RFP was cloned in the AAVS1 donor vector to validate the system.
Four AAVS1 target sequences were cloned in pCas-Guide vector:

<table>
<thead>
<tr>
<th>pCas-Guide-AAV1 T1</th>
<th>GTTAATGTGGCTCTGGTTCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCas-Guide-AAV1 T2</td>
<td>GAGAACCAGAGCCACATTAA</td>
</tr>
<tr>
<td>pCas-Guide-AAV1 T3</td>
<td>ACAGTGGGGCCACTAGGGAC</td>
</tr>
<tr>
<td>pCas-Guide-AAV1 T4</td>
<td>TGTCCCTAGTGGCCCCACTG</td>
</tr>
</tbody>
</table>
CRISPR/Cas Safe-harbor AAVS1 system

AAVS1 targeted by CRISPR/Cas

The gene with AAVS1 homologous seq as donor template DNA

The gene will be integrated at AAVS1 in the genome
RFP Fluorescence after cotransfection of pCas-Guide-AAVS1 and pAAVS1-RFP-DNR

2-day post transfection

Fluorescence before puromycin selection
- Scrambled + donor
- T1 + donor

Colony after Puromycin selection
- T1 + donor

23-day post transfection
Genomic PCR primers to detect the RFP cassette integration at AAVS1 site

RFP cassette Inserted at AAVS1

Present in Donor DNA

F: forward primer
R: reverse primer

F: forward primer
R: reverse primer
HEK293T cells were cotransfected with AAVS1 gRNA and RFP donor constructs. 5 days later, genomic DNA was extracted and nested genome PCR was performed to detect the integration of the RFP cassette.
HEK293T cells were cotransfected with AAVS1 gRNA and RFP donor vectors; cells were passaged for around 20 days, then applied to puro selection. 5 single cell colonies were isolated for each transfection group. Genomic PCR was performed using single cell colonies from pCas-Guide-AAVS1 T1 and RFP donor cotransfection. Two out 5 puro+ colonies amplified the correct PCR products.
AAVS1 Integration Was Confirmed By Sequencing