ZyCloning Master Mix (3x)

Catalog: RK30131
Size: 20 RXN / 100 RXN
Components:
ZyCloning Master Mix (3X) RM02990
ZyCloning Positive Control Vector/Insert Mix RM02998

Introduction

"Seamless cloning" refers to methodologies that allow gene construction without relying on restriction sites in a more streamlined workflow that is sequence-independent and scarless, without the retention of undesired superfluous sequences or scarred overhangs. Seamless cloning methods rely on homologous sequence assembly to join a vector and one or more insert DNA fragments to be joined precisely in the desired direction.

ABclonal's ZyCloning Master Mix (3X) is an optimized, ready-to-use reagent that allows simple, ultra-fast, and efficient homology-based seamless cloning with over 95% accuracy. ZyCloning confers maximum versatility for molecular cloning workflows, allowing the user to employ any vector and up to eight insert fragments for transformation into the preferred competent cell line for repair and propagation. Possible applications for ZYCloning including site-directed mutagenesis, production of expression vectors, and more.

Product Storage

Store at -20 °C. Product is stable at 4 °C for at least six days. Although ZyCloning is freeze-thaw resistant, user should avoid over 20 freeze-thaw cycles.

Required Equipment and Materials

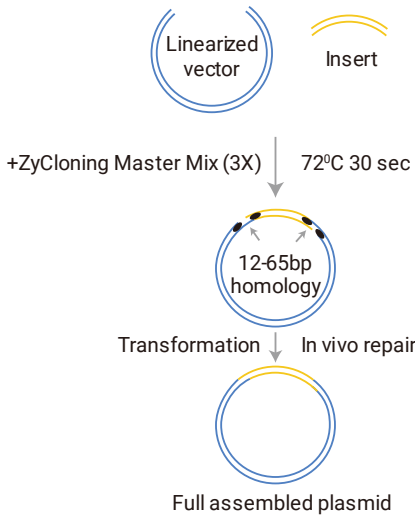
1. Thermal cycler, stable heat/cold blocks, or water bath at protocol prescribed temperatures
2. Thin-walled 0.2-0.3 mL PCR tubes
3. User-designed inserts and vectors

Please read this information sheet completely before beginning the protocol for best assembly and cloning workflow design to maximize product assembly yield.

Usage Notes

1. ZyCloning is a homology-based seamless cloning workflow that uses normal homology design to ensure accurate directional cloning.
2. Vector and inserts do not need to be further purified before assembly if T4 DNA kinase is used to add 5' phosphates to the vector and inserts.
3. Synthesized gene fragments are also suitable for direct use with ZYCloning but should be free of any additional adaptors.
4. Linearized vectors resulting from restriction enzyme digestion have higher efficiency in the ZYCloning system than vectors from PCR amplification due to the potential contamination from residual primers. Sequence overhangs from restriction digestion can remain in the reaction, providing user-designed homology regions are not homologous to the overhangs.

ZyCloning Assembly Workflow



Homologous Arm Design and Cloning Strategy Considerations

1. The homology-based methodology refers to the homology within the 5' overhang. Any mismatches (up to 14 base pairs in length) outside the homology region will be removed *in vivo* after transformation. Potential mismatches should not exceed one-third of the homology length.
2. Users electing to amplify gene fragments using crude primers should add six additional bases to the homology region.
3. The homology arms should be 12 to 65 base pairs (bp) in length. If multiple inserts are to be assembled, each homology region should be at least 24 bp.
4. Inserts should each be at least 131 bp in length.

Vector and Insert Considerations

1. There are designated vector and insert ranges that will improve the success rate of ZyCloning assembly. Assembly temperature may vary based on size and number of insert(s) and should be optimized if user elects to use homologous arm design outside the recommended parameters.
2. If the user adheres to the recommended fixed 1:3 molar ratio of vector-to-insert, then the full range of **vector** amount is between 1.5 to 12 ng/kb, with optimal range within 3 to 6 ng/kb.
3. If the user fixes the input vector amount at 3 ng/kb, the range for **single insert** is 2.3 to 36 ng/kb, with optimal range within 4.5 to 18 ng/kb. This ratio sets the **total amount of all DNA fragments within the reaction** to an acceptable range of 0.03 to 0.23 pmol, with optimal range between 0.07 to 0.14 pmol.
4. For multiple fragment assembly (adjust to 2 µL for the vector/insert(s) mixture):
   a) With two inserts, set vector : insert 1 : insert 2 to 1 : 1.5 : 1.5;
   b) With three inserts and above, set vector : insert 1 : … : insert n to equimolar amounts, with maximum seven inserts.
5. **NOTE**: if short oligonucleotides (less than the recommended 131 bp) are to be employed, add 2 µL of vector to 1 µL of ZYcloning Master Mix (3X) initially, incubate at 72 °C for 30 seconds, then add the 1 µL of oligo mixture. Incubate at 36 °C for 30 seconds, then follow the transformation procedure per step 4 below.

Standard Assembly Reaction Protocol

Based on the usage, primer design, and cloning considerations above, user can employ the following protocol for most assembly reactions:

1. Assemble the following into a single 0.2-0.3 mL thin-walled PCR tube:

Component	Volume
Linearized vector (3ng/kb)	1 µl
Insert (or mixture of inserts) (9ng/kb total)	1 µl
ZyCloning Master Mix (3X)	1 µl
Total volume	**3 µl**

2. Incubate the total reaction mixture at 72 °C for 30 seconds.
3. Cool on ice or at 4 °C on a thermal cycler or cold block.
4. Cooled mixture can be immediately transformed into competent cells* using conventional protocols.

Positive Control Assembly Reaction Protocol

User can ensure the ZYCloning protocol is working using the included positive control. Positive control is a mixture of ABclonal's ZyCloning control vector and insert, which when assembled is a kanamycin-resistant plasmid with pMB1 origin that constitutively expresses fuGFP. The successfully assembled gene will cause the colonies to glow green under a long wavelength UV light (i.e., a blacklight) at 320-400 nm.

1. Assemble the following into a single 0.2-0.3 mL thin-walled PCR tube:

Component	Volume
ZyCloning Positive Control Vector/Insert Mix	2 µl
ZyCloning Master Mix (3X)	1 µl
Total volume	**3 µl**

2. Incubate the total reaction mixture at 72 °C for 30 seconds.
3. Cool on ice or at 4 °C on a thermal cycler or cold block.
4. Cooled mixture can be immediately transformed into competent cells* using conventional protocols.
5. When colonies are observable, visualize successful assembly under long wave UV source to calculate reaction efficiency.

***NOTE:** ZYCloning system is not compatible with electrocompetent cells. Chemical competent cells from any source will work when following the recommended transformation procedure.

Troubleshooting:

1. **If there are no colonies after the transformation of the assembly reaction:**
   a. Ensure the control reaction was successful.
   b. Evaluate the homology region. In some cases, the homology region is too short. Additionally, avoid sequences which may be self-complementary.
   c. Reduce the antibiotic concentration to 40% of the normal concentration.
   d. Ensure sufficient competent cell volume is added. At least 50 µl is required for the 3 µl reaction mix.
   e. Use gel electrophoresis to check the DNA quality of the vector to make sure it is not degraded.

2. **If there are too many colonies present in the negative control:**
   a. There may be leftover uncut vector from restriction enzyme digestion; confirm complete digestion by gel electrophoresis.
   b. Template DNA concentration in PCR is too high or the DpnI digestion to remove the template after PCR was insufficient.
   c. Contamination may have occurred, particularly from supercoiled DNA with the same antibiotic resistance.
   d. The antibiotic in the agar plate is too old or the plate was prepared with too low concentration of the correct antibiotic.

3. **If there are too many colonies without correct insert:**
   a. Use an agarose gel to visualize insert quality and use gel purification to clean up the insert(s) to be used if the sample has excessive PCR side products.