

Supplemental information

**Combining CRISPR-Cas-mediated terminal
resolution with a novel genetic workflow
to achieve high-diversity adenoviral libraries**

Julian Fischer, Ariana Fedotova, Lena Jaki, Erwan Sallard, Anja Erhardt, Jonas Fuchs, and Zsolt Ruzsics

Supplemental Information

Table S1: Overview of exemplary HAdV-Species and applicable enzymes for HFR; *In silico* analysis of representative genomes for each human adenovirus species for applicability of HFR mutagenesis regarding applicable enzymes, insertion sites, and insert sizes.

Virus	GenBank	Candidate Enzyme	Forward Sites	Min. Distance	Max. Distance	Mean	Reverse Sites	Min. Distance	Max. Distance	Mean	Minimum possible insertions	Min. Size	Max. Size	Mean
A12	NC001460	SnaBI (TACGTA)	534	3	606	76,27	454	3	859	75	988	2	1004	113,49
		SwaI (ATTTAAAT)	231	4	1267	147,24	224	5	1109	174,11	454	2	1361	231,26
B11	AF532578	SwaI (ATTTAAAT)	174	4	1096	199,38	188	5	1040	184,53	361	2	1476	296,8
		PmeI (GTTTAAAC)	161	8	1632	213,99	189	4	1437	181,32	350	2	1906	345,75
B3	DQ086466	SwaI (ATTTAAAT)	144	5	1900	244,73	171	5	1657	206,09	315	3	2145	349,52
		PmeI (GTTTAAAC)	128	9	1665	272,95	164	4	2081	212,95	291	2	2176	407,09
C2	AC000007	SnaBI (TACGTA)	427	3	823	83,89	357	3	1100	100,34	785	2	1104	165,99
C5	AC000008	SwaI (ATTTAAAT)	125	6	2093	286,1	136	5	2423	262,3	262	5	3191	430,23
C5delE3		SwaI (ATTTAAAT)	112	6	2093	302,54	125	5	2432	270,36	238	6	3191	447,95
D17	AF108105	SwaI (ATTTAAAT)	98	4	3242	257,06	122	4	2828	286,82	217	2	3369	470,04
		PmeI (GTTTAAAC)	90	6	1852	389,08	133	8	2298	263,29	222	2	2592	536,46
		SnaBI (TACGTA)	368	3	1022	95,27	308	3	848	113,82	677	2	1096	192,49
E4	AY458656	SwaI (ATTTAAAT)	100	4	2293	359,21	109	4	3778	329,55	210	3	3947	561,12
F41	DQ315364.2	PmeI (GTTTAAAC)	192	5	1748	176,98	218	5	959	155,89	409	3	2000	275,49
G52	DQ923122.2	SwaI (ATTTAAAT)	123	4	2017	375,46	98	4	3249	345,16	221	2	4003	471,08

Table S2: Predicted genomic DNA fragments of HAdV-C5 and HAdV-C5-pIXZG

following DraIII digestion; Overview of expected fragments of genomic DNA extracted from HAdV-C5 and HAdV-C5-pIXZG following DraIII nuclease digestion, including respective start- and end-position and predicted fragment size. Fragment expected to exhibit a shift between different constructs is highlighted in red.

HAdV-C5			HAdV-C5-pIXZG		
Start position	End position (inclusive)	Fragment size	Start position	End position (inclusive)	Fragment size
1	953	953	1	953	953
954	1410	457	954	1410	457
1411	2993	1583	1411	2993	1583
2994	3367	374	2994	3367	374
3368	3455	88	3368	3455	88
3456	6414	2959	3456	7164	3709
6415	15976	9562	7165	16726	9562
15977	19006	3030	16727	19756	3030
19007	26665	7659	19757	27415	7659
26666	27518	853	27416	28268	853
27519	33906	6388	28269	34656	6388
33907	35938	2032	34657	36688	2032

Table S3: Overview of mutations relative to reference detected using NGS; Overview of variants detected during bacmid and rAd genome sequencing, relative to HAdV-C5-delE3 mutant virus reference sequence.

Position	Mutation	CDS
4952	SNP	pIVa2
8783	Ala -> Asp	pTP/Pol
11284	Tyr -> His	52K
14073	polyA	NCR
17387	Gly -> Arg	pV
18754	TGA*A -> TGA*	NCR
19483	SNP	Hexon
19513	SNP	Hexon
19657	SNP	Hexon
19658	Thr -> Ala	Hexon
20378	SNP	Hexon
21163	SNP	Hexon
21630	Arg -> Glu	Hexon
25995	SNP	100K
26726	Pro/Ala/Ala -> Pro	33K
	Gly/Gly/Ser -> Gly	22K
27161	SNP	NCR
27314	SNP	pVIII
27339	Leu -> Pro	pVIII
27650	SNP	pVIII
27651	Pro -> Ser	pVIII
30964	SNP	NCR
30966	SNP	NCR
33898	SNP	NCR

Table S4: Previous publications discussing methods for cloning and rescuing rAd libraries;
Values shown here are in reference to information found within the publications main text, where applicable

Publication	Correctly assembled	Diversity	Max. discussed diversity	Notes
Elahi, S.M. (2002)¹	Up to 100%	10 ⁴	10 ⁶	Diversity demonstrated by dilution of BFP/GFP expressing virus and lysates after rescue
Hillgenberg, M. (2006)²	44%	10 ⁶		Diversity and assembly demonstrated by functional screening of cDNA transcripts (ELISA)
Lupold, S.E. (2009)³	>99%	1.6x10 ⁴	10 ⁵	Diversity shown by dilution of library and counting of viral burst
McVey, D. (2003)⁴	>99%	4x10 ⁵		Diversity demonstrated on cosmid level and by dilution of GFP expressing virus
Hatanaka, K. (2003)⁵	26%	0.3x10 ⁴	3x10 ⁵	Efficiency determined on the level of Cre-recombination. Diversity demonstrated by dilution of GFP expressing virus
Miura, Y. (2007)⁶		2x10 ⁴		Diversity demonstrated by dilution of GFP expressing virus
Miura, Y. (2013)⁷		5x10 ⁹		Diversity demonstrated by dilution of plasmid and detection of GFP expressing virus
Yamamoto, Y. (2014)⁸	~63%	10 ⁴		Diversity demonstrated by dilution of plasmid and detection of GFP expressing virus

Table S5: Oligonucleotides used for diversification; Oligonucleotides used for generation of barcoding sequences. Diverse central regions are underlined. For 12N-library, oligonucleotide “Lib3_cap” was added to the mix to ensure that homologous regions would not be digested by 5’-Exonuclease.

Name	Sequence (5' -> 3')
Lib_Frag1	ATATGTTTTATGTATCCAGTAACCATTGT <u>TANNNNAT</u> GACTCTAGAGGATCCCCG GGTACCGAGCTC
Lib_Frag2	TACAATGGTTACTGGATACATAAAACATAT <u>TNNNNNTA</u> GTCCATCGGTTGCCCAA GTGTTAAGAT
Lib_Frag3	ATTGCGGGAAACGGCCCTAGGGGTGATAT <u>TANNNNAT</u> CTTAACACTTTGGGCAA CCGATGGACTA
Lib_Frag2_te rm	TACAATGGTTACTGGATACATAAAACATAT <u>TNNNNNTA</u> GACCTGCAGGCATGCAAG CTTGCGTAATC
Lib_Frag3_te rm	GATTACGCCAAGCTTGCATGCCTGCAGGTCT <u>TANNNNAT</u> CTTAACACTTTGGGCA ACCGATGGACTA
Lib_Frag4_te rm	TATATCACCCCTAGGGCCGTTTCCCGCAAT <u>TNNNNNTA</u> GACCTGCAGGCATGCAAG CTTGCGTAATC
Lib3_Cap	GACCTGCAGGCATGCAAGCTTGCGTAATC

Table S6: Primers; Primers used for cloning in this study.

Name	Sequence (5' - 3')
BWHC05for	TATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATGCCTCCGGGGTCCACTGCAA TTACTTCTCGACCAATTCTCATGTTTGAC
BWHC05rev	TATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATGCCTCCGGGGTCCACTGCAA TTATAAACTCGACAGCGACACACTTGC
C5-pIXKan_for	TTTGGGTAACAGGAGGGGGGTGTTCTACCTTACCAATGCAATTTAAATTCGTGTGGG CGGACAATAAAGTCTTAACTGAA
C5-pIXKan_rev	GCAAGACACTTGCTTGATCCAAATCCAAACAGAGTCTGGTTTTTTATTTAAATTGTGGG CGGACAAAATAGTTGG
fC5-Kan-L_for	GAATAAGAGGAAGTGAAATCTGAATAATTTAAATTCGTGTGGGCGGACAATAAAGTCT TAACTGAA
fC5-Kan-L_rev	TTCCACCCCTTAAGCCACGCCCACACATTTAAATAAATGTGGGCGGACAAAATAGTTGG
GHBfor	CCGCGTGTGTACCTCTACCTGGAGTTTTTCCACGGTGGA
GHBrev	TCCACCGTGGGAAAACTCCAGGTAGAGGTACACACGCGG
M13A_for	GACGGGTAAACGACGGCCAGT
M13A_rev	TAATGACTCAGTACAGGAAACAGCTATGAC
O6-AVT_for	AATGGAAGAGCTCCCATGTCAGCCGTTAAGTGTCCTG
O6-AVT_rev	CATTGAAGAGCTTAGAAAACTCATCGAGCATCAAATGAACTGCAA
O6-fC5_for	GCTCGATGAGTTTTTCTAAGCTCTTCAATGGAATAAGAGGAAGTGAAATCTGAATAATT TGACGGGTAAAACGACGGCCAGT
O6-fC5_rev	CTTAACGGCTGACATGGGAGCTCTTCCATTTCCACCCCTTAAGCCACGCCCACACATTT CAGTACCATAGAGCCCACCGCATCCC
PCR-LibBB_for	CATGCAAGCTTGCGTAATCATGGTCA
PCR-LibBB_rev	GATCCCCGGGTACCGAGCTC
pIXZsG_pIX_H3	CGTCACCGCATGTGAGCAGACTTCCTCTGCCCTCTCCGGAAACCGCATTGGGAGGGGA GGAAGCCT
pIXZsG_pIX_H5	CTACCTTACCAATGCAATTTGAGTCACACTAAGATATTGCT
pIXZsG_ZG_H3	CAGAGTCTGGTTTTTTATTTATGTTTCAGGGCAAGGCGGAGCCGGAG
pIXZsG_ZG_H5	TCTGCTCACATGCGGTGACGTGGAGGAGAATCCCGGGCCAGCCAGTCCAAGCACGG CCTGAC

Table S7: Plasmids; Plasmids used and generated during this study, including size and notable features.

Plasmid Name	Size	Features	Accession
pGPS1.1	4814 bp	GPS-1 Genome Priming System transposon donor; kanamycin resistance (KanR); tetracycline resistance (TetR)	10666445
pO6-A5-GFP	3457 bp	Constitutive GFP expression under human CMV promoter; HAdV-C5-targeted transfer vector, carrying part of packaging domain; kanamycin resistance (KanR) ⁹	
pKD46	6329 bp	red-recombineering plasmid; Arabia sugar dependent expression of lambda red phage exo, beta and gam protein; temperature sensitive; β -lactamase expression (AmpR)	10829079
pO6-fC5-GFP	3047 bp	Constitutive GFP expression under murine CMV promoter; HAdV-C5-targeted transfer vector, carrying part of packaging domain; empty multi cloning site; kanamycin resistance (KanR)	OR810920
pO6-fC5-8N-GFP	3089 bp	Constitutive GFP expression under murine CMV promoter; HAdV-C5-targeted transfer vector, carrying part of packaging domain; multi cloning site equipped with 2-oligo diversified barcode; kanamycin resistance (KanR)	OR810926
pO6-fC5-12N-GFP	3123 bp	Constitutive GFP expression under murine CMV promoter; HAdV-C5-targeted transfer vector, carrying part of packaging domain; multi cloning site equipped with 3-oligo diversified barcode; kanamycin resistance (KanR)	OR810927
pO6-fC5-16N-GFP	3157 bp	Constitutive GFP expression under murine CMV promoter; HAdV-C5-targeted transfer vector, carrying part of packaging domain; multi cloning site equipped with 4-oligo diversified barcode; kanamycin resistance (KanR)	OR810928
pBWH-C5-delE3	40498 bp	Genomic BACmid; carrying mutant HAdV-C5 genome (HH-Ad5-VI-wt ¹⁰), E3-region deleted; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	OR810922
pBWH-fC5-E1Kn	39014 bp	Genomic BACmid; carrying HAdV-C5 genome, E3-region deleted; E1-region replaced with KanR to facilitate HFR; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR); kanamycin resistance (KanR)	OR810925
pVfC5-8N	38946 bp	Genomic BACmid; carrying HAdV-C5 genome, E1/E3-region deleted; carrying 2-oligo diversified genetic barcoding region; Expression of GFP under murine CMV promoter; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	OR810929
pVfC5-12N	38980 bp	Genomic BACmid; carrying HAdV-C5 genome, E1/E3-region deleted; carrying 3-oligo diversified genetic barcoding region; Expression of GFP under murine CMV promoter; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	OR810930
pVfC5-16N	39014 bp	Genomic BACmid; carrying HAdV-C5 genome, E1/E3-region deleted; carrying 4-oligo diversified genetic	OR810931

		barcoding region; Expression of GFP under murine CMV promoter; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	
pAR-Int5-Cas9	6699 bp	Constitutive sgRNA expression under human U6 promoter, targeting 5' of HAdV-C5 ITR sequence; Constitutive SpCas9-FLAG-NLS expression under EF1 α -promoter; ampicillin resistance	-
pBWH-C5-pIX-Kan	43300 bp	Genomic BACmid; carrying HAdV-C5 genome; pIX locus replaced with KanR to facilitate HFR; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR); kanamycin resistance (KanR)	OR810923
pBWH-C5-pIXZG	43216 bp	Genomic BACmid; carrying HAdV-C5 genome; pIX protein C-terminally linked to ZsGreen ¹¹ via T2A self-cleaving peptide; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	OR810924
pBWH-C5	42376 bp	Genomic BACmid; carrying HAdV-C5 genome; ITRs flanked by ACT sequences; chloramphenicol resistance (CamR)	OR810921
pKSB2	6457 bp	Single-copy BACmid backbone; chloramphenicol resistance (CamR)	-

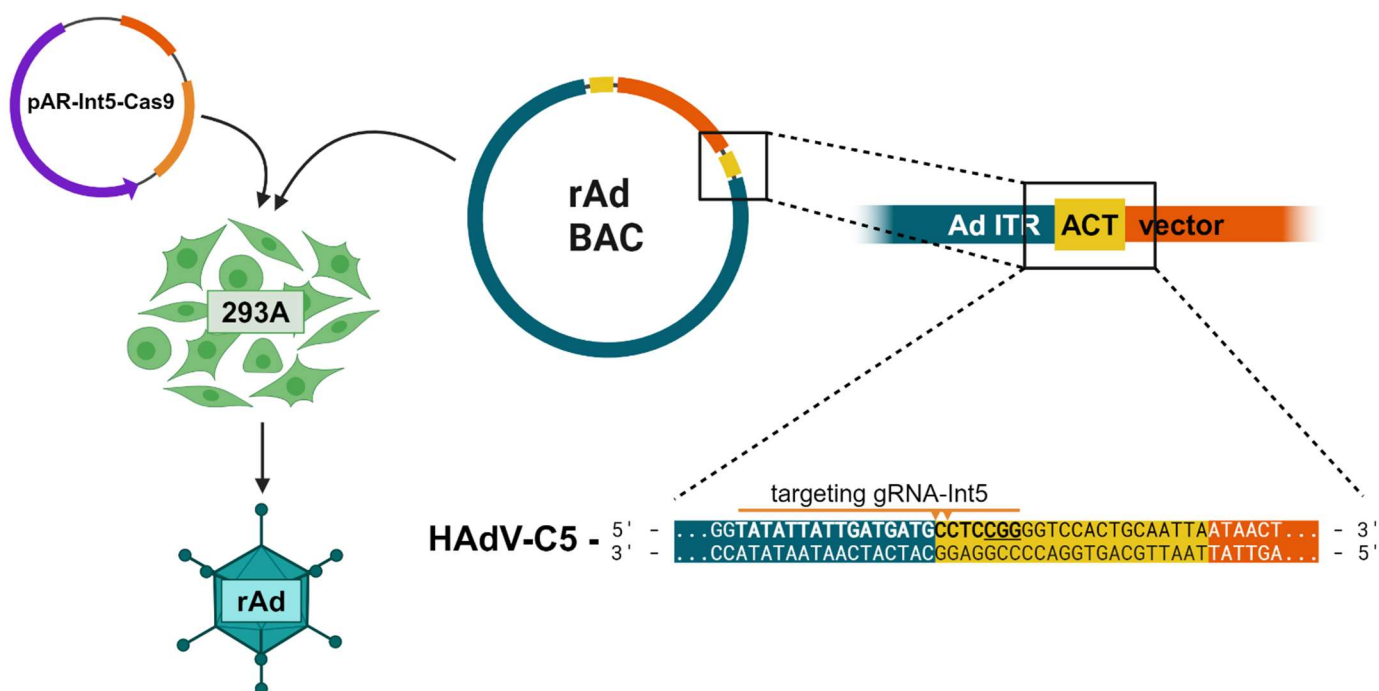


Figure S1: Schematic overview of CTR for reconstitution of rAd; Overview of rescue protocol utilizing CTR in this study; rAd bacmids are equipped with artificial CRISPR/Cas9 target sites (ACT, yellow boxes) between bacmid backbone (orange) and viral genomic DNA (blue). ACT allows binding of a specific gRNA (gRNA-Int5; light orange) to bind proximal to viral ITR (binding sequence presented bold, protospacer adjacent motif PAM underlined). When co-transfecting rAd bacmids and helper plasmid pAR-Int5-Cas9 into producer cells (e.g. 293A), coding for both Cas9 nuclease (purple) and specific gRNA-Int5, bacmid is cleaved within the cell and viral DNA released, allowing progeny virus to form.¹²

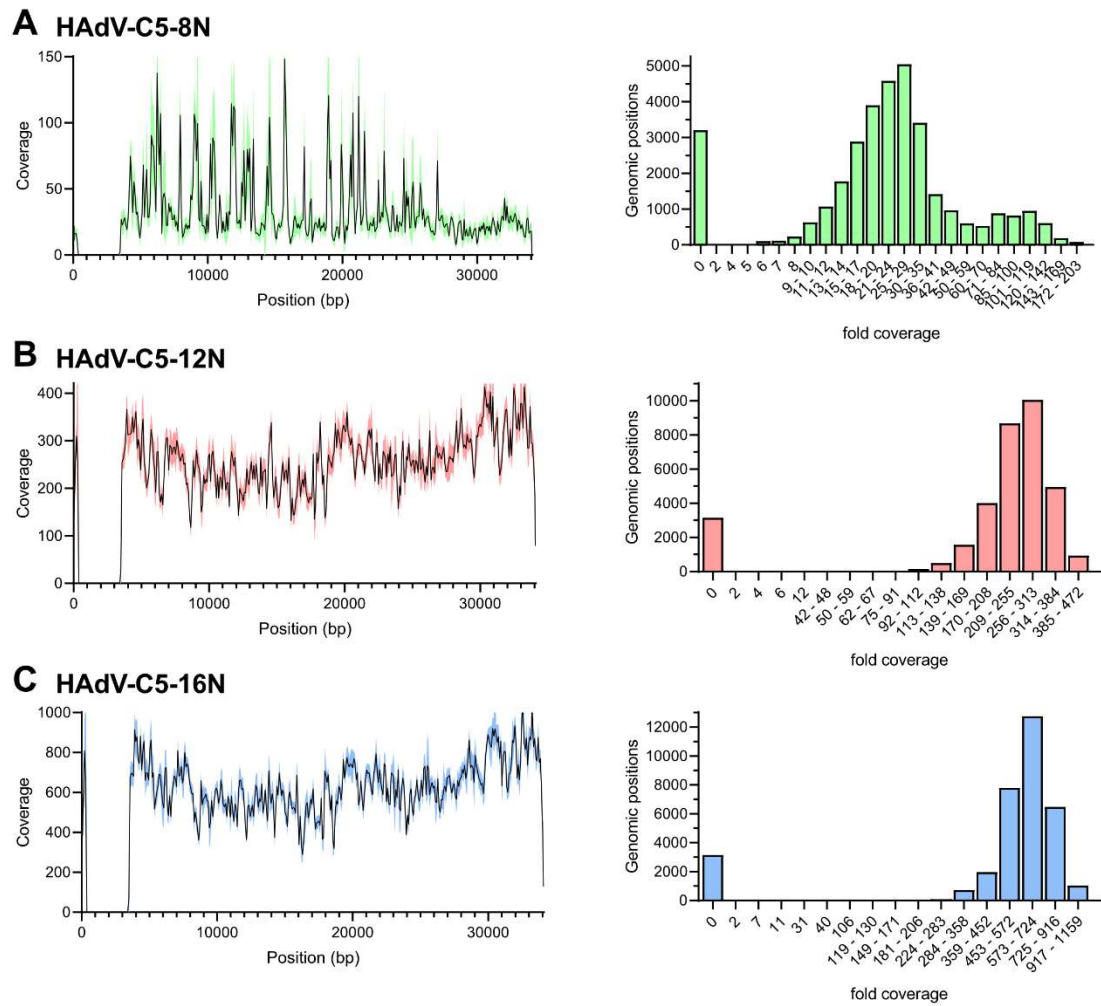


Figure S2: Mapping and coverage of genome reads obtained from library preparations against original virus; Mapping statistics for NGS reads obtained for viral genomic DNA directly after rescue. Data shows coverage of genetic region by mapping location (left) and nucleotide amount (right) for recombinant virus containing (A) 8N, (B) 12N- and (C) 16N-diversified viral libraries.

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