Development and Use of Ribonuclease Protection Assays

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Introduction

The objectives of this reciprocation session were to discuss the virtues of a ribonuclease protection assay (RPA) and provide detailed information about how to develop and effectively use an RPA for quantifying mRNA abundance. The ability to determine the level of gene expression in tissue or cells is dependent upon accurately quantifying the abundance of the desired mRNA transcript(s). One traditional approach in molecular biology has been to use Northern blot analysis to quantify the target mRNA. This involves isolating intact total RNA or mRNA and immobilizing the RNA on a solid support (such as nylon membrances) and then hybridizing the blot with a radio-labeled cDNA probe. This assay, however, is not effective when one is attempting to quantify low-abundance mRNA species. For example; in pig adipose tissue, the abundance of the insulin-regulated glucose transporter (GLUT4) mRNA is low and difficult to quantify accurately using Northern blot analysis (Chiu et al., 1994). Thus, other approaches are necessary to quantify target mRNAs that are present in very low abundance. The assay of choice to use when this problem arises is the RPA. In addition, as will be discussed, there are other advantages in using RPA to quantify mRNA levels.

Overview of Ribonuclease Protection Assay

The RPA is an exquisitely sensitive technique for the detection, quantification and characterization of RNA. A significant advantage of this assay is that it offers at least 10 times the sensitivity of Northern blot analysis, and it is not uncommon to realize a 50-fold gain in sensitivity. For example, in our laboratory we have developed RPAs for GLUT4, IGF-1 and the growth hormone receptor mRNAs, and can reliably detect 0.5 pg of target mRNA. In addition, the RPA allows the investigator to use multiple probes in a single assay to quantify different mRNAs. To accomplish this latter objective with Northerns requires repeated cycles of probing, stripping and reprobing blots, with loss of RNA (and, hence, loss of sensitivity and precision) with each cycle. Radio-labeled RNA probes and variations of the RPA are also used in mapping analyses, targeting exon/intron junctions and determining transcription initiation and termination sites.

In RPA, an excess of anti-sense RNA probe is first hybridized with sample RNA (usually total RNA, since the high sensitivity of RPA generally makes poly(A) selection unnecessary). After hybridization, probe not duplexed with target RNA is degraded by treatment with ribonucleases specific for single-stranded RNA. The "protected" duplex fragments are subsequently separated on a gel and exposed to X-ray film. In general, probes are designed to produce protected fragments that range in size from 100 to 600 bases. This size is selected for two reasons: 1) fragments this size can be quickly separated on high-resolution 5% polyacrylamide/8M urea gels and 2) synthesizing labeled anti-sense RNA probes larger than 1000 bases is more problematic than making smaller probes.

A virtue of RPA is that it can be effectively used to quantify multiple transcripts. In order to use RPA in this manner, it is first necessary to demonstrate that each probe will give a single clean band and that little or no background signal is produced by the probe in the absence of specific target. In addition, another important consideration is that when using multiple probes, they should not contain regions of intermolecular complementarity, i.e., regions that will permit probes to hybridize to each other. Using multiple probes clearly increases the quantity and efficience with which data can be generated. Furthermore, experimental variability is reduced by measuring levels of multiple-target mRNAs in the same sample. For example, one of the probes can be used as an internal control to quantify a mRNA species whose levels remain constant, such as ribosomal RNA. This enables the target mRNA transcripts to be normalized against the internal controls.

Important Considerations

There are three areas that are critical for developing a reliable and sensitive RPA: 1) Isolating intact RNA; 2) Synthesizing intact anti-sense RNA probe and 3) The detection analysis. After isolating RNA, it is imperative that all RNA samples be run on a gel and that the 18s and 28s ribosomal RNA species be visualized (by ethidium bromide) to verify that these bands are present and not degraded. Our experience has been that if these bands are not distinct, then the RNA has been degraded to some extent. As a result, the sample(s) should not be used for RPA.

Perhaps the single most important aspect of the RPA is synthesis of intact RNA probe. The details of purifying plas-

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mid DNA, cutting the plasmid with the appropriate restriction enzyme and synthesizing RNA by in-vitro transcription have been described in detail (Molecular Cloning, 1989) and will not be discussed herein. We would like to emphasize, however, that after the restriction enzyme digestion, it is important that the linearized DNA be repurified by running on an agarose gel and re-extracting the DNA. We have found that there are occasions where the in-vitro transcription does not work well if the DNA has not purified after the restriction enzyme digestion. Secondly, it is important that the labeled RNA probe be evaluated by running on a PAGE/8M Urea gel before use. This evaluation simply involves running a fraction of the probe on a gel and then visualizing the probe on film. This is done to verify that the probe is the correct size (i.e., premature cessation of in-vitro transcription did not occur). This precaution also verifies that the probe is not degraded and that the RNA probe synthesized is one distinct species. It is not uncommon when individuals are learning how to synthesize the probe that the gel shows a band at the expected size and then a number of smaller transcripts (i.e. a smear beneath the band). Should this occur, then the in-vitro transcription must be conducted again. The protocol used as a general guide is available from the authors. It is based on several assays that have been described previously (Melton et al., 1984; Starksen et al., 1986; Lowe et al., 1987). The last aspect, that cannot be over-emphasized, is that the RPA is absolutely dependent upon impeccable laboratory skills. RNA is remarkably sensitive to degradation; if one is not attentive, RNAse contamination of the sample can occur.

The typical approach we take in our laboratory in setting up an RPA is first to establish the assay using sense-strand RNA that we synthesize using in-vitro transcription. This offers several advantages. First, a large quantity of RNA (1 mg) can be synthesized and stored, obviating the need to work with RNA extracted from tissues or cells in developing the assay. Secondly, this can be used to establish the standard curve and it also provides a reliable means to determine the sensitivity of the assay. Lastly, the sense-strand RNA can be run (at several concentrations) on the gel with the unknown RNA samples providing the means to directly assess mass of the target mRNA from the internal standard curve.

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