MACS® qPCR LENTIcheck Kit (BaEV)
human

Order no. 130-139-240

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1. Description

This product is for research use only.

Components	**16 µL MACS qPCR LENTIcheck (BaEV) Primers & Probe** (80 µM per primer/probe): Probe is labeled with FAM dye. **20 µL MACS qPCR LENTIcheck (BaEV) Control** (10^8 copies/µL)
Capacity	For 96 reactions (1–2 test samples).
Product format	MACS qPCR LENTIcheck (BaEV) Primers & Probe are supplied in nuclease-free water. MACS qPCR LENTIcheck (BaEV) Control is supplied in nuclease-free water and poly-A-RNA carrier.
Storage	Store protected from light at +2 to +8 °C. If the reagents are not used within 3 months, store at –20 °C protected from light. When MACS qPCR LENTIcheck (BaEV) Control was stored at –20 °C, reheat at +50 °C for 20 minutes before use. The expiration date is indicated on the vial label.

1.1 Principle of the MACS qPCR LENTIcheck Kit (BaEV), human

The qPCR replication competent lentivirus (RCL) assay is intended to detect the lentiviral envelope gene sequence (baboon envelope (BaEV) gene) of potential RCLs that might arise, e.g., during unintended recombination of plasmids used in vector production or due to recombination of the viral vector with viral sequences in cells after transduction.

The MACS qPCR LENTIcheck Kit (BaEV), human provides specific primers to amplify an amplicon of the lentiviral envelope gene BaEV sequence. The envelope gene (BaEV) is the most suitable target for detecting RCLs for the usage of vectors that contain this gene, since the sequence must be incorporated to generate a replication competent virus.

A BaEV–specific probe comprising a fluorescent dye (FAM) and quencher moiety is additionally used. During the PCR amplification, the probe binds to the BaEV gene and gets hydrolyzed by the 5′-3′-exonuclease activity of the PCR polymerase. Thereby, the fluorescent dye is separated 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 amount of the respective gene in the sample.

For reliable quantification, the MACS qPCR LENTIcheck (BaEV) Control is used to determine a standard curve for the amplification of the BaEV gene. Additional positive and negative controls are used to ensure proper detection of the BaEV gene. No signals should be gained for the viral vector or for the transduced cells under investigation.

The primers included in the MACS qPCR LENTIcheck Kit (BaEV), human are intended to be used with the lentiviral vectors provided by Lentigen and Miltenyi Biotec. For information about compatibility with other lentiviral vectors, please contact Miltenyi Biotec Technical Support.

1.2 Background information

During the production of lentiviral vectors as well as in cells transduced with lentiviral vectors, there is the risk of an interplay between the viral vector and viral properties gained from other potentially present viruses or virus genes. Such an interplay might give rise to RCLs, also named replication competent retroviruses (RCRs). The MACS qPCR LENTIcheck Kit (BaEV), human has been developed to detect the gene encoding for the pseudotyping envelope protein BaEV. The BaEV gene is used to express the envelope protein during the production of lentiviral vectors to enable the packaging of the vector. However, BaEV should not be present in the viral vector. Therefore, the viral vector as well as the transduced cells are intended to be free of BaEV gene. The BaEV gene can be detected using the MACS qPCR LENTIcheck Kit (BaEV), human. The absence of the BaEV gene in *ex vivo* transduced cells according to the qPCR assay can be used for lot release testing, in particular, when time constrains are present.

1.3 Reagent and instrument requirements

- Genomic DNA purifed from 1×10^6 cells
- 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)

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- 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 qPCR protocols as recommended for the multicolor real-time PCR detection system by the respective supplier
- Adhesive sealing films
- 2× PCR workstations (1 for DNA-free work and 1 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.

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.

▲ The primers included in the MACS qPCR LENTIcheck Kit (BaEV), human are intended to be used with the lentiviral vectors provided by Lentigen and Miltenyi Biotec. For the compatibility with other lentiviral vectors please contact local Miltenyi Biotec Technical Support.

▲ All components of the MACS qPCR LENTIcheck Kit (BaEV), human (Primers & Probe and Control) are ready-to-use. When MACS qPCR LENTIcheck (BaEV) Control was stored at –20 °C, reheat at +50 °C for 20 minutes before use. Bring all reagents to room temperature (+19 to +25 °C) before use.

2.2 Protocol procedure

▲ When mixing DNA solutions with 2× master mix: Always work with solutions of lower DNA concentration first, followed by solutions of successively higher DNA concentrations.

1. Prepare a blank solution to serve as no-template control (NTC) in the DNA-free PCR workstation: Add 50 µL nuclease-free water to a new 1.5 mL microcentrifuge tube.
2. Prepare standards for a standard curve by serial dilution of the MACS qPCR LENTIcheck (BaEV) Control in the DNA-free PCR workstation according to table 1. Mix well.

Standard	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)	10	Nuclease-free water	90
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
ST 7	1 copy/µL	ST 6 (1×10^1 copies/µL)	20	Nuclease-free water	180
ST 8	0.5 copies/µL	ST 7 (1 copy/µL)	50	Nuclease-free water	50

Table 1: Dilution scheme for standard curve.

3. (Optional) Prepare additional quality control samples by serially diluting the standard 1 (ST 1) of the MACS qPCR LENTIcheck (BaEV) Control in the template DNA PCR workstation according to table 2. Mix well.

Quality Control	Target standard concentration	Component 1		Component 2	
		Name	Volume (µL)	Name	Volume (µL)
QC 1	5×10^5 copies/µL	ST 0 (1×10^7 copies/µL)	10	Nuclease-free water	190
QC 2	5×10^4 copies/µL	QC 1 (5×10^5 copies/µL)	10	Nuclease-free water	90
QC 3	5×10^3 copies/µL	QC 2 (5×10^4 copies/µL)	10	Nuclease-free water	90
QC 4	5×10^2 copies/µL	QC 3 (5×10^3 copies/µL)	10	Nuclease-free water	90
QC 5	5×10^1 copies/µL	QC 4 (5×10^2 copies/µL)	10	Nuclease-free water	90
QC 6	5 copies/µL	QC 5 (5×10^1 copies/µL)	10	Nuclease-free water	90

Table 2: Dilution scheme for quality control samples.

4. Discard QC 1, QC 2, and QC 5. QC 3, QC 4, and QC 6 will serve as quality controls.
5. Prepare genomic DNA from 1×10^6 cells. Elute the genomic DNA in 300 µL nuclease-free water. This starting material will be called "test sample" within the text.

   ▲ **Note:** Two replicates of the test samples should be analyzed with five replicate qPCR measurements for each replicate.

   To be able to control the assay performance, it is recommended to prepare additional control samples:

   - A negative control sample (non-transduced cells),
   - a DNA extraction negative control sample, and
   - a non-transduced cell sample with the MACS qPCR LENTIcheck (BaEV) Control spiked to the cells before genomic DNA extraction (DNA extraction positive control sample). Spiking 300 copies per 1×10^6 cells is equivalent to 50 copies per µg genomic DNA, assuming 6 µg of genomic DNA per 10^6 cells.

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Sample description	Cell number/ pellet	Spike	Volume of PBS for pellet resuspension (µL)
Test sample (transduced T cells)	1×10^6	No.	200
Negative control sample (non-transduced cells)	1×10^6	No.	200
DNA extraction negative control sample	0 (PBS only)	No.	200
DNA extraction positive control sample	1×10^6	Yes. Add 30 µL of ST 6 (10 copies/ µL MACS qPCR LENTIcheck (BaEV) Control)	170

Table 3: Overview of the test sample and the corresponding controls.

6. Prepare and aliquot a 2× master mix solution for the duplex assay in the DNA-free PCR workstation according to table 4, e.g., for one sample use 1188 µL Taqman Fast Advanced Master Mix and 12 µL MACS qPCR LENTIcheck (BaEV) Primers & Probe. Mix well.

Number of test samples incl. controls	Taqman Fast Advanced Master Mix (µL)	MACS qPCR LENTIcheck (BaEV) Primers & Probe (µL)
1	1188	12
2	1584	16

Table 4: Preparation of 2× master mix solution.

7. (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 5).

Number of test samples incl. controls	Taqman Fast Advanced Master Mix (µL)		MACS qPCR LENTIcheck (BaEV) Primers & Probe (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	1176	1164	12	12	24
2	1568	1552	16	16	32

Table 5: Preparation of 2× master mix solution including a reference standard dye with 50× or 100× concentrated stock solution.

8. In the DNA-free PCR workstations add 50 µL 2× master mix to the 1.5 mL microcentrifuge tubes containing the no-template control (NTC).

9. In the DNA-free PCR workstations add 50 µL 2× master mix to new 1.5 mL microcentrifuge tubes for the standard curve (8 tubes) and for the three quality control samples (3 tubes). A loading scheme is shown in table 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ST 8 (0.5 copies/ µL)			ST 7 (1 copy/µL)			ST 6 (1×10^1 copies/µL)			ST 5 (1×10^2 copies/µL)		
B	ST 4 (1×10^3 copies/µL)			ST 3 (1×10^4 copies/µL)			ST 2 (1×10^5 copies/µL)			ST 1 (1×10^6 copies/µL)		
C	DNA extraction negative control sample 1	Negative control sample 1 (non-transduced cells)	Replicate 1 of test sample 1 (transduced T cells)	Replicate 2 of test sample 1 (transduced T cells)	DNA extraction positive control sample 1	DNA extraction negative control sample 2	Negative control sample 2 (non-transduced cells)	Replicate 1 of test sample 2 (transduced T cells)	Replicate 2 of test sample 2 (transduced T cells)	DNA extraction positive control sample 2	-	-
D											-	-
E											-	-
F											-	-
G											-	-
H	NTC			QC 6 (5 copies/ µL)			QC 4 (5×10^2 copies/µL)			QC 3 (5×10^3 copies/µL)		

Table 6: Example of a loading scheme of a 96-well PCR plate.

10. In the DNA-free PCR workstations add 75 µL 2× master mix to new 1.5 mL microcentrifuge tubes for each of the two replicates of each test sample, the respective negative control sample (non-transduced cells), the DNA extraction negative control sample, and the DNA extraction positive control sample (5 tubes). A loading scheme is shown in table 6.

11. In the template DNA PCR workstation add 50 µL of each standard (table 1) to the 50 µL 2× master mix prepared in step 9. Mix well.

12. (Optional) In the template DNA PCR workstation, add 50 µL of the three quality control samples QC 3, QC4, and QC 6 (table 2) to the 50 µL 2× master mix prepared in step 9. Mix well.

13. In the template DNA PCR workstation add 75 µL of each of the two replicates of each of the test sample, the respective negative control sample (non-transduced cells), the DNA extraction negative control sample, and the DNA extraction positive control sample (table 3) to the respective 75 µL of 2× master mix prepared in step 10. Mix well.

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

15. In the template DNA PCR workstation, add 25 µL of each prepared solution into the 96-well PCR plate according to table 6. Each replicate of each test sample and the respective controls will be applied and analyzed in quintuple.

16. Seal the plate by using an adhesive sealing film and spin down before loading into the instrument. Run plate in a multicolor realtime PCR detection system under following conditions (table 7).

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Temperature (°C)	Time (seconds)	Data collection	Number of cycles
+50	120	No	1
+95	20	No	1
+95	5	No	40
+60	30	Yes (plate read)	

Table 7: Thermal cycling conditions.

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 baseline and threshold values, need to be defined. The primary data analysis will result in threshold cycle or crossing point (Ct) values for the dye of each sample.

Use the data of the serial dilution of the MACS qPCR LENTIcheck (BaEV) Control (table 1) to generate a standard curve for BaEV (FAM). Plot the Ct values against the log(10) of the number of copies used for the standard.

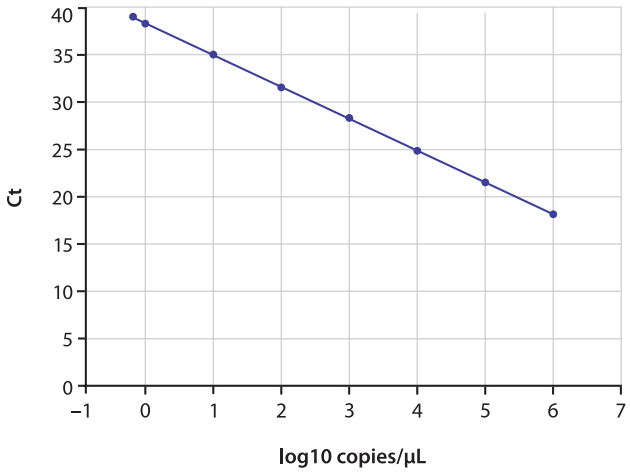


Figure 1: Example of a standard curve depicting the measured Ct value versus the log(10) of the copies per µL of the MACS qPCR LENTIcheck (BaEV) Control standard dilution (refer to table 1).

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

The linear equation of the standard is:

$$Ct = \text{slope} \times \log(\text{copies}/\mu L) + \text{Y-intercept}$$

Calculation example from figure 1:

$$Ct = (-3.333) \times \log(\text{copies}/\mu L) + 38.331$$

Accordingly, the copy number of BaEV in the respective sample can be calculated by transforming the equation:

$$\text{Copies}/\mu L = 10^{(Ct - \text{Y-intercept}) \div \text{slope}}$$

Calculation example from figure 1:

$$\text{Copies}/\mu L = 10^{(Ct - 38.331) \div (-3.333)}$$

4. Recommended acceptance criteria

Determine if the assay met system suitability acceptance criteria:

- The slope of the standard curve must be between –3.1 and –3.6.
- The R2 for the standard curve must be greater than or equal to 0.990.
- At least two wells containing the NTC must return an average Ct value of "N/A".
- The DNA extraction negative control sample and the negative control sample (non-transduced cells) must have no signal in five wells.
- The quality control sample QC 3 must be within 0.3 logs of the known quantity.
- The quality control samples QC 4 and 6 must be within 0.5 logs of their known quantities.

Valid options:

- It is valid to omit up to three wells from the standard curve that are greater than 0.5 Ct from the rest of the replicates.

All acceptance criteria must be met for the assay to be considered valid. If the assay fails to meet the acceptance criteria, the assay is invalid and must be repeated.

5. Recommended criteria for a positive result

▲ Results < 40 Ct are positive.

DNA extraction positive control sample:

- Sample will be deemed positive if at least three out of five wells are positive.
- If less than three wells shows amplification, it is recommended to repeat the assay.

Test sample:

- Sample will be deemed positive if at least three out of five wells are positive in duplicate samples.
- If less than three wells are positive for a replicate, that replicate will be deemed negative.
- If both replicates are negative, then the sample will be deemed negative.
- If one replicate is negative and one replicate is positive, it is recommended to repeat the assay.
  - If in the repeat assay both replicates are negative, then the sample will be reported as negative.
  - If in the repeat assay one or both replicates are positive, then the sample will be reported as positive.

6. Troubleshooting

Signal in negative control sample

Signals in negative control sample typically indicate contamination of the used material with MACS qPCR LENTIcheck (BaEV) Control 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.

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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 vortexing for 5–10 seconds.

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

MACS qPCR LENTIcheck (BaEV) Primers & Probe might have not been added when preparing the 2× master mix solution or they were exposed to excessive light or temperature for a 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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