PerkinElmer Life and Analytical Sciences

Phos-tools™

Phos-tag™ 540 Phosphoprotein Gel Stain
(PRD500A001KT)

Phos-tag™ 300/460 Phosphoprotein Gel Stain
(PRD400A001KT)

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

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I. PRODUCT NAME

Phos-tag™ 540 Phosphoprotein Gel Stain
(Cat. # PRD500A001KT)

Phos-tag™ 300/460 Phosphoprotein Gel Stain
(Cat. # PRD400A001KT)

II. INTENDED USE

Phos-tag™ Phosphoprotein Gel Stains are complete kits for the selective detection of phosphorylated proteins in SDS and 2D polyacrylamide gels.

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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 gel stains provide a high sensitivity approach for the selective detection of phosphoproteins separated by polyacrylamide gel electrophoresis. Selective staining of phosphoproteins with Phos-tag gel 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 Phos-tag gel stains may be used

to detect phosphoproteins from either SDS-polyacrylamide gels or 2D gels. The staining procedure is simple, reliable and robust, requiring as little as 3-4 hours to complete. Phos-tag phosphoprotein gel stains are capable of detecting as little as 1 ng of phosphoprotein per band, depending upon the phosphorylation state of the protein. The signal strength is linear when plotted as a function of the mass of phosphoprotein over three orders of magnitude. Phos-tag 540 Phosphoprotein Gel Stain has excitation/emission maxima of 540 nm and 570 nm, respectively. Phos-tag 300/460 Phosphoprotein Gel 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 gel 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 gel stains, the total protein profile may be detected using any of a variety of general protein stains, including silver stain, Coomassie Blue stain, or one of the numerous commercially available fluorescent gel stains. It is not necessary to destain the gel or repeat the fixing step before counterstaining. Phos-tag phosphoprotein gel stains are fully compatible with mass spectrometry, allowing further characterization of phosphoproteins, including protein identification by peptide mass profiling or tandem mass spectrometry-based sequencing. Additionally, once phosphorylated proteins are detected in polyacrylamide gels using a Phos-tag gel stain, phosphorylated peptides may subsequently be selectively isolated from proteolytic digests using Phos-trap™ Phosphopeptide Enrichment Kit (PRT301001KT), permitting localization of the phosphorylation sites to particular peptides.

IV. PRINCIPLES OF THE STAINING PROCEDURE

Phos-tag phosphoprotein gel 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 gel imaging platforms. Currently, two stains are available; Phos-tag 540 and Phos-tag 300/460 phosphoprotein gel stains.

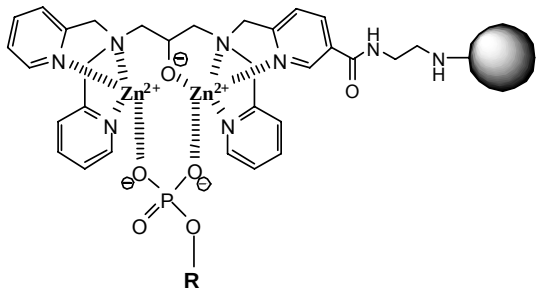

Figure 1. *The basic Phos-tag structure can readily be coupled to other functional groups, including various fluorophores.*

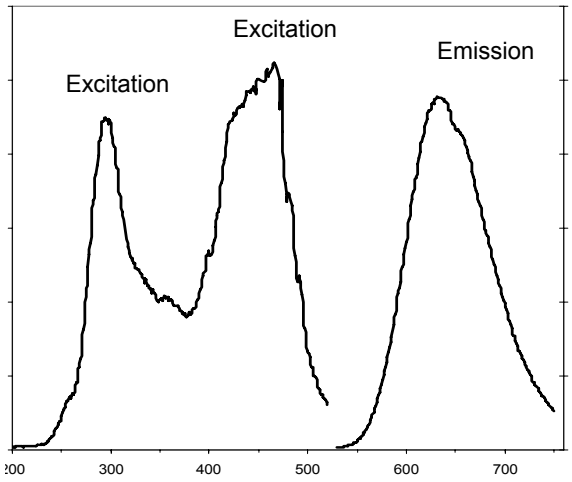

Figure 2. Excitation and Emission Spectra for Phos-tag 300/460

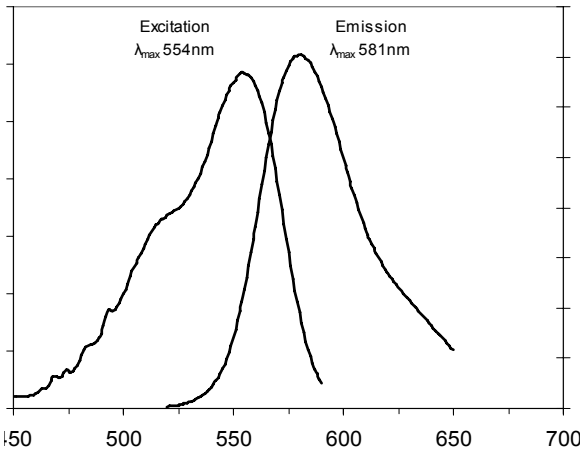

Figure 3. *Excitation and Emission Spectra for Phos-tag 540*

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.

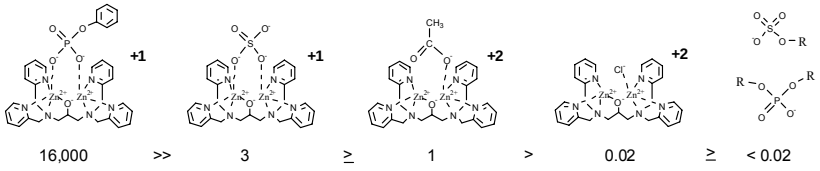

Figure 4. *Phos-tag™ reagent is highly selective for the phosphomonoester residues of phosphoserine, phosphothreonine and phosphotyrosine. There is exceptionally low competition from other charged species. (Data provided by NARD Institute, Ltd., Japan)*

V. KIT COMPONENTS

Reagents supplied are intended

FOR LABORATORY USE ONLY.

Phos-tag™ 540 Phosphoprotein Gel Stain

(Cat. # PRD500A001KT)

- Phos-tag 540 Stain Concentrate

  4 vials (each 1 mL vial is enough to stain 4 mini-gels)
- Phos-tag 540 Gel Stain Buffer (1X), 2 bottles, 200 mL each
- Phos-tag 540 Wash Buffer (2X), 2 bottles, 800 mL each

Phos-tag™ 300/460 Phosphoprotein Gel Stain

(Cat. # PRD400A001KT)

- Phos Tag 300/460 Stain Concentrate

  4 vials (each 1 mL vial is enough to stain 4 mini-gels)
- Phos-tag 300/460 Gel 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 gel 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 gel 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 MINI-GELS

The outlined protocol works reliably for standard Tris-glycine SDS-polyacrylamide gels, Bis-Tris gels and 2D gels. As required with any high sensitivity gel staining procedure, gels should never be handled with bare hands during processing. Powder-free disposable gloves should be worn during handling of the gels.

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 gels, circular staining dishes provide the best fluid dynamics on orbital shakers, resulting in less dye aggregation and better staining. For large-format 2-D gels, 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.

The volumes suggested in this protocol are the minimum recommended. The gel must be fully immersed and free-floating during the staining procedure.

Minimal staining volumes for typical gel sizes are as follows:

25 mL, for 8 cm × 10 cm × 0.75 mm gels (mini-gels)

170 mL, for 16 cm × 20 cm × 1 mm gels

250 mL, for 20 cm × 20 cm × 1 mm gels

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

For unambiguous interpretation of results, it is suggested that a lane of broad range molecular weight markers be included on each polyacrylamide gel intended for staining with Phos-tag Phosphoprotein Gel 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 Gel

1. Fix the mini-gel in 40 mLs of freshly prepared 50% methanol/10% acetic acid, using deionized water. Agitate in the fixative for 30 minutes. Discard the fixer.
2. Add 40 mLs of 50% methanol/10% acetic acid and repeat the fixation step for 1 hour to overnight.
3. Discard the fixer and incubate the gel in 100 mLs of deionized water with gentle agitation for 10 minutes. It is important that the gel be completely immersed in water in order to remove all of the methanol and acetic acid from the gel which can interfere with Phos-tag phosphoprotein gel staining.
4. Repeat the step 3 twice, for a total of three water washes.

B. Staining/Destaining the Gel

1. Prepare Phos-tag Stain by diluting 250 µL of Phos-tag Dye Concentrate 1:100 with Stain Buffer for a total of 25 mLs. If more stain than needed was reconstituted, unused stain may be stored at 4°C, protected from light, for one week.
2. Pour the staining solution into a small, clean plastic dish. Place the gel into the staining solution. Protect the gel and staining solution from light at all times by covering the container with a lid or with aluminum foil. Gently agitate the gel in the stain solution for 90 minutes at room temperature (50 rpm on an orbital shaker).
3. Dilute 60 mLs of Destain Buffer (2X) with 60 mLs of deionized water.
4. Discard the stain solution and agitate the gel in 40 mLs of Destain Buffer (1X) for 30 minutes.
5. Repeat step 4 twice, for a total of 3 washes with Destain Buffer.
6. Discard the Destain Buffer and agitate the gel in deionized water for 5 minutes. Repeat.

C. Imaging the Gel

Phos-tag dyes are compatible with a wide range of imaging systems (See appendix).

<u>Phos-tag 540 Phosphoprotein Gel Stain</u>

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

Emission: 570 nm

<u>Phos-tag 300/460 Phosphoprotein Gel Stain</u>

Excitation: 300 nm or 460 nm
- Transilluminator (300 nm)
- Blue LED laser (457 nm)
- SHG laser (473 nm)
- Argon laser (488 nm)
- Xenon lamp with 460 nm filter

Emission: 630 nm

For optimal performance, images should be acquired as soon as possible after completing the staining procedure. Gels may be stored protected from light and imaged the next day with good sensitivity.

Phos-tag 300/460 phosphoprotein gel stain is most suitable for direct visualization with a UV light transilluminator.

NOTE: Always use proper eye protection if directly viewing stained bands using a UV transilluminator. Do not view gels on a UV transilluminator without proper eye protection. UV light can cause eye injury and blindness.

Keep in mind that this mode of detection is less sensitive than camera or laser-based detection approaches. When viewing or imaging the gel with UV transillumination, place the gel directly on the transilluminator. Do not use plastic wraps or plastic backing. It is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (such as cheesecloth). Otherwise, fluorescent dyes will accumulate on the glass surface and cause high background fluorescence. The polyester backing on some pre-cast gels is highly fluorescent. For maximum sensitivity using a UV transilluminator, the gel should be placed polyacrylamide side down and an appropriate band-pass or long-pass emission filter should be used to screen out the blue fluorescence of the plastic. The use of a blue-light transilluminator or laser scanner will reduce the amount of fluorescence from the plastic backing so that the gel may be placed polyester side down. Noticeable photobleaching may occur after several minutes of exposure to ultraviolet light. It is possible to take advantage of the integrating capability of photographic or CCD cameras and use long exposure times to increase the detection sensitivity, often making bands detectable that are not visible to the naked eye. Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, try a 1

second exposure initially. CCD cameras and laser scanners provide the highest detection sensitivity. Images are best obtained by digitizing at about 1024 × 1024 pixels resolution with 14- or 16-bit gray scale levels per pixel. Contact the camera manufacturer for recommendations regarding filter sets. A CCD camera-based image analysis system or laser scanner can gather quantitative information that will allow comparison of fluorescence intensities among different bands or spots.

If the background is high or irregular, the gel should be washed in deionized water for an additional 20-30 minutes and re-imaged.

Storing Gels

Always store gels in the dark to prevent photobleaching. Depending upon the amount of protein in bands of interest, gels may retain a usable signal for many days. To dry a stained gel for permanent storage, incubate the gel in a solution of 2% glycerol for 30 min. Gels may be dried in a standard gel dryer between sheets of cellophane, although there is sometimes a slight decrease in sensitivity. Note that proteins present at very low levels may no longer be detectable after gel drying.

VIII. REPRESENTATIVE RESULTS

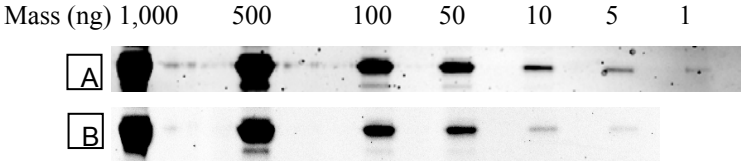

Figure 5 *After electrophoresis in 12% NuPAGE® gels, ovalbumin was stained with Phos-tag ™ 300/460 dye and the gels were imaged at each excitation wavelength: A) ProXPRESS™ Proteomic Imaging System: Ex 460nm / Em 650nm B) Geliance™ 600 Imaging System: Excitation 302nm*

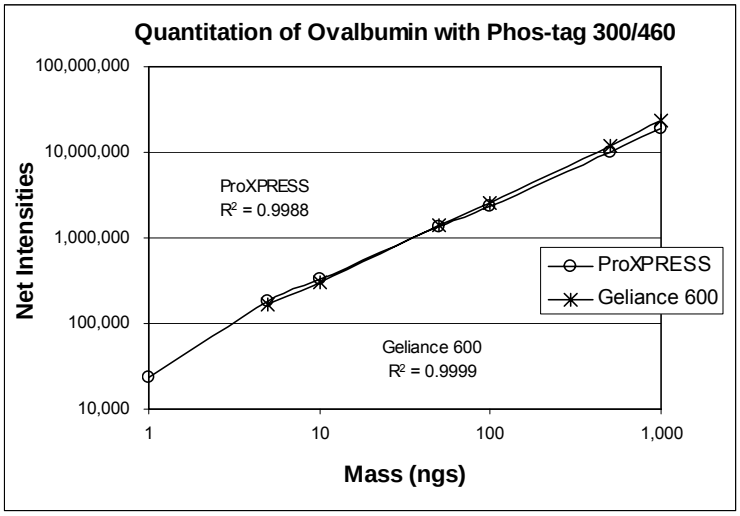

Figure 6. Ovalbumin, a phosphorylated protein, stained with Phos-tag™ 300/460 dye produced a linear signal when plotted as a function of protein mass at both excitation wavelengths. The ovalbumin signal intensity was quantified using Phoretix 1D software from Nonlinear Dynamics.

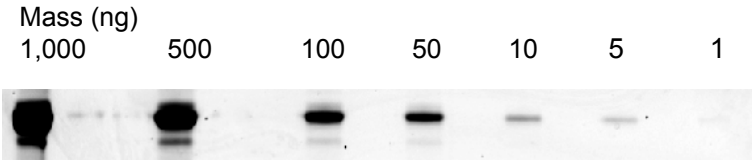

Figure 7 *After electrophoresis in 12% NuPAGE® gels, ovalbumin was stained with Phos-tag ™ 540 dye and the gel was imaged with a ProX-PRESS™ Proteomic Imaging System: Ex 540nm / Em 590nm.*

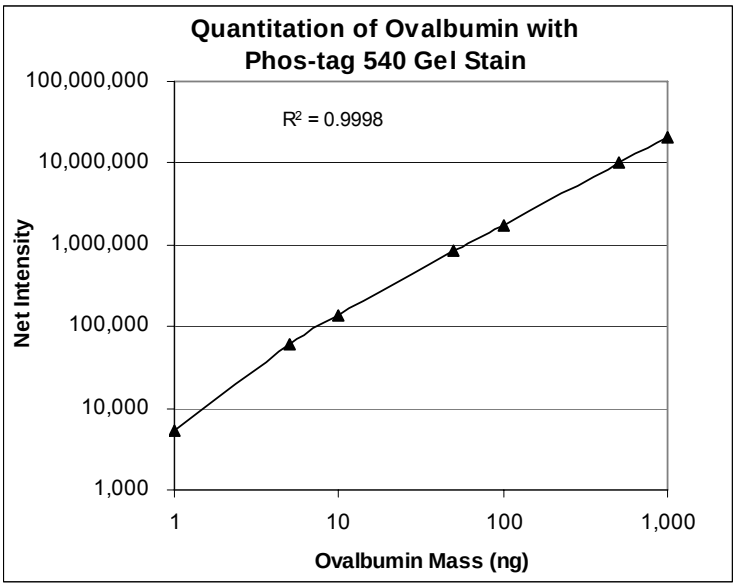

Figure 8*. Ovalbumin, a phosphorylated protein, stained with Phos-tag™ 540 dye, produced a linear signal when plotted as a function of protein mass. The gel was imaged on a ProXPRESS Proteomic Imaging System: excitation 540 nm / emission 590 nm. The ovalbumin signal intensity was quantified using Phoretix 1D software (Nonlinear Dynamics).*

IX. TROUBLESHOOTIING

Problem	Potential Causes and Remedies
No or only faint staining of phospho-proteins on the gels.	• Insufficient protein was loaded. Verify sufficient protein was applied per lane using a total protein gel 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 gels. • Improper storage of reconstituted stain. Reconstituted stain may be stored at 4° C, protected from light for up to one week. • Failure of detection reagents has occurred. Check expiration date on kit. Prepare fresh staining solutions. • Imaging instrument is not correctly configured. 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 gels.	• Residual SDS is interfering with staining. Fix the gel with 50% methanol / 10% 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 gels.	• Gel is not completely covered with stain or is folded onto itself. Ensure that the gel is completely submerged with enough staining solution, using containers that are large enough so that the gel can move freely in the solution during agitation. • More than one gel is being stained per container. Only stain one gel 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 gel.	Dust or other contamination of gel, imaging device or buffer solutions may have occurred. The gel 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 gels with clean powder-free gloves.

X. APPENDIX: SUGGESTED IMAGER CONFIGURATIONS

The availability and models of gel 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 stained gel.

A. Representative Imaging Configurations for Detecting Phos-tag 540 Phosphoprotein Gel 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 Healthcare/Amersham Biosciences)	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 Gel 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 bandpass) or CBB (~570 nm bandpass)
FLA-3000G, FLA-5100 (Fuji Photo Film Co., Ltd.)	473 nm laser	580 nm longpass
FluorImager (GE Healthcare/Amersham Biosciences)	488 nm laser	610/35 nm bandpass
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 longpass (Kodak Wratten #9)
LAS-1000 plus (Fuji Photo Film Co., Ltd.)	470 nm blue LED	515 nm longpass

Lumi-Imager F1 (Roche/Boehringer -Mannheim)	300 nm UV transillumination	600/20 nm band-pass
Molecular Imager FX (Bio-Rad Laboratories, Inc.	488 nm laser	640/35 nm band-pass
Nucleovision 920 (Nucleotech Corp.)	300 nm UV transillumination	Texas Red (~630 nm band-pass)
Storm860 (GE Healthcare /Molecular Devices)	450 nm blue LED	520 nm long-pass
TUI-6000 (Ultra-Lum Inc.)	300 nm UV transillumination	600 nm band-pass
Typhoon Trio+, Trio, 9200, 9210, 9400, 9410 (GE Healthcare/ Amersham Biosciences)	457 or 488 laser	560 nm long-pass or 610/30 band-pass
UV transilluminator, 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

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