

Supplementary Information for:

Signatures of native-like glycosylation in RNA replicon-derived HIV-1 immunogens

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Supplementary Table 1: Glycan composition analysis of replicon expressed env in HEK 293F cells. The upper table shows the categorized glycan compositions at each N-linked glycan site with the reported value the mean of three or more biological replicates. The global averages are shown in the right-hand table. The lower table further categorizes the glycan compositions into oligomannose-, hybrid-, and complex-type as well as the percentage of glycan compositions containing at least one fucose or one sialic acid residue and core-type glycan structures.

	N88	N133	N142	N156	N160	N185e	N185h	N197	N234	N262	N276	N295	N301	N332	N339	N355	N363	N386	N392	N398	N406	N411	N448	N462	N611	N618	N625	N637	Total
M9Glc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M9	0	0	0	78	24	0	0	13	26	39	0	86	0	72	14	0	38	42	28	0	1	1	30	0	0	0	0	1	18
M8	0	7	0	0	24	0	0	10	25	22	14	8	0	22	31	0	18	22	34	0	5	7	20	2	1	0	0	3	10
M7	2	0	0	22	10	0	0	3	11	9	15	0	0	3	12	1	7	12	10	0	5	19	8	1	2	0	0	21	6
M6	2	6	0	0	9	0	0	4	5	6	7	0	0	2	6	1	3	9	7	0	6	13	5	1	1	0	0	11	4
M5	11	21	0	0	9	2	1	10	9	15	29	6	0	1	6	42	7	10	11	3	34	23	9	6	14	10	0	17	11
M4	0	14	0	0	1	0	0	1	0	2	3	0	0	0	2	2	1	2	2	1	10	4	3	2	1	0	0	2	2
M3	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	2	3	0	0	0	0	3	1
FM	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	9	0	1
Hybrid	1	0	66	0	1	0	0	1	0	0	5	0	0	0	1	10	1	1	0	0	7	10	3	0	1	0	1	6	4
Fhybrid	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	3	1	0	0	1	4	0	0	0	1	0	0	3	1
HexNAc(3)(x)	3	0	6	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	4	0	1	1	3	0	1	0	1
HexNAc(3)(F)(x)	1	27	0	0	2	1	0	2	0	0	2	0	0	0	1	4	1	0	0	11	6	2	1	5	2	0	0	2	3
HexNAc(4)(x)	18	0	23	0	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	12	2	0	1	0	0	0	0	0	2
HexNAc(4)(F)(x)	27	0	4	0	2	3	0	12	7	5	7	0	0	1	2	15	5	0	2	52	14	0	7	46	10	25	1	12	9
HexNAc(5)(x)	8	0	0	0	0	0	82	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
HexNAc(5)(F)(x)	9	7	1	0	2	2	1	5	1	1	6	0	0	0	1	14	2	0	1	17	2	0	3	26	24	9	0	5	5
HexNAc(6+)(x)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HexNAc(6+)(F)(x)	7	18	0	0	0	0	7	1	0	0	3	0	0	0	0	2	0	0	0	1	0	0	0	5	13	5	0	1	2
UNOCC	9	0	0	0	15	89	0	34	12	0	0	0	0	0	21	4	14	0	0	0	0	17	1	0	25	50	87	14	14
core	1	0	0	0	2	1	3	1	3	2	1	0	0	0	2	0	1	2	4	0	0	1	7	4	3	0	0	1	1

%	N88	N133	N142	N156	N160	N185e	N185h	N197	N234	N262	N276	N295	N301	N332	N339	N355	N363	N386	N392	N398	N406	N411	N448	N462	N611	N618	N625	N637	Total
Mannose	15	47	0	100	77	3	5	41	77	92	69	100	0	99	72	48	75	97	93	5	61	70	77	11	19	12	10	58	51
Hybrid	1	0	66	0	1	0	0	3	0	0	5	0	0	0	1	13	2	1	0	1	10	10	3	1	1	0	1	8	5
Complex	73	53	34	0	5	6	93	20	8	6	25	0	0	1	3	35	8	1	3	94	29	3	12	84	52	39	2	19	29
Unoccupied	9	0	0	0	15	89	0	34	12	0	0	0	0	0	21	4	14	0	0	0	0	17	1	0	25	50	87	14	14
Fucosylation	44	53	5	0	5	6	12	21	9	6	19	0	0	1	4	38	9	1	3	83	26	2	11	83	50	40	10	22	21
Sialylation	25	8	93	0	0	1	10	6	4	3	10	0	0	0	0	13	0	0	0	57	12	1	2	33	9	9	0	4	11
core	1	0	0	0	2	1	3	1	3	3	0	0	0	0	2	0	1	2	4	0	0	1	6	4	3	0	0	1	1

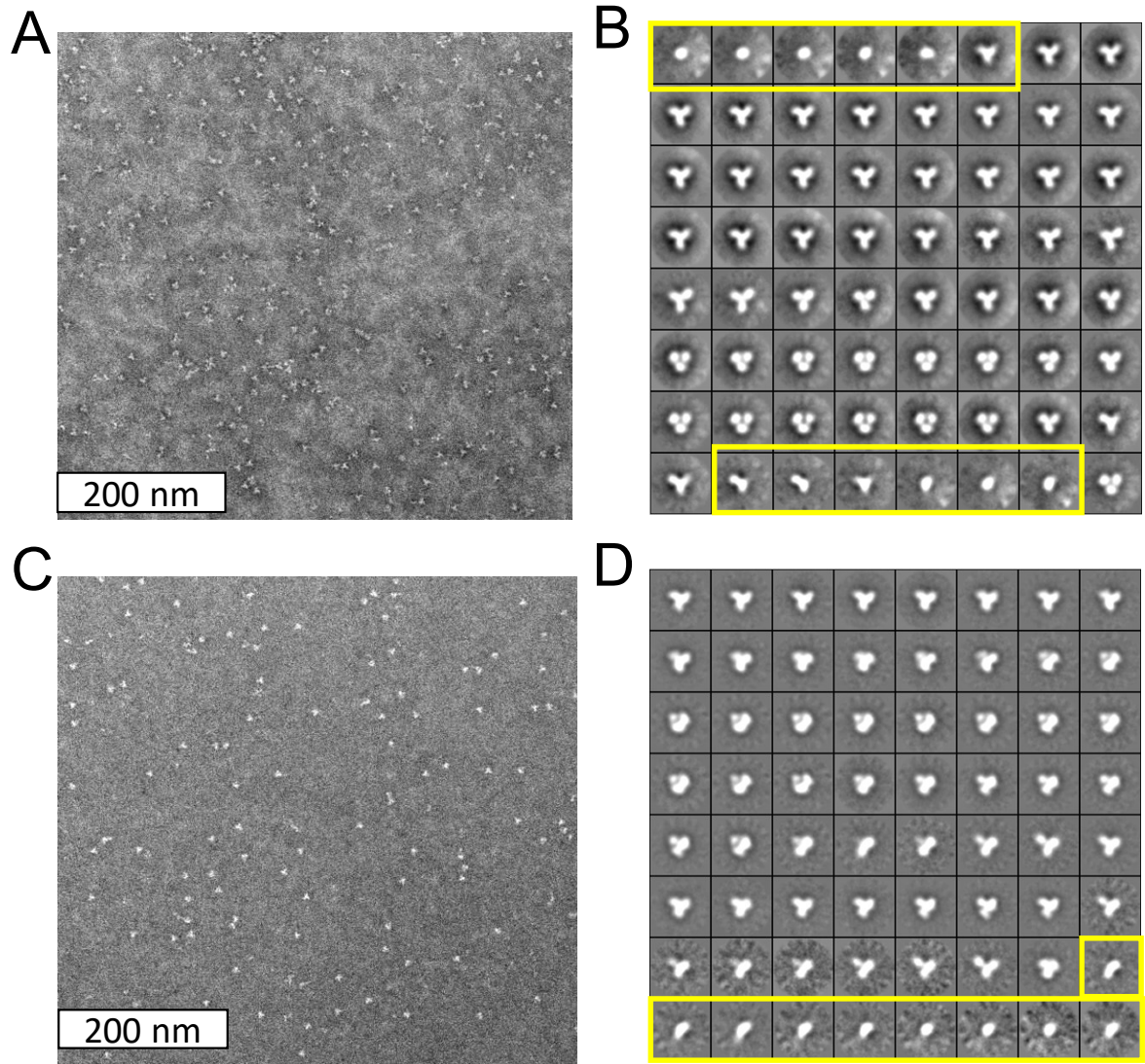
[illegible]

	N88	N133	N142	N156	N160	N185a	N185h	N197	N234	N262	N276	N295	N301	N332	N339	N355	N363	N366	N392	N398	N406	N411	N448	N462	N611	N618	N625	N637	Total	
M9Glc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M9Glc	0
M9	0	0	0	0	61	0	0	2	47	15	0	26	0	69	41	0	64	92	51	0	0	0	38	2	0	0	0	0	M9	19
M8	0	0	0	0	0	0	0	4	20	57	59	7	0	10	18	3	16	5	44	0	0	7	26	0	0	0	0	2	M8	10
M7	1	0	0	0	0	1	0	4	2	1	9	0	0	2	3	4	2	1	0	0	0	10	3	5	0	0	0	6	M7	2
M6	4	13	13	39	0	0	0	2	1	0	11	22	0	0	1	34	0	1	0	0	0	11	1	0	0	0	0	5	M6	6
M5	15	0	87	0	1	0	4	1	20	0	35	0	16	1	14	2	1	1	0	100	53	10	14	2	0	0	4	M5	14	
M4	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	M4	0	
M3	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M3	0	
FM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	FM	0	
Hybrid	32	0	0	0	0	0	1	0	1	0	1	0	0	0	8	0	0	0	4	0	2	0	0	0	0	0	16	Hybrid	2	
Fhybrid	0	N.D.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Fhybrid	0	
HexNAc(3)(x)	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HexNAc(3)(x)	0	
HexNAc(3)(F)(x)	0	0	0	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	12	HexNAc(3)(F)(x)	1		
HexNAc(4)(x)	23	0	0	0	0	0	0	2	0	2	0	0	67	0	7	1	0	0	0	0	0	0	0	4	0	0	HexNAc(4)(x)	9		
HexNAc(4)(F)(x)	5	0	0	0	0	0	2	26	0	20	0	0	0	0	15	0	0	0	99	0	0	62	0	21	0	1	HexNAc(4)(F)(x)	4		
HexNAc(5)(x)	0	50	0	0	0	0	1	0	0	0	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HexNAc(5)(x)	3		
HexNAc(5)(F)(x)	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	14	0	25	0	18	HexNAc(5)(F)(x)	2			
HexNAc(6+)(x)	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HexNAc(6+)(x)	0		
HexNAc(6+)(F)(x)	0	34	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	75	0	4	HexNAc(6+)(F)(x)	8			
UNOCC	11	0	0	0	94	0	80	0	7	0	0	0	0	34	5	15	0	0	0	0	14									

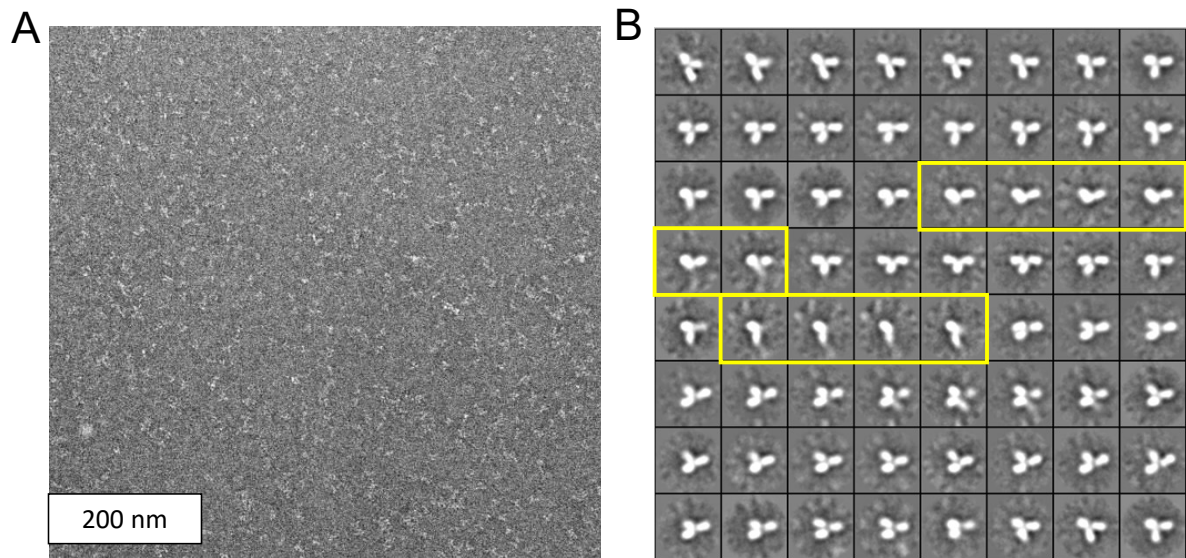
Supplementary Table 4: Glycan composition analysis of transiently expressed Env via plasmid DNA in HEK 293F cells

	N88	N133	N142	N156	N160	N185e	N185h	N197	N234	N262	N276	N295	N301	N332	N339	N355	N363	N386	N392	N398	N406	N411	N448	N462	N611	N618	N625	N637	Total		
M9Glc	0	0			0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	M9Glc	0	
M9	0	0			30	0	0	16	45	36	0	41		71	42	0	54	47	25	0	0	0	27	0	0	0	0	0	M9	17	
M8	0	0			17	0	0	11	25	32	16	17		19	27	0	11	19	43	0	0	4	25	0	0	0	0	6	M8	11	
M7	0	0			7	0	0	5	13	7	15	10		5	10	1	9	9	9	0	0	10	10	0	0	0	0	19	M7	6	
M6	1	0			3	0	0	5	5	9	8	23		2	5	1	6	8	8	0	0	13	5	0	0	0	0	18	M6	5	
M5	17	0			30	0	0	16	5	5	25	9		2	6	24	12	13	12	0	100	57	16	1	6	0	0	25	M5	15	
M4	0	0			2	0	0	0	0	1	9	0		0	2	1	1	1	1	0	0	1	3	0	0	0	0	2	M4	1	
M3	0	0			0	0	0	0	0	0	1	0		0	0	0	0	0	0	0	0	1	0	0	0	0	0	M3	0		
FM	0	0			0	0	0	0	0	0	0	0		0	0	1	0	0	0	0	0	0	0	0	0	0	0	FM	0		
Hybrid	1	0			1	0	0	0	0	0	14	0		0	0	7	2	1	0	0	0	8	3	0	0	0	5	11	Hybrid	2	
Fhybrid	0	0			0	0	0	0	0	0	0	0		0	0	10	1	0	0	1	0	1	1	0	0	0	0	2	Fhybrid	1	
HexNAc(3)(x)	1	0			0	0	0	0	0	0	1	0		0	0	1	0	0	0	0	0	2	0	0	0	0	1	0	HexNAc(3)(x)	0	
HexNAc(3)(F)(x)	1	37			0	1	0	3	0	0	0	0		0	0	14	2	0	0	0	6	0	1	1	1	0	0	1	HexNAc(3)(F)(x)	3	
HexNAc(4)(x)	11	0			0	0	100	0	0	0	2	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HexNAc(4)(x)	5	
HexNAc(4)(F)(x)	15	63			3	3	0	10	2	6	5	0		0	1	20	1	0	0	28	0	1	6	45	4	6	0	7	HexNAc(4)(F)(x)	9	
HexNAc(5)(x)	24	0			0	0	0	0	0	0	0	1	0		0	0	0	0	0	0	0	0	0	0	0	0	1	0	HexNAc(5)(x)	1	
HexNAc(5)(F)(x)	20	0			0	5	0	3	0	3	1	0		0	0	16	1	0	0	37	0	0	2	42	33	2	0	2	HexNAc(5)(F)(x)	7	
HexNAc(6+)(x)	1	0			0	0	0	0	0	0	0	0		0	0	0	0	0	0	29	0	0	0	0	0	0	0	0	HexNAc(6+)(x)	1	
HexNAc(6+)(F)(x)	1	0			0	1	0	0	0	0	2	0		0	0	2	0	0	0	0	0	0	9	31	0	0	2	HexNAc(6+)(F)(x)	2		
UNOCC	3	0			5	88	0	30	2	0	0	0		0	5	0	0	0	0	0	0	0	0	0	24	92	92	4	UNOCC	14	
core	6	0			1	0	0	1	1	1	1	0		0	0	2	0	0	0	0	0	0	1	0	2	1	0	0	1	core	1

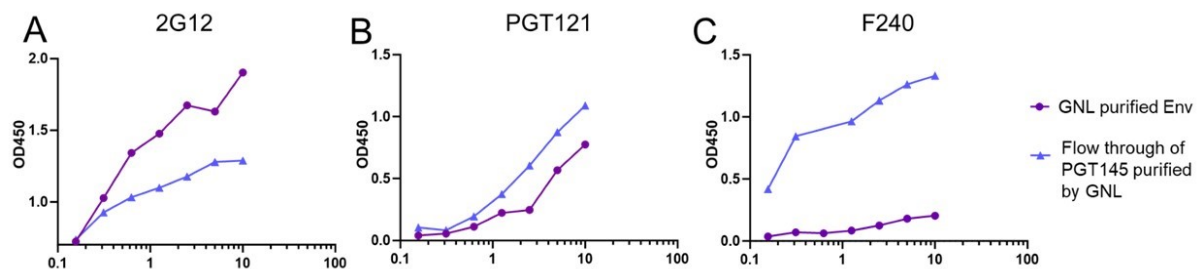
	N88	N133	N142	N156	N160	N185e	N185h	N197	N234	N262	N276	N295	N301	N332	N339	N355	N363	N386	N392	N398	N406	N411	N448	N462	N611	N618	N625	N637	Total	
Mannose	18	0			90	1	0	53	94	89	74	100		99	93	27	93	98	99	0	100	86	87	1	6	0	0	70	Mannose	49
Hybrid	1	0			1	0	0	0	0	0	14	0		0	0	18	2	1	0	1	0	9	4	0	0	0	5	13	Hybrid	3
Complex	72	100			3	10	100	17	3	10	11	0		1	1	54	4	1	0	99	0	4	9	97	69	8	3	13	Complex	25
Unoccupied	3	0			5	88	0	30	2	0	0	0		0	5	0	0	0	0	0	0	0	0	0	24	92	4	Unoccupied	12	
Fucosylation	36	100			3	10	0	17	3	10	8	0		1	1	63	4	1	0	71	0	3	10	97	68	8	1	14	Fucosylation	19
Sialylation	26	25			2	5	0	1	1	5	12	0		0	0	40	0	0	0	56	0	5	4	57	53	0	4	6	Sialylation	11
core	6	0			1	0	0	1	1	1	1	0		0	0	2	0	0	0	0	0	1	0	2	1	0	0	1	core	1



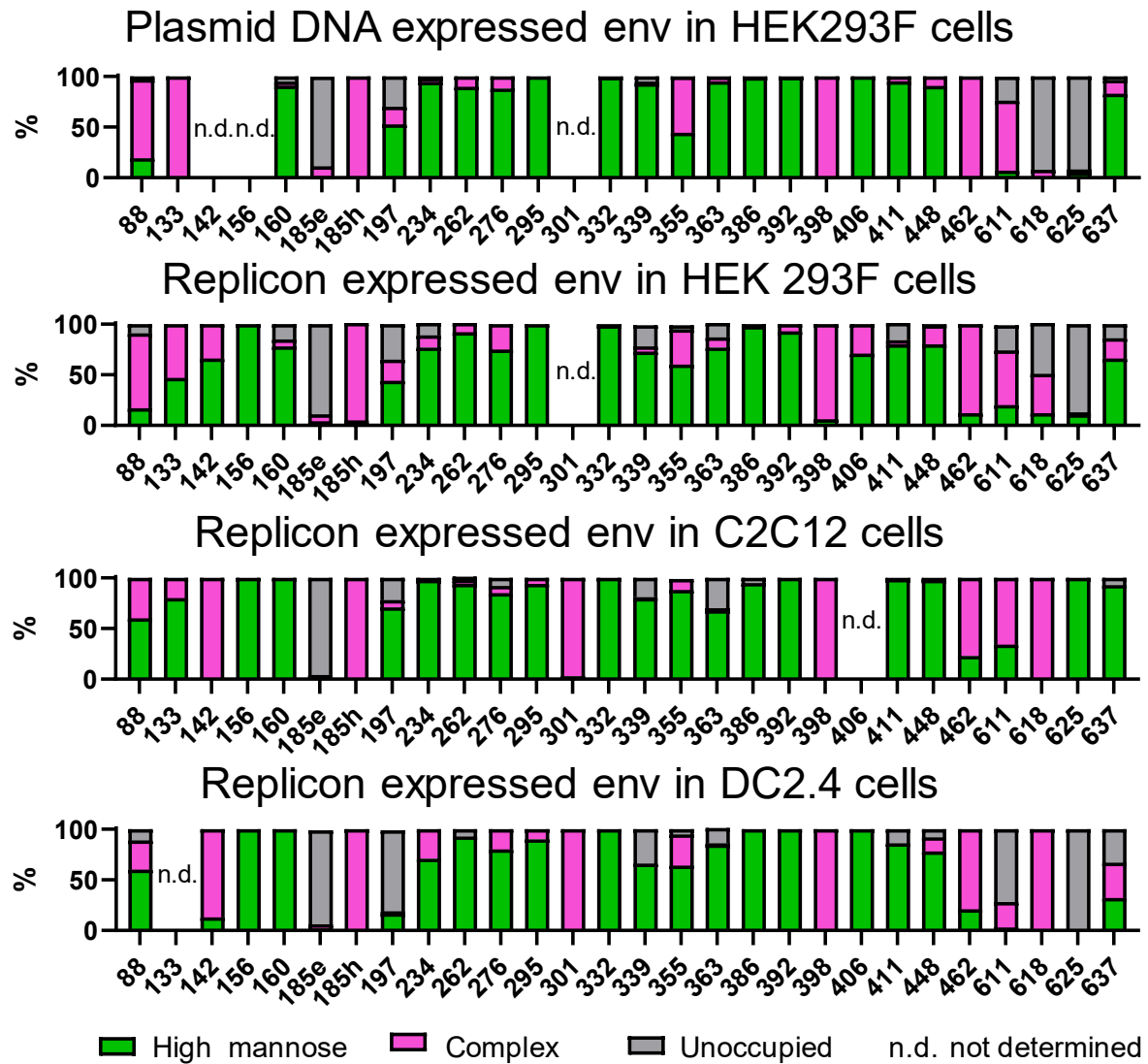
Supplementary Figure 1: Representative of NS-EM image of BG505.664 Env particles secreted by HEK293F cells transfected by plasmid DNA. A) NS-EM image of Env purified by GNL beads. B) 2D class averages of the particles shown in A. Amongst 8071 particles imaged, 20% of these particles fall with in class averages corresponding to non-native conformations shown in yellow. C) NS-EM image of Env purified by GNL followed by size-exclusion chromatography. D) 2D class averages of the particles shown in C. Amongst 6035 particles imaged, 14% of these particles fall with in the class averages corresponding to non-native conformations shown in yellow.



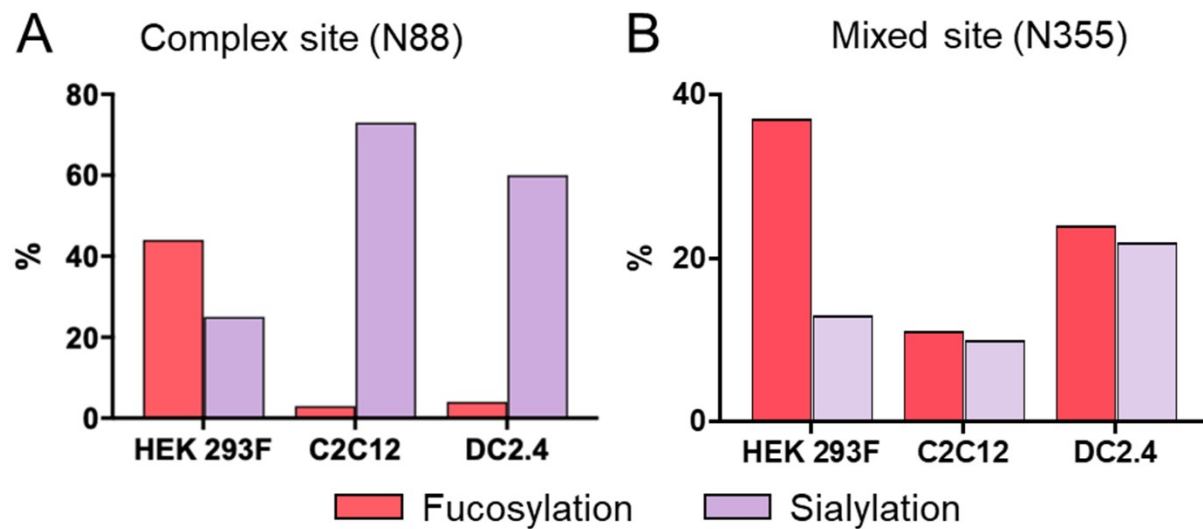
Supplementary Figure 2: Representative NS-EM image of BG505 NFL.664 Env particles secreted by C2C12 cells transfected by replicon RNA. A) NS-EM images of Env purified by VRC01 (CD4 binding site antibody). B) 2D class averages of the particles. Amongst 10679 particles imaged, 17% of these particles fall within the class averages corresponding to the non-native conformation of Env (highlighted, yellow).



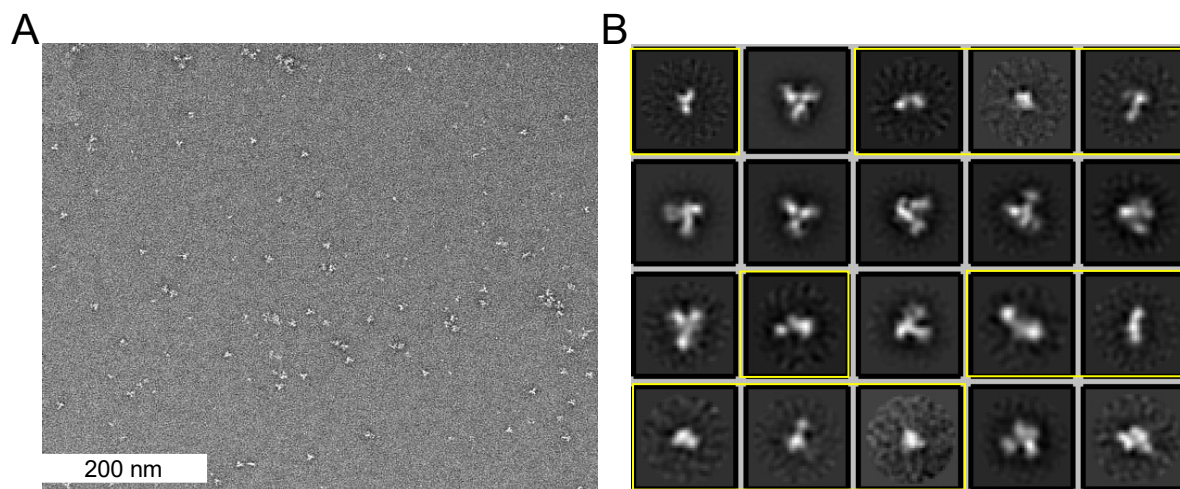
Supplementary Figure 3: Binding analysis of replicon expressed Env affinity-purified using GNL and PGT145. The binding analysis in purple represents the Env purified using GNL beads. The binding analysis in blue represents the non-trimeric conformation of Env which was eluted during PGT145 purification, subsequently purified using GNL. The ELISA was performed with the glycan binding bnAb (A) 2G12, V3 glycan dependent bnAb (B) PGT121, and non-nAb (C) F240. The assays were conducted in duplicate, with each sample being tested twice in independent wells to confirm consistency of results.



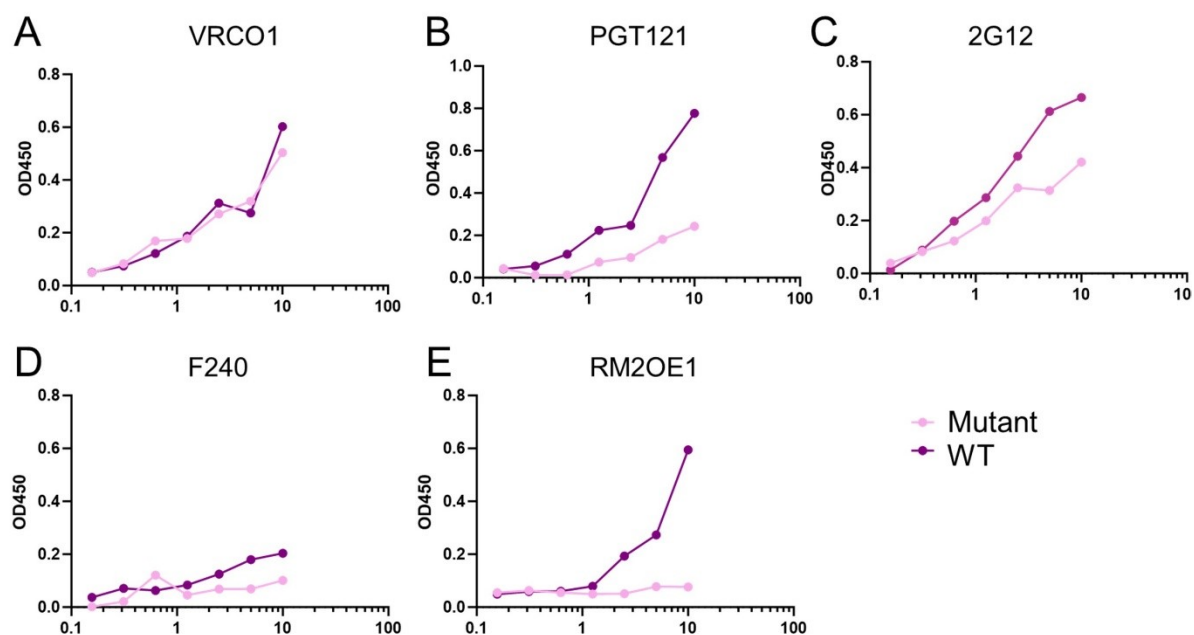
Supplementary Figure 4: Site-specific glycan composition of env via different production systems. The bar graphs represent the glycan composition observed at each N-glycan site with the average of three or more biological replications produced in different production systems. The glycan compositions are represented in three categories, underprocessed glycan structures which includes both oligomannose-type and hybrid-type glycans, complex-type which includes highly processed glycan compositions and core-truncated structures, and unoccupied which represents N-glycan site with no glycan on it. Sites annotated with n.d. represent sites where data was insufficient, and the glycan compositions were consequently not determined.



Supplementary Figure 5: Complex-type glycan abundances in replicon expression of env observed across different cell lines. The levels of fucosylation and sialylation observed on env expressed in HEK 293F, C2C12 and DC2.4 cells of the two N-linked glycan sites A) Complex site, N88 and B) Mixed site, N355, are displayed. The bar graphs represent the percentage of fucosylation and sialylation represented in pink and purple, respectively.



Supplementary Figure 6: A) Representative of NS-EM image of BG505 NFL.664 mutant Env particles secreted by HEK 293F cells transfected with replicon RNA. **B)** 2D class averages of the particles. Amongst all of the particles imaged, 55% fall within class averages corresponding to non-native conformations shown in yellow.



Supplementary Figure 7: Binding analysis of replicon expressed WT and Mutant BG505 NFL.664 with HIV-1 bnAbs and non-nAbs. The bnAbs shown in this figure are VRCO1 (A), PGT121 (B), and 2G12 (C). The non-nAbs shown here are F240 (D) and RM20E1 (E). The assay was performed twice to confirm consistency of the results.