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A Method for Preparing DNA Sequencing Templates Using a DNA-Binding Microplate

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A DNA-binding matrix was immobilized on the surface of a 96-well microplate and used for plasmid DNA preparation for DNA sequencing. The same DNA-binding plate was used for bacterial growth, cell lysis, DNA purification, and storage. In a single step using one buffer, bacterial cells were lysed by enzymes, and released DNA was captured on the plate simultaneously. After two wash steps, DNA was eluted and stored in the same plate. Inclusion of phosphates in the culture medium was found to enhance the yield of plasmid significantly. Purified DNA samples were used successfully in DNA sequencing with high consistency and reproducibility. Eleven vectors and nine libraries were tested using this method. In 10 μl sequencing reactions using 3 μl sample and 0.25 μl BigDye Terminator v3.1, the results from a 3730*xl* sequencer gave a success rate of 90–95% and read-lengths of 700 bases or more. The method is fully automatable and convenient for manual operation as well. It enables reproducible, high-throughput, rapid production of DNA with purity and yields sufficient for high-quality DNA sequencing at a substantially reduced cost.

KEY WORDS: rapid plasmid purification, DNA capture plate, genome, DNA library

High-throughput sample preparation is a particular challenge as a result of the large number of variables that can impact the process and the consequent difficulty in obtaining reproducible and consistent results. This is particularly true of sequencing by capillary electrophoresis (CE sequencing), which requires cloning, culture, and purification of a large number of plasmid clones. This process is complicated further by the fact that there are a large number of plasmid vectors used for the construction of genomic and cDNA libraries, which vary greatly in copy number and size.[1,2] Different platforms have been developed to address the demand for speed, low cost, quality, reliability, scalability, and automation.[3–6] Although much has been achieved in the implementation of high-throughput processes, there are needs for further improvements. Most plasmid purification methods are based on variations of the classic alkaline lysis method, which is very reliable but is a tedious multiple-step process.[7] High-speed centrifugation or filtration driven by vacuum or centrifugation is typically required to obtain a cleared lysate after neutralization. These procedures lead to sample transfer steps and pose a significant hurdle for cost reduction and automation. Alternative approaches had been investigated but not been applied reliably as a result of a number of inherent limita-

tions.[4,8] There is a need for a simplified platform that will have as few components and steps as possible and is flexible for operating manually as well as with automation on various universal robotic liquid-handling systems without using additional accessory parts such as a shaking stage, vacuum manifold, or magnet.

Technological advances in CE sequencing have improved resolution remarkably, enabling the use of a drastically reduced amount of DNA template in dye terminator sequencing reactions.[9] It has enabled direct sequencing of bacterial genomic DNA and plasmids of low copy number or low concentrations. As little as 5–10 ng high-quality pUC19 in a 10-μl sequencing reaction with 0.25 μl dye terminator reproducibly gives a sequencing read-length comparable with 100 ng or more pUC19.[10] Therefore, it is feasible to improve cost-effectiveness through reducing volume for the bacterial culture and purification reagents and through eliminating transfer steps and equipment to save operation time and cost on labor and consumables. Although the yield is expected to be lower than that obtained with conventional methods, the quantity and purity of purified plasmid will be sufficient for high-quality reads and consistent performance in DNA sequencing application.

Here, we describe a method for plasmid purification in a 96-well format that uses only one plate for all steps, including culture, purification, and storage. It uses a DNA-binding microplate and a complex buffer combining en-

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zymes and chemicals for simultaneous cell lysis and DNA capture. It consumes minimal amounts of reagents and time and has no transfer steps. The simplified procedure is likely able to meet the demands for speed and low cost on high-throughput plasmid production for DNA sequencing without compromising data quality.

MATERIALS AND METHODS

Reagents, Plasmids, DNA Libraries, and Bacterial Culture Media

Reagents and enzymes were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburg, PA). High copy number plasmid pUC19 (New England Biolabs, Beverly, MA), pGEM-3fZ(+) (Promega, Madison, WI), and low copy number plasmid pBR322 (New England Biolabs) were obtained from the manufacturer and transformed into electrocompetent *Escherichia coli* cells EB10B or EB5α (EdgeBio, Gaithersburg, MD). Genomic or cDNA libraries and open-reading frame (ORF) clones were purchased from commercial sources including Invitrogen (Carlsbad, CA), Amplicon Express (Pullman, WA), and Open Biosystems (Huntsville, AL). Terrific Broth (TB) was prepared as follows: autoclave 900 ml broth containing 12 g tryptone, 24 g yeast extract, and 4 ml glycerol and then add 100 ml sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4. Luria-Bertani (LB) and 2×yeast extract tryptone (YT) media were prepared as described previously.[11] Modifications to the media were made by the addition or removal of glycerol, phosphates, or both into LB and 2×YT or from TB.

Bacterial Culture and DNA Purification

Microplates for use in this method were prepared by immobilizing a DNA-binding matrix on the surface of a standard U-bottom 96-well microplate with a 350-μl-well volume (Costar, Corning, NY). In each well, 200 μl bacterial culture medium with appropriate antibiotics was inoculated from a freshly grown single colony or with 3 μl bacteria stock in 20% glycerol. The concentrations for antibiotics used in these studies are 50 μg/ml for ampicillin and spectinomycin and 25 μg/ml for kanamycin and chloramphenicol. Following inoculation with clones containing high copy number plasmids, the microplate was covered with a gas-permeable plate sealer and incubated at 37°C with shaking at 300 rpm for 17–19 h. For the low copy number plasmid pBR322, freshly grown single colonies or 3 μl glycerol stock were inoculated in 100 μl TB with ampicillin in the microplate and incubated at 37°C with shaking at 300 rpm for 8 h. Subsequently, 100 μl TB containing ampicillin and 40 μg/ml chloramphenicol (prewarmed to 37°C) were added to each well and incubated overnight (16 h). The cultured microplates were then cen-trifuged at 850 g for 3 min to harvest the cells. After decanting the supernatant, a laboratory microplate shaker, Titer Plate Shaker (Lab-Line Instruments, Inc., Melrose Park, IL), or Eppendorf MixMate (Eppendorf AG, Westbury, NY), operating at 1000 rpm for 2 min, was used to disperse the pelleted cells. These microplate mixers were used for all subsequent mixing steps. Enzyme mix/lysis buffer (100 μl) was added to each well, and the microplate was shaken for 5 min to lyse the bacteria and allow released plasmids to bind to the microplate, which was then washed with 150 μl Wash Buffer I and 150 μl Wash Buffer II. Each wash step was accomplished by 1 min mixing on a microplate shaker. The washed microplate was inverted on a clean absorbent pad and centrifuged at 850 g for 1 min to remove all liquid from the plate. Finally, 40 μl 10 mM Tris · HCl, pH 7.5 or 8.0, or water was added to each well and shaken for 1 min to elute plasmid DNA. Purified plasmid DNA was kept in the same plate and stored at 4°C or –20°C. For comparison, bacterial cultures grown in the microplate were transferred to microtubes, and plasmid DNA was purified with the PurElute Plasmid MiniPrep Kit (EdgeBio). This kit uses a classical alkaline lysis and silica absorption spin column to purify plasmid DNA.[12]

DNA Quantitation and Sequencing

Plasmid purity was analyzed by gel electrophoresis on 1% agarose gels. To obtain a rough estimate of concentration, high-purity pUC19 of known concentrations was loaded in wells adjacent to the samples. High-purity pUC19 was prepared using an ion exchange method with PurElute IEX Plasmid MaxiPrep Kit (EdgeBio) and then quantified by absorbance at 260 nm reading with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).[10] For accurate determination of DNA concentration, DNA samples were first incubated with 30 μg/ml DNase-free RNase A (Qiagen, Valencia, CA) for 30 min at 37°C and then assayed with Quant-iT PicoGreen dsDNA assay reagent (Invitrogen). DNA sequencing reactions were run in 96-well or 384 plates on a PTC-200 thermal cycler (MJ Research, Watertown, MA) using a thermal cycling program of 95°C for 15 s, 45°C for 5 s, and 60°C for 2 min for 35 cycles in a 10-μl reaction volume containing 0.25 μl BigDye Terminator v3.1 (Applied BioSystems, Foster City, CA). Unless otherwise stated, 3 μl plasmid sample was used in each reaction, regardless of DNA concentration. The primers used for sequencing were those listed in literature or recommended by the vector or clone suppliers. Excess dye terminator was removed by gel filtration with a Performa DTR V3 96-well short plate (EdgeBio) prior to analysis on an ABI 3730*xl* DNA analyzer. Sequencing output was analyzed with Phred software CodonCode InterPhace v2.3.1 (CodonCode, Dedham, MA).

RESULTS AND DISCUSSION
A Unique Plasmid Purification System

In the early days of genomic sequencing in large sequencing centers, classical plasmid purification by alkaline lysis was engineered for different platforms to facilitate high-throughput production.[1,13] These operations were highly controlled with strict limits on vectors and growth conditions that facilitated the development of fully automated systems. However, in the general research community, such strict limitations were not possible, and attempts to migrate the technologies from the large centers to the broader research community have been met with mixed success. Typically, various pieces of equipment are connected to a robotic liquid-handling system, including an orbital shaker, plate gripper, and vacuum manifold or magnetic plate. The typical procedure has a resuspension buffer, lysis solution, and neutralization/precipitation solution and then requires an alcoholic precipitation step or solid-phase absorption, followed by wash and elution steps, three or more different plates, and multiple operations, including a few plate transfers, for 45 min or longer. The procedure can be replicated manually but is tedious, labor-intensive, and prone to cross-contamination and mistakes.

In contrast, the present method uses only three solutions: low-reagent volumes, fewer steps, and much shorter time. It uses one plate for all of the steps from bacterial growth to sample storage, i.e., no plate transfer. The procedure only takes 20–25 min and is adapted readily for manual or automatic operation. For automation, no part other than a 200-μl vol pipette head is required, preferably in 96-channel format.

With the recognition that high-quality CE sequencing was compatible with low nanogram amounts of DNA, the use of coated surfaces for purification of plasmid DNA for sequencing became feasible.[10] The technical difficulty for isolation of plasmids on a DNA-binding surface in a 96-well plate format is the considerably limited surface space available for DNA capture. It becomes even more challenging when the cells are grown and later lysed in the same DNA-binding microplate. Not only do all of the reagents have to be compatible with the DNA-binding matrix, but also, the inhibitory contaminants from culture medium ingredients, bacterial metabolic byproducts, and cell components must be prevented from competing with plasmids for binding sites on the surface and be removed effectively during wash steps. To achieve the goal, several iterations were performed, which include selection of the best DNA-binding matrix, development of reproducible processes for immobilizing the matrix on the microplate, formulation of a complex enzyme mix/lysis buffer, and optimization of every solution and step in the procedure. The surface of the wells in the microplates was modified by adhering a DNA-binding matrix, which increased the available surface for DNA binding substantially. Unlike alkaline lysis-based protocols, which use three solutions and require lysate clarification, in this method, cell lysis and DNA capture were accomplished by using one enzyme mix/lysis buffer in a single step without lysate clarification. The enzyme mix of lysozyme and RNase lysed the cells and degraded RNAs, respectively. Lysozyme and lysis buffer selectively degrade the cell wall and *permeabilize* the cell membrane, allowing the plasmids to be released while retaining genomic DNA. Reagents in lysis buffer enhanced selective capture of the plasmid on the DNA-binding matrix in the plate.

The yield was determined and compared with conventional plasmid miniprep. The yield of a pCMV-SPORT6 cDNA clone grown in 200 μl TB in the plate, purified, and eluted in 40 μl 10 mM Tris · HCl, pH 7.5, was 75.1 ± 16.4 μg/ml or 3 μg total. The same sample, processed by the alkaline lysis method, gave a yield of 185.8 ± 14.6 μg/ml in 50 μl or 9.3 μg total. The relatively low yield for the plate-based system reflects the limitations imposed by the surface area of the well. The lower yield with this plate purification system indicates that the method may not be suitable for some downstream applications, such as transfections, which require a large quantity. Nevertheless, the yield is more than sufficient for DNA sequencing and interestingly, proved to be advantageous for sequencing applications. Very high concentration samples sequence poorly as a result of the rapid consumption of reagents during the early stages of cycling, causing a sharp decline in the signal strengths and short reads. Sequencing reactions containing low concentrations and quantities of BigDye are sensitive to DNA concentration; therefore, DNA often has to be diluted before use. The samples prepared in this plate-based purification system were used in 10 μl CE sequencing reactions with only 0.25 μl BigDye at a constant volume of 3 μl without the need to quantify the DNA. Results with a high degree of success were observed reproducibly across many samples and many vectors (data shown below).

Selection of Culture Media for Optimal Growth and Plasmid Yield

Three media—LB, 2×YT, and TB—and two ingredients—glycerol and phosphate buffer—were investigated to select the best bacterial growth medium for the system. TB was found to be the medium of choice for bacterial growth and gave the best yield. Although growth in TB medium tended to produce more cells than with 2×YT or LB, the differences were not large in the plate (Fig. 1A). However, plasmid yields from TB culture were significantly higher than 2×YT or LB cultures (Fig. 1B). In an effort to

A

Media	LB				2×YT				TB			
Glycerol	-	+	-	+	-	+	-	+	-	+	-	+
Phosphates	-	-	+	+	-	-	+	+	-	-	+	+

B

Media	LB				2×YT				TB			
Glycerol	-	+	-	+	-	+	-	+	-	+	-	+
Phosphates	-	-	+	+	-	-	+	+	-	-	+	+

C

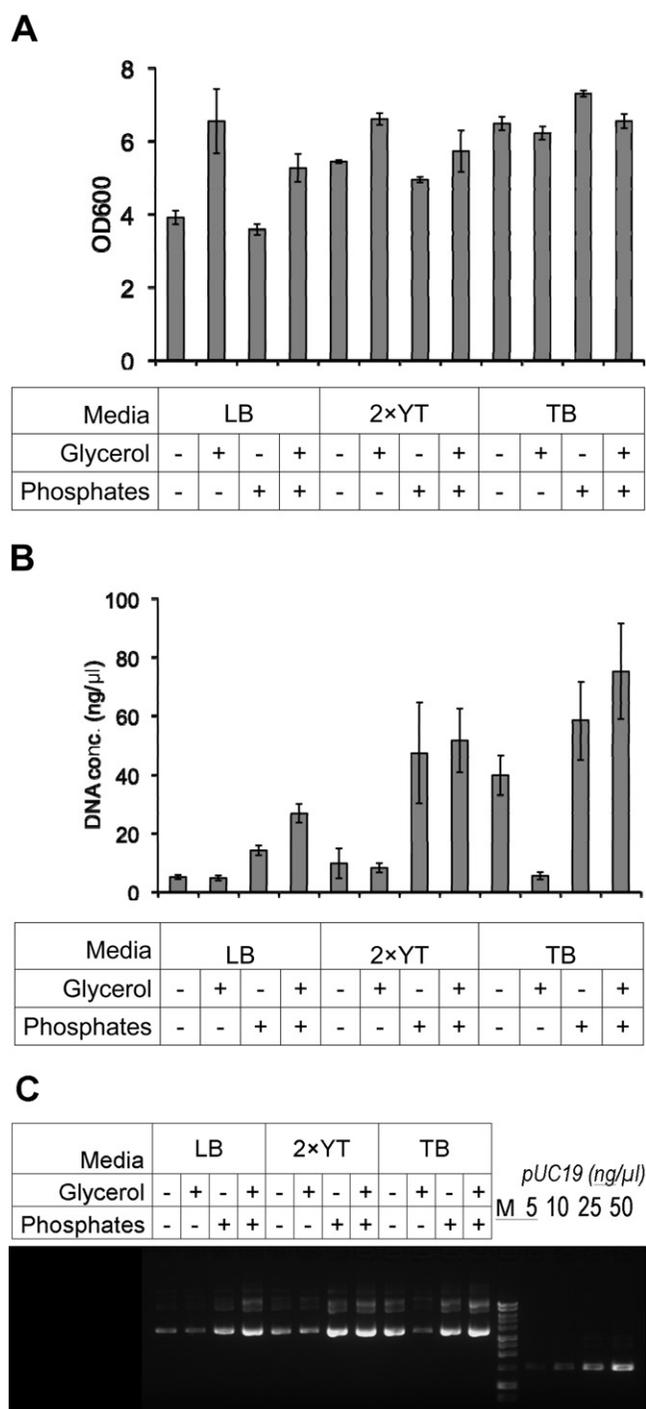| Media	LB				2×YT				TB			
Glycerol	-	+	-	+	-	+	-	+	-	+	-	+
Phosphates	-	-	+	+	-	-	+	+	-	-	+	+

pUC19 (ng/µl)
M 5 10 25 50

FIGURE 1

Bacterial growth and plasmid purification with the DNA-binding microplate. A pCMV-SPORT6 cDNA library clone was grown in the DNA-binding microplate with LB, 2×YT, or TB media, with or without the addition of glycerol, phosphate buffer, or both. Plasmids were purified in the same plate. (A) OD_{600} for the overnight culture grown in the plate. The results shown are mean ± SD from four wells. (B) DNA concentrations of the purified plasmids were determined by PicoGreen dsDNA assay. Results are mean ± SD from seven wells. (C) Agarose gel electrophoresis analysis of the purified plasmids. Each sample (5 µl) or pUC19 of known concentrations was loaded on a 1% agarose gel. M, 1 kb DNA ladder (Promega).

understand the origins of these differences, we explored supplementing 2×YT and LB with glycerol and phosphate, components of TB that are not present in the other two culture media, as well as removing these reagents from the TB. Glycerol increased cell density in LB culture significantly but not for 2×YT or TB. Interestingly, cultures containing glycerol but no phosphates produced a substantially reduced yield of plasmids. In contrast, phosphates, regardless of the addition of glycerol, increased plasmid yield significantly (Fig. 1B, C). Nevertheless, inclusion of glycerol and phosphate buffer obtained good bacterial growth as well as a good yield (Fig. 1). An alternative pH buffer, Tris · HCl (pH 7.5), was used instead of phosphate buffer in media without any enhancement on plasmid yield (data not shown). Sequencing results for samples from cultures using different media were consistent with the plasmid yields. Culture with TB medium, which contains glycerol and phosphate buffer, gave the highest signal intensity and read-length. Supplementing glycerol and phosphate into LB and 2×YT improved signal strength significantly (Fig. 2).

These data are consistent with previous observations of phosphate depletion in saturated cultures with high copy number plasmids.[14] It is speculated that synthesis of the large amount of plasmids depleted phosphate and led to suppression of plasmid copy number. It was also observed that the addition of extra nucleotides could enhance plasmid yield.[15] We suggest that supplementing standard culture media with glycerol and phosphate may be, in general, a convenient and effective way to improve yield for high copy number plasmids. Actual effect on plasmid yield for other preparation methods has yet to be confirmed. However, highly enriched media such as TB are not favored by many plasmid purification methods as a result of the tendency of the bacteria to overgrow during overnight culture and overload the purification system. In this method, overgrowth and cell death in overnight culture have not been observed to be a limiting issue. The possible reason may be the relatively smooth growth curve in a microplate. The dynamic mixing in microplate helps *E. coli* grow steadily; cell growth started quickly and began slowing down when cell density increased, and aeration gradually became a limiting factor for growth but still sufficient to keep cells from dying.

Consistent Performance in DNA Sequencing Application

Beside DNA concentration determination and agarose gel electrophoresis analysis, all of the purified plasmids were subjected to DNA sequencing analysis. As DNA sequencing output can be affected by many factors, and there are no parameters that can reliably indicate usefulness of a DNA sequencing template, we assessed the quality of plasmid
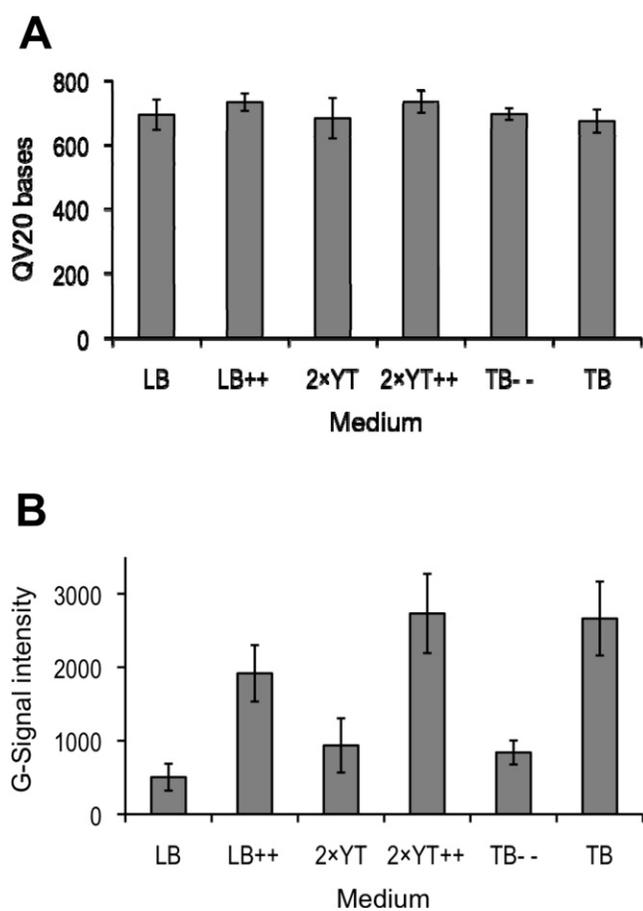
A

B

FIGURE 2

DNA sequencing using plasmids purified from the culture using different media. A pCMV-SPORT6 cDNA library clone was grown and processed in the DNA-binding plate. Purified plasmids were subjected to DNA sequencing for comparison. (A) Phred QV20 scores. (B) Intensity of G-signal. ++, Addition of glycerol and phosphate buffer into LB and 2×YT; –, removal of glycerol and phosphate buffer from TB.

based on its functionality on DNA sequencing application. Phred QV20 scores were consistent and reproducible among all samples, including genomic and cDNA library clones and ORF clones in high copy number plasmid vectors, high copy number plasmids pUC19 and pGEM-3fZ(+), and low copy number plasmid pBR322 (Table 1; Figs. 3A,B and 4). Variations among wells in a plate and from plate to plate, using the same clone or independent clones within a library, were evaluated for sequencing signal intensity and Phred QV20 scores. To do this, clones were grown independently and purified with three DNA-binding microplates and then sequenced and analyzed in parallel. Signal intensities varied from 500 to 2000 or higher. Read-length variation is quite small and favors long reads, as shown by QV20 scores in an average 777 ± 29 bases for plasmid pGEM-3fZ(+) (Fig. 3A). Similar results were obtained with all other plasmids tested. For replicates of individual library clones, read-length is highly reproducible. QV20 scores for two replicate plates for 96 individual clones from a rat cDNA library in pT7T3d-PacI were shown in Figure 3B, with a R^2 of 0.99 and a slope of 0.98. The result was consistent for all other library clones tested. The distribution of Phred QV20 scores for 760 clones from a human brain cDNA library in pCMV-SPORT6 is summarized in Figure 4. The overall success rate, as measured by the percentage of reads with a minimal length of 100 bases, was 92.2%. The average read-length for the reads of 100 or longer was 661 bases.

CONCLUSIONS

The method represents a significant advance for a multi-well plasmid preparation for CE sequencing by an all-steps-in-one-plate approach. It eliminated technical errors asso-
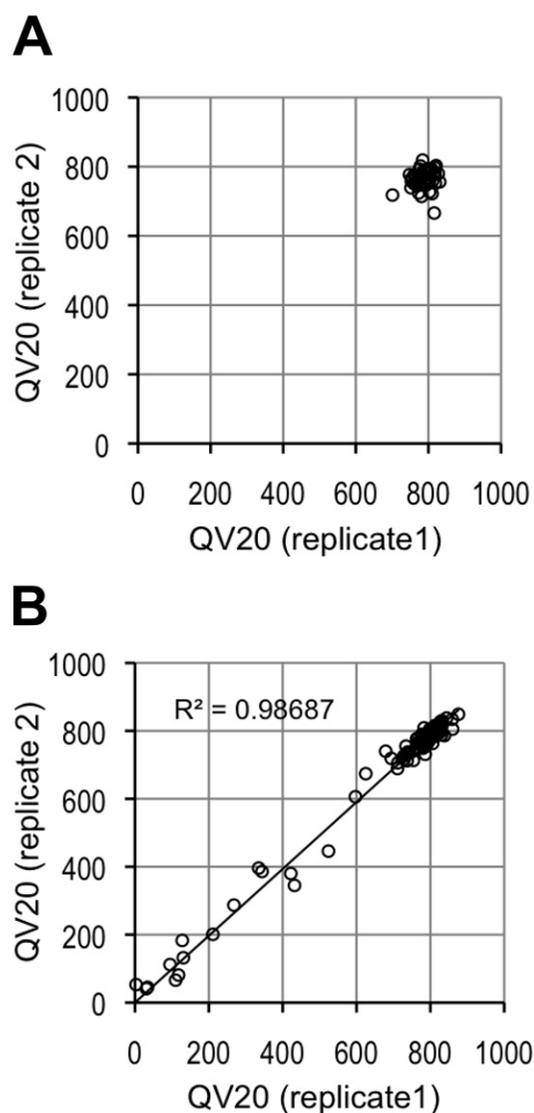
TABLE 1.

Plasmid Vectors and Clones Used in the Study

Vector Name	Clone Description	*E. Coli* Host	Number of Clones[a]
PUC19	high copy number vector	EB10B	1
pGEM-3fZ(+)	high copy number vector	EB10B	1
pBR322	low copy number vector	EB5α	1
pCMV-SPORT6	human brain cDNA library	DH5α	$>1 \times 10^9$
pSMART-HC Kan	Fugu shotgun genomic library	ELITE 10G	96
pDONR223	Human ORF library	DH5α	96
pDNR-LIB	Human cDNA library (IRAU)[b]	DH10B	96
pT7T3d-PacI	*Rattus norvegicus* cDNA library (IRAD)[b]	DH10B	96
pOTB7	Human small cell carcinoma cDNA library (IRAU)[b]	DH10B	96
pBluescript II KS(+)	Fugu hgmpF adult brain cDNA library (LLAM)[b]	DH10B TonA	384
PUC19	Human cDNA library (IRBS)[b]	XL10 Gold	59
pPCR-Script Amp SK(+)	Human mixed tissues cDNA library (IRAT)[b]	DH10B TonA, XL10 Gold	70

A number of *E. coli* strains harboring plasmid vectors or DNA library constructs were grown and processed with the DNA-binding microplate. [a]Number of clones acquired for the study; [b]clones from the I.M.A.G.E. Consortium and Mammalian Gene Collection program distributed by Open Biosystems.[16,17]

A

B

FIGURE 3

Variation and reproducibility of DNA sequencing results for plasmids purified with the DNA-binding microplate. Phred scores QV20 for two replicate plates of purified plasmids were plotted against each other. Each circle represents a pair of QV20 for two correspondent wells in the replicate plates. (A) In each plate, 48 wells of pGEM 3fZ(+) were purified and sequenced. (B) In each plate, a same set of 96 individual clones for a pT7T3d-PacI cDNA library was purified and sequenced. The straight line shows linear regression of the data. R^2, Coefficient of determination for the linear regression.

ciated with sample transfer and manipulation, reduced handling time, reagents, and plastics costs, and can be readily performed manually as well as on an automated liquid-handling platform. Plasmid DNA purified by the method is of sufficiently high quality that it can generate consistent high-quality sequence data with greatly reduced sequencing reagents. Furthermore, the DNA concentration produced by the system over a wide range of plasmids is in a sufficiently narrow range as to minimize or even
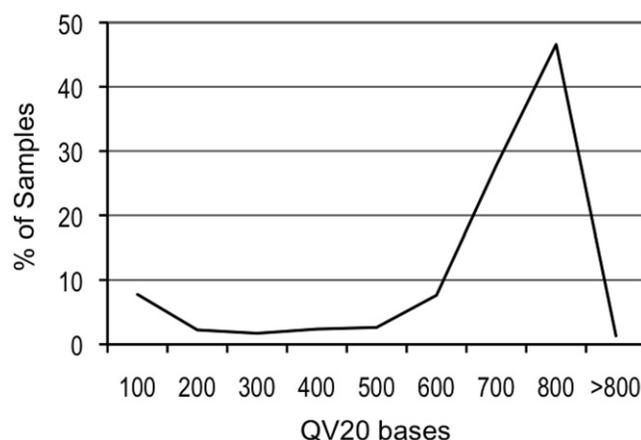

FIGURE 4

Distribution of DNA sequencing reading length for a pCMV-SPORT6 human brain cDNA library. Numbers of QV20 scores were counted in the interval of every 100 bases and divided by the total number to obtain the percentage distribution. The graph shows the analysis of sequencing data for 760 individual colonies.

obviate the necessity for downstream determinations of concentration prior to sequencing. The method is particularly suited for high-throughput DNA sequencing and may possibly find use in other applications such as transformation and DNA amplifications, where low quantities of good-quality plasmids are sufficient to meet the needs.

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