TITLE: DNA Ligation Sub-cloning

SOP Number: D-DEZ-PRO-009 Revision Number: 0

Effective Date: 07 Apr 2015

Author: _____ Date: _____

Reviewer: _____ Date: _____

QA Approval: _____ Date: _____

A. OBJECTIVE

This SOP is to provide information for the sub-cloning of DNA ligations in the DTVR analytical lab (Rm 4156 MERF).

B. APPLICABILITY

This procedure shall be performed by a trained scientist or technician in the DTVR analytical lab (Rm 4156 MERF).

C. REFERENCES

D-DEZ-PRO-011 NEB 5 Alpha Transformation Protocol
D-DEZ-PRO-012 CRISPR Cloning – 85nt Scaffold Bicistronic Backbone
D-DEZ-PRO-013 DNeasy gDNA Isolation from Cultured Cells

D. MATERIALS AND EQUIPMENT

Incubator: Lab Line Imperial III Incubator
Gel purification kit: QIAquick Gel Extraction Kit (Cat. No. 28706; Qiagen)
Restriction buffer and enzymes: University of Iowa Enzyme Core
Calf Intestinal Phosphatase (CIP): Alkaline Phosphatase, Calf Intestinal (CIP) (Cat. No. M0290S; New England Biolabs Inc.)
Calf Intestinal Phosphatase (CIP) buffer: Provided with Alkaline Phosphatase, Calf Intestinal (CIP) (Cat. No. M0290S; New England Biolabs Inc.)
Klenow Fragment: Klenow Fragment (3'->5' exo-) (Cat. No. M0212S; New England Biolabs Inc.)
deoxynucleotide triphosphates (dNTP): Deoxynucleotide Solution Mix (Cat. No. N0447S; New England Biolabs Inc.)
Polymerase Chain Reaction (PCR): University of Iowa Enzyme Core
transfer Ribonucleic Acid (tRNA)
Adenosine 5'-Triphosphate (rATP)
T4 DNA polymerase: T4 DNA Polymerase (Cat. No. M0203S; New England Biolabs Inc.)
T4 Polymerase Buffer: Provided with T4 DNA Polymerase (Cat. No. M0203S; New England Biolabs Inc.)
T4 polynucleotide kinase: T4 Polynucleotide Kinase (Cat. No. M0201S; New England Biolabs Inc.)
Dithiothreitol (DTT): DTT (Cat. No. R0861; Thermo Scientific)
5-alpha competent *E. coli* cells: One Shot Stbl3 Chemically Competent *E. coli* (Cat. No. C7373-03; Life Technologies)
Super Optimal Broth with added glucose (SOC): S.O.C. Medium (Cat. No. 1544-034; Life Technologies)
Selection plates

E. PREPARATION OF VECTOR

1. Cut 1µg DNA using appropriate restriction buffer and enzymes in a 20µl reaction.
2. Dephosphorylate vector by adding 2µl 10X CIP buffer and 1µl CIP. Incubate for 1 hour at 37°C (no matter what kind of ends the restriction enzyme produces).

3. Run reaction out on a 0.8% Ethidium bromide gel to visualize and confirm correct band size.
4. Gel purify and elute in H_2O.

F. PREPARATION OF INSERT

1. Cut 1µg DNA using appropriate restriction buffer and enzymes in a 20µl reaction.
2. Optional fill-in: Add 0.5µl Klenow and 2µl dNTPs (5mM), incubate 15min at 25°C. (Can usually be performed in the restriction buffer. Extract next if to be dephosphorylated). Can be inactivated at 75°C for 10min.
3. Run gel, gel purify and elute in H_2O as described above.
4. PCR fragments that contain a restriction site can be cut as in II.A.

   Note: If to be inserted blunt ended then it can be phosphorylated and filled in simultaneously as follows (add ingredients to tube with DNA or add DNA as part of water component. Make sure that DNA was not co-precipitated with tRNA-use glycogen instead):
   5µl dNTPs (10mM)
   5µl rATP (10mM)
   5µl 10X T4 Pol buffer (700mM Tris, pH7.5, 100mM MgCl2, 50mM DTT)
   27µl water
   4µl T4 DNA polymerase
   4µl T4 polynucleotide kinase

5. Incubate 30min at 37°C

G. LIGATION (OVERNIGHT)

1. Mix 5µl insert (or TE for control), 1µl cut vector, 1µl 10X ligation buffer, 1µl 1M DTT, 1µl 10mM ATP, and 1µl T4 DNA ligase. (Note: BRL Ligation buffer may be used instead).
2. Incubate at 16°C overnight.

H. NEB 5 ALPHA TRANSFORMATION PROTOCOL

1. Thaw a tube of NEB 5-alpha Competent E. coli cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
2. Add 1-5 µl containing 100 pg-1 µg of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 µl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.

TITLE: DNA Ligation Sub-cloning

SOP Number: D-DEZ -PRO-009 Revision Number: 0

Effective Date: 07 Apr 2015

8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
10. Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

I. History

Effective Date	Revision	Change
07 Apr 2015	0	PM-18 Rev. #3