Pichia Transformation Protocol.
Electroporation of BioGrammatics competent Pichia cells,
2017-06-01.
bG-Customer Protocol – 0XX.

Expectations

Each "one-shot" tube of BioGrammatics electroporation-competent Pichia pastoris (E-comp) cells has 25 – 30 ul of frozen competent cells.
Using the following protocol, electroporation with 2 ul of the 5 ng/ul control transformation control plasmid, pJAG-ARS, should generate ~1000 colonies. This is similar to number of transformants one can obtain with an expression vector linearized in the AOX1 promoter with PmeI to targeted integration into the Pichia genome at the AOX1 locus. Lower transformation frequencies are obtained targeting alternative loci, and with different forms of DNA.

E-comp cells can be stored at -80°C for up to 6 months without significant loss of competency.

Note, the following protocol uses cuvettes with a 1 mm gap width for electroporation; most published protocols for Pichia use 2 mm cuvettes. Furthermore, pre-programmed electroporation settings may be set for 2 mm cuvettes. Protocols for either type of cuvette are acceptable, however, the appropriate electroporation settings must match the cuvette gap width (1 mm or 2 mm). Higher voltages required for the 2 mm cuvettes will cause arching with the 1 mm cuvettes.

DNA

Expression vectors are most often electroporated into Pichia cells as linear DNA molecules. For example, the restriction enzyme Pme I recognizes the 5'-GTTTAAAC-3' site in the middle of the AOX1 promoter; expression vectors linearized with Pme I preferentially integrate into the AOX1 promoter in the Pichia genome. DNA should be cleaned and concentrated after restriction enzyme digestion, prior to transformation. Re-suspension of the DNA in a solution with low conductivity, i.e. in water, or in low concentrations of Tris/EDTA, is best for electroporation.

Electroporation Protocol.

1. Label and chill sterile 1 mm electroporation cuvettes in an ice/water slurry at least 5 minutes prior to electroporation. The cuvettes, and sample, should be as close to 0°C as possible at the time of electroporation. *Note, the settings provided in this protocol are for 1 mm cuvettes - not 2 mm cuvettes.*

2. Remove E-comp cells from -80°C freezer. Thaw and place on ice. Warming tubes by hand during transport from the freezer works well; rapid thawing may actually be slightly better than a slow thaw.

3. Add DNA to cells. Results with DNA volumes up to 5 ul per "one shot" tube (~30 ul) are similar; larger volumes may result in lower numbers of transformants. Most importantly, reduce the amount of salt/ions added to the E-comp cells to minimize conductivity of the sample during electroporation. Water or low concentrations of Tris are best.

Electroporation with 200 ng of a linear expression vector should generate 1000's of transformants if the AOX1 locus is targeted, efficiencies at other loci vary. More DNA will result in more transformants, up to ~1ug of DNA/sample.

4. Gently mix the DNA and E-comp cells, then, transfer the entire sample to a sterile, ice-cold 1 mm cuvette.
Make sure the sample is inserted between the metal plates.

5. Rapidly, place the cuvette between the electrodes in the "shock chamber" of your electroporation device, activate and discharge the device - all in ~5 seconds.

Note, warming of the cuvette/sample can significantly reduce the number of transformants. The time after the sample-cuvette is removed from the ice bath and placed in the Electroporator, until the current is discharged, is critical. This "Ice to Zap" time should be as short as possible.

<u>Electroporation settings:</u>

- BioRad (Gene Pluser Xcell®, MXcell®, II, and E. coli Pulser®): 10 uF capacitance, 600 ohms resistance, and 1150 volts.
- BTX machines (ECM 399 or 630) and the BioRad GenePuler I: 25 uF, 200 ohms and 1150 volts.
- Eppendorf (Eporator®, Multiporator®): 1150 volts (1200V if 1150 is not possible), capacitance and resistance are fixed.

In general, the electroporation should be conducted at a voltage as close to 1150 volts as possible with a theoretical time constant of 5-6 milliseconds (25 uF and 200 ohms, or 10 uF and 600 ohms). Actual time constants of 4 – 6 milliseconds are best.

6. After the electroporation discharge (zapping the cells), add ~1 ml Pichia Electroporation Recovery Solution (PERS, a 1:1 mixture of YPD and 1 M Sorbitol) to the cuvette and mix it with the cells. Transfer the sample from the cuvette to a tube for incubation. *The original E-comp cell tube works well. Inverting the cuvette to draw out the sample from between the electrodes with a pipet tip helps to extract more sample.*

7. Incubate samples at at room temperature up to 30°C, shaking at ~100 rpm for ~3 hr. Shorter recovery times will yield fewer transformants; longer times, for example overnight, can result in cell division/"sister" clones.

8. Spread the cells onto the appropriate YPD-agar plates for selection, and incubate the plates at 30°C for 2 - 3 days. Multiple dilutions are recommended For example, plate 10 ul of the sample on one plate, and the 100 ul of the sample on another plate. For "hard to target" loci more of the transformation sample can be plated. Centrifugation at 8k rpm in a microfuge for 30 sec will pellet cells from PERS for plating. [Drug concentrations for selection: G418 at 800-1000 ug/ml, and Nourseothricin (Nat), or Zeocin, at 100 ug/ml].

9. Carefully pick single colonies from the original selection plates and transfer/patch them to a second selection plate to ensure that any "picked" non-transformed cells will not grow. Single colony isolate transformed cells is also recommended. Incubate at 30°C overnight. Subsequent testing should be performed with cells originating from the 2nd selective plate. Furthermore, no selection is required for stably transformed cells in subsequent testing.

10. Glycerol stocks of select clones should be made from cells off the 2nd selective plate. Add sterile glycerol to an overnight YPD culture, (30% v/v glycerol).

Quick Electroporation Protocol Outline.

1) Linearize plasmid DNA (clean and concentrate).
2) Chill cuvette (ice-water slurry).
3) Add DNA to "one shot" E-comp cells; transfer to 1 mm cuvette.
4) Electroporate sample with settings of 1150 V and 5-6 ms (10 uF and 600 Ohms, or, 25 uF and 200 Ohms). Actual time constants of 4-5 ms should be obtained.
5) Add 1 ml of Pichia Electroporation Recovery Solution (PERS) to the cuvette and transfer the sample to a tube for incubation at 30°C, 100 rpm for ~3 hr.
6) Spread samples on selective plates; incubate at 30° C for 2-3 days.