2019

Working

One-pot Optimization of Genetic Circuits using Poly-transfections

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dx.doi.org/10.17504/protocols.io.k98cz9w

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Plasmid Design and Cloning

1 Poly-transfections can be performed either with the transfection marker included on the same plasmid or by cotransfecting a different transfection marker plasmid with each different gene of interest. For co-transfections, skip to step 6 but remember to mix the transfection marker plasmids with the plasmids encoding the genes of interest before making the poly-transfection complexes.

Determine the components of the circuit to be tested

One should plan in advance what the circuit(s) to be tested will contain. For example throughout this protocol we will plan an experiment to characterize the ability of Gal4VP16 to activate a new design for a Gal4VP16 activatable promoter. Our circuit components include:

1. constitutive expression of Gal4VP16
- Insulator = CTCF binding motif
- Promoter = CMV
- 5' UTR = inert sequence
- Gene = Gal4VP16
- 3' UTR = inert sequence
- Terminator = SV40 poly A

2. fluorescent protein output from a Gal4VP16 activatable promoter.
- Insulator = CTCF binding motif
- Promoter = Gal4VP16 activatable promoter
- 5' UTR = inert sequence
- Gene = Fluorescent protein (later determined to be mKO2)
- 3' UTR = inert sequence
- Terminator = SV40 poly A

2 Determine which fluorescent proteins to use in the experiment

One will need to determine the set of fluorescent proteins to be used, which may depend on what equipment is available to make fluorescent measurements. For the flow cytometer we used (BD Fortessa with 355nm laser + 379/28nm filter, 405 + 450/50, 488 + 530/30, 561 + 582/15, 640 + 710/50) the following sets of fluorescent proteins were demonstrated to work well:

- Two-color experiments = TagBFP + mNeonGreen or mKO2
- Three-color experiments = TagBFP + mNeonGreen + mKO2 or iRFP670 or iRFP720
- Four-color experiments = TagBFP + mNeonGreen + mKO2 + iRFP720
- Five-color experiments = Sirius + TagBFP + mNeonGreen + mKO2 + iRFP720

Use of additional colors is possible, especially with spectral analyzers. Though one will need to make sure that bleedthrough of signal from one fluorescent protein to another is minimal (after compensation if necessary).

For our example, we will use TagBFP as a transfection marker for Gal4VP16 expression, mKO2 as the output signal, and mNeonGreen as the transfection marker for the output plasmid. This design results in the following transfection marker components to be assembled:

3. constitutive expression of TagBFP
- Insulator = CTCF binding motif
- Promoter = CMV

- 5' UTR = inert sequence
- Gene = TagBFP
- 3' UTR = inert sequence
- Terminator = SV40 poly A

4. constitutive expression of mNeonGreen
- Insulator = CTCF binding motif
- Promoter = CMV
- 5' UTR = inert sequence
- Gene = mNeonGreen
- 3' UTR = inert sequence
- Terminator = SV40 poly A

Note that not all fluorescent proteins may be suitable for multi-color cytometry. We found that almost all red fluorescent proteins had significant signal in the 450/50 channel with a strange stochastic behavior. We believe this is due to a blue intermediate during folding of the red fluorescent proteins. For this reason, we chose to use an orange fluorescent protein (mKO2) instead which has some spillover into the 530/30 channel but has predictable behavior which can be accounted for with compensation. We also observed that yellow fluorescent proteins spill into the 379/28 channel so we used green fluorescent proteins instead. Finally, some red fluorescent proteins have signal in the 710/50 channel, limiting the choice of red/orange FP.

3 Clone needed plasmid level 0 (pL0) constructs

One should clone the needed parts (eg. promoters, coding sequences, etc) into pL0 plasmids with the appropriate Golden Gate assembly overhangs - or obtain existing pL0's from other researchers. pL0 plasmids generally have spectinomycin resistance and the backbones have LacZ which is displaced after assembly for blue/white screening. pL0 overhangs are as follows and are generated by the BbsI restriction enzyme for cloning into the pL0 backbone:

- pL0-I (insulator) = [GGAG] - [TACT]
- pL0-P (promoter) = [TACT] - [CAGA]
- pL0-5 (5' UTR) = [CAGA] - [AGGT]
- pL0-G (gene/coding sequence) = [AGGT] - [GCTT]
- pL0-3 (3' UTR) = [GCTT] - [CAAC]
- pL0-T (terminator/pA) = [CAAC] - [CGCT]

For example, a pL0-P with CMV can be obtained by generating the double-stranded DNA fragment below by PCR or synthesis and cloned into the pL0-P backbone vector with BbsI Golden Gate (bolded sequences correspond to BbsI recognition site and underlined sequences correspond to BbsI cut site):

NNN**GAAGAC**NNTACT - CMV sequence - CAGANN**GTCTTC**NNN

In general, Golden Gate reactions consist of the following:
- 40 fmol of each DNA part
- Water to a total reaction volume of 10 uL
- 1 uL of T4 ligase buffer (Promega)
- 0.4 uL of Type IIs restriction enzyme (NEB)
- 0.2 uL of T4 ligase HC (Promega)

Golden Gate thermocycling consists of the following:
1. 37C for 5min
2. 50 cycles of 37C for 2min, 16C for 5min
3. 50C for 5min
4. 80C for 10min
5. Hold at 4C

Golden Gate reactions can be transformed into chemically competent cells at relatively high efficiency.

4 Clone plasmid level 1 (pL1) constructs containing transcription units

Using the pL0 constructs, one should assemble transcription units (TUs) into pL1 backbones. These should contain one each of pL0's designated as I, P, 5, G, 3, and T. In most cases, circuit components should be assembled into the pL1-S1S2 backbone while fluorescent protein transfection markers should be assembled into the pL1-S2S3 backbone. Assembly of pL1 plasmids is conducted using BsaI-mediated Golden Gate assembly.

For our example, we will assemble:
1. pL1-S1S2 with TU for constitutive Gal4VP16

2. pL1-S1S2 with Gal4VP16 activatable promoter and mKO2 output
3. pL1-S2S3 with constitutive expression of TagBFP
4. pL1-S2S3 with constitutive expression of mNeonGreen

5 Clone plasmid level 2 (pL2 constructs) that contain one circuit TU and one transfection marker TU

Using Gibson assembly, one should assemble together pairs of constructs with one circuit TU and one transfection marker TU. In most assemblies the pL2-S3S1 backbone may be used, but other Gibson backbones with Weiss lab overlapping sequences can also be used. Each plasmid should be digested with I-SceI restriction enzyme, PCR purified or gel extracted, and the linear fragments used for a Gibson reaction. We obtained reaction mix from SGI as the Gibson Ultra Kit and followed the manufacturer's protocol for assembly. We electroporated assembly products electrocompetent cells (NEB 10-beta cells) as we generally found larger constructs harder to transform with chemically competent cells.

We will also deposit pL2 backbones already containing constitutive expression of different fluorescent proteins, such that only a single Golden Gate step is required to assemble functional pL2 vectors.

In our example, pL1-S1S2 with constitutive Gal4VP16 (#1) should be assembled with pL1-S2S3 with TagBFP (#3) and pL2-S3S1 to generate construct A. And also pL1-S1S2 with activatable promoter and mKO2 (#2) should be assembled with pL1-S2S3 with mNeonGreen (#4) and pL2-S3S1 to generate construct B. This should generate two final plasmids for poly-transfection. Both constructs should be midiprepped to obtain transfection quality DNA with reduced endotoxin concentrations.

Alternatively, it is possible to conduct poly-transfections using pL1's instead of pL2's. In this case, equimolar concentrations of pL1's should be mixed together in each lipid complexes, such that mixtures contain the same constructs as those assembled into pL2's listed here.

Cell Culture and Poly-transfections

6 Grow and maintain cells of interest

Cells of interested should be obtained from a reliable source, thawed into the appropriate growth medium, and verified to be free of mycoplasma or other contaminants. Cells should be regularly split into new flasks/dishes when appropriate confluence is reached (use the appropriate maintence conditions provided for the cells) using trypsin or other dissociation medium. For our study, we grew HEK293FT cells (Thermo Fisher) and HeLa cells (ATCC) in DMEM
DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Cellgro) and supplemented with 10% FBS (Cellgro) and at 37C / 5% CO_2. Before transfection, one should calculate approximately how many cells will be required for the experiment and determine if the necessary number of cells have grown. We use 200,000 HEK293FT cells per well or 150,000 HeLa cells per well in a 24-well plate and then scale that number according to plate surface area.

7 Prepare transfection mixtures

Transfection mixtures can be separately prepared according to the manufacturer's protocol, then simultaneously added to cells. We have mostly used lipofectamine 3000 for transfection, but have shown that poly-transfections work with a variety of transfection agents.

In our protocol, we diluted 900 ng of each input plasmid with 75 uL of Opti-MEM (Thermo Fisher), mixed in 1.5 uL of P3000 reagent, then mixed in 1.5 uL of Lipofectamine 3000 reagent. Each complex was mixed separately and incubated for 30 min before being added to cells plated in a 6-well format. Note that in a 24-well format, these volumes correspond to 150 ng of DNA in 12.5 uL of Opti-MEM, 0.25 uL of P3000, and 0.25 uL of lipofectamine per complex. If using transfection markers on a separate plasmid from the circuit component, one can use equimolar concentrations of each to a total of 150 ng of DNA (24-well format) or 900 ng (6-well) then add the corresponding amount of Opti-MEM and lipofectamine reagents.

8 Add transfection mixtures to cells

Add the desired complexes to cells. In our example, separate complexes prepared for constructs A and B would be applied to cells at the same time and cells would be allowed to grow in order to express the circuit components as well as fluorescent proteins. In our experiments we waited 48 hours between transfection and flow cytometry.

Flow Cytometry

9 Prepare cells for flow cytometry

Cells should be suspended into PBS or FACS buffer using methods appropriate for the cell lines tested. For our adherent cells, we trypsinized cells (0.2 mL in 24-well), resuspended them in growth media (0.5 mL in 24-well), spun then down at 100 x g, aspirated the media and resuspended in PBS (0.5 mL for 24-well).

10 Prepare flow cytometer and run calibrations

The flow cytometer should be operated according to the manufactuer's protocols. Single color controls should be run and PMT voltages set to obtain best dynamic range without saturation of highly expressing cells. We highly recommend running multi-peak fluorescent calibration beads (we have used Spherotech URCP-100-2H or RCP-30-5A) to make sure that the cytometer is running correctly and to enable more quantitative and reliable measurements that are reproducible across different cytometers.

11 Run samples through the flow cytometer

After running controls through the cytometer, samples should be run, making sure that a wide range of fluorescent color space is covered when fluorescent channels are plotted against each other. As many cells as necessary should be collected such that an adequate number of events are recorded across the fluorescent color space. Generally we used all cells from a well in a 24-well plate for 2-complex experiments, a 6-well plate for 3-complex experiments, and 2 pooled wells from a 6-well plate for 4-complex experiments.

12 Export data and finish cytometer session

Data should be exported as fcs files and the cytometer cleaned and shut down.

Analyze data

13 Set up existing analysis pipelines

We have developed code to quickly bin data along multiple dimensions using MATLAB. The code can be found at GitHub under https://github.com/jonesr18/MATLAB_Flow_Analysis

Additonal code may also be needed for downstream analysis in order to read in fcs files (Laszlo Balkay: https://www.mathworks.com/matlabcentral/fileexchange/9608-fca-readfcs), and logicle transform (Rachel Finck: https://www.mathworks.com/matlabcentral/fileexchange/45022-logicle-transformation).

14 Process data using the FlowData method

Use a MATLAB script to call the FlowData method for binning. See the attached zip file for an example m-file and fcs files.

15 Further analyze binned data to generate characterization or conclusions

If custom analysis needs to be conducted, binned or raw data can be analyzed and visualized in Matlab.