Bright Makes This Green Light Right

Monster Green™ Protein: A Brighter, Longer-Expressing Green Fluorescent Protein

By Brian D. Almond, Ph.D., Promega Corporation

Abstract

Promega recently introduced the Monster Green™ Fluorescent Protein, a novel gfp *gene cloned from* Montastrea cavernosa. *The Monster Green™ Fluorescent Protein reporter gene (hMGFP) has been "humanized" to increase expression and ensure reliability. Experimental results show that Monster Green™ Fluorescent Protein results in a brighter signal and expression that persists longer than other commercially available GFPs.*

Monster Green™ Fluorescent Protein is encoded by an improved synthetic version of the green fluorescent protein gene originally cloned from the great star coral *Montastrea cavernosa*.

Introduction

Green fluorescent protein (GFP) is commonly used to monitor gene expression and intracellular protein trafficking. GFP fusion proteins, which are easily visualized by standard fluorescence microscopy, are used to track real-time subcellular localization of proteins of interest.

Promega's Monster Green™ Fluorescent Protein is encoded by an improved synthetic version of the green fluorescent protein gene originally cloned from the great star coral *Montastrea cavernosa*. The native *Montastrea gfp* gene expresses a protein that photobleaches and produces a very faint fluorescent signal, making it unsuitable as a reporter.

To improve the fluorescent properties of the native GFP, random mutagenesis was performed, resulting in the generation of a MGFP clone. The clone expresses a GFP that is resistant to photobleaching and is brighter than the native gene.

The MGFP gene also contains many consensus transcription factor binding sites that can result in nonspecific transcriptional activation under certain experimental conditions. The numerous transcription factor binding sites compromise the reliability of MGFP as a reporter. To improve reliability of protein expression, the number of consensus transcription factor binding sites has been reduced from 67 in the MGFP gene to 3 in the synthetic hMGFP ("humanized" MGFP; Figure 1).

Translation of the MGFP gene involves codons not frequently used in mammalian cells, which can reduce MGFP expression efficiency in mammalian cells. To improve expression levels, the synthetic hMGFP gene uses only the highest usage mammalian codons.

To further increase mammalian expression efficiency, the Kozak sequence for translation initiation has been added to the beginning of the gene. As well, other undesirable elements, including eukaryotic polyadenylation signals (AATAAA) have been removed.

Vector Construction and Unique Restriction Sites

The Monster Green™ Fluorescent Protein gene has been cloned into a modified version of Promega's pCI Mammalian Expression Vector[c], resulting in the phMGFP Vector[a,b,c,d] (Cat.# E6421; Figure 2). The pCI Vector was modified to remove a *Nae* I site from the f1 origin of replication, thus the only *Nae* I site is present within the hMGFP gene.

The number of commonly used restriction sites has been minimized within the synthetic hMGFP gene. Restriction sites (*Eco*R V, *Sma* I and *Nae* I) have been added to allow convenient creation of protein fusions. The *Eco*R V and *Sma* I sites are located before the starting ATG codon of hMGFP. The *Nae* I site was added to the hMGFP open reading frame just before the stop codon. Each of these sites, when digested, results in blunt-ended fragments that preserve the translational reading frame of hMGFP (Figure 2). For cloning convenience, a nonunique *Nco* I and a unique *Xba* I restriction site have been added to the beginning and end, respectively, of the hMGFP gene. The second *Nco* I site, which could not be removed due to possible effects on the CMV enhancer/promoter, is located 561bp upstream of the Monster Green™ Protein open reading frame.

Spectral Properties

The spectral properties of the Monster Green™ Fluorescent Protein are slightly red-shifted compared to other commercially available GFPs. Peak excitation occurs at 505nm with a shoulder at 480nm, and the peak emission occurs at 515nm (Figure 3). We recommend using standard fluoroisothiocyanate (FITC) filters to visualize hMGFP fluorescence. Fluorescent microscopy analysis of hMGFP expression may be performed using an excitation filter of 470±20nm (470/40nm) and an emission filter of 515nm (long pass). For FACS™ analyses we recommend the standard FACS™ settings of an argon laser (488nm excitation) and filters of 530±15nm (530/30nm) for emission.

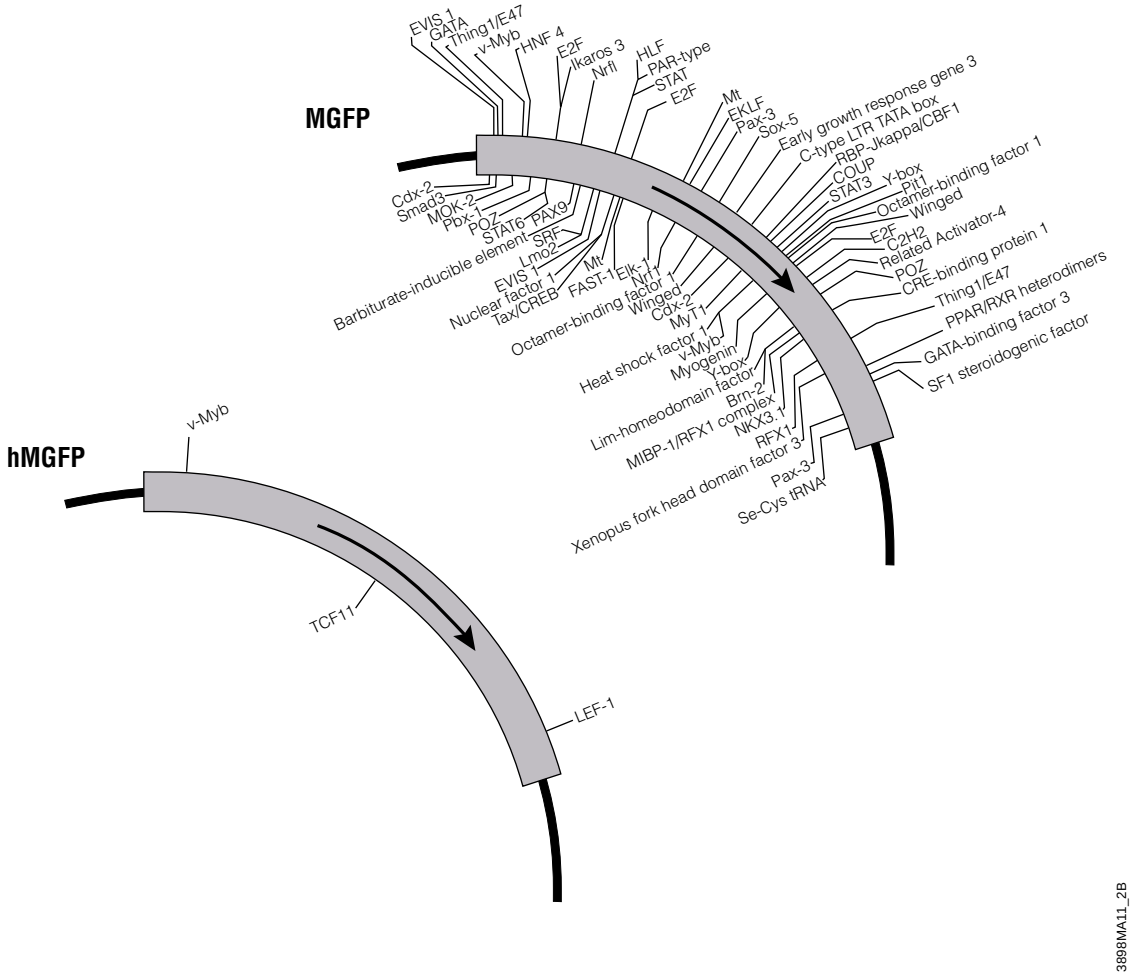


Figure 1. Transcription factor binding sites in the MGFP and synthetic hMGFP genes. Most (96%) of the transcription factor binding sites in the MGFP gene have been removed to create the synthetic hMGFP gene.

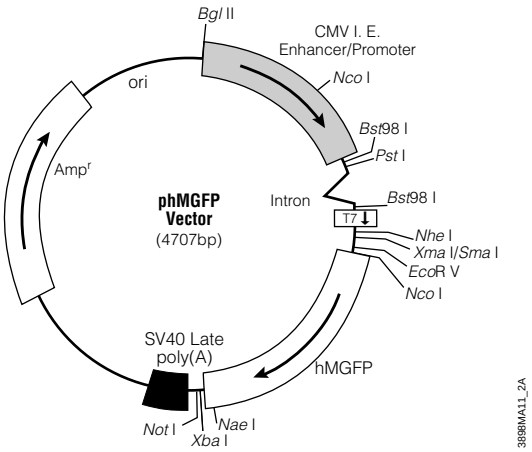


Figure 2. The phMGFP vector circle map and multiple cloning region. hMGFP is the open reading frame of hMGFP; Amp^r refers to the gene conferring ampicillin resistance in *E. coli*; ori is the origin of plasmid replication in *E. coli*. Arrows within the hMGFP and Amp^r genes indicate the direction of transcription.

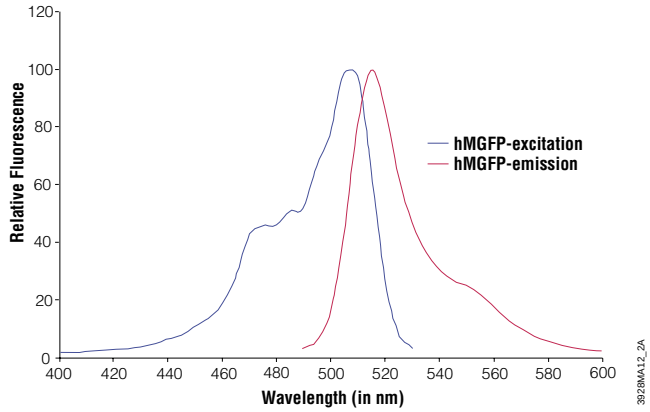


Figure 3. Excitation and emission spectra of the Monster Green™ Fluorescent Protein. CHO cells were transfected with the phMGFP Vector. Twenty-four hours after transfection cells were lysed in Glo Lysis Buffer, 1X (Cat.# E2661), and the excitation and emission spectra were generated using the Spex Fluorolog®-2 spectrofluorometer. The emission spectrum was collected on cell lysates excited at 480nm. Excitation data were collected at 400–530nm with an emission wavelength of 540nm.

Increased Fluorescence

A GFP with increased fluorescence intensity enables better fluorescent visualization and greater sensitivity for a variety of experimental applications. To determine the relative degree of fluorescence intensity of the Monster Green™ Fluorescent Protein, the hMGFP and other commercially available *gfp* genes were cloned into identical vectors, transfected into CHO and NIH 3T3 cells and analyzed by FACS™ analysis (Figure 4). Data were collected for three logs of fluorescence above background. Upon fluorescent microscopic analysis the transfected cells in the first log of fluorescence (green-shaded boxes of each graph in Figure 4) are very dimly fluorescent. Transfected cells in the second log of fluorescence (blue-shaded boxes of each graph in Figure 4) are bright. The cells in the third and highest log of fluorescence (red-shaded boxes of each graph in Figure 4) are extremely bright. The percentages listed in Figure 4 represent the number of transfected cells in each log of fluorescence.

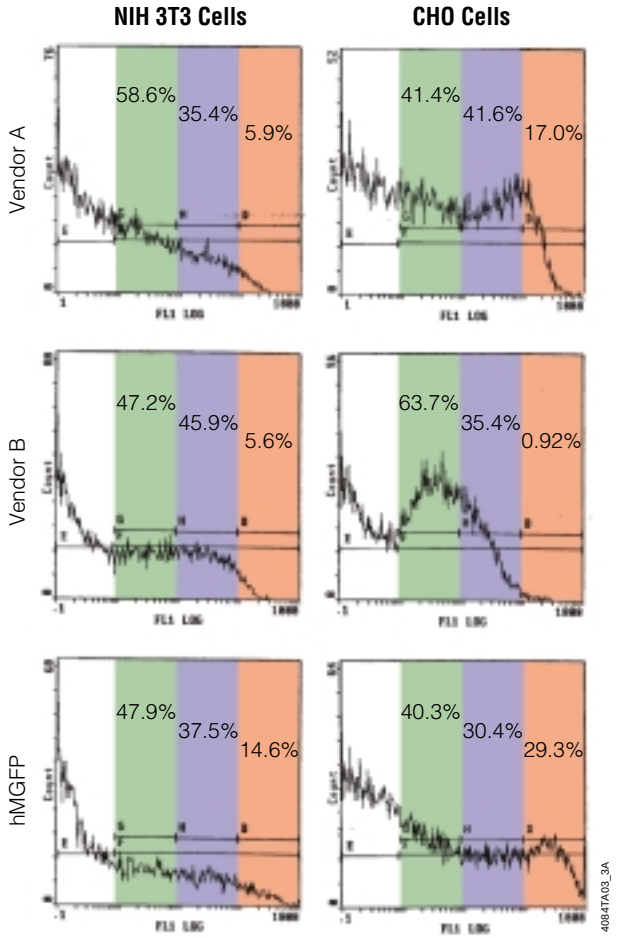


Figure 4. FACS data from cells transfected with various GFP-expressing vectors. All the *gfp* genes were cloned into the same mammalian expression vector (pCI-neo) and transfected into CHO or NIH 3T3 cells. Twenty-four hours after transfection cells were trypsin-treated, and a Coulter Elite™ FACS™ Instrument (standard argon laser (488nm) excitation and 530/30nm emission filter) was used to analyze 50,000 cells. The cell population was separated into fluorescent (transfected) and non-fluorescent (non-transfected) cells. Three logs of fluorescence above background were recorded for the transfected cells. The colored boxes represent the three logs of fluorescence, and the values within the boxes are the percentage of transfected cells within each log of fluorescence. Similar transfection efficiencies were noted for the vectors containing the three *gfp* genes (data not shown). The experiment was repeated generating very similar results.

As demonstrated by the data in Figure 4, more of the hMGFP-transfected cells are in the third or highest log of fluorescence (red box). For the CHO transfection 29.3% of the hMGFP-transfected cells are in the highest fluorescence log versus 17.0% and 0.92% from Vendors A and B, respectively. In the NIH 3T3 transfection, 14.6% of the cells transfected with hMGFP are in the highest log of fluorescence compared to 5.9% and 5.6% from Vendors A and B, respectively.

As a quantitative measure of fluorescence intensity the FACS™ data were analyzed as mean fluorescence per transfected cell. In the CHO cell transfections, hMGFP displays an increase of mean fluorescence of 40% and 238% per transfected cell when compared to Vendors A and B, respectively (Figure 5). Analysis of the NIH 3T3 cell transfection data demonstrates that hMGFP has an increase of 67% and 21% in mean transfected cell fluorescence when compared to transfections performed with GFPs from Vendors A and B, respectively.

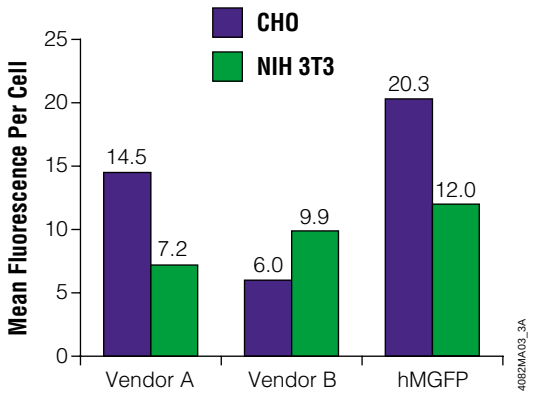


Figure 5. Mean fluorescence per transfected cells. The data from Figure 4 FACS experiments were analyzed as mean fluorescence per transfected cell.

Increased Persistence of Expression

Many commercially available GFPs, unfortunately, induce apoptosis when expressed in mammalian cells (1), resulting in reduced length of expression time. To determine the relative persistence of expression, the same hMGFP and Vendor A reporter gene constructs used for the FACS™ experiments (Figures 4 and 5) were used in the persistence of expression experiments. On Day 1 post-transfection, a difference in fluorescence intensity between cells transfected with hMGFP and the *gfp* from Vendor A can be seen (Figure 6). On Day 2, a dramatic increase in fluorescence intensity is seen for cells transfected with hMGFP when compared to cells transfected with the *gfp* gene from Vendor A. This

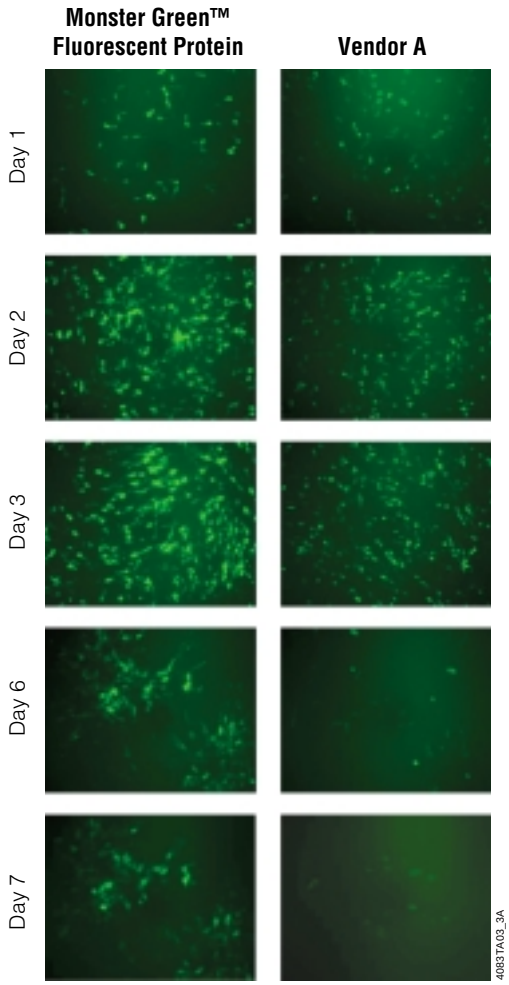


Figure 6. Persistence of expression. A mammalian expression vector containing either the *gfp* gene from Vendor A or hMGFP was independently transfected into NIH 3T3 cells. The medium was changed every two days; no selection agent was added. Microscopic analyses were performed with the aid of a Zeiss Axiovert® S100 fluorescent microscope using 470/40nm excitation filter and a 515nm long pass emission filter. The imaging data were collected using Spot Diagnostic Imaging equipment. The experiment was repeated with similar results.

dramatic difference in cellular fluorescence between cells transfected with hMGFP and Vendor A *gfp* continues for Day 3. Very few fluorescent cells can be detected in the Vendor A *gfp* transfection on Day 6. Conversely, in the hMGFP transfection fluorescent cells can be readily detected. Interestingly, beginning on Day 6 the hMGFP-transfected cells showed localized regions of fluorescent cells. Finally, on Day 7 only very dim fluorescent cells can be detected from cells transfected with the *gfp* gene from Vendor A. As seen on the previous day, localized regions of fluorescence intensity are found in the hMGFP-transfected cells on Day 7.

Conclusions

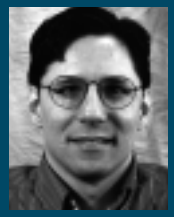
By generating photoresistance, removing consensus transcription factor binding sites and optimizing codon usage, the novel *gfp* gene originally cloned from *M. cavernosa* has been developed into a highly fluorescent, well-tolerated *gfp* reporter gene. Additionally, convenient restriction sites that allow the creation of N- and C-terminal fusion proteins have been added. Using FACS™ and fluorescent microscopic analysis, CHO and NIH 3T3 transfections with the Monster Green™ Fluorescent Protein gene (hMGFP) and other commercially available *gfp* genes demonstrate that Promega's hMGFP is brighter and has longer persistence of expression than another commercially available GFP.

Reference

1. Liu, H.S. *et al.* (1999) *Biochem. Biophy. Res. Comm.* **260**, 712–717.

Protocol

◆ *Monster Green™ Fluorescent Protein phMGFP Vector Technical Bulletin*, #TB320, Promega Corporation. (www.promega.com/tbs/tb320/tb320.html)



Brian Almond, Ph.D.
Research Scientist

Ordering Information

Product	Size	Cat.#
Monster Green Fluorescent Protein phMGFP™ Vector[a,b,c,d]	20µg	E6421

[a] Patent Pending.

[b] Certain applications of this product may require licenses from others.

[c] The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

[d] For Nonprofit Organization Research Use Only. With respect to research use by for-profit organizations, or any diagnostic or therapeutic uses, please contact Promega Corporation for supply and licensing information.