Optimized Quantitation of Marek’s Disease Virus using Multiplex QPCR

Thermo Scientific ABsolute Blue & ABsolute Fast QPCR master mixes

Introduction

Marek’s disease, an economically-important lymphoid neoplasm of chickens, is caused by oncogenic strains of Marek’s disease herpesvirus (MDV). The virus replicates in lymphocytes and the epithelia of the skin and feather tissues (1). Despite very successful vaccination with attenuated virus strains, vaccine failures do occur as field viruses evolve towards greater virulence. In the Avian Oncogenic Virus Group, led by Professor Venugopal Nair, our research is focused on mechanisms of oncogenesis, genes involved in oncogenicity and increased virulence, and mechanisms of vaccinal protection. Central to these studies is the ability to accurately quantify vaccine and virulent strains of MDV in chicken tissue samples.

Traditionally, MDV has been detected by time-consuming virus isolation from blood samples. We have developed, optimized and validated a sensitive, reproducible QPCR assay for quantitation of MDV in cultured cells and in chicken tissue samples (2). By targeting serotype-specific genes for PCR amplification, we are able to distinguish between the three serotypes of MDV. MDV is a cell-associated virus and thus it is important for us to be able to quantify the virus in the context of the number of chicken cells. To achieve this, we use a duplex QPCR assay to quantify both the chosen MDV target gene (FAM-labelled probe) and the chicken ovotransferrin (Ovo) gene (Yakima Yellow-labelled probe) in a single reaction (2). By using calibrated standard curves for the virus gene reaction and the Ovo gene reaction, we are able to accurately calculate MDV genome copy number per 10^4 chicken cells, permitting meaningful comparison between samples (3, 4).

Methodology

Approximately 100 ng sample DNA was used for each reaction, and samples were run in a 40-cycle PCR on an Applied Biosystems 7500 FAST real-time PCR system. The problem we have experienced with master mixes from other suppliers is that, when running duplex (virus gene/Ovo gene) reactions, the Ovo reaction was partially or fully inhibited in samples having high levels of virus DNA. This resulted in a falsely high value when calculating virus genome copy number per 10^4 cells. Dilution of the samples did not resolve the problem, and primer limitation for the virus gene reaction had a limited effect. Chicken embryo fibroblast cell culture monolayers were infected with a vaccine strain of MDV. At 0, 24, 48, 72, 96 and 120 hours post infection, the cells were harvested and DNA prepared. The samples were run in either duplex QPCR (to detect the virus gene and the Ovo gene) or in singleplex QPCR (to detect the Ovo gene only). For two years we used the machine in ‘standard mode’ but more recently the need to increase sample throughput prompted us to start using the ‘FAST mode. We compared Thermo Scientific ABsolute Blue master mix with a QPCR master mix from an alternative supplier (supplier Y) under standard thermal cycling conditions (Figure 1). In addition, we compared the Thermo Scientific ABsolute Fast QPCR mix with fast master mixes from two other suppliers (supplier W and supplier X) under fast thermal cycling conditions (Figure 2). Cycle threshold (Ct) values were subtracted from 40 (since a 40-cycle PCR was used) to obtain 40-Ct values (eg. Ct value = 5, 40-Ct value = 35), which are proportional to log10 amount of gene product detected.

Singleplex Assay Results

The performance of the master mixes in singleplex reactions was comparable, although reactions using supplier W’s fast master mix were significantly less sensitive. 40-Ct values for the virus gene (blue bars) increased with time in culture, consistent with virus replication. As expected, the level of the chicken Ovo gene, as measured by singleplex reaction (red bars) remained fairly constant (Figure 1 & 2). We chose to use the ABsolute Blue and ABsolute Fast QPCR mixes for all our assays because the blue color increased the contrast between the reagent which helped us to verify pipetting accuracy. (It is true that the blue color aids pipetting accuracy but, for us, that is of secondary importance to the good PCR results. >95% of our reactions are duplex reactions. The singleplex reactions were only run as a comparison to confirm inhibition of the Ovo reaction in duplex reactions).

Duplex Assay Results

Using Thermo Scientific master mixes, in either standard (ABsolute Blue) or fast mode (ABsolute Fast), the level of the Ovo gene measured in duplex reaction was very similar to that measured in...
singleplex reaction, indicating that there was minimal inhibition of the Ovo reactions, even when high levels of virus DNA were present. Conversely, in duplex reactions with master mixes from supplier X or supplier Y, the Ovo reaction was markedly inhibited when the virus gene reaction $40$-Ct exceeded a value of 19. Using master mix from supplier W, the Ovo reaction failed in all samples when run in duplex with the virus gene reaction (Figure 1 & 2).

Summary
We found that in samples containing very high levels of MDV DNA (e.g. Feather tips) the Ovo reaction is often inhibited. Consequently, we prefer to use the ABSolute Blue standard master mix for these samples (Figure 3) since standard thermal cycling conditions tend to favour assays containing high levels of MDV. However, for the vast majority of our chicken tissue DNA samples, which have lower virus levels (for example spleen and blood lymphocytes), we use the ABSolute Fast QPCR master mix.

References: