PCRmax Ltd ™ qPCR test

Bubalus bubalis Buffalo

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 Bubalus bubalis 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 Bubalus bubalis 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 Buffalo 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 Buffalo 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

- **Bubalus bubalis specific primer/probe mix (BROWN)**
  FAM labelled
- **Bubalus bubalis 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 -20ºC 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 Bubalus bubalis detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

Trademarks

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	
Bubalus bubalis primer/probe mix (BROWN)	110 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE)	500 µl
Post-PCR heat-sealed foil	
Bubalus bubalis Positive Control Template (RED) *	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 unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4µl 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 µl
Bubalus bubalis primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	3 µl
Final Volume	**15 µl**

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

3. **Pipette 5µl 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/µl. The concentration should not exceed 5ng/µl. 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 lyophilsed 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

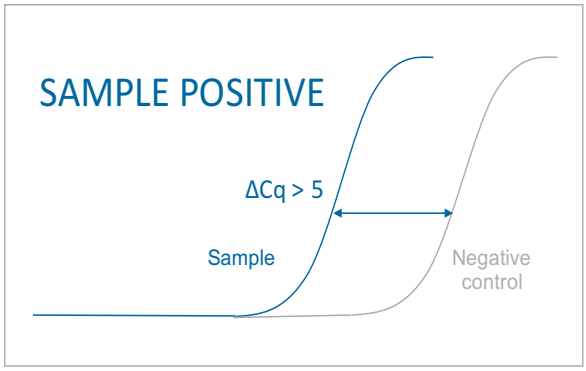
Interpretation of Results

For interpretation when speciation kit used independently

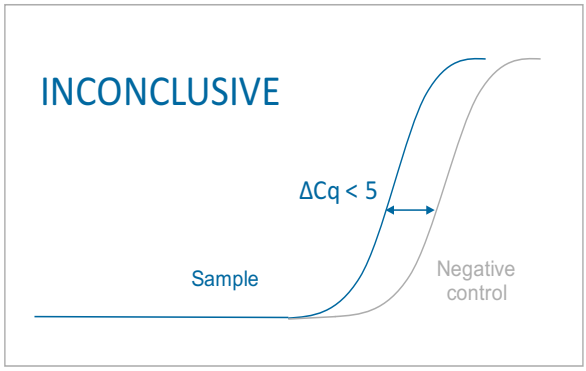
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 35	**+ / -**	**+**	**-**	**POSITIVE RESULT**
> 35 or -	**+**	**+**	**-**	**NEGATIVE RESULT**
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED** due to test contamination
+ / -	**+ / -**	**+**	**> 35**	*
> 35 or -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

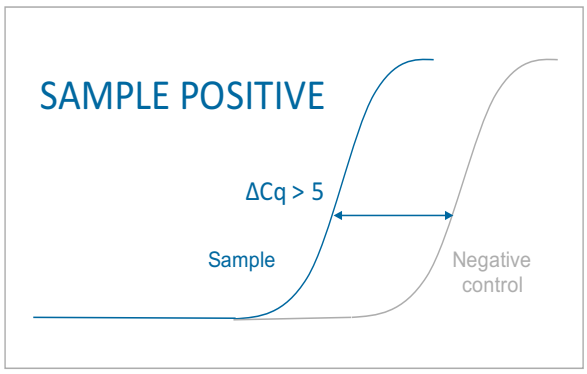
Interpretation of Results

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

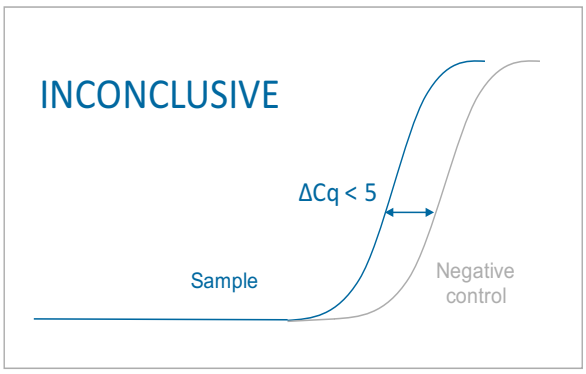
Target (FAM)	Internal control (VIC)	Universal signal (FAM)	Positive control (Target & Universal signal)	Negative control (Target only)	Interpretation
+	+ / -	+	+	-	**POSITIVE RESULT** calculate species % and check test sensitivity
-	+	+	+	-	**NEGATIVE RESULT**
-	-	+	+	-	**SAMPLE FAILED** repeat test
+	+ / -	-	+	-	**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)

Species % = (2^-[(Cq Bubalus bubalis [SAMPLE] – Cq Bubalus bubalis [+ve control]) - (Cq Universal meat [SAMPLE] – Cq Universal meat [+ve control])]) x 100

Worked example: test gives following Cq values:

Bubalus bubalis test on sample: 23.5
Bubalus bubalis test on positive control DNA: 24.1
Universal meat test on sample: 22.4
Universal meat test on positive control DNA: 22.2

Bubalus bubalis% =

(2^((23.5-24.1)-(22.4-22.2))) x 100 =

(2^((-0.6 - 0.2)) x 100 =

(2^ -0.8) x 100 = **57.4%**

n.b. In rare circumstances, some samples may produce a speciation % greater then 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 ≤ Cq < 23.2	0.1
23.2 ≤ Cq < 26.6	1
26.6 ≤ Cq < 30.0	10
30.0 ≤ Cq ≤ 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 Bubalus bubalis DNA is greater than the calculated test sensitivity then the quantitative result is accurate.
If the calculated percentage Bubalus bubalis 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 Bubalus bubalis 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 Bubalus bubalis DNA. But the percentage can only be assumed less than 10% rather than precisely 1%