EZCLONE SYSTEMS

GENERAL TIPS TO MAKE CLONING EASY!

1) Whenever possible, we strongly suggest the use of directional cloning strategies (i.e. cloning into a vector cut with two different restriction enzymes) over non-directional cloning approaches (cloning into single enzyme digested + phosphatase treated vector). Directional cloning strategies are straight forward, and are more efficient than non-directional approaches because a low background is achieved without phosphatase treatment (which can damage DNA ends). With the help of adaptors, it is almost always possible to come up with a great directional cloning strategy! Nevertheless, if you have to use a non-directional cloning strategy, the phosphatase should be carefully titrated to determine the optimal amount which prevents re-ligation of the vector with the least amount of damage to the vector DNA ends.

2) Strategies involving an end blunting step are significantly less efficient than those that don't involve end blunting. In conjunction with other inefficiencies in the same strategy (e.g. the use of 4 or 5 different fragments in a single ligation reaction), strategies involving an end blunting step can become intractable. End blunting strategies are almost completely avoidable by simply using an adaptor which converts the two otherwise incompatible ends. Because the ends of adaptors are not damaged in any way, their usage in a cloning strategy really doesn't reduce the overall cloning efficiency and this approach yields excellent results.

3) For multi-step cloning experiments, we always carry out the second step using mini prep DNA generated from the first cloning step (as opposed to preparing maxi prep DNA, which is time consuming). To date, we have found that mini prep DNA prepared by either the "Alkaline Lysis" method or by the "Boil Prep" method have been of high quality and are readily digested with any restriction enzyme necessary for the second step. For digestions with enzymes that require low salt conditions, however, the 70% Ethanol wash should be carried out adequately (i.e. 1. plenty of 70% Ethanol should be used, 2. after adding the 70% Ethanol, the tubes should be vortexed, and 3. as much residual ethanol should be removed as possible following centrifugation). The last issue that is germane to using mini prep DNA is that although we typically do not include an RNase digestion step in the mini prep procedure, we always add 2-3ul of high quality 10mg/ml RNase to the restriction digests.

4) Although most cloning methods work well, we recommend using the "Low Melt Agarose In-Gel Ligation" protocol - available for download at **www.ezclonesystems.com/Protocols.html**. Since DNA fragments don't have to be extracted from agarose, the Low Melt Agarose In-Gel Ligation protocol is fast. In addition, because the DNA fragments are not subjected to damaging chemical or mechanical stresses, cloning is generally more efficient than other methods and higher signal to noise ratios are typically achieved.

5) Other cloning tips can be found in our "Low Melt In-Gel Ligation Protocol" available for download at **www.ezclonesystems.com/Protocols.html**.

Use of Adaptors in Cloning Strategies

1) **Using one adaptor in a ligation reaction**. The optimal use of adaptors in cloning strategies involves the addition of only one adaptor in a single ligation reaction. In this approach, a strategy is developed in which one end of the insert is compatible with one end of the vector. The other end of the insert and vector can be made compatible using the appropriate adaptor. This strategy is highly efficient and because our adaptors are provided in an unphosphorylated format, the generation of adaptor multimers does not occur.

2) **Using two or more adaptors in a single ligation reaction**. Two or more adaptors can also be used in a single ligation reaction and we have had excellent success with this approach. However, there are two issues that must be taken into consideration. First, because our adaptors are not phosphorylated, the ligation of an adaptor between two pieces of DNA generates a staggered nick. This staggered nick can come apart during the 68C heating step which follows the ligation reaction in the low melt agarose ligation protocol. If there is only one staggered nick in a DNA molecule (i.e. if only one adaptor is used), the ends of the DNA molecule will come right back together when the temperature is brought back down. This uni-molecular reaction is very fast and in this setting, the cloning efficiency remains very high. However, if one fragment in the DNA molecule is flanked by two adaptors (i.e. with two staggered nicks), the insert will pop out during the heating step and the cloning will be very inefficient. This problem can be easily rectified, however, by the simple addition of 1ul of T4 polynucleotide kinase to the ligation reaction. T4 polynucleotide kinase works well under standard ligation conditions and this simple measure makes two adaptor cloning strategies work well. There is a chance of generating adaptor multimers but the probability is low because our adaptor format allows for the use of low molar amounts in the ligation reaction. Nevertheless, we recommend that if two adaptors are to be used in a single ligation reaction, the amount of each adaptor added should be decreased to 0.25 – 0.5ul each.

   The second issue that should be taken into consideration before carrying out an experiment with two adaptors in a single ligation reaction is the possible consequence from cross-annealing of trace amounts of unannealed adaptors. If the two different adaptors used in a single cloning experiment contain exact internal homology, then it is possible that the vector will be linked together directly (i.e. without the insert) through trace amounts of a cross-annealed adaptor. Because this direct linkage of the two ends of the vector with a cross-annealed adaptor is a bimolecular reaction whereas the strategy of interest requires the linkage of 4 fragments (i.e. vector, insert, and two adaptors), even small amounts of cross-annealed adaptor can cause significant background. Therefore, prior to initiating a cloning experiment, the internal sequences of the two adaptors to be used should be compared to ensure that possible cross-annealing cannot generate a new adaptor with the capacity to directly link the vector together.

3) **Cloning with XhoI adaptors**. Through a number of different cloning experiences, we have discovered that unphosphorylated XhoI ends clone with extremely low efficiency. This is not specific to the XhoI enzyme since unphosphorylated synthetic XhoI overhangs, or ends generated by the XhoI isoscizomer, PaeR7I, similarly clone with low efficiency. As shown in figure 1, while an unphosphorylated HindIII/EcoRI adaptor clones with high efficiency into a HindIII/EcoRI cut

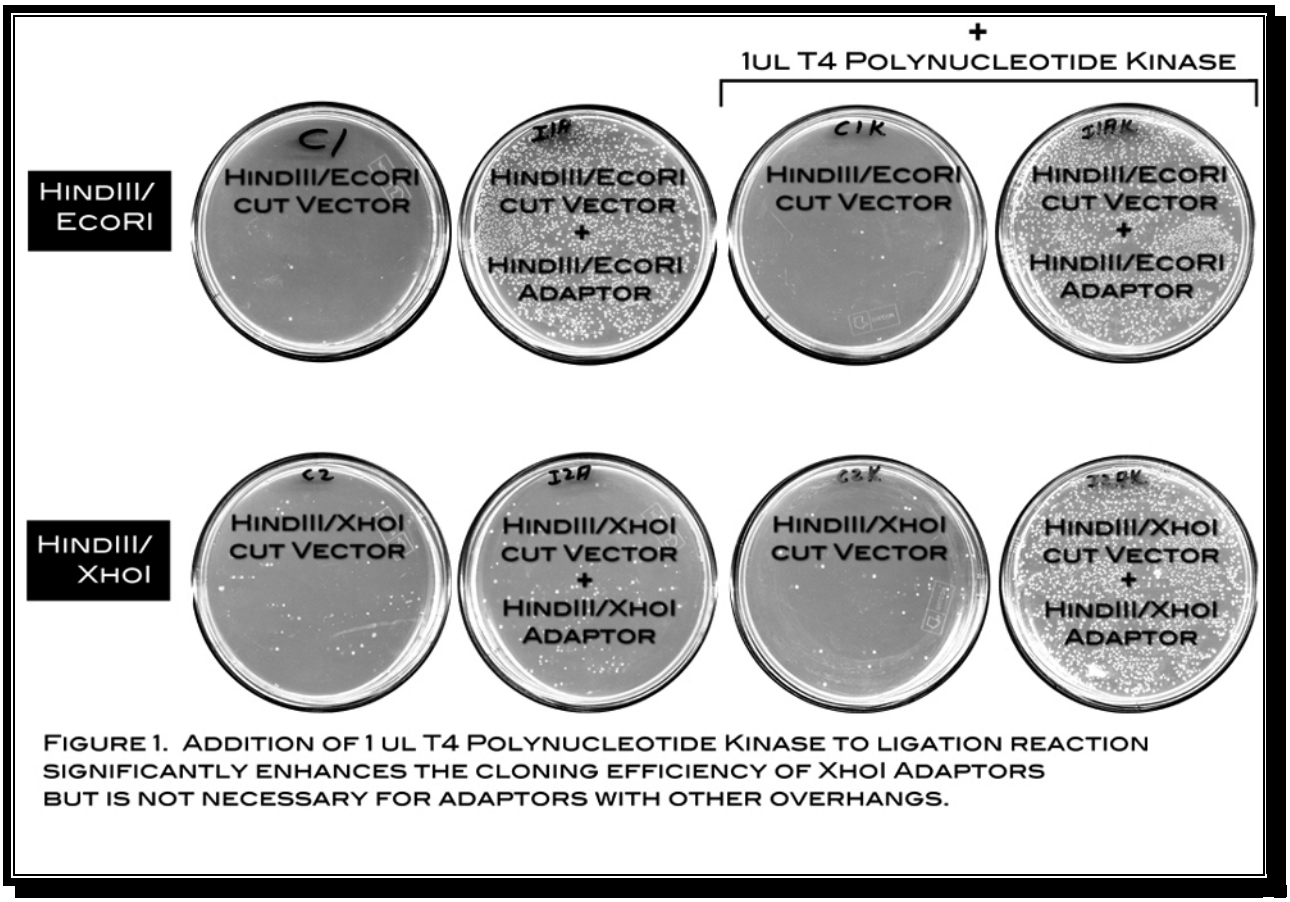

FIGURE 1. ADDITION OF 1 UL T4 POLYNUCLEOTIDE KINASE TO LIGATION REACTION SIGNIFICANTLY ENHANCES THE CLONING EFFICIENCY OF XHOI ADAPTORS BUT IS NOT NECESSARY FOR ADAPTORS WITH OTHER OVERHANGS.

vector, an unphosphorylated HindIII/XhoI adaptor clones very poorly into the same vector cut with HindIII/XhoI. Importantly, this problem can be easily rectified by the simple addition of 1ul of T4 polynucleotide kinase to the ligation reaction (see figure 1). This issue appears, to date, to be specific to XhoI adaptors (e.g. see figure 1) and we have not encountered this peculiarity with any other adaptor. This issue is also germane to investigators attempting to carry out non-directional cloning experiments into a vector cut with XhoI. Since the vector must be phosphatased, this strategy is extremely difficult and although it can work, it is very innefficient!

So, that's it! If you have any questions about these or any other issue, don't hesitate to contact us at **info@ezclonesystems.com** or fill out a technical question form on our web site **www.ezclonesystems.com**.

Good luck with your experiments!