NOYA 2x HRM Master Mix

Catalog number: N- 1221

« Research Use Only »

Overview

Sensitive and quantitative detection of DNA targets by real time PCR .NOYA 2x HRM master mix kit has been optimized for high resolution melt analysis perform after PCR reaction and mutation detection. This is a ready to use solution and fast experimental tool as well. It minimizes contamination due to reduced number of pipetting steps. The mix is optimized for efficient and reproducible experiments and qualitative results are guaranteed. It may be used for complex templates such as GC rich DNA sequences.

Storage:

Store at -20°C. Repeated freezing and thawing may reduce the sensitivity and should be avoided.

100 Reactions

Kit Content

- NOYA 2x HRM master mix, 1ml
- Hot-start Taq polymerase.
- Mgcl2 (25mM) 1ml.

Other supplies required

- Disposable gloves
- Precision pipette
- Aerosol resistant pipette tip
- Sterile 1.5ml tube
- Nuclease free water
- Plastic PCR tube or 96-well reaction plates
- Real Time PCR for quantitative PCR

Protocol

Set up the experimental reaction by adding in order the following components:

For the 1st reaction;		
	Volume/reaction	Final Concentration
Nuclease free water	Variable	-
NOYA 2x HRM Master Mix	10µl	1x
25mM Mgcl2*	Variable	up to 4mM
Upstream Primer	Variable	0.1-0.2µM
Downstream primer	Variable	0.1-0.2µM
DNA Template **	Variable	10-50ng of genomic DNA
Hot-start Taq polymerase	0.3µl	0.625 units
Total volume	20 µl	

*Mgcl2 25mM for easy optimization of PCR performance. It is well known that the magnesium concentration affects primer annealing, product specificity and enzyme activity. Careful optimization of the PCR conditions can usually reduce the production of primer –dimer and unspecific product.

** Quality of DNA template is too important .Utilize DNA template free from PCR inhibitors.

- Reaction Volume : use 10-50ng of genomic DNA in 20 µl reaction
- Primer design: use primer with matched Tm. Avoid inter- and intra-primer complementary sequences.
- Amplicon size: the best results are obtained with 100-200bp length.

For different volumes calculate all components proportionally.

1. Pipette all components on ice or cool box and minimize exposure of the Master Mix to the light. (Minimize pipetting error by avoiding very small volume (5 µl) and using calibrated pipette.
2. Gently mix the reactions while avoiding bubble (do not vortexing).
3. Dispense appropriate volume into PCR tubes or plates.

PCR cycle programs

Place the reaction in the instrument and run the appropriate PCR program as follows. The optimization of amplification protocol for each primer /template system is recommended.

Step	Time	Temp.	Additional comments
Initial activation step	10 min	95°C	Enzyme activation
Denaturation	15 s	95°C	
Annealing /Extension *	30-50 s	X°C	Approximately 3- 5°C below *Tm* of primers
Extension**	15-45 s	68-72 °C	
Cycle number 30-50			Cycle number depends on the amount of template DNA

*HRM procedure can be performed by two different approaches: A) First running on PCR machine and Running PCR product on real time system by an HRM profile .B) Running whole process on Real time PCR. In both cases for HRM profile refer to the instrument instruction. Since double stranded intercalating dye in master mix binds to all double stranded molecules, we highly recommend, routinely melt curve analysis step in order to verify specificity and identity of the PCR products. **For HRM analysis this step can be omitted.

Troubleshooting

Observation(s), Possible Cause(s), Solution(s)
«PCR»
Amplification of the majority of samples have a Ct > 30, resulting in the reaction not reaching plateau
Low template concentration • Increase concentration of template DNA.
Poor priming efficiency • Reduce annealing temperature. • Increase annealing time.
MgCl2 concentration is not optimal • Optimize the MgCl2 concentration (attempt 1.5 mM, 2.5 mM, 3.5 mM MgCl2).
DNA is damaged • Re-purify the DNA.
Ct of samples are inconsistent
Varying DNA concentrations • Re-check the DNA concentrations.
DNA is damaged • Re-purify the DNA.
Non-specific amplification
Mispriming • Increase the annealing temperature during amplification/extension. • Check for mispriming using a software package and redesign the primers if necessary. • Reduce primer concentration to minimize primer-dimers and non-specific priming.
MgCl2 concentration is not optimal for primer set • Optimize the MgCl2 concentration (attempt 1.5 mM, 2.5 mM, 3.5 mM MgCl2) for amplification.
Multiple melting peaks appear for one product
Two products of the same length • Check the products on an agarose gel. • Heterozygosity can result in two peaks.
A HRM melt uses more readings than a standard melt that can give the impression of 2 products on a melting peak • Check the products on an agarose gel. • Adjust the filter settings of the melt peak analysis to minimize noise. • Increase the annealing/extension temperature to help minimize non-specific amplification.
«HRM»
Replicate samples show a wide variance during HRM analysis
Variations in reaction mixture (e.g. salt) • Check the purity of the template solution. • Dilute all template samples in the same buffer (10 mM Tris-HCl, pH 8.5) to equalize salt content. • Re-purify the DNA using a new method. • Ensure all reagents and Master Mixes are vortexed well prior to reaction setup.

Concentrations of template are very different • Ensure all samples are the same DNA concentration for maximum resolution.
Rare or new generic variants may generate, results outside of the expected ranges • Sequence the unusual results. • Redesign primers to produce a shorter amplicon, hopefully avoiding the genetic variation. • Mixing the amplicons (9.5 µl from each of two amplicons) can help identify potential new variants.
Secondary structure in the amplicon • Use a Melting Profile software system to ensure no secondary structure are present (eg. http://mfold.rna.albany.edu/?q=DINAMelt).
Only one homozygote is detected during HRM
Difficult to detect mutation (typically Type IV, A/T) • May need to sequence to confirm, or use a probe or snapback primers.
Only one homozygote is present • Mix 9.5 µl of each of the homozygote controls and run a HRM analysis (include an initial melt at 95 °C form 3 min); the melting profile should change to the heterozygote, which is usually easier to detect.
Different genotypes cannot be detected during HRM
MgCl2 concentration not optimal • Perform amplification and HRM at 1.5, 2.5, 3.5 mM MgCl2 to find the optimal concentration for HRM analysis.
Non-specific amplification • Check the melting curve to see if multiple peaks are present (adjust filter settings if necessary). • Run product on a 2 - 3% agarose gel to confirm if multiple bands are present. • Reduce primer concentration to minimize primer-dimers and non-specific priming. • Increase the annealing/extension temperature. • Redesign primers.
Template contains HRM inhibitors (eg. salt) • Re-purify DNA template.
Difficult to detect mutation (typically Type IV, AT) • Shorter amplicons are better for detecting Type III and Type IV mutations. • Redesign the primers to produce a smaller amplicon. • Use a probe or snap-back primers.
Unusual appearance of melt curves
Multiple melt regions or secondary structure • Presence of mutation(s) can result in more complex melting curves due to the development of secondary structures. This can still provide reliable HRM data. • Long amplicons are more likely to develop secondary structures. • Redesign primers to amplify a shorter fragment.
Non-specific amplification • Increase the melting/annealing temperature during amplification. • Optimize the MgCl2 concentration (e.g. 1.5 mM, 2.5 mM, 3.5 mM MgCl2).

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