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ABOUT THE COVER: Photograph of a potato plant. Inset photographs were taken with a digital camera. See article on page 49.

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THERMOSCRIPT™ RT, A NEW AVIAN REVERSE TRANSCRIPTASE FOR HIGH-TEMPERATURE cDNA SYNTHESIS TO IMPROVE RT-PCR

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 Margret Nathan
 Ruo H. Xu
 Kalavathy Sitaraman
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 20849

The conversion of RNA to cDNA is accomplished by reverse transcriptase (RT). The ideal RT synthesizes long cDNA in high yield and at elevated temperatures. Performing cDNA synthesis at higher temperatures increases the specificity of priming and helps to increase the efficiency by reducing RNA secondary structures (1,2). RTs have been reported to have limited thermal stability (3,4). In addition, the RNase H activity associated with RTs has limited their utility in synthesis of long cDNA (5). Here we describe THERMOSCRIPT RT, a genetically engineered avian reverse transcriptase with greatly reduced RNase H activity and high thermal stability. Use of THERMOSCRIPT RT for high-temperature RT-PCR is demonstrated.

METHODS

Reverse transcriptases. THERMOSCRIPT RT is a cloned RT in which the active site of the RNase H domain has been mutated, reducing its RNase H activity by 99.5%. Native avian myeloblastosis virus RT (AMV RT) was used as a reference RT.

RNA. Synthetic 7.5-kb RNA was synthesized *in vitro* with T7 RNA polymerase (5). Total HeLa and rat brain RNA were isolated using TRIzol® Reagent.

Gel electrophoresis. Labeled first-strand cDNA was electrophoresed on an alkaline 1.2% (w/v) agarose gel (6). RT-PCR products (10 µl) were analyzed by electrophoresis on 0.8% to 1.5% (w/v) agarose gels in 1X TAE with 0.5 µg/ml ethidium bromide.

First-strand cDNA synthesis assay. To evaluate the performance of THERMOSCRIPT RT and AMV RT at increasing temperatures, first-strand cDNA synthesis from a 7.5-kb RNA template was performed. The optimized reaction for THERMOSCRIPT RT (20 µl) contained 50 mM Tris-acetate (pH 8.4); 75 mM K-acetate; 8 mM Mg-acetate; 5 mM dithiothreitol; 1 mM each of dATP, dTTP, dCTP, and dGTP; 0.5 µg oligo(dT)₂₀; 2.5 µg 7.5-kb RNA; 40 units RNASEOUT™ Recombinant RNase Inhibitor (Cat. No. 10777); and 15 units THERMOSCRIPT RT. The optimal reaction for AMV RT (7) contained 100 mM Tris-HCl (pH 8.3); 50 mM KCl; 10 mM MgCl₂; 10 mM dithiothreitol; 0.5 mM each of dATP, dTTP, dCTP, and dGTP; 15 units AMV RT and oligo(dT)₂₀; 7.5-kb RNA; and RNASEOUT RNase Inhibitor at concentrations indicated for THERMOSCRIPT RT. The first-strand cDNA was labeled using [α -³²P]dCTP (3,000 Ci/mmol) with specific activity 250 cpm/pmol. Reaction mixtures were preheated to the desired temperatures (2 min) before RT was added to initiate cDNA synthesis (hot start). Reactions were terminated by the addition of EDTA. Reaction yield was analyzed by TCA precipitation of a portion of the labeled cDNA, and product size was determined by gel electrophoresis.

RT-PCR. A schematic of the RT-PCR procedure for the THERMOSCRIPT RT-PCR System (Cat. No. 11146) is in figure 1. Total cell RNA

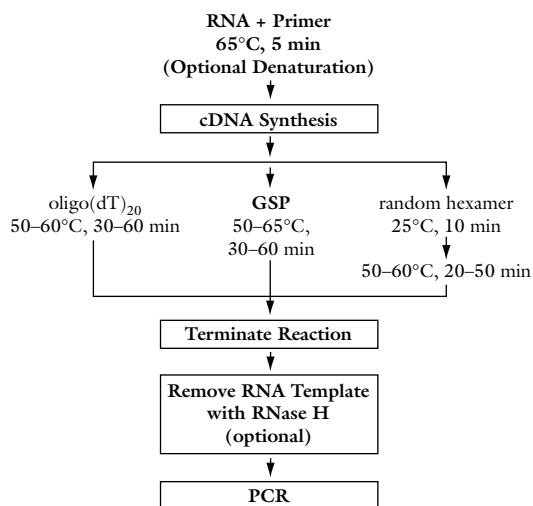


FIGURE 1. Schematic of RT-PCR.

Components of the THERMOSCRIPT RT-PCR System

THERMOSCRIPT RT	10 mM dNTP mix
oligo(dT) ₂₀	RNASEOUT RNase inhibitor
random hexamers	<i>E. coli</i> RNase H
5X cDNA synthesis buffer	DEPC-treated water
0.1 M DTT	

and oligo(dT)₂₀ were incubated at 65°C for 5 min and cooled on wet ice; then complete cDNA synthesis reaction mix [final: 50 mM Tris-acetate (pH 8.4); 75 mM K-acetate; 8 mM Mg-acetate; 5 mM dithiothreitol; 1 mM each of dATP, dTTP, dCTP, and dGTP; 40 units RNASEOUT RNase Inhibitor; and 15 units THERMOSCRIPT RT] was added. The reaction tubes were transferred to a prewarmed heat block and incubated for 50 min. Following RT inactivation, RNA was degraded by an RNase H treatment. Reverse transcription reactions with gene-specific primers (GSPs) were done using the same protocol except that a cDNA synthesis mix without RT was added to annealed RNA-GSP, the mixture was prewarmed for 2 min in a heat block to the appropriate temperature, and then RT was added to start synthesis (hot start cDNA synthesis).

For PCR, 2 µl of the 20-µl cDNA reaction was added to a 50-µl PCR mixture and incubated for 2 min at 94°C. PCR was 35 cycles of 94°C for 30 s, 55–60°C for 30 s, and 68–72°C for 1–15 min (~1 min/kb). ELONGASE® Enzyme Mix (8) was used for fragments >3 kb, while PLATINUM™ Taq DNA Polymerase (9) was used for fragments <3 kb. Primers used are in table 1.

RESULTS AND DISCUSSION

Full-length cDNA synthesis. To evaluate the efficiency of the RT activity of THERMOSCRIPT RT, first-strand cDNA synthesis was performed under stringent conditions (hot start) using

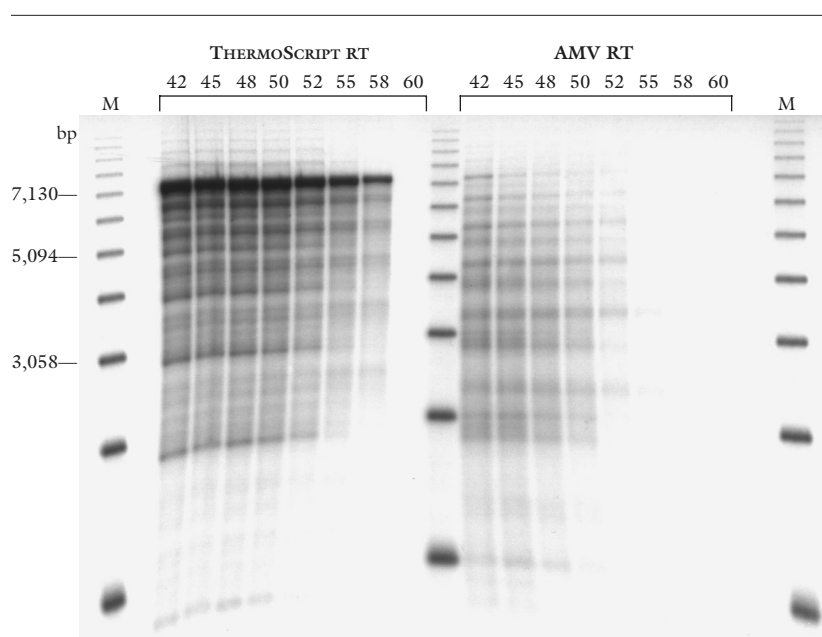


FIGURE 2. Comparison of first-strand cDNA products at various temperatures. cDNA was synthesized from 2.5 µg of 7.5-kb RNA by THERMOSCRIPT RT and AMV RT at 42°C to 60°C in the presence of [α -³²P]dCTP under stringent conditions (hot start). The cDNA was fractionated on a 1.2% alkaline agarose gel. Lane M is ³²P-labeled 1 Kb DNA Ladder.

a 7.5-kb poly(A)-tailed synthetic RNA. THERMOSCRIPT RT produced full-length cDNA between 42°C and 58°C (>20% of the total cDNA produced), whereas AMV RT produced only small amounts of full-length product between 42°C and 52°C (≤2% of the total cDNA produced) and produced little or no product of any length above 52°C (figure 2). In addition, the total yield and yield of full-length cDNA at all temperatures were greater for

TABLE 1. Primer sequences and expected product size.

Gene	Primer	Sequence	Expected Product Size (kb)
Rat Dynein	sense antisense	GCGGC GCTGG AGGAG AA AGGTG GCGGC TCAA CACAA AG	12.3
Human Adenomatous Polyposis coli (APC)	sense antisense	GCTGC AGCTT CATAT GATCA GTTGT TA ATACC AATTT TTCCC TGATG TAAGT TTAGT CA	8.9
Human Polymerase ε (Pol ε)	sense antisense	CGCCA AATTT CTCCC CTGAA CCGTA GTGCT GGGCA ATGTT C	6.8
Human Tuberos Sclerosis 2 (TSC-2)	sense antisense	GGAGT TTATC ATCAC CGCGG AAATA CTGAG AG TATTT CACTG ACAGG CAATA CCGTC CAAGG	5.3
Human Pol ε	sense antisense	AAGGC TGGCG GATTA CTGCC GATGC TGCTG GTGAT GTACT C	3.5
Human Pol ε	sense antisense	CAGCC CCTGT CACTG TGAAG GGATG CTGCT GGTGA TGTAC TCCAC G	2.0
Human Phosphatase 2A (PP2A)	sense antisense	GTTTCG ATGTC CAGTT ACTGT CT GAAAT ATCTT GCCCA AAGGT GTAAC CAGCT	0.53

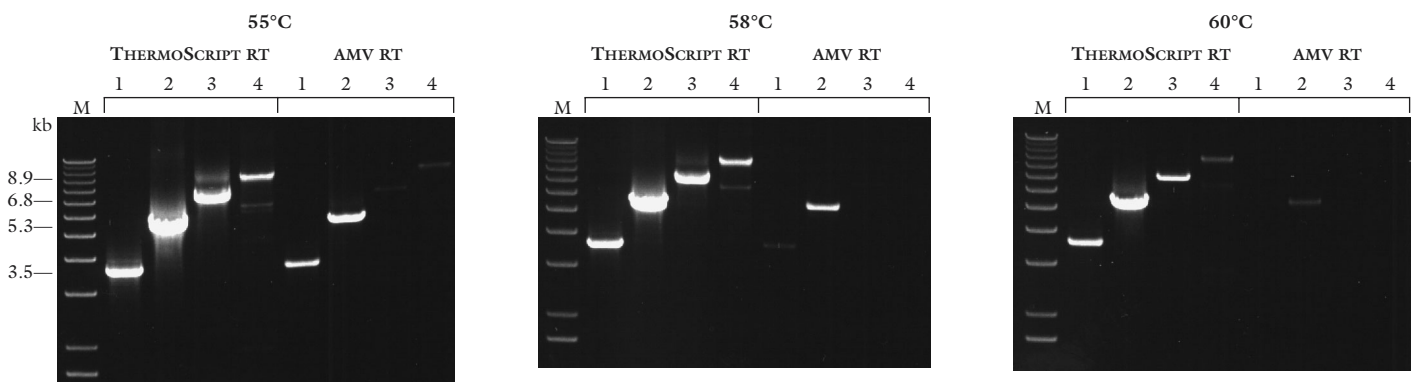


FIGURE 3. RT-PCR products. cDNA was synthesized with THERMOSCRIPT RT or AMV RT from total HeLa RNA (pol ϵ or TSE-2, 1 μ g; and APC, 5 μ g) at 55, 58, or 60°C using oligo(dT)₂₀. cDNA reactions were assembled on ice and placed in a prewarmed heat block. Lane 1 – pol ϵ , lane 2 – TSC-2, lane 3 – pol ϵ , and lane 4 – APC. Lane M is the 1 Kb PLUS DNA LADDER™.

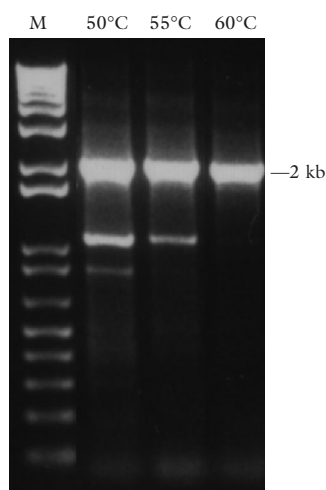


FIGURE 4. Improved specificity of RT-PCR. The human pol ϵ mRNA was amplified from cDNA produced by THERMOSCRIPT RT from 1 μ g of total HeLa RNA annealed to a GSP (GGATG CTGCT GGTGA TGTAC TCCAC G). Hot start was used for the cDNA synthesis reaction by adding the THERMOSCRIPT RT to prewarmed cDNA reactions. Lane M is the 1 Kb PLUS DNA LADDER.

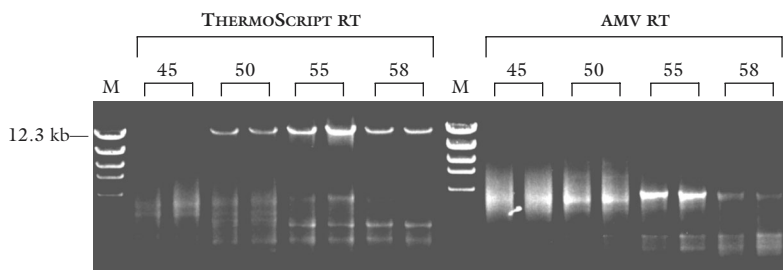


FIGURE 5. Long RT-PCR products at elevated temperatures. 5 μ g of total rat brain RNA was used for the cDNA synthesis by THERMOSCRIPT RT or AMV RT with a GSP (AGGTG GCGGC TCAAA CACAA AG) for rat dynein at 45°C to 58°C. Hot start was used for the cDNA synthesis by adding the RT to prewarmed cDNA reaction mixes. Lane M is the 1 Kb PLUS DNA LADDER.

THERMOSCRIPT RT than for AMV RT. The capability of producing a 7.5-kb cDNA under these stringent conditions demonstrates the thermostable RT activity of THERMOSCRIPT RT.

RT-PCR. The advantageous results of THERMOSCRIPT RT in stringent first-strand cDNA synthesis led to a series of RT-PCR experiments. The reaction conditions were more relaxed to reflect typical RT-PCR conditions. These include adding the RT to the reaction on ice and then transferring to a prewarmed heat block. THERMOSCRIPT RT resulted in high yields of RT-PCR products at all temperatures, whereas AMV RT produced significantly lower or no yield of PCR products (figure 3).

The thermostability of THERMOSCRIPT RT can be exploited even more when using a GSP to prime cDNA synthesis. Since a GSP can anneal to RNA at higher temperatures than oligo(dT), reverse transcription can be done at high temperatures and with a hot start for cDNA synthesis to improve priming specificity. For a human polymerase ϵ PCR product, non-specific products generated were reduced or eliminated by increasing the temperature of the cDNA synthesis reaction from 50°C to 60°C (figure 4). For extremely long 12.3-kb cDNA, the amount of nonspecific PCR products decreased when cDNA was synthesized at higher temperatures (figure 5). Furthermore, the yield of the 12.3-kb PCR product increased with the higher temperatures. AMV RT did not produce any long cDNA molecules under these reaction conditions.

THERMOSCRIPT RT is active at temperatures above 60°C. When priming cDNA synthesis

with a GSP with a high annealing temperature, yields of PCR products remained high up to 70°C (figure 6). Using a GSP and analyzing fragments <1.5 kb, THERMOSCRIPT RT is capable of efficient RT-PCR in which reverse transcription is carried out at temperatures as high as 70°C.

In summary, THERMOSCRIPT RT is capable of synthesizing cDNA at temperatures as high as 70°C for RT-PCR. The thermostability is useful to improve specificity and when RNA with a high degree of secondary structure is analyzed (1,2). Also, since THERMOSCRIPT RT has been engineered to reduce RNase H activity by 99.5%, the enzyme is capable of efficiently synthesizing long cDNA transcripts.

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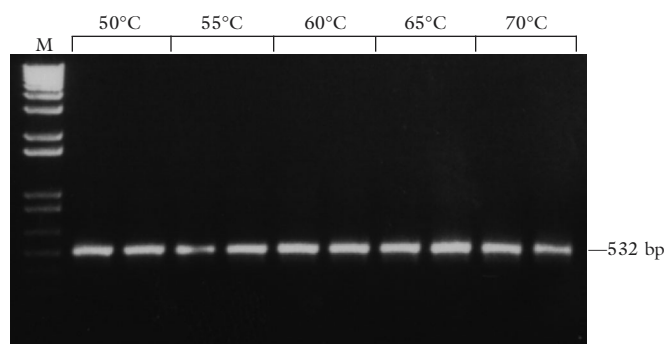


FIGURE 6. Yield of RT-PCR products at high temperatures. The human PP2A mRNA was amplified from cDNA synthesized by THERMOSCRIPT RT with a GSP (GAAAT ATCTT GCCCA AAGGT GTAAC CAGCT) from 100 ng of total HeLa RNA. Hot start was used for the cDNA synthesis by adding the RT to prewarmed cDNA reaction mixes. Lane M is the 1 Kb PLUS DNA LADDER.



"You're a mess. You gotta get your doctor to put you on the Caffeine Patch."

IMPROVED SENSITIVITY AND SPECIFICITY OF RT-PCR

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The sensitivity and reliability of RT-PCR are dependent on a variety of factors that influence the efficiency and specificity of reverse transcription and PCR, such as primer design and cycling conditions. A critical parameter is nonspecific annealing and extension of primers prior to the initial denaturation step of PCR. These artifacts can be minimized by utilizing hot start techniques (1–3). We have described PLATINUM™ *Taq* DNA Polymerase for automatic hot start PCR that improved specificity and sensitivity of PCR (4). Also, a novel thermostable reverse transcriptase, THERMOSCRIPT™ RT, increased the specificity of RT-PCR by efficient reverse transcription at high temperatures (5). In this paper, we present the combination of THERMOSCRIPT RT with two PLATINUM amplification products to improve RT-PCR results.

To evaluate automatic hot start, 21 primer sets targeting fragments between 158 bp and

3.5 kb from 6 different mRNAs in HeLa total RNA were analyzed. First-strand synthesis was performed using the THERMOSCRIPT RT-PCR System (Cat. No. 11146). Oligo(dT)₂₀ and random hexamer primers were tested for each target. Identical aliquots of first-strand cDNA were amplified by either *Taq* DNA Polymerase, PLATINUM *Taq* DNA Polymerase (Cat. No. 10966), or PLATINUM *Taq* DNA Polymerase High Fidelity (Cat. No. 11304). Amplification reactions containing PLATINUM enzymes were assembled at ambient temperature from master mixes. All other PCRs were assembled on ice from master mixes. Reactions were placed directly in a thermal cycler equilibrated at 94°C.

For PCR with *Taq* DNA polymerase, of the 21 primer sets examined, 3 were dependent on PLATINUM *Taq* DNA Polymerase, and 10 showed a marked improvement in product yield and/or specificity. Representative results are shown in figure 1 (panel A). Even for very

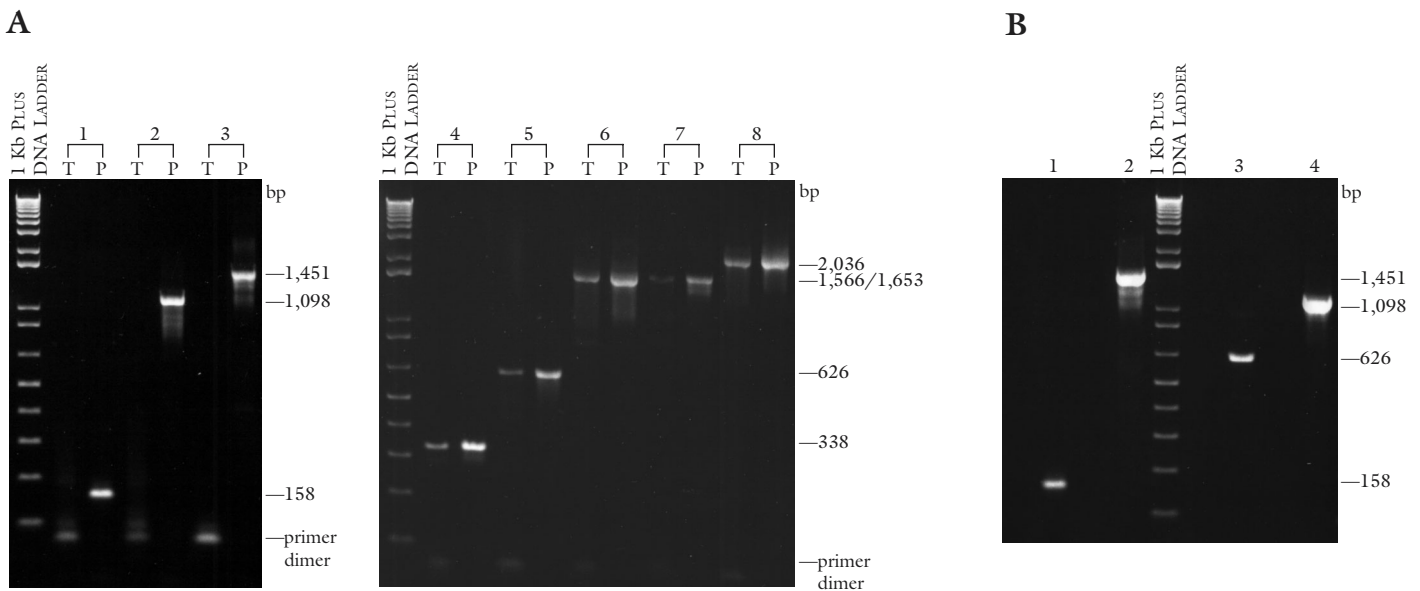


FIGURE 1. Comparison of yield and specificity in RT-PCR. cDNA synthesis was performed using the THERMOSCRIPT RT-PCR system with oligo(dT)₂₀ primer and HeLa total RNA (β -actin, 1 ng; RPA or PP2A, 100 ng; DNA pol ϵ , 1 μ g). Incubation was at 50°C for 30 min, followed by RNase H digestion for 20 min at 37°C. One-tenth (2 μ l) of the first-strand synthesis was used for PCR with *Taq* DNA polymerase (T) or PLATINUM *Taq* DNA Polymerase (P) (panel A); or PLATINUM *Taq* DNA Polymerase High Fidelity (panel B). Reactions were incubated at 94°C for 30 s and amplified for 35 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 1.5 min. One-tenth (5 μ l) of each PCR was analyzed by gel electrophoresis on 1.2% agarose, 0.5X TBE with 0.5 μ g/ml ethidium bromide. Products shown are 158-bp β -actin, 1,098-bp β -actin, 1,451-bp RPA, 338-bp RPA, 626-bp β -actin, 1,566-bp DNA pol ϵ , 1,653-bp β -actin, and 2,036-bp DNA pol ϵ (panel A, lanes 1–8, respectively).

abundant mRNA, such as β -actin, PLATINUM *Taq* DNA Polymerase eliminated primer-dimer artifacts, enabling efficient amplification of the desired fragment. Results were comparable for oligo(dT)₂₀ and random hexamer primed cDNA; however, yield and specificity were slightly better for the oligo(dT)₂₀.

Recently, a higher fidelity PLATINUM *Taq* DNA Polymerase has been developed. Besides 6-times better fidelity than *Taq* DNA Polymerase, PLATINUM *Taq* DNA Polymerase High Fidelity resulted in higher product yield than PLATINUM *Taq* DNA Polymerase in 13 of the 21 primer pairs (representative results in figure 1, panel B). These data demonstrate the importance of controlling the specificity and stringency of priming events in the early stages of PCR.

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The Help Box from Your Technical Support & Training Team

Factors Affecting Specificity in PCR

Primers

Primer design is critical to high specificity. PCR primers are typically between 18 to 25 nucleotides in length, with 40 to 60% GC content. When designing primers, avoid complementary sequences at the 3' end of the primer pairs to prevent primer-dimer formation. Avoid internal complementary sequences to minimize internal hairpins. It is best not to have a C or G as the 3' end base. For degenerate primers, it is preferable that the most unambiguous sequence be at the 3' end of the primer. The last 3 bases on the 3' end can be enough to initiate PCR.

Annealing Temperature

Annealing temperatures are usually set about 5°C below the T_m of the primers. It is best that both primers have a similar T_m. Melting temperatures in the range of 55°C to 70°C give the best results. There are several formulas for calculating the T_m. The calculated T_m can vary significantly (up to 20°C) depending on the formula used and the primer sequence. In some formulas, the calculated T_m is dependent on the input of salt concentration.

Hot Starting the PCR

For less than optimal primers, many times using hot start can improve specificity. Automatic hot start with PLATINUM products gives the added benefit of being able to assemble reactions at room temperature.

RNA ISOLATION WITH TRIZOL[®] REAGENT

Arno Sewall
Sharon McRae
Technical Services
Life Technologies, Inc.
Rockville, Maryland
20849

What are some possible stopping points when working with TRIzol Reagent?

- After homogenization, and before addition of chloroform, samples can be stored in TRIzol Reagent at -70°C for at least 1 month, or can stay at room temperature for several hours.
- The RNA can be stored in isopropanol overnight at 4°C before centrifugation.
- RNA pellet, before resuspension, may be stored in 75% ethanol at 4°C for 1 week, or -20°C for up to a year.

What type of tubes should I use with the TRIzol Reagent?

- Polypropylene. Do not use tubes sensitive to phenol.

What will be the approximate aqueous volume when using TRIzol Reagent?

- About 60% of the TRIzol Reagent volume becomes part of the aqueous phase after chloroform addition.

Can I use a table-top centrifuge when I use the TRIzol Reagent?

- Yes. Use $2,600 \times g$ at 4°C for 60 min for phase separation and 30 min for RNA precipitation.

If my tissue has a high content of proteoglycans and/or polysaccharides, what can I do to ensure that these compounds don't contaminate the RNA?

- Centrifuge following homogenization before adding chloroform at $12,000 \times g$ at 4°C for 10 min to pellet polysaccharides (also pellets genomic DNA). In addition, you may need to do a high salt isopropanol precipitation as follows.
- After collection of the aqueous phase, add 0.25 ml isopropanol and 0.25 ml of 0.8 M sodium citrate, 1.2 M NaCl per 1 ml TRIzol Reagent. Mix the solution, centrifuge, and proceed with isolation as described. This precipitates the RNA and maintains proteoglycans and polysaccharides in a soluble form. Samples known to have a high content of proteoglycans or polysaccharides include rat liver, rat aorta, and plants.

What can I do to increase the yield from small amounts of starting samples?

- Add 10 μg of RNase-free glycogen to <10 mg tissue or $<1 \times 10^6$ suspension cells. Glycogen, unlike salmon sperm DNA carrier, can be added when TRIzol Reagent is added to sample.

What can I do to avoid genomic DNA contamination of my RNA in RT-PCR applications?

- If you do not need to analyze the genomic DNA, centrifuge the sample following homogenization before adding chloroform at $12,000 \times g$ for 10 min at 4°C to pellet the DNA. Add chloroform to the supernatant and proceed with RNA isolation protocol. Also, after RNA isolation, treat with amplification grade DNase I.

Can isolations with TRIzol Reagent be scaled up?

- Yes. Scale up linearly for tissues and suspension cells. For monolayer cells, scale up linearly based on the surface area of the plate, NOT the amount of cells. Use at least 1 ml of TRIzol Reagent for every 10 cm^2 of surface area.

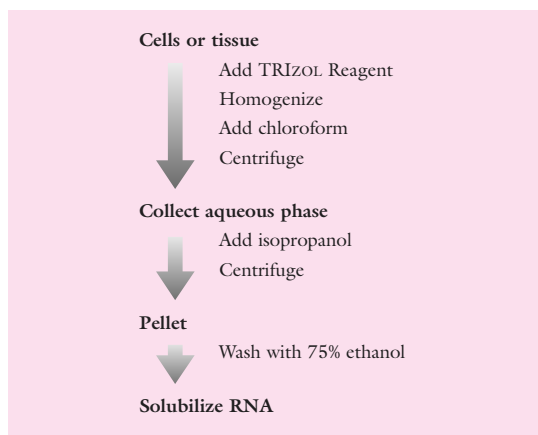


FIGURE 1. Summary of steps to isolate total RNA with TRIzol Reagent.

Can TRIzol Reagent be used to isolate RNA from small samples?

■ Yes. We have been successful with 10^4 cells using 0.4 ml of TRIzol Reagent and 10 μ g glycogen.

How can the stability of the isolated RNA be increased during storage?

■ Dissolve RNA in deionized formamide and store at -70°C for up to 1 year. To precipitate RNA from formamide, add NaCl to final concentration of 0.2 M followed by 4 volumes of ethanol. Incubate 3–5 min at room temperature and centrifuge at $10,000 \times g$ for 5 min.

MEASURING ABSORBANCE OF RNA SAMPLES

The A_{260}/A_{280} ratio is a commonly used criterion for nucleic acid purity. Values for pure RNA are usually >2.0 (1,2). However, the absorbance of nucleic acids at these wavelengths is dependent upon the ionic strength and pH of the medium. For example, the A_{260}/A_{280} ratio of the same RNA preparation differed depending on whether the RNA was diluted into water or TE (table 1).

An extensive study (3) of the effects of pH and ionic strength has shown that the change in the A_{260}/A_{280} ratio is primarily due to a decrease in the absorbance at 280 nm when the ionic strength or pH is increased. Wilfinger *et al.* also note that these same effects are seen when the A_{260}/A_{280} ratio of DNA is measured. This effect was independent of the method of purification. We recommend that RNA be diluted with TE for spectrophotometric assays.

The usefulness of the A_{260}/A_{280} ratio as a measure of purity for nucleic acid preparations has been questioned recently (4). However, it is a simple and useful parameter to help estimate purity. Although a high A_{260}/A_{280} ratio may not

indicate an extremely pure preparation, a low A_{260}/A_{280} ratio (1.7 for RNA in TE) may indicate that the preparation is not pure and may not be suitable for some applications.

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TABLE 1. Comparison of RNA absorbance. Total RNA was isolated with TRIzol Reagent. Aliquots of each preparation were diluted into distilled DEPC-treated water or TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] before absorbances were taken.

	Diluent	A_{260}	A_{280}	$A_{260/280}$
Cytoplasmic RNA	Water	0.381	0.223	1.71
Cytoplasmic RNA	TE	0.335	0.145	2.31
Total RNA	Water	0.585	0.328	1.79
Total RNA	TE	0.544	0.247	2.21

The Help Box from Your Technical Support & Training Team

Effect of Glycogen on PCR and RT-PCR

Glycogen (0.2 to 0.4 $\mu\text{g}/\mu\text{l}$) is commonly used as carrier in precipitation of small amounts of DNA, RNA, and oligonucleotides. The effect of glycogen on the enzymes used in RT-PCR or PCR is therefore important. No inhibition was seen in the ability of *Taq* DNA

polymerase to amplify GAPDH from 5, 10, and 50 ng of genomic DNA (from HeLa or K562 cells) using 20 μg glycogen in a 50- μl PCR. Also for RT-PCR, the addition of 20 μg glycogen to the PCR had no effect on the amount of amplified product produced. For the reverse transcription reaction, 40 μg glycogen in a 20- μl reaction had no effect on SUPERScript™ II RNase H⁻ RT [Gerard, G. (1994) *FOCUS* 16, 102]. Therefore, glycogen can be used for precipitation of RNA for first-strand synthesis of cDNA for RT-PCR. Also, no effect of glycogen has been seen in second-strand DNA synthesis.

TROUBLESHOOTING: SERUM-FREE CULTURE OF KERATINOCYTES

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If you have a low plating, cloning, or growth efficiency with primary cultures . . .

- After dissociation, count only those smaller and more regularly shaped basal cells; do not include the more flattened, irregularly shaped cornified cells. Use this count of smaller cells to determine appropriate seeding density of primary culture.
- Use a seeding density of 3×10^3 to 5×10^3 cells/cm² of surface area.
- Change medium every 2 to 3 days to maintain optimal growth conditions.
- If you need to seed with a low cell density ($<3 \times 10^3$) in Keratinocyte-SFM, titrate the bovine pituitary extract (BPE). Too much BPE inhibits cell growth.
- In Keratinocyte-SFM, both BPE and EGF should be titrated to determine optimal concentration. If EGF is too high, the growth will be abnormal or have an unhealthy morphology. A healthy morphology exhibits a nonstratified monolayer with a rounded, packed appearance, referred to as a “cobblestone appearance.”
- Start cultures from neonatal foreskins ≤ 5 days old.
- Check that the carbon dioxide level in the incubator is 5% to prevent the medium from becoming alkaline.
- Use only solutions (wash solutions, trypsin solutions, etc.) that are low calcium, or calcium- and magnesium-free. Calcium exposure encourages differentiation and decreases growth.

If other cells such as melanocytes or fibroblasts contaminate your primary cultures . . .

- Keratinocyte-SFM and Defined Keratinocyte-SFM were formulated to minimize fibroblast growth, and they do not contain mitogenic factors for melanocytes. Melanocytes may be seen in the cultures during the first week but will not survive long-term without the mitogenic factors.

If you have low plating, cloning, or growth after passaging cells . . .

- Passage cells at confluencies $\leq 75\%$. Higher confluencies may result in decreased growth or failure to adhere to the flask.
- For Defined Keratinocyte-SFM, minimize trypsin exposure. This medium is a very low protein formulation, so it is easy to over-trypsinize the cells. Although the cells may appear viable, they may adhere poorly or grow poorly.
- To trypsinize, use 0.05% trypsin, 0.53 mM EDTA. Check flasks after 2 min and then every 30 to 45 s under the microscope, until cells are rounded. Tap flask to dislodge remaining cells and dilute with soybean trypsin inhibitor immediately. *Note:* Do not use FBS to stop the trypsin, as it may cause differentiation.
- The cells may be reaching senescence. As cells approach their maximum passage number (6 to 8 passages), vacuoles and empty spaces begin appearing in the cytoplasm.

If you have difficulty transfecting keratinocytes . . .

- Use Keratinocyte-SFM without BPE, EGF, insulin, hydrocortisone, and T3. Transfection efficiencies of 40% to 50% have been obtained [Hawley-Nelson, P. and Ciccarone, V. (1996) *FOCUS* 18, 43].
- Do not use OPTI-MEM® I medium for cationic lipid reagent transfection. It contains enough calcium to induce differentiation.
- For optimal results, perform transfection of keratinocytes in the first or second passage.

FUN FACTS ON KERATINOCYTES

Establishing Cultures

- To lessen variability from donor to donor, pool the cells from 5 to 6 donors of neonatal foreskins.
- You can typically expect 1×10^6 to 3×10^6 cells from 4 to 6 foreskins.
- To achieve a purer primary keratinocyte population, use dispase to separate the dermis from the epidermis. After separation of the epidermis, use trypsin to further dissociate into single cells.
- Using gentamicin in place of penicillin/streptomycin gives added protection against mycoplasma contamination. (Note: Mycoplasma contaminants tend to be more common in primary cell culture.)
- Giving tissue a very short (≤ 5 s) alcohol wash will minimize contamination of the culture. Be cautious not to overexpose.
- Keratinocytes from older donors may have a lower plating efficiency and culture life span.

Maintaining Cultures

- To avoid differentiation, it is advantageous to use a serum-free system instead of FBS to culture keratinocytes. The calcium level in FBS is enough to cause keratinocytes to differentiate.
- To control the phenotype of cultured keratinocytes, Keratinocyte-SFM is available calcium-free to allow you to supplement calcium to suit culture requirements and experimental conditions.
- The calcium concentration in Keratinocyte-SFM and Defined Keratinocyte-SFM was optimized to maintain human keratinocytes in an undifferentiated state. Mouse keratinocytes require less calcium than human keratinocytes and will differentiate in standard serum-free formulations. Use calcium-free Keratinocyte-SFM and supplement 0.02 mM calcium to keep mouse keratinocytes from differentiating [Yuspa, S.H., Koehler, B., Kulesz-Martin, M., and Hennings, H. (1981) *J. Invest. Dermat.* 76, 144].

- For studies of growth regulation and differentiation, Defined Keratinocyte Medium (without BPE) is preferable to Keratinocyte-SFM (with BPE). There are no undefined materials to complicate experimental models or interpretation of results.
- Both Defined Keratinocyte-SFM and Keratinocyte-SFM grow human neonatal foreskins and human cervical epithelia. Researchers have reported success growing human oral epithelia in Keratinocyte-SFM.
- Keratinocyte-SFM and Defined Keratinocyte-SFM have been formulated to down-regulate fibroblast contamination in primary cultures.
- Once supplemented, Defined Keratinocyte-SFM can be stored ~15 weeks at 2°C to 8°C. Keratinocyte-SFM supplemented with BPE and EGF can be stored ~4 weeks at 2°C to 8°C.
- To stop trypsin activity when passaging keratinocytes, use soybean trypsin inhibitor. Do not use FBS because it can cause cell differentiation.
- You can expect an average of about 5 to 6 passages from human neonatal foreskins in primary culture.
- Titrate bovine pituitary extract (BPE) in your system to minimize variable results (figure 1).

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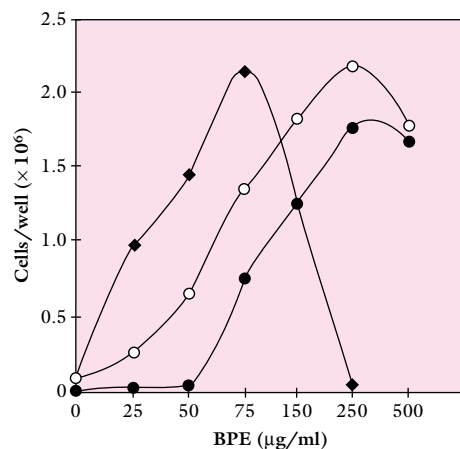
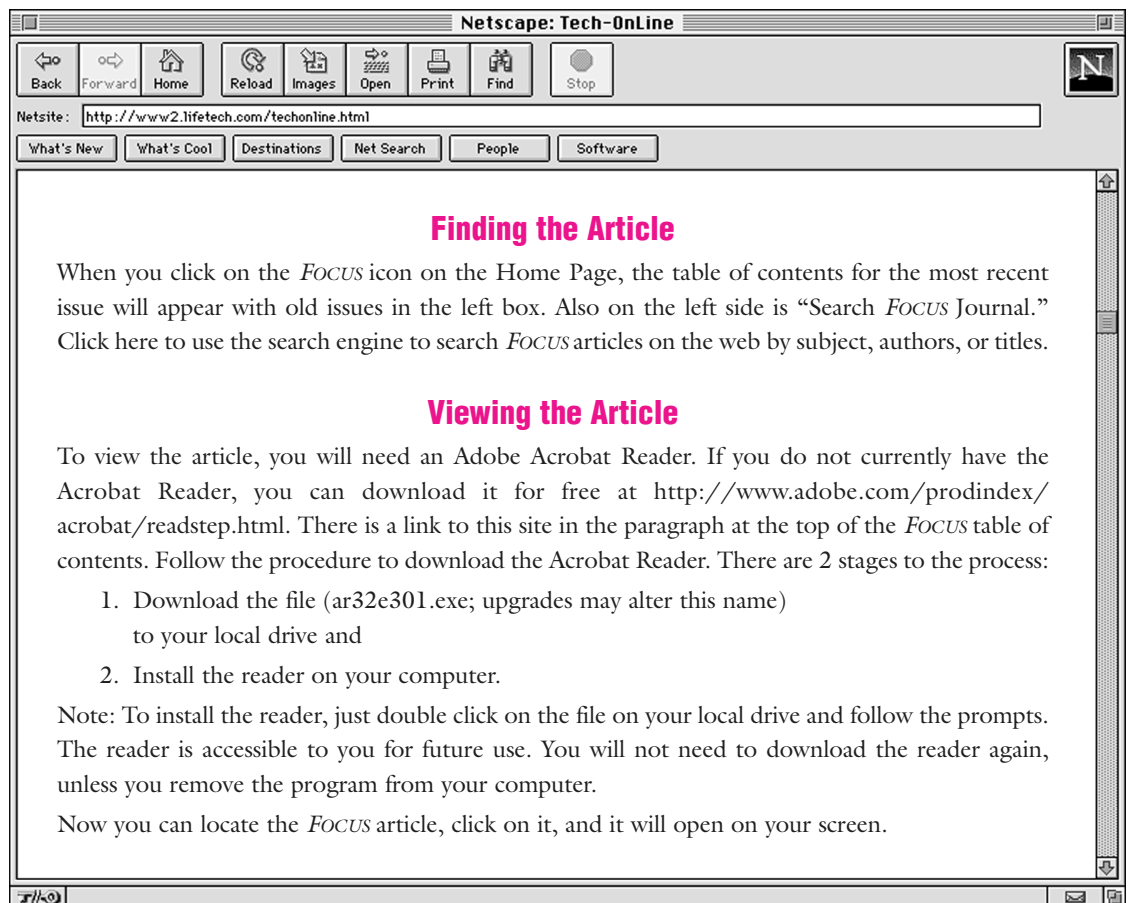


FIGURE 1. BPE variability. Growth of keratinocytes in Keratinocyte-SFM was compared by titrating 3 batches of BPE.

Did You Know . . .

- Vitamin A affects keratinocyte differentiation. Changes in keratin synthesis are regulated by vitamin A [Fuchs, E. and Green, H. (1981) *Cell* 25, 617].
- Potassium channels may moderate calcium influx in more differentiated keratinocytes and may play a central role in keratinocyte differentiation [Mauro, T., Dixon, D.B., Komuves, L., Hanley, K., and Pappone, P.A. (1997) *J. Invest. Dermat.* 108, 864].
- Differentiation in keratinocytes is marked by formation of cell connections (desmosomes) and expression of specific proteins such as filagrin and involucrin.
- Cultures of normal keratinocytes must attain confluence before KGF (Keratinocyte Growth Factor) promotes an increase in cell number relative to untreated controls. KGF inhibits cross-linked envelope formation and nucleosomal fragmentation in cultured human keratinocytes [Hines, M.D. and Allen-Hoffmann, B.L. (1996) *J. Biol. Chem.* 271, 6245].
- In cultured skin substitutes grafted to athymic mice, researchers have selectively depleted melanocytes from human keratinocyte cultures. The melanocytes can then be added at a selected density to the skin substitute. This enables a predictable pigment for improved cosmesis in healed wounds [Swope, V.B., Supp, A.P., Cornelius, J.R., Babcock, G.F., and Boyce, S.T. (1997) *J. Invest. Dermat.* 109, 289].

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A SERUM-FREE CULTURE MEDIUM FOR MONOCYTE/MACROPHAGE STUDIES

Human mononuclear phagocytes (MP) represent a very powerful arm of the immune effector cell population. Circulating monocytes can undergo phenotypic and functional alterations that allow them to effectively participate in a variety of localized host defense mechanisms. The mononuclear phagocyte system is composed of both circulating monocytes and resident, differentiated tissue macrophages found in such sites as the liver (Kupffer cells), lung alveoli, lymphatic organs and vessels, bone marrow, and skin (Langerhans cells). These cells play a central role in immunoregulatory and immune surveillance functions to protect against adventitious agents and neoplastic disease, are involved in inflammatory response to injury, actively clear damaged cells and immune complexes from circulation, and take part in tissue resorption and remodeling.

Studies of MP and the regulatory signals involved in triggering their participation in these functions have been somewhat hampered by the heterogeneous nature of this cell population, difficulties in isolation of pure MP subsets, and preserving them as unstimulated or nonactivated cells prior to experimentation. Conventional tissue culture techniques that frequently employ serum can artifactually stimulate cells by the introduction of microbes (bacteria, viruses, mycoplasma) or their products, and other biological response modifiers, including hormones, proteins, and other biomolecules. To complicate matters, no lot of serum is identical to another, and a considerable effort may need to be expended to find a "good lot." The use of a serum-free medium would circumvent these problems and is essential in *ex vivo* treatment of cancer by adoptive immunotherapy. Several recent clinical studies have utilized peripheral blood monocytes in this fashion (1-3).

We describe here some of the properties of a serum-free medium, Macrophage Serum-Free Medium (Macrophage-SFM), that supports human peripheral blood monocyte culture by maintaining viability and function *in vitro*. This

medium provides a superior alternative to serum-containing media for investigators studying these cells. A number of stringent requirements were met during development of this product due to its potential clinical utility. These requirements included: the medium must be defined; all protein constituents must be of human sequence origin and be pasteurized or recombinant in origin; endotoxin levels must be negligible (<0.1 ng/ml); and the medium must support human monocyte/macrophage culture and functional performance and must be devoid of components that may contribute to an artifactual cellular response.

This report discusses the utilization of Macrophage-SFM for routine culture and experimentation with mononuclear phagocytes.

MATERIALS AND METHODS

Monocyte isolation. Peripheral blood monocytes were isolated from buffy coats obtained from the American Red Cross (Buffalo, NY). Mononuclear cell isolation was conducted similar to the method of Boyum (4). Briefly, buffy coats were diluted 1:1 with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (D-PBS). The diluted buffy coats were layered over Lymphocyte Separation Medium, ($d = 1.077$) in a 2:1 ratio and centrifuged at $400 \times g$ for 20 min at 20°C . The mononuclear cell interface was removed and contaminating platelets and residual erythrocytes were removed by repeated centrifugations. Mononuclear cells were suspended in culture medium, and monocytes were isolated by their adherence to plastic tissue cultureware following a 1-h incubation at 37°C in a humidified 95% air, 5% CO_2 incubator. Monocytes isolated in this fashion were cultured in either conventional RPMI-1640 medium with 10% fetal bovine serum (FBS) or GIBCO BRL Macrophage-SFM. Cells were cultured with either of these media alone or supplemented with human recombinant GM-CSF (rGM-CSF).

MTT assay of macrophage/monocyte viability. Assay of macrophage/monocyte viability was performed according to the method of Mosmann

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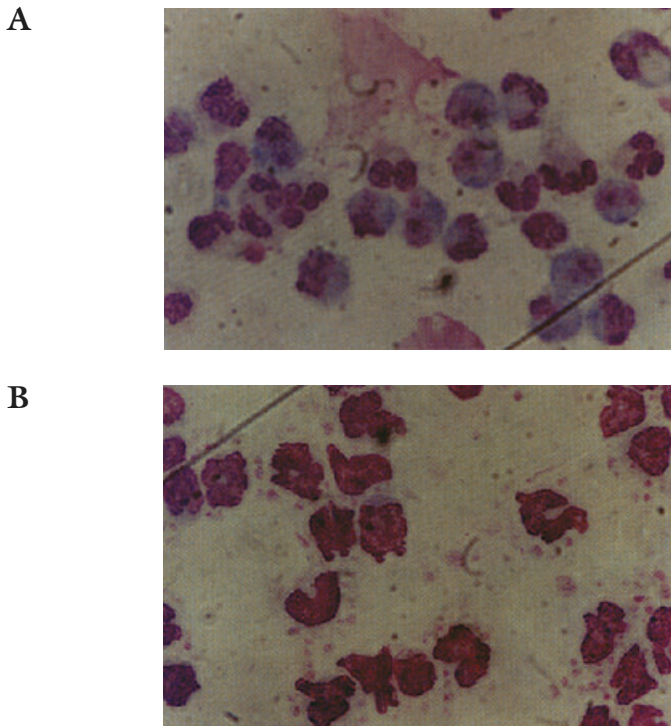


Figure 1. Photomicrographs of human monocytes incubated in either RPMI 1640 supplemented with 10% human serum (Panel A) or Macrophage-SFM (Panel B). Human peripheral blood monocytes were allowed to adhere to plastic cover slips for 1 h, were washed with D-PBS, and were stained with Wright's stain as described in Materials and Methods. (Magnification 1,000X)

(5). This assay utilizes the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)], which is converted enzymatically in mitochondria of viable cells to a blue dye that is insoluble in water. Mononuclear cells (1×10^6) in RPMI-1640 medium supplemented with 1% human serum albumin (HSA) (USP grade) were added to each well of 96-well round bottom plates. Non-adherent cells were removed by washing with warm RPMI-1640. Various media and supplements were then added for various culture times prior to assay. MTT was then added to achieve a final concentration of 0.4 mg/ml and incubated at 37°C for 4 h in a 95% air, 5% CO₂ incubator. The resulting crystalline formazan deposits were solubilized in 95% isopropanol:5% formic acid. Spectrophotometric analysis measured the relative absorbance at 595 nm.

Staining procedures. Several different staining procedures were utilized to monitor monocyte purity post-isolation and culture. Wright's stain and aceto-orcein staining were used to analyze

the morphology of isolated cells. Wright's staining was performed according to the conventional methodology. Slides or coverslips were placed in Wright's stain for 30 s, an equal volume of D-PBS was added for 3 min, and then the slides were washed with distilled water. Aceto-orcein [1% (w/v) in PBS] was added directly to slides and analyzed microscopically.

An anti-Leu-M3 monoclonal antibody (Becton Dickinson) that recognizes an antigen (CD14) expressed on mature monocytes was used to verify monocyte phenotype. Adherent cells were reacted with anti-Leu-M3 diluted in PBS containing 1% HSA followed by biotinylated goat anti-mouse antiserum (Immunon), each for 1 h at 37°C. Positive reactions were visualized with streptavidin-conjugated horseradish peroxidase using the substrate 3-amino-9-ethylcarbazole and a hematoxylin counterstain.

Enzyme immunoassay. A quantitative assessment of the cell surface markers was determined by enzyme immunoassay. Peripheral blood mononuclear cells attached to tissue culture grade 96-well plates were fixed in 1% paraformaldehyde, washed, and blocked with 1% bovine serum albumin (BSA) in PBS. Specific leukocyte monoclonal antibodies (MAb) including IOM2 (CD14, monocytes), IOM3 (monocyte antigen), ION2 (CD67, granulocytes), IOT56 (CD56, NK cells), and control nonspecific antibody were obtained from AMAC Inc. MAbs (10 µg/ml) were diluted in 0.1% BSA, 0.05% TWEEN 20®-containing PBS and incubated with attached, fixed cells for 1 h at 25°C. Affinity purified, alkaline phosphatase-conjugated goat anti-mouse IgG was added for 1 h after removal of primary antibody and washing of the plates with PBS containing 0.05% TWEEN 20. P-nitrophenyl phosphate (1 mg/ml) was used as a substrate in 0.1 M glycine (pH 10.4), 0.001 M ZnCl₂, and 0.001 M MgCl₂. The reaction was terminated with 5 N NaOH after 30 min at 20°C and absorbance was measured at 405 nm.

Phagocytosis assay. The ability of adherent cells to phagocytize was determined by exposing monocytes to fluorescent, 0.2-µm diameter, fluorescein isothiocyanate (FITC)-labeled beads (Fluoresbrite carboxylate microspheres, Polysciences, Inc.) or BODIPY-labeled opsonized *Saccharomyces cerevisiae* (Zymosan A Bioparticles, Molecular Probes, Inc.) in a manner similar to that previously described (6).

Confocal microscopy. The procedures used for preparing leukocytes for microscopic examination and the configuration of the confocal laser scanning microscope have been described (20).

RESULTS

Human peripheral blood monocytes were isolated by conventional methodology and analyzed for phenotypic and functional characteristics typical of these mononuclear phagocytic cells. Cellular performance levels were monitored and compared in the presence of conventional serum-supplemented medium or Macrophage-SFM.

Conventional methodology often uses the inherent ability of monocytes to attach to artificial substrata such as plastic or glass to isolate these cells from other mononuclear leukocytes. It was essential to document that the use of Macrophage-SFM would not adversely affect monocyte isolation and would not significantly alter the purity of this isolated subset. Figure 1 demonstrates that the isolated cells have the typical morphological features of human monocytes. No obvious differences were evident in the cells adhering in the presence of either RPMI 1640 + 10% human serum (Panel A) or Macrophage-SFM (Panel B). Additionally, figure 2 shows morphologically that these cells have the characteristically irregular nuclear appearance of monocytes (Panel A) and that they stain positively for the CD14 monocyte marker (Panel B).

An ELISA was performed to determine the purity of the isolated adherent cells (figure 3). These adherent leukocytes are strongly reactive with IOM3 and IOM2 monocytoid specific MAbs. Little or no reactivity was observed with CD67 granulocyte (ION2) or CD56 NK cell (IOT56) markers. Similar results are seen in cells cultured in serum-containing medium (data not shown).

Figure 4 are views obtained using a confocal laser scanning microscope of a freshly isolated normal human blood monocyte and of a monocyte-derived macrophage cultivated for 8 days in Macrophage-SFM. Panel A is a computer projected composite of nine serial optical sections (each 0.5- μ m thick) of a single monocyte ingesting numerous FITC-beads (green); the arrow illustrates a phagocytized particle that has been internalized. The horseshoe-shaped nucleus, a feature that is characteristic of many freshly isolated monocytes, has been stained with

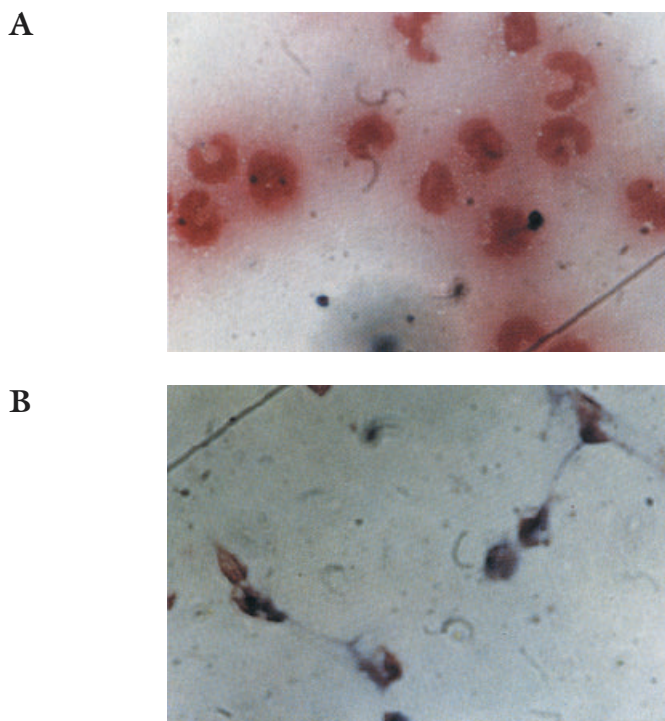


Figure 2. Photomicrographs of adherent human monocytes incubated in Macrophage-SFM. Adherent monocytes were stained with 1% aceto-orcein (Panel A) or with anti-Leu-M3 MAb (Panel B), as described in Materials and Methods. (Magnification 1,000X)

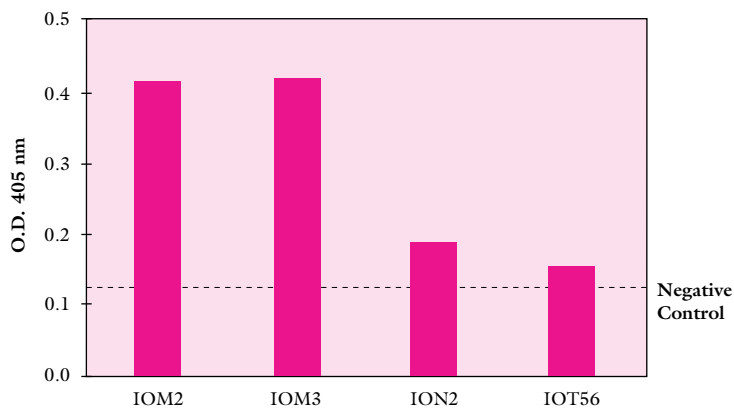


Figure 3. Cellular phenotype of mononuclear cells adhering to plastic dishes in the presence of Macrophage-SFM. Isolated mononuclear cells were placed in Macrophage-SFM, allowed to adhere to 96-well plates, and reacted with 10 μ g/ml of monocyte (IOM2 and IOM3), granulocyte (ION2), NK cell (IOT56), or negative control MAb to determine the molecular phenotypic profile of the adherent cells.

propidium iodide (red) under conditions that reflect the relative DNA content. Panel B is a single optical section (0.5- μ m thick) of a human macrophage that has been stained with a surface membrane dye (PKH26, Zynaxis) prior to phagocytizing opsonized *Saccharomyces cerevisiae*. Detection of the lipophilic membrane

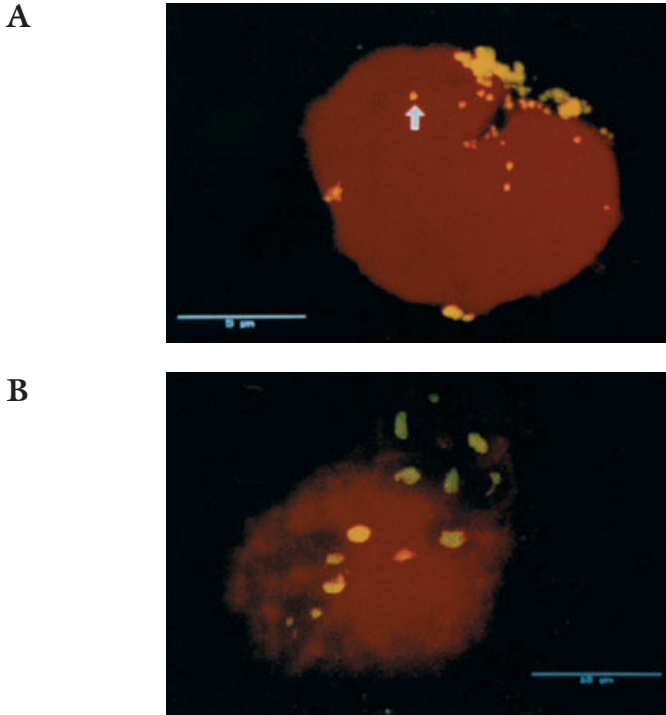


Figure 4. Confocal microscopy of phagocytizing cells. A human monocyte (panel A) and a macrophage (panel B) phagocytizing fluorescent particles.

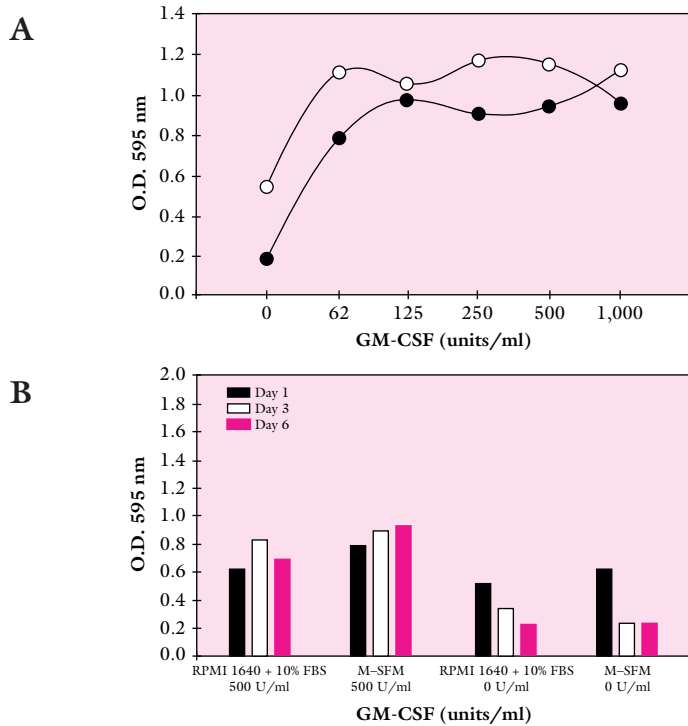


Figure 5. Human peripheral blood monocyte maintenance as a function of GM-CSF supplementation. Human peripheral blood monocytes were isolated as described and incubated for various times in either RPMI 1640 with 10% FBS or Macrophage-SFM in the presence or absence of human recombinant GM-CSF. Results shown are from an MTT assay used to measure monocyte viability. Panel A: Cells were cultured in either RPMI 1640 + 10% FBS (○) or Macrophage-SFM (●) for 7 days in the presence of various concentrations of rGM-CSF. Panel B: Monocytes were cultured with or without 500 U/ml rGM-CSF for 1, 3, or 6 days.

dye (red) is optimal with rhodamine filters. The nucleus is not stained as shown by the darkened area within the cell. The BODIPY-labeled yeast (green) can be seen ingested within phagocytic vacuoles. These particles have a fluorescein-like excitation and emission which facilitate multi-color fluorescence application. These data also show that monocyte function is not impaired in the presence of Macrophage-SFM. Similar results are seen in cells cultured in serum-containing medium (data not shown).

Figure 5 shows the effect of culture conditions on monocyte viability. Supplementation of the medium with rGM-CSF has a positive effect on monocyte viability in cultures of up to 7 days. The addition of rGM-CSF to monocytes cultured in Macrophage-SFM resulted in a comparable or higher total net increase in absorbance values than those cultured in serum-supplemented medium. Endogenous serum appears to provide a suitable concentration of biological response modifiers (BRMs) that are necessary to maintain monocytes in culture. Macrophage-SFM does not contain GM-CSF and for routine long-term culture it is recommended that GM-CSF or other BRMs be added as medium supplements.

DISCUSSION

The results of this study indicate that Macrophage-SFM can perform effectively for routine maintenance of isolated human peripheral blood mononuclear phagocytes. The culture conditions presented offer the advantage that isolated cells are cultured in a defined environment devoid of substances that can either trigger nonspecific stimulation or activation of mononuclear phagocytes or are antagonistic to normal cellular functions. Potentially troublesome factors found in serum such as immunoglobulins, prostaglandins, hormones, various cytokines, high endotoxin levels, and adventitious microbes are not present in this medium, providing an excellent medium to optimize experimentation.

Since the original description of mononuclear phagocytes and their potential immunological role in infectious disease by Metchnikoff (8), researchers have strived to better understand and regulate these cells. A serum-free, defined medium could potentially aid researchers studying the inflammation process because it is devoid of components such as IL-1, TNF- α , prostaglandins, and leukotrienes. These are all

known to be inflammatory mediators (9), and their underlying presence in medium can affect studies in this regard. Similarly, studies monitoring monocyte/macrophage stimulation or activation may be impeded by background excitation levels induced by endotoxin, a known monocyte activation agent (10). Human monocytes/macrophages have also been shown to have increased tumoricidal activity as a result of cytokines, such as interferon- γ (11), M-CSF (12), TNF- α (13), IL-1 (14), IL-2 (15), and IL-4 (16), and hormones (17). Trace serum levels of these BRMs could affect experimentation and data interpretation. The susceptibility of monocytes and macrophages to infection by HIV-1 has accentuated the critical role these cells may play in the pathogenesis of AIDS (18-19). Studies of viral infection and the purification of virus from these cells may be improved through the use of Macrophage-SFM. Also, studies of human monocytoid lineage cells such as U-937 and HL-60 may possibly be improved by evaluating differentiation in a defined environment.

The results of this study indicate that Macrophage-SFM supports human peripheral blood monocyte/macrophage function comparable to conventional serum-based media. Macrophage-SFM permits the cultivation of these cells under defined conditions devoid of potential pathogens and microbial products that can negatively impact experimental studies.

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A NEW REAGENT FOR SIMPLE ISOLATION OF PLANT GENOMIC DNA

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Successful isolation of plant genomic DNA relies on disruption of rigid cell walls to release the DNA and separation of cellular components from the genomic DNA. Use of liquid nitrogen for quick freezing of plant tissues combined with physical grinding of the frozen tissues disrupts cell walls in most plant tissues. Separation of genomic DNA from cellular components is achieved with chemical reagents and centrifugation.

Typically, chemical reagents inactivate enzymes that degrade genomic DNA and denature proteins to release DNA. CTAB (cetyltrimethylammonium bromide) is widely used by plant researchers for DNA isolation (1). However, organic solvents such as phenol and chloroform have to be included with CTAB to achieve satisfactory quality and yield of genomic DNA. Furthermore, CTAB cannot selectively isolate DNA from RNA. RNA contamination in genomic DNA isolated using CTAB requires an additional treatment to eliminate RNA. Here we describe a new chemical reagent—Plant DNAZOL[®] Reagent, a guanidine-detergent lysing solution—that is specifically formulated

for the isolation of genomic DNA from plants. Plant DNAZOL Reagent allows the selective precipitation of DNA from the lysate and hydrolyzes RNA (3). Several applications, including Southern hybridization, PCR, and the AFLP[™] technique, have been used to test the quality of DNA isolated by Plant DNAZOL Reagent.

METHODS

DNA isolation. Plant tissues from tobacco, *Arabidopsis*, rice, maize, and potato were frozen in liquid nitrogen and ground into a light-green powder using a mortar and pestle. 0.1 g of tissue powder was added to a microcentrifuge tube containing 300 μ l Plant DNAZOL Reagent. The solution was mixed thoroughly using a pipette tip and incubated for 5 to 10 min at room temperature with mixing by inversion or mechanical rotation. 300 μ l chloroform (1:1 ratio with Plant DNAZOL Reagent) was added and mixed vigorously for 15 s. For isolation of small amounts of DNA for PCR analysis, the addition of chloroform is optional. After incubation for 5 min at room temperature, the sample was centrifuged at 12,000 $\times g$ for 10 min. The upper aqueous layer was transferred to a clean tube. For *Arabidopsis*, a phenol extraction was performed. The DNA was precipitated with 0.75 volume of 100% ethanol. Then the DNA was collected at 5,000 $\times g$ for 4 min. The pellet was washed with 300 μ l Plant DNAZOL-ethanol wash solution (1 volume Plant DNAZOL Reagent + 0.75 volume 100% ethanol). The pellet was washed with 300 μ l of 75% ethanol and dried by inverting the tube for 5 min. The pellet was dissolved in 100 μ l of TE buffer (pH 8.0) and centrifuged at 12,000 $\times g$ to remove any undissolvable polysaccharides (figure 1). DNA isolation using the CTAB method was based on the protocol of Rogers and Bendich, but without any RNase digestion (2). DNA concentration was determined by fluorescence with Hoechst 33258 (4).

PCR. Amplification was performed with 1 μ g of rice GUS (β -glucuronidase) transgenic

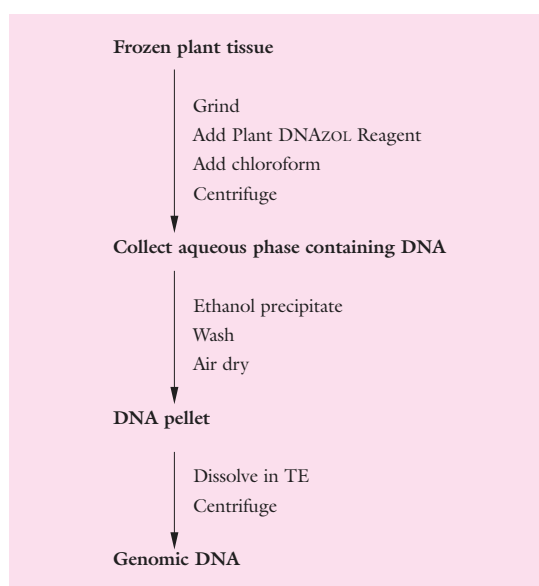


FIGURE 1. Genomic DNA isolation with Plant DNAZOL Reagent.

genomic DNA in 50 μ l of GIBCO BRL PCR SUPERMIX with 0.5 μ M of each GUS primer (AAA CGG CAA GAA AAA GCA GTC and TTA CAT TAC AAG ACG CTG CGA). PCR was 30 cycles at 94°C for 60 s, 55°C for 30 s, and 72°C for 3 min, with a final 72°C for 10 min.

Southern blot. Probe was prepared from pBI 121 plasmid DNA by random priming with [α -³²P]dCTP (3,000 Ci/mmol). 10 μ g of rice genomic DNA was digested with *Bam*H I and *Hind* III, transferred to a BIODYNE® B membrane, and hybridized (5).

AFLP assay. AFLP assays were performed using *Arabidopsis* genomic DNA with the GIBCO BRL AFLP Analysis System II (6).

RESULTS AND DISCUSSION

In plant genomic DNA isolation, RNA contamination is often observed unless an RNase treatment is included. DNA isolated from tobacco, rice, and maize leaves had no detectable RNA contamination using Plant DNAZOL Reagent without the need for RNase treatment. In contrast, heavy RNA contamination was seen using CTAB without RNase treatment (figure 2). The RNA contamination in the plant genomic DNA affects not only the accuracy of quantitative measurement of plant DNA but also the mobility of genomic DNA in gel electrophoresis (see figure 3). In addition with Plant DNAZOL Reagent, removal of polysaccharides was achieved based on the insolubility of polysaccharides in TE buffer (7).

In a broad range of plants, similar amounts of plant genomic DNA were isolated using Plant DNAZOL Reagent and CTAB (table 1). However, it takes 75 min using Plant DNAZOL Reagent, compared with 2.5 h using CTAB. In addition, by scaling up Plant DNAZOL Reagent linearly, >70 μ g potato genomic DNA was isolated from 1 g fresh weight using different growth stages of potato leaves. Also, DNA was isolated successfully with Plant DNAZOL Reagent from different tissues such as stems, roots, callus, and whole plant (data not shown).

Southern hybridization and PCR of genomic DNA are routinely used to check integration of foreign DNA into plants to make transgenic plants. DNA isolated with Plant DNAZOL Reagent performed well in both Southern hybridization (figure 3) and PCR (figure 4). As expected, no DNA bands were seen in genomic

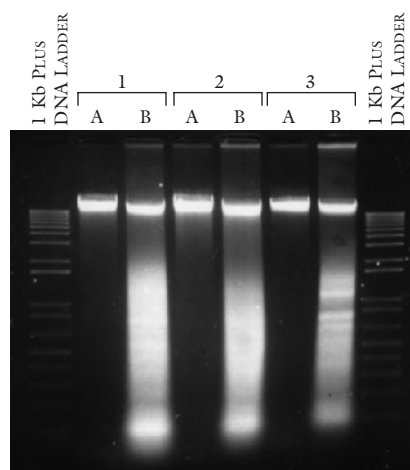


FIGURE 2. Comparison of genomic DNA quality. Genomic DNA was isolated with Plant DNAZOL Reagent (A) or a CTAB (B) from rice, maize, and tobacco (lanes 1–3, respectively). 250 ng of DNA was electrophoresed on an agarose gel.

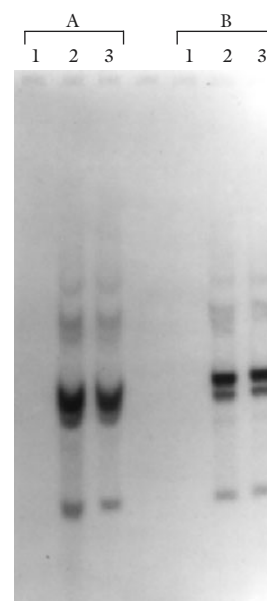


FIGURE 3. Southern hybridization. Genomic DNA isolated using CTAB (A) or Plant DNAZOL Reagent (B). *Bam*H I and *Hind* III digested genomic DNA from nontransformed rice (lane 1) and two different transgenic rice plants (lanes 2 and 3). The probe was to the transformed GUS gene.

TABLE 1. Comparison of genomic DNA yield. Results shown are mean \pm SD for duplicate samples from 3 plants.

	Tobacco (μ g)	Potato (μ g)	Rice (μ g)	Maize (μ g)
Plant DNAZOL Reagent	6 \pm 0.4	12 \pm 0.4	4.8 \pm 0.7	9 \pm 0.5
CTAB	6 \pm 0.7	12 \pm 0.6	4.8 \pm 0.5	10 \pm 0.7

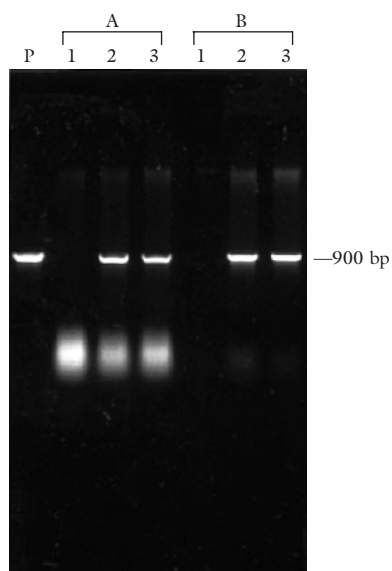


FIGURE 4. PCR products. Genomic DNA was isolated from rice plants or transgenic rice plants using CTAB (A) or Plant DNAZOL Reagent (B). PCR primers were designed to detect the GUS gene transformed into rice plants. PCR products from: plasmid DNA (lane P), nontransformed rice (lane 1), and duplicate samples of a transgenic rice (lanes 2 and 3).

DNA isolated from nontransformed plants. Also, typical AFLP patterns were obtained using DNA purified by Plant DNAZOL Reagent (figure 5).

In summary, genomic DNA isolated with Plant DNAZOL Reagent is high-quality DNA with yields similar to CTAB—but in half the time and without needing RNase treatment. The DNA can be used in many applications, including restriction endonuclease digestions, PCR, Southern blots, and AFLP analysis.

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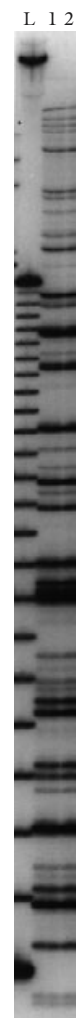


FIGURE 5. AFLP patterns. *Arabidopsis* genomic DNA was isolated using Plant DNAZOL Reagent. AFLP *EcoR* I+AA and *Mse* I+CAG primers were used for selection amplification. AFLP products from duplicate samples of *Arabidopsis* leaves (lanes 1 and 2). Lane L. 30-330 AFLP DNA Ladder.

COMPARING DIGITAL IMAGES TO CONVENTIONAL PHOTOGRAPHS

Conventional photographic cameras are widely used to document data. If the pictures need to be digitally analyzed or distributed, the photographs must be scanned and digitally edited. Digital cameras allow speed and flexibility for documenting data. With a digital camera, the film developing process is eliminated and the images are ready for further analysis and presentation.

Digital cameras rely on a Charge Coupled Device (CCD) to obtain the image from the lens. Light enters the camera lens and is magnified or minimized by the lens. Then the CCD interprets the light waves hitting its surface and converts them into an image through a computer.

Conventional cameras rely on a chemical process. Light enters the camera lens, hits the negative film, and the negative darkens when developed. This results in an image where all of the colors are shifted from the original. This negative is developed onto photographic paper, which reverses the negative and provides the correct colors for the print.

In this paper, we compare pictures of plant tissues and DNA gels taken by a conventional photographic camera to images taken by a digital camera. Furthermore, we demonstrate that the digital camera successfully documented an autoradiograph of AFLP™ results determined by chemiluminescent detection.

METHODS

Plant materials. Potato plants (Atlantic) were kindly provided by Dr. J. Saunders, Beltsville, Maryland. For *in vitro* tissue culture, potato stems were surface sterilized and incubated on Murashige and Skoog (MS) medium either with 0.5 µg/l of zeatine riboside for regeneration of shoots, or with 2 mg/l 2,4-D for callus formation. The plant materials were incubated with 16 h light and 8 h dark for one month. Conventional photographs were taken with a Minolta® X-700 camera and Kodak® Gold ISO 100 film.

Gel electrophoresis. Genomic DNA of *Agrobacterium tumefaciens* C58 was isolated as

described (1). PCR was performed with primers to the 16S RNA gene on 50 ng genomic DNA at 30 cycles of 94°C for 1 min, 56°C for 30 s, and 70°C for 3 min. PCR products were analyzed on a 1% (w/v) agarose gel. The gel images were taken either by a Polaroid® camera or by the Kodak Digital Science EDAS 120 System (Cat. No. 10947). For quantitative analysis, the Polaroid photographs were scanned with an HP ScanJet 4C, and TIF files were analyzed by Kodak Digital Science 1D Image Analysis Software.

AFLP assays. Chemiluminescent detection of AFLP bands was performed as described (2). The autoradiograph was taken by the Kodak Digital Science DC120 Camera.

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FIGURE 1. Images of potato plants and regeneration experiments. Panel A. Conventional photograph. Panel B. Digital image.

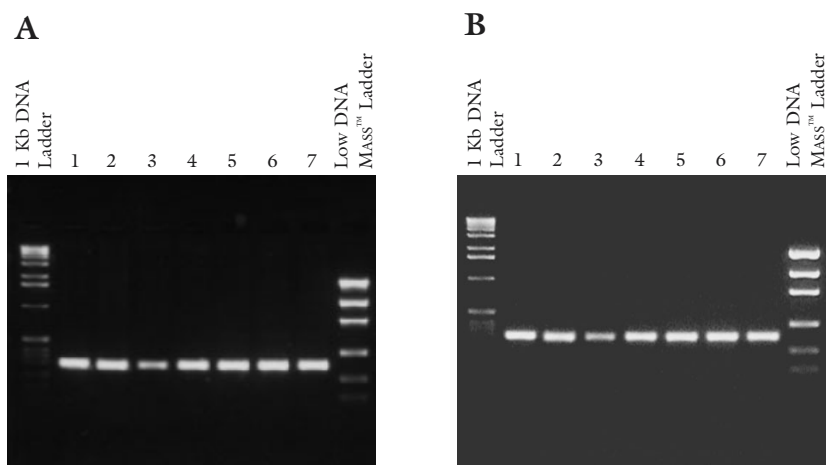


FIGURE 2. Agarose gel. The PCR products were analyzed on a 1% agarose gel and the image taken by: panel A, Polaroid camera or panel B, digital camera.

RESULTS AND DISCUSSION

Color images taken by a conventional camera were compared to digital images of potato plants grown in soil and in tissue culture medium. Both images clearly represented the potato plants (figure 1). In addition, only the desired images

were printed with the digital camera instead of developing multiple photographs to find the best image. Note that the quality of the printer and printing paper is critical to the quality of both printed images.

Besides color photographs of plants, we documented gel images. Images of agarose gels from both cameras were similar (figure 2). In addition, the integration of a computer with the digital camera makes it easier to modify the image background and legend. For further analysis, the images taken by the conventional camera were scanned into a computer. The molecular weight of the PCR products was calculated from both the digital camera image and the conventional camera scanned image, and the results were the same (less than the program's error range) (table 1).

AFLP analysis is a PCR-based DNA fingerprinting technique (1,3) resulting in complex autoradiographs to interpret polymorphisms. Similar AFLP patterns were observed from the images taken by the digital camera (figure 3) compared to scanning the autoradiograph into a computer (data not shown).

In summary, the digital camera system is able to document and analyze diverse images, including color images and gel images. The integration of the digital camera with a computer allows immediate analysis, and there is no need to develop multiple images to get a publishable-quality photograph. Moreover, digital images can be distributed electronically, quickly, and inexpensively.

TABLE 1. Determination of molecular weight of the DNA band in the gel.

Lane	Molecular Weight from Digital Image	Molecular Weight from Conventional Photograph
1	310.3	317.2
2	317.2	317.2
3	324.1	331.0
4	324.1	324.1
5	324.1	324.1
6	317.2	317.2
7	310.3	310.3
Average	318.2 ± 6.2	320.2 ± 6.7

Life Technologies Reaches Out to Local Students

We have two programs to give students interested in careers in biotechnology a chance to get some first hand experience in a life science company. These experiences can sometimes result in co-authorship of scientific manuscripts as in the above article.

For high school students, we offer a limited number of internships that range from clerical to information technology (IT) positions (*e.g.*, Lee Sheng, co-author on manuscript above, is currently interning in IT). For 1998, the internships have been filled by students from the Mark Twain School and Montgomery Youth Works (Montgomery County, Maryland).

For college students, a limited number of co-op positions are available for experience in a research and development laboratory (*e.g.*, Ryan Fleming, co-author on manuscript above, is a recent graduate of University of Maryland and started at Life Technologies as a co-op student).

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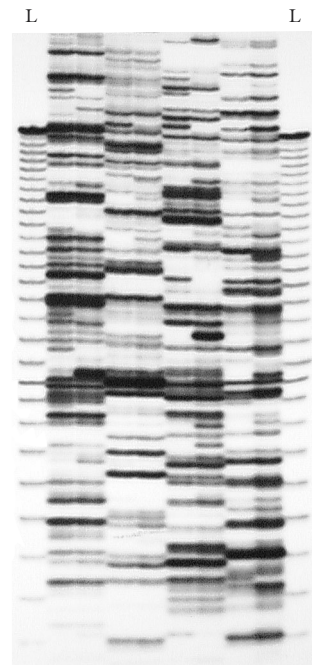


FIGURE 3. AFLP analysis. AFLP was performed with different varieties of rice and detected by chemiluminescence. The image was obtained with the digital camera. Lane L. 30-330 AFLP DNA Ladder.

USE OF ALKALINE PHOSPHATASES IN CLONING

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Alkaline phosphatases are used to dephosphorylate the 5' ends of DNA for radiolabeling with T4 polynucleotide kinase or to prevent self ligation of vector DNA during cloning. Ligation of DNA requires a 5' phosphate (1,2). Dephosphorylated vector DNA cannot self ligate under these conditions while a DNA insert, containing phosphates on 5' termini, can be efficiently ligated into the vector. Therefore, dephosphorylation of vector lowers the number of background colonies containing vector only.

Two commonly used alkaline phosphatases are bacterial alkaline phosphatase (BAP) and calf intestinal alkaline phosphatase (CIAP). A genetically engineered, mutant BAP called TsAP has been developed with the advantage of being heat inactivated at 65°C in the presence of EDTA (3). Although these enzymes catalyze the same reaction and have generally the same substrate specificity, the conditions for optimal removal of 5' phosphates from DNA differ (table 1). For example, BAP has a different unit activity definition, and although all the enzymes are zinc metalloenzymes, only CIAP is reported

to have a requirement for zinc ion in the reaction mixture (1,2). This paper investigates the use of alkaline phosphatases directly in restriction endonuclease digests and subsequent cloning.

METHODS

Alkaline phosphatase activity with purified substrate. pUC19 DNA was linearized with *EcoR* I, *Pst* I, or *Hinc* II to generate 5' overhangs, 3' overhangs, and blunt ends, respectively. The restriction endonucleases were removed by phenol extraction followed by ethanol precipitation. The DNA was dissolved in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]; 1 pmol was diluted in various REACT® buffers (1 through 4); and the alkaline phosphatase [CIAP (Cat. No. 18009), BAP (Cat. No. 18011), or TsAP (Cat. No. 10534)] was added in the recommended amounts prior to incubation (table 1). The alkaline phosphatase was removed by phenol extraction and ethanol precipitation. The DNA was dissolved in TE, split into two 10-µl aliquots for ligation. T4 DNA ligase was added to one tube and distilled water was added to the other (no ligase control). The reactions were incubated 16 h at room temperature (to ensure blunt-end ligations went to completion) to test the extent of 5' dephosphorylation. After the ligation was completed, 15 µl were electrophoresed on 0.8% agarose gels in 1X TAE. The other 5 µl were diluted with 5 µl of TE and used in transformation.

Alkaline phosphatase activity with restriction endonucleases. pUC19 DNA was linearized with restriction endonucleases (1 h, 37°C) in the recommended buffer. Alkaline phosphatase was added directly to the reaction and incubated as shown in table 1. The alkaline phosphatase was removed by phenol extraction and ethanol precipitation. The DNA was dissolved in 20 µl TE, split into two 10-µl aliquots for ligation as described above.

Transformation. One microliter of the diluted ligation reactions (~5 ng of DNA) was transformed into MAX EFFICIENCY® DH10B™ cells (Cat. No. 18297) according to the manufacturer's

TABLE 1. Comparison of reaction conditions.

Reaction Condition	BAP	CIAP	TsAP
Reaction temperature	65°C	37° (5' overhang) 50°C (blunt ends or 5' recessed)	65°C
Reaction time (min)	60 min	30 min (5' overhang) 60 min (blunt, 5' recessed)	15 min
Reaction pH	8.0 8-9.5	8.5 9.4-10.5	8.0 —
Metal ion requirement (Structural cofactor)	Zn ²⁺	Zn ²⁺	Zn ²⁺
Metal ion requirement (Catalytic)	—	—	Mg ²⁺
Inactivated by heating?	No	Probably	Yes
Amount of enzyme used (units)			
5' overhang	70	0.01	0.02
3' overhang (5' recessed)	70	1	0.2
blunt	70	1	0.2

Notes: Reaction conditions for BAP and CIAP vary with the manufacturer. Observations vary about the completeness of the heat inactivation of CIAP. Many researchers remove CIAP by proteinase K and phenol extraction.

recommendation. The efficiency of dephosphorylation was calculated as follows:

$$\text{Efficiency} = \frac{(+\text{AP}+\text{ligase}) - (+\text{AP}-\text{ligase})}{(-\text{AP}+\text{ligase})} \times 100\%$$

$$= \frac{(\text{Dephosphorylated and religated}) - (\text{background})}{(\text{Digested and religated DNA})} \times 100\%$$

Addition of excess alkaline phosphatase. Alkaline phosphatase was added to linearized vector at concentrations of 1X, 10X, and 50-2,800X (1 μ l, undiluted enzyme) with respect to recommended amount per picomoles of ends. The vector DNA was phenol extracted, ethanol precipitated, and dissolved in TE. Insert DNA purified from restriction-endonuclease-digested λ DNA or ϕ X174 was ligated into the vector and transformed into SUBCLONING EFFICIENCY™ DH5 α ™ Competent Cells.

RESULTS AND DISCUSSION

Activity of alkaline phosphatases following restriction digestions. The ability of CIAP, BAP, and TsAP to dephosphorylate 5' ends generated by restriction endonuclease digestion of a purified substrate was examined with and without restriction endonuclease in the most commonly used REACT buffers (table 2). The alkaline phosphatases were able to fully dephosphorylate the various 5' ends in the tested REACT buffers (table 3). These results were the same in the presence or absence (data not shown) of the restriction endonuclease used. Efficiency of dephosphorylation was also measured by decrease in the ability to transform bacterial cells after religation. All alkaline phosphatases showed a dephosphorylation efficiency of 97-99%.

Besides *EcoR* I, *Pst* I, and *Hinc* II; *Acc* I, *Bam*H I, *Sph* I, *Hind* III, and *Sal* I were tested because these enzymes have been reported to interfere with subsequent modifications of digested DNA. The dephosphorylation was complete except for *Sal* I. This inhibition of dephosphorylation occurred with cloned *Sal* I from different manufacturers. Inhibition was overcome by the addition of increased alkaline phosphatase (1 unit CIAP, 1 unit TsAP, and 150 units BAP) (figure 1). These results suggest that in most cases the purification of DNA after restriction endonuclease digestion is not necessary prior to dephosphorylation. However,

TABLE 2. REACT buffers.

Buffer	Tris-HCl (mM)	pH	MgCl ₂ (mM)	NaCl (mM)	KCl (mM)
REACT 1	50	8.0	10	—	—
REACT 2	50	8.0	10	50	—
REACT 3	50	8.0	10	100	—
REACT 4	20	7.4	5	—	50

inhibition can occur. Adding increased alkaline phosphatase, or purifying the DNA before dephosphorylation, will alleviate the inhibition.

Effect of excess alkaline phosphatase. The addition of excess alkaline phosphatase on DNA integrity or the ability to obtain adequate dephosphorylation was investigated. Although it is recommended that researchers calculate the number of ends to be dephosphorylated and add the appropriate amount of enzyme required (table 1), we usually add 1 μ l of enzyme because of the convenience. BAP, TsAP, and CIAP added at 2 times, 50 times, and 2,800 times the recommended concentration (1 μ l enzyme) did not decrease the level of dephosphorylation as measured by religation and cloning. Also,

TABLE 3. Activity of alkaline phosphatases in common REACT buffers. The extent of dephosphorylation was measured by the failure of the DNA to religate in the presence of T4 DNA ligase. Ligated products were detected by visual inspection on an ethidium bromide-stained agarose gel. (+) indicates that no religation was detected.

REACT Buffer	5' Overhang				3' Overhang				Blunt End			
	1	2	3	4	1	2	3	4	1	2	3	4
BAP	+	+	+	+	+	+	+	+	+	+	+	+
CIAP	+	+	+	+	+	+	+	+	+	+	+	+
TsAP	+	+	+	+	+	+	+	+	+	+	+	+

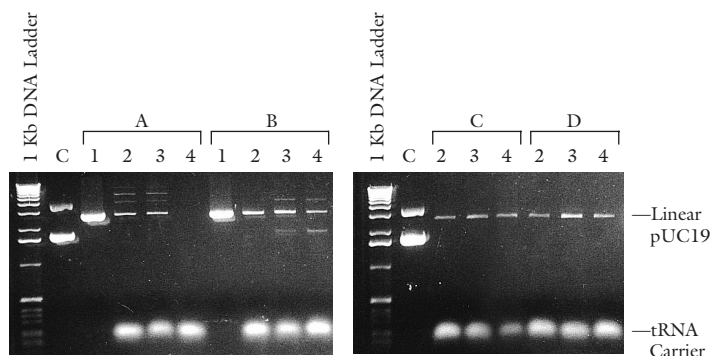


FIGURE 1. Effect of *Sal* I on alkaline phosphatase. pUC19 DNA was digested with *Sal* I (5' overhang); treated with recommended amounts of alkaline phosphatase (panels A and B) or excess alkaline phosphatase (panels C and D; 1 unit CIAP, 150 units BAP, 1 unit TsAP); purified; and religated. Lane C, uncut pUC19; lane 1, pUC19 digested with *Sal* I; lanes 2-4, religated DNA from CIAP, BAP, or TsAP samples, respectively. Panels A and C were one *Sal* I supplier and panels B and D were *Sal* I from a second supplier.

inserts were liberated from mini-prep DNA of transformed λ DNA fragments ligated into dephosphorylated vector DNA using the same restriction endonucleases to clone the insert, confirming the integrity of the ligated termini.

Based on these results, we conclude that alkaline phosphatases can be directly added to many restriction digests containing REACT buffers 1–4 to achieve dephosphorylation of the 5' ends. Furthermore, addition of excessive phosphatase did not negatively affect the reactions.

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LARGE-VOLUME TRANSFORMATION WITH HIGH-EFFICIENCY CHEMICALLY COMPETENT CELLS

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High-efficiency chemically competent cells are used for DNA library construction and transformations with subsaturating amounts of DNA. For these applications, a small aliquot of the transforming DNA is used in a standard 100- μ l transformation reaction. Several transformation reactions are required to generate a representative cDNA library ($\sim 1 \times 10^6$ colonies) or to obtain a sufficient number of clones from a limited amount of DNA. The number of reactions required to generate a sufficient number of clones can be reduced by increasing the volume of each transformation. Since the optimal heat-shock time is dependent on the surface-to-volume ratio of the cell suspension and the thermal conductivity of the tubes (1), the standard heat-shock time of 45 s at 42°C would not be optimal for large-volume transformations. In a previous report (2), it was noted that as the volume of the transformation was increased from 100 μ l to 500 μ l using LIBRARY EFFICIENCY[®] DH5 α [™] Chemically Competent Cells, the optimal heat-shock time also increased. The following paper expands that report's data to include the optimal heat-shock times for large-volume transformations with MAX EFFICIENCY[®] and ULTRAMAX[™] Chemically Competent Cells.

METHODS

Transformations. Chemically competent cells were transformed with subsaturating

(50 pg DNA/100 μ l cells) and saturating (25 ng DNA/100 μ l cells) amounts of pUC19 DNA in 50-ml polypropylene centrifuge tubes (see references 3 and 4 for how the DNA amounts were chosen). After mixing the cells and DNA, the tubes were incubated on ice for 30 min. Transformation volumes of 100, 250, 500, and 1,000 μ l of cells were each heat shocked for 30, 45, 60, 90, and 120 s at 42°C to determine the optimal condition for each volume of cells. The transformations were incubated on ice for 2 min after the heat shock. S.O.C. medium (900 μ l/100 μ l cells) was added and the cells were incubated for 1 h at 37°C while shaking (250 rpm). The transformations were then diluted in S.O.C. medium and plated in duplicate on LB Agar containing 100 μ g/ml ampicillin, followed by incubation for 12 to 16 h at 37°C. The MAX EFFICIENCY DH5 α (3) and DH10B[™] (4) cell transformations were diluted 1:100 for subsaturating conditions and 1:10,000 for saturating conditions; the ULTRAMAX DH5 α -FT[™] (5) reactions were diluted 1:500 and 1:100,000, respectively.

cDNA library construction. The cDNA library was derived from HeLa cell poly(A)⁺ RNA. The SUPERScript[™] Plasmid System (6) was used to generate double-stranded cDNA, which was ligated into pSPORT1. Ligation reactions were pooled before transformations. Transformations with the cDNA ligation [5 μ l (~ 15 ng DNA) ligation/100 μ l cells] were

performed at the optimal conditions determined for the saturating amount of DNA.

RESULTS AND DISCUSSION

Large-volume transformations can be highly efficient when the heat-shock time is increased as the volume of the reaction is increased. For subsaturating transformations performed at the optimal heat-shock condition for each volume, the cells performed at or above the efficiency specification of 1×10^9 transformants/ μg pUC19 DNA for MAX EFFICIENCY cells and 5×10^9 transformants/ μg pUC19 DNA for ULTRAMAX cells (table 1). A sample heat-shock curve is presented in figure 1 for subsaturating DNA.

The total colony output from transformations with saturating amounts of DNA was above the specifications (1×10^6 colonies/100 μl reaction for MAX EFFICIENCY cells and 1×10^7 colonies/100 μl reaction for ULTRAMAX cells) (table 2). The transformations with saturating amounts of DNA generally required a longer heat-shock time for optimal colony output than did those with subsaturating amounts of DNA.

For library construction, the colony output was within the expected range since efficiency is less with ligation mixtures than with purified DNA (table 3). With MAX EFFICIENCY cells, a 1,000- μl transformation was needed to produce sufficient clones for a representative library ($\sim 1 \times 10^6$ CFU); a 250- μl reaction with ULTRAMAX DH5 α -FT cells generated the same number of clones.

In summary, chemically competent cells can be transformed at high efficiency in large-volume reactions. This allows for the complete transformation of ligation reactions (up to 50 μl tested) in a single tube, making it possible to construct a cDNA library with a single large-scale transformation.

ACKNOWLEDGEMENTS

The authors thank Sandy Simms for providing purified HeLa cell poly(A)⁺ mRNA; and Robert Bebee, James Hartley, and Michael Smith for insightful comments and direction.

TABLE 1. Transformation efficiency with a subsaturating amount of DNA. 50 μg of pUC19 DNA was transformed per 100 μl of cells. The optimal heat-shock times are presented for each cell volume. Results are the mean \pm SE for ≥ 4 transformations.

Competent Cell	Cell Volume (μl)	Optimal Heat-Shock Time (s)	Transformation Efficiency (transformants/ $\mu\text{g} \times 10^9$)
MAX EFFICIENCY DH5 α Cells	100	45	2.2 ± 0.2
	250	60	1.04 ± 0.01
	500	90	1.39 ± 0.02
	1,000	90	1.23 ± 0.07
MAX EFFICIENCY DH10B Cells	100	45	1.09 ± 0.08
	250	45	1.0 ± 0.1
	500	60	1.44 ± 0.08
	1,000	60	1.22 ± 0.07
ULTRAMAX DH5 α -FT Cells	100	45	9.3 ± 0.6
	250	60	5.5 ± 0.6
	500	90	7.4 ± 0.2
	1,000	120	5.7 ± 0.3

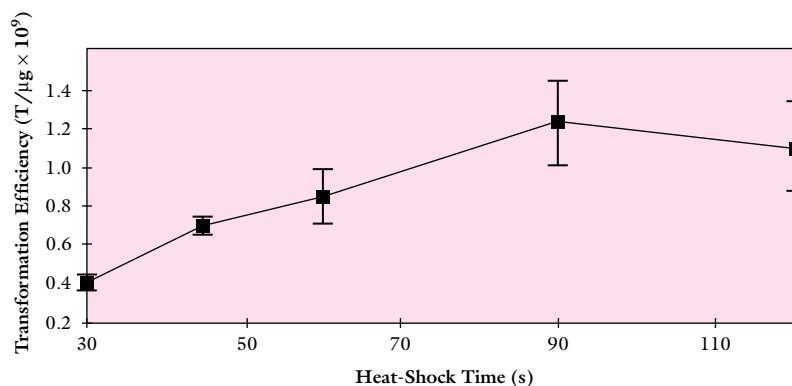


FIGURE 1. A heat-shock curve for subsaturating amounts of DNA. MAX EFFICIENCY DH5 α cells (1,000 μl) were transformed with pUC19 DNA at various heat-shock times. Results are \pm one standard deviation.

TABLE 2. Total colony output for transformations with saturating amount of DNA. 25 ng of pUC19 DNA was transformed per 100 μl cells. The optimal heat-shock times are presented for each cell volume. Results are the mean \pm SE for ≥ 4 transformations.

Competent Cell	Cell Volume (μl)	Optimal Heat-Shock Time (s)	Total Colony Output (CFU/ml $\times 10^6$)
MAX EFFICIENCY DH5 α Cells	100	60	11.1 ± 0.9
	250	90	7.6 ± 0.3
	500	120	10.30 ± 0.07
	1,000	60	5.20 ± 0.06
MAX EFFICIENCY DH10B Cells	100	30	4.9 ± 0.3
	250	60	3.80 ± 0.08
	500	60	4.3 ± 0.2
	1,000	60	5.5 ± 0.3
ULTRAMAX DH5 α -FT Cells	100	60	25 ± 4
	250	90	27 ± 2
	500	90	31.6 ± 0.9
	1,000	120	28.2 ± 0.8

TABLE 3. Total colony output for transformations with cDNA ligations. Results are the mean \pm SE for ≥ 4 transformations.

Competent Cell	Cell Volume (μ l)	Heat-Shock Time (s)	Total Colony Output (CFU/ml $\times 10^5$)	Total Volume (ml)	Total Colonies (CFU/RXN)
MAX EFFICIENCY	100	60	1.34 \pm 0.06	1	1.34 $\times 10^5$
DH5 α Cells	250	90	1.09 \pm 0.03	2.5	2.71 $\times 10^5$
	500	120	0.91 \pm 0.03	5	4.53 $\times 10^5$
	1,000	60	0.67 \pm 0.01	10	6.70 $\times 10^5$
MAX EFFICIENCY	100	30	0.86 \pm 0.05	1	0.86 $\times 10^5$
DH10B Cells	250	60	1.03 \pm 0.07	2.5	2.57 $\times 10^5$
	500	60	1.06 \pm 0.07	5	5.31 $\times 10^5$
	1,000	60	1.0 \pm 0.1	10	10.1 $\times 10^5$
ULTRAMAX	100	60	4.6 \pm 0.3	1	4.6 $\times 10^5$
DH5 α -FT Cells	250	90	4.0 \pm 0.3	2.5	10.0 $\times 10^5$
	500	90	3.6 \pm 0.5	5	18.1 $\times 10^5$
	1,000	120	3.4 \pm 0.6	10	34.3 $\times 10^5$

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The Help Box from Your Technical Support & Training Team

Frequently Asked Questions About Transforming Competent *E. Coli*

Q. How can troublesome DNA inserts be maintained in bacteria?

- A.** For toxic inserts:
 —Supplement the medium with extra nutrients (e.g., add 20 to 30 mM glucose to Terrific Broth)
 —Use a low copy number vector (e.g., pBR322)
 For unstable inserts:
 —Use a strain known to maintain unstable sequences, such as STBL2™ cells
 —Grow cells at 30°C
 —Do not let the cells reach stationary phase in liquid culture

Q. Is S.O.C. required when using competent cells?

- A.** Several media can be used to grow transformed cells, but S.O.C. is optimal for expression before plating the cells. The use of S.O.C. medium is critical for achieving the highest efficiency transformations.

Q. Does methylation of the DNA affect its ability to be cloned?

- A.** Yes. Cells will degrade incoming DNA that has a methylation pattern that is “foreign” relative to that of the host. Several bacterial strains have been modified to accept mammalian methylation patterns. Look for the markers *mcrA*, *mcrBC*, and *mrr* in the bacterial genome when cloning mammalian DNA.

Q. What size DNA inserts can pUC vectors hold?

- A.** DNA inserts can be up to 10 kb in pUC vectors. In general, large inserts are less stable in bacterial strains. For the larger inserts, use a strain designed for stabilizing inserts (such as STBL2 cells). Also, look for the *deoR* marker in the cell's genotype.