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Supplemental Information

Efficient Designer Nuclease-Based Homologous Recombination Enables Direct PCR Screening for Footprintless Targeted Human Pluripotent Stem Cells

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Supplemental Figures

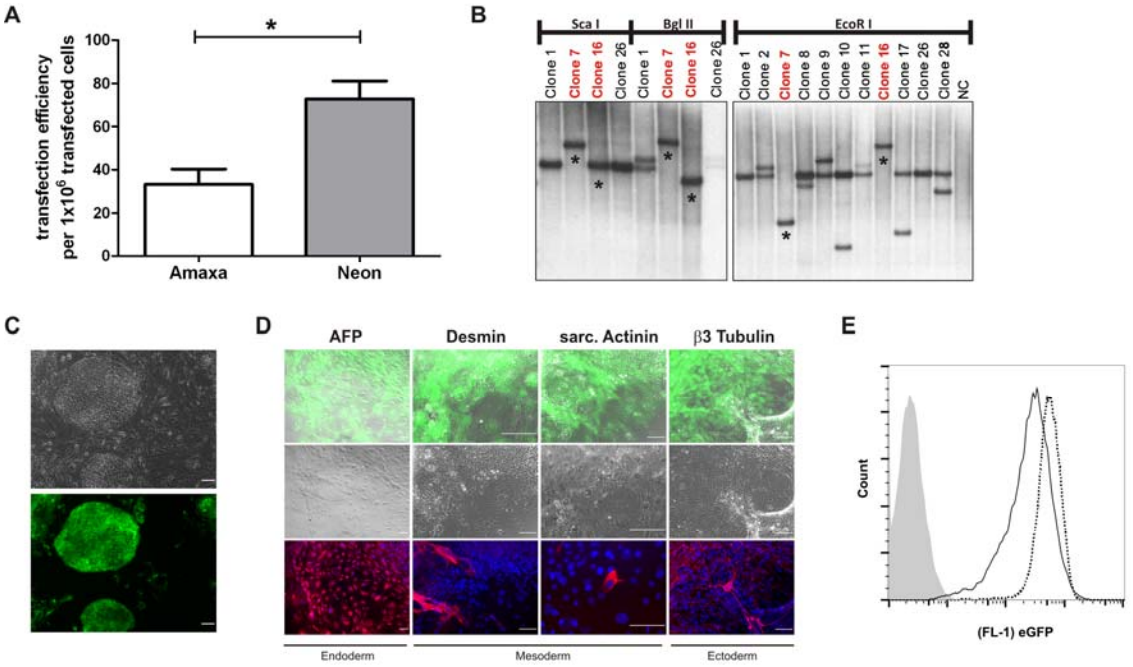

Figure S1. Optimization of transfection and characterization of hCBiPS2eGFP reporter cell clones, related to Figure 1. (A) Comparison of transfection efficiencies of hCBiPS2 and hES3 cells using our optimied protocol for the Neon® transfection system versus the optimized protocol of the Amaxa™ Nucleofection system recommended for hPSCs (mean ± SEM of 4 independent experiments). **(B)** Southern Blot analysis revealed 2 clones (depicted in red) with single eGFP integration. **(C)** Microscopy images of the transgenic $eGFP^{pos}$ iPSC clone hCBiPS2eGFPC7 on feeder cells. Scale bars represent 100 µm. **(D)** Immunostaining of clone hCBiPS2eGFPC7 cell derivatives on day 15 of differentiation showed expression of endodermal (AFP), mesodermal (sarc. alpha-Actinin, Desmin) and ectodermal (ß3-Tubulin) marker proteins (red). Nuclei are stained with DAPI (blue). Upper pictures show eGFP transgene expression. Scale bars represent 100 µm. **(E)** Flow cytometric analysis of derivatives of clone hCBiPS2eGFPC7 on day 28 of differentiation (continuous line) revealed consistent eGFP expression compared to undifferentiated cells (dotted line).

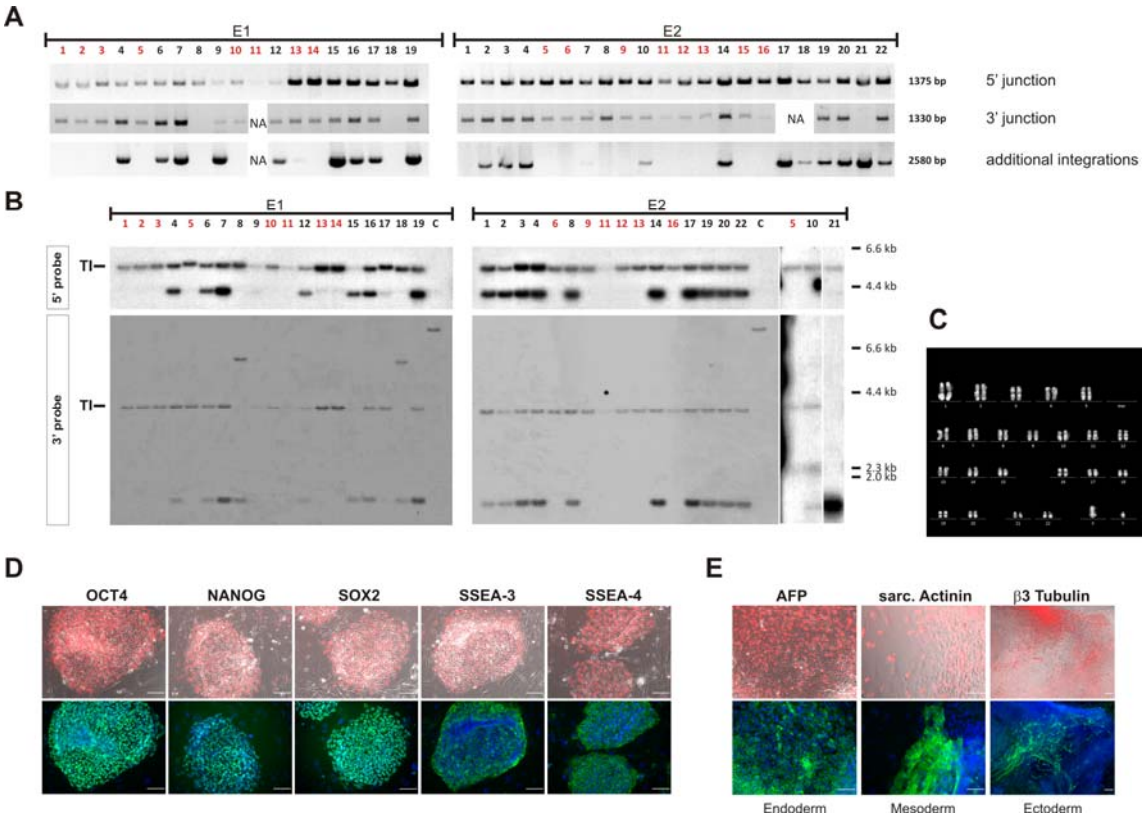

Figure S2. Genomic analysis of eGFP^{neg}RedStar^{pos} human iPS cell clones after eGFP-ZFN targeting and representative phenotypic characterization of one targeted iPSC clone, related to Figure 2. The analyses of two targeting experiments (E1, E2) applying different molecular ratios of ZFNs:Donor (1:1 or 1:9) are shown. **(A)** PCR analysis for 5' (upper row, primers p3 & p4) and 3' (middle row, primers p5 & p6) junctions as expected for correct targeted integration of the donor cassette into the eGFP locus. Bands in the lower row show the presence of additional integrations, as determined using primers p7 and p8 that bind to the donor plasmid backbone outside the homologous arms. NA, not assessed. See also figure 2A. **(B)** Southern blot analysis of hiPSeGFPC7 cells targeted with eGFP-ZFN and the 2A-RedStar donor plasmid. Genomic DNA was digested with BsmI and hybridized with the internal 5' probe (on top) and internal 3' probe (below). Correctly targeted clones without additional integrations are indicated in red. The 5' probe detects a 5.2 kb targeted fragment and a 4.1 kb fragment for random donor integration. The 3' probe detects a 3.5 kb targeted fragment and a 1.4 kb fragment for random donor integration. See also figure 2A. TI, targeted integration. C, control (hCBiPS2eGFPC7). **(C)** Clone E1_RSiPSC7 exhibits a normal karyotype (46,XY) after ZFN-mediated HR. **(D)** Assessment of pluripotency. Phase contrast images with overlay of nuclear RedStar fluorescence (upper row) and immunostaining for pluripotency markers (lower row, green) of undifferentiated E1_RSiPSC7 (passage 13 after HR and cloning). Nuclei are stained with DAPI (blue). Scale bars represent 100 µm. **(E)** Assessment of differentiation potential. Immunocytological detection of endodermal (AFP), mesodermal (sarc.alpha-Actinin) and ectodermal (ß3-Tubulin) marker proteins (green) in differentiated iPSC derivatives of clone E1_RSiPSC7 indicates the maintenance of pluripotency. Phase contrast images with overlay of nuclear RedStar fluorescence (upper row). Nuclei are stained with DAPI (blue). Scale bars represent 100 µm.

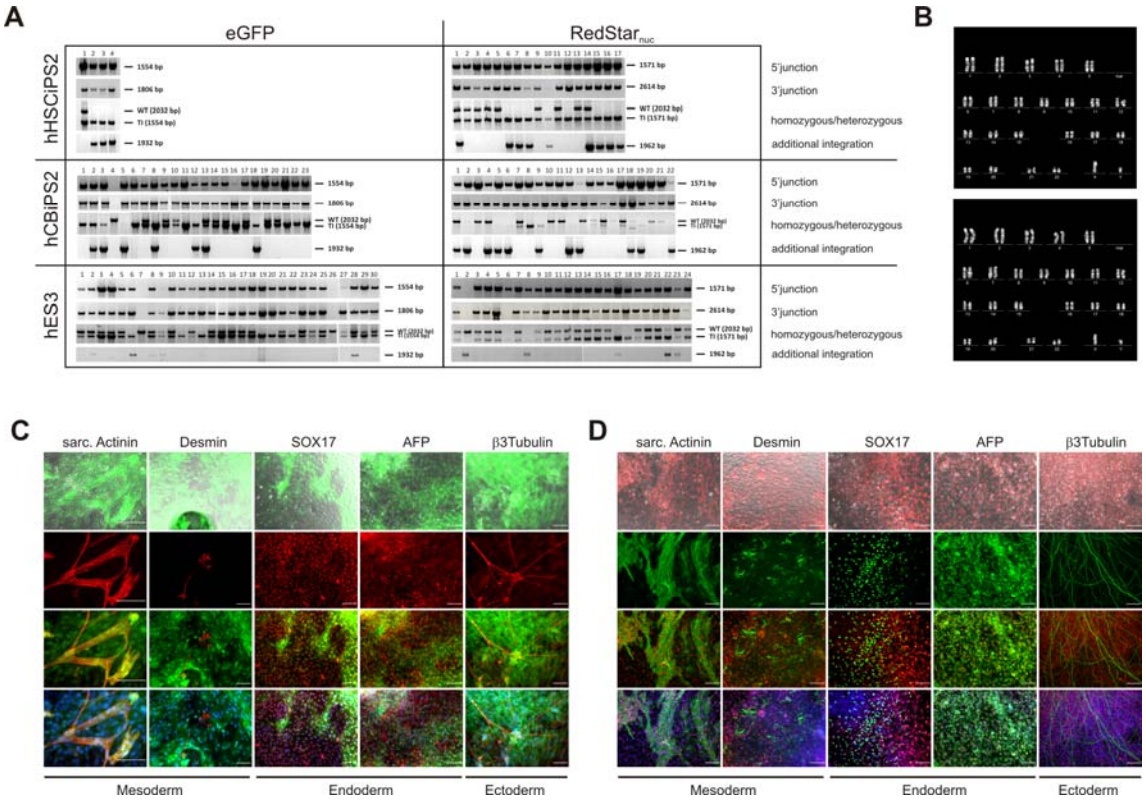

Figure S3. Genotyping and phenotypic characterization of AAVS1 targeted PSC clones, related to Figure 3. (A) PCR analysis of AAVS1-targeted CAG-eGFP or CAG-RedStar transgenic hiPSC and hESC clones for the 5' (primers p9 & p10) and 3' (primers p5/p1 & p12) junctions generated by targeted integration, for the determination of homozygous (primers p9 & p10 & p12, one band) versus heterozygous (primers p9 & p10 & p12, two bands of different size) transgene integration, and for the detection of additional donor integrations (primers p10 & p13). See the scheme in figure 3A and supplemental table S1 for details. **(B)** Karyotype. Human iPSC clones hCBiPS2_AAVS1eGFPC18 (upper picture) and hCBiPS2_AAVS1RedStarC8 (lower picture) exhibit a normal karyotype (46,XY). **(C)** Immunostaining of clone hCBiPS2_AAVS1eGFPC18 cell derivatives on day 24 of differentiation showed expression of mesodermal (sarc. alpha-Actinin, Desmin), endodermal (SOX17, AFP) and ectodermal (β3-Tubulin) marker proteins (red). Upper pictures show eGFP transgene expression. Nuclei are stained with DAPI (blue). Scale bars represent 100 µm. **(D)** Immunostaining of clone hCBiPS2_AAVS1RedStarC8 cell derivatives on day 24 of differentiation showed expression of mesodermal (sarc. alpha-Actinin, Desmin), endodermal (SOX17, AFP) and ectodermal (β3-Tubulin) marker proteins (green). The upper pictures show RedStar transgene expression. Nuclei are stained with DAPI (blue). The scale bars represent 100 µm.

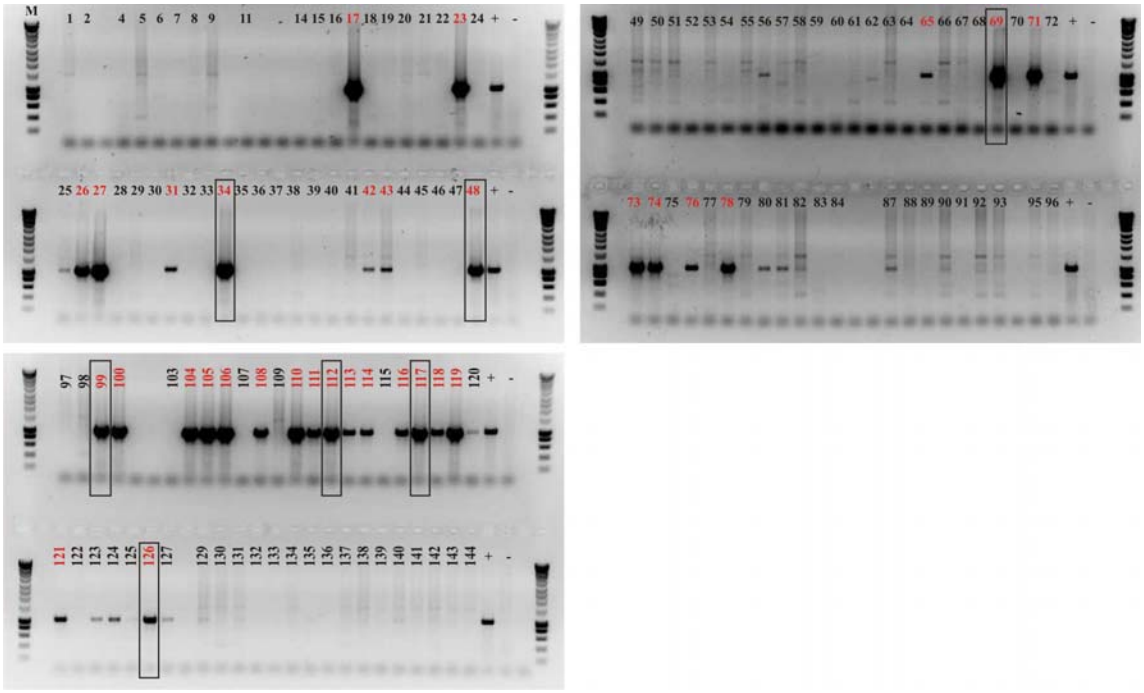

Figure S4. PCR screening for ssODN donor integration in 144 iPSC clones, related to Figure 4. HCBiPS2 cells were transfected with AAVS1 specific TALENs and the ssODN donor for incorporation of a HindIII site into the AAVS1 locus. On day 3 after transfection, 10 cells per well were seeded into 96 well plates. On average, one to five cells survived per well and PCR screening for targeted integration showed positive insertion of the ssODN in 32 out of 480 analysed pools (data not shown). Limiting dilution from nine positive pools resulted in 144 single cell clones. PCR analysis for the targeted integration of the ssODN revealed 33 positive clones (marked in red) from which we randomly chose 7 clones (framed) for further evaluation (see Fig. 4). M, DNA marker (Eurogentec).

Table S1. Oligonucleotides used for PCR, related to the Experimental Procedures

		Name	Sequence	Specificity	Product Size
eGFP targeting		**p1**	CAA CGT GCT GGT TAT TGT GC	EGFP sequencing	800 bp
		p2	GGC TTC ATG ATG TCC CCA TA		
		p3	GTC CCC TTC TCC CTC TCC AG	5'junction	1375 bp
		p4	CCT GGC AAT TGG ACT TGC TTC		
		p5	CAT CTG ACG GTC CAG TCA TGC	3'junction	1330 bp
		p6	TGT GGA ATT GTG AGC GGA TA		
		p7	GCC TGA ACA CCA TAT CCA TCC	additional integration	2580 bp
		p8	GCA GCT GAG AAT ATT GTA GGA GAT C		
AAVS1 targeting	CAG-eGFP donor	**p9**	CCA GCT CCC ATA GCT CAG TCT G	5'junction	1554 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
		p11	GGG CAC AAG CTG GAG TAC A	3'junction	1806 bp
		p12	GTG AGT TTG CCA AGC AGT CA		
		p9	CCA GCT CCC ATA GCT CAG TCT G	homo- vs. heterozygous	TI_1554 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
		p9	CCA GCT CCC ATA GCT CAG TCT G		WT_2032 bp
		p12	GTG AGT TTG CCA AGC AGT CA		
		p13	ATA ATA CCG CGC CAC ATA GC	additional integration	1932 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
	CAG-RedStar donor	**p9**	CCA GCT CCC ATA GCT CAG TCT G	5'junction	1571 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
		p1	CAA CGT GCT GGT TAT TGT GC	3'junction	2614 bp
		p12	GTG AGT TTG CCA AGC AGT CA		
		p9	CCA GCT CCC ATA GCT CAG TCT G	homo- vs. heterozygous	TI_1571 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
		p9	CCA GCT CCC ATA GCT CAG TCT G		WT_2032 bp
		p12	GTG AGT TTG CCA AGC AGT CA		
		p13	ATA ATA CCG CGC CAC ATA GC	additional integration	1962 bp
		p10	ATG GGG AGA GTG AAG CAG AA		
	ssODN	**p14**	ACA GTG GGG CCC C**aa gct t**G	HindIII detection	1024 bp
		p12	GTG AGT TTG CCA AGC AGT CA		
		p15	CTT GTA GGC CTG CAT CAT CA	PCR product for HindIII digestion	994 bp
		p16	GAA CAC CTA GGA CGC ACC AT		

Table S2. Primary Antibodies used for immunohistology, related to the Experimental Procedures

Name	Class	Species	Clonality	Vendor	Dilition
anti-OCT4	IgG2b	mouse	monoclonal	Santa Cruz Biotechnologie, CA, USA	1:100
anti-NANOG	IgG1	mouse	monoclonal	Abcam, Cambridge, USA	1:500
anti-SSEA3	IgM	mouse	monoclonal	Hybridoma Bank, Iowa City, USA	1:100
anti-SSEA4	IgG3	mouse	monoclonal	Hybridoma Bank, Iowa City, USA	1:70
anti-sarc. alpha Actinin	IgG1	mouse	monoclonal	Sigma, Missouri, USA	1:800
anti-alpha-Fetoprotein	IgG1	mouse	monoclonal	R&D Systems, Minneapolis, USA	1:300
anti-beta3 Tubulin	IgG2a	mouse	monoclonal	Upstate, NY, USA	1:400
anti-Desmin	IgG1	mouse	monoclonal	Progen, Heidelberg, DE	1:20
anti-SOX17	IgG	goat	monoclonal	Millipore, Darmstadt, DE	1:200
anti-TroponinT	IgG1	mouse	monoclonal	Thermo Scientific, St. Leon-Rot, DE	1:100

Supplemental Experimental Procedures

Sequences

AAVS1-specific ZFN sequence (right) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASPAAMAERPFQCRICMRNFSQSSNLARHIRTHT
GEKPFACDICGRKFARTDYLVDHTKIHTGSQKPFQCRICMRNFSYNTHLTRHIRTHTG
EKPFACDICGRKFAQGYNLAGHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIEL
IEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTK
AYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFK
GNYKAQLTRLNHVTNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific ZFN sequence left (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASPAAMAERPFQCRICMRNFSYNWHLQRHIRTH
TGEKPFACDICGRKFARSDHLTTHTKIHTGSQKPFQCRICMRNFSHNYARDCHIRTHT
GEKPFACDICGRKFAQNSTRIGHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIEL
IEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTK
AYSGGYNLPIGQADEMERYVEENQTRNKHANPNEWWKVYPSSVTEFKFLFVSGHFK
GNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific TALEN sequence (right) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASAPRRRAAQPSDASPAAQVDLRTLGYSQQQQE
KIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIAALPEATH
EAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAW
RNALTGAPLNLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASHD
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RLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN
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RNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEEL
LIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific TALEN sequence (left) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASAPRRRAAQPSDASPAAQVDLRTLGYSQQQQE
KIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIAALPEATH
EAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAW
RNALTGAPLNLTPQQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNG
GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNKGGKQALETVQRLLPVLCQAHGL
TPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQ
RLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASH
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SNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASHDGGRPALESIVAQLSRPDPALA
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GYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEEN
QTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVE
ELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

eGFP 2A-RedStar$_{nuc}$ donor DNA sequence

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TTTTTCCTAC AGCTCCTGGG CAACGTGCTG GTTATTGTGC TGTCTCATCA
TTTTGGCAAA GAATTCCTCG AGACCATGGT GAGCAAGGGC GAGGAGCTGT
TCACCGGGGT GGTGCCCATC CTGGTCGAGC TGGACGGCGA CGTAAACGGC
CACAAGTTCA GCGTGTCCGG CGAGGGCGAG GGCGATGCCA CCTACGGCAA
GCTGACCCTG AAGTTCATCT GCACCACCGG CAAGCTGCCC GTGCCCTGGC
CCACCCTCGT GACCACCCTG GCCTACGGCG TGCAGTGCTT CAGCCGCTAC
CCCGACCACA TGAAGCAGCA CGACTTCTTC AAGTCCGCCA TGCCCGAAGG
CTACGTCCAG GAGCGCACCA TCTTCTTCAA GGACGACGGC AACTACAAGA
CCCGCGCCGA GGTGAAGTTC GAGGGCGGCA CCCTGGTGAA CCGCATCGAG
CTGAAGGGCA TCGACTTCAA GGAGGACGGC AACATCCTGG GGCACAAGCT
GGAGTACAAC TACAACAGCC ACAACGTCTA TATCATGGCC GACAAGCAGA

eGFP

homology arm left

eGFP-2A-RedStar amino acid sequence

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVP
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FEGGTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIEVNFKIRHRG
TPRSGGGEGRGSLLTCGDVEENPGPRMSRSSKNVIKEFMRFKVKMEGTVNGHEFEIE
GEGEGRPYEGHNTVKLKVTKGGPLPFAWDILSPQFQYGSKVYVKHPADIPDYKKLSF
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RIEVNVELRKAKKDDQMLKRRNVSSFPDDATSPLQEN*