Highly Efficient Oligonucleotide Transfer into Intact Yeast Cells Using Square-Wave Pulse Electroporation

ABSTRACT

Here, we present a rapid and reproducible procedure based on square-wave pulse electroporation that allows efficient penetration of synthetic oligonucleotides into intact yeast cells. This procedure was successfully used to modify the yeast genome with small amounts of oligonucleotide.

INTRODUCTION

Oligonucleotides are potentially powerful tools for yeast cell modification; they can be used to modify the genome through homologous recombination and also used as antisense oligonucleotides to temporarily suppress the expression of a gene. However, poor penetration of oligonucleotides into intact yeast cells is a major limiting parameter for their use in this organism. One hundred micrograms of synthetic oligonucleotides were needed to obtain transformants through recombination using the lithium acetate (LiAc) procedure (6). Among the techniques used to allow the penetration of DNA in various organisms, electroporation is one of the most powerful. Two types of pulses can be delivered to cells: pulses decaying exponentially or square-wave pulses (7). Electroporation using exponential decays has been used to introduce plasmids into yeast cells (1,4). It was also used to transform yeast cells with synthetic oligonucleotides, but with a lower efficiency than with the LiAc procedure (8). The square-wave approach seems to be more efficient for a number of applications. Indeed, square-wave pulse electroporation (SqE) has been successfully used to introduce plasmids into yeast cells (2,5). Here, we have adapted the SqE procedure to introduce short synthetic oligonucleotides into yeast cells. This procedure dramatically improves the penetration of oligonucleotides as compared to lithium acetate, thus allowing their use for antisense or homologous recombination applications. We demonstrate that transformants can be obtained by homologous recombination with less than 1 µg of a properly designed oligonucleotide.

MATERIALS AND METHODS

Electroporation Procedure

Exponentially growing yeast cells (10⁷ cells/mL) were harvested and incubated in dithiothreitol (25 mM in YEPD, 10⁹ cells/mL) for 30 min at 30°C with shaking. Cells were then washed twice with electroporation buffer (EB; 10 mM Tris-HCl, pH 7.0, 270 mM sucrose, 1 mM MgCl₂) and resuspended in EB to give 10⁸ cells per assay. Oligonucleotides were added to reach a final volume of 50 µL. A single square-wave pulse was immediately delivered using a Model PS-15 Cell Electropulsator (JOUAN, St. Herblian, France) and 0.2-cm electroporation cuvettes. Pulses were checked using a digital oscilloscope (Model VC-6025; Hitachi, Tokyo, Japan). After the pulse, cells were allowed to recover for 1 h in YEPD at 30°C.

Flow Cytometry Analysis

Cellular uptake of oligonucleotides was monitored by cytofluorometry using as probe a 15-mer oligonucleotide coupled to fluorescein isothiocyanate (FITC) (Fluo-TH; Eurogentec Bel S.A., Seraing, Belgium). Fluo-TH-treated cells were incubated for 10 min with propidium iodide (PI) (5 µM in cold sterile water, 10⁸ cells/mL) and then washed twice with cold water. Analysis was performed on a FACS-Calibur™ (Becton Dickinson Immunocytometry, San Jose, CA, USA) using an argon 488-nm excitation laser. Uptake of the oligonucleotide was monitored by FITC green fluorescence (GF; 530 nm); whereas, PI red fluorescence (RF; 650 nm) was used to score dead cells. The proportion of oligonucleotide-positive live cells in the input population was calculated as the ratio between the number of cells that showed GF but did not show RF and the total number of cells. The proportion of oligonucleotide-positive cells in the surviving fraction was calculated as

Figure 1. Nucleotide and amino acid sequences of the URA3 alleles. Nucleotides that differ from the wild-type URA3 sequence are shown in lower case. Nucleotides of URA3::CTAG that differ from ura3::taa are in bold type. Useful restriction sites are underlined.

294 BioTechniques

Vol. 25, No. 2 (1998)
The ratio between the number of cells that showed GF but did not show RF and the total number of cells that did not show RF.

**Transformation Experiments**

Yeast strain Yfx15, bearing the defective *ura3::taa* allele (Figure 1), was derived from CmY826 (9). The *URA3::CTAG* mutant was obtained by directly transforming Yfx15 with a 45-mer oligonucleotide (Orec45pyr; Eurogentec) designed to correct *ura3::taa* through recombination. Replacement of a DraI site by an XbaI site was used to verify the transformants at the molecular level. Yeast cells treated with the Orec45pyr oligonucleotide were plated on medium lacking uracil to select for *URA3* transformants. The frequency of transformants was computed as the ratio between the number of viable cells and the amount of oligonucleotide used.

**RESULTS AND DISCUSSION**

Square-wave pulse electroporation was optimized for oligonucleotide penetration and increased cell survival. To achieve this, penetration of a fluorescent oligonucleotide (labeled with FITC) was monitored by cytofluorometry. As shown in Figure 2, with an electric field strength set to 2.8 kV/cm, increasing the pulse length increased the number of electroporated cells but dramatically reduced cell survival (curve □). Consequently, the total number of oligonucleotide-positive cells in the input population (curve ○) reached an optimum, with a pulse length of 8 ms. Interestingly, the proportion of oligonucleotide-positive cells in the surviving fraction (curve ▼) also reached an optimum, which suggests that a sub-fraction of cells is resistant to electroporation.

The efficiency of the procedure was next assessed using an oligonucleotide designed for yeast genomic modification through homologous recombination (Orec 45pyr), which reverts the *ura3* phenotype to *URA3*+. Using this assay, a pulse length of 8–12 ms gave a maximal number of transformants (Figure 3, ○), in good concordance with the results obtained by direct detection of the intracellular oligonucleotide using the FACS system. Even though approximately 40% of the cells showed a detectable amount of intracellular oligonucleotide as assessed by FACS analysis (Figure 2) using this setting, the genome of only a minor proportion of this population could be modified. This suggests that in these conditions, the recombination step is the limiting one. In addition, when no selection system is available and transformants have to be tested by colony hybridization screening, the frequency of transformants is the most critical value. The frequency of transformants is maximal at 12–16 ms (Figure 3, ▼) and could be further increased (to 2.10^4) by co-transformation with a plasmid bearing an auxotrophy marker and subsequent selection for this marker (not shown).

Using an electric field strength of 2.8 kV/cm and a pulse duration of 12 ms, we next investigated the effect of the amount of oligonucleotide. The highest number of *URA3*+ revertants was obtained with 10 µg of oligonucleotide (Table 1). Interestingly, transformants could also be obtained with as little as 100 ng of oligonucleotide, and 1 µg of oligonucleotide gave transformants in sufficient number for most applications. Increasing the amount of oligonucleotide to more than 20 µg resulted in lower efficiencies. A likely explanation is that the oligonucleotide's negative charges decreased the resistance of the solution during the electroporation procedure.

We finally compared SqE to other commonly used procedures such as LiAc treatment of cells (3) or electroporation using pulses with exponential decays (ExpE; Reference 4). Cell viability was best using LiAC or SqE (around 35% of living cells); whereas, ExpE was the worst method on that criterion (12% of living cells). Using

![Figure 2. Influence of pulse duration on cellular uptake of oligonucleotides. Yeast cells were electro-permeabilized in the presence of 10 µg of Fluo-TH. Electric field strength was set to 2.8 kV/cm, and pulse duration varied from 0–24 ms. Fluo-TH uptake was monitored by flow cytometry analysis.](image)

![Figure 3. Influence of pulse duration on transformation of yeast cells with an oligonucleotide. Yfx15 cells were transformed with 1 µg of Orec45pyr. Electric field strength was set to 2.8 kV/cm, and pulse duration varied from 0–24 ms.](image)

Table 1. Effect of the Amount of Oligonucleotide on *URA3*+ Reversion

<table>
<thead>
<tr>
<th>Amount of oligonucleotide (µg)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of <em>URA3</em>+ revertants</td>
<td>2</td>
<td>15</td>
<td>80</td>
<td>307</td>
<td>318</td>
<td>185</td>
<td>122</td>
</tr>
</tbody>
</table>

Yfx15 cells were transformed using SqE with increasing amounts of Orec45pyr. Electric field strength was set to 2.8 kV/cm, and time duration was set to 12 ms.
SqE, the optimal dose of oligonucleotide was only 1 µg, and it gave a very high transformation efficiency (80 transformants/µg; Figure 4). Using ExpE, 10 µg of oligonucleotide were needed to reach an optimal transformation efficiency, which was only 25% of that obtained with SqE (around 20 transformants/µg; Figure 4). Both techniques were far more efficient than lithium acetate, which, in our hands, gave only around 1 transformant/µg. In comparison, Moershell et al. (6) obtained approximately 5 transformants/µg of oligonucleotide with the LiAc procedure and 100 µg of oligonucleotide. Even so, SqE turns out to be the most efficient procedure.

In conclusion, square-wave pulse electroporation is a rapid and convenient method that allows efficient penetration of oligonucleotides into intact yeast cells without compromising cell viability. Consequently, this procedure dramatically reduces the amount of oligonucleotide needed for direct transformation of yeast.

REFERENCES


This work was supported by funds from the Agence Nationale pour la Recherche contre le SIDA and from Rhône-Poulenc. François-Xavier Barre, a student at the Institut de Formation Supérieure Biomédicale, is a recipient of a fellowship from the Agence Nationale pour la Recherche contre le SIDA. Address correspondence to Dr. Annick Harel-Bellan, CNRS UPR9079, IFCI, 7 rue Guy Moquet, BP8 94801 Villejuif, France. Internet: ahbellan@vif.cnrs.fr

Received 17 November 1997; accepted 25 March 1998.

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Figure 4. Influence of DNA transfer procedures on transformation efficiency. Yfx15 cells were transformed with the Orec45pyr oligonucleotide using various procedures. LiAc: lithium acetate treatment. ExpE: electroporation using pulses with exponential decay (192 Ω, 40 µF, 1.5 kV). SqE: square-wave pulse electroporation (2.8 kV/cm, 12 ms). For the control (0 µg), the background absolute number of transformants is shown.