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# High-throughput plasmid mini preparations facilitated by micro-mixing

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## ABSTRACT

**We have developed a reliable high-throughput plasmid isolation system using a 96-well plate format. This system combines a novel glass bead micro-mixing method with modified alkaline lysis and Sephacryl S-500 DNA purification procedures. Mechanical forces generated by vortexing glass beads inside each well of the 96-well plates ensure that the bacterial pellets are homogeneously resuspended, the cells are completely lyzed, and the resulting bacterial lysates are thoroughly mixed with the potassium acetate solution. The vortexing speed and duration for glass bead mixing have been standardized to facilitate plasmid DNA yields without significant adjustments.**

The efficient preparation of large numbers of high quality DNA templates with consistently high yield is essential for high-throughput molecular genetic analyses such as large scale genomic and cDNA sequencing and expression mapping. Several methodologies have been developed toward this goal (1–9). Here, we report significant improvements in high-throughput plasmid isolation. Specifically, we developed a glass bead micro-mixing system which enhances the homogeneous suspension of *Eschericheria coli* growing in narrow culture medium columns of 96-well plates. This mixing method facilitates the resuspension of bacterial pellets and the homogenization of bacterial lysates in a modified alkaline lysis plasmid mini-preparation procedure (10). Plasmids were subsequently purified by a simple two step 96-well Sephacryl-500HR filtration procedure (9). We used this high-throughput procedure to isolate recombinant plasmids for high density cDNA array expression mapping (11) and sequencing of both normal and malignant human ovarian epithelial cDNAs.

Poly(A)$^+$ RNA was isolated from primary cultures of normal human ovarian surface epithelia (HOSE) (12–14) and four fresh frozen ovarian tumors using a Stratagene messenger RNA isolation kit. Approximately 5 µg of oligo(dT)-selected RNA were used to construct each cDNA library using the ZAP Express cDNA cloning kit (Stratagene). Recombinant pBK-CMV phagemids containing the cDNAs were excised from ZAP-Express constructs by a massive excision procedure and propagated in *E.coli* strain XLOLR. The plasmids were isolated and purified as described below.

### Preparation of micro-mixing culture plates

The lid for the 96-deep-well titer plates (Beckman, cat. no. 267002 and 267007) has an indentation over each well that will hold two 3.5 mm glass beads (VWR, cat. no. 26396-521). After liberally pouring glass beads into the plate lid, the plate and lid are gently tilted so that only two beads are detained in each indentation. A 96-square-well titer plate (Beckman, cat. no. 140504) is then aligned upside down on top of the lid and the beads are transferred into the square-well plate by inverting the assembly.

### Plasmid mini-preparation

(i) Using a 96-channel pipettor (Costar cat. no. 7605), 1.2 ml of TYGPN (3,8) medium containing 50 µg/ml kanamycin are added into each well of an autoclaved 96-square-well plate with beads. Each well is inoculated with an *E.coli* transfectant colony using a sharp pointed toothpick. Toothpicks are left in the wells and the plates are incubated at room temperature for 15 min. The toothpicks are removed by pressing them into a styrofoam board and lifting the toothpicks out of the wells.

(ii) The plates are placed into a snug-fitting container (four plates fit in a 21.5 × 21.5 cm$^2$ plastic box) and covered with two layers of autoclaved Whatman 3MM Chromatography paper and a 2 cm thick soft foam cushion. The plates are then incubated at 37°C with vigorous agitation (~350 r.p.m.) for 20 h.

(iii) Bacteria are pelleted by centrifugation at 2350 *g* for 5 min at 4°C (Jouan CR422 centrifuge). After centrifugation one glass bead will be tightly embedded in each bacterial pellet. The supernatant is decanted along with the one loose bead from each well and the plates are gently blotted dry.

(iv) A 96-channel pipettor is used to add 100 µl GTE buffer (10) to each well. The plates are covered with paper towels to prevent inter-well mixing and the bacterial pellets are resuspended by vortexing on a multi-tube vortexer (VWR, cat. no. 58816-115) at speed 5 for 3 min.

(v) An aliquot of 200 µl of freshly prepared NaOH–SDS solution (10) is added to each well. The plates are vortexed at speed 3 for 10 s prior to incubation on ice for 5 min.

(vi) Ice-cold potassium acetate solution (150 µl) (10) is then added to each well. The plates are vortexed at speed 3.5 for 30 s and incubated on ice for 5 min. Centrifuge plates at 2350 *g* for 5 min

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at 4°C. Use a 12-channel pipettor to transfer the supernatant to clean square well plates.

(vii) To generate enough turbulence to mix the two solutions, 0.9 ml of ethanol are quickly dispensed into each well. Incubate the plates on ice for 2 min, centrifuge for 10 min, and decant the supernatant.

(viii) To wash the pellets, gently add 0.6 ml of 70% ethanol to each well without disturbing the pellets. Centrifuge for 5 min, decant the supernatant, and dry pellets in 55–60°C oven for 20–30 min.

(ix) Resuspend pellets in 50 µl (or less if necessary) of TE buffer containing 20 µg/ml RNaseA. Vortex at speed 2 for 30 s and incubate the plates at 4°C overnight.

## Purification of plasmid DNA

(i) Pre-equilibrate Sephacryl-500HR (Pharmacia) with three changes of an equal volume of TE buffer and store at 4°C.

(ii) Use a 96-channel pipettor to add 360 µl of the Sephacryl-500HR slurry into each well of a 96-well filtration plate (Silent Monitor, Pall, cat. no. SM045L50P). Centrifuge at 770*g* for 2 min at 4°C to remove the TE.

(iii) Transfer DNA solution into Silent Monitors, centrifuge at 770 *g* for 3 min, and collect the purified plasmids in polypropylene microtiter plates (Nalge Nunc, cat. no. 442587). Cover the plates with aluminum foil tape (Scotch, cat. no. 425, 5020-01-02) and store the DNA at –20°C.

Using this procedure, one person can isolate >3000 plasmids/week with limited assistance in transferring the supernatant after potassium acetate precipitation in order to avoid prolonged exposure of plasmids to this solution. The consistency of DNA yield was monitored by fractionating 1 µl (~1/50 of total volume) of supercoiled plasmid DNA from one or two of the plates prepared on a single day on agarose gels (Fig. 1A–D). To estimate the yield of plasmids, we digested some of the recombinant DNA with *Eco*RI and quantified the amount of DNA by comparing the restriction fragment band intensities with a DNA mass ladder (BRL, cat. no. 10068-013) (Fig. 1E). Typically 6–10 µg of recombinant plasmid DNA were isolated from each transfectant. The plasmids isolated by this procedure were used in high density cDNA array expression mapping assays and fluorescence nucleotide sequencing. Approximately 6–10 µl (1–1.5 µg) of the purified DNA were sufficient for a single set of dye primer sequencing reactions using *AmpliTaq* FS enzyme (ABI) (Table 1).

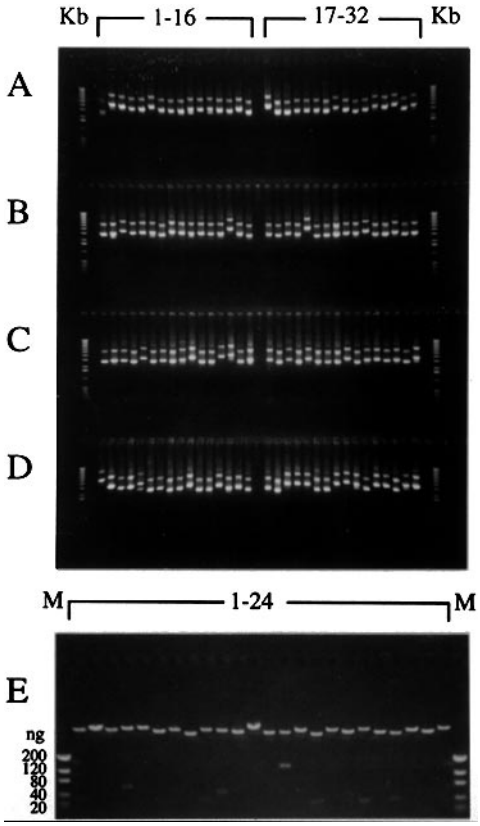


**Figure 1.** Electrophoresis of recombinant ovarian epithelial cDNAs in 0.7% agarose gel with 1× TAE and 0.2 µg/ml ethidium bromide. (**A**–**D**) Lanes 'Kb' contain 1 kb DNA ladder (100 ng, BRL) and each lane in lanes 1–32 contains 1 µl supercoiled plasmid DNA. (**E**) Lanes 'M' contain DNA mass ladder (470 ng, BRL) and each lane in lanes 1–24 contains 0.5 µl of *Eco*RI digested plasmid DNAs. The approximate amount of DNA in each mass ladder band is indicated.

**Table 1.** Summary of sequencing results of 64 randomly picked ovarian tumor cDNA

| Length of cDNA sequence determined[a] | No. of cDNA | % of total | Average no. of N per sequence |
|---|---|---|---|
| 501–550 | 15 | 23.4 | 0.6 |
| 451–500 | 18 | 28.1 | 0.9 |
| 401–450 | 10 | 15.6 | 0.7 |
| 351–400 | 8 | 12.5 | 1.5 |
| 301–350 | 4 | 6.2 | 0.8 |
| 251–300 | 2 | 3.1 | 0 |
| 201–250 | 0 | 0 | 0 |
| 151–200 | 2 | 3.1 | 0 |
| 101–150 | 1 | 1.6 | 0 |

Successful Rate: 93.8%
% >351 bases: 79.7%
[a]T3 fluorescent dye primers and *AmpliTaq* FS enzyme (ABI) were used in sequencing reactions. The sequencing ladders were fractionated in ABI 373 DNA sequencer. The reason for short reading length (<300 bases) in 7.8% of the cDNA was because these constructs contained small cDNA inserts.

There are several noteworthy advantages of the glass bead micro-mixing technique presented here. The technique ensures a more homogeneous preparation environment which increases the consistency of yield between each isolation. Therefore, subsequent sample processing is significantly simplified. For example, adjustments of the volume of templates used for each sequencing reaction are avoided. Each step of this micro-mixing preparation procedure can be easily standardized. Thereby, variations caused by factors such as the device, speed, duration for complete resuspension of cell pellets, and mixing of cell lysates and reagents can be reduced. In addition to high efficiency, another advantage of this preparation method was the cost savings. The major material expenses included the Sephacryl-500HR, Silent Monitor plates, polypropylene microtiter plates, 96-channel pipettor cartridges and pipette tips. Since the 96-square-well plates were reusable, they only imposed a one time expense. Overall, the expenditure per plasmid was <30 cents. Labor cost was significantly reduced by processing eight or twleve 96-well plates in each plasmid preparation.

The significant improvements in high-throughput plasmid isolation presented here are simple, economical, and ensure a consistent yield of high quality cDNA, a necessity for large-scale expression mapping assays and nucleotide sequencing.

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