BIOPROBES 57

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NOVEMBER 2008

The picture of cell health

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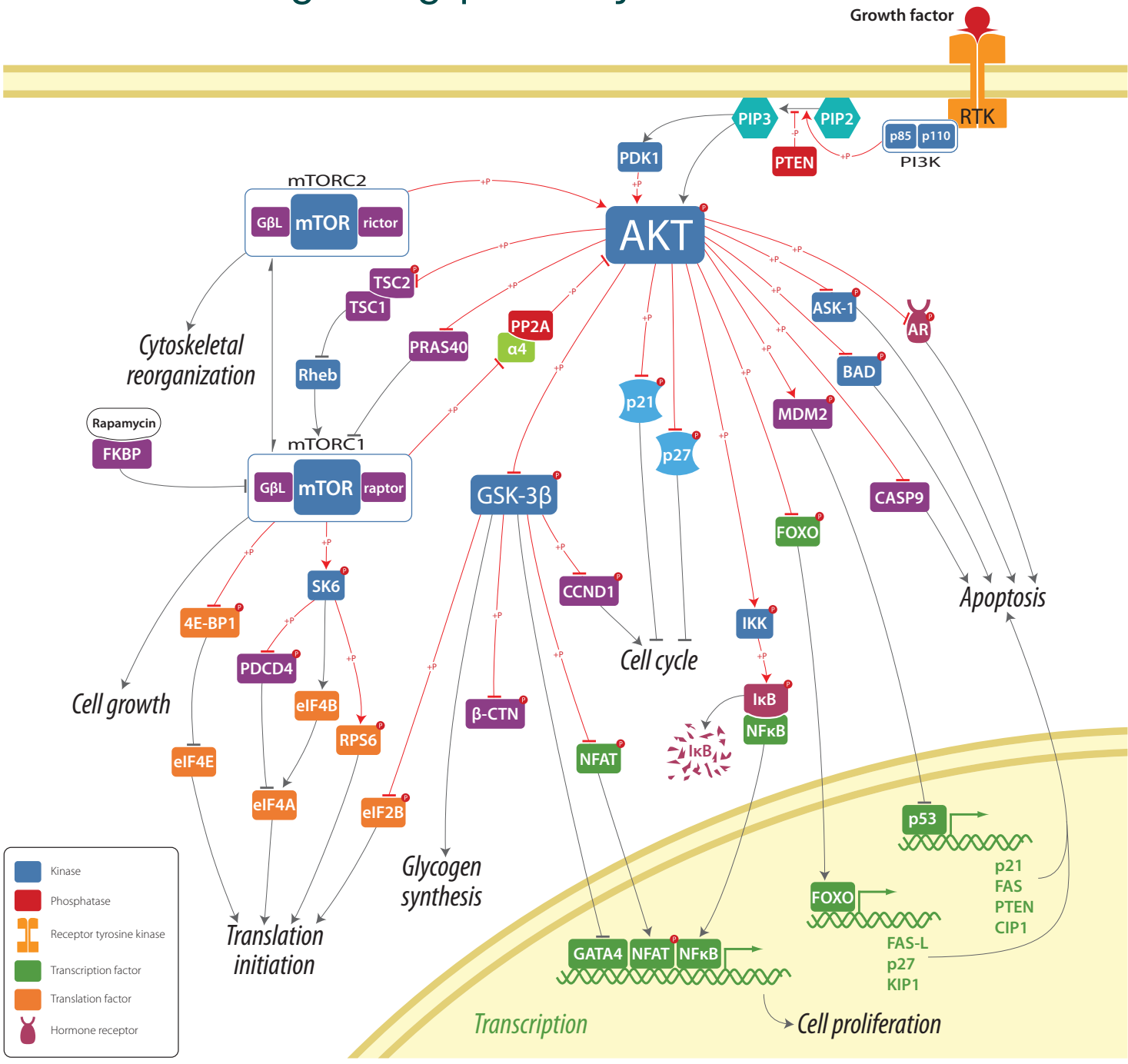
Akt/mTOR signaling pathway



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Published by Invitrogen
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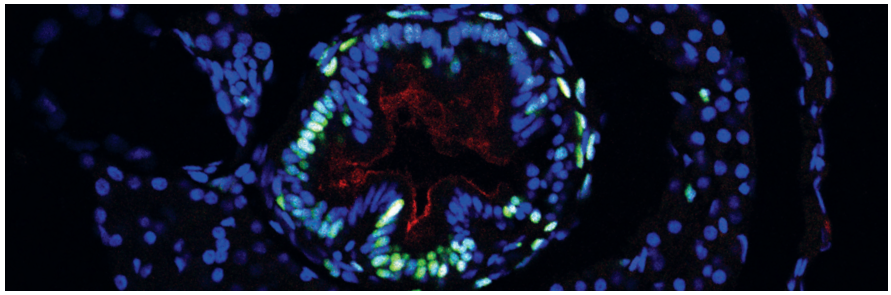

BioProbes 57

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2008 APEX® AWARDS FOR PUBLICATION EXCELLENCE

Features

Departments

Cell proliferation studies made simple

Salic, A. and Mitchison, T.J. (2008) A chemical method for fast and sensitive detection of DNA synthesis *in vivo*. *Proc Natl Acad Sci U S A* 105:2415–2420.

Accurate assessment of DNA synthesis in proliferating cells provides important information in a wide range of pharmacological and regulatory studies. Although the two principal methods currently used for measuring DNA synthesis have given researchers useful insights, each method has its limitations. [^{3}H] thymidine labeling is laborious and slow, offers poor resolution of sample structure, and suffers from all the potential health and waste-disposal concerns inherent in radioligand methodology. Labeling with 5-bromo-2-deoxyuridine (BrdU) is faster, more sensitive, and offers better structural resolution; however, the success of this approach necessitates extensive sample denaturation that can be difficult to reproduce and can significantly degrade sample structure.

In this study, Salic and Mitchison demonstrate the advantages of "click" chemistry—incorporation of 5-ethynyl-2´-deoxyuridine (EdU) followed by labeling with a fluorescent azide derivative—as an alternative to existing DNA synthesis assays. Their data show that EdU is extensively incorporated into the DNA of proliferating NIH 3T3, HeLa, and *Xenopus* cells. Subsequent visualization of the fixed cells with Alexa Fluor® 488 or Alexa Fluor® 594 azide derivatives revealed efficient and highly reproducible labeling regardless of the Alexa Fluor® dye used. Comparison with BrdU staining carried out on the same cells revealed that all cells showing BrdU labeling were also stained by the EdU method; however, EdU labeling yielded substantially more intense fluorescence, with excellent preservation of sample structure. The authors further demonstrate that the click methodology can be used to visualize proliferation in unfixed cells, large tissue/organ explants, and even in live animals, where Alexa Fluor® azide staining of tissue samples harvested from EdU-injected mice clearly identified sites of cell proliferation.

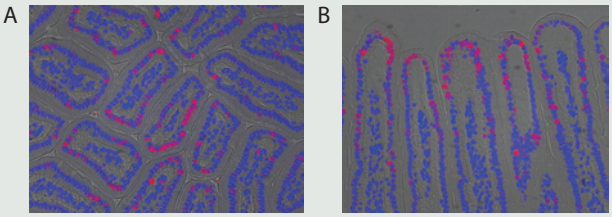

Figure 1—Labeling DNA *in vivo* by using EdU. An adult mouse was injected i.p. with 100 µg of EdU in PBS. Tissues were harvested, fixed, and sectioned 96 hr later. Tissue sections on slides were then reacted for 10 min with 10 µM Alexa Fluor® 568 azide. The images shown are overlays of a DIC image of the sectioned tissue, a fluorescent image of cellular DNA (Hoechst stain; blue), and a fluorescent image of EdU-labeled DNA revealed by reaction with Alexa Fluor® 568 azide (red). (**A** and **B**) Mouse small intestine. Villi are seen in transverse section in (**A**) and in longitudinal section in (**B**). The cells with red nuclei are descended from cells that had been in S phase during the EdU pulse and thus incorporated EdU into their DNA. Images reproduced from Salic, A. and Mitchison, T.J. (2008) *Proc Natl Acad Sci U S A* 105:2415–2420, © 2008 National Academy of Sciences, USA.

The authors propose that the brightness, specificity, and structural retention inherent in the EdU methodology could be combined with electron microscopy (by treatment with eosin–azide followed by photosensitized oxidation of diaminobenzidine) or with advanced, high-resolution optical imaging techniques (e.g., photoactivation localization microscopy, or PALM), greatly expanding the scope and utility of this already promising approach.

To learn more about Click-iT® EdU assays for cell proliferation, see "The picture of cell health" on pages 4–8, or visit **www.invitrogen.com/bp57**.

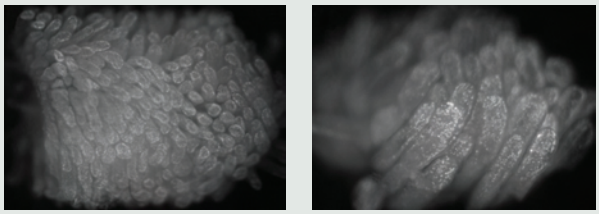

Figure 2—Exploring cell proliferation and tissue dynamics in animals using EdU. Whole-mount fluorescent images of mouse small intestine, stained to detect EdU incorporation. Two adult mice were each injected i.p. with 100 µg of EdU in PBS and their small intestines were removed after 24 and 96 hr, respectively. A freshly harvested 2 cm–long segment of the small intestine was opened with a longitudinal cut, rinsed, and immediately stained for 10 min with 100 µM tetramethylrhodamine (TMR) azide in the presence of Cu(I), without fixation or permeabilization. The intestine piece was then fixed, washed to remove unreacted TMR azide, and imaged at low magnification on a dissecting microscope equipped with fluorescence. After 24 hr, the EdU shows strong incorporation in cells located at the base of each villus. Ninety-six hours after the EdU pulse, the labeled cells have moved away from the base, near the tips of the villi. Images reproduced from Salic, A. and Mitchison, T.J. (2008) *Proc Natl Acad Sci U S A* 105:2415–2420, © 2008 National Academy of Sciences, USA.

Product	Quantity	Cat. no.
EdU (5-ethynyl-2´-deoxyuridine)	50 mg	A10044
Click-iT® EdU Alexa Fluor® 488 Imaging Kit	1 kit	C10083
Click-iT® EdU Alexa Fluor® 594 Imaging Kit	1 kit	C10084

FLUOROPHORE SELECTION GUIDE FOR FLOW CYTOMETRY

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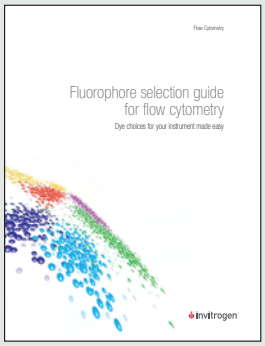

- Fluorophores listed by the laser(s) that excite them
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A comprehensive index provides catalog numbers and full product names. This selection guide is especially useful for scientists who have instruments equipped with multiple lasers. Download the PDF at **www.invitrogen.com/bp57**.

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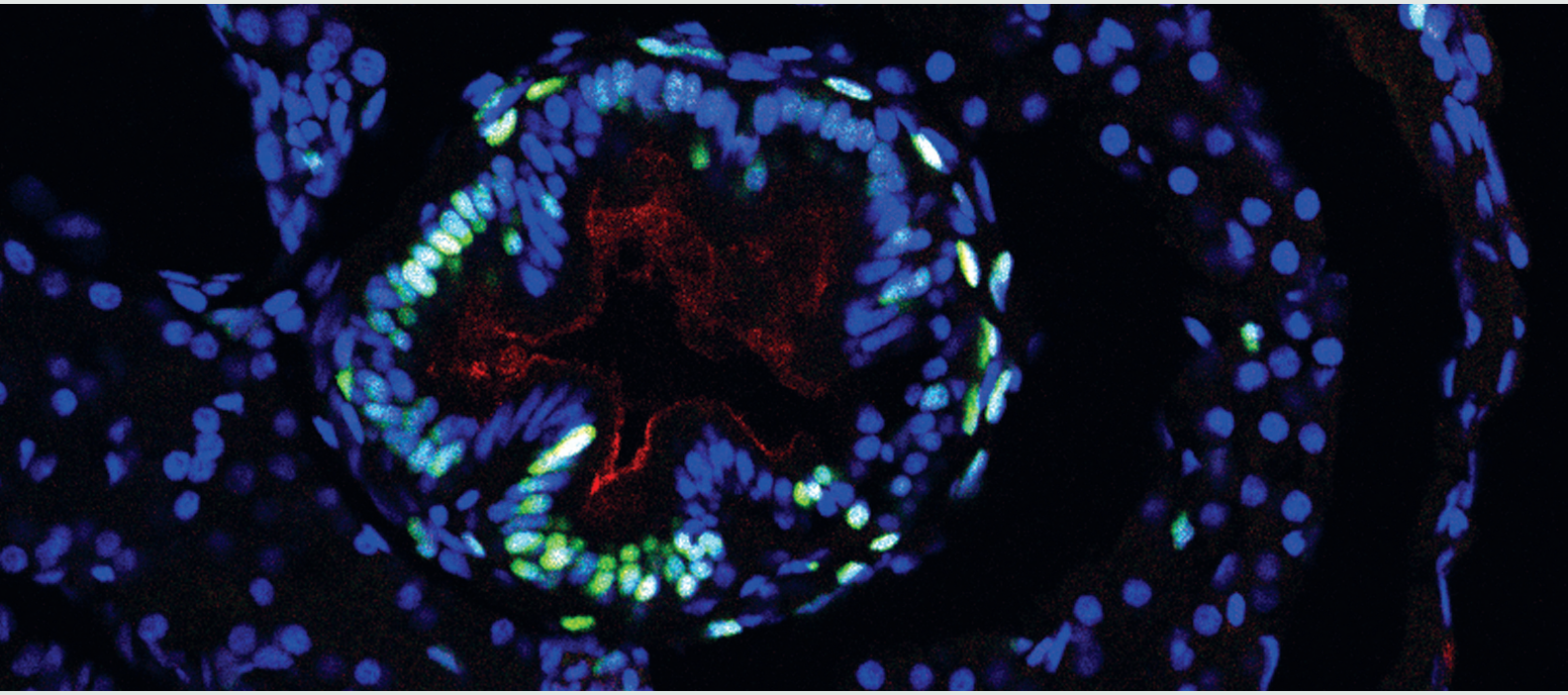

The picture of cell health

ALAMARBLUE®, CYQUANT® NF, AND CLICK-IT® EDU ASSAYS.

While there is no shortage of assays for cell health and prepackaged kits promising easy answers, we know it is really the question that matters. What are the important landmarks that define cell health in a particular experiment? Is it the metabolic activity of the cell population? The ability of the cell population to grow at expected rates? The capacity of individuals in the population to proliferate (Figure 1)? Taking a multiparametric approach to assessing cell health yields a much richer data set to mine, both for answers and for the next series of questions. The three assays described here (Table 1) each provide a different snapshot of cell health, and can individually or together form the basis of an assay for cell proliferation, cytotoxicity, or drug efficacy.

Figure 1 (above)—Cross section through the mid-intestine of a zebrafish larva. A 5-day-old zebrafish larva was exposed to a 16 hr pulse of 400 µM EdU and then fixed, embedded in paraffin, cut into 7 µm sections, and processed for detection using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Cat. no. C10083). Slides were subsequently stained with Alexa Fluor® 568 soybean agglutinin (SBA) and TO-PRO®-3 dye (Cat. no. T3605). Proliferating nuclei appear white due to labeling with both Alexa Fluor® 488 azide (green) and TO-PRO®-3 dye (blue). Goblet cells in the intestinal bulb, labeled with Alexa Fluor® 568 SBA, appear red. This 20x z-section image was captured using a CoolSNAP™ camera (Princeton Instruments) mounted on a Nikon D-Eclipse C1 confocal microscope and processed (including pseudocoloring) using Photoshop® software (Adobe, Inc.). Image submitted by Sarah Cheesman, Institute of Molecular Biology, University of Oregon, USA.

Table 1—Choose the Invitrogen assay that answers your question about cell health.

	Are the cells metabolically active?	Have the cells proliferated?	Which cells are actively proliferating?
Product	alamarBlue® reagent	CyQUANT® NF Cell Proliferation Assay	Click-iT® EdU Assay
What the product does	Measures metabolic activity in a cell population	Quantitates total DNA content of a cell population	Measures nascent DNA synthesis of individual cells within a population
Detection platform	Fluorescence or absorbance microplate reader	Fluorescence microplate reader	Fluorescence or absorbance microplate reader, fluorescence microscope or high-throughput imager (HCS), or flow cytometer
Protocol outline	Add alamarBlue® reagent directly to cell culture media, mix, and read	Remove media, add CyQUANT® NF reagent, mix, and read	Incubate cells with EdU, perform Click-iT® detection (and signal amplification for the microplate assay), and read

Are the cells metabolically active?

The alamarBlue® assay measures the ability of metabolically active cells to convert a nonfluorescent resazurin-based reagent into a fluorescent as well as a colorimetric indicator. Damaged and nonviable cells—which are also likely to be nonproliferating cells—have reduced metabolic activity and thus generate a proportionally lower signal. The alamarBlue® assay provides a measure of relative cell number that can be used to assess cytotoxicity or, when performed at multiple time points, can be used to indirectly determine the average proliferation rate of the population. Furthermore, the nontoxic alamarBlue® reagent permits long-term exposure of cells without measurably impacting cell growth (Figure 2), allowing extended studies of cell viability, cell proliferation, and cytotoxicity.

Of the three cell health assays described here, the alamarBlue® assay is by far the easiest, both in terms of time and convenience. This homogeneous assay has a simple mix-and-read protocol, eliminating washing, fixation, and extraction steps. The alamarBlue® reagent is added directly to either cell suspensions or attached cells in complete culture medium, and the metabolized product is then detected either by fluorescence (excitation/emission maxima ~571/585 nm) or by UV/Vis spectroscopy (absorbance maximum ~570 nm). With these different detection options and its homogeneous format, the alamarBlue® assay is particularly useful for high-throughput assays with either absorbance or fluorescence microplate readers.

Have the cells proliferated?

The CyQUANT® NF Cell Proliferation Assay Kit provides a convenient microplate-based method for quickly and easily counting cells in a population based on their DNA content. Because cellular DNA content is highly regulated, the CyQUANT® NF assay can be used to directly measure relative cell number at specific time points, which can be converted into an average rate of cell proliferation in a population. →

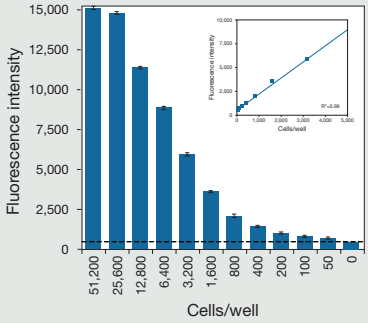

Figure 2—Sensitivity of alamarBlue® assay after an 18 hr incubation of cells with reagent. SH-SY5Y cells were plated in a 96-well plate at different cell densities, and the alamarBlue® reagent (Cat. no. DAL1100) was added to each well and allowed to incubate at 37°C for 18 hr. The horizontal line at ~450 RFU represents the background fluorescence in the experiment, which was calculated as three times the standard deviation of the "no cell" control. The inset shows that the alamarBlue® assay is linear over the range from 50 to 5,000 cells/well after an 18 hr incubation of cells with reagent. Error bars are shown as ±SEM.

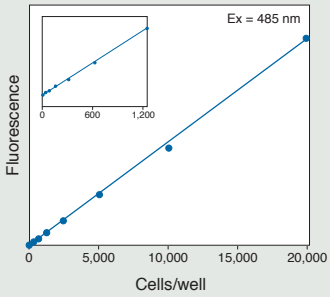

Figure 3—Cell quantitation using the CyQUANT® NF Cell Proliferation Assay Kit. CHO (M1WT3) cells were plated at densities of 0–20,000 per well in a standard 96-well microplate. Cells were incubated with CyQUANT® NF reagents, supplied in the CyQUANT® NF Cell Proliferation Assay Kit (Cat. no C35006), for 30 min at 37°C, according to the standard adherent cell analysis protocol, and fluorescence intensities were measured with a fluorescence microplate reader using excitation at 485 nm and emission at 530 nm. The linear range of the assay under these conditions is from 20 to 20,000 cells per 100 µl sample. The inset shows the linearity that can be obtained at very low cell numbers.

Assays that measure metabolic activities, such as the alamarBlue® assay, may exhibit cell number–independent variability in response to drug or chemical treatments.

Similar to the alamarBlue® assay, the CyQUANT® NF assay has a quick and easy workflow with no washing or solubilization steps required. The CyQUANT® NF assay eliminates the freeze–thaw cell lysis step in the original CyQUANT® Kit by employing a cell-permeant DNA-binding dye in combination with a plasma membrane permeabilization reagent. To perform the CyQUANT® NF assay, simply remove the culture media from the cell suspension or attached cells, add the CyQUANT® NF reagent, incubate the cells for 10–60 min, and measure the green-fluorescent signal (excitation/emission maxima ~497/520 nm). The CyQUANT® NF assay has an extremely wide linear detection range—from at least 100 to 10,000 cells per well—and can be used in either 96-well or 384-well microplate format. In some cell types (e.g., CHO cells) the assay is linear to as few as 20 and as many as 20,000 cells per well (Figure 3).

Which cells are actively proliferating?

The Click-iT® EdU cell proliferation assay improves upon traditional methods for detecting and quantitating newly synthesized DNA (Table 2). Active DNA synthesis was originally measured by following the incorporation of the radioactive nucleoside [^{3}H] thymidine. This method was later replaced by immunodetection of the incorporated nucleoside analog bromodeoxyuridine (BrdU) using an anti-BrdU antibody.

The Click-iT® EdU assay is also based on detecting the incorporation of a nucleoside analog into newly synthesized DNA, but in this case the nucleoside analog is EdU (5-ethynyl-2′-deoxyuridine) and it is detected not with an antibody but by a click reaction—a copper-catalyzed covalent reaction between an azide and an alkyne. In the Click-iT® EdU assay, the EdU nucleoside contains the alkyne and the fluorescent detection reagent contains the azide. The Click-iT® EdU signal can be directly visualized using fluorescence microscopy (Figures 1 and 4) or flow cytometry and is especially useful for high-content screening (HCS) assays.

Table 2—Click-iT® EdU assays.

Platform	Number of samples	Available fluorophores*	Cat. no.	Notes
Flow cytometry	50 assays based upon a 0.5 ml volume	Alexa Fluor® 488 dye	C35002	• Includes two cell-cycle dyes compatible with the detection fluorophore • Not interchangeable with the imaging assays
		Alexa Fluor® 647 dye	A10202	
		Pacific Blue™ dye	A10034	
High-throughput imaging (HCS)	2 x 96 tests (2-plate) 10 x 96 tests (10-plate)	Alexa Fluor® 488 dye	A10027,† A10028‡	• Includes Hoechst 33342 for cell registration or cell-cycle analyses • Not interchangeable with the flow cytometry assay
		Alexa Fluor® 594 dye	A10209,† C10082‡	
		Alexa Fluor® 647 dye	A10208,† C10081‡	
Fluorescence microscopy	50 coverslips	Alexa Fluor® 488 dye	C10083	• Includes the blue-fluorescent nuclear counterstain, Hoechst 33342 • Not interchangeable with the flow cytometry assay
		Alexa Fluor® 594 dye	C10084	
		Alexa Fluor® 647 dye	C10085	
Fluorescence microplate reader	400 assays in 96-well plate format	Oregon Green® 488 dye/ Amplex® UltraRed reagent	C10214	• Includes a stop reagent that stabilizes the signal for up to 24 hr

* Except for the Click-iT® EdU Microplate Assay Kit (Cat. no. C10214), each kit contains one fluorophore. † 2-plate assay. ‡ 10-plate assay.

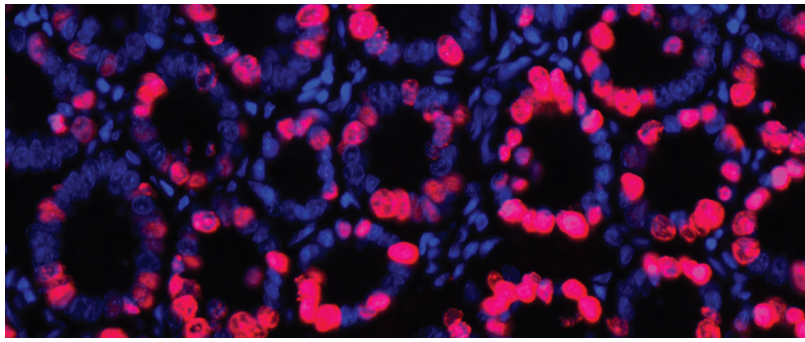

Figure 4—Proliferating cells in rat ileum. Cell proliferation in a rat ileum tissue section was detected with the Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Cat. no. C10084) using the protocol outlined in Figure 6. Click-iT® EdU staining of tissue sections is complete in 80 min, whereas BrdU protocols require harsh denaturation, long blocking steps, and an overnight incubation with anti-BrdU antibody followed by a secondary antibody detection step. Proliferating cells fluoresce red; nuclei are stained with the blue-fluorescent counterstain Hoechst 33342 (Cat. no. H1399).

In contrast to the BrdU detection protocol, the Click-iT® EdU detection protocol requires no harsh DNA denaturation step to allow the detection reagent access to the incorporated nucleoside. The small size of the fluorescent azide (MW <~1000) compared to that of an anti-BrdU antibody (MW ~150,000) enables efficient detection of the incorporated EdU using mild conditions (Figure 5). The HCl denaturation step in the BrdU detection protocol not only disrupts dsDNA integrity, which can affect nuclear counterstaining, but also potentially destroys cell morphology and antigen recognition sites.

Furthermore, for cell-based flow, microscopy, and HCS applications, the streamlined Click-iT® EdU detection protocol both reduces the total number of steps and significantly decreases the total amount of time required to perform a cell proliferation assay, especially with tissue samples (Figure 6). And unlike the BrdU assay, which relies →

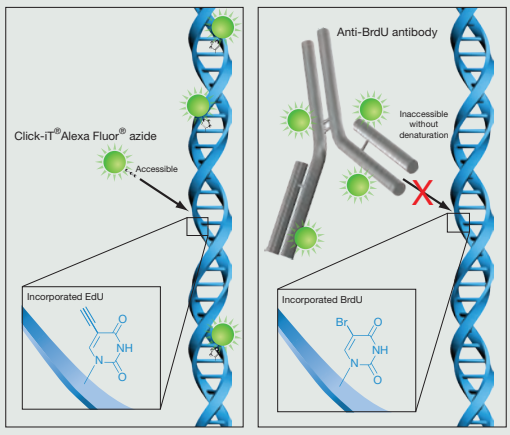

Figure 5—Detection of incorporated EdU with Alexa Fluor® 488 azide dye compared to detection of incorporated BrdU with an anti-BrdU antibody. The small size of the Alexa Fluor® 488 azide dye eliminates the need to denature DNA in order for the detection reagent to gain access to the modified base.

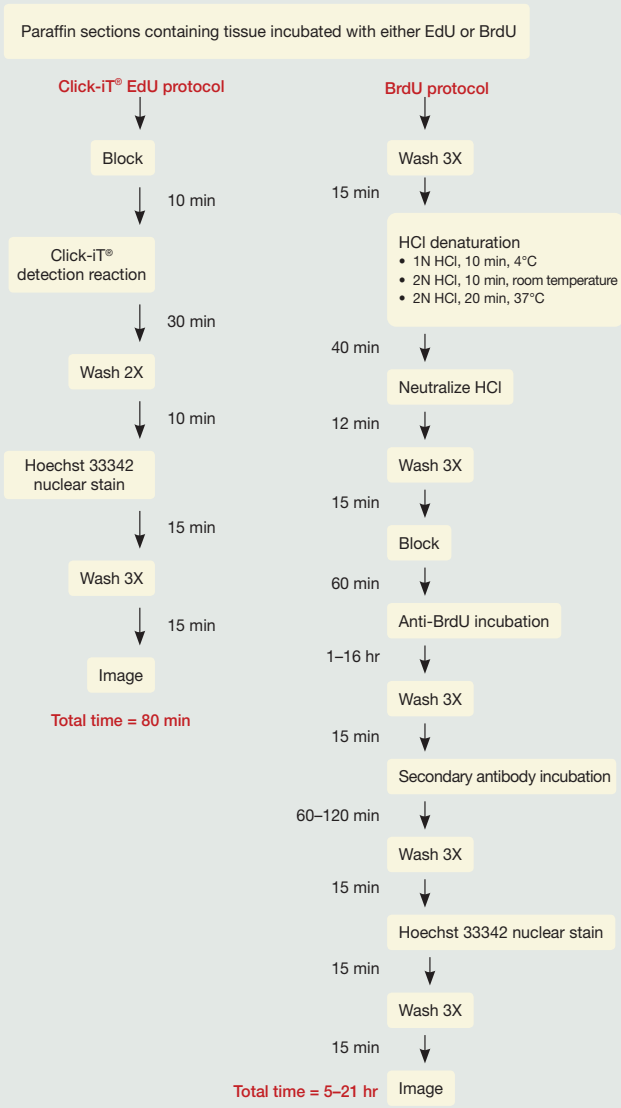

Figure 6—Comparison of Click-iT® EdU and BrdU protocols for the detection of cell proliferation in tissue. Click-iT® EdU reduces the total number of steps and significantly decreases the amount of time required to detect proliferating cells in tissue.

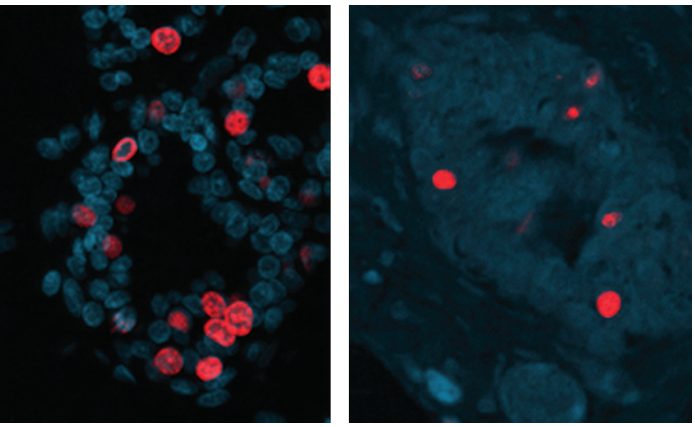

Figure 7—Comparison of EdU direct detection and BrdU secondary detection. Rats were treated with estradiol three days prior to a 2 hr pulse of EdU or BrdU administered intraperiotoneally (160 µg/g body weight). Paraffin sections (5 µm thick) were processed according to the protocol in Figure 6. Proliferating cells, labeled red, were detected either with a click reaction using Alexa Fluor® 594 azide supplied in the Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Cat. no. C10084) (left) or with anti-BrdU antibody followed by Alexa Fluor® 594 goat anti–mouse IgG secondary antibody (Cat. no. A21125) (right). Nuclei are stained with the blue-fluorescent counterstain Hoechst 33342 (Cat. no. H1399). In the BrdU detection method, denaturation of DNA with HCl results in a weaker nuclear signal.

on antibodies that can exhibit nonspecific binding, the Click-iT® EdU assay utilizes bioorthogonal or biologically unique moieties, producing low backgrounds and high detection sensitivities (Figure 7).

Click-iT® EdU assay now available in microplate format

As with the CyQUANT® NF assay, the Click-iT® EdU assay can also be used to measure the average proliferation rate of a cell population. In the new Click-iT® EdU microplate assay, the EdU nucleoside is incorporated into newly synthesized DNA and detected with an Oregon Green® azide by a click reaction. The Oregon Green® signal is then amplified using a horseradish peroxidase (HRP) conjugate of an anti–Oregon Green® dye antibody, in conjunction with the fluorogenic Amplex® UltraRed peroxidase substrate. Upon oxidation by HRP, the Amplex® UltraRed substrate is converted into a bright red-fluorescent product (excitation/emission maxima ~568/585 nm) with a high extinction coefficient and good quantum efficiency, delivering greater sensitivity and a broader assay range than other fluorogenic and colorimetric peroxidase substrates commonly used in microplate assays.

The Click-iT® EdU Microplate Assay Kit contains the key reagents for EdU incorporation into actively proliferating cells, click detection, and HRP amplification, as well as the Amplex® UltraRed stop reagent, which enables the HRP reaction to be terminated at a user-determined time point and stabilizes the signal for up to 24 hr. In contrast to the CyQUANT® NF microplate assay, which measures total DNA, the Click-iT® EdU microplate assay detects and quantifies only newly synthesized DNA. The Click-iT® EdU microplate assay has been successfully tested using HeLa, A549, U-2OS, and A541 cells treated with a variety of reagents that modulate DNA synthesis, including the DNA synthesis inhibitor aphidicolin and the mitotic inhibitor paclitaxel (Figure 8).

Learn more about the new Click-iT® EdU technology and its applications at **www.invitrogen.com/bp57**. ■

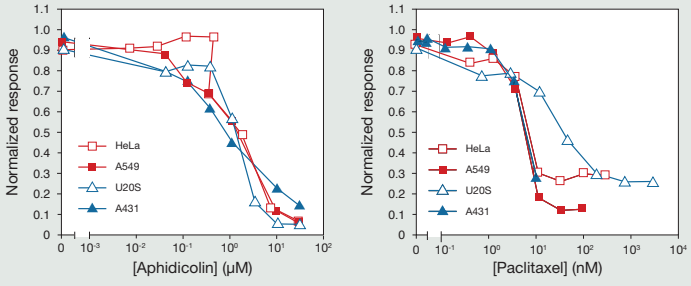

Figure 8—Detection of cell proliferation in aphidicolin- or paclitaxel-treated cells using the Click-iT® EdU microplate assay. HeLa, A549, U-2OS, and A541 cells were each treated with a serial dilution of (**A**) aphidicolin, a DNA synthesis inhibitor, or (**B**) paclitaxel, a mitotic inhibitor, for 24 hr and then pulsed with 10 µM EdU for 2 hr prior to fixation and subsequent click detection using the Click-iT® EdU Microplate Assay Kit (Cat. no. C10214). Fluorescence intensities were measured with a fluorescence microplate reader using excitation at 568 nm and emission at 584 nm.

alamarBlue® is a registered trademark of TREK Diagnostic Systems, Inc.

Product	Quantity	Cat. no.
alamarBlue® reagent	25 ml	DAL1025
alamarBlue® reagent	100 ml	DAL1100
CyQUANT® NF Cell Proliferation Assay Kit	1 kit, 200 assays	C35007
CyQUANT® NF Cell Proliferation Assay Kit	1 kit, 1000 assays	C35006
EdU (5-ethynyl-2′-deoxyuridine)	50 mg	A10044

See Table 2 for ordering information and product specifications for the Click-iT® EdU Kits.

Sensitive, easy protein detection

COMPARISON OF THE LATEST WESTERN BLOTTING PRODUCTS.

With the ability to measure specific protein expression levels directly from cells or tissue samples, western blots are not only extremely informative, but also easy to perform, quantitative, and, when coupled with innovations in western blot workflow, can be performed in record time with unparalleled sensitivity. Using a variety of techniques for detection (chemiluminescence, fluorescence, or colorimetric), protein expression can be detected at subnanogram levels (Figure 1). However, choosing from among the available techniques can be challenging—each is often claimed to be easiest to perform, most sensitive, and most compatible with easy means of detection. In addition, the product options for western blot detection have never been broader.

Here, some of the latest products based on these sensitive western blot detection methods—chemiluminescent, fluorescent, and colorimetric (Figure 2 and Table 1)—are compared. Chemiluminescence is the preferred detection method for the highest sensitivity. However, fluorescent nanocrystals that eliminate the need for film offer performance rivaling that of chemiluminescence. Researchers can also use colorimetric detection to measure protein expression at slightly lower sensitivity than fluorescence or chemiluminescence, obtaining a stable western blot that is visible to the human eye. Determine which of these western blot detection methods is right for you after considering sensitivity, ease of use, and detection compatibility.

Film-free western blot detection: WesternDot™ kits

WesternDot™ 625 western blot kits combine the unique properties of Qdot® 625 nanocrystals with the high-affinity streptavidin–biotin binding reaction to allow straightforward, picogram-sensitivity detection of proteins on blots (Figure 1). Incorporating a standard western blotting protocol, detection relies on a biotinylated secondary →

Table 1—How to detect chemiluminescent, fluorescent, or colored western blots.

			Means of detection			
	Detection method	Abs or Ex/Em*	Eye	Film	Gel imager	UV illuminator
NOVEX® ECL or WesternBreeze® chemiluminescent kit	Chemiluminescence	461		●	●	
WesternDot™ 625 kit	Fluorescence	254–488/625	●†		●	●
WesternBreeze® chromogenic kit	Colorimetric	550	●		●	

*Absorption (Abs), excitation (Ex), or emission (Em) maxima, in nm. † May not be able to detect lowest protein levels with eyesight alone.

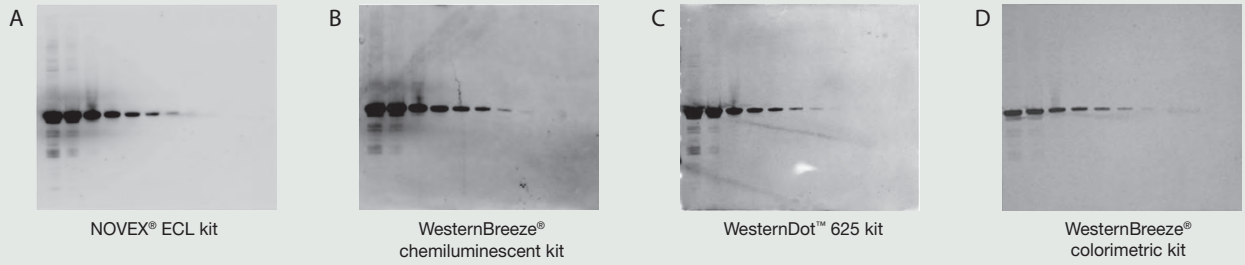

Figure 1—Comparison of chemiluminescent, fluorescent, and colorimetric western blot detection. Each PVDF blot contained a 3-fold dilution series of Jurkat cell extract with 5 µg in lane 1. The housekeeping gene GAPDH was detected with the (**A**) NOVEX® ECL kit, (**B**) WesternBreeze® chemiluminescent kit, (**C**) WesternDot™ 625 kit, or (**D**) WesternBreeze® chromogenic kit. Blots were imaged on a Fujifilm LAS-4000 with (**A**) 25 min, (**B**) 15 min, and (**C**) 5 sec exposures.

antibody (either anti-mouse or anti-rabbit) followed by the key component, Qdot® 625 streptavidin conjugate (Figure 3). Qdot® 625 nanocrystals can be excited either by UV or blue-light excitation, and the red-orange fluorescent signal can be detected with commonly used means of detection of DNA or protein gels (Figure 4). No specialized emission filters are required; simply use filters suitable for ethidium bromide, SYPRO® Ruby stain, or SYPRO® Red stain. The fluorescence-based WesternDot™ kits eliminate the hassle of using a darkroom and developing reagents, and the need to optimize film exposures, with little compromise to sensitivity.

Sensitivity is preserved because Qdot® nanocrystals are extremely efficient at generating fluorescence. WesternDot™ kit sensitivity results in part from the extraordinarily high extinction coefficient of the Qdot® 625 nanocrystal, combined with a good quantum efficiency. Qdot® 625 nanocrystals have extinction coefficients several orders of magnitude higher than traditional organic fluorophores and even other Qdot® nanocrystals (Table 2). Furthermore, the high photostability of Qdot® nanocrystals allows longer exposure times, providing increased sensitivity under conditions that lead to the deterioration of other fluorophores; blots can be visualized many times over the course of days, weeks, or even months.

Table 2—Extinction coefficients of several Qdot® nanocrystals and traditional organic dyes, in $cm^{-1}M^{-1}$.

	350 nm	405 nm	488 nm
Alexa Fluor® 405 dye	15,000	35,000	NA
Alexa Fluor® 488 dye	5,000	<1,000	73,000
Qdot® 565 nanocrystal	1,900,000	1,100,000	290,000
Qdot® 625 nanocrystal	18,000,000	9,900,000	2,700,000
Qdot® 800 nanocrystal	12,600,000	8,000,000	3,000,000

NA = not applicable.

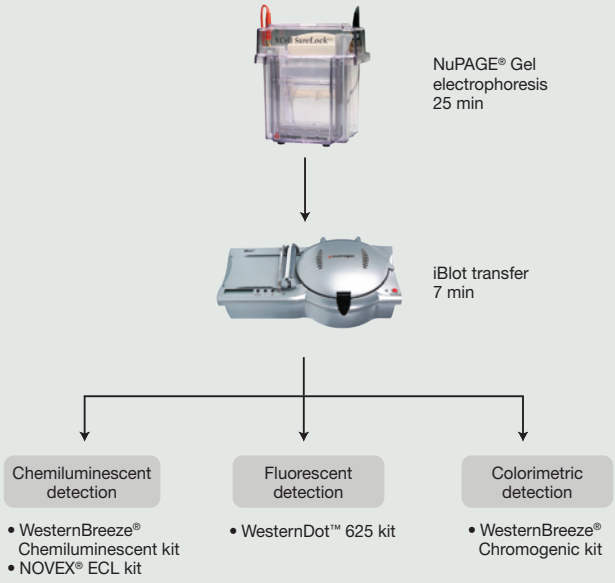

Figure 2—Products for western blotting.

Each WesternDot™ 625 kit contains everything required for detection: optimized, ready-to-use or ready-to-dilute reagents for immunodetection of proteins immobilized on nitrocellulose, PVDF, or nylon membranes, and two staining dishes for standard mini blots. For more information, visit **www.invitrogen.com/bp57.**

Sensitive detection in visible light: WesternBreeze® chromogenic kits

WesternBreeze® chromogenic kits yield high sensitivity results with extremely low background without the need for any additional equipment. Just watch the signal develop over a short period of time. The kit is based on the chromogenic phosphatase substrate BCIP

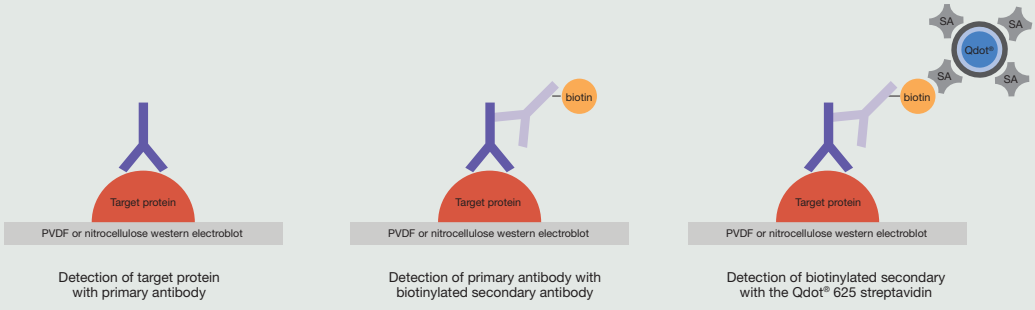

Figure 3—Schematic of the WesternDot™ kit. Detection of a biotinylated secondary antibody with Qdot® 625 streptavidin (SA).

Table 3—Comparison of western blot detection kits.

Features		NOVEX® ECL Chemiluminescent Substrate Reagent Kit	WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit	WesternDot™ 625 Western Blot Kit	WesternBreeze® Chromogenic Western Blot Immunodetection Kit
Number of mini blots per kit		25	20	20	20
Kit content	Substrate	Yes	Yes	Not needed	Yes
	Secondary antibody	No	Yes	Yes	Yes
	Qdot® 625 streptavidin	Not needed	Not needed	Yes	Not needed
	Wash buffer	No	Yes	Yes	Yes
	Blocking buffer	No	Yes	Yes	Yes
	Signal enhancer	Yes	Yes	Not needed	Not needed
	Staining dishes	No	Yes	Yes	Yes
Membrane compatibility		Nitrocellulose, PVDF	Nitrocellulose, PVDF	Nitrocellulose, PVDF, nylon	Nitrocellulose, PVDF
Signal stability		Hours	Days	Days to months	Months to years
Sensitivity		++++	++++	+++	++

(5-bromo-4-chloro-3-indolyl phosphate) and electron-transfer agent NBT (nitro blue tetrazolium). Hydrolysis of the indolyl phosphate followed by oxidation produces a dark blue precipitate at the precise site of enzymatic activity on the blot. The WesternBreeze® kit has been optimized to provide picogram sensitivity (Figure 1). Each kit includes ready-to-use or easy-to-dilute blockers, primary antibody diluent, wash solution, conjugated secondary antibody solution, substrate, and two convenient incubation trays.

For the greatest sensitivity: chemiluminescent kits

When you really need sensitivity from your western or dot blot membranes, nothing really outperforms chemiluminescence. Researchers can now choose between an entire kit, the WesternBreeze® chemiluminescent kit, or stand-alone substrate with enhancer. The enhanced chemiluminescent NOVEX® ECL substrate enables immunodetection of horseradish peroxidase (HRP)–conjugated secondary antibodies using film and X-ray developer or an imaging system. The simple two-part reagent system consists of reagent A (luminol) and reagent B (an enhancer) used in equal volumes to attain the most intense light emission. Researchers looking for even more convenience will appreciate the WesternBreeze® chemiluminescent kits, which contain the enzyme-conjugated secondary antibody, blocking reagent, and staining dishes, in addition to the substrate and enhancer. Whether you are using the NOVEX® ECL substrate or the WesternBreeze® chemiluminescent kit, the signal can be detected in as little as 2.5 hr and is stable for days. Longer exposures can help to increase sensitivity, and with either product, the blots can be stripped and reprobed.

More about Invitrogen's western blotting products

Many product features for the western blot detection kits described here are detailed in Table 3. For more information about these and other products for western blotting, visit **www.invitrogen.com/bp57**. ■

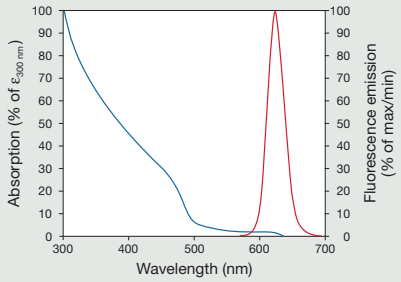

Figure 4—Absorption and emission spectra of the Qdot® 625 streptavidin conjugate.

Product	Quantity	Cat. no.
WesternDot™ 625 Goat Anti-Mouse IgG Western Blot Kit	20 mini blots	W10132
WesternDot™ 625 Goat Anti-Rabbit IgG Western Blot Kit	20 mini blots	W10142
WesternBreeze® Chromogenic Kit–Anti-Mouse	20 mini blots	WB7103
WesternBreeze® Chromogenic Kit–Anti-Rabbit	20 mini blots	WB7105
WesternBreeze® Chromogenic Kit–Anti-Goat	20 mini blots	WB7107
WesternBreeze® Chemiluminescent Kit–Anti-Mouse	20 mini blots	WB7104
WesternBreeze® Chemiluminescent Kit–Anti-Rabbit	20 mini blots	WB7106
WesternBreeze® Chemiluminescent Kit–Anti-Goat	20 mini blots	WB7108
NOVEX® ECL Chemiluminescent Substrate Reagent Kit	25 mini blots	WP20005

Faster, more flexible protein quantitation

THE NEW LUMINEX® 200™ ANALYZER WITH XPONENT® SOFTWARE.

By its very nature, fixed-array quantitation can't deliver flexibility in protein quantitation when you need it. Invitrogen's new Luminex® 200™ Analyzer with xPONENT® Software (Figures 1 and 2) enables you to measure multiple protein targets simultaneously, providing faster, more flexible protein quantitation. With the Luminex® 200™ instrument, xPONENT® software, multiplex bead-based Luminex® assays, and award-winning technical support, Invitrogen offers the total solution.

Advantages of Luminex® xMAP® technology

Luminex® xMAP® technology is based on proven technologies—flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry—that have been combined in a unique way. Three Luminex® xMAP® technologies are at the core of the Luminex® 200™ System. The first is Luminex® xMAP® microspheres—100 fluorescently dyed, 5.6 micron–sized polystyrene microspheres that act as both identifiers and solid surfaces for the assay. The second is a flow cytometry–based instrument, the Luminex® analyzer, which integrates such key Luminex® xMAP® detection components as lasers, optics, advanced fluidics, and high-speed digital signal processors. The third is xPONENT® software, designed for template-based data acquisition with robust data regression analysis—ideal for the clinical laboratory.

Figure 1—The Luminex® 200™ system. The new system performs up to 100 assays simultaneously in a single well of a microtiter plate.

With a flexible, open-architecture design, Luminex® xMAP® technology can be configured to perform a wide variety of bioassays quickly, cost effectively, and accurately. To accomplish this, the Luminex® instrument color-codes microspheres into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex® instrument, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, Luminex® xMAP® technology allows rapid and precise multiplexing of up to 100 unique assays on a single sample.

The Luminex® 200™ System

The Luminex® 200™ System includes the Luminex® 200™ instrument, the Luminex® XYP™ plate-handling platform, the Luminex® SDS sheath fluid delivery system, xPONENT® software, and a PC. This system allows multiplexing of up to 100 analytes in a single microtiter plate well and is compatible with kits designed for the Luminex® 100 instrument.

Several new features mark the Luminex® 200™ system. A new vertical probe height adjustment makes probe adjustment easier, with no need for tools. A precision-machined alignment plate through to the XYP™ platform slashes alignment time and makes sample-probe placement more reliable. In addition, a removable front bezel allows quick access to the instrument front and minimizes exposure to its inner workings. A painted fluidics compartment minimizes corrosion, and hard feet improve overall system alignment. Furthermore, a new air compressor provides quieter operations and improved reliability.

The Luminex® 200™ System has a device master file (DMF) at the U.S. Food and Drug Administration (FDA), and kits developed by Luminex® strategic partners that have received 510(k) clearance from the FDA can be used on the Luminex® 200™ system for diagnostic

purposes. The Luminex® system is fully compatible with partners' xMAP® technology–based 510(k) cleared assays and software applications.

Customize your xPONENT® software by adding modules

xPONENT® software, designed with customer input, brings the power of integration and automation to the laboratory to improve workflow efficiency. xPONENT® software combines ease of use and flexibility to allow both protein and nucleic acid testing on one Luminex® system. Users can customize their own software solution by adding individual modules, including modules for 21 CFR Part 11 compliance, security, LIS interface, and automation.

xPONENT® software provides a color touch screen that simplifies the workflow, and an intuitive graphical user interface that is easy to use. Users can calibrate the instrument for use with MagPlex® magnetic microspheres. xPONENT® software also provides optional technical controls for 21 CFR Part 11 compliance to provide an electronic signature audit trail, and automation compatibility for increased workflow efficiency. Data archiving allows users the flexibility to archive by batch, protocol, kits, and lots. New import and export functions simplify data exchange of these batches, protocols, kits, lot numbers, samples, and worklists. In addition, XML data output provides easy-to-capture data regression and more system log information.

Real-time system status indicators from the main menu facilitate immediate updates, all from the same screen, and a new graphical plate layout sets up an easier workflow preparing plates for testing. The plate layout report reduces errors, because it allows the user to print the report and use it as a guide when pipetting.

Figure 2—The Luminex® 200™ probe. Assays are carried out on a 96-well plate, with 100 tests per well. Fluidics based on flow cytometry cause suspended microspheres to line up single file before passing through the detection chamber, allowing discrete measurements.

The Luminex® total solution makes quantitation easy

Invitrogen offers several tools and services that together make up the Luminex® total solution:

- The Luminex® 200™ Analyzer
- Multiplex bead-based Luminex® assays
- xPONENT® software
- Exceptional technical support*

With the Luminex® total solution, you'll use less sample and gain more data. Experience the power of the complete multiplex analysis solution from Invitrogen. Learn more at **www.invitrogen.com/bp57**.

*Invitrogen is the winner of the 2008 Life Science Industry Awards for "Most Knowledgeable Technical Support" and "Most Responsive Customer Service".

Save time and get reliable results with the Luminex® assays

Invitrogen's BioSource™ brand brings together expertise in flow cytometry, conjugation chemistry, immunoassay development, and manufacturing in a robust line of multiplex bead-based Luminex® assays. Invitrogen's multiplex kits for the Luminex® platform have been among the most reliable and highest quality reagents available for the past 10 years. These assays provide accurate, reproducible, and sensitive quantification of multiplex proteins. In-house manufactured antibodies ensure excellent specificity and sensitivity, and fast, easy protocols allow you to perform an assay and analyze your data in less than a day.

Luminex® xMAP® technology provides multiplexing of up to 100 different extracellular or intracellular markers for simultaneous analysis, with similar reproducibility to ELISA methodology. Invitrogen now offers more than 150 kits to allow for multiplexing of markers within a given species (human, mouse, rat, and monkey) and is actively expanding the product line. Invitrogen has pioneered one of the most comprehensive analyte panels in the industry, and with the introduction of the Luminex® 200™ detection system, now offers the total solution.

Less sample, more data with the Luminex® total solution

Experience the power of Luminex® multiplex analysis from Invitrogen. For more information on the Luminex® 200™ instrument or to find Luminex® assays related to your research, visit **www.invitrogen.com/bp57**. To order the Luminex® 200™ System (which Includes the Luminex® 200™ Analyzer, Luminex® XYP™ Platform (plate handler), Luminex® SDS™ (Sheath Delivery System), and xPONENT® Software), visit **www.invitrogen.com/luminexinstrument**. ■

Measuring activation effects in response to interferon activity

UNCOVERING DIFFERENTIAL ACTIVATION PATTERNS USING THE STAT1, 3, 5a/b PHOSPHO 3-PLEX PANEL.

Multiplex bead immunoassays are finding increasing application in research. The STAT1, 3, 5a/b Phospho 3-Plex Panel illustrates some of the advantages of these assays for multiplex detection of signaling proteins.

Interferon (IFN) activation of signaling proteins

Cytokines are a diverse group of low molecular weight proteins that mediate signals between cells. One subset of this protein family is the IFNs, which are produced by the cells of the immune system in response to foreign agents such as viruses and tumor cells. Interaction of IFN-α, IFN-β, and IFN-γ with their receptors leads to activation of intracellular kinases, which transmit signals to the cell nucleus through a series of phosphorylation events. The best-characterized IFN signaling pathway activates members of the Janus kinase (JAK) family, which then phosphorylate substrate proteins known as signal tranducers and activators of transcription (STATs). The STAT family of proteins control the transcription of specific genes in response to cytokine stimulation. Phosphorylation of tyrosine residues promotes both STAT homo- and heterodimerization; the various STAT dimers complex with other nuclear proteins and bind to DNA. Given that diverse cellular responses to IFN exposure are mediated by a common pathway, it is likely that different STAT homo- and heterodimer combinations are responsible for various cell phenotypes through differential gene regulation. Monitoring the levels of individual STAT proteins in cells is one approach for determining the patterns of their activation by various IFNs and other signaling molecules.

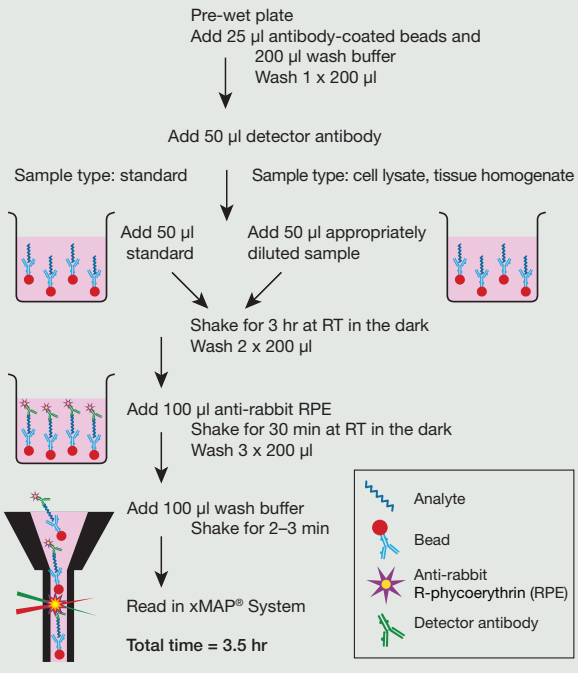

Figure 1—Summary of the protocol used for a multiplex bead immunoassay using the STAT1, 3, 5a/b Phospho 3-Plex Panel.

Luminex® multiplex assays: simultaneous protein measurements

Luminex® multiplex bead immunoassays allow the simultaneous measurement of target signaling proteins in cell lysates and tissue homogenates. They thus provide an alternative method to ELISA that offers significant savings of time and sample (Figure 1).

One of these multiplex assays, the STAT1, 3, 5a/b Phospho 3-Plex Panel, has been developed for the simultaneous detection of three members of the STAT family (STAT1, STAT3, and STAT5). To demonstrate the application of this panel, we used it to quantify the activation of these three proteins in a variety of cells exposed to IFN-α, IFN-β, or IFN-γ, and compared the results to those obtained with individual ELISAs. Three cell types were used for these experiments: K562 (human myelogenous leukemia cells), Jurkat (human T lymphocyte cells), and TF-1 (human erythroleukemia cells). Each cell line was grown in suspension to high density, serum starved for 24 hr, and then separated into four aliquots of equal volume for IFN treatment. Cell lysates were then made from each aliquot and the lysates were brought to an equivalent

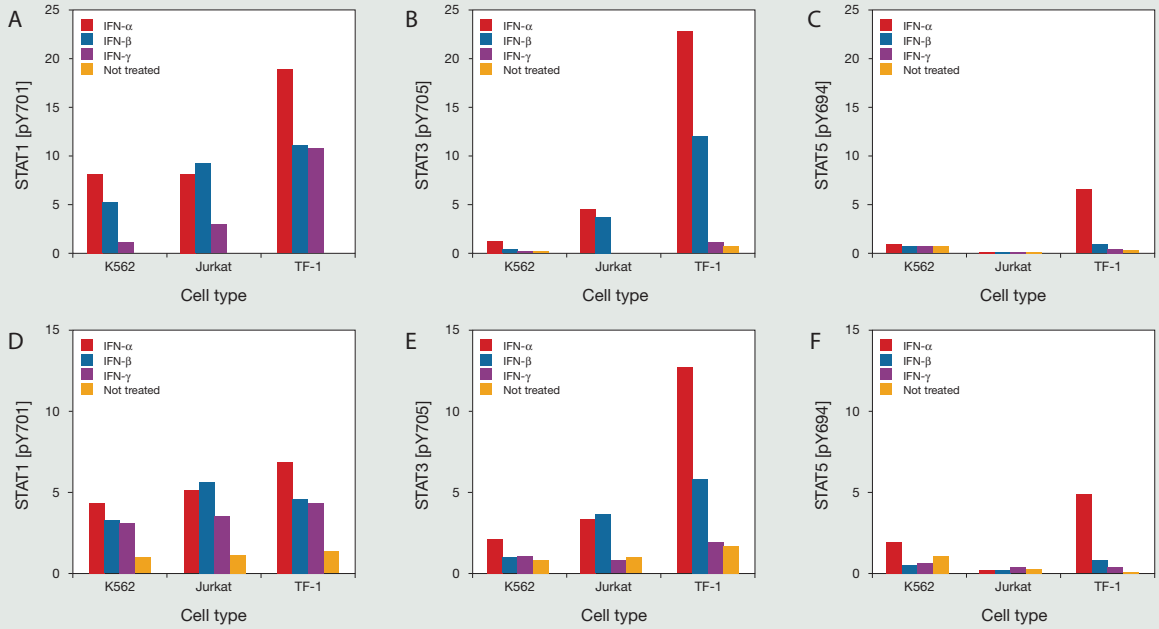

Figure 2—Differential STAT activation by cell type and IFN treatment. Quantitation of STAT1, STAT3, and STAT5a/b activation in three cell types. Cells were treated with 25 ng/ml IFN-α, 20 ng/ml IFN-β, or 100 ng/ml IFN-γ for 10 min, or were not treated and were used as controls. Cell extracts were prepared from each treatment and 40 µg/ml of each extract was analyzed using the STAT1, 3, 5a/b Phospho 3-Plex Panel (**A–C**) and ELISA (**D–F**).

concentration of total protein. Each cell lysate was analyzed for STAT1 [pY701], STAT3 [pY705], and STAT5a/b [pY694/699] levels using the STAT1, 3, 5a/b Phospho 3-Plex Panel and individual ELISA assays.

Detection of activation patterns of signaling proteins

Both the Luminex® assays and ELISA demonstrated distinct patterns of activation of STAT protein family members (Figure 2). Both methods of detection showed significant activation of STAT1 in all three cell types in response to IFN-α and IFN-β. These IFNs also activated STAT3 in Jurkat and TF-1 cells. Cellular response to IFN-γ treatment was mostly limited to STAT1 activation in Jurkat and TF-1 cells. Levels of STAT5a/b activation remained low in all cell types and treatment conditions with the exception of IFN-α treatment of TF-1 cells. Activation patterns by cell type were very similar for both IFN-α and IFN-β. This is consistent with established observations that both IFN-α and IFN-β bind to the same cell surface receptor. Distinct activation patterns for IFN-γ suggest binding to its own receptor. These results demonstrate that the pattern of STAT activation was distinct for each cell type. Differential activation of STAT1, STAT3, and STAT5a/b in the different cell types suggests the possibility of different STAT homo- and heterodimer populations in response to treatment with IFNs. For example, significant activation of both STAT1 and STAT3 in TF-1 cells in response to IFN-α suggests a greater population of STAT1/STAT3 heterodimers, in contrast to the response to IFN-γ, where only STAT1 was significantly activated and STAT1 homodimers are thus the most likely transcriptional regulators.

Comparable results to ELISA with less time and sample

The results using the STAT1, 3, 5a/b Phospho 3-Plex Panel were comparable to those obtained by ELISA (FIgure 2), but with significant savings in time, labor, and sample volume due to multiplexing (Figure 1). The ability to monitor the activation of all three proteins in a single well has the added advantage of allowing direct comparison of these different markers in the same sample.

Invitrogen is continually expanding the line of Luminex® assays, and has a STAT1, 3, 5a/b Total 3-Plex Panel in development. For more information about Luminex® assays and their application to the investigation of signaling proteins, visit **www.invitrogen.com/bp57**. ■

Product	Quantity	Cat. no.
STAT1, 3, 5a/b Phospho 3-Plex Panel	100 tests	LHO0005

Simultaneously analyze three key markers for cell death

THE APOPTOSIS 3-PLEX PANEL FOR MULTIPLEXED DETECTION AND QUANTIFICATION OF APOPTOSIS MARKERS.

Apoptosis, or programmed cell death, is a highly orchestrated, genetically controlled pathway in multicellular organisms that leads to cell ablation (Figure 1). The apoptotic pathway is responsible for the proper growth and development of the organism, as well as its immunological competence. Detecting and quantifying the key molecular events along this pathway can provide a more complete description of the state of a cell population and its stage of apoptosis.

Simultaneous detection of three apoptosis markers

Apoptosis has many morphological and biochemical hallmarks that distinguish it from cell death by injury (necrosis). In contrast with necrotic cells, apoptotic cells exhibit compaction of nuclear chromatin, shrinkage of the cytoplasm, and membrane blebbing, as well as DNA fragmentation and protease activation. Three well-established markers for apoptosis can be readily detected with the Apoptosis 3-Plex Panel. Designed for the Luminex® xMAP® platform (see pages 12–13 for more information on this technology), the Apoptosis 3-Plex Panel provides a bead-based immunoassay that simultaneously detects cytochrome *c*, cleaved caspase-3 [175/176], and cleaved poly(ADP-ribose) polymerase (PARP) [214/215] in whole cell lysates or individual cell fractions.

In the Apoptosis 3-plex immunoassay, the whole cell lysates or cell fractions are first incubated with analyte-specific antibody–coated beads, followed by a second incubation with detector antibodies, and then a third incubation with R-phycoerythrin (RPE) conjugates. During this final incubation, the RPE conjugates bind to the detector antibodies associated with the immune complexes on the beads, forming four-member, solid-phase sandwiches. After washing to remove unbound RPE conjugates, the beads are analyzed with the Luminex® 100™ or 200™ Analyzer (see pages 12–13), which examines the spectral properties of the beads and the amount of associated RPE fluorescence. From these data, the concentration of each of the three analytes can be determined.

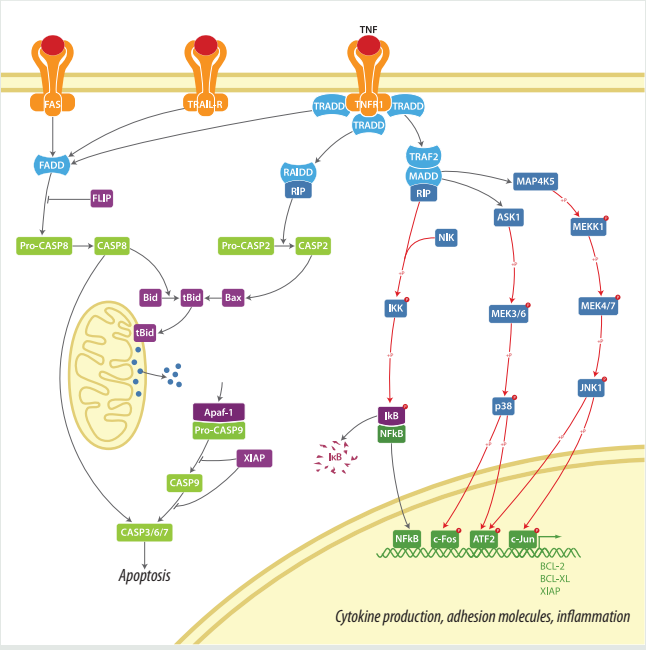

Figure 1—Apoptotic pathways.

The Apoptosis 3-Plex Panel delivers sensitivity with ease

Multiplexed detection of three apoptosis markers with the Apoptosis 3-Plex Panel not only saves time, effort, and sample, but produces results comparable to those obtained by ELISA. Figure 2 shows the detection range for each of the three analytes using both recombinant protein standards and native protein. Table 1 shows the sensitivity of the three assays using recombinant protein standards. Preparations of the individual recombinant protein standards were also used to demonstrate the absence of bead crosstalk among the components of the Apoptosis 3-Plex Panel (data not shown), an important requirement for any multiplexing assay.

Three notable markers for apoptosis

Apoptosis 3-Plex Panel simultaneously detects three reliable markers of apoptosis—cytochrome *c*, cleaved caspase-3 (between residues 175 and 176), and cleaved poly(ADP-ribose) polymerase (PARP) (between residues 214 and 215)—in whole cell lysates or individual cell fractions. Below is a brief description of each of these three markers:

- Cytochrome *c*, a 15 kDa oxidative phosphorylation protein, normally resides within the intermitochondrial space. In response to apoptotic stimuli, however, cytochrome *c* is released from the mitochondria into the cytosol. Cytochrome *c* determinations in apoptosis studies therefore have greatest utility when used in conjunction with cell fractionation techniques.
- Caspase-3 is an effector caspase synthesized as a 32 kDa inactive proenzyme. Cleavage by initiator caspases, such as caspase-9, produces a p12 subunit and a p20 subunit, which is further processed into a p17 subunit and a small polypeptide. The cleaved caspase-3 assay in the Apoptosis 3-Plex Panel detects the p20 fragment, an important marker of the early stages of apoptosis.
- PARP, a 116 kDa nuclear protein, plays a role in DNA repair as well as in other cellular processes, including DNA replication, cell proliferation, and differentiation. During apoptosis, ICE family proteases, such as caspase-3 and -7, cleave PARP to yield an 85 kDa and a 25 kDa fragment. The cleaved PARP assay in the Apoptosis 3-Plex Panel recognizes the 85 kDa fragment, and therefore serves as a reporter for caspase activation.

Comparing analysis in whole cell lysates versus cell fractions

We have tested this assay on Jurkat cell cultures that were either treated with staurosporine for 2 hr to induce apoptosis or were left untreated as a control. From these cell cultures, we prepared whole cell lysates, as well as cytosolic and mitochondrial cell fractions using the fractionation procedures outlined in the accompanying product manual.

When compared with the control cells, the staurosporine-treated Jurkat cells contained significantly elevated levels of cleaved caspase-3 [175/176] and cleaved PARP [214/215] (Figure 3), as expected for cells undergoing apoptosis. Furthermore, these increases were detectable in whole cell lysates, as well as in the cytosolic and mitochondrial fractions of the induced Jurkat cells. Thus, these two analytes can be simultaneously detected and quantified using any of these cell preparations. →

Table 1—Performance summary for the Apoptosis 3-Plex Panel.*

Assay	Bead region	Sensitivity	Linearity of dilution (cytosolic fraction)	Linearity of dilution (mitochondrial fraction)	Intra-assay precision	Inter-assay precision
Caspase-3 [175/176]	10	>5 pg/ml	0.99	0.99	3.9%	3.7%
Cytochrome *c*	16	>81 pg/ml	1.00	1.00	6.9%	9.5%
PARP [214/215]	21	>1 pg/ml	1.00	1.00	7.7%	8.0%

* Visit www.invitrogen.com/luminex for further details about Apoptosis 3-Plex Panel performance.

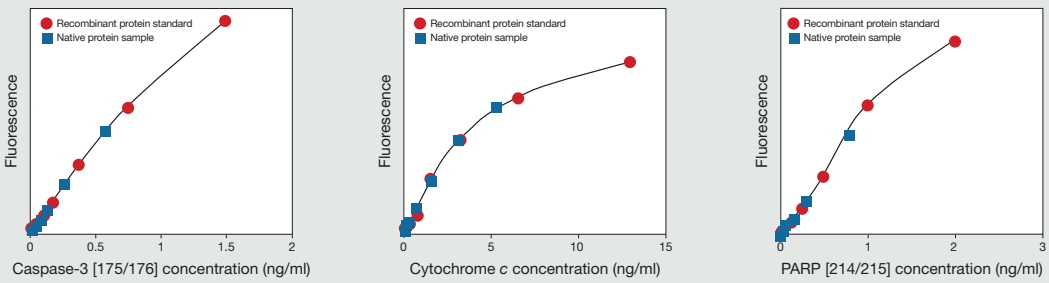

Figure 2—Standard curves for the Apoptosis 3-Plex Panel. Three standard curves were generated using the Apoptosis 3-Plex Panel (Cat. no. LHO0007) and either recombinant protein standards or native protein samples in cell lysates or fractions. For each of the three analytes—caspase-3 [175/176], cytochrome *c*, and PARP [214/215]—the standard curves generated with proteins from these two different sources do not show significant differences.

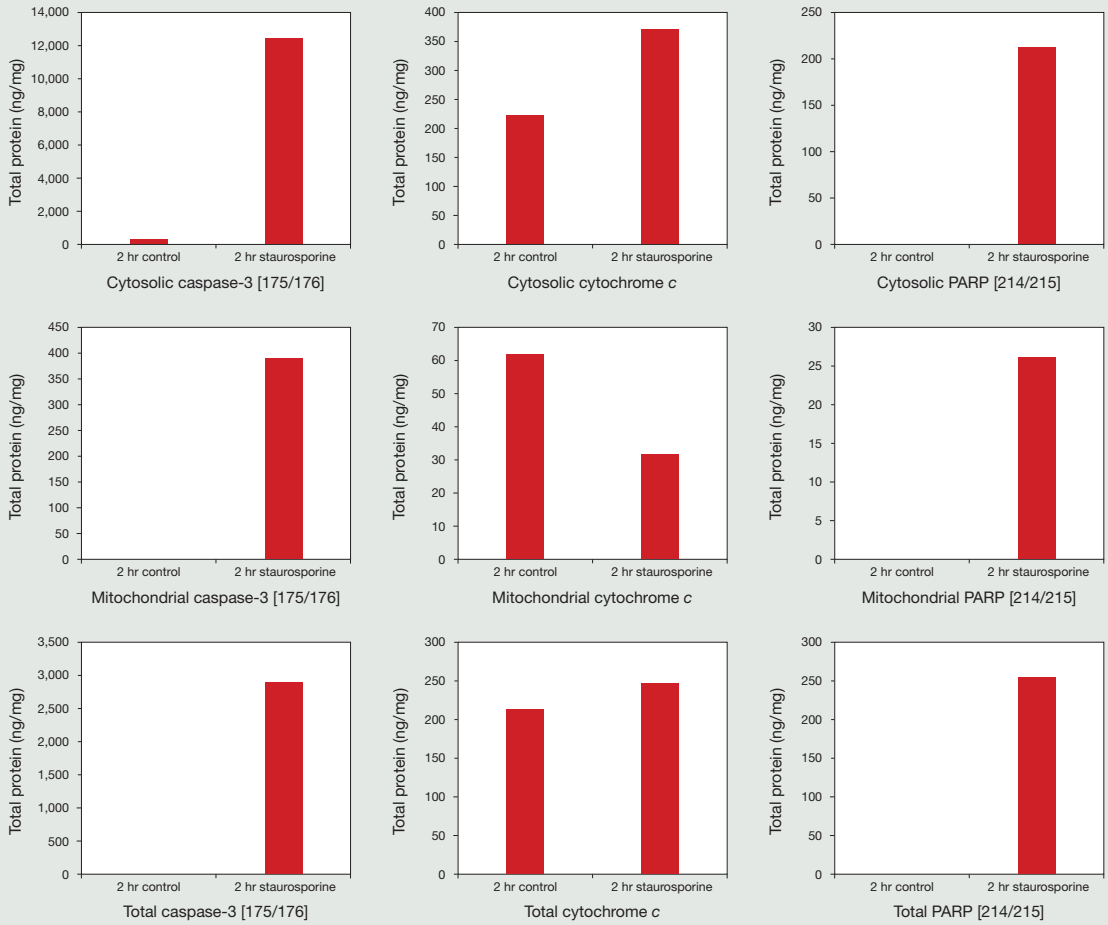

Figure 3—Detection of three apoptosis markers in various cell fractions using the Apoptosis 3-Plex Panel. Whole cell lysates, cytosolic fractions, and mitochondrial fractions were diluted in assay diluent (1:500, 1:20, and 1:50, respectively), and analyzed with the Apoptosis 3-Plex Panel (Cat. no. LHO0007).

In contrast, cytochrome *c* was detected in approximately equal concentrations in the whole cell lysates from the control cells and the staurosporine-treated cells (Figure 3). Cell fractionation was found to be necessary to observe changes in the profiles of this analyte. Cytochrome *c* was detected in the cytosolic fraction of both the control and the staurosporine-treated cells; however, the concentration of cytochrome *c* in the cytosol of the treated cells was approximately two times higher than the concentration of cytochrome *c* in the cytosol of the control cells. This increase in cytosolic cytochrome *c* in the treated cells was accompanied by a decrease in the mitochondrial compartment, consistent with the translocation of this analyte from the mitochondria during apoptosis. Therefore, to observe the apoptosis-specific signatures of each of the three analytes simultaneously, cell fractions should be used for analysis with the Apoptosis 3-Plex Panel.

For more information

The Apoptosis 3-Plex Panel is compatible with both the Luminex® 100™ and the Luminex® 200™ Analyzer. For more information about the Luminex® xMAP® platform and assays designed for this technology, see pages 12–13 or visit **www.invitrogen.com/bp57.** ■

Product	Quantity	Cat. no.
Apoptosis 3-Plex Panel	100 tests	LHO0007

Targeting and profiling lipid kinases for drug discovery

THE ADAPTA™ UNIVERSAL KINASE ASSAY.

The PI3K/Akt/mTOR pathway is a key regulator of cell growth and survival. Positive growth stimuli lead to activation of the mTOR-containing TORC1 complex, which regulates cellular growth and entry into the cell cycle via mechanisms that involve translation initiation. These same stimuli also lead to repression of apoptosis, primarily via activation of the mTOR-containing TORC2 complex, which phosphorylates and inactivates pro-apoptotic factors (Figure 1).

In many types of cancer, activity of the PI3K/Akt/mTOR pathway is upregulated due to amplification, mutation, or translocation of pathway components. As progress continues in kinase drug discovery, awareness has grown that success is, in many cases, linked to inhibition of multiple kinases, or to the specific inhibitory profile of a compound. This mechanism may also prove effective for compounds targeting the PI3K family, as simultaneous inhibition of mTOR and PI3Kα appears to be a promising means of targeting cancer. Therefore, understanding the selectivity of compounds within the PI3K family is crucial to understanding efficacy, in addition to avoiding off-target effects. The Adapta™ Universal Kinase Assay from Invitrogen provides a sensitive measure of kinase activity, and is an effective tool in the study of P13K kinases.

The PI3K family of kinases

The PI3K family of kinases includes 16 members, including 10 kinases that preferentially phosphorylate phosphoinositide substrates at either the 3-hydroxyl position (PI3Ks) or 4-hydroxyl position (PI4Ks) of the inositol moiety. The other 6 members (the class IV PI3K-like kinases, or PIKKs) preferentially phosphorylate protein substrates, and include the Akt pathway member mTOR, in addition to other kinases such as DNA-PK, a kinase involved in DNA repair. Because of the homology between family members and the diversity of their cellular functions, there is a strong need to understand the profile of an inhibitory compound among family members during the development of therapeutic agents that target PI3K family members within the Akt pathway.

Methods to detect kinase activity

Simple, nonradiometric methods to assess compound activity against the entire range of PI3K family members have been lacking, but recent advances in kinase assay technology have helped fill this need. Fluorescence assays to measure kinase activity against protein or peptide substrates have traditionally been developed using fluorescently labeled substrates that are recognized after →

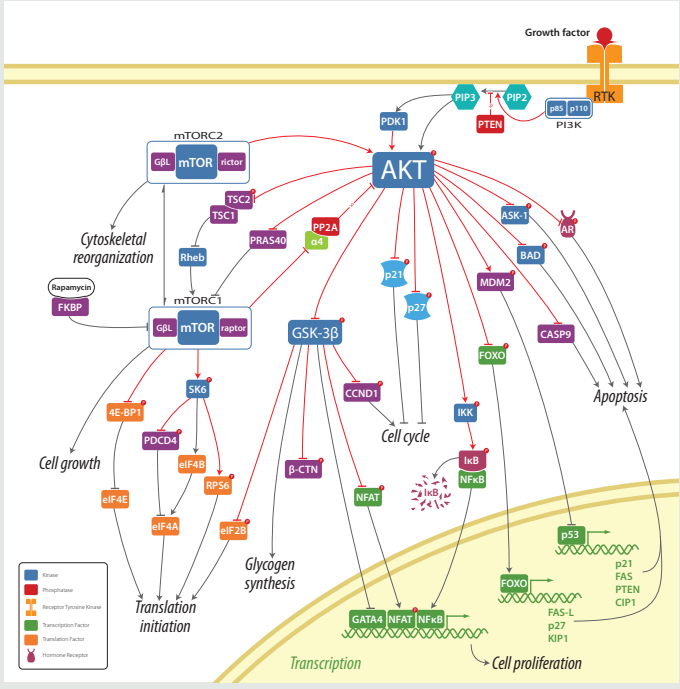

Figure 1—The P13K/Akt/mTOR signaling pathway.

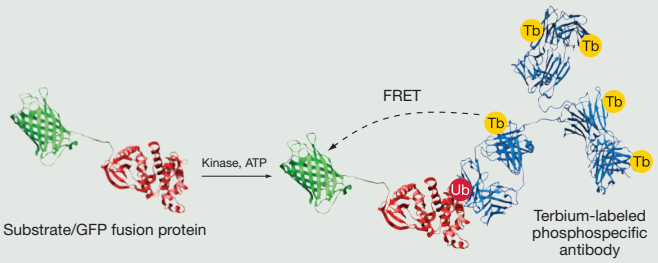

Figure 2—Schematic of a LanthaScreen™ TR-FRET–based kinase assay. Phosphorylation of a labeled protein or peptide substrate is detected by association with a phosphospecific antibody that is labeled with a terbium-based fluorophore. Because proximity between the fluorophores is dependent on phosphorylation, the TR-FRET signal correlates with kinase activity.

phosphorylation by binding to a phosphospecific antibody (Figure 2). When the phosphospecific antibody is labeled with an appropriate fluorescence resonance energy transfer (FRET) donor fluorophore (such as terbium when using fluorescein or GFP-labeled substrates), kinase activity is indicated by an increase in FRET signal, whereas kinase inhibitors cause a decrease in this signal. Because lanthanide-based fluorophores (such as terbium and europium) have long fluorescent lifetimes, FRET can be measured in a gated or "time-resolved" manner hundreds of microseconds after the sample is excited, thereby avoiding many types of optical interference such as that from precipitated or autofluorescent compounds (referred to as a "TR-FRET" assay readout). Although this strategy has been widely applied to protein kinases, it is less suited to the assay of lipid kinases because of the difficulty in preparing appropriately labeled substrates as well as in generating antibodies that show a suitable difference in affinity for the product of the kinase assay relative to affinity for the substrate itself.

The Adapta™ Universal Kinase Assay detects the formation of ADP, the common product of all kinase reactions, making the assay ideally suited for monitoring lipid kinases. The assay is based on the binding of an Alexa Fluor® 647 dye–labeled ADP analog (tracer) to a europium (Eu)-labeled anti-ADP antibody (Figure 3). When the Alexa Fluor® 647 and europium fluorophores are proximal to one another, they function as a FRET pair that can be measured in a time-resolved format (TR-FRET). When the tracer and antibody are added to an uninhibited kinase reaction, ADP formed from the kinase reaction displaces the tracer from the antibody, resulting in a decrease in FRET signal. When the kinase is inhibited, less ADP is formed, and the TR-FRET signal is maintained.

Kinase assays that measure a decrease in ATP consumption (by converting unused ATP to light in a luciferase-coupled reaction) suffer from a lack of sensitivity—to generate a substantial change in signal, the majority of the ATP present in the reaction must be converted to ADP. In contrast, the Adapta™ Universal Kinase Assay typically requires ~10% conversion of substrate to product to generate a robust assay window.

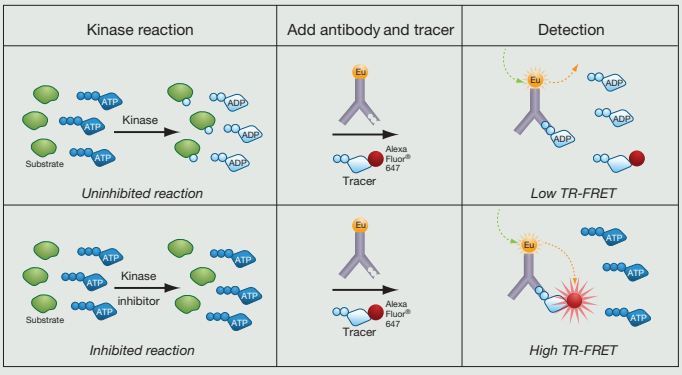

Figure 3—Schematic of the Adapta™ Universal Kinase Assay for measuring ADP formation. In the first step, the kinase reaction is performed in the presence or absence of inhibitor. In the second step, formation of ADP is detected by adding a europium-labeled anti-ADP antibody, Alexa Fluor® 647–labeled ADP, and EDTA. ADP formed by uninhibited kinase disrupts antibody–tracer interaction, resulting in a low TR-FRET signal. Inhibited kinase forms less ADP, resulting in an intact antibody–tracer interaction and a high TR-FRET signal.

Optimizing the Adapta™ and LanthaScreen™ assays for P13K kinases

To profile the small-molecule kinase inhibitor PI-103 against lipid kinase family members, assays were developed for the class I lipid kinases PIK3CA (p110α), PIK3CB (p110β), and PIK3CG (p110γ); the class III lipid kinase PIK3C3 (hVPS34); and the class IV protein kinases mTOR and DNA-PK. The class I and class III kinase assays were optimized as shown in Figure 4, using the Adapta™ assay format. A dilution series of the class I kinases was first assayed using 50 µM of their preferred PIP2:PS substrate (a mixture of phosphatidylinositol 4,5-bisphosphate, and phosphatidyl serine), at an ATP concentration of 10 µM. The class III kinase was assayed at the same concentration of ATP, using 100 µM of its preferred substrate, PI:PS (a mixture of phosphatidylinositol and phosphatidyl serine). After a 1 hr reaction, a 5 µl solution of EDTA, Eu-labeled anti-ADP antibody, and Alexa Fluor® 647 dye–labeled ADP (tracer) was added, and the mixture was allowed to equilibrate for 30 min before being analyzed. From these initial kinase titration experiments, the concentration of kinase required to generate sufficient ADP

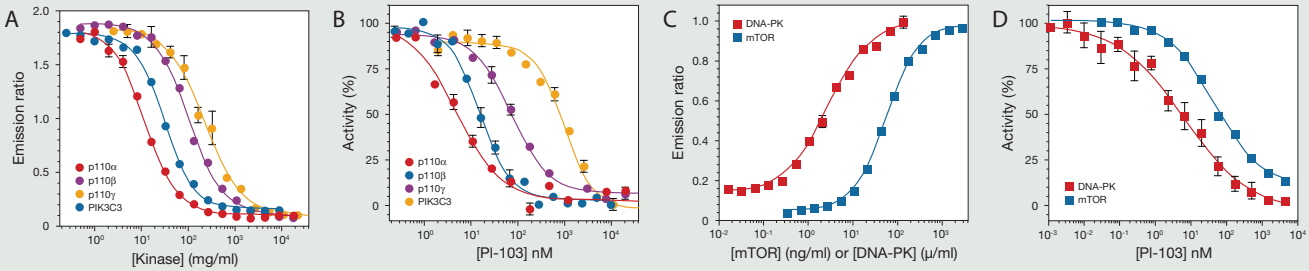

Figure 4—Optimized assays of class I, class III, and class IV PI3K family members. The inhibitor PI-103 was profiled against class I and class III P13K lipid kinases using the Adapta™ Universal Kinase Assay (**A,B**). Assays of class IV PI3K family members mTOR and DNA-PK were optimized using the LanthaScreen™ assay format (**C,D**).

to cause 80% of the tracer to be displaced from the antibody (the EC_{80} value) was determined to be 40 ng/ml for p110α, 400 ng/ml for p110β, 2 µg/ml for p110γ, and 150 ng/ml for hVPS34. Next, using these concentrations of kinases, the same reaction was performed in the presence of a dilution series of inhibitor, and IC_{50} values were calculated from the resulting TR-FRET values (Figures 4A and 4B, and Table 1).

Assays for the class IV protein kinases mTOR and DNA-PK were developed using the LanthaScreen™ TR-FRET assay format described in Figure 2. The mTOR assay was developed using a purified recombinant GFP fusion of the physiological mTOR substrate, 4E-BP1, and a terbium-labeled phosphospecific antibody that recognizes mTOR-catalyzed phosphorylation of Thr46 on 4E-BP1. The DNA-PK assay was developed using enzyme purified from HeLa nuclear extracts, a fluorescein-labeled peptide derived from the physiological DNA-PK substrate, p53, and a terbium-labeled phosphospecific antibody that recognizes DNA-PK–catalyzed phosphorylation of Ser15 on this substrate. As with the Adapta™ assay format, a dilution series of kinases was first assayed against substrate and ATP (400 nM GFP–4E-BP1 and 10 µM ATP for mTOR, or 1.6 µM Fl-p53 peptide and 4 µM ATP for DNA-PK) in a 1 hr reaction. Assays were developed by adding 10 µl of EDTA and appropriate terbium-labeled antibody, and allowing the mixture to equilibrate for 1 hr before being analyzed to determine the amount of kinase required to elicit a suitable change in the TR-FRET emission ratio (150 ng/ml for mTOR or 4 units/ml for DNA-PK). The kinase reactions were then performed in the presence of a dilution series of inhibitor, and IC_{50} values were calculated from the resulting TR-FRET values (Figures 4C and 4D, and Table 1). As with the assays performed in the Adapta™ format, there was excellent agreement between the IC_{50} values that were determined in the experiment relative to values that had been generated in radiometric assays and previously reported in the literature.

Table 1—IC_{50} values determined for the inhibitor PI-103 across class I, class III, and class IV PI3K family members.

Kinase	IC_{50} (observed)	IC_{50} (literature)*
P110α	6.3 nM	8 nM
P110β	22 nM	88 nM
P110γ	90 nM	150 nM
PIK3C3	1.4 µM	2.3 µM
FRAP1 (mTOR)	52 nM	20–80 nM
DNA-PK	7 nM	2 nM

* Previously reported literature values (Knight, Z.A. et al. (2006) *Cell* 125:733–747) were determined in a radiometric assay format.

A simple, fluorescence-based assay for lipid kinases

The study described here demonstrates the use of simple, fluorescence-based assays to accurately profile a kinase inhibitor across a range of PI3K family members. By specifically measuring ADP formation, the Adapta™ assay allows simple interrogation of a class of kinases that has traditionally been difficult to assay in a nonradiometric, fluorescent format. The Adapta™ assay is compatible with a low-volume, high-throughput screening (HTS) format and is performed like a traditional antibody-based TR-FRET kinase assay, making the assay easy to integrate into existing drug discovery workflows.

To learn more about the Adapta™ Universal Kinase Assay, or to view a complete list of Invitrogen™ products and services for interrogating the Akt/mTOR signaling pathway, visit **www.invitrogen.com/bp57**. While you're there, be sure to request your free Akt/mTOR pathway poster. ■

Product	Quantity	Cat. no.
Adapta™ Universal Kinase Assay Kit	800 rxns	PV5099

Signal preservation for live- and fixed-cell analysis

DYES AND ANTIFADE REAGENTS TO REDUCE PHOTOTOXIC EFFECTS AND MAXIMIZE YOUR SIGNAL.

One of the challenges of fluorescence imaging is minimizing the destructive effects of strong illumination and extended light exposure on dyes and cells. This is particularly true under the intense light associated with high-resolution imaging using broadband spectral output from arc lights. It has been estimated that the light intensity arriving on a sample using a 60x oil immersion objective in fluorescent mode with a mercury arc lamp or laser can be on the order of 30,000 times greater than that of standard bright field with a tungsten lamp. It's not hard to imagine that this high-intensity light exposure can have a phototoxic effect on cells and their associated labels.

Eliminating the harmful effects of light in live- and fixed-cell imaging requires different strategies. Harmful effects are not just due to signal loss or photoinstability, which is frustrating enough. As you focus, compose your shot, and refocus, your image is slowly fading, bleaching before you can capture a suitable shot. Even greater harm, especially for live-cell imaging, is due to phototoxic effects—equivalent to sunburning of the cells—that can readily occur without proper care. A bleached dye, especially a very rapidly bleaching one like FITC, can present a major concern: upon excitation of the dye, free radicals are often formed and denature proteins, cause membrane blebbing, uncouple mitochondria, trigger apoptosis, and cause many other side effects, some unknown. Fortunately, Invitrogen offers many of the best available options to greatly minimize these concerns.

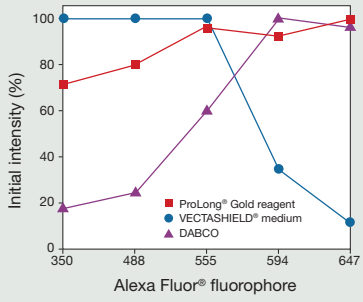

Figure 1—ProLong® Gold reagent outperforms competitors' antifades across the visible spectrum. Competitors' reagents preserve signal strength at only specific wavelengths, whereas ProLong® Gold reagent offers stability across the entire spectrum.

General strategies for live-cell work

In live-cell work, artful use of instrumentation can aid imaging. Especially critical are eliminating UV exposure, minimizing the light needed, maximizing photon detection and capture, and carefully selecting photostable dyes and controls. For example, Nipkow and Yokogawa spinning-disc confocal microscopes and related counterparts like swept-field confocal microscopes have dramatically reduced phototoxicity and greatly increased photostability in live-cell imaging. Any laser system can readily avoid UV light, whereas some imagers using traditional arc-light illumination have reduced or nearly eliminated the most harmful wavelengths—those 400 nm and shorter. UV-free illumination has also been achieved recently with diode illuminators, which provide a spectrally pure light source similar to laser lines at a fraction of the price. Consequently, the UV-free and diode systems are gaining rapid acceptance for live-cell work where light damage is a concern. Careful selection of the best organic dyes—and even fluorescent proteins, when used correctly—is providing outstanding results using these approaches.

Qdot® nanocrystals and Alexa Fluor® dyes maximize signal

Dye imaging has a reputation for being a rushed process because of the need to document results while they are still visible. Seeing your stained sample once is not sufficient—the goal is a crisp, bright image that can be shared with other scientists. Thus, for most applications with both live and fixed cells, using highly photostable fluorophores with high signal strength is important. Qdot® nanocrystals and Alexa

Fluor® dyes have high initial signal strength and photostability, and in the case of Qdot® nanocrystals, have eliminated concerns about photostability. Qdot® nanocrystals are rapidly gaining acceptance with their proven utility in live-cell work, especially in monitoring motor protein function and receptor trafficking. To learn more, visit **www.invitrogen.com/bp57.**

ProLong® and *SlowFade*® Gold antifades preserve initial brightness

"We were surprised at the increased signal we had simply from switching to the new formulations of SlowFade® *Gold and ProLong® Gold antifades."*

—Mike Mancini, Baylor School of Medicine

For fixed-cell work, the task of preserving the signal is much less complicated: using the proper dye along with antifade mountants can solve most problems. Although limiting light exposure helps, ProLong® Gold and *SlowFade*® Gold antifade mountants show remarkable benefits. Significant improvements in signal stability and initial intensity from blue to red dyes are achieved. These antifade mountants require no additional processing, can work with virtually any dye, and in the case of ProLong® Gold reagent, can preserve samples for years if stored in the dark at –20°C. *SlowFade*® Gold reagent preserves samples for 1–2 weeks. Added as the last step before a coverslip is placed, these formulations have refractive indices matched for most common coverslips, tissues, and immersion media.

Although many options for antifades exist, even the best home brew options, in our hands, were unable to maintain brightness and preserve signal strength across the entire visible spectrum as well as ProLong® Gold and *SlowFade*® Gold reagents do. As shown in Figure 1, ProLong® Gold reagent preserves initial signal strength across the entire visible spectrum, not just blue or red. Most striking is the retention of dye signal compared to, for example, VECTASHIELD® medium (Vector Laboratories) or the home brew DABCO formulation, where greater than 80% of the red or blue signal, respectively, is lost when the mountant is applied. An unappreciated and detrimental consequence of many antifades, especially some popular home brew formulations, is the suppression or quenching of initial signal strength. Although photostability is attained, there is less signal to begin with. Just as importantly, none of the other antifade options are as easy to make or use. ProLong® Gold and *SlowFade*® Gold formulations are merely removed from the refrigerator and applied to the sample, ready for consistent imaging results—no mixing, vortexing, or centrifugation required.

Choosing the right antifade mountant for your application

Both hard-mount ProLong® Gold reagent and glycerol-based *SlowFade*® Gold reagent are offered with or without DAPI in a selection of aliquot sizes. *SlowFade*® Gold reagent is useful when immediate viewing Is desired, or when glycerol-based material is preferred, such as with thick confocal-ready specimens, whereas ProLong® Gold reagent is recommended for all materials less than 20 µm in thickness (Table 1). The original ProLong® mountant is provided with two components—a mounting agent and an antifade reagent—that must be mixed and centrifuged before use. The mounting agent alone, without the antifade added, may be useful in applications where some degree of bleaching is desired, such as acceptor photobleaching in fluorescence resonance energy transfer (FRET) imaging. The original *SlowFade*® formulation is offered for samples that are sensitive to glycerol, such as carbocyanine dyes. Learn more about ProLong® Gold and *SlowFade*® Gold antifade reagents at **www.invitrogen.com/bp57**. ■

Table 1—Determining which antifade mountant is appropriate for your application.*

Product	Curing	Storage	Edge-sealing required	Compatible with broad range of fluorophores	Autofluorescence	Available with DAPI		Available without DAPI	
ProLong® Gold antifade reagent	Curing gel	Archivable	No	Yes	No	10 ml vial	P36931	10 ml vial	P36930
						2 x 5 ml dropper	P36935	2 x 5 ml dropper	P36934
SlowFade® Gold antifade reagent	Noncuring solution	Short-term use	Optional	Yes	No	10 ml vial	S36938	10 ml vial	S36936
						2 x 5 ml dropper	S36939	2 x 5 ml dropper	S36937

*The original ProLong® Antifade Kit (P7481) and *SlowFade*® Antifade Kit (S2828) may be useful for specific applications as described in this article.

Custom antibodies and peptides

START-TO-FINISH SOLUTIONS FOR CUSTOM ANTIBODY PRODUCTION AND PEPTIDE SYNTHESIS.

Invitrogen offers comprehensive custom antibody production services that are tailored to your needs. Our services include peptide synthesis, conjugation of peptides, antibody generation, serum collection, and ELISA analysis. Antibody and peptide services can be ordered separately, or you can take advantage of one of our convenient packages to get all the services you need.

Complete services

For complete services starting with peptide design, choose from one of our two rabbit protocols, or opt for production in other species including chicken, goat, sheep, guinea pig, mouse, or rat.

- PolyQuik™ protocol—An expedited protocol that runs 56 days and is perfect for quick antibody production.
- Premium protocol—Our premium protocol provides a longer immunization and additional bleeds for a higher antibody yield. This is a 70-day protocol with four immunizations to ensure an optimal immunoresponse.

Both protocols use specific pathogen-free animals. These animals are regarded as free of all primary pathogens in the test profile and suitable for any research protocol. The facility used is USDA certified, IACUC regulated, and AAALAC accredited and has NIH/OLAW/PHS assurance. Both protocols can meet your requirements for high degrees of certification and approval. For details on these protocols, visit **www.invitrogen.com/bp57**.

Convenient custom service packages are built around these protocols and include an optional peptide design service. We also offer a phosphorylation site–specific antibody package that builds on our premium services package and adds peptide affinity purification and cross-adsorption to ensure specificity and affinity.

Access to an online project tracking tool, EvoTrack™ Online, allows you to see the status of your custom antibody or peptide synthesis project, and view expected delivery dates. You can send instructions, receive milestone notifications by email, and even place orders on our secure site. EvoTrack™ Online is free to all Invitrogen custom antibody customers at **evotrack.invitrogen.com**.

Custom peptide synthesis

Invitrogen offers a full range of peptide synthesis options to meet your research needs. Custom peptides can be used for enzymatic assays, as antigens for the generation of peptide antibodies, or as components of crystallographic analysis. We offer high-quality resin-cleaved peptides made to specifications. Choose the quantity you need, including gram-scale synthesis (Table 1), and choose from crude peptide or a specific purity. You can order custom peptides HPLC purified from 70% up to 95% purity. All peptides are synthesized using Fmoc solid phase chemistry on automated column peptide synthesizers. Quality assurance is provided by mass spectral analysis and HPLC purity determination.

Most peptide antigens requested range from 12 to 16 residues. Peptides that are 9 residues or shorter have been effective antigens, but those longer than 12–16 amino acids may contain several epitopes.

Table 1—Peptide synthesis services.

Service	Description
Peptide scale	1, 5, 20, 50, or 100 mg, or up to gram scale
Peptide purification	HPLC purification to >70%, >80%, >90%, or >95%; crude peptide also available
Peptide length	Synthesis of peptides from 6 to 45 residues
Labels and modifications	Acetylation, amidation, biotinylation, formylation, methylation, myristylation, fluorescent dyes, phosphorylation (serine, tyrosine, threonine); inquire about others
Conjugations	Keyhole limpet hemocyanin, thyroglobulin, ovalbumin, bovine serum albumin, or multiple antigen peptide
Special amino acids	Peptide mix of 2–19 random amino acids in which an amino acid is altered (position, replacement amino acid, and ratio all specified by customer)
Cyclization	Disulfide bridge from cysteine to cysteine
Amino acid analysis	Digestion and analysis of amino acids

Related peptide services

Our peptide design service takes into account the many factors to consider when choosing a suitable part of a protein sequence for successful antibody production. The sequence, amino acid composition, and length of a peptide influence whether correct assembly, purification, and subsequent solubilization are feasible. Our peptide design service helps minimize solubility problems and maximize the chances of obtaining the antibody you need. Our scientists provide customized, detailed reports and sequences of the peptide to be synthesized for your project. Invitrogen peptide design includes:

- Protein/peptide sequence analysis by computer algorithms
- Multiple protein sequence alignment when needed; identification of potential glycosylation sites
- Advanced BLAST search of potential peptide candidates through relevant genome database; perusal of scientific publications relevant to peptide design when needed
- Final design of multiple peptide candidates

For designing your own peptide, PeptideSelect™ Online (Figure 1) is a web-based tool that aids in peptide design or epitope mapping. Explore PeptideSelect™ Online at **peptideselect.invitrogen.com**.

Custom antibody production

You can supply us with your own antigen; we can make antibodies against peptides (unconjugated and conjugated), fusion proteins, or recombinant proteins. Antigens can be lyophilized or provided in solution without organic solvents. Please inquire for required amounts.

Our hapten–carrier conjugation options include keyhole limpet hemocyanin, bovine serum albumin, thyroglobulin, and ovalbumin. Alternatively, we offer proprietary multiple antigenic peptide synthesis, which presents synthesized peptides in alternate, multiple conformations on a branched lysine core.

Choose the purity needed for your research. Peptide affinity chromatography produces highly functional monospecific antibodies with extremely high affinity. For site and modification specificity, select affinity purification with cross-adsorption. Protein A purification is also available. All purified antibodies are analyzed by ELISA and absorbance.

For more information about Invitrogen's complete solutions for custom antibody services or to place an order, contact customer service at **custom.services@invitrogen.com**, or visit **www.invitrogen.com/bp57**. ■

Custom antibody and protein labeling

Our custom conjugation service is efficient and confidential, and we guarantee the quality of our work. We are ISO 9001:2000 certified, with experience performing both small-scale and large-scale conjugations and purifications. Let us put our expertise to work for you. We can conjugate antibodies and other proteins to many labels, including:

- Alexa Fluor® dyes
- Qdot® nanocrystals
- Cascade Blue® dye, Oregon Green® dye, and other Molecular Probes® proprietary fluorophores
- Traditional fluorophores such as FITC, TRITC, and Texas Red® dye
- Biotins and other haptens
- R-phycoerythrin (RPE), allophycocyanin (APC), and Alexa Fluor® dye tandems
- Horseradish peroxidase (HRP), alkaline phosphatase, and other enzymes

Don't see the label you are interested in? Just ask—in most cases we can accommodate your request. For more information, including pricing and order forms, visit **www.invitrogen.com/bp57** or email us at MPCustoms@invitrogen.com

For help in choosing the optimal fluorophore for your conjugation, try the Fluorescence Spectra viewer (a link can be found at the web page mentioned above). This convenient online tool lets you compare the excitation and emission spectra of up to five fluorophores simultaneously. You can also consult the experts in our technical support department (see the back inside cover for contact information).

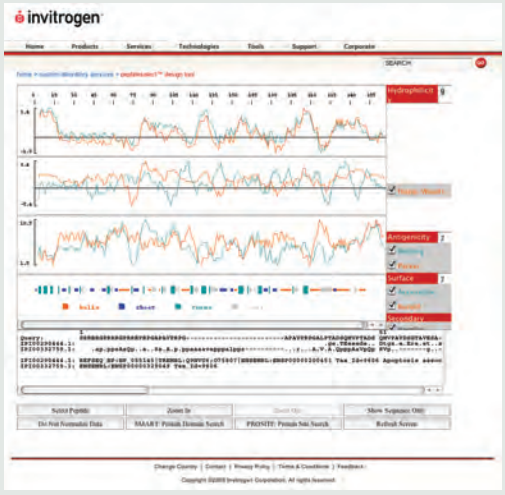

Figure 1—Example PeptideSelect™ Online display page.

Single-step isolation of functional human NK cells

USING THE NKp46 MARKER AND DYNABEADS® FLOWCOMP™ TECHNOLOGY.

NK cells contribute to a variety of innate immune responses against viruses, tumors, and allogenic cells.[1] The potential to exploit NK cells, for example in cancer immunotherapy, has encouraged the development of exciting research areas. NK cell research is today one of the most promising areas in therapeutic immunology.[2,3] Human NK cells usually express CD56; however, because this surface marker is not exclusive to NK cells, separation technology focusing on this marker potentially results in contamination by non-NK cells. In contrast, the natural cytotoxicity receptor NKp46 is selectively expressed on the surface of all peripheral blood NK cells in healthy individuals. This makes it a superior pan–NK cell specific marker conserved among human, mouse, and other mammalian species.[4,5] We therefore focused on development of this marker for isolation of human NK cells. The approach relies on technology using magnetic beads that bind to antibodies recognizing the NKp46 marker.

Isolation protocol for NK cells

We have developed an isolation protocol for NK cells using Dynabeads® FlowComp™ Human NKp46 (Figure 1). Based on Dynabeads® FlowComp™ technology, the procedure isolates target NK cells using antibodies to the human NKp46 marker. In humans, NKp46$^+$ NK cells can be isolated from peripheral blood mononuclear cells (PBMC) and comprise 3–12% of total lymphocytes (Figure 2). In this protocol (Figure 1), the NKp46 antibodies are conjugated to modified biotin and then incubated with PBMC at 2–8°C. Next, modified streptavidin-coated Dynabeads® are added to bind the antibody-labeled cells, which are then placed on a magnet to separate the bead-bound cells. Following resuspension in FlowComp™ release buffer, the NK cells are released from the beads. A similar protocol can be used to isolate a variety of other cell types using cell-specific markers.

Cells isolated in this manner are bead-free and can be analyzed by flow cytometry. They can also be used in a variety of other downstream applications such as proliferation, cytokine secretion, degranulation, and cytotoxicity assays as well as in cloning and gene expression profiling.

Cells isolated using the NKp46 marker are highly pure

We characterized the expression of several surface markers in the NK cells isolated using NKp46; the cells showed normal distribution [6] of

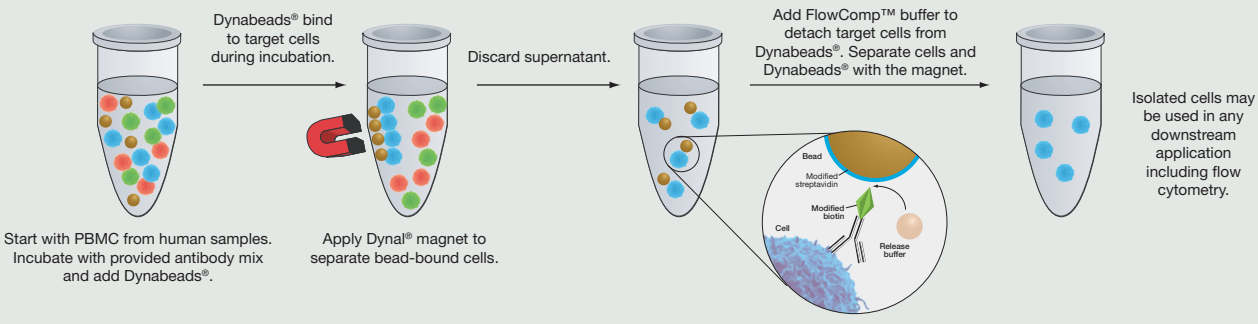

Figure 1—Schematic illustration of human NKp46$^+$ NK cell isolation using Dynabeads® FlowComp™ technology. Different starting samples or antibodies can be used for isolation of NK cells from mammalian species using the same principle.

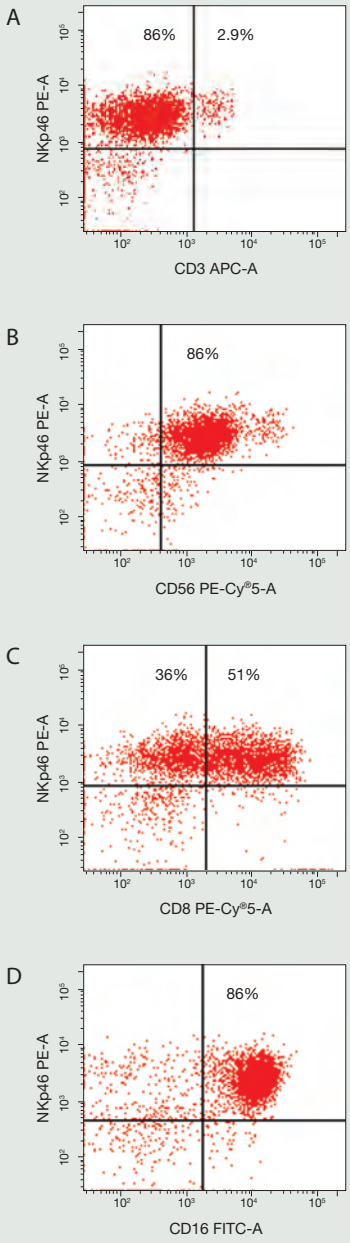

Figure 3—NK phenotype after isolation using Dynabeads® FlowComp™ Human NKp46. NKp46 is a pan–NK cell marker; all subsets of NK cells are represented in the isolated cells. Shown are (**A**) NKp46 expression, showing a uniform NKp46$^+$ population, (**B**) CD56 expression, showing both a bright and a dim population, (**C**) CD8 expression, confirming a CD8$^+$ and a CD8$^-$ subset, and (**D**) CD16 expression, illustrating that the majority of NK cells express CD16.

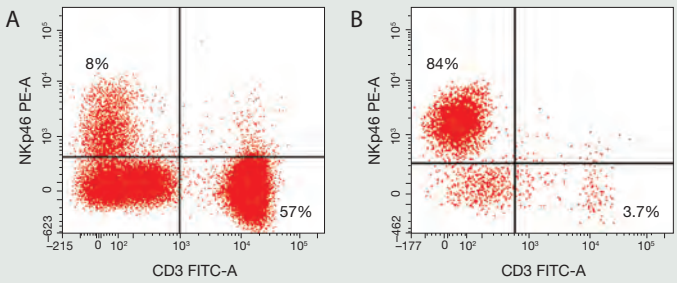

Figure 2—High purity of isolated NK cells using Dynabeads® FlowComp™ technology. (**A**) In human PBMC, about 8% of the cells were NKp46$^+$. (**B**) The isolated NKp46$^+$ cells were typically 84% pure. Contamination by CD^{3+} NK-T/T cells was minimal.

CD56^{bright}, CD56^{dim}, CD8$^+$, and CD8$^-$ subsets, and of CD16 expression (Figure 3). Expression of KIRs was found in the CD56^{dim} population and NKG2A in the CD56^{bright} population, as expected (Figure 4). Positive isolation by NKp46 represents a unique approach for cell isolation that results in high recovery of very pure NK cells in a single step. Only a few CD3$^+$ T/NK-T cells were present after isolation (Figure 2). If further CD3 depletion is required, Dynabeads® CD3 (Cat. no. 111-51D) can be used.

Isolated human NKp46$^+$ cells have upregulated LAMP-1a expression

NK cell activation, induced via receptors like NKp46, can lead to cytotoxicity mediated via polarized exocytosis of secretory lysosomes (degranulation) and interferon (IFN)-γ production. We studied cell surface mobilization of a secretory lysosome marker, LAMP-1 (CD107a), to monitor the functionality →

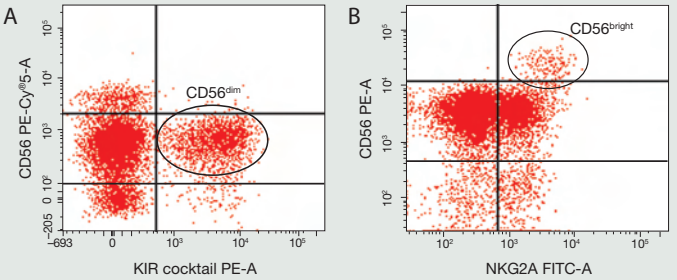

Figure 4—Expression of KIRs and NKG2A on isolated human NK cells. Only the CD56^{dim} subset stained positive for KIR receptors (PE-labeled CD158a and CD158b cocktail), whereas the CD56^{bright} subset expressed NKG2A.

of NK cells after isolation with Dynabeads®. Directly isolated or IL-2–expanded NK cells were cultured with MHC class I–deficient target cells for 4 hr prior to detection of LAMP-1. We observed increased mobilization of LAMP-1 on NK cells grown with target cells, compared to control cells (Figure 5).

Isolated NK cells efficiently kill target cells

Consistent with these findings, NK cells also killed target cells in a dose-dependent manner in a standard cytotoxicity assay (Figure 6). The isolated NK cells thus retained their functional characteristics after isolation with Dynabeads®.

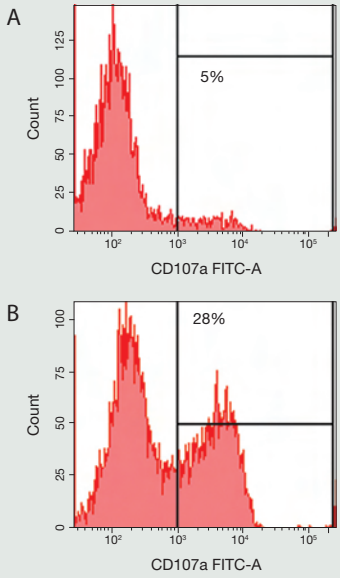

Figure 5—LAMP-1 expression by NK cells. (**A**) Human NKp46-isolated NK cells. (**B**) The human NK cells degranulated when stimulated with K562 target cells at an E/T ratio of 2:1, as measured by flow cytometry to detect LAMP-1 (CD107a) expression.

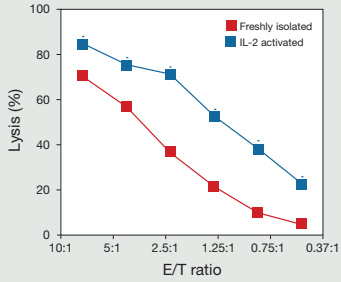

Figure 6—Capability of NK cells to kill target cells after Dynabeads® FlowComp™ isolation. Freshly isolated and IL-2–activated human NK cells efficiently kill MHC class I–deficient target cells (K562) in a 4 hr ^{51}Cr release assay.

Dynabeads® FlowComp™ technology yields highly viable cells

Isolating cells in a tube-based system is a gentle procedure giving viable and highly functional cells. Human NK cells were >95% viable after isolation, based on trypan blue and propidium iodide staining of the total sample; for comparison, NK cells isolated by an alternative column-based protocol had only 79% viability. Similar yields have been obtained for CD49b (DX5)-isolated mouse NK cells. Invitrogen plans to release a product for isolating mouse CD49b$^+$ cells, as well as an improved version of the Dynal® NK Cell Negative isolation Kit with higher isolation capacity and purity. Check our website frequently for updates.

For more information about Dynabeads® FlowComp™ technology and antibodies for cell isolation and cell staining, visit **www.invitrogen.com/bp57.** ■

References

1. Vivier, E. et al. (2008) *Nat Immunol* 9:486–494.
2. Ljunggren, H-G. et al. (2007) *Nat Rev Immunol* 7:329–339.
3. Terme, M. et al. (2008) *Nat Immunol* 9:503–510.
4. Pessino, A. et al. (1998) *J Exp Med* 188:953–960.
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6. Cooper, M. et al. (2001) *Trends Immunol* 22:633–640.

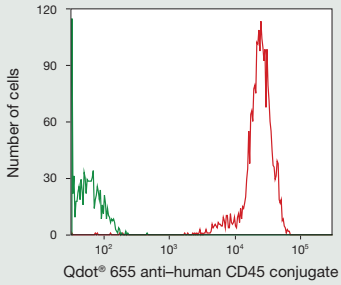
Product	Application	Quantity	Cat. no.
Dynabeads® FlowComp™ Human NKp46	One-step human NK cell isolation	3 ml, 2 x 10^9	113.64D
Dynal® NK Cell Negative Isolation Kit	Isolation of untouched NK cells	5 ml	113.15D
Dynabeads® FlowComp™ Flexi	Isolation of any cell type using desired monoclonal antibody	1 kit	110.61D
Dynabeads® CD3	Removal of residual human CD3$^+$ cells	5 ml	111.51D

QDOT® NANOCRYSTAL–CONJUGATED PRIMARY ANTIBODIES FOR FLOW CYTOMETRY

With new flow cytometry instrumentation allowing ever-expanding detection capabilities, Qdot® nanocrystals add an exciting new array of fluorescent labels, with greater flexibility and precision to design multicolor panels. Invitrogen offers a growing menu of human and mouse Qdot® conjugated primary antibodies, which offer:

- Flexibility—excited by 405 nm or 488 nm, allowing maximum use of violet lasers
- Compatibility—can be used in combination with existing organic dyes to increase the number of detectable parameters
- Stability—photostable because they are inorganic, and do not require protection from light
- Minimal single-laser compensation—narrow emission spectra allow for minimal compensation when using a single excitation source

Several of Invitrogen's newly released Qdot® conjugated primary antibodies are listed below. For more information and a complete list, visit www.invitrogen.com/bp57.



Human peripheral blood lymphocytes stained with Qdot® 655 anti–human CD45 conjugate. Flow cytometric analysis was performed using a BD™ LSR II cytometer (BD Biosciences) with a 655/20 nm bandpass filter.

Product	Quantity	Cat. no.
CD3, hamster anti-mouse, Qdot® 605 conjugate	100 µl	Q10090
CD4, rat anti-mouse, Qdot® 605 conjugate	100 µl	Q10092
CD10, mouse anti-human, Qdot® 800 conjugate	100 µl	Q10154
CD45, mouse anti-human, Qdot® 655 conjugate	100 µl	Q10155
CD45, mouse anti-human, Qdot® 800 conjugate	100 µl	Q10156
Rat IgG2a, Qdot® 605 conjugate	100 µl	Q10157
Rat IgG2a, Qdot® 655 conjugate	100 µl	Q10158
Hamster IgG1, Qdot®605 conjugate	100 µl	Q10159

NEW GROWTH FACTORS FOR STEM CELL AND CHEMOTAXIS RESEARCH

Hu Sonic Hedgehog C25II Mutant (Shh) is a member of a family of proteins that are key players for development in both vertebrates and invertebrates. There are three related genes (sonic, desert, and Indian) that share amino acid (aa) identity. The mutant form of SHH typically is significantly more bioactive than the nonmutant form.

Hu CXCL2/GRO Beta (39-107aa variant) GROa, GROb, and GROg are products of three distinct nonallelic human genes. These genes exhibit high homology, and all three are members of the alpha (C-X-C) subfamily of chemokines. GRO expression is inducible by PDGF and by inflammatory mediators, in monocytes, fibroblasts, melanocytes, and epithelial cells. GRO proteins are key neutrophil attractants and activators and can bind with high affinity to CXCR2. The 69 aa variant of human GRO beta is significantly more active than the 73 aa variant in inducing myeloperoxidase release from cytochalasin B–treated neurotrophils.

Hu CXCL13/BCA-1 is a potent chemoattractant for B lymphocytes. The BCA-1 receptor is heavily studied and known to be associated with a slower than normal development of Peyer's patches in mice.

Hu Cripto is the founding member of the epidermal growth factor-CriptoFRL1Cryptic (EGF-CFC) family of proteins that are significant players in cancer and multiple developmental processes. Key developmental processes include formation of the germ layers and dorsal organizer, specification of anterior–posterior and left–right axes, and heart muscle differentiation.

All proteins are manufactured under ISO13485 quality standards and validated using GIBCO® cell culture media. Learn more at www.invitrogen.com/bp57.

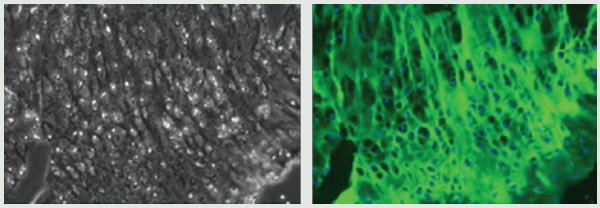
ANTIBODY FOR WESTERN BLOTTING

Protein phosphorylation is a key event in cell signaling and can activate or deactivate proteins. Invitrogen's phosphospecific antibodies are tested in peptide-blocking experiments to ensure phosphorylation site specificity. The newest phosphospecific antibody for western blotting is listed here. Learn more at www.invitrogen.com/bp57.

Product	Quantity	Cat. no.
ERB2 [pY1248] PAB Glyc	10 blot	44904G

FIVE NEW ANTIBODIES FOR STEM CELL RESEARCH

In the expanding field of stem cell research, researchers are discovering that many proteins thought to be expressed only in stem cells are also expressed in adult tumor cells. Five new antibodies for stem cell research are the newest additions to Invitrogen's comprehensive stem cell research portfolio. These antibodies are verified against multiple species and validated with multiple applications. For more information and a complete list of antibodies, visit www.invitrogen.com/bp57.



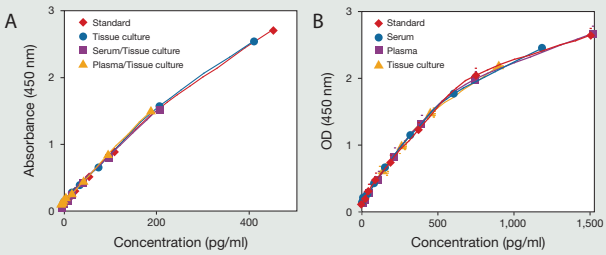
Cartilage tissue detected with Mouse Anti-Collagen II (5B2.5, Cat. no. 433130) and labeled with Alexa Fluor® 488 dye. The tissue was viewed with both phase-contrast (left) and fluorescence (right) microscopy. The green, sponge-like structure shown is consistent with the extracellular matrix. Nuclei were stained with DAPI (Cat. no. D1306, D3571, D21490, blue).

Product	Applications	Quantity	Cat. no.
Mouse Anti-A2B5 (105)	ICC, IHC, FC	100 µg	433110
Mouse Anti-Collagen II (2B1.5)	WB, ICC, IHC	100 µg	433120
Mouse Anti-Collagen II (5B2.5)	WB, IHC	100 µg	433130
Mouse Anti-GCTM2	WB, ICC, IHC, IP, FC	100 µg	433140
Mouse Anti-VRK1 (IF6)	WB, IP	100 µg	433150

ICC = immunocytochemistry; IHC = immunohistochemistry; FC = flow cytometry; WB = western blotting; IP = immunoprecipitation.

ELISA KITS

Invitrogen is dedicated to providing reliable ELISA kits, and now offers new kits for IL-17 and MMPs (matrix metalloproteinases). IL-17 is a therapeutic target for a number of diseases, and plays an important role in bridging innate and adaptive immunity. The IL-17 Mouse ELISA Kit provides quantitative measurements of mouse IL-17 in serum, plasma, and tissue culture medium. MMPs are a class of zinc-dependent proteolytic enzymes responsible for the degradation of extracellular matrix proteins and involved in an array of physiological and pathological processes, including development, morphogenesis, reproduction, wound healing, inflammation, angiogenesis, neurological disorders, and cancer cell invasion and metastasis. The MMP-9 Human ELISA Kit alllows quantitative measurements of human MMP-9 in serum, plasma, buffered solution, and tissue culture medium. Visit www.invitrogen.com/bp57 for a complete list of ELISA kits.



Demonstration of parallelism between natural and recombinant MMP-9 and IL-17. Random human or mouse serum, plasma, and tissue culture samples were serially diluted in standard diluent buffer and analyzed. The optical density of each dilution was plotted against the human MMP-9 standard curve (**A**) or the mouse IL-17 standard curve (**B**). The parallelism demonstrated by the figures indicates that the standard accurately reflects the human MMP-9 or mouse IL-17 content in natural samples.

Product	Quantity	Cat. no.
IL-17 Mouse ELISA Kit	96 tests	KMC3021
IL-17 Mouse ELISA Kit	192 tests	KMC3022
MMP-9 Human ELISA Kit	96 tests	KHC3061
MMP-9 Human ELISA Kit	192 tests	KHC3062

ANTIBODY BEAD KITS FOR THE LUMINEX® PLATFORM

Invitrogen has developed a number of multiplex bead-based Luminex® assays for quantification of intracellular and extracellular parameters, and neurobiology markers. The newest specificities are listed here; for the complete list, visit www.invitrogen.com/bp57.

Product	Quantity	Cat. no.
Apoptosis 3-Plex Panel	100 tests	LHO0007
STAT1, 3, 5a/b Phospho 3-Plex Panel	100 tests	LHO0005
PIM-1 (Total) Antibody Bead Kit	100 tests	LHO6561
PIM-1 [pS8] Antibody Bead Kit	100 tests	LHO6571
Guinea Pig Buffer Kit	100 tests	LGB0001
Guinea Pig TNF-α Antibody Bead Kit	100 tests	LGC3011
Monkey G-CSF Antibody Bead Kit	100 tests	LPC2031
Monkey IL-12 Antibody Bead Kit	100 tests	LPC0121

ANTIBODIES AND PerCP CONJUGATES FOR FLOW CYTOMETRY

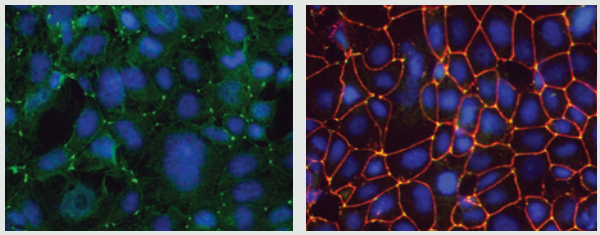
Invitrogen's large selection of high-quality, competitively priced antibodies for flow cytometry are designed for both clinical and research use. The newest specificities are listed here. In addition, Invitrogen now offers primary antibodies conjugated to peridinin chlorophyll protein (PerCP) for flow cytometry applications. PerCP, a fluorochrome commonly used as a third color on 488 nm laser–equipped flow cytometers, is excited by the 488 nm laser, with peak emission at 678 nm. To browse antibodies by specificity or application, visit www.invitrogen.com/bp57.

Product	Quantity	Cat. no.
Mouse anti-human CD11c PE-Cy5.5	0.5 ml	MHCD11c18
Rat anti-mouse IFN-γ Alexa Fluor® 647	1 ml	RM90021
Mouse anti-human CD3 PerCP	0.5 ml	MHCD0331
Mouse anti-human CD4 PerCP	0.5 ml	MHCD0431
Mouse anti-human CD8 PerCP	0.5 ml	MHCD0831
Mouse anti-human CD14 PerCP	0.5 ml	MHCD1431

Product	Quantity	Cat. no.
Mouse anti-human CD16 PerCP	0.5 ml	MHCD1631
Mouse anti-human CD19 PerCP	0.5 ml	MHCD1931
Mouse anti-human CD45 PerCP	0.5 ml	MHCD4531
Mouse anti-human CD45RO PerCP	0.5 ml	MHCD45RO31
Mouse IgG1 isotype control	0.5 ml	MG131
Mouse IgG2a isotype control	0.5 ml	MG2a31

CELL JUNCTION ANTIBODIES

Tight junctions form an important barrier of paracellular transport in epithelial cells, and the sealing of two adjacent cells at bicellular tight junctions is well described. The main structural components of bicellular tight junctions are claudins and occludin—tetra–membrane-spanning proteins. Less is known about the structure of tricellular tight junctions, a point where three adjacent cells are in contact with each other. Tricellulin is the first protein identified that specifically concentrates in tricellular tight junctions. Like claudins and occludins, tricellulin protein has four membrane-spanning domains, and is highly expressed in epithelium-derived tissues such as small intestine, kidney, and lung. Invitrogen offers the widest range of antibodies for cell junction research antibodies, including antibodies to specifically image tricellular junctions, and validated antibodies with multiple applications and species specificity. To browse antibodies by specificity or application, visit www.invitrogen.com/bp57.



Localization of tight junctions. Tricellular tight junctions are revealed using an antibody specific to the carboxy-terminus of tricellulin, shown in green (Cat. no. 48-8400). Bicellular tight junctions are revealed by the localization of occludin, shown in red (Cat. no. 33-1500).

Product	Application	Species reactivity	Clonality	Quantity	Cat. no.
Rabbit anti-tricellulin (C-term)	WB, ICC	Hu, Ms, Rt, Cn, Ch, Bv, Eq, Mk	Polyclonal, ZMD.699 (Rabbit IgG)	100 µg	48-8400
Rabbit anti-tricellulin (N-term)	WB, ICC	Hu, Ms, Rt	Polyclonal, ZMD.698 (Rabbit IgG)	100 µg	48-8300
Mouse anti-occludin	WB, E, IF	Hu, Ms, Rt, Cn	Monoclonal, OC-3F10 (Ms IgG1-κ)	100 µg	33-1500
Mouse anti-occludin-FITC	IF	Hu, Ms, Rt, Cn	Monoclonal, OC-3F10 (Ms IgG1-κ)	100 µg	33-1511
Mouse anti-occludin-HRP	WB, E	Hu, Ms, Rt, Cn	Monoclonal, OC-3F10 (Ms IgG1-κ)	100 µg	33-1520
Rabbit anti-occludin	WB, E, IP, IF, IHC, ICC	Hu, Ms, Rt, Cn	Polyclonal, Z-T22 (Rb IgG)	100 µg	71-1500
Mouse anti-ZO-1	WB, E, IF	Hu, Cn	Monoclonal, ZO1-1A12 (Ms IgG1-κ)	100 µg	33-9100
Mouse anti-ZO-1-FITC	WB, E, IF	Hu, Ms, Rt, Rb, Ch, Cn, X	Monoclonal, ZO1-1A12 (Ms IgG1-κ)	100 µg	33-9111
Rabbit anti-ZO-1	WB, E, IP, IF, IHC, ICC	Hu, Ms, Rt, Cn, Gp	Polyclonal, Z-R1 (Rb IgG)	100 µg	61-7300
Rabbit anti-ZO-1 (Mid)	WB, IF, IHC (frozen)	Hu, Ms, Rt, Cn	Polyclonal, ZMD.436 (Rb IgG)	100 µg	40-2200
Rabbit anti-ZO-1 (N-term)	WB, IF, IHC	Hu, Ms, Rt, Cn	Polyclonal, ZMD.437 (Rb IgG)	100 µg	40-2300

WB = western blot; ICC = immunocytochemistry; IP = immunoprecipitation; IHC = immunohistochemistry; IF = immunofluorescence; E = ELISA; Hu = human; Ms = mouse; Rt = rat; Rb = rabbit; Cn = canine; Ch = chicken; Bv = bovine; Eq = equine; Mk = monkey; Gp = guinea pig; X = *Xenopus*.

Recently published

A LOOK AT HOW YOUR FELLOW RESEARCHERS ARE USING INVITROGEN™ PRODUCTS.

Neuroscience: Correlating the effects of a neuroinhibitor with its distribution. Reversible inactivations resulting from localized infusions of drugs such as the $GABA_A$-agonist muscimol are an important alternative to permanent lesions for assessment of structure–function relationships in the brain. However, interpretations of the effects of muscimol infusions into the brain have been limited because of uncertainty about the distribution of the drug around the injection site. To clarify this uncertainty, a team of researchers from Yale University combined fluorescence imaging of BODIPY® TMR-X dye–conjugated muscimol in rat brain with electrophysiological and behavioral functional assessments. Their findings show that this technical approach in general, and the use of BODIPY® TMR-X conjugated–muscimol in particular, provide valuable insights into the spatial context of reversible brain inactivations.

Allen, T.A., Narayanan, N.S., Kholodar-Smith, D.B., Zhao, Y., Laubach, M., Brown, T.H. (2008) Imaging the spread of reversible brain inactivations using fluorescent muscimol. *J Neurosci Meth* 171:30–38.

Functional genomics: Imaging cell-surface expression of retinol-binding protein receptor. As part of the functional characterization of random mutants of STRA6, a 74-kDa high-affinity membrane receptor for plasma retinol-binding protein (RBP), Kawaguchi and coworkers from the UCLA School of Medicine used α-bungarotoxin binding site (BBS) tagging in combination with Alexa Fluor® 594 dye–conjugated α-bungarotoxin to screen for cell surface expression by fluorescence microscopy. The BBS tag is a small (13 amino acid) fusion insert that confers specific affinity for binding of fluorescent or biotinylated α-bungarotoxin conjugates. Insertion of BBS into an extracellular loop of STRA6 did not interfere with either cell surface expression or cellular retinol uptake (measured using ^{3}H retinol). Only two of 141 retinol uptake–deficient STRA6 mutants exhibited cell surface expression levels similar to the wild-type, demonstrating the dependence of retinol uptake activity on the cell surface expression of STRA6.

Kawaguchi, R., Yu, J., Wiita, P., Honda, J., Sun, H. (2008) An essential ligand-binding domain in the membrane receptor for retinol-binding protein revealed by large-scale mutagenesis and a human polymorphism. *J Biol Chem* 283:15160–15168.

Protein structure/function: Topographical analysis of the P-glycoprotein multidrug transporter. The cytosolic region of transmembrane helix 6 (TM6) has been identified as a key structural domain in the nucleotide-dependent multidrug efflux pumping activity of the ABC^{B1} (P-glycoprotein) transporter. Storm and coworkers constructed a series of ABC^{B1} isoforms containing unique cysteine residues within TM6 and measured the extent of covalent modification by 7-diethylamino-3-(4′-maleimidylphenyl) -4-methylcoumarin (CPM), BODIPY® 499/508 maleimide, and fluorescein-5-maleimide in the basal, ATP-bound, and vanadate-trapped conformations of each isoform. The physicochemical properties of these three probes are sufficiently different to make their reactivity with cysteine residues extremely sensitive to the local environment and protein conformational changes. Structural modeling of the covalent modification data indicated that the largest conformational changes in TM6 occur in the cytosolic extension, proximal to the nucleotide binding domain, supporting the postulated pivotal role of TM6 in coupling drug binding to nucleotide hydrolysis.

Storm, J., Modok, S., O'Mara, M.L., Tieleman, D.P., Kerr, I.D., Callaghan, R. (2008) Cytosolic region of TM6 in P-glycoprotein: Topographical analysis and functional perturbation by site-directed labeling. *Biochemistry* 47:3615–3624.

Immunohistochemistry: Enhanced GFP detection using tyramide signal amplification (TSA). Loss of fluorescence signal over time presents a significant problem in cell lineage tracing applications of GFP in transgenic animals. In the case of bone marrow stem cells, it appears that developmental progression from progenitor cells to progeny is often accompanied by epigenetic silencing of green-fluorescent protein (GFP) expression. To uncover low levels of expression below the threshold for direct GFP fluorescence detection, a team of researchers from the National Institutes of Health has developed amplified detection methods using anti-GFP antibodies in combination with tyramide signal amplification (TSA). Several 3- and 4-step procedures were successfully demonstrated. Maximal sensitivity was achieved with a 3-step procedure using rabbit anti-GFP followed by HRP–conjugated goat anti-rabbit IgG (SuperPicTure™ Polymer Detection kit) followed by Alexa Fluor® 350 tyramide or Alexa Fluor® 594 tyramide.

Toth, Z.E., Shahar, T., Leker, R., Szalayova, I., Bratincsák, A., Key, S., Lonyai, A., Németh, K., Mezey, E. (2007) Sensitive detection of GFP utilizing tyramide signal amplification to overcome gene silencing. *Exp Cell Res* 313:1943–1950.

Flow cytometry: Multiparametric detection of apoptosis. A detailed evaluation of SYTO® red-fluorescent nucleic acid stains for flow cytometric detection of apoptosis in human B-cell lymphoma cells has been conducted by Wlodkowic and coworkers. The SYTO® dyes were compared with well-established apoptotic markers including FITC annexin V, YO-PRO®-1, and fluorescently labeled inhibitors of caspases (FAM-VAD-FMK). SYTO® 62, detected using a typical allophycocyanin (APC) or Cy®5 excitation/emission configuration, gave the best results in terms of reproducing the live, apoptotic, and necrotic cell populations reported by the established markers in bivariate analyses in combination with propidium iodide. Used in conjunction with FAM-VAD-FMK or FITC annexin V, SYTO® 62 also proved to be amenable for multivariate kinetic analysis of apoptotic events.

Wlodkowic, D., Skommer, J., Hillier, C., Darzynkiewicz, Z. (2008) Multiparameter detection of apoptosis using red-excitable SYTO® probes. *Cytometry* 73A:563–569.

The publications summarized here are just a few of the recent additions to the 59,000+ references describing applications of Invitrogen™ products in our searchable bibliography database. Visit www.invitrogen.com/support to search for citations by product.

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