Pichia Spheroplast Kit

A Manual for the Preparation and Transformation of *Pichia pastoris* Spheroplasts

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Table of Contents

Important Information

Kit Contents

The *Pichia* Sphreoplast Kit contains 2 boxes. Store each box as detailed below:

Store Box 1 at +4°C.
Store Box 2 at -20°C.

Box 1 Reagents

Store Box 1 at +4°C.

Item	Components	Amount
SOS media	1 M Sorbitol, 0.3X YPD, 10 mM $CaCl_2$	20 ml
Sterile Water	--	2 x 125 ml
SE	1 M Sorbitol; 25 mM EDTA, pH 8.0	2 x 125 ml
SCE	1 M Sorbitol; 1 mM EDTA; 10 mM sodium citrate buffer, pH 5.8	2 x 125 ml
1 M Sorbitol	--	2 x 125 ml
CaS	1 M Sorbitol; 10 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$	2 x 60 ml
40% PEG	40% (w/v) PEG 3350 (Fisher Scientific grade) in water	25 ml
CaT	20 mM Tris-HCl, pH 7.5; 20 mM $CaCl_2$	25 ml

Box 2 Reagents

Store Box 2 at -20°C.

Item	Components	Amount
Zymolyase	3 mg/ml Zymolyase in water	10 x 20 µl
1 M DTT	1 M dithiothreitol in water	10 x 1 ml

Materials Supplied by User

You will need the following reagents and equipment.

- 30°C rotary shaking incubator
- Water baths capable of 37°C and 45°C
- Centrifuge suitable for 50 ml conical tubes (floor or table-top)
- Culture flask (500 ml)
- Conical tubes, 50 ml, sterile
- Snap-top tubes, 6 ml and 15 ml, sterile (Falcon 2059 or similar)
- UV-VIS Spectrophotometer
- Mini agarose gel apparatus
- Media for transformation, growth, screening and expression
- 5% SDS solution (10 ml required per transformation)

Methods

Overview

Introduction

The yeast *Pichia pastoris* is a microbial eukaryote which has been developed into a recombinant protein expression system. To express recombinant proteins in *Pichia pastoris*, a *Pichia* expression vector containing the gene of interest must be introduced into the host. Following introduction, the gene of interest is integrated into the host genome by a method known as gene replacement. Spheroplast transformation is an efficient method for introducing DNA into *Pichia pastoris* cells. The *Pichia* Spheroplast Kit was designed for the production of *Pichia pastoris* spheroplasts for subsequent transformation with recombinant vector constructs.

Explanation of Spheroplasting

Yeast cells have a cell wall which limits the efficiency of transformation using chemical techniques common to bacterial cells. To enable the uptake of foreign DNA into the *Pichia pastoris* cells it is necessary to partially remove the cell wall. This is done by treating the cells with the enzyme zymolyase and the reducing agent DTT. Zymolyase is a β-glucanase that hydrolyzes glucose polymers with β-1,3 linkages. Enzyme treatment is followed by several isotonic washes to remove the enzyme. To transform the cells, DNA is incubated with the spheroplasts. To facilitate cell wall regeneration a solution containing polyethylene glycol (PEG) is added followed by a 10 minute incubation. Following removal of the PEG, the cells are resuspended in an isotonic regeneration media and plated in top agar. The efficiency of *Pichia pastoris* transformation by the spheroplast method may vary, though it is typically in the range of 10^3 to 10^4 colonies per microgram of DNA.

The *Pichia* Spheroplast Kit

The *Pichia* Spheroplast Kit contains buffers, media and reagents required for 10 spheroplast preparations. Each preparation of spheroplasts can be used for up to 5 transformations. The formation of spheroplasts is a carefully controlled procedure. All of the components in the kit have been tested to ensure the production of 70% spheroplasts in less than 30 minutes of zymolyase treatment. In addition, reagents, stock solutions, and final solutions are tested for sterility.

Guidelines for Growing *P. pastoris*

The growth temperature for *P. pastoris* is 30°C for liquid cultures, plates and slants. Log phase cultures of *Pichia pastoris* have a doubling time of approximately 2 hours in YPD media. When methanol utilization plus (Mut^+) recombinant *Pichia* strains are grown on minimal methanol (MM) plates or in liquid MM media, it is advisable to add methanol every two days to compensate for methanol lost from the medium due to evaporation. For plates, add 100 µl of 100% methanol to the lid of the inverted plate. For liquid medium, add methanol directly to the liquid to a final concentration of 0.5%.

Guidelines for Storing *P. pastoris*

For medium-term storage of cells, use YPD medium or YPD agar slants. The cells can be stored in such media for several months at +4°C. For permanent storage, grow an overnight culture in YPD media. Add glycerol to the culture to a final concentration of 15%. Store the culture at -80°C.

Preparing the Transforming DNA and *Pichia* Cells

Introduction

This section provides guidelines for preparing your transforming DNA and *Pichia* cells for the transformation procedure.

Preparing the Plasmid DNA

Your gene of interest should be cloned into a *Pichia* expression vector and the correct construction confirmed by sequencing. The DNA needed to generate His^+ *Pichia* transformants does not need to be ultra-pure, but must be pure enough to be successfully digested with the appropriate restriction enzyme prior to transformation. Cleaner DNA will result in better transformation efficiencies. Additional guidelines are provided below for preparing your transforming DNA.

Digesting the Transforming DNA

Digest *Pichia* constructions with the appropriate enzyme to yield His^+ Mut^- (typically *Not* I or *Bgl* II) or His^+ Mut^+ (typically *Sal* I or *Stu* I) transformants.

The digested plasmid DNA can be generated ahead of time and stored frozen. It is not necessary to purify the portion of the plasmid containing the *AOX1* sequences and the gene of interest away from the rest of the plasmid prior to transformation.

We recommend that the success of the digestion of the plasmid be confirmed by agarose gel electrophoresis. Complete digestion is not critical, however, the number of His^+ transformants obtained will be reduced if the DNA is not completely digested.

We also recommend that you include a plasmid-only (no insert) negative control in your transformation experiment to determine the protein background during the SDS-PAGE analysis.

Media

Prepare the following media several days in advance and store at +4°C (see **Recipes**, pages 7-9 for details).

- YPD (Yeast extract Peptone Dextrose) medium, 1 liter
- YPD plates, 1 liter
- RDB (Regeneration Dextrose Base) plates, 1 liter
- RDHB (Regeneration Dextrose Histidine Base) plates, 1 liter

Prepare the following solution on the day of transformation and maintain at 45°C.

- 5% SDS solution in water
- RD (Regeneration Dextrose), molten agarose, 100 ml

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Preparing the Transforming DNA and *Pichia* Cells, continued

Growing the *Pichia* Cells

1. Streak *Pichia* strain onto a YPD plate such that isolated, single colonies will grow. Incubate the plate at 30°C for 2 days.
2. Inoculate 10 ml of YPD in a 50 ml conical tube or a 100 ml flask with a single colony of *Pichia* cells from the YPD plate and grow overnight at 30°C with vigorous shaking (> 200 rpm). This culture will be used as the inoculum in Step 3 and may be stored at +4°C for 3-4 days.
3. Place 200 ml of YPD in each of three 500 ml culture flasks. Inoculate the flasks with 5, 10, and 20 µl of cells from Step 2 and incubate them overnight with vigorous shaking at 30°C.
4. The next morning, equilibrate the transformation solutions (SE, SCE, Sterile Water, SOS, PEG, CaS, CaT, 1 M sorbitol) provided in the kit, the RDB plates (for plating transformants), and the RDHB plates (for viability control) by bringing them to room temperature.
5. Check the OD_{600} of each of the three culture flasks. Harvest the cells from the culture which has an OD_{600} between 0.2 and 0.3 (the optical density of the cells is important for the successful formation of spheroplasts). The cells are harvested by centrifugation at room temperature for 5-10 minutes at 1500 x g. Use these cells for preparing the spheroplasts for transformation. Decant the supernatant and discard the other cultures.

   Note: If the cells have grown beyond an OD_{600} of 0.3, dilute the culture with fresh media to an OD_{600} less than 0.1 and allow the cells to grow back up to the correct density. During log phase, the doubling time of *Pichia* cells is approximately 2 hours.

Preparing Spheroplasts

Before Starting

You should have a cell pellet from Step 5, previous page.

- Prepare 100 ml of molten RD agarose and keep at 45°C (see **Recipes**, page 9)
- Thaw one tube of 1 M DTT (provided)
- Prepare fresh SED for one batch of spheroplasts as follows:

  Using sterile technique, transfer 19 ml of SE (provided) to an appropriate sterile container (*e.g.* 50 ml conical tube). Add 1 ml of 1 M DTT and mix well. For best results this solution of SED should be made and used immediately.

Important

The quality and freshness of DTT is critical for a successful spheroplast preparation. The 1 M DTT provided is analytical reagent grade and must be stored at -20°C.

Washing the Cells

1. Wash the cells from Step 5, previous page by resuspending the pellet in 20 ml of sterile water (provided). Resuspend the pellet by swirling the tube. Transfer to a sterile, 50 ml conical tube.
2. Pellet the cells by centrifugation at 1500 x g for 5 minutes at room temperature. Decant and discard the supernatant. Use the cell pellet to prepare spheroplasts.
3. Wash the cell pellet once by resuspending in 20 ml of fresh SED, prepared above and centrifuge at 1500 x g for 5 minutes at room temperature.
4. Wash the cells once with 20 ml of 1 M sorbitol and centrifuge as described in Step 2.
5. Resuspend the cells by swirling in 20 ml of SCE buffer and divide the suspension into two 50 ml conical tubes (~10 ml each).
6. Remove one tube of Zymolyase from -20°C and place it on ice. Mix well by flicking the tube several times. Zymolyase is provided as a slurry and does not go into solution. It is important to mix the slurry thoroughly before each use to ensure addition of a consistent amount of Zymolyase.

Adding Zymolyase

You will use one tube of cells prepared above to determine the optimal time of digestion with Zymolyase to make spheroplasts. Once the optimal time is determined, use the other tube of cells to make spheroplasts.

Zymolyase digests the cell wall and makes the cells extremely fragile. Handle the sample **gently**. The moment after addition of Zymolyase, digestion of the cell wall begins.

- Prepare at least 20 ml of a 5% SDS solution (not provided) for.
- Set your UV-Vis spectrophotometer to 800 nm and blank with 800 µl 5% SDS and 200 µl SCE.
- Set up 17 sterile microcentrifuge tubes and label them 0, 2, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50. Add 800 µl of 5% SDS to each tube.

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Preparing Spheroplasts, continued

Adding Zymolyase, continued

1. From one tube of cells (Step 5, previous spage), withdraw 200 µl cells and add to the tube marked "0". This is your zero time point. Set the tube aside on ice.
2. Add 7.5 µl of Zymolyase to the same tube of cells, mix gently by inversion, and incubate the cells at 30°C. **Do not shake the sample.** This sample will be used to establish the incubation time for optimal spheroplasting. Keep the second tube of cells at room temperature for use in Step 6. Keep the remaining Zymolyase on ice.
3. Monitor the formation of the spheroplasts as follows: At time 2 minutes, withdraw 200 µl of cells (from the suspension in Step 2) and add to the tube marked "2". Repeat at time t= 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 minutes after adding Zymolyase. Read the OD_{800} for all samples.
4. Determine the percent of spheroplasting for each time point using the equation:

   % Spheroplasting = 100 - [(OD_{800} at time t/OD_{800} at time 0) x 100]

   For example: At time t = 0, the OD_{800} = 0.256
   At time t = 15, the OD_{800} = 0.032

   Calculation: % spheroplasting = 100-[(0.032/0.256) x 100]
   = 100 - [(0.125)x100]
   = 100 - 12.5
   = 87.5%
5. Determine the time of incubation that results in approximately 70% spheroplasting. This time of incubation is variable due to differences in lots of Zymolyase. In Invitrogen labs, it takes approximately 15-40 minutes of Zymolyase treatment to achieve optimal spheroplasting.

   Note: It is important to establish the minimum time required for the desired amount of spheroplasting. Prolonged incubation with Zymolyase is deleterious to spheroplasts and will result in lower transformation efficiency.
6. Add 7.5 µl Zymolyase to the remaining tube of cells as described in Step 1. Incubate the tube at 30°C for the time that was established in Step 5 to obtain the optimal level (70%) of spheroplasting.
7. Harvest the spheroplasts by centrifugation at 750 x g for 10 minutes at room temperature. Decant and discard the supernatant.
8. Wash the spheroplasts once with 10 ml of 1 M sorbitol (**gently** disperse the pellet by tapping the tube, **do not vortex**). Collect the spheroplasts by centrifugation at 750 x g for 10 minutes at room temperature.
9. Wash the spheroplasts once with 10 ml of CaS and centrifuge as in Step 7. **Gently** resuspend the spheroplasts in 0.6 ml of CaS. The spheroplasts must be used immediately (up to 30 minutes) for transformation. They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations.

Transformation

Before Starting

Make sure your RDB plates are at room temperature and that you have molten RD top agarose available. Thaw your linearized DNA and keep on ice.

Procedure

1. For each transformation, dispense 100 µl of the spheroplast preparation from Step 9 (previous page) into a sterile 15 ml snap-top Falcon 2059 tube (or equivalent).
2. Add 10 µg of DNA and incubate the tube at room temperature for 10 minutes.
3. During the 10 minute incubation, make a fresh PEG/CaT solution. Since each transformation requires 1.0 ml of the PEG/CaT solution, calculate the amount you need and prepare this volume by adding together equal volumes of 40% PEG and CaT (a 1:1 solution).
4. Add 1.0 ml of fresh PEG/CaT solution to the cells and DNA, mix gently, and incubate at room temperature for 10 minutes.
5. Centrifuge the tube at 750 x g for 10 minutes at room temperature and carefully aspirate the PEG/CaT solution. Invert the tube and tap it gently to drain the excess PEG/CaT solution.
6. Resuspend the pellet of transformed cells in 150 µl of SOS medium and incubate it at room temperature for 20 minutes.
7. Add 850 µl of 1 M sorbitol. Proceed to **Plating**, below.

Plating

Pichia spheroplasts need to be plated in top agarose or agar to protect them from lysis prior to selection.

1. Mix together 100-300 µl of each spheroplast-DNA solution from Step 7, above with 10 ml of molten RD agarose and pour on RDB plates. Allow the top agarose to harden. Note there is enough of the spheroplast-DNA solution to plate duplicate and triplicate plates.
2. Invert plates and incubate at 28-30°C. Transformants should appear in 4-6 days.
3. For cell viability: Mix 100 µl of spheroplasts with 900 µl of 1 M sorbitol.
4. Mix 100 µl of this diluted sample with 10 ml of molten RDH and pour on a RDHB plate. Allow top agarose to harden.
5. Invert plates and incubate at 28-30°C. Appearance of colonies after 4-6 days demonstrates that the spheroplasts can regenerate into dividing cells.

Appendix

Recipes

Stock Solutions

10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilize. Heat the solution to dissolve YNB completely. Store at +4°C. The shelf life of this solution is one year.

500X B (0.02% Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at +4°C. The shelf life of this solution is approximately one year.

100X H (0.4% Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water. Heat the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilize and store at +4°C. The shelf life of this solution is approximately one year.

10X D (20% Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

10X GY (10% Glycerol)

Mix 100 ml of glycerol with 900 ml of water. Sterilize either by filtering or autoclaving. Store at room temperature. The shelf life of this solution is greater than one year.

100X AA (0.5% of each Amino Acid)

Dissolve 500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine in 100 ml of water. Filter sterilize and store at +4°C. The shelf life of this solution is approximately one year.

YPD or YEPD

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)

1. Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. **Note**: Add 20 g of agar if making YPD slants or plates.
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 ml of 10X D.

Store the liquid medium at room temperature. Store YPD slants or plates at +4°C. The shelf life is several months.

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Recipes, continued

MGY and MGYH

Minimal Glycerol Medium ± Histidine (1 liter)

1.34% YNB
1% glycerol
4 x 10^{-5}% biotin
± 0.004% histidine

1. Combine aseptically 800 ml autoclaved water with 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X GY.
2. For growth of *his4* strains in this medium, a version can be made that contains histidine (called MGYH) by adding 10 ml of 100X H stock solution.

Store at +4°C. The shelf life of this solution is approximately two months.

RD and RDH Liquid Media

Regeneration Dextrose Medium ± Histidine (1 liter)

1 M sorbitol
2% dextrose
1.34% YNB
4 x 10^{-5}% biotin
0.005% amino acids
± 0.004% histidine

1. Dissolve 186 g of sorbitol in 700 ml of water and proceed to Step 2.
2. Autoclave 20 minutes on liquid cycle.
3. Cool and maintain the liquid medium in a 45°C water bath.
4. Prepare a prewarmed (45°C) mixture of the following stock solutions:

   100 ml of 10X D
   100 ml of 10X YNB
   2 ml of 500X B
   10 ml of 100X AA
   88 ml of sterile water

   Add to sorbitol solution.
5. For growth of *his4* strains you must add histidine to the media. Add 10 ml of 100X H (histidine) to the prewarmed mixture in Step 4. Store liquid medium at +4°C.

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Recipes, continued

RDB and RDHB Agar Plates

1. Dissolve 186 g of sorbitol in 700 ml of water and add 20 g of agar.
2. Autoclave 20 minutes on liquid cycle.
3. Place the autoclaved solution in a 60°C water bath prior to addition of prewarmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the prewarmed (45°C) mixture from **RD and RDH Liquid Media**, Step 4, previous page. Add to sorbitol/agar solution. **If you are selecting for His^+ transformants, do not add histidine.**
5. Pour the plates immediately after mixing the solutions in Step 4. The plates should be stored at +4°C and should last for several months.

RD and RDH Top Agar

1. Dissolve 186 g of sorbitol in 700 ml of water and add 10 g of agar or agarose.
2. Autoclave 20 minutes on liquid cycle.
3. Place the autoclaved solution in a 60°C water bath prior to addition of prewarmed mixture of stock solutions. This will keep the medium from becoming too thick to mix reagents.
4. Prepare the prewarmed (45°C) mixture from **RD and RDH Liquid Media,** Step 4, previous page. Add to sorbitol/agar solution. **If you are selecting for His^+ transformants, do not add histidine.**
5. Place the solution to 45°C after adding the solutions in Step 4. During transformation, use as a molten solution at 45°C.

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