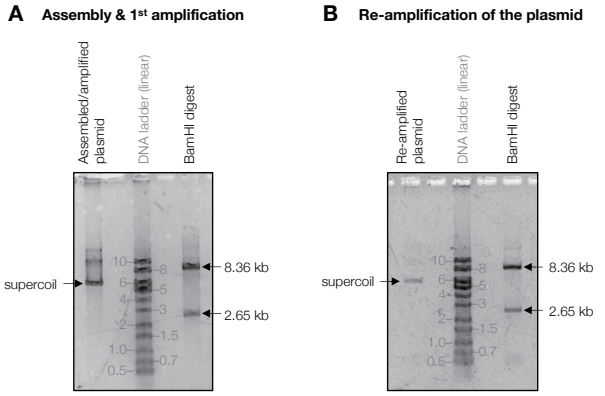

Figure 2: Agarose gel electrophoresis results of the experiment shown in Figure 1. **A**. Agarose gel electrophoresis results of the reaction solution in which the three PCR fragments were assembled and amplified. BamHI digestion with two cleavage sites produced two fragments of the expected size, yielding the target plasmid of approximately 11 kb. **B**. Agarose gel electrophoresis result of the passage amplification from 10^6-fold dilution of the obtained plasmid.

4. Conclusion

In this study, a plasmid containing NPC1, a gene that is unstable and difficult to amplify in *E. coli*, was constructed and amplified using the OriCiro® Cell-free Cloning Kit. We successfully constructed the target plasmid without any optimization nor special experimental conditions (the series of experiments were successfully performed by an undergraduate student who has just joined our laboratory). Although the plasmid yield was not high compared to the standard *E. coli*-based method, this cell-free method may offer a useful and unique alternative to *E. coli*-based cloning and amplification, especially for difficult-to-handle genes.

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