Guide to Functional Genetics in *Dictyostelium* Using Library Complementation/Multi-Copy Expression Analysis

includes a *Dictyostelium* Vegetative Growth Expression Library,
Library Design and Specifications and a *Dictyostelium* Transformation Optimization Protocol

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Douglas N. Robinson
Department of Cell Biology
Johns Hopkins School of Medicine
Baltimore, MD 21205

dnr@jhmi.edu

Description of the Experimental Approach

The goal of multi-copy suppression analysis and library complementation is to make available sensitive interaction "yeast-like" genetics including epistasis and suppression available for the cellular slime mold, *Dictyostelium discoideum*. The system should allow genetic interactions between molecules and pathways to be elucidated. One of the powerful applications of this approach is that the cell biological and developmental functions of redundant or essential molecules may be elucidated making these difficult to study classes of molecules tractable. Furthermore, by taking advantage of the intrinsic property of partial length clones found in cDNA libraries, one can simultaneously select or screen for dominant-negative, constitutively active and attenuated functions of molecules that lead to suppression of a phenotype. This fact permits interactions between pathways and epistasis within a pathway to be simultaneously uncovered.

Preparation and Handling of the Library DNA

The library comes in the form of 100 μl bacterial glycerol stocks and should be stored at –80^{o}C. The titer is 4.1x10^{10} cfu/ml. To prepare DNA for *Dictyostelium* transformations, the library can be amplified. I plate out 80 15-cm LB-Amp or carbenicillin plates at a density of 10,000 cfu/plate. The plates are allowed to grow at 30^{o}C until the colonies touch each other (24-30 hours). The cells are recovered by scraping. I pool the cells into two batches and prepare each batch by Qiagen Maxi-prep. In other words, I prepare approximately 400,000 colonies worth of cells per Qiagen Maxi-prep. After preparation, the two pools of purified plasmid DNA can be combined. The DNA can be digested for analysis on ethidium-stained agarose gel electrophoresis.

WARNING: The Library Cells Should Be Grown at 30^{o}C on Standard Luria Broth Agar with the Appropriate Drug Only to Insure Stability of cDNA Inserts. Higher Temperatures (37^{o}C) and Richer Medias Must Be Avoided.

NOTE: You should aliquot the library and refreeze aliquots at –80^{o}C the first time you thaw it. This is your lifetime supply of it.

Description of the construction of the cDNA library

1. I constructed a vector, pLD1A15SN, for use in a library. It is very similar to other Ddp2-based vectors except that it has a different polylinker and no ATG methionine.

2. I cloned GFP-myosin-II into this vector. It rescues myo-II null cell development and expresses GFP-myosin-II to similar levels as pBIG - *i.e.* the vector works as it should.

3. I purified polyA+ mRNA from total RNA generated from log phase Ax-2 cells grown on surfaces and in suspension. The total RNA was 50:50 from these two populations of cells.

4. I used a Superscript II kit from Gibco-BRL to do the first and second strand synthesis of cDNA and to prepare the cDNA for ligations. It was then ligated into pLD1A15SN.

5. The ligated cDNA was transformed into Max Efficiency STBL2 cells from Gibco-BRL. These cells are supposed to be the best available to stabilize unstable and repetitive DNA sequences.

6. A conservative estimate of 250,000 clones were recovered.

7. The library was analyzed by picking single colonies for analysis of the plasmids and by prepping library DNA for bulk analysis.
8. From the analysis, all plasmids had full-length vectors suggesting that the inserts were stabilized as hoped.

Specifications of the *Dictyostelium* cDNA library

From minipreps:

Total examined:	82
Number with inserts:	65
Proportion:	79%
Size range:	0.5 - 3 kb
Average size:	1.1 kb
Standard deviation:	0.4 kb

From maxi-prepped total library DNA:

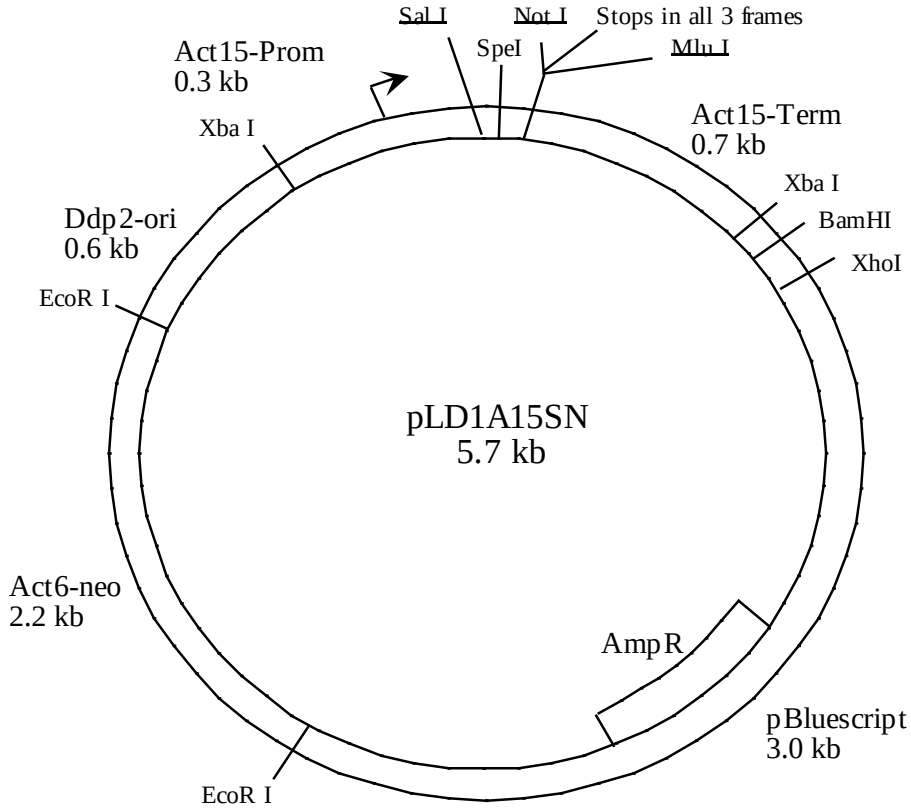
Size range:	0.5 - >4 kb
Peak ethidium bromide intensity:	1.3 kb

Conservative Complexity Estimate:

Assume 250,000 primary bacterial clones
x 79% = 198,170 with inserts

Thus, from the Poisson distribution, we can be 99% sure that we have covered clones that exist at 1 in 43,000. If in a *Dictyostelium* cell there are 250,000 transcripts, the library should have a copy of anything that exist in as little as 6 copies per cell. Full-length clones are a different matter of course.

Library vector:



....Act 15 promoter .... attt ttttttttt ttGTTATTTCTTAAAACAAATAAATTAAATTAAATAA

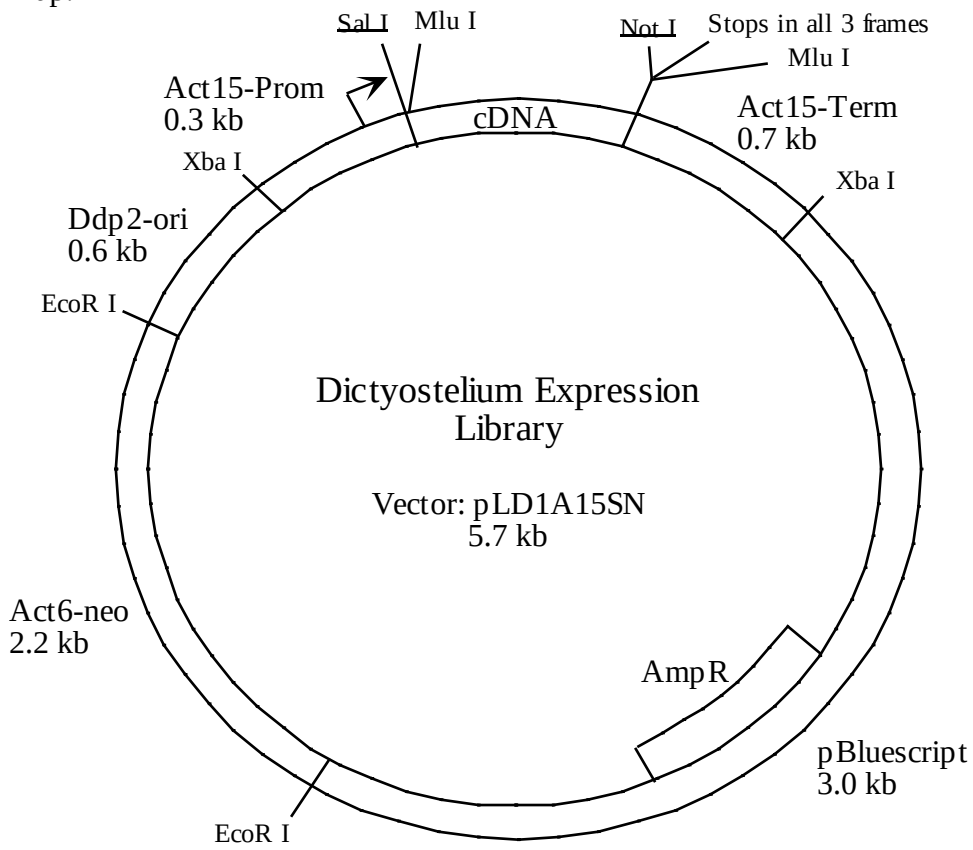
Sal I SpeI NotI Stops in 3 frames Mlu I

AAAAATAAAAGTCGAC ACT AGT T GCGGCCGC TAAA TAAA TAA ACGCGT

Underlined sites are unique.
Arrows identify transcripton start sites.
There is no translation initiator ATG in this vector.

pLD1A15SN is a Ddp2-based vector, meaning that the replicase orf is required in trans either as an integrant in the genome of your favorite *Dictyostelium* strain or on a second plasmid. I prefer it as an integrant, if possible.

Library Map:



Underlined sites are unique.

cDNA was generated from poly A^+ mRNA isolated from log phase suspension and surface grown Ax-2 cells.

cDNAs were cloned into the Sal I and Not I sites. cDNA may be removed as a Sal I - Not I fragment or as an Mlu I fragment.

The bacterial host cells are Gibco-BRL STBL2 cells. These cells should be propagated in LB-ampicillin or carbenicillin at 30C. In order to maintain stability of the insert, higher temperatures and rich media should be avoided.

The library titre is 4.1×10^{10} cfu/ml.

pLD1A15SN is a Ddp2-based vector, meaning that the replicase orf is required in trans either as an integrant in the genome of your favorite *Dictyostelium* strain or on a second plasmid. I prefer it as an integrant, if possible.

Library transformation protocol for *Dictyostelium*

1. 3-4 days before I am ready to do the transformation I plate cells so that on the day of the transformation they will be in log phase at a density of approximately 5-6 x 10^6 cells/ml. The cells are grown in Hans' complete HL-5 (500 mls HL-5, 75 mls 5X HL-5, 50 mls FM, 3.75 mls Penn/Strep (final conc. of P/S: 60.24 units/ml penn and 0.06 mg/ml strep)).

2. On transformation day, I harvest the cells and determine the total number of cells collected.

3. Cells are pelleted by centrifugation in a table top clinical centrifuge for 3 minutes at 1-2,000xg.

4. Resuspend cells in 12 mls ice cold E-pore buffer (50 mM sucrose, 10 mM sodium phosphate pH 6.5; filter sterilized).

4. Spin as in 3.

5. Resuspend cells at a density of 1 x 10^7 cells per 350 μl of E-pore buffer (350 μl is the transformation volume.). Add an appropriate amount of DNA to each transformation volume.

> [For a library transformation, I typically use 1 μg of library DNA per transformation. Remember to set aside 1-3 350 μl aliquots for "No DNA" control transformation.]
>
> *Remember: pLD1A15SN is a Ddp2-based vector, meaning that the replicase orf is required in trans either as an integrant in the genome of your favorite *Dictyostelium* strain or on a second plasmid. I prefer it as an integrant, if possible.*

6. Prepare 500 μl of ice cold minimal HL-5 per transformation; store on ice.

7. Chill an appropriate number of 0.4 cm cuvettes.

8. Transfer 350 μl of transformation cells to a cuvette and electroporate at desired voltage (see "optimizing field strength"), 3.0 μF. The τ should be between 1.5-2.0.

9. Immediately add 500 μl of ice cold HL-5. These cells may now be stored on ice until ready to plate.

10. Plate 850 μl aliquots of transformed cells on 9 mls of Hans' complete HL-5 media.

11. Allow cells to recover for 20-24 hours at 22C. Then, replace media with 10 mls Hans' complete media plus 15 μg/ml G418. Continue to grow at 22C.

12. Replace media with fresh Hans' complete media plus 15 μg/ml G418 every 2-3 days until clones are ready to be harvested (approx. 8 days).

Optimizing the field strength:

I have found that the optimal field strength is highly cell-line specific. So far, for the lines that I have tested the optimum is between 1.3 keV/0.4 cm (3250 V/cm) and 1.0 keV/0.4 cm (2500 V/cm). Before transforming a line with the library, I perform a test transformation in which I transform 5-8 samples at each setting, varying the voltage from 0.9-1.4 keV in 0.1 keV increments. The optimum should be easily discerned for use when transforming the library into the cells in large scale. Transformation efficiency that I have been able to achieve so far has been 1500 primary colonies/μg of DNA. There are actually many more colonies than that per μg of DNA, but I do not include small colonies in my count for fear that they may be secondary sibling colonies. I exclude those in my estimates since the point of determining the transformation efficiency is to be able to determine how well the library is represented. Therefore, 1500 colonies/μg of DNA might actually be a lower limit. That is fine in this case since more is better.

Recovery of plasmid DNAs from *Dictyostelium*:

After your selection and/or screen you will want to recover the plasmid DNA from the Dictyostelium cells. This may be easily done by using a Promega Wizard *Plus* Minipreps: DNA Purification System. You can use one ml of just sub-confluent cells and follow the manufacterer's protocol for bacterial mini-DNA prep. This will not give you enough DNA for analytical work. However, 3-5 microliters of the recovered DNA may be directly transformed into Gibco-BRL's STBL2 cells according to manufacterer's specifications. I usually examine up to 18 clones to get an estimate of the complexity of the DNA mix in the cells. If clonality is certain this is probably more than enough. If, however, you are selecting by competitive growth, you may want to examine more to make sure all of the "winners" have been recovered.

A side note: By following this protocol, I have never found a rearranged plasmid from *Dictyostelium* or the STBL2 bacteria.

Recommended primer sequences for analyzing the recovered library plasmids

5' end
LD1SM:
5'-AAA AGT CGA CCC ACG CGT CC-3'

3' end
LD13'
5'-CGC GTT TAT TTA TTT AGC GGC CGC CC-3'

These primer sequences will only work with the library, not with pLD1A15SN alone. Also, I find that LD1SM works really well, whereas LD13' generally gives lower quality sequence information presumably because of the poly T stretch (from the poly A tail) that has to be sequenced through.

Reference:

Robinson, D.N. and J.A. Spudich. 2000. Dynacortin, a genetic link between equatorial contractility and global shape control discovered by library complementation of a *Dictyostelium* cytokinesis mutant. J. Cell Biol. 150: 823-38.