Low Melt Agarose "In-Gel" Ligation Protocol

EZClone Systems
We Make Your Genes Fit!
(Largest Selection of Subcloning Adaptors Available Anywhere)
www.ezclonesystems.com

Plasmid Subcloning using Low Melt Agarose Ligation Protocol

Notes on Adaptor Use

- Do not thaw at 37°C. Thaw briefly at room temperature and keep on ice until use (store at –20°C after use).
- Use 1ul of adaptor per ligation reaction.
- When using two or more adaptors in a single ligation, 1ul of T4 polynucleotide kinase **MUST** be added to ligation reaction. We also recommend adding 1ul of T4 polynucleotide kinase to cloning experiments involving the use of any XhoI adaptor (see technical section or our web site for explanation).

Additional Note

- This protocol was modified on 7/30/02. This modified version is easier than the previous version and is compatible with competent cells made using a variety of methods.

Protocol

I. Plasmid Restriction Digestion

	Vector Plasmid	Plasmid Containing Insert
DNA	0.5ul (0.5ug)	1-5ul (1-5ug)
10X Restriction Buffer	3ul	3ul
Restriction Enzyme 1	1.5ul	1.5ul
Restriction Enzyme 2	1.5ul	-
Restriction Enzyme 3	-	1.5ul
H_2O	23.5ul	19-23ul
Total	30ul	30ul

Note: The amount of plasmid containing insert to be digested depends on the size of the insert and is primarily a consideration for detection issues. If the insert is between 150-800bp, digest around 5ug of plasmid. If the insert is 800-2000bp, digest 2-3ug. If the insert is larger than 2000bp, digest 1-2ug (if insert is similar in size to the vector, only 1ug should be used to allow for better separation).

II. Agarose Gel Electrophoresis

- Run digestions on a 1% Low Melting Point Agarose Gel in 1X TAE (never use TBE).
- Using a clean razor blade, isolate gel pieces containing fragments of interest and put each gel piece into a separate microfuge tube (1. Take as thin a slice as possible and quickly trim off any excess agarose – this increases the fragment concentration and minimizes contamination with trace amounts of unwanted DNA which may cause background. 2. Minimize exposure of fragments to UV light).

III. Ligation Reaction

- Put tubes containing gel pieces into a 70°C water bath for 5 minutes (Note: 65°C is not hot enough).
- When gel pieces are melted, take tubes out one at a time, mix briefly by flicking with finger, and immediately dole out appropriate amount to the “CNTL” and “TEST” tubes (see page 2).
- After adding appropriate amounts of each fragment, put CNTL and TEST tubes back into 70°C water bath for 3-5 minutes.
- While incubating CNTL and TEST tubes at 70°C, prepare Ligation Reaction Mix (see page 2).
- After CNTL and TEST tubes have been at 70°C for 3-5 minutes and the Ligation Reaction Mix has been prepared, transfer all of these tubes (CNTL, TEST, and Ligation Reaction Mix) to 37°C water bath. Keep at 37°C for 1 minute.
- Keeping all tubes in 37°C water bath, pipette up 10ul of ligation mix. Then pick up CNTL tube and immediately transfer the ligation mix into the CNTL tube. Mix immediately by flicking gently with finger and then leave at room temperature.
- Similarly, transfer 10ul of ligation mix to the TEST tube and then immediately add 1ul of appropriate adaptor(s). Mix by flicking gently with finger.
- Let ligations incubate at room temperature for 3hrs to overnight (3hrs for two part ligations, and overnight for three or more part ligations or ligation of blunt ends).

Important: Adaptors must be added immediately after adding ligation mix to ligation fragments (do not add adaptor at the same time that other fragments are added because this mixture is too warm and the adaptors will denature).

Ligation Setup Guide

For two part ligations:

CNTL tube = 10ul of vector gel piece	+ 10ul Ligation Reaction Mix
TEST tube = 5ul of vector gel piece + 5ul of insert gel piece	+ 10ul Ligation Reaction Mix

For two part ligation with an adaptor:

CNTL tube = 5ul of vector gel piece + 5ul of insert gel piece	+ 10ul Ligation Reaction Mix
TEST tube = 5ul of vector gel piece + 5ul of insert gel piece + 1ul of Adaptor	+ 10ul Ligation Reaction Mix

For three part ligations:

CNTL tube = 5ul of vector gel piece + 5ul of one of the insert gel pieces	+ 10ul Ligation Reaction Mix
TEST tube = 4ul of vector gel piece + 3ul of each of the two insert gel pieces	+ 10ul Ligation Reaction Mix

For three part ligations with an adaptor:

CNTL tube = 5ul of vector gel piece + 5ul of one of the insert gel pieces	+ 10ul Ligation Reaction Mix
TEST tube = 4ul of vector gel piece + 3ul of each of the two insert gel pieces + 1ul of Adaptor	+ 10ul Ligation Reaction Mix

Ligation Reaction Mix:

Per Reaction	3X	5X	7X	9X
6ul H_2O	18ul	30ul	42ul	54ul
2ul 10X Ligation Buffer	6ul	10ul	14ul	18ul
2ul Ligase	6ul	10ul	14ul	18ul
10ul				

Important notes:

- Warm Ligase buffer in 37°C water bath for a few minutes and vortex to ensure that DTT is in solution.
- When using two adaptors in a single ligation reaction, 1ul of T4 Polynucleotide Kinase must be added (per reaction) to the ligase mix. Use 1ul of each adaptor.

IV. Transformation of competent bacteria

- After ligation, put tubes at 70°C for 5 minutes.
- Transfer tubes to 37°C water bath for 1 minute.
- Add 10ul of ligation to 100ul competent cells (or 5ul of ligation to 50ul competent cells), mix by flicking gently with finger, and transfer immediately to ice.
- Incubate on ice for 30 minutes.
- Heat shock at 37°C for 2.5 minutes and then transfer back to ice bucket.
- Transfer the mixture to a sterile tube containing 500ul of 2xYT, mix briefly with finger, and incubate in 37°C water bath for 1 hour.
- Plate 300ul bacteria and put plates right side up in 37°C incubator.
- Next day, pick colonies and inoculate cultures for mini prep analysis.

Notes:

We recommend that you do a negative control (CNTL). However, if you get similar numbers of colonies on the TEST and CNTL plates (or even if you see fewer colonies on the TEST plate relative to the CNTL plate), your experiment still may have worked! The main reason for including a control is to allow us to gauge how many mini preps to analyze for each cloning experiment.

Troubleshooting

Please refer to our "Plasmid Subcloning Troubleshooting Guide" (available for download – www.ezclonesystems.com) for subcloning problems.

Solutions

10X Restriction Buffers – Use vendor recommended buffers.

Ligase Buffer – Use vendor recommended buffer. Note, however, that we haven't tested whether fast ligation buffers [those that contain polyethylene glycol (PEG)] are compatible with this protocol and we recommend avoiding these buffers until this issue is assessed.

50X TAE
1 L
242g Tris Base
57.1 ml Glacial Acetic Acid
100 ml 0.5M EDTA (pH 8.0)
- Add H_20 up to 1L.

2xYT	1L
tryptone	16g
yeast extract	10g
NaCl	5g

- Add H_20 up to 1L and autoclave.