Primerdesign™ Ltd

OnDemand

Advanced Kit

(Incl. DNA, RNA and Speciation)

Handbook

ONDEMAND

For Research Use Only. Not for use in diagnostic procedures

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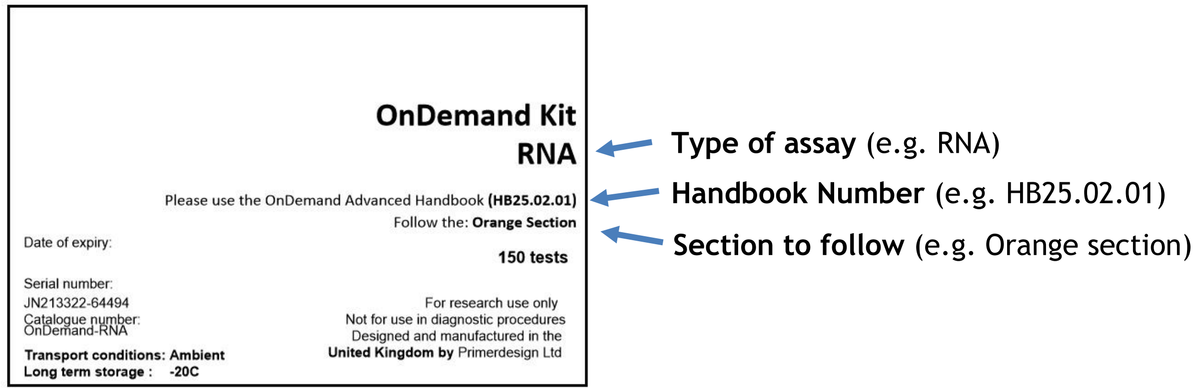
Where to find the correct section to follow:

Please refer to your assay format for correct handbook procedure to follow. The reference is located on front of the kits packaging material. Below is an example pouch label, the handbook number is referenced, and this number can be found on the footer of the document. The section to follow is also referenced via the colour section and type of assay (e.g. RNA).

Example Pouch label:



If not, or not accessible, the correct link should be supplied via the website and/or email correspondence. However, if still unclear or any other question please do not hesitate to contact us via enquiry@primerdesign.co.uk or (+44) 2380 748 830.

Introduction to OnDemand Kits

OnDemand Kits are Real-Time PCR Kits which Primerdesign have the expertise to design and develop. They are for 'hotspot' target organisms which have particular importance in their individual areas of interest and therefore we understand the importance of developing a Kit. The OnDemand Kits can be developed on request and to meet your specific requirements meaning you will be provided with a Kit which is of the highest quality and which works for you.

We offer specific qPCR Kit development including primer/probe design, PCR mix optimization and Kit development to ensure a very effective qPCR reaction. We have already developed a huge range kits as part of our ever expanding genesig catalogue. This service allows for the combination of bioinformatic design and development, in conjunction with laboratory testing and evaluation, to provide personally designed primers to match your exact needs and ensure high levels of sensitivity and specificity.

Specificity

The Primerdesign OnDemand Kits for each specific target are designed for the in vitro detection and/or quantification of the specific target. The Kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design. The dynamics of genetic variation means that new sequence information may become available after the initial design.

Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

Kit storage and stability

This kit is stable at room temperature but must be stored at -20ºC on arrival. Once the Lyophilised components have been resuspended they must not be exposed to temperatures above -20°C for longer than 30 minutes at a time and unnecessary repeated freeze/thawing must be avoided. The kit is stable for six months from the date of resuspension under these circumstances.
If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.
Primerdesign does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All known starting sample material which are 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 nonspecific 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 OnDemand detection Kits have very high priming efficiencies of >90% and can detect less than 100 copies of target template.

Quality Control

As part of our ISO9001 and ISO13485 certified quality assurance systems, all Primerdesign products are verified to ensure the highest levels of performance and reliability.

Principles of the test

Real-time PCR

The target's specific primer and probe mix is provided, and this can be detected through the specified channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target DNA /cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 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 qPCR platforms.

Positive control

For copy number determination and as a positive control for the PCR set up, the Kit contains a positive control template.
This can be used to generate a standard curve of target copy number / Cq value. Alternatively, the positive control can be used at a single dilution where full quantitative analysis of the samples is not required.

Each time the Kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target worked properly in that 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. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings a negative control reaction is recommended to 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 or RNA extraction, it is often advantageous to have an exogenous source of template that is spiked into the lysis buffer. This control template is then co-purified with the sample and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control template also indicates that PCR inhibitors are not present at a high concentration.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the HCV primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

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 Primerdesign genesig 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 Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease Kits 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.

Trademarks

Primerdesign™ is a trademark of Primerdesign Ltd.
genesig® is a registered trademark of Primerdesign Ltd.
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 Applera Genomics (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 Primerdesign ™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc

Reagents and equipment to be supplied by the user

- Real-time PCR Instrument
- DNA extraction Kit
  - This Kit is recommended for use with genesig Easy DNA/RNA extraction Kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.
- oasig™ Lyophilised or Precision®PLUS 2X qPCR Master Mix
  - This Kit is intended for use with oasig or PrecisonPLUS 2X qPCR Master Mix.
- Pipettors
- Tips
- Vortex
- Centrifuge
- Thin walled 1.5 ml PCR reaction tubes

Primerdesign™ Ltd

OnDemand Kit

DNA Kit

Advanced Kit

150 tests

For Research Use Only. Not for use in diagnostic procedures

Kit contents

*N.B. Once resuspended the Kits mustremain at **-20ºC** until ready to use.*

- **Target specific primer/probe mix (BROWN Lid)**
- **Target positive control template (RED Lid)**
- **Internal extraction control primer/probe mix (BROWN Lid)**
- **Internal extraction control DNA (BLUE Lid)**
- **Endogenous control primer/probe mix (BROWN Lid)**
- **RNase/DNase free water (WHITE Lid)**
- **Template preparation buffer (YELLOW Lid)**

ONDEMAND

Resuspension protocol

#PRIMERDESIGNTOPTIP

- 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. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
   - To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
Target primer/probe mix (BROWN Lid)	**165 µl**
Internal extraction control primer/probe mix (BROWN Lid)	**165 µl**
Endogenous control primer/probe mix (BROWN Lid)	**165 µl**

3. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
   - To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE Lid)	**600 µl**
Post-PCR heat-sealed foil	
Target Positive Control Template (RED Lid) *	**500 µl**

#PRIMERDESIGNTOPTIP

* 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

#PRIMERDESIGNTOPTIP

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 µl of the Internal extraction control DNA (BLUE Lid) to each sample in DNA lysis/extraction buffer per sample.**

2. **Complete DNA extraction according to the manufacturers protocols.**

qPCR Set-Up protocol

1. **For each DNA sample prepare a Reaction Mix according to the table below:**
    - Include enough reactions for positive and negative controls.
    - n = the total number of reactions you intend to run

Component	1 Reaction Volumes	n+3 Reaction Volumes
oasig™ or PrecisonPLUS™ 2X qPCR Master Mix	**10 µl**	**10 x (n+3) µl**
Target primer/probe mix (BROWN Lid)	**1 µl**	**1 x (n+3) µl**
Internal extraction control primer/probe mix(BROWN Lid)	**1 µl**	**1 x (n+3) µl**
RNase/DNase free water (WHITE Lid)	**3 µl**	**3 x (n+3) µl**
Final Volume	**15 µl**	**15 x (n+3) µl**

2. **(Optional) For each DNA sample prepare an endogenous control reaction according to the table below**
    - This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig or PrecisonPLUS 2X qPCR Master Mix	10 µl
Endogenous control primer/probe mix (BROWN Lid)	1 µl
RNase/DNase free water (WHITE Lid)	4 µl
Final Volume	**15 µl**

3. **Pipette 15 µl of each mix into individual wells according to your qPCR experimental plate set up.**

4. **Prepare sample DNA templates for each of your samples.**

5. **Pipette 5 µl of DNA 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 20 µl.

6. **(Optional)**
    1. **If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:**

Component	Volume
oasig or PrecisonPLUS 2X qPCR Master Mix	10 µl
Target primer/probe mix (BROWN Lid)	1 µl
RNase/DNase free water (WHITE Lid)	4 µl
Final Volume	**15 µl**

    2. **Preparation of standard curve dilution series.**

        1. Pipette **90 µl** of template preparation buffer into 5 tubes and label them tube 2 up to tube 6
        2. Pipette **10 µl** of Positive Control Template (RED Lid) into tube 2
        3. Vortex thoroughly
        4. Change pipette tip and pipette **10 µl** 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 Lid)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

a. **Pipette 5 µl of standard template into each well for the standard curve according to your experimental plate set up.**
   - The final volume in each well is 20µl

qPCR amplification protocol

Amplification conditions using oasig or PrecisonPLUS 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	Annealing/Extension *DATA COLLECTION* *	60 s	60 °C

** Fluorogenic data must be acquired during this step through the **FAM and VIC channels**. Ensure fluorescence is acquired at this step.*

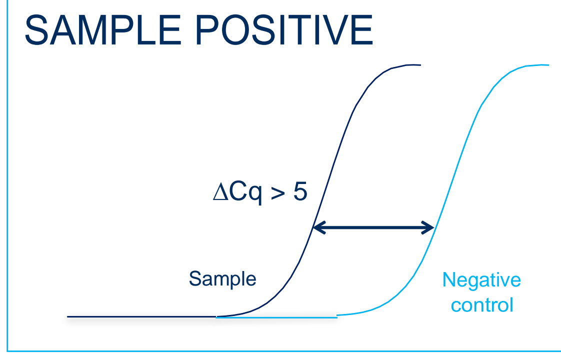
Interpretation of results

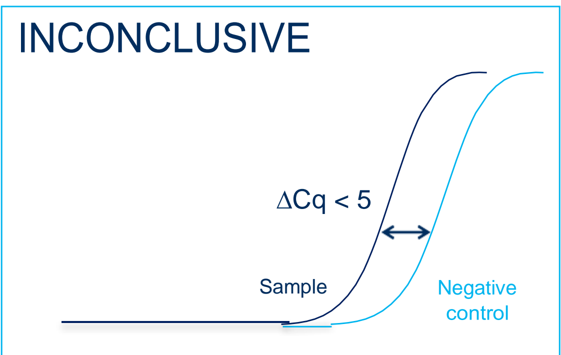
Table represents how to interpret your results for each of your samples tested. Please use the key supplied below for support. The numbers quoted in the table, refer to Cq values.

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
Positive results				
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
Negative results				
-	+	+	-	**NEGATIVE RESULT**
Run Failures				
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

Key	
+	Positive
-	Negative
+ / -	Positive or negative

*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 below:





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.

How do you interpret your controls data?

Control type	Description of result Interpretation
Positive control	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.
Internal PCR control	The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of **28±3** are within the normal range. When amplifying an DNA 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.
Endogenous control	The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in each sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.

PrimerdesignTM Ltd

OnDemand Kit

RNA Kit

Advanced Kit

150 tests

For Research Use Only. Not for use in diagnostic procedures

Kit contents

N.B. Once resuspended the Kits must remain at -20ºC until ready to use.

- **Specific primer/probe mix (BROWN Lid)**
- **Positive control template (RED Lid)**
- **Internal extraction control primer/probe mix (BROWN Lid)**
- **RNA Internal extraction control Template (BLUE Lid)**
- **Endogenous control primer/probe mix (BROWN Lid)**
- **RNase/DNase free water (WHITE Lid)**
- **Template preparation buffer (YELLOW Lid**

Resuspension Protocol

#PRIMERDESIGNTOPTIP

- 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. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied, according to the table below:**
    - To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
Target primer/probe mix (BROWN Lid)	**165 µl**
Internal extraction control primer/probe mix (BROWN Lid)	**165 µl**
Endogenous control primer/probe mix (BROWN Lid)	**165 µl**

3. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control RNA (BLUE Lid)	**600 µl**
Post-PCR heat-sealed foil	
Positive Control Template (RED Lid) *	**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.

RNA extraction

#PRIMERDESIGNTOPTIP

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

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

1. **Add 4 µl of the Internal extraction control RNA (BLUE Lid) to each sample in RNA lysis/extraction buffer per sample.**
2. **Complete RNA extraction according to the manufacturer's protocols.**

OneStep RT-qPCR detection protocol

#PRIMERDESIGNTOPTIP

A OneStep approach combining the reverse transcription and amplification in a single closed tube is the preferred method.

For optimum performance and sensitivity.

All pipetting steps and experimental plate set up should be performed on ice. After the plate is poured proceed immediately to the OneStep amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each RNA sample prepare a reaction mix according to the table below:**
    - Include enough reactions for positive and negative controls.
    - n = the total number of reactions you intend to run

Component	1 rxn	n + 3 rxns
oasig™ OneStep or PrecisonPLUS™ OneStep 2X RT-qPCR Master Mix	10 µl	10 x (n+3)
Target primer/probe mix (BROWN Lid)	1 µl	1 x (n+3)
Internal extraction control primer/probe mix (BROWN Lid)	1 µl	1 x (n+3)
RNase/DNase free water (WHITE Lid)	3 µl	3 x (n+3)
Final Volume	15 µl	15 x (n+3) µl

2. **(Optional) For each RNA sample prepare an endogenous control reaction according to the table below**
    - This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig OneStep or PrecisonPLUS OneStep 2X RT-qPCR Master Mix	10 µl
Endogenous control primer/probe mix (BROWN Lid)	1 µl
RNase/DNase free water (WHITE Lid)	4 µl
Final Volume	**15 µl**

3. **Pipette 15 µl of each mix into individual wells according to your qPCR experimental plate set up.**

4. **Pipette 5 µl of RNA template into each well, according to your experimental plate set up.**
    - The final volume in each well is 20 µl.

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

7. **(Optional)**

3. **If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:**

Component	Volume
oasig OneStep or PrecisonPLUS OneStep 2X RT-qPCR Master Mix	10 µl
Target primer/probe mix (BROWN Lid)	1 µl
RNase/DNase free water (WHITE Lid)	4 µl
Final Volume	**16 µl**

4. **Preparation of standard curve dilution series.**

   1. Pipette **90 µl** of template preparation buffer into 5 tubes and label them tube 2 up to tube 6
   2. Pipette **10 µl** of Positive Control Template (RED Lid) into tube 2
   3. Vortex thoroughly
   4. Change pipette tip and pipette **10 µl** 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 Lid)	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	20 per µl
Tube 6	3 per µl

a. **Pipette 5 µl of standard template into each well for the standard curve according to your experimental plate set up.**

   - The final volume in each well is 20 µl

OneStep RT-qPCR Amplification Protocol

Amplification conditions using oasig™ OneStep or PrecisonPLUS™ OneStep 2X RT-qPCR Master Mix.

	Step	Time	Temp
	Reverse Transcription	10 min	55 °C
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	Annealing/Extension ***DATA COLLECTION ****	60 s	60 °C

* Fluorogenic data must be acquired during this step through the **FAM and VIC channels**. Ensure fluorescence is acquired at this step

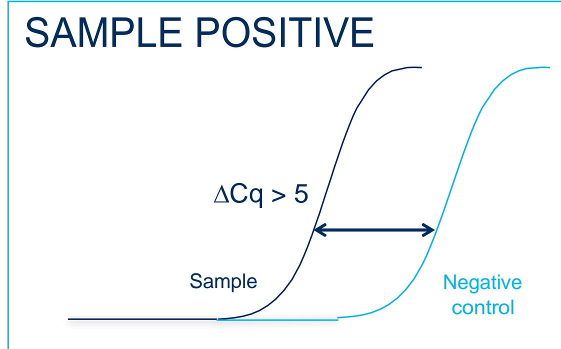
Interpretation of results

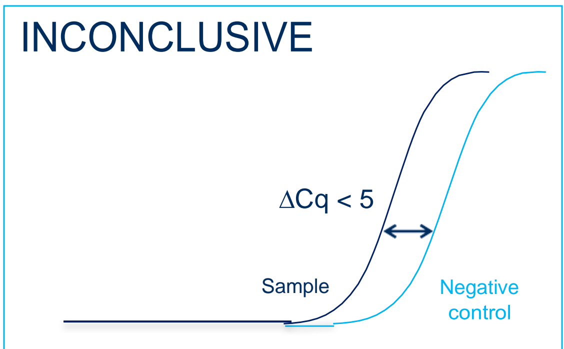
Table represents how to interpret your results for each of your samples tested. Please use the key supplied below for support. The numbers quoted in the table, refer to Cq values.

Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
Positive results				
≤ 30	**+ / -**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**+**	**+**	**-**	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	**-**	**+**	**-**	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
Negative results				
-	**+**	**+**	**-**	**NEGATIVE RESULT**
Run Failures				
+ / -	**+ / -**	**+**	**≤ 35**	**EXPERIMENT FAILED** due to test contamination
+ / -	**+ / -**	**+**	**> 35**	*
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

Key	
+	Positive
-	Negative
+ / -	Positive or negative

*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 below:





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

How do you interpret your controls data?

Control type	Description of result Interpretation
Positive control	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.
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 PCR reaction and the individual machine settings. Cq values of **28±3** are within the normal range. When amplifying an RNA 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.
Endogenous control	The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in each sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.

Primerdesign™ Ltd

Speciation handbook

Speciation Kit

100 tests

For Research Use Only. Not for use in diagnostic procedures

Kit contents

N.B. *Once resuspended the Kits must remain at **-20ºC** until ready to use.*

- **Species-specific primer/probe mix (BROWN Lid)**
- **Species-specific positive control template (RED Lid)**
- **Internal extraction control primer/probe mix (BROWN Lid)**
- **Internal extraction control DNA (BLUE Lid)**
- **RNase/DNase free water (WHITE Lid)**
- **Template preparation buffer (YELLOW)**

Resuspension protocol

#PRIMERDESIGNTOPTIP

- 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. **Resuspend the primer/probe mixes in the RNase/DNase free water supplied according to the table below:**
    - To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
Species-Specific primer/probe mix (BROWN Lid)	**110 µl**
Internal extraction control primer/probe mix (BROWN Lid)	**165 µl**

3. **Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:**
    - To ensure complete resuspension, vortex the tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE Lid)	**500 µl**
Post-PCR heat-sealed foil	
Species-Specific Positive Control Template (RED Lid) *	**500 µl**

#PRIMERDESIGNTOPTIP

* 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

#PRIMERDESIGNTOPTIP

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 µl of the Internal extraction control DNA (BLUE Lid) to each sample in DNA lysis/extraction buffer per sample.**
2. **Complete DNA extraction according to the manufacturers protocols.**

qPCR detection protocol

1. **For each DNA sample prepare a reaction mix according to the table below:**
   - Include enough reactions for positive and negative controls
   - n = the total number of reactions you intend to run

Component	1 Reaction Volumes	n+3 Reaction Volumes
oasig or PrecisonPLUS 2X qPCR Master Mix	**10 µl**	**10 x (n+3) µl**
Species-Specific primer/probe mix (BROWN Lid)	**1 µl**	**1 x (n+3) µl**
Internal extraction control primer/probe mix(BROWN Lid)	**1 µl**	**1 x (n+3) µl**
RNase/DNase free water (WHITE Lid)	**3 µl**	**3 x (n+3) µl**
Final Volume	**15 µl**	**15 x (n+3) µl**

2. **Pipette 15 µl of each mix into individual wells according to your qPCR experimental plate set up.**
3. **Pipette 5 µl of DNA template into each well, according to your experimental plate set up.**

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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.

qPCR amplification protocol

Amplification conditions using oasig or PrecisonPLUS 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	Annealing/Extension *DATA COLLECTION **	60 s	60 °C

* Fluorogenic data must be acquired during this step through the **FAM and VIC channels**. Ensure fluorescence is acquired at this step.

Interpretation of results

For data interpretation when:

1. **Speciation Kit is used independently of the universal meat or fish Kit**
    - Please refer to Data Interpretation **section 1**

2. **Speciation Kit used in conjunction with the universal meat or fish Kit**
    - Please refer to Data Interpretation **section 2**

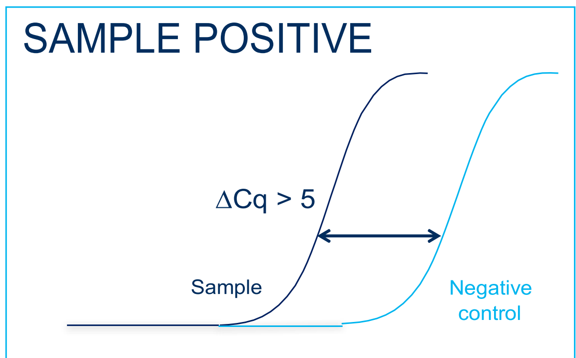
Section 1 Data Interpretation:

Table represents how to interpret your results for each of your samples tested. Please use the key supplied below for support. The numbers quoted in the table, refer to Cq values.

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

Key	
+	Positive
-	Negative
+ / -	Positive or negative

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:



INCONCLUSIVE

ΔCq < 5

Sample

Negative control

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.

How do you interpret your controls data?

Control type	Description of result Interpretation
Positive control	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.
Internal PCR control	The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of **28±3** are within the normal range. When amplifying an DNA 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.

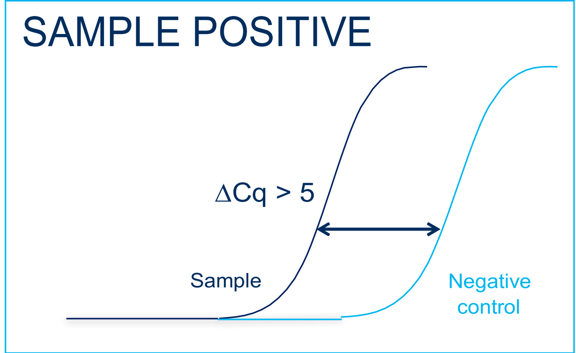
Section 2 Data Interpretation:

Table represents how to interpret your results for each of your samples tested. Please use the key supplied below for support. The numbers quoted in the table, refer to Cq values.

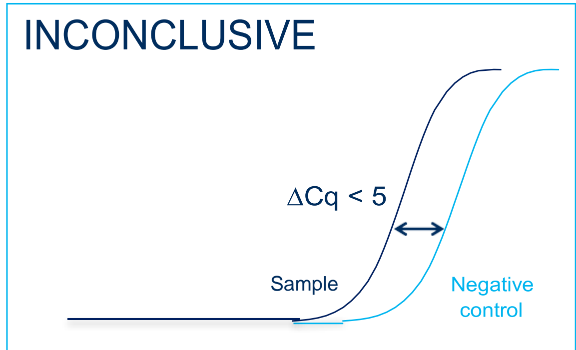
Target (FAM)	Internal control (VIC)	Universal signal (FAM)	Positive control (Target & Universal signal)	Negative control (Target only)	Interpretation
Positive Result					
+	+ / -	+	+	-	**POSITIVE RESULT** calculate species % and check test sensitivity
Negative Result					
-	+	+	+	-	**NEGATIVE RESULT**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

Key	
+	Positive
-	Negative
+ / -	Positive or negative

*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 below:



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 %

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N.B. a Microsoft Excel applet for automatic % calculation is available free of charge.
Contact: support@primerdesign.co.uk to request

Basic formula:

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

Worked example:

Test gives following Cq values: Bos taurus test

Cq of sample Bos Taurus: **24.1**
Bos taurus test on positive control DNA: **23.5**
Universal meat test on sample: **22.2**
Universal meat test on positive control DNA: **22.4**

Bos taurus% =

(2^-((24.1-23.5)-(22.2-22.4))) 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 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 dependent on the amount of DNA that has been successfully extracted from a given sample. The genesig advanced 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 support@primerdesign.co.uk 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 target species DNA is greater than the calculated test sensitivity, then the quantitative result is accurate.
If the calculated percentage target species 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

Worked Example

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