

## **Measurement of Green Fluorescent Protein in the SPECTRAmax® GEMINI-XS Spectrofluorometer.**

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### **INTRODUCTION**

GFP (Green Fluorescent Protein) is an extremely stable protein of 238 amino acids produced by the jellyfish *Aequorea victoria*<sup>1</sup> that fluoresces in the lower green region of the visible spectrum. Over the past few years GFP has become one of the most widely used tools in molecular and cell biology as the GFP gene can be expressed in non-homologous species. Additionally, the fact that GFP can generate a highly visible fluorescence that requires no additional substrates has made it valuable as a marker for gene expression, as a cell lineage tracer or as an *in vivo* covalently attached fluorescent label replacing more invasive and unwieldy fluorescent staining techniques. GFP can be monitored non-invasively and may be used as a quantitative marker in living cells. GFP can also be used as a tag for studying real time localization and movement of protein-GFP chimeras within cells<sup>2</sup>.

However, there have been some technical difficulties associated with the use of GFP. One major concern is the excitation and emission spectra of the protein. Wild type GFP has two excitation peaks, a major one at 395 nm (in the long UV range) and a smaller one at 475 nm (blue) with an emission peak at 509 nm (green). For wild type GFP, it has been found that exciting the protein at 395 nm causes rapid quenching of the fluorescence. Recently, some modified forms of the GFP gene have been produced with different spectral properties. For example, the major excitation peak has been red-shifted to 490 nm and the emission peak staying at 509 nm. Perhaps more importantly, these modifications generally increase the fluorescent output<sup>3</sup>.

As the GFP signal can be weak and hard to detect, especially when expressed at low levels, fluorescent cells are usually observed using a laser scanning confocal microscope or similar instrument. A weak signal thus precludes use in a microplate spectrofluorometer, which would allow increased throughput. The SPECTRAmax® GEMINI-XS is a dual-monochromator microplate fluorometer

system incorporating highly sensitive optics<sup>4</sup> that permit experimental determinations at low-level detection limits. This application note describes experimental protocols that enable the detection of relatively low numbers of cells expressing Green Fluorescent Protein (GFP) in a 96-well microplate using the SPECTRAmax GEMINI-XS spectrofluorometer.

## MATERIALS AND METHODS

Human embryonic kidney 293 cells (HEK-293) were transiently transfected with a wild-type GFP expression plasmid (pS65T, Clontech, discontinued product; cytomegalovirus immediate early promoter drives expression of GFP) using Lipofectamine as per manufacturer's instructions (Gibco/Invitrogen, Cat. No. 18324-012). Cells were grown to confluence in 162 cm<sup>2</sup> flasks under standard cell-culture conditions. Fluorescence microscopy revealed an expression level in excess of 75 %.

The GFP-expressing cells were removed with trypsin, and the cells were washed with medium (MEM Alpha, Gibco-RBL Cat. No. 22571-020). The appropriate number of cells was seeded in alternate columns of a 96-well microplate (Costar, black walled, clear bottomed Cat. No. 3603) and diluted with mock-transfected cells to maintain a constant cell number of 200,000 cells per well. This resulted in a final number of 6,250 (column 12) to 200,000 (column 2) GFP expressing cells per well in a volume of 100  $\mu$ l. These cells were seeded in alternate columns 2, 4, 6, 8, 10, and 12. In contrast, columns 1, 3, 5, 7, 9 and 11 were seeded with 200,000 mock-transfected cells per well. Then the plate was returned to the incubator for 48 hours.

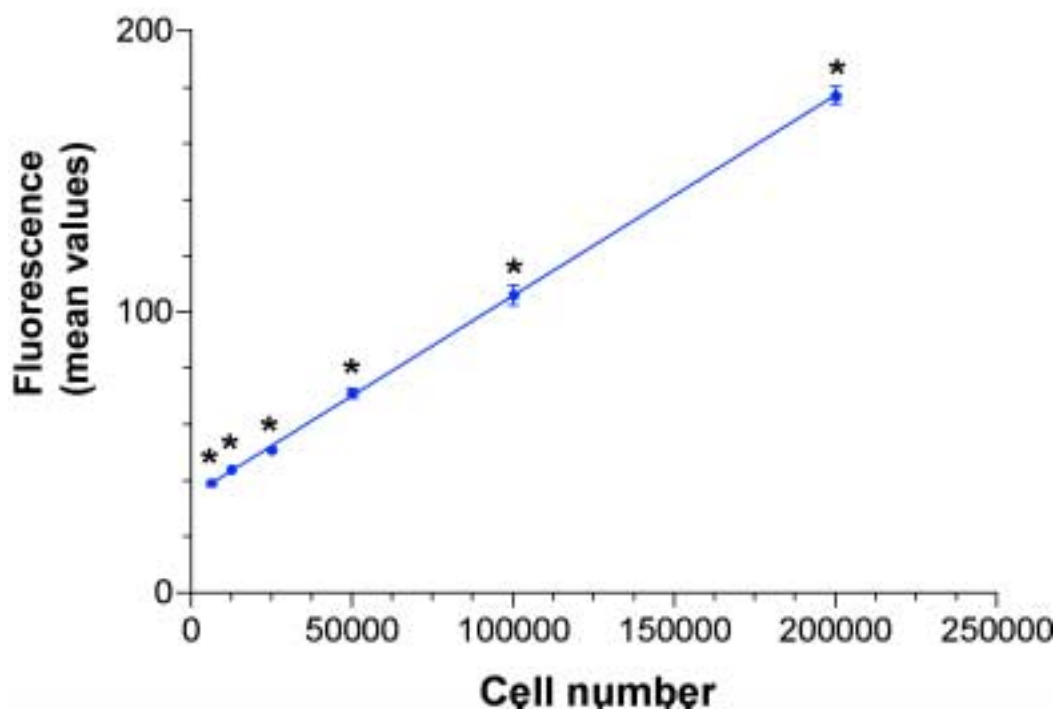
Initially, excitation and emission scans were run to select the optimum excitation wavelength and emission wavelength/cutoff filter combination for the maximum signal/background ratio<sup>5</sup>. The parameters selected for all subsequent experiments were: GFP;  $\lambda_{EX}$  – 472 nm,  $\lambda_{EM}$  – 512 nm with a 495 nm emission cutoff filter.

## RESULTS

Results show a linear relationship between fluorescence intensity and cell number (Fig.1) A maximal fluorescence intensity of  $177 \pm 3$  RFU was observed at the highest cell concentration (Table 1). SPECTRAmax GEMINI-XS spectrofluorometer was able to detect a specific GFP signal at all cell numbers as determined by One Way Analysis of Variance (ANOVA) (Table 1). This analysis indicated that, after 48 hours incubation, there was a significant difference ( $P < 0.01$ ) between the GFP-transfected and mock-transfected cells at an initial seeding density of 6,250 GFP- expressing cells per well (Table 1). Since approximately 75 % of the cells actually express GFP, this translates to a detection limit of roughly 4,690 GFP-expressing cells/well.

	Initial number of GFP expressing cells per well					
	200,000	100,000	50,000	25,000	12,500	6,250
Signal from GFP-expressing cells (RFU)	177 ± 9	106 ± 10	71 ± 5	51 ± 3	44 ± 2	39 ± 4
	200,000	200,000	200,000	200,000	200,000	200,000
Signal from mock-transfected cells (RFU)	30 ± 1	29 ± 2	31 ± 1	32 ± 2	34 ± 2	32 ± 2
ANOVA <i>P</i> Value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

**Table 1:** Specificity and Sensitivity of GFP Detection within Transfected Cells. Data shown is the relative fluorescence units (RFU) detected from GFP-expressing cells and mock transfected cells. Data is described as mean RFU ± standard deviation, n=8.



**Figure 1:** Linearity and sensitivity of GFP detection within transfected cells. Cells were serially diluted as described in Materials and Methods. Asterisk indicates significant difference between mock-transfected and GFP-expressing (●) cells. Data is shown as mean RFU ± standard deviation, n=8.

A set of experiments was designed to compare two types of microplates for their ability to detect a 1  $\mu$ M fluorescein solution compared to suspension of GFP-expressing cells (Table 2). Interestingly, the fluorescein signal increased from 11190 RFU in Greiner plates to 32787 RFU in ABgene Thermo-Fast® 96 skirted black PCR plates (Cat. No. AB-0800, specify *black* when ordering). More importantly, the signal detected from GFP-expressing cells increased by almost 3-fold when black ABgene PCR plates were used (Table 2). A possible explanation for the greater signal detectable using the black PCR plates is that the base of each

well of the PCR plate is higher and the wells are narrower than the standard microplate. This feature brings the sample closer to the optics and for a constant volume, effectively increases the path-length.

	<b>Fluorescein (150 <math>\mu</math>l)</b>	<b>Cell suspension (100 <math>\mu</math>l)</b>	<b>Buffer (100 <math>\mu</math>l)</b>
Greiner Fluorostar	11190 $\pm$ 36	17.1 $\pm$ 0.5	0.25 $\pm$ 0.03
ABgene Thermo-Fast	32787 $\pm$ 179	46.4 $\pm$ 1.9	0.80 $\pm$ 0.07
Fold-increase in signal	2.9	2.7	3.2

**Table 2:** Comparison of Fluorescent Signals Obtained with Two Different Microplates. Signals from a fluorescein solution and a GFP expressing cell suspension were compared in Greiner and ABgene plates. Representative data is shown as mean RFU  $\pm$  standard deviation (S.D.), n=8.

## DISCUSSION

GFP and its variants are convenient markers for use in the fluorescent detection of gene expression or protein localization in living cells because it eliminates the need to incubate with secondary reagents such as dyes or antibodies. Additionally, vectors encoding these fluorescent proteins are also commonly used as simple transfection markers. Furthermore, GFP fluorescence is stable, species-independent, and does not require any substrates or cofactors. With the burgeoning use of GFP in life sciences and drug discovery research, and ever increasing demands for higher assay throughputs, traditional detection technology such as fluorescence-activated cell sorting (FACS) and fluorescence microscopy can no longer satisfy demands. Transfer of an assay to 96-well microplate technology increases throughput significantly.

This report demonstrates that the SPECTRAmax GEMINI-XS microplate spectrofluorometer, with its dual scanning monochromator and patented optical sensitivity, can be used to detect a relatively low number of cells expressing GFP. The proprietary optical design with the use of elliptical mirrors instead of lenses enhances sensitivity and data reproducibility. Even at an initial seeding density of 6,250 GFP-expressing cells per well, the Gemini-XS is able to reliably detect the 1.2-fold increase in signal over mock transfected cells. Because the SPECTRAmax GEMINI-XS is able to monitor up to four different wavelengths during an experiment, it may be used for multiple color-labeling experiments such as those using the range of Clontech Living Colors™ Fluorescent Proteins.

In practical terms, possible uses of the SPECTRAmax GEMINI-XS with fluorescent proteins include discrimination of cellular apoptosis or necrosis (apoptosis induced decrease in fluorescence<sup>6</sup>), determination of bacterial survival rates<sup>7</sup> or measurement of homologous DNA recombination repair which plays a role in cell viability/genomic stability<sup>8</sup>.

## ACKNOWLEDGEMENTS

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## PATENT NOTE

Products containing DNA sequences coding for mutant *Aequorea victoria* green fluorescent protein (GFP) variants or proteins thereof require a license from Aurora Biosciences Corporation under U.S. Patent Nos. 5,625,048, 5,777,079, and 5,804,387 and other pending U.S. and foreign patent applications. For-Profit research institutes or entities that wish to use this product in non-commercial or commercial applications are required to obtain a license from Aurora Biosciences Corporation.

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