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CRISPR/Cas Gene Editing of a Large DNA Virus: African Swine Fever Virus

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[Abstract] Gene editing of large DNA viruses, such as African swine fever virus (ASFV), has traditionally relied on homologous recombination of a donor plasmid consisting of a reporter cassette with surrounding homologous viral DNA. However, this homologous recombination resulting in the desired modified virus is a rare event. We recently reported the use of CRISPR/Cas9 to edit ASFV. The use of CRISPR/Cas9 to modify the African swine fever virus genome resulted in a fast and relatively easy way to introduce genetic changes. To accomplish this goal we first infect primary swine macrophages with a field isolate, ASFV-G, and transfect with the CRISPR/Cas9 donor plasmid along with a plasmid that will express a specific gRNA that targets our gene to be deleted. By inserting a reporter cassette, we are then able to purify our recombinant virus from the parental by limiting dilution and plaque purification. We previously reported comparing the traditional homologous recombination methodology with CRISPR/Cas9, which resulted in over a 4 log increase in recombination.

Keywords: ASFV, African swine fever, ASF, CRISPR, CRISPR/Cas9

[Background] African Swine Fever (ASF) is a highly lethal contagious viral disease of swine caused by ASF virus (ASFV). The genome of ASFV consists of a double-stranded DNA genome of approximately 180-190 kilobase pairs. ASFV causes a spectrum of disease, from highly lethal to sub-clinical, depending on host characteristics and the virus strain (Tulman *et al*., 2009). There is no commercial vaccine for ASFV; experimentally, the only vaccines that have shown to protect against the current circulating strain from the outbreak in Georgia in 2007 (ASFV-G) are live attenuated vaccines that contain one or more deletions to the viral genome, for example: (O' Donnell *et al*., 2015). Traditionally, gene deletions for ASFV have been performed by homologous recombination where a donor plasmid containing homologous genomic sequences is used for gene deletion (O' Donnell *et al*., 2015), however this only occurs at a very low rate, making production of recombinant ASFV difficult (Borca *et al.,* 2018).

Recently we reported the use of CRISPR/Cas9 as an alternative approach to introduce gene deletions in ASFV, with a 4 log increase over traditional methods (Borca *et al.,* 2018). This increase, in recombination using CRISPR/Cas9, allows for easier production and purification of recombinant viruses from the parental wild-type. It is possible that using CRISPR/Cas9 will allow for viral protein mutations, expanding our abilities to dissect critical domains for virulence, possibly even in genes that have been previously determined to be essential. This approach has successfully been reported with other large

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DNA viruses including Orthopox (Okoli *et al.*, 2018), Vaccinia virus (Yuan *et al.,* 2015 and 2016), Herpes Simplex virus (Suenaga *et al.,* 2014*)* and Pseudorabies (Tang *et al.*, 2016).

Materials and Reagents

1. Pipette tips 10 µl, 200 µl, 1,000 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog numbers: 2140-05, 2160P, 2079)
2. Primaria™ 6-well plates (Corning, catalog number: 353846)
3. Primaria™ Tissue culture flasks T-75 filtered flasks (Corning, Falcon®, catalog number: 353824)
4. 50 ml Conical tube (Corning, Falcon®, catalog number: 352098)
5. Polypropylene microcentrifuge tubes
6. T-25 flask (Corning, catalog number: 3056)
7. T-150 flask (Corning, catalog number: 3151)
8. 0.22 µm filter (Corning, catalog number: 430769)
9. African swine fever virus field isolates such as ASFV-G (isolated from infected swine serum and adding serum to swine macrophages to propagate the virus. Titrations were done as described by Enjuanes *et al*., 1976).
10. Yorkshire pigs aged 3-12 months as blood donors
11. Cas9 donor plasmid (custom designed for target of interest by blue heron bio. See attached insertion cassette sequence (Supplemental file)
12. Fugene HD (Active Motif, catalog number: 32042)
13. gRNA plasmids (custom designed for target of interest by blue heron bio.)
14. OptiMEM (Thermo Fisher Scientific, Gibco™, catalog number: 31985062)
15. Phosphate Buffered Saline (PBS) 1x, pH 7.0-7.3 (Thermo Fisher Scientific, Gibco™, catalog number: 14190144)
16. 0.5 M EDTA pH 8.0 (Thermo Fisher Scientific, Invitrogen™, catalog number: 15575020)
17. Ficoll-Paque PLUS density gradient of 1.077 g/ml (GE Healthcare, catalog number: 17144002)
18. RPMI 1640 medium (Thermo Fisher Scientific, Gibco™, catalog number: 11875093)
19. Gamma Irradiated Fetal bovine serum (GE Healthcare, Hyclone™, catalog number: SH30071.03)
20. Antibiotic-Antimycotic (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 15240096)
21. Gentamycin (Thermo Fisher Scientific, Gibco™, catalog number: 15750060)
22. HEPES 1 M (Thermo Fisher Scientific, Gibco™, catalog number: 15630080)
23. L-Glutamine, 200 nM (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
24. L929 media (L cell, L929, derivative of Strain L) (ATCC, catalog number: CCL-1)
25. Heparin (Sagent Pharmaceuticals, catalog number: 400-10)
26. L929 conditioned media (see Recipes)
27. Macrophage Wash Media (see Recipes)
28. Macrophage complete media (see Recipes)

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29. PBS + EDTA (see Recipes)

Equipment

1. P1,000, P200, P20, P10 Pipettes (Gilson)
2. 2 Liter roller bottles (Corning, catalog number: 431329)
3. 250 ml centrifuge bottles (Thermo Fisher Scientific, Thermo Scientific™, Nalgene™, catalog number: 3141-0250)
4. Inverted Fluorescent Microscope (Carl-Zeiss)
5. Hematocytometer
6. Pipet aid (Drummond Scientific, catalog number: 4-000-101)
7. Tabletop Centrifuge (Eppendorf, model: 5810)
8. Floor Centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Sorvall® RC-4)
9. Sorval SLA-1500 fixed angle rotor (Thermo Fisher Scientific, model: SLA-1500)
10. Vortex mixer (Fisher Scientific, catalog number: 12-812)
11. -70 °C freezer (Thermo Scientific, model: ULT2586-9-A40)

Procedure

A. Macrophage isolation

1. Obtain 500 ml of swine blood by venipuncture and treat with heparin.
2. Incubate the blood at 37 °C for 1-2 h until you observe erythrocyte sedimentation and blood leukocyte enrichment into a supernatant plasma.
3. Collect plasma and float over Ficoll-Paque (35 ml plasma, 10 ml Ficoll-Paque) in a 50 ml Falcon tube.
4. Centrifuge in an Eppendorf 5810 at 367 *x g* for 30 min at room temperature.
5. Collect top layers and pool into a 250 ml centrifuge bottle.
6. Centrifuge at 7,500 *x g* in a Sorval SLA-1500 for 20 min at room temperature.
7. Collect mononuclear leukocytes and wash with macrophage media by adding an equal volume of media as collected cells, inverting several times to mix.
8. Centrifuge in an Eppendorf 5810 at 367 *x g* for 10 min at room temperature.
9. Remove supernatant, and wash cells by adding 10 ml macrophage media and centrifuging as in Step 8 again for a total of three washes.
10. Resuspend cells in 10 ml of Macrophage complete media.
11. Plate cells into five Primaria™ T-75 flasks (each with 1 ml of cells and 10 ml of macrophage media) and incubate overnight.
12. Adherent cells are now considered macrophages.
13. Detach cells using PBS EDTA, washing with 10 ml and incubating with 10 ml at 37 °C until cells detach (typically 15-20 min).

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14. Count macrophages using 10 µl of cell suspension in a hematocytometer.
15. Plate macrophages at a density of 5 x 10^6 per well in Primaria™ 6-well plates and allow to attach overnight.

B. Infection/Transfection with CRISPR/Cas9

1. Inoculate each well with a field isolate of ASFV, such as ASFV-G or other field isolates of ASFV, at a multiplicity of infection (MOI) of 1.0. Inoculate by adding virus directly to the media of each well containing 2 ml of media, gently shake plates side to side to mix and incubate at 37 °C under 5% CO_2 for 1 h.
2. Add 1.6 µg of donor plasmid and 1.6 µg of gRNA plasmid suspended in ddH_2O to 150 µl of optiMEM in polypropylene microcentrifuge tubes.
3. Carefully mix by pipetting or vortexing briefly.
4. Add 10 µl of Fugene HD mix by pipetting and incubate for 10 min.
5. Add dropwise 150 µl of complex to macrophages containing 2 ml of media.
6. Incubate at 37 °C under 5% CO_2 for 24 h.
7. Observe under a fluorescent microscope 24 h after transfection for your reporter gene to determine the rate of transfection. Calculate the percentage by observing both attached and unattached cells that are expressing the fluorescent marker.
8. Freeze plate at -70 °C, and store until ready to use.
9. Take 1 ml of frozen media from transfection to make serial 1:10 dilutions (1:10, 1:100, 1:1,000; *etc.*) in macrophage media.
10. Add 100 µl of dilutions to 6 well plates of macrophages prepared as in Step B7, in a final volume of 2 ml of media. Incubate at 37 °C under 5% CO_2.
11. Observe every 24 h for complete CPE (cytopathic effect) (typically 1-7 days), mark wells that are positive for your fluorescent marker. If complete CPE occurs quickly (< 48 h) without seeing your fluorescent marker in any of the dilutions, additional dilutions may be required–further diluting the transfection allows for expression of your reporter gene.
12. Freeze all plates as CPE occurs, and take the lowest dilution that has your recombinant gene expressed to purify further.
13. Continue diluting and passing recombinant virus until you can observe fluorescence in all cells showing CPE. At least 5 passages are recommended.
14. Confirm purity by the absence of the deleted gene by conducting PCR of an internal portion of the deleted gene or by Whole Genome Sequencing.

Data analysis

In our previous work (Borca *et al.,* 2018), we used CRISPR/Cas9 as an alternative approach to introduce gene deletions in ASFV in primary swine macrophage cell lines and compared the homologous recombination rate with traditional homologous recombination methods. It showed that

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using the approach as described in the above procedure, has a 4 log increase in the homologous recombination rate. This method will allow for manipulation of outbreak strains without tissue culture adaption (a timely process, that often results in genome instability), and customized production using new emerging ASFV isolates as the backbone for a live-attenuated vaccine.

Notes

Troubleshooting

1. Low levels of recombinant virus

   A high-transfection efficiency is required for recombination to occur with the viral genome. Observing a high transfection efficiency doesn't mean recombinant virus has been produced. Passage 1 of the virus determines recombination efficiency and the amount of recombinant virus in the sample. The amount of recombinant virus should be increasing with increasing passages. If this is not the case, go back to the previous passage.
2. Essential genes

   If the recombination efficiency is low, it is possible that the virus gene being targeted could be essential. If this is the case, try partial gene deletions or gene mutations. It is possible that you can observe a small amount of what appears to be recombinant virus in Passage 1 even if the gene is essential, if the gene can be complemented by the non-recombinant virus. It is unlikely that this will be observed in Passage 2 of the recombinant virus.
3. Poor infection of macrophages

   Improper handling of macrophages and possible animal variation can lead to isolation of cells that are not capable of supporting ASFV infection. Although this is rare, use of a different animal and careful handling of macrophages can improve infection.

Recipes

1. L929 conditioned media

   500 ml RPMI 1640 medium

   55 ml Fetal Bovine Serum-Gamma Irradiated

   12.5 ml HEPES 1 M

   5.5 ml L-Glutamine 200 nM

   5.5 ml Antibiotic-Antimycotic

   0.55 ml Gentamicin

   Condition media as follows:

   a. Seed a T-25 flask between 5 x 10^6 and 1 x 10^7 cells in total 15 ml of media
   b. Transfer to a T150 flask in total 35 ml volume when confluent
   c. When confluent, detach and resuspend cells in 12 ml media in T150
   d. Add 3 ml of cells to each of (d) 2 L Roller Bottles, and add 250 ml L929 media to each

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e. Allow growing for 11 days
f. Pour contents/media into 250 ml centrifuge bottles
g. Centrifuge at 5,500 *x g* in a Sorval SLA-1500 for 20 min
h. Collect supernatant and filter through a 0.22 µm filter
i. Freeze at -20 °C until use

2. Macrophage Wash Media
   250 ml RPMI 1640
   100 ml FBS
   5 ml Anti-Anti
   0.5 ml Gentamycin 50 mg/ml
3. Macrophage complete media
   250 ml RPMI 1640
   150 ml conditioned L929 media
   100 ml FBS (16.5% final concentration)
   5 ml Anti-Anti
   0.5 ml Gentamycin 50 mg/ml
   150 ml donor swine plasma
4. PBS + EDTA
   500 ml PBS
   10 ml 0.5 M EDTA

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Competing interests

The authors declare no conflicts of interest or competing interests.

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