

Replicase-Based DNA Vaccines for Allergy Treatment

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Summary

Replicase-based vaccines were introduced to overcome some of the deficiencies of conventional DNA- and RNA-based vaccines, including poor efficiency and low stability. At ultra-low doses, these alphavirus-derived vectors elicit cellular as well as humoral immune responses. Additionally, replicase-based vectors induce “self-removal” of the vaccine via apoptosis of transfected cells. This chapter describes the construction of a replicon-based DNA vaccine vector from commercially available plasmids. We present protocols for monitoring cellular immune responses following replicase-based immunization including measurement of allergen-specific proliferation of splenocytes, ELISPOT, a FACS-based cytokine secretion assay providing information about T-helper subsets, and a cytokine fluorescent bead immunoassay.

Key Words: DNA vaccine; genetic immunization; type I allergy; immunotherapy; alphavirus; replicase; replicon; apoptosis; Th1/Th2 responses.

1. Introduction

Despite the encouraging data from numerous animal studies, results from preclinical work with primates as well as from clinical trials imply that DNA vaccines are less effective as expected (*1–3*). Furthermore, with respect to DNA vaccines for allergy treatment, the high amount of plasmid DNA necessary for intramuscular (IM) or intradermal (ID) needle injection still remains a major shortcoming.

Replicase-based or “self-replicating” DNA vaccines were originally designed to prevail over the deficiencies of conventional DNA- and RNA-based vaccines including poor efficacy as well as low stability. Alphaviruses such as Semliki Forest virus, Sindbis virus, or Venezuelan equine encephalitis virus, contain a single positive-stranded RNA encoding its own RNA replicase. After infection of a cell, the viral RNA first translates the replicase complex, which then pursues its own RNA replication. A genomic negative-strand (anti-sense)

RNA is synthesized, functioning as a template for synthesis of genomic positive-strand RNA as well as subgenomic RNA encoding the structural virus proteins. By replacement of these genes with the coding sequence for an antigen of interest and insertion of a strong promoter to initiate transcription of the full-length RNA in the nucleus, replicase-based DNA vaccines can be constructed (4–7). At extremely low doses these vectors proved to be superior compared to conventional DNA vaccines in terms of immunogenicity and efficacy. Even at nanogram quantities, replicase-based DNA vaccines elicit cellular as well as humoral immune responses (5,6,8–10).

In contrast to expectations that “self-replicating” DNA and RNA vaccines would drastically enhance the level of antigen expression and presentation, some *in vitro* experiments demonstrated that the amount of produced antigen did not significantly differ compared to conventional DNA and RNA vectors (6,8,9,11).

The major difference between alphaviral vectors and conventional DNA vaccines is the RNA-replication inside the host, which mimics a viral infection of transfected cells, thereby triggering several “danger signals” (12). Similar to alphaviral infections, “self-replicating” vaccines cause apoptotic death of transfected cells. This phenomenon can be attributed to the requisite double-stranded RNA-intermediates generated during alphaviral replication (9,11). A recent publication demonstrates that the superior efficacy of replicon-based DNA vaccines *in vivo* is at least partly mediated by the induction of innate antiviral pathways triggered via double-stranded RNA (8). In addition, the context and the stimulus for the induction of apoptosis have an important impact on any immune response (13). Antigen presenting cells (APCs) can recognize and engulf apoptotic cells and either become activated (14) or induce silencing signals (15). One of the mechanisms underlying the enhanced immunogenicity of “self-replicating” vaccines seems to be the enhanced uptake of antigen from cells that die apoptotically by APCs (16,17) and the fact, that double-stranded RNA itself is a “danger signal,” functioning as an adjuvant to the T-cell specific stimulus of the encoded antigen (18).

Conventional and replicase-based DNA-vaccines seem to use different effector mechanisms. A stronger role of effector CD8⁺ cells induced by replicon-based vaccines could be at least in a tumor model (8). Intradermal injection of replicon-based construct at a nanogram dose induced a Th1-biased immune response, which protected from a subsequent allergen challenge and also redirected an established allergic response. Despite their excellent immunogenicity with respect to stimulation of CD4⁺ and CD8⁺ cells, replicase-based DNA vaccines stimulated weaker, or in some cases no antibody responses,

depending on the nature of the protein expressed, probably owing to apoptotic removal of potential targets for antibody producing B cells (19).

DNA replicons represent the latest generation of DNA vaccines and three aspects render them highly interesting candidates for effective and safety-optimized vaccine approaches against allergy: (1) the minimal amount of plasmid necessary for induction of anti-allergic immune responses will increase the acceptance of DNA vaccination for allergy treatment; (2) the induction of apoptosis of cells transfected with the DNA replicon vaccine leads to “self-removal” of the vaccine after triggering the immune response; and (3) DNA replicon vaccines gain their immunogenicity via viral danger signals and induction of anti-viral immune responses, which may be a way to overcome the poor immunogenicity of conventional DNA vaccines in humans.

2. Materials

2.1. Construction of a Replicon-Based DNA Vaccine

1. pSinRep5 plasmid (Invitrogen, Lofer, Austria).
2. pCI mammalian expression vector (Promega, Mannheim, Germany).
3. *Escherichia coli* strain XL1-blue competent cells (Stratagene, La Jolla, CA) and electroporator.
4. Standard Luria broth (LB) medium.
5. Ampicillin.
6. Oligonucleotide primers, deoxynucleotide triphosphate (dNTP) mix (25 mM each).
7. Pfu-polymerase, restriction enzymes, T4 DNA Ligase, corresponding buffers.
8. Polymerase chain reaction (PCR)-cyclers.
9. Agarose gel DNA electrophoresis equipment.
10. Gel extraction kit (e.g., NucleoSpin Extract; Macherey-Nagel, Easton, PA),

2.2. Immunization/Sensitization of Mice

See Chapter 18, Subheading 2.3.

2.3. Proliferation Cultures From Splenocytes

1. Earle's modified Eagle's medium (MEM) supplemented with 1% mouse serum (PromoCell, Heidelberg, Germany), 1% L-glutamine, 2.5% penicillin/streptomycin, 1 mM sodium pyruvate, 2% HEPES, 1% nonessential amino acids, 2 μ M 2-mercaptoethanol.
2. Recombinant protein.
3. Ammonium, chloride, potassium (ACK) lysis buffer: 0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2 ethylene-diamine tetraacetic acid (EDTA). Adjust pH to between 7.2 and 7.4 with 1 N HCl and sterilize.
4. 96-Well flat-bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ).
5. [^3H] thymidine.
6. Cell harvester (e.g., 96 Mach; IIM, Tomtec, CT).

7. Scintillation counter (e.g., Wallac MicroBeta TriLux; Perkin-Elmer, Finland).

2.4. ELISPOT

1. Complete medium, recombinant protein, and ACK lysis buffer as described in **Subheading 2.3**.
2. Polyvinylpyrrolidone formamide (PVDF) bottom plates with high binding capacity (MAIP S45 10; Millipore, Billerica, MA).
3. 70% EtOH.
4. Standard phosphate buffered saline (PBS).
5. Dried skim milk (blotting grade).
6. Bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany).
7. Tween-20 (Sigma).
8. 30% H₂O₂.
9. Coating antibody (clone AN-18.17.24 for interferon [IFN]- γ , clone TRFK5 for IL-5) (PharMingen, San Diego, CA).
10. Biotinylated detection antibody (clone R4-6A2 for IFN- γ , clone TRFK4 for IL-5) (PharMingen).
11. Streptavidin-HRP (Becton Dickinson).
12. AEC-stock: 4 mg/mL 3-amino-9-ethyl-carbazole (Acros, Geel, Belgium) in dimethylformamide (DMF).
13. AEC-substrate solution: dissolve 9.6 g citric acid in 500 mL H₂O. Dissolve 14.2 g Na₂HPO₄ in 500 mL H₂O. Add phosphate solution to citric acid solution until pH reaches 5.0.
14. 0.45- μ m Sterile filter and 10-mL syringe.

2.5. FACS-Based Cytokine Secretion Assay

1. FACS-buffer: PBS, 0.5% BSA, 2 mM EDTA.
2. Equipment for cell culture as described in **Subheading 2.2**. (medium, ACK-buffer, culture plates).
3. Specific peptide and/or recombinant protein.
4. Mouse cytokine secretion assay detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany), store in the dark at 4°C.
5. CD4-FITC and CD8-FITC (PharMingen).
6. CD45R/B220-PerCP™ (PharMingen).
7. Propidium iodide (PI).
8. Rotation device for tubes.

2.6. Cytokine Fluorescent Bead Immunoassay

1. Mouse cytokine fluorescent bead immunoassay basic kit for cell culture supernatants, based on the Luminex™ technology (Bender MedSystems, Vienna, Austria), store at 2–8°C.
2. Mouse simplex fluorescent bead immunoassays for IFN γ , IL-4, IL-5, IL-10, TNF α based on the Luminex technology (Bender MedSystems), store between 2 and 8°C.

3. Microplate shaker.
4. Luminex100 LabMAP™ System (Luminex, Austin, TX).
5. Clear PVDF filter plates, pore size 1.2 μm (e.g., MABV N12 50, Millipore; Billerica, MA).

3. Methods

3.1. Construction of a Replicon-Based DNA Vaccine (see Fig. 1)

Dubensky et al. have demonstrated how replicon-based DNA vaccines can be constructed from Sindbis virus RNA (5). Here, we demonstrate how such a vector can be built from the commercially available plasmids pCI (Promega) and pSinRep5 (Invitrogen). DNA manipulations were performed by standard recombinant DNA methods (20) and are not described in detail because of space limitations. PCR/restriction fragments were run on 1% agarose gels and excised bands were purified with a gel extraction kit. Plasmids were transformed into *Escherichia coli* XL1-blue by standard electroporation. The bacteria were plated on LB plates containing ampicillin (50–100 $\mu\text{g/mL}$) and incubated overnight at 37°C. Single colonies were selected and grown overnight in LB containing ampicillin. The plasmid DNA was then isolated and checked by restriction enzyme digestion and DNA sequencing (MWG Biotech, Ebersberg, Germany).

1. Synthesize PCR primers. The sin fw primer encompasses the 5'-end of the Sindbis replicase (shown below in *italic*). Attached to the 5'-end are 23 bp of the 3'-end of the cytomegalovirus (CMV) promoter of pCI. These 23 bp include a *SacI* restriction site (shown in **bold**) and the transcription start (shown in **bold underline**) (see Note 1).

The sin rv primer binds after 1407 bp at a *Eco47III* site (shown in **bold**) of the Sindbis replicase (of vector pSinRep5).

Sin fw: 5'-gca**GAGCTC**gtttagtgaaccg**Tattgacggc**gtagtacacactattg-3'

Sin rv: 5'-gaa**AGCGCT**aaaagaggtg-3'

2. PCR-amplify a 1436-bp fragment with sin fw/sin rv primers from 10 ng pSinRep5 template plasmid with Pfu-Polymerase (see Chapter 18, Subheading 3.1.1. for PCR protocol).
3. Restriction digest the PCR fragment with *SacI/Eco47III* and ligate it into the *SacI/SmaI* sites of pCI. The resulting plasmid is designated pCMV/sin-fragment.
4. Verify the sequence of the inserted fragment by sequencing (see Note 2).
5. Ligate the 6650bp *Eco47III/NotI* fragment of pSinRep5 into the respective restriction sites of pCMV/sin-fragment, thereby creating the functional vector pCMV/sin.
6. A gene of interest can now be ligated into the multiple cloning site (MCS) 3'-from the subgenomic promoter.

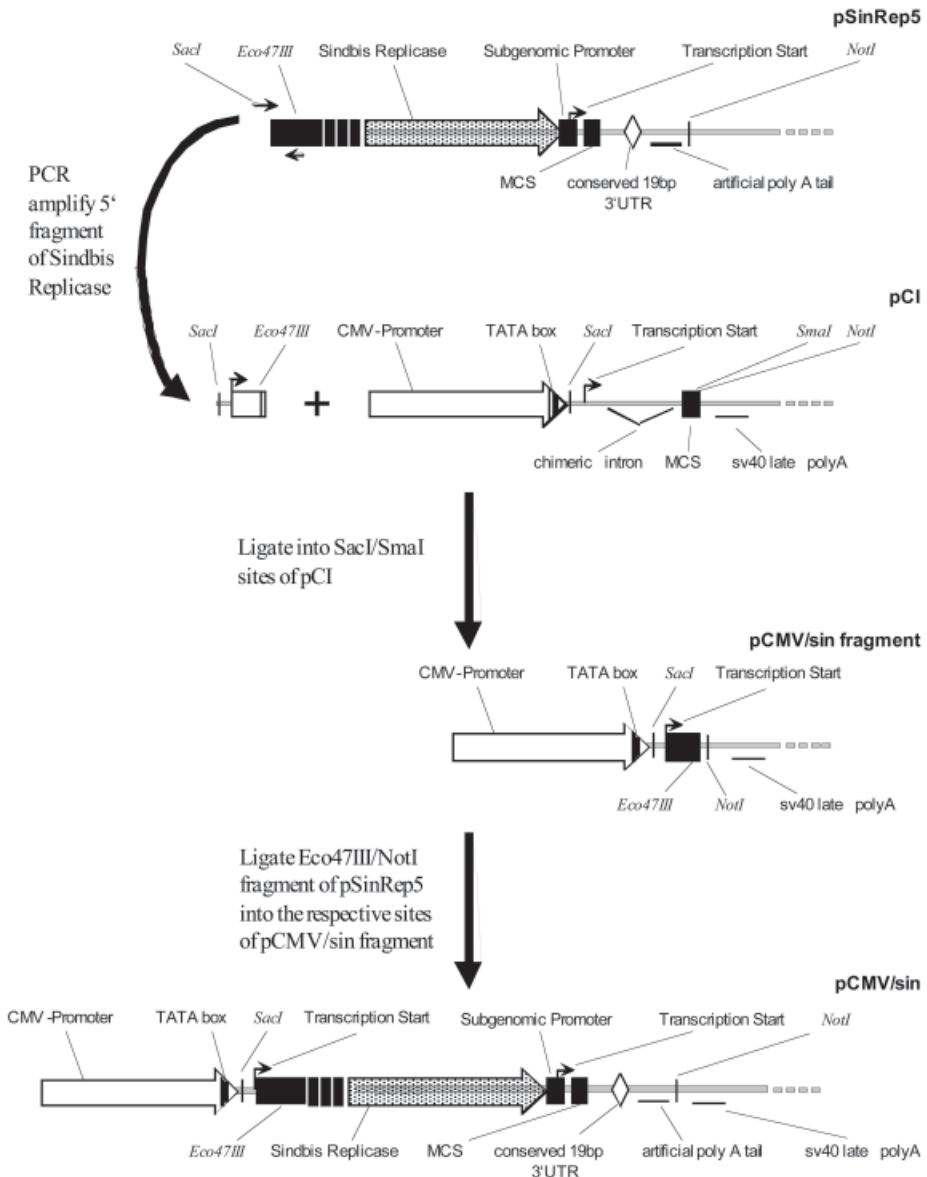


Fig. 1. Schematic drawing of cloning strategy for the construction of a Sindbis replicon based DNA vaccination vector.

3.2. Immunization/Sensitization of Mice

We recommend starting with an immunization dose of 1 µg replicase-based plasmid DNA per mouse. In our experience, increasing the amount of DNA

will not result in improved immunogenicity. For details of immunization/sensitization of mice *see* Chapter 18, **Subheading 2.3**.

3.3. Assays for Monitoring Cellular Immune Responses

Because of their viral danger signals, replicon-based DNA vaccines elicit typical “anti-viral” immune responses characterised by low antibody production and strong Th1/CD8⁺ cell activation. In this chapter, we will focus on assays for evaluating cellular immune responses, especially with respect to cytokine secretion. For detailed description of methods evaluating humoral immune responses after DNA-based immunization, please refer to Chapter 18 in this book.

3.3.1. Proliferation Cultures From Splenocytes

At the end of the experimental schedule, mice are sacrificed by cervical dislocation. Remove spleens aseptically, mince them in 500 μ L culture medium (*see* **Note 3**) and let aggregates sediment for 5 min. Take 400 μ L of single cell suspension supernatants and resuspend cells in 7 mL ACK lysis buffer. Lyse erythrocytes by incubation at room temperature for 7 min, add medium, and wash splenocytes twice by centrifugation at 300g. Resuspend cells in complete medium at 4×10^6 /mL and distribute 50 μ L of this solution into microtiter wells. Add 50 μ L of a 40- μ g/mL antigen solution in complete medium to each well. Prepare between three and five replicate wells per animal. Additionally, the same number of wells without addition of antigen must be prepared from each splenocyte solution to serve as negative control. Also include wells from naïve animals with and without protein stimulation. Incubate culture plates for 96 h at 37°C, 95% relative humidity, 7.5% CO₂. For the last 20 h, 0.5 μ Ci [³H] thymidine in a volume of 10–20 μ L medium is added to each well. Harvest the cells with a cell harvester and measure thymidine incorporation in a scintillation counter. Calculate stimulation indices from the averages of replicate wells according to the equation, [antigen_{cpm}/medium_{cpm}].

3.3.2. ELISPOT

3.3.2.1. COAT PLATES

1. Activate PVDF membrane by adding 200 μ L 70% EtOH per well. Incubate for 10 min at room temperature.
2. Wash plate three times with 200 μ L PBS per well. Incubate for 2 min between each washing step.
3. Add 100 μ L coating antibody per well (4 μ g/mL in PBS). Seal plate airtight and incubate overnight at 4°C.

3.3.2.2. BLOCKING AND SEEDING OF CELLS

1. Wash plate with 200 μ L PBS per well.

2. Block plate with 100 μL /well PBS, 2% skim milk. Incubate for 2 h at room temperature in humid chamber.
3. Wash plate with 200 μL PBS per well.
4. Add 50 μL of protein solution (40 $\mu\text{g}/\text{mL}$) (*see Subheading 3.2.1.*) per well. Add 50 μL of splenocytes ($4 \times 10^6/\text{mL}$) (*see Subheading 3.2.1.*) (*see Note 4*).
5. Incubate plate 24 h in a CO_2 incubator as described in **Subheading 3.3.1**. Do not move the plate during the incubation.

3.3.2.3. DEVELOP THE SPOTS

1. Wash three times with 200 μL PBS per well. Incubate 2 min between each washing step.
2. Wash three times with 200 μL PBS/0.1% Tween-20 per well. Incubate for 2 min between each washing step.
3. Add 100 μL biotinylated detection antibody (2 $\mu\text{g}/\text{mL}$ in PBS, 1% BSA). Incubate for 2.5 h at room temperature in humid chamber.
4. Wash four times with 200 μL PBS/0.1% Tween-20 per well. Incubate for 2 min between each washing step.
5. Add 100 μL streptavidine-horseradish peroxidase (HRP) (diluted 1:1000 in PBS, 1% BSA) and incubate for 2 h at room temperature in humid chamber.
6. Wash three times with 200 μL PBS/0.1% Tween-20 per well. Incubate for 2 min between each washing step.
7. Remove the bottom of the PVDF plate and thoroughly wash it with distilled water. Wash the underside of the membrane (backs of the wells) with PBS. Put the plate back on the bottom (*see Note 5*).
8. Wash four times with 200 μL PBS per well. Incubate for 2 min between each washing step.
9. Dilute AEC stock solution 1:15 in AEC substrate solution (solution becomes turbid). Filter through a 0.45- μm filter (solution becomes clear). Add 30% H_2O_2 (1:1500) before use.
10. Add 100 μL per well and observe spot formation (*see Note 6*).
11. After sufficient spot formation wash thoroughly with H_2O to stop the reaction. Remove bottom and let plate dry overnight at 4°C (spots will darken).

Spots can be counted manually (using a microscope) or with an ELISPOT-reader. If no ELISPOT-reader is available a convenient alternative is a standard flatbed scanner with a resolution of 1600 dpi or higher. Scanned plates can then be counted easily on the screen (count and tag). Images can also be analyzed with the program UTHSCSA ImageTool, which is freely distributed at <http://ddsdx.uthscsa.edu/dig/itdesc.html>.

3.3.3. FACS-Based Cytokine Secretion Assay

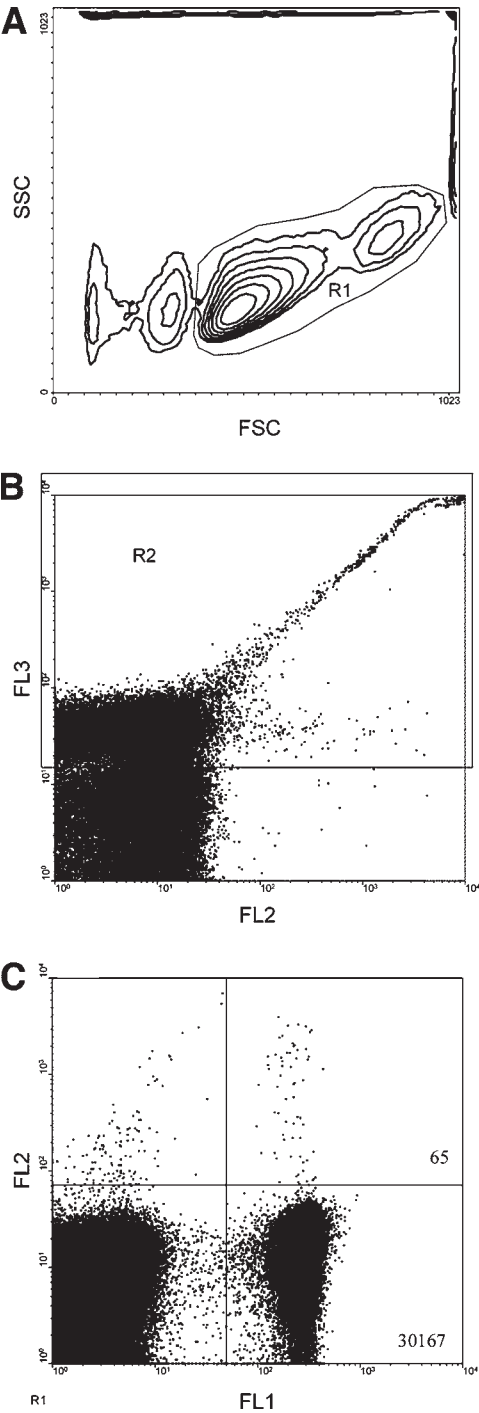
For quantitative analysis of antigen-specific murine leukocyte populations using a cytokine secretion assay, mouse splenocytes are restimulated for a short period of time with specific peptide or protein. Subsequently, a cytokine-

specific catch reagent is attached to the cell surface of all leucocytes. The cells are then incubated for a short period of time at 37°C to allow cytokine secretion. The secreted cytokine binds to the catch reagent on the positive, secreting cells. These cells can subsequently be labeled with a second cytokine-specific detection antibody conjugated to phycoerythrin (PE) for detection by flow cytometry.

Splenocytes are prepared as described in **Subheading 3.3.1.** and diluted to 2×10^7 /mL. Seventy-five microliters of this single cell suspension together with 75 μ L medium containing 40 μ g/mL recombinant protein or 20 μ g/mL specific peptide are incubated in wells of a 96-well culture plate for 3–6 h (peptide) or 6–16 h (protein) at 37°C, 95% relative humidity, and 7.5% CO₂ (see **Notes 7–9**).

Protocol for secretion assay:

1. Warm up 2 mL culture medium per sample.
2. Put FACS buffer and 100 μ L culture medium per sample on ice.
3. Prechill centrifuge to 4°C.
4. Prewarm a chamber or a room with a rotation device for tubes to 37°C.
5. Collect cells by pipetting up and down and put them on ice in a 4-mL closable tube.
6. Wash cells twice with 2 mL ice-cold FACS buffer by centrifugation at 300g for 10 min at 4°C.
7. Prepare 90 μ L cold culture medium plus 10 μ L cytokine catch reagent per sample.
8. Resuspend the pellet from the second wash step thoroughly in 95 μ L of diluted catch reagent.
9. Incubate on ice for 10 min.
10. Add 2 mL warm culture medium.
11. Incubate cells in closed tubes for 45 min at 37°C under slow continuous rotation, or turn tube every 5 min to resuspend settled cells (see **Note 10**).
12. Put the tubes on ice.
13. Fill them up with cold FACS buffer and wash by centrifugation at 300g for 10 min at 4°C.
14. Resuspend cells in 2 mL cold FACS buffer and split them up into two vials (one for CD4 labeling, one for CD8 labeling).
15. Centrifuge at 300g for 10 min at 4°C.
16. Prepare 5 μ L detection antibody plus 45 μ L cold FACS buffer per sample and add CD45R/B220-PerCP (1:100) (see **Note 11**). Add CD4-FITC (1:200) to half of the samples, CD8-FITC (1:200) to the other.
17. Resuspend each pellet in 48 μ L antibody solution.
18. Put cells on ice for 10 min.
19. Wash cells with 1 mL ice-cold FACS buffer.
20. Resuspend pellets in 120 μ L FACS buffer.
21. Add propidium iodide to a final concentration of 0.5 μ g/mL (see **Note 12**).
22. Acquire between 200,000 and 300,000 events from each sample.
23. Gate cells based on forward and side scatter (FSC/SSC) properties of lymphocytes (see **Fig. 2A**, *RI*)—this is best done on a density or contour plot.



24. Exclude dead cells and B cells according to PI- and CD45R/B220-PerCP™-staining in a fluorescence 2 vs fluorescence 3 plot (see **Fig. 2B**, R2)
25. Gate cells (AND R1, NOT R2) and analyze cytokine stained with PE vs CD4-FITC or CD8-FITC staining of viable lymphocytes (see **Fig. 2C**).
26. Calculate the percentage of cytokine secreting cells among total CD4⁺ or CD8⁺ cells according to the equation [no. of secreting CD8⁺ in the analyzed sample/no. of total CD8⁺ cells in the analyzed sample] × 100. The example in **Fig. 2C** shows 65 secreting CD8⁺ cells (upper right quadrant) and 30,167 nonsecreting CD8⁺ cells (lower right quadrant). The percentage of secreting CD8⁺ cells is therefore 65/(65 + 30,167) × 100.

3.3.4. Cytokine Fluorescent Bead Immunoassay

This method allows simultaneous measurement of multiple different cytokines in a sample. The principle of the test is as follows: microspheres classified by precise ratios of two fluorophores are coated by cytokine-specific antibody (see **Note 13**). The coated beads are incubated with the samples or standard. Cytokine molecules in the sample bind to the antibodies adsorbed to the fluorescent beads. After addition of biotin-conjugated secondary antibody, the specific antibody binds to the cytokine captured by the first antibodies. Streptavidin-phycoerythrin is added, binds to the biotin conjugate, and emits fluorescent signals.

For production of supernatants containing cytokines, splenocytes are prepared and stimulated as described in **Subheading 3.3.1.** except that 48-well culture plates are used and the total volume per well is 500 instead of 100 µL. After 4 d of culture, carefully take supernatants, which can be stored at -70°C for later examination.

Assay procedure (see **Note 14**):

1. Dilute the provided assay buffer 1:10 in deionized H₂O.
2. Dissolve standards in deionized H₂O as indicated on the respective vials and prepare a mixture of standards.
3. Make serial dilutions of the standard mixture sufficient for seven wells.
4. Dilute the fluorescent beads 1:10 in assay buffer resulting in a final volume of 3 mL/plate (see **Note 15**).
5. Add 25 µL of standard dilution 1–7 in designated wells of the plate.
6. Add 25 µL of assay buffer to the blank well.
7. Add 25 µL of each of your samples to separate wells.

Fig. 2. Example plots of an interferon (IFN)γ secretion assay. **(A)** Gating of lymphocytes in a contour blot; region 1 (R1). **(B)** Exclusion of B-cells and dead cells; region 2 (R2). **(C)** Analysis of IFNγ secreting CD8⁺ cells (upper right quadrant), and CD8⁺ nonsecreting cells (lower right quadrant).

8. Add 25 μL of diluted bead mixture to all wells, including the blank well.
9. Cover with a sealing tape, protect from light with an aluminum foil, and incubate at room temperature for 2 h on a microplate shaker at about 200 rpm.
10. Dilute biotin conjugate 1:10 with assay buffer to give a final volume of 6 mL/plate.
11. Add 50 μL biotin-conjugate to all wells.
12. Cover the plate with sealing tape, protect from light with aluminum foil, and incubate at room temperature for 1 h on a shaker.
13. Prepare streptavidin-PE by mixing 176 μL of the provided solution with 5324 μL assay buffer.
14. Add 50 μL streptavidin-PE to all wells.
15. Incubate as described in **step 12**.
16. Add 75 μL stop solution per well.
17. Start the Luminex data collector and warm up the Luminex100 LabMAP System for 30 min.
18. Prime with sheath fluid.
19. Perform alcohol flush with 1.2 mL 70% EtOH.
20. Calibrate only if the temperature is not within the range of $\pm 2^\circ\text{C}$ of the calibration temperature: apply three drops of reporter calibrator to well A1, three drops of classification calibrator to well B1, and fill up well C1 with sheath fluid. Press “calibration.” Fill in the correct lot numbers and calibrate twice; first with classification calibrator, second with reporter calibrator.
21. Load the plate into the platform of the Luminex100 LabMAP System. Set the instrument to remove 50 μL /well and to read 100 events per bead set. Fill in the correct numbers of the bead sets used. Start a new session.
22. After measurement, save your session, sanitize with 1.2 mL 70% EtOH, and fill the tray with H_2O (“soak”).
23. Create a standard curve by plotting the fluorescent intensity for each standard concentration on the ordinate against the corresponding concentration on the abscissa. Draw a best fit curve through the points of the graph (*see* **Note 16**).
24. Determine the concentration of analytes for each sample by interpolation (*see* **Note 17**).

4. Notes

1. It is important that transcription starts exactly at the 5'UTR (bp 1–59 of pSinRep5). Additional nucleotides added between transcription start and the 5'-UTR or changes in the 5'-UTR can be deleterious to replication, because this element forms a hairpin structure that is essential for binding of the viral replicase and promotes RNA replication (**21**).
2. The cloning of the Sindbis replicase into pCI is done in two steps. Only the first fragment (1436 bp) is cloned by PCR. Therefore, only the sequence of this short fragment has to be validated, avoiding sequencing of the whole 10-kb replicase gene.
3. Culture media are usually supplemented with fetal calf serum. In our experience, background levels (i.e., cultures from naïve animals or wells incubated with

medium containing no protein for restimulation can be drastically reduced by using mouse serum instead).

4. It is important to add the cells drop-wise to the center of the well, to assure even distribution of the cells. The last drop of splenocytes solution hanging on the pipet tip can be applied by touching the surface of the well with the tip.
5. This step greatly decreases the background from leakage through the underside of the well.
6. Duration of spot formation greatly varies depending on the amount of cytokine secretion and can take from 10 min up to 1 h. In contrast to the commonly used BCIP/NBT staining, AEC staining usually produces lower background and is less sensitive to over-exposure.
7. For the cytokine secretion assay it is not necessary to include replicate wells or wells stimulated with medium alone, but cultures from some naïve animals should be incubated to serve as negative controls. When setting up a new experiment, it can be helpful to include a positive control (e.g., a sample stimulated with 10 µg/mL of the superantigen Staphylococcal Enterotoxin B [Sigma, St. Louis, MO] for 3–16 h). Do not use mitogens such as PHA or PMA/Ionomycin, as the resulting high frequencies of cytokine secreting cells do not allow conclusions on the sensitivity of the secretion assay. For comparison of different experiments, the stimulation time should be kept constant.
8. The addition of costimulatory agents such as CD28 may enhance the response to the antigen.
9. Because of nonspecific stimulation, it is crucial not to use culture media containing any nonmurine proteins, such as BSA or FCS.
10. During this step it is crucial to prevent contact of cells to avoid cross-contamination with cytokines. If you still notice cross-contamination, work with up to 10 mL of medium during the secretion period.
11. For optimal sensitivity, labeling of undesired cells (e.g., B cells, with antibodies conjugated to PerCP is recommended). These cells can then be excluded together with PI stained dead cells by gating.
12. Add PI just prior to acquisition for exclusion of dead cells from flow cytometric analysis. Incubation with PI for longer periods will affect the viability of the cells. Do not fix the cells when using PI. Exclusion of dead cells from analysis reduces nonspecific background staining and increases sensitivity.
13. Do not expose kit reagents to light during storage or incubation, as the beads are photosensitive.
14. Washing steps with assay buffer between incubations as described in the manual only need to be performed when testing high biotin-containing culture media (i.e., RPMI).
15. It is recommended to adjust the volumes for preparation of fluorescent beads and biotin conjugates to the number of wells actually used on the plate. As only one filter plate is included in the basic kit, you have to buy additional plates to use remaining reagents.
16. Because exact conditions may vary from assay to assay, standard curves must be established for every run.

17. A control sample of known concentration should be established and run as an additional control with each assay to allow estimation of the validity of the assay results.

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