**PID 4893 – Avipoxvirus Materials and Methods**

***qPCR assay design and validation***

A qPCR assay was designed on the 4b core protein gene of avipoxvirus (AAPV). Two primers (vAAPV-124f ACGTCAACTCATGACTGGCAAT and vAAPV-246r TCTCATAACTCGAATAAGATCTTGTATCG) and an internal hydrolysis probe (vAAPV-159p-FAM-AGACGCAGACGCTATA-MGB, 5´ end, reporter dye FAM (6-carboxyfluorescein), 3´ end, quencher dye NFQMGB (Non-Fluorescent Quencher Minor Grove Binding)) were designed using Primer Express Software (Thermo Fisher Scientific, Carlsbad, CA).The 123 base pair amplicon was entered into Basic Local Alignment Search Tool (BLAST, NCBI) to confirm unique detection. The AAPV assay was validated for efficiency and sensitivity by running a 10-fold standard curve in triplicate off of serial dilutions made from pCR2.1 plasmid DNA (Eurofins Genomics LLC, Louisville, KY) containing the AAPV amplicon. The assay was 94.5% efficient and sensitive enough to detect as few as 10 copies of the target gene.

***Absolute quantitation***

To absolutely quantify the number of copies of AAPV target genes in scab or feather samples, a plasmid standard curve was prepared in triplicate using 10-fold serial dilutions. To determine molecules/l the following formula was used:

Avogadro’s number (6.02x1023 mol) X plasmid concentration (g/µl)

Molecular weight ((plasmid length + insert) X 660 g/mol)

To determine absolute number (*abs*) the following formula was used: Log10((Cq-*y*) ÷ *s*), where *y* is the y-intercept and *s* is slope obtained from a plotted standard curve. To determine number of copies per well the following formula was used: 10*abs* ÷ 2. The copy number per well (1µl DNA) was divided by two since there are two copies of the gene per cell.

***qPCR reaction***

Each qPCR reaction contained 20X primers and probe with a final concentration of 400nM for each primer and 80nM for the probe, commercially available PCR master mix (TaqMan™ Universal PCR Master Mix, Thermo Fisher Scientific, cat #4318157) containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl2, 2.5mM deoxynucleotide triphosphates, 0.625U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 5μl of diluted extracted TNA. qPCR was performed using an automated fluorometer (ABI PRISM 7900 HTA FAST, Thermo Fisher Scientific). The following amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals were collected during the annealing phase and Cq values extracted with a threshold of 0.2 and baseline values of 3-10. A no template control (DEPC-treated water) was run with all assays to ensure absence of non-specific binding of the primers and probes. Positive controls (AAPV plasmid or known positive sample) were run with the assay to ensure the assay was working properly.