

# Genetically-encoded photosensitizer KillerRed

- Genetically-encoded photosensitizer, direct expression in cells
- No exogenous chemical compounds or cofactors required
- No cell toxicity before light activation
- Induction by green light irradiation
- Successful performance in fusions
- Recommended for selective *in vivo* cell killing and CALI applications

## Description

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, all known to date photosensitizers are chemical compounds that must be introduced into living systems exogenously. This limitation severely constricts the area of their applications.

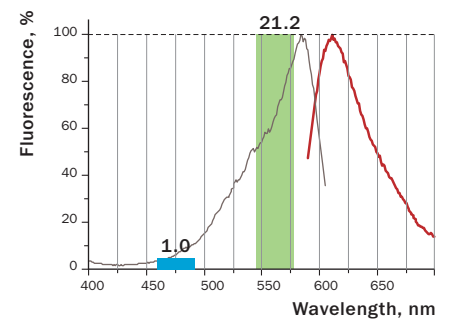
Developed from the Anthomedusae chromoprotein (Shagin *et al.*, 2004), red fluorescent protein KillerRed is the first genetically-encoded photosensitizer (Bulina *et al.*, 2006a). Unlike chemical analogs, KillerRed can be directly expressed by target cells, both individually and in fusion with a target protein. It shows no cell toxic effects before light activation. Upon green-light irradiation, KillerRed generates ROS that damage the neighboring molecules.

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.

## Main properties of KillerRed

Characteristic		
Molecular weight	27 kDa	
Polypeptide length	239 aa	
Structure	dimer	
Aggregation	no	
Maturation at 37°C	slow	
Activating light	green (e.g. 540-580 nm)	
	<b>before photoconversion</b>	<b>after photoconversion</b>
Fluorescence color	red	NO
Excitation max	585 nm	-
Emission max	610 nm	-
Quantum yield	0.25	<0.001
Extinction coefficient, M <sup>-1</sup> cm <sup>-1</sup>	45 000	no data
Brightness*	11.3	0
Cell toxicity	no	high

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



## Normalized excitation (grey line) and emission (red line) spectra for KillerRed.

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<sup>2</sup>. Numbers above the rectangles represent decrease in viable *E. coli* cells after 30-min irradiation (folds).

## 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.

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

KillerRed suitability to generate stably transfected cells has been proven by Marinpharm company ([www.marinpharm.com](http://www.marinpharm.com)). Various cell lines expressing KillerRed are commercially available.

**KillerRed suitability for light-induced killing of prokaryotic cells** has been demonstrated using *E. coli* XL1-Blue strain. 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<sup>2</sup>, 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

Following two ways have been found to be effective for killing the eukaryotic cells using KillerRed: (1) via 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

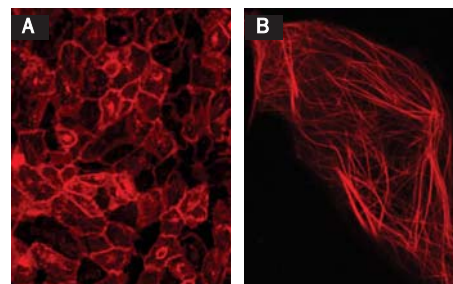
Mitochondrial localization increases the phototoxic effect of photosensitizers (primarily by provoking the apoptosis). Use of KillerRed targeted to mitochondria allows effective cell killing through apoptotic pathway as has been demonstrated in the following experiments:

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 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<sup>2</sup>). 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 hrs after illumination.

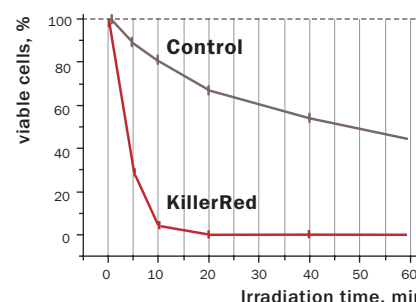
Apart from the immediate phototoxic effect, photosensitizers can mediate postponed cellular responses such as cell growth arrest or cell death via long-term apoptotic mechanism.

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<sup>2</sup>) 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.



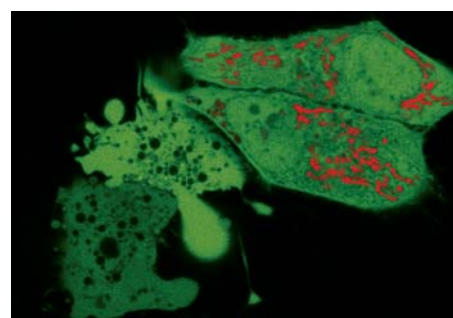
### KillerRed expression in mammalian cells.

A — Stably transfected ARPE-19 cells expressing membrane-targeted KillerRed. Image was kindly provided by Dr. Christian Petzelt (Marinpharm); B — Transiently transfected HeLa cells expressing KillerRed fusion with Tau34.



### Time-course of light-induced killing of *E. coli* expressing KillerRed.

Although white light illumination was applied in this experiment, only the green component of irradiation spectrum appeared to be crucial for induction of KillerRed phototoxicity. Strong cell killing effect was achieved upon green (540-580 nm) and almost zero effect upon blue (460-490 nm) light irradiation of equalized power (about 35mW/cm<sup>2</sup>).



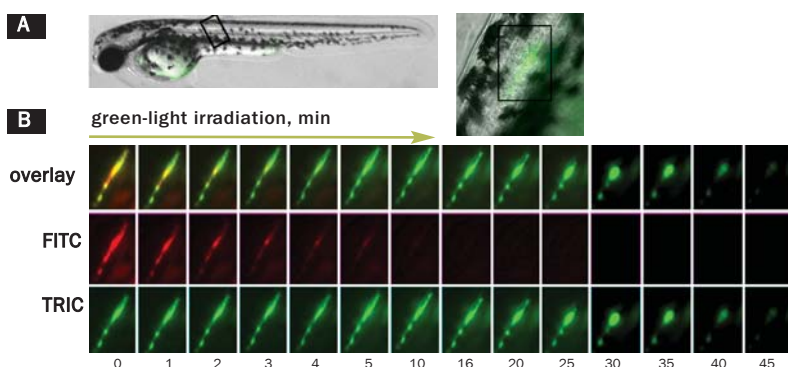
### Confocal image of HeLa cells expressing KillerRed in mitochondria (red) and TurboGFP in cytosol (green).

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

### Effects of KillerRed localized in membrane

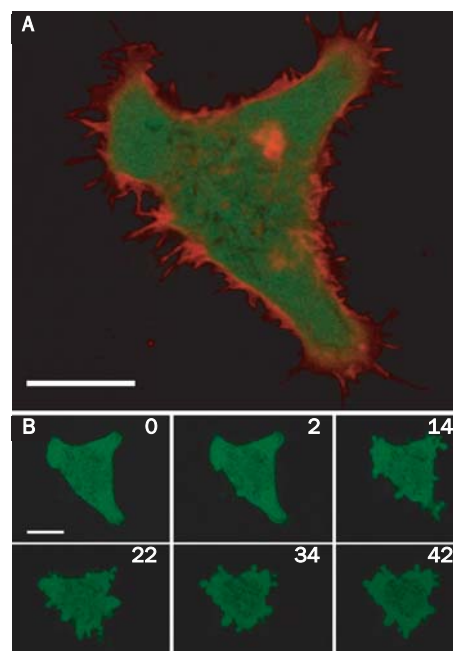
Comparing with the mitochondria-targeted KillerRed, irradiation of membrane-localized KillerRed causes a more effective and fast cell death with in 10-30 min, presumably because of lipid oxidation (Bulina *et al.*, 2006b).

In addition, 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.



### 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<sup>2</sup>) for 10 min. Numbers indicate time since irradiation, min. Scale bar, 10 μm. Figure was first published in (Bulina *et al.*, 2006b).

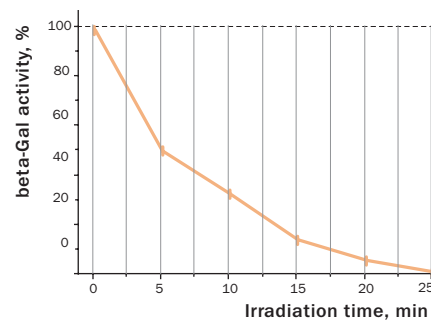
### KillerRed use for CALI applications

KillerRed use for CALI application has been demonstrated on the model of beta-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 beta-galactosidase (beta-gal) enzyme and expressed in *E. coli*. *In vitro* test showed that in *E. coli* cell extract beta-gal fused to KillerRed lost 99,4% of enzymatic activity within 25 min of white light exposure (1W/cm<sup>2</sup>), with half inactivation time of about 5 min. Irradiation of *E. coli* extracts containing unfused beta-gal protein alone or beta-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<sup>2</sup>) 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<sup>2</sup>), translocation of the PH domain into cytosol



### Time-course of CALI of beta-galactosidase.

Upon green-light irradiation (540-580 nm, 30 min, 360mW/cm<sup>2</sup>) beta-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 beta-gal gene.

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.

### Available variants and fusions

KillerRed codon usage is optimized for high expression in mammalian cells (Haas *et al.*, 1996), but it can be successfully expressed in other heterologous systems.

#### KillerRed-dMito fusion

Mitochondrial targeting sequence (MTS) was derived from subunit VIII precursor of human cytochrome C oxidase (Rizzuto *et al.*, 1989; Rizzuto *et al.*, 1995). Two MTS were fused to KillerRed N-terminus. When expressed in mammalian cells, this variant is localized in mitochondria. Mitochondrial localization of KillerRed increases its light-induced cell toxicity and makes it effective for cell killing presumably through apoptotic pathway.

**KillerRed-mem fusion** comprises KillerRed linked with membrane localization signal (MLS) of neuromodulin. The neuromodulin MLS (N-terminal 20 amino acid residues) contains a signal for posttranslational palmitoylation of cysteines 3 and 4 that targets KillerRed to cellular membranes (Skene & Virag, 1989). Irradiation of membrane-localized KillerRed leads to high effective and fast cell death, presumably due to lipid oxidation. Comparing to the mitochondrially targeted KillerRed, irradiation of membrane-localized KillerRed leads to even more effective and fast cell death (within 10-30 min). Membrane-targeted KillerRed was shown to be suitable for the light induced cell killing within a developing zebrafish.

### Recommended antibodies, filter sets and activating parameters

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 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. Importantly, we recommend that you use arc-lamp irradiation. Laser-light irradiation in confocal mode is much less efficient.

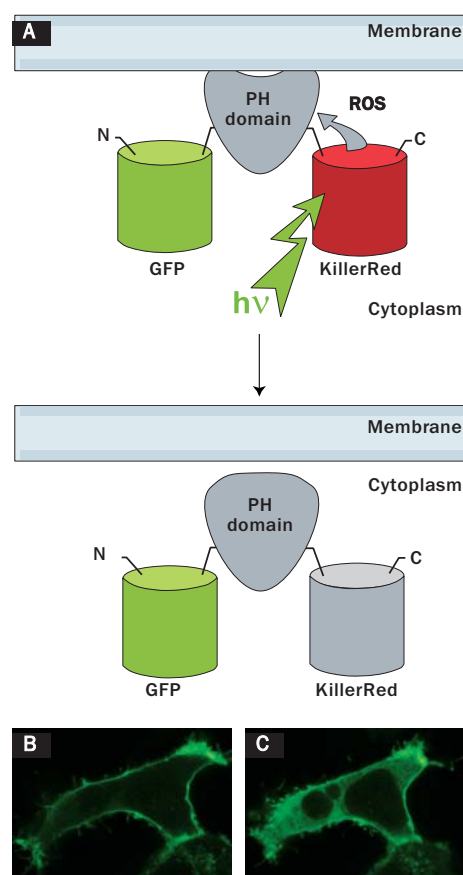
In CALI, mild illumination of KillerRed-tagged protein for a limited time results in precise inactivation of this protein only. Upon more prolonged and intensive irradiation, KillerRed can be effectively used for damaging the organelles and killing the target cells. Intensity of green light and irradiation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

### KillerRed licensing opportunities

Evrogen technology embodied in KillerRed proteins 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](mailto:license@evrogen.com).



### KillerRed-mediated light-induced inactivation of PLC delta-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.

### References

Bulina *et al.* (2006a) *Nat. Biotechnol.* 24(1): 95-99  
 Bulina *et al.* (2006b) *Nature Protocols* 1 (2): 947-953.  
 Haas *et al.* (1996) *Curr. Biol.* 6: 315-324.  
 Rizzuto *et al.* (1989) *J. Biol. Chem.* 264: 10595-10600.  
 Rizzuto *et al.* (1995) *Curr. Biol.* 5: 635-642.  
 Shagin *et al.* (2004) *Mol. Biol. Evol.* 21(5): 841-850.  
 Skene J.H.P. & Virag I. (1989) *J. Cell. Biol.* 108: 613-625.

## KillerRed-related products

Product	Cat.#	Description	Size
<b>KillerRed expression/source vectors</b>			
pKillerRed-C	FP961	Mammalian expression vector encoding humanized KillerRed and allowing KillerRed expression and generation of fusions to the KillerRed C-terminus	20 µg
pKillerRed-N	FP962	Mammalian expression vector encoding humanized KillerRed and allowing KillerRed expression and generation of fusions to the KillerRed N-terminus	20 µg
pKillerRed-B	FP963	Bacterial expression vector; source of the humanized 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
<b>Antibodies against KillerRed</b>			
Anti-KillerRed antibody	AB961 AB962	Rabbit polyclonal antibody against KillerRed and JRed	100 µg 200 µg

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

## Third party products: stably transfected cell lines expressing KillerRed

Cell line	Source	Description
m3-Killer	mouse	M3 mouse melanoma cells expressing membrane-targeted KillerRed
ARPE19-Killer	human	ARPE19 cells expressing membrane-targeted KillerRed

Cell lines are manufactured by Marinpharm GmbH (Berlin, Germany, [www.marinpharm.com](http://www.marinpharm.com)) under the Evrogen license.

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