High-Throughput Liberation of Water-Soluble Yeast Content by Irreversible Electropermeation (HT-irEP)

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The article describes a high-throughput method for the liberation of water-soluble cell contents by exploiting the phenomenon of irreversible membrane electropermeation (HT-irEP). The method is exemplified in recombinant proteins and plasmid liberation from yeast *Saccharomyces cerevisiae* on the detectable level. Obtained extracts are pure enough to be readily applied for further analytical analysis such as enzyme assay, PCR, and so on. From the same HT-irEP extract, one can measure activity of the target protein and perform amplification of the corresponding gene from the DNA vector by PCR for recombinant protein with intracellular expression. Therefore, the method is suitable for the high-throughput screening (HTS) of yeast libraries where extracellular expression of recombinant protein is problematic. The method can be easily automated and integrated into existing HTS systems. (*Journal of Biomolecular Screening* 2007:267-275)

Key words: high-throughput screening, yeast library, plasmid liberation, protein liberation, membrane electropermeation

INTRODUCTION

HIGH-THROUGHPUT SCREENING (HTS) of libraries expressing mutants of a recombinant protein is problematic if the protein can not be secreted as a completely functional molecule from a host cell. Otherwise, library screening requires clone-by-clone cell disintegration to gain access to proteins. This is further complicated due to the hard cell wall. There are a number of cell disintegration methods: mechanical (grinding with glass beads, high pressure, ultrasound) and chemical (cell lysis by enzymes and detergents).[1,2] However, there are engineering challenges in integrating mechanical methods of cell disruption in HTS systems due to mechanical constraints and reproducibility requirements. On the other hand, detergents and lytic enzymes are compatible with the HTS platform, but very often they also affect the target molecule. All these methods have a common disadvantage—they result in complete cell disintegration, which forms a complex mixture of cell debris, cell organelles, bulk cell proteins, proteases, lipids, genomic DNA, and so on (i.e., homogenate). Consequently, homogenates interfere with detection and analysis of target molecules (by enzyme assay, mass spectrometry, PCR, etc.) and inevitably require additional steps during sample preparation for further analysis.

The use of signal sequences for the extracellular expression of recombinant proteins is not always an alternative solution for high-throughput (HT) sample preparation because (1) it is difficult to find a successful combination of host organism, secretion pathway, and corresponding signal sequence.[3,4] It is especially crucial for proteins with a subunit ternary structure, which requires posttranslational modification for functional activity and, more important, for enzymes that have noncovalent bound cofactors such as glucose oxidase.[5] (2) For the purposes of mutant library screening, even successful extracellular expression of recombinant protein still requires further laborious recovery of the DNA vector for sequencing, and complexity of the procedure depends on the host organism. However, very often, we need to sequence the mutant just to know which mutation has improved enzyme traits or to exclude false positives.

Electropermeation (EP) of host cells is an alternative method for sample preparation where high-intensity rectangular pulses of the electric field cause reversible (rEP) or irreversible (irEP) membrane electropermeation, which depends on intensity, duration, frequency, and number of the pulses.[6,7] Reversible electropermeation is achieved by a series of exponentially decaying pulses (e.g., 5-μsec pulses within 15–100 μsec), and it is now routinely used for delivering foreign molecules into cells (electrotransfection or electroporation) (Eppendorf AG, Multiporator®, Hamburg, Germany). The exponentially decaying pulse prevents significant amounts of current from flowing through the cells

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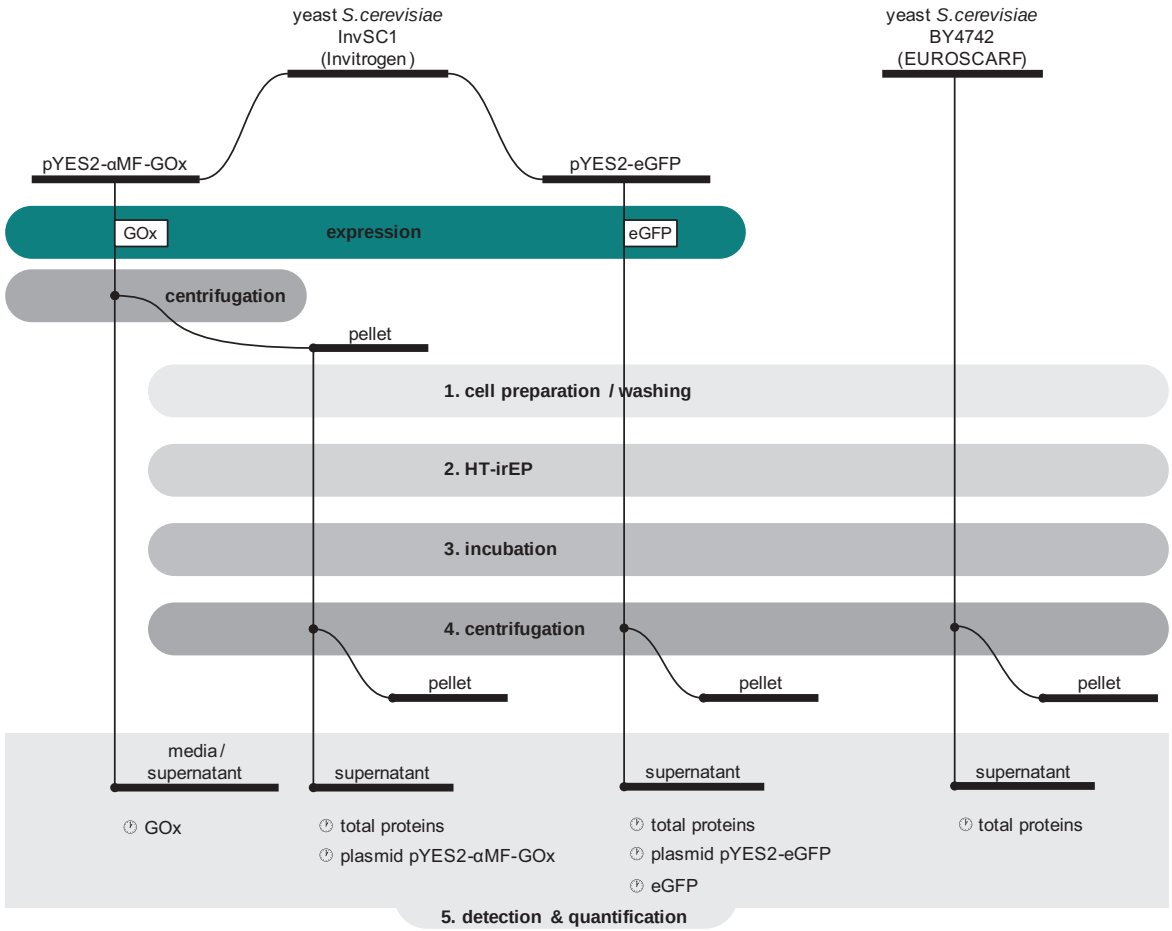


FIG. 1. Scheme of the experiments. Yeast transformed with pYES2-αMF-GOx secrete glucose oxidase (GOx) extracellularly, whereas yeast transformed with pYES2-eGFP express enhanced green fluorescent protein (eGFP) intracellularly. HT-irEP treatment steps: (1) cell preparation—quantitative transfer of cells into low-conductance media; (2) HT-irEP—high-throughput irreversible electropermeation; (3) incubation—postpulsation incubation in osmolarly stabilized buffer; (4) centrifugation—to remove cell remains; (5) detection and quantification—detection and quantification of indicated target molecules.

after the pores have formed, which improves cell viability. If the external voltage applied is on the milliseconds scale, high electrical currents flowing through the inside of the cells inflict severe damage and cause irreversible membrane electropermeation. This phenomenon can be exploited to liberate cytoplasmic content to surrounding media—ions and molecules with different sizes. Essentially, the irEP method is applicable to any types of biotechnologically important cells such as bacteria, yeasts, and mammalian cells.[8] However, working with yeasts in particular, the irEP method offers a remarkable advantage because cells can easily be removed by centrifugation because they are not disintegrated due to a hard wall. Optimized series of rectangular electric pulses have provoked the release of cytoplasmatic proteins from yeast without cell lysis and vacuole damage; therefore, there was no release of proteases.[9] The irEP treatment has been successfully applied for the liberation of 80% to 90% of cytoplasmatic-associated specific enzyme activity of yeast (*Saccharomyces cerevisiae PV3* [diploid], *Y47* [wild haploid], *SPY509*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe*) homologous enzymes (glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase, hexokinase, β-D-galactosidase, invertase, alcohol dehydrogenase, and glutathine reductase) without any further or previous treatment.[9-13] In general, the irEP method consists of the following principal steps: (1) replacement of nutrient media with low-conductance pulsation media, such as dH$_2$O (~3 μS/cm); (2) electropulsation; (3) postpulsation incubation in osmotically stabilized buffer; and (4) collection of the supernatant (**Fig. 1**).[10]

irEP is a very promising method for HT sample preparation (HT-irEP) because it (1) results in liberation of the water-soluble intracellular content (like proteins and short nucleic acids) in detectable amounts, (2) does not result in cell disintegration, (3) can be performed within a couple of handling steps, and

(4) therefore can be easily automated. Technically speaking, the HT-irEP can be performed using commercially available pulse generators for HT elecroporation, such as the PA-4000/PA-96WS PulseAgile® 96-Well Electroporation System (Cyto Pulse, Inc., Glen Burnie, MD; http://www.cytopulse.com) or the BTX® HT 96-Well Electroporation System (BTX, Holliston, MA; http://www.btxonline.com). However, unfortunately, the design of the commercial array of electrodes is not suitable to reach required field intensity to cause irEP of yeast. Furthermore, no data exist for the applicability of the 96-well electroporation system for the purposes of protein and nucleic acid liberation from yeast for the library screening. Therefore, we have performed this research to develop a method that (1) liberates cytoplasmatic water-soluble yeast content (proteins and nucleic acids) (2) without cell disintegration and (3) that is compatible with HTS platforms.

MATERIALS AND METHODS

Yeast

The yeast *S. cerevisiae* InvSC1 (Invitrogen, Carlsbad, CA; genotype: MATα, his3Δ1 leu2 trp1-289 ura3-52; phenotype: *His*$^-$, *Leu*$^-$, *Trp*$^-$, *Ura*$^-$) have been transformed[14] with either (1) the pYES2-αMF-GOx construct (GOx—glucose oxidase from *Aspergilus niger*, with upstream αMF–α-mating secretion factor in pYES2; Invitrogen) expressing glucose oxidase extracellularly or (2) pYES2-eGFP expressing enhanced green fluorescent protein (eGFP, from jellyfish *Aequorea victoria*) intracellularly. Yeast were grown aerobically in a 100-mL flask on selective minimal media at 30 °C and 250 rpm and induced by galactose[15] for 24 to 36 h, starting with an initial cell density of OD_{600} = 0.4 (Invitrogen cat. no. V825-20). The localization of the fluorescence of recombinant eGFP in live yeast was checked on a Carl Zeiss Laser Scanning Microscope 510 (λ_{ex} = 488 nm, λ_{ex} = 509 nm); images are published at SGD database (http://www.yeastgenome.org/yeast_images.shtml). Also, wild-type yeast *S. cerevisiae* BY4742 (EUROSCARF, genotype: MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) were used in the trials (**Fig. 1**). Yeast were maintained haploid and were always harvested at OD_{600} ~2.0 (early exponential phase). Cells were washed from nutrient media by triple precipitation by centrifugation (at least 3200 *g* for 5 min) in water and finally were resuspended in *d*H_2O (~3 μS cm^{-1} at 22 °C) to a final concentration of either 10 or 25 mg of wet cell biomass per milliliter.

HT-irEP

In total, 500 μL of yeast suspension was pipetted per well of the 96-deep-well microplate (2.2 mL well, with 8 × 8-mm squared cross section, U-bottom, polypropylene, nonsterile; BRAND, Wertheim, Germany), which was pulsed sequentially using a self-designed aluminium pair of electrodes (8 × 8-mm external perimeter with 2-mm electrode gap and 40-mm gap depth; see **Fig. 6**)[16] connected to a single-channel Cyto Pulse PA-3000S Laboratory PulseAgile® Electroporation System (Cyto Pulse, Inc.). The external dimensions of the electrodes were designed in a way to repeat the internal space and shape of the deep well in order to displace the whole contents into the gap between them; therefore, no more than 500 μL of yeast culture was in the deep well. Based on earlier publications[9-13] and on-site research, the following combination of pulsation parameters provides the optimal product yield: E = 3.75 kV cm^{-1} (or 750 V per 2 mm), pulse duration = 1.5 msec, pulse interval = 1 sec, and number of pulses = 20. Immediately after the pulsation, 100 μL of 6×-fold incubation buffer (IB) was added per well to form osmotically stabilizing buffer: 20 mM phosphate buffer (pH 7.2), 200 mM glycerol, 2 mM $MgCl_2$, and 2 mM dithiotrietol (DTT) (268 mOsmol kg^{-1}; 3.85 mS cm^{-1}). After repeated use of the electrodes, the organic matter accumulated on the electrode's surface; therefore, it was cleaned in 10% HCl for 10 to 15 sec, followed by intensive rinsing with *d*H_2O. Due to the single-channel prototype of the HT-irEP system, the electrodes were rinsed and regenerated after pulsation of each well. Then microplates were covered with lids and incubated at 30 °C and 900 rpm for different periods between 1 and 5 h. Up to 500 μL of supernatant per well can be collected after cell precipitation by centrifugation (at least 3200 *g* for 10 min) for further analysis (**Fig. 1**).

Protein assay

Specific GOx extracellular activity was monitored in expression media by coupled horseradish/ABTS assay.[5] Total proteins in HT-irEP extracts were quantified by both light absorption at 280 nm (in a range up to ~2.0 mg mL^{-1}) in the Hellma® quartz microplate using the Analytik Jena FLASHScan S12 microplate reader and by the NanoOrange® protein quantification kit (in a range up to 200 μg mL^{-1}; cat. no. N6666, Molecular Probes, Eugene, OR) using the TECAN Safire™ microplate reader. For purification of recombinant eGFP expressed intracellularly, the yeast cells were disintegrated by high-pressure homogenization (EmulsiFlex-C3, Avestin, Inc., Ottawa, Ontario, Canada; http://www.avestin.com) in the homogenization buffer (20 mM Tris [pH 7.5], 1 mM mercaptoethanol, and 0.1 mM PMSF), and further eGFP was purified by hydrophobic interaction chromatography in the linear gradient (0 → 100 %B) within 3.3 column volumes on the Toyopearl Butyl 650s in the KronLab column (15/125 mm) (buffer A: 20 mM phosphate [pH 8.0], 5 mM EDTA, 1.5 M $[NH_4]_2SO_4$; buffer B: 20 mM phosphate [pH 8.0], 1 mM EDTA), detecting eGFP at λ = 280 and 488 nm. The eGFP fraction further was desalted and concentrated using an ultrafiltration cell (Amicon-stirred ultrafiltration cell model 8200) with a 5-kDa cutoff PLCC membrane (cat. no. PLCC06210, Millipore,

Billerica, MA). The fluorescence of recombinant eGFP was quantified by the TECAN Safire™ (λ_{ex} = 488 nm, λ_{em} = 509 nm, gain = 100, Z position = 9000 µm; the same parameters of fluorescence detection were used to record eGFP fluorescence in live cells), whereas concentration of purified eGFP was quantified by the NanoOrange® protein quantification kit.

Plasmid assay

Plasmid liberation was detected and also quantified by either conventional or real-time PCR. The conventional PCR was performed in a Mastercycler gradient (Eppendorf AG) and had the following conditions: initial denaturation for 4 min at 94 °C, then 39 cycles at 94 °C for 1 min/56 °C for 1 min/72 °C for 2.25 min, and the final step 10 min at 72 °C. PCR mix in thin-wall PCR tubes (Multi-Ultra tubes 0.2 mL; Carl Roth, Karlsruhe, Germany) had a total volume of 50 µL and consisted of the following: 41.5 – x µL water, 5 µL buffer (10×), 1 µL dNTPs (10 mM each), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), x µL DNA template, and 0.5 µL *Taq* polymerase (5U/µL). For detection of the pYSE2-αMF-GOx construct by PCR, the forward (5´-catcggggtaccatgagatttc-ctt-3´) and reverse (5´-gaagctctagagctcactgcatgg-3´) primers were designed with an expected length of the PCR product of 2121 bp. For detection of the pYSE2-eGFP construct by PCR, the forward (5´-gcagctgtaatacgactcac-3´) and reverse (5´-ccggtggtgcagatgaactt-3´) primers were designed with an expected length of the PCR product of 245 bp. The real-time PCR was performed on a Stratagene M×3000P system, using the Qiagen QPCR SybrGreen-Kit. Real-time PCR conditions: initial denaturation for 15 min at 95 °C, then 40 cycles for 15 sec at 95 °C/30 sec at 55 °C/30 sec at 72 °C, and the final step 1 min at 95 °C. The reaction mixture was as follows: 12.5 µL of 2×QMM (Qiagen Mastermix; Qiagen, Hilden, Germany), 0.13 µL of forward primer (100 µM), 0.13 µL of reverse primer (100 µM), 7.25 µL of water, and 5 µL of sample. For detection/quantification of the pYSE2-αMF-GOx construct by real-time PCR, the forward (5´-ctggattgtcaaccacaacg-3´) and reverse (5´-gaacgtatcctcgggt-gaag-3´) primers were designed with an expected length of the PCR product of 107 bp. Obtained PCR products were cross-checked by gel electrophoresis (see **Fig. 5**).

Statistics

Prior to the statistical analysis, all data sets were tested for normality using the Kolmogorov-Smirnov (KS) test with a significance level at $p > 0.10$ ($\alpha = 0.05$) (STATISTICA, StatSoft, Inc., Tulsa, OK). The real-time PCR kinetic curves (see **Fig. 4**) were fit to the Boltzmann sigmoid equation and, halfway from bottom to top (V_{50} values), were used for the quantification of plasmid concentration. Nonlinear and linear regression analyses and data fit were performed using GraphPad Prism v4.0 (GraphPad, Inc., San Diego, CA).

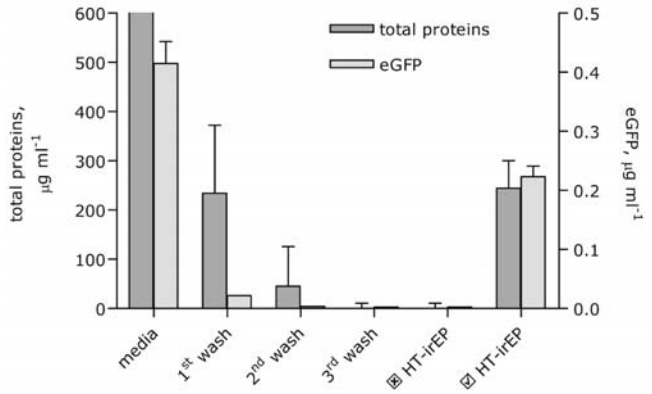


FIG. 2. Concentrations of total proteins and enhanced green fluorescent protein (eGFP) assessed in supernatants during high-throughput irreversible electropermeation (HT-irEP) processing according to **Figure 1**. Media: protein concentrations in expression media (concentration of total proteins in expression media has exceeded the upper detection limit of the method, > 2.0 mg mL^{-1}; eGFP fluorescence has been recorded in live cells). Washes 1-3: proteins concentrations in supernatants collected after consecutive washing steps to replace expression media with low-conductive pulsation media (i.e., dH_2O). ☒ HT-irEP: washed but non-pulsated yeast after incubation in dH_2O for 1 to 5 h at 30 °C and 900 rpm. ☑ HT-irEP: yeast were HT-irEP treated and incubated in incubation buffer for 1 to 5 h at 30 °C and 900 rpm.

RESULTS AND DISCUSSION

The experiments were carried out according to the chart presented in (**Fig. 1**). We have targeted the detection and quantification of 2 recombinant proteins (glucose oxidase and green fluorescent protein) expressed in yeast (*S. cerevisiae* InvSC1) using different strategies: extracellular (glucose oxidase) and intracellular (green fluorescent protein), as well as corresponding plasmids. In addition, quantification of total bulk homologous proteins liberated by HT-irEP treatment from yeast (*S. cerevisiae* InvSC1 and wild-type BY4742) was used as quantitative criteria of the extraction performance.

Cell preparation

In this trial, the triple washing of yeast cells with dH_2O was sufficient for the complete elimination of ion and protein background from the nutrient media to succeed with HT-irEP (**Fig. 2**). After the washing procedure, the yeast cells were concentrated in dH_2O either at 10 or 25 mg of yeast wet biomass per milliliter. This cell density is sufficient to detect released enzyme activity or nucleic acids in the HT-irEP extracts after 1 h of postpulsation incubation.

HT-irEP

The pulse parameters were investigated for the optimal combination using the liberation of bulk homologous proteins from

Table 1. Statistical Data Quality for Liberation of Target Molecules from HT-irEP-Treated (E = 3.75 kV cm^{-1}, W = 1.5 msec, I = 1 sec, and n = 20) Yeast *Saccharomyces cerevisiae*

	GOx Activity in Media (μmol glc min^{-1})	*Total Proteins in HT-irEP Extract (μg mL^{-1})*		*Total Proteins Liberated by HT-irEP from mg of Wet Yeast Biomass (μg mg^{-1})*		*eGFP in HT-irEP Extract (μg mL^{-1})*	*eGFP Liberated from HT-irEP mg of Wet Yeast Biomass (ng mg^{-1})*	*pYES2-αMF-GOx in HT-irEP Extracts (ng mL^{-1})*	*pYES2-αMF-GOx Liberated by HT-irEP per mg Wet Yeast Biomass (pg mg^{-1})*
Yeast concentration used for HT-irEP treatment (mg mL^{-1})		10		10		25	25	10	10
Postpulsation incubation time (h)		1		1		5	5	1	1
Detection/Quantification Method for Target Molecules	*HP/ABTS Assay*	A_{280}	$F_{NanoOrange}$	A_{280}	$F_{NanoOrange}$	F_{eGFP}	F_{eGFP}	*Real-Time PCR*	*Real-Time PCR*
Mean	6.489	244.07	106.96	29.289	12.836	0.223	10.695	2.096	251.5
Median	6.477	248.76	107.41	29.851	12.889	0.227	10.899	2.040	244.7
Standard deviation (±SD)	0.695	56.345	5.805	6.761	0.6965	0.018	0.851	0.502	60.21
Standard error of mean (±SEM)	0.071	8.399	2.902	1.008	0.3483	0.007	0.322	0.052	6.28
Sample size (n)	96	48	48	48	48	12	12	94	94
CV (%)	10.7	23.1	5.4	23.1	5.4	8.1	8.0	24.0	23.9
Normality test KS	0.0606	0.0793	0.0888	0.0793	0.0888	0.3319	0.3319	0.0890	0.0887
Normality test p value	> 0.10	> 0.10	> 0.10	0.0818	> 0.10	0.0191	0.0191	0.0691	0.0708
Passed normality test?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

HT-irEP, high-throughput irreversible electropermeation; GOx, glucose oxidase; glc, glucose; eGFP, enhanced green fluorescent protein; pYES2-αMF-GOx, plasmid construct used to express GOx in yeast and secrete it with α-mating factor; HP/ABTS assay, coupled horseradish peroxidase/ABTS assay; A_{280}, UV/Vis absorbance at 280 nm; $F_{NanoOrange}$, fluorescence intensity specific for NanoOrange® protein assay (λ_{ex} = 485 nm and λ_{em} = 590 nm); F_{eGFP}, fluorescence intensity specific for eGFP (λ_{ex} = 488 nm and λ_{em} = 509 nm); KS, Kolmogorov-Smirnov normality test; CV, coefficient of variation.

yeast as an indicator. The investigation of pulsation parameters ranged from the following:

Field intensity (E): 3.5-4.0 kV cm^{-1}
Pulse width (W): 1-2 msec
Pulse interval (I): 0.5-1 sec
Number of pulses (N): 10-20

There were 81 combinations of pulsation parameters in total; among them, the following combination of conditions (see Materials and Methods) appears to provide the highest total protein liberation during 1 h of postpulsation incubation (data not presented). Also, it is important to note that in the course of HT-irEP under mentioned optimal conditions, the temperature of the sample changes no more than 1 °C.

Postpulsation incubation

The osmotically stabilized incubation buffer enabled the preservation of the vacuolar integrity, whereas the reducing agent (DTT) served to increase the cell wall porosity as well as maintain enzyme activity during incubation.[17,18] The amount of liberated target molecules is a function of incubation time.[9] In all HT-irEP cases (**Table 1** and **Fig. 3**), the liberation of a detectable amount of target molecules has been already observed after 1 h of postpulsation incubation, which actually suits the requirements of any HTS assay.

Bioanalytics

Liberation of total proteins was detected by 2 methods: by light absorbance at 280 nm (A_{280}) and by the NanoOrange® fluorescent protein quantification kit ($F_{NanoOrange}$). The observed amount of liberated total proteins by HT-irEP treatment after 1 h of postpulsation incubation is estimated to be ~1.5% to 3% of wet yeast biomass (depending on the detection method). The amount of the cell's bulk proteins liberated is more than enough for any qualitative analysis, including assays of homologous yeast proteins. A mismatch between the results of both protein

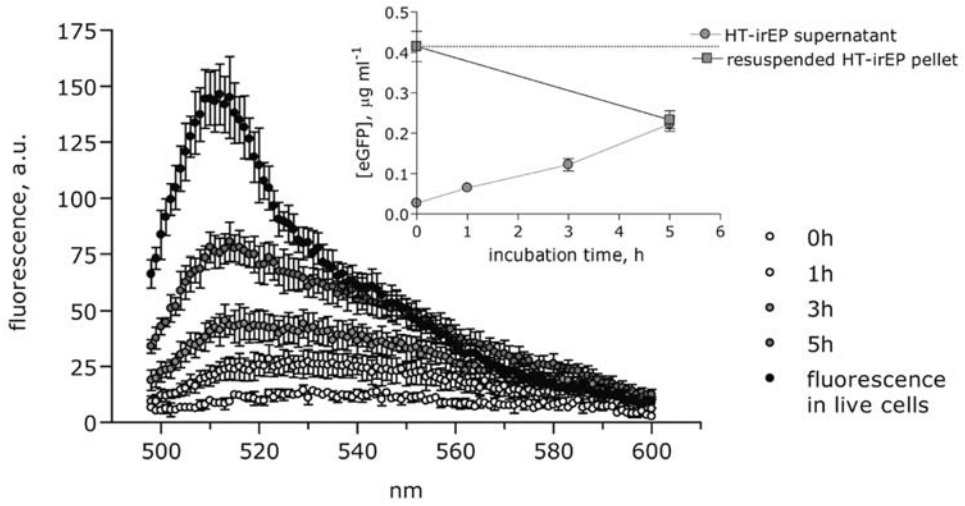


FIG. 3. Enhanced green fluorescent protein (eGFP) emission spectrum of live yeast *Saccharomyces cerevisiae* suspension (25 mg mL^{-1}) and emission spectra (λ_{ex} = 488 nm) of high-throughput irreversible electropermeation (HT-irEP) supernatant after a different time of post-EP incubation (0–5 h at 900 rpm and 30 °C). Spectra are averaged (±SD) from independently treated samples (n = 12). Embedded plot shows kinetics of eGFP liberation from yeast into supernatant (n = 12; ±SD). The supernatant was separated from the cells by centrifugation; the remaining pellet was resuspended in water back to its initial volume, and corresponding fluorescence was measured. After 5 h of postpulsation incubation, ~56% of eGFP-base fluorescence was registered in the supernatant; moreover, the sum of fluorescence intensities of the supernatant and resuspended pellet was equal to the initial fluorescence intensity in live yeast.

quantification methods is probably due to nonspecific light absorbance at 280 nm by cell components other than proteins. Therefore, quantification with the fluorescent probe is more specific and less variable (CV_F ~5% vs. CV_A ~23%; **Table 1**).

In general, low concentration of all compounds in the HT-irEP extracts (e.g., see total proteins in **Table 1**) allows direct detection and quantification of different target molecules right in the HT-irEP extracts, because of the absence of interfering effects from bulk compounds when sensitive analytical techniques are used.

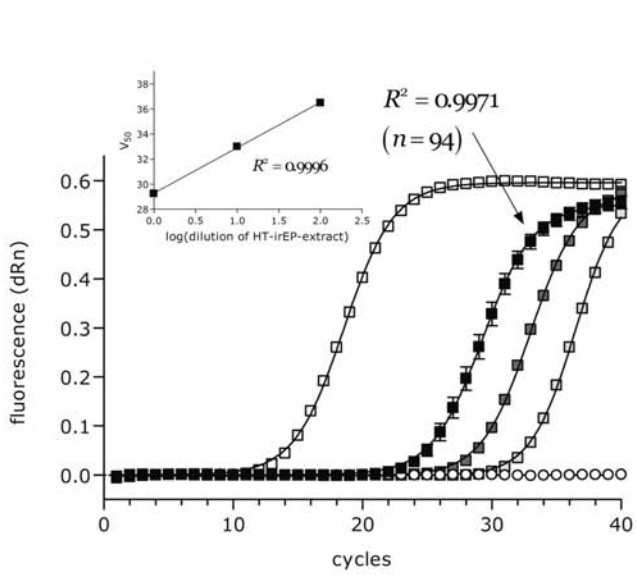
To detect the liberation of plasmids that bear genes of recombinant proteins, we have designed specific primers that allow amplification of specific fragments from corresponding DNA constructs (**Fig. 5**). Polymerase chain reaction, including real-time PCR, was successfully conducted directly in the HT-irEP extracts, indicating the presence of the plasmids in the HT-irEP extracts (**Fig. 4, 5**).

Table 1 presents quantitative analysis of target molecules (total proteins, GOx, eGFP, and plasmid pYES2-αMF-GOx) in HT-irEP extracts from yeast.

The GOx was expressed extracellularly (n = 96) into media, where its activity was measured—that is, before HT-irEP treatment (**Fig. 1**). The coefficient of variation (CV) for GOx activity was around 10%, and the data were normally distributed, which satisfies the initial criteria for any library manipulations (e.g., screening) for instance in course of directed protein evolution. Then, 94 samples from this library were HT-irEP treated, and liberation of the DNA vector-possessing gene of GOx was detected by PCR (randomly chosen n = 13; **Fig. 5A**) and also quantified by real-time PCR (n = 94; **Fig. 4, 5B** and **Table 1**). Statistical data were normally distributed, with CV of approximately 24%. It is important to note that the linearity of the plasmid determination in the diluted sample (embedded plot in **Fig. 4**) indicates that the content of the HT-irEP extracts does not interfere with the real-time PCR. Therefore, HT-irEP extracts can be directly applied for any type of PCRs, and the success completely depends on the primers and optimization of the reaction parameters.

The recombinant eGFP was expressed intracellularly, and its allocation has been verified by a laser-scanning confocal microscope. After HT-irEP and 1 h of postpulsation incubation, the eGFP is already liberated in a detectable amount (65 ± 11 ng mL^{-1}), and kinetics of liberation shows that after 5 h, around 56% (223 ± 18 ng mL^{-1}) of eGFP-originated fluorescence is liberated (**Fig. 3**). This observation matches earlier observations that irEP treatment liberates up to 60% to 90% of the specific activity of the following yeast cytoplasmatic homologous enzymes within 4 to 8 h of postpulsational incubation: glyceraldehyde 3-phosphate dehydrogenase,[9,13] 3-phosphoglycerate kinase,[9,10,13] hexokinase,[9] β-D-galactosidase,[9,11] invertase,[12] alcohol dehydrogenase,[10] and glutathine reductase.[10] Once again, the liberation of the corresponding DNA vector that bears the gene of the recombinant protein has been detected by conventional PCR directly in HT-irEP extracts (**Fig. 5C**).

The electropulsation is associated with very fast ion release from the first applied pulses. Therefore, it requires performing



	Number of cycles at V_{50} ± SEM	*n*	DNA template concentration
Positive control - pYES2-Amf-GOx	19.08 ± 0.025	2	3.5 ng µl^{-1}
HT-irEP-extract 1:1	29.24 ± 0.028	94	2.096 pg µl^{-1}
HT-irEP-extract 1:10	32.99 ± 0.038	1	0.227 pg µl^{-1}
HT-irEP-extract 1:100	36.48 ± 0.061	1	0.020 pg µl^{-1}
Negative control - water	-	2	-

SEM – standard error of mean
V_{50} – number of cycles at halfway between bottom (0) and top for the Boltzmann sigmoid

FIG. 4. pYES2-αMF-GOx liberation by the high-throughput irreversible electropermeation (HT-irEP) method (n = 94; ±SD). Different dilutions of the HT-irEP extract (1:1, 1:10, and 1:100) indicate that its content does not interfere with real-time PCR reaction, proved by linearity ($R^2 = 0.9996$) of the embedded plot. Positive control is with purified pYES2-αMF-GOx 3.5 ng µL^{-1}, and negative control is dH$_2$O.

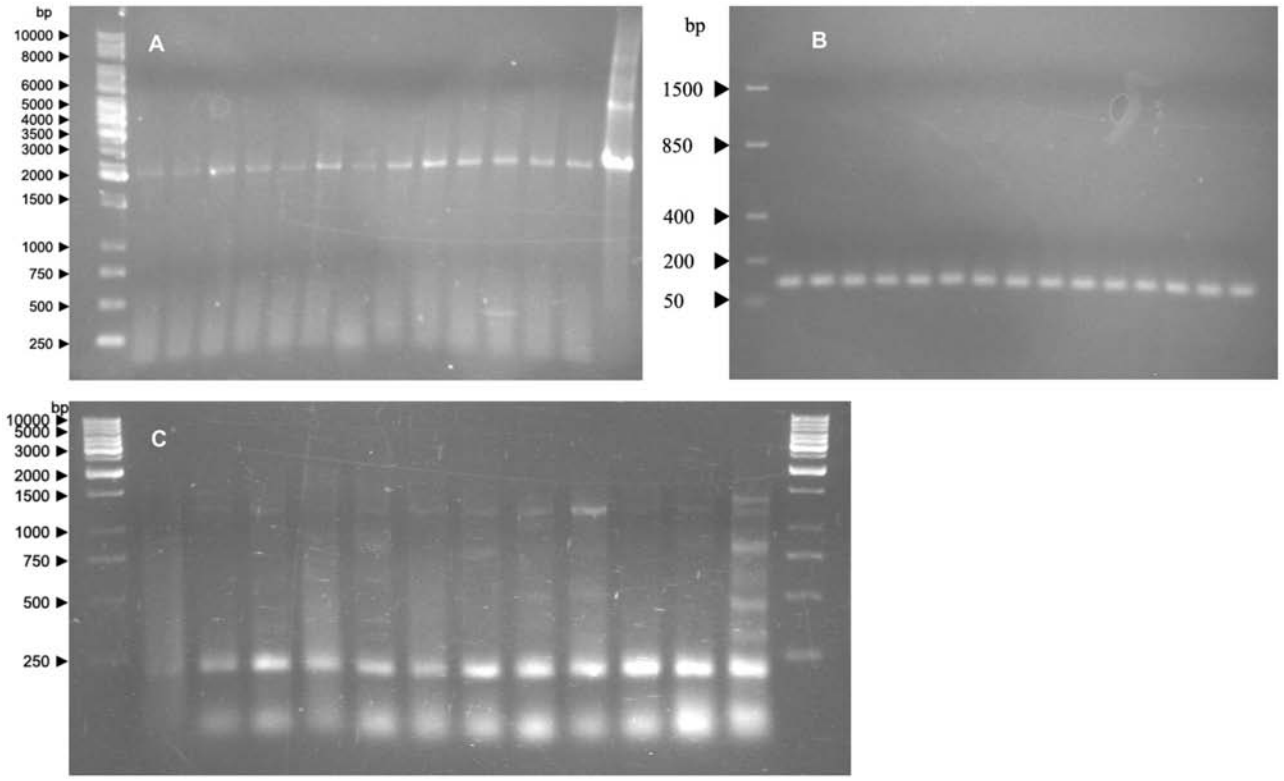


FIG. 5. DNA vector liberation from high-throughput irreversible electropermeation (HT-irEP)–treated yeast *Saccharomyces cerevisiae*. PCR products are obtained by (**A**) PCR of the HT-irEP extracts with pYES2-αMF-GOx (PCR product = 2121 bp, 1% agar gel, 100 V, 1.25 h) and (**B**) real-time PCR of the HT-irEP extracts with pYES2-αMF-GOx, with samples randomly chosen from n = 94 (PCR product = 107 bp, 2% agar gel, 72 V, 1.5 h), and (**C**) PCR of the HT-irEP extracts with pYES2-eGFP (PCR product = 245 bp, 1% agar gel, 85 V, 45 min). DNA ladders: MBI Fermentas GeneRuler™ #SM0311 and FastRuler™ #SM1103.

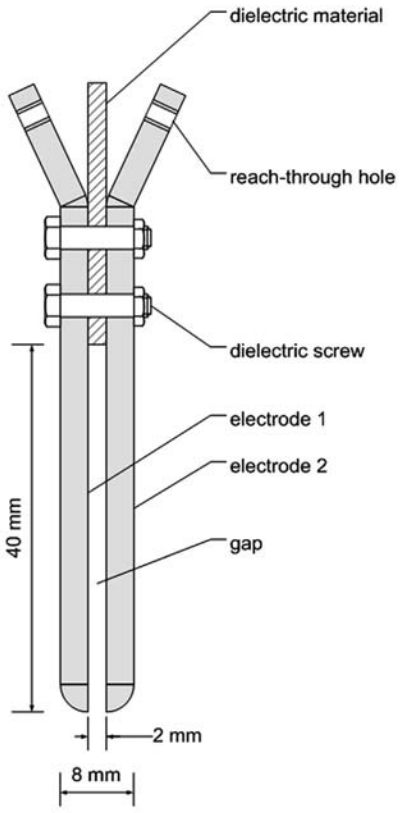


FIG. 6. Design drawing of the electrodes pair for the high-throughput irreversible electropermeation (HT-irEP) treatment of the yeast *Saccharomyces cerevisiae* library directly in the 96-deep-well microplate. The electrodes were connected to the single-channel Cyto Pulse PA-3000S Laboratory PulseAgile® Electroporation System (Cyto Pulse, Inc., Glen Burnie, MD). External dimensions of the electrodes exactly match the internal dimensions (8 × 8 mm) and the shape of the deep well; therefore, electrodes displace whole deep-well content (500 µL) into the gap between them.

electropulsation in low-conductivity media such as dH$_2$O (~µS cm^{-1}) to avoid a temperature increase due to Joule heating. In general, yeast easily survive a drastic change in an osmotic environment without cell lysis,[19] which is also evident in our observations (**Fig. 2**). Replacing nutrient media with low-conductivity media for irEP treatment is no methodological obstacle for the wide-ranging applications of the HT-irEP method because pulsation media can contain osmotic stabilizers such as sorbitol or mannitol, if necessary. Therefore, it is only a matter of the media optimization to apply it to any cell type (all types of bacteria or mammalian cells).[8]

HT-irEP treatment and following incubation result in the appearance of plasmids in the incubation buffer in sufficient amounts for PCR. However, incubation of yeast with IB, but without irEP treatment does not result in liberation of the plasmid in a detectable amount; therefore, the observed effect can be assigned to the HT-irEP treatment but not to cell lysis due to osmotic shock by the incubation buffer. Anyway, plasmid liberation by HT-rEP does not raise any conflicts if we take into account that plasmids are charged molecules, and usually they are delivered into the cell by a relative process—electroporation. Furthermore, recently, a direct transfer of plasmid between yeast and bacteria by high-intensity electric field pulses (electroduction) was carried out.[20] To our knowledge, this article presents the first evidence for the possibility of electroinduced liberation and detection of plasmid DNA from yeast into the incubation media.

One of the important statistical requirements for any HTS assay is low variability (CV < 15%).[21] However, in these HT-irEP trials, we had relatively high variability in some samples with CV ~20% to 25% (**Table 1**), which is high for an HTS method. However, there are 2 possible explanations for this fact: (1) the samples were treated by a single-channel prototype of the device in a sequential manual manner, and (2) organic matter accumulates on the anode surface during repeated use of the electrodes, which reduces the electrode's efficiency between sample-to-sample treatments. A relatively simple way to overcome both of these technical constraints and resolve the problem is to use an array of 96 electrodes (according to our design) connected to a system capable of accommodating an array of electrodes, such as the PA-4000/PA-96WS PulseAgile® 96-Well Electroporation System (Cyto Pulse, Inc.). Such an electropulsing system, in combination with our electrodes, will be capable of reducing the sample variability in the treated library due to the minimization of the systematic error. Therefore, the postpulsation electrode regeneration is required to recover the efficiency of the treatment. After the optimization of the HT-irEP, the method can be easily implemented for HTS systems.

The HT-irEP method is a reliable, robust, simple, and cheap method for the liberation of water-soluble cell content, including water-soluble proteins and DNA vectors at detectable quantities. HT-irEP extracts are diluted enough that they can be directly applied for further sensitive analysis, including enzyme assay, PCR, and possibly mass spectrometry. That is why HT-irEP sample preparation can be used for library screening where secretion of recombinant enzyme does not lead to sufficiently high content of the active enzyme. In addition, the HT-irEP method can be used alone for high-throughput plasmid liberation and fast amplification with subsequent sequencing of the PCR products. Also, HT-irEP can be used for the purposes of metabolic engineering for the screening of metabolic mutant libraries with different expression levels of yeast homologous enzymes. HT-irEP provides a real alternative to existing methods of HT sample preparation because it can be easily integrated into automated HTS systems.

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