Chapter 8

"System 48" High-Throughput Cloning and Protein Expression Analysis[1]

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Summary

We describe a plate-based cloning and expression strategy for efficient high-throughput generation of validated expression clones in *Escherichia coli*. The process incorporates 48- or 96-well plates at all stages including the cloning and colony selection phases which are often performed manually. A 48-grid agar growth plate has been integrated into the colony selection component to improve throughput at the cloning stage. The combinations of 48- and 96-well plate formats are compatible with automated liquid handlers and multichannel pipettes. This revised cloning and expression pipeline increases throughput significantly, and also results in a reduction in both time and material requirements. The system has been validated by the production and screening of several thousand clones at the Midwest Center for Structural Genomics.

Key words: Agar cloning; 48-Grid agar plate; Structural genomics; Automated cloning; High throughput; Protein expression; Automation; Robotics

8.1. Introduction

The abundance of data from genomic sequencing projects has led to an increased demand for the proteins encoded by these genomes *(1)*. This demand has stimulated the development of strategies for high-throughput (HTP) cloning and *in vitro*

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expression approaches for protein production *(2, 3)*. An assortment of *in vivo (3, 4)* and cell-free *(5, 6)* expression strategies are available which are compatible with the HTP approach. These approaches incorporate different methods utilizing recombinational *(4)*, restriction enzyme *(7)*, or ligation-independent cloning (LIC) methods *(8)* for the preparation of cloned material. An essential requirement for these approaches is the ability to use commercially available liquid handlers and other hardware that are compatible with standard microplate formats. The ability to automate the cloning and expression components for protein expression is essential for minimization of handling/tracking errors associated with a myriad of individual samples, meeting throughput requirements and to insure cost effectiveness.

The production pipeline from target selection to validated expression clone can be organized into component processes such as target selection, amplification (PCR), cloning, and expression and solubility analysis. Each component typically consists of one or more automation methods that use liquid handlers or other robots to improve throughput and reduce costs. The selection of individual colonies (clones) represents a small fraction of the process for production of validated expression clones. For most applications, however, clonality is an essential requirement since a single clone is desirable for most experimental endpoints. Recent progress in cloning systems have led to high-efficiency approaches for generation of vector constructs that require screening of only a minimal number of clones to obtain the desired construct *(9, 10)*. The effect of increased throughput at many steps in the cloning and expression process has increased demand for target proteins, resulting in HTP projects with requirements to screen clones for thousands of individual targets. However, most current cloning and expression processes usually transition from plate-based formats (automated) to individual culture plates (manual) at the cloning stage, resulting in a dramatic reduction in throughput. Several programs have described approaches to circumvent the cloning bottleneck by using plate formats to screen a block of twelve clones using specially constructed "cloning grills" *(11)* or plates *(12)*. We have developed a method that uses commercially available 48-grid plates to circumvent in a large part, this "transition" process and the many steps related to it, via our novel cloning method.

8.2. Materials

Robot hardware and reagent mixes are defined for a single 96-well plate procedure. In practice, most laboratories typically utilize several plates requiring linear scaling of materials and reagent mixes.

8.2.1. Preparation of T4 Polymerase Treatment of Amplified DNA Fragments and Vector

1. Make LIC Reaction mix sufficient for one 96-well plate by combining the following reagents:
   - 10X T4 polymerase buffer (included with polymerase; 465 µl)
   - dCTP (465 µl of 25 mM), molecular biology grade
   - Dithiothreitol (DTT) solution (228 µl of 100 mM)
   - Water (60 µl)
   - T4 DNA Polymerase (250 units) (LIC quality, ~2.5 units/µl, EMD Biosciences/Novagen)
   - Keep this mixture on ice and add the T4 DNA polymerase just before use. Pipette up and down several times to uniformly distribute the enzyme in the reaction mix. (*see* **Note 1**)
2. Preparation of LIC-compatible vector is described in a **Chapter 7**.

8.2.2. Transformation and Selection of Individual Colonies

1. Prepare Ampicillin stock solution (50 mg/ml) by addition of 1 g of ampicillin to 20 ml of sterile water. Working concentration for Lauria Bertaini (LB) broth and agar is 100 µg/ml ampicillin.
2. Lauria Bertaini (LB) Agar (500–550 ml), Miller with ampicillin (LB/Amp agar).
3. Lauria Bertaini (LB) Broth (200 ml), Miller.
4. Two sterile Q Tray 48-grid plates (Cat. # X6029, Genetix USA Inc) are labeled to allow mapping of the agar wells to the corresponding well on the transformation plate. Choose a clean level surface and then proceed to pour sufficient LB/Amp agar (250–275 ml of LB/Amp agar per plate) into the two previously labeled 48-grid agar plates. The media should be poured slowly into one of the peripheral rectangular openings (any outside opening other than one of the actual 48-grid slots; refer to the shaded areas within the graphic in **Fig. 8.1**, stage 2) to minimize bubbling in agar. Allow to solidify at room temperature for at least 20–30 min prior to use (*see* **Note 2**).
5. Chemically competent cells are prepared using the Z-Competent™ E. coli Transformation Buffer Set (ZYMO Research) according to the procedure recommended by the vendor (*see* **Note 3** regarding efficiency and strain selection). Competent cells are stored as 5-ml aliquots (sufficient for transformation of a single plate) at –70°C.
6. Sterilized, 1.25-in. precut glass rod rollers ("Sterilized, 1.25-in. precut glass rod rollers" should be prepared in advance by cutting 130-mm Borosilicate Glass rod Petri Dish Spreaders into pieces 1.25 in. in length.)

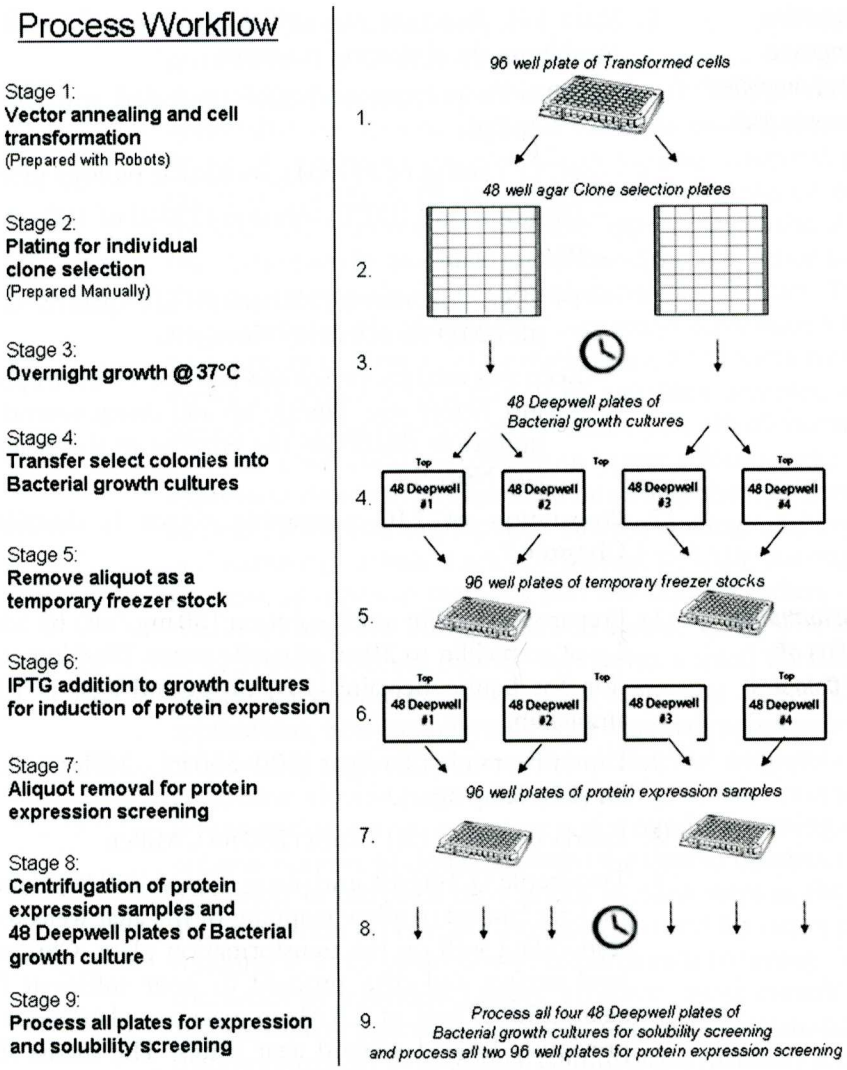

Fig. 8.1. Illustration depicting the process flow for production and screening of expression clones.

7. A 96-microwell plate suitable for heat shock using a controlled temperature heat block (*see* **Note 4**).

8.2.3. Bacterial Growth and Preparation of Soluble Lysate

1. Lauria Bertaini (LB) Broth (500 ml), Miller (Fisher cat. no. BP-1426).
2. Isopropyl-β-D-thiogalactopyranoside (IPTG; 100 mM).
3. Four 48-deepwell plates (*see* **Note 5**).
4. Lysis buffer (sufficient for 100 samples): add 80 µl of Bacterial Protease Inhibitors (Cat. # P8849, Sigma Chemical, St. Louis, MO) to 20 ml of 50 mM Sodium phosphate, pH 8.0, 300 mM NaCl. Add 80 µl of recombinant stabilized T4 lysozyme solution (Epicentre Technologies, Madison,

WI), 20 µl of Benzonase (EMD Biosciences, San Diego, CA 92121), and mix.

8.2.4. SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

1. Tris–Glycine Gradient gels (4–20%) (Cambrex Bio Science Rockland, MD).
2. SDS–PAGE running buffer (Sigma Chemical).
3. 2× SDS–PAGE sample buffer (Sigma Chemical).
4. Simply Blue protein stain (Invitrogen).

8.3. Methods

Although several universal cloning site systems are presently available, we selected the Ligation-Independent Cloning (LIC) method *(13, 14)* for implementation into our high-throughput protein production pipeline. This approach is one of several universal cloning site systems presently available which provides flexibility of expression with different fusion tags and protease cleavage sites. The rationale *(2)* for selection of this HTP cloning and expression approach as well as robotic methods for fragment generation has been previously described *(3, 10)*. A library of LIC-compatible vectors *(15, 16)* is available for distribution, and the protocols for preparation and use of these vectors are described in **Chapter 7**. Automated methods using a Beckman FX, Multimek, and Biomek 2000 have been developed for procedures outlined in this chapter and are available by request from the authors.

8.3.1. Preparation of T4 Polymerase-Treated DNA Fragments

1. Array 10.4 µl of the LIC reaction mix into a polypropylene 96-well plate. This plate will be used for transformation via a heat shock procedure and should be compatible with a controlled temperature heat block (*see* **Note 4**).
2. Add 30 µl of purified and diluted PCR fragment (*see* **Note 6** regarding suggested dilutions of PCR fragments for the annealing reaction) to the LIC reaction mix and pipette up and down several times to mix. Incubate at room temperature for 30 min (*see* **Note 7** regarding oil overlay or use of a thermocycler with heated lid).
3. Incubate on a heat block at 75°C for 20 min to inactivate the T4 DNA polymerase.
4. Following the heating process the LIC plates are stored in the refrigerator at 4°C until needed.

8.3.2. Vector Annealing and Cell Transformation (Fig. 8.1, Stage 1)

1. Take competent cells (BL21) and vector plates out of the freezer to thaw. Cells should be continuously stored on ice until use.

2. Array 4 µl of LIC-treated vector into a 96-well plate.
3. Add 4 µl of LIC-treated PCR fragment to the vector and pipette up and down several times to mix. After incubation at room temperature for 5 min, place the plate atop a cold block or in an ice bucket.
4. Add 45 µl of BL21 cold competent cells (*see* **Note 3**) to the plate, and BRIEFLY centrifuge this plate at 4°C to be sure the competent cells mix with the annealing reaction. Incubate at 4°C (usually on ice) for 5–15 min.
5. Heat shock the cells by placing the plate on the heat block set to 48°C for 1 min.
6. The plate is then transferred back to 4°C (usually on ice) for 2–10 min before adding 120 µl of LB to the transformation plate.
7. The plate is then incubated at 37°C for 30 min.

8.3.3. Plating for Individual Clone Selection (Fig. 8.1, Stage 2)

1. For one 96-microwell plate of transformants, label the bottom half of two empty 48-grid agar plates to correspond to the 96 samples of the transformation plate.
2. Predry the LB/Amp agar grid plates by incubation at 37°C for 15–20 min with the plate lids ajar. Remove the plates, uncover, and use flame-sterilized tweezers to place one sterilized, 1.25 in. precut glass rod roller into each grid location, preferably into the same uniform location (top, center, or bottom within the grid location). Then, for each well, place approximately 50–100 µl of the transformation plate solution within each corresponding grid location on the LB/Amp agar grid plate. It is recommended to dispense the transformant/solution onto the center of the rod, and to use the same single pipette tip to manipulate the same single sterilized, precut glass rod roller to spread the transformant uniformly throughout the grid location. Limit the spreading to 1–2 rolls, to maximize cell growth potential and alternatively to minimize cell destruction.
3. After processing the entire 48-grid, LB/Amp agar grid plate, cover the plate and incubate on the benchtop for 5–10 min at RT to allow the residual liquid to absorb into the agar. Uncover the plate and inspect each of the agar wells ensuring that there is very little liquid transformant remaining. If significant liquid remains, cover the plate and allow it to sit for another 10 min at RT, in order to *totally* absorb the transformant solution into agar. Once there is little-to-no liquid transformant remaining, quickly invert the entire 48-grid plate (lid and bottom) thus resulting in a bottom side up orientation.
4. Follow this up by gently tapping the bottom, in order to assist in the release of the glass rod rollers onto the inside of the

cover/lid. Lift the bottom of the 48-grid plate and place atop a new upside down, sterile 48-grid plate cover/lid.

5. Incubate at 37°C for 12–18 h. Be sure to incubate the LB/Amp agar grid plate in an inverted orientation, with the plate lids offset (cracked) for ventilation. Plates with volumes greater than 300 ml of agar and/or if volumes larger than 100 µl of the transformation solution are to be plated, and should be left to incubate with their lids offset. Plates with volumes less than 300 ml of agar should avoid being left to incubate with their lids offset.
6. Return to the used glass rod rollers lying inside of the upside down 48-grid plate cover/lid, and rinse both rod rollers and 48-grid plate cover/lid with H_2O. Follow this with a thorough 95% Ethanol rinse that should then be followed by another thorough H_2O rinse. Dry with paper towels or allow to air dry.

8.3.4. Colony Selection for Growth and Induction (Fig. 8.1, Stages 3 and 4)

1. For the 96-microwell plate of transformants, label four 48-deepwell plates (as illustrated in **Fig. 8.1**, stage 4) to correspond to the two colonies selected from the 48-grid, LB/Amp, agar plates.
2. To each of the four 48-deepwell plates, use a liquid-dispensing device (e.g., QFill2 station) to add 2 ml/well of LB/Amp broth (medium for bacteria) containing ampicillin. Note: Add antibiotic each time.
3. Using a 10-µl pipette tip, select colonies from the two 48-grid, LB/Amp, agar plates (look for single colonies) by scraping part of a colony from the plate and placing the tip in the corresponding well containing 2-ml LB/Amp broth. Be sure to laterally mix the pipette tips in each well for up to 5 s before incubating. Upon incubation, be sure to leave the tips in each of the wells to assist with agitation. Make sure the tips are fully immersed in the well and are touching the well bottom. Tips that are not flush with the well bottom may eject during incubation or adhere to the side of the well and may not properly mix in the LB/Amp. This may lead to a loss of sample, poor/no sample growth, and/or cause sample crosscontamination.
4. Place the cultures at 37°C in a shaking incubator, set at an rpm of no higher than 250.

8.3.5. Preparation of Temporary Freezer Stock (Fig. 8.1, Stage 5)

1. Temporary freezer stocks are prepared when the A_{600} of the bacterial growth culture is approximately 0.4 (*see* **Note 8**).
2. Remove the deepwell plates from the incubator and prepare a glycerol stock from the deepwell plate by manually removing 200 µl from each well of the four 48-deepwell plates, and place into each corresponding location within two additional 96 round-bottom plates.

3. Centrifuge these 96 round-bottom plates for 10 min at 3,000 × *g* and remove the growth media by inverting the plate and blotting the top surface on a paper towel.
4. Resuspend the pellet in 200 µl of LB + 30% glycerol, mix 1–2 times, and follow by freezing.

8.3.6. Analysis of Protein Expression (Fig. 8.1, Stages 6 and 7)

1. Use an automated liquid handler or multichannel pipette to add 30 µl of 100 mM IPTG (induces bacteria to express protein) to each of the wells and return the deepwell plates to a shaker incubator for an additional 2 h.
2. While waiting, label two 96-deepwell plates (**Fig. 8.1**, Stage 7).
3. After incubation is complete, use an automated liquid handler or multichannel pipette to remove 400 µl from each well of the four 48-deepwell plates and place into a corresponding location of the two 96-deepwell plates (*see* **Note 9**).
4. Centrifuge the two 96-deepwell plates for 10 min at 3,000 × *g* and remove the growth media by inverting the plate and then blotting the top surface on a paper towel.
5. Resuspend the pellets in 70 µl of 1× SDS–PAGE buffer using an automated liquid handler or multichannel pipette. The plates are then sealed with foil and vortexed to mix.
6. Boil for 4 min and again vortex well. Prior to analysis by SDS–PAGE, samples should be briefly centrifuged to collect the liquids at the bottom of the well.

8.3.7. Preparation of a Soluble Lysate (Fig. 8.1, Stages 7 and 8)

1. The growth culture (1.4 ml) remaining in the four 48-deepwell plates is used for solubility analysis. Pellet the cells by centrifugation for 10 min at 3,000 × *g* and discard the supernatant. Dry the pellets by inverting the plate and blotting the plate on a paper towel for 15–30 s. The plates should be stored at −20°C until solubility analysis.
2. Lyse the bacteria by suspension of the pellets in 180-µl lysis buffer and incubation at room temperature for 5 min. During the incubation, vortex the samples 1–2 times.
3. Transfer the suspension to a labeled 96-microwell plate and centrifuge at 3,000 × *g*, at (4°C) for 10 min.
4. After centrifugation, remove 50 µl of the supernatant and add to an awaiting labeled 96-microwell plate containing 60 µl/well of 2× SDS–PAGE sample buffer and boil for 3 min.

8.3.8. SDS–PAGE Analysis of Expression and Solubility

1. For denaturing gel analysis, load 5 µl of low molecular weight marker for each gel.
2. Load 8 µl of each sample prepared from the total growth culture for analysis of total expressed protein.

3. Load 12–15 µl of each sample prepared from the lysate sample for analysis of soluble protein.
4. Remove from gel station and stain for protein. Gels can be stained anywhere from 60 min to overnight. (*see* **Note 10** expression/solubility scoring.

8.4. Notes

1. T4 DNA polymerase reaction buffers supplied by most vendors can be substituted for the LIC reaction buffer described in the text. Our comparison of various common T4 DNA polymerase reaction buffers shows less than a 25% difference in the cloning efficiency of the final product.
2. Q tray plates prepared in this manner can be stored for at least 2 weeks at 4°C. Freshly poured plates should be dried prior to use by removal of the plate cover and incubation in 37°C for 30 min
3. The method presented in this chapter uses direct transformation of an expression capable BL21 strain. Typical transformation efficiencies for BL21 strains are in the range of 0.5–1 $\times 10^6$ colonies/µg of plasmid DNA. The observed number of colonies on the Q tray plates is a function of the overall efficiency of the annealing reaction and the level of competency of the bacterial cells. It is recommended that the investigator place various amounts or dilutions of several representative target samples prior to multiple plate screening.
4. The well geometry of the plates should match the instrument (thermal cycler of heat block) used for the heat shock procedure. We optimize transformation efficiency for each strain and plate by testing different times and temperatures.
5. A variety of 48-deepwell plates are available from standard scientific supply vendors. We prefer pyramidal bottom plates as these more effectively retain the pellet after centrifugation steps and facilitate pipetting operations that involve removal operation near the bottom of the plate.
6. Our studies of various fragment-to-vector ratios *(2)* indicate a wide tolerance for variation in the amount of target DNA fragment on the annealing reaction. This latitude eliminates the need for normalization of fragment concentrations prior to annealing, thus conserving time and simplifying the process for implementation of the method as an automated process.

7. The use of a thermal cycler with a heated lid is preferred since there are no additional components to add to the mix and the number of pipetting steps is reduced. An alternative is to layer 20–40 µl of mineral oil on the annealing mix prior to heat treatment.
8. Cultures grown in plates typically reach an A_{600} of 0.4–2.5 h after inoculation. An exact A_{600} measurement of 0.4 is not necessary for the preparation of temporary freezer stocks in plates. These freezer stocks should be viable for at least a month when stored at −70°C. Stock culture for the positive expression clones can also be prepared by streaking out a loopful of bacterial growth culture from each well onto an agar plate.
9. Transfer from the 48-well to a 96-well plate can be accomplished using the span-8 tool of a Biomek FX instrument or by selective loading of tips on a Beckman Multimek. Tips are arrayed in six staggered columns in a tip box using a multichannel pipette (odd columns in one rack and even columns in another rack). These tip boxes are then used for successive transfer into the corresponding new location within the 96-deepwell plate.
10. Targets are scores as "no expression" or "insoluble" based on the absence of a detectable stained protein band of the correct molecular weight observed after SDS–PAGE analysis. Targets can be scored as positive, based on an observation of a protein stained band of correct molecular weight. We use a relative ranking scale that compares staining of the target relative to the general intensity most of the *E coli* proteins. Target bands that are visible but with intensity level less than most of the *E coli* proteins are scored as level 1 or low expression/solubility. Levels 2 and 3 (moderate and high expression/solubility) have staining intensity comparable to that of highly expressed *E. coli* proteins or more prominent than any *E. coli* protein, respectively.

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