

cGPS[®] Custom HepG2 Full Kit DD

Fast and effortless targeted integration into native HepG2 cells

User Manual
11_03_24

Catalog #HG2-1111-10-DD and #HG2-1111-05-DD

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INFORMATION

Introduction

The cGPS Custom HepG2 Full Kit DD includes the hsRAG1 EF1a Meganuclease Plasmid DD, the hsRAG1 Integration Matrix EF1a Neo.2 for the cloning of your gene and the control Integration Matrix, the hsRAG1 Integration Matrix EF1a Neo.2 Luc. Refer to the individual components datasheets for a detailed description of the reagents.

Shipping / Storage

All cGPS Custom HepG2 reagents are shipped at room temperature.

Upon receipt, store the hsRAG1 Integration Matrices and hsRAG1 EF1a Meganuclease Plasmid DD preferably at -20°C.

Product Use Limitation

This product is not intended for consumption, administration or application to humans or animals.

The Customer shall use this Product for research purposes only. The research (“Research”) is understood as the generation of knowledge regarding natural biological mechanisms. All other uses outside the Research use, including without limitation activities of drug discovery, diagnostic and industrial production, the drug development, as well as commercial uses, require a license from Collectis bioresearch.

Any activities outside the Research require the Customer to obtain license from Collectis bioresearch under Collectis' patents and Institut Pasteur's patents.

Territory of use: World.

KIT CONTENT

The cGPS Custom HepG2 Full Kit DD (Catalog #HG2-1111-10-DD or #HG2-1111-05-DD) includes the following reagents:

| Reagent | Tube labels | Amount | Comments | Store |
|---|---|----------------|------------------|--------------|
| hsRAG1 EF1a Meganuclease Plasmid DD Catalog #EM-HS1-3-10-DD or Catalog #EM-HS1-3-05-DD | hsRAG1 EF1a Meganuclease Plasmid DD | 30 µg 15 µg | in TE, pH 8.0 | -20°C or 4°C |
| hsRAG1 Integration Matrix EF1a Neo.2 Catalog #IM-HS1-83 | pIM.RAG1.EF1a.Neo.2 Integration Matrix | 20 µg | in TE, pH 8.0 | -20°C or 4°C |
| hsRAG1 Integration Matrix EF1a Neo.2 Luc Catalog #IM-HS1-84 | pIM.RAG1.EF1a.Neo.2.Luc Integration Matrix | 20 µg | in TE, pH 8.0 | -20°C or 4°C |

Please refer to the components related datasheets a detailed description of each reagent. Each reagent can be purchased separately. For more information regarding ordering, please visit <http://www.collectis-bioresearch.com>.

Transfection Reagent

The cGPS Custom HepG2 Full Kit DD does not include any Transfection Reagent. However, we have obtained the best results with the FuGENE[®] HD reagent commercialized by Roche Diagnostics.

To obtain optimized results, it is strongly recommended to thoroughly follow the described protocol, even though it could slightly differ from the manufacturer's user manual.

Other reagents

Media, selection agents and other needed products are available from general suppliers and are not supplied in the kit.

DESCRIPTION OF cGPS CUSTOM HEPG2

Introduction

Meganuclease-induced homologous recombination is the most powerful targeted integration tool for genome engineering. Since the first gene targeting experiments in yeast more than 25 years ago (1, 2), homologous recombination has been used to insert, replace or delete genomic sequences in a variety of cells (3-5). However, targeted events occur at a very low frequency in mammalian cells. The frequency of homologous recombination can be significantly increased by a specific DNA double-strand break (DSB) at the targeted locus (6, 7). Such DSBs can be induced by meganucleases, sequence-specific endonucleases that recognize large DNA recognition sites (12 to 30 bp).

Because of their high specificity, these proteins can cleave a unique chromosomal sequence without affecting global genome integrity. Natural meganucleases are essentially represented by homing endonucleases, a widespread class of proteins found in eukaryotes, bacteria and archae (8). Early studies of the *I-SceI* and HO homing endonucleases have illustrated how the cleavage activity of these proteins initiates homologous recombination (HR) events in living cells and demonstrated the recombinogenic properties of chromosomal DSBs (9, 10). Since then, meganuclease-induced homologous recombination has been successfully used for genome engineering purposes in bacteria (11), mammalian cells (6, 7, 12-14), mice (15) and plants (16, 17).

Meganuclease-driven targeted integration is the basis of all Collectis bioresearch cGPS® (cellular Genome Positioning System) and cGPS® Custom products.

Description

The cGPS Custom HepG2 Full Kit DD is designed for the generation of isogenic stable HepG2 expression cell lines by taking advantage of meganuclease-driven targeted integration. This DNA recombination system uses a meganuclease and site-specific recombination (19, 20) to facilitate integration of a gene into a given site in the genome of higher eukaryotic cells.

In the cGPS Custom HepG2 Full Kit DD, two different vectors are used to generate isogenic stable HepG2 cell lines.

The first major component of cGPS Custom HepG2 Full Kit DD is the hsRAG1 Integration Matrix EF1a Neo.2 plasmid into which your gene will be cloned. Expression of the gene is controlled by the EF1a promoter. The plasmid contains two regions homologous to the endogenous native hsRAG1 gene, flanking a heterologous sequence that contains the selection and the gene expression cassettes.

The second major element is the hsRAG1 EF1a Meganuclease Plasmid DD which expresses the hsRAG1 engineered meganuclease that targets a 24bp endogenous sequence in the endogenous hsRAG1 gene.

The hsRAG1 Integration Matrix EF1a Neo.2 and hsRAG1 EF1a Meganuclease Plasmid DD are co-transfected into HepG2 cells. Upon co-transfection, the engineered hsRAG1 EF1a Meganuclease is expressed, recognizes its endogenous recognition site, binds to it and induces a DNA double-strand break at this precise site. The cell senses the DNA damage and triggers homologous recombination to fix it, using the co-transfected hsRAG1 Integration Matrix EF1a Neo.2 (used as a DNA repair matrix since it contains regions homologous to the broken DNA). The selection marker and the gene of interest, cloned into the hsRAG1 Integration Matrix EF1a Neo.2 in between the homology regions, get integrated at the meganuclease recognition site during this recombination event.

Thus, stable HepG2 targeted cell clones can be selected for the neomycin resistance and expression of the recombinant protein of interest. The hsRAG1 Integration Matrix EF1a Neo.2 Luc expresses luciferase and serves as a positive control for targeted integration.

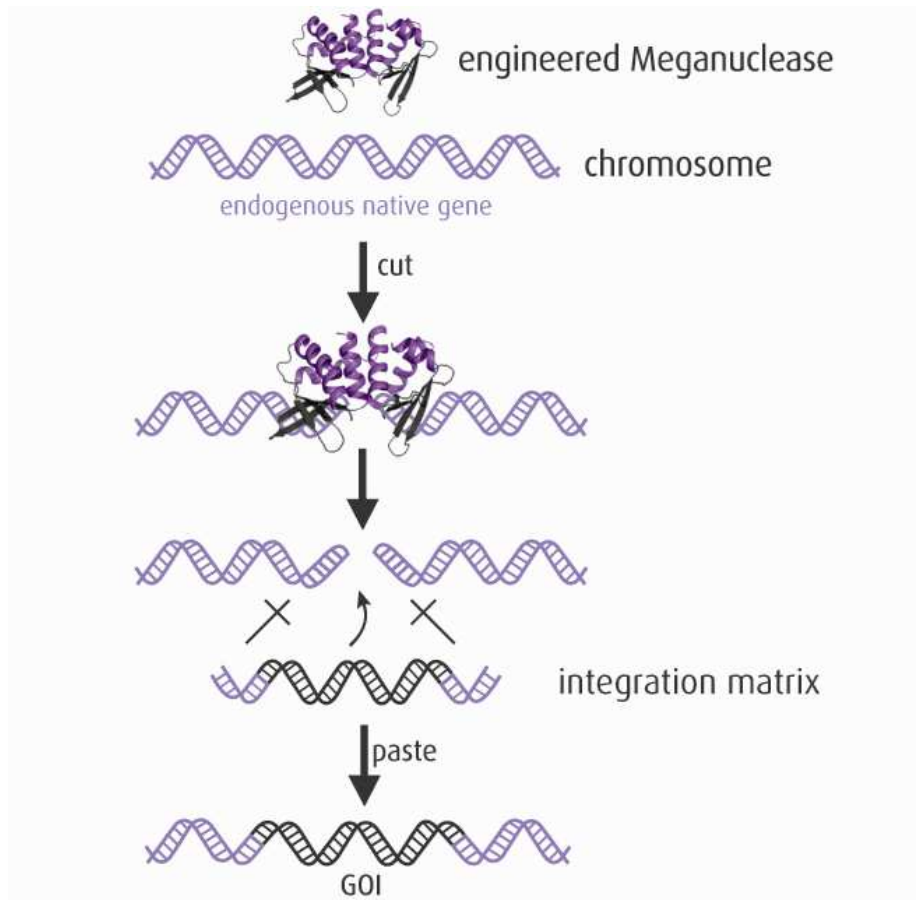


Fig.1: Principle of cGPS[®] Custom products

(GOI: Gene Of Interest)

Recombination process

The figure 2 illustrates the major genetic elements of the cGPS Custom HepG2 Full Kit DD:

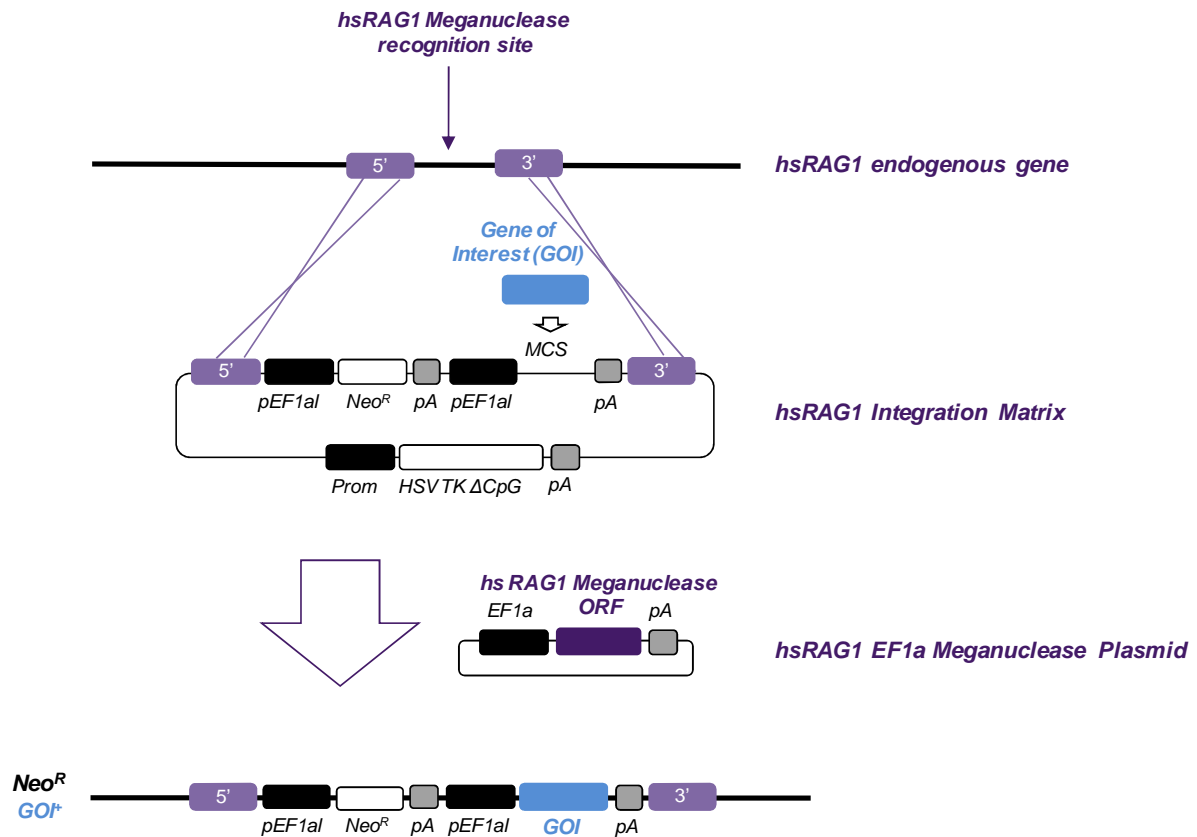


Fig.2: Schematic representation of targeted integration using the cGPS Custom HepG2 Full Kit DD

Benefits

Use of the cGPS Custom HepG2 Full Kit DD to generate stable expression cell lines provides a number of key benefits:

- enabled targeted integration into native HepG2 cell lines
- highly efficient targeted integration
- guaranteed expression at a transcriptionally active targeted locus
- sustained protein production
- clone isogenicity
- fast
- effortless

PROTOCOLS

To obtain optimized results, it is strongly recommended to thoroughly follow the described protocols, even though it could slightly differ from your own practices or dedicated reagent handbooks. All the following protocols have been extensively applied by our product development team and give optimal results.

Cloning of the gene of interest

The first step for generating a cGPS Custom HepG2 expressing cell line is to clone your gene into the hsRAG1 Integration Matrix EF1al Neo.2 (pIM.RAG1.EF1al.Neo.2). The expression of such gene is under the control of a EF1al promoter and the bovine growth hormone (BGH) polyadenylation signal. The pIM.RAG1.EF1al.Neo.2 plasmid contains all the characteristics to favor highly efficient homologous recombination at the endogenous hsRAG1 gene. The left and right homology arms of about 2kb are the regions of homology flanking the engineered meganuclease recognition site. These two homology arms are separated by (i) the neomycin resistance gene under the EF1al promoter control (ii) an EF1al promoter for the expression of your gene, (iii) a multiple cloning site for the cloning of your gene and (iv) a polyadenylation signal controlling the stability of your gene mRNA. The plasmid also bears an HSV TK negative selection marker to eliminate unwanted integration events - such as random integrations – by the use of ganciclovir (GCV).

Refer to the map of the Integration Matrix in the related datasheet to choose the restriction sites suitable for your cloning.

Positive control

The hsRAG1 Integration Matrix EF1al Neo.2 Luc (pIM.RAG1.EF1al.Neo.2.Luc) is provided as a positive control for targeted integration. It may also be used to assay for luciferase expression levels in HepG2 cells.

Transfection

HepG2 cells are co-transfected with the hsRAG1 Integration Matrix EF1al Neo.2 containing your cloned gene (or with the hsRAG1 Integration Matrix EF1al Neo.2 Luc) and the hsRAG1 EF1a Meganuclease Plasmid DD using the FuGENE[®] HD reagent.

Transfection procedure

One day prior transfection, HepG2 cells are seeded in a 10cm tissue culture dish (10^6 cells per dish) in complete medium (see Appendix for medium definition)

On transfection day (D), dilute 2 μ l of the hsRAG1 Integration Matrix EF1al Neo.2 (pIM.RAG1.EF1al.Neo.2) containing your gene of interest, or 2 μ l of the hsRAG1 Integration Matrix EF1al Neo.2 Luc (pIM.RAG1.EF1al.Neo.2.Luc) and 3 μ l of the hsRAG1 EF1a Meganuclease Plasmid DD in 500 μ l of EMEM medium only (serum-free and antibiotic-free). Vortex for 1 to 2 seconds.

The FuGENE[®] HD reagent must be warm-up at room temperature before use. Dilute 15 μ l of FuGENE[®] HD reagent in the diluted DNA mix.
Vortex for 2 to 5 seconds.

Incubate mix for 15 minutes at room temperature.

Dispense total transfection mix over plated cells.

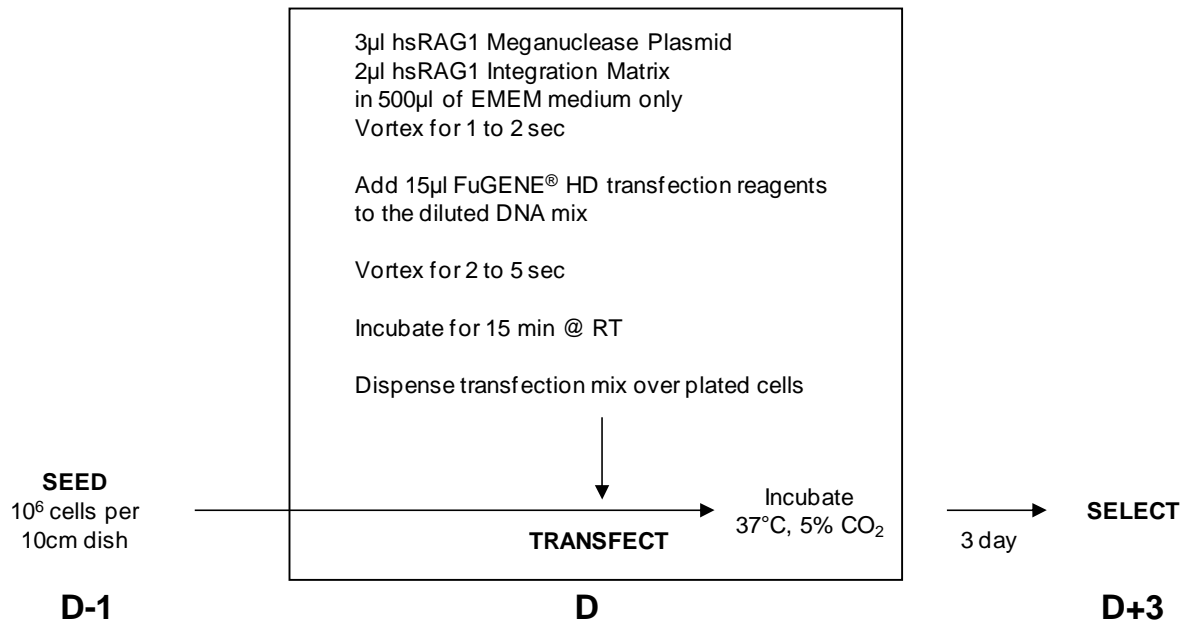


Fig.3: Principle of transfection procedure

Selection of targeted cell clones

NB: the selection of isogenic targeted clones is achieved through a drug-driven positive/negative selection process AND a molecular screen (PCR).

Clonal selection

Transfect HepG2 cells as described above.

1. 3 days after transfection (D+3), harvest cells by trypsinization and seed them into two dishes. Replace medium with fresh complete medium supplemented with 0.8mg/ml of G418.
2. Replace medium with fresh complete medium supplemented with 0.8mg/ml of G418 every 3 days during 10 days.
3. At D+13, replace medium with fresh complete medium supplemented with 0.8mg/ml of G418 and 50µM of ganciclovir (GCV) every 2 or 3 days for a total of 5 days.
4. At D+18, replace medium with fresh complete medium supplemented with 0.8mg/ml of G418 every 2 or 3 days for a total of 6 days. At this step cells are maintained in complete medium supplemented with G418 only.
5. At D+24, G418 resistant clones are picked in a 96-well plate.
6. Trypsinize cells in the 96w plate every 6 days during 12 days, for a better growth.
7. At approximately D+36 (when cells reach confluency), 96-well plates are duplicated: one plate for maintenance, the other one for genomic DNA extraction and PCR screening (see PCR screening protocol below).
8. PCR positive clones are then identified and amplified for downstream applications.
9. At this stage, targeted clones can be assayed for activity (in the case of the use of the control Integration Matrix, luciferase activity can be monitored (21)).

Transfection of HepG2 cells

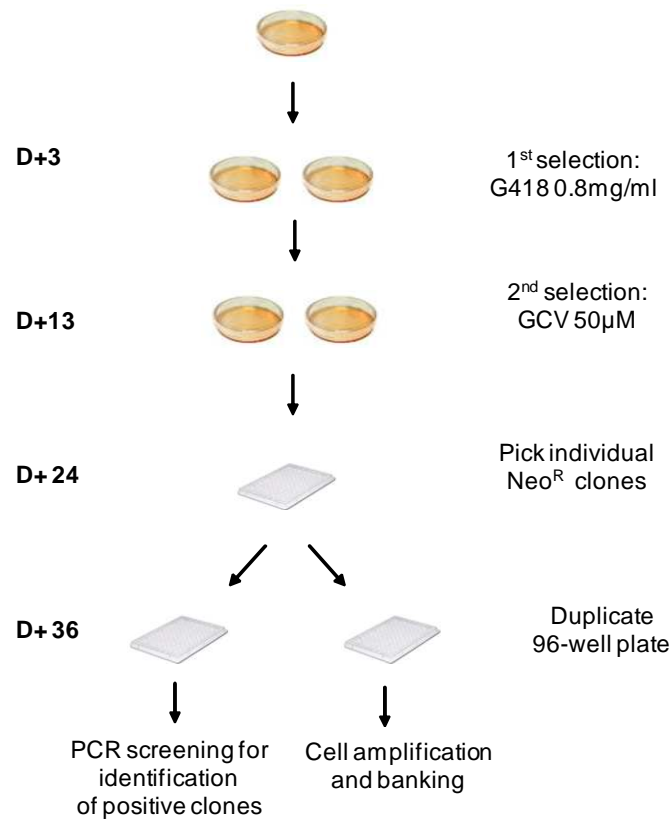


Fig.4: Principle of selection process

Molecular screen (PCR)

Expected results

Using the positive control hsRAG1 Luc Integration Matrix (pIM.RAG1.EF1aI.Neo.2.Luc), we routinely observe 10 to 15% of PCR positive clones (targeted integration events). However, according to your gene of interest, the percentage of PCR positive clones can be slightly different. Thus, it is advised to perform the following PCR screen on at least 96 G418 and GCV double resistant clones (one 96w plate).

gDNA extraction from 96-well plates

We recommend using the ZR-96 Genomic DNA Kit™ (Zymo Research) for genomic DNA extraction in 96-well plate format.

PCR screening

PCR primers and protocol have been carefully designed for a quick and robust identification of targeted clones (oligonucleotides are not provided along with the Full Kit), as highlighted in the figure below:

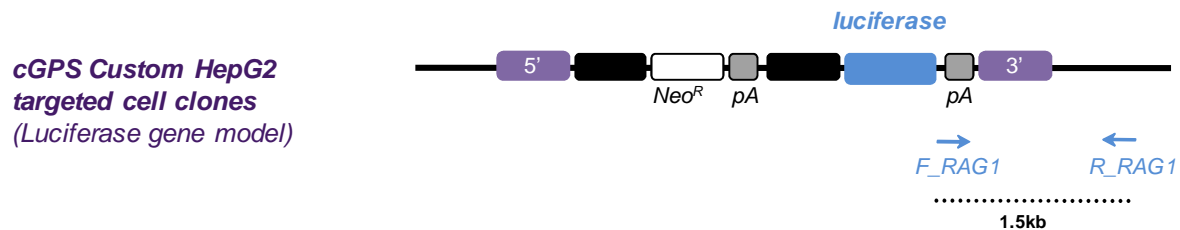


Fig.5: Schematic representation of the *hsRAG1* locus after targeted integration. Primers location and expected PCR band size are indicated.

F_RAG1: GGAGGATTGGGAAGACAATAGC
R_RAG1: CTTTCACAGTCCTGTACATCTTGT

We recommend using the Herculase® II Fusion DNA polymerase (Stratagene) for PCR screening.

PCR conditions:

Total volume reaction: 25µl
 gDNA: 5µl
 Primers: 0.25µM each
 dNTP: 1mM
 Herculase: 0.5µl

PCR program:

| Temperature (°C) | Time (minutes) | Cycle number |
|------------------|----------------|--------------|
| 95 | 5 | 1 |
| 95 | 1 | 30 |
| 55 | 1 | |
| 72 | 1.5 | |
| 72 | 10 | 1 |

Southern blotting

The drug-driven selection process and the PCR screen lead to 95% of success in the identification of isogenic stable clones (single copy targeted integration events).

Should you want to perform a full molecular characterization of the generated clones (Neo^R, GCV^R, PCR+), a Southern blot analysis can be performed.

For a full characterization, it is advised to check both sides of the targeted integration event. As an example, the targeted integration of the Luciferase gene can be checked in the 5' side with an *EcoRV* digestion and by hybridizing the resulting gDNA fragment with a Neo probe. The same approach is used

for the 3' side of the integration. gDNA is digested with *HindIII* and probed with a genomic probe located outside the 3' homology arm. Refer to the pIM.RAG1.EF1aL.Neo.2 restriction map for use of the adequate restriction enzymes.

Primers for a Neo probe:

- Forward oligo: TGGATTGCACGCAGGTTCTCCGG
- Reverse oligo: CAACGCTATGTCCTGATAGCGGTC

Primers for a genomic probe:

- Forward oligo: CAACATCTTCTGTGCGCTGACTC
- Reverse oligo: GTCAGACTCATCTGCCAGCATAAG

NB: genomic DNA (gDNA) is purified from 10^7 cells (about a nearly confluent 10cm dish). We recommend that 5 to 10 μ g of gDNA are digested with a 10-fold excess of restriction enzyme by overnight incubation.

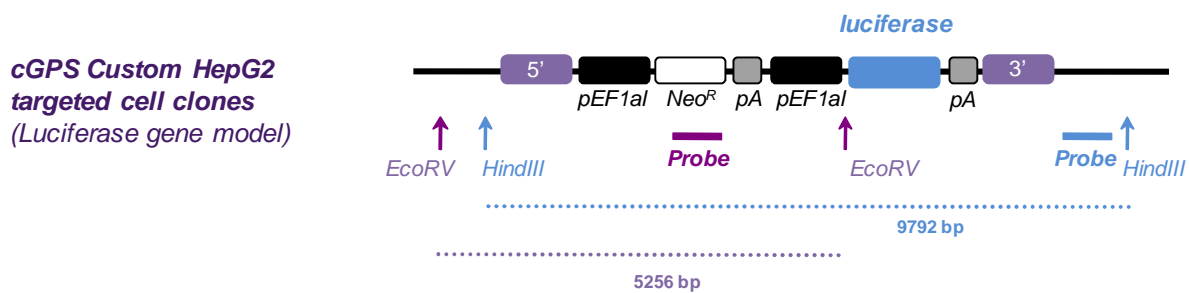


Fig.6: Schematic representation of the *hsRAG1* locus after targeted integration. Southern Blot probes, restriction enzymes cut sites location and expected hybridization band size are indicated.

APPENDIX

Methods

For assistance with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, refer to *Molecular Cloning: A Laboratory Manual* (21) or *Current Protocols in Molecular Biology* (22).

Culture medium

Complete medium: EMEM Medium supplemented with penicillin (100UI/ml), streptomycin (100µg/ml) and 10% fetal calf serum (FCS).

PRODUCT QUALIFICATION

The Meganuclease plasmids and the Integration Matrices used as positive control are prepared with Transfection Grade quality. The Integration Matrix into which your gene of interest is cloned is not prepared with Transfection Grade quality since post-cloning event of your gene imposes a new plasmid preparation.

All marks herein are the sole property of Collectis with the exception of McCoy 5A, property of Sigma Aldrich, FuGENE® HD, property of Roche Diagnostics, ZR-96 Genomic DNA Kit™, property of Zymo Research, and Herculase® II Fusion DNA polymerase, property of Stratagene.

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