# Vitality® hrGFP Mammalian Expression Vectors pIRES-hrGFP-1a, pIRES-hrGFP-2a, phrGFP-C, phrGFP-N1, phrGFP-1, and phrGFP

## INSTRUCTION MANUAL

Catalog #240031 (pIRES-hrGFP-1a)
#240032 (pIRES-hrGFP-2a)
#240035 (phrGFP-C)
#240036 (phrGFP-N1)
#240059 (phrGFP-1)
#240062 (phrGFP)

Revision #064007g

**For In Vitro Use Only**

*240031-12 064007G/*

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# Vitality® hrGFP Mammalian Expression Vectors pIRES-hrGFP-1a, pIRES-hrGFP-2a, phrGFP-C, phrGFP-N1, phrGFP-1, and phrGFP

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# Vitality® hrGFP Mammalian Expression Vectors pIRES-hrGFP-1a, pIRES-hrGFP-2a, phrGFP-C, phrGFP-N1, phrGFP-1, and phrGFP

## MATERIALS PROVIDED

| Material provided | Concentration | Quantity |
|---|---|---|
| pIRES-hrGFP-1a (Catalog #240031) | 1.0 µg/µl | 20 µg |
| pIRES-hrGFP-2a (Catalog #240032) | 1.0 µg/µl | 20 µg |
| phrGFP-C (Catalog #240035) | 1.0 µg/µl | 20 µg |
| phrGFP-N1 (Catalog #240036) | 1.0 µg/µl | 20 µg |
| phrGFP-1 (Catalog #240059) | 1.0 µg/µl | 20 µg |
| phrGFP (Catalog #240062) | 1.0 µg/µl | 20 µg |

## STORAGE CONDITIONS

**All Components:** –20°C

## ADDITIONAL MATERIALS REQUIRED

### Sterile Media and Reagents

T4 DNA ligase
Ligase buffer[§]
*Taq* DNA polymerase[†]
*Taq* DNA polymerase buffer[††]
TE buffer[§]

### Equipment

Falcon® 2059 polypropylene tubes (15-ml)
Water baths (37°C and 42°C)

## OPTIONAL ADDITIONAL MATERIALS

*Cre* recombinase [Stratagene Catalog #600270]
pExchange module EC-Hyg [Stratagene Catalog #211181]
pExchange module EC-Puro [Stratagene Catalog #211182]
pExchange module EC-Neo [Stratagene Catalog #211183]
XL1-Blue supercompetent cells [Stratagene Catalog #200236]

[§] See *Preparation of Media and Reagents*.
[†,††] See *Endnotes*.

Revision #064007g

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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*. *Aequorea* GFP forms weak homodimers at moderate to low concentrations, and is often cytotoxic when expressed at low levels. Due to this latter characteristic, researchers have often been frustrated in their attempts to produce stable GFP-expressing cells lines using the *Aequorea* protein.[3]

At Stratagene we have isolated a cDNA clone for GFP from a novel marine organism, and have fully humanized the gene using codons preferred in highly expressed human genes. The fluorescence spectrum for the cloned GFP protein is 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. We have expressed the protein in a wide range of human, rodent, and simian cell lines, and observed levels of fluorescence comparable to that for the red-shifted, humanized variant of *Aequorea* GFP (EGFP) in all cell-types tested. In viability experiments, we find that high level expression of functional fluorescent protein in retrovirus-transduced cells is substantially more consistent and less toxic over time and passage number for the humanized recombinant GFP (hrGFP) than for EGFP. (See http://www.stratagene.com/vectors/gfp/vitality_gfp.htm.) Thus the stable GFP-expressing cell lines are produced much more efficiently using Stratagene's hrGFP compared with EGFP.

Stratagene offers six Vitality® hrGFP vectors for mammalian expression. These vectors support a variety of expression configurations, thus providing ideal expression options for each specific application. The hrGFP allows expressed genes to be easily visualized using fluorescence microscopy or fluorescence-activated cell sorting (FACS).

## Description of the Vectors

### pIRES-hrGFP-1a and -2a Vectors

The pIRES-hrGFP vectors (see Figures 1 and 2) contain a dicistronic expression cassette in which the multiple cloning site (MCS) is followed by the EMCV-IRES linked to the hrGFP 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 on the same transcript. The gene of interest may be fused to three contiguous copies of either the FLAG® epitope (pIRES-hrGFP-1a) or the HA epitope (pIRES-hrGFP-2a). The vectors are derived from the vector pExchange-1, and thus take advantage of *Cre*-mediated site-specific recombination to allow quick and efficient directional insertion of prefabricated modules. With the pIRES-hrGFP-based vectors, a wide variety of drug-resistant markers may be readily substituted into the core hrGFP vector bearing the gene of interest.

Figures 1 and 2 show circular maps and locations of important features for pIRES-hrGFP-1a and pIRES-hrGFP-2a, respectively.

### phrGFP-C and -N1 Vectors

The vectors phrGFP-N1 and phrGFP-C allow fusion of hrGFP at either the N-terminus or the C-terminus of a protein of interest. phrGFP-C contains a copy of the hrGFP gene downstream of the MCS, allowing fusion of hrGFP to the C-terminus of the protein of interest (Figure 3). phrGFP-N1 contains a copy of the hrGFP gene that lacks a translational termination codon inserted upstream of a versatile MCS to allow fusion of hrGFP to the N-terminus of the protein of interest (Figure 4). The vectors are derived from the vector pExchange-1, and thus take advantage of *Cre*-mediated site-specific recombination to allow quick and efficient directional insertion of prefabricated modules.

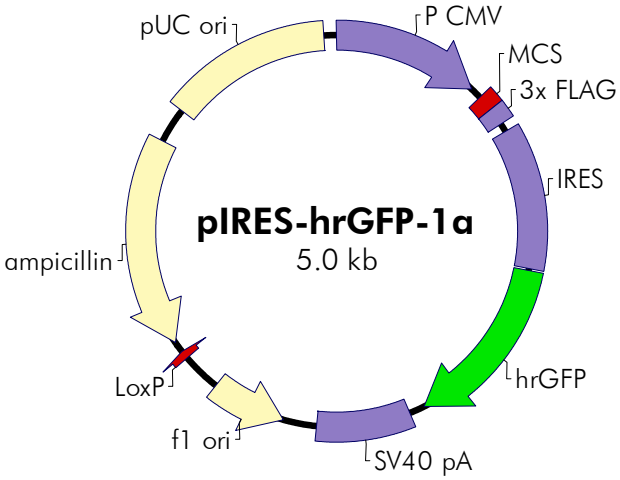
### phrGFP-1 Vector

The phrGFP-1 vector contains the hrGFP gene, which includes a Kozak consensus sequence and termination codons directly between two multiple cloning sites for easy transfer of the hrGFP module to new vectors (Figure 5). This vector is derived from the vector pExchange-1, and thus takes advantage of *Cre*-mediated site-specific recombination to allow quick and efficient directional insertion of prefabricated modules.

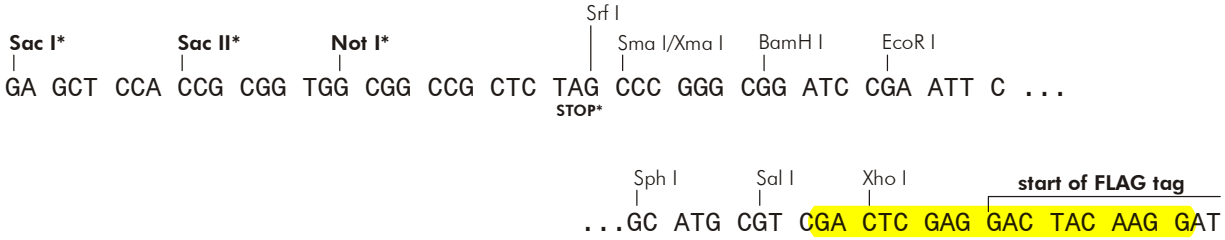
### phrGFP Vector

The phrGFP vector contains the hrGFP gene and the SV40 polyadenylation signal. A *LoxP* site allows easy integration of hygromycin, neomycin, or puromycin genes via pre-existing modules from the Exchanger system in an easy 30-minute reaction using *Cre* recombinase. The phrGFP vector lacks a eukaryotic promoter. Desired promoter/enhancer elements are inserted upstream of the hrGFP gene via the extensive multiple cloning site. The hrGFP gene itself contains a Kozak consensus sequence and termination codon. See Figure 6.

## The pIRES-hrGFP-1a Vector




## pIRES-hrGFP-1a Multiple Cloning Site Region
**(sequence shown 651–727)**

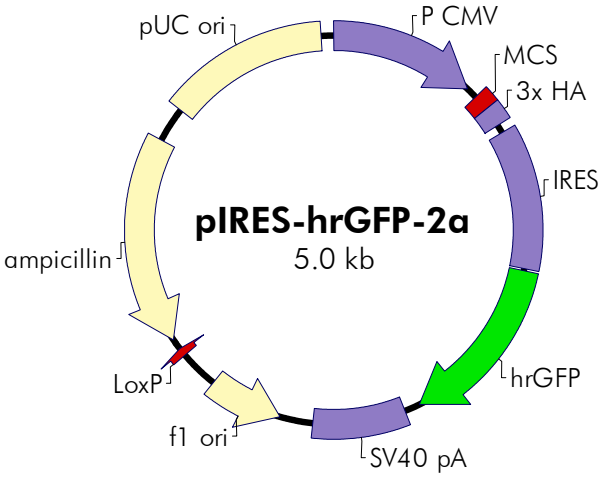



* The presence of a stop codon (TAG) in-frame with the 3×FLAG tag must be considered when inserting genes into the MCS. Do not use the *Sac* I, *Sac* II, or *Not* I sites for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.

| Feature | Nucleotide Position |
|---|---|
| CMV promoter | 1–602 |
| T3 primer binding site (for 5′ end of insert) [5′ AATTAACCCTCACTAAAGGG 3′] | 620–639 |
| multiple cloning site | 651–715 |
| stop codon (in frame with 3× FLAG tag) | 677–679 |
| 3× FLAG tag | 716–787 |
| primer binding site (for 3′ end of insert) [5′ GTCCTTATCATCGTCGTCTT 3′] | 747–766 |
| internal ribosome entry site (IRES) | 823–1397 |
| hrGFP ORF | 1407–2123 |
| T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′] | 2155–2176 |
| SV40 polyA signal | 2188–2571 |
| f1 origin of ss-DNA replication | 2709–3015 |
| LoxP sequence | 3178–3211 |
| ampicillin resistance (*bla*) ORF | 3256–4113 |
| pUC origin of replication | 4260–4927 |

**FIGURE 1** Features of the pIRES-hrGFP-1a Mammalian Expression Vector.

## The pIRES-hrGFP-2a Vector




## pIRES-hrGFP-2a Multiple Cloning Site Region
**(sequence shown 651–727)**

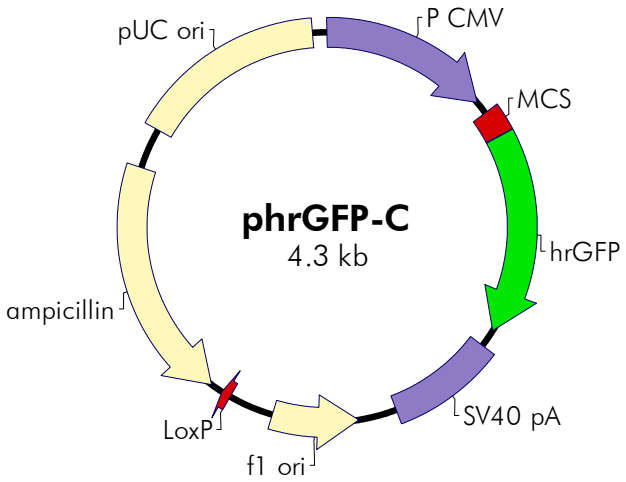
```
                                                    Srf I
Sac I*          Sac II*         Not I*              |   Sma I/Xma I   BamH I      EcoR I
|               |               |                   |   |             |          |
GA GCT CCA CCG CGG TGG CGG CCG CTC TAG CCC GGG CGG ATC CGA ATT C ...
                                   STOP*

                                  Sph I       Sal I       Xho I
                                  |           |           |          start of HA tag
...GC ATG CGT CGA CTC GAG TAC CCA TAT GAC
```

* The presence of a stop codon (TAG) in-frame with the 3×HA tag must be considered when inserting genes into the MCS. Do not use the *Sac* I, *Sac* II, or *Not* I sites for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.

| Feature | Nucleotide Position |
|---|---|
| CMV promoter | 1–602 |
| T3 primer binding site (for 5′ end of insert) [5′ AATTAACCCTCACTAAAGGG 3′] | 620–639 |
| multiple cloning site | 651–715 |
| Stop codon (in-frame with 3×HA tag) | 677–679 |
| 3×HA tag | 716–796 |
| Primer binding site (for 3′ end of insert) [5′ TAAGCGTAGTCAGGTACATC 3′] | 779–798 |
| internal ribosome entry site (IRES) | 832–1406 |
| hrGFP ORF | 1416–2132 |
| T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′] | 2164–2185 |
| SV40 polyA signal | 2197–2580 |
| f1 origin of ss-DNA replication | 2718–3024 |
| LoxP sequence | 3187-3220 |
| ampicillin resistance (*bla*) ORF | 3265–4122 |
| pUC origin of replication | 4269–4936 |

**FIGURE 2** Features of the pIRES-hrGFP-2a Mammalian Expression Vector.

## The phrGFP-C Vector



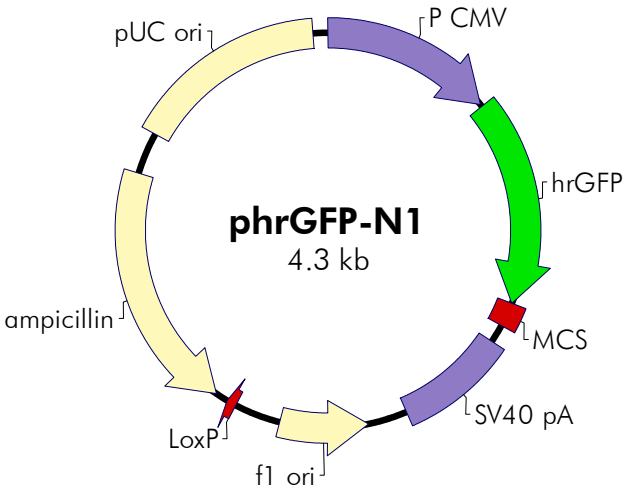

## phrGFP-C Multiple Cloning Site Region
**(sequence shown 646–761)**

```
          Sac I          Sac II         Not I                                  BamH I
AG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG GGC GGA TCC CCC GGG CTG...

                  EcoR I      EcoR V     Hind III    Cla I           Sal I      Xho I
...CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC CTC GAG ACC...

                                                  start of hrGFP
                                              ...ATG GTG AGC AAG CAG
                                                 START
```

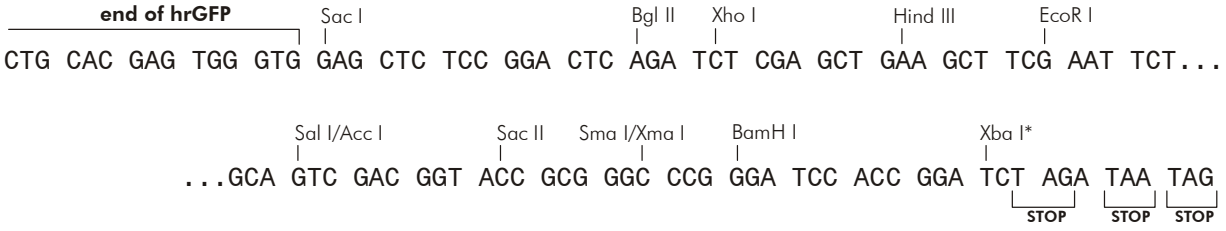
| Feature | Nucleotide Position |
|---|---|
| CMV promoter | 1–602 |
| T3 primer binding site (for 5′ end of insert) [5′ AATTAACCCTCACTAAAGGG 3′] | 620–639 |
| multiple cloning site | 651–743 |
| primer binding site (for 3′ end of insert) [5′ ACCTTGAAGCTCATGATCTC 3′] | 786–805 |
| hrGFP ORF | 747–1463 |
| T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′] | 1495–1516 |
| SV40 polyA signal | 1528–1911 |
| f1 origin of ss-DNA replication | 2049–2355 |
| LoxP sequence | 2518-2551 |
| ampicillin resistance (*bla*) ORF | 2596–3453 |
| pUC origin of replication | 3600–4267 |

**FIGURE 3** Features of the phrGFP-C Mammalian Expression Vector.

## The phrGFP-N1 Vector




## phrGFP-N1 Multiple Cloning Site Region
**(sequence shown 1308–1412)**

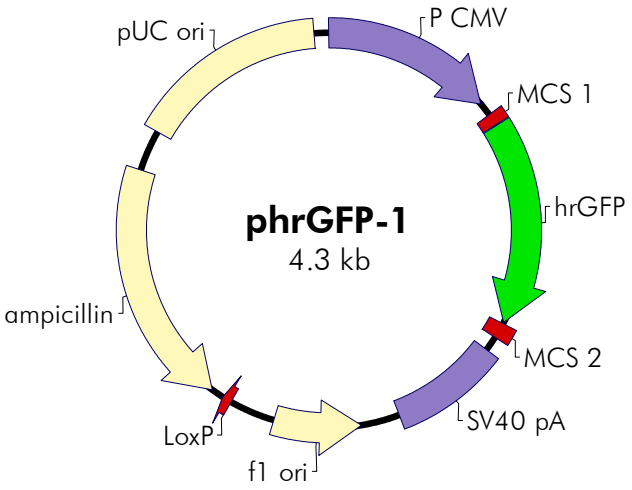



* If restriction using Xba I is desired, the phrGFP-N1 plasmid DNA must first be purified from a *dam*⁻*E. coli* strain.

| Feature | Nucleotide Position |
|---|---|
| CMV promoter | 1–597 |
| hrGFP ORF | 606–1322 |
| 5′ N1 primer binding site (for 5′ end of insert) [5′ CAGCTGACCAGCCTGGGCAAG 3′] | 1275–1295 |
| multiple cloning site | 1323–1406 |
| T7 primer binding site (for 3′ end of insert) [5′ TAATACGACTCACTATAGGG 3′] | 1439–1458 |
| SV40 polyA signal | 1471–1854 |
| f1 origin of ss-DNA replication | 1992–2298 |
| LoxP sequence | 2461–2494 |
| ampicillin resistance (*bla*) ORF | 2539–3396 |
| pUC origin of replication | 3543–4210 |

**FIGURE 4** Features of the phrGFP-N1 Mammalian Expression Vector.

## The phrGFP-1 Vector




### phrGFP-1 Multiple Cloning Site 1 Region
**(sequence shown 645–704)**

Sac I | Sac II | Not I | Srf I | Sma I/Xma I | BamH I | start of hrGFP

```
AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG GGC GGA TCC ACC ATG GTG AGC
```

KOZAK

### phrGFP-1 Multiple Cloning Site 2 Region
**(sequence shown 1407–1478)**

end of hrGFP | EcoR I | EcoR V | Hind III | Sal I | Xho I

```
TGG GTG TAA TAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC CTC GAG...
```

STOP STOP

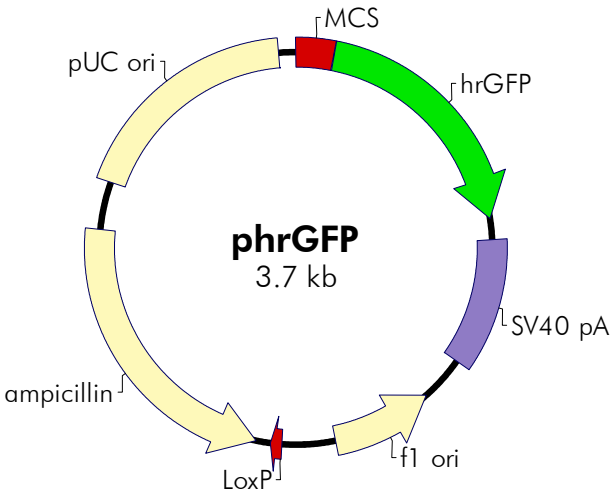
Kpn I

```
...GGG GGG CCC GGT ACC AGG TAA
```

| Feature | Nucleotide Position |
|---|---|
| CMV promoter | 1–602 |
| T3 primer binding site (for 5′ end of insert in MCS1) [5′ AATTAACCCTCACTAAAGGG 3′] | 620–639 |
| multiple cloning site 1 (Sac I to BamH I) | 651–692 |
| Kozak sequence | 691–699 |
| 5′ hrGFP primer (for 3′ end of insert in MCS1) [5′ ACCTTGAAGCTCATGATCTC 3′] | 735–754 |
| hrGFP ORF | 696–1412 |
| 3′ hrGFP primer (for 5′ end of insert in MCS2) [5′ CAGCTGACCAGCCTGGGCAAG 3′] | 1365–1385 |
| stop codons | 1413–1418 |
| multiple cloning site 2 (EcoR I to Kpn I) | 1419–1472 |
| T7 primer binding site (for 3′ end of insert in MCS2) [5′ TAATACGACTCACTATAGGG 3′] | 1493–1512 |
| SV40 polyA signal | 1525–1908 |
| f1 origin of ss-DNA replication | 2046–2352 |
| LoxP sequence | 2515–2548 |
| ampicillin resistance (*bla*) ORF | 2593–3450 |
| pUC origin of replication | 3597–4264 |

**FIGURE 5** Features of the phrGFP-1 Mammalian Expression Vector.

## The phrGFP Vector




## phrGFP Multiple Cloning Site Region
**(sequence shown 1–128)**

```
Nsi I       Bgl II      Xba I          Sac I          Sac II          Not I                Srf I
AT GCA TAG ATC TTC TAG ATA GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC CCG GGC...

   BamH I                          EcoR I      EcoR V     Hind III                 Sal I
...GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC...

                                  Xho I                start of hrGFP
...CTC GAG ACC ATG GTG AGC AAG
```

| Feature | Nucleotide Position |
|---|---|
| multiple cloning site | 1–113 |
| hrGFP ORF | 117–833 |
| 5′ hrGFP primer binding site [5′ CCAGGTTCACCTTGAAGCTCAT 3′] | 162–183 |
| T7 primer binding site [5′ TAATACGACTCACTATAGGG 3′] | 866–885 |
| SV40 polyA signal | 898–1281 |
| f1 origin of ss-DNA replication | 1419–1725 |
| LoxP sequence | 1888–1921 |
| ampicillin resistance (*bla*) ORF | 1966–2823 |
| pUC origin of replication | 2970–3637 |
| Primer binding site (for 5′ end of insert) [5′ TCACATGTTCTTTCCTGCGTTATCC 3′] | 3635–3659 |

**FIGURE 6** Features of the phrGFP Mammalian Expression Vector.

## OVERVIEW OF *CRE*-MEDIATED SITE-SPECIFIC RECOMBINATION

Stratagene's Vitality hrGFP reporter vectors utilize *Cre*-mediated site-specific recombination to allow the quick and efficient directional insertion of prefabricated modules. The site-specific recombination catalyzed by *Cre* recombinase is dependent on the presence and orientation of two *LoxP* sites. Each *LoxP* site has two 13-bp inverted repeats, which serve as recognition sites for the recombinase, on opposite sides of an 8-bp nonpalindromic region. The relative orientations of these *LoxP* sites determine the effects of the recombination event. Two sites oriented as tandem repeats results in the excision of the intervening sequence, while two sites in opposite orientation lead to an inversion of the DNA sequence between the two sites. In addition to these intramolecular reactions, site-specific recombination can be intermolecular, leading to the formation of a co-integrate, which is a new molecule formed by the insertion of one DNA molecule into another. A schematic diagram of the recombinant molecule formed by inserting a pExchange module into the pIRES-hrGFP-1a vector is shown in Figure 7. The same type of recombination reaction is possible using any of the three pExchange modules (see Figure 8) and any of the Vitality vectors.

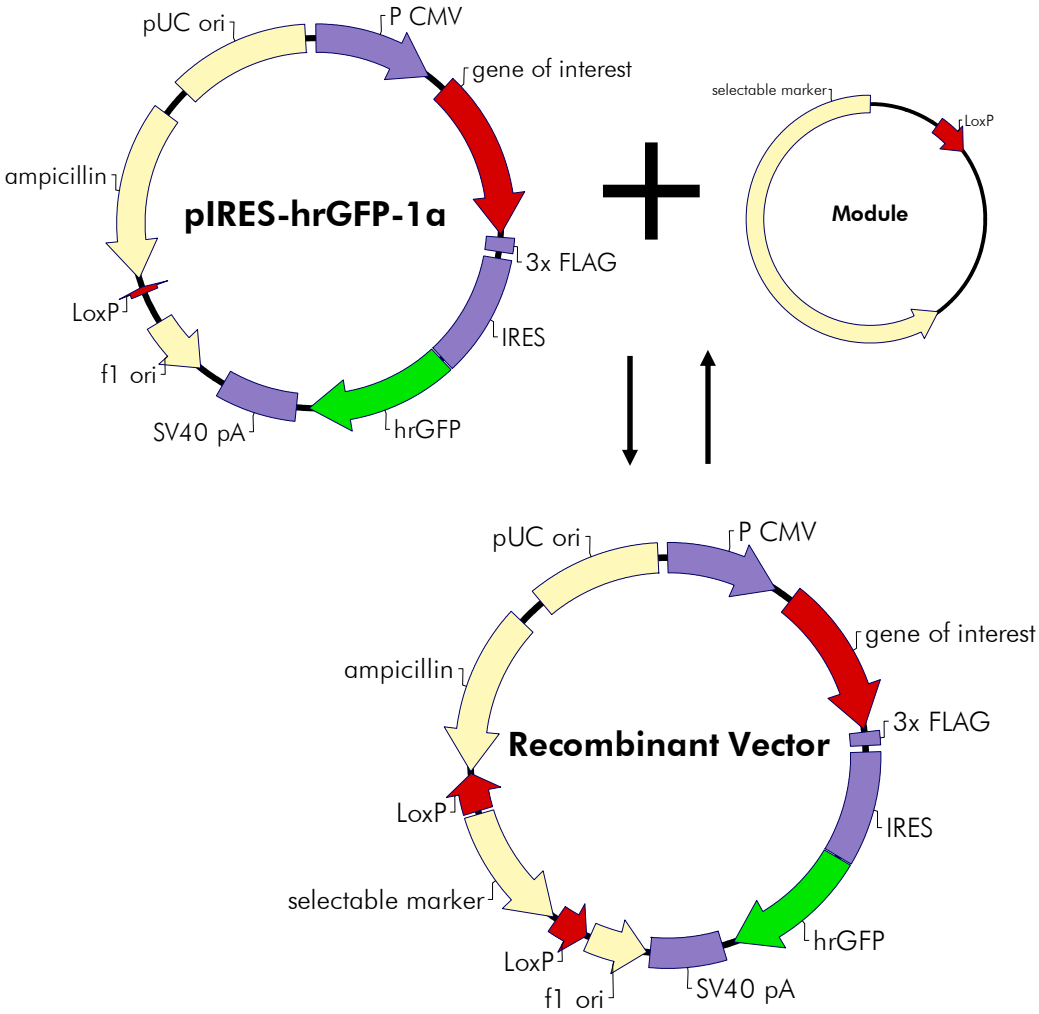



**FIGURE 7** *Cre*-mediated recombination for introduction of new resistance cassettes.

## MODULE DESCRIPTIONS

The Vitality hrGFP vectors allow for the introduction of a desired eukaryotic resistance gene into the core expression vectors. Each module, shown in Figure 8, contains a *LoxP* site for insertion of either the hygromycin-, puromycin-, or neomycin-resistance module into the *LoxP* site of any of the Vitality hrGFP vectors. The hygromycin- and puromycin-resistance modules also have a chloramphenicol-resistance marker for selection. Further sequence information for the pExchange modules is available at *www.stratagene.com*.

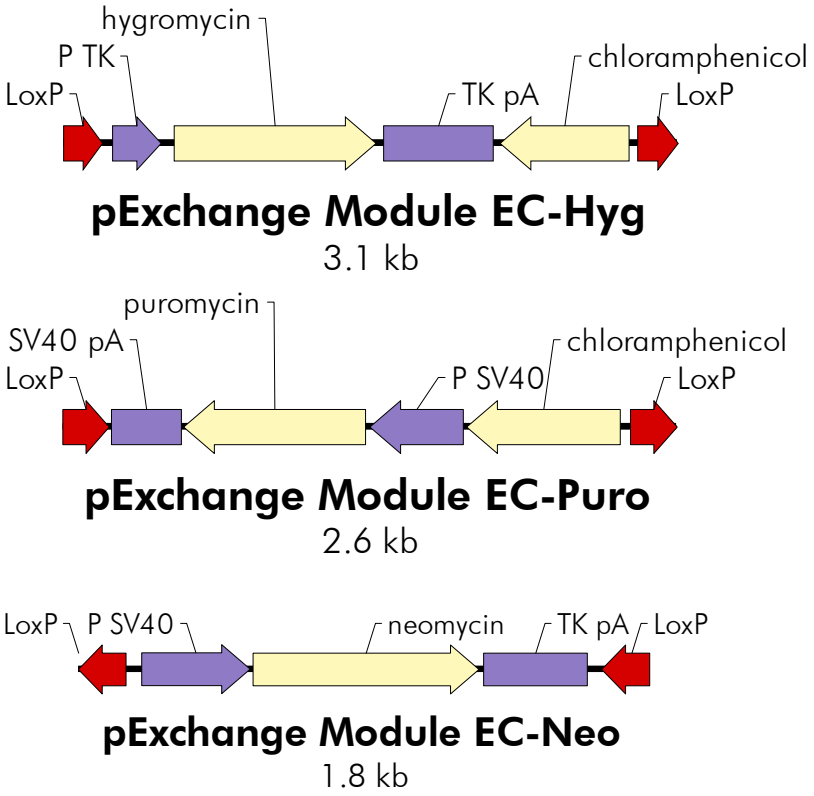



**FIGURE 8** The three pExchange modules, available separately from Stratagene.

**TABLE I**

**Features of pExchange Modules**

| pExchange Module EC-Hyg | | pExchange Module EC-Puro | | pExchange Module EC-Neo | |
|---|---|---|---|---|---|
| **Feature** | **Location** | **Feature** | **Location** | **Feature** | **Location** |
| *LoxP* sequence | 1–35 | *LoxP* sequence | 1–35 | *LoxP* sequence | 1–35 |
| HSV-TK promoter | 91–-339 | SV40 polyA signal | 168–137 | SV40 promoter | 94–483 |
| hygromycin | 343–1376 | puromycin | 1031–432 | neomycin | 484–1278 |
| HSV-TK polyA signal | 1375–1937 | SV40 promoter | 1273–1138 | HSV-TK polyA signal | 1266–1724 |
| chloramphenicol | 2895–2236 | chloramphenicol | 2394–1735 | *LoxP* sequence | 1763–1775 |
| *LoxP* sequence | 3104–3116 | *LoxP* sequence | 2603–2615 | | |

## PREPARING THE HRGFP VECTORS

***Important*** *When cloning into pIRES-hrGFP-1a and pIRES-hrGFP-2a, note the presence of a stop codon (TAG) in the MCS that is in-frame with the fusion tags (see the circular maps for these vectors in* Description of the Vectors*). Do not use the* Sac *I,* Sac *II, or* Not *I sites upstream of the stop codon for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.*

- Ensure that the coding sequence of the insert is in the correct reading frame and that it contains an initiation codon or Kozak sequence.[4] For gene fusions using the phrGFP-N1 vector, ensure that the gene of interest is inserted in frame with the hrGFP 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 4). For gene fusions using the phrGFP-C vector, ensure that the gene of interest lacks a termination codon, and reads in frame with the hrGFP sequence (see the MCS sequence in Figure 3).

- Stratagene suggests cloning the gene of interest into the appropriate Vitality vector before introducing a eukaryotic resistance marker into the vector by *Cre*-mediated recombination (see Figure 8). Introduction of the drug resistance marker to the vector may result in the loss of unique cloning sites in the vector MCS.

- Stratagene suggests 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:

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

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 vector being used; consult the circular vector maps for sizes.

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

| | Control | | | Experimental | |
|---|---|---|---|---|---|
| **Ligation reaction components** | **1**[a] | **2**[b] | **3**[c] | **4**[d] | **5**[d] |
| Prepared vector (0.1 µg/µl) | 1.0 µl | 1.0 µl | 0.0 µl | 1.0 µl | 1.0 µl |
| Prepared insert (0.1 µg/µl) | 0.0 µl | 0.0 µl | 1.0 µl | *Y* µl | *Y* µl |
| rATP [10 mM (pH 7.0)] | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl |
| Ligase buffer (10×)[e] | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl |
| T4 DNA ligase (4 U/µl) | 0.5 µl | 0.0 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| Double-distilled ($ddH_2O$) to 10 µl | 6.5 µl | 7.0 µl | 6.5 µl | *Z* µl | *Z* µl |

[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 agar plates (see *Preparation of Media and Reagents*) containing the appropriate antibiotic. Refer to references 5 and 6 for bacterial transformation protocols.

**Note** *Competent cells with transformation efficiencies ≥5 × $10^9$ cfu/µg are available from Stratagene.*

# 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 vectors may be determined by PCR amplification of DNA from individual colonies.

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

   4.0 µl of 10× *Taq* DNA polymerase buffer[††]
   0.4 µl of dNTP mix (25 mM each dNTP)
   40.0 ng of the appropriate 5´ primer (see table below for sequence)
   40.0 ng of the appropriate 3´ primer (see table below for sequence)
   0.4 µl of 10% (v/v) Tween® 20
   1.0 U of *Taq* DNA polymerase[†]
   $dH_2O$ to a final volume of 40 µl

### Primers for hrGFP Vectors

| Vector | Primer | Nucleotide sequence (5´ to 3´) |
|---|---|---|
| pIRES-hrGFP-1a | Forward (T3 promoter primer) | AATTAACCCTCACTAAAGGG |
| | Reverse | GTCCTTATCATCGTCGTCTT |
| pIRES-hrGFP-2a | Forward (T3 promoter primer) | AATTAACCCTCACTAAAGGG |
| | Reverse | TAAGCGTAGTCAGGTACATC |
| phrGFP-C | Forward (T3 promoter primer) | AATTAACCCTCACTAAAGGG |
| | Reverse (5′ hrGFP Primer) | ACCTTGAAGCTCATGATCTC |
| phrGFP-N1 | Forward (3′ hrGFP primer) | CAGCTGACCAGCCTGGGCAAG |
| | Reverse (T7 primer) | TAATACGACTCACTATAGGG |
| phrGFP-1 | MCS 1 Forward (T3 promoter primer) | AATTAACCCTCACTAAAGGG |
| | MCS 1 Reverse (5′ hrGFP Primer) | ACCTTGAAGCTCATGATCTC |
| | MCS 2 Forward (3′ hrGFP primer) | CAGCTGACCAGCCTGGGCAAG |
| | MCS 2 Reverse (T7 primer) | TAATACGACTCACTATAGGG |
| phrGFP | Forward | TCACATGTTCTTTCCTGCGTTATCC |
| | Reverse (5′ hrGFP Primer) | CCAGGTTCACCTTGAAGCTCAT |

[†,††] See *Endnotes*.

2. Stab the transformed colonies with a sterile toothpick and swirl the colony into reaction tubes. Immediately following inoculation into the reaction mixture, remove the toothpick and streak onto antibiotic-containing patch plates for future reference.

3. Gently mix each reaction, 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 | 94°C | 4 minutes |
| | 50°C | 2 minutes |
| | 72°C | 2 minutes/kb DNA |
| 30 cycles | 94°C | 1 minute |
| | 56°C | 2 minutes/kb DNA |
| | 72°C | 1 minute |
| 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 broth (see *Preparation of Media and Reagents*) containing the appropriate antibiotic. Grow overnight at 37°C with shaking.

6. The next morning, purify the plasmid DNA from the liquid cultures. You will need to prepare enough plasmid DNA so that *Cre*-mediated recombination may be performed if desired. Miniprep DNA or DNA purified using CsCl gradient centrifugation is of suitable quality.

1. To prepare the recombination reaction, add the following components to a 0.5-ml microcentrifuge tube:

   *X* µl of Vitality hrGFP vector containing the gene of interest (500 ng)
   *Y* µl of pExchange module (100 ng)
   1 µl of *Cre* recombinase
   1 µl of 10× *Cre* recombinase reaction buffer
   *Z* µl of distilled water ($dH_2O$) to a final volume of 10 µl

2. Incubate reaction for 30 minutes at 37°C. The reaction reaches equilibrium in ≤30 minutes.

3. Heat the reaction at 65°C for 20 minutes to denature the *Cre* recombinase. If not denatured, *Cre* recombinase will reduce the transformation efficiency.

Transform competent bacteria with 2.5 µl of the *Cre*-recombination reaction, and plate the transformed bacteria on LB agar plates containing the appropriate antibiotic. Refer to references 5 and 6 for bacterial transformation protocols.

**Note** *Competent cells with transformation efficiencies ≥5 × $10^9$ cfu/µg are available from Stratagene.*

## Confirming Recombination

The presence of recombinant plasmids can be confirmed by analyzing the miniprep DNA of a few colonies by restriction digestion. Ninety-five percent of the colonies are expected to contain recombinant plasmids.

## 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).[5]

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 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. The table below lists excitation and emission spectra for Stratagene's hrGFP as compared to EGFP.

## SPECIFICATIONS FOR HRGFP AND EGFP EXCITATION AND EMISSION SPECTRA

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

[a] Both 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 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*

## TROUBLESHOOTING

| Observation | Suggestion |
|---|---|
| Very few colonies following transformation with *Cre*-recombined vector | Transformation efficiency is inhibited by *Cre* recombinase that is not denatured by heat treatment. Heat the recombination reaction at 65°C for 20 minutes to denature the *Cre* recombinase |
| | Ensure that the *Cre*-recombinant transformations are plated on the appropriate agar plates |
| | Transformation is inefficient. Check transformation efficiency with a control plasmid |
| | DNA is degraded. Electrophorese the DNA to check its quality |
| Low percentage of colonies with recombinant plasmids | Increase the vector:module ratio in the *Cre*-mediated recombination protocol |
| 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

### LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized $H_2O$ 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)

### LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized $H_2O$ to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

### 10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

***Note*** *rATP is added separately in the ligation reaction*

### TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

## REFERENCES


1. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994) *Science* 263(5148):802–5.
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3. Hanazono, Y., Yu, J. M., Dunbar, C. E. and Emmons, R. V. (1997) *Hum Gene Ther* 8(11):1313–9.
4. Kozak, M. (1991) *J Biol Chem* 266(30):19867–70.
5. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6. Hanahan, D. (1983) *J Mol Biol* 166(4):557–80.


## ENDNOTES

† Purchase of these products is accompanied by a license to use them in the Polymerase Chain Reaction (PCR) process in conjunction with an Authorized Thermal Cycler. Stratagene's PCR products are sold under licensing arrangements with Roche Molecular Systems, Inc., F. Hoffmann-La Roche Ltd., and The Perkin-Elmer Corporation.

†† These products are optimized for use in the Polymerase Chain Reaction (PCR) process covered by patents owned by Hoffmann-La Roche. Purchase of these PCR-related products does not convey any rights under the PCR patents owned by Hoffmann-La Roche. A license to use the PCR process accompanies the purchase of certain reagents from Stratagene when used in conjunction with an Authorized Thermal Cycler.



## MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on Stratagene's website at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.