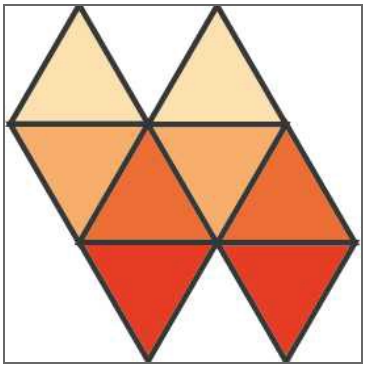

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Troubleshooting guide for DDNS V2

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ABSTRACT

This document provides general guidance for troubleshooting problems encountered in the DDNS method. It outlines the factors that might influence the outcome of the DDNS PCR assay run, the common problems encountered, possible causes of the problems, and provides recommendations on corrective action and preventative action.
This document is primarily developed for DDNS performed on stool samples, but included are other factors to consider for other sample types.

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DDNS troubleshooting guide

Purpose

To outline the most common sources of DDNS assay run failure / unacceptable results, reduced/lower sensitivity than expected and provide recommendation on how to avoid these pitfalls and improve performance.

Associated Forms & Documents

DDNS Protocol v2.0 (protocols.io)
Verification guidelines for the DDNS method for poliovirus direct detection (Verification guidelines for the DDNS method for poliovirus direct detection (protocols.io)
Quality Control and Data Recording for DDNS (protocols.io)
Poliovirus detection and nanopore sequencing FAQs (Poliovirus detection and nanopore sequencing FAQs (protocols.io))

Troubleshooting considerations.

Table1. Factors to consider when troubleshooting DDNS results.

	A	B
	Factor	**Considerations**
	Nucleic acid (RNA) template	• Stool sample condition (timeline of collection and processing, transport, and storage conditions) • Quality (nucleic acid extraction method, correct performance of procedure for extraction, handling, and storage of RNA extract) • Purity (presence of PCR Inhibitors and /or contaminants affecting RT-PCR amplification) • Number of freeze-thaw cycles (maximum of three freeze-thaws only)
	PCR and sequencing reagents/ kit	• Quality (Lot to lot variation because of manufacturing or transport and storage conditions) • Shelf life (expiration date, stability)

A	B
	• Integrity (storage and transport condition, packaging, number of freeze-thaw cycles) • Sequencing reagents compatible with the flow cell - device
Run controls (Positive control CVA20 and negative control Nuclease free water)	• Quality (lot to lot variation as result of manufacturing or transport conditions) • Shelf life (expiration date) • Stability (storage temperature, reconstitution of the positive control CVA20, aliquoted for single use only for positive control CVA20) • Integrity (storage and transport condition, packaging)
DDNS PCR assay programs	• Thermal profiles properly programmed in the instrument with temperature ramping speed consideration
Conventional end-point/ block-based PCR thermal cycler	• Compatibility of PCR consumables used (PCR plates, strip tubes, sealing film, caps etc that may affect PCR amplification if not sealed properly) • Equipment maintenance and performance monitoring
Nanopore sequencing	• Sequencing run settings • Sequencing reagent - flow cell - device – software compatibility
Operator error (manual, automated, result interpretation error)	• Procedure execution errors (consumables, protocol steps, volumes, device settings) • Analysis errors
Laboratory's internal quality control	• Standardized checkpoints to ensure proper execution and risks for failure in each step are minimised • Quality indicators in place to serve as tools for troubleshooting
DDNS Data analysis (using PiranhaGui Software)	• Software issue (version, compatibility, input/ output access) • Sample/run data input error
General laboratory practice	• Cleanliness of workspaces and equipment • Equipment and pipette calibration and maintenance • Labelling of samples, reagents, reactions

Below is a list of commonly encountered DDNS PCR assay run errors, possible causes, recommended corrective, and preventive actions.

No amplification detected / sequence obtained in samples previously identified as positive by culture or ITD-qPCR provided that positive and negative controls are valid

A	B	C	D
Problematic result	Possible cause	Recommended solution/ corrective action	Recommended preventive action
No amplification detected / seqeunce obtrained in samples previosuly identified as positive by culture or ITD-qPCR provided that positive and negtaive controls are valid	Erroneous RNA extraction procedure performed	To verify, repeat the DDNS PCR assay using the same RNA extract of the failed samples; If the result of the repeat test remains negative, repeat the RNA extraction method with the recommended kit	• Ensure trained personnel. • Prepare quick guides and extraction plate maps ready on hand

	A	B	C	D
	No amplification detected / seqeunce obtrained in samples previosuly identified as positive by culture or ITD-qPCR provided that positive and negtaive controls are valid	Extraction method used not recommended for DDNS method	Re-extract the stool supernatant using the MagMAX Viral RNA Isolation kit manually or automated; If this extraction method is not available in your lab, use either the Qiagen QIAamp Viral RNA Mini kit or Roche High Pure Viral RNA kit	Ensure that all the extraction method steps are followed according to protocol
	No amplification detected / seqeunce obtrained in samples previosuly identified as positive by culture or ITD-qPCR provided that positive and negtaive controls are valid	PCR inhibitors	Confirm presence of inhibitors by spiking the positive control virus (CVA20) into the sample; if the positive control does not amplify, then inhibitors are suspected; if inhibitors are suspected, use BSA in the mastermix to help overcome PCR inhibitors; alternatively, if inhibitors are suspected, dilute existing stool supernatant or eluted RNA 10-fold and repeat the RT-PCR. If a signal; is observed using the diluted sample, inhibitors are suspected	• Ensure that all the extraction method steps are followed adequately. • Use BSA in the RT-PCR mastermix to help overcome PCR inhibitors.
	No amplification detected / seqeunce obtrained in samples previosuly identified as positive by culture or ITD-qPCR provided that positive and negtaive controls are valid	Sample mislabelling	Check sample labelling, with crosschecking EPID and Lab ID numbers	Ensure correct sample labelling and recoding
	No amplification detected / seqeunce obtrained in samples previosuly identified as positive by culture or ITD-qPCR provided that positive and negtaive controls are valid	Poor specimen quality or RNA template may be damaged/ degraded	Confirm by repeating the test from nucleic acid extraction to PCR; if the issue persists, use a semi-nested PCR approach which has a shorter first round PCR product	Ensure that collected samples are properly stored and processed.

	A	B	C	D
			so likely to amplify a bit better	

Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive and negative controls for all targets are valid.

	A	B	C	D
	Problematic result	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive and negative controls for all targets are valid.	Inconsistent pipetting technique in template addition	Repeat the DDNS assay for the concerned samples using proper pipetting technique; merely repeating the sample may also lead to additional detection	• Ensure trained personnel. • Always check liquid volumes in pipette tips for each addition • Ensure pipettes are services and calibrated
	Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive and negative controls for all targets are valid.	RNA samples in a PCR plate/run were extracted using different methodologies with different performance characteristics	Re-extract and repeat the DDNS PCR assay on the concerned samples using the MagMAX Viral RNA isolation kit or QIAamp Viral RNA Mini Kit or Roche High Pure Viral RNA kit	Ensure trained personnel
	Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive and negative controls for all targets are valid.	Failure to extract viral target RNA due to presence of virus below the detectable limit (LOD) of the DDNS assay	• Perform the semi-nested PCR approach as it is abit more sensitive that the nested PCR. • Perform DDNS on culture isolates of the stool supernatant suspected to contain virus levels below the DDNS LOD	Ensure trained personnel
	Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive	Sample mislabelling	Check sample labelling, with crosschecking EPID and Lab ID numbers	Ensure correct sample labelling and recording

	A	B	C	D
	and negative controls for all targets are valid.			
	Negative result for some samples: amplification detected / sequence obtained in some but not all samples that tested positive by culture or ITD. Provided that positive and negative controls for all targets are valid.	PCR machine failure. Consider if PCR reagents/ kit performance verified prior to use and PCR reagents/kit lot passed QC prior to use and all samples including run controls show no amplification	• Retest the whole plate using different PCR machine (previously verified to work with the assay) • Contact technical service 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 • Ensure all users are trained on proper use of machines

No amplification detected/ sequence obtained in all samples including positive and negative controls

	A	B	C	D
	Problematic result	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	No amplification detected/ sequence obtained in all samples including positive and negative controls	Extraction Reagent/ PCR kit lot issue	• Check that the reagents/ PCR kit lot used has not expired. • 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 duplicate. If the reactions using 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 results, then this confirms that a bad reagent lot was used; Retest the whole plate using new reagent lot of QC passed negative template control material;	• Only use reagents/ PCR kits within date • Verify if reagents were transported and received 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)

	A	B	C	D
			Prepare a report and contact the supplier/ manufacturer regarding the issue and request for technical assistance	
	No amplification detected/ sequence obtained in all samples including positive and negative controls	One or more of the mastermix components from either the PanEV (First round PCR) or VP1 PCR (second round PCR) are limiting the reaction due to missed addition, incorrect calculation, or expired reagents.	• Re-check calculation on worksheet • Repeat test using new stock reagents; if it is a case of a bad reagent lot caught in the lot-testing, then contact the manufacturer/supplier	• Ensure that all reagents /Kits used are within expiry. • Record kit and reagent lot numbers on worksheet • Record/tick every addition of components while preparing mastermixes • Record/tick addition steps during protocol i.e. forward primer, barcoded primers, first PCR product
	No amplification detected/ sequence obtained in all samples including positive and negative controls	Incorrect PCR program or cycling conditions were used	Repeat amplification from RT step	Record/tick each step in the protocol to ensure correct thermocycler programs and cycling conditions are used
	No amplification detected/ sequence obtained in all samples including positive and negative controls	Failed RNA extraction	• Re-extract and repeat the DDNS assay on all samples using the MagMAX Viral RNA isolation Kit or QIAamp Viral RNA Mini Kit or Roche High Pure Viral RNA kit.	• Ensure that all steps of the extraction protocol are followed adequately. • Control RNA extraction and first PCR separately by introducing a separate positive control for the first PCR in the form of a previously extracted RNA that successfully amplified before • Check that all recommendations and notes in the RNA extraction protocol are considered.

Identical sequences appearing at low read numbers (10-100) over multiple samples

	A	B	C	D
	Problematic result	Possible cause	Recommended solution/ corrective action	Recommended preventive action

	A	B	C	D
	Identical sequences appearing at low read numbers (10-100) over multiple samples	Contamination of equipment due to repeated DDNS performance	• Perform a deep clean of workstations, pipettes and equipment with nucleic acid degradation solutions (e.g. DNAZap).	• Clean workstation and pipettes between runs • Perform a routine deep clean every five DDNS runs • Separate work areas for pre- and post-amplification steps

Amplification is detected but no sequences are produced

	A	B	C	D
	Problematic result	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Amplification is detected but no sequences are produced	Incorrect sequencing run settings used	• Make sure you have selected the correct sequencing and barcoding kits in the run options. • If pod5 files are being produced, you can re-call the data post-run. These files may be in pod5_fail, or pod5_skip	When setting up the run, ensure you select SQK-LSK114, and EXP-PBC096
	Amplification is detected but no sequences are produced	Incompatible kit reagents used	• If you have multiple ONT kits, it may be possible that you took the adapter mix from an incompatible kit for the final adapter ligation step. • You would need to repeat the library preparation, ensuring that you use the correct reagents from the SQK-LSK114 kit	• Keep ONT reagents in their original packaging and make sure you only take from that kit box when you are doing the library preparation.
	Amplification is detected but no sequences are produced	Samples missed during pooling	• If individual samples are accidentally missed during pooling, there will be no reads for that barcode.	• Take extra care to pool all barcoded samples and check volume in pipette tip for each addition.

For Issues during the sequencing run please see some suggested causes and solution on the Nanopore page here

MinKNOW reports a lower number of pores at the start of sequencing than the number reported by the Flow Cell check

	A	B	C	D
	Observed issue	Possible cause	Recommended solution/ corrective	Recommended preventive action

	A	B	C	D
			action	
	MinKNOW reports a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	• After the Flow cell check, it is important to remove any air bubbles near the priming before priming the flow cell. if not removed, the air bubble can travel to the nannopore array and cause irreversible damage to the nanopores • Avoid introducing air as this will permanently damage the intehrity of the pore membranes"	use good practice pipetting to prevent introducing air into the center array which could have a detrimental effect on the membranes and subseqeunctly the active pore count.
	MinKNOW reports a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the seqeuncing run, remove the flow cell from the device and insert it again ensuring that the flow cell is firmly seated in the device and that it has reached the target temperature. if applicable, try a different position on the seqeuncing device (GridION/PromethION)	Correctly insert the flow cell into the seqeuncing device, ensure that it is firmly seated in the device and that is has reached the target temperature
	MinKNOW reports a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminants in the library have damaged the membranes or blocked the pores and therefore unable to seqeunce. This manifests itself as a build-up of "Unavailable" pores over time.	If, despite the channel blocking, the library is still producing a sufficient number reads, carry on with the sequencing experiment. Otherwise, stop the sequencing run in MinKNOW. Then wash out the library from the flow cell using the instructions for the Flow Cell Wash Kit.Then prepare another library and load it on the flow cell.	Ensure to follow the DDNS protocol adequately to avoid carry over of chemical components that could contaminate the RNA sample and subseqeunently have a significant effect on downstream library preparation efficiency, and therefore sequencing throughput.

MinKNOW script failed.

	A	B	C	D
	Observed issue	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. if the issue persists, please collect the MinKNOW log files and contact technical support	

Shorter than expected read length

	A	B	C	D
	Observed issue	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Shorter than expected read length	unwanted fragmentation of the DNA sample during library preparation	During library preparation, avoid pipetting and vortexing when mixing reagents.	Flick or invert the tube when mixing reagents during library preparation

Below is a list of commonly encountered errors when installing and running PIRANHA GUI

Docker installation errors

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Docker installation errors	Error pops up asking to update WSL2 (Windows Subsystem for Linux)	If Docker is using WSL2 backend it may need updating, you can download the executable for doing this on the Microsoft website.	Install this update before installing docker, but it is also ok to wait until it shows the error, in case you do not need to do this.
	Docker installation errors	Error pops up mentioning HyperV, HyperVisor, or virtualisation capabilities	• Go to “Turn Windows features on or off” and make sure HyperV is enabled, you may need to restart the computer after this. • If you are not able to turn HyperV on and it says, “Virtualisation not enabled in firmware”, you need to start up your device in bios settings and enable virtualisation here before turning it on as above.	Check virtualisation is enabled in Windows features before starting up Docker for the first time.

Docker failed to start error

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Docker failed to start	Hyper-V is not enabled or not installed	Enable Hyper-V, by following the below steps: a) Open the Control Panel on your Windows system. b) Click on “Programs and Features” and then click on “Turn Windows features on or off”. c) Scroll down the list of features and look for the “Hyper-V” option. Check the box next to it to enable Hyper-V. d) Click on “OK” to save your changes. e) Restart your computer to apply the changes. Once Hyper-V is enabled, try starting Docker Desktop again	Ensure that Hyper-V is enabled and installed
	Docker failed to start	Virtualization is not enabled in BIOS	Enable virtualization in the BIOS. see the link here on how to enable virtualisation: https://collabnix.com/error-docker-failed-to-start-docker-desktop-for-windows/.	Ensure that virtualisation in the BIOS is enabled

PIRANHA GUI says Docker is not installed when it is

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	PIRANHA GUI says Docker is not installed when it is	Docker is not open	Close PIRANHA GUI, open Docker, then reopen PRIANHA GUI	Open Docker first before opening the PIRANHA GUI
	PIRANHA GUI says Docker is not installed when it is	Docker is not starting up properly or failed to start	Make sure that Hyper-V is installed and enabled on your system to allow Docker to start properly	Ensure that Hyper-V is enabled and installed

You start analysis but it immediately stops with an error

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action

	A	B	C	D
	You start analysis but it immediately stops with an error	The column headers for the barcode csv are incorrect	Make sure the first two columns are labelled “barcode” and “sample” all lower case and with no accidental spaces added	Double check the barcode csv file before running PIRANHA, and you can reuse csv files that have worked before rather than creating new each time.
	You start analysis but it immediately stops with an error	The csv is saved as the wrong file type	PIRANHA GUI is very sensitive to the different csv file types. Ensure that it is saved as a csv (comma delimited)	If you have a csv that has worked previously, you can copy this to your desired location and reuse it, ensuring that the correct information for the current run is input.

You run analysis but the output is empty or all 0 reads

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	You run analysis but the output is empty or all 0 reads	The sample/barcode associations in the barcode csv are incorrect	Double check in the barcodes csv that you have put the correct barcodes to the correct samples. You can also check your fastq_pass files to double check the barcodes detected if you have doubts	As soon as you have added barcodes to a sample, take note and log this in the barcodes csv
	You run analysis but the output is empty or all 0 reads	The file path for the fastq_pass is incorrect	Go back to the start page and check the location selected. Make sure you click all the way through to where the barcode directories are and not to a higher-level directory.	Click through all the way to a barcode directory when selecting the fastq_pass folder in the GUI.
	You run analysis but the output is empty or all 0 reads	Error during transfer of your fastq files	Check inside your fastq_pass barcode directories to make sure they contain fastq files and the number of files match the number in the original fastq_pass folder created by MinKNOW.	Make sure everything has finished copying before removing any external hard drives or USB sticks and eject properly before removing.
	You run analysis but the output is empty or all 0 reads	The GUI cannot access your fastq files	Sometimes, due to read/write permissions, the GUI cannot access the fastq files you point it to. Try moving your files to another destination on your	• When transferring fastq files, put them into a directory within your documents rather than on the desktop or a shared drive.

	A	B	C	D
			device and see if that helps.	You can move the analysis output and fastq files elsewhere after running if you wish. • Check during the initial phase of the analysis to see how many fastq files were located by PIRANHA to see if they were accessed
	You run analysis but the output is empty or all 0 reads	Incorrect analysis settings were used	Check the analysis settings in PIRANHA options as incorrect read length settings can lead results in 0 mapped reads	It is recommended to check the PIRANHA analysis option settings to ensure they are all appropriate for the actual analysis

Your controls have not been recognised in the run report

	A	B	C	D
	Error/Issue raised	Possible cause	Recommended solution/ corrective action	Recommended preventive action
	Your controls have not been recognised in the run report	You have not named your controls to match the default	PIRANHA looks for samples named “positive” and “negative” in the barcodes.csv. Rename your controls appropriately.	Name your controls as positive or negative when you first fill in the details in the barcodes.csv
	Your controls have not been recognised in the run report	Rename your controls appropriately.	• You can tell PIRANAH GUI the names of your controls in the Run Options if you are not staying with the default. • If what you write in does not match the name in the barcodes csv, PIRANHA will not be able to make the association. Ensure that if you specify control names that are different from the default that you match this correctly to the names in the barcodes.csv file	Keep to the default nomenclature of positive and negative