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Postulated WIV Bat-CoV-X Full-length Clone Construction Process

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Quote from Zeng *et al.*, 2016

- From Materials and Methods, Virus and cells section
  "All experiments using live virus was conducted under biosafety level 2 (BSL2) conditions."
  From the first paragraph of the Discussion
  "In this study, we have developed a fast and cost-effective method for reverse genetics of coronaviruses by combining two approaches developed by others (29, 30). Our method allows the genomes of coronaviruses to be split into multiple fragments and inserted into a BAC plasmid with a single step. Recombinant viruses can then be efficiently rescued by direct transfection of the BAC construct. As the genomes can be divided into multiple short fragments, mutations can be introduced into individual fragments easily (31). Using this method, we successfully rescued three recombinant viruses derived from SL-CoV WIV1 (rWIV1, rWIV1-DX, and rWIV1-GFP-DX)."

The next page is withheld in full citing (b)(1) and (b)(3) 50 USC 3024(i) and is not provided.

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WIV SARS-CoV Reverse Genetics System

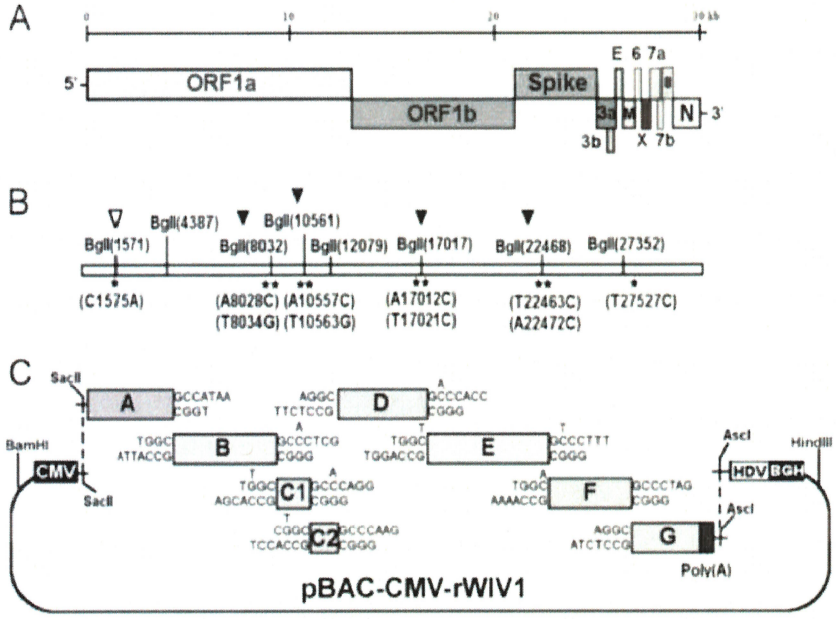

FIG 1 Strategy for construction of an infectious WIV1 BAC clone. (A) Genomic structure of WIV1. (B) The mutations are indicated under the stars. C1575A was used to ablate a natural BglI site at nucleotide 1571 (▽), and T27527C was used to disrupt a potential T7 stop site. The others were for introducing BglI sites (▼). (C) The WIV1 genome was split into eight contiguous cDNAs (A to G): A, nt 1 to 4387; B, nt 4388 to 8032; C1, nt 8033 to 10561; C2, nt 10562 to 12079; D, nt 12080 to 17017; E, nt 17018 to 22468; F, nt 22469 to 27352; G, nt 27353 to 30309. Unique BglI sites were introduced into the fragments by synonymous mutations to make these fragments capable of unidirectional ligation along with native BglI sites in the genome. The original nucleotides are shown above the flanking sequences of corresponding fragments. A poly(A) sequence was added to the 3′ terminus of fragment G. A CMV promoter, HDV ribozyme, and BGH transcriptional terminal signal were inserted into pBeloBAC11 between BamHI and HindIII sites. SacII and AscI sites were introduced between the CMV promoter and ribozyme. Fragments A to G were inserted into the pBAC-CMV plasmid in a single step.

Zeng et al., 2016

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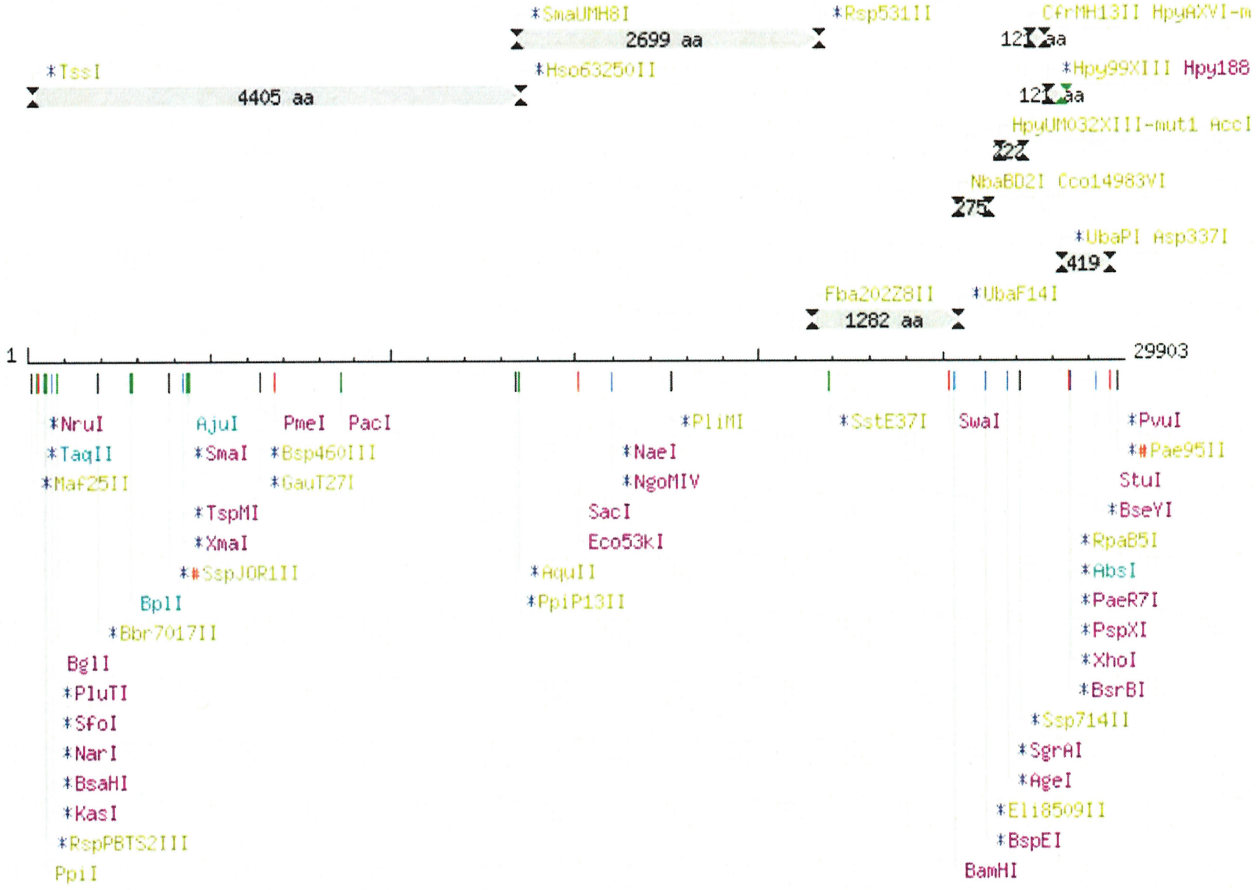
SARS-COV-2 GENOME RESTRICTION MAP



Type IIS Restriction Enzymes

- BsmBI (Plus strand)
  5′ – CGTCTCNNNNN-3′
  3′ – GCAGAGNNNNN – 5′
- BsaI (Plus strand)
  5′ – GGTCTCNNNNN-3′
  3′ – CCAGAGNNNNN – 5′
- SARS-CoV-2 genome does not have any SacII or AscI restriction sites

- BsmBI (Minus strand)
  5′ – NNNNNGAGACG-3′
  3′ – NNNNNCTCTGC-5′
- BsaI (Minus strand)
  5′ – NNNNNGAGACC-3′
  3′ – NNNNNCTCTGG – 5′

[redacted]

(b)(1); (b)(3):50 USC 3024(i); Sec. 1.4(c); Sec. 1.4(e)

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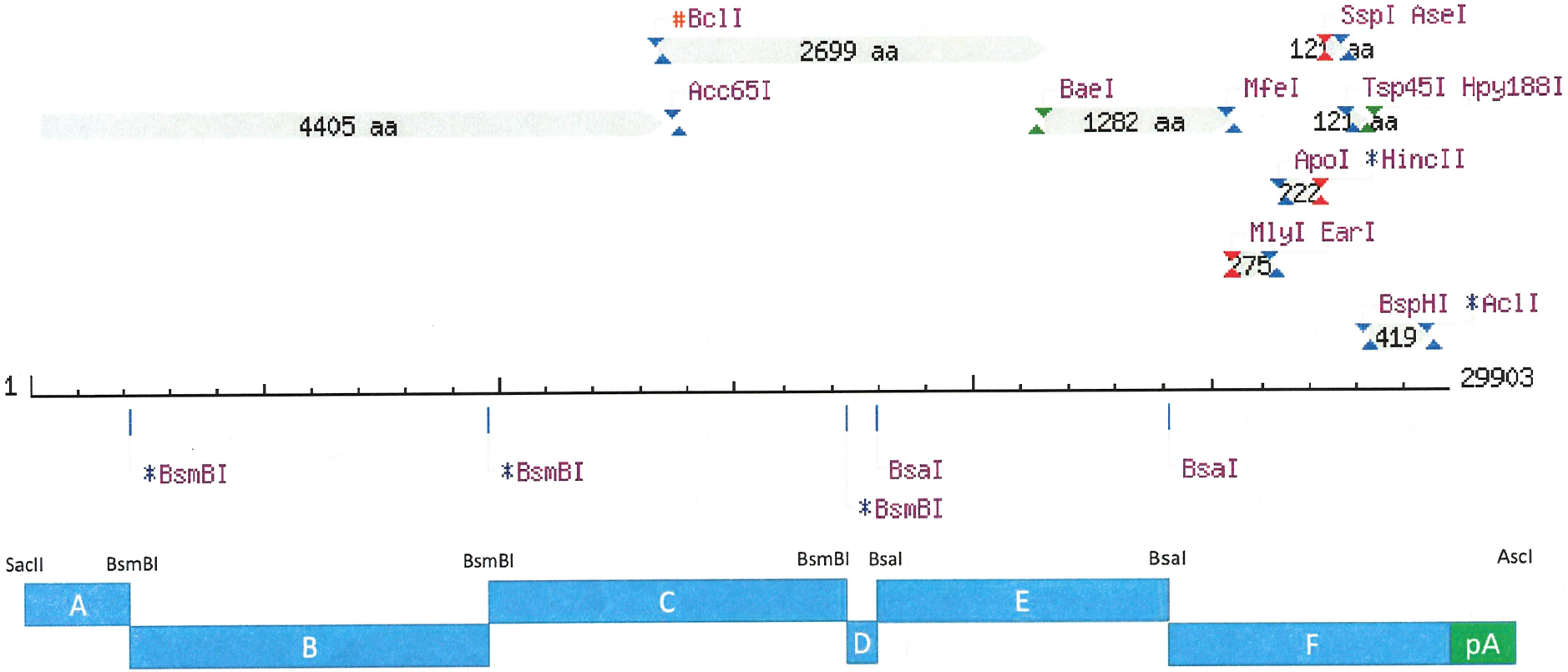
RE-based Fragment Build Option – *Bsm*BI/*Bsa*I (4 nt overhangs)



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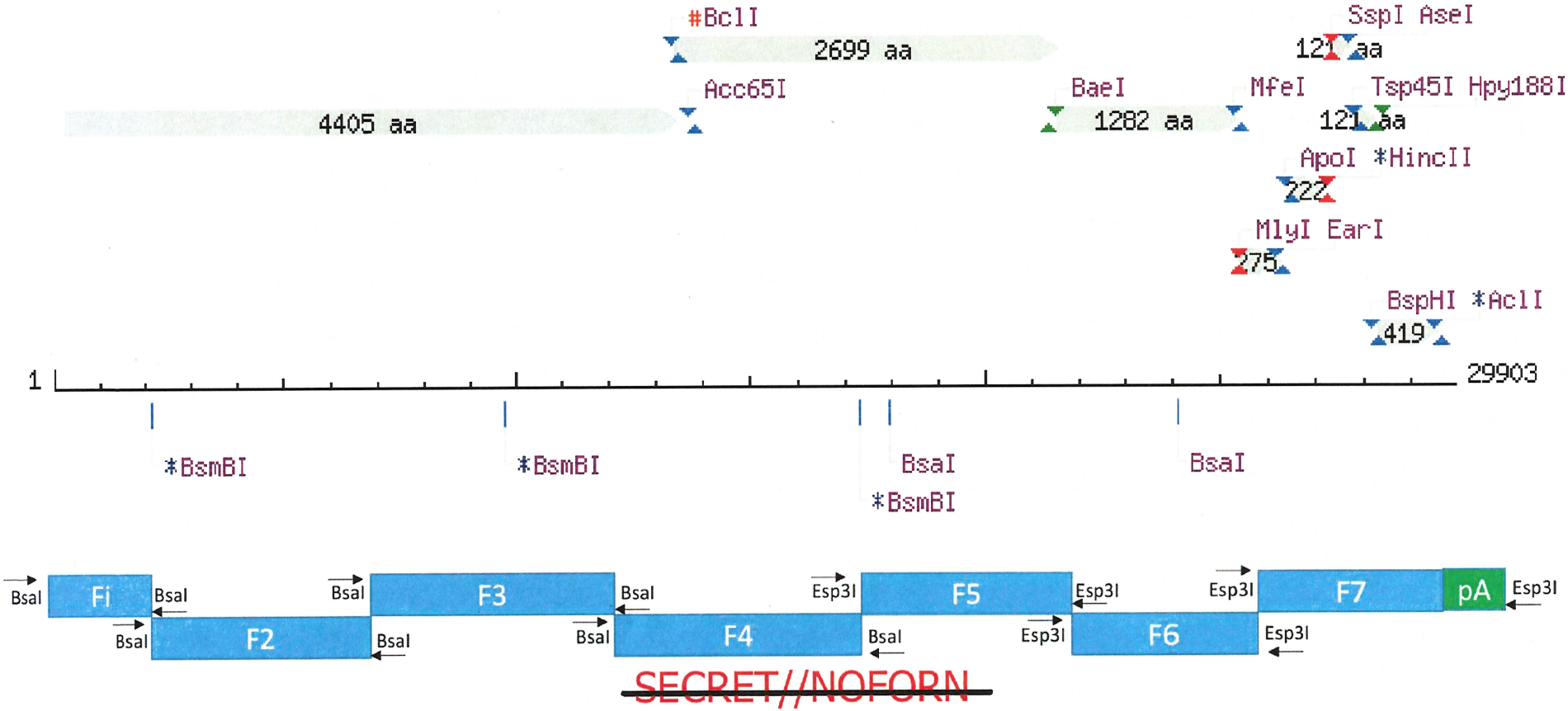
RE-based Fragment Build Option – *BsaI*/*Esp3*I (Invisible restriction sites)



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Xie et al., 2020 SARS-CoV-2 FLC Assembly

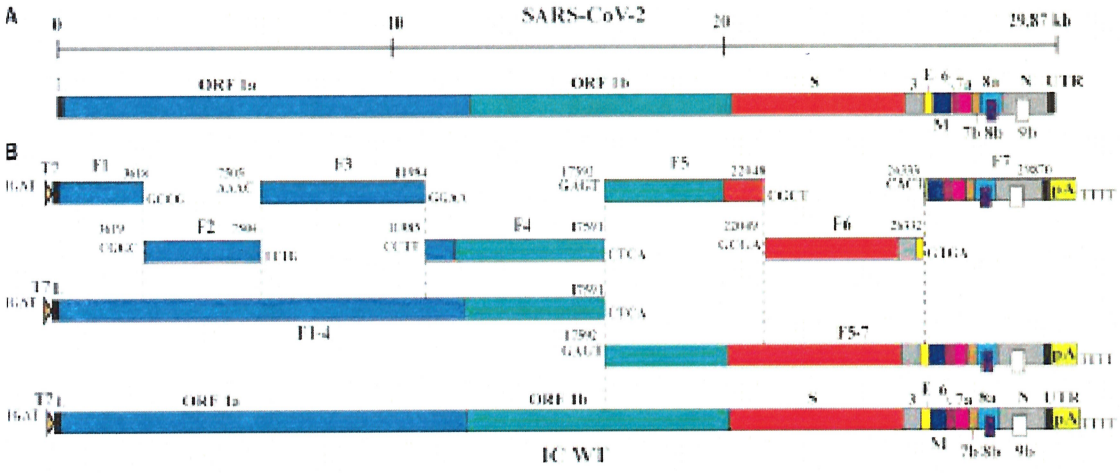

Figure 1. Assembly of a Full-Length SARS-CoV-2 Infection cDNA Clone

(A) Genome structure SARS-CoV-2. The open reading frames (ORFs) from the full genome are indicated.

(B) Strategy for *in vitro* assembly of an infectious cDNA clone of SARS-CoV-2. The nucleotide sequences and genome locations of the cohesive overhangs are indicated. The WT full-length (FL) cDNA of SARS-CoV-2 (IC WT) was directionally assembled using *in vitro* ligation.

(C) Diagram of the terminal sequences of each cDNA fragment recognized by BsaI and Esp3I.

(D) Gel analysis of the seven purified cDNA fragments. Individual fragments (F1–F7) were digested from corresponding plasmid clones and gel purified. Seven purified cDNA fragments (50–100 ng) were analyzed on a 0.6% native agarose gel. The 1-kb DNA ladders are indicated.

(E) Gel analysis of cDNA ligation products. About 400 ng of purified ligation product was analyzed on a 0.6% native agarose gel. Triangle indicates the FL cDNA product. Circles indicate the intermediate cDNA products.

(F) Gel analysis of RNA transcripts. About 1 μg of *in-vitro*-transcribed (IVT) RNAs were analyzed on a 0.6% native agarose gel. DNA ladders are indicated. Because this is a native agarose gel, the DNA size is not directly correlated to the RNA size. Triangle indicates the genome-length RNA transcript. Circles show the shorter RNA transcripts.

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Spike Gene

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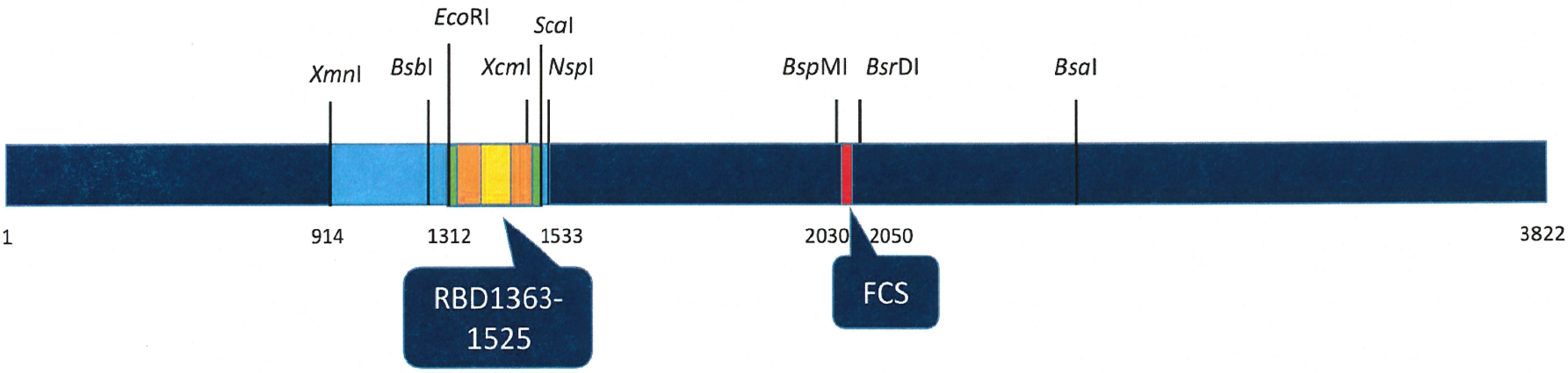
SARS CoV-2 SPIKE GENE



Highest homology toRaTG13Pangolin CoV

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