A reliable external control for ribonuclease protection assays
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ABSTRACT

A method is described for generating an external spiked human RNA control to enhance the reliability of assessment of gene expression in tumour extracts. Spiking with an external standard RNA controls for all subsequent steps of analysis on a lane by lane basis and allows for uniform comparison of the gene of interest as a fraction of total RNA, particularly when multiple samples are not available. The antisense probe that is being used to detect endogenous gene expression is also used as an external control. A sense riboprobe is made from the same vector. Because of the flanking RNA polymerase sites incorporated in both probes, hybridization with the sense riboprobe at a much lower concentration than the antisense probe generates a larger product that can be readily separated from the endogenous protected fragment. This method is generally applicable to any riboprobe that has a T3 and T7 RNA polymerase site and allows any externally added riboprobe use for assessing endogenous gene expression to be used as the external spike control.

Ribonuclease protection assays are commonly used to assess quantitative expression of genes in human cancer and relate tumour biology to gene expression. To standardise for unequal sample extraction, measuring levels of an internal control gene co-expressed in the sample offers a means of ensuring that equal amounts of each sample are recovered, as well as equal amounts are loaded and detected during the subsequent biochemical analysis. Variation in the level of the internal control may be due to poor extraction or losses within the experiment after the initial aliquotting of the RNA. This requires that the internal control gene be expressed at a constant level under all conditions, and one used frequently is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1–5). Several problems exist in the use of GAPDH as an internal control, particularly for measuring gene expression in RNA extracted from tumours. GAPDH is known to be regulated in a biphasic manner during the cell cycle where 19-fold variations in mRNA have been observed (6). It is regulated by a number of factors including cellular proliferation (7), hypoxia (8), dihydroxyvitamin D in breast carcinoma cells (9) and by hormones such as dexamethasone in vivo (10).

An external control could be used as an additional way to assess gene expression quantitatively. Spiking with an external standard mRNA after extraction would control for all subsequent steps of analysis on a lane-by-lane basis. Thus, samples with low internal standard gene expression could be due to low expression in the tumour, degradation during extraction or losses in the experiment. Use of the spike control would show if low expression is due to a step subsequent to extraction. The RNA loading is already standardised by spectrophotometry and ethidium bromide staining of RNA samples run on gels first. If the level of the spike control was uniform and the internal control was low this would suggest endogenous variation of the internal control mRNA. Since multiple samples are not available for repeated extractions and comparisons in primary tumours or many human samples, the use of the spike control allows for a uniform comparison of expression of the gene of interest as a fraction of total RNA.

In order to use both the endogenous control gene and the external spike control with the minimum number of different probes we have developed a method that utilises the existence of two RNA polymerases within most vectors. When an antisense riboprobe is transcribed from the DNA construct, in addition to the insert that is transcribed, the vector sequence between the RNA polymerase and the start of the insert is also transcribed. Most common vectors have two RNA polymerases flanking either side of the polycloning site—T7 and T3 RNA polymerases in the case of Bluescript. The first strand of, for example, the GAPDH riboprobe is generated by linearising the Bluescript/GAPDH construct at the 5′ end (or amino-terminus) of the insert with HindIII and transcribing from the opposite end with the T3 RNA polymerase to form an antisense strand for probing endogenous mRNA (a protected fragment size of 120 bases). By linearising the 3′ end of the insert and using the T7 RNA polymerase, a complementary sense strand is formed which hybridises to the first probe generated, and also competes with endogenous GAPDH. If the second riboprobe is designed, not to cut immediately after the insert but several bases further downstream, the additional vector sequence transcribed overlaps with, and is complementary to the first probe generated and thus will also hybridise (Fig. 1). The protected fragment (150 bases) from such a hybridisation satisfies the two main requirements: that it is larger than the endogenous protected fragment, thereby providing a distinct band for quantification (while also assaying and demonstrating variations in the endogenous GAPDH expression at the same time).

This is illustrated in Figure 2 where a series of primary human carcinomas have been analysed for expression of a member of the wingless gene family (wnt4). A subgroup of cases express the

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Figure 1. The generation of an extended sense riboprobe using T7 RNA polymerase to act as an external control. This riboprobe, which has 30 bases more of the Bluescript vector sequence, hybridises to the antisense riboprobe in competition with endogenous GAPDH, resulting in a protected control fragment that is 30 bp longer than the endogenous fragment (Fig. 2).

Figure 2. A ribonuclease protection assay demonstrating the variability of the endogenous GAPDH signal in 28 human tumour samples compared with the constant level of the external control formed from the hybridisation of the GAPDH sense and antisense riboprobes. The upper arrow indicates the protected band for the wtnt4 gene. The middle arrow demonstrates the spike GAPDH control showing uniformity of the protection assay. The lower arrow shows the endogenous GAPDH expression which varies between cancers. The two lanes marked with • had degraded ribosomal RNA.

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