Troubleshooting Guide –

HERA RT-qPCR Kit

HERA SYBR® Green RT-qPCR kit

The following guide can be used to troubleshoot any reactions using the *HERA* RT-qPCR Kit or *HERA* SYBR® Green RT-qPCR kit.

If you require any additional information, please email us at info@willowfort.co.uk.

Problem	Causes	Possible Actions
No or low Amplification	Poor quality RNA	• Reduce the freeze-thaw cycles, to reduce degradation • Check the RIN score before cDNA synthesis • Ensure a clean working area to minimise RNase contamination • Use nuclease-free water
	Poor RNA purity	• Consider sample specific purification kits for RNA • Measure RNA purity using a UV spectrophotometer. • Reduce the concentration of inhibitors by dilute RNA in nuclease free water • Remove salts and inhibitors by repeating purification.
	Low quantity of RNA	• Check RNA concentrations using a UV spectrophotometer. • Consider using reagents and reverse-transcriptase with increased sensitivity to low RNA concentrations.
	High GC content	• Use thermostable reverse transcriptase to allow for higher temperatures, breaking the GC bonds.
	High secondary structure content	• Increase temperature during reverse transcription to prevent hairpin structures. • Before reverse transcription, prevent secondary structures by incubating RNA at a high temperature, followed by a transfer to ice.
	Insufficient reverse transcriptase	• Increase cDNA quantity • Consider a reverse transcriptase with increased sensitivity • Consider a reverse transcriptase efficient against potential inhibitors

Problem	Causes	Possible Actions
No or low Amplification continued…	Insufficient time and temperature for optimum Reverse Transcriptase reaction	• Check RT-PCR machine for correct time and temperature
	Error in primer design	• Choose primers specific to RNA i.e. if using degraded RNA, random primers would be beneficial rather than oligo(dT) primers
	Reagent quality	• Refer to manufacturer's storage suggestions • Only use fresh reagents with reverse transcriptase • Reduce the number of freeze-thaw cycles • Ensure a complete mix of reagents • Check for any precipitation in any reagents.
Non-Specific Amplification	DNA contamination	• Use a control e.g. No-RT control • Treat RNA samples before beginning reverse transcription
	Error with primer design	• Increase primer length to increase specific amplification. • Increase annealing temperature to improve specificity of primer binding.
	Poor RNA quality	• Check RNA concentration before cDNA synthesis • Reduce the number of freeze-thaw cycles • Include a RNase inhibitor during reverse transcription • Use nuclease-free water buffer when storing RNA • Include a genomic DNA step during RNA extraction
Truncated cDNA	High secondary structure content	• Increase temperature during reverse transcription to prevent hairpin structures. • Before reverse transcription, prevent secondary structures by incubating RNA at a high temperature, followed by a transfer to ice.
	Errors with primers	• Choose primers specific to RNA i.e. if using degraded RNA, random primers would be beneficial rather than oligo(dT) primers • Use oligo(dT) when synthesizing full-length cDNA • Optimise primer concentration

Problem	Causes	Possible Actions
Higher Ct value than expected	RNA template is damaged or degraded	• Review RNA extraction protocol
	RNase contamination	• Ensure a clean work area. Use 70% ethanol and dH_2O to clean all equipment and space. • Add a RNase inhibitor after cDNA synthesis
	RT inhibition present	• Use an additional wash of ethanol to remove inhibitors during RNA extraction.
	Poor cDNA synthesis	• Check correct temperature on Thermocycler • If necessary, increase cDNA synthesis temperatures
Low Ct value than expected or lower Ct value than positive control	Template contamination	• Identify source of contamination by checking and replacing reagents. • Use separate pipettes for pre- and post-cDNA reactions • Ensure a clean work area. Use 70% ethanol and dH_2O to clean all equipment and space
	Concentration of RNA sample are too high	• Confirm the RNA concentration. • Decrease concentrations of RNA in PCR set-up
PCR Reaction Not Reproducible or Reaction Stopped Working	Different cycling conditions	• Use the same thermal cycler for optimization and all future experiments.
	dNTPs, primers or sample degraded	• Aliquot components to reduce number of freeze-thaw cycle.
	Error in set up	• Ensure correct reagents have been added • Check correct thermal cycler program has been selected.
	Change in component	• Check for new components added to the master mix (e.g. new batch of primers)
	Inhibitors in template	• Decrease template concentration.