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A rapid and simple procedure for high-efficiency lithium acetate transformation of cryopreserved *Schizosaccharomyces pombe* cells

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Abstract

A rapid, simple, convenient, and highly efficient transformation of the fission yeast *Schizosaccharomyces pombe* has been developed. Freezing fission yeast cells in glycerol, a permeating cryoprotectant, with lithium acetate improved remarkably the transformation efficiency by one to two orders of magnitude. The optimum concentration of glycerol was found to be 30%, which is higher than that (10–15%) in the conventional cryopreservation of yeast cells. Glycerol not only played a role in cryopreserving the competent cells but also improved the transformation efficiency of the process. The thawed cell suspension with glycerol and lithium acetate was immediately mixed with carrier DNA, plasmid DNA and polyethylene glycol. Next, the mixture was heat shocked and directly spread on a selection plate. This simple procedure yielded more than 10^6 transformants/μg plasmid DNA, reducing the time required to only 20 min in total, including the thawing time. Furthermore, the frozen competent cells were stored long-term for more than 3 months without any significant loss of efficiency. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: ***Schizosaccharomyces pombe*; transformation; cryopreservation; glycerol**

Introduction

The fission yeast *Schizosaccharomyces pombe* resembles higher eukaryotes in the structure and function of its genes and proteins. The possibility of cloning mammalian genes based on the complementation of *Sz. pombe* mutants has been demonstrated (Lee and Nurse, 1987), making *Sz. pombe* an attractive model of eukaryotic cells for molecular genetic studies.

The transformation of *Sz. pombe* mainly involves two methods of introducing foreign DNA into cells: electroporation and lithium acetate (LiAc) methods. Although electroporation methods are efficient ($10^5 - 10^7$ transformants/μg plasmid DNA), simple and rapid, an electro-pulse generator is required (Prentice, 1992; Hood and Stachow, 1990; Ishiguro and Kobayashi, 1995; Suga and Hatakeyama, 2001; Suga *et al*., 2004). On the other hand, LiAc methods yield $10^4 - 10^5$/μg cheaply and require no special equipment, but they are laborious and rather time-consuming (3–4 h) because of the multiple-step procedures involved (Bröker, 1987; Okazaki *et al*., 1990; Chua *et al*., 2000). Therefore, a simple LiAc method that optimizes and partly omits multiple-step procedures was developed (Kanter-Smoler *et al*., 1994). Colonies grown on agar plates were directly used in another LiAc method for situations not requiring a high-efficiency transformation (Elble, 1992; Morita and Takegawa, 2004). In addition, there have been many attempts to maximize the convenience and rapidity of cryopreserving competent cells, because of both the need to store cells for a long time and the desire to not have to prepare fresh cells for each transformation (Dohmen *et al*., 1991; Jimenez, 1991;

Bröker, 1993; Zhao *et al*., 1993; Akada *et al*., 2000; Suga *et al*., 2000; Suga and Hatakeyama, 2003). Therefore, it is useful to cryopreserve highly competent cells for transformation in routine work.

In this paper, we describe a rapid and simple LiAc method for fission yeast that greatly increases transformation efficiency after freezing and thawing through the addition of LiAc and the use of a simple freezing process in glycerol, a permeating cryoprotectant.

Materials and methods

Yeast strains and plasmids

The *Sz. pombe* strains ATCC38399 (*h*$^-$ *leu1-32*), ATCC38436 (*h*$^-$ *ura4-294*) and TK107 (*h*$^-$ *phh1::ura4*$^+$ *ura4-D18 leu1-32*) were used as the recipient for plasmids pAL7 and pAU5 (Okazaki *et al*., 1990; Kato *et al*., 1996). Plasmids were isolated and purified using the QIAGEN Plasmid Mini Kit (Qiagen).

Cryopreservation of competent cells

This lithium acetate protocol for *Sz. pombe* was based on the methods previously described by Okazaki *et al*. (1990) and Kanter-Smoler *et al*. (1994). *Sz. pombe* cells were grown in SLD medium with a low carbon source (0.67% Bacto yeast nitrogen base, 0.5% glucose) supplemented with 150 μg/ml of leucine (for *leu*$^-$) or uracil (for *ura*$^-$) to a density of approximately 1×10^7 cells/ml at 30 °C. The culture was placed on ice for 15 min just before harvesting. The cells were collected by centrifugation at $1600 \times g$ for 5 min, and the resulting pellet was washed three times with ice-cold sterilized water. This pellet was suspended in ice-cold glycerol solution with 0.1 M lithium acetate (LiAc), pH 4.9, to give 10^9 cells/ml. Aliquots of 50 μl of the cell suspension were dispensed into 1.5 ml microcentrifuge tubes. The aliquots were placed on ice for 30 min to let the glycerol permeate the cells, and then frozen and stored by placing them directly into a −80 °C freezer.

Transformation with cryopreserved competent cells

For each transformation, the frozen competent cells were quickly thawed in a water bath at 30 °C for 2 min. The thawed cell suspension was mixed with 5–10 ng purified plasmid DNA and 145 μl polyethylene glycol (PEG) solution (50% PEG-4000), and then the mixture was vortex-mixed and incubated at 30 °C for 60 min. The cell suspension was heat-shocked at 43 °C for 15 min and allowed to cool at room temperature for 10 min. The cells were collected by a brief centrifugation ($1600 \times g$, 3 min) and resuspended in 500 μl 1/2YE medium (0.25% yeast extract, 1.5% glucose) supplemented with 30 μg/ml leucine or uracil and incubated at 30 °C for 60 min while being shaken. The cell suspension was spread directly onto minimal selection plates (0.67% yeast nitrogen base without amino acids, 2% glucose and 2% agar). Transformant colonies appeared in 4–6 days at 30 °C.

Results and discussion

Effect of cryoprotectant on transformation efficiency with cryopreservation

The freezing step is important for maintaining the competence of cells in the transformation procedure, since LiAc methods are multi-step processes. We modified Okazaki *et al*.'s (1990) LiAc protocol for *Sz. pombe* and also froze the competent cells at various steps of these procedures. First, a 60-min pre-incubation procedure with LiAc before the addition of PEG is often omitted in simplified LiAc methods (Kanter-Smoler *et al*., 1994; Akada *et al*., 2000; Morita and Takegawa, 2004). We found that this pre-incubation was not effective in our procedure and rarely led to a decrease in the transformation efficiency (data not shown). In this study, alternatively, 0.1 M LiAc (pH 4.9) was added to cryoprotectant and frozen with the cells. Thus, the freezing step was positioned just after washing the cells with water and before the addition of PEG.

Figure 1 shows the effects of various concentrations of cryoprotectants on the transformation efficiency of *Sz. pombe* (*leu*$^-$) following freezing and thawing, within the range of 5–40% glycerol or dimethylsulphoxide (DMSO) tested. A transformation efficiency of $3.5 \pm 2.1 \times 10^4$ transformants/μg DNA was routinely achieved without cryopreservation process. The transformation efficiency of cells frozen in glycerol increased greatly when the concentration of glycerol was increased to 30%. Freezing in 30% glycerol increased the

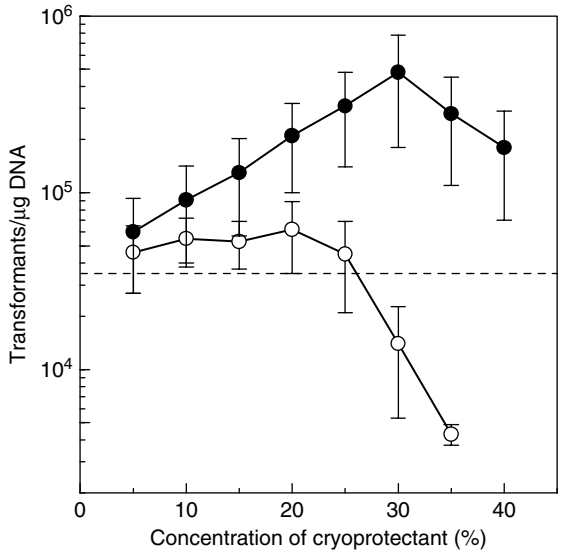

Figure 1. Effect of concentration of cryoprotectants on the transformation efficiency of *Sz. pombe* (*leu*$^-$) with cryopreservation. (●) glycerol; (○) DMSO. The broken line shows the transformation efficiency without cryopreservation; 5 ng pAL7 was used for each transformation. Data represent the average values ±SD of five independent experiments

efficiency more than 14-fold compared to that in control cells without cryopreservation. On the other hand, the efficiency in DMSO increased slightly at 10–20% concentration, but efficiency dropped markedly at concentrations above 25%. Similar to the phenomenon observed in *Sz. pombe* (*ura*$^-$), the cells frozen in 30% glycerol resulted in an approximately 220-fold increase in efficiency, reaching a maximum value of $2.6 \pm 1.3 \times 10^6/\mu g$. However, in the osmosensitive mutant strain, *Sz. pombe* TK107 (Δ*phh1*), freezing in more than 20% glycerol decreased the efficiency because of hyperosmotic stress (data not shown). Further, no improvement in efficiency with cryopreservation was observed in budding yeast, *Saccharomyces cerevisiae* (data not shown).

In general, permeating cryoprotectants are commonly used in 10–15% glycerol and 5% DMSO for the cryopreservation of competent yeast cells in LiAc methods (Ju and Warner, 1991; Bröker, 1993; Tan *et al*., 1998; Akada *et al*., 2000). In this study, a higher concentration of glycerol (30%) was suitable for the cryopreservation of highly competent *Sz. pombe* cells, except for the osmosensitive strain. This cryopreservation method was more efficient than previously described methods (Dohmen *et al*., 1991; Bröker, 1993; Akada *et al*., 2000).

Effect of cryoprotectant on transformation efficiency without cryopreservation

The addition of DMSO to a final concentration of 5–10% prior to a heat-shock step increases the transformation efficiency of *S. cerevisiae* (Hill *et al*., 1991; Soni *et al*., 1993). Further, the addition of glycerol improved the efficiency in *S. cerevisiae* cells picked up from colonies on plates, but the effect of glycerol on transformation efficiency was not intimately examined (Keszenman-Pereyra and Hieda, 1988). Thus, we investigated the effect of these permeating cryoprotectants on the transformation efficiency without freezing, as shown in Figure 2. Note that the concentrations of cryoprotectants indicated are not final concentrations, but those before the addition of PEG. *Sz. pombe* (*leu*$^-$) cells treated with 30% glycerol (final conc. 6%) increased the efficiency by approximately 10-fold compared to that without glycerol. The optimum concentrations of glycerol were similar with or without cryopreservation (Figures 1 and 2). On the

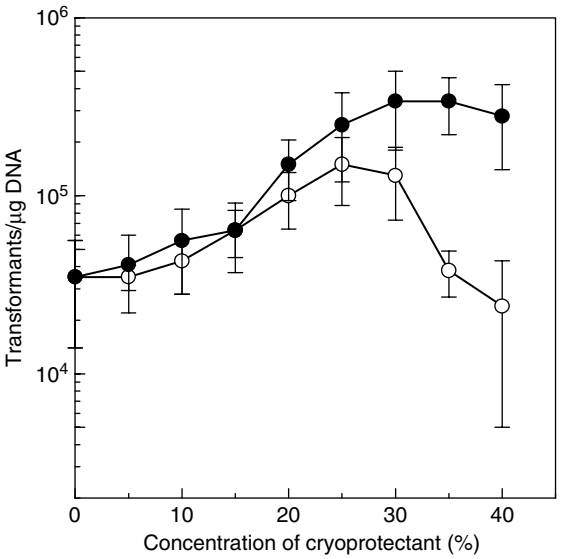

Figure 2. Effect of concentration of cryoprotectants on the transformation efficiency of *Sz. pombe* (*leu*$^-$) without cryopreservation. (●) glycerol; (○) DMSO. The indicated concentrations of cryoprotectants were before the addition of PEG; 5 ng pAL7 was used for each transformation. Data represent the average values ±SD of five independent experiments

other hand, 25% DMSO (final conc. 5%) increased the efficiency by approximately fourfold. This optimum concentration of DMSO for *Sz. pombe* was consistent with that previously reported for *S. cerevisiae* (Hill *et al*., 1991; Soni *et al*., 1993). These permeating agents have an ability not only to cryoprotect cells but also to improve transformation efficiency, and glycerol was a more effective agent for *Sz. pombe* cells. Thus, the thawed competent cells could be used directly for transformation without removing the glycerol because the presence of glycerol in the transformation mixture was important.

We found that electro-transformation of *Sz. pombe* was increased by pre-incubation with 20% glycerol for 30 min before an electric application (data not shown). Similarly, high osmotic stress of 2.0 M sorbitol or 1.5 M NaCl improves electro-transformation of *Sz. pombe* (Suga *et al*., 2003). Although glycerol is a permeating agent, it acts as a transiently osmotic stabilizer for a short period. Therefore, a high concentration of glycerol might improve LiAc transformation due to the high osmotic stress is causes.

Effect of other factors on transformation efficiency with cryopreservation

The growth medium is an important factor in both efficient transformation and cryopreservation, so we examined the effect of growth media on transformation efficiency with cryopreservation, as shown in Table 1. MB medium (with 0.5% glucose) is the optimum synthetic medium for a highly efficient LiAc method of *Sz. pombe* (Okazaki *et al*., 1990). Freezing cells grown in MB medium with 30% glycerol greatly increased the transformation efficiency by approximately 14-fold, which was similar to the increase obtained with SLD medium (with 0.5% glucose). On the other hand, the transformation efficiency increased by threefold when *Sz. pombe* cells grown in rich medium (YPD) were frozen with 10% glycerol (Bröker, 1993). However, freezing these cells with 30% glycerol had no increase in the efficiency (Table 1). In a simple LiAc method using cells picked up from colonies grown on an SD agar plate, the colonies could be stored at 4 °C for up to 1 week (Morita and Takegawa, 2004). Although freezing these cells with 30% glycerol did not increase the efficiency (Table 1), freezing with 15% glycerol slightly increased the efficiency by threefold (data not shown). These results show that it is suitable to grow the cells in synthetic liquid medium with a low glucose for cryopreservation of high-competency *Sz. pombe* cells.

LiAc was added to glycerol solution and frozen with the cells in this study. Further, we investigated the influence of cryopreservation without LiAc on transformation efficiency, as shown in Table 2. The cells were frozen in 30% glycerol alone, and then 0.1 M LiAc was added to a thawed cell suspension just after thawing. We found that the transformation efficiency decreased to about 20% compared to that obtained by freezing in glycerol with LiAc. This result suggests that to improve the efficiency after freezing, the presence of LiAc during freezing and thawing is essential.

The cooling and warming rates are also important factors in the cryopreservation of cells, because the formation of intracellular ice crystals or dehydration disrupts cell membranes (Mazur and Schmidt, 1968; Mazur, 1977). The cells were slowly frozen by being placed directly in a −80 °C

Table 1. Effect of growth media on the transformation efficiency of *Sz. pombe* (*leu*$^-$)

Growth medium	Transformants/μg DNA	
	None	Freezing with 30% glycerol
SLD (0.5% glucose)	$3.8 \pm 2.8 \times 10^4$	$5.2 \pm 3.2 \times 10^5$
MB (0.5% glucose)	$2.3 \pm 1.8 \times 10^4$	$3.2 \pm 1.5 \times 10^5$
YPD (2% glucose)	$2.0 \pm 1.2 \times 10^4$	$2.8 \pm 3.3 \times 10^4$
SD agar plate (2% glucose)	$5.6 \pm 5.0 \times 10^2$	$6.8 \pm 3.5 \times 10^2$

The cells in liquid media were grown to a density of 1×10^7 cells/ml at 30 °C. The cells on an SD plate (with 2% glucose, 2% agar) at 30 °C were grown for 3 days. The frozen cells were contained with 30% glycerol and 0.1 M LiAc; 5 ng pAL7 was used for each transformation. Data represent the average values ± SD of five independent experiments.

Table 2. Effect of the presence of LiAc during freezing and thawing on the transformation efficiency of *Sz. pombe* (*leu*$^-$)

Freezing agent	Transformants/μg DNA
30% glycerol	$8.8 \pm 4.5 \times 10^4$
30% glycerol + 0.1 M LiAc	$4.3 \pm 0.8 \times 10^5$

The cells were frozen in 30% glycerol with or without 0.1 M LiAc; 5 ng pAL7 was used for each transformation. Data represent the average values ±SD of five independent experiments.

Table 3. Effect of cryopreservation period at −80 °C on the transformation efficiency of *Sz. pombe* (*leu*⁻) frozen in 30% glycerol and 0.1 M LiAc

Cryopreservation period (days)	Transformants/μg DNA
7	1.6×10^5
14	1.5×10^5
21	2.0×10^5
28	2.3×10^5
56	2.2×10^5
84	2.0×10^5

Aliquots were cryopreserved simultaneously, and thawing and transformation were routinely carried out on a portion of the aliquots. The thawed cells incubated with PEG were diluted with TE buffer and spread directly onto plates; 5 ng pAL7 was used for each transformation. Data represent the average values ±SD of three experiments.

freezer. Thus, it was possible to freeze competent cells without an apparatus to control the cooling rate. The transformation efficiency increased when we increased the thawing temperature up to 40 °C, but efficiency decreased above 45 °C. The efficiency of rapid thawing at 40 °C was twice as high as that with slow thawing on ice. The procedure of slow freezing and rapid thawing was adequate for the cryopreservation of competent yeast cells. We investigated the storage stability of the cryopreserved competent cells in 30% glycerol and 0.1 M LiAc, as shown in Table 3. The frozen competent cells stored at −80 °C maintained high competency for more than 3 months without any loss of transformation efficiency.

Rapid and simple transformation with cryopreserved cells

Incubation with 1/2YE medium after removal of LiAc and PEG is often omitted in simplified LiAc protocols (Bröker, 1993; Kanter-Smoler *et al*., 1994; Akada *et al*., 2000; Morita and Takegawa, 2004). Actually, this omission of the incubation and the direct spread of the cell suspension contained with LiAc and PEG did not affect transformation efficiency (data not shown). Thus, there was no need to remove LiAc and PEG by centrifugation. As a result, a rapid and simple transformation with cryopreserved cells was possible. A thawed cell suspension (involving 30% glycerol and 0.1 M LiAc) was directly mixed with plasmid DNA and PEG solution, and the mixture was immediately heat-shocked. The cell suspension was then diluted with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and spread directly onto selection plates. This protocol of *Sz. pombe* (*leu*⁻) cells yielded an efficiency of $2.3 \pm 0.8 \times 10^5$/μg. Furthermore, the addition of 50 μg carrier DNA (salmon sperm DNA, sheared, boiled) to the thawed *Sz. pombe* cells increased the efficiency by about one order of magnitude. Finally, a maximum transformation efficiency of $2.1 \pm 0.7 \times 10^6$/μg was obtained using this rapid and simple procedure (Table 4), which had a higher yield than previously described LiAc methods (Okazaki *et al*., 1990; Kanter-Smoler *et al*., 1994). All manipulations can be done in only 20 min total, including the thawing time.

This method saves time and effort by eliminating the need for fresh cell preparation, and it helps to fulfil the demand for high efficiency, simplicity and rapidity in gene expression and function analyses.

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Table 4. Protocol of rapid and simple transformation with cryopreserved *Sz. pombe* cells

Cryopreservation of competent cells

1. Cells are grown in SLD medium (0.67% Bacto yeast nitrogen base, 0.5% glucose) supplemented with amino acid to a density of 1×10^7 cells/ml at 30 °C
2. The culture is placed on ice for 15 min just before harvesting. The cells are collected by centrifugation at 1600 × *g* for 5 min, and washed three times with ice-cold sterilized water
3. This pellet is suspended in ice-cold 30% glycerol and 0.1 M LiAc (pH 4.9) at 10^9 cells/ml. Then, 50 μl aliquots of the cell suspension are dispensed into 1.5 ml microcentrifuge tubes
4. The aliquots are placed on ice for 30 min, then frozen and stored by placing them directly into a −80 °C freezer

Transformation with cryopreserved competent cells

1. The frozen cells are quickly thawed in a water bath at 40 °C for 2 min
2. The thawed cell suspension is mixed directly with 5 μl carrier DNA (10 mg/ml), plasmid DNA and 145 μl 50% PEG-4000, and the mixture is immediately heat-shocked at 43 °C for 15 min
3. The cell suspension is diluted with TE buffer (pH 7.5) and spread directly onto a selection plate

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