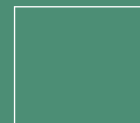

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ABOUT THE COVER: Photograph of the Grand Canyon in Yellowstone Park by Robert W. Oberfelder. See Dr. Oberfelder's latest manuscript on page 33.

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New UltraMAX DH5α-FT™ Chemically Competent Cells for Demanding Cloning Experiments

Robert A. Donahue, Jr.
Robert L. Bebee
Research and Development
Life Technologies, Inc.
Gaithersburg, Maryland 20877

High efficiency chemically competent and electrocompetent *E. coli* cells are routinely used for transformations with little DNA and for library construction. In some applications (*e.g.*, the construction of large representative cDNA libraries), the required efficiencies could only be achieved with electroporation. Now with UltraMAX DH5α-FT™ Competent Cells (patent pending), transformation efficiencies (>5 × 10^9 transformants/µg) rivaling electroporation can be achieved. DH5α-FT cells are a derivative of DH5α™ cells (1). UltraMAX DH5α-FT cells can be used to generate cDNA libraries, clone small amounts of DNA, select cDNA clones with the GeneTrapper™ cDNA Positive Selection System, and generate single-stranded DNA (ssDNA) for sequencing.

Methods

Transformation. The genotype of the DH5α-FT strain is F′ φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1/F′ *pro*AB$^+$ *lac*I^{q}ZΔM15 Tn10(tet^r). Transformation of UltraMAX DH5α-FT cells (Cat. No. 10643) was performed as recommended by the manufacturer. The samples were diluted 1/400 before plating in duplicate on LB medium containing 100 µg/ml ampicillin followed by incubation for 12 to 16 h at 37°C.

cDNA library construction. The cDNA library was derived from total RNA extracted from a rat neuronal cell line (graciously provided by Paul Rosenberg, Children's Hospital, Boston, MA). The poly(A)$^+$ message was purified from total RNA with the Gibco BRL MessageMaker™ System (2). The SuperScript™ Plasmid System (3) was used to produce double-stranded cDNA, which was ligated into pCMV•SPORT2. 5 µl of the cDNA ligation was directly transformed into UltraMAX DH5α-FT cells in quadruplicate. The cells were diluted and plated on LB medium containing ampicillin and incubated 12 to 16 h at 37°C.

cDNA clone selection. Following the GeneTrapper System instructions (4), 1 µl of pSPORT 1-CAT control DNA was added to 50 µl of cDNA prepared from a human cell line. The CAT oligonucleotide was used for the cDNA capture and repair steps. The repaired dsDNA was either electroporated into ElectroMAX DH10B™ cells or transformed into the UltraMAX DH5α-FT cells. 100 µl of cell culture was plated on LB medium containing either 100 µg/ml ampicillin or 100 µg/ml ampicillin and 7.5 µg/ml chloramphenicol. The plates were incubated 12 to 16 h at 37°C.

Preparation of ssDNA. Phagemid production and ssDNA preparation were carried out essentially as described (5) with minor modifications (6). A plasmid derivative of a gp6-display vector (7) was transformed into UltraMAX DH5α-FT cells and a single colony isolate was cultured overnight at 30°C in TBG (8) broth containing 100 µg/ml carbenicillin and 10 µg/ml tetracycline. The overnight culture was diluted 1/200 into fresh medium and incubated at 37°C with shaking until the OD_{550} of

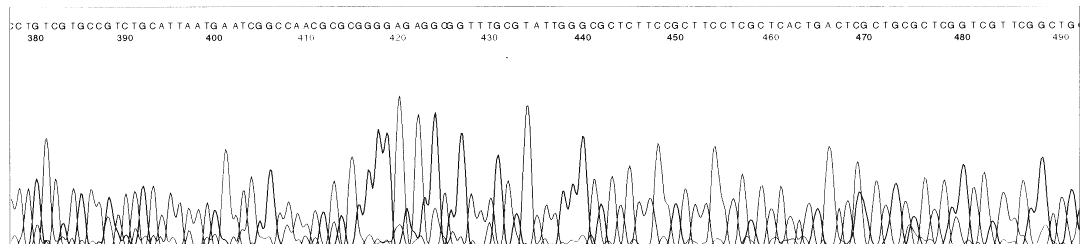

FIGURE 1. Automated sequencing of ssDNA isolated from DH5α-FT cells.

the cells reached ~0.1. Phagemid rescue was carried out by addition of M13K07 helper phage to the culture at 1×10^9 to 2×10^9 pfu/ml (an MOI of 10 to 20 pfu/cell) followed by a 30-min incubation at 37°C, 100 rpm. The culture was centrifuged (10 min at 4,000 × *g*, 4°C) and the supernatant filtered (0.2 µ). Phage particles were precipitated with 1/4 volume of 20% (w/v) polyethylene glycol 8000 (PEG 8000), 3.5 M ammonium acetate and incubation at 4°C overnight. Phage was collected by centrifugation (10 min at 10,000 × *g*, 4°C), resuspended in 1 ml TBS, and reprecipitated with PEG/ammonium acetate. ssDNA was isolated from the resuspended phage by extraction with phenol and collected by ethanol precipitation. ssDNA was sequenced using an automated dye-terminator reaction and gene 6-specific primer.

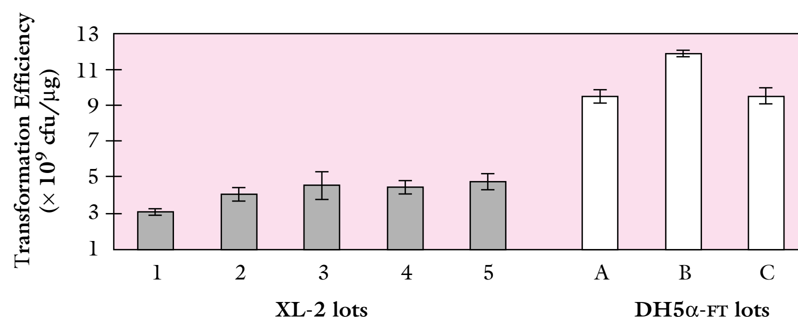

FIGURE 2. Comparison of transformation efficiencies between ultracompetent cells. Several lots of XL-2 and DH5α-FT competent cells were transformed with control DNA following the manufacturers' recommendations. Results are the mean ± SEM for ≥6 transformations for each lot.

Results and Discussion

For library construction, UltraMAX DH5α-FT Chemically Competent Cells produced $5 \pm 1 \times 10^5$ cfu/transformation using 5 µl of a ligation mix. Thus, 2 to 3 transformations with UltraMAX DH5α-FT cells generated a representative cDNA library (>10^6 clones). To generate a representative cDNA library with chemically competent cells (efficiency of 1×10^9 cfu/µg pUC) required at least 20 transformations (C. Gruber, personal communication). Unlike electroporation, ethanol precipitation of the ligated cDNA was not required before transformation of UltraMAX DH5α-FT cells. This reduced the time and manipulations required and the cDNA loss that may occur when preparing the samples for electroporation.

Until now, for optimal results, electrocompetent cells were recommended with the GeneTrapper System to screen a library for the cDNA of interest. A comparable number of clones containing the captured sequence were generated using transformation of UltraMAX DH5α-FT cells as compared to electroporation (table 1). UltraMAX DH5α-FT cells allowed transformation of 5 µl of selected cDNA in a single reaction without special equipment or chambers.

ssDNA purified from DH5α-FT cells infected with M13K07 helper phage was successfully used for sequencing (figure 1). Sequencing reactions generated over 550 bases of sequence without ambiguities. Since the DH5α-FT cells readily produced phagemid DNA and the strain contains the *supE* marker, UltraMAX DH5α-FT cells can also serve as a host for phage display applications (5,7).

UltraMAX DH5α-FT Chemically Competent Cells were compared to the XL-2 strain. Transformations with UltraMAX DH5α-FT cells consistently generated higher numbers of colonies than Ultracompetent XL-2 cells (figure 2).

In summary, UltraMAX DH5α-FT Competent Cells were used to generate cDNA libraries, to select cDNA clones with the GeneTrapper System, and to prepare ssDNA for automated sequencing. UltraMAX DH5α-FT cells can be used in place of electrocompetent cells in applications requiring high efficiency cells or cells that generate a large clonal output.

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TABLE 1. CAT clones selected with the GeneTrapper System. The positive colonies grow on plates containing both ampicillin and chloramphenicol and represent repaired and selected pSPORT1-CAT DNA.

	Total Colonies	Positive Colonies	Percent Positives
UltraMAX DH5α-FT cells	294	244	83
ElectroMAX DH10B cells	262	220	84

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Stable Maintenance of a Tandem Array of Four R67 Dihydrofolate Reductase Genes

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Eliminating minor deletions or rearrangements of plasmid inserts is a major concern when studying the gene products of directly repeated DNA sequences. Recombination of direct repeats often occurs in strains that do not contain the *rec*A mutation. Our lab has used Stbl2™ *E. coli* cells to stably maintain a tandem array of four R67 dihydrofolate reductase (DHFR) genes (1). Since R67 DHFR is a homotetramer, this four-gene array produces a monomeric protein possessing the essential tertiary structure of the native tetramer. Attempts to maintain this tandem array in JM107 and SURE *E. coli* strains resulted in high levels of gene recombination and reduction of the insert size. Stbl2 cells *[F⁻mcrA Δ(mcrBC-hsdRMS-mrr) recA1 endA1 gyrA96 thi supE44 relA1*

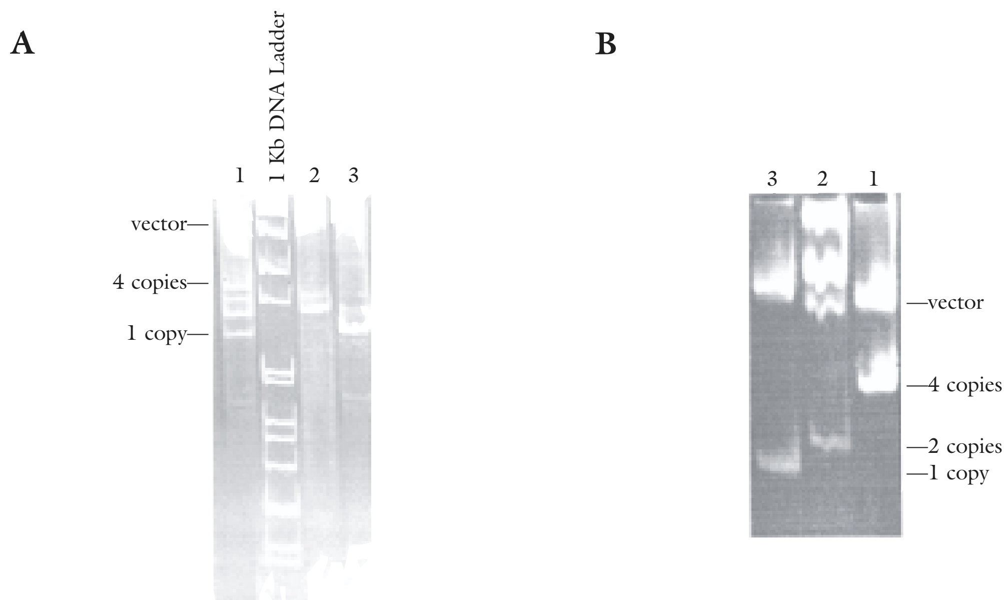

FIGURE 1. Stability of plasmid miniprep DNA. Plasmid DNA was extracted from SURE cells (Panel A) or Stbl2 cells (Panel B), digested with *Eco*R I, and analyzed on a 6% polyacrylamide gel. Lane 1. *Eco*R I digest of the quadruplicated R67 DHFR gene propagated in cells. Lane 2 Control: *Eco*R I digest of plasmid DNA containing the duplicated R67 DHFR gene (5). Lane 3 Control: *Eco*R I digest of plasmid DNA containing a single copy of the R67 DHFR gene (6).

λ⁻Δ*(lac-proAB)]* (2) propagated a plasmid containing the tandem array of four R67 DHFR genes without recombination.

Methods

A tandem array of four in frame copies of the R67 DHFR gene was constructed and ligated into pUC8 (1). As controls, single and duplicate copies were also cloned. The construct was then transformed into SURE, JM107, and STBL2 (MAX EFFICIENCY STBL2 Competent Cells, Cat. No. 10268) *E. coli* strains utilizing standard protocols (3). Each of the transformed strains was plated on YT plates containing 200 µg/ml ampicillin and 20 µg/ml trimethoprim. All plates were incubated overnight at 37°C, except for those corresponding to STBL2 cells, which were incubated at 30°C. Ampicillin- and trimethoprim-resistant colonies were inoculated into 10 ml of YT medium containing 200 µg/ml ampicillin and 20 µg/ml trimethoprim and the cultures grown overnight. Plasmid DNA was isolated using the boiling miniprep method (4), *Eco*R I digested, and analyzed on a 6% polyacrylamide gel in TBE buffer.

Results and Discussion

The ability of a tandem array of R67 DHFR genes to be maintained in JM107 (data not shown), SURE, and STBL2 *E. coli* cells was examined. Since two *Eco*R I sites lie directly outside the coding sequence, the size of the *Eco*R I fragment allowed determination of the insert size. With SURE cells, a series of bands were seen, consistent with homologous recombination between the four gene copies. In contrast, the *Eco*R I-digested plasmid DNA from STBL2 cells resulted in a single full-length band for the insert (figure 1). An additional confirmation of the stable propagation of the four in frame gene copies can be seen by the size of the resulting proteins. Figure 2 shows the 8,430- and 33,720-kDa proteins produced by the one- and four-gene-copy constructs. The four-copy construct was propagated in STBL2 cells. Both these DNA and protein results show that STBL2 cells maintained the tandem array of four R67 DHFR genes.

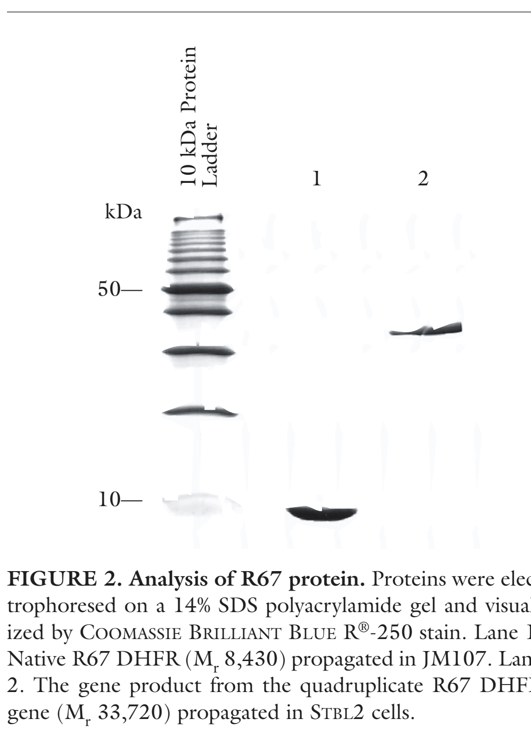

FIGURE 2. Analysis of R67 protein. Proteins were electrophoresed on a 14% SDS polyacrylamide gel and visualized by COOMASSIE BRILLIANT BLUE R®-250 stain. Lane 1. Native R67 DHFR (M_r 8,430) propagated in JM107. Lane 2. The gene product from the quadruplicate R67 DHFR gene (M_r 33,720) propagated in STBL2 cells.

Acknowledgement

This research was supported by NIH grant GM35308 (to E.E.H.).

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Transformation of *E. coli* Using a Microtiter Plate Format

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Abstract

Bacterial transformation with recombinant plasmids has been facilitated considerably by affordable commercial preparations of highly competent bacteria. While the availability of these bacteria has reduced the workload required to clone, it remains time-intensive to transform a large number of samples. We describe a protocol allowing transformation of bacteria with 96 DNA samples in a microtiter plate format. This method permits the rapid handling of cloning steps where tens of nanograms of recombinant plasmid are available.

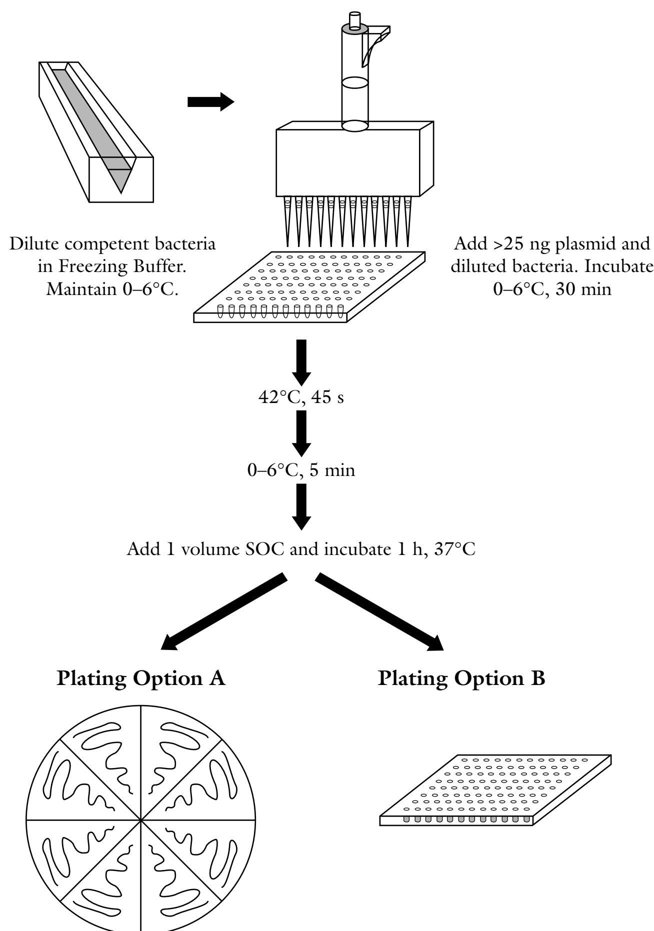

FIGURE 1. Schematic of microtiter plate format transformation.

A considerable body of work has been devoted to generating bacterial cells with high transformation competency (1–3), permitting the use of ever decreasing amounts of DNA for transformation. A number of techniques (*in vitro* mutagenesis, recircularization of derivatized plasmids, retransformation of isolated plasmid DNA stocks, sequencing, etc.) would benefit greatly from high-throughput bacterial transformations to allow the rapid and inexpensive generation of a few clones from each of a number of individual input samples.

For a high-throughput bacterial transformation protocol to be cost-effective, the process must be performed on multiple samples using a minimum of reagents. We describe here a modification of the Hanahan protocol (1,2) in which a single 200-µl aliquot of commercially prepared frozen competent cells is diluted and used in the microtiter plate format for transformation of 96 plasmids. The choice of one of two plating options is dictated by the downstream processing requirements of the resulting clones.

Methods

Manual transformation. A 200-µl aliquot of Library Efficiency DH5α™ Competent Cells (>10^8 colony forming units/µg, Cat. No. 18263) was thawed on ice and added to 5 ml ice-cold sterile Hanahan competent cell freezing buffer [FB; 100 mM potassium chloride, 50 mM calcium chloride, 10% w/v glycerol, 10 mM potassium acetate (pH 6.2)] (2) in a multichannel pipette reservoir held at 0°C (figure 1). An 8- or 12-channel pipette was used to dispense 50-µl aliquots into the wells of a prechilled thin-walled polyvinylchloride V-bottom microtiter plate. One to five microliters of plasmid DNA (>25 ng of pUC18 clones containing a 1.2-kb HIV-1 envelope gene segment) were added to each well, and the plate was incubated at 0°C for 30 min. The cells were heat-shocked for 45 s at 42°C, then returned to

0°C for 5 min. Fifty microliters of GIBCO BRL SOC Medium were added, and the plate was incubated at 37°C for 30 to 60 min. Ten microliters of the transformed cells were plated on LB plates supplemented with 100 µg/ml ampicillin and 1% agar. Transformed bacteria were grown in a Petri dish or spread across the surface of the wells of a microtiter plate filled with solid medium. The cells were incubated at 37°C for 16 to 18 h.

Semi-automated transformation. The diluted competent bacteria described above were dispensed into the wells of a microtiter plate cooled to 6°C in a Robocycler Thermal Cycler. Note: Conventional thermal cyclers using a single heating/cooling unit were not successful due to the time required to ramp from 6°C to 45°C and back to 6°C in the heat shock. Following addition of >25 ng of plasmid, the plate was incubated as follows: 6°C for 30 min, 42°C for 45 s, 6°C for 5 min, 37°C for 60 min. SOC Medium (50 µl) was added immediately prior to the 37°C phase. Plating and growth of the cells were performed as described above (figure 1).

RESULTS

We initially considered a direct inoculation of liquid selective growth medium with the transformed cells but felt that inactivation of the selection antibiotic could lead to a mixed population of resistant and sensitive bacteria. Two strategies were used successfully to isolate individual colonies of transformed bacteria on a solid medium so as to avoid the presence of antibiotic-sensitive satellite colonies. Initially the 10 µl of cells was applied to the periphery of a Petri dish divided into 8 equal pie-shaped segments. The cells were then streaked within the wedge with a sterile inoculating loop to disperse the cells into individual colonies. Incubation of up to 20 h allowed for the isolation of individual colonies with sterile inoculating loops (figure 2). This strategy offered the greatest ease of isolating individual colonies but required 12 plates and a moderate amount of time (~20 min/96 samples).

An alternative higher throughput strategy used a multichannel pipette to aliquot 10 µl of cells into the wells of a microtiter plate containing solid selective growth medium. The plate was agitated to disperse the inoculum over the surface of the agar. Following 16 h of incubation, the antibiotic-resistant colonies in the center of the wells remained sufficiently separated to isolate individual colonies with an inoculation needle. Incubation for more than 20 h resulted in merging of the colonies within the well.

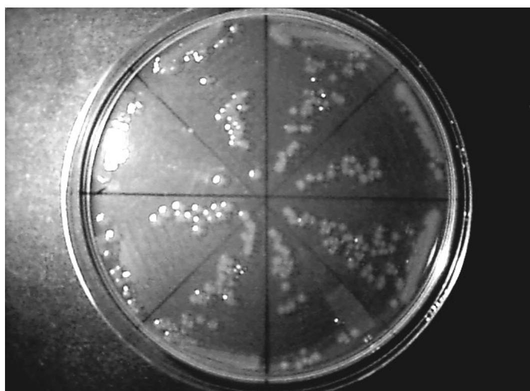

FIGURE 2. Results of plating option A.

DISCUSSION

The manual implementation of this protocol using ice and water baths for the transformation protocol and manual dispensing of cells onto Petri dishes offered a considerable decrease in costs and time over the conventional transformation of an equal number of clones. Further time savings were achieved through the use of a thermal cycler. Operator intervention was limited to the initial assembly of cells and plasmid DNA, an intermediate addition of SOC, and the terminal aliquoting of cells onto selective medium.

In summary, with this protocol, several hundred DNA samples can be introduced into competent bacteria and plated with a minimum of effort and reagents in a 2-h period.

ACKNOWLEDGEMENTS

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MAX EFFICIENCY DH10B™: A HOST FOR CLONING METHYLATED DNA

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E. *coli* is capable of degrading foreign DNA sequences that contain methylated cytosine and adenine residues. Restriction systems that function in this way have been identified and are designated McrA, McrB, and Mrr. The McrA and McrB (*m*odified *c*ytosine *r*estriction) systems restrict DNA that contains methylated cytosine residues, while the Mrr (*m*ethylated adenine *r*ecognition and *r*estriction) system restricts DNA that contains methylated adenine residues (1-3).

The McrA gene product appears to restrict the sequence $C^{5m}CGG$, which is modified by the *Hpa* II methylase; however, other unknown methylation patterns may also be recognized (1). The McrB system is the best-defined of the methyl-specific restriction systems. Its specificity appears to be $Pu^{me}C$ (1, 4). The McrB gene product recognizes three different cytosine modifications in this consensus sequence: 5-methylcytosine; N-4-methylcytosine; and 5-hydroxymethylcytosine (1, 2, 5, 6). It has been reported that the McrB function is provided by the products of at least two genes, *mcr*B and *mcr*C (7-10). The Mrr system restricts the sequences $G^{N6m}AC$ and $C^{N6m}AG$, which are modified by the *Hha* II and *Pst* I methylases, respectively (3). These methyl-specific restriction systems are found in many of the commonly used *E. coli* cloning hosts, as indicated in two recent publications (11, 12).

The use of a host strain that does not restrict DNA at sequences containing methylated cytosine and adenine bases is clearly a requirement for the construction of representative genomic libraries, as well as for other procedures that involve the introduction of methylated DNA into *E. coli*, such as cDNA cloning (12-14) or plasmid rescue (15). A recent study by Woodcock, *et al.* (11) showed that some *mcr*B-strains are more permissive than others, indicating that some *mcr*B mutations may be "leaky". In addition, this study showed that strains that are *mcr*A⁻ and carry a deletion through the *mrr-hsd-mcr*B region have the highest levels of methylation tolerance.

Life Technologies has constructed a strain for high efficiency cloning of methylated DNA, MAX EFFICIENCY DH10B™ cells. This strain is *mcr*A, *mcr*B, *mcr*C, and *mrr* due to the introduction of an *mcr*A mutation and the deletion of the entire *mrr-hsd*RMS-*mcr*BC region. The absence of these restriction systems allows DH10B cells to produce more colonies/µg of methylated DNA than other *E. coli* hosts.

Table 1 demonstrates the effect of transforming competent *E. coli* cells with methylated DNA. The probability of recovering a clone of interest

Table 1. Transformation with *in vitro*-methylated pBR322 DNA.[a]

Host	*mcrA*	*mcrB*	*Control monomer pBR322 (T/µg)*	*Control unmethylated dimer pBR322 (T/µg)*	*Hpa II-methylated dimer pBR322 (T/µg)*	*Hha I-methylated dimer pBR322 (T/µg)*
MAX EFFICIENCY DH10B cells	–	Δ	1.4×10^9	3.4×10^8	2.5×10^8	2.9×10^8
LIBRARY EFFICIENCY DH5αMCR cells	–	Δ	2.4×10^8	7.8×10^7	3.0×10^7	3.1×10^7
MAX EFFICIENCY HB101 cells	+	–	5.2×10^8	6.5×10^7	3.0×10^4	5.9×10^7
MAX EFFICIENCY DH5α cells	+	+	1.2×10^9	2.4×10^8	4.0×10^4	7.3×10^5

Δ indicates the deletion of this gene.

[a] Aliquots of dimer pBR322 DNA were methylated *in vitro* with *Hpa* II methylase, which methylates the sequence $C^{5m}CGG$ that is restricted by McrA and *Hha* I methylase, which methylates the sequence $G^{5m}CGC$ that is restricted by McrB. The competent cells were transformed according to the supplied protocols with the methylated plasmids (500 pg each), unmethylated dimer (500 pg), and monomer (50 pg) pBR322 DNA as controls for transformation efficiency. Ampicillin resistant colonies were counted and results are presented as transformants per µg (T/µg).

that contains methylated cytosine residues is significantly enhanced when the host is both *mcr*A and *mcr*BC, as shown by the >1,000-fold increase in transformation efficiency of DH10B cells relative to DH5α cells.

In addition to the markers that make DH10B an ideal host for cloning methylated DNA, the DH10B strain contains the following genetic markers: *rec*A1, which increases the stability of inserts; *end*A1, which improves the quality of DNA from mini-preps; and *hsd*RMS, which allows cloning of DNA without cleavage by endogenous restriction endonucleases. The DH10B strain also contains the φ80d*lac*ZΔM15 marker that provides α-complementation of the β-galactosidase gene from pUC or similar vectors for blue/white screening on plates containing X-gal.

MAX EFFICIENCY DH10B Competent Cells are guaranteed to yield >1 $\times$ 10^9 transformants/µg pUC19 and are supplied in convenient 200-µl aliquots. As with all GIBCO BRL competent cells products, MAX EFFICIENCY DH10B Competent Cells are supplied with control DNA (monomer pUC19) and a transformation protocol.

Genotype: F^- *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G.

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COMPETENT CELLS: LARGE VOLUME TRANSFORMATIONS

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LIBRARY EFFICIENCY® (1-3) and MAX EFFICIENCY® (4) Competent Cells provide high transformation efficiencies, $>1 \times 10^8$ and $>1 \times 10^9$ transformants/µg monomer supercoiled plasmid DNA, respectively, for construction of cDNA libraries. The recommended transformation protocol for frozen competent cells uses 100 µl of cells and a 45 s heat shock (3). For the construction of large libraries, some researchers may want to use a larger volume of cells to reduce the number of individual transformation reactions necessary to produce a complete library. According to Hanahan (5), the optimal length of heat shock for a given volume of competent cells is related to the surface to volume ratio of the cell suspension and the thermal conductivity of the tubes used for the transformation.

In this study, the length of heat shock was investigated to determine the conditions for using larger volumes of cells. Transformations were performed using 100-, 250-, and 500-µl aliquots of LIBRARY EFFICIENCY DH5α™ Competent Cells with 50, 125, and 250 pg, respectively, of pUC19 monomer DNA in 11×17-mm polypropylene tubes (Falcon 2059). This DNA concentration is well below the saturation level and within the range where the relationship between number of transformants and DNA concentration is linear (3). The recommended protocol was followed, except that heat shock time was varied from 30 to 90 s. The cells were incubated on ice with the DNA for 30 min, followed by the heat shock. The tubes were returned to ice for 2 min, after which 0.9 ml of S.O.C. medium per 100 µl of cells was added. The tubes were then incubated in a shaking water bath (200 rpm) for 1 h. The cultures were diluted 1:10 in S.O.C., and 100-µl aliquots were plated in triplicate on LB plates containing 100 µg/ml ampicillin. Ampicillin-resistant colonies were counted after overnight growth at 37°C, and transformation efficiencies were calculated based on the average of the three plates.

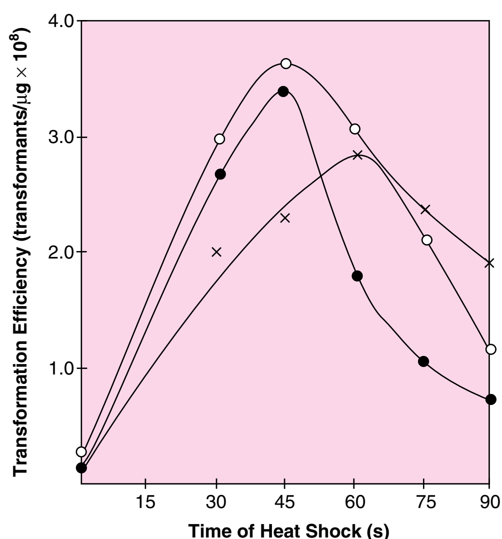

Figure 1. Effect of volume and heat shock length on transformation efficiency. Transformations were performed using 100 µl (●), 250 µl (○), or 500 µl (x) of LIBRARY EFFICIENCY DH5α Competent Cells as described in the text. Each point represents the transformation efficiency calculated from the average number of colonies on three plates.

The results shown in figure 1 indicate that the transformation efficiency using both the 100- and 250-µl volume of competent cells in the 11×17-mm polypropylene tube peaks at a heat shock time of 45 s. In this experiment, the standard 100-µl transformation yielded 3.6×10^8 transformants/µg while the 250-µl volume was approximately the same. Peak transformation efficiency for the 500-µl volume required a 60 s heat shock. Although the transformation efficiency was reduced to 78% of the level achieved with the 100-µl volume, the value was still in excess of 1×10^8 transformants/µg.

In summary, high transformation efficiencies may be maintained with frozen competent cells in large volume transformation reactions provided the appropriate heat shock conditions are employed. Although not investigated in this study, the shape of the tube may also affect the transformation efficiency (5). It is advisable to determine a saturation curve using a small scale reaction for each cDNA preparation prior to scaling up to generate a complete library (5).

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TOP FIFTEEN 15 Fun Facts & Helpful Hints for Competent Cells

Did you know:

▶ You can recover clones from dried agar plates by using the "Lazarus Technique" [see Eissenberg, J.C. (1993) *Focus 15*, 53].

▶ Glycogen and tRNA will not interfere with transformation efficiencies in chemically competent or electrocompetent cells. For tRNA, ≤50 µg/ml may be used. With chemically competent cells, ≤8 µg of glycogen; and with electrocompetent cells, ≤5 µg may be used.

▶ Unopened DH5α cells are stable for up to 1 year, when stored at –70°C.

▶ The transformation mix (after the 1-h incubation in S.O.C.) is stable at 4°C overnight, so you can plate it out the day after doing the transformation. In addition, the transformation mix can be stored at –70°C as a 15% glycerol stock.

▶ Reducing agents such as DTT and BME are not needed with Life Technologies competent cells. With other commercially available cells, these reducing agents need to be added to reach the stated transformation efficiency. When reducing agents are used with Life Technologies competent cells, the transformation efficiency does not change.

▶ When scaling up the volume of cells in transformation reactions, the heat shock kinetics change. If scaling up, use longer incubation times [see Karger, B.D., Rollins, S., and Link, D.S. (1988) *Focus 10*, 34].

▶ You can cure *E. coli* of a plasmid. Dilute cells in a yeast extract, tryptone broth to 10^4 cells/ml. Add 1,2,...5 µg/ml of coumermycin. The next day, streak out the last dilution that shows growth. Check for the presence of plasmid by replica plating with and without antibiotics.

▶ Cotransformation (2 plasmids in the same cell) can be performed in bacteria. Use saturating amounts of DNA (10 ng of each plasmid). Make sure that the origin of replication is different on each plasmid so that they can both be maintained in the cell. If the *ori* is the same, the plasmids will compete for replication and the one with even a slight disadvantage will be lost.

▶ IPTG is not needed with pUC and pUC-based vectors for blue/white screening in DH5α cells, because this host does not contain *lac*I^q [see Sunday, G.J. (1987) *Focus 9:3*, 16]. IPTG is needed in DH5αF´IQ™ or DH5α-FT cells where the *lac*I^q marker is present.

▶ Stbl2 cells may be used to stabilize inserts with direct repeats and retroviral sequences. In addition, 30°C incubation in terrific broth can help prevent deletions.

▶ Using an *end*A1 positive strain is recommended for generating ss M13 DNA to remove any dsDNA plasmids that co-precipitate with the packaged ssDNA. The endonucleases digest the dsDNA, while the packaged ssDNA is protected by the protein coat.

▶ Strains that are MCR minus allow better representation with methylated DNA; hence, these strains are best for constructing genomic DNA libraries.

▶ Strains that are *deo*R can be transformed 10 to 20 times more efficiently by large plasmids [40–60 kb; see Hanahan, D. (1985) *DNA Cloning Vol. I* (Glover, D.M., ed.), p. 111, IRL Press, Oxford].

▶ Biotinylated DNA can be transformed and does not affect the efficiency of transformation. Biotin end-labeled DNA has been ligated and transformed successfully into DH5α cells.

▶ The doubling time of DH5α cells at 37° in rich medium is ~30 min; in minimal medium it is ~90 min. At 30°C the doubling time is ~60–90 min in rich medium and 2.5 h in minimal medium. DH5αF´™ cells will be slower by 5 to 10 min because of the F´ episome.

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Troubleshooting Transformation

If you are getting low transformation efficiency with chemically competent cells:

- There were impurities in the DNA. Remove phenol, protein, detergents, and ethanol, by ethanol precipitation or GLASSMAX® DNA Isolation Systems.
- There was excess DNA. Use no more that 1–10 µg of DNA in ≤5 µl volume/100 µl of cells.
- The cells were handled improperly. Thaw cells on ice, and use immediately. Refreezing can decrease the efficiency. Do not vortex cells.
- Nonoptimized tubes were used. Use 17 × 100-mm polypropylene tubes. The heat shock step is calibrated to the size and material of these tubes. If different tubes are used, the heat transfer to the cells may not be optimal.

If you are getting low transformation efficiency with electrocompetent cells:

- Salts and buffers inhibited electroporation—and may have caused arcing. Use DNA in water or TE (10 mM Tris-HCl, 1 mM EDTA).
- The apparatus settings were not optimal. When using the GIBCO BRL CELL-PORATOR® System, use 0.15-cm gap chambers and 400 V on the CELL-PORATOR unit, 330 µF; low ohm setting; fast charge rate; 4,000 ohm resistance on the Voltage Booster. On the GIBCO BRL *E. coli* CELL-PORATOR Apparatus, use 0.15-cm chambers with the medium setting.
- The microelectroporation chambers, cells, and DNA were not chilled on ice. Also, equilibrate the control compartment of the chamber safe or single safe to 4°C prior to electroporation.
- An excessive volume of cells was used. Use <25 µl and place the cells between the indented bosses.

If you are getting all white colonies, but your clone does not have an insert:

- IPTG was not used with vectors containing the *lac*I^q marker. Spread 30 µl of 100 mM IPTG solution on the surface of the plate.
- The X-gal did not diffuse into the agar properly. Spread 50 µl of 2% X-gal on the surface of the plate or add X-gal to cooled medium before plating at a final concentration of 50 µg/ml. Plates with X-gal should be stored at 4°C in the dark. Do not use plates with X-gal in agar that are >4 months old.
- The color did not develop fully. Incubate at 37°C for 12 to 16 h.
- Small linkers and adapters ligated into the vector. Gel purify the insert and vector prior to ligation.
- The vector used did not allow for α-complementation. Check that the vector contains the α-peptide of the *lac*Z (β-galactosidase) gene.

If you are getting all blue colonies with recombinant DNA:

- The insert was cloned in frame with the α-peptide. Another screening method, such as PCR, is necessary to find recombinants.
- The *E. coli* strain used had an intact β-gal gene. Use an *E. coli* host containing the *lac*ZΔM15 partial deletion of *lac*Z that allows for α-complementation.

If performing large-scale plasmid preps, you have cell death or low cell growth with decreased plasmid yield:

- The insert produced a protein that was toxic to the cells. Decrease transcription of the protein, by adding 20 to 30 mM glucose to terrific broth. Decrease plasmid copy number by changing to a non-pUC-based vector or by incubation at 30°C instead of 37°C.

If you see an opaque colony:

- The protein/RNA transcripts being expressed from the cloned insert altered the morphology of *E. coli* colonies (opaque, instead of translucent). This is gene dependent [see Barik, S. (1997) *BioTechniques 22.1*, 112].

If your restriction endonuclease is not digesting DNA:

- The restriction endonuclease may be *dam* and *dcm* sensitive. If so, propagate the DNA in a *dam* and *dcm* minus strain, such as DM1 Cells (Cat. No. 18268).

If you get extra bands that migrate faster than supercoiled DNA on an agarose gel and are resistant to cleavage by restriction endonucleases:

- The DNA was irreversibly denatured. This has been observed when plasmids were isolated by the alkaline lysis method. To minimize denaturation, use 0.1 N NaOH instead of 0.2 N NaOH and decrease the time of NaOH exposure.

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Protein Analysis with the BenchMark™ Protein Ladders

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Protein molecular weight standards are used to determine the molecular weight of proteins as well as to monitor electrophoresis and Western transfers. BenchMark™ Protein Ladders are recombinant proteins that have been cloned, expressed, and affinity purified. They contain numerous evenly spaced bands from 10 to 220 kDa. The Protein Ladder contains highlight bands at 20 and 50 kDa, and the Prestained Ladder contains a pink highlight band to provide easy orientation. This article provides recommendations for protein electrophoresis and visualization of the BenchMark Ladders.

Methods

SDS polyacrylamide gel electrophoresis (SDS-PAGE). The single-percentage gels were prepared as described by Laemmli (1) using the Gibco BRL SDS-Polyacrylamide Gel System, and the Gibco BRL Protein Gel-Mix® Running Mate concentrated TGS diluted 10 times. The BenchMark Protein Ladder (Cat. No. 10747) is supplied ready to load in 50 mM Tris (pH 6.8), 10% (v/v) glycerol, 2 mM EDTA, 10 mM DTT, 2% SDS; the BenchMark Prestained Protein Ladder (Cat. No. 10748) is supplied in 50 mM Tris (pH 6.8), 10% (v/v) glycerol, 5 mM EDTA, 10 mM DTT, 1% SDS. Both ladders were warmed to room temperature before loading (Do not boil the standards) onto the SDS-PAGE gels. Electrophoresis was at constant voltage (150 V) with the Gibco BRL Model 500 Power Supply until the dye front was 1 cm from the bottom of the gel.

Visualization. The unstained BenchMark Ladder was stained with 0.2% (w/v) Coomassie Brilliant Blue R®-250 in 40% (v/v) methanol, 10% (v/v) acetic acid for 1 to 2 h, then destained in 10% (v/v) methanol, 20% (v/v) acetic acid until the background was clear. Silver staining was performed as described (2).

Electrophoretic transfer. After electrophoresis, the gel was equilibrated for 15 min with Towbin (3) transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% (v/v) methanol). The proteins were transferred onto 0.2-µm nitrocellulose membrane, at 100 V for 60 min in transfer buffer.

Results

To assess the molecular weight range of separation on SDS-PAGE, the BenchMark Protein Ladder was electrophoresed on several percentage gels. For 8, 12, and 16% SDS-PAGE gels, the useful molecular weight ranges were 40-200 kDa, 15-100 kDa, and 5-50 kDa, respectively (figure 1). On an 8% gel, proteins with molecular weights <30 kDa migrated with the dye front. The 16% gel compressed proteins >60 kDa. Use of a 4-20% gradient gel resulted in the most uniform distribution from 10 to 220 kDa, permitting screening of samples with a wide molecular weight range. Also, the sharp bands and orientation bands were maintained with silver staining using 5 to 10% of the standard load.

The BenchMark Protein Ladder was used as a standard to generate apparent

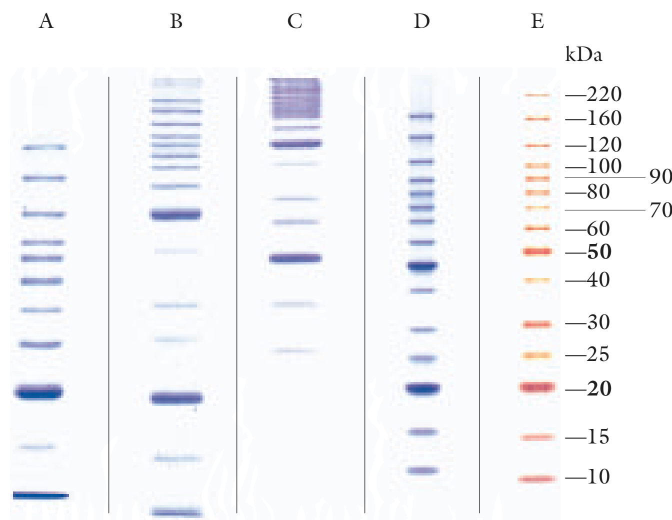

FIGURE 1. Protein separation on various percentage gels. The BenchMark Protein Ladder (5 µl) was electrophoresed on 8%, 12%, 16%, and 4-20% minigels (Panels A - D, respectively) and Coomassie Brilliant Blue R-250 stained. Gels in panels A - C were 1-mm-thick with 5-mm lanes. Gels in panels D and E were 1.5-mm-thick with 3-mm lanes. Panel E. 0.25 µl of the ladder was electrophoresed on a 4-20% gel and silver stained.

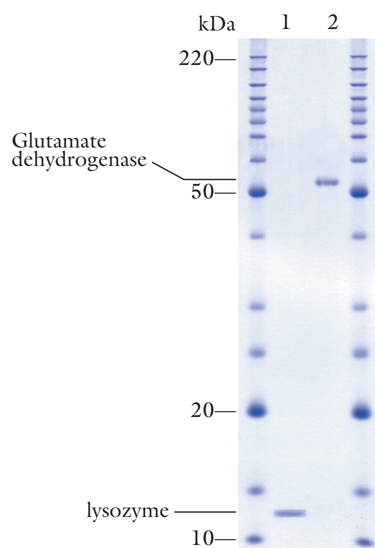

FIGURE 2. Electrophoretic mobility comparison. The BENCHMARK Protein Ladder (10 µl), lysozyme (lane 1) and glutamate dehydrogenase (lane 2) were electrophoresed on a 12% SDS-PAGE gel (15 × 17 cm) and stained with COOMASSIE BRILLIANT BLUE R-250.

molecular weights for glutamate dehydrogenase (55.5 kDa) and lysozyme (14.3 kDa) (figure 2). The apparent molecular weight of glutamate dehydrogenase was 53.1 kDa and for lysozyme was 13.2 kDa.

Prestained protein standards have been used to monitor the progress of proteins through an SDS-PAGE gel and to assess the quality of electrophoretic transfer onto a membrane in a Western blot. Prestained standards are not designed to be used for molecular weight determination, since modification of the proteins with the dyes results in changes in the apparent molecular weight of each protein. The apparent molecular weight of each prestained protein is determined for each lot using the unstained ladder (figure 3). Typical ranges for the apparent weights were ~10-220 kDa.

The prestained ladder can be used to monitor transfer of proteins to membranes for Western blots (figure 4). The amount of ladder needed to see the bands after transfer was ~ half that required to observe the ladder directly in the gel during electrophoresis. Electrophoretic transfer conditions required for complete transfer vary depending upon the apparatus, gel thickness, and percent acrylamide. Follow the recommended conditions of the transfer apparatus manufacturer. Under the conditions used in figure 4, the 220-kDa band was only partially transferred.

RECOMMENDATIONS

Some general recommendations for use of the BENCHMARK Protein Ladders are below. A key parameter is the amount of protein to use. Factors that affect the optimal loading amount include lane width, gel thickness, gel size, percent polyacrylamide, and whether the proteins will be transferred to a membrane. Table 1 shows recommendations for the prestained ladder. If the proteins will be electrophoretically transferred, then only half the recommended amount is required.

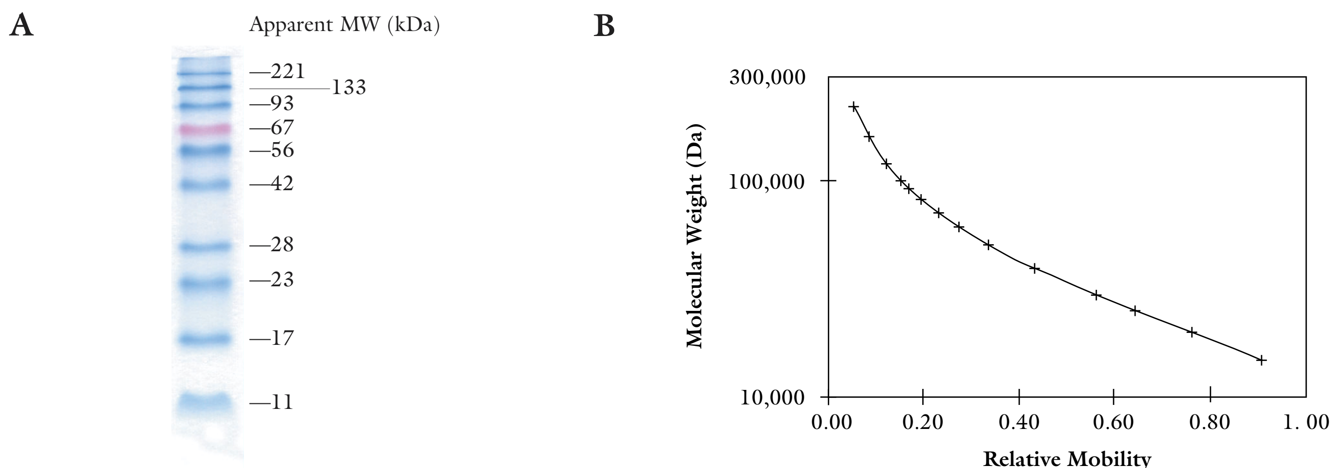

FIGURE 3. Apparent molecular weight of the BENCHMARK Prestained Protein Ladder. Panel A. The Prestained Protein Ladder (15 µl) was electrophoresed on a 12.5% SDS-PAGE gel. Panel B. Apparent molecular weights were calculated by interpolation of a curvilinear fit of the standard curve generated by plotting the log of the known molecular weights of the bands in the unstained BENCHMARK Protein Ladder and the relative mobility (R_f) of each protein.

Providing recommendations for the unstained ladder is more difficult due to the variety of protein stains available. The age of the stain and destain, the staining time, the destaining time, and the composition of the stain and destain can affect the intensity of the unstained ladder. Empirical data are critical to obtain optimal results.

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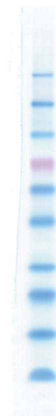
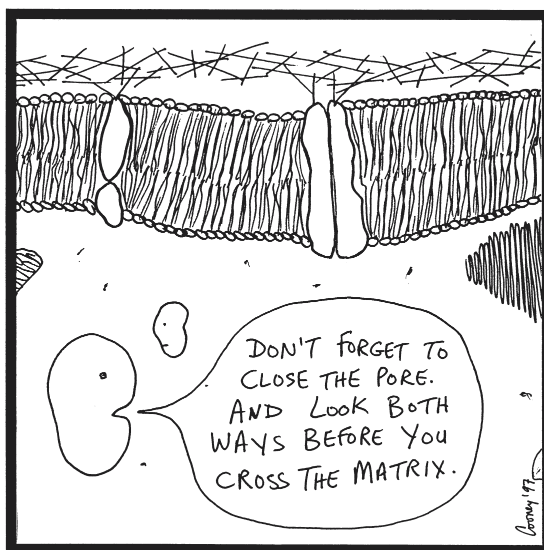


FIGURE 4. Electrophoretic transfer. The BenchMark Prestained Protein Ladder (5 µl) was electrophoresed on a 1.5-mm-thick 4-20% SDS-PAGE (3-mm lane width). The proteins were transferred to nitrocellulose.

TABLE 1. Gel loading recommendations for the BenchMark Prestained Ladder.

Lane Width	Gel Thickness	Gel Size	Acrylamide (%)	Recommended Load (µl)
4 mm	1.0 mm	Minigel	12	10
6 mm	1.0 mm	Minigel	12	15
4 mm	1.5 mm	Minigel	12	15
4 mm	1.0 mm	15 × 17 cm	12	20
4 mm	1.0 mm	Minigel	8	20

The first line in the table is the point of reference and the underlined parameter is the altered parameter in each row. The minigel is 8 × 10 cm.



When exocytotic vesicles leave home.

CHEMILUMINESCENT DETECTION OF AFLP™ FINGERPRINTS

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ABSTRACT

Chemiluminescent detection of AFLP™ patterns was compared with AFLP patterns detected by ^{32}P-labeled AFLP primers. DNA bands detected with ^{32}P-labeled primers also were detected with chemiluminescent detection using a small denaturing sequencing gel (15 × 17 cm). Moreover, chemiluminescent detection of AFLP patterns using a universal AFLP nonradioactive probe was achieved in both eukaryotes and prokaryotes with various genome sizes and complexity.

Recently, a novel PCR-based DNA fingerprinting technique, Amplified Restriction Fragment Polymorphism (AFLP), has been described as a powerful technique to identify molecular markers for both plant and bacterial DNA (1,2). AFLP is performed by 1) restriction endonuclease digestion of genomic DNA and ligation of specific adapters; 2) amplification of the subpopulation of genomic DNA by PCR using primer pairs containing common sequences of the adapter and one to three arbitrary nucleotides; and 3) gel electrophoresis analysis of the amplified fragments. The combination of different restriction endonucleases, the choice of selective nucleotides in the primers, and resolution of sequencing gels makes the results highly reproducible and able to detect multiple polymorphic DNA markers (2–4). Therefore, the AFLP technique has become a well-accepted DNA fingerprinting technique for the construction of genetic linkage maps in plants and molecular typing for both eukaryotes and prokaryotes (5–8).

Typically, detection of AFLP bands requires radioisotope-labeled primers. Nonradioisotopic detection of AFLP can use fluorescent-labeled primers and DNA sequencing instruments, but it requires heavy capital investment and the expense of fluorescent-labeled primers. *In situ* gel detection using silver stain is an alternative nonradioisotopic technique for detection of AFLP (9). However, both strands of the PCR products are detected, resulting in the formation of many doubly banded patterns. This complicates the interpretation of AFLP results. Moreover, low sensitivity limits the detection, especially with the small DNA fragments.

Chemiluminescence is a sensitive nonradioisotopic detection technique (10). In general, chemiluminescent detection involves blotting nucleic acids onto membranes, followed by hybridization of a probe to add an enzyme to activate the chemiluminescent substrate. In this paper, we describe a simple nonradioisotopic detection of AFLP patterns using chemiluminescence. The AFLP products were separated on a small (15 × 17-cm) denaturing sequencing gel, blotted onto a nylon membrane, hybridized with an alkaline phosphatase-labeled probe, and detected by chemiluminescence. The technique has been applied in eukaryotes such as soybean and in prokaryotes such as *Agrobacterium*.

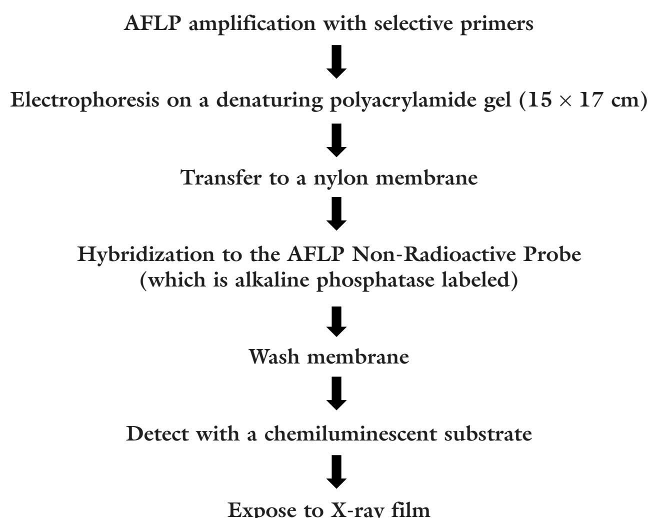

FIGURE 1. Scheme of chemiluminescent detection of AFLP.

METHODS

Genomic DNA. Agrobacterium strains, Tm4 and S4, were kindly supplied by Dr. E. Nester (University of Washington). Single colonies were generated on GIBCO BRL YM agar plates at 30°C for 24 h. Single colonies from different

strains were inoculated into 3 ml of YM medium. The cells were grown at 30ºC for 24 h with constant shaking. Genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation as described (1).

The genomic DNA of two soybean ecotypes, Noir and Barc, was from the lab of Dr. Matthews at the USDA in Beltsville, Maryland.

AFLP reaction. The AFLP reaction was performed by following the manufacturer's instructions in the AFLP Analysis System I and II manuals. Briefly, 200 to 250 ng of genomic DNA were digested with 5 units of *Eco*R I and *Mse* I at 37ºC for 2 h and incubated at 70ºC for 15 min. The DNA fragments were then ligated to *Eco*R I and *Mse* I adapters at 20ºC for 2 h.

For preselective amplification, 5 µl of a 10-fold diluted ligation mixture were amplified using *Eco*R I +0 and *Mse* I +0 primers for *Agrobacterium* samples, or using the premade pre-amplification mixture supplied in the AFLP Core Reagent Kit for soybean samples.

The AFLP *Eco*R I +C and *Mse* I +G primers were used for the selective amplification of *Agrobacterium* samples. For the soybean samples, *Eco*R I +AAA and different *Mse* I +3 primers—+CCT or +CGA, —were used for the selective amplification.

AFLP gel electrophoresis and blotting. The AFLP samples were heated at 90ºC for 3 min before loading on a 5% polyacrylamide sequencing gel. The soybean samples amplified by *Eco*R I +AAA and *Mse* I +CCT or +CGA were electrophoresed for 3 h at 200 V on a 15 × 17-cm gel or 105 min at 2,015 V on a 30 × 39-cm gel. After gel electrophoresis, the DNA was transferred to a GIBCO BRL Biodyne B membrane overnight and baked at 80ºC for 1 h.

Hybridization. The membranes were prehybridized at 42ºC for 20 min in the ACES Prehybridization Buffer [500 mM $NaPO_4$ (pH 7.2), 0.1% SDS] and hybridized with 6.5 µl of the AFLP Non-Radioactive Probe (Cat. No. 10822) at 42ºC for 30 min in the ACES Hybridization Buffer [500 mM $NaPO_4$ (pH 7.2), 1% Hammersten grade casein, 0.1% SDS]. The membranes were washed 2 times in 50 mM $NaPO_4$ (pH 7.2), 0.1% SDS at 42ºC and 2 times in the final wash buffer [10 mM Tris-HCl (pH 8.6), 150 mM NaCl]. The membranes were incubated with CDP-Star at room temperature for 5 min and exposed to

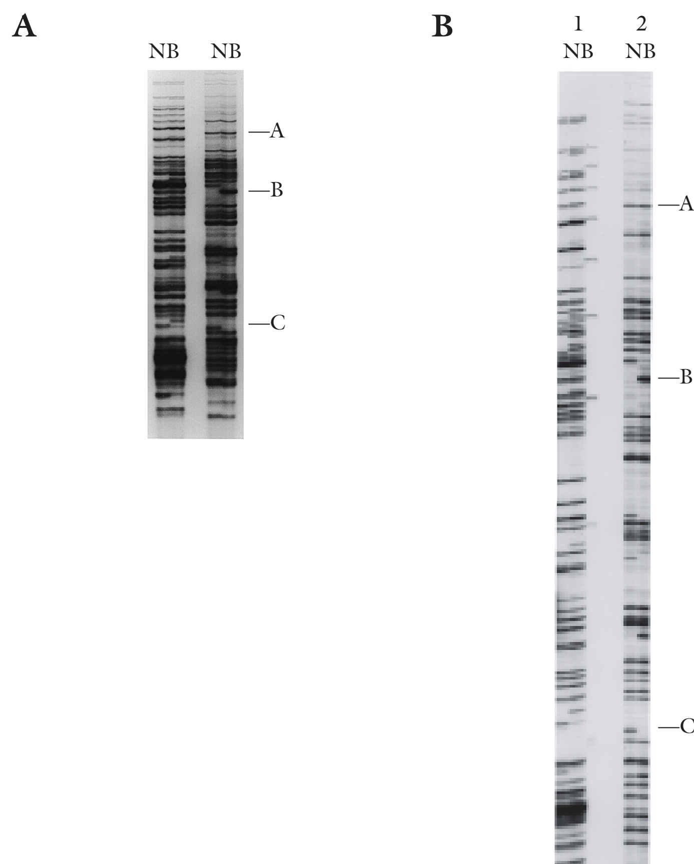

FIGURE 2. AFLP patterns on large and small acrylamide gels. Soybean genomic DNA was isolated from two ecotypes, Noir (N) and Barc (B). The ^{32}P-labeled *Eco*R I primer used for all the samples was +AAA. The *Mse* I primers were +CCT and +CGA for lanes 1 and 2, respectively. Panel A. 15 × 17-cm gel. Panel B. 30 × 39-cm gel. Representative bands are marked on each gel (A–C).

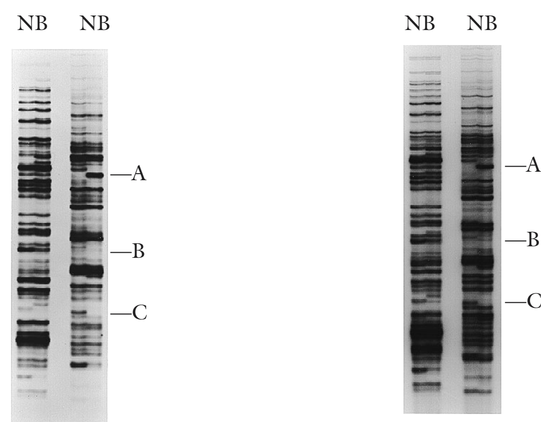

FIGURE 3. Comparison of radioisotopic and chemiluminescent detection. Soybean genomic DNA was isolated from two ecotypes, Noir (N) and Barc (B). The *Eco*R I +AAA primer was used for all the samples. The *Mse* I primers were +CCT and +CGA for lanes 1 and 2, respectively. Panel A. Chemiluminescent detection. Panel B. Radioisotopic detection. Representative bands are marked on each gel (A–C).

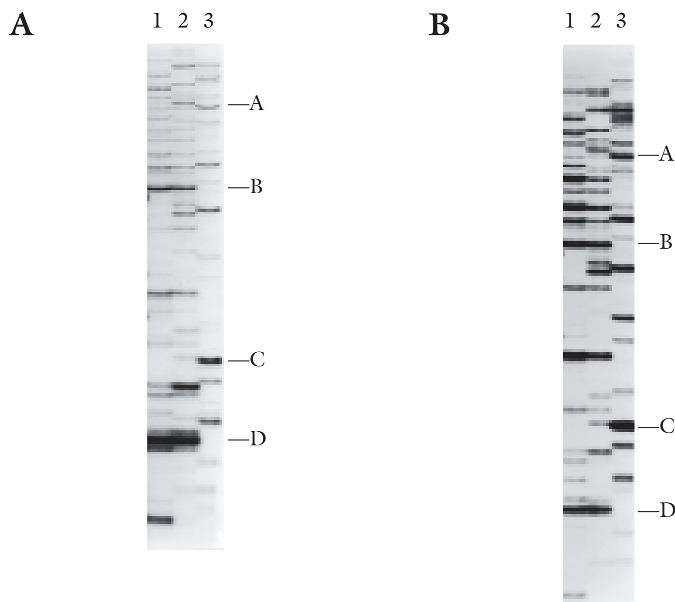

FIGURE 4. Comparison of AFLP pattern between radioisotopic and chemiluminescent detection for *Agrobacterium* strains. AFLP primers were *Eco*R I +G and *Mse* I +C. Lane 1: Tm4; lane 2: S4; lane 3: LBA4404. Panel A. Radioisotopic detection. Panel B. Chemiluminescent detection. Representative bands are marked on each gel (A–D).

X-ray film at room temperature for 45 min.

Results and Discussion

The scheme of chemiluminescent detection of AFLP is illustrated in figure 1. Initially, large sequencing gels (30 × 39 cm) were used to obtain maximum data points with the AFLP technique (1,2). AFLP resolution on a 15 × 17-cm sequencing gel was evaluated with soybean using a ^{32}P-labeled primer (figure 2). Polymorphic DNA bands in the small gel were similar to those bands detected in the large gel. Resolution of AFLP using a 15 × 17-cm sequencing gel makes chemiluminescent detection by blotting and hybridization feasible.

Chemiluminescent detection was performed on AFLP products from 2 soybean ecotypes (figure 3). Similar AFLP patterns were observed in those performed by a ^{32}P-labeled primer and in those detected by chemiluminescent detection. Although chemiluminescent detection did not detect some of the faint DNA bands, the dominant polymorphic bands were seen using chemiluminescent detection.

AFLP patterns are determined by the genome complexity and the numbers of selective nucleotides in the primer combinations (1,2,4). Chemiluminescent detection was tested for AFLP analysis in genomes with different complexity using the same AFLP Non-Radioactive Probe and compared to using ^{32}P-labeled primers (figure 4). For *Agrobacterium,* similar patterns were seen with nonradioactive and radioactive detection as seen in soybean. These results demonstrate that chemiluminescent detection can be applied in organisms with different genome complexity as well as with different AFLP primer combinations using the AFLP Non-Radioactive Probe.

In summary, chemiluminescent detection of AFLP using the AFLP Non-Radioactive Probe (directly labeled with alkaline phosphastase) offered AFLP results comparable to those detected by ^{32}P-labeled primers in both prokaryotes and eukaryotes. Moreover, the requirement for a simple gel apparatus and power supply for chemiluminescent detection offers the advantage of using several gels simultaneously for multiple sample analysis. The AFLP Non-Radioactive Probe can be used for organisms with different genome sizes and genome complexity.

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A Highly Sensitive Method for One-Step Amplification of RNA by Polymerase Chain Reaction

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ABSTRACT

Using SuperScript™ II RT (1) and *Taq* DNA polymerase (2), we developed a convenient and sensitive SuperScript One-Step RT-PCR System. The system uses two premixed solutions: 1) an optimized mixture of SuperScript II RT and *Taq* DNA polymerase and 2) a 2X reaction mix containing buffers, dNTPs, and $MgSO_4$. With this system, 10 copies of an *in vitro* transcript RNA and β-actin mRNA from 100 fg of total HeLa RNA were detected. The high sensitivity and premixed format of the One-Step RT-PCR System make it an easy and convenient tool for rapid and routine screening of RNA expression.

Coupling reverse transcription and polymerase chain reaction (RT-PCR) is a sensitive and powerful method to detect RNA (3). Using SuperScript II RT for cDNA synthesis improves the efficiency and sensitivity of RT-PCR as compared to MMLV RT or AMV RT (4). RT-PCR can be carried out either in two-step or one-step formats. In two-step RT-PCR, cDNA synthesis is first performed with RT in an appropriate buffer. The RT step is followed by PCR amplification with a thermostable DNA polymerase in another appropriate buffer (5). This two-step format requires opening the reaction tube after cDNA synthesis to either remove a cDNA aliquot for subsequent PCR or to add PCR reagents. The method is widely used and effective for cDNA cloning and characterization, RACE techniques, and cDNA library construction, as well as gene expression detection.

In the one-step RT-PCR method, reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both the RT and DNA polymerase without opening the tube. This simplifies the procedure and minimizes the potential for cross-sample contamination. One-step RT-PCR is suitable for routine and high-throughput screening of gene expression. When using SuperScript II RT, the One-Step RT-PCR System detects RNA molecules present in low abundance.

Methods

RNAs. The 891-bp CAT mRNA was a run-off transcript of pTEPA-CAT plasmid DNA by T7 RNA polymerase. The CAT RNA was treated with DNase I, Amplification Grade (Cat. No. 18068), for removal of DNA template, followed by phenol extraction and ethanol precipitation. Total HeLa RNA was isolated by TRIzol® Reagent or the GlassMAX® RNA Microisolation Spin Cartridge System (6).

One-step RT-PCR. One-step RT-PCR was carried out using the Gibco BRL SuperScript One-Step RT-PCR System (Cat. No. 10928). Reactions (50 µl final volume) were assembled by mixing 25 µl of 2X reaction mix [2X buffer includes 2.4 mM $MgSO_4$, 400 µM dNTPs each, and 4 µg/ml BSA], 1 µl of enzyme mix [SuperScript II RT and recombinant *Taq* DNA polymerase in 20 mM Tris-HCl (pH 7.5 at 25°C), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol (v/v), and stabilizer],

TABLE 1. Primer sequences.

Gene	Primer		Product size (bp)
CAT	sense	CGACCGTTCAGCTGGATATTAC	500
	antisense	TTGTAATTCATTAAGCATTCTGCC	
β-actin	sense	TGAAGTACCCCATCGAGCACG	174
	antisense	CAAACATGATCTGGGTCATCTTCTC	
β-actin	sense	CAGGGCGTGATGGTGGGCA	253
	antisense	CAAACATGATCTGGGTCATCTTCTC	
β-actin	sense	GCTCGTCGTCGACAACGGCTC	353
	antisense	CAAACATGATCTGGGTCATCTTCTC	
β-actin	sense	TGAAGTACCCCATCGAGCACG	755
	antisense	AGTGATCTCCTTCTGCATCCTGT	
β-actin	sense	GCTGGTCGTCGACAACGGCTC	976
	antisense	AGGAGCAATGATCTTGATCTTCATT	
β-actin	sense	ATGGCCACGGCTGCTTCCAGCTCC	1,026
	antisense	ATTCAACTGGTCTCAAGTCAGTGTA	
β-actin	sense	GCTCGTCGTCGACAACGGCTC	1,684
	antisense	ATTCAACTGGTCTCAAGTCAGTGTA	
β-actin	sense	GCCAGCTCACCATGGATGATGAT	1,715
	antisense	ATTCAACTGGTCTCAAGTCAGTGTA	

* Primer sequences for the polε, RPA, PP2A, and CBP PCR products are listed in the Internet version of this article at http://www.lifetech.com/focus/192039.pdf.

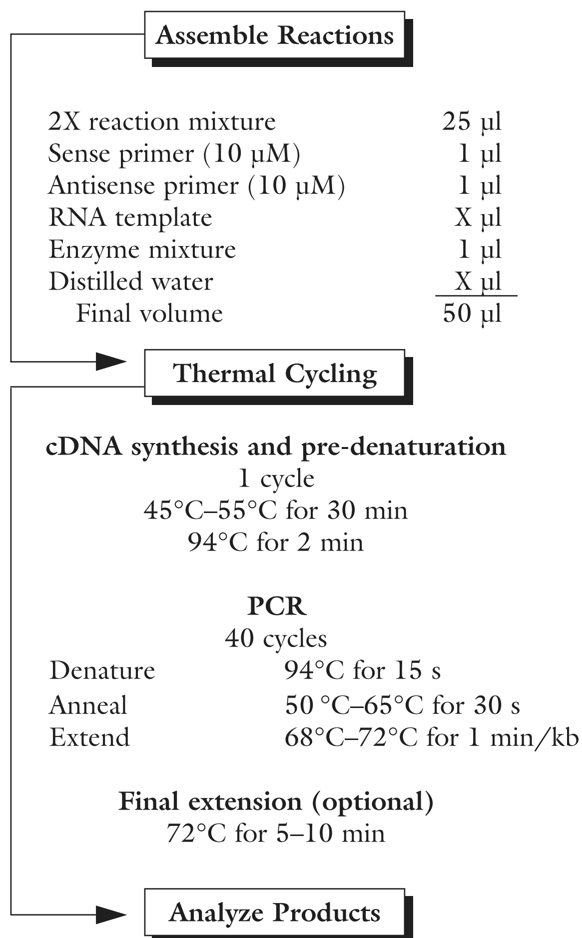

FIGURE 1. The SuperScript One-Step RT-PCR protocol.

200 nM of each primer (table 1), and the appropriate amount of sample RNA. Alternatively, for experiments utilizing the same primer or target RNA, a master mix of enzyme and buffer with primer or target RNA was made. The samples were incubated at 45°C–55°C for 30 min; then 94°C for 2 min followed by amplification of 40 cycles of 94°C for 15 s, 50°C–65°C (depends on primer set) for 30 s, and 68°C–72°C for 1–3 min (1 kb/1 min); followed by one cycle of 72°C for 5–10 min. PCR products (10 µl) were analyzed on 0.8%–1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide.

Results and Discussion

Several reports have suggested inhibition of amplification when RT was mixed with *Taq* DNA polymerase for one-step RT-PCR (7). From our studies (data not shown), the inhibition appears to be related to the amount of enzyme and buffer conditions. By examining the ratio of enzymes in combination with a variety of buffers, a one-step RT-PCR system was developed that permits optimal activity for both SuperScript II RT and *Taq* DNA polymerase. The procedure is shown in figure 1.

The system detected 10 copies of a 500-bp CAT product (figure 2). No PCR products were

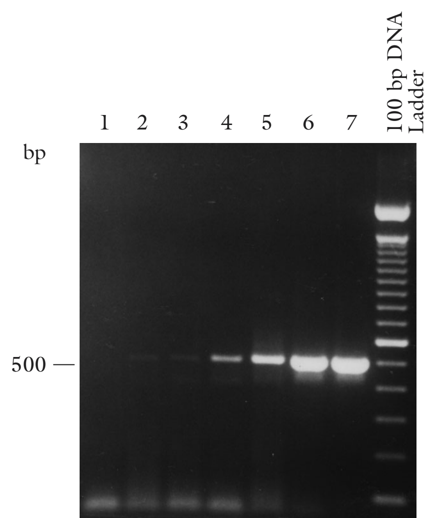

FIGURE 2. Amplification of CAT mRNA. Reactions were incubated at 45°C for 30 min; 94°C for 2 min; then 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 90 s; followed by 68°C for 5 min. Lane 1. No RNA template. Lanes 2 to 7 contain 5, 10, 10^2, 10^3, 10^4, and 10^5 copies of CAT mRNA, respectively.

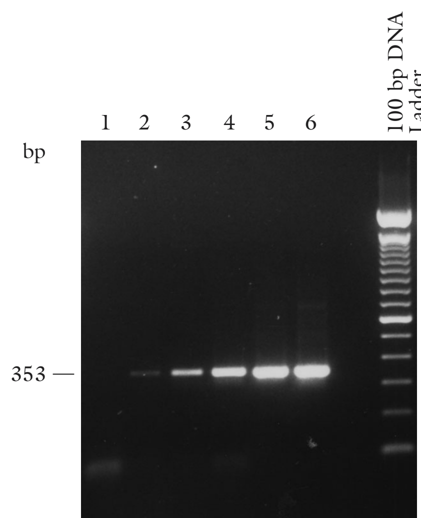

FIGURE 3. Amplification of β-actin mRNA. The incubations were as in figure 1 except the cDNA synthesis was at 50°C and the annealing temperature was 55°C. Lane 1. No RNA template, Lanes 2 to 6 contain 0.1, 1, 10, 10^2, and 10^3 pg total HeLa RNA.

observed from control reactions that omitted RT with up to 10^9 copies of CAT mRNA (data not shown). Application of one-step RT-PCR to samples containing limited quantities of total cellular RNA was tested. A 353-bp β-actin fragment was detected from 0.1 pg total HeLa RNA (figure 3).

SuperScript II RT improves the versatility of the SuperScript One-Step System. The RT reaction can be performed between 42°C and 55°C (1). This may facilitate amplification of RNAs with secondary structure. Cosolvents such as dimethyl sulfoxide and glycerol that may help RT-PCR (8,9) were excluded from the reaction buffer, since no significant improvement was observed for the amplicons tested (data not shown). Since SuperScript II RT is highly efficient in the One-Step RT-PCR buffer, incubation times may be decreased for short templates (<300 bp) to 1–2 min at 45°C. A 10-min incubation was sufficient for detection of the 1.68-kb β-actin mRNA target (figure 4). The 30-min RT incubation was chosen to permit efficient cDNA synthesis for a wide range of primer sets. Decreased yield of specific product and increased nonspecific bands were observed with some of the primer sets with incubation times beyond 30 min (data not shown).

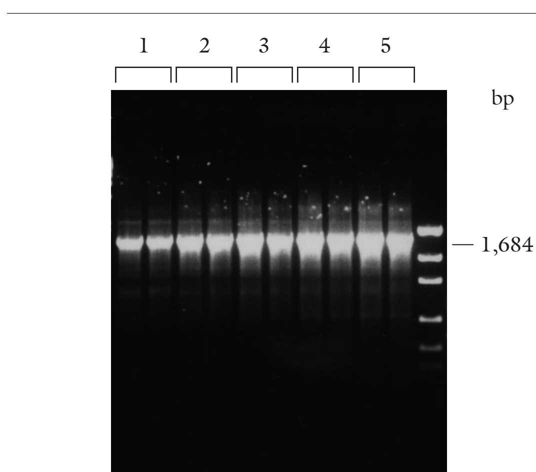

FIGURE 4. Incubation time for cDNA synthesis. The 1,684-bp β-actin fragment amplified after RT incubation at 45°C for 2, 5, 10, 15, and 20 min, respectively, in duplicate (lanes 1–5).

The SuperScript One-Step System was used with RNA targets ranging from 100 bp to 3.5 kb (figure 5). The RNA targets included β-actin (10), DNA polymerase ε (polε) (11), cap binding protein (CBP) (12), replication protein A (RPA) (13), and phosphatase 2A (PP2A) (14), representing genes with different levels of abundance. The system detected specif-

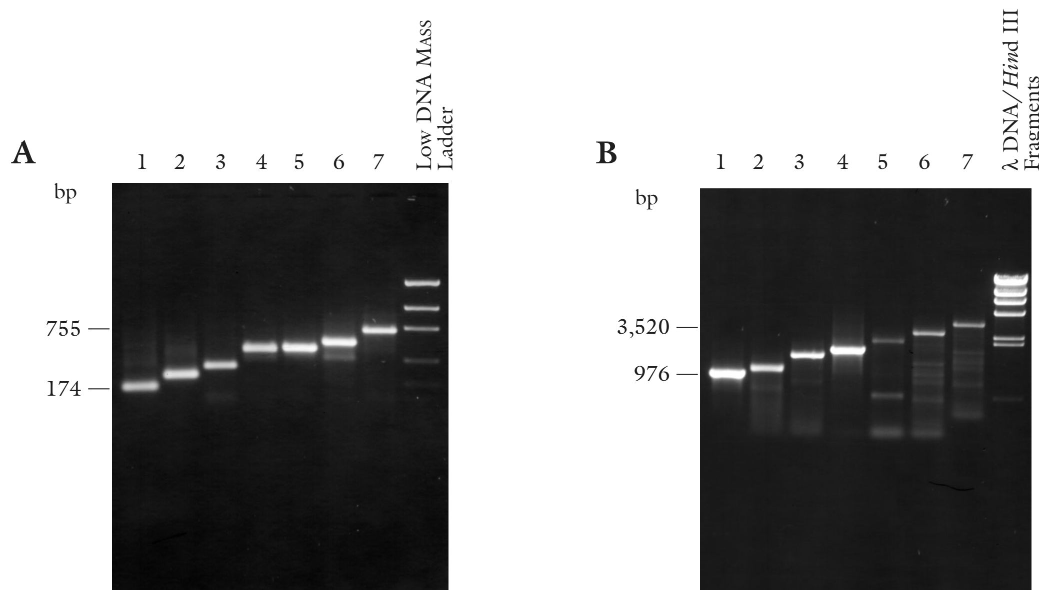

FIGURE 5. RT-PCR products of different sizes. 4–8 µl of RT-PCR products were loaded on a 1.5% (Panel A) or 1.0% (Panel B) agarose gel containing ethidium bromide. Panel A. Lanes 1 and 2. β-actin, 174 and 253 bp. Lane 3. PP2A, 331 bp. Lane 4. CBP, 495 bp. Lane 5. RPA, 514 bp. Lane 6. polε, 606 bp. Lane 7. β-actin, 755 bp. Panel B. Lane 1. β-actin, 976 bp. Lanes 2 and 3. polε, 1,081 and 1,475 bp. Lane 4. β-actin, 1,715 bp. Lanes 5–7. polε, 2,036, 2,531, and 3,520 bp, respectively.

ic mRNA targets using total RNAs from a variety of sources, including HeLa cells, human tissue (submaxillary salivary gland cell), rat tissue (liver, brain, and spleen), and tobacco plant leaves (data not shown). In addition, one-step RT-PCR has a large capacity for RNA, since as much as 5 µg total RNA template was used, which can be useful for the detection of very rare mRNAs.

One important parameter for PCR is the magnesium concentration. Optimal concentration can vary depending on the primer sets. Analysis of >600 RT-PCRs with 40 different primer sets designed for 11 different genes (tested at 1 to 2 mM magnesium) showed that the 1.2-mM magnesium concentration of the SuperScript One-Step System detected these targets. Only 4 primer sets showed low yield and a slightly higher magnesium optimum (1.4 to 2.0 mM). These reactions were easily optimized by addition of magnesium ion. (These data are available in the Internet version of this article at http://www.lifetech.com/focus/192039.pdf.)

The data presented use a gene-specific primer for cDNA synthesis. Use of oligo(dT) is not recommended for the one-step procedure since this system uses higher temperatures (45°C–55°C), which would give poor yield of cDNA with oligo(dT). If oligo(dT) is necessary, a two-step system is recommended.

In this paper, we have described the SuperScript One-Step RT-PCR System for rapid screening and sensitive amplification of RNA in a one-step protocol. A total of 40 primer sets for 11 separate mRNAs of varying abundance successfully amplified and detected different regions ranging between 100 bp and 3.5 kb.

Acknowledgements

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Passive PCR?

Chained to the reaction
absorbed into the fundamentals
the truth cannot escape

Poly Poly Poly
it's my race
the recipe to repeat

Primed more specific
to the pur breed of
More More More

Still chained to the reaction
Science isn't mere science
anymore…

—Lynn Shooks

RT-PCR of Difficult Templates Using the SuperScript One-Step™ RT-PCR System

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The simplicity and sensitivity of the SuperScript One-Step™ RT-PCR System have been shown for templates up to 3.5 kb (1). In this article, we present the amplification of mRNAs up to 8.9 kb from total HeLa RNA using the One-Step RT-PCR System in combination with eLONGase™ Enzyme Mix. In addition, SuperScript II RT was used to reverse transcribe RNA templates at up to 55°C.

Methods

Total HeLa RNA was isolated with TRIzol® Reagent. The SuperScript One-Step RT-PCR System (Cat. No. 10928) was used as described in the previous paper (1).

For the temperature study of SuperScript II RT employed in the One-Step System, identical reactions were assembled and incubated at 45°C to 55°C in duplicate. The total HeLa RNA used varied from 1 ng to 100 ng, depending on the abundance of the mRNA. After the RT incubation for 30 min, the reactions were incubated at 94°C for 2 min and then 40 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 60 s, followed by 68°C for 5 min.

For larger RT-PCR products, the magnesium concentration was increased to 1.8 mM from the standard 1.2 mM, and 1 µl of eLONGase Enzyme Mix (Cat. No. 10481) was added to each reaction. The final 50-µl reaction consisted of 1X buffer, 1.8 mM $MgSO_4$ and other salts, 200 µM dNTPs each, 2 µg/ml BSA, 0.2 µM of primers, 100 ng of total HeLa RNA, 1 µl of One-Step RT-PCR Enzyme Mix, and 1 µl of eLONGase Enzyme Mix. For the experiments using the same template or primers, a master mix of buffer, enzyme mixes, and primers or template was made to ensure consistency. The samples were incubated at 50°C for 30 min and then 94°C for 2 min. Amplification was performed with 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 6 to 9 min (1 min/kb). All primers were Gibco BRL Custom Primers. The products were resolved and visualized on 0.8 or 1.0% (w/v) agarose-TAE gels containing ethidium bromide.

Results and Discussion

Temperature of the RT reaction. SuperScript II RT has improved temperature stability compared to MMLV RT (2). To test the effect of temperature on RT-PCR products, 36 primer sets (representing different genes from human, rat, plant, and one *in vitro* transcript) were examined. With many fragments (figure 1), product yield and specificity were good at 45°C and increased temperatures made no difference. For some template-primer combinations (figure 2A), mispriming that was characteristic at 45°C disappeared with increased temperature. In some cases (figure 2B), there was an increase in product yield with elevated temperature.

Long RT-PCR products. The SuperScript One-Step RT-PCR System was designed for sensitivity and ease of use for high-throughput RT-PCR. Typically, these products are <3.5 kb and the combination of SuperScript II RT and *Taq* DNA polymerase offers the best combination of enzymes for a highly sensitive system (1). For studies of full-length coding sequences or

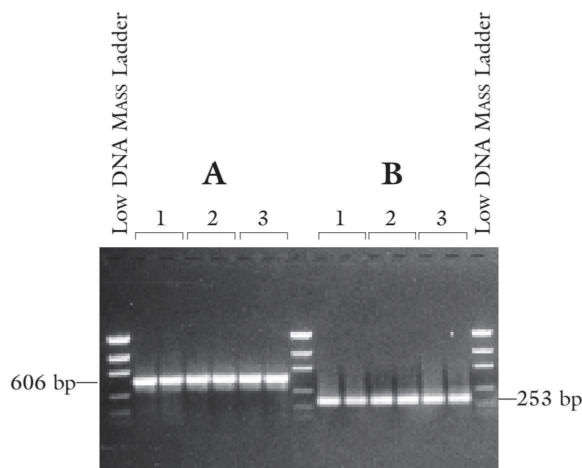

FIGURE 1. Templates not affected by RT reaction temperatures. The RT incubation was at 45°C, 50°C, and 55°C (lanes 1–3, respectively). For the 606-bp pol ε product (Panel A), 100 ng and for the 253-bp β-actin product (Panel B), 10 ng of total HeLa RNA were used. Duplicate samples were analyzed on a 1.0% agarose-tris acetate gel.

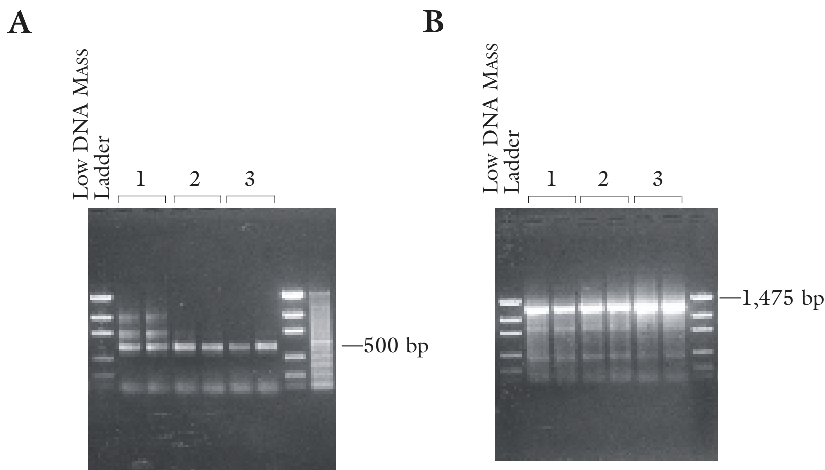

FIGURE 2. Improved RT-PCR products at higher RT reaction temperatures. Panel A. The 500-bp GAPDH product was amplified from 10 ng of tobacco total RNA. Panel B. The pol ε 1,475-bp product was amplified from 100 ng of total HeLa RNA. The RT incubation was at 45°C, 50°C, and 55°C (lanes 1–3, respectively). Duplicate samples were analyzed on a 1.0% agarose-tris acetate gel.

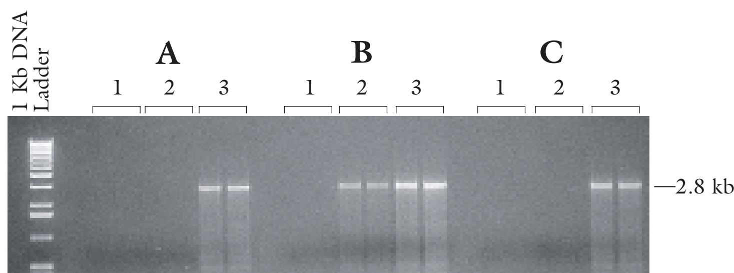

FIGURE 3. Sensitivity of RT-PCR for larger products. 1 ng, 10 ng, and 100 ng (lanes 1–3, respectively) of total HeLa RNA were used in 50-µl RT reactions incubated at 50°C. Primers for a 2.78-kb tuberous sclerosis II (TSC II) fragment were used. Duplicate samples were analyzed on a 0.8% agarose-tris acetate gel. Panel A. SuperScript One-Step RT-PCR System. Panel B. One-Step System with 1 µl of eLONGase Enzyme Mix. Panel C. Supplier A's kit (contains a polymerase enzyme mix for long PCR).

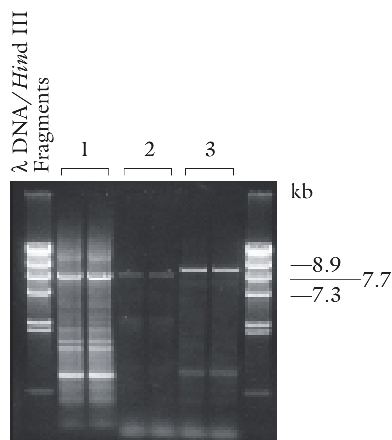

FIGURE 4. Long RT-PCR products. 100 ng of total RNA were used with a 50°C RT reaction temperature. 1 µl of eLONGase Enzyme Mix was added to the One-Step System. Three primer sets resulting in 7.3, 7.7, and 8.9 kb (lanes 1–3, respectively) PCR products of the adenomatous polyposis coli (APC) gene were used. 10 µl of the 7.3-kb and 20 µl of the 7.7- and 8.9-kb fragments were analyzed in duplicate on a 0.8% agarose gel.

for amplification of long segments of RNA, the use of the One-Step RT-PCR System supplemented with eLONGase Enzyme Mix was tested. The SuperScript One-Step RT-PCR System and Supplier A's one-tube kit (figure 3) were able to amplify a 2.8-kb product from 100 ng of total RNA, but not from 10 ng. The addition of eLONGase Enzyme Mix to the One-Step RT-PCR System not only increased the product yield with 100 ng of total RNA but also increased the sensitivity to allow amplification of long templates from 10 ng of total RNA. Even though Supplier A's one-tube kit contained a polymerase enzyme mix designed for long templates, it was not able to amplify the 2.8-kb target from 10 ng of total RNA.

The SuperScript One-Step System supplemented with eLONGase Enzyme Mix produced RT-PCR products up to 8.9 kb (figure 4). The variation in product yield is at least partially due to different primer sets. The One-Step protocol also resulted in production of nonspecific bands. This is not uncommon for long templates. Even though the One-Step System can amplify large RT-PCR products, the conventional two-step protocol using the SuperScript Preamplification System and eLONGase Enzyme Mix is recommended for this application. This allows the flexibility to optimize both the RT and PCR reactions individually. The two-step protocol utilizes oligo(dT) for reverse transcription to produce full-length cDNA, RNase H treatment, and then amplification using gene-specific primers (3). The RNase H treatment has been shown to be essential for some long templates and primer combinations (3).

In summary, the addition of eLONGase Enzyme Mix has made it possible to amplify long and rare mRNAs directly from total RNA preparations. Further, the thermostability of SuperScript II RT, allowing RT reactions up to 55°C, can increase the specificity and product yield for some templates and primer sets.

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