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SARS-COV-2 GENOME ANALYSIS

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

June 25, 2020

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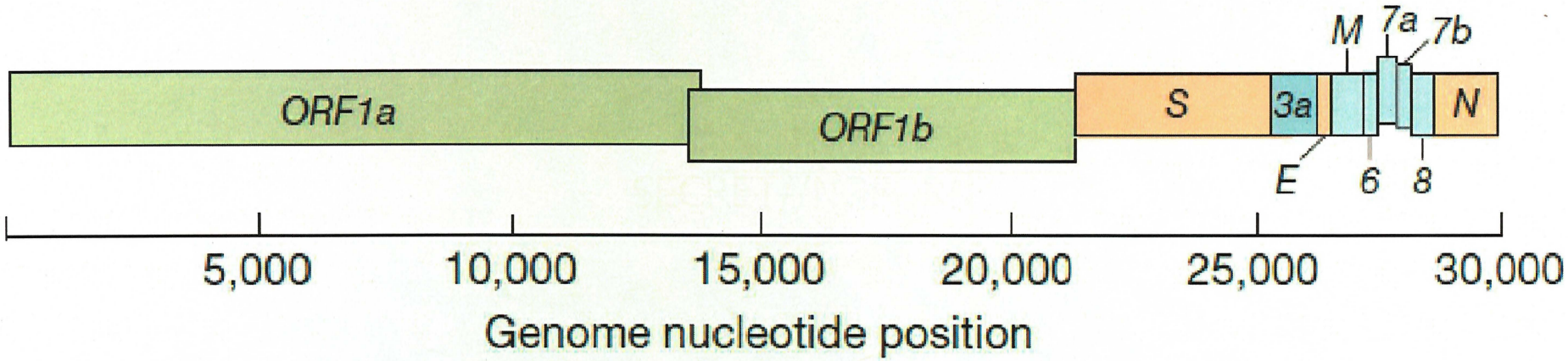
SARS-CoV-2 Genome



The next two pages are DIF citing (b)(1) and (b)(3) and are not provided.

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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 CoronavirusBuild a 5' transcription initiation fragment"stitch" the fragments together using an infectious clone technology 3' to the transcription initiation fragmentRestriction-enzyme-based fragment cloning systemOverlapping Fragment systemGuided RNA RecombinationClone in a suitable host (E. coli, yeast, etc.)Sequence verify cloned insert

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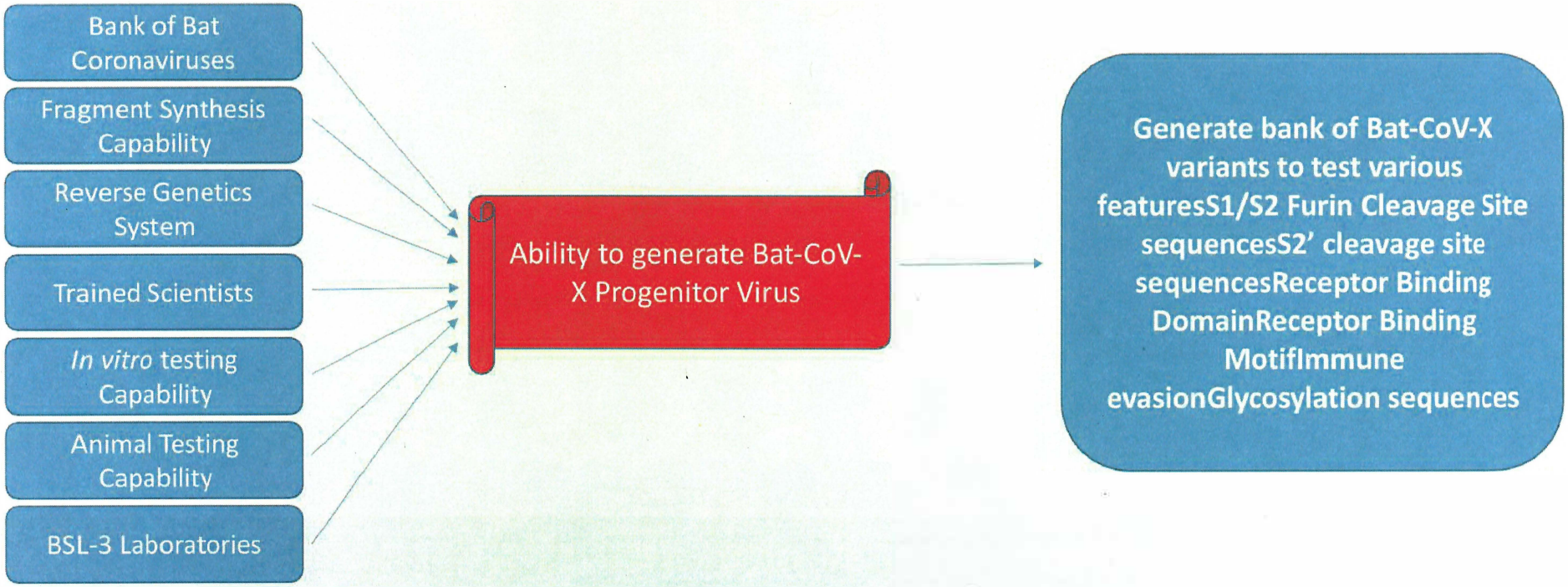
CAPABILITIES NEEDED TO CONSTRUCT A BAT-LIKE CORONAVIRUS INFECTIOUS CLONE



The next page is DIF citing (b)(1) and (b)(3) and is not provided.

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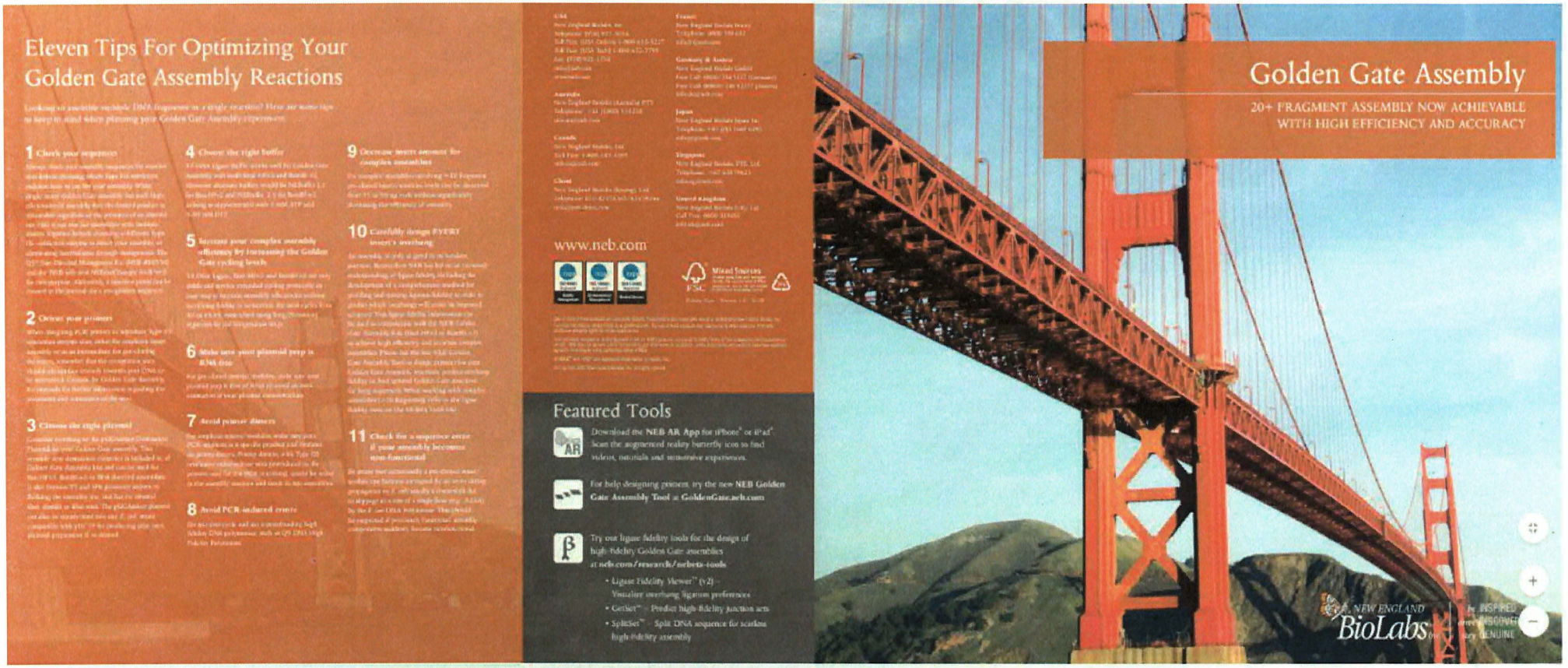
Type IIS Restriction Enzymes and Golden Gate Assembly System

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'



Eleven Tips For Optimizing Your Golden Gate Assembly Reactions

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

1 Check your sequences

2 Order your primers

3 Choose the right plasmid

4 Choose the right buffer

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

6 Make sure your plasmid prep is RNA free

7 Avoid primer dimers

8 Avoid PCR-induced errors

9 Decrease insert amount for complex assemblies

10 Carefully design EVERY insert's overhang

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

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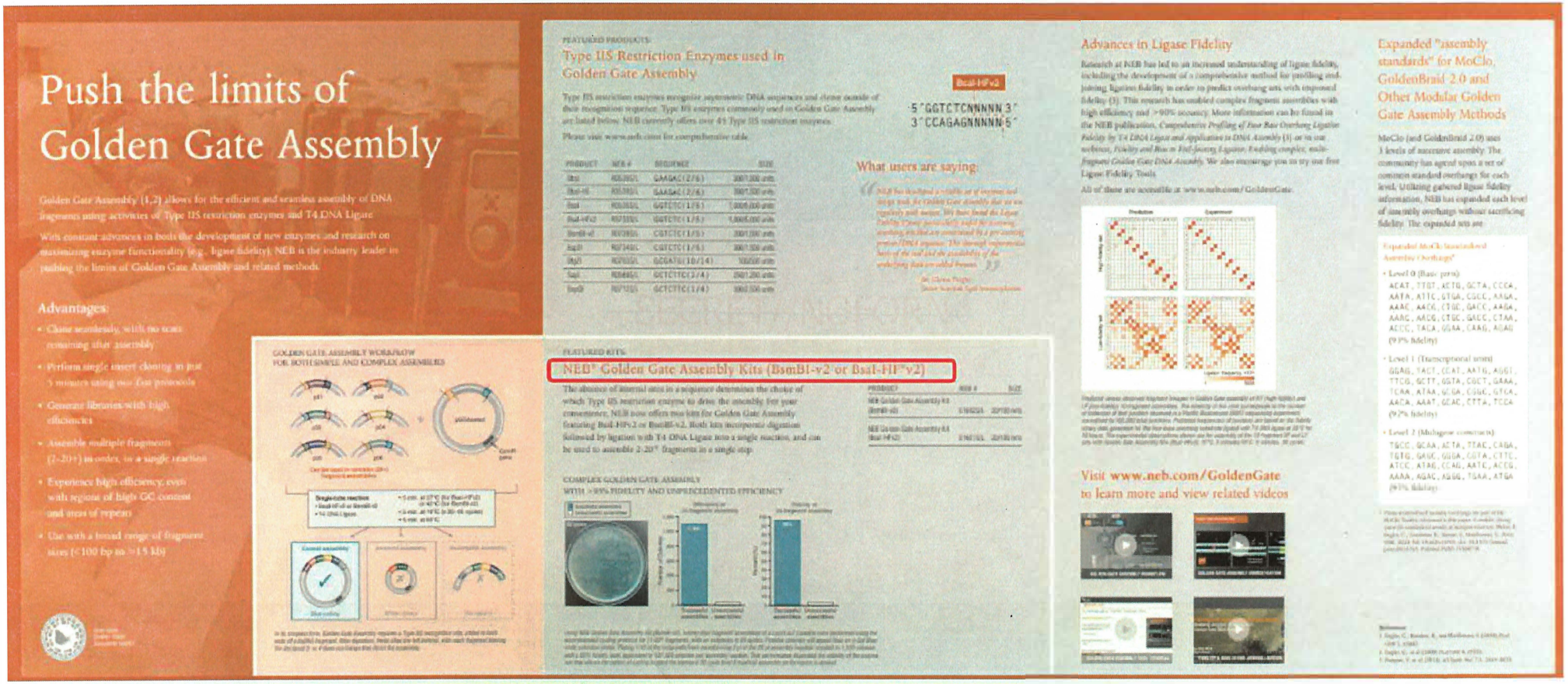
Featured Tools

Download the **NEB AR App** for iPhone® or iPad® Scan the augmented reality butterfly icon to find videos, tutorials and immersive experiences.

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

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

- Ligase Fidelity Viewer™ (v2) – Visualize overhang ligation preferences
- GetSet™ – Predict high-fidelity junction sets
- SplitSet™ – Split DNA sequence for scarless high-fidelity assembly



The next page is DIF citing (b)(1) and (b)(3) and is not provided.

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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 ChinaGe et al., 2013Yang et al., 2016Hu et al., 2017Five-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	*R. sinicus* (1)
October, 2011	anal swab	8	3	3	*R. sinicus* (3)
May, 2012	anal swab & feces	54	9	4	*R. sinicus* (4)
September, 2012	feces	39	20	19	*R. sinicus* (16)
					R. ferrumequinum (3)
April, 2013	feces	52	21	16	*R. sinicus* (16)
July, 2013	anal swab & feces	115	9	8	*R. sinicus* (8)
May, 2014	feces	131	8	4	*A. stoliczkanus* (3)
					R. affinis (1)
October, 2014	anal swab	19	4	4	*R. sinicus* (4)
May, 2015	feces	145	3	0	
October, 2015	anal swab	25	6	5	*R. sinicus* (5)
Total		*602*	*84*	*64*	*R (61) A (3)*

Extracted from Hu et al., 2017

The next page is DIF citing (b)(1) and (b)(3) and is not provided.

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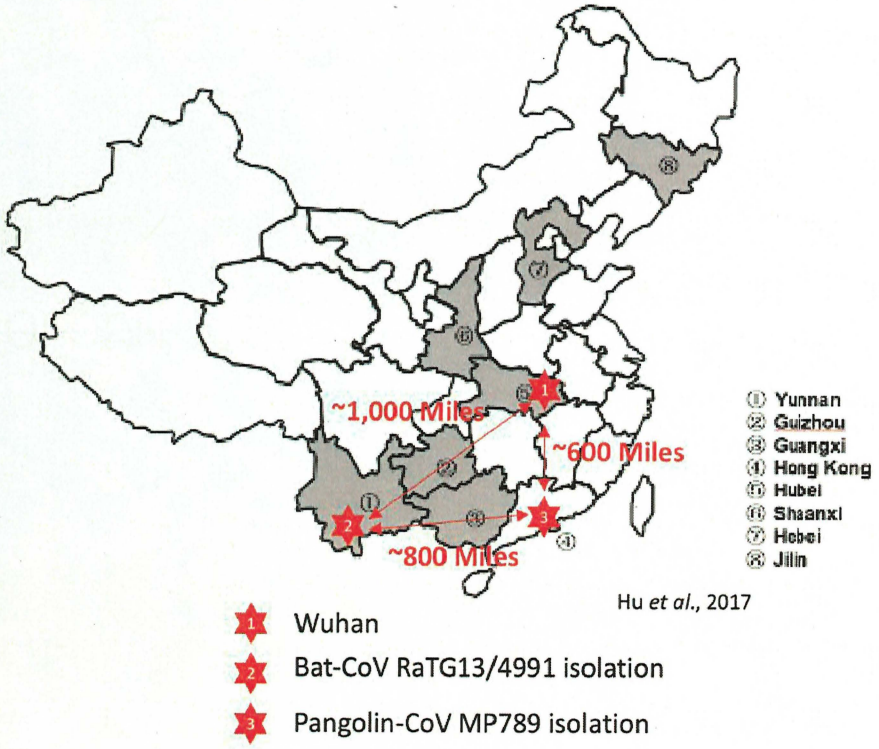
Isolation Locations for RaTG13 and MP789

- RaTG13/4991 isolated from a cave in Yunnan ProvinceRaTG13/4991 is a RdRp lineage 1 BetaCoVMP789 was isolated from diseased Pangolins in Guangdong ProvinceMP789 is a RdRp lineage 2 BetaCoV~800 miles separate these two locationsWIV also collected CoV's from Guandong 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?



Hu *et al.*, 2017

Yu *et al.*, 2019

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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 DIF citing (b)(1) and (b)(3) and is not provided.

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WIV SARS-CoV Reverse Genetics System

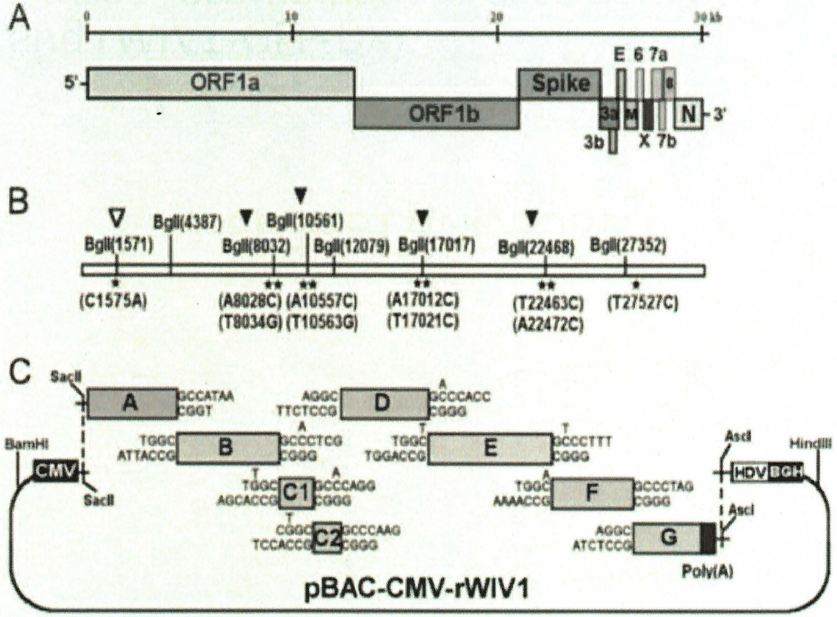

FIG 1 Strategy for construction of an infectious WIV1 BAC clone. (A) Genomic structure of WIV1. (B) The mutations are indicated under the stars. C1575A was used to ablate a natural BglI site at nucleotide 1571 (▽), and T27527C was used to disrupt a potential T7 stop site. The others were for introducing 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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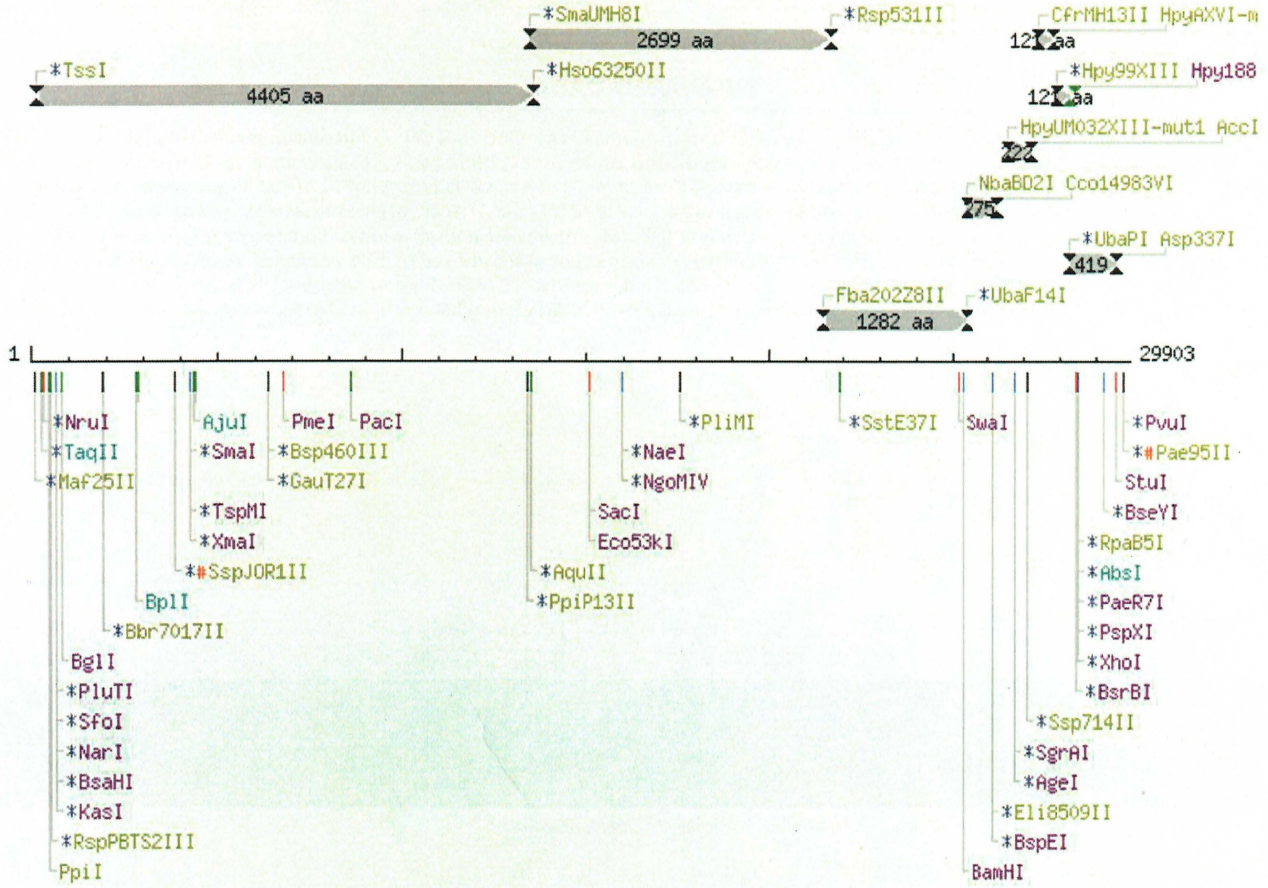
SARS-COV-2 GENOME RESTRICTION MAP



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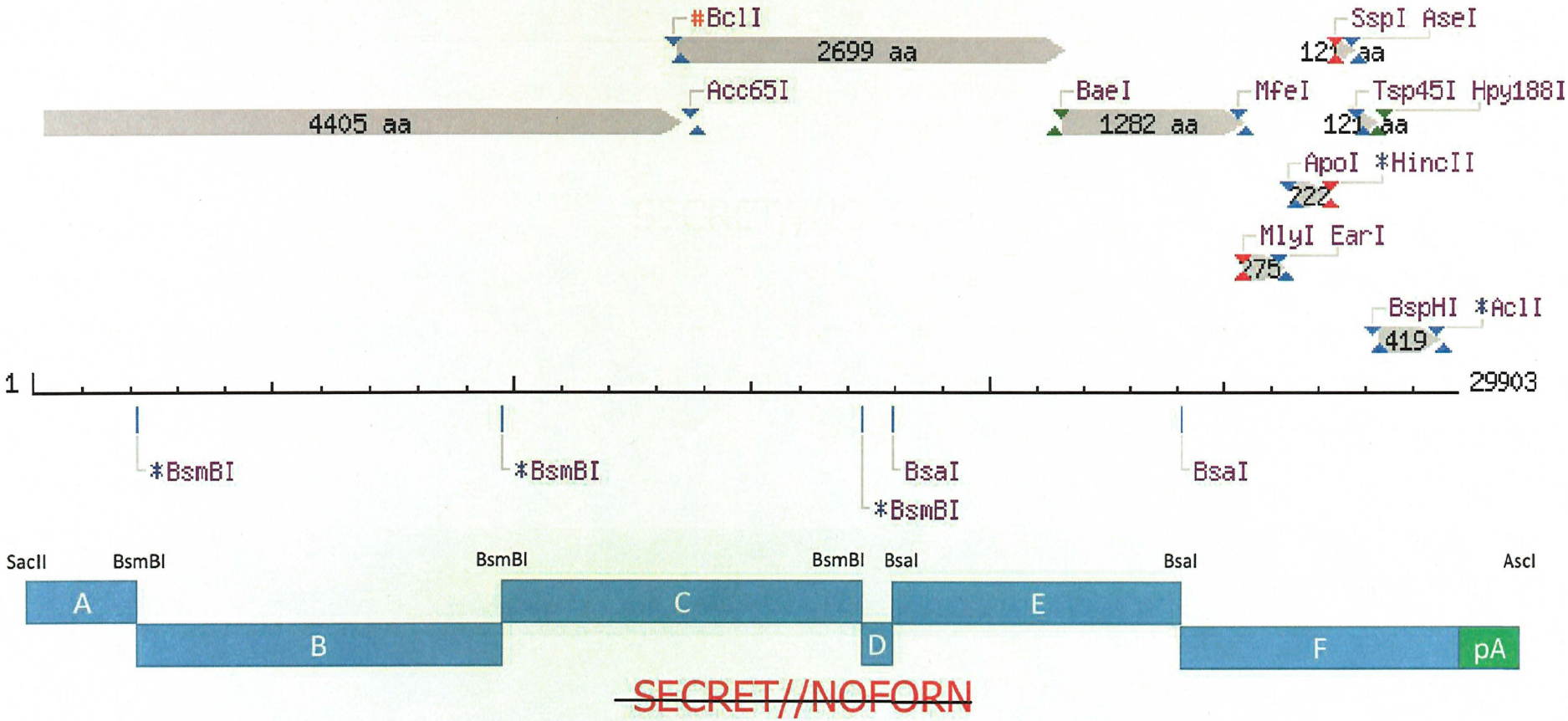
RE-based Fragment Build Option – *Bsm*BI/*Bsa*I (4 nt overhangs)



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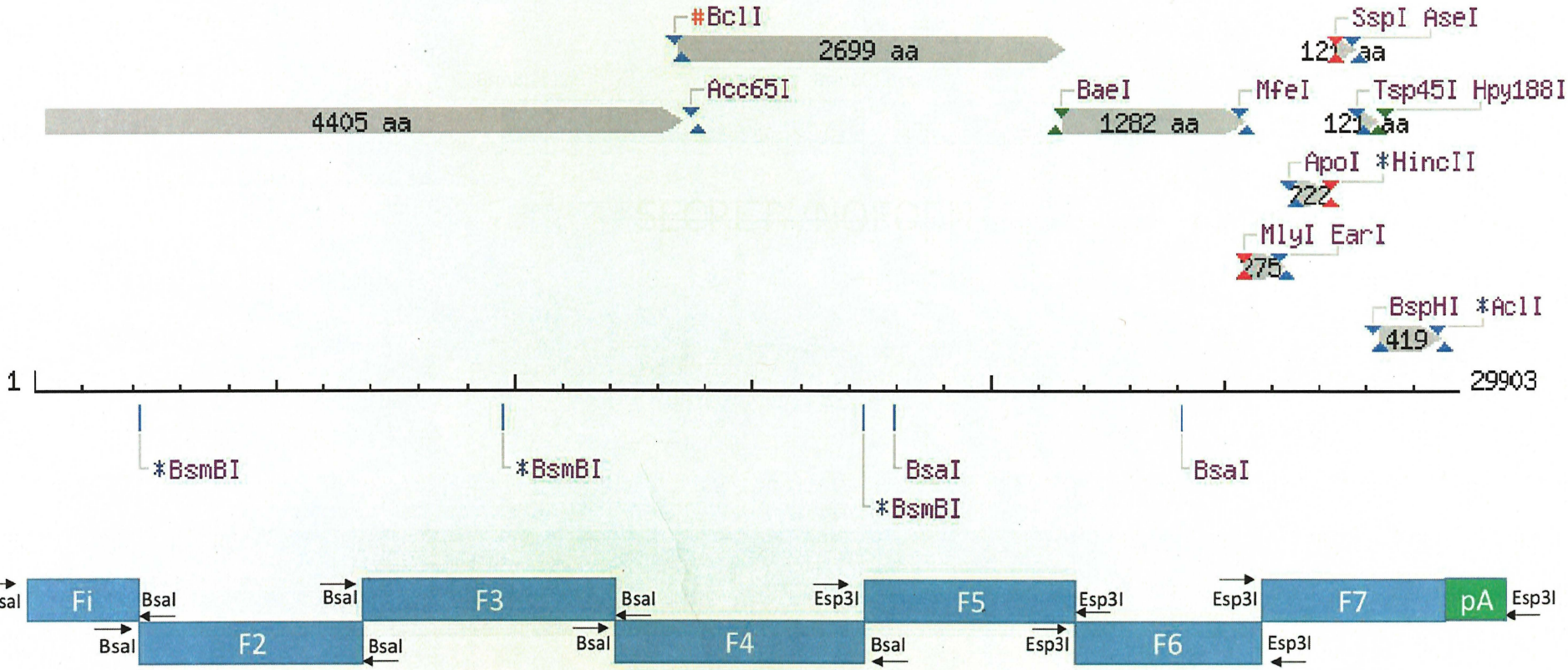
RE-based Fragment Build Option – *BsaI*/*Esp3*I (Invisible restriction sites)



Xie *et al.*, 2020

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

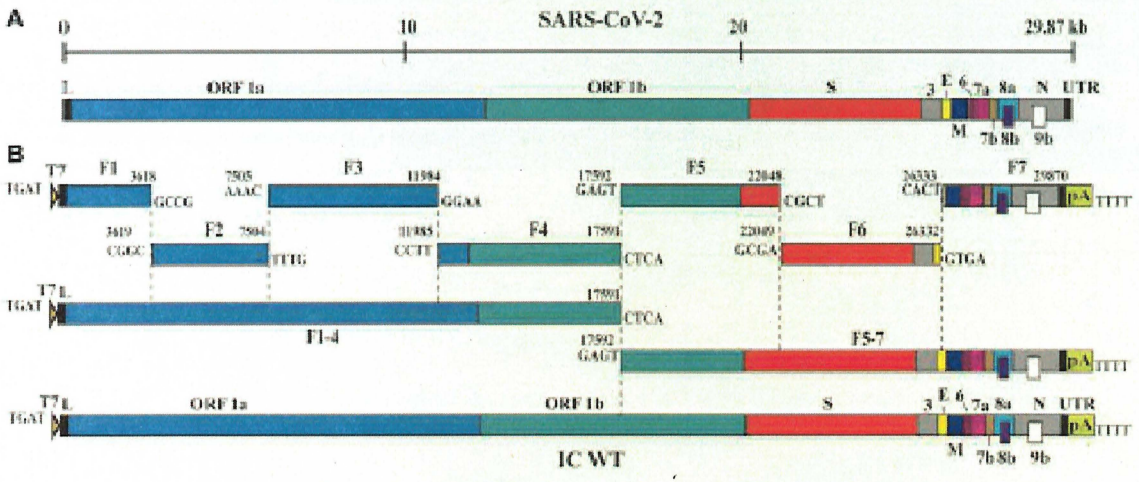


Figure 1. Assembly of a Full-Length SARS-CoV-2 Infection cDNA Clone

(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.

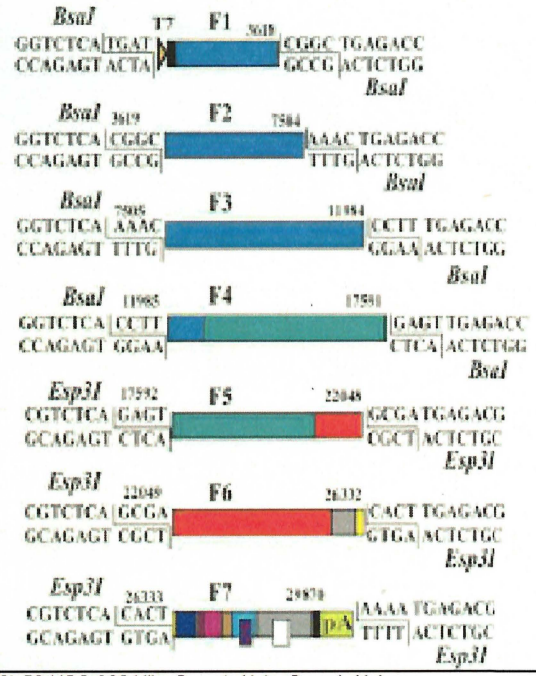
(C) Diagram of the terminal sequences of each cDNA fragment recognized by BsaI and Esp3I.

(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 0.6% 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.

C Fragment Terminal Sequence



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

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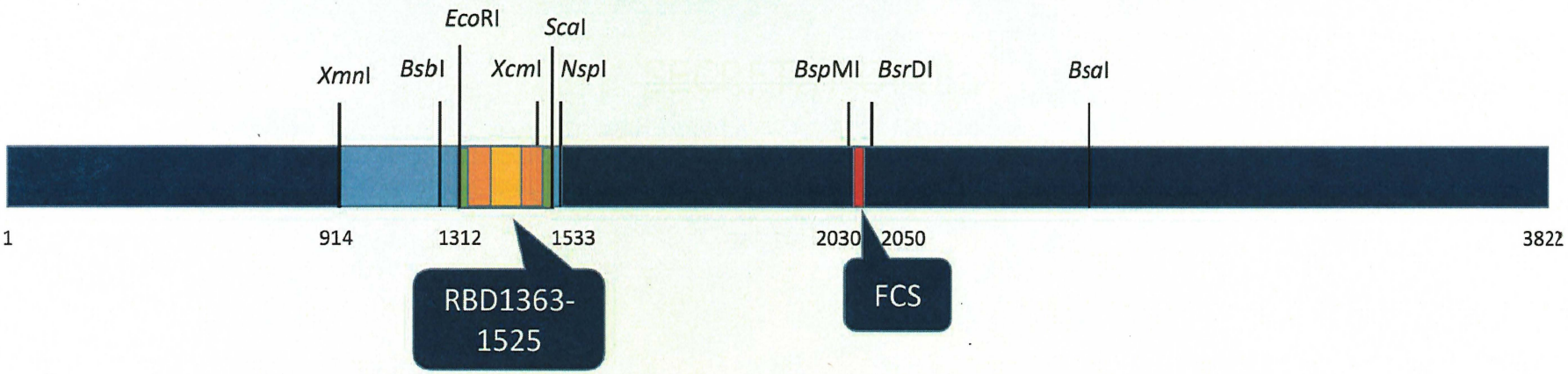
Spike Gene

SARS CoV-2 SPIKE GENE



Highest homology toRaTG13Pangolin 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:Rs4231Rs7327Rf4075Rs4081Rs4085Rs4235As6526Rp3

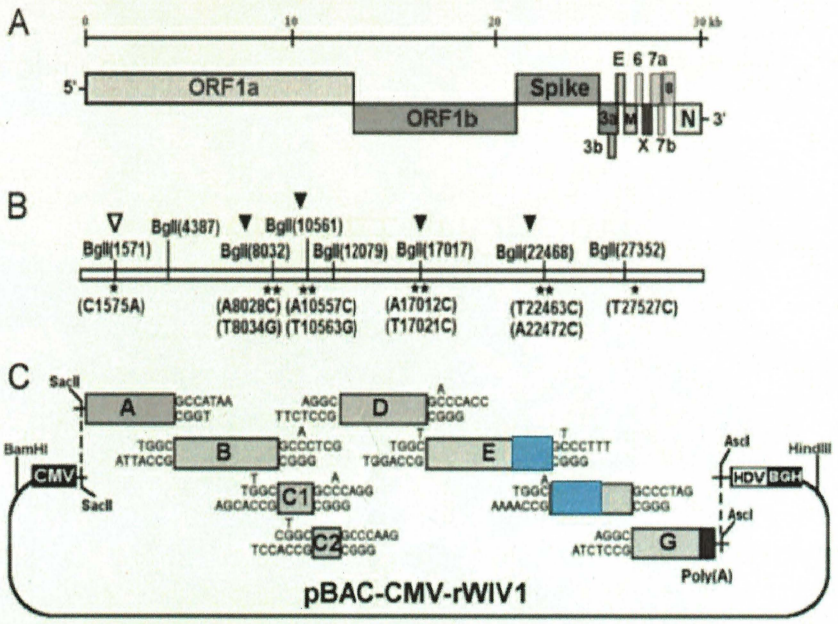

FIG 1 Strategy for construction of an infectious WIV1 BAC clone. (A) Genomic structure of WIV1. (B) The mutations are indicated under the stars. C1575A was used to ablate a natural BglI site at nucleotide 1571 (▽), and T27527C was used to disrupt a potential T7 stop site. The others were for introducing 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., 2016Hu et al., 2017

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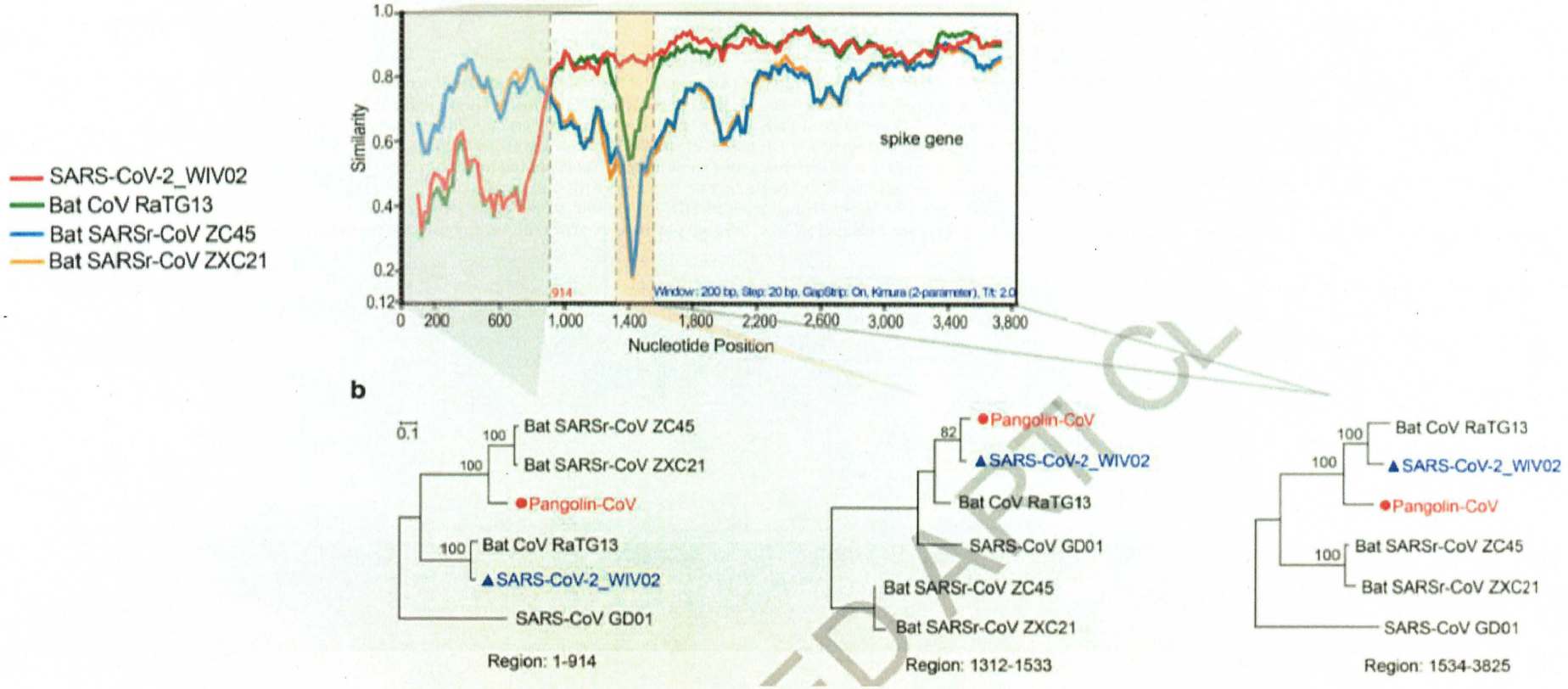
SARS-COV-2 SPIKE GENE SEGMENTS QUERY: PANGOLIN-COV



Xiao *et al.*, 2020

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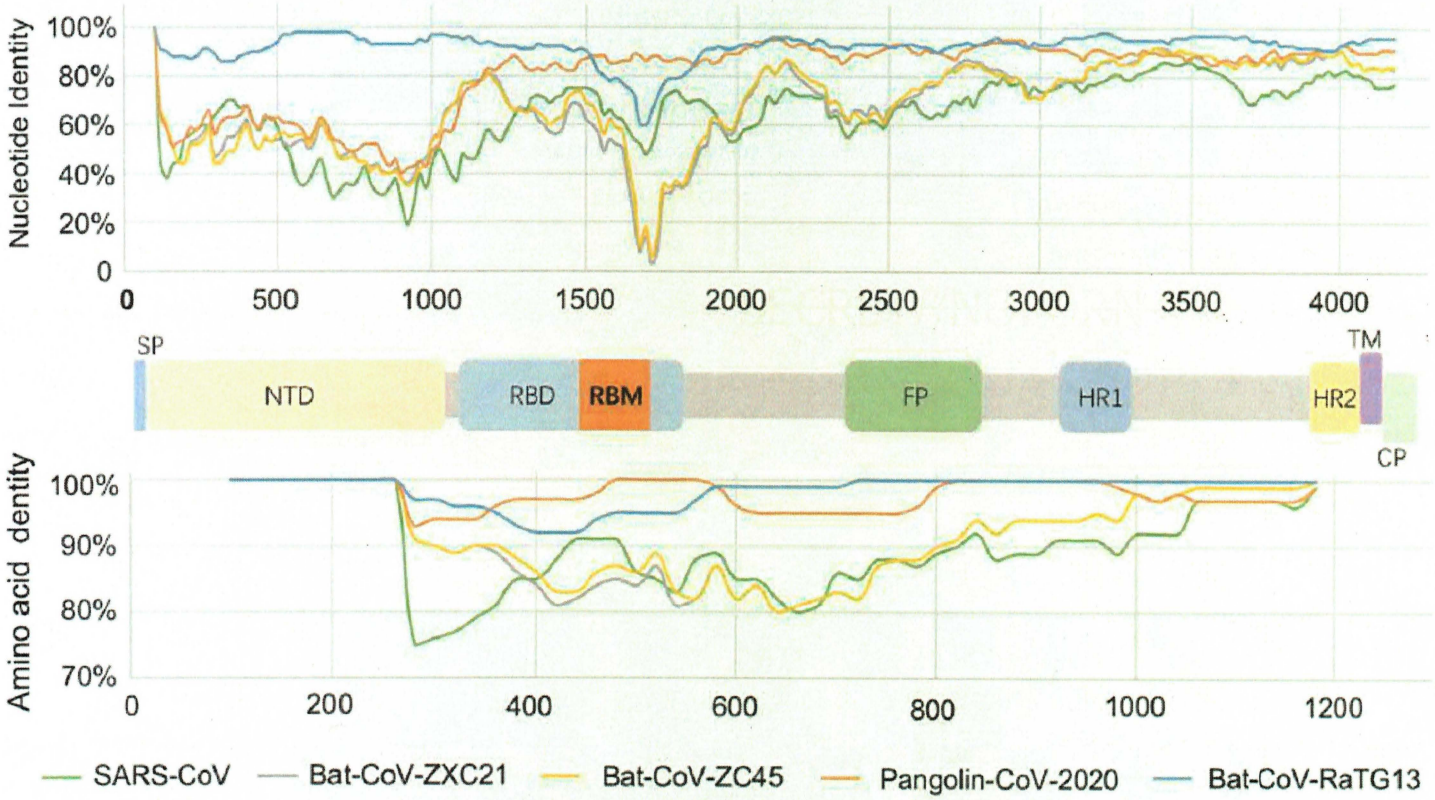
SARS-CoV-2 SimPLOT



- "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."

Liu *et al.*, 2020

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Ren *et al.*, 2008

JOURNAL OF VIROLOGY, Feb. 2008, p. 1899–1907 Vol. 82, No. 4
0022-538X/08/$08.00+0 doi:10.1128/JVI.01085-07

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

Wuze Ren,[1]† Xiuxia Qu,[2]† Wendong Li,[1]‡ Zhenggang Han,[1] Meng Yu,[3] Peng Zhou,[1] Shu-Yi Zhang,[4] Lin-Fa Wang,[3]* Hongkui Deng,[2] and Zhengli Shi[1]*

State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China[1]; Key Laboratory of Cell Proliferation and Differentiation of the Ministry of Education, College of Life Sciences, Peking University, Beijing, China[2]; CSIRO Livestock Industries, Australian Animal Health Laboratory and Australian Biosecurity Cooperative Research Center for Emerging Infectious Diseases, Geelong, Australia[3]; and School of Life Science, East China Normal University, Shanghai, China[4]

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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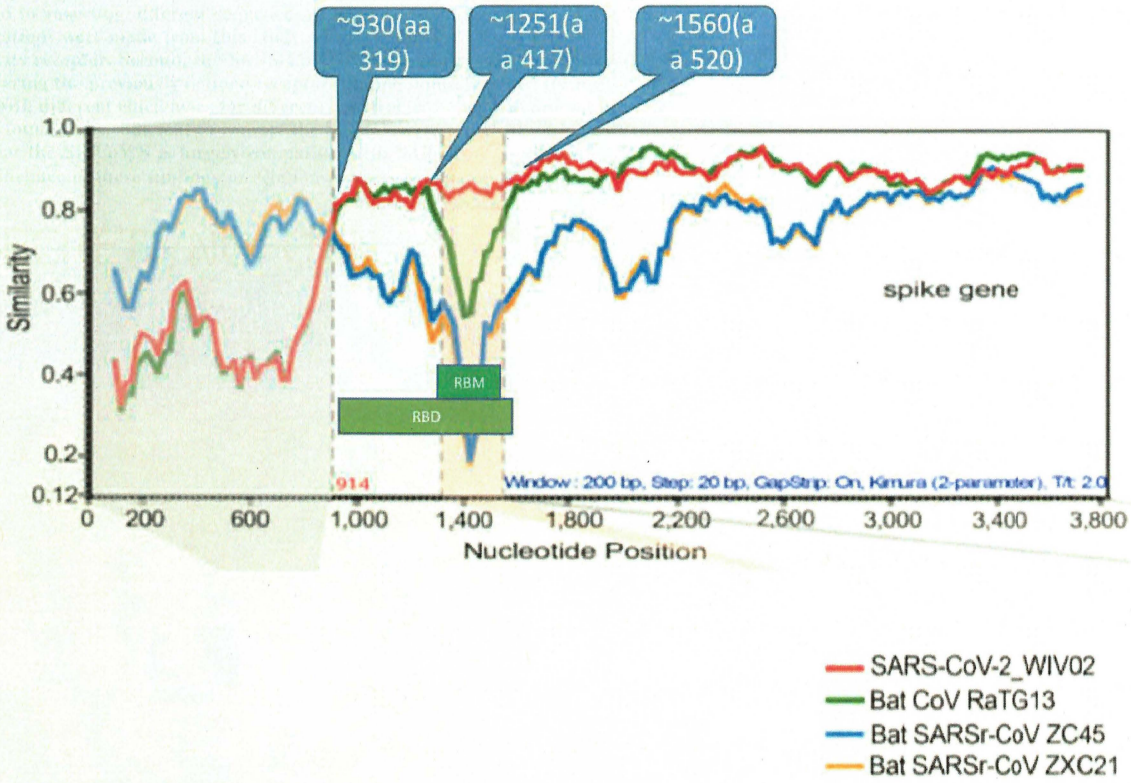
Minimal Receptor Binding Domain Cassette

- WIV scientists previously defined the minimal Receptor Binding Domain cassette that could functionally transfer ACE2 binding capability from one Spike protein to anotherSARS Nucleotide: 930-1554SARS Amino Acid: 310-518Receptor Binding MotifSARS Nucleotide: 1251-1482SARS Amino Acid: 417-494Homology 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 CATGTCGACACTTCTTATGAGTGCGACATTCCTATTGGAGCTGGCATTTGTGCTAGTTAC
1980 H V D T S Y E C D I P I G A G I C A S YSARS-CoV-2
CATGTCAACAACTCATATGAGTGTGACATACCCATTGGTGCAGGTATATGCGCTAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S YBCoV 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
CAGACTCAAACTAATTC----------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

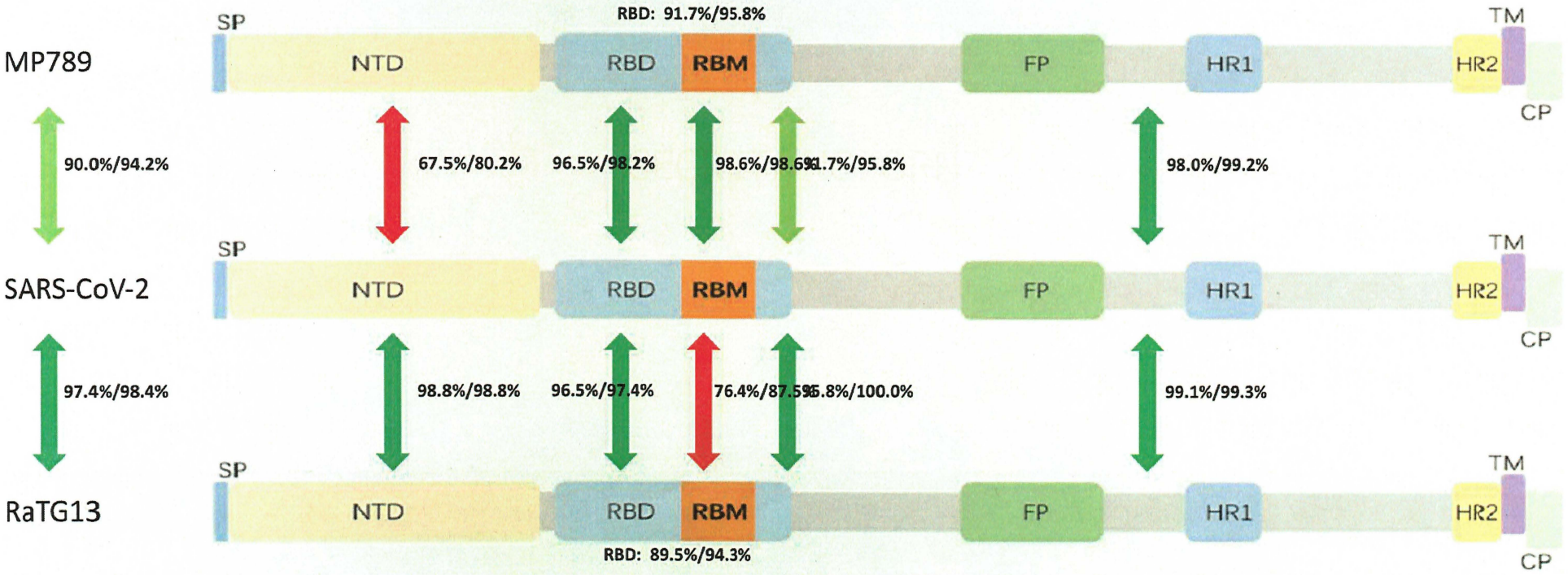
- The percent GC of the furin cleavage site insert is 77% compared to ~40% of the surrounding DNAContains an NmeAIII restriction siteThe other CoV's with FCS have a %GC of <55%

Virus	Nucleotide and Amino Acid Sequences		
SARSCoV2 (47%)	G G T A T A T G C G C T A G T T A T C A G A C T C A G A C T A A T T C	T C C T C G G C G G G C A	C G T A G T G T A G C T A G T C A A T C C A T C A T T G C C T A C A C T A T G
	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 A Y T M
	%GC: 14/35 = 40%	%GC: 10/13 = 77%	%GC: 17/39 = 44%
MERS-CoV (54%)	C T C T G T G C T C T T C C T G A C A C A C C T A G T A C T C T C A C	A C C T C G C A G T G T G	C G C T C T G T T C C A G G T G A A A T G C G C T T G G C A T C C A T T G C T
	L C A L P D T P S T L T	P R S V	R S V P G E M R L A S I A
	%GC: 18/35 = 51%	%GC: 8/13 = 62%	%GC: 21/39 = 54%
BatCoV-HKU5 (47%)	G G T C A A T C A C T T T G T G C T A T T C C A C C A A C T A C T T C	T T C A C G C G T T C G A	C G T G C T A C T T C T G G T G C A T C T G A T G T G T T T C A A A T C G C C
	G Q S L C A I P P T T S	S R V R	R A T S G A S D V F Q I A
	%GC: 15/35 = 43%	%GC: 7/13 = 54%	%GC: 19/39 = 49%
IBV-Beaudette (34%)	[illegible]	[illegible]	[illegible]
	F N [illegible] F Y [illegible] K [illegible] T N [illegible]	[illegible]	[illegible] P Y V
	%GC: 12/35 = 31%	%GC: 5/13 = 38%	%GC: 13/39 = 33%

Influenza viruses convert from low path to high path by addition of a poly-basic 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

% Identity:>95

Spike Protein Regions



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

```
SARSCoV2          TRFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPBCoV_RaTG13
TRFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPMP789            TKFRTLLTIHRGDPMP---
NNGWTVFSAAYYVGYLAPRTFMLNYNENGTITDAVDCALDP                                        NTD<>RBDSARSCoV2
LSETKCTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRBCoV_RaTG13
LSETKCTLKSFTVEKGIYQTSNFRVQPTDSIVRFPNITNLCPFGEVFNATTFASVYAWNRMP789/Manis
LSEAKCTLKSLTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATTFASVYAWNR SARSCoV2
KRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTBCoV_RaTG13
KRISNCVADYSVLYNSTSFSTFKCYGVSPTKLNDLCFTNVYADSFVITGDEVRQIAPGQTMP789/Manis
KRISNCVADYSVLYNSTSFSTFKCYGVSPTKLNDLCFTNVYADSFVVRGDEVRQIAPGQT
>RBMSARSCoV2          GKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQABCoV_RaTG13
GKIADYNYKLPDDFTGCVIAWNSKHIDAKEGGNFNYLYRLFRKANLKPFERDISTEIYQAMP789/Manis
GRIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQA
RBM<                    RBD<SARSCoV2
GSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKBCoV_RaTG13
GSKPCNGQTGLNCYYPLYRYGFYPTDGVGHQPYRVVVLSFELLNAPATVCGPKKSTNLVKMP789/Manis
GSTPCNGVEGFNCYFPLQSYGFHPTNGVGYQPYRVVVLSFELLNAPATVCGPKQSTNLVK SARSCoV2
NKCVNFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVBCoV_RaTG13
NKCVNFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVMP789
NKCVNFNFNGLTGTGVLTESSKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGV
```

ACE2 Critical ContactACE2

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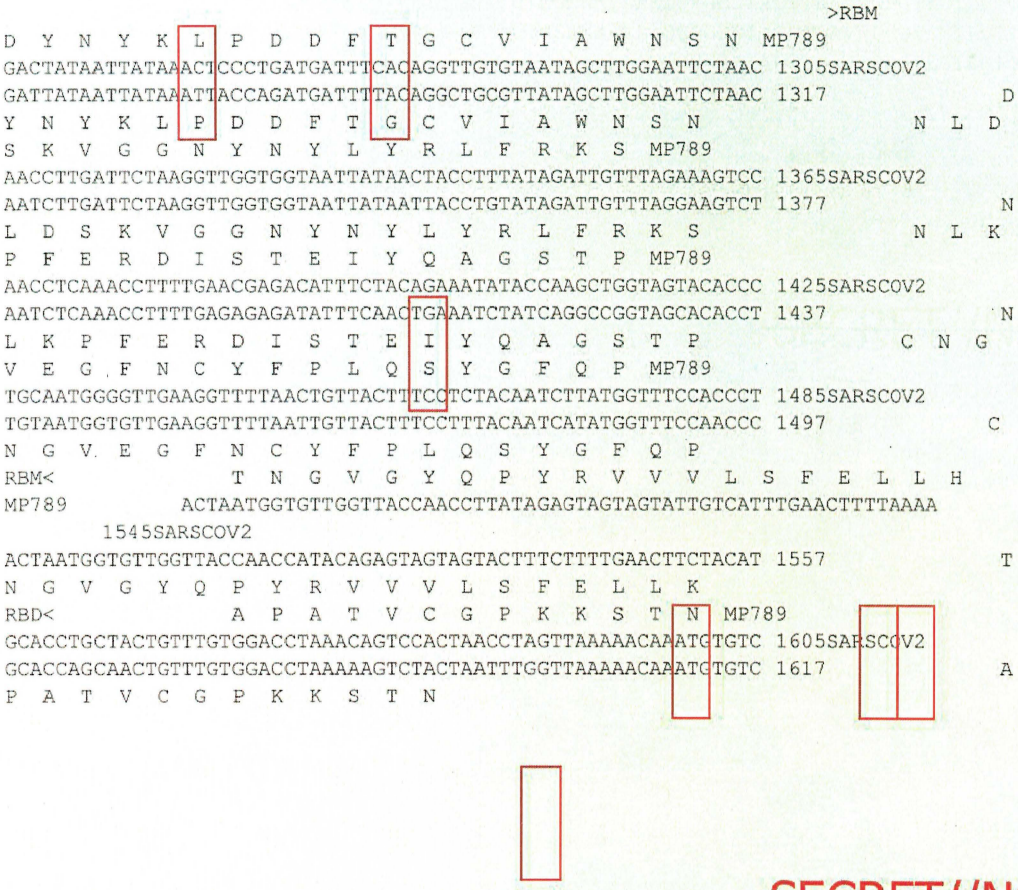
SARS-COV-2 RBD vs PCoV MP789 RBD



- 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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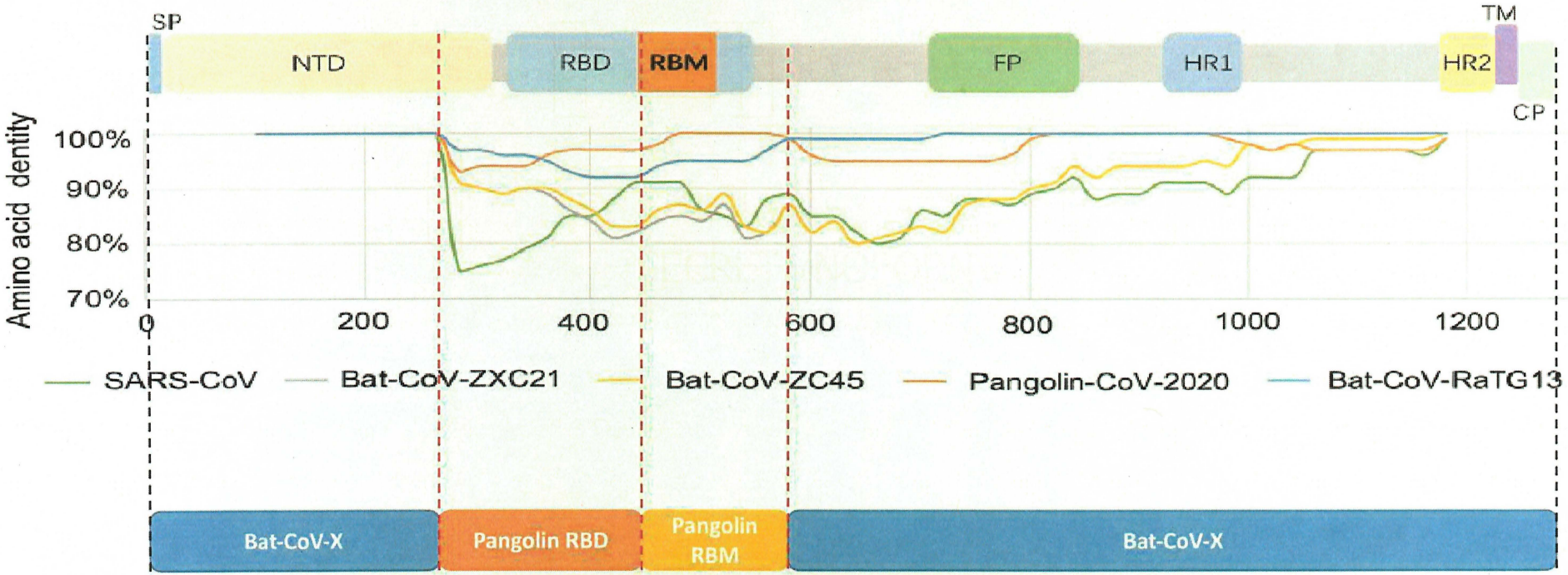
SARS-CoV-2 Spike Appears to be a Chimera



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

Liu *et al.*, 2020

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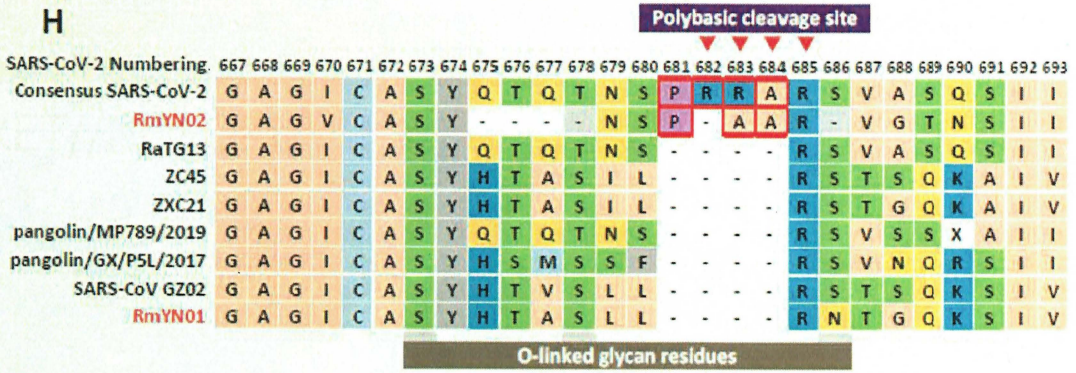
RmYN02 - A Red Herring?

- 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



Zhou *et al.*, 2020

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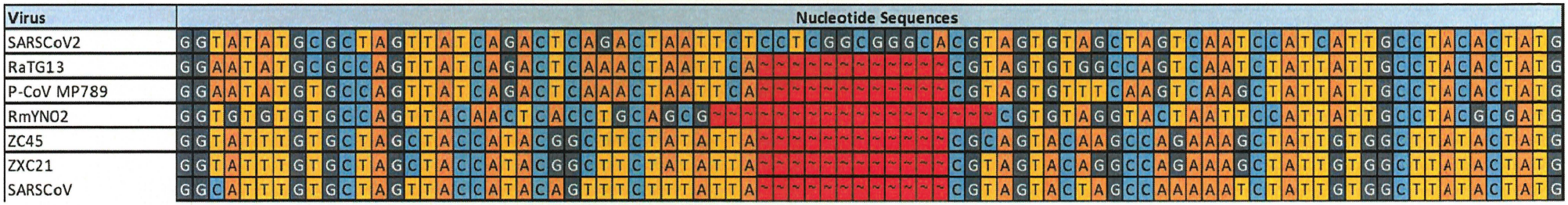
RmYN02 - A Red Herring?



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HIV Epitopes

Perez, 2020

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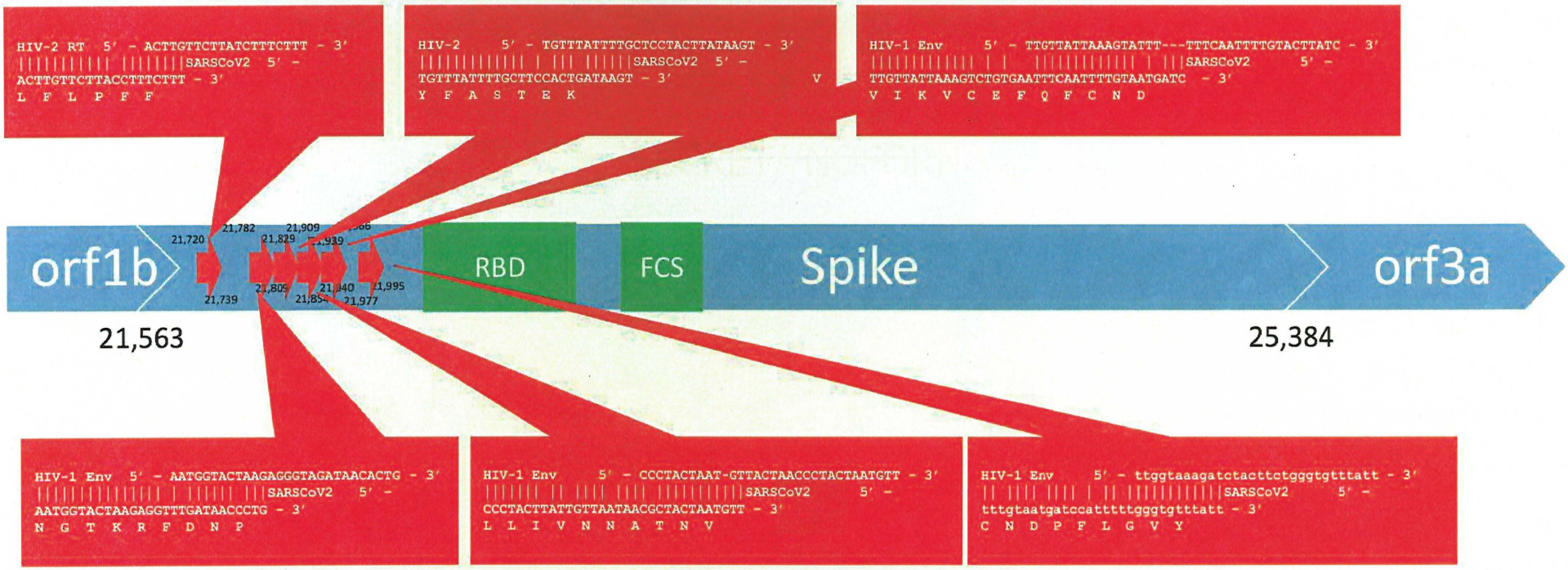
HIV Sequences in the SARS-CoV-2 Spike Gene



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 KQLQARILAVERYLKDQQLLGG - 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

The next 2 pages are DIF citing (b)(1) and (b)(3) and are not provided.

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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 Chimerc 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

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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BACK-UP SLIDES

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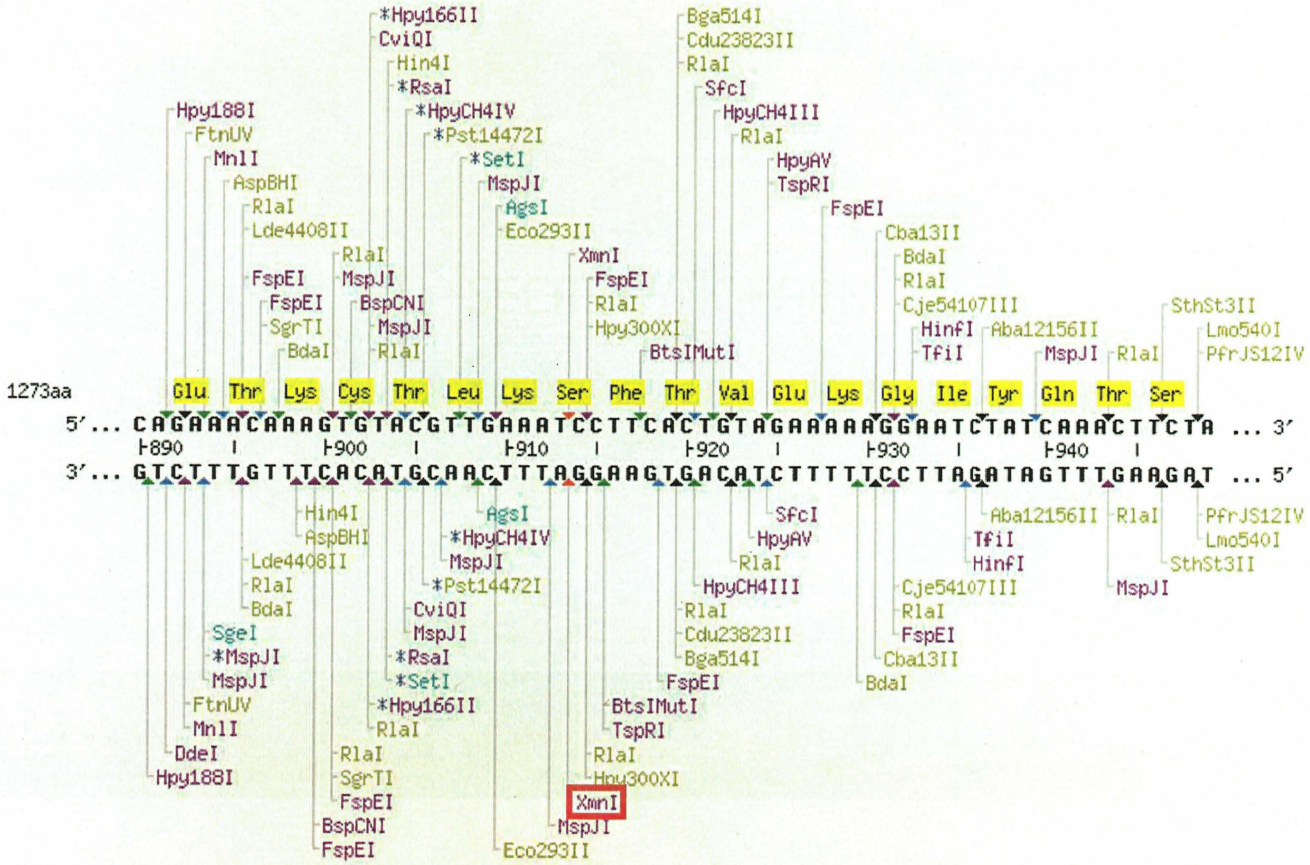
Nucleotide 914 Region



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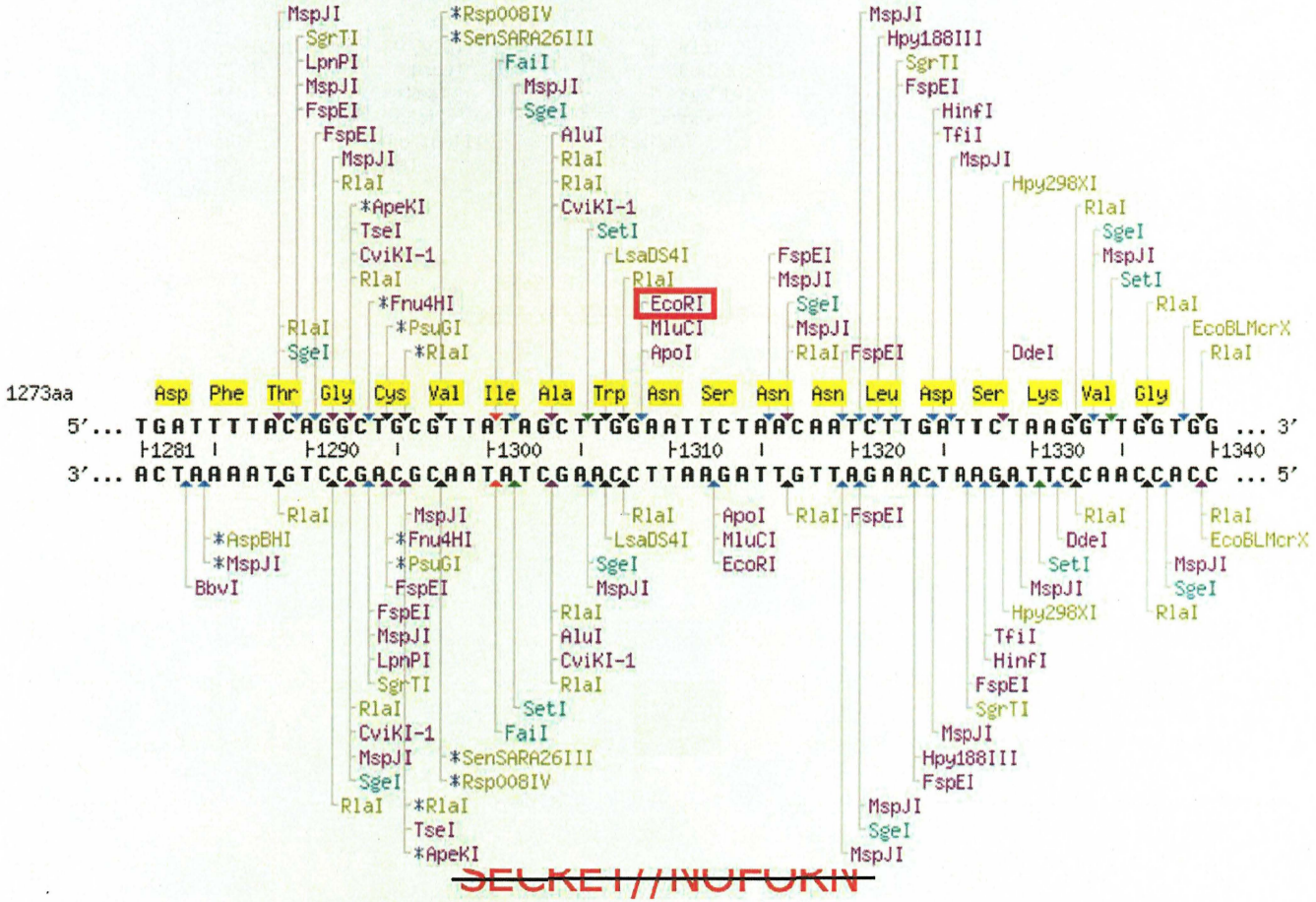
Nucleotide 1312 Region



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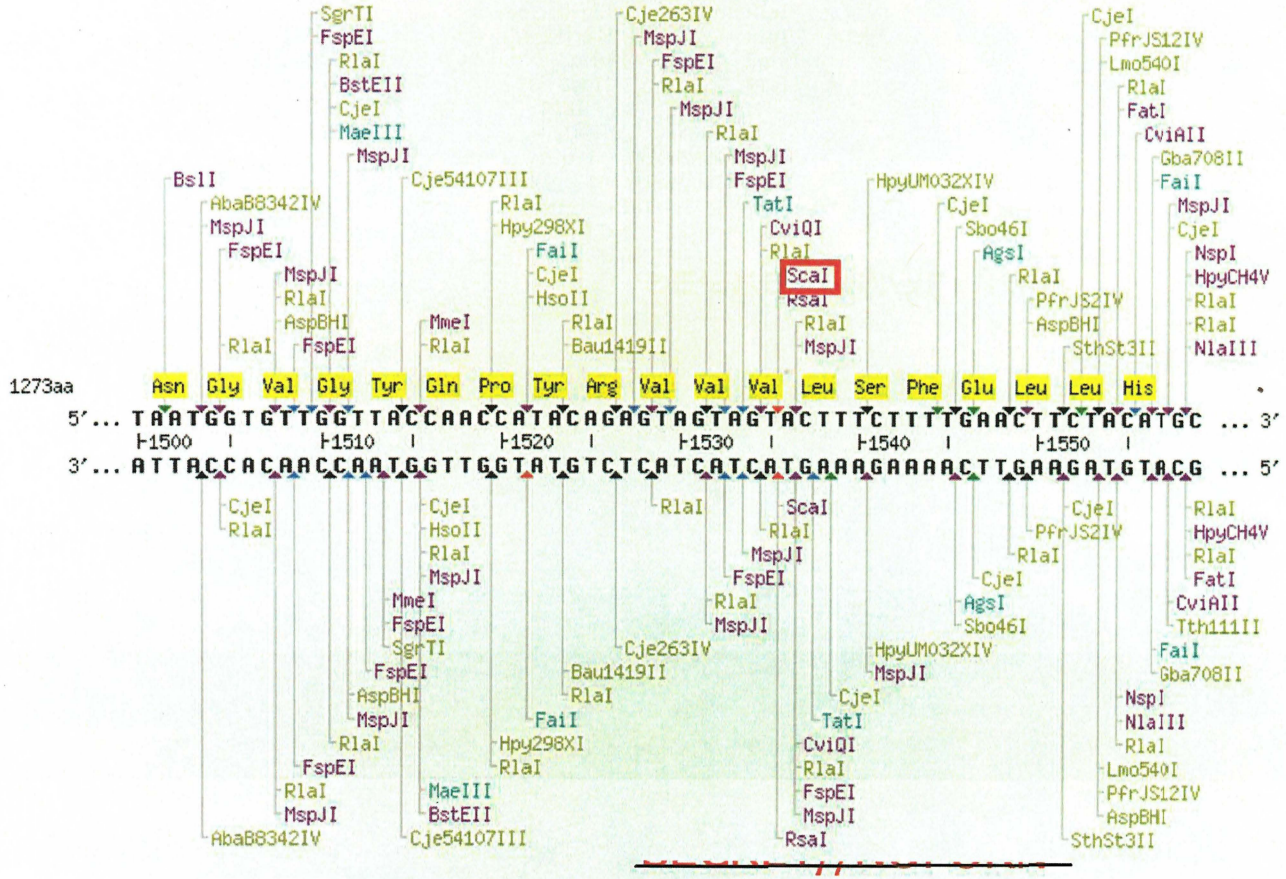
Nucleotide 1535 Region




SARS-CoV CATGTCGACACTTCTTATGAGTGCGACATTCCTATTGGAGCTGGCATTTGTGCTAGTTAC
1980 H V D T S Y E C D I P I G A G I C A S YSARS-CoV-2
CATGTCAACAACTCATATGAGTGTGACATACCCATTGGTGCAGGTATATGCGCTAGTTAT 2022
H V N N S Y E C D I P I G A G I C A S YBCoV 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
CAGACTCAAACTAATTC----------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