

T CELL COMPANY

T-TRACK[®]
CMV

T-Track[®] CMV INSTRUCTIONS FOR USE

T-Track[®] CMV is a test for the determination of the functionality of cell-mediated immunity (CMI) of CMV seropositive patients.

The test enables a semiquantitative evaluation of the CMV-specific immunocompetence of these patients. The test is not suitable to detect a CMV infection.

Sufficient for 12 tests
In vitro diagnostic device

REF 11001003

IVD **CE**



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COMPONENTS OF THE T-TRACK® CMV KIT

	Kit component		Unit	Amount	
1	K50001	Ready-MTP	Piece	1	Microtiterplate (12 strips)
2	K50003	T-activated® IE-1	Volume	70 µl	Concentrate
3	K50002	T-activated® pp65	Volume	70 µl	Concentrate
4	K50073	PHA ¹⁾	Volume	70 µl	Concentrate
5	K50005	mAb-AP	Volume	70 µl	Concentrate
6	K50006	DB	Volume	12 ml	Ready to use
7	K50007	Stain	Volume	6 ml	Ready to use
8	K50008	WB1	Volume	200 ml	Ready to use
9	K50009	WB2	Volume	70 ml	Ready to use
10	K50010	Instructions for use	Piece	1	

¹⁾ Positive control material 

STORAGE AND STABILITY

Storage

Store all kit components at 2-8 °C. The Stain solution is light sensitive and should be stored protected from light. Unused test strips should be replaced in the original packaging under sterile working conditions and stored at 2-8 °C protected from light.

Stability

See kit box label for the expiry date. Kit components should be stored under the recommended conditions. Components used repeatedly should be stored at 2-8 °C until further usage. They are stable for up to 3 months after initial opening of the kit, at most up to the expiry date of the kit.

Transport and storage of blood samples

To ensure the functionality of blood leukocytes, blood samples should be transported or stored at room temperature (18-25 °C) and analyzed within 24 hours after blood withdrawal.

Heparinized blood samples should not be refrigerated or frozen.

SAFETY WARNINGS AND PRECAUTIONS



Care should be taken when handling material of human origin. All blood samples should be considered potentially infectious. Handling, use, storage, and disposal of blood samples and assay components should follow national biohazard safety guidelines and regulations. Care should be taken when working with chemicals. All chemicals should be considered potentially hazardous.

For further information, request the corresponding Material Safety Data Sheet (MSDS) via Request@Lophius.com.

LIMITATIONS/REMARKS

- Read the assay instructions carefully before use.
- For professional use only.
- The test is not suitable to detect a CMV infection.
- Results should be used and interpreted only in the context of the overall clinical picture.
- Store the kit at 2-8 °C. The kit should not be used beyond the expiry date.
- Do not mix components of different kit lots.

- Use sterile conditions whenever recommended and precautionary measures all along the assay to avoid contamination of reagents, test strips, cell suspensions and cell culture media.
Note: The expression “sterile condition” means a controlled working environment to avoid contaminations.
- Variations to the stated pipetting and washing steps, incubation times and/or temperatures may influence the results. The recommendations in the instructions for use **10** must be followed.
- Do not refrigerate or freeze whole blood samples.
- For the comparability of the test results, a validation of cell counting and measurement devices (e.g. measuring range, measuring protocol) is recommended.
- Consider the limitations of the measurement devices.

ADDITIONAL EQUIPMENT AND MATERIALS

- Blood collection tubes, S-Monovette® Li-Heparin 7.5 ml (Sarstedt, Cat.-No. 01.1608.001)
- Centrifugation tubes (15 ml and 50 ml, Greiner, Cat.-No. 188271 and Cat.-No. 227261)
- Reaction tubes (1.5 ml, Greiner, Cat.-No. 616201)
- Sterile pipettes and pipette tips
- Sterile Pasteur pipettes
- Sterile PBS (Lonza, Cat.-No. BE17-516F)
- Ficoll® (Pancoll, PAN Biotech, Cat.-No. P04-60500)
- Trypan blue (optional, Sigma, Cat.-No. T8154)
- Sterile serum-free cell culture medium (AIM-V®, Invitrogen, Cat.-No. 31035-025)
- Tap water
- Centrifuge with a swing-out rotor (capable of at least 1000 x g, able to maintain the samples at room temperature (18-25 °C) and to operate without a brake mechanism) for the preparation of PBMC

- Class II microbiological cabinet
- Neubauer counting chamber with microscope and trypan blue or validated cell counting device (recommended)
- Humidified incubator (37 °C, 5 % CO₂)
- ELISpot reader (recommended)

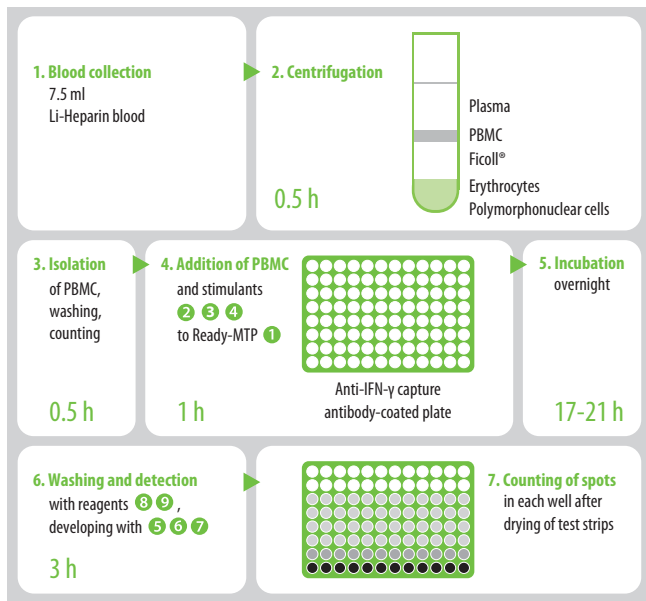
PRINCIPLE OF T-TRACK® CMV

The T-Track® CMV assay is based on the highly sensitive Enzyme-linked Immunosorbent Spot (ELISpot) technique. In principle, the ELISpot method is a solid-phase ELISA using a PVDF-membrane microtiter plate. T-Track® CMV enables a highly specific and sensitive detection of CMV-specific reactivated (IFN- γ -secreting) effector cells at a single-cell level.

Isolated PBMC are stimulated for 17-21 hours with selected T-activated® CMV proteins (IE-1, pp65). The cytokine IFN- γ , secreted by CMV-reactive cells, is bound by IFN- γ -specific capture antibodies immobilized on the PVDF-membrane. Cells are removed and the captured IFN- γ is detected using IFN- γ -specific detection antibodies coupled to alkaline phosphatase.

Following addition of a soluble substrate, an enzymatic reaction produces an insoluble colored precipitate and spots are revealed. Thereby one spot represents the footprint of a single antigen-reactive IFN- γ -secreting effector cell.

The spots can be enumerated either manually by light microscopy or using an automated imaging system (ELISpot reader).



For the measurement of one patient sample, the following measurements are performed:

- **Negative control** (2 replicates): non-stimulated PBMC for the detection of nonspecifically activated effector cells
- **Assay marker 1** (2 replicates): **CMV T-activated® immediate-early-1 (IE-1) protein** ② antigen-stimulated PBMC
- **Assay marker 2** (2 replicates): **CMV T-activated® phosphoprotein 65 (pp65)** ③ antigen-stimulated PBMC
- **Positive control** (cell functionality, 1 replicate): phytohemagglutinin-L (PHA) ④-stimulated PBMC
- **Operator control** (correct assay performance, 1 replicate): capture-antibody saturated with IFN- γ

PROCEDURE

This test should be performed in strict adherence to the steps described in these instructions for use **10**. PBMC isolation and setup of test strips should be carried out under sterile conditions.

Preliminary remarks

Blood collection should be performed by qualified persons according to local instructions for blood withdrawal. To carry out a test, one 7.5 ml Li-Heparin tube, completely filled with blood, is needed per patient to gain sufficient PBMC both from immunocompetent and immunosuppressed patients. To ensure blood leukocyte functionality during incubation, the PBMC isolation procedure should be validated. In addition, the PBMC enumeration procedure should be validated. Cell counting can vary in a non-validated process and lead to unreliable measurement results.

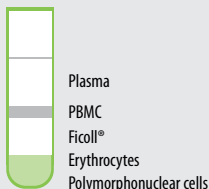
The procedure of spot enumeration must also be validated by the user. To ensure a standardized evaluation of test result, the use of a validated and calibrated ELISpot reader with corresponding software is recommended. Visual inspection of spot counts should be performed to verify concordance with reader output. Reader's spot counting parameters must be defined and the same settings should be used for multiple readings.

Reagent preparation

The cell culture medium must be pre-warmed to room temperature before use. Allow the kit to equilibrate to room temperature before use. Mix solutions gently (do not vortex) and centrifuge the vials briefly.

The packaging of material used under sterile working conditions must be disinfected before use.

PBMC preparation (under sterile conditions)



- 1 Dispense 15 ml Ficoll® in a 50 ml polypropylene tube.
- 2 Dilute 7.5 ml whole blood with 7.5 ml sterile PBS in another 50 ml polypropylene tube.
- 3 Overlay the Ficoll® carefully and slowly with 15 ml of diluted whole blood.
- 4 Centrifuge for 30 min at 880 x g in a swing-out rotor without brake.
- 5 Collect the interphase (cloudy PBMC-containing layer between Ficoll® and plasma) with a sterile Pasteur pipette.
- 6 Transfer the PBMC in a new 50 ml polypropylene tube.
- 7 Fill up to a volume of 50 ml with sterile PBS.
- 8 Centrifuge for 10 min at 300 x g.
- 9 Discard the supernatant (washing step).
- 10 Resuspend the cell pellet in 1 ml sterile PBS.
- 11 Fill up to a volume of 50 ml with sterile PBS.
- 12 Centrifuge for 10 min at 300 x g.
- 13 Discard the supernatant (washing step).
- 14 Resuspend the cell pellet in 500 µl sterile, serum-free AIM-V® medium.
- 15 Count the living cells in a Neubauer counting chamber under the microscope (trypan blue staining) or using a validated cell counting device (recommended).
- 16 Adjust the counted cells to 2×10^6 cells/ml AIM-V® medium. For one test, 1.6×10^6 cells in 800 µl AIM-V® medium are needed.

Preparation of working solutions

Note: The following volumes refer to one test for one patient (corresponding to 1 test strip). When analyzing a larger number of patient samples, all volumes of the reagents and working solutions must be adjusted accordingly.

	Stock solution	Medium (AIM-V®)	Working solution
Negative control	-	125 µl	125 µl
2 T-activated® IE-1	5 µl	120 µl	125 µl
3 T-activated® pp65	5 µl	120 µl	125 µl
4 PHA	5 µl	50 µl	55 µl
Operator control	-	175 µl	175 µl









Preparation of test strips (under sterile conditions)

Per test (for one test person) **1 test strip** (8 wells) is needed.

- 1 Remove Ready-MTP 1 from the packaging.
- 2 Remove surplus test strips from the plastic frame of the Ready-MTP.

Note: Test strips are labeled by numbers. Place the unused test strips back into the aluminium pouch and securely close. Store at 2-8 °C until further use and protect against mechanical damage.

- 3 Pipetting of working solutions beginning with the negative control into the wells:
 - Negative control: 2 times 50 µl cell culture medium
 - IE-1 2: 2 times 50 µl T-activated® IE-1 working solution
 - pp65 3: 2 times 50 µl T-activated® pp65 working solution
 - PHA 4: 1 time 50 µl PHA working solution
 - Operator control: 1 time 150 µl medium

A		50 µl negative control (medium) per well	Add 100 µl PBMC solution per well
B			
C		50 µl T-activated® IE-1 working solution per well	Add 100 µl PBMC solution per well
D			
E		50 µl T-activated® pp65 working solution per well	Add 100 µl PBMC solution per well
F			
G		50 µl PHA working solution	Add 100 µl PBMC solution
H		Operator control: 150 µl medium	-

Stimulation

- Mix the adjusted cell suspension (see page 9: PBMC preparation) immediately before use.
- Carefully add 100 µl of cell suspension (2×10^5 cells) to each well. Starting with the negative control, add the cell suspension from top to bottom. Caution: NO cell suspension is added to the operator control well.
Note: Change pipette tip after each well to avoid cross-contamination.
- Cover the Ready-MTP **1** with the lid.
- Incubate the Ready-MTP **1** for 17-21 hours at 37 °C, 5 % CO₂ in a humidified incubator.

Detection of IFN-γ-producing effector cells

(sterile working conditions are no longer necessary)

Note: The volumes given below refer to one single test.

- Discard the cell suspension and the medium.
- Add 200 µl WB1 **8** per well.
- Discard WB1 and repeat the washing step 5 more times.
Note: The procedure can be interrupted at this point; remove WB1 from the last washing step and store the plate at -20 °C for up to 48 hours.

- 4 Prepare the mAb-AP working solution by mixing 5 µl mAb-AP 5 with 900 µl DB 6.
- 5 Add 100 µl of mAb-AP working solution per well.
- 6 Place the lid on the Ready-MTP.
- 7 Incubate the covered test strips for 2 hours at room temperature (18-25 °C).
- 8 Discard the mAb-AP working solution.
- 9 Add 200 µl of WB1 8 per well.
- 10 Discard WB1 and repeat the WB1 wash step 2 more times.
- 11 Add 200 µl of WB2 9 per well.
- 12 Discard WB2 and repeat the WB2 wash step 2 more times.
- 13 Add 50 µl of stain 7 per well.
- 14 Place the lid on the Ready-MTP.
- 15 Incubate the test strips for 6-7 minutes at room temperature (18-25 °C) protected from light.
Note: Avoid a longer incubation period as this may increase background coloration and interfere with a reliable spot enumeration.
- 16 Pour out the Stain.
- 17 Rinse the test strip 3 times with tap water to stop the staining reaction.
- 18 Remove the bottom of the plate and gently tap the wells on a paper towel to remove any remaining liquid.
- 19 Dry the test strips overnight at room temperature (18-25 °C) or by placing the plate for at least one hour in the air stream of a laminar flow cabinet.
Note: After drying, the staining is stable for several weeks if protected from light.

READ-OUT OF RESULTS

The developed spots are counted and the analysis, interpretation, and documentation of the test results are carried out according to the instructions in the following chapters.

QUALITY CONTROL

The negative control (unstimulated condition) is typically expected to have no or few spots (< 10 spots per 2×10^5 cells). An increased spot count in the negative control might indicate a subclinical CMV reactivation or a general unspecific stimulation, resulting in a decreased analytical sensitivity.

The positive control (PHA) ④ is a measure of PBMC functionality and should show a strong response with a high spot count (usually > 200 spots per 2×10^5 cells in immunocompetent individuals). A reduced spot count in the positive control might indicate immunosuppression of the patient or improper storage or treatment of the blood sample. The underlying reason should be clarified by other appropriate methods.

The operator control must show a homogenous staining of the entire membrane. This staining is independent of the quality of the patient's sample and indicates a correct test performance. In case of a negative or inhomogeneous result of the operator control, test results are not analyzable.

RESULTS INTERPRETATION

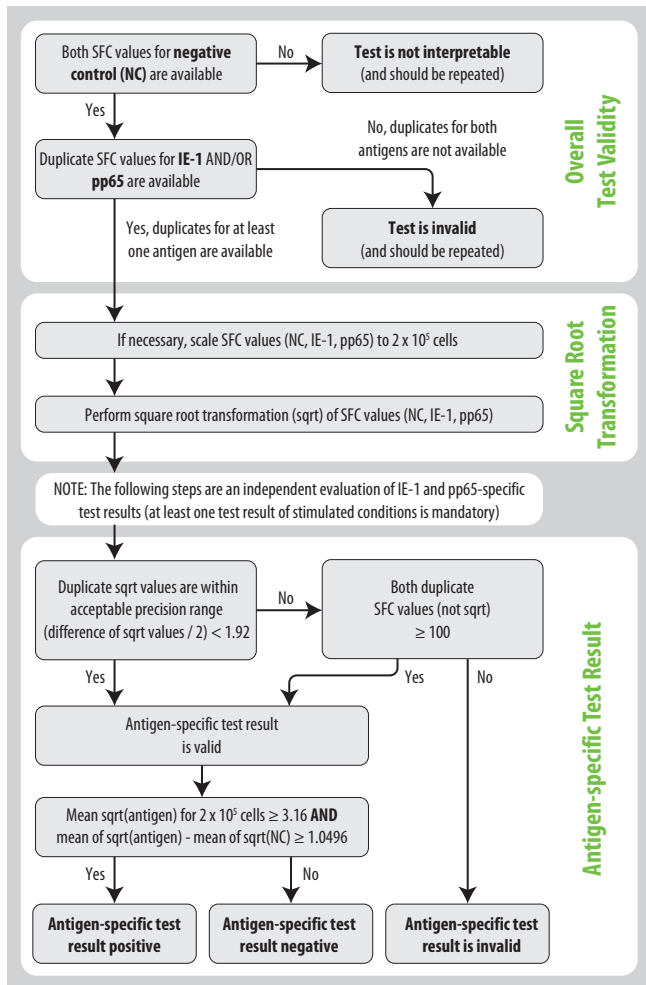
The functionality of the cell-mediated immunity is determined by the frequency of spot-forming cells (SFC, representing the signature of IFN- γ -producing cells) in specifically-stimulated (T-activated® IE-1 ② and T-activated® pp65 ③) and non-stimulated (negative control) conditions.

For the **validation** and **qualitative evaluation** of the results, the respective duplicate values of SFC (per 2×10^5 cells) from stimulated and unstimulated conditions are square-root-transformed (sqrt) (see Footnote 1). We recommend to use the provided **T-Track® CMV Calculator** software (freeware) for result validation and qualitative evaluation.

To be **valid**, a test result must fulfill the following requirements (see also Decision Tree on page 15):

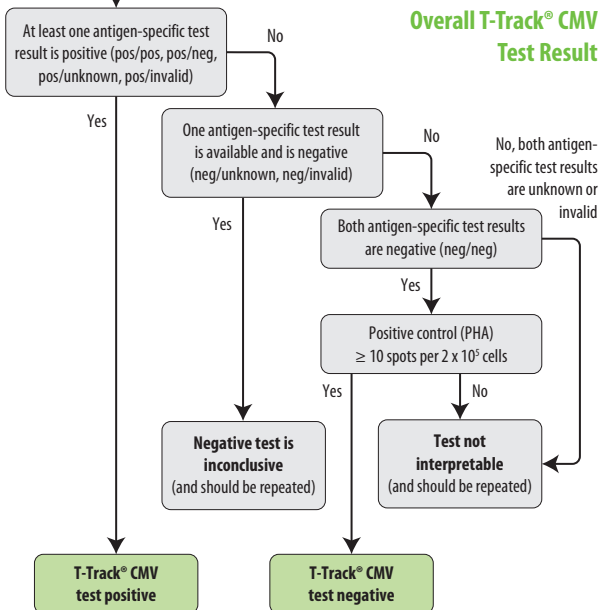
- At least one test result of stimulated conditions (T-activated® IE-1 ② and/or T-activated® pp65 ③) is mandatory.
- Both replicate SFC values from the respective conditions (negative control, T-activated® IE-1 ② and T-activated® pp65 ③) are mandatory.
- Duplicate SFC values of the respective stimulated conditions should lie within an acceptable precision range, whereby the (difference of duplicate sqrt values / 2) < 1.92 (see Footnote 2)
- In case of imprecise duplicate SFC values of stimulated conditions, the test result remains qualitatively valid if both SFC duplicate values ≥ 100 . The quantitative evaluation remains however invalid (due to “imprecise measurements”).

-
1. based on reassessment of clinical study results considering duplicate measurements by an independent statistician.
 2. where $1.92 = 3 \times 0.64$, with 0.64 corresponding to the intraserial standard deviation determined from a large number of measurements obtained from five independent cohorts of both immunocompetent and immunocompromised individuals.



NOTE: The following steps are a combined evaluation of IE-1 and pp65-specific test results

Overall T-Track® CMV Test Result



The test is **positive** if at least one of the stimulated conditions (T-activated® IE-1 ② or/and T-activated® pp65 ③) fulfills the two following criteria:

A, mean of $\sqrt{\text{stimulated}}$ for 2×10^5 cells ≥ 3.16 (see Footnote 3)

AND

B, mean of $\sqrt{\text{stimulated}}$ - mean of $\sqrt{\text{unstimulated}}$ ≥ 1.0496 (see Footnote 4)

The test is **confirmed negative** if neither of the stimulated conditions fulfill these two criteria **AND** the positive control (PHA) ④ concurrently shows ≥ 10 spots per 2×10^5 cells.

The test **cannot be evaluated** if in a negative test the positive control (PHA) ④ presents < 10 spots per 2×10^5 cells. However, in case a test is interpreted as positive based on the above criteria (A and B) and the positive control (PHA) ④ presents < 10 spots per 2×10^5 cells, the test is still considered **positive**.

In case of missing or invalid measurement for one of the two stimulated conditions (T-activated® IE-1 ② or T-activated® pp65 ③):

- a **positive** test result is **confirmed positive**, given that it fulfills the above criteria (A and B)
- a **negative** test result is **inconclusive** (in the absence of knowledge of the second measurement). Confirmation of test result should be performed by repeating the assay.

3. where '3.16' = $\sqrt{10}$, with '10' being the spot count.

4. where '1.0496' is based on a one-sided z-test for 2 replicates with an intraserial standard deviation (SD) of 0.64 (SD = 0.64 was calculated from a large number of measurements obtained from five independent cohorts of both immunocompetent and immunocompromised individuals).

If a T-activated® antigen-stimulated well shows more spots than the read-out device can resolve, a 1:2 to 1:3 dilution of the blood sample (considering the measuring range of the ELISpot reader) should be re-tested for a more accurate quantification of the spot number.

T-TRACK® CMV CALCULATOR SOFTWARE

We recommend using the provided **T-Track® CMV Calculator** software (freeware) for an accurate validation and qualitative evaluation of the results. Please, refer to the corresponding **T-Track® CMV Calculator** Instructions for Use.

As additional information the **T-Track® CMV Calculator** displays the mean SFC counts of duplicate SFC values (rounded to the nearest spot count), calculated with the following formula:

$[\text{mean of } \sqrt{\text{SFC}}]^2$

PERFORMANCE CHARACTERISTICS OF T-TRACK® CMV

Clinical sensitivity

The clinical sensitivity was assessed by stimulating freshly isolated PBMC from 67 CMV-seropositive, non-immunosuppressed hemodialysis patients with T-activated® IE-1 ② and T-activated® pp65 ③ and the subsequent calculation of the percentage of positive test results. The clinical sensitivity of the T-Track® CMV assay was overall 90% based on 4 replicate measurements and the evaluation of the geometric mean of SFC values (Barabas *et al.*, 2017; Banas *et al.*, 2017). A new evaluation of these results based on all six possible duplicate combinations (four-to-two-replicate simulation), square root transformation of SFC values and applying the QC rules described above resulted in an overall sensitivity of the assay of 90%.

Measuring range and linearity

The assay performance characteristics and linearity depend in part on the measurement device used. To determine all assay performance characteristics the Bioreader® 5000 Ea was used. In a range of 10 to 1,000 spots, the spot count is proportional to the amount of antigen-reactive effector cells. However, if more than 400 spots per well are detected, a retesting of the blood sample with a dilution of 1:2 to a maximum of 1:3 can be required, depending on the sensitivity of the measuring device used.

In terms of number of PBMC seeded, linearity of the IFN- γ ELISpot assay was demonstrated for 6×10^4 to 2×10^5 cells per well.

Repeatability

The repeatability of the assay was determined with PBMC from 11 individual donors. For this, blood was taken from each donor for three independent measurements. A total of 33 PBMC samples were isolated and independently tested with T-Track® CMV by one operator using the same reagents and equipment. The determined variation

of the repeated measurements based on 2 replicate SFC values did not exceed 35% [median CV of 14.4% (range 2.8 - 34.9%)].

Reproducibility

The reproducibility of the assay was assessed on PBMC from 3 individual donors. Two blood samples were collected per donor. A total of 6 PBMC samples were isolated in parallel and independently tested with T-Track® CMV. Only one of the following parameters varied per experiment:

- Inter-operator variation (3 different operators; same reagents, equipment and site)
- Inter-batch variation (3 different kit lots; same operator, equipment and site).

Using a validated cell-counting method, variability between operators and kit batches did not exceed 38% in a four-to-two-replicate simulation considering all 6 possible combinations of duplicates [median CV of 9.8% (range 0.1 - 37.1%) inter operators and of 9.1% (range 0.6 - 36.6%) inter batches].

REFERENCES












- Barabas *et al.* (2008). Urea-mediated Cross-Presentation of Soluble Epstein-Barr Virus BZLF1 Protein. *PLoS Pathog.* 4(11):e1000198.
- Barabas *et al.* (2017). An optimized IFN- γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. *BMC Immunol.* 18:14.
- Banas *et al.* (2017). Validation of T-Track[®] CMV to assess the functionality of cytomegalovirus-reactive cell-mediated immunity in hemodialysis patients. *BMC Immunol.* 18:15.
- Reuschel *et al.* (2017). Functional impairment of CMV-reactive cellular immunity during pregnancy. *J. Med. Virol.* 89:324-331.

ABBREVIATIONS

AP	Alkaline phosphatase
Cat.-No.	Catalog number
CO ₂	Carbondioxide
CMV	Cytomegalovirus
CV	Coefficient of variation
DB	Dilution buffer
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunosorbent Spot
IE-1	Immediate-early-1
IFN- γ	Interferon- γ
Li	Lithium
mAb	Monoclonal antibody
MSDS	Material safety data sheet
MTP	Microtiter plate
NC	Negative control
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin-L
pp65	Phosphoprotein 65
PVDF	Polyvinylidenefuorid
SD	Standard deviation
SFC	Spot forming cells
sqrt	Square root transformation
WB	Wash buffer

NOTES


GLOSSARY OF SYMBOLS

	Sufficient for "n" tests
	Consult instructions for use
	Caution
	Manufacturer
	Order number
	Lot number
	Use by / date of expiry
	Storage temperature limitation
	Positive control material
	In vitro diagnostic device
	CE conformity mark

This product is covered by the Lophius Biosciences General Terms and Conditions of Sale, which are available at www.Lophius.com

T-Track® CMV is an IFN- γ ELISpot assay based on Lophius Biosciences' proprietary T-activation technology.

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