# Engineering an AAV viral vector production cell line: SV40 T antigen locus removal in HEK293T via Mad7 genome editing

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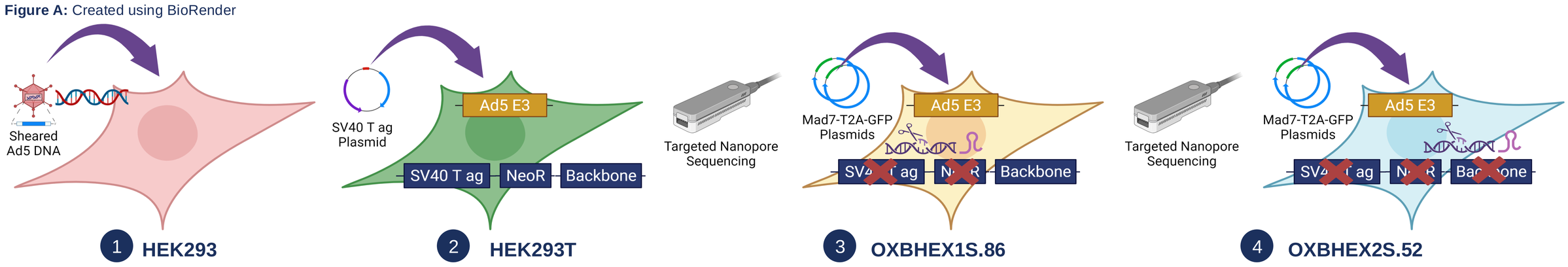
## Introduction

Integrating the Simian Virus 40 T antigen (SV40 T ag) into the Human Embryonic Kidney clone 293 (HEK293, (**A1**)) generated the HEK293T cell line (**A2**). HEK293T cells have increased growth kinetics and higher transfectability than HEK293. However, theoretical safety concerns of the SV40 proteins make it undesirable to use HEK293T cells for AAV and Ad production. The SV40 T ag integrated cassette also contains an undesirable neomycin resistance marker (NeoR). OXB previously generated a knock-out HEK293T cell line in which large portions of the SV40 T ag locus were deleted, retaining the beneficial characteristics of HEK293T cells (OXBHEX1S.86, (**A3**)). SV40 T ag and NeoR protein expression was ablated in OXBHEX1s.86 but T ag DNA sequences and backbone from the integrated plasmids remained.

Further Mad7-based genome editing was performed on OXBHEX1S.86 using multiple crRNAs to target remaining regions of the SV40 T ag integrated cassette and surrounding genomic regions, resulting in complete removal of the SV40 T ag and NeoR integrated cassette. Clones isolated from the edited cell population were screened for AAV production, and by PCR to determine successful target deletions. Genome editing was further confirmed in top clones by targeted nanopore sequencing. The removal of the SV40 T Ag and NeoR cassette was confirmed in the top clone (OXBHEX2S.52 (**A4**)) using PacBio whole genome sequencing with further confirmation by mass spectrometry. OXBHEX2S.52 retains the fast-growing phenotype of HEK293T cells and achieves AAV9 yields that are 3-fold higher at bioreactor scale compared to the HEK293 process, while also maintaining an excellent purity profile. OXBHEX2S.52 was generated in a specialised cell line development laboratory and is ready for GMP cell banking and subsequent GMP manufacturing of AAV vectors.

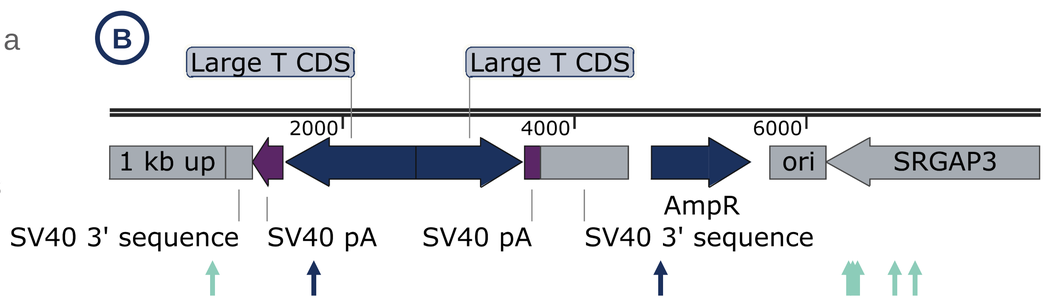
A **Figure A:** Created using BioRender




## Strategy for SV40 T ag KO using Mad7-based gene editing

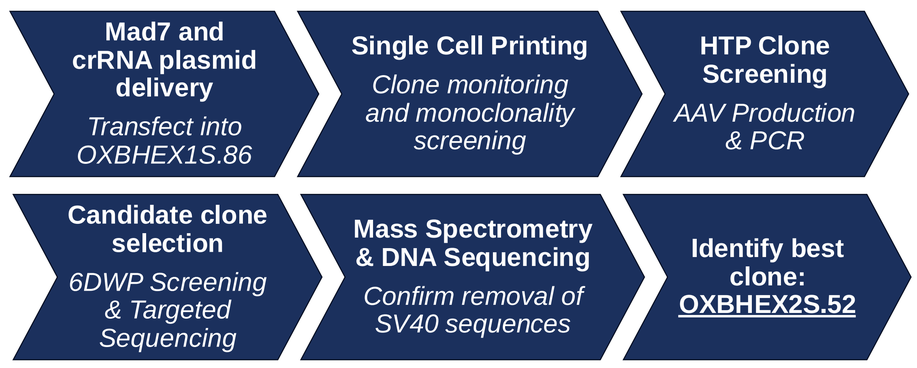
- The SV40 T ag/NeoR cassette is integrated in HEK293T cells on Chromosome 3 upstream of the SRGAP3 gene region.
- Initial gene editing removed a large portion of the SV40 T ag/NeoR cassette in lead clone OXBHEX1S.86.
- The remaining cassette was determined by targeted Nanopore sequencing **(B)**.

B




- OXBHEX1S.86 underwent a second round of genome editing using multiple crRNAs targeting the remaining SV40 T ag cassette (blue arrows) and flanking genomic regions (green arrows) **(B)**.
- Following gene editing, clones were isolated and screened for AAV9 production **(C)**. OXBHEX1S.86 underwent a second round of Mad7-based genome editing using multiple crRNA sequences.

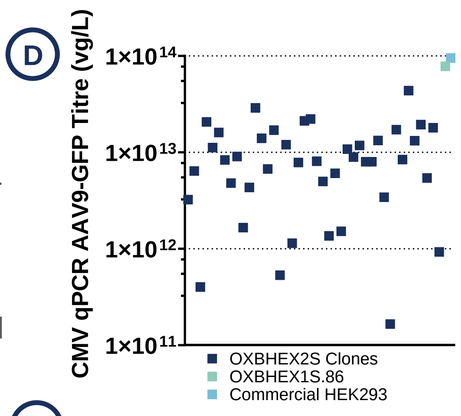
C




## Genome editing produced a diverse population of clones

- Clones were screened for AAV production at 96 well scale.
- Productivity ranged 100-fold with top clones producing AAV yields within 2-fold of a commercial HEK293 cell line **(D)**.

D



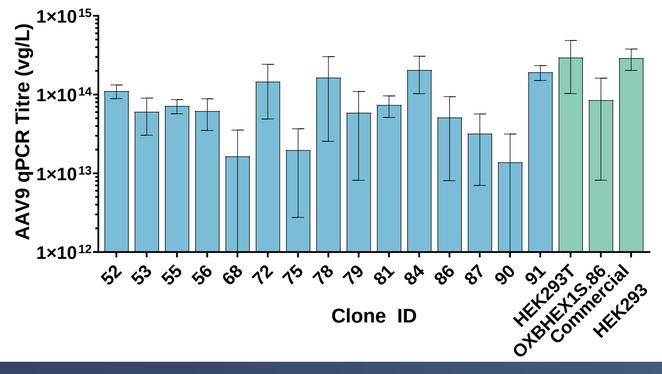

E

| Clone ID | 48 | 49 | 50 | 51 | 52 | 53 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5'SV40 T | | | | | | | | | | | | | | |
| AmpR | | | | | | | | | | | | | | |
| SRGAP3 | | | | | | | | | | | | | | |
| Clone ID | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 74 | 75 | 76 | 77 |
| 5'SV40 T | | | | | | | | | | | | | | |
| AmpR | | | | | | | | | | | | | | |
| SRGAP3 | | | | | | | | | | | | | | |
| Clone ID | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 |
| 5'SV40 T | | | | | | | | | | | | | | |
| AmpR | | | | | | | | | | | | | | |
| SRGAP3 | | | | | | | | | | | | | | |

| | |
|---|---|
| | No amplification– indicates genomic disruption |
| | Amplification– genomic region remains intact |
| | Not tested – poor titre or amplifies in other targets |

- Gene editing was highly efficient.
- Key regions of the SV40 T ag cassette were disrupted in most clones **(E)**.
- Promising clones were screened for AAV production in suspension culture **(F)**.

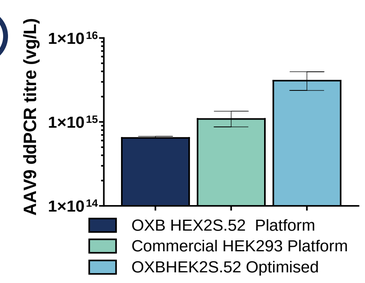
F



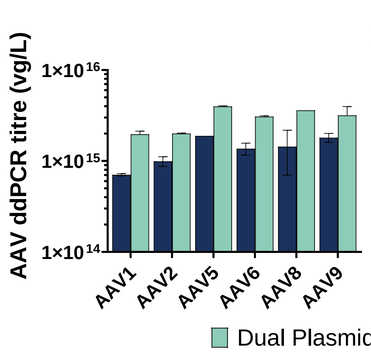

## Top clone produced high AAV titre following process optimization

- Top AAV producing clones were selected for targeted nanopore sequencing and **OXBHEX2S.52** was chosen as the lead candidate.
- After process optimization clone OXBHEX2S.52 outperformed a commercial HEK293 cell line by 3-fold at bioreactor scale **(G)**.
- Excellent AAV titre **(H)** and % full capsid **(I)** were achieved in bioreactors across various AAV serotypes, using both dual and triple transfection processes.

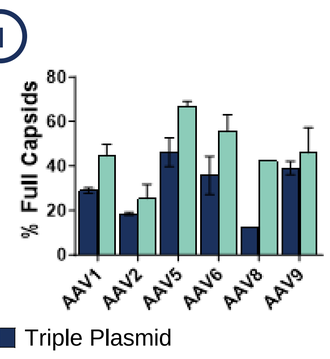
G




H




I




## WGS and Mass Spectrometry confirmed complete KO

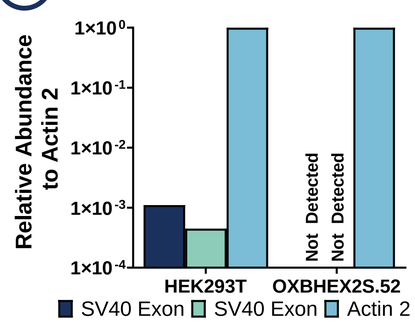
- PacBio whole genome sequencing (WGS) of OXBHEX2S.52 achieved 37-fold coverage.
- No reads mapped to sequences unique to the SV40 T ag cassette or the Mad7 plasmids, indicating a complete knockout without off-target integration.

**Table 1 – Whole Genome Sequencing**

| Sequencer | Raw Reads | CCS reads | Assembler |
|---|---|---|---|
| PacBio Sequel II | 23,591,180 | 11,867,283 | Hifiasm |
| **Coverage** | **Scaffold N50** | **Longest Scaffold** | **SV40/Mad7/GFP sequences identified** |
| 37.6x | 57.52 Mb | 133.23 Mb | 0 |

- OXBHEX2S.52 underwent protein mass spectrometry
- No SV40 T ag peptides were detected in OXBHEX2S.52 **(J)**.

J




## Conclusion

Genome editing of HEK293T has resulted in cell line **OXBHEX2S.52** that is:

- **More productive:** AAV titre exceeded OXB's previous platform which used a commercial cell line.
- **Generates high quality AAV:** Up to 60% full capsids depending on serotype.
- **Contains no SV40 T ag or Mad7 sequences / proteins:** Confirmed by 37-fold WGS and Mass Spectrometry.

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