

Quantification of green fluorescent protein fluorescence using real-time PCR thermal cycler

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Fluorescent proteins have become a widely used transgenic marker in the last decade because they spontaneously form fluorophores without the need for other gene products, they do not require external substrates, and they enable real-time monitoring in intact organisms. The currently available fluorescent proteins belong to either one of two families: the green fluorescent protein (GFP) family, originating from the jellyfish *Aequorea victoria*, and the reef coral fluorescent protein (RCFP) family, originating from reef corals. These fluorescent proteins and their mutants with altered spectral characters span emission spectra from 480–620 nm.

There are two broad groups of applications for fluorescent proteins as transgenic markers. The first group includes the labeling of whole organisms (1), the subcellular localization of cells and proteins (2), the analysis of tissue specificity of gene expression, and fluorescence-activated cell sorting. These applications require

semiquantitative detection in space or time and use imaging technologies, such as macroscopic fluorescence imaging systems (3) and confocal fluorescence microscopy (4), to detect the fluorescent targets. The second group of applications consists of bioassays that require accurate quantification of fluorescence in either solution or in living cells. For example, bacteria and yeast that synthesize GFP after contact with chemicals that bind to human estrogen receptors have been developed as bioassays for environmental estrogens (5–7). Such bioassays are usually applied to a large number of samples, requiring the use of a fluorometer that can read microtiter plates. Since microtiter plate fluorometers are not common equipment found in a molecular biology laboratory, we determined that real-time thermal cyclers can replace fluorometers for the quantification of fluorescent proteins.

Real-time thermal cyclers monitor the progress of PCR by measuring the fluorescence of an intercalating dye

or a fluorophore attached to a DNA hybridization probe. Most thermal cyclers use heating blocks that have a standard 96-well microtiter dish format, so they also can be used as microtiter plate fluorometers—provided that the excitation and emission wavelengths of the fluorescent protein in question match the specification of the thermal cycler, and that the fluorescence reading is returned in a digital form. Thermal cyclers that have monochromatic light sources are inherently limited in their excitation wavelength. Systems with a halogen lamp for its light source are more flexible, having full control of the spectral characters possible through the choice of filter sets. Regardless of the light source, the suitability of any real-time thermal cycler for the quantification of a particular fluorescent protein can be evaluated by comparing the spectral characters of the fluorescent protein with those of the DNA fluorophores for which the thermal cycler was designed. The most widely used fluorescent proteins are listed in Table 1 along with their matching DNA fluorophores.

We compared the ability of a standard microtiter plate fluorometer (Synergy™ HT, Bio-Tek Instruments GmbH, Friedrichshall, Germany) and a real-time thermal cycler (iCycler iQ®, Bio-Rad Laboratories, Hercules, CA, USA) to detect GFP (Roche Diagnostics GmbH, Penzberg, Germany) (Figure 1). The sensitivity of both systems was similar to the lower limit of detection at approximately 1 mg/mL. Although a limited solubility of

Table 1. Matching Fluorescent Proteins with DNA Fluorophores

Fluorescent Protein	Source of Wild-Type Gene	Matching DNA Label
rGFP, GFP _{UV} (395/509, 475/509); EGFP (488/507), Class 2 GFPs ^a	jellyfish <i>Aequorea victoria</i>	Cy™2 (489/506)
S65T-GFP ^b (489/509)	jellyfish <i>A. victoria</i>	6-FAM (494/516), Oregon Green® 500 (499/519), SYBR Green I (497/520)
Ds Red Monomer (556/586)	<i>Discosoma</i> sp. reef coral	Cy3 (552/570), TAMRA (565/580)
ZsGreen1 (493/505)	<i>Anthozoa</i> reef coral	Cy2 (489/506)
AsRed2 (576/592)	<i>Anthozoa</i> reef coral	Cy3.5 (581/596)
HcRed1 (588/618)	<i>Heteractis crispa</i> reef coral	ROX™ (581/607), Texas Red® (589/610)

Excitation/emission wavelengths are specified in nanometers in parentheses. GFP, green fluorescent protein.
^aReference 8.
^bReference 9.

GFP has been encountered frequently in published reports, we were able to generate a calibration curve up to the concentration of 10 mg/mL. The form of the curves was slightly sigmoidal, rather than linear, for both systems (Figure 1).

In many applications, GFP has to be measured in heterogeneous systems such as tissue cultures or bacterial cells rather than in cell-free solutions. In order to ensure that real-time thermal cycle is suitable for this task as well, we measured fluorescent signal generated by germinating spores of a filamentous fungus responding to the mycotoxin zearalenone (Figure 2). The bioassay exploits the ability of *Gliocladium roseum* to sense and respond to the presence zearalenone by synthesizing a zearalenone-specific lactonase. The bioassay strain was constructed by fusing the esterase gene in *G. roseum* with a GFP gene (Utermark and Karlovsky, unpublished). The fluorescence signal can be quantified by any real-time thermal cyclers able to detect FAM and/or SYBR® Green dyes. Unlike fluorometers, real-time thermal cyclers are available in many molecular biology laboratories.

According to our findings, real-time thermal cyclers are a viable alternative to microtiter plate-compatible fluorometers for measuring the amount of GFP and other fluorescent proteins within living cells and as purified proteins.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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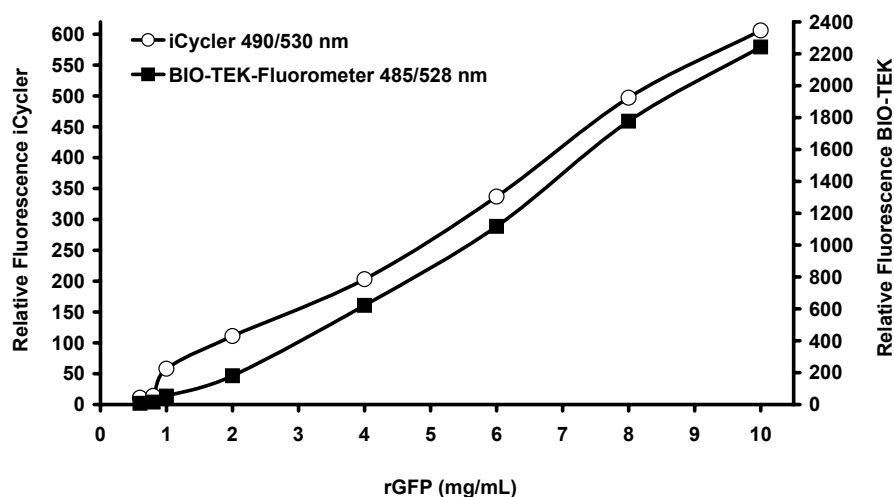


Figure 1. Comparison of green fluorescent protein (GFP) determination with the help of a fluorometer and real-time thermal cyclers. Solutions of purified recombinant GFP (rGFP) were analyzed in fluorometer Synergy HT with published excitation and emission wavelength for the fluorophore and in an iCycler real-time thermal cyclers with excitation and emission filters for SYBR Green I.

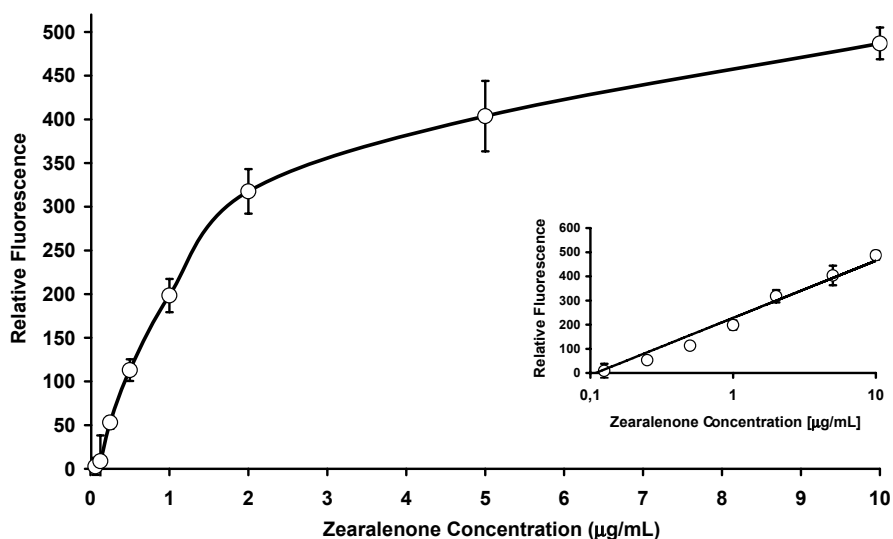


Figure 2. Determination of zearalenone using a bioassay based on a green fluorescent protein (GFP) fusion. *Gliocladium roseum* strain carrying a fusion of zearalenone esterase with GFP was incubated with increasing concentrations of zearalenone. The fluorescence of GFP was measured in an iCycler real-time thermal cyclers. Inset: the same data plotted on a semilogarithmic scale. Points are averages of 5 measurements with error bars of ± 1 standard deviation.

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