Chapter 16

Determining How Coronaviruses Overcome the Interferon and Innate Immune Response

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Abstract

All viruses have to overcome the innate immune response in order to establish infection. Methods have been developed to assay if, and how, viruses overcome these responses, and many can be directly applied to coronaviruses. Here, in vitro methods to determine how coronaviruses overcome this response are described.

Key words Coronaviruses, Innate immunity, Interferon, Antagonism of innate immunity, Virus protein function

1 Introduction

One of the key interactions in the pathogenesis of any viral infection is that between the virus and the host innate immune response. An effective innate immune response can clear the virus before infection is established. Therefore, most viruses have evolved mechanisms to block and/or antagonize the innate immune response.

The innate immune response is the first line of defence against an invading pathogen, including viruses. Once recognized as pathogens by pattern recognition receptors (PRRs), viruses often trigger innate immune signaling pathways that lead to the production of the type I interferons (IFNs), IFNα and IFNβ. Examples of the signaling pathways that trigger these responses are the retinoic acid-inducible gene (RIG)-1 and the interferon regulatory factor (IRF)-3 pathways. RIG-I is a PRR that recognizes viral RNA (single or double stranded) carrying uncapped 5’ triphosphate groups [1]. IRF3 is a key signaling molecule in a number of virus-triggered innate signaling pathways, including the RIG-I pathway [2].

Tumor necrosis factor (TNF)-α is a cytokine induced upon viral infection and induces upregulation of various antiviral mechanisms, including apoptosis of infected cells. The TNFα signaling pathway
signals via the transcription factor, nuclear factor kappa light chain enhancer of activated B cells (NFκB).

Coronaviruses have long been studied for their ability to induce and block innate immune responses. In recent years, we have performed methods to determine the innate immune response to Middle East respiratory coronavirus (MERS-CoV) in vitro [3] and in vivo [4]. Here, methods established for MERS-CoV will be discussed, but can be applied to any other coronaviruses.

2 Materials

2.1 Cloning of Coronavirus Genes

1. Thermocycler.
2. Sample of coronavirus RNA.
3. Optional: RNA extraction reagent, e.g., TRIzol™ reagent.
4. Optional: RNA purification kit suitable for use with TRIzol™ or similar samples.
5. High-fidelity reverse transcriptase (RT)-PCR kit to convert viral RNA to cDNA.
6. Primers, as follows:
   (a) The 5′ primer is designed as follows:
       [GATC][5′ restriction site (sense)]AACATG[10–15 bases of gene (sense)].
   (b) The 3′ primer is designed as follows:
       [GATC][3′ restriction site (antisense)][10–15 bases of gene (antisense)].
7. Taq DNA polymerase kit for the amplification of cDNA.
8. Agarose.
9. 50× TAE buffer: 50 mM EDTA, 2 M Tris, 1 M glacial acetic acid. Also commercially available.
10. Microwave.
11. Agarose gel apparatus.
12. Gel extraction kit.
13. Restriction enzymes and buffers corresponding to restriction sites designed in primers.
14. DNA Ligase.
15. Competent bacteria.
16. Materials for transformation of bacteria (e.g., by heat shock or electroporation).
17. LB-agar plates containing suitable antibiotic for selection.
18. Mini and Maxiprep kits.
2.2 Luciferase Assays for Innate Immune Signaling

1. HEK293T cells.
2. HEK293T cell media: Dulbecco’s modified Eagles media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.
3. Cell culture plates.
4. 37 °C CO₂ incubator.
5. Plasmids as follows:
   (a) Reporter plasmids: a plasmid containing the luciferase gene under the control of either an IFNβ promoter or an NFκB promoter.
   (b) Plasmid containing MERS-CoV gene (or empty plasmid).
   (c) Inducer plasmids: a plasmid containing N-RIG-I or IRF-3.
6. Transfection reagent. Various methods and commercially available kits are available and HKE293T cells are relatively easy to transfect.
7. Recombinant TNFα.
8. A luciferase reporter assay. Various commercial options are available.

2.3 Virology Assay

1. HIVΔVpu containing plasmid.
2. HEK293T cells.
3. HEK293T cell media: Dulbecco’s modified Eagles media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.
4. Cell culture plates.
5. 37 °C CO₂ incubator.
6. Plasmid containing MERS-CoV gene (or empty plasmid).
7. Transfection reagent.
8. HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit.
9. Plate reader capable of reading ELISA output.

3 Methods

In any discussion of the innate immune response, it is always worth noting that the immune response is complex and can, often, only be seen in full in animal models. However, methods in this area are necessarily highly specific for specific coronaviruses and hosts. For example, we were able to use a novel mouse model to determine the immune cell infiltration and immune gene activation induced by MERS-CoV infection [4]. Here, methods for the in vitro assessment of virus: host interactions are described.
3.1 Cloning of Individual Coronavirus Genes into Plasmids

To determine specific virus: host interactions, it is usually necessary to clone and express individual coronavirus genes into expression plasmids. We have had much success in this with MERS-CoV [3]. This method used the AAV-CAGGS-EGFP expression plasmid, but others can be used.

1. Obtain a sample of MERS-CoV RNA.
   (a) Commercially available RNA samples, such as from the American Type Culture Collection.
   (b) RNA extraction from MERS-CoV infected cells using, e.g., TRIzol™ reagent (or similar) according to the manufacturers’ instructions. Infection with MERS-CoV requires containment level (CL) 3 and has been described previously [5].

2. Perform cDNA synthesis using commercially available high-fidelity RT-PCR kit.

3. Perform DNA synthesis using primers designed to amplify gene of interest and allow insertion into plasmid.

4. Run DNA mixture on 1% agarose (in 1× TAE buffer) gel using electrophoresis.

5. Cut out band that corresponds to expected size of target gene and purify using a gel extraction kit, following manufacturer’s instructions.

6. Cut out band and plasmid using appropriate restriction enzymes and buffers.

7. Run DNA mixtures on 1% agarose gel using electrophoresis.

8. Cut out band that corresponds to expected size of target gene and the empty plasmid and purify using a gel extraction kit, following manufacturer’s instructions.

9. Ligate gene into plasmid.

10. Expand plasmid by bacterial transformation and miniprep methods using a suitable antibiotic for selection.

11. Verify plasmid by sequencing.

12. Expand plasmid using maxiprep methods.

3.2 Luciferase Assays for Innate Immune Signaling

To determine the inhibition of innate immune signaling, reporter assays can be used. We have reported assays for three innate immune signaling pathways: RIG-I-dependent IFNβ promoter activation, IRF-3-dependent IFNβ promoter activation, and TNF-α-dependent NFKB promoter activation [3].

1. Seed HEK293T cells into 48-well plates at a density sufficient to achieve 90–95% confluency the following day.

2. Culture overnight at 37 °C in 5% CO₂.
3. Co-transfect cells with the following plasmids (a total of 600 ng of plasmid DNA) using an appropriate transfection reagent (see Note 1):
   (a) 200 ng luciferase reporter plasmid.
   (b) 200 ng viral gene expression plasmid or empty vector control.
   (c) 200 ng inducer plasmid (if assaying IFNβ promoter activation).

4. Culture for 18 h at 37 °C in 5% CO₂.

5. For TNFα-dependent NFκB promoter activation only: treat cells with 30 ng recombinant TNFα for 6 h.

6. Perform luciferase assay using the luciferase reporter assay according to the manufacturers’ instructions (see Note 2).

7. Calculate the relative luciferase expression in viral gene expressing cells compared to the empty plasmid controls.

3.3 Virology Assays Using Sensitive Viruses

A valuable tool in the determination of the function of viral proteins is to use reporter viruses that are blocked by the pathway of interest. A good example of this is the use of HIVΔVpu that is restricted by the anti-viral restriction factor, tetherin [6].

HIVΔVpu has been used to identify viral proteins as antagonists of tetherin, or at least that a virus encodes at least one tetherin antagonist [7]. Because tetherin tethers HIV virions to the cell surface, cell-associated HIVΔVpu will be increased, and supernatant HIVΔVpu will be lower in tetherin-positive cells compared to tetherin-negative cells. HIV concentration can be easily quantified using commercially available assays for HIV p24.

3.3.1 HIVΔVpu Stock Generation

1. Seed HEK293T cells in 10 cm plates at a density sufficient to achieve 90–95% confluency the following day.
2. Culture overnight at 37 °C in 5% CO₂.
3. Transfect cells with plasmid containing HIVΔVpu (see Note 3).
4. Replace media 6–18 h after transfection.
5. Culture for 2 days at 37 °C in 5% CO₂.
6. Harvest supernatant from transfected cells, clarify by centrifugation at 500 × g for 5 min, aliquot, and store at −80 °C.
7. Determine titer of HIVΔVpu stock (see Notes 4 and 5).

3.3.2 Virology Assay

1. Seed a tetherin-positive, HIV-sensitive cell line (this may require transfection/transduction of cells with tetherin and/or CD4) into 24-well plates (see Note 6).
2. Culture overnight at 37 °C in 5% CO₂.
3. Transfect cells with MERS-CoV gene expression plasmid or an empty plasmid control.
4. Culture overnight at 37 °C in 5% CO₂.
5. Infect cells with a fixed titer of HIVΔVpu (see Note 7).
6. Culture for up to 3 days at 37 °C in 5% CO₂ (see Note 7).
7. Harvest supernatant and cell lysate from infected cells.
8. Quantify the levels of HIV p24 using commercially available ELISA assays (see Note 5).
9. Compare supernatant levels of HIV-1 p24 in cells expressing coronavirus proteins to the empty vector control.

3.4 Coronavirus Infectious Clones

Instead of using a true reporter virus, such as HIVΔVpu, it is possible to use coronavirus mutants directly, as in Taylor et al. [8]. Infectious clones have been synthesized for various coronaviruses, and methods for these have been described elsewhere for MERS-CoV [9, 10]. When mutants are created, these can be used in lieu of the HIVΔVpu described above, along with appropriate methods of quantification of output [5]. However, this method relies on the ability to handle viruses at the full containment level of the parent virus (e.g., CL3 for MERS-CoV), so may be inappropriate.

4 Notes

1. Methods of transfection are deliberately not discussed as these vary significantly by laboratory. HEK293T cells are very easy to transfect and a wide range of methods work with these cells.
2. There are various commercially available luciferase assays. Luciferase reagent can also be “homemade,” and recipes are available online.
3. One commercially available example of a HIVΔVpu strain is pNL-U35 available from the NIH AIDS Reagent Program.
4. There are various published methods for quantifying HIV concentration using commercially available reagents—including p24 ELISA (see Note 5) or GHOST cell titration [11].
5. When purchasing HIV-1 p24 ELISA, take note of the upper and lower limits of the assay, as some are highly sensitive, but may have too low an upper detection limit. In contrast, the AIDS Vaccine Program produces an ELISA with a wide range of detection, but is not as useful at low concentrations.
6. One example of a constitutively tetherin-positive cell line is HeLa cells [6].
7. Exact titer and time points will depend on infectibility of cells and sensitivity of p24 assay (see Note 5) and should be worked out in preliminary experiments.
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