

PLASMID SUBCLONING TROUBLESHOOTING GUIDE



EZCLONESYSTEMS
WE MAKE YOUR GENES FIT!
(Largest Selection of Cloning Adaptors Available Anywhere)

Few or no colonies on control or test plate:

1) Some procedural mistake in processing during one or more cloning steps. This is probably not likely unless investigator is relatively inexperienced.

Solution. Study procedure again and repeat cloning.

2) Cells have lost competency.

Solution. If this is suspected, test competency by transfecting 0.01ng of supercoiled plasmid (should yield at least 5 colonies). If efficiency is low, make new competent cells.

3) Low fragment concentrations.

A. Low Melting Point In-Gel Ligation Protocol.

- Gel slices are too big. This results in a low concentration of DNA in agarose leading to low colony numbers.

Solution. Repeat digest, run samples on Low Melting Point Agarose gel. Cut a thinner slice. After cutting, lay slice on its side and quickly trim excess agarose that doesn't contain DNA. Your slices should be between 30-90 ul.

B. Standard fragment purification procedure.

- Loss of material during purification. Run small amount of purified fragment on gel to determine whether significant loss occurred during purification.

Solution. Digest, run gel, and purify fragments again. Alternatively, use "Low Melt In Gel Ligation Protocol" [can be downloaded on "Protocols" page of our web site (www.ezclonesystems.com)].

4) Using XhoI adaptors without adding T4 polynucleotide kinase. Adding 1ul of T4 polynucleotide kinase results in approximately 100 fold higher ligation efficiency with any XhoI adaptor. This may also be moderately helpful for some other adaptors if low colony numbers are obtained.

Solution. Add 1ul of T4 polynucleotide kinase to standard ligation reaction.

Note: We have not tested whether T4 polynucleotide kinase works in quick ligation buffers so we recommend using standard ligation conditions for these experiments and ligate overnight.

5) Using two adaptors in a single ligation without adding T4 polynucleotide kinase. T4 polynucleotide kinase is required for this situation.

Solution. Add 1ul of T4 polynucleotide kinase to standard ligation reaction.

Note: We have not tested whether T4 polynucleotide kinase works in quick ligation buffers so we recommend using standard ligation conditions for these experiments and ligate overnight.

6) Using a difficult cloning strategy such as those that require enzymatic blunting of ends, the cloning of very large fragments (e.g. >5kb), non-directional strategies, or strategies involving the ligation of 5 or more fragments in a single ligation reaction. Although any one of these inefficient strategies alone may work, the combination of any two of these in a single cloning experiment is almost intractable.

Solution. a. Try to develop a simpler strategy. Use a two step cloning strategy if necessary. b. Instead of blunting ends, use cloning adaptors (available at www.ezclonesystems.com) to make ends compatible. c. Cloning of large fragments may be facilitated by using electroporation instead of conventional competent cells. d. If using a non-directional cloning strategy, it is best to re-design strategy using a directional approach. If a non-directional approach must be used, carry out a titration to determine the least amount of phosphatase treatment needed to yield vector that gives less than 20 colonies (without insert). Use this vector sample to ligate to insert.

7) There is a mistake in the strategy or the map for the insert plasmid has inaccuracies. This is much more common than most investigators realize and explains more than half of the cloning problems encountered in our technical services department. Plasmid map errors are most likely in plasmids obtained from other investigators and with plasmids where the insert has been transferred multiple times into a succession of different plasmid polylinkers. An example of the most common situation is shown in figure 1. In this case, there is a second BamHI site immediately inside the HindIII site which is mistakenly omitted on the map. Although the insert plasmid is cut with BamHI and HindIII, the excised fragment is a BamHI/BamHI fragment which migrates at the expected size of the BamHI/HindIII fragment but which cannot be inserted into the BamHI/HindIII cut vector.

Solution. If this possibility is suspected, it may be a good idea to sequence across the ends of the insert plasmid. You can also carry out a diagnostic digest to help address whether the scenario in figure 1 is a problem. In this case, the insert plasmid should be digested with HindIII alone, with BamHI alone and with HindIII and BamHI. If either the HindIII digest or the BamHI digest yields a fragment that is the same size as the HindIII/BamHI fragment, this is likely the problem. Note, however, that it is still possible that the second BamHI site is outside the HindIII site in which case the cloning strategy should still work. To definitively assess the location of the second BamHI site, it would be necessary to sequence across this region. If it is determined that this is the problem, a new strategy should be developed.

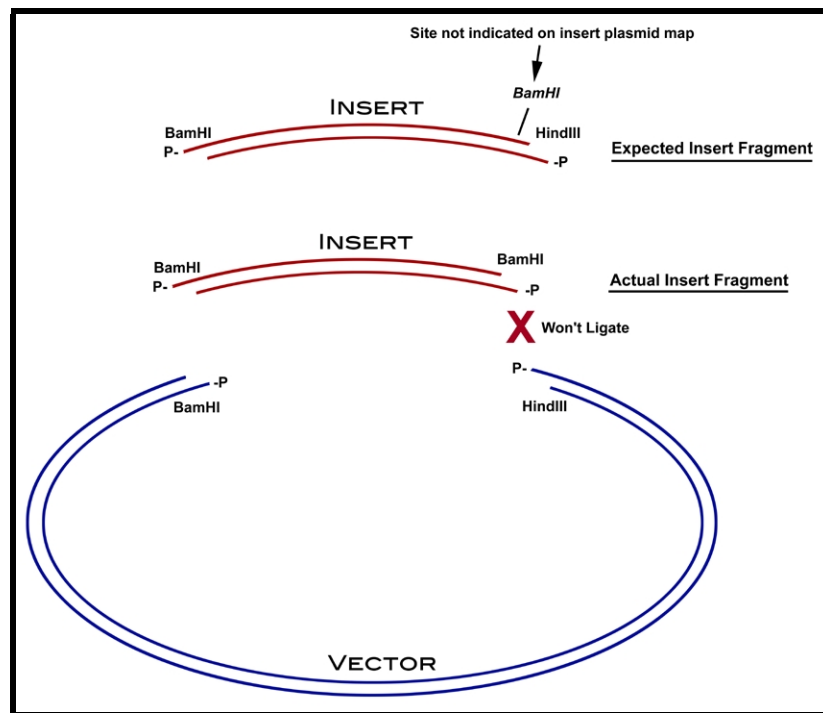


Figure 1

Low colony number and 6 or more mini preps are negative.

- See points 1, 3, 4, 5, 6, and 7 above.

8) This also may happen occasionally when using two adaptors in a single ligation. You may have to screen around 10 colonies.

High numbers of colonies on both the Test and the Control plates (i. e. usually 100 or more on the control plate unless cells are very competent).

A) For Non-Directional Strategies.

9) If the strategy is a non-directional approach using phosphatase treatment of the vector, this high background is most likely due to inefficient phosphatase treatment.

Solution. Carry out a titration to determine the least amount of phosphatase treatment needed to yield vector that gives less than 20 colonies (without insert). Use this vector sample to ligate to insert.

B) For Directional Strategies. Directional strategies (i.e. the vector is cut with two different enzymes) should result in low background since the two incompatible ends cannot religate. If a high background is observed, one of the following may be the cause.

10) One of the enzymes used to cut the vector is not digesting or is inactive.

Solution. Test whether this is a problem by cutting the vector with each enzyme alone. If one enzyme is not cutting, the enzyme may have gone bad (typically not likely), the site may be inhibited by overlapping dam methylation, the enzyme has significant site specificity, or the digestion conditions are not favorable for that particular enzyme. Refer to vendor catalog to determine whether any of these might be applicable for the enzyme being used.

11) Although not that likely, it is possible that the DNA sample or some other solution is contaminated with uncut plasmid.

Solution. This can be tested by setting up ligation without ligase and determining the number of colonies that arise. If this results in a high number of colonies, leave out solutions one by one until the contamination is identified and replace appropriate solution.

12) The two restriction sites being digested in the vector are extremely close together (i.e. 0-3 bp apart). Many restriction enzymes do not cut well if the site is close to a DNA end. Therefore, after one enzyme cuts, the other may not because it is now close to the end.

Solution. In this case, it is best to select different sites in the polylinker for cloning. If this is not possible, you may try digesting the vector with one enzyme first and then the second enzyme (and vice versa) and set up ligations without insert. One of the enzymes may not be as susceptible to the end proximity problem and therefore cutting with the other enzyme first may allow a more complete digest.

13) There is a mistake in the strategy or the vector map has errors. This is similar to the issue discussed in no. 9 above except that in this case, there is a mistake in the vector plasmid. For example, the vector is cut with BamHI and HindIII but there is an unaccounted for second HindIII site or BamHI site on the other side of the BamHI or HindIII sites, respectively. In this case, although the vector is digested with BamHI and HindIII, the vector would be left with either BamHI/BamHI ends or with HindIII/HindIII ends which would ligate together without an insert and would result in very high backgrounds.

Solution. Sequence across polylinker of vector to ensure that polylinker map is correct. Alter strategy accordingly.