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Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity

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ABSTRACT

The ratio of absorbance at 260 and 280 nm (the $A_{260/280}$ ratio) is frequently used to assess the purity of RNA and DNA preparations. Data presented in this report demonstrate significant variability in the RNA $A_{260/280}$ ratio when different sources of water were used to perform the spectrophotometric determinations. Adjusting the pH of water used for spectrophotometric analysis from approximately 5.4 to a slightly alkaline pH of 7.5–8.5 significantly increased RNA $A_{260/280}$ ratios from approximately 1.5 to 2.0. Our studies revealed that changes in both the pH and ionic strength of the spectrophotometric solution influenced the $A_{260/280}$ ratios. In addition, the ability to detect protein contamination was significantly improved when RNA was spectrophotometrically analyzed in an alkaline solution. UV spectral scans showed that the 260-nm RNA absorbance maximum observed in water was shifted by 2 nm to a lower wavelength when determinations were carried out in Na_2HPO_4 buffer at a pH of 8.5. We found RNA $A_{260/280}$ ratios to be more reliable and reproducible when these spectrophotometric measurements were performed at pH 8.0–8.5 in 1–3 mM Na_2HPO_4 buffer.

INTRODUCTION

As early as 1942, Warburg and Christian (15) reported that the $A_{260/280}$ ratio could be used to evaluate nucleic acid contamination during protein purification. The high nucleic acid molar extinction at 260 and 280 nm compared to that of protein made the $A_{260/280}$ ratio a sensitive indicator of nucleic acid contamination. Over the last 20 years, molecular biologists have reversed the original application of the $A_{260/280}$ ratio and have increasingly relied on this determination for a quantitative and qualitative assessment of nucleic preparations. Although most investigators consider the $A_{260/280}$ ratio to be a useful parameter, a number of recent reports have raised concerns about the reliability of these assessments (8–11).

Historically, molecular biology manuals (6,7,13) have stated that an $A_{260/280}$ ratio of 1.8 for DNA or 2.0 for RNA is indicative of a "pure" nucleic acid preparation (i.e., protein free). However, we have noted that simply changing the source of the laboratory water used in these determinations can alter the $A_{260/280}$ ratio of the same RNA preparation. Since factors other than protein must be contributing to these changes, studies were initiated to identify the source of this variability. Data presented in this report demonstrate that the pH and the presence of salts in the solutions used in these spectrophotometric evaluations significantly affect the $A_{260/280}$ ratio as well as the quantitative assessment of nucleic acid preparations.

MATERIALS AND METHODS

RNA was extracted from frozen (-70°C) rat tissues using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) or with previously tested commercial reagents (5). Extractions were performed according to the manufacturer's protocol. In some experiments, RNA was isolated using the single-step method (3) or the CsCl-ultracentrifugation method (2). RNA was solubilized in either diethyl pyrocarbonate (DEPC)-treated water or stabilized formamide [FORMAzol®; Molecular Research Center; (4)] and stored frozen. Before spectrophotometric analysis, RNA samples were thawed and heated for 10 min at 55°C.

Solubilized RNA was diluted in the respective solutions as described in the text and spectrophotometrically analyzed. Absorbance values were measured with a GeneQuant™ RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ, USA) or a Model DR/4000 U Spectrophotometer (Hach Company, Loveland, CO, USA). An appropriate blank solution was used to zero the spectrophotometer. Conductivity and pH measurements were performed with a Model 340 pH Meter (Corning Costar, Cambridge, MA, USA) and Model

HI8033 Conductivity Meter (Hanna Instruments, Woonsocket, RI, USA).

Analysis of Variance and Student-Newman-Keuls' multiple range test were used to evaluate simple treatment effects (14). Treatment means identified with different superscript letters are significantly different (P <.05), and all data are reported as mean ±1 standard error of the mean (SEM).

RESULTS

In an initial experiment, we evaluated the UV absorbance of RNA diluted in water obtained from three different purification systems and from a commercial source (HPLC grade). The results presented in Table 1 reveal significant changes in calculated RNA content when A_{260} measurements were performed in water from these sources. Moreover, the $A_{260/280}$ ratio fluctuated significantly (P <.05). Since the RNA aliquots came from the same sample, protein or other contamination should not have influenced these determinations. In an effort to identify the source of variability in the A_{260} and $A_{260/280}$ ratio, pH and conductivity determinations were performed on the water samples. The analysis revealed differences in both the pH and conductivity of the water samples. As expected, distilled water had an acidic pH. The pH and conductivity of the water samples were inversely related to the calculated RNA content and positively related to the $A_{260/280}$ ratio. These findings suggested that the UV absorbance of RNA might be sensitive to fluctuations in pH or to the presence of minerals in the water.

To evaluate this phenomenon further, Na_2HPO_4 solutions (0.1–10.0 mM) were used as a buffering agent for spectrophotometric determinations of RNA. The resulting pH of the Na_2HPO_4 solutions is depicted on the right ordinate in the top panel of Figure 1. The RNA $A_{260/280}$ ratio increased parallel to the increases in pH and Na_2HPO_4 concentration, with the sharpest increase occurring between pH values 6.0–7.6 and Na_2HPO_4 concentrations 0.01–0.2 mM (top panel, Figure 1). Additional studies on the kinetics of the pH-induced shift in the $A_{260/280}$ ratio indicated that it occurs

Table 1. Effect of Water Purification Method on the Quantitation and $A_{260/280}$ Ratio of RNA

Water Source	Calculated RNA Content (µg/µL)	$A_{260/280}$ Ratio	Conductivity (µS/cm)	pH
A	0.579^{c} ± 0.008	1.973^{a} ± 0.014	6.3	6.10
B	$0.643^{b,c}$ ± 0.005	1.685^{b} ± 0.013	5.5	5.47
C	0.664^{b} ± 0.012	1.565^{c} ± 0.005	2.6	5.31
D	0.697^{a} ± 0.008	1.554^{c} ± 0.011	2.0	5.20

Spectrophotometric analysis of RNA was performed in water purified by: (A) double distillation plus single-column deionization; (B) commercial vendor, HPLC Grade; (C) single distillation plus four-column deionization and (D) single distillation. RNA content was calculated assuming $A_{1\ cm}$/0.1% at 260 nm = 25. Results are presented as mean ± SEM, n = 4. Means identified with the same superscript are not significantly different (P >.05).

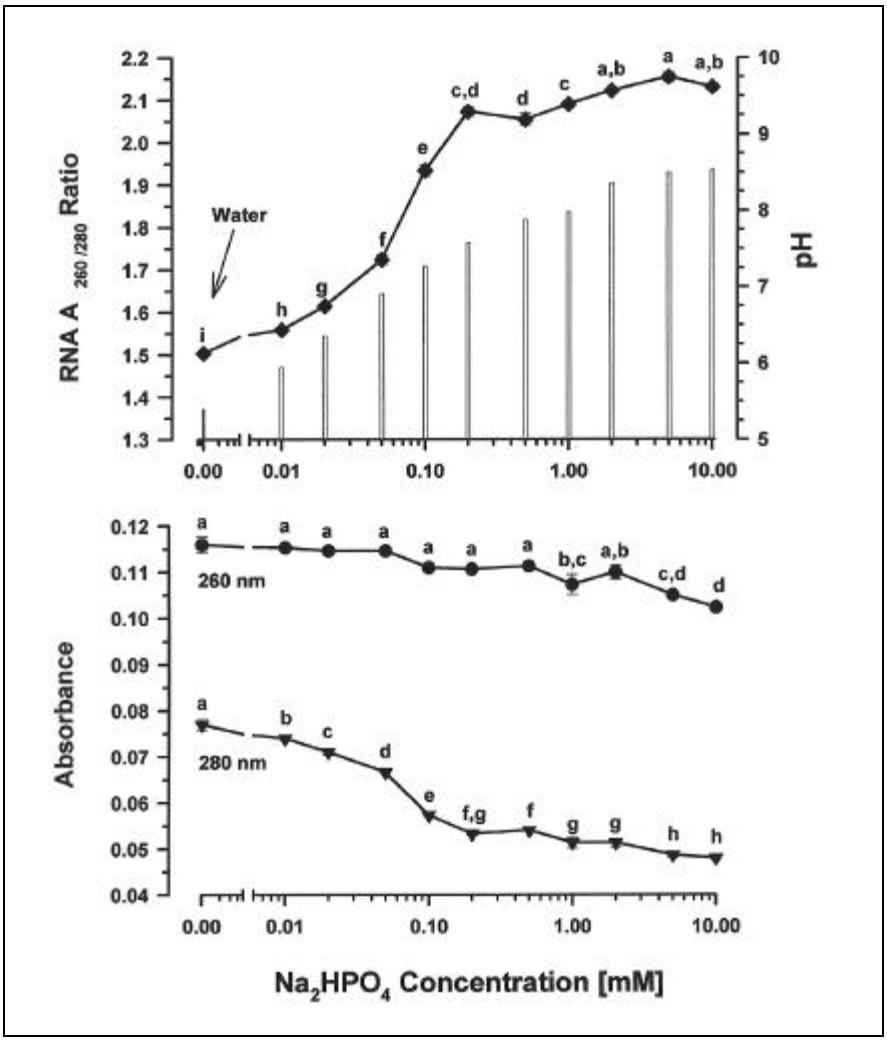

Figure 1. The effect of pH and Na_2HPO_4 concentration on the $A_{260/280}$ ratio and absorbance of RNA. 10 mM of Na_2HPO_4 solution was prepared and diluted as indicated in the Figure. The resulting pH of the diluted buffer is depicted on the right ordinate of the top panel (open bars). The 260 and 280 nm absorbance measured in the presence of the indicated Na_2HPO_4 concentration is presented in the bottom panel. n = 3.

within 10–15 s as the pH is increased from 5.4–8.6 (data not shown). The bottom panel of Figure 1 reveals that no significant difference in the A_{260} was detected at Na_2HPO_4 concentrations ranging from 0–0.5 mM; however, buffer concentrations above 1 mM resulted in small decrements (4%–12%) in absorbance. In contrast, the 280 nm absorbance significantly decreased with incremental increases in Na_2HPO_4 concentration. These results indicate that the increase in the $A_{260/280}$ ratio is primarily due to a pH-dependent decrease in absorbance at 280 nm.

To evaluate the effect of ionic strength on RNA absorbance at a constant pH of 5.4, we performed additional spectrophotometric determinations in NaCl solutions (data not shown). Increasing NaCl concentration from 0.1–1000 mM produced a significant decrease in absorbance at both 260 and 280 nm and incremental increases in the RNA $A_{260/280}$ ratio from 1.533–2.129. This response was not ion-specific since concentration-dependent responses were also obtained with potassium acetate and EDTA solutions. Increases in ionic strength may significantly reduce RNA absorbance, thereby affecting quantitative determinations based on A_{260}.

To determine whether the pH and ionic strength were influencing the ability to detect protein contamination, RNA absorbance was evaluated in the presence of protein (liver powder) using 0.01–10 mM Na_2HPO_4 solutions. The results presented in Figure 2 demonstrate that the effect of proteins on the $A_{260/280}$ ratio of RNA is much more pronounced at pH 8.5 than at pH 6.0. The decrease in the $A_{260/280}$ ratio, following the addition of protein to the RNA, amounted to 7% in 0.01 M Na_2HPO_4 at pH 6.0 vs. the 26% decrease observed in 1.0 mM Na_2HPO_4 at pH 8.5. Identical results were observed when RNA absorbance was evaluated in the presence of human plasma protein (data not shown). These results demonstrate that protein contaminants are more likely to be detected using spectrophotometric solutions in which the pH is maintained at a slightly alkaline range.

To obtain more detailed information on the change in the $A_{260/280}$ ratio, UV spectral scans were performed on RNA diluted in water (pH 5.4), 1 mM Na_2HPO_4 (pH 8.5) and a buffer solution (TNE) containing 10 mM Tris, 1 mM EDTA, 0.2 mM NaCl, pH 7.4. TNE buffer was included in this study since it is frequently recommended for nucleic acid spectrophotometric determinations (6,7). The data obtained from this experiment are summarized in Figure 3. In water, RNA has an absorbance maximum of approximately 260 nm. In the presence of 1 mM Na_2HPO_4 or TNE buffer, the absorbance maximum is shifted to a wavelength of approximately 258 nm, and absorbance is decreased by 5% and 16%, respectively. RNA absorbance is even more reduced relative to water and 1 mM Na_2HPO_4, possibly because of the higher ionic strength of TNE buffer. The shift in the RNA absorbance spectra correlates to the pH and ionic strength of the spectrophotometric solutions used for these determinations.

The data presented in Figure 3 demonstrate that ionic strength and pH can shift the RNA UV absorbance to lower wavelengths. Therefore, it was of interest to determine whether RNA ratios should be computed at wavelengths other than 260 and 280 nm. To address this question, we have computed RNA ratios ranging from $A_{257/277}$–$A_{263/283}$, based on the absorbance data presented in Figure 3. The results are plotted in the inset of Figure 3. Each point represents a difference of 20 nm between two wavelengths. The selection of a lower wavelength for the calculation of the RNA ratio, such as $A_{258/278}$, lowers the absolute value of the ratio relative

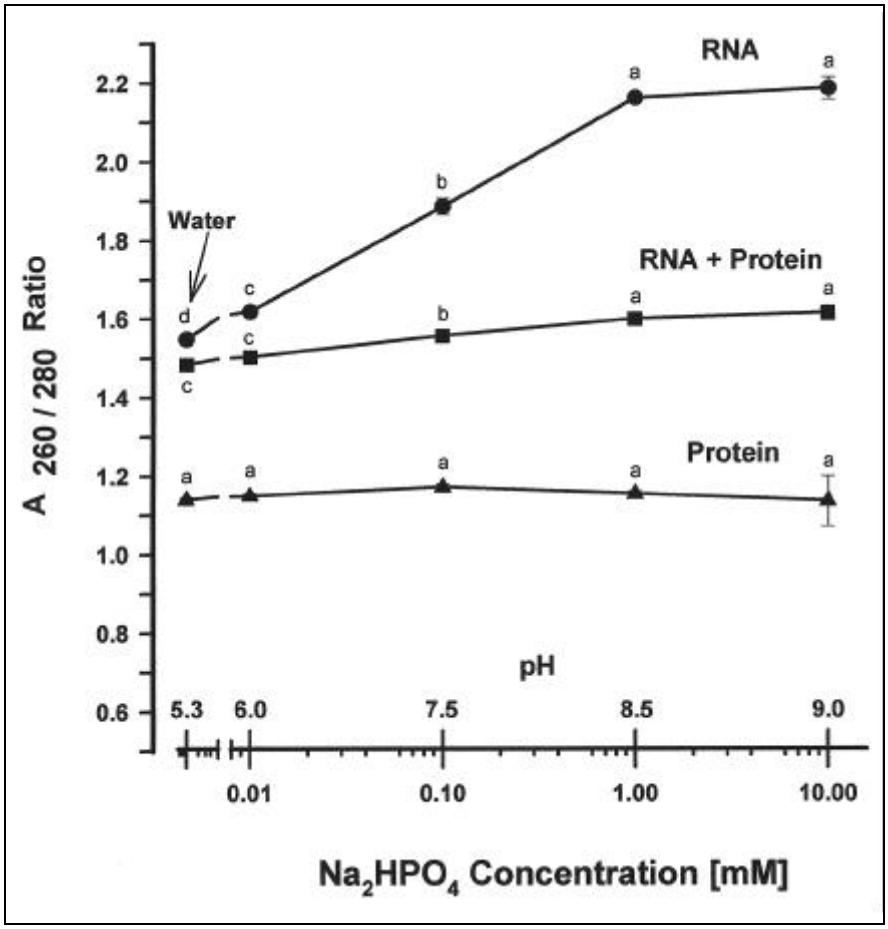

Figure 2. The effect of pH and ionic strength on the $A_{260/280}$ ratio of RNA in the presence or absence of solubilized rat liver powder protein. Rat liver powder was solubilized in water and insoluble protein was removed by centrifugation. Aliquots of RNA (0.62 µg/µL) and protein (0.02 µg/µL) were mixed with Na_2HPO_4 (0.01–10.0 mM), and the absorbance at 260 and 280 nm was spectrophotometrically evaluated. The concentration and pH of the Na_2HPO_4 solution is depicted along the abscissa. $n = 3$.

to that computed at 260 nm, but does not alter the relative differences that were observed among the three treatment groups. Therefore, computing RNA absorbance ratios at wavelengths other than 260 and 280 nm would have little benefit.

In other experiments, the effects of pH and ionic strength were evaluated with RNA isolated by the CsCl-ultracentrifugation method (2), the single-step method (3) or with other commercial reagents (5). The results were the same as those observed for RNA isolated with TRI Reagent. In addition, the magnitudes of the pH- and salt-induced changes in absorbance and the $A_{260/280}$ ratio were identical for RNA solubilized in water or formamide.

Although this study focused on the influence of pH and ionic strength on RNA spectrophotometric analysis, the observed results are also applicable to DNA. The DNA $A_{260/280}$ ratio was consistently approximately 1.4 in water (pH 5.4) compared to 1.7–1.8 in 1 mM Na_2HPO_4 (pH 8.5). The pH and ionic strength of spectrophotometric solutions also have a significant influence on DNA quantitation and the $A_{260/280}$ ratio. Moreover, increasing ionic strength tends to reduce the 260 nm absorbance more than it does for RNA.

DISCUSSION

Although many investigators have raised concerns about the use of the $A_{260/280}$ ratio to assess nucleic acid

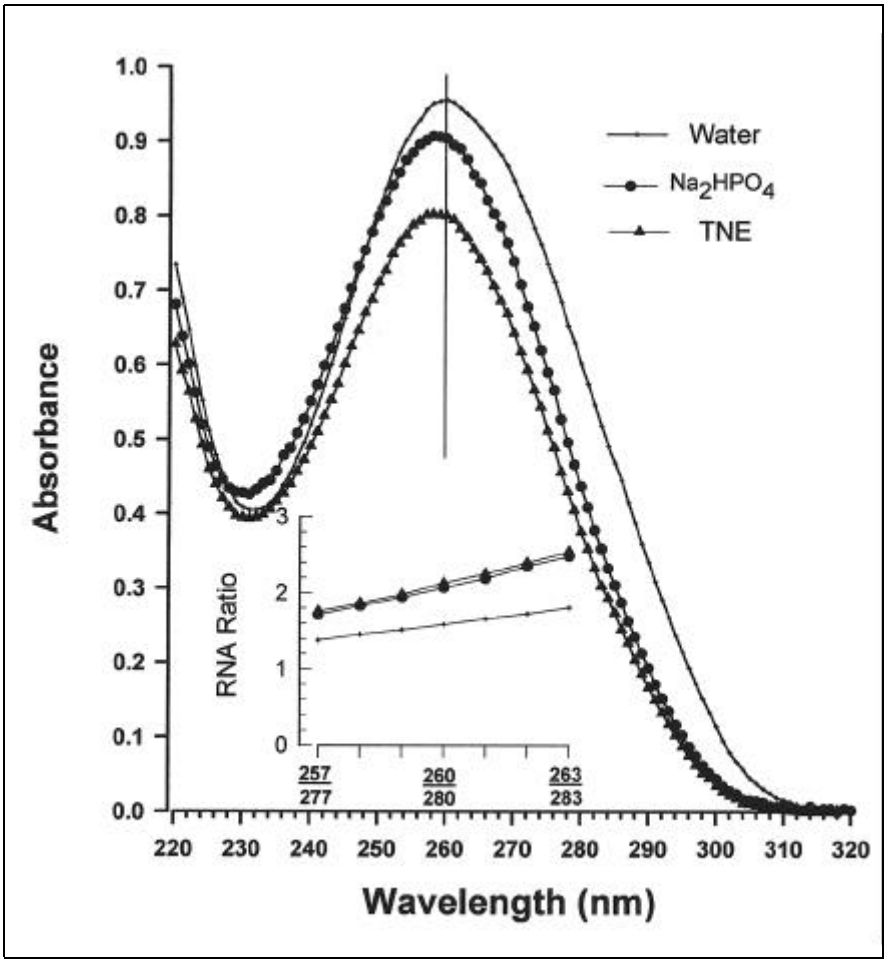

Figure 3. UV absorbance scans of rat kidney RNA analyzed in water (pH 5.4), 1 mM Na_2HPO_4 (pH 8.5) or TNE buffer (pH 7.4). Scans were performed at 1-nm intervals with 36-µg aliquots of RNA. The inset panel depicts the absorbance ratios ranging from $A_{257/277}$ to $A_{263/283}$ computed with RNA data obtained in water, Na_2HPO_4 and TNE. Each point represents a difference of 20 nm between the two wavelengths.

purity (8–11), molecular biologists generally view this simple and straightforward determination as a useful parameter in the evaluation of RNA and DNA preparations (6,7,12,13). The studies outlined in this report demonstrate that the pH and ionic strength of the spectrophotometric solutions significantly alter the $A_{260/280}$ ratio of nucleic acids. We observed that the acidic pH of distilled water used in the spectrophotometric analysis significantly decreases the $A_{260/280}$ ratio of the RNA, thereby confounding the assessment of the quantitative and qualitative characteristics of the preparation. This phenomenon occurred consistently with RNA isolated with different methods and with RNA solubilized in either formamide or water.

It has been known for many years that the resonance structures of pyrimidine and purine bases are responsible for the absorption maxima in the 260–280 nm region of the UV spectra. Although it has been well established that the absorption spectra for nucleic acid bases, nucleosides and nucleotides are strongly pH-dependent because of the degree of ionization of the bases at different pHs (1), this point has not been adequately emphasized in molecular biology literature. The issue is further complicated by the fact that RNA and DNA molar extinction coefficients are computed at a neutral pH. Since the $A_{260/280}$ ratio displays less variability at pH 8.0 than at pH 7.0, it might be useful to evaluate nucleic acid molar extinction coefficients at the higher pH.

The data presented in this report demonstrate that the pH and ionic strength of the solutions used for spectrophotometric analysis can substantially influence the qualitative and quantitative determinations of nucleic acids. Although RNA preparations are routinely solubilized in a variety of solutions, diluents for spectrophotometric determinations should contain sufficient buffering capacity to maintain a pH >8.0. Investigators who use buffers for spectrophotometric analysis of nucleic acids should recognize that the ionic strength and pH of these solutions may influence A_{260} absorbance, and thereby affect quantitative estimates of calculated nucleic acid content. To maintain a slightly alkaline pH, we have found a 1–3 mM Na_2HPO_4 buffer at pH 8.0–8.5 to be a useful solution for the spectrophotometric evaluation of nucleic acids. Na_2HPO_4 buffer is simple to prepare, and unlike Tris-based buffers, it can be treated with DEPC to inactivate RNase. Spectrophotometic analysis of nucleic acids in an alkaline Na_2HPO_4 buffer provides reproducible quantitative results and an increased capability to detect protein contamination compared to water.

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PCR-Based Method for Isolation of Full-Length Clones and Splice Variants from cDNA Libraries

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ABSTRACT

Most cDNA library screening procedures do not distinguish between full-length and incomplete clones and therefore may yield incomplete cDNA fragments. Thus, there is a widespread need for a method allowing the efficient selection of full-length clones. I present a rapid, PCR-based method that allows the simultaneous screening of >10^6 cDNAs. The longest cDNA is identified in the first step so that incomplete clones may be eliminated from study at this stage to save time. The method also facilitates the identification and isolation of rare splice variants from a background of a more abundant variant.

INTRODUCTION

Most cDNA library screening procedures yield cDNA fragments that may be incomplete. For example, polymerase chain reaction (PCR) with degenerate primers, or the two-hybrid system (4), will normally give cDNAs encoding only part of a protein. Database searches for related members of a gene family often identify short expressed sequence tags (ESTs). The standard approach is then to use this fragment as a probe for hybridization to a high-quality library thought to contain a reasonable proportion of long clones. However, cDNAs isolated from libraries by hybridization with a gene-specific probe are often incomplete because of degradation of the template mRNA, incomplete first-strand synthesis, use of internal primers or incomplete methylation of cloning sites. Considerable time is often expended purifying several new clones, only to find that none of them contain the full open reading frame, usually because of a lack of the 5′ end. Therefore, there is a widespread need for a method allowing the efficient selection of full-length cDNAs from a library. Such a method should *(i)* allow the screening of a large number of clones (10^5–>10^6), because full-length cDNAs may be very rare in the library; *(ii)* require only a short fragment or small amount of sequence initially; *(iii)* allow the rejection of shorter clones as early as possible in the procedure; and *(iv)* avoid sub-cloning PCR products (because of low fidelity of *Taq* DNA polymerase). Following is a screening method that meets these criteria.

Table 1. Creating cDNA Pools

1. Transform with library.
2. After recovery at 37°C to establish antibiotic resistance, add glycerol to 15% and store in aliquots at -80°C.
3. Thaw one aliquot and titrate on plates containing LB and ampicillin overnight at 37°C.
4. Set up 100× 3 mL TB and ampicillin in sterile, numbered tubes.
5. Inoculate each TB tube with known numbers of transformed cells [colony-forming units (cfu)] from the frozen aliquots. See below for discussion of the appropriate number of cfu per culture.
6. Shake at 30°C to optical density (OD) 0.1–0.7.
7. Store a sample from each culture by taking an aliquot, adding glycerol to 15% and freezing at -80°C.
8. Arrange the tubes in a 10 × 10 grid.
9. Take half of each culture and combine into 10 pools, corresponding to the rows of the grid. For example, half of each of cultures 31–40 will be combined into pool “row 4” (Figure 1).
10. Take the remaining half of each culture and combine into 10 pools, corresponding to the columns of the grid. For example, the remainder of cultures 10, 20, 30, ..., 100 will be combined into pool “column 10”.
11. Purify DNA from the row and column pools (20 preps). The quality and quantity of DNA required depends on the method of screening to be used.
12. Combine an aliquot of each pool to give a total library pool containing DNA from every clone in the library.

MATERIALS AND METHODS

An aliquot of a cDNA library is titrated and subdivided into smaller aliquots. DNA is purified from pools of these aliquots and screened for the presence of appropriate clones. These procedures are detailed below for a cDNA library in a plasmid vector encoding β-lactamase. Simple modifications can be made to accommodate the use of a wide range of libraries. A method for establishing arrays of phage clones is described in Reference 6.

Creating cDNA pools

Table 1 describes the procedure for creating cDNA pools. The DNA extracted from the row and column pools can now be screened by PCR or South-