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Note

Desalting DNA by Drop Dialysis Increases Library Size upon Transformation

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It is often desirable to obtain gene libraries with the greatest possible number of variants. We tested two different methods for desalting the products of library ligation reactions (silica-based microcolumns and drop dialysis), and examined their effects on final library size. For both intramolecular and intermolecular ligation, desalting by drop dialysis yielded approximately 3–5 times more transformants than microcolumn purification.

Key words: directed evolution; drop dialysis; library construction; ligation; microcolumn

In many molecular biology protocols, it is useful, or even essential, to maximize the number of colonies that result from a cloning experiment (*i.e.*, ligation, desalting, and transformation). This is especially true in the field of directed evolution, in which the Darwinian principles of random mutagenesis and selection are used to identify proteins with new or improved properties. The first step in a directed evolution experiment is to introduce molecular diversity into parental gene sequences. Many random mutagenesis protocols have been developed,[1,2] including methods for random point mutagenesis (*e.g.*, error-prone PCR), random homologous recombination (*e.g.*, DNA shuffling), and random non-homologous recombination (*e.g.*, Incremental Truncation for the Creation of Hybrid enzYmes, ITCHY). The next step is to capture the diversity that has been generated, by cloning the pool of mutagenized DNA molecules into an appropriate expression vector. Transformation of a suitable host, typically *Escherichia coli*, with the cloned DNA yields a library that can be stored for use in downstream screening or selection steps. Often, the library screen or selection is very high-throughput; it is common to design directed evolution experiments with the capacity to interrogate millions, or even billions, of variants. In these high-throughput cases, it is critical to optimize the library cloning and transformation steps, because large, diverse libraries are the most likely to include variants with improvements in the desired property.[3]

Electroporation is the method of choice for transforming *E. coli* with the products of library ligation reactions. Very high transformation efficiencies can be obtained,[4] resulting in large libraries. However, the high electric field strengths that are used (12–18 kV/cm) mean that efficient transformation requires low-conductivity samples, to prevent arcing. Hence, each library ligation reaction must be desalted prior to electroporation. This purification step is commonly carried out with silica-based microcolumns. A previous study that tested electroporation efficiencies with intact plasmid DNA (rather than with the products of ligation reactions) showed the microcolumn desalting method to be highly effective.[5] However, an older study showed that drop dialysis, in which a 5–100 µL drop is placed on a floating membrane filter, can also result in effective desalting, with DNA recovery rates of 98–99%.[6] Here we present a rigorous comparison of the two desalting methods, and show that drop dialysis is preferred in the construction of large libraries.

First, two parental sequences were randomly recombined using ITCHY: (i) the *trpF* portion of the bifunctional *E. coli trpCF* gene (GenBank accession no. NP_415778); and (ii) a cDNA encoding residues 36-367 of the β2 subunit of the *Rattus norvegicus* voltage-gated potassium channel (Kvβ2; GenBank accession no. NM_017304). The TrpF and Kvβ2 proteins share the same $(\beta\alpha)_8$ barrel fold, but their sequences are highly divergent ($<$10% sequence identity). The genes were each cloned into vector pInSALect[7] and then recombined onto the same linearized vector molecule by PCR, as described previously.[8] Our library was constructed using ITCHY with time-dependent truncation.[9]

At the final stage of library construction, three identical intramolecular ligations (90 µL) were set up, each of which comprised blunt-ended DNA from the ITCHY protocol (180 ng), 1× Fermentas T4 DNA Ligase Buffer, and 30 units of T4 DNA ligase (Fermentas, Vilnius, Lithuania). The ligation reactions were incubated at 16 °C for 16 h, and then heat inactivated (65 °C, 10 min). After heat inactivation, each of the three reactions was split into three 30-µL aliquots. One aliquot was desalted using a microcolumn (EZNA MicroElute Cycle Pure kit; Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's guidelines. The desalted DNA was eluted from the column with 30 µL of elution buffer (10 mM Tris, pH 8.5). The second aliquot was desalted by drop dialysis. A standard Petri dish was half-filled with 30 mL of deionized (Milli-Q) water. A mixed cellulose ester membrane filter (pore size 0.025 µm, diameter 25 mm, MF-Millipore, Billerica, MA, USA) was floated on the water. The 30-µL aliquot of the ligation reaction was pipetted onto the membrane, covered with the lid of the Petri dish, and left to dialyze for 1 h. After dialysis, the desalted sample was recovered

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Abbreviations: ITCHY, incremental truncation for the creation of hybrid enzymes; PCR, polymerase chain reaction

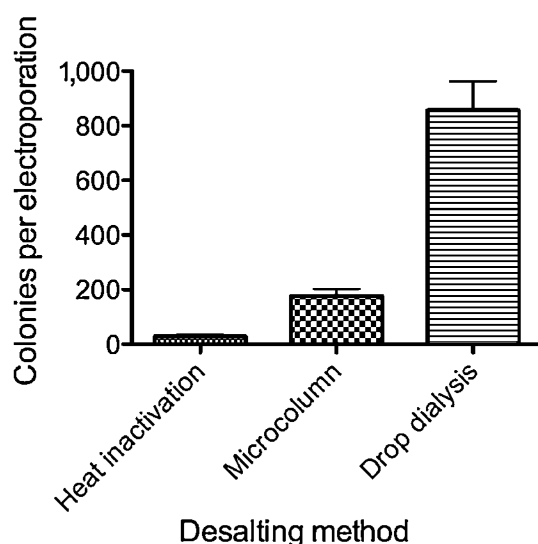

Fig. 1. Comparison of DNA Desalting Methods in the Construction of ITCHY Libraries.

Intramolecular ITCHY ligation reactions were heat inactivated only, purified with a silica-based microcolumn, or purified by drop dialysis. Aliquots of the desalted reaction products were used to transform *E. coli* by electroporation, and the numbers of colonies on dilution plates were used to estimate the total number of transformants resulting from each electroporation. Colony counts plotted are mean ± SEM for three independent ligation reactions.

from the top of the membrane, and the volume of the sample was adjusted to 30 µL with water. The third 30-µL sample from each ligation was not desalted further. Aliquots (2 µL) of each desalted library ligation and the heat inactivated controls were used to transform 50-µL aliquots of *E. coli* DH5α-E (Invitrogen, Carlsbad, CA, USA), by electroporation. SOC medium (500 µL) was added to each aliquot of cells immediately after pulsing. The transformed cells were allowed to recover at 37 °C with shaking for 1 h, and then aliquots were spread on LB-agar plates containing chloramphenicol (34 µg/mL). Colonies were counted after 16 h of incubation at 37 °C.

Figure 1 shows the mean numbers of colonies that resulted when 6.7% of each desalted sample (2 µL of a 30 µL total volume) was used to transform *E. coli* by electroporation. On average, microcolumn purification yielded 6.4-fold more colonies than heat inactivation (without further desalting). However, drop dialysis yielded the greatest number of colonies (4.8-fold more than microcolumn purification).

Had the desalted samples from the triplicate ligations been pooled, there would have been 84 µL of each sample (heat inactivated, microcolumn purified, and dialyzed) remaining. Transforming more aliquots of electrocompetent *E. coli* with all of this material would have yielded libraries with total sizes of approximately 1.3×10^3 variants (heat-inactivated ligation), 8.0×10^3 variants (microcolumn purification), and 3.9×10^4 variants (drop dialysis) respectively. The total number of possible variants in an ITCHY library is given by the product of the lengths of the two parental genes. In this case, the number of possible variants is 597 bp × 996 bp = 594,612. Our library analysis program, GLUE,[10] estimates that 1.8×10^6 clones would be required in our ITCHY library in order to sample 95% of all possible variants. In this example, none of the three desalting methods led to a library of that size. However,

Table 1. Comparison of Methods for Desalting the Products of Intermolecular (Vector + Insert) Ligation Reactions

Desalting method	Colonies per electroporation*
Microcolumn	870 ± 50
Drop dialysis	2,930 ± 540

*Mean ± SEM for three independent ligation reactions.

our analysis with GLUE suggests that the library from drop dialysis will include approximately 6.3% of all possible variants, which is certainly preferable to the other alternatives: 1.3% of all possible variants when microcolumn purification is used; and only 0.2% of all possible variants when the ligation is heat-inactivated but not desalted.

In a second experiment, we tested the effect of varying the desalting method on the outcome of intermolecular (vector + insert) ligation. This mimics the construction of an error-prone PCR library. A 338-bp DNA fragment was ligated with the 4.3-kb expression vector pLAB101[11] after each had been digested with restriction enzymes NdeI and SpeI (both New England Biolabs, Ipswich, MA, USA). Three ligation reactions were performed in a total volume of 60 µL per reaction. Each reaction contained 150 ng of vector DNA, 35 ng of insert DNA (a 3-fold molar excess of insert over vector), 1× T4 DNA Ligase Buffer (Fermentas), and 20 units of T4 DNA ligase (Fermentas). The reactions were incubated at 16 °C for 18 h and then heat inactivated (65 °C, 10 min). Each of the three ligation reactions was split into two 30-µL aliquots. One of the two 30-µL aliquots was desalted with a microcolumn, and the other was desalted by drop dialysis (see above). Aliquots (2 µL) of the desalted reactions were used to transform *E. coli* DH5α-E by electroporation, as described above. Dilutions of the transformed cells were spread on LB-agar plates containing carbenicillin (100 µg/mL) and colonies were counted after 16 h of incubation at 37 °C. Desalting the intermolecular ligation reactions by drop dialysis yielded 3.4-fold more colonies than microcolumn purification (Table 1). This is similar to the 4.8-fold improvement observed for intramolecular ITCHY ligations (Fig. 1).

Our data indicate that drop dialysis is a highly effective method for desalting DNA, confirming earlier results.[6] In both intramolecular and intermolecular ligation tests, we obtained approximately 3–5 times more transformants when we desalted by drop dialysis, as compared with the more commonly used silica-based microcolumn purification. In this study, we tested a single brand of microcolumn (the EZNA MicroElute Cycle Pure kit from Omega Bio-Tek). However, in preliminary desalting tests with intact plasmid DNA (rather than with library ligations), conducted as described previously,[5] we found that this microcolumn and its associated purification protocol yielded identical results to a well-known but more expensive alternative (the QIAquick PCR Purification Kit, Qiagen, Valencia, CA). Hence, drop dialysis remains the superior protocol for desalting ligation reactions, regardless of the microcolumn to which it is compared. Further, a previous study found that varying the membrane filter from one with an average pore diameter of 0.01 µm to one with an average pore diameter of 0.05 µm did not change the

effectiveness of the drop dialysis protocol, although the use of membranes with very small pore diameters can increase the time required for complete removal of buffer salts.[6] The membranes used in our experiments (average pore diameter, 0.025 µm) allow for rapid dialysis while minimizing the likelihood that ligation products are lost.

Finally, drop dialysis requires less hands-on time than microcolumn purification. The membrane filter discs do require careful pipette handling as the samples are loaded onto the membrane. They are also more expensive than microcolumns (NZ$7.48 per membrane *versus* NZ$3.40 per microcolumn at the time of writing), although expert users can reduce the cost by desalting 2–4 reactions on a single membrane simultaneously. Overall, the extra care and costs required (as compared with microcolumn purification) are likely to be warranted for practitioners of directed evolution, for whom the largest possible libraries are often desirable.

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