Restriction Site Analysis of SARS-CoV-2 Demonstrates the signature of a synthetic virus

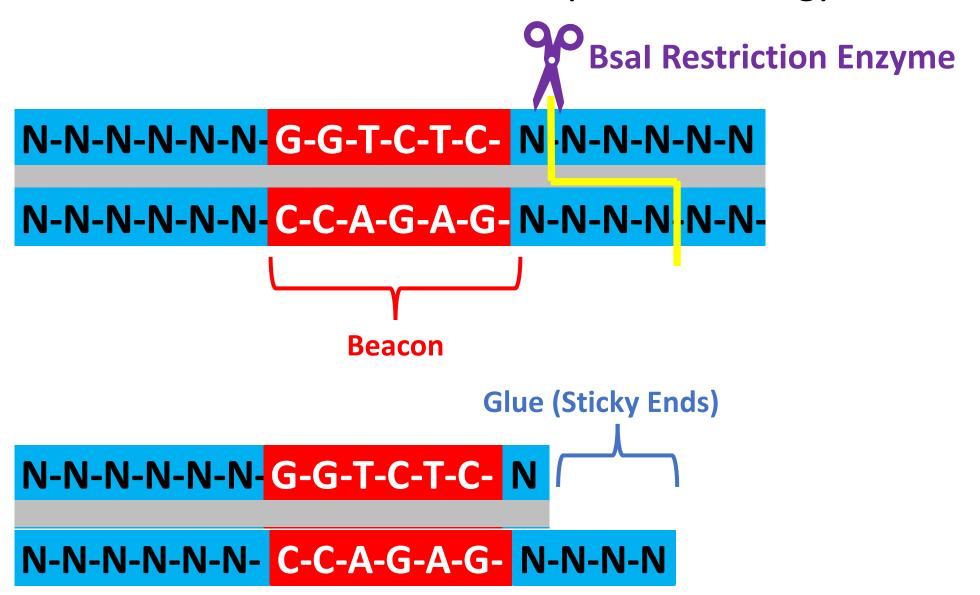
The likelihood SARS-CoV-2 is a natural sarbecovirus is less than one in a billion based on this analysis



Introduction

- Two hypotheses of the origin of SARS-CoV-2 exist:
 - A spillover from an animal host somewhere outside a laboratory
 - A laboratory-related accident
- Finding features within the genome to address these hypotheses would advance the investigation without requiring the cooperation of thirdparties
- Previous analyses of the unprecedented, first ever furin cleavage site in a sarbecovirus and the exceptional pre-adaption of the receptor binding domain to human ACE2 have been challenged because they provide an evolutionary advantage to the virus and, no matter how seemingly unnatural, as supporting a natural process
- Here I perform an analysis of the number, location, genome pattern, and sequences of two restriction sites, Basl and BsmBI, that helps distinguish natural viruses from synthetic viruses
 - These two Type IIC restriction enzymes are the workhorses of synthetic coronavirus research and part of Baric's "No See 'Em" technology
 - An advantage of this analytical approach is that, in synthetic biology, the manipulation of these small, six nt sequences is the foundation of reverse genetics but their small size and random location within a genome, unrelated to genes, makes it extremely unlikely that they could provide an evolutionary advantage
- Finding multiple patterns within SARS-CoV-2's genome to be both a) consistent with man-made chimeric virus genomes and b) demonstrably extremely inconsistent with all observed natural sarbecovirus genomes, this could greatly advance the investigation of COVID-19's origins."

How Do Restriction Enzymes Work? The Beacon and Glue of Synthetic Biology



SARS1 Assembly Process: I

 Top: Human Urbani strain with three natural BgII sites noted (in black)

 Bottom: Synthetic assembly uses three human-designed Bgll sites (red), two Bgll sites (black), with Bgll (1577) extinguished

Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus

Boyd Yount*[†], Kristopher M. Curtis*[†], Elizabeth A. Fritz[‡], Lisa E. Hensley[‡], Peter B. Jahrling[‡], Erik Prentice[§], Mark R. Denison[§], Thomas W. Geisbert[‡], and Ralph S. Baric*[†]

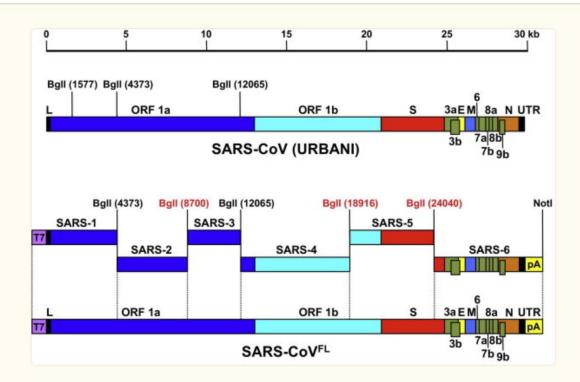


Fig. 2

Systematic assembly of a SARS-CoV full-length cDNA clone by in vitro ligation. A full-length cDNA of the SARS-CoV Urbani strain (SARS-CoV FL) was assembled by in vitro ligation of six contiguous cDNA fragments (SARS-1 to SARS-6) spanning the entire viral genome, which were flanked by native (in black) or engineered (in red) unique BglI restriction endonuclease sites. The assembled full-length cDNA contained a T7 RNA polymerase promoter (T7) at the 5'-end and a poly(A) tail (pA) at the 3'-end, allowing for in vitro transcription of full-length, capped polyadenylated transcripts. The viral genes and relevant restriction sites are indicated.

Figure adapted from Yount et al. (2003).

SARS1 Assembly Process: II

Actual changes to create restriction sites:

- 4,419: Natural restriction site used without change
- 8,749, 12,133, 24,088:
 Synthetic restriction site created with a single nt change (small letter t or g)
- 18,966: Sometimes 4 nt changes are needed to give restriction site and preserve amino acid code

B SA	RS Junctions
SARS A/B	GCCATAATGGC4,419
Junction	CGGTATTACCG
SARS B/C	GCCAGCGTGGC t8,749
Junction*	CGGTCGCACCG
SARS C/D	GCCCAAGAGGC 12,113
Junction	CGGGTTCTCCG
SARS D/E Junction*	at g t 18,966 GCCCTCCTGGC CGGGAGGACCG
SARS E/F Junction*	t 24,088 GCCTACACGGC CGGATGTGCCG



PLOS PATHOGENS



"The original fragments E and F were shortened to leave spike gene as an independent fragment. The new fragments were designated as Es and Fs.

Bsal or **BsmBl** sites were introduced into the junctions of Es/Spike and Spike/Fs.

Then any spike could be substituted into the genome of SARSr-CoV WIV1 through this strategy."

-Ben Hu, Peter Daszak, Zhengli-Li Shi

Note (right) that the Es restriction site is on the sense strand and the Fs restriction site is on the anti-sense strand. That rotational symmetry is the basis for the elimination of the designed restriction sites after ligation in the final clone.

The 'No See 'Em' technique

 PLoS Pathog.
 2017 Nov; 13(11): e1006698.
 PMCID: PMC5708621

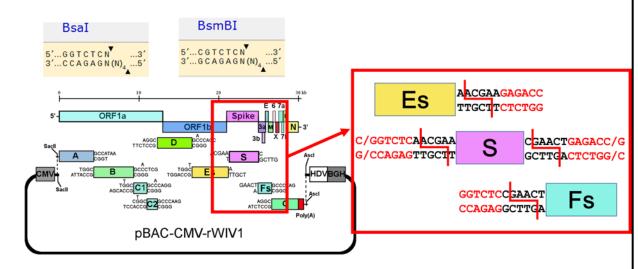
 Published online 2017 Nov 30. doi: 10.1371/journal.ppat.1006698
 PMID: 29190287

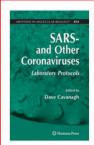
Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus

Ben Hu, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft,**1 Lei-Ping Zeng, Investigation, Methodology,**1 Xing-Lou Yang, Investigation, Resources,**1 Xing-Yi Ge, Formal analysis, Resources,**1 Wei Zhang, Investigation,**1 Bei Li, Investigation,**1 Jia-Zheng Xie, Investigation,**1 Xu-Rui Shen, Investigation,**1 Yun-Zhi Zhang, Resources,**2.3 Ning Wang, Investigation,**1 Dong-Sheng Luo, Investigation, Resources,**1 Xiao-Shuang Zheng, Investigation,**1 Mei-Niang Wang, Resources,**1 Peter Daszak, Funding acquisition, Writing – review & editing,**5 Jie Cui, Conceptualization, Formal analysis, Funding acquisition, Software, Writing – review & editing,**1 and Zheng-Li Shi, Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – review & editing**1.**

Christian Drosten, Editor

Spike substitution strategy





SARS- and Other Coronaviruses pp 293-315 | Cite as

Systematic Assembly and Genetic Manipulation of the Mouse Hepatitis Virus A59 Genome

Authors Authors a	nd affiliations
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Eric F. Donaldson, Amy C. Sims, Ralph S. Baric

Protocol

First Online: 01 August 2008



Part of the Methods in Molecular Biology book series (MIMB, volume 454)

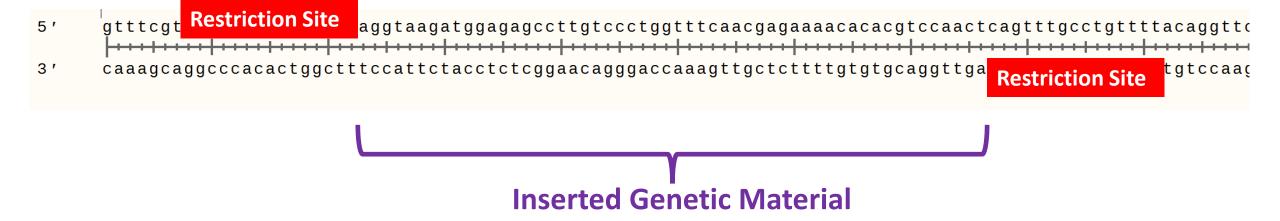
Abstract

We have developed a DNA assembly platform that utilizes the nonspecific, highly variable sequence signatures of type IIs restriction enzymes to assemble a full-length molecular clone murine hepatitis coronavirus (MHV) strain A59. The approach also allows changes to be engineered into a DNA fragment by designing primers that incorporate the restriction site and the mutations of interest. By adding the type IIs restriction site in the proper orientation, subsequent digestion removes the restriction site and leaves a sticky end comprising the mutation of interest ready to ligate to a second fragment generated in parallel as its complement. In this chapter, we discuss the details of the method to assemble a full-length infectious clone of MHV and then engineer a specific mutation into the clone to demonstrate the power of this unique site-directed "No See'm" mutagenesis approach.

The 'No See 'Em' Gene Manipulation Assembly Platform

While it is true this technique hides the ligation seams in the finished synthetic virus, having access to potential precursor viruses, fragments or families of related sarbecoviruses allows the assembly path to be observed by the absence of certain genetic features.

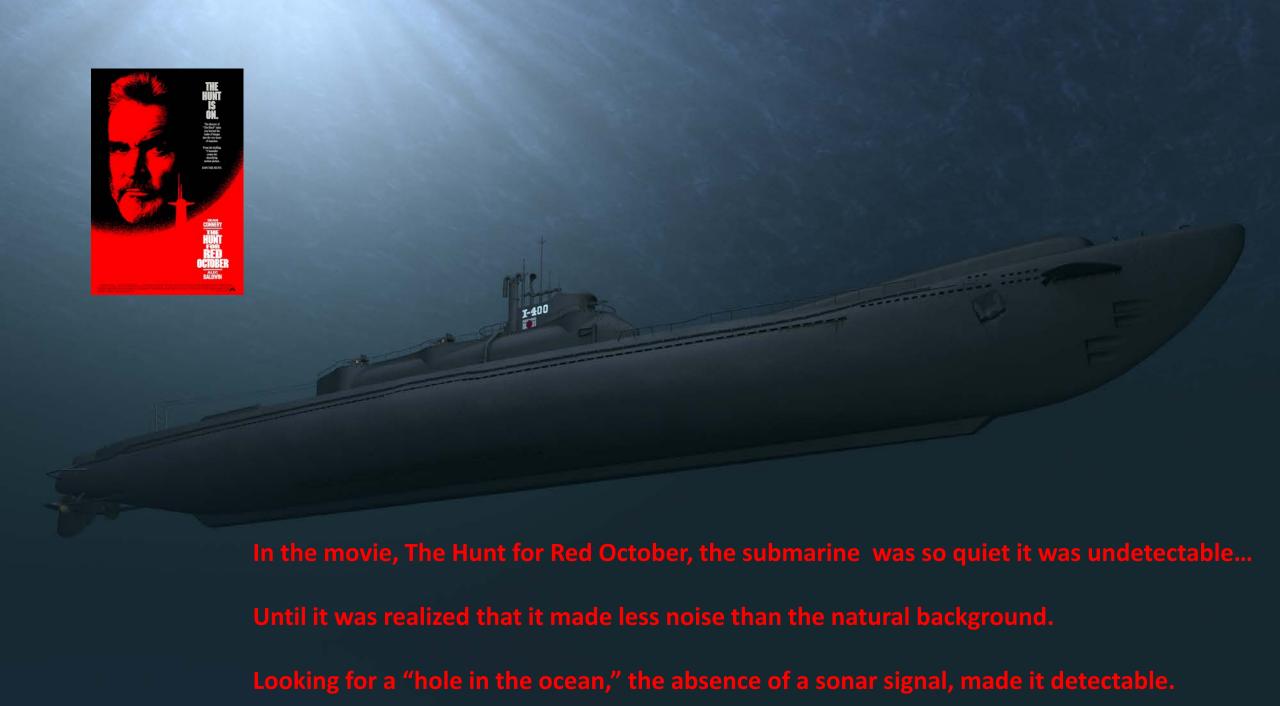
August 2008



Signature of 'No See 'Em' Synthetic Assembly in Precursor:

<u>Addition</u> of Symmetrical Restriction Sites on 5' (Positive Sense) and 3' (Negative Sense) Strands

Removal of Native Restriction Sites Where Needed



Hypothesis: Can "holes in the genome" with respect to restriction sites provide a signature of synthetic assembly of a virus?

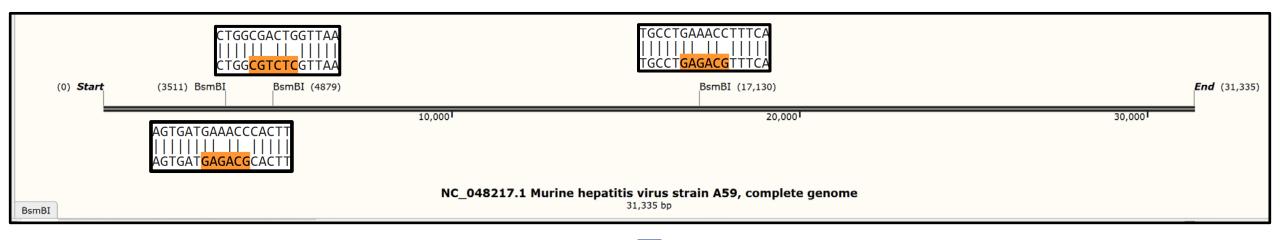
- How many of these two restriction sites are expected to be found in natural sarbecoviruses?
 - Any given hexamer sequence will occur every 4096 nts or 7.3/30k genome
 - Given the G:C bias of these sequences and a 40% G:C content of SARS-CoV-2, it should occur every 6944 or 4.3/30k genome
 - Therefore, a combined total of between 8.6 and 14.6 BsmBl and Bsal restriction sites per genome is predicted

<u>BsaI</u>

```
5′... GGTCTC (N)₁ ▼... 3′
3′... CCAGAG (N)₅ ... 5′
```

Esp3I-BsmB1

```
5′... CGTCTC (N)₁ ▼... 3′
3′... GCAGAG (N)₅ ↓... 5′
```



Example of Synthetic Biology Workflow

 Destruction of native BsmBI restriction sites by the introduction of point mutations is often the first step in the laboratory synthetic biology workflow and reverse genetics of coronaviruses Here, three native BsmBI restriction sites (above) are removed by making two 3rd position in the codon, synonymous substitutions, yielding a synthetic virus with no remaining BsmBI restriction sites (below)



SARS-CoV-1, SARS-CoV-2, and related viruses are different



Coronavirus Enzymes



ARTICLE



https://doi.org/10.1038/s41467-021-22905-7

OPEN

SARS-CoV-2 gene content and COVID-19 mutation impact by comparing 44 *Sarbecovirus* genomes

Irwin Jungreis

1,2

Rachel Sealfon

3 & Manolis Kellis

1,2

Manolis Kellis

1,3

Manolis Kellis

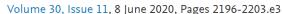
1,4

Manolis Kellis

M

42 Sarbecoviruses from above paper in green

Current Biology





Report

A Novel Bat Coronavirus Closely Related to SARS-CoV-2 Contains Natural Insertions at the S1/S2 Cleavage Site of the Spike Protein

Hong Zhou ^{1, 8}, Xing Chen ^{2, 8}, Tao Hu ^{1, 8}, Juan Li ^{1, 8}, Hao Song ³, Yanran Liu ¹, Peihan Wang ¹, Di Liu ⁴, Jing Yang ⁵, Edward C. Holmes ⁶, Alice C. Hughes ² $\stackrel{\triangle}{\sim}$ Xyuhai Bi ⁵ $\stackrel{\triangle}{\sim}$ Weifeng Shi ^{1, 7, 9} $\stackrel{\triangle}{\sim}$ X

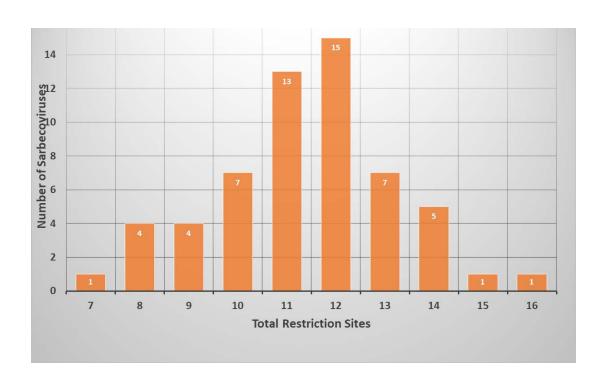
16 Sarbecoviruses from above paper in red

	Restriction Sites				
Coronavirus Identifier	Esp3I-BsmB1 E			al	Total
	Start to 15,000	15,001 to end	Start to 15,000	15,001 to end	
>KY417151_Bat_SARS_like_CoV_Rs7327	3	1	4	1	9
>KY417152_Bat_SARS_like_CoV_Rs9401	3	2	4	1	10
>KY417144_Bat_SARS_like_CoV_Rs4084	3	2	4	1	10
>KF367457_Bat_SARS_like_CoV_WIV1	3	3	4	1	11
>KU973692_UNVERIFIED_SARS_related_CoV_F46	3	1	2	3	9
>KF569996_Rhinolophus_affinis_CoV_LYRa11	3	1	3	2	9
>NC_004718_SARS_CoV Tor2	3	3	5	1	12
>MK211376_CoV_BtRs_BetaCoV_YN2018B	3	2	4	2	11
>KJ473816_BtRs_BetaCoV_YN2013	3	3	4	2	12
>KY770858_Bat_CoV_Anlong_103	3	3	4	2	12
>FJ588686_Bat_SARS_CoV_Rs672_2006	3	1	3	3	10
>KJ473815 BtRs BetaCoV GX2013	4	3	3	3	13
>JX993987 Bat CoV Rp Shaanxi2011	3	3	3	3	12
	2	3	4	3	
>KT444582_SARS_like_CoV_WIV16					12
>KY417145_Bat_SARS_like_CoV_Rf4092	3	2	4	3	12
>KY417143_Bat_SARS_like_CoV_Rs4081	2	1	4	3	10
>MK211378_CoV_BtRs_BetaCoV_YN2018D	3	2	4	3	12
>MK211377_CoV_BtRs_BetaCoV_YN2018C	3	2	4	3	12
>KY417142_Bat_SARS_like_CoV_As6526	3	1	4	3	11
>KY417147_Bat_SARS_like_CoV_Rs4237	2	2	4	3	11
>KY417148_Bat_SARS_like_CoV_Rs4247	2	2	4	3	11
>MK211375_CoV_BtRs_BetaCoV_YN2018A	3	2	4	3	12
>JX993988_Bat_CoV_Cp_Yunnan2011	3	3	5	2	13
>KJ473814_BtRs_BetaCoV_HuB2013	1	2	2	5	10
>DQ412043_Bat_SARS_CoV_Rm1	1	3	4	3	11
>KY417146_Bat_SARS_like_CoV_Rs4231	3	2	4	4	13
>KY417149 Bat SARS like CoV Rs4255	2	2	4	4	12
>KP886808 Bat SARS like CoV YNLF 31C	1	2	5	3	11
>GQ153542_Bat_SARS_CoV_HKU3_7 GQ153542	1	2	5	3	11
>DQ022305_Bat_SARS_CoV_HKU3_1	1	2	4	4	11
>GQ153547 Bat SARS CoV HKU3 12 GQ153547	1	2	4	4	11
>MG772933_Bat_SARS_like_CoV_bat_SL_CoVZC45		2	4	5	14
		2	4	5	14
>MG772934_Bat_SARS_like_CoV_bat_SL_CoVZXC2	4	2	4	5	15
>DQ071615_Bat_SARS_CoV_Rp3					
>MK211374_CoV_BtRl_BetaCoV_SC2018	2	1	4	5	12
>KY938558_Bat_CoV_strain_16BO133	4	1	5	4	14
>KY770860_Bat_CoV_Jiyuan_84	3	2	4	5	14
>DQ412042_Bat_SARS_CoV_Rf1	2	1	5	5	13
>DQ648856_Bat_CoV_BtCoV_273_2005	2	1	5	5	13
>KJ473812_BtRf_BetaCoV_HeB2013	2	2	5	5	14
>KY352407_SARS_related_CoV_strain_BtKY72	1	1	7	4	13
>NC_014470_Bat_CoV_BM48_31_BGR_2008	2	1	5	8	16
>AY278489 SARS-CoV-1 GD01	3	3	5	1	12
>AY304486.1 Civet SARS CoV SZ3	3	3	5	1	12
>AY390556 SARS-CoV-1 GZ02	3	3	5	1	12
>AY485277.1 SARS-CoV-1 Sino1-11	3	3	5	2	13
>AY508724 SARS-CoV-1 NS-1	3	3	5	1	12
>DQ084200 SARS-like CoV HKU3-3	1	2	4	4	11
>DQ648857 SARS-like CoV BtCoV/279/2005	1	2	4	3	10
>KF294457 SARS-related bat CoV Longquan-140	1	2	3	5	11
>KY770859.1 SARS CoV Anlong-112	3	3	3	2	11
Cambodian Bat RShSTT182 EPI_ISL_852604	1	3	3	1	8
Cambodian Bat RShSTT200 EPI_ISL_852605	1	3	3	1	8
Japanese Rc-o319	4				
		2	3	1	10
Pangolin Guangdong 1	2	1	2	4	9
Pangolin GuangxiP2V 2017	1	2	2	3	8
RMYN02	2	1	2	3	8
Thai bat RacC203	3	1	1 1	2	7

Summary of Natural Sarbecoviruses

- Two data sets were used to establish the properties of natural sarbecoviruses
 - 58 sequenced genomes from before 2020 which are documented to be sourced from nature
 - A number of published synthetic sarbecoviruses
- Natural sarbecoviruses have, on average, 11.8 restriction sites (RS) per genome (combining Bsal and BsmBl)
 - No natural virus has fewer than seven total RSs
- When dividing the genome in half, at nt 15,000, natural sarbecoviruses have a distribution of RS between the two halves in which there is never a segment without at least one of each RS
- In examining Bsal and BsmBl RSs from the 5' to 3' of the genome, no pattern or order of RSs is seen
 - They alternate in an apparent random order

Total Number of Restriction Sites in 58 Sarbecoviruses



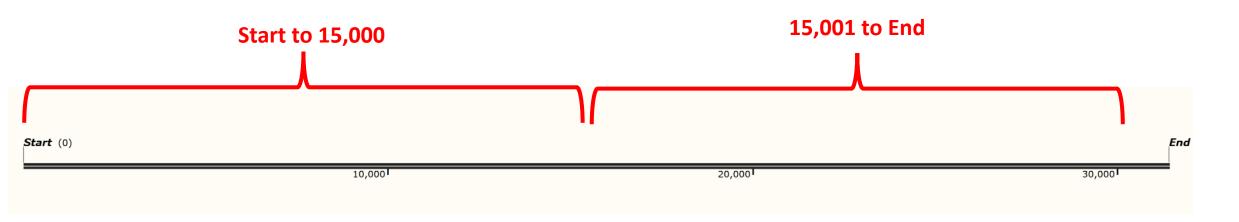
- Mean = 11.4 + 2.1 per genome (combined BsmBl & Bsal)
- No natural sarbecoviruses has been found with fewer than seven per genome
- Based on observed number and distribution the probabilities shown below can be calculated

Number of sites (X)	Probability ≤ X
≤4	0.00016
≤5	0.0009
≤6	0.004

Summary of Synthetic Viruses & SARS-CoV-2

- Synthetic viruses can routinely have fewer than seven total restriction, a number never before seen in a natural virus
- Synthetic viruses often have one or more genome segments that are missing a restriction site, a
 distribution never seen in natural viruses
- In examining Bsal and BsmBl RSs from the 5' to 3' of the genome, a pattern of segregation of restriction sites can be observed. This has never been seen in a natural virus
- SARS-CoV-2 has five total restriction sites, with a likelihood of 0.0009 for a natural virus
- SARS-CoV-2 has no Bsal sites in the first 17,320 nt with a likelihood of 0.0013 for a natural virus
- SARS-CoV-2 has a "segregated" RS pattern: BsmBI-BsmBI-BsmBI-BsaI-BsaI, never seen in a natural virus and with a likelihood of 0.05
- SARS-CoV-2 has seven RSs that differ from natural sarbecoviruses, a likelihood of 0.000014 to have occurred naturally by random mutations within the 42 nts of the RSs in a total genome of 30,000
- The likelihood these four features occurred together in a natural sarbecoviruses is one in greater than 1.2 billion

Genome Segmental Distribution of Restriction Sites in 58 Sarbecoviruses



Restriction	Genome Section			
Enzyme	Start to 15,000 15,001 to Er			
BsmBl	2.4 <u>+</u> 0.9	2.0 <u>+</u> 0.7		
Bsal	3.9 <u>+</u> 1.0	3.0 <u>+</u> 1.5		

- The 58 viruses have 232 total restriction site segments (two segments and two enzymes per virus)
- All of the 232 segments have at least two restriction sites, at least one of each enzyme
- Thus, the 95% confidence upper bound for the probability of zero restriction sites at a given region of the genome is given by the approximation (Rule of Threes) as 3/232 = <0.013

Genome Distribution Pattern of Restriction Sites in 58 Sarbecoviruses

Hypothesis:

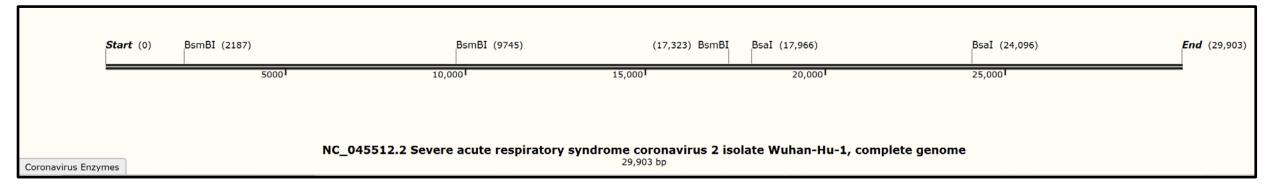
- A natural sarbecovirus will have a "random" distribution of BsmBI and BsaI restriction sites when viewing the 5' to 3' genome sequence
- A synthetic virus may display a "segregated" distribution of BsmBI and BsaI restriction sites when viewing the 5' to 3' genome sequence. This facilitates one step, unidirectional assembly of cloned segments



Random Distribution: (RS1)a-(RS2)b-(RS1)c-(RS2)d, where a-d equal 1-5 and RS1 and RS2 can be either BsmBI or BsaI

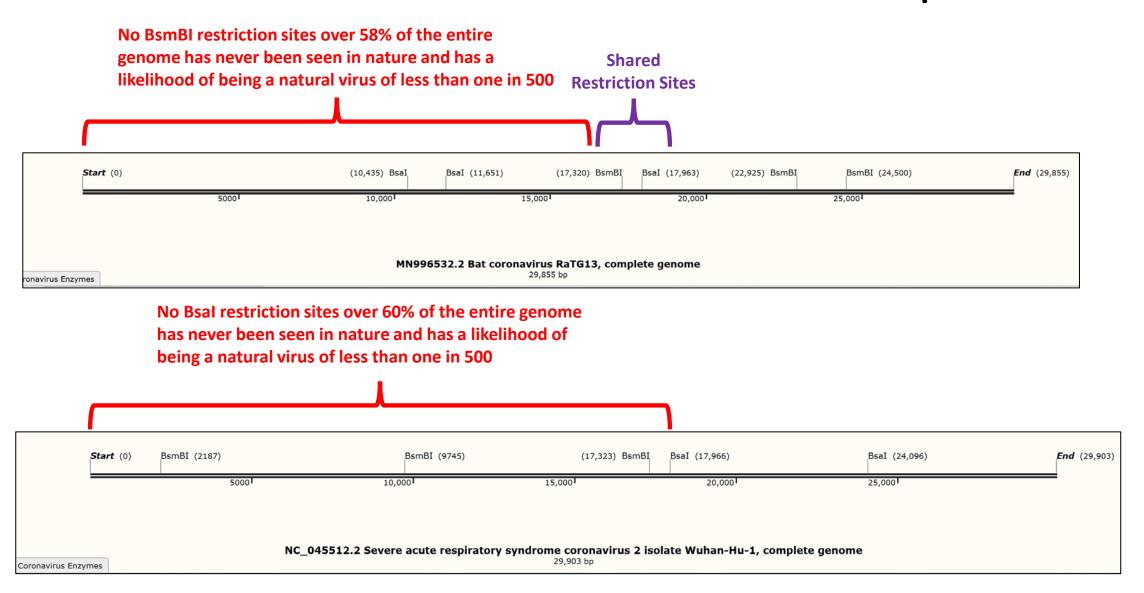
Segregated Distribution: (RS1)a-(RS2)b where a and b equal 1-5 and RS1 and RS2 can be either BsmBI or BsaI

SARS-CoV-2 restriction sites number and pattern have never been observed in a natural sarbecovirus



- SARS-CoV-2 has a total of five Bsal and BsmBl restriction sites
 - A natural sarbecovirus has never been observed with less than seven
 - The likelihood SARS-CoV-2 is not a natural virus, based on RS number, is >99.9%
- SARS-CoV-2 has no Bsal restriction sites in the first 60% of the genome and no BsmBl restriction sites in the remaining 40% of the genome
 - This "segregated" distribution has never been seen in a natural virus, making the likelihood SARS-CoV-2 is not a natural virus, based on this pattern, at 95%

SARS-CoV-2 and RaTG13 Share Two Restriction Sites and Have Seven Unique Sites



What is the probability that the seven-restriction site changes between SARS-CoV-2 & RaTG13 occurred randomly?

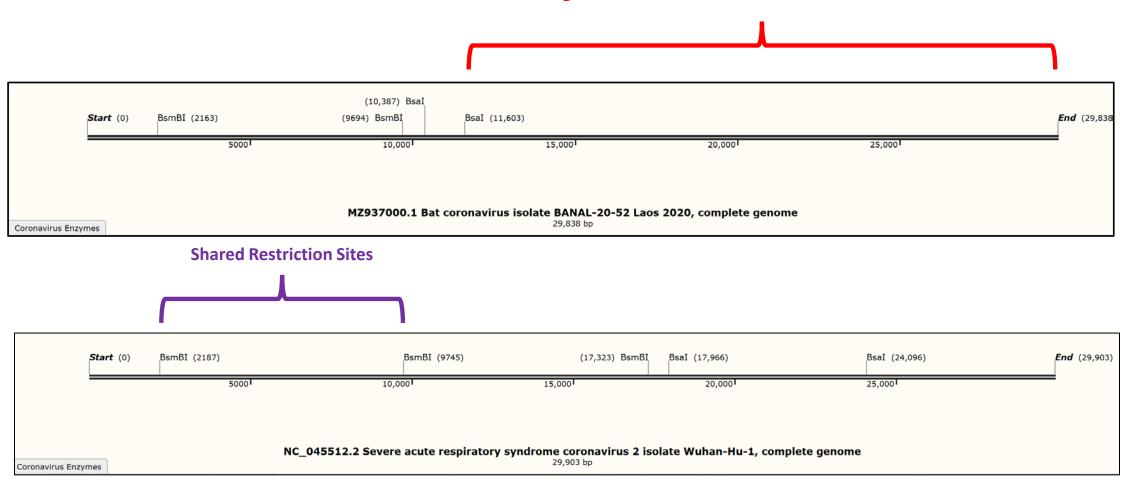
- SARS-CoV-2 has a genome of 29,903 nts
- SARS-CoV-2 and RaTG13 are 96.2% identical at the genome level
- Assumptions
 - The viruses differ by seven restriction sites of six nt each
 - Random probability of six nts being identical is (0.962) to the 6th power or 0.793
 - Probability of at least one nt difference in one restriction site is 1.0 0.793 = 0.207
 - Probability of all 7 restriction sites with at least 1 mutation is (0.202) to the 7th power =

0.000014 or one in >72,990

This pattern of 7 RS changes could not have been random

SARS-CoV-2 and BANAL-52 Share Two Restriction Sites and Have Five Unique Sites

No Bsal or BsmBl restriction sites over 61% of the entire genome has never been seen in nature and has a likelihood of being a natural virus of less than one in 50,000



What is the probability that the five restriction site changes between SARS-CoV-2 & BANAL-52 occurred randomly?

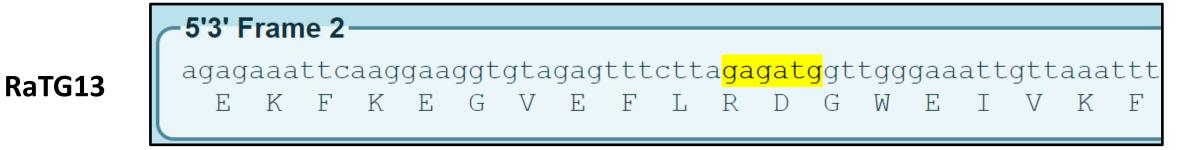
- SARS-CoV-2 has a genome of 29,903 nts
- SARS-CoV-2 and BANAL-52 are 96.9% identical at the genome level
- Assumptions
 - The viruses differ by five restriction sites of six nt each
 - Random probability of six nts being identical is (0.969) to the 6th power or 0.828
 - Probability of at least one nt difference in one restriction site is 1.0 0.828 = 0.172
 - Probability of all 5 restriction sites with at least 1 mutation is (0.172) to the 5th power =

0.000151 or one in >6,600

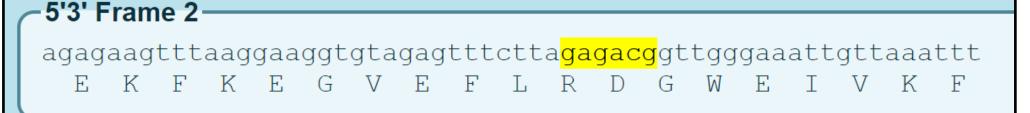
This pattern of 5 RS changes could not have been random

SARS-CoV-2 BsmBl Restriction Site 2187





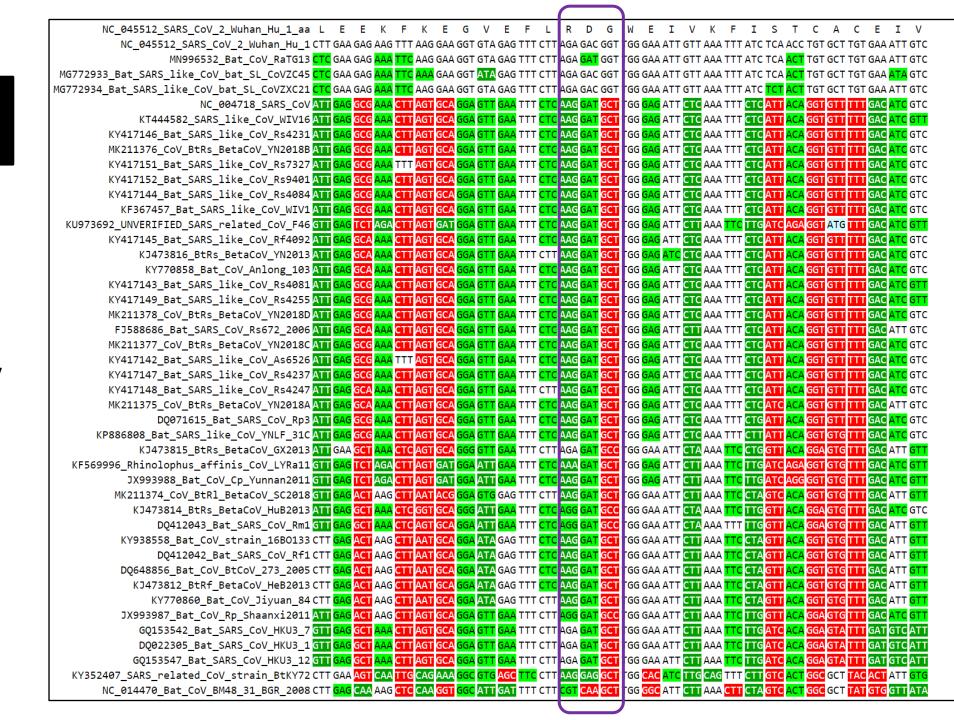
CoV-2



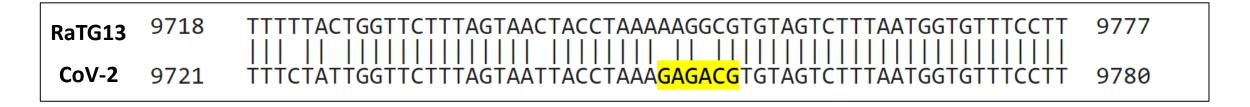
Synonymous mutation in homologous peptide region

SARS-CoV-2 BsmBl Restriction Site 2187

The out-of-frame GAGACG restriction site is found in only three of 58 sarbecoviruses, including SARS-CoV-2 and excluding RaTG13.



SARS-CoV-2 BsmBl Restriction Site 9745





Synonymous mutation in homologous peptide region

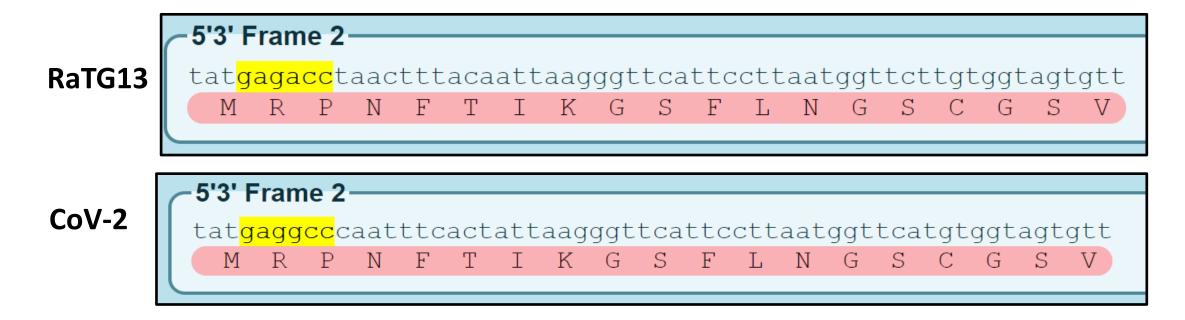
SARS-CoV-2 BsmBl Restriction Site 9745

The out-of-frame GAGACG restriction site is found in only three of 58 sarbecoviruses, including SARS-CoV-2. It is absent in RaTG13.



RaTG13 Bsal Restriction Site 10,435





Synonymous mutation in homologous peptide region

RaTG13 Bsal Restriction Site 10,435

The out-of-frame GAGACC is found in RaTG13 and 52 out of 57 of the other sarbecoviruses.

Only five of 58 viruses do NOT have this restriction site, including SARS-CoV-2.

```
NC_045512_SARS_CoV_2_Wuhan_Hu_1_aa C
                                           NC 045512 SARS CoV 2 Wuhan Hu 1 TGT GCT ATG AGG CCC AAT TTC
MG772934 Bat SARS like CoV bat SL COVZXC21TGT GCC ATG AGA CCT AAT TTT ACT ATT AAG GGT TCA TTC CTT AAT GGT TCA TGT GGT AGT GTT GGT TTT AAT AAT ATA
                                                                                                                                                                                                                                                                                                                                     <mark>CT</mark> TTC CTT AAT <mark>GGA</mark> TCA TGT GGT AGT GTT GGT TTT <mark>AAT</mark>
                                                     GQ153547 Bat SARS COV HKU3 12 TGT GCC ATG AGA CCT AAT TAC ACC ATT AAG GGT TCC TTC AAT GGT TCA TGT GGT AGT GTT GGT TTT AAT ATT GAT TAT G
            KY352407 SARS related CoV strain BtKY72 TGT GCA ATG AGA CCT AGC TTT ACT ATT AAA GGT TCA TTC CTT AAT GGT TCT TGT GGT AGT GTT GGT TTC AAC ATA GAC TAT GAC TGT GTC TGT GGT TCT TGT GGT AGT GTT GGT TTC AAC ATA GAC TGT GAC TGT GTC TGT GTC TGT GGT AGT GTT GGT TGT GAC ATA GAC TGT GAC TGT GTC TGT GTC TGT GTC TGT GGT TGT GTC TG
                               NC_014470_Bat_CoV_BM48_31_BGR_2008 TGT GCA ATG AGG TCC AAC ACT ATT AAG GGT TCA TTC CTT AAT GGT TCT TGT GGT AGT GTT GGT TTT AAC ATT GAC TAT GAC TGC GTG
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RaTG13 Bsal Restriction Site 11,651





Synonymous mutation in homologous peptide region

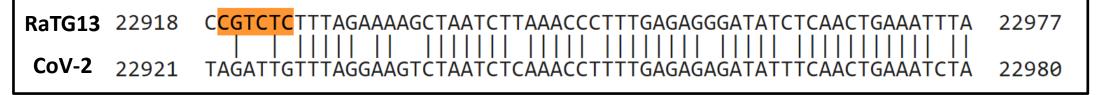
RaTG13 Bsal Restriction Site 11,651

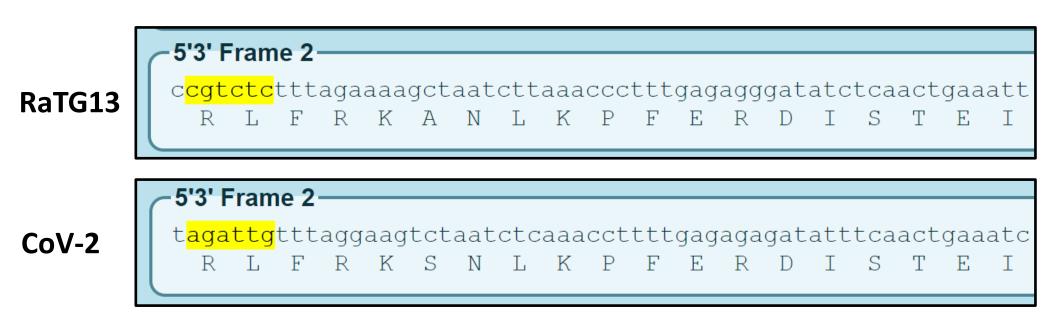
The in-frame GGTCTC site is found in only three of 57 sarbecoviruses, including RaTG13 but not SARS-CoV-2.

55 of 58 sarbecoviruses, including SARS-CoV-2, have this restriction site extinguished.



RaTG13 BsmBl Restriction Site 22,925





Synonymous mutation in homologous peptide region

RaTG13 BsmBl Restriction Site 22,925

The in-frame CGTCTC restriction site in RaTG13 is unique among sarbecoviruses, including SARS-CoV-2.

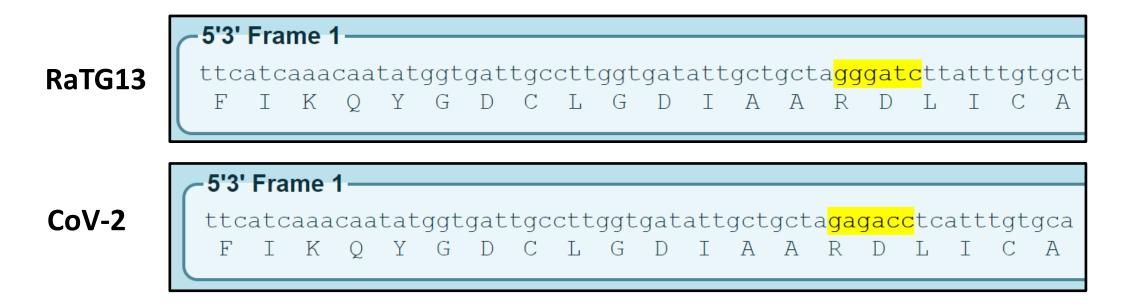
The leucine amino acid in the restriction site is not found in 56 out of 58 sarbecoviruses.

The consensus dipeptide is RS, not RL.

```
NC 045512 SARS CoV 2 Wuhan Hu 1 aa
            NC_045512_SARS_CoV_2_Wuhan_Hu_1 TAC CTG TAT AGA TTG TTT AGG AAG TCT
MG772933 Bat SARS like CoV bat SL CoVZC45 TAC AGG
MG772934 Bat SARS like CoV bat SL CoVZXC21 TAT AGG
         KY417146 Bat SARS like CoV Rs4231 TAT TTA TAT AGA TGG
                                                  AAA TAT AGA TCT
            KF367457 Bat SARS like CoV WIV1 TAT AAA TAT AGA TCT
                                                                  TC AGA CAT GGC AAG CTT AGG CCT TTT GAG AGA GAT ATT TCT AAT
                                                                                 AAG CTT AAA CCA TTC GAG CGA GAT CTT TCA TCT GAT GA - --A AAC GGT GTA CGT ACA CTG AGG
  KU973692 UNVERIFIED SARS related CoV F46 TAT TTT TA
                KY770858 Bat CoV Anlong 103 TAT TTT TAT AGA TCT AT CGC TCC ACC AAA TTA AAG CCA TTT GAG CGT GAT GTT TCT TCT
         KY417143 Bat SARS like CoV Rs4081 TAT TAT TAT AGA TCC
         KY417149_Bat_SARS_like_CoV_Rs4255 TAT TAT TAT AGA TCC
         MK211378 CoV BtRs BetaCoV YN2018D TAT TAT TAT AGA TCC
          FJ588686 Bat SARS CoV Rs672 2006 TAT TAT TAT AGA TCC
         MK211377 CoV BtRs BetaCoV YN2018C TAT TAT TAT AGA TCC
         KY417142 Bat SARS like CoV As6526 TAT TAT TAT AGA TCC
                                                                  AC CGT AAG ACT AAG CTC AAG CCT TTT GAG AGA GAT CTA
   KF569996 Rhinolophus affinis CoV LYRa11 TAT AAA TAT AGA TCT
             JX993988_Bat_CoV_Cp_Yunnan2011 TAT TAT TAC AGA TCA
                                                                  TAC <mark>AGA</mark> AAG <mark>GAA AAA</mark> CTC AAA CCT TTT GAG <mark>AGG</mark> GAT TTG TCA TCT G-- --- --- ---
            DQ648856 Bat CoV BtCoV 273 2005 TAT TTT TA
                 KY770860 Bat CoV Jiyuan 84 TAT TTT TA
               DQ022305 Bat SARS CoV HKU3 1 TAT TAC TAL AGA TCT
              GQ153547 Bat SARS CoV HKU3 12 TAT TAC TAL AGA TCT
   KY352407_SARS_related_CoV_strain_BtKY72 TA- --- -- AGG CTT TTT AGA CAT GGG AAG ATC AAA CCT TAT GAA CGC GAT ATT TCC AAT GTC CTT TAT AAT TCA GGT GGT GGT
        NC_014470_Bat_CoV_BM48_31_BGR_2008 TTC TTT TAL AGG AGA TTC AGA CAT GGA AAG ATT AAA CCT TAT GGG CGT GAT GTT GTT GTT TAT AAC CCT TCA GGT AGA TGT
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SARS-CoV-2 Bsal Restriction Site 24,096





Synonymous mutation in homologous peptide region

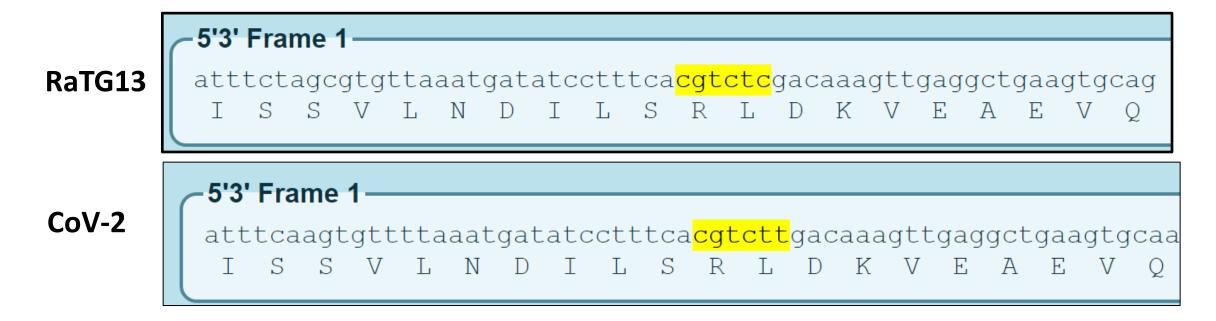
SARS-CoV-2 Bsal Restriction Site 24,096

The out-of-frame GAGACC restriction site is found in 18 out of 57 but not RaTG13.

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NC 045512 SARS CoV 2 Wuhan Hu 1 aa L G
           NC 045512 SARS COV 2 Wuhan Hu 1 CTT GGT GAT ATT GCT GCT AGA GAC CT CATT TGT GCA CAA AAG TTT AAC GGC CTT ACT GTT TTG CCA CCT TTG CTC ACA GAT GAA ATG ATT
                     MN996532 Bat CoV RaTG13 CTT GGT GAT ATT GCT GCT
                                                                      AGG GAT CTT ATT TGT GCT CAA AAG TTC AAT GGC CTT ACT GTT CTG CCA CCT TTG CTC ACA GAT GAA ATG ATC
MG772934 Bat SARS like CoV bat SL COVZXC21 TTG GGT GAT ATT TCT GCT AGA GAT TTG ATT TGT GCT CAA AAG TTC AAT GGA CTC ACT GTC TTA CCA CCA TTG CTC ACA GAT GAA ATG ATC
                          NC 004718 SARS COVICTA GGT GAT ATT AAT GCT AGA GAT CTIC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG TTG CCA CCT CTG CTC ACT GAT GAT ATG ATT
               KT444582 SARS like COV WIV16 CTA GGT GAT ATT AAT GCT AGA GAT CT C ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG CTG CCC CCT CTC CTC ACT GAT GAT ATG ATT
          KY417146 Bat_SARS_like_Cov_Rs4231 CTA GGT GAT GTT AAT GCT AGA GAT CTC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG TTG CCA CCT CTG CTC ACT GAT GAT ATG ATT
         MK211376 COV Btrs BetaCov YN2018B CTA GGT GAT ATT AAT GCT AGA GAT CTC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG TTG CCA CCT TTG CTC ACT GAT GAT ATG ATT
          KY417151 Bat SARS like Cov Rs7327 CTA GGT GAT ATT AAT GCT AGA GAT CT C ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG TTG CCA CCT CTG CTC ACT GAT GAT ATG ATT
          KY417152 Bat SARS like CoV Rs9401 CTA GGT GAT ATT AAT GCT AGA GAT CTC ATT TGT
          KY417144 Bat SARS like Cov Rs4084 CTA GGT GAT ATT AAT GCT AGA GAT CTC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG CTG CCA CCT CTG CTC ACT GAT GAT ATG ATT
           KF367457 Bat SARS like Cov WIV1 CTA GGT GAT ATT AAT GCT AGA GAT CTC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG CTG CCA CCT CTG CTC ACT GAT GAT ATG ATT
  KU973692_UNVERIFIED_SARS_related_CoV_F46 CTA GGT GAT ATT AGT GCT AGA GAC CTC ATT TGT GCC CAA AAG TTC AAT GGA CTT ACA GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
          KY417145_Bat_SARS_like_CoV_Rf4092 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAG AAG TTC AAC GGA CTT ACT GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
               KJ473816 Btrs Betacov yn2013 CTA GGC GAT ATT AGT GCT AGA GAC CT C ATT TGT GCT CAG AAG TTC AAC GGA CTT ACT GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
                KY770858_Bat_CoV_Anlong_103 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAG AAG TTC AAT GGA CTT ACT GTC CCA CCA TTG CTC ACA GAT GAA ATG ATT
          KY417143 Bat SARS like CoV Rs4081 CTA GGT GAT ATT AAT GCT AGG GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC CTG CCA CCT TTA CTC ACC GAT GAC ATG ATT
          KY417149 Bat SARS like CoV Rs4255 CTA GGT GAT ATT AAT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC TTG CCA CCT TTA CTC ACC GAT GAC ATG ATT
          MK211378 COV Btrs Betacov YN2018D CTA GGT GAT ATT AAT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC CCA CCT TTA CTC ACC GAT GAC ATG ATC
          FJ588686 Bat SARS COV Rs672 2006 CTA GGC GAT ATT AGT GCT AGA GAC CT C ATT TGT GCT CAG AAG TTC AAC GGA CTT ACT GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
          MK211377_COV_BtRs_BetaCoV_YN2018C CTA GGT GAT ATT AAT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC CTT CCA CCT TTA CTC ACA GAC GAC ATG ATT
          KY417142 Bat_SARS_like_CoV_As6526 CTA GGT GAT ATT AAT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC CTT CCA CCT TTA CTC ACA GAC GAC ATG ATT
          KY417147 Bat SARS like CoV Rs4237 CTA GGT GAT ATT AAT GCT AGG GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC TTG CCA CCT TTA CTC ACC GAT GAC ATG ATT
          KY417148 Bat SARS like CoV Rs4247 CTA GGT GAT ATT AAT GCT AGA GAT CTC ATT TGT GCG CAG AAG TTC AAT GGA CTT ACA GTG TTG CCA CCT CTA CTC ACA GAT GAC ATG ATT
         MK211375 COV Btrs BetaCov YN2018A CTA GGT GAT ATT AAT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGC CTT ACT GTC CTT CCA CCT TTA CTC ACA GAT GAC ATG ATT
                   D0071615 Bat SARS COV Rp3 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAG AAG TTT AAT GGA CTT ACT GTC CTA CCA CCA CTG CTC ACA GAT GAA ATG ATT
       KP886808 Bat SARS like COV YNLF 31C TTG GGT GGT ATT AAC GCT AGA GAT CTC ATC TGT GCT CAA AAG TTT AAC GGA CTT ACA GTC CTA CCA CCT TTG CTC ACT GAT GAT ATG ATT
               KJ473815_Btrs_BetaCoV_GX2013 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAG AAG TTC AAT GGA CTT ACT GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
   KF569996_Rhinolophus_affinis_CoV_LYRa11 CTA GGC GAT ATT AGT GCA GAC CT C ATT TGT GCG CAA AAG TTT AAT GGA CTT ACT GTC CTT CCA CCT TTG CTC ACT GAC GAA ATG ATT
             JX993988 Bat CoV Cp Yunnan2011 CTA GGC GAT ATT AGT GCT AGA GAT CT CATT TGT GCG CAG AAG TTC AAT GGA CTC ACT GTC CTT CCA CCT CTA CTC ACC GAT GAA ATG AAT
          MK211374 COV BtR1 BetaCoV SC2018 CTA GGT GAT ATT AGT GCT AGA GAC CTC ATC TGT GCA CAA AAG TTC AAT GGA CTT ACT GTC CTA CCA CCT TTA CTC ACC GAT GAC ATG ATT
              KJ473814 Btrs BetaCov HuB2013 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAA AAA TTC AAT GGA TTG ACT GTT CTA CCA CCA TTG CTC ACA GAT GAA ATG AAT
                  DQ412043 Bat SARS COV Rm1 CTA GGC GAT ATT AGT GCT AGA GAC CTC ATT TGT GCT CAA AAG TTT AAT GGA CTT ACT GTC CTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
            KY938558_Bat_CoV_strain_16B0133 TTG GGT GGT ATT AAC GCA AGA GAT CTC ATC TGT GCT CAA AAG TTT AAT GGG CTG ACA GTC TTA CCA CCT TTG CTC ACT GAT GAC ATG ATT
                  DQ412042_Bat_SARS_COV_RF1 CTG GGT GGT ATT AAC GCA AGA GAT CTC ATC TGT GCT CAA AAG TTT AAT GGA CTG ACA GTC TTA CCA CCT TTG CTC ACT GAT GAC ATG ATT
           DQ648856_Bat_CoV_BtCoV_273_2005 CTG GGT GGT ATT AAC GCA AGA GAT CTC ATC TGT GCT CAA AAG TTT AAT GGA CTG ACA GTC TTA CCA CCT TTG CTC ACT GAT GAC ATG ATT
              KJ473812 Btrf_BetaCov_HeB2013 TTG GGT GGT ATT AAC GCA AGA GAT CTC ATC TGT GCT CAA AAG TTT AAT GGA CTC ACA GTC TTA CCA CCT TTG CTC ACT GAT GAC ATG ATT
                 KY770860 Bat CoV Jiyuan 84 TTG GGT GGT ATT AAC GCA AGA GAT CTC ATC TGT GCT CAA AAG TTT AAT GGA CTC ACA GTC TTA CCA CCT TTG CTC ACT GAT GAC ATG ATT
            JX993987 Bat CoV Rp Shaanxi2011 CTA GGC GAT ATT AGT GCT AGA GAT CT C ATT TGT GCA CAA AAA TTC AAC GGA CTG ACT GTC TTA CCA CCA TTG CTC ACA GAT GAA ATG ATT
               GO153542 Bat SARS COV HKU3 7 CTA GGT GAT GTT AGT GCT AGA GAC CTT ATC TGT GCC CAG AAG TTC AAT GGA CTT ACT GTG CTA CCA CCA CTG CTC ACA GAT GAG ATG ATT
               DO022305 Bat SARS COV HKU3 1 CTA GGT GAT GTT AG GAC GAT GAG GAC CTT ATC TGT GCC CAG AAG TTC AAT GGA CTT ACT GTG CTA CCG CCA CTG CTC ACA GAT GAG ATG GTT
              GQ153547_Bat_SARS_COV_HKU3_12 CTA GGT GAT GTT ACT GAG GAC CTT ATC TGT GCC CAG AAG TTC AAT GGA CTT ACT GTG CTA CCA CCA CCA CTC CTT ACA GAT GAG ATG ATT
   KY352407_SARS_related_CoV_strain_BtKY72 TTA GGT GGT ATA AAT GCT AGA GAC CTT ATT TGT GCT CAA AAG TTC AAT GGT CTC ACC GTT TTG CCA CCT TTA CTT ACA GAC GAC ATG ATT
        NC_014470 Bat_Cov_BM48_31_BGR_2008 TTA GGT GGT GGT GAT GAT GCT CGT GAC CTC ACT GAC GGA CAA AAG TTC AAT GGG CTC ACA GTA CTC CCC CTA CTC ACT GAT GAA ATG ATT
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RaTG13 BsmBl Restriction Site 24,500





Synonymous mutation in homologous peptide region

RaTG13 BsmBl Restriction Site 24,500

- The in-frame restriction site CGTCTC is found only in RaTG13 and in no other sarbecoviruses
- The SARS-COV-2 sequence CGT-CTT is found in BANAL-52, -103, and -236 but in no other sarbecoviruses

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NC 045512 SARS CoV 2 Wuhan Hu 1 aa T L V K Q L
            NC 045512 SARS COV 2 Wuhan Hu 1 ACG CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTT TTA AAT GAT ATC CTT TCA
                                                                                                                                     CGT CTT 🖟 AC AAA GTT GAG GCT GAA
                     MN996532_Bat_CoV_RaTG13 ACG CTT GTT AAA CAA CTT AGC TCC AAT TTT GGA GCT ATT TCT AGC GTG TTA AAT GAT ATC CTT TCA CGT CTC GAC GAA GTT GAG GCT GAA
 MG772933_Bat_SARS_like_CoV_bat_SL_CoVZC45 ACG CTT GTT AAA CAA CTT AGT TCC AAT TTT GGT GCA ATT TCA AGC GTG TTG AAT GAC
MG772934 Bat SARS like CoV bat SL COVZXC21 ACG CTT GTT AAA CAA CTT AGT TCC AAT TTT GGT GCA ATT TCA AGC GTG TTG AAT GAT ATT CTT TCA CGC CTT AC AAA GTC GAG GCT GAC
                           NC 004718 SARS COVI<mark>ACA</mark> CTTIGTT AAA CAA CTTIAGC TCT AAT TTTIGGT GCA ATTITCA AGTI<mark>GTG CTA</mark> AAT GATIATC CTTI<mark>TCC</mark>
                KT444582 SARS like CoV WIV16 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCG ATT TCA AGT GTG CTA AAT GAT ATC CTT
          KY417146 Bat SARS like Cov Rs4231 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GTG TTA AAT GAT ATC CTT TCC CGA CTT GAT AAA GTC GAG GAG
          MK211376 COV BtRs BetaCov YN2018B ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAT ATC
          KY417151 Bat SARS like Cov Rs7327 ACA CTT GTT AAA CAA CTT AGC TCT AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAT ATC CTT TCC CGA CTT GAT AAA GTT GAG GCG GA
          KY417152 Bat SARS like Cov Rs9401 ACA CTT GTT AAA CAA CTT AGC TCT AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT ATC CTT
          KY417144 Bat SARS like Cov Rs4084 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCG ATT TCA AGT GTG TTA AAT GAT ATC CTT
            KF367457 Bat SARS like Cov WIV1 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCG ATT TCA AGT GTG CTA AAT GAT ATC CTT TCC CGA CTT GAT AAA GTC GAG GCA GAC
  KU973692 UNVERIFIED SARS related CoV F46 ACA CTT GTT AAA CAA CTC AGC TCC AAT TTT GGT GCT ATT TCA AGT GTT TTA AAT GAC ATT TTA TCA CGA CTT GAC AAA GTT GAG GCA GAA
          KY417145 Bat SARS like Cov Rf4092 ACA CTT GTT AAA CAG CTT AGC TCC AAT TTC GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA GGA CTA 🗚 CAA GTC GAG GCA GAC
                KJ473816 Btrs Betacov yn2013 ACA CTT GTT AAA CAG CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA CGA CTA GAC AAA GTC GAG GCA GAC
                 KY770858_Bat_CoV_Anlong_103 ACA CTT GTT AAA CAG CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA
          KY417149 Bat SARS like Cov Rs4255 ACG CTT GTT AAA CAG CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA CGA CTA AAT AAA GTC GAG GAC
           FJ588686 Bat SARS COV Rs672 2006 ACA CTT GTT AAA CAG CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA CGA CTA GAC AAT GTC GAG GCA GAI
          MK211377 COV Btrs BetaCov YN2018C ACG CTT GTT AAA CAG CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA
          KY417142 Bat SARS like Cov As6526 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATC CTT TCA GGC CTA GAT AAA GTC GAG GCA GA
          KY417147 Bat SARS like Cov Rs4237 ACA CTT GTT AAA CAG CTT AGC TCT AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA
          KY417148 Bat SARS like Cov Rs4247 ACA CTT GTT AAA CAG CTT AGC TCT AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATT CTT TCA CGA CTA GAC AAG GTC GAG GCA GAC
          MK211375 COV Btrs BetaCov YN2018A ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAT ATC CTT TCA
                   DQ071615_Bat_SARS_COV_RP3 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATC CTG TCA GAC ATA GAC AAA GTC GAG GCA GAC
        KP886808 Bat SARS like Cov YNLF 31C ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GTT TTA AAT GAC ATC CTC TCA CGA CTT GAC AAA GTT GAG GCA GAC
                KJ473815 Btrs BetaCov GX2013 ACA CTT GTA AAA CAA CTT AGC TCC AAT TTT GGT GCT ATC TCA AGT GTA TTA AAT GAC ATT CTA TCC AGA CTT GAC AAA GTC
   KF569996 Rhinolophus affinis CoV LYRa11 ACA CTT GTT AAA CAA CTA AGC TCC AAC TTT GGT GCG ATT TCA AGT GTT TTA AAT GAC ATT CTG TCA CGA CTT GAC AAA GTT GAG GCA GAA
             JX993988_Bat_CoV_Cp_Yunnan2011 ACA CTT GTT AAA CAA CTA AGT TCT AAC TTT GGT GCA ATT TCA AGT GTT TTA AAT GAC ATT CTG TCT CGA CTT GAC AAA GTT GAG GCT GAA
           MK211374_COV_BtR1_BetaCoV_SC2018 ACA CTT GTC AAA CAA CTT AGT TCC AAC TTT GGT GCT ATT TCA AGT GTT TTA AAT GAT ATT CTT TCA CGA CTT GAT AAA GTC GAA GCA GAC
               KJ473814 BtRs BetaCoV HuB2013 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCG AGT GTT CTA AAC GAC ATT CTT CCA GGA CTC GAC AAA GTC GAG GCA GAA
                   DQ412043 Bat SARS COV Rm1 ACA CTT GTT AAA CAA CTT AGC TCC AAT TTT GGT GCA ATT TCA AGT GTG CTA AAT GAC ATC CTT TCA CGA CTA GAC AAA GTC GAG GCA GAC
            KY938558_Bat_CoV_strain_16B0133 ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GCT TTG AAT GAT ATC CTC TCA CGA CTT ♦AC AAA GTT GAG GCA GAC
                   DQ412042 Bat SARS COV Rf1 ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GCT TTG AAT GAC ATC CTC TCA CGA CTT GAC AAA GTT GAG GCA GAC
            DQ648856 Bat COV BtCoV 273 2005 ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GCT TTG AAT GAC ATC CTC TCA CGA CTT GAC AAA GTT GAG GCA GAG
              KJ473812 Btrf Betacov HeB2013 ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GCT TTG AAT GAT ATC CTC TCA CGA CTT GAC AAA GTT GAG GCA GAG
                  KY770860_Bat_CoV_Jiyuan_84 ACA CTT GTC AAA CAA CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GCT TTG AAT GAT ATC CTC TCA CGA CTT GAC AAA GTT GAG GCA GAC
            JX993987 Bat CoV Rp Shaanxi2011 ACA CTT GTC AAA CAG CTT AGC TCC AAT TTT GGT GCT ATT TCA AGT GTT TTG AAT GAC ATT CTT TCA CGA CTT HAT AAA GTT GAG GCA GAA
                GO153542 Bat SARS COV HKU3 7 ACG CTT GTT AAA CAG CTT AGC TCT AAT TTT GGA GCT ATT TCG AGT GTG TTA AAT GAT ATT CTT TCT CGA CTT GAT AAA GTT GAG GCG GAG
                DQ022305_Bat_SARS_COV_HKU3_1 ACG CTT GTT AAA CAA CTC AGC TCT AAT TTT GGA GCT ATC TCA AGT GTG TTA AAT GAT ATT CTC TCT CGC CTT GAT AAA GTT GAG GCA GAA
               GO153547 Bat SARS COV HKU3 12 ACG CTT GTT AAA CAA CTT AGC TCT AAT TTT GGA GCT ATC TCA AGT GTG TTA AAT GAC ATT CTC TCT CGC CTT AAT AAA GTT GAG GCA GAA
   KY352407 SARS related CoV strain BtKY72 ACT CTT GTA AAG CAA CTT AGC TCC AAT TTT GGC GCT ATT TCA AGT GTG TTA AAT GAC ATT CTT TCT AGA CTT GAT AAA GTT GAG GCT GAA
         NC 014470 Bat COV BM48 31 BGR 2008 ACA CTA GTT AAA CAG CTT AGC TCC AAT TTT GGT GCT ATT TCT AGT GTA CTG AAT GAT ATT CTG TCT CGA CTT GAC AAA GTA GAG GCC GAA
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- A blast of Amplicon #1 against RaTG13 showing the Bsal restriction site in the amplicon that is changed in RaTG13
- The amplicon has a 2% overall difference from RaTG13 but within the restriction site one of six or 17% are changed

Range	1: 669	to 1761 GenBank	Graphics		▼ Next N	<u> 1atch</u> ▲ <u>F</u>
Score 1897 b	oits(102	Expect 0.0	Identities 1075/1096(98%)	Gaps 12/1096(1%)	Strand Plus/Pl	lus
Query Sbjct	14 669			GGCGACGAGCTTGGCACTGAT	CCTTATG	71 728
Query Sbjct	72 729	AAGATTTTCAAGAA	AACTGGAACACTAAACATA	AGCAGTGGTGTCACCCGTGAT	CTCATGC	131 788
Query Sbjct	132 789			STCGATAACAACTTCTGTGGC		191 848
Query Sbjct	192 849	GCTACCCTCTTGAG	TGCATTAAAGACCTTCTAG	GCTCGTGCTGGTAAAGCTTCA	TGCACTT	251 908
Query Sbjct	252 909	TGTCCGAACAACTG	GACTTTATTGACACTAAAA	AGAGGTGTATACTGCTGTCGT	GAACATG	311 968
Query Sbjct	312 969			SAAAAGAGCTATGAATTGCAG		371 1028
Query Sbjct	372 1029	TTGAAATTAAATTG	GCAAAGAAATTTGACACAT	TTTAATGGGGAATGTCCAAAT	TTTGTAT	431 1088
Query Sbjct	432 1089			CCAAGGGTTGAAAAGAAAAAG		491 1148
Query Sbjct	492 1149			STTGCTTCACCAAATGAATGC		551 1208
Query Sbjct	552 1209	TGTGCCTTTCAACT	CTCATGAAGTGTGATCAT	TGTGGTGAAACTTCATGGCAG	ACAGGCG	611 1268
Query Sbjct	612 1269			ACTGAAAATTTGACTAAAGAG		671 1328
Query Sbjct	672 1329			STCAAAATTTATTGTCCAGCA		731 1388
Query Sbjct	732 1389	ATCCAGAAGTAGGA		GAGTATCATAATGAATCTGGC	TTGAAAC	791 1448
Query Sbjct	792 1449			TTTGGAGGCTGTGT <mark>GGTCTC</mark> C		851 1508
Query Sbjct	852 1509	GTTGCTACAATAAG	TGTGCCTATTGGATTCCAG	CGTGCTAGCGCCAACATAGGT	TGCAATC	911 1568
Query Sbjct	912 1569			TTAATGAT-ACCTTCTGGAA		969 1628
Query Sbjct	970 1629			GACTTTGAACTTGATGGAAGA		1027 1686
Query Sbjct	1028 1687	CATTATTTTGGCAT		A-TGCTTTTGTGGAAACTGGG		1086 1745
Query Sbjct	1087 1746	TG-ATAATAAAA-A				

- The site of the Bsal restriction site that was present in Amplicon #1 and removed in amplicon #2 is shown (purple box)
- No sarbecovirus has a restriction site at this position, suggesting this was a laboratory-designed restriction site
- The finding of a restriction site in an amplicon that is not found in nature and its removal in an overlapping amplicon is consistent with reverse genetics workflow
- A blast of Amplicon #1
 identifies only RaTG13 and
 SARS-CoV-2, both with the
 GTTCTC non-restriction site
 sequence



Caveats

- Not seeing this pattern does not mean it is natural
- All you have to do is add back restriction sites after 'No See 'Em' technology and...poff

