Special Fluorescent Technologies

Photoactivatable fluorescent proteins:

- Tracking of movements of individual cells, cellular organelles and protein fractions
- Monitoring of protein degradation
- Superresolution imaging

Genetically encoded biosensors:

- Real time monitoring of changes in concentration of Ca^{2+} and H_2O_2 in various subcellular compartments
- Early detection of apoptosis

Genetically encoded photosensitizer:

- Selective light-induced cell killing
- Precise light-induced inactivation of proteins

Contents

Overview

In addition to basic fluorescent proteins traditionally used as fluorescent labels (see page 97), Evrogen offers specialized fluorescent protein-based tools including:

Photoactivatable fluorescent proteins (see page 58)

Photoactivatable fluorescent proteins (PAFPs) are the genetically encoded reporters that change spectral properties in response to irradiation with specific light. The main application of PAFPs is the precise labeling and tracking of movements of individual cells, cellular organelles and protein fractions. The object of interest tagged by PAFP can be photoconverted using a beam of focused light and monitored directly within the living tissue or cell [Patterson and Lippincott-Schwartz 2002; Chudakov et al. 2004; Gurskaya et al. 2006].

Other applications utilizing photoactivatable fluorescent proteins include real-time monitoring of protein degradation at the single cell level and superresolution imaging by PALM technique [Zhang et al. 2007; Shroff et al. 2007].

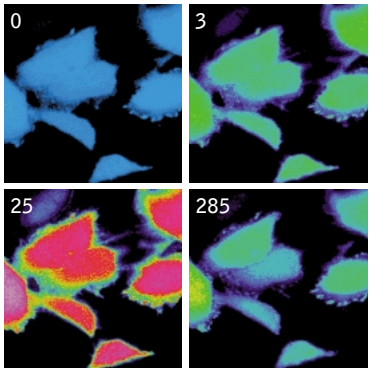
Fluorescent biosensors (see page 70)

Evrogen offers genetically-encoded fluorescent indicators for monitoring activity of caspase-3 (Casper3-BG, Casper3-GR) and changes in intracellular concentration of calcium ions (Case 12) and hydrogen peroxide (HyPer). All indicators demonstrate clearly detectable alterations in spectral properties in response to a cell event of interest [Subach et al. 2008; Shcherbo et al. 2009; Souslova et al. 2007; Belousov et al. 2006], allow precise targeting into various subcellular compartments and real time measuring of signals in natural intracellular surroundings.

Fluorescent photosensitizer (see page 87)

Photosensitizers are chromophores that generate reactive oxygen species (ROS) upon light irradiation. They can be used for precise inactivation of selected proteins in chromophore-assisted light inactivation (CALI) technique and for the light-induced cell killing, for example in photodynamic therapy.

Red fluorescent protein KillerRed is the first genetically-encoded photosensitizer [Bulina et al. 2006]. Besides KillerRed, all currently known photosensitizers are chemical compounds that must be introduced into living systems exogenously. Unlike chemical analogs, KillerRed can be directly expressed by target cells, both individually and in fusion with a target protein. It shows no cytotoxic effects before light activation. Upon green-light irradiation, KillerRed generates ROS that damage the neighboring molecules.



Ratiometric imaging of HyPer response to H_2O_2 in HeLa cells. HeLa cells expressing HyPer in cytoplasm were plated to glass bottom dishes and challenged with 180 μM H_2O_2. Images were acquired by Leica AF 6000 LX with 0.5 Hz frequency by sequential illumination of cells via CFP/YFP (excitation/emission) and YFP/YFP filters. Resulting images were obtained by dividing of YFP/YFP images to CFP/YFP images followed by pseudo coloring. Time after H_2O_2 addition (sec) is indicated at the top.

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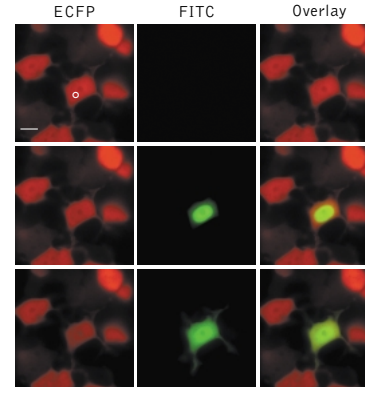
Subach et al. (2008). Chemistry & Biology, 15 (10): 1116–1124 / pmid: 18940671

Zhang et al. (2007). Biotechniques, 42 (4): 446–450 / pmid: 17489230

Photoactivatable Fluorescent proteins

Photoactivatable fluorescent proteins (PAFPs) represent an effective tool for monitoring cellular events. These reporters are capable of pronounced changes in their spectral properties in response to irradiation with light of a specific wavelength and intensity. KFP-Red converts from a non-fluorescent (dark) to a bright fluorescent state (photoactivation), whereas PS-CFP2 changes fluorescence color (photoswitching or photoconversion).

PAFPs provide a more precise and less damaging way to study cell migration and protein movements than photobleaching techniques such as fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP). In contrast to the observation of fluorescently tagged objects by constant imaging, tracking with PAFPs do not require the continual visualization. This feature greatly extends the spatiotemporal limits of studies of biological dynamics, and reduces the photobleaching and phototoxicity problems of imaging procedures.



PS-CFP photoswitching in the cell nucleus
PS-CFP was uniformly expressed in mammalian cells and its subpopulation was selectively photoswitched in the nucleus of one cell. Further imaging has revealed a gradual decrease in the green signal and an increase in the cyan signal within the nucleus due to the movement of photoswitched PS-CFP through nuclear pores into the cytoplasm and of non-photoswitched protein in the reverse way. This resulted in their equilibration through the cell within several minutes. Signals in ECFP and FITC channels are shown in red and green, respectively. Circle outlines the photoswitched region. Scale bar, 10 μg.
PS-CFP is a parental version of PS-CFP2.

Main properties of Evrogen photoactivatable FPs:

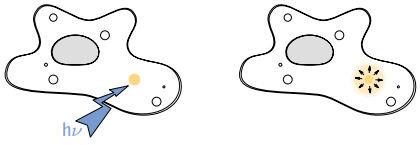
	PS-CFP2	KFP-Red
	before / after activation	before / after activation
Fluorescence color	cyan / green	No / red
Excitation maximum, nm	400 / 490	580 / 580
Emission maximum, nm	468 / 511	600 / 600
Quantum yield	0.2 / 0.23	$<$0.001 / 0.07
Extinction coefficient, $M^{-1}cm^{-1}$	43 000 / 47 000	123 000 / 59 000
Brightness*	8.6 / 10.8	0 / 4.1
pKa	4.3 / 6.1	no data / no data
Activating light	UV-violet (360-420 nm)	green (530-560 nm)
Photoswitching	irreversible	reversible
Calculated contrast, fold	up to 2000	35 (irreversible kindling) 70 (reversible kindling)
Structure	monomer	tetramer
Cell toxicity	not observed	not observed
Aggregation	no	no
Maturation rate at 37°C	fast	medium
Molecular weight, kDa	27	26
Polypeptide length, aa	238	238

*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.

Applications of photoactivatable FPs

Protein tracking

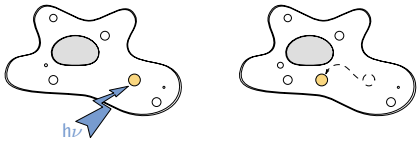
Recomended protein:
PS-CFP2



Parameters determined:
- Movement rate and direction,
- Diffusion coefficient,
- Mobile and immobile fractions,
- Time parameters of compartmental residency and exchange between compartments,
- Rate of turnover.

Organelle tracking

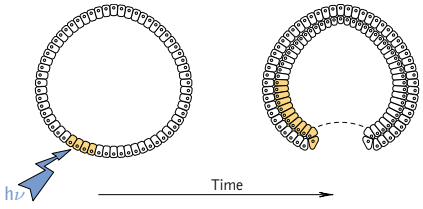
Recomended proteins:
PS-CFP2, KFP-Red



Parameters determined:
- Movement rate and direction,
- Rate of content interchange,
- Fission and fusion events.

Cell tracking

Recomended proteins:
PS-CFP2, KFP-Red



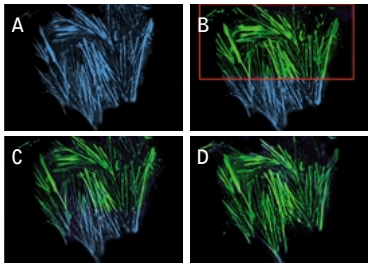
Parameters determined:
- Movement rate and direction,
- Cell localization,
- Rate of cell division,
- Shape and volume of cells.

Three levels of spatio-temporal labeling with photoactivatable fluorescent proteins.
A focused beam of light (blue arrows) is used to activate photoactivatable fluorescent proteins (orange zones) in a region of interest in a cell, tissue or organism. Migration of the labeled object (protein, organelle or cell) can then be monitored over time. A number of qualitative and quantitative parameters can be determined in each case.

Monitoring of cell migration: PAFPs provide an unique opportunity for non-invasive labeling and tracking the movements of specific cells in living organisms and tissues. The obvious examples include studying of embryogenesis, metastasis and tumor formation, the migration of small parasites within a host, and the taxis reactions of free unicellular organisms. Use example is described in [Chudakov et al. 2003] and on page 66 of this catalog.

Tracking of protein and organelle movement: Monomeric PS-CFP2 is proved to be suitable for generation of fusions. It makes it the ideal photoactivatable tags for selective labeling of the proteins and cellular organelles and tracking their movements in the living cells. Examples of use are described in [Chudakov et al. 2003; Chudakov et al. 2007] and on page 62 of this catalog.

Monitoring protein turnover: Another application of monomeric PAFPs is the careful determination of protein half-life. In the method proposed, cells are transfected with a construct coding for target protein fused with a PAFP. A steady-state concentration of the fusion protein and corresponding fluorescent signal (e.g. cyan) depends on protein synthesis and maturation rates as well as protein degradation rate. After photoconversion of the PAFP in a whole cell, a pool of distinct fluorescent molecules (e.g. green) appears. This process is independent on the synthesis and maturation of the new PAFP molecules. Thus, the decay of the activated fluorescence directly corresponds



Photoconversion of a PS-CFP2 fusion with human β-actin. 405-nanometer diode laser was used for imaging and conversion, argon-ion 488-nanometer spectral line was used for imaging and tracking of the photoconverted protein. (A) PS-CFP2 fusion with β-actin was expressed in opossum kidney epithelial cells. A portion of the actin cytoskeletal network (B; red box) was then illuminated at 405-nanometers with 40-percent laser power. After 10 minutes (C), the photoconverted actin has begun to be incorporated into filaments outside the region of interest, and at 30 minutes (D) much of the cytoskeletal network is labeled with the optical highlighter. The image is reproduced from MicroscopyU web site http://www.microscopyu.com with kind permission of Michael W. Davidson (Florida State University).

to the degradation of the PAFP-tagged protein. Time-lapse imaging of the activated signal allows for quantification of degradation process in real-time at the single cell level [Zhang et al. 2007].

Superresolution imaging: Photoactivated localization microscopy (PALM) using PAFPs allows imaging of intracellular proteins at nanometer spatial resolution. Two-color PALM utilizing spectrally distinct PAFPs fused to the proteins of interest is used for determination of ultrastructural relationship between these proteins. It has been demonstrated that cyan-to-green photoactivatable PS-CFP2 is perfectly suitable for such application [Shroff et al. 2007].

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Zhang et al. (2007). Biotechniques, 42 (4): 446–450 / pmid: 17489230

Cyan-to-green photoswitchable fluorescent protein PS-CFP2

- Monomer, succesful perfomance in fusions
- Irreversible photoconversion from a cyan to a green fluorescent form
- High contrast of photoconversion
- High pH stability allowing labeling of acidic organelles
- Recommended for tracking cell, organelle, and protein movement, monitoring the protein turnover and superresolution imaging

PS-CFP2 is an improved mutant of the photoswitchable monomeric fluorescent protein PS-CFP [Chudakov et al. 2004]. PS-CFP2 exhibits faster maturation and a brighter fluorescence both before and after photoswitching than its parental variant.

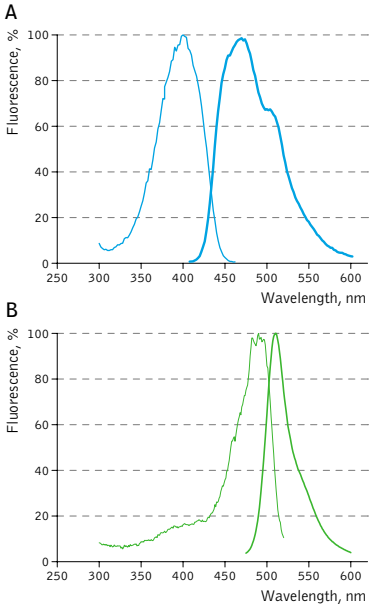
PS-CFP2 is capable of irreversible photoconversion from cyan to green fluorescent form in response to 405 nm light irradiation. It is recommended for real-time *in vivo* tracking movement of individual cells, organelles, and protein fractions [Chudakov et al. 2007]. It can also be applied for monitoring of the protein turnover at the single cell level [Zhang et al. 2007] and superresolution imaging by PALM [Shroff et al. 2007]. In addition, PS-CFP2 can be used as a routine cyan fluorescent tag (excitation maximum at 400 nm and emission maximum at 468 nm) at moderate excitation intensities and as a donor in FRET applications [Souslova and Chudakov 2006].

PS-CFP2 can be easily expressed and detected in a wide range of organisms. Mammalian cells transiently transfected with PS-CFP2 expression vectors display an evenly distributed cyan signal within 10-12 hrs after transfection. No cytotoxic effects are observed.

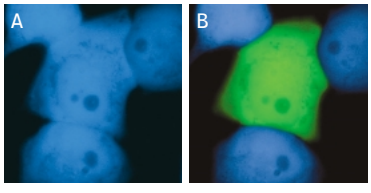
Main properties of PS-CFP2

Characteristic	before / after photoactivation
Fluorescence color	cyan / green
Excitation maximum, nm	400 / 490
Emission maximum, nm	468 / 511
Quantum yield	0.2 / 0.23
Extinction coefficient, $M^{-1}cm^{-1}$	43 000 / 47 000
Brightness*	8.6 / 10.8
pKa	4.3 / 6.1
Activating light	UV-violet (e.g. 405 nm)
Photoswitching	irreversible
Calculated contrast, fold	up to 2000
Structure	monomer
Cell toxicity	not observed
Aggregation	no
Maturation rate at 37°C	fast
Molecular weight, kDa	27
Polypeptide length, aa	238

*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



PS-CFP2 normalized excitation (thin line) and emission (thick line) spectra.
(A) before activation; (B) after activation. Complete PS-CFP2 spectra in Excel format can be downloaded from the Evrogen Web site at www.evrogen.com / support / FP-tech-pa.shtml



PS-CFP2 photoconversion in transiently transfected mammalian cells.
Central cell expressing PS-CFP2 was irradiated by intense 405 nm light that resulted in PS-CFP2 photoswitching. Before photoswitching no detectable green fluorescence at FITC excitation was seen in cells expressing PS-CFP2. In contrast, high-level signal was observed in cyan channel. Upon irradiation with a 10-15 micro Joules (about 20-30 W/cm^2) violet dye laser (405 nm) for a few seconds a fluorescence increase of more than 300-fold was observed in FITC channel. (A) before photoconversion; (B) after photoconversion.

PS-CFP2 successful performance has been proven in many fusions including that with cytoplasmic β-actin, BH3 interacting domain death agonist (BID), nucleolar protein fibrillarin, and dopamine transporter (hDAT).

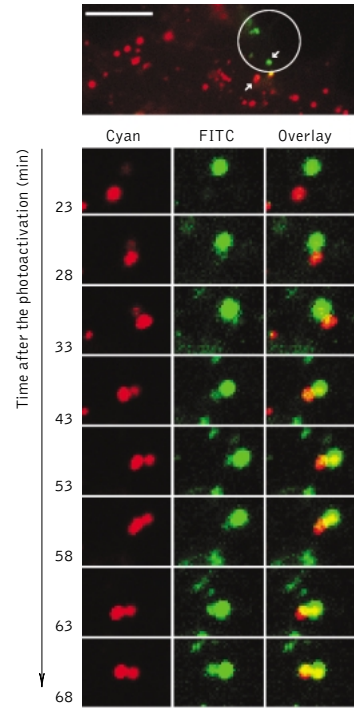
High pH stability: Before photoactivation, PS-CFP2 exhibits a high pH stability with a pKa of 4.3. No changes were observed either in the shape or in the amplitude of fluorescence spectra within a pH range of 5.0 and 9.0. This makes it possible to target PS-CFP2 to acidic organelles such as endosomes and lysosomes. After photoswitching, PS-CFP2 has a pKa of 6.1, similar to that of other GFP-like proteins with a phenolate anion chromophore (e.g. EGFP).

High contrast of photoconversion: In response to intense 400 nm light irradiation, PS-CFP2 undergoes irreversible photoconversion expressed in a decrease in cyan fluorescence and appearance of a 490 nm excitation peak with emission maximum at 511 nm. After complete photoconversion, green fluorescence of PS-CFP2 increases more than 400 times, whereas the level of cyan fluorescence drops more than 5.5 times lower. Thus, the increase in the green-to-cyan fluorescence ratio accounts for more than a 2000-fold contrast. Considerable decrease of cyan fluorescence during PS-CFP2 photoconversion provides a molecular tool to simultaneously track both the movement of the photoactivated protein and its replacement with the non-activated form.

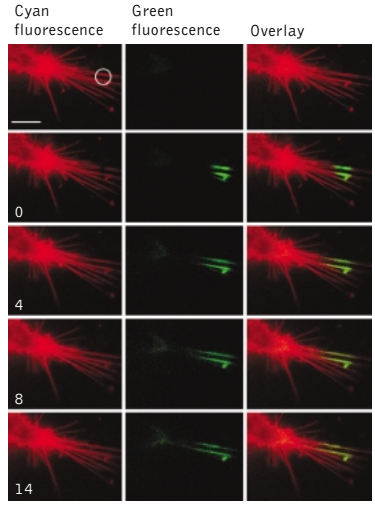
Suitability for tracking protein traffic has been demonstrated on example of PS-CFP (the parental variant of PS-CFP2) fused with the human dopamine transporter, hDAT [Chudakov et al. 2004]. PS-CFP-tagged hDAT was expressed in HEK293 cells. As expected, the fusion protein was localized in the cellular membranes. Then PS-CFP-hDAT was selectively photoswitched in the middle parts of two filopodia by short pulse of 404 nm laser irradiation. High contrast of photoconversion allowed monitoring hDAT movement precisely within thin filopodia in the vicinity of a big non-switched PS-CFP-hDAT pool at the filopodia base. At the same time, a decrease in the cyan fluorescence during photoswitching allowed monitoring non-switched PS-CFP-hDAT molecules entering the activated region.

When expressed heterologously, hDAT is capable of endocytosis. To test whether early endosomes are able to exchange cargo proteins such as hDAT, PS-CFP-hDAT fusion was selectively photoswitched in several endosomes. Then the endosomes (both photolabeled and intact) were monitored within the whole cell for more than an hour. They exhibited fast and rather chaotic intracellular movement. Eventually two endosomes drew together to form a doublet. One of them contained photoswitched PS-CFP-hDAT and soon after their contact PS-CFP-hDAT mutual exchange between the endosomes occurred: cyan fluorescence of the activated endosome recovered while green fluorescence of the non-switched endosome grew.

PS-CFP2 use in superresolution imaging: The performance of PS-CFP2 in photoactivated localization microscopy (PALM) was demonstrated in [Shroff et al. 2007]. Using PS-CFP2 as a second label in two-color PALM it was shown that paxillin and zyxin proteins, which seem co-localized when viewed by conventional TIRF, in fact form separate nanoscale clusters.



PS-CFP-hDAT interchange between two endosomes. Signals in cyan and FITC channels are shown in red and green pseudocolors, respectively. Circle outlines the photoswitched region. Arrows point to the endosomes tracked. Scale bar, 10 μm.



Tracking of PS-CFP-hDAT fusion within filopodia of HEK293 cells. Signals in cyan and FITC channels are shown in red and green pseudocolors, respectively. Circle outlines the photoswitched region. Scale bar, 10 μm. Time after the photoactivation (min) is indicated on left.

Recommended antibodies, filter sets and laser lines

PS-CFP2 can be recognized using Anti-Tag(CGY)FP antibody (Cat.# AB121-AB122) available from Evrogen.

Visualization before photoswitching: Before activation, PS-CFP2 produces cyan fluorescence with excitation and emission maxima at 400 and 468 nm, respectively. Standard levels of excitation do not cause significant photoswitching of cyan or photobleaching of green fluorescence.

PS-CFP2 excitation spectrum is absolutely different from that of common cyan fluorescent proteins, such as TagCFP, ECFP or Cerulean. Therefore, common CFP filter sets are not optimal for PS-CFP2 visualization and photoactivation.

Recommended filter sets are: XF119-2*, XF131, XF06, XF03, XF11, XF129-2, XF05-2 (Omega Optical); DAPI-5060B* and DAPI-1160A (Semrock); 31037, 31041, 31016, 31021, 31000v2, 1009v2, 31013v2, 11005v2, 31047 (Chroma Technology Corp.).
* - preferred filter sets

Photoswitching: PS-CFP2 undergoes irreversible photoconversion (in response to intense 405 nm light irradiation) expressed in a decrease in cyan fluorescence and appearance of a 490 nm excitation peak with emission maximum at 511 nm.

Visualization after photoswitching: Standard GFP filter sets (e.g. Omega Optical XF100-2) can be used for visualization of green fluorescence of photoactivated PS-CFP2. Importantly, the filter should not pass excitation light with wavelengths shorter than 450 nm to avoid cross-excitation of non-photoactivated PS-CFP2.

The detailed protocol is published in Chudakov et al. 2007.

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Chudakov et al. (2007). Nat Protoc, 2 (8): 2024–2032 / pmid: 17703215

Shroff et al. (2007). Proc Natl Acad Sci USA, 104 (51): 20308–20313 / pmid: 18077327

Souslova and Chudakov (2006). Microsc Res Tech, 69 (3): 207–209 / pmid: 16538627

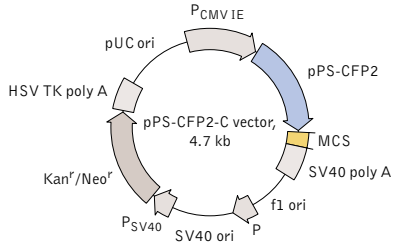
Zhang et al. (2007). Biotechniques, 42 (4): 446–450 / pmid: 17489230

PS-CFP2-related products

Product	Cat.#	Description	Size	Page(s)
PS-CFP2 expression/source vectors				
pPS-CFP2-C	FP801	Mammalian expression vector encoding humanized PS-CFP2 and allowing its expression and generation of fusions to the PS-CFP2 C-terminus	20 μg	64
pPS-CFP2-N	FP802	Mammalian expression vector encoding humanized PS-CFP2 and allowing its expression and generation of fusions to the PS-CFP2 N-terminus	20 μg	64
Antibodies against PS-CFP2				
Anti-Tag(CGY)FP	AB121 AB122	Rabbit polyclonal antibody against TagCFP, TagGFP, TagGFP2, TagYFP, PS-CFP2, and EGFP	100 μg 200 μg	101

Please contact your local distributor for exact prices and delivery information.

pPS-CFP2-C vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter(s)	PS-CFP2
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	Reporter expression in mammalian cells; generation of fusions to the reporter C-terminus

Product	Cat.#	Reporter	Color	Size
pPS-CFP2-C	FP801	PS-CFP2	cyan-to-green	20μg

Please contact your local distributor for exact prices and delivery information.

pPS-CFP2-C vector MCS

PS-CFP2 → BspE I | Xho I* | Hind III | Pst I | Kpn I | Apa I | BamH I | STOPs

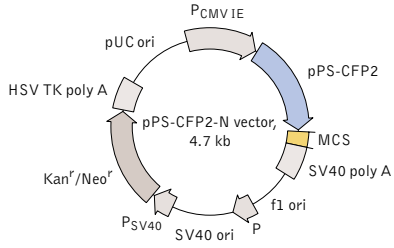
. . . TCC.GGA.CTC.AGA.TCT.CGA.GCT.CAA.GCT.TCG.AAT.TCT.GCA.GTC.GAC.GGT.ACC.GCG.GGC.CCG.GGA.TCC.ACC.GGA.TCT.AGA.TAA.CTG.ATC.A . . .

Bgl II | Sac I | EcoR I | Sal I | Sac II | Sma I/Xma I | Xba I# | Bcl I#

* — not unique sites.

\# — sites are blocked by *dam* methylation. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

pPS-CFP2-N vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter(s)	PS-CFP2
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	Reporter expression in mammalian cells; generation of fusions to the reporter N-terminus

Product	Cat.#	Reporter	Color	Size
pPS-CFP2-N	FP802	PS-CFP2	cyan-to-green	20μg

Please contact your local distributor for exact prices and delivery information.

pPS-CFP2-N vector MCS

Afe I | Xho I* | Hind III | Pst I | Kpn I | Apa I | BamH I → PS-CFP2

. . . GCT.AGC.GCT.ACC.GGA.CTC.AGA.TCT.CGA.GCT.CAA.GCT.TCG.AAT.TCT.GCA.GTC.GAC.GGT.ACC.GCG.GGC.CCG.GGA.TCC.ACC.GGT.CGC.CAC.CAT.GA . . .

Nhe I | Bgl II | Sac I | EcoR I | Sal I | Sac II | Sma I/Xma I | Age I

* — not unique sites.

Notice to Purchaser:

Photoactivatable FP-related products are intended to be used by academic (non-commercial) entities and for research purposes only. Any use of the proprietary nucleic acid or protein other than for research use or by a commercial entity is strictly prohibited. Transfer of this product by purchaser to any other party is specifically prohibited.

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839, and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

MATERIAL SAFETY DATA SHEET INFORMATION: To the best of our knowledge, these products do not require a Material Safety Data Sheet. However, all the properties of these products (and, if applicable, each of their components) have not been thoroughly investigated. Therefore, we recommend that you use gloves and eye protection, and wear a laboratory coat when working with these products.

Kindling red fluorescent protein KFP-Red

- Reversible or irreversible photoactivation
- Activated by green light that does not damage cells and tissues
- Quenching by blue light
- Recommended for tracking cells and cellular organelle movements

KFP-Red (also referred to as KFP1) is a photoactivatable GFP-like protein generated on the basis of *Anemonia sulcata* chromoprotein, asFP595 [Lukyanov et al. 2000; [Chudakov et al. 2003*a*; Chudakov et al. 2003*b*]. KFP-Red switches from a non-fluorescent to a red fluorescent form (with excitation/emission maxima at 580 nm and 600 nm, respectively) under the exposure to intense green light irradiation.

KFP-Red can be used for *in vivo* monitoring cell and cellular organelle movement. It was successfully expressed and tested in various experimental models, including bacteria, *Xenopus* embryo, and cultured mammalian cells.

Like other Anthozoa GFP-like proteins, KFP-Red is a tetramer. This restricts the wide use of KFP-Red as a fusion partner for cellular proteins.

Reversible or irreversible kindling: Depending on the kindling light intensity KFP-Red can be photoactivated reversibly or irreversibly allowing the monitoring of both short- and long-term cell processes.

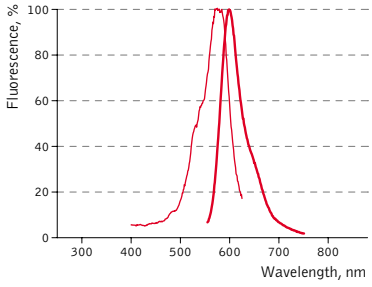
A reversibly kindled KFP-Red relaxes to the initial non-fluorescent form ($t_{1/2}$ = 50 sec.), or can be quenched instantly by blue light (430-490 nm). Reversible kindling results in about 70 times increase of the red fluorescence intensity comparing to unkindled protein.

Reversible kindling and quenching can be repeated many times.

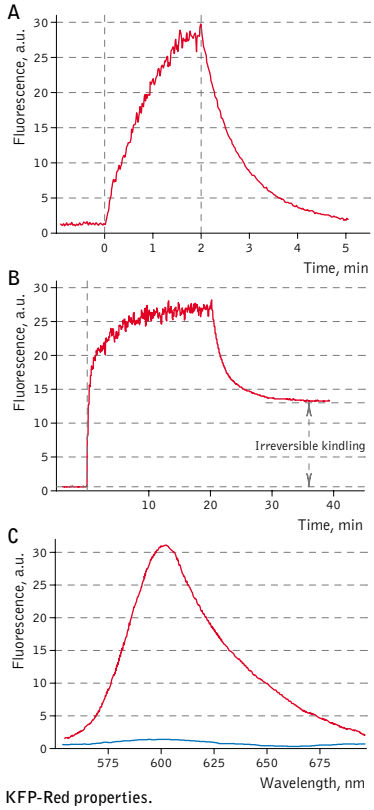
Main properties of KFP-Red

Characteristic	before / after photoactivation
Fluorescence color	No / red
Excitation maximum, nm	580 / 580
Emission maximum, nm	600 / 600
Quantum yield	<0.001 / 0.07
Extinction coefficient, $M^{-1}cm^{-1}$	123 000 / 59 000
Brightness*	0 / 4.1
Activating light	green (530-560 nm)
Photoswitching	reversible
Calculated contrast, fold	35-70
Structure	tetramer
Cell toxicity	not observed
Aggregation	no
Maturation rate at 37°C	medium
Molecular weight, kDa	26
Polypeptide length, aa	238
Possible limitations	Limited applicability for fusions generation

*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



KFP-Red normalized excitation (thin line) and emission (thick line) spectra.
Complete KFP-Red spectra in Excel format can be downloaded from the Evrogen Web site at www.evrogen.com / support / FP-tech-pa.shtml



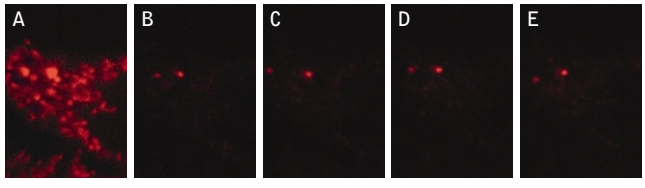
KFP-Red properties.
(A) KFP-Red reversible kindling and relaxation kinetics. Zero time is set at the commencement of irradiation with kindling light (532 nm laser light, 1% power). Kindling irradiation was stopped after 2 minutes. (B) KFP-Red irreversible kindling. Zero time is set at the commencement of irradiation with kindling light (532 nm laser light, 20% power). Kindling irradiation was stopped after 20 min. (C) Irreversibly kindled (red line) and "unkindled" (blue line) KFP-Red fluorescence spectra and brightness ratio.

An irreversibly kindled KFP-Red gives stable red fluorescence which is at least 35 times brighter than that of the protein before kindling. An irreversibly kindled KFP-Red remains stable and brightly fluorescent for more than 72 hrs in living cells and for at least a year in protein samples.

An irreversibly kindled KFP-Red can be partially quenched by blue light, but then it restores its brightness within several minutes. Therefore, in some applications, blue light can be used to quench a reversibly kindled KFP-Red, whereas an irreversibly kindled KFP-Red remains fluorescent.

Application of KFP-Red to track cell migration was demonstrated using embryonic fate mapping as an example. Xenopus embryos were taken at the stage of two blastomeres and KFP-Red mRNA was microinjected into the animal poles of both blastomeres. At the early neurula stage, a round-shaped group of cells within the neural plate was kindled irreversibly. Irradiated cells became brightly fluorescent and their migration in the developing embryo was monitored. Longitudinal extension accompanied by transversal convergence of the labeled group of cells was visible after the first two hours after kindling. At the end of neurulation, the labeled spot appeared as a thin stripe on the surface of the left neural fold.

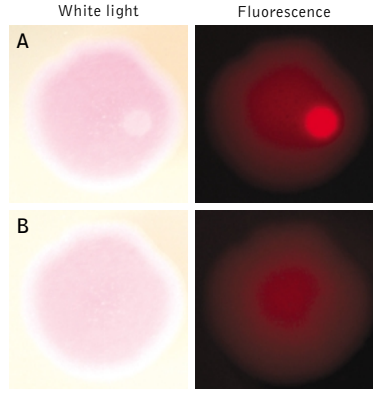
KFP-Red suitability for tracking movement of cell organelles was demonstrated on PC12 cells transfected with a mitochondria-targeted KFP-Red expressing vector. After 25 hours of incubation, mitochondria remained non-fluorescent (no kindling observed) upon irradiation using a 1% power scanning green laser (HeNe laser line 543 nm, 1 mW, once per 10 seconds; the number of scans is not limited). After several scans with a 5-10% power laser, mitochondria became brightly fluorescent and were observed using a 1% power laser for several minutes. Brief irradiation (about 20 seconds in fast mode) with a 30% power green laser light induced irreversible kindling of KFP-Red in mitochondria within the irradiated field. Irreversibly kindled mitochondria were monitored.



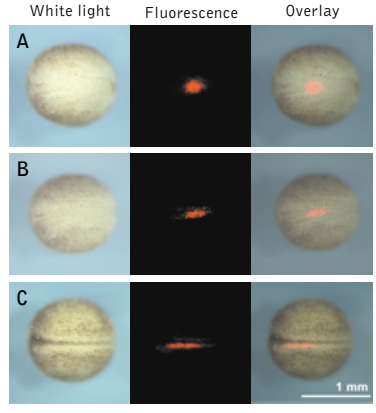
Monitoring of mitochondrial movement using KFP-Red in PC12 cells.
(A) Reversibly and irreversibly kindled mitochondria. Irradiation with weak blue laser light caused instantaneous quenching of reversibly kindled mitochondria, while the irreversibly kindled mitochondria (compare A and B) remained fluorescent; (B-E) Irreversibly kindled mitochondria tracking using a 1% power green laser.

Recommended filter sets and laser lines

KFP-Red is non-fluorescent before light activation. Upon green-light irradiation, the protein kindles to its red fluorescent form. Green light of low intensity (e.g. 1% power scanning green laser, HeNe laser line 543 nm, 1 mW, scan per 10 seconds; the number of scans is not limited) does not cause kindling and may be used as excitation light for KFP-Red visualization.



Reversible photoactivation of KFP-Red in *E. coli*.
The round-shaped part of the *E. coli*. colony expressing KFP-Red was irreversibly kindled with intense green light. This region fluoresces brightly, while its absorption is low. After several minutes, the kindled protein relaxed to the non-fluorescent state, while its absorption recovered. (A) immediately after kindling; (B) five minutes later.



Monitoring of cell migration during *Xenopus* neural plate development using KFP-Red.
(A) At the early neurula stage, a round-shaped group of cells within the neural plate was irreversibly "kindled"; (B) longitudinal extension of the labeled group of cells after two hours after kindling; (C) thin stripe of the labeled cells at the end of neurulation.
Experimental data were presented by Dr. A. Zaraisky (Institute of Bioorganic Chemistry RAS, Moscow, Russia).

Scanning with about 5-10% power laser results in reversible kindling of KFP-Red. More intensive-light irradiation is required for irreversible KFP-Red kindling (e.g. irradiation for 20 seconds in fast mode with a 30% power green laser light induces irreversible kindling of KFP-Red in mitochondria within the irradiated field). Irradiation with weak blue laser light causes instantaneous quenching of reversibly kindled KFP-Red, whereas for the irreversibly kindled KFP-Red, quenching is not so pronounced.

TRITC filter set or similar can be used for visualization of activated KFP-Red. Omega Optical filter sets QMAX-Red and XF174 are recommended.

Kindling effect depends on temperature. Light intensity required for kindling goes down when the temperature decreases and goes up when the temperature rises. This property can be used to achieve kindling at lower light intensities by sample cooling.

REFERENCES

Chudakov et al. (2003*a*). J Biol Chem, 278 (9): 7215–7219 / pmid: 12496281

Chudakov et al. (2003*b*). Nat Biotechnol, 21 (2): 191–194 / pmid: 12524551

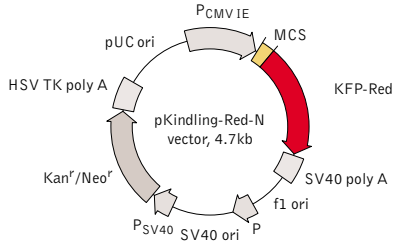
Lukyanov et al. (2000). J Biol Chem, 275 (34): 25879–25882 / pmid: 10852900

KFP-Red-related products

Product	Cat.#	Description	Size	Page(s)
KFP-Red expression/source vectors				
pKindling-Red-N	FP301	Mammalian expression vector encoding humanized KFP-Red and allowing its expression and generation of fusions to the KFP-Red N-terminus	20 μg	68
pKindling-Red-B	FP302	Bacterial expression vector; source of the KFP-Red coding sequence	20 μg	68
pKindling-Red-mito	FP401	Mammalian expression vector encoding humanized KFP-Red targeted to mitochondria	20 μg	69
Vector sets				
Mito-tracker vector set	FPM01	Mammalian expression vectors for fluorescent labeling of mitochondria: pTurboGFP-mito (FP517), pPhi-Yellow-mito(FP607), and pKindling-Red-mito(FP517)	20 μg each	47,69
Recombinant protein				
rKFP-Red	FP351	Purified recombinant kindling red fluorescent protein	100 μg	69

Please contact your local distributor for exact prices and delivery information.

pKindling-Red-N vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter(s)	KFP-Red
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	Reporter expression in mammalian cells; generation of fusions to the reporter N-terminus

Product	Cat.#	Reporter	Color	Size
pKindling-Red-N	FP301	KFP-Red	no-to-red	20μg

Please contact your local distributor for exact prices and delivery information.

Multiple cloning site (MCS)

Nhe I / Afe I — Bgl II / Xho I — Sac I / Hind III — EcoR I / Pst I — Sal I / Kpn I — Sac II / Apa I — Sma I/Xma I / BamH I — Age I — KFP-Red / Nco I*

```
... GCT.AGC.GCT.ACC.GGA.CTC.AGA.TCT.CGA.GCT.CAA.GCT.TCG.AAT.TCT.GCA.GTC.GAC.GGT.ACC.GCG.GGC.CCG.GGA.TCC.ACC.GGT.CGC.CAC.CAT.GG...
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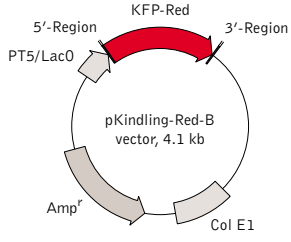
* — not unique sites.

pKindling-Red-B vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	bacterial expression vector
Reporter(s)	KFP-Red
Reporter codon usage	mammalian
Promoter	T5 promoter/lac operator
Host cells	prokaryotic
Selection	ampicillin
Replication	ColE1 ori
Use	Source of the reporter coding sequence; reporter expression in bacterial cells

Product	Cat.#	Reporter	Color	Size
pKindling-Red-B	FP302	KFP-Red	no-to-red	20μg

Please contact your local distributor for exact prices and delivery information.

5' Region

BamH I / Nco I* — KFP-Red

```
RBS ATG.AGA.GGA.TCG.GGA.TCC.ATG.G...
```

3' Region

STOP / Hind III

```
... TGA.AGC.TT...
```

* — not unique sites.

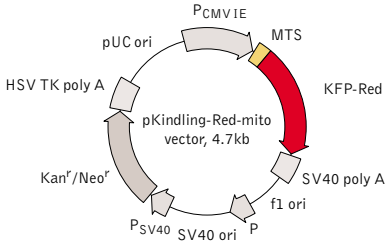
Notice to Purchaser:

Photoactivatable FP-related products are intended to be used by academic (non-commercial) entities and for research purposes only. Any use of the proprietary nucleic acid or protein other than for research use or by a commercial entity is strictly prohibited. Transfer of this product by purchaser to any other party is specifically prohibited.

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839, and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

MATERIAL SAFETY DATA SHEET INFORMATION: To the best of our knowledge, these products do not require a Material Safety Data Sheet. However, all the properties of these products (and, if applicable, each of their components) have not been thoroughly investigated. Therefore, we recommend that you use gloves and eye protection, and wear a laboratory coat when working with these products.

pKindling-Red-mito vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

MTS - Mitochondrial targeting sequence

Vector type	mammalian expression vector
Reporter(s)	KFP-Red
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	fluorescent labeling of mitochondria

Product	Cat.#	Reporter	Color	Size
pKindling-Red-mito	FP401	KFP-Red	no-to-red	20μg

Please contact your local distributor for exact prices and delivery information.

rKFP-Red recombinant protein

Product	Cat.#	Reporter	Color	Size
rKFP-Red	FP351	KFP-Red	no-to-red	100μg

Please contact your local distributor for exact prices and delivery information.

Use

- Microinjection into cells and tissues
- Control for fluorescence microscopy
- Calibration of fluorimeters and FACS machines
- Standard on protein gels and Western blots

Description

Recombinant KFP-Red (rKFP-Red) is a 26-kDa photoactivatable colored non-fluorescent protein. It has spectral properties identical to those of the expressed KFP-Red.

rKFP-Red can be kindled by green light. Irreversibly kindled purified rKFP-Red retains red fluorescence for many months and can be used as a standard on protein gels and Western blots; control for fluorescence microscopy and for calibration of fluorometeres and FACS machines. Moreover, rKFP-Red may be microinjected into cells and tissues of interest, kindled, and used as a marker of these particular objects.

rKFP-Red is purified from transformed *E. coli* using acetone precipitation.

Storage: at +4°C in the dark place (before photoactivation).

Fluorescent biosensors

Sensor	Intended use	Detection	Advantages	Page
HyPer	Monitoring changes in intracellular H_2O_2 concentration	Rise in H_2O_2 concentration leads to decrease in reporter excitation peak at 420 nm and proportional increase in excitation peak at 500 nm	Ratiometric measurement; High specificity and sensitivity; Capability of targeting the sensor to the specific cell compartments	71
Case12	Monitoring changes in intracellular Ca^{2+} concentration	Rise in Ca^{2+} concentration leads to significant increase of the reporter brightness	High dynamic range; Relatively high pH stability; Capability of targeting the sensor to the specific cell compartments	76
Casper3-BG	Monitoring caspase-3 activity in living cells; Early detection of apoptosis	Activation of caspase-3 leads to elimination of FRET between TagBFP and TagGFP2, resulting in the decrease of green and increase of blue fluorescence	High sensitivity	81
Casper3-GR	Monitoring caspase-3 activity in living cells; Early detection of apoptosis	Activation of caspase-3 leads to elimination of FRET between TagGFP and TagRFP, resulting in the decrease of red and increase of green fluorescence	High sensitivity; Proven suitability for FLIM-based screenings	84

Hydrogen peroxide sensor HyPer

- Ratiometric detection of intracellular H_2O_2 level changes
- High selectivity and sensitivity, no artifactual ROS generation
- Direct expression in cells, easy targeting to various subcellular compartments
- No exogenous chemical compounds required
- Recommended for monitoring H_2O_2 production inside living cells

Reactive oxygen species (ROS) are tightly involved in normal cell functions as well as in development of a wide variety of pathologies. Commonly used for ROS detection, dichlorofluorescein (DCF) derivatives have several serious disadvantages: they are not specific (i.e. they are sensitive to multiple types of ROS); they cannot be targeted to specific intracellular compartments; and, most importantly, they can produce ROS upon light exposure, which results in artifactual ROS generation and signal amplification.

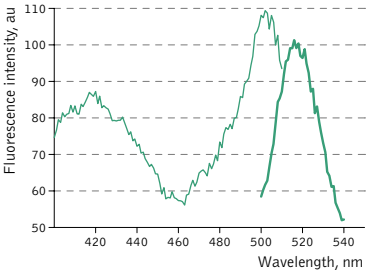
HyPer is the first fully genetically encoded fluorescent sensor capable of detecting intracellular hydrogen peroxide (H_2O_2), one of the main ROS generated by cells [Belousov et al. 2006]. Developed on the basis of yellow fluorescent protein inserted into the regulatory domain of *E. coli* protein OxyR (OxyR-RD) [Choi et al. 2001], HyPer demonstrates submicromolar affinity to hydrogen peroxide and is insensitive to other oxidants tested, such as superoxide, oxidized glutathione, nitric oxide, and peroxinitrite. HyPer does not cause artifactual ROS generation and can be used for detection of fast changes of H_2O_2 concentration in different cell compartments under various physiological and pathological conditions.

Without H_2O_2 HyPer has two excitation peaks with maxima at 420 nm and 500 nm, and one emission peak with maximum at 516 nm. Upon exposure to H_2O_2, the excitation peak at 420 nm decreases proportionally to the increase in the peak at 500 nm, allowing ratiometric measurement of H_2O_2. Similarly to wild-type OxyR, oxidized HyPer can be reduced inside cells.

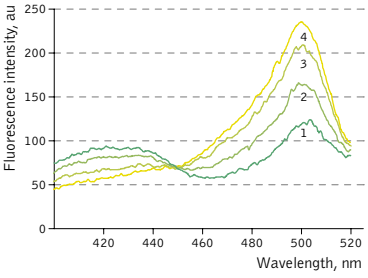
Main properties of HyPer

Characteristic	
Emission maximum, nm	516
Excitation maximum, nm	420 and 500
Fluorescence color	green
Polypeptide length, aa	478
Molecular weight, kDa	52
Specificity	H_2O_2
Sensitivity	submicromolar H_2O_2 concentrations
pKa	8.5
Structure	monomer
Aggregation	no
Maturation rate at 37°C	fast

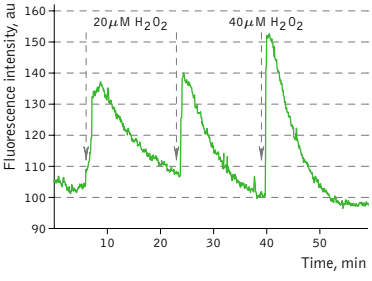
*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



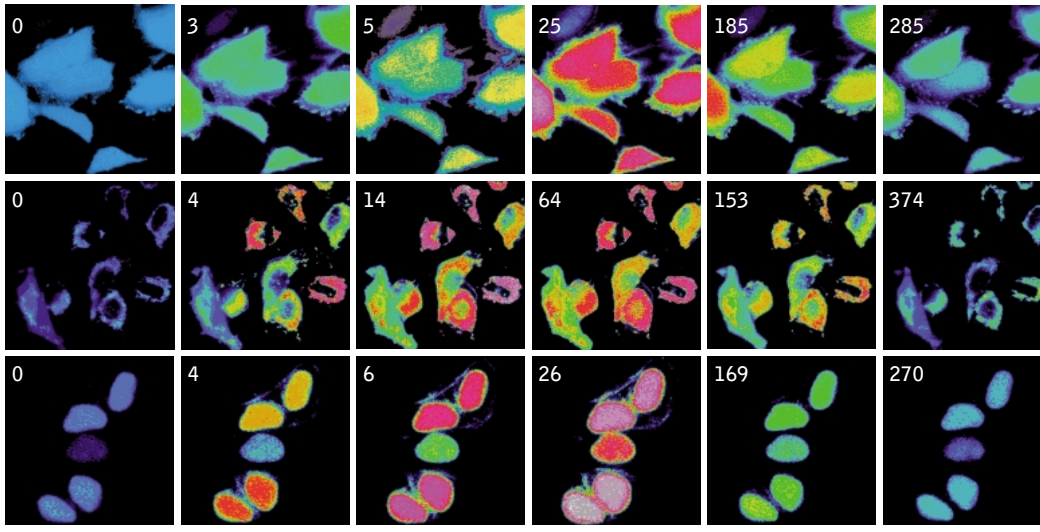
HyPer normalized excitation (thin line) and emission (thick line) spectra.
Complete HyPer spectra in Excel format can be downloaded from the Evrogen Web site at www.evrogen.com/support/FP-tech-biosensors.shtml



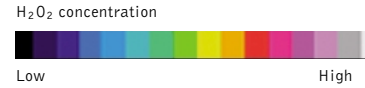
Changes in the excitation spectrum of isolated HyPer in response to H_2O_2 addition.
Changes in the excitation spectrum of isolated HyPer in response to H_2O_2 addition. Trace 1 - without H_2O_2; trace 2 - 25 nM H_2O_2; trace 3 - 100 nM H_2O_2; trace 4 - 250 nM H_2O_2. Emission was measured at 530 nm.



Kinetics of fluorescence (excitation at 490 nm, emission at 530 nm) of HyPer in *E. coli* cell suspension in the presence of 50 U/ml catalase in response to three successive additions of hydrogen peroxide.



Monotoring of cell response of mammalian cells to hydrogen peroxide addition using HyPer directed to various cellular compartments. HeLa cells expressing HyPer localized in cytoplasm (top line), mitochondria (medium line) and nucleus (bottom line) were plated to glass bottom dishes and challenged with 180 μM H_2O_2. Images were acquired by Leica AF 6000 LX with 0.5 Hz frequency by sequential illumination of cells via CFP/YFP (excitation/emission) and YFP/YFP filters. Resulting images were obtained by dividing of YFP/YFP images to CFP/YFP images followed by pseudocoloring. Time after H_2O_2 addition (sec) is indicated at the top of each image.



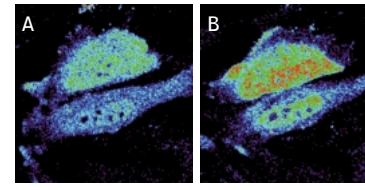
Violet and blue excitation light should be applied for monitoring HyPer green emission changes caused by intracellular H_2O_2 production. Excitation light intensity must be individually determined for a particular biological system and instrumentation used.

HyPer can be directly expressed by target cells individually or in fusion with a specific localization signal. It successfully folds and remains highly sensitive to hydrogen peroxide both in bacteria and in mammalian cells. HyPer suitability to generate stably transfected cells has been proven by Marinpharm company. Various cell lines expressing HyPer are commercially available.

HyPer effectiveness has been demonstrated on various models including detection of low concentrations of H_2O_2 generated upon physiological stimulation of mammalian cells by growth factors [Belousov et al. 2006, Markvicheva et al. 2009], monitoring of H_2O_2 production during Apo2L/TRAIL-induced apoptosis [Belousov et al. 2006], demonstration of hydrogen peroxide mediated insulin-induced calcium increase in skeletal muscle cells [Espinosa et al. 2009], and visualization of a tissue-scale gradient of hydrogen peroxide mediating rapid wound detection in zebrafish [Niethammer et al. 2009].

Recommended filter sets

Recommended Omega Optical filter sets for HyPer are QMAX-Green, XF100-2, and XF100-3. It can also be detected using Chroma Technology Corp. filter set 41001 FITC/ RSGFP/ Bodipy/ Fluo 3/ DiO or the similar.



Imaging of H_2O_2 induction in HeLa cells stimulated with EGF.
Pseudocolored images of HeLa cells expressing HyPer in cytoplasm at the time point of (A) and 2 h after (B) EGF addition.

REFERENCES

Belousov et al. (2006). Nat Methods, 3 (4): 281–286 / pmid: 16554833

Choi et al. (2001). Cell, 105 (1): 103–113 / pmid: 11301006

Espinosa et al. (2009). J Biol Chem, 284 (4): 2568–2575 / pmid: 19028699

Markvicheva et al. (2009). Methods Mol Biol., 476: 76–83 / pmid: 19253046

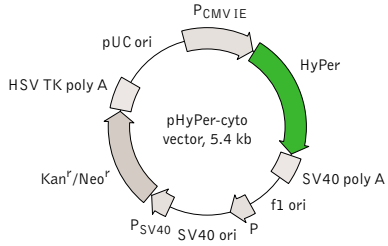
Niethammer et al. (2009). Nature, : doi:10.1038 / nature08119 / pmid: 19494811

HyPer-related products

Product	Cat.#	Description	Size	Page(s)
HyPer expression/source vectors				
pHyPer-cyto	FP941	Mammalian expression vector allowing HyPer expression in cytosol under the control of CMV promoter	20 µg	74
pHyPer-dMito	FP942	Mammalian expression vector encoding mitochondria-targeted HyPer	20 µg	74
pHyPer-nuc	FP944	Mammalian expression vector encoding nuclear-targeted HyPer	20 µg	75
Gateway® HyPer-AS	FP943	Gateway® entry clone for transfer of HyPer into Gateway® destination vectors; HyPer codon usage is optimized for expression in *Arabidopsis* and *Saccharomyces*	20 µg	75
Vector sets				
HyPer	FPS01	pHyPer-cyto and pHyPer-dMito vectors encoding cytosolic and mitochondria-targeted forms of HyPer	20 µg earch	74

Please contact your local distributor for exact prices and delivery information.

pHyPer-cyto vector



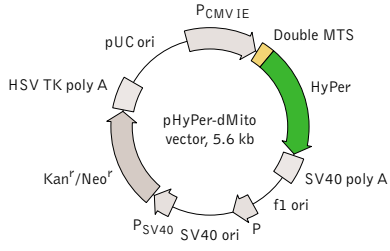
For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter	HyPer
Reporter codon usage	mammalian / *E. coli*
Promoter for HyPer	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	HyPer expression in mammalian cells under the control of CMV promoter; source of HyPer coding sequence

Product	Cat.#	Reporter	Color	Size
pHyPer-cyto vector	FP941	HyPer	green	20 μg

Please contact your local distributor for exact prices and delivery information.

pHyPer-dMito vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

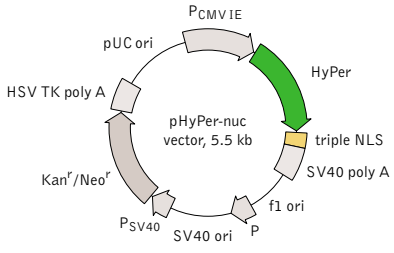
MTS - Mitochondrial targeting sequence

Vector type	mammalian expression vector
Reporter	HyPer
Reporter codon usage	mammalian / *E. coli*
Promoter for HyPer	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of mitochondria-targeted HyPer in mammalian cells under the control of CMV promoter; source of mitochondria-targeted HyPer coding sequence

Product	Cat.#	Reporter	Color	Size
pHyPer-dMito vector	FP942	HyPer	green	20 μg

Please contact your local distributor for exact prices and delivery information.

pHyPer-nuc vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

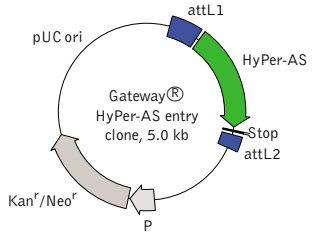
NLS - nuclear localization signal

Vector type	mammalian expression vector
Reporter	HyPer
Reporter codon usage	mammalian / *E. coli*
Promoter for HyPer	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of nuclear-targeted fluorescent hydrogen peroxide sensor HyPer in mammalian cells under the control of CMV promoter; source of nuclear-targeted HyPer coding sequence

Product	Cat.#	Reporter	Color	Size
pHyPer-nuc vector	FP944	HyPer	green	20 μg

Please contact your local distributor for exact prices and delivery information.

Gateway® HyPer-AS entry clone



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	Gateway® entry clone
Reporter	HyPer
Reporter codon usage	*Arabidopsis* and *Saccharomyces*
Promoter for HyPer	No
Host cells	prokaryotic
Selection	kanamycin
Replication	pUC ori
Use	Transfer of HyPer codon variant optimized for expression in plants and yeast into Gateway® destination vectors

Product	Cat.#	Reporter	Color	Size
Gateway® HyPer-AS entry clone	FP943	HyPer	green	20 μg

Please contact your local distributor for exact prices and delivery information.

Calcium ion sensor Case12

- High dynamic range detection of intracellular Ca^{2+} level changes
- High selectivity and sensitivity, relatively high pH stability
- Fast maturation, high brightness of fluorescent response
- Direct expression in cells, easy targeting to various subcellular compartments
- No exogenous chemical compounds required
- Recommended for monitoring changes in Ca^{2+} concentration inside living cells

Case12 is a high dynamic range genetically encoded fluorescent sensor for direct measurement of changes of intracellular Ca^{2+} under various physiological and pathological conditions [Souslova et al. 2007]. The sensor is sensitive to changes of calcium concentration in a physiological range from a hundred nanomoles to micromoles with a high signal-to-noise ratio. Binding of Ca^{2+} is fast and reversible, allowing monitoring of high-frequency Ca^{2+} oscillations. In response to Ca^{2+} concentration rise, Case12 shows up to 12-fold increase of fluorescence brightness. Fluorescence of Case12 is characterized by single excitation/emission maxima peaked at 491/516 nm.

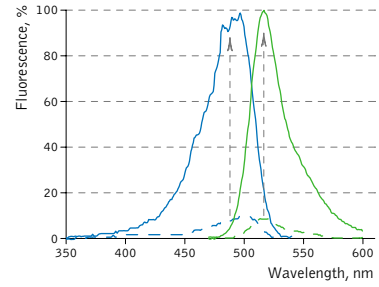
The common weak point of conventional calcium sensors is their low pH stability. For example, pKa (meaning of pH at which fluorescence brightness is 50% of maximum) for Pericams reaches as high as 8.0. Therefore, at physiological pH (7.2-7.5) such sensors exhibit low brightness and dynamic range [Nagai et al. 2001]. In contrast, the pKa of Case12 is 7.2 (in the presence of 10 μM Ca^{2+}) close to that reported for G-CaMP [Nakai et al. 2001]. This relatively high pH stability makes Case12 well suitable for *in vivo* use.

Case12 is characterized by fast maturation at 37°C. It can be directly expressed by target cells, both individually and in fusion with a specific localization signal. Case12 suitability to generate stably transfected cells has been proven by Marinpharm company. Various cell lines expressing Case12 are commercially available.

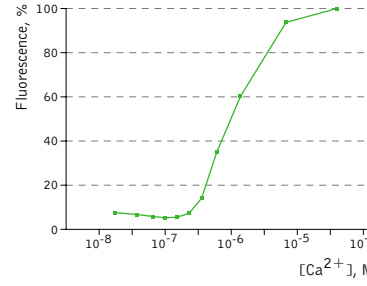
Main properties of Case12

Characteristic	
Emission maximum, nm	516
Excitation maximum, nm	491
Fluorescence color	green
Polypeptide length, aa	415
Molecular weight, kDa	46.4
Specificity	Ca^{2+}
Kd for Ca^{2+}	1 μM
pKa	7.2
Structure	monomer
Aggregation	no
Maturation rate at 37°C	fast

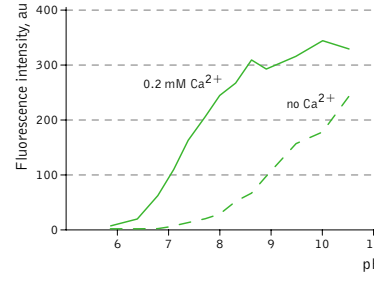
*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



Case12 normalized excitation (blue line) and emission (green line) spectra without Ca^{2+} (dashed lines) and in the presence of 1 mM of Ca2+ (solid lines)[Souslova et al. 2007].
Case12 shows multi-fold brightness increase of fluorescence in the response to 1 mM Ca^{2+}. Complete Case12 spectra in Excel format can be downloaded from the Evrogen Web site at www.evrogen.com/support/FP-tech-biosensors.shtml



Ca^{2+} titration curves [Souslova et al. 2007].
The apparent Kd for Ca^{2+} binding was found to be 1 μM, which lies within the physiological range of Ca^{2+} concentrations.



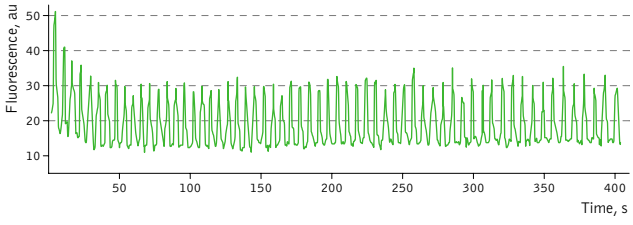
Dependence of Case12 fluorescence on pH in the presence (solid line) and in the absence (dashed line) of Ca^{2+} [Souslova et al. 2007].

Monitoring changes in green emission of Case12 in response to intracellular changes of Ca^{2+} concentration should be carried out by excitation by blue light (488 nm laser line or standard GFP filter set). Emission can be collected at approximately 500-540 nm. Intensity of excitation light should be individually determined for particular biological system and instrumentation. In general, we recommend that you minimize excitation light intensity and duration.

Note: Yellow fluorescent core of Case12 undergoes partial photoconversion to a dark state upon irradiation with blue light. It means that an apparent "bleaching" effect occurs at the beginning of time series imaging of cells expressing Case12 protein. Unlike the real bleaching, in the case of Case12, signal drops to the level of dynamic equilibrium between fluorescent and dark state of the chromophore, and then remains stable.

Maximum dynamic range in HeLa cells: HeLa cells transfected with Case12 show relatively weak green fluorescence. Addition of 20 μM calcium ionophore A23187, allowing calcium to enter cells (2 mM Ca^{2+} in the medium), resultes in 5-6-fold increase in green fluorescence brightness. Subsequent addition of 20 mM EGTA removes Ca^{2+} and decreases the fluorescence signal close to baseline level, with the final contrast of 11-12-fold.

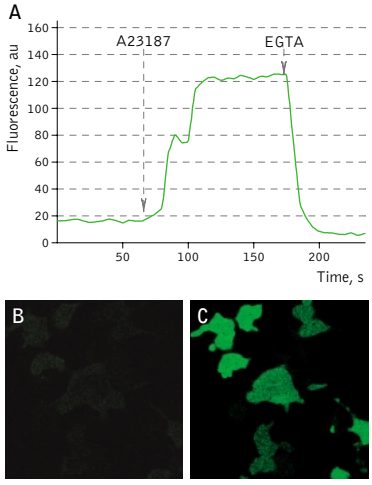
Monitoring of Ca^{2+} changes under physiological conditions: Mammalian cells expressing Case12 display a nice high dynamic range response upon addition of ATP at a final concentration of 100 μM. This experiment clearly showes that Case12 fluorescence response to Ca^{2+} oscillations is fast and reversible. It also demonstrates that the sensor responds to changes in Ca^{2+} concentration in living cells in the nanomolar range.



Fluorescence changes of human melanoma-derived M21 cells expressing Case12 in response to 100 μM ATP. Images were captured every 0.294 sec on the confocal microscope.

Compatibility with existing filter sets

We recommend standard GFP filter sets. Appropriate Omega Optical filter sets for Case12 are QMAX-Green, XF100-2 and XF100-3. It can also be detected using Chroma Technology Corp. filter sets 41001, 41017, 41020, 41025 or similar.



Testing Case12 in living cells [Souslova et al. 2007].
(A) Typical response of HeLa cells expressing Case12 to calcium ionophore A23187. (B, C) HeLa cells expressing Case12 shown before (B) and after (C) ionophore addition.
Leica microscope DM IRE2, confocal TCS-SP2, objective HCX-PL-APO-63x/1.40-0.60/OIL.

REFERENCES

Nagai et al. (2001). Proc Natl Acad Sci U S A, 98 (6): 3197–3202 / pmid: 11248055

Nakai et al. (2001). Nat Biotechnol, 19 (2): 137–141 / pmid: 11175727

Souslova et al. (2007). BMC Biotechnol, 7 (1): 7366–7375 / pmid: 17603870

Case12-related products

Product	Cat.#	Description	Size	Page(s)
Case12 expression/source vectors				
pCase12-Cyto	FP991	Mammalian expression vector allowing Case12 expression in cytosol under the control of CMV promoter	20 μg	79
pCase12-mito	FP992	Mammalian expression vector encoding mitochondria-targeted Case12	20 μg	80
pCase12-mem	FP993	Mammalian expression vector encoding Case12 targeted to cell membrane	20 μg	80

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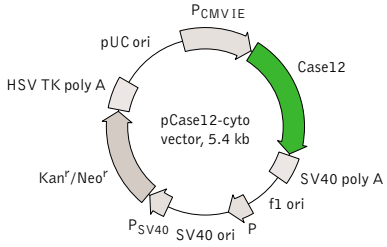
The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839, and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

pCase12-cyto vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter	Case12
Reporter codon usage	mammalian
Promoter for Case12	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of fluorescent Ca^{2+} sensor Case12 in mammalian cells under the control of CMV promoter; source of Case12 coding sequence

Product	Cat.#	Reporter	Color	Size
pCase12-cyto vector	FP991	Case12	green	20 μg

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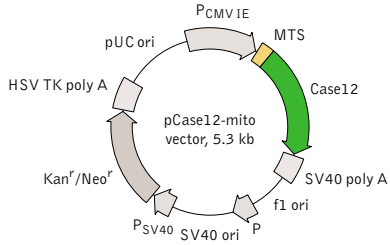
pCase12-mito vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

MTS - Mitochondrial targeting sequence

Vector type	mammalian expression vector
Reporter	Case12
Reporter codon usage	mammalian
Promoter for Case12	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of mitochondria-targeted fluorescent Ca^{2+} sensor Case12 in mammalian cells under the control of CMV promoter; source of mitochondria-targeted Case12 coding sequence

Product	Cat.#	Reporter	Color	Size
pCase12-mito vector	FP992	Case12	green	20 μg

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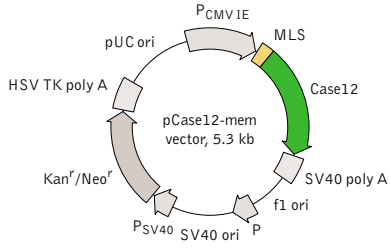
pCase12-mem vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

MLS - Membrane localization signal

Vector type	mammalian expression vector
Reporter	Case12
Reporter codon usage	mammalian
Promoter for Case12	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of membrane-targeted fluorescent Ca^{2+} sensor Case12 in mammalian cells under the control of CMV promoter; source of membrane-targeted Case12 coding sequence

Product	Cat.#	Reporter	Color	Size
pCase12-mem vector	FP993	Case12	green	20 μg

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MATERIAL SAFETY DATA SHEET INFORMATION: To the best of our knowledge, these products do not require a Material Safety Data Sheet. However, all the properties of these products (and, if applicable, each of their components) have not been thoroughly investigated. Therefore, we recommend that you use gloves and eye protection, and wear a laboratory coat when working with these products.

Caspase-3 apoptosis sensor Casper3-BG

- Early detection of Caspase-3 activity onset
- High sensitivity
- Direct expression in cells
- No exogenous chemical compounds required
- Recommended for early detection of apoptosis

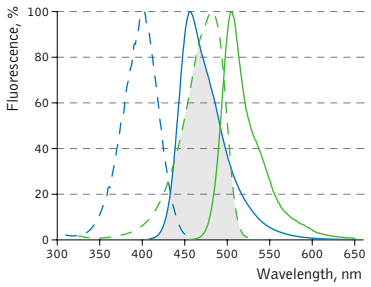
Casper3-BG is a FRET based sensor that can be used for detection of caspase-3 mediated apoptosis in living cells. The sensor consists of blue and green fluorescent proteins, TagBFP (see page 12) and TagGFP2 (see page 16), connected by the linker containing caspase-3 cleavage sequence DEVD. Good overlap between the emission spectrum of TagBFP and the absorbance spectra of TagGFP2 ensures efficient FRET between these proteins. The activation of caspase-3 during apoptosis leads to cleavage of DEVD sequence and elimination of FRET that can be detected as a decrease in green emission of TagGFP2 and a simultaneous increase in blue emission of TagBFP.

TagBFP / TagGFP2 pair is superior to other BFP / GFP pairs. The calculated Forster distance and FRET efficiency for TagBFP / TagGFP2 pair is larger than those reported for the standard ECFP / EYFP and mCyPet / mYPet pairs. Moreover, TagBFP and TagGFP2 proteins lack the ability to form heterodimers, which results in more than 6-fold lower background for FRET analysis than in case of weakly dimerizing FRET pairs, such as ECFP / EYFP [Subach et al. 2008].

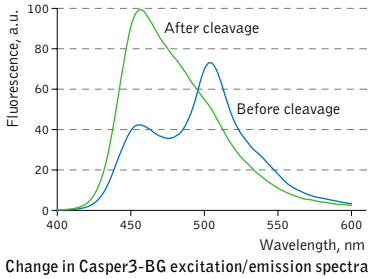
Main properties of Casper3-BG

Characteristic	
Calculated Forster distance R_0	5.25
FRET efficiency E	0.57
Specificity	caspase-3 activity
Response	elimination of FRET
Polypeptide length, aa	490
Molecular weight, kDa	55
FRET donor	TagBFP
Fluorescence color	blue
Excitation maximum, nm	402
Emission maximum, nm	457
Brightness, % of EGFP	99
pKa	2,7
FRET acceptor	TagGFP2
Fluorescence color	green
Excitation maximum, nm	483
Emission maximum, nm	506
Brightness, % of EGFP	105
pKa	5.0

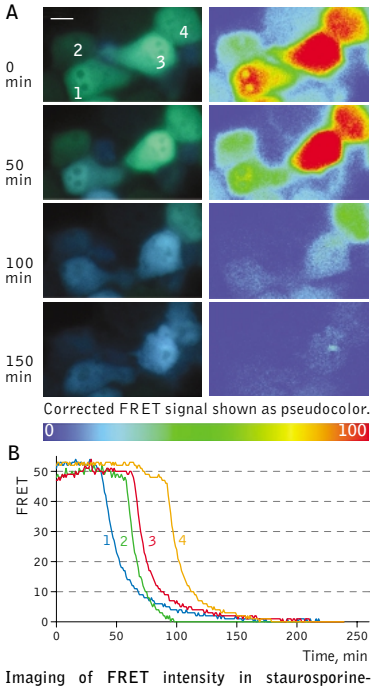
*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



Excitation (dashed lines) and emission (solid lines) spectra of TagBFP (blue) and TagGFP2 (green) are shown individually. Spectral overlap is filled with gray.



Change in Casper3-BG excitation/emission spectra upon the cleavage of DEVD sequence *in vitro*.



Imaging of FRET intensity in staurosporine-treated HeLa cells: (A) Fluorescent images of the cells after staurosporine treatment (left). The corrected FRET signals are shown as pseudocolor images (right). Scale bar, 10 mm. (B) time course of corrected FRET normalized per donor fluorescence observed in four cells indicated in (A).

The excellent performance of Casper3-BG sensor has been demonstrated *in vivo* on the example of HeLa cells staurosporine-induced apoptosis [Subach et al. 2008]. The two-filter method of sensitized FRET measurements [Gordon et al. 1998] on a pixel-by-pixel basis was applied, as described in [Galperin, E et al. 2004]. The initial mean FRET efficiency *in vivo* normalized to donor fluorescence was 51.5%.

Following 40-80 min exposure to 1 mM staurosporine, the FRET gradually dropped to zero before the shrinking of cells characteristic to apoptosis. The large FRET efficiency of the TagBFP / TagGFP2 pair enabled the detection of even weak proteolitic activity in each cell at the beginning of apoptosis, when only a fraction of the substrate was cleaved.

Recommended filter sets

The set of filters from Chroma (403/12 nm exciter, part #74673, 457/50 nm emitter, part #66974, and dichroic mirror, part #86100) or similar.

REFERENCES

Galperin, E et al. (2004). Nat Methods, 1 (3): 209217 / pmid: 15782196

Gordon et al. (1998). Biophys J, 74 (5): 2702–2013 / pmid: 9591694

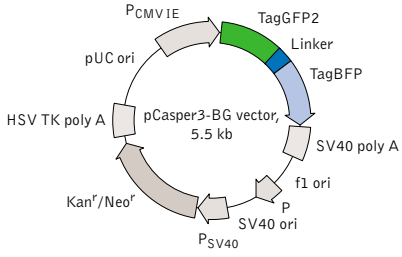
Subach et al. (2008). Chemistry & Biology, 15 (10): 1116–1124 / pmid: 18940671

Casper3-BG-related products

Product	Cat.#	Description	Size	Page(s)
pCasper3-BG	FP970	Mammalian expression vector encoding Casper3-BG	20 μg	83

Please contact your local distributor for exact prices and delivery information.

pCasper3-BG vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Linker - caspase-3 cleavage sequence

Vector type	mammalian expression vector
Reporter	Casper3-BG
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of fluorescent caspase-3 apoptosis sensor Casper3-BG in mammalian cells under the control of CMV promoter; source of Casper3-BG coding sequence

Product	Cat.#	Reporter	Color	Size
pCasper3-BG vector	FP970	Casper3-BG	blue/green	20 μg

Please contact your local distributor for exact prices and delivery information.

Caspase-3 apoptosis sensor Casper3-GR

- Early detection of Caspase-3 activity onset
- High sensitivity
- Direct expression in cells
- No exogenous chemical compounds required
- Proven suitability for FLIM-based screenings
- Recommended for early detection of apoptosis

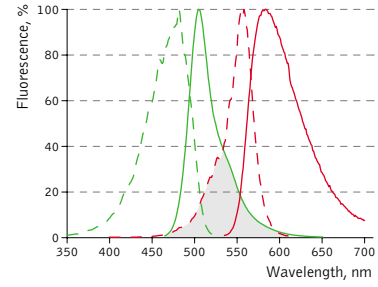
Casper3-GR is a FRET based sensor that can be used for detection of caspase-3 mediated apoptosis in living cells. The sensor consists of green and red fluorescent proteins TagGFP and TagRFP connected by the linker containing caspase-3 cleavage sequence, DEVD. The high fluorescence quantum yield of TagGFP along with the high molar extinction coefficient of TagRFP and excellent overlap of donor emission and acceptor excitation spectra result in highly effective FRET between these fluorescent proteins. The activation of caspase-3 during apoptosis leads to cleavage of DEVD sequence and elimination of FRET that can be detected as decrease in the red emission of TagRFP and a simultaneous increase in green emission of TagGFP.

The calculated Forster distance (R_0 = 5.7 nm) for TagGFP / TagRFP pair is one of the largest among the values reported. At the same time, since TagGFP and TagRFP emission peaks are spaced by as much as 79 nm, the emission signal for these two proteins can be easily separated in any imaging system. As an additional advantage, shifting the wavelengths toward

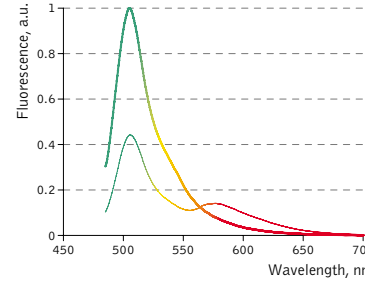
Main properties of Casper3-GR

Characteristic	
Calculated Forster distance R_0	5.70
FRET efficiency E	0.50
Specificity	caspase-3 activity
Response	elimination of FRET
Polypeptide length, aa	484
Molecular weight, kDa	54
FRET donor	TagGFP
Fluorescence color	green
Excitation maximum, nm	482
Emission maximum, nm	505
Brightness, % of EGFP	104
pKa	4,7
FRET acceptor	TagRFP
Fluorescence color	red
Excitation maximum, nm	555
Emission maximum, nm	584
Brightness, % of EGFP	148
pKa	3,8

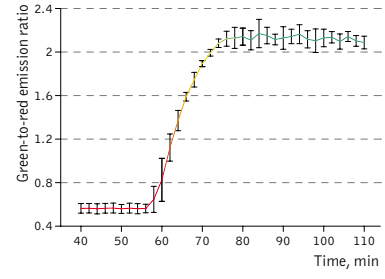
*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



Excitation (dashed lines) and emission (solid lines) spectra of TagGFP (green) and TagRFP (red) are shown individually. Spectral overlap is filled with gray.



Emission spectra of Casper3-GR before (thin line) and after digestion by Caspase-3 (thick line).



Green-to-red emission ratio change of Casper3-GR upon staurosporine-induced apoptosis. Approximately 40-50 min after staurosporine infusion, cells demonstrated pronounced changes in fluorescence signal ratio. Emission ratio shown for 5 cells, time point aligned to the median of ratio changes, individual for each cell. Excitation at 488 nm, emission was detected at 500-530 nm and 560-600 nm.

the red part of the spectrum (comparing to traditional cyan and yellow FRET partners) reduces input of cellular autofluorescence.

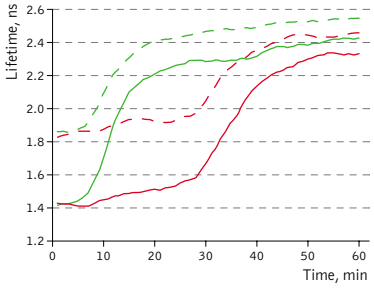
The activation of caspase-3 during apoptosis leads to cleavage of DEVD sequence and elimination of FRET that can be detected as decrease in red emission of TagRFP and a simultaneous increase in green emission of TagGFP. Direct monitoring of the donor/acceptor emission ratio demonstrated up to 5-fold ratio changes upon cleavage by recombinant caspase 3 *in vitro*. The increase in donor fluorescence intensity was at least 2-fold corresponding to a FRET efficiency of at least 50%.

The excellent performance of Casper3-GR sensor has been demonstrated *in vivo* on staurosporine-induced apoptosis of HeLa cells [Subach et al. 2008]. Living cells were monitored at 37°C with Leica SP2 confocal microscope (excitation using 488 nm laser line, emission collected at 500-530 nm and 560-650 nm). The fluorescence was evenly distributed in the cytosol and nucleus with no aggregation or non-specific localization observed. Importantly, both green and red signals were reliably stable under various irradiation conditions for hours. No reversible or irreversible fluorescence bleaching or photoconversion was observed. Approximately 30-40 min after 2 μM staurosporine infusion, cells demonstrated rapid (within 10 min) and pronounced changes in green-to-red fluorescence signal ratio, indicating activation of caspase-3. Later these cells demonstrated characteristic membrane blebbing. The average contrast in living cells (calculated as donor/acceptor emission ratio change for 5 cells, time point aligned to the median of ratio changes, individual for each cell) reached 3.8-fold.

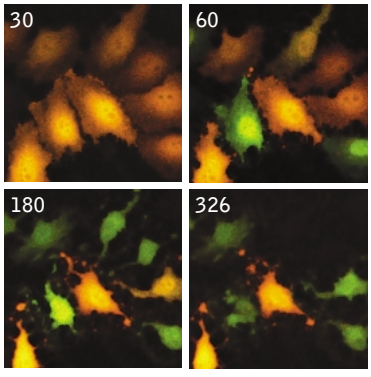
Measurement of Casper3-GR apoptosis induced FRET changes by FLIM revealed the dramatic increase of TagGFP fluorescence lifetime from 1.5 ns to 2.5 ns. The FRET efficiency of the uncleaved Casper3-GR (38% based on the phase lifetime) is among the highest measured by FLIM. Since the FRET efficiency of the cleaved substrate is zero, the dynamic range of the sensor is rather high, indicating that Casper3-GR can be successfully used for the high content FLIM based screenings on living cells.

Recommended filter sets

The excitation wavelength required to visualize FRET changes of Casper3-GR by ratio-imaging is provided by an ordinary FITC/GFP excitation filter or ubiquitous 488 nm laser line, and the two emission signals are acquired using a 500-530 nm (FITC/GFP emission filter) bandpass filter and a 560-600 nm bandpass filter (Cy3/DsRed emission filter) or a 560LP longpass filter.



TagGFP fluorescence phase lifetime (solid lines) and average modulation lifetime (dashed lines) changes for Casper3 during staurosporine-induced apoptosis. Excitation was at 488 nm and donor fluorescence emission was passed through a 500-530 nm bandpass filter.



Two channel fluorescence imaging of Casper3-GR upon staurosporine-induced apoptosis in HeLa cells. Time (in min.) is shown after staurosporine infusion.

REFERENCES

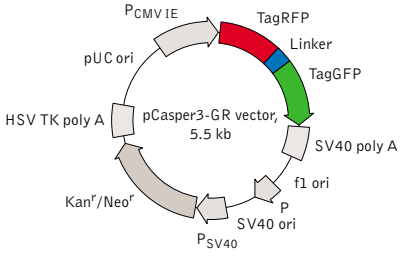
Subach et al. (2008). Chemistry & Biology, 15 (10): 1116–1124 / pmid: 18940671

Casper3-GR-related products

Product	Cat.#	Description	Size	Page(s)
pCasper3-GR	FP971	Mammalian expression vector allowing Casper3-GR expression in cytosol under the control of CMV promoter	20 μg	86

Please contact your local distributor for exact prices and delivery information.

pCasper3-GR vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Linker - caspase-3 cleavage sequence

Vector type	mammalian expression vector
Reporter	Casper3-GR
Reporter codon usage	mammalian
Promoter	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of fluorescent caspase-3 apoptosis sensor Casper3-GR in mammalian cells under the control of CMV promoter; source of Casper3-GR coding sequence

Product	Cat.#	Reporter	Color	Size
pCasper3-GR vector	FP971	Casper3-GR	green/red	20 μg

Please contact your local distributor for exact prices and delivery information.

Genetically-encoded photosensitizer KillerRed

- Light-induced production of reactive oxygen species
- Direct expression in cells, easy targeting to various subcellular compartments
- No exogenous chemical compounds required
- Not toxic before activation by green light irradiation
- Recommended for selective light-induced protein inactivation and cell killing

KillerRed is a red fluorescent protein capable of light-induced production of reactive oxygen species (ROS) [Bulina et al. 2006*a*]. It can be directly expressed both individually and in fusion with a target protein in various experimental systems, including bacteria, *Xenopus*, zebrafish, and mammalian cells. Upon green-light irradiation, KillerRed generates ROS that damage the neighboring molecules.

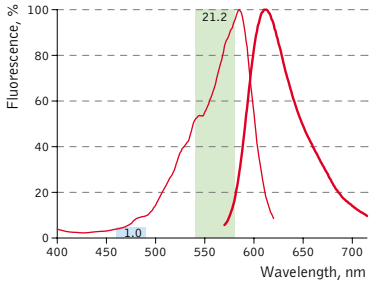
Mild illumination of cells expressing protein of interest fused to KillerRed results in precise inactivation of this protein only. Using KillerRed variants targeted to cell membrane or to mitochondria in combination with more prolonged and intensive green light irradiation it is possible to kill the selected cells in culture or inside a thin layer of transparent tissue. Intensity of green light and irradiation time must be individually determined for particular biological system and instrumentation.

KillerRed-mediated ROS production is accompanied by profound KillerRed photobleaching. The resulting cell events (cell fate after irradiation, effect on protein localization) should be monitored using another fluorescent reporter, for example a green fluorescent protein. We recommend that you use TurboGFP (page 27) for cell and organelle, or TagGFP2 (page 16) for protein labeling.

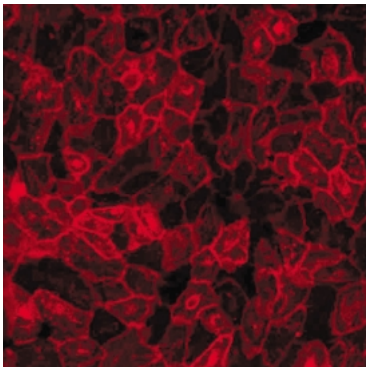
Main properties of KillerRed

Characteristic	
Molecular weight, kDa	27
Polypeptide length, aa	239
Structure	dimer
Aggregation	no
Maturation rate at 37°C	slow
Activating light	green (e.g. 540-580 nm)
Fluorescence color	red
Excitation maximum, nm	585
Emission maximum, nm	610
Quantum yield	0.25
Extinction coefficient, $M^{-1}cm^{-1}$	45 000
Brightness*	11.3

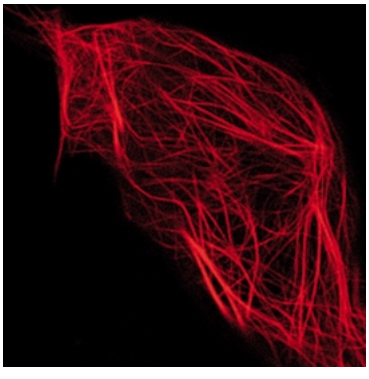
*Brightness is a product of extinction coefficient and quantum yield, divided by 1000.



KillerRed normalized excitation (thin line) and emission (thick line) spectra.
Blue and green rectangles show relative phototoxic effect from irradiation with blue (460-490 nm) and green (540-580 nm) light of 35 mW/cm^2. Numbers above the rectangles represent decrease in viable *E. coli* cells after 30-min irradiation (folds).



Expression of membrane-targeted KillerRed in stably transfected ARPE-19 cells.
Photograph of stably transfected cell line was provided by Dr. Christian Petzelt (MARINPHARM).



Transiently transfected HeLa cells expressing KillerRed fusion with Tau34.

Despite its dimerization capacity, KillerRed demonstrates successful performance in many fusions including that with mitochondrial targeted signal, cytoplasmic β-actin, fibrillarin, dopaminee transporter, Tau34, etc.

KillerRed suitability to generate stably transfected cells has been proven by Marinpharm company. Various cell lines expressing KillerRed are commercially available.

KillerRed's suitability for light-induced killing of prokaryotic cells has been demonstrated using *E. coli* XL1-Blue strain. KillerRed killed 96% of bacterial cells after 10 min and almost 100% of cells after 20 min of irradiation with white light.

KillerRed-mediated killing of eukaryotic cells: The following two ways have been found to be effective for killing the eukaryotic cells using KillerRed: (1) via an apoptotic pathway using KillerRed targeted to mitochondria, and (2) via membrane lipid oxidation using membrane-localized KillerRed. Irradiation of KillerRed localized in cell cytosol, has a weak effect on cell survival.

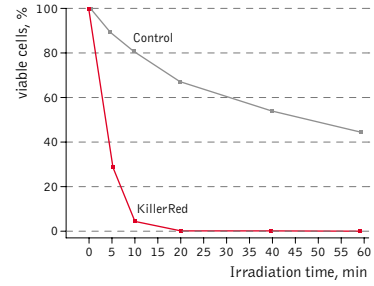
(1) Effects of KillerRed localized in mitochondria: Use of KillerRed targeted to mitochondria allows effective cell killing through an apoptotic pathway as has been demonstrated in the following experiment: HeLa cells expressing cytoplasmic TurboGFP and mitochondria-localized KillerRed-dMito were generated. 10-min irradiation of selected cells with green light resulted in profound KillerRed photobleaching. 60 min after irradiation, cells had an abnormal shape and "bubbles" typical of apoptotic pathway. These cells disrupted within the next 30-60 min.

In another experiment, nearly 100% of B16 melanoma cells expressing KillerRed targeted to mitochondria died within 45 min after 15-min of irradiation (40x objective, 535-575 nm excitation filter, 3.3 W/cm^2). It is noticeable that when preincubated with the pancaspase inhibitor zVAD-fmk (10 μM), the cells were resistant to the same green-light exposure and held their shape for at least 1.5 hours after illumination.

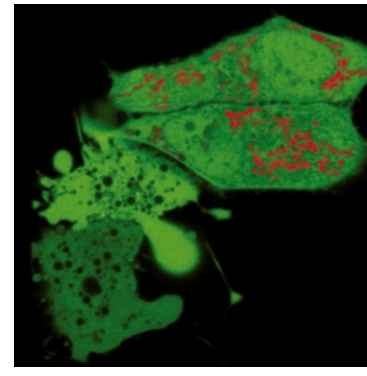
Apart from the immediate phototoxic effect, photosensitizers can mediate postponed cellular responses such as cell growth arrest or cell death via long-term apoptotic mechanism. In the experiment from [Bulina et al. 2006*a*], B16 melanoma cells expressing mitochondria-targeted KillerRed or EGFP were mixed together and irradiated by green light of low intensity (3.7x objective, 535-575 nm excitation filter, 115 mW/cm2) for 45 min. No red fluorescent cells were observed in 16 hrs after irradiation, whereas green fluorescent cells remained viable. It confirms that mitochondria-localized KillerRed can mediate cell death through long-term mechanisms in response to low-intensity illumination. This effect can be used in different applications.

(2) Effects of KillerRed localized in membrane: Comparing with the mitochondria-targeted KillerRed, irradiation of membrane-localized KillerRed causes a more effective and fast cell death within 10-30 min, presumably because of lipid oxidation [Bulina et al. 2006*b*].

Moreover, membrane-targeted KillerRed was shown to be an effective tool for the light-induced cell killing within a developing zebrafish. Zebrafish embryo was microinjected with a mixture of vectors driving expression of membrane-targeted KillerRed and a green fluorescent protein at the single- cell stage. A muscle cell expressing both proteins was irradiated with green light (40x objective, TRITC filter set, 10 min) at 48 hrs after

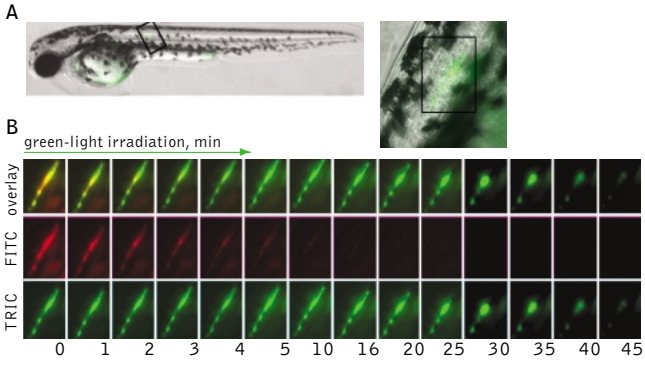


Time-course of light-induced killing of *E. coli* expressing KillerRed.
A single *E. coli* colony was picked out, diluted into 1 ml of PBS buffer and divided into two equal portions. One of them was irradiated with white light (1W/cm^2, light source Fiber-Light from Dolan-Jenner Industries, Inc) for different periods of time, whereas the other was kept in darkness. Both sample aliquots were plated onto Petri dishes at different dilutions. The number of growing colonies corresponded to the number of bacterial cells surviving after irradiation (i.e. colony forming units, CFU). CFU number for the irradiated *E. coli* portion was compared with the non-irradiated one, thus allowing estimation of the relative phototoxic effect for KillerRed. In control experiments, *E. coli* cells expressing different fluorescent and non-fluorescent proteins were used.



Confocal image of HeLa cells expressing KillerRed in mitochondria (red) and TurboGFP in cytosol (green).
Lower left cells were pre-irradiated with green light (515-560 nm, 7W/cm^2) light for ten minutes. This led to profound KillerRed photobleaching. Cells are shown 60 min after irradiation. It is clearly visible that irradiated cells have abnormal shape and "bubbles", characteristic for apoptotic cell-death pathway.

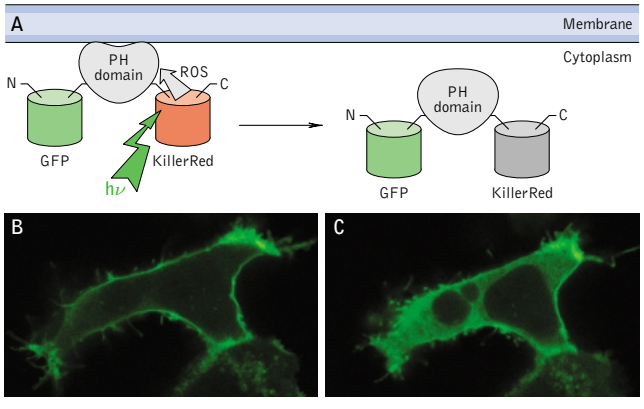
fertilization. By the end of 10-min irradiation, the cell already started to change its shape. Within 20 min after irradiation was stopped, the cell was disrupted completely. Mitochondria targeted KillerRed was shown to be of low efficiency in similar experiments.



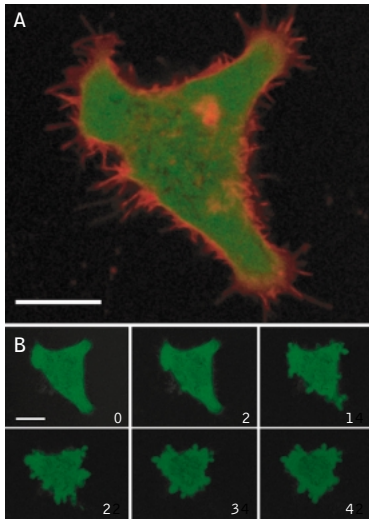
Light-induced killing of a muscle cell within a developing zebrafish embryo. (A) A region expressing membrane-targeted KillerRed and green fluorescent marker; (B) time course of light-induced killing of a muscle cell within a developing zebrafish. Fluorescence was collected using standard FITC and TRITC filter sets.

KillerRed use for protein inactivation: KillerRed use for chromophore-assisted light inactivation (CALI) of proteins has been demonstrated on the model of β-galactosidase inactivation in bacterial cells and inactivation of pleckstrin homology (PH) domain of phospholipase C δ-1 (PLC δ-1) in mammalian cells. In the first experiment, KillerRed was fused to β-galactosidase (β-gal) enzyme and expressed in *E. coli*. Effective suppression of β-gal activity was demonstrated in living *E. coli* streaks and *E. coli* cell extract.

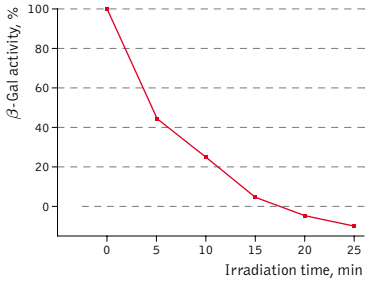
In the second experiment, a triple EGFP-PH-KillerRed fusion protein that allows both protein visualization and CALI was transiently expressed in mammalian cell line. Intracellular localization of EGFP signal was evaluated before and after CALI of the PH domain using confocal and fluorescence microscopy. In intact cells, the fusion is located predominantly at the plasma membrane because of specific affinity of PH domain to phosphatidylinositol 4,5-bisphosphate.



KillerRed-mediated light-induced inactivation of PLC δ-1 PH domain. (A) Schematic outline of the experimental system; (B,C) confocal images of a cell expressing EGFP-PH-KillerRed triple fusion (EGFP green fluorescent signal) before (B) and after (C) 10-s irradiation with green light. Note considerable increase in cytoplasmic signal.



Light-induced killing of HeLa cell using membrane-targeted KillerRed. (A) Confocal image of HeLa cells expressing membrane-localized KillerRed (red) and TurboGFP in cytosol (green); (B) time-course of cell fragmentation induced by green-light irradiation (63x objective, mercury lamp, 515-560 nm excitation filter, 7 W/cm^2) for 10 min. Numbers indicate time since irradiation, min. Scale bar, 10 μm. Figure was first published in [Bulina et al. 2006*a*].



Time-course of CALI of β-galactosidase. In the model CALI experiment KillerRed was fused to β-galactosidase (β-gal) enzyme and expressed in *E. coli*. Upon green-light irradiation (540-580 nm, 30 min, 360mW/cm^2) β-gal activity was effectively suppressed in living *E. coli* streaks. On the contrary, no effect of green light on the enzyme activity was detected in control cells containing unmodified β-gal gene. *In vitro* test showed that in *E. coli* cell extract β-gal fused to KillerRed lost 99.4% of enzymatic activity within 25 min of white light exposure (1W/cm^2), with half inactivation time of about 5 min. Irradiation of *E. coli* extracts containing unfused β-gal protein alone or β-gal mixed with KillerRed had no effect on enzyme activity. To verify specificity of KillerRed phototoxic effect, horse-radish peroxidase (HRP) was added to the sample. Upon 15 min of illumination (white light, 1W/cm^2) only 2% of HRP activity was lost, showing high specificity of the phototoxic effect.

Irradiation with intense green light led to KillerRed-mediated ROS production, PH domain damage, and fusion protein dissociation from the membrane. After 10 sec of green-light irradiation (63x objective, mercury lamp, 515-560 nm filter, 7W/cm^2), translocation of the PH domain into cytosol was clearly visible. When irradiated for a longer period of time, considerable amount of PH domain translocated into cytosol, increasing the cytoplasm-to-membrane green fluorescent signal ratio to 0.5-0.9.

In the negative control experiments, the cellular location of a DsRedExpress (Clontech) containing construct, GFP-PH-DsRedExpress, showed no dependence on green-light irradiation. Similarly, no detectable CALI of the PH domain was achieved when KillerRed was expressed in the cell separately from PH domain, in either the membrane or cytosol.

Recommended antibodies, filter sets, and activating lasers

KillerRed can be recognized using Anti-KillerRed antibody (Cat.# AB961-AB962) available from Evrogen.

Before light activation, KillerRed can be detected using TRITC filter set or similar. Recommended Omega Optical filter sets are QMAX-Red and XF174.

KillerRed phototoxicity is induced by green-light irradiation at 540-580 nm and depends on light intensity irradiation time and KillerRed concentration. Arc-lamp irradiation is strongly recommended; laser-light irradiation in confocal mode is much less efficient.

REFERENCES

Bulina et al. (2006*a*). Nat Biotechnol, 24 (1): 95–99 / pmid: 16369538

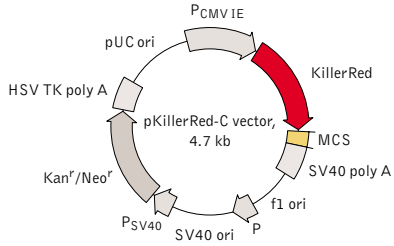
Bulina et al. (2006*b*). Nat Protoc, 1 (2): 947–953 / pmid: 17406328

KillerRed-related products

Product	Cat.#	Description	Size	Page(s)
KillerRed expression/source vectors				
pKillerRed-C	FP961	Mammalian expression vector encoding humanized KillerRed and allowing its expression and generation of fusions to the KillerRed C-terminus	20 μg	92
pKillerRed-N	FP962	Mammalian expression vector encoding humanized KillerRed and allowing its expression and generation of fusions to the KillerRed N-terminus	20 μg	92
pKillerRed-B	FP963	Bacterial expression vector; source of the KillerRed coding sequence	20 μg	93
pKillerRed-dMito	FP964	Mammalian expression vector encoding mitochondria-targeted KillerRed	20 μg	93
pKillerRed-mem	FP966	Mammalian expression vector encoding membrane-targeted KillerRed	20 μg	94
Vector sets				
Cell-Killer	FPK01	pKillerRed-dMito vector encoding mitochondria-targeted KillerRed and pTurboGFP-N vector allowing cytoplasmic expression of bright green fluorescent protein TurboGFP for monitoring cell fate	20 μg each	93, 45
Membrane-Killer	FPK02	pKillerRed-mem vector encoding membrane-targeted KillerRed and pTurboGFP-N vector allowing cytoplasmic expression of bright green fluorescent protein TurboGFP for monitoring cell fate	20 μg each	94, 45
Double-Killer	FPK03	pKillerRed-mem vector encoding membrane-targeted KillerRed, pKillerRed-dMito vector encoding mitochondria-targeted KillerRed and pTurboGFP-N vector allowing cytoplasmic expression of bright green fluorescent protein TurboGFP for monitoring cell fate	20 μg each	93,94,45
Antibodies against KillerRed				
Anti-KillerRed	AB961 AB962	Rabbit polyclonal antibody against KillerRed	100 μg 200 μg	106

Please contact your local distributor for exact prices and delivery information.

pKillerRed-C vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter	KillerRed
Reporter codon usage	mammalian
Promoter for KillerRed	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	KillerRed expression in mammalian cells; generation of fusions to the KillerRed C-terminus

Product	Cat.#	Reporter	Color	Size
pKillerRed-C	FP961	KillerRed	red	20 μg

Please contact your local distributor for exact prices and delivery information.

pKillerRed-C vector MCS

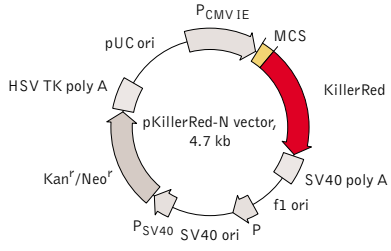
KillerRed → BspE I | Xho I | Hind III | Pst I | Kpn I | Apa I | BamH I | STOPs

... TCC.GGA.CTC.AGA.TCT.CGA.GCT.CAA.GCT.TCG.AAT.TCT.GCA.GTC.GAC.GGT.ACC.GCG.GGC.CCG.GGA.TCC.ACC.GGA.TCT.AGA.TAA.CTG.ATC.A ...

Bgl II | Sac I | EcoR I | Sal I | Sac II | Sma I/Xma I | Xba I# | Bcl I#

— sites are blocked by *dam* methylation. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

pKillerRed-N vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	mammalian expression vector
Reporter	KillerRed
Reporter codon usage	mammalian
Promoter for KillerRed	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori; eukaryotic - SV40 ori
Use	KillerRed expression in mammalian cells; generation of fusions to the KillerRed N-terminus

Product	Cat.#	Reporter	Color	Size
pKillerRed-N	FP962	KillerRed	red	20 μg

Please contact your local distributor for exact prices and delivery information.

pKillerRed-N vector MCS

Nhe I | Bgl II | Sac I | EcoR I | Sal I | Sac II | Sma I/Xma I | Age I | KillerRed →

... GCT.AGC.GCT.ACC.GGA.CTC.AGA.TCT.CGA.GCT.CAA.GCT.TCG.AAT.TCT.GCA.GTC.GAC.GGT.ACC.GCG.GGC.CCG.GGA.TCC.ACC.GGT.CGC.CAC.CAT.GG ...

Afe I | Xho I | Hind III | Pst I | Kpn I | Apa I | BamH I | Nco I*

* — not unique sites.

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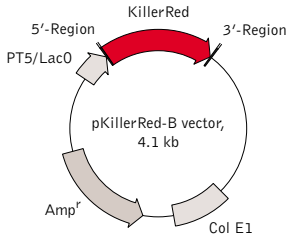
pKillerRed-B vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

Vector type	bacterial expression vector
Reporter	KillerRed
Reporter codon usage	mammalian
Promoter for KillerRed	T5 promoter/lac operator
Host cells	prokaryotic
Selection	ampicillin
Replication	ColE1 ori
Use	Source of the KillerRed coding sequence; KillerRed expression in bacterial cells

Product	Cat.#	Reporter	Color	Size
pKillerRed-B	FP963	KillerRed	red	20 μg

Please contact your local distributor for exact prices and delivery information.

5' Region

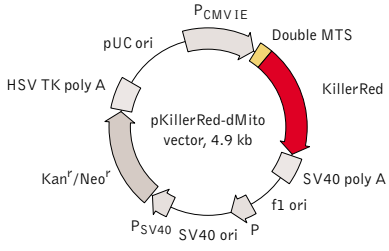
BamH I KillerRed
RBS ATG.AGA.GGA.TCG.GGA.TCC.ATG.G...
Nco I*

3' Region

STOP
...TGA.AAG.CTT...
Hind III

* — not unique sites.

pKillerRed-dMito vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

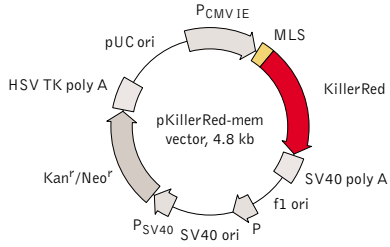
MTS - Mitochondrial targeting sequence

Vector type	mammalian expression vector
Reporter	KillerRed
Reporter codon usage	mammalian
Promoter for KillerRed	P$_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of mitochondria-targeted KillerRed in mammalian cells under the control of CMV promoter; source of mitochondria-targeted KillerRed coding sequence

Product	Cat.#	Reporter	Color	Size
pKillerRed-dMito	FP964	KillerRed	red	20 μg

Please contact your local distributor for exact prices and delivery information.

pKillerRed-mem vector



For vector sequence, please visit our Web site at http://www.evrogen.com/support/vector-info.shtml

MLS - Membrane localization signal

Vector type	mammalian expression vector
Reporter	KillerRed
Reporter codon usage	mammalian
Promoter for KillerRed	$P_{CMV\ IE}$
Host cells	mammalian
Selection	prokaryotic - kanamycin eukaryotic - neomycin (G418)
Replication	prokaryotic - pUC ori eukaryotic - SV40 ori
Use	Expression of membrane-targeted KillerRed in mammalian cells under the control of CMV promoter; source of membrane-targeted KillerRed coding sequence

Product	Cat.#	Reporter	Color	Size
pKillerRed-mem	FP966	KillerRed	red	20 μg

Please contact your local distributor for exact prices and delivery information.