Living Colors®
User Manual

PT2040-1 (PR1Y691)
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I. Introduction

The bioluminescent jellyfish Aequorea victoria produces light when energy is transferred from Ca^{2+}-activated photoprotein aequorin to green fluorescent protein (GFP; Shimomura et al., 1962; Morin & Hastings, 1971; Ward et al., 1980). The cloning of the wild-type GFP gene (wt GFP; Prasher et al., 1992; Inouye & Tsuji, 1994a) and its subsequent expression in heterologous systems (Chalfie et al., 1994; Inouye & Tsuji, 1994a; Wang & Hazelrigg, 1994) established GFP as a novel genetic reporter system. When expressed in either eukaryotic or prokaryotic cells and illuminated by blue or UV light, GFP yields a bright green fluorescence. Light-stimulated GFP fluorescence is species-independent and does not require any cofactors, substrates, or additional gene products from A. victoria. Additionally, detection of GFP and its variants can be performed in living cells and tissues as well as fixed samples.

Clontech makes GFP and GFP variants available for research purposes through its line of **Living Colors® Fluorescent Proteins** (Figure 1). Living Colors proteins are excellent reporters for gene expression and protein localization studies. Following is a list of the Living Colors GFP variants currently offered. You will find that these variants are well suited for fluorescence microscopy and flow cytometry.

- GFP excitation variants:
  - **EGFP,** enhanced green fluorescent protein (GFPmut1; Cormack et al., 1996), and **destabilized EGFP (dEGFP**; Li et al., 1998) have red-shifted excitation spectra. When excited with blue light, they fluoresce 4–35-fold more brightly than wt GFP.
- GFP emission variants:
  - **EYFP** (enhanced yellow fluorescent protein) contains four amino acid substitutions that shift the emission from green to yellowish-green. The fluorescence level of EYFP is roughly equivalent to that of EGFP.
  - **ECFP** (enhanced cyan fluorescent protein) contains six amino acid substitutions, one of which shifts the emission spectrum from green to cyan (Heim & Tsien, 1996; Miyawaki et al., 1997). It is a suitable donor molecule in fluorescence resonance energy transfer (FRET) studies and can be used for dual-labeling fluorescence microscopy in conjunction with EYFP (Green et al., 2000).
- UV-Optimized GFP variant:
  - **GFPuv** is reported to be 18 times brighter than wt GFP when expressed in E. coli and excited by standard UV light (Crameri et al., 1996). This variant contains additional amino acid mutations which also increase the translational efficiency of the protein in E. coli.

I. Introduction continued

- Red Fluorescent Protein:
  - We also offer **DsRed2**, a red fluorescent protein that derives from a coral of the Discosoma genus, referred to as Discosoma sp. (Ds). Because of its distinct red emission, DsRed2 is a perfect complement to the green, yellow, and cyan fluorophores. With the development of filters designed specifically for detecting EGFP, EYFP, ECFP, and DsRed, it's now possible to use these reporters in combination to carry out two-, three-, or, even, four-color analyses. The Red Fluorescent Protein, DsRed, is more carefully described in a separate User Manual, PT3404-1, which can be downloaded from our website at **www.clontech.com**.

In addition to their chromophore mutations, the enhanced GFP variant genes EGFP, EYFP, and ECFP contain more than 190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (Haas, et al., 1996). Furthermore, upstream sequences flanking the coding regions have been converted to a Kozak consensus translation initiation site (Kozak, 1987), and potentially inhibitory flanking sequences in the original cDNA clones (lgfp10 & pGFP10.1; Chalfie et al., 1994) were removed. All of these changes enhance the translational efficiency of the mRNA—and, consequently, the expression of the GFP variant—in mammalian and plant systems.

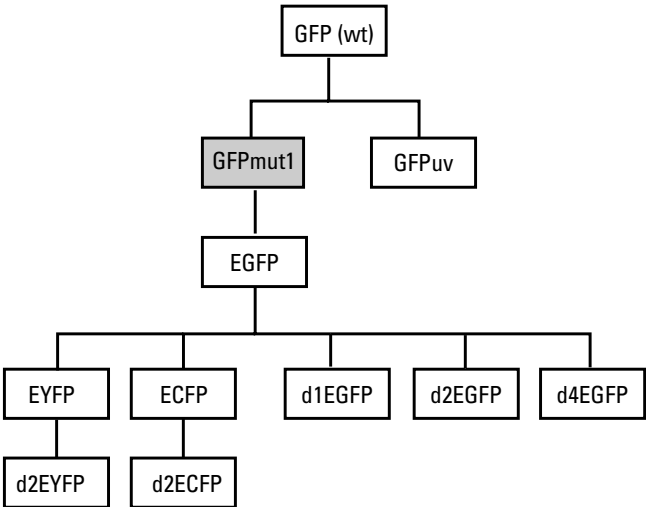

Figure 1. Genealogy of GFP proteins. GFPmut1 (Cormack et al., 1996), the red-shifted variant used to create the variants shown, is not sold by Clontech.

II. Properties of GFP and GFP Variants

A. The GFP Chromophore: Structure and Biosynthesis

The GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly (amino acids 65–67) in the primary protein sequence (Cody et al., 1993) and is only fluorescent when embedded within the complete GFP protein. Of the complete 238-amino-acid polypeptide, amino acids 7–229 are required for fluorescence (Li et al., 1997). The crystal structure of GFP has revealed a tightly packed β-can enclosing an α-helix containing the chromophore (Ormö et al., 1996; Yang, F. et al., 1996). This structure provides the proper environment for the chromophore to fluoresce. Nascent GFP is not fluorescent, since chromophore formation occurs post-translationally. The chromophore is formed by a cyclization reaction and an oxidation step that requires molecular oxygen (Heim et al., 1994; Davis, D. F. et al., 1995). These steps are either autocatalytic or use factors that are ubiquitous, since fluorescent GFP forms in a broad range of organisms. Chromophore formation may be the rate-limiting step for maturation of the fluorescent protein (Heim et al., 1994; Davis, D. F. et al., 1995). Wild-type GFP absorbs UV and blue light and emits green light.

B. GFP Excitation Variants

The red-shifted variants EGFP and dEGFP (destabilized for rapid turnover) contain mutations in the chromophore (Figure 2) which shift the maximal excitation peak to ~490 nm (Figure 3).

wt GFP: Phe^{64} Ser Tyr Gly Val Gln^{69} . . . Ser^{72} . . . Tyr^{145} Asn^{146} . . . Met^{153} . . . Val^{163} . . . Thr^{203}

EGFP: Leu^{64} Thr Tyr Gly Val Gln^{69}

EYFP: Phe^{64} Gly Tyr Gly Leu Gln^{69} . . . Ala^{72} . . . . . Tyr^{203}

ECFP: Leu^{64} Thr Trp Gly Val Gln^{69} . . . . . Ile^{146} . . . . Thr^{153} . . . . Ala^{163}

Figure 2. Amino acid sequence differences between wt GFP and its variants. Amino acid substitutions are underlined.

The major excitation peak of the red-shifted variants encompasses the excitation wavelength of commonly used filter sets. Similarly, the argon-ion laser used in most FACS machines and confocal scanning laser microscopes emits at 488 nm, so excitation of the red-shifted GFP variants is much more efficient than excitation of wt GFP. In practical terms, this means the detection limits in both microscopy and FACS are considerably lower with the red-shifted variants. Dual-labeling experiments can be performed by selective excitation of wt GFP and red-shifted GFP (Kain et al., 1995; Yang, T. T. et al., 1996b). It is also possible to use EGFP and GFPuv in double-labeling experiments.

II. Properties of GFP and GFP Variants continued

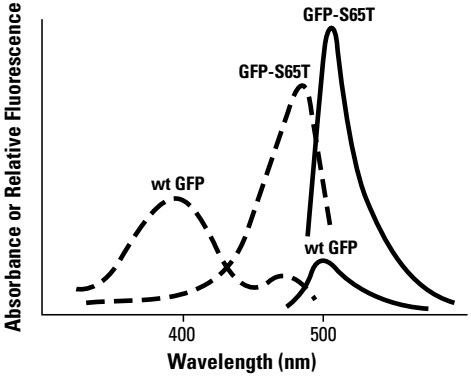

Figure 3. Excitation and emission spectra of wt GFP and a red-shifted GFP excitation variant (GFP-S65T). Excitation: dashed lines. Emission: solid lines. The emission data for wt GFP were obtained by exciting the protein at 475 nm. (The emission peak for wt GFP is several-fold higher when illuminated at 395 nm). The emission data for GFP-S65T were obtained by exciting the protein at 489 nm (Heim et al., 1995). EGFP and GFP-S65T have similar spectra.

- **EGFP** contains two amino acid substitutions: F64L and S65T (Figure 2). Based on spectral analysis, EGFP fluoresces 35-fold more intensely than wt GFP when excited at 488 nm (Cormack et al., 1996; Yang, T. T. et al., 1996a) due to an increase in its extinction coefficient (E_m; Table I) and a higher efficiency of chromophore formation. EGFP chromophore formation is much more efficient at 37°C than wt GFP; 95% of the soluble EGFP protein contains the chromophore when expressed at this temperature (Patterson et al., 1997).

C. GFP Emission Variants

- **Cyan Emission Variant (ECFP)**

  The **ECFP** variant contains six amino acid substitutions (Figure 2). One of these, Tyr-66 to Trp, shifts the chromophore's excitation maxima to 433 nm (major peak) and 453 nm (minor peak), and the emission maxima to 475 nm with a small shoulder at 501 nm (Table I; Figure 4). The other five substitutions enhance the brightness and solubility of the protein in a manner similar to the other EGFP variants.

- **Yellow Emission Variant (EYFP)**

  The **EYFP** variant contains four different amino acid substitutions (Figure 2) that shift the fluorescence from green (509 nm) to yellow-green (527 nm; Table I; Figure 4). Although EYFP's fluorescence excitation maximum is 513 nm, it can be efficiently excited at 488 nm, the standard laser line for an argon-ion laser.

II. Properties of GFP and GFP Variants continued

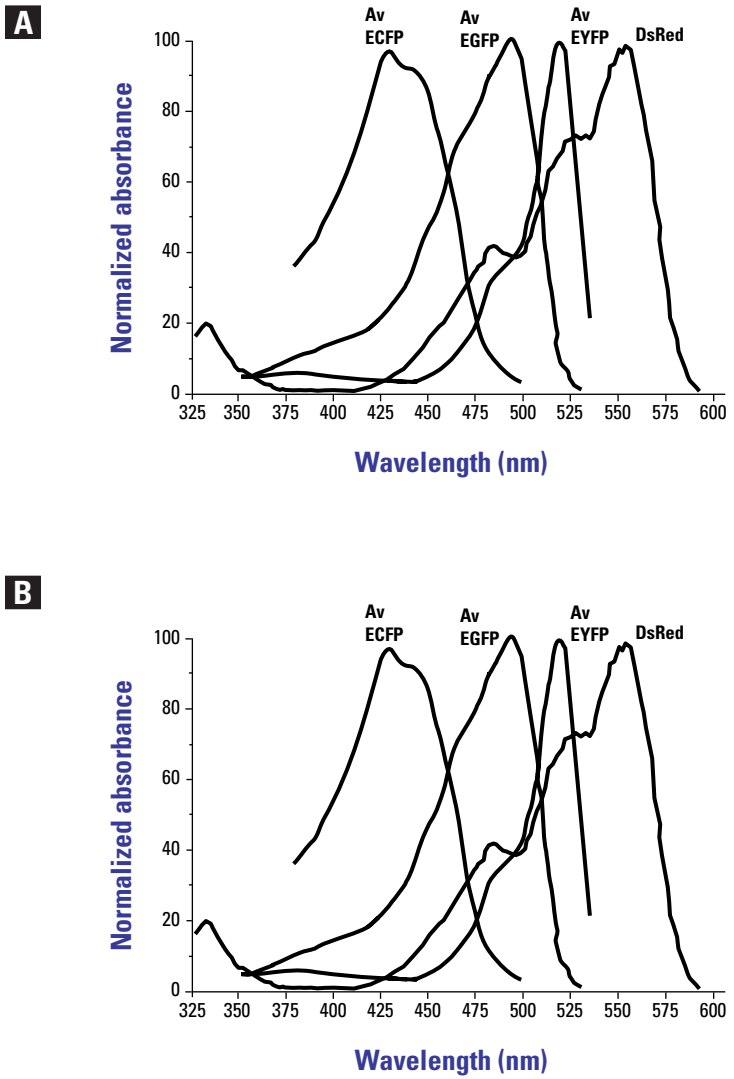

Figure 4. DsRed, EGFP, ECFP, and EYFP have distinct absorption and emission spectra. Panel A. Absorbance. **Panel B.** Emission. Curves are normalized; the height of each curve is not an indication of relative signal strength per fluorophore. EGFP, ECFP, and EYFP are all variants of the green fluorescent protein from Aequorea victoria, denoted as Av in the figure. DsRed is derived from a coral of the Discosoma genus referred to as Discosoma sp. (Ds). (Spectra for EGFP, ECFP, and EYFP were provided by D. W. Piston, Vanderbilt University.)

II. Properties of GFP and GFP Variants continued

D. UV-Optimized GFP Variant

GFPuv is optimized for maximal fluorescence when excited by UV light (360–400 nm) and for higher bacterial expression. The GFPuv variant is ideal for experiments in which GFP expression will be detected using UV light for chromophore excitation (e.g., for visualizing bacteria or yeast colonies). GFPuv was developed by Crameri et al. (1996).

GFPuv contains three amino acid substitutions (Phe-99 to Ser, Met-153 to Thr, and Val-163 to Ala [based on the amino acid numbering of wt GFP]), none of which alter the chromophore sequence. These mutations make E. coli expressing GFPuv fluoresce 18 times brighter than wt GFP (Crameri et al., 1996). While these mutations dramatically increase the fluorescence of GFPuv through their effects on protein folding and chromophore formation, the emission and excitation maxima remain at the same wavelengths as those of wt GFP (Crameri et al., 1996; Figure 3; Table I). However, GFPuv has a greater propensity to dimerize than wt GFP.

GFPuv expressed in E. coli is a soluble, fluorescent protein even under conditions in which the majority of wt GFP is expressed in a nonfluorescent form in inclusion bodies. This GFP variant also appears to have lower toxicity than wt GFP; hence, the E. coli containing GFPuv grow two to three times faster than those expressing wt GFP (Crameri et al., 1996). Furthermore, the GFPuv gene is a synthetic GFP gene in which five rarely used Arg codons from the wt gene were replaced by codons preferred in E. coli. Consequently, the GFPuv gene is expressed very efficiently in E. coli.

E. Destabilized GFP Variants

In mammalian cells, destabilized variants (dEGFP, dECFP, and dEYFP) are degraded more rapidly than wt GFP. In order to create the destabilized variants, EGFP, ECFP, and EYFP were fused to amino acid residues 422–461 of mouse ornithine decarboxylase (MODC). This C-terminal region of MODC contains a PEST (Pro-Glu-Ser-Thr) amino acid sequence that targets the protein for rapid intracellular degradation. The d1, d2, and d4 variants exhibit half-lives of 1, 2, or 4 hours, respectively, compared to EGFP, ECFP, and EYFP, which have half-lives of >24 hours. Half-lives were determined by flow cytometry (Figure 5), fluorescence microscopy, and Western blot analysis (Li et al., 1998; July 1999 & April 1998 Clontechniques).

The fluorescence intensity and spectral properties of destabilized variants are identical to those of the original chromophore, but because of their shortened half-lives, destabilized variants can be used to more accurately measure the kinetics of promoter activity or the transient expression of a protein to which they are fused.

II. Properties of GFP and GFP Variants continued

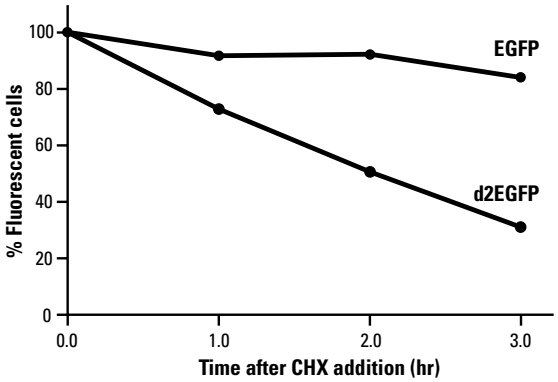

Figure 5. Flow cytometry analysis of d2EGFP stability. CHO-K1 Tet-Off™ cells were transfected with pTRE-EGFP or pTRE-d2EGFP. After 24 hr, the transfected cells were treated with 100 µg/ml cycloheximide (CHX) for 0, 1, 2, or 3 hr to inhibit protein synthesis. The CHX-treated cells were harvested with EDTA, and 1 x 10^5 cells were used for flow cytometry analysis. The percentages of cells with fluorescence above background are plotted on the y-axis. Values are normalized to fluorescence levels at the zero time point, defined as 100%. After 3 hr, fluorescence intensity of the cell population expressing d2EGFP was reduced to 30% of initial intensity.

F. Acquisition and Stability of GFP Fluorescence

1. Protein solubility

In bacterial expression systems, much of the expressed wt GFP is found in an insoluble, nonfluorescent form in inclusion bodies. In contrast, almost all EGFP and GFPuv is expressed as a soluble, fluorescent protein.

2. Photobleaching

GFP fluorescence is very stable in a fluorometer (Ward, pers. comm.). Even under the high-intensity illumination of a fluorescence microscope, GFP is more resistant to photobleaching than is fluorescein (Wang & Hazelrigg, 1994; Niswender et al., 1995). The fluorescence of wt GFP and EGFP is quite stable when illuminated with 450–490 nm light (the major excitation peak for the red-shifted excitation variants, but the minor peak for wt GFP). Some photobleaching occurs when wt GFP is illuminated near its major excitation peak with 340–390 nm or 395–440 nm light (Chalfie et al., 1994; Niswender et al., 1995). The rate of photobleaching of wt GFP and its green variants also varies with the organism being studied; for example, GFP fluorescence is quite stable in Drosophila (Wang & Hazelrigg, 1994) and zebrafish. In C. elegans, 10 mM NaN_3 accelerates photobleaching (Chalfie et al., 1994). Studies of the photobleaching characteristics of GFPuv have not been performed to date.

II. Properties of GFP and GFP Variants continued

When using ECFP, photobleaching does not pose a major problem, and we recommend using this variant if photobleaching interferes with your studies. (Rapid photobleaching has been a problem with previously described blue variants of GFP.) However, if you decide to fix and mount your specimens, commercially available mounting solutions that do not contain antibleaching reagents can cause severe photobleaching within seconds when the slides are exposed to light during fluorescence microscopy. Be sure to keep mounted slides in the dark at 4°C.

3. Stability to oxidation/reduction

GFP needs to be in an oxidized state to fluoresce because chromophore formation is dependent upon an oxidation of Tyr-66 (Heim et al., 1994). Strong reducing agents, such as 5 mM $Na_2S_2O_4$ or 2 mM $FeSO_4$, convert GFP into a nonfluorescent form, but fluorescence is fully recovered after exposure to atmospheric oxygen (Inouye & Tsuji, 1994b). Weaker reducing agents, such as 2% β-mercaptoethanol, 10 mM dithiothreitol (DTT), 10 mM reduced glutathione, or 10 mM L-cysteine (Inouye & Tsuji, 1994b), or moderate oxidizing agents (Ward, pers. comm.), do not affect the fluorescence of GFP.

4. Stability to pH

wt GFP retains fluorescence in the range pH 5.5–12; however, fluorescence intensity decreases between pH 5.5 and pH 4, and drops sharply above pH 12 (Bokman & Ward, 1981). EGFP exhibits a reduced range of pH stability. For this variant, fluorescence is stable between pH 7.0 and pH 11.5, drops sharply above pH 11.5, and decreases between pH 7.0 and pH 4.5, retaining about 50% of fluorescence at pH 6.0 (Patterson et al., 1997; Ward, pers. comm.). Because GFP is so sensitive to pH, it can be used as an intracellular pH indicator in living cells (Kneen et al., 1998).

5. Stability to chemical reagents

GFP retains its fluorescence in mild denaturants, such as 1% SDS or 8 M urea, and after fixation with glutaraldehyde or formaldehyde. Fully denatured GFP is not fluorescent. GFP is sensitive to some nail polishes used to seal coverslips (Chalfie et al., 1994; Wang & Hazelrigg, 1994); therefore, use molten agarose or rubber cement to seal coverslips on microscope slides. Fluorescence is also quenched by the nematode anesthetic phenoxypropanol (Chalfie et al., 1994). GFP fluorescence is irreversibly destroyed by 1% H_2O_2 and sulfhydryl reagents such as 1 mM DTNB (5,5'-dithiobis-[2-nitrobenzoic acid]) (Inouye & Tsuji, 1994b). Many organic solvents can be used at moderate concentrations without abolishing fluorescence; however, the absorption maximum may shift.

II. Properties of GFP and GFP Variants continued

GFP is nonfluorescent in absolute ethanol and is unlikely to withstand the complete dehydration required for paraffin or plastic embedding.

6. Protein stability

in vitro:

GFP is exceptionally resistant to heat (T_m=70°C), alkaline pH, detergents, chaotropic salts, organic solvents, and most common proteases, except pronase (Bokman & Ward, 1981; Ward, 1981). Some GFP fluorescence can be observed when nanogram amounts of protein are resolved on native or 1% SDS polyacrylamide gels (Inouye & Tsuji, 1994a).

Fluorescence is lost if GFP is denatured by high temperature, pH extremes, or guanidinium chloride, but can be partially recovered if the protein is allowed to renature (Bokman & Ward, 1981; Ward & Bokman, 1982). A thiol compound may be necessary to renature the protein into the fluorescent form (Surpin & Ward, 1989).

in vivo:

GFP appears to be stable when expressed in various organisms; EGFP has an estimated half-life of >24 hours (Li et al., 1998).

7. Temperature sensitivity of GFP chromophore formation

Although fluorescent GFP is highly thermostable (see above), it appears that the formation of the GFP chromophore is temperature sensitive. In yeast, GFP fluorescence was strongest when the cells were grown at 15°C, decreasing to about 25% of this value as the incubation temperature was raised to 37°C (Lim et al., 1995). However, GFP and GFP-fusion proteins synthesized in S. cerevisiae at 23°C retain fluorescence despite a later shift to 35°C (Lim et al., 1995). It has also been noted that E. coli expressing GFP show stronger fluorescence when grown at 24°C or 30°C compared to 37°C (Heim et al., 1994; Ward, pers. comm.). Mammalian cells expressing GFP have also been seen to exhibit stronger fluorescence when grown at 30–33°C compared to 37°C (Pines, 1995; Ogawa et al., 1995).

All the enhanced GFP variants show little difference in fluorescence when expressed at either 25°C or 37°C. The mutations that increase the efficiency of protein folding and chromophore formation (Cormack et al., 1996, Crameri et al., 1996) also suppress the thermosensitivity of chromophore formation.

8. Dimerization of GFP

GFP is fluorescent either as a monomer or as a dimer. The ratio of monomeric and dimeric forms depends on the protein concentration and environment. wt GFP dimerizes via hydrophobic interactions at protein concentrations above 5–10 mg/ml and in high-salt conditions.

II. Properties of GFP and GFP Variants continued

Dimerization results in a 4-fold reduction in the absorbance at 470 nm and a concomitant increase in absorbance at 395 nm (Ward, pers. comm.). GFPuv has a greater propensity to dimerize than wt GFP; the 470-nm excitation peak is suppressed at protein concentrations about 5-fold lower than for wt GFP (Ward, pers. comm.). In most experimental systems using wt GFP as a reporter, the monomeric form will predominate; however, when GFPuv is used as the reporter, dimerization may be evident even at moderate expression levels.

G. Sensitivity and fluorescence intensity

1. General considerations

GFP fusion proteins have been found to give greater sensitivity and resolution than staining with fluorescently labeled antibodies (Wang & Hazelrigg, 1994). GFP fusions are more resistant to photobleaching and exhibit low background fluorescence (Wang & Hazelrigg, 1994).

Because GFPuv, EGFP, and dEGFP exhibit higher extinction coefficients than wt GFP, they provide significantly increased sensitivity as reporter molecules. Furthermore, subcellular localization of EGFP (e.g., to the cytoskeleton) can produce an even more intense signal by concentrating the signal to a specific area within the cell. However, for some applications, the sensitivity of GFP may be limited by autofluorescence or limited penetration of light. Studies with wt GFP expressed in HeLa cells (Niswender et al., 1995) have shown that the cytoplasmic concentration must be greater than ~1.0 µM to obtain a signal that is twice the autofluorescence. This threshold for detection is lower with the red-shifted excitation variant: ~100 nM for EGFP (Piston, pers. comm.).

2. As a quantitative reporter

The signal from GFP and its variants does not have any enzymatic amplification; hence, the sensitivity of GFP will probably be lower than that for enzymatic reporters such as β-galactosidase, SEAP, and firefly luciferase. However, GFP signals can be quantified by flow cytometry, confocal scanning laser microscopy, and fluorometric assays. Purified GFP and GFP variants can be quantified in a fluorometer-based assay in the low-nanogram range. Furthermore, using destabilized GFPs as reporters can increase sensitivity by preventing accumulation of GFP under basal or non-induced expression. Therefore, destabilized GFPs effectively extend the induction window by increasing fold-induction.

II. Properties of GFP and GFP Variants continued

TABLE I. LIVING COLORS® FLUORESCENT PROTEINS: SPECTRAL PROPERTIES

Protein	**Excitation Maxima** (nm)	**Emission Maxima** (nm)	$\mathbf{E_m}$ (cm^{-1} M^{-1})	**Quantum Yield** (%)
wt GFP	395 (470)	509 (540)	9,500[a]	~80[a]
EGFP[d]	488	507	55,000[b]	~60[b]
ECFP[d]	433 (453)	475 (501)	26,000[b]	~40[b]
EYFP[d, e]	513	527	84,000[b]	~61[b]
GFPuv	395	509		
DsRed[e]	558	583	75,000[c]	~70[c]

[a] Patterson et al., 1997

[b] Dave Piston, pers. comm.

[c] Baird et al., 2000; Slightly lower values are reported by Patterson et al., 2001.

[d] The destabilized variant (dEGFP, dECFP, or dEYFP) has identical excitation and emission maxima, but a shorter half-life.

[e] EYFP and DsRed can also be excited at 488 nm. See Figure 4.

III. Expression of GFP and GFP Variants continued

A. Suitable Host Organisms and Cells

GFP fluorescence is species-independent. Fluorescence has been reported from many different types of GFP-expressing hosts. Below is a partial listing of organisms that can express fluorescent GFP; a more extensive listing with references can be found at **www.clontech.com/gfp.**

1. Microbes

Agrobacterium tumefaciens
Anabaena
Bacillus subtilis
Bartonella henselae
Candida albicans
Caulobacter crescentus
Dictyostelium
Escherichia coli
Helicobacter pylori
Mycobacterium
Myxococcus xanthus
Polysphondylium pallidum
Pseudomonas
Rhizobium meloliti
Saccharomyces cerevisiae
Salmonella
Schizosaccharomyces pombe
Ustilago maydis
Yersinia

2. Invertebrates

Aedes aegypti
Caenorhabditis elegans
Diamondback moth
Drosophila melanogaster
Lytechinus pictus

3. Vertebrates

Fish, avian, and mammalian cell lines (specific cell lines listed on web)
Mouse
Xenopus
Zebrafish

4. Plants

Arabidopsis thaliana (thale cress)
Citrus sinensis (L.) Osbeck cv. Hamlin (an embryogenic sweet-orange cell line, H89)
Glycine max (soybean)
Hordeum vulgar (barley)
Nicotiana benthamiana & Nicotiana clevelandii (tobacco)
Onion
Zea mays (maize)

III. Expression of GFP and GFP Variants continued

B. Expression of GFP Fusion Proteins

GFP has been expressed as a fusion to many different proteins (Table II). In many cases, chimeric genes encoding either N- or C-terminal fusions to GFP retain the normal biological activity of the heterologous partner, as well as maintaining fluorescent properties similar to native GFP (Flach et al., 1994; Wang & Hazelrigg, 1994; Marshall et al., 1995; Stearns, 1995). The use of GFP and its variants in this capacity provides a "fluorescent tag" on the protein, which allows for in vivo localization of the fusion protein. GFP fusions can provide enhanced sensitivity and resolution in comparison to standard antibody staining techniques (Wang & Hazelrigg, 1994), and the GFP tag eliminates the need for fixation, cell permeabilization, and antibody incubation steps normally required when using antibodies tagged with chemical fluorophores. Lastly, use of the GFP tag permits kinetic studies of protein localization and trafficking (Flach et al., 1994; Wang & Hazelrigg, 1994).

TABLE II. PARTIAL LIST OF PROTEINS EXPRESSED AS FUSIONS TO GFP

Protein	Host Cell or Organism	Localization	References
CotE	Bacillus subtilis	Forespore	Webb et al., 1995
DacF, SpoIVA	Bacillus subtilis	Prespore/mother cell	Lewis & Errington, 1996
Coronin	D. discoideum	Phagocytic cups	Maniak et al., 1995
Myosin	D. discoideum		Moores et al., 1996
YopE cytotoxin	Yersinia		Jacobi et al., 1995
Histone H2B	Yeast	Nucleus	Flach et al., 1994; Schlenstedt et al., 1995
Tubulin	Yeast	Microtubules	Stearns, 1995
Nuf2p	Yeast	Spindle-pole body	Silver, 1995; Kahana et al., 1995
Mitochondrial matrix targeting signal	Yeast	Mitochondria	Cox, 1995
Npl3p	Yeast	Nucleus	Corbett et al., 1995
Nucleoplasmin	Yeast	Nucleus	Lim et al., 1995
Cap2p	Yeast		Waddle et al., 1996
Swi6p	Yeast		Sidorva et al., 1995

III. Expression of GFP and GFP Variants continued

TABLE II continued. PARTIAL LIST OF PROTEINS EXPRESSED AS FUSIONS TO GFP

Protein	Host Cell or Organism	Localization	References
HMG-R	Yeast		Hampton et al., 1996
Bud10p	Yeast		Halme et al., 1996
Pmp47	Yeast	Peroxisome	Dyer et al., 1996
Act1p, Sac6p, Abp1p	Yeast	Cytoskeleton	Doyle et al., 1996
p93dis1	Fission yeast	Spindle-pole body & microtubules	Nabeshima et al., 1995
Exu	Drosophila	Oocytes	Wang & Hazelrigg, 1994
Mei-S332	Drosophila	Centromere	Kerrebrock et al., 1995
Tau	Drosophila	Microtubules	Brand, 1995
β-galactosidase	Drosophila	cell movement	Shiga et al., 1996
Streptavidin		Insect cells	Oker-Blom et al., 1996
ObTMV movement protein	Tobacco	Filaments	Beachy, 1995; Heinlein et al., 1995
Potato virus X coat protein	Potato plant	Viral infection	Santa Cruz et al., 1996
GST	Zebrafish	Cytoplasmic(?)	Peters et al., 1995
11β-HSD2	Mammalian cells	Endoplasmic reticulum	Náray-Fejes-Tóth et al., 1996
MAP4	Mammalian cells	Microtubules	Olson et al., 1995
Mitochondrial targeting signal	Mammalian cells	Mitochondria	Rizzuto et al., 1995
Cyclins	Mammalian cells	Nucleus, microtubules, or vesicles	Pines, 1995
PML	Mammalian cells		Ogawa et al., 1995
Human glucocorticoid receptor	Mammalian cells		Ogawa et al., 1995 Carey et al., 1996
Rat glucocorticoid receptor	Mammalian cells		Rizzuto et al., 1995 Htun et al., 1996
Chromogranin B	HeLa cells	Secreted	Kaether et al., 1995
HIV p17 protein	HeLa cells	Nucleus	Bian et al., 1995
NMDAR1	HEK 293 cells	Membrane	Marshall et al., 1995
CENP-B	Human cells	Nucleus	Sullivan et al., 1995

III. Expression of GFP and GFP Variants continued

C. GFP Expression in Mammalian Cells

Appropriate vectors may be transfected into mammalian cells by a variety of techniques, including those using calcium phosphate (Chen & Okayama, 1988), DEAE-dextran (Rosenthal, 1987), various liposome-based transfection reagents (Sambrook et al., 1989), and electroporation (Ausubel et al., 1994). Any calcium phosphate transfection procedure may be used, but we recommend using the CalPhos™ Mammalian Transfection Kit (#K2051-1) for high calcium phosphate-mediated transfection efficiencies with an incubation of only 2–3 hours. Likewise, any liposome-mediated transfection procedure may be used, but we recommend using CLONfectin™ (#8020-1) for high, reproducible transfection efficiencies in a variety of cell types. For further information on cell culture techniques, we recommend Culture of Animal Cells, Third Edition, R. I. Freshney (1993, Wiley-Liss).

The efficiency of a mammalian transfection procedure is primarily dependent on the host cell line. Therefore, when working with a cell line for the first time, it is advisable to compare the efficiencies of several transfection protocols. This can best be accomplished using one of the GFP vectors which has the CMV immediate early promoter for high-level expression in most cell lines.

In most cases, GFP expression may be detected by fluorescence microscopy, FACS analysis, or fluorometer assays 24–72 hours post-transfection, depending on the host cell line used. If you used electroporation, wait until 48 hours post-transfection to assay or begin selection to allow cells to recover from the electroporation procedure. To visualize GFP-expressing cells by fluorescence microscopy, grow the cells on a sterile glass coverslip placed in a 60-mm culture plate. Alternatively, an inverted fluorescence microscope may be used for direct observation of fluorescent cells in the culture plate.

D. GFP Expression in Plants

In Arabidopsis, we recommend using EGFP instead of wt GFP, because the wt coding sequence contains a region recognized in Arabidopsis as a cryptic plant intron (bases 400–483). This intron is spliced out, resulting in a nonfunctional protein (Haseloff & Amos, 1995). Haseloff and Amos report successful expression in Arabidopsis of a modified version of GFP in which the cryptic intron sequences have been altered. The cryptic intron has also been removed by the changes to codon usage in EGFP, ECFP, and EYFP. A modified GFP gene with the same codon usage as EGFP has been used to express GFP in many plant systems using a variety of transfection techniques (Chiu et al., 1996). Functional wt GFP has been transiently expressed in several other plant species, indicating that the cryptic intron is unrecognizable in other plant species.

III. Expression of GFP and GFP Variants continued

E. GFP Expression in Yeast

wt GFP and GFPuv all express well in yeast. For expression in yeast, the GFP variant contained in Clontech's vectors must first be isolated (by PCR or by excision from the plasmid) and inserted into an appropriate yeast expression vector. Note that GFP genes with human codon usage (i.e., enhanced variants such as EGFP) are not efficiently expressed in yeast (Cormack et al., 1997). Also note that S. cerevisiae strains carrying the ade2 mitochondrial mutation accumulate a red pigment, which gives the cells an autofluorescence that interferes with detection of GFP (Smirnov et al., 1967; Weisman et al., 1987).

Our Yeast Protocols Handbook (PT3024-1) contains additional information on yeast growth and maintenance, preparation of yeast competent cells, and transformation of yeast cells. To obtain a copy, please visit our web site at **www.clontech.com**.

Additionally, we recommend Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G. R. Fink eds. (Vol. 194 in Methods in Enzymology, 1991, Academic Press).

IV. Detection of GFP, GFP Variants, and DsRed

A. Microscopy

1. Conventional microscopy

a. Filter sets

Although you can achieve excellent results using standard filter sets, such as FITC filters to detect EGFP, and rhodamine or propidium iodide filters to detect DsRed, optimized filter sets for detecting GFP, GFP variants, and DsRed have been developed by Omega Optical Inc. and Chroma Technology Corp. On their websites (**www.omega-filters.com**; **www.chroma.com**), you will find detailed information about how to detect Living Colors® Fluorescent Proteins. Optical properties, recommended uses, and sample data are all carefully described so that you can choose the filters that best suit your needs. With researchers increasingly interested in multi-color analysis, each company has done extensive testing to find the optimal filter combinations for separating cyan, green, yellow, and red fluorescence. You can read about the test results, including suggestions for experimental design and data collection, and view color images by visiting each of these websites.

If you should use fluorescein isothiocyanate (FITC) filters, keep in mind that these filter sets usually transmit excitation light at 450–500 nm—well above the major excitation peak (395 nm) of wt GFP. These filters, therefore, produce a fluorescent signal from wt GFP that is several-fold less than would be obtained with filters that transmit at 395 nm. However, it is not advisable to excite at 395 nm because at this wavelength, GFP rapidly photoisomerizes and loses the fluorescent signal.

In contrast to wt GFP, EGFP has a single, strong, red-shifted excitation peak at 488 nm, making it well suited for detection in commonly used equipment which utilize FITC optics. Because living cells tolerate longer wavelengths better due to the lower energies, the fluorescent signal obtained by illuminating EGFP at ~488 nm is more stable and less toxic than that of wt GFP.

b. Light source

The emission intensity from a light source can vary with wavelength. Because the intensity of the excitation light directly affects the brightness of a reporter, it is important to compare the excitation spectra of your chosen reporters with the emission spectrum of the light source. Conventional fluorescence microscopes are equipped with either a mercury- or xenon-arc lamp. Both lamps are suitable for exciting fluorescent proteins. But be aware that, whereas the light intensity from a xenon lamp deviates very little across the blue, green, yellow, and red regions, the emission from a mercury lamp has sharp peaks in the cyan and red regions. Therefore, fluorophores excited

IV. Detection of GFP, GFP Variants, and DsRed continued

by cyan and red light may appear brighter when excited by a mercury lamp. Check the manufacturers' specifications for details about your lamp(s).

c. Detection systems

In conventional microscopy, fluorescence is usually detected with either a photographic (35-mm) or digital camera (which frequently harbors a cooled charge-coupled device, a CCD). One advantage of a 35-mm camera is that it can record the "true" color (as seen by eye through the microscope) of a single fluorescent protein as well as the mixed color of colocalized fluorescent proteins. But recording an image with a 35-mm camera may require a longer-than-average exposure time, effectively photobleaching the sample. Digital cameras, on the other hand, are usually more sensitive than 35-mm cameras but (unless you use a color digital camera) require image analysis software to produce "pseudo-colored" data.

d. Multicolor analysis

With the development of optimized filter sets and the introduction of our red fluorescent protein, DsRed, it's now possible to separate as many as three fluorescent reporters (cyan, yellow, and red) by microscopy. Some investigators have even distinguished all four colors (cyan, green, yellow, and red) using flow cytometry (Hawley et al., 2001). In fact, many examples of multicolor analysis can now be found in the literature (a few of which are listed in Table III), and we recommend you survey the literature for help in deciding which combination will work best for you. Here, we offer the following recommendations for two-color analysis:

- For dual-labeling experiments, we recommend using DsRed2 with EGFP. One advantage of using DsRed2 with EGFP is that an overlay of the proteins' emissions appears yellow, so you can distinguish between the individual proteins and the overlap.
- ECFP and EYFP can also be used together to carry out double-labeling experiments (Green et al., 2000; Ellenberg et al., 1999; July 1999 Clontechniques). We tested the optical separation of ECFP and EYFP with conventional fluorescence microscopy. In one study, we examined HeLa cells expressing ECFP and EYFP, targeted to the mitochondria and nucleus, respectively (Green et al., 2000). To visualize the fluorescence, we used a Zeiss Axioskop equipped with a 100W mercury arc lamp and filter sets for detecting ECFP (Omega Optical's XF114) and EYFP (Omega Optical's XF104). Images were captured with a 35-mm camera. Using this set up, the two colors are well separated; no bleed-through was evident. During the initial exposure, (i.e., during the first ~15 sec), these proteins display

IV. Detection of GFP, GFP Variants, and DsRed continued

similar intensities; however, because ECFP photobleaches faster than EYFP, unequal intensities become obvious after several more seconds of illumination.

TABLE III. MULTI-COLOR ANALYSIS WITH LIVING COLORS® PROTEINS

Type of Analysis	Application	Recommended Combination	References*
Two-color	Microscopy	DsRed + EGFP (GFP)	October 1999 Clontechniques Handler & Harrell, 2001
		ECFP + EYFP	Green et al., 2000 Stuurman et al., 2000 October 1999 Clontechniques
Three-color	Microscopy	DsRed + ECFP + EYFP	Bloemberg et al., 2000 October 1999 Clontechniques
Four-color	Flow cytometry	DsRed + EGFP + EYFP + ECFP	Hawley et al., 2001 July 2001 Clontechniques

* Also, see Patterson et al., (2001).

2. Laser-scanning microscopy

If you are using a laser-scanning confocal fluorescence microscope, your excitation source will usually be an argon-ion or krypton-ion laser, or both. With the 488-nm line of the argon laser, you can excite EGFP, EYFP, and DsRed. But you'll need the 458-nm line from an argon laser or 413-nm line from a krypton laser to produce strong emissions from ECFP (Hawley et al., 2001; Ellenberg et al., 1999). The krypton-ion laser may also be used to excite DsRed; its 568-nm line is close to DsRed's excitation maximum (Hawley et al., 2001). With so many laser configurations now available, we recommend you consult the manufacturer(s) to obtain more detailed information about a particular set up. Compare the laser specifications with the excitation spectra of the fluorescent proteins you plan to use.

3. Multi-photon laser-scanning microscopy

Multi-photon excitation microscopy has won the approval of many researchers who seek other alternatives for resolving fluorescently labeled proteins in vivo. Its advantages have been widely reported (Piston, D. W., 1999; Potter, S. M., 1996; Marchant, J. S., 2001). Chief among them is the ability to excite fluorophores with low-energy infrared light. IR light not only penetrates tissue more effectively than visible light, it also minimizes photobleaching and causes less photodamage. In principle, all Living Colors Fluorescent Proteins, including DsRed (Jakobs et al., 2000), are suitable for use in multi-photon applications.

IV. Detection of GFP, GFP Variants, and DsRed continued

4. Fluorescence Microscopy

The protocol provided below is one possible microscopy procedure. Other equally suitable and more detailed microscopy procedures may be found elsewhere (e.g., Ausubel et al., 1995 et seq.)

Materials required:

- 70% Ethanol
- Dulbecco's Phosphate buffered saline (DPBS; pH 7.4)
- DPBS/4% paraformaldehyde (pH 7.4–7.6)
  Add 4 g of paraformaldehyde to 80 ml of DPBS. Heat to dissolve. Once the solution has cooled, readjust the pH if necessary, then dilute to a final volume of 100 ml. Store at –20°C.
- Rubber cement, molten agarose, or commercial mounting medium (e.g., ProLong® Antifade Kit, Molecular Probes)

Fluorescence microscopy procedure

In a tissue culture hood:

a. Sterilize a glass coverslip with 70% ethanol.

b. Place the coverslip in a sterile tissue culture dish.

c. Plate and transfect cells in the tissue-culture dish containing the coverslip.

Note: Some cell types may not adhere to the glass coverslip. In these cases, you may need to pre-treat the glass coverslip with a substrate that promotes cell adhesion (e.g., lamin, or poly-D-lysine, or both). As an alternative to glass coverslips, you might try culturing cells directly on BD Falcon™ CultureSlides.

c. At the end of the culture period, remove the tissue culture media and wash once with DPBS.

d. Fixing cells

i. After cells have been washed with DPBS, add freshly made DPBS/4% paraformaldehyde directly to the coverslip.

ii. Incubate cells in solution at room temperature for 30 min.

iii. Wash cells twice with DPBS. Allow cells to soak in DPBS for 10 min during each wash.

e. Mounting the coverslip onto a glass microscope slide

i. Carefully remove the coverslip from the plate with forceps. Pay close attention to which side of the coverslip contains the cells.

ii. Place a tiny drop of commercial mounting solution (e.g., ProLong® Antifade Kit, Molecular Probes) on the slide, and allow the coverslip to slowly contact the solution and to lie down on the slide, cell side down.

IV. Detection of GFP, GFP Variants, and DsRed continued

iii. Carefully aspirate the excess solution around the edge of the coverslip using a Pasteur pipette connected to a vacuum pump.

iv. If desired, seal the coverslip to the microscope slide using molten agarose, rubber cement, or black nail polish.

v. Allow to dry. The drying time may vary depending on the mounting solution used.

vi. Examine slides by fluorescence microscopy. Once fixed, cells can be stored in the dark at 4°C.

B. Flow Cytometry

1. Multicolor analysis and cell sorting

Several investigators have sorted GFP-expressing cells by flow cytometry (Cheng & Kain, 1995; Ropp et al., 1995; Yu & van den Engh, 1995). FACS analyses have been carried out with plant protoplasts, yeast, E. coli, and mammalian cells expressing GFP (Atkins & Izant, 1995; Fey et al., 1995; Sheen et al., 1995; Cheng et al., 1996). Maximal emission is achieved when EGFP is excited at 488 nm, which corresponds perfectly to the 488-nm line of argon-ion lasers used in many flow cytometers. In fact, this variant was selected specifically on the basis of its increased fluorescence in flow cytometry assays.

Since EGFP, EYFP, and DsRed can all be excited by a single laser excitation wavelength (488 nm), two-color (EGFP/EYFP; Lybarger et al., 1998) and three-color (Hawley et al., 2001) flow cytometric analyses are possible. ECFP and EYFP are not recommended for dual-color analysis with flow cytometry because the argon laser, used for excitation in most flow cytometers, does not efficiently excite ECFP. To excite ECFP, your flow cytometer must be equipped with a krypton laser tuned to 413 nm (Ellenberger et al., 1998 & 1999) or an argon laser tuned to 458 nm (Hawley et al., 2001).

EGFP and DsRed are perhaps the best combination for cell sorting and two-color analysis by flow cytometry. These proteins can be co-excited by the 488 nm line of the argon laser and their emissions can be conveniently recorded through the FL-1 (green) and FL-2 (red) channels. The analysis can be further refined by using optimized filters sets and by adjusting the compensation settings.

IV. Detection of GFP, GFP Variants, and DsRed continued

2. Detecting fluorescent proteins in ethanol-treated cells

When expressed as aqueous soluble products, fluorescent proteins may leak from ethanol-treated cells; ethanol permeabilizes the plasma membrane. To avoid this problem, we suggest you use paraformaldehyde if you plan to fix fluorescent protein-expressing cells. Otherwise, if you wish to measure transfection efficiency, but plan to fix cells with ethanol, we recommend using EGFP-F, ECFP-Mem, or EYFP-Mem, described below.

In some cases, you may notice a gradual increase in background fluorescence in ethanol-treated cells during flow cytometry. A rinse with PBS supplemented with 1% BSA after ethanol fixation, or long fixation times (18–24 hr), eliminates this problem (D. Ucker, pers. comm.).

a. EGFP-F is post-translationally farnesylated, so it remains attached to the inner face of the plasma membrane even during treatment with ethanol. EGFP-F contains a short, C-terminal farnesylation signal from c-Ha-Ras (Aronheim et al., 1994; Hancock et al. 1991). The signal targets EGFP-F to the plasma membrane without altering the intrinsic fluorescent properties of the EGFP chromophore (Jiang & Hunter, 1998).

b. ECFP-Mem & EYFP-Mem are N-terminal fusions that contain the N-terminal 20 amino acids of neuromodulin (GAP-43; Moriyoshi et al., 1996). During post-translational processing, a palmitoyl group is added to the neuromodulin domain of the fusion protein. The modification targets the fluorescent protein primarily to the inner face of the plasma membrane. A small amount of the fusion does bind other cellular membranes.

IV. Detection of GFP, GFP Variants, and DsRed continued

C. Quantitative Fluorometric Assay

Recombinant GFP (or EGFP) can be used in fluorometric assays to confirm and measure GFP expression. After generating a standard curve using known amounts of recombinant protein, compare the fluorescence intensities of your experimental samples to the standard curve to determine the amount of GFP expressed in the sample. A detailed method for quantitation of wt GFP levels using the TD-700 fluorometer is available from Turner Designs, Inc (**www.turnerdesigns.com**).

When generating the standard curve, the recombinant protein should be diluted in the same buffer as the experimental samples. In some expression systems, GFP can be detected directly in the fluorometer (simply suspend cells in an isotonic buffer such as PBS); in other cases, cells will need to be disrupted in order to optimally detect GFP fluorescence.

Figure 6 shows a comparison of the fluorescence intensity of GFP detected in sonication buffer and in a cleared bacterial cell lysate. These results illustrate that the sensitivity and the linear range of detection of GFP are relatively unaffected by the cell lysate background.

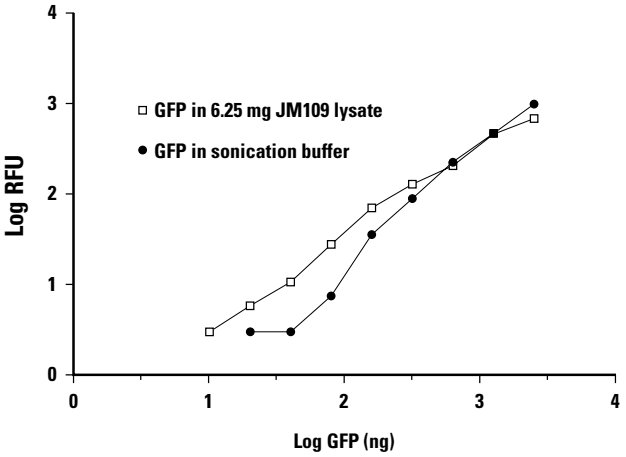

Figure 6. Comparison of fluorescence intensity of GFP in sonication buffer versus GFP in cell lysates. LB was inoculated with a culture of untransformed JM109 cells and incubated overnight at 30°C. Cell lysates were prepared as described in the protocol. Serial dilutions of rGFP were prepared in both sonication buffer and JM109 cell lysate. Fluorescence intensity was measured in a modified Hoefer Pharmacia Biotech, Inc. DyNA Quant 200 Fluorometer using an excitation filter of 365 nm and an emission filter of 510 nm. (RFU = relative fluorescence units)

IV. Detection of GFP, GFP Variants, and DsRed continued

The following procedures have been developed using our Recombinant GFP Protein (rGFP #8360-2). Use an excitation filter of 365 nm and an emission filter of 510 nm when assaying wt GFP or GFPuv, and an excitation filter of 450–490 nm and an emission filter of 510 nm when assaying EGFP.

1. Materials required
   - Sonication buffer (pH 8.0)
     50 mM NaH_2PO_4
     10 mM Tris-HCl (pH 8.0)
     200 mM NaCl
     Adjust pH to 8.0. Store at room temperature.
   - PBS (pH 7.4)
2. Generating a GFP standard curve:
   a. Prepare serial dilutions of rGFP in sonication buffer, deionized H_2O, or 10 mM Tris-HCl (pH 8.0).
      Note: Samples can be stored at –20°C.
   b. Assay dilutions in a fluorometer.
   c. Plot the log of the relative fluorescence units (RFU) as a function of the log of fold-dilution of each sample such as the standard curve shown in Figure 7.
3. Measuring fluorescence intensity of bacterial samples:
   a. Pellet 20 ml of bacterial cell culture.
   b. Remove the supernatant and freeze pellet at –70°C.
   c. Wash pellet once with 1.6 ml of PBS.
   d. Resuspend pellet in 1.6 ml of sonication buffer.
   e. Prepare 2-fold serial dilutions in sonication buffer.
   f. Assay dilutions in a fluorometer.
   g. Compare results with those of the standard curve to determine the amount of GFP protein expressed in the experimental samples.
4. Preparation of bacterial cell lysates:
   a. Pellet 100 ml of bacterial cell culture and remove the supernatant.
      Note: Cell pellets may be stored at –70°C for later use.
   b. Wash pellet twice with 4 ml of PBS.
   c. Resuspend pellet in 4 ml of sonication buffer.
   d. Freeze/thaw sample in a dry ice/ethanol bath five times.
   e. Vortex sample for 1 min, then incubate on ice for 1 min.
   f. Repeat Step e two more times.
   g. Run sample through an 18-gauge needle several times.

IV. Detection of GFP, GFP Variants, and DsRed continued

h. Transfer cell lysate to a 1.5-ml microcentrifuge tube and centrifuge at 12,000 rpm for 5 min at 4°C.

i. Collect the supernatant and determine the protein concentration using a standard assay (e. g., Bradford, Lowry, etc.; Scopes, 1987).

j. Assay the dilutions in a fluorometer.

k. Compare the results with the standard curve to determine the amount of GFP expressed in the experimental samples.

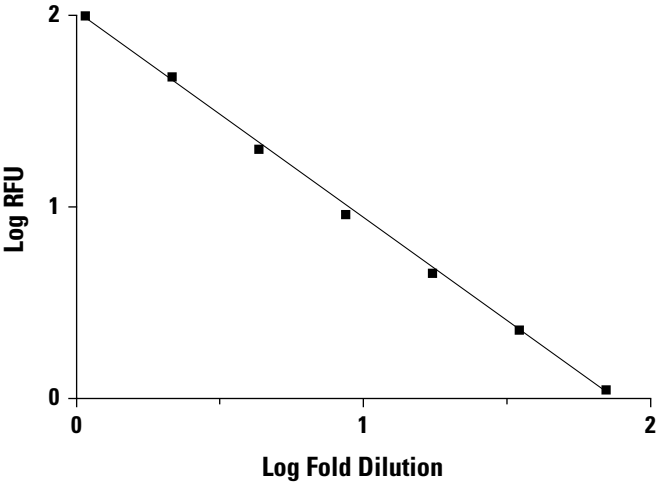

Figure 7. Relative fluorescence intensity of two-fold serial dilutions of a suspension of GFP-transformed cells in sonication buffer. LB was inoculated with JM109 cells transformed with a plasmid encoding wt GFP and incubated overnight at 30°C. Fluorescence intensity was measured by reading 5-µl samples of 2-fold dilutions in a modified Hoefer Pharmacia Biotech, Inc. DyNA Quant 200 Fluorometer using an excitation filter of 365 nm and an emission filter of 510 nm.

V. Purified Recombinant GFP and GFP Variants

A. General Information

Our Recombinant Green Fluorescent Protein (rGFP; #8360-2), Recombinant EGFP (rEGFP; #8365-1), and Recombinant GFPuv (rGFPuv; #8366-1) are purified from transformed E. coli using a method which ensures optimal purity of the recombinant protein and maintenance of GFP fluorescence. All proteins are 27-kDa monomers with 239 amino acids. The excitation and fluorescence emission spectra for the rGFP is identical to GFP purified from Aequorea victoria (Chalfie et al., 1994). The purified proteins retain their fluorescence capability under many harsh conditions (Section II.F) and are suitable as control reagents for GFP expression studies using the Living Colors line of GFP reporter vectors.

Applications of purified rGFP and rGFP variant proteins include use as standards for SDS or two-dimensional PAGE, isoelectric focusing, Western blot analysis, calibration of fluorometers and FACS machines, fluorescence microscopy, and microinjection of GFP into cells and tissues.

B. rGFP and rGFP Variants as Standards in Western Blots

When used as a standard for Western blotting applications in conjunction with the Living Colors A.v. Peptide Antibody (#8367-1, -2), the recombinant proteins can be used to correlate GFP expression levels to fluorescence intensity or to differentiate problems with detection of GFP fluorescence from expression of GFP protein.

Use a standard procedure for discontinuous polyacrylamide gel electrophoresis (PAGE) to resolve the proteins on a one-dimensional gel (Laemmli, 1970). If another electrophoresis system is employed, the sample preparation should be modified accordingly. Just prior to use, allow rGFP protein to thaw at room temperature, mix gently until solution is clear, and then place tube on ice.

On a minigel apparatus, 25–75 µg of lysate protein per lane is typically needed for satisfactory separation (i.e., discrete banding throughout the molecular weight range) of a protein mixture derived from a whole cell or tissue homogenate. If rGFP is to be used as an internal standard in a Coomassie blue-stained minigel, we recommend loading 500 ng of rGFP per lane. If rGFP is added to a total cell/tissue lysate or other crude sample, the amount of total protein loaded per lane must be optimized for the particular application.

For Western blotting applications, we recommend loading 40–400 pg of rGFP (or rGFP variant) per lane for a strong positive signal.

Generally, rGFP will not fluoresce on an SDS gel or a Western blot.

V. Purified Recombinant GFP and GFP Variants continued

C. rGFP and rGFP Variants as a Control for Fluorescence Microscopy

The following two protocols are for use of rGFP or rGFP variants as a control on microscope slides in fluorescence microscopy. The purified proteins may be used to optimize lamp and filter set conditions for detection of GFP fluorescence, or as a qualitative means to correlate GFP fluorescence with the amount of protein in transfected cells.

1. Unfixed samples

Use this method for live cell fluorescence or other cases where a fixation step is not desired.

a. Perform 1:10 serial dilutions of the 1.0 mg/ml rGFP or rGFP variant stock solution with 10 mM Tris-HCl (pH 8.0) to yield concentrations of 0.1 mg/ml and 0.01 mg/ml.

Notes:

- These dilutions should suffice as a positive control. The 1.0 mg/ml solution will give a very bright fluorescent signal by microscopy.
- The diluted samples can be aliquoted and stored frozen at –70°C for up to 1 yr with no loss of fluorescence intensity. Avoid repeated freeze-thaw cycles with the same aliquot.

b. Using a micropipette, spot 1–2 µl of diluted protein onto the microscope slide. If using a slide that contains a mounted coverslip, position the spot several millimeters away from the sample such that a second coverslip can be added over the protein spot.

c. Allow the protein to air-dry for a few seconds, and mark the position of the spot on the other side of the slide to aid in focusing.

d. Add a coverslip over the spot using a 90% glycerol solution in 100 mM Tris-HCl (pH 7.5).

e. Fluorescence from the spot is best viewed at low magnification, using either a 10X or 20X objective lens.

2. Fixed samples

In some cases it may be necessary to fix the recombinant protein to the microscope slide prior to microscopy. This can be done by dipping the section of the microscope slide containing the air-dried protein spot (after Step 1.c above) into 100% methanol for 1 min. Allow the slide to dry completely and place a coverslip over the sample as in Step 1.d above.

VI. GFP Antibodies

A. Applications for GFP Antibodies

We offer three GFP-specific antibodies:

- Living Colors® A.v. Peptide Antibody (#8367-1, -2) detects all GFP variants. The A.v. Peptide Antibody, which is also available conjugated to horseradish peroxidase (HRP), is recommended for Western analysis.
- Living Colors® A.v. (JL-8) Monoclonal Antibody (#8371-1, -2), a mouse monoclonal (subclass IgG_2a), recognizes all Living Colors GFP variants, including our enhanced and destabilized proteins, fusions to these proteins, and purified recombinant GFP. The JL-8 Monoclonal is ideal for Western and ELISA applications.
- Living Colors® Full-Length A.v. Polyclonal Antibody (#8372-1, -2) was specifically developed and tested for immunoprecipitating GFP and GFP variants. It also detects destabilized variants and fusions to all Living Colors GFP proteins.

We also offer DsRed-specific antibodies:

- Living Colors® D.s. Peptide Antibody (#8370-1, -2) recognizes wt DsRed and DsRed variants such as DsRed1-E5 and DsRed2. The antibody also binds DsRed fusions when the protein of interest is fused to the N-terminus of DsRed or one of its variants. Because the D.s. Peptide epitope often becomes hidden when proteins are fused to the C-terminus of DsRed, you may have difficulty detecting C-terminal fusions with this antibody. Raised in rabbits and affinity-purified, this polyclonal antibody is recommended for use in Western blotting and immunoprecipitation. The D.s. Peptide Antibody does not cross-react with any Aequorea victoria GFP variants.
- Living Colors® DsRed Monoclonal Antibody (#8374-1, -2) recognizes denatured forms of wild-type DsRed and its variants, including DsRed1, DsRed2, and DsRed1-E5. Recommended for Western blotting applications only, this antibody (mouse IgG) binds DsRed1 and DsRed2 even when the proteins are expressed as fusions to other proteins. Both N- and C-terminal fusions are recognized.

VI. GFP Antibodies continued

B. Immunoprecipitation with the Living Colors A.v. Peptide Antibody

1. Solutions Required

- PBS
- Lysis buffer
  PBS containing 0.5% Triton X-100, 5 mM EDTA, and the following protease inhibitors (all available from Sigma): PMSF, 0.1 mM; pepstatin A, 10 µM; leupeptin, 10 µM; aprotinin, 25 µg/ml.
- Living Colors Peptide Antibody
- Protein A or Protein G agarose beads (Pierce or BioRad)
  Use 20–30 µl of beads per sample. Spin down beads to remove the ethanol, then wash beads twice in PBS. Aspirate the supernatant from the pellet of beads.

2. Procedure

a. Spin down ~ 1 x 10^6 cells (or cells from one 100-mm dish) and wash twice in PBS. Resuspend in 1.2 ml of lysis buffer.
b. Incubate at 4°C for 30 min with continuous gentle inversion.
c. Clear the lysate by centrifuging in a microcentrifuge at 12,000 rpm for 20 min at 4°C.
d. Transfer 1 ml of supernatant to a 1.5-ml microcentrifuge tube.
e. Add 5 µg of Living Colors A.v. Peptide Antibody to the supernatant. Incubate at 4°C for 2–3 hr with continuous gentle inversion.
f. Add the antibody/lysate mixture to the pellet of Protein A (or Protein G) agarose beads.
g. Vortex and incubate at 4°C for 2 hr with continuous gentle inversion.
h. Wash the beads 5 times with PBS. For each wash, add 1 ml of PBS and incubate for 2 min with continuous gentle inversion. Aspirate the supernatant from the pellet of beads.

3. Sample analysis:

a. Wash the beads with 1 ml of Lysis Buffer.
b. Repeat this wash procedure four times.
c. Add 30 µl of 2X SDS-PAGE sample buffer to beads.
d. Vortex, then boil for 2 min.
e. Repeat step d.
f. Centrifuge in a microcentrifuge at 12,000 rpm for 1 min at room temperature.
g. Electrophorese 10–20 µl of supernatant on an SDS-polyacrylamide gel (a 12% gel will resolve GFP at 27 kDa). Analyze the resolved proteins on a Western blot, by silver or Coomassie blue staining, or by autoradiography.

VII. Troubleshooting Guide

A. Potential Difficulties in Using GFP Fluorescence

- Variability in the intensity of GFP fluorescence has been noted. This may be due in part to the relatively slow formation of the GFP chromophore and the requirement for molecular oxygen (Heim et al., 1994).
- The slow rate of chromophore formation and the apparent stability of wt GFP may preclude the use of GFP as a reporter to monitor fast changes in promoter activity (Heim et al., 1994, Davis, I. et al., 1995). This limitation is reduced by use of EGFP and destabilized GFPs, which acquire fluorescence faster than wt GFP (Heim et al., 1994).
- The wt GFP coding sequences contain a cryptic plant intron (between nucleotides 400 and 483) that is efficiently spliced out in Arabidopsis and results in a nonfunctional protein (Haseloff & Amos, 1995). Functional wt GFP has been transiently expressed in several other plant species indicating that the cryptic intron may not be recognized or recognized less efficiently in these species. EGFP, ECFP, and EYFP do not contain the cryptic intron and can be used for expression in plants.
- Some people have put GFP expression constructs into their system and failed to detect fluorescence. There can be numerous reasons for failure, including use of an inappropriate filter set, expression of GFP below the limit of detection, and failure of GFP to form the chromophore. Recombinant GFP Protein and a GFP-specific antibody, such as the Living Colors A.v. Peptide Antibody, may be used to troubleshoot GFP expression in these cases. Since GFPuv and EGFP fluoresce brighter than wt GFP, they are better alternatives for expression of GFP.
- GFP targeted to a low pH environment may lose fluorescence.
- Ethanol-fixed cells will lose fluorescence because the soluble fluorescent protein leaks through the permeabilized plasma membranes. Use pEGFP-F, pECFP-Mem, or pEYFP-Mem Vectors to avoid this problem.

B. Requirements for GFP Chromophore Formation

- Formation of the chromophore in wt GFP appears to be temperature sensitive; however, the mutations in EGFP, EYFP, ECFP, and GFPuv suppress this thermosensitivity. In some cases, E. coli, yeast, and mammalian cells expressing wt GFP have shown stronger fluorescence when grown at lower temperatures (Heim et al., 1994; Ward, pers. comm.; Lim et al., 1995; Pines, 1995; Ogawa et al., 1995). Hence, incubation at a lower temperature may increase the fluorescence signal obtained when using wt GFP.
- GFP chromophore formation requires molecular oxygen (Heim et al., 1994; Davis, D. F. et al., 1995); therefore, cells must be grown under aerobic conditions.

VII. Troubleshooting Guide continued

C. Photobleaching or Photodestruction of Chromophore

- Excite at 470 nm for wt GFP, 360–400 nm for GFPuv, and 488 nm for the red-shifted GFP excitation variants. Excitation at the 395-nm peak for wt GFP may result in rapid loss of signal. For GFPuv, use the longest possible excitation wavelength to minimize the rate of photobleaching.
- A tungsten-QTH or argon light source is preferable. Mercury and xenon lamps produce significant UV radiation which will rapidly destroy the chromophore unless strongly blocked by appropriate filters.

D. Autofluorescence

- Some samples may have a significant background autofluorescence, e.g., worm guts (Chalfie et al., 1994; Niswender et al., 1995). A band-pass emission filter may make the autofluorescence appear the same color as GFP; using a long-pass emission filter may allow the color of the GFP and autofluorescence to be distinguished. Use of DAPI filters may also allow autofluorescence to be distinguished (Brand, 1995; Pines, 1995).
- Most autofluorescence in mammalian cells is due to flavin coenzymes (FAD and FMN; Aubin, 1979) which have absorption and emission maxima at 450 and 515 nm respectively. These values are very similar to those for wt GFP and the red-shifted GFP variants, so autofluorescence may obscure the GFP signal. The use of DAPI filters when using 450/515 nm light for excitation may make autofluorescence appear blue while the GFP signal remains green. In addition, some growth media can cause autofluorescence. When possible, perform microscopy in a clear buffer such as PBS, or medium lacking phenol red. Also, when selecting for GFP expression in mammalian cells, you may want to exclude the highest expressing clones, which will exhibit more background fluorescence in culture (D. Piston, pers. comm.).
- Some cell types can produce a speckled autofluorescence pattern which is likely due to mitochondrially bound NADH (Aubin, 1979). This problem is minimized with excitatory light around 488 nm, as opposed to UV excitation (Niswender et al., 1995). Therefore, we recommend using EGFP for expression in these systems.
- For mammalian cells, autofluorescence can increase with time in culture. For example, when CHO or SCI cells were removed from frozen stocks and reintroduced into culture, the observed autofluorescence (emission at 520 nm) increased with time until a plateau was reached around 48 hours (Aubin, 1979). Therefore, in some cases it may be preferable to work with freshly plated cells.

VII. Troubleshooting Guide continued

- Always use a mock-transfected control to gauge the extent of autofluorescence.
- For fixed cells, autofluorescence can be reduced by washing with 0.1% sodium borohydride in PBS for 30 min after fixation.

E. Considerations for Mammalian Expression

- Use an enhanced fluorescent variant (ECFP, EGFP, or EYFP) instead of wt GFP. The brighter fluorescence and improved translation of these variants makes them easier to detect.
- Verify your GFP variant plasmid construct and concentration with a restriction digest. Verify that all subcloning steps have been done correctly, keeping in mind specific restriction sites in some vectors which are inactivated by methylation. Our EGFP Sequencing Primers may be used to verify sequence junctions.
- Be sure the vector is compatible with your cell type. Find out if the CMV promoter has been used previously for transient expression in your cells.
- Use another assay to estimate transfection efficiency. Transfect with a second reporter plasmid that contains the same promoter element. For example, use pEGFPLuc (#6169-1) or pHygEGFP (#6014-1). With pEGFPLuc (#6169-1), you can determine transfection efficiency by fluorescence microscopy and obtain quantitative data using a standard luciferase assay.
- Expression of GFP protein can be verified by Western analysis using either the Living Color A.v. Peptide Antibody (#8367-1, -2) or the Living Colors Full-Length A.v. Polyclonal Antibody (#8373-1, -2).

F. Optimizing Microscope/FACS Applications

- In general, optimal visual detection of GFP fluorescence by microscopy is achieved in a darkened room, after your eyes have adjusted.
- Choose the filter set that is optimal for the GFP variant that you are using. In general, conditions used for fluorescein should give some signal with all variants except GFPuv. Autofluorescence may be a problem in some cell types or organisms. For more information on the filter sets recommended for GFP and its variants, see Section IV.
- To check the microscope setup, spot a small volume of purified recombinant GFP protein (e.g., rGFP, rEGFP, or rGFPuv) on a microscope slide. As a crude substitute, any source of fluorescein can be used, such as a fluorescein-conjugated antibody or avidin-fluorescein.
- GFP fluorescence is very sensitive to some nail polishes used to seal coverslips (Chalfie et al., 1994; Wang & Hazelrigg, 1994). In place of nail polish for mounting coverslips, we recommend molten agarose, rubber cement, or commercial mounting solution.

VII. Troubleshooting Guide continued

- Exciting GFP intensely for extended periods may generate free radicals that are toxic to the cell. This problem can be minimized by excitation at 450–490 nm.

G. Anomalous Band in some GFP Plasmid Preparations

We have occasionally observed a band running at approximately 500 bp in some (but not all) plasmid preparations of the GFP vectors that have the backbone carrying the kanamycin resistance gene and the f1 origin (i.e., pEGFP-1, pEGFP-N1, -N2, -N3, -C1, -C2, -C3, pEYFP-C1, -N1, pEYFP-1, and pECFP-N1, -C1, and -1). The presence and level of this band varies greatly from one plasmid DNA preparation to another. We have not thoroughly characterized this band; however, it appears to be a small circular DNA, possibly single-stranded, generated as a by product of plasmid replication. As far as we can ascertain, this band does not interfere with ligating inserts into the vectors, does not affect transformation or transfection efficiencies, and does not have any deleterious effect on mammalian cells. However, if you wish to avoid the presence of this band in your plasmid preps, try any non-alkaline bacterial lysis procedure (Sayers et al., 1996) or gel-purify the major band after alkaline lysis.

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IX. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

We offer a variety of vectors for expression of GFP and its variants. The Vector Information Packet that accompanies any purchased vector provides a map, multiple cloning site (MCS), complete sequence, and additional information about the vector. Much of this information is also available at our web site.

Bacterial Expression Vectors

Protein can be overexpressed in bacteria using these vectors. The coding sequence can be excised using restriction sites in the flanking MCS regions; or the coding sequence can be amplified by PCR.

- pEGFP Vector 6077-1
- pEYFP Vector 6004-1
- pECFP Vector 6075-1
- pGFP Vector 6097-1
- pGFPuv Vector 6079-1

Mammalian Expression Vector

- pCMS-EGFP 6101-1

N-Terminal Protein Fusion Vectors

- pECFP-N1 6900-1
- pEYFP-N1 6006-1
- pEGFP-N1/2/3* 6085-1, 6081-1, 6080-1

C-Terminal Protein Fusion Vectors

- pECFP-C1 6076-1
- pEYFP-C1 6005-1
- pEGFP-C1/2/3* 6084-1, 6083-1, 6082-1

*Each vector has the MCS in one of three possible reading frames

Promoterless Mammalian Expression Vectors

Used for monitoring the activity of promoters or promoter/enhancer combinations inserted into the MCS upstream of the gene encoding the GFP variant.

- pECFP-1 6901-1
- pEGFP-1 6086-1
- pEYFP-1 6007-1

Destabilized Mammalian ExpressionVectors

- pd2EGFP 6010-1

IX. Related Products continued

- pd2ECFP-1 6910-1
- pd2EGFP-1 6008-1
- pd2EYFP-1 6912-1

Destabilized N-Terminal Protein Fusion Vectors

- pd1EGFP-N1 6073-1
- pd2EGFP-N1 6009-1
- pd4EGFP-N1 6072-1
- pd2ECFP-N1 6911-1
- pd2EYFP-N1 6913-1

Subcellular Localization Vectors

These vectors target the GFP variant to a specific organelle or subcellular structure. You can visualize the localized fluorescence in living or fixed mammalian cells using fluorescence microscopy.

- pEGFP-Actin 6116-1
- pEYFP-Actin 6902-1
- pECFP-ER 6907-1
- pEYFP-ER 6906-1
- pEGFP-F (farnesylated) 6074-1
- pECFP-Golgi 6908-1
- pEYFP-Golgi 6909-1
- pECFP-Mem 6918-1
- pEYFP-Mem 6917-1
- pECFP-Mito (mitochondria) 6903-1
- pEYFP-Mito 6115-1
- pECFP-Nuc (nucleus) 6904-1
- pEYFP-Nuc 6905-1
- pEGFP-Peroxi (peroxisome) 6932-1
- pECFP-Peroxi 6931-1
- pEYFP-Peroxi 6933-1
- pEGFP-Tub (tubulin) 6117-1
- pEYFP-Tub 6118-1
- pECFP-Endo (endoplasmic reticulum) 6934-1
- pEGFP-Endo 6935-1
- pEYFP-Endo 6936-1

IX. Related Products continued

Bicistronic Expression Vectors

This vector allows for the simultaneous expression of EGFP and another recombinant protein from one mRNA transcript.

- pIRES2-EGFP 6029-1
- pIRES-EYFP 6032-1

Cotransfection Marker Vectors

- pHygEGFP 6014-1
- pEGFPLuc 6169-1

Retroviral Expression Vectors

- pLEGFP-N1 6059-1
- pLEGFP-C1 6058-1

Tet Expression Vector

- pBI-EGFP Tet 6154-1

Creator™ System Vectors

- pLP-ECFP-C1 Acceptor 6343-1
- pLP-EGFP-C1 Acceptor 6342-1
- pLP-EYFP-C1 Acceptor 6341-1
- pLP-IRES2-EGFP Acceptor 6345-1
- pDNR-d2EGFP Donor Reporter 6356-1
- pDNR-EGFP Donor Reporter 6357-1

Tet Expression Vectors

- pTRE-d2EGFP 6242-1
- pRevTRE2-d1EGFP 6139-1

Mercury™ Pathway Profiling Vectors

- pCRE-d2EGFP 6034-1
- pGRE-d2EGFP 6033-1
- pNF-kB-d2EGFP 6054-1

IX. Related Products continued

Supporting Products

• Living Colors A.v. Peptide Antibody	8367-1, -2
• Living Colors A.v. Peptide Antibody-HRP Conjugate	8369-1
• Living Colors A.v. (JL-8) Monoclonal Antibody	8371-1, -2
• Living Colors Full-Length A.v. Polyclonal Antibody	8372-1, -2
• EGFP-N Sequencing Primer	6479-1
• EGFP-C Sequencing Primer	6478-1
• EFP PCR Primer Set	9120-1
• Recombinant GFP Protein	8360-2
• Recombinant EGFP Protein	8365-1
• Recombinant GFPuv Protein	8366-1

DsRed Products

• pDsRed2 Vector	6943-1
• pDsRed2-1 Vector	6944-1
• pDsRed2-C1 Vector	6974-1
• pDsRed2-N1 Vector	6973-1
• pTimer Vector	6941-1
• pTimer-1 Vector	6942-1
• Living Colors® D.s. Peptide Antibody	8370-1, -2
• Living Colors® DsRed Monoclonal Antibody	8374-1, -2
• DsRed-N Sequencing Primer	6480-1
• DsRed-C Sequencing Primer	6484-1
• DsRed1-N Sequencing Primer	6482-1
• DsRed1-C Sequencing Primer	6483-1

Appendix: Recommended Resources & Reading

A. Fluorescent Proteins Newsgroup

The bionet category of internet news groups includes a group for the discussion of fluorescent proteins. This newsgroup provides a forum for discussing bioluminescence, promoting development of reporter proteins from bioluminescent organisms, and facilitating their application to interesting biological questions. You can access the newsgroup at www.bio.net/hypermail/FLUORESCENT-PROTEINS.

B. GFP: Green Fluorescent Protein Properties, Applications, and Protocols

Martin Chalfie & Steven Kain, Editors, Wiley Press, 1998

This book provides the first thorough description of GFP and its applications as a reporter. It includes detailed case studies and advice on successfully engineering reporter constructs to achieve maximal expression in a variety of organisms. It also provides step-by-step protocols with tips for using GFP in molecular biology research.

C. Methods in Cell Biology: Green Fluorescent Proteins

Kevin F. Sullivan and Steve A. Kay, Editors, Academic Press, 1998

This book is a practical guide to working with fluorescent proteins, and contains detailed protocols for the expression and visualization of fluorescent proteins in living cells. (ISBN: 0126760756)

Notes

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