Convenient and Rapid Ribonuclease Protection Assay for Use with Primary Cell Cultures

The ribonuclease protection assay (RPA) is a commonly used method for the detection and quantitation of specific RNA species from total RNA preparations. This highly sensitive technique allows for the specific detection of target RNA species and is useful for evaluating large numbers of samples. Most standard RPA protocols recommend using 5–20 µg total RNA that is typically purified. However, when dealing with finite numbers of cells (i.e., primary myeloid leukocytes isolated from whole blood or small tissue sections) or using cells that have intrinsically low mRNA levels [i.e., lymphocytes or anucleated human platelets (4)], current RPA methods become limiting with regard to RNA yield. This aspect, accompanied with potential sample loss occurring through multiple RNA precipitation steps, limits the use of valuable RNA samples for other investigations, particularly if the sample is from an individual with a specific disease. We report here a simple modification of the ribonuclease protection assay that is especially useful for mRNA analysis of primary cell cultures where cell numbers and RNA yield are limiting factors. This convenient adaptation facilitates the rapid handling of large sample numbers through the elimination of multiple RNA precipitation steps without sacrificing the detection of multiple RNA species within a single sample and is of use when assaying changes in transcript levels [i.e., lymphocytes or anucleated human platelets (4)], as previously described (5). Cell disruption and RNA isolation from 1 × 10^6 monocytes, 1 × 10^6 HUVEC, and 1 × 10^6 platelets was accomplished using 1 mL TRIZOL® (Invitrogen, Carlsbad, CA, USA). In the standard RPA method, total RNA is precipitated out of the aqueous phase with isopropanol, brought up into double-distilled water treated with 0.1% DEPC, and quantitated to determine the yield. The typical yield of total RNA using these cell isolation conditions was 5.8 ± 0.5 µg from 1 × 10^6 monocytes, 10.5 ± 0.9 µg from 1 × 10^6 HUVEC, and 6.2 ± 0.5 µg from 1 × 10^6 platelets. Both the sample RNA and low specific activity antisense riboprobes for β-actin, GAPDH, and 28S RNAs were co-precipitated with ethanol as described (1). With the rapid RPA method, riboprobes are directly added to the extracted RNA-containing aqueous phase, and both the sample RNA and probes were co-precipitated with isopropanol; this allows for the quantitative recovery of cellular RNA to be monitored. To maintain equal amounts of RNA between reactions, a fixed volume of aqueous phase (500 µL) was added to the extracted RNA-containing aqueous phase. The riboprobes for human β-actin, human GAPDH, and 28S (276, 404, and 173 nucleotides in length, respectively) were synthesized from pTRI-β-actin, pTRI-GAPDH, and pTRI RNA 28S vectors (Ambion). In vitro transcription was accomplished using the MAXIscript™kit (Ambion) in the presence of [α-32P]UTP (800 Ci/mmol, 10 mCi/mL; ICN Biomedicals, Costa Mesa, CA, USA). To generate low specific activity [32P]-labeled antisense riboprobes for β-actin and GAPDH, the total UTP concentrations were 0.5 mM UTP and 0.6 µM [α-32P]UTP, whereas the total UTP concentration for the 28S RNA riboprobe was 1 mM UTP and 0.3 µM [α-32P]UTP. In the standard RPA method (labeled Std), RNA was precipitated and brought up into DEPC-treated water, and riboprobes to β-actin (5 × 10^4 cpm), GAPDH (5 × 10^4 cpm), and 28S RNA (1 × 10^5 cpm) were added to the sample RNA; both the probe and sample RNA were co-precipitated by adding ammonium acetate to 0.5 M and 2.5 volumes of 100% ethanol. In reactions using the rapid method (labeled Rapid), the riboprobes were added directly to the RNA-containing aqueous phase from TRIZOL extraction and co-precipitated by adding 500 µL isopropanol. The RNA/riboprobe pellet was dissolved into 20 µL hybridization buffer containing 80% formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), 1 mM EDTA, and incubated overnight at 43°C. Subsequent RNase A and T1 digestions were done using the RPA II kit according to the manufacturer’s instructions. The protected fragments for β-actin, GAPDH, and 28S RNAs (245, 316, and 115 nucleotides in length, respectively) were separated on a 6% denaturing polyacrylamide gel and detected by autoradiography. The protected RNA for GAPDH was detected as a doublet of equal intensities. The protected RNA species from total RNA preparations were visualized on autoradiograms with exposure times ranging from 3 h to overnight; exposure times for reactions using 1 × 10^6 platelets were from overnight to 48 h. Quantitation of the relative RNA levels was accomplished by phosphor imager analysis using the Storm™ 860 system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
μL) was maintained. Although not used in this study, the addition of carrier RNA or glycogen can be added to facilitate RNA precipitation and maximize recovery (2). In both methods, the RNA/riboprobe pellet was dissolved into hybridization buffer and incubated overnight at 43°C. Subsequent RNase A and T1 digestions were done according to the RPA Kit (Ambion, Austin, TX, USA), and the protected riboprobe fragments were separated by denaturing PAGE.

As shown in Figure 1, both the standard and rapid methods yielded similar levels of protected riboprobe products for β-actin, GAPDH, and 28S RNAs when using total RNA isolated from 1 × 10⁶ monocytes or HUVECs, and comparable findings were seen between these two methods using 5 × 10⁵ cells (data not shown). It should be noted that, similar to previous findings demonstrating the presence of RNA transcripts in platelets by PCR and northern blot analysis (3,4), we were also able to easily detect β-actin, GAPDH, and 28S RNAs using only 1 × 10⁸ platelets by both RPA methods. It appears that the rapid method is more efficient at retaining low levels of total RNA and, thus, is effective at RNA detection when using low cell amounts. As shown using 1 × 10⁶ platelets (0.06 μg total RNA), the rapid method yielded approximately 1.5-fold greater signals for β-actin and 28S RNAs. Detection of GAPDH mRNA was not deleted under these conditions, demonstrating the limitations of both RPA methods in the detection of moderate-to-low abundance targets when using very low amounts of total RNA. While still maintaining the heightened specificity of the RPAs, these results demonstrate the ability of this simple modification to improve the sensitivity and efficiency of the RPA.

REFERENCES


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An E-Box in pGL3 Reporter Vectors Precedes Their Use for the Study of Sterol Regulatory Element-Binding Proteins

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The analysis of eukaryotic enhancers and promoters is generally performed by using reporter systems such as luciferase, β-galactosidase, or chloramphenicol acetyl transferase genes.

This approach is based on a highly sensitive chemiluminescent or radioactive detection, depending on promoter/enhancer activity. One of the luciferase reporter gene vectors, the pGL3 vector series (Promega, Madison, WI, USA), is very useful in gene regulation studies because of its high sensitivity (2,6). These vectors were improved in many levels compared to the previous generation of luciferase reporter pGL2 vector. However, these luciferase reporter vectors still often generate a relatively high background activity, which is generally due to the interaction of transcription factors with cryptic promoter/enhancer elements located in the vector sequence (3,4,9–11). In this report, we have identified a binding site for the basic helix-loop-helix transcription factor sterol regulatory element-binding proteins (SREBPs) in the multiple cloning site of the promoterless pGL3 luciferase reporter vectors, which can interfere with the interpretation of transfection studies.

To construct the pGL3 ΔE-box vector, double-stranded oligonucleotides corresponding to the multiple cloning site of pGL3 basic vector without SacI, MluI, NheI, and Smal restriction sites (i.e., 5′-CCTCGAGATCTCGAGATCT-3′ and 5′-AGTTACTTGAATCGCGATCTTCGACGTAC-3′) were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), annealed, and ligated to the pGL3 basic vector digested by KpnI/HindIII. Positive clones were screened and sequenced to verify that the E-box was omitted. The pCMV control vector and the pCMV-SREBP-1a and pCMV-SREBP-2 expression vectors were described elsewhere (8) and were co-transfected with pGL3 basic vector or pGL3 ΔE-box. HepG2 cells were maintained in DMEM, supplemented with 10% fetal calf serum, L-glutamine, and antibiotics, at 37°C in a humidified atmosphere of 5% CO₂/95% air. Transfection assays using the CaCl₂ precipitation technique and luciferase assays were carried out as described previously (8). All luciferase activity measurements were normalized to β-galactosidase activity to correct for differences in transfection efficiency, and the values represent the mean of three independent ex-