



Vitality hrGFP II Mammalian Expression Vectors

Instruction Manual

Catalog #240143 (p hrGFP II-1)
#240144 (p hrGFP II-C)
#240145 (p hrGFP II-N)
#240157 (pIRES-hrGFP II)

Revision E.0

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240143-12



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Vitality hrGFP II Mammalian Expression Vectors

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Vitality hrGFP II Mammalian Expression Vectors

MATERIALS PROVIDED

| Materials provided | Concentration | Quantity |
|--|---------------|----------|
| p hrGFP II-1 mammalian expression vector (Catalog #240143) | 1.0 µg/µl | 20 µg |
| p hrGFP II-C mammalian expression vector (Catalog #240144) | 1.0 µg/µl | 20 µg |
| p hrGFP II-N mammalian expression vector (Catalog #240145) | 1.0 µg/µl | 20 µg |
| pIRES-hrGFP II mammalian expression vector (Catalog #240157) | 1.0 µg/µl | 20 µg |

STORAGE CONDITIONS

All Components: -20°C

ADDITIONAL MATERIALS REQUIRED

Anti-FLAG® M2 antibody (Catalog #200471 or 200472)

Ligase buffer[§]

T4 DNA ligase

Taq2000 DNA polymerase

Taq DNA polymerase buffer

TE buffer[§]

[§] See *Preparation of Media and Reagents*.

Revision E.0

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INTRODUCTION

The green fluorescent protein (GFP) has become an extremely versatile tool for tracking and quantifying biological entities in the fields of biochemistry, molecular and cell biology, as well as high throughput screening and gene discovery.^{1,2} GFPs have been identified in a wide range of coelenterates, and while recently the number of cloned GFPs has expanded, to date the best characterized proteins are those from the jellyfish *Aequorea victoria* and the anthozoan *Renilla reniformis*.

We have isolated a cDNA clone for the *R. reniformis* GFP, and have fully humanized the gene using codons preferred in highly expressed human genes. The humanized recombinant GFP (hrGFP) cDNA was then subjected to random mutagenesis to generate the brighter variant, hrGFP II. The fluorescence spectra for hrGFP and hrGFP II are essentially identical to the published spectrum for the purified native protein, with the major excitation peak at 500 nm and the emission peak at 506 nm (see Figure 8).³

We have expressed the hrGFP II in a wide range of human, rodent, and simian cell lines (see Figures 1 and 2), and observed levels of fluorescence higher than that for the red-shifted, humanized variant of *Aequorea* GFP (EGFP) in all cell-types tested (see Figure 3). In addition, stable GFP-expressing cell lines are produced much more efficiently using the Agilent hrGFP compared with EGFP, since *Aequorea* GFP is often cytotoxic when expressed at low levels.⁴

Agilent offers four Vitality hrGFP II vectors* for mammalian expression. These vectors support a variety of expression configurations, thus providing ideal expression options for each specific application. The hrGFP II allows expressed genes to be easily visualized using fluorescence microscopy or fluorescence-activated cell sorting (FACS). We also offer a Vitality full-length hrGFP polyclonal antibody (Catalog #240141 and #240142) which recognizes the wild type green fluorescent protein, hrGFP, and the hrGFP II mutant as well as N- and C- terminal fusions to these proteins. Applications include Western blotting, immunoprecipitation, and flow cytometry. Because hrGFP is derived from a different organism, the hrGFP antibody does not cross react with the *Aequorea victoria* GFP variant, EGFP.

* U.S. Patent No. 7,083,931.

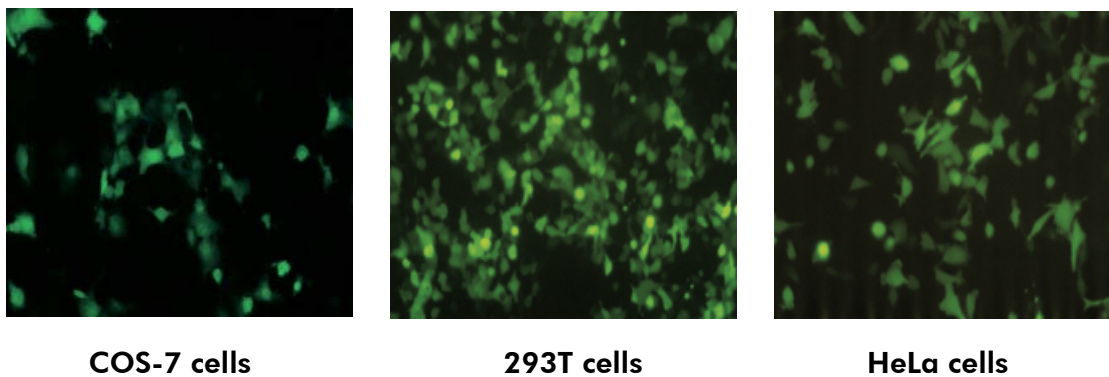


FIGURE 1 The *Renilla reniformis* humanized recombinant green fluorescent protein II (hrGFP II) was expressed in a range of mammalian cell types by transfection with the phrGFP II-C vector. Cells were plated at $2.4\text{--}6 \times 10^5$ cells per well in 6-well plates the day before transfection. Transfection was performed using a cationic lipid reagent at DNA:reagent (w/v) ratios of 1:1.25 (HeLa) and 1:2 (COS-7 and 293T) and incubated for 5 hours. All images were taken with a 10-second exposure time.

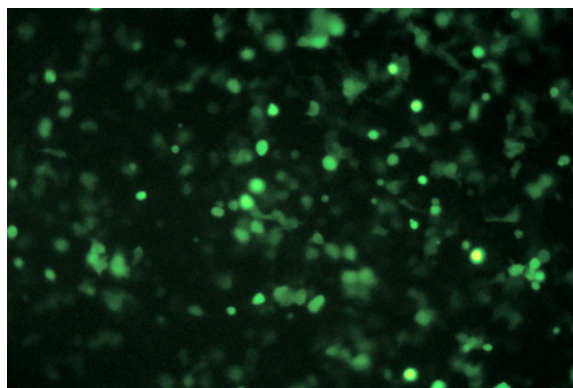


FIGURE 2 The *Renilla reniformis* humanized recombinant green fluorescent protein II (hrGFP II) was expressed in 293T cells by transfection with the pRES-hrGFP II vector. Cells were plated at 6×10^5 cells per well in a 6-well plate the day before transfection. Transfection was performed using a cationic lipid reagent at DNA:reagent (w/v) ratio of 1:2 and incubated for 5 hours. The image was taken 48 hours post transfection with a 10-second exposure time.

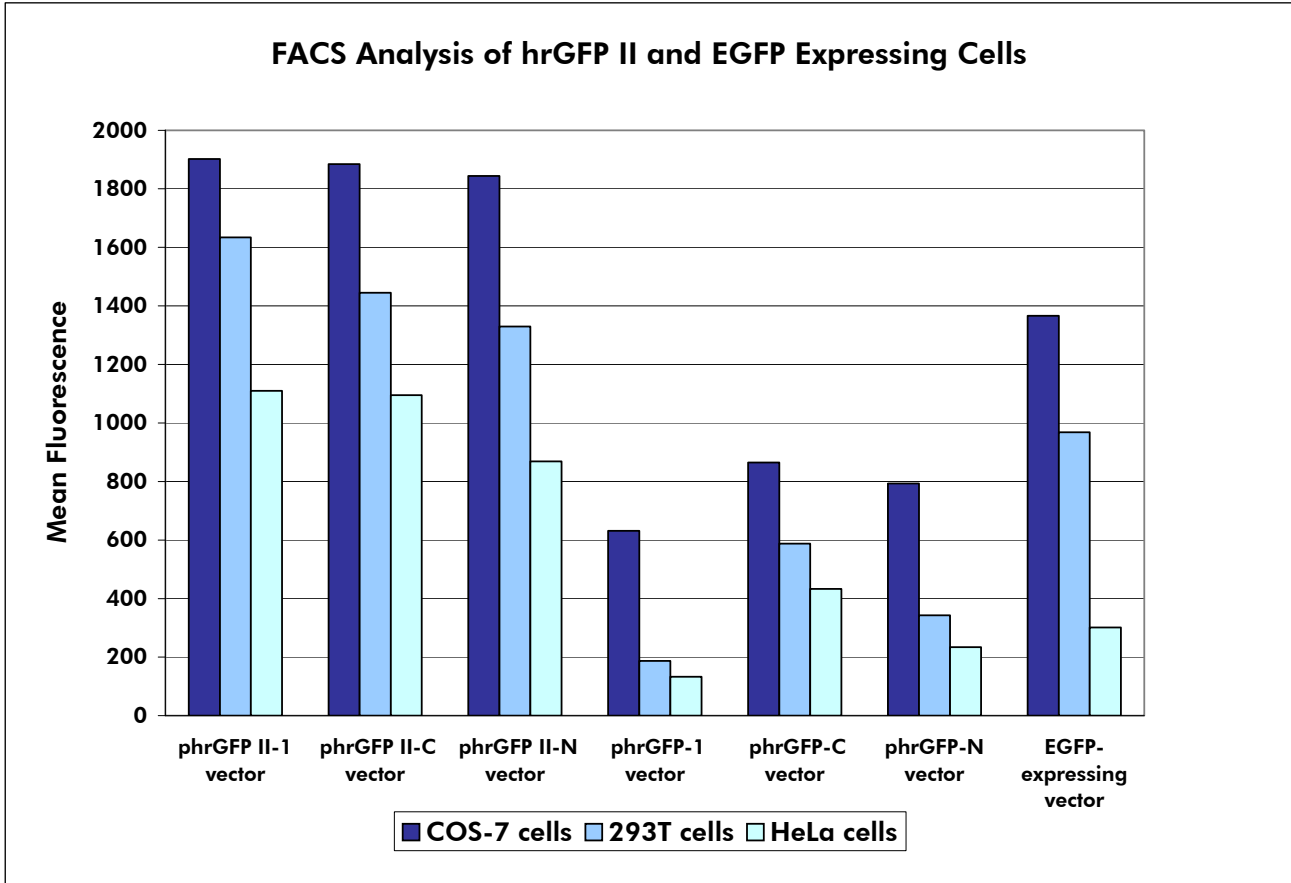


FIGURE 3 FACS analysis of reporter brightness. Transient transfections of COS-7, 293T, and HeLa cells with various GFP-expressing vectors were performed using a cationic lipid reagent at DNA:reagent (w/v) ratios of 1:1.25 (HeLa) and 1:2.5 (COS-7 and 293T). The cells were collected at 24 hours post-transfection for FACS analysis. Mean fluorescence is defined by the total fluorescence in the marker region (M1) divided by the total cells collected in the marker region (M1).

Description of the Vectors

The phrGFP II-C, phrGFP II-N, pIRES-hrGFP II, and phrGFP II-1 mammalian expression vectors contain the CMV promoter, which directs constitutive, high-level expression of hrGFP II RNA transcripts of the hrGFP II reporter alone, the co-expressed hrGFP II reporter and gene of interest, or as fusion proteins in many cell lines. These cell lines include COS-7, 293T, HeLa, and CHO-K1.

Each vector contains the neomycin/kanamycin-resistance gene under control of the β -lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells. In addition, all four hrGFP II vectors contain the bovine growth hormone polyadenylation sequence (BGHpA) for improved stability and translatability of mRNA.

phrGFP II-C and -N Vectors

The vectors phrGFP II-C and phrGFP II-N allow fusion of hrGFP II at either the C-terminus or the N-terminus of a protein of interest. phrGFP II-C contains a copy of the hrGFP II gene downstream of the MCS, allowing fusion of hrGFP II to the C-terminus of the protein of interest (see Figure 4). phrGFP II-N contains a copy of the hrGFP II gene that lacks a translational termination codon inserted upstream of the MCS to allow fusion of hrGFP II to the N-terminus of the protein of interest (see Figure 5).

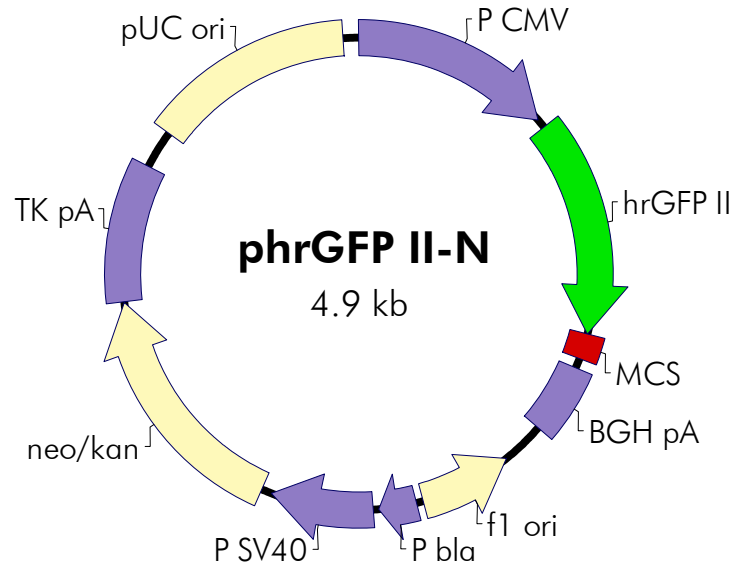
pIRES-hrGFP II Vector

The pIRES-hrGFP II vector contains a bicistronic expression cassette in which the MCS is followed by the EMCV-IRES linked to the hrGFP II coding sequence. This design allows the expression of a gene of interest to be monitored at the single-cell level due to expression of hrGFP II on the same transcript. The gene of interest may be fused to three contiguous copies of the FLAG[®] epitope, provided by the vector (see Figure 6). For FLAG tag fusions, Western blot analysis using the anti-FLAG antibody at a 1:500 dilution will detect the protein of interest.

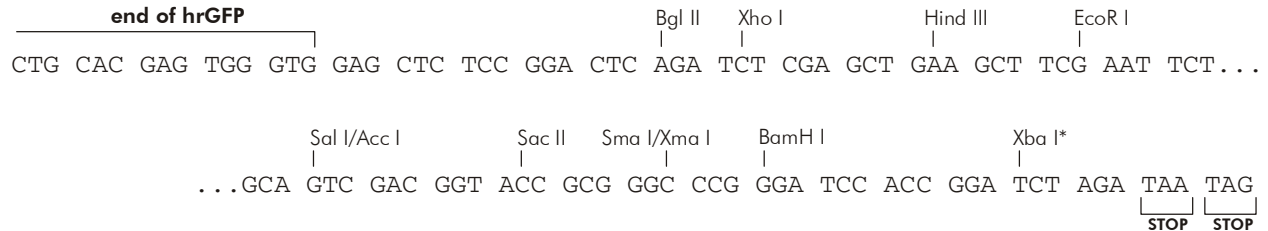
phrGFP II-1 Vector

The phrGFP II-1 vector contains the hrGFP II gene, which includes a Kozak consensus sequence and termination codons directly between two multiple cloning sites for easy transfer of the hrGFP II module to new vectors (see Figure 7).

phrGFP II-N Vector Map



phrGFP II-N Multiple Cloning Site Region (sequence shown 1398–1502)

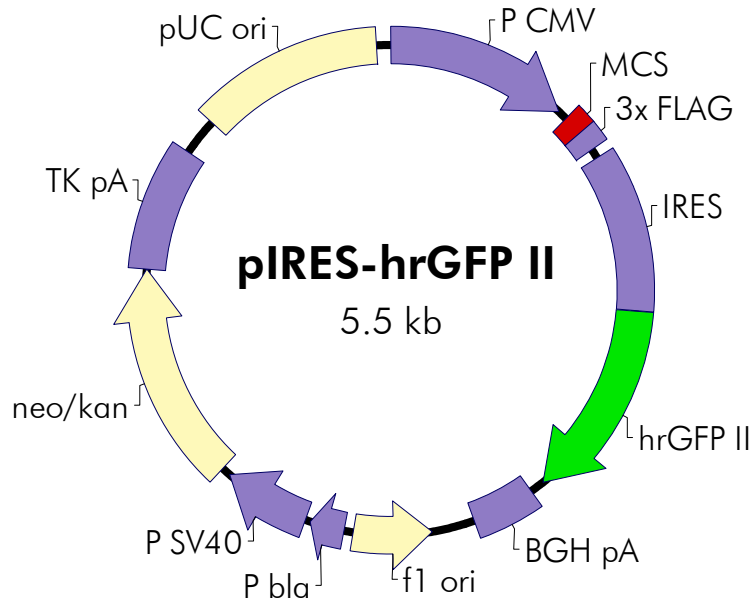


* If restriction using Xba I is desired, the phrGFP II-N plasmid DNA must first be purified from a *dam*⁻ *E. coli* strain.

| Feature | Nucleotide Position |
|---|---------------------|
| CMV promoter | 1–665 |
| T7 primer binding site [5' TAATACGACTCACTATAGGG 3'] | 665–684 |
| hrGFP II ORF | 696–1412 |
| forward sequencing primer binding site (for 5' end of insert) [5' GAGTACCACTTCATCCAGCA 3'] | 1281–1300 |
| multiple cloning site (Sac I to Xba I) | 1413–1496 |
| stop codons | 1497–1502 |
| reverse sequencing primer binding site (for 3' end of insert) [5' AAGGACAGTGAGAGTGGCAC 3'] | 1620–1639 |
| bovine growth hormone (BGH) polyA signal | 1530–1761 |
| f1 origin of ss-DNA replication | 1906–2212 |
| <i>bla</i> promoter | 2237–2361 |
| SV40 promoter | 2381–2719 |
| neomycin/kanamycin resistance ORF | 2754–3545 |
| HSV-thymidine kinase (TK) polyA signal | 3549–4004 |
| pUC origin of replication | 4133–4800 |

FIGURE 5 Features of the phrGFP II-N mammalian expression vector.

pIRES-hrGFP II Vector Map



pIRES-hrGFP II Multiple Cloning Site Region (sequence shown 685–795)

AGA CCC AAG CTT CTG GAG ^{Srf I} GCC CGG GCT TTC AGG GTA CCG AAG AAG GAT CCA AGG AGG...
^{Sma I/Xma I} ^{BamH I} ^{EcoR I}

...AAT TCT GCA GAT ATC CAT CAC ACT GGC GGC CGC GAC TAC AAG GAT GAC GAT GAC
^{EcoR V} ^{Not I} **start of FLAG tag**

| Feature | Nucleotide Position |
|---|---------------------|
| CMV promoter | 1–665 |
| forward sequencing primer binding site (for 5' end of insert) [5' ATGGGCGGTAGGCGTGTA 3'] | 576–593 |
| T7 primer binding site [5' TAATACGACTCACTATAGGG 3'] | 665–684 |
| multiple cloning site (<i>Srf I</i> to <i>Not I</i>) | 703–774 |
| 3× FLAG tag | 775–846 |
| reverse sequencing primer binding site (for 3' end of insert) [5' ATGCAGTCGTCGAGGAATTG 3'] | 853–872 |
| internal ribosome entry site (IRES) | 882–1456 |
| hrGFP II ORF | 1460–2176 |
| T3 primer binding site [5' AATTAACCCTCACTAAAGGG 3'] | 2209–2228 |
| bovine growth hormone (BGH) polyA signal | 2230–2460 |
| f1 origin of ss-DNA replication | 2617–2901 |
| <i>bla</i> promoter | 2937–3061 |
| SV40 promoter | 3081–3398 |
| neomycin/kanamycin resistance ORF | 3433–4224 |
| HSV-thymidine kinase (TK) polyA signal | 4225–4674 |
| pUC origin of replication | 4812–5479 |

FIGURE 6 Features of the pIRES-hrGFP II mammalian expression vector.

| Feature | Nucleotide Position |
|--|----------------------------|
| CMV promoter | 1–665 |
| forward sequencing primer binding site (for 5' end of insert in MCS 1) [5' AAATGGGCGGTAGGCGGTACGGTG 3'] | 574–598 |
| T7 primer binding site [5' TAATACGACTCACTATAGGG 3'] | 665–684 |
| multiple cloning site 1 (<i>Hind</i> III to <i>Bam</i> H I) | 691–734 |
| Kozak sequence | 733–741 |
| reverse sequencing primer binding site (for 3' end of insert in MCS 1) [5' ACCAGCTGGTTGCCGAACAG 3'] | 861–880 |
| hrGFP II ORF | 738–1454 |
| forward sequencing primer binding site (for 5' end of insert in MCS 2) [5' GAGTACCACTTCATCCAGCA 3'] | 1323–1342 |
| stop codons | 1455–1460 |
| multiple cloning site 2 (<i>Eco</i> R I to <i>Apa</i> I) | 1461–1508 |
| T3 primer binding site [5' AATTAACCCTCACTAAAGGG 3'] | 1513–1532 |
| stop codons | 1518–1537 |
| reverse sequencing primer binding site (for 3' end of insert in MCS 2) [5' AAGGACAGTGAGAGTGGCAC 3'] | 1624–1643 |
| bovine growth hormone (BGH) polyA signal | 1534–1764 |
| f1 origin of ss-DNA replication | 1910–2216 |
| <i>bla</i> promoter | 2241–2365 |
| SV40 promoter | 2385–2723 |
| neomycin/kanamycin resistance ORF | 2758–3549 |
| HSV-thymidine kinase (TK) polyA signal | 3553–4008 |
| pUC origin of replication | 4137–4804 |

FIGURE 7 Features of the phrGFP II-1 mammalian expression vector.

PREPARING THE HRGFP II VECTORS

- ◆ Ensure that the coding sequence of the insert is in the correct reading frame and that it contains an initiation codon or Kozak sequence.⁵ For gene fusions using the phrGFP II-C vector, ensure that the gene of interest lacks a termination codon, and reads in frame with the hrGFP II sequence (see the MCS sequence in Figure 4). For gene fusions using the phrGFP II-N vector, ensure that the gene of interest is inserted in frame with the hrGFP II coding sequence. If the insert lacks its own termination codon, termination codons at the 3' end of the MCS may be used (see the MCS sequence in Figure 5).
- ◆ When cloning into the pIRES-hrGFP II vector, the gene of interest may be fused to three contiguous copies of the FLAG epitope, provided by the vector. For fusion with the FLAG tag, ensure that the gene of interest lacks a termination codon and that it reads in frame with the FLAG tag sequence (see the MCS sequence in Figure 6). A stop codon exists at the end of the FLAG tag sequence. Alternatively, to avoid fusion to the FLAG tag, include a termination codon at the end of the gene of interest.
- ◆ We recommend dephosphorylation of the digested vector with CIAP prior to ligation with the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and removing the desired plasmid band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.
- ◆ After purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the plasmid DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

LIGATING THE INSERT

The ideal insert-to-vector molar ratio of DNA is variable; however, a reasonable starting point is a 2:1 insert-to-vector ratio. The ratio is calculated using the following equation:

$$X \text{ } \mu\text{g of insert} = \frac{(\text{Number of base pairs of insert}) (0.1 \text{ } \mu\text{g of Vitality hrGFP II vector})}{\sim Y \text{ bp of Vitality hrGFP II vector}}$$

where X is the quantity of insert (in micrograms) required for a 1:1 insert-to-vector molar ratio. Multiply X by 2 to get the quantity of insert required for a 2:1 ratio. Y is the size (in base pairs) of the Vitality hrGFP II vector being used; consult the circular vector maps for sizes.

1. Prepare three control and two experimental 10- μ l ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

| Ligation reaction components | Control | | | Experimental | |
|---|----------------|----------------|----------------|----------------|----------------|
| | 1 ^a | 2 ^b | 3 ^c | 4 ^d | 5 ^d |
| Prepared vector (0.1 μ g/ μ l) | 1.0 μ l | 1.0 μ l | 0.0 μ l | 1.0 μ l | 1.0 μ l |
| Prepared insert (0.1 μ g/ μ l) | 0.0 μ l | 0.0 μ l | 1.0 μ l | Y μ l | Y μ l |
| rATP [10 mM (pH 7.0)] | 1.0 μ l | 1.0 μ l | 1.0 μ l | 1.0 μ l | 1.0 μ l |
| Ligase buffer (10 \times) ^e | 1.0 μ l | 1.0 μ l | 1.0 μ l | 1.0 μ l | 1.0 μ l |
| T4 DNA ligase (4 U/ μ l) | 0.5 μ l | 0.0 μ l | 0.5 μ l | 0.5 μ l | 0.5 μ l |
| Double-distilled (ddH ₂ O) to 10 μ l | 6.5 μ l | 7.0 μ l | 6.5 μ l | Z μ l | Z μ l |

^a This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies if the digestion and CIAP treatment are effective.

^b This control indicates whether the plasmid is cleaved completely or whether residual uncut plasmid remains. Expect an absence of transformant colonies if the digestion is complete.

^c This control verifies that the insert is not contaminated with the original plasmid. Expect an absence of transformant colonies if the insert is pure.

^d These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies to represent recombinants.

^e See *Preparation of Media and Reagents*.

2. Incubate the reactions for 2 hours at room temperature (22°C) or overnight at 4°C. For blunt-end ligation, reduce the rATP to 5 mM and incubate the reactions overnight at 12–14°C.

TRANSFORMATION

Transform competent bacteria with 1–2 μl of the ligation reaction, and plate the transformed bacteria on LB–kanamycin agar plates (see *Preparation of Media and Reagents*). Refer to references 6 and 7 for bacterial transformation protocols.

Note *Competent cells with transformation efficiencies $\geq 5 \times 10^9$ cfu/ μg are available from Agilent.*

VERIFICATION OF INSERT PERCENTAGE, SIZE, AND ORIENTATION

Individual colonies can be examined to determine the percentage of vectors with inserts and the insert size and orientation by PCR directly from the colony or by restriction analysis.

PCR Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in any of the Vitality hrGFP II vectors may be determined by PCR amplification of DNA from individual colonies.

1. Prepare a PCR amplification reaction containing the following components:

5.0 μl of 10 \times *Taq* DNA polymerase buffer
0.4 μl of dNTP mix (25 mM each dNTP)
100.0 ng of the appropriate 5' primer (see table below for sequence)
100.0 ng of the appropriate 3' primer (see table below for sequence)
0.5 μl of 10% (v/v) Tween[®] 20
2.5 U of *Taq2000* DNA polymerase
dH₂O to a final volume of 50 μl

Primers for hrGFP II Vectors

| Vector | Primer | Nucleotide sequence (5' to 3') |
|----------------|---------------|---------------------------------|
| p hrGFP II-C | Forward | 5' AAATGGGCGGTAGGCGTGTACGGTG 3' |
| | Reverse | 5' ACCAGCTGGTTGCCGAACAG 3' |
| p hrGFP II-N | Forward | 5' GAGTACCACTTCATCCAGCA 3' |
| | Reverse | 5' AAGGACAGTGAGAGTGGCAC 3' |
| pIRES-hrGFP II | Forward | 5' ATGGGCGGTAGGCGTGTA 3' |
| | Reverse | 5' ATGCAGTCGTCGAGGAATTG 3' |
| p hrGFP II-1 | MCS 1 Forward | 5' AAATGGGCGGTAGGCGTGTACGGTG 3' |
| | MCS 1 Reverse | 5' ACCAGCTGGTTGCCGAACAG 3' |
| | MCS 2 Forward | 5' GAGTACCACTTCATCCAGCA 3' |
| | MCS 2 Reverse | 5' AAGGACAGTGAGAGTGGCAC 3' |

2. Touch the transformed colonies with a sterile toothpick and streak onto antibiotic-containing patch plates for future reference. Incubate the patch plates at 37°C overnight. Immediately following the streak onto plates, swirl this same toothpick into a separate PCR reaction tube.
3. Gently mix each reaction. If not using a cycler with a hot top, overlay each reaction with 30 µl of mineral oil and perform PCR using the following cycling parameters:

Cycling Conditions for RoboCycler Temperature Cyclers and Single-Block Thermal Cyclers

| Number of cycles | Temperature | Length of time |
|------------------|-------------|------------------|
| 1 cycle | 92°C | 2 minutes |
| 30 cycles | 95°C | 30 seconds |
| | 56°C | 30 seconds |
| | 72°C | 2 minutes per kb |
| 1 cycle | 72°C | 5 minutes |

4. Analyze the PCR products for the sizes of the gene inserted into the expression construct using standard 1% (w/v) agarose gel electrophoresis. Additional information can be obtained by further restriction analysis of the PCR products.
5. Following identification of colonies containing the correct insert, return to the patch plates made in step 2 above and pick a portion of each of the positive colonies into 5-ml aliquots of LB-kanamycin broth (see *Preparation of Media and Reagents*). Grow overnight at 37°C with shaking.
6. The next morning, purify the plasmid DNA from the liquid cultures.

MAMMALIAN CELL TRANSFECTION

When the correct recombinant plasmids are confirmed, prepare enough DNA of appropriate purity for the mammalian cell transfection procedure to be carried out. Protocols for transfection of mammalian cell lines can be found in Sambrook, *et al.* (1989).⁶

The efficiency of transfection will vary depending on the host cell line used. In most cases, mammalian host cell lines transfected with plasmids should show expression of hrGFP II 24–72 hours after transfection. Fluorescing cells growing in tissue culture dishes can be observed using an inverted fluorescence microscope. Fluorescence of populations of harvested cells can also be measured using FACS analysis or fluorometer assays. Table I lists excitation and emission spectra for Agilent hrGFP II and original hrGFP, as compared to EGFP. See Figure 8 for plotted hrGFP II spectra.

SPECIFICATIONS FOR GFP EXCITATION AND EMISSION SPECTRA

TABLE I

GFP Spectra Maxima

| GFP Form ^a | Excitation/Emission Spectra Maxima (nm) |
|-----------------------|---|
| hrGFP II | 500/506 |
| hrGFP | 500/506 |
| EGFP | 488/509 ^b |

^a All forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

^b The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note Filter sets compatible with the detection of hrGFP II, hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see www.omegafilters.com):

Exciter filter: XF1073

Emitter filter: XF3084

Beam splitter: XF2010

Microscope cube set with the exciter filter, emitter filter and beam splitter: XF100-2

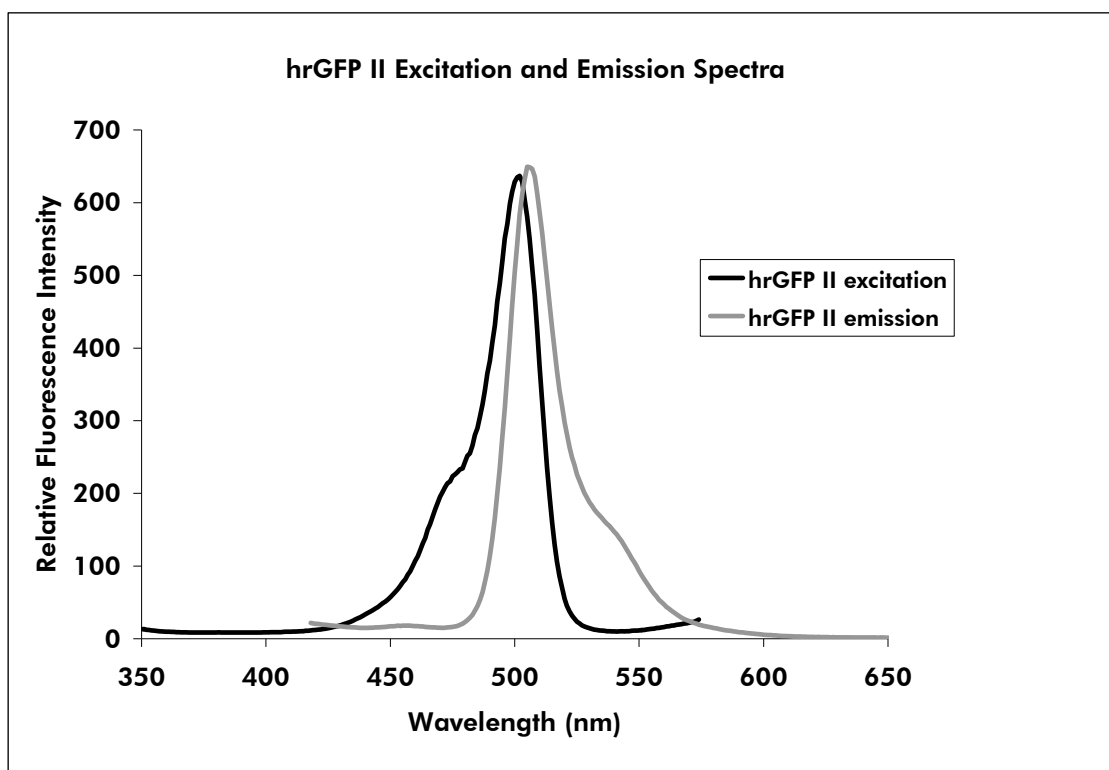


FIGURE 8 Excitation and emission spectra of hrGFP II (excitation maximum = 500 nm; emission maximum = 506 nm).

TROUBLESHOOTING

| Observation | Suggestion |
|---|--|
| No signal detected in the flow cytometry F1 channel | Verify that the transfection was successful by fluorescence microscopy before harvesting the cells for flow cytometry. |
| Western analysis does not detect fusion protein | Insert is cloned out of frame. Sequence to ensure correct reading frame. Reclone if insert is out of frame. |
| | Assay is not sufficiently sensitive or is being performed incorrectly. Use a positive control. |

PREPARATION OF MEDIA AND REAGENTS

| | |
|--|--|
| <p>LB Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Add deionized H₂O to a final volume of 1 liter Adjust pH to 7.0 with 5 N NaOH Autoclave Cool to 55°C. Add appropriate antibiotic. Pour into petri dishes (~25 ml/100-mm plate)</p> | <p>LB Broth (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract Add deionized H₂O to a final volume of 1 liter Adjust pH to 7.0 with 5 N NaOH Autoclave</p> |
| <p>LB-Kanamycin Agar (per Liter) Prepare 1 liter of LB agar Autoclave Cool to 55°C Add 5 ml of 10-mg/ml filter-sterilized kanamycin Pour into petri dishes (~25 ml/100-mm plate)</p> | <p>LB-Kanamycin Broth (per Liter) Prepare 1 liter of LB broth Autoclave Cool to 55°C Add 5 ml of 10-mg/ml filter-sterilized kanamycin</p> |
| <p>10× Ligase Buffer 500 mM Tris-HCl (pH 7.5) 70 mM MgCl₂ 10 mM dithiothreitol (DTT)</p> <p>Note <i>rATP is added separately in the ligation reaction</i></p> | <p>TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA</p> |

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at <http://www.genomics.agilent.com>. MSDS documents are not included with product shipments.