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CULTURE OF HUMAN KERATINOCYTES IN DEFINED SERUM-FREE MEDIUM

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A variety of systems have been developed to culture human keratinocytes. Early work used serum-supplementation with media such as Medium 199 (1) and NCTC 168 (2). Keratinocyte growth and colony formation were improved by plating cells on lethally irradiated 3T3 fibroblasts and adding epidermal growth factor (EGF) and hydrocortisone to the medium (3). One of the first serum-free formulations developed was based on Medium 199 containing a growth factor cocktail that included bovine brain extract (4). Serum-free culture of human keratinocytes without 3T3 fibroblast feeder layers became widely accepted with the development of MCDB-153 (5). Serum-free MCDB-153 medium included trace elements, ethanolamine, phosphoethanolamine, hydrocortisone, EGF, and bovine pituitary extract (BPE). This medium and several enhanced versions have been used widely for human keratinocyte cultivation (6–8).

Serum-free medium containing BPE as the primary mitogen has several drawbacks. The undefined composition of BPE complicates experimental models and interpretation of results. It may stimulate or inhibit human keratinocyte cultures, depending on the concentration and the presence of other components (9). In addition, BPE requires titration in different systems and its stability in

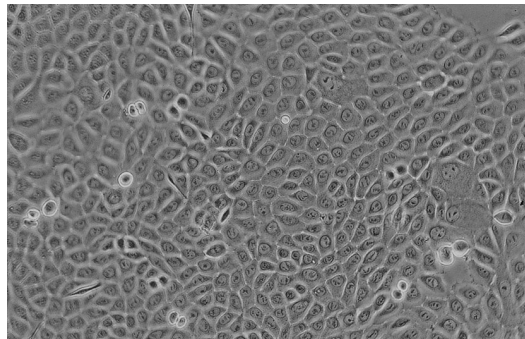
medium is limited to ~4 weeks under normal use conditions.

Defined Keratinocyte-SFM eliminates BPE by inclusion of defined growth promoting additives including insulin, EGF, and FGF. The medium is designed for the isolation and initiation of primary keratinocytes as well as expansion of keratinocyte cultures. Defined Keratinocyte-SFM demonstrates superior primary cell growth while maintaining morphology and physiological markers.

METHODS

Isolation and culture of human keratinocytes. Unless otherwise indicated, all media and reagents were GIBCO BRL brand. Neonatal foreskins were placed in serum-free medium (without growth factors) containing 5 µg/ml gentamycin and stored at 4°C. Foreskins can be stored in this manner for ~5 days without significant loss of cell viability. Foreskins were briefly rinsed in 70% isopropanol and then placed in Dulbecco's phosphate buffer saline (DPBS), without Ca⁺⁺ and Mg⁺⁺, containing 20 µg/ml gentamycin for 60 min. Foreskins were cut into halves or quarters, depending on the size of the tissue, and the pieces were transferred, dermis side down, to a petri dish containing 25 units/ml dispase and incubated 18 to 24 h at 4°C. Epidermal sheets were separated from the full-thickness skin with

A



B

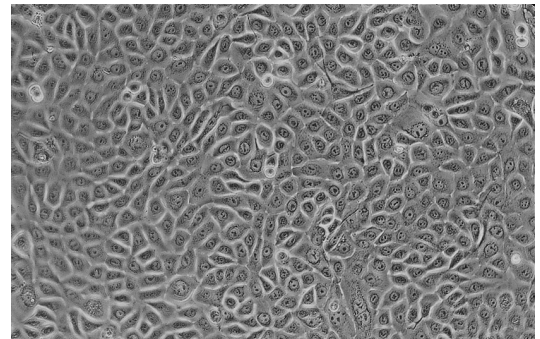


FIGURE 1. Phase contrast microscopy of human keratinocytes. Cells were cultured in Defined Keratinocyte-SFM (panel A) and Keratinocyte-SFM (contains BPE) (panel B) (100X).

forceps, pooled in 60-mm culture dishes containing 5 to 7 ml of 0.05% trypsin/0.53 mM EDTA, and incubated at 37°C for 15 to 20 min with gentle pipetting to aid in tissue dissociation. Pooling of tissue specimens is performed to reduce the effects of donor-to-donor growth variation. Trypsin activity was terminated by addition of soybean trypsin inhibitor (10 mg/ml in DPBS). Any remaining pieces of epidermal sheets were carefully removed and discarded. The cell suspension was transferred to a sterile centrifuge tube and the cells pelleted by centrifugation at $40 \times g$ for 5 min (22°C) and washed once with SFM. The supernatant was discarded, the cell pellet resuspended in the appropriate medium, and cell densities determined using a hemocytometer. Cells were plated in culture flasks or dishes.

Secondary cultures were established by removing the spent medium, briefly washing the cell monolayer with Versene (1:5,000), and adding an appropriate volume of 0.05% trypsin/0.53 mM EDTA. Cells were incubated at 37°C until they became round (~5 min), trypsin was removed, and the cells were incubated at 37°C until they detached from the culture surface with gentle tapping (~5 min). Trypsin activity was stopped by addition of 10 mg/ml soybean trypsin inhibitor solution; cells were pelleted by centrifugation at $40 \times g$ for 5 min (22°C), washed once with SFM, and resuspended in the appropriate medium. Trypsinization times are critical to the performance of any keratinocyte medium. Human keratinocytes that remain in trypsin too long have lower plating efficiencies and may be induced to differentiate. Secondary cell cultures were also established from primary keratinocytes obtained from Cell Systems Corporation with results comparable to those found with cultures established from neonatal foreskins. Cultures were incubated at 37°C in a humidified atmosphere consisting of 5% CO₂/95% air. Stock cultures were maintained at a split ratio of 1:2 to 1:3 and subcultured at 70% to 80% confluence. Keratinocytes at passage 0 through 4 were used for experimental evaluation.

Morphology and growth assays. Morphological analysis and immunostaining of cells were performed in 8-chamber glass culture slides. Keratinocytes were plated at 2×10^4 cells/cm² in a total volume of 400 µl/0.8-cm²

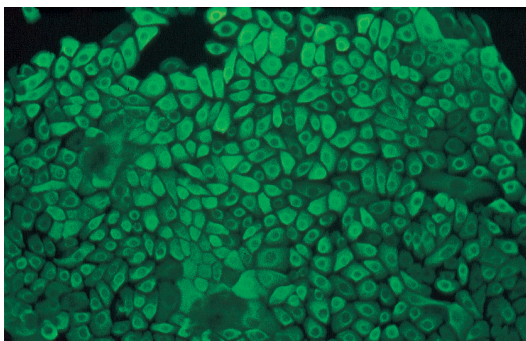


FIGURE 2. Expression of keratin 14. Human keratinocytes were cultured in Defined Keratinocyte-SFM (100X).

chamber. Cells were incubated for 24 h, then fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton® X-100 in DPBS, and allowed to react with rabbit anti-cytokeratin 14 antibody (1:200 dilution). Cells labeled with antibodies were visualized using goat anti-rabbit F(ab')₂ FITC conjugate (1:50 dilution).

Human keratinocyte growth assays were performed in 24-well culture dishes (2 cm² growth area) utilizing a seeding density of 1×10^4 cells/cm². Endpoint growth assays were assessed at 6 days postseeding for primary cells and 72 h for secondary cells. Growth kinetic assays were counted at 24-h intervals over 96 h without media replacement. Single-cell cloning assays were performed in 96-well tissue-culture-treated plates by serial dilution of cell suspensions to 5 cells/ml in the appropriate medium and plating 100 µl/well. Plates were incubated for 5 days before observation.

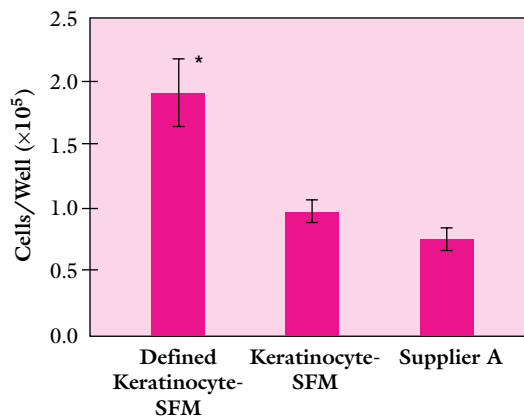


FIGURE 3. Growth of primary human keratinocytes. Growth was determined 6 days postseeding. Values represent the mean ± SEM, n = 7. * p < 0.05 versus Keratinocyte-SFM and Supplier A.

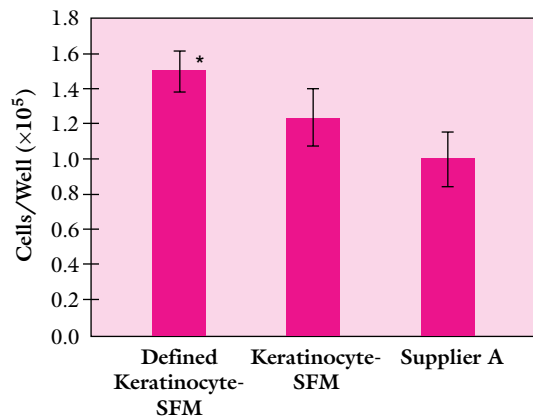


FIGURE 4. Growth of secondary human keratinocytes. Growth was determined 72 h postseeding. Values represent the mean \pm SEM, $n = 7$. * $p < 0.05$ versus Supplier A.

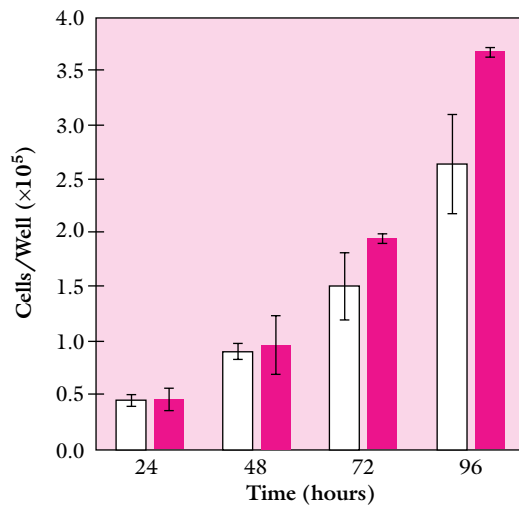


FIGURE 5. Growth kinetic analysis of human keratinocytes. Secondary cells were cultured in Defined Keratinocyte-SFM (■) or Keratinocyte-SFM (□). Values represent the mean \pm SD, $n = 2$.

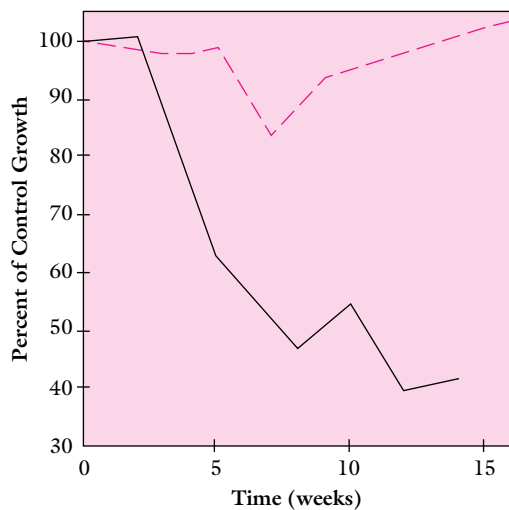


FIGURE 6. Evaluation of media shelf life using primary human keratinocytes. Cells were cultured in Keratinocyte-SFM (solid line) or Defined Keratinocyte-SFM (dashed line) over a 15-week period. Cells were counted after 6 days in medium stored for given times and compared to cells cultured in fresh medium.

Supplier A is a defined medium for human keratinocytes. Keratinocyte-SFM is a BPE-containing formulation (10).

RESULTS AND DISCUSSION

Human keratinocytes cultured in Defined Keratinocyte-SFM exhibited the same contact-inhibited, “crazy paving” pattern morphology (11) as cells grown in the presence of BPE (figure 1). Monolayer cultures had distinct borders and prominent nuclei. All cultures stained positive for keratin 14, a standard marker for basal human keratinocytes (figure 2).

Primary cultures established in Defined Keratinocyte-SFM demonstrated significantly better growth when compared to other keratinocyte media (figure 3). Population doubling times (PDTs) were: Defined Keratinocyte-SFM, 46.3 ± 5.9 h, Keratinocyte-SFM, 66.6 ± 12.8 h, and Supplier A, 83.5 ± 19.1 h.

Growth of secondary cultures was similar between Defined Keratinocyte-SFM and Keratinocyte-SFM, although better cell growth was achieved in Defined Keratinocyte-SFM than in Supplier A’s defined medium (figure 4). PDTs for secondary keratinocytes cultured were: Defined Keratinocyte-SFM, 25.0 ± 1.1 h, Keratinocyte-SFM, 29.0 ± 1.6 h, and Supplier A, 35.4 ± 4.1 h. Daily growth kinetic experiments using secondary cultures confirmed that cells cultured in defined medium proliferated at a rate comparable to BPE-containing medium (figure 5, $p > 0.05$). Cloning efficiencies of ~40% have been achieved with human keratinocytes cultured in Defined Keratinocyte-SFM in single-cell cloning experiments and are comparable to those found for cells cultured in BPE-containing medium. Cultures can be maintained for at least 6 passages in Defined Keratinocyte-SFM with split ratios of 1:2 performed twice weekly. Fully supplemented medium had a shelf life of >14 weeks, considerably longer than medium containing BPE (figure 6).

Culture systems to propagate human keratinocytes have evolved to reduce the undefined components and to increase culture longevity and cell yields. The results presented here demonstrate that BPE can be replaced without adversely affecting cellular proliferation rates and general physiology of human keratinocytes. The removal of BPE as a

medium component while maintaining medium performance represents a step forward in human keratinocyte culture by providing a more standardized and controlled culture environment (12).

ACKNOWLEDGEMENT

We thank Carl Soderland (Cell Systems Corp.) for providing human keratinocytes.

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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 MgSO₄. 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. XXXXX). Reactions (50 μl final volume) were assembled by mixing 25 μl of 2X reaction mix [2X buffer, 2.4 mM MgSO₄, 400 μM dNTPs each, and 4 μg/ml BSA], 1 ml 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	AGTGATCTCCTTCTGCATCTGT	
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	GCCAGTCCACATGGATGATGAT	1,715
	antisense	ATTCAACTGGTCTCAAGTCAGTGTA	

* Primer sequences for the pole, RPA, PP2A, and CBP PCR products are listed in the Internet version of this article at <http://www.lifetech.com/focus/1910xx.pdf>.

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 range 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 observed from control reactions that omitted

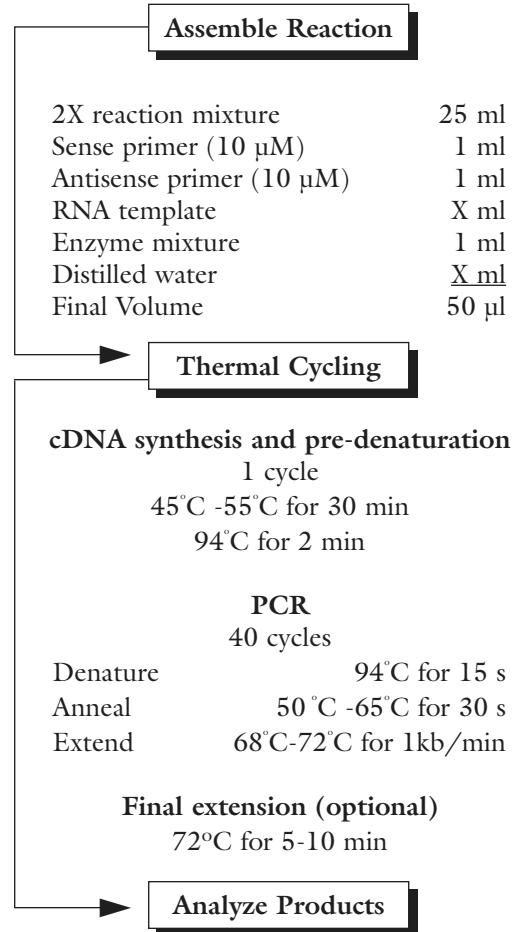


FIGURE 1. The SUPERSCRIPT ONE-STEP RT-PCR protocol.

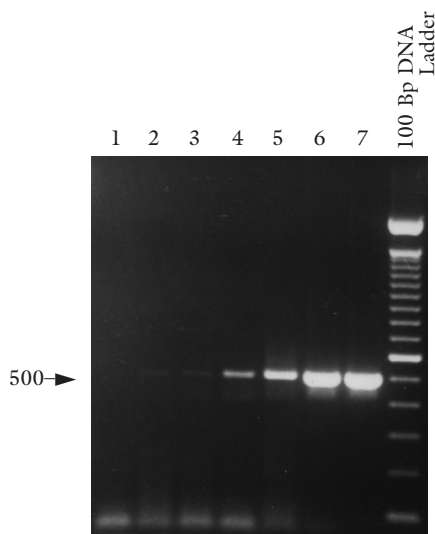


FIGURE 2. Amplification of CAT mRNA. Reactions were incubated at 45°C for 30 min; 94°C for 2 min; then 40 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 90 s; followed by 68°C for 5 min. Lane 1. No RNA template. Lanes 2 to 7 contain 5, 10, 10², 10³, 10⁴, and 10⁵ copies of CAT mRNA, respectively.

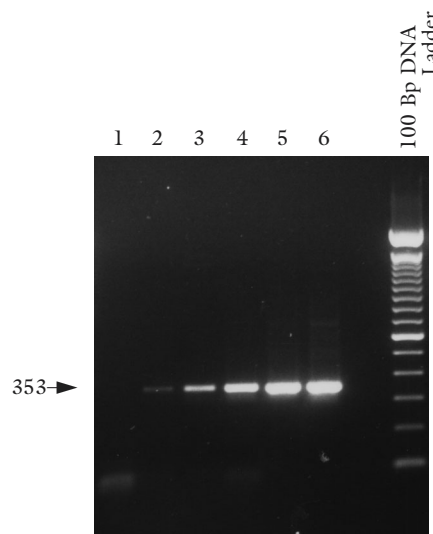


FIGURE 3. Amplification of β -actin mRNA. The incubations were as in figure 1 except the cDNA synthesis was at 50°C and the annealing temperature was 55°C. Lane 1. No RNA template, Lanes 2 to 6 contain 0.1, 1, 10, 10², and 10³ pg total HeLa RNA.

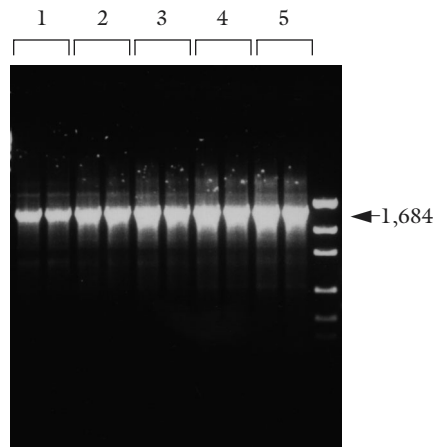


FIGURE 4. Incubation time for cDNA synthesis. The 1,684-bp β -actin fragment amplified after RT incubation at 45°C for 2, 5, 10, 15, and 20 min, respectively, in duplicate (lanes 1-5).

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).

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 (*polE*) (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 specific mRNA targets using total RNAs from a variety of sources, including HeLa cells, human

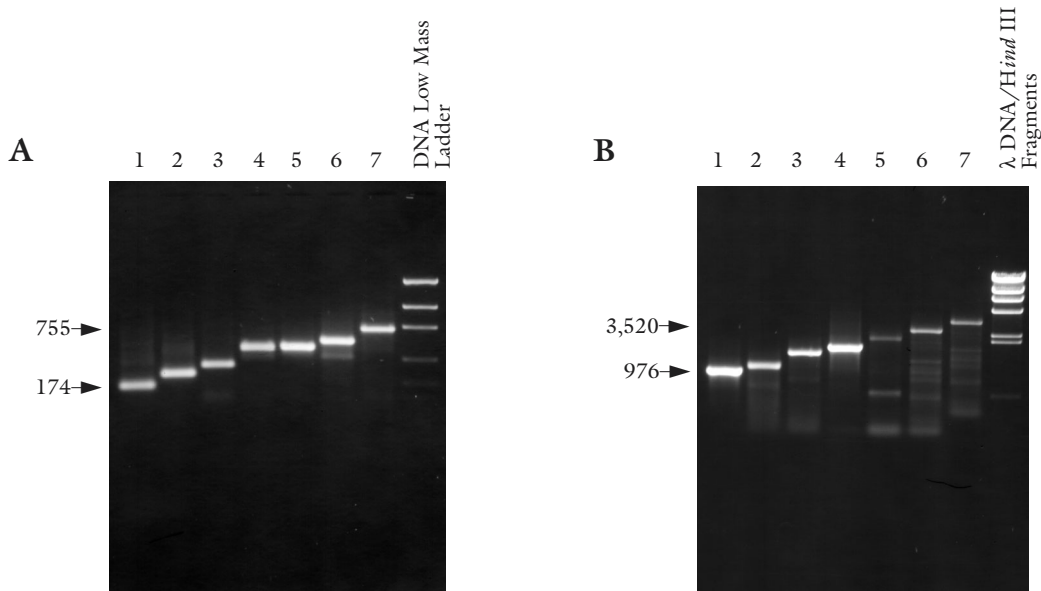


FIGURE 5. RT-PCR products of different sizes. 4-8 μ l of RT-PCR products were loaded on a 1.5% (Panel A) or 1.0% (Panel B) agarose gel containing ethidium bromide. Panel A. Lanes 1 and 2. β -actin, 174 and 253 bp. Lane 3. PP2A, 331 bp. Lane 4. CBP, 495 bp. Lane 5. RPA, 514 bp. Lane 6. *polE*, 606 bp.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

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/1910xx.pdf>).

The data presented uses 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.

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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

A NEW BACULOVIRUS EXPRESSION VECTOR FOR THE SIMULTANEOUS EXPRESSION OF TWO HETEROLOGOUS PROTEINS IN THE SAME INSECT CELL

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The BAC-TO-BAC™ Baculovirus Expression System was developed to simplify the generation of recombinant baculoviruses. This system is based on site-specific transposition of an expression cassette from the recombinant donor plasmid into a shuttle vector of baculovirus DNA (bacmid) that is propagated in *E. coli* (1). Recombinant bacmid DNA is rapidly isolated from *E. coli* cells, transfected into insect cells, and viral stocks (>10⁷ pfu/ml) are harvested from insect cells for protein expression, purification, and analysis.

In this paper, a new vector, pFASTBAC™ DUAL, which allows for the cloning and simultaneous expression of two heterologous proteins, is presented. This vector has two late promoters, the polyhedrin promoter (*polh*) and the p10 promoter. By inserting separate genes in the 2 multiple cloning sites (MCS), it is possible to generate a recombinant baculovirus that produces 2 heterologous proteins in the same insect cell. This is particularly useful in the investigation of protein-protein interactions or the expression of multisubunit proteins.

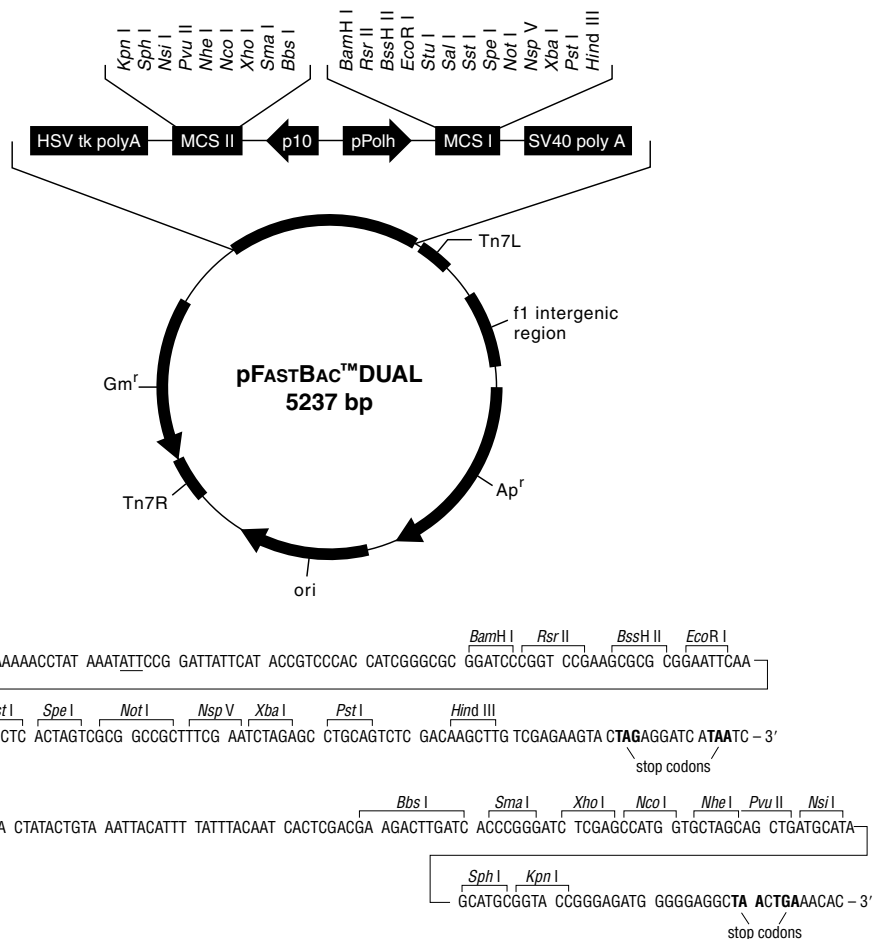


FIGURE 1. Map of pFASTBAC DUAL expression vector. For MCS I, +1 corresponds to the transcriptional start for the polyhedrin (*polh*) promoter. The ATG site of original *polh* start was mutated to ATT. The stop codons are shown in bold. For MCS II, +1 corresponds to the transcriptional start for the p10 promoter. Digestion at the *Bbs* I site generates a *Bam*H I compatible overhang.

METHODS

The cloning vector pFASTBAC DUAL (figure 1, Cat. No. 10712) comes with control DNA that contains the chloramphenicol acetyltransferase (CAT) gene cloned into the *polh* MCS of pFASTBAC DUAL at the *Bam*H I and *Pst* I sites and the β -glucuronidase (*gus*) gene cloned into the p10 MCS at the *Nco* I and *Nsi* I sites. pFASTBAC DUAL control DNA was transformed into MAX EFFICIENCY DH10BAC™ Competent Cells, and the cells were selected as described previously (2). Recombinant bacmid DNA, isolated as described in the BAC-TO-BAC system manual, was transfected into *Spodoptera frugiperda* (Sf9) cells using CELLFECTIN™ Reagent, and virus was collected after 72 h. The expression of *gus* was demonstrated *in situ* (2).

For expression of reporter genes, 1×10^6 cells (Sf9, Sf21, or BTI-TN-5B1-4) were seeded into a 35-mm dish. The cells were allowed to attach for 1 h at 27°C and then infected with recombinant baculovirus at an MOI of 5. Sf9 and Sf21 cells were cultured at 27°C in Sf-900 II SFM (Cat. No. 10902), and BTI-TN-5B1-4 cells were cultured at 27°C in EXPRESS FIVE™ SFM (Cat. No. 10486). All media were supplemented with 50 U/ml penicillin and 50 μ g/ml streptomycin. All cell culture media and reagents were GIBCO BRL brand. At appropriate time points postinfection, cells were collected by centrifugation, washed one time in PBS, and resuspended in 50 μ l TE buffer. Cells were lysed by a rapid freeze/thaw at -70°C, then an equal volume of 2X SDS loading buffer [4% SDS, 125 mM Tris-HCl (pH 6.7), 30% glycerol, 0.002% bromophenol blue, 2% 2-mercaptoethanol] was added. Samples were boiled for 5 min and analyzed by SDS-PAGE.

To quantify expression of reporter genes, infections were set up as described above. At appropriate time points, the dishes were washed one time with PBS and then 1 ml of lysis buffer [0.1 M Tris-HCl (pH 8.0) containing 0.1% Triton® X-100] was added to the dish. Dishes were stored at -70°C for 2 h, thawed at 37°C, and chilled on ice. Cell lysates were clarified by centrifugation at $12,000 \times g$ and divided into two equal-volume samples in fresh tubes. One set of tubes was heat treated, then stored at -70°C until they were assayed for CAT activity (3). The other set of lysates was stored at -70°C

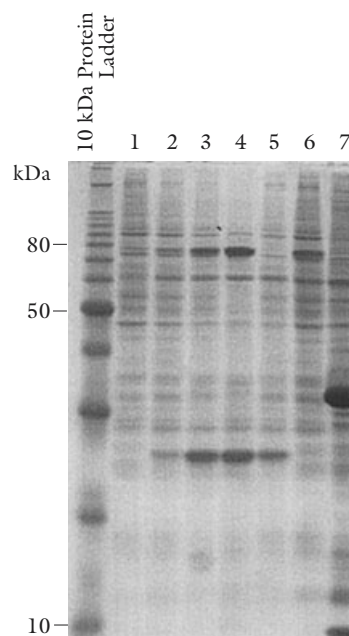


FIGURE 2. SDS-PAGE analysis of cell extracts. Sf9, Sf21, and BTI-TN-5B1-4 cells were infected at an MOI of 5. Samples were analyzed by SDS-PAGE on a 12.5% gel. For each sample, 25 μ g of total protein was loaded. Lane 1. Uninfected Sf9 cells. Lane 2. Sf9 cells infected with both genes (48 hpi). Lane 3. Sf21 cells infected with both genes (48 hpi). Lane 4. BTI-TN-5B1-4 cells infected with both genes (72 hpi). Lane 5. Sf9 cells infected with CAT (48 hpi). Lane 6. Sf9 cells infected with *gus* (48 hpi). Lane 7. Sf9 wild-type AcNPV infected cells (48 hpi).

without further treatment and assayed for *gus* activity (4).

RESULTS AND DISCUSSION

The simultaneous expression of 2 heterologous proteins has been achieved using the pFASTBAC DUAL vector and the BAC-TO-BAC System. For expression from the *polh* promoter, an in-frame ATG must be provided by the cloned gene. For the p10 promoter, cloning into the *Bbs* I, *Sma* I, or *Xho* I sites requires an ATG sequence for translation initiation. When cloning into the *Nco* I site or sites downstream, make sure the reading frame of the gene of interest is in-frame relative to the ATG sequence of the *Nco* I site.

The expression of CAT and *gus* was measured from virus expressing 1 or both proteins to verify that the expression pattern and the total activity were not affected by the expression of two proteins. The appropriate protein bands at ~73 kDa for *gus* (4) and ~26 kDa for CAT were observed in the 3 commonly used insect cell lines (figure 2). Neither of these bands was observed in uninfected or wild-type infected cells.

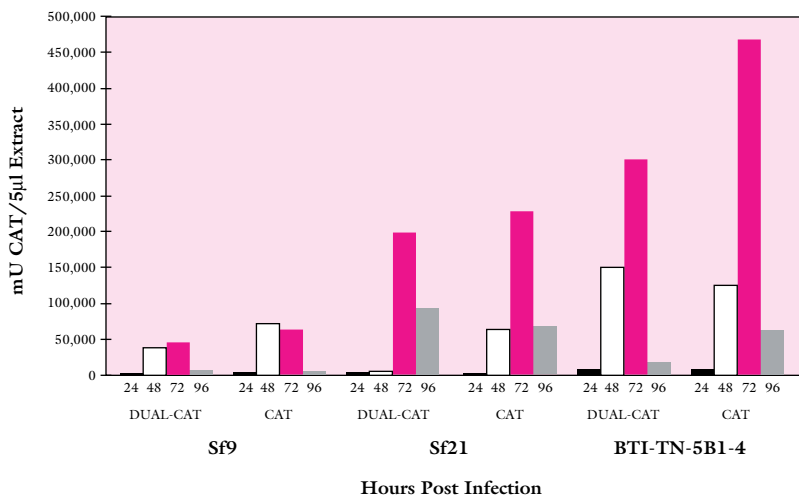


FIGURE 3. Total CAT activity for *gus*/CAT- and CAT-infected cells. Sf9, Sf21, and BTI-TN-5B1-4 cells were infected at an MOI of 5. CAT transcription is controlled by the polyhedrin promoter.

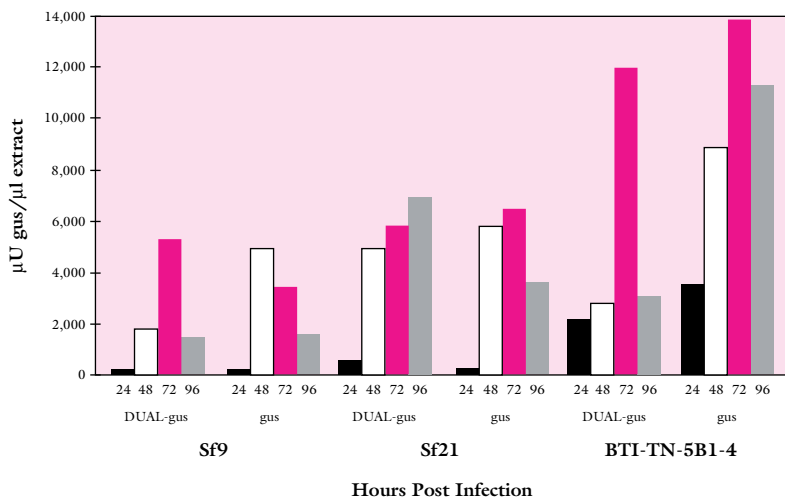


FIGURE 4. Total *gus* activity for *gus*/CAT- and *gus*-infected cells. Sf9, Sf21, and BTI-TN-5B1-4 cells were infected at an MOI of 5.

The activity of CAT was quantitated for virus expressing CAT or CAT and *gus* over a 96-h time course. Maximal activity was at 72 h (figure 3). The CAT gene is under the control of the *polh* promoter in the same context in both recombinant viruses. Comparable total CAT activity was observed for the single protein CAT virus (CAT) and the dual CAT/*gus* construct in Sf9 and Sf21 cells, indicating no detrimental effect of expressing two proteins simultaneously. For BTI-TN-5B1-4 cells, there appeared to be more activity for the single-gene construct than the DUAL construct. This may be a

function of the high level of protein expression in these cells. Similar results were seen with the *gus* expression for virus expressing *gus* or CAT and *gus* (figure 4). The *gus* gene is 13 bp closer to the p10 promoter in the single construct than it is in the DUAL recombinant virus.

Differences in total activity were observed for the various cell lines. The Sf9 cells had the lowest CAT activity (figure 3). The level of CAT activity observed from infections with the DUAL construct was 4.5 times higher in Sf21 cells and 7 times higher in BTI-TN-5B1-4 cells compared to Sf9 cells. The specific activity for CAT from Sf9 cells was 36 U/µg. The specific activity was 2 times higher for Sf21 cells and 3 times higher for BTI-TN-5B1-4 cells. For *gus* activity, Sf9 cells also had the lowest activity (figure 4). However, the difference between Sf9 and Sf21 cells was not as great.

These data demonstrated that the pFASTBAC DUAL Expression Vector used with the BAC-TO-BAC System produced large quantities of 2 heterologous proteins in the same cell. Under the control of the same promoter, comparable levels of gene expression can be obtained when the recombinant virus directed the expression of 2 heterologous proteins as compared to when one protein was expressed. The expression of proteins in different cell lines and at different time points indicated the importance of fully characterizing these parameters to optimize the levels of protein obtained.

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USING DNA LADDERS AS SIZE STANDARDS FOR POLYACRYLAMIDE GEL ANALYSIS OF DNA

DNA ladders were designed to size DNA on agarose gels. DNA ladders have more bands, balanced band intensities, and orientation bands when compared with DNA standards derived from restriction digests. There are applications where polyacrylamide gel electrophoresis (PAGE) may be advantageous for resolution of small fragments. Since migration of DNA fragments on polyacrylamide is influenced by sequence as well as size (1–3), we have examined several DNA ladders to determine their usefulness in sizing DNA in PAGE gels. A DNA ladder and a restriction endonuclease digest of a known DNA were used to calculate the sizes of DNA fragments separated on a native PAGE gel.

METHODS

DNA size standards evaluated on a 6% nondenaturing polyacrylamide gel included Φ X174 DNA digested with *Hae* III; pBR322 DNA digested with *Msp* I; 25 bp DNA Ladder (Cat. No. 10597); 50 bp DNA Ladder (Cat. No. 10416); 100 bp DNA Ladder (Cat. No. 15628); and 123 bp DNA Ladder (Cat. No. 15613). Each standard was diluted in loading buffer [final concentration 1 mM Tris-HCl (pH 7.5), 1 mM EDTA, 6.5% sucrose, 0.03% bromophenol blue], and 300 ng were loaded onto the gel (well width, 2.5 mm; gel thickness, 1 mm).

A restriction digest with a DNA ladder on one side, and the other restriction digest on the other side was electrophoresed at 6 V/cm for 90 min in 1X TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) and stained in 1X TBE containing 1 μ g/ml ethidium bromide for 10 min at room temperature. The migration distance of each band was determined by measuring the distance from the bottom of the well to the middle of the DNA band. Standard curves were constructed (figure 1) with the two flanking lanes and used to calculate the apparent sizes of the digest in the middle lane. The semilogarithmic plots of the 25 bp DNA Ladder, 50 bp

DNA Ladder, and pBR322/*Msp* I were used to calculate the apparent molecular weight of fragments of FX174/*Hae* III. The semilogarithmic plots of the 100 bp DNA Ladder, 123 bp DNA Ladder, and Φ X174/*Hae* III were used to calculate the apparent molecular weight of fragments of pBR322/*Msp* I. Finally, the values calculated using the DNA ladders or the restriction digests and were plotted against the known fragment sizes for either Φ X174/*Hae* III or pBR322/*Msp* I (figure 2).

RESULTS AND DISCUSSION

Several DNA ladders were examined to determine their usefulness in sizing DNA on native PAGE. Figure 2 shows photographs of the gels, and the data derived from them. The line represents perfect correspondence between the calculated and actual sizes of the fragments in the center lane of each photograph. The points represent the fragment size determined using the DNA ladder or the restriction digest as the standard. The point closest to the line represents the most accurate estimate of the size of that fragment. For the lower range (25 to 250 bp) the 25 bp DNA Ladder was most accurate, and for the higher range (250 to 800 bp)

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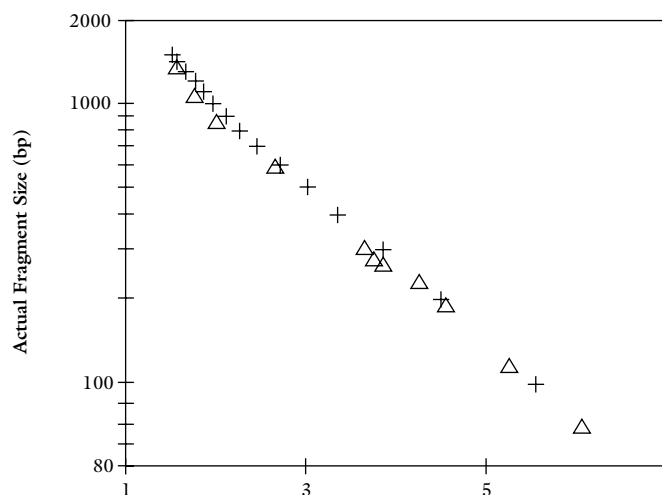


FIGURE 1. A representative standard curve for the 100 Bp DNA Ladder (+) and Φ X174/*Hae* III fragments (Δ).

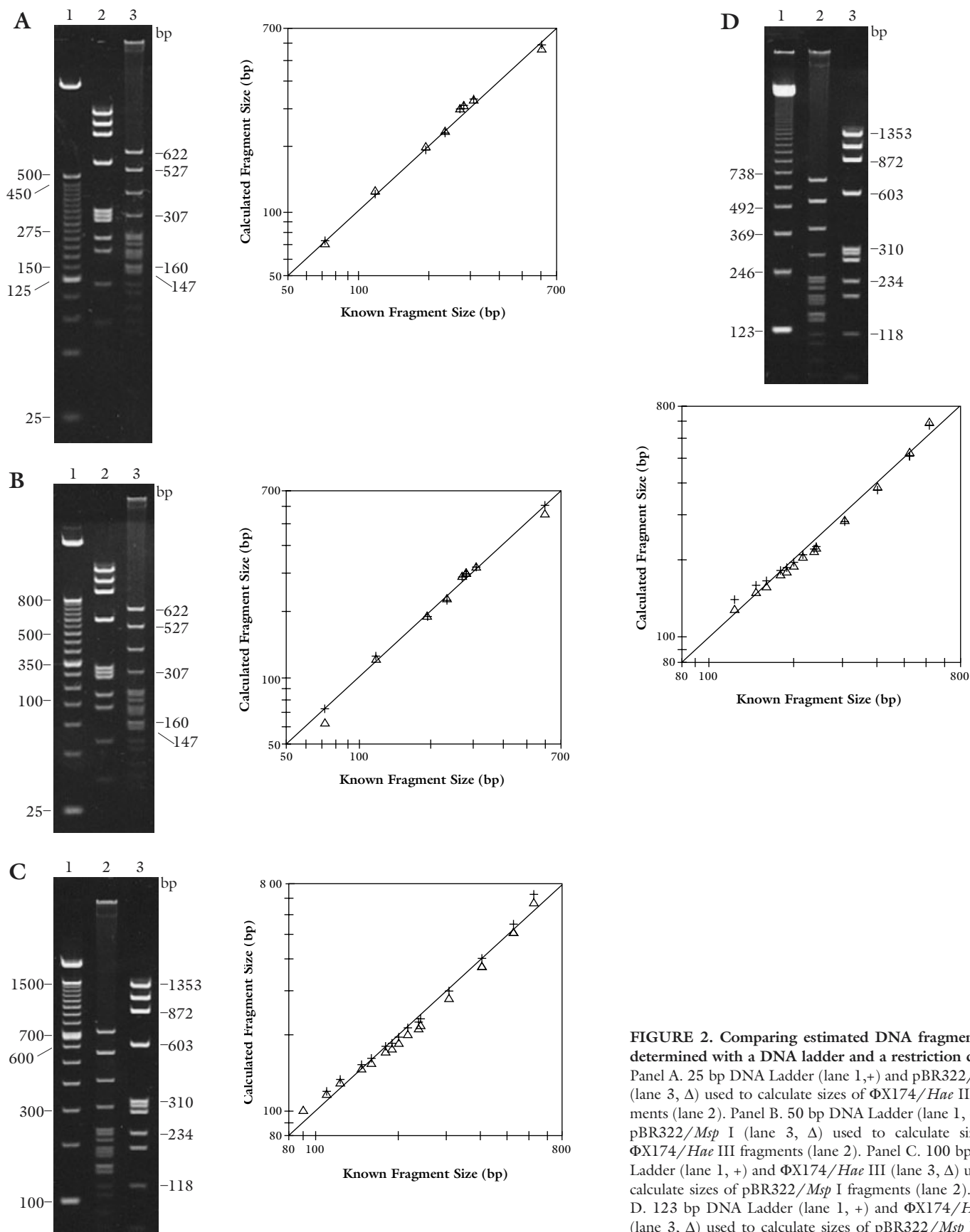


FIGURE 2. Comparing estimated DNA fragment size determined with a DNA ladder and a restriction digest. Panel A. 25 bp DNA Ladder (lane 1, +) and pBR322/*Msp* I (lane 3, Δ) used to calculate sizes of Φ X174/*Hae* III fragments (lane 2). Panel B. 50 bp DNA Ladder (lane 1, +) and pBR322/*Msp* I (lane 3, Δ) used to calculate sizes of Φ X174/*Hae* III fragments (lane 2). Panel C. 100 bp DNA Ladder (lane 1, +) and Φ X174/*Hae* III (lane 3, Δ) used to calculate sizes of pBR322/*Msp* I fragments (lane 2). Panel D. 123 bp DNA Ladder (lane 1, +) and Φ X174/*Hae* III (lane 3, Δ) used to calculate sizes of pBR322/*Msp* I fragments (lane 2).

the 50 and 100 bp DNA Ladders were most accurate. The 100 bp DNA Ladder was better than the 123 bp DNA Ladder in closeness to the actual value, especially in the low range. The DNA ladders were as accurate as the restriction digests in determining the size of an unknown fragment. The increased number of bands in the DNA ladders allows for more accurate size determination in the appropriate range.

With the 100 bp DNA Ladder (panel C) the migration of the highlight band was reduced. This fragment migrated very close to the 700-bp band, when its actual size is 600 bp (4). Somewhat reduced migration of the highlight bands of the other DNA ladders on polyacrylamide has also been observed (data not shown), but it was not obvious on the gel photographs here. The highlight bands of each ladder are composed of sequences that are not

related to the repeated fragments of the rest of the ladder. Their behavior in polyacrylamide gels demonstrates the potential influence of particular DNA sequences upon electrophoretic mobility (3). It has also been reported that electrophoresis at lower temperatures (5°C) can enhance anomalous migration in PAGE (2). From the data presented, we conclude that DNA ladders and restriction digests gave similar results when used to estimate the sizes of DNA fragments in native PAGE gels.

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DNA FINGERPRINTING IN COTTON USING AFLPs

Amplified fragment length polymorphism (AFLP) is based on the selective amplification of restriction fragments from total genomic DNA with different primer pairs (1). Fine variation among samples can be distinguished by AFLP on a DNA sequencing gel. The AFLP method has been used for producing high-density genetic maps in many crops (2). In this paper, we apply AFLP to the cotton genome.

Genomic DNA from young leaves of *Gossypium barbadense* (Pima 3-79) was extracted following the modified method of Wagner (3). AFLP was performed using the GIBCO BRL AFLP System I (Cat. No. 10544) following the manufacturer's protocol (2) with minor modifications. The use of 800 ng of the genomic DNA of cotton in the initial reaction provided the best results in comparison to the 250 ng of DNA suggested in the instructions. A high amount of DNA might be necessary because the high levels of polyphenolic and secondary products in cotton make it difficult to get pure DNA for selective amplification.

Cotton DNA and control tomato DNA were digested with 3 µl and 2 µl of *EcoRI*/*MseI* (1.25 units/µl each), respectively, at 37°C for 3 h, and the fragments were ligated with the *EcoRI* and *MseI* adapters at 18°C for 3 h. Each ligation reaction was diluted 1:10 with TE buffer. After selective amplification, 4 µl of each reaction mixture was electrophoresed on a 6% DNA sequencing gel and at 1,800 V until the xylene cyanole dye migrated two-thirds of the way down the gel. The gel was dried and exposed to x-ray film (Biomax-MR) for 16 h.

On average, the primer pairs used in AFLP provided about 60 bands, ranging from 30 bp to 600 bp (table 1). The results, using the same sample, demonstrated that AFLP bands vary

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TABLE 1. Number of bands seen with different primer combinations in AFLP analysis of the cotton genome.

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	87	63	64	79	27	67	26	None
E-ACA	13	65	60	73	48	38	43	20
E-ACG	25	33	60	45	-	-	-	-

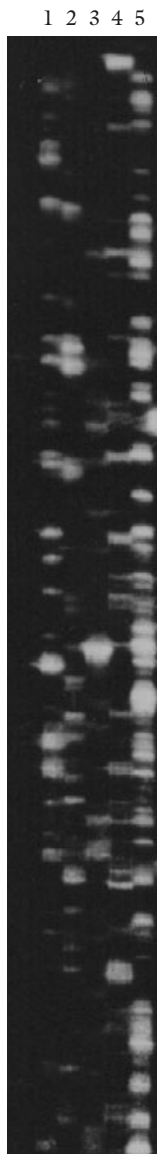


FIGURE 1. Screening of AFLP primer pairs from Pima-3 79 cotton. Lane 1. Control tomato DNA. Lanes 2-5. E-AAC primer paired with M-CAA, M-CAC, M-CAG, and M-CAT, respectively.

among the different primer pairs, indicating that each primer pair amplified different segments of the same genome (figure 1). Also, each lane contained some major bands and some minor bands. The major bands indicated the presence of more copies of similar sequence in the cotton genome in comparison to that of the minor bands.

In a comparison of the AFLP to RFLP and RAPD methods (4), AFLP identified more polymorphic bands than RAPD in soybean. Also, our previous genetic analysis (5) in cotton showed that AFLP provides more bands compared to the RFLP or RAPD technique. Our results demonstrate that AFLP is a strong tool for genetic dissection of the cotton genome.

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ETHANOL PRECIPITATION: AMMONIUM ACETATE AS AN ALTERNATIVE TO SODIUM ACETATE

Ethanol precipitation is frequently used to concentrate DNA following enzymatic reactions. In addition, ethanol precipitation is used to remove salts or reaction products and often follows phenol and chloroform extractions. The use of sodium salts for precipitating DNA is common in most laboratories, and the precipitation characteristics of DNA in sodium acetate were recently re-examined (1). Ammonium acetate, at a final concentration of 2.5 M, also has been used for ethanol precipitation of DNA. There are two instances when ammonium acetate is used frequently: the removal of unincorporated nucleotides following a DNA labeling reaction (2) and the removal of protein from DNA in mini-plasmid preparation protocols (3). The effectiveness of ammonium acetate for precipitating DNA or removing proteins or nucleotides has not been previously reported.

A study was undertaken to determine the effects of incubation time, incubation temperature, centrifugation time, and centrifugation temperature on ethanol precipitation of DNA using ammonium acetate in place of sodium acetate. This study also quantitatively examined the efficiency of removal of proteins and free nucleotides from DNA by ethanol precipitation in the presence of ammonium acetate.

Methods

Preparation of DNA. Supercoiled pUC19 DNA was digested with *Eco*R I and the 3' recessed termini were filled-in with dTTP, dGTP, dCTP, and [α - 32 P]dATP using the large fragment of DNA polymerase I. Herring sperm DNA was sonicated to give an average size of 200–400 bp.

Ethanol Precipitations. All precipitations were performed in a 200- μ l volume. Each tube contained 1 μ l of labeled DNA (1 ng), 10 μ l of herring sperm DNA (1 ng/ μ l, 10 ng/ μ l, or 100 ng/ μ l), and 190 μ l 10 mM Tris-HCl (pH 7.6), 1 mM Na₂EDTA (TE). To precipitate the DNA, 100 μ l of 7.5 M ammonium acetate (0.5 volumes) and 750 μ l of 95% ethanol (2.5 volumes) were added to the tubes. The tubes were inverted 10 times to mix the contents and incubated for the specified

period of time. The temperature of the ethanol added to the vial was the same as the incubation temperature. The -70°C incubation took place in a dry ice/ethanol bath, the -20°C incubation was in a -20°C ethanol bath, the 0°C incubation was performed on wet ice, and the 22°C incubation was at room temperature. After the appropriate incubation time, the solutions were centrifuged at $16,000 \times g$ in a fixed angle microcentrifuge at 4°C or room temperature. The supernate was removed and the pellets were rinsed with 200 μ l of 95% ethanol. The amount of radiation in the pellets was determined by Cerenkov counting in a scintillation counter. Data points represent the averages of at least two samples.

Removal of Free Nucleotide. The efficiency of removing free nucleotides was monitored by precipitating nick-translated pUC19 in the presence of unincorporated nucleotides. Supercoiled pUC19 was labeled using the Nick Translation System with 65 μCi [α - 32 P]dATP. Acid precipitable counts and total counts were determined before and after two sequential ethanol precipitations.

Removal of Protein. The efficiency of removing protein from DNA containing solutions was monitored using the ^{14}C -labeled Protein Molecular Weight Standards. Ammonium acetate was added to a final concentration of 2.5 M to solutions containing 50 $\mu\text{g}/\text{ml}$ or 1,000 $\mu\text{g}/\text{ml}$ of BSA and 37.5 $\mu\text{g}/\text{ml}$ ^{14}C -labeled proteins. These protein solutions contained 1 μg of DNA in a 50 μl volume (20 $\mu\text{g}/\text{ml}$) and were incubated for 0 or 30 min at 0°C or 22°C prior to centrifugation at $16,000 \times g$ for 15 min at room temperature. After the resulting supernate was transferred to a fresh tube, ethanol was added to a concentration of 70%, and the solution centrifuged at $16,000 \times g$ for 15 min at room temperature. After each centrifugation, 2 μl of the supernate was removed and counted in 10 ml of a scintillation cocktail.

RESULTS

Incubation Temperature. The effect of incubation temperature on the efficiency of ethanol precipitation of DNA in the presence of

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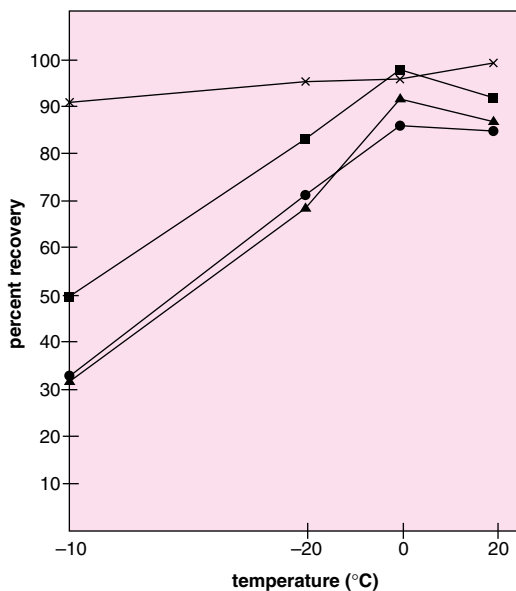


Figure 1. Effect of incubation temperature on ethanol precipitation with ammonium acetate. All solutions were incubated overnight at the designated temperature and centrifuged for 15 min at 22°C. DNA concentrations were 5 µg/ml (x), 0.5 µg/ml (■), 0.05 µg/ml (▲), and 0.005 µg/ml (●).

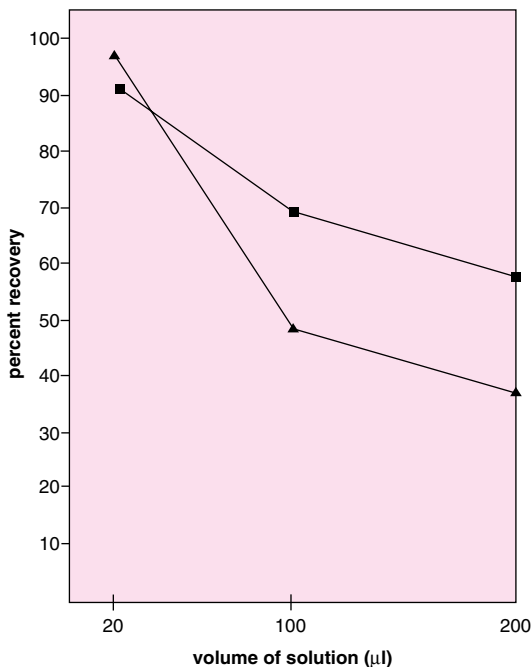


Figure 2. The effect of volume on the recovery of DNA by ethanol precipitation with ammonium acetate. DNA at two concentrations, 0.5 mg/ml (■) and 0.05 mg/ml (▲), was precipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol (at 0°C). Samples were centrifuged for 15 min at 22°C.

ammonium acetate was determined by incubating DNA solutions ranging from 0.005 µg/ml to 5 µg/ml at -70°C, -20°C, 0°C, and 22°C for 0, 10, and 30 min and overnight. In general, the temperature of incubation (and ethanol) did not have a dramatic effect on the recovery of DNA by ethanol precipitation for incubation times ranging from 0 to 30 min (table 1). The yield of DNA incubated at -70°C was slightly reduced, in agreement with previous studies (1). The most dramatic effect of temperature was seen when the ethanol precipitations were allowed to incubate overnight (figure 1 and table 1). Although incubation temperature had little effect on more concentrated DNA (5 µg/ml), DNA concentrations ≤ 0.5 µg/ml showed a marked improvement in percentage recovery at 0°C and 22°C incubation temperatures.

Incubation Time. The effect of incubation time on the efficiency of DNA precipitation was determined at all four of the incubation temperatures described above. The same general trend was observed for all of the incubation times (table 1). For DNA concentrations of 5 µg/ml, the extended incubation did not increase yields. Although there appears to be little effect of incubation time from 0 to 30 min, extended incubation did increase the percentage of DNA precipitated in the presence of 2.5 M ammonium acetate and 70% ethanol for DNA concentrations of ≤ 0.5 µg/ml.

Centrifugation Time and Temperature. Centrifugation of ethanol precipitates following incubation is commonly performed at 4°C. To determine the effect of centrifugation time and temperature, a 0.05 µg/ml DNA solution was incubated at 0°C for 10 min, and centrifuged for 15 or 30 min at 4°C or room temperature. The recovery of DNA increased with the extended centrifugation time from 37% to 57% for centrifugation at 22°C and from 22% to 39% for centrifugation at 4°C. In addition to achieving higher recoveries with 30 min centrifugations, it is noteworthy that recoveries were improved by centrifugation at room temperature.

Volume. The effect of volume on the recovery of DNA precipitated with ammonium acetate and ethanol was determined with DNA concentrations of 0.05 µg/ml and 0.5 µg/ml in volumes of 20, 100, and 200 µl (figure 2). By reducing the volume, the yield of precipitated DNA at a given concentration was improved. To

Table 1. Effect of time and temperature on ethanol precipitation with ammonium acetate

DNA Concentration	Percent DNA recovered															
	- 70°C				- 20°C				0°C				22°C			
	0 min	10 min	30 min	over-night	0 min	10 min	30 min	over-night	0 min	10 min	30 min	over-night	0 min	10 min	30 min	over-night
5 mg/ml	85	80	89	91	87	78	91	96	88	94	94	96	88	97	93	100
0.5 mg/ml	62	46	52	50	57	52	65	83	60	58	63	98	62	64	65	92
0.05 mg/ml	28	29	30	32	35	33	49	69	36	33	38	92	47	40	36	87
0.005 mg/ml	25	27	38	33	41	38	49	72	37	33	39	86	40	35	38	85

Note: Data shown in bold type had $\Delta 80\%$ recovery.

control for the ability to remove the supernate reproducibly, each pellet was monitored before and after a 70% ethanol rinse. No changes in the amount of radioactivity associated with the pellet were noted upon washing.

Removal of Free Nucleotides. Two types of experiments were performed to monitor the removal of free nucleotides by ethanol precipitation. In the first set of experiments, a labeled nucleotide (80,000 cpm of [α - 32 P]dATP in the presence of 20 μ M cold dNTPs) was added to varying concentrations of DNA. Room temperature ethanol was added to the samples, and they were immediately centrifuged for 15 min at room temperature. In the presence of 2.5 M ammonium acetate and 70% ethanol, approximately 7% of the free nucleotides precipitated out of solutions containing 100 ng, 1 μ g, or 5 μ g of DNA in a 50 or 200- μ l volume (data not shown). The amount of nucleotide precipitated was independent of the DNA concentration, and under these conditions, greater than 90% of the DNA was precipitated.

In the second set of experiments, pUC19 (1 μ g) was labeled by nick translation. The acid precipitable and total counts were determined after nick translation and after the first and second ethanol precipitations in the presence of ammonium acetate or sodium acetate. The ratio of precipitable counts to total counts increased from 68% to 87% after the first precipitation with sodium acetate and from 61% to 90% after the first precipitation with ammonium acetate (data not shown). After two precipitations with either salt, the precipitable counts equaled the total counts, indicating that unincorporated nucleotides were removed efficiently in both cases.

Removal of Proteins. Some rapid plasmid preparation protocols use 2.5 M ammonium

acetate followed by centrifugation to remove protein from the solution. To test the efficiency of protein removal from a DNA-containing solution, 14 C-labeled protein and BSA were mixed with a 20 μ g/ml DNA solution. This concentration is the same as a standard nick translation or restriction digestion (1 μ g of DNA in 50 μ l). Ammonium acetate was added, and the solution was mixed and incubated for 0 or 30 min at 0°C or 22°C prior to centrifugation at 22°C. In all cases, approximately 90% of the labeled protein precipitated out of solution (data not shown). The addition of ethanol to the supernate precipitated the DNA but failed to precipitate the 14 C-labeled proteins that had remained in the first supernate. The same experiment was performed with labeled DNA and unlabeled protein. Again, a protein pellet was observed following ammonium acetate addition and centrifugation, but no labeled DNA was associated with this material. Once again, following the addition of ethanol, greater than 90% of the DNA was recovered (data not shown).

DISCUSSION

When using ammonium acetate as described here for ethanol precipitation of DNA, the incubation temperature and length of incubation time do not have an effect when DNA concentrations are ≥ 5 μ g/ml. However, at lower DNA concentrations, incubation at 0°C and 22°C resulted in higher yields of DNA, especially as the length of incubation increased to overnight.

Other factors that affected the recovery of precipitated DNA were the centrifugation speed and temperature, the length of centrifugation, and the volume of the solution. The recovery of DNA was improved when solutions were centrifuged for 15 min at maximum speed in a fixed angle microcentrifuge at 16,000 $\times g$ compared to a horizontal microcentrifuge at 8,800 $\times g$ (data not shown). All results reported here were obtained using a fixed angle microcentrifuge. A

greater percentage of DNA was also recovered when samples were centrifuged at 22°C rather than 4°C and for 30 min in comparison to 15 min. The volume of the solution also had an effect on recovery, with much better recoveries being observed for small volumes.

Recovery of DNA by ethanol precipitation can be thought of as taking place in two steps: precipitation and collection of the precipitate. The precipitation appears to take place equally well at temperatures ranging from -70°C to 22°C, and decreased temperature does not substitute for incubation time (table 1). The collection of the precipitate requires centrifugation of the DNA through the 70% ethanol solution. At reduced temperature (4°C versus 22°C) this solution will be more viscous, making it more difficult for precipitates to reach the bottom of the tube. Longer centrifugation time improves the efficiency of recovery because it allows precipitates to reach the bottom of the tube. Likewise, smaller volumes decrease the time required for the precipitate to reach the bottom of the tube and can improve the efficiency of recovery.

For the removal of unincorporated nucleotides by ethanol precipitation, ammonium acetate is slightly more efficient than sodium acetate. In instances where there is a substantial amount of unincorporated nucleotides (*i.e.*, kinase reactions), the difference in the absolute amount of radio-activity can be considerable. However, when two successive precipitations are done, the difference in the efficiency between salts is negligible. Dilution of the DNA solutions prior to precipitation did not reduce the amount of unincorporated label that precipitated.

When ammonium acetate is added to a concentration of 2.5 M, proteins can be efficiently removed by centrifugation of the sample prior to the addition of the ethanol. Reduced temperature and/or increased incubation times did not have an effect on the precipitation of the ¹⁴C-labeled proteins. Experiments with labeled DNA indicated that the DNA was not precipitated or trapped during the protein removal. The DNA can then be recovered from the supernate by ethanol precipitation.

In general, ethanol precipitations with ammonium acetate can be performed by making the DNA-containing solution 2.5 M in ammonium acetate, adding 2.5 volumes of room temperature ethanol, and centrifuging immediately at 16,000 × *g* for 15 min at room temperature. Since DNA is recovered more efficiently in reduced volumes and contaminants such as unincorporated nucleotides are removed just as efficiently at high or low DNA concentrations, there is no need to dilute samples to greater than 50 μl prior to the addition of salt and ethanol. A 70% ethanol wash is recommended after precipitations to remove residual salt and to dilute the small amount of liquid that is difficult to remove from the pellet.

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