Primer Design

DynaExpress Hetero-Stagger PCR Cloning method needs four PCR primers. Two PCR primers contain eight or nine extra bases at the 5', which are complementary to the pHST vector single-strand extension, as shown in Figure 2. The other two primers have no extra bases.

The primers containing extra bases should be added the following sequences to each 5' end of the gene-specific primers:

5' (Forward) Primer: 5'- TACAGTGCC - NNNN----NNN-3' *

3' (Reverse) Primer: 5'- TACAGTGGG - NNNN----NNN-3'

* NNNN----NNN ; gene specific sequences

If a proofreading thermostable DNA polymerase, for example *Pfu, $Vent_R$, and Deep $Vent_R$,* is used, both whole extra bases should be attached to the primers. While non-proofreading DNA polymerase (*Taq, Tth and Tfl*) is used, the red T at the 5'-ends of both extra bases should not be attached to the primers. In the case of the latter, we recommend a final extension step for 10min at 72℃ for efficient adding of an A overhang during the PCR reaction.

The orientation of the insert within the vector can be changed by swapping the above extra bases between the two primers or using pHST-2 vector which has reverse directional single-strand extensions compared to pHST-1.

DNA Amplification

Two PCR reactions are set up to generate two PCR products containing different extra terminal sequences which are complementary to the single-strand extensions of pHST.

One PCR reaction should be performed using Forward Primer with extension sequence and Reverse Primer without extension sequence. The other PCR reaction should be performed using Forward Primer without extension sequence and Reverse Primer with extension sequence.

* NNNN----NNN ; gene specific sequences

PCR Reaction 1:	5' (Forward) Primer:	5'- TACAGTGCC- NNNN----NNN-3' *
	3' (Reverse) Primer:	5'- NNNN----NNN-3'
PCR Reaction 2:	5' (Forward) Primer:	5'- NNNN----NNN-3'
	3' (Reverse) Primer:	5'- TACAGTGGG- NNNN----NNN-3'

The efficiency of cloning is known to depend on the purity of PCR fragments. After amplification, the PCR product should be analyzed by the agarose gel electrophoresis. We recommend quantifying the amount of DNA by using a known standard weight marker (DynaMaker DNA Low D #DM112 or High D #DM122) run onto the gel. Purification method of PCR products depends on the results of the electrophoresis. If a sufficient amount of homogeneous band of the desired size is observed on the gel, it can be directly used for the annealing procedure. If PCR products show smear or multiple bands on the gel, we recommend gel-purification of the band by use of Gel Indicator DNA extraction kit (#DM550).

Even if a single and discrete band of expected size is observed, the PCR sample contains a complex mixture such as primer-dimmers, primers, nucleotides, enzymes and salts. So, we recommend removing these contaminants by silica-based PCR purification kit such as PCR cleaning Kit (Q-Biogene), Rapid PCR Purification Kit (Marligen), MiniElute PCR Purification Kit and Quick PCR Purification Kit (Qiagen). Removing the contaminants results in a 2-3 fold higher yield of recombinant colonies.

If your PCR template is a plasmid DNA containing Ampicillin resistance gene, we recommend cutting the template plasmid by incubating 50-100 µl PCR reaction mixture with 10 - 20 units of *Dpn*I for 30 min at 37℃ before purifying your PCR products.

Annealing Protocol

1. Perform the two separate PCR reactions to produce two PCR products.
2. Set up the 15 µl annealing mixture on ice*[1] as follow:

PCR product 1 and PCR product 2	X µl*[2, 3]
Annealing buffer	8 µl
pHST1 vector (15ng/µl)	2 µl
Distilled water	variable
Total volume	15 µl

3. Heat to 95℃ for 5 min and cool gradually to room temperature for 5 min.*[4, 5, 6]
4. Transform chemically competent E.coli cells JM109 with 5 µl of annealing mixture.*[7]

*[1] We recommend setting up the annealing mixture on ice.
*[2] If using non-purified PCR product, do not add more than a total 3 µl PCR product. Addition of each PCR product should be separately at the end in order to avoid mixing both PCR products directly.
*[3] We recommend using molar ratio of 2 times more total PCR product DNAs than pHST vector DNA (30 ng, 0.017 pmol). For example, more than 20 ng (0.033 pmol) of 1,000 bp PCR products, which contain 10 ng PCR product 1 and 10 ng PCR product 2, is used. However, less PCR product also be sufficient because of the high cloning efficiency of this kit as shown in “Example” page 6. We recommend using roughly equal amounts of each PCR product DNA from the gel analysis.
*[4] If necessary, the annealing mixture should be covered by mineral oil before heating.
*[5] If using non-purified PCR product containing DNA polymerase, do not gradually heat the annealing mixture. Put the tube directly in a heat block or PCR machine heated to 95℃ in advance.
*[6] After annealing, alternatively, 1 µl of Ligase Mixture may be added to the annealing mixture and incubated for 5 min at 25℃ to make covalently linked recombinant molecules. The Annealing buffer contains other materials needed for ligation reaction, such as ATP and Mg. The efficiency of the transformant is increased up to about two times.
*[7] Because of the long complementary extensions, the vector and insert form stable recombinant molecules without ligation reaction. The mixture of annealed vector and PCR products can be used directly for the transformation of chemical competent cells. The annealing mixture can be stored at –20℃, until transformtion.

Transformation protocol

1. Thaw the competent cells on ice (50 µl in a tube of each transformation).
2. Add 5 µl of the annealing mixture directly into the competent cells and mix by flicking gently*[1].
3. Incubate the tube on ice for 20 minutes.
4. Heat Shock the cell by placing the tube in a 42°C water bath for 45 seconds. Do not mix or shake.
5. Remove tube from the 42°C bath and place it on ice for 2 min.
6. Transfer the cell to 15 ml sterilized culture tubes containing 250 µl of SOC medium (pre-warmed from room temperature to 37°C). Culture the cell at 37°C for 1 hr in a shaker.

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7. Spread aliquot of the cell to an LB agar plate containing ampicillin.
   To perform blue-white color screening, spread 25µl of 20 mg/ml X-Gal and 50 µl of 100 mM IPTG onto the LB agar plates and allow these reagents to be absorb 30 minutes prior to inoculating the cells.
8. Incubate the plate at 37°C overnight.

*[1] Do not add more than 7.5 µl of annealing mixture to 50 µl of competent cells.

Clone Screening

Pick at least 10 white colonies and grow overnight in 3-5 ml LB medium containing 100 µg/ml of ampicillin. Isolate plasmid and analyze by restriction enzyme digestion or sequencing.

Alternatively colony PCR can be performed to screen the transformants by the sequence primers supplied with the kit or your PCR primers of the target gene.

Sequencing

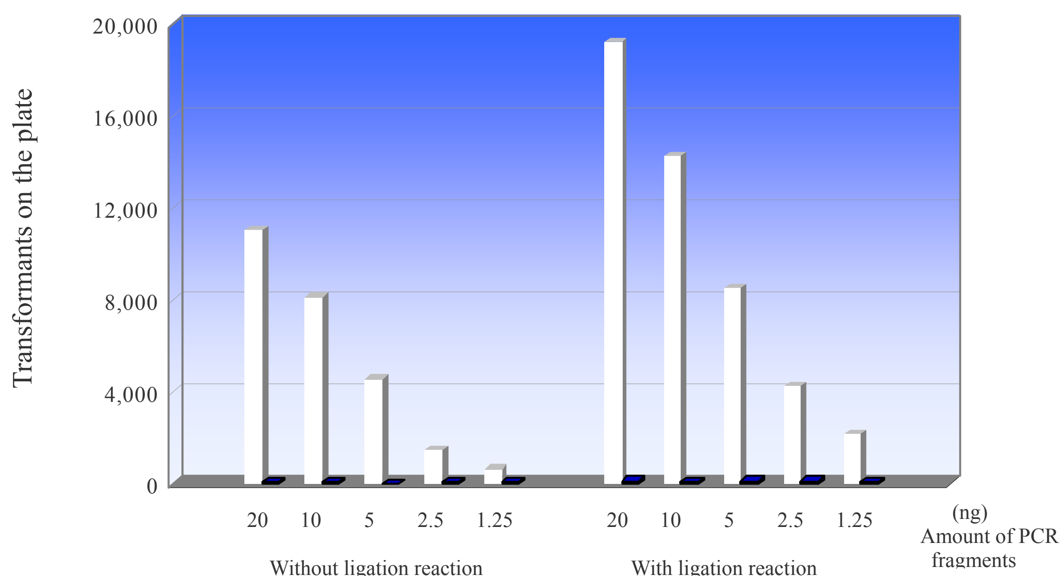
For sequencing your insert in pHST vector, two sequence primers are provided with the Kit.

pHST Forward Sequence Primer; 5'- AAGGCGATTAAGTTGGGTAACGCCA-3'
pHST Reverse Sequence Primer; 5'- CTTTACACTTTATGCTTCCGGCTCG -3'

pHST Forward Sequence Primer anneals to pHST vector 40 bases upstream of *Eco*RI site. While pHST Reverse Sequence Primer anneals to pHST vector 68 bases downstream of *Hin*dIII site.

Example

Several amounts of about 1 kb PCR fragments were cloned according to the standard protocol using the [DynaExpress] Hetero-Stagger PCR Cloning Kit. Half of the amounts of transformed competent cells (150 µl) were spread onto LB agar plates. The white bars and the blue bars show the numbers of white colonies and blue colonies, respectively.



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Troubleshooting

Problem	Possible Cause	Solution
Few or no colonies	The cells have lost competence due to improper storage or shipping conditions.	Check transformation efficiency using a plasmid such as pUC19.
	Incorrect primer sequence	Check the primer sequence.
	Perform PCR reactions with incorrect PCR primer sets.	Check each primer set in the PCR reaction 1 and PCR reaction 2. See “DNA Amplification” page 4.
	Incorrect antibiotic.	Check selective growth medium.
Low number of white colonies	Incorrect DNA polymerase used for PCR	Check whether your thermostable DNA polymerase is proofreading or non-proofreading and your primer designs. There are differences in primer designs between proofreading and non-proofreading DNA polymerase. See “Primer Designs” page 4.
	Not enough PCR fragment	Check the amount of PCR fragment by electrophoresis or spectrophotometory and use a sufficient amount. Often spectrophotometer measurement of DNA following spin-column purification is inaccurate due to some contamination. We strongly recommend checking by the electrophoresis.
	Presence of inhibitor in PCR products.	Gel-purify the PCR product using Gel Indicator DNA extraction kit (code No. DM550). Alternatively, purify the PCR product just by silica-based spin column.
	PCR fragment has been damaged by UV during the gel-purification.	We recommend the Gel Indicator DNA extraction kit (code No. DM550) to obtain gel-purified the PCR products without UV exposure.
	Too much non-purified PCR products were added to the annealing mixture.	If using non-purified PCR product, do not add more than total 3 μl PCR products to the annealing mixture. If you want to add more amount of PCR fragment, we recommend purifying the PCR products at least by silica-based spin column.
	Gradually heat the annealing mixture, even if it contain non-purified PCR product including DNA polymerase.	Do not gradually heat the annealing mixture. Put the tube directly in a heat block or PCR machine heated to 95℃ in advance.
	Cloned insert is not tolerated by *E.coli.*	Incubate the plate and liquid culture not at 37 ℃ but at room temperature. Alternatively, the orientation of insert within the vector should be changed by swapping the vector–specific terminal sequences between the two primers containing extra bases or using

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		pHST-2 Vector, linearized (code No. DS153) whose single-strand extensions are reverse direction compared to pHST-1.
	Excess volume of the annealing mixture was added to competent cell.	The volume of the annealing mixture should be no more than 15 % that of chemical competent cell.
Only white colonies	No IPTG or X-Gal in plate	Check that the plate contains sufficient IPTG or X-Gal.
	Contamination of plasmid DNA containing Amp^r used as PCR template	We recommend cutting template plasmid with *Dpn*I in PCR reaction mixture or gel-purifying the PCR products. See "DNA Amplification" page 4.
	Inactivation of antibiotic	Use the fresh selective growth medium.
White colonies with blue center or light blue colonies	Leaky expression of the *lacZ* fragment	Pick these types of colonies and check inserts as some of them may contain insert. In this case, conversely clear white colonies may contain no insert.
Do not grow in liquid culture	These are satellite colonies.	Be sure to pick large white colonies and check the Ampicillin plate.
	Keep the plate long before picking colonies.	Plasmids containing the insert are shed from cell or kill cell on the plate as cloned insert is not tolerated by *E.coli* in some degree. Pick colonies from fresh plate.
White colonies do not contain insert.	Primer-dimmers or Non-specific PCR products were cloned.	Improve the PCR reaction condition to obtain single and discrete band on the gel. Alternatively, gel-purify the PCR fragment with the Gel Indicator DNA extraction kit (#. DM550)
White colonies do not contain plasmid.	Cloned insert is not tolerated by *E.coli* to some degree.	Plasmids containing the insert are rapidly shed from cell immediately after running out of Ampicillin in the liquid culture. Add more Ampicillin in the culture medium. In rare cases, even if plasmids containing the insert are rapidly shed from cell, the cells grow in liquid culture containing enough Ampicillin. We think that the only Ampicillin resistance gene of plasmids may remain in the cell by integration into chromosome. In the case of the latter, incubate the plate and liquid culture not at 37℃ but at room temperature. Alternatively, the orientation of insert within the vector should be changed by swapping the vector–specific terminal sequences between the two primers containing extra bases or using pHST-2 Vector, linearized (code No. DS153) whose single-strand extensions are reverse direction compared to pHST-1.

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