Growth and Quantification of MERS-CoV Infection

Christopher M. Coleman¹ and Matthew B. Frieman¹

¹University of Maryland School of Medicine, Microbiology and Immunology, Baltimore, Maryland

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging highly pathogenic respiratory virus. Although MERS-CoV only emerged in 2012, we and others have developed assays to grow and quantify infectious MERS-CoV and RNA products of replication in vitro. MERS-CoV is able to infect a range of cell types, but replicates to high titers in Vero E6 cells. Protocols for the propagation and quantification of MERS-CoV are presented. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

MERS-CoV was first identified and described in November 2012 (van Boheemen et al., 2012; Zaki et al., 2012) from a case of pneumonia in Saudi Arabia. MERS-CoV is an enveloped virus containing a 30-kb single-stranded, positive-sense RNA genome. Genomic analysis revealed that MERS-CoV is a novel member of the Coronaviridae closely related to two known bat coronaviruses (BtCoV), BtCoV-HKU4 and BtCoV-HKU5, so MERS-CoV has been placed with these viruses in lineage C of the Betacoronavirus genus (van Boheemen et al., 2012).

The development of assays for growth and quantification of MERS-CoV has been helped by previous work on other human and animal coronaviruses. In the wake of the outbreak of severe acute respiratory syndrome (SARS)-CoV in 2003, a number of quantitative methods were developed for SARS-CoV, and these have been used as a starting point for development of assays for MERS-CoV.

MERS-CoV readily infects a range of cell types (Chan et al., 2013), making it possible to develop assays for MERS-CoV in vitro. While MERS-CoV does not replicate in small animals (Coleman et al., 2013; de Wit et al., 2013a), it does replicate in rhesus macaques (de Wit et al., 2013b) and marmosets (Falzarano et al., 2014). However, these are much more difficult and expensive animals to handle, so the ability to test the efficacy of these assays on samples from in vivo MERS-CoV infection is limited. Here we describe methods for growing (Basic Protocol 1) and quantifying (Basic Protocols 2-4) MERS-CoV in vitro.

CAUTION: MERS-CoV is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See UNIT 1 A.1 and other pertinent resources (APPENDIX 1B) for more information.
GROWTH OF MERS-CoV

MERS-CoV productively infects a number of cell lines. New stocks of MERS-CoV can be created by simple infection and collection of supernatant. However, it is important to note that different strains of MERS-CoV may grow at different rates, which affects the point at which supernatant should be harvested for maximum yield. MERS-CoV causes damage to and, ultimately, death of infected cells, therefore a good guide to the success of the virus growth is observation of cell death, termed the cytopathic effect (CPE).

Materials

- Vero E6 cells (ATCC #1568)
  - *Keep aliquots of Vero E6 cells frozen in liquid nitrogen.*
- Vero E6 cell growth medium (see recipe)
- MERS-CoV (GenBank accession #KC776174.1, MERS-CoV-Hu/Jordan-N3/2012)
  - MERS-CoV can be obtained as a frozen stock from Kanta Subbarao (NIH, Bethesda, MD).
- 175-cm² (T-175) tissue culture flasks
- 1.5-ml screw-cap tubes

Additional reagents and equipment for basic cell culture techniques including trypsinization (Phelan, 2006)

1. Remove Vero E6 cells from long-term storage, and, using basic cell culture methods, maintain cells in continuous culture for at least 1 week before use. Trypsinize (Phelan, 2006) and resuspend cells in Vero E6 cell growth medium.
2. Seed approximately $1 \times 10^7$ Vero E6 cells into a T-175 flask so that they will be 90% to 95% confluent the following day.
3. Culture overnight at 37°C in 5% CO₂.
4. Remove medium so that 5 ml remains.
5. Thaw MERS-CoV at 37°C just prior to use. Add 1 ml MERS-CoV to the flask.
  - *For virus growth experiments, add an MOI (multiplicity of infection) of 3 to the plated cells (the volume will depend on the virus titer).*
6. Distribute virus over cells and incubate for 1 hr at 37°C in 5% CO₂.
7. Replenish medium up to a total volume of 20 ml.
8. Incubate flask for 48 to 72 hr at 37°C in 5% CO₂, or until significant CPE is observed.
  - *The appearance of CPE can be strain specific or dependent on the starting titer of the seed stock. We observed significant CPE with MERS-EMC at 48 hr post-infection, and significant CPE with MERS-Jordan at 72 hr post-infection. We would recommend checking the flask daily from 48 hr post-infection onwards.*
9. Collect supernatant from the infected flask and centrifuge 5 min at 500 × g, room temperature, to remove any cellular debris.
10. Aliquot appropriate volumes (100 to 1000 μl) of the clarified supernatant (without any cryo-additives) into 1.5-ml screw-cap tubes and store at −80°C until needed.
  - *The titer will be unknown at this stage; however, we find that 100 μl aliquots are efficient for small volume (see the next note on freeze-thawing of stocks) and 1000 μl aliquots are sufficient for re-infection of T175 flasks for further rounds of propagation.*
Each tube should only be used once for subsequent infections, because multiple freeze–thaw cycles can significantly decrease the infectious titer. Tubes containing 1 ml of supernatant are useful for re-infecting for the next round of growth, though bear in mind that MERS-CoV—in common with all RNA viruses—will mutate as it is passaged.

**QUANTIFICATION OF MERS-CoV BY TCID\textsubscript{50}**

The tissue culture infective dose that causes 50% cytotoxicity (TCID\textsubscript{50}) assay is a quantitative method for assessing the infectivity of a virus stock. One TCID\textsubscript{50} is defined as the amount of pathogen that causes death of 50% of cells (Reed and Muench, 1938), so TCID\textsubscript{50} depends on the ability of the virus to kill the cells in culture. Here we describe a method for crystal violet staining of a TCID\textsubscript{50}. However, there are other vital cell dyes or methods for determining cell viability that can be used instead.

**Materials**

- Vero E6 cells (ATCC #1568)
  - Keep aliquots of Vero E6 cells frozen in liquid nitrogen.
- Vero E6 cell growth medium (see recipe)
- MERS-CoV (see Basic Protocol 1)
- 4% paraformaldehyde in H\textsubscript{2}O
- 0.05% (w/v) crystal violet in 20% methanol
- Tissue culture-treated flat-bottomed 96-well plates
  - Optional: Untreated round-bottomed 96-well plates for sample dilution
  - Optional: Light box to view the plates
- Additional reagents and equipment for basic cell culture techniques (Phelan, 2006)

1. Resuspend $1 \times 10^6$ cells in 10 ml Vero E6 cell growth medium per 96-well plate to be used. Seed $1 \times 10^4$ Vero E6 cells per well into 96-well plates in normal Vero E6 cell growth medium and culture overnight at 37°C in 5% CO\textsubscript{2}.

   *Phelan (2006) describes basic cell culture techniques.*

2. Thaw MERS-CoV at 37°C just prior to use. Dilute MERS-CoV sample(s) in Vero E6 cell growth medium, and then continue diluting down a 5- or 10-fold dilution series, ideally in duplicate or triplicate.

   *For ease, viral samples can be diluted in a duplicate 96-well plate without cells. Change tips between each dilution.*

3. Transfer 100 μl diluted MERS-CoV sample to each well of cells in the 96-well plate.

   *The same tip(s) can be used when going up the dilution series.*

4. Incubate cells with the MERS-CoV sample dilutions for 48 to 96 hr.

   *CPE can be strain specific. We observed the best TCID\textsubscript{50} results at 48 hr post-infection for MERS-EMC and 96 hr for MERS-Jordan.*

5. Remove medium from cells and fix cells in 100 μl ice-cold 4% paraformaldehyde for 5 min at room temperature.

6. Remove paraformaldehyde and stain cells with 100 μl 0.05% (w/v) crystal violet stain in 20% methanol for 30 min at room temperature.

7. Wash cells twice in tap water.

   *BSL-3 regulations require that this be done by adding 100 μl of tap water to each well and then removing into an appropriate disinfecting solution.*
8. Tap plate dry on a tissue.

9. Examine plate and score wells.

   Each well should be scored as **positive** for MERS-CoV (i.e., MERS-CoV has killed all of the cells, so there is no crystal violet stain) or **negative** for MERS-CoV (i.e., the cells have all survived, so the well is stained violet).

   The crystal violet stain should be visible to the naked eye, but a light box is useful to discriminate wells that have light staining.

10. Calculate TCID\textsubscript{50} using the following formula:

\[
\log_{10} \text{TCID}_{50} = 2 \times [X_p + (0.5 \times D) - (D \times S_p)]
\]

   where:

   \(X_p\) = last sample where all sample replicates are positive

   \(D\) = \(\log_{10}\) of serum dilution

   \(S_p\) = sum of the proportion of replicates at all dilutions where positives are seen (starting with the \(X_p\) dilution)

   There are TCID\textsubscript{50} calculators available online.

**QUANTIFICATION OF MERS-CoV BY PLAQUE ASSAY**

Plaque assays, which are used to quantify a wide range of viruses, depend on the ability of a virus to cause cell lysis and spread to neighboring cells, both of which MERS-CoV is able to do. The basic premise of a plaque assay is that the virus infects a single cell and then spreads out in a radial pattern from that initial infection. The radial spreading occurs if the medium covering the cells is semi-solid, thereby preventing free diffusion of the virus in the medium. The area of cell death caused by this spread is named a plaque, and each plaque can be assumed to have developed from a single cell infection by a single virion, hence the titer from this assay is in plaque forming units (pfu) per ml.

**Materials**

- Vero E6 cells (ATCC #1568)

  *Keep aliquots of Vero E6 cells frozen in liquid nitrogen.*

- Vero E6 cell growth medium (see recipe)

- MERS-CoV (see Basic Protocol 1)

- Phosphate-buffered saline (PBS; APPENDIX 2A)

- 2× supplemented MEM (see recipe)

- 1.6% agarose, suitable for tissue culture (see recipe)

  *Optional:* 0.5% neutral red in PBS with 0.85% NaCl

- Tissue culture-treated flat-bottomed 6-well plates

- Titer tubes or other tubes suitable for sample dilution

**Additional reagents and equipment for basic cell culture techniques (Phelan, 2006)**

1. Seed Vero E6 cells in 6-well plates in 3 ml Vero E6 cell growth medium and culture overnight at 37°C in 5% CO\textsubscript{2}.

   *Seed one plate per viral sample.*

   *Phelan (2006) describes basic cell culture techniques.*

2. Dilute MERS-CoV samples in PBS down a 10-fold dilution series, giving enough to add 200 μl per remaining volume.
3. Add 200 μl of each dilution to one well of a 6-well plate.
4. Incubate plate at 37°C in 5% CO₂ for 1 hr, shaking every 15 min.
5. Mix the 2 × supplemented MEM with 1.6% melted agarose in a 1:1 ratio, and allow the mixture to cool at room temperature for 2 min.
6. Add 3 ml of MEM–agarose mix into each well of the 6-well plates.
   
   Work quickly, but smoothly, to ensure the agarose mix does not set prior to addition.
7. Incubate plates at 37°C in 5% CO₂ for 3 days.
   
   Optional: To aid in visualizing the plaques, add 3 ml 0.5% neutral red to each well and incubate for 1 to 2 hr.
8. Visualize and count plaques. Count plates that contain 10 to 100 plaques.
   
   For those used to counting plaques of SARS-CoV, MERS-CoV are smaller and more difficult to make out.
9. Calculate titer in pfu/ml using the following formula:

   \[ \text{titer (pfu/ml)} = \frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume of virus added to cells in a well (ml)}} \]

QUANTIFICATION OF MERS-CoV RNA PRODUCTS OF REPLICATION

Coronaviruses contain a single-stranded positive-sense RNA genome, which is transcribed into negative-sense RNA intermediates for replication and protein production. For basic coronavirus detection, primers can be designed to part of the genomic RNA, which will detect both the genomic RNA and the corresponding mRNA for that open reading frame (ORF).

Coronavirus mRNA is made as a sub-genomic positive-sense RNA that contains a common 5’ primer leader sequence derived from the 5’ end of the genomic RNA, followed by the ORF of the viral gene (reviewed in Pasternak et al., 2006). Therefore, it is possible to discriminate coronavirus mRNA from genomic RNA by PCR by designing the following primers: (1) a forward primer containing part of the leader sequence, and (2) a reverse primer in a gene that is not immediately 3’ to the leader sequence, so that the genomic RNA is not detected (i.e., anything other than the sequence for the ORF1a replicase polyprotein).

MERS-CoV is currently not classified as a Select Agent, which means that RNA-containing solutions from MERS-CoV infected cells can be handled under BSL-2 conditions as long as it is confirmed that all live MERS-CoV have been destroyed. For this assay, we have designed TaqMan primer/probe sets to an area upstream of the MERS-CoV envelope gene (UpE), to the membrane protein (M) mRNA are shown in Table 15E.2.1. An appropriately labeled endogenous control can also be added to the mix to make a triplex assay. For example, we have successfully used an ABY-labeled human transferrin receptor protein 1 (TFRC) primer/probe set on human and monkey samples.

Materials

Cell culture extract or homogenized tissue in Trizol (or similar) from which RNA will be obtained
RNA extraction reagents or kit
Optional: Reagents for cDNA synthesis (e.g., RevertAid reverse transcriptase; Thermo Scientific)
MERS-CoV primer/probe sets described in Table 15E.2.1
TaqMan master mix
Table 15E.2.1 Primer and Probe Sequences for Detection of MERS-CoV UpE, MERS-CoV M mRNA and Human TFRC

| MERS-CoV UpE | Forward | GCAACGCAGCAATTCAGTT |
| MERS-CoV M mRNA | Forward (Leader sequence) | CTATCTCATTCCCTGTCCTC |
| | Reverse | GGAAGATGGGCCATAGGAGCC |
| Example endogenous control (human TFRC) | Forward | TGGTACCAGCAACTTCAAGGTT |
| | Reverse | CCGGATGCTTCACATTTTGCA |
| | Probe (FAM)CTGAGGCGCAGATTATTGCC(QSY) |
| | Probe (VIC)CTCTTCACATAATCGCCCCGAGCTCG(QSY) |
| | Probe (ABY)CTGCCAGCCAACTGTTGTATACGC(QSY) |

*Probes dyes can be switched and/or replaced with another appropriate dye as needed.

Optical 96-well plates for PCR, and sealing tape
qPCR machine capable of reading at least 3 TaqMan probe dyes

*The method in this protocol utilizes ABY, FAM, and VIC probe dyes, however, other dye combinations may be used based on the investigator’s preference.*

1. Extract RNA from sample according to the instructions provided with RNA extraction reagent.

*There are multiple methods available for RNA extraction, the only requirement being that it is of sufficient quality for PCR. For Trizol reagent samples, we have used this method with RNA isolated by chloroform extraction according to the manufacturer’s instructions. Or, for example, the PureLink RNA mini kit (Life Technologies) is a column-based kit that can be used with Trizol samples.*

2. Set up reactions in an optical 96-well plate according to the manufacturer’s instructions.

*We use TaqMan Fast Virus 1-Step Master Mix (Life Technologies), which works with RNA directly and does not require a separate cDNA synthesis step. If your TaqMan procedure requires that you use cDNA, perform cDNA synthesis according to the manufacturer’s instructions before setting up the PCR reactions.*

3. Run on a PCR machine capable of reading FAM, VIC and ABY. Temperature and cycle time run parameters may vary with different TaqMan reagents, so follow instructions provided with the reagent.

*We have successfully used the primers and probes described in Table 15E.2.1 using the Taqman Fast Virus 1-Step Master Mix without modification; specifically, reverse transcription at 50°C for 5 min; inactivation of reverse transcriptase and initial denaturation at 95°C for 20 sec; a 40-cycle amplification at 95°C for 3 sec followed by 60°C for 30 sec.*

4. Calculate ΔΔCt using the following calculation.

a. Determine ΔCt.

ΔCt[MERS-CoV UpE or M mRNA] – ΔCt[endogenous control]

b. Determine ΔΔCt.

ΔCt[positive or negative control sample] – ΔCt[sample of interest]
c. Transform to relative expression.

Relative MERS-CoV expression (compared to mock control) = $2^{\Delta \Delta C_t}$

Readout is the relative expression of MERS-CoV UpE or M mRNA compared to control.

**REAGENTS AND SOLUTIONS**

*Use tissue–culture grade water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Agarose, 1.6%**

Add 1.6% powdered agarose to H$_2$O. Microwave the agarose solution for 1 to 2 min to melt the agarose, and place in a 65°C water bath to maintain as a liquid until ready for use.

**MEM with phenol red, 2×**

Minimum essential medium (MEM)
20% heat-inactivated FBS
2% L-glutamine
2% penicillin/streptomycin
1.5 ml phenol red
Store at 4°C for ≤6 months

*Medium that contains phenol red is usually at 1× concentration, so mixing it with the agar will cause the phenol red concentration in the medium to be too dilute. Therefore, we prefer to purchase phenol red-free medium and supplement it with 2× phenol red.*

**Vero E6 cell growth medium**

Minimal essential medium (MEM)
10% heat-inactivated FBS
1% L-glutamine
1% penicillin/streptomycin
Store at 4°C for ≤6 months

**COMMENTARY**

**Background Information**

ERS-CoV is an emerging human beta-coronaviruses that was first detected in Saudia Arabia in November 2012 (Zaki et al., 2012). As of December 2014, there have been 904 confirmed MERS-CoV cases, with 347 deaths. MERS-CoV infections have been detected in 23 countries, primarily in the Middle East, but also in Europe, Southeast Asia, and North America ([http://www.coronamap.com](http://www.coronamap.com)).

Members of the *Coronaviridae* virus family are enveloped viruses with a large single-stranded positive-sense RNA genome. The coronavirus genome encodes the membrane (M), spike (S), envelope (E), and nucleocapsid (N) structural proteins; the ORF1a and ORF1b replicase polyproteins; and one to eight accessory proteins that perform important functions in coronavirus replication and pathogenesis in vivo, such as blocking innate immune signaling pathways (Frieman and Baric, 2008). In the case of MERS-CoV, there are five accessory proteins (van Boheemen et al., 2012) for which mechanisms of action have begun to be characterized (Niemeyer et al., 2013; Yang et al., 2013; Matthews et al., 2014; Siu et al., 2014).

MERS-CoV is not the first highly pathogenic coronavirus to emerge, or indeed the first pathogenic coronavirus. In 2002, highly pathogenic SARS-CoV emerged in China and spread around the world before disappearing again in 2003. There are also less pathogenic human coronaviruses, such as hCoV-nl-63 and hCoV-OC43. Therefore, the coronaviruses have become an important group of viruses for scientific research. Given the background of coronavirus research and that MERS-CoV replicates in a wide range of cell types in vitro, assays for MERS-CoV growth and quantification have been rapidly developed.
Troubleshooting

Tissue culture problems

MERS-CoV relies on healthy cells in order to propagate, so any issues with cell culture can dramatically affect the MERS-CoV yield. Bacterial and fungal contamination of cell culture media can be avoided by adding antibiotics (e.g., penicillin and streptomycin) and/or anti-fungals to the medium. Observing proper aseptic tissue culture technique, such as wearing gloves and other appropriate personal protective equipment, spraying surfaces with 70% ethanol, and not waving hands over uncapped tubes or tissue culture bottles, should reduce contamination. Stored cell culture media should be regularly inspected for signs of contamination (cloudiness or fungal outgrowth) and disposed of if found to be contaminated.

Vero E6 cells do not overgrow plates as readily as other cell types, because once they become confluent, cell division slows down. However, if you neglect them, they will overgrow and die; so, remain vigilant with cells in culture, and if they are over-confluent prior to infection, re-seed a fresh flask or plate of cells.

No detectable MERS-CoV by TCID_{50} assay (Basic Protocol 2)

We have found that the TCID_{50} assay (Basic Protocol 2) is significantly less sensitive than the plaque assay (Basic Protocol 3) for detection of MERS-CoV (see Anticipated Results). So, if a given MERS-CoV preparation does not have detectable cell death by Basic Protocol 2, we recommend performing the plaque assay before concluding that there is no MERS-CoV present.

Poor quality RNA: No detectable endogenous control in Basic Protocol 4

Input RNA of good quality is required for Basic Protocol 4. The endogenous control in this assay should always be detectable, and thus is a good proxy for assessing whether the quality of the RNA is good enough.

When handling RNA or RNA-containing solutions, ensure that the workspace, equipment (e.g., filtered pipette tips and gloves), and solutions (e.g., water for resuspension) are certified RNase-free, or are first cleaned in 70% ethanol or an RNase-removing cleaning solution.

Anticipated Results

MERS-CoV yields of 1 × 10^7 to 1 × 10^8 pfu/ml are typically obtained from Basic Protocol 1. When comparing MERS-CoV titers determined using Basic Protocol 2 and Basic Protocol 3, we have determined that the TCID_{50} is approximately 1000- to 10,000-fold less sensitive than the plaque assay, that is, a MERS-CoV stock of 2 × 10^6 TCID_{50}/ml from Basic Protocol 2 might have 1 × 10^3 pfu/ml in Basic Protocol 3.

The MERS-CoV RNA detection assay described in Basic Protocol 4 is very sensitive and we have been able to detect MERS-CoV RNA in cells that are less susceptible to MERS-CoV.

Time Considerations

All protocols (Basic Protocols 1, 2, and 3) involving the handling of live MERS-CoV must be completed under BSL-3 conditions. Preparing to enter a BSL-3 environment can take 10 to 20 min, because careful preparation is required to collect the reagents, equipment, and cells to be taken into the BSL-3 laboratory. Under current regulations, Basic Protocol 4 can be performed under BSL-2 conditions once the Trizol has been harvested from cells. If MERS-CoV becomes a Select Agent, however, then MERS-CoV RNA will have to be handled under BSL-3 or Select Agent BSL-2 conditions, which will add time to Basic Protocol 4.

For Basic Protocols 1, 2, and 3, the greatest amount of time will be spent waiting for the cytopathic effect (CPE) in infected cells, which can take 3 to 4 days for MERS-CoV, depending on the strain. However, for MERS-CoV replication assays, we have detected infectious, though low titer, MERS-CoV in the supernatant from infected cells as early as 24 hr post-infection. For Basic Protocol 4, we have been able to detect MERS-CoV products of replication as early as 12 hr post-infection in MERS-CoV infected cells.

The infection of cells for Basic Protocol 1 should take no longer 90 min, with the majority of the time taken up with the 1-hr initial infection. Harvesting of virus in Protocol 1 includes aspiration of medium, centrifugation, and aliquotting into appropriate tubes, which takes around 60 min total. The dilutions required for Basic Protocols 2 and 3 can take anywhere from 10 to 60 min, depending on the number of samples. A multichannel pipettor significantly shortens the time that would otherwise be required to do each sample individually. Basic Protocol 3 requires a 1-hr incubation with MERS-CoV prior to adding the agarose gel solution to the plates. The crystal violet stain for Basic Protocol 2 takes 1 to
2 hr, depending on the number of plates, including the 30 min required for the stain itself. Reading of plaques for Basic Protocol 3 takes approximately 10 to 30 min, depending on the number of plates.

RNA isolations for Basic Protocol 4 can take anywhere from 10 min to 2.5 hr depending on the number of samples and the RNA isolation method used. If cDNA is required prior to PCR in Basic Protocol 4, this can take approximately 2 hr, depending on the number of samples and reagents used. The resulting cDNA can be left at 4°C at least overnight, so the reaction can be set up late in the day and left overnight with an infinite 4°C step as the last step. Similarly, preparing and running the PCR plate for Basic Protocol 4 can take anywhere from 1.5 to 3 hr, depending on the number of samples and the Taqman reagent used.

Acknowledgements

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