

Analysis of mRNA Expression by Real-Time PCR

Stephen A. Bustin and Tania Nolan

Abstract

The last few years have seen the transformation of the fluorescence-based real-time reverse transcription polymerase chain reaction (RT-PCR) from an experimental tool into a mainstream scientific technology. Assays are simple to perform, capable of high throughput, and combine high sensitivity with exquisite specificity. The technology is evolving rapidly with the introduction of new enzymes, chemistries and instrumentation and has become the “Gold Standard” for a huge range of applications in basic research, molecular medicine, and biotechnology. Nevertheless, there are considerable pitfalls associated with this technique and successful quantification of mRNA levels depends on a clear understanding of the practical problems and careful experimental design, application and validation.

Introduction

The conventional reverse transcription polymerase chain reaction (RT-PCR) (Simpson *et al.*, 1988; Vrieling *et al.*, 1988) is unrivalled as a sensitive assay for the rapid, inexpensive and simple detection of RNA. Importantly, the ability to amplify several templates in a single reaction (multiplexing) (Edwards *et al.*, 1994) has made it a truly high throughput assay (Willey *et al.*, 1998).

However, for a long time quantitative RT-PCR was perceived as a qualitative assay capable of answering yes/no questions only, since even minor variations in reaction components and thermal cycling conditions can greatly affect the yield of any amplified product (Wu *et al.*, 1991). However, the publication of several ground-breaking reports addressing the fundamental theoretical and practical issues associated with quantification (Kelley *et al.*, 1993; Nedelman *et al.*, 1992; Raeymaekers, 1993; Siebert *et al.*, 1992) led to a more general recognition of the inherent quantitative capacity of the PCR assay (Halford *et al.*, 1999). This development was advanced by the obvious need for quantitative data, *e.g.* for measuring viral load in HIV patients (Kappes *et al.*, 1995), monitoring of occult disease in cancer (Bustin *et al.*, 1998) or examining the genetic basis for individual variation in response to therapeutics through pharmacogenomics (Jung *et al.*, 2000).

Conventional quantitative protocols rely either on competitive techniques where known amounts of RT-PCR-amplifiable competitors are spiked into the RNA samples prior to the RT step (Freeman *et al.*, 1999) or on non-competitive methods where the target is co-amplified with a second RNA molecule with which it shares neither the primer recognition sites nor any internal sequence (Reischl *et al.*, 1999). However, the lack of standardisation produces strikingly inconsistent results (Apfalter *et al.*, 2001; Mahony *et al.*, 2000; Smieja *et al.*, 2001), with variable reproducibility (Henley *et al.*, 1996; Souzae *et al.*, 1996), false positive rates as high as 28% (Johnson *et al.*, 1999) and error rates ranging between 10% and 60% dependent on the analysis method (Souzae *et al.*, 1996; Zhang *et al.*, 1997a; Zhang *et al.*, 1997b). Equally as important, conventional quantification protocols

