CALR MutaPrime FA Kit 4 **ENG**

INSTRUCTION

CALR MutaPrime FA Kit is a diagnostic system for identification of insertion and deletion mutations in the CALR gene. The appropriate PCR primers are labeled with fluorescent dye 6-FAM, detection of mutations is based on separation of the PCR products by capillary gel electrophoresis, followed by analysis of resulting fluorescence peaks by means of fragment analysis technique.

Calreticulin (CALR) is a multifunctional protein that acts as a major Ca(2+)-binding protein in the lumen of the cellular endoplasmic reticulum. It is, suggested to play a role in transcriptional regulation. Calreticulin is encoded by the *CALR* gene on the chromosome19. Somatic mutations in exon 9 of *CALR* are the second most prevalent acquired nucleotide changes in Ph-negative myeloproliferative neoplasms (MPNs), except of polycythaemia vera (PV). The two specific mutations are most common, L367fs*46 (type 1 mutation) which represents a 52-bp deletion flanked by 7 base pairs of identical sequence and a K385fs*47(type 2), which results from a 5-bp insertion, and representing an inverse duplication of the five nucleotides preceding the insertion. Overall, these two frameshift mutations types are found in more than 80% of all patients with mutant CALR.
Capillary gel electrophoresis is method of PCR products separation in electric field inside a capillary pre filled with polymer. When high voltage is applied, the fluorescently labeled PCR-products and other components start to pass move inside the capillary at the speed depending on their electric charge and mass and, therefore, arrive to the detection area at different times. The detected PCR-products are displayed as a sequence of fluorescent peaks. Therefore, migration time is the key quality characteristic to the molecule, whereas peak heights and areas comprise quantitative parameters of the sample.
To detect these CALR mutations, one should take the samples of genomic DNA extracted from peripheral blood or bone marrow.

There are three stages in CALR mutations identification:

1. DNA extraction from the clinical samples
2. PCR amplification of the target DNA locus
3. Detection step: capillary gel electrophoresis followed by a fragment analysis

DNA extraction from patient clinical material samples

DNA extraction could be performed with DNA extraction kit of any manufacturer. Nevertheless, more optimal results will be possible if the DNA is extracted by magnetic nanoparticles or using sorbent method with columns.
Extracted DNA must be stored at -20^{0}C
The optimum range of DNA concentration is 1.0 to 4.0 ng per single PCR.

PCR amplification of the target DNA locus

Reagents for PCR amplification:

Nuclease free water	User-supplied
Taqman® Universal PCR Mastermix	User-supplied Thermo Fisher Scientific (4364338)
Tubes with lyophilized primers	Kit component: CALR Primer Mix
Sample/tubes with lyophilized positive controls for: Wilde type CALR control DNA Type1 CALR mutation control DNA Type2 CALR mutation control DNA	Kit components: CALR WT CALR T1 CALR T2

Dilution of positive controls

For positive (**CALR T1&T2**) and wild type controls (**CALR WT**) dilution add 30 µl clean nuclease free water (user-supplied) to each of three control tubes (kit components). **Mix well!** Diluted controls must be stored at -20^{0}C.

Dilution of Primer Mix

For the **Primer Mix** dilution, add 20 µl clean nuclease-free water (user-supplied). **Mix well!** The diluted controls must be stored at -20^{0}C.

PCR mix preparation for one reaction:

№	Reagent name	Volume, µl
1	Taqman® Universal PCR Mastermix	12.5
2	Nuclease free water	7.5
3	DNA sample (1-4 ng)/ *diluted* positive controls (type1 or type 2) / negative control (WT)	3
4	*Diluted* Primer Mix	2

Recommended total volume of PCR reaction is 25 µl

A reaction sample without DNA is recommended for each PCR run.

PCR protocol:

50°C for 2 min
95°C for 10 min,
followed by:
95°C for 10 sec
64°C for 20 sec
72°C for 30 sec
} x40 cycles
72°C for 7 min
10°C hold

This PCR Protocol is adapted for any model of solid state thermocyclers (produced by Bio-Rad, Thermo Fisher Scientific etc.)

Capillary gel electrophoresis

The last stage of CALR mutations detection (fragment analysis) should be performed by genetic analyzer. Performing of capillary gel electrophoresis is recommended by Life Technologies (3130, 3500, 3500xl) genetic analysers with POP7 polymer.

Fragment analysis by Genetic Analyzer ABI PRISM 3500 (Thermo Fisher Scientific):

GeneMapper, Peak Scanner, GeneMarker programs can serve the fragment analysis for CALR mutations detection.

Sample preparation

In the 1,5 ml tube premix:

		x1	x16	x24l
Hi-DiTM Formamide	User-supplied Thermo Fisher Scientific (4311320)	10 µl	170 µl	250 µl
LIZ600 dye size standart	User-supplied Thermo Fisher Scientific (4366589)	0.2 µl	3.4 µl	5 µl

1. Vortex and briefly centrifuge the 1,5 ml tube to remove drops the inside of the lid
2. Add 10 µl of the Formamide and LIZ600 mix to each well of the 96 well plate (user-supplied)
3. Vortex PCR products and add 1 µl to Formamide and LIZ600 mix in the each well of the plate
4. Stick down the plate by cover up
5. Denaturate samples in the plate by PCR thermocycler 1 cycle 95^{0}C for 3 min
6. Get out the plate from PCR thermocycler and place it to the ice for 5 min
7. Get out the cover up from the plate and close it by rubber sept. Fix the plate in the genetic analyzer carriage and close the lid.

NOTE.Samples Diluted in the Hi-DiTM Formamide could be stored no more than 24h. Hi-DiTM Formamide and PCR products should be stored at 20^{0}C, LIZ600 should be stored at +4^{0}C.

Results analysis
Examples of the CALR gene mutation analysis

- The wild-type allele in DNA sample is determined as a 263-bp peak at the electrophoretic pattern (Fig.1).
- Detection of a 211-bp peak characterizes the type 1 CALR mutation (-52 bp deletion) (Fig.2).
- Presence of a 268-bp peak characterizes the type 2 mutation (+5-bp insertion) (Fig.3).

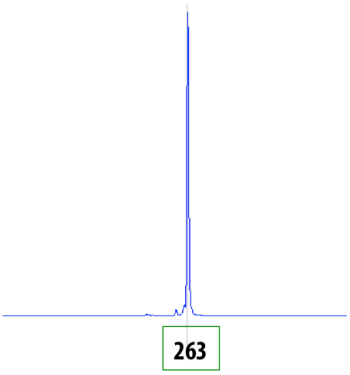

Figure 1. WT CALR

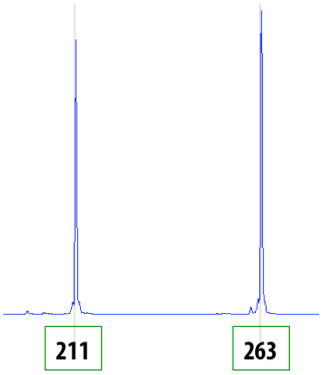

Figure 2. CALR type 1 mutation

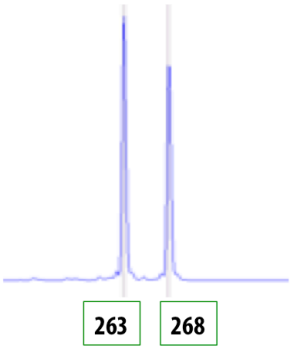

Figure 3. CALR type 2 mutation

It is possible to calculate the level of CALR mutant allele burden by a formule: mutCALR/(mutCALR+wtCALR)x100%, where mutCALR and wtCALR are the areas of the corresponding peaks on the electrophoregram.

Related publications:

1. Nangalia J, Massie CE, Baxter EJ, Nice FL et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med. 2013 Dec 19;369(25):2391-405.
2. Klampfl T1, Gisslinger H, Harutyunyan AS, Nivarthi H et al. Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms. N Engl J Med. 2013 Dec 19;369(25):2379-90.
3. Lavi N. Calreticulin mutations in myeloproliferative neoplasms. Rambam Maimonides Med J. 2014 Oct 29;5(4):e0035.