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 F.0

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240031-12

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VITALITY HRGFP MAMMALIAN EXPRESSION VECTORS

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CONTENTS

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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

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
Water baths (37°C and 42°C)

OPTIONAL ADDITIONAL MATERIALS

XL1-Blue supercompetent cells [Agilent Catalog #200236]

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

Revision F.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*. *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]

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. Thus the stable GFP-expressing cell lines are produced much more efficiently using Agilent's hrGFP compared with EGFP.

Agilent 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).

The Vitality hrGFP vectors include the ampicillin resistance marker, making them suitable for transient transfection of a variety of mammalian cells. If your experiments require stable hrGFP-expressing cell lines, you can cotransfect your cells with an additional plasmid that expresses a different selectable marker, such as a neomycin or hygromycin resistance gene. Refer to reference 4 for a protocol on isolating stable cell lines using a cotransfection approach.

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

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).

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).

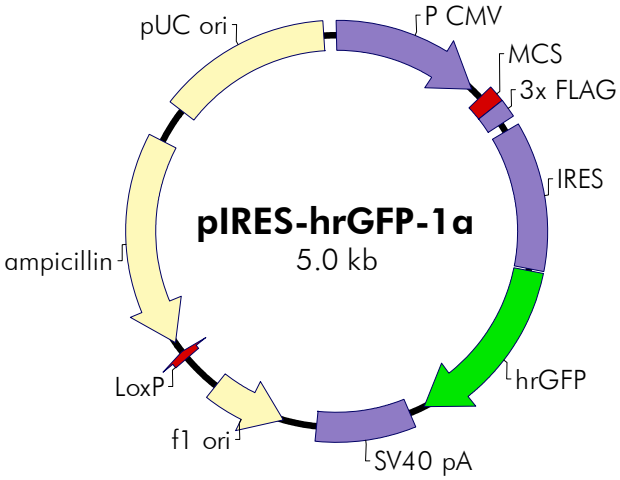
phrGFP-1 Vector

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. 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)

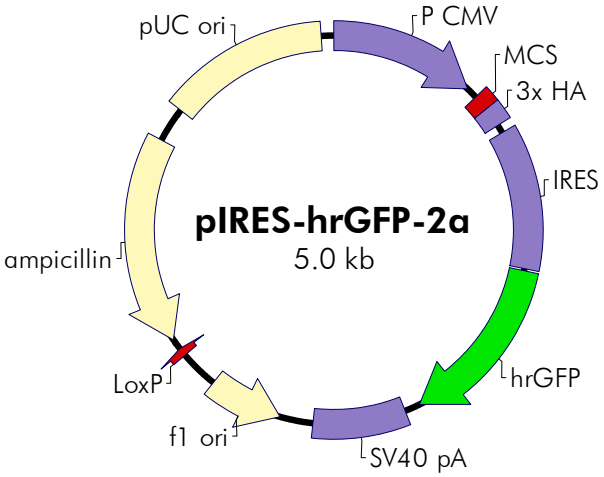
```
                                                    Srf I
Sac I*          Sac II*         Not I*              |   Sma I/Xma I   BamH I      EcoR I
|               |               |                   |   |             |          |
GA GCT CCA CCG CGG TGG CGG CCG CTC TAG CCC GGG CGG ATC CGA ATT C ...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

                                        Sph I       Sal I       Xho I       start of FLAG tag
                                        |           |           |           |
...GC ATG CGT CGA CTC GAG GAC TAC AAG GAT
```

* 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)

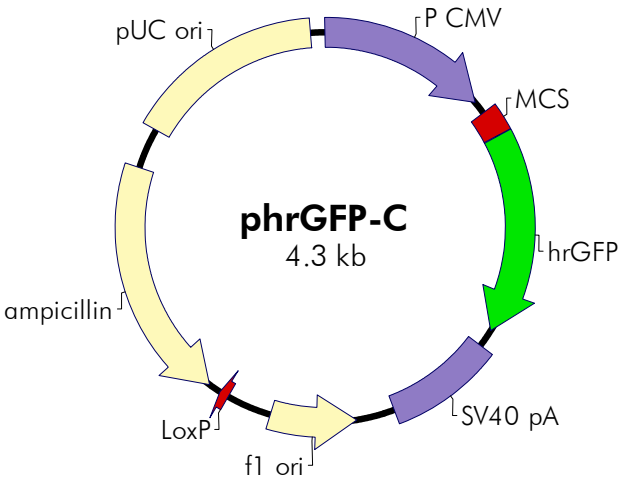
```
                                                    Srf I
Sac I*          Sac II*         Not I*              |   Sma I/Xma I   BamH I      EcoR I
|               |               |                   |   |             |          |
GA GCT CCA CCG CGG TGG CGG CCG CTC TAG CCC GGG CGG ATC CGA ATT C ...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



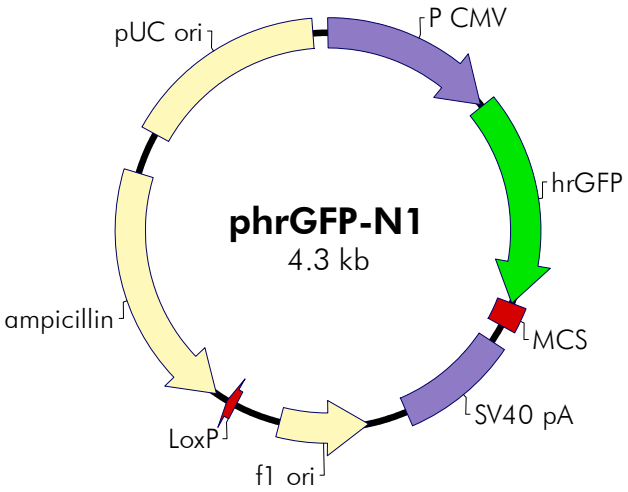
phrGFP-C Multiple Cloning Site Region
(sequence shown 646–761)

```
          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
```

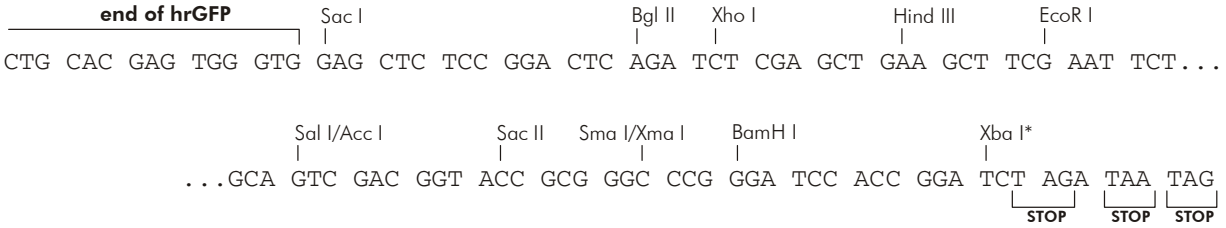
Feature	Nucleotide Position
CMV promoter	1–602
T3 primer binding site (for 5′ end of insert) [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
multiple cloning site	651–743
primer binding site (for 3′ end of insert) [5′ ACCTTGAAGCTCATGATCTC 3′]	786–805
hrGFP ORF	747–1463
T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 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)

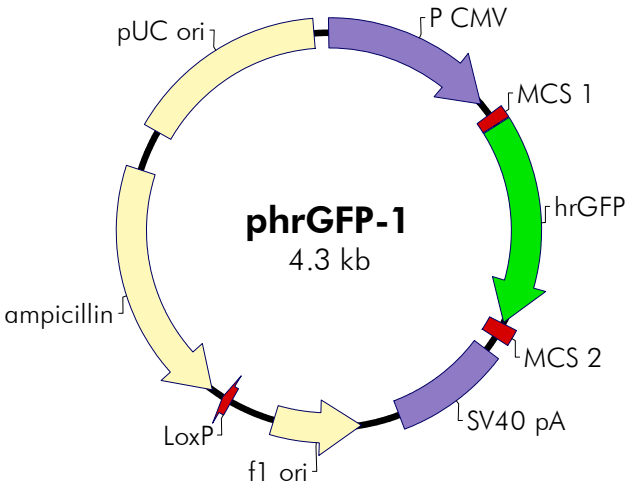


* If restriction using Xba I is desired, the phrGFP-N1 plasmid DNA must first be purified from a *dam*⁻*E. coli* strain.

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

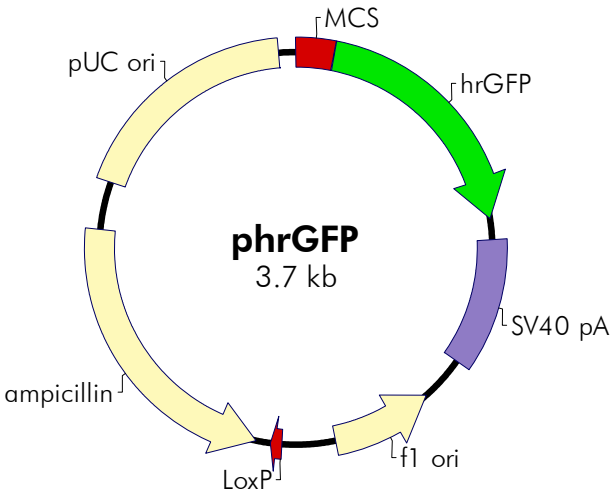
Kpn I

...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.

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.[5] 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).

- 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:

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

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 [6] and [7] for bacterial transformation protocols.

Note *Competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg are available from Agilent. Visit www.genomics.agilent.com for more information.*

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

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

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 using a miniprep or CsCl gradient protocol.

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 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 Agilent'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
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

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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.