

For life science research only.  
Not for use in diagnostic procedures.  
For in vitro use only.



## *Human Cytokeratin-19 (CK-19) Quantification Kit*

Real-time Polymerase Chain Reaction (real-time PCR) based method for the quantitative determination of human CK-19 expression.

**Cat. No. MDX-0601**

**Kit for 32 reactions**

### Note

- Store the unopened kit at  $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$  in the dark until the expiration date
- Protect CK-19 Detection mix (vial 1, brown cap) from light
- Avoid repeated freezing and thawing

*Instruction Manual*

*May 2007, version 1*

### KIT CONTENTS

Vial/Cap	Label	Contents/Function
1 Brown cap	CK-19 Detection mix*	<ul style="list-style-type: none"><li>• 140 <math>\mu\text{L}</math></li><li>• Ready-to-use Primer and Hybridization Probe mix, specific for human CK-19 mRNA</li></ul>
2 Green cap	DNA Standard	<ul style="list-style-type: none"><li>• 10 <math>\mu\text{L}</math></li><li>• CK-19 DNA standard</li><li>• Contains approx. <math>10^7</math> copies/<math>\mu\text{L}</math></li></ul>
3 Red cap	BSA Solution	<ul style="list-style-type: none"><li>• 20 <math>\mu\text{l}</math></li><li>• For use in PCR reaction</li></ul>
4 Colorless cap	dH <sub>2</sub> O for RT-PCR	<ul style="list-style-type: none"><li>• 500 <math>\mu\text{l}</math></li><li>• Nuclease-free, high purity dH<sub>2</sub>O for dilution of the DNA standard and for use in PCR reaction</li></ul>

\* Protect CK-19 Detection mix from light!

# INTRODUCTION

## Background information

The development of distant metastases is the primary cause of death in breast cancer patients. About 50% of patients have no detectable metastases at the time of diagnosis and are usually considered cured after receiving primary treatment. However, 20-30% will experience relapse within 5-10 years suggesting early tumor cell dissemination to secondary sites.<sup>(1,2)</sup> Since standard diagnostic techniques lack sufficient sensitivity and specificity required for early detection, a significant research effort has been directed to the development of accurate molecular tests to detect occult tumor cells.

The intermediate filament cytokeratin-19 (CK-19) is a useful molecular marker for the detection of occult tumor cells in biological samples due to its specific expression in normal and cancerous epithelial cells but not in hemopoietic cells.<sup>(1,3)</sup> Furthermore, in breast cancer patients, detection of CK-19-positive tumor cells before the initiation of any adjuvant treatment is a significant adverse prognostic factor associated with an increased risk of disease relapse and decreased survival.<sup>(3,4)</sup> The presence of tumor cells in the peripheral blood or bone marrow has been correlated with poor prognosis of patients with other types of cancer, including prostate<sup>(5)</sup> and colorectal cancer.<sup>(6)</sup> CK-19 may be a useful marker for these malignancies as well.

For the analysis of molecular tumor markers, Real-Time PCR methods are a favourable option.<sup>(7,8)</sup> The *Medexis Human Cytokeratin-19 (CK-19) Quantification Kit* provides a specific and sensitive method for quantifying CK-19 mRNA positive cells in cell cultures, scientific biopsy material and other biological samples, such as peripheral blood, lymph nodes or bone marrow of patients with solid cancers. Results are expressed as CK-19 copies/ $\mu$ L using a calibration curve.

## Kit principle

The *Medexis Human Cytokeratin-19 (CK-19) Quantification Kit* provides all detection reagents required to quantify CK-19 expression. The Kit provides an assay that is highly specific for human CK-19 expression and it is designed to avoid amplification and detection of genomic DNA and CK-19 pseudogenes. The Detection mix consists of CK-19-specific primers and hybridization probes. A CK-19 DNA standard containing approximately  $10^7$  copies/ $\mu$ L is also provided for use as (1) a positive PCR control and (2) external standard to make a calibration curve to quantify human CK-19 in unknown samples.

Quantification is based on Real-Time monitoring during PCR amplification of fluorescently labeled hybridization probes that are specific for human CK-19. One probe is labeled at the 3' end with fluorescein ( $\lambda_{exc} = 495 \text{ nm} - \lambda_{em} = 520 \text{ nm}$ ), and the other at the 5' end with LC Red 640 ( $\lambda_{exc} = 625 \text{ nm} - \lambda_{em} = 640 \text{ nm}$ ).

## Sample material

- Most typically, the test sample is derived from peripheral blood, bone marrow or lymph nodes. However, any biological sample is suitable for PCR.
- For total RNA extraction from biological samples and cDNA synthesis you may use any commercially available kit or reagent. However, we recommend the following reagents from Invitrogen (Carlsbad, CA, USA): the TRIZOL® LS reagent for the RNA extraction step; the Superscript™ III Platinum® Two-Step qRT-PCR kit or the ThermoScript™ RT-PCR System for cDNA synthesis; and the Platinum® Taq DNA Polymerase for Real-Time PCR reaction. In addition, positive enrichment of circulating epithelial cells by using immunomagnetic beads increases the sensitivity of the assay.<sup>(9)</sup>
- **The same quantity of total RNA should be used for all samples.**

## Analytical characteristics

- Dilutions ranging from  $10^4$  to 10 copies of CK-19 DNA standard per  $\mu$ L showed linearity over the entire quantification range with correlation coefficients larger than 0.99 indicating a precise log-linear relationship.
- The inter-assay precision ranges from 1.8% (n=3) to 1.7% (n=3) for 10 copy and  $10^4$  copies, respectively
- Between-assays precision ranges from 1.0% (n=5) to 2.0% (n=4) for 10 copy and  $10^4$  copies, respectively
- Stability: at least nine (9) months at  $-20^\circ \text{C}$  if unopened
- In selecting the primer pair for CK-19, we added an intron spanning site in the forward primer to avoid non-specific target detection. This feature is important when the biological sample has traces of contaminating genomic DNA. The primers were also designed to distinguish the human CK-19 specific sequence from the two known CK-19 pseudogenes.
- The main advantage of this assay, as in all Real-Time PCR assays, is that quantification is based on the exponential phase of the PCR instead of using the endpoint accumulation of PCR product at the end of the stationary phase of the PCR.

## PROCEDURE

### Precautions

To reduce risk of contamination:

- RNA extraction, cDNA synthesis, preparation of the Real-Time PCR steps and thermocycling should be performed in separate rooms
- Preparation of the PCR mixture should be done in a PCR-hood
- In every extraction or synthesis step during the whole procedure, filter tips, RNase- DNase-free reaction vials and calibrated precision pipettes dedicated for each step should be used
- Wear gloves while performing the assay

### Additional equipment required

The Kit is specifically designed and adapted for use with the LightCycler® Instrument (Roche Diagnostics, Indianapolis, IN, USA), but it can also be used with other Real-Time PCR instruments.

### Preparation of PCR master mix

Component	Volume/reaction (µL)
dH <sub>2</sub> O for RT-PCR (colorless cap vial)	10
10x PCR Rxn buffer (-MgCl <sub>2</sub> )	2
MgCl <sub>2</sub> (50 mM)	1
dNTPs (10 mM)	0,4
BSA (red cap vial)	0,4
Taq DNA Polymerase (5 U/µL)	0,2
CK-19 Detection mix (brown cap vial)	4
<b>Total volume</b>	<b>18</b>

- The above PCR master mix protocol allows the addition of 2 µL cDNA or CK-19 DNA standard, while cDNA volumes up to 5 µL may be added to make a final volume of 20 µL by adjusting the water volume.
- The Kit is designed for 32 reactions with a final volume of 20 µL each. Each PCR run, apart from the unknown samples, should contain a PCR negative control (by replacing the template cDNA with nuclease-free water), and four dilutions of the DNA standard (corresponding to 10-10<sup>4</sup> copies/µL), to create a calibration curve. To help avoid sampling discrepancies, prepare the master mixes by multiplying the amount in the “Volume” column by the number of reactions to be cycled plus one additional reaction per 10 reactions.
- CK-19 DNA standard dilutions should be done in the dH<sub>2</sub>O for RT-PCR supplied with the kit.
- Mix all components and cDNAs gently before use and mix the master mix carefully by pipetting after addition of the last component.

#### Note

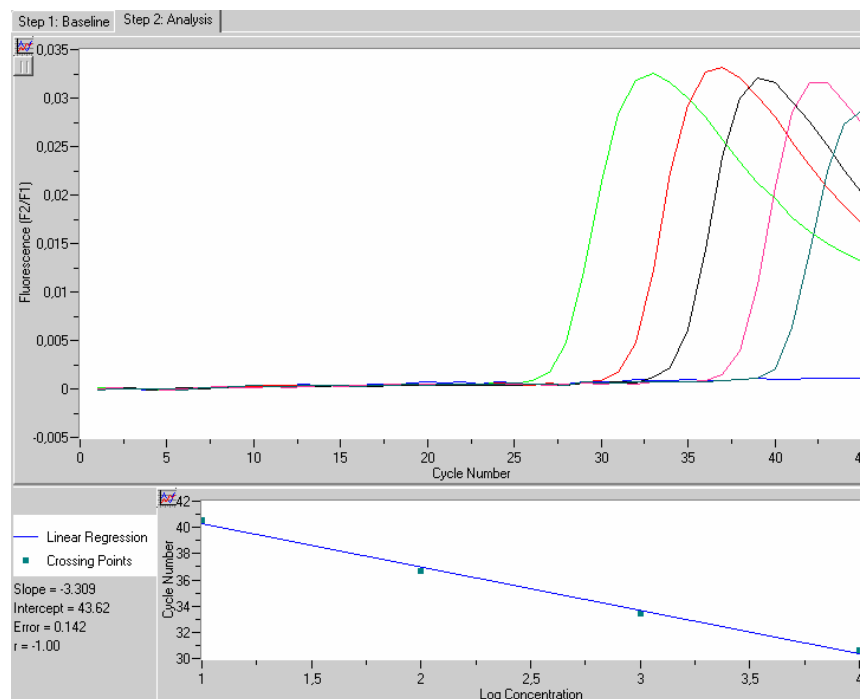
- Keep all components on ice while performing the assay
- Protect CK-19 Detection mix from light

### Real-Time PCR protocol

Step	Temperature	Time	Cycles
denaturation	95 °C	5 min	1
denaturation	95 °C	10 sec	50
annealing	55 °C	20 sec	
extension	72 °C	20 sec	
cooling	40 °C	30 sec	1

## DATA ANALYSIS

For quantification of CK-19 mRNA expression level, a calibration curve should be generated by using serial dilutions of the CK-19 DNA standard in dH<sub>2</sub>O, corresponding to 10-10<sup>4</sup> copies/µL. **These dilutions must be freshly prepared from the standard stock solution (10<sup>7</sup> copies/µL).** This calibration curve should be created by plotting the number of CK-19 copies corresponding to each external DNA standard versus the value of its crossing point (Cp). **Figure 1** shows an indicative Real-Time PCR calibration graph generated using the CK-19 DNA standard of the kit with the LightCycler® System. Quantification is based on Real-Time monitoring during PCR amplification of fluorescently labeled hybridization probes specific for CK-19. The point where the fluorescence rises above background noise (crossing point, Cp) is best quantified through the LightCycler® software as the second derivative maximum of the curve.



**Figure 1.** A typical graph of human CK-19 standard DNA copies by Real-Time PCR amplification with the LightCycler® system. *Top:* Logarithmic plot of fluorescence signal (F2/F1) during amplification. Serial dilutions of the standard were used as external standards. The curves from right to left correspond to 10, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup> CK-19 copies/μL, while the flat line represents the negative control. *Bottom:* The graph shows the crossing points (Cycle number) plotted versus the log of the number of copies/μL.

The number of CK-19 transcripts for all the unknown samples are expressed as copies per μL of sample. Results can be normalized in respect to:

- Total RNA, expressed as copies/μg RNA or
- The expression of a housekeeping gene in the same samples, e.g. porphobilinogen deaminase (PBGD), to obtain a normalized transcript value for each sample.

## REFERENCES

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## NOTICE TO KIT PURCHASER:

The MEDEXIS Human Cytokeratin-19 (CK-19) Quantification Kit ("Kit") is designed for research use using the Polymerase Chain Reaction (PCR). The PCR process is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-LaRoche, Ltd (Roche). No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser of the Kit.

The Kit is designed for use with Authorized Thermal Cyclers and Authorized PCR Reagents. In particular, the Kit is optimized for use with the LightCycler® 2.0 Instrument manufactured by Roche Diagnostics (Roche Diagnostics P.O. Box 50414 9115 Hague Rd. Indianapolis, IN 46250-0414, USA). Authorized Reagents to practice the PCR process (including Real-Time PCR) can be obtained from Invitrogen, Inc. (1600 Faraday, Avenue P.O. Box 6482 Carlsbad, CA 92008, USA). Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Drive, Foster City, CA 94404, USA or Roche Molecular Systems Inc., 1145 Atlantic Avenue, Alameda, CA 94501, USA.

The Kit is sold for research use only (RUO) within the European Union (EU). Sale, resale or use outside the EU is not authorized. The safety and efficacy of the Kit in diagnostic or other clinical uses has not been established.

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