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DIAGNOSTIC KIT FOR THE DETECTION OF CIRCULATING TUMOR CELLS IN PANCREATIC CANCER

Introduction

One of the well-established methods for circulating tumor cells (CTCs) detection in patients with solid tumors is a methodology based on the real-time RT-PCR (Real time reverse-transcription polymerase chain reaction) principle. The detection of the epithelial cancer genes in compartments of mesenchymal origin is the basic idea of this method. RT-PCR is an indirect method which enables to prove cancer cells genes in compartments distant from primary tumor by detection of the markers' overexpression. Total RNA purified from patients' samples (blood, bone marrow and other body fluids containing cell material) is transcribed by reverse transcriptase into complementary DNA (cDNA).

Specific epithelial gene expression levels in the sample are determined by the standardization curve using specific hydrolysis TaqMan probes in real-time RT-PCR. The real-time PCR is an enzymatic reaction using primers, a specific fluorescently labeled probes, and thermostable DNA-polymerase, which is monitored by a real-time PCR thermal cycler in real-time manner and based on the detection of fluorescence variations.

Kit contains:

- **CEA sense 0.015 mM and CEA antisense 0.015 mM specific primers**
- **CEA probe 1.25 цM TaqMan probe**
- **CEA standard (10^7 copies CEA in 1цl)**
- **CEA positive control – cDNA sample with positive expression of CEA**
- **EGFR sense 0.005 mM and EGFR antisense 0.005 mM specific primers**
- **EGFR probe 1.25 цM TaqMan probe**
- **EGFR standard (10^7 copies EGFR in 1цl)**
- **EGFR positive control – cDNA sample with positive expression of EGFR**
- **hTERT sense 0.005 mM and hTERT antisense 0.005 mM specific primers**

- **hTERT probe 1.25 µM TaqMan probe**
- **hTERT standard (10^7 copies hTERT in 1µl)**
- **hTERT positive control – cDNA sample with positive expression of hTERT**
- **dNTP 10mM mix (mix dTTP, dATP, dCTP, dGTP in ratio 1:1:1:1)**
- **DEPC-treated H_2O**

Other necessary chemicals and equipment:

- **HotStart DNA polymerase with 5‘- 3‘ exonuclease activity**
- **Vortex**
- **Minicentrifuge**
- **Real-time PCR thermal cycler**
- **PCR working box**
- **PCR tubes**
- **2.0 ml tubes**
- **tips**

Procedures:

Store chemicals between –18°C and –30°C. Thaw these chemicals on ice before starting procedure: CEA sense 0.015 mM, CEA antisense 0.015 mM, CEA probe 1.25 µM, EGFR sense 0.005 mM, EGFR antisense 0.005 mM, EGFR probe 1.25 µM, hTERT sense 0.005 mM, hTERT antisense 0.005 mM, hTERT probe 1.25 µM dNTP 10 mM mix, DEPC treated water. Use appropriate HotStart Taq polymerase with suitable buffer and $MgCl_2$. Shortly vortex and spin down thawed chemicals. HotStart Taq polymerase should be added directly from a freezer (only spin down shortly). Turn on the real-time PCR thermal cycler.

1. Preparation of the standards: CEA standard with 10^7 copies CEA/µl is included in the kit. Perform 10 times dilution up to 10^1 copies CEA/µl. Prepare standards for EGFR and hTERT in the same way.
2. Prepare PCR tubes suitable for your cooling rack.
3. Perform PCR with 100 ng cDNA (respectively total RNA entering the previous reverse transcription) in 25 µl volume.
4. Prepare MasterMix for CEA quantification. Pipette these reagents into 2 ml tube according to tab. 1: 0.5 µl dNTP 10 mM mix, 0.5 µl CEA sense 0.015 mM, 1 µl CEA antisense 0.015 mM, 4 µl CEA probe 1.25 µM, reaction buffer, $MgCl_2$ and DNA polymerase according to the manufactured protocol and DEPC-treated water into overall volume 25 µl per reaction. Mentioned volume is for one sample. Shortly vortex MasterMix and spin it down.
5. Aliquot 24 µl MasterMix into prepared PCR tubes. Add 1 µl of the samples (cDNA), 1 µl of water as a NTC (no template control), 1 µl CEA positive control and 1 µl of diluted specific standards (10^1-10^7 copies CEA/µl).
6. Insert PCR tubes into the thermal cycler and run appropriate program:
    1. Step: activation of DNA polymerase and cDNA denaturation
       96 °C / 15 minutes
    2. Step: two-steps amplification
       95 °C / 15 sec - 65 °C / 15 sec
       detection in JOE channel at 65 °C.
7. Prepare MasterMix for EGFR quantification. Pipette these reagents into 2 ml tube according to tab. 2: 0.5 µl dNTP 10 mM mix, 2 µl EGFR sense 0.005 mM, 2 µl EGFR antisense 0.005 mM, 4 µl EGFR

probe 1.25 µM, reaction buffer, $MgCl_2$ and DNA polymerase according to the manufactured protocol and DEPC-treated water into overall volume 25 µl per reaction. Mentioned volume is for one sample. Shortly vortex MasterMix and spin it down.

8. Aliquot 24 µl MasterMix into prepared PCR tubes. Add 1 µl of the samples (cDNA), 1 µl of water as a NTC (no template control), 1 µl EGFR positive control and 1 µl of diluted specific standards (10^1-10^7 copies EGFR/µl).
9. Insert PCR tubes into thermal cycler and run appropriate program:
    1. Step: activation of DNA polymerase and cDNA denaturation
       96 °C / 15 minutes
    2. Step: two-steps amplification
       95 °C / 15 sec - 62 °C / 15 sec
       detection in JOE channel at 62 °C.
10. Prepare MasterMix for hTERT quantification. Pipette these reagents into 2 ml tube according to tab. 3: 0.5 µl dNTP 10 mM mix, 2 µl hTERT sense 0.005 mM, 2 µl hTERT antisense 0.005 mM, 4 µl hTERT probe 1.25 µM, reaction buffer, $MgCl_2$ and DNA polymerase according to manufactured protocol and DEPC-treated water into overall volume 25 µl per reaction. Mentioned volume is for one sample. Shortly vortex MasterMix and spin it down.
11. Aliquot 24 µl MasterMix into prepared PCR tubes. Add 1 µl of the samples (cDNA), 1 µl of DEPC treated water as a NTC (no template control), 1 µl hTERT positive control and 1 µl diluted specific standards (10^1-10^7 copies hTERT/µl).
12. Insert PCR tubes into thermal cycler and run appropriate program:
    1. Step: activation of DNA polymerase and cDNA denaturation
       96 °C / 15 minutes
    2. Step: two-steps amplification
       95 °C / 15 sec - 60 °C / 15 sec
       detection in JOE channel at 60 °C
13. When reactions are done, set up standard curves and analyze the data.

Table 1.

Reaction volume: 25µl	Volume	Concentration	Conc. per reaction	Final concentration
CEA sense 0.015mM	0.5 µl	0.015 mM	7.5 pmol	300 nM
CEA antisense 0.015mM	1 µl	0.015 mM	15 pmol	600 nM
CEA probe 1.25µM	4 µl	1.25 µM	5 pmol	200 nM
dNTP 10mM mix	0.5 µl	10 mM	5 nmol	200 µM
DNA polymerase	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Mg^{2+}	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Buffer	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
cDNA	1 µl	0.1µg cDNA/µl	100 ng	4 ng/µl
H_2O	Do 25µl	/	/	/

Table 2.

Reaction volume: 25µl	Volume	Concentration	Conc. per reaction	Final concentration
EGFR sense 0.005mM	2 µl	0.005 mM	10 pmol	400 nM
EGFR antisense 0.005mM	2 µl	0.005 mM	10 pmol	400 nM
EGFR probe 1.25µM	4 µl	1.25 µM	5 pmol	200 nM
dNTP 10mM mix	0.5 µl	10 mM	5 nmol	200 µM
DNA polymerase	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Mg^{2+}	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Buffer	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
cDNA	1 µl	0.1µg cDNA/µl	100 ng	4 ng/µl
H_2O	To 25µl	/	/	/

Table 3.

Reaction volume: 25µl	Volume	Concentration	Conc. per reaction	Final concentration
hTERT sense 0.005mM	2 µl	0.005 mM	10 pmol	400 nM
hTERT antisense 0.005mM	2 µl	0.005 mM	10 pmol	400 nM
hTERT probe 1.25µM	4 µl	1.25 µM	5 pmol	200 nM
dNTP 10mM mix	0.5 µl	10 mM	5 nmol	200 µM
DNA polymerase	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Mg^{2+}	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
Buffer	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.	Accord. to the manufact.
cDNA	1 µl	0.1µg cDNA/µl	100 ng	4 ng/µl
H_2O	To 25µl	/	/	/

Possible problems and their solutions:

Problem	Probably reason	Possible solution
All fluorescence curves are horizontal	Poorly prepared MasterMix	Prepare a new MasterMix
Fluorescence curve of the sample is not exponential	PCR inhibition	Prepare a new MasterMix Perform reverse transcription again Request new sample RNA
	Degradation of primers	Dilution of new primers Ordering a new synthesis of primers
Fluorescence curve in negative control	PCR contamination	Replacement of all components, cleaning of pipettes, decontamination of work place and repeating of the whole procedure of PCR

Evaluation of results:

The automatic output from the apparatus. The evaluation will follow these rules:

- Assembling of standard curve with the value of efficiency above 0.85 and a correlation coefficient above 0.9.
- Automatic plotting and recording samples into standard curve.
- Normalization of data for amount of RNA input.
- The circulating tumor cells are present in the samples if the expression of the cancer specific markers is above the limits: CEA above 200 copies of CEA/μg RNA in systemic blood, CEA above 350 copies of CEA/μg RNA in bone marrow, EGFR above 250 copies of EGFR/μg RNA in systemic blood, EGFR above 30 000 copies of EGFR/μg RNA in bone marrow, hTERT above 2500 copies of hTERT/μg RNA in systemic blood and hTERT above 20 000 copies of hTERT/μg RNA in bone marrow.

Safety precautions:

Hazardous substances: biological material

Any biological material is treated as potentially infectious. During work, it is necessary to use appropriate protection (disposable gloves). In addition, general principles of occupational safety in molecular biology laboratory apply.