RealPCR* Technical Guide

Test **with Confidence**™

IDEXX

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Polymerase chain reaction (PCR)

All living organisms contain DNA or RNA as their genetic material. Based on the sequence of the genetic material, it is possible to identify specific organisms and/or viruses in a sample. The amount of DNA is usually too low to be detected directly from a sample; PCR is used to amplify DNA to detectable levels.

An RNA template is first converted to complementary DNA (cDNA), followed by PCR. This process is termed reverse-transcription PCR (RT-PCR).

There are several basic components necessary for PCR:

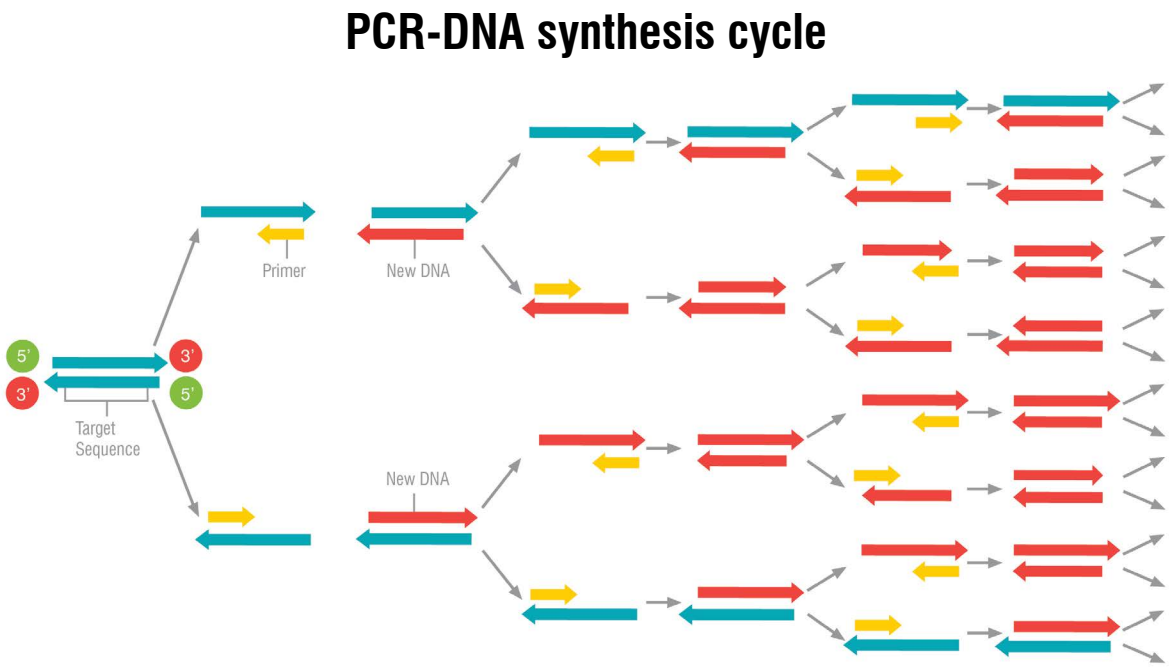
1. Master mix: DNA polymerase (enzyme capable of synthesizing DNA), free dNTPs (DNA building blocks for a new DNA strand), buffer (containing $MgCl^2$) and reverse transcriptase (in the case of RNA targets)
2. Primers: Short synthetic DNA oligonucleotides that initiate elongation and specify the region of DNA to be amplified
3. Positive control: Contains the test target and confirms that the PCR worked
4. Negative control (PCR grade water): Indicates contamination if positive

PCR is a stepwise process that takes 1–2 hours to complete, depending on the program and the PCR instrument:

1. Melting: The double-stranded DNA in the sample is denatured at high temperature to yield single-stranded DNA.
2. Annealing: Specific primers bind to predetermined regions of the single-stranded DNA.
3. Elongation: DNA polymerase extends the primers by adding free dNTPs.

After the PCR, the starting sample DNA (parental DNA) has been amplified exponentially and can be detected (figure 1). In traditional PCR, detection is accomplished by gel electrophoresis and DNA staining. In real-time PCR, the detection happens at the same time as the amplification by monitoring fluorescence after each heating and cooling cycle.

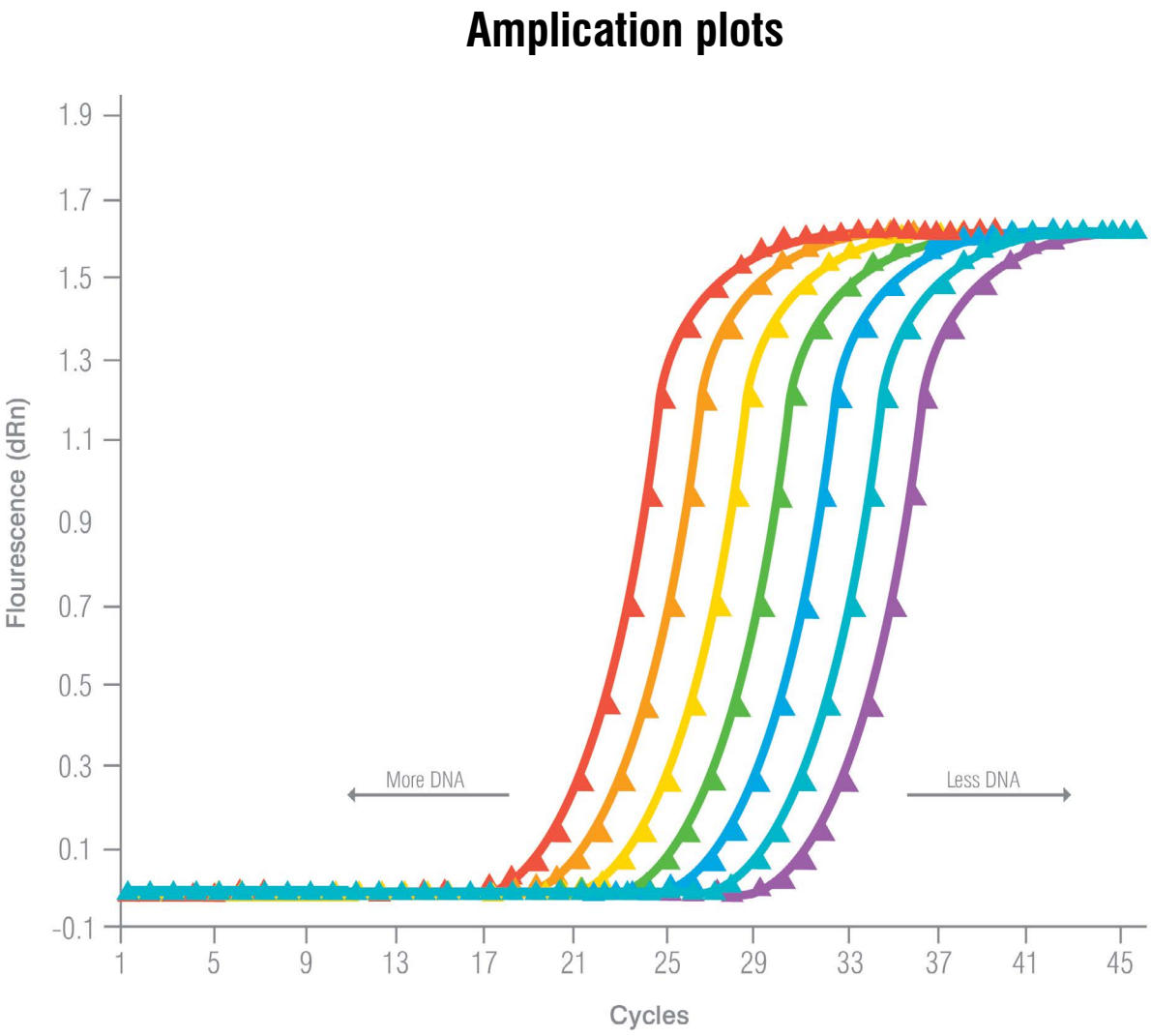
Figure 1. Polymerase chain reaction (PCR)



Real-time PCR (qPCR)

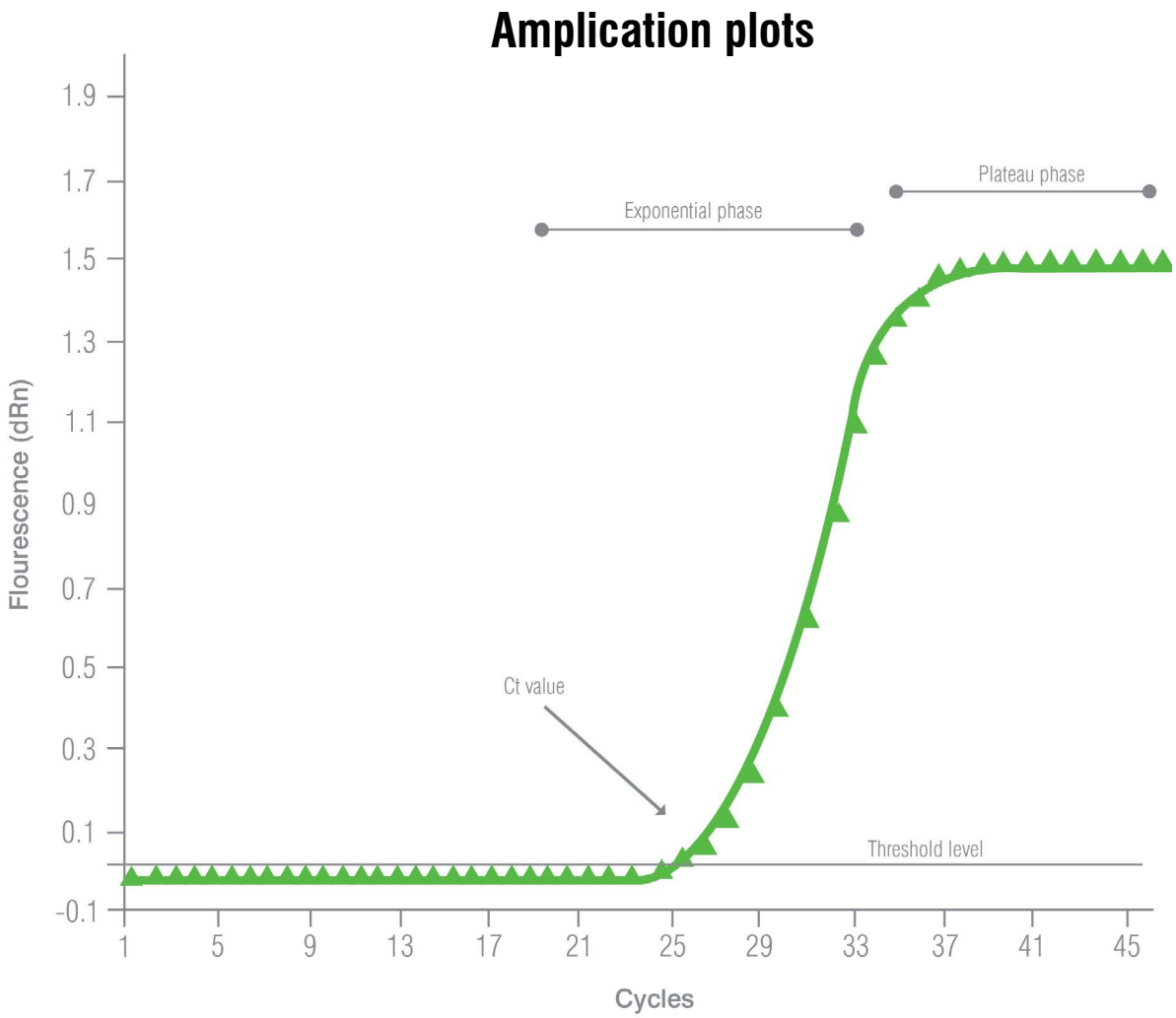
Real-time PCR, also referred to as quantitative or qPCR, utilizes both primers and probes for detecting target nucleic acid in a sample. Probes are similar to primers but contain attached fluorescent dyes used for detection of the amplified target. After each qPCR cycle the fluorescence is monitored in an amplification curve.

Figure 2. Real-time PCR amplification curve



Typically 40–45 cycles are needed in real-time PCR to detect the smallest amount of target nucleic acid from the sample. As shown in figure 3, the PCR cycle at which the fluorescence reaches a preset threshold level is termed the Ct value (or Cq or Cp value). The more target nucleic acid initially present in the sample, the earlier the Ct value will be.

Figure 3. Characteristics of an amplification curve



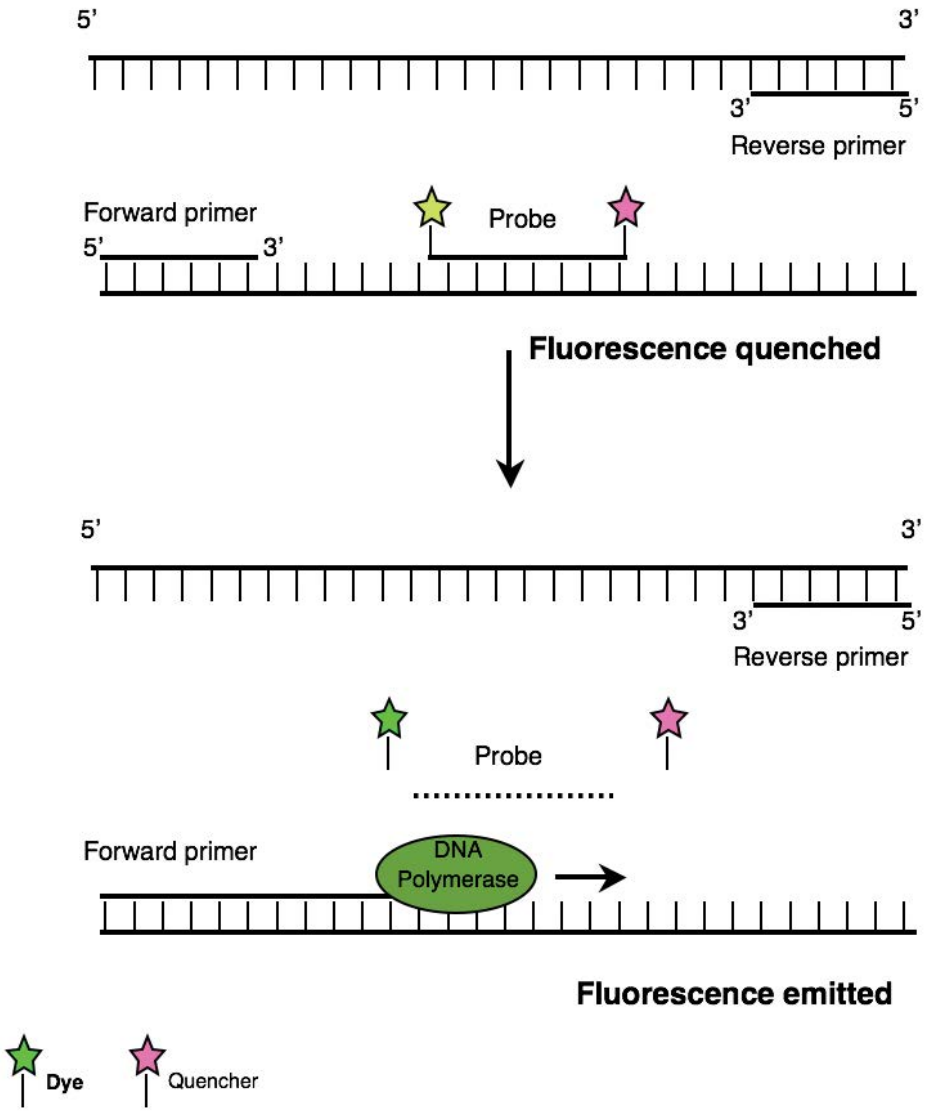
Real-time PCR detection formats

Fluorescence is the emission of light by a substance that has absorbed light. Real-time PCR utilizes fluorescent dyes to signal the presence of amplified DNA template. Thus, a real-time PCR instrument exposes the reaction mix to a specific wavelength of light (excitation), and the reaction emits light (emission) of a different wavelength. The detection/filter channel within a real-time PCR instrument reads a specific wavelength while blocking others. All common fluorescent dyes used in real-time PCR have specific excitation and emission spectra (for instance, the FAM* excitation maximum is 495 nm and its emission maximum is 520 nm). When using different fluorescent dyes, it is possible to simultaneously detect multiple targets within the same PCR well (multiplex PCR).

Hydrolysis (TaqMan*) probes are the most common form of qPCR probes and are widely used in human, veterinary, and environmental diagnostics. These probes utilize a fluorescent dye at one end of the DNA oligonucleotide and a quencher at the other (see figure 4). During PCR, the probe specifically anneals to the target DNA sequence (from sample), which is flanked by the two primers.

As DNA polymerase extends the new DNA strand, the probe is degraded by the 5' to 3' exonuclease activity of the polymerase, resulting in the fluorophore being separated from the quencher and emitting fluorescence. The more DNA present in the reaction, the earlier the fluorescence reaches a detectable level—resulting in earlier Ct values.

Figure 4. Hydrolysis probes



Nucleic acid extraction

PCR is adversely affected by inhibitors in the sample; nucleic acid extraction is required to purify DNA or RNA from other components in the sample. PCR inhibitors include detergents, chelators, and nucleases that can affect the enzyme(s) in the reaction mix. The extracted sample contains purified DNA or RNA for the target of interest (when present), as well as host nucleic acid. The extraction method is an important consideration for each sample type and real-time PCR test.

Purification methods

Binding columns: Binding columns contain silica gel membranes that bind nucleic acids while allowing unwanted material to pass through. This filtrate is pulled through by vacuum, centrifugation, or gravity, while RNA/DNA sticks to the filter and is eluted separately.

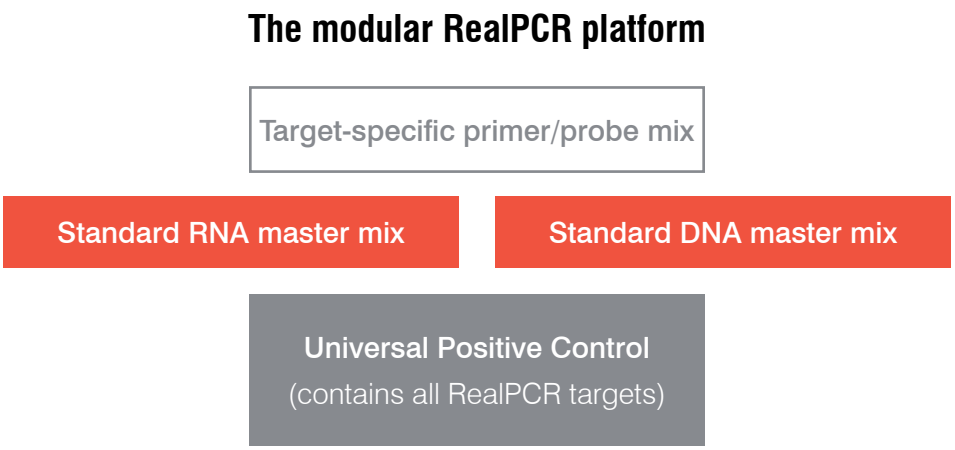
Magnetic beads: Magnetic beads are glass or silica material that binds RNA and/or DNA in the sample. The unwanted material is washed away, and the nucleic acids can be released from the beads into a clean container.

Quick preparations: Quick preparations include boiling and rapid lysis buffers that replace the purification protocol. For rapid lysis procedures, the sample is added to an enzyme-containing solution and heated at approximately 65°C for 30–60 minutes. A subsequent incubation at 97°C for 10–15 minutes inactivates enzymes in the rapid lysis solution. The cooled solution is used as the sample in the real-time PCR test. Quick preparation methods are not universally used because of potential inhibitors. Only certain tests and sample types are compatible with quick preparation methods.

The modular RealPCR platform

The RealPCR platform has target-specific primer/probe mixes for use with shared standard master mixes and a single positive control (figure 5). In addition to shared reagents, the RealPCR platform also features a standardized PCR cycling protocol for all RNA and DNA tests. The benefits of this platform are standardized reagents, a single cycling protocol for all RealPCR mixes and flexible reagent management. The RealPCR platform is also compatible with the RealPCR Lab Monitoring Tools.

Figure 5. RealPCR platform



Standardized protocol for DNA and RNA

Whether testing for the presence of RNA or DNA, the RealPCR platform uses a single cycling protocol. Therefore, multiple tests can be run at the same time using the same PCR instrument.

Real-time PCR instrument recommendations

RealPCR assays have been validated on the following real-time PCR instruments:

- Applied Biosystems* 7500
- Applied Biosystems* QuantStudio*
- Applied Biosystems* ViiA* 7
- Agilent AriaMx*
- Agilent Mx3000P*
- Agilent Mx3005P*
- Roche LightCycler* 480
- Bio-Rad CFX96*
- QIAGEN* Rotor-Gene* (72 well rotor)

RealPCR assays may work with other PCR instruments but should be validated.

Most real-time PCR instrument software programs have similar work flows that include defining targets (for example, BVDV, *Mycoplasma gallisepticum*, bluetongue virus) and other parameters.

Reaction setup

- PCR plate formats vary by instrument but can include; 96-well plates, 8-strip tubes (in 96-well holder), or 384-well plates.
- Assign or define the following parameters: sample name, sample positions, cycling protocol, and filter channels.

Analysis

- Most instruments offer multiple analysis modes.
- Instrument software calculates baseline and threshold and determines Ct values.
  - Each instrument's software uses different algorithms for Ct calculations.
  - It is possible to get different Ct values from the same sample on different instruments.
  - It is possible to get different Ct values from the same sample on the same instrument using a different analysis algorithm.
  - Although Ct values can vary, a positive sample should always have a characteristic amplification curve in comparison to the PCR negative control.
- Target nucleic acid quantity can be approximated based on the Ct value.
  - Earlier Ct value = higher amounts of target
  - Later Ct value = lower amounts of target
- Visual inspection of the amplification curves is important to confirm the functionality of the instrument software.

Export

- Most instrument software has the ability to export data for data management.

Calibration

- Instrument optics and thermal parameters should be calibrated according to the instrument manufacturer's recommendations. In some regions, an outside company may need to conduct the thermal calibration. When using a Roche LC480 real-time PCR instrument, contact IDEXX Technical Service for instructions on how to create a color compensation file often required for multiplex tests.

ROX* passive reference and ROX normalization

ROX is required for some real time PCR instruments (i.e. Applied Biosystems) or considered as optional for other instruments (i.e. Agilent). ROX, a passive reference dye, is used to normalize fluorescent signals. ROX normalization is used by the software to eliminate nonamplification-related fluorescence, such as air bubbles or well-to-well volume differences. Additionally, ROX helps with perimeter effects, in which instrument cameras have difficulty reading fluorescence originating near the perimeter of a 96-well plate. The RealPCR master mixes contain a ROX reference dye.

RealPCR sample extraction methods

Details for sample extraction methods validated for each RealPCR mix are listed in the respective RealPCR target mix product inserts. Validated methods typically include a column-based procedure and a magnetic bead method for higher test volumes. Some mixes also have a validated quick preparation method. Table 1 lists the product codes and insert versions for the commercial extraction methods referred to in the RealPCR product inserts.

Table 1. Commercial extraction methods

Extraction kit	Company	Catalog number	Product insert version
RealPCR* DNA/RNA Magnetic Bead Kit	IDEXX	99-56102 (n = 384) 99-56106 (n = 96)	06-56102-00
RealPCR* DNA/RNA Spin Column Kit	IDEXX	99-56103 (n = 50)	06-56103-00
RealPCR* Rapid Lysis Buffer	IDEXX	99-56370 (n =100)	06-56370-02
RNeasy* Mini Kit	QIAGEN	74104 (n = 50) 74106 (n = 250)	June 2012
QIAamp* Viral RNA Mini Kit	QIAGEN	52904 (n = 50) 52906 (n = 250)	December 2014
QIAamp* DNA Mini Kit	QIAGEN	51304 (n = 50) 51306 (n = 250)	May 2016
MagMAX*-96 Viral RNA Isolation Kit	Life Technologies	AM1836 (n = 96) AMB18365 (n = 5 x 96)	1836M Rev. H
MagMAX* Pathogen RNA/DNA Kit	Life Technologies	4462359 (n = 480)	4463379 Rev. B
MagMAX* Total Nucleic Acid Isolation Kit	Life Technologies	AM1840 (n = 100)	4385118 Rev. C
NucleoMag* VET Magnetic Extraction Kit	Macherey-Nagel	744200.1 (n = 96) 744200.4 (n = 4x96)	January 2017/Rev. 05
NucleoSpin* Virus	Macherey-Nagel	740983.50 (n = 50) 740983.250 (n = 250)	February 2015/Rev. 02
NukEx Pure RNA/DNA	Gerbion*	G05004-50 (n = 50) G05004 (n = 200)	Version 3.1/ November 2, 2015
High Pure PCR Template Prep Kit	Roche	11796828001 (n = 100)	Version 20 October 2012

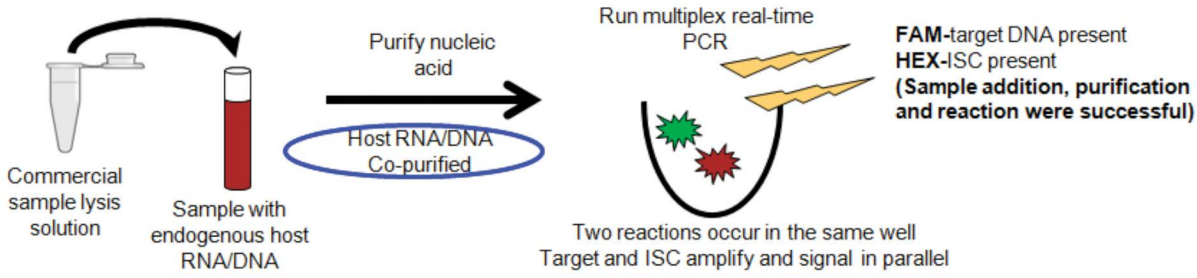
RealPCR controls

Internal sample control (ISC)

The primary internal control approach used in the modular RealPCR platform is an ISC. An ISC internal control design targets endogenous RNA or DNA from the host sample. The ISC target varies depending on the sample type, host species, and test design for the pathogen target. For example, the RealPCR* BVDV RNA Mix detects the presence of bovine RNA (ISC) as well as the presence of BVDV in the sample. Thus, there is no need for the addition of an internal positive control (IPC) to be added to sample extraction when using most RealPCR mixes.

A different fluorophore is used for the ISC probe such that the pathogen target and ISC reactions run as a multiplex in the same well (figure 6). A positive ISC signal confirms sample collection technique, sample addition to PCR reaction, and sample quality. Further, detection of the ISC validates the nucleic acid purification, reverse transcription, and amplification steps.

Figure 6. Internal sample control



The ISC primers and probe target host endogenous genetic material. Most sample types will contain the host ISC target and test positive. In some cases a very strong positive sample may outcompete the ISC reaction, resulting in little or no ISC signal. This is a valid test result as the presence of target confirms adequate sample addition, extraction, and PCR. Some sample types lack the ISC target (for example, environmental samples); therefore, the RealPCR IPC is recommended.

RealPCR* Internal Positive Control (IPC)

Some RealPCR mixes use an IPC as the internal control. In this case, the RealPCR IPC is added to the extraction lysis solution and then copurified with the sample. The RealPCR IPC is a pool of all RealPCR internal control targets, including the ISC endogenous host targets. Therefore, the RealPCR IPC can be used to supplement ISC detection for RealPCR target mixes that use an ISC internal control.

RealPCR* Positive Control (PC)

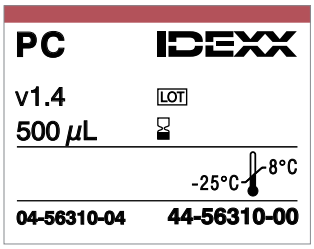
The PC is a single vial containing all RealPCR and ISC targets and can be used with any RealPCR target mix. It verifies the amplification of the target nucleic acid, thereby confirming that the target mix and master mix are working properly. Additionally, the PC contains the IDEXX Signature Sequence, which can be used to monitor for potential PC laboratory contamination (see details below).

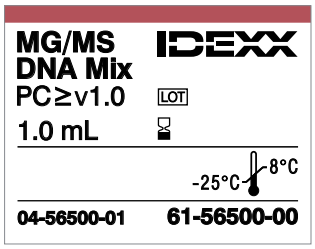
The PC and IPC are dried components that are stored at -25°C to 8°C and reconstituted with PCR grade water when ready to use. The PC and IPC should be aliquoted as appropriate to avoid repeated freeze/thaws and stored at less than -15°C.

Version control of the RealPCR PC and IPC

The RealPCR PC and IPC have version numbers (such as v1.0). When new target-specific mixes are developed for the RealPCR product line, the target sequences are added to the PC and the PC version number increases (for example, v1.2 increases to v1.3). Similarly, the IPC versions are updated when new internal control targets are added to the IPC. It is possible to have multiple control versions in inventory at IDEXX. Obsolete control versions are removed with the launch of a new RealPCR target mix. When a laboratory starts using a new RealPCR target mix, it is important to confirm the versions of the PC and IPC.

Figure 7. Example of version identification for the RealPCR Positive Control





Tables 2 and 3 summarize commonly used real-time PCR controls.

Table 2. RealPCR controls

Quality control item	Type of control	Process controlled	Recommended frequency
Positive Control (PC)	Functional	qPCR: primers, probes, and master mix	Minimum of one for each target mix
Negative Target Control (NC)—Water	Contamination	Contamination of qPCR reagents, carryover contamination	Minimum of one for each target mix
Internal Sample Control (ISC)—host DNA or RNA	Functional	Sample addition, sample integrity, extraction, and qPCR	Part of the target mix (primers and probes included)
Internal Positive Control—DNA and RNA (IPC)	Functional	Extraction and qPCR	Required for some tests; optional for test that use ISC

Table 3. Suggested laboratory controls

Quality control item	Type of control	Process controlled	Recommended frequency
Negative Extraction Control—Water	Contamination	Contamination that occurs during extraction	One for every 20 samples extracted
RealPCR* Signal Check DNA Mix	Positive control for laboratory monitoring	Contamination of laboratory environment	Weekly to monthly
RealPCR* PC Tracker DNA Mix	Contamination	Detects PC contamination in the laboratory environment	Weekly to monthly
Laboratory Contamination Monitoring—Target Mixes	Contamination	Detects contamination in the laboratory environment.	Weekly to monthly

The RealPCR* Lab Monitoring Set

RealPCR PC Tracker DNA Mix

The RealPCR PC Tracker DNA Mix contains primers and probe that amplify and detect the IDEXX Signature Sequence (a unique sequence not found in nature) included in all IDEXX RealPCR positive controls. The RealPCR PC Tracker DNA Mix is intended to detect cross-contamination originating from the inappropriate use of positive control material. As a part of routine laboratory contamination monitoring, the tracker mix can be used to identify the presence of contamination originating from the RealPCR Positive Control(s). See the RealPCR Lab Monitoring Set insert for additional information.

RealPCR Signal Check DNA Mix

The RealPCR Signal Check DNA Mix contains primers and probe that amplify and detect a conserved bacterial DNA sequence. The RealPCR Signal Check DNA Mix will test positive with water due to the inherent presence of bacterial DNA and therefore can be used as a PCR-positive control during routine laboratory contamination monitoring. See the RealPCR Lab Monitoring Set insert for additional information.

Laboratory contamination monitoring procedure

Laboratory contamination monitoring is a swab-based procedure for detecting the presence of nucleic acid contamination on laboratory surfaces, equipment, or environment.

It is recommended that the frequency of laboratory contamination monitoring (weekly to monthly) be proportionate to the volume of PCR testing performed.

Samples for laboratory monitoring should be tested with all target mixes used in a real-time PCR diagnostic laboratory. To minimize contamination risk during laboratory monitoring, a positive target sample should be not included. The RealPCR Signal Check DNA Mix is used as the positive control.

Process for swabbing work areas

1. Use one cotton-tipped swab for each workspace that is to be tested for contamination.
2. Briefly dip each swab into a 1.5 mL tube containing approximately 300 µL PCR grade water. Remove excess water from the swab by pressing the tip into the wall of the tube. Wipe swab across approximately 30 cm of work surface 2–3 times with a rotating motion of the swab.
3. Place the tip back into the tube and twirl vigorously for 10–15 seconds to elute captured nucleic acid on the swab.
4. Remove water from the swab by pressing the tip into the wall of the tube; discard the swab and use the remaining liquid as sample for each RealPCR target mix to be tested.

Example: Laboratory contamination monitoring template

		RealPCR target mix				
		A (Test A)	**B (Test B)**	**C (Test C)**	**D (Test D)**	**E (Test E)**
Sample: Swab eluate/NTC	**Extraction (Area 1)**	Area 1 Test A	Area 1 Test B	Area 1 Test C	Area 1 Test D	Area 1 Test E
	PCR mix (Area 2)	Area 2 Test A	Area 2 Test B	Area 2 Test C	Area 2 Test D	Area 2 Test E
	Sample/controls (Area 3)	Area 3 Test A	Area 3 Test B	Area 3 Test C	Area 3 Test D	Area 3 Test E
	qPCR instrument (Area 4)	Area 4 Test A	Area 4 Test B	Area 4 Test C	Area 4 Test D	Area 4 Test E
	Water (NTC)	Area 5 Test A	Area 5 Test B	Area 5 Test C	Area 5 Test D	Area 5 Test E

Decontamination procedure

Decontamination of a laboratory space is recommended following the detection of nucleic acid contamination that resulted in a positive Ct value for a target tested as part of the laboratory monitoring procedure. The procedure for decontamination and retesting is as follows:

1. For each positive laboratory location, use an appropriate decontamination reagent such as a 10% bleach solution† (~0.5% sodium hypochlorite), DNA AWAY*, DNAZap*, or equivalent to fragment the contaminating nucleic acid. Spray enough fluid to wet the entire surface area.
2. Allow solution to sit for a minimum of 5 minutes.
3. Decontaminate the area by wiping off with paper towels and rinsing the site twice with deionized water.
   Important: Sodium hypochlorite has the potential to react on contact with certain components found in nucleic acid extraction kits. It is important that surfaces are rinsed thoroughly.
4. Retest the decontaminated site(s) following the laboratory monitoring procedure; use target mixes that tested positive in the first round of laboratory contamination monitoring. Include the RealPCR Signal Check DNA Mix as a control to confirm a positive PCR reaction.
5. Record results in the laboratory contamination monitoring program tracking file.
6. If the site is still positive for the pathogen, repeat the decontamination and retesting procedure.

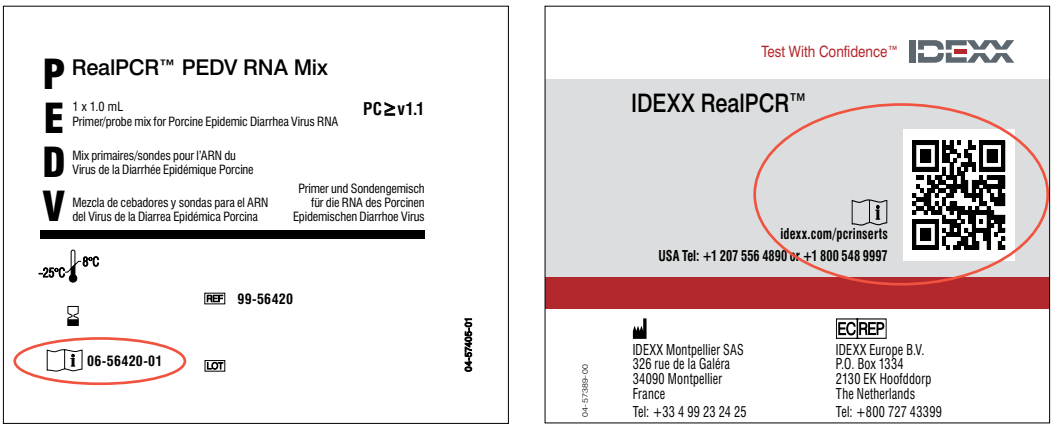
†Sodium hypochlorite degrades quickly over time. To ensure maximum effectiveness, prepare new decontamination solution every 2–4 weeks using distilled or deionized water.

Certificates of analysis and product inserts

Product inserts are available online for each individual RealPCR target mix at **idexx.com/PCRinserts**.

The outer package for all RealPCR products directs users to a website that has a collection of electronic product inserts. This allows for improved version control and visibility of changes as well as reduced waste. The insert part number for each target mix is identified next to the **i** symbol on the outer pouch label. Consult the IDEXX website (idexx.com) to obtain a certificate of analysis for any RealPCR reagent lot.

Figure 8. Where to find RealPCR product inserts



Component storage requirements

Table 4. RealPCR target mixes materials and storage

Identification	Cap color	Quantity	Storage at receipt	Storage after reconstitution	Freeze/thaw cycles
RealPCR DNA target mixes (DNA TMx), dried	Green	1 x 1 mL	-25°C to 8°C	-25°C to -15°C	≤6
RealPCR RNA target mixes (RNA TMx), dried	Yellow	1 x 1 mL	-25°C to 8°C	-25°C to -15°C	≤6

- It is important to protect target mixes from light.
- Reconstitute the RealPCR target mixes with PCR grade water when ready to use and aliquot as appropriate to avoid repeated freeze/thaws; store remaining material at less than -15°C.

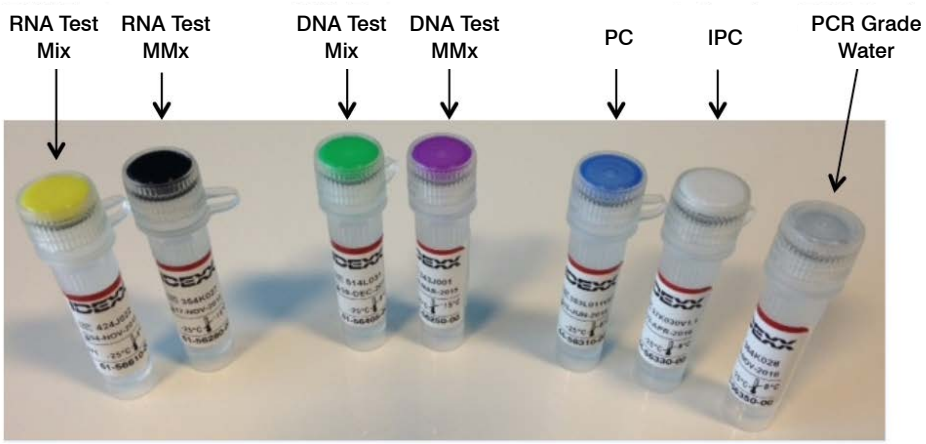
Table 5. RealPCR standard reagents materials and storage

Identification	Cap color	Quantity	Storage at receipt	Storage after reconstitution	Freeze/thaw cycles
RealPCR DNA* Master Mix (DNA MMx)	Purple	1x 1 mL	-25°C to -15°C (shipped at 2°C–8°C)	N/A	≤6
RealPCR* RNA Master Mix (RNA MMx)	Black	1 x 1 mL	-25°C to -15°C (shipped at 2°C–8°C)	N/A	≤6
RealPCR Internal Positive Control (IPC), dried	White	1 x 500 µL	-25°C to 8°C	-25°C to -15°C	≤6
RealPCR Positive Control (PC), dried	Blue	1 x 500 µL	-25°C to 8°C	-25°C to -15°C	≤6
RealPCR* PCR Grade Water	Clear	2 x 1 mL	-25°C to 8°C	N/A	N/A

- It is important to protect the master mixes from light.
- The RealPCR master mixes are particularly viscous; refer to individual product inserts for guidance on handling.
- Reconstitute the RealPCR Positive Control and the RealPCR Internal Positive Control with PCR grade water at use, and aliquot as appropriate to avoid repeated freeze/thaws; store remaining material at less than -15°C.

Packaging—component labeling and cap color system

Figure 9. RealPCR labeling and cap color system



Assay preparation

PCR mixes are prepared by adding the target mix and the master mix at the appropriate volumes for the number of samples to be tested.

Table 6a. The RealPCR reaction

Component	Volume
Master mix	10 µL
Target mix	10 µL
Sample	5 µL
Total reaction mix	**25 µL**

Table 6b. The RealPCR standard DNA/RNA cycling protocol

	Temperature	Time	Cycles
Reverse transcription (RT)	50°C	15 minutes	1
Denaturation	95°C	1 minute	1
Amplification	95°C	15 seconds	45
	60°C	30 seconds	

Note: Ensure the instrument is set to record fluorescence following the 60°C amplification step.

Real-time PCR analysis and interpretation

The RealPCR cycling protocol is standardized for running the RNA and DNA master mixes. After each individual cycle, a reading is taken to monitor the fluorescence. The initial readings collect the background fluorescence. This will vary from instrument to instrument, but many instruments use cycles 3 through 15 to calculate the background.

When multiple RealPCR reactions are performed in a single PCR run, assign a unique identifier for each target and internal control used in the PCR run. For example, when testing porcine epidemic diarrhea virus (PEDV) RNA and transmissible gastroenteritis virus (TGEV) RNA on a single plate, the PEDV wells must be analyzed independently from the TGEV wells. The same is true for the internal control used for each target mix. Refer to the specific instrument's user manual for guidance on how to analyze data. To set the threshold, use the Auto Ct setting. On the Agilent Mx3000P and Mx3005P, ensure the background-based threshold fluorescence method is being used for analysis. For the Roche LightCycler 480, use the Abs Quant/2nd Derivative Max method. For the QIAGEN Rotor-Gene instrument, manually adjust the threshold line above background in the linear phase of exponential amplification. This is best done on log view plots and is needed for each reporter in the target mix.

In certain situations, the software will assign a threshold value that is well below background, resulting in no Ct values for samples with typical amplification curves. In this case, it is necessary to manually adjust the threshold. Refer to the instrument manufacturer guidelines for recommendations on threshold adjustment.

A positive sample result for the RealPCR reagent system will have a positive Ct value and characteristic amplification curve in comparison to the PCR negative control.

Frequently asked questions

Modular RealPCR platform and components

Q: Why are the components shipped with ice packs while some components have a -25°C to -15°C storage requirement?

A: The master mixes have been tested for proper performance and maintain full activity after shipment on ice. To ensure long-term life of these reagents, the master mixes should be stored at -25°C to -15°C upon receipt, while the other components may be kept at 2°C to 8°C. Once dried components are reconstituted, these must also be stored at -25°C to -15°C.

Q: Do I need to have experience in PCR in order to run the RealPCR reagents?

A: No previous experience is needed. IDEXX Technical Service provides training for all new laboratories starting to use the modular RealPCR platform.

Q: Do we need to have separate rooms for real-time PCR and nucleic acid extraction?

A: A separate clean room for preparation of the real-time PCR mix and a separate room for nucleic acid extraction are optimal. The minimum requirement is to have separate benches and equipment (pipettes and so forth) for these two steps.

Q: Where can I get the performance data for the RealPCR reagents?

A: Validation reports for individual tests are available from IDEXX Technical Service.

Q: What is the time to results with the RealPCR platform?

A: The real-time PCR run takes ≤70 minutes (depending on the instrument). Nucleic acid extraction usually takes 1–2 hours, depending on the extraction method and the number of samples to be processed.

Q: What is the shelf life of the RealPCR components?

A: All components have a minimum 12-month shelf life from the date of manufacture.

Q: What is the cost for setting up a real-time PCR laboratory?

A: The setup cost can range from $20,000 to $70,000 (€15,000 to €50,000), depending on the level of automation for nucleic acid extraction and the choice of real-time PCR instrument.

Q: How much laboratory space is needed for running real-time PCR?

A: Approximately 30–40 ft^2 (3–4 m^2) of bench space is needed.

Q: What equipment is needed to set up a real-time PCR laboratory?

A: Contact IDEXX Technical Service for a reference sheet of required equipment.

Q: Are there Material Safety Data Sheets (MSDS) available for the modular RealPCR components?

A: Yes. Please contact IDEXX Customer Support to request any MSDS.

Q: What regulatory agencies have approved the reagents or tests?

A: Contact IDEXX Technical Service for information on current regulatory approvals.

Q: Reagents arrived frozen at my laboratory. Are they still okay to use?

A: Yes. All of the RealPCR components are stable frozen.

Q: Reagents arrived at my laboratory at ambient temperature. Are they still okay to use?

A: Depending upon the length of time, arrival at ambient temperature is fine for some reagents. Contact IDEXX Technical Service for guidance.

Extraction

Q: Can archived samples be used?

A: Yes. However, for RealPCR target mixes that use an ISC, extended storage time may degrade host RNA/DNA. This may result in a negative ISC result. This is also true for nucleic acid and for tissue stored for an extended period of time.

Q: Does the ISC target for an RNA reagent detect DNA as well?

A: No. RealPCR RNA target mixes that use an endogenous host ISC target will detect host RNA only.

Q: Can RealPCR target mixes detect ISC targets in serum?

A: Yes. RealPCR target mixes that have a serum claim have good detection of the ISC target in serum.

Q: What is the difference between the PCR negative control and the negative extraction control?

A: The PCR negative control is used to ensure no contamination is present in any of the PCR reagents. It can also be an indicator of contamination occurring during sample addition to the PCR plate. The negative extraction control monitors potential contamination from strong positive samples into negative samples during nucleic acid extraction.

Q: Which sample type can be used with the RealPCR reagents?

A: Samples types are identified in the product insert for each RealPCR target mix.

Q: Can pooled samples be used with the RealPCR reagents?

A: Yes. Pooled samples can be used for some RealPCR target mixes and sample types. Please refer to the product inserts for detailed information.

Q: What is the risk for contamination when doing real-time PCR?

A: The risk for contamination is increased when reaction mixes are prepared in close proximity to nucleic acid extractions or sample handling. IDEXX provides guidance for setting up a laboratory environmental contamination monitoring program.

Q: Can I use extraction kits or real-time PCR instruments other than those validated by IDEXX for use with the RealPCR reagents?

A: Individual product inserts list validated extraction methods and instruments. It is recommended that customers using protocols not listed in the product insert should first validate the method. For customers in North America, contact IDEXX Technical Service to request a performance data report that summarizes studies for each reagent set.

Q: Can I use the RealPCR Rapid Lysis Buffer for all nucleic acid extractions?

A: No, the RealPCR Rapid Lysis Buffer is only validated for use with specific RealPCR target mixes and sample types.

PCR

Q: Why are there 45 cycles in the RealPCR cycling protocol?

A: The RealPCR platform was developed with 45 cycles to be compatible with as many instruments as possible. While not all tests and/or instruments require 45 cycles, RealPCR is a standardized system; therefore, all tests have been validated with the same protocol.

Q: Should components be added to the reaction mix in a specific order?

A: Yes, the PCR mix is prepared by adding the appropriate master mix to a target-specific mix. For the RNA reagents, it is recommended that the RNA master mix be pipetted into the target-specific mix so that the pipette tip can be rinsed. The PCR mix should be prepared before handling samples.

Q: Why do the ISC and IPC have smaller amplification curves?

A: The internal controls use a different dye, which has a lower quantum yield.

Q: What can cause the ISC to be negative?

A: Many factors may result in a negative ISC signal:

- Improper sample collection
- A failed extraction procedure
- An extraction with excess PCR inhibitors
- A strong target that outcompetes the ISC amplification
- Sample was not added to the PCR reaction

In the event of a negative target/negative ISC (invalid result), it is important to troubleshoot the problem. A positive target/negative ISC result is still a valid reagent result.

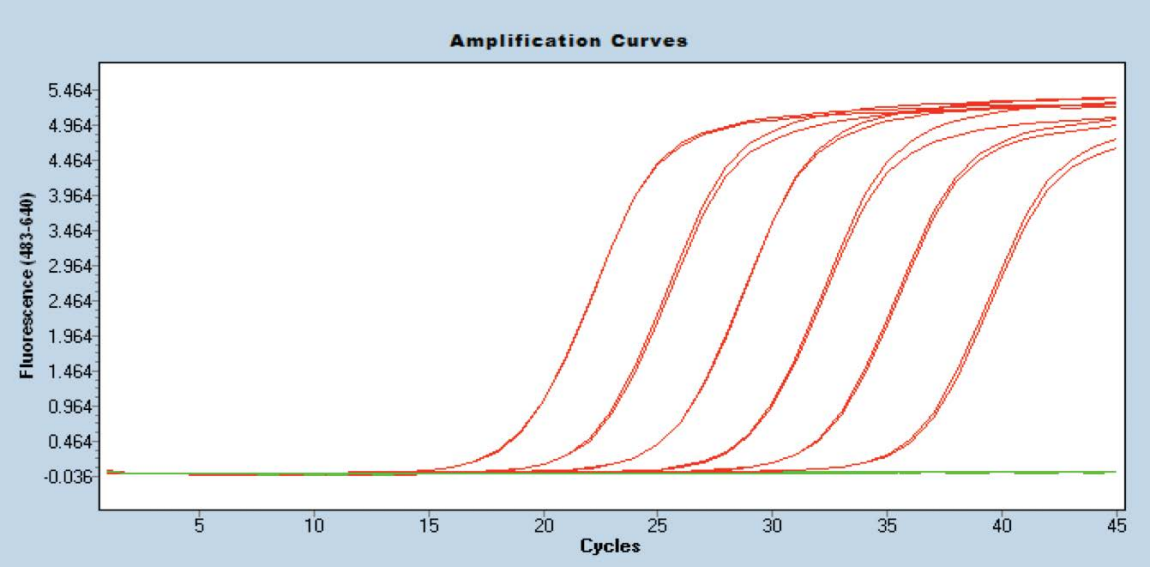
Q: I am consistently getting positive sample results in wells that only contain reaction mix and water. Why is this?

A: The RealPCR reagents are extremely sensitive to the presence of nucleic acids. Any contamination can easily result in a false-positive sample. Below are recommendations to control contamination.

- Attention to proper pipetting techniques; avoid producing aerosols.
- Maintain separate laboratory areas and equipment for preparing PCR mixes and sample addition.
- Control work flow of personnel to minimize tracking contamination into the PCR mix clean area.
- Dispose of completed reaction plates in receptacles located in a different area from the PCR mix preparation area.

Q: What should a normal amplification curve look like?

A: A typical amplification curve should have a characteristic sigmoidal shape. The figure below depicts the amplification curves for a tenfold serial dilution of template.



Q: Most of my amplification curves have a smooth, sigmoidal shape. However, occasionally I see irregular curves. Are these really positive results?

A: Rarely, a negative amplification curve can be detected by the thermocycler software as positive while lacking the characteristic sigmoidal appearance that is indicative of a true exponential increase in DNA that occurs during a successful PCR. These irregular curves can often be attributed to nonamplification-based fluorescence. IDEXX recommends retesting late Ct value samples that lack an amplification curve.

Q: Why does the free probe not emit signal?

A: The free or unbound probe actually does emit a signal. However, the fluorophore is normally quenched and therefore undetectable by the real-time PCR instrument's optical system. Only after degradation of the probe will the fluorophore become unquenched and allow the signal to reach the instrument's detector.

Q: Can the probe be degraded even if it is not annealed to target (i.e., a negative sample)?

A: If the detection mix becomes contaminated, then it is possible for the probe to become degraded and therefore result in the unquenching of the fluorophore. In this case, the signal intensity would be very high, even in the early cycles of the PCR stage. For this reason IDEXX recommends aliquoting the detection mix, thus minimizing handling, and checking that the amplification curves have a sigmoidal shape.

Q: What happens if there is a bubble in the well of the PCR? What happens to the curve?

A: A quick jump can be seen in the amplification curve. Normalization with ROX can often fix the problem for instruments that use ROX.

Q: What is the appropriate response to a late-positive Ct value (such as Ct = 42)?

A. Evaluate the amplification curve before a final result is reported. If there is no characteristic amplification-based signal, then it may be a negative sample. If the result is unclear, then the sample should be retested.

Q: What is the Ct cutoff for positive samples?

A: A specific Ct cutoff is not given for most RealPCR reagents because of the variability in instrument software algorithms for Ct value determination. Amplification curves should be visually inspected, in particular for samples identified with a weak Ct value.

Q: Some tests have a suspect cutoff indicated; what is the recommendation for these samples?

A: Samples with a Ct value later than the suspect cutoff may be positive or the result of contamination. Analysis of the amplification curve may indicate whether the sample is a true positive. Re-extraction may be necessary to rule out contamination. If contamination is suspected, see page 14 for guidance on setting up routine laboratory monitoring.

Thermal cycler requirements

Q: What PCR plates and covers do I use with the RealPCR platform?

A: The brand of the PCR plate and cover depends on the real-time instrument that is used. Please follow the manufacturer's recommendations.

Q: What are the calibration recommendations for real-time PCR instruments?

A: Instrument optics and thermal parameters should be calibrated according to the instrument manufacturer's recommendations. In some regions, an outside company may need to conduct the thermal calibration. Please contact IDEXX Technical Service for additional information on calibration.

Q: When programming the instrument, what should be used for quencher if Black Hole Quencher* (BHQ*) is not listed as an option?

A: You can also use nonfluorescent quencher (NFQ) or simply select "none."

Q: Which filters are required in the qPCR instrument to run the RealPCR reagents?

A: All RealPCR reagents require FAM and HEX. The Cy5 channel is required for two-target multiplex target mixes, and the ROX channel is used by some instruments to normalize signal.

Q: Is separate software required for results interpretation?

A: No, use the real-time PCR instrument software for results interpretation.

Definitions

Amplicon: The product of a PCR amplification.

Amplification: Exponentially multiplying the target DNA using PCR.

Annealing: Occurs when complementary sequences of single-stranded DNA or RNA pair by hydrogen bonds to form double-stranded DNA.

Annealing temperature: The temperature at which annealing occurs (see *Annealing* above).

Channel filter: Selects the appropriate wavelengths for emission and excitation in real-time PCR instruments according to the fluorophores used in the reaction.

Complementary strand: Either of the two chains that make up a double helix of DNA, with corresponding positions on the two chains being composed of a pair of complementary bases.

Ct value = Cq value = Cp value: The number of cycles needed to reach a set threshold fluorescence signal level.

Denaturing: Heating the reaction to cause the DNA strands to separate by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Deoxyribonuclease (DNase): An enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone, thus degrading DNA.

DNA: Deoxyribonucleic acids encode the genetic information of living organisms and many viruses.

DNA polymerase: The enzyme that synthesizes DNA strands from nucleotides.

Emission: The act of a real-time PCR fluorophore producing light, in response to absorbing a specific wavelength of light (see *Excitation*).

Endogenous: In the case of the internal sample control (ISC), *endogenous* refers to the host's own genetic material. The use of an endogenous host target such as the ISC ensures control of sample addition, sample quality, proper extraction, and successful amplification.

Excitation: In real-time PCR, refers to the wavelength of light used to induce a fluorophore to emit light (see *Emission*).

Extension: The step or process in which DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand in 5' to 3' direction.

Fluorescence: The emission of light by material that has absorbed light. In real-time PCR, the fluorescence is measured to determine the relative amount of DNA in a reaction.

Hold: A single temperature step in the qPCR instrument.

Hydrolysis probe: A short synthetic oligonucleotide designed to identify a certain target DNA sequence. Includes a reporter and a quencher and emits fluorescence once cleaved by the DNA polymerase during amplification.

Internal control (IC): A target RNA or DNA sequence that is spiked into a prepared PCR mix. The IC provides a positive control for the reverse transcriptase and DNA polymerase activities.

Internal positive control (IPC): A target RNA or DNA sequence that is spiked into the sample lysis solution and copurified with sample nucleic acids. The IPC controls for proper nucleic acid extraction and proper functioning of reverse transcriptase (RNA targets) and DNA polymerase.

Internal sample control (ISC): A target RNA or DNA sequence endogenous or inherent to the host sample. The ISC controls for the presence of sample, sample quality, extraction efficacy, reverse transcriptase, and DNA polymerase activities.

Master mix: PCR master mixes are premixed, ready-to-use solutions containing DNA polymerase, dNTPs, MgCl2, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. RNA master mixes also include a reverse transcriptase to convert RNA to cDNA.

Melting temperature: The temperature at which 50% of all DNA molecules of a given sequence are present as double-stranded and 50% are present as single-stranded.

Definitions (continued)

Negative control (NC): PCR negative control verifies that there is no cross-contamination between samples or contamination of PCR reagents, instruments, or environment.

Negative extraction control (NEC): A mock sample, usually water, which can be used to detect the presence of cross-contamination between samples during an extraction process. It is recommended to include 1 NEC for every 19 samples being processed for nucleic acid purification.

Polymerase chain reaction (PCR): A process by which DNA molecules are exponentially copied by changing the temperature in the reaction in order to attain a detectable level of DNA.

Positive control (PC): PCR positive control verifies that all PCR reagents and equipment are functioning properly.

Primer: A short synthetic DNA strand that serves as a starting point for DNA amplification.

Ramp: The time it takes for the temperature to increase/decrease as part of the PCR cycling protocol.

Real-time PCR: A common tool for the amplification, detection, and simultaneous quantification of a specific DNA target molecule.

Ribonuclease (RNase): A nuclease that catalyzes the degradation of RNA.

RNA: Ribonucleic acid strands convey genetic instructions from DNA to the translation of proteins. RNA also comprises the genetic information of many viruses.

ROX: A passive reference dye that is used to normalize the fluorescent reporter signal in real-time quantitative PCR and whose fluorescence does not change during the reaction.

RT-PCR: Reverse transcription of the target RNA to cDNA, using the enzyme reverse transcriptase; the cDNA can then be used for PCR.

Standard cycling program: A single PCR program that can be used for multiple RNA and DNA targets.

Threshold: A preset level slightly above the fluorescent background for the detection of DNA-based fluorescence.

Wavelength: The physical characteristic used to describe the excitation and emission of the fluorescent dyes used in real-time PCR. Unit of measure is nanometers (nm).

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