Fluorochrome Absorption and Emission Spectra

Allophycocyanin (APC) is an accessory photosynthetic pigment found in bluegreen algae. Its molecular weight is approximately 105 kDa. APC has 6 phycocyanobilin chromophores per molecule, which are similar in structure to phycoerythrobilin, the chromophore in R-PE. It has a 650 nm wavelength absorption maximum and a 660 nm fluorescence emission maximum. Using a 660 ± 10 nm BP filter will give optimum detection for this fluorochrome. APC can be used in flow cytometers equipped with dual lasers for multi-color analysis. It can be excited by laser light between 600-640 nm. For this, we recommend a He-Ne laser at 633 nm, or a tunable dye laser tund between 600-640 nm.

PE-Cy5 is a tandem conjugate system, with an absorption maximum of approximately 650 nm which combines R-phycoerythrin and a cyanine dye (MW 1.5 kDa). *This product was previously known as **BD Cy-Chrome**.* When excited by 488-nm light, the excited fluorochrome (PE) is able to transfer its fluorescent energy to the cyanine molecule, which then fluoresces at a longer wavelength. The resulting fluorescent maximum is approximately 670 nm Using a 650-nm longpass filter will give optimum detection for this fluorochrome. Compared to other fluorescence energy-transfer systems used in flow cytometry (e.g., RED613™ , EDC, PerCP), PE-Cy5 is a superior fluorochrome for third color analysis because of its high emission intensity and broad spectrum. As with our PE conjugates, an average of one PE-Cy5 molecule is coupled per antibody or protein. Because of its broad absorption range, PE-Cy5 is not recommended for use with dual-laser flow cytometers where excitation by both lasers is possible.

Precautions for flow cytometry: PE-Cy5 tandem fluorochromes have been reported to bind some classes of human macrophages and granulocytes via Fc receptors, and PE has been reported to bind to mouse B lymphocytes via Fc receptors. Preincubation of mouse leukocytes with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 can reduce the non-specific binding of PE-Cy5-conjugated reagents to mouse B cells. **However, PE-Cy5 conjugated reagents should not be used to stain splenocytes of SJL, NOD, and MRL mice as B lymphocytes and/or other leukocytes have been reported to non-specifically stain regardless of the use of Mouse BD Fc Block™.** Reagents conjugated to PE, PerCP, PerCP-Cy5.5, APC, and APC-Cy7 tandem fluorochrome can be used on leukocytes from these mouse strains.

Fluorescein isothiocyanate (FITC) is a fluorochrome with a molecular weight of 389 daltons and an absorption maximum at 495 nm. Its excitation by 488-nm light leads to a fluorescence emission maximum around 520 nm. Using a 530 ± 15 nm bandpass (BP) filter will give optimum detection for this fluorochrome. The isothiocyanate derivative (FITC) is the most widely used form for

conjugation to antibodies and proteins, but other derivatives are available. FITC has a high quantum yield (efficiency of energy transfer from absorption to emission fluorescence) and approximately half of the absorbed photons are emitted as fluorescent light. The number of FITC molecules per conjugate partner (antibody, Avidin, Streptavidin, etc.) is usually in the range of three to five molecules.

R-phycoerythrin (PE) is an accessory photosynthetic pigment found in red algae. In vivo, it functions to transfer light energy to chlorophyll during photosynthesis. In vitro, it is a 240-kDa protein with 34 phycoerythrobilin fluorochromes per molecule. The large number of fluorochromes per PE molecule make it an ideal pigment for flow cytometry applications. Its absorption maximum is 564 nm. When excited by 488-nm light, its fluorescence emission maximum is approximately 575 nm. For single-laser flow cytometer use, we recommend using a 585 ± 21 nm BP filter for optimal detection. When performing multi-color analysis with a dual-laser system, a tighter window of detection is required to compensate for the other conjugates being used (e.g.,Texas Red ™). For this, we recommend using a 575 ± 13-nm BP filter. Our conjugation chemistry yields an average of one PE molecule per antibody or protein. The emitted light is collected in the fluorescence-2 (FL2) channel.

PE-Texas Red™ is a tandem conjugate system which combines PE and Texas Red™ and has an absorption maximum of approximately 564 nm. When excited by 488-nm light, the excited fluorochrome (PE) is able to transfer its fluorescent energy to the Texas Red™ molecule, which then fluoresces at a longer wavelength. The resulting fluorescent emission maximum is approximately 615 nm. Special care must be taken when using PE-Texas Red™ conjugates in conjunction with PE as there is considerable spectral overlap in the emission profiles of both fluorochromes.

Peridinin chlorophyll protein (PerCP) is a component of the photosynthetic apparatus found in the dinoflagellate,*Glenodinium* . PerCP is a protein complex with a molecular weight of approximately 35 kDa. When excited by light at 488 nm from an argon-ion laser, PerCP has a excitation maximum around 490 nm, with an emission spectrum which peaks at 675 nm. The emitted light is collected in the fluorescence-3 (FL3) channel. Due to its photobleaching characteristics, PerCP conjugates are not recommended for use on stream-in-air flow cytometers.

PerCP-Cy5.5 is a tandem conjugate system than combines PerCP with a cyanine dye (Cy5.5™) and has an absorption maximum of approximately 490 nm. When excited by 488-nm light, the excited fluorochrome (PerCP) is able to transfer its fluorescent energy to the cyanine molecule, which then fluoresces at a longer wavelength. The resulting fluorescent emission maximum is approximately 694 nm. Using a 650 nm longpass filter will give optimum detection for this fluorochrome. The emitted light is collected in the

fluorescence-3 (FL3) channel. PerCP-Cy5.5 is recommended for use with stream-in-air flow cytometers.

APC-Cy7 is a tandem conjugate system that combines APC and a cyanine dye (Cy7™) and has an absorption maximum of approximately 650 nm. When excited by light from a dye or HeNe laser, the excited fluorochrome (APC) is able to transfer its fluorescent energy to the cyanine molecule, which then fluoresces at a longer wavelength. The resulting fluorescent emission maximum is approximately 767 nm. It is recommended that a 750-nm longpass filter be used along with a red-sensitive detector such as the Hammatsu R3896 PMT for this fluorochrome. Special filters are required when using APC-Cy7 in conjunction with APC. It is recommended that special precautions be taken with PharRed conjugates, and cells stained with them, to protect the fluorochrome from long-term exposure to visible light.

Texas Red™ is a sulfonyl chloride derivative of sulforhodamine 101 with a molecular weight of 625 daltons. BD Biosciences Pharmingen offers Texas Red™ , conjugated to avidin, as a useful second step for multi-color analysis. Because it emits in the long wavelengths of the deep red region , Texas Red™ has little spectral overlap with FITC. When performing multi-color analysis involving both Texas Red™ and PE, BD Biosciences Pharmingen recommends excitation of Texas Red™ using a dual-laser

flow cytometer equipped with a tunable dye laser to avoid "leaking" into the PE detector. If a krypton laser, emitting light at 568 nm, is used, the laser light will "leak" into the PE channel. Texas Red™ can be used in conjunction with APC for multi-color analysis when both dyes are excited in the 595-605 nm range with a dye laser. Texas Red™ has an absorption maximum of 596 nm . Its emission maximum, when excited by 595-600-nm laser light, is 615 nm . Using a 620 ± 10-nm bandpass filter will give optimum detection for this fluorochrome .

Comparison of individual fluorochromes with single and dual laser flow cytometry.

Fluorochrome	Laser Excitation Wavelength (nm)	FACScan™ FACSCalibur™ (1 laser)	FACSCalibur™ (2 lasers)	FACStar™ FACStarPlus™ FACSVantage™ (1 laser)	FACSVantage™ SE FACStarPlus™ (2lasers)
Fluorescein	488	YES	YES	YES	YES
Phycoerythrin	488	YES	YES	YES	YES

(PE)					
PE-Texas Red	488	YES	YES	YES	YES
PE-Cy5	488	YES	YES	YES	NO[$]
Propidium Iodide	488 & 595	YES	YES	YES	YES
Peridinin Chlorophyl Protein (PerCP)	488	YES	YES	YES[*]	YES[*]
Texas Red	595	NO	NO	NO	YES[++]
Allophycocyanin (APC)	595 & 633	NO	YES	NO	YES[$]
APC-Cy7	595 & 633	NO	YES[#]	NO	YES

[*]PerCP is highly sensitive to photobleaching and must be used with laser power <150 mW

[++]Can only be used with a dye laser

[#]Not recommended (dull)

[$]PE-Cy5 and APC cannot be simultaneously used on instruments lacking cross-beam compensation.