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(54) **RT-LATE-PCR**

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2006.

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(57) **ABSTRACT**

An assay comprising more than one primer pair and more
than one detection probe, a low copy number synthetic ampli-
con corresponding to each of the primer pairs. The assay can
detect and distinguish between various sub-types and strains
of an influenza virus using any suitable nucleic acid amplifi-
cation technique. Related kits and methods are also
described.

Fig. 1

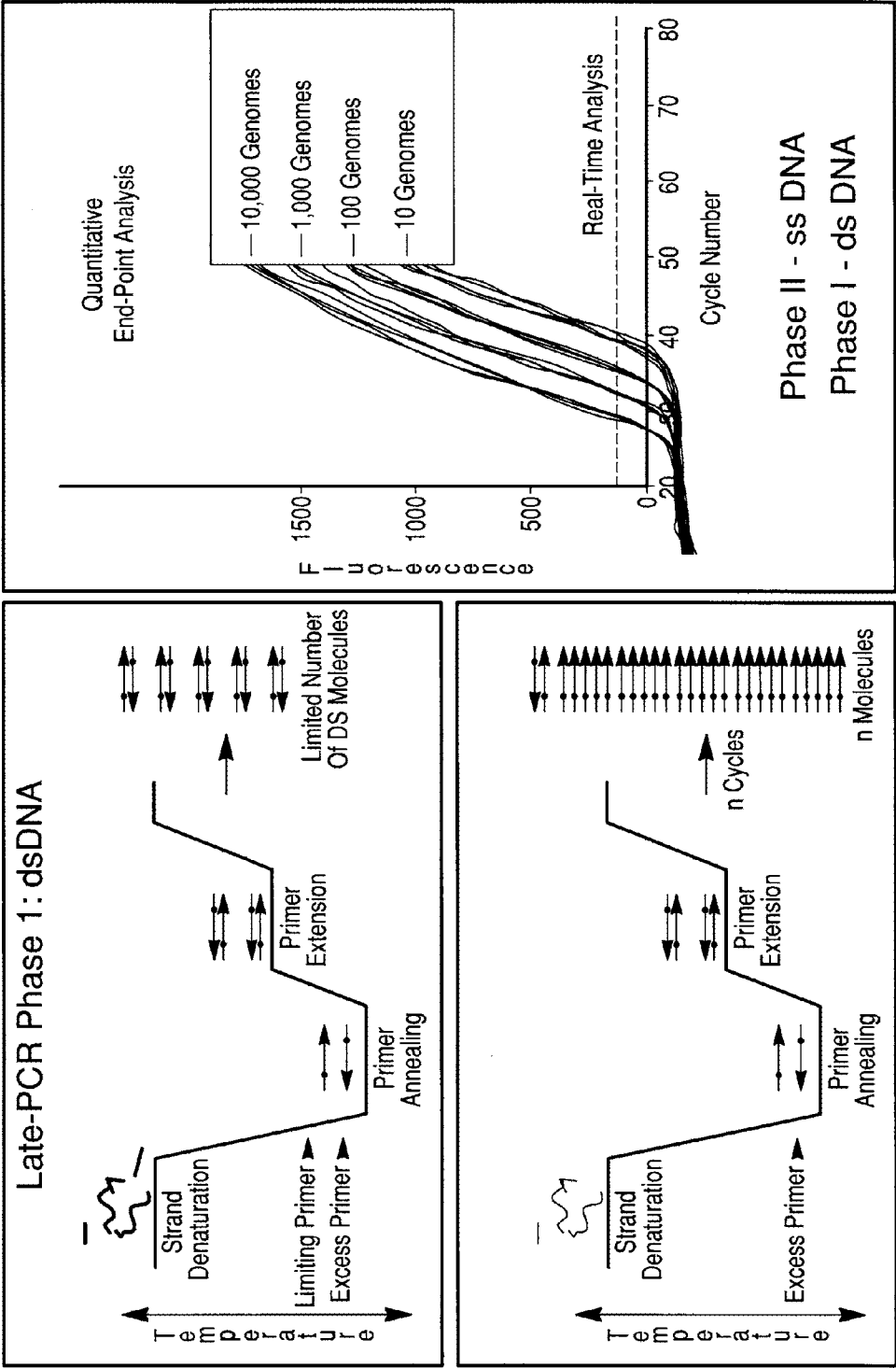


FIGURE 2

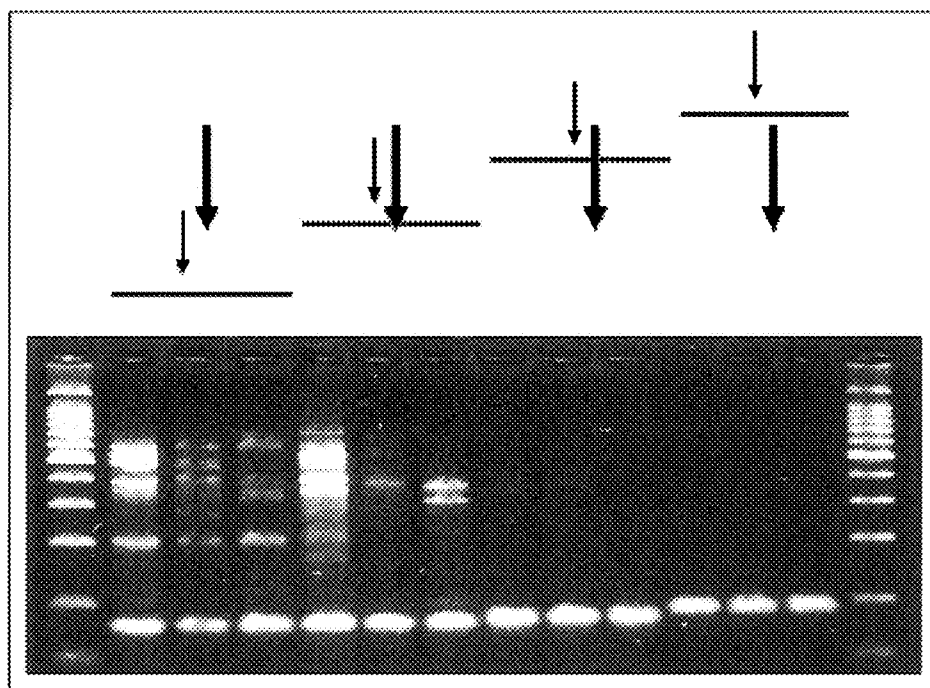


FIGURE 3

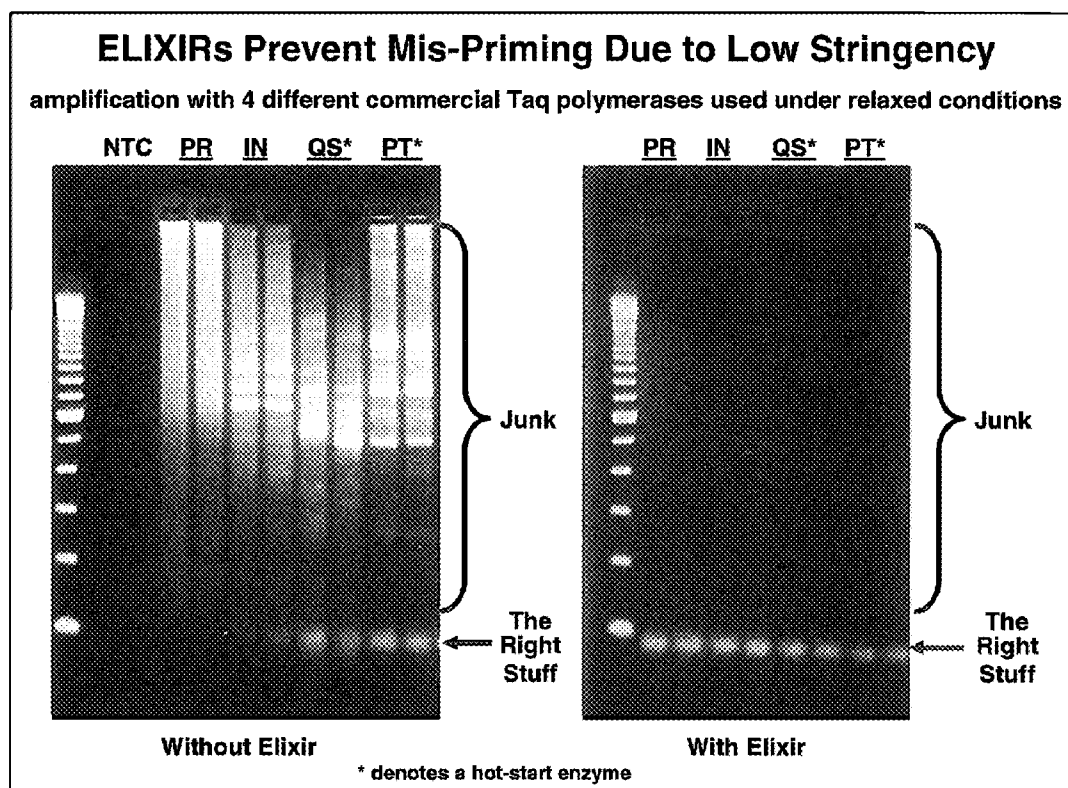
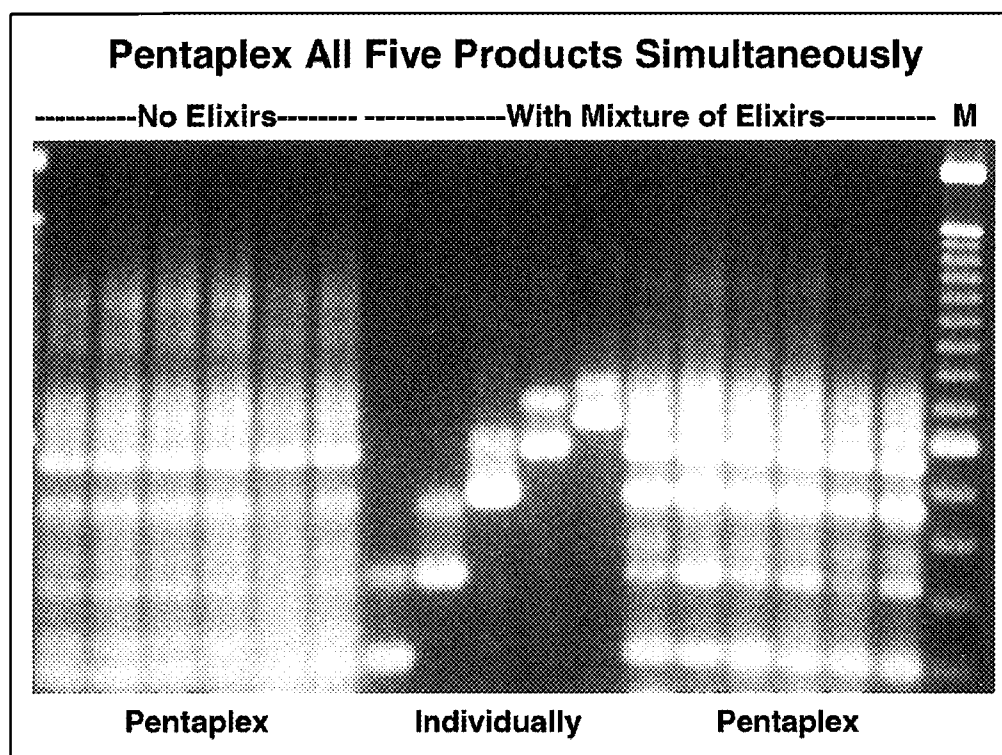


FIGURE 4



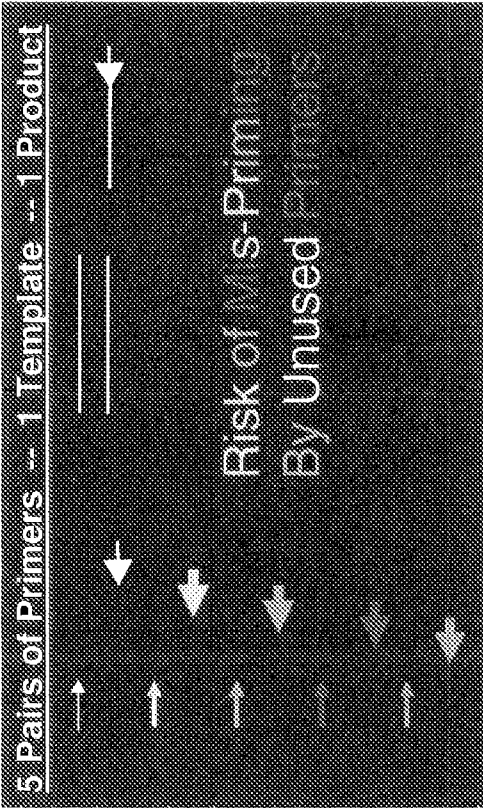
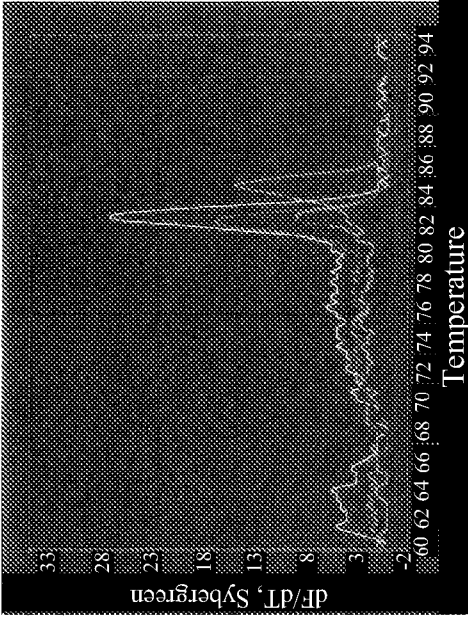
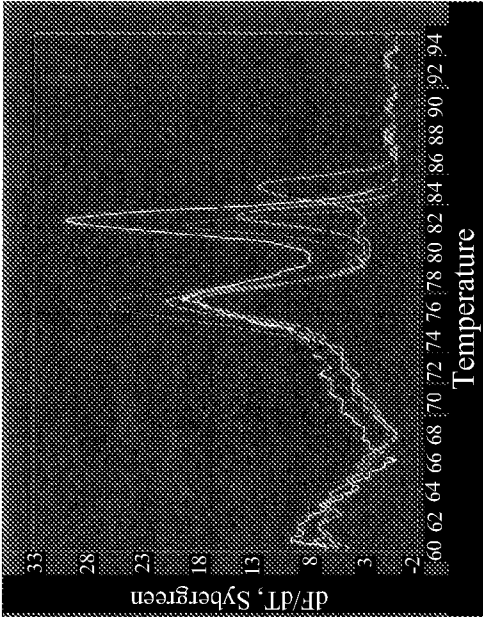


FIGURE 5a

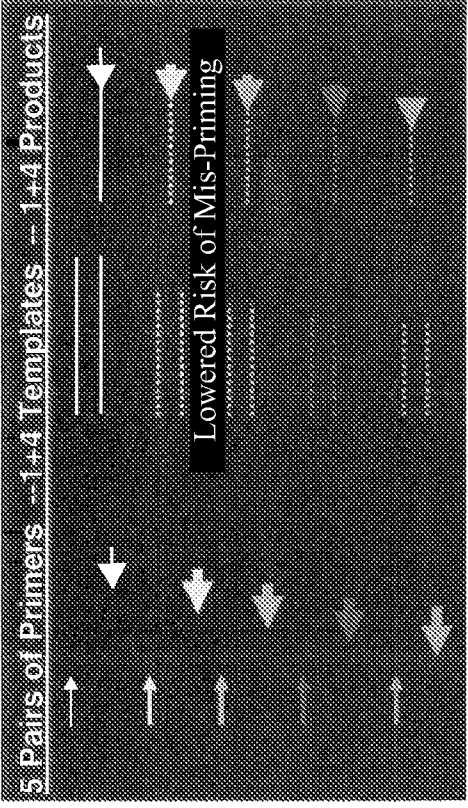


FIGURE 5b

FIGURE 6

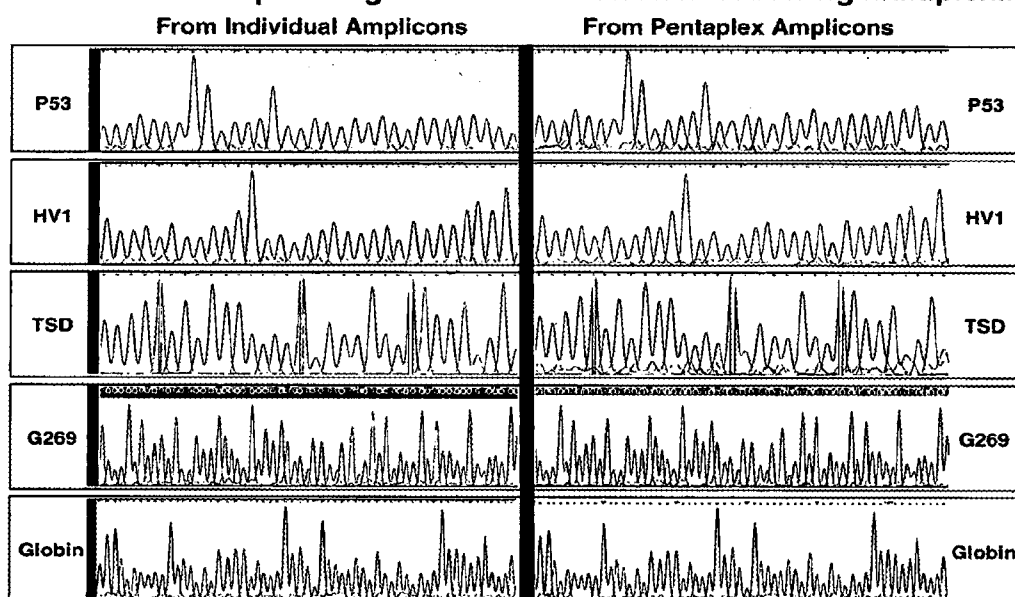
Dilute-'N'-Go Sequencing for Product Detection following Multiplexing

Fig. 7

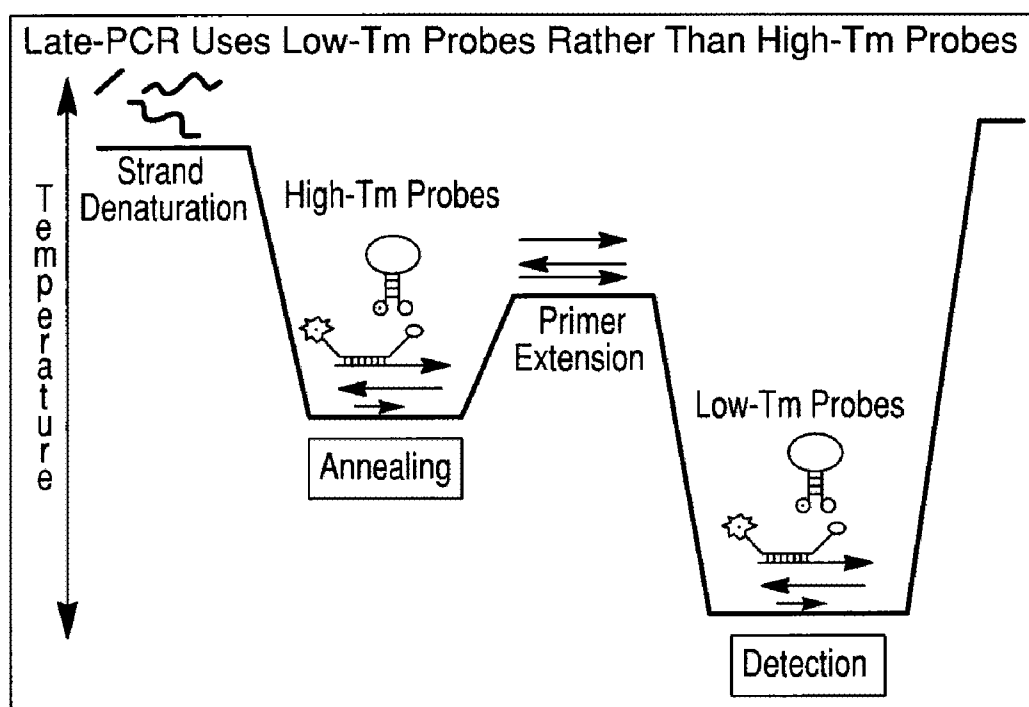
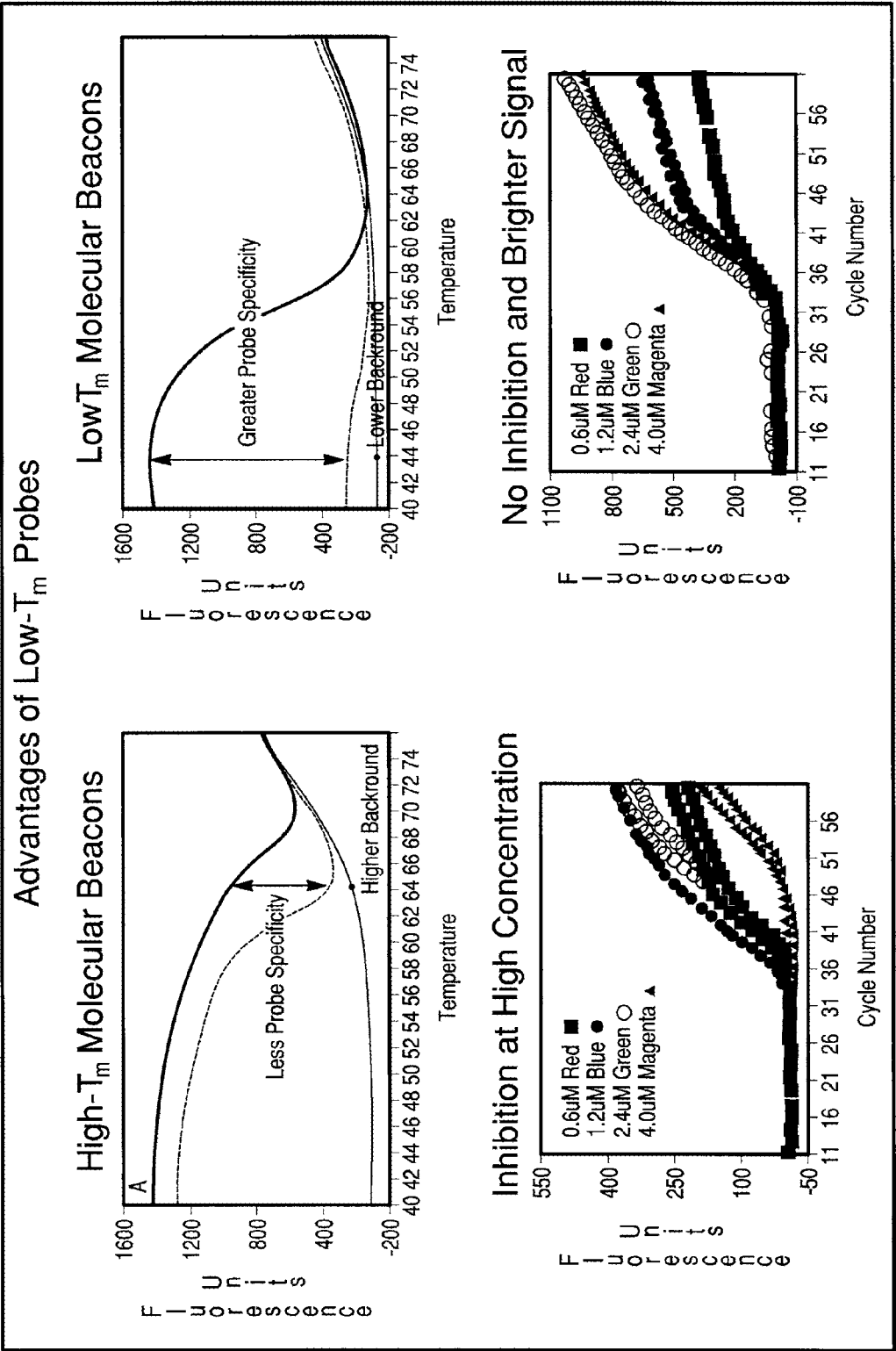


Fig. 8



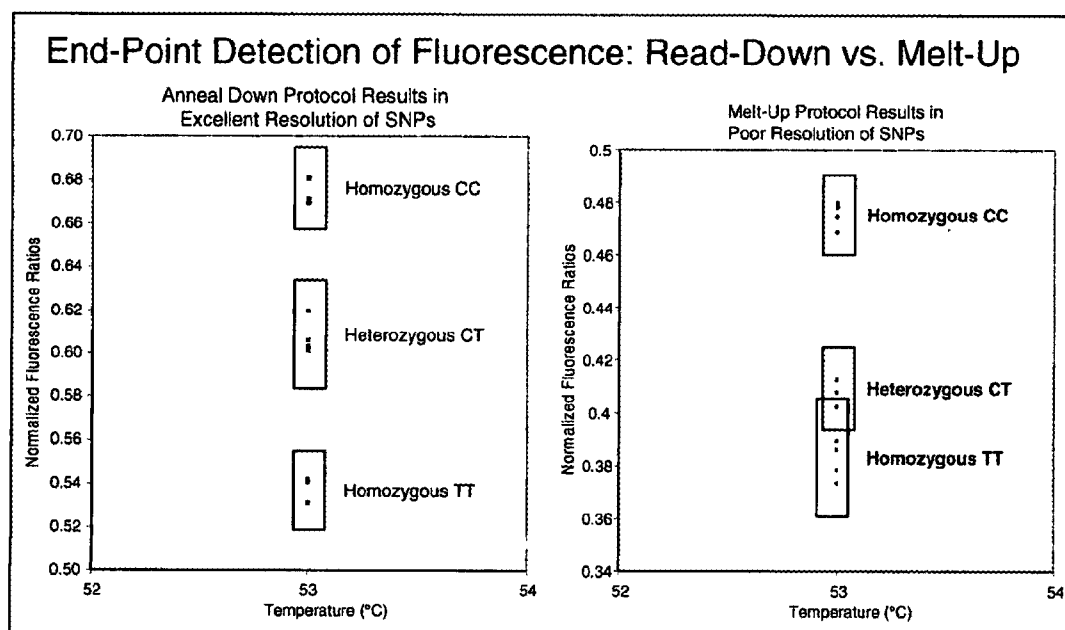


FIGURE 9

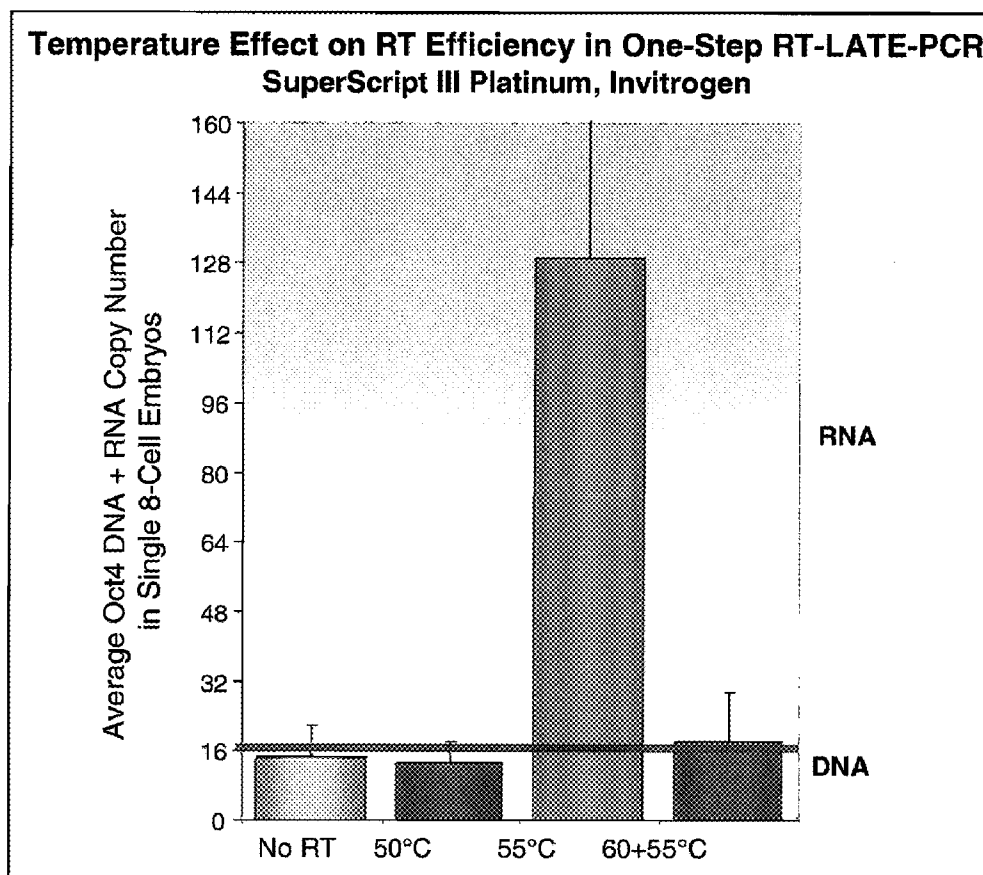


FIGURE 10

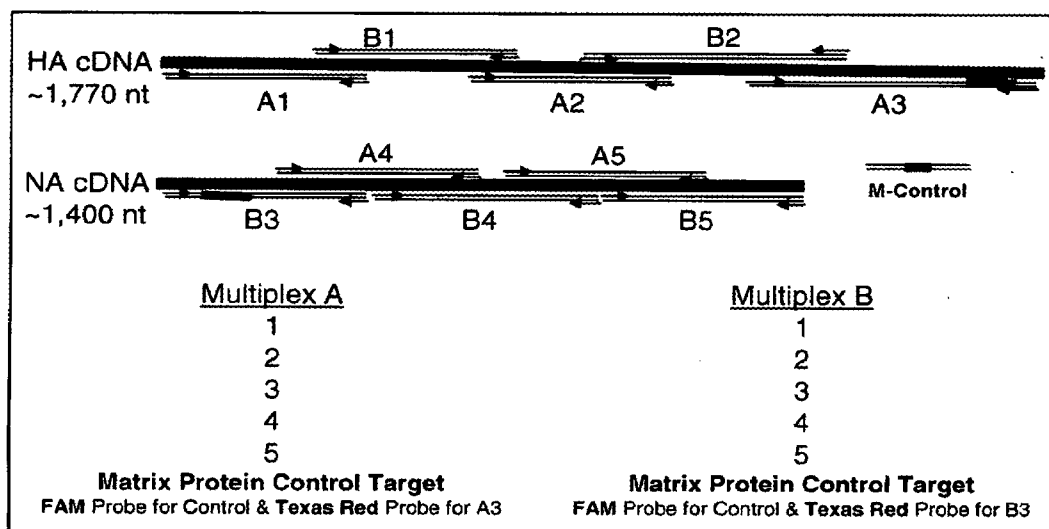


FIGURE 15

FIGURE 11

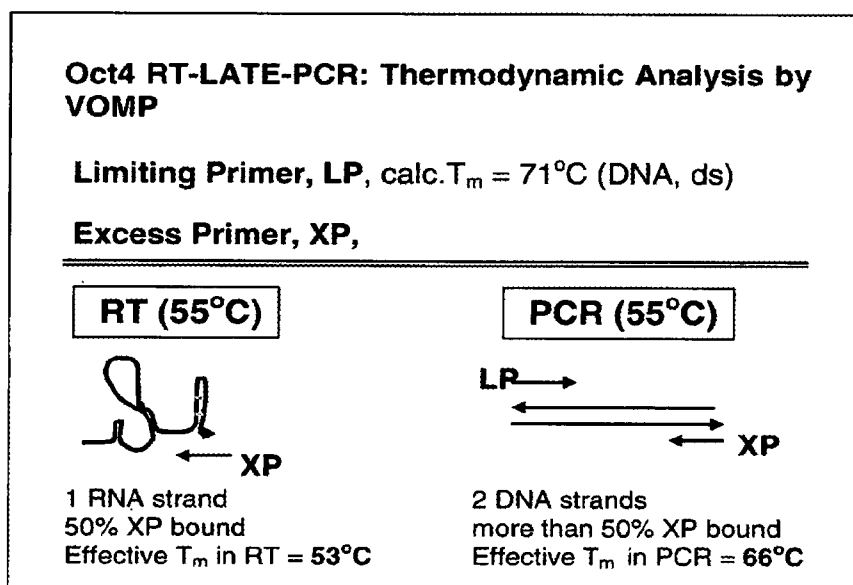


Fig. 12

Clustal comparison of Influenza Virus M RNA Sequences M RNA Segment

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	H3N2M	989	2	H1N1M	985	91
1	H3N2M	989	3	H5N1M	1039	86
1	H3N2M	989	4	BM	1076	5
2	H1N1M	985	3	H5N1M	1039	87
2	H1N1M	985	4	BM	1076	5
3	H5N1M	1039	4	BM	1076	4

H3N2M	-----TAGATATTGAAAGATGAGCCTTCTAACCGAGGTCGAAACGTATGTTCT	48
H1N1M	-----ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCT	35
H5N1M	AGCAAAAGCAGGTAGATGTTGAAAGATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCT	60
BM	-----ATGTCGCTGTTTGGAGACACAATTGCCTACCTGCT	35
	*** ** *	

H3N2M	CTCTATCGTTCATCAGGCCCTCAAAGCCGAAATCGCGCAGAGACTTGAAGATGTCTT	108
H1N1M	CTCTATCGTCCCGTCAGGCCCTCAAAGCCGAGATCGCACAGAGACTTGAAATGTCTT	95
H5N1M	CTCTATCATCCCGTCAGGCCCTCAAAGCCGAGATCGCGCAGAACTTGAAGATGTCTT	120
BM	TTCATTGACAGAAGATGGAGAAGGCAAAGCAGAACTAGCAGAAAAATTACACTGTTGGTT	95
	** * ** *	

H3N2M	TGCTGGGAAAAACACAGATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCCT	168
H1N1M	TGCTGGAAAGAATACCGATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCCT	155
H5N1M	TGCAGGAAAGAACACCGATCTCGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCT	180
BM	CGGTGGGAAAGAATTTGACCTAGACTCTGCCCTGGAATGGATAAAAAACAAAGATGCTT	155
	* ** * ** *	

H3N2M	GTCACCTCTGACTAAGGGGATT--TTGGGGTTTGTGTTACGCTCACCGTGCCAGTGAG	226
H1N1M	GTCACCTCTGACTAAGGGGATT--TTAGGATTTGTGTTACGCTCACCGTGCCAGTGAG	213
H5N1M	GTCACCTCTGACTAAGGGGATT--TTGGGATTTGTATTACGCTCACCGTGCCAGTGAG	238
BM	AACTGATAT-ACAAAAAGCACTAATTGGTGCCTCTATCTGC-TTTTAAACCCAAAGAC	213
	* * * * * * * * * * * * * * * * * *	

H3N2M	CGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTCAATGGGAATGGGGATCCAAAT	286
H1N1M	CGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTTAATGGGAATGGGGATCCAAAT	273
H5N1M	CGAGGACTGCAGCGTAGACGCTTTGTCCAGAATGCCCTAAATGGAAATGGAGATCCAAAT	298
BM	CAGGAAAGAAAAAGAAGATTGAT---CACAGAGCCTCTATCAGGAATGGGAACAACAGC	269
	* * * * * * * * * * * * * * * * * *	

H3N2M	AACATGGACAAAGCAGTTAACTGTAT----AGAAAACCTTAAGAGGGAGATAACATTCC	341
H1N1M	AATATGGACAGAGCAGTTAACTGTAT----CGAAAGCTTAAGAGGGAGATAACATTCC	328
H5N1M	AATATGGATAGAGCAGTCAAGCTATAT----AAGAAGCTGAAAAGAGAAATAACATTCC	353
BM	AACA-AAAAAGAAAGGCCTGATTCTAGCTGAGAGAAAAATGAGAAGATGTGTGAGCTTTC	328
	** * * * * * * * * * * * * * * * *	

Fig. 12 (cont.)

H3N2M ATGGGGCCAAAGAAATAGCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGCATGGGCC 401
H1N1M ATGGGGCCAAAGAAATAGCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGTATGGGAC 388
H5N1M ATGGGGCTAAGGAGGTGCACTCAGCTACTCAACCGGTGCACTTGCCAGTTGCATGGGTC 413
BM ATGAAGCATTTGAAATAGCAGAAGGCCATGAAAGCTCAGCGCTACTATACTGTCTCATGG 388
*** ** * * * * * * * * * * * * * *

H3N2M TCATATACAATAGGATGGGGCTGTAACCACCGAAGTGGCATTGTCCTGGTATGTGCAA 461
H1N1M TCATATACAACAGGATGGGGCTGTGACCACCGAATCAGCATTGTCCTGGTATGTGCAA 448
H5N1M TCATATACAACAGGATGGGAACGGTGACTACGGAAGTGGCTTTTGGCCTAGTGTGTGCCA 473
BM TCATGTACCTGAATCCTGGAAATTATTCAATGCAAGTAAACTAGGAACGCTCTGTGCTT 448
**** * * * * * * * * * * * * * *

H3N2M CATGTGAACAGATTGCTGACTCCCAGCACAGGTCTCATAGGCAAATGGTGGCAACAACCA 521
H1N1M CCTGTGAACAGATTGCCGACTCCCAGCATAAGTCTCATAGGCAAATGGTAAACAACAACCA 508
H5N1M CTTGTGAGCAGATTGCAGATTCACAGCATCGGTCTCACAGACAGATGGCAACTACCACCA 533
BM TGTGCGAGAAACAAGCATCACATTACACAGGGCTCATAGCAGAGCAGCGAGATCTTCAG 508
* * * * * * * * * * * * * * * * *

H3N2M ATCCATTAATAAAACATGAGAACAGGATGGTTTGGCCAGCACTACAGCTAAAGCTATGG 581
H1N1M ACCCATTAATAAGACATGAGAACAGAATGGTTCTGGCCAGCACTACAGCTAAGGCTATGG 568
H5N1M ACCCACTAATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTACAGCTAAGGCTATGG 593
BM TGCCCTGGAGTGAGACGAGAAATGCAGATGGTCTCAGCTATGAACACAGCAAAAACAATGA 568
* * * * * * * * * * * * * * * * *

H3N2M AGCAAATGGCTGGATCAAGTGA-----GCAGGCAGCGGAGGCCATGGAGATTGCTAGTC 635
H1N1M AGCAAATGGCTGGATCGAGTGA-----ACAAGCAGCTGAGGCCATGGAGGTTGCTAGTC 622
H5N1M AGCAGATGGCAGGATCAAGTGA-----GCAGGCAGCGGAAGCCATGGAGATCGCTAATC 647
BM ATGGAATGGGAAAAGGAGAAGACGTCCAAAACACTGGCAGAAAGAGCTGCAAAGCAACATTG 628
* * * * * * * * * * * * * * * * *

H3N2M AGG--CCAGG--CAAATGGTGCAGGCAATGAGAACC-GTTGGGACTCATCCTAGCTCCAG 690
H1N1M AGG--CCAGG--CAGATGGTGCAGGCAATGAGAGCC-ATTGGGACTCATCCTAGCTCTAG 677
H5N1M AGG--CTAGG--CAGATGGTGCAGGCAATGAGGACA-ATTGGGACTCATCCTAACTCTAG 702
BM GAGTACTGAGATCTCTTGGGGCAAGTCAAAGAATGGAGAAGGAATTGCAAAGGATGTAA 688
* * * * * * * * * * * * * * * * *

H3N2M TACTGGTCTAAGAGATGATCTTCTTGAAATTTGCAGACCTATCAGAAACGAATGGGGGT 750
H1N1M CACTGGTCTGAAAAATGATCTCCTTGAAATTTGCAGGCCTATCAGAAACGAATGGGGGT 737
H5N1M TGCTGGTCTGAGAGATAATCTTCTTGAAATTTGCAGGCCTACCAGAAACGAATGGGAGT 762
BM TGGAAGTGCTAAAGCAGAGCTCTATGGGAAATT-CAG--CTCTTGTGAAGAAATATCTAT 745
* * * * * * * * * * * * * * * * *

H3N2M GCAGATGCAACGATTCAAGTGACCTGCTTGTGTTGCTGCGAGTATCATTGGGATCTTGC 810
H1N1M GCAGATGCAACGATTCAAGTGATCCTCTTGTGTTGCCGCAAGTATAATTGGGATTTGTC 797
H5N1M GCAGATGCAACGATTCAAGTGATCCTATTGTTGTTGCCGCAAGTATCATTGGGATCTTGC 822
BM AATGCTCGAACCATTTCAG--ATTCTTTCAATTTGTTCTTTTATCTTATCAG--CTCTCC 801
* * * * * * * * * * * * * * * * *

H3N2M ACTTGATATTGTGGATTCTTGATCGTCTTTTTTTTCA-AATGCATCTATCGACTCTTCAA 869
H1N1M ACCTGATATTGTGGATTATTGATCGCCTTTTTTTCCA-AAAGCATTATCGTATCTTTAAA 856
H5N1M ACTTGATATTGTGGATTCTTGATCGTCTTTTTCTTCA-AATGCATTATCGTCGCCTTAAA 881

Fig. 12 (cont.)

```
BM      ATTTTCATGGCTTGGACAATAGGGCATTGAATCAAATAAAAAGAGGAGTAAACATGAAAA 861
        *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
H3N2M   CACG-----GCCTGAAAAGAGGGCCTTCTACGGAAGGAGTACCTGAGTCTATGAGGG 921
H1N1M   CACG-----GTTTAAAAAGAGGGCCTTCTACGGAAGGAGTACCAGAGTCTATGAGGG 908
H5N1M   TACG-----GTTTAAAAAGAGGGCCTGCTACGGCAGGGGTACCTGAGTCTATGAGGG 933
BM      TACGAATAAAAAGTCCAAACAAAGAGACAATAAACAGAGAGGTATC--AATTT-TGAGAC 918
        ***          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
H3N2M   AAGAATATCGAAAGGAACAGCAGAATGCTGTGGATGCTGACGGCAGT-CATTTTGTTCAGC 980
H1N1M   AAGAATATCGAGAGGAACAGCAGAATGCTGTGGATGCTGACGATGGT-CATTTTGTTCAGC 967
H5N1M   AAGAGTACCGGCAGGAACAGCAGAGTGCTGTGGATGTTGACGATGGT-CATTTTGTCAAC 992
BM      ACAGTTACCAAAAAGAAATCCAGGCCAAAG-AAACAATGAAGGAGGTACTCTCTGACAAC 977
        *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
H3N2M   ATAGAGCTG----- 989
H1N1M   ATAGAGCTG--GAGTAAAAA----- 985
H5N1M   ATAGAATTG--GAGTAAAAAACTACCTTGTCTACTAATACGGAAGAC----- 1039
BM      ATGGAGGTATTGAGTGACCACATAATAATTGAGGGGCTTTCTGCCGAAGAGATAATAAAA 1037
        *  *  *  *
H3N2M   -----
H1N1M   -----
H5N1M   -----
BM      ATGGGTGAAACAGTTTTTGGAGATAGAAGAATTGCATTAA 1076
```

Fig. 13

Clustal comparison of Influenza Virus HA RNA Sequences

HA RNA Segment

SeqA	Name	Len (nt)	SeqB	Name	Len (nt)	Score
=====						
1	H3N2HA	1718	2	H5N1HA	1736	28
1	H3N2HA	1718	3	H1N1HA	1692	37
1	H3N2HA	1718	4	BHA	1038	3
2	H5N1HA	1736	3	H1N1HA	1692	64
2	H5N1HA	1736	4	BHA	1038	4
3	H1N1HA	1692	4	BHA	1038	5
=====						
H5N1HA	-----TCATCTGTCAAATGGAGAAAATAGTGCTTCTTTTTTG					36
H1N1HA	-----ATGAAA--GCAAAACTACTGGTCCTGTTAT					28
H3N2HA	TATTAACCATGAAGACTATCATTGCTTTGAGCTACATTCTATGTCTGGTTTTCGCTCAAA					60
BHA	-----					
H5N1HA	CAATAGTCAGTCTTGTTAAAAGTGATCAGA---TTTGCATTGGTTACCATGCAAACAAC					93
H1N1HA	GTACATTTACAGCTACATATGCAGACACAA---TATGTATAGGCTACCATGCCAACAACT					85
H3N2HA	AACTTCCCGGAAATGACAACAGCACGGCAACGCTGTGCCTTGGGCACCATGCAGT--ACC					118
BHA	-----					
H5N1HA	CGACAGA--GCAGGTTGACACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGA					151
H1N1HA	CAACCGA--CACTGTTGACACAGTACTTGAGAAGAATGTGACAGTGACACACTCTGTCAA					143
H3N2HA	AAACGGAACGATAGTGAAAACAATCACGAATGACCAAATTGAAGTTACTAATGCTACTGA					178
BHA	-----					
H5N1HA	CATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAAT					211
H1N1HA	CCTACTTGAGGACAGTCACAACGGGAAACTATGTCTACTAAAAGGAATAGCCCCACTACA					203
H3N2HA	GCTGGTTTCAGAGTTCCTCAACAGGTGGAATATGCGA--CAGTCCTCATCAGATCCTTGAT					236
BHA	-----					
H5N1HA	TTTGAGAGATTGTAGTGTAGCTGGATGG-CTCCTCGGAAACCCAATGTGTGACGAATTCA					270
H1N1HA	ATTGGGTAATTGCAGCGTTGCCGGATGG-ATCTTAGGAAACCCAGAATGCCGAATTACTGA					262
H3N2HA	GGAGA-AACTGCA-CACTAATAGATGCTCTATTGGGAGACCCTCAGTGTGATGGCTTCC					294
BHA	-----					
H5N1HA	TCAATGTGCCGGAATGGTCCTACATAGTGGAGAAGGCCAATCCAGTCAATGACCTCTGTT					330
H1N1HA	TTTCCAAGGAATCATGGTCCTACATTGTAGAAACACCAAATCCTGAGAATGGAACATGTT					322
H3N2HA	AAAATAAGAAATG--GGACCTTTTTGTGAACGCAGCAAAGCCT---ACAGCAACTGTT					348

Fig. 13 (cont.)

BHA -----

H5N1HA ACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTATTGAGCAGAATAAACCAT 390
H1N1HA ACCCAGGGTATTTTCGCCGACTATGAGGAACTGAGGGAGCAATTGAGTTCAGTATCTTCAT 382
H3N2HA ACCCTTATGATGTGCCGGATTATGCCTCCCTTAGGTCAGTGTGCCTCATCCGGCACAC 408
BHA -----

H5N1HA TTGAGAAAATTCAGATCATCCCCAAA--AGTTCCTGGTCCAGTCATGAAGCCTCATTAG 447
H1N1HA TTGAGAGATTCGAAATATTCGCCAAAGAAAGCTCATGGCCCAACCAC--ACCGTAACCG 439
H3N2HA TGGAGTTTAAACAATGAAAGCTTCAA-----TTGACTGGAGTC--ACTCAAAATG 456
BHA -----GATCGAATCTGCACTG 16

H5N1HA GGGTGAGCTCAGCATGTCCATACCAGAGAA--AGTCCTCCTTTTTCAGA--AATGTGGTA 503
H1N1HA GAGTATCAGCATCATGCTCCCATAATGGGA--AAAGCAGTTTTTACAGA--AATTTGCTA 495
H3N2HA GAACAAGCTCTGCTTGTAAGAGGAGATCTA--ATAACAGTTTCTTTAGT--AGATTGAAT 512
BHA GGATAACATCTTCAAACCTCACCTCATGTGGTCAAAACAGCTACTCAAGGGGAGGTCAATG 76
* * * * *

H5N1HA TGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAAC 563
H1N1HA TGGCTGACGGGGAAGAATGGTTTGTACCCAAACCTGAGCAAGTCCTATGTAAACAACAAA 555
H3N2HA TGGTTGACCCACTTAAATTCAAATACCCAGCATTGAACGTGACTATGCCAAACAATGAA 572
BHA TGAAGTGTGTGATACCACTGACAACAAC-ACCAACAAAATCTTATTTTGCAAATCTCAAA 135
** * * * *

H5N1HA CAAGAAGATCTTTTGGTACTGTGGGGGATTACCATCCTAATGATGCGGCAGAGCAGACA 623
H1N1HA GAGAAAGAAGTCCTTGTAATGAGGGGTGTTTCATCACCCTGCTAATAGGGGACCAAGG 615
H3N2HA AAATTTGACAAATTGTACATTTGGGGGGTTTACCACCCGGGTACGGACAATGACCAAAAT 632
BHA GGAACAAGGACCAGAGGGGAAACTATGCCAGACTGTCTCAACTGTACAGATCTGGATGTG 195
* * *

H5N1HA AAGCTCTATCAAAACCCAACCACCTATATT---TCCGTTGGGACATCAACACTAAACCAG 680
H1N1HA GCCCTCTATCATAAGAAAATGCTTATGTC---TCTGTAGTGTCTTCA-CATTATAGCAG 671
H3N2HA AGCCTATAT--GCTCAAGCTTCAGGAAGA--ATCACAGTCTCTACCAAAAGAAGCCAA 686
BHA GCCTTGGGCAGGCCAATGTGTGTGGGGACCACACCTTCTGCGAAAGCTTCAATACTCCAC 255
* * * *

H5N1HA A-GATTG--GTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATG 737
H1N1HA AAGATTG--ACCCAGAAAATAGCCAAAAGACCCAAAGTAAGAGATCAGGAAGGAAGAATC 729
H3N2HA CAAACTGTAATCCCGAATATCGGATCTAGACCCAGGGTAAGGGATATCCCGAGCAGAATA 746
BHA GAAGTCA--GACCTGTTACATCCGGGTGCTTTCCTATAATGCACGACAGAACAAAAATC 312
* * * *

H5N1HA GAGTTCTTCTGGACAATTTTAAAAACGAATGATGCAATCAACTTCGA--GAGTAATGGAA 795
H1N1HA AACTACTACTGGACTCTGCTGGAACCTGGGGATACAATAATATTTGA--GGCAAATGGAA 787
H3N2HA AGCATCTATTGGACAATAGTAAAACCGGGAGACATACTTTTGATTAA--CAGCACAGGGA 804
BHA AG--GCAACTAGCCAATCTTC--TCAGAGGATATGAAAATATCAGGTTATCAACCCAAA 367
* * * *

Fig. 13 (cont.)

H5N1HA ATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATTATGA 855
H1N1HA ATCTAATAGCGCCATGGTATGCTTTTGCAGTCTGAGTAGAGGCTTTGGATCAGGAATCATCA 847
H3N2HA ATCTAATTGCTCCTCGGGTTACTTCAAAATACGAAGTGGGAAAAGCTCAATAA---TGA 861
BHA ACGTTATCGATGCAGAAAAGGCACAGGAGGACCCTACAGACTTGGAACTCAGGATCTT 427
* * * * *

H5N1HA AAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACCTCCAATGGGGGCGATAA 915
H1N1HA CCTCAAATGCACCAATGGATGAATGTGATGCGAAGTGTCAAACACCTCAGGGAGCTATAA 907
H3N2HA GATCAGATGCACCCATGGCAAATGCAATTCTGAATGCATCACTCCAAATGGAAGCATTTC 921
BHA GCCCTAACGC---TACCAGTAAAGCGGATTT---TTCGCAACAAATGGCTTGGGCTGTCC 481
* * * * *

H5N1HA ACTCTAGTATGCCATTCCACAATATACACCCCTCTCACCATCGGGGAATGCCCCAAATATG 975
H1N1HA ACAGCAGTCTTCTTTCCAGAATGTACACCCAGTCACAATAGGAGAGTGTCCAAAGTATG 967
H3N2HA CCAATGACAAACCATTTCAAAATGTAAACAGGATCACATATGGGCGCTGTCCCAGATATG 981
BHA CAAAGGACAACAACAAAATGCAACGAAACCCACTAACAGTAGAAG--TACCATACATTTG 539
* * * * *

H5N1HA T-GAAATCAAACAGATTAGTCTTGGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGA 1034
H1N1HA T-CAGGAGTGCAAAATTAAGGATGGTTACAGGACTAAGGAACATCCC-----A 1014
H3N2HA T-TAAGCAAAACACTCTGAAATTGGCAACAGGGATGCGAAATGTACC-----A 1028
BHA TACAGAAGGGGAAGACCAAATTACTGTTTGGGGGTTCCATTTCAGATA-----AC 588
* * * * *

H5N1HA AGAAGAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTATAGAGGGAGGATGGCAG 1094
H1N1HA TCCATTCAATCCAGAGGTTTGTGGAGCCATTGCCGGTTTCATTGAAGGGGGGTGGACT 1074
H3N2HA GAGAAACAACTAGAGGCATATTTGGCGCAATCGCGGGTTTCATAGAAAATGGTTGGGAG 1088
BHA AAAACCCAAATGAAGAACCTCTATGGAG----ACTCAAATCCTCAAAGTTACCTCAT 643
* * * * *

H5N1HA GGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTACGCT 1154
H1N1HA GGAATGGTAGATGGGTGGTATGGTTATCATCATCAGAATGAGCAAGGATCTGGCTATGCT 1134
H3N2HA GGAATGGTGGATGGTTGGTACGGTTTCAGGCATCAAAATTCTGAGGGAATAGGACAAGCA 1148
BHA CTGCTAATGGAGTAACCACACA-TTATGTTTCTCAGATTGGCGGCTTCCCAGATCAAACA 702
* * * * *

H5N1HA GCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGATC 1214
H1N1HA GCAGATCAAAAAAGTACACAAAATGCCATTAACGGGATTACAAACAAGGTGAATTCTGTA 1194
H3N2HA GCAGATCTCAAAGCACTCAAGCAGCAATCAACCAAATCAATGGGAAGCTGAATAGGTTG 1208
BHA GAAGACGGAGGACTACCACAAAGCGGCAGAATTGTCTGTTGATTACATGATGCA---AAAA 759
* * * * *

H5N1HA ATTGACAAAATGAACACTCAGTTTGTAGGCCGTTGGAAGGGAATTTAACTTAGAAAGG 1274
H1N1HA ATTGAGAAAATGAACACTCAATTCACAGCTGTGGGCAAAGAATTCACAAAATTGGAAAGA 1254
H3N2HA ATCGGGAAAACCAACGAGAAATCCATCAGATTGAAAAAGAATTCAGAAGTAGAAGGG 1268
BHA CCTGGGAAAACAGGAAC--AATT---GTCTATCAAAGAGGTGTTTGTGTCCTCAAAAGG 814
* * * * *

H5N1HA AG-AATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAA 1333
H1N1HA AG-GATGGAAAACTTAAATAAAAAAGTTGATGATGGGTTTCTAGACATTTGGACATATAA 1313
H3N2HA AG-AATTGAGGACCTCGAGAAATATGTTGAGGACACTAAATAGATCTCTGGTCATACAA 1327
BHA TGTGGTGCAGGAGTGGCAGGAGCAAAGTAATAAAAGGGTCCTTGCTTTT--AATTGGTGA 872

Fig. 13 (cont.)

* * * * *

H5N1HA TGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTTCATGACTCAAATGT 1393
H1N1HA TGCAGAATTGTTGGTTCTACTGGAAAATGAAAGGACTTTGGATTTCCATGACTCCAATGT 1373
H3N2HA CGCGGAGCTTCTTGTGGCCCTGGAGAACCAACATACAATTGATCTAACTGACTCAGAAAT 1387
BHA AGCAGA-----TTGCCCTTCATGAAAAAT-----ACGGTGGATTAAACAAAAGCAAGCCT 921
* * * * *

H5N1HA CAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAAC TGGGTAA 1453
H1N1HA GAAGAATCTGTATGAGAAAGTAAAAAGCCAATTAAAGAATAATGCCAAAGAAATAGGAAA 1433
H3N2HA GAACAAACTGTTTGAAAGAACAAGAAGCAACTGAGGGAAAATGCTGAGGATATGGGCAA 1447
BHA TA-----CTACACAGGAGAACATGCAAA-AGCCATAGGAAATTGCCCA--ATATGGGTGA 973
* * * * *

H5N1HA CGGTTGTTTCGAGTTCTATCA-TAAATGTGATAATGAATGTATGGAAAGTGTAAGAAACG 1512
H1N1HA CGGGTGTTTTGAATTCTATCA-CAAGTGTAACAATGAATGCATGGAGAGTGTGAAAAATG 1492
H3N2HA TGGTTGTTTCAAAAATATACCA-CAAATGTGACAATGCCTGCATAGGGTCAATCAGAAATG 1506
BHA AAACACCTTTGAAGCTTGCCAATGGAACCAATATAGACCTCCTGCAAAACTATTAAAGG 1033
* * * * *

H5N1HA GAACGTATGACTACCCGCACTATTTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGTG 1572
H1N1HA GAACTTATGACTATCCAAAATATTCCGAAGAATCAAAGTTAAACAGGGAGAAAATTGATG 1552
H3N2HA GAACTTATGACCATGATGTATACAGAGATGAAGCATTAAACAACCGTTCCAGATCAAAG 1566
BHA AAAGG----- 1038
* *

H5N1HA GAGTAAATTTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCGA 1632
H1N1HA GAGTGAAATTTGGAATCAATGGGAGTCTATCAGATTCTGGCGATCTACTCAACTGTGCGCA 1612
H3N2HA GTGTTGAGCTGAAGTCAGGATACAAAGATTGGATTCCTATGGATTTTCTTTGCCATATCAT 1626
BHA -----

H5N1HA GTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGGT 1692
H1N1HA GTTCCCTGGTTCTTTTGGTCTCCCTGGGGGCAATCAGCTTCTGGATGTGTTCCAATGGGT 1672
H3N2HA GTTTTTTGCTTTGTGTTGTTTGTGGGG--TTCATCATGTGGGCCTGCCAAAAGGCA 1683
BHA -----

H5N1HA CGTTACAATGCAGAATTTGCATTTAAATTTGTGAGTTCAGATTG 1736
H1N1HA CTTTGCAGTGCAGAATATGC----- 1692
H3N2HA ACATTAGGTGCAACATTTGCATTTGAGTGCATTAA----- 1718
BHA -----

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
=====						
1	H1N1NA	1409	2	H5N1NA	1392	75
1	H1N1NA	1409	3	H3N2NA	1410	42
1	H1N1NA	1409	4	BNA	1463	16
2	H5N1NA	1392	3	H3N2NA	1410	33
2	H5N1NA	1392	4	BNA	1463	7
3	H3N2NA	1410	4	BNA	1463	18
=====						
H1N1NA	-----ATGAATCCAAATCAAAAAATAATAA					25
H5N1NA	TTATTGGTCTCAGGGAGCAAAAGCAGGAGTTCAAAATGAATCCAAATAAGAAGATAATAA					60
H3N2NA	-----ATGAATCCAAATCAAAAGATAATAA					25
BNA	-----CCAAATGAACAA					13
					** ** **	
H1N1NA	CCATTGGATCAATCAGTATAGCAATCGGAATAATTAGTCTAATGTTGCAAAATAGGAAATA					85
H5N1NA	CCATCGGATCAATCTGTATGGTAACTGGAATGGTTAGCTTAATGTTACAAATTGGGAACT					120
H3N2NA	CGATTGGCTCTGTTTCTCTCACCATTTCACAATATGCTTCTTCATGCAAATTGCCATCT					85
BNA	T-GCTACCTTCAACTATACAAACGTTAACCTTATTCTCACATCAGGGGAGTATTATTA					72
	*		*		* * *	
H1N1NA	TTATTTCAA-TATGGGCTAGTCACTCAATCCAAACTGGAAGTCAAAACCACACTGGAGTA					144
H5N1NA	TGATCTCAA-TATGGGTCACTCATTCAATTCACACAGGGAATCAACACAAAGCTG----					174
H3N2NA	TGATAACTACTGTAACATTGCATTTCAGCAATAT--GAATTCAAATCCCCCCCCAA--					140
BNA	TCACTATATGTGTCAGCTTCATTGTCATACT-TACTATATTTCGGATATATTGCTAAAAAT-					130
	* *		* *		*** * *	
H1N1NA	TGCAACCAAGAATCATCACATATGAAAAC-AGCACCTGGGTGAA-TCACACATATGTTA					202
H5N1NA	---AACCAA-----					180
H3N2NA	--CAACCAAG---TGATGCTGTGTGAACCA-ACAATAATAGAAAG-AAACATAACAGAGA					193
BNA	TCCCATCAACAGAAATTACTGCACCAACAATGCCATTGGATTGTGCAACCGCATCAATG					190
		*	***			
H1N1NA	ATATTAACAACACTAATGTTGTTGCTGGAAAGGACAAA-----ACTTCAG--TGACATT					254
H5N1NA	---TCAGCAATACTAATTTTCTTACTGAGAAAGCTGTG-----GCTTCAG--TAAAAAT					229
H3N2NA	TAGTGTATCTGACCAACACCACCATAGAGAAGGAAATAT-----GCCCAAACTAGCAGA					248
BNA	TTCAGGCTGTGAACCGTTCTTGCACAAAAGGGGTGACACTTCTTCTCCCAGAACCGGAGT					250
		*			* ** *	
H1N1NA	GGCCGGCAATTTCATCT-----CTTTGTTCTATCAGTGGATGGGCTATATACACAAAAG					307

Fig. 14 (cont.)

H5N1NA AGCGGGCAATTCATCT-----CTTTGCCCCATTAATGGATGGGCTGTATACAGTAAGG 282
H3N2NA ATACAGAAATTGGTCAAAGCCG-CAATGTAACATTACAGGATTTGCACCTTTTTCTAAGG 307
BNA GGACATACCCGCGTTTATCTTGCCCCGGCTCAACCTTTTCAGAAAGCACTCCTAATTAGCC 310
* * * * *

H1N1NA ACAACAGCATAAGAATTGGCTCCAAAGG--AGATGTTTTTGTCTATAAGAGAACCTTTCA 364
H5N1NA ACAACAGTATAAGGATCGGTTCCAAGGG--GGATGTGTTTGTATAAGAGAGCCATTCA 339
H3N2NA ACAATTCGATTAGGCTTTCCGCTGGTGG--GGACATCTGGGTGACAAGAGAACCTTATG 364
BNA CTCATAGATTTCGGAGAAACCAAGGAAACTCAGCTCCCTTGATAATAAGGGAACCTTTTA 370
* * * * *

H1N1NA TATCATGTTCTCACTTGGAATGCAGAACCTTTTTTCTGACCCAAGGTGCTCTATTAAATG 424
H5N1NA TCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTGAAGTCTCAGGGAGCCTTGCTGAATG 399
H3N2NA TGTCATGCGATCCTGACAAGTGTTATCAATTTGCCCTTGGGCAGGGAACAACACTAAACA 424
BNA TTGCTTGTGGACCAAAGGAATGCAACACTTTGCTCTAACCCTATGCAGCCCAACCAG 430
* * * * *

H1N1NA ACAAACATTCAAATGGGACCGTTAAGGACAGAAGTCCTTATAGGGCCTTAATGAGCTGTC 484
H5N1NA ACAAGCACTCCAATGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGTTGTC 459
H3N2NA ACGTGCATTCAAATGACACAGTACATGATAGGACCCCTTATCGGACCCTATTGATGAATG 484
BNA GGGGATACTACAATGGAACAAGAGGAGACAGAAACAAGCTGAGGCATCTAATTTTCAGTCA 490
* * * * *

H1N1NA CTCTAGGTGAAGCTCCGTCCCCATACAATTCAAAGTTTGAATCAGTTGCATGGTCAGCAA 544
H5N1NA CTGTGGGTGAGGCTCCCTCCCCATATAACTCAAGTTTGTAGTCTGTTGCTTGGTCAGCAA 519
H3N2NA AGTTAGGT--GTTCCATTTTCATCTGGGGACCAAGCAAGTGTGCATAGCATGGTCCAGCT 541
BNA AATTGGGCAAAATCCCAACAGTAGAAAACCTCCATTTTCCACATGGCAGCATGGAGCGGGT 550
* * * * *

H1N1NA GCGCATGCCATGATGGCATGGGCTGGTTAACAATCGGAATTTCTGGTCCAGACAATGGAG 604
H5N1NA GTGCTTGCCATGATGGCACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGG 579
H3N2NA CAAGTTGTACGATGGAAAAGCATGGCTGCATGTTTGTGTAAACGGGGGATGATAAAAATG 601
BNA CCGCATGCCATGATGGTAAGGAATGGACATATATCGGAGTTGATGGCCCTGACAATAATG 610
* * * * *

H1N1NA CTGTGGCTGTACTAAAATACAACGGCATAATAACTGAAACCATAAAAAAGTTGGAAAAAGC 664
H5N1NA CTGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGGAATA 639
H3N2NA CAACTGCTAGCTTCATTTACAATGGGAGGCTTGTAGATAGTATGTTTCATGGTCCAAAG 661
BNA CATTTGCTCAAAATAAAATATGGAGAAGCATATACTGACACATACCATTCTATGCAAACA 670
* * * * *

H1N1NA GAATATTAAGAACACAAGAGTCTGAATGTGTCTGTGTGAACGGGTGATGTTTACCATAA 724
H5N1NA ACATACTGAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAA 699
H3N2NA AAATCCTCAGGACCCAGGAGTCAGAATGCGTTTGTATCAATGGAACCTGTACAGTAGTAA 721
BNA ACATCCTAAGAACACAAGAAAGTGCCCTGCAATTGCATCGGGGGAATTTGTTATCTTATGA 730
* * * * *

Fig. 14 (cont.)

* * * * ** * * * *

H1N1NA GGACTGTATAAGACCTTGCTTCTGGGTTGAGTTAGTCAGAGGACTGCCTAGAGAAAATAC 1304
H5N1NA AGATTGCATAAGACCTTGTCTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAGCAC 1279
H3N2NA AAGCTGCATCAATCGGTGCTTTATGTGGAGTTGATAAGGGGAAGAAAAGAGGAACTGA 1304
BNA GAAATGTGATGTCCCCTGTATTGGGATAGAGATGGT-ACATGATGGTGGAAAAGAGACTT 1324
* * * * *

H1N1NA AACAACTCTGGACTAGTGGGAGCAGCATTTCTTTTTGTGGCGTAAATAGTGATACTGCAAA 1364
H5N1NA AA---TTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAATAGTGACACTGTGGG 1336
H3N2NA AGTCTTGTGGACCTCAAACAGTATTGTTGTGTTTTGTGGCACCTCAGGTACATATGGAAC 1364
BNA GGCACTCAGCAGCTACAGCCAT-TTACTGTTTAATGGGCTCAGGACAGCTGCTGTGGGAC 1383
* * * * *

H1N1NA CTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTACCATTTG-ACAA----- 1409
H5N1NA TTGGTCTTGGCCAGACGGTGCTGAATTGCCATTACCATTTG-ACAAGTAGTTGTTCA--- 1392
H3N2NA AGGCTCATGGCCTGATGGGGCGGACATCAATCTCATGCCTATATAA----- 1410
BNA ACTGTCACAGGT-GTTAATATGGCTCTGTAATGGAGGAATGGTTGAGTCTGTTCTAAACC 1442
* * * * *

H1N1NA -----
H5N1NA -----
H3N2NA -----
BNA CTTTGTTCCCTATTTTGTTTGA 1463

Fig. 16a

One Temperature End-Point Reading
Four colors with single color probes, some colors used twice
All probes read at 45C except as noted below
for internal controls which are read at 25C

I.	1	ic @ 25C	ic @ 25C	ic @ 25C	ic @ 25C	ic @ 25C	Most	Sign. @ 40C
II	1	Matrix	ic @ 25C	ic @ 25C	ic @ 25C	ic @ 25C	Unlikely	● ○ ○ ○
III	1 2	Matrix	H5 (no N1)	ic @ 25C	ic @ 25C	ic @ 25C		● ● ○ ○
IV	1 3	Matrix	ic @ 25C	H1 (no N1)	ic @ 25C	ic @ 25C		● ○ ● ○
V	1 4	Matrix	ic @ 25C	ic @ 25C	H3 (no N1)	H3 (no N1)		● ○ ○ ●
VI	1 55	ic @ 25C	ic @ 25C	B virus	B virus	B virus		● ○ ● ●
VII	1 2/6	Matrix	H5+N1 (H5/1 subtype)	ic @ 25C	ic @ 25C	ic @ 25C	H5N1	● ● ○ ○
VIII	1 3/6	Matrix	N1 (H5/1 subtype)	H1	ic @ 25C	ic @ 25C	H1N1	● ● ● ○
IX	1 6/4	Matrix	N1 (H5 subtype)	ic @ 25C	H3	H3	Unlikely	● ● ○ ●
X	1 6	Matrix	N1 (no HA)	ic @ 25C	ic @ 25C	ic @ 25C		● ● ○ ○
XI	1 4/7	Matrix	ic @ 25C	N2 (H3 subtype)	H3	H3	H3N2	● ○ ● ●
XII	1 7	Matrix	ic @ 25C	H1+N2 (H3)	ic @ 25C	ic @ 25C	Unlikely	● ● ● ○
XIII	1 7	Matrix	ic @ 25C	N2 (no HA)	ic @ 25C	ic @ 25C		● ● ● ○

FIGURE 16b

Condition	Probe Result	Assay Result
No Virus of Type A or Type B Internal Control indicates amplification has occurred.	 40°C 25°C	No virus
Type B HA or Type B NA detected, not both Plus Internal Control	 40°C 25°C	Type B virus HA or NA
Type B HA and Type B NA detected Plus Internal Control fully saturated	 40°C 25°C	Type B virus HA and NA
Type A H5N1 (of H5/H1 subtypes) Plus Internal Control and Type A M gene	 40°C 25°C	H5N1
Type A H1N1 (of H5/N1 subtypes) Plus Internal Control and Type A M gene	 40°C 25°C	H1N1
Type A H3N2 (of H3 subtype) Plus Internal Control and Type A M gene	 40°C 25°C	H3N2
Type A H5N2 (of H3 subtype) Plus Internal Control and Type A M gene	 40°C 25°C	H5N2
Type A H3N1 (of H5/N1 subtypes) Plus Internal Control and Type A M gene	 40°C 25°C	H3N1
Type A H1N2 (of H3 subtype) Plus Internal Control and Type A M gene	 40°C 25°C	H1N2
Type A no detectable Type A HA or NA Plus Internal Control and Type A M gene	 40°C 25°C	Type A Virus Serotype ?
Type A H5, no detectable NA Plus Internal Control and Type A M gene	 40°C 25°C	H5, no NA
Type A H1, no detectable NA Plus Internal Control and Type A M gene	 40°C 25°C	H1, no NA
Type A H3, no detectable NA Plus Internal Control and Type A M gene	 40°C 25°C	H3, no NA
Type A no detectable HA, N1 (of H5/N1 subtype)	 40°C 25°C	
	 40°C 25°C	

FIGURE 16b (Cont.)

Plus Internal Control and Type A M gene

N1, no HA

Type A no detectable HA, N2 (of H3 subtype)
Plus Internal Control and Type A M gene

N3, no HA

FIGURE 17

1. H5

Probe Test Amplicon (H5N1), Tm=79.7

**GTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTA
CAGTGGCGAGTTCCTAGCACTGGCAATCATGGTAGCTGGTCTATCC**

Primer Amplicon (H5N1), Tm=77.2

**GTGGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTA
CAGTGCACTGGCAATCATGGTAGCTGGTCTATCC**

Limiting Primer (reverse complement)

**GGATAGACCAGCTACCATGATTGCC, Tm=66.8 (H5N1), 21.5(H3N2), 30.3 (H1N1),
14.2 (B)**

Excess Primer-RNA 63.2 linear

**GTGGAGTAAAATTGGAATCAATAGG, Tm=63.6(H5N1), 15.7(H3N2), 53.8(H1N1),
34.6(B)**

Beacon Probe (reverse complement) Texas Red, Stem=58.1

**Texas Red-CGCGACTAGGGAAGCTCGCTCGCG -Dabsyl, Tm=52.7 (H5N1), 8.0 (H3N2),
24.9(H1N1), 13.0(B)**

2. H1

Probe Test Amplicon (H1N1), Tm=76.1

**CCATGACTCCAATGTGAAGAATCTGTATGAGAAAGTAAAAAGCCAATTAAAGAA
TAATGCCAAAGAAATAGGAAACGGGTG**

Primer Amplicon (H1N1), Tm=73.5

**CCATGACTCCAATGTGAAGAATCTGTATAAAGAATAATGCCAAAGAAATAGGAA
ACGGGTG**

Limiting Primer (reverse complement)

**CACCCGTTTCCTATTTCTTTGGCATTATTC, Tm=22.5(H5N1), 21.6(H3N2),
66.7(H1N1), 30.2(B)**

Excess Primer, RNA 64.2 linear

CCATGACTCCAATGTGAAG, Tm=36.1(H5N1), 30.1(H3N2), 63.8(H1N1), 20.3(B)

Beacon Probe (reverse complement) CY3, Stem=59.3

**CY3-CGCGGATTGGCTTTTTACTTTCTCACC GCG-Dabsyl, Tm=7.8(H5N1),
20.1(H3N2), 56.6(H1N1), 12.7(B)**

FIGURE 17 (Cont.)

3. N1

Probe Test Amplicon (H5N1), T_m=87.4

**GCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACT
GACAGGACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGC
GGCCCAAAGAGAGCACAATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGT
GTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGACGGTGCTG**

Primer Amplicon (H5N1), T_m=

**GCAATAACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACT
GACAGGACTAGATTGCATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGC
GGCCCAAAGAGAGCACAATTTGGACATCTTTTGTGGTGTAATAGTGACACTG
TGGGTTGGTCTTGGCCAGACGGTGCTG**

Limiting Primer (reverse complement)

CAGCACCGTCTGGCCAAGAC, T_m=69.3(H5N1), 69.3(H1N1), 41.0(H3N2), 5.7(B)

Excess Primer, RNA 66.3 linear

GCAATAACTGATTGGTCAGG, T_m=62.9 (H5N1), 62.7(H1N1), 29.9(H3N2), 40.4(B)

Beacon Probe (reverse complement) FAM, Stem=63.6

**FAM- GGCGGATGCTGCTCCCACTACCGCC -Dabsyl, T_m=56.3(H5N1), 56.3(H1N1),
12.1(H3N2), 25.8(B)**

4. H3

Probe Test Amplicon (H3N2), T_m=78.0

**CCATCAGATTGAAAAAGAATTCTCAGAAGTAGAAGGGAGAATTCAGGACCTCG
AGAAATATGTTGAGGACACTAAAATAGATCTCTGGTCATACAACG**

Primer Amplicon (H3N2), T_m=75.5

**CCATCAGATTGAAAAAGAATTCTCAGAAGTAGAAGGGAGAATTTATGTTGAGG
ACACTAAAATAGATCTCTGGTCATACAACG**

Limiting Primer (reverse complement)

**CGTTGTATGACCAGAGATCTATTTAGTGCCT, T_m=12.2(H5N1), 67.9(H3N2),
4.6(H1N1), -0.6(B)**

Excess Primer, RNA 59.7 linear

**CCATCAGATTGAAAAAGAATTCT, T_m=29.3(H5N1), 62.7(H3N2), 21.5(H1N1),
22.2(B)**

EXO-R Probe (reverse complement) CY5, Stem=66.8

FIGURE 17 (Cont.)

**CY5-CGCTGAAAGCGTTTCTCGAGGTCCTG-BHQ1, Tm=9.9(H5N1), 54.5(H3N2),
15.4(H1N1), 9.1(B)**

4/5. B(HA)

Probe Test Amplicon, Tm=84.4

**CGGTGGATTAAACAAAAGCAAGCCTTACTACACAGGGGAACATGCAAAGGCCA
TAGGAAATTGCCCAATATGGGTGAAAACACCCTTGAAGCTGGCCAATGGAACC
AAATATAGACCTCCTG**

Primer Amplicon, Tm=83.2

**CGGTGGATTAAACAAAAGCAAGCCTTACTACGGCCATAGGAAATTGCCCAATA
TGGGTGAAAACACCCTTGAAGCTGGCCAATGGAACCAAATATAGACCTCCTG**

Limiting Primer (reverse complement)

**CAGGAGGTCTATATTTGGTTCCATTGGC, Tm=14.4(H5N1), 21.0(H3N2), 24.8(H1N1),
67.5(B)**

Excess Primer, RNA 58.9 linear

CGGTGGATTAAACAAAAGCA, Tm=12.1(H5N1), 26.0(H3N2), 20.3(H1N1), 62.4(B)

Beacon Probe 1 (reverse complement) Texas Red, Stem=54.0

**Texas Red- GCGAGTTTGCATGTTCTCCTGTCTCGC -Dabsyl, Tm=19.2(H5N1),
16.2(H3N2), 15.3(H1N1), 52.1(B)**

Beacon Probe 2 (reverse complement) CY5, Stem=54.0

**CY5- GCGAGTTTGCATGTTCTCCTGTCTCGC -Dabsyl, Tm=19.2(H5N1), 16.2(H3N2),
15.3(H1N1), 52.1(B)**

4/5. B(NA)

Probe Test Amplicon, Tm=83.3

**CATGGGCTGACAGTGATGCCCTTGCTTTTAGTGAGTAATGGTTTCAATGGAA
GAACCTGGTTGGTACTCCTTTGGCTTCGAAATAAAAGACAAGAAATGTGATGTC
CCCTGTATTGGG**

Primer Amplicon, Tm=

**CATGGGCTGACAGTGATGCCCTTGCTTTTAGTGGAAGAACCTGGTTGGTACTC
CTTTGGCTTCGAAATAAAAGACAAGAAATGTGATGTCCCCTGTATTGGG**

Limiting Primer (reverse complement)

**CCCAATACAGGGGACATCACATTTCTTG, Tm=10.9(H5N1), 16.2(H1N1),
-4.6(H3N2), 68.9(B)**

FIGURE 17 (Cont.)

Excess Primer, RNA 69.7 just linear

CATGGGCTGACAGTGAT, Tm=38.7(H5N1), 43.2(H1N1), 42.5(H3N2), 63.7(B)

Beacon Probe 1 (reverse complement) Texas Red, Stem=58.1

Texas Red- GCCGCTCCATTGAAACCATTACGCGGC -Dabsyl, Tm=26.3(H5N1), 27.9(H1N1), 21.2(H3N2), 53.1(B)

Beacon Probe 2 (reverse complement) CY5, Stem=58.1

CY5- GCCGCTCCATTGAAACCATTACGCGGC -Dabsyl, Tm=26.3(H5N1), 27.9(H1N1), 21.2(H3N2), 53.1(B)

6. M

Probe Test Amplicon (H5N1) Tm=87.4

**CTAACCGAGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCTCAAA
GCCGAGATCGCGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCT
CGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACC**

Primer Amplicon (H5N1), Tm=87.8

**CTAACCGAGGTCGAAACGTACCATCCCGTCAGGCCCCCTCAAAGCCGAGATCG
CGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCTCGAGGCTCTC
ATGGAGTGGCTAAAGACAAGACCAATCCTGTCACC**

Limiting Primer (reverse complement)

GGTGACAGGATTGGTCTTGTCTTTAGC, Tm=67.3 (H5N1), 67.3(H3N2), 67.3(H1N1), 14.4(B)

Excess Primer

CTAACCGAGGTCGAAAC, Tm=62.2(H5N1), 62.2(H3N2), 62.2(H1N1), 20.6(B)

Probe (reverse compliment) CY3, Stem=61.2

CY3- GCGCTATAGAGAGAACAGCGC -Dabsyl, Tm=33.8 (H5N1), 33.8(H3N2), 33.8(H1N1), 9.6(B)

7. N2

Probe Test Amplicon (H3N2), Tm=80.6

**GGTCCAACCCTAATTCCAAATTGCAGATAAATAGGCAAGTCATAGTTGACAGAG
GTAATAGGTCCGGTTATTCTGGTATTTTCTCTGTTGAAGGCAAAAGCTGCATC**

Primer Amplicon (H3N2), Tm=

**GGTCCAACCCTAATTCCAAATTGCAGATAAATAGGCAAGTCATAGTTTATTCT
GGTATTTTCTCTGTTGAAGGCAAAAGCTGCATC**

FIGURE 17 (Cont.)

Limiting Primer (reverse complement)

GATGCAGCTTTTGCCTTCAACAGAG, Tm=29.4(H5N1), 16.7(H1N1), 67.4(H3N2), 15.6(B)

Excess Primer, RNA 69.4 just before hairpin

GGTCCAACCCTAATTCCAA, Tm=13.6(H5N1), 22.0(H1N1), 63.4(H3N2), 22.7(B)

Probe (reverse complement) FAM, Stem=61.2

FAM- GGCCGCCTATTACCTCTCGGCC -Dabsyl, Tm=30.0(H5N1), 27.7(H1N1), 38.9(H3N2), 20.6(B)

8. H3 Control

Probe Test Amplicon (H3N2), Tm=77.0

**CCATCAGATTGAAAAAGAATTCTCAGAAGTAGAAGGGAGAATTCAGGAACTCT
AGAAATATGTTGAGGACACTAAAATAGATCTCTGGTCATACAACG**

Limiting Primer (reverse complement)

CGTTGTATGACCAGAGATCTATTTAGTGTCT, Tm=12.2(H5N1), 67.9(H3N2), 4.6(H1N1), -0.6(B)

Excess Primer

CCATCAGATTGAAAAAGAATTCT, Tm=29.3(H5N1), 62.7(H3N2), 21.5(H1N1), 22.2(B)

Probe (reverse complement) CY5, Stem=66.8

**CY5- CGCTGAAAGCGTTTCTCGAGGTCCTG -BHQ1, Tm=32.9 vs. modified amplicon
sequence CAGGAACTCTAGAAA**

9. H5 Delete Region

Probe Test Amplicon(H5N1), Tm=79.0

**CGACTGGGCTCAGAAATAGCCCTCAAAGAGAGAGAAGAAGAAAAAAGAGAGGA
TTATTTGGAGCTATAGCAGGTTTATAGAGGGAGG**

Primer Amplicon (H5N1), Tm=77.4

**CGACTGGGCTCAGAAATAGCCCTCAAAGAGTTATTTGGAGCTATAGCAGGTTT
ATAGAGGGAGG**

FIGURE 17 (Cont.)

Limiting Primer (reverse complement)

**CCTCCCTCTATAAAACCTGCTATAGCTCCAAA, Tm=69.7(H5N1), 14.3(H3N2),
20.5(H1N1), 6.5(B)**

Excess Primer

CGACTGGGCTCAGAAA, Tm=62.9(H5N1), 17.4(H3N2), 29.3(H1N1), 17.4(B)

Probe Sequence(reverse complement), Stem Tm=

**Fluor-stemTCCTCTCTTTTTCTTCTTCTCTstem-Dabsyl, Tm=58.9(H5N1), -3.8(H3N2),
20.4(H1N1), 13.4(B)**

RT-LATE-PCR

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. patent application Ser. No. 11/822,536, filed Jul. 6, 2007, which claims priority to U.S. Provisional Patent Application Ser. No. 60/819,000, filed Jul. 7, 2006, each of which is hereby incorporated by reference in their entireties.

BACKGROUND

[0002] Rapid detection and typing of influenza virus and identification of its various strains is critical to identification and control of a potential human pandemic. Influenza virus is composed of eight single-stranded RNA molecules (HA, NA, PB2, PB1, PA, NS, M, NP) that code for eleven specific proteins. The RNA for the matrix protein (M) is relatively conserved and is therefore used to detect and distinguish a Type A virus. M can also be used to detect and distinguish H5N1.

[0003] The hemagglutinin protein (HA) and neuraminidase protein (NA) are grouped into 16 and 9 subtypes, respectively, both have high sequence variability even within subtypes and thus provide an effective means of monitoring changes that might occur in a virus. The HA protein protrudes from the surface of the virus and allows it to attach to a cell to begin the infection cascade. The NA protein is also located on the surface of the virus and allows the release of new particles within the infected cell.

[0004] Currently the Eurasian H5N1 virus infects only the lower lungs in human and is therefore less readily transmitted human-to-human than annual strains of human influenza that infect the upper respiratory track. But, mutations within the HA and NA RNAs are frequent and alter viral infectivity and lethality in different hosts and their tissues. In addition, gene assortment among the different viral subtypes is another very worrisome feature of influenza and could result in recombining RNA sequences for high infectivity in humans with high lethality.

SUMMARY

[0005] Accordingly, there is a need for an informative influenza assay that can be performed in the field, i.e., at the point of care ("POC"). Moreover, in order to save both time and money it will also be important to make POC assays compatible with more extensive laboratory analysis, such as sequencing of, for example, HA and NA. In this way, the evolution of a viral disease and viral genomics can be analyzed in real-time.

[0006] One embodiment is directed to an assay comprising a plurality of primer pairs, a plurality of probes, and a low copy number synthetic amplicon corresponding to each of the plurality of primer pairs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] FIG. 1 illustrates an embodiment of LATE PCR (left) and provides fluorescence curves produced by LATE PCR.

[0008] FIG. 2 is an agarose gel showing four sets of reactions, performed in triplicate, each reaction using the same Excess Primer (thick arrow), plus a different own Limiting Primer (thin arrow). The melting temperature of the four different Limiting Primers increases from left to right and the

annealing temperature used for each set of reactions is 2° C. below the melting temperature of the Limiting Primer.

[0009] FIG. 3 shows agarose gels of identical LATE-PCR without ELIXIR (left) and with ELIXIR (right). The replicate reactions are prepared using four different preparations of commercially available Taq polymerases, both with and without a hot start. The reactions were incubated at room temperature for 30 minutes before amplification.

[0010] FIG. 4 is an agarose gel of a pentaplex LATE-PCR without ELIXIR (left six lanes), monoplex LATE-PCR with ELIXIR (middle five lanes), and pentaplex with ELIXIR (right six lanes). A molecular weight ladder is shown in far right lane. In the pentaplex reactions, all five targets are amplified simultaneously.

[0011] FIG. 5 shows reaction design (left panels) and dF/dT in the presence of SybrGreen (right panels) where five primer pairs and (a) one template or (b) one template corresponding to one primer pair+four low copy number amplicons corresponding to the remaining four primer pairs are included.

[0012] FIG. 6 shows pyrosequencing of a LATE-PCR monoplex reaction (left panel) and a multiplex reaction (right panel). The post-LATE-PCR reactions are split into five aliquots each and pyrosequencing performed in the presence of a sequencing primer corresponding to each amplicon.

[0013] FIG. 7 shows an embodiment of a Low- T_m Probe detection approach.

[0014] FIG. 8 compares amplification and detection of a high- T_m molecular beacon probe and a low- T_m molecular beacon probe.

[0015] FIG. 9 compares resolution of single nucleotide polymorphism in heterozygous CC, heterozygous CT, and homozygous TT using an anneal down protocol (left) and a melt-up protocol (right).

[0016] FIG. 10. LATE-PCR The captions under the bars indicate the temperature used for RT prior to LATE-PCR. No RTase (light blue bar), the inactivated RTase (blue bar), 30 min at 50° C. (green bar), 30 min at 55° C.; 10 min at 60° C.+20 min at 55° C. (orange bar). LATE-PCR is identical for all samples.

[0017] FIG. 11. LATE-PCR protocol with 50 nM limiting primer (LP) and 2 μ M RT primer and LATE-PCR excess primer (XP).

[0018] FIG. 12 is a clustal comparison of Influenza Virus M RNA sequences for H3N2, H5N1, H1N1, and B.

[0019] FIG. 13 is a clustal comparison of Influenza Virus HA RNA sequences for H3N2, H5N1, H1N1, and B.

[0020] FIG. 14 is a clustal comparison of Influenza Virus NA RNA sequences for H3N2, H5N1, H1N1, and B.

[0021] FIG. 15 shows a schematic of an embodiment of an assay

[0022] FIG. 16 shows the layout and an possible outcomes for an exemplary assay.

[0023] FIG. 17 provides primer, probe, and amplicon sequences that can be used in an embodiment of an influenza virus assay.

DETAILED DESCRIPTION

[0024] All references cited are incorporated herein by reference.

[0025] An influenza virus assay can detect and distinguish between various sub-types and strains of an influenza virus using any suitable nucleic acid amplification technique. This assay can be performed in a single reaction vessel with all reagents present at the start of an assay. An assay can use more

than one primer pairs in combination with one or more probes to amplify and detect specific target nucleic acid sequences of influenza. Using the information obtained from an amplification reaction it is possible to distinguish between various sub-types and strains of the influenza virus. Specifically, an assay can provide a positive or negative (yes/no) determination of the likely presence or absence of influenza virus types A and B, and sub-types H1N1, H3N2, and H5N1 in a sample. An assay also can be used to monitor for one or more mutations in an influenza virus strain. Mutations in an influenza virus, within, for example the HA and NA, can alter viral infectivity and lethality in different hosts and different tissues.

[0026] A sample can be any material to be tested, such as, for example, a biological or environmental sample. Biological samples can be obtained from any organism. In one embodiment, a sample is obtained from a mammal, such as a human, or a bird. In one embodiment, a sample comprises a nasopharyngeal aspirate, blood, saliva, or any other bodily fluid.

[0027] A nucleic acid amplification method used in an assay can be a thermal cycling technique, such as a polymerase chain reaction ("PCR") or an isothermal technique. Standard PCR amplification methods are described in, for example, U.S. Pat. Nos. 4,683,195 and 4,683,202 (both of which are incorporated herein by reference). In one embodiment, the nucleic acid amplification method is linear after the exponential ("LATE") PCR ("LATE-PCR"), as described in U.S. patent application Ser. No. 10/320,893, which is incorporated by reference. An embodiment of LATE-PCR is illustrated in FIG. 1. LATE-PCR is an asymmetric PCR method, which uses unequal concentrations of primers and can yield single-stranded primer-extension products, or amplicons. LATE-PCR amplifications and assays typically include at least 30 cycles, at least 60 cycles, or at least 70 cycles. FIG. 2, which shows an agarose gel showing that amplification product production is less specific when the melting temperature of the Limiting Primer is below that of the Excess Primer, demonstrates the specificity advantage that can be afforded by a properly designed LATE-PCR. An Excess Primer can misprime at a low annealing temperature, but the reaction can become very specific when the melting temperature of the Limiting Primer is above the melting temperature of the Excess Primer ($T_{mL[0]} - T_{mX[0]} \geq 0$).

[0028] As used interchangeably herein, the terms "nucleic acid primer", "primer molecule", "primer", and "oligonucleotide primer" include short, (for example, between about 16 and about 50 bases) single-stranded oligonucleotides which, upon hybridization with a corresponding template nucleic acid molecule, serve as a starting point for synthesis of the complementary nucleic acid strand by an appropriate polymerase molecule. Primer molecules can be complementary to either the sense or the anti-sense strand of a template nucleic acid molecule. A primer can be composed of naturally occurring or synthetic oligonucleotides, or a mixture of the two. If the primers in a pair of PCR primers are used in unequal concentrations, as is the case in LATE-PCR, primer added at a lower concentration is a "Limiting Primer", and primer added at a higher concentration is an "Excess Primer."

[0029] As used herein "amplification target sequence" used interchangeably with "target sequence" and "target nucleic acid" refers to a DNA sequence that provides a template for copying by the steps an amplification technique. An amplification target sequence can be single-stranded or double-stranded. If the starting material is RNA, for example mes-

senger RNA, the DNA amplification target sequence is created by reverse transcription of RNA to create complementary DNA, and the amplification target sequence is a cDNA molecule. Thus, in a PCR assay for RNA, a hybridization probe signals copying of a cDNA amplification target sequence, indirectly signifying the presence of the RNA whose reverse transcription produced the cDNA molecules containing the amplification target sequence. An amplification target sequence typically is bracketed in length by a pair of primers used to amplify it. An extension product (or amplicon), whether double-stranded or, in non-symmetric PCR, single-stranded, is the exponentially amplified amplicon, bracketed by the primer pair.

[0030] An amplification target sequence can be a single nucleic acid sequence. In some cases an amplification target sequence will contain allelic variations or mutations and, thus, will not be a single sequence, even though amplified by a single primer pair. An assay for an amplification target sequence containing variations may use one detection probe for all variations, a single allele-discriminating probe for one variant, or multiple allele-discriminating probes, one for each variant.

[0031] Any suitable primer pair can be used, such as, for example, one of more of the following primer pairs:

H5
Limiting Primer (reverse complement)
GGATAGACCAGCTACCATGATTGCC, $T_m = 66.8$ (H5N1),
21.5 (H3N2), 30.3 (H1N1), 14.2 (B)

Excess Primer-RNA 63.2 linear
GTGGAGTAAATGGAATCAATAGG, $T_m = 63.6$ (H5N1),
15.7 (H3N2), 53.8 (H1N1), 34.6 (B)

H1
Limiting Primer (reverse complement)
CACCCGTTTCTATTCTTTGGCATTATTC, $T_m = 22.5$ (H5N1),
21.6 (H3N2), 66.7 (H1N1), 30.2 (B)

Excess Primer, RNA 64.2 linear
CCATGACTCCAATGTGAAG, $T_m = 36.1$ (H5N1), 30.1 (H3N2),
63.8 (H1N1), 20.3 (B)

N1
Limiting Primer (reverse complement)
CAGACCGTCTGGCCAAGAC, $T_m = 69.3$ (H5N1), 69.3 (H1N1),
41.0 (H3N2), 5.7 (B)

Excess Primer, RNA 66.3 linear
GCAATAACTGATTGGTCAGG, $T_m = 62.9$ (H5N1),
62.7 (H1N1), 29.9 (H3N2), 40.4 (B)

H3
Limiting Primer (reverse complement)
CGTTGTATGACCAGAGATCTATTTAGTGTCCT,
 $T_m = 12.2$ (H5N1), 67.9 (H3N2), 4.6 (H1N1), -0.6 (B)

Excess Primer, RNA 59.7 linear
CCATCAGATTGAAAAGAATTCT, $T_m = 29.3$ (H5N1),
62.7 (H3N2), 21.5 (H1N1), 22.2 (B)

B (HA)
Limiting Primer (reverse complement)
CAGGAGGTCTATATTGGTTCCATTGGC, $T_m = 14.4$ (H5N1),

-continued

21.0 (H3N2), 24.8 (H1N1), 67.5 (B)

Excess Primer, RNA 58.9 linear
CGGTGGATTAAACAAAAGCA, Tm = 12.1 (H5N1), 26.0 (H3N2),

20.3 (H1N1), 62.4 (B)

B (NA)

Limiting Primer (reverse complement)
CCCAATACAGGGGACATCACATTCTTG, Tm = 10.9 (H5N1),

16.2 (H1N1), -4.6 (H3N2), 68.9 (B)

Excess Primer, RNA 69.7 just linear
CATGGGCTGACAGTGAT, Tm = 38.7 (H5N1), 43.2 (H1N1),

42.5 (H3N2), 63.7 (B)

M

Limiting Primer (reverse complement)
GGTGACAGGATTGGTCTGTCTTTAGC, Tm = 67.3 (H5N1),

67.3 (H3N2), 67.3 (H1N1), 14.4 (B)

Excess Primer
CTAACCGAGGTCGAAAC, Tm = 62.2 (H5N1), 62.2 (H3N2),

62.2 (H1N1), 20.6 (B)

N2

Limiting Primer (reverse complement)
GATGCAGCTTTTGCTTCAACAGAG, Tm = 29.4 (H5N1),

16.7 (H1N1), 67.4 (H3N2), 15.6 (B)

Excess Primer, RNA 69.4 just before hairpin
GGTCCAACCTAATTCCAA, Tm = 13.6 (H5N1), 22.0 (H1N1),

63.4 (H3N2), 22.7 (B)

H3 Control

Limiting Primer (reverse complement)
CGTTGTATGACCAGAGATCTATTTAGTGCCT,

Tm = 12.2 (H5N1), 67.9 (H3N2), 4.6 (H1N1), -0.6 (B)

Excess Primer

CCATCAGATTGAAAAAGAAATCT, Tm = 29.3 (H5N1),

62.7 (H3N2), 21.5 (H1N1), 22.2 (B)

H5 Delete Region

Limiting Primer (reverse complement)
CCTCCCTCTATAAACCTGCTATAGCTCCAA,

Tm = 69.7 (H5N1), 14.3 (H3N2), 20.5 (H1N1), 6.5 (B)

Excess Primer

CGACTGGGCTCAGAAA, Tm = 62.9 (H5N1), 17.4 (H3N2),

29.3 (H1N1), 17.4 (B)

[0032] In one embodiment all of the above primer pairs are used.

[0033] A target sequence can be present at a starting concentration of greater than or equal to approximately 1,000,000 copies/sample. In one embodiment, a target sequence is present at a concentration of approximately 10 to approximately 1,000,000 copies/sample. In another embodiment a target sequence is present at a concentration of less than 10 copies/sample, less than 100 copies/sample, less than 1,000 copies/sample, less than 10,000 copies/sample, less than 100,000 copies/sample, less than 500,000 copies/sample, or less than 1,000,000 copies/sample.

[0034] In one embodiment, a target sequence can produce an amplicon as provided in FIG. 17.

[0035] An assay includes reagents for an amplification reaction, such as those used in LATE-PCR, a symmetric PCR amplification, or an isothermal amplification method. For example, the assay mixture can include each of the four deoxyribonucleotide 5' triphosphates (dNTPs) at equimolar concentrations, a thermostable polymerase, a divalent cation, and a buffering agent. An assay mixture can include additional ingredients, such as, for example, a separate reverse transcriptase enzyme. Non-natural dNTPs can be used. For instance, dUTP can be substituted for dTTP and used at three-times the concentration of the other dNTPs due to the less efficient incorporation by Taq DNA polymerase.

[0036] An assay also can include reagents that can suppress mispriming. In one embodiment, a reagent capable of suppressing mispriming is a single-stranded oligonucleotide capable of forming a stem-and-loop structure, commonly referred to as a "hairpin" structure such as those described in, for example, U.S. patent application Ser. No. 11/252,506, (referred to as an "ELIXIR™"), which is incorporated by reference herein. FIG. 3 demonstrates the ability of an ELIXIR™ to inhibit Taq polymerase, reducing mispriming and increasing generation of target product. FIG. 4 shows the ability of an ELIXIR™ to reduce mispriming in a multiplex reaction.

[0037] An assay also can include an amplicon corresponding to a primer pair that is capable of suppressing mispriming. In one embodiment, one or more copies of an amplicon corresponding to a primer pair, where there are less targets present than primer pairs, is (referred to herein as "mono-multiplex"). For example, an assay can have the capacity of amplify any one of several different amplicons, but in any particular assay it is possible that either none or only a few viral target sequences will be present in a sample. Such reactions mono-multiplex reactions can be difficult to construct, because they have to suppress mispriming among the unused primers, while still allowing amplification of the correct product from any pair of primers. To prevent such mis-priming, it can be advantageous to add low copy numbers of synthetic amplicons for each primer pair. In one embodiment, approximately 20 copies of synthetic amplicons per primer pair can be included in an assay. FIG. 5 demonstrates how inclusion of low levels of synthetic amplicons can suppress mispriming in a mono-multiplex reaction. The difference between the design of unsuppressed and "internally suppressed mono-multiplex" reaction is illustrated in FIG. 5a and the present and absence of mis-primed products is illustrated in FIG. 5b.

[0038] In another embodiment, reactions that do not contain influenza viral sequences have an internal control that rules out false negatives. This internal control can be observed at a single wavelength. In one embodiment, the internal control can be observed at 25° C. and can be generated by amplification of an internal control target possessing a variant of H3 flanked by the H3 primers. Detection can be accomplished using any suitable probe, including, for example, a mismatch-tolerant probe.

[0039] Amplification products can be detected by an end-point analysis or using a real-time analysis. As used herein, the term "real time," with respect to an amplification reaction, refers to the method by which the amplification reaction is detected. In a "real-time" amplification reaction, accumulation of amplicon or product is measured during the progression of the reaction, as opposed to solely after the reaction is complete, the latter being "end-point" analysis. In one embodiment detection is quantitative.

[0040] The assays can use any suitable means to detect amplification, including, but not limited to dyes, such as intercalating dyes, DNA binding agents, and probe molecules. As used interchangeably herein, the terms “nucleic acid probe”, “probe molecule”, and “oligonucleotide probe” and “hybridization probe” include defined nucleic acid sequences complementary to a target nucleic acid sequence to be detected such that the probe will hybridize to the target. Probes can be detectably labeled, such that hybridization of a probe to a target sequence can be readily assessed. A “detectable label” includes moieties that provide a signal that can be detected and, in some embodiments, quantified. Such labels are well known to those in the art and include chemiluminescent, radioactive, metal ion, chemical ligand, fluorescent, or colored moieties, or enzymatic groups which, upon incubation with an appropriate substrate, provide a chemiluminescent, fluorescent, radioactive, electrical, or colorimetric signal. Methods of detection of such signals are also well known in the art.

[0041] Probes can be composed of naturally occurring or synthetic oligonucleotides and include labeled primers. Some hybridization probes, for example molecular beacon probes, emit an increased detectable signal upon hybridizing to their complementary sequence without enzymatic action to hydrolyze the probes to generate a signal. We refer to such probes as probes that hybridize to their target and “signal upon hybridization.” Other probes, for example TaqMan™ dual fluorescently labeled random coil probes are cut, or hydrolyzed, during the amplification reaction, and hydrolysis leads to a detectable signal change. Probes that rely on hydrolysis as part of signal generation are not probes that “signal upon hybridization.”

[0042] In one embodiment, an assay uses a “molecular beacon probe,” which is a single-stranded oligonucleotide, typically 25-35 bases-long, in which the bases on the 3' and 5' ends are complementary. Molecular beacon probes are discussed in, for example, U.S. Pat. Nos. 5,925,517, 6,037,130, 6,103,476, 6,150,097, and 6,461,817, and U.S. Patent Appl. Pub. No. 2004/0023269A1, all of which are incorporated by reference. A molecular beacon probe can form a hairpin structure at temperatures at and below those used to anneal the primers to the template (typically below about 60° C.). The double-helical stem of the hairpin brings a fluorophore attached to one end (often, but not necessarily the 5' end) of a probe in proximity to a quencher attached to the other end of the probe (typically, but not necessarily, the 3' end). In the hairpin configuration, probe fluorescence is quenched. If a probe is heated above the temperature needed to melt the double stranded stem apart, or a probe is allowed to hybridize to a target oligonucleotide that is complementary to a sequence within the single-strand loop of a probe, fluorophore and quencher are separated, and the resulting conformation shows increased fluorescence. The strength of a fluorescent signal can increase in proportion to the amount of a molecular beacon hybridized to an amplicon. Molecular beacons with different loop sequences can be conjugated to different fluorophores in order to monitor increases in amplicons that differ by as little as one base (Tyagi, S. and Kramer, F. R. (1996) “Molecular Beacons: Probes That Fluoresce Upon Hybridization,” Nat. Biotech. 14:303-308; Tyagi, S. et al., (1998) “Multicolor Molecular Beacons for Allele Discrimination,” Nat. Biotech. 16: 49-53; Kostrikis, L. G. et al., (1998) “Spectral Genotyping of Human Alleles,” Science 279: 1228-1229).

[0043] Any suitable fluorophore/quencher pair can be used in a molecular beacon probe. In one embodiment, four probes are used each with a single fluorophore, wherein the fluorophores are texas red, CY3, CY5, and FAM. Any suitable quencher can be used, such as, for example, Black Hole™ quenchers, dabsyl, and BHQ1. In one embodiment, an assay include one or more fluorophore/quencher pair, wherein the pair can be any of texas red/dabsyl, CY5/dabsyl, FAM/dabsyl, CY5/BHQ1, and CT3/dabsyl. In another embodiment, an assay uses one or more of the following probes:

H5
Texas Red-CGCGACTAGGGAACCTCGCTCGCG-Dabsyl,
Tm = 52.7 (H5N1), 8.0 (H3N2), 24.9 (H1N1), 13.0 (B)

H1
CY3-CGCGGATTGGCTTTTACTTTCTCACCGCG-Dabsyl,
Tm = 7.8 (H5N1), 20.1 (H3N2), 56.6 (H1N1), 12.7 (B)

N1
FAM-GGCGGATGCTGCTCCCACTACCGCC-Dabsyl,
Tm = 56.3 (H5N1), 56.3 (H1N1), 12.1 (H3N2), 25.8 (B)

H3
CY5-CGCTGAAAGCGTTTCTCGAGGTCCTG-BHQ1,
Tm = 9.9 (H5N1), 54.5 (H3N2), 15.4 (H1N1), 9.1 (B)

B (HA)
Beacon Probe 1
Texas Red-GCGAGTTTGCATGTTCTCCTGTCTCGC-Dabsyl,
Tm = 19.2 (H5N1), 16.2 (H3N2), 15.3 (H1N1), 52.1 (B)

Beacon Probe 2
CY5-GCGAGTTTGCATGTTCTCCTGTCTCGC-Dabsyl,
Tm = 19.2 (H5N1), 16.2 (H3N2), 15.3 (H1N1), 52.1 (B)

B (NA)
Beacon Probe 1
Texas Red-GCCGCTCCATTGAAACCATTACGCGGC-Dabsyl,
Tm = 26.3 (H5N1), 27.9 (H1N1), 21.2 (H3N2), 53.1 (B)

Beacon Probe 2
CY5-GCCGCTCCATTGAAACCATTACGCGGC-Dabsyl,
Tm = 26.3 (H5N1), 27.9 (H1N1), 21.2 (H3N2), 53.1 (B)

M
CY3-GCGCTATAGAGAGAACAGCGC-Dabsyl, Tm = 33.8
(H5N1), 33.8 (H3N2), 33.8 (H1N1), 9.6 (B)

N2
FAM-GGCCGCCTATTACCTCTCGGCC-Dabsyl,
Tm = 30.0 (H5N1), 27.7 (H1N1), 38.9 (H3N2), 20.6 (B)

H3 Control
CY5-CGCTGAAAGCGTTTCTCGAGGTCCTG-BHQ1,
Tm = 32.9 vs. modified amplicon sequence
CAGGAACCTAGAAA

H5 Delete Region
Fluor-stemTCTCTCTTTTCTCTCTCTstem-Dabsyl,
Tm = 58.9 (H5N1), -3.8 (H3N2), 20.4 (H1N1), 13.4 (B)

[0044] In another embodiment, all of the above molecular beacon probes are used in an assay. In a further embodiment,

an assay can include nine sequence-specific molecular beacons that are capable of detecting seven influenza virus targets. In a further embodiment, three molecular beacons probes, each detectable at a different wavelength, can form a probe-target hybrid at T_m 45° C. and two additional molecular beacon probes, each detectable at a different single wavelength, can form a probe-target hybrid at T_m 30° C. Further, two additional molecular beacon probes, each detectable at two different wavelengths, can form a probe-target hybrid at T_m 45° C. An additional mis-match tolerant probe can also be used to detect one of the viral targets at 40° C. and a variant of that sequence present in an internal control at 25° C.

[0045] In one embodiment, an assay can use a “Low- T_m Probe.” A Low- T_m Probe is discussed in U.S. patent application Ser. No. 10/320,893 and refers to a labeled hybridization probe that signals upon hybridization to its target, which in a LATE-PCR is the Excess Primer-Strand generated by extension of the Excess Primer, and that has a $T_{m[O]}$ at least 5° C. below or at least 10° C. below the $T_{m[0]}$ of the primer that hybridizes to and extends along the Excess Primer-Strand, which in a LATE-PCR is the Limiting Primer. FIG. 7 shows an embodiment of a Low- T_m Probe detection approach.

[0046] As shown in FIG. 8, Low- T_m Probes can be more specific over a wider temperature range and can display lower backgrounds. Low- T_m Probes also show less amplification at higher concentrations than High- T_m Probes.

[0047] In another embodiment, an assay can use a “Super-Low- T_m Probe.” This probe also is discussed in U.S. patent application Ser. No. 10/320,893.

[0048] An assay can include more than one probe. In one embodiment, multiple molecular beacon probes are used. In another embodiment, the probes are capable of forming probe-target hybrids at more than one temperature. In a further embodiment, multiple probes can be used, where a first probe forms a probe-target hybrid at a first temperature and a second probe forms a probe-target hybrid at a second temperature. In one embodiment, five molecular beacon probes can form a probe-target hybrid at a temperature of greater than 45° C. and can be detected at 40° C. and two molecular beacon probes can form a probe-target hybrid at 30° C. and can be detected at 25° C.

[0049] An assay can also include mismatch tolerant probes, such as, for example, fluorescent probes. In one embodiment, an assay uses a mismatch tolerant probes. An assay also can detect probe-target hybrids as a sample is cooled after PCR amplification (“anneal down”). This approach can be used in end-point fluorescence detection. This anneal-down approach can be more sensitive and provide better resolution than cooling first and then reading during warm-up (melt-up), because the read-during-cooling approach can minimize formation of hairpin structures in a target sequence. FIG. 9 compares resolution of single nucleotide polymorphism in heterozygous CC, heterozygous CT, and homozygous TT using an anneal down protocol (left) and a melt-up protocol (right). In one embodiment, the temperature of an assay reaction is changed from less than 95° C. to less than 65° C. than 45° C., to less than 25° C.

[0050] An influenza viral assay (or an assay of any RNA virus) can involve reverse transcription (RT) as a first step of a detection reaction. During reverse transcription RNA sequences are converted to complementary DNA (cDNA), providing a cDNA template for PCR amplification.

[0051] An approach to RT-PCR is the use of a “One-Step RT-PCR system.” In a system of this type, reagents for both

RT and PCR can be added to a sample in a single mixture and the reaction tube can be sealed and placed in a thermocycler. The RT and PCR enzyme-catalyzed reactions are carried out sequentially in the thermocycler, taking advantage of the different thermostabilities of the enzymes involved (typically, a reverse transcriptase and a thermostable DNA polymerase) and by setting an appropriate thermal profile. An initial incubation at non-denaturing temperature allows RT to occur first. The temperature then can be raised to initiate PCR; at this temperature, the reverse transcriptase can be inactivated, but the DNA polymerase is not. When a “hot start” is used, DNA polymerase is kept inactive during the RT step by interaction with a specific antibody. When the temperature is elevated, the antibody can be denatured and the DNA polymerase activated. In one embodiment, a multi-functional enzyme, having both a RTase activity and a DNA polymerase activity, can be used. In a further embodiment, a multi-functional enzyme having RTase activity, DNA polymerase activity, and exonuclease activity can be used, where the exonuclease activity can cleave double-stranded DNA in TaqMan-type detection method.

[0052] The temperature and duration of RT and PCR steps can be readily determined by one of skill in the art. In one embodiment, an RT step can be performed for from less than 2 minutes to more than 60 minutes at a temperature of from approximately 50-60° C. and a DNA-polymerase step can be performed as a thermocycle at approximately 95° C. for several cycles as discussed elsewhere.

[0053] An assay can be performed using any suitable device, such as a thermal cycler. In one embodiment, an assay is performed using a portable device, a man-portable device, or a handheld device, such as, for example, a Bioseq II. In another embodiment, an assay is performed using a bench-top device, including, for example, an ABI Prism 7700 Sequence Detector (Applied Biosystems, Inc., CA) machine, a Cepheid Smart Cycler, and a Primus PCR thermocycler.

[0054] An assay can be performed in less than or equal to 30 minutes, less than or equal to 20 minutes, less than or equal to 15 minutes, or less than or equal to 10 minutes.

[0055] Assay reagents can be provided as a kit or a consumable. The reagents can be supplied as a lyophilized preparation. Each reagent can be supplied separately or as a mixture of one or more reagents. Reagents also can be supplied on a substrate, such as a bead. A lyophilized reagent can be stable for more than one year.

[0056] An assay can yield single-stranded products for further use, for example as starting material for DNA sequencing or as probes in other reactions, or can be used in other assays. In one embodiment, single stranded DNA produced by an assay (assay product) can be sequenced using any suitable sequencing method, such as, for example, the dideoxy-method or pyrosequencing (Salk et al. (2006) Anal. Biochem. 353:124, incorporated by reference) by diluting a fraction of the assay reaction products into a sequencing reaction mixture. Assay product can be diluted by approximately 1:10, approximately 1:20, approximately 1:50, approximately 1:100, or approximately 1:200 or more for use in a sequencing reaction. FIG. 6 shows a LATE-PCR multiplex reaction, in which one sample is split into five aliquots each spiked with a different sequencing primer, and sequenced.

[0057] An assay can distinguish Influenza Type B and Type A virus. In one embodiment, an assay distinguishes Influenza Type B and Type A virus on the basis of sequences in the HA and NA genes. Within the Type A viruses an assay can dis-

tinguish between subtypes H5 (with or without N1 or N2), H1 (with or without N1 or N2), and H3 (with or without N1 or N2). In one embodiment, an N1 target sequence used is conserved for the H5 and H1 subtypes and can be useful for detecting H5N1 and H1N1. In another embodiment, H3N1 can be determined and such a determination can indicate viral reassortment. The N2 target sequence used is characteristic of the H3N2 subtype, thus, detection of H5N2 or H1N2 can indicate viral reassortment. FIGS. 12-14 shows clustal comparisons of influenza virus M, HA, and NA proteins for virus H1N1, H5N1, H3N2, and B. Such analysis is useful in interpreting data obtained from an assay and from subsequent sequencing of assay products. Using information obtaining from an assay, it is possible to monitor mutations in a known virus strain, which allows for detection of and prediction of changes in virulence and infectivity.

[0058] An exemplary avian influenza assay and possible results of this exemplary assay are provided in Table I and FIG. 15. FIG. 16 shows a schematic of an embodiment of an assay and FIG. 17 provides primer, probe sequences, and amplicon sequences that can be used in an embodiment of an influenza virus assay. In one embodiment, an assay include all of the primers and probes of FIG. 17 in a mono-multiplex assay. The features of such a mono-multiplex are summarized in Table I and the 15 possible outcomes of the reaction are illustrated 16.

TABLE I

Position Tm	Amplicon Primers	Target Sequence	Probe Type	Color(s)	Melting
1	H5 45 C.	H5	M. Beacon	Red	
2	H1 45 C.	H1	M. Beacon	Yellow	
3	N1 45 C.	N1	M. Beacon	Green	
4	H3 45 C.	H3	EXO-R	Blue	
6	Type A M gene 30 C.	M gene	M. Beacon	Yellow	
7	N2	N2 30 C.	M. Beacon	Green	
4/5	Type B HA only 45 C.	Type B HA	M. Beacon	Blue	
	Type B HA only 45 C.	Type B HA only	M. Beacon	Red	
4/5	Type B NA only 45 C.	Type B NA only	M. Beacon	Blue	
	Type B NA only 45 C.	Type B NA only	M. Beacon	Red	
4/5	Type B HA + NA 45 C.	Type B HA + NA	M. Beacon	Blue + Blue	
	Type B HA + NA 45 C.	Type B HA + NA	M. Beacon	Red + Red	
8	H3 int. control 30 C.	mis-matched H3	EXO-R	Blue	
	H5 int. control	no matches	no probe		
	H1 int. control	no matches	no probe		
	N1 int. control	no matches	no probe		
	N2 int. control	no matches	no probe		
	M int. control	no matches	no probe		
	Type B HA i.c.	no matches	no probe		
	Type B NA i.c.	no matches	no probe		

[0059] The exemplary assay described in this table contains:

- [0060]** 8 pairs of primers
- [0061]** 3 Molecular Beacons with 45° C.
- [0062]** 2 Molecular Beacons with 30° C.

- [0063]** 2 pairs of Molecular Beacons both at 45° C.
- [0064]** Total=9 Molecular Beacons
- [0065]** 1 mismatch-tolerant probe
- [0066]** 1 detectable internal control
- [0067]** 7 undetected internal controls

Example 1

[0068] Starting with samples of purified RNA, the HA RNA (1770 nucleotides long) and the NA RNA (1400 nucleotides long) are both reverse transcribed in toto using random hexamers in a highly efficient two step RT-procedure. Each reaction also contains low levels of an M-Gene control DNA. The resulting control and cDNA molecules are amplified in two parallel multiplex LATE-PCR assays that each generate six amplicons. The presence of Eurasian H5N1 strain in a sample is established by probing for M, N1 and two different H5 sequences that are likely to be crucial for human-to-human transmission and for virulence. Reactions that do not generate either signal for H5 Eurasian will nevertheless produce a control DNA signal, proving that they are not false negatives. Reactions that do signal the presence of the H5 Eurasian strain from either of two independent probes (one in Multiplex A and one in Multiplex B) also generates a strong M-gene signal in both Multiplex A and Multiplex B. However, some samples may generate a signal for an M protein and only one of two possible HA signals. This is regarded as an indication of viral evolution. All samples that generate either one or two HA signals, or an N1 signal plus an M-gene signal are immediately be processed further for analysis. The amplicons for the all portions of HA and NA already are present in the LATE-PCR multiplex reactions. All 10 HA and NA amplicons are 300-500 by in length and are processed for parallel pyrosequencing sequencing.

Example 2

[0069] This example is directed to an RT-LATE-PCR assay for the quantification of Oct4-specific sequences in embryonic mouse cells. Oct4 is a gene expressed in totipotent and pluripotent cells and, therefore, preimplantation embryos contain considerable levels of Oct4 RNA. In addition, each cell contains two copies of the Oct4 gene (Oct4 genomic DNA). In the experiments presented in this example, Oct4 RNA and DNA are co-purified and co-quantified, according to a method previously published by this laboratory (Hartshorn C, Anshelevich A, Wang L. J. Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. BMC Biotechnol 2005; 5:2). Briefly, single embryos at the 8-cell stage are transferred to tiny droplets of dry lysing reagents placed on the lid of PCR tubes. After cell lysis, which occurs very rapidly, the tube is placed on the lid and inverted. One-step RT-PCR is performed by adding the reagents in the same tube already containing the lysed sample. Thus, both RNA and DNA are present in each sample. LATE-PCR primers are designed within an exon, also according to our published strategy, so that amplicons generated by Oct4 cDNA molecules and Oct4 genomic DNA molecules are identical and detected by the same fluorescent probe, a sequence-specific molecular beacon conjugated to the TET fluorescent dye. The final volume of these assays is 50 µl, according to the instruction of the One-Step RT-PCR kit, but the volume can be decreased to 25 µl.

[0070] The RT-LATE-PCR reaction is carried out in an ABI Prism 7700 Sequence Detector (Applied Biosystems, CA)

and quantification of “total DNA” (cDNA+genomic DNA) copy numbers for each sample is achieved by comparison with standard scales prepared with serial dilutions of commercially available genomic DNA. (Because the Oct4 primers are designed to amplify equally cDNA and genomic DNA, standard scales of genomic DNA are amplified exactly with the same efficiency as unknown cDNA samples, ensuring accurate quantification.) The total number of Oct4 templates in each 8-cell embryo includes 16 copies of genomic DNA (two copies of the gene per cell, one on each chromosome 17) while all the other copies are due to the presence of cDNA and, thus, reflect the Oct4 RNA content of the embryo.

[0071] Several One-Step RT-PCR kits are tested using this assay and the efficiencies for Oct4 RNA quantification are compared. FIG. 10 shows the effect of temperature on SuperScript III reverse transcriptase (Invitrogen).

[0072] The blue and orange bars in the figure are comparable to the light blue “No RT” bar, indicating that under these temperature conditions RT does not take place and only genomic Oct4 DNA is amplified in the samples (16 copies per embryo, as expected). At 55° C. (green bar), however, RT occurs and cDNA is efficiently generated. Considering that the reverse transcriptase used for these experiments is active in the 42–60° C. range, this narrow window of activity is unexpected. To clarify this point, the thermodynamics of the Oct4 primers during RT is analyzed and compared to their behavior during PCR.

[0073] Visual OMP 5.0 software (VOMP) is used for this analysis and the results are summarized in FIG. 11. The two primers used for a LATE-PCR assay (limiting primer, LP, and excess primer, XP) have different T_m s and concentrations. In the Oct4 RT-LATE-PCR assay the most abundant primer (XP) is also the RT primer, being antisense to Oct4 RNA. As shown in FIG. 11, the effective T_m of this primer calculated in the presence of double-stranded DNA during the PCR annealing step (at 55° C.) is of 66° C., very close to the calculated T_m of 67° C. The effective T_m of this same primer, however, drops dramatically to 53° C. in the presence of single-stranded RNA, even if the temperature of RT is also set at 55° C. This change results in a much lower percent of primer hybridized during RT than during the PCR annealing step, although the temperature is the same during the two steps. Although only 50% of the available primer is hybridized to target at 55° C., the primer was present at high concentration (2 μ M) which allowed efficient RT. Additional VOMP analyses show that Oct4 XP's effective T_m during RT does not change in the 50–60° C. range, which explains why increasing RT temperature in this case led to RT failure (much less primer was bound to target at 60° C. than at 55° C. and, contrary to the manufacturer's indications, the RTase was completely denatured after the initial 10 minutes at 60° C., see next section). On the other hand, the failure of RT at temperature lower than 55° C. (when, based solely on T_m , more primer should be hybridized) is probably due to increased levels of secondary structure of the target RNA interfering with the ability of the reverse transcriptase to progress along the template strand.

[0074] These results indicate that primers designed for PCR or LATE-PCR also should be analyzed in terms of their thermodynamic modification of a primer's design so that its T_m can meet the necessary requirements during both RT and PCR. In cases where this is not possible due to restraints intrinsic to the sequence, a third primer—designed to hybridize only during the RT step—could be added to the one-step mixture. In addition, the characteristics of LATE-PCR are

advantageous to promote RT priming in a one-step assay. In fact, by designating the XP to be also the RT primer we are able to use higher RT primer concentrations than those used under standard conditions in RT-PCR assays.

Example 3

[0075] This example demonstrates optimization of RT reaction parameters.

[0076] Satisfactory RT results are obtained for two different genes shortening the RT step from 30 to 5 minutes, although a slight loss of sensitivity is observed. Further reducing RT to 2 or 3 minutes still yields acceptable results. The reverse transcriptase used was SensiScript by Qiagen. (Raja et al., 2002. Temperature-controlled Primer Limit for Multiplexing of Rapid, Quantitative Reverse Transcription-PCR Assays: Application to Intraoperative Cancer Diagnostics. *Clinical Chemistry* 48:8, 1329-1337.)

[0077] RT also performed with SuperScript II (Invitrogen) for 5 minutes. (Raja et al., 2005. Technology for Automated, Rapid, and Quantitative PCR or Reverse Transcription-PCR Clinical Testing. *Clinical Chemistry* 51:5, 882-890.)

[0078] RT is successfully carried out for just 1 minute with either MMLV (Moloney Murine Leukemia Virus RT) or SuperScript III. (Stanley and Szewczuk, 2005. Multiplexed tandem PCR: gene profiling from small amounts of RNA using SYBR Green detection. *Nucleic Acids Research* 33:20, e180.)

[0079] Based on the above studies, a One-Step RT-PCR assay for detection of avian flu is designed that will encompass a RT step of no more than 5 minutes and as low as 1 minute. In doing so, we are aware that the optimal length of the RT reaction depends on several factors, including, but not limited to, efficient primer binding (see Example 2). The intrinsic thermostability of the RT enzyme also comes into play when choosing the temperature for RT, because the half-life of any enzyme sharply decreases at increasing temperatures, although some enzymes are more stable than others.

[0080] A clear example is provided by the following table posted on the web by the manufacturer Invitrogen

Summary of RT Half Lives at 50, 55, and 60° C.

[0081]

Temperature	SuperScript™ II RT (min)	SuperScript™ III RT (min)
50° C.	6.1	220
55° C.	2.2	24
60° C.	ND	2.5

[0082] From this table it follows that, when working with SuperScript III at 60° C. or with SuperScript II at 55° C., the optimal RT step duration is no more than 5 minutes in any case, independently from the primer T_m s, because the enzyme is completely denatured in this period of time.

[0083] Newer RTases with broader thermostability ranges are commercially available. For example, StrataScript 5.0 from Stratagene, has a half-life of 35 minutes at 55° C. There is also a number of polymerases commercialized by Roche and derived from thermophilic bacteria, which have both RTase and DNA polymerase activity.

[0084] We note that it is important to designing gene-specific RT primers with T_m s precisely calculated for optimal hybridization to target at a temperature elevated enough to minimize the secondary structure of single-stranded, GC-rich RNA molecules such as those present in viral genomes, but at the same time allowing a sufficient half-life of the chosen enzyme. The importance and the subtleties of this approach are not widely recognized, as shown by the suggestion: "Primers for real-time RT-PCR should be designed according to standard PCR guidelines" (Platinum Quantitative RT-PCR ThermoScript One-Step System instruction sheet, included with product purchased in 2006).

Example 4

[0085] This example demonstrates the use of a Smiths Detection Bio-Seeq II instrument as a portable, point-of-care assay device. The Bio-Seeq II used in this example is comprised of four independently operating thermal cycling units, each encasing a long thin-walled sample tube having a sample volume of 25 μ l. The primers and probes provided in FIG. 17 are used. Each sample is viewed using four-color fluorescence optics for dyes that emit at 520 nm, 580 nm, 625 nm, 680 nm. All colors can be viewed simultaneously without moving parts, a feature of the BioSeeq that reduces sampling time and lowers the risk of mechanical failure. To make full use of the broad detection temperature range available in LATE-PCR each unit can ramp up at 10° C./sec between 25-95° C., and is actively cooled at a rate of at least 2.5° C./sec between 95-25° C. The tolerance for thermal variance at any chosen hold temperature is $\pm 1^\circ$ C. The unit is AC or battery powered.

[0086] Each of the LATE-PCR mono-multiplex assays described below is designed to detect and distinguish any one of 15 possible outcomes in a single closed-tube. See FIGS. 15 and 16. These assays are easy to use "in the field" and provide rapid definitive yes/no answers as to the absence or presence of Influenza Virus sub-types H1N1, H3N2, B and H5N1. The assays also detect the presence of a Type B virus, or Type A influenza virus of unknown sub-type and include internal controls to rule out false negatives.

[0087] Each mono-multiplex reaction includes internal control target sequences, as shown in FIG. 17, at low copy number (approximately 20) to insure that all primer pairs are engaged in amplifying either a viral target sequence or an internal control. Accordingly, the reaction described below utilizes eight pairs of primers and eight internal controls.

[0088] Each mono-multiplex reaction is read at end-point by dropping the temperature to 40° C. and then to 25° C. Nine sequence-specific molecular beacons are used in this reaction to detect 7 of the possible viral targets. Three molecular beacons, each of a single color, form probe-target hybrids at T_m 45° C. Two additional molecular beacons, each of a single color, form probe-target hybrids at T_m 30° C. Two additional molecular beacons, each with two colors, form probe-target hybrids at T_m 45° C.

[0089] One mis-match tolerant probe is used to detect one of the viral targets at 40° C. and a variant of that sequence present in an internal control at 25° C. Seven of eight internal controls go undetected because they possess no targets for any probe.

[0090] Each mono-multiplex reaction is designed to distinguish Influenza Type B and Type A viruses on the basis of sequences in the HA and NA genes. Within the Type A viruses the reaction distinguishes between subtypes H5 (with or without N1 or N2), H1 (with or without N1 or N2), and H3 (with or without N1 or N2). The N1 target sequence used is conserved for the H5 and H1 subtypes and therefore is useful for detecting H5N1 and H1N1. Detection of H3N1 would indicate viral reassortment. The N2 target sequence used is characteristic of the H3N2 subtype. Therefore detection of H5N2 or H1N2 would indicate viral reassortment. The mono-multiplex reaction described here is a one-step RT-LATE-PCR reaction. The chemical features of this one-step process are described elsewhere.

[0091] One assay provides a reliable means of detecting the Eurasian subtype of H5. Specimens that test positive for H5 Eurasian in the field are sent to an analytical laboratory for complete multiplex analysis and sequencing.

[0092] In a second assay, the H5 amplicon produced in the field includes the region of the RNA known to code for high pathogenicity of the Eurasian sub-type. This region is less conserved, but very important. Under these circumstances the tube that tests positive in the field for Eurasian H5 is sent to the laboratory for immediate sequencing of the H5 amplicon. There is no need to transport the specimen itself or additional amplification

[0093] The next step involves in-depth laboratory analysis of influenza genes using LATE-PCR multiplexing and nucleic acid sequencing. Starting with samples of purified RNA, the HA RNA (1770 nucleotides long) and the NA RNA (1400 nucleotides long) are both reverse transcribed in toto using random hexamers in a highly efficient two step RT-procedure. Each reaction also contains low levels of the same M-Gene control DNA describes for the BioSeeq POC assays above. The resulting control and cDNA molecules are amplified in two parallel multiplex LATE-PCR assays that each generate six amplicons (FIG. 15).

[0094] The possible presence of Eurasian H5N1 strain in a sample will be established by probing for M, N1 and two different H5 sequences which are likely to be crucial for human-to-human transmission and for virulence. Reactions that do not generate either signal for H5 Eurasian still produce a Control DNA signal, proving that they are not false negatives. Reactions that do signal the presence of the H5 Eurasian strain from either of two independent probes (one in Multiplex A and one in Multiplex B) also generate a strong M-gene signal in both Multiplex A and Multiplex B. However, some samples may generate a signal for the Matrix protein and only one of the two possible HA signals. This is regarded as an indication of viral evolution. All samples that generate either one or two HA signals, or an N1 signal plus an M-gene signal will immediately be processed further for analysis. Amplicons for the all portions of HA and NA are already be present in the LATE-PCR multiplex reactions. All 10 HA and NA amplicons are 300-500 by in length and are processed for parallel pyrosequencing as described above.

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atcaggcccc ctcaaagccg aaatcgcgca gagacttgaa gatgtctttg ctgggaaaaa 120

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cacagatctt gaggtctctca tggaatggct aaagacaaga ccaatcctgt cacctctgac	180
taaggggatt ttggggtttg tgttcacgct caccgtgccc agtgagcgag gactgcagcg	240
tagacgcttt gtccaaaatg ccctcaatgg gaatggggat ccaataaca tggacaaagc	300
agttaaactg tatagaaaac ttaagaggga gataacattc catggggcca aagaaatagc	360
actcagttat tctgctggcg cacttgccag ttgcatgggc ctcatataca ataggatggg	420
ggctgtaacc accgaagtgg catttggcct ggtatgtgca acatgtgaac agattgctga	480
ctcccagcac aggtctcata ggcaaatggg ggcaacaacc aatccattaa taaaacatga	540
gaacaggatg gttttggcca gcactacagc taaagctatg gagcaaatgg ctggatcaag	600
tgagcaggca gcgaggcca tggagattgc tagtcaggcc aggcaaatgg tgcaggcaat	660
gagaaccgtt gggactcatc ctagtccag tactggtcta agagatgatc ttcttgaaaa	720
tttgcagacc tatcagaaac gaatgggggt gcagatgcaa cgattcaagt gacctgcttg	780
ttgttgctgc gagtatcatt gggatcttgc acttgatatt gtggattctt gatcgtcttt	840
ttttcaaatg catctatcga ctcttcaaac acggcctgaa aagagggcct tctacggaag	900
gagtacctga gtctatgagg gaagaatato gaaaggaaca gcagaatgct gtggatgctg	960
acggcagtc ttttgtcagc atagagctg	989

<210> SEQ ID NO 30

<211> LENGTH: 985

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 30

atgagtcttc taaccgaggt cgaaacgtac gttctctcta tcgtcccgtc aggccccc	60
aaagccgaga tcgcacagag acttgaaaat gtctttgctg gaaagaatac cgatcttgag	120
gctctcatgg aatggctaaa gacaagacca atcctgtcac ctctgactaa ggggatttta	180
ggatttgtgt tcacgctcac cgtgcccagt gagcgaggac tgcagcgtag acgctttgtc	240
caaaatgccc ttaatgggaa tggggatcca aataatatgg acagagcagt taaactgtat	300
cgaaagctta agaggagat aacattccat ggggccaaag aaatagcact cagttattct	360
gctggtgcac ttgccagttg tatgggactc atatacaaca ggatgggggc tgtgaccacc	420
gaatcagcat ttggccttat atgcgcaacc tgtgaacaga ttgccgactc ccagcataag	480
tctcataggc aaatggtaac aacaaccaac ccattaataa gacatgagaa cagaatggtt	540
ctggccagca ctacagctaa ggctatggag caaatggctg gatcgagtga acaagcagct	600
gaggccatgg aggttgctag tcaggccagg cagatggtgc aggcaatgag agccattggg	660
actcactcta gctctagcac tggctgaaa aatgatctcc ttgaaaattt gcaggcctat	720
cagaaacgaa tgggggtgca gatgcaacga ttcaagtgat cctcttgttg ttgccgcaag	780
tataattggg attgtgcacc tgatattgtg gattattgat cgcctttttt caaaagcat	840
ttatcgtatc tttaaacacg gtttaaaaag agggccttct acggaaggag taccagagtc	900
tatgagggaa gaatatcgag aggaacagca gaatgctgtg gatgctgacg atggtcattt	960
tgtcagcata gagctggagt aaaaa	985

<210> SEQ ID NO 31

<211> LENGTH: 1039

<212> TYPE: DNA

-continued

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 31

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agcaaaagca ggtagatgtt gaaagatgag tcttctaacc gaggtcgaaa cgtacgttct    60
ctctatcadc ccgtcaggcc ccctcaaagc cgagatcgcg cagaaacttg aagatgtctt    120
tgcaggaaaag aacaccgatc tcgaggctct catggagtgg ctaaagacaa gaccaatcct    180
gtcacctctg actaaaggga ttttgggatt tgtattcacg ctcaccgtgc ccagtgcgag    240
aggactgcag cgtagacgct ttgtccagaa tgccttaaat ggaaatggag atccaaataa    300
tatggataga gcagtcaagc tatataagaa gctgaaaaga gaaataacat tccatggggc    360
taaggaggtc gcactcagct actcaaccgg tgcacttgcc agttgcatgg gtctcatata    420
caacaggatg ggaacggtga ctacggaagt ggcttttggc ctagtgtgtg ccacttgtga    480
gcagattgca gattcacagc atcgggtctc cagacagatg gcaactacca ccaaccact    540
aatcagacat gagaacagaa tgggtgctggc cagcactaca gctaaggcta tggagcagat    600
ggcaggatca agtgagcagg cagcggaagc catggagatc gctaatacagg ctaggcagat    660
gggtgcaggca atgaggacaa ttgggactca tccctaactct agtgctggtc tgagagataa    720
tcttcttgaa aatttgacag cctaccagaa acgaatggga gtgcagatgc agcgattcaa    780
gtgatcctat tgttgttgcc gcaaataatca ttgggatctt gcacttgata ttgtggattc    840
ttgatcgtct tttcttcaaa tgcatttato gtcgccttaa atacggtttg aaaagagggc    900
ctgctacggc aggggtacct gagtctatga gggaagagta ccggcaggaa cagcagagt    960
ctgtggatgt tgacgatggt cattttgtca acatagaatt ggagtaaaaa actaccttgt   1020
ttctactaat acggaagac                                     1039

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<210> SEQ ID NO 32

<211> LENGTH: 1076

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 32

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atgtcgctgt ttggagacac aattgcctac ctgctttcat tgacagaaga tggagaaggc    60
aaagcagaac tagcagaaaa attacactgt tggttcggtg ggaagaatt tgacctagac    120
tctgccctgg aatggataaa aaacaaaaga tgcttaactg atatacaaaa agcactaatt    180
ggtgctctta tctgcttttt aaaacccaaa gaccaggaaa gaaaaagaag attcatcaca    240
gagcctctat caggaatggg aacaacagca acaaaaaaga aaggcctgat tctagctgag    300
agaaaaatga gaagatgtgt gagctttcat gaagcatttg aaatagcaga aggccatgaa    360
agctcagcgc tactatactg tctcatggtc atgtacctga atcctggaaa ttattcaatg    420
caagtaaaac taggaacgct ctgtgctttg tgcgagaaac aagcatcaca ttcacacagg    480
gctcatagca gagcagcgag atcttcagtg cctggagtga gacgagaaat gcagatggtc    540
tcagctatga acacagcaaa aacaatgaat ggaatgggaa aaggagaaga cgtccaaaaa    600
ctggcagaag agctgcaaa caacattgga gtactgagat ctcttggggc aagtcaaaag    660
aatggagaag gaattgcaaa ggatgtaatg gaagtgttaa agcagagctc tatgggaaat    720
tcagctcttg tgaagaata tctataatgc tcgaaccatt tcagattctt tcaatttgtt    780
cttttatctt atcagctctc catttcatgg cttggacaat agggcatttg aatcaataa    840

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aaagaggagt aaacatgaaa atacgaataa aaagtccaaa caaagagaca ataaacagag	900
aggtatcaat tttagacac agttacaaa aagaaatcca ggccaaagaa acaatgaagg	960
aggtactctc tgacaacatg gaggtattga gtgaccacat aataattgag gggctttctg	1020
ccgaagagat aataaaaatg ggtgaacag ttttgagat agaagaattg cattaa	1076

<210> SEQ ID NO 33

<211> LENGTH: 1736

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 33

tcactgtca aatggagaaa atagtgttc ttttgcaat agtcagtctt gttaaaagt	60
atcagatttg cattggttac catgcaaaa actcgacaga gcaggttgac acaataatgg	120
aaaagaacgt tactgttaca catgccaag acatactgga aaagacacac aacgggaagc	180
tctgcgatct agatggagtg aagcctctaa ttttgagaga ttgtagtga gctggatggc	240
tcctcgga cccaatgtgt gacgaattca tcaatgtgcc ggaatggtcc tacatagtgg	300
agaaggccaa tccagtcaat gacctctgtt acccagggga tttcaatgac tatgaagaat	360
tgaaacacct attgagcaga ataaaccatt ttgagaaaat tcagatcatc cccaaaagtt	420
cttggccag tcataagacc tcattagggg tgagctcagc atgtccatac cagagaaagt	480
cctcctttt cagaaatgtg gtatggctta tcaaaaagaa cagtacatac ccaacaataa	540
agaggagcta caataatacc aaccaagaag atcttttggg actgtggggg attcaccatc	600
ctaattgatgc ggcagagcag acaaagctct atcaaaaccc aaccacctat attccgttg	660
ggacatcaac actaaaccag agattggtac caagaatagc tactagatcc aaagtaaacg	720
ggcaaagtgg aaggatggag ttcttctgga caattttaaa accgaatgat gcaatcaact	780
tcgagagtaa tggaaatttc attgtccag aatatgcata caaaattgtc aagaaagggg	840
actcaacaat tatgaaaagt gaattggaat atggtaactg caacaccaag tgtcaaaactc	900
caatgggggc gataaactct agtatgcat tccacaatat acaccctctc accatcgggg	960
aatgcccaca atatgtgaaa tcaaacagat tagtccttgc gactgggctc agaaatagcc	1020
ctcaaagaga gagaagaaga aaaaagagag gattatttgg agctatagca ggttttatag	1080
agggaggatg gcaggaatg gtagatggtt ggtatgggta ccaccatagc aatgagcagg	1140
ggagtgggta cgctgcagac aaagaatcca ctcaaaaggc aatagatgga gtcaccaata	1200
aggtcaactc gatcattgac aaaatgaaca ctcagtttga ggccgttgga agggaattta	1260
acaacttaga aaggagaata gagaatttaa acaagaagat ggaagacggg ttcttagatg	1320
tctggactta taatgctgaa cttctggttc tcatggaaaa tgagagaact ctgactttc	1380
atgactcaaa tgtcaagaac ctttacgaca aggtccgact acagcttagg gataatgcaa	1440
aggaaactgg taacggttgt ttcgagttct atcataaatg tgataatgaa tgtatggaaa	1500
gtgtaagaaa cggaacgtat gactaccgc agtattcaga agaagcaaga ctaaaaagag	1560
aggaaataag tggagtaaaa ttggaatcaa taggaattta ccaaaactg tcaatttatt	1620
ctacagtggc gagttcccta gcaactggca tcatggtagc tggctctatc ttatggatgt	1680
gctccaatgg gtcgttacaa tgcagaattt gcatttaaat ttgtgagttc agattg	1736

<210> SEQ ID NO 34

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<211> LENGTH: 1692

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 34

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atgaaagcaa aactactggt cctgttatgt acatttacag ctacatatgc agacacaata    60
tgtataggct accatgccaa caactcaacc gacactgttg acacagtact tgagaagaat    120
gtgacagtga cacactctgt caacctactt gaggacagtc acaacggaaa actatgtcta    180
ctaaaaggaa tagcccccact acaattgggt aattgcagcg ttgccggatg gatcttagga    240
aaccagaat gcgaattact gatttccaag gaatcatggt cctacattgt agaaacacca    300
aatcctgaga atggaacatg ttaccagggt tatttcgccg actatgagga actgagggag    360
caattgagtt cagtatcttc atttgagaga ttcgaaatat tcccaaaga aagctcatgg    420
cccaaccaca ccgtaaccgg agtatcagca tcatgctccc ataatgggaa aagcagtttt    480
tacagaaatt tgctatggct gacggggaag aatggtttgt acccaaacct gagcaagtc    540
tatgtaaaca acaaagagaa agaagtcctt gtactatggg gtgttcacac cccgcctaac    600
ataggggacc aaagggccct ctatcataca gaaaatgctt atgtctctgt agtgtcttca    660
cattatagca gaagattcac ccagaaaata gccaaaagac ccaaagtaag agatcaggaa    720
ggaagaatca actactactg gactctgctg gaacctgggg atacaataat atttgaggca    780
aatggaaatc taatagcgcc atggtatgct tttgcaactga gtagaggctt tggatcagga    840
atcatcacct caaatgcacc aatggatgaa tgtgatgcga agtgtcaaac acctcaggga    900
gctataaaca gcagtcttcc tttccagaat gtacaccag tcacaatagg agagtgtcca    960
aagtatgtca ggagtgcata attaaggatg gttacaggac taaggaacat cccatccatt   1020
caatccagag gtttgtttgg agccattgcc ggtttcattg aaggggggtg gactggaatg   1080
gtagatgggt ggtatgggta tcatcatcag aatgagcaag gatctggcta tgctgcagat   1140
caaaaaagta cacaaaatgc cattaacggg attacaaaca aggtgaattc tgtaattgag   1200
aaaaatgaaca ctcaattcac agctgtgggc aaagaattca acaaatgga aagaaggatg   1260
gaaaacttaa ataaaaaagt tgatgatggg tttctagaca tttggacata taatgcagaa   1320
ttgttggttc tactggaaaa tgaaaggact ttggatttcc atgactccaa tgtgaagaat   1380
ctgtatgaga aagtaaaaag ccaattaaag aataatgcca aagaaatagg aaacgggtgt   1440
tttgaattct atcacaagtg taacaatgaa tgcattggaga gtgtgaaaaa tggaaacttat   1500
gactatccaa aatattccga agaatcaaag ttaaacaggg agaaaattga tggagtga    1560
ttggaatcaa tgggagtcta tcagattctg gcgatctact caactgtgc cagttccctg    1620
gttcttttgg tctccctggg ggcaatcagc ttctggatgt gttccaatgg gtctttgcag    1680
tgcagaatat gc                                         1692

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<210> SEQ ID NO 35

<211> LENGTH: 1718

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 35

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tattaacat gaagactatc attgctttga gctacattct atgtctgggt ttcgctcaaa    60
aacttcccg aaatgacaac agcacggcaa cgctgtgcct tgggcacat gcagtaccaa    120

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acggaacgat agtgaaaaca atcacgaatg accaaattga agttactaat gctactgagc	180
tggttcagag ttctcaaca ggtggaatat gcgacagtcc tcatcagatc cttgatggag	240
aaaaatgcac actaatagat gctctattgg gagaccctca gtgtgatggc ttccaaaata	300
agaaatggga cctttttgtt gaacgcagca aagcctacag caactgttac ctttatgatg	360
tgccggatta tgccctccctt aggtcactag ttgcctcatc cggcacactg gagtttaaca	420
atgaaagctt caattggact ggagtcactc aaaatggaac aagctctgct tgtaaaagga	480
gatctaataa cagtttcttt agtagattga attggttgac ccacttaaaa ttcaaatacc	540
cagcattgaa cgtgactatg ccaaacaatg aaaaatttga caaattgtac atttgggggg	600
ttcaccaccc gggtacggac aatgacaaa ttagcctata tgctcaagct tcaggaagaa	660
tcacagtctc taccaaaaga agccaacaaa ctgtaatccc gaatatcgga tctagacca	720
gggtaaggga tatccccagc agaataagca tctattggac aatagtaaaa cggggagaca	780
tacttttgat taacagcaca gggaatctaa ttgctcctcg gggttacttc aaaatacgaa	840
gtgggaaaag ctcaataatg agatcagatg caccatttgg caaatgcaat tctgaatgca	900
tcactccaaa tggaagcatt cccaatgaca aaccatttca aaatgtaaac aggatcacat	960
atggggcctg tcccagatat gttaagcaaa acactctgaa attggcaaca gggatgcgaa	1020
atgtaccaga gaaacaaact agaggcatat ttggcgcaat cgcgggttcc atagaaaatg	1080
gttgggaggg aatggtggat ggttggtacg gtttcaggca tcaaaattct gagggaatag	1140
gacaagcagc agatctcaaa agcactcaag cagcaatcaa ccaaatcaat gggaagctga	1200
ataggttgat cgggaaaacc aacgagaaat tccatcagat tgaaaaagaa ttctcagaag	1260
tagaaggagg aattcaggac ctcgagaaat atgttgagga cactaaaata gatctctggt	1320
catacaacgc ggagcttctt gttgccctgg agaaccaaca tacaattgat ctaactgact	1380
cagaaatgaa caaactgttt gaaagaacaa agaagcaact gagggaaaat gctgaggata	1440
tgggcaatgg ttgtttcaaa atataccaca aatgtgacaa tgctgcata gggatcaatca	1500
gaaatggaac ttatgacct gatgtataca gagatgaagc attaaacaac cggttccaga	1560
tcaaaggtgt tgagctgaag tcaggatata aagattggat cctatggatt tcctttgcc	1620
tatcatgttt ttgtctttgt gttgttttgt tggggttcat catgtgggcc tgccaaaaag	1680
gcaacattag gtgcaacatt tgcatttgag tgcattaa	1718

<210> SEQ ID NO 36

<211> LENGTH: 1038

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 36

gatcgaatct gcactgggat aacatcttca aactcacctc atgtggtcaa aacagctact	60
caaggggagg tcaatgtgac tgggttgata ccactgacaa caacaccaac aaaatcttat	120
tttgcaaatc tcaaaggaa aaggaccaga gggaaactat gccagactg tctcaactgt	180
acagatctgg atgtggcctt gggcaggcca atgtgtgtgg ggaccacacc ttctgcgaaa	240
gcttcaatac tccacgacct gttacatccg ggtgctttcc tataatgcac gacagaacaa	300
aaatcgaagt caaggcaact agccaatctt ctgagggat atgaaaatat caggttatca	360
acccaaaacg ttatcgatgc agaaaaggca ccaggaggac cctacagact tggaacctca	420

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ggatcttgcc ctaacgctac cagtaaaagc ggatttttcg caacaatggc ttgggctgtc	480
ccaaaggaca acaacaaaaa tgcaacgaac cactaacag tagaagtacc atacatttgt	540
acagaagggg aagaccaaat tactgttttg gggttccatt cagataacaa aaccctaatg	600
aagaacctct atggagactc aaatcctcaa aagttcacct catctgctaa tggagtaacc	660
acacattatg tttctcagat tggcggcttc ccagatcaa cagaagacgg aggactacca	720
caaagcggca gaattgtcgt tgattacatg atgcaaaaac ctgggaaaac aggaacaatt	780
gtctatcaaa gaggtgtttt gtgcctcaa aaggtgtggt gcgcgagtgg caggagcaaa	840
gtaataaaag ggtccttgcc tttaatttgt gaagcagatt gccttcatga aaaatacggc	900
ggattaacaa aaagcaagcc ttactacaca ggagaacatg caaagccat aggaaattgc	960
ccaatatggg tgaaaacacc tttgaagctt gccaatggaa ccaatatag acctcctgca	1020
aaactattaa aggaaagg	1038

<210> SEQ ID NO 37

<211> LENGTH: 1409

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 37

atgaatccaa atcaaaaaat aataaccatt ggatcaatca gtatagcaat cggaataatt	60
agtctaattgt tgcaaatagg aaatattatt tcaatatggg ctagtctctc aatccaaact	120
ggaagtcaaa accacactgg agtatgcaac caaagaatca tcacatatga aaacagcacc	180
tgggtgaatc acacatatgt taatattaac aacactaatg ttgttgctgg aaaggacaaa	240
acttcagtga cattggccgg caattcatct ctttgttcta tcagtggatg ggctatatac	300
acaaaagaca acagcataag aattggctcc aaaggagatg tttttgtcat aagagaacct	360
ttcatatcat gttctcactt ggaatgcaga accttttttc tgaccaagg tgctctatta	420
aatgacaaac attcaaatgg gaccgttaag gacagaagtc cttatagggc cttaatgagc	480
tgctctctag gtgaagctcc gtcccatac aattcaaagt ttgaatcagt tgcattgtca	540
gcaagcgcac gccatgatgg catgggctgg ttaacaatcg gaatttctgg tccagacaat	600
ggagctgtgg ctgtactaaa atacaacggc ataataactg aaaccataaa aagttggaaa	660
aaagcgaatat taagaacaca agagtctgaa tgtgtctgtg tgaacgggtc atgtttcacc	720
ataatgaccg atggcccgag taatggggcc gcctcgtaaa aaatcttcaa gatcgaaaag	780
gggaagggtta ctaaatcaat agagttgaat gcacccaatt ttcattatga ggaatgttcc	840
tgttacccag aactggcac agtgatgtgt gtatgcaggg acaactggca tggttcaaat	900
cgaccttggg tgtcttttaa tcaaaacctg gattatcaaa taggatacat ctgcagtggg	960
gtgttcgggtg acaatccgcg tcccaaagat ggagagggca gctgtaatcc agtgactgtt	1020
gatggagcag acggagtaaa ggggttttca tacaatatg gtaatggtgt ttggatagga	1080
aggactaaaa gtaacagact tagaaagggg tttgagatga tttgggatcc taatggatgg	1140
acagataccg acagtgtatt ctcatgaaa caggatgttg tggcaataac tgattggtca	1200
gggtacagcg gaagtctcgt tcaacatcct gagttaacag gattggactg tataagacct	1260
tgcttctggg ttgagttagt cagaggactg cctagagaaa atacaacaat ctggactagt	1320
gggagcagca tttctttttg tggcgtaaat agtgatactg caaactggtc ttggccagac	1380

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 ggtgctgagt tgccattcac cattgacaa 1409

<210> SEQ ID NO 38
 <211> LENGTH: 1392
 <212> TYPE: DNA
 <213> ORGANISM: Influenza virus

<400> SEQUENCE: 38

ttattggtct cagggagcaa aagcaggagt tcaaatgaa tccaaataag aagataataa	60
ccatcggatc aatctgtatg gtaactggaa tggtagctt aatgttaca attgggaact	120
tgatctcaat atgggtcagt cattcaatc acacaggga tcaacacaaa gctgaaccaa	180
tcagcaatac taattttctt actgagaaag ctgtggcttc agtaaaatta gcgggcaatt	240
catctctttg ccccattaat ggatgggctg tatacagtaa ggacaacagt ataaggatcg	300
gttccaaggg ggatgtgttt gttataagag agccattcat ctcatgctcc cacttggaat	360
gcagaacttt ctttttgact cagggagcct tgctgaatga caagcactcc aatgggactg	420
tcaaagacag aagccctcac agaacattaa tgagttgtcc tgtgggtgag gctccctccc	480
catataactc aaggtttgag tctgttgctt ggtcagcaag tgcttgccat gatggacca	540
gttggttgac aattggaatt tctggcccag acaatggggc tgtggctgta ttgaaataca	600
atggcataat aacagacact atcaagagt ggaggaataa catactgaga actcaagagt	660
ctgaatgtgc atgtgtaaat ggctcttgct ttactgtaat gactgacgga ccaagtaatg	720
gtcaggcatc acataagatc ttcaaatgg aaaaaggga agtgggttaa tcagtcgaat	780
tggatgctcc caattatcac tatgaggaat gctcctgtta tctgatgcc ggcgaaatca	840
catgtgtgtg cagggataat tggcatggct caaatcgcc atgggtatct ttcaatcaaa	900
atttgagta tcaaatagga tatatatgca gtggagtttt tggagacaat ccacgcccc	960
atgatggaac aggtagtgtt ggtccggtg cctctaacgg ggcatatgg gtaaaaggt	1020
tttcatttaa atacggcaat ggtgtctgga tcgggagaac aaaaagcact aattccagga	1080
gcggctttga aatgatttgg gatccaaatg ggtggactga aacggacagt agcttttcag	1140
tgaacaaga tctgtagca ataactgatt ggtcaggata tagcgggagt ttgtccagc	1200
atccagaact gacaggacta gattgcataa gacctgttt ctgggttgag ttgatcagag	1260
ggcggcccaa agagagcaca atttggacta gtgggagcag catatctttt tgtggtgtaa	1320
atagtgcac tgtgggttgg tcttggccag acggtgctga attgccattc accattgaca	1380
agtagttgtt ca	1392

<210> SEQ ID NO 39
 <211> LENGTH: 1410
 <212> TYPE: DNA
 <213> ORGANISM: Influenza virus

<400> SEQUENCE: 39

atgaatccaa atcaaaagat aataacgatt ggctctgttt ctctcaccat ttccacaata	60
tgcttcttca tgcaaatgc catcttgata actactgtaa cattgcattt caagcaatat	120
gaattcaact ccccccaaa caaccaagtg atgctgtgtg aaccaacaat aatagaaga	180
aacataacag agatagtgtg tctgaccaac accaccatag agaaggaaat atgccccaaa	240
ctagcagaat acagaaattg gtcaaagccg caatgtaaca ttacaggatt tgcacctttt	300

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tctaaggaca attcgattag gctttccgct ggtggggaca tctgggtgac aagagaacct	360
tatgtgtcat gcgacctga caagtgttat caatttgccc ttgggcaggg aacaacacta	420
aacaacgtgc attcaaatga cacagtacat gataggaccc cttatcggac cctattgatg	480
aatgagttag gtgttccatt tcatctgggg accaagcaag tgtgcatagc atgggtccagc	540
tcaagttgtc acgatggaaa agcatggctg catgtttgtg taacggggga tgataaaaat	600
gcaactgcta gcttcattta caatgggagg cttgtagata gtattgtttc atgggtccaaa	660
gaaatcctca ggacccagga gtcagaatgc gtttgatca atggaacttg tacagtagta	720
atgactgatg ggagtgttc aggaaaagct gatactaaaa tactattcat tgaggagggg	780
aaaaatcggtc atactagcac attgtcagga agtgctcagc atgtcgagga gtgctcctgc	840
tatcctcgat atcttgggtg cagatgtgtc tgcagagaca actggaaagg ctccaatagg	900
cccatagtag atataaacat aaaggattat agcattgttt ccagttatgt gtgctcagga	960
cttgttggag acacaccag aaaaaacgac agctccagca gtagccattg cttggatcct	1020
aacaatgaag aaggtgtgca tggagtgaag ggctgggcct ttgatgatgg aatgacgtg	1080
tggatgggaa gaacgatcag cgagaagta cgctcaggat atgaaacctt caaagtcatt	1140
gaaggctggt ccaacccata ttccaaattg cagataaata ggcaagtcatt agttgacaga	1200
ggtaaatagg cgggttatct tgggtatttc tctgttgaag gcaaaagctg catcaatcgg	1260
tgcttttatg tggagtgtat aagggaaga aaaggagaaa ctgaagtctt gtggacctca	1320
aacagtattg ttgtgttttg tggcacctca ggtacatatg gaacaggctc atggcctgat	1380
ggggcggaca tcaatctcat gcctatataa	1410

<210> SEQ ID NO 40

<211> LENGTH: 1463

<212> TYPE: DNA

<213> ORGANISM: Influenza virus

<400> SEQUENCE: 40

ccaaatgaa caatgtacc ttcaactata caaacgttaa ccctatttct cacatcaggg	60
ggagtattat tatcactata tgtgtcagct tcattgtcat acttactata ttccgatata	120
ttgctaaaaat tcccataaac agaaattact gcaccaacaa tgccattgga ttgtgcaaac	180
gcatacaatg ttcaggctgt gaaccgttct gcaacaaaag gggtgacact tcttctccca	240
gaaccggagt ggacataccc gcgtttatct tgcccgggct caacctttca gaaagcactc	300
ctaattagcc ctcatagatt cggagaaacc aaaggaaact cagctccctt gataataagg	360
gaacctttta ttgcttggg accaaaggaa tgcaaacact ttgctctaac ccactatgca	420
gccccaccag ggggatacta caatggaaca agaggagaca gaaacaagct gaggcatcta	480
atttcagtca aattgggcaa aatcccaaca gtagaaaact ccattttcca catggcagca	540
tggagcgggt ccgatgccca tgatggtaag gaatggacat atatcggagt tgatggccct	600
gacaataatg cattgtctca aataaaatat ggagaagcat atactgacac ataccattcc	660
tatgcaaaaca acatcctaag aacacaagaa agtgccctgca attgcatcgg gggaaattgt	720
tatcttatga taactgatgg ctacagcttca ggtgttagtg aatgcagatt tcttaagatt	780
cgagagggcc gaataataaa agaaatatcc ccaacaggaa gaataaaaca tactgaagaa	840
tgacatgcg gatttgctag caataaaacc atagaatgtg cctgtagaga taacagttac	900

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acagcaaaaa gaccctttgt caaattaaac gtggagactg atacagcaga aataagattg    960
atgtgcacag agacttatth ggacaccccc agaccagatg atggaagcat aacagggcct    1020
tgtgaatcta atggggacaa aggggagtga ggcatcaagg gaggatttgt ccatcaaaga    1080
atggcatcca agattggaag gtggtactct cgaacgatgt ctaaaactaa aaggatgggg    1140
atggggctgt atgtcaagta tgatggagac ccatgggctg acagtgatgc ccttgctttt    1200
agtggagtaa tggtttcaat ggaagaacct ggttggtact cctttggctt cgaaataaaa    1260
gacaagaaat gtgatgtccc ctgtattggg atagagatgg tacatgatgg tggaaaagag    1320
acttggcact cagcagctac agccatttac tgtttaatgg gctcaggaca gctgctgtgg    1380
gacactgtca caggtgttaa tatggctctg taatggagga atggttgagt ctgttctaaa    1440
ccctttgttc ctattttgtt tga                                           1463

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<210> SEQ ID NO 41
<211> LENGTH: 101
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

```

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<400> SEQUENCE: 41

```

```

gtggagtaaa attggaatca ataggaattt accaaatact gtcaatttat tctacagtgg    60
cgagttccct agcactggca atcatggtag ctggtctatc c                        101

```

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<210> SEQ ID NO 42
<211> LENGTH: 88
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

```

```

<400> SEQUENCE: 42

```

```

gtggagtaaa attggaatca ataggaattt accaaatact gtcaatttat tctacagtgc    60
actggcaatc atggtagctg gtctatcc                                           88

```

```

<210> SEQ ID NO 43
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

```

```

<400> SEQUENCE: 43

```

```

ccatgactcc aatgtgaaga atctgtatga gaaagtaaaa agccaattaa agaataatgc    60
caaagaaata ggaaacgggt g                                                81

```

```

<210> SEQ ID NO 44
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

```

```

<400> SEQUENCE: 44

```

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ccatgactcc aatgtgaaga atctgtataa agaataatgc caaagaaata ggaaacgggt 60

g 61

<210> SEQ ID NO 45

<211> LENGTH: 202

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 45

gcaataactg attggtcagg atatagcggg agttttgtcc agcatccaga actgacagga 60

ctagattgca taagaccttg tttctgggtt gagttgatca gagggcggcc caaagagagc 120

acaatttga ctagtgggag cagcatatct tttgtggtg taaatagtga cactgtgggt 180

tggtcttggc cagacggtgc tg 202

<210> SEQ ID NO 46

<211> LENGTH: 187

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 46

gcaataactg attggtcagg atatagcggg agttttgtcc agcatccaga actgacagga 60

ctagattgca taagaccttg tttctgggtt gagttgatca gagggcggcc caaagagagc 120

acaatttga catctttttg tgggtgtaaat agtgacactg tgggttggtc ttggccagac 180

ggtgctg 187

<210> SEQ ID NO 47

<211> LENGTH: 98

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 47

ccatcagatt gaaaaagaat tctcagaagt agaagggaga attcaggacc tcgagaaata 60

tgttgaggac actaaaatag atctctggtc atacaacg 98

<210> SEQ ID NO 48

<211> LENGTH: 83

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 48

ccatcagatt gaaaaagaat tctcagaagt agaagggaga atttatgttg aggacactaa 60

aatagatctc tggtcataca acg 83

<210> SEQ ID NO 49

<211> LENGTH: 122

<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 49

cggtggatta aacaaaagca agccttacta cacaggggaa catgcaaagg ccataggaaa 60
ttgcccaata tgggtgaaaa cacccttgaa gctggccaat ggaaccaa atagacctcc 120
tg 122

<210> SEQ ID NO 50
<211> LENGTH: 105
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 50

cggtggatta aacaaaagca agccttacta cgcccatagg aaattgcca atatgggtga 60
aaacaccctt gaagctggcc aatggaacca aatatagacc tcctg 105

<210> SEQ ID NO 51
<211> LENGTH: 119
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 51

catgggctga cagtgatgcc cttgcttcta gtggagtaat ggtttcaatg gaagaacctg 60
gttggtactc ctttggtctc gaaataaaag acaagaaatg tgaatgtccc tgtattggg 119

<210> SEQ ID NO 52
<211> LENGTH: 102
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 52

catgggctga cagtgatgcc cttgcttcta gtggaagaac ctggttggtg ctcctttggc 60
ttcgaaataa aagacaagaa atgtgatgtc cctgtattg gg 102

<210> SEQ ID NO 53
<211> LENGTH: 152
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 53

ctaaccgagg tcgaaacgta cgttctctct atcatcccggt caggccccct caaagccgag 60
atcgcgcaga aacttgaaga tgtctttgca ggaaagaaca ccgatctcga ggctctcatg 120
gagtggctaa agacaagacc aatcctgtca cc 152

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<210> SEQ ID NO 54
<211> LENGTH: 141
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 54

ctaaccgagg tcgaaacgta ccatcccgtc agggccctc aaagccgaga tcgcgcagaa 60
acttgaagat gtctttgcag gaaagaacac cgatctcgag gctctcatgg agtggctaaa 120
gacaagacca atcctgtcac c 141

<210> SEQ ID NO 55
<211> LENGTH: 107
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 55

ggtccaaccc taattccaaa ttgcagataa ataggcaagt catagttgac agaggtaata 60
ggtccggtta ttctggtatt ttctctgttg aaggcaaaag ctgcatc 107

<210> SEQ ID NO 56
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 56

ggtccaaccc taattccaaa ttgcagataa ataggcaagt catagtttta ttctggtatt 60
ttctctgttg aaggcaaaag ctgcatc 87

<210> SEQ ID NO 57
<211> LENGTH: 98
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 57

ccatcagatt gaaaaagaat tctcagaagt agaagggaga attcaggaac tctagaaata 60
tggtgaggac actaaaatag atctctgtgc atacaacg 98

<210> SEQ ID NO 58
<211> LENGTH: 88
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 58

cgactgggct cagaaatagc cctcaaagag agagaagaag aaaaaagaga ggattatttg 60
gagctatagc aggttttata gagggagg 88

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<210> SEQ ID NO 59
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      oligonucleotide

<400> SEQUENCE: 59

cgactgggct cagaaatagc cctcaaagag ttatttggag ctatagcagg ttttatagag      60
ggaggg                                           65

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1. (canceled)
2. A reverse transcription-polymerase chain reaction (PCR) method comprising:
 - a) adding to a reaction vessel a reaction mixture comprising at least one RNA comprising a target sequence, reverse transcriptase, DNA amplification reagents that include an excess primer and a limiting primer for the target sequence, and DNA polymerase, wherein the concentration of the excess primer is 500-2000 nM and at least five times higher than the concentration of the limiting primer, and wherein the concentration-adjusted melting temperature of the limiting primer to the target sequence is equal to or greater than the concentration-adjusted melting temperature of the excess primer to the target sequence;
 - b) incubating the reaction mixture at a first temperature to reverse transcribe the target sequence from the RNA utilizing the excess primer and the reverse transcriptase to create a cDNA template for LATE-PCR amplification, said first temperature being sufficiently high to disrupt secondary structure of the RNA but sufficiently low to provide a reverse-transcriptase half-life of at least 2.2 minutes, wherein the effective melting temperature of the excess primer to the RNA is not lower than 2° C. below said first temperature; and
 - c) thermally cycling the reaction mixture to amplify the cDNA template using the excess primer, the limiting primer and the DNA polymerase to produce both double-stranded amplification product and single-stranded amplification product.
3. The method of claim 2 wherein the concentration of the excess primer is at least ten times higher than the concentration of the limiting primer.
4. The method of claim 2 wherein the concentration of the excess primer is at least twenty times higher than the concentration of the limiting primer.
5. The method of claim 1 wherein the RNA is added to the reaction mixture as at least one lysed cell or at least one lysed virus.
6. The method of claim 1 wherein the reverse transcriptase and the DNA polymerase are a single enzyme that has both reverse transcription activity and DNA polymerase activity.
7. The method of claim 1 wherein the reaction mixture includes a fluorescently-labeled hybridization probe for detection of the single-stranded amplification product.
8. The method of claim 1 wherein said at least one RNA comprises at least two target sequences, and the DNA amplification reagents include an excess primer and a Limiting primer for each target sequence.
9. The method of claim 1 wherein said first temperature is in the range of 50° C. to 60° C.
10. A reaction mixture comprising:
 - (a) at least one RNA, wherein said RNA comprises a target sequence;
 - (b) reverse transcriptase;
 - (c) DNA amplification reagents including an excess primer and a limiting primer for the target sequence, wherein the concentration of the excess primer is 500-2000 nM and at least five times higher than the concentration of the limiting primer, and wherein the concentration-adjusted melting temperature of the limiting primer to the target sequence is equal to or greater than the concentration-adjusted melting temperature of the excess primer to the target sequence; and
 - (d) DNA polymerase.
11. The reaction mixture of claim 10 wherein the concentration of the excess primer is at least ten times higher than the concentration of the limiting primer.
12. The reaction mixture of claim 11 wherein the concentration of the excess primer is at least twenty times higher than the concentration of the limiting primer.
13. The reaction mixture of claim 10 wherein the RNA is added to the reaction mixture as at least one lysed cell or at least one lysed virus.
14. The reaction mixture of claim 10 wherein the reverse transcriptase and the DNA polymerase are a single enzyme that has both reverse transcription activity and DNA polymerase activity.
15. The reaction mixture of claim 10 further comprising a fluorescently-labeled hybridization probe for detection of the single-stranded amplification product.
16. The reaction mixture of claim 10 wherein said at least one RNA comprises at least two target sequences, and the DNA amplification reagents include an excess primer and a Limiting primer for each target sequence.

* * * * *