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rate controls on serial sections for both immunocytochemistry and in situ hybridization. We have found that dextran sulfate, which is used in the prehybridization and hybridization solutions, is notoriously unpredictable, with considerable batch-to-batch variation, and can be at least partially responsible for the artifact, because changing this component solved the problem. Changing the nuclear track emulsion from Kodak NTB-2 to K-5 Nuclear Research Emulsion (Ilford, Paramus, NJ, USA) produced similar results (data not shown). Stains used to visualize cell morphology or to detect horseradish peroxidase activity should also be controlled for nonspecific chemographic artifacts when used together with the nuclear track emulsion (4).

Our results point out the importance of adequate controls for combined immunocytochemistry and in situ hybridization analyses. False-positive signals can be avoided by performing these analyses simultaneously and separately on serial sections. Also, lot controls for key reagents, such as dextran sulfate and stains used for immunocytochemistry, are recommended.

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Received 11 November 1997; accepted 19 January 1998.

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DNase I Treatment of Total RNA Improves the Accuracy of Ribonuclease Protection Assay

BioTechniques 24:732-734 (May 1998)

The ribonuclease protection assay is commonly used to determine the abundance of specific mRNA transcripts from total RNA preparations. Along with evaluating the levels of endogenous gene expression, this highly sensitive assay can be used to assess the transcriptional activity of promoters linked to reporter genes (i.e., luciferase, chloramphenicol acetyltransferase [CAT], β -galactosidase) and elucidate posttranscriptional control mechanisms of transfected cDNAs. Similar to problems seen with quantitative RNA-PCR studies (1,2), false-positive results can be obtained in the ribonuclease protection assay when the total RNA sample is contaminated by genomic or plasmid DNA. We report a convenient method for the complete removal of contaminating DNA from total RNA preparations. This simple modification of the ribonuclease protection assay is especially useful for mRNA analysis of transfected cells that contain high copy numbers of the gene of interest.

To illustrate the effect DNA contamination has on the ribonuclease protection assay, we transfected cells with luciferase cDNA reporter gene plasmids (pGL3 vectors; Promega, Madison, WI, USA) and then analyzed a total RNA preparation by ribonuclease protection assay for luciferase mRNA production and protein expression by measuring luciferase enzymatic activity. The pGL3-Basic plasmid lacking a eukaryotic promoter and the pGL3-Control plasmid containing the simian virus 40 (SV40) promoter and enhancer were transfected separately into COS-7 cells using LIPOFECTAMINE® (Life Technologies, Gaithersburg, MD, USA). Total RNA was isolated with TRIzol® (Life Technologies) following the manufacturer's RNA isolation protocol after 48 h and brought up into diethyl pyrocarbonate (DEPC)-treated H₂O. Each RNA sample (5 μ g) was then incubated at 37°C in

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1× NEBuffer 2 (New England Biolabs, Beverly, MA, USA) with or without 2 U of RNase-free DNase I (Ambion, Austin, TX, USA). Alternatively, any commercial 1× in vitro transcription reaction buffer or a buffer containing 10 mM Mg²⁺ ion (3) can also be used. After 15 min, riboprobe was added, and both the sample RNA and probe were co-precipitated with ethanol. Extraction of the sample RNA from DNase I is not necessary because all subsequent steps involving RNA-RNA interactions are unaffected by DNase I. The RNA/ribo- probe pellet was dissolved into hybridization buffer and incubated overnight at 43°C. Subsequent RNase A and T1 digestions were done according to the RPA II Kit (Ambion), and the protected

riboprobe fragments were separated by polyacrylamide gel electrophoresis. Luciferase enzymatic activity was measured by chemiluminescence-based detection using the Luciferase Assay System (Promega) on a MicroLumat LB96P luminometer (EG&G Berthold, Gaithersburg, MD, USA).

As shown in Figure 1, both the total RNA preparations from transfections of pGL3-Basic and pGL3-Control plasmids yielded a protected luciferase fragment when DNase I treatment was omitted. The apparent presence of luciferase mRNA detected resulted from hybridization of the riboprobe to contaminating plasmid DNA contained in the RNA preparation because the promoterless pGL3-Basic plasmid pro-

duces only trace amounts of luciferase mRNA. To demonstrate that this explanation was correct, we measured luciferase activity from each transfection. As shown, minimal activity was detected from the pGL3-Basic transfection, which demonstrates that detection of RNA was a false positive. Upon treatment of the sample RNA with DNase I, a protected fragment was detected only in the pGL3-Control transfection. Densitometric analysis of the signal derived from mRNA hybridization is approximately equal to the difference in the signals detected from pGL3-Basic and pGL3-Control samples not treated with DNase I (data not shown). These results clearly demonstrate that contaminating DNA in total RNA preparations can result in false-positive signals and that addition of DNase I treatment of the total RNA preparations improves the specificity of ribonuclease protection assays.

Figure 1. DNase I treatment of total RNA preparations eliminates false-positive results in a ribonuclease protection assay. Total RNA isolated from COS-7 cells transfected with either pGL3-Basic or pGL3-Control was incubated with buffer alone (-DNase I) or 2 U of RNase-free DNase I (+DNase I) for 15 min at 37°C; as a control, RNA extracted from nontransfected cells (n.t.) was also included. Then a ribonuclease protection assay was performed by adding 5 × 10⁴ counts per minute (cpm) of ³²P-labeled luciferase antisense riboprobe to the sample RNA, and both the probe and sample RNA were co-precipitated by adding ammonium acetate to 0.5 M and 2.5 vol of 100% ethanol. The radioactive riboprobe (475 nucleotides in length) was synthesized by in vitro transcription of a 381-bp fragment of the luciferase cDNA from pGL3-Basic cloned into pCR®2.1 (Invitrogen, Carlsbad, CA, USA) with T7 RNA polymerase (Ambion) in the presence of [³²P]UTP. The RNA/ribo- probe pellet was dissolved into 20 µL of 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, and the protected fragments were separated on a 6% denaturing polyacrylamide gel and detected by autoradiography. In parallel samples, lysates of cells transfected with the same plasmids were assayed for luciferase activity; enzymatic activity is reported as relative light units (RLU) normalized to protein content.

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Received 26 November 1997; accepted 23 February 1998.

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