PerkinElmer Life and Analytical Sciences

Phos-tools™

Phos-tag™ 540 Phosphoprotein Blot Stain
(PRD510A001KT)

Phos-tag™ 300/460 Phosphoprotein Blot Stain
(PRD410A001KT)

For Laboratory Use Only
Caution: Research Chemicals for Research Purposes Only

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I. PRODUCT NAME

Phos-tag™ 540 Phosphoprotein Blot Stain

(Cat. # PRD510A001KT)

Phos-tag™ 300/460 Phosphoprotein Blot Stain

(Cat. # PRD410A001KT)

II. INTENDED USE

Phos-tag™ phosphoprotein blot stains are complete kits for the selective detection of phosphorylated proteins transferred to PVDF electroblot membrane.

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III. INTRODUCTION

Most cellular processes are regulated by reversible phosphorylation of proteins, which in turn play a critical role in the regulation of cell signaling, metabolic maintenance, gene expression, cell division, signal transduction, and apoptosis. PerkinElmer's proprietary Phos-tag phosphoprotein blot stains provide a high sensitivity approach for the selective detection of phosphoproteins separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. Selective staining of phosphoproteins

with Phos-tag blot stain permits their simple identification, which is critically important for the characterization of protein kinase substrates, as well as for revealing the on/off state of various signal transduction pathways. The staining procedure is simple, reliable and robust, requiring as little as 2-3 hours to complete. Phos-tag phosphoprotein blot stains are capable of detecting as little as 5 ng of phosphoprotein per band, depending upon the phosphorylation state of the protein. The signal strength is linear over three orders of magnitude when plotted against the mass of phosphoprotein. Phos-tag 540 Phosphoprotein Blot Stain has excitation/emission maxima of 540 nm and 570 nm, respectively. Phos-tag 300/460 Phosphoprotein Blot Stain has dual excitation maxima of 300 and 460 nm, with a broad emission maximum centered at approximately 630 nm. Thus, the two stains provide detection solutions for the majority of imaging devices available. Counterstaining with a total protein stain is an excellent way to differentiate between an abundant protein that is lightly phosphorylated and a low-abundance protein that is heavily phosphorylated. Once phosphoproteins are detected with one of the Phos-tag phosphoprotein blot stains, the total protein profile may be detected using any of a variety of general protein stains. Numerous total protein stains for electroblot membranes are commercially available, including Ponceau Red, Amido Black, Coomassie Blue, colloidal gold and various fluorescent stains. It is not necessary to destain the blot or repeat the fixing step before counterstaining. Block the membrane according to the protocol for the counterstain. Alternatively, antibody-based immunodetection of specific proteins may be performed after staining with a Phos-tag phosphoprotein blot stain. For example, once total phosphoproteins have been identified, phosphotyrosine-containing phosphoproteins may be highlighted using an anti-phosphotyrosine monoclonal antibody and Western Lightning Western Blot Chemiluminescence Reagent (NEL105001EA). The Western Lightning Chemiluminescence Re-

agent Plus is a non-radioactive light-emitting system designed to detect proteins immobilized on a membrane. The method provides a sensitivity of 1 - 10 pg of protein and yields fast, permanent, hard-copy results on Kodak X-Omat Blue Autoradiography Film or on a commercially available imaging workstation.

IV. PRINCIPLES OF THE PROCEDURE

Phos-tag phosphoprotein blot stains are a new family of phosphoprotein-selective dyes based upon 1,3-bis[bis(pyridin-2-ylmethyl) amino] propan-2-ol, a highly selective Zn(II) ion chelator operating at neutral pH. The chelator is readily coupled to a variety of fluorophores, offering the ability to match spectral properties of a particular stain with a wide variety of available fluorescent electroblot membrane imaging platforms. Currently, two stains are available; Phos-tag 540 and Phos-tag 300/460 Phosphoprotein Blot Stains.

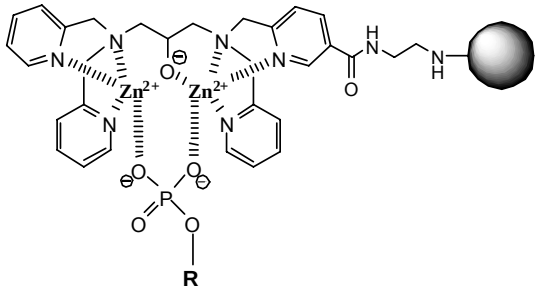

Figure 1. *The basic Phos-tag structure can readily be coupled to other functional groups, including various fluorophores.*

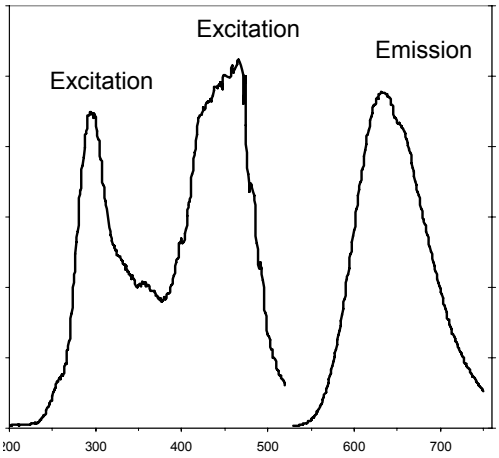

Figure 2. Excitation and Emission Spectra for Phos-tag 300/460

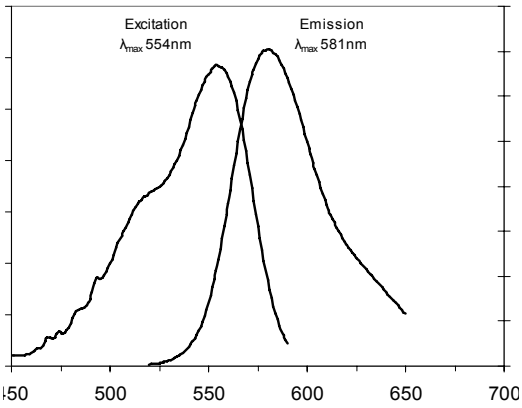

Figure 3. *Excitation and Emission Spectra for Phos-tag 540*

Phos-tag selectively binds to the phosphomonoester residues of phosphoserine, phosphothreonine and phosphotyrosine residues via a charge-based coordination of chelated Zn^{2+} cations. Interaction with other anionic residues, including carboxylate residues on proteins, is insignificant. Binding to the phosphomonoester group of phosphoproteins minimizes sequence context differences in binding,

often encountered using antibody-based detection approaches, making Phos-tag stain a universal detector of protein phosphorylation status. The binding affinity of the Phos-tag reagent is roughly 25 nm, making it more than three orders of magnitude stronger than other approaches based upon conventional immobilized metal affinity interactions involving Ga^{3+} or Fe^{3+} cations.

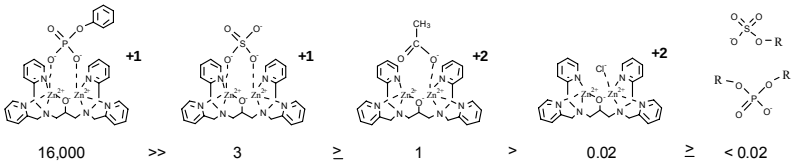

Figure 4. Phos-tag™ reagent is highly selective for the phosphomonoester residues of phosphoserine, phosphothreonine and phosphotyrosine. There is exceptionally low competition from other charged species. (Data provided by NARD Institute, Ltd., Japan)

V. KIT COMPONENTS

Reagents supplied are intended FOR LABORATORY USE ONLY.

Phos-tag™ 540 Phosphoprotein Blot Stain

(Cat. # PRD510A001KT)

- Phos-tag 540 Stain Concentrate

  4 vials (each 1 mL vial is enough for 4 mini-gel blots)
- Phos-tag 540 Blocking Buffer (4X), 1 bottle, 100 mL
- Phos-tag 540 Blot Stain Buffer (1X), 2 bottles, 200 mL each
- Phos-tag 540 Wash Buffer (2X), 2 bottles, 800 mL each

Phos-tag™ 300/460 Phosphoprotein Blot Stain

(Cat. # PRD410A001KT)

- Phos-tag 300/460 Stain Concentrate

  4 vials (each 1 mL vial enough for 4 mini-gel blots)
- Phos-tag 300/460 Blocking Buffer (4X), 1 bottle, 100 mL
- Phos-tag 300/460 Blot Stain Buffer(1X), 2 bottles, 200 mL each
- Phos-tag 300/460 Wash Buffer (2X), 2 bottles, 800 mL each

Storage and Stability

Kits are shipped at ambient temperature. Upon receipt, Phos-tag stain concentrates should be stored at 4°C, protected from light. All other buffers in the kit may be stored at ambient temperature. When stored properly, the stain will be stable through the expiration date printed on the package.

VI. ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Reagents and consumables:

- Methanol, Reagent Grade or higher
- Glacial Acetic Acid, Analytical Grade or higher
- Deionized water, 18 megaohm-cm or equivalent
- Powder-free latex or vinyl gloves
- Staining containers, polypropylene, polyvinyl chloride or polycarbonate

Equipment:

- Rotary shaker

Phos-tag 540 dye-compatible electroblot membrane imagers with appropriate excitation source (see Appendix for imager setting recommendations):

- Solid State YAG Laser; 532 nm
- HeNe Laser; 543 nm
- Xenon lamp with 540 nm excitation filter

Phos-tag 300/460 dye-compatible electroblot membrane imagers with appropriate excitation source. (see Appendix for imager setting recommendations):

- UV Transilluminator 302 nm
- Blue LED Laser; 457 nm
- Argon Laser; 488 nm
- Xenon lamp with 460 nm excitation filter

VII. REAGENT PREPARATION AND ASSAY PROTOCOL FOR PVDF BLOTS

The outlined protocol works reliably for PVDF membranes. This kit is not suitable for staining nitrocellulose electroblot membranes. As required with any high sensitivity staining procedure, membranes should never be handled with bare hands or metal forceps during processing. Powder-free disposable gloves should be worn during handling of the blots.

Polypropylene dishes, such as Rubbermaid Servin' Savers® or Stain Shield® containers, are optimal for staining because the high-density plastic adsorbs only a minimal amount of the dye. Clean and rinse the staining containers well before use, as detergent will interfere with staining. Ideally, use containers dedicated to Phos-tag staining, but minimally it is a good idea to briefly rinse the containers with 70% ethanol and allow them to air dry prior to their use. For small blots, circular staining dishes provide the best fluid dynamics on orbital shakers, resulting in less dye aggregation and better staining. For large membranes, polyvinyl chloride photographic staining trays, such as Photoquip Cesco-Lite 8 × 10 inches photographic trays work well. Glass dishes are not recommended as they have a tendency to bind dye.

If a staining tray is used that is significantly larger than the dimensions of the blot, more solution is required to compensate.

The volumes in this protocol are the minimum volumes required. The blot should be free-floating, protein side down with solutions covering both sides of the membrane. If a large tray is used, more solution is required.

For unambiguous interpretation of results, it is suggested that a lane of broad range molecular weight markers be included on each polyacrylamide gel which is transferred to PVDF and detected with Phos-tag Phosphoprotein Blot Stain. Markers should be selected that contain the phosphoprotein ovalbumin (45 kDa) in them to serve as a positive control. Typically, other proteins in the marker mix, such as bovine serum albumin (67 kDa) serve as negative controls for the staining procedure. Pre-stained molecular weight markers are not suitable for this application.

A. Fixing the Membrane

1. After the transfer of a gel by standard electroblotting methods, allow the membrane to air dry.
2. To re-wet the membrane, soak briefly in 100% ethanol or methanol. Discard the alcohol and then agitate the membrane in deionized water for a few minutes to completely hydrate the membrane.
3. Fix the mini-gel electroblot in 40 mL of freshly prepared 10% methanol/ 7% acetic acid. Agitate in the fixative with the membrane oriented protein side down for 15 minutes.
4. Discard the fixer and wash the blot in 40 mL of deionized water for 5 minutes to remove fixing solution.
5. Repeat wash step 3 times with fresh deionized water for a total of 4 washes. It is important that the blot be completely immersed in the deionized water in order to remove all of the methanol and acetic acid from the membrane which can interfere with Phos-tag phosphoprotein blot staining.

B. Blocking the Membrane

1. Dilute 6.25 mL of Blocking Buffer (4X) with 18.75 mL of deionized water to make 25 mL of 1X Blocking Buffer.
2. Agitate the membrane in Blocking Buffer (1X) for 1 hour to overnight. The blot should be free-floating, and oriented protein side down with blocking solution covering both faces of the membrane.

C. Staining the Membrane

1. Prepare Phos-tag Stain by diluting 250 µL of Phos-tag Dye Concentrate 1:100 with Stain Buffer for a total of 25 mL. Protect the stain from light until use. If more stain than needed was

reconstituted, unused stain may be stored at 4°C, protected from light, for one week.
2. Wash membrane briefly in deionized water to remove excess blocking reagent.
3. Agitate the membrane oriented protein side down in prepared stain for 1 hour in the dark.
4. Dilute 60 mL of Destain Buffer (2X) with 60 mL of deionized water.
5. Discard stain solution and agitate the membrane in 40 mL of Destain Buffer (1X) for 5 minutes.
6. Discard Destain Buffer and repeat step 5 twice for a total of 3 washes.
7. Rinse the membrane briefly in deionized water.
8. Allow the membrane to air-dry before imaging.

D. Imaging the Membrane

Phos-tag dyes are compatible with a wide range of imaging systems (See appendix). Typically, electroblots are best imaged in reflective rather than transmission mode.

Phos-tag 540 Stains

Excitation: 540 nm
Solid State YAG Laser (532 nm)
HeNe Laser (543 nm)
Xenon lamp with 540 nm filter

Emission: 570 nm

Phos-tag 300/460 Stains

Excitation: 300 nm or 460 nm
Transilluminator (300 nm)

Blue LED Laser (457 nm)

Argon Laser (488 nm)

Xenon lamp with 460 nm filter

Emission: 630 nm

VIII. REPRESENTATIVE RESULTS

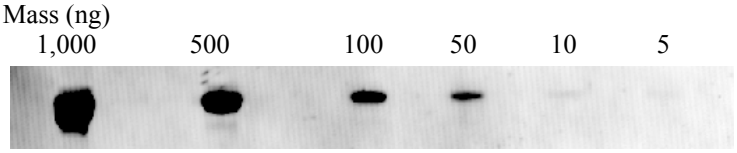

Figure 5. *After electrophoresis in 12% NuPAGE® gels, ovalbumin was transferred to Immobilon-FL PVDF membrane and stained with Phos-tag ™ 540 Phosphoprotein Blot Stain. The membrane was imaged with a ProXPRESS™ Proteomic Imaging System: Ex 540nm / Em 590nm.*

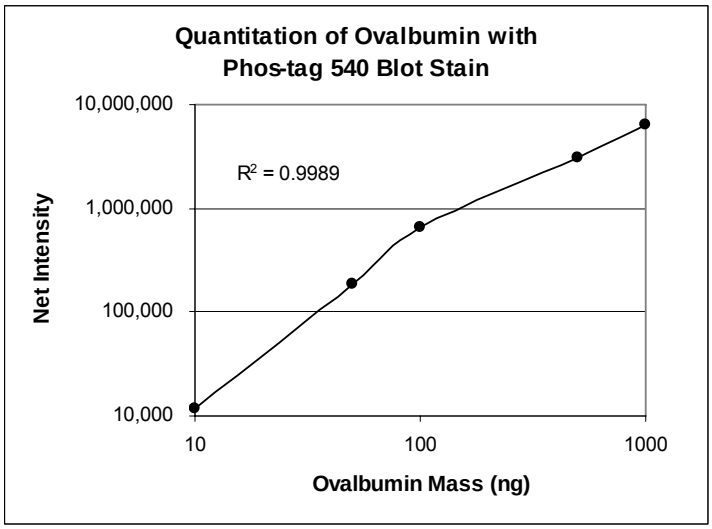

Figure 6*. Ovalbumin, a phosphorylated protein, stained with Phos-tag™ 540 Phosphoprotein Blot stai, produced a linear signal when plotted as a function of protein mass. The membrane was imaged on a ProXPRESS Proteomic Imaging System: excitation 540 nm / emission 590 nm. The ovalbumin signal intensity was quantified using Phoretix 1D (Nonlinear Dynamics) software.*

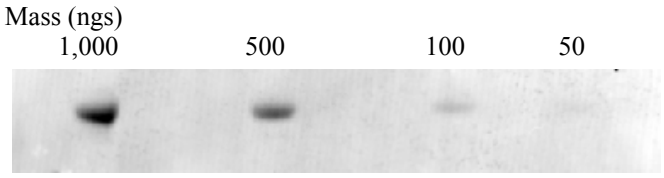

Figure 7. *After electrophoresis in 12% NuPAGE® gels, ovalbumin was transferred to an Immobilon-FL PVDF membrane and stained with Phos-tag ™ 300/460 Phosphoprotein Blot stain. The membrane was imaged with a ProXPRESS™ Proteomic Imaging System: Ex 460nm / Em 650nm.*

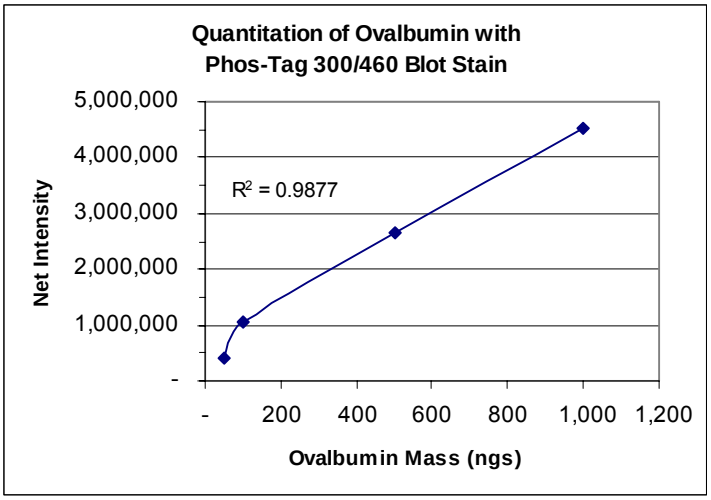

Figure 8*. Ovalbumin, a phosphorylated protein, stained with Phos-tag™ 300/460 Phosphoprotein Blot Stain produced a linear signal when plotted as a function of protein mass. The membrane was imaged on a ProXPRESS Proteomic Imaging System: excitation 460 nm / emission 650 nm. The ovalbumin signal intensity was quantified using Phoretix 1D (Nonlinear Dynamics) software.*

IX. TROUBLESHOOTING

Problem	Potential Causes and Remedies
No or only faint staining of phosphoproteins on the membrane.	• Insufficient protein was loaded. Verify sufficient protein was applied per lane using a total protein stain. (Typically 20-1,000 ng of a complex protein sample should be loaded per lane.) • Phos-tag dye was diluted excessively. Ensure that you use the stain at the recommended 1:100 dilution. Greater dilution will result in lower fluorescence intensity. • Reconstituted Phos-tag stain was reused to stain additional membranes. • Improper storage of reconstituted stain. Reconstituted stain may be stored at 4°C, protected from light. • Failure of detection reagents has occurred. Check expiration date on kit. Prepare fresh staining solutions. • Verify that the imaging instrument is equipped with correct excitation/ emission filters, PMT/CCD setting and light source for detecting the selected Phos-tag stain. • Imaging system is malfunctioning. Check the instrument manual for troubleshooting.

High background or nonspecific staining is observed on membrane.	• Residual SDS is interfering with staining. Fix the membrane with 10% methanol / 7% acetic acid and rehydrate with deionized water, according to the staining protocol. • Ineffective washing after staining due to poor mixing, inadequate volume or oversized tray. Use sufficient volumes and use a rotary shaker to ensure proper fluid dynamics.
Irregular or non-uniform staining is observed on membranes.	• Membrane is not completely covered with stain or is folded onto itself. Ensure that the membrane is completely submerged with enough staining solution, using containers that are large enough so that the membrane can move freely in the solution during agitation. • More than one membrane is being stained per container. Only stain one membrane per container. • Ineffective washing after staining, due to poor mixing or an improperly sized tray. Use sufficient stain and perform staining on a rotary shaker to ensure proper fluid dynamics.

Streaks or speckles observed on the membrane.	• Dust or other contamination of gel, membrane, imaging device or buffer solutions may have occurred. The gel or membrane may have been handled with bare hands or contaminated with powdered glove residue. High sensitivity staining procedures require careful attention to cleanliness. When running gels, use freshly made and filtered buffers. Use high quality deionized water (≥ 18 megaohm-cm). Wash glassware thoroughly. Clean the surface of the imaging platen with 10-100% ethanol, followed by deionized water. Always handle membranes with clean powder-free gloves.

X. APPENDIX: SUGGESTED IMAGER CONFIGURATIONS

The availability and models of imaging instruments, as well as their specifications, are continually changing. Please consult your instrumentation manufacturer with regard to optimal settings for imaging a Phos-tag stain.

A. Representative Imaging Configurations for Detecting Phos-tag 540 Phosphoprotein Blot Stain

Instrument (Manufacturer)	Excitation Source	Emission filter
ProXPRESS Proteomic Imaging System (PerkinElmer Life and Analytical Sciences, Inc.)	Xenon arc lamp with 540/25 nm excitation filter	590/35 nm band-pass
FLA-3000G, FLA-5100 (Fuji Photo Film Co., Ltd.)	532 nm laser	570 nm long-pass
FluorImager (GE Health-care/Amersham Biosci-ences)	514 nm laser	570 nm band-pass
Molecular Imager FX (Bio-Rad Laboratories, Inc.	532 nm laser	555 nm long-pass
Typhoon Trio+, Trio, 9200, 9210, 9400, 9410 (GE Healthcare/Amersham Biosciences)	532 nm laser	560 nm long-pass

B. Representative Imaging Configurations for Detecting Phos-tag 300/460 Phosphoprotein Blot Stain

Instrument (Manufacturer)	Excitation Source	Emission filter
Geliance (PerkinElmer Life and Analytical Sciences)	302 nm UV	EtBr/UV filter
ProXPRESS (PerkinElmer Life and Analytical Sciences, Inc)	Xenon arc lamp with 460/80 nm excitation filter	650/150 nm
Dark Reader (Clare Chemical Research)	490 nm blue light box	600/35 nm (or the system's amber lid)
Eagle-Eye II (Stratagene)	300 nm UV transilluminator	Ethidium bromide (~600 nm band-pass) or CBB (~570 nm band-pass)
FLA-3000G, FLA-5100 (Fuji Photo Film Co., Ltd.)	473 nm laser	580 nm long-pass
FluorImager (GE Healthcare/Amersham Biosciences)	488 nm laser	610/35 nm band-pass
FOTO/Analyst Archiver CCD system (Fotodyne Inc.)	300 nm UV transilluminator	618 nm band pass
FOTO/UV 450 (Fotodyne Inc.)	300 nm UV transilluminator	490 nm long-pass (Kodak Wratten #9)

LAS-1000 plus (Fuji Photo Film Co., Ltd.)	470 nm blue LED	515 nm long-pass
Lumi-Imager F1 (Roche/Boehringer -Mannheim)	300 nm UV tran-sillumination	600/20 nm band-pass
Molecular Imager FX (Bio-Rad Labo-ratories, Inc.	488 nm laser	640/35 nm band-pass
Nucleovision 920 (Nucleotech Corp.)	300 nm UV tran-sillumination	Texas Red (~630 nm band-pass)
Storm860 (GE Healthcare /Mo-lecular Devices)	450 nm blue LED	520 nm long-pass
TUI-6000 (Ultra-Lum Inc.)	300 nm UV tran-sillumination	600 nm band-pass
Typhoon Trio+, Trio, 9200, 9210, 9400, 9410 (GE Healthcare/ Amer-sham Biosciences)	457 or 488 laser	560 nm long-pass or 610/30 band-pass
UV transillumina-tor, Visi-Blue plate and Polaroid or Kodak film camera system	300 nm UV light or 480 nm blue light (using Visi-Blue plate)	600 ± 35 nm or 490 nm long-pass (Kodak Wratten #9 filter)

XI. LICENSING

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Phos-tag™ is a trademark of the NARD Institute, Ltd.
NuPAGE® is a registered trademark of the Invitrogen Corp.

XII. NAME AND PLACE OF MANUFACTURE

For further technical information or to place an order, call:

U.S. and Canada:
PerkinElmer Life & Analytical Sciences, Inc.
710 Bridgeport Ave.
Shelton, CT 06484-4794 USA
1-800-762-4000

techsupport@perkinelmer.com

Europe:
PerkinElmer Life & Analytical Sciences, Inc.
Imperiastraat 8
B-1930 Zaventem
Belgium
+32 2 717 7911

Techsupport.europe@perkinelmer.com

For customers outside of the U.S. and Europe: Please contact your local distributor.
Website: www.perkinelmer.com

PerkinElmer Life and Analytical Sciences, Inc.
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
(800) 762-4000 or (+1) 203-925-4602
Www.perkinelmer.com

For a complete list of global offices, visit www.perkinelmer.com/lasoffices

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