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Supplementary Materials for

Transient RNA structures cause aberrant influenza virus replication and innate immune activation

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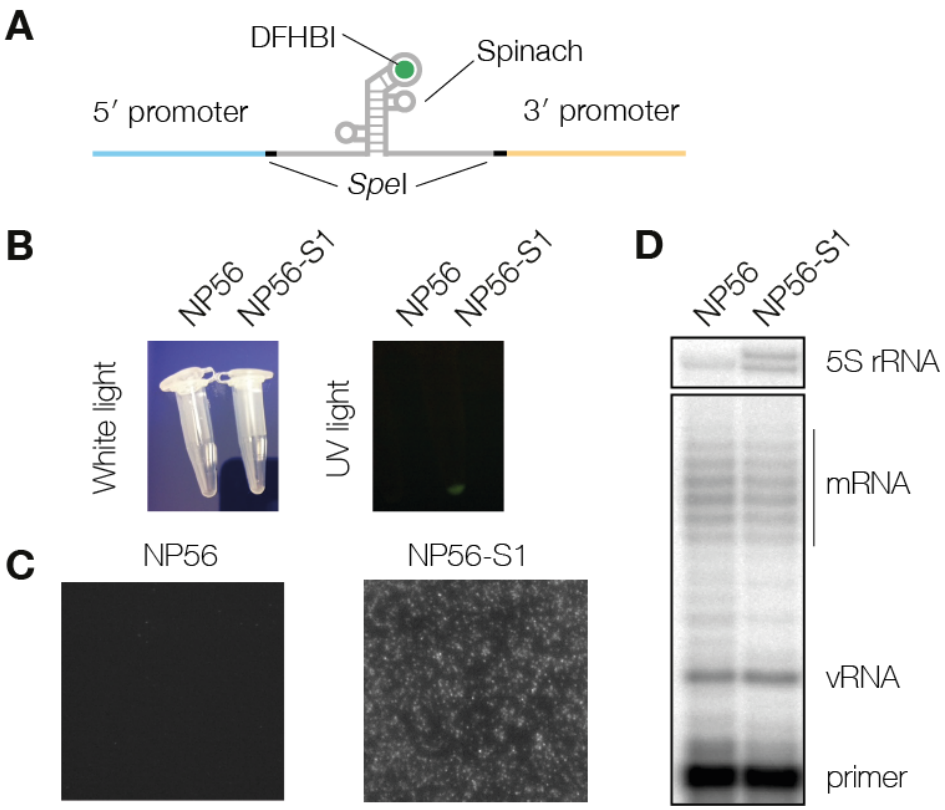

Figure S1. Effect of a single spinach aptamer on mvRNA replication. A) To rule out that a single RNA structure upstream of the RNA polymerase reduces activity, we engineered the NP56 mvRNA template and inserted the RNA aptamer Spinach, creating NP56-S1. B) A Spinach-containing mvRNAs generated in vitro demonstrated fluorescence after addition of DFHBI relative to the 56-nt control mvRNA, confirming that the aptamer folds properly in the context of the IAV promoter sequence. C) TIRF microscopy image of a 56-nt mvRNA or Spinach-containing mvRNA generated in vitro in the presence of DFHBI. D) After transfection of plasmids encoding the Spinach-containing mvRNA NP56-S1 into HEK293T cells and analysis of the RNA produced using primer extension, no difference in replication was observed relative to our NP56 control mvRNA.

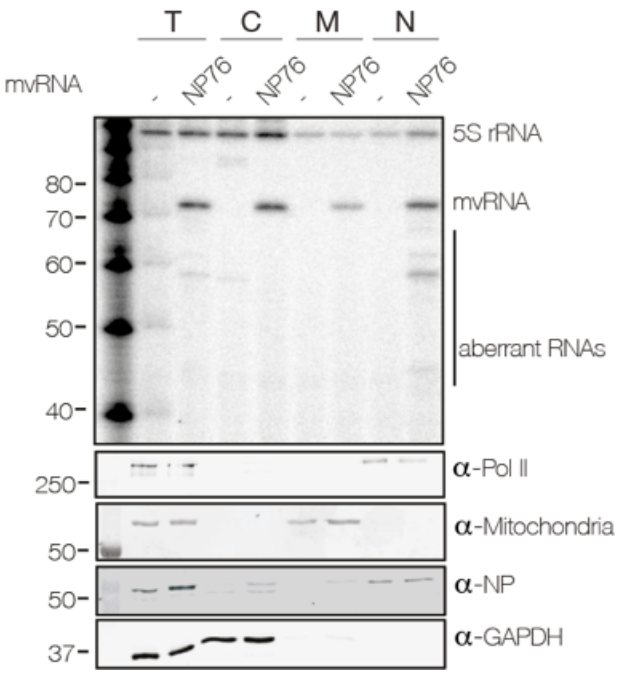

Figure S2. Fractionation of HEK 293T cells transfected with IAV RNA polymerase and a 76 nt-long segment 5-derived mvRNA. Top panel shows primer extension analysis of RNA extracted from fractions. Bottom four panels show western blot analysis. For each lane, comparable amounts were loaded.

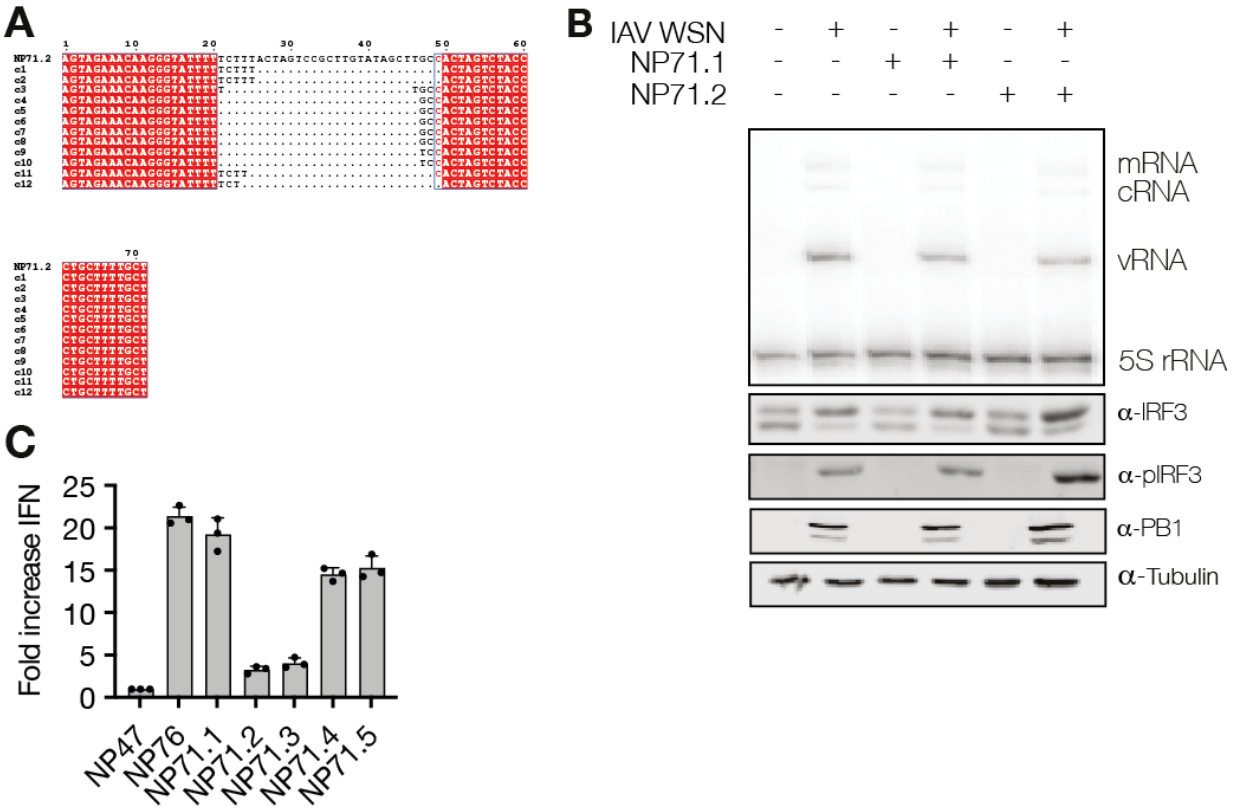

Figure S3. Aberrant RNA sequences, effect of exogenous mvRNAs on infection, and retransfection of total RNA. A) Alignment of aberrant RNA products isolated from in vitro replication assays. B) IFN-β promoter activity induced by the retransfection of total RNA isolated from HEK 293T cells transfected with plasmids expressing segment 5-derived mvRNAs and the viral RNA polymerase subunits. Luciferase signal was normalized to the NP47 mvRNA, which does not trigger innate immune responses. E) Induction of IRF3 phosphorylation by pre-transfected, exogenous mvRNAs during infection with IAV A/WSN/33 (H1N1). mvRNA amplification could not be detected by primer extension, likely because exogenous templates poorly compete with endogenous viral RNAs for replication.

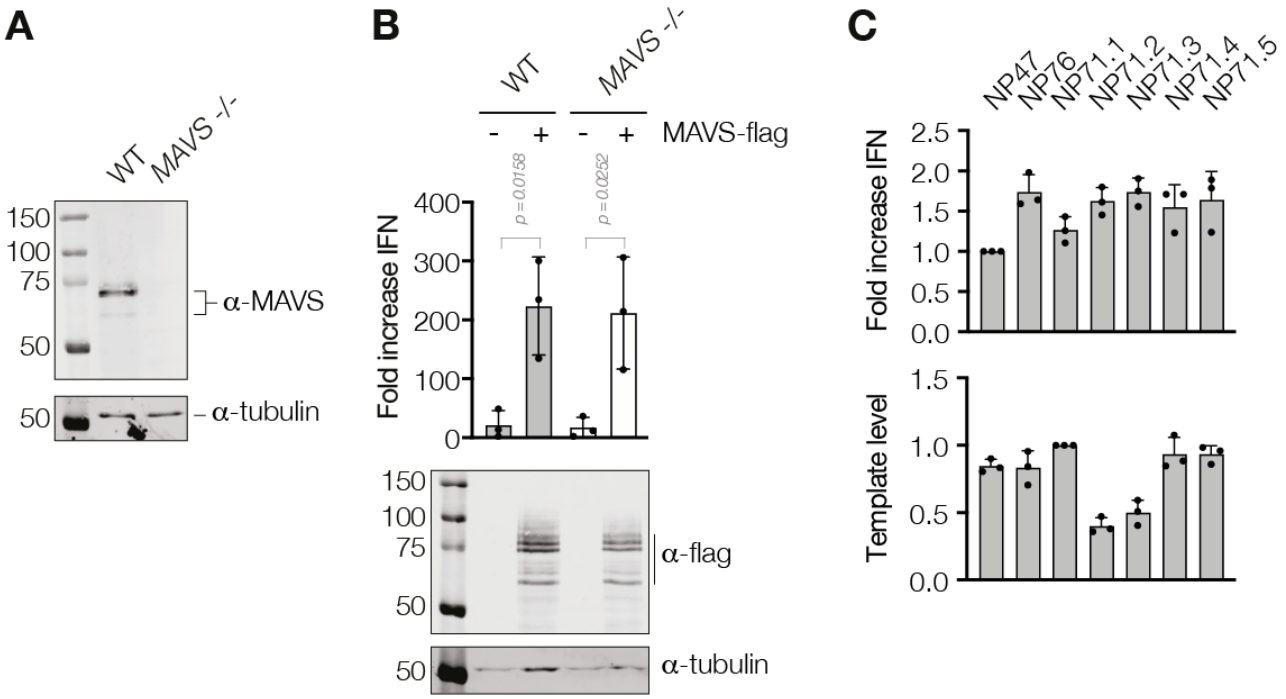

Figure S4. Characterization of *MAVS*$^{-/-}$ HEK 293 cells and mvRNA replication in *MAVS*$^{-/-}$ HEK 293 cells. A) Western blot analysis of MAVS expression in wildtype or *MAVS*$^{-/-}$ HEK 293 cells. B) IFN-β promoter activity in wildtype or *MAVS*$^{-/-}$ HEK 293 cells following transfection of a plasmid expressing MAVS-flag. Bottom panel shows western blot of MAVS-flag expression. C) IFN-β promoter activity of HEK 293 *MAVS*$^{-/-}$ cells expressing mvRNAs NP71.1-NP71.5. and primer extension analysis of mvRNA NP71.1-NP71.5 replication in HEK 293 *MAVS*$^{-/-}$ cells. P-values were computed using student t-test.

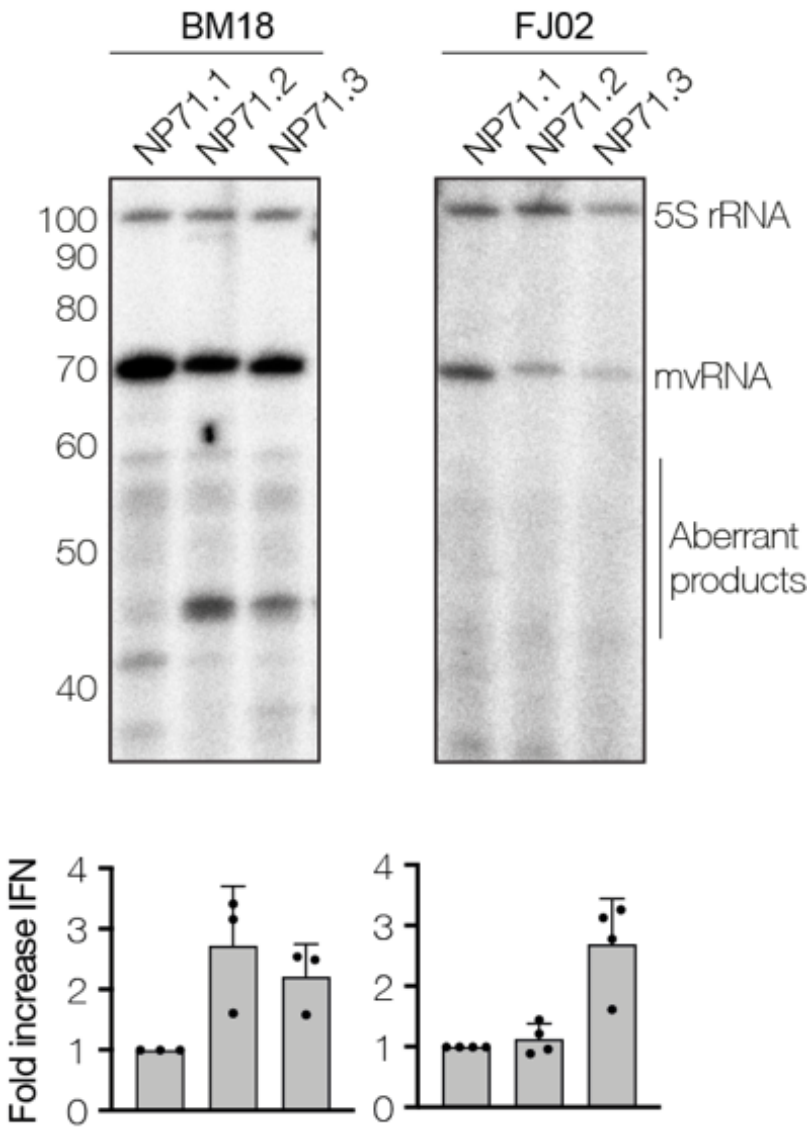

Figure S5. Replication of model mvRNAs in HEK 293T cells by the IAV A/Brevig Mission/1/18 (BM18) or A/duck/Fujian/01/02 (FJ02) RNA polymerases. The ability of these reactions to induce IFN-β promoter activity was analyzed using a luciferase reporter assay.

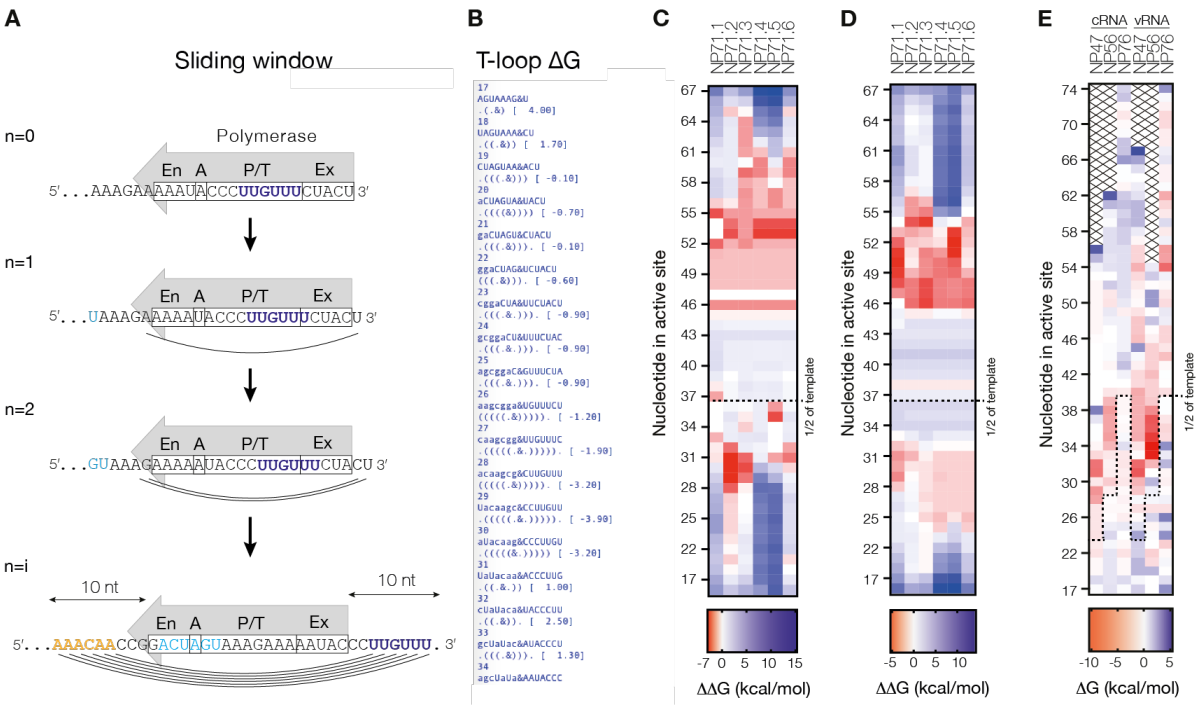

Figure S6. Sliding-window analysis of the free energy (ΔG) of transient secondary RNA structure formation near the influenza A virus RNA polymerase. A) To calculate the ΔG for a t-loop, 10 nt on either side of the polymerase were allowed to fold using the duplex-fold algorithm of the ViennaRNA package. To calculate the ΔG for alternative structures, cofold of the ViennaRNA package was used. The ΔΔG for the likelihood of t-loop formation was calculated by subtracting the upstream and downstream ΔG values from the t-loop ΔG value. B) Example of Python script output. C) ΔΔG values for positive sense 71-nt long mvRNA templates. D) ΔΔG values for negative sense 71-nt long mvRNA templates. E) t-loop ΔG values for positive sense and negative sense NP47, NP56 and NP76 mvRNA templates.

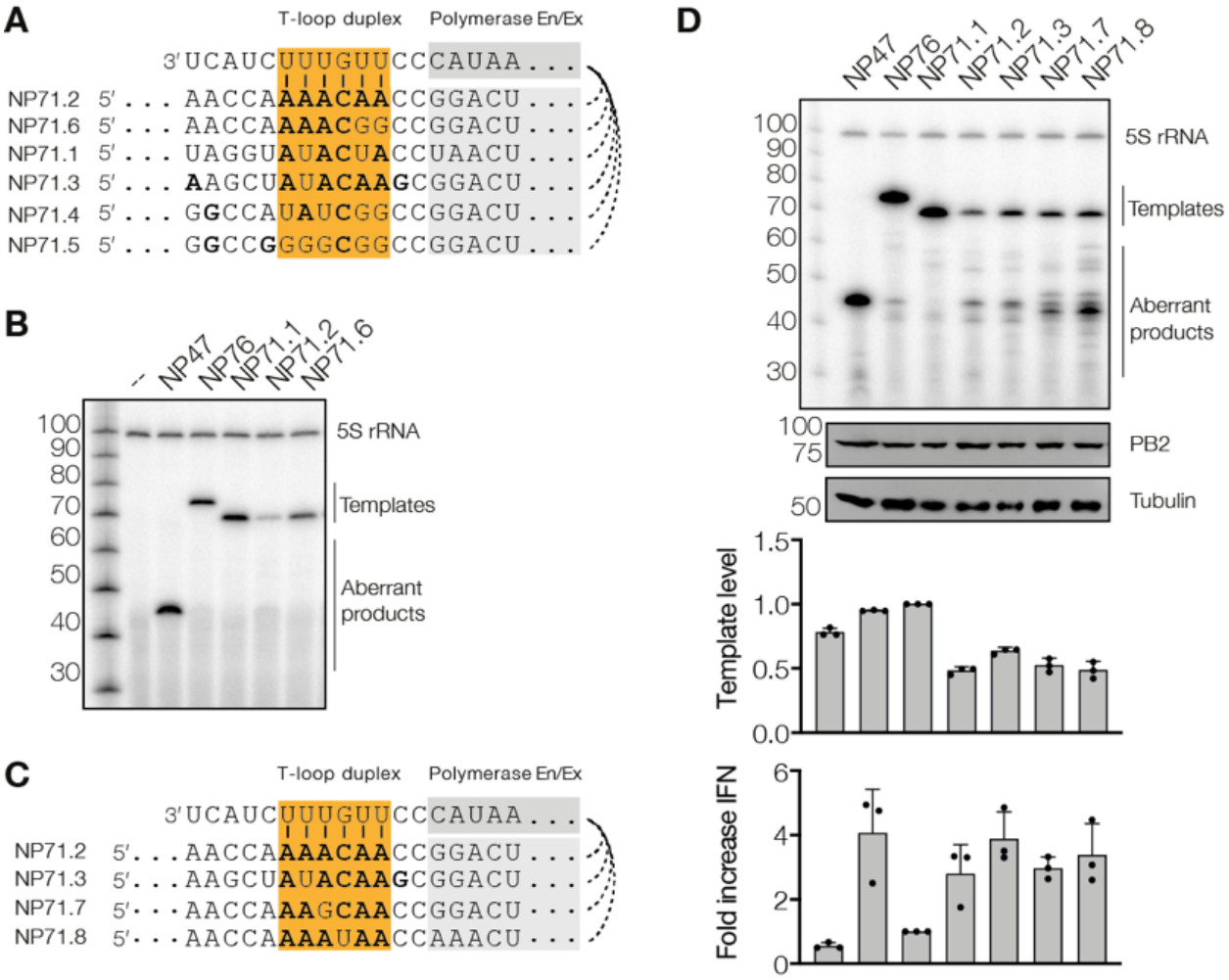

Figure S7. Replication of engineered mvRNAs and their ability to induce IFN-β promoter activity. A) Alignment of t-loops (shaded orange) in NP71 mvRNA templates. Location of RNA polymerase entry (En) and exit (Ex) channel is shaded gray. B) Primer extension analysis of RNA extracted from HEK 293T cells expressing NP71 mvRNA templates. C) Alignment of t-loops (shaded orange) of additional NP71 mvRNA templates. Location of RNA polymerase entry and exit channel is shaded gray. D) Primer extension analysis of RNA extracted from HEK 293T cells expressing NP71 mvRNA templates. Second and third panel show western blot analysis. Top graph shows quantification of mvRNA template level, while bottom graph shows IFN-β promoter activity analysis using a luciferase reporter.

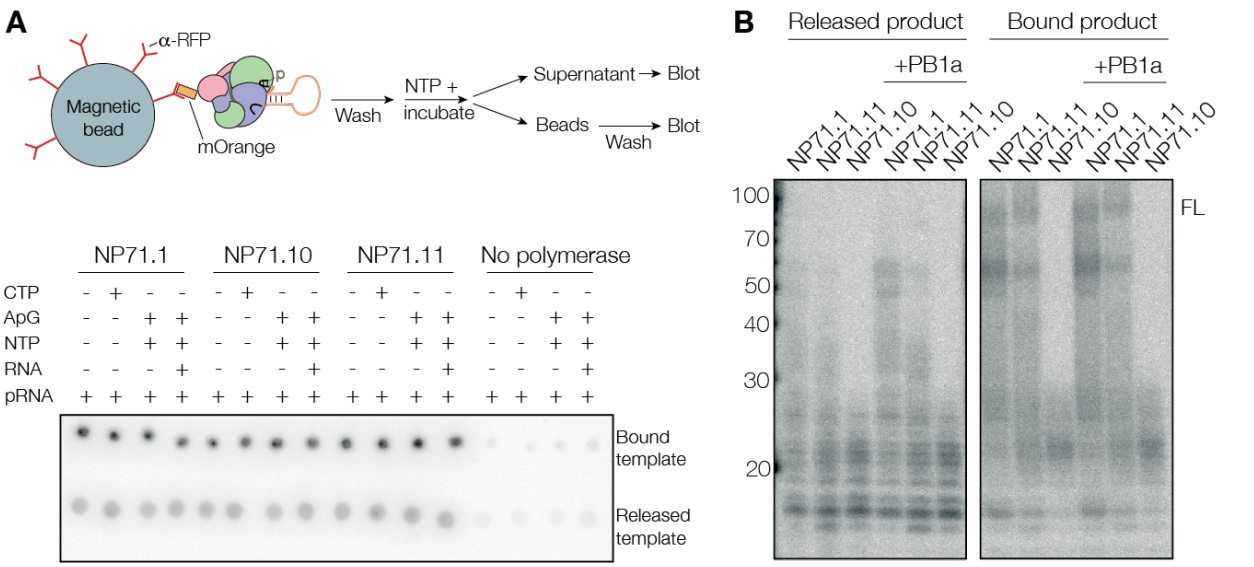

Figure S8. Analysis of IAV RNA polymerase template binding and release on different mvRNA templates in vitro. A) Binding of radiolabeled RNA templates by mOrange-tagged, immobilized IAV RNA polymerase in the absence and presence of ApG and NTPs. B) Binding of radiolabeled RNA products by mOrange-tagged, immobilized IAV RNA polymerase in the absence and presence of free inactive IAV RNA polymerase (PB1a).

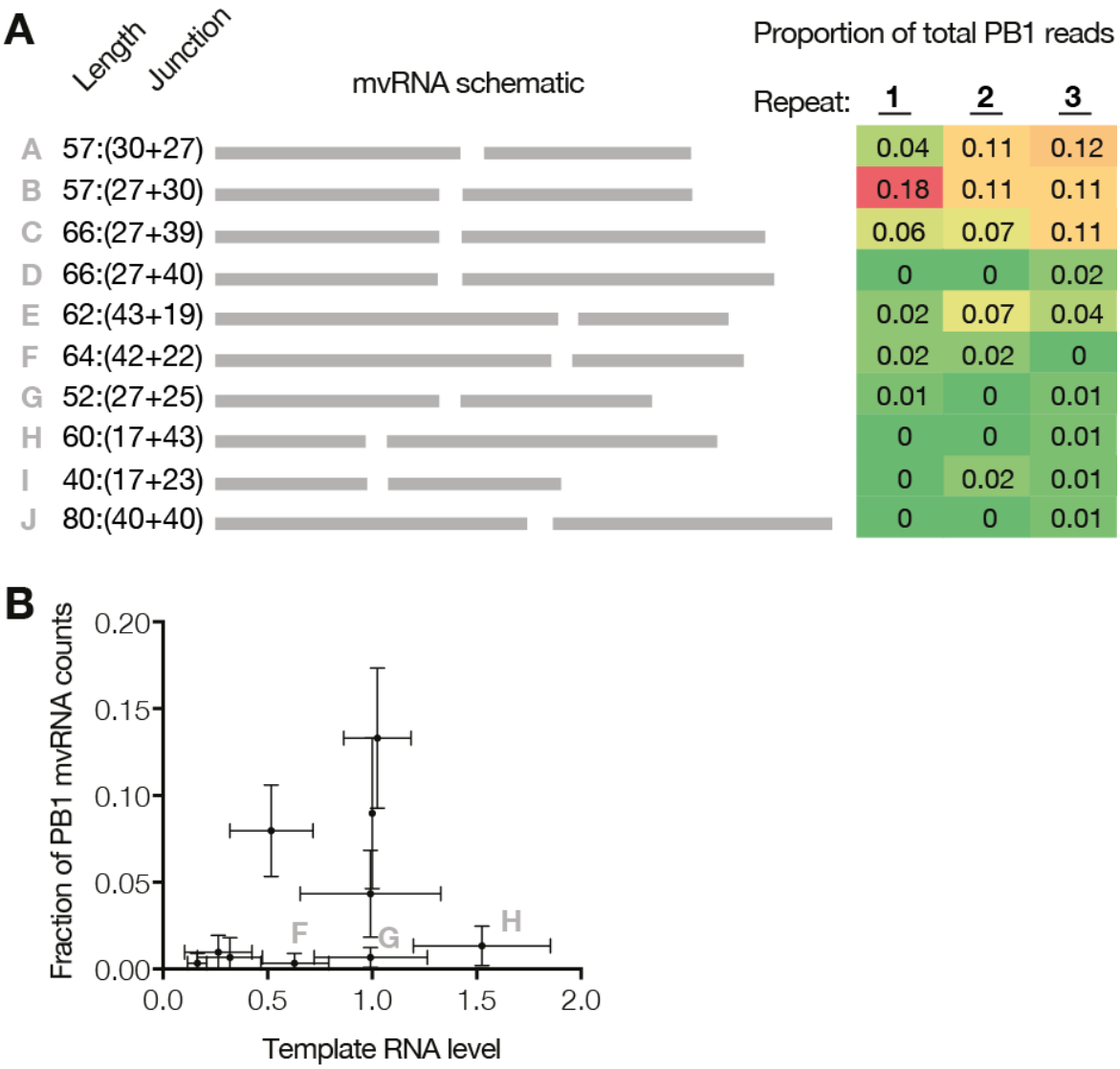

Figure S9. Selection of segment 2 mvRNAs. A) Segment 2 mvRNA sizes and abundance in three next generation sequencing experiments. B) Relation between mvRNA level in transfection assay and mvRNA read counts in next generation sequencing. The mvRNAs F, G and H are indicated.

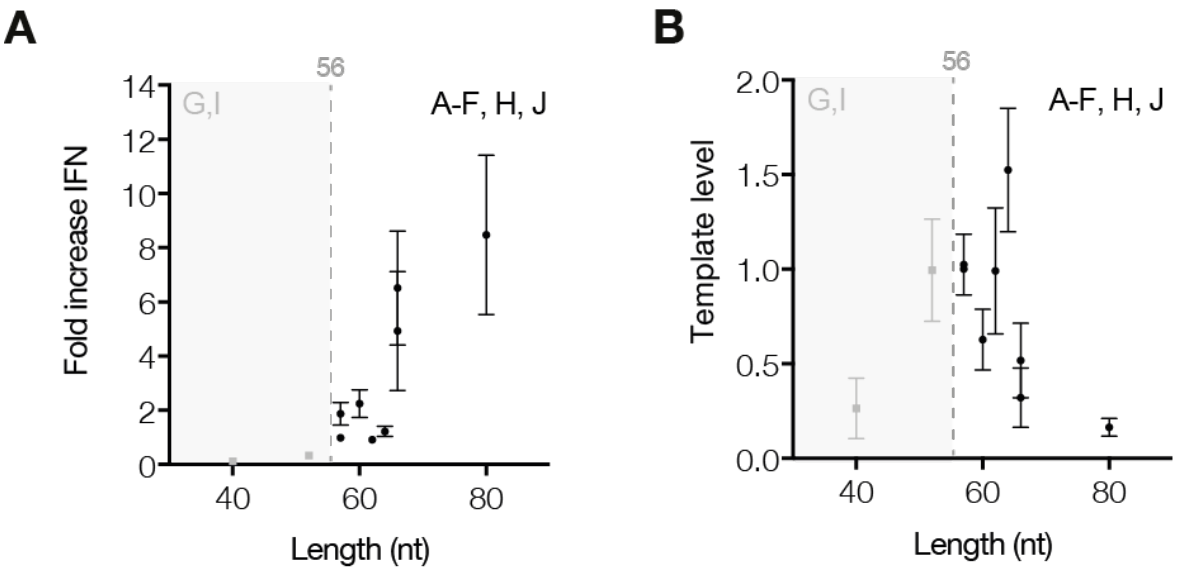

Figure S10. Relation between the A) mvRNA template length and the IFN-β promoter activity induction, or B) the mvRNA template length and mvRNA replication.

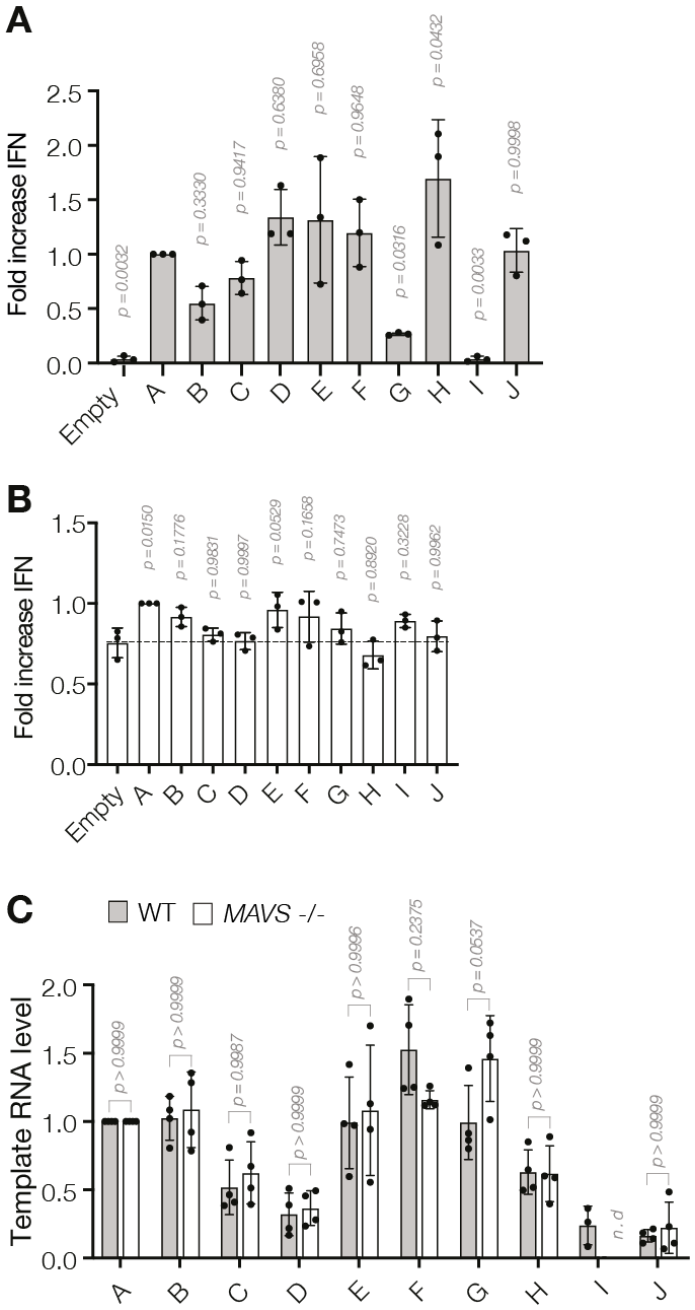

Figure S11. Segment 2 mvRNA replication in *MAVS*$^{-/-}$ HEK 293 cells. A) IFN-β promoter activity measured after retransfection of total RNA extracted from HEK 293T cells expressing segment 2 mvRNAs into HEK 293T cells. B) IFN-β promoter activity induced by expression of segment 2 mvRNAs in *MAVS*$^{-/-}$ HEK 293 cells. C) Primer extension analysis of segment 2 mvRNAs levels in wildtype and *MAVS*$^{-/-}$ HEK 293 cells. P-values were computed using one-way ANOVA with multiple-corrections relative to template A or the empty plasmid control.

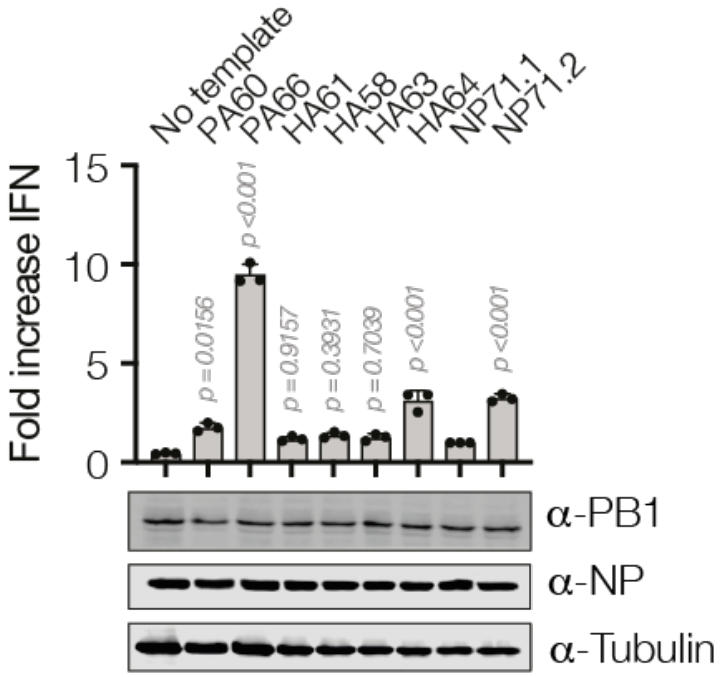

Figure S12. Induction of IFN beta promoter activity by segment 3 and 4 mvRNAs. P-values were computed using one-way ANOVA with multiple-corrections relative to the NP71.1 mvRNA template.

Table S1. Sequences of mvRNA templates used.

Name	Internal lab reference	Sequence 5' and 3' (vRNA)
NP71.1	GC33	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUUAGGUAGUAUA CCUAGUAACUAGUCUACCCUGCUUUUGCU
NP71.2	GC50.1	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGUUGUUU UGGUUGCCACUAGUCUACCCUGCUUUUGCU
NP71.3	GC50.2	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGCUUGUAU AGCUUGCCACUAGUCUACCCUGCUUUUGCU
NP71.4	GC67	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGCCGAUA UGGCCGCCACUAGUCUACCCUGCUUUUGCU
NP71.5	GC83	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGCCGCCC CGGCCGCCACUAGUCUACCCUGCUUUUGCU
NP71.6	GC50.9	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGCCGUUU UGGUUGCCACUAGUCUACCCUGCUUUUGCU
NP71.7	GC50.3	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGUUCUUU UGGUUGCCACUAGUCUACCCUGCUUUUGCU
NP71.8	GC50.4	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCCGGUUGCUU UGGUUGCCACUAGUCUACCCUGCUUUUGCU
NP47	NP47	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUCUACCCUGCU UUUGCU
NP56	NP56	AGUAGAAACAAGGGUAUUUUUCUUUCUCGAGCGUACUAGUC UACCCUGCUUUUGCU
NP76	NP76	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGAUUUCGAUG UCACUCUGUGAGUGAUUAUCUACCCUGCUUUUGCU
NP71.10	GC50_13	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGGCAGCAAAAG CAGGGUAACUAGUCUACCCUGCUUUUGCU
NP71.11	GC50_15	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGGCAGCAAAAG CACCCAUACUAGUCUACCCUGCUUUUGCU
NP71.12	GC50_16	AGUAGAAACAAGGGUAUUUUUCUUUACUAGUGGCUCUAAAAG CACCCAUACUAGUCUACCCUGCUUUUGCU

Table S2. Cloned PB1 WSN mvRNAs.

Name	Length (nt)	Sequence 5' and 3' (vRNA)
PB1 A	57	AGUAGAAACAAGGCAUUUUUUCAUGAAAUCCAUUCAAAUGGU UUGCCUGCUUUCGCU
PB1 B	57	AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAUUCAAAUGGU UUGCCUGCUUUCGCU
PB1 C	66	AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAAGCUAAACAU UCAAAUGGUUUGCCUGCUUUCGCU
PB1 D	67	AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAAGCUAAAUCA UUCAAAUGGUUUGCCUGCUUUCGCU
PB1 E	62	AGUAGAAACAAGGCAUUUUAAGUCGGAUUGACAUCCAUUCAA AUGGUUUGCCUGCUUUCGCU
PB1 F	64	AGUAGAAACAAGGCAUUUUUUCAGUCGGAUUGACAUCCAUUC AAAUGGUUUGCCUGCUUUCGCU
PB1 G	52	AGUAGAAACAAGGCAUUUUUUCAUGCAUUCAAAUGGUUUGCC UGCUUUCGCU
PB1 H	60	AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAAGCUAAAUUC AGUUUGCCUGCUUUCGCU
PB1 I	40	AGUAGAAACAAGGCAUUUUUUCAGUUUGCCUGCUUUCGCU
PB1 J	80	AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAAGCUAAAUUC GGAUUGACAUCCAUUCAAAUGGUUUGCCUGCUUUCGCU

Table S3. Cloned mvRNAs based on t-loop analysis.

Name	Sequence 5' and 3' (vRNA)
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PA60	AGUAGAAACAAGGUACUUUUUUGGACAGUAUGCCAUUUUGAAUCAGUAC CUGCUUUCGCU
HA61	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUAAUCUUCC CCUGCUUUUGCU
HA58	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUAUUCCCCU GCUUUUGCU
HA63	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUGUUUUUAUUUU CCCCUGCUUUUGCU
HA64	AGUAGAAACAAGGGUGUUUUUCCUUAUAUUUCUGAAAUCCUAAUCUCAU UCCCCUGCUUUUGCU

Supplemental Fasta file 1. WSN mvRNA sequences.

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>BM18_NA,1,1437,38:(17+21)
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>BM18_PB1,1,2321,39:(18+21)
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>BM18_PB1,1,2322,38:(18+20)
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AGCRAAAGCAGGCAAACAATGCCTTGTTTCTACT
>BM18_PB1,1,2317,42:(17+25)
AGCRAAAGCAGGCAAACCATGAAAAAATGCCTTGTTTCTACT

Supplemental script – Python script for sliding window analysis

```
#Influenza virus RNA polymerase simulation script that searches for t-loops and checks up/down stream for intermol bp
#Uses Python 3.8, biopython, openpyxl, and Vienna RNA 2.47; side packages were installed with Anaconda3
#By AJ te Velthuis, Sept 2020

import sys
sys.path.append("/users/USER/opt/anaconda3/lib/python3.8/site-packages")

##input sequence. Paste sequence between quotation marks below.

RNA = "PASTE SEQUENCE HERE"

Name ="Template"

##output bubbles in txt file
f= open("deltaGvalues.txt","w+")

##specify polymerase properties

Footprint = 20

##specify NP footprint

NP = 24

TloopDuplex = 48

Duplex = int(TloopDuplex / 2)

##specify window and other comparisons

Uloop = "&" #Use & for co-fold to compute long-range interactions between upstream and downstream sequences.

Swindow = 1  #size of sliding window


##########################################

##invert input sequence to start at 3' end

NegRNA = RNA[::-1]

Length  = len(NegRNA)

##########################################

##polymerase bubble properties

Bubble = Footprint + Duplex

##iteration start point of simulation; start at nt 2 otherwise downstream sequence is empty for co-fold
i = 1
```

```
##end bubble sequence and add 1, because sequence count starts at 0

End = int((Length - Footprint + 1) / Swindow)


##find polymerase bubble sequence; allow for small 3' part to emerge, but
##then cap how long the emerging sequence can be by assuming that every 24 nt will be bound by NP
##unclear if NP binds in chunks or progressively. Assume that at least 24 nt are needed based on data from Ortin lab.
##stops 1 nt from end to avoid having no sequence in cofold

for i in range(1, End-1):

    if i <= NP:
        Upstream = i
        Downstream = 0
    else:
        Upstream = NP
        Downstream = i-NP

    #find upstream and downstream sequence of the bubble for intermolecular folding check

    Ahead = NegRNA[Footprint+i:Footprint+NP+i]

    Aheadinv = Ahead[::-1]

    #currently only takes 1 nt of downstream as 0 gives an error in cofold.
    Down = NegRNA[Downstream:i]

    Downinv = Down[::-1]

    ##find the two duplex ends and invert them so they are 5' to 3'

    if i <= Duplex:
        Prime3 = NegRNA[0:Upstream]
    else:
        Prime3 = NegRNA[i-Duplex:i]

    Prime3inv = Prime3[::-1]

    if i <= End:
        Prime5 = NegRNA[Footprint+i:Bubble+i]
    else:
        Prime5 = NegRNA[Footprint+i:Length]

    Prime5inv = Prime5[::-1]

    #compute A/U content of nucleotides in active site. Assume window of 6 before and after active site. Various print options are inactivated, but can be used for checking if script works.

    ActiveSite = i + 16

    #print("location of bubble is ", i+1)

    #print("3 prime end 3' to 5' is ", Prime3)
```

```
    #print("5 prime end 5' to 3' is ", Prime5inv)

    ActiveSiteSeqUp = NegRNA[ActiveSite-1:ActiveSite+4]

    ActiveSiteSeqDown = NegRNA[ActiveSite-5:ActiveSite]

    #seq_up_list = list(ActiveSiteSeqUp)
    seq_up_list = list(ActiveSiteSeqDown)
    at_count_up = seq_up_list.count("a") + seq_up_list.count ("t") + seq_up_list.count("A") + seq_up_list.count ("T") + seq_up_list.count ("u") + seq_up_list.count ("U")
    at_frac_up = float(at_count_up)/5
    total_at_up = 100 * at_frac_up

##saving the A/U content is inactivated below
#print(total_at_up)
    #f.write("%f\n" % (total_at_up))

    ##Vienna RNA package for RNA structure prediction
    import RNA

    Test = Prime3 + Uloop + Prime5

    NegTest = Test[::-1]

    #Output = (">"+Name+"_polymerase_bubble_number_%d\n" % (i+1))

    ##To write bubble sequence to .txt file
    #f.write(Output)
    #f.write(NegTest)
    #f.write("\r\n")

    #use duplex fold to compute t-loop from Vienna package because it ignores intermol bp
    duplex = RNA.duplexfold(Prime5inv, Prime3inv)

    #print("%s\n%s [%6.2f]" % (NegTest, duplex.structure, duplex.energy))
    ##print("%6.2f" % (duplex.energy))

    #use cofold from Vienna package to check for bp in sequence upstream and downstream of t-loop. Various options can be checked separately, including just upstream seq, just downstream seq, or both seq
    Other = Aheadinv + Uloop + Downinv
    #Other = Aheadinv
    #Other = Downinv

    (ss, mfe_dimer) = RNA.cofold(Other)
    #print("%s\n%s  [%6.2f]" % (Other, ss, mfe_dimer))

    DDeltaG = duplex.energy - mfe_dimer ##compute DeltaDeltaG

    ##check if deltaG of t-loop is lower than deltaG of other structures
    if duplex.energy >= mfe_dimer:
        DeltaG = mfe_dimer * -1
    #elif duplex.energy >= 0:
        #DeltaG = 0
    else:
        #DeltaG = duplex.energy
```

```
        DeltaG = duplex.energy

    #DeltaG = mfe_dimer
    #DeltaG = duplex.energy

    #print(DeltaG)
    #print(DDeltaG)
    #print(duplex.energy)

    #print("%s\n%s [ %6.2f ]" % (NegTest, ss, mfe))

    ##to write deltaG values to .txt file. Various options are available depending on what is being analyzed.
    f.write("%f\n" % (duplex.energy))
    #f.write("%f\n" % (DeltaG))
    #f.write("%f\n" % (mfe_dimer))

    #print(NegTest)
    #print("-----")

    i =+ Swindow

#########################################################

##close .txt file that deltaG values were written to
f.close()
print ("Done %s" % (Name))
```