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SHORT COMMUNICATION

An improved yeast transformation method for the generation of very large human antibody libraries

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Antibody library selection by yeast display technology is an efficient and highly sensitive method to identify binders to target antigens. This powerful selection tool, however, is often hampered by the typically modest size of yeast libraries ($\sim 10^7$) due to the limited yeast transformation efficiency, and the full potential of the yeast display technology for antibody discovery and engineering can only be realized if it can be coupled with a mean to generate very large yeast libraries. We describe here a yeast transformation method by electroporation that allows for the efficient generation of large antibody libraries up to 10^{10} in size. Multiple components and conditions including $CaCl_2$, $MgCl_2$, sucrose, sorbitol, lithium acetate, dithiothreitol, electroporation voltage, DNA input and cell volume have been tested to identify the best combination. By applying this developed protocol, we have constructed a 1.4×10^{10} human spleen antibody library essentially in 1 day with a transformation efficiency of 1–1.5×10^8 transformants/μg vector DNA. Taken together, we have developed a highly efficient yeast transformation method that enables the generation of very large and productive human antibody libraries for antibody discovery, and we are now routinely making 10^9 libraries in a day for antibody engineering purposes.

Keywords: antibody discovery/antibody engineering/antibody libraries/yeast electroporation

Therapeutic antibodies generated by animal immunizations *in vivo* or by recombinant antibody display technologies *in vitro* have been successful in the clinic and as such have validated these technologies as effective drug discovery techniques. Although it is typically expected that monoclonal antibodies derived from animal immunizations are of sufficiently high affinity to achieve therapeutic efficacy, developing therapeutic antibodies from animal immunizations requires either humanizing the non-human antibodies or utilizing transgenic animals expressing human antibodies. Direct selection of fully human antibodies from pre-established antibody libraries by *in vitro* display technologies (phage, bacterial, yeast, mammalian cell and ribosomal displays; Becker and Guarente, 1991; Griffiths *et al.,* 1994; Gietz *et al.,* 1995; Feldhaus *et al.,* 2003; Chao *et al.,* 2006; Gai and Wittrup, 2007) offers a valuable parallel approach and may present the best alternative in cases where the target antigen fails to elicit a productive immune response *in vivo*.

Human antibody libraries have been engineered to display full-length antibodies or various antibody fragments such as Fab, scFv and dAb. The libraries are generally constructed by capturing antibody diversities from B cell repertoires into the libraries, sometimes with additional synthetic diversities in the variable regions (He and Khan, 2005). Antibody libraries have also been constructed by synthetically introducing randomized CDR residues in limited human antibody frameworks (Helmuth *et al.,* 2001). Because the antibody heavy and light chains are separately amplified and reformatted into the library display format, new pairings between VH and VL are formed during this process. Although this VH–VL shuffling creates novel antigen-binding sites, it also very significantly increases the theoretical library diversity. Several strategies have been developed to engineer better and more productive antibody libraries by reducing the theoretical library diversity and the actual library size required to effectively sample the library. These strategies include intelligently designed synthetic or semi-synthetic libraries (Helmuth *et al.*, 2001), and immune (He and Khan, 2005) or pseudoimmune (Hoet *et al.*, 2005) libraries generated from less diverse but potentially more reactive B cell repertoires. Though these approaches may be effective in significantly reducing the theoretical library diversity by several or even many logs, the need for large library sizes and methods to generate them will always complement library designs and increase the chance to identify antibody hits regardless of their rarity.

Yeast surface display of antibody fragments provides exquisite selection power to enrich high-affinity antigen binders by employing fluorescence-activated cell sorting (FACS). Although yeast library sizes in the 10^9 range have been previously reported (Hoogenboom, 2002, 2005; He and Khan, 2005), typical yeast display library sizes ($\sim 10^7$) tend to be significantly smaller than those achieved by other display technologies (Griffiths *et al.*, 1994; Gietz *et al.*, 1995). We describe here a method by which very large yeast libraries can be easily constructed within a short period of time. This simple yet highly effective electroporation method allows the construction of non-immune human antibody libraries up to 10^{10} in size. Pilot library selections have identified fully human antibodies to TNFα with low nanomolar affinities, thereby demonstrating the productivity of this library.

Abbreviations: scFv, single-chain fragment variable; VH, variable heavy; Vκ, variable light, kappa; hTNFα, human tumor necrosis factor-α.

High-efficiency transformation of yeast by electroporation

Foreign DNA is transformed into yeast in the early logarithmic growing phase by electroporation. It is thought that the electric field delivered as an exponentially decaying pulse induces transient pore formations on the cell membrane through which the DNA molecules are able to pass into the cells (Lee *et al.*, 2006). The transformation efficiency is affected by the electric field strength, yeast strain, cell number and condition, electroporation volume and buffer (Becker and Guarente, 1991; Neumann *et al.*, 1996; Sblattero and Bradbury, 1998, 2000; Lipovsek *et al.*, 2007; Segal *et al.*, 2007; Rothe *et al.*, 2008). We developed our electroporation protocol for *Saccharomyces cerevisiae* by extensively optimizing several conditions to identify the best combination for maximal transformation efficiency (Fig. 1 and Supplementary material, available at *PEDS* online). These include the combination of lithium acetate (LiAc) and dithiothreitol (DTT) as cell conditioning agents, both of which have been used to enhance the frequency of yeast transformation (Simon, 1993; Neumann *et al.*, 1996; Segal *et al.*, 2007), and the inclusion of sorbitol and calcium chloride (Neumann *et al.*, 1996; Lipovsek *et al.*, 2007; Rothe *et al.*, 2008) in the electroporation buffer. The combination of these reagents consistently resulted in a transformation efficiency of $3 \pm 1 \times 10^7$ transformants using 1 μg vector DNA and 100 μl yeast cells (Fig. 1A, black column). Assuming 1 OD_{600} equals 10^7 cells and cell density during electroporation is 1.6×10^9 cells/ml, we estimated that roughly 2% of yeast cells were successfully transformed. We next tested if the cell volume per cuvette could be scaled up to increase the electroporation yield. Surprisingly, the transformation efficiency was significantly enhanced to over 1×10^8 transformants/μg vector DNA when the cell volume was increased from 100 to 200 μl per cuvette. As we further increase the cell volume to 300 and 400 μl with the same cell density and cell to DNA ratio, the transformation efficiency improved 5-fold to about 1.3×10^8, and as large as 5×10^8 yeast library size could be easily made (Fig. 1B). Similarly, high transformation efficiency and large library sizes were reproduced by different operators and also on two different electroporators (BioRad Gene Pulser model # 1652076 and Gene Pulser II model # 1652108).

One interpretation of the improved transformation efficiencies and yield is that the increased cell volumes results in a higher degree of survival of all yeast cells after electroporation. Though we did observe a slight 20% increase in cell viability when cell volume was increased from 100 to 200 μl, this increase along could not completely explain the significant improvement in electroporation efficiency by more than 3-fold. It is our view that the significant transformation improvement is the combined product of more transformed cells and better cell survival. As more cells are electroporated by the same energy (25 μF), it is conceivable that there will be less transformed cells, but the better cell viability could maintain a similar or better apparent transformation efficiency. Indeed, at 400 μl cell per cuvette, the viability of cells after electroporation is improved by 3-fold while the transformation efficiency is improved by 5-fold.

The effect of DNA quantity on transformation efficiency was next examined by electroporating 400 μl yeast suspension with 1, 2, 3, 4 or 8 μg of vector DNA while maintaining the same 1:3 vector to insert ratio. Interestingly, the transformation efficiency did not significantly change when up to 4 μg vector DNA was used for each transformation, and some decrease was observed with 8 μg vector DNA input (Fig. 2A). As expected, the yield of the transformed library size correspondingly increased with increasing amounts of DNA input (Fig. 2B). Overall, the use of 4 μg linearized vector appears to be the most efficient condition. To explore whether the 12 μg DNA insert (to achieve a vector to insert ratio of 1:3) can be reduced, different vector to insert ratios were tested. Our results showed that the ratio could be reduced to 1:1.5 without any significant impact on the transformation efficiency (Fig. 2C). However, lower transformation efficiencies were observed when the vector to insert ratio was further reduced to below 1:1 or increased to above

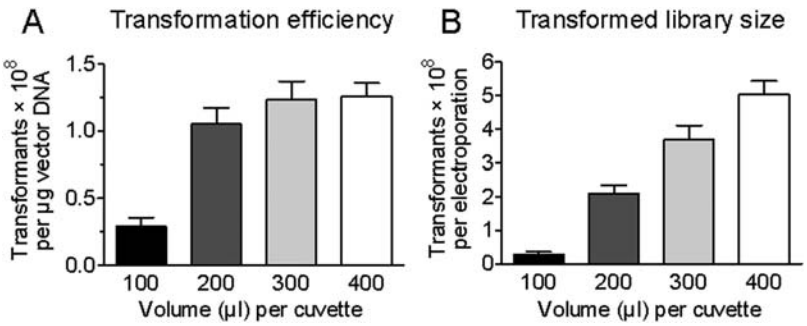

Fig. 1. Electroporation efficiency is significantly improved by increasing the volume per cuvette. *Saccharomyces cerevisiae* were grown overnight to stationary phase (OD_{600} of ~3). An aliquot of the culture was inoculated into 100 ml of YPD media (10 g/l yeast nitrogen base, 20 g/l peptone and 20 g/l D-(+)-glucose) to reach OD_{600} of ~0.3. The cells were grown until OD_{600} reached ~1.6 before collecting by centrifugation. The cells pellet was washed twice with 50 ml of cold water, and once with 50 ml electroporation buffer (1 M sorbitol/1 mM $CaCl_2$). Cells were conditioned in 20 ml of 0.1 M LiAc/10 mM DTT by incubation in culture flask with shaking (225 rpm) for 30 min at 30°C, washed one more time with 50 ml of electroporation buffer, then suspended in 100–200 μl of the same buffer to reach 1 ml volume. This corresponds to $\sim 1.6 \times 10^9$ cells/ml. The scale up of culture and washes volumes should be proportional if more electrocompetent yeast suspension is needed. 100, 200, 300 or 400 μl of cell suspension were used for electroporation per cuvette and 1, 2, 3 or 4 μg linearized vector with corresponding 3, 6, 9 or 12 μg DNA insert (vector to insert ratio = 1:3) were used. Cells were electroporated at 2.5 kV and 25 μF in a BioRad GenePulser cuvette (0.2 cm electrode gap). Typical electroporation time constant ranged from 3.0 to 4.5 ms. After electroporation, cells were suspended in 10 ml of 1:1 mix of 1 M sorbitol: YPD media and incubated at 30°C for 1 h. Finally, the cells were collected and cultured in SD-UT media (-ura, -trp) which contained 20 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids, 5.4 g/l Na_2HPO_4, 8.6 g/l $NaH_2PO_4{\cdot}H_2O$ and 5 g/l casamino acids. Number of transformants was determined by plating 10-fold serial dilutions of transformed cells at 30°C on selective SD-UT plates and counting colonies after 3 days. The transformation efficiencies (**A**) were expressed as transformants/μg vector DNA (mean ± standard deviation). The yields (**B**) were expressed as total number of transformants per cuvette (mean ± standard deviation).

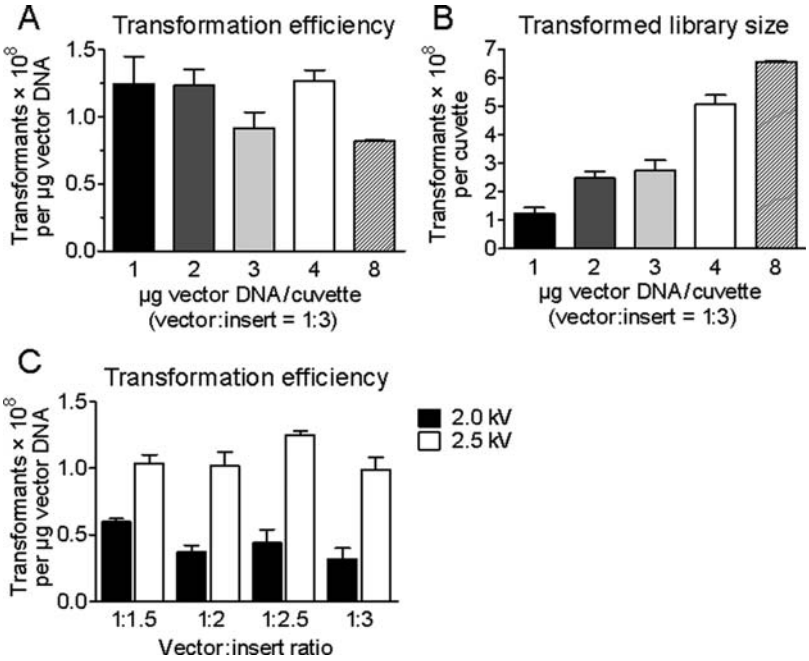

Fig. 2. Increased DNA input and high voltage, but not vector to insert ratio, are critical for maximal transformation efficiency. *Saccharomyces cerevisiae* were treated as described in Fig. 1. Four hundred microliters of yeast cell suspension (1.6×10^9 cells/mL) were electroporated using 1, 2, 3 or 4 µg vector DNA per cuvette (vector to insert ratio is always maintained at 1:3). After 72 h the numbers of colonies were determined and represented as (**A**) transformation efficiency, transformants/µg vector DNA (mean ± standard deviation) and (**B**) transformation yield, total number of transformants per cuvette (mean ± standard deviation). Additionally, (**C**) 400 µl yeast suspension were electroporated by 2 or 2.5 kV and different vector to insert DNA ratios. The transformation efficiencies are expressed as in (A).

Table I. Enhanced electroporation efficiencies of *S.cerevisiae* by combining LiAc, DTT, sorbitol and $CaCl_2$

Treatment[a,b]	Electroporation efficiency[c] $\times 10^7$	Yield per cuvette[d] $\times 10^7$	% efficiency[e]	% loss in efficiency	Maximal library size[f]
1. Combined protocol	13.5 ± 2.6	54.2 ± 1.1	100	0	1.4×10^{10}
2. No LiAc	1.7 ± 0.1	6.7 ± 0.4	12	88	1.7×10^9
3. No DTT	0.81 ± 0.02	3.3 ± 0.1	6	94	8.1×10^8
4. Sorbitol/$MgCl_2$	11.4 ± 0.9	45.5 ± 3.5	84	16	1.1×10^{10}
5. Sorbitol only	9.0 ± 1.8	36.0 ± 7.1	66	34	9.0×10^9
6. Sucrose/$CaCl_2$	0.4 ± 0.3	1.6 ± 1.5	3	97	4.1×10^8
7. Sucrose/$MgCl_2$	0.09 ± 0.01	0.4 ± 0.1	1	99	8.9×10^7

[a]Combined protocol as described in Fig. 1 and Supplementary material available at *PEDS* online. Electroporation of 400 µl of yeast suspension (1.6×10^9/ml) with 4 µg of vector and 12 µg of DNA insert.
[b]For the other treatments, the combined protocol was followed as in footnote a except for the following changes: (2) cells were pre-treated only with DTT; (3) cells were pre-treated only with LiAc; (4) $MgCl_2$ was used instead of $CaCl_2$. (5) $CaCl_2$ was excluded from electroporation buffer; (6) 270 mM sucrose was used instead of 1 M sorbitol; and (7) 270 mM sucrose/1 mM $MgCl_2$ was used as electroporation buffer.
[c]Number of transformants/µg of vector DNA.
[d]Total number of transformants per cuvette electroporation reaction.
[e]Assuming the transformation efficiency obtained using our protocol equal to 100%.
[f]Assuming average efficiency of 1.35×10^8 transformants/µg vector DNA and 25 cuvette electroporation reactions in a single experiment.

1:5 (data not shown). Furthermore, the 2.5 kV electroporation voltage was found to be critical as reduction of electroporation voltage from 2.5 to 2 kV or lower resulted in a significant loss of transformation efficiency, and this loss in transformation efficiency could not be rescued by reducing the cell volume to 100 or 300 µl (Fig. 2C and data not shown).

Since the yeast cells were pretreated with LiAc and DTT followed by electroporation in the presence of $CaCl_2$ and sorbitol, each of these components were examined for their importance in achieving maximal transformation efficiency (Table I). As shown, the elimination of LiAc or DTT pretreatment resulted in a respective 88% or 94% loss in transformation efficiency. Similarly, elimination of sorbitol or replacing it by sucrose led to a loss of >97% efficiency (Table I and data not shown). This is consistent with the previous findings that the ability of sorbitol to stabilize osmotic pressure and support yeast membrane integrity after electroporation was at least partially responsible for achieving higher transformation efficiency (Lee *et al.*, 2006; Lipovsek *et al.*, 2007). The presence of $CaCl_2$ during electroporation, presumably to facilitate DNA adhering to cell membrane (Rothe *et al.*, 2008), appeared to be the least critical parameter as its elimination only decreased the transformation efficiency by 34% and its substitution by $MgCl_2$ only resulted in a small 16% decrease.

Our data are based on transforming yeast cells by linearized vector backbone and library DNA inserts and the transformed

yeast cells homologously recombine or gap-repair the vector backbone with the library DNA insert. We believe that a similar improvement in transformation efficiency could be observed if yeast cells are transformed by intact circular DNA, but it remains to be demonstrated experimentally. It should also be noted that though we derived the library size by plating transformed yeast cells onto SD-UT plates and count colonies after 3 days, we have found that by sequencing DNAs from individual colonies roughly 85% of colonies plated immediately after transformation will have multiple sequences as determined by sequencing reaction chromatograms. This sequence multiplicity suggests that many individual cells have been transformed by multiple copies of DNA during electroporation. Alternatively transformed yeast cells after electroporation may not be easily diluted into single cell suspension such that many colonies were actually grown from cell clumps rather than individual cells. It is therefore possible that the transformation efficiency and library size reported here could be actually significantly higher. To increase the useful sequences for library diversity analysis, we routinely plate the yeast library again after two passages in liquid cultures to resolve the sequence multiplicity before colony PCR and sequencing analysis. These two additional passages in liquid culture and the subsequent plating of library cells significantly resolve the sequence multiplicity and reduce the number of sequence to one per colony.

Generation of a 10^{10} naïve human spleen antibody library for yeast display

We applied this optimized electroporation protocol in constructing a large human antibody library with a size approaching to those of typical phage libraries (Gietz *et al.*, 1995; Suga and Hatakeyama, 2003). Human VH and Vκ cDNA fragments were separately amplified by polymerase chain reactions (PCR) from spleen polyA RNA using previously reported primers (Thompson *et al.*, 1998). The Vκ fragments were proportionally pooled together such that each germline family would be equally represented in the library. scFv fragments were separately prepared from VH and pooled Vκ fragments by overlapping PCR. Multiple yeast libraries were generated from these scFv fragments mixed with linear vector DNA by electroporation and proportionally pooled together to maintain equal representations of all VH germline sequences. A large number of yeast colonies were sequenced to analyze the scFv germline composition after two passages in liquid cultures and the result confirmed a high diversity. With the exception of the rare VH7 germline genes, all the germline families were identified and their representations were roughly proportional to their germline gene numbers in humans (Fig. 3A).

We next used the pooled scFv library DNA to scale up library transformation to show that the new electroporation protocol can be scaled to generate very large yeast libraries. Indeed, in this experiment, we were able to generate a 1.4×10^{10} yeast library in 1 day simply by increasing the electroporation to 20 identical cuvettes. It is routine now in our laboratory to generate 1 to 2×10^9 size yeast libraries for antibody affinity maturation or other purposes by performing yeast transformation in triplicated cuvettes.

To demonstrate that the constructed libraries can produce antibodies with reasonably good affinity, the human spleen antibody library was selected against human TNFα. We first did two rounds of magnetic-activated cell sorting to reduce the library size to $\sim 10^7$ cells, followed by several rounds of FACS essentially as previously described (Fig. 3B; Weaver

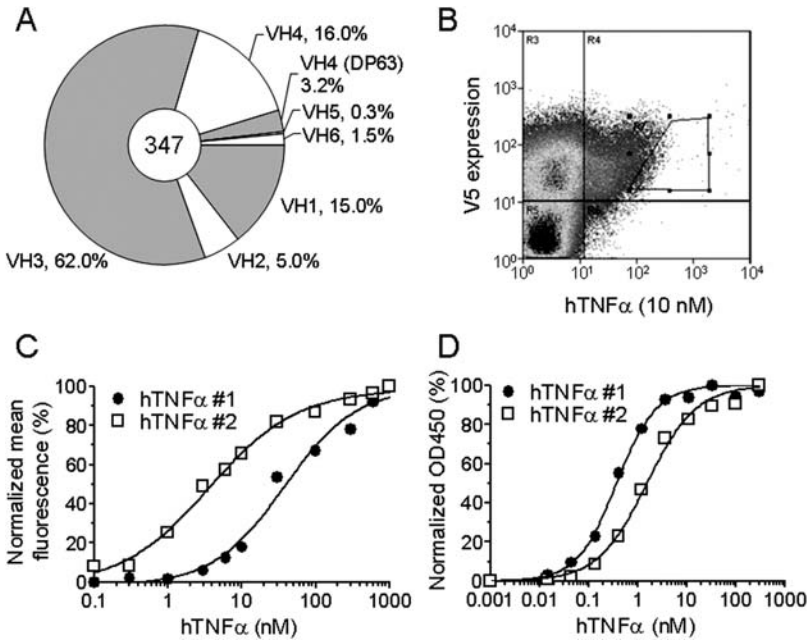

Fig. 3. Naïve human spleen antibody library diversity and productivity. (**A**) Observed VH library composition. The yeast library was plated on selective SD-UT (-ura, -trp) plates after passage of the original culture. Sequences were obtained from 347 colonies. The VH regions were aligned to the human VH germline sequences to determine their origins. (**B**) Representative flow cytometry dot plot after two rounds of TNFα selection. Yeast cells are doubled-labeled by biotinylated hTNFα (10 nM) and mouse anti-V5 IgG followed by Alexa 488-conjugated chicken anti-mouse IgY and R-PE (R-Phycoerythrin) conjugated streptavidin. (**C**) Affinity of hTNFα-binding scFv measured on the yeast cell surface. The mean fluorescence intensity as determined by flow cytometry is plotted against increasing concentrations of biotinylated antigen and fitted to a sigmoidal binding curve after normalization of the data. (**D**) Affinity of the hTNFα-binding antibodies after IgG conversion measured by ELISA.

et al., 1988; Becker and Guarente, 1991). Fifty percent or more of the clones expressed the carboxyl-terminal V5 tag in every round, indicating that the majority of the library is expressed as a full-length scFv on the yeast surface in a format suitable for screening for antigen-binding activity. This is comparable to the literature standards for non-immune scFv libraries (Weaver *et al.*, 1988). Multiple binders to TNFα were identified. By sequence analysis, we have identified TNFα-binding svFv containing VH from VH1, VH3 and VH4 human germline sequences and Vκ from Vκ1 and Vκ2 germline sequences, indicating that the library contains antigen binders from a diverse antibody pool. One of the scFv showed a 4 nM affinity and another showed a 40 nM affinity to TNFα on yeast surface (Fig. 3C). Upon IgG conversion, the 4 nM binder maintained a similar binding affinity (1.5 nM by ELISA), whereas the 40 nM binder showed a significant increase in affinity (0.3 nM by ELISA) (Fig. 3D). This increase in apparent affinity could be due to the avidity of the converted IgG interacting with two TNFα in the same homotrimeric TNFα protein.

In summary, a highly efficient and reproducible yeast electroporation procedure has been developed that enables the construction of very large ($>10^{10}$) human antibody libraries for yeast display. This new procedure removes a significant bottleneck in applying yeast display technology as a practical tool to sample a much larger antibody diversity space previously unexplored, and the library is now only limited by the size of yeast culture that can be grown in laboratories.

Supplementary data

Supplementary data are available at *PEDS* online.

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