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Application Note

Quantitation of Green Fluorescent Protein in Microplates using the FL600

The assessment of gene expression is an important tool in molecular and cellular biology. Several methods have been developed, but most cannot provide real-time information concerning expression. Here we report the quantitation of cellular extracts from cells expressing the gene for green fluorescent protein (GFP) using the FL600 in order to demonstrate the feasibility of GFP to assess "real-time" gene expression.

Introduction

The assessment of gene expression has become one of the most utilized tools in molecular and cellular biology today. The expression of transiently and/or permanently transfected cloned DNA sequences allows the investigator to determine the transcriptional activity of promoters. Unfortunately, in most instances the natural product of the promoter cannot be assayed in a quantitative manner. In the past, the promoter was joined to a reporter gene which encoded for a protein with unique enzymatic activity, such as b-galactosidase or chloramphenicol acetyltransferase, that could be assayed easily. The level of gene activity would then be monitored as a function of that enzymatic activity. These assays, while easy to perform and generally quite quantitative, suffer from their inability to be measured in "real time". In these assays it was generally necessary to make cell lysates and perform the reaction at some later time on the lysates. The Achilles' heel of these experiments is the stability of the enzyme throughout storage and during the actual assay. Recently, the use of inherently fluorescent proteins, such as green fluorescent protein (GFP), has been described as a means to assess gene expression and transfection efficiency.

Green fluorescent protein (GFP) from the jellyfish Aequorea victoria is a 27-kDalton monomer protein consisting of 238 amino acids which is naturally fluorescent (1). Its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light (10). The wild type green fluorescent protein (wtGFP) has an absorbance/excitation peak at 395 nm with a minor peak at 475 nm (UV to blue light), and has a peak emission at 508 nm, with a shoulder at 540 nm (green light). The crystal structure of the protein suggests a closely packed b-can enclosing a a-helix (9). The chemical structure necessary for fluorescence has been determined and the data suggest a hexapeptide that contains a cyclic-tripeptide moiety, serine, dehydro-tyrosine, glycine, which is covalently linked through the protein's peptide backbone (2). The exact mechanism of formation of this unique structure is unknown, but it takes place post-translationally and requires molecular oxygen (6). Interestingly, the hexapeptide is contained within the a-helix portion of the protein suggesting that the b-can provides the means to exclude solvents and molecular oxygen, which tend to quench fluorescent signal (9). Unlike other bioluminescent reporters, which require additional proteins, substrates, or cofactors to emit light, GFP is inherently fluorescent and its fluorescence is not species-specific.

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As shown in Table 1, several mutations have been made to the wild type protein (wtGFP) to improve one or more characteristics of the protein. The most widely used variants are the red-shifted GFPs. These mutants have a single excitation peak around 488 nm rather than the primary excitation peak and minor peak found in the wild type protein (Table 2). These variants do however, have the same emission spectra as wild type protein. EGFP, also known as GFPmut 1:23, has two mutations in the chromophore region (Table 1) and is reported to have a single, red-shifted excitation peak at 488 nm and fluoresces with greater intensity than wild type protein (3). The GFP-S65T mutant, with the Serine at position 65 mutated to a Threonine, also has a single red shifted excitation peak, fluoresces more intensely than wild type, and has an added advantage of acquiring fluorescence approximately four times faster than wild-type GFP (4).

Several mutants have been optimized for expression in bacterial cells. For example, GFPuv, which has been optimized to fluoresce when excited with ultra violet (UV) light contains three amino acid substitutions, none of which alter the chromophore sequence. These substitutions alter protein folding and chromophore formation. When expressed in E. coli GFPuv is more soluble than the wtGFP, which is primarily found in inclusion bodies in a nonfluorescent insoluble form. Also, five rarely used Arg codons from the wild type gene have been replaced by codons preferred in E. coli in order to increase expression efficiency. This protein does have a propensity to dimerize, which forms as a result of hydrophobic interactions and results in a four-fold reduction in absorbance at 470 nm and an increase in absorption at 395 nm. Despite the changes, the excitation and emission maxima remain the same as wild type. The Stemmer mutant is quite similar to the GFPuv, with the exception of the Arg codon changes.

Mutations have also been used to create blue emission GFP variants. The Y66H variant has a histidine residue at position 66 instead of a tyrosine. This results in excitation and emission maxima of 382 and 459 nm respectively. Other improvements on the blue emitting proteins have been made to improve their brightness. For example EBFP also contains amino acid changes at positions 64, 65, and 145 that improve brightness and resulting in an excitation and emission maxima of 380 and 440 respectively.

Other substitutions have been made to improve protein folding and chromophore formation. Besides the obvious amino acid substitutions previously described many different silent mutations have been made to the wild type sequence. Clontech has made over 150 sequence substitutions to the native DNA sequence in order to change codon usage to reflect the intended host's codon bias. Upstream sequences flanking the coding region have also been converted to a Kozak consensus translation initiation site in order to increase translation initiation efficiency.

Expression of GFP as a transgene has opened many exciting new windows of investigation in cell, developmental, and molecular biology. Fluorescent GFPs have been expressed in bacteria (1, 3) yeast (11), slime mold (12), plants (13), drosophilia (14), and mammalian cells (17). GFPs can function as a protein tag, tolerating either an N- or a C-terminus fusion to a broad variety of proteins, many of which have been shown to maintain native function (18). The enormous flexibility of these proteins as a noninvasive real-time marker in living cells allows for numerous applications. Here we describe several experiments demonstrating the use of fluorescence to quantitate various Green Fluorescent Proteins (GFPs) using the FL600 microplate fluorescence reader.

Table 1. GFP mutants and their amino acid substitutions

	Amino acid position										
Mutant	64	65	66	67	68	69	….	99	145	153	163
wt GFP	Phe^{64}	Ser	Tyr	Gly	Val	Gln	….	Phe^{99}	Tyr^{145}	Met^{153}	Val^{163}
EGFP[# &]	Leu^{64}	Thr	Tyr	Gly	Val	Gln	….	Phe^{99}	Tyr^{145}	Met^{153}	Val^{163}

GFP-S65T[# &]	Phe[64]	Thr	Tyr	Gly	Val	Gln	….	Phe[99]	Tyr[145]	Met[153]	Val[163]
EBFP[# &]	Leu[64]	Thr	His	Gly	Val	Gln	….	Phe[99]	Phe[145]	Met[153]	Val[163]
Y66H	Phe[64]	Ser	His	Gly	Val	Gln	….	Phe[99]	Tyr[145]	Met[153]	Val[163]
GFPuv[@]	Phe[64]	Ser	Tyr	Gly	Val	Gln	….	Ser[99]	Tyr[145]	Thr[153]	Ala[163]
Stemmer	Phe[64]	Ser	Tyr	Gly	Val	Gln	….	Ser[99]	Tyr[145]	Thr[153]	Ala[163]
	Chromophore location										

These mutants also contain over 150 silent base mutations that correspond to human codon-usage preferences. Upstream sequences flanking the coding region have been converted to a Kozak consensus translation initiation site. Five rarely used Arg codons from the wild type gene have been replaced by codons preferred in E. coli in order to increase expression efficiency.

Materials and Methods

The 96 well clear microplates, catalogue number CFCP N96, were purchased from Biosearch, (Bedford, MA). Purified recombinant proteins for wtGFP, EGFP, GFP-S65T, and GFPuv were purchased from Clontech. Partially purified extracts containing recombinant wtGFP protein, as well as the EGFP, Y66H, and Stemmer mutant proteins were a generous gift of Daniel González (Rutgers, New Brunswick, NJ). A series of dilutions of each protein extract were made using Tris-EDTA buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) as the diluent. Fluorescent determinations were made using an FL600 Fluorescence Microplate reader (BioTek Instruments, Winooski, Vermont) with KC4 data reduction software on an external PC controlling reader function and data capture (BioTek Instruments, Winooski, VT). Filter sets varied depending on the variant of GFP used. Determinations of wtGFP were made using a 400 nm, 30 nm bandpass excitation filter and a 508 nm, 20 nm bandpass emission filter. Stemmer, mutant and GFPuv were read using a 360 nm, 40 nm bandpass excitation filter and a 508 nm, 20 nm bandpass emission filter. The red-shifted mutants EGFP and GFP-S65T were determined using a 485 nm, 20 nm bandpass excitation filter and a 530 nm, 25 nm bandpass emission filter,while the “blue” emitting mutant, Y66H, was investigated using a 360 nm, 40 nm bandpass excitation filter with a 460 nm, 40 nm bandpass emission filter. Table 2 indicates the excitation and emission maxima of several of the proteins used. Protein concentrations for each GFP variant were determined using absorbance at appropriate wavelength (lmax) and the concentration calculated using the reported extinction coefficients (e) for each variant (15, 16).

Table 2. Excitation and emission data of GFP variants

GFP variant	Excitation max (nm)	Emission max (nm)	Extinction coefficient (ε) ($cm^{-1}M^{-1}$)
wtGFP[@]	395	509	27,6000
	470	509	12,000
EGFP	488	509	61,000
GFP-S65T	489	509	56,000
GFPuv	395	509	27,000
Y66H	382	459	25,000
Stemmer[@]	397	509	27,000
	475	509	12,000

Note that both excitation peak wavelengths are indicated.

Results

Concentration curves were made using several GFP variants and the fluorescence determined. In all cases a linear correlation to concentration was observed. The fluorescence of partially purified wtGFP protein was measured from 0 to 140 ng. Determination of total protein content by the method of Lowry indicates that wtGFP represents 3% of the total protein. As demonstrated in Figure 1, using an excitation wavelength of 400 nm and an emission wavelength of 508 a linear response was observed (r2=0.998). In terms of detection limits as little as 5 ng per well of

wtGFP can be detected. Using purified recombinant wtGFP obtained commercially, similar results were obtained in regards to linearity and detection limits.

When the fluorescence of the red-shifted proteins was determined, a linear relationship between concentration and fluorescence was also observed for either EGFP or GFP-S65T (Figure 2). With the fluorescein filter set, the increase in the extinction coefficient (Table 2) for EGFP results in a brighter fluorescence than with the wild-type protein. This may account for the lower detection limit of 1 ng per well we observed with this protein (data not shown). Quantitation of EGFP in extracts using a calibration curve determined by linear regression can be used with a high degree of confidence ($r^2 = 0.998$).

As demonstrated in Figure 3, the Stemmer mutant and GFPuv can utilize the same filter set. As with all the other GFP variants examined both proteins demonstrate a linear relationship between concentration and fluorescence signal (Figure 3). The detection limit of the Stemmer mutant was determined to be 2.5 ng (data not shown). These data are in close agreement with the extinction coefficient data that indicates that the Stemmer mutant is half as bright as the EGFP variant. Although they are optimized for excitation using UV light and in these experiments were excited at 360 nm, they can be quantitated very well with the same filter set as wtGFP, which utilized a 400 nm excitation filter.

The fluorescence from a serial dilution of the GFP-Y66H protein extract was performed. Although the Y66H blue emitting mutant sample was not pure enough to quantitate protein content using absorbance, there was sufficient protein to detect a fluorescent signal. With dilution of the protein extract, a linear relationship between dilution and fluorescence was observed (Figure 4).

As demonstrated in Figure 5 using the appropriate filter set is important in order to maximize the signal. Use of the red-shifted mutants such as EGFP allows the use of the traditional "fluorescein" filter set (485 nm excitation, 530 nm emission). When filters that maximize the signal with wtGFP (400 nm excitation and 508 nm emission) are used to detect EGFP, the signal returned is greatly diminished, despite having the same sensitivity setting. Unfortunately, filter overlap precludes the use of the 485 nm excitation filter with a 508 nm emission filter. Wild-type GFP, as previously described has a primary excitation centered at 395 nm with a shoulder located at 485 nm. With the traditional "fluorescein" filter set than a loss of fluorescent signal is observed as compared to the result when a 400 nm excitation and 508 nm emission is used. Due to the secondary excitation peak at 370 nm with wtGFP, the degree of loss is alleviated somewhat as compared to EGFP, which only has a single excitation peak. In the case of GFPuv, which has been optimized for excitation with UV light, the loss of fluorescent signal is much more profound when the "fluorescein" filter set is used (data not shown).

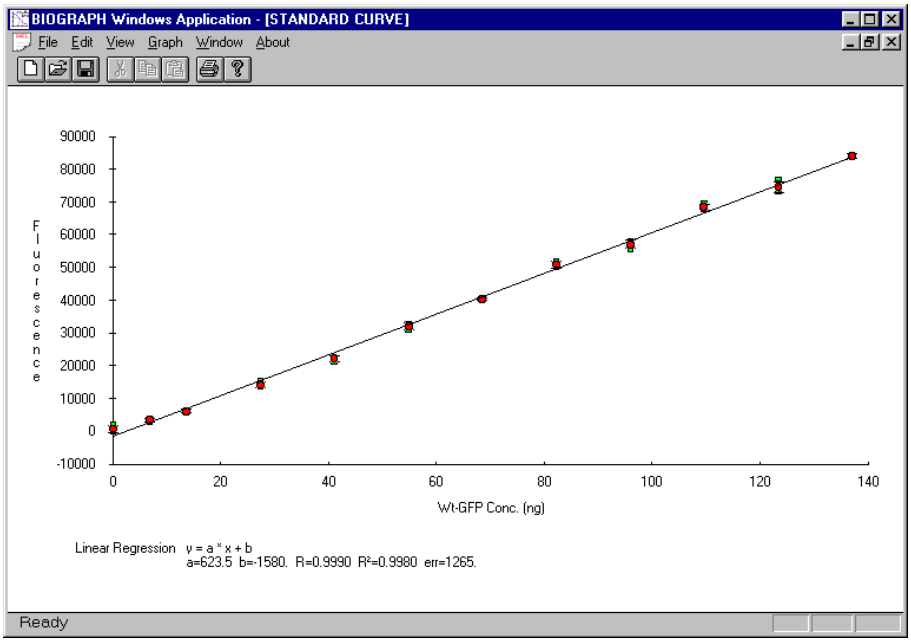


Figure 1. Concentration curve using partially purified wtGFP protein. Dilutions of wtGFP protein were made using 10 mM Tris, 10 mM EDTA buffer as the diluent. Samples were read using an FL600 fluorescence plate reader with reader function controlled by KC4 data reduction software on an external PC. Fluorescence was determined using a 400 nm, 30 nm bandpass excitation filter and a 508 nm, 20 nm bandpass emission filter with an instrument sensitivity setting of 175.

A B

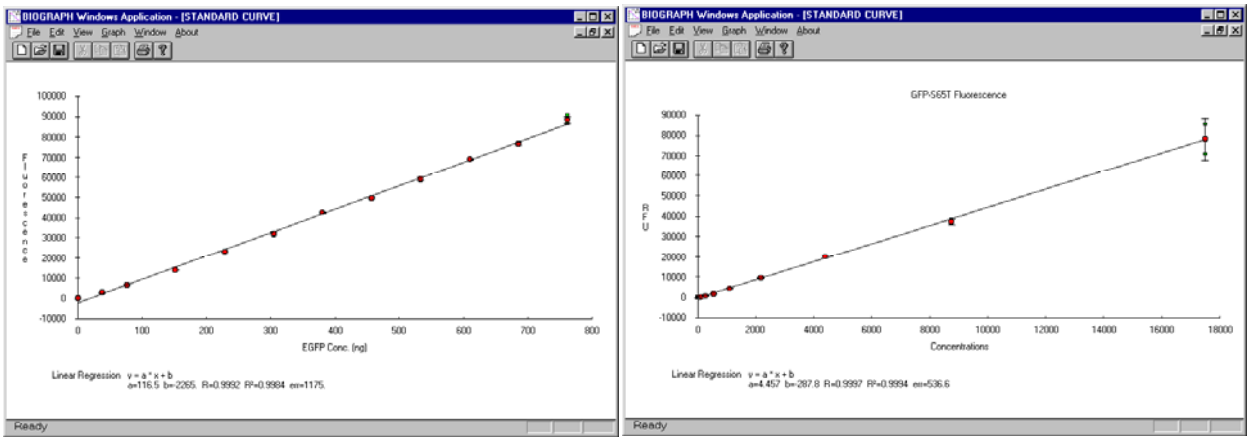


Figure 2. Concentration curves of red-shifted GFP proteins. Dilutions of (A) EGFP or (B) GFP-S65T proteins were made using 10 mM Tris, 10 mM EDTA buffer as the diluent. Samples were read using an FL600 fluorescence plate reader with reader function controlled by KC4 data reduction software on an external PC. Fluorescence was determined using a 485 nm, 20 nm bandpass excitation filter and a 530 nm, 25 nm bandpass emission filter with an instrument sensitivity setting of 135.

A B

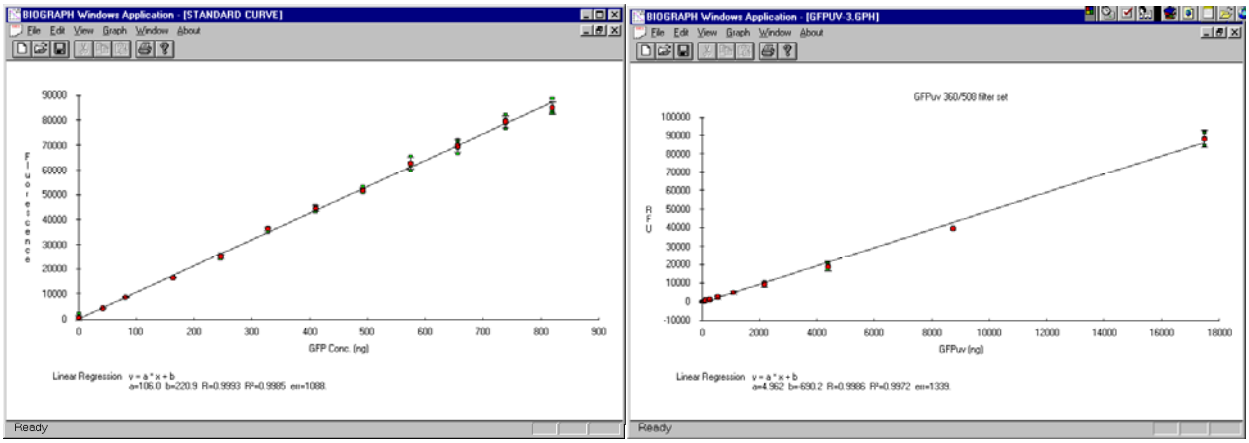


Figure 3. Concentration curves using partially purified Stemmer mutant GFP and rGFPuv proteins. Dilutions of partially purified Stemmer-mutant GFP protein (A); or rGFPuv protein (B) were made using 10 mM Tris, 10 mM EDTA buffer as the diluent. Samples were read using an FL600 fluorescence plate reader with reader function controlled by KC4 data reduction software on an external PC. Fluorescence was determined using a 360 nm, 40 nm bandpass excitation filter and a 508 nm, 20 nm bandpass emission filter with an instrument sensitivity setting of 140.

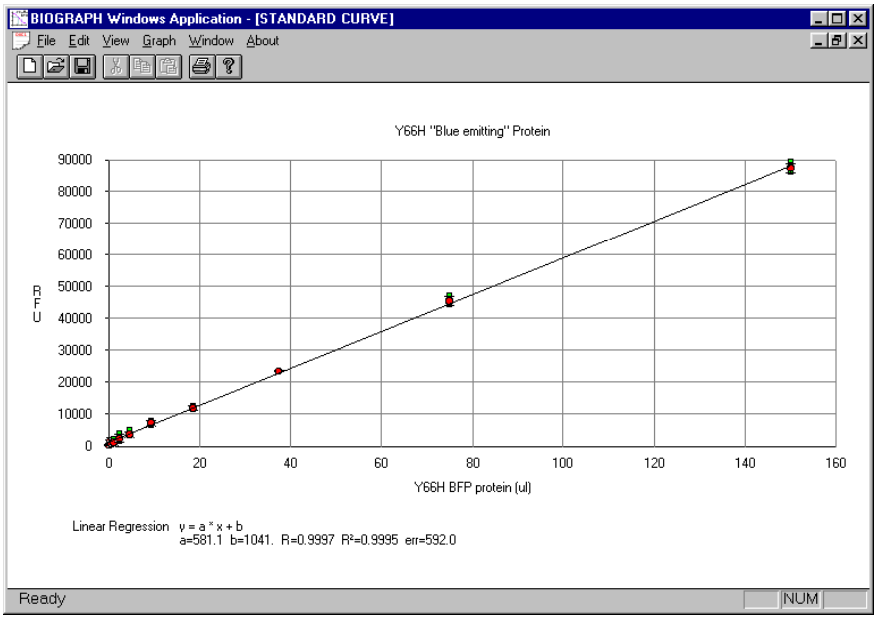
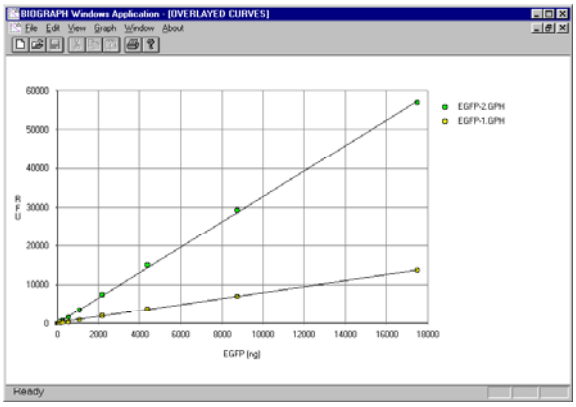


Figure 4. Concentration curve using partially purified GFP-Y66H mutant protein. Dilutions of mutant GFP-Y66H protein were made using 10 mM Tris, 10 mM EDTA buffer as the diluent. Samples were read using an FL600 fluorescence plate reader with reader function controlled by KC4 data reduction software on an external PC. Fluorescence was determined using a 360 nm, 40 nm bandpass excitation filter and a 460 nm, 45 nm bandpass emission filter with an instrument sensitivity setting of 150.

A



B

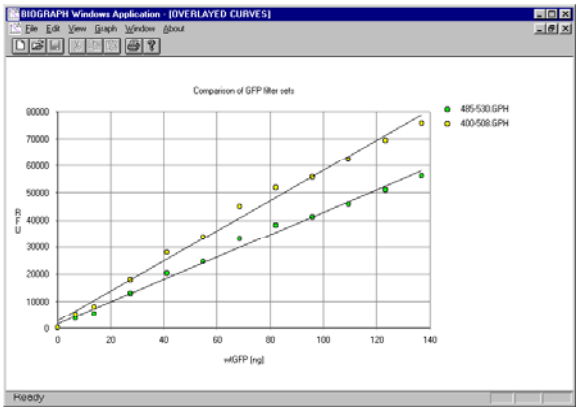


Figure 5. Loss of signal when using alternative filter sets. Dilutions of (A) EGFP protein or (B) wtGFP were made using 10 mM Tris, 10 mM EDTA buffer as the diluent. Samples were read using an FL600 fluorescence plate reader with reader function controlled by KC4 data reduction software on an external PC. Fluorescence was determined using either a 485 nm, 20 nm bandpass excitation, 530 nm, 25 nm bandpass emission filter set or a 400 nm, 30 nm bandpass excitation, 508 nm, 20 nm bandpass emission filter set. For EGFP and wtGFP determinations using either filter set, a sensitivity setting of 170 was used.

Discussion

The utility of green fluorescent proteins has become increasingly obvious. Detection only requires the addition of near UV to blue light and is not limited by the availability of substrates, therefore providing information concerning gene expression and cellular localization in real time. Also GFPs do not interfere with cell growth in a wide variety of organisms and as such, are a convenient indicator of transformation. In this application note we describe the direct quantitation of several different mutant forms of GFP using fluorescence.

Because GFPs are actually proteins rather than small fluorescent molecules, the physical properties of GFPs as peptides must be taken into account. Despite being a peptide, GFP is resistant to denaturation. GFP, with a Tm=70°C, is quite resistant to heat denaturation once formed. In vivo it appears that the formation of GFP is somewhat temperature sensitive. In yeast GFP fluorescence is reported to be maximal at 15°C and decreases to about 25% of maximal when the incubation temperature is raised to 37°C (7). It has been demonstrated that mutations that increase efficiency of protein folding, as with EGFP and GFPuv, suppress the sensitivity of fluorescence to growth incubation temperature (3). In regards to redox status, GFP needs to be in an oxidized state in order to fluoresce, as the formation of the cyclic tripeptide chromophore requires molecular oxygen (6). Strong reducing agents, such as 5 mM $Na_2S_2O_4$ or 2 mM $FeSO_4$ convert GFP into a nonfluorescent moiety. Fortunately, weaker reducing agents such as, 2% b-mercaptoethanol, 10 mM dithiotheitol (DTT), 10 mM glutathione, or 10 mM L-cysteine, do not seem to affect the fluorescence of GFP (16). Strong oxidizing agents such as 1% H_2O_2 will also abolish fluorescence as will complete denaturation. GFP is quite resistant to pH changes. For example, wtGFP is fluorescent over a very broad pH range (pH 5.5-12) with rapid loss of fluorescence with pH levels either above or below this range. The red-shifted mutants have been reported to have a narrower range of pH stability and exhibit fluorescence between pH 7.0 and 11.5.

In regards to fluorescence, GFP offers some advantages that one would not expect from proteins. Unlike many proteins, activity is not lost and fluorescence is maintained after fixation with either glutaraldehyde or formaldehyde. Most importantly, GFP and its most of its variants are reported to be quite resistant to photobleaching. Chaotropic agents such as 8M urea, and low concentrations of detergents (1% SDS) also do not effect fluorescence of the protein. There are several issues that have to be kept in mind when using GFP as a reporter molecule. Expression of exogenous proteins in organisms can lead to solubility problems. Wild-type GFP,

like many other proteins when expressed in bacteria, is primarily found in inclusion bodies in an insoluble form and is nonfluorescent. Mutations that increase protein folding and translation efficiency have been found to eliminate many of the solubility problems. For example, E. coli expressing GFPuv, with its mutations to improve protein folding and chromophore formation, are reported to be 18 times brighter than bacteria expressing wtGFP mostly due to increased protein solubility. In regards to the use of GFP as a means to measure transcription, the rate of chromophore formation and the apparent stability of wtGFP may preclude the use of GFP as a reporter to monitor rapid changes. Chromophore formation takes place post-translationally and requires molecular oxygen. Cells, bacteria in particular, grown in anaerobic conditions will be refractory to GFP fluorescence. Likewise if very rapid changes in transcription rates are being investigated, the lag between protein production and chromophore formation may preclude the use of GFP. Similarly, GFP proteins once produced are quite stable and resistant to degradation, again reducing their utility as a reporter for rapid changes. Another important point to consider in regards to using GFP as quantitative reporters is the lack of signal amplification. The signal associated with GFP does not have any enzymatic activity associated with it, therefore there is no opportunity for amplification as would be the case for reporters such as b-galactosidase, luciferase, or alkaline phosphatase.

Another problem associated with GFP fluorescence is background autofluoresence. Most autofluoresence in mammalian cells is due to the presence of the flavins FAD and FMN. These compounds have excitation and emission maxima of 450 nm and 515 nm respectively and tend to cause problems primarily with the red-shifted variants. Likewise, NADH, another commonly found cellular cofactor, has an excitation of 365 nm and an emission of 445 nm and may result in background fluorescence when wtGFP fluorescence is quantitated. If background autofluorescence is a problem, it may be necessary to tailor the GFP mutant to the type of autofluorescence encountered. It has been estimated that cytoplasmic concentration must be 1.0 mM in order for the wtGFP signal to be twice that generated from autofluorescence. Red-shifted variants, because they are brighter, have a lower threshold of 100-200 nM (16).

Although most experiments using GFP are performed using either fluorescence microscopy or flow cytometry, these data presented in this application note, demonstrate the utility of direct measurement of GFP using a fluorescence microplate reader. Microscopy can provide information on the number of cells expressing GFP, but is quite subjective in regards to quantitation. Flow cytometry can provide quantitative information, but throughput is quite limited. Determination of fluorescence using a microplate fluorometer allows quantitative information as well as high throughput.

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