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SARS-COV-2 GENOME ANALYSIS

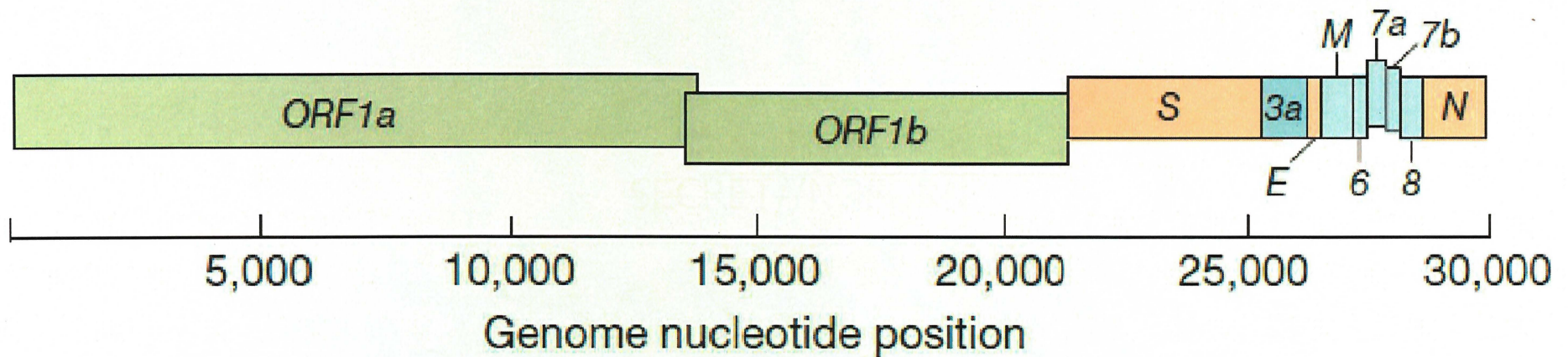
(b)(3):10 USC 424; (b)(6)

June 25, 2020

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SARS-CoV-2 Genome



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Methods to construct a Coronavirus Full-Length Clone

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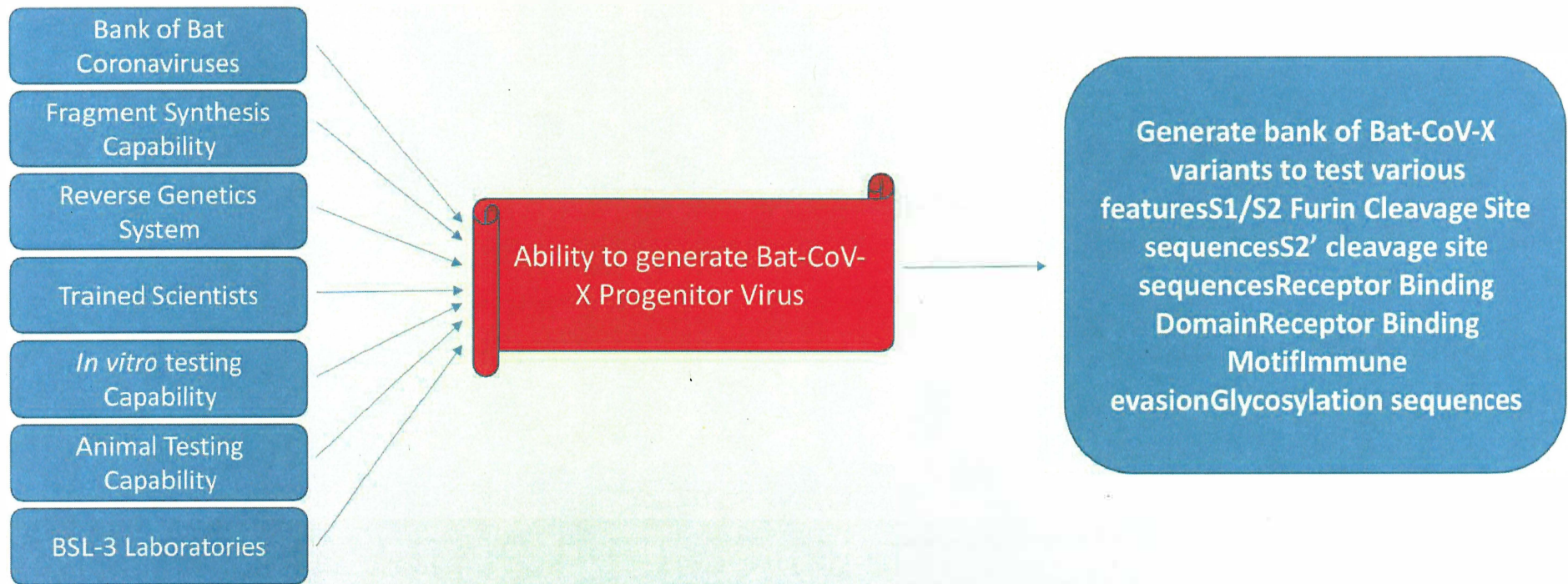
CORONAVIRUS INFECTIOUS CLONE CONSTRUCTION

- 1) Synthesize or PCR amplify 6-10 segments of a Bat Coronavirus
Build a 5' transcription initiation fragment
"stitch" the fragments together using an infectious clone technology
3' to the transcription initiation fragment
Restriction-enzyme-based fragment cloning system
Overlapping Fragment system
Guided RNA Recombination
Clone in a suitable host (E. coli, yeast, etc.)
Sequence verify cloned insert

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CAPABILITIES NEEDED TO CONSTRUCT A BAT-LIKE CORONAVIRUS INFECTIOUS CLONE



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Type IIS Restriction Enzymes and Golden Gate Assembly System

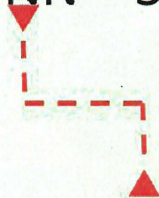
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Type IIS Restriction Enzymes

- Non palindromic recognition siteCuts at sites outside of recognition siteEach digested location has unique nucleotide overhangsExampleBsaI

5' – GGTCTCNNNNN – 3'
3' – CCAGAGNNNNN – 5'



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Eleven Tips For Optimizing Your Golden Gate Assembly Reactions

Looking to assemble multiple DNA fragments in a single reaction? Here are some tips to help you avoid when planning your Golden Gate Assembly experiment.

1 Check your sequences

Always check and carefully inspect the sequence of each DNA fragment. Check for any restriction sites that may interfere with the assembly. Also check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

2 Optimize your primers

When designing PCR primers to amplify a specific DNA fragment, make sure the primers are designed to amplify the correct sequence. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

3 Choose the right plasmid

Choose a plasmid that is compatible with the restriction enzymes you are using. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

4 Choose the right buffer

Choose a buffer that is compatible with the restriction enzymes you are using. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

5 Increase your complex assembly efficiency by increasing the Golden Gate cycling levels

Increasing the number of cycles in the Golden Gate assembly reaction can increase the efficiency of the reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

6 Make sure your planned prep is BSA free

BSA (bovine serum albumin) can interfere with the Golden Gate assembly reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

7 Avoid primer dimers

Primer dimers can interfere with the Golden Gate assembly reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

8 Avoid PCR-induced errors

PCR-induced errors can interfere with the Golden Gate assembly reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

9 Decrease insert amount for complex assemblies

Decreasing the amount of insert DNA can increase the efficiency of the Golden Gate assembly reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

10 Carefully design EVERY insert's overhang

Designing the overhangs of the insert DNA carefully can increase the efficiency of the Golden Gate assembly reaction. Check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

11 Check for a sequence error if your assembly becomes non-functional

If your Golden Gate assembly reaction is non-functional, check for any potential issues with the sequence of the fragments, such as the presence of a stop codon or a start codon. If you are using a standard restriction enzyme, make sure the sequence is compatible with the enzyme. If you are using a custom restriction enzyme, make sure the sequence is compatible with the enzyme.

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Featured Tools

Download the NEB App for iPhone® or iPad®. Scan the augmented reality butterfly icon to find videos, tutorials and interactive experiments.

For help designing primers, try the new NEB Golden Gate Assembly Tool at neb.com/gga.

Try our ligase fidelity tools for the design of high-fidelity Golden Gate assemblies at neb.com/research/fidelity-tools.

- **Ligase Fidelity Viewer™ (v2)** - Visualize overhang ligature preferences.
- **GenSet™** - Predict high fidelity junction sets.
- **SplitSet™** - Split DNA sequences for seamless high-fidelity assembly.

Golden Gate Assembly

20+ FRAGMENT ASSEMBLY NOW ACHIEVABLE
WITH HIGH EFFICIENCY AND ACCURACY

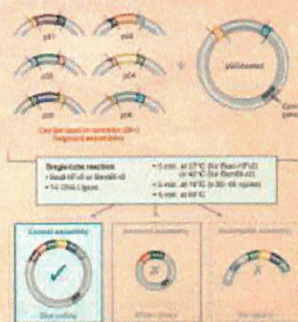
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Push the limits of Golden Gate Assembly

With constant advances in both the development of new enzymes and research on maximizing enzyme functionality (e.g., ligase fidelity), NEB is the industry leader in pushing the limits of Golden Gate Assembly and related methods.

GOLDEN GATE ASSEMBLY WORKSHOP
FOR BOTH SIMPLE AND COMPLEX ASSEMBLIES



In its simplest form, Kasten-Kast Assembly requires a Type 40 rectangular hole, either in a flat wall or a drilled segment. This design, which also is left-handed, will work against almost any direction of force, so it does not require the drilled hole assembly.

FEATURED PRODUCTS

Type IIS Restriction Enzymes used in Golden Gate Assembly

Type IIS restriction enzymes recognize asymmetric DNA sequences and cleave outside of their recognition sequence. Type IIS enzymes commonly used in Golden Gate Assembly are listed below. NEB currently offers over 45 Type IIS restriction enzymes.

Please visit www.aeds.com for comprehensive table

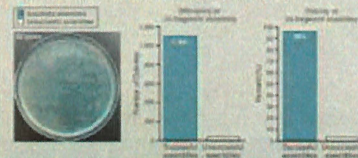
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FLAVONOIDS

NED® Golden Gate Assembly Kits (BsmBI-v2 or BsaI-HF™v2)

The absence of internal sites in a sequence determines the choice of which Type II restriction enzyme to drive the assembly for your convenience. NEB now offers two kits for Golden Gate Assembly featuring BsaI-HFv2 or BsmBI-v2. Both kits incorporate digestion followed by ligation with T4 DNA Ligase into a single reaction, and can be used to assemble 3-20⁴ fragments in a single step.

COMPLEX GENE GATE ASSEMBLY
WITH > 99% FIDELITY AND UNPRECEDENTED EFFICIENCY



Using the United States Agency for Global-Aid, Internet the highest percentage of a country's population were interviewed using the questionnaire (table presented in 11-2017) reported with an estimate of 20 minutes. Further, interviewers will spend time on the day after each interview session. During 11-2017, the highest number of respondents for the 20 countries reported included in 11-2017 included a 100% survey rate, and approximately 100,000 respondents per country. Further, the information regarding the estimate of the sample size (table presented in 11-2017) is shown in the table of 20 countries that were included in the survey.

Real-HP v2

5'GGTCTCNNNNN3
3'CCAGAGNNNNN5

What users are saying

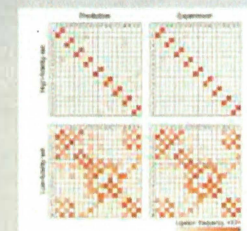
NIJ has cultivated a reliable set of scientists and design firms. The Chicago-based scientists also are an important part of success. They have found the "finger printing" of each individual, based on the varying lengths of the DNA segments by a previously patented DNA sequence. The thorough implementation of the test and the availability of the technology have enabled success.

Dr. Klaus Fichter
Senior Hospital Staff Physician

Advances in Ligase Fidelity

Research at NER has led to an increased understanding of ligase fidelity, including the development of a comprehensive method for profiling and joining ligases *in vitro* in order to predict overlapping sites with imposed fidelity (3). This research has enabled complex fragment assemblies with high efficiency and >90% accuracy. More information can be found in the NER publication, *Comprehensive Profiling of Two-Base Overhang Ligation Fidelity by T4 DNA Ligase and Application to DNA Assembly* (4), or in our website, *Fidelity and Bias in Full-Scale Ligation, End-filling complex, multi-fragment Golden Gate DNA Assembly*. We also encourage you to try our first Ligation Fidelity Tools.

All of these are available at www.mhhe.com/CollegeCape

[illegible]

Visit www.neb.com/GoldenGate
to learn more and view related videos



Expanded "assembly standards" for MoClo, GoldenBraid 2.0 and Other Modular Golden Gate Assembly Methods

McClure (and Goldenfild 2.0) uses 3 levels of accuracy assembly. The community has agreed upon a set of common standard overhangs for each level. Utilizing gathered ligase fidelity information, NEB has expanded each level of assembly overhangs without sacrificing fidelity. The expanded set are

Expanded MitoChloroplasted
Assembling Cholesterol?

- Level 0 (Basic proof)
ACAT, TTGT, ATGT, GCTA, CCCA,
AATA, ATTG, GTGA, CGCC, AGCA,
AAAC, AACG, TBCG, GAAC, CAGA,
AANC, AACG, CTCG, GACG, ATGA,
ACCG, TACA, GAAA, CAAG, AGAA
(93% identity)
- Level 1 (Transcription units)
GAGG, TACT, CCAT, AATA, AGGT,
TTGG, CGGT, GCTA, CGCT, GAAA,
TCGA, ATAA, GAGA, CGGC, GTCA,
AAGT, AACT, GCAC, CTTA, TACA
(92% identity)
- Level 2 (Multigene conserved)
TAGC, GCAA, ACTA, TTAC, CAGA,
TGTG, GAAC, GAGA, GATA, CTTC,
ATCC, ATAG, CCAU, AACT, ACCG,
AANA, AGAC, AGGG, TGA A, ATGA
(89% identity)

1. *Pharmaceuticals and the Environment: The Role of the FDA*. Washington, DC: American Enterprise Institute for Public Policy Research, 2004.

1. Hughes, C., Boudier, E., and McMillan, G. (1998) *Proc. 1998*, 170-183.

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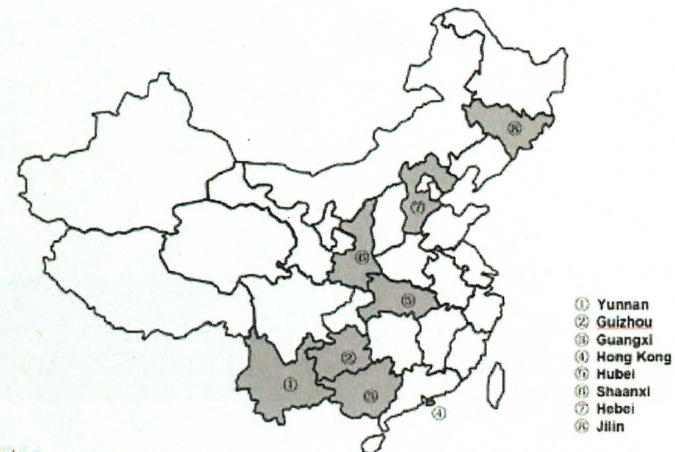
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WIV Bat Coronavirus Collection Efforts

- WIV possesses a large bank of Bat Coronaviruses isolated from various bat species in Yunnan Province China Ge et al., 2013 Yang et al., 2016 Hu et al., 2017 Five-year longitudinal study to isolate Bat Coronaviruses (April 2011 – October 2015) Only a few sequences have been published

Table 1. Summary of SARSr-CoV detection in bats from a single habitat in Kunming, Yunnan.

Sampling time	Sample type	Sample Numbers			SARSr-CoV + bat species (No.)
		Total	CoV +	SARSr-CoV +	
April, 2011	anal swab	14	1	1	<i>R. sinicus</i> (1)
October, 2011	anal swab	8	3	3	<i>R. sinicus</i> (3)
May, 2012	anal swab & feces	54	9	4	<i>R. sinicus</i> (4)
September, 2012	feces	39	20	19	<i>R. sinicus</i> (16)
					<i>R. torunnequinum</i> (3)
April, 2013	feces	52	21	18	<i>R. sinicus</i> (16)
July, 2013	anal swab & feces	115	9	8	<i>R. sinicus</i> (8)
May, 2014	feces	131	8	4	<i>A. stoliczkanus</i> (3)
					<i>R. affinis</i> (1)
October, 2014	anal swab	19	4	4	<i>R. sinicus</i> (4)
May, 2015	feces	145	3	0	
October, 2015	anal swab	25	6	5	<i>R. sinicus</i> (5)
Total		602	84	64	<i>R</i> (61) <i>A</i> (3)



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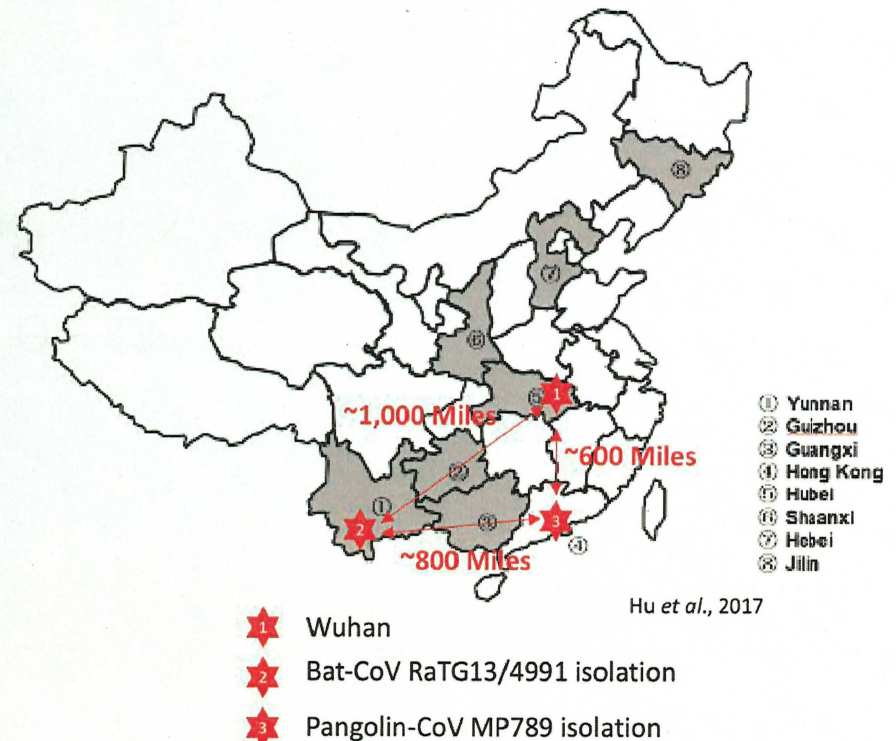
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Extracted from Hu et al., 2017

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Isolation Locations for RaTG13 and MP789

- RaTG13/4991 isolated from a cave in Yunnan Province
RaTG13/4991 is a RdRp lineage 1 BetaCoV
MP789 was isolated from diseased Pangolins in Guangdong Province
MP789 is a RdRp lineage 2 BetaCoV
~800 miles separate these two locations
WIV also collected CoV's from Guangdong and may have a MP789-related virus in their bank
"All the genomic constituents of SARS-CoV including the hypervariable regions S and ORF8 were discovered from different bat SARSr-CoVs in the same cave in Yunnan, with evidence of recombination events detected between these bat SARSr-CoVs..." (Yu et al., 2019)
Question: How would a Pangolin RBD from 800 miles away in Guangdong Province recombine into a BatCoV in Yunnan Province?



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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).”

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WIV SARS-CoV Reverse Genetics System

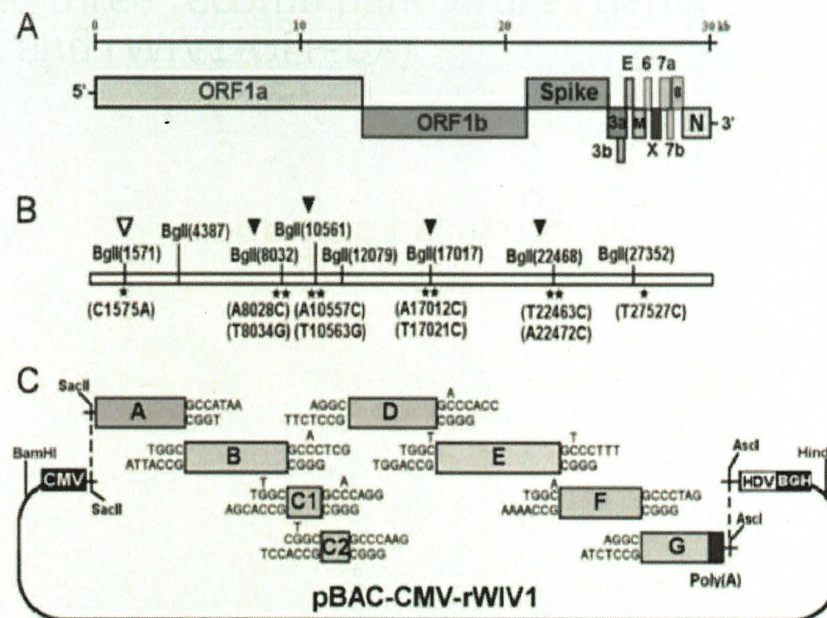
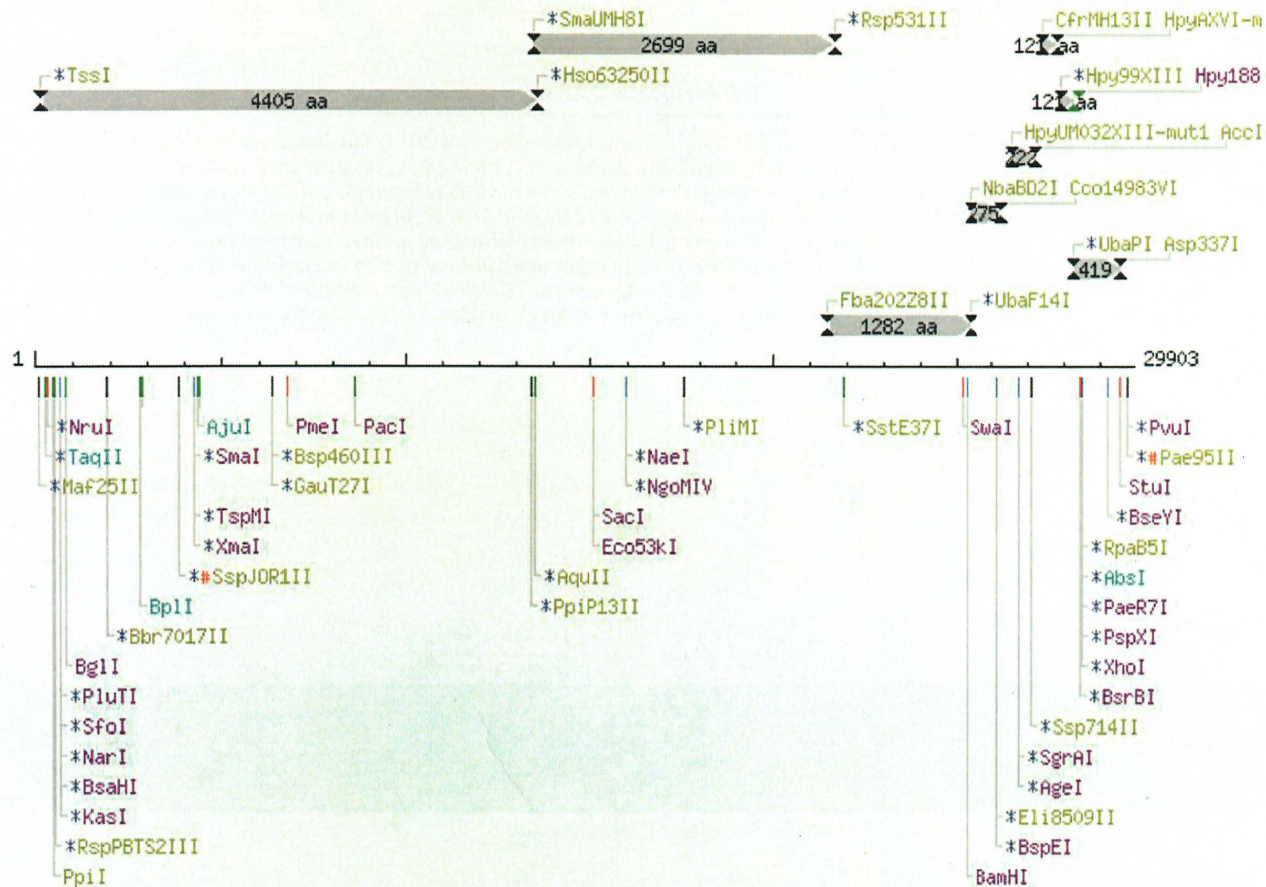


FIG 1 Strategy for construction of an infectious WIV1 BAC clone. (A) Genomic structure of WIV1. (B) The mutations are indicated under the stars. C1575A was used to ablate a natural BglII site at nucleotide 1571 (▽), and T27527C was used to disrupt a potential T7 stop site. The others were for introducing BglII 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 BglII sites were introduced into the fragments by synonymous mutations to make these fragments capable of unidirectional ligation along with native BglII 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 Ascl sites were introduced between the CMV promoter and ribozyme. Fragments A to G were inserted into the pBAC-CMV plasmid in a single step.

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SARS-COV-2 GENOME RESTRICTION MAP



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Type IIS Restriction Enzymes

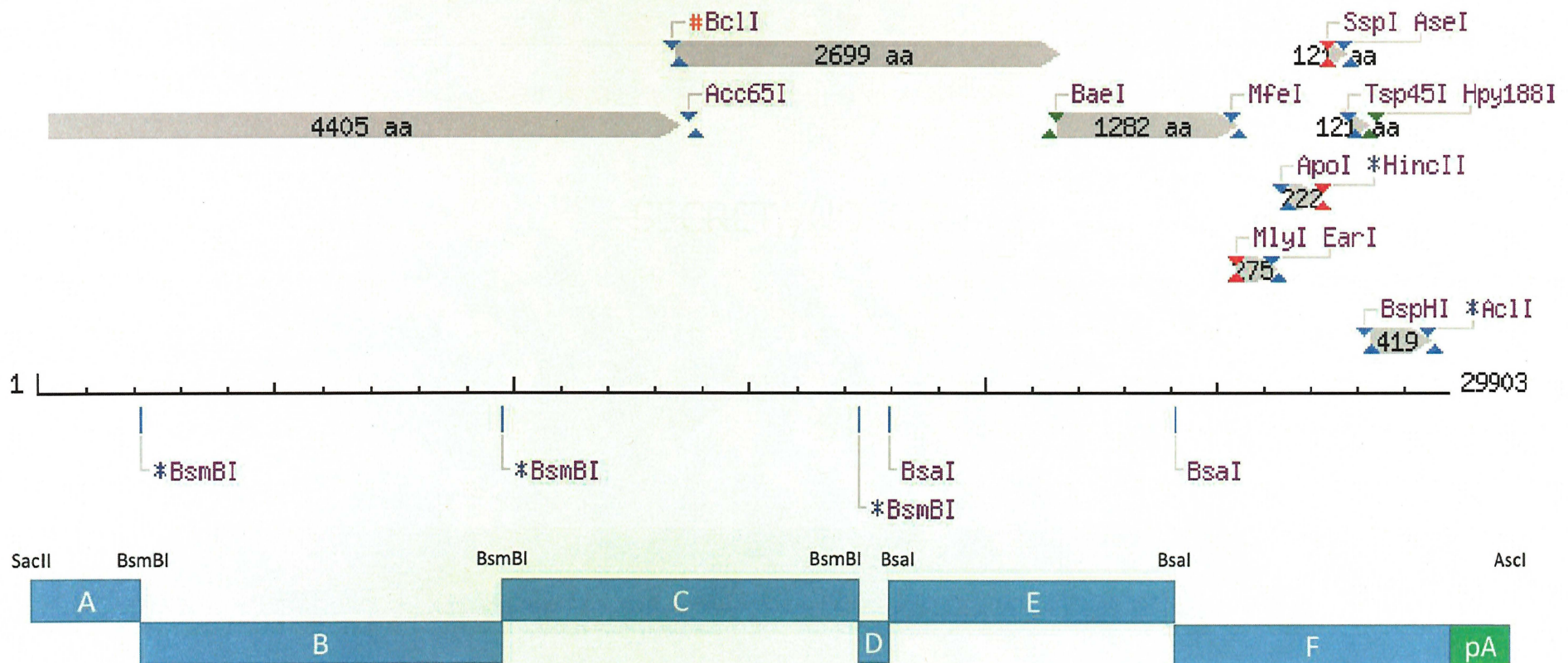
- BsmBI (Plus strand)
5' - CGTCTCNNNNN-3'
3' - GCAGAGNNNNN - 5' BsaI (Plus strand)
5' - GGTCTCNNNNN-3'
3' - CCAGAGNNNNN - 5' SARS-CoV-2 genome does not have any SacII or AscI restriction sites
- BsmBI (Minus strand)
5' - NNNNNGAGACG-3'
3' - NNNNNCTCTGC-5' BsaI (Minus strand)
5' - NNNNNGAGACG-3'
3' - NNNNNCTCTGG - 5'

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

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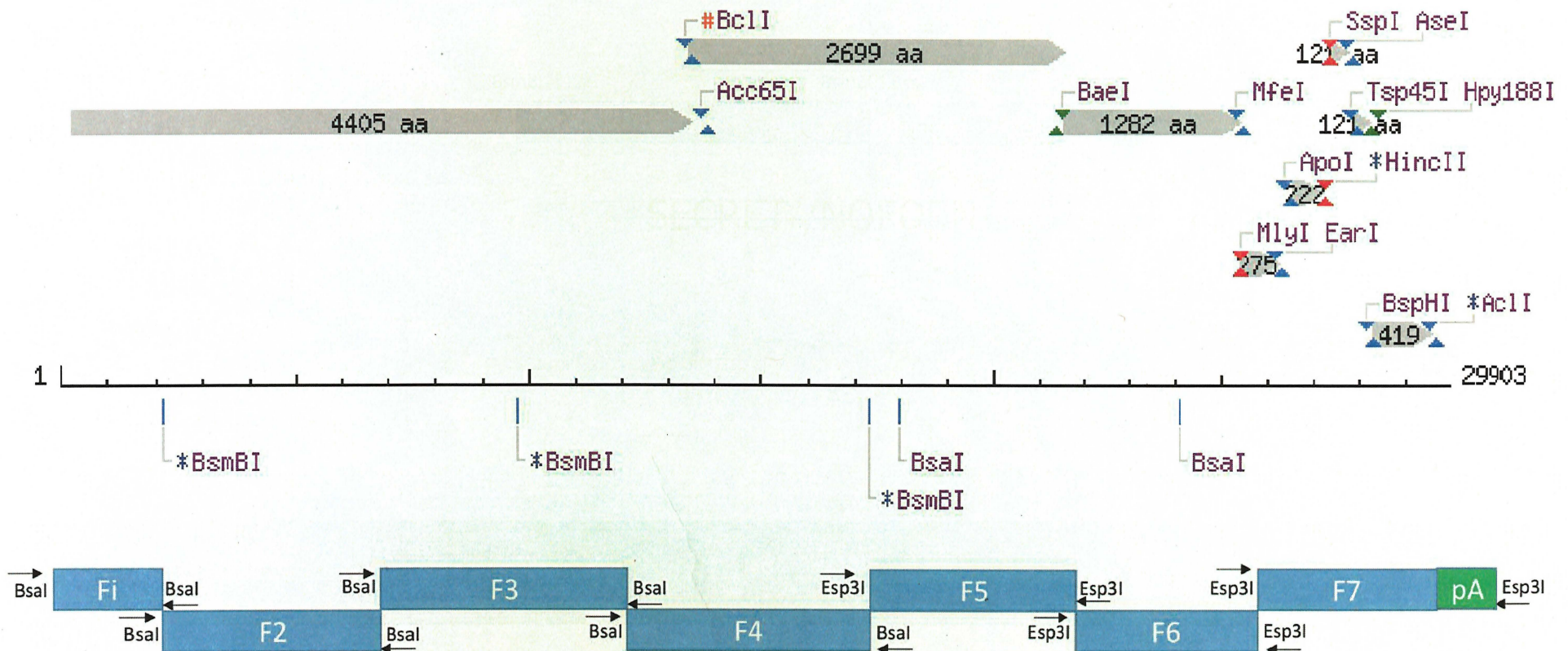
RE-based Fragment Build Option – *BsmBI*/*BsaI* (4 nt overhangs)



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RE-based Fragment Build Option – *BsaI/Esp3I* (Invisible restriction sites)



Xie et al., 2020

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

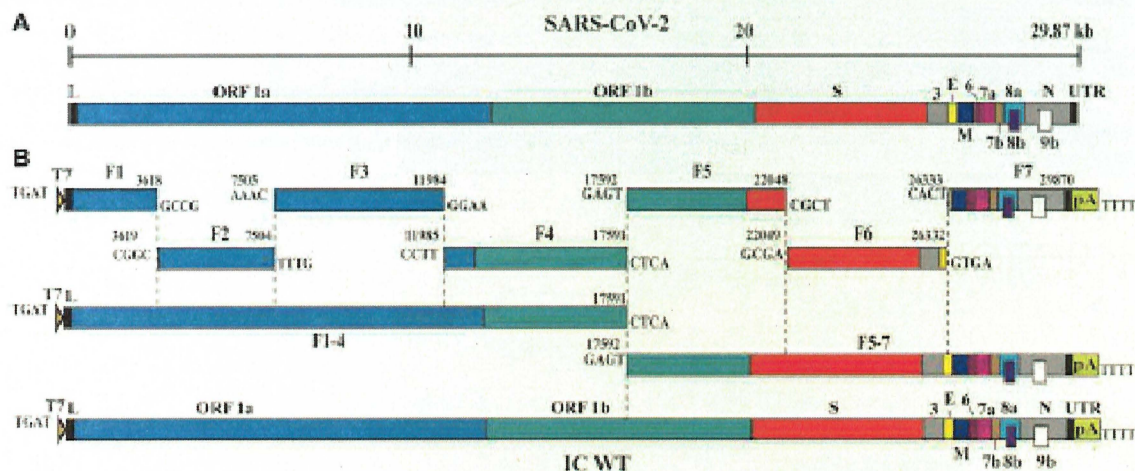


Figure 1. Assembly of a Full-Length SARS-CoV-2 Infection cDNA Clone

(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.

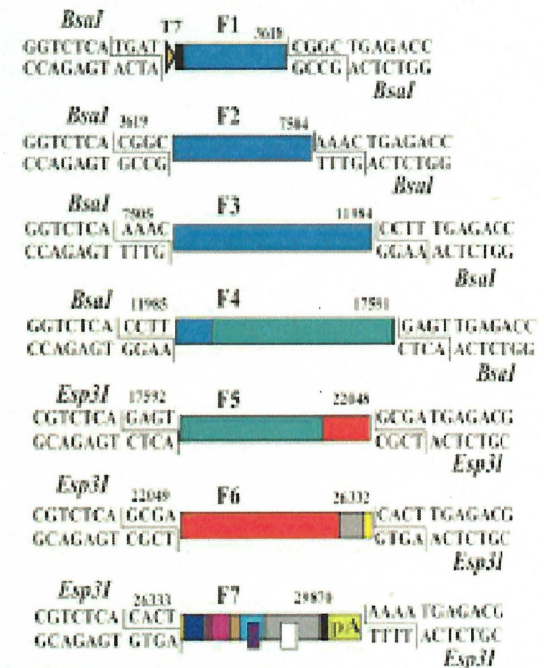
(C) Diagram of the terminal sequences of each cDNA fragment recognized by *BsaI* and *Esp3I*.

(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 0.8% 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 0.8% 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 0.8% 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.

C Fragment Terminal Sequence



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

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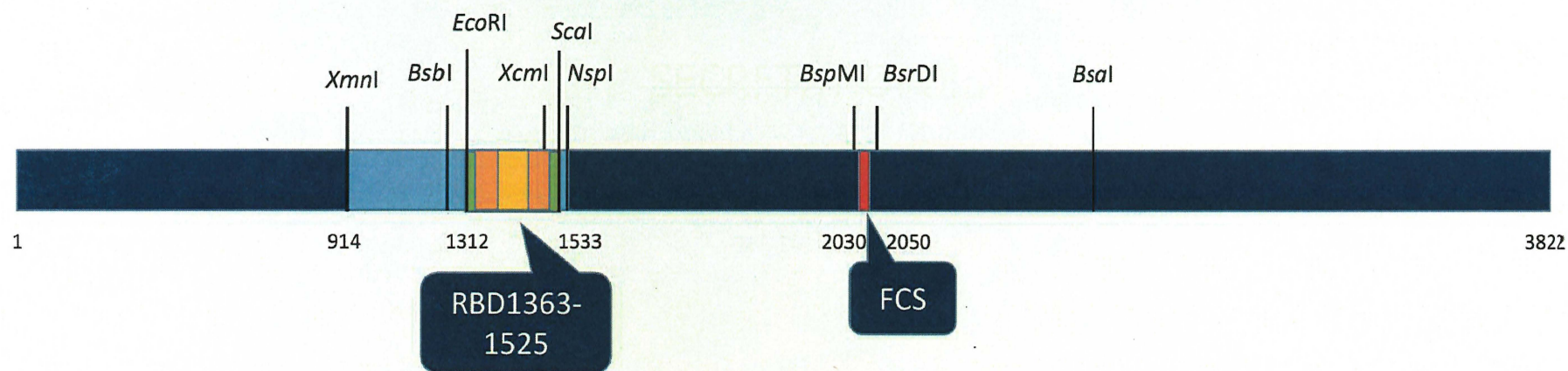
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Spike Gene

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SARS CoV-2 SPIKE GENE



Highest homology to RaTG13 Pangolin CoV

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Spike Gene Swapping Using the WIV SARS-CoV Reverse Genetics System

- Hu et al., 2017 swapped out the WIV1 spike gene for the spike gene of the following: Rs4231Rs73 27Rf4075Rs4081Rs4085Rs4235As6526Rp3

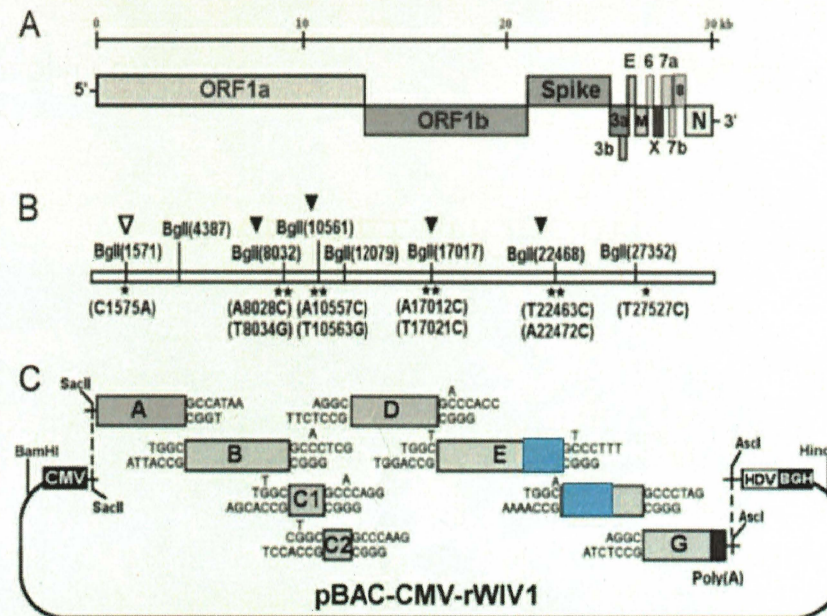
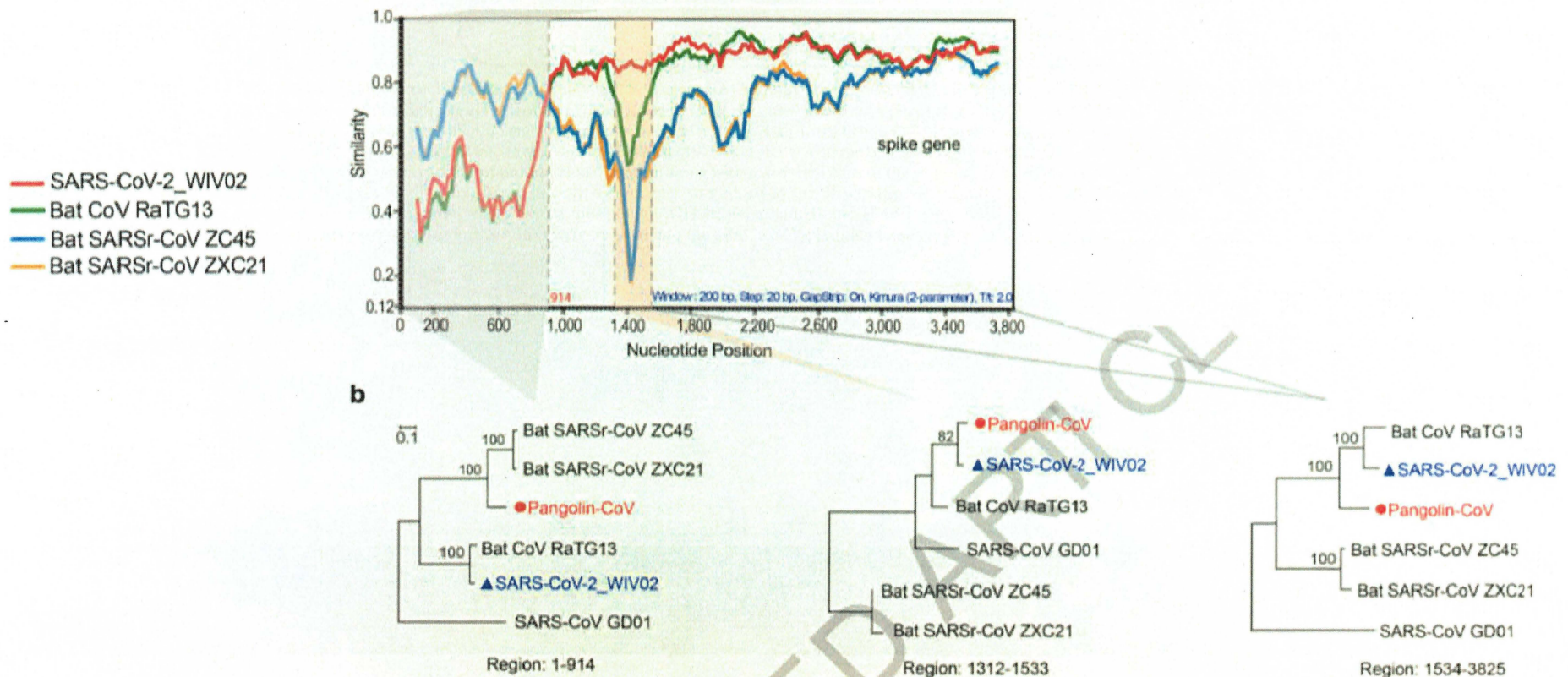


FIG 1 Strategy for construction of an infectious WIV1 BAC clone. (A) Genomic structure of WIV1. (B) The mutations are indicated under the stars. C1575A was used to ablate a natural BglI site at nucleotide 1571 (▽), and T27527C was used to disrupt a potential T7 stop site. The others were for introducing 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 the 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.

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SARS-COV-2 SPIKE GENE SEGMENTS

QUERY: PANGOLIN-COV

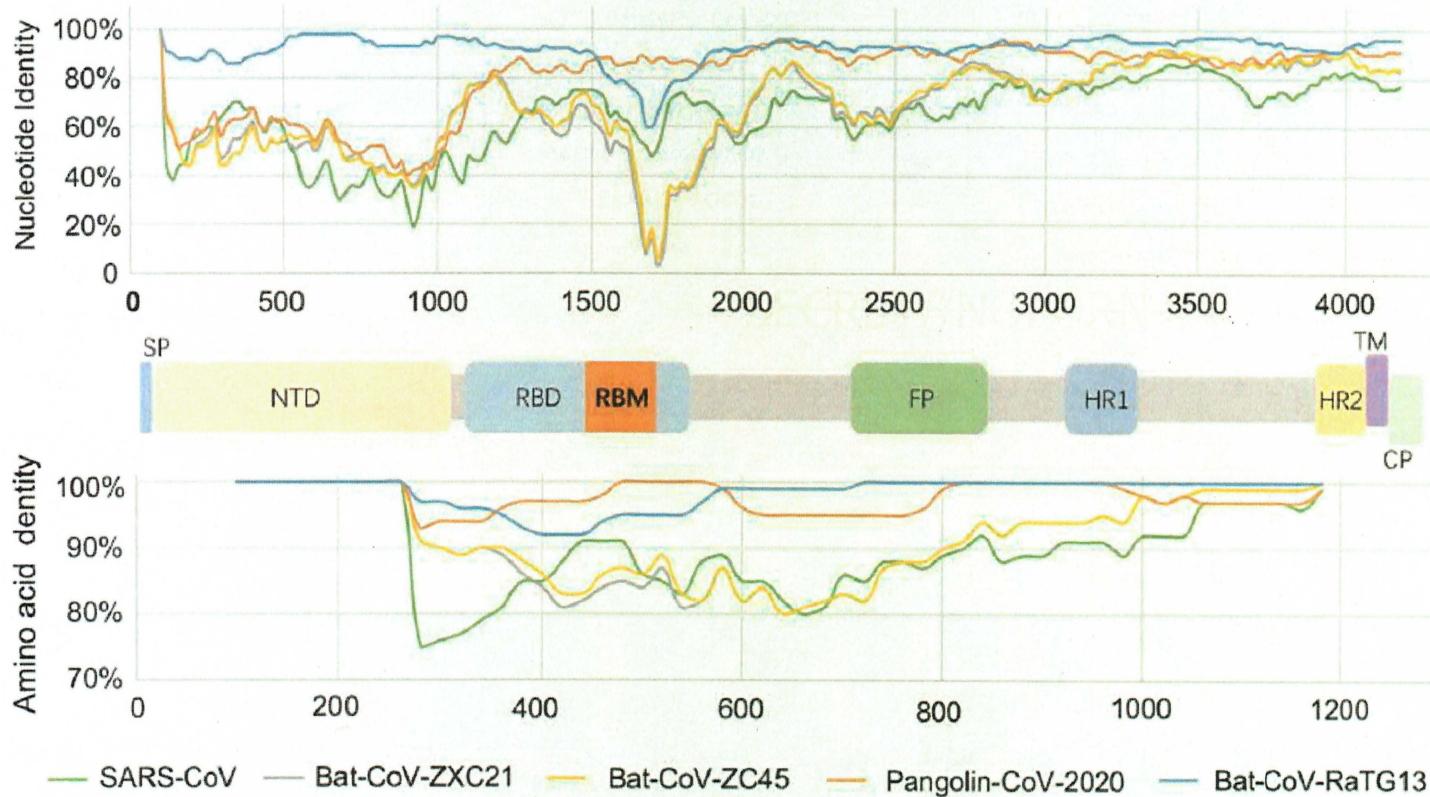


Xiao *et al.*, 2020

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SARS-CoV-2 SimPLOT



- "A recent study found that a human ACE2-binding ridge in SARS-CoV-2 RBD takes a more compact conformation compared with the SARS-CoV RBD; moreover, several residue changes in SARS-CoV-2 RBD may also enhance its human ACE2-binding affinity [13]. The core residues in RBM which may related to higher human ACE2-binding affinity than SARS-CoV are 100% identical between SARSCoV-2 and CoV-Pangolin-2020. Therefore, pangolin-CoV-2020 (CoV-pangolin/GD) potentially recognizes human ACE2 better than the SARS-CoV."

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Ren *et al.*, 2008

JOURNAL OF VIROLOGY, Feb. 2008, p. 1899–1907
0022-538X/08/S08.00+0 doi:10.1128/JVI.01085-07
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Vol. 82, No. 4

Difference in Receptor Usage between Severe Acute Respiratory Syndrome (SARS) Coronavirus and SARS-Like Coronavirus of Bat Origin[▽]

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Lin-Fa Wang,^{3*} Hongkui Deng,² and Zhengli Shi^{1*}

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Received 20 May 2007/Accepted 15 November 2007

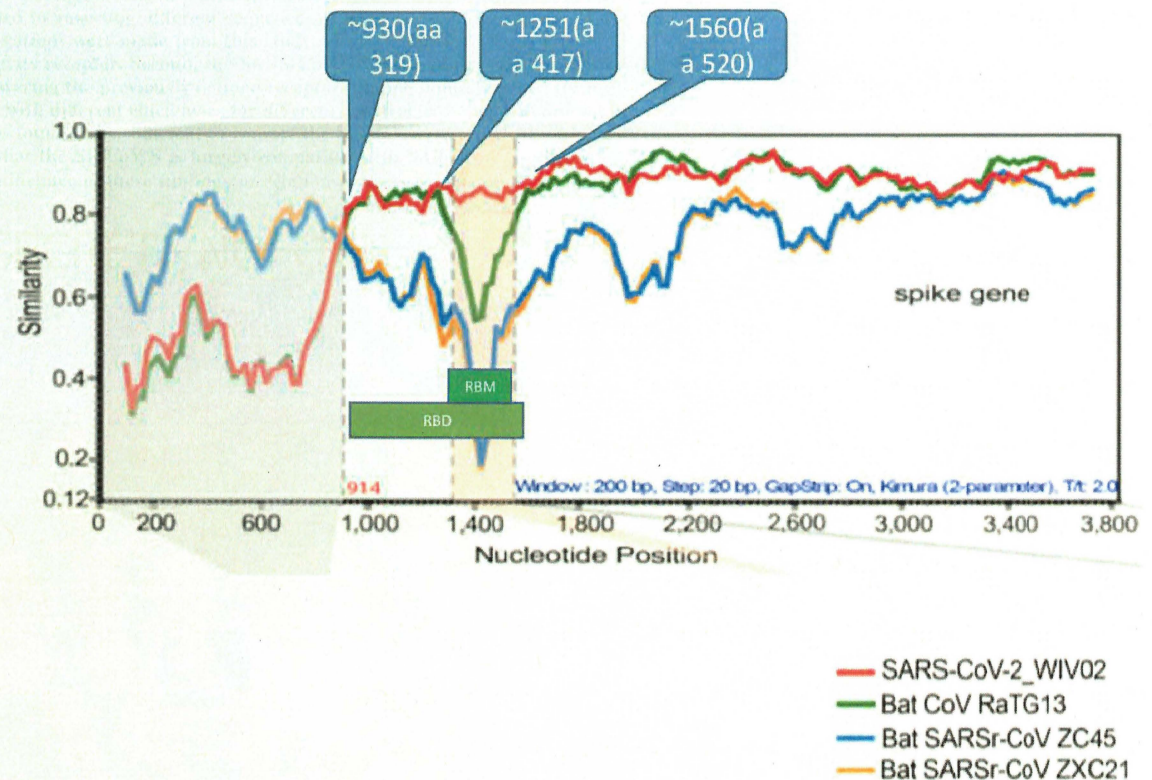
Severe acute respiratory syndrome (SARS) is caused by the SARS-associated coronavirus (SARS-CoV), which uses angiotensin-converting enzyme 2 (ACE2) as its receptor for cell entry. A group of SARS-like CoVs (SL-CoVs) has been identified in horseshoe bats. SL-CoVs and SARS-CoVs share identical genome organizations and high sequence identities, with the main exception of the N terminus of the spike protein (S), known to be responsible for receptor binding in CoVs. In this study, we investigated the receptor usage of the SL-CoV S by combining a human immunodeficiency virus-based pseudovirus system with cell lines expressing the ACE2 molecules of human, civet, or horseshoe bat. In addition to full-length S of SL-CoV and SARS-CoV, a series of S chimeras was constructed by inserting different sequences of the SARS-CoV S into the SL-CoV S backbone. Several important observations were made from this study. First, the SL-CoV S was unable to use any of the three ACE2 molecules as its receptor. Second, the SARS-CoV S failed to enter cells expressing the bat ACE2. Third, the chimeric S covering the previously defined receptor-binding domain gained its ability to enter cells via human ACE2, albeit with different efficiencies for different constructs. Fourth, a minimal insert region (amino acids 310 to 518) was found to be sufficient to convert the SL-CoV S from non-ACE2 binding to human ACE2 binding, indicating that the SL-CoV S is largely compatible with SARS-CoV S protein both in structure and in function. The significance of these findings in relation to virus origin, virus recombination, and host switching is discussed.

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Minimal Receptor Binding Domain Cassette

- WIV scientists previously defined the minimal Receptor Binding Domain cassette that could functionally transfer ACE2 binding capability from one Spike protein to another
SARS Nucleotide: 930-1554
SARS Amino Acid: 310-518
Receptor Binding Motif
SARS Nucleotide: 1251-1482
SARS Amino Acid: 417-494
Homology cut points of SARS-CoV-2 coincide with WIV-identified borders of RBD and RBM



Xiao *et al.*, 2020 & Ren *et al.*, 2008

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Furin Cleavage Site

SARS-CoV 1980 CATGTCGACACTTCTTATGAGTGCGACATTCCTATTGGAGCTGGCATTGTGCTAGTTAC
H V D T S Y E C D I P I G A G I C A S Y SARS-CoV-2
CATGTCAACAACATCATATGAGTGTGACATACCCATTGGTGCAGGTATATGCGCTAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S Y BCoV RaTG13
CATGTCAATAACTCGTATGAGTGTGACATACCTATTGGTGCAGGAATATGCGCCAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S Y SARS-CoV CATAAGTTTCTTTATT-----
ACGTAGTACTAGCCAAAAATCTATTGTGGCT 2028 H T V S L L R S T S Q K S I
V ASARS-CoV-2 CAGACTCAGACTAATTCTCCTCGGCGGGCACGTAGTGTAGCTAGTCAATCCATCATTGCC
2082 Q T Q T N S P R R A R S V A S Q S I I ABCoV RaTG13
CAGACTCAAACATAATTC-----ACGTAGTGTGGCCAGTCAATCTATTATTGCC 2070 Q T Q
T N S R S V A S Q S I I AFurin Cleavage SiteNmeAIII Restriction Site

A unique restriction site facilitates
identifying the correct *E. coli* clone

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SARS-CoV-2 Furin Cleavage Site GC Content

- The percent GC of the furin cleavage site insert is 77% compared to ~40% of the surrounding DNA. Contains an NmeAIII restriction site. The other CoV's with FCS have a %GC of <55%

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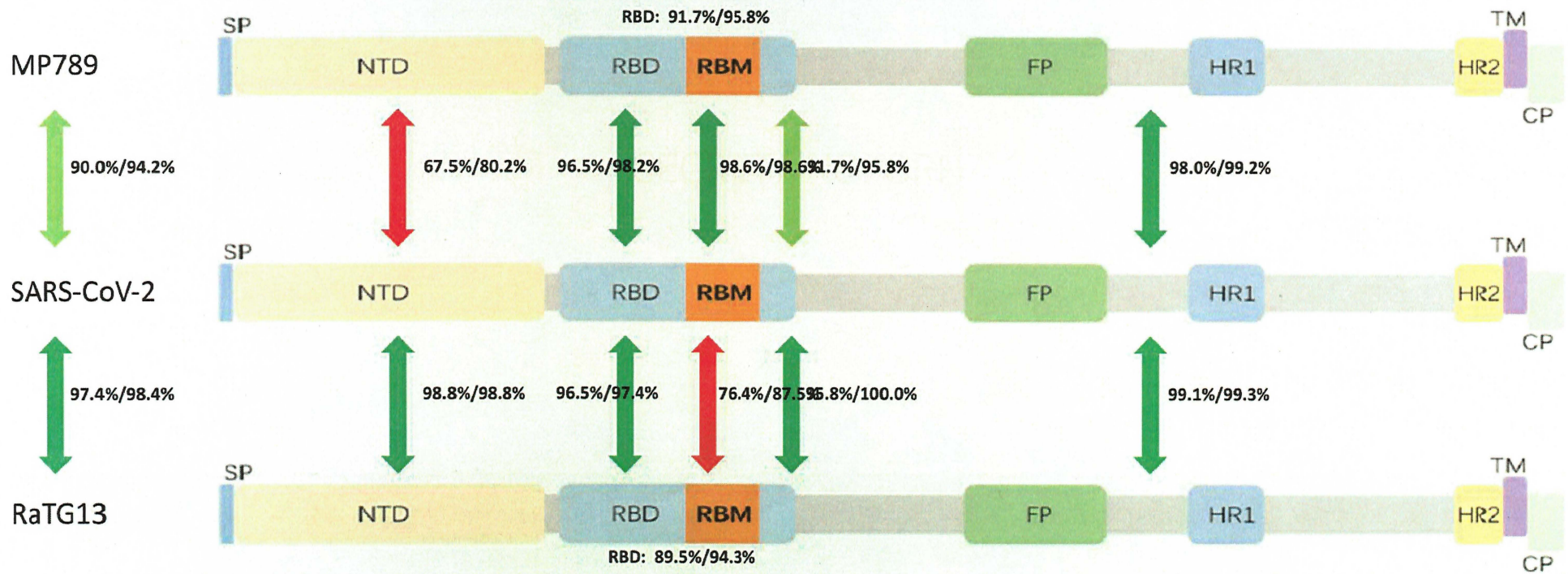
Influenza viruses convert from low path to high path by addition of a polybasic cleavage site by virtue of RNA Polymerase stuttering which adds preferentially A's and T's – this is not the case with SARS-CoV-2

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% Identity:>95

Spike Protein Regions



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SARS-CoV-2 Spike RBD Alignment: Possible RBM Cassette Insertion

```
SARSCoV2      TRFQTLALHRSYLT PGDSSSGWTAGAAAYVGYLQPRTFLLKYNENGTITDAVDCALDPBCoV_RaTG13
TRFQTLALHRSYLT PGDSSSGWTAGAAAYVGYLQPRTFLLKYNENGTITDAVDCALDPMP789      TKFRTLTIHRGDPMP---
NNGWTVFSAAYVGYLAPRTFMLNYNENGTITDAVDCALDP      NTD<>RBDSARSCoV2
LSETKCTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRCoV_RaTG13
LSETKCTLKSFTVEKGIYQTSNFRVQPTDSIVRFPNITNLCPFGEVFNATTFASVYAWNRM789/Manis
LSEAKCTLKSLTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATTFASVYAWNRCoV_RaTG13
KRISNCVADYSVLYNSASFSTFKCYGVSP TKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTB CoV_RaTG13
KRISNCVADYSVLYNSTSFSTFKCYGVSP TKLNDLCFTNVYADSFVITGDEVQRQIAPGQTM789/Manis
KRISNCVADYSVLYNSTSFSTFKCYGVSP TKLNDLCFTNVYADSFVVRGDEVQRQIAPGQT
>RBMSARSCoV2      GKIADYNYKLPDDFTGCVIAWNSNNLDSKVGNYNYLYRLFRKSNLKPFFERDISTEIQABCoV_RaTG13
GKIADYNYKLPDDFTGCVIAWNSKHIDAKEGGNFNYLYRLFRKANLKPFFERDISTEIQAMP789/Manis
GRIADYNYKLPDDFTGCVIAWNSNNLDSKVGNYNYLYRLFRKSNLKPFFERDISTEIQ
RBM<      RBD<SARSCoV2
GSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKBCoV_RaTG13
GSKPCNGQTGLNCYYPYRYGYPTDGVGHQPYRVVLSFELLNAPATVCGPKKSTNLVKMP789/Manis
GSTPCNGVEGFNCYFPLQSYGFHPTNGVGYQPYRVVLSFELLNAPATVCGPKQSTNLVK SARSCoV2
NKC VN FNFLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVBCoV_RaTG13
NKC VN FNFLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVMP789
NKC VN FNFLTGTGVLTESSKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGV
```

ACE2 Critical ContactACE2

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SARS-COV-2 RBD vs PCoV MP789 RBD

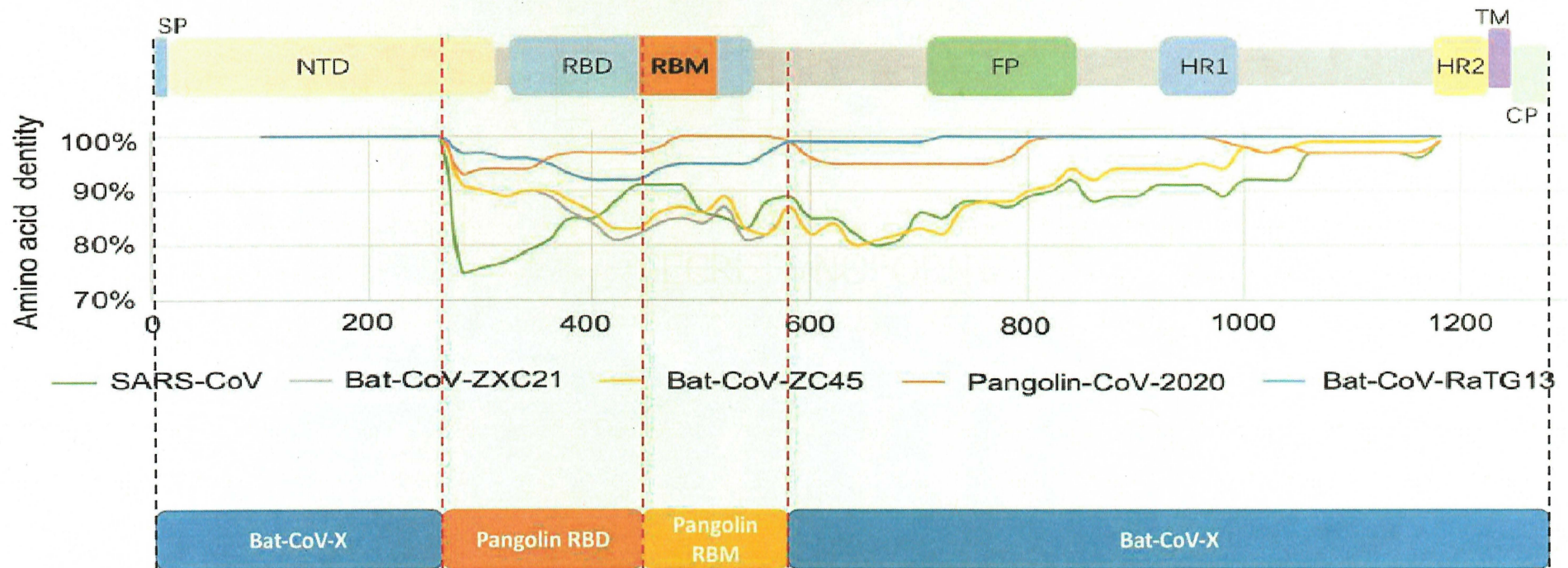
>RBM
D Y N Y K L P D D F T G C V I A W N S N MP789
GACTATAATTATAAACTCCCTGATGATTTACAGGTTGTGTAATAGCTTGAATCTAAC 1305SARSCOV2
GATTATAATTATAAACTACCAGATGATTTACAGGCTGCGTTATAGCTTGAATCTAAC 1317 D
Y N Y K L P D D F T G C V I A W N S N N L D
S K V G G N Y N Y L Y R L F R K S MP789
AACCTTGATTCTAAGGTTGGTGGTAATTATAACTACCTTTATAGATTGTTAGAAAGTCC 1365SARSCOV2
AATCTTGATTCTAAGGTTGGTGGTAATTATAATTACCTGTATAGATTGTTAGGAAGTCT 1377 N
L D S K V G G N Y N Y L Y R L F R K S N L K
P F E R D I S T E I Y Q A G S T P MP789
AACCTCAAACCTTTTGAACGAGACATTTCTACAGAAATATACCAAGCTGGTAGTACACCC 1425SARSCOV2
AATCTCAAACCTTTTGAAGAGATATTTCAACTGAAATCTATCAGGCCGGTAGCACACCT 1437 N
L K P F E R D I S T E I Y Q A G S T P C N G
V E G F N C Y F P L Q S Y G F Q P MP789
TGCAATGGGGTTGAAGGTTTAACTGTTACTTTCCTCTACAATCTTATGGTTTCCACCCCT 1485SARSCOV2
TGTAATGGTGTGAAGGTTTAAATTGTTACTTTCCTTTACAATCATATGGTTTCCAACCC 1497 C
N G V E G F N C Y F P L Q S Y G F Q P
RBM< T N G V G Y Q P Y R V V V L S F E L L H
MP789 ACTAATGGTGTGGTTACCAACCTTATAGAGTAGTAGTATTGTCATTTGAACCTTTAAAA
1545SARSCOV2
ACTAATGGTGTGGTTACCAACCATAACAGAGTAGTAGTACTTTCTTTTGAACCTTCTACAT 1557 T
N G V G Y Q P Y R V V V L S F E L L K
RBD< A P A T V C G P K K S T N MP789
GCACCTGCTACTGTTTGTGGACCTAAACAGTCCACTAACCTAGTTAAAAACAPATGTGTC 1605SARSCOV2
GCACCAGCAACTGTTTGTGGACCTAAAAAGTCTACTAATTTGGTTAAAAACAPATGTGTC 1617 A
P A T V C G P K K S T N

- 38 codon differencesFirst: 4Second: 0Third: 31First and third: 31 results in an amino acid changePangolin RBD cassette appears to be a codon optimized insert

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SARS-CoV-2 Spike Appears to be a Chimera



Break points align with those identified by WIV Scientists in 2008 (Ren *et al.*, 2008)

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RmYN02 - A Red Herring?

- Zhou et al., 2020 publish paper describing Bat CoV rmYN02Next generation sequencing was done on pooled bat samples to develop two genome sequences – RmYN01 and RmYN02Claim that RmYN02 contains inserted nucleotides at the S1/S2 cleavage siteAssert that the SARS-CoV-2 FCS is therefore of natural originNo virus is available for peer confirmation

H

Polybasic cleavage site

SARS-CoV-2 Numbering: 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693

Consensus SARS-CoV-2	G	A	G	I	C	A	S	Y	Q	T	Q	T	N	S	P	R	R	A	R	S	V	A	S	Q	S	I	I
RmYN02	G	A	G	V	C	A	S	Y	-	-	-	-	N	S	P	-	A	A	R	-	V	G	T	N	S	I	I
RaTG13	G	A	G	I	C	A	S	Y	Q	T	Q	T	N	S	-	-	-	-	R	S	V	A	S	Q	S	I	I
ZC45	G	A	G	I	C	A	S	Y	H	T	A	S	I	L	-	-	-	-	R	S	T	S	Q	K	A	I	V
ZXC21	G	A	G	I	C	A	S	Y	H	T	A	S	I	L	-	-	-	-	R	S	T	G	Q	K	A	I	V
pangolin/MP789/2019	G	A	G	I	C	A	S	Y	Q	T	Q	T	N	S	-	-	-	-	R	S	V	S	S	X	A	I	I
pangolin/GX/P5L/2017	G	A	G	I	C	A	S	Y	H	S	M	S	S	F	-	-	-	-	R	S	V	N	Q	R	S	I	I
SARS-CoV GZ02	G	A	G	I	C	A	S	Y	H	T	V	S	L	L	-	-	-	-	R	S	T	S	Q	K	S	I	V
RmYN01	G	A	G	I	C	A	S	Y	H	T	A	S	L	L	-	-	-	-	R	N	T	G	Q	K	S	I	V

O-linked glycan residues

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Zhou et al., 2020

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RmYN02 - A Red Herring?

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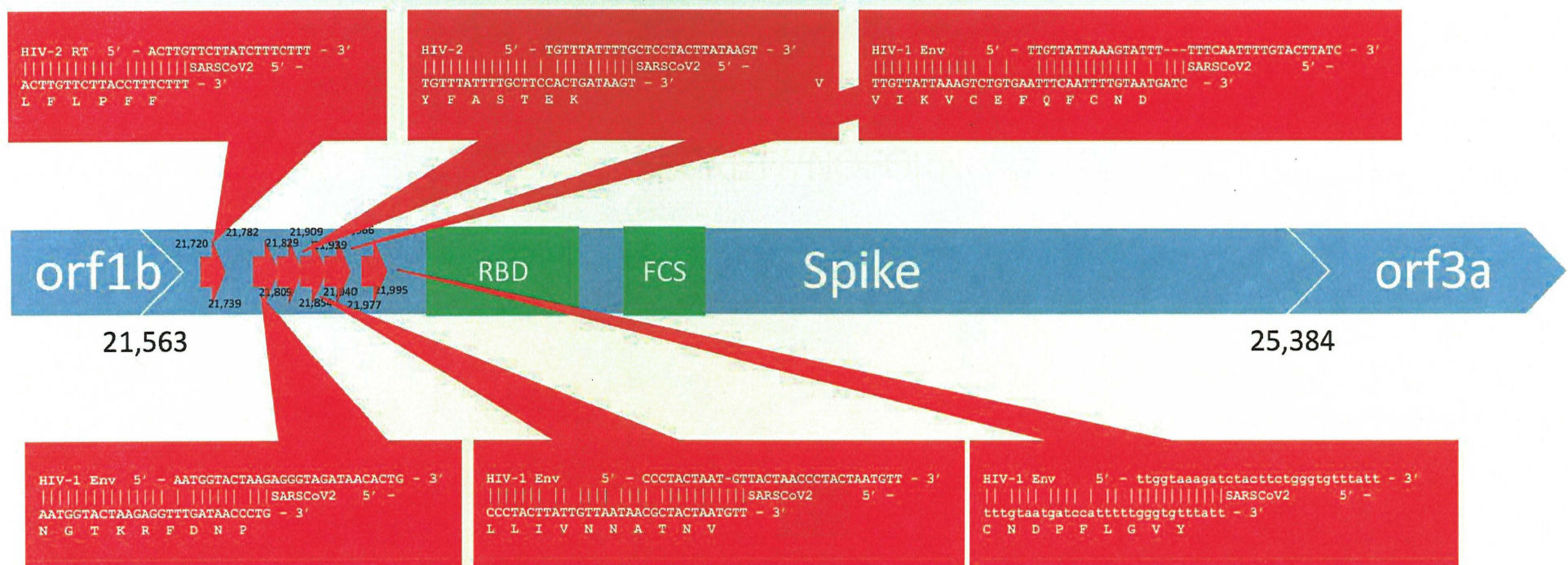
HIV Epitopes

Perez, 2020

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HIV Sequences in the SARS-CoV-2 Spike Gene



Adapted from Perez, 2020

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Perez, 2020 Scientific Challenges

- None of the six proposed regions are identical at either the nucleotide or amino acid level with the corresponding HIV/SIV segmentsNone of the six peptides are related to identified immunosuppressive regions of HIV and SIV (Retroviral ISU Domains)The HIV gp41 Immunosuppressive (ISU) Domains sequence is KQLQARILAVEYLKDDQQLGG - this sequence does not match any of the sixFour of the six regions either perfectly or almost perfectly match corresponding peptides in multiple Pangolin CoVs - Perez did not account for Pangolin genomes in the paperSeveral are only found in Pangolin CoV Spike sequences and not in Bat CoV Spike sequences, indicating that the SARS-CoV-2 Spike NTD region originated from a Pangolin CoV template

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Alternative Scenario

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Hypothetical Laboratory Origin of SARS-CoV-2

- WIV conducted a longitudinal studies to isolate a large number of bat Coronaviruses from multiple locations in China (2011-2015)WIV Developed Reverse Genetic System, assembled WIV1 full-length infectious clone, and created chimeric viruses exchanging the WIV1 spike gene with the spike gene from other bat Coronaviruses (2015-2017)WIV and other Chinese scientists conduct gain of function studies on SARS, MERS, IBV, and PEDV to insert furin cleavage sites demonstrating increased virulence of the chimeric virusesWIV conducted in vivo and in vitro studies to characterize the bank of bat CoronavirusesWIV conducted the live bat Coronavirus studies under BSL2 conditionsChinese BSL2 and US BSL2 conditions are differentChinese labs have had a history of virus escapes from BSL2 laboratoriesHypothesis: Between 2017 and 2019, WIV created a full-length infectious clone in pBAC-CMV using an unpublished bat Coronavirus genome as template (BatCoVX)Hypothesis: Between 2017 and 2019, WIV created chimeric Bat-CoV-X viruses using the pBAC-CMV-BCoVX backbone and swapping out key cassettes with other bat Coronaviruses (RBD, RBM, etc.) and adding additional features such as a furin cleavage siteHypothesis: In 2018-2019, WIV conducted in vitro and in vivo studies to characterize the BatCoVX chimeric viruses under BSL2 conditionsHypothesis: In mid-2019, one of the not fully characterized Bat-CoV-X chimeric viruses escaped from the WIV facilities and begins infecting civilians in the city of WuhanHypothesis: Starting in mid-2019 through present, WIV and other Chinese laboratories conduct studies to characterize the Chimeric BCoVX virus that escaped (now called SARS-CoV-2)WIV (Zhou et al., 2020) publishes the 2019-nCoV genome sequence showing relatedness to RaTG13 (a previously unpublished genome)BatCoVX likely highly related to RaTG13Hypothesis: Beginning in early 2020, WIV and other government controlled agencies begin to publish obfuscation information to drive the narrative that SARS-CoV-2 is of natural origin and resulted from natural recombinationRaTG13RMYN02Pangolin CoV's

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CONCLUSION

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Concluding Points

- WIV possesses a bank of Bat Coronavirus isolatesWIV has scientists experienced in Coronavirology and Coronavirus Infectious Clone generationWIV Scientists generated chimeric SARS CoV and Bat CoV Spike genes to identify minimal Spike Receptor Binding Domain cassette that could transfer receptor binding specificity (Ren et al., 2008)WIV possesses an existing and published Coronavirus Reverse Genetics System (Zeng et al., 2016) utilizing their pBAC-CMV plasmidWIV has utilized the pBAC-CMV-WIV1 Full-length clone to generate chimeras with Bat CoV spike genes (Hu et al., 2017)WIV has BSL2/BSL3/BSL4 animal facilitiesWIV has multiple in vitro assays (apoptosis, IFN-B induction, etc.) to characterize their Bat Coronaviruses and chimeric Bat CoronavirusesWIV and other Chinese researchers have conducted Gain of Function studies in SARS, MERS, IBV, and PEDV to add Furin Cleavage Sites to CoV Spike proteinThe absence of a published progenitor virus for SARS-CoV-2 only indicates that it has not been published, not that it does not existThe genomic sequence of SARS-CoV-2 has Type IIS restriction sites that are consistent with being generated by the Golden Gate Cloning system utilizing the published pBAC-CMV plasmidThe SARS-CoV-2 genome has several break points where homology jumps from Bat Coronaviruses to Pangolin Coronaviruses which is consistent with a synthesized chimeric virusThe SARS-CoV-2 Spike protein similarity with RaTG13 and Pangolin CoV Spike proteins may also be explained by use of cassettes swapped into the base virus – these break points align with those identified by WIV scientists (Ren et al., 2008)The Pangolin RBD cassette is 100% identical at the amino acid level while the DNA sequence appears to be codon optimizedThere are no other published SARS lineage Betacoronaviruses that possess a Furin Cleavage Site in their Spike protein (RmYN02 does not have an insertion) and the SARS-CoV-2 FCS does not appear to be inserted via the same mechanism that drives Influenza virus insertions of polybasic cleavage sitesZeng et al., 2016 stated that “All experiments using live virus was conducted under biosafety level 2 (BSL2) conditions” which would make an accidental release of a pathogenic Bat CoV capable of binding human ACE2 more likelyA chimeric virus comprised of segments from natural Bat CoV genomes would appear like a recombined virus

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The molecular biology capabilities of WIV and the genome assessment are consistent with the hypothesis that SARS-CoV-2 was a lab-engineered virus that was part of a bank of chimeric viruses in Zhen-Li Shi's laboratory at WIV that escaped from containment

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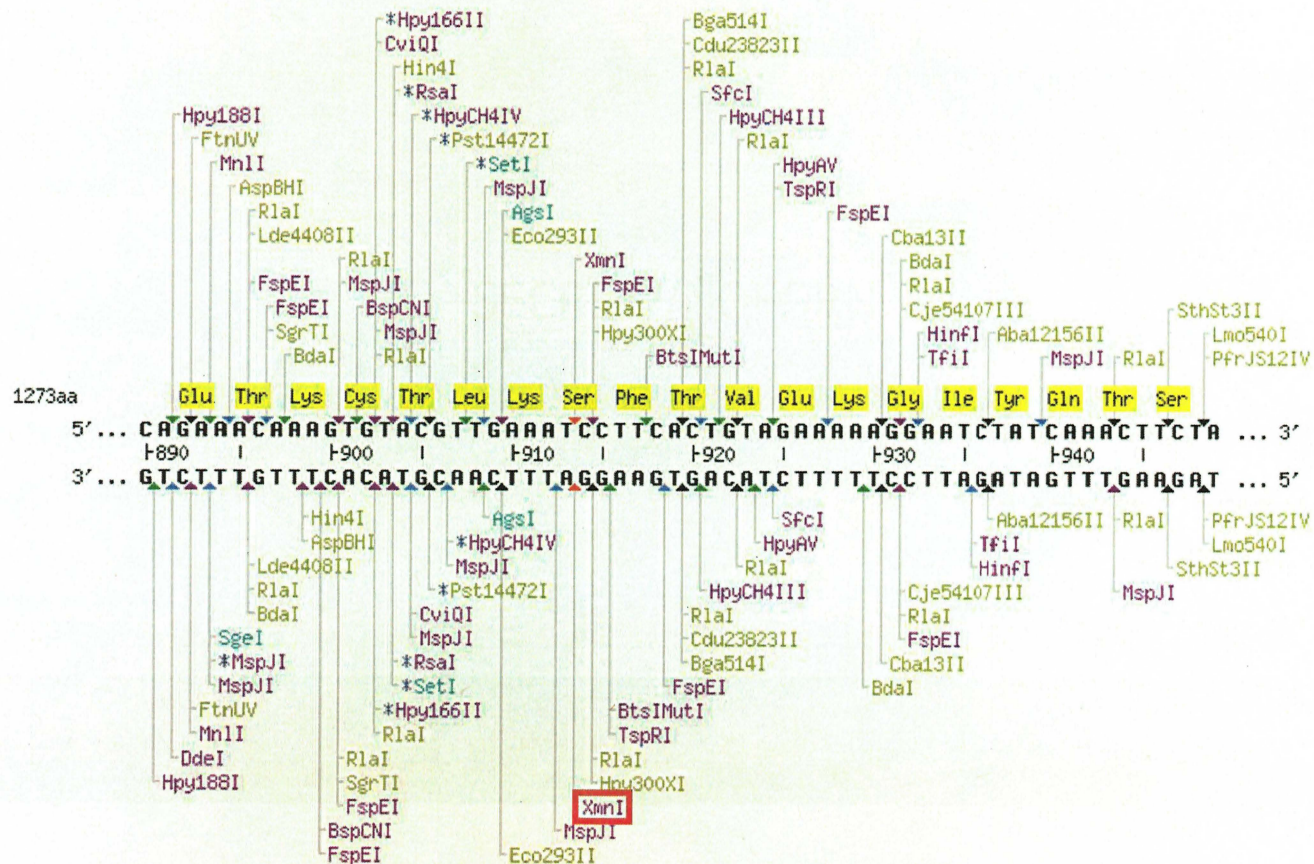
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BACK-UP SLIDES

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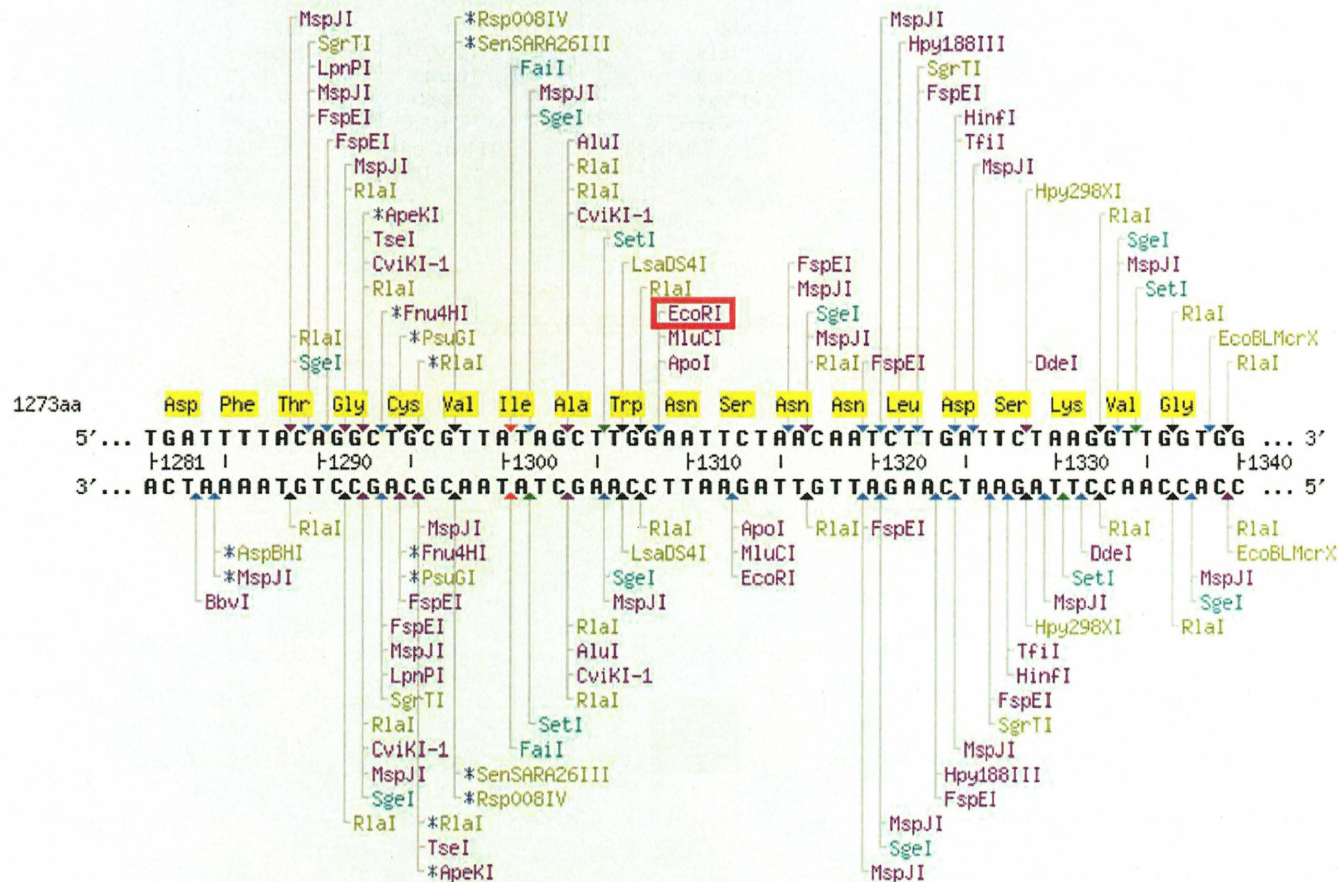
Nucleotide 914 Region



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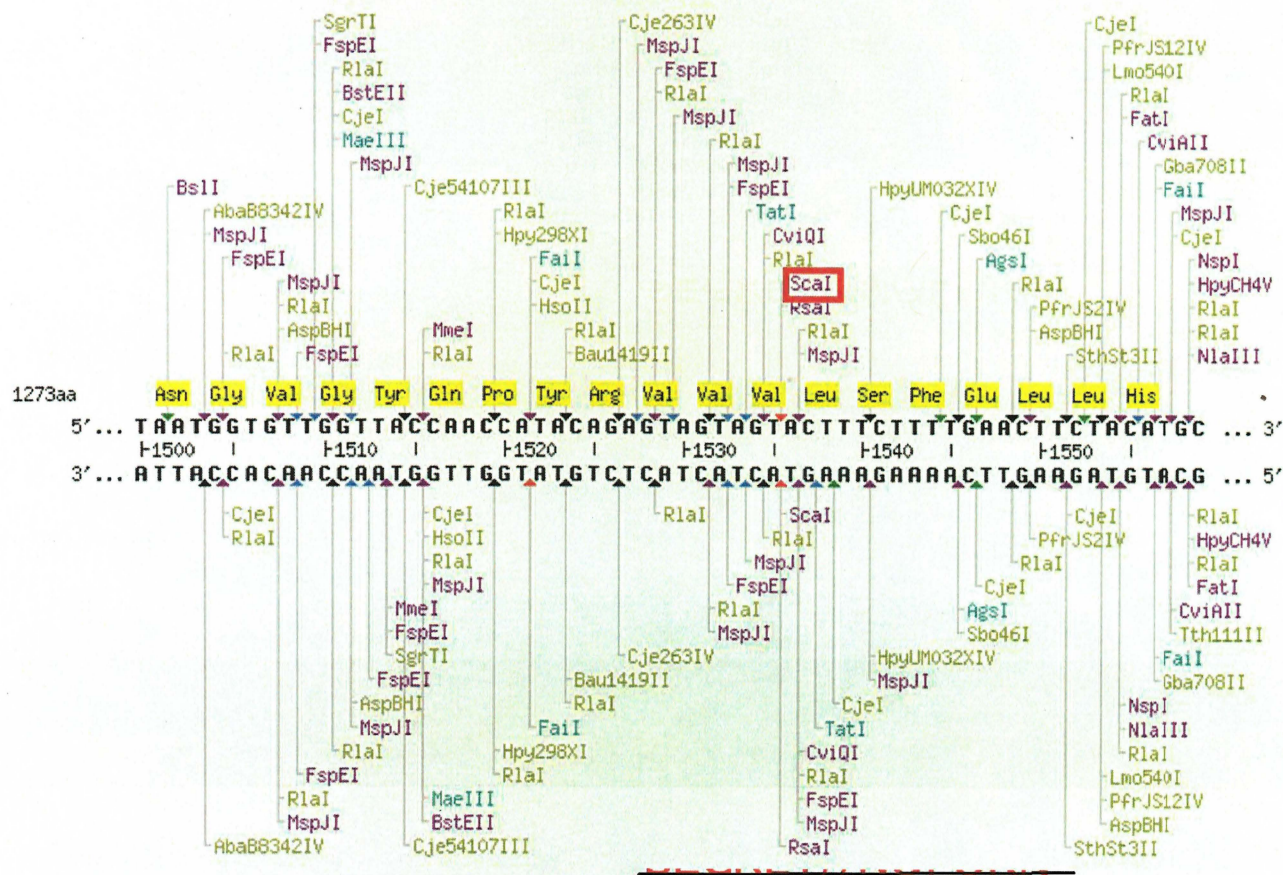
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Nucleotide 1312 Region



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Nucleotide 1535 Region



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SARS-CoV CATGTCGACACTTCTTATGAGTGCGACATTCCTATTGGAGCTGGCATTGTGTGCTAGTTAC
1980 H V D T S Y E C D I P I G A G I C A S Y SARS-CoV-2
CATGTCAACAACATCATATGAGTGTGACATACCCATTGGTGCAGGTATATGCGCTAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S Y BCoV RaTG13
CATGTCAATAACTCGTATGAGTGTGACATACCTATTGGTGCAGGAATATGCGCCAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S Y SARS-CoV CATACAGTTTCTTTATT-----
ACGTAGTACTAGCCAAAAATCTATTGTGGCT 2028 H T V S L L R S T S Q K S I
V ASARS-CoV-2 CAGACTCAGACTAATTCTCCTCGGCGGGCACGTAGTGTAGCTAGTCAATCCATCATTGCC
2082 Q T Q T N S P R R A R S V A S Q S I I ABCoV RaTG13
CAGACTCAAACATAATTC-----ACGTAGTGTGGCCAGTCAATCTATTATTGCC 2070 Q T Q
T N S R S V A S Q S I I AFurin Cleavage SiteBspMI Restriction SiteNmeAIII Restriction SiteBsrDI
Restriction Site

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