Miltenyi Biotec

MACS® COPYcheck Kit
human

Order no. 130-128-157

Contents

1. Description

This product is for research use only.

Components	**MACS COPYcheck Primers and Probes Mix** (80 µM each): Components are labeled with FAM and JOE dyes. **MACS Check Positive Control (pLTG2713)** (gag'-VSVg-RRE-PTBP2) (10^8 copies/µL)
Capacity	For 96 reactions.
Product format	MACS COPYcheck Primers and Probes Mix is supplied in a buffered solution of 10 mM TRIS-Cl and 1 mM EDTA, at pH 8.0, using nuclease-free water. MACS Check Positive Control (pLTG2713) is supplied in a buffered solution of 5 µM TRIS-Cl and 0.5 µM EDTA, at pH 8.0, using nuclease-free water.
Storage	Store protected from light at –20 °C. The expiration date is indicated on the vial labels.

1.1 Principle of the MACS COPYcheck Kit

The vector copy number (VCN) assay is intended to determine the average copies of integrated lentiviral vectors per cell genome after *ex vivo* transduction of human cells. After isolating genomic DNA from the transduced cells, the copies of the lentiviral gag gene are determined in relation to the human reference gene PTBP2 by quantitative PCR.
The MACS COPYcheck Kit provides specific primers to amplify an amplicon of the lentiviral gag gene and the human reference gene PTBP2, respectively. In addition, specific probes comprise fluorescent dyes (JOE/FAM) and quencher moieties are used. During the PCR amplification, the probes bind to the respective target gene and get hydrolyzed by the 5′-3′-exonuclease activity of the PCR polymerase. Thereby, the fluorescent dye separates from the quencher leading to the emission of a fluorescence signal. The threshold cycle (Ct) value determined during the quantitative PCR is related to the copy number of the respective gene in the sample.
For reliable quantification, a plasmid standard MACS Check Positive Control (pLTG2713) is used to determine a standard curve for the amplification of gag and PTBP2, respectively. According to the standard curve, the number of copies in the genomic DNA for gag and PTBP2 can be calculated from the respective Ct values. As the human genomic DNA comprises two copies of the PTBP2 gene, the ratio of the copy number of gag and PTBP2 can then be used to calculate the average copy number of the viral vector in the genomic DNA of the transduced cells.
Please note that the primers included in the MACS COPYcheck Kit, human are only compatible with the lentiviral vectors provided by Lentigen and Miltenyi Biotec.

1.2 Background information

The MACS COPYcheck Kit has been developed for the determination of vector copy numbers in transduced cells, i.e., to determine the amount of integrated lentiviral vector per transduced cell as characterization of cellular products or as in-process control in production processes using the CliniMACS Prodigy® or other transduction systems.

1.3 Reagent and instrument requirements

- Genomic DNA purifed from 1×10^6 cells (approximately 10–150 ng/reaction). This starting material will be called "test sample" within the text.
- Nuclease-free water.
- Taqman™ Fast Advanced Master Mix (Thermo Fisher Scientific Cat. No. 4444556) or similar.
- Multicolor real-time PCR detection system (thermal cycler) for the detection of FAM (excitation maximum 495 nm, emission maximum 520 nm) and JOE (excitation maximum 525 nm, emission maximum 555 nm) dyes.
- Depending on the thermal cycler, a reference standard dye, such as ROX Reference Dye (Thermo Fisher Scientific Cat. No. 12223-012) or similar.
- 1.5 mL microcentrifuge tubes.
- 96-well PCR plates for fast PCR protocols as recommended for the multicolor real-time PCR detection system by the respective supplier.
- Adhesive sealing films.
- 2× PCR workstations (DNA-free and for handling template DNA).
- 2× 1000 µL pipettes (DNA-free and for handling template DNA) with disposable filter tips.
- 2× 100 µL pipettes (DNA-free and for handling template DNA) with disposable filter tips.
- 2× 20 µL pipettes (DNA-free and for handling template DNA) with disposable filter tips.
- Vortex mixer.
- Microcentrifuge for 1.5 mL tubes.
- Microcentrifuge tube racks.

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2. Protocol

2.1 Reagent preparation

▲ Mix all solutions briefly with a vortex mixer before use and perform a short spin before opening the tube.

▲ All components of the MACS COPYcheck Kit (Primers and Probes Mix and Positive Control (pLTG2713)) are ready-to-use. Bring all reagents to room temperature (19–25 °C) before use.

2.2 Protocol procedure

1. Prepare and aliquot 2× master mix solution for the duplex assay in the DNA-free PCR workstation according to table 1, e.g., for 1-8 samples use 792 µL Taqman Fast Advanced Master Mix and 8 µL MACS COPYcheck Primers and Probes Mix. Mix well.
   ▲ **Note:** The amount of 2× master mix, which is needed for the preparation of the standard and negative controls, is already included in the volumes given in table 1.

Number of test samples	Taqman Fast Advanced Master Mix (µL)	MACS COPYcheck Primers and Probes Mix (µL)
1–8	792	8
9–16	1188	12
17–24	1584	16

Table 1: Preparation of 2× master mix solution.

2. (Optional) In case a reference standard dye, e.g. ROX, is required for the multicolor real-time PCR detection system, the respective amounts of standard dyes should be added to the 2× master mix. Typically, the reference standard dye is provided as 100× or 50× concentrated stock solution (table 2).

Number of test samples	Taqman Fast Advanced Master Mix (µL)		MACS COPYcheck Primers and Probes Mix (µL)	Reference standard dye stock solution (µL)	
	Concentration of reference standard dye stock solution			Concentration of reference standard dye stock solution	
	100×	50×		100×	50×
1–8	784	776	8	8	16
9–16	1176	1164	12	12	24
17–24	1568	1552	16	16	32

Table 2: Preparation of 2× master mix solution including a reference standard dye with 50× or 100× concentrated stock solution.

3. Add 50 µL 2× master mix to new 1.5 mL microcentrifuge tubes for the standard curve (6 tubes), no-template control (NTC; 1 tube), and one tube for each test sample (= starting material). A loading scheme is shown in table 4.

4. Prepare a blank solution to serve as NTC in the DNA-free PCR workstation: Add 90 µL nuclease-free water to a new 1.5 mL microcentrifuge tube.

5. Prepare the standards for the standard curve by serial dilution of the MACS Check Kit Positive Control (pLTG2713) standard stock solution in the template DNA PCR workstation according to table 3. "ST" is the abbreviation of "standard". Mix well.

Vial name	Target standard concentration	Component 1		Component 2	
		Name	Volume (µL)	Name	Volume (µL)
ST 0	1×10^7 copies/µL	Stock (1×10^8 copies/µL)	5	Nuclease-free water	45
ST 1	1×10^6 copies/µL	ST 0 (1×10^7 copies/µL)	10	Nuclease-free water	90
ST 2	1×10^5 copies/µL	ST 1 (1×10^6 copies/µL)	10	Nuclease-free water	90
ST 3	1×10^4 copies/µL	ST 2 (1×10^5 copies/µL)	10	Nuclease-free water	90
ST 4	1×10^3 copies/µL	ST 3 (1×10^4 copies/µL)	10	Nuclease-free water	90
ST 5	1×10^2 copies/µL	ST 4 (1×10^3 copies/µL)	10	Nuclease-free water	90
ST 6	1×10^1 copies/µL	ST 5 (1×10^2 copies/µL)	10	Nuclease-free water	90

Table 3: Dilution scheme for standard curve.

6. Add 50 µL of test samples to 50 µL of 2× master mix (table 1) in the template DNA PCR workstation. Mix well.

7. Add 50 µL of NTC to 50 µL of 2× master mix in the template DNA PCR workstation. Mix well.

8. Add 50 µL of each standard curve DNA solutions (table 3) to 50 µL of 2× master mix in the template DNA PCR workstation. Mix well.
   ▲ **Note:** When mixing DNA solutions with 2× master mix: Work always with solutions of lower DNA concentration first, followed by solutions of successively higher DNA concentrations.

9. Briefly before use, vortex all prepared solutions thoroughly.

10. Add 25 µL of each prepared solution into the 96-well PCR plate according to table 4. Each sample will be applied and analyzed in triplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1			Sample 2			Sample 3			Sample 4		
B	Sample 5			Sample 6			Sample 7			Sample 8		
C	Sample 9			Sample 10			Sample 11			Sample 12		
D	Sample 13			Sample 14			Sample 15			Sample 16		
E	Sample 17			Sample 18			Sample 19			Sample 20		
F	Sample 21			Sample 22			Sample 23			Sample 24		
G	ST 6 (1×10^1 copies)			ST 5 (1×10^2 copies/µL)			ST 4 (1×10^3 copies/µL)			ST 3 (1×10^4 copies/µL)		
H	ST 2 (1×10^5 copies/µL)			ST 1 (1×10^6 copies/µL)			—			NTC		

Table 4: Example of a loading scheme of a 96-well PCR plate.

11. Seal the plate by using an adhesive sealing film and spin down before loading into instrument. Run plate in a multicolor real-time PCR detection system under following conditions (table 5).

Temperature (°C)	Time (seconds)	Data collection	Number of cycles
50	120	No	1
95	20	No	1
95	5	No	40
56	20	No	
65	20	Yes (plate read)	

Table 5: Thermal cycling conditions.

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3. Data acquisition and analysis

Perform the primary data analysis according to the recommendations for the used multicolor real-time PCR detection system. Typically, analysis settings, e.g., for the baseline and threshold values, need to be defined. The primary data analysis will result in threshold cycle (Ct) values for each dye of each sample.

Use the data of the plasmid standard (table 3) to generate standard curves for PTBP2 (JOE) and gag (FAM), respectively. Plot the Ct value against the log(10) of the number of copies used for the standard.

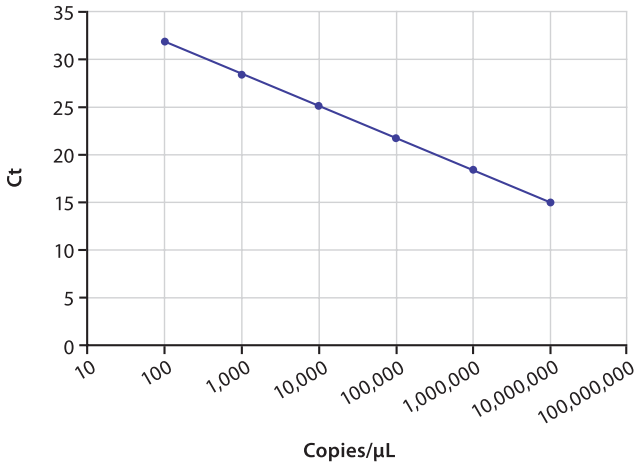

Figure 1: Example of a standard curve depicting the measured Ct value versus the log(10) of the copies per µL of the MACS Check Positive Control (pLTG2713) standard dilution (refer to table 3).

The mean Ct value for each triplicate sample is used to determine the log(10) of the number of copies/µL according to the respective standard curve. The determined value to the power of 10 gives the copy number per µL of PTPB2 or gag in the respective sample.

▲ The factor two in the calculation takes into account the two copies of the PTBP2 gene in the diploid human genome.

Formula for the calculation of the VCN:

$$\text{Copy number per transduced cell} = \frac{\text{qPCR copy number/µL (gag)}}{\text{qPCR copy number/µL (PTBP2)}} \div \frac{\text{transduction frequency (\%)}}{100\,\%} \times 2$$

Calculation example:

$$\text{Copy number per transduced cell} = \frac{300}{800} \div \frac{30\%}{100\%} \times 2 = 2.5$$

4. Troubleshooting

Signal in negative control sample

Signals in negative control sample typically indicate contamination of the used material with genomic DNA or PCR products from previous runs. It is recommended to set up the PCR reaction in a different area separated from PCR reactions or analysis. Dedicated equipment (pipettes, tips etc.) should be used for preparing the PCR mixes. To reduce the risk of cross-contaminations, filter tips should be used. Avoid aerosols that might be generated, e.g., by vortexing, rigorous pipetting or during centrifugation. The area and the equipment for preparing the PCR reactions should be regularly cleaned.

Deviation of replicate samples

Uniform mixtures are important to gain reproducible results. During set up of the PCR reactions, the components should be mixed well, e.g., by thorough pipetting.

Pipetting errors can give rise to increased variation. It is recommended to regularly calibrate the pipettes for PCR set up.

Uneven temperature or data collection on the multicolor real-time PCR detection system can also cause inconsistent results. The used instrument should be checked regularly.

No signal detected

The amount of genomic DNA used for the assay might be insufficient. For 1×10^6 cells, about 1–6 µg genomic DNA can be expected. In case the yield should be much lower, check the respective genomic DNA isolation protocol.

Primers and Probes Mix might have not been added when preparing 2× master mix solution or they were exposed to excessive light or temperature for prolong period.

Store kit components protected from light, minimize exposure to room temperature, and mix well before master mix preparation.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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