

THE J. CRAIG VENTER INSTITUTE
CENTER FOR STRUCTURAL GENOMICS OF INFECTIOUS DISEASE
Standard Operating Procedure

TITLE: PCR Amplification and Ligation Independent Cloning (LIC)		PAGE: 1 of 7
SOP #: CSG-003	REVISION LEVEL: .002	EFFECTIVE DATE: 07/31/2009
AUTHORS: Saul Sarria, Sarah Grimshaw		PRIMARY REVIEWER: Jeremy Hasseman

1. OVERVIEW

1.1. Purpose

This SOP describes the procedures for the Ligation Independent Cloning (LIC) system including: PCR Amplification, PCR product dilution, T4 DNA polymerase treatment and annealing reaction (see Appendix for cloning diagram).

1.2. Scope

These methods have been developed by the Center for Structural Genomics of Infectious Disease (CSGID) team at the J. Craig Venter Institute. It is the responsibility of all research associates in this group to read, understand, and adhere to this document.

1.3. Related Documents

- 1.3.1. LIC Novagen manual
\\netvenus\pfgrc\CSGID\Various_Manuals\LIC_novagen_manual.pdf
- 1.3.2. Vector pMCSG7 paper
\\netvenus\pfgrc\CSGID\LIC_vectors\pMCSG7paper.pdf
- 1.3.3. Vector pMCSG19c paper \\netvenus\pfgrc\CSGID\LIC_vectors\pMCSG19_PEP2006.pdf
- 1.3.4. PicoGreen macro \\netvenus\pfgrc\CSGID\Templates\Picogreen_macro\PicoGreenScript.xls

2. REQUIREMENTS

2.1. Materials

- 2.1.1. LabChip DNA 5K Assay Chip (Caliper Life Science; Cat # 760395)
- 2.1.2. Phusion PCR Kit (New England Biolabs; Cat # F-530 L)
- 2.1.3. HIFI PCR Supermix (Invitrogen; Cat # 12532-016)
- 2.1.4. MJ Research Hard-shell 96-well plates (BioRad; Cat # HSP-9641)
- 2.1.5. PCR rubber cap mat (In-house)
- 2.1.6. MicroAmp® Clear Adhesive Films (Order In-house)
- 2.1.7. T4 DNA polymerase (3000U/mL) (NEB; Cat # M0203S)
- 2.1.8. Genomic DNA or Amplified genomic DNA (see SOP: CSG-001)
- 2.1.9. Forward & reverse PCR primers (Invitrogen: stock [0.3 ug/ul])
- 2.1.10. Millipore 96 well filtration plate (Millipore; Cat # LSKS09624)
- 2.1.11. Quant-it PicoGreen dsDNA assay (Invitrogen; Cat # P7589)

TITLE: **PCR Amplification and Ligation Independent Cloning (LIC)**

SOP #: CSG-003

REVISION LEVEL: .002

PAGE: 2 of 7

- 2.1.12. dGTP (Invitrogen; Cat # 10297-018)
- 2.1.13. dCTP (Invitrogen; Cat # 10217-016)
- 2.1.14. DTT (Fisher scientific; Cat # BP172-5)
- 2.1.15. Milli-Q water (In-house)

2.2. Equipment

- 2.2.1. Centrifuge capable of spinning plates
- 2.2.2. Thermocycler with 96 well head
- 2.2.3. Biomek 96 and Span-8 robots
- 2.2.4. Caliper LC 90

3. PCR AMPLIFICATION

Before starting: Primer plates/wells should be organized so that PCR reactions for each strain/genome are clustered in neighboring wells. Make a separate master mix for each genome and dispense into the corresponding wells, enzyme being added last. This is immediately followed by the addition of the primer mix. DNA used in PCR should be an amplified product from a whole genomic amplification reaction (refer to SOP#: CSG-001_Repli-G).

- 3.1. Thaw out PCR primers. If kept frozen at -80°C they can be left out on the bench overnight to thaw.
- 3.2. Forward and reverse primers are diluted from original stock concentrations of 0.3 µg/µl to obtain a final working concentration of 200 ng/µl. Dilute and mix forward and reverse primers using the following biomek program:

\\Netvenus\PFGR\Biomek_Central\Biomek_Multi96\Multi96_Methods\CSGID\1. PCR Setup\PCR Primer Mix Plate Preparation (MJR 96).bmf.

- 3.3. Perform an initial round of PCR using Phusion on all samples. After Caliper analysis, failures will be rearranged and amplified using HiFi Supermix as an alternative enzyme.
- 3.4. Thaw out PCR components and prepare a master mix in a large enough vessel, mixing components well before use.
- 3.5. Phusion PCR (1st round)
 - 3.5.1. Phusion Master Mix:

THE J. CRAIG VENTER INSTITUTE
 CENTER FOR STRUCTURAL GENOMICS OF INFECTIOUS DISEASE
Standard Operating Procedure

TITLE: **PCR Amplification and Ligation Independent Cloning (LIC)**

SOP #: CSG-003

REVISION LEVEL: .002

PAGE: 3 of 7

	1 Reaction	1 96-well plate (+ 10%)
5 x buffer (GC or HF)	5.0 µl	530 µl
10 mM dNTP	2.0 µl	212 µl
dH2O	14.5 µl	1537 µl
Phusion DNA polymerase	0.5 µl	53 µl
Genomic DNA (50 ng/ul)	1.0 µl	106 µl
Master Mix volume	23 µl	2438 µl
Primers	2 µl	2 µl
Total reaction volume	25 µl	25 µl/reaction

3.5.2. Phusion Cycling Conditions:

Temperature	Time	Cycle Repetitions
98°C	1 min	1
98°C	10 sec	
55°C	30 sec	25
72°C	3 min/kb*	
72°C	10 min	1

3.6. HiFi Supermix PCR (2nd round)

3.6.1. HiFi Supermix PCR Master Mix:

	1 Reaction	1 96-well plate (+ 10 %)
HiFi Supermix	28 µl	2968 µl
dH2O	1 µl	106 µl
Genomic DNA (50 ng/ul)	1 µl	106 µl
Master Mix volume	30 µl	3180 µl
Primers	2 µl	2 µl
Total reaction volume	32 µl	32 µl/reaction

Note: The following Biomek program can be used to add primers to the Master Mix:

\\Netvenus\PFGR\Biomek_Central\Biomek_Multi96\Multi96_Methods\CSGID
 \I. PCR Setup\PCR Primer Transfer (MJR 96 to MJR 96).bmf.

3.6.2. HiFi Supermix thermocycling conditions:

THE J. CRAIG VENTER INSTITUTE
 CENTER FOR STRUCTURAL GENOMICS OF INFECTIOUS DISEASE
Standard Operating Procedure

TITLE: PCR Amplification and Ligation Independent Cloning (LIC)

SOP #: CSG-003

REVISION LEVEL: .002

PAGE: 4 of 7

Temperature	Time	Cycle Repetitions
98°C	1 min	1
94°C	10 sec	
55°C	30 sec	
68°C	1min/kb*	30
72°C	10 min	1
4°C	forever	

*Extension times are based on the largest fragment present in each individual plate. Thermocycler programs are pre-set with appropriate names that correspond with desired extension times (Example below).

PCR program name	Largest size	Extension time
SUP-2 min	2 kb	2 min
PHU-3 min	3 kb	3 min

3.7. PCR product purification

- 3.7.1. Transfer PCR products into a 96-well Millipore filter plate. Place plate on a vacuum system for 10 minutes or until the wells on the plate are dry. Re-suspend PCR in 35-45 µl of sterile Milli-Q water and transfer to a new 96-well MJR plate. Shake for 20 minutes on a bench shaker at room temperature.
- 3.7.2. Analyze results by running amplified products on a Caliper LC 90 (Refer to GW003-Caliper SOP for guidance). Any results determined to be a failure repeat PCR using Phusion enzyme (refer to section 3.7). Upon completion of all rounds of PCR, proceed to section 4 (below).

4. T4 DNA POLYMERASE TREATMENT

Note: Separate T4 DNA polymerase reactions will be performed on both the LIC vectors and PCR products to create complementary overhands on each.

PCR Dilutions will be calculated via the Cypress LIMS system using found Caliper concentrations to obtain a final PCR product concentration of 0.2 pmol/reaction. The following Biomek Span-8 program can be utilized to carry out dilutions:

\\Netvenus\PFGR\Biomek_Central\Biomek_Span8\Span8 - Methods\CSGID\262-PCR Dilution (LIC) -1plate_111908_Template.bmf

THE J. CRAIG VENTER INSTITUTE
 CENTER FOR STRUCTURAL GENOMICS OF INFECTIOUS DISEASE
Standard Operating Procedure

TITLE: **PCR Amplification and Ligation Independent Cloning (LIC)**

SOP #: CSG-003

REVISION LEVEL: .002

PAGE: 5 of 7

Note: The following tables are for one single T4 DNA polymerase reaction. Adjust the final volume based on total reactions needed for the particular project. Do not pipette these volumes, scale up for accuracy.

4.1. T4 DNA polymerase treatment for PCR products:

Reaction Component	[Initial]	Volume (uL)	[Final]
PCR products	Variable [x ng/μl]	12 μl (final vol) [0.2 pMol]	0.01 pMol/μl
T4 buffer (NEB #2)	10x	2.0 ul	1x
DEPC- treated water	N/A	Variable	N/A
T4 Polymerase	3 U/μl	0.2 ul	0.04 U/reaction
dCTP	10 mM	5.0 ul	2.5 mM
DTT	100 mM	0.8 ul	4 mM
Total Reaction Volume:		20.0 ul	

4.2. T4 DNA polymerase treatment for LIC Vectors:

Reaction Component	[Initial]	Volume (uL)	[Final]
Vector (digested)	Variable [x ng/μl]	12 μl (final vol) [0.2 pMol]	0.01 pMol/μl
T4 buffer (NEB #2)	10x	2.0 μl	1x
DEPC- treated water	N/A	Variable	N/A
T4 Polymerase	3 U/μl	0.2 μl	0.04 U/reaction
dGTP	10 mM	5.0 μl	2.5 mM
DTT	100 mM	0.8 μl	4 mM
Total Reaction Volume:		20.0 μl	

4.3. Mix the reactions very gently by pipetting up and down. Incubate on a thermocycler at 22°C for 30 minutes (refer to Novagen protocol).

4.4. Inactivate enzyme at 75°C for 20 minutes on thermocycler.

5. LIC REACTION-ANNEALING

5.1. Pipette 2 μl of the completed T4 PCR products into a new MJ Research PCR Plate. Pipette 1.0 μl of completed T4 vector to each well, mix by gentle stirring with the pipette. Quick spin the plate in a centrifuge then incubate reactions at 22 °C for 5 minutes on a thermocycler.

THE J. CRAIG VENTER INSTITUTE
CENTER FOR STRUCTURAL GENOMICS OF INFECTIOUS DISEASE
Standard Operating Procedure

TITLE: **PCR Amplification and Ligation Independent Cloning (LIC)**

SOP #: CSG-003

REVISION LEVEL: .002

PAGE: 6 of 7

- 5.2. Remove the plate and add 1.0 μ l of 25 mM EDTA to each sample, mixing by gently stirring with the pipette. Quick spin the plate and again incubate at 22°C for 5 minutes on a thermocycler.
- 5.3. Proceed with transformation into chemically competent cells (refer to SOP#: CSG-004_Trans, FC and OE).

TITLE: **PCR Amplification and Ligation Independent Cloning (LIC)**

SOP #: CSG-003

REVISION LEVEL: .002

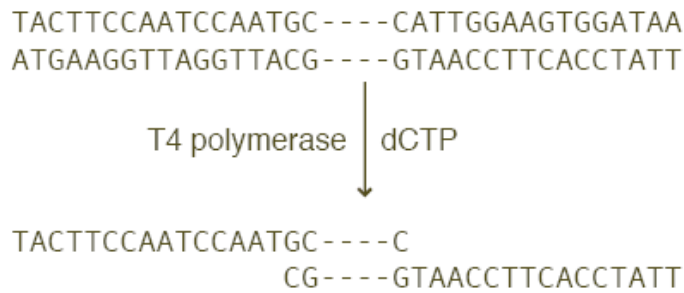
PAGE: 7 of 7

1. APPENDIX

Vector



PCR product



Annealed



Figure provided by Mark Donnelly, Argonne National Laboratory