1. PURPOSE

1.1. This SOP describes the transformation of LIC vectors into *E. coli* expression cells, frozen culture preparation and overexpression of recombinant proteins.

2. SCOPE

2.1. These methods have been developed by the Center for Structural Genomics of Infectious Diseases (CSGID) group. It is the responsibility of all research associates in this group to read, understand, and adhere to this document.

3. REQUIREMENTS

3.1. Material Requirements

3.1.1. Chemically competent *E. coli* expression cells (BL21(DE3)Magic or BL21(DE3)pLysS) (In-house made)
3.1.2. LIC plasmids from LIC Reaction (SOP#: CSG-003).
3.1.3. SOC media (In-house made)
3.1.4. 48-well Q-trays (2XYT, 0.8% glucose, 100 μg/ml AMP, 30 μg/ml Kan) (In-house made)
3.1.5. Ampicillin antibiotic (Stock concentration 100 mg/ml)
3.1.6. Kanamycin antibiotic (Stock concentration 50 mg/ml)
3.1.7. Chloramphenicol antibiotic (Stock concentration 34 mg/ml)
3.1.8. 50% glycerol (In-house made)
3.1.9. Deep-well Block (ABgene#AB0932)
3.1.10. Clear polystyrene, flat bottom 96-well plate (Fisher Scientific; Cat.# 12565-501)
3.1.11. Hardshell PCR 96-well plate, clear-well (BioRad; Cat# HSP-96)

3.2. Equipment Requirements

3.2.1. 12-channel Rainin pipettor
3.2.2. SpectraMax 96-well plate Spectrophotometer (MD)
3.2.3. Multitron II Shaking Incubator (ATR)
3.2.4. 37^{o}C Standing incubator
3.2.5. Sterile rubber PCR covers (VWR; Cat # 40002-002)

3.2.6. Thermocycler
3.2.7. Timer

4. PROCEDURE

Note: Please be extremely meticulous when handling plasmids, culture, and/or isolated proteins to avoid well-to-well cross-contamination. Cross-contamination can happen at any step and will lead to protein mixtures inevitably complicating downstream expression and solubility screening.

4.1 Chemical Transformation of the expression plasmids

4.1.1 Thaw a 96-well plate of chemically competent BL21(DE3)Magic cells on ice (~10 min). Label the plate and mark the top left corner with an "X".

4.1.2 While on ice, add 2 µl of the expression plasmids to the cells using a 12-channel Rainin pipettor or a Biomek program under the directory \\Netvenus\pfgrc\Biomek_Central\Biomek_Multi96\Multi96_Methods\CSGID\3. Transformation.

Note: Make sure that the orientation of the plasmid plate and the cells is consistent. **Do not pipette cells up and down.** Gently swirl once or twice with the pipette tip to mix.

4.1.3 Cover the plate with a sterile rubber PCR cover and incubate on ice for 30 min.

4.1.4 Heat-shock cells by quickly placing the plate on a preheated 42ºC thermocycler block for 45 seconds. (Extending the heat-shock past 45 s can damage the cells.)

4.1.5 Place plate immediately on ice for 2 minutes.

4.1.6 Add 70 µl of room temperature SOC media to plate. Gently swirl once or twice with the pipette tip to mix. **Do not pipette up and down.** Cover with breathable Airpore cover and incubate statically for 1 hour at 37 ºC.

4.1.7 Place Q-trays out of 4 ºC approximately 30 minutes before plating in 37 ºC. Wipe away any moisture on the inside of the lids using a paper towel.

Leave the lids slightly ajar for 30 minutes, allowing the moisture on the plate to dry.

4.1.8 Organize and label your Q-trays according to the plate layout so that one well in the transformation reaction plate equates to one square-well in the Q-tray. Label the back of the plate as shown in the diagram below.

A	6					1
B	6					1
C	6					1
D	6					1
E	6					1
F	6					1
G	6					1
H	6					1

A	12					7
B	12					7
C	12					7
D	12					7
E	12					7
F	12					7
G	12					7
H	12					7

(bottom view)

Note: Mix the cells gently before plating.

4.1.9 Plate 35 µl of the transformation reaction on corresponding Q-tray well containing LB (with added 0.8% glucose and specific antibiotic for LIC vectors) or use a Biomek program under the directory \\Netvenus\pfgrc\Biomek_Central\Biomek_Span8\Span8_Methods\CSGID\T4 - Annealing - Plating Transformants

4.1.10 To spread culture within the wells, carefully tilt the plate in a circular motion and place on a plate rotator at low setting until dry.

4.1.11 Place Q-trays inverted in static incubator at 37 ºC for 12-16 hours.

Note: Colony growth in LB may take longer than in 2XYT.

4.1.12 The next day examine the colonies and score the results using the colony count sheet under the directory \\Netvenus\pfgrc\CSGID\Templates-Colony Count Blank Sheet.xls. (See Appendix 5.5).

4.2 Inoculation and OD_{600} Readings

4.2.1 For liquid culture mix LB media and the appropriate antibiotic(s) in a sterile E-flask as the table mentioned below. This is enough for two blocks.

Note: Antibiotics must be made fresh. Add the appropriate antibiotic depending on the strain and the vector you are using (see Appendix 5.2 and 5.3). For two blocks of culture, mix recipe below and dispense 500 µl into each well of 2 mL deep-well block.

Liquid Media	Volume (mL)	Final concentration
LB	100	-
Ampicillin (100 mg/mL)	0.1	100 µg/mL
Kanamycin (50mg/mL)	0.06	30 µg/mL
Chloramphenicol (34 mg/mL)	0.1	34 µg/mL

4.2.2 From the Q-trays pick a single colony by carefully stabbing a colony using a sterile toothpick and transfer to a well containing 500 µl LB media, Ampicillin and Kanamycin for BL21(DE3)Magic cells (or Ampicillin and Chloramphenicol for BL21(DE3)pLysS strain).

4.2.3 Cover with Airpore strip and place block(s) in Multitron shaking incubator at 37ºC at 900RPM for 1 hr.

4.2.4 While incubating, turn on SpectraMax plate reader.

4.2.5 Pipette 150 µl LB media in all the wells of a 96-well flat bottom plate. This is the "Blank" plate.

4.2.6 Open SOFTmax Pro software (spf).

4.2.7 Open a new file and select the 'Experiment#1' header and the 'Plate#1' subheader.

4.2.8 Select 'Setup' button then 'Endpoint' button.

4.2.9 Set wavelength to '600nm' and fill checkbox for 'Pre-Read Plate'.

4.2.10 Place blank plate in reader and select 'Read' button in toolbar.

Note: The blank data will be applied to every subsequent read as long as you keep the experiment window open; every subsequent sample read will overwrite the previous one so save the data after each reading. The default OD readings from the SpectraMax instrument are skewed and need to be adjusted before interpretation. Use \\Netvenus\pfgrc\CSGID\Templates-spectromax-vs-real-OD (background data)-150ul.xls to adjust values for the entire plate.

4.2.11 Label three clear polystyrene, flat bottom 96-well plates as follows: Frozen Culture, Overexpression-M9 and Overepression-2XYT.

4.2.12 After 1 hr of incubation transfer 150 µl culture into the three plates or use the Biomek method under the directory: \\Netvenus\pfgrc\Biomek_Central\Biomek_Multi96\Multi96_Methods\CSGID-Culture Transfer - Growth Blocks to Three Flatbottoms.bmf. Take OD_{600} reading.

Note: Make sure that the orientation of the plates is consistent.

Note: Our goal is to monitor the average growth of each plate and halt growth within the following range:

Plate	**OD_{600}**
Frozen culture	0.7 - 0.8
Overexpression in M9	0.5 – 0.6
Overexpression in 2XYT	0.5 - 0.6

Note: Replace Airpore strip after each reading.

4.3 Overexpression in M9 and 2XYT media (Changing media)

4.3.1 Once the average corrected OD_{600} value reaches to 0.5 – 0.6, pellet the culture plate by centrifugation at 2200 RCF for 20 minutes at 4°C.

4.3.2 Remove supernatant by pipetting carefully. Do not disturb pellet.

Note: Don't decant as this may result in cross contamination across neighboring wells.

4.3.3 Add 150 µl of M9 media to the plate labeled M9, and 2XYT media to the plate labeled 2XYT. Make sure that the proper antibiotic is added to the media (Ampicillin and Kanamycin with a final concentration of 100 µg/ml and 30 µg/ml respectively). Resuspending the pellet is not necessary.

4.3.4 Incubate the plates at 37ºC at 900 RPM for 30 minutes. Record the optical density to check how the growth progress. The average corrected OD_{600} value after 30 minutes for M9 is ~ 0.6 and for 2XYT is ~ 0.8.

4.3.5 Induce the cultures with 5 µl IPTG (30 mM) to final 1mM concentration (see Appendix 5.4 for IPTG preparation).

Note: IPTG solution must be made fresh every time the overexpression is performed. Once you weigh out enough IPTG powder, put the lid on tight to prevent moisture from entering into the bottle.

4.3.6 Incubate plates at 25ºC for 18-20 hours (overnight) at 900 RPM using Multitron. Go to step 4.5.

4.4 Frozen Culture (FC)

4.4.1. Monitor cell density until the average corrected OD_{600} value for the whole plate is in the range between 0.7 and 0.8.

4.4.2. Add 30 µl of 50 % glycerol (final 10 %) to each well and mix by pipetting repeatedly or use the Biomek method under the directory \\Netvenus\pfgrc\Biomek_Central\Biomek_Multi96\Multi96_Methods\CSGID-Glycerol to Expression FC Plates.bmf

4.4.3. Seal plates and store at -80ºC.

4.5 Overexpression continued

4.5.1. After ~19 hours of incubation measure the final cell density (OD_{600}) for the two plates and record. The average corrected OD_{600} values after 19 hours for both M9 and for 2XYT media are above 2.

4.5.2. Split the culture into two Hardshell PCR 96-well plates (~ 65 µl per well).

4.5.3. Pellet the culture at 2200 RCF for 45 minutes at 4ºC.

4.5.4. Decant the supernatant from the plates into a reservoir containing 10 % bleach.

Note: When decanting invert plate quickly and cleanly to minimize any cross contamination between wells.

4.5.5. Get rid of any residual media by placing an inverted plate onto an absorbent towel.

4.5.6. Seal one of the plates, and store pellet at -80ºC (as a back-up). Proceed to Lysis of Expression Cells by Sonication (SOP: #CSG-005) with the other plate or store it at -80ºC until ready to sonicate.

5. APPENDIX

5.1. Antibiotics

Antibiotic	Mass of Antibiotic (g)	Solvent	mL	Concentration
Ampicillin	0.1	Milli-Q water	1.0	100 mg/ml
Chloramphenicol	0.034	100 % Ethanol	1.0	34 mg/ml
Kanamycin	0.05	Milli-Q water	1.0	50 mg/ml
Tetracycline	0.0125	Milli-Q water	1.0	12.5 mg/ml

5.2. *E. coli* Expression Strains

Strains	In House	Antibiotics
DH10B	No	None

DH5α	Yes	None
BL21(DE3)pLysS	Yes	Chloramphenicol
BL21(DE3)Magic	Yes	Kanamycin

5.3. Expression Vectors

Strains	Antibiotics
pMCSG 7	Ampicillin
pMCSG 19C	Ampicillin

5.4. Reagents

Reagent	Formula weight	Mass of Reagent (g)	Solvent	mL	Concentration (Stock)
IPTG	238.3	0.107	Milli-Q water	15.0	30 mM
DTT	154.25	0.154	Milli-Q water	1.0	1 M

5.5. Colony Count Sheet

Plate Name_____ Date_____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												