

PrimerDesign™ Ltd

# Quantification of Avian adenovirus (Egg Drop Syndrome) genomes

DNA polymerase gene

For general laboratory and research use only

**Advanced kit**





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## Introduction to Avian adenovirus

Avian Adenovirus (Egg drop syndrome), a viral disease of chickens and quail, is characterized by a decrease in egg production accompanied by a reduction in egg quality. This disease is primarily of economic importance, as the birds do not become ill. Avian adenovirus was first described in chickens in the 1970s. The causative virus, Avian adenovirus, has its reservoir in Avians and geese. The initial outbreak in chickens was probably caused by a contaminated Marek's disease vaccine grown in Avian embryo fibroblasts. This virus infected breeding flocks and spread to other flocks through infected eggs. Although it was eradicated from most commercial breeders, Avian adenovirus became endemic in chickens in many parts of the world. Rare outbreaks of Avian adenovirus are also caused by virus transmission from Avians and geese, either directly or through contaminated water. Until recently, Avian adenovirus was thought to be avirulent in Avians and geese. However, in 2001, this virus was isolated from an outbreak of respiratory disease in young goslings, and the disease was reproduced by experimental infection of 1-day old birds.

## Specificity

The *PrimerDesign™ Quantification Kit for Avian adenovirus (EDS) Genomes* is designed for the *in vitro* quantification of all Avian adenovirus. The primers have 100% homology with the avian and duck EDS sequences in the NCBI database and therefore have a very broad detection profile. However, due to the inherent instability of viral genomes, it is not possible guarantee quantification of all clinical isolates.



## Kit Contents

- Pathogen specific primer/probe mix (150 reactions **BROWN**)
- Pathogen positive control template (for Standard curve **RED**)
- Internal extraction control DNA (150 reactions **BLUE**)
- Internal extraction control primer/probe mix (150 reactions **BROWN**)
- RNase/DNase free water

## Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**  
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x Precision™ Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

## Kit storage

This kit is stable at room temperature. Once the lyophilized components have been re-suspended the kit should be stored at -20°C. Unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



## Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

## Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between  $1 \times 10^8$  and  $1 \times 10^2$  copies of target template.



# Principles of the test

## Real-Time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan<sup>®</sup> principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real time PCR platforms.

## Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

## Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



### **Internal DNA extraction control**

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the pathogen target DNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

### **Carry-over prevention using UNG (optional)**

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



## Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Barrier tips are recommended for all pipetting steps.

### 1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

### 2. Reconstitute the kit components according to the table below

To ensure complete reconstitution, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

| Component   | Volume |
|---|--------|
| <b>Pre-PCR box</b>  |        |
| Primer/Probe mix ( <b>BROWN</b> )                             | 165 µl |
| Internal extraction control DNA ( <b>BLUE</b> )               | 600 µl |
| Internal extraction control primer/probe mix ( <b>BROWN</b> ) | 165 µl |
| <b>Post-PCR bottle</b>  |        |
| Positive Control Template ( <b>RED</b> ) *                    | 500 µl |

\* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

## DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

**DO NOT add the internal extraction control DNA directly to the biological sample as this can lead to degradation and a loss in signal strength.**

1. Add 4µl of Internal extraction control DNA (**BLUE**) to each sample in DNA lysis/extraction buffer

2. Complete DNA extraction according to the manufactures protocols



## Real-time PCR detection

### 1. Prepare a reaction mix according to the table below

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

#### Pathogen detection mix

| Component   | 1 reaction   |
|---|--------------|
| 2X Precision™ Mastermix                                       | 10 µl        |
| Pathogen primer/probe mix ( <b>BROWN</b> )                    | 1.0 µl       |
| Internal extraction control primer/probe mix ( <b>BROWN</b> ) | 1.0 µl       |
| RNase/DNase Free water  | 3.0 µl       |
| <b>Final volume</b>   | <b>15 µl</b> |

### 2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

### 3. Prepare sample DNA templates for each of your samples (suggested concentration 5ng/µl) in RNase/DNase free water.

If the concentration of DNA is not known, then dilute your DNA sample reactions 1:20 (10 µl of sample DNA and 190µl of water)

### 4. Pipette 5µl of diluted template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl



## 5. Preparation of standard curve dilution series

- 1) Pipette 900 $\mu$ l of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100 $\mu$ l of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100 $\mu$ l from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

| Standard Curve                         | Copy Number                 |
|--|-----------------------------|
| Tube 1 Positive control ( <b>RED</b> ) | $2 \times 10^7$ per $\mu$ l |
| Tube 2                                 | $2 \times 10^6$ per $\mu$ l |
| Tube 3                                 | $2 \times 10^5$ per $\mu$ l |
| Tube 4                                 | $2 \times 10^4$ per $\mu$ l |
| Tube 5                                 | $2 \times 10^3$ per $\mu$ l |
| Tube 6                                 | $2 \times 10^2$ per $\mu$ l |
| Tube 7                                 | 20 per $\mu$ l              |
| Tube 8                                 | 2 per $\mu$ l               |

## 6. Pipette 5 $\mu$ l of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20 $\mu$ l.



# Amplification Protocol

## Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

|           | Step                               | Time    | Temp |
|-----------|------------------------------------|---------|------|
|           | UNG treatment (if required) **     | 15 mins | 37°C |
|           | Enzyme Activation (if required)*** | 10 mins | 95°C |
| 50 cycles | Denaturation                       | 10s     | 95°C |
|           | <b>DATA COLLECTION*</b>            | 60s     | 60°C |

\* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels

\*\* Required if your mastermix includes UNG to prevent PCR carryover contamination

\*\*\* Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



## Interpretation of Results

| Pathogen | Internal control | Negative control | Positive control | Interpretation |
|----------|------------------|------------------|------------------|----------------|
| +ve      | +ve              | -ve              | +ve              | +ve            |
| +ve      | -ve              | -ve              | +ve              | +ve            |
| -ve      | +ve              | -ve              | +ve              | -ve            |
| -ve      | -ve              | -ve              | -ve              | Exp Fail       |
| +ve      | +ve              | +ve              | +ve              | Exp Fail       |

### Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency and that 1:20 of extracted DNA is used in the reaction, a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. CT values of  $26 \pm 3$  are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.



## Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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