

PCRmax Ltd TM qPCR test

Equus asinus **Donkey**

Speciation Kit

100 tests

For general laboratory and research use only



Principles of the test

Real-time PCR

This kit provides a method for detecting *Equus asinus* mitochondrial DNA that may be present in a food 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 specific detection of the desired species whilst excluding detection of other 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 hydrolysis probe ("Taqman-style") which is degraded during PCR, releasing fluorescence. The fluorescence trace can be used to both detect and quantify the number of copies of *Equus asinus* mitochondrial DNA present in the sample.

Positive control

The kit provides a positive control template which should be used on every run to prove that your reaction conditions are working correctly. Please note the positive control template poses a significant risk of contamination and should be handled carefully in a separate post PCR environment.

Negative control

To validate any positive findings 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.

Internal 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 Donkey target DNA even when present at low copy number. The Internal control is detected through the VIC channel.

Sensitivity

Under optimal PCR conditions the kit provides exceptional sensitivity. Priming efficiency is guaranteed >95% and the kit can detect less than 100 copies of the target mtDNA. Assuming 50 copies of mtDNA per cell this equates to a detection sensitivity limit of 1-2 muscle cells within a sample.

Specificity

The kit is designed to specifically detect Donkey species that are relevant to the food industry and to give negative detection on other possible animal species.

If you have a query about the detection status of a specific species or sub-species please enquire: enquiry@pcrmax.com

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 0.2U 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.

Kit Contents

- **Equus asinus specific primer/probe mix (BROWN)**
FAM labelled
- **Equus asinus positive control template (RED)**
- **Internal extraction control primer/probe mix (BROWN)**
VIC labelled as standard
- **Internal extraction control DNA (BLUE)**
- **RNAse/DNAse free water (WHITE)**

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

DNA extraction kit

This kit is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

Lyophilised 2x qPCR Mastermix

This kit is designed to be compatible with all commercially available Mastermixes that run with standard cycling conditions.

Pipettors and Tips

Vortex and centrifuge

Thin walled 1.5 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

PCRmax does not recommend using the kit after the expiry date stated on the pack.

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 (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 DNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PCRmax Equus asinus detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period PCRmax 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. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applied Biosystems Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U. S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM® GeneAmp® and MicroAmp® are registered trademarks of the Applied Biosystems (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the PCRmax reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.

Reconstitution 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. Filter 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 in the RNase/DNase free water supplied according to the table below:**
To ensure complete resuspension, vortex each tube thoroughly.

Component	Volume
Pre-PCR pack	
Equus asinus primer/probe mix (BROWN)	110 μ l
Internal extraction control primer/probe mix (BROWN)	165 μ l
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE)	500 μ l
Post-PCR heat-sealed foil	
Equus asinus Positive Control Template (RED) *	500 μ l

* 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 unprocessed biological sample as this will lead to degradation and a loss in signal.

- 1. Add 4 μ l of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**
- 2. Complete DNA extraction according to the manufacturers protocols.**

Real-time PCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below:**
Include sufficient reactions for positive and negative controls.

Component	Volume
Lyophilised 2x qPCR Mastermix	10 μ l
Equus asinus primer/probe mix (BROWN)	1 μ l
Internal extraction control primer/probe mix (BROWN)	1 μ l
RNAse/DNAse free water (WHITE)	3 μ l
Final Volume	15 μl

2. **Pipette 15 μ l of each mix into individual wells according to your real-time PCR experimental plate set up.**

3. **Pipette 5 μ l of DNA template into each well, according to your experimental plate set up.**

To obtain a strong signal, the ideal concentration of DNA is 1-3ng/ μ l. The concentration should not exceed 5ng/ μ l. Substitute sample DNA for RNAse/DNAse free water as a negative control. Substitute sample DNA for positive control template as a positive control.

Amplification Protocol

Amplification conditions using lyophilised 2x qPCR Mastermix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37 °C
	Enzyme activation	2 mins	95 °C
Cycling x50	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

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

** Required if your Mastermix includes UNG to prevent PCR carryover contamination

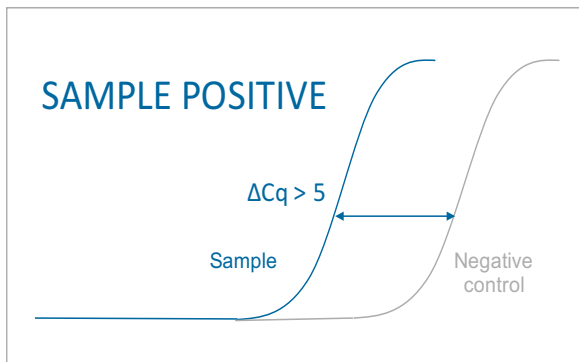
Interpretation of Results

For interpretation when speciation kit used independently

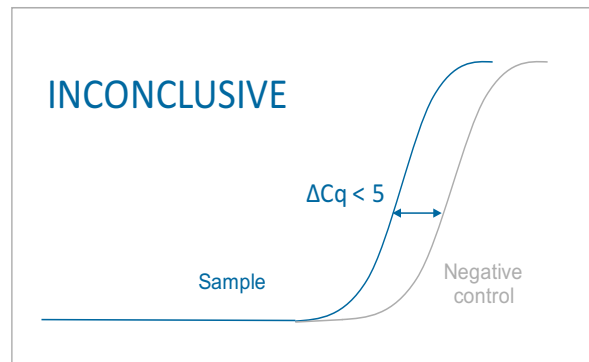
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 35	+ / -	+	-	POSITIVE RESULT
> 35 or -	+	+	-	NEGATIVE RESULT
+ / -	+ / -	+	≤ 35	EXPERIMENT FAILED due to test contamination
+ / -	+ / -	+	> 35	*
> 35 or -	-	+	-	SAMPLE PREPARATION FAILED
+ / -	+ / -	-	+ / -	EXPERIMENT FAILED

Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35 , the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. Cq values of 28 ± 3 are within the normal range. When amplifying a Donkey sample with a 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.

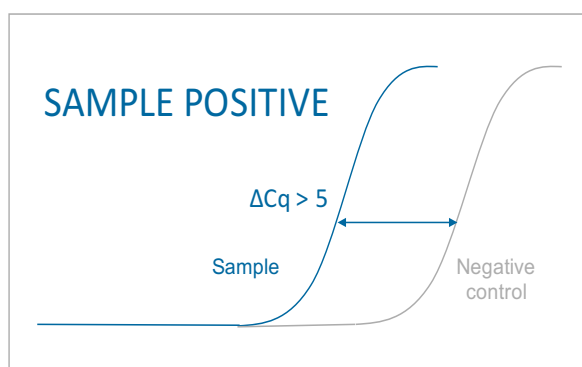
Interpretation of Results

For interpretation when speciation kit used in conjunction with the universal meat or fish kit

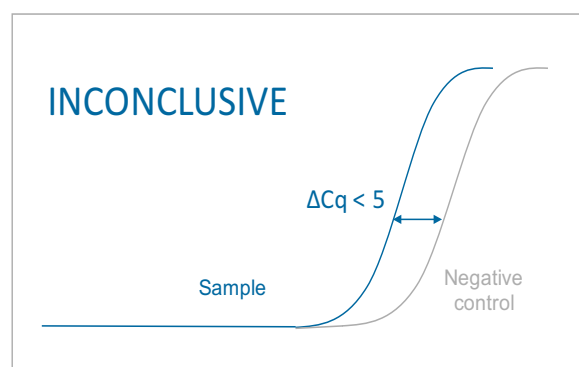
Target (FAM)	Internal control (VIC)	Universal signal (FAM)	Positive control (Target & Universal signal)	Negative control (Target only)	Interpretation
+	+/-	+	+	-	POSITIVE RESULT calculate species % and check test sensitivity
-	+	+	+	-	NEGATIVE RESULT
-	-	+	+	-	SAMPLE FAILED repeat test
+	+/-	-	+	-	SAMPLE FAILED repeat test
+/-	+/-	+/-	+	≤ 35	EXPERIMENT FAILED due to test contamination
+/-	+/-	+/-	+	> 35	*
-	+	-	+	-	NO ANIMAL DNA DETECTED
-	-	-	+	-	SAMPLE PREPARATION FAILED
+/-	+/-	+/-	-	+/-	EXPERIMENT FAILED

Positive control template (**RED**) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised

*Where the test sample is positive and the negative control is positive with a Cq > 35 , the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Calculating species %

(n.b. a Microsoft Excel applet for automatic % calculation is available free of charge. Contact enquiry@pcrmax.com to request)

$$\text{Species \%} = (2^{-(\text{Cq Equus asinus [SAMPLE]} - \text{Cq Equus asinus [+ve control]}) - (\text{Cq Universal meat [SAMPLE]} - \text{Cq Universal meat [+ve control]})}) \times 100$$

Worked example: test gives following Cq values:

Equus asinus test on sample: 23.5

Equus asinus test on positive control DNA: 24.1

Universal meat test on sample: 22.4

Universal meat test on positive control DNA: 22.2

Equus asinus% =

$$(2^{((23.5-24.1)-(22.4-22.2))}) \times 100 =$$

$$(2^{(-0.6 - 0.2)}) \times 100 =$$

$$(2^{-0.8}) \times 100 = \mathbf{57.4\%}$$

n.b. In rare circumstances, some samples may produce a speciation % greater than 100. This is usually due to the presence of PCR inhibition affecting the multiplex reaction and should be reported as 100%. If the reported speciation is greater than 400% then the level of PCR inhibition is likely too great for accurate speciation reporting. Samples such as these should be re-extracted with extra washes to remove PCR inhibitors.

Calculating test sensitivity

The sensitivity of a speciation test is dependant on the amount of DNA that has been successfully extracted from a given sample. The speciation kits have the unique ability to provide information on this sensitivity to empower the user to interpret their data with more precision.

Precise calculations on test sensitivity can be carried out using the Microsoft Excel applet for automatic % calculation that is available free of charge. (Contact enquiry@pcrmax.com to request). But as a rule of thumb the sensitivity of a given test can be estimated based upon the Cq value achieved from the Universal meat primer/probe.

Universal test Cq	Test sensitivity %
$Cq < 19.8$	0.01
$19.8 \leq Cq < 23.2$	0.1
$23.2 \leq Cq < 26.6$	1
$26.6 \leq Cq < 30.0$	10
$30.0 \leq Cq \leq 35.0$	Level of animal DNA is too low for accurate speciation testing
$Cq > 35.0$	Level of animal DNA is too low for analysis to proceed

If the calculated percentage of *Equus asinus* DNA is greater than the calculated test sensitivity then the quantitative result is accurate.

If the calculated percentage *Equus asinus* DNA is less than the calculated test sensitivity then the quantitative result is not accurate and a qualitative positive result equal to the reported % sensitivity should be reported

e.g. If your calculated percentage *Equus asinus* DNA is 1% but the calculated test sensitivity is only 10% then the quantitative result can not be assumed to be accurate. The qualitative result is still true however. i.e. the sample does contain *Equus asinus* DNA. But the percentage can only be assumed less than 10% rather than precisely 1%