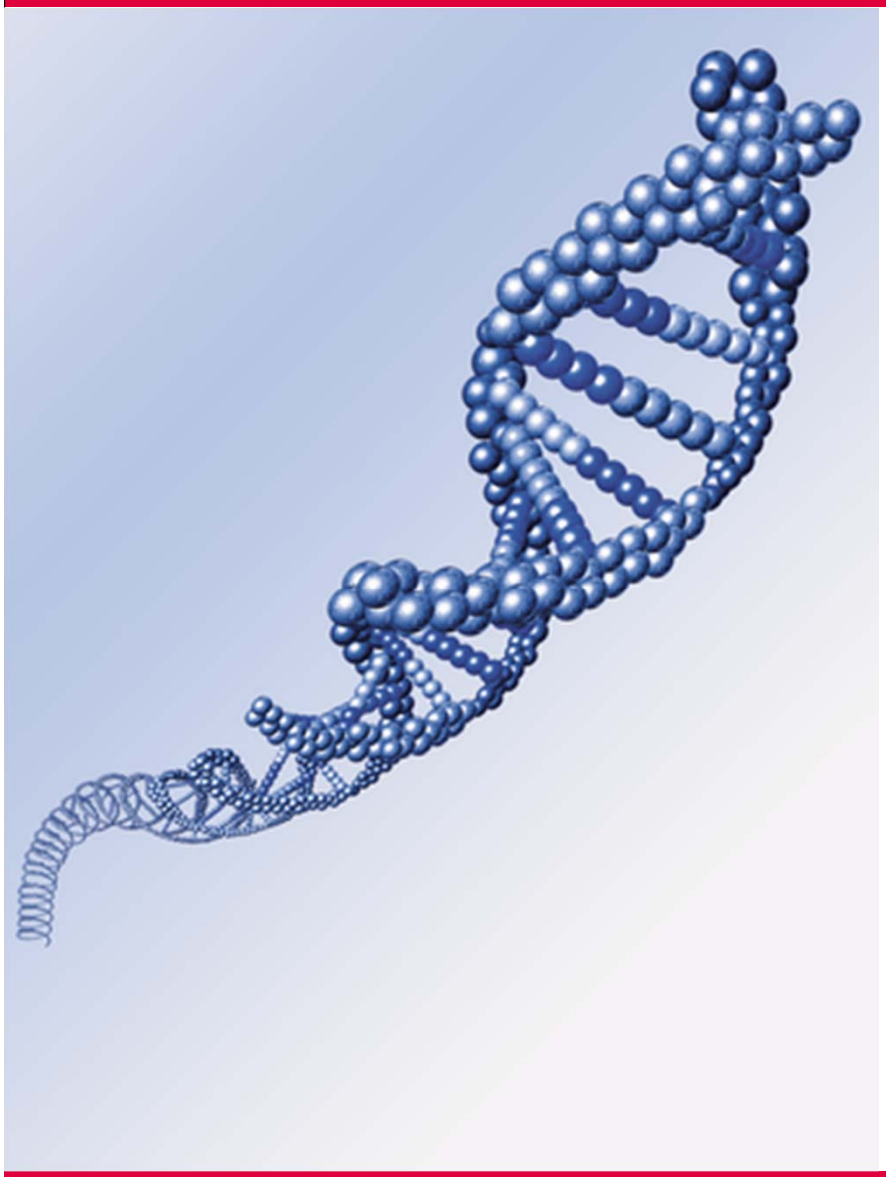

Internal controls for real-time PCR-based pathogen identification

QIAGEN
Dr. Lillian Roth
Lead Scientist Veterinary R&D Team
Global R&D Competence Center
Applied Testing

QIAGEN webinar June 1, 2011

- Introduction
- Internal Controls
- QIAGEN Internal Control
- Examples of applications
- Conclusion

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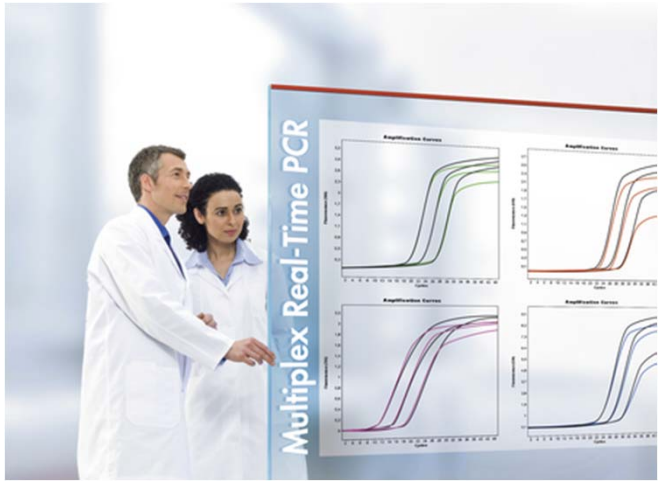
Introduction

Real-time PCR-based pathogen identification — interpreting results

Real-time PCR – sequence amplification and real-time detection using sequence-specific probes



- Provides rapid, sensitive and specific identification of pathogens in biological samples
- Requires appropriate controls for correct interpretation of results

  - Controls:
    - Prevent false positives and false negatives
    - Give you confidence in your results

Adequate controls are essential for real-time PCR-based pathogen identification

Introduction

Real-time PCR-based pathogen identification — interpreting results

Positive result

True or false positive?

Introduction

Real-time PCR-based pathogen identification — interpreting results

Positive result

True or false positive?

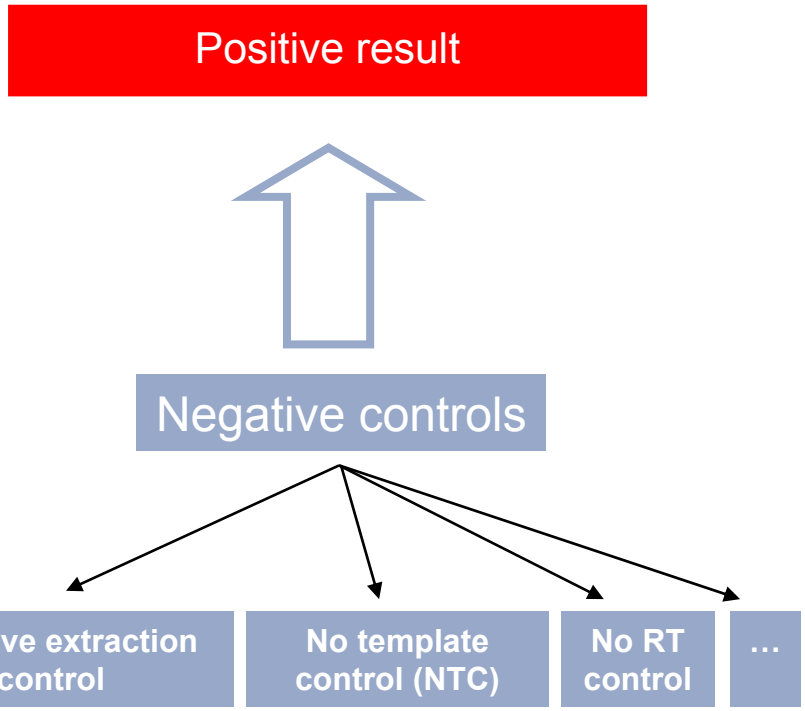
Negative controls

- Negative extraction control
- No template control (NTC)
- No RT control
- …

Negative controls for contamination control

Introduction

Real-time PCR-based pathogen identification — interpreting results



Negative controls for contamination control

Introduction

Real-time PCR-based pathogen identification — interpreting results

Negative result

True and false negative?

Introduction

Real-time PCR-based pathogen identification — interpreting results

Negative result

True and false negative?

Possible reasons for false negative results

- Errors in sample extraction
- Thermal cycler malfunction
- Reverse transcription failure and/or PCR failure (e.g., due to inhibition by heme, bile salts, urea, nucleic acids, …)

How can you identify PCR inhibition?

- Spike one aliquot of the sample with target sequence
- Extract separately
- Detect target sequence in a separate reaction

High workload, too many samples, doubled costs

Introduction

Real-time PCR-based pathogen identification — interpreting results

Negative result

True and false negative?

Internal control (IC)

Simultaneous extraction and/or amplification
of the pathogen target and an internal positive control
within the same tube in a duplex reaction

Internal Control rules out PCR inhibition and other malfunctions

Introduction

Real-time PCR-based pathogen identification — interpreting results

Negative result

True and false negative?

External positive control

Internal control (IC)

Simultaneous extraction and/or amplification
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Real-time PCR-based pathogen identification — interpreting results

Negative result

External positive control

Internal control (IC)

Simultaneous extraction and/or amplification
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Internal Control rules out PCR inhibition and other malfunctions

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Internal Controls
Technical approaches

Endogenous IC

- Occurs naturally in test specimen
- Sequence of host genome or normal microflora genomes (e.g., ß-actin, 16s)

Exogenous IC

- IC template is spiked into sample
  - During nucleic acid extraction
  - Before PCR amplification

Exogenous ICs	
Homologous IC	Heterologous IC
▪ Artificial template ▪ Primers identical to pathogen assay primers ▪ Differentiation by probe	▪ Artificial or natural template ▪ Primers and probe different from pathogen primer/probe

Internal Controls
Technical approaches

Endogenous IC

Pro
▪ Present in specimen – no need to spike IC into sample ▪ Controls host cell lysis and extraction (no control of pathogen lysis) – validation of correct sampling for some applications (e.g. swabs)

Contra
▪ Amount of IC template varies depending on sample type and sampling technique ▪ Cellular pathology associated with the disease may influence expression level (RNA only) ▪ Different host species or sample types require different IC designs ▪ Not possible to differentiate between failure of extraction and amplification

Exogenous IC	
Homologous	**Heterologous**

Internal Controls
Technical approaches

Endogenous IC	Exogenous IC	
	Homologous	Heterologous

Endogenous IC

Pro

- Present in specimen – no need to spike IC into sample
- Controls host cell lysis and extraction (no control of pathogen lysis) – validation of correct sampling for some applications (e.g. swabs)

Contra

- Amount of IC template varies depending on sample type and sampling technique
- Cellular pathology associated with the disease may influence expression level (RNA only)
- Different host species or sample types require different IC designs
- Not possible to differentiate between failure of extraction and amplification

Exogenous IC – Homologous

Pro

- Uses same primers as pathogen target assay - Identical amplification efficiency (Questionable!)

Contra

- Uses same primers as pathogen target assay – Primer competition ("Competitive IC")
- Limited options for IC design/IC assay optimization
- Complex design and synthesis procedure
- New IC design required for each assay (template & primer/probe)

Internal Controls
Technical approaches

Endogenous IC	Exogenous IC	
	Homologous	Heterologous

Risk of impaired assay sensitivity due to PCR competition

extraction (no control of pathogen lysis) – validation of correct sampling for some applications (e.g. swabs)	(Questionable!)
Contra	**Contra**
▪ Amount of IC template varies depending on sample type and sampling technique	▪ Uses same primers as pathogen target assay – Primer competition ("Competitive IC")
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Internal Controls
Technical approaches

Endogenous IC	Exogenous IC	
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Risk of impaired assay sensitivity due to PCR competition

Tedious IC design and/or limited versatility

Contra	Contra
▪ Amount of IC template varies depending on sample type and sampling technique	▪ Uses same primers as pathogen target assay – Primer competition ("Competitive IC")
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Internal Controls
Technical approaches

Endogenous IC	Exogenous IC	
	Homologous	Heterologous
Risk of impaired assay sensitivity due to PCR competition		Pro
		▪ Defined and consistent amount of IC template ▪ Unrestricted design options for optimization of IC properties ▪ One IC can be used for several assays
Tedious IC design and/or limited versatility		
Contra	Contra	Contra
▪ Amount of IC template varies depending on sample type and sampling technique	▪ Uses same primers as pathogen target assay – Primer competition (“Competitive IC”)	▪ No validation of sampling procedure (swabs for intracellular pathogens)
▪ Cellular pathology associated with the disease may influence expression level (RNA only)	▪ Limited options for IC design/IC assay optimization	
▪ Different host species or sample types require different IC designs	▪ Complex design and synthesis procedure	
▪ Not possible to differentiate between failure of extraction and amplification	▪ New IC design required for each assay (template & primer/probe)	

Internal Controls
Technical approaches

Endogenous IC	Exogenous IC	
	Homologous	Heterologous
Risk of impaired assay sensitivity due to PCR competition		
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		▪ Defined and consistent amount of IC template ▪ Unrestricted design options for optimization of IC properties ▪ One IC can be used for several assays
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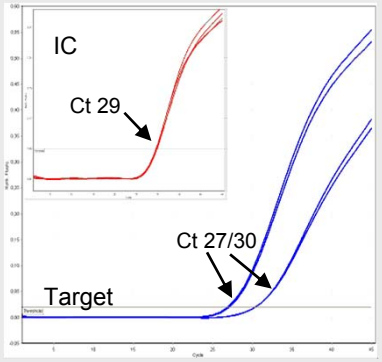
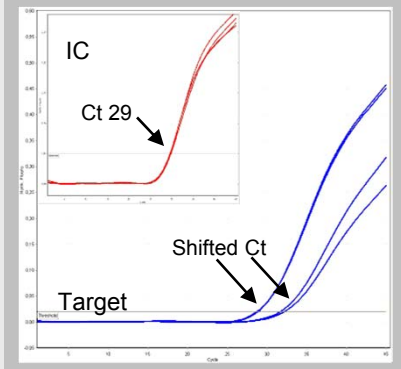
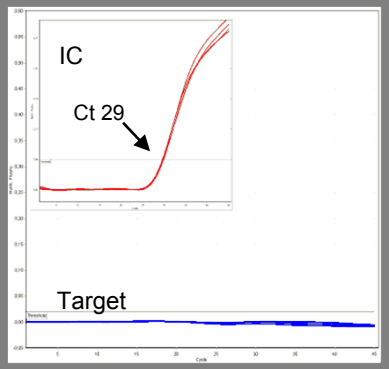
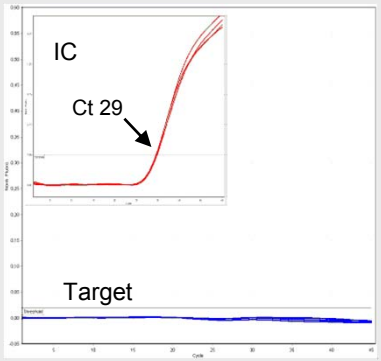
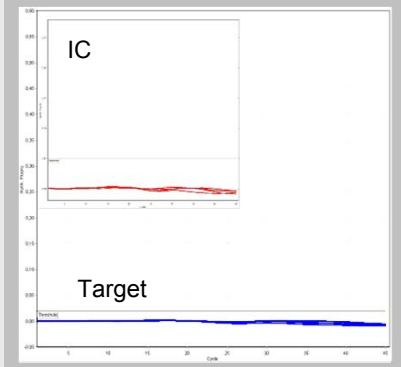
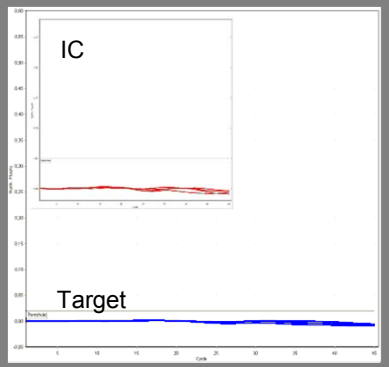
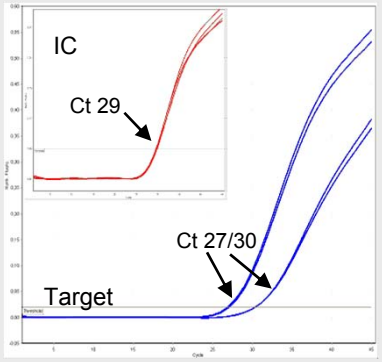
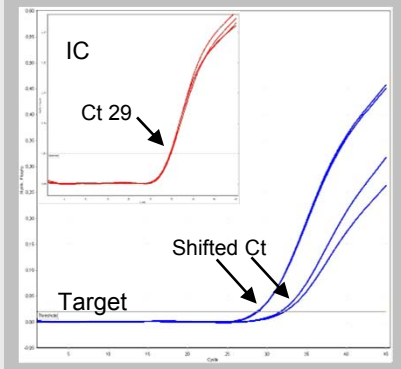
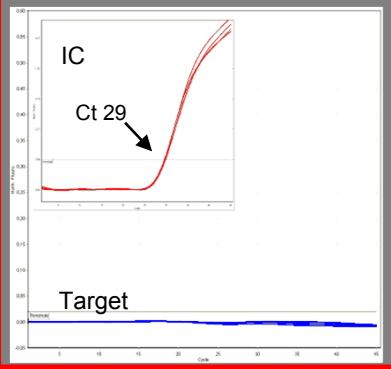
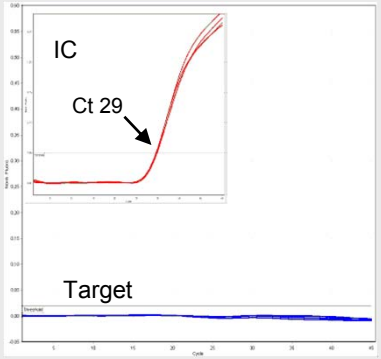
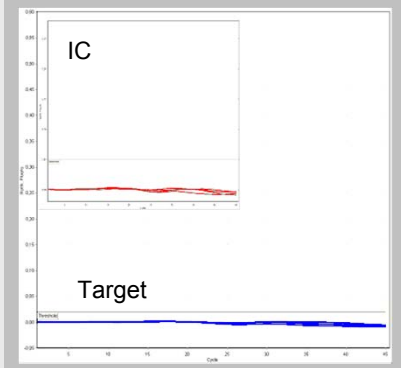
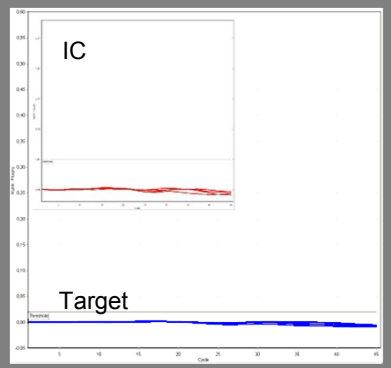
▪ Different host species or sample types require different IC designs	▪ Complex design and synthesis procedure	
▪ Not possible to differentiate between failure of extraction and amplification	▪ New IC design required for each assay (template & primer/probe)	
		Enables non-competitive IC design and easy implementation of new assays

Internal Controls
Design requirements

	No inhibition	Low inhibition	Strong inhibition
IC too strong			
IC too weak			

Poor design or setup can corrupt IC function

Internal Controls
Design requirements

	No inhibition	Low inhibition	Strong inhibition	
IC too strong				**Risk of false negative results**
IC too weak				

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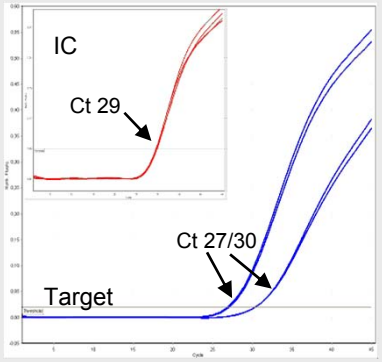
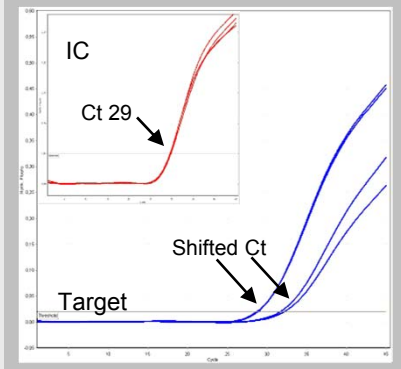
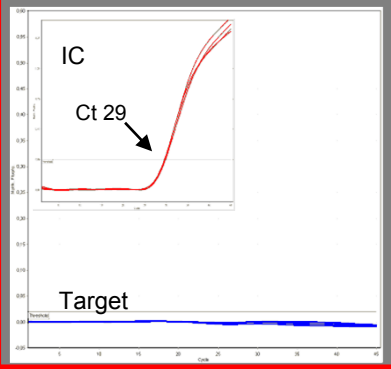
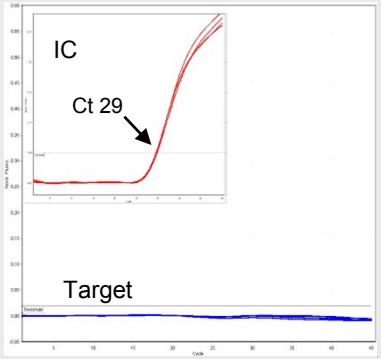
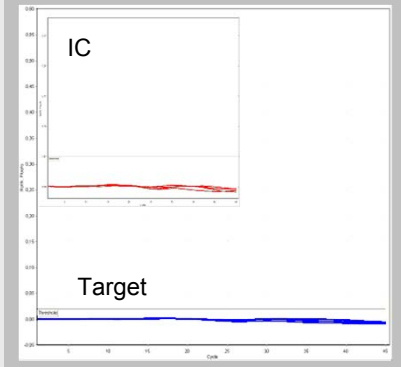
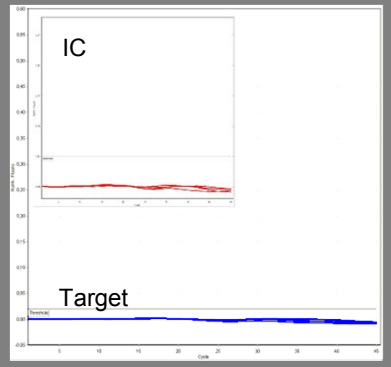
IC too strong			
IC too weak			

Risk of false negative results

Poor design or setup can corrupt IC function

Internal Controls
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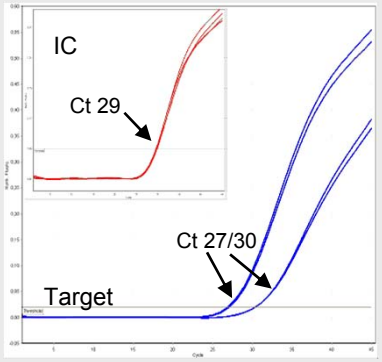
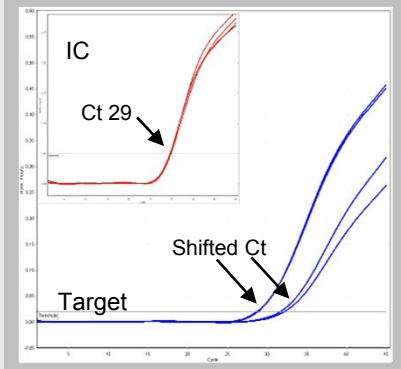
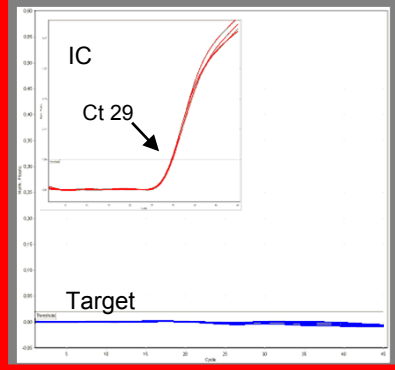
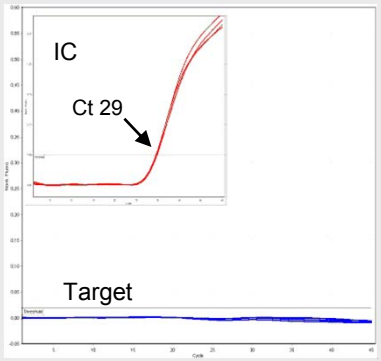
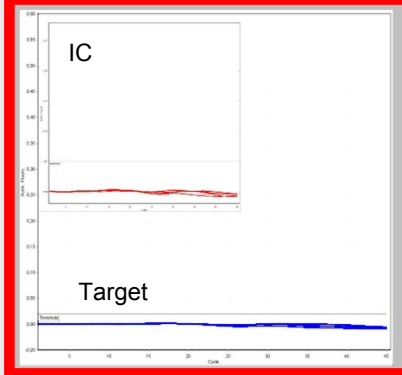
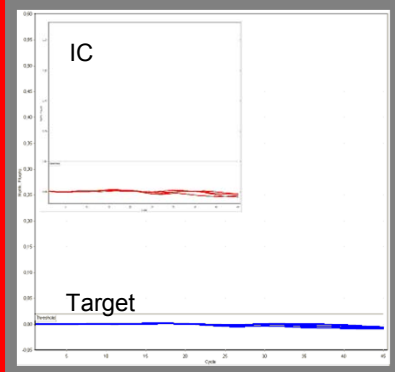
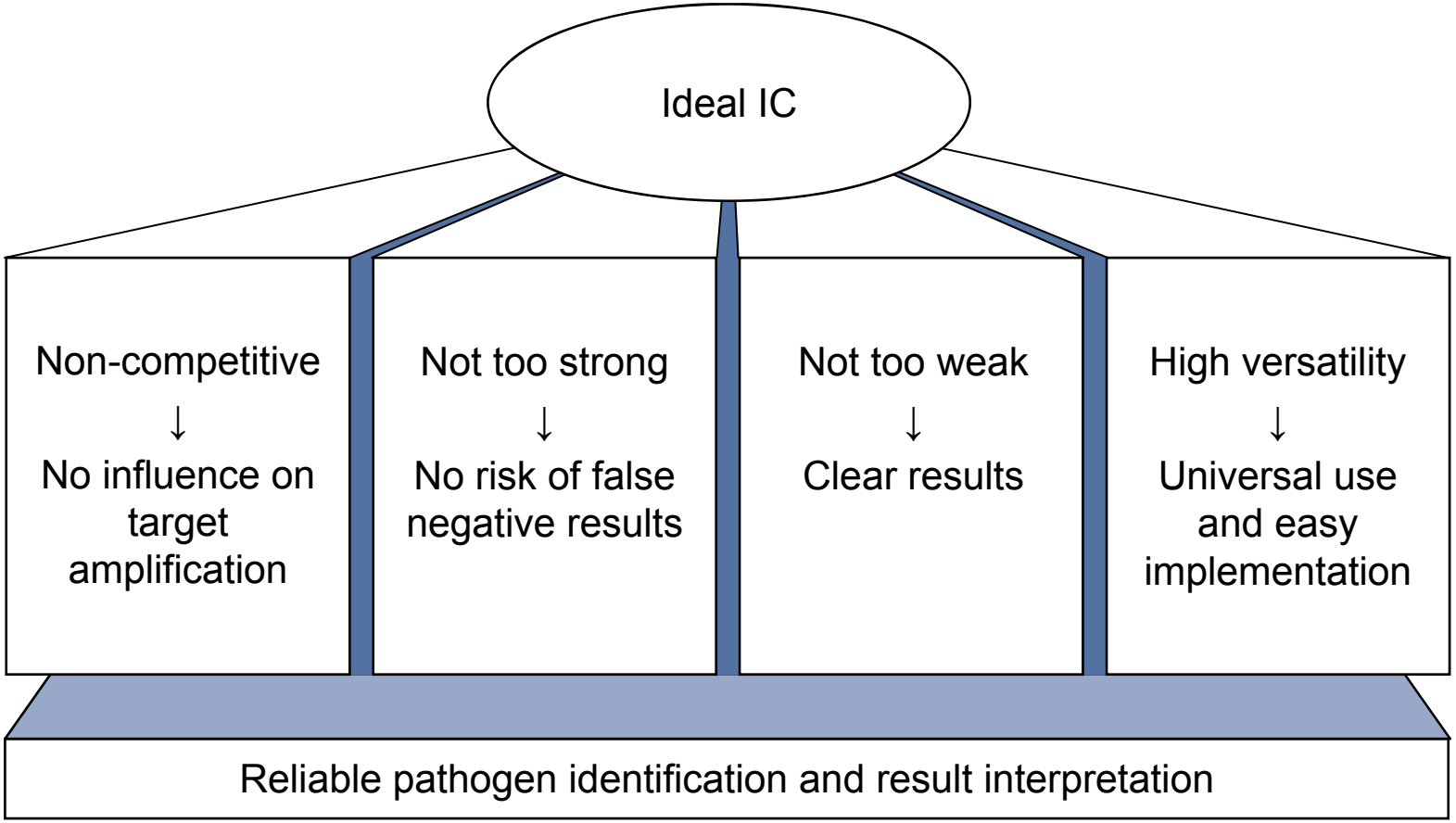
IC too strong				**Risk of false negative results**
IC too weak				**True negatives give questionable results ↓ Re-testing**

Poor design or setup can corrupt IC function

Internal Controls
Design requirements



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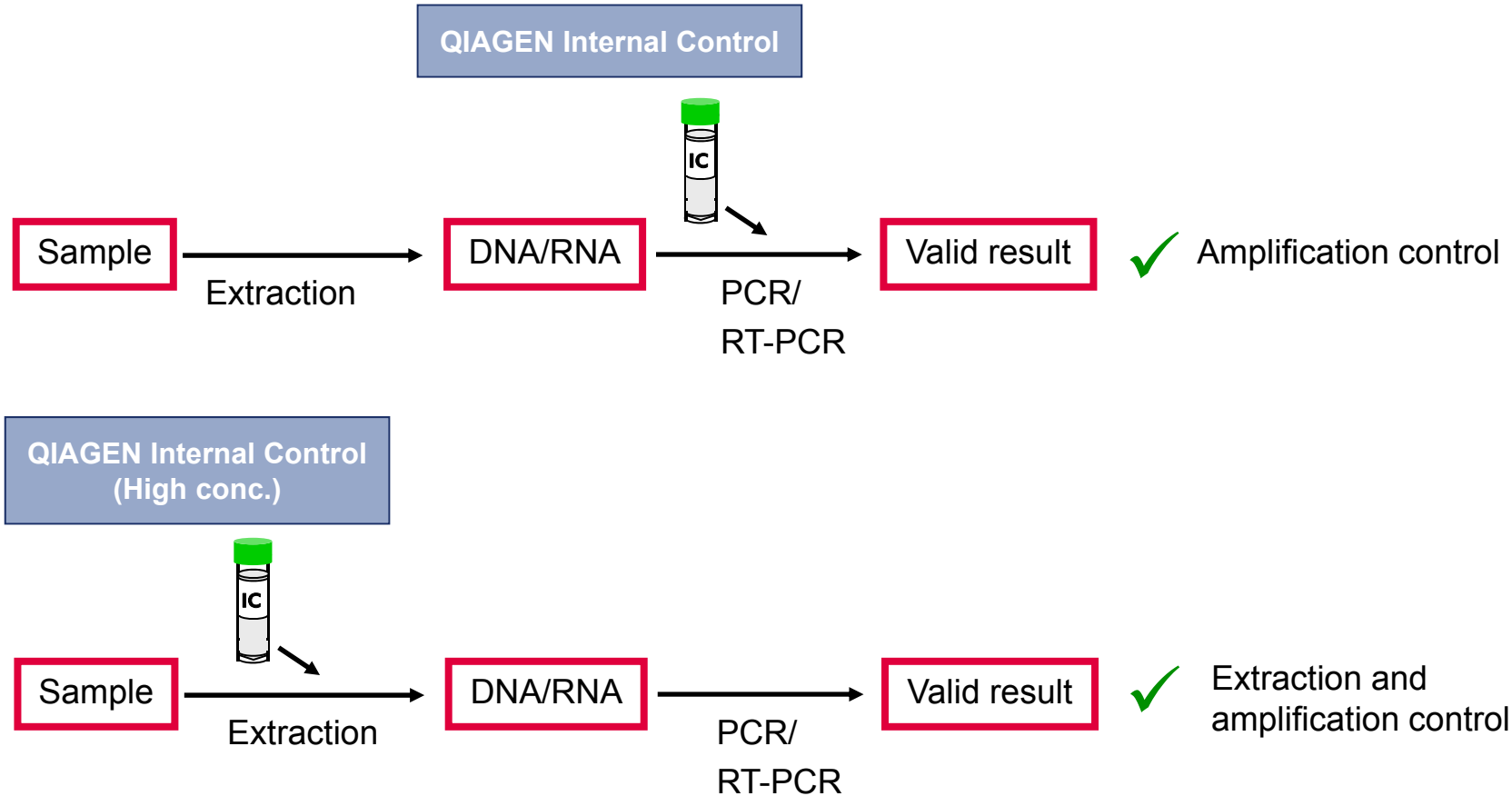
QIAGEN Internal Control
QuantiFast Pathogen +IC Kits — a new generic IC concept

- Highly sensitive real-time PCR or one-step RT-PCR chemistry
- Co-amplification of pathogen target and QIAGEN IC
- QIAGEN IC:
  - Exogenous IC
  - Unique artificial sequence
  - No competition of pathogen target and IC in duplex PCR
    - Non-competitive design of IC assay
    - Pre-optimized conditions with new chemistry
  - Correct indication of inhibition
  - Universal use with existing or new dual-labeled probe-based assays
  - Implementation without need for optimization
  - Two variants:
    - DNA IC for detection of viral and bacterial DNA
    - RNA IC for detection of viral RNA

New generic IC concept – ready-to-use with dual-labelled probe-based assays

QIAGEN Internal Control
Flexible use



Flexible use — control of extraction and/or amplification

QuantiFast Pathogen +IC Kits
Kit content

QuantiFast Pathogen PCR +IC Kit (100, 400)

- 5x QuantiFast Pathogen MasterMix
- Internal Control Assay (lyophilized), Buffer TE
- RNAse-free water
- QuantiTect NA Dilution Buffer
- 50x ROX Dye Solution
- 50x High-ROX Dye Solution
- Internal Control DNA (lyophilized)

QuantiFast Pathogen RT-PCR +IC Kit (100, 400)

- 5x QuantiFast Pathogen MasterMix
- 100x QuantiFast Pathogen RT Mix
- Internal Control Assay (lyophilized), Buffer TE
- RNAse-free water
- QuantiTect NA Dilution Buffer
- 50x ROX Dye Solution
- 50x High-ROX Dye Solution
- Internal Control RNA (lyophilized)

Internal Control DNA (High conc.)

Internal Control RNA (High conc.)

QuantiFast Pathogen +IC Kits
Which assays can be used with the QIAGEN IC?

- IC assay with MAX labeled probe – HEX/JOE/VIC equivalent for generic use with dual-labelled probe-based assays
- Pathogen assay design specifications for optimal performance of co-amplification with IC
  - Typical assay design to work at 60°C combined annealing/extension protocol
  - Follow standard real-time PCR assay design specifications
    - Using specialized design software (Primer3, Primer Express, …)
    - Using standard algorithm parameters and reaction conditions
      - Tm of primers 58-63°C and within 2°C of each other
      - Tm of probes 5-10°C higher than the Tm of the primers
  - Amplicon size ≤ 150 bp
  - FAM + non-fluorescent quencher recommended

Compatible with established, published, and new assays for pathogen identification

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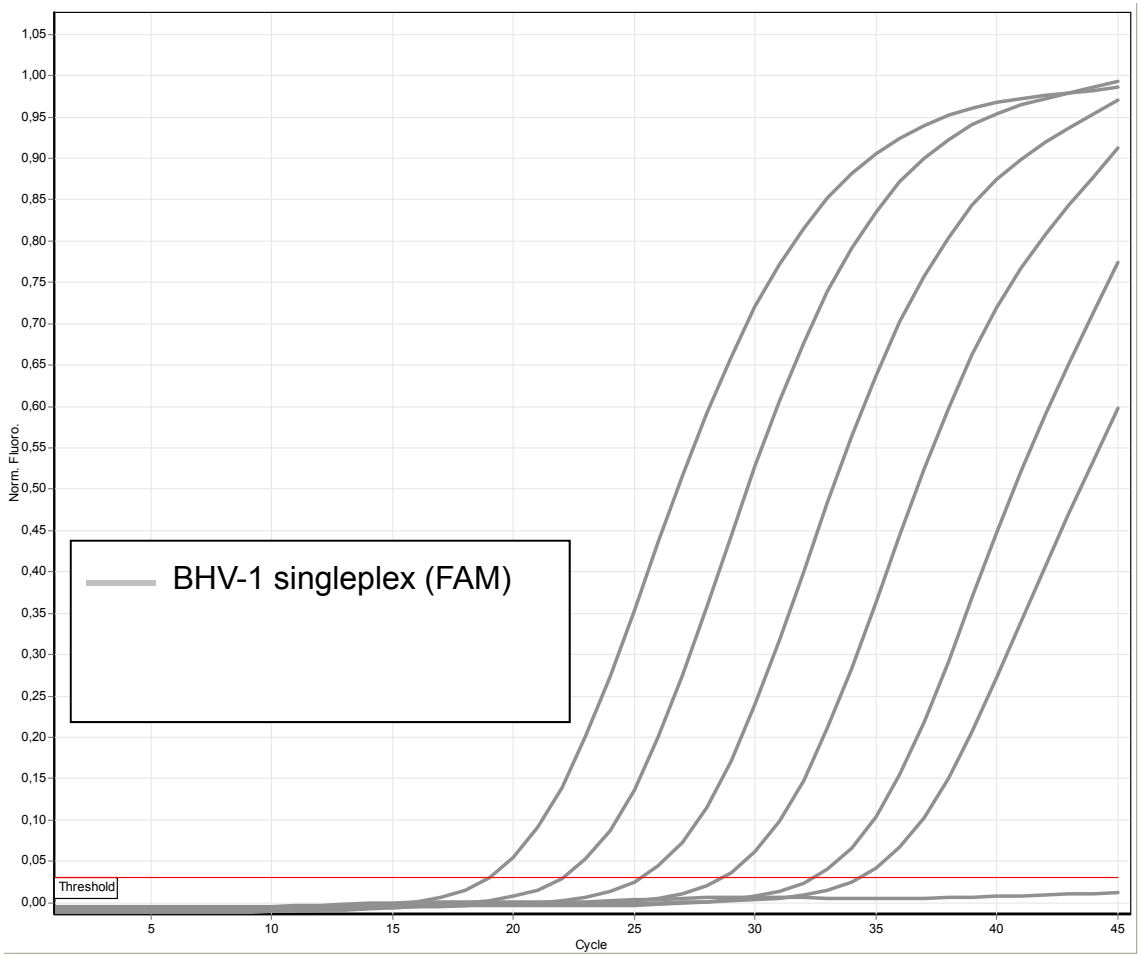
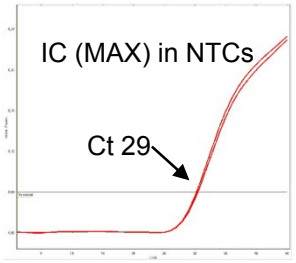
Examples of applications
Comparison co-detection with IC vs. singleplex detection

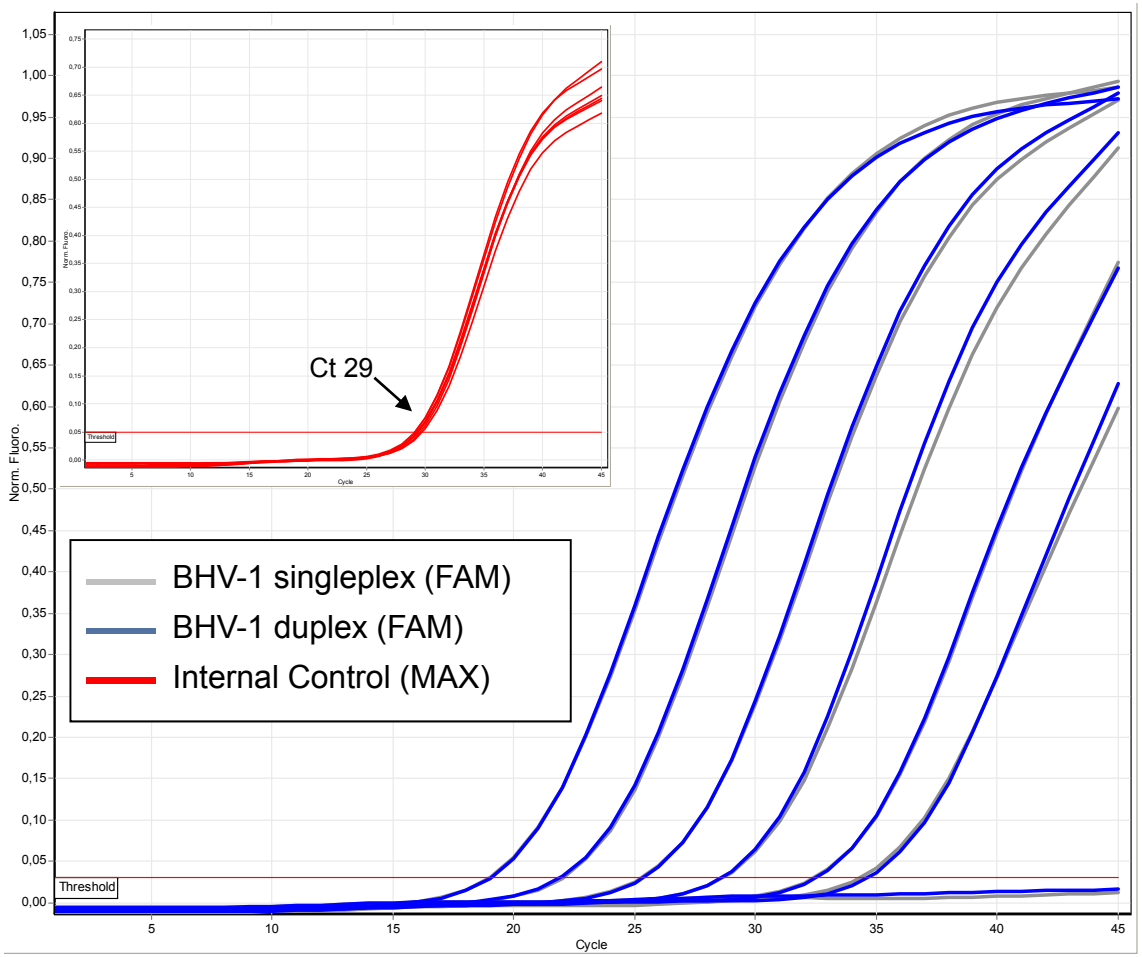


- Serial dilution of Bovine herpes virus type 1 DNA from viral culture
- Detection with QuantiFast Pathogen chemistry in singleplex or duplex reactions with IC on Rotor-Gene Q
- Setup according to handbook, without any PCR optimization
- One of 3 replicates is shown
- IC signal in NTCs serves as reference



Identical sensitivity and Ct values of BHV-1 co-detection with IC compared to singleplex detection

Examples of applications
Comparison co-detection with IC vs. singleplex detection



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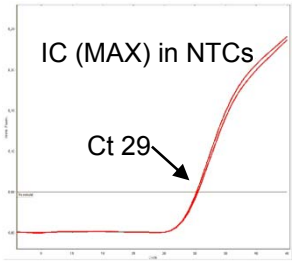
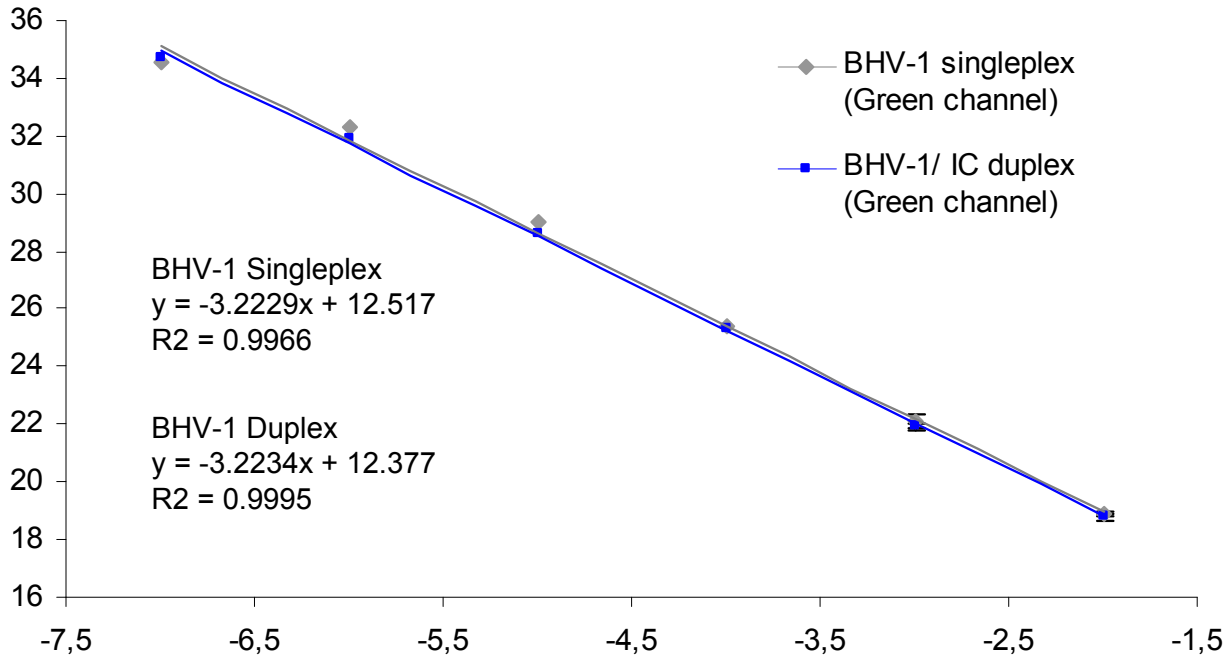


Identical sensitivity and Ct values of BHV-1 co-detection with IC compared to singleplex detection

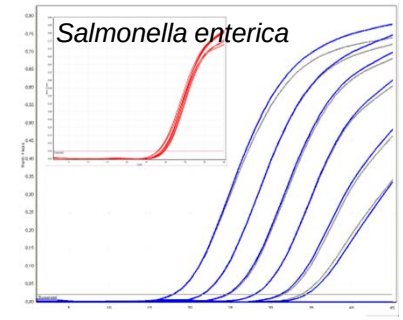
Examples of applications
Comparison co-detection with IC vs. singleplex detection

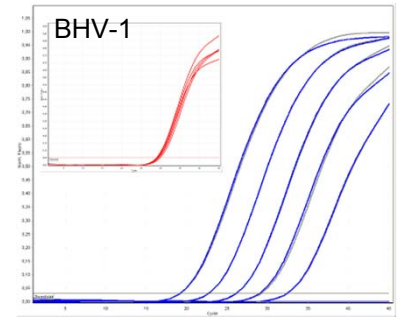


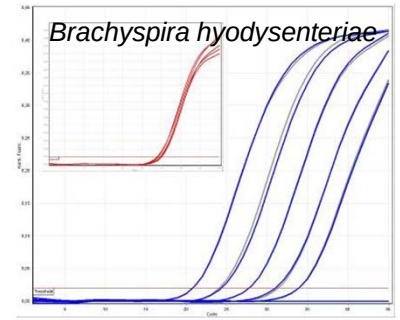
- Corresponding standard curves plotting log template dilution vs. mean Ct values
- Error bars represent ±1 SD of 3 replicates
- High and identical efficiency, linearity and precision

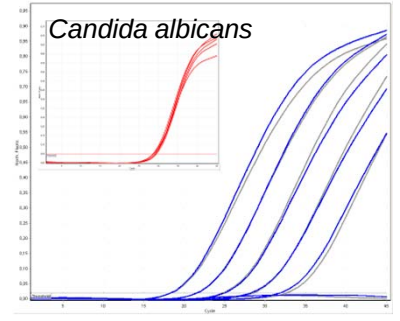
Identical precision, efficiency and linearity of BHV-1 co-detection with IC compared to singleplex detection

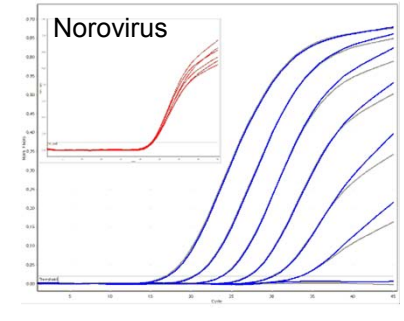
Examples of applications
Identical singleplex and duplex performance for various targets

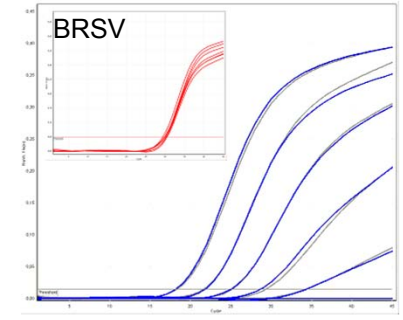


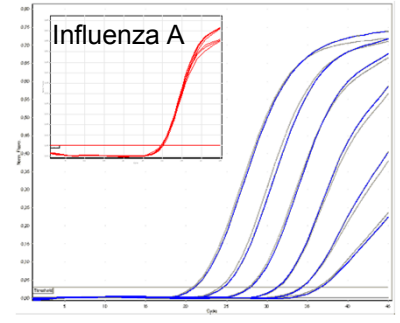


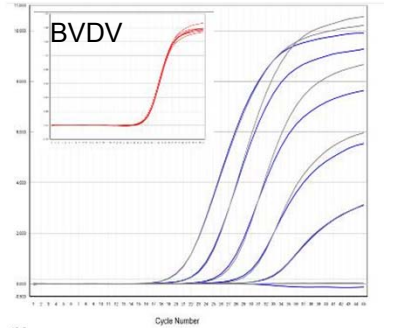








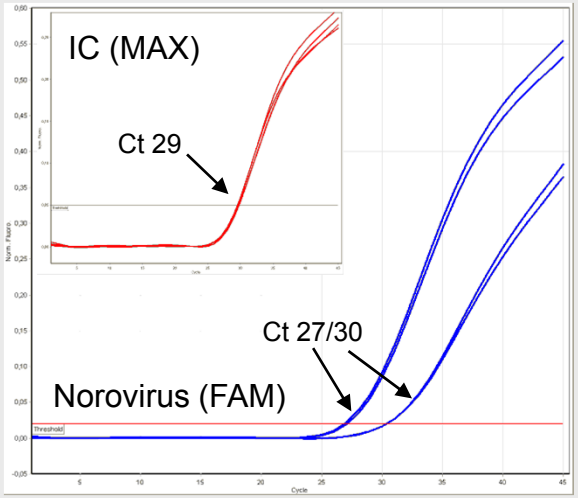




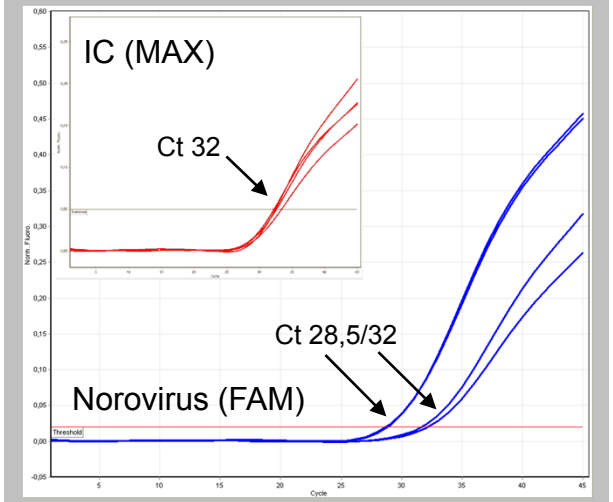
Identical performance of co-detection with IC compared to singleplex detection for DNA and RNA targets

Examples of applications
Reliable indication of inhibition

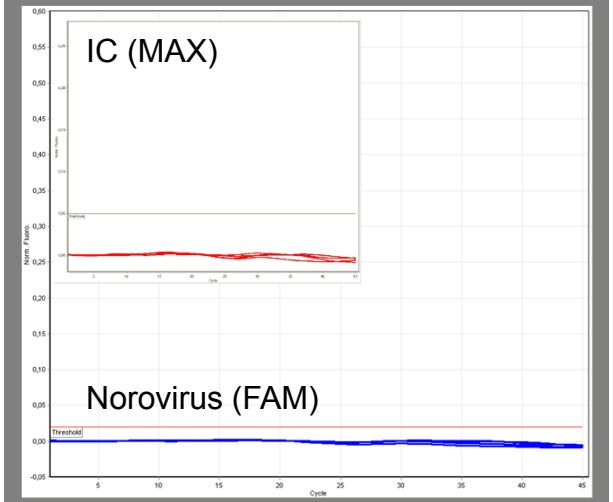
No inhibition



Low inhibition
25 ng humic acid

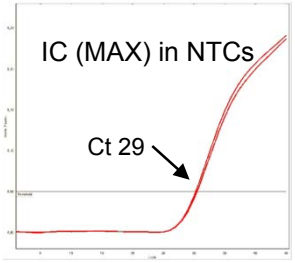


High inhibition
100 ng humic acid

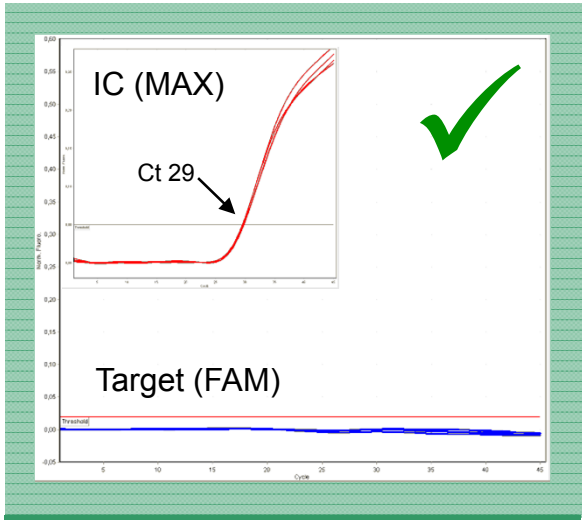


Reliable indication of inhibition by correctly designed QIAGEN IC –
IC shift or failure reflects target inhibition

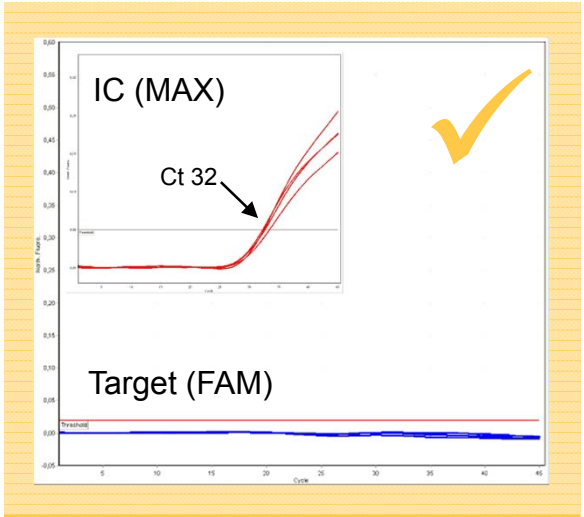
Examples of applications
Correct interpretation of negative results in unknown samples



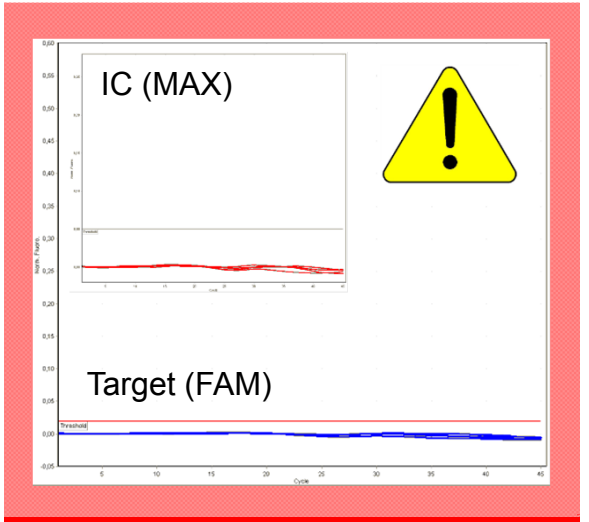
- IC signal in NTCs or negative purification controls serve as a reference



Valid negative result



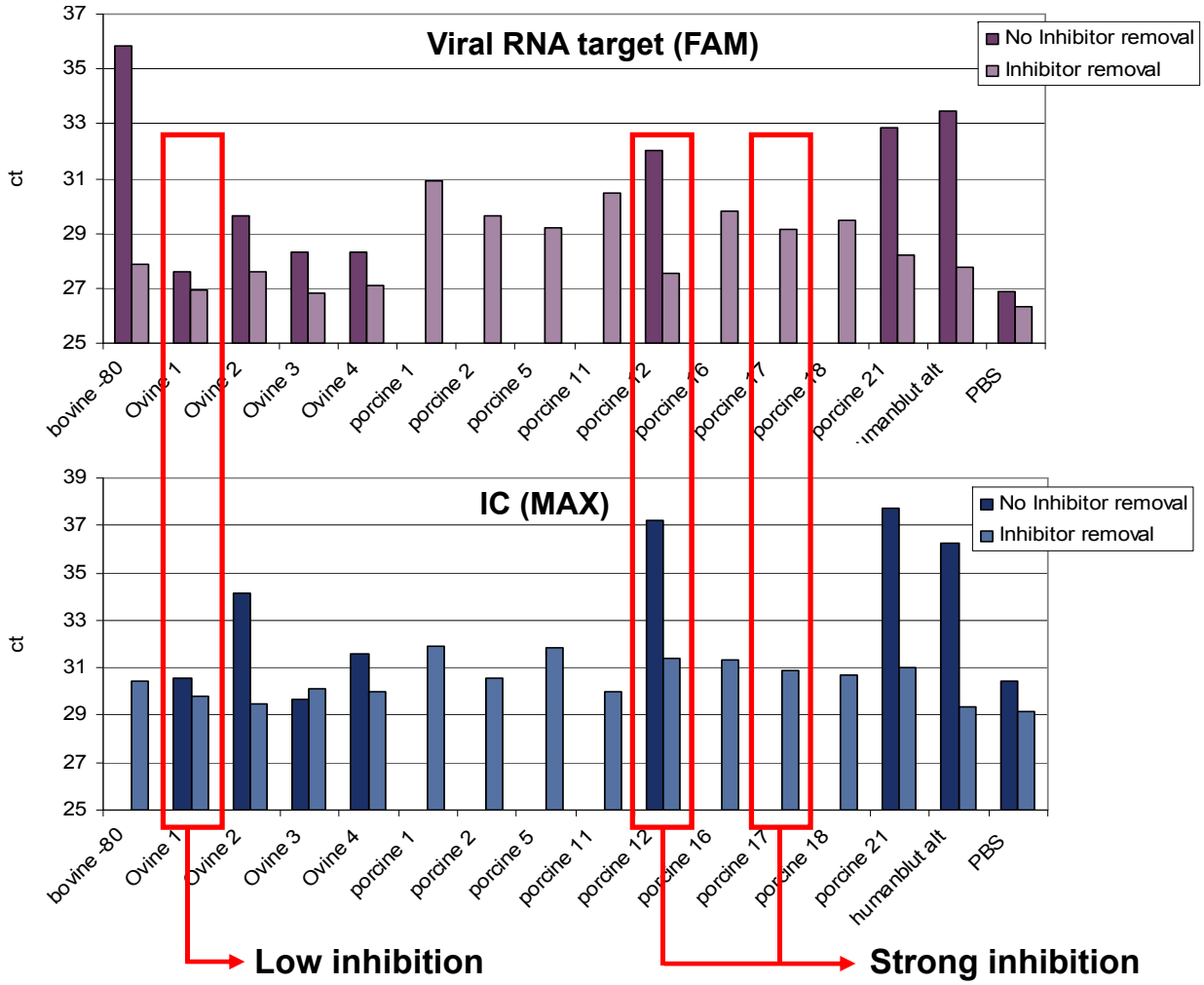
Slight inhibition;
very low target concentrations
might be influenced



Reaction failed

Correct interpretation of negative results – IC shift or failure warns against inhibition

Examples of applications
Identification of negative effects on target identification



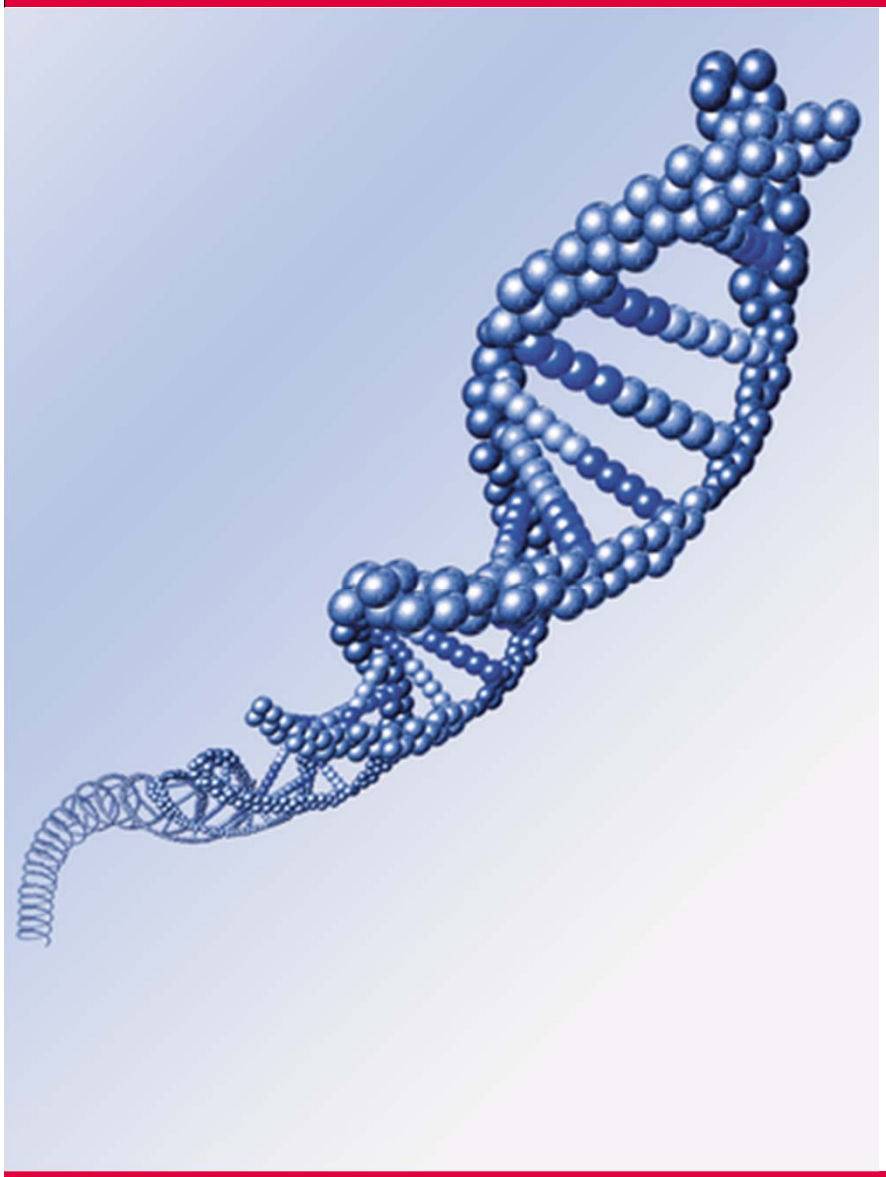
- Various inhibitory blood samples spiked with RNA virus from cell culture supernatant
- Automated viral RNA isolated with or without inhibitor removal on the QIAxtractor
- Detection with QuantiFast Pathogen RT-PCR +IC Kit and target-specific primer/ probe performed on Rotor-Gene Q

IC reflects low or strong inhibition of the target (FAM)

- Introduction
- Internal Controls
- QIAGEN Internal Control
- Application Examples
- Conclusion

QuantiFast Pathogen +IC Kits
Conclusion

- Valid real-time PCR results require the use of adequate controls
- Careful IC design and set-up is the prerequisite of a functional IC system
- QuantiFast Pathogen +IC Kits offer
  - Highly sensitive and rapid real-time PCR or one-step RT-PCR
  - Increased process safety — correct interpretation of negative results through co-amplification of user-defined pathogen target and IC
  - Pre-optimized QIAGEN IC
    - Non-competitive design and set-up
    - Accurately reflects inhibition of the target system
    - Is compatible with most standard real-time PCR assays
    - Eliminates the need for optimization of duplex set-up with the target
    - Flexible to use as PCR and/or extraction IC
  - Fast universal two-step protocols
  - Parallel read-out of different pathogen assays and easy implementation of new assays



Thank you!

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Global R&D Competence Center
Applied Testing

QIAGEN webinar June 1, 2011