Viale Piceno 137/f
61032 Fano PU (IT)
Telephone + 39 (0)721830605
FAX +39 (0)721837154
e-mail:info@diatheva.com
www.diatheva.com

コスモ・バイオ株式会社

ERCC1 Gln504Lys HRM kit

MBK0041
50 Reactions (42 tests)
Store at -20°C

Intended use

For identification of ERCC1 Gln504Lys polymorphism, due to a G>T transition (rs3212986), by High Resolution Melt (HRM).

Introduction

ERCC1 gene encodes for a protein involved in the DNA repair pathway by nucleotide excision, and it is required for the repair of DNA lesions such as those induced by UV light or formed by electrophilic compounds including cisplatin. ERCC1 protein forms a heterodimer with the XPF endonuclease, and the heterodimeric endonuclease catalyzes the 5' incision in the process of excising the DNA lesion. The heterodimeric endonuclease is also involved in recombinational DNA repair and in the repair of inter-strand crosslinks. Polymorphisms that alter expression of this gene may play a role in carcinogenesis

ERCC1 Gln504Lys polymorphism is associated with nephrotoxicity in patient receiving a cisplatin-cyclophosphamide regimen[1] and with outcome in patient treated with platinum-based chemotherapy[2].

Product description

The ERCC1 Gln504Lys HRM kit is an easy-to-use master mix dedicated for the use with the Rotor-gene 6000 instrument (Corbett Research) and Rotor-Gene Q (Qiagen). The kit contains reagent, enzyme and genotype controls for the detection of ERCC1 Gln504Lys SNP located in the of ERCC1 gene by HRM.

Kit contents

1 x ERCC1 Gln504Lys 2X master mix (650 µl)
1 x DNA polymerase (10 µl)
2 X ERCC1 Gln504Lys T/T CONTROL (10 µl)
2 X ERCC1 Gln504Lys T/C CONTROL (10 µl)
2 X ERCC1 Gln504Lys C/C CONTROL (10 µl)

Storage

The product should be stored at -20°C immediately upon arrival. When stored under the recommended conditions and handled correctly, the kit is stable up to the expiry date indicated in the attached label.
Note: The product is guaranteed for two thawing steps. For an intermittently use we suggest to subdivide ERCC1 Gln504Lys 2X master mix in small aliquots.

Precautions

The user should always pay attention to:

- use pipette tips with aerosol-preventive filters, deionized DNA-free water and gloves;
- store positive material (plasmidic DNA controls) separately from all other reagents and, if possible, add it to the reaction mix in a separated space;
- do not use the same precision pipettes for reaction mix and DNA;
- thaw all components samples at room temperature before starting an assay;
- when thawed, mix the components and centrifuge briefly.
- Protect x ERCC1 Gln504Lys 2X master mix from light.

Procedure

PREPARING HRM GENOTYPIC CONTROLS
Genotype controls must be prepared by 1:10 dilution of stock solution in PCR –grade water (not provided).
Genotype controls are provided to characterize the HRM curves of unknown amplicons.

PCR SETUP
Total volume per reaction is 25 µl.

Before each use, thaw all reagents completely, mix and centrifuge.
Pipet mastermix and the Taq polymerase in the quantity needed for the planned reactions into a 1.5 ml reaction tube and mix as indicated in table 1 .

Moreover include provided positive controls for each assay as references for genotype assignment.

Table 1

	1 reaction	50 reaction
PCR Mastermix 2X	12.5 µl	625 µl
Taq Diatheva (5U/µl)	0.125 µl	6,25 µl
H_2O	10,375	518,75
Total volume	23 µl	1150 µl

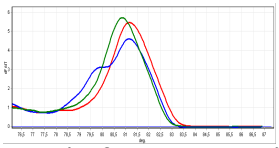
Aliquot 23 µl of master mix into each PCR reaction tube before adding 2 µl sample DNA*, or 2 µl of each provided positive controls (ERCC1 Gln504Lys T/T-T/C-C/C genotypes).

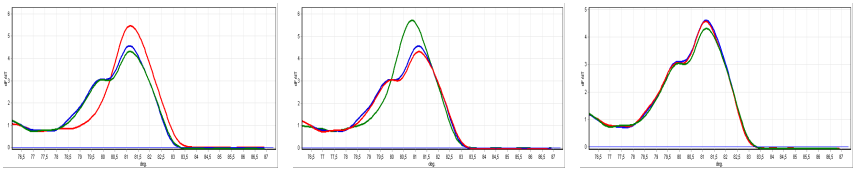
*Employ 10 ng DNA/reaction (2 µl of a 5ng/µl concentrated DNA).
DNA extracted employing **QIAamp DNA Blood Mini Kit** and **DNeasy Blood & Tissue Kit** –QIAGEN can be used without quantitation step, simply diluting the sample 1:2 in water.
(DNA quality is a fundamental factor for HRM genotyping: kit has been optimized with DNA extracted by **DNeasy Blood & Tissue Kit** - **QIAamp DNA Blood Mini Kit -**QIAGEN and **Blood genomic DNA isolation mini Kit –** NORGEN).

!! Attention !! Final elution step must be carried out using distilled water instead the elution buffer supplied with the respective kits.

Alternative isolation methods have to be validated from the user. In any case we suggest to elute purified DNA in water.

Negative controls (NTCs) are not required for this analysis since contaminations can be easily detected through positive controls curves behaviour: if the reaction is not contaminated positive controls generate 3 distinct curves, while if contaminated, controls generate two or three heterozygous curves (see the graphs below)


Example of not contaminated mix: the 3 positive controls generate 3 different curves.


Example of contaminated mix: the 3 positive controls generate 2 or 1 curves only.

THERMAL PROFILE
HRM analysis setting is a prerequisite for accurate results. For details, please refer to the manual provided with your HRM real-time PCR instrument.
Program the HRM instrument according to the operator's manual and table 2

Table 2: optimized cycling protocol for rotor-gene 6000

STEP	TEMPERATURE	TIME	CICLES
Initial denaturation	95°C	10 min	1X
Denaturation	95°C	10 sec	40 X
Annealing / Extension	59°C	40 sec	
Acquire on the GREEN Channel in the Annealing / Extension step			
High Resolution melting	Ramp 83°-94°C	Rise temp 0.2°C/step; wait for 90 sec pre-melt conditions; wait for 2 sec for each step afterwards; For optimal acquisition of fluorescence data, set the gain to 70% of saturation in the highest fluorescent signal.	
Acquire on the HRM channel			

ANALYSIS SETUP

For HRM analysis set confidence level at 90%.

Due to the comparison between controls and unknown samples HRM profiles, the software instrument performs an automatic genotyping showing a confidence level for each sample. Results with a confidence level lower than 90% are defined "variation" and have to be reject.

Troubleshooting

No signal, poor Rn value (PCR or HRM) or signal detected late in PCR	Pipetting error or missing reagent	Check the storage conditions of the reagents, Repeat the assay.
	Problems with starting template DNA	Check the concentration, storage conditions, and quality of the template and control DNA ($\lambda_{260}/\lambda_{280}$ ratio of DNA samples must be over 1,7.) Efficient removal of PCR inhibitors is essential for optimal results; purify nucleic acids from your sample using an appropriate purification method. Insufficient or degraded template DNA, increase the amount of template DNA if possible.

References

- Khrunin AV et al.**Genetic polymorphisms and the efficacy and toxicity of cisplatin-based chemotherapy in ovarian cancer patient** Pharmacogenomics J. 2010 Feb;10(1):54-61
- Kalikaki A et al. **DNA repair gene polymorphisms predict favorable clinical outcome in advanced non-small-cell lung cancer** Clin Lung Cancer. 2009 Mar;10(2):118-23**.**

Example images for HRM analysis Images

A-A

A-C

C-C

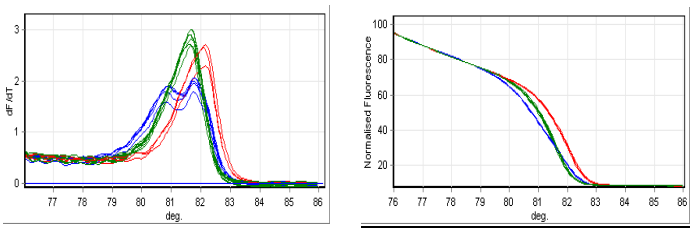


Fig. 1: Graphical visualizations for the identification of the MTHFR A1298C polymorphisms.
A) Melting curves show heteroduplexes formation in the A-C population (in blue) and temperature shift between A-A and C-C genotypes (green and red curves, respectively).
B) Normalized melting curves of the three genotypes.

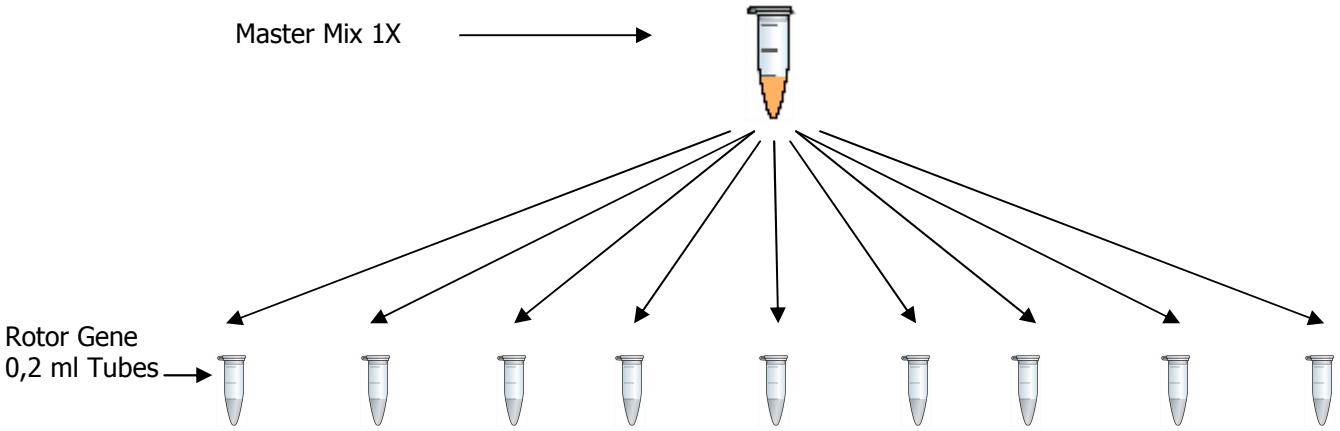
Example of experiment set-up for 6 unknown samples:

1) **Prepare each positive controls and samples as described in procedure paragraph and keep them on ice.**

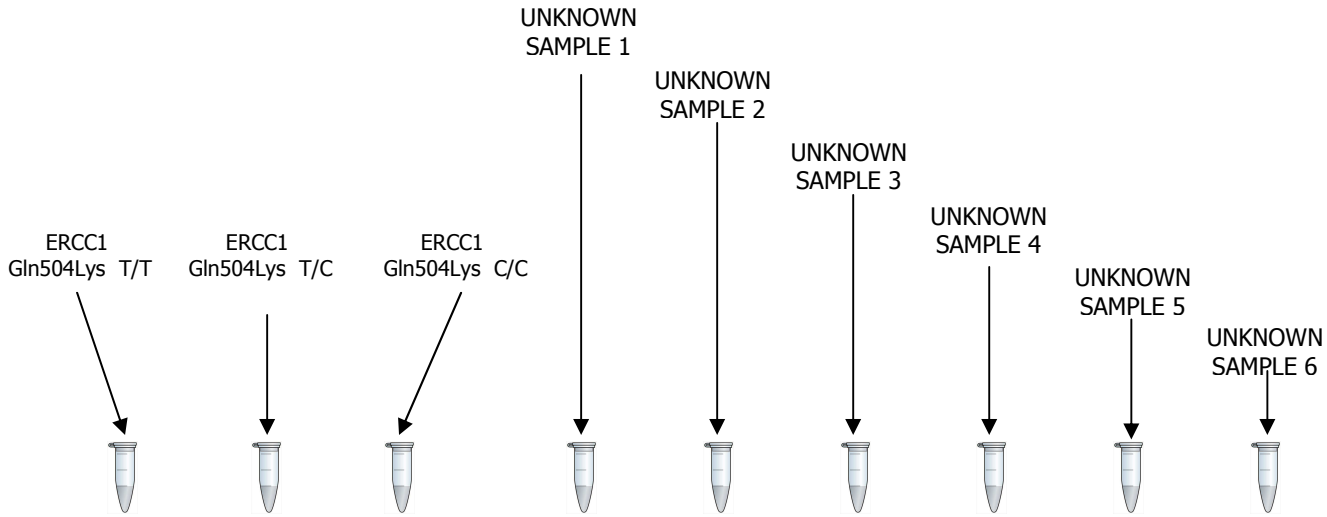
2) **prepare Master Mix 1X for 10* reaction (the 3 positive controls + 6 unknown samples + 1):**

	1 reaction	10 reaction
GSTP1 Ile105Val 2X PCR Master Mix	12.5 µl	1255 µl
DNA Polymerase (5U/µl)	0.125 µl	1,25 µl
H_2O	10.375	103,75 ul
Total volume	23 µl	230 µl

3) **aliquot 23 µl in each rotor-gene 0,2 ml tubes.**



4) **Dispense in each PCR tubes 2 µl of positive controls or samples prepared in step 1.**



5) **Start thermal cycling as described in procedure paragraph.**

*In order to eliminate pipetting errors we suggest to consider a reaction excess for every Master Mix constitution.