PLOS ONE

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A precise gene delivery approach for human induced pluripotent stem cells using Cas9 RNP complex and recombinant AAV6 donor vectors

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Citation: Chupradit K, Thongsin N, Tayapiwatana C, Wattanapanitch M (2022) A precise gene delivery approach for human induced pluripotent stem cells using Cas9 RNP complex and recombinant AAV6 donor vectors. PLoS ONE 17(7): e0270963. https://doi.org/10.1371/journal.pone.0270963

Editor: Xiaoping Bao, Purdue University, UNITED STATES

Received: February 28, 2022

Accepted: June 17, 2022

Published: July 7, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by Thailand Research Fund (grant no. RSA6280090 to MW), the National Research Council of Thailand (NRCT): NRCT5-RGJ63012-126 to NT and MW, and the Distinguished Research Professor Grant (NRCT 808/2563 to MW), the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (grant no. B05F630080 to

Abstract

Genome editing in human induced pluripotent stem cells (hiPSCs) offers a potential tool for studying gene functions in disease models and correcting genetic mutations for cell-based therapy. Precise transgene insertion in hiPSCs represents a significant challenge. In the past decade, viral transduction has been widely used due to its high transduction efficiency; however, it can result in random transgene integration and variable transgene copy numbers. Non-viral-based strategies are generally safer but limited by their low transfection efficiency in hiPSCs. Recently, genome engineering using adeno-associated virus (AAV) vectors has emerged as a promising gene delivery approach due to AAVs' low immunogenicity, toxicity, and ability to infect a broad range of cells. The following protocol describes the workflow for genome editing in hiPSCs using the CRISPR/Cas9 ribonucleoprotein (RNP) complex combined with the recombinant AAV serotype 6 (AAV6) donor vectors to introduce a gene of interest (GOI) fused with mCherry fluorescent reporter gene into the *AAVS1* safe harbor site. This approach leads to efficient transgene insertion and is applicable to precise genome editing of hiPSCs or other types of stem cells for research purposes.

Introduction

Genome editing in hiPSCs offers a potential strategy for studying gene function and treating diseases. Unlike other gene-editing techniques, CRISPR/Cas9 is the most efficient, easy to perform, and amenable to multiplex gene editing [1, 2]. The CRISPR/Cas9 system consists of a Cas9 nuclease and a single guide RNA (sgRNA), which form a ribonucleoprotein (RNP) complex. Once the RNP complex binds to the target DNA, it generates double-stranded break

MW and B05F630102 to CT), and Mahidol University under the New Discovery and Frontier Research Grant to MW. NT is supported by Siriraj Graduate Scholarship, Faculty of Medicine Siriraj Hospital, Mahidol University. MW is supported by Chalermphrakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University. The funders had and will not have a role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

(DSBs). The cells harness two endogenous DNA repair mechanisms, including the error-prone non-homologous end joining (NHEJ), which results in target gene disruption, and homology-directed repair (HDR), which results in precise genome editing [3–5]. The HDR-mediated gene correction or transgene insertion requires a DNA donor template in the form of a plasmid or single-stranded oligonucleotide (ssODN) containing left and right homology arms flanking the desired insertion.

In the past decade, CRISPR/Cas9-mediated gene editing has been utilized to correct genetic mutations in several disease-specific iPSCs, for example, thalassemia [6–8], hemophilia [9], primary hyperoxaluria type 1 (PH1) [10], and sickle cell disease [11]. Even though the ssODNs and plasmid vectors can introduce several base-pair mutations or transgene insertion, the gene targeting efficiency usually decreases with the larger transgene inserts [12, 13]. Recently, genome engineering using adeno-associated virus (AAV) vectors has emerged as a promising gene delivery tool due to its low immunogenicity and the ability to infect multiple human cell types in dividing and non-dividing cells [14]. A combination of CRISPR/Cas9 and AAV vectors provided an efficient knock-in of a DsRed reporter gene at the NRL locus in the human embryonic stem cells [15]. Furthermore, a highly efficient bi-allelic correction of sickle cell disease (SCD) mutation was reported using the Cas9 RNP complex combined with AAV6 transduction in a patient-derived iPSC line [16] and Townes-SCD mouse hematopoietic stem cells (HSCs) [17]. Notably, stable hemoglobin-A production was observed after autologous transplantation into Townes-SCD mice [17].

AAV is a single-stranded DNA virus comprising 4.7-kilobase (kb) genome in length. The AAV genome consists of *rep* (replication) and *cap* (capsid) genes flanked by two 145-bp inverted terminal repeats (ITRs) [18]. There are up to 12 serotypes of AAV vectors available [19, 20]. Previous studies demonstrated that AAV6 is the most efficient serotype for the transduction of primary human HSCs [21–24]. Recently, a successful genome editing in HSCs from sickle cell patients was achieved by using the AAV6 vectors [25]. Since the AAV is a replication-defective virus, it requires helper viruses such as adenovirus or herpes simplex virus for productive infection [26]. In the presence of the helper viruses, the AAVs can randomly integrate into the host chromosome. On the other hand, in the absence of the helper viruses, the AAVs preferably integrate into a specific site called *AAVS1* on human chromosome 19 [27]. For transgene knock-in, the transgenes are placed between the two ITRs in the AAV donor plasmid while the *rep*, *cap*, and helper genes are supplied in the helper plasmid. Production of recombinant helper-free AAV vectors requires co-transfection of the AAV donor and helper plasmids. Since the total size of the two ITRs is 290 bp and the homology arm size is 600 bp, the transgene size is limited to 3.8 kb for proper packaging efficiency [28].

In this protocol, we describe a step-by-step procedure to deliver the gene of interest (GOI) tagged with the mCherry reporter protein into the *AAVS1* safe harbor site in hiPSCs. Our protocol includes AAV6 vector production, purification, titration, nucleofection into hiPSCs, and clonal isolation. This approach could offer an efficient gene-editing platform for disease modeling and novel therapeutic strategies for genetic diseases.

Materials and methods

The protocol described in this article is published on protocols.io, https://dx.doi.org/10.17504/protocols.io.yxmvmn2d9g3p/v3 and is included for printing as S1 File with this article.

Results

In this protocol, we first transfected a pAAV donor plasmid and a pDGM6 helper plasmid (Fig 1A) into HEK293T cells for AAV6 production. The transfected HEK293T cells were harvested

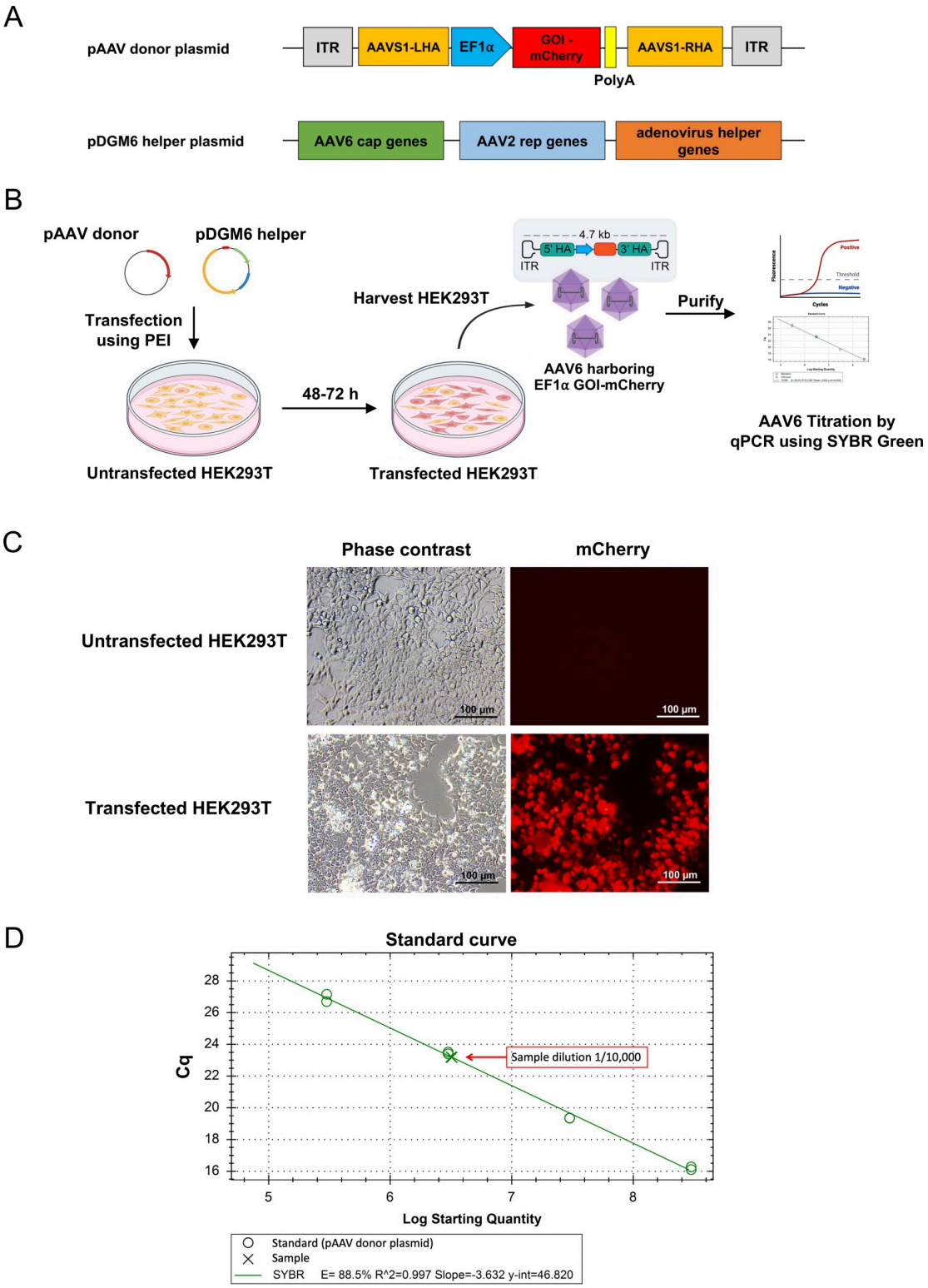


Fig 1. Production of AAV6 vectors in HEK293T cells. (A) The components of the pAAV donor plasmid and pDGM6 helper plasmid. The pAAV donor plasmid vector comprises ITR, left homology arm of *AAVS1* gene (AAVS1-LHA), EF1α promoter, the gene of interest (GOI) tagged with mCherry, polyA tail, right homology arm of *AAVS1* gene (AAVS1-RHA) and ITR. The pDGM6 helper plasmid consists of the AAV6 cap genes, the AAV2 rep genes, and the adenovirus helper genes. (B) Schematic of AAV6 production by co-transfection of the pAAV donor and pDGM6 helper plasmid vectors into HEK293T cells. (C) Fluorescence microscopic analysis of the untransfected and transfected HEK293T cells at 3 days post-transfection. Scale bar = 100 μm. (D) The qPCR standard curve was created by plotting the logarithmic DNA concentrations against Cq values.

https://doi.org/10.1371/journal.pone.0270963.g001

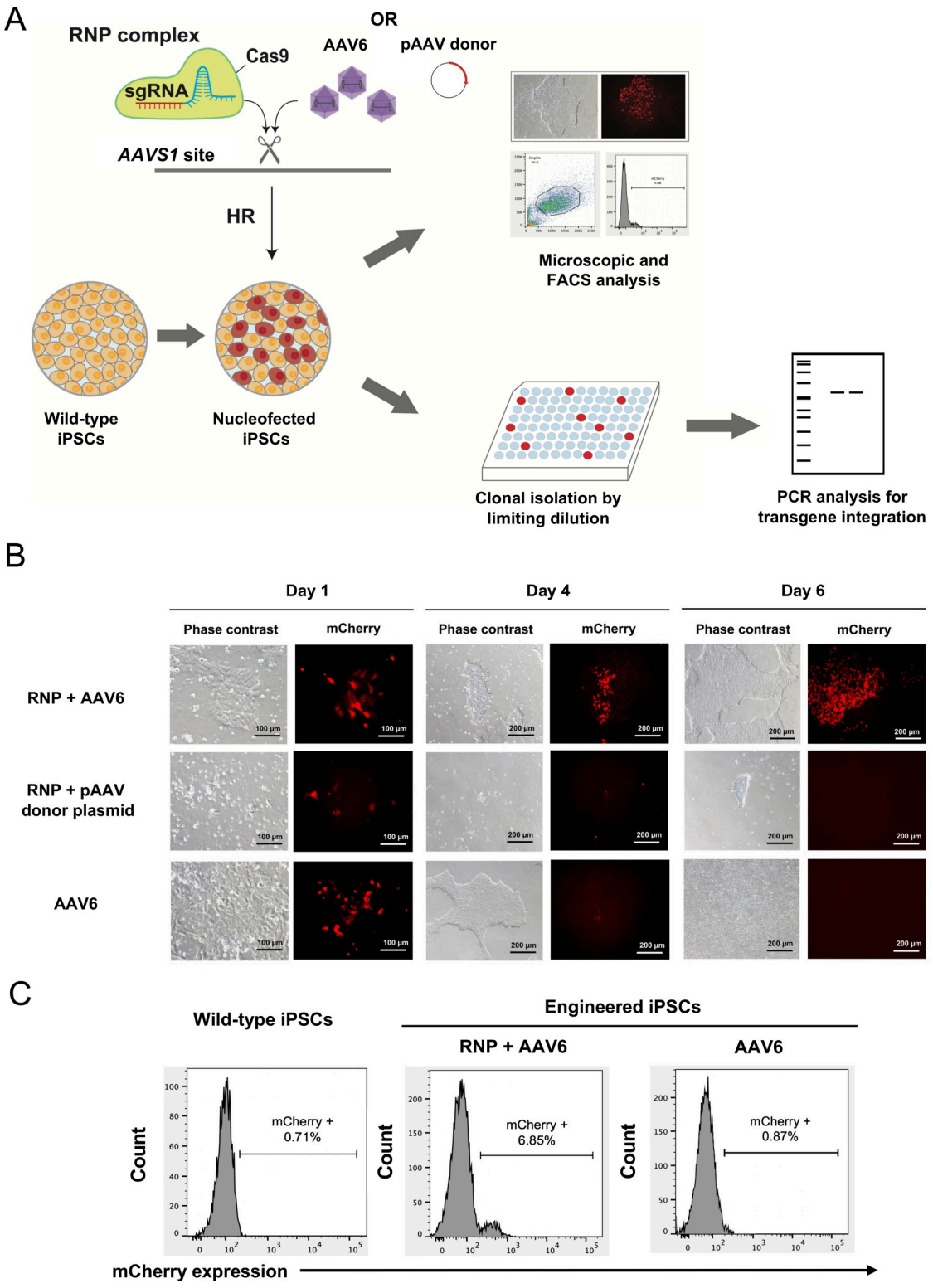
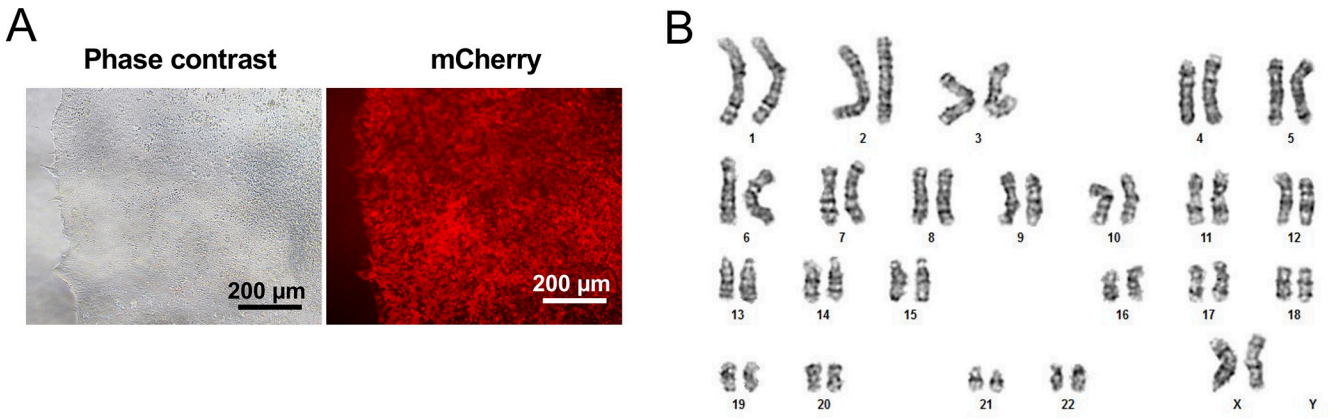


Fig 2. Nucleofection of human iPSCs. (A) Schematic diagram of the gene-editing strategy targeting the *AAVS1* locus using the CRISPR/Cas9 RNP complex and the recombinant AAV6 vectors in human iPSCs. HR = Homologous Recombination. (B) Microscopic fluorescence analysis of human iPSCs shows mCherry expression on days 1, 4 and day 6 post-nucleofection from three different conditions. Scale bar = 100 μm (Day 1) and 200 μm (Days 4 and 6). (C) The percentage of mCherry^{+} cells on day 6 post-nucleofection as analyzed by flow cytometry.

https://doi.org/10.1371/journal.pone.0270963.g002


A
Phase contrast
mCherry
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200 μm
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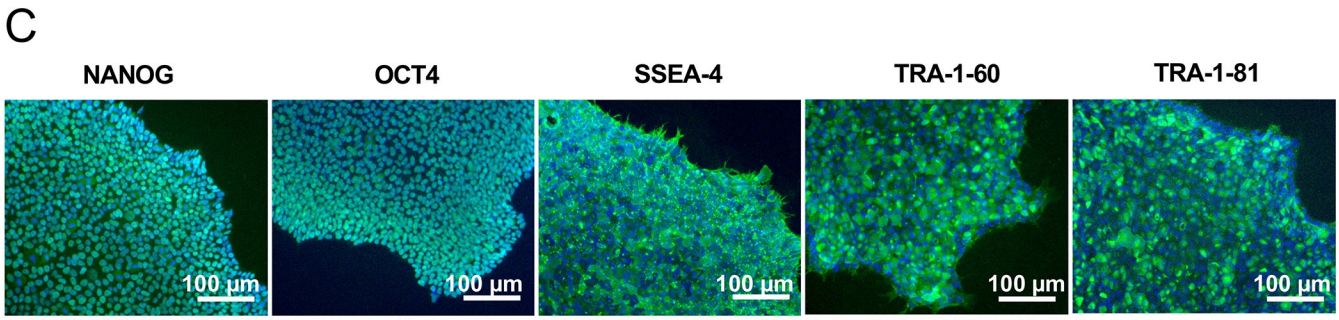
C
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OCT4
SSEA-4
TRA-1-60
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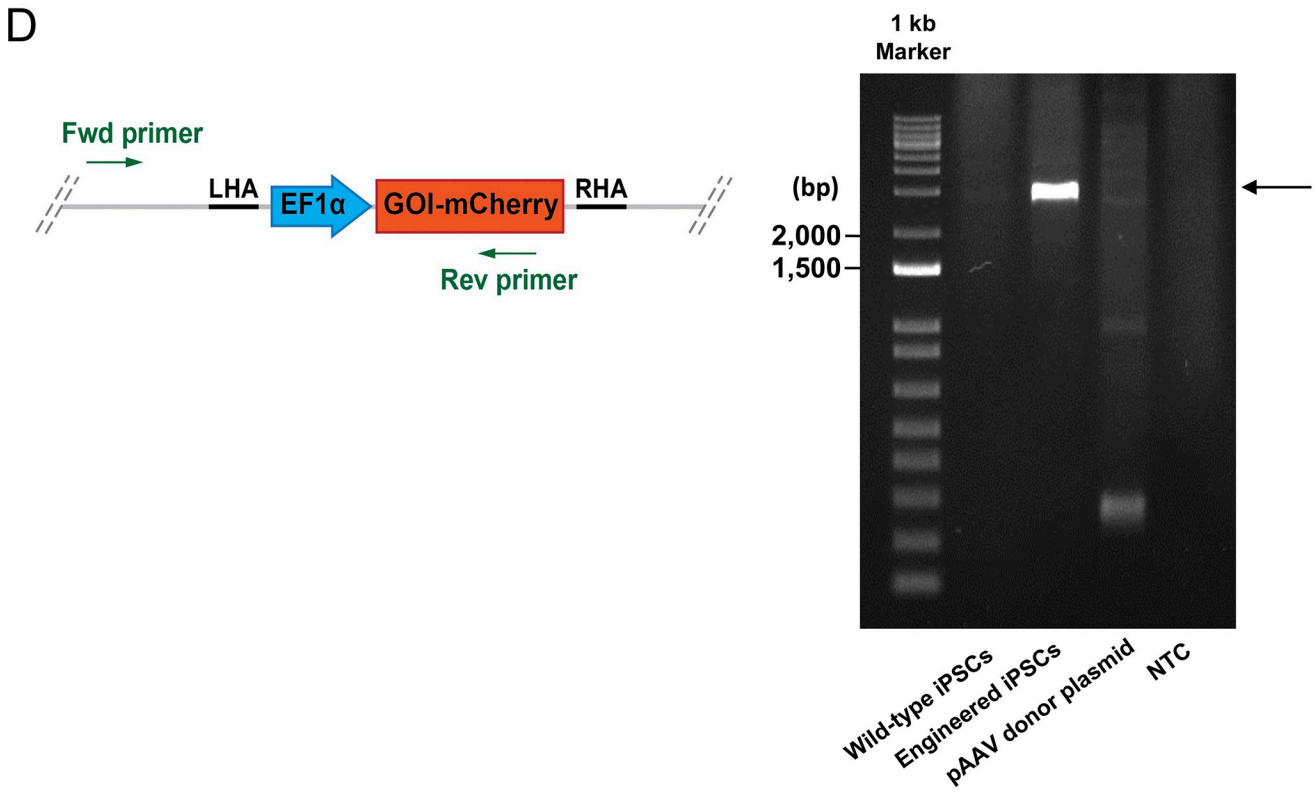
D
Fwd primer
LHA
EF1α
GOI-mCherry
RHA
Rev primer
1 kb
Marker
(bp)
2,000
1,500
Wild-type iPSCs
Engineered iPSCs
pAAV donor plasmid
NTC

Fig 3. Clonal isolation and characterization of the genetically engineered human iPSCs. (A) The mCherry expression of human iPSCs after limiting dilution. Scale bar = 200 μm. (B) Karyotype analysis by standard G banding demonstrated that the engineered human iPSCs exhibited normal karyotype (46, XX). (C) Immunofluorescence staining of pluripotent markers NANOG, OCT4, SSEA-4, TRA-1-60 and TRA-1-81. The nuclei were stained with DAPI. Scale bar = 100 μm. (D) PCR amplification of genomic DNA extracted from the wild-type iPSCs, genetically-engineered iPSCs, pAAV donor plasmid and non-template control (NTC). The major band indicates a successful transgene knock-in at the *AAVS1* locus.

https://doi.org/10.1371/journal.pone.0270963.g003

for isolation and purification of AAV6 (Fig 1B). Three days post-transfection, most of the transfected HEK293T cells expressed mCherry compared to the untransfected control (Fig 1C). We then harvested the AAV6 vectors from the HEK293T cells and purified them using the AAVpro® Purification kit. The AAV6 titer was determined using primers specific to the ITR regions by quantitative PCR analysis. The standard curve was prepared by plotting the logarithmic DNA concentrations against the mean values of the quantification cycle (Cq). We obtained the correlation coefficient (R^2) of the standard curve of 0.997 (slope −3.632) (Fig 1D). The sample data from dilution 1/10,000 was selected for calculating the AAV6 titer. From this experiment, we obtained the AAV6 titer of 6.36×10^9 genome copies/μl.

For knock-in of the GOI-mCherry gene into the *AAVS1* locus of the hiPSCs, we nucleofected the Cas9 RNP complex followed by adding the purified recombinant AAV6 vectors at the MOI of 100,000 (RNP + AAV6) (Fig 2A and 2B). For experimental controls, we delivered the transgene by nucleofecting the RNP complex with 0.5 μg of the pAAV donor plasmid (RNP + pAAV donor plasmid) or transducing the AAV6 vectors at the MOI of 100,000 alone (AAV6). On day 1 post-nucleofection, the $mCherry^+$ cells were observed in all conditions. On days 4 and 6 post-nucleofection, most of the cells in the RNP + pAAV donor plasmid condition died while the cells in the RNP + AAV6 and AAV6 conditions survived and grew larger in size. However, the $mCherry^+$ cells were observed only in the RNP + AAV6 condition. Flow cytometric analysis revealed that there were approximately 6.85% of $mCherry^+$ cells under the RNP + AAV6 condition, while there were no $mCherry^+$ cells under the AAV6 condition (Fig 2C). These results indicated that the combination of RNP complex and AAV6 vectors resulted in successful transgene delivery into human iPSCs. In contrast, the use of RNP complex with the donor plasmid resulted in poor transgene delivery and massive cell death while the transduction of AAV6 alone led to transient mCherry expression, which progressively diluted out during cell division.

We next performed clonal isolation by limiting dilution and characterized the genetically engineered human iPSCs. After clonal isolation, the engineered iPSCs exhibited a homogeneous fluorescence distribution (Fig 3A). The engineered cells were expanded for karyotype analysis. The results showed that the cells exhibited normal karyotype (46, XX) (Fig 3B) and expressed pluripotent markers, including NANOG, OCT4, SSEA-4, TRA-1-60, and TRA-1-81 (Fig 3C). We performed PCR using the primers that amplify the upstream region of the *AAVS1* left-homology arm and the mCherry reporter protein as indicated by the green arrow (Fig 3D). The results demonstrated a successful transgene knock-in at the *AAVS1* safe harbor locus on human chromosome 19 (Fig 3D). Taken together, the CRISPR/Cas9 RNP complex in combination with the recombinant AAV6 vectors provide a precise gene delivery method for human iPSCs. The knowledge obtained from this study can be applied for transgene knock-in for both research and therapeutic applications.

Supporting information

S1 File. Step-by-step protocol, also available on protocol.io.
(DOCX)

S2 File. Uncropped gel of Fig 3D.
(PDF)

Acknowledgments

The authors would like to thank Dr. Thaweesak Chieochansin for his assistance with the pAAV donor plasmid construction.

Author Contributions

Conceptualization: Koollawat Chupradit, Nontaphat Thongsin, Methichit Wattanapanitch.

Data curation: Koollawat Chupradit.

Formal analysis: Koollawat Chupradit, Nontaphat Thongsin, Methichit Wattanapanitch.

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Investigation: Koollawat Chupradit, Nontaphat Thongsin.

Methodology: Koollawat Chupradit, Nontaphat Thongsin.

Project administration: Methichit Wattanapanitch.

Resources: Chatchai Tayapiwatana, Methichit Wattanapanitch.

Supervision: Chatchai Tayapiwatana, Methichit Wattanapanitch.

Validation: Koollawat Chupradit, Nontaphat Thongsin.

Visualization: Koollawat Chupradit.

Writing – original draft: Koollawat Chupradit.

Writing – review & editing: Nontaphat Thongsin, Methichit Wattanapanitch.

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