Pro Ligation-Free Cloning Kit

Application Handbook

Cat. No. E086 / E087

Notice to Purchasers

abm's products are guaranteed to meet their required quality control standards at the time of shipment. Notice of problematic products must be made to **abm** within 10 days of receipt. This product warranty limits **abm**'s liability to the replacement of the product only.

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Pro Ligation-Free Cloning Kit: Quick-Start Protocol

To download the full manual, visit Cat. No. E086 or E087 on *www.abmgood.com*.

Design and Amplify Overlapping PCR Products

1. Design primers to amplify insert fragments with 15-25 bp of overlapping homology to each other and to the linearized vector.
2. PCR amplify the fragments and visualize on an agarose gel.

Critical Step: For more details on primer design, see page 4 of the manual.

Purification Steps (Recommended)

3. Column purify your products, or gel extract if the PCR amplification contained non-specific bands.
- Alternatively, add 0.5 µl of Assembly Enhancer and 1.0 µl Cloning Optimizer to 50 µl of PCR product. Incubate at 37°C for 5 minutes, then at 80°C for 20 minutes.

Note: Gel extraction will help remove any residual PCR template plasmid, reducing background colony growth.

Assembly Reaction

4. Quantify the insert and vector DNA by a UV spectrophotometer or by comparing the target band against a known molecular weight marker run on the same gel.

5. Set up the following reaction on ice:

Reagent	Cloning Reaction	Example Reaction
2X Pro Ligation-Free MasterMix	10 µl	10 µl
Linearized Vector	100 ng	100 ng (5 kb vector)
Each Insert	3:1 insert-vector molar ratio	60 ng (1 kb insert)
Nuclease Free H_2O	Up to 20 µl	Up to 20 µl

To calculate amount of insert to add:

$$\text{insert (ng)} = 3 \times \frac{\text{insert (bp)}}{\text{vector (bp)}} \times \text{vector (ng)}$$

6. For 1 insert, incubate at 50°C for 15 minutes. For multiple inserts or difficult assemblies, incubate at 50°C for 1 hour.
7. Transform immediately, or store samples at -20°C until transformation.

ProClone™ Chemical Transformation

8. Add reaction mix to 60 µl of ProClone™ competent cells (Cat. No. E003).
9. Incubate on ice for 30 minutes.
10. Heat-shock for 45 seconds at 42°C, then chill on ice for 2 minutes.
11. Add 150 µl of LB Medium without antibiotics.
12. Shake at 37°C for 1 hour for recovery.
13. Spread cells onto pre-warmed LB agar plates with an appropriate antibiotic.
14. Incubate plates overnight at 37°C.

Troubleshooting: If no colonies are present, check: primer design, purity and concentration of DNA inserts, vector-insert ratio, antibiotic used, and transformation efficiency.

Screening and Analysis

15. Screen by colony PCR, or grow up colonies overnight in LB medium containing the appropriate antibiotic.
16. Isolate plasmid DNA. We recommend using our Plasmid Mini-Prep Kit (D504).
17. Confirm correct assembly by restriction analysis or sequencing.

Troubleshooting: If too many colonies are present or clones do not pass screening, consider the following possibilities: vector was not completely digested, presence of residual PCR template plasmid, antibiotics are old, or amplification was not specific.

Table of Contents

1. Introduction

The Pro Ligation-Free Cloning Kit allows for the robust assembly of multiple DNA fragments in one step. DNA fragments generated with 15-25 bp overlaps are assembled seamlessly without concern for restriction enzyme site availability, thus allowing for a simple, yet versatile method to rapidly clone highly complex, multiple component constructs.

Advantages over Traditional Cloning:

1. Rapidly assemble as many as 6 fragments into any vector in one step.
   → Eliminates time-consuming, multi-step traditional cloning methods.
2. Seamlessly clone your inserts anywhere in any vector.
   → Cloned products are free of unwanted base pairs.
   → Eliminates dependence on available restriction sites and compatible ends.

Improvements on Ligation-Free Cloning Kit (Cat. No. E001/E002):

1. Achieve robust assembly in one step.
   → Use Pro Ligation-Free for difficult, multi-fragment assemblies.
2. Faster, more reliable cloning with the addition of the Assembly Enhancer.
   → Eliminates time-consuming gel extraction or column purification steps.

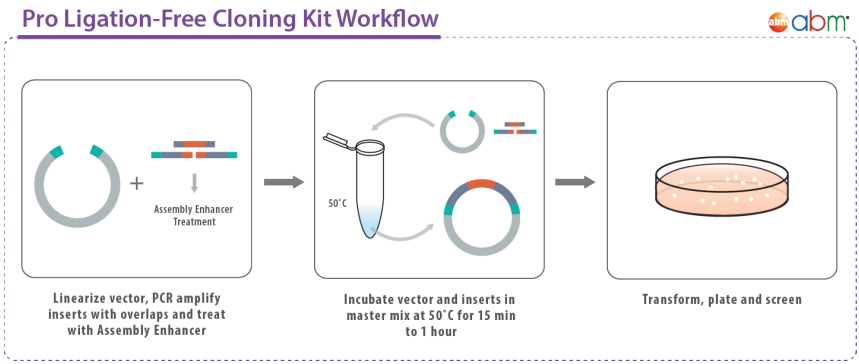

Figure 1. General workflow for seamless cloning assembly of multiple inserts using the Pro Ligation-Free Cloning Kit.

2. List of Components

Pro Ligation-Free Cloning Kit (20 reactions):

Part No.	Components	E086
E086-1	2X Pro Ligation-Free Cloning MasterMix	200 µl
E086-2	Positive Control Insert	4 µl
E086-3	Positive Control Vector	16 µl
E086-4	Assembly Enhancer	10 µl
E086-5	Cloning Optimizer	20 µl
	Size	**20 reactions**

Pro Ligation-Free Cloning Kit (50 reactions):

Part No.	Components	E087
E087-1	2X Pro Ligation-Free Cloning MasterMix	500 µl
E087-2	Positive Control Insert	4 µl
E087-3	Positive Control Vector	16 µl
E087-4	Assembly Enhancer	25 µl
E087-5	Cloning Optimizer	50 µl
	Size	**50 reactions**

3. Storage Conditions

Store all components at -20°C. All components are stable for one year from the date of shipping when stored and handled properly. The 2X Pro Ligation-Free Cloning MasterMix, Assembly Enhancer, and Cloning Optimizer should be kept on ice when not stored at -20°C and should be aliquoted to avoid repeated freeze/thaws.

4. Additional Materials Required

	Material	Purpose	Recommended Products	Cat. No.
1	High-fidelity DNA polymerase	PCR Amplify Insert and/or Vector	Precision™ DNA Polymerase	G078 G124
2	Agarose gel DNA stain	View PCR products	Agarose Safe-Green™	G060-2 G108-G
3	Purification kit*	Purify inserts, remove unwanted amplicons, primers, buffer, etc.	DNA Gel Extraction Kit Column-Pure PCR Clean-Up Kit	D507 D509
4	Competent cells	Transform assembled vector into *E. coli*	ProClone™ Competent Cells	E003
5	LB agar plates with appropriate antibiotic	Selection of cells harboring the assembled vector	LB agar Ampicillin Kanamycin	G247 G021 G022
6	Plasmid purification kit	Purify cloned plasmids before restriction digest screening	Column-Pure Plasmid Mini-prep Kit	D504

*If gel shows no additional unwanted amplicons, the Assembly Enhancer can be used on PCR products without need for column purification kits.

5. Preparation of the Inserts

5.1 Design of PCR Primers for Inserts

Accurate primer design is critical for a successful seamless assembly reaction. The following figures (Figures 2-5) are a guideline for designing these primers. We recommend first designing a DNA sequence file outlining how inserts will be assembled in your vector. Next, PCR primers should be designed that incorporate a small portion of the sequence of each adjacent fragment. Ultimately each primer should have a sequence overlapping with the adjacent fragment or vector at the 5' end, and an insert-specific region at the 3' end.

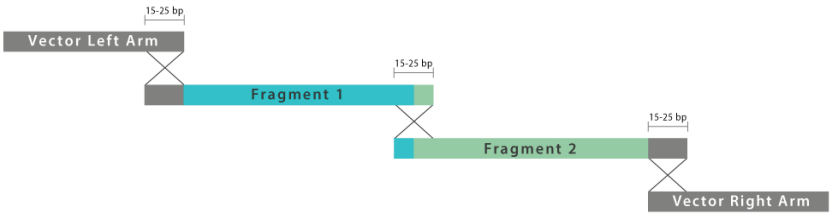

Figure 2. Schematic outlining the assembly of PCR amplified fragments with overlapping adjacent sequences.

5.1.1 Primer Design for Multiple Fragments

•Identify the junctions between each insert.

•Choose a 15-25 bp overlap between each junction point (Figure 3 boxed in purple). The overlap sequences should be unique to each fragment pair, in order to preserve the desired order of assembly.

•Design the insert-specific region of the primer (excluding the 5' overlap to the adjacent fragment) with an appropriate melting temperature (T_m) for PCR and minimal secondary structures (Figure 3, the 3' segment of the arrows).

•To each primer, add the 5' overlap region of the primer which should be complementary to the adjacent fragment (Figure 3, the 5' segment of the arrows).

•For design of outermost primers, see Section 5.1.2 or 5.1.3.

•Amplify Fragment 1 with F1-FP + F1-RP, Fragment 2 with F2-FP + F2-RP and Fragment 3 with F3-FP + F3-RP.

Figure 3. Primer design for multiple inserts. The theoretical construct of a 3 fragment assembly is shown, where insert-specific sequences (3' of arrows) are in their corresponding colours, with overlap sequences (5' of arrows) coloured according to their adjacent fragments. The specific 15-25 bp overlap region between adjacent fragments is boxed in purple.

5.1.2 Design of Outermost Primers for a PCR-Linearized Vector

•Identify the junctions between the insert and vector.

•Choose 15-25 bp overlap directly up and downstream of the insert – these will be the vector left and right overlaps (Figure 4, boxed in purple). The overlap sequences should be unique to each fragment pair, in order to preserve the desired order of assembly.

•Design the primers for amplifying the vector with an appropriate T_m. These primers should include the overlap region chosen in the previous step and should orient in opposing directions in order to achieve an inverse PCR of the vector (See Figure 4). Alternatively, overlap vector primers can be designed using the same logic described in Section 5.1.1.

•For how to design primers to amplify the fragment, see Section 5.1.1.

•PCR-amplify the vector with VP-FP + VP-RP and the fragment with F1-FP and F1-RP.

Figure 4. Primer design for inserts using a PCR-linearized vector. The theoretical construct of a 1 fragment assembly is shown, where the insert-specific sequence (3' of arrows) is in blue, and overlap sequences (5' of arrows) are in grey. The specific 15-25 bp overlap region between adjacent fragments is boxed in purple.

5.1.3 Design of Outermost Primers when using a Restriction Enzyme-Digested Vector

Primer design when using a restriction enzyme digested vector is identical as when using a PCR-linearized vector, except that the overlap region must include the restriction site.

•Identify the junctions between the insert and the vector to be digested with restriction enzymes.
•Choose a 15-25 bp region excluding each restriction site; these will be the vector overlaps (Figure 5, boxed in purple). The overlap sequences should be unique to each fragment pair, in order to preserve the desired order of assembly.
•For how to design primers to amplify the fragment, see Section 5.1.1.
•PCR amplify the fragment with F1-FP and F1-RP.
•Digest the vector with the chosen restriction enzymes (Figure 5, example restriction sites boxed in pink).

Figure 5. Primer design for inserts using a restriction enzyme-digested vector. The theoretical construct of a 1 fragment assembly is shown, where the insert-specific sequence (3' of arrows) is in blue, and overlap sequences (5' of arrows) in grey. The restriction enzyme sequences are boxed in pink and the specific 15-25 bp overlap regions between fragment and vector is boxed in purple.

5.1.4 Additional Notes on Primer Design

•The 3' portion of the primer is the insert specific region which must have a suitable T_m for PCR (the region excluding the 5' overlap with the adjacent fragment).
•When possible we recommend designing the 5' overhang sequence to have a T_m of ~50°C.
•The T_m 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.

5.2 PCR Amplification of Inserts

We recommend using a high-fidelity DNA polymerase (**abm** Precision™ DNA Polymerase [Cat. No. G078, or G124 in MasterMix format]) to amplify all inserts and/or vector. These enzymes have enhanced proofreading ability and will greatly reduce mutations that are generated during PCR amplification. If PCR inserts will not be purified by gel extraction, it is important to use a minimal amount of template DNA in the PCR reaction as excess plasmid DNA can be carried forward and lead to the formation of background colonies. Less than 10 ng of plasmid template is optimal, but if large amounts of template DNA must be used, the PCR product may be treated with Cloning Optimizer (see Section 5.3) to prevent the formation of background colonies.

Suggested PCR Set Up	
Reaction Components	**Volume**
2X PCR Precision™ Mastermix (G124)	25 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Template DNA	1 µl (10 ng)
Nuclease Free H_2O	22 µl
Total Volume	50 µl

After the PCR reaction, analyze the amplicons by electrophoresis on an agarose gel using SafeGreen™ (Cat. No. G108-G) to confirm target DNA amplification. If a single, specific target DNA fragment is amplified, the product can be purified using a PCR Clean-Up Column Kit (Cat. No. D509) or by using the Assembly Enhancer. If non-specific background or multiple bands are visible on the agarose gel, isolate your target fragment by gel extraction. For high-throughput preparation of PCR products, treat each PCR product with the Assembly Enhancer (see Section 5.3 for details).

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

5.3 Optional: Assembly Enhancer and Cloning Optimizer Treatment of Inserts

The Assembly Enhancer can streamline your cloning projects by eliminating further manual work and use of additional consumables. The Assembly Enhancer is suitable for high-throughput cloning applications as it can be added directly to PCR products and incubated in a programmed thermal cycler. This treatment can also reduce PCR product loss, typical of column-based purification kits (>50% loss).

The Cloning Optimizer cleaves only *E. coli* Dam-methlyated DNA but does not cleave PCR amplified DNA (as it is not methylated). Therefore it can be employed to degrade plasmid template DNA which could otherwise lead to negative downstream effects (see Section 9).

a. Add 0.5 µl of Assembly Enhancer and 1 µl Cloning Optimizer to 50 µl of PCR product.
b. Incubate at 37°C for 5 min, followed by 80°C for 20 min in a thermal cycler or heating block.
c. Cool to room temperature before adding into the Pro Ligation-Free Cloning Reaction mixture.

6. Preparation of the Linearized Vector

Complete digestion of the vector is critical to achieving high cloning efficiency, as incomplete digestion will lead to high incidence of background colonies. Complete linearization can be achieved by either restriction enzyme digest or by PCR amplification and treatment with the Cloning Optimizer.

6.1 Restriction Enzyme Digest Method

To ensure a complete digestion of vector DNA, a longer digestion time and high enzyme:DNA ratio is required. Double restriction enzyme digestion and use of enzymes which generate incompatible sticky ends is recommended whenever possible as this prevents vector self-ligation.

a. Set up a restriction enzyme digestion, as follows:

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

*Or upscale as necessary.

b. Incubate at the required temperature for 1-4 hours, depending on enzyme efficiency.

c. Perform gel electrophoresis, then isolate and purify the linearized vector with a DNA Gel Purification Kit (Cat No. D507). Use 20 µl to elute the provided Positive Control Vector and 30 µl to elute the cloning vector sample.

d. Check the completion of your digestion by transforming 100 ng (or ~1 µl) 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.

6.2 PCR Linearization Method

Alternatively, PCR primers can be designed to amplify your plasmid DNA and yield a linearized vector with ends comprising the same 15-25 bp overlap that was introduced during the insert primer design (see Figure 4). When using this method, it is possible to become completely independent of restriction enzymes, with the ultimate freedom to clone your insert into any location within the vector DNA. However, use of a high-fidelity DNA polymerase is essential in order to prevent undesired backbone mutations. This method is not recommended for plasmids over 8 kb in length.

a. Set up a PCR reaction using a high-fidelity DNA polymerase (**abm**'s Precision™ Taq DNA Polymerase [Cat. No. G078, or G124 in MasterMix format]) using ~10 ng of template vector. It is important to use as small an amount of template plasmid as possible in order to reduce the number of background colonies.

b. Perform gel electrophoresis to visualize the linearized vector.

c. Isolate and purify the DNA either with a PCR Clean-Up Column Kit (Cat. No. D509) or by gel extraction (Gel Extraction Kit [Cat. No. D507]) if non-specific or multiple bands are visible.

d. If you are concerned about background colonies formed by leftover template plasmid, treat the PCR reaction with Cloning Optimizer (see Section 5.3 for details).

e. Check the completion of your linearization by transforming 100 ng (~1 µl) of the linearized and purified vector with ProClone™ Competent cells (Cat. No. E003).

7. Pro Ligation-Free Cloning Reaction

Included with this kit is a Positive Control that will help to successfully establish the Pro 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 6.1 before continuing with the cloning reaction.

a. Setup the cloning reaction in a 0.2 ml PCR tube on ice.

Pro Ligation-Free Cloning Reaction Set-up			
Reaction Components	**Cloning Reaction**	**Positive Control Reaction**	**Negative Control Reaction**
2X Pro Ligation-Free Cloning MasterMix	10 µl	10 µl	10 µl
Digested Vector (from Section 6.1)	100 ng	9 µl Digested Positive Control Vector	100 ng
Inserts	3:1 insert-vector molar ratio*	1 µl Positive Control Insert	-
Nuclease Free H_2O	Up to 20 µl	-	Up to 20 µl
Total Volume	20 µl**	20 µl	20 µl

* Calculate the required amount of each insert to add using the following formula:

$$\text{insert (ng)} = 3 \times \frac{\text{insert (bp)}}{\text{vector (bp)}} \times \text{vector (ng)}$$

** If the volume of the reaction is greater than 20 µl, use additional 2X Pro Ligation-Free MasterMix and dilute to 1X of the total volume (eg. 15 µl of MasterMix in a total volume of 30 µl).

b. Pipette mix the reaction and incubate at 50°C for the following:
15 minutes for 1 insert
1 hour for multiple inserts or difficult assemblies

c. Transform immediately, or store samples at -20°C unti ready to transform.

8. ProClone™ Chemical Transformation

a. Perform the transformation by adding the cloning reaction mixes described previously (20 µl reactions) to 60 µl of ProClone™ Competent Cells (Cat. No. E003). Mix gently.

b. Incubate the mixture on ice for 30 minutes.

c. Heat-shock for 45 seconds at 42°C, then chill on ice for 2 minutes.

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

e. Recover the cells by vigorous shaking (250 rpm) at 37°C for 1 hour.

f. Spread the transformed cells onto pre-warmed LB plates with appropriate antibiotic selection. Note: the Positive Control Vector confers Kanamycin resistance.

g. Incubate plates overnight at 37°C.

h. Assess plates the following day. The negative control plate should have a minimal number of colonies.

i. Screen colonies either by colony PCR using primers that flank the assembled product or restriction digest following plasmid extraction (Column-Pure Plasmid Mini-Prep Kit [Cat. No. D504]). Note, screen the positive control reaction by digesting with EcoRI and XhoI. Successful clones will have a 0.7 kb insert and 4.8 kb vector band after EcoRI/XhoI digestion.

9. 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 Actions
No Colonies	Low transformation efficiency	•Perform a positive control transformation to optimize. •Ensure the cells have not been previously thawed and re-frozen. Cells that settle immediately after thawing are likely dead. •Ensure you are using the correct antibiotic against your selection marker. •Test the efficiency of your competent cells by transforming uncut pUC19 and plating on Ampicillin plates.
	Sub-optimal cloning conditions	•Check primer design. Ensure the 5' overlap sequence is 15-25 bp and that the T_m of the overlap sequence is ~50°C when possible. •Check the molar ratio of insert:vector (3:1). •Re-quantify the vector and insert.
	Impurity of the vector or insert DNA	•Either treat the PCR product vector and inserts with the Assembly Enhancer or use a DNA purification kit to remove any potential inhibitors. •Gel purify inserts and vectors whenever possible.
Lawn of Colonies	Plates are old	Be sure that your antibiotic plates are fresh (<2 weeks old).
	Reaction was highly efficient	•Compare your reaction plate to the negative control. •If # of colonies on the reaction plate > # of colonies on the negative control plate, then the assembly was likely successful. late several dilutions of the recovery mixture. •If # of colonies on the reaction plate = # of colonies on the negative control plate, then the assembly was likely unsuccessful. See below for more details.

Problem	Possible Cause	Suggested Actions
No Insert Present	Incomplete linearization of vector or residual circular template DNA from PCR	•Perform a complete digestion of vector (increase amount of enzyme and digestion time). •Perform a gel extraction of PCR linearized vector, followed by treatment with Cloning Optimizer (see Section 5.3).
Incorrect Plasmid	Contamination of PCR plasmid template with DNA carrying the same selection marker	•Perform a gel extraction of PCR linearized vector, followed by treatment with Cloning Optimizer (see Section 5.3). •Re-transform and isolate a monoclonal population of your target vector to remove other co-isolated plasmids; confirm presence of correct vector by sequencing, then re-start cloning procedure.
Incorrect Insert	PCR amplification of insert not specific, multiple PCR products present	•Either optimize the PCR or perform gel extraction of the correct band. • Sequence the PCR product to confirm the identity of the fragment before proceeding.
	Inserts may be toxic to *E. coli*	•Consider cloning insert behind an inducible promoter or switching to a low copy plasmid.

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