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Yeast Functional Analysis Report

High efficiency transformation of *Schizosaccharomyces pombe* pretreated with thiol compounds by electroporation

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Abstract

A highly efficient method for transformation of the fission yeast *Schizosaccharomyces pombe* by electroporation has been developed. Significantly higher transformation efficiency was obtained when intact cells grown in SD medium (0.67% Bacto yeast nitrogen base without amino acids, 2% glucose) were pretreated with thiol compounds before an electric pulse was applied to the cells. Among the thiol compounds tested, dithiothreitol (DTT) was the most effective for pretreatment. A high transformation efficiency was obtained when the cells were pretreated with 25 mM DTT at 30°C for 15 min in an osmotically adjusted buffer, since the cells were sensitive to osmotic pressure. It was important to exclude glucose from the DTT pretreatment buffer, as it caused a drastic decrease in efficiency. The optimal cell concentration and amount of DNA during the electric pulse were 1×10^9 cells/ml and 10 ng, respectively. The maximum transformation efficiency, 1.2×10^7 transformants/μg plasmid DNA, was obtained when an electric pulse of 11.0 kV/cm was applied for 5 ms. Furthermore, the high competency of cells pretreated with DTT was maintained by freezing them in a non-permeating cryoprotectant such as sorbitol. **Copyright © 2001 John Wiley & Sons, Ltd.**

Keywords: ***Schizosaccharomyces pombe*; transformation; electroporation**

Introduction

The fission yeast *Schizosaccharomyces pombe* resembles higher eukaryotes in the structure and function of genes and proteins. The possibility of cloning mammalian genes based on the complementation of *Sz. pombe* mutants has thus been demonstrated (Lee and Nurse, 1987). For yeast transformation, several methods are used to introduce foreign DNA into cells, including lithium acetate methods (Ito *et al*., 1983; Bröker, 1987), electroporation methods (Hashimoto *et al*., 1985; Hood and Stachow, 1990), and spheroplast methods (Beach *et al*., 1982; Karube *et al*., 1985). One lithium acetate method for *Sz. pombe* is widely used and gives 10^5–10^6 transformants/μg plasmid DNA, but this method is also laborious and rather time-consuming because of the multiple-step procedures involved (Okazaki *et al*., 1990; Moreno *et al*., 1991; Alfa *et al*., 1993). On the other hand, electroporation for *Sz. pombe* has achieved similar efficiencies, along with several technical advantages over the lithium acetate method, including simplicity, rapidity and reproducibility (Prentice, 1992; Ishiguro and Kobayashi, 1995). In the lithium acetate and electroporation methods, macromolecular movement, such as DNA uptake of yeast, is trapped by the rigid cell wall. Although spheroplasts obtained by enzymatic digestion of the cell wall improve transformation efficiency, preparation and regeneration procedures are required (Karube *et al*., 1985; Stowers *et al*., 1995). Ito *et al*. (1984) found that *S. cerevisiae* cells treated with thiol compounds can uptake plasmid DNA. Thiol compounds such as dithiothreitol (DTT) and 2-mercaptoethylamine (2-MEA), used as cell-wall weakeners, have improved transformation efficiency by electroporation for *S. cerevisiae* (Meilhoc *et al*., 1990; Ganeva *et al*., 1994), *Schwanniomyces occidentalis* (Costaglioli *et al*., 1994), *Hansenula polymorpha* (Faber *et al*., 1994), *Kluyveromyces lactis* (Sánchez *et al*., 1993) and *Candida albicans* (Thompson *et al*., 1998; De Backer

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et al., 1999). Furthermore, although there have been many attempts to prepare frozen yeast cells pretreated with DTT for a simple and rapid procedure using permeating cryoprotectants such as glycerol and dimethyl sulphoxide (DMSO), drastic decreases in transformation efficiency are inevitable in such cases (Meilhoc *et al.*, 1990; Costaglioli *et al.*, 1994; Faber *et al.*, 1994). We reported previously that a high efficiency was maintained for a long period by freezing intact yeast cells in sorbitol, a non-permeating cryoprotectant (Suga *et al.*, 2000). In this paper, we describe a highly efficient method with a rapid procedure for *Sz. pombe* transformation by electroporation. This method gave up to 1.2×10^7 transformants/μg plasmid DNA by pretreating the intact cells grown in a synthetic medium with DTT. Moreover, the highly competent cells were cryopreserved without any loss of transformation efficiency.

Materials and methods

The *Sz. pombe* strains ATCC38399 (*h*$^-$ *leu1-32*) and ATCC38436 (*h*$^-$ *ura4-294*) were used as the recipients for plasmids pAL7 and pAU5, respectively (Okazaki *et al.*, 1990). The plasmids were isolated from *E. coli* hosts and were purified using Qiagen Plasmid Kits (Qiagen).

Yeast cells were grown in SD medium (0.67% Bacto yeast nitrogen base without amino acids, 2% glucose) supplemented with 150 μg/ml leucine (for *leu*$^-$) or uracil (for *ura*$^-$) to a density of approximately 1×10^7 cells/ml at 30°C. The cells were collected by centrifugation at $1600 \times g$ for 5 min. The pellet was then suspended in one-tenth the initial volume of DTT buffer (25 mM dithiothreitol, 0.6 M sorbitol, 20 mM Hepes, pH 7.5) and incubated at 30°C for 15 min. The cells were washed three times with ice-cold sterilized 1 M sorbitol by centrifugation, because an extremely low conductivity was necessary in order to avoid arcing. This pellet was suspended in ice-cold 50 μl 1 M sorbitol to a final cell density of approximately 10^9 cells/ml. The cell suspension was mixed with 0.5–10 ng purified plasmid DNA and then transferred to a chilled cuvette with a 0.2 cm electrode gap. A high electric pulse was applied to the cell suspension at 2.2 kV, 25 μF, 200 Ω, using the BioRad Gene Pulser II with Pulse Controller Plus. The electroporated cells were immediately diluted in 1 ml ice-cold 1 M sorbitol and a 0.1–0.2 ml aliquot was spread on minimal selection plates (0.67% Bacto yeast nitrogen base without amino acids, 2% glucose, 2% agar). Transformant colonies appeared in 4–6 days at 30°C.

The numbers of viable cells were counted by spreading the dilutions of cell suspension on YPD plates (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, 2% agar).

Results and discussion

Effect of thiol compounds on transformation efficiency

Thiol compounds, including DTT and 2-MEA, used as cell-wall weakeners, have dramatically increased transformation efficiency by electroporation for several yeast species (Meilhoc *et al.*, 1990; Sánchez *et al.*, 1993; Costaglioli *et al.*, 1994; Faber *et al.*, 1994; De Backer *et al.*, 1999). These disulphide-bond reducers are effective in improving transformation efficiency, since they increase cell-wall porosity (De Nobel *et al.*, 1989; Ganeva *et al.*, 1995). The effect of thiol compounds on the transformation of *Sz. pombe*, however, has not been reported until now. Table 1 shows the effect of various thiol compounds, including DTT, 2-MEA, 2-mercaptoethanol and 3-mercapto-1,2-propanediol, on the transformation efficiency of *Sz. pombe* (*leu*$^-$) at the optimum concentrations for 15 min incubation. Cell viability was not affected under these conditions. The optimum concentration for transformation efficiency differed for each thiol compound: a low concentration for 2-mercaptoethanol,

Table 1. Effect of various thiol compounds on transformation efficiency at optimum concentrations. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with various thiol compounds included in 20 mM Hepes at 30°C for 15 min

Thiol compound	Concentration (mM)	Transformants/μg DNA[a]
None	–	$1.5 \pm 0.2 \times 10^6$
Dithiothreitol	25	$7.8 \pm 0.7 \times 10^6$
2-mercaptoethylamine	1	$4.5 \pm 1.8 \times 10^6$
2-mercaptoethanol	10	$4.7 \pm 1.3 \times 10^6$
3-mercapto-1,2-propanediol	100	$5.0 \pm 0.5 \times 10^6$

[a]The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments.

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but a high concentration for 3-mercapto-1,2-propanediol. DTT was the most effective among the thiol compounds tested, and improved the efficiency more than five-fold compared to that in control cells treated with no thiol compounds, as shown in Table 1. On the other hand, the transformation efficiency of *Sz. pombe* (*ura*$^-$) with no treatment was $1.9 \pm 0.5 \times 10^5$/μg, while that pretreated with DTT was greatly increased to $2.6 \pm 0.2 \times 10^6$/μg. Although transformation efficiency was dependent on each strain, strains pretreated with DTT certainly showed higher efficiency.

The transformation efficiency increased linearly at concentrations of DTT up to 25 mM, but no further increase was observed at concentrations of 50 mM or more, as shown in Figure 1. As shown in Table 1, the best efficiency was obtained when cells were pretreated with 25 mM DTT for 15 min, while the efficiency decreased for periods of 30 min or longer. Both the transformation efficiency and the viability of cells decreased after incubation for 15 min (data not shown). Optimum conditions of DTT pretreatment for *Sz. pombe* were in good agreement with those previously reported for other yeasts (Meilhoc *et al.*, 1990; Costaglioli *et al.*, 1994; Faber *et al.*, 1994).

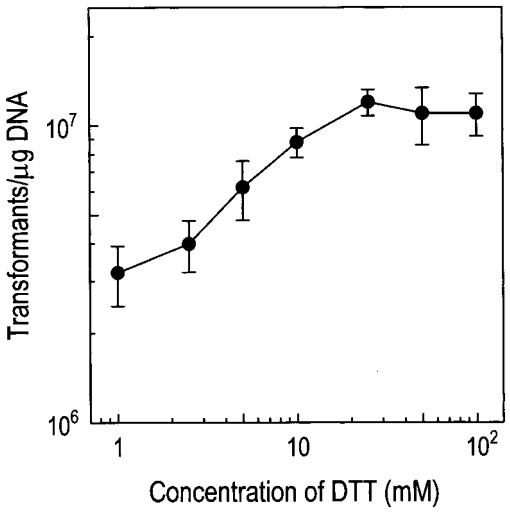

Figure 1. Effect of DTT concentration on transformation efficiency. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with DTT included in 0.6 M sorbitol and 20 mM Hepes at 30°C for 15 min. The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments

Effects of osmotic stabilization and culture medium on cells pretreated with DTT on transformation efficiency

The competency and viability of *S. cerevisiae* cells pretreated with 2-mercaptoethanol have been improved by including osmotic stabilizers in the suspension buffer (Brzobohatý and Kováč, 1986). Thus, the effect of osmotic stabilization in *Sz. pombe* (*leu*$^-$) cells pretreated with DTT on transformation efficiency was investigated for electroporation. As shown in Table 2, the isotonic (0.6 M sorbitol) and hypertonic (1.0 M sorbitol) conditions included in DTT pretreatment buffers showed higher transformation efficiencies than were achieved without an osmotic stabilizer. These results suggest that the cells treated with thiol compounds were sensitive to osmotic pressure. Similar results using 0.6 M sorbitol were obtained in *S. cerevisiae* cells pretreated with DTT by electroporation (data not shown).

The DTT buffer for pretreatment must be removed by washing the cells with a non-electrolyte solution before applying the high electric pulse to them, in order to avoid arcing in the cuvette. The transformation efficiency dropped to 20% or less when the pretreated *Sz. pombe* cells were washed with a hypotonic solution such as water. The high efficiency was maintained, however, when the pretreated cells were washed with 1 M sorbitol. It was necessary to provide continuous osmotic support for *Sz. pombe* cells pretreated with DTT until the electric pulse was applied.

Table 3 shows the influence of culture medium in DTT buffer for pretreatment on transformation efficiency. In electroporation procedures for several yeast species, DTT buffer for pretreatment has contained culture medium (Meilhoc *et al.*, 1990;

Table 2. Effect of osmotic stabilizer in DTT pretreatment buffer on transformation efficiency. Cells of *Sz. pombe* (*leu*$^-$) were pretreated at 30°C for 15 min

Pretreatment condition	Transformants/μg DNA[a]
25 mM DTT + 20 mM Hepes	$6.1 \pm 0.4 \times 10^6$
25 mM DTT + 0.6 M Sorbitol + 20 mM Hepes	$1.1 \pm 0.3 \times 10^7$
25 mM DTT + 1.0 M Sorbitol + 20 mM Hepes	$9.3 \pm 3.8 \times 10^6$

[a]The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments.

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Table 3. Influence of culture medium in DTT pretreatment buffer on transformation efficiency. Cells of *Sz. pombe* (*leu*$^-$) were pretreated at 30°C for 15 min

Pretreatment condition	Transformants/μg DNA[a]
25 mM DTT + 0.6 M Sorbitol + 20 mM Hepes	$1.2 \pm 0.3 \times 10^7$
25 mM DTT + 0.6 M Sorbitol + SD medium + 20 mM Hepes	$4.1 \pm 1.7 \times 10^5$
25 mM DTT + 0.6 M Sorbitol + 0.67% yeast nitrogen base + 20 mM Hepes	$8.8 \pm 4.4 \times 10^6$
25 mM DTT + 0.6 M Sorbitol + 2% Glucose + 20 mM Hepes	$1.5 \pm 1.0 \times 10^5$
2% Glucose	$4.8 \pm 2.0 \times 10^5$

[a]The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments.

Sánchez *et al*., 1993; Iborra, 1993; Costaglioli *et al*., 1994). For *Sz. pombe*, however, when SD medium was added to DTT pretreatment buffer, the transformation efficiency dropped to about 30% compared to that with no treatment, as shown in Table 3. The influence of the components of SD medium in DTT pretreatment buffer on transformation efficiency for *Sz. pombe* (*leu*$^-$) was therefore investigated. Concentrations of nitrogen and carbon sources in the medium in DTT buffer were the same as those in SD medium. The transformation efficiency was not affected by the presence of a nitrogen source, but was drastically decreased by the presence of a carbon source, glucose. Furthermore, pretreatment in 2% glucose alone drastically decreased transformation efficiency. Ganeva *et al*. (1994) have reported that incubation before and after applying an electric pulse with glucose drastically decreases the transformation efficiency for *S. cerevisiae*. Hence, it would be important in the electroporation of yeast that glucose be excluded from the DTT pretreatment buffer.

Effects of other factors on transformation efficiency

The growth phase of the cell is an important factor in the transformation of yeast. Cells were grown to mid-log (7×10^6 cells/ml), late-log (1×10^7 cells/ml) and stationary phases (2.5×10^7 cells/ml). Prior to electroporation, the cells in each growth phase were concentrated by centrifugation and suspended in 1 M sorbitol to achieve a constant cell concentration (1×10^9 cells/ml). The transformation efficiencies in all the growth phases in SD medium were improved by DTT pretreatment, with the best efficiency obtained in the late-log phase, as shown in Table 4. The optimum growth phase in SD medium pretreated with DTT was in agreement with that not pretreated (Ishiguro and Kobayashi, 1995).

Sz. pombe cells grown in synthetic medium such as SD medium instead of natural medium increase transformation efficiency by electroporation (Ishiguro and Kobayashi, 1995; Suga *et al*., 2000). EMM medium for the growth of *Sz. pombe* cells has been widely used (Moreno *et al*., 1991; Alfa *et al*., 1993), but in the present study it improved transformation efficiency only slightly, as shown in Table 4. Therefore, it was necessary to grow the *Sz. pombe* cells in SD medium for pretreatment with DTT.

In many electroporation experiments, the most critical factors affecting transformation efficiency are electric-field strength and pulse duration. When the time constant was fixed at 5 ms in all electric fields ranging from 7.0 to 12.0 kV/cm, the transformation efficiency in *Sz. pombe* (*leu*$^-$) cells pretreated with DTT was consistently about 10-fold higher than in cells with no pretreatment. The optimum conditions for the electric pulse were almost the same regardless of DTT pretreatment. The best efficiency, approximately 1.2×10^7/μg, was achieved in the case of an electric field of 11.0 kV/cm (data not shown). The best conditions of electric pulse for *Sz. pombe* (*ura*$^-$) were similar to those obtained for *Sz. pombe* (*leu*$^-$).

For other yeasts pretreated with DTT, incubation with culture medium for cell recovery after applying the electric pulse improves efficiency (Meilhoc *et al*., 1990; Sánchez *et al*., 1993; Iborra, 1993; Costaglioli

Table 4. Effect of growth phase grown in SD or EMM medium on transformation efficiency. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with 25 mM DTT included in 0.6 M sorbitol and 20 mM Hepes at 30°C for 15 min

Growth phase	Transformants/μg DNA[a]	
	SD medium	EMM medium
Mid-log	$3.9 \pm 0.5 \times 10^6$	$1.3 \pm 0.6 \times 10^6$
Late-log	$1.1 \pm 0.3 \times 10^7$	$2.0 \pm 0.9 \times 10^6$
Stationary	$7.1 \pm 2.3 \times 10^6$	$1.8 \pm 1.2 \times 10^6$

[a]The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments.

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et al., 1994; Faber *et al.*, 1994). However, no such improvement for *Sz. pombe* was observed when the cells applied the electric pulse were incubated with SD medium or EMM medium at 30°C for 30 min (data not shown).

Effects of cell concentration and amount of DNA on transformation efficiency

Cell concentration during application of the electric pulse greatly affected transformation efficiency. Figure 2 shows that the cell concentrations of *Sz. pombe* (*leu*$^-$) varied from 6×10^7 to 3×10^9 cells/ml, and the cells were transformed with 0.5 ng of plasmid pAL7. Cell suspension at concentrations above 4×10^9 cells/ml was physically impossible to obtain. Transformation efficiency increased significantly in proportion to cell concentration up to 1.3×10^9 cells/ml; higher concentrations gave no further improvement. This saturation may have been caused by the heterogeneity of the applied electric field due to cell-to-cell contact.

The maximum efficiency for intact cells of *Sz. pombe* has been reported to require 500 ng plasmid DNA (Ishiguro and Kobayashi, 1995). However, when amounts of the plasmid pAL7 ranging from 1 to 500 ng were added to *Sz. pombe* (*leu*$^-$) cell suspensions of 1×10^9 cells/ml pretreated with DTT, as shown in Figure 3, the transformation efficiency was constant with up to 10 ng DNA, but decreased drastically with 25 ng or more. On the other hand, the total number of transformants increased with DNA amounts up to 100 ng, but gradually decreased with 250 ng or more. Similar profiles relating the transformation efficiency and the number of transformants with the amount of DNA were obtained with *Sz. pombe* (*ura*$^-$) and pAU5 (data not shown). Our results were in good agreement with those previously reported for *S. occidentalis* pretreated with DTT (Costaglioli *et al.*, 1994). Therefore, the optimal amount of DNA to obtain transformants of the cells pretreated with DTT was considered to be less than 100 ng.

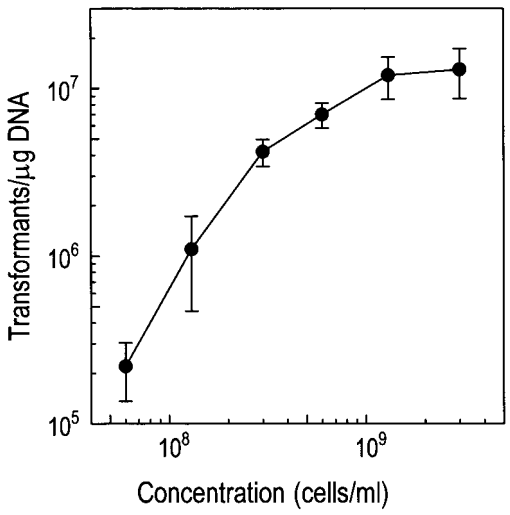

Figure 2. Effect of cell concentration during an electric pulse on transformation efficiency. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with 25 mM DTT included in 0.6 M sorbitol and 20 mM Hepes at 30°C for 15 min. The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments

Cryopreservation of highly competent cells pretreated with DTT

Although pretreatment of yeast cells with DTT has improved transformation efficiency, freezing pretreated cells in a permeating cryoprotectant such as glycerol or DMSO does not maintain their high efficiency (Meilhoc *et al.*, 1990; Costaglioli *et al.*,

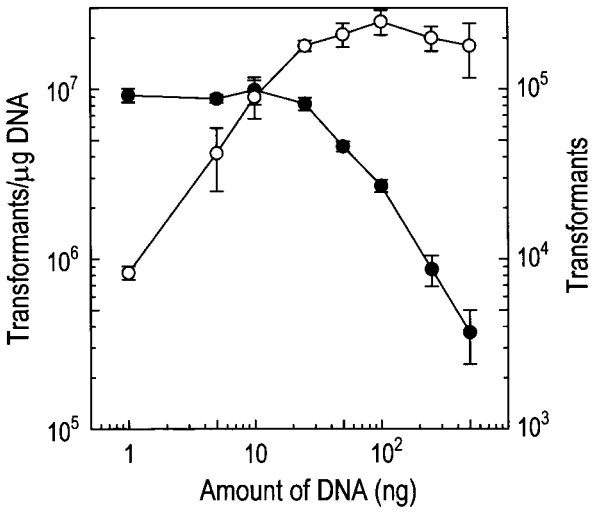

Figure 3. Effect of amounts of plasmid DNA during an electric pulse on transformation efficiency and total number of transformant colonies. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with 25 mM DTT included in 0.6 M sorbitol and 20 mM Hepes at 30°C for 15 min. (●) transformation efficiency; (○) total number of transformant colonies. The plasmid pAL7 was added to each pretreated cell suspension of 10^9 cells/ml. The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments

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1994; Faber *et al.*, 1994). We previously reported that the transformation efficiency after cryopreservation is maintained by electroporation when intact yeast cells are frozen in 2 M sorbitol, a non-permeating cryoprotectant (Suga *et al.*, 2000). *Sz. pombe* (*leu*$^-$) cells pretreated with DTT were washed three times with 1 M sorbitol, resuspended in cryoprotectant, and slowly frozen (cooling rate, approximately 10°C/min) by being placed directly in a −80°C freezer. For each electroporation, the frozen cells were quickly thawed (warming rate, approximately 200°C/min) in a water bath at 30°C, and then washed once by centrifugation with ice-cold 1 M sorbitol and electroporated as described in Materials and methods. We previously reported that the transformation efficiency of intact *Sz. pombe* cells when frozen in 1.0 M and 1.5 M sorbitol decreased because of thawing injuries to membranes caused by water influx and efflux under non-equilibrium conditions between extracellular and intracellular osmotic pressure (Suga *et al.*, 2000). However, the frozen cells pretreated with DTT were able to maintain high efficiency around 1 M sorbitol, as shown in Figure 4. A similar result as shown for 1 M sorbitol was obtained with *Sz. pombe* (*ura*$^-$) (data not shown). These results may be attributed to decreases in thawing injuries to membranes brought about by the pretreatment of cells with DTT. Furthermore, the highly competent cells stored at −80°C were used for 6 months or more without any loss of transformation efficiency. This cryopreservation saved time and effort, since the need to prepare fresh cells and pretreat them with DTT before each electroporation was eliminated.

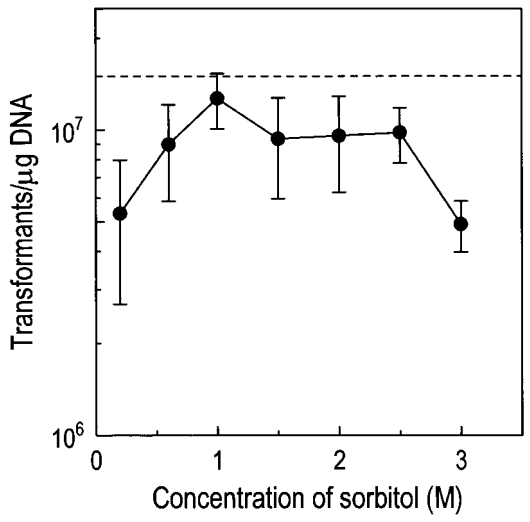

Figure 4. Effect of concentration of sorbitol on transformation efficiency after cryopreservation. Cells of *Sz. pombe* (*leu*$^-$) were pretreated with 25 mM DTT included in 0.6 M sorbitol and 20 mM Hepes at 30°C for 15 min, and then were suspended in sorbitol and cryopreserved at −80°C. The broken line shows the transformation efficiency before cryopreservation. The electric pulse was held constant at 11 kV/cm for 5 ms. Data represent the average values ± SD of five independent experiments

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