Research Institute for Tropical Medicine - Department of Health
9002 Research Drive, Filinvest Corporate City, Alabang, Muntinlupa City, 1781 Philippines
Tel Nos.: (632) 8809-7599 / 8807-2631/32/37 | Website: www.ritm.gov.ph

TROUBLESHOOTING GUIDE FOR REAL-TIME PCR RUNS FOR SARS-CoV-2 DETECTION

2nd revision 16 November 2020

A well-designed and -optimized polymerase chain reaction (PCR)-based assay has the potential to be a highly sensitive and highly specific nucleic acid target detection method, relative to other diagnostic methods. However, even good PCR assays have limitations as there are many factors which have to be controlled to ensure the success of a PCR run. As such, problems encountered in PCR runs require careful analysis and stepwise investigation, considering the said factors. This guidance document outlines the factors influencing the outcome of Taqman-based Real-Time PCR runs, the common problems/challenges encountered, practical troubleshooting procedures, and provides recommendations on how to address these issues.

Error recognition and correction are important and these should be integrated in the laboratory's Quality Management System (LQMS), spanning the pre-analytic, analytic and post-analytic phases of testing, to ensure that the final output – laboratory test results – are as accurate and reliable as possible.

TROUBLESHOOTING CONSIDERATIONS

Table 1. Factors to consider when troubleshooting Real-Time PCR results.

FACTOR	CONSIDERATIONS
1. DNA/RNA Template	• Source (specimen type, manner of specimen collection, timing of collection, etc) • Quantity (too little may be below the assay's limit of detection, too much may result in failed PCR run due to PCR inhibition) • Integrity (storage and transport conditions, leakage, etc) • Quality (nucleic acid extraction methodology, correct performance of procedure for extraction, handling and storage of nucleic acid extract) • Purity (presence of PCR inhibitors and/or contaminants) • RNA quality (should match the reverse transcription priming protocol) • Number of freeze-thaw cycles (maximum of three freeze-thaws only)
2. PCR Reagents / PCR Kit	• Quality (lot to lot variation as a result of manufacturing or transport conditions during delivery, assay design, certified to have no contaminants/inhibitors) • PCR assay design (efficiency, sensitivity, specificity, reproducibility) • Indicated to work and has been tested for the specimen type to be used • Analytical sensitivity/limit of detection (PCR assay) • Analytical specificity/target analyte cross-reactivity (target sequence-specific primers) • Shelf life (expiration date, stability) • Integrity (storage and transport condition, packaging, number of freeze-thaw cycles) • Ease of use (monoplex vs multiplex format and PCR reagent packaging (i.e. premixed vs. non-premixed, or lyophilized vs. ready-to-use) affects the number of pipetting steps and the risk for errors during PCR mastermix assembly/preparation) • PCR assay type (in-house vs. commercial kit) • Compatibility with available/existing instruments in the laboratory
3. PCR Run Controls	• Quality (lot to lot variation as a result of manufacturing or transport conditions during delivery, well-optimized, certified to have no contaminants/inhibitors) • Concentration (if too concentrated or diluted, it might not serve as an indicator of PCR mastermix quality) • Shelf life (expiration date)

	• Stability (storage temperature, dilution, aliquoted depending on frequency of use) • Integrity (storage and transport condition, packaging, number of freeze-thaw cycles) • Compatibility with the PCR assay's target analytes (contains the correct sequence targeted by the PCR assay's primers & probes)
4. PCR Profile / Assay program	• Well-optimized • Properly-programmed in the instrument • Baseline setting
5. Real-Time PCR thermal cycler	• Compatibility with the PCR assay (contains appropriate dye channels for excitation and detection of fluorophores) • Appropriateness of Real-Time PCR software used with the thermal cycler • Minimum and maximum reaction volume capacity of the PCR machine • Compatibility of PCR consumables used (plates, strips, sealing film, caps, etc that may affect optical detection) • Equipment maintenance and performance monitoring
6. Operator Errors **- Manual Errors** **- Analysis Errors**	• Procedure execution errors • Analysis errors
7. Laboratory's Internal Quality Control	• Standardized checkpoints to ensure proper execution and risks for failure in each step are minimized • Quality indicators in place to serve as tools for troubleshooting

COMMON REAL-TIME PCR RUN ERRORS

Table 2. Commonly encountered Real-Time PCR run errors, possible causes, recommended corrective and preventive actions.

PROBLEM ENCOUNTERED	POSSIBLE CAUSES	RECOMMENDED SOLUTION/ CORRECTIVE ACTION	RECOMMENDED PREVENTIVE ACTION
1. **Invalid positive control** and **invalid test unknowns::** No amplification detected in all tested unknowns	1.1. Error in the assay master mix preparation.	• Verify if volume computation for each PCR mastermix component is correct • Verify if all components of the PCR mastermix components were added to the mix • Check for reagent pipetting (aspiration / dispensing / pipettor-pipette tip combination) errors during PCR mastermix preparation	• Strictly follow work instructions and good clinical laboratory practices • Employ standard worksheets to guide laboratory staff in mastermix preparation, template addition, PCR loadind and results analysis

and controls for all targets		• **Invalidate the run and repeat test:** Retest affected samples with a new preparation of the PCR mastermix	
	1.2. Expired / nonviable / compromised PCR mastermix reagents	• **Invalidate the run and repeat test:** Retest affected samples using a new set (QC-checked) of reagents	• Integrate a flagging mechanism in reagent inventory system for near-expiry and expired reagents • QC check new lots by running positive and negative controls using a lot verified to be working and the incoming reagent lot (old vs new)
	1.3. Incorrect PCR profile used	• Verify the rRT-PCR assay profile and correct if necessary • **Invalidate the run and repeat test:** Retest affected samples using the correct PCR profile.	• Integrate controls to avoid inadvertent editing of the PCR profile • Train/remind lab staff to check PCR profile before starting the run
	1.4. Problems with plate layout for the PCR run (software) such as incorrectly set dyes or unset dyes or wrong sample type assignment	• Check the PCR run file for plate layout assignment errors based on the layout assignment in the worksheet • Edit the run file by setting the correct dyes (if PCR analysis software allows) and/or samples to the plate layout	• Train/ remind lab staff to double-check PCR plate layout against the PCR worksheet prior to starting the run
2. **Invalid positive control:** No amplification detected in the positive control for any or all targets	2.1. Incorrect or degraded positive control	• Trace which positive control tube was used, check whether the same positive control was used in previous runs and if there is evidence of degradation (*i.e.* trend of increasing Cq value across runs which utilized the affected control material)	• QC check new lots by running positive and negative controls using a lot verified to be working and the incoming reagent lot (old vs new)

Valid negative control: No amplification detected in the negative control for all targets		• **Invalidate the run and repeat test:** Retest affected samples using a new/different (QC-checked) positive control tube/lot along with the positive control material used in the failed run	• Prepare single-use aliquots of the QC-passed positive control material • QC-passed positive controls should be stored according to temperature indicated by the manufacturer • Monitor the performance of QC-passed lots of positive control material across runs
	2.2. Positive control dispensed at the wrong well	• Check if samples and controls were correctly dispensed in the correct well based on the PCR worksheet; • **Invalidate the run and repeat test:** If verified to be incorrectly dispensed, repeat the run ensuring proper placement of samples and controls in the correct well/s	• When adding the template, always check the placement of samples and controls against the worksheet
	2.3. Incorrect orientation of PCR plate loaded onto PCR machine	• Check if the PCR plate/tubes/strips were incorrectly loaded onto the PCR machine block based on the orientation indicated in the PCR worksheet; • If incorrectly loaded onto the block, edit the sample and control layout (if supported by PCR machine's analysis software) based on the orientation indicated in the PCR worksheet (which served as reference during template	• Double-check the placement/orientation of the plate/tube/strip tube on the PCR block, based on the PCR worksheet before starting the run

		addition) and re-analyze the run using correct orientation; if editing the layout after the run is not supported by the PCR machine's analysis software, repeat the run ensuring proper orientation of the plate/tube/strip	
3. **Invalid negative control:** Amplification detected in the negative control for any or all targets	3.1. Cross-contamination from a positive sample / pipetting error	• Immediately perform surface decontamination (10% bleach followed by 70% Ethanol, at least 10 minutes contact time) of all lab surfaces used in template addition, including pipettors, forceps and other materials used • Improve pipetting skills, especially during template addition • Check if pipette tip used during template addition is leaking • Check if tips used for template addition are aerosol-barrier tips • Check if the correct pipettor-pipette tip combination was used during template addition • **Invalidate the run and repeat test:** Retest the whole plate. Practice caution in dispensing samples and controls during template addition	• Strengthen pipetting skills • Strictly observe/enforce decontamination procedures before and after use of a work station
	3.2. Contamination from amplicons in the environment/amplicon contamination *Suspect if: two or more PCR runs conspicuously*	• Immediately perform surface decontamination (freshly prepared 10% bleach followed by 70% Ethanol, at least 10 minutes contact time respectively) of all lab surfaces, including pipettors, forceps and other materials used	• Strict implementation of work compartmentalization (reagent handling, template addition, specimen handling/nucleic acid extraction, PCR) and theunidirectional workflow (lab

	display too many positives including NTCs and repeatedly occurs even when repeated by different technicians / successive run days)	• **Invalidate the run and repeat test:** Retest the whole plate. Monitor the succeeding plates; if amplification in NTC is persistent, decontaminate all Pre-PCR areas by performing at least 3 rounds of wipe-down using 10% bleach and 70% ethanol.
		• To rule out/confirm which areas are affected: perform environmental testing in pre-PCR areas (reagent preparation, specimen handling, template addition, anterooms to each):
		  ○ Swab each area under investigation (eg. Interior surface of BSC/PCR cabinet, pipettor, door knob, refrigerator/freezer handle, etc)
		  ○ Extract the nucleic acid for each sample representing an area under investigation
		  ○ Perform PCR for each environmental sample
		• Concentrate decontamination activities on areas with target-positive environmental samples
		• Isolate all potentially-exposed reagents and consumables to amplicon; Use new (non-exposed) reagents and consumables in succeeding PCR runs
			traffic/work is from clean to dirty where dirty areas are those where amplicons are generated); monitor staff compliance to this policy and make sure PPE, equipment, lab re-usables, lab consumables are dedicated for each work area
			• Do not open PCR strip tubes/plates after PCR run.
			• Do not use previously ran PCR strip tubes as balancer in the PCR machine.
			• Perform regular (before and after use of a work station) surface decontamination procedures
			• Use freshly-prepared decontamination solutions (decontamination activities may be ineffective if decontamination solutions are no longer viable)

	3.3. Negative control template used is contaminated	• Immediately perform surface decontamination (freshly prepared 10% bleach followed by 70% Ethanol, at least 10 minutes contact time	• Prepare single-use aliquots of the QC-passed negative template control material

		respectively) of all lab surfaces, including pipettors, forceps and other materials used • **Invalidate the run and repeat test:** Retest the whole plate using a new lot of QC-passed negative template control material • To confirm: Test the working negative control that failed against a fresh negative control with a different lot number	• QC check new NTC lots/batches (old vs new)
	3.4. Reagent lot issue *If issue was caught during routine QC-check/lot testing prior to routine use, request supplier for replacement (if possible)*	• Invalidate the run and repeat test: Retest the whole plate using a new lot of QC-passed negative template control material • prepare report, contact the supplier manufacturer regarding the issue and request for technical assistance	• Verify if reagents were transported according to manufacturer recommendations • Check if reagents are stored according to manufacturer recommendations • QC check new lots by running positive and negative controls using a lot verified to be working and the incoming reagent lot (old vs new)
4. **Invalid positive control:** No amplification detected in the positive control for any or all targets. **Invalid negative control:** Amplification detected in the	4.1. Possibility of switching controls during template addition.	Check if samples and controls were correctly dispensed in the correct well based on the PCR worksheet;	• · When adding the template, always check the placement of samples and controls against the worksheet

negative control for any or all targets			
5. **Invalid negative control** and **flagged tested unknowns:** Amplification detected in all tested samples and negative control for SARS-CoV-2 target/s	5.1. Contaminated mastermix reagents	If reagent set/test kit was QC-checked prior to routine use then a violation of the unidirectional workflow may have occurred, contaminating consumables with amplicons • Isolate reagents suspected to be contaminated to avoid being used again for succeeding runs • Perform surface decontamination (freshly-prepared 10% bleach followed by 70% ethanol with at least 10 mins contact time) of the reagent preparation area including reagent storage compartments • **Invalidate the run and repeat test:** Retest the whole plate. Use a new set/lot (QC-passed) of reagents If reagent set/test kit was not QC-checked prior to routine use: • **Invalidate the run and repeat test:** Retest whole plate. Use a new set/lot (QC-passed) of reagents • investigate for possibility of contaminated lot (manufacturing issue) delivered by supplier by running QC-passed negative controls using reagents suspected to be	• Strict implementation of the unidirectional workflow (lab traffic/work is from clean to dirty where dirty areas are those where amplicons are generated); monitor staff compliance to this policy and make sure PPE, equipment, lab reusables, lab consumables are dedicated for each work area • Do not open PCR strip tubes/plates after PCR run. • Do not use previously ran PCR strip tubes/plates as balancer in the minifuge/plate minifuge or PCR machine. • Perform regular (before and after use of a work station) surface decontamination procedures • Use freshly-prepared decontamination solutions (decontamination activities may be ineffective if decontamination solutions are no longer viable)QC check new lots by running positive and negative controls using a lot

		contaminated and reagents confirmed (QC-checked) to be non-contaminated • prepare report, contact the supplier manufacturer regarding the issue and request for technical assistance and possibility of lot replacement If the issue was caught during QC-checking of incoming lots: • investigate for possibility of contaminated lot (manufacturing issue) delivered by supplier by running QC-passed negative controls using reagents suspected to be contaminated and reagents confirmed (QC-checked) to be non-contaminated • prepare report, contact the supplier manufacturer regarding the issue and request for technical assistance and possibility of lot replacement	verified to be working and the incoming reagent lot (old vs new) • QC check new lots by running positive and negative controls using a lot verified to be working and the incoming reagent lot (old vs new)
	5.2. Contaminated consumables (tips, PCR plates, PCR tubes, PCR strips, etc)	Possible violation of the unidirectional workflow or work compartmentalization, contaminating consumables with amplicons • Isolate the consumables suspected to be contaminated to avoid being used again for succeeding runs • **Invalidate the run and repeat test:** Retest the whole plate using a different set of consumables.	• Strict implementation of work compartmentalization (reagent handling, template addition, specimen handling/nucleic acid extraction, PCR) and the unidirectional workflow (lab traffic/work is from clean to dirty where dirty areas are those where amplicons are generated); monitor

		• To rule out/confirm which areas are affected: perform environmental testing in pre-PCR areas (reagent preparation, specimen handling, template addition, anterooms to each):
		  ○ Swab each area under investigation (eg. Interior surface of BSC/PCR cabinet, pipettor, door knob, refrigerator/freezer handle, etc)
		  ○ Extract the nucleic acid for each sample representing an area under investigation
		  ○ Perform PCR for each environmental sample
		• Concentrate decontamination activities on areas with target-positive environmental samples
		• Isolate all potentially-exposed reagents and consumables to amplicon; Use new (non-exposed) reagents and consumables in succeeding PCR runs
			staff compliance to this policy and make sure PPE, equipment, lab reusables, lab consumables are dedicated for each work area
			• Do not open PCR strip tubes/plates after PCR run.
			• Do not use previously ran PCR strip tubes/plates as balancer for the minifuge/plate minifuge or in the PCR machine.
			• Perform regular (before and after use of a work station) surface decontamination procedures
			• Use freshly-prepared decontamination solutions (decontamination activities may be ineffective if decontamination solutions are no longer viable)

	5.3. Contamination from the environment/amplicon contamination	• Immediately perform surface decontamination (freshly prepared 10% bleach followed by 70% Ethanol, at least 10 minutes contact time respectively) of all lab surfaces, including pipettors, forceps and other materials used • **Invalidate the run and repeat test:** Retest the whole plate. Monitor the succeeding plates; if	• Strict implementation of work compartmentalization (reagent handling, template addition, specimen handling/nucleic acid extraction, PCR) and the unidirectional workflow (lab traffic/work is from clean to dirty

		amplification in NTC is persistent, decontaminate all Pre-PCR areas by performing at least 3 rounds of wipe-down using 10% bleach and 70% ethanol. • To rule out/confirm which areas are affected: perform environmental testing in pre-PCR areas (reagent preparation, specimen handling, template addition, anterooms to each): ○ Swab each area under investigation (eg. Interior surface of BSC/PCR cabinet, pipettor, door knob, refrigerator/freezer handle, etc) ○ Extract the nucleic acid for each sample representing an area under investigation ○ Perform PCR for each environmental sample • Concentrate decontamination activities on areas with target-positive environmental samples • Isolate all potentially-exposed reagents and consumables to amplicon; Use new (non-exposed) reagents and consumables in succeeding PCR runs	where dirty areas are those where amplicons are generated); monitor staff compliance to this policy and make sure PPE, equipment, lab reusables, lab consumables are dedicated for each work area • Do not open PCR strip tubes/plates after PCR run. • Do not use previously ran PCR strip tubes as balancer in the PCR machine. • Perform regular (before and after use of a work station) surface decontamination procedures Use freshly-prepared decontamination solutions (decontamination activities may be ineffective if decontamination solutions are no longer viable)
6. Invalid result for all samples: No amplification detected in all tested samples for the <u>internal control target</u>	6.1. Collection media incompatible with extraction method	• Re-extract the specimen using an extraction method that has been validated with the collection media of the specimen. If no other suitable extraction method is available, request for a repeat collection from the patient with a	• Ensure that the extraction method to be used is verified to be compatible with the collection media used • Integrate a QC step in the specimen reception procedure for

Provided that positive and negative controls for all targets are valid.		collection media that is suitable to the existing extraction methods	noting down the media type to help address compatibility issues • List down suitable specimen types for the extraction methods available in your lab
	6.2. Erroneous extraction procedure performed	• To verify, repeat the SARS-CoV-2 PCR assay using the same RNA extract. • If the result of the repeat test remains invalid, repeat the extraction (and inactivation) method using a new clinical specimen aliquot (if available).	• Ensure trained personnel • Have quick guides ready on hand
	6.3. Poor specimen quality	• If results for re-extracted and repeat-tested samples remain invalid, release as '**invalid**' with a comment for '**poor specimen quality, repeat collection recommended**'	• Clear protocols for specimen acceptance and rejection criteria must be established and consistently implemented
7. Invalid result for some samples: Amplification detected in some but not all tested unknowns for the internal control target Provided that positive and negative controls for all targets are valid.	7.1. Inconsistent pipetting technique in template addition	• Repeat the SARS-CoV-2 PCR assay for the concerned samples using proper pipetting technique and stricter adherence to good clinical laboratory practices.	• Ensure trained personnel
	7.2. RNA samples in a PCR plate/run were extracted using different methodologies with different	• Re-extract and repeat the SARS-CoV 2 PCR assay on the concerned samples using an extraction method that has been verified to be of good performance with your lab.	• Ensure trained personnel • Ensure appropriateness of extraction methodology for the specimen type • Ensure appropriateness of extraction methodology for the media type

	performance characteristics		
8. Samples that **have Cq values** but have **no sigmoidal amplification curve**	8.1. Software analysis error: For flat, slanted plots have baselines set to end too early	• **Contact the PCR machine supplier for technical assistance on proper baseline adjustment.** • For plots that are discernably leveled (flat), set the baseline end to # of cycles -1 (e.g. baseline end at 44 for a PCR assay of 45 cycles) • Document this incident and file a record; If this problem persists, request for technical assistance from the equipment supplier to determine if there is an issue with the thermalcycler unit.	• Request for end-user training from the equipment supplier on baseline adjustment for troubleshooting purposes, particularly for laboratory staff involved in PCR analysis and result verification • Properly document equipment-related incidents and maintain records
	8.2. High background noise throughout the PCR run	• Repeat the SARS-CoV-2 PCR assay on the test unknown. Make sure that there are no bubbles in the well after template addition. • Document this incident and file a record; If this problem persists, request for technical assistance from the equipment supplier to determine if there is an issue with the thermalcycler unit.	
9. Tested samples **with sigmoidal amplification curve** but **no assigned Cq value**	9.1. Software analysis error: Baseline end is set too late	• **Contact the PCR machine supplier for technical assistance on proper baseline adjustment.** • Repeat the PCR run using the extracted nucleic acid for the concerned sample	• Request for end-user training from the equipment supplier on baseline adjustment for troubleshooting purposes, particularly for

			laboratory staff involved in PCR analysis and result verification • Properly document equipment-related incidents and maintain records
	9.2. Biological variability: Template amount for target is too low and fluorescence plateau is below the threshold line	• Repeat the PCR run using the extracted nucleic acid for the concerned sample in two reaction wells: 1 reaction well containing the assay manufacturer-recommended RNA template volume, 2nd reaction well containing an increased RNA template volume.	
10. **Skewed and/or non-horizontal baselines** of amplification curves	10.1. Software analysis error: Baseline end is set too late	• **Contact the PCR machine supplier for technical assistance on proper baseline adjustment.** , • Once the required adjustment in baseline setting has been made, repeat the PCR run using the extracted nucleic acid for the concerned sample	• Integrate controls to prevent unauthorized/uncommunicated adjustments in the PCR machine's software analysis settings • Properly document equipment-related incidents and maintain records
	10.2. Software detection error: Early cycles have high background noise	• **Contact the PCR machine supplier for technical assistance.**	• Properly document equipment-related incidents and maintain records

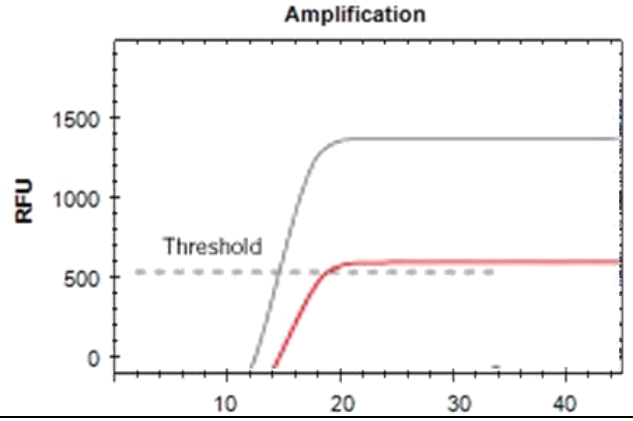
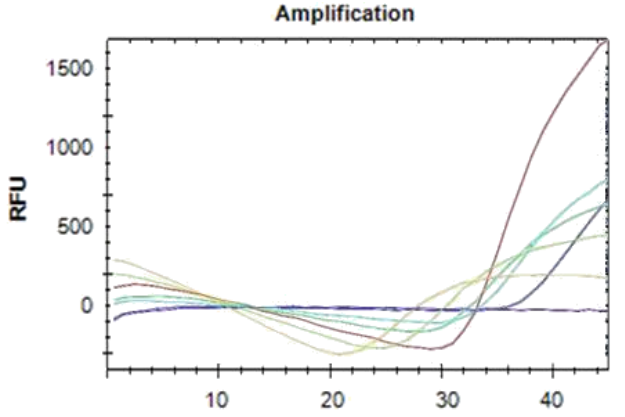
11. For PCR assays with 2 or more SARS-CoV-2 targets: **Amplification of only a single SARS-CoV-2 target** in a tested unknown	11.1. Biological variability	• For kits with multiple SARS-CoV-2 targets, refer to the manufacturer's instructions for use (IFU) for their algorithm to interpret results. If the assay's/kit's algorithm cannot resolve an inconclusive result, repeat the assay on the concerned sample. This might be due to differences between the limit of detection by the assay's/kit's individual gene targets leading to uncertainty in the classification of a positive and negative result for a sample. • If the repeat test gives the same inconclusive result, release as **'inconclusive.' with a comment 'repeat collection recommended'**	• Have a back-up PCR kit/assay utilizing a different set of SARS-CoV-2 gene target/s, preferably with a higher analytical sensitivity/limit of detection, which can be used as a confirmatory testing assay.
12. Non-ideal amplification curves (non-sigmoidal shape)	12.1. Poor specimen quality *Consider if the run controls displayed sigmoidal curves*	• Confirm by repeating the test using the same RNA extract; if the issue persists, repeat from nucleic acid extraction to PCR; if the issue persists, repeat from specimen inactivation, nucleic acid extraction to PCR; if the issue still persists, release as **'invalid due to poor specimen quality'** with a comment to recollect **'repeat collection recommended'**	• If specimen collection is performed by the testing lab, ensure staff performing specimen collection are properly trained and the appropriate media type is used for the specimen type and extraction methodology to be used b the testing lab; ensure that collected samples are properly stored (based on manufacturer recommendations – media); ensure that the transport conditions are appropriate • If specimens were referred (collected off-site and transported to the testing laboratory), ensure

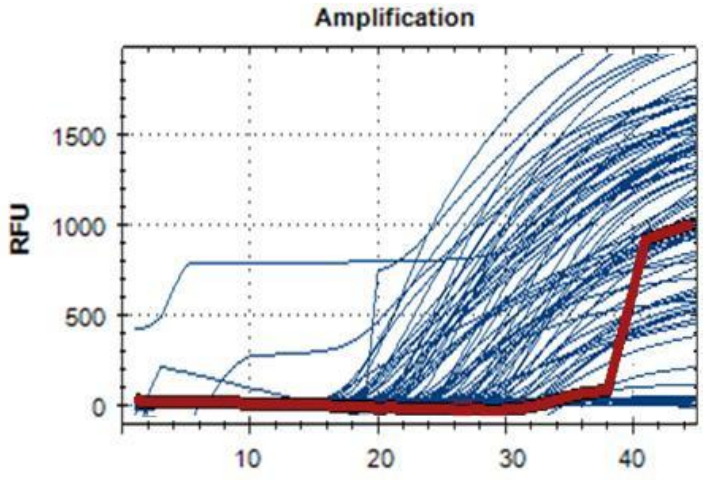
			that incoming specimens are evaluated for quality and sufficiency, following institutional acceptance and rejection criteria and the protocol for specimen reception.
	12.2. Incorrect PCR profile used	• Verify the rRT-PCR assay profile and correct if necessary • Retest affected samples using the correct PCR profile.	• Integrate controls to avoid inadvertent editing of the PCR profile • Train/remind lab staff to check PCR profile before starting the run
	12.3. PCR machine failure *Consider if PCR assay/kit performance-verified prior to routine use **and** PCR assay/kit lot passed QC prior to routine use **and** all samples including controls in the run display non-sigmoidal curve*	• Retest the whole plate using a different PCR machine (previously verified to work with the assay) • Contact biomedical engineer and equipment supplier for technical assistance	• Ensure preventive maintenance plan for the equipment is strictly followed • Only use PCR machines with valid calibration certificates for diagnostics • Ensure all users are trained on proper equipment use • Ensure usage of the equipment is documented
	12.4. Reagent/PCR kit lot issue *If issue was caught during routine QC-*	• Verify this by parallel-testing a reagent lot that has been previously confirmed to be working vs the reagent lot suspected to give problematic results. Use positive and negative control for the parallel-test, in triplicate. If the reactions using	• Verify if reagents were transported and received according to manufacturer recommendations.

	check/lot testing prior to routine use, request supplier for replacement (if possible)	the reagent lot previously confirmed to be working provides the expected results and the reactions for the suspected problematic lot does not provide the expected result, then this confirms that a bad lot was used. • Retest the whole plate using a new lot of QC-passed negative template control material • prepare report, contact the supplier manufacturer regarding the issue and request for technical assistance	• Check if reagents are stored according to manufacturer recommendations • QC check new lots by running positive and negative controls using a lot verified to be working and the incoming reagent lot (old vs new)

Table 3. Commonly-encountered issues with Real-Time PCR amplification curves generated and basic troubleshooting procedures

REAL-TIME PCR RESULT ISSUES	POSSIBLE PROBLEM	TROUBLESHOOTING
Lower plateau than expected		
	One or more of the mastermix components are limiting the	Re-check calculation on worksheet

	reaction due to incorrect calculation or degradation	Repeat test using new stock reagents If it's a case of a bad lot caught in lot-testing, contact supplier
Slanted curves		
	Baseline end is set too late	Manually set the baseline of the machine to a cycle range that has no visible detected amplification. Contact supplier for assistance on how to manually set baseline.
Erratic curves		

	Presence of optical obstacles (bubbles, fingerprints)	Ensure bubbles are gone by spinning down plate. Use powder-free gloves Use lint-free wipes to wipe plate seal and the bottom of the plate or tube.

Research Institute for Tropical Medicine - Department of Health
9002 Research Drive, Filinvest Corporate City, Alabang, Muntinlupa City, 1781 Philippines
Tel Nos.: (632) 8809-7599 / 8807-2631/32/37 | Website: www.ritm.gov.ph

GENERAL GOOD CLINICAL LABORATORY PRACTICES WHEN PERFORMING DIAGNOSTIC REAL-TIME PCR ASSAYS

A. Prevent contamination

- Use molecular-grade reagents and consumables
- Be consistent and strict in decontamination protocols in terms of schedule and implementation
- Maintain separate areas and dedicated equipment (eg. pipettes, microcentrifuges) and supplies (eg. microcentrifuge tubes, pipette tips, gowns and gloves) for assay reagent setup and handling of extracted nucleic acids
- Workflow must always be from the clean area to the dirty area.
- Wear proper PPE in all laboratory areas and ensure PPE used are segregated according to work compartmentalization
- Wear clean disposable gowns and new, previously unworn, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever contamination is suspected.
- Ensure unidirectional workflow is implemented, such that likelihood of amplicons in the PCR area are carried over to the clean areas (reagent preparation, specimen handling and template addition areas) is minimized
- Do not bring extracted nucleic acid or PCR products into the assay setup area.
- Keep reagent tubes and reactions capped as much as possible
- Use aerosol barrier (filter) pipette tips only

B. Biosafety Cabinets and PCR Hoods

- Perform surface decontamination properly before and after use using freshly-prepared decontamination solutions; never assume that the BSCs and PCR hoods are clean prior to working
- Turn off light when handling probes for real-time PCR

C. Pipetting skills

- Ensure staff performing the diagnostic assay is properly trained and equipped with the necessary pipetting skills
- Check that pipettor volumes are set correctly
- Ensure that consistent volumes are drawn
- Dispense everything on the pipette tip
- Avoid using contaminated tips on sterile reagents

D. Organization in work

- Use standard worksheets to document samples processed and plate layout
- Ensure correct worksheet is used (inspect mastermix volumes, thermocycling conditions)
- Label aliquoted reagents with date prepared
- Label sample tubes properly and implement techniques to prevent sample switching
- Fill the worksheet correctly and properly.
- Organize your hands' workflow inside the hood to avoid going above open tubes and samples
- Vortex mix and spin down reagents and samples before use
- Keep stock reagents separate from the working set
- Always check recommended storage conditions for PCR reagents.

E. QC and QA

- Ensure staff are properly trained and competent to perform diagnostic PCR assays
- Ensure SOPs are in place to guide all laboratory staff accordingly
- Ensure standard forms (worksheets) are used in all critical procedures in the analytic phase for documentation purposes and to serve as a guide to the staff performing the test
- Perform lot-testing incoming reagents, particularly PCR reagents
- Assay controls should be run concurrently with all test samples. If using a commercial kit, check if these are already included in the kit. PTC – positive template control with an expected Cq value range NTC – negative template control added during rRT-PCR reaction set-up RP – all clinical samples should be tested for human RNAse P (RNP) gene to assess specimen quality
- Monitor performance of controls to prevent use of degraded controls
- Monitor expiration dates of all reagents to avoid use of expired reagents
- Ensure equipment are properly maintained, with the preventive maintenance plan followed accordingly to avoid use of uncalibrated equipment
- Participate in External Quality Assurance Programs

Prepared by:
The RITM Molecular Biology Laboratory

References:

Valones M. A. A., Guimaraes R. L., Brandao L. A. V., de Souza P. R. E. (2009). Principles and Applications of PCR in Medical Diagnostic Fields: A Review. Br. J. Micro. 40, 1-11

Heid, CA,Stevens J, Livak, KJ, Williams, PM. (1996). Quantitative Real Time PCR. Genome Res 6:986-994. PMID: 8908518

Patrino G., Ansorge W. (2005). Molecular Diagnostics. Elsevier Science Academic Press.

Steegen, K., Demecheleer, E., De Cabooter, N., Nges, D., Temmerman, M., Ndumbe, P., … Verhofstede, C. (2006). A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance. Journal of Virological Methods, 133(2), 137–145.

Yang S., Rothman R. E. (2004) PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. The Lancet Infectious Diseases, Volume 4, Issue 6, 337 - 348