Comparison of Methods for Quantitation of Radioactivity in Protected Hybrids in RNase Protection Assays


Several methods exist for the detection and quantitation of RNA, including Northern analysis, in situ hybridization, solution hybridization/RNase protection, dot blot and reverse transcriptase polymerase chain reaction (RT-PCR), and each has unique advantages. The solution-hybridization/RNase-protection assay is a highly sensitive and reproducible method of quantitating levels of specific mRNA transcripts. Its sensitivity offers the advantage of detecting low message levels and permits the use of relatively small amounts of starting tissue. Using the trichloroacetic (TCA) precipitation protocol described here, levels of mRNA lower than 1 pg can be accurately quantitated, allowing for the quantitation of mRNA at levels not detected by other means (9,12).

The solution-hybridization/RNase-protection assay uses a radiolabeled, antisense probe complementary to the RNA transcript to be measured. Total RNA isolated from tissues or cells is hybridized to excess radiolabeled probe, and the solution is treated with ribonucleases specific for single-stranded RNA, leaving double-stranded hybrids intact.

Following RNase digestion, the most widely used protocol for measurement of protected hybrids involves gel electrophoresis followed by autoradiography and densitometry. An alternative method has been described in which hybrids are precipitated with TCA, filtered and analyzed by scintillation counting (1,3,4,6,7). The TCA precipitation method has several advantages. The protocol involves fewer steps and requires significantly less time to perform. A simplification of the experimental procedures reduces the number of steps in which human errors may be introduced. Finally, the ability to assay 100–300 samples in a single assay offers an advantage over gel electrophoresis, which is limited by the number of available wells in the gel; commercially available apparatuses are commonly limited to 40–60 samples.

We conducted studies to compare the TCA precipitation method to the more widely used electrophoresis and autoradiography technique. Solution-hybridization/RNase-protection assays were analyzed in parallel by autoradiography with densitometry assessment and by TCA precipitation with scintillation counting. We also directly analyzed the agarose gels after autoradiography by excising bands from the gels for scintillation counting.

Solution-hybridization/RNase-protection assays were carried out using probes for preproenkephalin (ppEnk) and proopiomelanocortin (POMC) mRNA. We measured ppEnk mRNA in total RNA samples isolated from the guinea pig caudate putamen, and we used the rat POMC probe to measure POMC mRNA from total RNA isolated from the rat anterior pituitary. All transcription templates were cloned in both sense and antisense orientations into the SP6P64 or SP6P65 plasmid vector (Promega, Madison, WI, USA). These vectors have the SP6 RNA transcription promoter located immediately 5’ to a multiple cloning site. The same promoter was used for transcription of both the antisense RNA riboprobes and sense transcript RNA that was used to construct the standard curves. The guinea pig ppEnk template was a 680-bp fragment from the coding region of the gene (8), and the rat POMC template was a 760-bp fragment from the cDNA (a generous gift of Dr. J. Roberts, Mount Sinai Medical Center). Plasmid DNA was isolated using alkaline lysis and centrifugation in cesium chloride, ethidium bromide equilibrium gradients and TCA precipitation (10). Transcription templates were prepared by digestion of plasmid DNA using HindIII for ppEnk and BamHI for POMC, cleaving 3’ with respect to the cloned fragment and leaving a 5’ overhang to prevent snap-back priming. Templates were then treated for 15 min at 37°C with 100 µg/mL proteinase K in the presence of 0.5% sodium dodecyl sulfate (SDS) to inactivate any ribonucleases present, followed by phenol extraction and ethanol precipitation.

The radiolabeled probe was synthesized by the procedure of Melton et al. (11). Reaction conditions were as follows: 500 µM each of rCTP, rUTP and rATP, 8 µM rGTP, 1.25 µM [32P]GTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA, USA), 0.5 µg of linearized plasmid, SP6 RNA polymerase (20 U; Promega), transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 5 mM NaCl), 10 mM dithiothreitol (DTT) and RNase inhibitor (40 U; Promega) in a total volume of 20 µL. Nonradioactive GTP was added to increase the GTP concentration to reduce premature termination of the transcript (11). After a 1 h incubation at 37°C, 10 µg of tRNA (Type XX; Sigma Chemical, St. Louis, MO, USA) were added to aid RNA precipitation, and the plasmid was digested with 1 U of RNase-free DNase I (Promega) for an additional 15 min at 37°C. The reaction product was diluted in 1 mL TSE buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) and phenol-extracted. One volume of 100% ethanol was added to the aqueous phase to bring the solution to 50% ethanol.

Single-stranded RNA was then purified by cellulose chromatography (5). One hundred thirty grams of CF11 cellulose (Whatman, Clifton, NJ, USA) were added to 1 L 0.1 M NaOH, washed 10 times with 1 L distilled (d)H2O and stored in 1 L 2 mM EDTA at room temperature. A Poly-Prep® column (Bio-Rad, Hercules, CA, USA) was cast with a 0.2-mL bed of CF11, and the following washes were carried out: (i) 1 mL 50% ethanol, (ii) 1 mL TSE, (iii) 1 mL 50% ethanol, (iv) 8 mL dH2O and (v) 3 mL 50% ethanol. Following the final 50% ethanol wash, the RNA sample was loaded onto the column, and the flow-through was collected. The column was washed with 3 mL 35% ethanol, and RNA was subsequently eluted with 3x 0.9-mL aliquots of 22% ethanol and 3x 0.9-mL aliquots of dH2O. All ethanol solutions were prepared with TSE and degassed in a vacuum chamber before application to the column. The purification process was monitored by spotting 10-µL samples from each step (input, flow-through, wash and each eluate) onto cellulose filter paper circles (Whatman), adding 4 mL liquid scintillation cocktail and measuring counts per
minute (cpm) in a scintillation counter. Twenty micrograms of tRNA were added to the first 22% ethanol eluate, which contained the majority of the labeled probe. The sample was ethanol precipitated, collected by centrifugation and resuspended in 100 µL dH2O. One microliter of the resuspension was spotted on filter paper and counted. The radiolabeled probe was stored at -80°C and used for solution hybridization either immediately or within 24 h to minimize radiolysis of the 32P probe.

Sense-strand RNA transcripts complementary to the radiolabeled RNA probe were used to generate a standard curve. Concentration of the sense transcripts was determined by absorbance at 260 nm. The standards were then diluted to final concentrations ranging from 0–40 pg per 10 µL in water containing 1 mg/mL tRNA. Ten microliters of each standard were assayed in duplicate for both gel electrophoresis and TCA precipitation for each experiment. Total RNA was isolated from guinea pig or rat tissues by the technique of Chomczynski and Sacchi (2). Ten micrograms of tRNA were added to each tube, and the RNA was precipitated with ethanol, air-dried for 25 min and resuspended in 4 µL dH2O. The samples were prepared for electrophoresis and run on a 1.7% agarose gel as previously described (10) with gel electrophoresis and TCA precipitation. All tubes were vortex mixed, and the precipitated RNA was collected onto glass microfiber filter (Grade 33AH) paper using a cell harvester (both from Brandel Scientific, Gaithersburg, MD, USA). The filters were rinsed with 5% TCA, and the filter was dried for 15 min under a heat lamp. Filter circles were placed into scintillation vials, 4 mL of liquid scintillation cocktail were added and the samples were measured for radioactivity in a scintillation counter.

For gel electrophoresis, 40 µL 10% SDS and 10 µL proteinase K (10 mg/mL) were added to each reaction and incubated at 37°C for 15 min to inactivate the ribonucleases after RNase treatment. All samples were then extracted with 0.4 mL 50% phenol-50% chloroform. Traces of phenol were removed with an additional chloroform extraction. Ten micrograms tRNA were added to each tube, and the RNA was precipitated with ethanol, air-dried for 25 min and resuspended in 4 µL dH2O. The samples were prepared for electrophoresis and run on a 1.7% agarose gel as previously described (10) with the following exceptions: (i) 0.5× MOPS buffer (0.02 morpholinopropanesulfonic acid, pH 7.0, 5 mM sodium acetate, 0.5 mM EDTA) was used for the gel, and (ii) the samples were denatured for electrophoresis by heating for 2 min at 100°C, 20 min at 68°C and an additional 2 min at 100°C. This rigorous treatment was necessary to completely denature the long, perfectly complementary double-stranded RNA hybrids. After electrophoresis, the gel was dried at 80°C under a vacuum for 90 min and then exposed to XAR-5 film (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) for 8 h to overnight.

Densitometric analysis was carried out with a Micro Computer Imaging Device (Imaging Research, Ontario, Canada). The relative optical density (ROD) was determined in areas of identical size containing the fragment to be measured. For the zero values of the standard curve (background level), the ROD was measured in an identical area corresponding to the band position in lanes that contained reactions with no sense transcript. A standard curve was constructed by linear regression, plotting ROD values against known quantities of RNA (0–40 pg). In addition to the single band of the protected hybrid, electrophoresis often revealed smears under the major hybridized species. Since TCA precipitation would include these species, the smears on the autoradiograms beneath the major hybridized species were also analyzed and values obtained were added to values acquired for the major hybridized species to generate data for the whole lane.

For excision and direct counting of gels, an autoradiographic film was used as a template to align the agarose gel. Bands of identical area were cut from each lane containing both standards and samples, and the gel fragments were analyzed by scintillation counting in liquid-scintillation cocktail. The major hybridized species and the adjacent regions under each band were excised and analyzed separately, and values were combined to generate data for the whole lane. A standard curve was constructed by linear regression plotting cpm values against known quantities of RNA (0–40 pg).

Figure 1 displays representative standard curves acquired for the ppEnk probe for each method used. Pearson’s correlation coefficients of nearly one for each standard curve indicate linearity over a wide range of RNA-calibration standards. The standard curves were used to quantitate picogram values of RNA in each tissue sample, and Figure 2A shows the resulting mean values for determinations of ppEnk mRNA. Figure 2B displays the mean values of determinations of POMC transcripts from the rat anterior pituitary. Differences among data generated from each method for each probe were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc tests of significance. No significant differences were found among methods using the ppEnk probe, and only one significant difference was found between the data from densitometry of the major hybridized species and gel excision of the entire lanes for the POMC probe. Since the method requires exacting precision, this difference may be
due to human error during excision of the hybridized species from the gel. To evaluate whether or not this significant difference was consistent across experiments, we performed a two-way ANOVA comparing methods by probes for the ppEnk and POMC studies. This analysis showed no significant differences among methods.

Gel electrophoresis and autoradiographic analyses are useful techniques to determine the size of selected transcripts, which can vary because of alternative splicing, transcription start sites or endonucleolytic cleavage. It is important to analyze the products of RNase protection using electrophoresis to confirm the size of the bands before the use of TCA precipitation to measure incorporated radioactivity in hybrids. The TCA precipitation method, however, has several advantages over electrophoresis, autoradiography and densitometry. TCA precipitation can be carried out on 100–300 samples per assay, whereas the number of samples that can be assayed by electrophoresis is limited to the number of available wells in the gel, usually 40–60 wells, and also the number of gels that can be run simultaneously. Additionally, the protocol for TCA precipitation requires considerably fewer steps, thereby allowing one to complete the analysis in as little as one-tenth the time required to run a gel, expose a film and conduct densitometric analysis or excision and counting analysis.

Simplifying the experimental procedure significantly reduces the number of steps in which human errors may be introduced. Densitometric analysis and gel excision require consistency in the definition and selection of bands. If excision is not performed with precision, errors can also be introduced while cutting the gel. Thus, densitometric analysis and gel excision are both techniques in which the consistency of the data depends upon individual judgments as well as performance. On the other hand, TCA analysis consists of a simple protocol that minimizes human error. We were able to demonstrate in these studies that the various methods of gel electrophoresis and autoradiography, direct gel excision and TCA precipitation are all useful techniques to determine the size of selected transcripts, which can vary because of alternative splicing, transcription start sites or endonucleolytic cleavage. It is important to analyze the products of RNase protection using electrophoresis to confirm the size of the bands before the use of TCA precipitation to measure incorporated radioactivity in hybrids. The TCA precipitation method, however, has several advantages over electrophoresis, autoradiography and densitometry. TCA precipitation can be carried out on 100–300 samples per assay, whereas the number of samples that can be assayed by electrophoresis is limited to the number of available wells in the gel, usually 40–60 wells, and also the number of gels that can be run simultaneously. Additionally, the protocol for TCA precipitation requires considerably fewer steps, thereby allowing one to complete the analysis in as little as one-tenth the time required to run a gel, expose a film and conduct densitometric analysis or excision and counting analysis.

Figure 1. Representative standard curves from five methods of quantitating radioactivity of protected hybrids in RNase protection assays using the guinea pig ppEnk probe. (A and B) Relative optical density values obtained from autoradiographic densitometry. (C and D) Cpm measured from scintillation counting of excised gel fragments. (E) Cpm measured in filtered TCA precipitates. All measurements of radioactivity are plotted against known picogram amounts of sense-standard RNA transcripts. The standard curves were determined by linear regression and have Pearson’s correlation coefficients close to 1.0. Standards were assayed in duplicate.

Figure 2. Comparison of mean values of ppEnk RNA and POMC RNA quantitated by five methods. The five methods used were (I) autoradiographic densitometry of the major protected band, (II) autoradiographic densitometry of the entire lane, (III) gel excision and scintillation counting of the major protected band, (IV) gel excision and scintillation counting of the entire lane and (V) scintillation counting of protected hybrid RNA collected by filtration of TCA-precipitated macromolecules. (A) Mean values ± standard error of the mean (SEM) for ppEnk mRNA measured in the guinea pig caudate putamen. Analysis by one-way ANOVA found no significant differences among data from any group [F(4,5) = 1.03, p = 0.47]. (B) Mean values ±SEM for POMC mRNA measured in the rat anterior pituitary. Statistical analysis by one-way ANOVA, followed by Newman-Keuls Post Hoc test of significance, of the results acquired by each detection method found a significant difference between densitometry of the major protected band (group I) and gel excision of the entire lane (group IV) [F(4,9) = 4.90, p = 0.02]. However, further analysis by two-way ANOVA comparing methods by probes for POMC and ppEnk found no significant difference among any of the five methods [F(4,14) = 2.23, p = 0.12].
tion yielded comparable results in solution-hybridization/RNase-protection assays. This validation of the methods, along with the benefits associated with TCA analysis, will allow investigators to use the TCA precipitation method for mRNA quantitation. TCA precipitation is particularly useful in biological and neurobiological studies of gene regulation in which one is interested in investigating small regions of tissue, such as specific regions of the brain.

REFERENCES


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Sticky-End PCR: New Method for Subcloning


Among various applications of the polymerase chain reaction (PCR), cloning of a PCR product into plasmid vectors is one of the most commonly used. Conventional methods to subclone a PCR product rely on restriction sites introduced by specific primers (1) or the addition of an extra adenine to the 3' end of the product by the terminal transferase activity of Taq DNA polymerase (2). Both methods are limited because: (i) it is not always suitable to cleave a PCR product with the restriction enzyme whose sites match those available in the vector, and (ii) low ligation and transformation efficiency is usually associated with the method adding extra adenine (3). Recently, a ligase-free approach has been reported to overcome the difficulties of these methods (4); however, it can result in a number of wrong products because of the heterologous annealing step and the subsequent long extension step. Furthermore, the ligase-free method involves direct transformation with PCR-generated linear plasmid DNA, and a low yield of transformants was often observed using regular E. coli host cells (3, 4).

We have developed a new method termed “sticky-end PCR”, which generates PCR products bearing cohesive ends compatible with any intended restriction sites. This PCR product can serve as a DNA linker and can be directly ligated into a plasmid vector without restriction cleavage. As illustrated in Figure 1, this method requires four PCR primers and reactions in two separate tubes. After combining, denaturing and annealing both PCR products, about 25% of the final products carry cohesive ends and are ready to be ligated into corresponding sites of the vector. The whole procedure requires only one round of PCR and does not require purification or any other treatments of the PCR products. Therefore, a cloning procedure, including ligation and transformation, can be completed within a day. The method described here is especially useful in cases where cleavage of the PCR product is not feasible as in the presence of internal restriction sites in the amplified sequence. We have successfully used this method in the construction of plasmid vector containing the tandem TAR-RRE gene for human immunodeficiency virus type 1 (HIV-1) gene therapy. To construct the tandem TAR-RRE gene, the conventional approach requires that the TAR-RRE PCR product be cleaved by BglII and BamHI and then introduced into the BamHI site, which is located immediately downstream of the first copy of TAR-RRE in the plasmid vector pLTR-TAR-RRE (unpublished). However, the TAR-RRE gene embeds several internal BglII sites, precluding the use of BglII during cloning. Two pairs of PCR primers differing only in the 5’ ends were designed to amplify the TAR-RRE region (Table 1). The difference in the 5’ termini of the primers matched the overhangs generated by the BglII and BamHI cleavage. PCR was performed in two separate tubes using primer pairs 1 and 3 and 2 and 4, respectively. To prevent the template-independent terminal transferase activity of Taq DNA polymerase, which often resulted in an additional adenine to the 3’ end of the product (2), Vent® DNA Polymerase (New England Biolabs, Beverly, MA, USA) was used in the PCR. The resulting products differ only in the 5’ ends, as illustrated in Figure 1. The two products were then mixed, followed by denaturation and annealing, which resulted in half of the mixed products bearing cohesive ends. Among those bearing cohesive ends, half could serve as BglII/BamHI linkers and were ready for ligation with a pLTR-TAR-RRE vector digested by BamHI. Note that in this example, the BamHI-digested vector was treated by shrimp alkaline phosphatase, and the PCR product was phosphorylated at its 5’ end before the ligation. As a general technique for two different restriction sites, the phosphorylation and dephosphorylation steps can be omitted. In the case of using one sticky- and one blunt-end ligation, only three primers are necessary. It is recommended that the dNTP and Vent DNA polymerase in the PCR mixture be separated from the PCR products by gel or column purification before the mixing and annealing.