Applied StemCell, Inc.
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Datasheet

# TARGATT™ Human Induced Pluripotent Stem Cell (hiPSC) Master Cell Line & Knockin Kit

## Product Information

**Catalog Number** AST-1600

**Description**

The TARGATT™ hiPSC Master Cell Line and transgenic kit were designed for fast and site-specific knockin in human iPSC cells, using an easy-to-use gene knockin approach. The master cell line provided in this kit contains an "attP" integrase-recognition landing pad engineered into the hH11 safe harbor locus in the genome. The kit also contains a cloning plasmid containing a corresponding "attB" sequence into which any gene of interest can be cloned (under control of the CAG promoter). The expression of the integrase (provided as an integrase plasmid) mediates the stable integration of the transgene into the master cell line (Figure 1). The TARGATT™ integrase technology enables highly efficient site-specific integration after antibiotic enrichment, without disruption of internal genes. The TARGATT™ iPSC master cell line and knockin kit are ideal for single transgene knockin and uniform, stable expression of your protein*.

The TARGATT™ iPSC Master Cell Line has been engineered from our well-characterized, karyotype normal, control iPSC line, ASE-9211. The ASE-9211 parental iPSC line was reprogrammed using episomal factors from fibroblasts obtained with full consent from a neonatal, African-American male donor. Both the parental ASE-9211 line and the TARGATT™ iPSC Master Cell Line have been characterized for pluripotency biomarkers, normal karyotype, and directed-differentiation to three germ layers as a validation of functional pluripotency. In addition, the ASE-9211 parental iPSC line is also used by the NIST Genome Editing Consortium to generate benchmark materials to establish standards for genome editing and stem cell research data.

The TARGATT™ iPSC master cell line and knockin kit are suitable for research applications involving site-specific large transgene knockin, gene overexpression, and other stable cell line generation applications*.

**We also have TARGATT™ master cell lines in HEK293 and CHO backgrounds. TARGATT™ master cell lines can also be generated in any cell line. Please contact Applied StemCell for TARGATT™ cell line engineering services to generate a master cell line in a specific cell line-of-choice.*

**Advantages of using TARGATT™ Master Cell Lines for gene knockin:**

- Site-specific and high efficiency integration (>50%)
- Stable knockin cell line generation
- Single copy gene integration into safe harbor locus
- Gene expression from an active, intergenic locus
- Low off-target integration

**Parental Line** Control human iPSC (ASE-9211); p15

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Age: Neonate
Gender: Male
Ethnicity: African-American
Tissue Source: Dermal Fibroblasts
Reprogramming Method: Episomal
Culture Conditions: Feeder-free

**Clinical information**

Healthy (with no known disease phenotypes)

**Contents**

All cell lines and plasmids provided in this kit are sufficient for 9 transfections (i.e., 3 triplicate transfections) according to the given protocol:

- TARGATT™ hiPSC Master Cell line
- TARGATT™ 22.2 CAG-MCS-attB; Cloning Plasmid
- TARGATT™ CMV-integrase Plasmid

**Quality Control**

A certificate of analysis (COA) with detailed quality control information for the cell line and components of the kit will be provided with each shipment.

**Shipping**

Dry ice

**Storage and Stability**

Store the TARGATT™ master cell line in liquid nitrogen freezer immediately upon receipt. Store the plasmids at -20°C. Do not freeze-thaw plasmids repeatedly. The products provided in this kit are stable for at least 6 months from the date of receiving when stored as directed.

**Safety Precaution**

**PLEASE READ BEFORE HANDLING ANY FROZEN VIALS**. Please wear the appropriate Personal Protective Equipment (lab coat, thermal gloves, safety goggles and a face shield) when handling the cells. Handle the frozen vials with due caution. Please be aware that the following scenario can occur: Liquid nitrogen can leak into the vials when the vials are submerged in liquid nitrogen. Upon thawing, the liquid nitrogen returns to the gas phase, resulting in a dangerous build-up of pressure within the vial. This can result in the vial exploding and expelling not only the vial contents but also the vial cap and plastic fragments of the vial.

**Restricted Use**

This product is for research use only and not intended for human or animal diagnostic or therapeutic uses.

## Schematic Representation of TARGATT™ Knockin Strategy

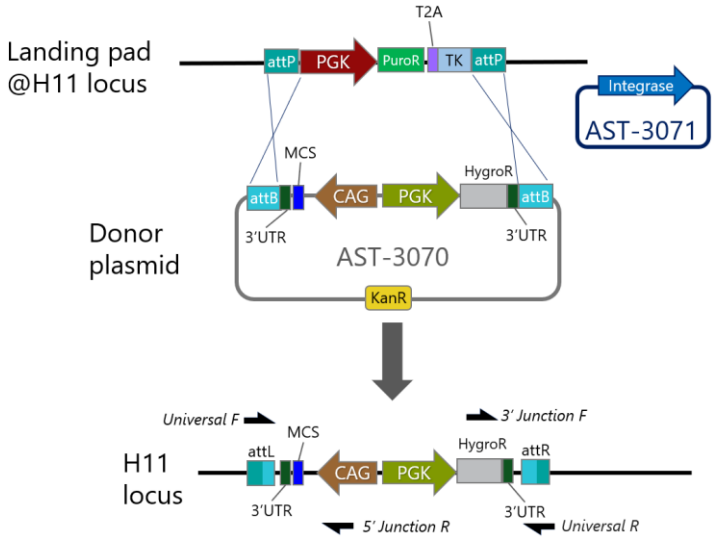



**Figure 1. Schematic representation of TARGATT™ site-specific transgene integration mediated by integrase.** The TARGATT™ hiPSC Master Cell Line was engineered with an "attP" landing pad in the hH11 safe harbor locus. The TARGATT™ 22.2 plasmid containing the integrase recognition site, attB and a multiple cloning site (MCS) downstream of the CAG promoter is used for cloning the transgene (gene of interest) and to generate the donor plasmid. The integrase generated by the co-transfected integrase plasmid catalyzes an irreversible reaction between the attP site in the genome and the attB site in the donor vector, resulting in the integration of the gene of interest at the selected H11 locus. The transgene can integrate into the landing pad. The genotyping primer sets for confirming integration of the transgene are also shown (black half arrows). The cells containing the gene of interest can be enriched using hygromycin and ganciclovir drug selection for the hygromycin resistance (HygroR) gene and thymidine kinase (TK), respectively.

## Media and Material

| Catalog # | Component | Amount |
|---|---|---|
| ASE-9211-TGT-PH3 | TARGATT™ hiPSC Master Cell Line | 1 x 10^6 cells |
| AST-3070 | TARGATT™ 22.2 CAG-MCS-attB Cloning Plasmid*# | 5 µg |
| AST-3071 | TARGATT™ CMV-integrase Plasmid#† | 15 µg |

** Amplify the cloning plasmid to clone your gene of interest; # Aliquot into single-use quantities and store at -20°C; do not freeze-thaw the plasmid repeatedly. † The integrase plasmid is proprietary material and should not be amplified. The provided quantity is sufficient for 9 transfections (i.e., 3 triplicate transfections). Additional quantities of these plasmids are available for purchase.*

## Materials Needed but not Provided

| Material | Vendor | Catalog# |
|---|---|---|
| mTeSR™ Plus | Stem Cell Technologies | 05825 |
| Matrigel® hESC-Qualified Matrix, LDEV-free | Corning | 354277 |
| Rock Inhibitor Y-27632 | Segment | 04-0012-02 |
| CryoStor® CS10 Freeze Media | Stemcell Technologies | 210102 |
| 0.5mM EDTA in PBS | Life Technologies | 15575-020 |
| TrypLE™ Express Enzyme (1X), No Phenol Red | Gibco | 12604013 |
| Neon™ Transfection System (100 µL) Kit | ThermoFisher | MPK10096 |
| Hygromycin | InvivoGen | Antihg-1 |
| Penicillin-Streptomycin (100X) | Gibco | 15140-122 |
| Ganciclovir (GCV) | Selleck Chemicals | S1878 |

*The above reagents are recommended based on our culture protocols. If you are using a similar/ alternative reagent, we recommend that you perform a small-batch test using your preferred reagents.*

## Protocol

### 1. Thawing and culturing cryopreserved cells

To ensure the highest level of viability, thaw the vial containing the cells and culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1.1 Prepare 4 mL of mTeSR™ Plus media (containing 1X Pen/Strep) with 10 µM Rock Inhibitor (mTeSR™ Plus media + Rock Inhibitor).
1.2 Add 1 mL of mTeSR™ Plus media + Rock Inhibitor in one well of a Matrigel® coated 6-well plate. Prepare 2 wells for each vial of frozen cells.
1.3 Use dry ice to bring one vial of frozen ASE-9211-TGT-PH3 cells to the cell culture room. Quickly thaw it in a 37°C water bath by gently shaking the cryovial continuously until only a small piece of ice remains.
1.4 Wipe the cryovial with a paper towel sprayed with 70% ethanol and place in a biosafety hood.
1.5 Add 9 mL of mTeSR™ Plus in a 15 mL-conical tube.
1.6 Use a 5 mL serological pipette to transfer the cells to the 15 mL tube.
1.7 Centrifuge the cells at 200 x g for 5 minutes at 4°C.
1.8 Aspirate the medium and add 2 mL of mTeSR™ Plus media + Rock Inhibitor.
1.9 Gently flick the conical tube to resuspend the cells and transfer them to the 2 wells of the Matrigel® coated plate.
1.10 Place the plate in the incubator and move the plate back-and-forth and side-to-side twice to spread the clumps evenly in the wells.

### 2. Sub-culturing procedure (culture maintenance)

2.1 The next day, aspirate the medium and add 2 mL of fresh mTeSR™ Plus into each well.
2.2 Change the medium every day. For medium change, aspirate off the spent medium and add 2 mL of fresh mTeSR-plus medium in each well.
2.3 When the colonies are big enough or close to merge, the cells need splitting.
*Note: To ensure the best quality of cells, the cell culture should be passaged every 4-6 days.*
2.4 Aspirate the medium from the culture dish.
2.5 Wash once with 1 mL of 0.5 mM EDTA (in PBS).
2.6 Aspirate the PBS, add 1 mL of 0.5 mM EDTA per well and incubate the cells for 3 minutes in a 37°C incubator.
2.7 Observe the cells under the microscope. The cells at the edge of the colonies will start to separate and round up.
2.8 With the cells still attached, aspirate the EDTA and add 1 mL of mTeSR™ Plus medium.
2.9 Scrape the cells from the bottom of the well until all the colonies are floating; pipette up and down 2-3 times to break the colonies into small clumps.
2.10 Make a 1:10 dilution and transfer the cells to the wells of the new Matrigel® coated plates.
2.11 Place the plate in the $CO_2$ incubator and move the plate back-and-forth and side-to-side twice to spread the clumps evenly in the wells.

### 3. Cryopreserving cells

3.1 Label the cryovials as needed, based on 2 vials per well of a 6-well plate, and pre-chill them in a 4°C freezer.
3.2 Aspirate the medium from the hiPSC culture.
3.3 Wash once with 1 mL of 0.5 mM EDTA (in PBS).
3.4 Aspirate the PBS and add 1 mL of 0.5 mM EDTA per well in 6-well dish and incubate the cells for 3 minutes in a 37°C incubator.
3.5 Observe the cells under microscope until the cells at the edge of the colonies start to separate and round up.
3.6 With the cells still attached, aspirate the EDTA and add 2 mL of CryoStor® CS10 medium.
3.7 Scrape the cells from the bottom of the well until the colonies are all floating.
3.8 Aliquot the cell suspension in 2 pre-chilled and labeled cryovials: 1 ml in each vial.
3.9 Place the cryovial in a CoolCell® Freezing Container or in a Styrofoam rack at - 80°C overnight, and transfer to liquid nitrogen the next day.

## 4. Transfection procedure

### 4.1 Plasmid amplification and cloning with TARGATT™ 22.2 CAG-MCS-attB cloning plasmid

4.1.1 Amplify the plasmids using NEB® 10-beta Competent E. coli (High Efficiency) competent cells
*Note: Do not use 5-alpha competent cells to transform the plasmids.*
4.1.2 Clone the gene of interest into the TARGATT™ 22.2 CAG-MCS-attB Cloning Plasmid to make the donor plasmid.
4.1.3 Aliquot into single-use quantity and store at -20°C. Do not freeze-thaw the plasmid repeatedly.
*Note: We recommend using 5 µg of the plasmid for each transfection*

### 4.2 Transfection using Neon® electroporation transfection system

4.2.1 Culture the ASE-9211-TGT-PH3 cells until the cells reach a 50% confluency.
4.2.2 Replace the complete medium with warm medium without Pen/Strep medium and culture overnight.
4.2.3 Aspirate the medium.
4.2.4 Wash the cells once with 1 mL PBS.
4.2.5 Add 1 mL TrypLE into each well and incubate for 5 minutes at 37°C.
4.2.6 Mix the cells by pipetting about 5 times and transfer the cells into a 15 mL conical tube.
4.2.7 Add 5 mL of mTeSR™ Plus medium to neutralize the enzyme.
4.2.8 Centrifuge at 200 x g for 5 minutes at 4°C.
4.2.9 Aspirate the supernatant and resuspend the cells in 1 mL PBS and count cell density.
4.2.10 Transfer 1 million cells into a 1.5 mL Eppendorf tube® and centrifuge at 200 x g for 5 minutes at 4°C.
4.2.11 Aspirate the supernatant and resuspend the cells in 100 µL transfection mixture (Table 1) containing the gene of interest cloned into the TARGATT™ 22.2 plasmid (donor plasmid) and 1 µg of integrase plasmid (AST-3071) and Neon™ buffer R.

**Table 1. Preparation of the transfection mixture**

| Reagent | Amount |
|---|---|
| Donor plasmid containing transgene | 4 µg |
| TARGATT™ CMV-integrase plasmid (AST-3071) | 1 µg |
| Neon Buffer R | to 100 µL |
| **Final volume** | **100µL** |

4.2.12 Load the cell-DNA mixture into the Neon™ pipette tip and electroporate the cells using the Neon™ electroporation system and the following conditions: 1250V, 30ms, 1 pulse.
*Note: If using other transfection methods, please optimize your protocol accordingly.*
4.2.13 After electroporation, transfer the cells into 3 wells of a Matrigel® coated 6-well plate, culture in non-Pen/Strep mTeSR™ Plus medium + Rock inhibitor for 24 hours
4.2.14 After 24 hours, the medium is refreshed with complete mTeSR™ Plus medium.
4.2.15 The cells will recover in 3-4 days.

### 4.3 Enrichment using hygromycin selection

**Selection strategy using hygromycin and ganciclovir (recommended):**
The ganciclovir (GCV) treatment (negative selection) and hygromycin treatment (positive selection) can remove the non-transfected cells and off-target cells as well as enrich for cells with transgene integrated into the H11 locus.

4.3.1 Three (3) days after electroporation, add 25 µg/mL hygromycin to the culture medium for 3 days.
4.3.2 At the end of day 3, passage the cells using 1 mL of Trypsin LE for 5 minutes into single cell colonies (following step 4.2.5).
4.3.3 Transfer all the cells into a fresh Matrigel® coated 6-well dish without dilution.

4.3.4 Add 4 µM ganciclovir on day 7-9 post-electroporation.
4.3.5 Add 25 µg/mL hygromycin again on day 10-12 post-electroporation.

**Optional selection strategy using only Hygromycin:**
4.3.6 Three (3) days after electroporation, add 25 µg/mL hygromycin to the culture medium for 9 days (day 12 post-electroporation).
*Note: If the cells reach 70% confluency around/before day 12 post-electroporation, dissociate the cells using EDTA (steps 2.5 – 2.11) and passage the cells at a 1:10 dilution into a fresh Matrigel® coated 6-well plate.*

### 4.4 Single cell cloning

4.4.1 On day 12 post-electroporation, dissociate the cells using 1 mL Trypsin LE for 5 minutes into single cells (following step 4.2.5).
4.4.2 Seed the single cell iPSCs into a Matrigel® coated 96-well plate with 80 µL of mTeSR™ Plus medium + Rock Inhibitor, and sort the cells using BD-Melody FACS machine.
*Note 1: If you are using a FACS machine by a different manufacturer, please optimize your sorting protocol.*
*Note 2: If you do not have a FACS instrument or do not want to use FACS for single cell sorting, you could also do a serial dilution.*
4.4.3 When the single cell clones grow into 4-8 cell clusters in about 4-5 days, replace the spent medium with 100 µL fresh mTeSR™ Plus medium.
4.4.4 Change the medium every 2 days. For medium change, aspirate off the spent medium and add 100 µL of fresh mTeSR-plus medium in each well.
4.4.5 When the cells reach 10% confluency, transfer each well of cells from the 96-well plate into one-well of a fresh Matrigel® coated 48-well plate.
4.4.6 When the cells reach 60% confluency, passage 50% of the cells into fresh Matrigel® coated 6-well plates and use the other 50% of the cells for genotyping.

## Supporting Data

### Characterization of the TARGATT™ hiPSC Master Cell Line, ASE-9211-TGT-PH3

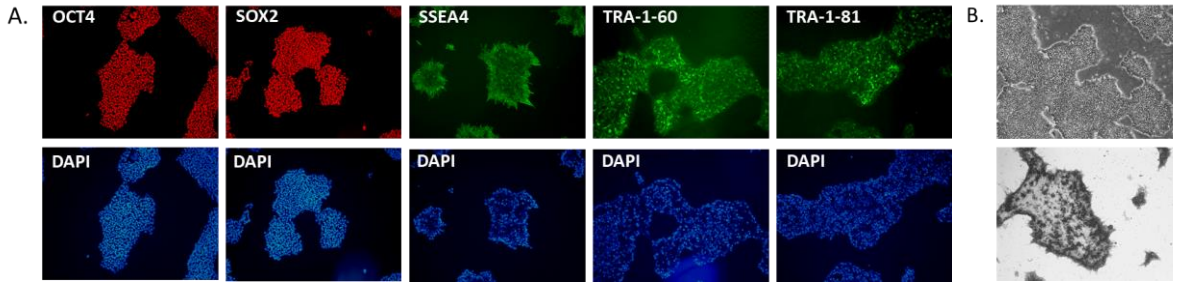
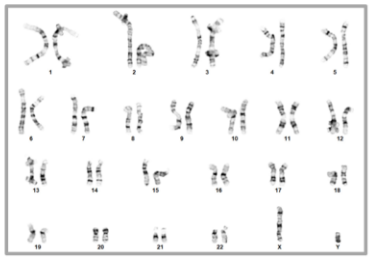



**Figure 2. Expression of pluripotency markers.** (A) The TARGATT™ hiPSC Master Cell Line, ASE-9211-TGT-PH3 expresses common iPSC biomarkers (top row: OCT-4, SOX2, SSEA-4, TRA-1-60, and TRA-1-81). Bottom row: Corresponding DAPI nuclear staining. (B) The TARGATT™ hiPSC Master Cell Line stains positive for Alkaline Phosphatase (AP; bottom). A typical unstained colony (top) was used to gauge the extent of the AP staining. All images were taken at 100x magnification.

A.



46, XY[19]: Apparently normal male karyotype

B.

| | | | | | |
|---|---|---|---|---|---|
| Amelogenin | X | Y | D18S51 | 12 | 18 |
| vWA | 16 | 17 | Penta E | 13 | |
| D8S1179 | 12 | 14 | D5S818 | 12 | 13 |
| TPOX | 6 | 9 | D13S317 | 12 | 13 |
| FGA | 22 | 24 | D7S820 | 10 | |
| D3S1358 | 15 | 17 | D16S539 | 11 | 12 |
| THO1 | 7 | 8 | CSF1PO | 11 | |
| D21S11 | 27 | 31 | Penta D | 7 | 8 |

**Figure 3. Karyotype (G-banding) and Short Tandem Repeat (STR) Analysis.** (A) The TARGATT™ hiPSC Master Cell Line demonstrates a normal male karyotype. (B) STR Profiling for ASE-9211-TGT-PH3 Master iPSC Line, was performed using a Powerplex 16-kit to authenticate the genetic footprint of the cell line. The experiment was run in duplicates and blinded to the interpreter to confirm the results.

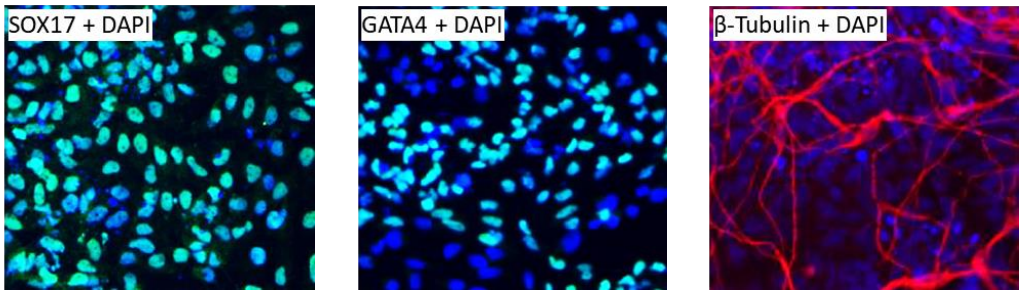
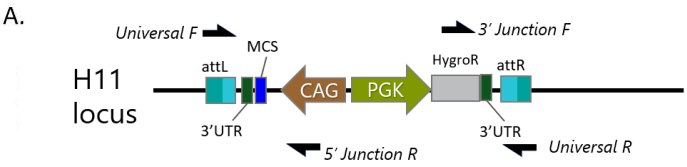


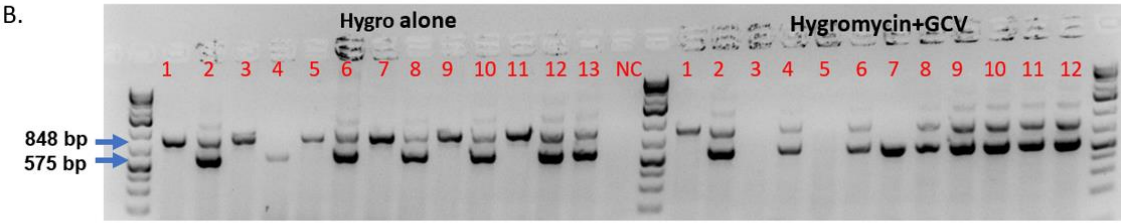

**Figure 4. Directed differentiation of TARGATT™ hiPSC Master Cell Line, ASE-9211-TGT-PH3 to the three germ layers.** The ASE-9211-TGT-PH3 hiPSC line was differentiated to specific lineages of the germ layers using well-established and optimized protocols. Immunostaining for biomarkers of each lineage was performed to confirm lineage commitment. Cells were also co-stained with nuclear marker, DAPI (blue). Images show co-localization of biomarker with DAPI. Endoderm (EN) marker: SOX17 (green); Mesoderm (ME) marker: GATA4 (green); Ectoderm (EC) marker: β-Tubulin (red).

## Confirmation of Site-specific CAG-MCS Plasmid Integration

A.




B.



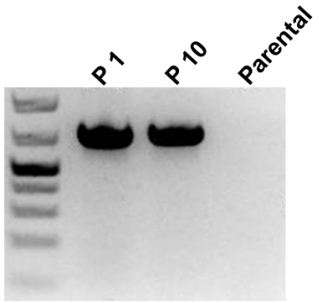

C.

| | Hygromycin only | Hygromycin + GCV |
|---|---|---|
| Positive clone # | clone#2, 6, 8,10, 12,13 | clone#2, 4, 6, 8, 9, 10, 11,12 |
| Locus specific integration (%) | 46% | 67% |

**Figure 5.** PCR gel electrophoresis to confirm the knockin of TARGATT™ 22.2 CAG-MCS-attB plasmid mediated by the TARGATT™ CMV-Integrase plasmid, after transfection into the TARGATT™ hiPSC Master Cell Line. 2 sets of primers were used to confirm knockin in 5` and 3`junctions (575 and 848 bp). A. Genotyping strategy; B. Image of the PCR gel electrophoresis; and C. Summary of the genotyping results. The integration efficiency into the TARGATT™ hiPSC master cell lines was 46% when the transfected cell pools were enriched with hygromycin only and 67% integration efficiency when enriched with hygromycin + GCV.

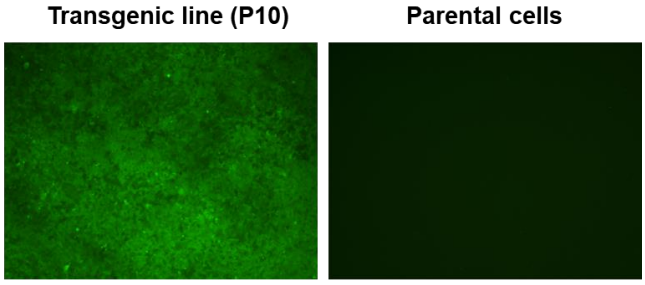
## Confirmation of Stable Transgene Expression After Locus-specific Integration into TARGATT™ hiPSC Master Cell Line

A.




3' Junction PCR

B.




GFP channel

**Figure 6.** Integration of GFP transgene into TARGATT™ hiPSC Master Cell Line and expression analysis after 10 passages. **(A)** PCR gel electrophoresis for GFP in passage 1 (P1), passage 10 (P10) and the parental line (control) in cells transfected and confirmed by genotyping PCR for integration of the GFP transgene. The result shows that the integration of the GFP transgene into the H11 locus is stable for 10 passages. (B) The GFP fluorescence signal was uniform and constant in passage 10 of the transgenic line but was not detected in the parental line indicating that the transgene (GFP) is stably expressed after 10 passages in culture.

## Appendix

The following PCR method was used to perform the genotyping analysis to confirm integration of the AST-3070 plasmid:

(i) Set up PCR reaction on ice according to manufacturer's instruction for the *Taq* polymerase being used. Below is an example for PCR solution using the NEB® Hot Start *Taq* polymerase:

| Component | Amount |
|---|---|
| Nuclease-free water | 40.50 µL |
| 10X NEB Standard *Taq* Buffer | 5.00 µL |
| 10 mM dNTP (NEB) | 1.00 µL |
| 10µM Universal primer | 1.00 µL |
| 10µM 3junction F primer | 1.00 µL |
| 10µM 5junction R primer | 1.00 µL |
| gDNA | 1.00 µL |
| NEB Hot Start *Taq* Polymerase | 0.50 µL |
| **Total Volume** | **50.00 µL** |

(ii) Primer sets: Please see Figure 1 (schematic) for location of primers

| | | |
|---|---|---|
| **5' Junction Primers:** | Universal F: 5'-ACTGGGGTAACCTTTGGGCTC-3`<br>5`junction R: 5'-GGGCAACGTGCTGGTTATTG-3` | 575 bps |
| **3' Junction Primers:** | 3`junction F: 5'-CCAATACGAGGTCGCCAACA-3`<br>Universal R: 5'-ACTGGGGTAACCTTTGGGCTC-3` | 848 bps |

(iii) Perform PCR amplification using the following programs for each primer sets.

| Step | # of Cycles | 5'+3` Junction | | 3' Junction | |
|---|---|---|---|---|---|
| | | Temp | Time | Temp | Time |
| 1 | 1 | 95°C | 30 s | 95°C | 30 s |
| 2<br>(Main PCR) | 35 | 95°C<br>60°C<br>72°C | 15 s<br>15 s<br>60 s | 95°C<br>60°C<br>72°C | 15 s<br>15 s<br>60 s |
| 3 | 1 | 72°C | 5 min | 72°C | 5 min |
| 4 | | 4°C | Hold | 4°C | Hold |