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Assembly of multiple full-size genes or genomic DNA fragments on Human Artificial Chromosomes using the iterative integration system

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Abstract

Human Artificial Chromosomes (HACs) are gene delivery vectors that have been used for decades for gene functional studies. HACs have several advantages over viral-based gene transfer systems, including stable episomal maintenance in a single copy in the cell and the ability to carry up to megabase-sized genomic DNA segments. We have previously developed the alphoid^{tetO}-HAC, which has a single gene acceptor loxP site that allows insertion of an individual gene of interest using Chinese hamster ovary (CHO) hybrid cells. The HAC, along with a DNA segment of interest, can then be transferred from donor CHO cells to various recipient cells of interest via microcell-mediated chromosome transfer (MMCT). Here, we detail a protocol for loading multiple genomic DNA segments or genes into the alphoid^{tetO}-HAC vector using an iterative integration system (IIS) that utilizes recombinases Cre, ΦC31, and ΦBT. This IIS-alphoid^{tetO}-HAC can be used for either serially assembling genomic loci or fragments of a large gene, or for inserting multiple genes into the same artificial chromosome. The insertions are executed iteratively, whereby each round results in the insertion of a new DNA segment of interest. This is accompanied by changes of expression of marker fluorescent proteins, which simplifies screening of correct clones, and changes of selection and counterselection markers, which constitutes an error-proofing mechanism that removes mis-incorporated DNA segments. In addition, the IIS-alphoid^{tetO}-HAC carrying the genes can be eliminated from the cells, offering the possibility to compare the phenotypes of human cells with and without functional copies of the genes of interest. The resulting HAC molecules may be used to investigate biomedically-relevant pathways or the regulation of multiple genes, and to potentially engineer synthetic chromosomes with a specific set of genes of interest. The IIS-alphoid^{tetO}-HAC system is expected to be beneficial in creating multiple-gene humanized models with the purpose to understand complex multi-gene genetic disorders.

Basic Protocol 1: Integration of the first DNA segment of interest into the IIS-alphoid^{teto}-HAC

Basic Protocol 2: Integration of a second DNA segment of interest into the IIS-alphoid^{teto}-HAC

Basic Protocol 3: Integration of a third DNA segment of interest into the IIS-alphoid^{teto}-HAC

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Conflict of Interest Statement
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Support Protocol 1: Fluorescence In Situ Hybridization (FISH) analysis for the circular IIS-alphoid^{tetO}-HAC

Keywords

Human Artificial Chromosome; HAC; Iterative Integration System; IIS; DNA assembly; genes delivery

INTRODUCTION

Human Artificial Chromosomes (HACs) are vectors for gene delivery and expression in target cells. HACs are highly stable and behave as an extra chromosome in host cells,, and are maintained independently from the host cell genome. Because HACs can carry megabase (Mb)-sized DNA segments, they have been successfully used for gene expression studies, the development of animal models of human diseases and cell re-programming, and have potential for use in gene therapy (Hiratsuka et al., 2011; Oshimura, Uno, Kazuki, Katoh, & Inoue, 2015; Katona, 2015; Kouprina, Earnshaw, Masumoto, & Larionov, 2013; Kouprina et al., 2014; Moriwaki et al., 2020; Ikeno, & Hasegawa, 2020; Moralli, & Monaco, 2020; Sinenko, Ponomartsev, & Tomilin, 2021).

HACs are constructed either by a “top down” or “bottom up” approach. The “top down“ HACs are engineered from natural chromosomes by telomere-directed truncation of the p- and q-arms using telomere-containing vectors, which leads to the replacement of natural telomeres by synthetic telomere repeats (Farr et al., 1995; Heller et al., 1996). The “bottom up” HACs, on the other hand, are *de novo* artificial chromosomes generated from BACs carrying high-order centromeric DNA repeats (HORs) (Harrington et al., 1997; Ikeno et al., 1998). After transfection into human cells, BAC DNA undergoes multimerization (20–30-fold) and a functional kinetochore is assembled, leading to HAC formation. Such HACs have a circular structure.

A decade ago, we devised the “bottom up” alphoid^{tetO}-HAC from a synthetic alphoid DNA array (Nakano et al., 2008). The array consists of a 343 bp synthetic dimer unit, amplified by rolling circle amplification (RCA) and then by transformation-associated recombination (TAR) in yeast (Ebersole et al., 2005) to up to 50 kb in size. The array was then multimerized up to 1.1 Mb after transfection into human HT1080 cells, leading to alphoid^{tetO}-HAC formation (Figures 1a, 1b) (Nakano et al., 2008; Kouprina et al., 2012). Into each dimer, approximately 3,000 copies of the 42 bp tetracycline operator (tetO) sequence, the binding site for *E. coli* tetracycline repressor (tetR), are incorporated in place of the CENP-B box, which can then be targeted specifically with tetR-fusion proteins. Targeting of the alphoid^{tetO} array with specific tetR fusion proteins disturbs kinetochore function, leading to HAC loss (Figure 1c). Such a unique feature of the alphoid^{tetO}-HAC to be eliminated from the cells along with the loaded gene gives researchers the possibility to compare the phenotypes of human cells with and without a gene of interest (Kim et al., 2011; Kononenko et al., 2014; Kouprina et al., 2018). This provides a proper interpretation of gene complementation analysis and a control for phenotypic changes attributed to expression of HAC-encoded genes. The original alphoid^{tetO}-HAC-based gene

delivery vector contains a single gene loading loxP site for the site-specific integration of one segment of exogenous DNA (Iida et al., 2010). The HAC is fitted with a Cre-loxP HPRT reconstitution system. While this system was quite successful at creating transgenic cells for gene functional studies (Kim et al., 2011; Kononenko et al., 2014; Ponomartsev et al., 2020), it has some limitations. For instance, this system can be used for integrating only a single genomic DNA segment into the HAC molecule at time (Liskovykh, Larionov, & Kouprina, 2021).

The assembly of multiple genes on the same HAC molecule, and the subsequent transfer of this vector into desired recipient cells, could have multiple applications in functional genomics. Therefore, construction of an alphoid^{tetO}-HAC vector containing multi-integration sites, allowing insertion of an unlimited number of genomic DNA fragments or genes, was our next goal. For this, we built the iterative integration system (IIS), which utilizes three recombinases, Cre, ΦC31, and ΦBT1, and combined it with the alphoid^{tetO}-HAC (Lee et al., 2018). This IIS-alphoid^{tetO}-HAC carries an integration platform cassette consisting of the SFM promoter (SV40 enhancer plus Feritin promoter) driving the expression of the GHT marker, a loxP site present between the promoter and the marker, and the attB^{ΦC31} site for the ΦC31 integrase (Figure 1d). The GHT marker is a fusion protein composed of a mutually exclusive selection marker, a mutually exclusive counterselection marker, and a mutually exclusive fluorescent marker. The GHT marker is a fusion of green fluorescent protein (eGFP), Hygromycin-B-phosphotransferase (hph), and thymidine kinase (TK). Such IIS-alphoid^{tetO}-HAC can be used to either serially assemble large Mb-sized genomic loci or genes from multiple smaller manageable segments of a very large gene or to serially insert multiple genes into the same HAC molecule. Such HAC molecules may be used for investigating different biomedically-relevant pathways or the regulation of multiple genes. The IIS-alphoid^{tetO}-HAC system may be used to create multiple-gene humanized models and has potential for gene therapy for polygenic diseases (Figure 2).

The iterative integration system is illustrated in Figure 3. It is a cyclic system, where two markers, GHT and PCF, substitute each other as a new genomic DNA segment is added to the integration sites. The PCF marker is a fusion protein composed of a mutually exclusive selection marker, a mutually exclusive counterselection marker, and a mutually exclusive fluorescent marker. The PCF marker is a fusion of puromycin-N-acetyltransferase (Puro), a red fluorescent protein (mCherry), and cytosine deaminase-uracil phosphoribosyl transferase (FcyFur). The PCF marker makes the cells appear red upon fluorescence microscopy, and resistant to Puromycin and sensitive to 5-Fluorocytosine. The GHT marker, on the other hand, makes the cells appear green, and resistant to Hygromycin B and sensitive to Ganciclovir. A 2A self-cleaving peptide sequence is placed between each element. As a consequence, these three proteins are transcribed as a single mRNA but are produced as separate proteins. As mentioned, the IIS-alphoid^{tetO}-HAC system uses three recombinase enzymes, i.e., Cre, ΦC31, and ΦBT1. Cre is a bidirectional enzyme that catalyzes the recombination between two substrate loxP sites and generates two product loxP sites. Recombinases ΦC31 and ΦBT1 are unidirectional enzymes that recombine an attachment bacteria (attB) site and an attachment phage (attP) site to produce attR and attL sites that are not substrates for further reaction.

The IIS-alphoid^{tetO}-HAC system starts with the HAC carrying the integration platform cassette. The HAC is propagated in donor hamster CHO cells (Figure 3a). The cells express eGFP. To insert the first genomic DNA segment of interest (hereafter referred to as DNA1) into the HAC, the cells are co-transformed with two plasmids, i.e., the A139 plasmid that expresses ΦC31 integrase and Cre recombinase (Figure 4a) and the Type I carrier vector A167 (Figure 4b) that contains the PCF marker and carries DNA1 (Figure 3a). Expression of ΦC31 and Cre causes two recombination events, loxP-loxP and attB$^{\Phi C31}$-attP$^{\Phi C31}$, correspondingly, between the Type I carrier vector and the integration platform cassette of the HAC (Figure 3b). The recombination reaction removes the GHT marker from the SFM promoter and replaces it with the PCF marker, loxP, and attB$^{\Phi BT1}$ sites, and the DNA1 segment from the Type I carrier vector A167, while deleting all other vector components (Figure 3c). The promoter within the platform cassette now drives the PCF marker. The cells that successfully completed both recombination reactions lose green fluorescence (eGFP) and sensitivity to Ganciclovir, and gain red fluorescence (mCherry), resistance to Puromycin, and sensitivity to 5-Fluorocytosine.

To insert a second DNA fragment of interest (DNA2) into the HAC, the cells are co-transformed with the A135-JH vector expressing ΦBT1 integrase and Cre recombinase (Figure 4c), and the Type II carrier vector A169 (Figure 4d), which contains DNA2 (Figure 3c). Cre and ΦBT1 expression causes two recombination events, loxP-loxP and attB$^{\Phi BT1}$-attP$^{\Phi BT1}$, respectively, between the Type II vector and the platform cassette (Figure 3d). This leads to the replacement of the PCF marker by the GHT marker and the attB$^{\Phi BT1}$ site, followed by the insertion of DNA2 from the Type II carrier vector. As a result, the platform cassette in the HAC will now contain a loxP site, an expressed GHT marker (eGFP), and an attB$^{\Phi BT1}$ site (Figure 3e). Selection with Hygromycin B and counterselection with 5-Fluorocytosine ensures that only cells that have correctly undergone the second round of assembly will survive. Untransformed parental cells and cells with incomplete recombination are killed by this double selection.

After two rounds of recombination, the integration platform cassette is once again where it started, with the exception that two genomic DNA segments of interest (DNA1 and DNA2) have now been integrated into the HAC (Figure 3e). The GHT marker is expressed, and the cells once again express GFP, and are resistant to Hygromycin B and sensitive to Ganciclovir. Further rounds of DNA fragment insertions can be repeated indefinitely as required (DNA3, DNA4…DNAn). The final IIS-alphoid^{tetO}-HAC carrying the required number of genomic DNA fragments can then be successfully moved from hamster donor CHO cells to different recipient cells by microcell-mediated chromosome transfer (MMCT) (Liskovykh, Lee, Larionov, & Kouprina, 2016; Liskovykh, Larionov, & Kouprina, 2021).

In this article, we describe three Basic Protocols (Figure 5). In Basic Protocol 1, the user will integrate the Type I carrier vector A167, carrying the first genomic DNA fragment of interest (DNA1), into the IIS-alphoid^{tetO}-HAC. In Basic Protocol 2, the user will integrate the Type II carrier vector A169, carrying a second genomic DNA fragment (DNA2), into the IIS-alphoid^{tetO}-HAC. In Basic Protocol 3, the user will integrate the Type I carrier vector A167, carrying a third genomic DNA fragment (DNA3), into the IIS-alphoid^{tetO}-HAC. The recombinant assay vectors will be transfected into hamster donor CHO cells containing the

IIS-alphoid^{tetO}-HAC, using a combination of either A167 plus A139 (ΦC31 integrase and Cre recombinase) vectors or A169 plus A135-JH (ΦBT1 integrase and Cre recombinase) vectors. By following these protocols, the user will be able integrate three genomic DNA fragments into the same IIS-alphoid^{tetO}-HAC molecule.

STRATEGIC PLANNING

Before experiments with the IIS-alphoid^{tetO}-HAC system, the user must first determine if the alphoid^{tetO}-HAC containing the “empty” integration platform cassette can be transferred via MMCT technique from the donor CHO cells into the recipient cells of interest, as HAC transfer to some cell lines may challenging. We recommend the user to apply the improved MMCT protocol (Liskovykh, Larionov, & Kouprina, 2021), which has been found to be efficient for many cell cultures, human immortalized mesenchymal stem cells, pluripotent cells (ES, iPS), and mouse embryonic fibroblast primary cultures.

The iterative integration system described in this article is carried out in hamster CHO cells, but it may be performed directly in the recipient cells of interest as well. If the user desires to conduct the iterative integration of the genomic DNA fragments within the cell line of interest, the user has to confirm that after MMCT transfer of the IIS-alphoid^{tetO}-HAC into the cell line of interest, the HAC is maintained in an autonomous form and as a single copy per cell. Such control experiment can be carried out by Fluorescence In Situ Hybridization (FISH), as previously described (Kim et al., 2011; Kononenko et al., 2014) (see also Support Protocol 1). This experiment is important because some cell lines used in the lab are very karyotypically unstable, displaying a wide variation of chromosome number even among cells of the same colony. This large-scale chromosome instability spills over to the HAC and may lead to multiple copies of the alphoid^{tetO}-HAC carrying the integration platform cassette, making the iterative integration system unworkable. The karyotype of the cell line can be determined by a standard FISH. If over 80% of the cells are able to maintain the HAC as a single copy, further work can be performed.

The user should remember that the described IIS-alphoid^{tetO}-HAC system is a cyclic system where two markers, GHT and PCF, substitute each other as a new genomic DNA segment is added to the integration sites. At each round of DNA integration, only a single copy of a selection/counterselection marker is present in each cell. As such, the development of cell resistance may be slower when a selection marker is changed. In some cell lines, resistance to the selection agents (Hygromycin B and Puromycin) may be lower than expected. The counterselectable markers (TK and FcyFur) are also more vulnerable to silencing. Therefore, at each round of DNA integration, when a counterselectable marker is changed, it is advisable to apply the counterselection as soon as possible (see details in Critical Parameters, “Selection and counterselection agent concentration”).

Before starting the Basic Protocols, the user should insert genomic DNA fragments of interest into the Type I and Type II carrier vectors A167 and A169. We recommend the user to apply the CRISPR/Cas9-mediated TAR cloning approach to isolate the desired DNA fragments (DNA1, DNA2, DNA3…DNAn) (Kouprina, Kim, & Larionov, 2021). TAR cloning allows selective and efficient isolation of full-size genes or chromosomal regions up

to 300 kb in size from total genomic DNA as circular YAC/BAC molecules in *S. cerevisiae* (Kouprina, & Larionov, 2008). For these experiments, Type I A167 and Type II A169 carrier vectors may be used to construct TAR vectors (Figure 4f). The targeting hook sequences homologous to 5' and 3' ends of the target genomic regions/genes may be inserted into the AscI/PacI and FseI/NotI sites of A167 and A169 vectors, respectively. Before TAR cloning, the A167 and A169 vectors containing the hooks should be linearized by PacI/FseI to expose the hooks for recombination with the genomic sequences homologous to the hook sequences. Because the A167 and A169 vectors contain YAC [the HIS3 marker and CEN6 (centromere from chromosome 6)] and BAC (F' origin of replication) cassettes (see Figures 4b, 4d), TAR-cloned molecules may be isolated in yeast cells and then moved directly to bacterial cells. In *E. coli* cells, the BAC molecules are then isolated to provide enough material for transfections into hamster CHO cells carrying the IIS-alphoid^{tetO}-HAC gene delivery vector. Alternatively, the DNA fragments of interest may be added to A167 or A169 vectors via ligation into the unique 8 bp restriction sites (AscI/PacI/FseI/NotI) (Figure 4e). Type I carrier vector A167 delivers the 1st genomic DNA fragment (DNA1) of interest and is then used for every subsequent odd-numbered round of DNA integration into the IIS-alphoid^{tetO}-HAC. Type II carrier vector A169 delivers the 2nd genomic DNA fragment (DNA2) and is then used for every subsequent even-numbered round of DNA integration into the IIS-alphoid^{tetO}-HAC.

Basic Protocol 1: INTEGRATION OF THE FIRST DNA SEGMENT OF INTEREST INTO THE IIS-alphoid^{tetO}-HAC

Here, we describe insertion of the Type I carrier vector carrying the first DNA segment of interest (Type I DNA1) into the integration platform cassette of the IIS-alphoid^{tetO}-HAC, propagated in hamster CHO cells (Figure 5 and Figure 6). At the starting point, the cells should express GFP, and be resistant to Hygromycin B and sensitive to Ganciclovir. A PCR against the HAC carrying the "empty" integration platform cassette with the diagnostic primers B072/B074 should yield a fragment of 559 bp (Figure 5).

To perform Basic Protocol 1, the user will co-transfect CHO cells carrying the IIS-alphoid^{tetO}-HAC with the Type I carrier vector A167 carrying DNA1 and the A139 vector. Vector A139 expresses ΦC31 integrase and Cre recombinase. Then, the user will culture the cells in Puromycin/Blasticidin S medium. After approximately 10 days of selection, the user will pick up the colonies exhibiting red fluorescence (mCherry is expressed) and culture them in Puromycin/Ganciclovir/Blasticidin S medium.

To verify recombination at the loxP site, the user will carry out a PCR reaction with the primer pairs B072/B071 and B074/B072 (Figure 5). B071 primer corresponds to the PCF marker. B072 corresponds to the SFM promoter sequence. B074 primer corresponds to the GHT marker. After the 1st and every next odd-numbered round of integration, a PCR reaction with B072/B071 primers should give a 447 bp product, while PCR with B074/B072 primers should be negative. Thus, the user will confirm the insertion of the PCF marker and elimination of the GHT marker.

To verify recombination between attB$^{\Phi C31}$ and attP$^{\Phi C31}$ sites and integration of DNA1 into the IIS-alphoid^{tetO}-HAC, the user should choose a forward primer complementary to a 3'-end region of DNA1 (F_{DNA1}) (Figure 5). B678 primer corresponding to the HAC backbone sequence can be used as a reverse primer (e.g., R3/B678 primers for VHL gene insertion; see Figures 7a, 7b).

To verify the integrity of the newly inserted attB$^{\Phi BT1}$ site, the user will need to select a reverse primer complementary to a 5'-end region of DNA1 (R_{DNA1}) (Figure 5). B485, complementary to the PCF sequence, can be used as a forward primer.

Materials

A139 vector expressing ΦC31 integrase and Cre recombinase

Type I carrier vector A167 carrying DNA1 (Type I DNA1, see STRATEGIC PLANNING)

> Concentration of the vectors should be 0.1–0.5 μg/μl. All vectors are available under request from the Developmental Therapeutics Branch, National Cancer Institute (NIH).

Hypoxanthine phosphoribosyltransferase (HPRT)-deficient Chinese hamster ovary (CHO) cells (JCRB0218) carrying the IIS-alphoid^{tetO}-HAC (alphoid^{tetO}-HAC containing the integration platform cassette, i.e., the promoter and the GHT marker components, and the recombinase recognition sites, loxP and attB$^{\Phi C31}$. The GHT compound marker is composed of a fusion of Green Fluorescence Protein (eGFP), P2A self-cleaving peptide, Hygromycin phosphotransferase (hph), and viral Thymidine Kinase (TK).

> CHO cells carrying the IIS-alphoid^{tetO}-HAC are available under request from the Developmental Therapeutics Branch, National Cancer Institute (NIH).

CFM (cell culture freezing medium) (see Reagents and Solutions)

F12 growth medium (see Reagents and Solutions)

F12 round I selection medium (see Reagents and Solutions)

Blasticidin S HCl (10 mg/mL) (Thermo Fisher Scientific, cat. no. A1113903)

Ganciclovir solution 10 mg/ml (see Reagents and Solutions)

Opti-MEM (Thermo Fisher Scientific, cat. no. 51985034)

Viafect (Promega, cat. no. E4981)

PBS (Thermo Fisher Scientific cat. no. 10010–023)

0.25 % Trypsin (Thermo Fisher Scientific, cat. no. 25200056)

6-well culture plates (Thermo Fisher Scientific, cat. no. 140675)

96-well culture plates (Thermo Fisher Scientific, cat. no. 167008)

24-well culture plates (Thermo Fisher Scientific, cat. no. 142475)

Cloning cylinders (Fisher Scientific, cat. no. 09–552-20)

Cryovial (Thermo Fisher Scientific, cat. no. 5012–0012)

GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific, cat. no. SM1331)

Nuclease-free water (Quality Biological, cat. no. 351–029-721)

DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504)

TaKaRa Ex Taq® DNA Polymerase (Takara Bio, cat. no. RR001C)

Agarose (Sigma-Aldrich, cat. no. A9539)

1.7-ml microcentrifuge tubes (Thomas Scientific, cat. no. 1159M35)

15-ml centrifuge tubes (Corning Falcon, cat. no. 352196)

50-ml centrifuge tubes (Corning Falcon, cat. no. 352070)

10-ml disposable pipettes (Corning Falcon, cat. no. 356551)

10-cm culture dishes (Thermo Fisher Scientific, cat. no. 174902)

Diagnostic primers to confirm insertion of DNA fragments:

B072 5’-CCAGTTGCGTGCGTGGAA-3’

B071 5’-CGCACCGTGGGCTTGTA-3’

B074 5’-GCCGGACACGCTGAACTT-3’

B485 5’-GTGCAAGAAGATTATGAAGCAG-3’

R_{DNA1} designed by the user

F_{DNA1} designed by the user

B678 5’-GCCTCTCTCTTTTATGAAGCTTCC-3’

Cell culture incubator

−80°C freezer

Liquid nitrogen tank

Standard Hemocytometer (Weber Scientific, cat. no. 3048–12)

PCR thermocycler

Nanodrop spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)

Refrigerated centrifuge

Refrigerated microcentrifuge

Inverted fluorescence microscope with filters of eGFP and mCherry (e.g., Zeiss AXIO)

Sub-Cell GT Horizontal Electrophoresis System, (BioRad, cat. no. 1704401)

Gel documentation system

Protocol Steps

1. Remove the cryovial containing the frozen CHO cells carrying the IIS-alphoid^{tetO}-HAC from a liquid nitrogen storage and immediately place it into a 37°C water bath for 1 min to thaw.
2. Transfer the thawed cells into a 15-ml centrifuge tube. Add 5 ml of pre-warmed F12 growth medium. Centrifuge at 1200 rpm for 3 min at room temperature.
3. Discard the supernatant. Resuspend the cell pellet in 10 ml of pre-warmed F12 growth medium supplemented with 5 μg/ml of Blasticidin S. Transfer the cell suspension onto a 10-cm culture dish. Incubate the dish for 2–4 days in a cell culture incubator at 37°C in 5% CO_2 atmosphere until the cells reach 50–80% confluency.
4. Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Plate 1×10^5 CHO cells in one well of a 6-well plate in 2 ml of F12 growth medium without antibiotics. Incubate the plate at 37°C in 5% CO_2 atmosphere overnight so that the cells are about 70–80 % confluent at the time of transfection (i.e. the next day, Figure 6a).
5. The next day, mix 1 μg of the Type I DNA1, 0.1 μg of A139, and 200 μl of Opti-MEM medium without serum in a sterile 1.7-ml tube. Mix gently by tapping.
6. Mix the Viafect transfection reagent gently before use, then add 10 μl directly to the mix from Step 5.
7. Mix gently and incubate for 5 min at room temperature.
8. After incubation, add the mixture from the previous step directly to the well with the cells, from Step 4.

   Do not mix by pipetting, just add drop by drop into the well.

   Changing the medium before transfection is not necessary.
9. Shake the plate vigorously backward-forward, left-right before placing back into the incubator.

10. Incubate the cells overnight (typically 16–18 hrs) at 37 °C in 5% CO_2 atmosphere (Figure 6b).

11. The next day, wash the cells with 1 ml of PBS once, add 300 µl of 0.25% Trypsin, and incubate for 5 min at 37 °C.

12. Add 4 ml of the F12 round I selection medium, resuspend the cells by pipetting up and down 5–7 times, and transfer the suspension onto a 10-cm culture dish containing 6 ml of F12 round I selection medium.

13. Let the cells grow until individual colonies become visible by the naked eye when you remove the medium from the dish. This usually takes 10–14 days. Change the F12 round I selection medium every 2–3 days.

14. With a permanent marker, make circles on the bottom of the culture dish around colonies that are well isolated from other colonies (Figure 6e).

15. Check the fluorescence of the cells in the circled colonies under the microscope. Mark only those colonies that have cells exhibiting red but no green fluorescence.

    The number of colonies formed on the dish may vary from one to one hundred depending on recombination efficacy. It is advisable to pick up at least 10 colonies for further analysis. Scale-up Steps 4–15 if you do not have enough colonies.

16. Pick up several individual colonies. To do this, wash the cells once with 5 ml of PBS, apply a cloning cylinder around a colony, and add 30 µl of 0.25% Trypsin into the cylinder's well. Incubate cells for 5 min at 37 ^{0}C. Add 150 µl of F12 round I selection medium supplemented with 5 µg/ml of Ganciclovir into the cylinder. Resuspend the cells well by pipetting 5–7 times and transfer the suspension into one well of a 96-well plate. Incubate the plate at 37°C in 5% CO_2 atmosphere.

17. Continue to grow the cells for an additional 3–7 days, until the cultures reach 90–100% confluency, changing the F12 round I selection medium supplemented with 5 µg/ml of Ganciclovir every 3 days. Once confluent, wash the wells once with 100 µl of PBS and add 50 µl of 0.25% Trypsin. Incubate the cells for 5 min. Transfer the cell suspension from each well of a 96-well plate to a separate well of a 24-well plate. Add 0.5 ml of F12 round I selection medium with 5 µg/ml Ganciclovir to each well of the 24-well plate with cells. Grow the cells until 90–100% confluency, changing the medium every 2–3 days (Figure 6g).

    Addition of Ganciclovir allows to select against the cells with an incorrect insertion (Figure 8b). Approximately half of the initially picked colonies can be selected against at this step.

18. Wash the cells once with 200 µl of PBS, add 100 µl of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times and transfer 80 µl of the suspension into a new separate 1.7-ml tubes. Add

0.5 ml of the F12 round I selection medium to each well to regrow the cells. Continue culturing the cells in the plate at 37°C in 5% CO_2 atmosphere.

It is important not to cross-contaminate the culture. Use separate pipet tips for each individual colony.

19. While the cells are growing, check whether the fragment DNA1 from Type I carrier vector was inserted into the IIS-alphoid^{tetO}-HAC. Spin the cells in the 1.7-mL tubes (from Step 18) down at 1200 rpm for 3 min at room temperature and isolate genomic DNA using the DNeasy Blood & Tissue Kit, per the manufacturer's instructions. Elute genomic DNA in 50 μl of water.

20. Use each genomic DNA sample to run four PCR reactions:

- **i.** with diagnostic primer pairs B071/B072;
- **ii.** with B074/B072, to confirm loxP recombination;
- **iii.** with F_{DNA1} and B678, to confirm attP$^{\Phi C31}$/attB$^{\Phi C31}$ recombination;
- **iv.** with forward B485 and R_{DNA1}, to confirm integrity of attB$^{\Phi BT1}$ site.

Set up PCR reactions with TaKaRa Ex Taq DNA Polymerase, per the manufacturer's instructions, using 1 μl of the genomic DNA solution (from Step 19) as a template. Set up 25-μl total reactions and use the following PCR conditions after initial denaturation: 94°C 20 sec, 60°C 30 sec, 72°C 30 sec, for 30 cycles (Figure 6i).

21. Run the samples in a 1.5% agarose gel.

PCR with B071/B072 should give a 447 bp product, while PCR with B074/B072 primers should be negative. PCRs with F_{DNA1}/B678, and with B485/R_{DNA1} should give products based on the user's design. It is advisable to sequence PCR products to ensure PCR accuracy.

22. Expand each colony with the PCR-confirmed DNA1 fragment insertion (left growing in Step 18) on a 10-cm culture dish, until the cells reach about 70–80% confluency (Figure 6k). Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Use 1×10^6 cells to start FISH (see Support Protocol 1).

FISH analysis on a metaphase spread is needed to confirm that the IIS-alphoid^{tetO}-HAC carrying a DNA1 fragment from A167 vector remains autonomous and has not integrated into the chromosomes.

23. Use 2×10^6 cells to prepare frozen stocks. Spin the cells down at 1200 rpm for 3 min at room temperature. Discard the supernatant. Resuspend the cell pellet in 1 ml of the F12 growth medium and add 1 ml of CFM (2x). Mix well and add 0.5 ml of the mixture to four cryovials. Place cryovials into the freezing box and place it at −80°C. For a long-term storage, place the cells in the liquid nitrogen tank the next day.

After the 1st round of insertion, the recombination reaction removes the GHT marker from its promoter and replaces it with the PCF marker, loxP and attB$^{\Phi BT1}$ sites, and a DNA fragment from the Type I carrier vector A167 (DNA1), while deleting all other vector components. The promoter within the platform cassette now drives the PCF marker. The cells that successfully completed recombination reactions lose green fluorescence (eGFP), resistance to hygromycin (hph), and sensitivity to Ganciclovir (TK), and gain red fluorescence (mCherry), resistance to Puromycin (Puro), and sensitivity to 5-Fluorocytosine (FcyFur) (Figure 5).

Colonies that have passed PCR and FISH verification, are suitable for the 2nd round of DNA insertion (Basic Protocol 2).

Basic Protocol 2: INTEGRATION OF A SECOND DNA SEGMENT OF INTEREST INTO THE IIS-alphoid^{tetO}-HAC

Here, we describe insertion of the Type II carrier vector A169 carrying a second DNA segment of interest (DNA2) into the IIS-alphoid^{tetO}-HAC, after the 1st round of DNA insertion (Basic Protocol 1) (Figure 5 and Figure 6). The HAC is propagated in hamster CHO cells. To perform Basic Protocol 2, the user will co-transfect the Type II carrier vector carrying DNA2 and the A135-JH vector. The A135-JH vector expresses ΦBT1 integrase and Cre recombinase. The user will culture the cells in Hygromycin B/Blasticidin S medium. After 10 days of selection, the user will pick up the colonies expressing green fluorescence (i.e. eGFP is expressed) and then expand them in medium containing Hygromycin B/5-Fluorocytosine/Blasticidin S.

After the 2nd round of DNA insertion and every subsequent even-numbered round of integration, a PCR reaction with B072/B074 primers should give a 559 bp product, while PCR with B071/B072 primers should be negative (Figure 5). Thus, the user will confirm the insertion of the GHT marker and elimination of the PCF marker.

To verify recombination between attB$^{\Phi BT1}$ and attP$^{\Phi BT1}$ sites and integration of DNA2, the user should choose primers from the sequence around a newly formed junction. A forward primer should correspond to a 3’-end region of DNA2 (F_{DNA2}). A reverse primer should anneal to a 5’-end region of the DNA fragment inserted in the 1st round (DNA1) (R_{DNA1}) (e.g., F23/R23 primers after the 2nd round of VHL gene integration; see Figure 7b).

To verify the integrity of the newly inserted attB$^{\Phi C31}$ site, the user should choose a reverse primer complementary to a 5’-end region of DNA2 (R_{DNA2}) (Figure 5). B075, corresponding to the GHT sequence, can be used as a forward primer.

Materials

A135-JH vector expressing ΦBT1 integrase and Cre recombinase

Type II carrier vector A169 carrying DNA2 (Type II DNA2, see STRATEGIC PLANNING)

Concentration of the vectors should be 0.1–0.5 μg/μl. All vectors are available under request from the Developmental Therapeutics Branch, National Cancer Institute (NIH).

CHO cells carrying the IIS-alphoid^{tetO}-HAC with DNA1 fragment inserted, from Basic Protocol 1.

CFM (cell culture freezing medium) (see Reagents and Solutions)

F12 growth medium (see Reagents and Solutions)

F12 round I selection medium (see Reagents and Solutions)

F12 round II selection medium (see Reagents and Solutions)

5-Fluorocytosine solution 10 mg/ml (InvivoGen, cat. no. sud-5fc)

Opti-MEM (Thermo Fisher Scientific, cat. no. 51985034)

Viafect (Promega, cat. no. E4981)

PBS (Thermo Fisher Scientific cat. no. 10010–023)

0.25 % Trypsin (Thermo Fisher Scientific, cat. no. 25200056)

6-well culture plates (Thermo Fisher Scientific, cat. no. 140675)

96-well culture plates (Thermo Fisher Scientific, cat. no. 167008)

24-well culture plates (Thermo Fisher Scientific, cat. no. 142475)

Cloning cylinders (Fisher Scientific, cat. no. 09–552-20)

Cryovial (Thermo Fisher Scientific, cat. no. 5012–0012)

GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific, cat. no. SM1331)

Nuclease-free water (Quality Biological, cat. no. 351–029-721)

DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504)

TaKaRa Ex Taq® DNA Polymerase (Takara Bio, cat. no. RR001C)

Agarose (Sigma-Aldrich, cat. no. A9539)

1.7-ml microcentrifuge tubes (Thomas Scientific, cat. no. 1159M35)

15-ml centrifuge tubes (Corning Falcon, cat. no. 352196)

50-ml centrifuge tubes (Corning Falcon, cat. no. 352070)

10-ml disposable pipettes (Corning Falcon, cat. no. 356551)

10-cm culture dishes (Thermo Fisher Scientific cat. no. 174902)

Diagnostic primers to confirm the insertions of DNA fragments:

B072 5’-CCAGTTGCGTGCGTGGAA-3’

B071 5’-CGCACCGTGGGCTTGTA-3’

B074 5’-GCCGGACACGCTGAACTT-3’

B075 5’-GGCTCCATACCGACGATAT-3’

R_{DNA2} designed by the user

F_{DNA2} designed by the user

R_{DNA1} designed by the user

Cell culture incubator

−80°C freezer

Liquid nitrogen tank

Standard Hemocytometer (Weber Scientific, cat. no. 3048–12)

PCR thermocycler

Nanodrop spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)

Refrigerated centrifuge

Refrigerated microcentrifuge

Inverted fluorescence microscope with filters of eGFP and mCherry (e.g., Zeiss AXIO)

Sub-Cell GT Horizontal Electrophoresis System, (BioRad, cat. no. 1704401)

Gel documentation system

Protocol Steps

1. Remove the cryovial containing the frozen CHO cells carrying the IIS-alphoid^{tetO}-HAC with DNA1 inserted from a liquid nitrogen storage and immediately place it into a 37°C water bath for 1 min to thaw.
2. Transfer the thawed cells into a 15-ml centrifuge tube. Add 5 ml of pre-warmed F12 growth medium. Centrifuge at 1200 rpm for 3 min at room temperature.
3. Discard the supernatant. Resuspend the cell pellet in 10 ml of pre-warmed F12 round I selection medium. Transfer the cell suspension onto a 10-cm

culture dish. Incubate the dish in the cell culture incubator at 37°C in 5% CO_2 atmosphere until the cells reach 50–80% confluency (Figure 6b).

4. Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Plate 1 × 105 CHO cells in one well of a 6-well plate in 2 ml of F12 growth medium without antibiotics. Incubate the plate at 37°C in 5% CO2 atmosphere overnight so that the cells are about 70–80 % confluent at the time of transfection (i.e. the next day).

5. The next day, mix 1 μg of the Type II DNA2, 0.1 μg of A135-JH, and 200 μl of Opti-MEM medium without serum in a sterile 1.7-ml tube. Mix gently by tapping.

6. Repeat Steps 6–11 of Basic Protocol 1.

7. Add 4 ml of F12 round II selection medium, resuspend the cells by pipetting up and down 5–7 times, and transfer the suspension onto a 10-cm culture dish containing 6 ml of the F12 round II selection medium (Figure 6d).

8. Let the cells grow until individual colonies become visible by the naked eye when you remove the medium from the dish. This usually takes 10–14 days. Change the F12 round II selection medium every 2–3 days.

9. With a permanent marker, make circles on the bottom of the culture dish around colonies that are well isolated from other colonies.

10. Check the fluorescence of the cells in the circled colonies under the microscope. Mark those colonies with the cells exhibiting green but not red fluorescence (Figure 6f).

    The number of colonies formed on the dish may vary from one to one hundred depending on recombination efficacy. It is advisable to pick up at least 10 colonies for further analysis. Scale-up Steps 4–10 if you do not have enough colonies.

11. Pick up several individual colonies. To do this, wash the cells once with 5 ml of PBS, apply a cloning cylinder around a colony, and add 30 μl of 0.25% Trypsin into the cylinder's well. Incubate the cells for 5 min. Add 150 μl of the F12 round II selection medium supplemented with 100 μg/ml of 5-Fluorocytosine into the cylinder. Resuspend the cells by pipetting 5–7 times and transfer the suspension into one well of a 96-well plate. Incubate the plate at 37°C in 5% CO_2 atmosphere.

12. Continue to grow the cells for an additional 3–7 days until the culture reaches 90–100% confluency, changing the F12 round II selection medium supplemented with 100 μg/ml of 5-Fluorocytosine every 3 days. After the cells reach confluency, wash the wells once with 100 μl of PBS and add 50 μl of 0.25% Trypsin. Incubate the cells for 5 min. Transfer the cell suspension from each well of the 96-well plate to a separate well of a 24-well plate. Add 0.5 ml of the F12 round II selection medium with 100 μg/ml 5-Fluorocytosine to each well of the

24-well plate with the cells. Grow the cells until 90–100% confluency, changing the medium every 2–3 days (Figure 6h).

Addition of 5-Fluorocytosine allows to select against the cells with incorrect insertion (Figure 8d). Approximately half of the initially picked colonies can be selected against at this step.

13. Wash the cells once with 200 µl of PBS, add 100 µl of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times and transfer 80 µl of each suspension into separate 1.7-ml tubes. Add 0.5 ml of the F12 round II selection medium to each well to regrow the cells. Continue culturing the cells in the plate at 37°C in 5% CO_2 atmosphere.

14. While the cells are growing, check whether the DNA2 fragment from the Type II carrier vector has been inserted into the IIS-alphoid^{tetO}-HAC. Spin down the cells in the 1.7-mL tubes (from Step 13) at 1200 rpm for 3 min at room temperature and isolate genomic DNA using DNeasy Blood & Tissue Kit, per the manufacturer's instructions. Elute genomic DNA in 50 µl of water (Figure 6j).

15. Use each genomic DNA sample to run four PCR reactions:

- **i.** with diagnostic primer pairs B071/B072;
- **ii.** with B074/B072, to confirm loxP recombination;
- **iii.** with F_{DNA2} and R_{DNA1}, to confirm attP$^{\Phi BT1}$/attB$^{\Phi BT1}$ recombination;
- **iv.** with forward B075 and R_{DNA2}, to confirm integrity of attB$^{\Phi C31}$ site.

Set up PCR reactions with TaKaRa Ex Taq DNA Polymerase, per the manufacturer's instructions, using 1 µl of the genomic DNA solution (from Step 14) as a template. Total volume of the reaction is 25 µl. Use the following PCR conditions after initial denaturation: 94°C 20 sec, 60°C 30 sec, 72°C 30 sec; 30 cycles.

16. Run the samples in a 1.5% agarose gel.

PCR with B074/B072 primers should give a 559 bp product, while PCR with B071/B072 primers should be negative. PCRs with F_{DNA2}/R_{DNA1} and with B075/R_{DNA2} should give products based on the user's design. It is advisable to sequence PCR products to ensure PCR accuracy.

17. Expand each colony with the PCR-confirmed DNA2 fragment insertion (on the plate, == from Step 13) on a 10-cm culture dish, until the cells reach about 70–80 % confluency (Figure 6l). Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Use 1×10^6 cells to start FISH (see Support Protocol 1).

FISH analysis on a metaphase spread is needed to confirm that the IIS-alphoid^{tetO}-HAC carrying DNA1, and DNA2 fragments remains autonomous and has not integrated into the chromosomes.

18. Prepare frozen stocks as described in Basic Protocol 1, Step 23.

After the 2nd round of integration, the IIS-alphoid^{tetO}-HAC will carry the DNA fragments from the first set of Type I and Type II carrier vectors (DNA1 and DNA2). The GHT marker will be expressed. Therefore, the cells will once again express eGFP, and will be Hygromycin resistant (hph) and Ganciclovir sensitive (TK) (Figure 5).

Clones that have passed PCR and FISH verification, are suitable for a 3^{d} round of insertion.

Basic Protocol 3: INTEGRATION OF A THIRD DNA SEGMENT OF INTEREST INTO THE IIS-alphoid^{tetO}-HAC

Here, we describe insertion of the Type I carrier vector A167 carrying a third DNA segment of interest (DNA3) into the IIS-alphoid^{tetO}-HAC after two previous rounds of DNA insertion (Basic Protocol 1 and Basic Protocol 2) (Figure 5 and Figure 6). The HAC is propagated in hamster donor CHO cells. After Basic Protocol 2, if the users want to add an additional DNA segment, they will essentially repeat Basic Protocol 1 with some modifications (see below). Such cycle (Basic Protocols 1–3) can then be repeated for any number of segments. To perform Basic Protocol 3, the user will co-transfect and select the cells as in Basic Protocol 1. As such, after the procedure, the cells will exhibit red fluorescence (mCherry is expressed).

After the 3rd round of DNA insertion and every subsequent odd-numbered round of integration, a PCR reaction with B071/B072 primers should give a 447 bp product, while PCR with B072/B074 primers should be negative (Figure 5). Thus, the user will confirm the insertion of the GHT marker and elimination of the PCF marker.

To verify recombination between attB$^{\Phi C31}$ and attP$^{\Phi C31}$ sites and integration of DNA3, the user should choose primers from the sequence around a newly formed junction. A forward primer should correspond to a 3'-end region of DNA3 (F_{DNA3}). A reverse primer should anneal to a 5'-end region of DNA2 (R_{DNA2}) (Figure 5) (e.g., F12/R12 primers for VHL gene insertion; see Figure 6b).

To verify the integrity of the newly inserted attB$^{\Phi BT1}$ site, the user should choose a reverse primer complementary to a 5'-end region of DNA3 (R_{DNA3}). B485, corresponding to the PCF sequence, can be used as a forward primer (Figure 5).

As mentioned, this protocol is almost identical to Basic Protocol 1, with the following important differences: i) the starting cell culture is the cells derived from a previous round of insertion (Basic Protocol 2), ii) Type I carrier vector contains a new DNA fragment (DNA3), and iii) a new pair of diagnostic primers ($F_{DNA3/}$ R_{DNA2}) is used.

Materials

A135-JH vector expressing ΦBT1 integrase and Cre recombinase

Type I carrier vector A167 carrying DNA3 (Type I DNA3)

Concentration of the vectors should be 0.1–0.5 μg/μl. All vectors are available under request from the Developmental Therapeutics Branch, National Cancer Institute (NIH).

CHO cells carrying the IIS-alphoid^{tetO}-HAC with DNA1 and DNA2 fragments inserted, from Basic Protocol 2.

CFM (cell culture freezing medium) (see Reagents and Solutions)

F12 growth medium (see Reagents and Solutions)

F12 round I selection medium (see Reagents and Solutions)

F12 round II selection medium (see Reagents and Solutions)

Ganciclovir solution 10 mg/ml (see Reagents and Solutions)

Opti-MEM (Thermo Fisher Scientific, cat. no. 51985034)

Viafect (Promega, cat. no. E4981)

PBS (Thermo Fisher Scientific cat. no. 10010–023)

0.25 % Trypsin (Thermo Fisher Scientific, cat. no. 25200056)

6-well culture plates (Thermo Fisher Scientific, cat. no. 140675)

96-well culture plates (Thermo Fisher Scientific, cat. no. 167008)

24-well culture plates (Thermo Fisher Scientific, cat. no. 142475)

Cloning cylinders (Fisher Scientific, cat. no. 09–552-20)

Cryovial (Thermo Fisher Scientific, cat. no. 5012–0012)

GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific, cat. no. SM1331)

Nuclease-free water (Quality Biological, cat. no. 351–029-721)

DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504)

TaKaRa Ex Taq® DNA Polymerase (Takara Bio, cat. no. RR001C)

Agarose (Sigma-Aldrich, cat. no. A9539)

1.7-ml microcentrifuge tubes (Thomas Scientific, cat. no. 1159M35)

15-ml centrifuge tubes (Corning Falcon, cat. no. 352196)

50-ml centrifuge tubes (Corning Falcon, cat. no. 352070)

10-ml disposable pipettes (Corning Falcon, cat. no. 356551)

10-cm culture dishes (Thermo Fisher Scientific cat. no. 174902)

Diagnostic primers to confirm the insertions of DNA fragments:

B072 5'-CCAGTTGCGTGCGTGGAA-3'

B071 5'-CGCACCGTGGGCTTGTA-3'

B074 5'-GCCGGACACGCTGAACTT-3'

B485 5'-GTGCAAGAAGATTATGAAGCAG-3'

R_{DNA3} designed by the user

F_{DNA3} designed by the user

R_{DNA2} designed by the user

Cell culture incubator

−80°C freezer

Liquid nitrogen tank

Standard Hemocytometer (Weber Scientific, cat. no. 3048–12)

PCR thermocycler

Nanodrop spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)

Refrigerated centrifuge

Refrigerated microcentrifuge

Inverted fluorescence microscope with filters of eGFP and mCherry (e.g., Zeiss AXIO)

Sub-Cell GT Horizontal Electrophoresis System, (BioRad, cat. no. 1704401)

Gel documentation system

Protocol Steps

1. Remove the cryovial containing the frozen CHO cells carrying the IIS-alphoid^{tetO}-HAC with DNA1 and DNA2 fragments from a liquid nitrogen storage and immediately place it into a 37°C water bath for 1 min to thaw.

2. Transfer the thawed cells into a 15-ml centrifuge tube. Add 5 ml of the pre-warmed F12 growth medium. Centrifuge at 1200 rpm for 3 min at room temperature.

3. Discard the supernatant. Resuspend the cell pellet in 10 ml of pre-warmed F12 round II selection medium. Transfer the cell suspension onto a 10-cm culture dish. Incubate the dish in a cell culture incubator at 37°C in 5% CO_2 atmosphere until the cells reach 50–80% confluency.

4. Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Plate 1 × 105 CHO cells in one well of a 6-well plate in 2 ml of F12 growth medium without antibiotics. Incubate the plate at 37°C in 5% CO2 atmosphere overnight so that the cells are about 70–80 % confluent at the time of transfection (i.e. the next day).

5. The next day, mix 1 μg of the Type I DNA3, 0.1 μg of A139, and 200 μl of Opti-MEM medium without serum in a sterile 1.7-ml tube. Mix gently by tapping.

6. Repeat Steps 6–17 of Basic Protocol 1.

7. Wash the cells once with 200 μl of PBS, add 100 μl of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times and transfer 80 μl of the suspension into a new separate 1.7-ml tubes. Add 0.5 ml of the F12 round I selection medium to each well to regrow the cells. Continue culturing the cells in the plate at 37°C in 5% CO_2 atmosphere.

   It is important not to cross-contaminate the culture. Use separate pipet tips for each individual colony.

8. While the cells are growing, check whether the fragment DNA3 from the Type I carrier vector was inserted into the IIS-alphoid^{tetO}-HAC. Spin the cell suspension in the 1.7 mL tube (from Step 7) down at 1200 rpm for 3 min at room temperature and isolate genomic DNA using the DNeasy Blood & Tissue Kit, per the manufacturer's instructions. Elute genomic DNA in 50 μl of water.

9. Use each genomic DNA sample to run four PCR reactions:

   i. with diagnostic primer pairs B071/B072;

   ii. with B074/B072, to confirm loxP recombination;

   iii. with F_{DNA3} and R_{DNA2}, to confirm attP$^{\Phi C31}$/attB$^{\Phi C31}$ recombination;

   iv. with forward B485 and R_{DNA3}, to confirm integrity of attB$^{\Phi BT1}$ site.

   Set up PCR reactions with TaKaRa Ex Taq DNA Polymerase, per the manufacturer's instructions, using 1 μl of the genomic DNA solution (from Step 7) as a template. Total volume of the reaction is 25 μl. Use the following PCR conditions after initial denaturation: 94°C 20 sec, 60°C 30 sec, 72°C 30 sec; 30 cycles.

10. Run the samples in a 1.5% agarose gel.

    PCR with B071/B072 should give a 447 bp product, while PCR with B074/B072 primers should be negative. PCRs with F_{DNA3}/R_{DNA2}, and with $B485/R_{DNA3}$ should give the products based on the user's design. It is advisable to sequence PCR products to ensure PCR accuracy.

11. Expand each colony with the PCR-confirmed DNA3 fragment insertion (from the plate in Step 7) on a 10-cm culture dish until the cells reach about 70–80 % confluency. Wash the cells once with 2 ml of PBS, add 1.5 ml of 0.25% Trypsin, and incubate for 5 min at 37°C. Resuspend the cells by pipetting up and down 5–7 times. Count the cells with a hemocytometer. Use 1×10^6 cells to start FISH (see Support Protocol 1).

    FISH analysis on a metaphase spread is needed to confirm that the IIS-alphoid^{tetO}-HAC carrying DNA1, DNA2, and DNA3 fragments remains autonomous and has not integrated into the host chromosomes.

12. Prepare frozen stocks as described in Basic Protocol 1, Step 23.

    After the 3rd round of integration, the IIS-alphoid^{tetO}-HAC will carry two fragments derived from the Type I carrier vector (DNA1 and DNA3) and a fragment from the Type II carrier vector (DNA2). The PCF marker will be expressed. Therefore, the cells will exhibit red fluorescence (mCherry), and will be Puromycin resistant (Puro) and 5-Fluorocytosine sensitive (FcyFur) (Figure 5).

Support protocol 1: FLUORESCENCE IN SITU HYBRIDIZATION (FISH) ANALYSIS FOR THE CIRCULAR IIS-alphoid^{tetO}-HAC

This protocol describes the steps to confirm the presence of the circular IIS-alphoid^{tetO}-HAC in an autonomous form in cells. The IIS-alphoid^{tetO}-HAC contains a unique tetO sequence, allowing detection via Fluorescence In Situ Hybridization (FISH). Hybridization with a fluorophore-labeled Peptide Nucleic Acid (PNA) probe (Alexa488-OO-ACCACTCCCTATCAG) on metaphase spreads is a robust method to visualize the HAC.

Materials

Cells from Basic Protocol 1, Step 22; from Basic Protocol 2, Step 17; from Basic Protocol 3, Step 11

F12 growth medium (see Reagents and Solutions)

0.25 % Trypsin (Thermo Fisher Scientific, cat. no. 25200056)

PBS (Thermo Fisher Scientific cat. no. 10010–023)

Nuclease-free water (Quality Biological, cat. no. 351–029-721)

DI water

KaryoMAX™ Colcemid™ Solution in PBS (Thermo Fisher Scientific, cat. no. 15212012)

KaryoMAX™ Potassium Chloride Solution (Thermo Fisher Scientific, cat. no. 10575090)

KCl (Sigma-Aldrich, cat. no. P9541)

Methanol (Sigma-Aldrich, cat. no. 322415)

Absolute Ethanol, 200 proof, (Thermo Fisher Scientific, cat. no. T038181000)

Acetic acid (Sigma-Aldrich, cat. no. 695092)

Microscope slides (Denville, cat. no. M1021)

Micro Cover Glass #1 22mm × 50mm (Electron Microscopy Sciences, cat. no. 72200–40)

Formaldehide fixation solution (see Reagents and Solutions)

Hybridization buffer (see Reagents and Solutions)

Wash solution (see Reagents and Solutions)

50 μM PNA probe (see Reagents and Solutions)

VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories, cat. no. H-1800)

Parafilm® M (Sigma-Aldrich, cat. no. P7793)

1.7-ml microcentrifuge tubes (Thomas Scientific, cat. no. 1159M35)

15-ml centrifuge tubes (Corning Falcon, cat. no. 352196)

10-ml disposable pipettes (Corning Falcon, cat. no. 356551)

10-cm culture dishes (Thermo Fisher Scientific, cat. no. 174902)

Slide warmer (Thermo Fisher Scientific, cat. no. 12–594)

Wheaton Coplin staining jars (Sigma-Aldrich, cat. no. S5516)

Slide holder (Jaece Industries, Inc., cat. no. L500-C)

Pipetman L P200L, 20–200 μL (Gilson, cat. no. FA10005M)

Cell culture incubator

−80°C freezer

Refrigerated centrifuge

Refrigerated microcentrifuge

Inverted fluorescence microscope with filters of DAPI and Alexa488 (e.g., Zeiss AXIO)

Protocol Steps

1. Incubate 1×10^6 HAC-containing CHO cells at 37°C in 5% CO_2 atmosphere in 10 ml of F12 growth medium in a 10-cm culture dish for 18–24 hrs.
2. Remove old medium from the culture dish and add 10 ml of F12 growth medium supplemented with 100 μl of KaryoMAX™ Colcemid. Incubate the cells at 37°C in 5% CO_2 atmosphere for 4 hrs.
3. Collect the medium from the culture dish to a 15-ml centrifuge tube.
4. Add 2 ml of PBS to the dish, swirl, and collect PBS to the same 15-ml centrifuge tube.
5. Add 1.5 ml of 0.25% Trypsin to the cells and incubate for 5 min at 37 °C. Collect the cell suspension to the same 15-ml centrifuge tube.
6. Spin down the cells in the 15-ml centrifuge tube at 1200 rpm for 3 min at room temperature.
7. Discard the supernatant. Resuspend the cells in 10 ml of PBS. Spin down at 1200 rpm for 3 min at room temperature.
8. Discard the supernatant. Gently resuspend the cells in 10 ml of KaryoMAX™ Potassium Chloride Solution pre-warmed to 37°C
9. Incubate the cells at 37°C for 20 min.
10. Prepare the desired amount of fresh fixative solution by mixing 3 volumes of Methanol with one volume of Acetic acid. Put it on ice.

    You will need 5 ml per sample.
11. Transfer the tube with the cell suspension into ice. Add 50 μl of the fixative solution. Mix by inverting the tube.
12. Centrifuge at 1500 rpm for 5 min in a pre-cooled (4°C) centrifuge.

    It is important to use centrifugation without break to avoid cell attrition, preventing loss of material.
13. Carefully pipette out as much supernatant as possible, taking care not to touch the cell pellet. Slowly add 1 ml of fixative solution to the tube. Do not mix. Incubate the cells on ice for 30 min.
14. Gently re-suspend the pellet by finger tapping, and centrifuge the suspension at 1500 rpm for 5 min at 4°C.
15. Carefully pipette out as much supernatant as possible, taking care not to touch the cell pellet. Gently re-suspend the pellet in 1 ml of fresh fixative solution. Centrifuge the suspension at 1500 rpm for 5 min at 4°C.

16. Repeat Step 15 two more times.

17. Finally, re-suspend the cells in 1 ml of fixative solution. Cells can be stored at −20°C in tightly closed tubes for up to 1 year.

    This is a safe stopping point.

18. Treat slides with 50 ml of 40% methanol in Coplin jar for 1 hr.

    Slides can be stored in 40% (v/v) methanol in Coplin jar sealed with parafilm at 4°C.

19. Replace methanol with 50 ml of 100% ethanol and incubate for 1 hr. Put the slides in a slide holder and let them air dry at room temperature.

20. Drop 20 µl of the fixed cells suspension from Step 17 onto microscope slides from about 10 cm using a Gilson Pipetman. Place the slide on a slide warmer (55°C) where they can be left for 1hr or longer.

21. Examine the slides under a phase contrast microscope to determine if the spreads are suitable for staining and counting. Metaphase chromosome spreads should be easy to find and well separated from each other.

    The slides can be stored at −20°C in a slide box for up to 1 month.

22. Put the slides into a Coplin jar with 50 ml of PBS for 15 min to rehydrate.

23. Replace PBS with 50 ml of Formaldehyde fixation solution for 2 min.

24. Wash the slides in a Coplin jar with 50 ml of PBS three times for 2 min.

25. Soak the slides in a Coplin jar with 50 ml of 70% (v/v) ethanol for 2 min.

26. Soak the slides in a Coplin jar with 50 ml of 85% (v/v) ethanol for 2 min.

27. Soak the slides in a Coplin jar with 50 ml of 100% ethanol for 2 min.

28. Put the slides in a slide holder and let them air dry at room temperature.

29. Pre-heat the slide warmer to 85°C.

30. For each slide, mix 0.2 µl of the PNA probe in 20 µl of hybridization buffer to a final concentration of 500 nM.

31. Pre-warm the slides on a slide warmer for 5 min.

32. Heat the hybridization buffer containing the PNA probe at 85°C for 5 min.

33. Add 20 µl of the hybridization buffer containing the PNA probe onto each slide. Cover with a coverslip.

34. Heat the slides in a slide warmer at 85°C for 10 min.

35. Place the slides in a box onto wet paper towels. Incubate in the dark at room temperature for 2 hrs.

36. Immerse the slides in Wash solution to remove coverslip.

37. Wash the slides in 50 ml of Wash solution in a Coplin jar twice at 60°C for 10 min.
38. Wash the slides with the Wash solution at room temperature for 10 min.
39. Wash with 50 ml of PBS in a Coplin jar three times for 5 min.
40. Soak in a Coplin jar with 70% (v/v) ethanol for 5 min.
41. Soak in a Coplin jar with 85% (v/v) ethanol for 5 min.
42. Soak in a Coplin jar with 100% ethanol for 5 min.
43. Dry the slides at room temperature.
44. Add 25 μl of VECTASHIELD mounting media with DAPI to the slides. Cover with a coverslip. Place the slides in the dark at room temperature overnight to let the mounting media solidify.

    Mounted slides can be stored in dark at −20°C for up to a month.
45. Observe the slides under the fluorescence microscope with filters for DAPI and Alexa488.

    The intact HAC should be visible as a single separate dot in both channels and should be separated from the host chromosomes (Figure 1b, white arrow).

REAGENTS AND SOLUTIONS

F12 growth medium

- 500 ml of F12 medium (Thermo Fisher Scientific, cat. no. 31765035)
- 50 ml of Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, cat. no. 26140)
- 5 ml of PenStrep (Thermo Fisher Scientific, cat. no. 15070063)
- Store at 4°C for up to one month

F12 round I selection medium

- 100 ml of F12 growth medium
- 40μl of Puromycin Dihydrochloride (10 mg/ml) (Thermo Fisher Scientific, cat. no. A1113803)
- 40μl of 10 mg/ml Blasticidin S HCl (10 mg/mL) (Thermo Fisher Scientific, cat. no. A1113903)
- Store at 4°C for up to one month

F12 round II selection medium

- 100 ml of F12 growth medium
- 200 μl of Hygromycin B (50 mg/mL) (Thermo Fisher Scientific, cat. no. 10687010)

- 40 µl of 10 mg/ml Blasticidin S HCl (10 mg/mL) (Thermo Fisher Scientific, cat. no. A1113903)
- Store at 4°C for up to one month

Ganciclovir solution 10 mg/ml

- 1 g of Ganciclovir (Thermo Fisher Scientific, cat. no. 461710010)
- 90 ml of cell culture grade water (Thermo Fisher Scientific, cat. no. MT25055CV)
- 10 ml of HCl 1N (Sigma-Aldrich, cat. no. H9892)
- Store at −20°C in 1 ml aliquots for up to one year
- Thawed aliquot can be stored at 4°C for up to one week

CFM (2x) medium

- 60 ml of F12 medium (Thermo Fisher Scientific, cat. no. 31765035)
- 20 ml of FBS (Thermo Fisher Scientific, cat. no. 26140)
- 20 ml of DMSO (Sigma, cat. no. D2650–100ML)
- Store at 4°C for up to one year

PNA probe

- Custom PNA probe corresponding to a tetO sequence (PNA BIO Inc). It comes as lyophilized powder. Probe sequence is Alexa488-OO-ACCACTCCCTATCAG.
- Resuspend a 5 nmol lyophilized PNA powder stock in 100 µl of Formamide DI (American Bioanalytical, cat. no. AB00600) to make a 50 µM stock.
- Store in 10 µl aliquots at −70°C, protected from light, for up to one year.
- After thawing, heat at 55°C for 5 min to ensure complete dissolution.

Formaldehyde fixation solution

- 10 ml of Formaldehyde solution for molecular biology, 36.5–38% in H_2O (Sigma-Aldrich, cat. no. F8775)
- 90 ml of PBS (Thermo Fisher Scientific cat. no. 10010–023)
- Store at room temperature for up to one month

Hybridization buffer

- 200 µl of 1M Tris, pH 7.4 (KD Medical, cat. no. RGF-3340),
- 3.8 ml of nuclease-free water (Quality Biological, cat. no. 351–029-721)
- 6 ml of Formamide DI (American Bioanalytical, cat. no. AB00600),

- 50 mg of Blocking reagent (Roche, cat. no. 11096176001)
- Store at 4°C for up to one year.

Wash solution

- 10 ml of 20 x SSC (Quality Biological, cat. no.351–003-131)
- 90 ml of DI water
- 100 μl of Tween-20 (Bio-Rad, cat. no. 170–6531)
- Store at room temperature for up to one month

COMMENTARY

Background Information

There are several methods to produce transgenic cells for gene functional studies. One of the methods relies on transfection of BAC DNA carrying a gene(s) of interest into host cells (Illenye et al., 2004; Tsuji et al., 2006; Hibbitt et al., 2007; Head et al., 2007; Hall et al., 2008; Poser et al., 2008; Arii et al., 2009; Auriche et al., 2010). Another popular approach is based on transduction with viruses or virus-based delivery vectors carrying small-sized genes (not bigger than 5 kb) or cDNA (Mijanović et al., 2020; Ma et al., 2021; Gimpel et al., 2021; Immidisetti et al., 2021; Wang et al., 2021; Huang et al., 2021; Zhang et al., 2021). Both methods, however, may lead to random integrations of BACs or viruses into host chromosomes. As a result, expression of the cloned genes may be subject to position effects and the number of copies integrated. Even nonintegrated adeno-associated recombinant viruses (rAAVs), which currently are the most attractive viral vectors, along with retro- and lentiviral vectors, have several serious disadvantages for use in gene therapy and even in gene functional studies, such as low cloning capacity and lack of long-term transgene expression. Another method is based on integration of a gene into a “hot spot” of a mammalian genome using a bacteriophage P1-derived Cre recombinase or ΦC31 integrase (Luo et al., 2013). However, efficiency of gene integration with such system is very low. In addition, all of these approaches are typically applicable to a single gene or DNA fragment.

In this article, we describe a protocol for the IIS-alphoid^{tetO}-HAC system, which addresses most of the problems associated with virus-based gene delivery vectors and BACs because the HAC-based vectors, including the alphoid^{tetO}-HAC, replicate and segregate as natural chromosomes, independently from the host genome, and have the ability to carry Mb-size gene/fragment inserts. The IIS-alphoid^{tetO}-HAC system includes the iterative integration system (IIS), which potentially allows adding an unlimited number of genomic DNA fragments. This feature makes the IIS-alphoid^{tetO}-HAC a more versatile vector, an aim that was shared by several groups in the field that also developed their own multi-integrase systems in combination with artificial chromosome-based vectors (Yamaguchi et al., 2011; Suzuki et al., 2014; Toth et al., 2014; Yoshimura et al., 2015; Honma et al., 2018). The IIS-alphoid^{tetO}-HAC system has several notable advantages compared to some of these other multi-integrase systems. Firstly, any desired number of genomic DNA fragments can be inserted into the IIS-alphoid^{tetO}-HAC. There is no inherent upper limit to the system, unlike

other structurally simpler multi-integration systems. Secondly, each step of gene/fragment insertion is accompanied by a visible change in cell fluorescence, which simplifies the screening of correct clones. Thirdly, the IIS-alphoid^{tetO}-HAC system can assemble large genes from intron-exon-intron segments, because it only integrates the DNA segments of interest without extraneous plasmid DNA and leaves only a small 35–55 bp scar site between adjacent DNA segments (e.g. between the first and second DNA segments of interest). Fourthly, to ensure integrity of assembly, the IIS-alphoid^{tetO}-HAC system has an error-proofing mechanism that selects against colonies in which the recombination reaction did not proceed to completion (see below for details and Figure 8). Finally, the alphoid^{tetO}-HAC may be removed from the cells, offering a unique possibility to compare the phenotypes of human cells with and without functional copies of the genes under study.

At its core, the IIS is a marker capture-switching system. Each round of integration causes the marker in the promoter-marker pair to be exchanged (see Figure 3), and this change in the marker allows for the screening and selection of successful integration events. While this feature added complexity, the re-use of markers means an unlimited number of integrations of DNA fragments could be accomplished by using only two markers, and the number of recombinase proteins in the system is limited to only three, i.e., Cre, ΦC31, and ΦBT1. In the IIS-alphoid^{tetO}-HAC system, each step of gene/DNA fragment loading uses the recombinase Cre and ΦC31 or Cre and ΦBT1, and is accompanied by an exchange between the GHT (eGFP-hph-TK) or PCF (PAC-mCherry-FcyFur) marker. These markers are compound markers that consist of three parts, a positive selection marker (hph) to select for cells when a new DNA fragment is added to the HAC, a counter-selection marker (TK or FcyFur) to exclude the cells when this event does not happen, and a fluorescence marker (eGFP or mCherry) to monitor the recombination reaction (exchange between the GHT and PCF markers). The three components of the compound markers are transcribed as a single mRNA molecule but produced as separate proteins due to the presence of the 2A self-cleaving peptide between each component..

There are several reasons to use this marker-capture-switching system vs other multi-integrase systems combined with HAC-based vectors (Yamaguchi et al., 2011; Suzuki et al., 2014; Toth et al., 2014; Yoshimura et al., 2015; Honma et al., 2018). Firstly, there are a limited number of commercially available selection agents for use in mammalian cell cultures (blasticidin, hygromycin, puromycin etc.). Multi-integration systems that use a new marker for each DNA insertion would rapidly run out of useable markers and be unable to go beyond four or five insertions. Such systems also leave no marker for the user to use in other projects aside from gene integration within that cell line. Our IIS system is a cyclic system, where only two markers, GHT and PCF, substitute each other as a new genomic DNA segment is added to the integration sites. Secondly, at the time when the IIS was built, the number of recombinase proteins that were optimized for expression and efficient activity in vertebrate cells was extremely limited. Only Cre was widely used; ΦC31 had just begun to be used in mammalian cells, and ΦBT1 had yet to see much use or optimization in yeast, and much less in vertebrate cells. These integrases are bacteriophage proteins that in their native state become less effective at temperatures higher than room temperature, in which vertebrate cells are typically cultured at.

A disadvantage of our marker-capture-switching system, on the other hand, is complexity. Compared to other multi-integration systems (Yamaguchi et al., 2011; Suzuki et al., 2014; Toth et al., 2014; Yoshimura et al., 2015; Honma et al., 2018), the IIS uses two recombinase proteins per integration cycle, and have many more moving parts. However, we hope that as the IIS has a limited number of recombinases in use, optimized conditions could be found and, with time, mutant recombinases with improved tolerance to elevated temperatures and lower rates of protein aggregation would eventually be developed.

The fluorescence color component was added to the compound marker to simplify the screening of correct clones that have successfully undergone the integration reaction. This is necessary, as screening of mammalian colonies is far more time-consuming and labor-intensive than in yeast or bacteria. In addition, the counterselectable markers were added to the compound markers to form an error-proofing mechanism in the IIS-alphoid^{tetO}-HAC system. Recombinase-mediated reactions can fail to go to completion, and growth, maintenance, screening, and storage of candidate vertebrate colonies is far slower, more labor-intensive, and space-limited than with either yeast or bacteria. It was thus desirable to remove as many faulty colonies as possible by drug selection and visual inspection, keeping only the best candidates for subsequent detailed characterization. If either of the two recombination reactions fails, this failure event can be selected against and screened out by the error-proofing design of the IIS-alphoid^{tetO}-HAC system (Figure 8). As illustrated, the backbone of each carrier vector has its own constitutively active compound marker. Hence, if recombination by Cre fails but ΦC31 occurs (Figure 8a), the Type I carrier A167 vector will integrate into the platform cassette but the PCF marker it carries will remain promoterless. Cells carrying this error are removed by selection with Puromycin and counter-selection with Ganciclovir. Alternatively, if recombination by ΦC31 fails but Cre occurs (Figure 8b), the Type I carrier vector A167 will integrate into the construction platform, and the SFM promoter will capture the PCF marker, leaving the original GHT marker promoterless. However, the backbone of the Type I carrier vector A167 is retained, and a fully expressed GHT marker under the CAGG promoter remains. Hence, cells generated by such a failure event are Puromycin resistant and have both red and green fluorescence. These cells can be removed either by counter selection using Ganciclovir against the thymidine kinase component (TK) of the GHT marker or by visual inspection when the colonies are formed. To counteract the loss of TK gene activity by heterochromatin silencing, the GHT marker is protected by flanking murine tDNA insulators (Ebersole et al., 2011; Lee et al., 2013). Similarly, the products of incomplete recombination between Type II carrier plasmid A169 with the active PCF cassette may be identified and removed. Selection with Hygromycin and counter selection with 5-Fluorocytosine ensures that only cells that have correctly undergone the second round of assembly will survive (Figures 8c, 8d). Untransformed parental cells and cells with incomplete recombination are killed by this double selection.

The IIS-alphoid^{tetO}-HAC system is expected to be beneficial in various fields of study, such as multiple-gene humanized models, polygenic disease models, reprogramming, and gene expression systems. In addition, the IIS-alphoid^{tetO}-HAC system opens new opportunities for synthetic biology in multicellular eukaryotes. It provides a unique tool to engineer synthetic chromosomes carrying large segments of genomic DNA, such as

segments containing long-range genetic elements required for appropriate regulation of gene expression, or multiple copies of genes (Moriwaki et al., 2020).

Critical Parameters

The IIS-alphoid^{tetO}-HAC system should be always checked before proceeding to the next step—Due to the large time investment associated to vertebrate cell culture, the user should proceed cautiously. All recombination recognition sites (loxP, attB$^{\Phi C31}$, attB$^{\Phi BT1}$) should be sequence-verified after each round of recombination, as in the presence of recombinase proteins, these sites experience higher than normal rates of DNA sequence changes.

Maintenance of the IIS-alphoid^{tetO}-HAC and its copy number—While the IIS-alphoid^{tetO}-HAC is stable within a cell line over the short term, it is advised that continuous Blasticidin S selection be used to ensure that the majority of cells in the culture retain the HAC over the long term. Furthermore, when restarting a cell culture from colonies, we advise that a FISH analysis be done to ascertain that only a single copy of the IIS-alphoid^{tetO}-HAC is present within the cell. While rare, we have, on occasion, recovered colonies that have spontaneously increased the HAC copy number. These colonies should be discarded as the IIS-alphoid^{tetO}-HAC system is designed to function as a single copy per cell.

Selection and counterselection agent concentration—Our iterative integration system includes two counterselectable markers (Thymidine Kinase and FcyFur) that can be selected against and removed by Ganciclovir and 5-Fluorocytosine, respectively. However, in vertebrate cells, the counterselectable marker's expression may also be lost via heterochromatin silencing, allowing the cell to survive without physically losing the counterselectable marker gene. It is recommended that the user determines an upper tolerance limit of the cell line of interest to these counterselectable agents. Ideally, the maximum tolerated concentration of counterselectable agents should be used in the experiments. However, if the cost of the counterselectable agents is an issue, the user has to determine the minimum concentration of the counterselectable agents needed to kill the cells containing the IIS-alphoid^{tetO}-HAC on a confluent 10-cm petri dish before spontaneous silencing of the counterselectable markers occurs, which would lead to a significant background of resistant colonies.

Note that the issue described above should be taken into account when the IIS system is carried out in the cell line of interest different from hamster CHO cells. Determination of the minimum concentration of the counterselectable agents in the cell line of interest is a complicated multistep process. First, the IIS-alphoid^{tetO}-HAC should be transferred from the donor hamster CHO cells to the recipient cell line of interest using MMCT (Liskovykh, Larionov, & Kouprina, 2021). In this cell line, the HAC will be maintained using Blasticidin S, as the HAC contains the *bsr* gene in its backbone (Nakano et al., 2008). The HAC also carries the empty integration platform cassette that contains the GHT marker (eGFP-hph-TK). Once the HAC transfer is completed, the user should use this cell line to determine the minimum concentration of Ganciclovir needed to kill all the cells

on a 10-cm confluent petri dish before the spontaneous emergence of resistant colonies. It is highly recommended that the experiment be extended to at least 2 to 3 weeks to determine how frequent spontaneous resistance colonies emerge at specific concentrations of Ganciclovir used, and when. These data are very important to determine the background of false positives the user may experience. It is also recommended that the cells be detached from the plate, and allowed to reattach, 3 days after selection, to help remove dead cells.

The next step is to test the PCF compound marker, which is a fusion of Puro-mCherry-FcyFur, and to determine the minimum concentration of 5-Fluorocytosine needed to kill the cells expressing this marker. For this purpose, the user should obtain cells with the HAC carrying a single copy of the PCF marker. The most time-efficient manner to obtain such cell line is to use the IIS system in the HAC and integrate an empty Type I carrier vector A167 into the platform cassette. This will replace the GHT marker by the PCF marker and also will help determine the concentration of the ΦC31-Cre expressing vector (A139) needed to trigger the recombination event. The user should determine the minimum concentration of 5-Fluorocytosine on a confluent 10-cm petri dish before the spontaneous emergence of resistant colonies. The experiment should be extended to at least 2 to 3 weeks to determine if there is any late emergence of spontaneous resistant colonies. Removal of dead cells can be aided by detaching the cells from the plate and allowing to reattach, 3 days after selection started.

Checking the IIS-alphoid^{tetO}-HAC system (optional)—Once the concentration of the Cre-integrase vectors have been determined, we recommend that users familiarize themselves with the IIS and attempt a trial run of the system in the cell line of interest or in hamster CHO cells. Due to IIS complexity, the user should integrate an empty Type I carrier vector A167 into the platform cassette of the HAC with the ΦC31-Cre expressing vector (A139), and then integrate the Type II carrier vector A169 into the platform cassette with the ΦBT1-Cre vector (A135-JH). It is easier to integrate empty carrier vectors, as their smaller sizes makes them less difficult to purify in larger quantities and more molecules can be transformed per unit mass DNA transfected.

Quantity of Cre-integrase vectors—The site-specific integrases ΦC31 and ΦBT1 function as tetramers and when overexpressed, they tend to form nonfunctional protein aggregates. Hence, overexpression of integrase proteins can result in reduced recombination activity. As a promoter's activity changes between cell lines, it is recommended that the user tests different concentrations of Cre-integrase vectors with a set amount of the empty Type I and Type II carrier vectors (A167 and A169) to determine the optimum concentrations that will yield the highest amount of recombination activity, being mindful that "less can be more".

Transfection with the carrier vectors A167 and A169 carrying large genomic DNA fragments—If the transfecting carrier vectors with the inserted genomic DNA fragments are 10–20 times larger than the empty vectors, we recommend the user to re-optimize the DNA-transfection agent ratio. A BAC carrying the *eGFP* gene of similar size to the loaded carrier vectors would be a good test subject to find the optimal DNA-transfection agent ratio.

Endotoxin-free DNA—The negative impact of endotoxins varies by cell line. We recommend the ΦC31-Cre (A139) and ΦBT1-Cre (A135-JH) vectors as well as Type I and Type II carrier vectors (A167 and A169) carrying the genomic DNA fragments to be purified using commercially available endotoxin-free kits. However, it has been observed that the buffers and solutions of some endotoxin-free kits are not sterile. We thus highly recommend the user to filter-sterilize all solutions before use, to avoid downstream fungal contamination of the cell culture. In addition, the use of *E. coli* strains with reduced endotoxins, such as Endotoxin-free ClearColi™ BL21(DE3) Electrocompetent Cells (BIOSEARCH Technologies, cat. no. 60810–1), could be helpful.

High DNA purity and quality—Before transfection, Type I and Type II carrier vectors (A167 and A169) carrying genomic DNA fragments larger than 30 kb (BAC DNA) should be checked by contour-clamped homogeneous electric field gel electrophoresis (CHEF). This step is necessary to confirm that the BAC DNA is intact and has been isolated predominantly as covalently closed circular molecules (ccc DNA). All measures should be taken to ensure that only circular BAC DNAs are transfected into the cells. This includes preserving DNA integrity during preparation using cold solutions and removal of nicked and linearized DNA molecules with exonucleases. In addition, linear molecules have a higher rate of random integration into the host genome compared to covalently closed circular supercoil DNA, which would reduce the efficiency of the IIS system and lead to a higher background.

Furthermore, the toxicity of transfection agents limits the quantity of DNA that can be transfected into a cell. Thus, BAC DNA preparation has to be pure, without any contaminating bacterial genomic DNA that will reduce the actual amount of BAC DNA that can be transfected into the cells and integrated into the IIS-alphoid^{tetO}-HAC. This limitation becomes a significant factor when we transfect large BAC DNA molecules, as each microgram of BAC DNA has fewer BAC molecules. Removal of bacterial genomic DNA can be accomplished by exonuclease treatment.

Troubleshooting

See Table 1 for a list of common problems with the protocols, their causes, and potential solutions.

Understanding Results

Usually, the average number of colonies with correct fluorescence (+mCherry/-eGFP or +eGFP/-mCherry) after co-transfection of 1 μg of Type I carrier vector A167 and 0.1 μg of A139 vector or 1 μg Type II carrier vector A169 and 0.1 μg of A135-JH vector into ~1 × 10^5 CHO cells carrying the IIS-alphoid^{tetO}-HAC, is equal to 20–40. After PCR analysis with the corresponding diagnostic primers, the number of colonies with the correct insertion is ~10–20. Typically, the yield of colonies for the Basic Protocols depends on the quality of BAC DNA and the length of the desired DNA fragments to be inserted. Usually, the average number of colonies with correct fluorescence after co-transfection of 1 μg of BAC DNA of 30–50 kb in size (A167 or A169 vectors carrying genomic DNA fragments of interest) and 0.1 μg of A139 or A135-JH vectors and ~1 × 10^5 CHO cells is 10–15. The

user should expect fewer colonies if transfection is performed using a BAC larger than 50 kb. After negative selection and PCR with the corresponding diagnostic primers, the number of colonies with the correct insertion will typically be about 2–5. Usually, FISH analysis reveals that 80%−90% of the colonies contain the intact HAC that stably propagates in the cells and is maintained as one copy per cell. About 10%−20% of the colonies may possess HAC aberrations such as integration of the HAC into the host chromosomes or HAC instability or multiplication.

Time Considerations

The full procedure depends on the number of genomic DNA fragments of interest to be inserted into the IIS-alphoid^{tetO}-HAC. Each round of DNA insertion described in the Basic Protocols can be completed within 4 weeks. The user should start with exponentially growing hamster CHO cells. For each round of insertion of the genomic DNA fragments of interest into the IIS-alphoid^{tetO}-HAC, the transfection itself takes 1 day to perform. The rest of the time is required for selection and growth of the colonies. The speed of colony formation and growth depends on the characteristics of the cells used, either hamster CHO cells or another recipient cells. Generally, rapidly proliferating cells form colonies faster. The user should be aware that the selective agents (Puromycin, Ganciclovir or Hygromycin B and 5-Fluorocytosine) affect cell growth, and colony formation may be significantly slower under these conditions. The user should also consider that the preparation steps for the protocols may take 2–3 weeks. These steps include either TAR cloning of the genomic DNA fragments of interest or ligation of them into Type I and Type II carrier vectors A167 and A169 (see STRATEGIC PLANNING).

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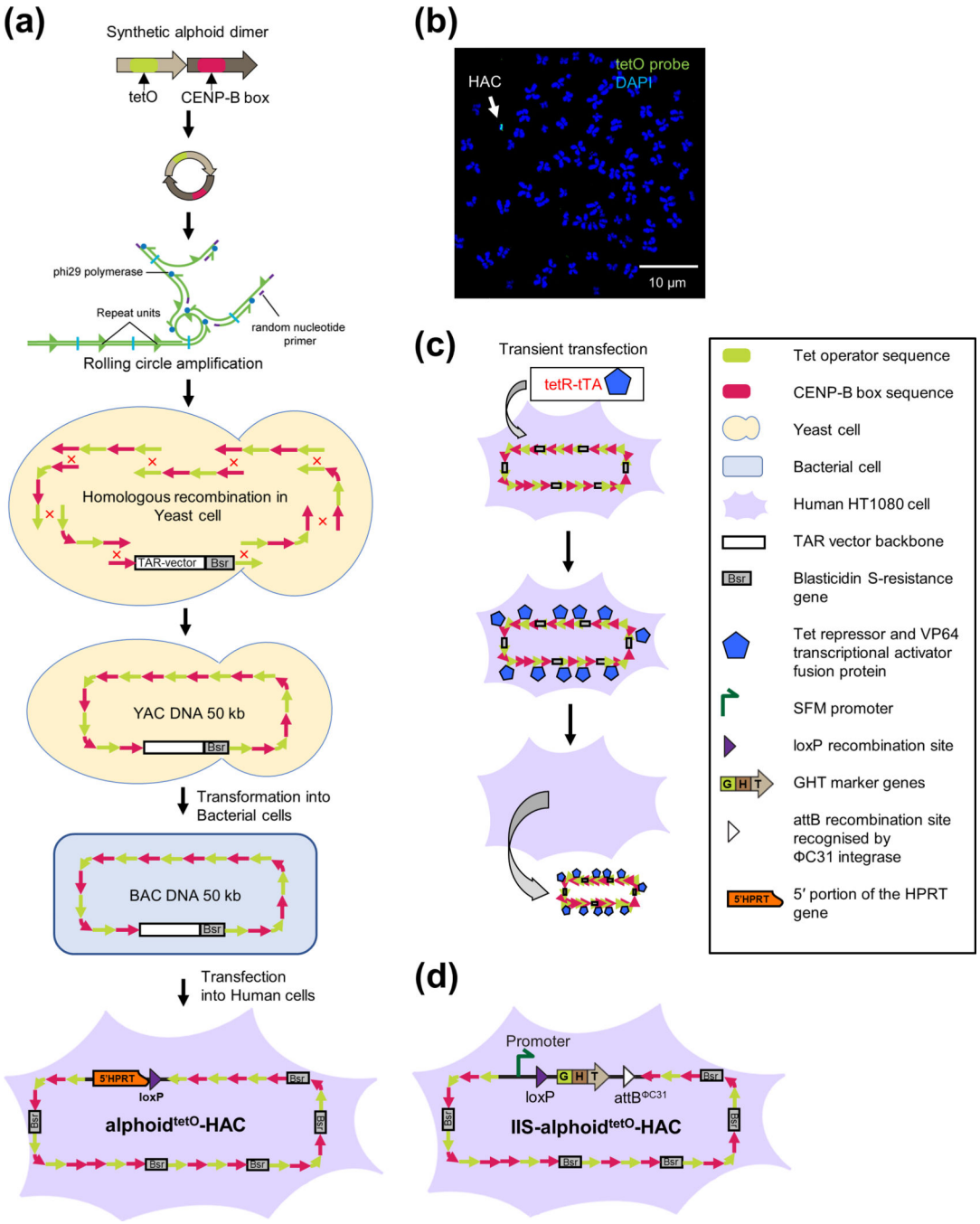

Figure 1.

Generation of the alphoid^{tetO}-HAC using a synthetic alphoid DNA array. **(a)** A 343 bp synthetic alphoid dimer consists of two monomers. One monomer is derived from a chromosome 17 alphoid type I 16-mer unit and contains a CENP-B box, a nucleotide motif involved in centromere formation. The second monomer is a wholly synthetic sequence derived from alphoid DNA consensus, with the sequence corresponding to the CENP-B box replaced by a 42 bp tetO motif. A dimer is amplified up to ~3–5 kb in size by rolling circle amplification (RCA) *in vitro* using phi29 DNA polymerase. Then,

the RCA-amplified fragments are assembled by transformation-associated recombination (TAR) cloning in yeast, leading to formation of a ~50 kb synthetic array cloned into a YAC/BAC vector. A hybrid circular YAC/BAC vector contains a blasticidin resistance marker (a *bsr* gene). The YAC/BAC molecules are then moved from yeast to bacterial cells for further BAC DNA isolation. After transfection of 50 kb input BAC DNA into human HT1080 cells, the alphoid^{tetO}-HAC is formed. Formation of the alphoid^{tetO}-HAC is accompanied by multimerization of input 50 kb DNA up to 1.1 Mb. **(b)** FISH analysis of the alphoid^{tetO}-HAC in human HT1080 cells. FISH analysis was performed using a fluorescein PNA (peptide nucleic acid)-labeled probe for the tetO sequence (see Support Protocol 1). A white arrow indicates the HAC (green), while the endogenous chromosomes are labeled blue (DAPI). **(c)** Loss of the alphoid^{tetO}-HAC from recipient cells may be induced by the transcriptional activator (tTA) fused with the tet-repressor (tetR) targeting the tetO-HAC kinetochore (Kim et al., 2011; Kononenko et al., 2014). **(d)** The IIS-alphoid^{tetO}-HAC was developed after insertion of the integration platform cassette into the alphoid^{tetO}-HAC. The integration platform cassette consists of the SFM promoter driving the expression of the GHT marker, a loxP site present between the promoter and the marker, and the attB$^{\Phi C31}$ site for the ΦC31 integrase. The GHT marker is a fusion of green fluorescent protein (eGFP), Hygromycin-B-phosphotransferase (hph), and thymidine kinase (TK).

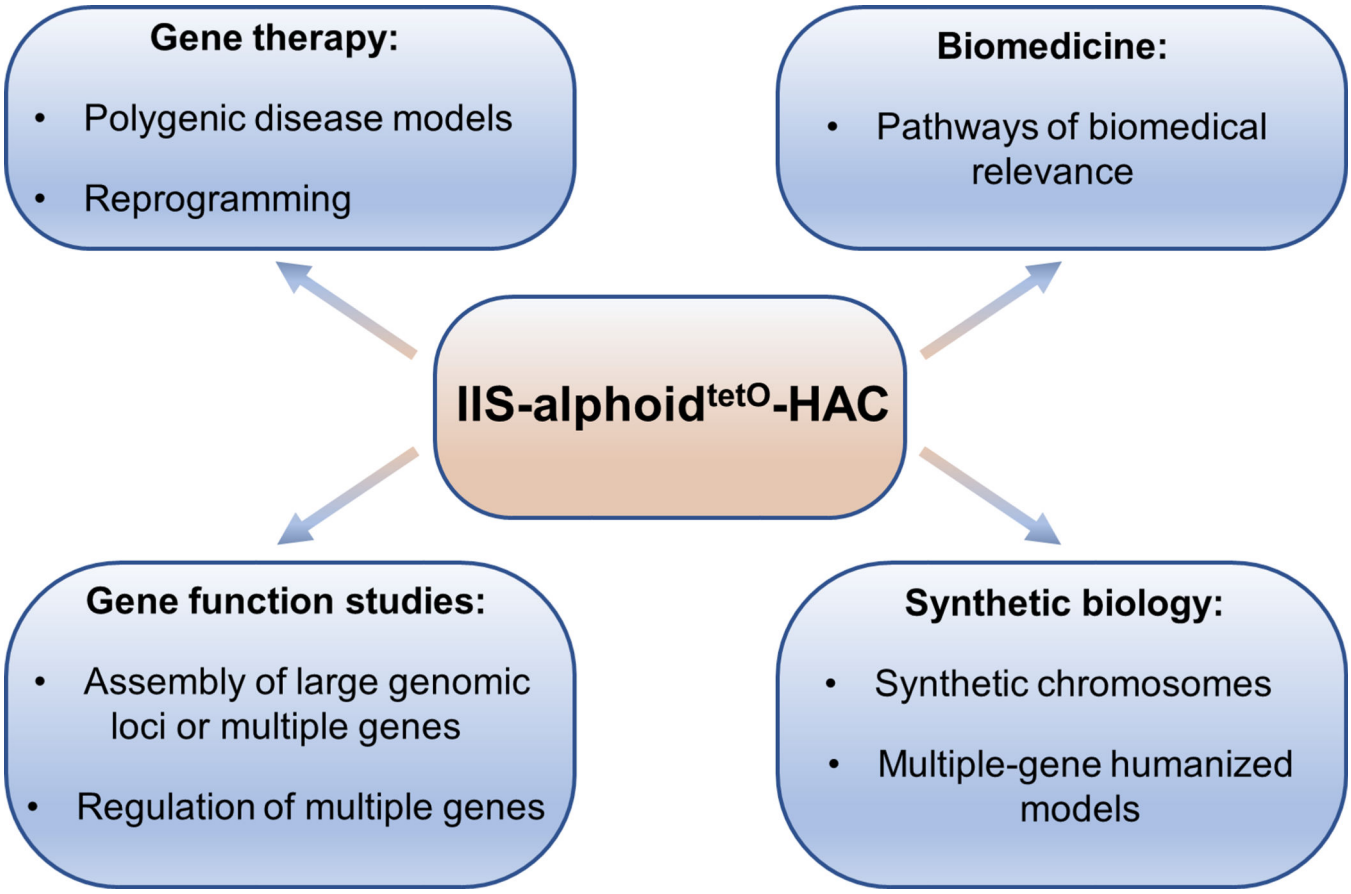

Figure 2.
Multiple applications of the IIS-alphoid^{tetO}-HAC system.

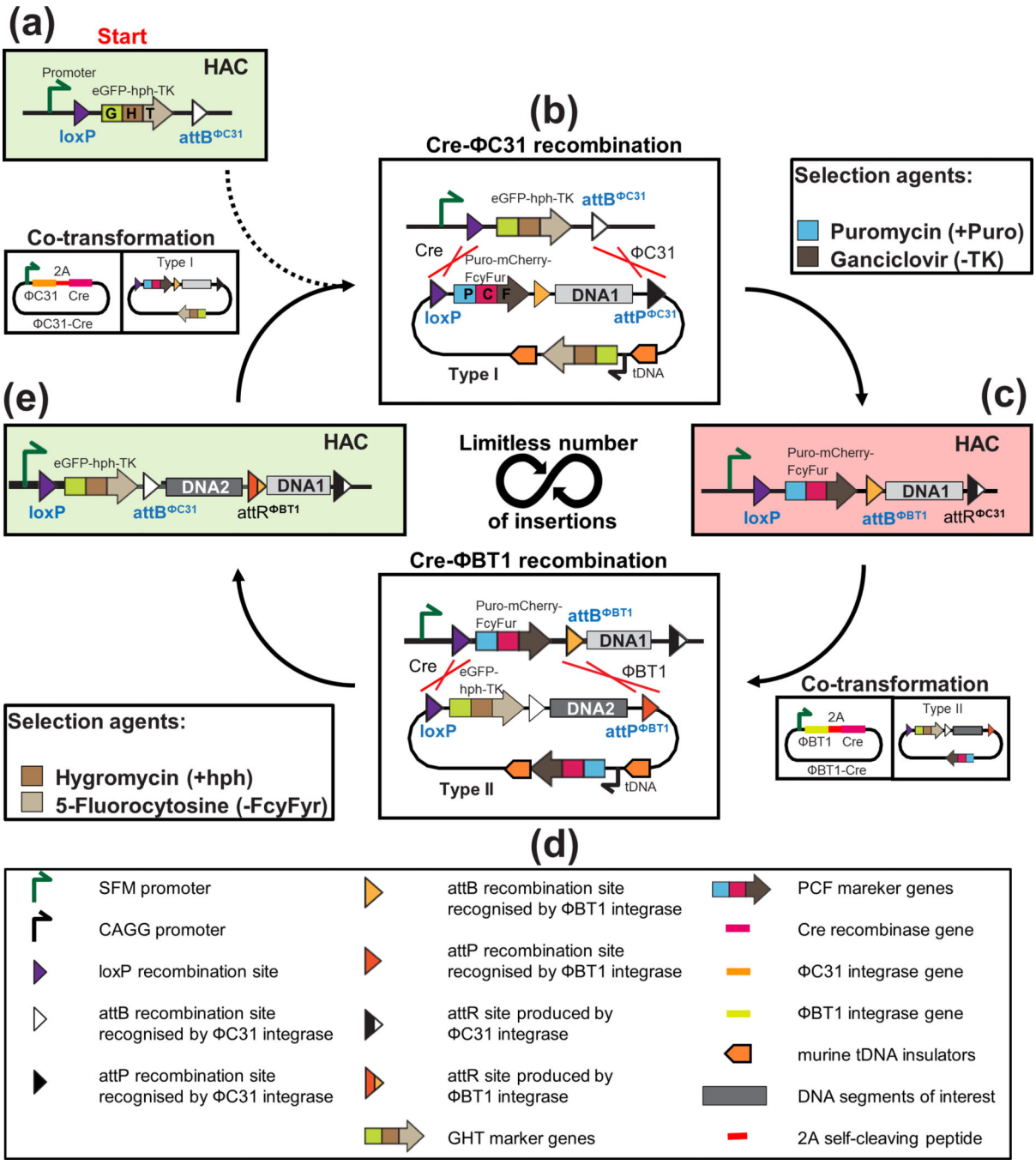

Figure 3.

Schematic of the perpetual serial integration of DNA segments into the IIS-alphoid^{tetO}-HAC by the iterative integration system (IIS). **(a)** The starting integration platform cassette on the HAC. In the empty platform cassette, the SFM promoter drives the expression of the GHT (eGFP-hph-TK) marker. The cells express green fluorescence protein (eGFP) (the cells look green), and are Hygromycin resistant (hph) and Ganciclovir sensitive (TK). **(b)** Type I carrier vector bearing the first DNA segment of interest (DNA1) is integrated into the platform cassette of the HAC by Cre recombinase and ΦC31 integrase, which are

themselves expressed from the plasmid A139. Recombination between a Type I carrier vector and a platform cassette by Cre recombinase and ΦC31 integrase leads to replacement of the GHT marker by the PCF (Puro-mCherry-FcyFur) marker and integration of the DNA of interest (DNA1) into the platform cassette. Cells with correct integration are selected for using Puromycin and Ganciclovir. **(c)** Structure of the platform cassette after the 1st round of DNA integration. The PCF marker is expressed. Therefore, the cells express red fluorescence (mCherry; the cells now look red), and are Puromycin resistant (Puro) and 5-Fluorocytosine sensitive (FcyFur). **(d)** Recombination between a Type II carrier vector bearing the second DNA segment of interest (DNA2) and a platform cassette by Cre recombinase and ΦBT1 integrase, which are expressed from the plasmid A135-JH, leads to replacement of the PCF marker by the GHT marker and DNA2 integration into the platform cassette. The integration event is selected for using Hygromycin and 5-Fluorocytosine. (**e**) Structure of the platform cassette after the 2nd round of DNA integration. The cells express the GHT marker and, thus, the green florescence protein eGFP (the cells look green). They once again become Hygromycin resistant (hph) and Ganciclovir sensitive (TK). This structure is identical to the starting cassette aside from the integration of DNA segments of interest, DNA1 and DNA2.

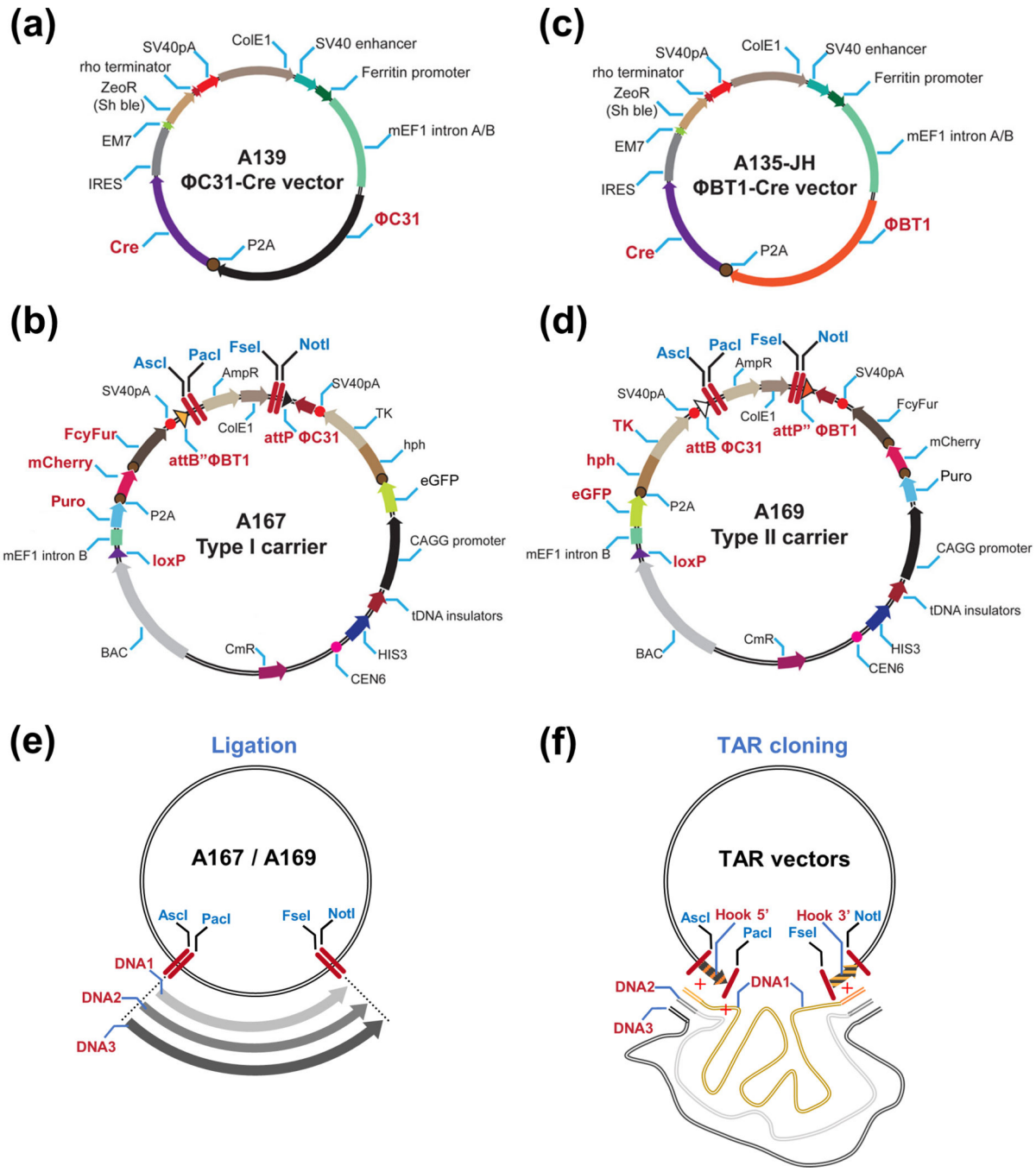

Figure 4.

Scheme of the vectors used in the IIS-alphoid^{tetO}-HAC system. (**a**) A139 vector expressing ΦC31 integrase and Cre recombinase and (**b**) Type I carrier vector A167 to deliver the 1st genomic DNA fragment (DNA1) and to perform every odd-numbered round of DNA integration. A167 vector has a promoterless PCF marker (Puro-mCherry-FcyFur) and a constitutively active GHT marker (eGFP-hph-TK) under the CAGG promoter in its vector backbone. (**c**) A135-JH vector expressing ΦBT1 integrase and Cre recombinase and (**d**) Type II carrier vector A169 to deliver the 2nd genomic DNA fragment (DNA2) and to

perform every even-numbered round of DNA integration. A169 vector has a promoterless GHT marker and a constitutively active PCF marker under the CAGG promoter in its vector backbone. A139 and A135-JH expression vectors carry Zeomycin resistance (Zeo^R). Both Type I and Type II carrier vectors are used to integrate DNA segments of interest into the platform cassette of the IIS-alphoid^{tetO}-HAC. These vectors contain both a BAC cassette containing F1 origin of replication (low-copy maintenance) and a pBR322 origin of replication (ColE1) to make the vectors multicopy. ColE1 origin is removed once a large DNA segment of interest (DNA1, DNA2 or DNAn) is added to the vector. **(e)** DNA fragments are inserted into Type I and Type II carrier vectors A167 and A169 via ligation into unique 8 bp restriction sites, i.e., AscI/PacI/FseI/NotI (marked in blue). **(f)** DNA fragments are inserted into Type I and Type II carrier vectors A167 and A169 by TAR cloning in yeast *S. cerevisiae* (Kouprina, Kim, & Larionov, 2021). In this case, AscI/PacI and FseI/NotI sites are used to insert the hook sequences homologous to the 5' and 3' ends of DNA segments of interest. For TAR isolation of DNA segments of interest from total genomic DNA, A167 and A169 vectors contain *CEN6* (a yeast centromere sequence) and *HIS3* (a yeast selectable marker) for proper propagation and selection of the TAR-cloned material in yeast. A BAC cassette allows to direct transfer of the TAR-cloned DNA material from yeast to bacterial cells for further BAC DNA isolation.

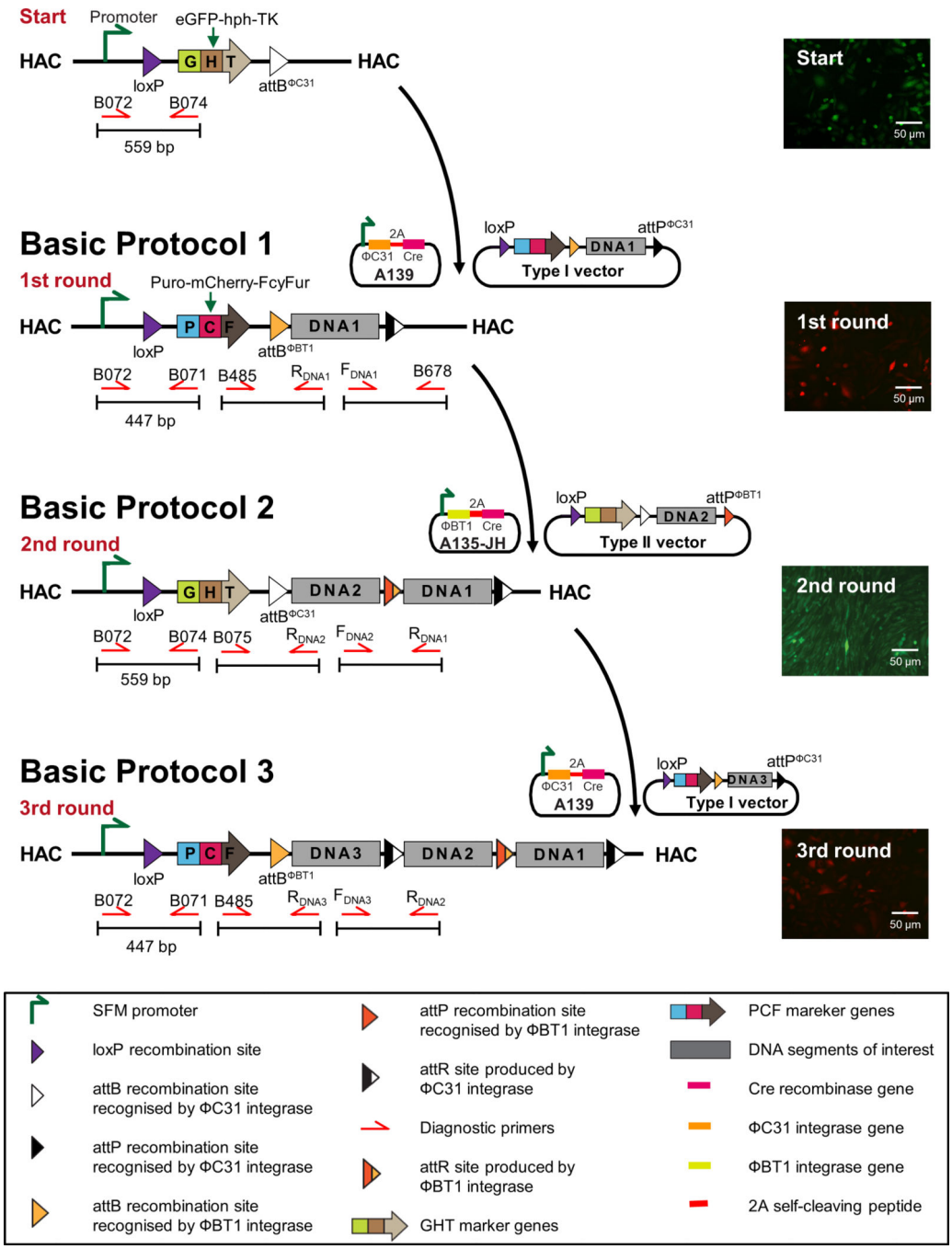

Figure 5.

Schematic of serial integration of three DNA fragments into the IIS-alphoid^{tetO}-HAC using the Type I carrier vector A167 carrying either DNA1 or DNA3 and the Type II carrier vector A169 carrying DNA2. **Starting point:** The empty integration platform cassette on the IIS-alphoid^{tetO}-HAC includes the SFM promoter (green arrow to the right) that drives expression of the GHT marker (eGFP-hph-TK). Containing cells exhibit green fluorescence, and are Hygromycin resistant (hph) and Ganciclovir sensitive (TK). **Basic Protocol 1:** structure of the platform cassette after the 1st round of DNA integration. The

1st round involves integration of the Type I carrier vector A167 carrying DNA1 into the platform cassette by ΦC31 integrase and Cre recombinase. Recombination between the Type I carrier vector A167 and the platform cassette leads to replacement of the GHT marker by the PCF marker (Puro-mCherry-FcyFur) and insertion of the 1st genomic DNA segment (DNA1). After the 1st round of DNA integration, the PCF marker is expressed. Therefore, the cells exhibit red fluorescence (mCherry), and are Puromycin resistant (Puro) and 5-Fluorocytosine sensitive (FcyFur). **Basic Protocol 2:** structure of the platform cassette after the 2nd round of DNA integration. The 2nd round involves recombination of the Type II carrier vector A169 carrying DNA2 and the platform cassette by ΦBT1 integrase and Cre recombinase. The PCF marker is replaced by the GHT marker and insertion of the 2nd genomic DNA fragment (DNA2) into the platform cassette. The integration event is selected using Hygromycin (hph) and 5-Fluorocytosine (FcyFur). The cells are exhibit green fluorescence. **Basic Protocol 3:** structure of the platform cassette after the 3rd round of DNA integration. The GHT marker is replaced by the PCF marker and the 3rd genomic DNA fragment (DNA3) is inserted into the platform cassette. The cells exhibit red fluorescence (mCherry), and are once again Puromycin resistant (Puro) and 5-Fluorocytosine sensitive (FcyFur).

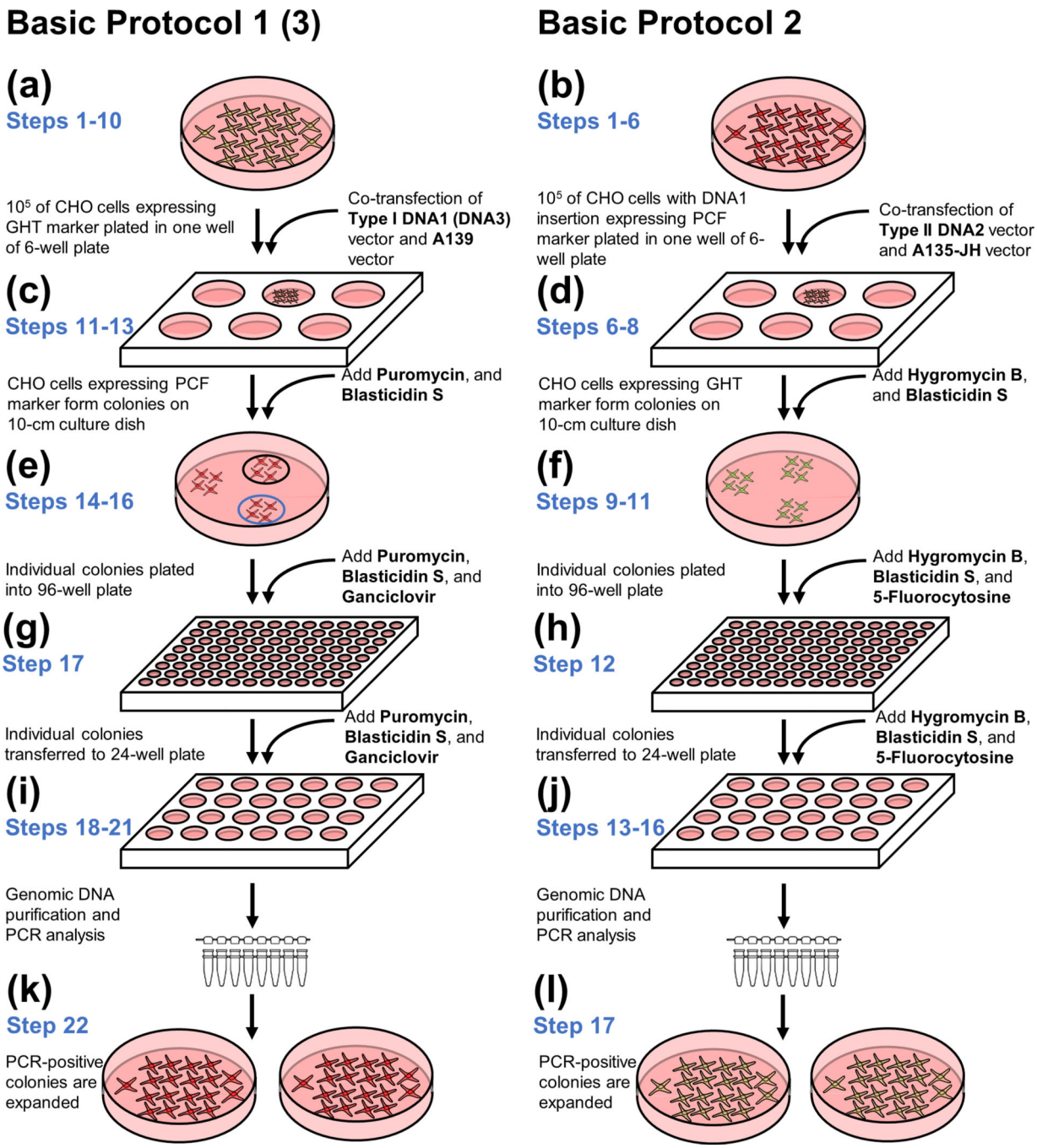

Figure 6.

Cell culture steps in the Basic Protocols. Each round of integration requires similar cell culture procedures. Herein, the steps of the protocols are presented side by side to highlight the key differences between them. The 1st round starts with hamster CHO cells carrying the IIS-alphoid^{tetO}-HAC. Afterwards, any subsequent round starts with the cells obtained during the preceding round. **(a-b)** The first procedure in each round is co-transfection of a specific carrier vector carrying a genomic DNA fragment, i.e., Type I DNA1, Type II DNA2, and Type I DNA3, along with either **(a)** A139 or **(b)** A135-JH vector. **(c-d)**

Cells in with correct integration has occurred exhibit a change in the expression of marker genes (eGFP to mCherry or mCherry to eGFP) and can form colonies under selection with either **(c)** Puromycin and Blasticidin S or **(d)** Hygromycin B and Blasticidin S. **(e-f)** Individual colonies with proper fluorescence are transferred into a 96-well plate. Additional counterselection agents, i.e, **(e)** Ganciclovir or **(f)** 5-Fluorocytosine, are supplemented to remove cells with incorrect integration. **(g-h)** The colonies are transferred to a 24-well plate and grown under selection. **(i-j)** Genomic DNA is purified from individual colonies and PCR-analyzed to confirm proper integration. **(k-l)** Colonies with PCR-confirmed integration of the DNA fragment are transferred to 10-cm dishes for FISH analysis and for preparation of frozen stocks.

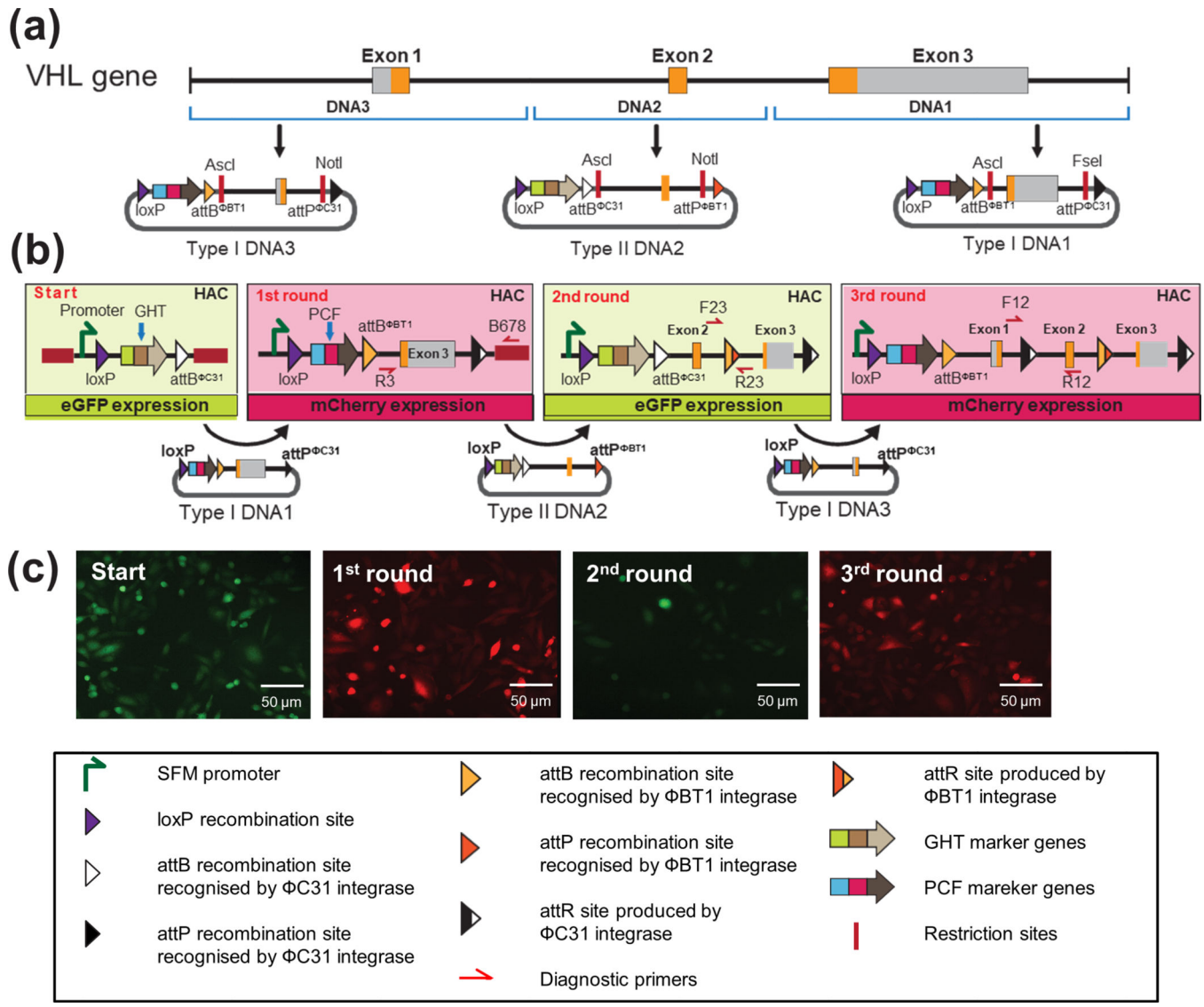

Figure 7.

Schematic of *VHL* gene reconstitution by serial integration into the IIS-alphoid^{tetO}-HAC. The *VHL* gene is located on chromosome 3 (positions 10137959–10154492; GHCH38/hg38). The gene contains three exons. Mutations in the gene are associated with the Von Hippel-Lindau (VHL) syndrome, a dominantly inherited hereditary cancer syndrome predisposing to a variety of malignant and benign tumors of the eye, brain, spinal cord, kidney, pancreas, and adrenal glands. **(a)** Construction of Type I vectors carrying genomic fragments DNA1 and DNA3,encompassing exon 3 and exon 1, respectively, and Type II vector carrying a genomic DNA2 fragment encompassing exon 2 of the *VHL* gene. DNA1 (exon 3) and DNA3 (exon 1) were inserted into the AscI/NotI sites of the Type I carrier vector A167. DNA2 (exon 2) was inserted into the AscI/NotI sites of the Type II carrier vector A169. **(b)** Three rounds of insertion of the *VHL* fragments into the IIS-alphoid^{tetO}-HAC carrying the integration platform cassette are shown. *Round 1*: A diagnostic PCR for DNA1 insertion was performed with primers R3/B678. *Round 2:* A diagnostic PCR for

DNA2 insertion was performed with primers F23/R23. *Round 3:* A diagnostic PCR for DNA3 insertion was performed with diagnostic primers F12/R12. The position of the primer pairs are shown. **(c)** Representative images of how cell fluorescence changes after each round of DNA insertion are shown.

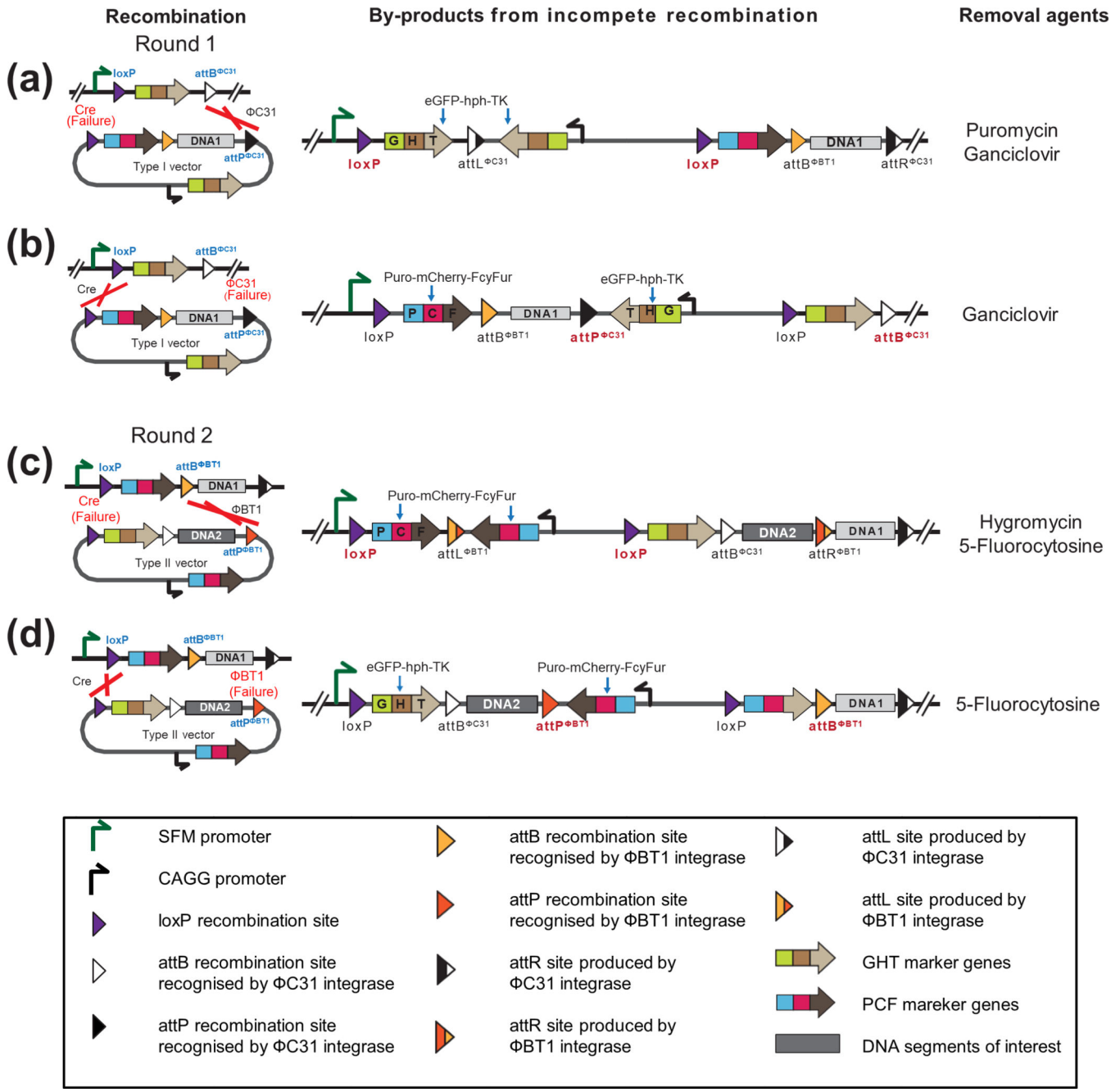

Figure 8.

Error-catching mechanism within the IIS-alphoid^{tetO}-HAC system. Recombination reactions mediated by Cre, ΦC31, and ΦBT1 do not always go to completion. Because maintenance, storage, and screening of vertebrate cell colonies is very labor-intensive compared to that for bacteria or yeast colonies, an error catching system was designed into the IIS to lighten the workload. **(a-b)** Products of incomplete recombination reactions involving the Type I carrier vector A167 retain an actively expressed GHT marker. **(c-d)** Products of incomplete recombination reactions between the Type II carrier vector A169 retain an expressed PCF

marker. The selection agents to remove each misassembled product are listed. This figure was adapted from (Lee et al., 2018).

Table 1

Troubleshooting

Problem	Possible Cause	Solution
Sectored colonies. A single colony is composed of red and green cells.	Proper integration into the IIS-alphoid^{tetO}-HAC occurred late during the colony's growth and did not occur in all cells.	Cells with the correct marker can be rescued by dispersing the colony to single cells and sub-cloning.
	Concentration of counterselection agent insufficient to kill cells with incorrect marker.	Concentration of counterselection agent should be increased.
Cells are simultaneously red and green.	This can happen if ΦC31 fails during the 1st round of integration (Figure 8b) or if ΦBT1 fails during the 2nd round of integration (Figure 8d). In that case, a single cell expresses both PCF and GHT markers. Those cells can be eliminated only by Ganciclovir during the 1st round or by 5-Fluorocytosine during the 2nd round (Figures 8b and 8d).	Increase concentration of counterselection agent used, until two-coloured cells are dead. Then, restart experiment from the beginning, using this higher concentration of counterselection agent.
	Concentration of counterselection agent, Ganciclovir or 5-Fluorocytosine, is insufficient to kill the cells expressing the wrong marker.	Increase concentration of the appropriate selection agent.
Colourless (non-fluorescent) colonies.	Insufficient concentration of selection agent (Hygromycin or Puromycin) to prevent marker silencing due to hetrochromatin spread.	Slowly increase concentration of the appropriate selection agent in a stepwise manner. Check for reappearance of fluorescence as selection agent selects for cells with increased marker expression.
	Selection agents degraded.	If fluorescence does not reappear with increased concentration of selection agent AND the colony remained viable, check that the selection agent has not degraded from prolonged storage at 4°C or excessive heating from repeated freeze-thaw cycles. Discard old selection agent, and aliquot fresh stock of selection agents to vials of smaller volume before use. If selection agents are kept frozen, storage at −80°C is suggested.
Phenotype not matching expected genotype: 'Fakes'	Heterochromatin silencing of one marker, giving the impression that one of the markers has been lost.	The colonies under investigation should be split, with one half preserved and other half for study.
		Screen out colonies that are 'faking' their genotype by using gradually increasing concentrations of selection agents to select for gene reactivation.
		Alternatively, apply HDAC inhibitor to (i.e., trichostatin a) to help reverse heterochromatin silencing. Be aware that the HDAC concentration used may kill the cells or/and cause HAC. destabilization.
Large numbers of 'fake' colonies.	Insufficient concentration of counterselection agent used.	Increase concentration of counterselection agent used.
	Too much time given for counterselection marker to degrade, allowing emergence of marker silencing by heterochromatin.	Decrease the time between transfection and application of counterselectable agent.
On application of selection agent, cells look unhealthy but do not detach from plate.	Lower than normal concentration of puromycin or hygromycin used.	Perform transformation in one well of a 6 well plate, then, 1 day after selection has begun, disperse cells to a 10 cm plate. Dying cells do not reattach.
		Increase concentration of selection agent, if possible.

Problem	Possible Cause	Solution
Few colonies of the correct fluorescence obtained.	If carrier vector has a large genomic insert, there may be insufficient molecules being transfected into host cell.	Instead of co-transfecting the integrase plasmid and carrier vector, these transfections can be done sequentially. First transfect the host cells at lower than normal confluence with the integrase plasmid. Then, allow the cell culture to recover for a day before transfecting the same cells with the carrier vector. Instead of co-transfecting the integrase plasmid and carrier vector, these transfections can be done sequentially. First transfect the host cells at lower than normal confluence with the integrase plasmid. Then, allow the cell culture to recover for a day before transfecting the same cells with the carrier vector.
No colonies obtained.	HAC lost.	Maintain HAC selection with blasticidin.
	Selection agents.	Give sufficient time for building up of new selection marker and degradation of old counterselection marker. Both the timing and concentration are factors.
	Too much integrase expression vector used, leading to overexpression of integrase protein which form inactive protein aggregates. Too little carrier vectors used.	Restart experiment with empty vector and optimize quantity of integrase expression plasmid or carrier vector used before proceeding to actual experiment.
	Mutation in attB$^{\Phi C31}$, attB$^{\Phi BT1}$, or loxP sites.	DNA-sequence the platform cassette to make sure the integrase attachment sites have not mutated. Pick a different colony to use if mutations have occurred.
	Endotoxin contamination of DNA used in transfection.	Repurify vector with endotoxin free kit. Remember to use wash buffer and elute buffer that are both endotoxin free and filter sterilized. *E. coli* strains with reduce endotoxin may be used to produce the DNA.
	Standard 37°C incubation temperature of mammalian cell culture reduces activity of ΦC31 and ΦBT1 bacteriophage integrase	Lower post-transformation incubation temperature to 30°C for 2 days to promote recombinase activity before returning to normal temperature. Optimum temperature may vary with cell line.