

ELF® 97 Cytological Labeling Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
Wash buffer (Component A)	200 mL	10X	<ul style="list-style-type: none"> • ≤-20°C • Protect from light 	When stored as directed, kit components are stable for ~6 months.
Blocking buffer (Component B)	50 mL, containing 2 mM sodium azide	NA		
Substrate additive 1 (Component E)	200 µL in DMSO			
Developing buffer (Component C)	200 mL			
ELF® 97 phosphatase substrate (Component D)	1 mL			
Substrate additive 2 (Component F)	200 µL			
Mounting medium (Component G)	15 mL			
Streptavidin–alkaline phosphatase conjugate * (Component H)	300 µL		<ul style="list-style-type: none"> • 2–6°C • Do not freeze • Protect from light 	

* This streptavidin–alkaline phosphatase conjugate has been specially formulated and tested for use in ELF® applications; it is distinct from our product S921.

Approximate fluorescence excitation/emission maxima: 345/530 nm

Introduction

Invitrogen's Molecular Probes™ ELF® 97 Cytological Labeling Kit (E6603) provides a unique signal amplification technology to facilitate the detection of cellular targets, including cell-surface receptors and subcellular structures. The ELF® 97 Kit contains our patented ELF® 97 phosphatase substrate, which yields a brightly fluorescent yellow-green precipitate at the site of enzymatic activity—a process we call **Enzyme-Labeled Fluorescence (ELF®)**.¹ Essentially any cellular structure that can be specifically recognized and bound to a hapten-bearing ligand can be labeled using our ELF® 97 Cytological Labeling Kit. For example (Figure 1), target antigen can be labeled by the sequential application of a specific primary antibody, biotinylated secondary antibody, streptavidin-alkaline phosphatase, and ELF® 97 phosphatase substrate. The ELF® 97 precipitate signal exhibits an extremely photostable fluorescent signal that has an unusually large Stokes shift (Figure 2), making the kit ideal for detecting cellular structures in autofluorescent samples or as part of a multiparameter labeling experiment in which several fluorophores are detected simultaneously.²⁻⁵

The ELF® amplification technology offers several advantages over conventional colorimetric and radioactive detection methods. ELF® signal localization has been optimized by visualizing fine subcellular structures, including microtubules and actin filaments. Using the kit, we have found that microtubules and actin filaments show ultrafine microscopic resolution, rivaling and often surpassing that achieved with direct fluorophore-labeled probes. Furthermore, labeling intracellular structures or cell-surface receptors with ELF® 97 precipitate results in signals that are many times brighter and more photostable than those produced by direct fluorophore conjugates (Figure 3).⁶ In our experiments, ELF® precipitate–labeled samples typically require only a one-second exposure for photography, whereas fluorescein-labeled samples require at least a 45-second exposure to achieve a similar brightness. Moreover the short exposure times produce bright ELF® signals with negligible background fluorescence, while the long exposures of fluorescein-labeled samples often result in unacceptably high background levels.

The ELF® 97 Cytological Labeling Kit provides a complete set of reagents for using the ELF® 97 phosphatase substrate to detect binding of biotinylated or hapten-labeled ligands. It also provides a streptavidin–alkaline phosphatase conjugate for detecting biotinylated antibodies (B2763, B2770), biotinylated ligands, or fluoresceinated probes used in combination with biotinylated anti-fluorescein antibodies (A982) or dinitrophenylated probes used in combination with biotinylated anti-dinitrophenyl (A6435). The enzyme conjugate is included. The kit provides researchers the flexibility of choosing a wide array of labeled probes for specific applications.

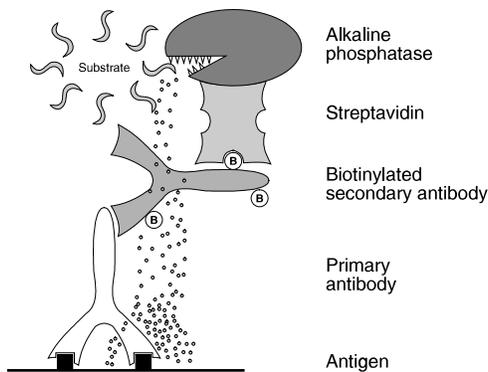


Figure 1. Schematic diagram of the methods employed in our ELF® 97 Cytological Labeling Kit. Samples are probed with biotinylated or haptenylated target-specific probes such as antibodies or ligands. Next, alkaline phosphatase conjugates of streptavidin or the hapten-specific probe are applied. The sample is then incubated with the ELF® 97 phosphatase substrate, which forms an intense yellow-green fluorescent ELF® 97 alcohol precipitate at the site of alkaline phosphatase activity.

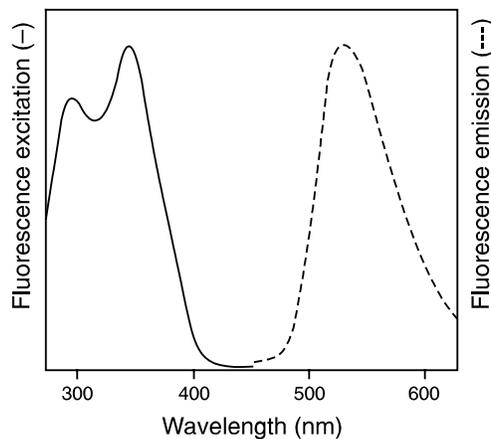


Figure 2. The normalized excitation and emission spectra of the ELF® 97 alcohol precipitate, which is generated by enzymatic cleavage of the soluble ELF® 97 phosphatase substrate.

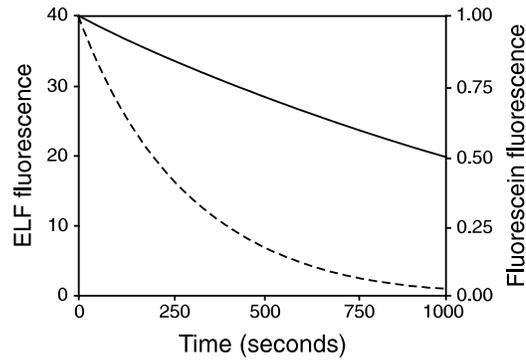


Figure 3. Comparison of the fluorescence output for direct fluorescein conjugates (---) versus that of ELF® 97 precipitate (—) in the detection of actin stress fibers. Acetone-fixed mouse fibroblasts were treated with Triton X-100 and then incubated with either biotin phalloidin followed by detection using our ELF® 97 Cytological Labeling Kit or with fluorescein phalloidin. ELF® 97 alcohol-labeled samples were illuminated with a 100-Watt mercury-arc lamp using a 60X 1.4 NA Planapochromat lens (Nikon) and the ELF® 97 filter set (O5705); Star 1 CCD camera (Photometrics) images were acquired at 100-second intervals with 0.4-second exposures and the camera gain at 1. Fluorescein-labeled samples were imaged using the same lens and a standard fluorescein filter set; images were acquired at 96-second intervals with 4-second exposures and a camera gain of 4. All samples were continuously illuminated for a total of 10 minutes, while the mercury lamp was attenuated with a 2.0 OD neutral-density filter. The fluorescence intensity at each time point was integrated over the frame, with the threshold of the signal set above background using Image-1 software (Universal Imaging Corp.). Curves were fit to single exponential functions derived from the averaged decay rates. The average decay rate for fluorescein under these conditions was constant, while the average decay rate for the ELF® 97 signal appeared to vary continuously at a rate that may be related to crystal size and heterogeneity. Thus, the ELF® 97 curve represents the average of these decay rates.

Before You Begin

Caution

Component E (substrate additive 1) is dissolved in DMSO and should be handled with appropriate safety precautions and disposed of in accordance with all local regulations.

Materials Required but Not Provided

- Levamisole, if significant endogenous phosphatase activity is anticipated
- Spin filtration filters (such as Invitrogen Cat. no. E6606)
- Cytoskeletal-stabilizing buffer (see step 3.3 and step 4.3 for *Labeling Actin Filaments* and *Labeling Microtubules*)
- Biotin phalloidin (Invitrogen Cat. no. B7474 for *Labeling Actin Filaments*)
- Counterstain such as DAPI or Hoechst 33342 for *Labeling Actin Filaments* and *Labeling Microtubules*
- Paclitaxil (optional for *Labeling Microtubules*, see step 4.2)
- Anti-tubulin antibody (Invitrogen Cat. no. A11126 for *Labeling Microtubules*)
- Biotinylated goat anti-mouse IgG (Invitrogen Cat. no. B2763 for *Labeling Microtubules*)
- Primary antibody, biotinylated primary antibody, biotinylated lectin, or biotinylated receptor probes for *Labeling Cell-Surface Receptors on Adherent Cells* and *Labeling Cell-Surface Receptors on Cells in Suspension*
- Secondary antibody for *Labeling Cell-Surface Receptors on Adherent Cells* and *Labeling Cell-Surface Receptors on Cells in Suspension*

Overview and General Considerations

The ELF® 97 Cytological Labeling Kit provides the reagents necessary for detecting haptently-ated phallotoxins, antibodies, lectins, or receptor probes bound to specific cellular structures. This kit is appropriate either when the primary detection reagent is biotinylated, as in the case of biotinylated receptor probes, or when the secondary detection reagent is biotinylated, as in the case of an unlabeled primary antibody used in conjunction with a biotinylated secondary antibody.

The following four protocols are provided as examples of using the kit to detect: actin filaments with biotin phalloidin (*Labeling Actin Filaments*), microtubules with anti-tubulin antibody in conjunction with biotinylated secondary antibody (*Labeling Microtubules*) and cell-surface receptors on fixed cells with biotinylated receptor probes (*Labeling Cell-Surface Receptors on Adherent Cells* and *Labeling Cell-Surface Receptors on Cells in Suspension*).

Do not allow the slides or coverslips to dry completely during any of the incubation steps.

If the sample is expected to contain significant endogenous phosphatase activity, we recommend incubating samples, just prior to adding the streptavidin–alkaline phosphatase conjugate, for 1 hour in 1 mM levamisole diluted in the blocking buffer. Remove the solution and apply the streptavidin–alkaline phosphatase conjugate as described.

Preparing the ELF® Reagent

- 2.1 **1X Wash buffer.** Dilute the 10X wash buffer (Component A) 10-fold with sterile deionized water. Store at 2–6°C in a sterile container. This solution can be stored for up to six months.
- 2.2 **Substrate working solution.** Dilute the amount of ELF® 97 phosphatase substrate (Component D) needed for the day's experiments 20-fold into developing buffer (Component C). Filter the resulting solution into a sterile vial using a 0.2 µm pore-size filter. Invitrogen offers 0.2 µm pore-size spin-filtration devices—ELF® spin filters (E6606)—that allow a very small volume to be filtered without significant loss of sample. The solution is simply added to the ELF® spin filter and then centrifuged in a microcentrifuge for 10 seconds. For filtering larger volumes, we recommend a syringe filter. After filtration, dilute substrate additive 1 (Component E) and substrate additive 2 (Component F) each 1:1000 into the diluted substrate working solution. Vortex the solution well and use it immediately, or store it at 2–6°C in a sterile container for up to 48 hours. For best results, prepare the substrate working solution fresh for each day's experiments. If the solution is stored for more than one day, it should be refiltered through a 0.2 µm pore-size filter to remove any precipitate before use.

Note: We provide an excess of the developing buffer and substrate additives.

Experimental Protocols

Labeling Actin Filaments

- 3.1 Grow adherent cells on slides or coverslips according to standard procedures. Rinse well with phosphate-buffered saline (PBS).
- 3.2 Fix the cells for 5 minutes at room temperature by immersing the slides in a glass staining dish containing acetone. Air-dry the slides. Rehydrate by incubating in PBS for 10–15 minutes at room temperature. At this stage, it is useful to examine the cells under the microscope to check for healthy morphology and adequate cell density.

- 3.3 Permeabilize the membranes further by incubating the slides in a tissue culture dish containing ice-cold cytoskeletal-stabilizing buffer (60 mM PIPES, 25 mM HEPES, pH 6.9, 10 mM EDTA, 1 mM MgCl₂) that has been supplemented with 0.2% Triton X-100. Note that the Triton X-100 must be vortexed vigorously in the cytoskeletal-stabilizing buffer to ensure that it is fully dissolved. Use 3 mL of the solution for a 60 mm tissue culture dish or 5 mL of the solution for a 100 mm dish. Incubate for 5 minutes with gentle agitation.
- 3.4 Wash the slides three times, for 5 minutes each time, with ice-cold cytoskeletal-stabilizing buffer.
- 3.5 Reconstitute biotin phalloidin (B7474) in methanol as indicated in the product manual for that product. Withdraw an appropriate aliquot to a small vial and evaporate all methanol using nitrogen gas; ensure that the sample is completely dry, as residual methanol can interfere with labeling. Resuspend the biotin phalloidin in PBS at a ratio of 100 µL PBS per unit of phalloidin. Apply biotin phalloidin to the slides and incubate for 15–30 minutes at room temperature in a humid chamber.
- 3.6 Wash the slides three times, for 5 minutes each time, with PBS. Drain off excess PBS.
- 3.7 Dip the slides in 1X wash buffer to remove traces of PBS. Drain briefly.
- 3.8 To each sample, apply 100 µL of a 1:50 dilution of streptavidin–alkaline phosphatase conjugate in the blocking buffer (dilute Component H 1:50 in blocking buffer). Incubate for 15 minutes at room temperature in a humid chamber.
- 3.9 Wash the slides three times, for 5 minutes each time, in 1X wash buffer.
- 3.10 Drain off the excess 1X wash buffer, using a tissue to wick all possible buffer from slides.
This step is very important.
- 3.11 To each sample, apply 100 µL of substrate working solution (see step 2.2). Incubate for 4 minutes. Remove excess solution before flipping coverslips or viewing slides.
- 3.12 To preserve the signal, wash the slides quickly with 1X wash buffer. Post-fix samples by incubating the slides in PBS/2% formaldehyde containing 20 mg/mL BSA for 15–30 minutes without agitation. This post-fixation solution can be used immediately or filter-sterilized and stored at 2–6°C.
- 3.13 Mount the samples with mounting medium (Component G), using ~1 drop per coverslip or 2–3 drops per slide. When counterstaining the samples, please note that this mounting medium reduces the signals from the blue fluorescent nucleic acid stain DAPI so higher staining concentrations are recommended. Alternatively, Hoechst 33342 is very compatible with this mounting medium.

Labeling Microtubules

The following protocol is provided as an example of using the ELF[®] 97 Cytological Labeling Kit to detect cellular structures that have been specifically labeled with an antibody. We recommend following your own immunological labeling protocol for the primary antibody incubation and then continuing with the protocol described below at step 4.6. We have successfully used this protocol to detect Golgi membranes, nuclei, and mitochondria using organelle-specific antibodies.

- 4.1 Perform steps 3.1–3.4 exactly as above.
- 4.2 Place the slides or coverslips in a tissue culture dish containing a final concentration of 2.5 µM paclitaxel (P3456) and 10 µg/mL GTP in cytoskeletal-stabilizing buffer. Use 3 mL of this solution for a 60 mm tissue culture dish or 5 mL of the solution for a 100 mm dish. Incubate for 1 hour at room temperature with gentle agitation.

Note: Paclitaxel is not absolutely required for labeling microtubules; however, for many cell types, paclitaxel stabilizes microtubules so that they can be visualized. For example, we have found that approximately 10-fold more microtubule structures are visible in mouse fibroblasts treated with paclitaxel than in untreated fibroblasts. Other cell types, such as neurofilaments and brain tissues, have abundant microtubules and do not require such stabilizers.

- 4.3 Wash the slides three times with cytoskeletal-stabilizing buffer (60 mM PIPES, 25 mM HEPES, pH 6.9, 10 mM EDTA, 1 mM MgCl₂).
- 4.4 To block nonspecific binding sites, apply 100 µL of 1% BSA in PBS containing 0.1% Tween 20 to each slide. Incubate for 30 minutes at 37°C in a humid chamber. Remove the blocking solution.
- 4.5 Add 100 µL of anti-tubulin (A11126) diluted in PBS/1% BSA to each slide. The appropriate dilution of antibody for optimal labeling must be determined empirically. Incubate for 1 hour at room temperature.
- 4.6 Wash the slides or coverslips three times with PBS.
- 4.7 Apply 100 µL of a 1:50 dilution of biotinylated goat anti-mouse IgG (B2763) in PBS/1% BSA to each slide. Incubate for 30 minutes at room temperature.
- 4.8 Wash three times, for 5 minutes each time, with PBS/0.1% Tween 20.
- 4.9 Dip the slides or coverslips briefly in 1X wash buffer.
- 4.10 Apply 100 µL of a 1:50 dilution of the streptavidin–alkaline phosphatase conjugate in the blocking buffer to each slide (dilute Component H 1:50 in blocking buffer). Incubate the slides for 15 minutes at room temperature.
- 4.11 Wash the slides three times, for 5 minutes each time, at room temperature with the 1X wash buffer. Drain slides briefly.
- 4.12 Apply 100 µL of the substrate working solution (see step 2.2) to each slide. Incubate for 5 minutes at room temperature. Remove excess substrate by draining the slides and touching the edges to absorbent tissue before viewing.
- 4.13 If samples are to be preserved, post-fix as described in step 3.12.
- 4.14 Mount samples as described in step 3.13.

Labeling Cell-Surface Receptors on Adherent Cells

The following protocol was designed for fixed cells. Because many of the ELF® 97 Kit buffers have high ionic strength, they may negatively affect cell physiology and morphology of live cells.

Note: If you are developing a protocol for detecting cell-surface receptors in live cells, we recommend omitting the Tween 20 from the PBS/1% BSA solutions.

- 5.1 Grow adherent cells on slides or coverslips according to standard procedures.
- 5.2 Fix the cells according to standard methods. We suggest incubating the cells in PBS containing 3.7% formaldehyde for 15 minutes at room temperature.
- 5.3 To block nonspecific binding sites, apply 100 µL PBS/1% BSA/0.1% Tween 20 to each slide and incubate for 30 minutes at room temperature. Remove the blocking solution by draining the slides and touching the edges to absorbent tissue before viewing.
- 5.4 Apply the appropriately diluted primary antibody, biotinylated primary antibody, biotinylated lectin, or biotinylated receptor probe in 100 µL PBS/1% BSA/0.1% Tween 20 to each slide. Incubate the slides for 30–60 minutes at room temperature.

- 5.5 Wash the slides three times with PBS. If a biotinylated primary detection reagent is used in step 5.4, then proceed to step 5.8. If the primary detection reagent is not biotinylated, proceed to step 5.6.
- 5.6 Apply an appropriately diluted secondary antibody in PBS/1% BSA/0.1% Tween 20 to each slide and incubate for 30–60 minutes more at room temperature.
- 5.7 Wash the slides three times with PBS and then dip the slides briefly in the 1X wash buffer to remove residual PBS.
- 5.8 Apply 100 μ L of a 1:50 dilution of the streptavidin–alkaline phosphatase conjugate in the blocking buffer to each slide (dilute Component H 1:50 in blocking buffer). Incubate for 15 minutes at room temperature.
- 5.9 Wash the slides three times, for 5 minutes each time, at room temperature with 1X wash buffer.
- 5.10 Apply 50 μ L of the substrate working solution (see step 2.2) to each sample. Incubate for 5 minutes at room temperature prior to viewing.
- 5.11 To preserve the samples, post-fix as described in step 3.12.
- 5.12 Mount samples as described in step 3.13.

Labeling Cell-Surface Receptors on Cells in Suspension

The following protocol was designed for fixed cells. Because many of the ELF® 97 Kit buffers have high ionic strength, they may negatively affect cell physiology and morphology of live cells.

Note: If you are developing a protocol for detecting cell-surface receptors in live cells, we recommend omitting the Tween 20 from the PBS/1% BSA solutions.

- 6.1 Grow the cells in suspension or on tissue culture dishes according to standard procedures.
- 6.2 If the cells are adherent, trypsinize using standard methods and wash 2–3 times with PBS to remove trypsin solution.
- 6.3 Fix the cells according to standard methods. We suggest incubating in PBS containing 3.7% formaldehyde for 15 minutes at room temperature.
- 6.4 Pellet the cells and resuspend in PBS/1% BSA/0.1% Tween 20 to achieve a concentration of about 10^6 cells/mL. Incubate the cells for 60 minutes at the appropriate temperature to block nonspecific binding sites.
- 6.5 Add the appropriately diluted primary antibody, biotinylated primary antibody, biotinylated lectin or biotinylated receptor probe directly to cell suspension and mix well but gently. Incubate for 60 minutes at the appropriate temperature.
- 6.6 Pellet the cells at 1,100 rpm for 10 minutes in a clinical tabletop centrifuge. Remove the supernatant and resuspend the cell pellet very gently in 1–2 mL PBS. Repeat the centrifugation and resuspension twice to wash the cells.
- 6.7 If the primary detection reagent is biotinylated, proceed to step 6.11. If the primary detection reagent is not biotinylated, proceed to step 6.8.
- 6.8 Resuspend the cell pellet in PBS/1% BSA/0.1% Tween 20 at a concentration of 10^6 cells/mL. Incubate the cells for 30 minutes at the appropriate temperature.
- 6.9 Add an appropriate dilution of the biotinylated secondary antibody directly to the cell suspension. Incubate the suspension for 60 minutes at the appropriate temperature.

- 6.10 Wash the cells three times by centrifuging at 1,100 rpm in a clinical tabletop centrifuge, followed by decanting the supernatant and gently resuspending the cells in 1–2 mL PBS.
- 6.11 Resuspend the final cell pellet at a concentration of 4×10^6 cells/mL in blocking buffer. Incubate the cells for 30–60 minutes at room temperature.
- 6.12 Remove 0.25 mL of the cell suspension to a fresh tube for ELF® 97 phosphate labeling.
- 6.13 Add 5 µL of the streptavidin–alkaline phosphatase conjugate (Component H) to the cell suspension. Mix gently. Incubate the suspension for 15 minutes at room temperature.
- 6.14 Wash the cells three times by centrifugation and resuspension as in step 6.10.
- 6.15 Resuspend the final cell pellet in 0.5 mL substrate working solution (see step 2.2). Incubate the cells for 5 minutes at room temperature.
- 6.16 Pellet the cells by centrifugation at 1,100 rpm for 10 minutes in a clinical tabletop centrifuge. Remove the supernatant.
- 6.17 Resuspend the cell pellet very gently in 1–2 mL PBS, taking care to not dislodge the crystals. Centrifuge again, as described above. Wash the cells two more times with 1–2 mL PBS each time.
- 6.18 Visualize the cells using a microscope or analyze using a flow cytometer.

References

1. U.S. Patent No. 5,316,906; 2. J Histochem Cytochem 43, 77 (1995); 3. J Clin Microbiol 34, 798 (1996); 4. J Histochem Cytochem 45, 345 (1997); 5. Trends Genet 12, 385 (1996); 6. Mol Biol Cell Suppl 4, 226a, abstract #1313 (1993).

Product List

Current prices may be obtained from our website or from our Customer Service Department.

Cat #	Product Name	Unit Size
E6603	ELF® 97 Cytological Labeling Kit *with streptavidin, alkaline phosphatase conjugate* *50 assays*	1 kit
E6606	ELF® spin filters *20 filters*.....	1 box

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probetech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License No. 223: Labeling and Detection Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2006, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.