Ethanol Precipitation: Ammonium Acetate as an Alternative to Sodium Acetate

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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 miniplasmid 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-µl volume. Each tube contained 1 µl of labeled DNA (1 ng), 10 µl of herring sperm DNA (1 ng/µl, 10 ng/µl, or 100 ng/µl), and 190 µl 10 mM Tris-HCl (pH 7.6), 1 mM Na_2EDTA (TE). To precipitate the DNA, 100 µl of 7.5 M ammonium acetate (0.5 volumes) and 750 µl 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 x g (14,000 rpm) in a fixed angle microcentrifuge at 4°C or room temperature. The supernate was removed and the pellets were rinsed with 200 µl 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 BRL 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 BRL ^{14}C-labeled Protein Molecular Weight Standards. Ammonium acetate was added to a final concentration of 2.5 M to solutions containing 50 µg/ml or 1,000 µg/ml of BSA and 37.5 µg/ml ^{14}C-labeled proteins. These protein solutions contained 1 µg of DNA in a 50 µl volume (20 µg/ml) and were incubated for 0 or 30 min at 0°C or 22°C prior to centrifugation at 16,000 x 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 x 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 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° 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 incubations 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 centrifu-

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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 µg/ml	**85**	**80**	**89**	**91**	**87**	**78**	**91**	**96**	**88**	**94**	**94**	**96**	**88**	**97**	**93**	**100**
0.5 µg/ml	62	46	52	50	57	52	65	**83**	60	58	63	**98**	62	64	65	**92**
0.05 µg/ml	28	29	30	32	35	33	49	69	36	33	38	**92**	47	40	36	**87**
0.005 µg/ml	25	27	38	33	41	38	49	72	37	33	39	**86**	40	35	38	**85**

Note: Data shown in bold type had ≥80% recovery.

Ethanol Precipitation, *continued*

gations, 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 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.

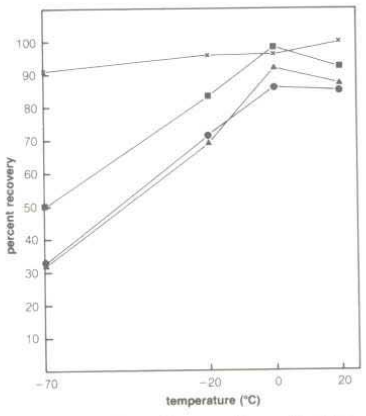

Figure 1. Effect of incubation temperature on ethanol precipitation with ammonium acetate. All solutions were incubated overnight at the designated temperature and centrifuged for 15 min at 22°C. DNA concentrations were 5 μg/ml (X), 0.5 μg/ml (■), 0.05 μg/ml (▲), and 0.005 μg/ml (●).

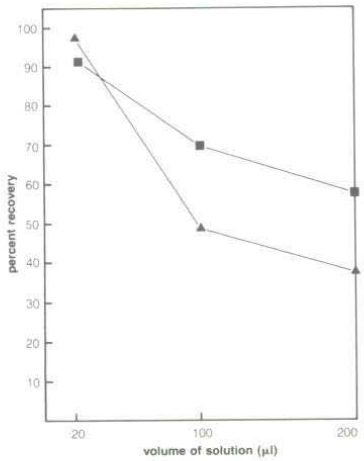

Figure 2. The effect of volume on the recovery of DNA by ethanol precipitation with ammonium acetate. DNA at two concentrations, 0.5 μg/ml (■) and 0.05 μg/ml (▲), was precipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol (at 0°C). Samples were centrifuged for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

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 μM 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 μg or 5 μg of DNA in a 50 or 200-μl 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 μg) 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 μg/ml DNA solution. This concentration is the same as a standard nick translation or restriction digestion (1 μg of DNA in 50 μl). 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 μg/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 x g (14,000 rpm) compared to a horizontal microcentrifuge at 8,800 x g (11,500 rpm) (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 radioactivity 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

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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 ^{14}C-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 x 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.

References:

1. Zeugin, J.A. and Hartley, J.L. (1985) *Focus* 7:4, 1.
2. Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* 65, 499.
3. *Focus* (1982) 4:3, 12.

Making Effective Use of Cloned M-MLV Reverse Transcriptase

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With the introduction of the BRL RNA Ladder, it has become possible to readily assess the ability of reverse transcriptase preparations to synthesize apparent full-length cDNA copies of long heteropolymeric RNA. The components of this RNA molecular weight standard each contain a 3′ poly(A) tail and therefore can serve as templates in cDNA synthesis reactions (1).

A study of BRL's Cloned Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (2,3) was performed with the RNA Ladder components as templates in order to investigate potential problems in cDNA synthesis reactions. I have examined cloned M-MLV RT for properties such as shelf-life, stability at 4°C, sensitivity to inhibition by rRNA and tRNA, activity in different reaction volumes, and sensitivity to the use of reaction conditions that deviate from optima. The results of these experiments are presented here.

Materials and Methods

RNA Templates. RNAs used as templates included the BRL RNA Ladder, which contains components of 0.3, 1.35, 2.4, 4.4, 7.5 and 9.5 kb, each with a 3′ poly(A) tail, and the 7.5 kb component alone.

cDNA Synthesis. Unless indicated otherwise, reaction mixtures (50 µl) contained 10 µl of 5X M-MLV RT Buffer [final concentration of 50 mM Tris-HCl (pH 8.3 at 22°C), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT], 100 µg/ml nuclease-free BSA, 50 µg/ml actinomycin D, 500 µM each of dCTP, dTTP, dGTP and [α-^{32}P]dATP (300 cpm/pmol), 0.5 to 5 µg oligo$(dT)_{12-18}$, 1 to 10 µg poly(A)-tailed RNA, and 200 units of BRL Cloned M-MLV RT per µg of RNA. Incubation was at 37°C for 1 hr. Aliquots were removed to determine specific activity of the labeled nucleotide in the reaction mixture and to determine the amount of acid-insoluble product synthesized. The remainder of the cDNA product was ethanol precipitated and fractionated by electrophoresis on a vertical 1.4% alkaline agarose gel (4). ^{32}P-labeled BRL 1 Kb DNA Ladder was used as the molecular weight standard. The dried gel was exposed to X-ray film for 2 hr at room temperature without a screen. In some cases, an autoradiograph of the dried gel was used as a template to excise portions of the gel. The amount of radioactivity in gel sections was determined by counting Cerenkov radiation.

Results and Discussion

Criteria for Acceptable RT Performance. Based upon evaluation of a number of BRL Cloned M-MLV RT preparations and comparison with avian myeloblastosis virus reverse transcriptase (AMV RT) (2), we have defined acceptable performance standards for M-MLV RT. One criterion is the synthesis of clearly discernible 9.5 and 7.5 kb cDNA, in addition to 1.35, 2.4 and 4.4 kb cDNA, from the BRL RNA Ladder. This criterion is evaluated by alkaline agarose gel electrophoresis. The other criterion is the synthesis of at least 20 ng of full-length 7.5 kb cDNA from 1 µg of 7.5 Kb poly(A)-tailed RNA. Both criteria were used in this study.

M-MLV RT Stability During Storage and at 4°C. Ten different preparations of cloned M-MLV RT stored at −20°C at 200 units/µl for 1 to 24 months were tested for their ability to synthesize 7.5 and 9.5 kb cDNA from the BRL RNA Ladder (figure 1). All 10 preparations performed equally well and synthesized 7.5 and 9.5 kb cDNA. In addition, keeping three of the cloned M-MLV RT preparations which were 10 to 24 months old at 4°C for 7 days had no apparent adverse effect upon the ability of the preparations to synthesize 7.5 and 9.5 kb cDNA from the RNA Ladder (data not shown). These results indicate that purified cloned M-MLV RT has a shelf-life at −20°C in excess of 2 years and is not adversely affected by exposure to 4°C during shipping or manipulations in the laboratory.

Effect of Contaminating RNAs. Preparations of mRNA purified by oligo(dT)-

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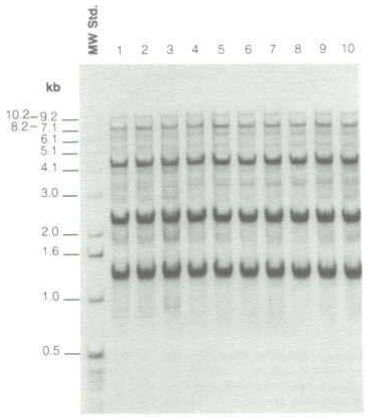

Figure 1. Test of BRL Cloned M-MLV RT stability. The autoradiograph shows cDNA synthesized from the BRL RNA Ladder by different lots of cloned M-MLV RT. All lots were stored at −20°C at a concentration of 200 units/µl. The lanes show the products synthesized by M-MLV RT preparations with the following ages in months: (1) 24, (2) 22, (3) 15, (4) 13, (5) 12, (6) 10, (7) 8, (8) 5, (9) 1, and (10) 7. The molecular weight standard is the BRL 1 Kb Ladder.