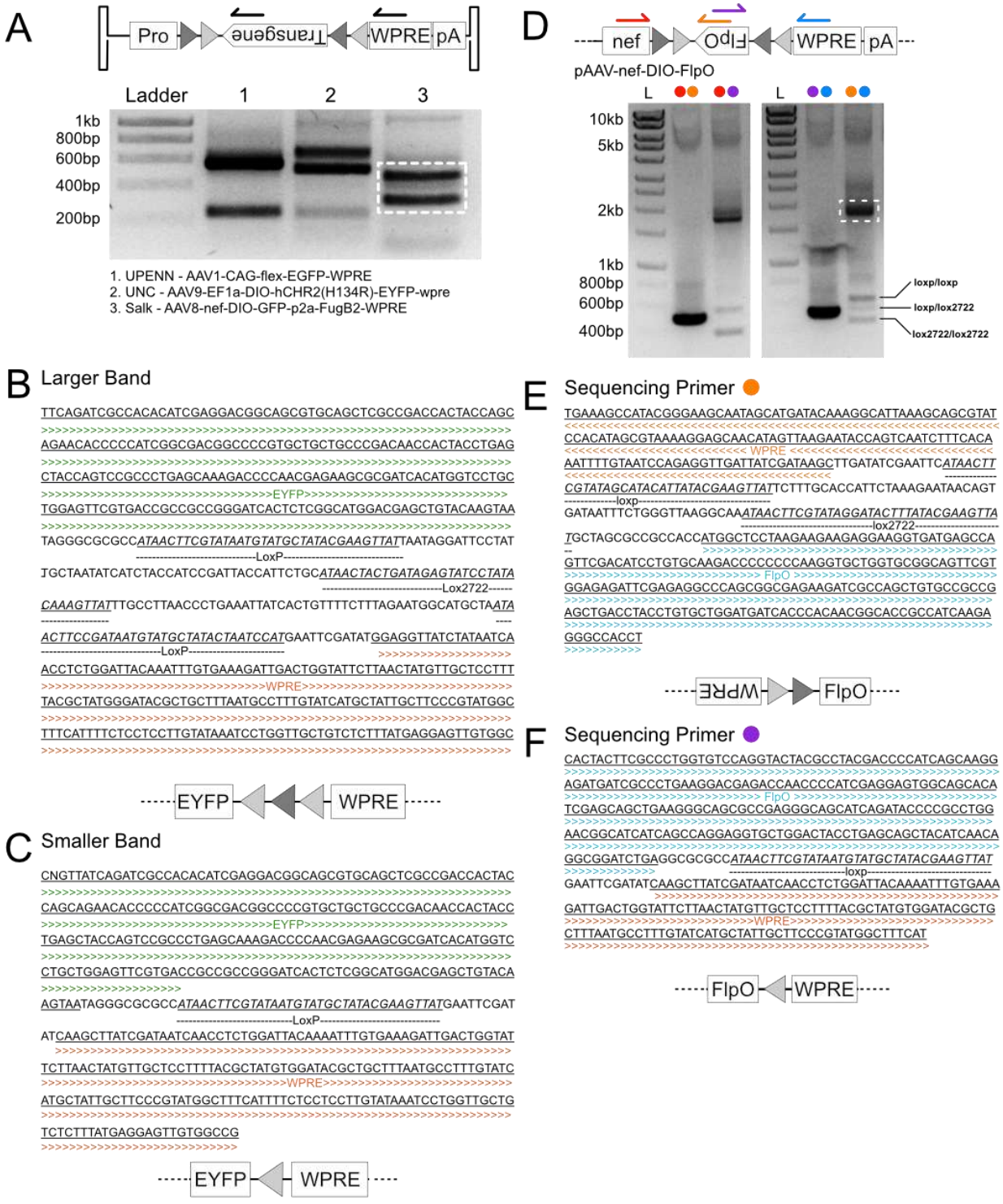
Supplementary Figures



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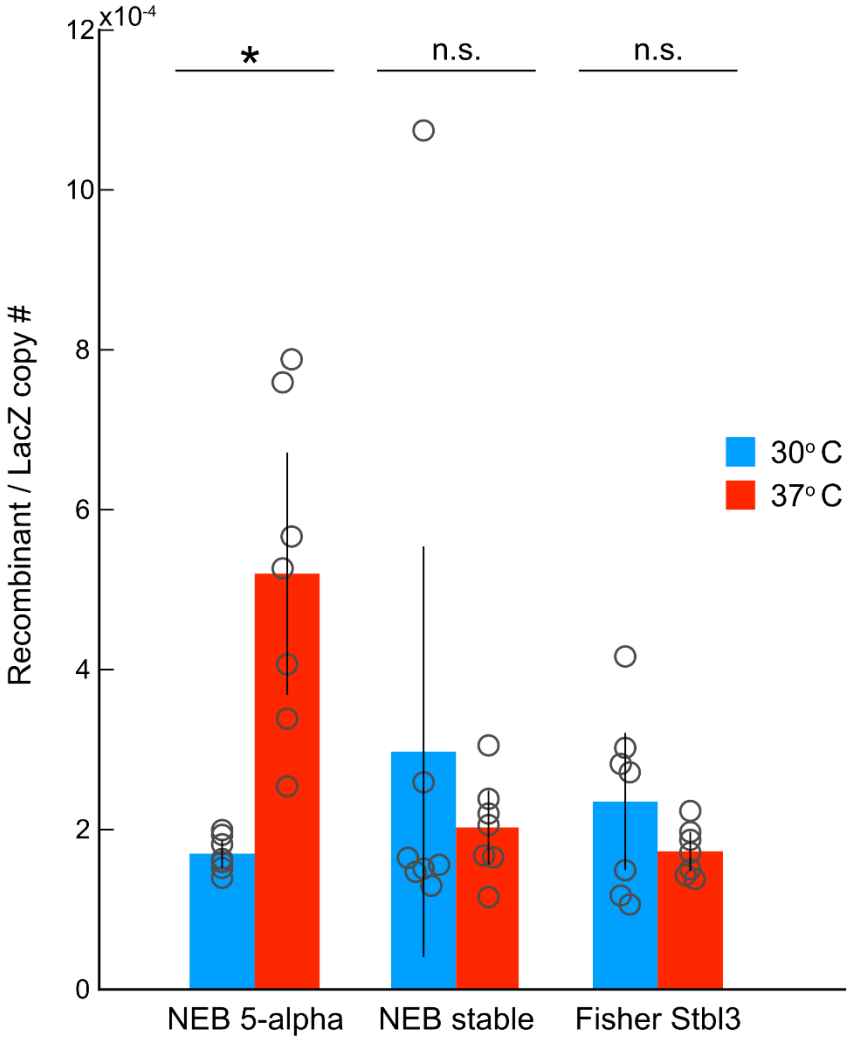
Supplementary Figure 1: PCR products from in-sense primers across recombinase sites reveal reverted transgenes in AAV genomes and plasmids

(A) PCR experiments using four sets of primers with either sense or antisense orientation in reference to the expected design (top) of the template pAAV-nef-DIO-FlpO plasmid. Antisense primers (lanes 2 and 5) produce a band at the expected size. In-sense primer pairs (lanes 3 and 6) produce doublet or triplet bands less 1kb. Cre-recognition site pairs labelling each band (bottom right) indicate the lox sites across which recombination occurred to produce the sequence found for each of these bands. The sequences in (B) and (C) are from the band found in the dotted box in (A) using the flp-specific primers shown. (D) PCR of FLEX and DIO AAV genomes using in-sense primers across recombinant sites creates short band doublets. General recombinase-dependent DIO or FLEX AAV design (top) with in-sense PCR primers (black). Gel of PCR products using the AAVs listed with sources (below). Dotted box in lane 2 denotes products used for sequencing. Sequencing of (E) larger and (F) smaller of the two bands show the transgene in-sense with the WPRE element and separated by three or one recombinase sites (triangles).

21

22

23


x10-4
12
10
8
6
4
2
0
*
n.s.
n.s.
Recombinant / LacZ copy #
30° C
37° C
NEB 5-alpha
NEB stable
Fisher Stbl3

Supplementary Figure 2: Effects of incubation temperature on spontaneous recombination rates across *loxp* sites in competent cell lines used for AAV plasmid production.

Recombination rates in minipreps of pUC19-*loxp*-inv_LacZ plasmids grown at 30°C for 21hrs or at 37°C for 16hrs as measured by qPCR. N=7 per condition. P values for to be 0.00068 for NEB 5-alpha, 0.4875 for NEB stable, and 0.1911 for Fisher Stbl3 using a two-tailed independent t-test.

A

Cre

	Position 1	Position 2
loxp	ATAACTTCGTATA GCATACAT TATACGAAGTTAT	ATAACTTCGTATA GCATACAT TATACGAAGTTAT
lox2722	ATAACTTCGTATA GGATACTT TATACGAAGTTAT	ATAACTTCGTATA GGATACTT TATACGAAGTTAT
loxn	ATAACTTCGTATA GTATACCT TATACGAAGTTAT	ATAACTTCGTATA GTATACCT TATACGAAGTTAT
loxp$^{66/71}$	ATAACTTCGTATA GCATACAT TATACGAACGGTA	TACCGTTCGTATA GCATACAT TATACGAAGTTAT
loxp$^{75/76}$	ATAACTTCGTATA GCATACAT TATACGCCCGGTA	TACCGGGCGTATA GCATACAT TATACGAAGTTAT
loxp$^{43/44}$	ATAACTTCGTATA GCATACAT TATAGGTACCGAG	AATGCATGCTATA GCATACAT TATACGAAGTTAT
loxp/2722	ATAACTTCGTATA GCATACAT TATACGAAGTTAT	ATAACTTCGTATA GGATACTT TATACGAAGTTAT

Flp

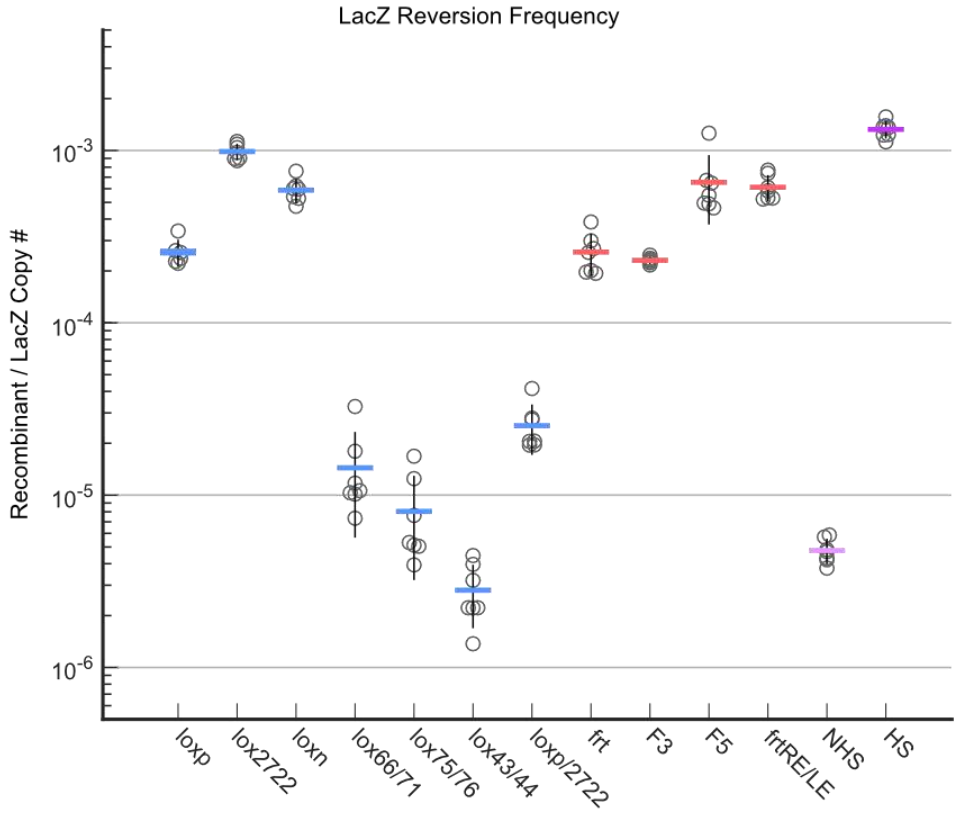
	Position 1	Position 2
frt	GAAGTTCCTATTC TCTAGAAA GTATAGGAACTTC	GAAGTTCCTATTC TCTAGAAA GTATAGGAACTTC
F3	GAAGTTCCTATTC TTCAAATA GTATAGGAACTTC	GAAGTTCCTATTC TTCAAATA GTATAGGAACTTC
F5	GAAGTTCCTATTC TTCAAAAG GTATAGGAACTTC	GAAGTTCCTATTC TTCAAAAG GTATAGGAACTTC
frtRE/LE	GAAGTTCATATTC TCTAGAAA GTATAGGAACTC	GAAGTTCCTATTC TCTAGAAA GTATATGAACTTC

Controls

	Position 1	Position 2
homologous shuffle (HS)	AAATTCGATGAATACATTATGAGCTTACTCAATT	AAATTCGATGAATACATTATGAGCTTACTCAATT
non-homologous shuffle (NHS)	ATATCTAATAGAGCGTAATAGTAGTACATTTTAT	ACCTTAGCAGAAATTCATATTATTAAGTCTAGTC

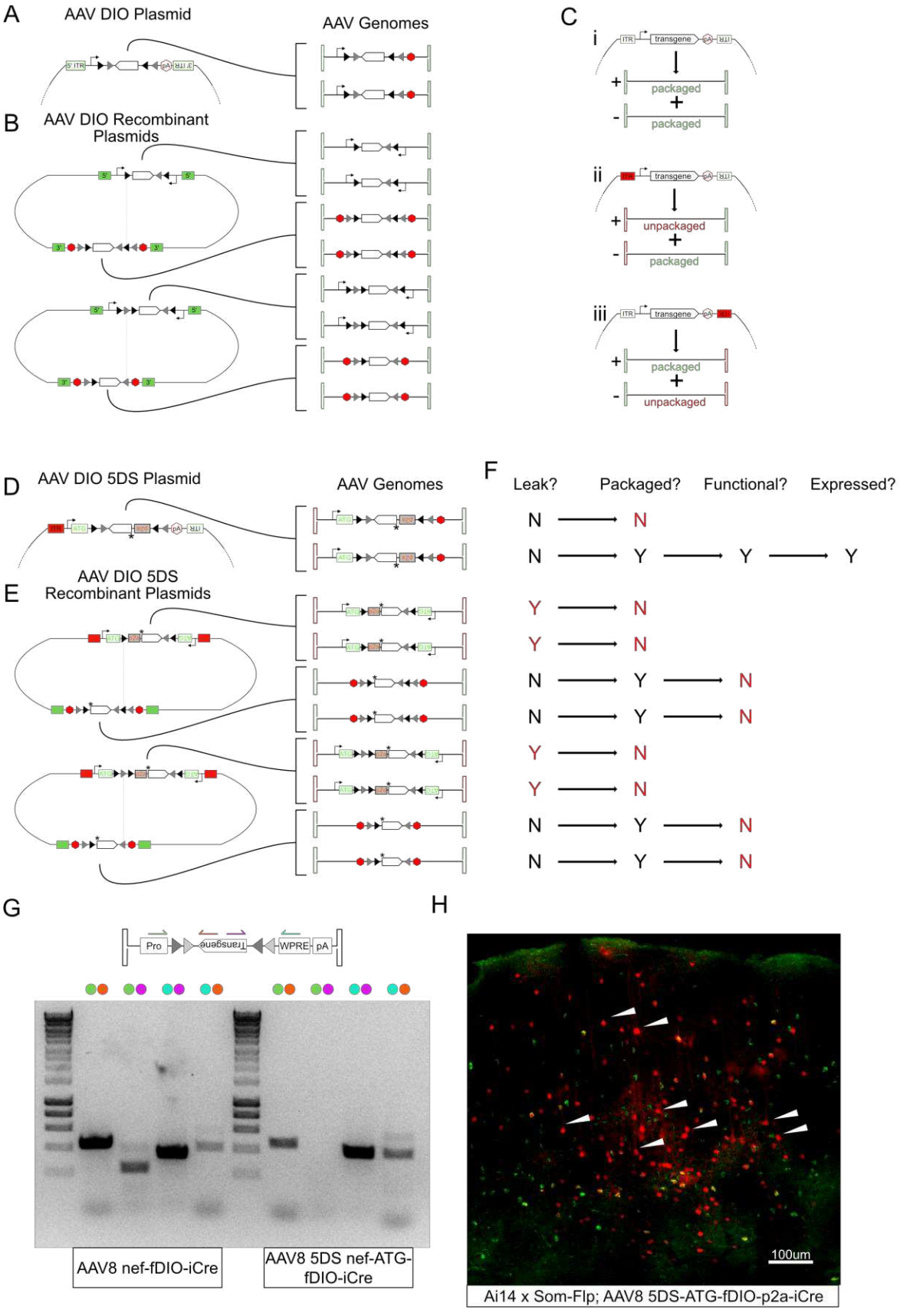
Within-pairs sequence differences

B



Supplementary Figure 3: All tested recombinase recognition site pairs and associated recombination rates as measured by qPCR

(A) Table of recombination recognition site pairs used to bookend inverted LacZ sequences in pUC19 experiments. Red text signifies within-pair sequence differences. (B) Accumulated LacZ recombination frequencies of all tested recombination recognition site pairs. Horizontal lines signify mean value across minipreps (circles), and vertical lines are standard deviation.


A
AAV DIO Plasmid
AAV Genomes
B
AAV DIO Recombinant Plasmids
C
i
transgene
packaged
packaged
ii
unpackaged
packaged
iii
packaged
unpackaged
D
AAV DIO 5DS Plasmid
AAV Genomes
E
AAV DIO 5DS Recombinant Plasmids
F
Leak?
Packaged?
Functional?
Expressed?
N N
N Y Y Y
Y N
Y N
N Y N
N Y N
Y N
Y N
N Y N
N Y N
G
Pro
Transgene
WPRE
pA
AAV8 nef-fDIO-iCre
AAV8 5DS nef-ATG-fDIO-iCre
H
100um
Ai14 x Som-Flp; AAV8 5DS-ATG-fDIO-p2a-iCre

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Supplementary Figure 4: Disruption of 5' ITR D-sequence packaging signal blocks AAV packaging of genomes containing reverted transgenes but not leak expression

(A) Typical recombinase dependent AAV plasmid design and AAV genome. (B) Expected first-order recombinant plasmids and resultant AAV genomes resulting from recombination events across like recombinase-recognition sites. (C) Packaging profile of ssDNA AAV genomes from plasmids containing unmodified ITR D-sequences (i), 5' modified ITR (ii, in red), and 3' modified ITR (iii, in red). (D) Plasmid design and resulting AAV genomes from a recombinase-dependent AAV design with both a disrupted ORF and 5' ITR modified D-sequence, in red. (E) Expected first-order recombinant plasmids and resultant AAV genomes from the plasmid in (D). (F) Flowchart of each AAV genomes' propensity to leak, be packaged, and functionally express the transgene in the presence of recombinase. (G) PCR of AAV genomes using primers targeting all possible transgene orientations following recombination. Templates, listed at bottom, are AAV8 nef-fDIO-iCre and the ITR modified AAV8 5DS-nef-ATG-fDIO-iCre. (H) Example image of an AAV8-5DS-ATG-fDIO-p2a-iCRE injection in primary visual cortex of an Ai14 x SomFlp mouse. White arrows indicate off target non-somatostatin cre expression. Anti-somatostatin staining in green, tdTomato in red.