Ligation-Free Cloning System

Application Handbook

Cat. No. E001 / E002

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Introduction

The Ligation-Free Cloning System from **abm** Inc. offers the most versatile strategy available to meet the demands of both your routine and most challenging cloning projects. The progressive design of the Ligation-Free Cloning System has now eliminated the requirement for unique restriction sites within the vector and intended inserts. Furthermore, the extremely high efficiency of this system (yielding >90% positive clones) coupled with a shorter set-up and reaction time has now made traditional restriction enzyme-based recombinant DNA cloning obsolete.

At the foundation of Ligation-Free Cloning technology is **abm**'s proprietary Ligation-Free Cloning MasterMix, which reliably facilitates conjugation between multiple inserts (PCR-generated sequences) and a linearized vector by recognizing a 15 bp overlap region at both ends (Figure 1).

This 15 bp overlap can be engineered by designing primers to amplify your desired sequences. To eliminate the possibility of mutations associated with PCR amplification, scientists at **abm** Inc. have developed Precision™ DNA Polymerase (Cat. No. G078) with the highest proof-reading capability, to facilitate reliable and error-free amplification. Additional advantages of using **abm**'s Ligation-Free Cloning System include:

- Multiple fragment cloning and assembly.
- Cloning of any insert into any location of your chosen vector.
- No dependence on restriction site availability, phosphatase treatment and ligation steps.
- Inserts free from any redundant or unwanted base pairs.
- Save over 50% on reagents costs in comparison to other kits currently available.

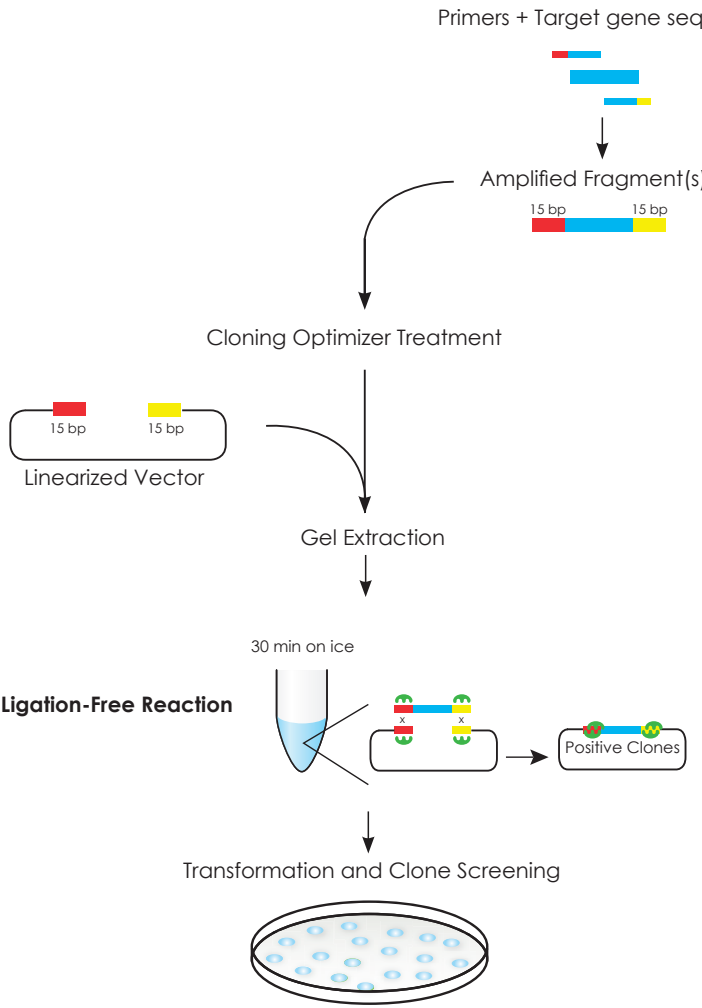

Figure 1. The Ligation-Free Cloning Procedure

Primers are designed to amplify the fragment with a 5' 15 bp overlap to the desired location within the vector. PCR is performed to amplify the fragment of interest and a Cloning Optimization Step is performed to remove contaminating template DNA (this step is only performed if the fragment of interest is amplified from plasmid DNA carrying the same selection marker as the target vector). The vector DNA is also linearized, either by restriction enzyme digest or by PCR, and is gel purified. The Ligation-Free Cloning reaction is then set up and left on ice for 30 minutes. The entire cloning reaction mix is then transformed and plated on an appropriate selection plate.

Table of Contents

1. List of Components

Store all components at -20 °C		Ligation-Free Cloning Kit
Part No.	**Components**	**E001**
E001-1	5X Ligation-Free Cloning MasterMix	100 µl
E001-2	Positive Control Insert	5 µl
E001-3	Positive Control Vector	15 µl
E001-4	Cloning Optimizer	25 µl
	Size	**25 rxns**

Store all components at -20 °C		Ligation-Free Cloning Kit
Part No.	**Components**	**E002**
E002-1	5X Ligation-Free Cloning MasterMix	400 µl
E002-2	Positive Control Insert	5 µl
E002-3	Positive Control Vector	20 µl
E002-4	Cloning Optimizer	100 µl
	Size	**100 rxns**

2. Additional Materials Required

1. General transformation materials including LB Agar (Cat. No. G247), an appropriate antibiotic for selection (e.g. Ampicillin Cat. No. G021 or Kanamycin Cat. No. G022) and LB Medium.
2. Proclone™ Competent Cells (Cat. No. E003).
3. DNA Gel Extraction Kit (Cat. No. D507) and PCR-Sure™ Kit (Cat. No. G065).
4. 2X PCR Precision™ MasterMix (Cat. No. G124).
5. SafeView™ DNA Stain (Cat. No. G468).

3. Storage Conditions

Store all components at -20°C in a non-frost-free freezer. All components are stable for one year from the date of shipping when stored and handled properly.

4. Insert and Vector Preparation

4.1. PCR Primer Design for the DNA Insert

Accurate primer design is the most critical factor in a successful Ligation-Free reaction. The following figure (Figure 2) is a guideline for designing these primers. In general, each primer should have a region of homologous sequence to the vector at the 5' end, and a gene-specific region at the 3' end. The 15 base pairs towards the 5' end of the primer must match the linear end of the DNA vector or fragment sequence to which it will be joined.

The 3' portion of the primer is the gene-specific region which must have a melting temperature (Tm) suitable for PCR (not including the homologous primer region). The optimal Tm should be around 55-65°C and can be adjusted by changing the length of the gene-specific region of the primer. The Tm difference between the forward and reverse primers should be ≤5°C to ensure successful amplification – alternatively, when complete freedom in your primer design is not possible, the use of our PCR Sure™ Kit (Cat. No. G065) will ensure full amplification of all fragments in your PCR reaction.

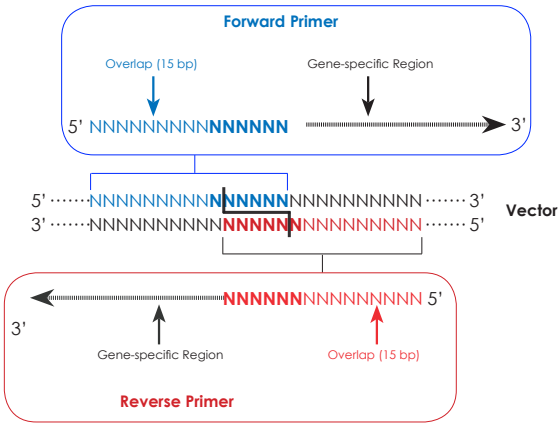

Figure 2a. Primer design when vector is linearized with a single restriction site

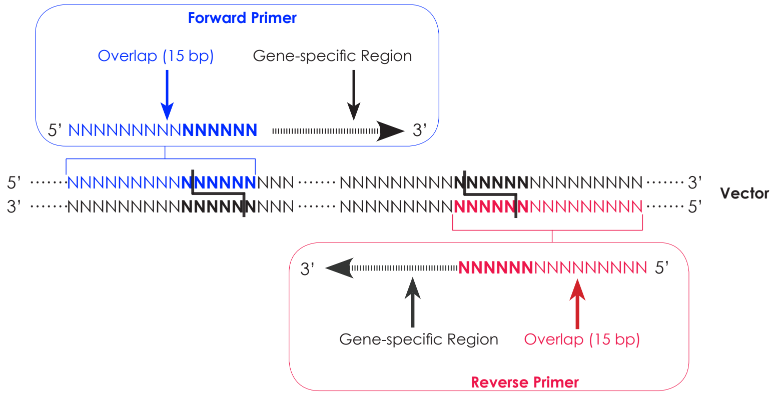

Figure 2b. Primer design when vector is linearized with double restriction sites

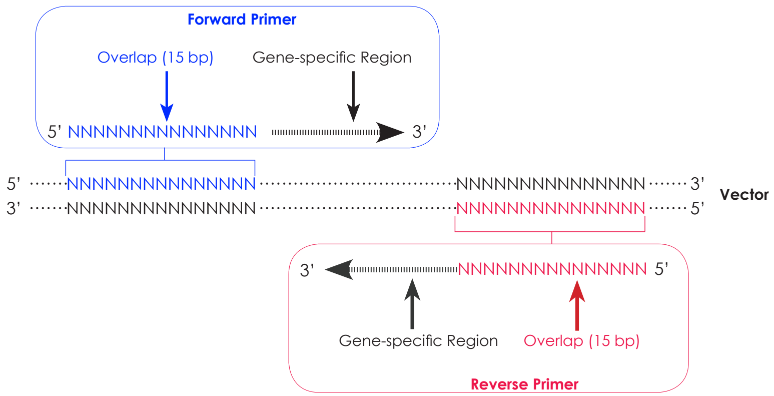

Figure 2c. Primer design when vector is PCR linearized

4.2. PCR Amplification of the DNA Insert

To ensure the Ligation-Free cloning reaction efficiency, it is important to use a minimal amount of DNA as your template during PCR amplification; 10-50 ng of plasmid template is optimal. A cDNA pool can also be used as template and (depending on the expression level of the gene) its quantity in the PCR reaction can be adjusted.
When plasmid DNA with the same resistance marker as the intended cloning vector is used, a Cloning Optimizer Treatment following PCR amplification will help to completely eliminate background colonies. Trace amounts of plasmid template DNA from the PCR step can easily be transformed and yield colonies. With this treatment, >90% positive colony yield is expected, while only ~30-40% clones will be positive when this treatment is omitted.

Cloning Optimizer Protocol:
a. Add 1µl of Cloning Optimizer (Cat No. E004) to 40 µl of PCR product.
b. Incubate at 37°C for 30 minutes, followed by 80°C for 15 minutes in a PCR Thermal Cycler or heating block.
c. Perform gel electrophoresis, then isolate and purify the DNA with a Gel Extraction Kit (Cat No. D507) or PCR clean-up kit.

In addition, it is critical to use the highest fidelity DNA Polymerase to avoid possible mutations associated with PCR amplification. **abm**'s Precision™ Taq DNA Polymerase (Cat No. G078) is the preferred choice with enhanced proofreading capability over other available polymerases.

Once the PCR reaction is complete, analyze the amplified product by electrophoresis on an agarose gel containing SafeView Plus™ (Cat No. G468) to confirm target DNA amplification. If a single specific target DNA fragment is amplified, you could simply purify the product by using a PCR Clean-Up Column Kit (Cat. No. D509) for direct cloning. If non-specific background or multiple bands are visible on your gel, isolate your target fragment by gel extraction.

Finally, quantification of the purified insert DNA is required before continuing with the Ligation-Free Cloning reaction. Quantify the amount of DNA by UV Spectrometry, NanoDrop or comparing the target band against a known molecular weight marker run on the same gel.

4.3. Preparation of the Linearized Vector

Complete digestion of the vector increases Ligation-Free cloning efficiency, as incomplete digestion will lead to high incidence of background colonies. Complete linearization can be achieved in one of two ways: by restriction enzyme digest or by PCR amplification.

4.3.1 Restriction Enzyme Digest Method

To ensure a complete digestion of a designated vector, a longer digestion time and high enzyme:DNA ratio is required. Double restriction enzyme digestion is preferred wherever possible. Perform vector linearization as follows:

a. Set up a restriction enzyme digestion

Restriction Enzyme Digest		
Reaction Components	**Cloning Vector**	**Positive Control Vector**
Vector	2 - 5 µg	4 µl (400 ng)
10X Reaction Enzyme Buffer	4 µl	CutSmart Buffer: 2 µl
Restriction Enzyme I	5 U	EcoRI-HF 1 µl
Restriction Enzyme II (if required)	5 U	XhoI 1 µl
Nuclease Free H_2O	up to 40 µl	12 µl
Total Volume	40 µl	20 µl

b. Incubate at the required temperature for a minimum of 3 hours. Overnight digestion is recommended.

c. Perform gel electrophoresis then isolate and purify the linearized vector with a DNA Gel Purification Kit (Cat No. D507). Use 10 µl to elute the Positive Control and 30 µl to elute the Cloning Vector sample.

d. Check the completion of your digestion by transforming 5-10 ng of the linearized and purified vector with ProClone™ Competent Cells (Cat No. E003). If the background is still too high (> 50 colonies) repeat the digestion of the vector with more enzyme before another round of gel extraction and purification.

4.3.2 PCR Linearization Method

Alternatively, your PCR primers could be designed to amplify your plasmid DNA and yield a linearized vector with ends representing the same 15 base pair homology that was introduced during the insert primer design.
Figure 3 describes the primer design process in more detail.

When using this method, it is possible to become completely **restriction enzyme free**, with the ultimate freedom to clone your insert into any location within the plasmid DNA.

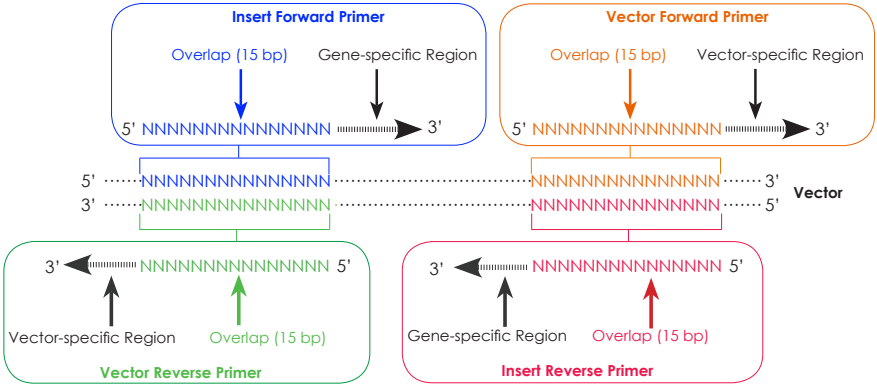

Figure 3. Primer design for linearization of vector DNA

Set up a PCR reaction with 10-50 ng of template vector. Use **abm**'s Precision™ Taq DNA Polymerase (Cat. No. G078) to ensure that no mutations are introduced to the vector DNA sequence during PCR amplification.
Following the PCR step, a Cloning Optimizer Treatment is recommended to help eliminate non-linearized, remaining circular template.

a. Add 1 µl of Cloning Optimizer to 40 µl of PCR product.
b. Incubate at 37°C for 30 minutes, followed by 80°C for 15 minutes in a PCR Thermal Cycler or heating block.
c. Perform gel electrophoresis, then isolate and purify the DNA with a Gel Extraction Kit (Cat. No. D507).
d. Check the completion of your digestion by transforming 5-10 ng of the linearized and purified vector with ProClone™ Competent cells (Cat No. E003). If background is still too high (> 50 colonies) repeat the Cloning Optimization step of the vector before another round of gel extraction and purification.

5. Ligation-Free Cloning Procedure

Included with this kit is a Positive Control that will help to successfully establish the Ligation-Free Cloning procedure in your laboratory. The Positive Control consists of a circular vector of 4.8 kb and a 0.7 kb purified insert for the cloning reaction.

Note: Digest the Positive Control Vector with EcoRI and XhoI as described in section 4.3.1 before continuing with the cloning reaction.

a. Setup the cloning procedure as follows:

Ligation-Free Reaction Set-up		
Reaction Components	**Cloning Reaction**	**Positive Control Reaction**
Purified DNA insert (from 4.2)	10 - 200 ng*	1 µl Positive Control Insert
Linearized Vector (from 4.3)	50 - 200 ng	10 µl Digested Positive Control Vector from 4.3
5X Ligation-Free Cloning MasterMix	4 µl	4 µl
Nuclease Free H_2O	To a final volume of 20 µl	5 µl
Total Volume*	20 µl	20 µl

* The molar ratio between the vector : insert should be 1:3. This can be calculated as follows:

The optimal amount of cloning vector = [0.02 x cloning vector bose pair number] ng (0.03 pmol).

The optimal amount of insert = [0.06 x insert base pair number] ng (0.09 pmol).

b. Mix well and incubate on ice for 30 minutes.

c. Perform the transformation by adding the cloning reaction mix described above (20 µl) to 60 µl of ProClone™ competent cells (Cat. No. E003). Mix gently.

d. Incubate the mixture on ice for 30 minutes.

e. Heat-shock for 45 seconds at 42°C, followed by further incubation on ice for 2 minutes.

f. Add 150 µl of LB Medium (without antibiotics and at room temperature) to the transformed cells.

g. Recover the cells by shaking at 37°C for one hour.

h. Spread the transformed cells onto pre-warmed LB plates containing an appropriate antibiotic for selection. **Note:** the Positive Control Vector has a Kanamycin resistance marker.

i. Incubate plates overnight at 37°C.

j. The next day, select 3 colonies and screen for positive clones using the appropriate restriction enzyme digestion. **Note**: Screen the Positive Control Reaction with EcoRI and XhoI.

6. Experimental Examples

The following two experimental examples have been provided to help illustrate the simplicity and potential of **abm**'s Ligation-Free Cloning system.

6.1 Single Fragment Cloning

Experimental Goal: To clone the 700 bp RFP gene into the restriction site EcoRI and XhoI on pShuttle (+) (Cat. No. A002).

6.1.1 Insert Primer Design

The primer design method (for the RFP insert) is illustrated in the following figure (Figure 4).

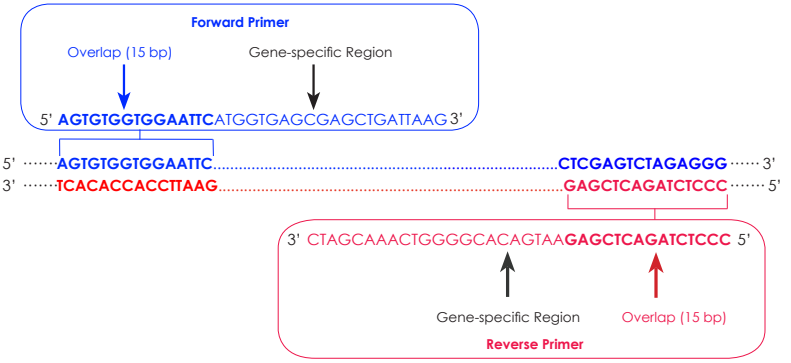

Figure 4. Primer Design for RFP Amplification

6.1.2 Linearization of pShuttle (+) vector

The following Restriction Enzyme digest was set up:

Restriction Enzyme Digest	
Reaction Components	**Volume**
pShuttle (+)	5 µl (2.5 µg)
NEB CutSmart™ Buffer*	4 µl
EcoRI-HF	1 µl
XhoI	1 µl
Nuclease-free dH_2O	29 µl
Total Volume	40 µl

*CutSmart™ Buffer is provided with EcoRI-HF and XhoI enzymes from NEB.

The reaction was incubated at 37°C overnight. The digested vector was visualized on an agarose gel by gel electrophoresis and gel purified using **abm**'s DNA Gel Extraction Kit (Cat. No. D507). The purified vector was quantified using Maestrogen's NanoDrop Instrument. The concentration of the vector was recorded as 50 ng/µl.

6.1.3 PCR Amplification of the Insert

Using **abm**'s PCR Precision™ MasterMix (Cat. No. G124), the RFP insert was PCR amplified from a plasmid template. The PCR reaction was set up as follows:

PCR Reaction Set-up	
Reaction Components	**Volume**
PCR Precision™ MasterMix	25 µl
Forward Primer (10mM)	1 µl
Reverse Primer (10mM)	1 µl
Plasmid Template	1 µl (50 ng)
Nuclease-free dH_2O	22 µl
Total Volume	50 µl

After the PCR reaction, Cloning Optimization was carried out to eliminate the template plasmid DNA used during this PCR amplification:

a. 1 µl of Cloning Optimizer was added to 40 µl of PCR product.
b. The mixture was incubated at 37°C for 30 minutes, followed by 80°C for 15 minutes in a PCR Thermal Cycler.

The product was visualized on an agarose gel and gel purified using **abm**'s DNA Gel Extraction Kit (Cat No. D507). The purified DNA was quantified using Maestrogen's NanoDrop Instrument. The concentration of the insert was recorded as 45 ng/µl.

6.1.4 Ligation-Free Cloning Reaction

The optimal concentration of vector and insert was calculated as follows:
Optimal amount of the cloning vector = [0.02 × 4800] ng = 96 ng
Optimal amount of insert = [0.06 × 700] ng = 42 ng

The Ligation-Free Cloning Reaction was setup as follows:

Ligation-Free Reaction Set-up		
Reaction Components	**pShuttle (+)/RFP Reaction**	**Negative Control Reaction**
Purified RFP Insert (From 6.1.3)	1 µl	-
Linearized Vector (From 6.1.2)	2 µl	2 µl
5X Ligation-Free Cloning MasterMix	4 µl	4 µl
Nuclease Free H_2O	13 µl	14 µl
Total Volume	20 µl	20 µl

The above reaction mix was incubated on ice for 30 minutes.

6.1.5 Transformation of Colony Screening

60 µl of chemically competent TOP10 cells were added directly to each reaction mix and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds followed by further incubation on ice for 2 minutes. 150 µl of LB Medium was added and the cells were allowed to recover at 37°C for 1 hour. The cells were then plated on pre-warmed Kanamycin plates and incubated at 37°C overnight.

a) **b)**

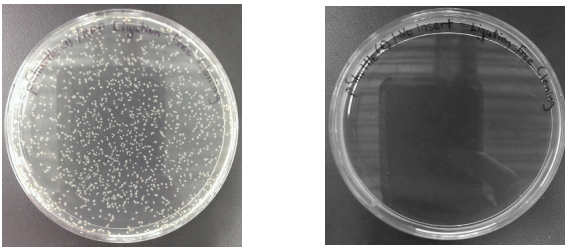

Figure 5. Picture of plates grown overnight a) pShuttle(+)/RFP Reaction b) Negative Control Reaction

The next day, 4 colonies were selected for miniprep and screened using EcoRI and XhoI digestion:

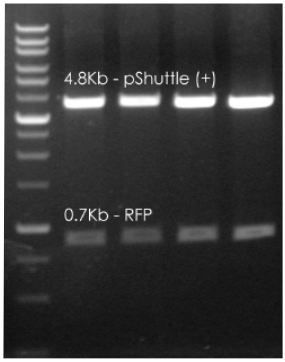

Figure 6. Miniprep screening results confirming a cloning efficiency of 4/4

6.2 Multiple Fragment Cloning

Experimental Goal: To clone the 2346 bp ZNF217 UTR (NM_006526) into the restriction sites EcoRI and XhoI on pLenti-UTR-Luc (Cat. No. MT-h29038).

6.2.1 Insert Primer Design

The UTR sequence was divided into three fragments: Fragment 1 (817 bp), Fragment 2 (925 bp) and Fragment 3 (703 bp). Three sets of primers were designed to amplify these fragments with the following features (see Figure 8 over page):

Fragment 1–FP: 15 bp overlap with the pLenti-UTR-Luc vector
Fragment 1–RP: 15 bp overlap with 5' end of Fragment 2

Fragment 2–FP: 15 bp overlap with 3' end of Fragment 1
Fragment 2–RP: 15 bp overlap with 5' end of Fragment 3

Fragment 3–FP: 15 bp overlap with 3' end of Fragment 2
Fragment 3–RP: 15 bp overlap with the pLenti-UTR-Luc vector

The sequences were as follows:

Fragment 1-FP:
TGGTGGCCTGCAGGT**GAATTC**AGTCTTGGTGGATGTCAGTG
Fragment 1-RP:
CAACGTGTGGCTGCTC

Fragment 2-FP:
GAGCAGCCACACGTTG
Fragment 2-RP:
CTAGTCACAGCAAGCTCTCTG

Fragment 3-FP:
CAGAGAGCTTGCTGTGACTAG
Fragment 3-RP:
GACGTCGTATGGGTA**CTCGAG**CAGGCATGATGGCTCGATA

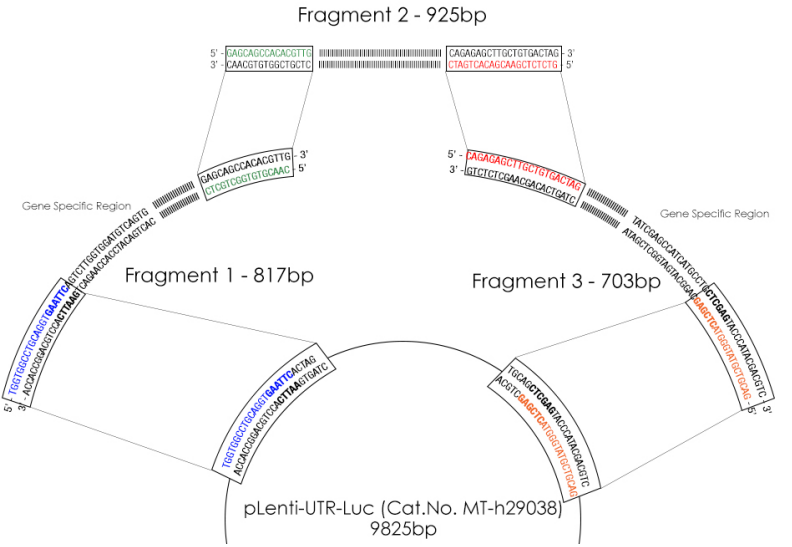

Figure 7. Primer Design for multiple fragment cloning

6.2.2 Preparation of the Three Fragments and the Vector

Insert and vector preparation was conducted as described in Section 4.2 and 4.3

6.2.3 Ligation-Free Cloning Reaction

Optimal concentrations of vector and insert were calculated as follows:

Optimum amount of the cloning vector = [0.02 × 9825] ng = 196.5 ng
Optimum amount of Fragment 1 = [0.06 × 817] ng = 49 ng
Optimum amount of Fragment 1 = [0.06 × 925] ng = 56 ng
Optimum amount of Fragment 1 = [0.06 × 703] ng = 42 ng

The Ligation-Free Cloning Reaction was setup as follows:

Ligation-Free Reaction Set-up	
Reaction Components	**Positive Control Reaction**
pLenti-UTR-Luc (75 ng/µl)	2.7 µl
Fragment 1 (16 ng/µl)	3 µl
Fragment 2 (12 ng/µl)	4.9 µl
Fragment 3 (28 ng/µl)	1.5 µl
5X Ligation-Free Cloning MasterMix	4 µl
Nuclease Free H_2O	3.9 µl
Total Volume	20 µl

The above reaction mix was incubated on ice for 30 minutes.

6.2.4 Transformation and Cloning Efficiency

Transformation and colony screening was carried out as described in Section 6.1.5.

The results of this cloning experiment were as follows:

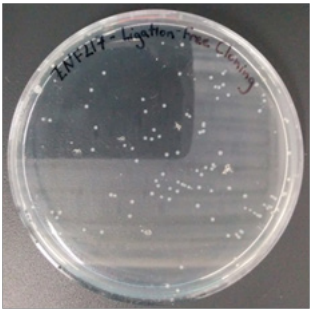

Figure 8. Plate Picture after Overnight Incubation

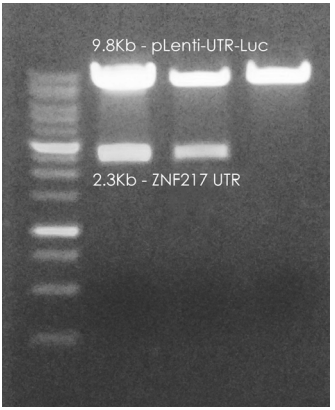

Figure 9. Miniprep DNA obtained from 3 colonies was digested with EcoRI/ XhoI showing a cloning efficiency of 2 out of 3

7. Special Notes for Consideration

1. **Please store the kit at -20°C upon arrival.** All kit components are sensitive to temperature change.

2. **Complete linearization of the cloning vector incerases ligation-free cloning efficiency.** During the vector linearization, try to use two restriction enzymes to digest the cloning vector. Increase the incubation time to more than 3 hours and when possible perform overnight digestion. Use a high restriction enzyme to DNA ratio during the digest. Gel purification of the linearized vector is also a critical step.

3. **A high-fidelity DNA polymerase is required** to generate the linearized vector or amplify the insert with PCR, to ensure no mutations are introduced during the amplification process. For this purpose we recommend our Precision™ DNA Polymerase (Cat No. G078) with very high fidelity and high amplification processivity. This polymerase guarantees a correct product with high efficiency which are both important for this application.

4. **Try to use pre-linearized DNA as your template when generating the linearized vector with PCR** (in order to eliminate possible interference from the trace amount of circular DNA). In a situation where circular DNA must be used as the template, treat the amplified PCR product with Cloning Optimizer before proceeding to the cloning reaction.

5. **To prepare the insert DNA with PCR, always try to use a minimal amount of DNA as template**, especially if the template DNA is from a circular plasmid carrying the same antibiotic selection marker as the cloning vector. Do not use more than 50 ng DNA.

6. **Purification of inserts smaller than 4 kb from the PCR reaction is NOT mandatory**. If the template is a linear DNA and the PCR product is clean (i.e. when ran on a gel, it shows only one band corresponding to the desired product) the end user can skip the purification step and go directly to the cloning reaction. Otherwise, either a PCR column purification or a gel recovery purification will be required to ensure a high cloning efficiency.

7. **When setting up the cloning reaction, it is critical to calculate and use the exact amount of linearized vector and insert as described in this manual.** The optimal cloning result is usually from a vector to insert ratio of 1:3 (Molar ratio).

8. Troubleshooting Guide

If you do not obtain the expected results from the provided protocols, use the following table to help troubleshoot your experiments more effectively:

Problem	Possible cause	Suggested Action
No Colonies	Low transformation efficiency	Perform a positive control transformation to optimize. Ensure you are using the correct antibiotic against your selection marker.
	Sub-optimal cloning conditions	Check the primer design, especially the homologous sequence. Check the molar ratio of vector to insert (1:3). Re-quantify the concentration of the vector and insert.
	Impurity of the vector or insert DNA	Purify the vector or insert with a DNA purification kit to remove any potential inhibitors. Use deionized water to replace the TE or elution buffer in column purification. Limit the volume of unpurified DNA to <1/5 of the cloning reaction.
Lawn of Colonies	Plates are old	Be sure that your antibiotic plates are fresh (<1 month old).
No Insert present	Incomplete linearization of your vector or circular DNA template left over from PCR	Perform a complete digestion of vector (add more enzyme, increase digestion time). Treat amplified PCR products with Cloning Optimizer.
Incorrect Plasmid	Contamination of PCR plasmid template DNA with the same selection marker	Perform a Cloning Optimizer Treatment after PCR amplification.
Incorrect Insert	PCR amplification of insert not specific, multiple PCR products present	Optimize PCR condition for specific target amplification.

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