LigandLink™

Universal Labeling Technology

(version B1)

Product	Catalog No.
LigandLink™ pLL-1 Kit	34001
LigandLink™ Covalent pLL-1 Kit	34007
Premade LigandLink™ Kits	34002-34006
LigandLink™ Fluorescein Label	34101
LigandLink™ Hexachlorofluorescein Label	34104
LigandLink™ Covalent Fluorescein Label	34107
LigandLink™ Covalent Hexachlorofluorescein Label	34108

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Overview

Active Motif's LigandLink™ Universal Labeling Kit enables specific and flexible labeling of proteins in living cells. In the LigandLink method, the gene of interest is cloned in frame with the gene for *E. coli* dihydrofolate reductase (eDHFR) in the pLL-1 vector. The vector is then transfected into mammalian cells and used to express fusion protein. After transfection, the protein of interest can be labeled simply by adding the LigandLink Label of choice to the cell medium (Figure 1). Depending on the cell type and the characteristics of the label, cells can be imaged in as little as ten minutes.

product	format	catalog no.
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ Covalent pLL-1 Kit	1 kit	34007
Premade LigandLink™ Kit	1 kit	34002-34006
LigandLink™ Fluorescein Label	300 rxns	34101
LigandLink™ Hexachlorofluorescein Label	300 rxns	34104
LigandLink™ Covalent Fluorescein Label	300 rxns	34107
LigandLink™ Covalent Hexachlorofluorescein Label	300 rxns	34108

Each LigandLink Kit contains 20 µg of vector, 20 µg of LigandLink pLL-1 NLS positive control, 100 reactions of LigandLink Fluorescein Label and one vial of DMF + acetic acid.
Each reaction of LigandLink Label is sufficient to label a single well of a 96-well plate.

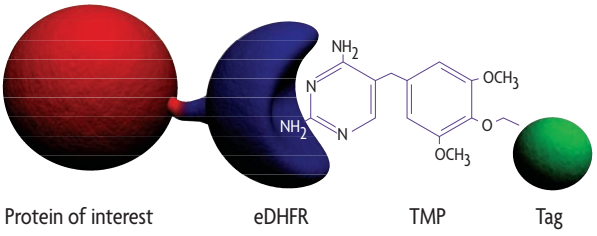

Figure 1: Specific protein labeling using LigandLink.

The gene of interest is cloned into pLL-1 in frame with the vector's *E. coli* dihydrofolate reductase (eDHFR) gene. After transfection into cells, the protein of interest is expressed as a fusion to eDHFR. Addition of cell-permeable LigandLink Label to the medium results in rapid, specific binding of the label by the fusion protein.

Introduction

Use of green fluorescent protein (GFP) has become commonplace in modern biology. However, biologically fluorescent proteins (FP) are limited by their inherent properties. For example, FPs have a relatively low quantum yield, and it is difficult to engineer their spectral properties to suit specific applications. Fluorescent dyes are a potential alternative to biological FPs as they are available in a broad variety of formats and can be engineered easily to ensure desired spectral properties. However, because fluorescent dyes are synthetic molecules it has not been possible to use them as a general tool for labeling specific proteins within a cell.

Active Motif's LigandLink Universal Labeling technology overcomes these limitations by providing a small ligand that can carry a variety of functional tags, including fluorescent dyes. Expression of your protein of interest as a fusion enables you to specifically label the protein of interest *in vivo* simply by adding one of the LigandLink labels to the medium*. To change the properties of your tag all you need to do is add a different LigandLink label. Thus LigandLink enables you to create a single protein fusion that can be labeled with a variety of tags, depending on the needs of your experiment.

The LigandLink Universal Labeling technology uses *E. coli* form of DHFR (eDHFR) and a number of trimethoprim (TMP) derivatives as a non-covalent ligand-receptor pair to provide a variety of functionalities[1]. The eDHFR protein was chosen as a fusion protein because it is a relatively small, monomeric protein (18 kDa vs 27 kDa for GFP) that has been shown to have a high affinity for the ligand TMP. TMP non-covalently binds with high specificity to eDHFR (K_1 = ~1 nm), and a substantially lower affinity for endogenous DHFR (K_1 = ~4 µm). This is because TMP is an antibiotic that was designed to specifically inhibit the bacterial enzymes responsible for the production of folic acid while not interacting with mammalian proteins. As there is minimal binding of LigandLink Labels to non-tagged mammalian proteins, this results in extremely low background. Moreover, TMP can be derivatized to carry a number of tags without substantially altering its affinity and specificity for eDHFR. Cell-permeable non-covalent LigandLink Labels are available with Fluorescein and Hexachlorofluorescein fluorescent dyes.

To complement our non-covalent LigandLink Universal Labeling technologies, Active Motif also offers covalent versions of our LigandLink ligand-receptor pairs. The covalent modifications are based on the principle of proximity-induced reactivity between two functional groups[1]. The covalent system uses a pLL eDHFR vector containing a cysteine mutation (eDHFR:L28C) that, when expressed, behaves as a nucleophile. The nucleophilic functional group is uniquely positioned to rapidly and irreversibly bind to an acrylamide electrophilic modification that has been appended to the LigandLink Covalent Label. A stronger covalent bond means prolonged labeling of your protein of interest, thus making this technology suitable for applications requiring a sustainable signal, such as single-molecule imaging and pulse-chase labeling. LigandLink Covalent Labels are available with Fluorescein and Hexachlorofluorescein fluorescent dyes.

* Patent pending.

LigandLink pLL-1 Vector Map

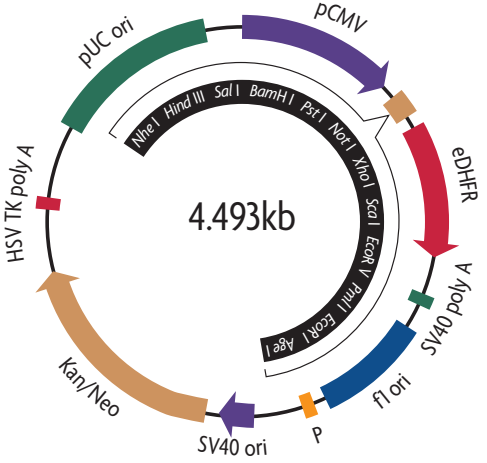

Figure 2: The LigandLink pLL-1 vector.

The LigandLink vector, pLL-1, was designed for ease of use. It features a CMV promoter for high level expression of eDHFR fusion proteins, with Neomycin for selection of stable cell lines. The multiple cloning site (MCS) was designed to facilitate cloning, whatever method you use. In addition to many popular restriction sites, the MCS includes three blunt-cutting restriction enzymes towards the 3´ end, each in a different reading frame with the eDHFR gene. This enables a number of PCR and restriction enzyme cloning strategies.

pLL-1 Vector Sequence Reference Points

Human cytomegalovirus (CMV) immediate early promoter	1-589
MCS	591-662
eDHFR	670-1170
SV40 early mRNA polyadenylation signal	1312-1345
f1 single-strand DNA origin	1409-1864
Bacterial promoter for expression of Kan^r gene	1926-1954
SV40 origin of replication	2205-2340
Kanamycin/neomycin resistance gene	2389-3183
Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal	3420-3437
pUC plasmid replication origin	3768-4411
pLL-1 FWD: 5´-TATTAGTCATCGCTATTACCATGGTG-3´	342-367
pLL-1 REV: 5´-CAGGTTCCACGGCATGGCGTT-3´	742-762

Sequence of pLL-1 MCS

Figure 3: The sequence of the pLL-1 vector illustrating the cloning and primer hybridization sites.

LigandLink pLL-1 NLS Vector Map

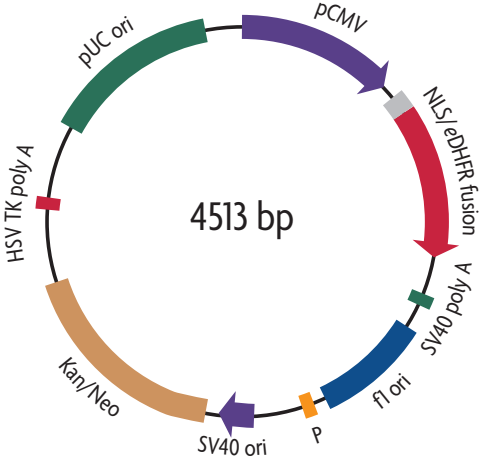

Figure 4: The LigandLink pLL-1 NLS vector.

To generate pLL-1 NLS, three copies of the canonical simian virus 40 T-antigen nuclear localization sequence (DPKKKRKV) were fused to the N-terminus of eDHFR in pLL-1 by preparing a double stranded oligonucleotide and ligating the NLS sequence into the *Nhe* I and *Age* I sites. This vector can be used as a positive control for nuclear staining as the NLS/eDHFR protein will translocate to the nucleus.

pLL-1 NLS Vector Sequence Reference Points

Human cytomegalovirus (CMV) immediate early promoter	1-589
Nuclear localization site (NLS) coding sequence:	603-677
eDHFR	690-1190
SV40 early mRNA polyadenylation signal	1332-1365
f1 single-strand DNA origin	1429-1864
Bacterial promoter for expression of Kan^r gene	1946-1974
SV40 origin of replication	2205-2340
Kanamycin/neomycin resistance gene	2409-3203
Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal	3440-3457
pUC plasmid replication origin	3788-4431
pLL-1 FWD: 5´-TATTAGTCATCGCTATTACCATGGTG-3´	342-367
pLL-1 REV: 5´-CAGGTTCCACGGCATGGCGTT-3´	762-782

Note: Covalent pLL-2 NLS is the covalent version of the pLL-1 NLS vector and has similar features to those annotated for pLL-1 NLS. pLL-1 FWD and pLL-1 REV primers are compatible with both pLL-1 NLS and Covalent pLL-2 NLS vectors.

Labeling of nuclear localized eDHFR by LigandLink Fluorescein

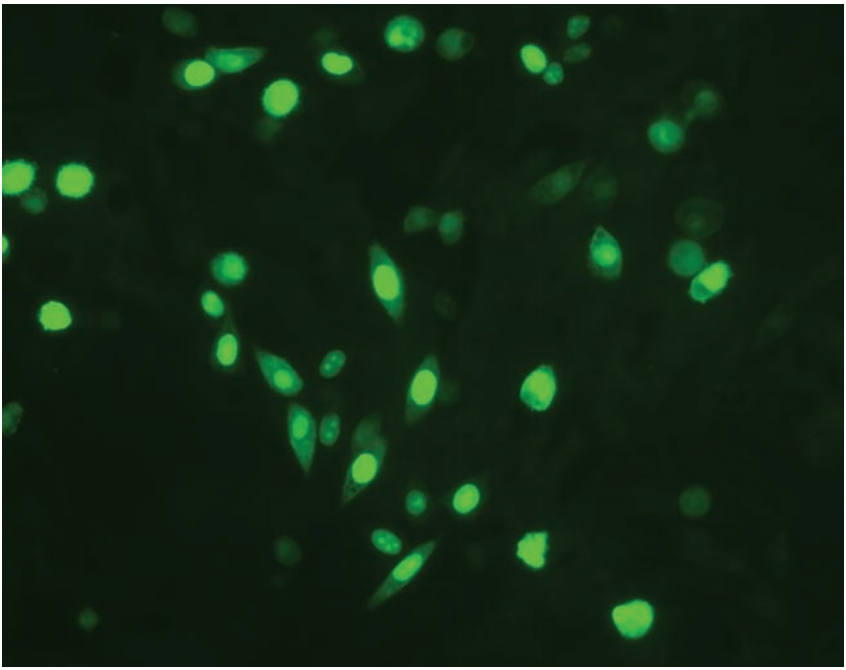

Figure 5: Labeling of nuclear localized eDHFR by LigandLink Fluorescein.

A nuclear localization sequence was cloned into pLL-1 and transfected into CHO cells. Twenty-four hours post transfection, 5 µM LigandLink Fluorescein was added to the cells for 2 hours at 37°C. Following wash steps to remove unbound label, the above image was taken.

Kit Components and Storage

Kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

LigandLink pLL-1 Kit	Quantity	Storage / Stability
pLL-1 Vector	20 µg	-20°C for 6 months
pLL-1 NLS (positive control vector for nuclear translocation)	20 µg	-20°C for 6 months
LigandLink Fluorescein Label	100 rxns	-20°C for 6 months
DMF + acetic acid	50 µl	4°C for 6 months

LigandLink Covalent pLL-1 Kit	Quantity	Storage / Stability
Covalent pLL-1 Vector	20 µg	-20°C for 6 months
Covalent pLL-2 NLS (positive control vector for nuclear translocation)	20 µg	-20°C for 6 months
LigandLink Covalent Fluorescein Label	100 rxns	-20°C for 6 months
DMF + acetic acid	50 µl	4°C for 6 months

Premade LigandLink Kits	Quantity	Storage / Stability
pLL-1 eDHFR Fusion	20 µg	-20°C for 6 months
pLL-1 NLS (positive control vector for nuclear translocation)	20 µg	-20°C for 6 months
LigandLink Fluorescein Label	100 rxns	-20°C for 6 months
DMF + acetic acid	50 µl	4°C for 6 months

LigandLink Labels*	Quantity	Storage / Stability
LigandLink Fluorescein Label	300 rxns	-20°C for 6 months
LigandLink Hexachlorofluorescein Label	300 rxns	-20°C for 6 months
DMF + acetic acid	50 µl	4°C for 6 months

LigandLink Covalent Labels*	Quantity	Storage / Stability
LigandLink Covalent Fluorescein Label	300 rxns	-20°C for 6 months
LigandLink Covalent Hexachlorofluorescein Label	300 rxns	-20°C for 6 months

* Each order of LigandLink Label is supplied with a DMF + acetic acid (150 µl). This may be stored at 4°C for 6 months. Each reaction of LigandLink Label is sufficient to label a single well of a 96-well plate.

The LigandLink Universal Labeling Kit is for research use only. Not for use in diagnostic procedures.

Additional Materials Required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Chambered covered glass slide or 96-well plate (or alternate seeding plate)
- Transfection Reagent
- Endotoxin-free (transfection-grade) plasmid DNA
- Fetal bovine serum (FBS)
- Serum-free cell culture medium
- Phosphate buffered saline (PBS, pH 7.4) containing Ca^{2+} and Mg^{2+}
- Glucose
- Sterile distilled water (dH_2O)
- Fluorescent microscope equipped with standard FITC filters
- 37°C cell culture incubator

Component Preparation and Recommendations

Prior to starting the assay please prepare the following:

Preparation of Plasmid DNA

Stock Solution: The pLL-1, pLL-1 NLS control, Covalent pLL-1, Covalent pLL-2 NLS control, and all premade pLL-1 vectors are supplied lyophilized as 20 µg aliquots. Prepare the DNA Stock Solution by resuspending the lyophilized DNA in 20 µl of sterile dH_2O in the provided vial. Stock concentration is 1 µg/µl. This Stock Solution can be stored at -20°C for 6 months.

Working Solution: Prepare the amount of plasmid DNA required for the assay by diluting the plasmid DNA in sterile dH_2O. For example, for an 8 well chamber slide, mix 1.5 µl of stock DNA with 23.5 µl of sterile dH_2O to generate a 0.06 µg/µl Working Solution.

Resuspension of LigandLink Fluorescent Label

The LigandLink Fluorescent Label is supplied lyophilized. The quantity of label supplied is 30 nmoles*. Prepare the LigandLink Fluorescent Label Stock Solution by resuspending the lyophilized label in 30 µl of DMF + acetic acid in the provided amber vial. This generates a 1 mM stock solution. This Stock Solution can be stored at -20°C for 6 months.

Note: Fluorescent dyes are light sensitive. Avoid exposing the cells to light during the ligand labeling and wash steps.

* For the 300-reaction aliquots of LigandLink Labels, three vials each with 30 nmoles of lyophilized label are provided.

Resuspension of LigandLink Covalent Fluorescent Label

The LigandLink Covalent Fluorescent Label is supplied lyophilized. The quantity of label supplied is 30 nmoles*. Prepare the LigandLink Covalent Fluorescent Label Stock Solution by resuspending the lyophilized label in 30 µl of DMF + acetic acid in the provided amber vial. This generates a 1 mM stock solution. This Stock Solution can be stored at -20°C for 6 months.

Note: Fluorescent dyes are light sensitive. Avoid exposing the cells to light during the ligand labeling and wash steps.

* For the 300-reaction aliquots of LigandLink Covalent Labels, three vials each with 30 nmoles of lyophilized label are provided.

Mammalian Cell Transfection

These conditions are recommended as guidelines only. The protocol below has been optimized for transfection of CHO-K1 cells (ATCC Cat. No. CCL-61) in an Lab Tek® 8-well chambered covered glass slide (Nalge Nunc Cat. No. 177402) using FuGENE® 6 Transfection Reagent (Roche Cat. No. 11 814 443 001). Please note that other transfection reagents may be used. You should empirically optimize the cell culture protocol, transfection conditions, ligand concentration and labeling protocol for your experimental system.

1. In an 8-well chambered covered glass slide, seed 1.6 x10^4 cells per well in 250 µl of complete growth medium.
2. Incubate the cells at 37°C in a humidified atmosphere containing 5% CO_2 until the cells are 90-95% confluent. For CHO-K1 cells this requires 16-20 hours.
3. In a microcentrifuge tube mix 88 µl of a serum-free culture media without additives or supplements with 4.4 µl FuGENE® 6 Transfection Reagent.

   Note: Do not touch the plastic walls of the tubes.
4. Tap the tube gently. Incubate at room temperature for 5 minutes.
5. Add 22 µl of the working DNA solution (see Preparation of Reagents above).
6. Tap the tube gently. Incubate at room temperature for 15 minutes.
7. Tap the tube gently again. Overlay 10 µl onto each well.
8. Incubate cells at 37°C in a humidified atmosphere containing 5% CO_2 overnight.

Cell Staining with LigandLink Label

This staining protocol is suitable for both non-covalent and covalent LigandLink Fluorescein Labels and LigandLink Hexachlorofluorescein Labels.

Note 1: After removing the LigandLink Label from -20°C, we recommend placing it at room temperature for 5 minutes before use. This minimizes moisture from entering the vial upon opening.

Note 2: Fluorescent dyes are light sensitive. Avoid exposing the cells to light during the labeling and wash steps.

Note 3: Only prepare the amount of diluted label required for the assay and use only freshly prepared diluted LigandLink Label.

Note 4: The concentration of LigandLink Label, the time of labeling, the wash conditions and the microscope exposure need to be optimized for each cell line.

Note 5: LigandLink Labels can be added directly to serum-containing medium.

Note 6: Use a standard FITC filter set to detect proteins labeled with LigandLink Fluorescein Label. Set excitation of 488 nm and emission between 500 and 550 nm. The optimal excitation and emission wavelengths for LigandLink Hexachlorofluorescein Label are 535 and 565 nm, respectively.

Note 7: Due to the acidity of the resuspended label, a slight color change may be observed in media that contains phenol red. This does not affect the results.

1. Dilute the LigandLink Label stock solution 1-5 µM in cell medium.
2. Add 200 µl of diluted label to each well of the 8-well chambered covered glass slide.
3. Incubate 2 hours at 37°C in a humidified atmosphere containing 5% CO_2.
4. Wash cells twice with 200 µl of PBS.
5. Add 200 µl of PBS supplemented with 10 mM glucose to each well of the 8-well chambered covered glass slide. We recommend that you use a PBS that contains both Ca^{2+} (~0.9 mM) and Mg^{2+} (~0.49 mM) to maintain healthy cells.
6. Transfer the chambered cover glass slide to a microscope and capture images.

References

1. Miller L.W. *et al.* (2005) *Nature Methods* 2(4): 255-257.
2. Gallagher S.S. *et al.* (2009) *ACS Chem. Biol.* 4(7): 547-556.

Appendix

Section A. Troubleshooting Guide

Problem/question	Possible Cause	Recommendation
No signal or weak signal	Fusion protein not expressed or expressed only at low levels	Check the reading frame of your construct by sequence analysis. Optimize transfection conditions and use high-quality, endotoxin-free DNA. Culture cells for a longer period of time before labeling to ensure that you have adequate protein expression. Protein expression can be improved by optimizing the health of the cells. Increase the seeding density or time of culture to allow cells to proliferate and adhere more tightly.
	Fusion protein stop codon not removed prior to cloning into pLL-1	Because the pLL-1 vector contains an MCS that is upstream of the eDHFR coding sequence, make sure the stop codon is removed from the protein of interest before cloning to ensure that the gene of interest will be in frame with the downstream eDHFR coding sequence (which contains its own stop codon).
	Insufficient LigandLink Label	Optimize cell-labeling protocols. Increase cell labeling time. Optimize the ligand concentration. Increase the exposure time while viewing cells under the microscope.
	Loss of labeling activity	Store the LigandLink Labels at -20°C and protect them from light so that they do not lose labeling activity. Use freshly prepared labeling solution to label your cells.
	Incorrect filter set	Ensure that you are using the correct filter set for viewing. Use a standard FITC filter set to detect proteins labeled with the LigandLink Fluorescein Label. Set excitation of 488 nm and emission between 500 and 550 nm. The optimal excitation and emission wavelengths for LigandLink Hexachlorofluorescein Label are 535 and 565 nm respectively.
	Ligand photobleaching	To prevent LigandLink Labels from photobleaching, analyze the fluorescent signal for only a short period of time.
High background fluorescence	Inadequate washing	Increase time for washing unbound ligand. Reduce label concentration. Reduce labeling time. Reduce exposure time while viewing cells under the microscope.
Cells detach from surface		When imaging live cells, handle cells carefully to ensure that they stay attached to the surface.
Altered cell morphology		Label cells in the dark so they are not exposed to intense light during labeling.

Section B. Related Products

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ Flexi NFκB Family	2 x 96-well plates	43298
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ NFκB p50 Chemi	1 x 96-well plate	41097
TransAM™ Flexi NFκB p50	1 x 96-well plate	41098
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p52 Chemi	1 x 96-well plate	48197
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ NFκB p65 Chemi	1 x 96-well plate	40097
TransAM™ Flexi NFκB p65	1 x 96-well plate	40098
TransAM™ STAT Family	2 x 96-well plates	42296
TransAM™ STAT3	1 x 96-well plate	45196

For a complete, up-to-date list of over 40 TransAM™ Kits, please visit **www.activemotif.com/transam**

In-cell Phospho-specific ELISAs	Format	Colorimetric Kit Catalog No.	Chemi Kit Catalog No.
FACE™ AKT	1 x 96 rxns	48120	48220
FACE™ NFκB p65 Profiler	3 x 96 rxns	48300	48400
FACE™ STAT2	1 x 96 rxns	48310	48410
FACE™ STAT4	1 x 96 rxns	48320	48420
FACE™ STAT6	1 x 96 rxns	48330	48430
FACE™ Maker	1 x 96 rxns	48000	48050
Suspension Cell FACE™	2 x 96 rxns	48305	48405

For a complete, up-to-date list of over 25 FACE™ Kits, please visit **www.activemotif.com/face**

Competent Cells	Format	Catalog No.
RapidTrans™ TAM1 Competent *E. coli*	1 x 96 rxns	43296
	5 x 96 rxns	43298

Competent Cells	Format	Catalog No.
Chariot™	25 rxns	30025
	100 rxns	30100

Fluorescent Dyes	Excitation / Emission	Format	Catalog No.
Chromeo™ 488 Carboxylic Acid	488 nm / 517 nm	1 mg	15510
Chromeo™ 488 NHS-Ester	488 nm / 517 nm	1 mg	15511
Chromeo™ 488 Antibody Labeling Kit	488 nm / 517 nm	1 kit	15090
Chromeo™ 494 Carboxylic Acid	494 nm / 628 nm	1 mg	15110
Chromeo™ 494 NHS-Ester	494 nm / 628 nm	1 mg	15111
Chromeo™ 494 Antibody Labeling Kit	494 nm / 628 nm	1 kit	15091
Chromeo™ 505 Carboxylic Acid	505 nm / 526 nm	1 mg	15610
Chromeo™ 505 NHS-Ester	505 nm / 526 nm	1 mg	15611
Chromeo™ 546 Carboxylic Acid	545 nm / 561 nm	1 mg	15210
Chromeo™ 546 NHS-Ester	545 nm / 561 nm	1 mg	15211
Chromeo™ 546 Antibody Labeling Kit	545 nm / 561 nm	1 kit	15092
Chromeo™ 642 Carboxylic Acid	642 nm / 660 nm	1 mg	15310
Chromeo™ 642 NHS-Ester	642 nm / 660 nm	1 mg	15311
Chromeo™ 642 Antibody Labeling Kit	642 nm / 660 nm	1 kit	15093

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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