INSTRUCTION MANUAL

Cytokeratin-19 LC Kit Cat.# OL10100 Not for diagnostic use.

MATERIALS PROVIDED

Materials Provided	Quantity for 24 / 48 / 96 kit size			Color Code
Positive Control	1x 48 µl	2x 48 µl	4x 48 µl	Red
Negative Control	1x 200 µl	2x 200 µl	4x 200 µl	Blue
One-Step Reaction Mix (2X)	1x 240 µl	2x 240 µl	4x 240 µl	Green
Enzyme Mix (20X)	1x 24 µl	2x 24 µl	4x 24 µl	Yellow
Primer & Probe Mix	1x 24 µl	2x 24 µl	4x 24 µl	Purple

STORAGE CONDITIONS

The kit is shipped on room temperature. This product should be stored at -20°C, protected from light, and has a shelf-life of at least 6 months when stored under these conditions. To minimize repeated freeze/thaw cycles, we recommend the preparation of aliquots after the initial use.

ADDITIONAL MATERIAL REQUIRED

RNA template
Microcentrifuge tubes (for reaction setup)
PCR tubes or PCR plates, including seals
qPCR instrument
Color Compensation CK-19 610-670 Kit (Cat.# OL0010006)
Standard laboratory equipment

WARNINGS & PRECAUTIONS

Before using this kit, carefully read the whole instruction manual. Never heat kit components in order to thaw them. Always include the provided controls to verify your results. Treat and dispose of waste using proper precautions and in accordance with local, state, and federal regulations. Safety data sheets can be found on the manufacturer's homepage. Visit oncolab.at for further information.

INTRODUCTION

One-Step RT-qPCR is a fast and convenient method for RNA detection. The enzyme mix, supplied in 20X concentration, contains a reverse transcriptase that converts RNA into cDNA. The reverse transcriptase is compatible with various RNA sample types, such as total RNA or poly(A)-RNA. The cDNA is consequently amplified by a DNA polymerase, enabling RNA quantification, all in one tube.

The One-Step Reaction Mix is supplied at 2X concentration and contains all necessary buffer components, a non-fluorescent visible dye and Taq DNA Polymerase. The visible dye is for setup monitoring purposes only and does not interfere with real-time detection.

The included Primer & Probe Mix contains primers and hybridization probes specific for Cytokeratin-19 (CK-19) and GAPDH. CK-19 is a type I cytokeratin found mostly in epithelial tissues with high plasticity such as stem cells, transforming cells or tumorous cells. In addition, CK-19 can also be found in circulating tumor cells in peripheral blood. The addition of GAPDH serves as an internal control since it should be present in almost all tissues and cell types.

PREPROTOCOL CONSIDERATIONS

RT-qPCR is a very sensitive method. A sterile, RNase-free work environment and proper pipetting techniques are crucial to avoid contaminations and to achieve reliable results. Always wear powder-free disposable gloves and change them frequently. Try not to touch surfaces that might cause RNase carryover. Only use RNase-free aerosol-blocking filter tips and only use reagents supplied in this kit.

High RNA quality is essential for the synthesis of full-length cDNA. We recommend the Trizol RNA purification method and RNA samples should be stored at -70°C or below. For optimal PCR efficiency we recommend an input range of 100 ng – 10 pg total RNA. To further optimize PCR results a DNAse treatment step is advisable.

Make sure all kit components are completely thawed and mixed prior to the reaction setup. Keep thawed components on ice.

When pipetting into strips or PCR plates, try to avoid air bubbles. If bubbles occur, a quick centrifugation step can help resolving them.

Note: The instrument LC480II requires Color Compensation to minimize crosstalk between the emission channels. This is a critical step for the multiplex HybProbe reactions that allows segregation of the fluorescence signals designated for the dominant channel. This is addressed by creating a Color Compensation file that needs to be applied for each data analysis after data acquisition on the LC480II instrument. The Color Compensation file needs to be generated only once and can be saved for future reuse.

ONE-STEP RT-qPCR PROTOCOL

The kit is specifically designed and adapted for the use on the LightCycler (LC) 480II instrument (Roche, CH) in combination with Roche Multiwell plates 96 (white) and a HybProbe detection format. For other real-time instruments, the reaction conditions and the thermal cycler program may need to be adapted and changed according to manufacturer instructions. This kit is not suitable to be used with a hydrolysis probe ("TaqMan") protocol.

REACTION SETUP:

Component	20 µl Reaction	Final Concentration
One-Step Reaction Mix (2X)	10 µl	1X
Enzyme Mix (20X)	1 µl	1X
Primer & Probe Mix (20X)	1 µl	1X
Template RNA	variable	Variable (< 1 µg total)
Molecular grade water	to 20 µl	n/a

1. Thaw the One-Step Reaction Mix and other reaction components at room temperature, then place them on ice. After thawing is complete, briefly vortex each component or mix by inversion or pipetting.
2. Determine the total volume for the appropriate number of reactions, adding 10% overage, and prepare the reaction mix of all components except for the RNA template accordingly. Mix thoroughly but gently by pipetting or vortexing. Briefly spin the tube to collect the reaction mix to the bottom.
3. Distribute the reaction mix into qPCR tubes or plate. Try to pipette accurately and minimize bubbles.
4. Add RNA template (e.g. 8 µl Positive Control) to qPCR tubes or plate. Tightly seal the tubes with flat, optically transparent lids; seal plates with optically transparent foil. Take care to properly seal the plate edges and corners to prevent evaporation.
5. Spin tubes or plates briefly to remove bubbles and collect liquid.
6. Using the Filter Combination Selection Tool adjust the filter settings as follows.

Filter Combination	Excitation Filter	Emission Filter
CK-19	498 nm	610 nm
GAPDH	498 nm	660 nm

7. Program the real-time instrument with the indicated thermocycling protocol (see table below).

Program Step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 Minutes	1
Initial Denaturation	95 °C	1 Minute	1
Denaturation Extension	95 °C 63 °C	10 Seconds 30 Seconds	50
Cooling	40 °C	30 Seconds	1

DATA ANALYSIS

Analyse data according to real-time instrument manufacturer instructions. If you are measuring RNA derived from biological material, you should always get a signal for GAPDH. A negative GAPDH signal is an indication that the quality of the used RNA is insufficient. 8 µl of the included Positive Control should give a CK-19 Cp value below 32 using the AbsQuant 2nd derivative max analysis mode (LightCycler 480II).

DISPOSAL

Dispose of in accordance with local regulations. Dispose of waste in accordance with environmental legislation.

TROUBLESHOOTING

Problem	Possible Cause	Solution
Low or no RT-qPCR signal	RNA template is degraded	• Confirm template input amount • Verify proper storage of RNA and Kit reagents • Re-check the RNA isolation protocol • Always wearing a lab coat, gloves and mask when working with RNA and use RNA-grade reagents and materials • Re-check the expiration date of the kit
	RNase contamination	• Add RNase inhibitor
	Wrong channel selected	• Re-check the manual for proper channel selection
	Wrong cycling protocol	• Re-check the manual for the correct cycling steps
	Wrong RT step temperature	• Use 55°C for RT step
Positive signal in negative control	RNA carry-over contamination	• Use separate pipettes for RT-qPCR reactions • Always change pipette tips after each step • Consider replacing all reagents • Clean all equipment and setup areas
Unspecific signal in the adjacent channel	Color Compensation incorrect	• Follow the manual included in the separate Color Compensation Kit CC610-670 (OL0010006)

DISCLAIMER

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

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