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Supplementary Materials for

Synthetic Generation of Influenza Vaccine Viruses for Rapid Response to Pandemics

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/5/185/185ra68/DC1)

HANABOD (HA and NA BARDA Oligo Designer) software, version 5.2, and documentation.

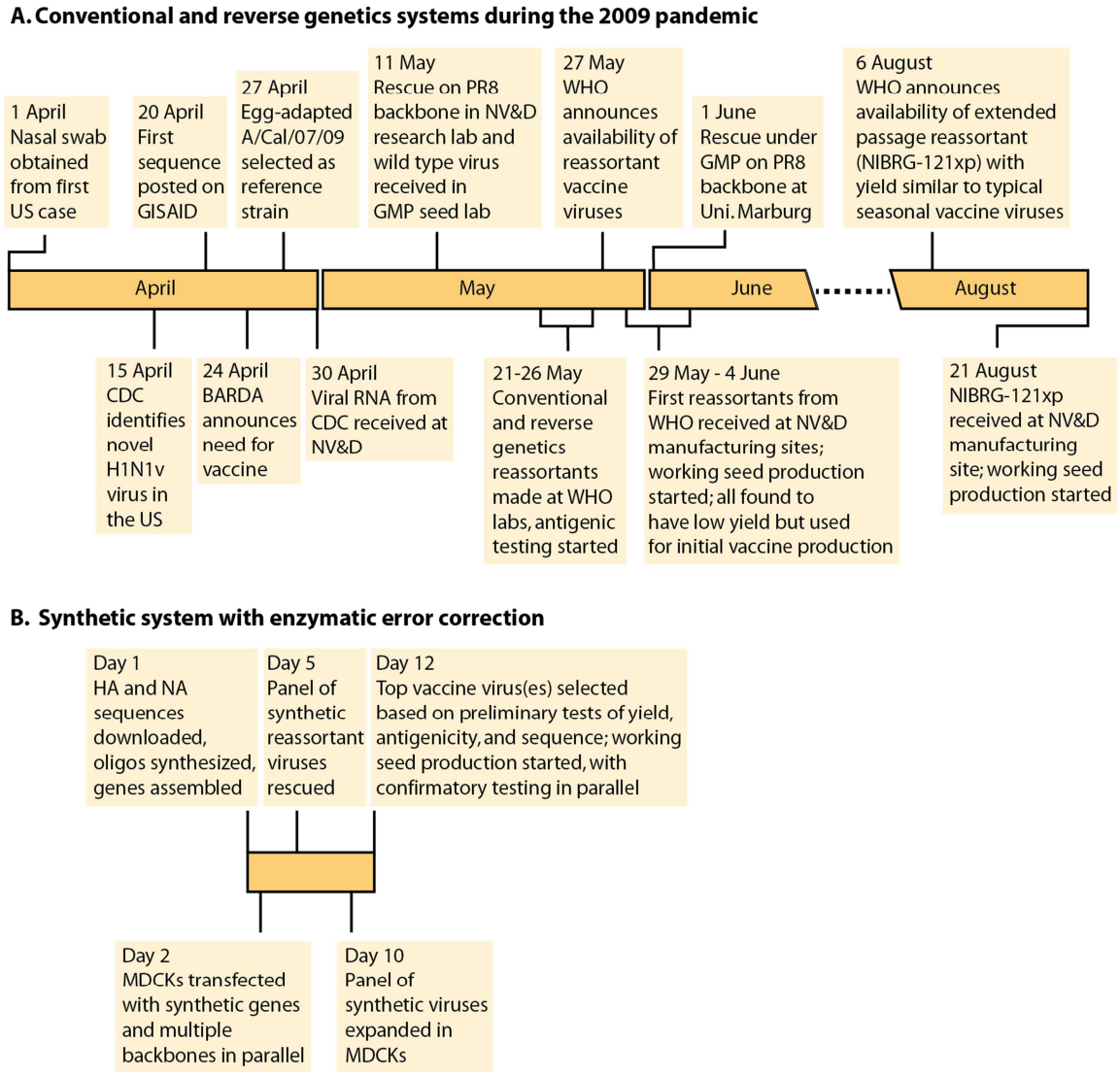
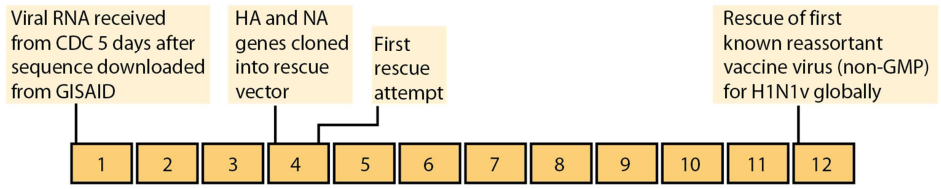
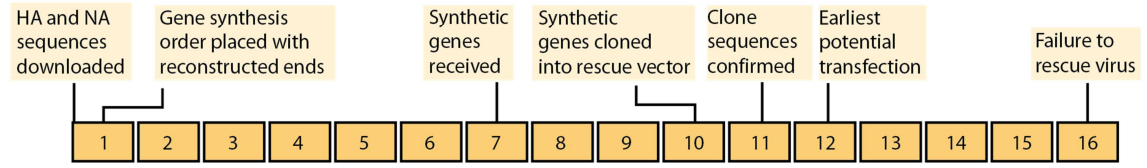


Figure S1. Timelines to the availability of influenza vaccine viruses at manufacturing sites (*2*, *22*, *23*, *25*). (**A**) The 2009 timeline from the detection of the H1N1 pandemic strain in the US to the receipt at a Novartis manufacturing site of a vaccine virus with a yield equivalent to that typical for a seasonal vaccine virus. The timeline includes conventional and reverse genetics efforts from WHO Collaborating Centers, Novartis, and Philipps-Universität Marburg. (**B**) Projected future timeline for generation of a vaccine virus using the synthetic system with enzymatic error correction. This timeline assumes virus generation at a manufacturing site, eliminating shipping delays. The projected timeline in **(B)** is aligned with the actual 2009 timeline in (**A**) based on HA and NA gene synthesis being initiated at the time when BARDA announced the need for a vaccine (April 24, 2009). The projected time savings with the synthetic approach relative to receipt at any Novartis manufacturing site of any reassortant vaccine virus in 2009 is 23 days. The time savings relative to receipt in 2009 of a vaccine virus with a yield typical of seasonal vaccine viruses would be 107 days.

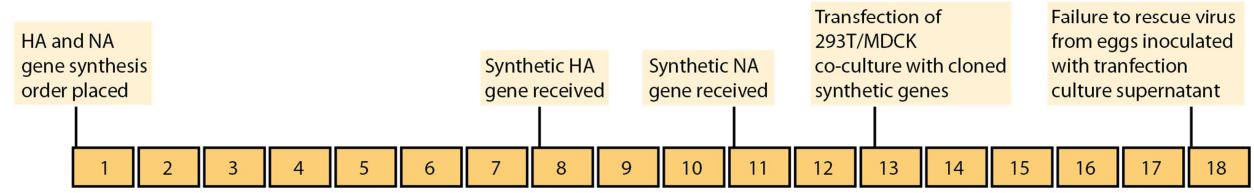
A. NV&D rescue of a reverse genetic reassortant from cloned genes - 2009



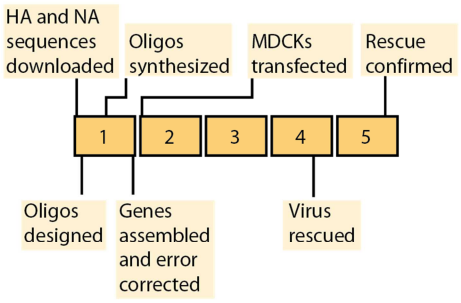
B. NV&D attempt to rescue virus with commercially synthesized genes - 2009



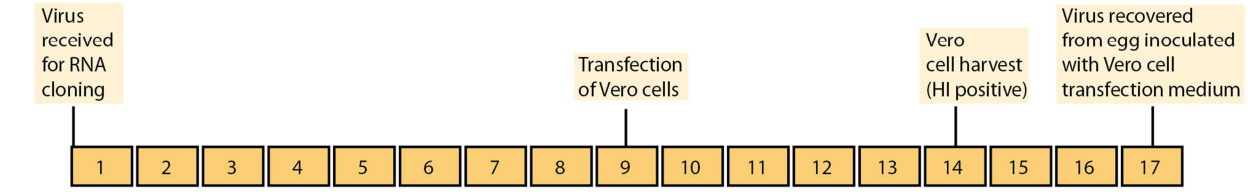
C. CSL attempt at virus rescue with commercially synthesized genes - 2009



D. Rescue with synthesized genes and enzymatic error correction



E. NIBSC rescue of A/Fujian/411/02 by reverse genetics with a quality system



F. Reverse genetic rescue with genes cloned from respiratory specimens

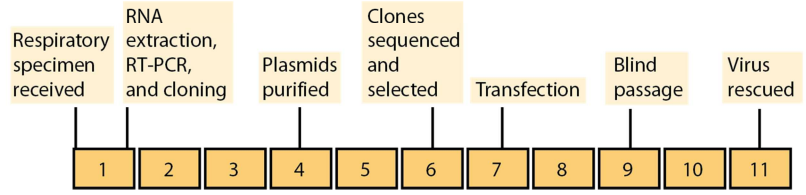


Figure S2. Timelines for synthetic and conventional reverse genetic influenza virus rescue. For comparison, the generation of a conventional reassortant vaccine virus requires approximately 21 days from the time that a wild type virus is received (*3*). Each box represents one day.

(**A**) NV&D rescue of a reverse genetic reassortant from cloned genes – 2009. The timeline begins with the receipt of H1N1v RNA from the CDC, 5 days after the HA and NA sequences were downloaded from GISAID, and 10 days after H1N1v sequences were first posted on GISAID. Therefore, for accurate comparison to the synthetic technique, 5 to 10 days must be added to the reverse genetics timeline. This effort resulted in the first PR8 reassortant potential H1N1v vaccine virus globally. The reassortant was not used for vaccine manufacture because the virus was rescued in 293T/MDCK co-cultures under research conditions. The rescue was repeated with manufacturing-suitable MDCK cells alone under highly controlled conditions, but this virus also was not used for vaccine manufacture, due to the regulatory hurdles to manufacturing with a vaccine virus produced by a new process.

(**B**) NV&D attempt at rescue with commercially synthesized genes – 2009. During the pandemic response, synthetic HA and NA genes were received 6 days after they were ordered from a commercial supplier. Because the supplying and receiving sites were both in Germany, shipping did not contribute significantly to the timeline. Initial attempts at rescue with the synthetic genes were not successful, although subsequent attempts after process optimization did succeed.

(**C**) CSL Limited (CSL) attempt at rescue with commercially synthesized genes – 2009 (*3*). The failure of the synthetic virus generation attempt was attributed to lack of egg-adaptive mutations in the sequences available. A subsequent recombinant addition of egg-adaptive mutations led to rescue in the system, which involves a final egg passage. Information on the timeline of the rescue of the virus with the egg-adaptive mutations was not provided. Because this technique includes rescue in 293T cells, which are not qualified for vaccine manufacture, the technique is not suitable for generating viruses to be used to produce vaccines for human use.

(**D**) Rescue with synthesized genes and enzymatic error correction. This timeline differs from the synthetic rescue timeline in Fig. 4 because shipping between sites is eliminated (by consolidating all activities at a single manufacturing site), and the time for virus rescue is extended (because rescue in MDCK cells alone is less rapid than rescue in a mixed 293T/MDCK cell co-culture). Because the synthetic technique allows the rescue of many viral variants in parallel (including coding sequence, genome segment end, and backbone variants), the probability of obtaining a high yield, antigenically correct strain is increased.

(**E**) National Institute for Biological Standards and Control (NIBSC) rescue of A/Fujian/411/02 by reverse genetics with a quality system (*10*). The timeline starts with receipt of a wild type virus. For comparison to the synthetic timeline, the interval from the public availability of sequence data to the receipt by a manufacturer of a sample of an emerging virus must be added to the reverse genetics timeline.

(**F**) Reverse genetic rescue with genes cloned from respiratory specimens (*26*). This technique requires receipt of a shipped respiratory specimen rather than an electronically transmitted gene sequence.

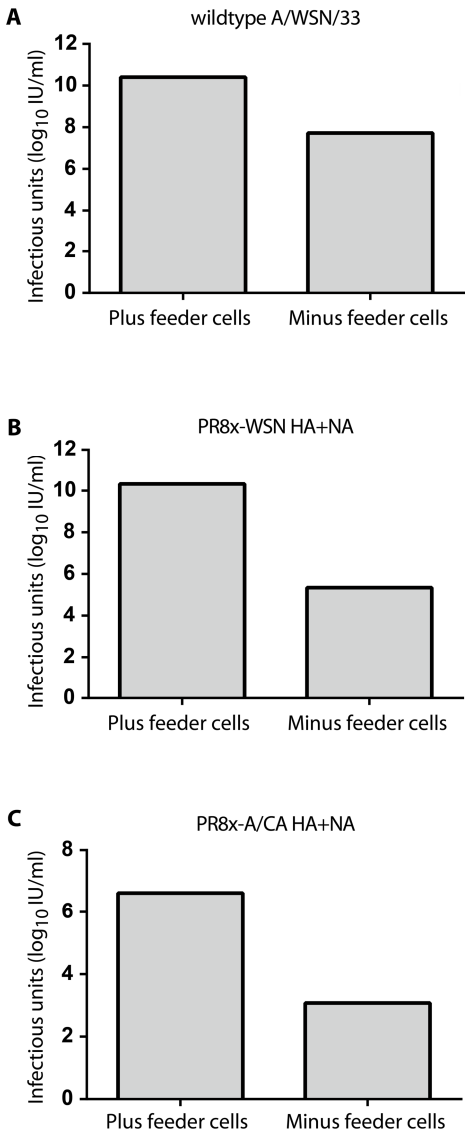


Figure S3. Effect of MDCK feeder cell addition on titers of viruses recovered from DNA-transfected cells. Fresh MDCK 33016PF cells were added after DNA transfection of MDCK 33016PF cells in serum-free medium. Titers of (**A**) wild type virus A/WSN/1933, or recombinant viruses containing the PR8x backbone with HA and NA segments from (**B**) A/WSN/1933 or (**C**) A/California/04/2009 were measured 72 hours after transfection by a focus formation assay. Data in each panel are from one experiment with each measurement made in duplicate.

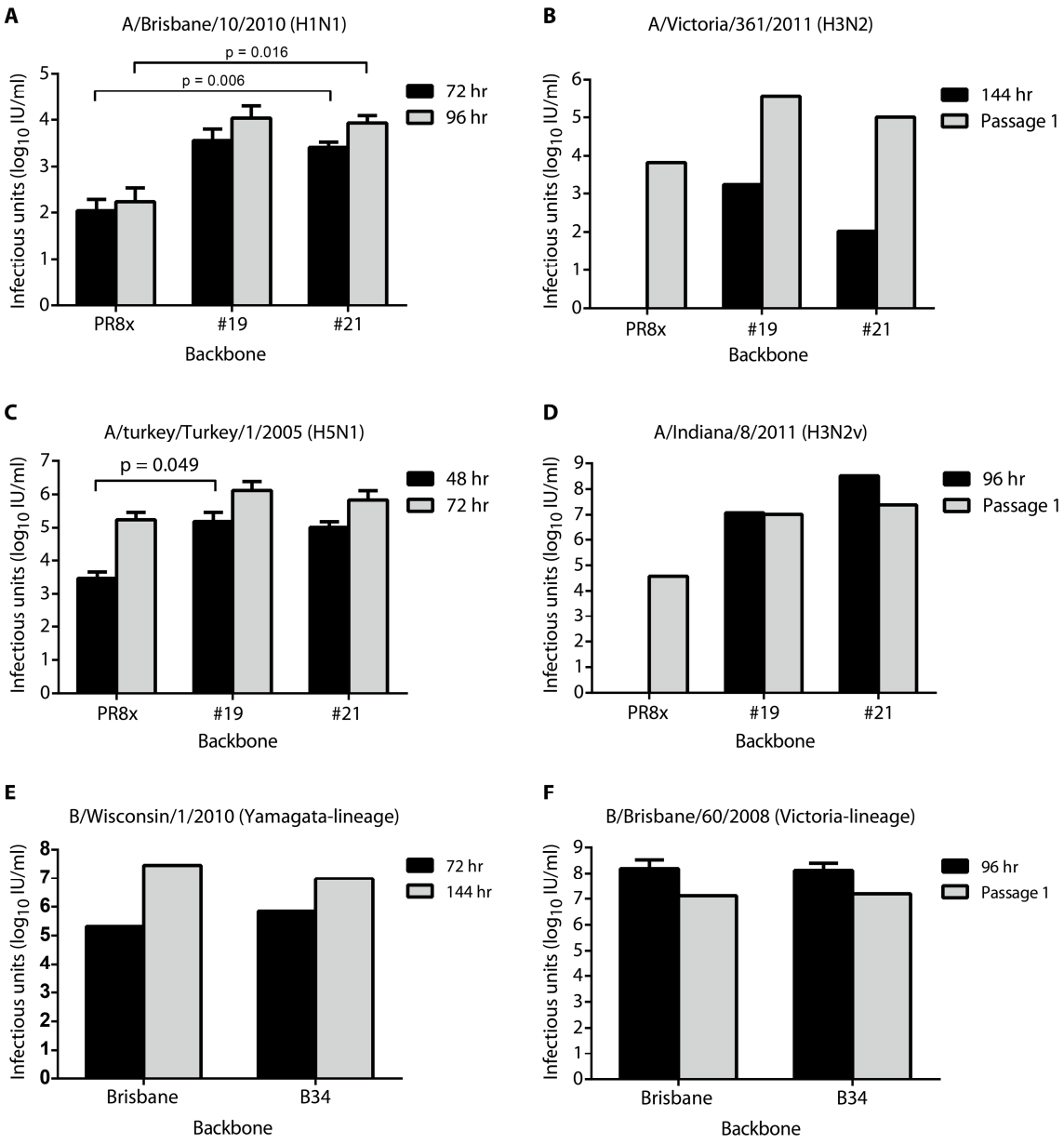


Figure S4. Effect of optimized backbones on synthetic influenza virus rescue efficiencies. Influenza viruses with the indicated backbones and synthetic HAs and NAs were detected in MDCK 33016PF cell culture fluid harvested at the indicated times after transfection or harvested 24-48 hrs after a blind passage of 500 µl of the primary culture fluid on fresh MDCK 33016PF cell monolayers (Passage 1). Viral titers were determined with a focus formation assay for **(A)** an H1N1 strain (3 independent experiments for each time point), **(B)** an H3N2 strain (2 independent experiments for each time point), **(C)** an attenuated H5N1 strain (4 or 5 independent experiments, depending on the backbone, for the 48 hr time point and 5 or 6 independent experiments, depending on the backbone, for the 72 hr time point), **(D)** a swine origin H3N2v strain (one experiment), **(E)** a B/Yamagata lineage strain (2 independent experiments), and **(F)** a B/Victoria lineage strain (3 independent experiments for Brisbane; 4 independent experiments

for B34). All measurements were made in duplicate (with the exception of one experiment in panel C at the 48 hr time point, for which single measurements were made). Error bars indicate the standard error of the mean. For experiments with sufficient replicates to allow reliable statistical evaluation, the significance of the difference between backbones and time points that produced the highest and lowest infectious titers was assessed by a one-way ANOVA, Fisher's LSD.

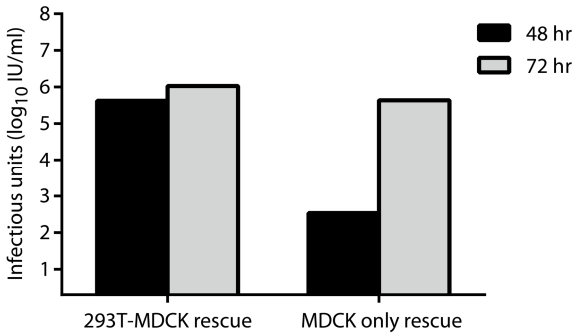


Figure S5. Synthetic H7N9a virus rescue efficiency from MDCK-supplemented 293T cells or from MDCK cells only. Influenza viruses were detected in culture fluid harvested 48 and 72 hrs after transfection with the #19 backbone plasmids and synthetic H7 HA and N9 NA gene constructs. Viral titers were determined on MDCK cell monolayers with a focus formation assay. Data are from a single experiment with 2 replicate measurements.

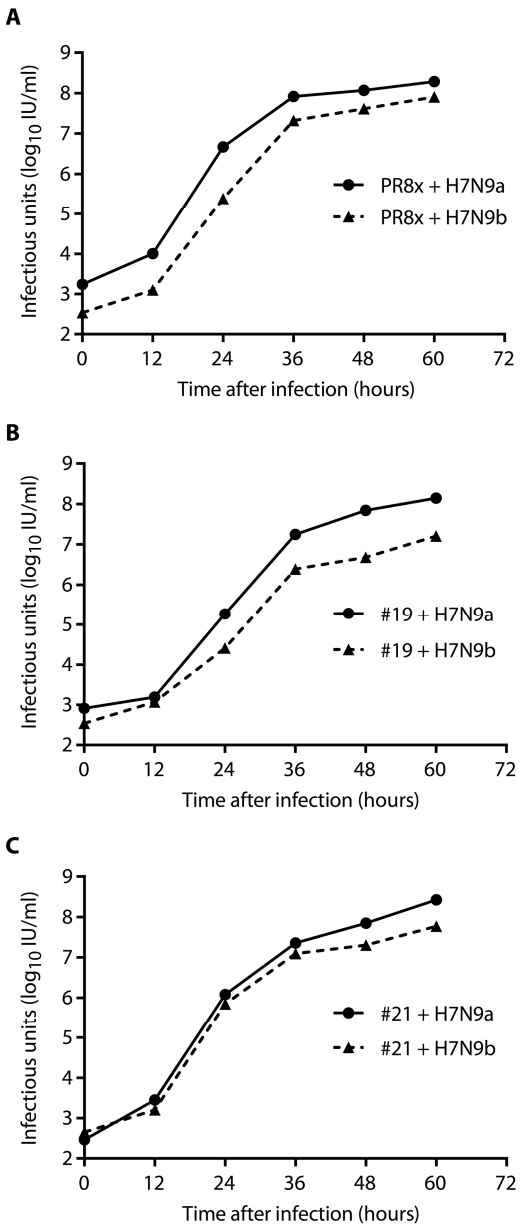


Figure S6. Replication kinetics of synthetic H7N9 viruses with alternative NA UTRs and different backbones. The NA UTR in the H7N9a set of viruses had C1434 in the positive sense; the NA UTR in the H7N9b set of viruses had U1434. The tested viruses had PR8x (**A**), #19 (**B**), or #21 (**C**) backbones and were propagated in MDCK 33016PF suspension cultures. The starting multiplicity of infection was 0.001. Each line depicts the results of 2 independent experiments. All measurements were made in duplicate.

Table S1. **GenBank accession codes of backbone gene sequences.**

Strain	Gene	GenBank accession code
A/Puerto Rico/8/1934	NS	KC866599
	M	KC866600
	NP	KC866598
	PA	KC866595
	PB1	KC866596
	PB2	KC866597
A/Hessen/105/2007	NP	KC866612
	PB1	KC866610
	PB2	KC866611
A/California/7/2009	PB1	KC866601
B/Brisbane/60/2008	NS	KC866606
	M	KC866606
	NP	KC866605
	PA	KC866602
	PB1	KC866603
	PB2	KC866604
B/Panama/45/1990	NS	KC866608
	N	KC866609

Table S2. Virus titers and HA yields from influenza viruses with optimized backbones relative to conventional vaccine viruses when propagated in embryonated chicken eggs. HA yields in mass per volume of allantoic fluid are normalized and shown as fold-improvement relative to yields from reference strains, which are set to 1.0. Guinea pig red blood cell (GP-RBC) agglutination, RP-HPLC, or lectin-capture ELISA was used to detect HA. Reference strains were obtained from the US CDC or the UK NIBSC.

Strain with alternative backbone	Reference strain	FFA titer	HA titer by GP-RBC agglutination	HA yield by RP-HPLC	HA yield by ELISA	Backbone
A/Christchurch/16/2010 (H1N1)[a,b]	NIB74	3.0	3.5	18	8.4	#21
A/Victoria/210/2009 (H3N2)[a,b]	X187	0.9	1.3	n/t	1.2	PR8x
A/Victoria/361/2011 (H3N2)[c,d]	IVR-165	6.4	2.6	n/t	3.4	#21
A/Indiana/8/2011 (H3N2v)[b,c]	X213	n/t	3.0	1.6	n/a	PR8x
B/Wisconsin/1/2010 (Yam)[c,d]	wild-type	4.7	3.4	n/t	3.5	Brisbane
B/Brisbane/60/2008 (Vic)[c,d]	wild-type	1.1	0.8	n/t	0.8	Brisbane

[a]virus contains HA and NA genome segments from plasmids

[b]HA yields determined from virus purified from egg allantoic fluid by a sucrose density gradient

[c]virus contains synthetic HA and NA genome segments

[d]HA yields determined directly from egg allantoic fluid

n/t, not tested

n/a, data not available because strain-specific antisera were not available for ELISA

Yam, B/Yamagata lineage

Vic, B/Victoria lineage

Table S3. Diversity of synthetic influenza virus strains rescued. All H1N1 strains listed are of the post-2009 H1N1 pandemic lineage.

Seasonal serotype A virus		Backbone		
Source of synthetic HA, NA genes	**Subtype**	**PR8X**	**#19**	**#21**
A/Brisbane/10/2010	H1N1	+	+	+
A/Christchurch/16/2010 (NIB74)	H1N1	+	+	+
A/Christchurch/16/2010 NIB74-K170E	H1N1	n/a	n/a	+
A/Christchurch/16/2010 NIB74-K171E	H1N1	n/a	n/a	+
A/Christchurch/16/2010 NIB74-G172E	H1N1	n/a	n/a	+
A/Christchurch/16/2010 NIB74-G173D	H1N1	n/a	n/a	+
A/Uruguay/716/2007	H3N2	+	+	+
A/Victoria/210/2009 (X187)	H3N2	+	+	+
A/Victoria/361/2011 (CDC E3)	H3N2	+	+	+
A/Victoria/361/2011 (WHO E3)	H3N2	+	+	+
A/Victoria/361/2011 (MDCK)	H3N2	+	+	+
A/Berlin/93/2011 (egg-derived)	H3N2	+	+	+
A/Berlin/93/2011 (cell-derived)	H3N2	+	+	+
A/Brisbane/402/2011	H3N2	+	+	+
A/Victoria/304/2011 NVD p2/E3	H3N2	-	-	+
A/Brisbane/256/2011 MDCK P2	H3N2	+	+	+
A/Brisbane/256/2011 P2/E3	H3N2	-	+	+
A/South Australia/34/2011	H3N2	-	+	+
A/Brisbane/299/2011 (IVR164)	H3N2	+	+	+
A/Brisbane/299/2011 (E5)	H3N2	+	+	+
A//South Australia/3/2011	H3N2	+	+	+
A/Wisconsin/1/2011	H3N2	+	+	+

Seasonal serotype B virus		Backbone	
Source of synthetic HA, NA genes	**Lineage**	**Brisbane**	**B34**
B/Hubei-Wujiangang/158/2009	Yam	+	+
B/Wisconsin/1/2010	Yam	+	+
B/Brisbane/3/2007	Yam	+	+
B/Jiangsu/10/2003	Yam	+	+
B/Johannesburg/05/1999	Yam	+	+
B/Yamanashi/166/1998	Yam	+	+
B/Yamagata/16/1988	Yam	+	+
B/Texas/6/2011	Yam	+	-
B/Brisbane/36/2012	Yam	-	+
B/New Hampshire/1/2012	Vic	+	+
B/Malaysia/2506/2004	Vic	+	+
B/Brisbane/32/2002	Vic	+	+
B/Brisbane/60/2008 (cell)	Vic	+	+
B/Brisbane/60/2008 (egg)	Vic	+	n/a
B/Nevada/3/2011	Vic	+	+

Pre-pandemic viruses		Backbone		
Source of synthetic HA, NA genes	**Subtype**	**PR8X**	**#19**	**#21**
A/Hubei/1/2010	H5N1	+	+	+
A/Egypt/N03072/2010	H5N1	+	+	+
A/Turkey/Turkey/1/2005	H5N1	+	+	+
A/goose/Nebraska/11-017097-4/2011	H7N9	+	+	+
A/Indiana/8/2011	H3N2v	+	+	+

n/a, not attempted; +, virus recovered in ≤6 days post-transfection; -, virus not recovered by 6 days post-transfection; Yam, Yamagata; Vic, Victoria.

Table S4. Oligonucleotide sequences for HA gene assembly in the proof-of-concept test

Well	Name	Sequence
A1	082911_test_HA_1	agtactggtcgacctccgaagttgggggggagcaaaagcaggggatacaaaatgaacactcaaatttt
A2	082911_test_HA_2	tttagctccaatcagcatacaagcaatgagtgccaaaatttgagtgttcattttgtatcccctgcttt
A3	082911_test_HA_3	ggcactcattgcttgtatgctgattggagctaaaggagataaaatatgtcttgggcaccatgctgtgg
A4	082911_test_HA_4	ctctctgttaatgtgttcacttttgttccatttgccacagcatggtgcccaagacatattttatctcc
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B12	082911_test_HA_24	ttgctctgtcaggagctatgaatgccccattgaaagtgaaggtcactgtgtcattgggatcaaggagt
C1	082911_test_HA_25	ttcaatggggcattcatagctcctgacagagcaagtttctttagaggggagtcactaggagttcagag
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D11	082911_test_HA_47	gagtcattgcagaatagaatacagatagacccagtgaaattgagtagtggatacaaagacataatc
D12	082911_test_HA_48	aagaaaacatgatgccccgaagctaaaccataagattatgtctttgtatccactactcaatttcac
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E2	082911_test_HA_50	aatagtgcaccgcatgtttccattctttatgcaaatgaaaaccaatcccattgcaatggctagaag
E3	082911_test_HA_51	tgcataaagaatggaaacatgcggtgcactatttgtatatagtttgagaaaaacacccttgtttct
E4	082911_test_HA_52	cgagtcggcattttgggccgccgggttattagtagaaacaagggtgtttttctcaaactatataca

Table S5. Oligonucleotide sequences for NA gene assembly in the proof-of-concept test

Well	Name	Sequence
A1	082911_test_NA_1	agtactggtcgacctccgaagttgggggggagcaaaagcagggtcaagatgaatccaaatcagaagat
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B2	082911_test_NA_14	ggatctatcgtgtattgttccatttgagtgtttccctctaatcgttgttccttggctgagagcataga
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D2	082911_test_NA_38	taaaaacacgctcggtagcactccccttcagcccaatagtccatgaaggacccactgtagccactc
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