PrimerDesign Ltd

GMO event quantification

Equus caballus Mitochondrial DNA quantification

Detection and quantification of Equus caballus mitochondrial DNA by real-time PCR

Species Specific Detection

detection by real time PCR

Contents

Introduction

Principles of the test

This kit provides a method for detecting Equus caballus mitochondrial DNA that may be present in a food or pharmaceutical sample. The kit is based on the PCR amplification of a unique species specific tag present in the mitochondrial genome of that species. The mitochondrial genome is an ideal target since it has been sequenced for many different species. This allows comprehensive bioinformatics analysis followed by careful design to ensure detection of all strains whilst excluding detection of other closely related species. Furthermore, since there are multiple copies of each mitochondrial genome within each cell, the detection sensitivity for this kit is up to 100 times greater than that of a test which targets a single copy locus within the nuclear DNA genome.

PCR amplification is detected by means of a double dye probe (Taqman® probe) which is degraded during PCR, releasing fluorescence. The fluorescence trace can be used to both detect and quantify the number of copies of Equus caballus mitochondrial DNA present in the sample.

Species specific primers and probe

The kit provides species specific primers and probe that detect the species of interest. The primers and probe are read through the FAM channel and detect the presence of the mitochondrial DNA belonging to that particular species.

The Equus caballus kit has been designed to detect Equus caballus but not Equus asinus (donkey).

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to enquiries@primerdesign.co.uk and our bioinformatics team will answer your question.

Positive control sequences

The kit provides a copy number standard curve so that quantification of the species specific mitochondrial DNA can be performed for your samples.

Internal extraction control

The kit provides internal extraction control primers/probe and template. When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis extraction 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 amplification of the control DNA also indicates that PCR inhibitors are not present at high concentrations.

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 species specific target DNA even when present at low copy number. The internal control primer/probe is detected through the VIC channel and gives a CT value of 28+/-3.

Kit Contents

- Species specific primer/probe mix (100 reactions BROWN)
- Internal extraction control primer/probe mix (150 reactions BROWN)
- Internal extraction control template (BLUE)
- Positive control template (RED)

Reagents and Equipment to Be Supplied by 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.
- Pipettes and Tips
- Vortex and centrifuge
- Thin walled 0.2 ml PCR reaction tubes

Kit Storage

This kit is stable at room temperature but should be stored at -20°C on arrival. Freeze/thawing cycles should be kept to a minimum once resuspended. Under these conditions reagents are stable for six months from the date of resuspension.

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 DNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace test sample DNA with RNAse/DNAse free water.

Licensing Agreement and Limitations of Use

PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of PrimerDesign kits does not include or provide licence with respect to any patents owned by Hoffman-La Roche or others.

PrimerDesign Satisfaction Guarantee

PrimerDesign takes pride in the quality of all our products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, PrimerDesign will replace the item free of charge.

Quality Control

As part of our ISO9001 quality assurance system, all PrimerDesign products are monitored to ensure the highest levels of performance and reliability.

Bench-side Protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting should be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps. The positive control template is a significant contamination risk and should therefore be pipetted after the negative control and sample wells.

1. **Pulse-spin each tube in a centrifuge before opening**
   This will ensure the lyophilized primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Resuspend lyophilised primer and probe mix in RNAse/DNAse free water provided.**
   To ensure complete resuspension, vortex each primer and probe mix thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Species specific Primer/probe mix (BROWN)	110 µl
INT extraction control/probe mix (BROWN)	165 µl
INT extraction control template (BLUE)	500 µl
Foil wrapped	
Positive Control Template (RED) *	500 µl

* This component contains a high concentration of genomic DNA and is a significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

There is a 10% over pipette in each of the primer/probe kits

Perform DNA extraction on test samples.

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

Do NOT add the internal extraction control directly to the unprocessed biological sample as this could lead to degradation and a loss in signal.

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

2. **Complete DNA extraction according to the manufacturer's protocols.**

3. **Prepare complete reaction mixes for samples**
   Reaction mixes will contain the primer/probe mix to detect the species of interest and the internal extraction control primer/probe. Include an additional reaction for the negative control well. Make up the reaction mix according to the volumes in the table below.

Component	1 reaction
PrimerDesign 2X *Precision*® Mastermix	10 µl
Species specific probe mix (BROWN)	1 µl
INT control probe mix (BROWN)	1 µl
RNAse/DNAse free water	3 µl
Final volume	**15 µl**

4. **Dispense 15µl of the reaction mix for each sample according to your plate layout**

5. **Prepare 5 reactions for the standard curve**

Component	7 reaction
PrimerDesign 2X *Precision*® Mastermix	70 µl
Species specific probe mix (BROWN)	7 µl
RNAse/DNAse free water	28 µl
Final volume	**105 µl**

6. **Dispense 15µl of reaction mix for each of the 5 standard curve reactions**

7. **Dispense 5µl of extracted sample DNA according to your plate set up**
   To obtain a strong signal, the ideal concentration of DNA is 1-3ng/µl. The concentration should not exceed 5ng/µl. Substitute sample DNA for RNase/DNase free water as a negative control.

8. Preparation of 1:10 standard curve dilution series of positive control DNA

If you do not require species specific quantification testing then you do not need to run all of the points on the standard curve. However you should always include at least one positive reaction as a control for the PCR reaction set up.

1) Pipette 450µl of RNAse/DNAse free water into 4 tubes and label 2 - 5
2) Pipette 50µl of Positive Control Template (RED) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 50µl 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 (RED)	2 x 10^5 per µl
Tube 2	2 x 10^4 per µl
Tube 3	2 x 10^3 per µl
Tube 4	2 x 10^2 per µl
Tube 5	2 x 10^1 per µl

9. Dispense 5µl of each standard curve dilution according to your plate set up

Amplification Protocol

1. Amplification conditions using Precision® mastermix

	Step	Time	Temp
	Enzyme activation	10min	95°C
	Denaturation	15s	95°C
Cycling x50	DATA COLLECTION*	60s	60°C

*Fluorogenic data should be collected during this step through the FAM and VIC channels.

Interpretation of Results

Internal PCR control

When used according to the above protocols, CT values of 30±3 are within the expected range. When amplifying a sample with a high mitochondrial 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.

Setting a data cut off point for quantitative analysis

The copy number standard curve reports on the number of mitochondrial genomes from the test species present in the sample. It is important to remember that on average there will be 100 mitochondrial genomes for every complete nuclear genome copy in the sample. Results that indicate that there are less than 100 mitochondrial genome copies, as determined from the standard curve, may indicate that there is less than one nuclear genome copy in the PCR reaction.