

Reverse Transcription Qualitative Real-Time PCR (RT-qPCR)

SegoliP Unit

Reuben K. Mwangi

International Livestock Research Institute
ILRI Biosciences

Viral Pathogen Genome Sequencing and Bioinformatics Analysis
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What are the Key Expectations from an RT-qPCR Procedure?

- ① Disease diagnosis
 - Positive or Negative
- ② Quantitative test - absolute quantify the amount of viral copies / titres
- ③ Determine success of sequence coverage - Based on the Cq Value
- ④ Quantify libraries
- ⑤ Gene expression



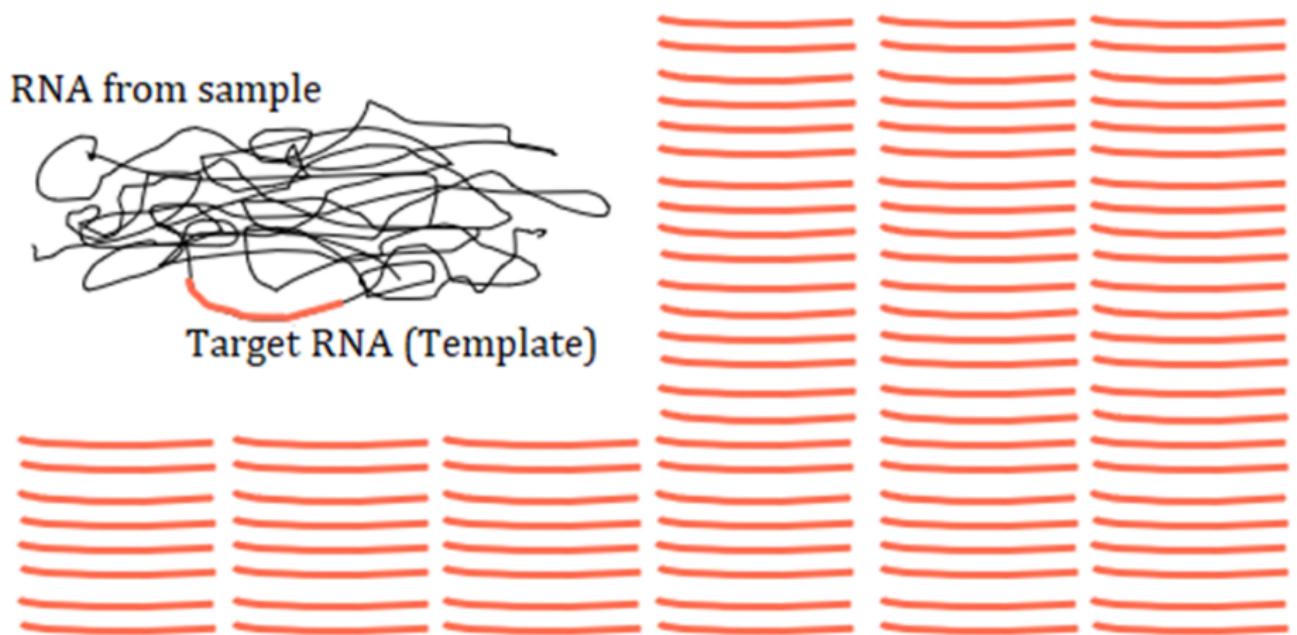
RT-qPCR and Genome Coverage [example]

The RT-qPCR result may be an indicator on the level of viral genome sequence coverage.

Sample	lineage	scorpio_call	nextclade_clade	Genome fraction (%)
COVM04948	GE.1	Omicron (XBB-like)	22F	91.5
COVM04949	FY.4.1	Omicron (XBB-like)	22F	96.4
COVM04950	XBB.1.22.1	Omicron (Unassigned)	22F	89.8
COVM04952	XBB.1.22.1	Omicron (Unassigned)	22F	87.1
COVM04953	Unassigned	Probable Omicron (Unassigned)	22F	40.4
COVM04954	GE.1	Omicron (XBB-like)	22F	91.1
COVM04955	GE.1	Omicron (XBB-like)	22F	95.1
COVM04956	GE.1	Omicron (XBB-like)	22F	91.2
COVM04957	Unassigned	Probable Omicron (Unassigned)	22F	68
COVM04958	GE.1	Omicron (XBB-like)	22F	89.4
COVM04959	XBB	Omicron (XBB-like)	22F	92.2
COVM04960	FY.4.1	Omicron (XBB-like)	22F	92.6
COVM04961	GE.1	Probable Omicron (XBB-like)	22F	90.3
COVM04962	Unassigned		22F	10.1
COVM04964	FY.4.1	Omicron (XBB-like)	22F	94.6
COVM04965	GE.1	Omicron (XBB-like)	22F	94.5

What is PCR?

The polymerase chain reaction (PCR) is a relatively simple in vitro technique to amplify (make multiple copies of) a specific sequence (i.e. a small region or fragment) of DNA from a complex mixture of DNA.

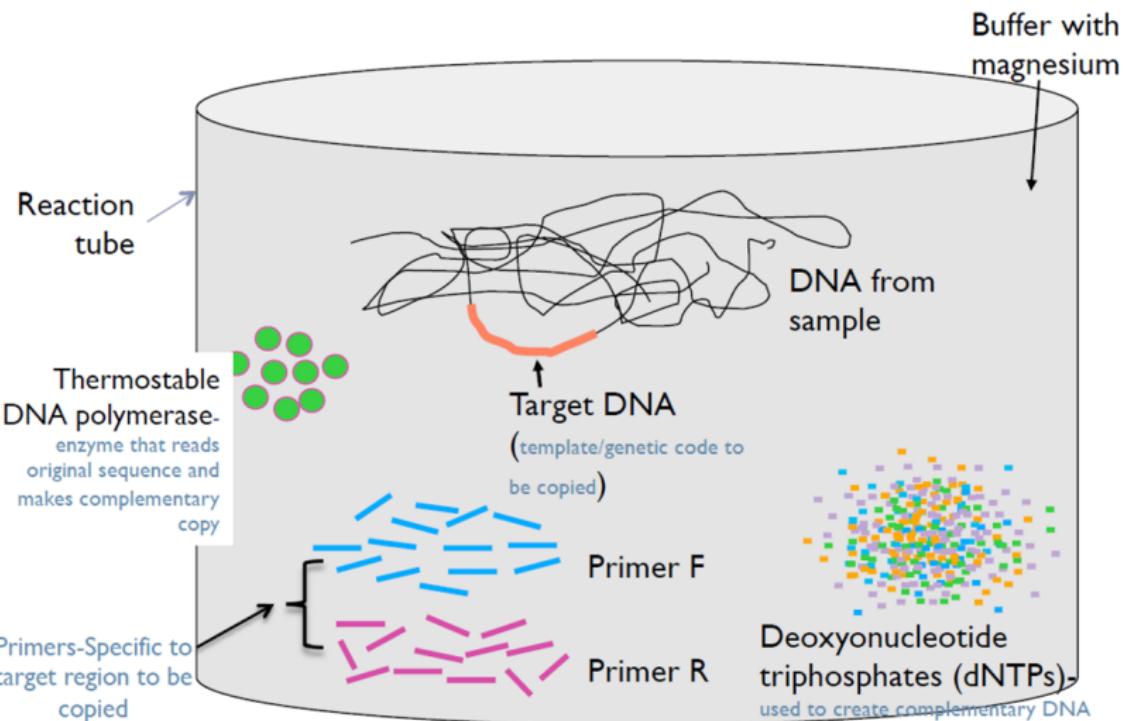


qPCR, RT-PCR, RT-qPCR

- Quantitative real-time PCR(qPCR) – PCR amplification of DNA in real time, measured by a fluorescent probe, enabling quantitation of the PCR products.
- Reverse transcription PCR (RT-PCR) – Allows the use of RNA as a template to generate complementary DNA (cDNA) and amplification of specific RNA targets using PCR.
- Reverse transcription real-time PCR (RT-qPCR) – combines RT-PCR with qPCR to enable the measurement of RNA levels through the use of cDNA in a qPCR reaction.



PCR reaction components



Steps in RT - Real-Time PCR

RT-qPCR is a quantitative PCR that characterizes and detects amplicons in a progressive manner, using Kinetics at the exponential phase from an RNA target.

- ① Reverse transcription of RNA to cDNA
- ② double-stranded DNA (cDNA) is heat denatured,
- ③ Probe anneals to single DNA strand; Primers anneal aligned to Fwd/Rev strand.
- ④ The primers are extended by DNA polymerase, resulting in a duplicate strand.
- ⑤ The DNA Polymerase with exonuclease activity cleaves off bases and reporter dye
- ⑥ Process of denaturation, annealing, and elongation repeated 20-40 times. Real-Time PCR detection takes place at the exponential phase



qPCR Chemistries

Currently, most qPCR experiments commonly employ the **dsDNA intercalating dye** (e.g., SYBR® Green) or **hydrolysis probes** (e.g., TaqMan®) chemistries to monitor amplification.

qPCR Setup

Chemistry Type **Hydrolysis Probes**

Targets

Name	Target
Type here to add	

Intercalating Dye
Hydrolysis Probes
Dual Hybridisation Probes
Molecular Beacon
LUX Primers

African Union 
AFRICA CDC  **ASLM**
AFRICAN SOCIETY FOR LABORATORY MEDICINE

File Edit Analysis Tools Help

Properties Method Plate Run Results Export

Experiment Properties

Name: 2021-05-26_090014
Barcode: Barcode - optional
User name: User name - optional
Instrument type: QuantStudio™ 5 System
Block type: 96-Well 0.2-mL Block
Experiment type: Standard Curve
Chemistry: TaqMan® Reagents
Run mode: Standard

Reporter Dye

- FAM™
- FAM™
- Fluorescein
- JOE™
- HEX™
- CAL Fluor® Orange 560
- VIC®
- Cy™ 3
- NED™
- ROX™
- CAL Fluor® Red 610
- Texas Red®
- Cy™ 5
- Quasar® 670
- LC® Red 670
- Allelic Target

File Edit Analysis Tools Help

Properties Method Plate Run Results Export

Experiment Properties

Name: 2021-05-26_090044
Barcode: Barcode - optional
User name: User name - optional
Instrument type: QuantStudio™ 5 System
Block type: 96-Well 0.2-mL Block
Experiment type: Standard Curve
Chemistry: SYBR® Green Reagents
Run mode: Standard

Reporter Dye

- SYBR® Green
- SYBR® Green
- LC Green®
- Eva Green®
- SYTO® 9
- SYTO® 81
- SYTO® 62

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qPCR Chemistries - Hydrolisis Probes Principle

Uses two Principles-

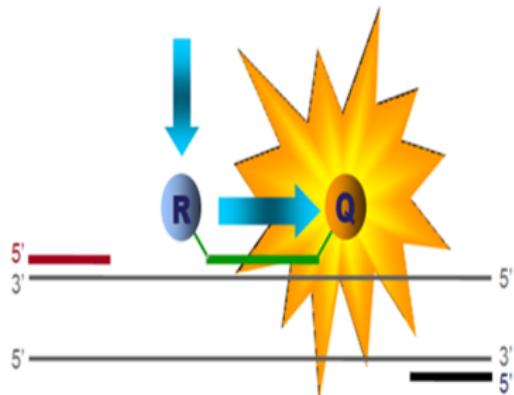
- a 5' Nuclease activity of DNA Polymerase some DNA Polymerases harbor “proof-reading” capacity to **nick out bases** i.e., 3' to 5' exonuclease activity(Hydrolysis) in addition to the usual 5' to 3' activity of adding bases
- b Fluorescence Resonance Energy Transfer- FRET The emission(Reporting) energy of a molecule is captured by the excitation band of an adjacent molecule(Quencher)



qPCR Chemistries - Hydrolisis Probes Principle cont...

The Reporter Dye e.g., FAM is excited by light energy from the instruments lamp. However, it is in very close proximity with another molecule(Quencher) that absorbs its emission energy and thus the emission of FAM dye is not seen by a detector.

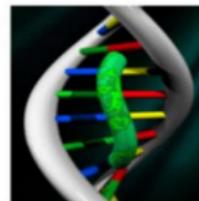
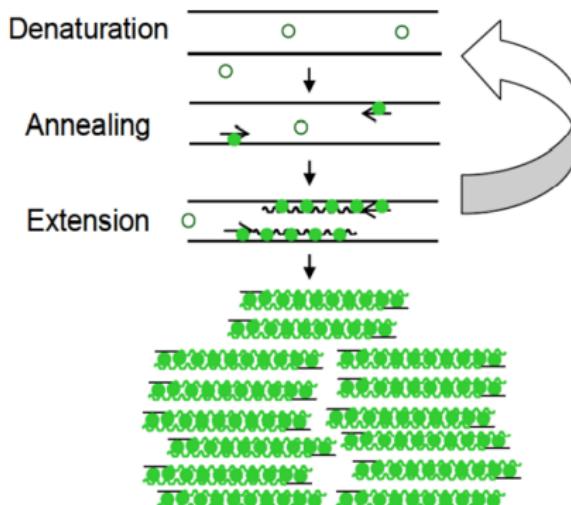
Software is used to discern the emission per cycle. The light emitted is proportional to the amplicons produced.



- Fluorescence Resonance Energy Transfer (FRET) from high energy (Reporter) to low energy (Quencher) dye
- No reporter signal with intact probe

qPCR Chemistries - Intercalating dyes

Examples - SYBR ® Green, Eva Green®, SYTO 9®



ThermoFisher
SCIENTIFIC



Terminology in qPCR Results Analysis

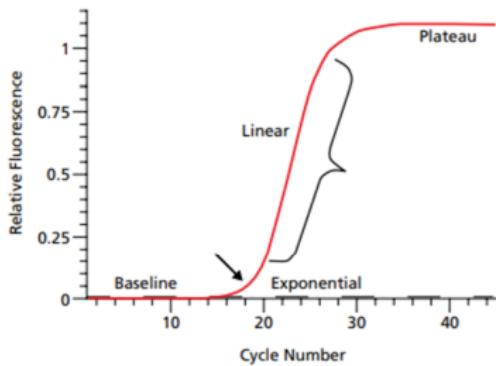
- **Cq (quantification cycle)** A generic term which includes Ct, (see below), crossing point (Cp), and all other instrument specific terms referring to the cycle used to quantify the concentration of target in the qPCR assay.
- **Ct (threshold cycle)** The Ct is defined as the number of cycles required to produce a constant emission of fluorescence. The constant fluorescent emission is recorded relative to a defined threshold setting and the cycle number at which the fluorescence generated crosses the threshold is the reaction Ct. **(N.B. The MIQE guidelines propose that Ct be replaced by Cq.)**



Visualizing qPCR Results

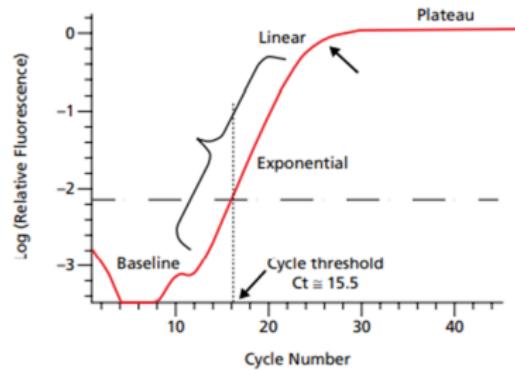
Standard Curve					
Equation	$y = -3.57x + 11.36$				
Efficiency	0.91				
R ²	0.9994				
Standards Results					
Well	Sample Name	C _q	Given Concentration (ng/μL)	Calculated Concentration (ng/μL)	Variation
25	Std1	6.55	20	22.25	11.25%
26	Std1	6.61	20	21.46	7.29%
27	Std1	6.66	20	20.74	3.7%
28	Std3	14.04	0.2	0.1783	10.83%

Standard X-Y Plot



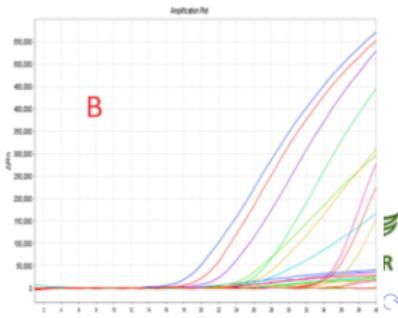
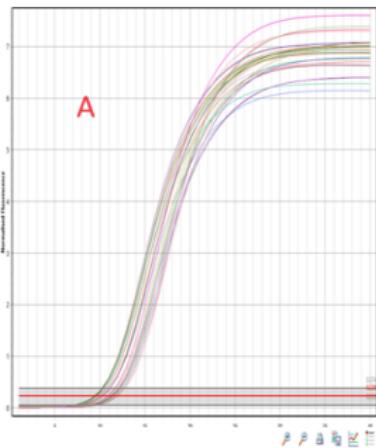
Afric
Uni

Semi-log Plot



Analysis of qPCR Results

	Intercalating Dye Chemistry	Hydrolysis Probe Chemistry
Linearity	$R^2 \geq 0.98$	$R^2 \geq 0.98$
Reproducibility	Replicate curves shall not vary by more than $1 C_q$.	Replicate curves shall not vary by more than $1 C_q$.
RFU Consistency	Maximum plateau fluorescence signal for all curves shall be within 20% of the mean. Fluorescence signal shall not be jagged.	Increase of fluorescence signal shall be consistent for all curves, exhibiting parallel slopes. Fluorescence signal shall not be jagged.
Curve Steepness	Curves shall rise from baseline to plateau within $10 C_q$ values or less.	Curves shall rise from baseline to 50% maximum RFU within $10 C_q$ values or less.
Curve Shape	Curves shall exhibit a sigmoidal shape, resulting in a plateau of fluorescence signal.	Curves need not be sigmoidal, but shall appear to be reaching a horizontal asymptote by the last PCR cycle.



Use of qPCR in Next-generation Sequencing

- The most **accurate and sensitive** method to **quantify libraries** is quantitative real-time PCR, which measures only the number of amplifiable DNA molecules with complete sequencing adaptors at both ends, even in very dilute libraries.
- **Each library** must be properly quantified before pooling and/or loading onto a flow cell to **ensure equimolar amounts of indexed libraries** in multiplexed applications and an **optimal cluster density**.
- **Underestimation of library concentration** may result in higher than desired cluster density (**over-clustering**), which hinders cluster resolution, while overestimation may generate lower cluster density (**under-clustering**).



Absolute Quantification of libraries example

Sample name ¹	dsDNA concentration (pM) ^{2,3}
Std 1	20
Std 2	2
Std 3	0.2
Std 4	0.02
Std 5	0.002
Std 6	0.0002

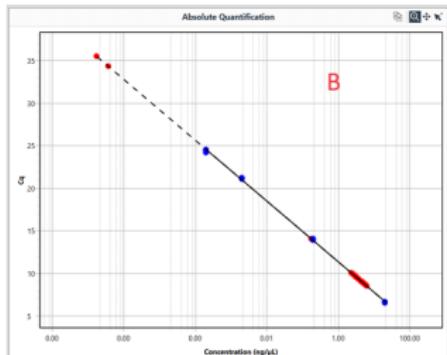
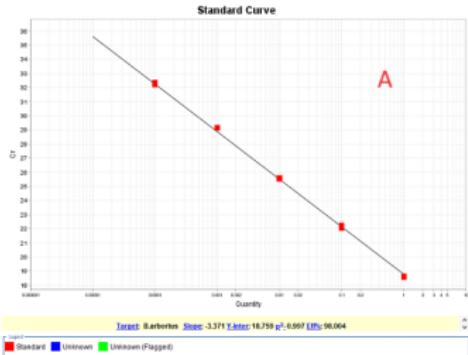
Sample Name	Reporter	Quencher	Quantity Mean	Quantity SD
Lib 1	SYBR	None	0.820	0.040
Lib 2	SYBR	None	6.286	0.614
Lib 3	SYBR	None	2.631	0.338
Lib 4	SYBR	None	1.312	0.132
Lib 5	SYBR	None	0.794	0.119
Lib 6	SYBR	None	1.096	0.171
Lib 7	SYBR	None	1.121	0.030
Lib 8	SYBR	None	1.183	0.031
Lib 9	SYBR	None	1.192	0.016
Lib 10	SYBR	None	1.676	0.085

Library name	Conc. in pM calculated by qPCR instrument (triplicate data points)			Avg. conc. (pM)	Size adjusted concentration (pM)		Conc. of undiluted library stock (pM)
Library 1:1000	A1	A2	A3	A	A x $\frac{452}{\text{Avg. fragment length}}$	= W	W x 1000
Library 1:2000	B1	B2	B3	B	B x $\frac{452}{\text{Avg. fragment length}}$	= X	X x 2000
Library 1:4000	C1	C2	C3	C	C x $\frac{452}{\text{Avg. fragment length}}$	= Y	Y x 4000
Library 1:8000	D1	D2	D3	D	D x $\frac{452}{\text{Avg. fragment length}}$	= Z	Z x 8000



Figure: KAPA DNA Library Quantification

Standard Curves examples



Standard Curve Characteristics

Equation: $y = -3.371x + 18.739$ [Report...](#)

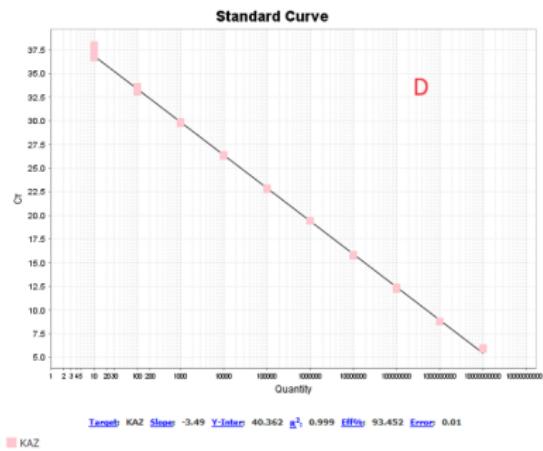
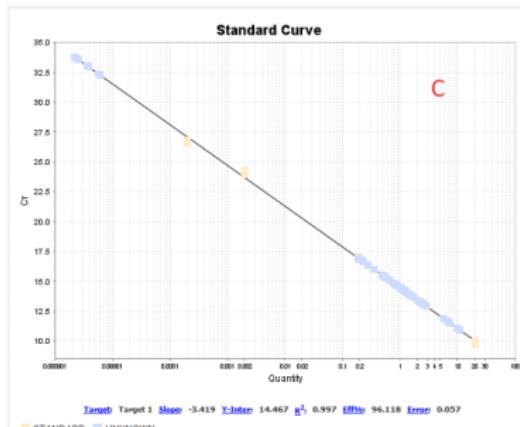
R²: 0.997 R_s: 0.984

Standard Curve Results

Well	Cq	Calculated Concentration (ng/L)	% Var.
25	8.55	20	11.25
26	8.60	20	21.48
27	6.64	20	38.74

Sample Results

Well	Cq	Calculated Concentration (ng/L)	% Var.
13	13.90	0.1952	
14	13.96	0.1875	
15	14.05	0.1771	



qPCR Efficiency

- Amplification efficiency is calculated using the formula: $E=10^{-1/\text{slope}}$
- Ideally, a PCR reaction in the exponential phase will double its amplicon amount at each cycle, i.e. $E=2$ for the reaction.
- Using efficiency equal to 2 in the equation above, $2=10^{-1/\text{slope}}$, the optimal slope of the standard curve will be -3.32 .
- In most cases the efficiency of the reaction is represented in percentage and it is calculated by using the following formula:
$$\% \text{Efficiency} = (E - 1) \times 100$$



qPCR Efficiency

- An efficiency close to **100%** is a good indicator of a **reliable, reproducible** and **robust** assay.
- In practice **good % E values** vary between **90%** and **105%**.
- Usually **low efficiencies** are caused by poor primer design or poorly optimised reaction conditions.
- **Higher % E values (>105%)** would be an indicator of (i) pipetting errors when performing the dilution series, (ii) or co-amplification of non-specific products, (iii) or presence of primer-dimers.



The linear standard curve: correlation coefficient (r) and coefficient of determination (R^2)

- These two values represent the **linearity** of the experimental data.
- Measures the variability across the replicates, and whether the **amplification efficiency** is the same in the different dilution series (different starting template concentrations).
- Slight differences in the CT values between replicates will lower r or R^2 values.
- A good standardised assay will normally produce an r whose absolute value is **> 0.990** or an R^2 value **> 0.980**.



Questions

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Shukran!

Thank You!

Me da wo ase!

Ngiyabonga kakhulu!

Webare!

Merci!

Jere-jef!

Asante sana!

E dupe!

Natotela Sana!



Better lives through livestock

