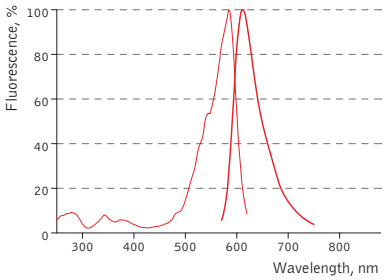
Genetically-encoded photosensitizer KillerRed

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



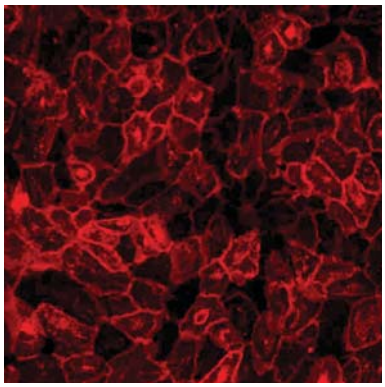
KillerRed normalized excitation (thin line) and emission (thick line) spectra.
Complete KillerRed spectra in Excel format can be downloaded from the Evrogen Web site at http://www.evrogen.com

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. Besides KillerRed protein (and its derivative KillerOrange), all known to date photosensitizers require chemical compounds that should be introduced into living systems exogenously. Red fluorescent protein KillerRed is the first genetically-encoded photosensitizer [Bulina, Chudakov, et al. 2006]. Unlike chemical analogs, KillerRed can be directly expressed by target cells, both individually and in fusion with a target protein. It shows no cell toxic effects before light activation. Upon green or orange light irradiation, KillerRed generates ROS that damage the neighboring molecules.

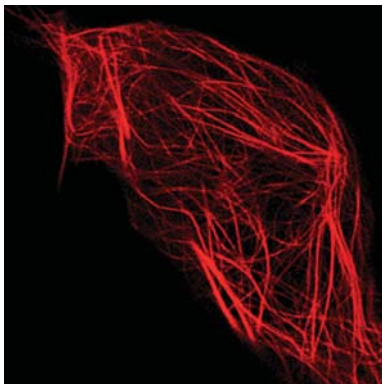
Main properties of KillerRed

Characteristic	
Molecular weight, kDa	27
Polypeptide length, aa	239
Structure	dimer
Aggregation	no
Maturation rate at 37°C	fast
Activating light	green or orange (e.g. 540-590 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 1 000.



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.

Performance and use

KillerRed can be used for *in vivo* killing of selected cells and CALI applications. It can be expressed and induced in various experimental systems, including bacteria, *Xenopus*, zebrafish, and mammalian cells.

KillerRed is 2000-fold more phototoxic than EGFP; however, it is not as effective as chemical probes. KillerRed-mediated ROS production is accompanied by profound KillerRed photobleaching.

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

KillerRed suitability to generate stably transfected cells has been proven by Marinpharm company. 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. A single *E. coli* colony expressing KillerRed 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 then plated onto Petri dishes at different dilutions. The number of growing colonies corresponded to the number of bacteria cells surviving after irradiation (i.e. colony forming units, CFU). CFU number for the irradiated *E. coli* portion was compared with 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.

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.

Effects of KillerRed localized in mitochondria: Mitochondrial localization increases the phototoxic effect of photosensitizers (primarily by provoking the apoptosis). Use of KillerRed targeted to mitochondria allows effective cell killing through an apoptotic pathway as has been demonstrated in the following experiments:

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.

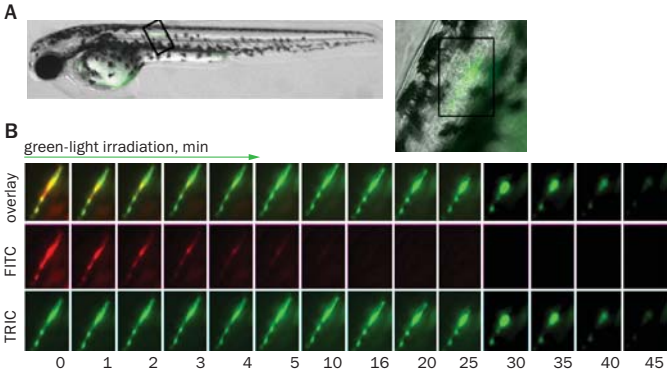
In another experiment, nearly 100% of B16 melanoma cells expressing KillerRed targeted to mitochondria died within 45 min after 15-min of irradiation (40x objective, 535-575 nm excitation filter, 3.3 W/cm^2). It is noticeable that when preincubated with the pancaspase inhibitor zVAD-fmk (10 μM), the cells were resistant to the same 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:**

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/cm^2) for 45 min [Bulina, Chudakov, et al. 2006]. 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.

Effects of KillerRed localized in membrane: Comparing to 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, Lukyanov, et al. 2006].

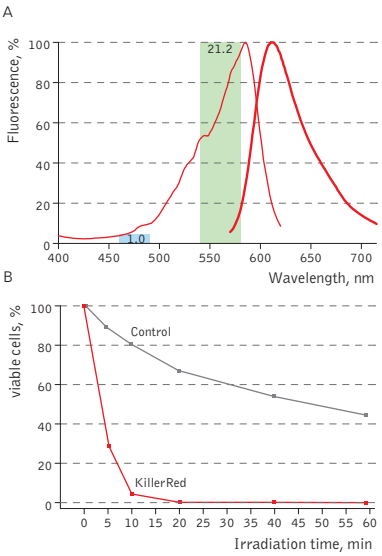
Moreover, membrane-targeted KillerRed was shown to be an effective tool for the light-induced cell killing within a developing zebrafish. Zebrafish embryo was microinjected with a mixture of vectors driving expression of membrane-targeted KillerRed and a green fluorescent protein at the single- cell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



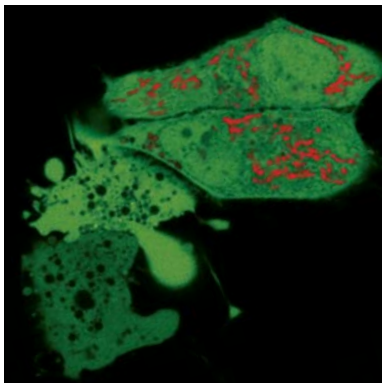
Light-induced killing of a muscle cell within a developing zebrafish embryo.
(A) A region expressing membrane-targeted KillerRed and green fluorescent marker; (B) time course of light-induced killing of a muscle cell within a developing zebrafish. Fluorescence was collected using standard FITC and TRITC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

KillerRed use for CALI applications: KillerRed use for CALI application has been demonstrated on the model of β-galactosidase inactivation in bacterial cells and inactivation of pleckstrin homology (PH) domain of phospholipase C delta-1 (PLC delta-1) in mammalian cells.

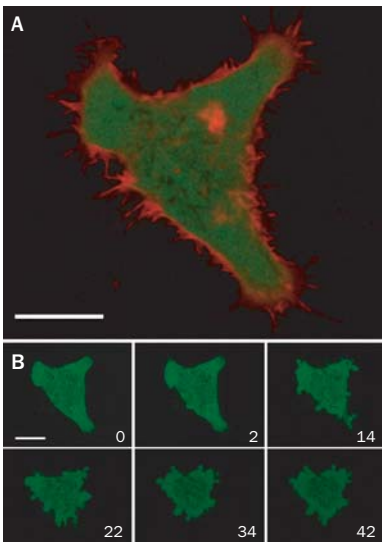
In the first experiment, KillerRed was fused to β-galactosidase (beta-gal) enzyme and expressed



KillerRed-mediated killing of bacteria cells.
(A) 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). (B) Time-course of light-induced killing of *E. coli* expressing KillerRed.



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.



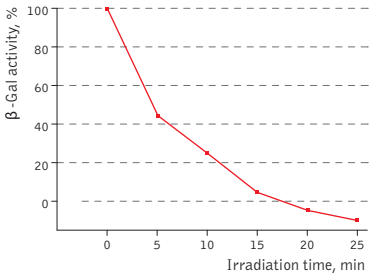
Light-induced killing of HeLa cell using membrane-targeted KillerRed. (A) Intact cell features red fluorescent cellular membrane and green fluorescent cytosol due to membrane-targeted KillerRed and TurboGFP expression, respectively. (B) time-course of cell fragmentation induced by 10-min green light irradiation. Scale bar, 10 μ m. Figure was first published in [Bulina, Chudakov, et al. 2006].

in *E. coli*. Upon green light irradiation (540-580 nm, 30 min, 360 mW/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.

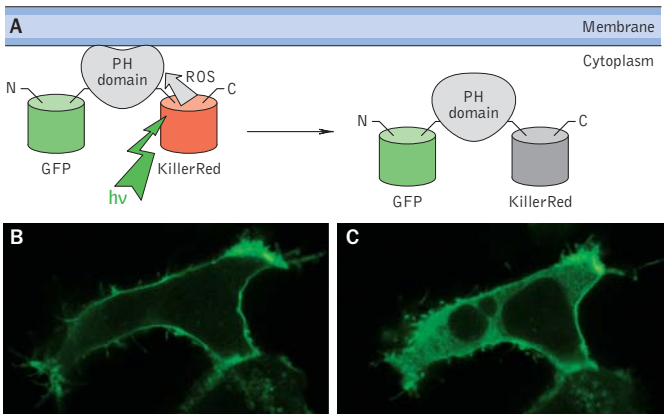
In the second experiment, a triple EGFP-PH-KillerRed fusion protein that allows both protein visualization and CALI was transiently expressed in mammalian cell line. Intracellular localization of EGFP signal was evaluated before and after CALI of the PH domain using confocal and fluorescence microscopy.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

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 DsRed-Express (Clontech) containing construct, GFP-PH-DsRed-Express, 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.



Time-course of CALI of β-galactosidase.



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.

Recommended antibodies, filter sets, and activating parameters

KillerRed can be recognized using Anti-KillerRed antibody (Cat. # AB961) 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 or orange light irradiation at 540-590 nm and depends on light intensity irradiation time and KillerRed concentration and localization. Arc-lamp irradiation or LEDs light is strongly recommended; laser-light irradiation in confocal mode is much less efficient.

In CALI, mild illumination of KillerRed-tagged protein for a limited time results in precise inactivation of this protein only. Upon more prolonged and intensive irradiation, KillerRed can be effectively used for damaging the organelles and killing the target cells. Light intensity and irradiation time should 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) can be monitored using another fluorescent reporter, for example a green fluorescent protein. We recommend that you use TurboGFP for cell and organelle, or TagGFP2 for protein labeling.

Available variants and fusions

KillerRed mammalian expression vectors contain KillerRed coding sequence with codon usage optimized for high expression in mammalian cells, i.e. humanized [Haas, Park, and Seed 1996].

Humanized KillerRed can also be expressed in *E. coli* and some other heterological systems upon subcloning into appropriate vector.

The available vectors encoding KillerRed variants and fusions are listed below in the section KillerRed-related products. For most updated product information, please visit Evrogen website www.evrogen.com.

If you need KillerRed codon variant or fusion construct that is not listed on our website, please contact us at product@evrogen.com.

Licensing opportunities

Evrogen technology embodied in KillerRed is available for expanded and commercial use with an adaptable licensing program. Benefits from flexible and market driven license options are offered for upgrade and novel development of products and applications. For licensing information, please contact Evrogen at license@evrogen.com.

References

Bulina, M.E., D.M. Chudakov, et al. (2006). Nat Biotechnol, 24 (1): 95–99 / pmid: 16369538

Bulina, M.E., K.A. Lukyanov, et al. (2006). Nat Protoc, 1 (2): 947–953 / pmid: 17406328

Haas, J., E. C. Park, and B. Seed (1996). Curr Biol, 6 (3): 315–324 / pmid: 8805248

KillerRed-related products

Product	Cat.#	Description	Size
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
pKillerRed-N	FP962	Mammalian expression vector encoding humanized KillerRed and allowing its expression and generation of fusions to the KillerRed N-terminus	20 µg
pKillerRed-B	FP963	Bacterial expression vector; source of the KillerRed coding sequence	20 µg
pKillerRed-dMito	FP964	Mammalian expression vector encoding mitochondria-targeted KillerRed	20 µg
pKillerRed-mem	FP966	Mammalian expression vector encoding membrane-targeted KillerRed	20 µg
Antibodies against KillerRed			
Anti-KillerRed	AB961	Rabbit polyclonal antibody against KillerRed, ArrestRed, and JRed	100 µg

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