Nucleic acid based multi-target approach for effective silencing of influenza A virus

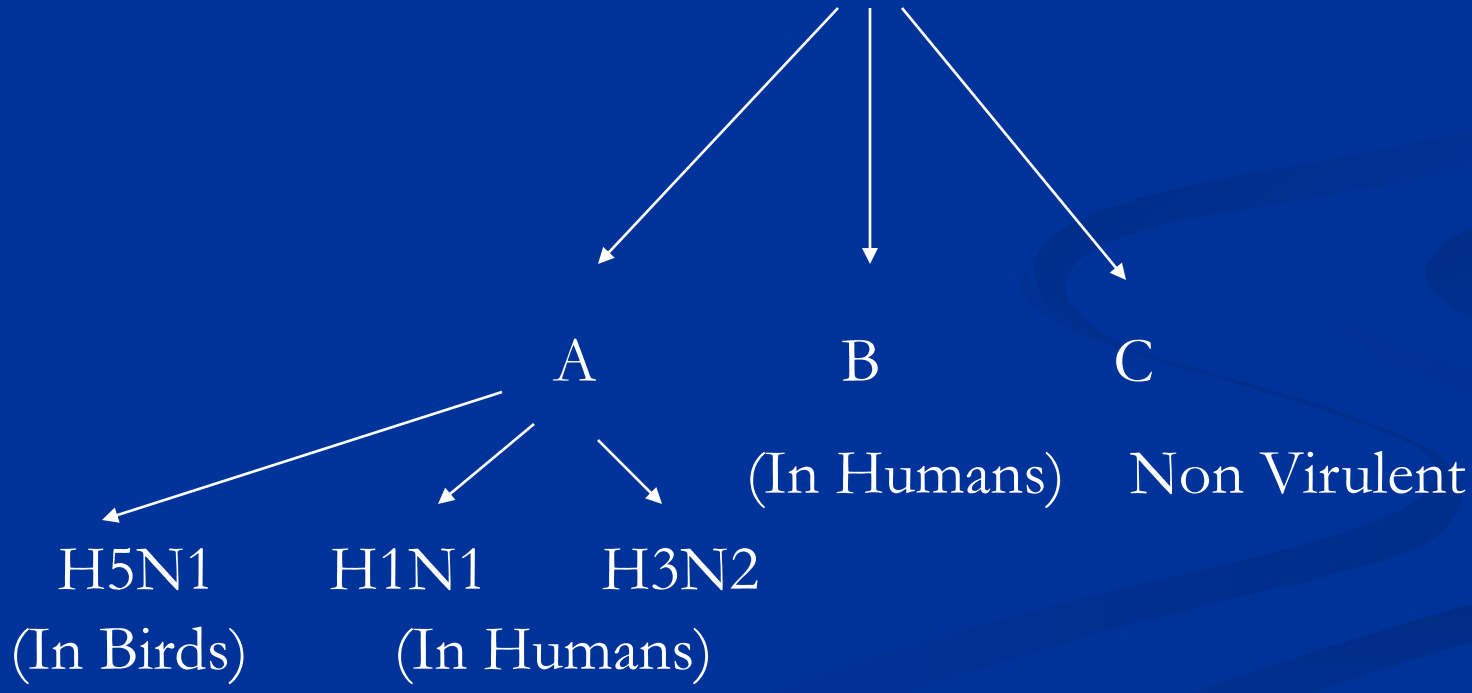
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Amity University Uttar Pradesh

INFLUENZA

- One of the major Respiratory Virus
- Causative agent of one of the most common disease i.e. common cold/ fever
- Most easily transmissible disease- through aerosol
- Strains keep on changing every season
- Cause of over 2,00,000 deaths every year

INFLUENZA

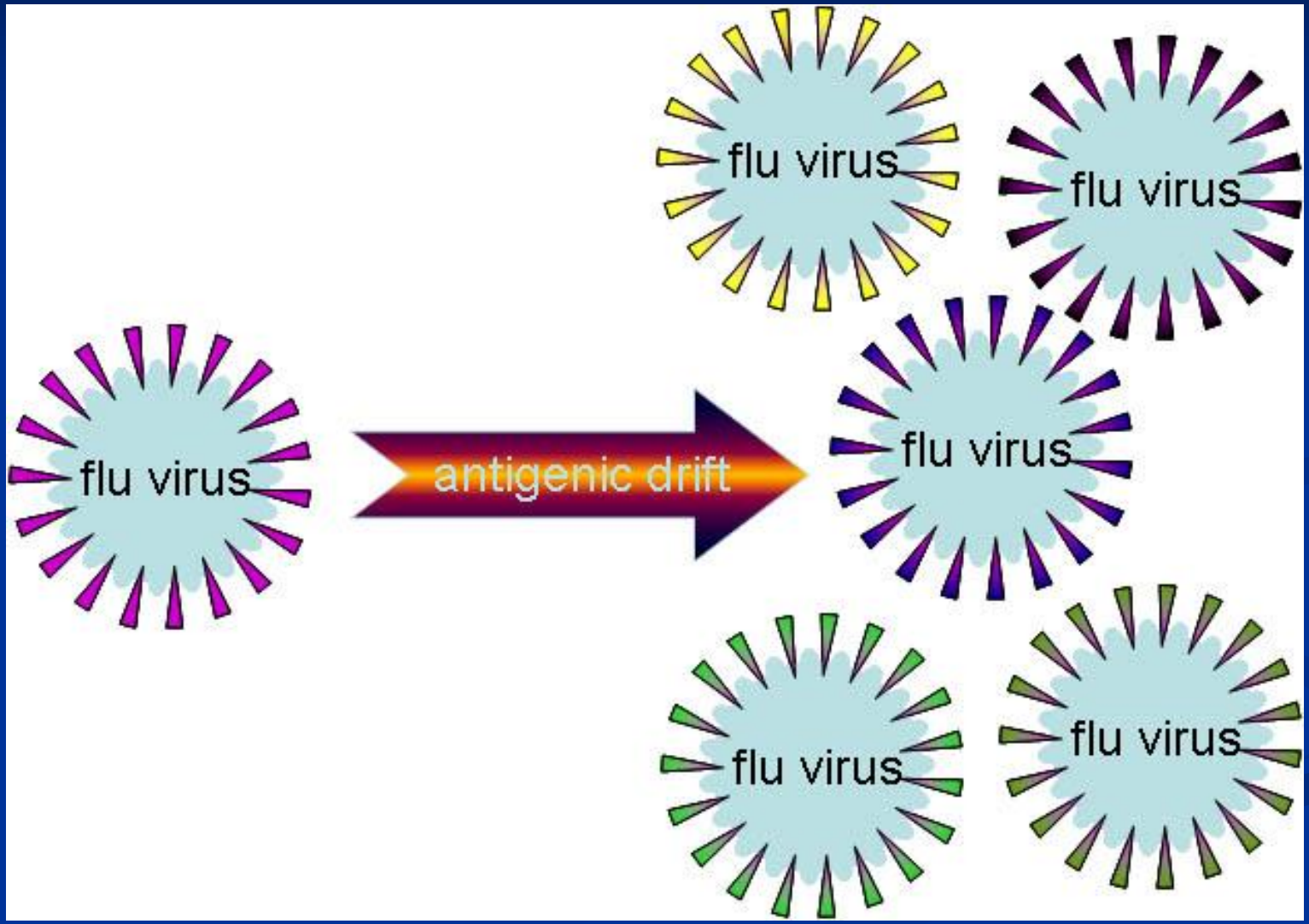
Types of Influenza Virus

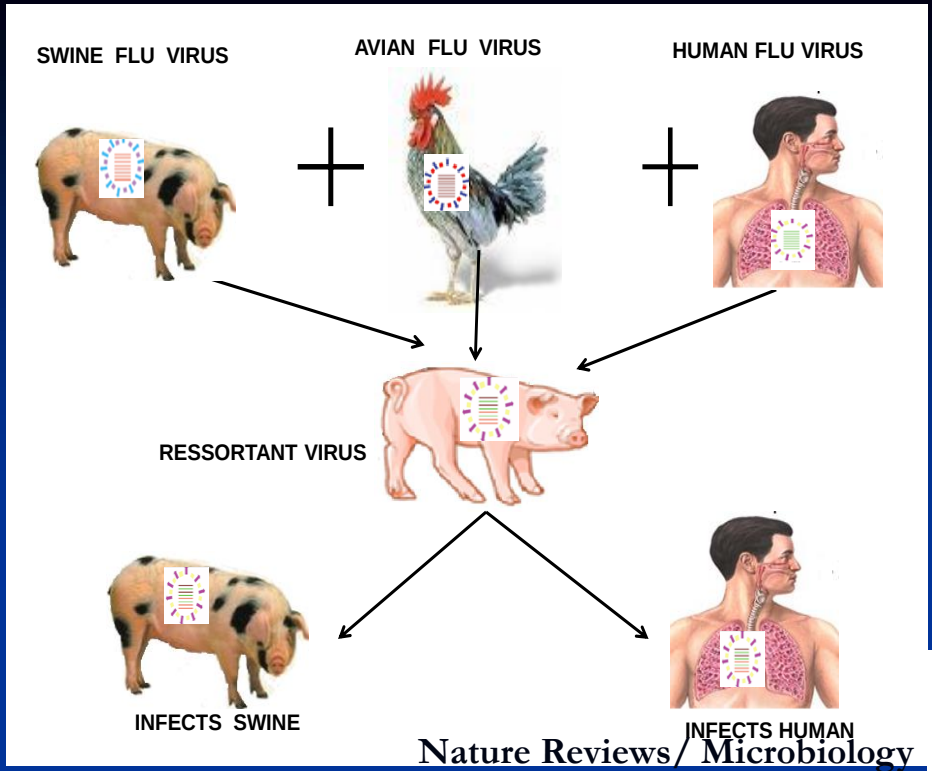


HOST RANGE:

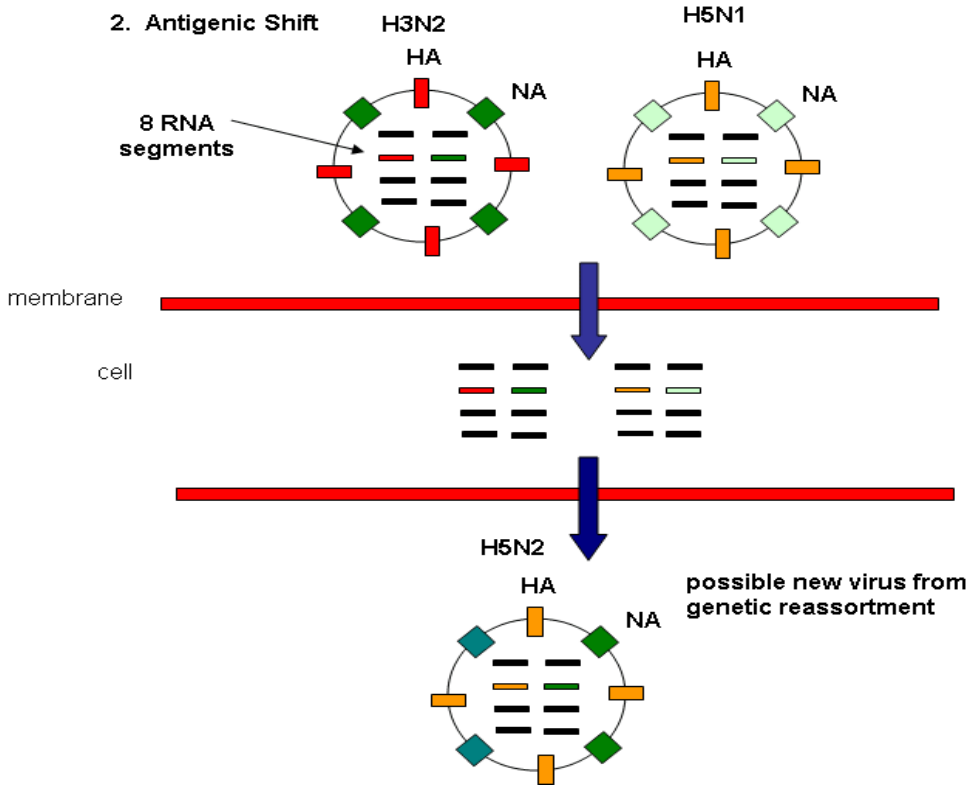
- Humans
- Swine
- Birds (Wild aquatic birds-Reservoir)
- Horses
- Whales
- Seals

Influenza Virus-Prone to Mutation



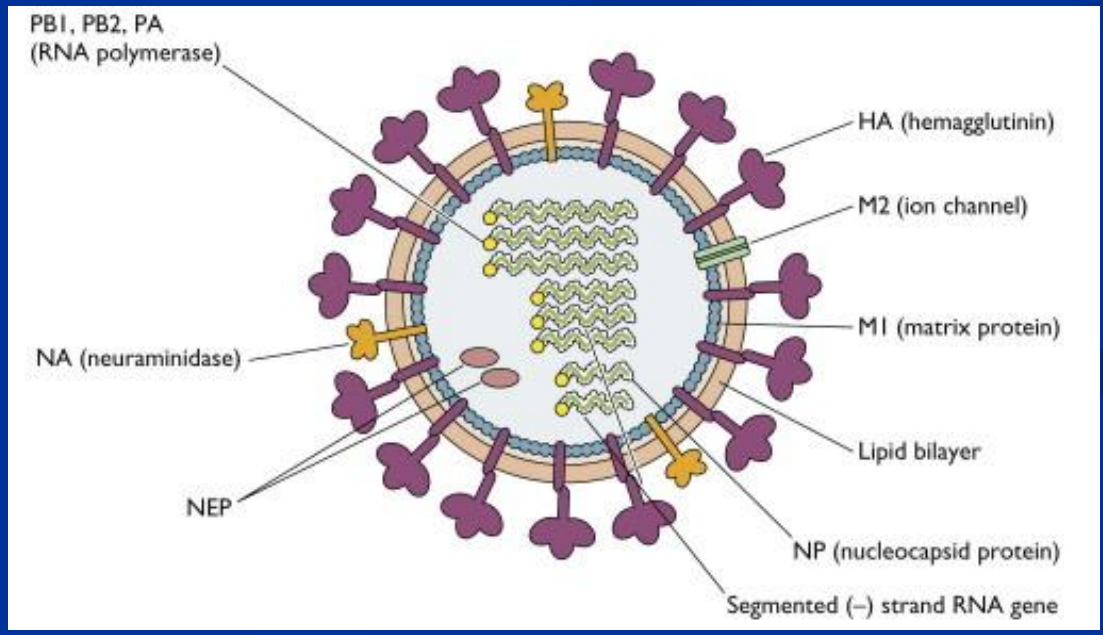


Nature Reviews/ Microbiology

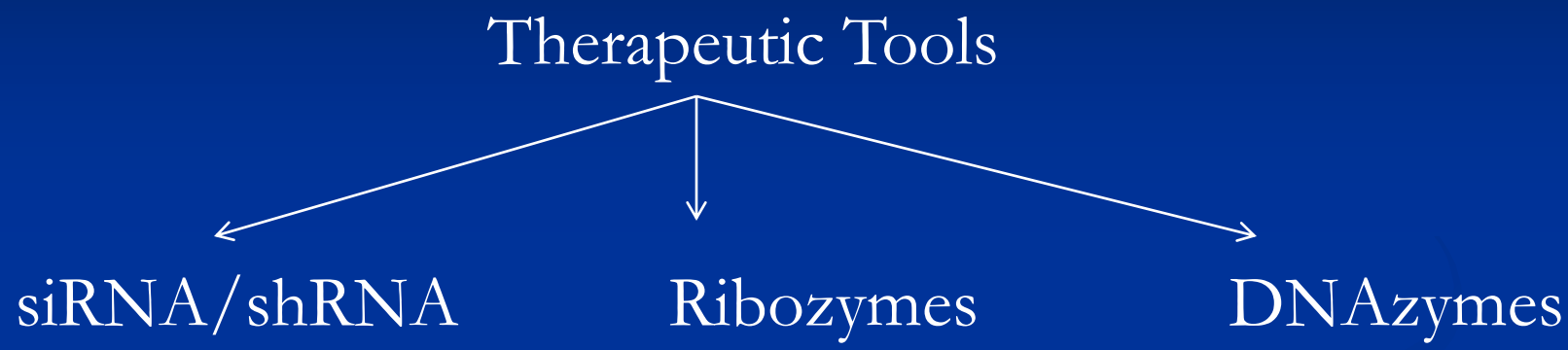


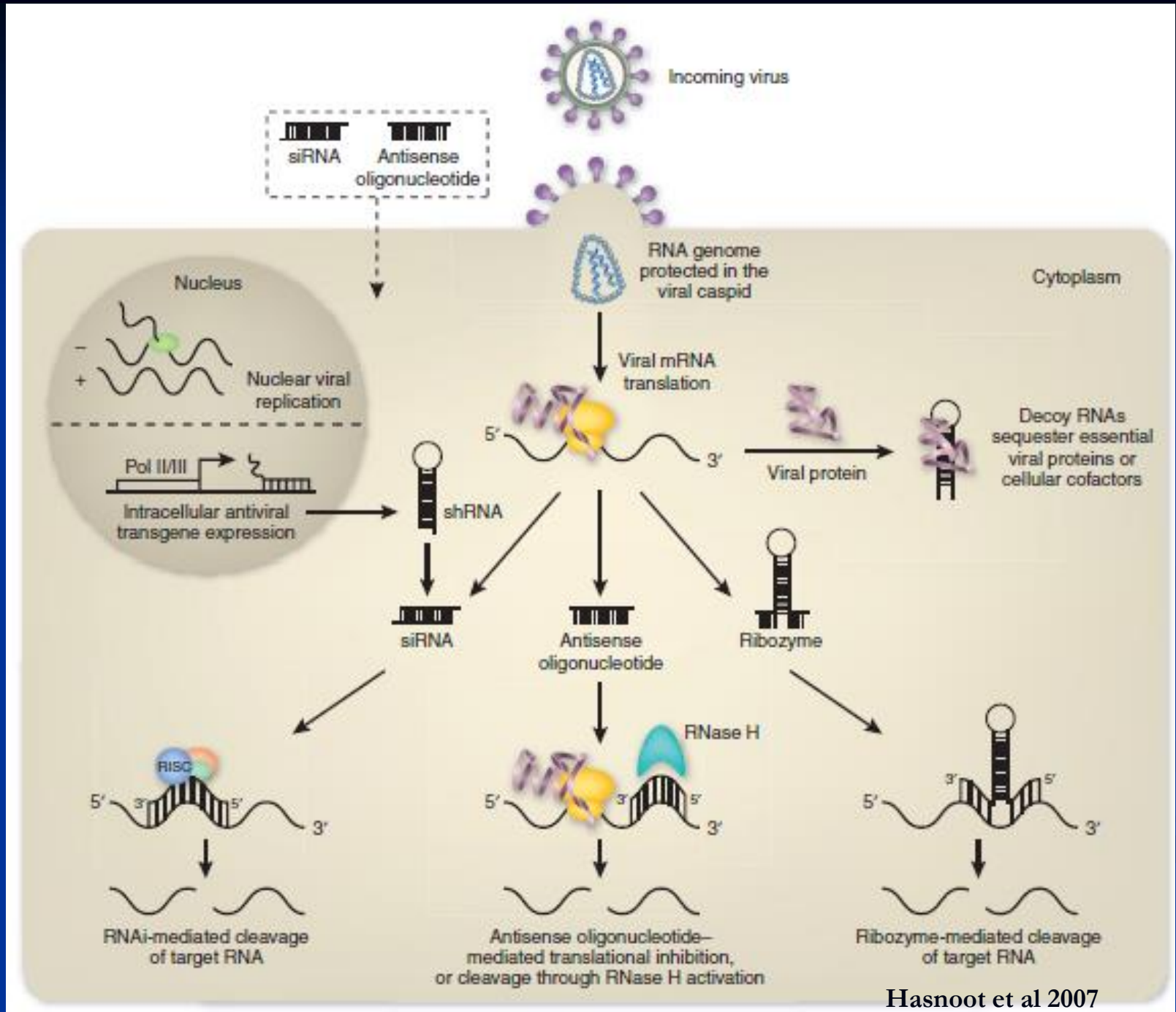
Control Options for Influenza:

- Vaccines: Seasonal vaccine (Trivalent/ Quadrivalent)
- Drugs:
  - Oseltamivir (Tamiflu)
  - Zanamivir (Relenza)
  - Peramivir (Rapivab)
  - Amantadine
  - Rimantadine



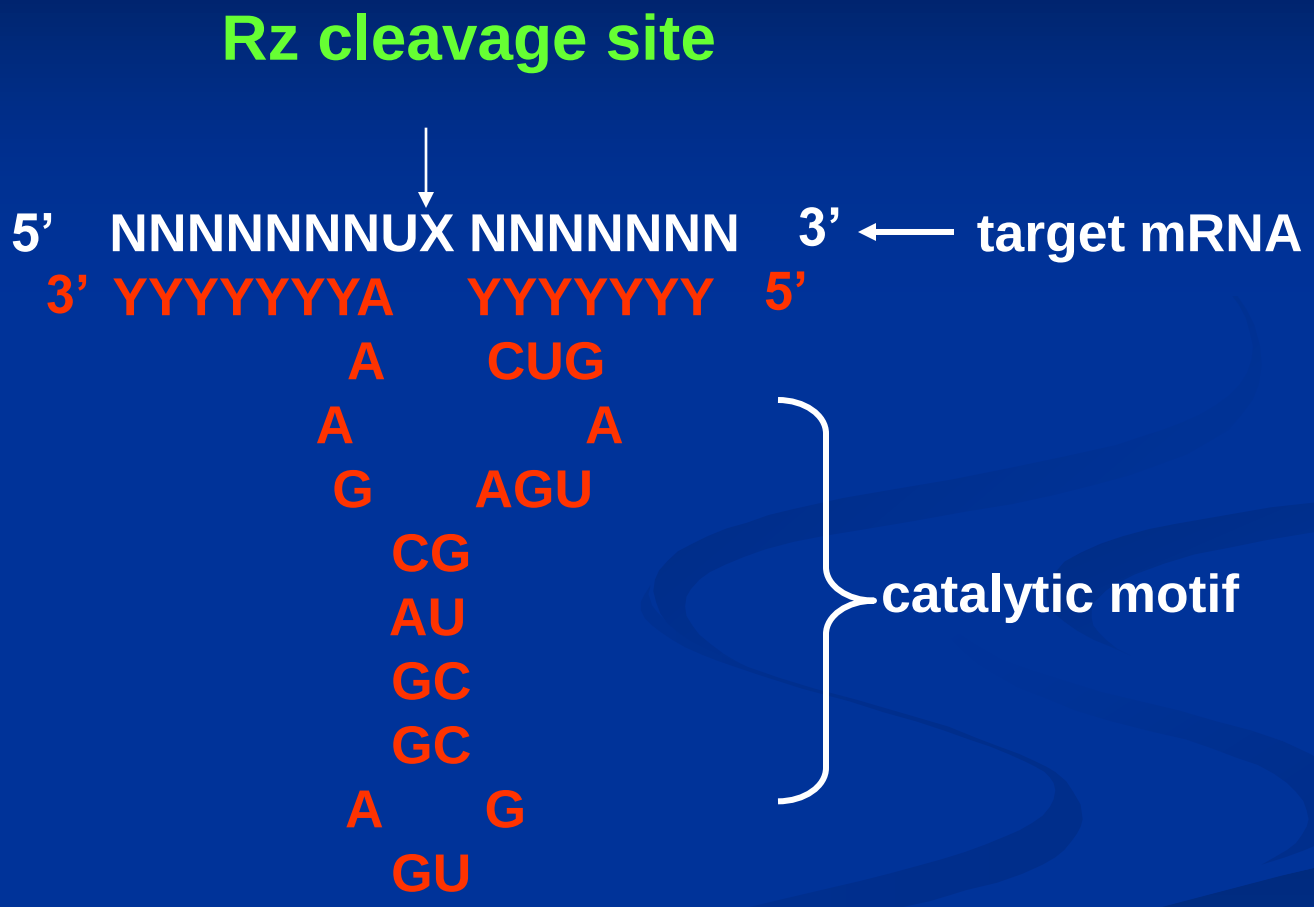
Nucleic Acids based therapeutics





Hasnoot et al 2007

Designing of Hammer-head Ribozyme



Designing of siRNA

Rules for selecting siRNA targets on mRNA sequences:

- Targets should be located 50-100 nt downstream of the start codon (ATG).
- Search for sequence motif $AA(N_{19})TT$ or $NA(N_{21})$, or $NAR(N_{17})YNN$, where N is any nucleotide, R is purine (A, G) and Y is pyrimidine (C, U).
- Target sequences should have a G+C content between 35-60%.
- Avoid stretches of 4 or more nucleotide repeats.
- Avoid 5'URT and 3'UTR, although siRNAs targeting UTRs have been shown to successfully induce gene silencing.
- Avoid sequences that share a certain degree of homology with other related or unrelated genes.

Segment	Size (nt)	Polypeptide(s)	Function
1	2341	PB2	Transcriptase: cap binding
2	2341	PB1	Transcriptase: elongation
3	2233	PA	Transcriptase: protease activity (?)
4	1778	HA	Haemagglutinin
5	1565	NP	Nucleoprotein: RNA binding; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	Neuraminidase: release of virus
7	1027	M1	Matrix protein: major component of virion
		M2	Integral membrane protein - ion channel
8	890	NS1	Non-structural: nucleus; effects on cellular RNA transport, splicing, translation. Anti-interferon protein.
		NS2	Non-structural: nucleus+cytoplasm, function unknown

Influenza A virus (A/Puerto Rico/8/34(H1N1)) nucleoprotein gene, complete cds

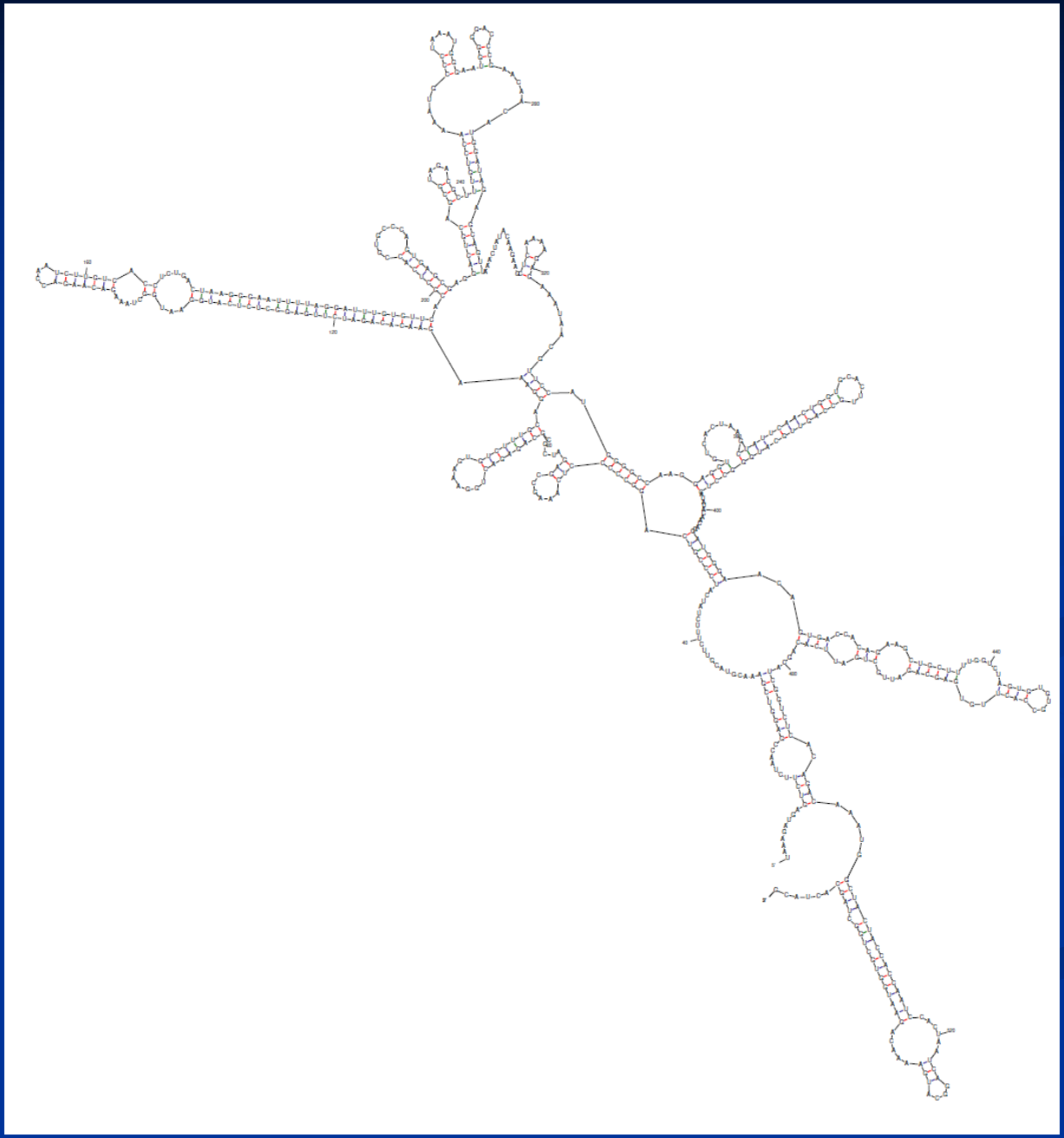
GenBank: M38279.1

GenBank Graphics

>gi|324691|gb|M38279.1|FLANPGENE Influenza A virus (A/Puerto Rico/8/34(H1N1)) nucleoprotein gene, complete cds

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TCTTACGAACAGATGGAGACTGATGGAGAACGCCAGAATGCCACTGAAATCAGAGCATCCGTCGGAAAA
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CGGTTGATCCAAAACAGCTTAACAATAGAGAGAATGGTGCTCTCTGCTTTTGACGAAAGGAGAAATAAA
TACCTTGAAGAACATCCCAGTGCGGGGAAAGATCCTAAGAAAACTGGAGGACCTATATACAGGAGAGTA
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SELECTION OF TARGET SITES



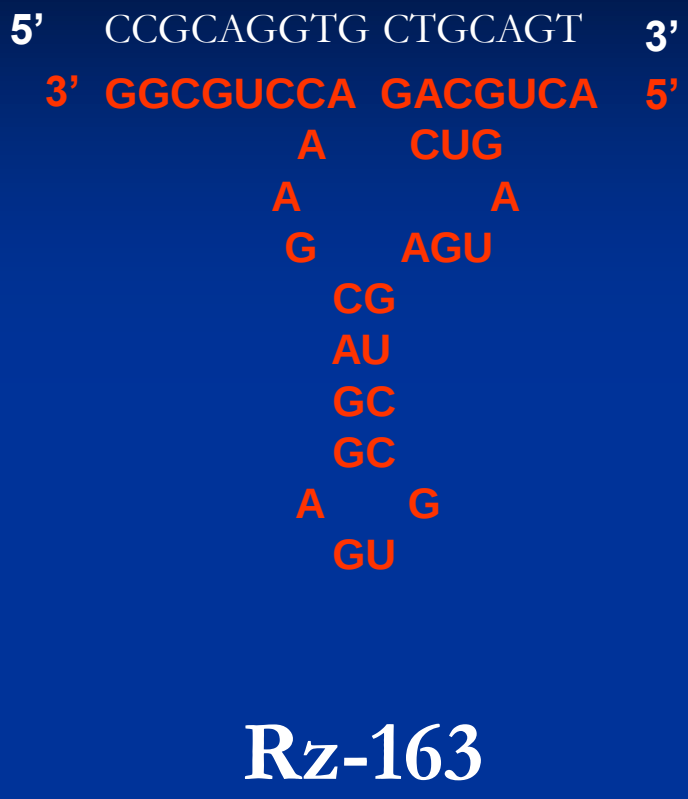
siRNAs designed against NP gene of influenza A virus

S.No	Target position	Target sequence 21nt target + 2nt overhang	RNA oligo sequences 21nt guide (5'→3') 21nt passenger (5'→3')
1	144-166	TGGTGGAATTGGACGATTCTACA	UAGAAUCGUCCAAUUCCACCA GUGGAAUUGGACGAUUCUACA
2	214-236	ATCCAAAACAGCTTAACAATAGA	UAUUGUUAAGCUGUUUUGGAU CCAAAACAGCUUAACAAUAGA
3	362-384	GAGAACTCATCCTTTATGACAAA	UGUCAUAAAGGAUGAGUUCUC GAACUCAUCCUUUAUGACAAA
4	690-712	AAGAATTGCTTATGAAAGAATGT	AUUCUUUCAUAAGCAAUUCUU GAAUUGCUUAUGAAAGAAUGU
5	715-737	AACATTCTCAAAGGGAAATTTCA	AAAUUUCCCUUUGAGAAUGUU CAUUCUCAAAGGGAAAUUUCA
6	1051-1073	GCCGCATTTGAAGATCTAAGAGT	UCUUAGAUCUUCAAAUGCGGC CGCAUUUGAAGAUCUAAGAGU
7	1061-1083	AAGATCTAAGAGTATTAAGCTTC	AGCUUAAUACUCUUAGAUCUU GAUCUAAGAGUAUUAAGCUUC
8	1476-1498	TTCCTTTGACATGAGTAATGAAG	UCAUUACUCAUGUCAAAGGAA CCUUUGACAUGAGUAAUGAAG
9	1542-1564	AAGAAAAATACCCTTGTTTCTAC	AGAAACAAGGGUAUUUUUCUU GAAAAAUACCCUUGUUUCUAC

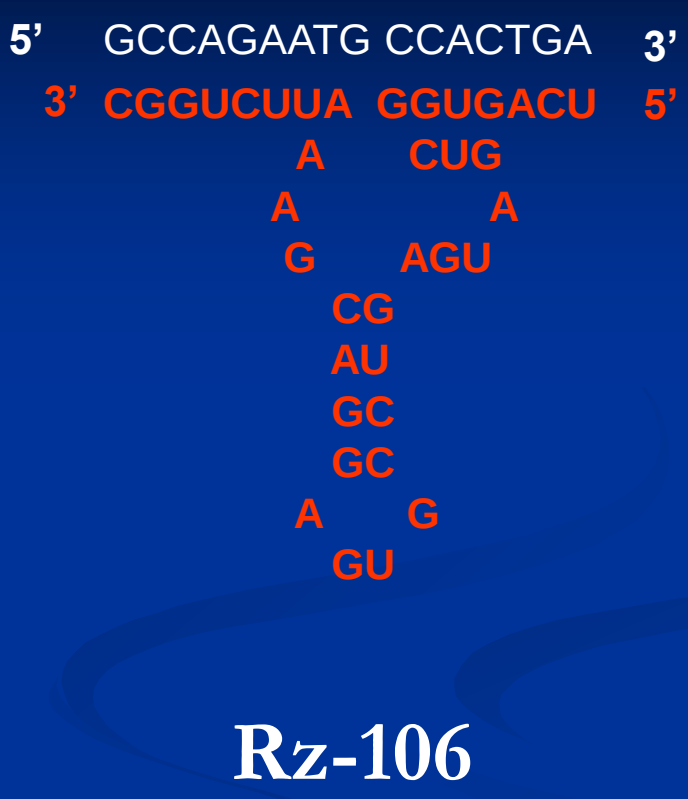
siRNAs designed against M gene of influenza A virus

S.No	Target position	Target sequence 21nt target + 2nt overhang	RNA oligo sequences 21nt guide (5'→3') 21nt passenger (5'→3')
1	144-166	AAGACAAGACCAATCTTGTCACC	UGACAAGAUUGGUCUUGUCUU GACAAGACCAAUCUUGUCACC
2	229-251	AGCGTAGACGCTTTGTCCAAAAT	UUUGGACAAAGCGUCUACGCU CGUAGACGCUUUGUCCAAAAU
3	298-320	AACTATACAAGAAGCTCAAAAGA	UUUUGAGCUUCUUGUAUAGUU CUAUACAAGAAGCUCAAAAGA
4	427-449	AAGCTGCTTTTGGTCTAGTGTGT	ACACUAGACCAAAAGCAGCUU GCUGCUUUUGGUCUAGUGUGU
5	503-525	TACTACCACCAATCCACTAATCA	AUUAGUGGAUUGGUGGUAGUA CUACCACCAAUCCACUAAUCA
6	627-649	CAGACTAGGCAGATGGTACATGC	AUGUACCAUCUGCCUAGUCUG GACUAGGCAGAUGGUACAUGC
7	694-716	AAGATGACCTTCTTGAAAATTTG	AAUUUUCAAGAAGGUCAUCUU GAUGACCUUCUUGAAAAUUUG

Ribozymes for M gene of influenza A virus



Rz-163



Rz-106

Cloning of NP and M gene of influenza virus in pcDNA3

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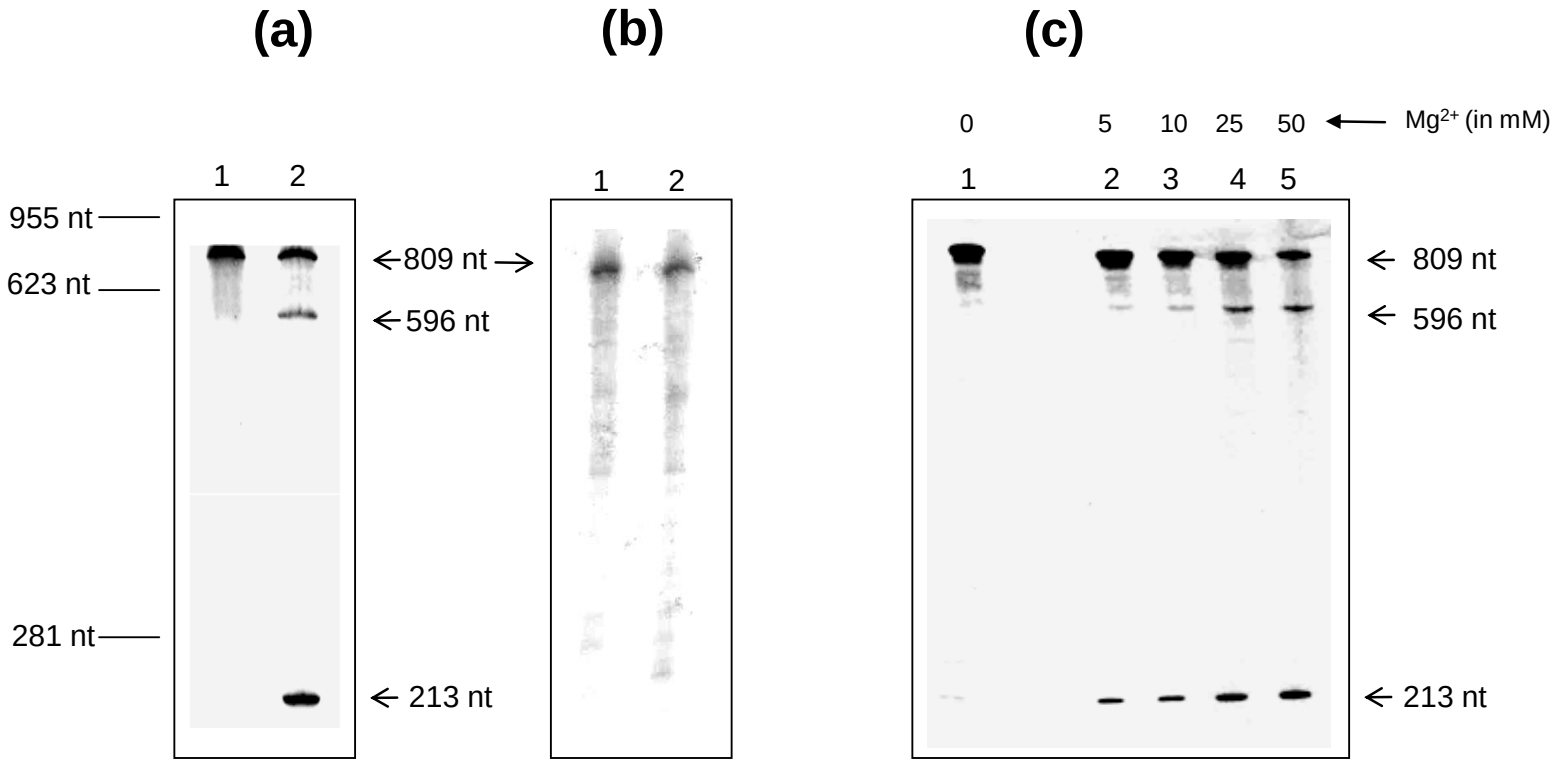
Co-transfection of HEK cells with siRNAs and cloned construct

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In-vitro transcription of M gene construct

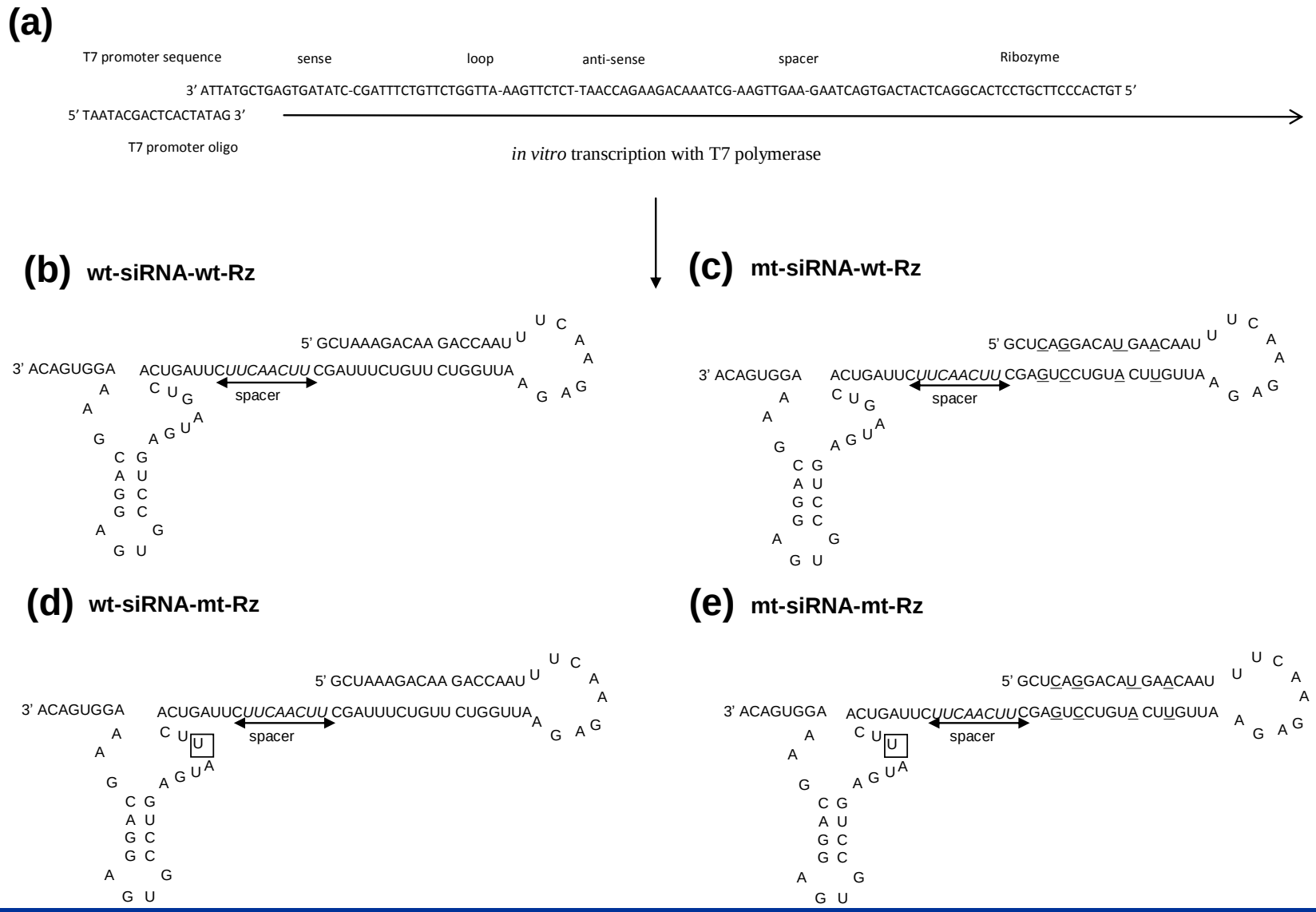
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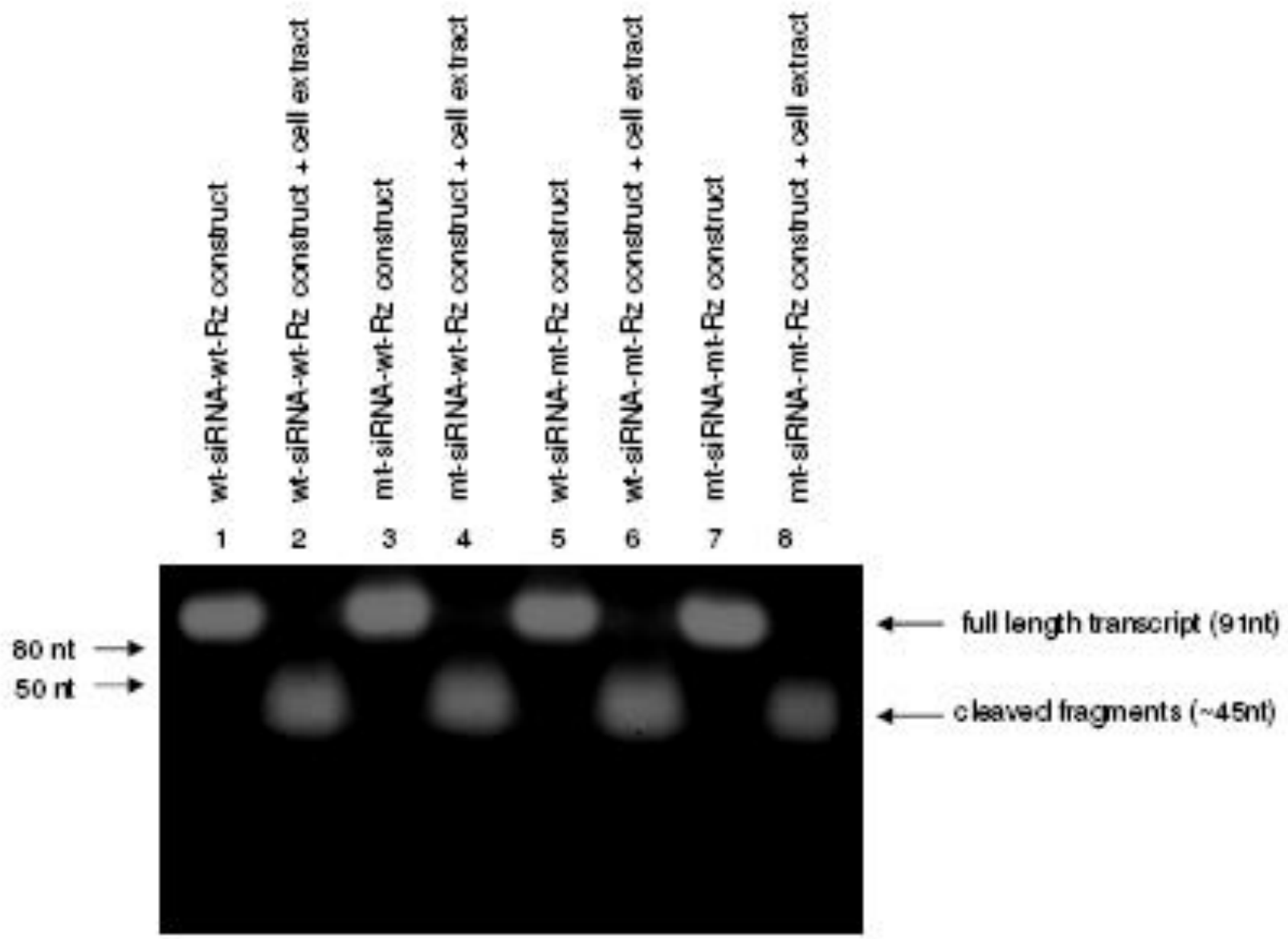
Analysis of the activity of Rz-163



Cleavage of target M1 RNA with Rz-163: Panel a: Autoradiograph showing full-length M1 RNA (lane 1) and cleaved fragments of target RNA with Rz (lane 2). Numbers on the left indicate size of RNA marker (cat # G3191, Promega). Panel b: lane 1 shows full length M1 RNA as before. Mutant Rz-163 failed to cleave the target RNA (lane 2). Panel c: The same transcript of M1 RNA was subjected to cleavage by Rz-163 in the presence of varying amounts of Mg2+ concentrations as shown on top of each lane.

Synthesis of novel Rz-chimeric-siRNA constructs and its mutatnts





In vitro synthesized chimeric constructs (50ng) were incubated with cytoplasmic protein extract (20 μg) for the period of 5 min at room temperature and cleavage products were analyzed by gel electrophoresis. Lane 1,3,5 and 7 represents uncleaved product and lane 2,4,6 and 8 represents cleaved product of 50 and 45 nucleotides.

MDCK cells grown to 70% confluency

↓

co-transfection with 500 ng of M1-pcDNA3 plasmid DNA and various concentrations ranging from 1.5 to 5g of siRNA-chimeric-Rz constructs

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Total RNA isolation at 24h post transfection

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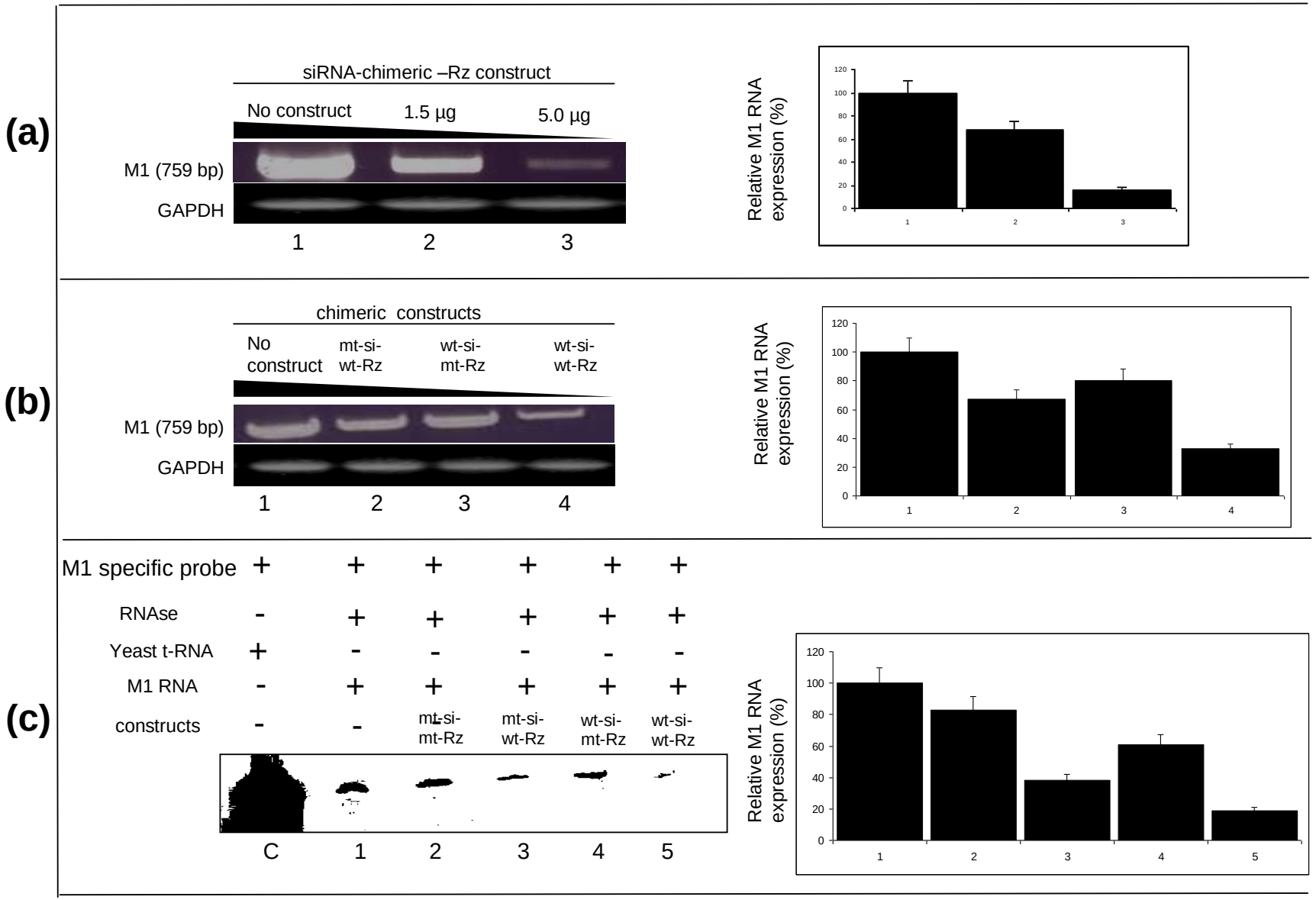
RT PCR analysis with 1μg RNA

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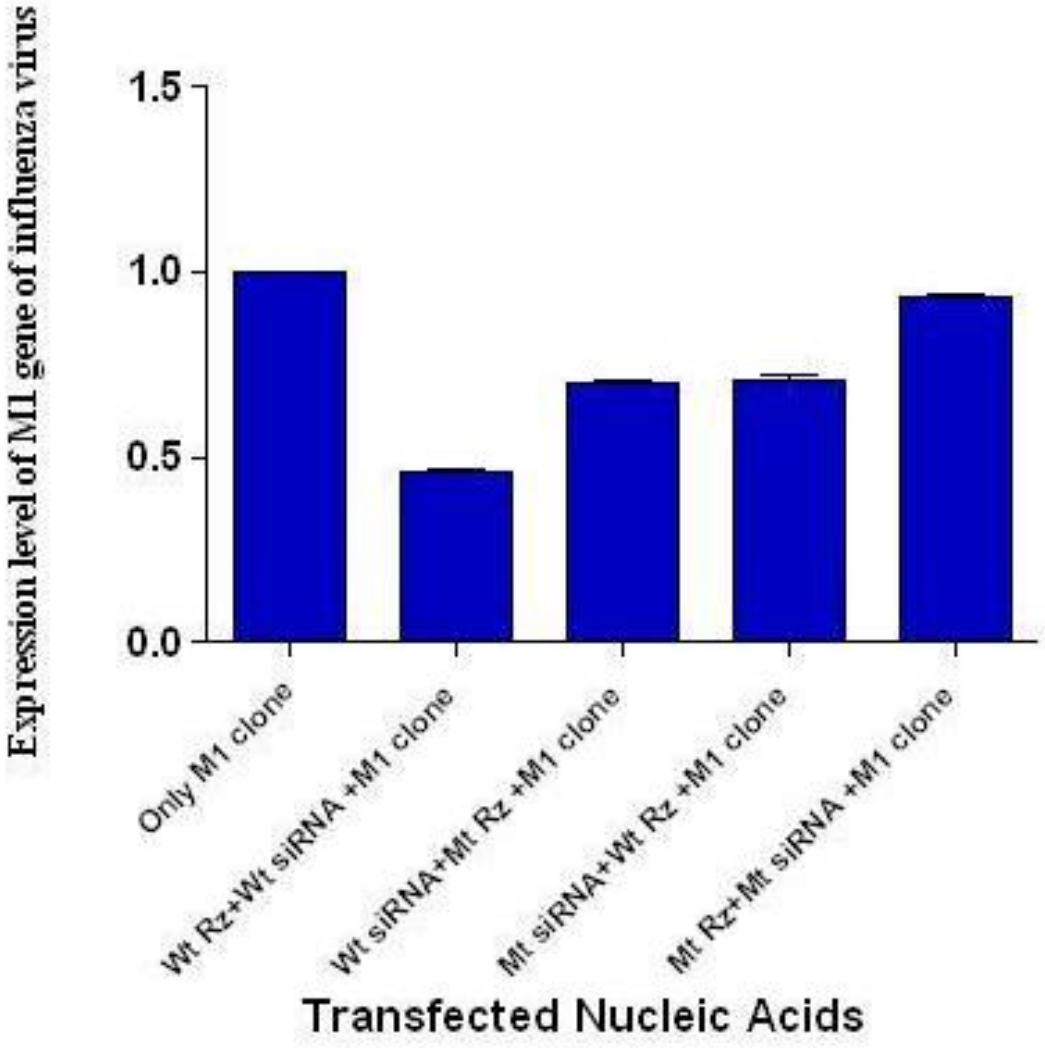
Real Time PCR analysis with 20ng RNA

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Ribonuclease Protection Assay with 5μgRNA



Intracellular reduction in expression of M1 RNA with chimeric constructs in MDCK cells: RNA level in MDCK cells were shown by RT PCR and RPA.



Real Time RT PCR to show the modulation of expression level of M1 gene of influenza virus in presence of various siRNA-Rz constructs: SYBR Green based real time RT PCR was performed in triplicate and the level of M1 RNA in various experiments was compared by calculating ΔΔCt value for each experiment. The RNA level of M1 in cells transfected with only M1 clone was taken as the reference whose value was "1" and RNA levels in all other transfected cells were compared with it.

MDCK cells grown to a confluency of 70-80% in 6 well plate

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Transfection with 5 μg of siRNA-Rz construct

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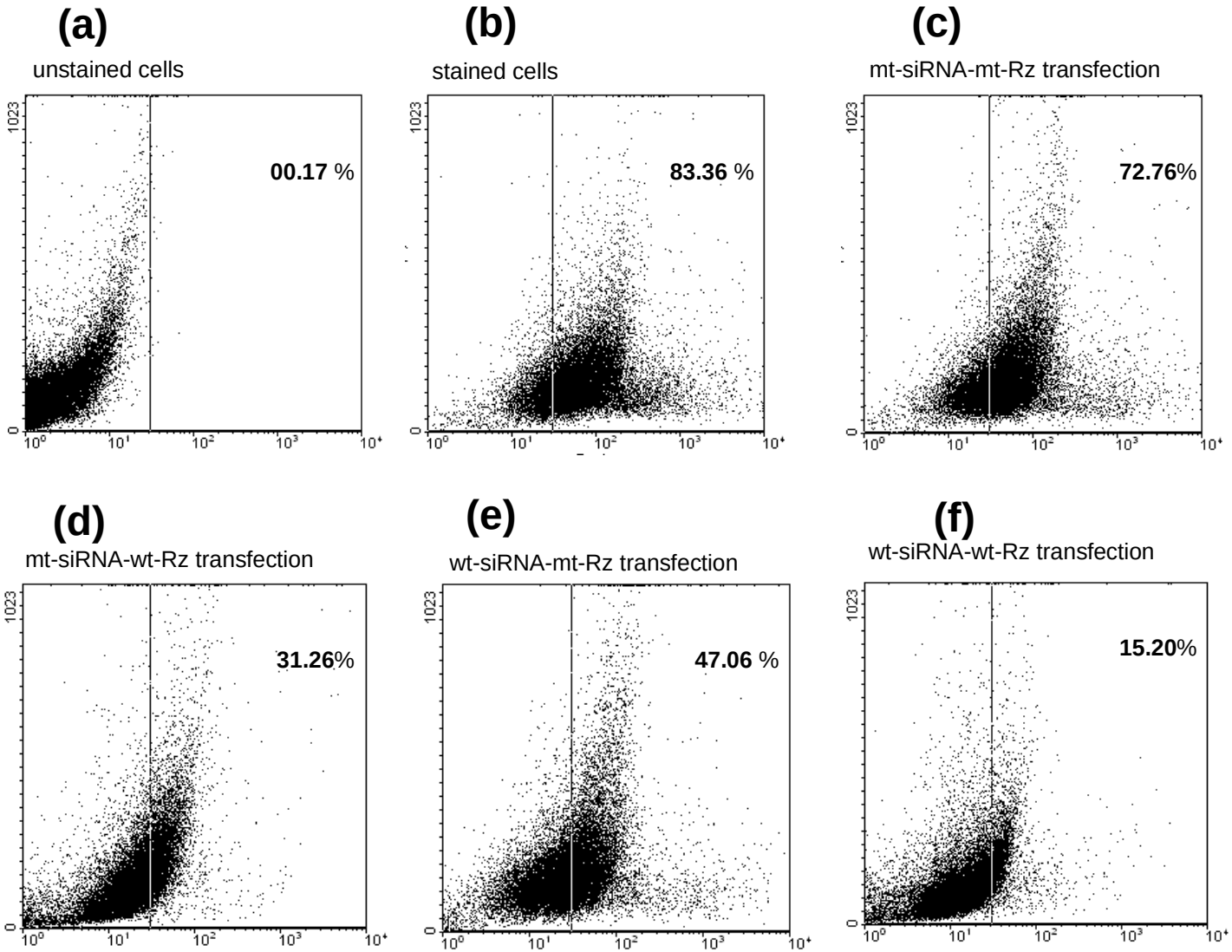
Infection of the transfected cells with inffluenza A virus (A/PR/8/34) at 24h post transfection

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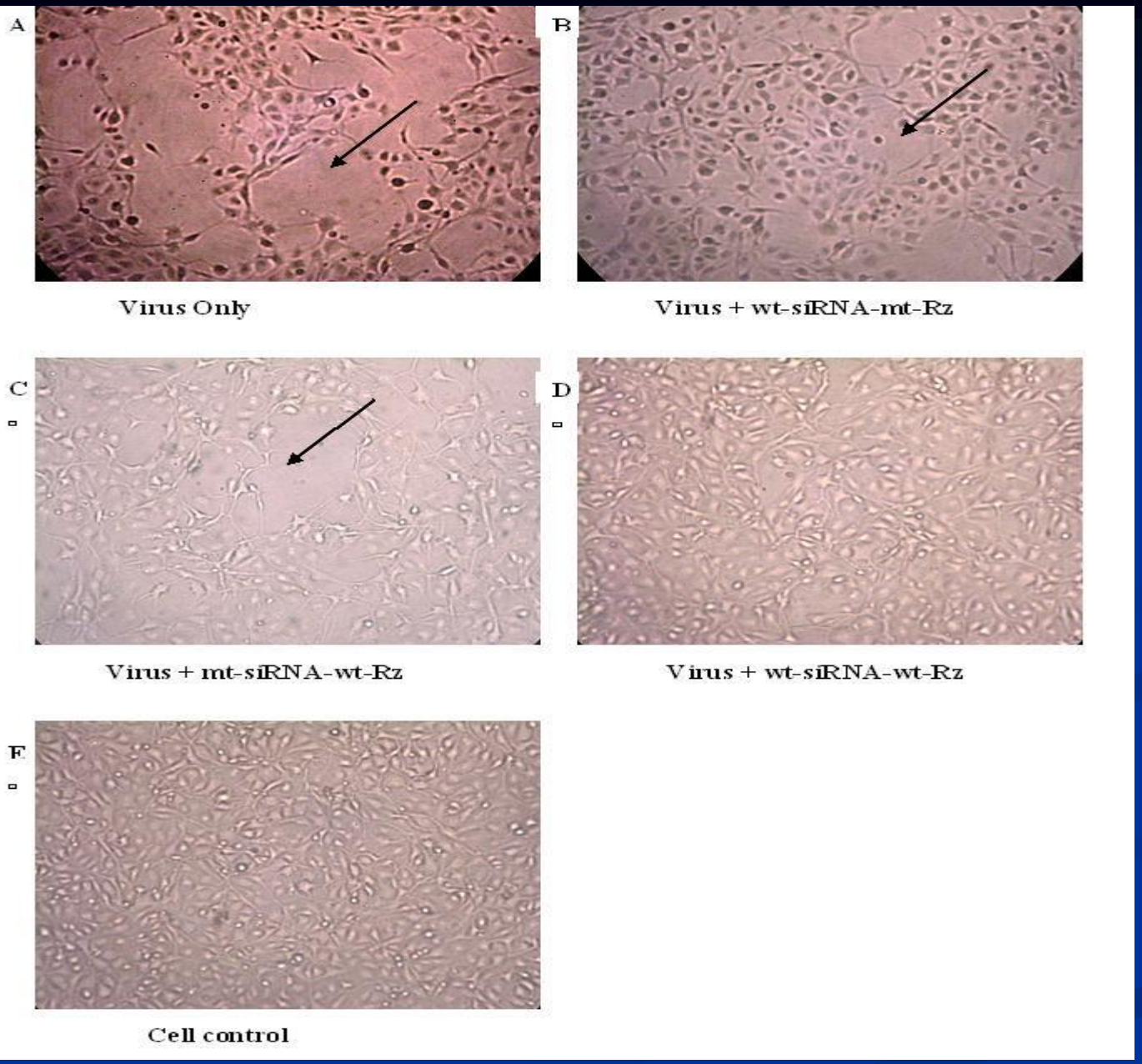
FACS analysis of the infected cells at 10h post infection for viral protein expression using M protein specific antibodies

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Another group of cells observed for the cytopathic effects



Wild-type mutant constructs show inhibition of virus-specific immuno-fluorescence: Protection against Influenza A/PR8/34 virus challenge using chimeric constructs was studied using MDCK cells. The various siRNA-chimeric-Rz constructs (5µg/ml) were transfected as indicated at the top of each panel and then challenged with a fixed dose of virus (MOI of 0.1). The cells were harvested 10 hours post infection and viral protein levels were monitored by FACS analysis. Panels a to f represent the type of constructs used for transfection and the levels were monitored by FACS analysis



Treatment of cell line with chimeric constructs inhibits the multiplication of virus (infected at a MOI of 0.1) and reduces its cytopathic effect (CPE).

Acknowledgement

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Department of Science & Technology, G.O.I


Thank You