AUTOFLUORESCENT PROTEINS

AFP's™

Applications Manual

Manual AFP-Manual (version AFP11NO98).doc

1801 de Maisonneuve Blvd West, 8th floor, Montreal, Quebec, Canada, H3H 1J9
Tel: 888-DNA-KITS (888-362-5487) 514-935-2200
Fax: 514-935-7541 e-mail: info@qbi.com
Internet: www.qbi.com

Foreword

This manual contains information concerning the AutoFluorescent Proteins (AFPs™), the vectors used and their variants, their general applications, methods of cloning, transfection and detection. This manual is intended to provide general guidelines for cloning and transfection using either the Green Fluorescent Protein (GFP) or the Red-shifted variant and the Blue Fluorescent Protein (BFP). Specific data on excitation/emission spectra are provided to efficiently detect native GFP, Red-shifted GFP, and BFP in microscopy and FACS applications. Quantum's AFPs™ have been monitored by transfection of mammalian cells with recombinant plasmids containing the autofluorescent protein coding sequences ensuring reliable quality of the fluorescence levels in standard conditions.

Notice to customer

The products included in this kit are for research use only and are not intended for use in humans or for diagnostic procedures. Experiments performed using the products in this kit should comply with the NIH guidelines or any relevant safety regulations. Usual safety precautions should be observed when handling hazardous materials.

Storage conditions

Upon receipt, store the AFPs™ vectors at -20°C. Do not store in a frost free freezer.

Shelf Life

The vectors are stable for at least 2 years when stored under the recommended conditions.

Table of Contents

Upgrades : In order to keep your protocol up to date on new applications, products or for a customized technical service, we invite you to bookmark our Web site at : http://www.qbi.com .

1. INTRODUCTION

The AutoFluorescent Proteins (AFPs™) are engineered mutants derived from native Green Fluorescent Protein (GFP). Originally isolated (Shimomura and Johnson, 1962) from the Pacific North West jellyfish *Aequoria victoria* (Ward, 1979), GFP demonstrates unique properties making it an ideal candidate for universal, non-isotopic, real-time tagging of biological molecules in living systems. Several key advantages are true for all native and engineered AutoFluorescent Proteins: no substrate is needed, the proteins are very stable and function in virtually any fixed or living cell or tissue. Since its cloning (Prasher *et.al*, 1992), the heterologous expression of GFP cDNA has triggered its widespread use as a reporter molecule for gene expression, and protein localization and trafficking studies in a broad variety of organisms (Cubitt *et al.*, 1995 ; Prasher 1995 ; Ward 1981) . The particular spectral properties of native GFP have led to its use as "single-tag system". However, the presence of two excitation peaks for wtGFP have limited its utility in situations where multiple tags are required. This limitation has been overcome by the introduction of spectrally shifted AFPs™. These engineered mutants, red-shifted GFP (rsGFP) and Blue Fluorescent Protein (BFP) show increased specific autofluorescence and tighter spectral properties with no overlap in their respective excitation and emission spectra. Use of both BFP and rsGFP as reporter genes and/or as fluorescent 'tags' allows for simultaneous multicolor reporting in multiparameter analyses while avoiding the interference of cellular autofluorescence observed at short wavelengths (Ormö *et al.* 1996). Quantum's AFPs™ offer a powerful <u>D</u>ual <u>C</u>olor <u>S</u>creening <u>S</u>ystem (DCSS) (Stauber, 1996) where mutants, having different spectral properties, can be used individually or in combination to tag different proteins expressed in the same cell to identify the intracellular localization of recombinant proteins as well as to study intracellular trafficking.

2. GENERAL APPLICATIONS OF AFPs™

2.1 Fluorescent Tags

Fluorescent molecules have long been a powerful research tool for the localization, identification and isolation of specific biological targets; proteins, nucleic acids, sub-cellular components and whole cells. Unfortunately, the use of these techniques has generally either required significant perturbations of the biological system being studied (the fixation and permeabilization of cells for analysis of internal proteins in FISH and FACS for example), or the scope of the observations has been severely limited, (as in the recognition of markers present only on the cell surface during FACS based live-cell sorting). The ideal **vital** fluorescent 'tag' should be detectable in the context of normal physiological conditions in a wide variety of cell types and organisms, be targetable to virtually any subcellular region and should not disturb either the normal function of the target molecule into which it is incorporated or the normal physiology of the organism in which it is expressed.

Since the isolation and cloning of the GFP gene (Prasher *et al.*, 1992) a number of studies have shown that the GFP can serve as an effective and polyvalent fluorescent protein tag since a broad variety of proteins have been shown to retain native function in the context of N-terminal and C-terminal GFP-fusions as well as maintaining the fluorescent properties of the GFP moiety. Furthermore the elucidation of the "β-can" molecular structure of GFP (Ormö *et al.*, 1996, Yang *et al.*, 1996) offers an explanation of these observations by suggesting that the AFPs are likely to function as autonomous "β-can" units even in the context of N- or C-terminal fusion proteins.

2.2 Genetic reporter systems

Genetic reporter systems, another common research tool, are used to observe and measure the impact of *cis*-acting nucleotide sequences (as in the identification and isolation of promoter and enhancer sequences), and *trans*-acting factors (as in the development of inducible expression systems), as well as for the selection of rare events (as in the selection of gene targeting events and stably transformed cell lines). The ideal reporter gene/activity should be absent from the host, detection of the reporter acitivity should be simple, rapid, and cost effective, and expression of the reporter gene should not affect the physiology of the host.

Since the demonstration by Chalfie and coworkers (Chalfie *et al.*, 1994) that GFP can be expressed as a functional transgene, *A. victoria* GFP and variants have been expressed in a wide variety of organisms including bacteria, yeast, slime mold, plants, *Drosophila*, zebrafish, and mammalian cells. Detection of Quantum's AFPs™ is simple, rapid, and cost effective, and the expression of AFPs has not been reported to disturb normal function of any organism in which it has been expressed.

2.3 AutoFluorescent Proteins

Clearly, Quantum's AFPs™ conform to essentially all of the criteria desired for **both** vital fluorescent tags **and** genetic reporter genes, and are suitable for all applications requiring either.

Although GFP, rsGFP, or BFP are powerful individual tools for applications requiring single fluorescent tags or genetic markers, the recent introduction of brilliant red-shifted GFP mutants and mutants with modified spectral properties like the Blue Fluorescent Protein (BFP) has widened the potential use of the AFPs™ to multiparameter analysis. The fact that the mutated GFP and BFP proteins have different excitation (473nm vs 387nm) and emission (509nm vs 450nm) spectra with no overlap allows the independent detection of BFP and GFP even when coexpressed in the same cell as fluorescent protein tags or genetic markers or any combination thereof.

AFPs™ can be used individually in transient expression, trafficking and protein localization studies, protein purification, environmental monitoring of bacterial strains, as Promoter Reporter Vectors, and as positive selection vectors in mammalian gene expression, or as Dual Color Screening System (DCSS) in co-localization, double-labeling, coexpression, transgene expression studies for stable cell line and transgenic animal and in protein-protein interaction studies in trafficking analysis.

Notably, GFP can be observed to fluoresce in native polyacrylamide gels photographed on standard laboratory transilluminators or in protein purification columns, and should thus also have a place in such *in vitro* analyses as bandshift assays, and in simplifying fusion protein purification procedures.

3. PRODUCT INFORMATION

	Excitation peak(s)	Emission peak
wtGFP:	395nm (473nm, minor)	509nm
rsGFP:	473nm	509nm
BFP:	387nm	450nm

Table 1: Available Products

Vector	Construct	Promoter	Quantity	Code
pQBI25	rsGFP	CMV	40 µg	AFP 4041
pQBI25	rsGFP	CMV	100 µg	AFP 9941
pQBI50-BFP	BFP	CMV	40 µg	AFP 4001
pQBI50-BFP	BFP	CMV	100 µg	AFP 9901
pQBI25 & pQBI50-BFP	rsGFP & BFP	CMV	40 µg ea	AFP 4401
pQBI63	rsGFP	T7	40 µg	AFP 4042
pQBI67-BFP	BFP	T7	40 µg	AFP 4002
pQBI63 & pQBI67-BFP	rsGFP & BFP	T7	40 µg ea	AFP 4402
pQBI-PGK	rsGFP-neo	PGK	40 µg	AFP 4043
pQBI-PolII	rsGFP-neo	PolII	40 µg	AFP 4044

4. CHARACTERISTICS OF THE AFPs ™

4.1 General

GFP is a 238 amino acid, 28 kDa protein with remarkable resistance to physico-chemical denaturing conditions. GFP fluorescence is even observed at temperatures greater than 65°C, and over a pH range of 5.5 to 12.2, although fluorescent intensity is reduced below a pH of 7.0 (Bokman and Ward, 1981). It retains its native fluorescent activity in 6 M guanidium hydrochloride, 8 M urea, or 1% SDS and in moderate concentrations of organic solvents. Tissues fixed with paraformaldehyde or glutaraldehyde continue to fluoresce, although activity appears to be more sensitive to photobleaching under these conditions. The protein can be denatured with chaotropic agents and strong acid or base (pH <4 or >11), or very high temperatures, however, it will largely regain its activity when brought back to physiological conditions. GFP resists most proteases, with the exception of pronase.

Attempts to reduce the size of the GFP determined that truncation of more than seven amino acids from the C-terminus, or more than the N-terminal Met resulted in a total loss of fluorescence (Dopf, J and Horagan, T, 1996). Based on the crystallographic structure of GFP, Ormö *et al.* hypothesized that there are no large segments that could be deleted while preserving the structural integrity of the protein (Ormö *et al.*, 1996).

4.2 Chromophore

The wtGFP chromophore consists of the tripeptide formed by the amino acids Ser65, Tyr66, and Gly67. Nascent GFP is not fluorescent, and fluophore formation requires post-tranlational modifications in the presence of molecular oxygen (Davis *et al.*, 1995, Heim *et al.*, 1994). These steps would appear to be autocatalytic, since functional GFP forms in a broad range of organisms, as well as in crude protein lysates (P. Trudel, unpublished observations). Chromophore formation requires the cyclation of the three amino acids, oxidation of the tyrosine, and proper folding of the protein chain. The final structure of the GFP has been likened to paint in a can (Yang *et al.*, 1996), with an outer structure (the can) composed of a series of eleven strands of ß-sheets that form the walls of a cylinder, and two α-helices that cap the top and bottom of the can, and provide a scaffold for the fluophore, which is held near the center of the can.

Because GFP must undergo a postranslational oxidation step, physiological conditions have an impact on the efficiency and rate of chromophore formation. When GFP is expressed in bacteria under anaerobic conditions, no fluorescence is observed until the bacteria are exposed to aerobic conditions. Furthermore, generation of the fluophore is somewhat temperature sensitive, the yield of fluorescent to total GFP protein decreases as temperatures increase above 30°C (Yang *et al.*, 1996), and a greater fluorescence intensity has been observed when *S. cerivisiae* or *E.coli* are cultured below 30°C, however fluorescence developed at lower temperatures is stable even following a subsequent shift to 37°C (Lim *et al.*, 1995; Heim *et al.*, 1994). A similar temperature response has been reported in mammalian cells expressing GFP (Pines, 1995; Ogawa *et al.*, 1995).

4.3 Variants

A wide range of mutations in and around the chromophore structure of GFP have been described (Table 2). The mutations result in modifications to the spectral properties, the speed of chromophore formation, the extinction coefficient, and the physical characteristics of the AFP. In the hexapeptide region surrounding the chromophore, the only amino acid that apparently cannot be substituted is Gly^{67} (Delagrave *et al.*, 1995).

Table 2: **GFP variants**

	aa	64	65	66	67	68	69	163	168	203	ref.
Variant											
wtGFP		Phe	Ser	Tyr	Gly	Val	Gln	Val	Ile	Thr	
blue				**His**							Heim *etal.*, 1994
>soluble								**Ala**			Kahana and Silver
brighter		**Leu**	Thr								Cormack *et al.* 1996
rsYellow			Gly			Leu				Tyr	Ormö *et al.*, 1996
S65T			Thr								Heim *et al.*, 1995
rsGFP		**Leu**	**Cys**						Thr		Quantum
BFP		**Leu**		**His**				**Ala**			Quantum

A number of variants resulting in modifications of the spectral properties of the fluorescent proteins have been described. Wild type GFP has a major excitation peak at 395 nm, and a minor peak at 475 nm, and emits green light maximally at 508 nm (Ward *et al.*, 1980). Modifications of Ser^{65} to Thr or Cys result in GFPs that continue to emit maximally at ~510 nm but which have a single excitation peak red-shifted to 488 nm and 473 nm respectively. This has several advantages in that this brings the excitation peaks more in line with those already used with fluorescent microscopes and fluorescence activated cell sorters (FACS) for FITC. Furthermore, chromophore formation of these mutants is more rapid and the extinction coefficient is greater than that of wtGFP which results in a stronger fluorescent signal (Heim *et al.*, 1995). The fluorescent signal generated by these proteins also appears to be less subject to fading, perhaps because of the decreased exposure to the shorter wavelength light. Changing Phe^{64} to Leu also leads to brighter fluorescence (Cormack *et al.* 1996). A combination of mutations leads to fluorescent proteins with emission peaks at > 520nm, however the excitation maximum is also increased to >510nm (Ormö *et al.*, 1996). Tsien and coworkers identified a mutation (Y66H) which results in a protein which absorbs long wave ultraviolet light and emits blue light, however the intensity is diminished by about 50% compared to that of the wild type GFP (Heim *et al.*, 1994). Finally, mutation of Val^{163} to Ala results in a GFP that is significantly more soluble and seems to form its chromophore faster than the wild type (Kahana and Silver, 1993), characteristics that have been hypothesized to also lead to improved fluorescence (Youvan, DC, and Michel-Beyerle, ME, 1996).

A number of GFPs with various degrees of codon usage modifications have been created for use in mammalian cells, including one totally ‘humanized’ version of GFP. While these mutants are perhaps more efficiently translated in mammalian cells, little real increase in fluorescence has been attributable to these modifications; amino acid modifications appear to have a much greater impact on fluorescence intensity.

4.4 Quantum’s AFPs™

Quantum has both red-shifted GFP (rsGFP) and its **exclusive** commercial Blue fluorescent protein (BFP). The rsGFP and BFP have been generated by selecting and combining mutations designed to give increased fluorescent intensity and longevity, increased solubility, and optimal spectral properties. An astute selection of mutations has ensured that Quantum’s rsGFP is the brightest GFP available, that it is expressed well in a broad range of organisms, and resists photobleaching. rsGFP contains more than 30 silent mutations which render selected codons optimal for translation in mammalian cells, however this GFP is still extremely well expressed in all organisms tested to date, including bacteria, thus obviating the need for multiple vectors, each dedicated to expression in a particular organism. rsGFP is very stable and highly resistant to photobleaching; fluorescence can still be detected in fixed tissues as long as three to four weeks following fixation, when stored under appropriate conditions.

Like rsGFP, our **exclusive** commercial BFP is significantly brighter than wtGFP and contains over 25 silent mutations for selected codon usage. The mutations in BFP result in a protein with a single excitation peak at 387 and a maximal emission peak at 450. Since the excitation and emission spectra of the rsGFP and BFP do not overlap, they are ideally matched for simultaneous use as individual markers in a Dual Colour Screening System allowing detection of both markers in the same cell. Furthermore, rsGFP and BFP can be used to simultaneously detect and select for live cells marked with either or both markers using FACS machines.

5. METHODS

5.1 Production of fusion proteins with AFPs™

The pQBI25, pQBI50-BFP, pQBIpolII[a] and pQBI-PGK[a] vectors can be used to generate fusion proteins between the C-terminus of a target protein and the N-terminus of the AFP. Cloning is achieved using the unique NheI site located at the beginning of the coding sequence of AFP (see the Appendices for vector maps, sequences).

```
      Nhe I site
         ⇓
AA-ATG-GCT-AGC-AAA
   Met  Ala  Ser  Lys
```

We suggest that in most cases, the simplest method for generating a fusion protein will be to use PCR®[b] to amplify the coding sequences (cds) of the protein to be fused to the AFP™. In the design of the amplification primers, an Nhe I-compatible restriction site is added to the 5' end of both primers, and optimally, an additional three to five bases is added. These short 5' extensions allow for a more efficient restriction enzyme cleavage following amplification, and allow the user to adjust the reading frame (see above for reading frame adjustment) of the cloned protein to that of the AFP™. The cDNA is then amplified with these specific primers, purified away from excess primers, and cleaved in order to generate Nhe I-compatible cohesive ends. Ligation of Nhe I compatible ends is generally an efficient process. (See troubleshooting to avoid difficulties).

Restriction enzymes generating NheI compatible cohesive ends are[c]:
Avr II, **Nhe I**, **Spe I**, **Sty I** , and **Xba I.** (Enzymes available from Quantum are shown in **bold**.)

Alternatively, blunt-ended fragments (generated by PCR®[b] or otherwise) can be cloned into Nhe I sites that have been rendered blunt with **Klenow fragment of DNA polymerase I**.

5.2 Replacement of the CMV promoter in pQBI25 and pQBI50-BFP.

Given the utility of the AFPs™ as reporter genes, replacement of the powerful CMV promoter present in the pQBI25 and pQBI50-BFP vectors with test or tissue specific promoters is a common procedure. The CMV promoter initiates transcription at position 893 while translation starts at the ATG at position 979 (Appendix A). Deletion of the CMV promoter can be most easily achieved by digesting with **Bgl II** and Sac II. Since **Sac II** cuts at position 965, the AFP intiation codons are not disturbed.

---

[a] **NOTE:** that pQBIpolII and pQBI-PGK already generate GFP-neo fusion proteins.

[b] **NOTE:** for the amplification of coding sequence (cds) fragments to be subcloned for the generation of fusion proteins, the use of thermostable polymerases with low mutagenic rates or proofreading activities may be advisable, to reduce the risk of mutation during the amplification process.

[c] **NOTE:** that the cutting efficiency near the ends of a DNA fragment are: Xba I (>90%) > Nhe I (50%) > Spe I.

5.3 Excision of the expression cassettes from pQBI25 and pQBI50-BFP.

The expression cassettes in pQBI25 and pQBI50-BFP can be excised as complete or partial units for specific uses. A complete expression cassette including the CMV promoter, the AFP™ coding sequences, and the BGH polyadenylation site can be excised using **Bgl II** (position 12) and Dra III (2337). Alternatively, an expression cassette with additional 3' sequences (including most of the SV40 promoter) can be excised with **Bgl II** and Avr II (2861) or **Sma I** (Xma I, 2884). An expression cassette consisting of only the CMV promoter and the AFP™ coding sequences can be excised using **Bgl II** and **BamH I** (1699), **EcoR I** (1730), **EcoR V** (1742), **Not I** (1757), **Xba I** (1775) or **Apa I** (1785). Finally, the AFP™ coding sequences can be removed using **Sac II** (962) and **BamH I** (1699), **EcoR I** (1730), **EcoR V** (1740), **Not I** (1757), **Xba I** (1775) or **Apa I** (1785). (Enzymes available from Quantum are shown in **bold**.)

5.4 Expression of AFPs™ in bacteria.

Vectors pQBI63 and pQBI67-BFP are designed to allow the efficient expression of either rsGFP or BFP in bacteria under the control of the powerful T7 promoter and serve primarily as a source for fluorescent protein. The plasmids can be introduced into any bacteria expressing the T7 RNA polymerase, using any of the standard techniques. Recombinant AFP™ can be isolated using standard procedures for generating crude protein extracts. At Quantum, we routinely use 3 rounds of freeze-thaw (-80°C, 37°C) in a 10 mM Tris buffer. Crude protein isolated this way is fairly pure, but can be further purified by column chromatography if necessary. Fluorescence of the rsGFP is such that transformed colonies can be recognized by their green fluorescence under normal laboratory lighting. Introduction of sequences which disturb the GFP coding sequences should allow identification of recombinant colonies by the absence of this green fluorescence. The entire expression cassette can be excised in its smallest form using **Bgl II** and **BamH I**, although other combinations of enzymes can also be used (Appendices, E and F). The GFP coding sequences can be removed (without the ATG initiation codon) with **BamH I** and **Nhe I**.

5.5 Generation of stable transformants.

Quantum supplies a choice of vectors for the generation of stable transformants of mammalian cells expressing rsGFP. Both vectors express rsGFP-neo fusion proteins for the efficient selection of GFP expressing cells. Since the neomycin resistance gene is fused to the C-terminus of the rsGFP gene, selection of cells resistant to the antibiotic G418 virtually guarantees 100% of rsGFP expressing cells. In pQBI-PGK the rsGFP-neo fusion cassette expression is driven by the murine phosphoglycerokinase (pgk) promoter linked to the adenovirus tripartite leader sequence. The murine polII promoter without leader sequences, drives the rsGFP-neo fusion gene expression in pQBIpol II. Both of these plasmids contain the bovine growth hormone (BGH) poly-adenylation signal sequences for efficient RNA processing, as well as the SV40 poly-adenylation signal sequences on the opposite strand to prevent the generation of anti-sense messages which may otherwise interfere with the expression of the selectable markers.

6. DETECTION

6.1 General

Like fluorescein, the quantum yield (QY = the efficiency of the conversion of input energy to output energy) of the AFPs™ is about 80% (Ward, 1981), however the extinction coefficient (ε = the efficiency of energy absorption) of wtGFP is about 3-fold smaller than the extinction coefficient of FITC (fluorescein isothiocyanate). Since the intensity of the fluorescent signal correlates to the product of ε x QY, the signal obtained per molecule of wtGFP is about 3-fold less intense than the signal obtained using FITC. The use of the GFP variants largely compensates for this difference, however, since the extinction coefficients of the brighter GFP variants has been improved with respect to the ε of the wtGFP. Furthermore, GFP fusion proteins have been found to give greater sensitivity and resolution than staining with fluorescently labeled antibody (Wang and Hazelrigg, 1994). The AFPs™ are more resistant to photobleaching than FITC-labeled antibodies, and backgrounds due to non-specific binding of primary and secondary antibodies are avoided. Contrary to AFP™-fusions, there exists the potential for signal amplification when using antibodies for detection (through the binding of multiple antibodies to a single target), however this is offset somewhat because neither antibody labeling nor target hybridization are 100% efficient.

6.2 Excitation sources.

Most fluorescent microscopes are equipped with mercury arc light sources. Mercury lamps do not emit equal amounts of light across their entire spectral range, but rather have peak intensities at 337, 365, 405, 435, 546, and 577nm. Although the peaks at 365 and 435nm do not correspond precisely to the optimal excitation wavelengths of the wtGFP, BFP and rsGFP, 100 W mercury arc lamps are generally suitable for most applications. Xenon arc lamps generally produce much less light than the mercury counterparts, however the light output is more spectrally uniform than that of the mercury sources, and this across the entire excitation ranges of the AFPs™. As a result, even lower power xenon arc lamps work extremely well for visualizing the AFPs™.

The standard laser provided with a flow cytometer will depend on the manufacturer and the particular model but many cytomoters (from Becton-Dickinson for example) come with multiline lasers (coherent enterprise lasers) as standard equipment. These multiline lasers provide both 488nm and UV (360-380nm) excitation wavelengths that are very appropriate for use with the AFPs™. Furthermore, some suppliers can provide these kinds of lasers as after market options. The 488nm line of a krypton/argon ion laser is suitable for exciting rsGFP.

6.3 Microscopy

The GFP and BFP signals are obtained with FITC-fluorescence filter sets and DAPI-filter sets respectively. New filter sets for GFP have recently been introduced by different suppliers. Table 3 shows nine current filter sets used with GFP and BFP. Media containing phenol-red has been observed to autofluoresce under the conditions used for the observation of the AFPs™. Therefore we recommend performing microscopy in phenol-red free media or in PBS to reduce background fluorescence, especially when using BFP. The fluorescence of rsGFP is easily observable even with media included.

When two fluorescent proteins are expressed simultaneously in the same cell, they can be observed with the BFP filter sets if they are in different cell compartments or structures. If they are present in the same cell compartment as, for example the cytoplasm, the green fluorescence will predominate. To observe the blue fluorescence, a special filter set equipped with a band-pass filter (465/20 nm) can be used first. The green fluorescence can then be observed with any appropriate filter set.

6.3.1 Inverted microscopy

The simplest way to observe the expression of AFPs™ in transfected cells is to use an inverted fluorescence microscope. In most cases transformed cells can be observed directly by inverted fluorescence microscopy, thus minimizing post-transformation manipulations

6.3.2 Up-right microscopy

To efficiently observe the fluorescence of living cells with an up-right microscope, seed cells into Lab-Tek chamber slides (Nunc) or the equivalent, and observe cells after their adhesion to the slides. Cells can also be fixed with 2-4% paraformaldehyde in PBS for 30 minutes on ice (add 1ml of 4% paraformaldehyde to 1ml cell suspension in PBS). Cover fixed cells with a coverslip and seal. [Although there have been reports of GFP fluorescence being sensitive to some nail polishes, we have not observed this problem at Quantum. Nonetheless, it may be prudent to test a given batch, or to use molten agarose or rubber cement to seal the coverslips.]

Table 3 Filter sets

AFP™	Manufacturer	Excitation filter	Beam splitter	Emission filter
rsGFP (FITC)	Zeiss 09	450-490nm	510nm	>520nm
	Leica I3	450-490nm	510nm	>515nm
	Leica H3	420-490nm	510nm	>515nm
	Olympus U-MSWB	420-480nm	500nm	>515nm
	Olympus U-MSWB	450-480nm	500nm	>515nm
BFP (DAPI)	Zeiss 02	365nm	460nm	>470nm
	Leica 0	355-425nm	455nm	>470nm
	Leica A	340-380nm	400nm	>425nm
	Olympus U-MNU	360-370nm	420nm	>400nm

NOTE: The choice of filter sets should be a function of the light source. Bear in mind when selecting a filter set that the Xenon arc lamp has a uniform intensity of emission over the spectrum used to excite the AFPs™ but that mercury arc lamps have strong “peaks” at 365, 405, 435, 546, and 577nm. For GFP, filter sets of 420-490 nm take advantage of the strong peak at 435 nm of the mercury arc lamps while filter sets with excitation filters of 450-490 nm are more appropriate for use with Xenon arc source lamps. The nature of the light source is less important with the BFPs since excitation spectra and intensity of high spectrum coincide.

6.4 FACS analysis and sorting of live cells transfected with BFP/GFP

Cells transfected with plasmids expressing GFP or BFP individually or in combination can be analyzed and/or sorted by FACS. An argon ion laser at a wavelength of 488nm is used with a 500nm longpass to excite GFP, whereas a multiline UV laser (350-363nm) with a 444nm/20nm emission filter is used for detection of BFP. Using the argon and UV-laser for the detection of GFP and BFP respectively, it is possible to distinguish positive green and blue cell populations from each other. The two color system can thus be used as a live genetic marker to distinguish between different cell populations.

7. TROUBLESHOOTING GUIDE

PROBLEM	POSSIBLE CAUSE	REMEDY
A- Low fluorescence:		
a) with Quantum vector	Slow rate of chromophore formation.	1. Expose cells to aerobic conditions for a few minutes. The chromophore formation requires oxygen. 2. Observe cells after 3 to 4 hours. There may be a lag of up to 3 hours between protein synthesis and chromophore formation in mammalian cells. 3. Grow the cells at lower temperature; the formation of chromophore is more efficient below 37°C.
	Improper detection settings.	Reduction of magnification from 100X to 60X at constant numerical aperture (N.A) results in roughly a two fold increase in intensity. For mammalian cells a 40X, ≥ 1.0 N.A lens should be adequate for all situations.
b) with an AFP-fusion recombinant.	Fusion protein may induce shift in excitation spectrum	Use a different filter set.
	Proteolysis or rapid turnover of fusion protein	Confirm the synthesis of protein by immuno-blotting. The weight of fused protein will be shifted by approximately 28 kDa.
	Expression of GFP fusion protein below limit of detection	Use the Quantum vector as a positive control.
B. Level of expression	Inappropriate promoter.	The CMV promoter functions well in most cells, nonetheless, it may be necessary to use an alternative promoter in certain cells. Replace the CMV promoter as described above (section 5.2).
C. Photobleaching or destruction of chromophore.	Over-excitation with UV light.	Short UV will destroy the chromophore and induce the formation of free radicals. Use a light source of lower energy or a xenon lamp which produces less short-wavelength light than does a mercury lamp.
D. Autofluorescence	High concentration of coenzymes like flavin or aromatic compound in studied cells.	1. Use a mock-transfected control to gauge the extent of autofluorescence. 2. 2. Try a band-pass excitation or emission filter to eliminate the autofluorescence without affecting the AFP fluorescence.

E. Difficulty generating fusion-protein recombinants.	Poor ligation efficiencies.	1. Ensure that the amplified fragment is first purified away from the excess primer before cleavage with a restriction enzyme, to ensure efficient RE digestion. 2. Purify the cleaved fragment away from the short overhangs that were liberated by the RE

PROBLEM	POSSIBLE CAUSE	REMEDY
		digestion. These very short fragments contain compatible cohesive ends and compete very efficiently for the vector.

8. REFERENCES

Cormack BP, Valdivia RH, and Falkow S. (1996) FACS-optimized mutants of the green fluorescent protein.(GFP). *Gene* **173**:33-38

Cubitt AB, Heim T, Adams SR, Boyd AE, Gross LA, and Tsien TY. (1995) Understanding, improving and using green fluorescent proteins. *Trends Biochem.* **20**: 448-455

Delagrave S, Hawtin RE, Silva CM, Yang MM, and Youvan DC. (1996) Bio/Technology **13**: 151-154

Dopf J, and Horiagan T. Deletion mapping of the *Aequorea victoria* green fluorescent protein. (1996) *Gene* **173**: 39-44

Heim R, Cubitt,AB, and Tsien, RY, (1995) Improved green fluorescence. *Nature* **373**:663-664

Heim R., Prasher DC, and Tsien RY., (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci.*, **91**:12501-12504

Kahana JA, and Silver PA, in 'Current Protocols in Molecular Biology" (1993) eds. Ausubel GM, Brent T, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. John Wiley & Sons, Inc.

Lim CR, Kimata Y, Oka M, Nomaguchi K, and Kohno K. (1995) The sensitivity of GFP fluorescence utilized to reveal novel nuclear-like compartments in a mutant nucleosporin Nsp1. J. Biochem. **118**: 13-17

Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, and Remington SJ. (1996) Crystal structure of *A. victoria* GFP. *Science* **273**: 1392-1395

Prasher DC. (1995) Using GFP to see the light. *Trends Genet*. **11:** 320-323

Prasher DC, Eckenrode VK, Ward WW, Pendergast FG, and Cormier MJ (1992) Primary structure of the *A. victoria* green fluorescent protein. *Gene* **111**: 229-233

Shimomura O, Johnson FH, and Saiga Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell. Comp. Physiol.* **59:** 223-227

Stauber RH, Gaitanaris GA, and Pavlakis GN. (1996) A dual colour screening system in molecular biology: green and blue autofluorescent proteins - Ideas and Applications. *QuantumNotes*, **1** #2: 1

Wang S and Hazerigg T. (1994) Implications for bcd mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* **639**: 400-403

Ward W.W. (1979) Energy transfer processes in bioluminescence. In: *Photochem. Photobiol. Rev*. **4:** 1-57, ed. Smith KC. (Plenum, NY)

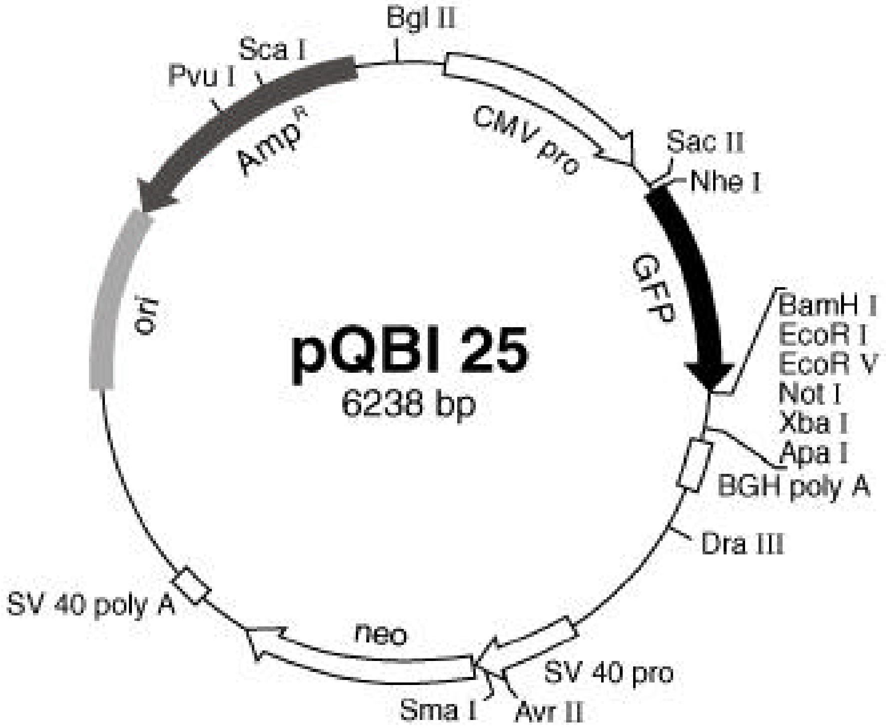
Ward WW, Cody CW, Hart RC, and Cormier MJ, (1980) Spectrophotometric identity of the energy transger chromophores in *Renilla* and *Aequorea. Photochem. Photobiol*. **31**:611-615

Ward WW. (1981) Properties of the Coelenterate GFPs. In *Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical applications.* Eds. DeLuca, M. And McElroy, WD. (Academic Press, Inc., NY) pg. 235-242

Youvan DC, Michel-Beyerle ME. (1996) Structure and fluorescence mechanism of GFP. *Nature Biotechnology* **14**: 1219-1220

Appendix A

pQBI25



Feature Locations:

CMV promoter:	**242-967**
Transcription start	**893**
GFP coding sequences	**979-1696**
chromophore	**1159-1176**
SP6 promoter/primer	**1791-1808**
BGH polyA	**1835-2058**
SV40 promoter	**2582-2907**
neo coding sequences	**2944-3734**
SV40 polyA	**3901-4031**
pUC ori	**4717-5188**
ampicillin	**6103-5245**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

The following restriction enzymes do not cut pQBI25:

Acc III; Acc65 I; Age I; Asc I; BsiW I; Bst98 I; **BstE II**; Bsu36 I; **Iso-Cla I**; **Eco47 III**; EcoN I; Fse I; **Hind III**; **Kpn I**; **Mlu I**; **Nru I**; Pac I; Pme I; PpuM I; PshA I; SanD I; **Sfi I**; Sgf I; SgrA I; Srf I; Sse8387 I; Swa I; Van91 I; Xcm I

pQBI25: Single-cut Restriction Enzymes:

Enzyme (position)
Apa I (1785); Avr II (2861); **BamH I** (1699); Bbe I (3074); Bcg I (5820); **Bgl II** (12); BsaM I (3991); BseR I (2857); Bsg I (1107); Bsp120 I (1781); BspLU11 I (4422); Csp I (3587); Dra III (2337); EclHK I (5315); Eco72 I (1306); **EcoR I** (1730); **EcoR V** (1742); Ehe I (3072); Esp3 I (899); **Hpa I** (1047); Kas I (3070); Nar I (3071); **Nhe I** (982); **Not I** (1757); **Pvu I** (5685); **Sac II** (965); **Sca I** (5795); SexA I (2628); **Sma I** (2884); **SnaB I** (650); **Ssp I** (6119); Tth111 I (3189); **Xba I** (1775); Xma I (2882)

pQBI25: Two-cut Restriction Enzymes:

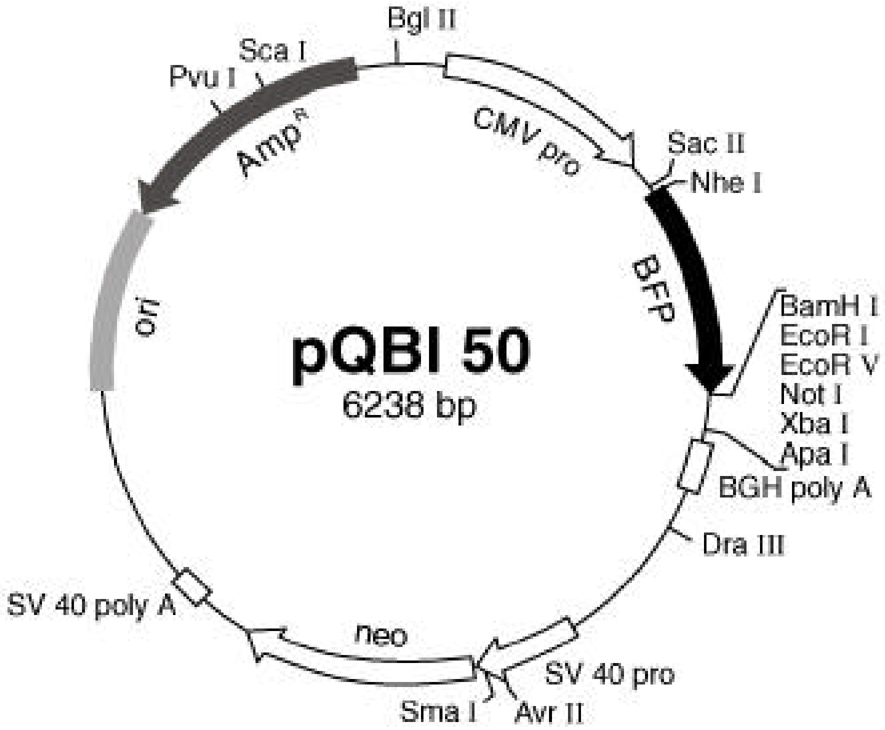
Enzyme (positions)
Afl III, (1303, 4422); ApaL I, (4736, 5982); Bbs I, (944, 2023); **Bcl I**, (1821, 2912); Bsa I, (1619, 5376); BsaB I, (1744, 2930); BspH I, (5142, 6150); **BssH II**, (968, 3468); Bst1107 I, (1431, 4043); **BstX I**, (1726, 1752); Csp45 I, (1603, 3753); EcoICR I, (876, 1815); **EcoO109 I**, (1781, 1782); Fsp I, (3173, 5537); Mun I, (161, 1541); **Nde I**, (544, 1211); Psp1406 I, (5541, 5914); **Pst I**, (1739, 3124); **Sac I**, (878, 1817); **Stu I**, (216, 2860); **Xho I**, (210, 1763); Xmn I, (2532, 5914)

pQBI25: Three-cut Restriction Enzymes:

Enzyme (positions)
AlwN I, (80, 1660, 4838); **Ava I**, (210, 1763, 2882); Bal I, (221, 1152, 3153); BspM I, (2958, 3339, 3789); BsrG I, (253, 1257, 1688); Dra I, (5181, 5200, 5892); Eco52 I, (1713, 1757, 2977); **Nae I**, (2231, 3573, 3856); NgoM I, (2229, 3571, 3854); **Nsi I**, (1774, 2611, 2683); Ppu10 I, (1770, 2607, 2679); **Sal I**, (32, 4048, 6236); Sap I, (3415, 3625, 4306); **Spe I**, (309, 1158, 1705)

Appendix B

pQBI50-BFP



Feature Locations:

CMV promoter:	**242-967**
Transcription start	**893**
BFP coding sequences	**979-1696**
chromophore	**1159-1176**
SP6 promoter/primer	**1791-1808**
BGH polyA	**1835-2058**
SV40 promoter	**2582-2907**
neo coding sequences	**2944-3734**
SV40 polyA	**3901-4031**
pUC ori	**4717-5188**
ampicillin	**6103-5245**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

The following restriction enzymes do not cut pQBI50-BFP:

Acc III; Acc65 I; Age I; Asc I; BsiW I; Bst98 I; **BstE II**; Bsu36 I; **Iso-Cla I**; **Eco47 III**; EcoN I; Fse I; **Hind III**; **Kpn I**; **Mlu I**; **Nru I**; Pac I; Pme I; PpuM I; PshA I; SanD I; **Sfi I**; Sgf I; SgrA I; Srf I; Sse8387 I; Swa I; Van91 I; Xcm I

pQBI50-BFP: Single-cut Restriction Enzymes:

Enzyme (position)
Apa I (1785); Avr II (2861); **BamH I** (1699); Bbe I (3074); Bcg I (5820); **Bgl II** (12); BsaM I (3991); BseR I (2857); Bsg I (1107); Bsp120 I (1781); BspLU11 I (4422); Csp I (3587); Dra III (2337); EclHK I (5315); Eco72 I (1306); **EcoR I** (1730); **EcoR V** (1742); Ehe I (3072); Esp3 I (899); **Hpa I** (1047); Kas I (3070); Nar I (3071); **Nhe I** (982); **Not I** (1757); **Pvu I** (5685); **Sac II** (965); **Sca I** (5795); SexA I (2628); **Sma I** (2884); **SnaB I** (650); **Ssp I** (6119); Tth111 I (3189); **Xba I** (1775); Xma I (2882)

pQBI50-BFP: Two-cut Restriction Enzymes:

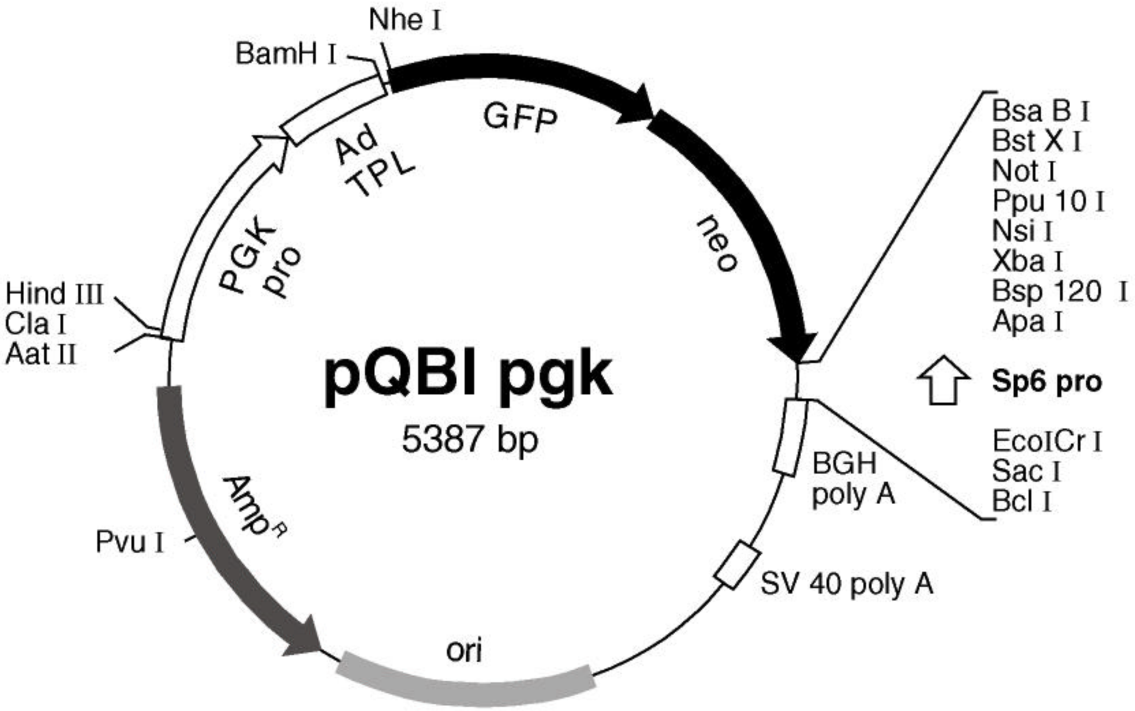
Enzyme (positions)
Afl III, (1303, 4422); ApaL I, (4736, 5982); Bbs I, (944, 2023); **Bcl I**, (1821, 2912); Bsa I, (1619, 5376); BsaB I, (1744, 2930); BSpH I, (5142, 6150); BsrG I, (253, 1257); **BssH II**, (968, 3468); Bst1107 I, (1431, 4043); **BstX I**, (1726, 1752); Csp45 I, (1603, 3753); EcoICR I, (876, 1815); **EcoO109 I**, (1781, 1782); Fsp I, (3173, 5537); Mun I, (161, 1541); **Nde I**, (544, 1211); Psp1406 I, (5541, 5914); **Pst I**, (1739, 3124); **Sac I**, (878, 1817); **Stu I**, (216, 2860); **Xho I**, (210, 1763); Xmn I, (2532, 5914)

pQBI50-BFP: Three-cut Restriction Enzymes:

Enzyme (positions)
AlwN I, (80, 1660, 4838); **Ava I**, (210, 1763, 2882); Bal I, (221, 1152, 3153); BspM I, (2958, 3339, 3789); Eco52 I, (1713, 1757, 2977); **Nae I**, (2231, 3573, 3856); NgoM I, (2229, 3571, 3854); **Nsi I**, (1774, 2611, 2683); Ppu10 I, (1770, 2607, 2679); **Sal I**, (32, 4048, 6236); Sap I, (3415, 3625, 4306); **Spe I**, (309, 1158, 1705)

Appendix C

pQBI-PGK



Feature Locations:

PGK promoter:	**17-559**
Ad TPL:	**583-969**
GFP:	**983-1699**
4 a.a. spacer	**1700-1711**
neo	**1712-2503**
chromophore	**1169-1187**
SP6 promoter/primer	**2582-2560**
BGH polyA	**2608-2832**
SV40 polyA	**3060-3190**
pUC ori	**3639-4336**
ampicillin	**5251-4394**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

The following restriction enzymes do not cut pQBI-PGK:

Acc65 I; Asc I; Avr II; BsiW I; Bst98 I; **BstE II**; Bsu36 I; Dra III; **Eco47 III**; EcoN I; Fse I; **Kpn I**; **Mlu I**; **Nru I**; Pac I; Pme I; PshA I; **Sac II**; SanD I; SexA I; **Sfi I**; Sgf I; SgrA I; **Sma I**; **SnaB I**; Srf I; Sse8387 I; Swa I; Van91 I; Xcm I; Xma I

pQBI-PGK: Single-cut Restriction Enzymes:

Enzyme (position)
Aat II (5386); Acc III(471); Age I(163); **Apa I**(2559); **BamH I**(963); Bbs I(2797); BcgI(4969); **Bcl I**(2595); BsaB I(2518); Bsp120 I (2555); BspLU11 I(3571); **BstX I**(2526); **Iso-Cla I**(26); Csp I(2353); Csp45 I(1607); EclHK I(4464); Eco72 I(1310); EcoICR I(2589); **Hind III**(31); **Hpa I**(1051); Mun I(1545); **Nde I**(1215); **Nhe I**(986); **Not I**(2531); **Nsi I**(2548); Ppu10I (2544); **Pvu I**(4834); **Sac I**(2591); **Ssp I**(5268); **Stu I**(346); Tth111 I(1955); **Xba I**(2549); Xmn I(5063)

pQBI-PGK: Two-cut Restriction Enzymes:

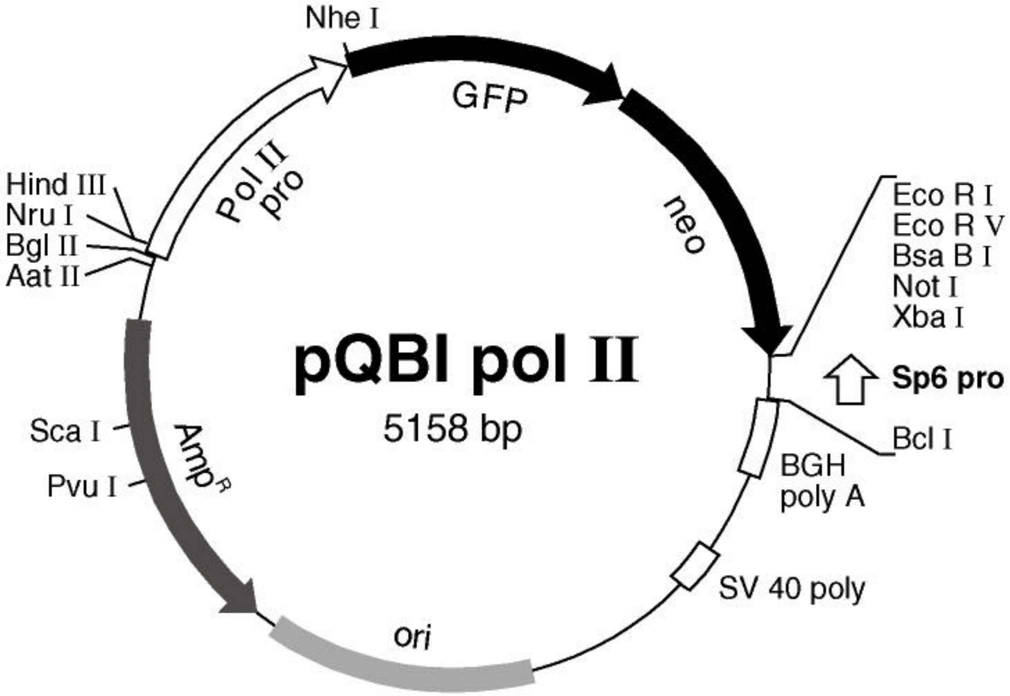
Enzyme (positions)
ApaL I, (3885, 5131); **Ava I**, (720, 2537); Bbe I, (460, 1840); **Bgl I**, (352, 4584); **Bgl II**, (12, 885); Bsa I, (1623, 4525); BsaM I, (483, 3140); BseR I, (208, 515); BspH I, (4291, 5299); BsrG I, (1261, 1692); **BssH II**, (972, 2234); Bst1107 I, (1435, 3192); Eco52 I, (1743, 2531); **EcoR I**, (43, 2504); **EcoR V**, (39, 2516); Ehe I, (458, 1838); Esp3 I, (297, 842); Fsp I, (1939, 4686); Hinc II, (1051, 3199); Kas I, (456, 1836); **Nae I**, (2339, 3005); Nar I, (457, 1837); **Nco I**, (1151, 2269); NgoM I, (2337, 3003); PpuM I, (466, 681); Psp1406 I, (4690, 5063); **Sal I**, (3197, 5385); **Sca I**, (630, 4944); **Spe I**, (297, 1162); **Xho I**, (720, 2537)

pQBI-PGK: Three-cut Restriction Enzymes:

Enzyme (positions)
Acc I, (1434, 3191, 3198); **Afl II**I, (566, 1307, 3571); AlwN I, (401, 1664, 3987); Apo I, (43, 2504, 3110); Bal I, (892, 1156, 1919); BsaA I, (569, 1310, 2141); BspM I, (561, 1724, 2105); Dra I, (4330, 4349, 5041); Drd I, (1637, 1864, 3679); Dsa I, (767, 1151, 2269); **Pst I**, (558, 1890, 2513); Sap I, (2181, 2391, 3455); **Sty I**, (73, 1151, 2269); Vsp I, (3342, 3401, 4636)

Appendix D

pQBIpolII



Feature Locations:

pol II promoter:	**30-740**
GFP coding sequences	**754-1470**
4 a.a. spacer	**1471-1482**
neo coding sequences	**1483-2275**
chromophore	**940-957**
SP6 promoter/primer	**2353-2331**
BGH polyA	**2379-2603**
SV40 polyA	**2831-2961**
pUC ori	**3410-4107**
ampicillin	**5022-4165**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

The following restriction enzymes do not cut pQBIpolII:

Acc65 I; Age I; Asc I; Avr II; **BamH I**; BsiW I; Bst98 I; **BstE II**; Bsu36 I; **Iso-Cla I**; Dra III; **Eco47 III**; EcoN I; Esp3 I; Fse I; **Kpn I**; **Mlu I**; Pac I; Pme I; PshA I; **Sac II**; SanD I; SexA I; **Sfi I**; Sgf I; SgrA I; **Sma I**; **SnaB I**; Srf I; Sse8387 I; **Stu I**; Swa I; Van91 I; Xcm I; Xma I

pQBIpolII: Single-cut Restriction Enzymes

Enzyme (position)
Aat II(5157); Acc III(244); Bbe I(1611); Bbs I(2568); Bcg I(4740); **Bcl I**(2366); **Bgl I**(4355); **Bgl II**(12); BsaB I(2289); BsaM I(2911); Bsg I(882); BspLU11 I(3342); Csp I(2124); Csp45 I(1378); EclHK I(4235); Eco72 I(1081); **EcoR I**(2275); **EcoR V**(2287); Ehe I(1609); **Hind III** (29); **Hpa I**(822); Kas I(1607); Nar I(1608); **Nde I**(986); **Nhe I**(757); **Not I**(2302); **Nru I**(25); PpuM I(250); **Pvu I**(4605); **Sca I**(4715); **Spe I**(933); Tth111 I(1726); **Xba I**(2320); Xmn I (4834)

pQBIpolII: Two-cut Restriction Enzymes:

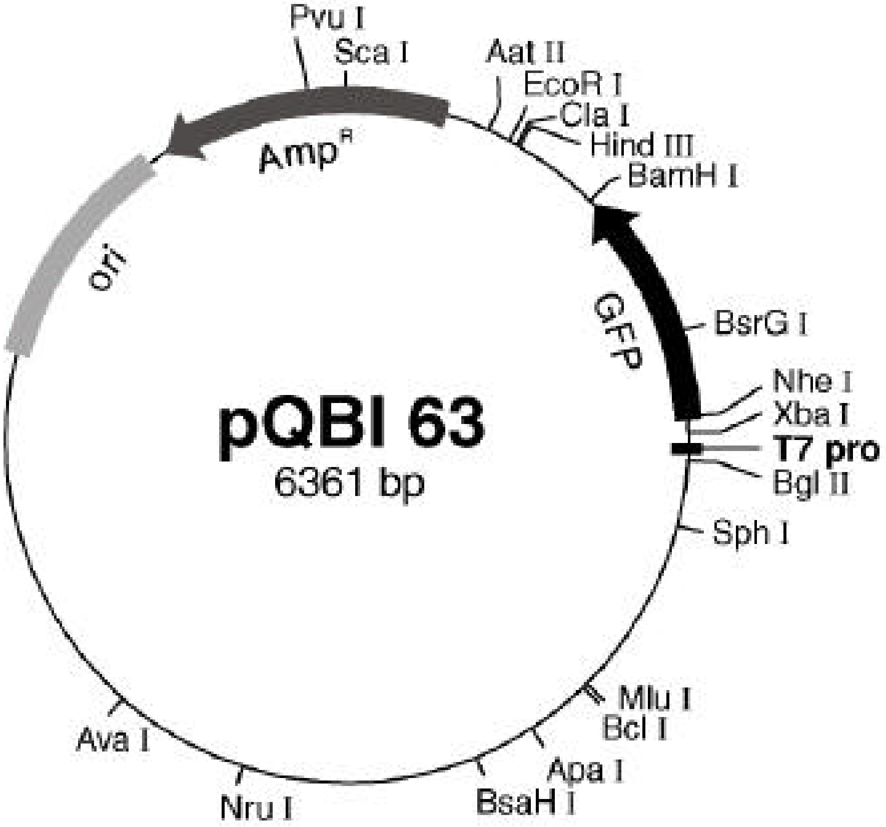
Enzyme (positions)
Afl III, (1078, 3342); AlwN I, (1435, 3758); **Apa I**, (263, 2330); Apo I, (2275, 2881); **Ava I**, (16, 2308); Bal I, (927, 1690); Bsa I, (1394, 4296); BsaA I, (1081, 1912); Bsp120 I, (259, 2326); BspM I, (1495, 1876); BsrG I, (1032, 1463); Bst1107 I, (1206, 2963); Dsa I, (922, 2040); Eco52 I, (1514, 2302); EcoICR I, (21, 2360); Fsp I, (1710, 4457); Mun I, (130, 1316); **Nae I**, (2110, 2776); **Nco I**, (922, 2040); NgoM I, (2108, 2774); **Nsi I**, (166, 2319); Ppu10 I, (162, 2315); Psp1406 I, (4461, 4834); **Pst I**, (1661, 2284); **Sac I**, (23, 2362); **Sal I**, (2968, 5156); **Ssp I**, (186, 5039); **Sty I**, (922, 2040); **Xho I**, (16, 2308)

pQBIpolII: Three-cut Restriction Enzymes:

Enzyme (positions)
Acc I, (1205, 2962, 2969); ApaL I, (416, 3656, 4902); BseR I, (450, 453, 632); BspH I, (189, 4062, 5070); BsrD I, (1841, 4296, 4470); BssS I, (2200, 3515, 4899); **BstX I**, (98, 136, 2297); Dra I, (4101, 4120, 4812); Drd I, (1408, 1635, 3450); Hinc II, (516, 822, 2970); Ple I, (3236, 3721, 4224); Sap I, (1952, 2162, 3226); **Sph I**, (2013, 2317, 2579); Vsp I, (3113, 3172, 4407)

Appendix E

pQBI63



Feature Locations:

GFP coding sequences	**1040-325**
chromophore	**858-841**
T7 promoter	**1132-1116**
pUC ori	**4541-5238**
ampicillin	**6153-5293**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

Restriction Enzymes that do not cut pQBI63:

Acc65 I; Age I; Asc I; Avr II; BseR I; BsiW I; Bst98 I; Bsu36 I; Csp I; Dra III; EcoICR I; **EcoR I**; Fse I; **Kpn I**; **Not I**; **Nsi I**; Pac I; Pme I; Ppu10 I; **Sac I**; **Sac II**; **Sal I**; SanD I; SexA I; **Sfi I**; Sgf I; **Sma I**; **SnaB I**; Srf I; Sse8387 I; **Stu I**; Swa I; **Xho I**; Xma I

pQBI63:Single Cut Restriction Enzymes

Enzyme (position)
Aat II (6288); **Apa I**(2080); Apo I(2144); **Ava I**(3423); **BamH I**(319); **Bcl I**(1883); **Bgl II**(1147); BsaM I(3357); Bsp120 I(2076); BspLU11 I(4473); BspM I (3052); **BssH II**(2280); **BstE II**(2050); **Iso-Cla I**(24); Csp45 I(417); EclHK I(5366); Eco52 I(2937); Eco72 I(716); EcoN I(1404); **Hind III**(29); **Mlu I**(1869); Mun I(477); **Nco I**(871); **Nhe I**(1036); **Nru I**(2972); PshA I(2714); **Pst I**(5611); **Pvu I**(5736); Sap I(4357); **Sca I**(5846); SgrA I(1188); **Spe I**(860); **Sph I**(1344); **Ssp I**(6170); Tth111 I(4218); **Xba I**(1081)

pQBI63:Two Cut Restriction Enzymes

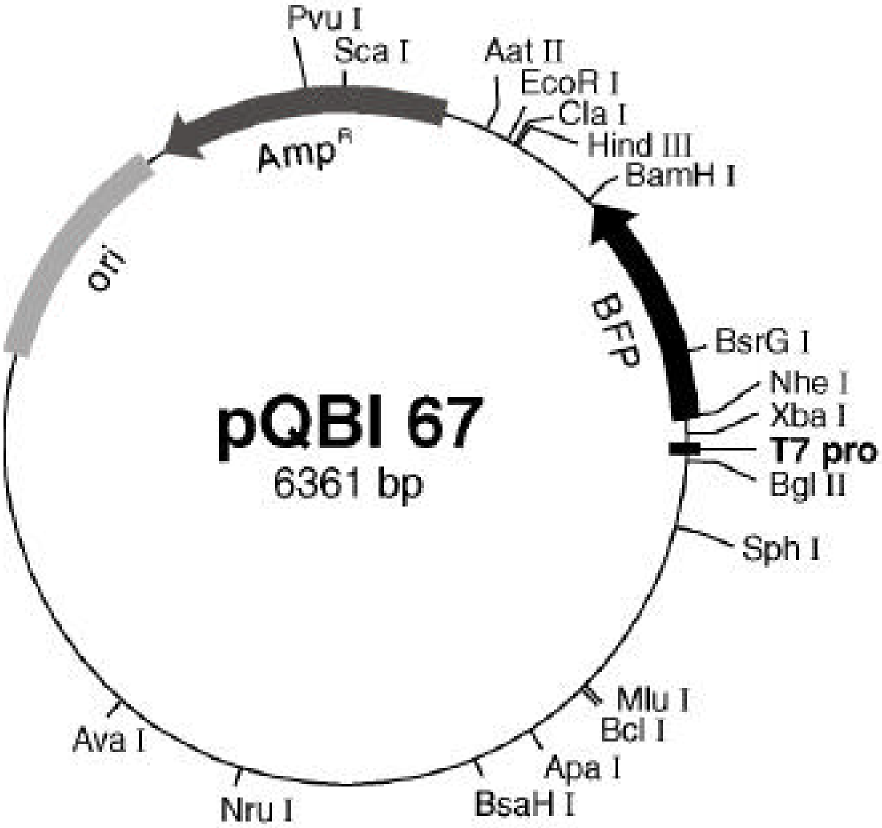
Enzyme (positions)
Acc I, (590, 4243); Acc III, (189, 3662); AlwN I, (365, 4889); Bal I, (870, 3444); Bsa I, (399, 5427); BsaA I, (716, 4225); BsrG I, (330, 761); Bst1107 I, (591, 4244); **EcoR V**, (187, 2319); Esp3 I, (2484, 4114); **Hpa I**, (975, 2375); **Nde I**, (809, 1043); PpuM I, (3437, 3479); Xmn I, (4031, 5965)

pQBI63:Three Cut Restriction Enzymes

Enzyme (positions)
Afl III, (715, 1869, 4473); **Ban II**, (1253, 1267, 2080); **Bgl I**, (2933, 3167, 5486); BsaB I, (1146, 1152, 3670); BsrB I, (1102, 4406, 6207); BssS I, (4646, 6030, 6337); **BstX I**, (1671, 1800, 1923); Dra I, (5232, 5251, 5943); Drd I , (391, 4166, 4581); Dsa I , (871, 1306, 3445); **Eco47 III**, (1274, 2775, 3727); Fsp I, (3356, 3454, 5588); Hinc II, (975, 2375, 5907); **Sty I** , (244, 871, 3367); Van91 I, (1451, 3319, 3368); Xcm I, (1725, 2241, 2259)

Appendix F

pQBI67-BFP



Feature Locations:

BFP coding sequences	**1044-325**
chromophore	**858-841**
T7 promoter	**1132-1116**
pUC ori	**4541-5238**
ampicillin	**6153-5293**

Restriction Enzyme sites:

The following restriction site lists should be used as a general reference only. The complete sequence of the AFP™ vectors are available to our customers through our technical support service at 1-888-DNA-KITS (1-888-362-5487) or by e-mail at info@qbi.com. These restriction maps were generated using DNA Star Edit Seq and Map Draw.
(Commercially available enzymes only).
Enzymes shown in bold are available through Quantum Biotechnologies.

Restriction Enzymes that do not cut pQBI67-BFP:

Acc65 I; Age I; Asc I; Avr II; BseR I; BsiW I; Bst98 I; Bsu36 I; Csp I; Dra III; EcoICR I; **EcoR I**; Fse I; **Kpn I**; **Not I**; **Nsi I**; Pac I; Pme I; Ppu10 I; **Sac I**; **Sac II**; **Sal I**; SanD I; SexA I; **Sfi I**; Sgf I; **Sma I**; **SnaB I**; Srf I; Sse8387 I; **Stu I**; Swa I; **Xho I**; Xma I

pQBI67-BFP: Single Cut Restriction Enzymes

Enzyme (position)
Aat II (6288); **Apa I**(2080); Apo I(2144); **Ava I**(3423); **BamH I**(319); **Bcl I**(1883); **Bgl II**(1147); BsaM I(3357); Bsp120 I(2076); BspLU11 I(4473); BspM I(3052); BsrG I(761); **BssH II**(2280); **BstE II**(2050); **Iso-Cla I**(24); Csp45 I(417); EclHK I(5366); Eco52 I(2937); Eco72 I(716); EcoN I(1404); **Hind III**(29); **Mlu I**(1869); Mun I(477); **Nco I**(871); **Nhe I**(1036); **NruI**(2972); PshA I(2714); **Pst I**(5611); **Pvu I**(5736); Sap I(4357); **Sca I**(5846); SgrA I(1188); **Spe I**(860); **Sph I**(1344); **Ssp I**(6170); Tth111 I(4218); **Xba I**(1081)

pQBI67-BFP: Two Cut Restriction Enzymes

Enzyme (positions)
Acc I, (590, 4243); Acc III, (189, 3662); AlwN I, (365, 4889); Bal I, (870, 3444); Bsa I, (399, 5427); BsaA I, (716, 4225); Bst1107 I, (591, 4244); **EcoR V**, (187, 2319); Esp3 I, (2484, 4114); **Hpa I**, (975, 2375); **Nde I**, (809, 1043); PpuM I, (3437, 3479); Xmn I, (4031, 5965)

pQBI67-BFP: Three Cut Restriction Enzymes

Enzyme (positions)
Afl III, (715, 1869, 4473); **Ban II**, (1253, 1267, 2080); **Bgl I**, (2933, 3167, 5486); BsaB I, (1146, 1152, 3670); BsrB I, (1102, 4406, 6207); BssS I, (4646, 6030, 6337); **BstX I**, (1671, 1800, 1923); Drd I, (391, 4166, 4581); Dsa I, (871, 1306, 3445); **Eco47 III**, (1274, 2775, 3727); Fsp I, (3356, 3454, 5588); Hinc II, (975, 2375, 5907); **Sty I**, (244, 871, 3367); Van91 I, (1451, 3319, 3368); Xcm I, (1725, 2241, 2259);