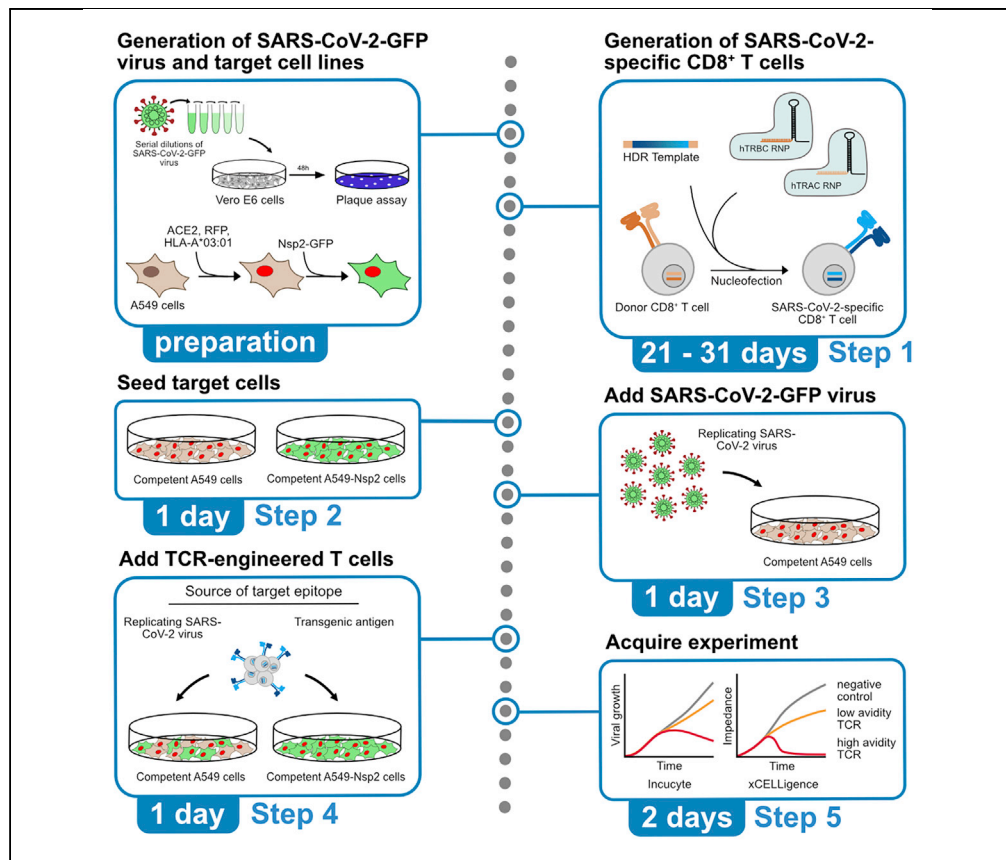


Protocol

SARS-CoV-2 CD8⁺ T cell killing assays using replicating viruses and transgenic antigens



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Highlights

The established killing assays use replicating SARS-CoV-2 virus or transgenic antigens

Near-physiological antigen processing and epitope presentation is ensured

Protocol can also be adapted for other applications, e.g., TCR therapy for cancer

The quality of an antigen-specific CD8⁺ T cell repertoire is crucial for the clearance of intracellular pathogens, in particular for viral infections. Here, we describe killing assays to determine the function of CD8⁺ T cells engineered with SARS-CoV-2-specific T cell receptors in a near-physiological system for antigen presentation. We detail the use of target cells either infected with replicating SARS-CoV-2 virus or engineered with SARS-CoV-2 open reading frames.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

SARS-CoV-2 CD8⁺ T cell killing assays using replicating viruses and transgenic antigens

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SUMMARY

The quality of an antigen-specific CD8⁺ T cell repertoire is crucial for the clearance of intracellular pathogens, in particular for viral infections. Here, we describe killing assays to determine the function of CD8⁺ T cells engineered with SARS-CoV-2-specific T cell receptors in a near-physiological system for antigen presentation. We detail the use of target cells either infected with replicating SARS-CoV-2 virus or engineered with SARS-CoV-2 open reading frames. For complete details on the use and execution of this protocol, please refer to Moosmann et al. (2022) and Wagner et al. (2022).

BEFORE YOU BEGIN

This protocol describes two different approaches in which target cells either infected with a replicating SARS-CoV-2 virus or transduced with SARS-CoV-2-derived open reading frames (ORFs) can be effectively killed by SARS-CoV-2-specific CD8⁺ T cells in Incucyte or xCELLigence-based assays.

To generate a suitable target cell line, the A549 human lung cancer cell line was engineered to express the angiotensin-converting enzyme 2 (ACE2), a histone (H2B) fused to a red fluorescent protein (RFP) and the HLA-A*03:01 molecule, which allows viral infection, live-cell imaging acquisition and appropriate epitope presentation, respectively. For infection of the resulting A549-ACE2-RFP-HLA-A*03:01 cells (from now on referred to as competent A549 cells), a genetically modified green fluorescent protein (GFP)-expressing SARS-CoV-2 virus was used. Thus, the target antigen is physiologically expressed and processed for epitope presentation. In addition, this killing assay with a replicating virus is relatively cost effective as no special consumables are needed.

For the SARS-CoV-2 ORF-transduction assay, competent A549 cells were additionally engineered to express the non-structural protein 2 (Nsp2) of the ancestral SARS-CoV-2 virus fused to GFP via a T2A tag, which guarantees polycistronic expression and post-translational separation (from now on referred to as competent A549-Nsp2 cells). Despite ectopic expression, the system ensures the expression of the entire target antigen as well as natural peptide cleavage and epitope presentation. Thus, it serves as an alternative near-physiological killing assay without the need of using infectious virus material and related biosafety infrastructures. The SARS-CoV-2 ORF-transduction assay can be safely used under BSL1 and BSL2 conditions.



For both killing assays, two previously characterized HLA-A*03:01-restricted, SARS-CoV-2-specific TCRs against epitope ORF1_VTN (S13: highly functional and S32: low functional) (Wagner et al., 2022) were integrated into CD8⁺ T cells via orthotopic T-cell receptor replacement (OTR) and the engineered T cells were expanded as described in 1)–12) of section “[generation of OTR-engineered SARS-CoV-2-specific CD8⁺ T cells](#).” The target epitope ORF1_VTN, which is part of the Nsp2, will be presented by HLA-A*03:01 molecules on the cell surface of either SARS-CoV-2 infected competent A549 cells or competent A549-Nsp2 cells.

SARS-CoV-2-specific CD8⁺ T cell receptor (TCR) sequences were derived from convalescent mild COVID-19 patients as described in (Wagner et al., 2022). For the generation of TCR-transgenic CD8⁺ T cells, OTR was performed by using CRISPR-Cas9-mediated homology-directed repair similar to (Moosmann et al., 2022).

Alternatives: This protocol describes specific steps for using A549 cells. Some of the described experiments, however, have also been performed in Calu-3 and K562 cell lines. Nevertheless, any target cell line can theoretically be used after proper titrations.

The source of T cells can also be flexible. In this protocol we are describing killing assays with SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells generated with CRISPR-Cas9-mediated OTR. We envision that also naturally polyclonal T cell populations from SARS-CoV-2 infected individuals could be used either directly *ex vivo* or post antigen-specific T cell expansion to enlarge reactive populations.

Institutional permissions

Usage of the blood samples for CRISPR-Cas9-mediated editing was approved by the local Institutional Review Board (Ethikkommission der Medizinischen Fakultät der Technischen Universität München). The study conforms to the standards of the Declaration of Helsinki.

Generation of A549-ACE2-RFP-HLA-A*03:01 (Nsp2-GFP) cells

Integration of ACE2 and RFP into A549 cells

- ⌚ Timing: 1 h (hands-on) + 1 day (additional cell culture) for step 1
- ⌚ Timing: 2 h (hands on) + 48 h (additional cell culture) for step 2
- ⌚ Timing: 30 min - 1 h (hands on) + 6 days (additional cell culture) for step 3
- ⌚ Timing: 30 min - 1 h (hands on) + 7 days (additional cell culture) for step 4
- ⌚ Timing: 1 h (hands-on) + 1 day (additional cell culture) for step 5
- ⌚ Timing: 2 h (hands-on) + 2 days (additional cell culture) for step 6
- ⌚ Timing: 30 min (hands-on) + 1 day (incubation) + 2–3 days (additional cell culture) for step 7
- ⌚ Timing: 2 h (hands on) for step 8

1. Seeding of HEK293T cells.
 - a. From a T75 cm³ flask, wash adherent cells once with PBS.
 - b. Add 3 mL of trypsin and incubate for 3 min in the incubator (37°C, 5% CO₂).
 - c. Add 10 mL of complete DMEM medium (10% FCS, 5% supplementary medium components (SC+ supplement)) (see [materials and equipment](#)) to inactivate trypsin and homogenize the cell suspension.

- d. Centrifuge for 5 min at $480 \times g$ at 20°C – 22°C , discard supernatant and resuspend the cell pellet in 10 mL complete DMEM medium.
- e. Count cells and resuspend at 0.4×10^6 cells/mL.
- f. Seed 2 mL of cell suspension (0.8×10^6 cells) for one well of a 6-well plate (6-WP).

Note: On the day of seeding, HEK293T cells should be 80%–90% confluent and a seeding cell number of 0.8×10^6 cells should be sufficient to reach the desired cell confluency of 70%–80% in the 6-WP on the following day. However, since growth behavior of HEK293T cells may vary among different laboratories and over time of culture, it is recommended to titrate cell numbers before performing transfection experiments. The here described steps of adherent cell line detachment and handling will not be described again in detail for other adherent cell lines used in this protocol (e.g., HeLa, RD114 cells, A549 cells). Please note that parameters like trypsin incubation time or seeding number should be adjusted.

2. Production of H2B-RFP and ACE2 lentiviruses via polyethylenimine (PEI)-mediated transfection.
 - a. Prepare plasmid DNA. For one well in a 6-WP, add the packaging plasmids pMD2G-VSVG (350 ng) and psPAX2 (750 ng), and the target plasmids pCI H2B-RFP (1500 ng) and pWPI ACE2-HA (1,500 ng), respectively, to room temperature (20°C – 22°C) OptiMEM medium (fill up to 100 μL).
 - b. Prepare transfection reagent. For one well in a 6-WP, add 9 μL PEI reagent (1 mg/mL) to 91 μL OptiMEM medium (20°C – 22°C).
 - c. Mix plasmid DNA and transfection reagent, vortex for 10 s and incubate for 20 min at 20°C – 22°C .
 - d. Replace medium for HEK293T cells with 2 mL of 37°C warm complete DMEM medium.
 - e. Add transfection reagent/plasmid DNA mixture dropwise to HEK293T cells, carefully sway the plate and place in the incubator (37°C , 5% CO_2).

Note: From now on, continue working under BSL2 conditions as lentivirus particles will be formed after this step.

- f. 6 h post transfection, replace medium with 2 mL of fresh 37°C warm complete DMEM medium.
- g. Harvest supernatant 48 h post transfection and filter through 0.45 μm PVDF filters.

Note: Lentiviruses can only be stored in the fridge for a very short time (max 1 day). Store the virus containing supernatant at -80°C until further use.

3. Titration of lentiviruses by colony-forming assay.
 - a. Seed 8×10^3 HeLa cells in 96-well plates (96-WP) in 100 μL 37°C warm complete DMEM medium.
 - b. Prepare two tubes for each lentivirus to titrate (1:40 and 1:1,000 dilutions in complete DMEM medium in order to cover an expected viral titer ranging from 4×10^3 to 2×10^6 colony forming units).
 - c. Add 10 μL of each dilution to the plated cells. Incubate cells overnight in the incubator (37°C , 5% CO_2).
 - d. Remove the medium and add 100 μL of complete DMEM medium containing antibiotics depending on the plasmid selection cassette (e.g., 1 $\mu\text{g}/\text{mL}$ puromycin or 100 $\mu\text{g}/\text{mL}$ zeocin).
 - e. Four days post transduction, remove the medium and add 100 μL of fresh complete DMEM medium containing antibiotics.
 - f. Six days post transduction, wash the cells with 100 μL PBS, and add 50 μL of a 1.25% crystal violet/25% ethanol solution (see [materials and equipment](#)).
 - g. Incubate for 10 min at 20°C – 22°C .
 - h. Rinse wells twice with 200 μL distilled water.
 - i. Remove all residual liquid from the wells and proceed to colony forming unit (CFU) calculation. Calculate the concentration of lentiviruses as following:

$$\frac{CFU}{mL} = CFU * dilution\ factor$$

The multiplicity of infection (MOI) is calculated as the ratio of infectious virus (e.g., CFU) per cell to be added.

4. Transduction and selection of A549-ACE2-RFP cells.

- a. Seed A549 cells one day prior transduction in the plate format of your choice (from 24-WP to 6-WP).

Note: A cell number of 5×10^4 A549 cells in a 24-WP was sufficient to reach an ideal cell confluence of 50%–70% for transduction. For best performance, it is recommended to titrate cell numbers.

- b. Add H2B-RFP and ACE2 lentivirus considering transduction with a MOI > 1 (i.e., a minimum of one lentiviral particle per cell) in order to transduce all cells in the well.
- c. One day after transduction, selection can be applied with 2.5 μ g/mL puromycin or 200 μ g/mL zeocin for one week.

Note: In the absence of a selection cassette in your target plasmid (e.g., H2B-RFP), cells can be FACS-sorted 3 days post-transduction similarly to 8) in the section “before you begin.”

Integration of HLA-A*03:01 into A549-ACE2-RFP cells

5. Seeding of RD114 cells in 6-WP.

Note: A seeding cell number of $0.8 - 1.0 \times 10^6$ cells was sufficient to reach the desired cell confluency of 30%–50% on the following day for transfection. For best performance it is recommended to titrate cell numbers.

6. Transfection of RD114 cells via calcium precipitation.

For the production of retroviral particles, RD114 cells were transfected with the pMP71 expression vector (containing the HLA heavy chain construct, gag/pol and amphotropic envelope) by calcium phosphate precipitation.

- a. Prepare transfection buffer (see [materials and equipment](#)).
- b. Make 3.31 M CaCl₂ solution by adding 1.8375 g CaCl₂ to 5 mL ddH₂O. Filter through 0.22 μ m PVDF filter.

Note: The solution must get warm.

- c. Prepare plasmid DNA-CaCl₂ solution. For one well in a 6-WP, add 18 μ g of plasmid DNA to ddH₂O (fill up to 150 μ L) in a sterile 1.5 mL Eppendorf tube and mix. Add 15 μ L of CaCl₂ solution and mix.

Note: It is recommended to use a maxiprep plasmid as source material for retroviral transfection as less volume is needed for the transfection reaction.

- d. For transfection of one well in a 6-WP, add 150 μ L of transfection buffer in round-bottom polystyrene test tubes.
- e. Add plasmid DNA-CaCl₂ solution to transfection buffer slowly under vortexing.

Note: The solution should become slightly cloudy.

- f. Incubate plasmid-transfection solution for 30 min at 20°C–22°C. Vortex once in between.
- g. Add plasmid-containing solution dropwise to RD114 cells, carefully sway the plate and place in the incubator (37°C, 5% CO₂).

Note: From now on, continue working under BSL2 conditions as retrovirus particles will be formed after this step.

- h. After 6 h, carefully perform medium exchange with 3 mL warm complete RPMI medium.
 - i. After 48 h, harvest retrovirus-containing supernatant and filter through 0.45 μm PVDF filters.
7. Transduction of A549-ACE2-RFP cells with HLA-A*03:01 retrovirus.
- a. Aliquot RetroNectin stock solution in 100 μL aliquots (1 μg/μL) to avoid freeze/thawing cycles and store at –20°C.
 - b. Dilute RetroNectin stock solution 1:100 in PBS to reach 0.01 μg/μL.
 - c. Coat Corning® Costar® non-treated 24-well plates (24-WP) with 400 μL of 0.01 μg/μL RetroNectin per well and incubate overnight in the fridge.

Note: Alternatively, RetroNectin coating can be performed for 2 h at 20°C–22°C. It has been shown that using RetroNectin-coated plates also improves the transduction efficiency for cell lines. This step, however, is optional as good transduction rates have also been achieved without RetroNectin coating.

- d. Remove the RetroNectin solution and wash all RetroNectin-coated wells once with 1 mL of 20°C–22°C PBS.

Note: Do not allow RetroNectin-coated wells to dry out.

- e. Add virus to 24-WP and centrifuge for 1 h at 2,000 × *g* at 32°C.

Note: 0.5 mL of retrovirus showed to be sufficient to generate good transduction efficiencies.

- f. Add 1 × 10⁵ A549-ACE2-RFP cells per retrovirus-containing well in 50 μL complete DMEM medium.
 - g. Centrifuge for 30 min at 2,000 × *g* at 32°C and place cells back in the incubator (37°C, 5% CO₂).
 - h. In case you have more than one replicate, pool cells when confluency is reached (after 2–3 days).
8. Fluorescent activated cell sorting (FACS) for HLA-A*03:01-expressing A549-ACE2-RFP cells.
- a. Detach transduced A549-ACE2-RFP cells from a T75 cm³ cell culture flask and take up in 10 mL medium similarly to 1) Seeding of HEK293T cells.
 - b. Centrifuge cells for 5 min at 480 × *g* at 20°C–22°C.
 - c. Resuspend cell pellet in 10 mL FACS buffer and centrifuge for 5 min at 480 × *g* at 20°C–22°C.
 - d. Perform one more washing step with 10 mL FACS buffer.
 - e. Resuspend A549-ACE2-RFP cells in staining buffer (APC-conjugated anti-HLA-A3 antibody, 1:200 in FACS buffer) at a density of 1–4 × 10⁷ cells/mL for 20 min on ice in the dark.
 - f. Add propidium iodide (PI) (1:100 in FACS buffer) (for generation of PI stock solution, see [materials and equipment](#)) and incubate for 1 min on ice in the dark.
 - g. Wash cells twice with 10 mL FACS buffer, filter through 112 μm polyamid sieve cloth and resuspend cells at 10–20 × 10⁶ cells/mL for cell sorting.
 - h. Purity sort with any flow cytometry sorter for HLA-A*03:01 (APC)-positive cells.

Alternatives: If no suitable antibody for the transduced HLA type is commercially available, positive selection can be performed by antibiotic selection if antibiotic-resistance is encoded

in the plasmid. The HLA-A*03:01 expressing retroviral plasmid used in this protocol carries a puromycin resistance. Therefore, antibiotic selection can be performed by using 3 µg/mL puromycin for at least one week with addition of fresh puromycin after every cell passage.

Integration of Nsp2-GFP into competent A549 cells

The retroviral plasmid mp72-Nsp2-T2A-GFP was previously generated in the laboratory. For this, the Nsp2 ORF (NC_045512.2, NCBI) was Gibson cloned from a pWPI vector (pWPI Puro_SARS-CoV2_nsp2, a kind gift from Prof. Andreas Pichlmair, Institute of Virology, Technical University Munich, Germany) into a mp72 vector. The mp72 vector was modified for specific restriction sites in the multiple cloning site *in house* from the mp71 vector (a kind gift from W. Uckert, Max-Delbrück-Centrum für Molekulare Medizin, Berlin). The T2A linker (Liu et al., 2017) and enhanced GFP (GFP) (C5MKY7, Uniprot) were also previously integrated into the mp72 vector in a custom synthesis with Twist Biosciences. Following cloning, the Nsp2-T2A-GFP protein can be produced by retroviral transfection and transduction as described below.

9. Seeding of RD114 cells in 6-WP. Protocol identical to 5) of section “before you begin.”
10. Transfection of RD114 cells via calcium precipitation. Protocol identical to 6) of section “before you begin”
11. Transduction of competent A549 cells with Nsp2 retroviruses. Protocol identical to 7) of section “before you begin.”
12. Sorting of GFP-expressing cells. Protocol similar to 8) of section “before you begin.” As GFP is endogenously expressed, no additional antibody staining is necessary.

Target cell line titrations for Incucyte and xCELLigence experiments

Titration of competent A549 cells for Incucyte experiment

⌚ Timing: 2 h (hands on) + 3 days (additional cell culture)

13. Seed 5,000 to 15,000 competent A549 cells in 100 µL complete RPMI medium per well of a 96-WP.

Note: Use 80%–90% confluent competent A549 cells. T cells are more sensitive to culture medium and are cultivated in complete RPMI medium. It is thus recommended to perform medium exchange for A549 cells from complete DMEM medium to complete RPMI medium two passages before the assay.

14. Choose the seeding cell concentration with a starting confluence of approximately 20%–40%, which will ultimately reach 100% confluency after 72 h (Figure 1).

Note: Titration steps need to be adjusted according to the cell line used.

Background measurement of xCELLigence plate

⌚ Timing: 1 h

15. Add 100 µL of complete RPMI medium per well in xCELLigence E-Plate VIEW 96 plates (E-Plate VIEW) and leave E-Plate VIEW under a sterile hood for 30 min at 20°C–22°C.
16. Place the E-Plate VIEW in the Agilent xCELLigence RTCA eSight instrument (xCELLigence eSight) (37°C, 5% CO₂) and set up the experiment according to instrument operational guidelines.
17. Take out the plate to add competent A549-Nsp2 target cells.

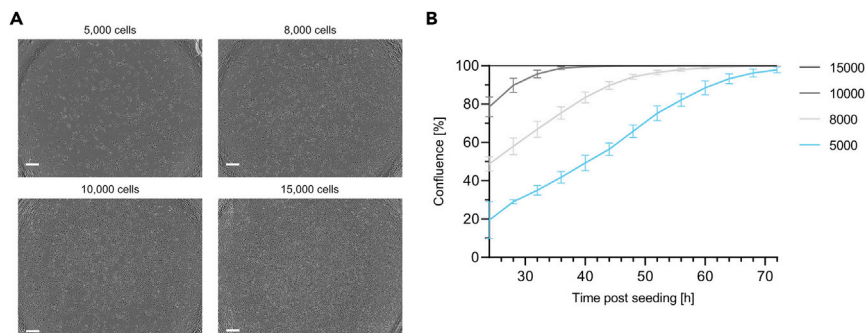


Figure 1. Target cell line titration in Incucyte S3 instrument

(A) Phase contrast images of competent A549 cell line titration from 5,000 to 15,000 target cells. Images were taken 24 h post seeding. Scale-bar: 200 μ m.

(B) The confluence [%] of the cell line titration was measured using phase contrast images (for more information refer to the “[quantification and statistical analysis](#) section”). 5,000 cells were selected as seeding amount for future applications (blue line). Data are shown as mean \pm SD of three to six technical replicates. Related to step 14) of ‘[titration of competent A549 cells for Incucyte experiment](#)’.

Titration of competent A549-Nsp2 cells for xCELLigence experiments

⌚ Timing: 2 h (hands on) + 3 days (additional cell culture)

18. Seed from 5,000 to 20,000 target cells in 100 μ L complete RPMI per well of an E-Plate VIEW. The outermost wells should only contain PBS to prevent evaporation.
19. Leave the plate under a sterile hood for 30 min to allow cells to settle down.
20. Place the E-Plate VIEW in the Agilent xCELLigence eSight instrument (37°C, 5% CO₂).
21. Choose the intensity of fluorescence channels e.g., red: 500 ms, green: 1,200 ms.
22. Setup the experiment so that impedance is measured every 30 min and real-time images are captured every 60 min for 72 h.
23. Select seeding cell concentration with cellular impedance between 1 and 3 after 24 h. For instance, 24 h post seeding, 10,000 competent A549-Nsp2 cells have an impedance value of 2.6 (Figure 2B).

Note: When using the xCELLigence eSight instrument, image acquisition can be additionally used to visualize cell growth with the brightfield channel (Figure 2A), and to calculate the confluence [%] (Figure 2C) (see [quantification and statistical analysis](#)).

Generation of SARS-CoV-2-GFP virus

Production and quantification of GFP-expressing SARS-CoV-2 virus

⌚ Timing: 4 h (hands on) + 4 days (additional cell culture) for step 24

⌚ Timing: 3 h (hands on) + 5 days (additional cell culture) for step 25

Performed as previously described in (Stukalov et al., 2021) and (Thi Nhu Thao et al., 2020).

24. Propagation of recombinant SARS-CoV-2-GFP strain.
 - a. Seed 2×10^7 Vero E6 cells in a T175 cm³ flask and complete DMEM medium one day prior virus production.
 - b. The SARS-CoV-2-GFP virus strain (kind gift of Prof. Volker Thiel, Institute of Virology and Immunology, Bern, Switzerland) was produced by infecting Vero E6 cells for 2 days with previously obtained virus stock (MOI of 0.01).

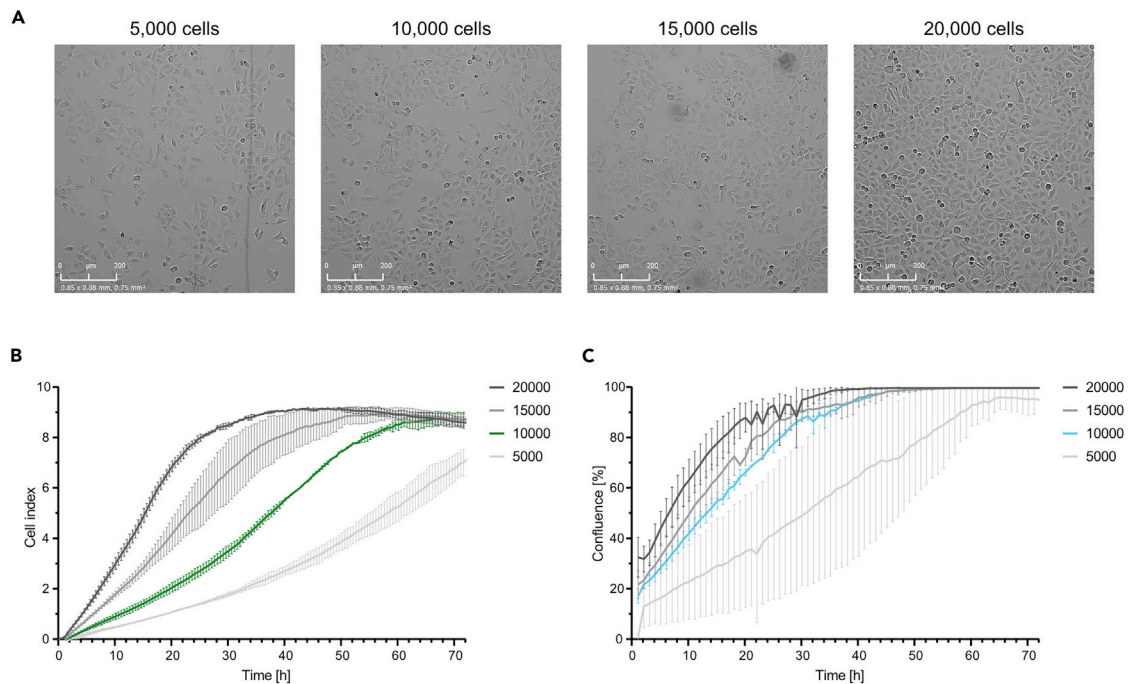


Figure 2. Target cell line titration in xCELLigence eSight instrument

(A) Brightfield images of competent A549-Nsp2 cell line titration from 5,000 to 20,000 target cells. Images were taken 24 h post seeding. Scale bar: 200 μm.

(B) Cellular impedance measurements (cell index) of competent A549-Nsp2 cells. 10,000 cells were selected as seeding amount for future applications (green line).

(C) The confluence [%] of the cell line titration was measured using brightfield images (for more information refer to the “[quantification and statistical analysis](#)” section). Data are shown as mean ± SD of two technical replicates. Related to step 23) of ‘[titration of competent A549-Nsp2 cells for xCELLigence experiments](#)’.

- c. In order to clear viral stock from cell debris, viral supernatant was collected and spun twice (10 min at 1,000 × g at 20°C). 1 mL aliquots were prepared and stored at –80°C.
25. Titration of viral stocks by plaque assay.
 - a. Seed 2×10^4 Vero E6 cells in a 96-WP one day prior plaque assay.
 - b. Infect confluent monolayers of Vero E6 cells with serial five-fold dilutions (recommended dilution factors should range from 1:20 to 1:1.56 × 10⁶) of virus supernatants for 1 h at 37°C.
 - c. Remove the inoculum and replace with serum-free MEM medium containing 0.5% carboxymethylcellulose.
 - d. Two days after infection, fix cells for 20 min at 20°C–22°C with formaldehyde added directly to the medium to a final concentration of 5%.
 - e. Wash fixed cells twice with PBS before staining with water containing 1% crystal violet and 10% ethanol for 20 min.
 - f. Rinse fixed cells twice with PBS.
 - g. Count the number of plaques at a dilution step for which you can easily make out individual plaques (pfu/mL, plaques forming unit per mL) and calculate the virus titer.
 - h. Calculate the virus titer as following:

$$\frac{PFU}{mL} = PFU * dilution\ factor$$

Note: Usually, you can expect the lowest dilutions to contain 100% of dead cells (not countable) while the highest dilutions won’t have plaques.

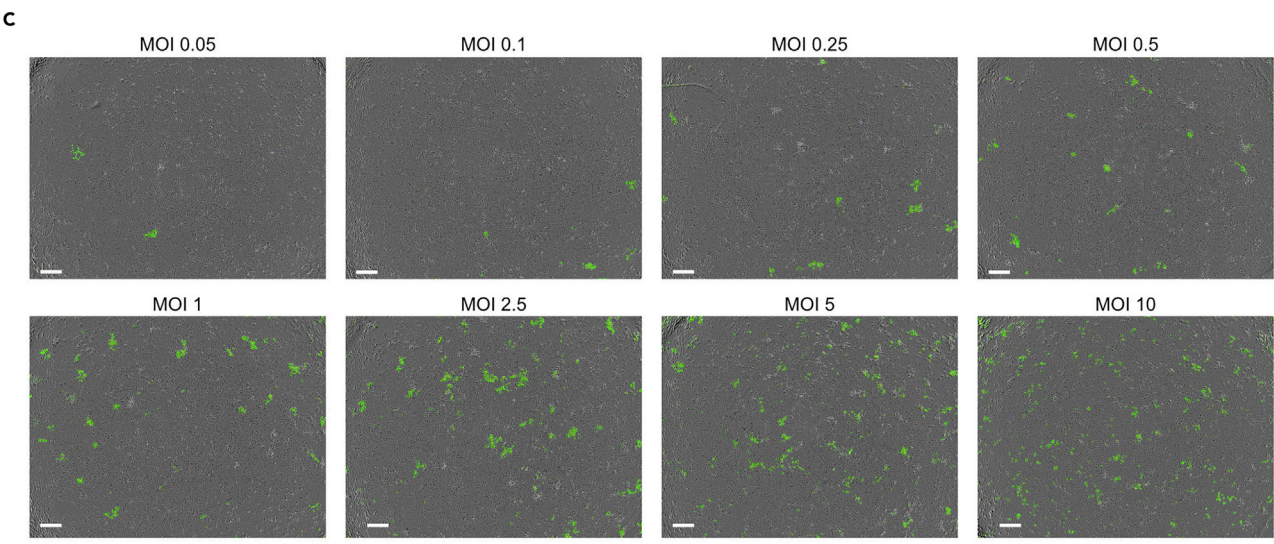
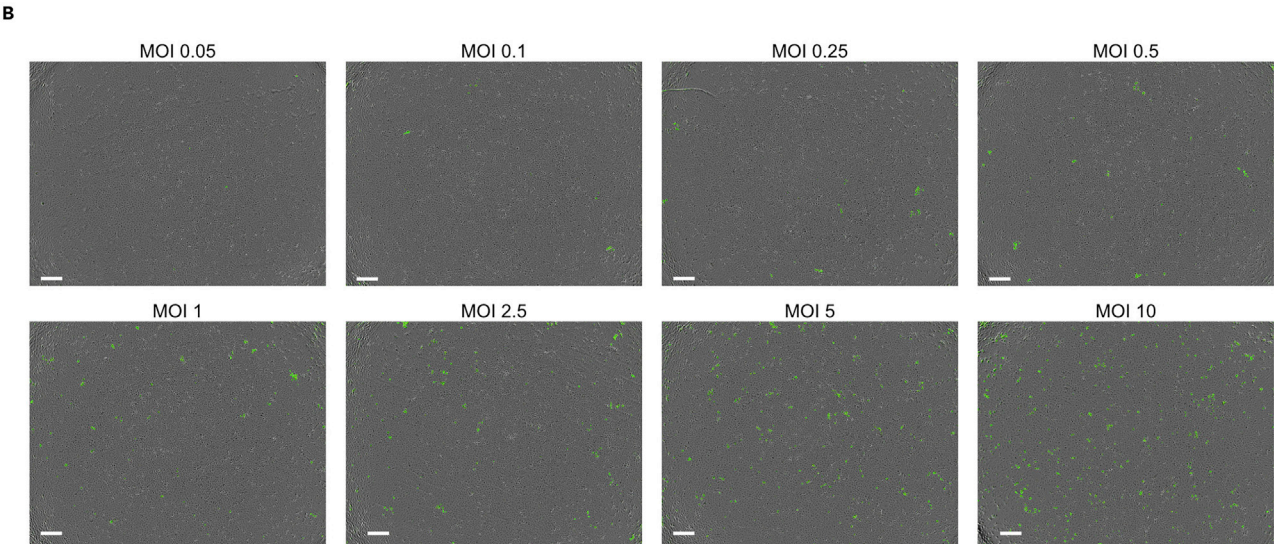
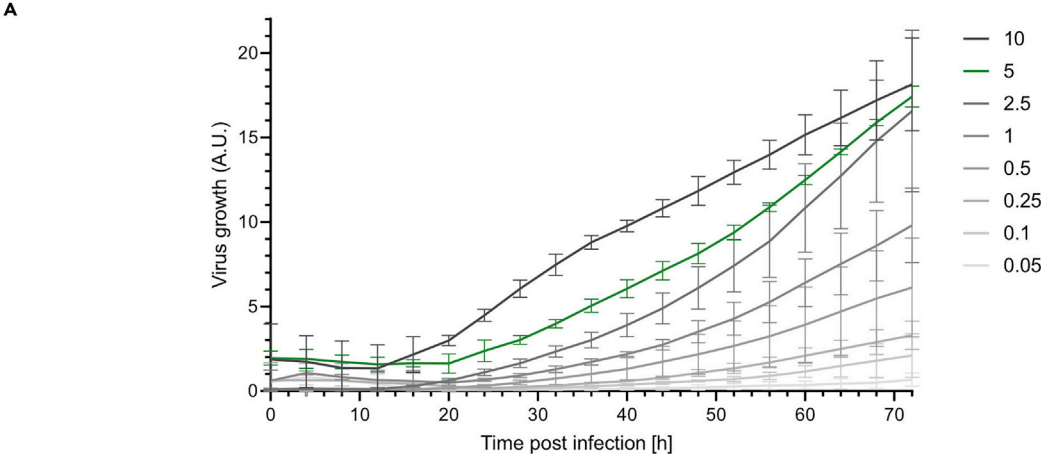


Figure 3. Determination of optimal SARS-CoV-2-GFP MOI

(A) 5×10^3 competent A549 target cells were seeded per well into 96-WP. 24 h post seeding, SARS-CoV-2-GFP virus was added from MOI 0.05 to MOI 10. Virus growth is monitored continuously every 4 h for 72 h. MOI 5 was chosen as virus concentration for future applications (green line). Data are shown as mean \pm SD of three technical replicates.

(B and C) Phase contrast and GFP images 48 h (B) and 72 h (C) post viral infection. Scale-bar: 200 μ m. Related to step 29) of 'selection of the appropriate MOI for Incucyte measurement'.

Selection of the appropriate MOI for Incucyte measurement

⌚ **Timing: 1 h (hands on) + 4 days (additional cell culture)**

26. Seed 5×10^3 competent A549 cells cultured in complete DMEM medium in a 96-WP one day prior infection.
27. Infect cells with SARS-CoV-2-GFP virus at multiple MOIs in order to reach either saturation or limit of GFP detection such as MOI 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05.
28. Monitor cell confluence and GFP signal every 4 h for 3 days by using the Incucyte S3 Live-Cell Analysis System (Incucyte S3).
29. Select a MOI that gives the highest GFP signal 72 h post-infection without inducing excessive stress on the target cells (Figure 3).

Note: Infection of competent A549 cells with MOI 10 showed the highest GFP signal 72 h post-infection. However, one should consider not adding too much of the viral stock supernatant to the recipient cells to avoid cell stress and dampen cell growth. This is why a MOI of 5 was chosen for the experiments described below.

⚠ CRITICAL: SARS-CoV-2 is a human respiratory virus that is contagious in humans. All described steps in this protocol containing SARS-CoV-2-GFP virus were approved by the required governmental administration and performed with appropriate safety measures under BSL3 conditions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD19-ECD (J3-119) (1:100)	Beckman Coulter	Cat#: A07770
CD3-BV421 (SFK7) (1:100)	BD Biosciences	Cat#: 56797
CD8-PE (3B5) (1:600)	Life Technologies	Cat#: MHCD0804
HLA-A3-APC (REA950) (1:200)	Miltenyi Biotec	Cat#: 130115-795
Human TCR α/β -FITC (IP26) (1:50)	BioLegend	Cat#: 306706
mTRBC-APC/Fire750 (H57-597) (1:50)	BioLegend	Cat#: 109246
Propidium iodide (PI) (1:100)	Thermo Fisher Scientific	Cat#: P1304MP
Bacterial and virus strains		
SARS-CoV-2 GFP	In house production	N/A
Biological samples		
Human blood/PBMCs	Healthy volunteers	Institut for Medical Microbiology, Immunology and Hygiene, Technical University Munich
Chemicals, peptides, and recombinant proteins		
Agencourt CleanSEQ magnetic beads	Beckman Coulter	Cat#: A63881
Alt-R® Cas9 Electroporation Enhancer	Integrated DNA Technologies	Cat#: 1075916
Alt-R® S.p. HiFi Cas9 Nuclease V3	Integrated DNA Technologies	Cat#: 1081061
Ampicillin	Roth	Cat#: K029.1

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biotin	IBA Lifesciences	Cat#: 6-6996-001
Carboxymethylcellulose	Sigma-Aldrich	Cat#: 21904
Corning® Costar® not treated 24 well plates	Sigma-Aldrich	Cat#: CLS3738-100EA
Crystal violet	Sigma-Aldrich	Cat#: C3886
Dulbecco's modified Eagle's medium (DMEM)	Life Technologies	Cat#: 10938025
Dulbecco's phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat#: D8537-500ML
DMSO	Merck	Cat#: D8418
DNA LoBind tubes	Sigma-Aldrich	Cat#: EP0030108051, EP0030108078, EP0030124359
Fetal calf serum	Biochrom	N/A
Formaldehyde 35% Technical	VWR	Cat#: VWRC90240.5000
Gentamicin	Life Technologies	Cat#: 15750-037
HEPES	Life Technologies	Cat#: 15630056
Herculase II fusion DNA polymerase	Agilent Technologies	Cat#: 600679
Human serum	In house	N/A
L-Glutamine	Sigma-Aldrich	Cat#: G8540-100G
Nuclease-free duplex buffer	Integrated DNA Technologies	Cat#: 11-01-03-01
Opti-MEM	Gibco	Cat#: 31985-062
PANCOLL human (Ficoll) (1.077 g/mL)	PAN Biotech	Cat#: P04-601000
Penicillin-streptomycin (10,000 U/mL)	Life Technologies	Cat#: 10378016
PEI transfection reagents	Polysciences	Cat#: 24765
Propidium iodide (PI)	Life Technologies	Cat#: P1304MP
Recombinant human IL-15	PeptoTech	Cat#: 200-15
Recombinant human IL-2	PeptoTech	Cat#: 200-02
Recombinant human IL-7	PeptoTech	Cat#: 200-07
RetroNectin (1 µg/µL)	TAKARA	Cat#: T100B
Round-bottom polystyrene test tubes	Thermo Fisher Scientific	Cat#: 352054
RPMI 1640 Gibco	Sigma-Aldrich	Cat#: R0883
Serum-free MEM medium	Gibco	Cat#: 31095-029
TipOne RPT filter pipette tips	Starlab	Cat#: S1181-3810, S1180-8710, S1182-1730
Trypsin 0.25%	Sigma-Aldrich	Cat#: T4049-100ML
β-mercaptoethanol (50 mM)	Life Technologies	Cat#: 31350010
Critical commercial assays		
P3 Primary Cell 4D-Nucleofector™ X Kit L	Lonza	Cat#: V4XP-3024
Experimental models: Cell lines		
A549 cells	from Georg Kochs (Universitätsklinikum, Freiburg)	N/A
A549-ACE2-RFP-HLA-A*03:01	In house production	N/A
A549-ACE2-RFP-HLA-A*03:01-Nsp2-GFP	In house production	N/A
A549-ACE2-RFP-HLA-A*03:01-Gaussia-GFP	In house production	N/A
HEK293T cells	from Georg Kochs (Universitätsklinikum, Freiburg)	N/A
HeLa S3 cells	ATCC	Cat#: CCL-2.2
RD114	In house	N/A
Vero E6 cells	ATCC	Cat#: CRL-1586
Oligonucleotides		
Alt-R® CRISPR-Cas9 crRNA 5'-AGAGTCTCTCAGCTGGTACA-3' for TRAC	Integrated DNA Technologies	N/A
Alt-R® CRISPR-Cas9 crRNA 5'-GGAGAATGACGAGTGGACCC-3' for TRBC	Integrated DNA Technologies	N/A
Alt-R® CRISPR-Cas9 tracrRNA	Integrated DNA Technologies	Cat#: 1072532
HDR template PCR primer fwd 5'-CTGCCTTACTCTGCCAGAG-3'	Merck	N/A
HDR template PCR primer rev 5'-CATCATTGACCAGAGCTCTG-3'	Merck	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
MP72 vector for retrovirus generation in RD114 cells	In house	N/A
psPAX2 packaging vector for lentivirus generation	In house	N/A
pMD2. VSV-G envelope expressing plasmid for lentivirus production	In house	N/A
Lentivirus pCI H2B-RFP vector backbone: pCI Gene/Insert name: IRES H2B RFP	Addgene	Cat#: 92398
Lentivirus pWPI ACE2-HA vector backbone: pWPI Gene/Insert name: human ACE2-HA	In house	N/A
Retrovirus pMXs HLA-A*03 Vector backbone: pMXs Gene/Insert name: HLA-A*03	In house	N/A
Software and algorithms		
Affinity Designer 1.9	Serif	https://affinity.serif.com/
FlowJo V10	FlowJo LLC	https://www.flowjo.com
GraphPad Prism 9	GraphPad	https://www.graphpad.com
Incucyte® S3 Software	Sartorius AG	https://www.sartorius.com
Microsoft Excel	Microsoft	N/A
RTCA xCELLigence Software	Agilent	https://www.agilent.com
Other		
4D-Nucleofector	Lonza	Cat#: AAF-1002B, AAF-1002X
CytoFlex S Cell Analyzer	Beckman Coulter	Cat#: B75408
E-Plate VIEW 96	Agilent	Cat#: 300601010
Incucyte® S3 Live-Cell Analysis System	Sartorius	Cat#: 4647
MoFlo Astrios EQ	Beckman Coulter	Cat#: B25982
SimpliAmp Thermal Cycler	Applied Biosystems, Darmstadt, Germany	Cat#: A24811
xCELLigence® RTCA eSight	Agilent	Cat#: 380601600

MATERIALS AND EQUIPMENT

Fixation and staining solution for lentivirus titration

Reagent	Final concentration	Amount (for 100 mL stock)
Crystal violet	1.25%	1.25 g
Ethanol solution	25%	25 mL
PCR grade water	n/a	75 mL
Total	n/a	100 mL

Store at 20°C–22°C for several months.

PCR master mix

Reagent	Final concentration	Amount (for 100 µL reaction)
PCR grade water	n/a	65 µL
5× Herculase II Reaction Buffer	1×	20 µL
hTRAC HDR genomic forward primer (10 mM)	0.4 mM	4.0 µL
hTRAC HDR genomic reverse primer (10 mM)	0.4 mM	4.0 µL
dNTPs (10 mM)	0.5 mM	5.0 µL
Herculase II Fusion DNA Polymerase	1.0%	1.0 µL
DNA (prediluted to 60 ng/µL)	600 ng/mL	1.0 µL
Total	n/a	100 µL

PCR master mix is freshly prepared on ice for direct use.

PI stock solution

Reagent	Final concentration	Amount
Propidium Iodide (PI)	0.2 mg/mL	5 mg
dPBS	n/a	25 mL
Total	n/a	25 mL

Dissolve PI in dPBS, vortex and aliquot in dark 1 mL Eppendorf tubes as PI is light sensitive. Keep at -20°C for several months and at 4°C for several weeks.

SC+ supplement

Reagent	Final concentration	Amount
β -Mercaptoethanol (50 mM)	1 mM	0.5 mL
Gentamicin (50 mg/mL)	1 mg/mL	0.5 mL
HEPES	23.83 g/L	0.595 mL
L-Glutamine	4.0 g/L	0.1 g
Penicillin-Streptomycin (10,000 U/mL)	2,000 U/mL	5 mL
RPMI 1640 Medium	n/a	19 mL
Total	n/a	25 mL

Filter through 0.22 μm PVDF filter and store at -20°C for several months.

SC- supplement

Reagent	Final concentration	Amount
β -Mercaptoethanol (50 mM)	1 mM	2.4 mL
HEPES	23.83 g/L	2.86 g
L-Glutamine	4.0 g/L	0.48 g
RPMI 1640 Medium	n/a	116.8 mL
Total	n/a	120 mL

Store at -20°C for several months.

Antibiotic mix (as supplemented for SC- medium)

Reagent	Final concentration	Amount
Gentamicin (50 mg/mL)	1 mg/mL	0.4 mL
Penicillin-Streptomycin (10,000 U/mL)	2,000 U/mL	4.0 mL
RPMI 1640 Medium	n/a	15.6 mL
Total	n/a	20 mL

Store at -20°C for several months.

Complete RPMI medium or complete DMEM

Reagent	Final concentration	Amount
RPMI 1640 Medium or DMEM medium	n/a	500 mL
Heat-inactivated Fetal Bovine Serum (FBS)	10%	50 mL
SC+ supplement	5%	25 mL
Total	n/a	575 mL

Store at 4°C for several weeks.

Complete RPMI-HS medium

Reagent	Final concentration	Amount
RPMI 1640 Medium	n/a	45 mL
Human serum	5%	2.5 mL
SC+ supplement	5%	2.5 mL
Total	n/a	50 mL

Store at 4°C for several weeks. As usually only smaller amounts of complete RPMI-HS medium are needed, it is also possible to make RPMI SC+ medium and to add 5% HS as needed.

FACS buffer

Reagent	Final concentration	Amount
PBS buffer, pH 7.4	n/a	500 mL
BSA	0.5%	2.5 g
Total	n/a	500 mL

Store at 4°C for 2–4 weeks.

Transfection buffer

Reagent	Final concentration	Amount
NaCl	274 mM	1.6 g
KCl	9.9 mM	0.074 g
Na ₂ HPO ₄	3.5 mM	0.050 g
HEPES	41.9 mM	1.0 g
PCR grade water		100 mL
Total	n/a	100 mL

- Dissolve reagents step by step in 80 mL H₂O.
- Stir for a couple of hours until all reagents are dissolved.
- Adjust pH to 6.76 with HCl and NaOH (0.5 N NaOH, about 1–2 mL) at 20°C–22°C.
- Fill up to 100 mL with PCR grade water.
- Sterile filter through 0.22 μm PVDF filter, aliquot and store at –20°C.

STEP-BY-STEP METHOD DETAILS

Generation of OTR-engineered SARS-CoV-2-specific CD8⁺ T cells

In this step, CRISPR-Cas9-mediated homology-directed repair is used to introduce a transgenic TCR orthotopically in the TCR endogenous locus of primary human T cells. This engineering technology is called ‘orthotopic TCR replacement’ (OTR). For a detailed protocol description please refer to (Moosmann et al., 2022). In this protocol, we mainly summarized the different steps to provide an overview of the whole procedure.

Preparation

⌚ Timing: ~ 1 week for step 1

⌚ Timing: 30 – 60 min for step 2

⌚ Timing: 1 day for step 3

1. DNA template design.
 - a. Design DNA template as described in (Moosmann et al., 2022).

- b. HDR constructs for this protocol were designed containing the murine constant region for both T cell receptor alpha and beta chains to simplify sorting of OTR-engineered cells.
2. Reconstitute reagents.
 - a. Reconstitute tracrRNA, hTRAC crRNA and hTRBC crRNA to 80 μ M.
 - b. Reconstitute the electroporation enhancer to 400 μ M in nuclease-free duplex buffer.
 - c. Reconstitute IL-2 to 10 μ g/mL, and IL-7 and IL-15 to 25 μ g/mL.
 - d. Aliquot biotin to 100 μ M stocks.
 - e. Aliquot and store all reagents at -20°C .
3. Double-stranded DNA production.
 - a. Make PCR master mix for amplification of HDR templates (see [materials and equipment](#)).
 - b. Run PCR according to the following protocol:

PCR cycling conditions			
Steps	Temperature	Time	Cycle
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 s	34 cycles
Annealing	62°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	3 min	1
Hold	4°C	unlimited	

- c. Run 2 μ L per HDR PCR reaction on a 1% agarose gel to control for successful amplification.
- d. Perform DNA purification using the MinElute PCR Purification Kit and store purified HDR template at 1 μ g/ μ L at -20°C .

Note: Usually, the HDR template was generated in eight tubes of a PCR-strip tubes (8 \times 100 μ L). For PCR purification, all eight PCR reactions were pooled. A very good yield would be 50–70 μ g, a good yield 30–50 μ g.

Methods

- ⌚ Timing: 3 h for step 4
 - ⌚ Timing: 30 min (hands-on) + 2 days (additional cell culture) for step 5
 - ⌚ Timing: 30 min for step 6
 - ⌚ Timing: 60 – 90 min for step 7
 - ⌚ Timing: 5 min for step 8
 - ⌚ Timing: 30 – 60 min (hands-on) + 12 – 16 h (additional cell culture) for step 9
 - ⌚ Timing: 10 min (hands-on) + 3 days (additional cell culture) for step 10
 - ⌚ Timing: 2 h for step 11
 - ⌚ Timing: 4 h (hands-on) + 14–21 days (additional cell culture) for step 12
4. Isolation of peripheral blood mononuclear cells (PBMCs) from fresh blood by Ficoll gradient.

- a. Dilute fresh blood 1:0.4 with PBS (e.g., mix 25 mL of blood with 10 mL PBS).
- b. Add 15 mL of Ficoll in 50 mL tubes.
- c. Carefully layer blood:PBS dilution on top of Ficoll.

Note: Tilt tube at a 45 degree angle and add blood:PBS mixture dropwise on top. Do not add the blood mixture directly on Ficoll but on the side of the tube.

- d. Centrifuge for 15 min at $1,000 \times g$ at 20°C – 22°C with deceleration break turned off.
 - e. Take off the plasma up to ~ 0.5 cm over the PBMC layer.
 - f. Isolate the PBMC layer in 50 mL tube, fill up with PBS to 50 mL and centrifuge for 10 min at $480 \times g$ at 20°C – 22°C .
 - g. Wash isolated PBMCs two more times with 50 mL PBS.
 - h. Count cells with hemocytometer.
5. T cell activation.
- a. Seed cells at a density of 1×10^6 cells/mL in complete RPMI medium supplemented with 300 U/mL IL-2, 5 ng/mL IL-7, 5 ng/mL IL-15 and $1.5 \mu\text{L}/10^6$ cells CD3/CD28 expamer (Poltorak et al., 2020).
 - b. Incubate at 37°C , 5% CO_2 for two days.

Note: Titrate CD3/CD28 expamer before experiments to find the best concentration for editing.

Alternatives: Anti-CD3 and anti-CD28 antibodies can be used alternatively for plate coating at a final concentration of $1 \mu\text{g}/\text{mL}$ in dPBS as described in (Moosmann et al., 2022).

6. Preparation of electroporation.
- a. Take off PBMCs and centrifuge for 10 min at $480 \times g$ at 20°C – 22°C .
 - b. Resuspend PBMCs in 1 mM biotin for 20 min at 20°C – 22°C (50 mL cell culture volume can be resuspended in 25 mL of 1 mM biotin solution).
 - c. After incubation, fill up the tube to 50 mL with PBS and centrifuge for 10 min at $480 \times g$ at 20°C – 22°C .
 - d. Perform one more wash step with 30 mL PBS and centrifuge for 10 min at $480 \times g$ at 20°C – 22°C .
 - e. Perform one final wash step with 50 mL complete RPMI medium.
 - f. Count PBMCs.
 - g. Split total cell number for different experimental conditions and divide appropriate volumes in 15 mL tubes. Place cells in the incubator (37°C , 5% CO_2) until electroporation.

Note: The number of input cells added per 100 μL Nucleocuvette™ Vessels has an impact on the editing efficiency. Depending on the HDR construct and cell concentration during electroporation, the knock-in rate can range from 1%–10%.

7. RNP production.
- a. Mix equal volumes of tracrRNA with hTRAC or hTRBC crRNA (80 μM) respectively to prepare 40 μM hTRAC or hTRBC gRNA. Mix well by pipetting.
 - b. Heat gRNA for 5 min at 95°C . Allow gRNA to cool to 20°C – 22°C . Then store on ice if not used immediately.
 - c. Add 20 μM electroporation enhancer to gRNA.
 - d. Dilute Cas9 nuclease (stock: 61 μM) to 6 μM with commercial PBS. Store on ice if not used immediately.
 - e. Mix very slowly equal volumes of 6 μM Cas9 protein and 40 μM gRNA (= tracrRNA + crRNA). Move pipette tip in cycles while adding Cas9, always add Cas9 protein to gRNA solution, not the other way around, final ribonucleoprotein (RNP) concentration: 20 μM .

- f. Incubate RNP complexes for 15 min at 20°C–22°C.
8. Prepare P3 electroporation buffer (delivered with nucleofector kit) according to manufacturer's protocol (Lonza).
9. Electroporation.

Volumes in this protocol are adjusted for 10×10^6 cells.

- a. Perform electroporation in 100 μ L Nucleocuvette™ Vessels.
- b. Mix together: 10 μ L of TRAC RNP, 10 μ L of TRBC RNP and 10 μ L of HDR template (10 μ g of a 1 μ g/ μ L HDR stock solution).
- c. Incubate assembled RNP solution for at least 30 s at 20°C–22°C.
- d. Centrifuge cells for 10 min at $480 \times g$ at 20°C–22°C and resuspend in 60 μ L P3 buffer, assuming a cell pellet volume of 10 μ L.
- e. Add PBMCs resuspended in P3 buffer to assembled RNP solution and mix to reach a final volume of 100 μ L.
- f. Transfer cells to 100 μ L Nucleocuvette™ Vessels and electroporate (program: EH100).
- g. Transfer cells to pre-warmed flasks containing the appropriate volume of RPMI-SC- medium (RPMI medium, 10% FCS, SC- supplement) (see [materials and equipment](#)) to reach a concentration of 1×10^6 cells/mL.

Note: To reduce the time cells spend in P3 buffer, perform electroporation only with two samples at a time.

10. Addition of antibiotics.
 - a. 12–16 h after electroporation, supplement RPMI-SC- medium with 5% antibiotic mix (see [materials and equipment](#)).
 - b. Incubate cells for 3 days at 37°C, 5% CO₂.
11. Validation of transgenic TCR replacement by flow cytometry analysis.
 - a. Take a small aliquot (~ 100 μ L) from the cell culture and perform antibody staining to determine endogenous TCR knockout (KO) and transgenic TCR knockin (KI).
 - b. Make antibody mix containing CD3-BV421 (1:100), CD8-PE (1:600), hTCR-FITC (1:50), mTRBC-APC/Fire750 (1:50) in FACS buffer and incubate for 20 min on ice in the dark.
 - c. Add PI (1:100) and incubate for 1 additional min on ice in the dark.
 - d. Wash 2 \times with FACS buffer and filter through 112 μ m polyamid sieve cloth.
 - e. Acquire a minimum of 10,000 events at CytoFlex S Cell Analyzer.
 - f. Analyze data using FlowJo V10.
12. FACS and feeder cell-based T cell expansion.
 - a. On ~day 5 post CRISPR editing, perform antibody staining for the transgenic TCR and sort SARS-CoV-2-specific TCR engineered CD8⁺ T cells in 1 mL FCS in 15 mL tubes.
 - b. Fill up the tube with complete RPMI-HS medium (5% HS, 5% SC+) (see [materials and equipment](#)), centrifuge for 10 min at $480 \times g$ at 20°C–22°C and take off supernatant.
 - c. Resuspend cells in complete RPMI-HS medium + 1 μ g/mL PHA + 180 IU/mL IL-2 to reach a final concentration of 1×10^6 cells/mL.

Note: If less than 200,000 cells are sorted, fill up to 200,000 cells with feeder cells. For this, use irradiated feeder cells (35 Gy–20 min) from an allogeneic donor. Expansion of low numbers of CD8⁺ T cells (< 200,000) with feeder cells has been shown to be very efficient. You can expect between 5×10^6 and 30×10^6 cells after three weeks of culture.

- d. After 7 days, remove 100 μ L of medium and add fresh complete HS-RPMI medium + 1 μ g/mL PHA + 180 IU IL-2/mL (final concentration).
- e. Add fresh medium (supplemented with cytokines) as soon as medium turns yellow (every 1–3 days). An exemplary time schedule for the feeder cell expansion protocol of sorted primary CD8⁺ T cells can be found in [Table 1](#).

Table 1. Feeder cell expansion protocol for sorted primary CD8⁺ T cells

Time	Observation	Cell culture	Total volume
Day 0	/	Take up cells in 200 μ L complete medium + 1 \times cytokines.	200 μ L
Day 7	Medium color is yellow (visible proliferation clusters).	Carefully remove 100 μ L of medium and add complete RPMI medium + 2 \times cytokines.	200 μ L
Day 8	Medium color is yellow.	Move cells from 96-WP to 48-WP and add 200 μ L complete RPMI medium + 1 \times cytokines	400 μ L
Day 10	Medium color is yellow.	Move cells from 48-WP to 24-WP and add 400 μ L complete RPMI medium + 2 \times cytokines	800 μ L
Day 13	Medium color is yellow.	Move cells from 24-WP to 12-WP and add 1,200 μ L complete RPMI medium + 2 \times cytokines	2,000 μ L
Day 15	Medium color is yellow.	Move cells from 12-WP to 6-WP and add 1,000 μ L complete RPMI medium + 3 \times IL-2 (no PHA at this step)	3,000 μ L

Note: When adding new medium less than 2 days apart, e.g., day 7 to day 8, only add cytokines for the added medium to avoid overstimulation.

- f. On day 15 of expansion (see Table 1), add 1 mL of complete RPMI medium + 3 \times IL-2 to 2 mL of culture medium and cells in a 6-WP. Addition of PHA from this point onward is not necessary anymore. Leave cells for \sim 7 days without PHA addition (+ addition of IL-2 every 2–3 days) before performing experiments.

Note: Usually the medium will turn yellow for every split as cells now expand exponentially. You can double the volume and move to the respective bigger plate at every step. Only transfer cells to a bigger plate when the medium is yellow and proliferation clusters are visible or when cells cover the entire well. Cell proliferation can vary from donor to donor. If cells do not show metabolic activity on day seven, add another 100,000 irradiated feeder cells from the same donor.

Alternatives: If enough CD8⁺ T cells have already been sorted for planned experiments, there is no need for further expansion.

Replicating virus killing assay using the Incucyte instrument

This protocol describes a near-physiological SARS-CoV-2 killing assay in which competent A549 target cells are infected with SARS-CoV-2-GFP virus and co-cultured with SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells. A schematic of the workflow can be found in Figure 4.

Seeding of competent A549 cells

⌚ Timing: 30 min - 1 h (hands on) + 24 h (additional cell culture)

13. Seed 5×10^3 competent A549 cells in complete RPMI medium in 100 μ L per well of a 96-WP. The outermost wells should only contain PBS to prevent evaporation.

Note: Use \sim 70% confluent competent A549 cells. A549 cells are normally cultivated in complete DMEM medium. Switch medium to complete RPMI medium two passages before the killing assay as T cells, which are cultured in complete RPMI medium, are more sensitive to culture conditions than tumor cells.

Infection of competent A549 cells with SARS-CoV-2-GFP virus

⌚ Timing: 30 min - 1 h (hands on) + 24 h (additional cell culture)

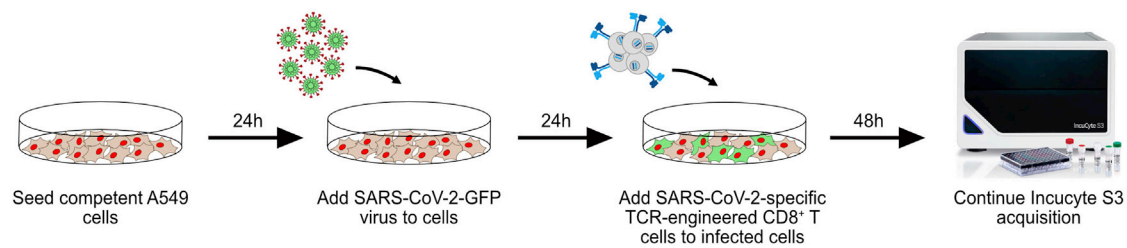


Figure 4. Workflow of Incucyte killing assay

Competent A549 cells are seeded in 96-WP. After 24 h, SARS-CoV-2-GFP virus is added to the cells and infected plates are monitored by the Incucyte S3 live-cell imaging platform. 24 h post infection, SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells are added and Incucyte S3 acquisition is continued for additional 48 h. Related to steps 13) - 23) of ‘[replicating virus killing assay using the Incucyte instrument](#)’.

14. 24 h post seeding, add SARS-CoV-2-GFP virus (MOI 5) in 100 μ L of complete RPMI medium.
15. Place the plate in the Incucyte S3 instrument (37°C, 5% CO₂) where real-time images of mock (RFP channel) and infected (GFP and RFP channel) cells are captured every 3 h for 72 h.

Note: The right controls for this experiment are target cells alone and target cells on which only TCR-engineered T cells are added without virus infection.

Pipet effector to target (E:T) dilutions with SARS-CoV-2-specific T cells

⌚ Timing: 1–2 h

16. Resuspend TCR-engineered T cells at a concentration of 3×10^6 cells/mL in complete RPMI medium + 100 IU/mL IL-2 (2 \times the final working concentration of 50 IU/mL).
17. Prepare and pipet E:T dilution series by always diluting the respective higher dilution in complete RPMI medium + 100 IU/mL IL-2. Perform all E:T titration steps in triplicates. Examples are shown in [Tables 2](#) and [3](#).

Addition of SARS-CoV-2-specific T cells

⌚ Timing: 1–2 h

18. 24 h post-infection, take the plate out of the Incucyte S3 instrument.
19. Remove 100 μ L of medium with a multichannel pipette.
20. Add 100 μ L of respective E:T SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells.
21. Place the plate back in the Incucyte S3 instrument and continue acquisition for additional 48 h.
22. At the endpoint (72 h post infection), remove supernatant and T cells by washing wells once with PBS.
23. Add fresh medium before final picture acquisition.

Table 2. Target and effector cell numbers for replicating SARS-CoV-2 virus killing assay

E:T ratio	Competent A549 cells [total cell count]	SARS-CoV-2-specific CD8 ⁺ T cells [total cell count]	SARS-CoV-2-specific CD8 ⁺ T cells [cells/mL]
30:1	5×10^3	1.5×10^5	1.5×10^6
10:1	5×10^3	5×10^4	5×10^5
3:1	5×10^3	1.5×10^4	1.5×10^5
1:1	5×10^3	5×10^3	5×10^4

Suggested range of titration steps for competent A549 target cells and SARS-CoV-2-specific CD8⁺ T effector cells from [E:T] 30:1 to 1:1.

Table 3. Effector T cell pipetting scheme for replicating SARS-CoV-2 virus killing assay

E:T ratio	Final cell number added	Dilution factor (from respective previous dilution)	Volume to take from previous cell dilution [μ L]	Add medium [μ L]	Total volume [μ L]
30:1	1.5×10^5			0	600
10:1	5×10^4	3	200	400	600
3:1	1.5×10^4	3,3333	180	420	600
1:1	5×10^3	3	200	400	600

Exemplary pipetting steps for serial dilution starting at 1.5×10^6 cells for [E:T] 30:1.

Transgenic antigen killing assay using the xCELLigence instrument

This protocol describes how competent A549 cells, transduced to express the SARS-CoV-2 protein Nsp2 linked to GFP via a T2A tag, can be used for natural presentation of the HLA-A*03:01 epitope of interest and subsequent killing assays with SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells. A schematic of the workflow can be found in [Figure 5](#).

Background measurement of xCELLigence plate

24. Perform background measurement as described in 15) –17) of section “before you begin.”

Seeding of competent A549-Nsp2 cells

⌚ Timing: 30 min - 1 h (hands on) + 24 h (additional cell culture)

25. Perform seeding of competent A549-Nsp2 cells similar to 18) – 23) of section “before you begin.”

Note: Competent A549 cells serve as non-epitope presenting control cell line for competent A549-Nsp2 cells. As an additional control condition, it is possible to use competent A549 cells that were transduced with a non-relevant protein like the Gaussia luciferase protein (competent A549-Gaussia cells).

Pipet E:T dilution series of SARS-CoV-2-specific T cells

⌚ Timing: 1–2 h

26. Resuspend SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells at a concentration of 3×10^6 /mL in complete RPMI medium + 100 IU/mL IL-2 (2× the final working concentration of 50 IU/mL).

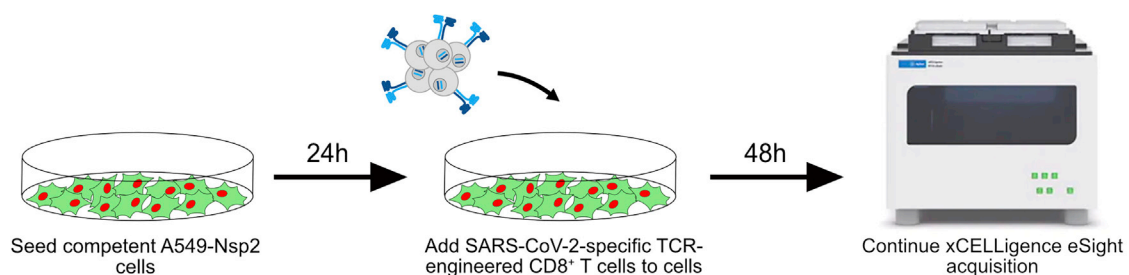


Figure 5. Workflow of xCELLigence killing assay

Competent A549-Nsp2 cells are seeded on an E-Plate VIEW 96-WP and impedance measurement as well as image acquisition is monitored by the xCELLigence eSight instrument. 24 h post seeding, SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells are added and acquisition is continued for additional 48 h. Related to steps 24) - 32) of ‘transgenic antigen killing assay using the xCELLigence instrument’.

Table 4. Target and effector cell numbers for transgenic antigen killing assay

E:T ratio	Competent A549-Nsp2 cells [total cell count]	SARS-CoV-2-specific CD8 ⁺ T cells [total cell count]	SARS-CoV-2-specific CD8 ⁺ T cells [cells/mL]
30:1	1×10^4	3×10^5	3×10^6
10:1	1×10^4	1×10^5	1×10^6
3:1	1×10^4	3×10^4	3×10^5
1:1	1×10^4	1×10^4	1×10^5

Suggested range of titration steps for competent A549-Nsp2 target cells and SARS-CoV-2-specific CD8⁺ T effector cells from [E:T] 30:1 to 1:1.

27. Prepare and pipet E:T dilution series by always diluting the respective higher dilution in complete RPMI medium + 100 IU/mL IL-2. Perform all E:T titration steps in triplicates. Examples are shown in Tables 4 and 5.

Addition of SARS-CoV-2-specific T cells

⌚ Timing: 1–2 h

28. 24 h post-seeding, 'Pause' the experiment and take the E-Plate VIEW out of the xCELLigence eSight instrument.
29. Remove 100 μ L of medium with a multichannel pipette.
30. Add 100 μ L of SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells according to the respective E:T ratio.
31. Include medium only condition as negative control and 2% Triton X as positive control for each cell line.
32. Place the E-Plate VIEW back in the xCELLigence eSight instrument and 'Start/Continue' acquisition for additional 48 h.

Note: Addition of large amounts of effector cells dampens target cell growth. To ensure that reduction in target cell growth is specific, it is recommended to include the following controls:

- a. Medium only control - As this shows the growth of target cells without dampening or unspecific cytotoxic effects following the pure addition of T cells.
- b. Non-epitope-presenting target cells – Target cells that do not present the respective epitope recognized by the TCR-engineered T cells, e.g., competent A549 cells that do not have the Nsp2 protein transduced.
- c. Non-epitope-specific effector cells - TCR-engineered T cells that are expected to not show target-specific recognition of the epitope under investigation, e.g., TCR-engineered T cells that are specific for another SARS-CoV-2 epitope or an unrelated viral or tumor-specific epitope.

Table 5. Effector T cell pipetting scheme for transgenic antigen killing assay

E:T ratio	Final cell number added	Dilution factor (from respective previous dilution)	Volume to take from previous cell dilution [μ L]	Add medium [μ L]	Total volume [μ L]
30:1	3×10^5			0	600
10:1	1×10^5	3	200	400	600
3:1	3×10^4	3,3333	180	420	600
1:1	1×10^4	3	200	400	600
0.1:1	3×10^5	10	60	540	600

Exemplary pipetting steps for serial dilution starting at 1.5×10^6 cells for [E:T] 30:1.

Alternatives: As an alternative assay, competent A549 cells can be peptide-pulsed with the epitope of interest before seeding on xCELLigence plates. For this, target cells are pulsed at a concentration of 3×10^6 /mL for 2 h with 10^{-5} M peptide at 20°C–22°C on a roller. Peptide-pulsed cells are washed 3× with full RPMI medium and respective amounts of target cells are plated on the E-Plate VIEW. As control for this assay, unpulsed competent A549 cells with the same E:T titration steps can be used. Be aware that epitope presentation is not always reflective to natural antigen processing.

EXPECTED OUTCOMES

DNA template generation by HDR PCR and purification should ideally yield 30–70 µg. Endogenous TCR KO and transgenic TCR KI efficiencies are expected to range, respectively, from 95%–99% and from 1%–10%. Sorting of transgenic TCR-expressing CD8⁺ T cells can range from 1×10^3 – 1×10^5 cells starting from $5 - 10 \times 10^6$ PBMCs, and good expansion was seen for the entire range (always with addition of feeder cells to a final starting cell number of 2×10^5). Within three weeks after sorting, $5 - 30 \times 10^6$ T cells are expected.

The replicating virus killing assay provides high sensitivity for CD8⁺ T cell-mediated antigen-specific killing as target cells and infection of target cells can be tracked with RFP and GFP expression, respectively. For a high functional TCR (S13) you can expect virus growth (GFP⁺ area) as well as survival of infected target cells [%] to be dampened in an E:T dependent manner. For a low functional TCR (S32) you can expect that virus growth and survival of infected target cells do not show a titration effect. At the same time, survival of non-infected target cells should not be affected for high or low functional TCRs (Figure 6).

Similarly, for the transgenic antigen killing assay, you can expect the cellular impedance values to rapidly shrink after addition of T cells engineered with a highly functional SARS-CoV-2-specific TCR (S13) in accordance to the E:T ratio. On the contrary, the impedance curve of a low-functional TCR (S32) should show only minor declines, most likely only for high E:T. Negative controls are expected to have a continuous growth irrespective of the target cell addition (competent A549 cells, competent A549-Gaussia cells and medium controls), unless too high E:T ratios are used. Indeed, starting from an E:T of 5:1, the pure addition of T cells may limit oxygen and nutrient availability for target cells and might induce unspecific growth inhibition. Cells at a low E:T ratio (1:1) will thus show a faster proliferation curve than cells at a high E:T ratio (10:1) (Figure 7). Thus, when comparing conditions from different E:T ratios, it is recommended to consider also the behavior of the negative controls to avoid misinterpretation and/or overestimation of cytotoxic ability. The positive control (2% Triton X) is expected to show immediate cell death.

The total integrated intensity ($RRI \times \mu\text{m}^2/\text{image}$) analysis should result in data curves that show a similar trend than the impedance curves. The RFP signal is expected to decline after addition of a high-functional TCR (S13) while the RFP signal after addition of a low-functional TCR (S32) should behave like the controls (Figure 8). Please refer to the “[quantification and statistical analysis](#)” section for detailed analysis descriptions.

Progression or decline of tumor cell growth can also be visualized in a video by combining images of the same well position over the time course. After the addition of a high-functional TCR (S13), tumor cells are expected to detach from the well surface and killing of tumor cells can be observed. On the contrary, the addition of a low-functional TCR (S32) should show no T-cell mediated killing. The co-culture of TCR-engineered T cells to a tumor cell line expressing a non-relevant target epitope (Gaussia) should not result in target cell killing. Here we compare killing after addition of S13 TCR and S32 TCR expressing CD8⁺ T cells at an E:T of 5:1 to A549-Nsp2 and A549-Gaussia cells ([supplemental information](#), [Methods videos S1](#), [S2](#), [S3](#), and [S4](#)).

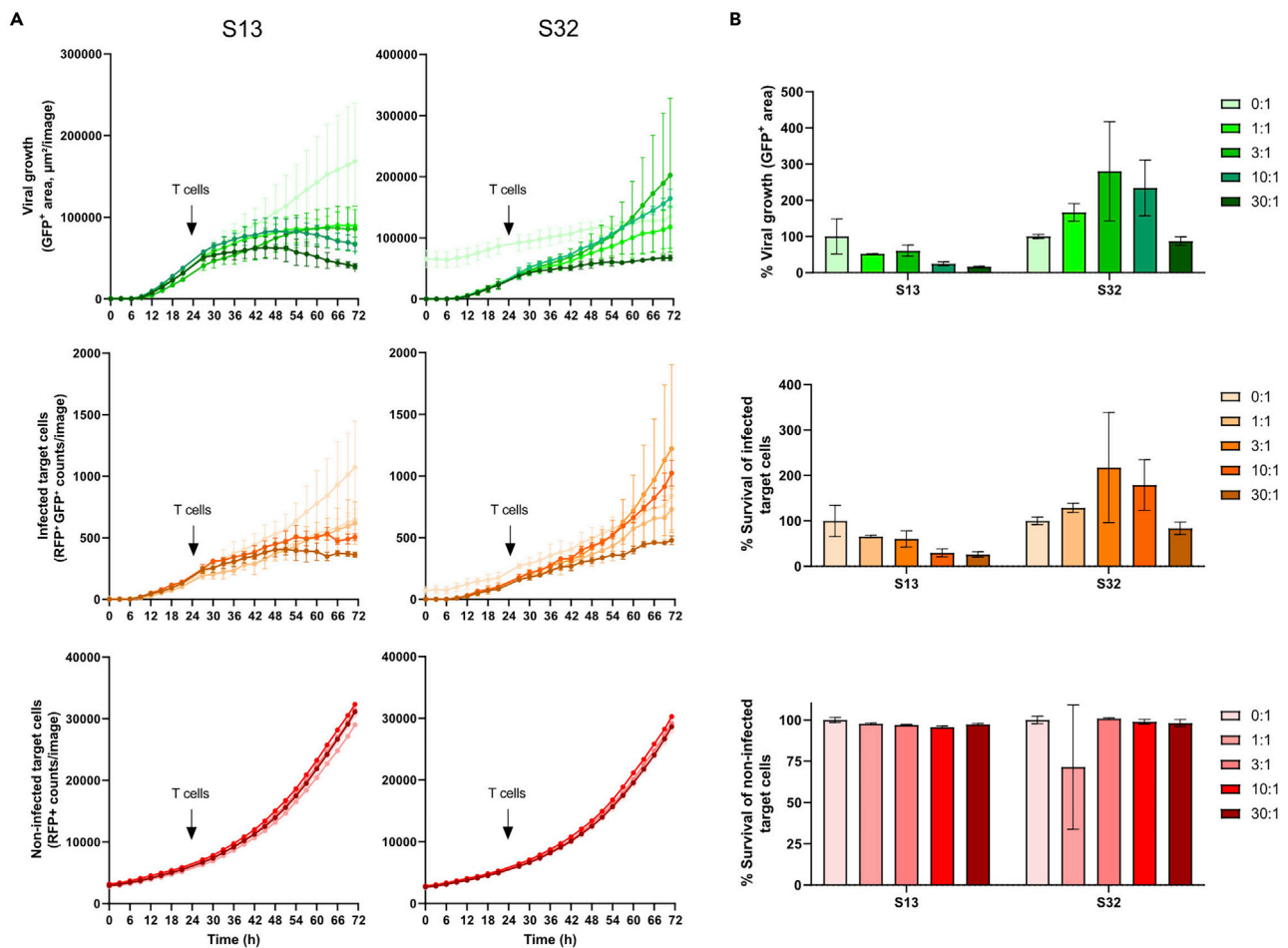


Figure 6. Replicating virus killing assay

5,000 competent A549 cells were seeded on 96-WPs and placed in the Incucyte S3 instrument. 24 h post seeding, cells were infected with SARS-CoV-2-GFP virus (MOI 5). 24 h post infection, either SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells (E:T ratio 1:1 to 30:1) or medium (0:1) was added to the target cells and Incucyte measurement was continued for another 48 h.

(A and B) Live tracking (A) and endpoint quantification (B) of viral growth (GFP⁺), infected target cells (GFP⁺ RFP⁺) and non-infected target cells (RFP⁺). For (A), data are shown as mean \pm SD of two technical replicates. For endpoint analyses (B), infected cells were washed before signal acquisition. Data are shown as mean \pm SD and are representative of one out of three independent experiments. Data are normalized to the 0:1 condition (Figure reprinted with permission from Wagner et al., 2022). Related to section "expected outcomes."

QUANTIFICATION AND STATISTICAL ANALYSIS

Incucyte data analysis

Cell viability (mock and infected), virus growth and infected target cells were respectively assessed as the cell number per image (RFP⁺ objects), total GFP area per image (μm^2) and overlap of object counts per image (RFP⁺ GFP⁺ objects) using the Incucyte S3 Software. Data are displayed using GraphPad Prism (V9.1). Statistical and correlation analyses were performed in GraphPad Prism.

1. Start the analysis type 'Basic analyzer' on the Incucyte S3 software and select the appropriate channels (e.g., phase, GFP, RFP) to be analyzed.

Note: If RFP-labelled cells and GFP-expressing SARS-CoV-2 virus are used, it is recommended to also select the 'Overlap' option.

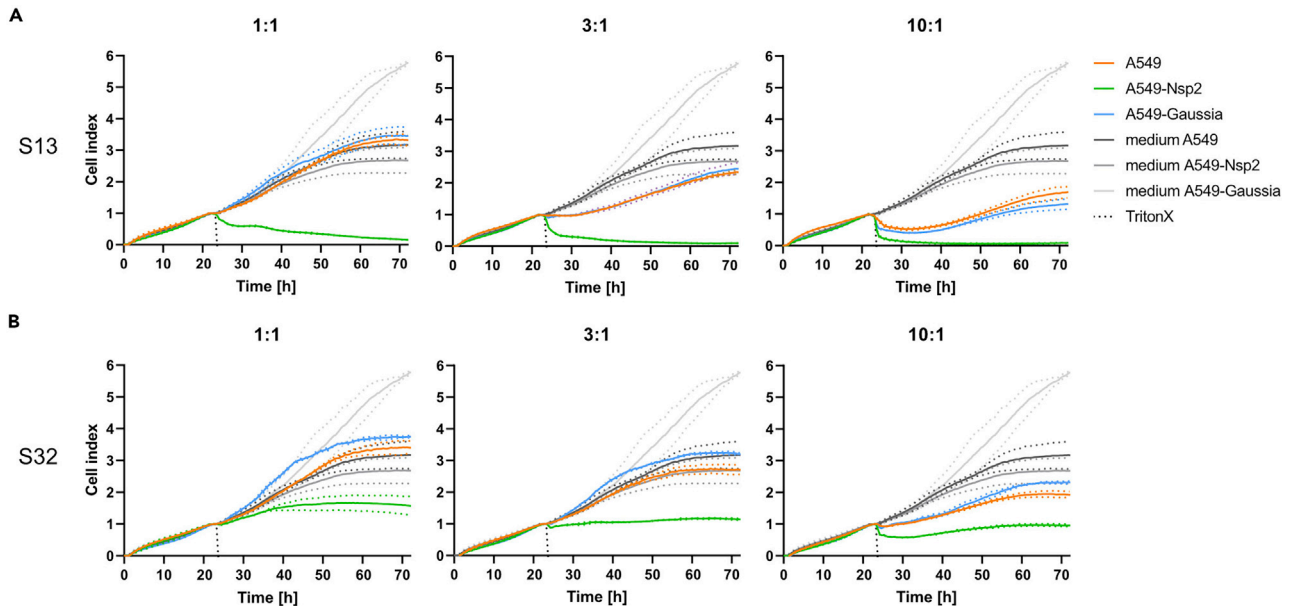


Figure 7. Transgenic antigen killing assay - impedance measurements

(A and B) 10,000 competent A549-Nsp2 cells were seeded on an E-Plate VIEW and, 24 h post seeding, either SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells (E:T ratio 1:1, 3:1 and 10:1) (green line) or medium (middle grey line) was added. As negative controls, competent A549 cells and competent A549-Gaussia cells were seeded and either SARS-CoV-2-specific, TCR-engineered CD8⁺ T cells (E:T ratio 1:1, 3:1 and 10:1) (orange and blue line, respectively) or medium (light grey and dark grey line, respectively) was added. Triton X served as positive control (tight dotted black line). xCELLigence acquisition is continued for additional 48 h. Cellular impedance values (cell index) are normalized to a value of 1 at the time point of T cell addition (24 h) and are shown for (A) TCR S13 (functional) and (B) TCR S32 (low-functional). Data are shown as mean \pm SD of two to four technical replicates. SD is shown as color-matched dotted line in both directions. Related to section “[expected outcomes](#).”

2. Make a selection of representative images from your experiments for previewing and refining the analysis.

Note: In order to train the software, it is recommended to select images with low and high confluence, as well as images with and without fluorescent signal. Overall, use between four and eight pictures to train the software.

3. Define the segmentation parameters and possible filters for the phase channel on one picture so the confluence mask overlaps all observed cells, and preview all images.

Note: As an example, segmentation adjustment of 1.1 and a minimum area filter of 1,000 μm^2 was used for the phase contrast analysis. It is necessary to adjust parameters for every experiment according to cell behavior.

4. Choose your segmentation options, threshold and filtering options for the green and red channels to obtain precise masking abilities, and preview all images.

Note: It is recommended to use a background subtraction method for the segmentation, preferentially a ‘Top-Hat model’. As an example, a ‘Top-Hat’ segmentation with a radius of 80 μm or 50 μm and a GCU threshold of 1.7 or 1 was used for the GFP or RFP channel, respectively. It is necessary to adjust parameters for every experiment according to fluorescence intensity and distribution.

5. Once all masks are fitting the different objects perfectly, proceed to scan times and wells selection.

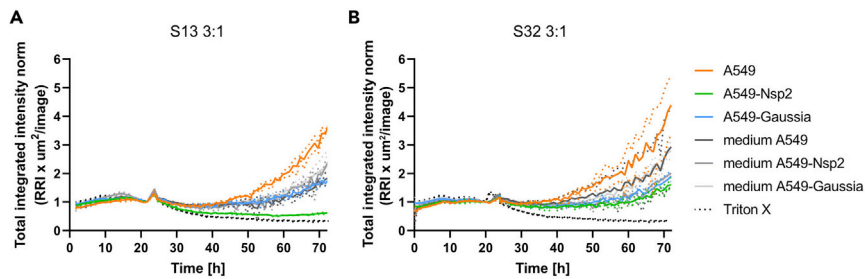


Figure 8. Transgenic antigen killing assay - target cell fluorescence analysis

Killing experiment was set up as described in [Figure 7](#).

(A and B) Total integrated intensity ($RRI \times \mu m^2/image$) was normalized to a value of 1 for all conditions at the time point 24 h for (A) TCR S13 (functional) and (B) TCR S32 (low-functional). Data are shown as mean \pm SD of two to four technical replicates. SD is shown as color-matched dotted line in both directions. Related to steps 15) - 25) of 'xCELLigence data analysis - Images'.

Note: If the experiment is still ongoing during the analysis definition, you can select 'analyzed future scans' for the next scans to be analyzed upon acquisition of the images.

6. Name your definition and start the analysis process.
7. Once the analysis is done, verify your masks on a random selection of images and scans.
8. Visualize and export the graph metrics of your choice (including the overlap mask).

Note: Normalization of metrics can be done on 'Confluence' if non-RFP labelled cells are used, or with 'Red Object Count' in the case of RFP-labelled cells.

9. If similar cell lines and growth conditions are used on other plates, reuse the same analysis definition.

xCELLigence data analysis - Impedance

Cell index (impedance) was assessed using the RTCA xCELLigence software. Data are displayed using GraphPad Prism (V9.1). Statistical and correlation analyses were performed in GraphPad Prism.

10. Open the tab 'Plot' and select the wells to be analyzed in the window 'Choose Wells to Draw' either manually or by dragging, then click 'Add'. Click 'Add All' if you want to analyze the entire plate. The impedance curves will appear in the plotting area.
11. Select the Time (in hour) by selecting 'From' and 'To' in the window 'Axis Scale', e.g., 'From' 0:00:00, 'To' 72:00:00.
12. Normalize data to the time point of T cell addition: in the window 'Plot Selection' for 'Y Axis' move 'Cell Index' to 'Normalized Cell Index'. Then select 'Normalize Time' to the last measurement before T cell addition, e.g., 24:00:00.
13. Right click on the plotting area and 'Copy Data in List Format'.
14. Paste data in excel and continue analysis.

Note: Instead of normalizing data you can also export raw data directly by right clicking on the plotting area and the 'Copy Data in List Format'.

xCELLigence data analysis - Images

15. Open the 'Image Browser' tab to create 'New Training Images'.
16. Select the channels to be analyzed (brightfield, red, green, blue) and select ten representative pictures per condition.
17. Move to the 'Image Processing' tab and create a 'New Processing Method'.

18. In the 'Analysis Mask' choose which channel to analyze first and deselect all other channels, e.g., confluence mask and select outlines and inner mask.

Note: Only select one channel at a time for analysis as masks will overlap and outlines will be difficult to distinguish.

19. Choose an outline width. Then move to the brightfield (Analyze) and adjust segmentation adjustment, cleanup and filters until all training images show good separation of parameters.
20. Move to the next channel and adjust parameters.

Note: As an example, segmentation adjustment of 1.1, cleanup Hole Fill (μm^2) of 300 or 0 and adjust size of 0 was used for brightfield analysis and red analysis, respectively.

21. Save your processing method and move to the 'Image analysis' task.
22. Launch analysis task, select the generated processing method and move all time points of interest from 'Exp Time' to 'Selected Time'.
23. You can now choose the wells to analyze and start the run.
24. When the analysis is finished, the program asks if you want to open it right now. Click 'Yes' and move from 'Analysis Task' to 'Analysis Result' (vertical tab on the top left side of the screen) to view analysis.
25. Export image analysis result by selecting the time points and the analysis for export.

In [Figure 8](#) is displayed the normalized total integrated intensity ($\text{RRI} \times \mu\text{m}^2/\text{image}$) for an E:T ratio of 3:1 for S13 and S32 TCR-engineered CD8⁺ T cells. The total integrated intensity is calculated by the maximal intensity multiplied by the area of fluorescence signal. As cells shrink and loose fluorescence signal when they are killed, the total integrated intensity can be used to track target cell killing. Comparable to [Figure 7](#), specific killing can be shown for competent A549-Nsp2 cells treated with S13 CD8⁺ T cells ([Figure 8A](#)); no such effect can be seen for S32 CD8⁺ T cells ([Figure 8B](#)).

LIMITATIONS

One might consider the origin of the desired cell line to use in this approach, as variability of infection rate from one cell line to the other is likely to occur. It is furthermore recommended to define the virus kinetic within the selected cell line beforehand in order to avoid low infection rates. The GFP-expressing virus used here is less infectious compared to virus obtained from clinical isolates and can lose its ability to express GFP after several passages. Therefore, viral stocks should be plaque-purified and viral purity should be assessed.

In the case of A549 cells, the target epitope ACE2 is not naturally expressed. Epitope density of virally transduced ACE2 can thus not directly be compared to physiological levels. However, also natural ACE2-expressing cell lines show a high degree of heterogeneity in the cellular level of the ACE2 protein ([Sherman and Emmer, 2021](#)).

Due to the low infection rate of the SARS-CoV-2-GFP virus, and thereby the overall low number of actually infected cells, small differences among TCRs might not be detected. To appreciate such small differences in functionality, more aggressive virus strains e.g., WT virus might be recommended. However, the use of clinical isolates involves the measurement of the cytotoxic activity at only one given end-point, at which staining of infected cells is performed.

Another general limitation of this protocol is the time needed to generate cell lines and TCR-transgenic CD8⁺ T cells. Indeed, the throughput of this protocol is limited. To obtain information about the global functionality of a CD8⁺ TCR repertoire more quickly, a polyclonal T cell population could

be used instead of a single TCR-transgenic CD8⁺ T cell population. However, readout might be challenging as epitope-specific effector CD8⁺ T cells will be low in numbers.

TROUBLESHOOTING

Problem 1

The TCR KI rate was lower than expected.

Potential solution

Use less or more cells in 100 μ L Nucleocuvette™ Vessels. Titrate the number of cells to be electroporated in one reaction for best KI efficiency.

Note: For additional troubleshooting issues concerning the KI efficiencies please refer to ([Moosmann et al., 2022](#)).

Problem 2

After sorting, transgenic TCR-engineered CD8⁺ T cells don't expand as expected.

Potential solution

Try using a different donor for feeder cells. Feeder cells from some donors provide a better growth environment for T cells than others.

Do not disturb the T cells for the first seven days of expansion. Try to stress cells as little as possible during the expansion phase. Therefore, do not centrifuge cells for medium exchange. To expand T cells from low cell numbers, do not split cells too early. Move cells to a bigger vessel when the entire surface area of the well is covered by cells. Add more medium only when it changes color in the culture, which is indicative of metabolic activity. Make sure to add cytokines in a regular manner similar as described in 12) in the section "[generation of OTR-engineered SARS-CoV-2-specific CD8⁺ T cells](#)." Addition of cytokines every 2–3 days ensures that T cells are stimulated enough for proliferation but not overstimulated.

Problem 3

The transgenic TCR KI rate after expansion is not good enough, i.e., < 85%.

Potential solution

Sometimes it can happen that the KI rate after expansion is too low. This might be caused by a sub-optimal performance of the cell sorting. When cells are expanded enough, it is possible to perform an additional sort to generate a purer population of transgenic TCR-expressing cells. As cell sorting is stressful to the cells, wait for a few days before performing experiments.

Alternatively, you can adjust the amount of total T cells used in the experiment according to the purity of SARS-CoV-2-specific CD8⁺ T cells. For instance, if the purity of SARS-CoV-2-specific CD8⁺ T cells is only 80%, you can add 125% of bulk effector cells to still have the same amount of effector cells eventually. It is however advised to not perform the experiment with too low TCR KI rates (< 85%) as the addition of more T cells can affect the experimental readout and bias the comparison among different TCRs.

Problem 4

The infection rate of SARS-CoV-2-GFP virus is very low.

Potential solution

The MOI might be calculated wrong. Repeat the virus titration assay (see 25) in the section "[generation of SARS-CoV-2-GFP virus](#)."

It is also possible that the susceptibility of target cells to SARS-CoV-2 infection is low. A potential reason could be the level of ACE2 expression on the cell surface. If cell lines that endogenously express ACE2 are used, check for ACE2 surface expression by flow cytometry or protein production by western blot. In case of cell lines virally transduced with an ACE2 transgene, perform another selection round for ACE2 and check for surface expression or protein production.

If GFP negative dead cells are observed, the produced virus might have lost its ability to express GFP. Plaque-purify the virus before production of a new viral stock and repeat the experiment.

Problem 5

The masks obtained during the Incucyte analysis are not overlapping all the objects, especially for the green channel.

Potential solution

Restart a new analysis definition with more representative images of all conditions and possible controls (4 to 8 images are optimal) (see 33) - 41) in the section "[quantification and statistical analysis](#)".

The 'hole-filling' option can be considered during the analysis definition if small areas are not covered by the masks. Most cell culture media contain autofluorescent components. Initial background should be tolerated as it will be corrected once the real fluorescence appears.

Problem 6

Dead cells due to virus infection still remain GFP⁺ and are biasing the analysis.

Potential solution

As dead cells are smaller and rounder compared to live cells, filtering option based on area can be considered during the analysis definition to define a minimal size of your object of interest.

Problem 7

Control target cells in the transgenic antigen killing assay (xCELLigence) show early growth arrest.

Potential solution

The impedance measurement of the transgenic antigen assay measured with the xCELLigence eSight instrument can be directly correlated to cell proliferation. If the impedance curves of control conditions show an early growth arrest, this could be an indication of suboptimal experimental conditions. Make sure that target cells are at a density of 70%–90% during culturing and before seeding onto an E-Plate VIEW. The pure addition of T cells on top of target cells at high E:T ratios can dramatically reduce oxygen and nutrient availability for target cells. Strong TCRs are expected to show an effect at low E:T ratios, i.e., 1 to 5 (see 24) - 27) in the section "[transgenic antigen killing assay using the xCELLigence instrument](#)".

Problem 8

The amount of GFP⁺ A549-Nsp2 target cells is low.

Potential solution

The Nsp2 protein is linked to GFP via a T2A tag to ensure polycistronic expression of the two proteins (see 9) - 12) in the section "[generation of A549-ACE2-RFP-HLA-A*03:01 \(Nsp2-GFP\) cells](#)". A low percentage of GFP⁺ cells would mean that only a proportion of A549 target cells also expresses the Nsp2 protein. It is thus recommended to repeat the cell sorting for GFP⁺ cells for bulk or single clone selection. If you additionally have an antibiotic resistance encoded on the Nsp2-T2A-GFP plasmid, you can also perform antibiotic selection for a few days.

Be advised that the transcription strength might be different for different constructs. We observed that overall GFP mean fluorescent intensity can be lower for some transduced products like for competent A549 cells expressing the SARS-CoV-2 spike protein. This might be correlated to the size of the transduced protein (Spike: 3,822 bp vs Nsp2: 1,914 bp).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Elvira D'ippolito (elvira.dippolito@tum.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data sets supporting the protocol and further examples of OTR are described by (Moosmann et al., 2022), (Schober et al., 2019) and (Müller et al., 2021). Examples of SARS-CoV-2-GFP killing assays are described by (Wagner et al., 2022). A holistic virus-host interaction analysis of SARS-CoV-2 with the lung-derived human cell line A549 is described by (Stukalov et al., 2021).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101699>.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.D., A.P., and D.H.B.; Methodology, L.M.M., E.D., V.G., and A.P.; Validation, L.M.M. and V.G.; Investigation, L.M.M., V.G., and E.D.; Writing – Original Draft, L.M.M. and V.G.; Writing – Review & Editing, E.D., A.P., and D.H.B.

DECLARATION OF INTERESTS

D.H.B. is co-founder of STAGE Cell Therapeutics GmbH (now Juno Therapeutics, a Bristol-Myers Squibb Company) and T cell Factory B.V. (now Kite, a Gilead Company). D.H.B. has a consulting contract with and receives sponsored research support from Juno Therapeutics. D.H.B. is member of the Scientific Board of Immatics. All other authors declare no competing interests.

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