

# HRM Analysis Kit (EvaGreen)

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Professional reagent for high resolution  
melting curve analysis

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# HRM Analysis Kit (EvaGreen)

Cat. No. 4992776/4992873

## Kit Contents

Contents	4992776 20 µl×125 rxn	4992873 20 µl×500 rxn
2× HRM Analysis PreMix (EvaGreen)	1.25 ml	4×1.25 ml
50× ROX Reference Dye	250 µl	1 ml
RNase-Free ddH <sub>2</sub> O	2×1 ml	5×1 ml
Handbook	1	1

## Storage

The HRM Analysis Kit (EvaGreen) can be stored at -30~-15°C for one year. It should be stored immediately after receipt at -30~-15°C, protected from light. Thaw the 2× HRM Analysis PreMix and 50× ROX Reference Dye and mix thoroughly before use. If the 2× HRM Analysis PreMix and 50× ROX Reference Dye are thawed but not used, it is important to thoroughly mix them prior to refreezing. The delamination of salts during the thawing process and subsequent crystallization during freezing will damage the enzyme and decrease product performance. For frequent use, HRM Analysis PreMix can be stored at 2-8°C for 3 months. Repeated freeze-thaw cycles should be avoided.

## Introduction

HRM analysis Kit (EvaGreen) combines the advantages of saturation dye EvaGreen and antibody modified enzyme, and is specially designed to perform High Resolution Melting (HRM) analysis. A 2×pre-mixed solution is included in this kit which greatly simplifies the preparation of qPCR reaction mixture. It is highly sensitive and could be used to analyze different types of templates.

Unlike SYBR Green, high concentration of EvaGreen dye will not inhibit PCR reaction, but could increase dsDNA products binding, so it is called “saturation dye”. In contrast with SYBR Green, “dye rearrangement” will not happen when using EvaGreen, so it could properly distinguish the difference on single base of amplification products.

This Kit can be used in known SNP analysis, unknown mutant gene scan and Methylation Specific PCR (MS-PCR).

## Features

1. HRM Analysis PreMix uses antibody modified hot start DNA polymerase, along with the specially optimized buffer solution, provides a high sensitive and high efficient analysis method with wide sample range.
2. HRM Mix includes EvaGreen saturation dye, which has high melting resolution under saturation condition, so it could detect the mutation of single base.
3. The unique buffer system in this kit strengthens the stability of melting curve, increasing the specificity of amplification.
4. This Kit includes ROX Reference Dye, which can eliminate the interference of signal background and revise the deviation of fluorescence signal between different wells.

## Principle of Operation

SYBR Green is the most widely used fluorescent dye in Real Time PCR, but its concentration need to be very low in the PCR reaction mixture since it has inhibition effect toward the PCR reaction. The binding condition of SYBR Green and dsDNA is not saturated, so it is called “unsaturation dye”. This trait of SYBR Green will lead to the “dye rearrangement” effect, and then influence the resolution of melting curve. In this condition the difference between single bases cannot be distinguished by analyzing melting curve when using SYBR Green as fluorescent dye.

EvaGreen is a novel fluorescent dye which can be used both in Real Time PCR and HRM analysis. This dye can selectively bind to dsDNA through a specific mechanism called “release according to requirement”. This mechanism not only maximally reduces the PCR inhibition effect of fluorescent dye, but also allows the HRM analysis to detect single base differences when the fluorescent dye is under saturation condition.

### Important Notes

1. This Kit includes the fluorescent dye EvaGreen. Store the reagent in dark and avoid direct exposure to strong light during the preparation of PCR reaction mixtures.
2. Performance of reagents may reduce without mixing well. Please gently mix the reagents by inverting the tubes and centrifuge briefly prior to use. Do NOT vortex and avoid producing bubbles.
3. The purity of primers is important for the specificity of PCR. Primers purified by PAGE or more superior methods are recommended.
4. In a 20  $\mu$ l reaction volume, the amount of genomic DNA template is usually less than 100 ng. Please use the same template amount between different reactions. Template purity should be:  $OD_{260}/_{280}$  1.6-2.0,  $OD_{260}/_{230}$  1.5-2.0.
5. Since HRM have extreme high sensitivity, so 50  $\mu$ l reaction volume is recommended. Large reaction volume could increase the experimental repeatability, and reduce the negative effect of experimental error to melting curve.
6. Primer design: Shorter PCR product could increase the HRM resolution. So beside the common principles of primer design, please make the length of PCR product within 80-120 bp. SNP site should be designed in the middle of PCR product sequence.

## Protocol

### <1> Set up the HRM PCR reaction system

**Note: 2× HRM Analysis PreMix and 50× ROX Reference Dye should be stored protected from light.**

1. Thaw 2× HRM Analysis PreMix (if stored at -30~-15°C), 50× ROX Reference Dye, template, primers and RNase-Free ddH<sub>2</sub>O. Completely mix and equilibrate reagents to room temperature before use.
2. All the HRM PCR reaction steps should be operated on ice.

Reaction system

Component	50 µl volume	20 µl volume	Final concentration
2× HRM Analysis PreMix	25 µl	10 µl	1×
Forward Primer (10 µM)	1.5 µl	0.6 µl	0.3 µM*
Reverse Primer (10 µM)	1.5 µl	0.6 µl	0.3 µM*
Template**	-	-	-ng
50×ROX Reference Dye <sup>△</sup>	-	-	-
RNase-Free ddH <sub>2</sub> O	Up to 50 µl	Up to 20 µl	-

Regular reaction volume is 20 µl, but please use 50 µl reaction volume if possible. Large reaction volume could reduce the negative effect of experimental error to melting curve.

\*A final primer concentration of 0.3 µM is optimal for most applications. Higher primer concentration can be used when the amplification efficiency is not favorable. If non-specific amplification is observed, the primer concentration should be reduced. For further optimization, a primer titration of 0.2-0.5 µM can be performed.

\*\*Since the HRM analysis is extremely sensitive, please use the same template amount between different reactions.

<sup>△</sup> The optimal concentration of ROX Reference Dye for commonly used Real-Time PCR instruments:

Instrument	Final Concentration
ABI 7900HT	5× (e.g. 5 µl ROX/ 50 µl volume)
ABI 7500 Fast	1× (e.g. 1 µl ROX/ 50 µl volume)
Roche LightCycler 480, Qiagen Rotor-Gene	No need

## <2> Real-Time Amplification

Typically, two-step PCR is recommended. However, if two-step PCR does not yield favorable results (e.g. non-specific amplification caused by low template concentration or reduced amplification efficiency induced by low T<sub>m</sub> value) three-step PCR is recommended.

Two-step PCR:

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C	2 min	Initial denaturation	N
PCR	40×	95°C	10 sec	Denaturation	N
		60°C <sup>△1</sup>	30 sec	Annealing/Extension	Y
Melting/Dissociation Curve Stage <sup>△2</sup>					

## Three-step PCR

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C	2 min	Initial denaturation	N
PCR	40×	95°C	10 sec	Denaturation	N
		60°C	20 sec	Annealing	N
		72°C	30 sec	Extension	Y
Melting/Dissociation Curve Stage <sup>△2</sup>					

<sup>△1</sup> An Annealing/Extension temperature of 60°C for 30 sec is optimal for most applications. However, if further optimization on reaction specificity is required, it is recommended to optimize the temperature in the range of 60°C-66°C

<sup>△2</sup> During the process of HRM Melting Curve analysis, please set the data collection frequency to every 0.02-0.1°C.

3. Close the tubes and mix samples gently. Brief centrifugation can be performed to collect residual liquid from the walls of the tubes.
4. Place the PCR tubes in the thermal cycler and then start the PCR cycle.

## Case Study

Retrieve known SNP from NCBI database for analysis, the information of SNP is as follows:

RefSNP number: rs174538

Gene Name: FEN1 (flap structure-specific endonuclease 1)

Sequence: CGCTCCGCCACCGAAGAACACGTCG[A/G]CAGGAGCAGGCGCCTA  
GCACAACCG

Allele Frequency: A=0.314, G=0.686

Design the primer according to the sequence of the both sides of SNP sites:

FEN1\_F: CCTCAACGCTCTCACCATTTTG;

FEN1\_R: GGCACTTCCTTTCCGGTTGTG;

The length of amplified PCR product is 108 bp.

Setup the PCR reaction, run the PCR program on Roche LightCycler480, 24 samples are analyzed.

Reaction system:

Component	20 $\mu$ l volume
2 $\times$ HRM Analysis PreMix	10 $\mu$ l
Forward Primer (10 $\mu$ M)	0.6 $\mu$ l
Reverse Primer (10 $\mu$ M)	0.6 $\mu$ l
Template	1 $\mu$ l (~50 ng)
50 $\times$ ROX Reference Dye*	0 $\mu$ l
RNase-Free ddH <sub>2</sub> O	Up to 20 $\mu$ l

\*ROX Reference Dye is not required on LightCycler 480

Reaction Program:

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1 $\times$	95°C	2 min	Initial denaturation	N
PCR	40 $\times$	95°C	10 sec	Denaturation	N
		60°C	30 sec	Annealing/ Extension	Y
Melting/Dissociation Curve Stage					

## Experiment Result

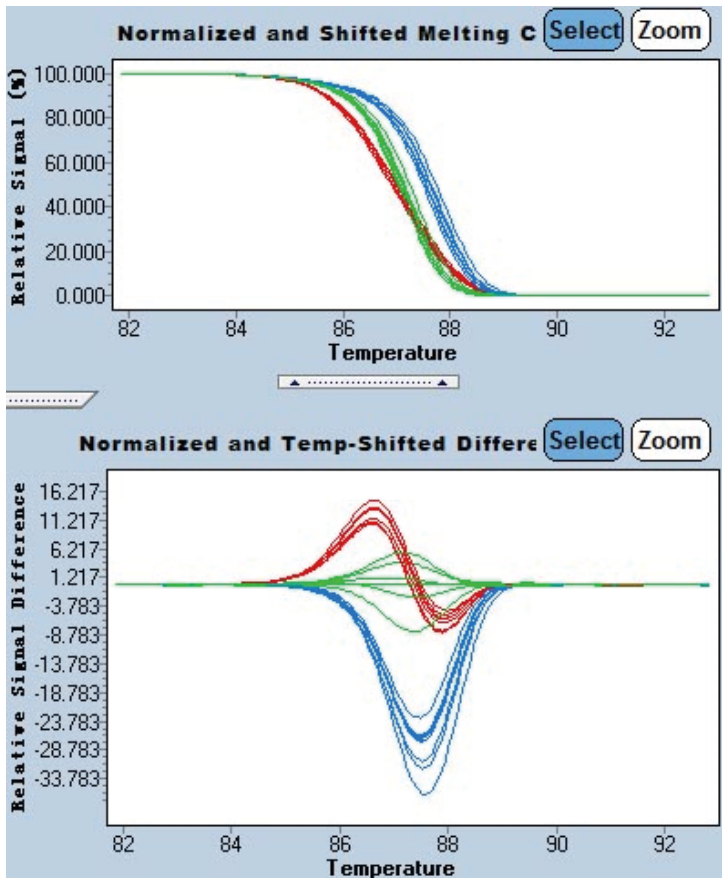


Figure 1: Result of a known SNP analysis, NCBI Assay ID is ss869699; length of PCR product is 108 bp; 24 samples were analyzed. Roche LightCycler 480 was used to collect dissociation curve signal data; Ramp Rate 0.05; Acquisition 12; Gene Scanning analysis. The result shows that HRM Analysis Kit can properly distinguish samples which belong to different genotypes, melting curve of samples belong to same genotype have high repeatability, no error occurred.