



Operation Guide of HRM Analysis Kit (EvaGreen) (FP210)

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Experiment Preparation

1. cDNA sample
2. Pipette and matching pipette tips (RNase-free)
3. 1.5 ml centrifuge tube (RNase-free) and 200 μ l PCR tube (RNase-free)
4. Vortex oscillator, centrifuge and metal bath/thermal cycler



Step 1



Thaw 2×HRM Analysis PreMix, 50×ROX Reference Dye, DNA template, primer and RNase-Free ddH₂O and place it on ice after equilibrating all reagents to room temperature and mixing thoroughly. Before use, mix each solution evenly by vortex oscillation, and briefly centrifuge to collect the liquid remaining on the tube wall.

Step 2

It is recommended to prepare Real Time PCR reaction solution on ice according to the following table

Component	50 μ l system	25 μ l system	20 μ l system	Final concentration
2 \times HRM Analysis PreMix	25 μ l	12.5 μ l	10 μ l	1 \times
Forward primer (10 μ M)	1.5 μ l	0.75 μ l	0.6 μ l	0.3 μ M
Reverse primer (10 μ M)	1.5 μ l	0.75 μ l	0.6 μ l	0.3 μ M
DNA template	—	—	—	—
50 \times ROX Reference Dye	—	—	—	—
RNase-Free ddH ₂ O	To 50 μ l	To 25 μ l	To 20 μ l	—



Tips

1. When preparing the quantification mixture, the required reaction volume shall be determined first. Calculate the total volume of reagents required and increase the volume by 10%-20% to compensate for the pipetting loss, thus to ensure the solution is sufficient for desired numbers of reactions. For example, when a total of 5 quantification reactions are required, the number of system preparations is at least 6; when a total of 10 quantification reactions are required, the number of system preparation is at least 11; when a total of 20 quantification reactions are required, the number of system preparations is at least 22. And so on.
2. The required amount of components except cDNA template and ddH₂O should be calculated first according to the number of reactions. . Prepare all components into the same tube on ice, thoroughly mix, and centrifuge for a short time.
3. The required volume of cDNA template and ddH₂O to be added per sample shall be calculated. If the required ddH₂O volume for each sample is the same, the overall required ddH₂O volume can be calculated. Add the ddH₂O to the mixture and mix well completely.

Reagent	1 50 µl system Usage amount	6 50 µl systems Usage amount	11 50 µls ystems Usage amount	22 50 µl systems Usage amount
2×HRM Analysis PreMix	25 µl	150 µl	275 µl	550 µl
Forward primer (10 µM)	1.5 µl	9 µl	16.5 µl	33 µl
Reverse primer (10 µM)	1.5 µl	9 µl	16.5 µl	33 µl
50×ROX Reference Dye	Calculate and add according to the actual situation.			
RNase-free ddH ₂ O	Calculate and add according to the actual situation.			

4. Divide the mixture into each detection tube/well, and add the samples in the order of mixture-cDNA-ddH₂O (if necessary) to prepare the system and thoroughly mix evenly.

Tips

The method of distributing premixed Mix can effectively improve the repeatability of the experiment. Please operate on ice when preparing and dispensing. Please avoid the strong light exposure when preparing the PCR system.

The usage amount of genomic DNA template is generally less than 100 ng in the 20 μ l reaction system. It is recommended to keep the same template amount between different reactions. The template purity requirement is: OD_{260/280}: 1.6-2.0, OD_{260/230}: 1.5-2.0.

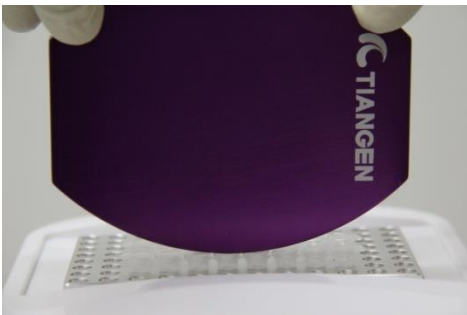
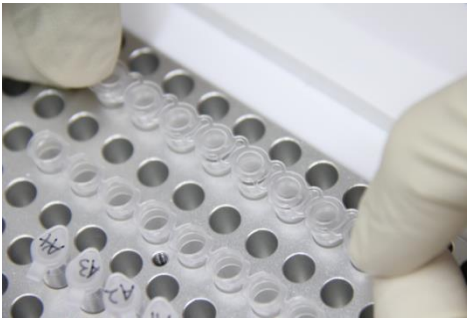
50 μ l reaction system is recommended to use under the appropriate conditions due to the high sensitivity of HRM. A large reaction system can improve the repeatability of the reaction and reduce the negative influence of experimental errors on the melting curve.

When the final concentration of the primer is 0.3 μ M, the amplification conditions are good in most systems. In case of lower amplification efficiency, improve the primer concentration in the PCR reaction system. The primer concentration in PCR reaction system can be appropriately reduced when the non-specific amplification occurs. It can be adjusted in the range of 0.2-0.5 μ M if needing to further optimize the primer concentration.

The optimum ROX Reference Dye concentrations for several common instruments are shown in the following table:

Instrument	Final concentration
ABI 7900HT	5 \times (e.g., 5 μ l ROX/50 μ l system)
ABI 7500Fast, Viia7, etc.	1 \times (e.g., 1 μ l ROX/50 μ l system)
Roche instrument, Bio-Rad instrument, Eppendorf instrument, etc.	No need to add

Step 3



When using the 8-tubes strip, cover the tube cover and compact with a capper after prepared and sub-package of the reaction system. Mark on both ends of the tube cover. Don't mark the tube cover directly above the detection well, or it will affect the fluorescence reading.

When using the N96 well plate, seal the plate with plate cover and compacted after prepared and sub-package of reaction system, and the marks shall be marked around the well plate or the detection well without samples.

Step 4



Use a plate centrifuge to briefly centrifuge N96 well plate or 8-tube strip. Please note to make the bottom of the plate outward and keep balance.

Place the 8-tube strip on the tube rack and fix it with a fixing piece to centrifuge when centrifuging the 8-tube strip.

Step 5



Stage	Circles	Temperature	Time	Content	Fluorescence signal acquisition
Initial denaturation	1×	95°C	2 min	Initial denaturation	No
PCR reaction	40×	95°C	10 sec	Denaturation	No
		60°C	30 sec	Annealing/Extension	Yes
Melting Curve Analysis (Melting/Dissociation Curve Stage)					

Determine if the thermal cycler is applicable to the HRM experiment before starting the reaction.

Transfer the samples to the real time PCR instrument and set the program to start the qPCR reaction.