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Entry Vector Cloning Protocol

The open reading frame (ORF) of the clone must be PCR amplified in order to clone the ORF into an Entry Vector. Please view the **Primer Design and PCR Amplification of ORFs Protocol** before moving forward.

Step 1, Electrophoresis:

1. Confirm the size of the amplification product by agarose gel electrophoresis and purify the remainder of the reaction using a purification column or similar method.

Step 2, Digestion:

1. Elute the DNA from the purification column in 26 µl of 10 mM Tris buffer. Set up a digestion reaction as described below, substituting other restriction enzymes as appropriate.

Component	Volume
10X restriction buffer	3µl
Sgf I (10U/µl)	0.6µl
Mlu I (10U/µl)	0.6µl
Purified PCR product	26µl
Total volume	**~30µl**

Mix well, and incubate at 37°C for 3 hrs.

2. Purify the digestion reaction using a purification column and elute in 18 µl of 10 mM Tris buffer.

3. Quantitate the DNA by UV at A260.

4. Digest the pCMV6-Entry vector with the restriction enzymes corresponding to the sequences added to the ORF. The pCMV6-Entry Vector is available from OriGene as 10 µg lyophilized DNA (**Cat# PS100001**). Resuspend the lyophilized DNA in 100 µl dH2O, and incubate for at least 30 min *before use*. Set up a digestion reaction as described below, substituting other restriction enzymes as appropriate.

Component	Volume
10X restriction buffer	3µl
Sgf I (10U/µl)	0.8µl
Mlu I (10U/µl)	0.8µl
Nuclease-free water	15.4 µl
Vector DNA	10 µl
Total volume	**30µl**

Incubate at 37°C for 3 hrs, then add 1 μl antarctic phosphatase (units used according to the manufacturer's protocol), and continue the incubation at 37°C for another 30 min. Dephosphorylation of the digested vector is essential to eliminate self-ligation.

5. Purify the desired vector fragment by running the digestion reaction on an agarose gel, and isolating the appropriate band using a gel purification column. Elute the digested plasmid vector in 40 μl of 10 mM Tris buffer.

Step 3, Ligation:

1. Set up a ligation reaction with the purified vector and insert fragments:

Component	**Volume**
10X ligase buffer	1μl
Nuclease-free water	3.5μl
T4 DNA Ligase	0.5μl
Vector Fragment	2μl (approx. 10ng)*
PCR product	3μl (approx. 30ng)*
Total volume	**10μl**

Incubate the ligation reaction at room temperature for 30-60 minutes. * Alternate ratios may need to be tested to obtain optional ligation efficiency.

Step 4, Transformation:

1. Transform 1 μl of the ligation mixture using 20 μl high efficiency competent E. coli cells (ideally 1x108 CFU/ug). Following transformation, resuspend cells in 200 uL LB.

2. Plate the entire transformation reaction on a standard LB-agar plate containing 25 μg/ml kanamycin. Incubate at 37oC overnight.

3. Pick at least 4-8 independent colonies to do miniprep from each ligation. Confirm the insert by restriction digestion and/or vector primer sequencing.