

Lab Report: Molecular Cloning by Gateway Cloning and Gibson (HiFi) DNA Assembly
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Introduction

Molecular cloning is a basic and critical technique in synthetic biology. It is used to amplify a DNA fragment of interest in living cells and very often to express the gene within the cloned DNA as well. It involves the cleavage of double strand DNA and the ligation of it to another double strand DNA to form the recombinant DNA. Usually, the DNA of interest is called as insert and the place it inserts into is called as vector, when a linear DNA is putting into a circular plasmid DNA. Since fundamentally, all cells from three domains share the same chemical structure and functional mechanism, e.g. replication and transcription, of DNA, the DNA fragments can move across organisms and still be functional. Molecular cloning makes use of this characteristic to clone DNA from complex sources, e.g. human cells, whose biological pathways are not yet fully explored or understood, to relatively simple targets, such as *E. coli*, whose biological pathways are well studied and whose phenotype can be predicted and controlled. By this manner, scientists can store a DNA of interest, can study a transcript of the DNA or the protein after translation, can massively produce the protein the DNA encodes, which are fundamentals to nowadays molecular and cellular biology research as well as the very hot immunotherapy and gene therapy which lies on the DNA recombination technology.

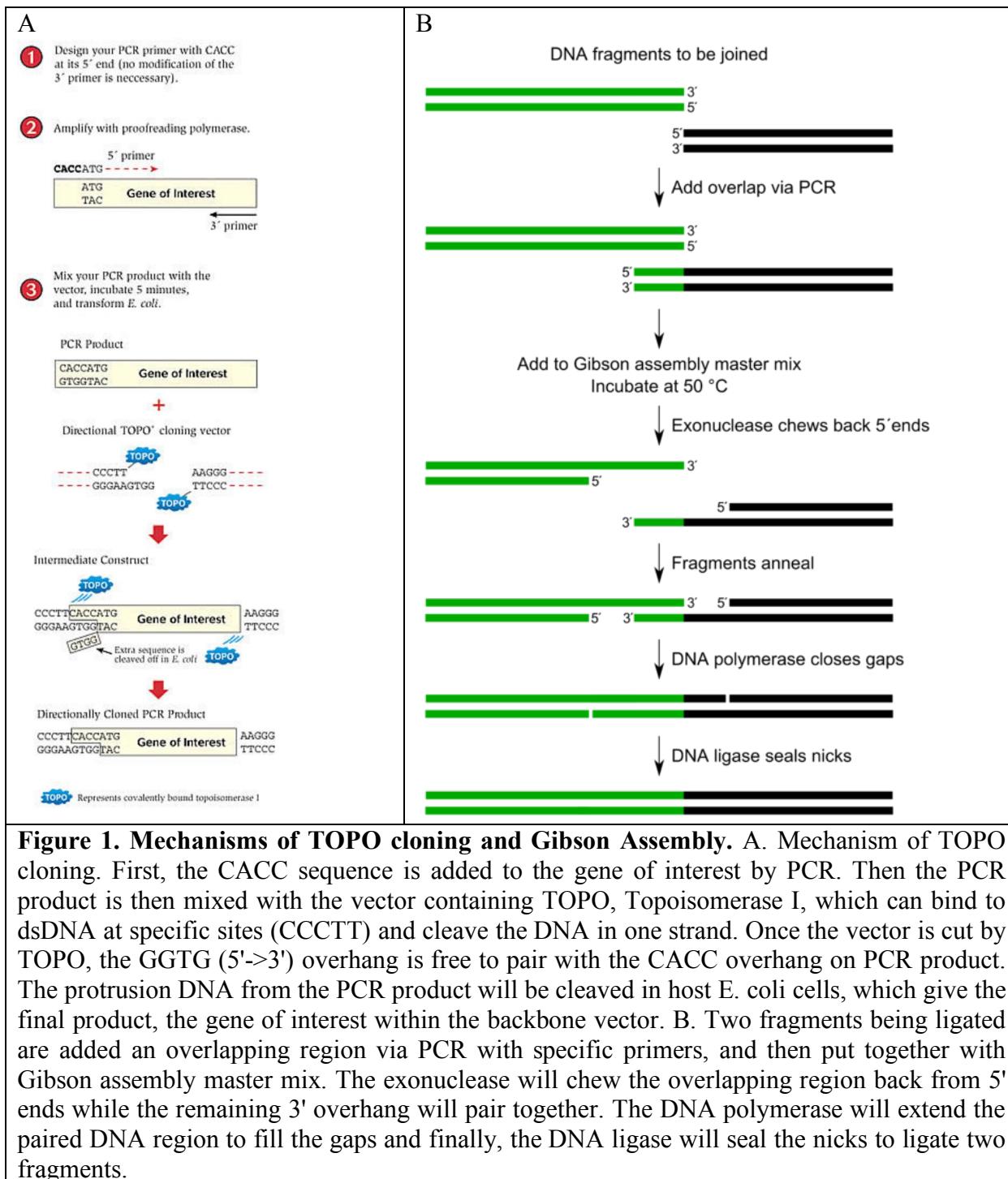
Gateway cloning is a patented biotechnology available from Invitrogen, which is widely used in the life science community because of its simplicity and high efficiency. Two main steps are involved in Gateway cloning: (1) Gateway BP reaction, where the DNA fragment flanked with attB sites, usually produced by PCR with specific primers, is mixed with the donor vector containing attP sites as well as the BP clonase to generate the Gateway Entry clone that contains attL sites flanking the inserted DNA fragment. (2) Gateway LR reaction, where the Entry clone with attL sites produced in BP reaction is mixed with the destination vector containing attR sites as well as the LR clonase to generate the Expression clone that contains attB sites.

Usually, the Entry clone also has ORI which enables the vector to be replicated and hence the inserted gene of interest be kept and stored, one or two antibiotics resistant gene which served as the selection marker after transformation and cultured with medium containing the corresponding antibiotics, and a ccdB toxic gene to *E. coli*, which is a routine choice of host, for the selection of actual inserted vector and elimination of those vectors with no insertion. As for the destination vector, it usually has the features mentioned for the entry clone and very importantly, a strong promoter used to boost the expression of the inserted gene.

One of the main advantages of the relatively expensive Gateway cloning technology apart from its simplicity and efficiency is that once the production of the entry clone is done which may take more time than other technologies, it can be transferred to many different destination vectors for various applications. One can also transfer entry clones with many different inserts into one type of destination vectors. There are also standardized Gateway entry clone archives, such as human cDNA libraries that save time for the researchers.

There are other technologies to prepare an entry clone, such as restriction enzyme-based methods. ThermoFisher commercialized a kit called pENTR™/D-TOPO™ Cloning Kit, which is capable of

cloning a blunt end DNA fragment into a specially designed vector to form the entry clone in Gateway cloning in 5 minutes.



Shown in Figure 1A (adapt from ThermoFisher) is the mechanism of TOPO cloning. First, the CACC sequence is added to the gene of interest by PCR. Then the PCR product is then mixed with the vector containing TOPO, Topoisomerase I, which can bind to dsDNA at specific sites (CCCTT) and cleave the DNA in one strand. Once the vector is cut by TOPO, the GGTG (5'->3') overhang is free to pair with the CACC overhang on PCR product. The protrusion DNA from the PCR product will be cleaved in host E. coli cells, which give the final product, the gene of interest within the backbone vector. The backbone vector is specially designed in a way that it contains attL sites flanking the gene of interest after insertion as well as antibiotics resistant genes to qualify it as an entry clone.

One of the other widely used molecular cloning technologies is Gibson assembly, which is capable of joining of multiple DNA fragments in a single pot, isothermal reaction. The 20-40 base pair overlap helps with the ligation of two adjacent fragments. The details are shown in Figure 1B. Two fragments being ligated are added an overlapping region via PCR with specific primers, and then put together with Gibson assembly master mix. The exonuclease will chew the overlapping region back from 5' ends while the remaining 3' overhang will pair together. The DNA polymerase will extend the paired DNA region to fill the gaps and finally, the DNA ligase will seal the nicks to ligate two fragments. This method can combine up to 15 fragments at one time, which will need the two-step method that the exonuclease and annealing steps happen first and then the DNA polymerase and ligation steps while in the up to 5 fragments reaction all of them are mixed in one step. The advantage of Gibson assembly including its cost-effectiveness and no involvement with restriction enzymes, which allows the full control of all the sequences in the ligation joints. NEB adapted the Gibson assembly to develop a method called "HiFi DNA Assembly", which "utilizes a high-fidelity polymerase with higher accuracy". Their protocols are similar, but the HiFi one typically generates more colonies.

VirD2 is gene first identified in *Agrobacterium tumefaciens* that are capable of transferring a DNA fragment (T-DNA) to plant cell nucleus. Given in the lab manual, It is involved in almost all of the transferring steps, including the cleavage of T-DNA from the source, the formation of the complex with T-DNA and helps with its export, and transportation to the plant cell nucleus and the integration into the plant cell's genome. Tau proteins are nowadays believed take crucial part in the neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [3]. It spans 133953bp on chromosome 17, 45894382..46028334 in GRCh38. Tau proteins are most found in neuron cells and believed to help stabilize axonal microtubules by interact with tubulin [4]. Neurofibrillary tangles will be formed from hyperphosphorylated tau proteins, which is further involved in the pathogenesis of above mentioned neuro diseases. The Tau proteins have several isoforms and Tau6 is one of them.

Statement of the purpose

This lab session is part of the Synthetic Biology and Biotechnology course. Mainly, it focuses on giving a hands-on experience of how a molecular cloning work is done on the bench. We use two different cloning methods, Gateway cloning (with TOPO cloning to generate entry clone) and Gibson cloning (the NEB adapted version, HiFi DNA Assembly); and two different genes of interest, VirD2 and Tau mentioned above. We practice and compare these methods by cloning VirD2 to pET32a-GW destination vector via Gateway cloning and cloning Tau6 to pE-EUMOStar destination vector.

Significance of the study

As students, it is important to get a sense of how things really work when learning the theoretical knowledge. Both cloning methods we study here are widely used in nowadays lab and commercially available and also very efficient. These two methods are supposed to be still viable for years and hence would give us a very high-level impression of the field and the current technologies.

Scope of the study

This lab session focuses on two main methods while there are other cloning methods such as restriction digestion based methods are not studied here. The transformation after cloning, plasmid extraction, restriction digestion of the plasmid, and sanger sequencing of the inserted fragment are used to verify the successfulness of the cloning of both methods. However, due to the limited time here in 4 lab sessions, most of the lab procedures of HiFi DNA assembly are done by the instructor and watched by us students; and the expression of the proteins after the cloning of the corresponding genes are not carried out here.

In silico simulation

The procedures in this lab session mostly involve with the DNA sequence, which can be simulated on computer to generate the theoretical results. The simulation has been performed with the help of SnapGene. The whole procedure has been detailed in the supplementary PDF file, “Molecular Cloning Simulation in SnapGene”. The final results, the cloning history for both Gateway cloning and Gibson cloning, are shown in Figure 2 and Figure 3.

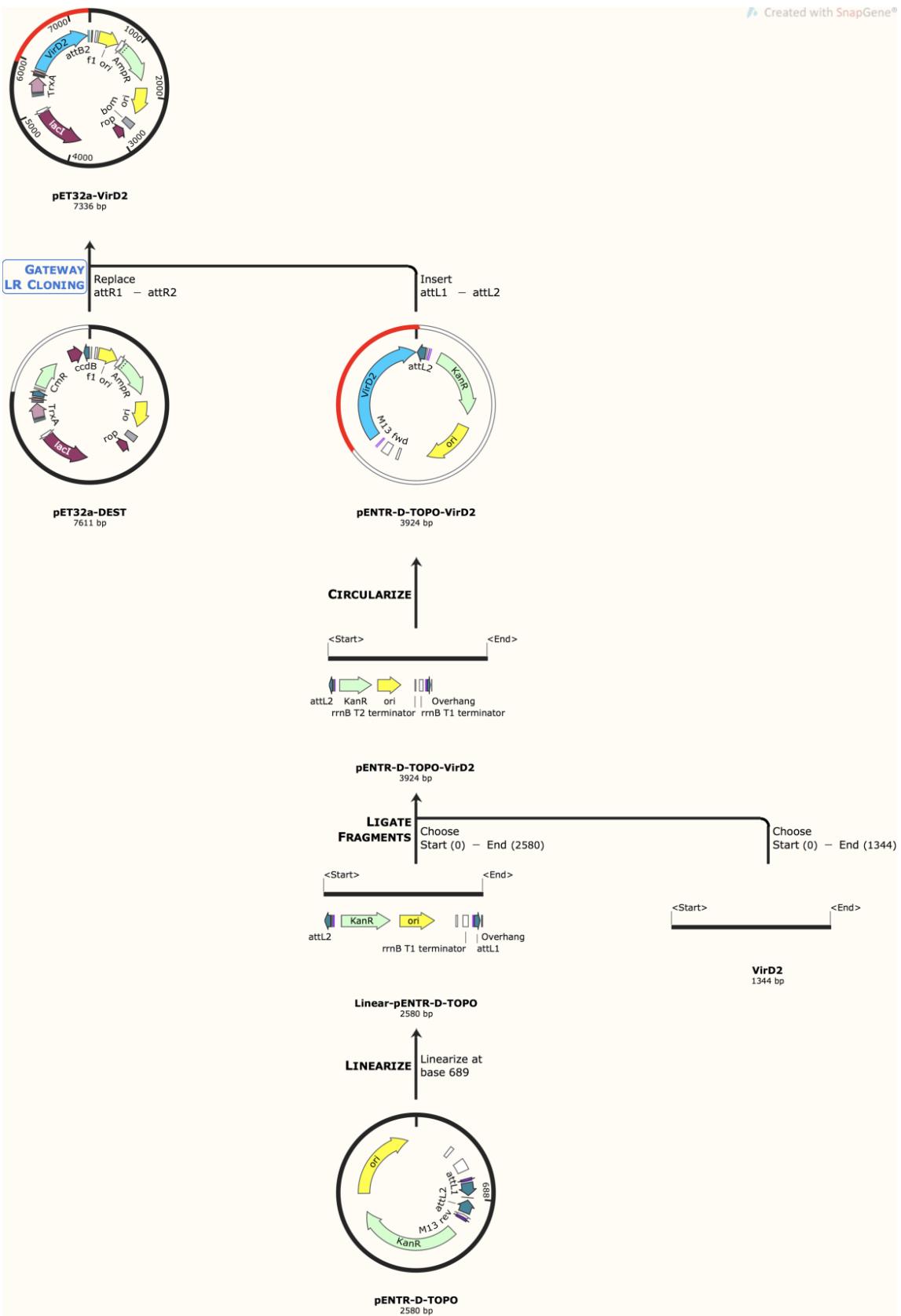


Figure 2. The history of cloning VirD2 into pET32a, simulation in SnapGene.

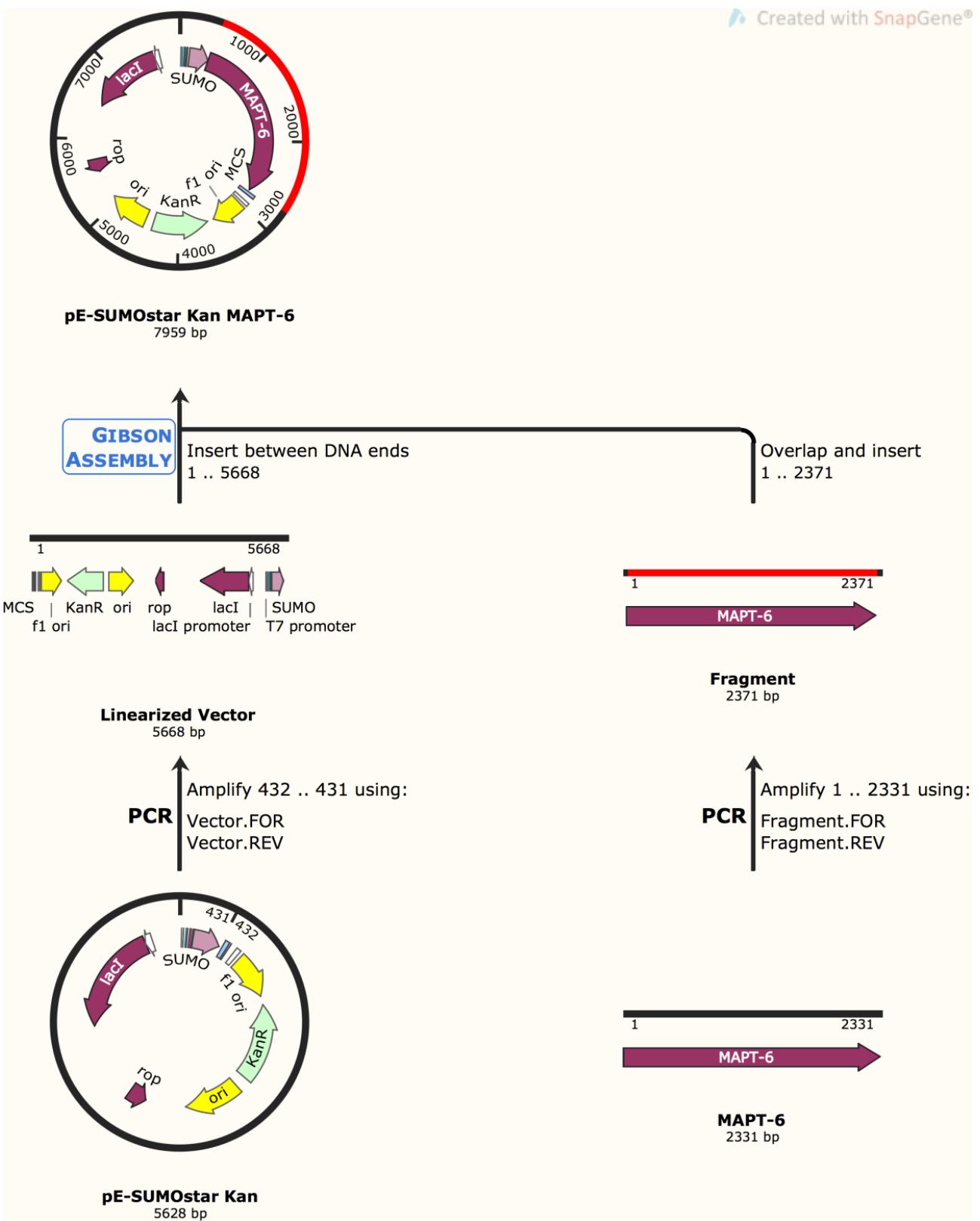


Figure 3. The history of cloning MAPT6 into pE-SUMOstar, simulation in SnapGene.

Methods

Part I: Cloning of the agrobacterium VirD2 into *E. coli* expression vector by gateway cloning method

PCR amplification of VirD2 was performed by specially designed forward and reverse primers, with CACC on forward primer for topo directional cloning. The reaction mix and PCR run configuration are shown in Table 1.1 and Table 1.2. Fifteen ul of the PCR product was run on 1% agarose gel. The rest of the PCR product was purified by PCR purification kit according to the protocol, following by the measurement of DNA concentration by Nanodrop (175ng/ul). DNA entry clone was produced by directional topo cloning method (pENTR™/D-TOPO™ Cloning Kit, ThermoFisher Invitrogen™, Catalog number: K240020). The PCR product, VirD2 DNA fragment, was mixed with the topo cloning reaction mix as shown in Table 1.3. The reaction mix was then incubated in thermal cycler at 23C for 2 hours, followed by the transformation into DH5 α *E. coli* cells as indicated in Box 1's protocol. For the sake of extracting plasmids from the transformed *E.coli* cells, a single colony from the plate was picked and inoculated into 5ml LB broth and grew overnight at 37C. After the miniprep according to the kit's protocol, the DNA concentration was measured by Nanodrop (165ng/ul) and restriction digestion was performed using EcoRV and NotI to confirm the presence of the insert, reaction mix is shown in Table 2. Upon the size confirmation of restriction digestion, a portion of the plasmid was sent for Sanger sequencing to confirm on the sequence level.

Table 1.1 PCR reaction mix		Table 1.2 PCR run configuration			Table 1.3 Topo cloning reaction mix	
Reagents	Vol	Stage	Temp C	Duration	Reagents	Volume
Forward primer	1.0ul	Pre heat	98	3min	Entry vector	1ul
Reverse primer	1.0ul	Denature	98	15s	Purified PCR product	50g (1ul)
Template DNA		Annealing	60	20s	Salt solution	1ul
Template DNA	1.0ul	Extension	72	20s	Deionized H ₂ O	3ul
10mM DNTPs mixture	1.0ul	Final extension	72	10min	Total Vol	6ul
DMSO	1.50ul	/				/
HF buffer (5x)	10.0ul					
Autoclaved MiliQ H ₂ O	34.5ul					

Box 1. Transformation of topo cloning reaction to DH5 α *E. coli*

1. Transform 2ul of assembly reaction mixture into DH5 α *E. coli* cells
2. Incubate the cells on ice for 10 minutes.
3. Perform heat-shock at 42 C for 30 seconds.
4. Transfer the tubes back on ice for 2 minutes.
5. Add 200 ul of SOC to the tubes
6. Incubate in shaking incubator at 37 C for 1hour.

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|--|
| 7. Spread 200 ul of culture on LB agar plates containing kanamycin antibiotic. |
| 8. Incubate the plates overnight at 37 C. |

Table 2. Restriction digestion to confirm the presence of the insert in the entry clone.

Reagents	Vol
DNA (Plasmid after miniprep)	5ul
EcoRV	0.3ul
NotI	0.3ul
10X Buffer (CutSmart)	2ul
Water	12.4ul

LR gateway cloning was performed using the pET32a as destination vector. The reaction mix is shown in Table 3. After mix all the reagents for LR cloning, the mix was incubated at 25C for 2 hours. The transformation of the product of LR cloning was the same as Box1's protocol except the ampicillin was used as antibiotics rather than kanamycin. For the sake of extracting plasmids from the VirD2 inserted destination vector transformed *E.coli* cells, a single colony from the plate was picked and inoculated into 5ml LB broth and grew overnight at 37C. After the miniprep according to the kit's protocol, the restriction digestion was performed using ScaI and SphI to confirm the presence of the insert, reaction mix is shown in Table 4. Upon the size confirmation of restriction digestion, a portion of the plasmid was sent for Sanger sequencing to confirm on the sequence level.

Table 3. LR gateway cloning reaction mix

Reagents	Vol set	Vol Finalized
Entry vector	50 -150 ng	0.5ul (165ng/ul)
Destination vector	150 ng	1ul
TE buffer pH8	Xul	2.5ul
LR clonase	2ul	2ul
Total volume	6ul	6ul

Table 4. Restriction digestion to confirm the presence of the insert in the destination clone.

Reagents	Vol
DNA (Plasmid after miniprep)	5ul
ScaI	0.3ul
SphI	0.3ul
10X Buffer (CutSmart)	2ul
Water	12.4ul

Part II: Molecular cloning of human microtubule associated protein TAU into *E. coli* expression vector by DNA assembly cloning method

PCR amplification of TAU6 gene was performed by specially designed forward and reverse primers, with flanking sequence (showing in Box2) homologous to the destination vector pE-SUMOstar on both forward and reverse primers. pE-SUMOstar was linearized by PCR with the primers listed in Box2. Both reactions' mix and PCR run configuration are shown in Table 5 and

Table 6. Fifteen ul of the PCR product was run on 1% agarose gel. Remaining pE-SUMOstar PCR product was treated with Dpn1 enzyme and incubate overnight at 37 C. Both products, TAU and pE-SUMOstar, were then purified by PCR purification kit according to the protocol, following by the measurement of DNA concentration by Nanodrop. The DNA assembly ligation reaction was then performed as shown in Table 7 and incubated in thermal cycler at 50C for 2 hours, followed by the transformation into DH5 α *E. coli* cells as indicated in Box 3's protocol. For the sake of extracting plasmids from the transformed *E.coli* cells, a single colony from the plate was picked and inoculated into 5ml LB broth and grew overnight at 37C. After harvesting the plasmid pE-SUMOstar_TAU6 by miniprep according to the kit's protocol, the DNA concentration was measured by Nanodrop and restriction digestion was performed using XbaI and HindIII to confirm the presence of the insert, reaction mix is shown in Table 8. Upon the size confirmation of restriction digestion, a portion of the plasmid was sent for Sanger sequencing to confirm on the sequence level.

Box2. TAU6 amplification primers and pE-SUMOstar amplification primers

MAPT6_fwd: ACCGCGAACAGATTGGAGGTATGTGGAGCCACCCGCAG

MAPT6_rev: CGGTACCACTAGTGGTCTACTATTATCACAGACCTGTTGCCAG

pE-SUMOstarKan_fwd: TGAGACCACACTAGTGGTACCGGTCTCAC

pE-SUMOstarKan_rev: ACCTCCAATCTGTTCGCGGTGAGCCTC

The single and double underlined segments are reverse complementary to each other correspondingly. They are the homology region of the following DNA assembly reaction.

Table 5. PCR reaction mix to amplify TAU6 gene / pE-SUMOstar

Reagents	Quantity/molarity
Forward primer	0.5ul
Reverse Primer	0.5ul
Template DNA	1.0ul
10mM DNTPs mixture	1.5ul
MgSO ₄	1.0ul
Pfx amplification buffer (10x)	5.0ul
PCR enhancer (10x)	5.0ul
Autoclaved MiliQ H ₂ O	35.0ul
Platinum Pfx DNA polymerase	0.5ul
Total reaction volume	50ul

Table 6. PCR run configuration for TAU6 gene / pE-SUMOstar

Stage	Temp C	Duration
Pre heat	95	3min
Denature	95	15s
Annealing	65	30s
Extension	72	60s
Final extension	72	5min

Table 7. DNA assembly reaction for TAU and pE-SUMOstar

Reagents	Vol
Linearized pE-SUMOstar(vector)	50-75ng (1.5ul)
PCR amplified TAU gene: 3x in molar ratio to vector	1ul
DNA Assembly Master mix (2X) 10ul.	10ul
Deionized H2O: Xul	7.5ul
Total volume: 20ul	20ul

Box 3. Transformation of DNA assembly reaction to DH5α *E. coli*

1. Transform 2ul of assembly reaction mixture into DH5a *E. coli* cells.
2. Incubate the cells on ice for 30 minutes.
3. Perform heat-shock at 42 C for 60 seconds.
4. Transfer the tubes back on ice for 2 minutes.
5. Add 950 ul of LB broth to the tubes
6. Incubate in shaking incubator at 37 C for 1hour.
7. Spread 200 ul of culture on LB agar plates containing kanamycin antibiotic.
8. Incubate the plates overnight at 37 C.

Table 8. Restriction digestion to confirm the presence of the insert in the plasmid pE-SUMOstar_TAU6

Reagents	Vol
DNA (Plasmid after miniprep)	16ul
XbaI	1ul
HindIII	1ul
10X Buffer	2ul
Water	20ul
Incubate at 37C for 1-2 hours	

Results

Part I: Cloning of the agrobacterium VirD2 into *E. coli* expression vector by gateway cloning method

Colony plate for entry clone



Figure 4. Entry clone colonies after spreading on agar plates with kanamycin. The colonies grow evenly. The number of colonies is high. Result from TA.

Restriction digestion of entry clone (EcoRV+NotI)

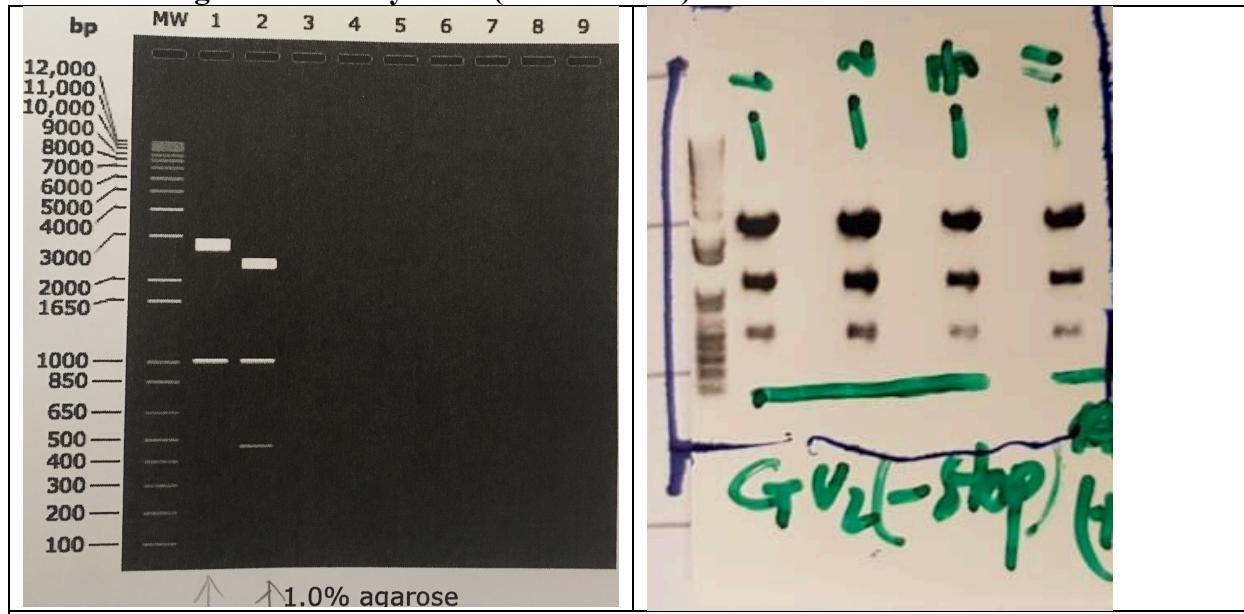


Figure 5. Restriction digestion of entry clone with EcoRV and NotI. Left side: theoretical bands, lane 2 represents the EcoRV and NotI cutting. Right side: gel result from TA.

From the theoretical bands we can know that there are three bands after cleavage of EcoRV and NotI. The gel image from TA shows three bands in good consistence with the theoretical ones, which means the fragment, VirD2 gene, has been successfully inserted into the backbone vector.

Sanger sequencing results for entry clone

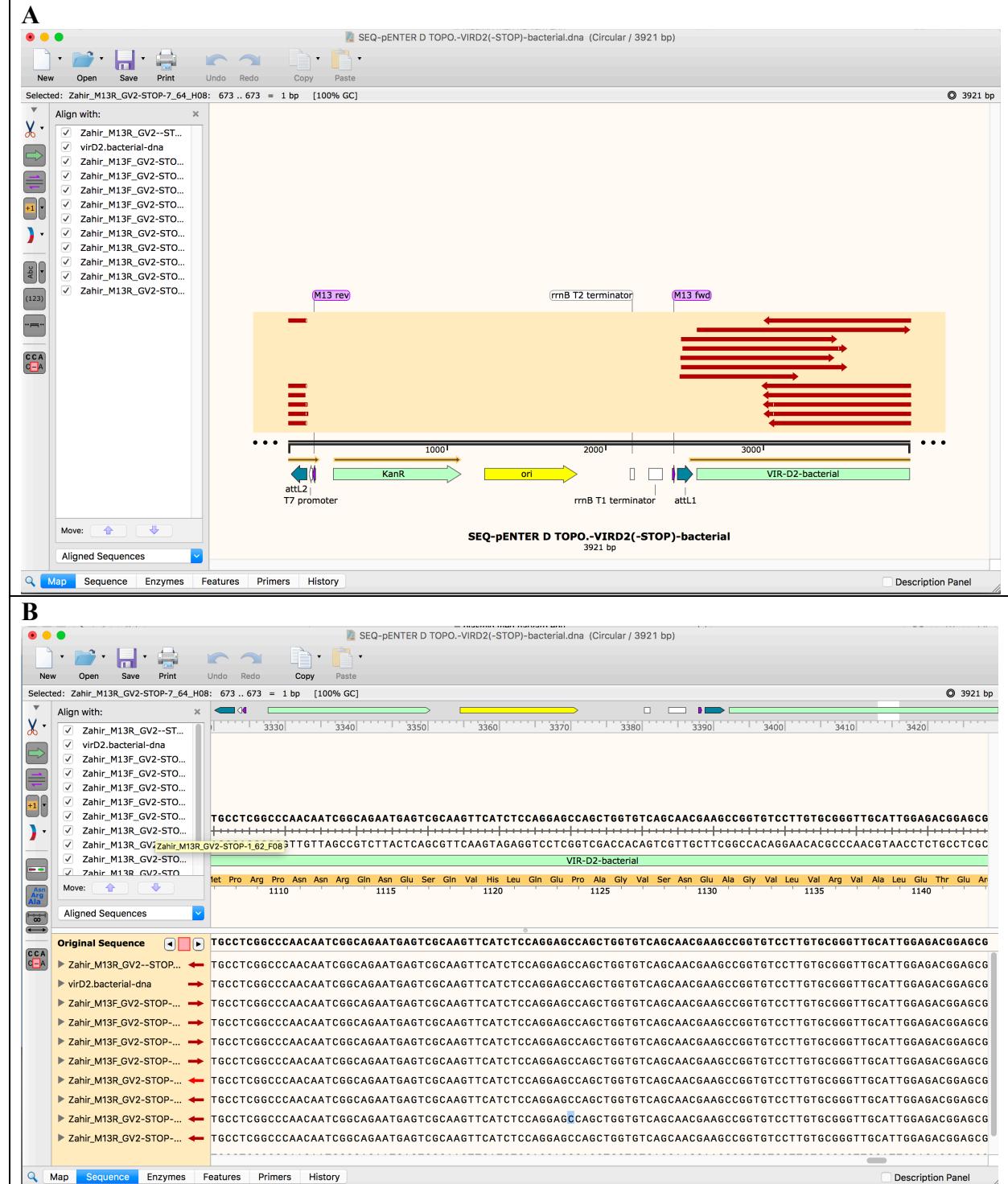


Figure 6. Sanger sequencing results show exact alignment to the insert. A. The alignment overview. B. The details of the alignment on base pair level. The M13 forward and M13 reverse primer were used as sequencing primers. All 6 forward sequences and 6 reverse sequences show perfect match to the VirD2 gene, which further confirms the successful insertion of the fragment.

Colony plate for expression clone



Figure 7. Expression clone colonies after spreading on agar plates with ampicillin. The colonies grow evenly. The number of colonies is high. Result from my colleague.

Restriction digestion for expression clone (ScaI + SphI)

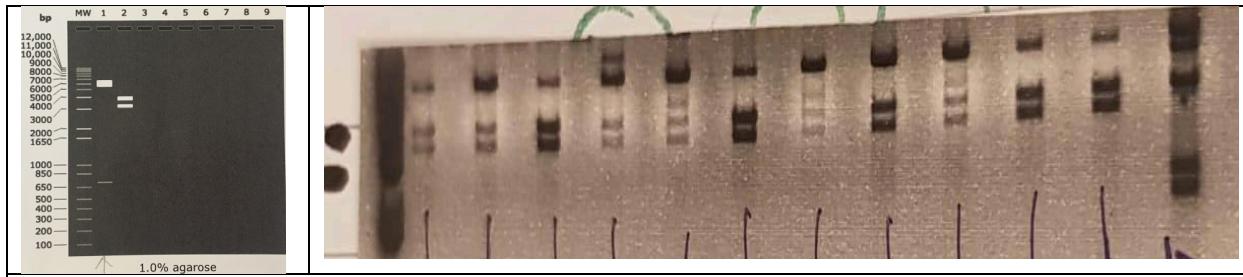
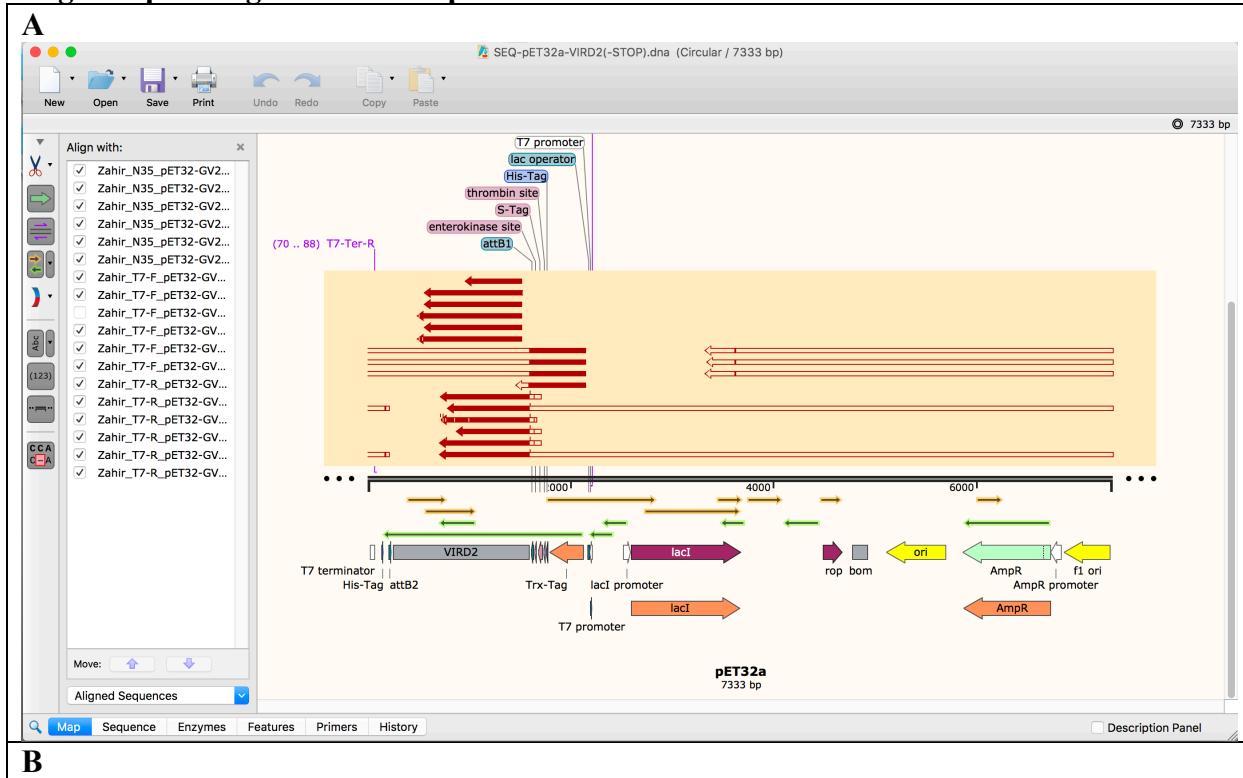


Figure 8. Restriction digestion of entry clone with ScaI and SphI. Left side: theoretical bands, lane 2 represents the ScaI and SphI cutting. Right side: gel result from TA.

From the theoretical bands we can know that there are two bands after cleavage of ScaI and SphI. Most lanes in the gel image from TA shows two bands in good consistence with the theoretical ones, which means the fragment, VirD2 gene, has been successfully inserted into the destination vector to form the expression vector by Gateway LR cloning. The top band is the expression vector itself. Some lanes that have three bands, where in the middle of top band and the two expected bands there is the other one, probably are due to DNA degradation.

Sanger sequencing results for expression clone



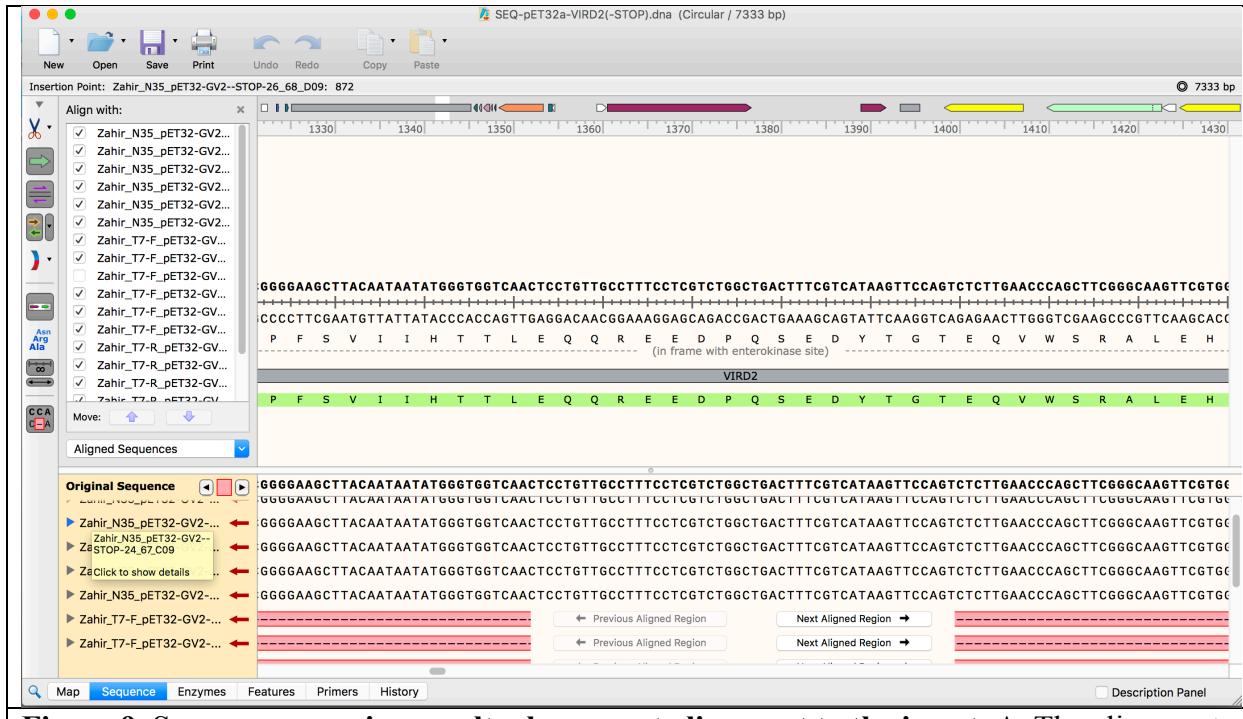


Figure 9. Sanger sequencing results show exact alignment to the insert. A. The alignment overview. B. The details of the alignment on base pair level. The T7 forward primer in its promoter region and T7 reverse primer in its terminator region were used as sequencing primers. The 4 sequences, shown in B and represented as solid dark lines, show perfect match to the VirD2 gene, which further confirms the successful insertion of the fragment. (I do not quite understand the soft light lines in the overview image as well as the red lanes with black dash lines. I do not have time to figure out more on this so I just leave it here.)

Part II: Molecular cloning of human microtubule associated protein TAU into E. coli expression vector by DNA assembly cloning method

Colony plate after transformation of the assembly



Figure 10. Colony of the HiFi DNA assembly spreading on agar plates with kanamycin. There is only one colony on the very left side of the plate. This is probably not the colony we want but some contaminant by chance resistant to kanamycin. And this is probably because our DNA assembly reaction preparation had some problems, like bad pipetting resulting into the missing of some reagents.

Gel electrophoresis of PCR product of insert, vector, and the assembly mix



Figure 11. Gel electrophoresis of PCR product of insert, vector, and the assembly mix. A. All lanes are the linearized vector. B. Four lanes are labeled, 1, 2, 3, 4. Result 1 to 3 are from my colleague Aldo Martinez Banderas, where 1 is the DNA assembly mix but only the insert is seen, and 2 is the vector, showing a very strong signal at the top and a smear which may be the degraded DNA, 3 is the insert but no signal. The Result 4 is from our group's result, where there is no signal, which reflects the agar plate spreading result too.

Gel electrophoresis of the assembled plasmid pE-SUMOstar_TAU6

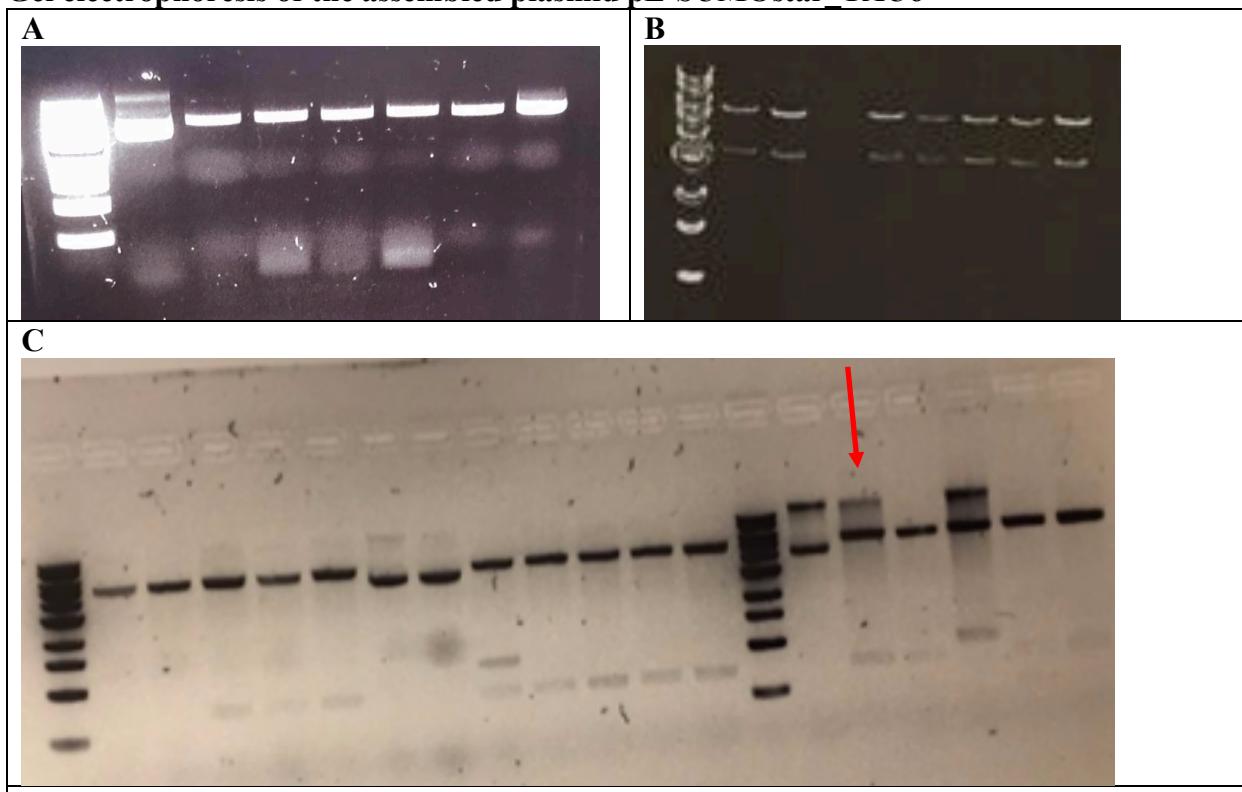


Figure 12. Gel electrophoresis of the assembled plasmid pE-SUMOstar_TAU6. A. The un-cut plasmid, result from TA. B. The digested plasmid, result from TA. C. The digested plasmid from students, the red arrowed one is mine.

The total length of the assembled plasmid is 8004bp. After the digestion, there are supposed to be two bands, one 5271bp mainly containing pE-SUMO, the other 2733bp mainly containing the insert TAU6. TA's results show good consistence with the expected size. Ours, however, there is a band in the top which is the full-length plasmid, and a main band at about 5000bp, which should be the one with 5271bp. The other band could have degraded as there is a smear in the lane, so does the full-length plasmid. Almost all students' groups show only one band which is wired. There might be some systematic error, like that from the enzyme.

Discussion

Overall, we have experienced all the procedures in Gateway cloning, mainly watching each step; and the procedures in HiFi DNA Assembly, mainly doing by ourselves. We got expected results from TA in Gateway cloning as shown in the gel electrophoresis and the sanger sequencing. However, the results from HiFi DNA Assembly was not as expected probably due to errors happened in the quick processing where many students involved. But indeed, we have practiced with the fundamental steps in the lab session and it have given me a good impression of how things should work which helps me in my research lab already.

In the topo cloning manual, it advertises as 5 min is good enough for the cloning. But 2 hours is recommended from TA, as incubating for 5 min sometimes fails but for 2 hours never fails. SOC was used in the transformation steps to facilitate the recovery of bacterial competent cells after the transformation because the rich medium in SOC helps to neutralize the harsh competent environment created by chemicals and provide better ambient than normal medium. In Gateway cloning kanamycin antibiotics used in entry clone was changed to ampicillin in transformation after LR reaction because the selectable marker in destination vector is the ampicillin resistant gene. The ccdB gene in the destination vector between the two attR sites serves as a toxic marker to help select against the colonies with the vector that has no insertion of DNA fragment of interest, in this case, the VirD2 gene. The mechanism behind the toxic is “ccdB codes for the toxic protein (CcdB) that acts as a DNA gyrase poison, locking up DNA gyrase with broken double stranded DNA and ultimately causing cell death” (From Addgene Blog [7]).

In the HiFi DNA assembly, primer sequence homologous to the destination vector pE-SUMOStar is right before the stop codon, so that the protein gets translated following the SUMO protein and then get a stop codon right after its translation. Basically, when designing the primers, start codon, stop codon, and the frame, must be well considered. In my simulation, the final product is 7959bp rather than 8004bp. This is because I cannot find TAU6 open reading frame who has a length of 2376bp. I checked all the 7 consensus coding sequences (CCDS) mentioned in UniPort (<https://www.uniprot.org/uniprot/P10636#sequences>), but not found the one with the same size. I was using Homo sapiens microtubule associated protein tau (MAPT), transcript variant 6, mRNA with a length of 2331bp (https://www.ncbi.nlm.nih.gov/nuccore/NM_001123066.3). Other minor differences in the simulation may also result from the not exact sequences in the backbone vectors. MgSO₄ was used in PCR because first, Mg²⁺ is a cofactor of thermostable PCR polymerase, and second, Mg²⁺ concentration in the PCR buffer stabilize the double strand DNA and hence affects the melting temperature and therefore needs to be well controlled for the specificity and efficiency of the PCR. PCR enhancer was used because we were amplifying large fragments, for TAU6, 2376 bp and for the backbone vector 5628 bp. The Dpn1 restriction enzyme was used right after the PCR of backbone vector pE-SUMOstar because it can digest the methylated old vector, which were all the circular DNA molecules in the reaction, while keep the newly synthesized unmethylated DNA un-cut, so that we got pure linearized vector for assembly reaction.

In both molecular cloning methods, we were cloning fragments into backbone vector containing a T7 promoter, which was then called an expression vector. T7 polymerase is a transcription polymerase with very high specificity that only recognize the T7 promoter, which in hence contributes to the high specificity and high efficiency of the expression of inserted gene.

Acknowledgements

I would like to say thank you to the course instructors, professor Magdy Mahfouz and professor Charlotte Hauser for the teaching of all lectures where the mechanisms of the lab session was covered; to TAs in both lab sessions; to all the students within the group I involved and other groups because we did labs all tighter and shared some results; to core labs where the sanger sequencing happened; and to Aldo Martinez Banderas specially who helped me understand some gel results after long time when I forgot some details.

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