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Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris

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Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* has gained widespread acceptance as a system of choice for heterologous protein expression in part because of the simplicity of techniques required for its molecular genetic manipulation (1). Several different procedures are available for introducing DNA into *P. pastoris*—spheroplast generation (2), electroporation (3), alkali cation (3,4), or polyethylene glycol (PEG) treatment (5). Here we describe a condensed protocol for cell preparation and transformation that works reliably with either auxotrophic markers or antibiotic selection.

The introduction of exogenous DNA into an organism requires two steps: (*i*) the preparation of competent cells for DNA uptake and (*ii*) the transformation of the cells with the DNA. Transformation of *P. pastoris* by electroporation is a quick procedure. However, preparation of conventional electroporation-competent cells requires hours of work involving several washes, incubations, and centrifugations. In contrast, competent cell preparation for the heat-shock method is short, but transformation requires approximately 2 h (4). The heat-shock procedure gives approximately 100-fold lower transformation efficiency than electroporation with plasmids containing auxotrophic marker genes such as *HIS4*. Additionally, the selection of zeocin-resistant transformants using the heat-shock transformation protocol does not work reliably.

We have modified the preparation of competent cells from the heat-shock procedure (5) and combined it with transformation by electroporation (3) to yield a condensed protocol that works consistently with auxotrophic markers or antibiotic selection. The main modification of the heat-shock procedure is the addition of a step in which *P. pastoris* cells are incubated in an optimized concentration of dithiothreitol (DTT). The cells prepared by this "hybrid" method are then electro-

Table 1. Comparison of Transformation Protocols

Electroporation (3)	Heat Shock (5)	Condensed
Cell Preparation (2 h 45 min) a. Grow 500 mL cells to desired A_{600} b. Centrifuge cells[a] for 10 min c. Add 100 mL YPD/0.02 M HEPES d. Add 2.5 mL 1.0 M dithiothreitol (DTT) dropwise e. Incubate for 15 min with shaking at 30°C f. Add water to 500 mL g. Centrifuge cells for 10 min h. Resuspend in 500 mL water i. Centrifuge cells for 10 min j. Resuspend in 250 mL water k. Centrifuge cells for 10 min l. Resuspend in 20 mL 1.0 M sorbitol m. Pellet by centrifugation for 10 min n. Resuspend in 1 mL 1.0 M sorbitol o. Aliquot into individual 1.5 mL tubes p. Place in -80°C freezer until needed Transformation (15 min) a. Mix DNA with cells in cuvette b. Incubate for 2 min on ice c. Pulse d. Add recovery medium (optional incubation for 1–3 h) e. Plate	Cell Preparation (25 min) a. Grow 10 mL cells to desired A_{600} b. Centrifuge cells[b] for 5 min c. Resuspend in 10 mL BEDS solution d. Centrifuge cells for 5 min e. Resuspend cells in 1 mL BEDS f. Aliquot into individual 1.5 mL tubes g. Place in -80°C freezer until needed Transformation (1 h 45 min) a. Mix DNA with cells b. Add 1.4 mL 40% polyethylene glycol (PEG), 200 mM bicine, pH 8.3 c. Incubate for 60 min at 30°C d. Heat shock at 42°C for 10 min (optional recovery for 1–3 h) e. Pellet cells for 5 min f. Resuspend cells in 150 mM NaCl, 10 mM bicine, pH 8.3 g. Repeat steps e and f i. Plate	Cell Preparation (30 min) a. Grow 50 mL cells to desired A_{600} b. Centrifuge cells[b] for 5 min c. Resuspend in 9 mL BEDS + 1 mL 1.0 M dithiothreitol (DTT) d. Incubate for 5 min with shaking e. Centrifuge cells for 5 min f. Resuspend cells in 1 mL BEDS g. Aliquot into individual 1.5 mL tubes h. Place in -80°C freezer until needed Transformation (15 min) a. Mix DNA with cells in cuvette b. Incubate for 2 min on ice c. Pulse d. Add recovery medium (optional incubation for 1–3 h) e. Plate

BEDS solution is composed of 10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) dimethyl sulfoxide (DMSO), and 1 M sorbitol. YPD media is composed of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose.
[a]All centrifugation steps were at 4000× *g* at 4°C.
[b]All centrifugation steps were at 500× *g* at room temperature.

Table 2. Condensed Protocol: Preparation of Competent Cells and Transformation

1. Grow a 5-mL overnight culture of *Pichia pastoris* cells in YPD in a 30°C shaking incubator.
2. The next day, dilute the overnight culture to an A_{600} of 0.15–0.20 in a volume of 50 mL YPD in a flask large enough to provide good aeration. (Starting volumes can be scaled up or down.)
3. Grow yeast to an A_{600} of 0.8–1.0 in a 30°C shaking incubator. Based on a generation time of 100–120 min, yeast should reach 0.8–1.0 in 4 to 5 h.
4. Centrifuge the culture at 500× *g* for 5 min at room temperature and pour off the supernatant.
5. Resuspend the pellet in 9 mL of ice-cold BEDS solution [10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) (dimethyl sulfoxide) DMSO, and 1 M sorbitol] supplemented with 1 mL 1.0 M dithiothreitol (DTT). Note that various concentrations (0–200 mM) of DTT were tested, but the amount used in this procedure (100 mM) yielded the most transformants.
6. Incubate the cell suspension for 5 min at 100 rpm in the 30°C shaking incubator.
7. Centrifuge the culture again at 500× *g* for 5 min at room temperature and resuspend the cells in 1 mL (0.02 volumes) of BEDS solution without DTT. We have also found transformation efficiency may be increased by resuspending cells in smaller volumes (0.005–0.01 volumes) of BEDS solution.
8. The competent cells are now ready for transformation. Alternatively, freeze cells slowly in small aliquots at -80°C by placing the aliquots inside a styrofoam box. Competent cells can be stored for at least 6 months at this temperature.
9. Mix approximately 4 µL (50–100 ng) of linearized plasmid DNA with 40 µL of competent cells in an electroporation cuvette. Incubate for 2 min on ice.
10. Electroporate samples using the following parameters:
    (*i*) ECM® 630 electroporator (BTX, San Diego, CA, USA): cuvette gap, 2.0 mm; charging voltage, 1500 V; resistance, 200 Ω; capacitance, 50 µF.
    (*ii*) Gene Pulser® II electroporator (Bio-Rad Laboratories, Hercules, CA, USA): cuvette gap, 2.0 mm; charging voltage, 1500 V; resistance, 200 Ω; capacitance, 25 µF.
11. Immediately after electroporation, resuspend samples in 1 mL cold 1.0 M sorbitol and then plate on selective media (YNB, 2% dextrose + 1.0 M sorbitol) for auxotrophic strains. Alternatively, if using zeocin-based plasmids, resuspend samples in 0.5 mL 1.0 M sorbitol and 0.5 mL YPD, incubate in a 30°C shaker for 1 h, and then plate on media containing increasing concentrations of zeocin (100, 250, 500, or 1000 µg/mL) for the selection of multicopy integrants. Note that increased numbers of transformants can be achieved for both types of selectable markers by incubating the resuspended cells in a 30°C shaker for longer periods of time (1–3 h). However, this is partly due to replication of transformants.

YPD media is composed of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose. YNB media is composed of 0.17% (w/v) yeast nitrogen base without amino acids and 0.5% (w/v) ammonium sulfate.

Table 3. Transformation Efficiencies

	Typical Transformation Efficiency (transformants per microgram DNA)	
Method	**Auxotrophic Marker Selection**	**Zeocin Selection (100 µg/mL)**
Electroporation	1×10^5	4×10^4
Heat Shock	1×10^3	0–20
Condensed Protocol	$\mathbf{3.5 \times 10^4}$	$\mathbf{2 \times 10^3}$

porated using the same parameters as conventional electroporation.

Transformation efficiencies using the condensed protocol are comparable to the conventional electroporation procedure using auxotrophic markers but are approximately 20-fold lower using the zeocin resistance marker. However, the condensed protocol provides sufficient transformants, including multicopy integrants, for protein expression studies and has several advantages over the conventional electroporation and heat-shock methods. Table 1 compares the steps in cell preparation and transformation for conventional electroporation, heat shock, and our condensed protocol. Compared to the heat-shock method, the condensed protocol requires less time for the transformation step and provides much higher transformation efficiencies. Compared to the electroporation procedure, the new procedure saves both reagents and time during cell preparation. In addition, the fewer number of steps during the cell preparation of the condensed protocol reduce the chance of contamination of competent yeast cells. Furthermore, unlike the electroporation cell preparation procedure, our condensed protocol does not require a large, refrigerated centrifuge. We use a small, nonrefrigerated centrifuge capable of spinning six 50-mL conical tubes at a time, enabling us to quickly prepare competent cells of six different strains simultaneously. This is significant because some *P. pastoris* strains (i.e., protease deficient, methanol utilization deficient) can express a given protein more efficiently than others, and it is often necessary to transform the same expression plasmid into various strains to determine empirically which strain gives the highest expression. Thus, the condensed protocol (Table 2) enables a researcher to prepare and transform multiple samples of highly competent *P. pastoris* cells in a short time with minimal equipment or effort.

Using *HIS4*-based and zeocin-based plasmids based on typical *P. pastoris* cloning vectors, pHILA1 and pPICZB (Invitrogen, Carlsbad, CA, USA), and strains such as JC100 (wild-type) or GS115 (*his4*), we obtained the results summarized in Table 3. These results are average transformation efficiencies calculated from at least five separate transformations.

The condensed protocol utilizes the most efficient portions of the electroporation and heat-shock transformation protocols to yield a procedure for *P. pastoris* cells that produces high transformation efficiencies while saving time, effort, and reagents.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Brief heat shock increases stable integration of lipid-mediated DNA transfections

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Lipid mediated gene transfer (lipofection) has been widely used to transfer genes into various cell types (1–4). Lipofection works very well in many cell lines, resulting in high transient transfection efficiencies (our observations). However, the rate of DNA integration into the genome following lipid-mediated transfection is relatively low (5) as compared to other methods, such as retroviral systems. This inefficient integration has been thought to be a major disadvantage of plasmid vectors and has limited their use in gene therapy trials.

We have attempted to overcome this hurdle by achieving higher rates of stable integrants in lipid-mediated transfections through treating the transfected cells with a mild heat shock. Others have attempted to increase stable integration by methods such as γ-irradiation (5), but the required doses resulted in 90% cell death. DNA damaging agents like hydrogen peroxide have also been used to increase stable integration of plasmid DNA. Doses of hydrogen peroxide that caused a significant effect on integration also caused 90% cell death (6). Treatment of cells with glycerol (7), dimethylsulfoxide (8), choroquine (9), and cell synchronization to the late G2/M phase of the cell cycle (10) enhances transfer of DNA into the cytoplasm and subsequently, incorporation into the nucleus. Our method uses a heat treatment of 10 min at 42°C immediately following lipid transfections, which results in up to a 100% increase in stable integrants assayed as colonies resistant to G418 antibiotic. Transient transfection efficiencies, monitored by flow cytometry using the enhanced green fluorescent protein (EGFP), were also found to be increased by a brief heat treatment.

A human lung carcinoma cell line (A549), human colon carcinoma cell line (SW480), human breast carcinoma cell line (MCF-7), and two murine cell lines, B-16 (melanoma cell line) and 4T1 (mammary tumor cell line), were used in the experiments. The cells were plated in 60-mm^2 tissue culture dishes (BD, Franklin Lakes, NJ, USA) at a density of 0.5×10^6 cells/dish in RPMI media, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin/streptomycin, and 1 mM sodium pyruvate (all from Invitrogen, Carlsbad, CA, USA). The cells were incubated overnight at 37°C in a 5% CO_2 incubator. The cells were transfected with the plasmid pCMV-EGFP-1, which was created by inserting the human cytomegalovirus (CMV) promoter into the multiple cloning site of pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA, USA). The neomycin gene in pCMV-EGFP-1 is under the control of the simian virus 40 (SV40) promoter. Transfections were performed using the lipid DMRIE-C (Invitrogen) at a ratio of 1:4 µg DNA:µL lipid. One microgram DNA was diluted in 500 µL reduced serum Opti-MEM® (Invitrogen), and 4 µL DMRIE-C were diluted in 500 µL Opti-MEM in another tube. The two tubes were mixed and incubated for 30–45 min at room temperature. Before the addition of the transfection mixture, the cells were washed twice in Opti-MEM. Four hours after transfection, the tissue culture dish was covered with Parafilm® and fully immersed in a water bath maintained at 42°C for 10–30 min as specified. After removal from the water bath, the outside of the plate was sterilized with 70% alcohol, and the transfection media was replaced with fresh supplemented RPMI media. Controls were handled similarly, but without any heat treatment. Cells