Determination of Mutation Frequency During Viral DNA Replication

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[Abstract] This protocol is a simple method for evaluating mutation frequency during African swine fever virus (ASFV) replication, although it could be used also for other DNA viruses (poxvirus, herpesvirus, mimivirus, *etc*) with minor modifications. In the original Carrascosa *et al*. (1982), the protocol was carried out with two cloned viruses, BA71Vc (a purified clone from BA71V wild type strain) and vΔpolX (lacking the reparative polymerase, pol X, gene), and two different cell types that can be infected by ASFV, Vero cells and swine macrophages. To facilitate the sequence comparison, a genome fragment containing the B646L gene was amplified by PCR and blunt-end cloned. This gene codes for the major capsid protein (p72) and multiple sequences can be found in the database, so the mutations found could be compared with natural gene variations. The cloned fragment can be either sequenced directly from bacteria colonies or from miniprep purified DNA.

Materials and Reagents

1. Virus supernatants (previously titrated)
2. XL-1 Blue competent bacterial cells (Stratagene, catalog Number: 200249)
3. Dulbecco modified Eagle medium (DMEM) (Sigma-Aldrich, catalog number: D5523)
4. Fetal Bovine Serum (Sigma-Aldrich, catalog number: F7524)
5. Vero Cells (ATCC, catalog number: CCL-81™)
6. Alveolar swine macrophages (Carrascosa *et al*., 1982)
7. Swine serum (obtained during the macrophages purification)
8. Kapa HiFi polymerase (Kapa Biosystems, catalog number: KK2101)
   Note: This polymerase kit includes buffer and dNTPs.
9. Primer B646L forward (Sigma-Aldrich)
   5'ATGGCATCAGGAGGAGCTTTTTGTCTTATT
10. Primer B646L reverse (Sigma-Aldrich)
    5'TTAGGTACTGTAACGCAGCACAGCTGAACCG
11. CloneJet PCR Cloning Kit (Thermo Fisher Scientific, catalog number: K1231)
    Note: This kit includes the pJET plasmid, DNA ligase and reaction buffer.

12. Agarose for DNA electrophoresis (Lonza, SeaKem® LE Agarose, catalog number: 50000)
13. TAE buffer (Sambrook and Russell, 2001)
14. TE buffer (pH 8.0) (Sambrook and Russell, 2001)
15. 5x Agarose Gel loading dye (QIAGEN, catalog number: 239901)
16. QIAquick gel extraction kit (QIAGEN, catalog number: 28704)
17. LB-agar plates (Sambrook and Russell, 2001)
18. Ampicillin (Sigma-Aldrich, catalog number: A0166)
19. Double-distillated or Milli-Q water

Equipment

1. MW24 plates (BD Biosciences, Falcon®, catalog number: 353047)
2. 1.5 ml Eppendorf tubes
3. Cell culture incubator
4. Refrigerated benchtop centrifuge (*e.g.* Hettich 200 R)
5. Thermal-cycler for PCR
6. Electrophoresis system for agarose gels and UV-documentation device to visualize the DNA bands
7. Waterbath

Software

1. Diverse bioinformatics software (*e.g.*, Geneious or CLC Genomics Workbench) (optional)
2. MS Excel, Prism Graph Pad or SPSS (optional)

Procedure

A. Sample preparations

1. Plate Vero cells at about 30,000 cells/cm^2 and swine macrophages at 60,000 cells/cm^2 in multi-well 24 plate in DMEM-10% fetal-bovine or swine sera, respectively. Leave overnight in a cell incubator at 37 °C with 7.5% CO_2.
   Note: For more details about ASFV infection protocols see Redrejo-Rodriguez et al (2013).
2. Next day infect cultures at an moi = 5 pfu/cell.
3. Prepare a negative control of uninfected cells and an extra well to monitor the infection success by titration. In the case of ASFV, we titrate the virus as in Redrejo-Rodriguez *et*

al. (2013). Other approaches could be used to evaluate the virus replication, such as qPCR.

4. At 20 h post infection, when total cytopathic effect is achieved, harvest the cells and media in 1.5 ml Eppendorf tubes.
5. Centrifuge 30 min at 14,000 rpm (about 20,000 *x g*) in Hettich (4 °C).
6. Wash the pellets with ice-cold TE (about 200 µl) and spin again for 5 min.
7. Suspend the pellet in 50 µl double-distilled water and boil for 20 min in a waterbath at 95 °C.
8. Spin briefly and use supernatant as sample.
9. Prepare the PCR tubes

Component	Per reaction (µl)
5x Buffer	5
10 mM dNTPs	0.75
5 µM Primer forward	5
5 µM Primer reverse	5
Kapa HiFi DNA polymerase	0.25
Sample	0.5
Water	8.5

B. Set up the PCR program

1. Adjust the annealing temperature according to the primer pair used. Increasing melting temperature may be required to avoid unspecific amplification products. Additionally, if there is no or very low amounts of amplified product an extra PCR step with 2-5 cycles at lower melting temperature may improve the results.
2. Denaturation temperature and incubation time were optimized following the Kapa HiFi polymerase recommendations.

Step 1		1 cycle
5 min	95 °C	
Step 2 (optional)		3 cycles
1 min	94 °C	
1 min	47 °C	
5 min	72 °C	
Step 3		20 cycles
30 sec	98 °C	
30 sec	54 °C	
4 min	72 °C	
Step 4		1 cycle
15 min	72 °C	

3. Run a small aliquot (2 µl) in agarose DNA electrophoresis to verify and quantify the amplification product.
4. Run the PCR product in 0.7% high quality agarose, running the gel in 1x TAE buffer. Purify the PCR product by single band gel extraction using the QIAquick gel extraction kit (this step could be avoided, but the cloning efficiency might decrease).

C. Ligation

Use about 100 ng of PCR product and 0.5-1 µl pJET plasmid. The CloneJet kit includes 2x reaction buffer and T4 DNA ligase, although any ligase may be used. Manufacturer's instructions recommended 5-30 min incubation at RT, but longer reactions (up to 2 h) may be done to increase the ligation yield.

D. Bacteria transformation

1. Transform XL-1 cells with the ligated plasmids and plate in LB-Agar plates with 100 µg/ml ampicillin.
2. After 12-16 h incubation at 37 °C, pick colonies and suspend them in 5 µl TE for direct sequencing [performed as in Sambrook and Russell (2001)]. Take at least 15 colonies of each sample.

   Optional: Although ideally the cloning efficiency is about 80%, false positives for PCR fragments not purified from single band can be produced. In those cases, it is highly recommendable to inoculate 5-10 ml LB medium for miniprep purification of plasmids and to check the presence of the insert by restriction digestion (the plasmid contains BglII sites surrounding the ligation site).

E. Data analysis

Sequences can be checked by hand or assembled with diverse bioinformatics software. We recommend Geneious or CLC Genomics Workbench, which allow simultaneous display of the alignments and all sequencing peaks in order to differentiate the “mixed peaks” from true base changes.

Example [see Table 1 in Carrascosa *et al.* (1982)]: We found 3 TA-CG transitions out of 56202 sequenced nucleotides, which give rise to a frequency of mutation of 5.34×10^{-5}.

The number of mutations is usually low and statistic analysis should be made with non-parametric test, like Mann-Whitney U, which is implemented in many different software packages, including MS Excel, Prism Graph Pad or SPSS.

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