Reference-based viral sequence annotation using VADR

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GenBank has a lot of sequence data

D94–D99 *Nucleic Acids Research, 2019, Vol. 47, Database issue* *Published online 26 October 2018*
doi: 10.1093/nar/gky989

GenBank

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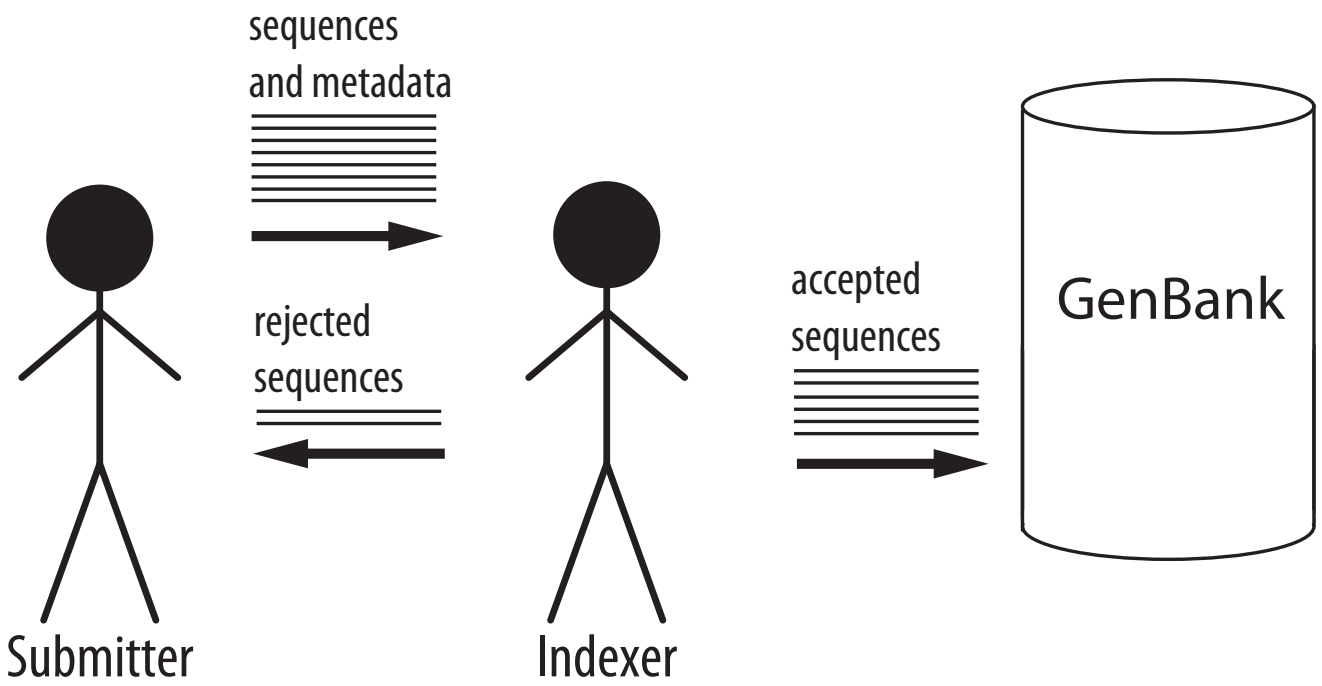
Table 1. Growth of GenBank divisions (nucleotide base-pairs)

Division	Description	Release 227 (August 2018)	Annual increase (%)[a]
MAM	Other mammals	6 214 774 850	60.47%
WGS	Whole genome shotgun data	3 204 855 013 281	42.93%
UNA	Unannotated	296 706	42.25%
PLN	Plants	23 027 832 426	37.21%
BCT	Bacteria	53 541 127 504	36.93%
TSA	Transcriptome shotgun data	225 520 004 678	35.01%
PHG	Phages	463 029 085	34.38%
VRL	Viruses	4 073 816 676	16.99%
PAT	Patent sequences	22 019 723 131	14.57%
VRT	Other vertebrates	10 441 689 546	12.90%
ENV	Environmental samples	5 818 999 756	4.09%
HTC	High-throughput cDNA	721 454 983	3.57%
PRI	Primates	8 262 441 252	2.96%
SYN	Synthetic	1 192 279 390	1.62%
GSS	Genome survey sequences	26 339 143 098	1.40%
EST	Expressed sequence tags	42 988 632 150	0.82%
HTG	High-throughput genomic	27 770 730 435	0.45%
ROD	Rodents	4 534 815 151	0.31%
STS	Sequence tagged sites	640 879 986	0.00%
INV	Invertebrates[b]	8 597 126 159	−50.09%
TOTAL	All GenBank sequences	3 677 023 810 243	39.52%

[a]Measured relative to Release 221 (August 2017).
[b]The decrease in INV data resulted from the suppression of 36 nematode-related genomes. See the release notes for Release 227 for more details (ftp.ncbi.nlm.nih.gov/genbank/release.notes/gb227.release.notes).

Manual NCBI GenBank indexing does not scale

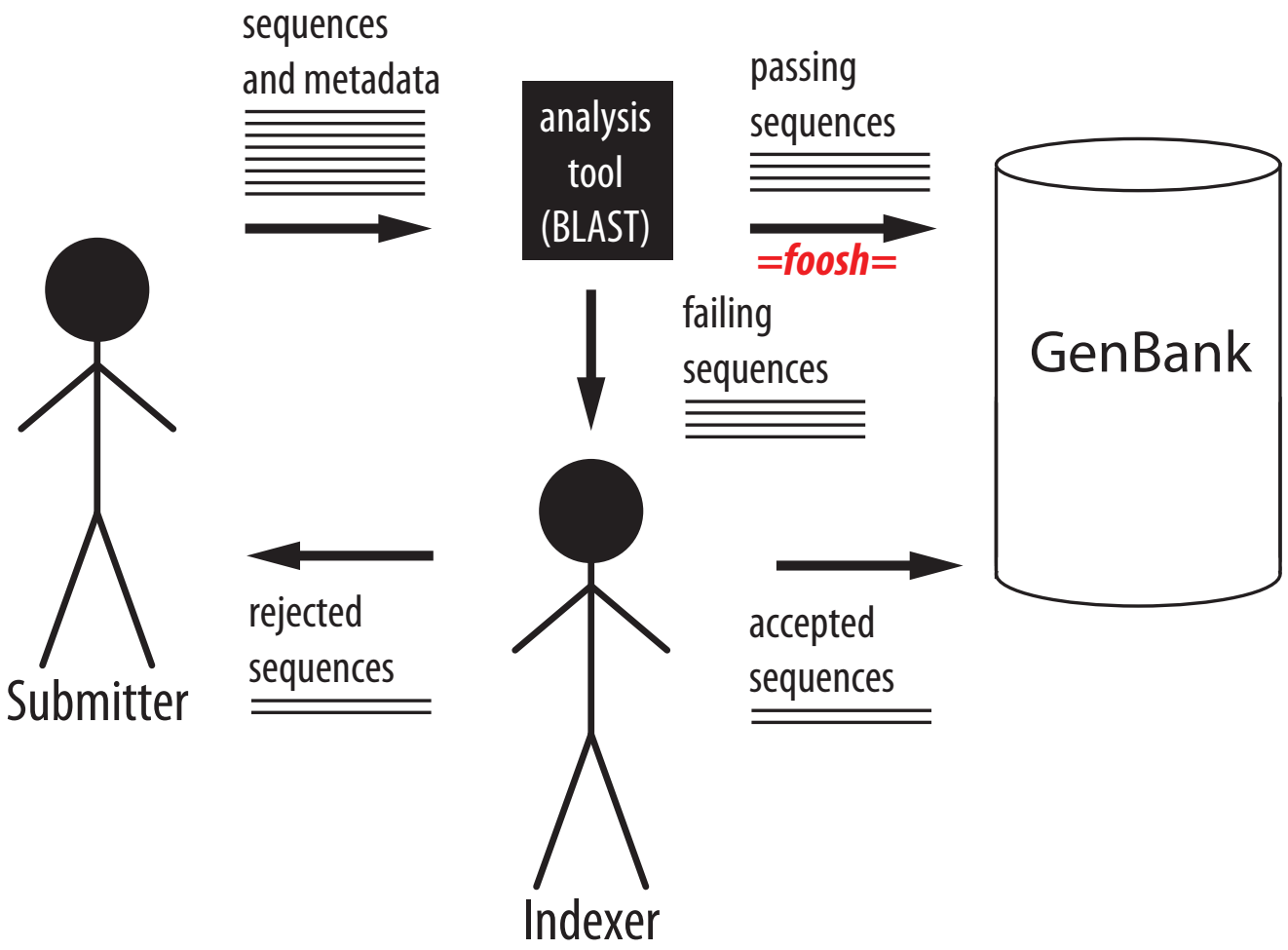


Submissions can be grouped and analyzed based on sequence type

- Many submissions are of *marker genes*, used to characterize environments (microbiome, soil), which are automatically analyzed by BLAST or specialized tools.

marker gene/ sequence type	2018 # seqs	total # seqs
16S rRNA	333,121	8,015,297
COX1	35,517	1,349,957
23S rRNA	74,287	275,014
ITS1	27,279	359,380
ITS2	24,144	184,515
ITS1+ITS2	26,734	445,721
Influenza	74,868	665,464

NCBI GenBank Indexers use BLAST



- Foosh pipelines exist for 16S, 23S, ITS (BLAST-based) and Influenza (FLAN)

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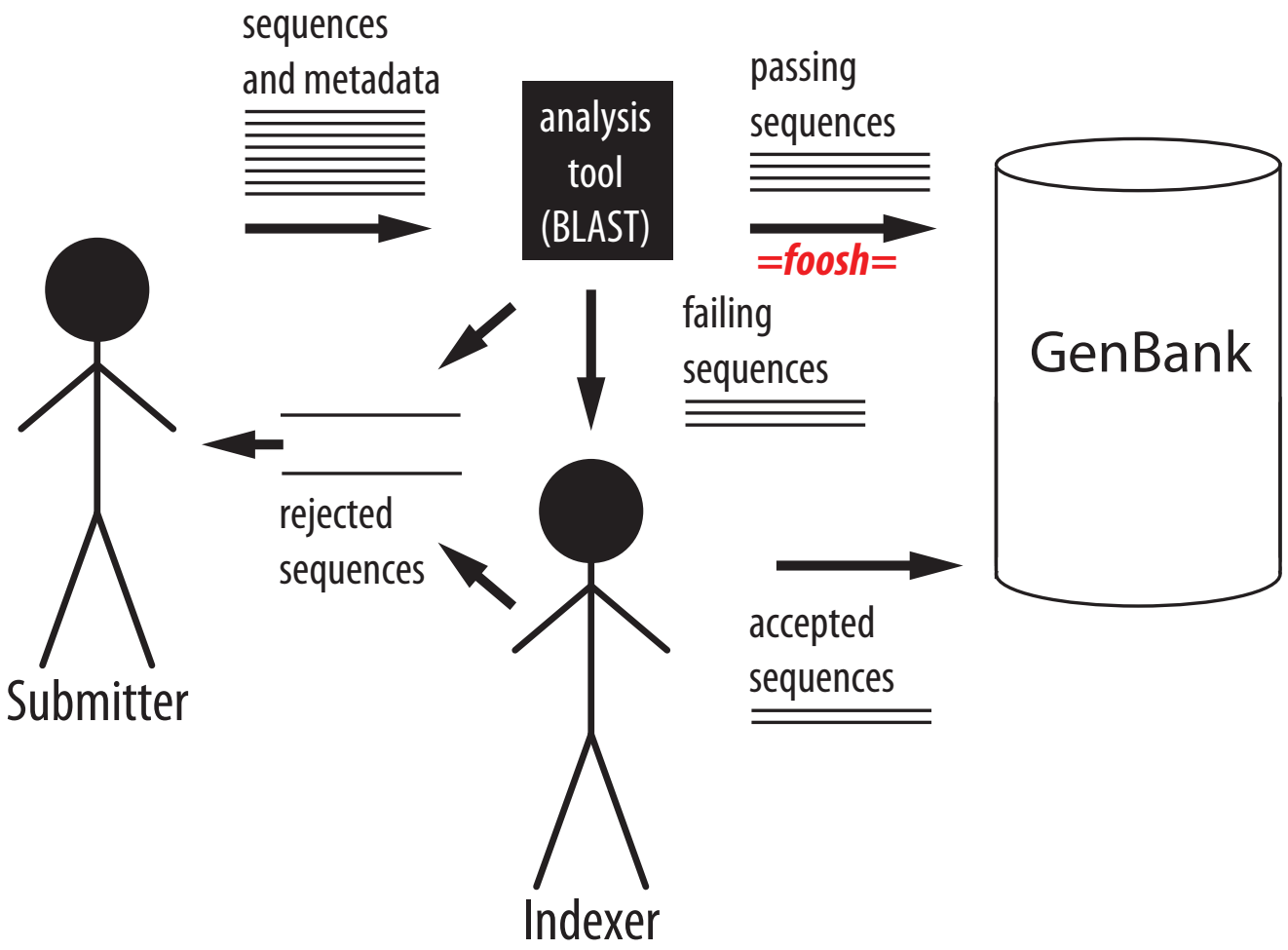


- Foosh pipelines exist for 16S, 23S, ITS (BLAST-based) and Influenza (FLAN)

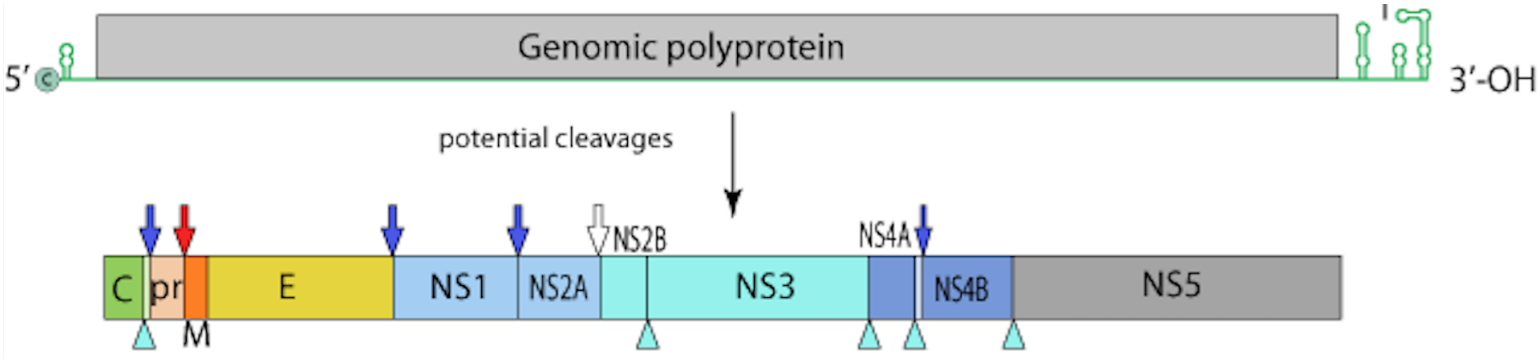
Viruses with highest number of sequences in GenBank*

species	#seqs	family
HIV-1	850,115	*Retroviridae*
Influenza A virus	684,026	*Orthomyxoviridae*
Hepacivirus C	244,533	*Flaviviridae*
Hepatitis B virus	114,306	*Hepadnaviridae*
Influenza B virus	100,373	*Orthomyxoviridae*
Rotavirus A	73,375	*Reoviridae*
SIV	44,374	*Retroviridae*
Norovirus (Norwalk virus)	40,925	*Caliciviridae*
Enterovirus A	31,478	*Picornaviridae*
PRRSV	29,081	*Arteriviridae*
Dengue virus	28,564	*Flaviviridae*
Human orthopneumovirus	24,384	*Pneumoviridae*
Enterovirus B	23,865	*Picornaviridae*
Rabies lyssavirus	23,771	*Rhabdoviridae*
West Nile virus	21,563	*Flaviviridae*
Measles morbillivirus	17,233	*Paramyxoviridae*

*as of October, 2019.

Viral sequences are not systematically or thoroughly annotated

- Genome annotation of the Zika virus:



- Zika's genome encodes a single polyprotein that is cleaved into 14 mature peptides.
- Zika RefSeq annotation (NC_012532) includes CDS and mature peptide annotation.

figure from ViralZone, Hulo et al. NAR. 2011 Jan;39:D576-82

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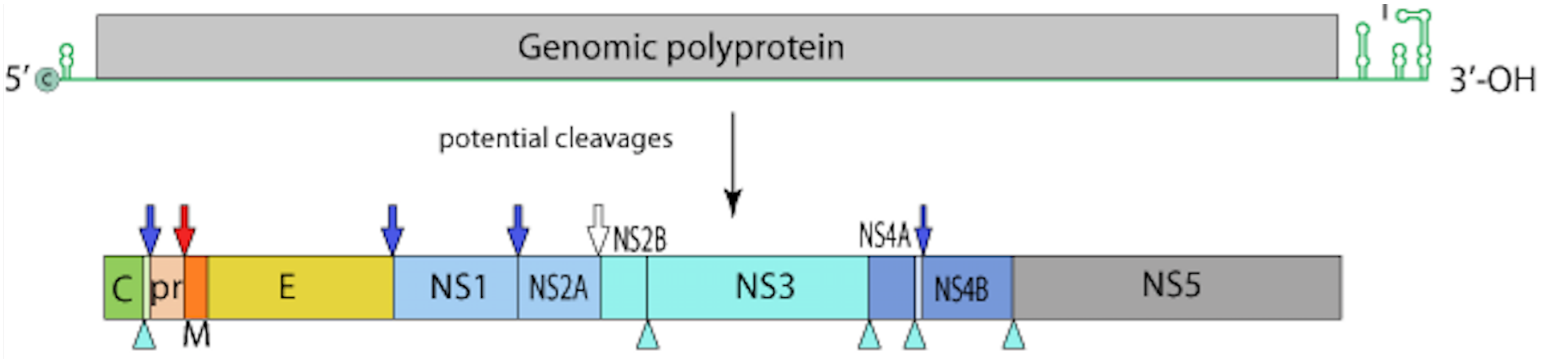

- Zika's genome encodes a single polyprotein that is cleaved into 14 mature peptides.
- Zika RefSeq annotation (NC_012532) includes CDS and mature peptide annotation.
- About 84% of Zika virus sequences have CDS annotation.
- Less than 25% of Zika virus sequences have mature peptide annotation.
- Less than 7% of Dengue virus sequences have mature peptide annotation.
- Less than 2% of Norovirus sequences have mature peptide annotation.

figure from ViralZone, Hulo et al. NAR. 2011 Jan;39:D576-82

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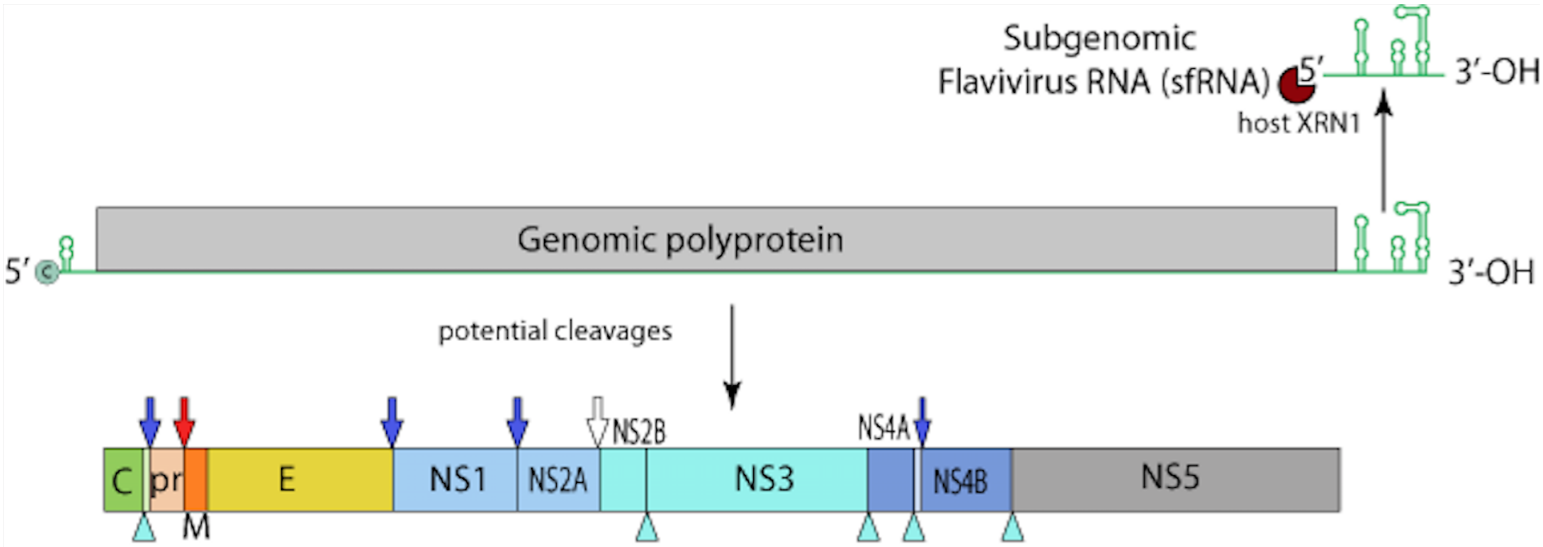


- RNA structures in the 3' UTR halt host exonuclease leading to an accumulation of 300-500nt subgenomic flavivirus RNAs (sfRNAs) are related to pathogenicity.

These RNA structures are not annotated in the Zika genome RefSeq (NC_012532)

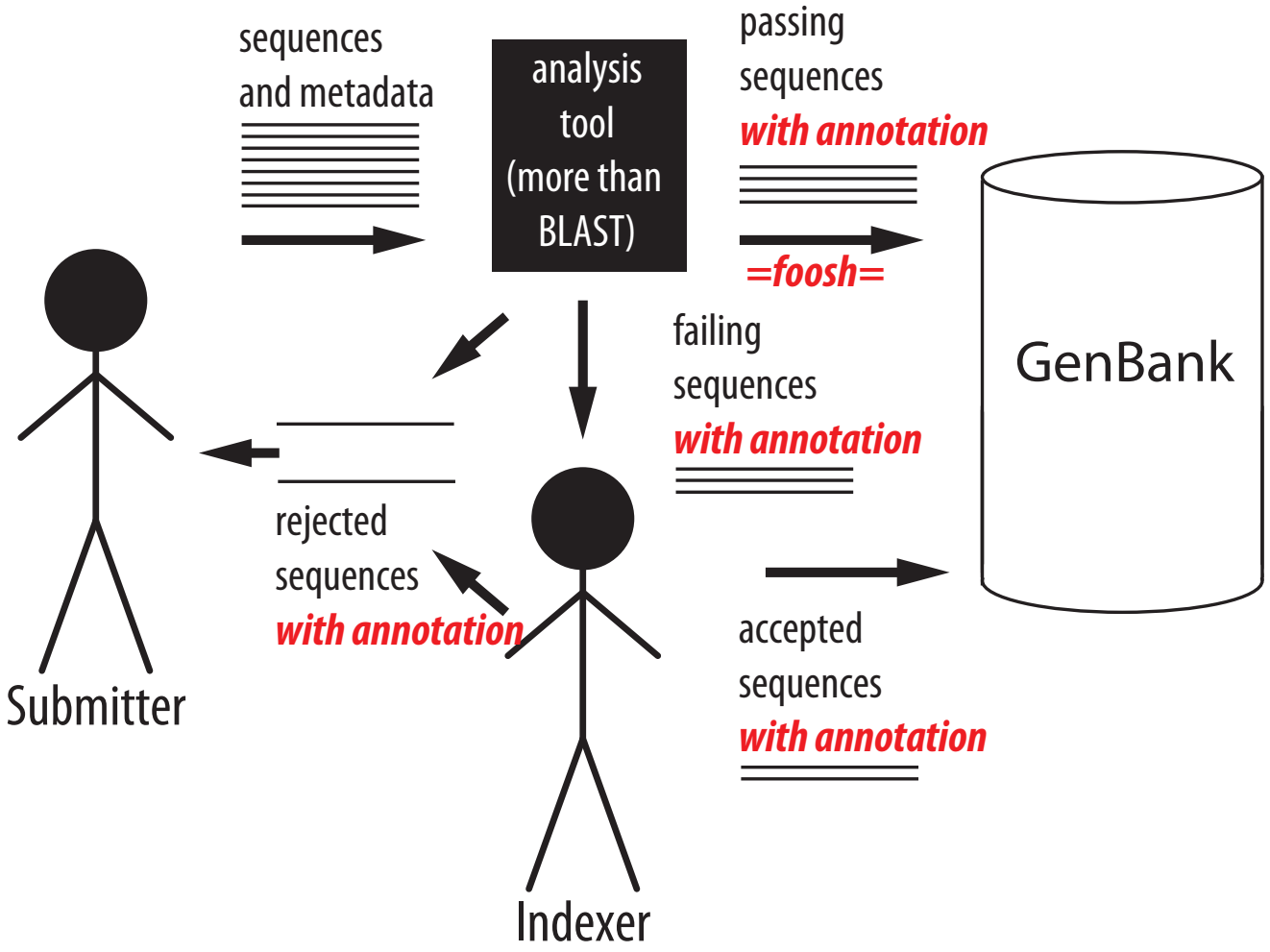
figure from ViralZone, Hulo et al. NAR. 2011 Jan;39:D576-82

Viral sequences are not systematically or thoroughly annotated

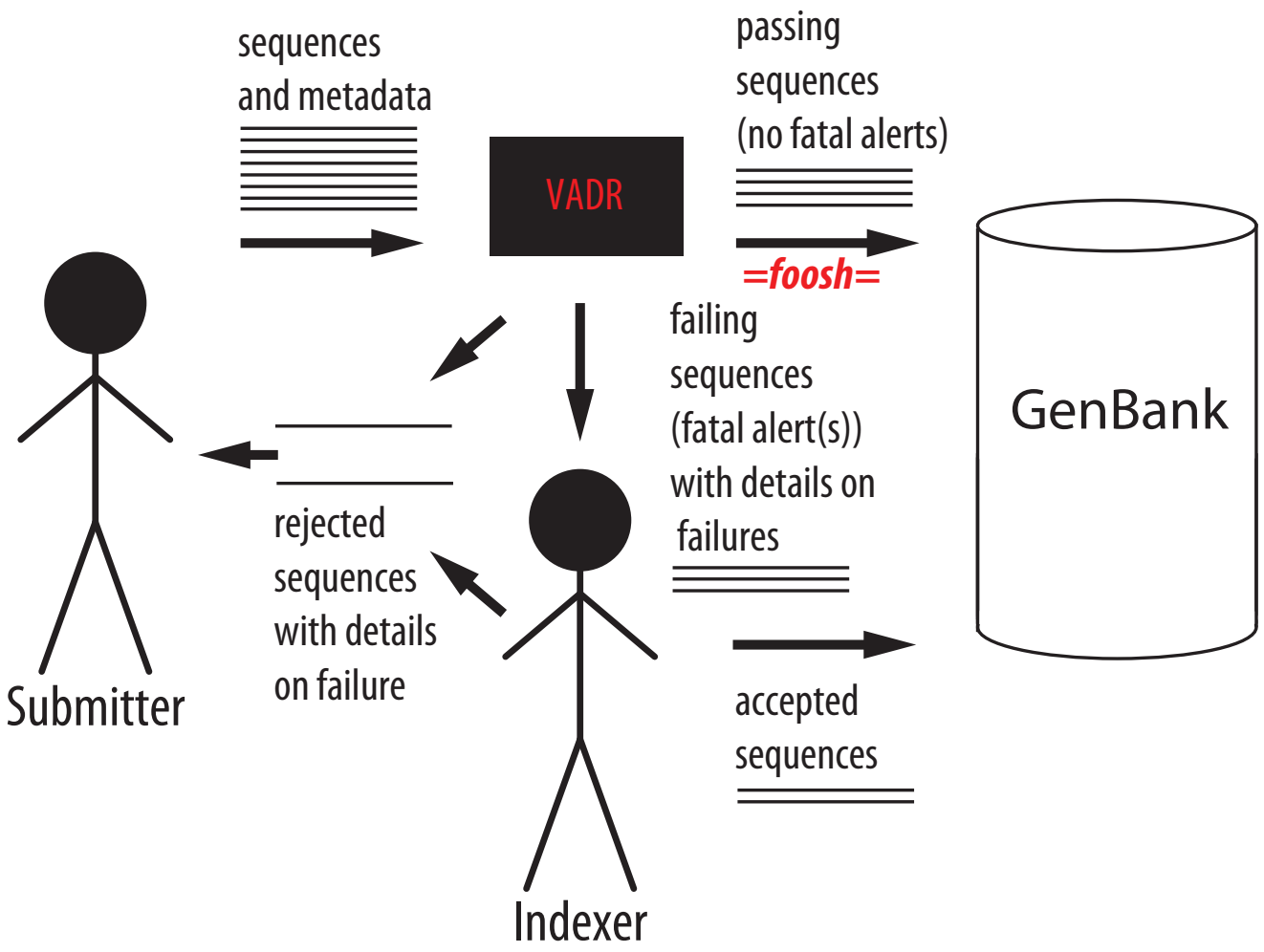
- CDS are not always annotated
- Mature peptides are rarely annotated
- Rfam families are rarely to never annotated in viral genomes (roughly 200 families)

Systematic and complete annotation would benefit viral researchers (facilitate comparative analyses)

Annotation and validation should be coupled



VADR (Viral Annotation DefineR)
uses RefSeqs to validate and annotate viral sequences



- Unexpected characteristics are reported as *alerts* (e.g. early stop codon)
- Some alerts are *fatal* and cause sequences to *fail*

Norovirus and Dengue virus chosen as first viruses for VADR testing

species	#seqs	family
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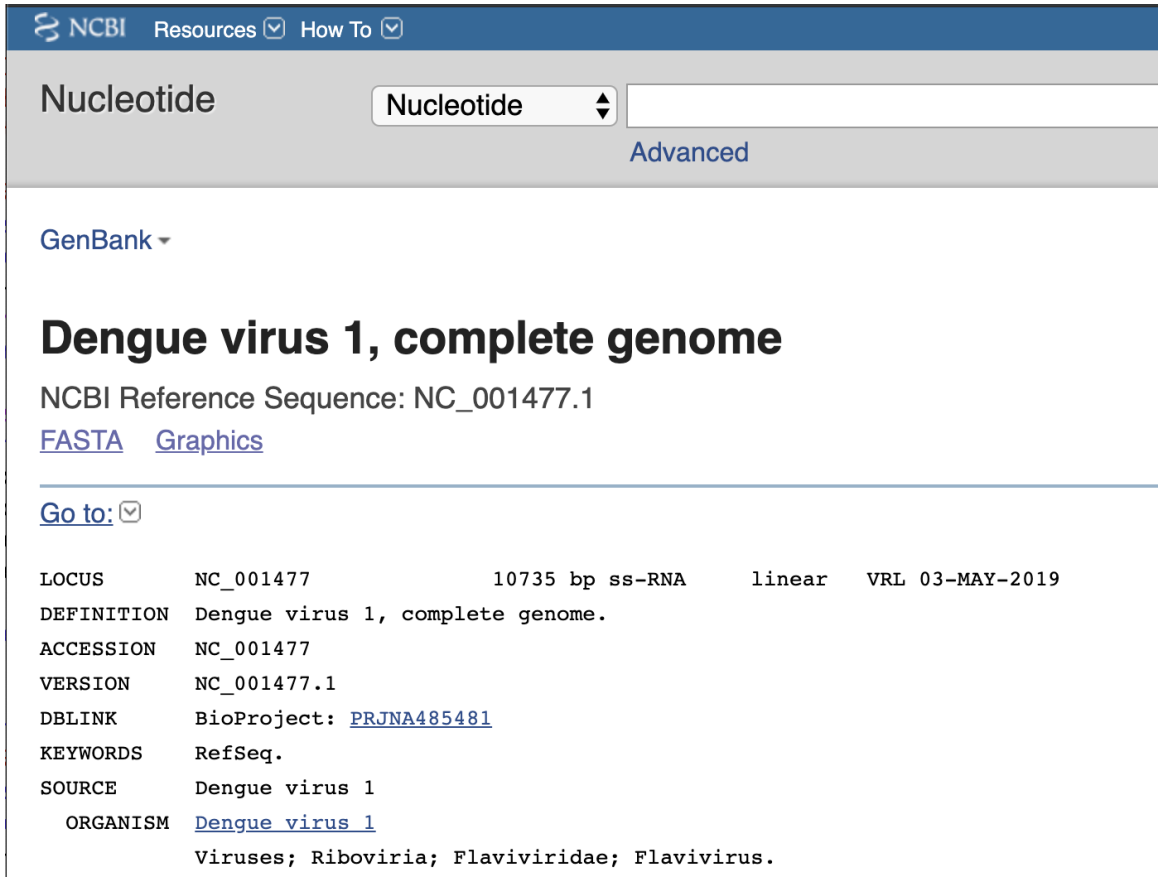
VADR build step (`v-build`) builds a homology model (covariance model (CM)) of a RefSeq and stores feature information



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```
FEATURES             Location/Qualifiers
     source          1..10735
                     /organism="Dengue virus 1"
                     /mol_type="genomic RNA"
                     /db_xref="taxon:11053"
                     /clone="45AZ5"
                     /type="1"
     5'UTR           1..94
     stem_loop       2..69
                     /note="stem-loop A (SLA)"
     regulatory      70..78
                     /regulatory_class="other"
                     /note="oligo U track spacer"
     regulatory      79..94
                     /regulatory_class="promoter"
                     /note="5' upstream AUG region (UAR)"
     stem_loop       79..93
                     /note="stem-loop B (SLB)"
     gene            95..10273
                     /gene="POLY"
                     /locus_tag="DV1_gp1"
                     /gene_synonym="polyprotein gene"
                     /db_xref="GeneID:5075725"
     CDS             95..10273
                     /gene="POLY"
                     /locus_tag="DV1_gp1"
                     /gene_synonym="polyprotein gene"
                     /codon_start=1
                     /product="polyprotein"
                     /protein_id="NP_059433.1"
                     /db_xref="GeneID:5075725"
                     /translation="MNNQRKKTGRPSFNMLKRARNRVSTVSQLAKRFSKGLLSGQGPM
                     KLVMAFIAFLRFLAIPPTAGILARWGSFKKNGAIKVLRGFKKEISNMLNIMNRRKRSV
```

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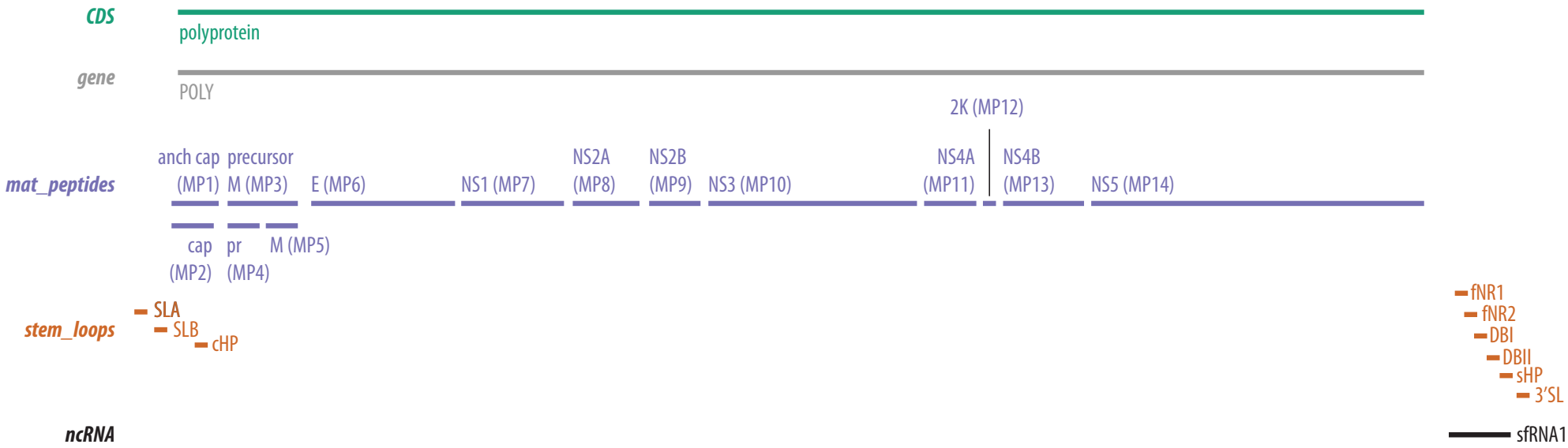
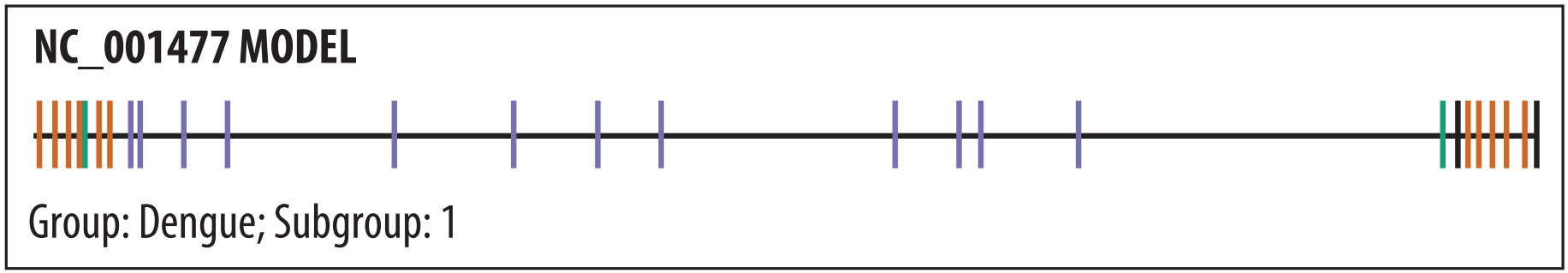
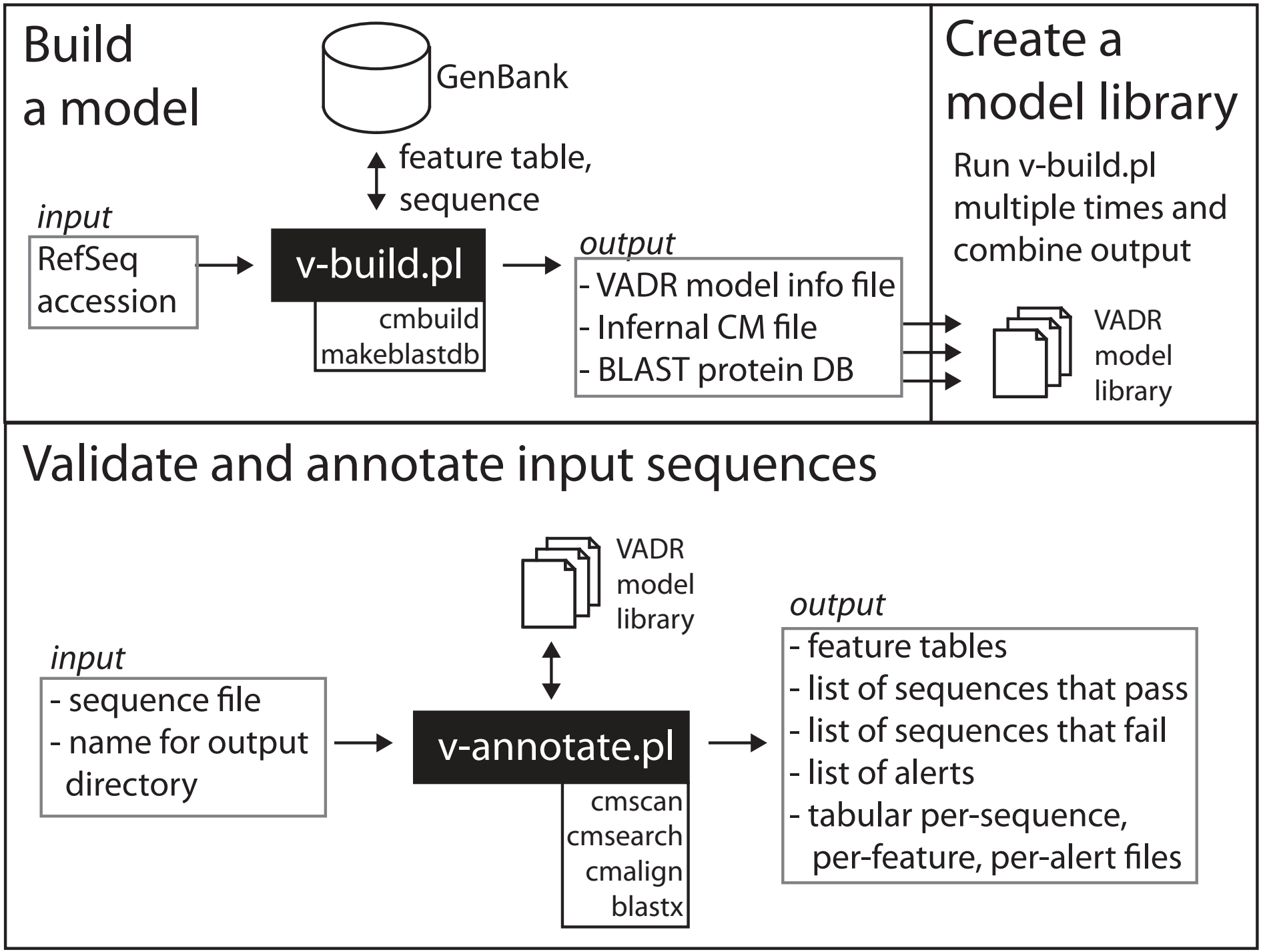
```
mat_peptide     95..436
                /gene="POLY"
                /locus_tag="DV1_gp1"
                /gene_synonym="polyprotein gene"
                /product="anchored capsid protein ancC"
                /protein_id="NP_722457.2"
                /db_xref="VBRC:35735"
mat_peptide     95..394
                /gene="POLY"
                /locus_tag="DV1_gp1"
                /gene_synonym="polyprotein gene"
                /product="capsid protein C"
                /note="added by NCBI staff following more recent
                annotations of this virus sequence"
                /protein_id="NP_722466.2"
                /db_xref="VBRC:67793"
mat_peptide     437..934
                /gene="POLY"
                /locus_tag="DV1_gp1"
                /gene_synonym="polyprotein gene"
                /product="membrane glycoprotein precursor prM"
                /protein_id="NP_733807.2"
mat_peptide     437..709
                /gene="POLY"
                /locus_tag="DV1_gp1"
                /gene_synonym="polyprotein gene"
                /product="protein pr"
                /note="peptide pr"
                /protein_id="YP_009164956.1"
```

VADR build step (`v-build`) builds a homology model (covariance model (CM)) of a RefSeq and stores feature information






Build
a model
GenBank
feature table,
sequence
input
RefSeq
accession
v-build.pl
cmbuild
makeblastdb
output
- VADR model info file
- Infernal CM file
- BLAST protein DB
Create a
model library
Run v-build.pl
multiple times and
combine output
VADR
model
library
Validate and annotate input sequences
VADR
model
library
input
- sequence file
- name for output
directory
v-annotate.pl
cmscan
cmsearch
cmalign
blastx
output
- feature tables
- list of sequences that pass
- list of sequences that fail
- list of alerts
- tabular per-sequence,
per-feature, per-alert files

VADR 1.0 model library

- 38 *Caliciviridae* models:
  - 9 Norovirus models
  - 7 Sapovirus models
  - 4 Vesivirus models
- 156 *Flaviviridae* models:
  - 10 Pegivirus models
  - 8 HCV models
  - 7 Pestivirus models
  - 4 Dengue virus models
  - 2 West Nile virus models
  - 2 Zika virus models

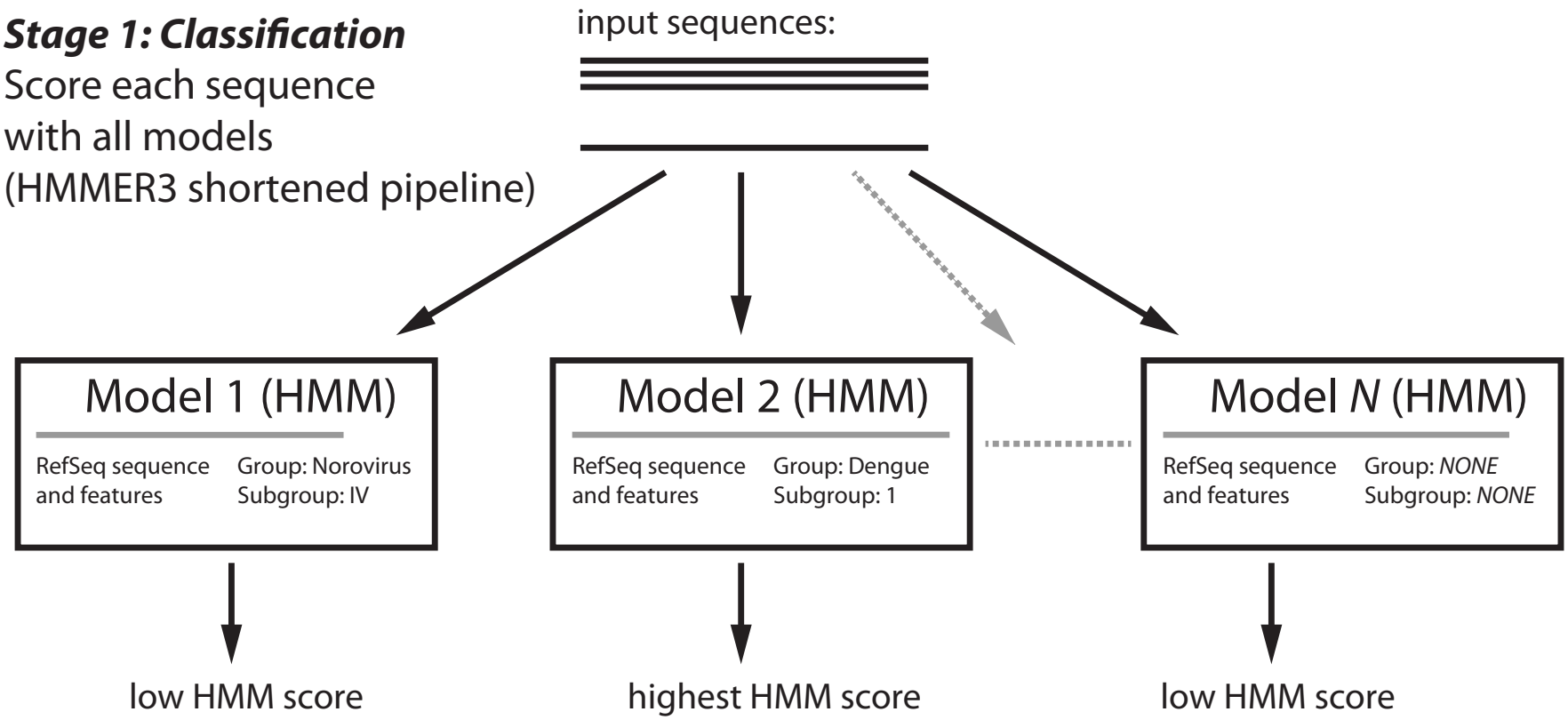
`v-annotate` annotates each sequence using its best-matching model

- For each sequence S:
  1. **Classification**: compare S to all models to find best matching model M
  2. **Coverage determination**: search M against S to find 'hits'
  3. **Alignment**: align S to M and map features from M to S
  4. **Protein validation**: compare predicted CDS in S to proteins from M using BLASTX

Different types of alerts are identified and reported at each stage

Stage 1: Classification

Score each sequence
with all models
(HMMER3 shortened pipeline)



Stage 1: Classification
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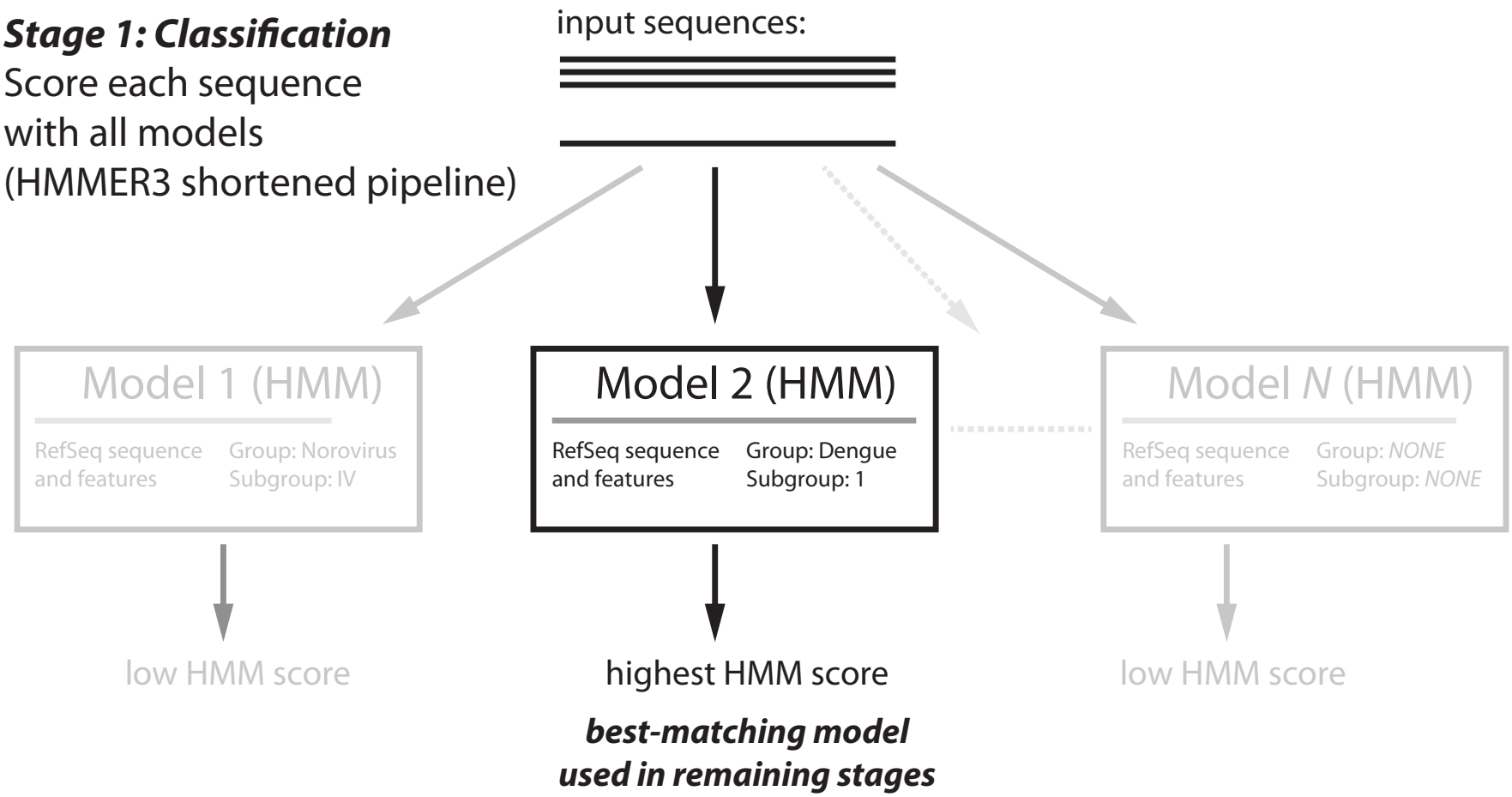
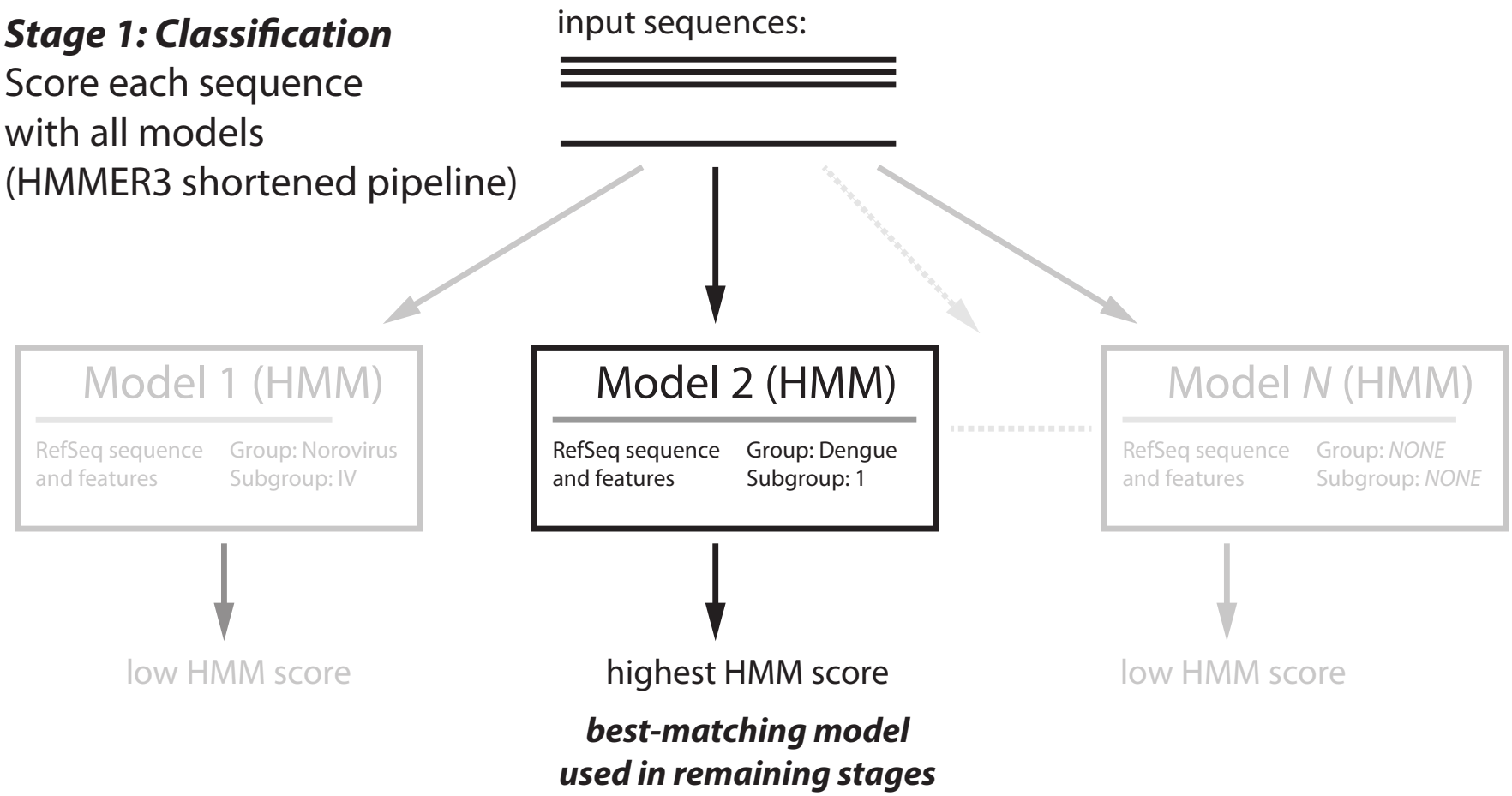
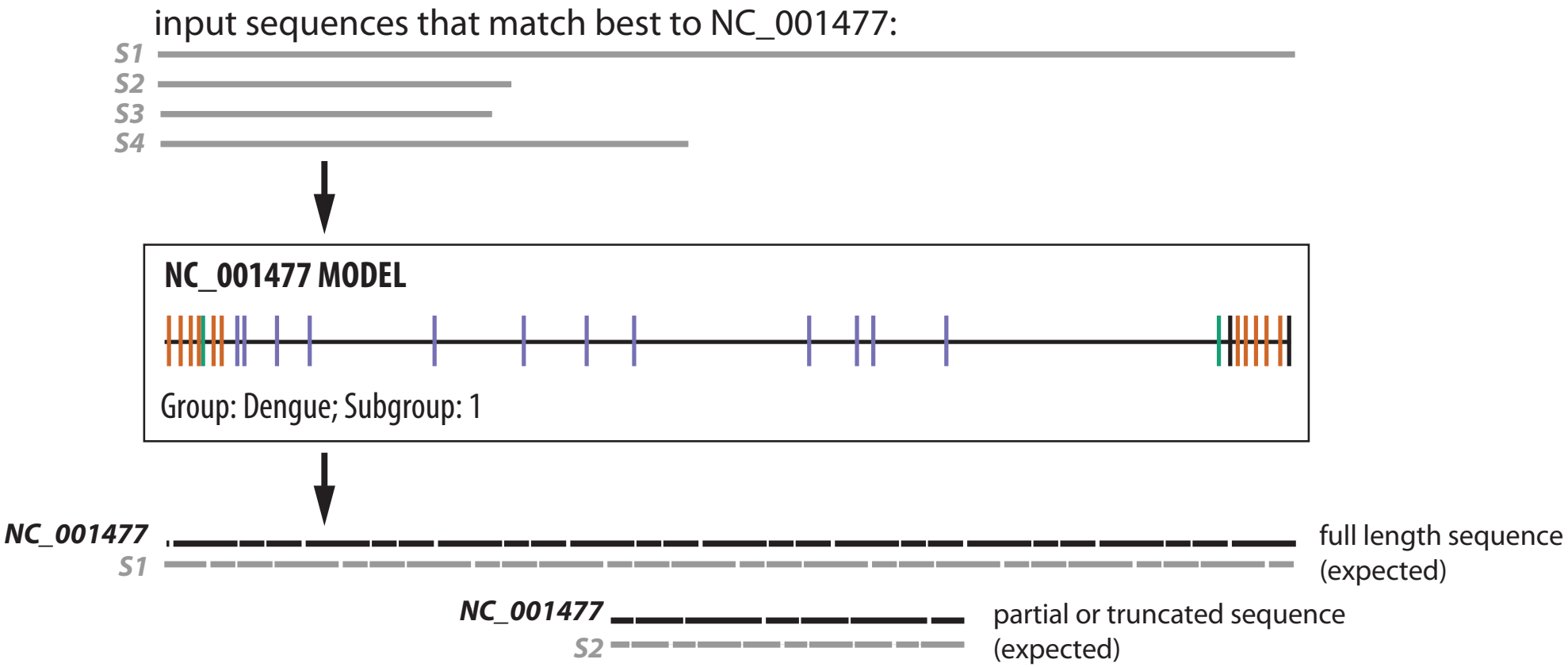




code	S/F	error message	description
Fatal alerts detected in the classification stage			
noannotn*	S	NO_ANNOTATION	no significant similarity detected
revcompl*	S	REVCOMPLEM	sequence appears to be reverse complemented
incsbgrp	S	INCORRECT_SPECIFIED_SUBGROUP	score difference too large between best overall model and best specified subgroup model
incgroup	S	INCORRECT_SPECIFIED_GROUP	score difference too large between best overall model and best specified group model
Non-fatal alerts detected in the classification stage			
qstsbgrp	S	QUESTIONABLE_SPECIFIED_SUBGROUP	best overall model is not from specified subgroup
qstgroup	S	QUESTIONABLE_SPECIFIED_GROUP	best overall model is not from specified group
indfclas	S	INDEFINITE_CLASSIFICATION	low score difference between best overall model and second best model (not in best model's subgroup)
lowscore	S	LOW_SCORE	score to homology model below low threshold

Stage 2: Coverage determination

Search each sequence with best-matching model (HMMER3 full pipeline)



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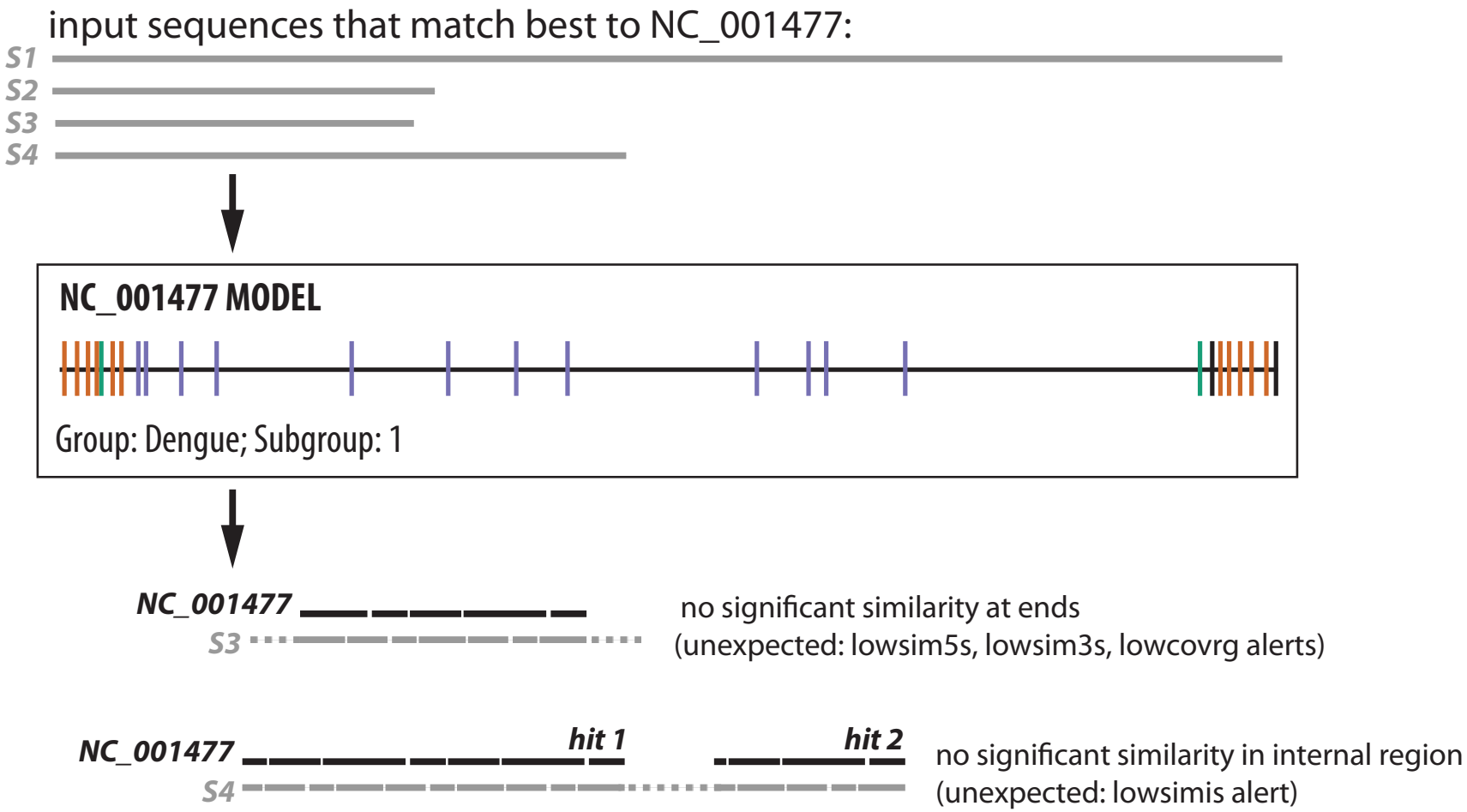
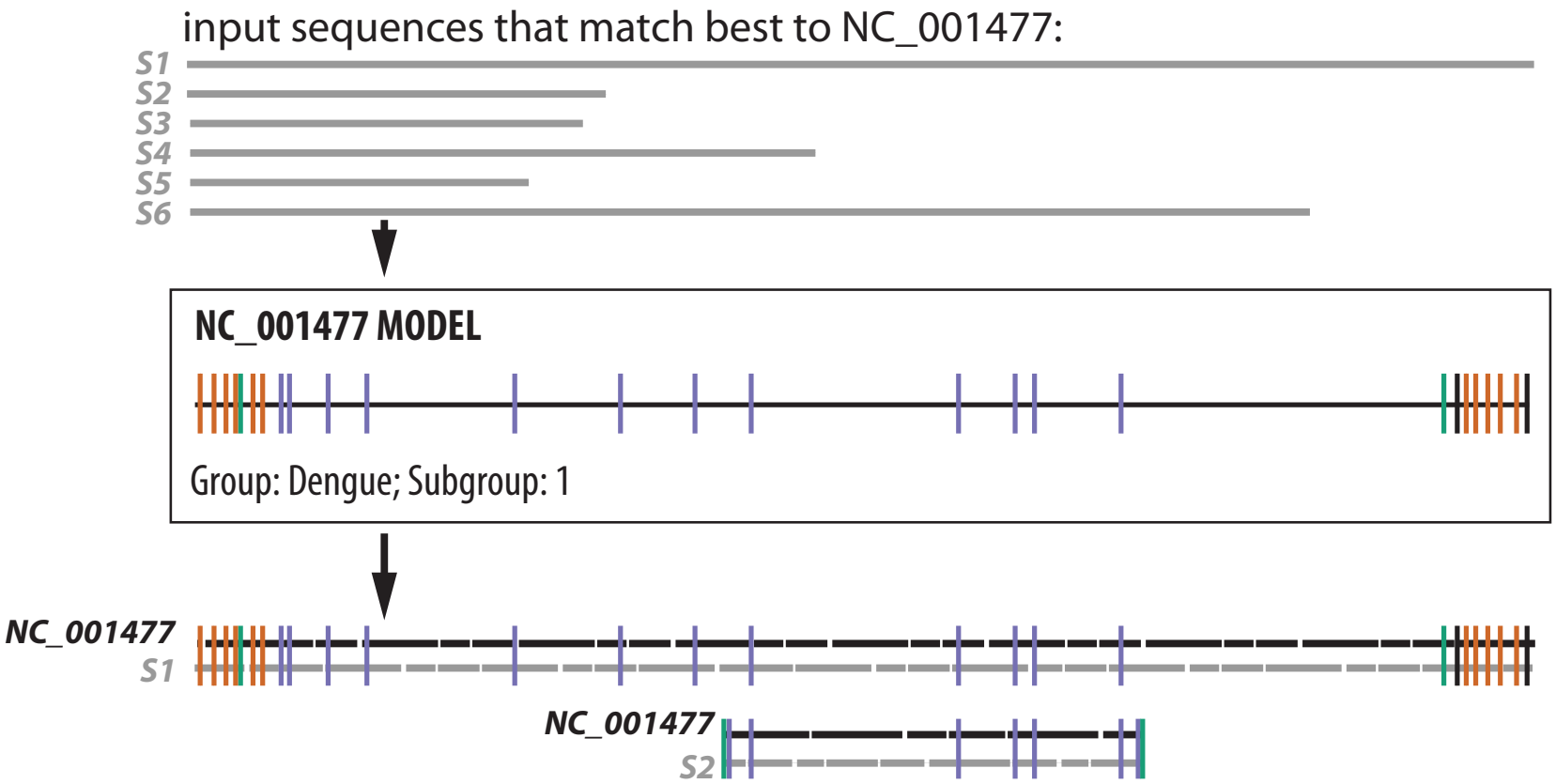


code	S/F	error message	description
Fatal alerts detected in the coverage stage			
lowcovrg	S	LOW_COVERAGE	low sequence fraction with significant similarity to homology model
dupregin	S	DUPLICATE_REGIONS	similarity to a model region occurs more than once
discontn	S	DISCONTINUOUS_SIMILARITY	not all hits are in the same order in the sequence and the homology model
indfstrn	S	INDEFINITE_STRAND	significant similarity detected on both strands
lowsim5s	S	LOW_SIMILARITY_START	significant similarity not detected at 5' end of the sequence
lowsim3s	S	LOW_SIMILARITY_END	significant similarity not detected at 3' end of the sequence
lowsimis	S	LOW_SIMILARITY	internal region without significant similarity
Non-fatal alerts detected in the coverage stage			
biasdseq	S	BIASED_SEQUENCE	high fraction of score attributed to biased sequence composition

Stage 3: Alignment and feature mapping

Align each sequence to its best-matching model (Infernal's cmalign)



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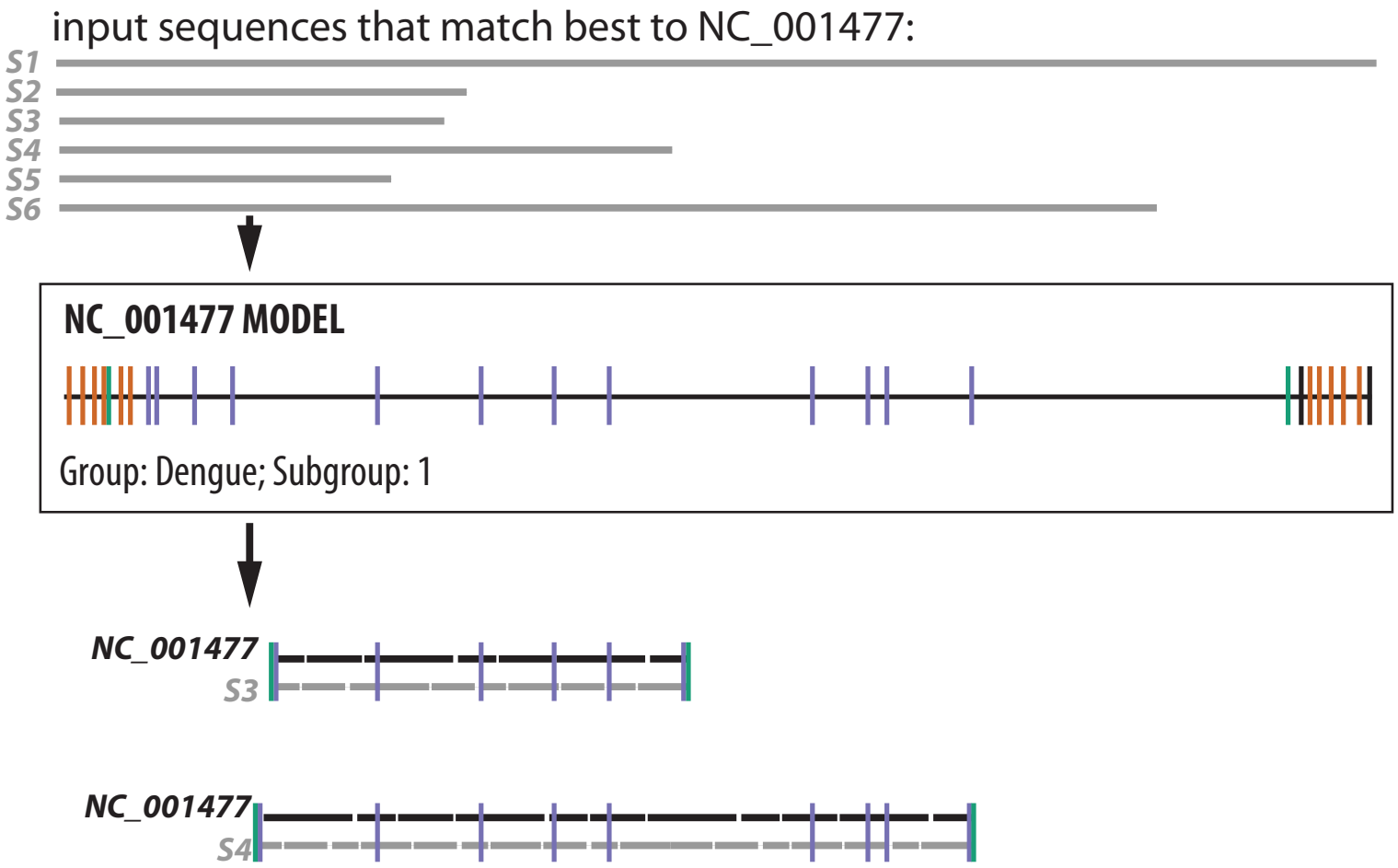

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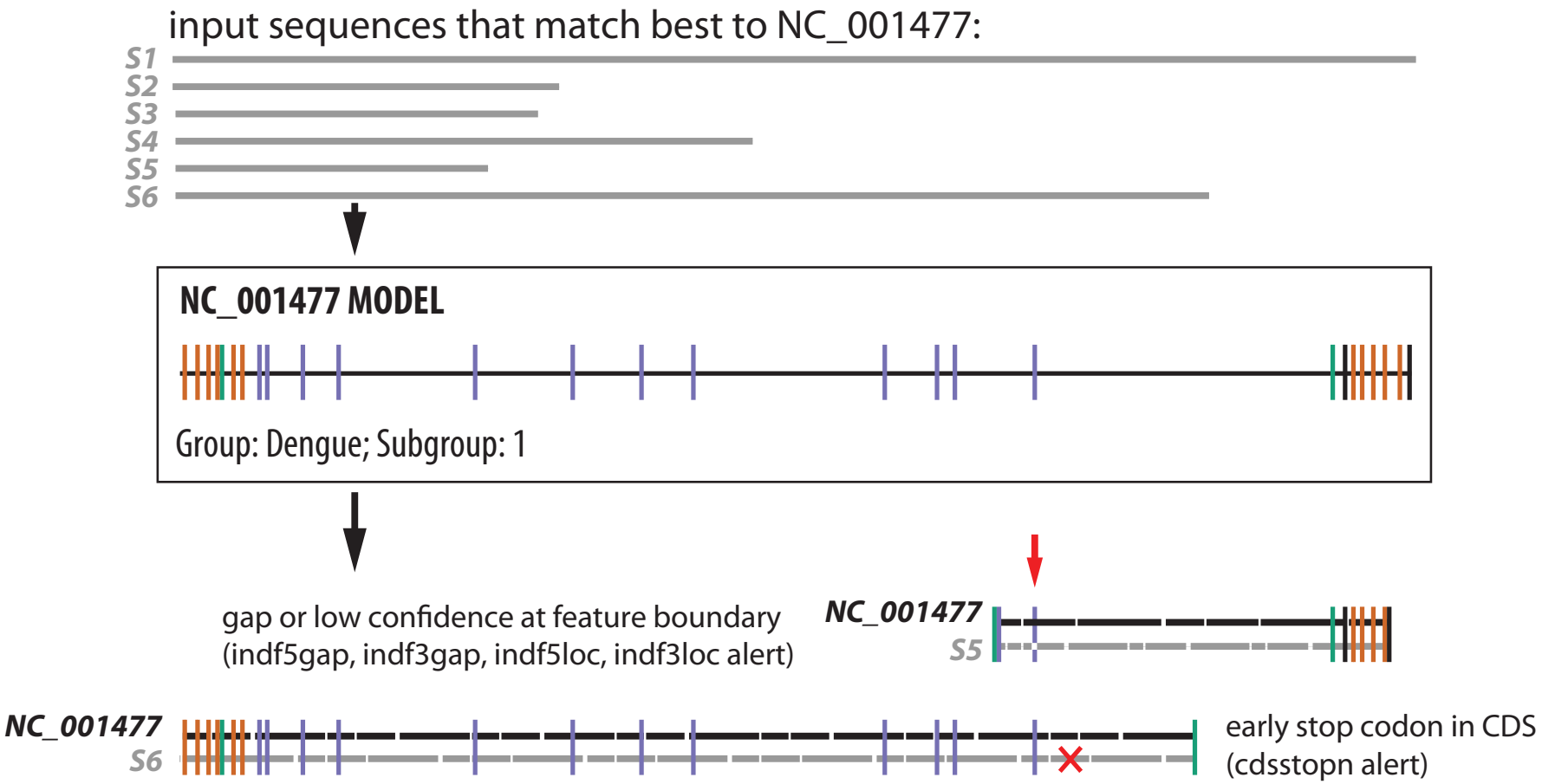


Stage 3: Alignment and feature mapping

Align each sequence to its best-matching model (Infernal's cmalign)

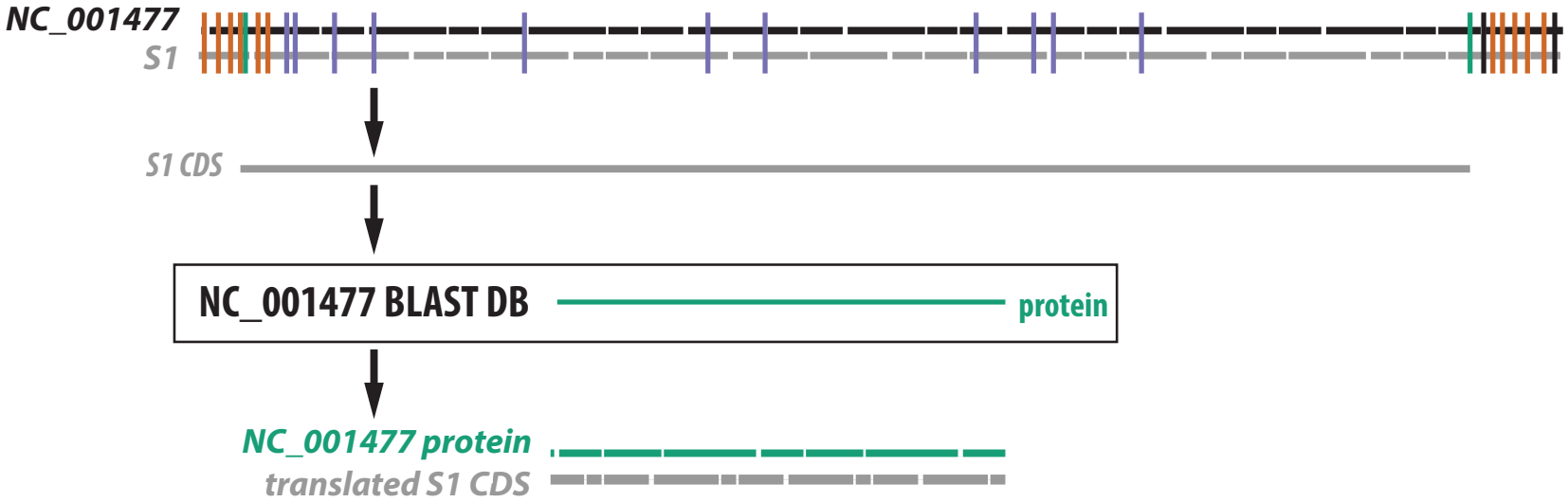
gap or low confidence at feature boundary
(indf5gap, indf3gap, indf5loc, indf3loc alert)

early stop codon in CDS
(cdsstopn alert)

code	S/F	error message	description
Fatal alerts detected in the annotation stage			
unexdivg*	S	UNEXPECTED_DIVERGENCE	sequence is too divergent to confidently assign nucleotide-based annotation
noftrann*	S	NO_FEATURES_ANNOTATED	sequence similarity to homology model does not overlap with any features
mutstart	F	MUTATION_AT_START	expected start codon could not be identified
mutendcd	F	MUTATION_AT_END	expected stop codon could not be identified, predicted CDS stop by homology is invalid
mutendns	F	MUTATION_AT_END	expected stop codon could not be identified, no in-frame stop codon exists 3' of predicted valid start codon
mutendex	F	MUTATION_AT_END	expected stop codon could not be identified, first in-frame stop codon exists 3' of predicted stop position
unexleng	F	UNEXPECTED_LENGTH	length of complete coding (CDS or mat_peptide) feature is not a multiple of 3
cdsstopn	F	CDS_HAS_STOP_CODON	in-frame stop codon exists 5' of stop position predicted by homology to reference
peptrans	F	PEPTIDE_TRANSLATION_PROBLEM	mat_peptide may not be translated because its parent CDS has a problem
pepadjcy	F	PEPTIDE_ADJACENCY_PROBLEM	predictions of two mat_peptides expected to be adjacent are not adjacent
indfantn	F	INDEFINITE_ANNOTATION	nucleotide-based search identifies CDS not identified in protein-based search
indf5gap	F	INDEFINITE_ANNOTATION_START	alignment to homology model is a gap at 5' boundary
indf5loc	F	INDEFINITE_ANNOTATION_START	alignment to homology model has low confidence at 5' boundary
indf3gap	F	INDEFINITE_ANNOTATION_END	alignment to homology model is a gap at 3' boundary
indf3loc	F	INDEFINITE_ANNOTATION_END	alignment to homology model has low confidence at 3' boundary
lowsim5f	F	LOW_FEATURE_SIMILARITY_START	region within annotated feature at 5' end of sequence lacks significant similarity
lowsim3f	F	LOW_FEATURE_SIMILARITY_END	region within annotated feature at 3' end of sequence lacks significant similarity
lowsimif	F	LOW_FEATURE_SIMILARITY	region within annotated feature lacks significant similarity

Stage 4: Protein validation (Alejandro Schäffer)

Compare each predicted CDS to model (RefSeq) proteins with BLASTX



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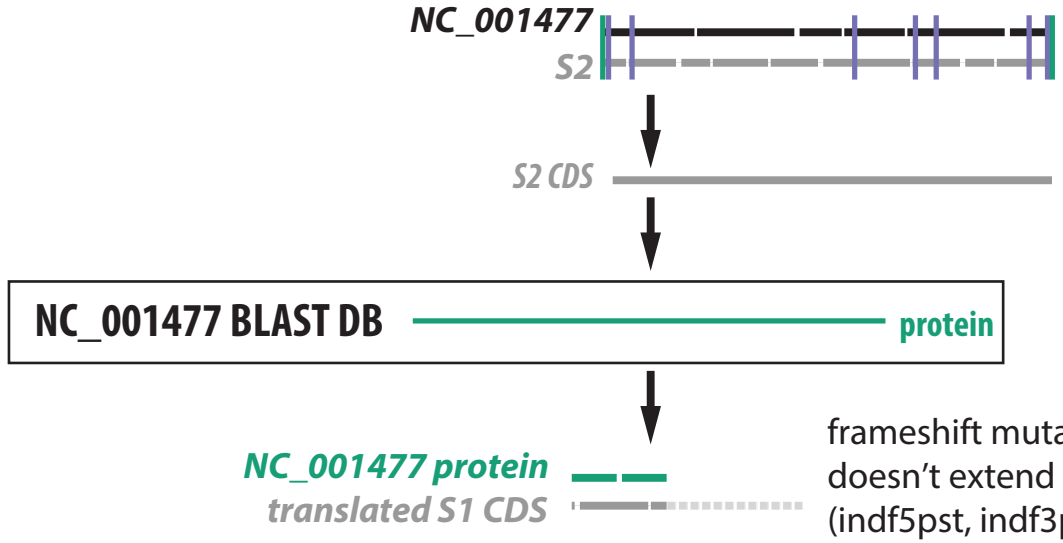

frameshift mutation; BLASTX alignment doesn't extend full length of CDS prediction (indf5pst, indf3pst alerts)

code	S/F	error message	description
Fatal alerts detected in the protein validation stage			
cdsstopp	F	CDS_HAS_STOP_CODON	stop codon in protein-based alignment
indfantp	F	INDEFINITE_ANNOTATION	protein-based search identifies CDS not identified in nucleotide-based search
indf5plg	F	INDEFINITE_ANNOTATION_START	protein-based alignment extends past nucleotide-based alignment at 5' end
indf5pst	F	INDEFINITE_ANNOTATION_START	protein-based alignment does not extend close enough to nucleotide -based alignment 5' endpoint
indf3plg	F	INDEFINITE_ANNOTATION_END	protein-based alignment extends past nucleotide-based alignment at 3' end
indf3pst	F	INDEFINITE_ANNOTATION_END	protein-based alignment does not extend close enough to nucleotide -based alignment 3' endpoint
indfstrp	F	INDEFINITE_STRAND	strand mismatch between protein-based and nucleotide-based predictions
insertnp	F	INSERTION_OF_NT	too large of an insertion in protein-based alignment
deletinp	F	DELETION_OF_NT	too large of a deletion in protein-based alignment

SOFTWARE

VADR: validation and annotation of virus sequence submissions to GenBank

Alejandro A Schäffer[1,2], Eneida L Hatcher[2], Linda Yankie[2], Lara Shonkwiler[2,3], J Rodney Brister[2], Ilene Karsch-Mizrachi[2] and Eric P Nawrocki[2*]

- compared VADR to VAPiD and VIGOR programs on Norovirus and Dengue virus
- VADR caught all the problems detected by VAPiD and VIGOR plus additional problems
- 2809 of 3143 norovirus sequences (89%) tested with VADR passed (could foosh)
- 1602 of 1702 Dengue sequences (94%) tested with VADR passed (could foosh)

Limitations

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- model (RefSeq) must be 'representative'
  - divergent sequences, regions, introns, gene order are problematic

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- slow
  - Norovirus complete: 30 seconds/sequence (8X/6X slower than VAPiD/VIGOR)
  - Dengue complete: 90 seconds/sequence (20X slower than VAPiD)
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- 190 CPU hours for all Norovirus and Dengue seqs (1 day on 8 core machine)

VADR was designed to be flexible

- Any conserved sequence region $<$ 20Kb can be modeled
- VADR models are more powerful as profiles built from *alignments* (RefAlign?)
- Currently in testing for COX1:
  - Started with 9000 vetted COX1 protein sequences (Susan Schafer)
  - Split based on taxonomy and aligned with MUSCLE
  - Derived 43 *alignment-based* models (e.g. *Porifera*, *Amphibia*) covering 5 genetic codes
  - Classification stage compares against 43 models
  - Protein validation stage compares against 9000 proteins

Future directions

- alignment-based models for viruses (testing with HCV)
- profile-based protein validation to replace BLASTX
- extend to more viruses and genes, including ribosomal RNAs and possibly ITS sequences

Acknowledgements

NCBI - viral annotation
Alejandro Schäffer
Rodney Brister
Ilene Mizrachi
Eneida Hatcher
Linda Yankie
Vincent Calhoun
Alex Kotliarov
Susan Schafer
Lara Shonkwiler
Sophia Hu

NCBI - leadership
David Landsman
Kim Pruitt
Jim Ostell
David Lipman

NLM - leadership
Patti Brennan
Jerry Sheehan

HMMER
Sean Eddy
Travis Wheeler

VADR results on all Norovirus and Dengue sequences

dataset	# seqs	# pass	# fail	fraction pass
Norovirus complete	1,384	1,157	227	0.836
Dengue complete	4,580	4,171	409	0.911
Norovirus partial	32,190	29,488	2,702	0.916
Dengue partial	20,973	17,276	3,697	0.824

alert code	error message	NC 1384 seqs ct(seqs)	NP 32190 seqs ct(seqs)	DC 4580 seqs ct(seqs)	DP 20973 seqs ct(seqs)	total 59127 seqs ct(seqs)
peptrans	PEPTIDE_TRANSLATION_PROBLEM	516(86)	716(535)	1330(95)	4051(1065)	6613(1781)
noannotn	NO_ANNOTATION	-	512(512)	5(5)	2236(2236)	2753(2753)
indf3pst	INDEFINITE_ANNOTATION_END	82(70)	1059(1029)	56(56)	600(593)	1797(1748)
indf5pst	INDEFINITE_ANNOTATION_START	59(57)	940(876)	16(16)	660(574)	1675(1523)
indf3loc	INDEFINITE_ANNOTATION_END	85(48)	185(90)	206(98)	293(136)	769(372)
incgroup	INCORRECT_SPECIFIED_GROUP	19(19)	302(302)	30(30)	286(286)	637(637)
indf5loc	INDEFINITE_ANNOTATION_START	19(15)	66(35)	222(135)	286(144)	593(329)
lowcovrg	LOW_COVERAGE	3(3)	217(217)	60(60)	279(279)	559(559)
unexleng	UNEXPECTED_LENGTH	42(34)	66(55)	105(49)	318(182)	531(320)
indf5gap	INDEFINITE_ANNOTATION_START	6(3)	23(12)	117(100)	220(127)	366(242)
indf3gap	INDEFINITE_ANNOTATION_END	4(2)	83(71)	15(14)	237(133)	339(220)
lowsim3f	LOW_FEATURE_SIMILARITY_END	-	-	272(88)	20(9)	292(97)
cdsstopp	CDS_HAS_STOP_CODON	7(5)	112(111)	15(15)	153(153)	287(284)
revcompl	REVCOMPLEM	3(3)	85(85)	35(35)	120(120)	243(243)
cdsstopn	CDS_HAS_STOP_CODON	96(93)	72(71)	58(58)	5(4)	231(226)
insertnp	INSERTION_OF_NT	50(43)	151(138)	-	2(2)	203(183)
lowsim5f	LOW_FEATURE_SIMILARITY_START	-	-	101(101)	79(39)	180(140)
lowsim3s	LOW_SIMILARITY_END	61(61)	80(80)	2(2)	5(5)	148(148)
mutstart	MUTATION_AT_START	13(11)	58(58)	8(8)	35(27)	114(104)
mutendcd	MUTATION_AT_END	52(50)	47(46)	6(6)	5(4)	110(106)
discontn	DISCONTINUOUS_SIMILARITY	-	8(8)	25(25)	35(35)	68(68)
dupregin	DUPLICATE_REGIONS	-	6(6)	33(33)	25(25)	64(64)
indfstrn	INDEFINITE_STRAND	1(1)	4(4)	56(56)	2(2)	63(63)
deletinp	DELETION_OF_NT	22(20)	26(25)	-	12(6)	60(51)
lowsimif	LOW_FEATURE_SIMILARITY	-	-	29(14)	18(9)	47(23)
indf3plg	INDEFINITE_ANNOTATION_END	1(1)	40(40)	-	2(2)	43(43)
indfantn	INDEFINITE_ANNOTATION	1(1)	23(23)	-	18(17)	42(41)
lowsim5s	LOW_SIMILARITY_START	12(12)	-	6(6)	20(20)	38(38)
noftrann	NO_FEATURES_ANNOTATED	-	1(1)	-	26(26)	27(27)
indf5plg	INDEFINITE_ANNOTATION_START	-	10(10)	-	-	10(10)
indfantp	INDEFINITE_ANNOTATION	-	3(3)	-	6(6)	9(9)
pepadjcy	PEPTIDE_ADJACENCY_PROBLEM	-	3(3)	-	6(6)	9(9)
mutendex	MUTATION_AT_END	2(2)	5(5)	1(1)	-	8(8)
mutendns	MUTATION_AT_END	1(1)	5(5)	-	-	6(6)