BD Living Colors™ EGFP Calibration Beads User Manual

Cat. No. 8375-1
PT3614-1 (PR27687)
Published 07/29/02

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I. Introduction

BD Living Colors™ EGFP Calibration Beads are a set of EGFP (Enhanced Green Fluorescent Protein) standards for flow cytometry calibration (GFPmut1; Cormack *et al*., 1996). A reference blank population of microbeads is also provided. These fluorescent standards allow you to calibrate the flow cytometer and quantitate the fluorescence intensity of an experimental sample. The fluorescent standards consist of four populations of microbeads, each coated with EGFP at a different density. These beads have fluorescence intensity (FI) as well as excitation and emission spectra matching specimens labeled with EGFP. This kit makes possible the conversion of fluorescence intensity of a sample to the number of *molecules of equivalent soluble fluorochrome (MESF)* of EGFP—a value that can be compared between instruments, between samples, or between experiments (Vogt, *et al*., 1989; Schwartz, *et al*., 1998). The EGFP Calibration Beads are manufactured exclusively for BD Biosciences Clontech by Bangs Laboratories, Inc.

These calibration standards make it possible to convert the fluorescence intensity to the number of *molecules of equivalent soluble fluorochrome (MESF)* of EGFP. MESF refers to the density of EGFP at which each population of microbeads has been coated. The MESF for each population in your kit is reported on the bottle label. Once your flow cytometry data have been converted to MESF, then data from different instruments or experiments can be accurately compared. The intensity of each fluorescence quantization standard has been calibrated against solutions of fluorescent dyes in units of MESF per microbead. The kit includes a reference blank necessary to measure the threshold fluorescence level of the instrument.

Correct use of this kit permits:

- Quantitative measurement of the fluorescence intensity of samples that are in the range of the standards.
- Determination of an instrument's fluorescent threshold.
- Determination of linearity and stability of the instrument's readouts.
- Data comparison between experiments as well as between instruments.

MESF and Fluorescence

In order to quantitate fluorescence using a flow cytometer, the peak or mean fluorescence intensity for each MESF bead population must be determined. Figure 1 shows a log fluorescence histogram on which the fluorescence intensity of the singlet populations of calibration beads has been recorded versus the number of beads. This plot was created from the original, two-dimensional plot by gating around the singlet populations. Each peak in Figure 1 corresponds to an MESF bead population. The peak closest to the origin of the axis is the blank, which gives the fluorescence threshold for the flow cytometer at those particular settings.

I. Introduction *continued*

Once the mean fluorescence of each calibrated standard has been determined from the histogram, a calibration curve can be constructed by simply plotting the log mean fluorescence intensity for each bead population versus the log MESF (Figure 2). After flow cytometry results are converted to MESF, results from different instruments can be compared. If any instrument settings are changed, the relationship between fluorescence and MESF will be changed. In that case, the mean fluorescence for each bead population must be redetermined using the new settings. These EGFP standards will give a linear curve ($r^2 > 0.98$) if the flow cytometer is operating properly.

Microbead Properties

EGFP Calibration Beads consist of five standard preparations of 7–10 µm diameter microbeads. All microbeads within a standard set produce the same amount of light scatter and appear within the same window of analysis that is used for human peripheral lymphocytes. One of the five microbead standards is an unlabeled blank, and the remaining four are EGFP-labeled microbeads with specific fluorescence intensities (FI). Each standard FI is expressed in terms of MESF units. See the bottle labels for the exact MESF values in your lot of standards.

EGFP Properties

EGFP is a red-shifted excitation variant, and human codon-optimized version of the Green Fluorescent Protein that was originally isolated from the jellyfish *Aequorea victoria* (Chalfie, *et al*., 1994; Haas, *et al*., 1996; Prasher, *et al*., 1992). EGFP contains chromophore mutations that increase its fluorescence intensity, resulting in a fluorescence that is 35 times more intense than the wild-type version (Yang, *et al*., 1996). EGFP can be detected at expression levels as low as 100 nM. This translates to about 10^4 molecules in a cell's cytoplasm or about 2 x 10^3 molecules on a cell surface. Therefore, a standard curve measuring up to 100,000 molecules of EGFP is adequate for most applications. In addition, EGFP's fluorescence is ideally suited for most flow cytometers—EGFP's excitation maximum is at 488 nm, and its emission maximum is at 507 nm. For more information on EGFP structure, function and applications, please refer to the Living Colors® User Manual (PT2040-1), available at **www.bdbiosciences.com.** Also see *Green Fluorescent Protein: Properties, Applications, and Protocols*, edited by Martin Chalfie and Steven Kain.

Technical Support

For technical support with EGFP Calibration Beads, please contact Bangs Laboratories directly at:

Phone: (317) 570-7020 or (800) 387-0672
E-mail: info@bangslabs.com

Customers in Europe, the Middle East, and Africa should contact their local BD Biosciences office for support.

I. Introduction *continued*

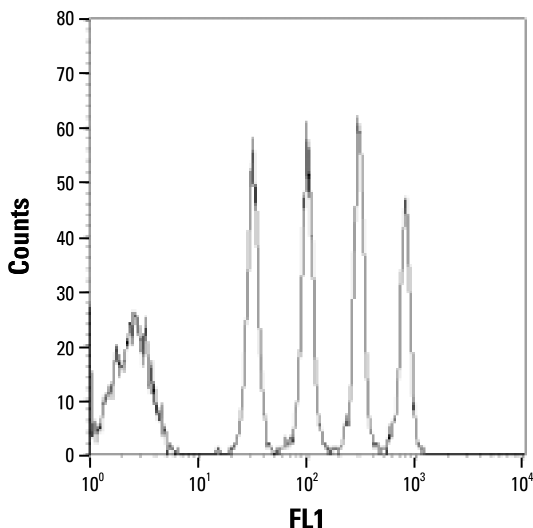

Figure 1. Log fluorescence histogram of EGFP standards. When the standards are run through the flow cytometer a log histogram of the fluorescent peaks can be created by gating around the singlet populations of beads. Each peak has a mean fluorescence value that should be consistent for those flow cytometer settings. For EGFP the Excitation maximum is 488 nm and the emission maximum is 507 nm.

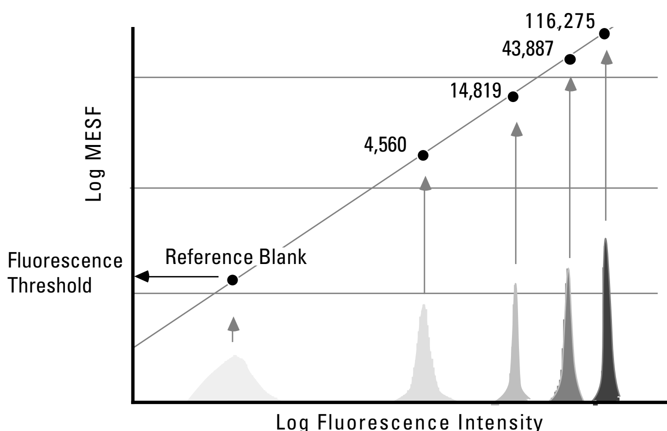

Figure 2. MESF standard curve. Mean fluorescence for each EGFP Standard is determined from the peaks of the log fluorescence histogram (Figure 1). When these values are plotted versus the molecules of equivalent soluble fluorochrome (MESF) for EGFP, a standard curve is generated that can be used to determine the MESF value for an unknown sample when read with the same flow cytometer settings. Representative MESF values are shown, see the bottle labels for the values for your lot of calibration beads.

II. List of Components

Store all EGFP-conjugated microbeads **in the dark** at 4–8°C.

Exposing these reagents to light, even for a limited period of time, will substantially reduce their performance.

Do not freeze.

The following reagents are suitable for 20 calibrations.

- **1 ml Blank Bead Population**
- **1 ml Bead 1 Population**
- **1 ml Bead 2 Population**
- **1 ml Bead 3 Population**
- **1 ml Bead 4 Population**

III. Additional Materials Required

The following materials are required but not supplied:

- **Phosphate Buffered Saline** (PBS; pH 7.5)

	Final conc.	To prepare 2 L of solution
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O. Adjust to pH 7.5 with 0.1 N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature.

- **[Optional] QuickCal® Software**

QuickCal® for Macintosh (Bangs Laboratories, Cat. #350)

QuickCal® for Windows/PC (Bangs Laboratories, Cat. #351)

This software assists in plotting the log mean fluorescence versus log MESF, calibration plot and in determining MESF values for experimental samples. For further information, contact Bangs Laboratories, Inc., www.bangslabs.com.

IV. General Considerations

Several factors can affect the fluorescence of EGFP. These points should be considered when designing and interpreting experiments.

- **Photobleaching**

  GFP fluorescence is very stable in a fluorometer (Ward, pers. comm.). Even under the high-intensity illumination of a fluorescence microscope, GFP is more resistant to photobleaching than is fluorescein (Wang & Hazelrigg, 1994; Niswender *et al.,* 1995). The fluorescence of wt GFP and EGFP is quite stable when illuminated with 450–490 nm light (the major excitation peak for EGFP, but the minor peak for wt GFP). Some photobleaching occurs when wt GFP is illuminated near its major excitation peak with 340–390 nm or 395–440 nm light (Chalfie *et al.,* 1994; Niswender *et al.,* 1995). The rate of photobleaching of wt GFP and its green variants also varies with the organism being studied; for example, GFP fluorescence is quite stable in *Drosophila* (Wang & Hazelrigg, 1994) and zebrafish. In *C. elegans,* 10 mM NaN_3 accelerates photobleaching (Chalfie *et al.,* 1994).

- **Protein stability**

  GFP is exceptionally resistant to heat (T_m=70°C), alkaline pH, detergents, chaotropic salts, organic solvents, and most common proteases, except pronase (Bokman & Ward, 1981; Ward, 1981; Robart & Ward, 1990).

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

- **Expression patterns**

  Some expression patterns may affect the MESF numbers that you detect in your samples. Subcellular localization of EGFP (e.g., to the cytoskeleton) can produce an even more intense signal by concentrating the fluorescence to a specific area within the cell. However, for some applications, the sensitivity of GFP may be limited by autofluorescence or limited penetration of light. Studies with wt GFP expressed in HeLa cells (Niswender *et al.,* 1995) have shown that the cytoplasmic concentration must be greater than ~1.0 µM to obtain a signal that is twice the autofluorescence. This threshold for detection is lower for EGFP (~100 nM, Piston, pers. comm.).

V. EGFP Calibration Procedure

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Microbead standards must be resuspended immediately before use. Before beginning, fully align the flow cytometer. Ensure that all settings are identical to those for the EGFP samples that you wish to quantitate.

A. Fluorescence Intensity Calibration

1. Vigorously shake or vortex the bottle of the reference blank standard to achieve a uniform suspension of microbeads.

   Note: Avoid production of foam when shaking or vortexing beads.
2. Add one drop of the reference blank standard to 0.5 ml of PBS (pH 7.2).
3. Run the beads through the flow cytometer. Adjust the flow rate so that the count rate is optimal for your instrument.
4. Create a two-dimensional histogram for EGFP gated on forward scatter versus side scatter for isolating the singlet populations of beads based on fluorescence.
5. Verify that the reference blank appears near the origin of the histogram.
6. Resuspend each of the four other standards to achieve a uniform suspension immediately before use. Add one drop of each of the four standard suspensions to the suspension already containing the blank standard.
7. When all the standards are in the suspension, run this preparation on the flow cytometer to establish a calibration plot.
   - Do not make any adjustments to the flow cytometer settings, as they will affect the plot distribution.
   - Record the mean fluorescence intensity for each of the five standards, as well as the instrument settings (e.g., amplifier gains, PMT voltages, etc.).
8. Plot the log of the mean fluorescence versus the log MESF (obtained from the bottle labels) for each population.

   Notes:
   - If data are collected on a linear fluorescence setting, then a log-plot of mean fluorescence versus log MESF should be plotted on a log-log graph, and the line generated should have a slope of 1.
   - If data are gathered on a log fluorescence setting, then they should be plotted on a semi-log graph. In this case, the standard curve may not have a slope of 1.
   - If you wish, you may purchase the QuickCal® Data Diskette (See Additional Materials Section) to help in generating this calibration plot.

B. Threshold Fluorescence Determination

1. Complete the Fluorescence Intensity Calibration procedure.
2. Determine the peak (median) channel number of the reference blank.

V. EGFP Calibration Procedure *continued*

3. Use the calibration plot to determine the MESF value associated with the fluorescence channel number of the reference blank. This is the fluorescence threshold of the instrument at these instrument settings.

C. Fluorescence Quantitation of an Unknown Sample

1. Complete the Fluorescence Intensity Calibration procedure to generate a calibration plot.
2. Analyze the unknown sample on the flow cytometer and record the mean fluorescence intensity.

   Note: The settings on the flow cytometer must remain the same as for the Calibration procedure, otherwise the relationship between mean fluorescence and MESF will not be the same.
3. Determine the MESF value for the sample by finding its value on the calibration plot.

VI. Troubleshooting Guide

For technical support, please refer to the contact information specified in the Introduction.

A. Significant Drop in Fluorescence Peaks

- If EGFP is exposed to light, for even limited periods of time, it can lose fluorescence via irreversible photobleaching. This loss of fluorescence will substantially affect performance.

B. Microbead Aggregation

- Microbead aggregation may occur if the singlet population in your forward scatter versus side scatter histogram decreases dramatically.
- Thoroughly resuspend microbeads by vigorously shaking the bottle immediately prior to use.
- If microbead aggregation occurs, add a mild detergent such as 0.01 % Tween 20 or SDS, and carefully vortex or sonicate on ice (be sure the sample is not denatured by heating during sonication).

C. MESF Standard Curve

- If plot of data is not linear, check instrument settings.
- Variations between experiments can sometimes be explained by differences between setup protocols (Zenger, *et al.,* 1998).

D. Photobleaching or Photodestruction of Chromophore

- Excite EGFP at 488 nm for maximum fluorescence.
- A tungsten-QTH or argon light source is preferable. Mercury and xenon lamps produce significant UV radiation which will rapidly destroy the chromophore unless strongly blocked by appropriate filters.

E. Optimizing FACS Applications

- Optimal detection of GFP fluorescence is achieved in a darkened room.
- EGFP has a single, strong, red-shifted excitation peak at 488 nm, making it well suited for detection with the commonly used FITC optics. Maximal emission is achieved when EGFP is excited at 488 nm, which corresponds perfectly to the 488-nm line of argon ion lasers used in many flow cytometry machines. In fact, this variant was selected specifically on the basis of its increased fluorescence in flow cytometry assays because the filter sets commonly used in fluorescence microscopy or fluorometry illuminate at 450–500 nm. In practical terms, the detection limits of EGFP with excitation at 488 nm should be considerably lower than for other GFP variants.

  Living cells and animals tolerate longer wavelengths better due to the lower energies; therefore, the fluorescent signal obtained with illumination of EGFP at ~488 nm is more stable and less toxic than the fluorescence obtained with wt GFP excited at 395 nm.

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VIII. Related Products

For a complete listing of all BD Biosciences Clontech products,
please visit **www.bdbiosciences.com**

Living Colors™ Vectors

Product	Cat. #
• pEGFP Vector	6077-1
• pEGFP-1 Promoter Reporter Vector	6086-1
• pEGFP-N1 N-terminal Protein Fusion Vector	6085-1
• pEGFP-C1 C-terminal Protein Fusion Vector	6084-1
• pBI-EGFP Tet Vector	6154-1
• pEGFPLuc Vector	6169-1
• pHygEGFP Vector	6014-1
• pCMS-EGFP	6101-1
• pIRES-EGFP	6029-1
• pPKCζ-EGFP	6927-1
• pPKCθ-EGFP	6925-1

ApoAlert™ Apoptosis Products

Product	Cat. #
• ApoAlert Annexin V-EGFP	8137-1
• ApoAlert Annexin V-EGFP Apoptosis Kit	K2019-1, -2

Transfection Reagents

Product	Cat. #
• CLONfectin™	8020-1
• CalPhos™ Mammalian Transfection Kit	K2051-1

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