

# **cGPS<sup>®</sup> Custom HEK-293 Full Kit**

*Fast and effortless targeted integration into native HEK-293 cells*

**User Manual**  
10\_07\_08

**Catalog #293-1111-10 and #293-1111-05**

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## INFORMATION

### Introduction

The cGPS Custom HEK-293 Full Kit includes the hsRAG1 Meganuclease Plasmid, the hsRAG1 Integration Matrix CMV Neo for the cloning of your gene and a hsRAG1 Integration Matrix CMV Neo. Refer to the individual components datasheets for a detailed description of the reagents.

### Shipping / Storage

All cGPS Custom HEK-293 reagents are shipped at room temperature.

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

### Product Use Limitation

The cGPS Custom HEK-293 products is not intended for consumption, administration or application to humans or animals.

**The cGPS Custom HEK-293 Full Kit shall be used for research purposes only. The term research (“Research”) is understood as the generation of knowledge regarding natural biological mechanisms, excluding specifically all activities of related to screening, diagnostics and industrial production, as well as all commercial activities based on the results of the Research.**

All activities outside of Research, including without limitation, all activities involving screening, diagnostics and industrial production, as well as all commercial activities based on the results of said Research, require a license from Collectis.

**Territory of use:** World.

## KIT CONTENT

The cGPS Custom HEK-293 Full Kit (Catalog #293-1111-10 or #293-1111-05) includes the following reagents:

Reagent	Tube labels	Amount	Comments	Store
<b>hsRAG1 Meganuclease Plasmid</b> Catalog #EM-HS1-1-10 or Catalog #EM-HS1-1-05	hsRAG1 Meganuclease Plasmid	30 µg 15 µg	in TE, pH 8.0	-20°C or 4°C
<b>hsRAG1 Integration Matrix CMV Neo</b> Catalog #IM-HS1-1	pIM.RAG1.CMV.Neo Integration Matrix	20 µg	in TE, pH 8.0	-20°C or 4°C
<b>hsRAG1 Integration Matrix CMV Neo Luc</b> Catalog #IM-HS1-3	pIM.RAG1.CMV.Neo.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 HEK-293 Full Kit does not include any Transfection Reagent. However, we have obtained the best results with the Lipofectamine™ 2000 reagent commercialized by Invitrogen.

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 HEK-293

### 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 HEK-293 Full Kit is designed for the generation of isogenic stable HEK-293 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 HEK-293 Full Kit, two different vectors are used to generate isogenic stable HEK-293 cell lines.

The first major component of cGPS Custom HEK-293 Full Kit is the hsRAG1 Integration Matrix CMV Neo plasmid into which your gene will be cloned. Expression of the gene is controlled by the CMV 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.

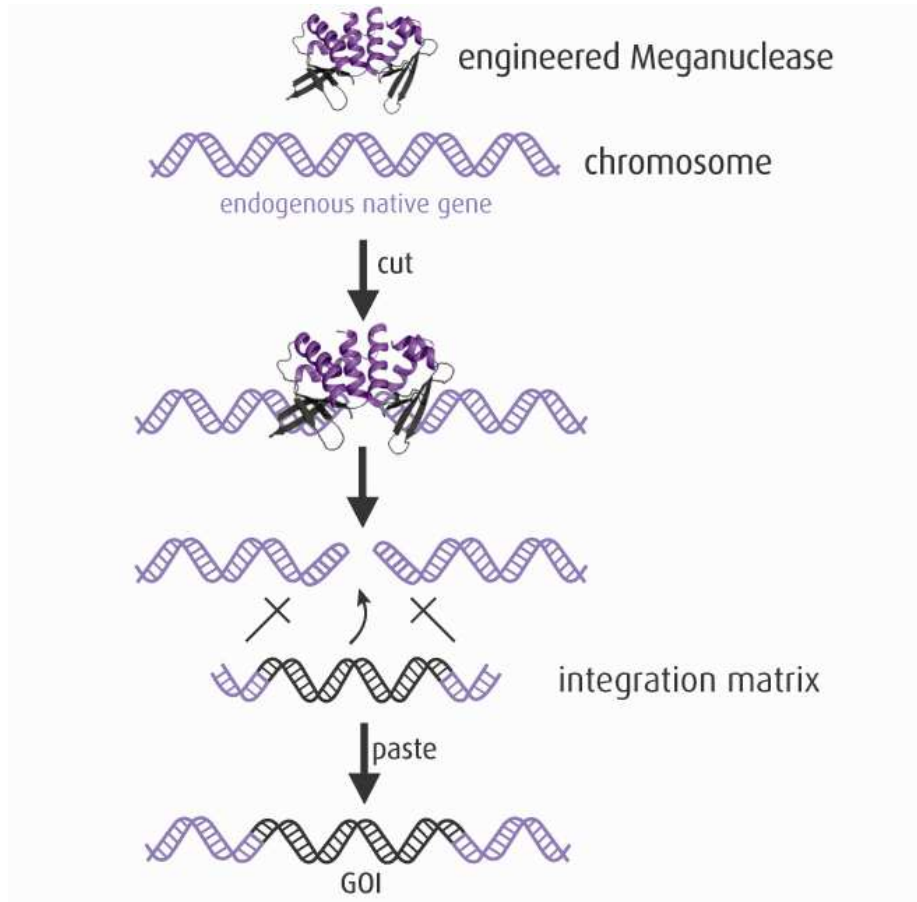
The second major element is the  $\eta\sigma$ RAG1 Meganuclease Plasmid which expresses the hsRAG1 engineered meganuclease that targets a 24bp endogenous sequence in the endogenous hsRAG1 gene.

The hsRAG1 Integration Matrix CMV Neo and  $\eta\sigma$ RAG1 Meganuclease Plasmid are co-transfected into HEK-293 cells. Upon co-transfection, the engineered hsRAG1 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 CMV Neo (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 CMV Neo in between the homology regions, get integrated at the meganuclease recognition site during this recombination event.

Thus, stable HEK-293 targeted cell clones can be selected for the neomycin resistance and expression of the recombinant protein of interest. The hsRAG1 Integration Matrix CMV Neo Luc expresses luciferase and serves as a positive control for targeted integration.

**The cGPS Custom HEK-293 Full Kit reagents can be used with any human-derived cell line that contains the 24bp hsRAG1 Meganuclease recognition site.** However, growth and transfection conditions, selection agent concentrations and other parameters might be different to achieve optimized

targeted integration. Should you want to apply these reagents to other cell lines, please refer to the cGPS Custom Human Full Kit user manual or contact us at [cGPS@collectis-bioresearch.com](mailto:cGPS@collectis-bioresearch.com).

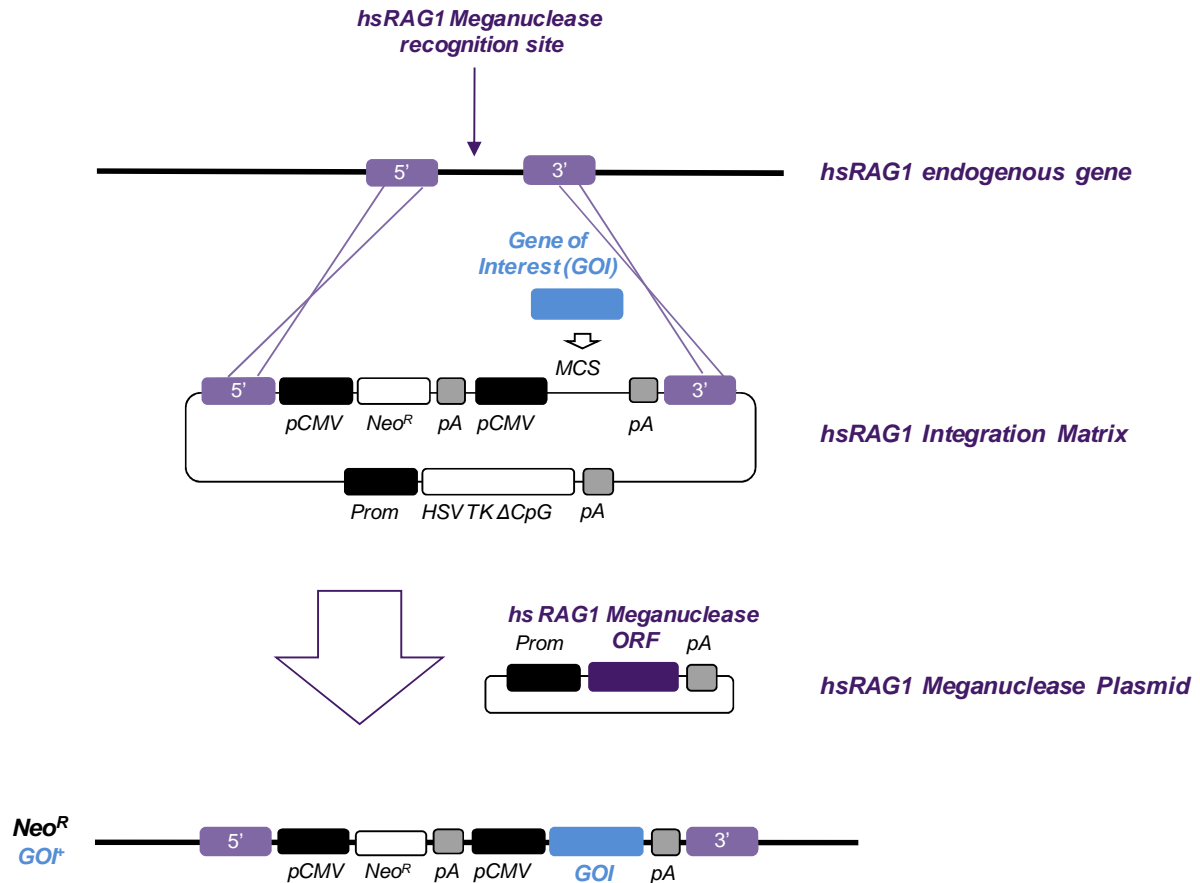


***Principle of cGPS<sup>®</sup> Custom products***

***(GOI: Gene Of Interest)***

## Recombination process

The figure below illustrates the major genetic elements of the cGPS Custom HEK-293 Full Kit:



## Benefits

Use of the cGPS Custom HEK-293 Full Kit to generate stable expression cell lines provides a number of key benefits:

- enabled targeted integration into native HEK-293 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 HEK-293 expressing cell line is to clone your gene into the hsRAG1 Integration Matrix CMV Neo (pIM.RAG1.CMV.Neo). The expression of such gene is under the control of a CMV promoter and the bovine growth hormone (BGH) polyadenylation signal. The pIM.RAG1.CMV.Neo 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 CMV promoter control (ii) a CMV 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.

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 (pIM.RAG1.CMV.Neo.Luc) is provided as a positive control for targeted integration. It may also be used to assay for luciferase expression levels in HEK-293 cells.

### Transfection

HEK-293 cells are co-transfected with the hsRAG1 Integration Matrix CMV Neo containing your cloned gene (or with the hsRAG1 Integration Matrix CMV Neo Luc) and the hsRAG1 Meganuclease Plasmid using the Lipofectamine™ 2000 reagent.

#### Transfection procedure

One day prior to transfection, HEK-293 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 2µg of the hsRAG1 Integration Matrix CMV Neo (pIM.RAG1.CMV.Neo) containing your gene of interest, or 2µg of the hsRAG1 Integration Matrix CMV Neo Luc (pIM.RAG1.CMV.Neo.Luc) and 3µg of the hsRAG1 Meganuclease Plasmid in 300µl of DMEM GlutaMAX™ medium only (serum-free and antibiotic-free). Dilute 10µl of Lipofectamine™ 2000 reagent in 290µl of DMEM GlutaMAX™ medium only (serum-free and antibiotic-free).

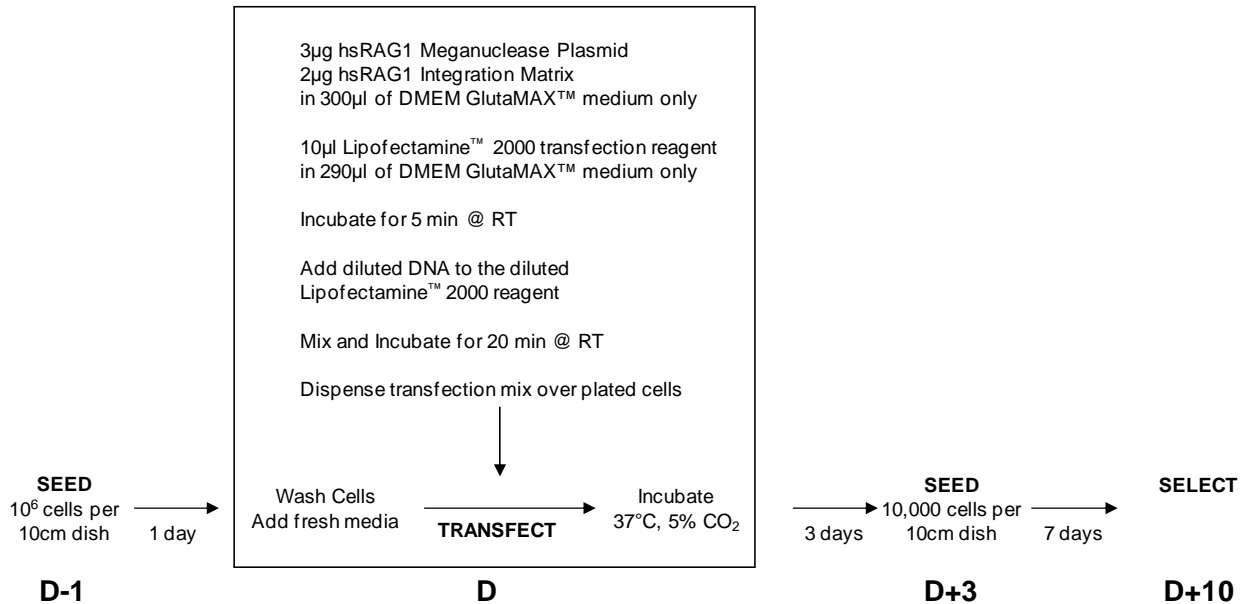
Incubate both mixes separately for 5 minutes at room temperature.

Add the diluted DNA to the diluted Lipofectamine™ 2000 reagent (**and never the other way around**), cap tubes and homogenize by gentle tube inversion.

Incubate for 20 minutes at room temperature.

Dispense total transfection mix over plated cells.

Incubate dish in a 37°C, 5% CO<sub>2</sub> humidified incubator.

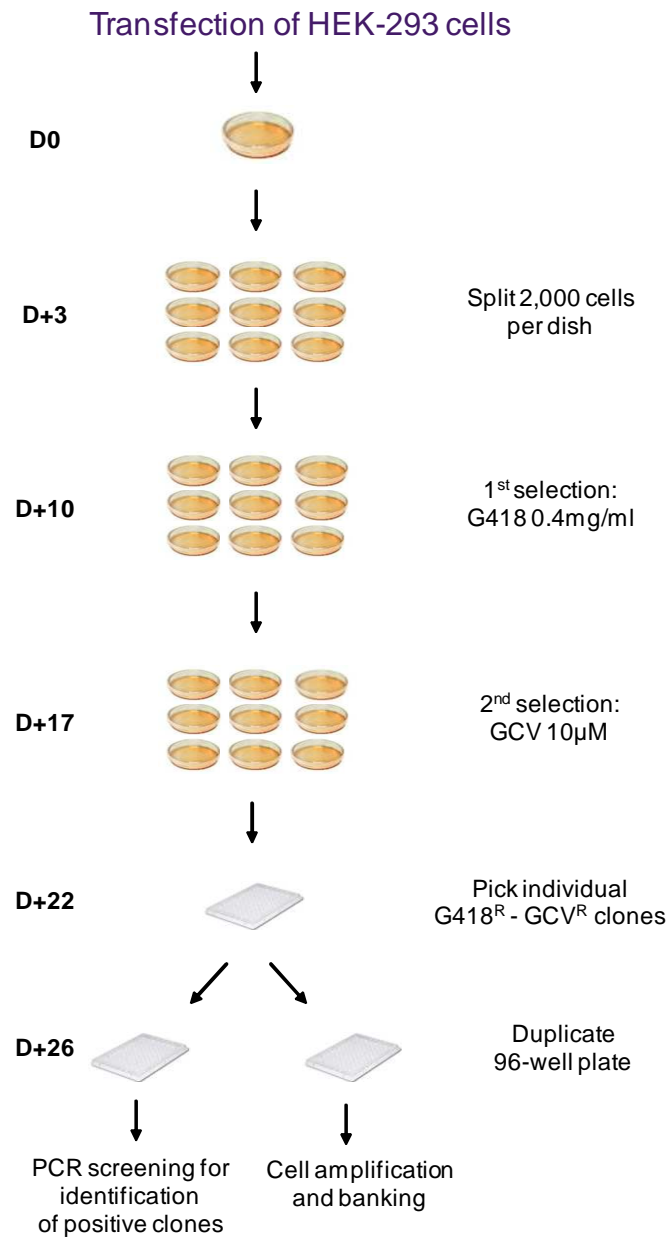


## Selection of targeted cell clones

### Clonal selection

1. Transfect HEK-293 cells as described above.
2. 3 days after transfection (D+3), harvest and count transfected cells.
3. Seed ten 10cm dishes with 2,000 transfected cells per 10cm dish in complete medium.
4. 7 days later (D+10), replace medium with fresh complete medium supplemented with 0.4mg/ml G418.
5. Replace with fresh complete medium supplemented with 0.4mg/ml G418 every 2 or 3 days for a total of 7 days.
6. At D+17, add 10µM of ganciclovir (GCV) while maintaining G418 selection.
7. Replace medium with fresh complete medium supplemented with 0.4mg/ml G418 and 10µM of GCV every 2 or 3 days for a total of 5 days.
8. 5 days later (D+22), G418 resistant and GCV resistant clones are picked in a 96-well plate.
9. After a few days (D+26), 96-well plates are duplicated: one plate for maintenance, the other one for genomic DNA extraction and PCR screening (see PCR screening protocol below).
10. PCR positive clones are then identified and amplified from downstream applications
11. Positive control clones can be assayed for luciferase activity (21).

Observed PCR positive clone frequency: 30 to 50%.



## Characterization of targeted cell clones

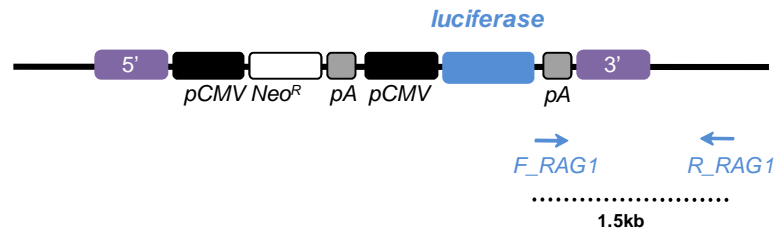
### gDNA extraction from 96-well plates

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:

**cGPS Custom HEK-293 targeted cell clones**  
(Luciferase gene model)



**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**

A correct targeted integration event in G418 and GCV double resistant clones can also be identified at the molecular level by Southern blot analysis.

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.CMV.Neo restriction map for use of the adequate restriction enzymes.

Primers for a Neo probe:

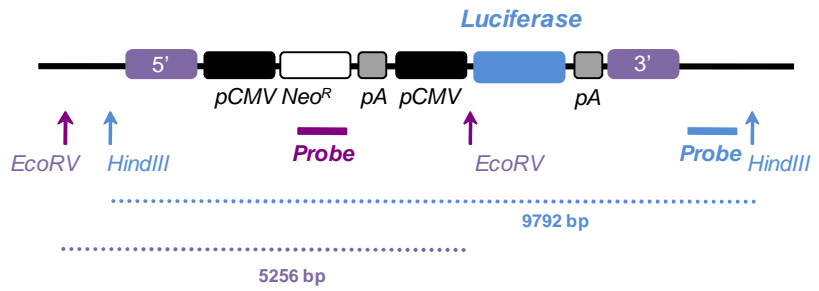
- Forward oligo: TGGATTGCACGCAGGTTCTCCGG
- Reverse oligo: CAACGCTATGTCCTGATAGCGGTC

Primers for a genomic probe:

- Forward oligo: CAACATCTTCTGTCGCTGACTC
- 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  $\mu$ g of gDNA are digested with a 10-fold excess of restriction enzyme by overnight incubation.

**cGPS Custom HEK-293  
targeted cell clones  
(Luciferase gene model)**



## 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: DMEM GlutaMAX™ medium supplemented with penicillin (100UI/ml), streptomycin (100µg/ml), amphotericin B (Fongizone) (0.25µg/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 Lipofectamine™ 2000 and GlutaMAX™, properties of Invitrogen, ZR-96 Genomic DNA Kit™, property of Zymo Research, and Herculase® II Fusion DNA polymerase, property of Stratagene.

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