Published in final edited form as:

Methods Mol Biol. 2009 ; 510: 403–413. doi:10.1007/978-1-59745-394-3_30.

Determination of Hepatitis C Virus-Specific T-Cell Activity

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Abstract

Magnitude and the breadth of T-cell responses against HCV are associated with the outcome of HCV infection. Parameters of HCV-specific T-cell responses that are frequently assessed in clinical immunological studies include proliferation of T cells in response to HCV antigens, frequency of HCV-specific T cells secreting cytokines, and changes in antigen specificity during the course of HCV infection. Common techniques for assessing these parameters, such as ³H-thymidine incorporation assay, cytokine ELISpot assay, and strategies for epitope mapping and identification of minimal optimal epitopes are outlined, and detailed protocols are described.

Keywords

hepatitis C virus; T cell; proliferation assay; ELISpot assay; epitope mapping

1. Introduction

HCV is a noncytopathic, hepatotropic virus that elicits immune-mediated liver injury. Because the persistency rate of HCV infection is as high as 75 to 85% (1), its impact on public health is considerable around the world (2).

The outcome of HCV infection is determined by viral factors and by host factors that include innate immune response and adaptive immune response (2–4). Spontaneously recovered patients usually mount vigorous multi-epitope-specific CD4⁺ and CD8⁺ T-cell responses that are readily detectable in the peripheral blood. In contrast, patients with persistent HCV infection develop late, transient, or narrowly focused T-cell responses (5–8) that select for viral escape mutations (2, 4).

Studies on HCV-specific T-cell activity have been conducted with the aim to increase our understanding of the natural course of HCV infection, the outcome of hepatitis C, and the effect of treatment. The analysis of HCV-specific T-cell responses includes the assessment (i) of T-cell proliferation in response to HCV antigens, (ii) of the frequency of cytokine-secreting HCV-specific T-cells, and (iii) of changes in T-cell specificity and viral sequence during the infection. Here, we describe methods for assessing those parameters.

Proliferation assays are methods for measuring clonal proliferation of lymphocytes in peripheral blood mononuclear cells (PBMCs) in response to HCV antigens. The ^3H -thymidine incorporation assay has been the most frequently used assay, because of extensive published data available for comparison, the small number of cells required, and its high throughput (9). The amount of proliferation is detected after culture with antigens by measurement of incorporation of ^3H -thymidine into the DNA of proliferating lymphocytes.

The enzyme-linked immunospot (ELISpot) assay is a method for identification and enumeration of cytokine-secreting T cells. The assay employs the principle of the sandwich enzyme-linked immunosorbent assay (ELISA), with some modifications for investigation of cytokine-secreting cells rather than secreted cytokines in the supernatant. The ELISpot assay has the advantage of a high sensitivity, capacity for semiautomation, and good results even when cryopreserved and thawed PBMCs are used. Its sensitivity is 10- to 100-fold higher than that of intracellular cytokine staining (ICS) and flow cytometry (9). In the ELISpot assay, cytokine-secreting T cells are cultured for 24–40 h with individual peptides, pools of overlapping peptides, and recombinant proteins in culture plates coated with cytokine-specific antibodies, so that the number of cytokine spots formed by single cytokine-secreting T cell can be determined.

The matrix approach is an epitope screening method that employs peptide pools in a matrix array in ELISpot assays (10). The identity of individual, recognized peptides can be deduced from the pattern of recognized peptide pools. Minimal optimal epitopes can subsequently be determined from the peptides by performing IFN- γ ELISpot assays with individual truncated peptides. This process can be aided by computational prediction of potential epitopes by scanning HCV polypeptide sequences for specific HLA binding motifs.

2. Materials

2.1. ^3H -Thymidine Incorporation Assay with Recombinant HCV Proteins

1. Peripheral blood mononuclear cells (PBMCs): See ref. 11 for protocols regarding isolation of PBMCs. See ref. 9 for collection, shipment, freezing, and thawing of blood samples.
2. Hepatitis C proteins: Recombinant HCV core, NS3, helicase, NS4, NS5A, NS5B proteins and protein buffer (Mikrogen, Neuried, Germany). Dilute with sterile 1 $\times$ phosphate-buffered saline (PBS; Mediatech, Herndon, VA) to 100 $\mu\text{g}/\text{mL}$.
3. Positive controls: phytohemagglutinin (PHA; Sigma, St. Louis, MO) as mitogen, tetanus toxoid (Calbiochem/EMD, San Diego, CA) as recall antigen.
4. Complete medium: RPMI 1640 medium (Mediatech) supplemented with 10% fetal bovine serum (FBS; U.S. Bio-Technologies, Pottstown, PA), 2 mM L-glutamine (Mediatech), 100 IU/mL penicillin (Mediatech), and 100 $\mu\text{g}/\text{mL}$ streptomycin (Mediatech) (see Note 1).
5. 96-well round-bottom tissue culture plate (Nunc, Roskilde, Denmark).

6. Tritiated thymidine (^3H -thymidine; MP Biomedicals, Solon, OH): To prepare a working solution, dilute stock solution with RPMI 1640 or sterile $1\times$ PBS to 50 $\mu\text{Ci/mL}$.
7. Cell harvester (PerkinElmer, Wellesley, MA).
8. 96-well harvest plate (Millipore, Bedford, MA).
9. Harvest plate sealing tapes, clear and opaque (Millipore).
10. Scintillation cocktail for microplate scintillation counters (MicroScint 40; PerkinElmer).
11. Microplate scintillation counter (TopCount; PerkinElmer).
12. Class II biological safety cabinet.

2.2. Ex vivo IFN- γ ELISpot Assay with Overlapping HCV Peptides and Recombinant Proteins

1. Overlapping HCV peptides (OLPs): 15-mer peptides overlapping by 10 amino acids and covering the complete HCV genotype 1 polyprotein sequence (Mimotopes, Raleigh, NC) (*see Note 2*). Dissolve individual OLPs with sterile dimethylsulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) at 20 mg/mL and dilute with sterile $1\times$ PBS to 1 mg/mL (1 mg OLP per 1 mL 5% DMSO/PBS). To prepare OLP pools, pool 50 μL of each OLP solution (1 mg/mL) and adjust total volume with 5% DMSO/PBS to 2100 μL (42-fold dilution). This concentration of each individual OLP in the pool is 24 $\mu\text{g/mL}$.
2. Hepatitis C proteins: Core, NS3, helicase, NS4, NS5A, NS5B and protein buffer as negative control.
3. Positive controls: PHA (mitogen) and CMV control peptide pool, EBV control peptide pool, and Influenza control peptide pool (CEF pools; recall antigens).
4. Coating antibody: anti-IFN- γ monoclonal antibody (M700A; Endogen/Pierce, Rockford, IL).
5. Detection antibody: biotinylated anti-IFN- γ monoclonal antibody (M701B; Endogen/Pierce) (*see Note 3*).
6. Alkaline phosphatase-conjugated streptavidin (streptavidin-AP; Dako, Carpinteria, CA) (*see Note 4*).
7. Alkaline phosphatase (AP) conjugates substrate kit (containing NBT/BCIP substrate; Bio-Rad, Hercules, CA).
8. Complete medium: RPMI 1640 medium supplemented with 10% human AB serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin.
9. Sterile $1\times$ PBS.
10. PBS/Tween: Tween-20 (Sigma) in $1\times$ PBS (1:2000).

11. 1% BSA/PBS: 1% bovine serum albumin (BSA; MP Biomedicals) in 1× PBS, sterile filtered.
12. 1% BSA/PBS/Tween: 1% BSA in PBS/Tween.
13. ELISpot plates (MultiScreen-IP; Millipore).
14. Automated plate washer (Nunc).
15. Squirt bottles containing 1× PBS or PBS/Tween.
16. ELISpot reader system (AID, Strassberg, Germany).
17. Class II biological safety cabinet.

2.3. Strategies for Epitope Mapping and Identification of Minimal Optimal Epitopes

1. Matrix pools of hepatitis C overlapping peptides: Pool OLPs according to a matrix map (for example, Fig. 1), following step 1 in section 2.2.
2. Materials for IFN- γ ELISpot assay in section 2.2.

3. Methods

3.1. ^3H -Thymidine Incorporation Assay with Recombinant HCV Proteins

3.1.1. Day 0: Assay Setup—Perform steps in a class II biological safety cabinet using aseptic techniques.

1. Predilute antigens to 2 $\mu\text{g}/\text{mL}$ with complete medium in sterile 1.5-mL polypropylene tubes.
2. Resuspend PBMCs at 2×10^6 cells/mL with complete medium in a sterile 15-mL polypropylene tube.
3. Add 100 μL PBMC suspension to each well (2×10^5 cells/well) (*see Note 5*).
4. Add 100 μL of each antigen dilution at a final concentration of 1 $\mu\text{g}/\text{mL}$ in quadruplicate to the 96-well round-bottom tissue culture plate.
5. Set up positive control wells using 1 $\mu\text{g}/\text{mL}$ PHA and 1 $\mu\text{g}/\text{mL}$ tetanus toxoid and negative control wells using protein buffer.
6. Incubate the plate at 37°C in a CO₂ incubator for 5 d.

3.1.2. Day 5: Pulsing ^3H -thymidine—Follow standard radiation safety procedures when working with ^3H -thymidine solution and ^3H -thymidine-labeled cells. Perform steps in a class II biological safety cabinet using aseptic techniques.

1. Pulse the plate by adding 20 $\mu\text{L}/\text{well}$ (1 $\mu\text{Ci}/\text{well}$) ^3H -thymidine.
2. Incubate the plate at 37°C in a CO₂ incubator for 18 h.

3.1.3. Day 6: Harvesting and Counting (*see Note 6*)

1. Harvest cells on a 96-well harvest plate with a cell harvester.

2. Dry the harvest plate in an oven at 56°C.
3. Seal the back of the plate with an opaque sealing tape, add 30 µL/well scintillation cocktail, and seal the front of the plate with a clear sealing tape.
4. Count the plate with a microplate scintillation counter.

3.1.4. Calculations and Reporting of Results

1. Data can be calculated and reported in two ways (*see* Note 7): (a) counts per minute (cpm) (mean cpm of experimental wells – mean cpm of background wells) and (b) Stimulation index (SI) (mean cpm of experimental wells / mean cpm of background wells).
2. On the basis of analysis of immune responses of healthy, HCV-uninfected control persons, a stimulation index of 3 to 5 is typically considered significant.

3.2. *Ex vivo* IFN- γ ELISpot Assay with HCV Overlapping Peptides and Recombinant Proteins

Ex vivo ELISpot assays for cytokines other than IFN- γ and for other T cell markers such as granzyme and TRAIL can be performed if appropriate coating and detection antibodies are used. The incubation time of the ELISpot cultured may need adjustment, depending on the cytokine to be analyzed.

3.2.1. Day –1: Coating—Perform steps in a class II biological safety cabinet using aseptic techniques.

1. Prepare coating antibody solution by adding 5 µL (5 µg) anti-IFN- γ monoclonal antibody to 10 mL sterile 1× PBS in a sterile 15-mL polypropylene tube (0.5 µg/mL final concentration) (*see* Note 3). Vortex.
2. Add 100 µL antibody solution to each well of an ELISpot plate (*see* Note 8).
3. Incubate the plate at 4°C overnight in a humidified box (*see* Note 9).

3.2.2. Day 0: Assay Setup (*see* Note 10)—Perform steps in a class II biological safety cabinet using aseptic techniques.

1. Wash the coated plate by adding 200 µL sterile 1× PBS to each well using a multichannel pipette and flicking off PBS. Repeat the wash step three more times. Blot the plate on paper towel once after the last wash.
2. Immediately after blotting, block the plate with 200 µL/well 1% BSA/PBS for 1 h at room temperature in a class II biological safety cabinet. Do not let the plate dry before adding 1% BSA/PBS (*see* Note 11).
3. Predilute antigens at 2 µg/mL in sterile 1.5-mL polypropylene tubes with complete medium. For OLP pools, use 8 µL each pool per 200 µL complete medium. Each OLP in the pool will be diluted to 2 µg/mL.
4. Resuspend PBMCs at 3×10^6 cells/mL with complete medium in a sterile 15-mL polypropylene tube.

5. Wash the plate twice with PBS.
6. Block the plate 30 min to 1 h with 200 μ L/well complete medium at room temperature in a class II biological safety cabinet.
7. Discard medium and blot the plate once on a paper towel.
8. Add 100 μ L PBMC suspension to each well (3×10^5 cells/well) (*see* Note 12).
9. Add 100 μ L of each antigen dilution at least in duplicate, preferably in triplicate to the plate. Final concentration of each antigen will become 1 μ g/mL.
10. Set up positive control wells using 1 μ g/mL PHA and 1 μ g/mL CEF control peptide pools and negative control wells using 5% DMSO/PBS and protein buffer (*see* Note 13).
11. Incubate the plate at 37°C in a CO₂ incubator for 30 h (*see* Note 14).

3.2.3. Day 1: Detection Antibody—Aseptic techniques are no longer needed from this point.

1. Prepare detection antibody solution by adding 5 μ L (2.5 μ g) biotinylated anti-IFN- γ monoclonal antibody to 10 mL 1% BSA/PBS/Tween in a 15-mL polypropylene tube (0.25 μ g/mL final concentration) (*see* Note 3). Vortex.
2. Wash the plate 3 times with PBS and 4 times with PBS/Tween using an automated plate washer (*see* Note 15).
3. Blot the plate once on a paper towel after the last wash.
4. Add 100 μ L detection antibody solution to each well.
5. Incubate the plate at 4°C overnight in a humidified box (*see* Note 16).

3.2.4. Day 2: Development

1. Wash the plate 4 times with PBS/Tween using an automated plate washer.
2. Take the back cover off the plate, and wash the back of the plate membrane once with PBS/Tween using a squirt bottle. Blot dry. Reassemble the plate.
3. Prepare streptavidin-AP solution by adding 5 μ L streptavidin-AP to 10 mL 1% BSA/PBS/Tween (1:2000 dilution).
4. Add 100 μ L streptavidin-AP solution to each well.
5. Incubate the plate at room temperature for 1 h (*see* Note 17).
6. Prepare 1 $\times$ AP development buffer by adding 400 μ L 25 $\times$ AP development buffer to 10 mL deionized water at room temperature. Vortex.
7. To prepare the AP development solution, add 100 μ L color reagent A and 100 μ L color reagent B to 10 mL 1 $\times$ AP development buffer. Vortex.
8. Allow the AP development solution to equilibrate to room temperature (*see* Note 18).

9. Wash the plate four times with PBS (no Tween-20, no BSA) using an automated plate washer (*see* Note 19).
10. Take the back cover off the plate. Rinse the back of the membrane extensively with PBS using a squirt bottle. Blot dry. Reassemble the plate.
11. Add 100 μ L AP development solution to each well.
12. Wait for about 10 to 20 min until spots appear in positive control wells.
13. Rinse the plate under running tap water.
14. Remove the back cover. Blot dry. Dry the plate completely in inverted position in a laminar flow hood for 60 to 90 min or overnight in the dark (*see* Note 20).
15. Read the plate and count the spots using an automated ELISpot reader. Spots should be evaluated on the basis of size, shape, contrast, and density.

3.2.5. Reporting of Results (*see* Note 21)

1. Data can be reported as number of spot forming cells (SFC) per 3×10^5 PBMCs.
2. Number of SFC against specific antigen can be calculated as (mean SFC of experimental wells – mean SFC of background wells).

3.3. Strategies for Epitope Mapping and Identification of Minimal Optimal Epitopes

3.3.1. Screening of Epitopes by the Matrix Approach (IFN- γ ELISpot Assay with Matrix Peptide Pools)

1. Follow the standard IFN- γ ELISpot assay protocol in section 3.2. Use matrix pools instead of individual OLPs or OLP pools.
2. Find wells showing significant numbers of spots (*see* Note 21).
3. Using the matrix map, locate specific peptides responsible for the putative responses (Fig. 1).
4. Repeat the IFN- γ ELISpot assay with the individual peptides that yielded positive responses in the matrix setting to confirm the putative response.
5. Compare the sequence of the confirmed peptides to those of previously described epitopes and identify known epitopes in the peptides. If no previously described epitopes appear among the confirmed peptides, determine the minimal optimal sequence by testing shorter, truncated peptides (Fig. 1).

3.3.2. Prediction of Minimal Optimal Epitopes with HLA Binding Motifs Databases

1. Scan the recognized peptides for HLA binding motifs using the Los Alamos National Laboratory's *HCV Databases* (http://hcv.lanl.gov/content/immuno/motif_scan/motif_scan) (*see* Note 22).
2. Find potential minimal optimal epitopes from the scan output.

3.3.3. Identification of Minimal Optimal Epitopes with Truncated Peptides

1. Synthesize truncated peptides of the confirmed peptides that contain potential epitopes.
2. Perform IFN- γ ELISpot assays using the truncated peptides and identify the shortest peptides that still induce T cell responses. These are called minimal optimal epitopes (see Note 23).

4. Notes

1. Alternatively, use human AB serum instead of FBS.
2. Alternatively use 20-mer peptides. Both 15- and 20-mer peptides can be used to detect CD4⁺ and CD8⁺ T-cell responses, because breakdown products shorter than the original peptides are typically recognized by CD8⁺ T cells, but 20-mer peptides are considered better for detection of CD4⁺ T-cell responses, whereas much shorter peptides (less than 12-mer) are considered optimal for detection of CD8⁺ T-cell responses (9).
3. Titrate each batch of coating and detection antibody to find the optimal concentration. Use a monoclonal antibody for coating to ensure specific binding to the cytokine of interest. For detection, however, use a polyclonal antibody that binds to several epitopes of the cytokine to amplify the signal.
4. Alternatively, horseradish peroxidase-conjugated streptavidin (streptavidin-HRP) and AEC substrate can be used for development, but red spots resulting from AEC fade faster than blue spots resulting from NBT/BCIP.
5. Alternatively, add as few as 5×10^4 cells/well.
6. Alternatively, harvest using glass-fiber filters and an appropriate cell harvester. After harvesting, dry the filters and punch them into scintillation vials. Add scintillation fluid to the vials and count with a scintillation counter.
7. Because different scintillation counters differ in the efficiency of counting, SI is better than cpm when data from different laboratories are to be compared, but variations in the negative control can substantially affect the SI. Low cpm of the negative control may cause a false increase of SI, and high cpm of the negative control may cause a false decrease of SI. Therefore, we recommend indicating total cpm and/or the range of cpm of the negative controls in addition to the SI in publications (9).
8. If PVDF membrane plates, such as MultiScreen-IP, are used, prewetting membranes (wells) with 35% ethanol may be worthwhile. Prewetting enhances protein binding to the membrane. Before adding the coating antibody, prewet each well with 15 μ L 35% ethanol for one minute, decant ethanol, and rinse 3 times with 200 μ L sterile 1 $\times$ PBS.

9. Alternatively, place a wet paper towel under the plate and wrap the plate with plastic wrap. Incubate at 4°C overnight. Generally, coated, wet plates can be stored at 4°C for about one week.
10. If necessary, preincubate PBMCs with antigens in a 96-well culture plate before setting up the ELISpot assay.
11. Alternatively, block wells with 200 µL/well complete medium for 2 hours at 37°C.
12. Alternatively, add 200,000 cells/well. Using fewer than 200,000 cells/well is not recommended.
13. In addition to positive control and negative control, we recommend running other controls: background control (no cells, no antigens), detection antibody control (PBS instead of detection antibody).
14. Do not disturb or move the plate during incubation. Doing so may cause streaks (cytokine trails caused by moving cells), irregular spots, or double spots. To ensure equal heat distribution, do not stack the plates during incubation. The incubation time may vary according to the cytokine to be detected. Perform a time kinetic and adjust incubation time accordingly.
15. If an automated plate washer is not available, add PBS/Tween using a multichannel pipette and completely flick off PBS/Tween.
16. Alternatively, incubate the plate at 37°C for 2 h or at room temperature for 4 h.
17. An incubation time of more than 1 h may results in increased background color. Dark background color prevents an automated ELISpot reader from recognizing spots.
18. Because alkaline phosphatase activity is reduced at low temperature, equilibration to room temperature prevents underdevelopment of the plate.
19. Washing the plates with PBS in the absence of Tween-20 is important because Tween-20 interferes with spot formation.
20. Spots may fade when they are exposed to light. The AP-developed, completely dried plate can be stored for at least six months in the dark. If AEC is used for development, the dried plate can be stored for up to 5 five mo.
21. The absolute number of spots does not always indicate a strong response, because of the possibility that the number of background spots is high. In addition, data from infected individuals should be compared with data from normal donors to reveal whether responses to specific antigens are significant. Therefore, setting a cut-off is necessary for reporting of the results. Internal cut-off values are defined as 2-fold higher than the background. External cut-off values are calculated as mean plus 2 or 3 standard deviations (SD) of number of SFC for normal donors (uninfected individuals). Ideally, both internal and external cut-off values should be used (9).

22. Alternatively, use the *SYFPEITHI Database of MHC Ligands, Peptide Motifs and Epitope Prediction* (<http://www.syfpeithi.de/>).
23. If T-cell responses to minimal optimal epitopes are strong, direct *ex vivo* ICS/flow cytometry can be performed by stimulation of PBMCs with peptides containing the novel epitopes and gating on CD4⁺ and CD8⁺ T-cells. If the T-cell responses are weak, ICS/flow cytometry can be performed with peptide-stimulated T-cell lines. HLA restriction may be determined by ICS/flow cytometry or cytotoxicity assay with partially HLA-matched and -mismatched heterologous B cell lines. For more details, see ref. 10.

Acknowledgements

This study was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

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	A	B	C	D	E	F	G	H	I	J
AA	1	2	3	4	5	6	7	8	9	10
BB	11	12	13	14	15	16	17	18	19	20
CC	21	22	23	24	25	26	27	28	29	30
DD	31	32	33	34	35	36	37	38	39	40
EE	41	42	43	44	45	46	47	48	49	50
FF	51	52	53	54	55	56	57	58	59	60
GG	61	62	63	64	65	66	67	68	69	70
HH	71	72	73	74	75	76	77	78	79	80
II	81	82	83	84	85	86	87	88	89	90
JJ	91	92	93	94	95	96	97	98	99	100

OLP 23: hvdllvgaaa**fcsamyvgdl**

OLP 24: vgaaa**fcsamyvgdl**cgsvf

OLP 25: **fcsamyvgdl**cgsvfhdvga

Fig. 1.

Matrix approach for epitope mapping. In this case, each matrix pool (A to J and AA to JJ) consists of five HCV overlapping peptides. A specific peptide is included in only one of the horizontal matrix pools (A to J) and only one of the vertical matrix pools (AA to JJ).

Therefore, if pools D and CC yield positive responses, peptide 24 triggered the response. If pools C, D, E, and CC induce formation of a significant number of spots in the ELISpot assay, the responsible epitope probably consists of the shared sequence of peptides 23, 24 and 25 (**fcsamyvgdl**).