Practical Guidelines for Collection, Manipulation and Inactivation of SARS-CoV-2 and COVID-19 Clinical Specimens

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SARS-CoV-2 is a novel coronavirus that causes the acute respiratory disease—Coronavirus disease 2019 (COVID-19)—which has led to a global health crisis. Currently, no prophylactics or therapies exist to control virus spread or mitigate the disease. Thus, the risk of infection for physicians and scientists is high, requiring work to be conducted in Biosafety Level-3 (BSL-3) facilities if virus will be isolated or propagated. However, inactivation of the virus can enable safe handling at a reduced biosafety level, making samples accessible to a diverse array of institutions and investigators. Institutions of all types have an immediate need for guidelines that outline safe collection, handling, and inactivation of samples suspected to contain active virus. Here we provide a practical guide for physicians and researchers wishing to work with materials from patients who are COVID-19 positive or suspected positive. © 2020 Wiley Periodicals LLC.

Basic Protocol 1: Practical guidelines for the safe collection and handling of specimens collected from COVID-19 and suspected COVID-19 patients
Basic Protocol 2: Inactivating SARS-CoV-2

Keywords: SARS-CoV-2 • COVID-19 • safety • plaque assay

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INTRODUCTION

Careful handling of samples from *in vitro* and *in vivo* studies that use infectious SARS-CoV-2 virus, as well as samples collected from suspected and/or confirmed COVID-19 patients, is critical to ensure safety of research personnel, prevent accidental release into the environment, and protect the community surrounding the research entity. A risk assessment must be performed, and appropriate mitigation measures identified as part of an institution's comprehensive biosafety program before beginning work with human pathogens. The NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (National Institutes of Health) classifies commonly circulating human coronaviruses (e.g. alpha coronaviruses 229E and NL63 and beta coronaviruses

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OC43, and HKU1), other than SARS-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome virus (MERS-CoV), as Risk Group 2 infectious agents. However, current risk assessment guidance from the NIH and CDC (CDC, 2019) recommends that manipulation of both SARS-CoV and MERS-CoV be performed using BSL-3 and ABSL-3 facilities and work practices, as both of these coronaviruses can cause moderate to severe disease in healthy adults. In addition, due to the potential for person-to-person transmission, capability of producing severe disease, and potential risk to the health and safety of humans, the Federal Select Agent Program regulates SARS-CoV as a Select Agent and Toxin (Federal Select Agent Program, 2020). The novel 2019 coronavirus, designated as SARS-CoV-2, seems to be similar to SARS-CoV, with documented cases of person-to-person transmission (Ghinai et al., 2020; Li et al., 2020) and potential to cause severe disease (COVID-19) in healthy adults. Laboratory biosafety guidance continues to evolve rapidly, however; current CDC, NIH, and WHO guidelines recommend that isolation and culture of SARS-CoV-2, and/or intentional infection of animals, be performed under BSL-3 and ABSL-3 (CDC, 2019). However, there is great need for research with samples isolated from COVID-19 patients, and for practical biosafety recommendations to facilitate this work.

Procedures that involve or have the potential to result in isolation or propagation of SARS-CoV-2 virus, or intentional infection of animal models, should be performed at BSL-3/ABSL-3, as currently recommended by CDC guidance (CDC, 2020b). The CDC and WHO have generated detailed guidance for clinical diagnostic laboratories (CDC, 2020b) , but this guidance is not always directly applicable to research laboratories, particularly in the case of research procedures that are difficult to perform inside of a primary containment device (e.g. imaging and flow cytometry studies). In many cases, handling of clinical specimens from suspected or confirmed COVID-19 patients can be performed in BSL-2 facilities with some enhanced work practices (CDC, 2020b; Iwen, Stiles, & Pentella, 2020), including some of the following types of work: processing, aliquoting, or preparing specimens for downstream assays or storage, chemical or heat fixation of specimens, extraction of nucleic acids, preparation of inactivated specimens, and performing diagnostic tests (e.g. serology) that do not involve potential propagation or concentration of virus.

Once COVID-19 patient samples have been appropriately inactivated, or nucleic acids have been extracted from the sample, most laboratory procedures can be performed safely at BSL-2. Traditional fixatives used for laboratory and clinical specimens will generally inactivate the virus by destroying proteins and the lipid envelope (Goldstein et al., 2007; Lehmann & Kreipe, 2001; McKinney, Moon, Kulesh, Larsen, & Schoepp, 2009; Start, Cross, & Smith, 1992). However, the viral genomic material generally remains intact. Since SARS-CoV-2 is a positive-sense single-stranded RNA virus, it is critical to laboratory safety to understand that the viral genome mimics a cellular messenger RNA (Gamarnik & Andino, 1998). Thus, the SARS-CoV-2 genome is sufficient to initiate viral replication, and therefore may cause productive infection in the case of a percutaneous, mucous membrane, or other potential exposure for research personnel (Bisht et al., 2014). Although there is no evidence that the SARS-CoV-2 genome has caused human infection, it is common laboratory practice for related viruses to maintain viral clones and replenish laboratory stocks by initiating the production of mRNA viral genome equivalents *in vitro* (Almazán et al., 2006; Scobey et al., 2013). The Centers for Disease Control and Prevention (CDC) considers positive-strand RNA virus genomes immediate precursors to virus infection (CDC, 2019), and in the case of the closely related SARS-associated coronavirus (SARS-CoV), the full-length genomic RNA is regulated as a select agent (Federal Select Agent Program, 2020). Thus, isolated genomic RNA of SARS-CoV-2 should be considered potentially infectious, warranting elevated precautions even when

nucleic acids are manipulated at BSL-2, and risk mitigation strategies should include evaluation of any potentially aerosol-producing procedures or use of sharps.

STRATEGIC PLANNING

Some specific administrative controls, work practices, and PPE recommendations for work with COVID-19 patient samples are described further in this section. This information is meant to be general guidance, and researchers are strongly encouraged to consult with their entity's biosafety professionals to perform a research- and entity-specific risk assessment. All manipulation of potentially infectious materials from COVID-19 patients must be performed in a certified biosafety cabinet (BSC) or other primary containment device (e.g. sealed biosafety centrifuge safety cups or rotors that are loaded and unloaded in the BSC). This is especially critical for all procedures with the potential to produce aerosols. Whenever possible, equipment known to generate aerosols during use (e.g. sonicators, tissue homogenizers, vortexes, etc.) should be moved into the BSC, taking care not to disrupt the airflow in the cabinet. Personnel working with COVID-19 patient samples should be appropriately trained or authorized for the work, and restricting use of common rooms or equipment shared with other researchers while COVID-19 samples are being manipulated is recommended. Appropriate EPA-registered disinfectants for coronavirus must be used to decontaminate work surfaces and solid and liquid wastes (US EPA, 2020). Standard personal protective equipment for work at BSL-2 includes use of a laboratory coat, gloves, and mucous membrane/eye protection for procedures with a risk of splash or spray (CDC, 2019). Researchers should consider using enhanced personal protective equipment consisting of either a dedicated laboratory coat with a disposable liquid-resistant barrier (e.g. disposable apron) or a closed-front liquid barrier gown and double gloves for work with COVID-19 patient samples. If proposed laboratory procedures cannot be performed inside a BSC, your institution's biosafety specialist should be consulted to determine if risk can be mitigated by substitution of inactivated samples for the procedure, addition of respiratory protection, or if none of these options are considered to be sufficient, relocation of the proposed work to BSL-3.

Research personnel should demonstrate proficiency in BSL-2 laboratory practices prior to beginning work with COVID-19 patient samples, and should be provided with appropriate occupational health information (e.g. monitoring for symptoms, exposure response, and reporting of symptoms consistent with COVID-19 to supervisor and institutional occupational health services as applicable).

PRACTICAL GUIDELINES FOR THE SAFE COLLECTION AND HANDLING OF SPECIMENS COLLECTED FROM PATIENTS WITH COVID-19 AND SUSPECTED COVID-19

BASIC PROTOCOL 1

Primary human specimens are crucial to the global research effort during the SARS-CoV-2 global pandemic. Therefore, guidelines to maximize safety during collection of clinical specimens are an urgent need. Prior to sample collection, the research teams must obtain informed consent and verify that there are no significant risks to the patient undergoing the procedure. Samples should be collected or overseen by a physician on the research team. All personnel must wear personal protective equipment (PPE) in accordance with the healthcare facility's local infection control protocol. CDC guidance for PPE includes a gown, double gloves, hair net, an N95 respirator, and eye protection (face shield or goggles; CDC, 2020c). Substitution of a powered air-purifying respirator (PAPR) is appropriate for personnel unable to wear an N95 respirator (CDC, 2020a). In accordance with institutional policies, samples should be de-identified at the time of collection to comply with HIPAA privacy laws. Upon collection, the outer set of gloves is removed and discarded in an acceptable waste container per local practice, to decrease the risk of contamination with samples having high viral load. Peripheral blood can be obtained via

arterial or venous catheter, which is often present in intubated patients with COVID-19. Samples should be placed in a plastic biohazard bag, which is then wiped down with an approved disinfectant and placed into a separate plastic biohazard bag, which is also cleaned with an approved disinfectant. Upon collection, samples are transported to an appropriate BSL-2 laboratory facility and handled in primary containment devices by trained personnel using enhanced work practices (described further below) for inactivation of virus.

Differences will exist among institutions concerning the specific use of PPE and acceptable practices for working at specific BSLs. For example, our institution has implemented enhanced PPE consisting of either a dedicated laboratory coat with a disposable liquid-resistant barrier (e.g. disposable apron) or a closed-front liquid barrier gown and double gloves for this work, as well as specific training and work practice enhancements, and has designated this biosafety level as "BSL-2+" (University of Pittsburgh, 2020). Other institutions may use different designations to describe a specific set of enhancements in addition to standard BSL-2 recommendations (e.g. BSL-2 with enhancements). The following protocol is to provide general guidance for safely collecting samples from patients with COVID-19. After viral inactivation, which is further described in Basic Protocol 2, samples can be further processed for downstream assays in standard BSL-2 facilities.

Materials

Personal protective equipment for sample collection (CDC, 2020c):
- Gown (AAMI level 3 or 4 recommended if available; Medline, NONLV325 or NON26276)
- Double gloves (Medline, FG100L)
- Hair bonnet (Medline, CRI1001)
- N95 filtering face piece respirator or PAPR
- *Personnel should consult with institutional infection control specialists or an industrial hygienist regarding appropriate respiratory protection as described in the institution's OSHA Respiratory Protection Plan (*CDC, 2020c*). Personnel should be fit-tested for an appropriate N95 respirator. Face-seal fit varies between N95 manufacturers and specific models. Likewise, there is wide variation between PAPR makes and models.*

Eye protection:
- Face shield (Medline; NONFS300) *or*
- Safety glasses (Medline; NON24774) *or*
- Safety goggles (Medline; NON24776)

Materials required for safe collection of specimens (will vary widely based upon clinical specialties):
- Facility-specific EPA-registered disinfectant effective for coronaviruses (US EPA, 2020)
- Biological specimen bags (8 × 10−inch: Fisher Scientific, 22-311-102; 11 × 18−inch: Fisher Scientific, 22-311-101)
- Leak-proof secondary container for sample transport (Fisher Scientific, 7135-0001)

Personal protective equipment for sample processing:
- Standard laboratory coat dedicated to sample processing area (Fisher Scientific, 19-181-561 or similar)
- Disposable liquid-barrier apron (VWR, 470156-174)
- Disposable liquid-barrier solid-front gown (AAMI level 3 or 4 recommended if available; Medline, NONLV325 or NON26276)
- *This type of gown may be substituted for combination of laboratory coat and apron.*
- Double gloves (Medline, FG100L)

Eye and mucous membrane protection:

Face shield (Medline, NONFS300) *or*
Safety glasses (Medline, NON24774) or safety goggles (Medline, NON24776) in combination with mask (Medline, NON27378)
Facility-specific EPA-registered disinfectant effective for coronaviruses (US EPA, 2020)
Certified Biological Safety Cabinet (BSC)

1. Confirm acquisition of informed consent and clinical stability of patient prior to collection of specimens.
2. Defer sample collection if any concern for patient safety is raised by the clinical team.
3. Prepare all collection materials.

   Specimen containers should have a leak-proof lid that can be securely closed.

4. Put on PPE (CDC, 2020d; also see Materials, above).

   Collection protocols required for specific clinical specialties may vary greatly. In all cases, standard safety guidance for sharps, blood-borne pathogens, etc., should be observed in addition to the recommended PPE for interacting with COVID-19 patients.

5. Collect the specimen(s).

   Ideally, specimens should be placed directly into an inactivating agent (i.e. paraformaldehyde or formalin).

6. Ensure the collection container is securely closed, then place into biohazard bag.

   We recommend de-identifying samples at the time of collection to comply with HIPAA privacy laws.

7. Remove outer gloves and discard into an acceptable waste container in accordance with infection control practices

   Using and properly disposing of the pair of outer gloves decreases the risk of self-contamination with respiratory secretions that have a high viral load.

8. Wipe the outer surface of the biohazard bag with an EPA-registered disinfectant or disinfectant wipe, then place into a second plastic biohazard bag. After sealing the second biohazard bag, wipe the outer surface with an EPA-registered disinfectant or disinfectant wipe.
9. With the help of an assistant outside the patient room, place the disinfected biohazard bag into a large sealable clear plastic bag.
10. Properly take off PPE per local protocol.
11. Place bag with specimen in an appropriately labeled secondary container for transport to a BSL-2+ laboratory space.

    A trained person should at all times be in possession of specimens during transport. PPE for laboratory personnel handling specimens includes a face shield, safety glasses, or goggles for eye protection, surgical mask for mucous membrane protection, two sets of gloves, and either a liquid-resistant closed-front gown or a COVID-19 sample−dedicated lab coat in conjunction with a disposable liquid resistant apron.

12. Upon receipt in the BSL-2+ facility, put on appropriate PPE.
13. Wipe the sealed specimen bag with a disinfectant and move the disinfected bag into a biosafety cabinet (BSC).
14. Open the specimen bag inside the BSC.

15. Within the BSC, specimens can be manipulated according to the specific protocol. Care should be taken to minimize the generation of aerosols. All liquids generated should be immediately disinfected using an approved disinfectant. Before removal from the BSC, all solid surfaces should be disinfected using an approved disinfectant.

BASIC PROTOCOL 2

INACTIVATING SARS-CoV-2 VIRUS IN PATIENT SPECIMENS

Inactivation of the SARS-CoV-2 virus should result in the absence of viable infectious material, rendering the treated samples non-infectious (Darnell, Subbarao, Feinstone, & Taylor, 2004). Proper inactivation is critical to safely working with patient samples isolated from the clinic or materials removed from BSL-3. Proper inactivation ensures that the virus cannot establish an infection should there be an accidental exposure. Since the specific use cases for SARS-CoV-2 samples may vary widely, a variety of inactivation conditions could be required. For example, pathologic analysis by histology, immunofluorescence, or RNA isolation may require that samples be placed in formalin, paraformaldehyde (PFA), or a phenol-based solution for nucleic acid extraction. Some examples of published inactivation methods for enveloped viruses, including coronaviruses, are use of UV or gamma irradiation (Darnell et al., 2004; Kumar et al., 2015), photochemical reactions in combination with UV irradiation (Ruane et al., 2004; Schneider et al., 2015; Singh et al., 2006), heat inactivation (Darnell et al., 2004), phenol and guanidine isothiocyanate−based nucleic acid extraction reagents (e.g. TRIzol; Darnell et al., 2004; Kumar et al., 2015), extreme alkaline (pH>12) or acidic (pH<3) conditions (Darnell et al., 2004), or fixatives (e.g. formaldehyde, formalin, paraformaldehyde, or glutaraldehyde; Darnell et al., 2004; Kumar et al., 2015; Muller et al., 2016).

Prior to moving SARS-CoV-2 virus or COVID-19 patient samples to BSL-2 containment, care must be taken to verify that the chosen inactivation method has successfully resulted in the absence of infectious material. When feasible, each unique tissue or sample type to be collected should be tested to ensure that each intended inactivation method was successful prior to removal from BSL-3 or BSL-2+ to a lower biosafety level. Ultimately, the success of any inactivation procedure is determined by whether it renders 100% of detectable virus incapable of replication. In this section, we will discuss general guidance for sample inactivation; the Support Protocol describes a method to use a plaque assay to ensure that inactivation has been successful. For enveloped viruses, such as SARS-CoV-2, use of a plaque assay is the gold standard for verifying that an inactivation protocol was successful.

General guidance when inactivation cannot be verified

Institutions are encouraged to validate all inactivation procedures locally, even those that are published in the literature, to ensure that the procedure is reproducible using entity-specific equipment, reagents, etc. Developing a laboratory-specific inactivation method, documenting the method in a standard operating procedure, and performing an appropriate viability assay capable of detecting infectious materials in the inactivated samples is the most effective means of ensuring that the method works and assuring the physician or scientist that handling the sample at BSL-2 is safe. Since plaque assays require the propagation of SARS-CoV-2 as a positive control, and since unvalidated inactivation procedures could result in manipulation of potentially infectious samples, they should only be conducted within BSL-3 containment. However, some institutions may not have ready access to the BSL-3 laboratory space or the expertise to carry out validation. As general guidance, infected tissues can be rendered safe with standard clinical and laboratory fixatives or nucleic acid extraction reagents. For example, tissues that will be preserved for imaging can be immersed in 10% formalin or phosphate-buffered saline containing 4% paraformaldehyde (PFA) for at least 24 hr. 24-hr fixation is generally sufficient for tissue

samples as large as 2 cm^3, although fixative permeability rates vary for larger samples of tissue and therefore should be validated prior to removal of larger tissue blocks to a lower biosafety level. The volume of the fixative should be 20 times the volume of the tissue. Fixatives should always be stored in appropriate conditions to discourage degradation (formalin at room temperature; PFA at 4°C and in the dark). Following fixation, samples can be moved to a suitable buffer for downstream manipulation [e.g., phosphate-buffered saline (PBS), etc.]. If phenol- and guanidine isothiocyanate−based reagents (e.g. TRIzol) will be used for nucleic acid extraction, samples should be immersed in 3 times the reagent volume. Solid tissues should be pulverized in an approved biosafety cabinet, in the TRIzol or other appropriate reagent, using pestles or disposable tissue homogenizers (Darnell et al., 2004; Kumar et al., 2015). Ensure that the sample and reagent are thoroughly mixed. Samples should sit for at least 1 hr, and can be frozen indefinitely at −80°C before completing the remainder of the extraction procedure. The above guidelines will be acceptable for most experimental protocols. However, due to the diverse nature of research, novel inactivation methods may be required that are not discussed above. Broadly, any method of inactivation may be acceptable if it has been validated using a plaque assay (see Support Protocol). Since plaque assays require propagation of virus for a positive control, all novel inactivation protocols and validation testing must be conducted at BSL-3. Institutional guidelines must always be followed, and institutional biosafety professionals should be consulted before beginning any procedures.

PLAQUE ASSAY

SUPPORT PROTOCOL

A plaque assay enables visualization of individual replication-competent virions by the unaided eye. Viruses that have infected individual cells in a tissue culture monolayer are observed by the accumulation of cell death immediately surrounding the site of infection. SARS-CoV-2 forms 2- to 5-mm plaques on Vero E6 cell monolayers with only 2-3 days of viral growth. By proxy, each plaque represents an individual virus particle, making the assay quantitative. By recording the volumes used during infection, the specific number of infectious virions can be measured. Millions of infectious virions can be measured by plaquing across many dilutions of the virus, making it feasible to count plaques from at least one dilution and extrapolate to the original value. Thus, the number of infectious virions in each sample is referred to as the 'titer' of the virus. In addition to this support protocol, a detailed plaque assay for SARS-CoV-2 is described in the Current Protocols article Mendoza, Manguiat, Wood, & Drebot (2020).

Materials

Vero E6 cell line (ATCC; CRL-1586)
Vero cell culture medium (see recipe)
Samples to be assayed for virus (refer to step 3, below, for details)
0.5% CMC overlay (see recipe)
Facility-specific EPA-registered disinfectant effective for coronaviruses (US EPA, 2020)
Crystal violet stain (see recipe)
1× phosphate-buffered saline [PBS; optional; mix 50 ml of 10× PBS (Fisher Scientific, 70-011-044 with 450 ml distilled H_2O]

Biological safety cabinet
6-well plates (Fisher Scientific, 07-200-83)
Tissue culture incubator, humidified, 5% CO_2, 37°C
Microtiter tubes (Fisher Scientific, 02-681-382)

NOTE: Steps 1-4 can be completed prior to moving to BSL-3.

1. Plate 5×10^5 Vero E6 cells per well in 6-well tissue culture plates.

Cells should be confluent the following day; however, this can vary among laboratories. Ideally, the number of cells to be plated should be determined based on the observed rate of cell growth and tested empirically.

2. Grow until cells are nearly confluent in a humidified, 5% CO_2, 37°C tissue culture incubator.

3. Create a 1:10 dilution of samples by adding 120.0 µl of the following samples to 1080.0 µl of Vero cell culture medium in autoclaved microtiter tubes:

   a. Positive control:

   Infectious virus: if available and if can be safely handled, *or*
   Untreated sample if infectious virus is not available: samples that have not been subjected to the inactivation procedure

   b. Negative control: A sample consisting of the medium or buffer used to collect original samples that is subjected to the inactivation procedure.

   c. Inactivated samples.

   The design of the plaque assay to be used in validation testing and inclusion of appropriate control samples is critical to ensure that proper safety testing is accomplished. The positive control or untreated sample is used to ensure that the Vero E6 cells used in the plaque assay can form plaques in the presence of infectious virus. The negative control is crucial, particularly for chemical inactivation methods. This negative control should consist of the same medium, sample buffer, and/or solvent used to collect samples to be inactivated, and should be processed through the inactivation SOP and be diluted out and plated on the Vero E6 cells in conjunction with the other samples. This negative control will be used to determine the dilution factor needed to ensure that any residual chemical does not result in death of the Vero E6 cell monolayer. Without simultaneous processing of a positive or untreated control and a negative control, no conclusions can be drawn if no plaques are observed in the serially diluted inactivated samples.

4. Make at least five additional 10-fold serial dilutions (dilutions 2-6) of the mixtures from step 3 by adding 120.0 µl volumes from higher dilution to 1080.0 µl of Vero cell culture medium (pre-aliquoted into microtiter tubes).

   Mix well between dilutions by repeatedly pipetting up and down at least five times.

5. Aspirate or decant medium from 6-well plates containing Vero E6 monolayers. Two 6-well plates will be required per sample that is being tested. Thus, a minimum of six 6-well plates will be required to test a single set of the samples listed in step 3.

6. Add 500.0 µl of dilutions from step 4 to duplicate wells of 6-well plates with Vero E6 monolayers.

7. Incubate 1 hr, in a tissue culture incubator, humidified, 5% CO_2, 37°C.

   Due to the low volume of virus inoculum, plates should be gently agitated every 10 min to ensure that cells do not dry out and that all cells maintain maximum exposure to infectious virus.

8. After 1 hr, add 2 ml of 0.5% CMC overlay directly to each well.

9. Incubate undisturbed in a tissue culture incubator, humidified, 5% CO_2, 37°C, for 2-3 days.

   It is important that the plates not be moved after applying CMC overlay. Disturbing the plates (including bumps or rearranging) will result in deformed plaques, which can make interpretation of the plaque assay ambiguous or impossible.

   It may be desirable to adjust the size of the plaques for easier counting. Plaque size may naturally vary among SARS-CoV-2 viral isolates. Experimental conditions that determine plaque size are the concentration of CMC overlay and time of growth. To increase plaque

size, consider decreasing the concentration of CMC overlay and/or increasing plaque growth time. To decrease the plaque size, consider increasing the concentration of CMC overlay and/or decreasing the plaque growth time. In general, it will be unnecessary to use a concentration of greater than 1% CMC overlay.

10. Remove the overlay by decanting into disinfectant.
11. *Optional*: Wash each well with 2 ml of PBS and remove the PBS after washing.
12. Add 1 ml crystal violet stain to each well. Incubate at room temperature for 15 min
13. Decant the crystal violet stain into disinfectant.

    The crystal violet stain includes 4% formaldehyde, which will render the virus non-infectious. Plates can be removed from the biological safety cabinet after wiping with an approved disinfectant. Plaques can be counted immediately, or the plates can be stored indefinitely prior to counting after being allowed to dry.

14. The assay is valid ONLY if all of conditions a-c are satisfied:
    a. Plaques are present in the positive sample (step 3a) at any dilution, thus demonstrating that the assay is capable of detecting presence of infectious virus.
    b. The Vero E6 cell layer is intact in the negative control sample (step 3b), thus demonstrating that the dilution factor ensures that any residual chemical does not kill the Vero E6 monolayer.
    c. Plaques are absent in ALL inactivated sample wells (i.e., all dilutions; step 3c) AND at least one serial dilution demonstrates both the presence of plaques in the positive control (step 14a) and an intact Vero E6 monolayer in the negative control (step 14b).

    When possible, inactivation conditions should be tested using high-titer virus grown in culture, to ensure that virus is present in the samples and at known titers. High titer also ensures that inactivation occurs even in the presence of high titers.

15. *Optional*: Determine the titer of the virus by averaging the counts for plaques in duplicate wells of the lowest dilution for which the plaques can be confidently delineated. D is an integer from steps 3 or 4. P is the average plaque count. Titer (T) in plaque forming units (PFU) per milliliter can be determined using the formula: $T = 2*P*10^D$.

    For example: From dilution 4, you count 5 and 8 plaques. $P = (5 + 8)/2 = 6.5$ and $D = 4$. Thus, $T = 2*6.5*10^4 = 1.3 \times 10^5$ PFU/ml.

REAGENTS AND SOLUTIONS

CMC overlay, 0.5% (100 ml)

90 ml Vero cell culture medium (see recipe)
10 ml 5% CMC stock solution (see recipe)
Prepare fresh and use within 1 hr
Maintain sterility

CMC stock solution, 5% (∼200 ml)

10 g carboxymethylcellulose (CMC) sodium salt (high viscosity; Sigma, C5013)
Distilled water to 200 ml
Shake to wet CMC
Autoclave
Allow to cool to room temperature
Stir overnight until dissolved
Solution will be very viscous
Store up to 6 months at room temperature

Lower concentrations of CMC stock solutions can be used as an alternative.

Crystal violet stain (100 ml)

1 g crystal violet (Fisher Scientific, C581)
20 ml methanol (Fisher Scientific, A412)
40 ml 10% (v/v) buffered formaldehyde (Fisher Scientific, 31901)
6 ml 10× PBS (Fisher, 70-011-044)
34 ml distilled water
Store up to 1 week at 4°C

Vero cell culture medium (525 ml)

To one 500-ml bottle of DMEM with L-glutamine, 4.5 g/L glucose, and sodium pyruvate (Fisher Scientific, MT10013CV) add:
10 ml fetal bovine serum (FBS; Fisher Scientific, FB12999102)
5 ml MEM nonessential amino acids solution (100×; Fisher Scientific; 11-140-050)
5 ml penicillin-streptomycin-glutamine (100×; Fisher Scientific, 10-378-016)
5 ml HEPES (Fisher Scientific, 15-630-106)
Store up to 1 month at 4°C
Maintain sterility

COMMENTARY

Time Considerations

The time required to complete Basic Protocol 1 is largely dependent on the specific protocol required for sample collection, and must be determined by the clinical team. Basic Protocol 2 is expected to require 5-7 days to complete. The time frame allows for Vero E6 cells to reach confluency and 2-3 days for plaque growth. Preparing the plaque assay after the Vero E6 cells become confluent is expected to take 2-3 hr, including sample dilutions, infection and application of CMC overlay. Plaque staining and counting are expected to require 1-2 hr.

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