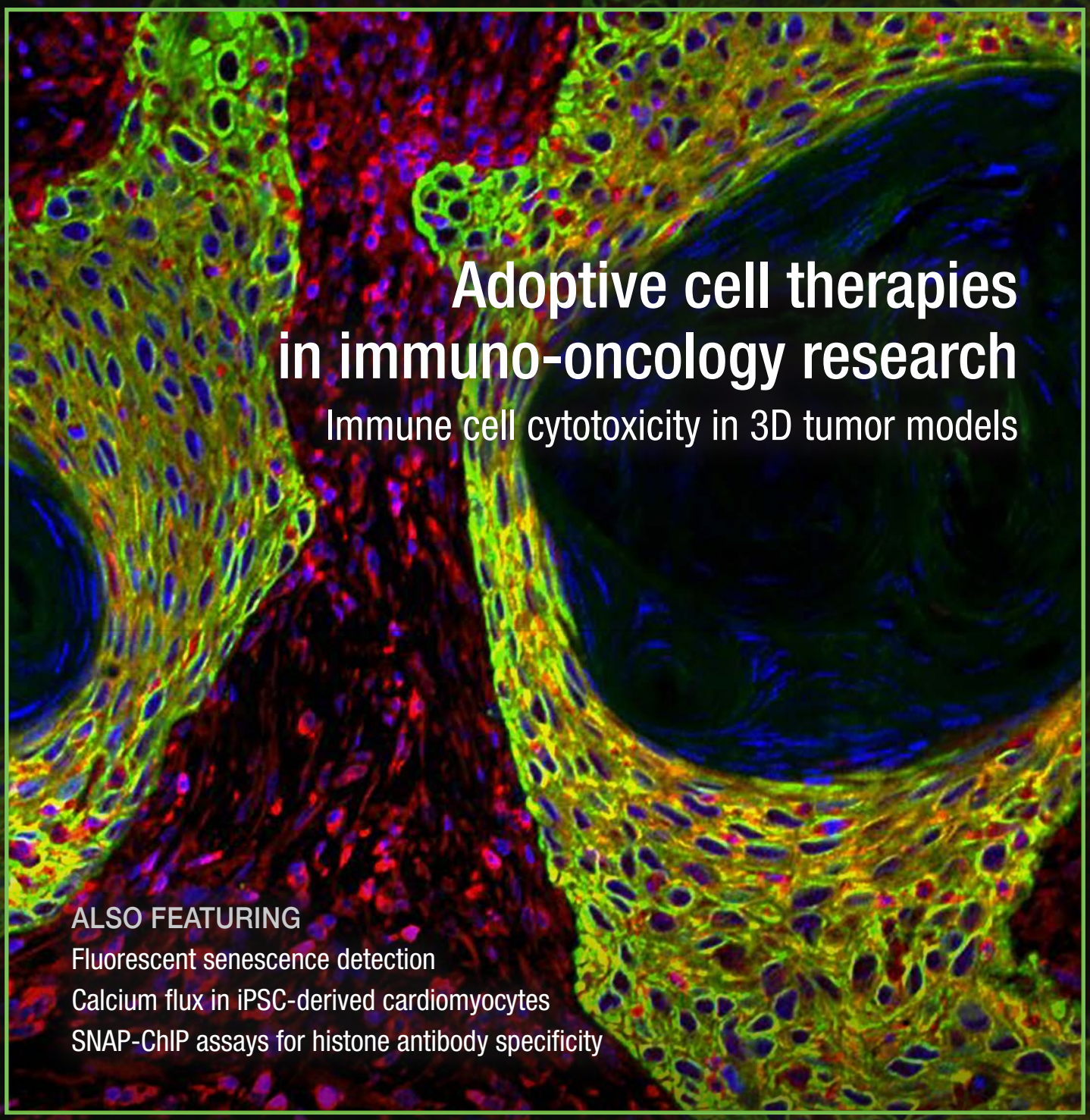
BIOPROBES 79

JOURNAL OF CELL BIOLOGY APPLICATIONS SUMMER 2019



Adoptive cell therapies in immuno-oncology research

Immune cell cytotoxicity in 3D tumor models

ALSO FEATURING

Fluorescent senescence detection

Calcium flux in iPSC-derived cardiomyocytes

SNAP-ChIP assays for histone antibody specificity

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by Thermo Fisher Scientific

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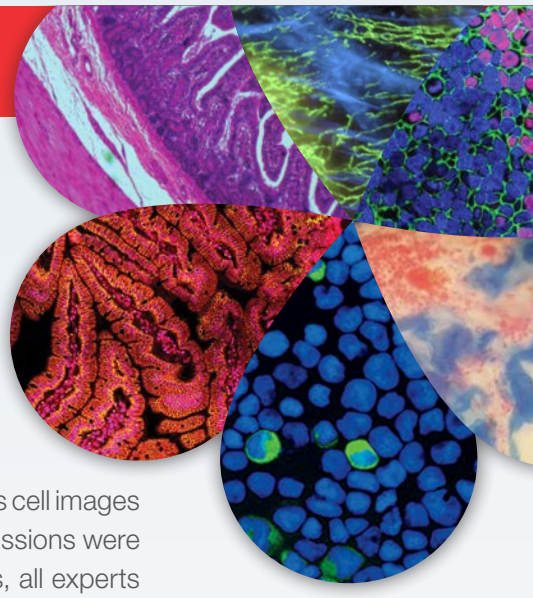
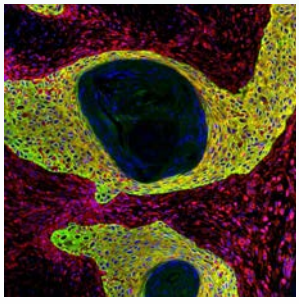


Cell-ebrate Science
Imaging Contest winners

We asked scientists in Singapore, Taiwan, and the Southeast Asia region to send us cell images showcasing their research using Invitrogen™ fluorescent reagents, and the submissions were stunning. After careful review by a panel of Thermo Fisher Scientific researchers, all experts in the field of fluorescence microscopy, three winning images were chosen.

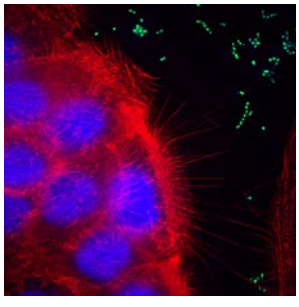
Congratulations to our 1st, 2nd, and 3rd place winners for their brilliant "beautiful science" images.



1st place winner

Mohsin Bin Bashir, A*STAR Institute of Medical Biology (IMB), Singapore

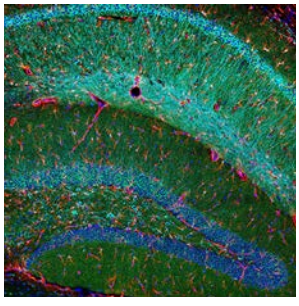
Hit the bull's eye. A human skin cancer section was probed with antibodies for keratin 14 and diaphanous 1, followed by detection with Invitrogen™ Anti–Mouse IgG Secondary Antibody, Alexa Fluor™ 488 (for keratin 14) and Invitrogen™ Anti–Rabbit IgG Secondary Antibody, Alexa Fluor™ 568 (for diaphanous 1). The section was counterstained with DAPI nucleic acid stain. The image was acquired using an Olympus™ FLUOVIEW™ FV1000 confocal laser-scanning microscope.



2nd place winner

Tay Wei Hong, Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore

A thorny defense. Spontaneously transformed keratinocyte (HaCaT) cells were infected with *Enterococcus faecalis* to delineate the mechanisms of enterococcal persistence during wound infections. The filopodia-like protrusions appear to prevent *E. faecalis* from latching onto the surface. Cells were labeled with Hoechst™ 33342 (blue, nuclei), Invitrogen™ anti-GFP antibody, Alexa Fluor™ 488 (green, *E. faecalis*), and Invitrogen™ Alexa Fluor™ 568 Phalloidin (red, F-actin). The image was acquired using a Zeiss™ Elyra PS.1 super-resolution microscope with a 63x/1.4NA oil objective.



3rd place winner

Wei Lee Lim, Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

Hippocampus—Where memories begin. A coronal section of mouse hippocampus, comprising the dentate gyrus and cornu ammonis (CA1–CA3), was immunostained with mouse anti-GFAP antibody and Invitrogen™ Goat Anti–Mouse IgG, Alexa Fluor™ 555 (red, astrocytes), chicken anti-MAP2 antibody and Invitrogen™ Goat Anti–Chicken IgY, Alexa Fluor™ 647 (cyan, neurons), and rabbit anti-CRTC2 antibody and Invitrogen™ Goat Anti–Rabbit IgG, Alexa Fluor™ 488 (green, CRTC2 protein), and counterstained with Hoescht™ 33342 (blue, nuclei).

Explore our imaging resources at **thermofisher.com/cellimaging**

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BioProbes 79

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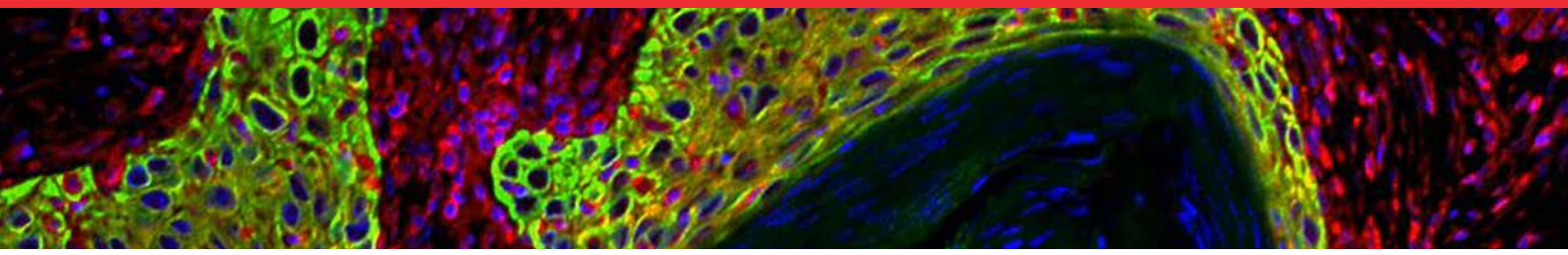

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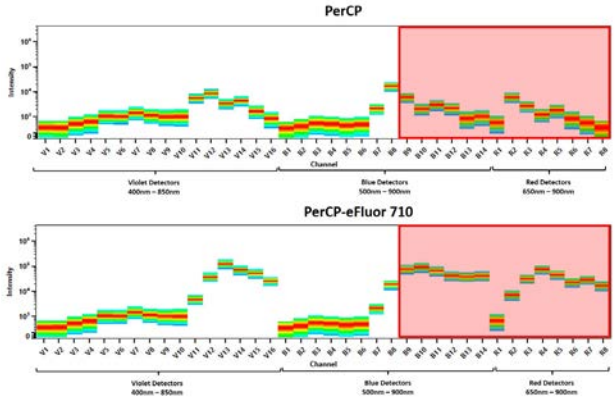
ThermoFisher SCIENTIFIC

Across the spectrum: Supporting spectral flow cytometry applications

Spectral flow cytometry exploits the inherent emission pattern of each fluorescent molecule to generate a unique spectral signature. By relying on the discrimination of unique spectral signatures rather than specific emission channels for detection, spectral flow cytometry enables the use of many fluorescent combinations that were previously difficult or impossible to separate, allowing for increasingly complex multicolor experiments.

The Invitrogen™ portfolio of flow cytometry antibodies, assays, and reagents addresses the needs of your spectral flow cytometry experiment, whether you are an immunologist investigating the role of regulatory T cells (Tregs), a cell biologist interested in fluorescently labeling RNA for downstream applications, or a microbiologist studying endocytosis, viability, or proliferation. To learn more about fluorescent reagents and assays for use on spectral flow cytometers, or for help with designing multicolor panels for this platform, visit thermofisher.com/spectralflowcytometry.



Comparison of cells labeled with PerCP and the tandem fluorophore PerCP–eFluor 710. Although the emission profiles are similar, the unique patterns in the far-red channels allow for the two fluorophores to be discriminated using spectral flow cytometry.

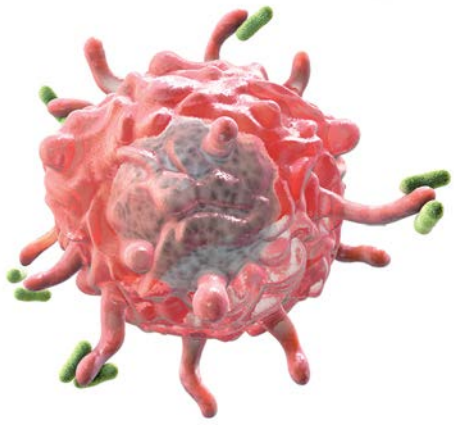
Behind the Bench blog: Macrophages and more macrophages

Want to learn a few basics about macrophages? Interested in hearing about the newest antibody markers available for studying macrophages? The Invitrogen™ Behind the Bench blog has four new blog posts that cater to your macrophage research.

Get macrophage basics directly from a Thermo Fisher Scientific R&D scientist in the blog post "Macrophages by flow cytometry: New insights through novel antibody reagents". Or, for a description of available antibody markers for studying macrophages, read three new blog posts from the R&D scientists who developed them:

- Anti–mouse RELM alpha monoclonal antibody (clone DS8RELM)
- Anti–mouse CD163 monoclonal antibody (clone TNKUPJ)
- Anti–human/mouse arginase-1 monoclonal antibody (clone A1exF5)

Each blog post provides insights into the performance of the antibody markers and their applications in the immunophenotyping of macrophages using flow cytometry. Read all about it at thermofisher.com/flowblog.



Watch: How to use the Pierce Chromogenic Endotoxin Quantitation Kit

The video "How to use the Pierce Chromogenic Endotoxin Quantitation Kit" brings to life the simple protocol of the Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit. In just over 10 minutes, you will learn how this kit accurately detects and measures endotoxin (lipopolysaccharide) in a protein, peptide, nucleic acid, or antibody sample using an endpoint amebocyte lysate assay. The Pierce Chromogenic Endotoxin Quant Kit enables endotoxin contamination detection with two linear sensitivity ranges: 0.01–0.1 EU/mL and 0.1–1.0 EU/mL. It is also:

- Highly specific—no interference from β-glucans and suitable for a wide range of samples
- Fast—perform assay in as little as 20 minutes
- Chromogenic—measure this endpoint assay using a standard spectrophotometer or plate reader at 405–410 nm

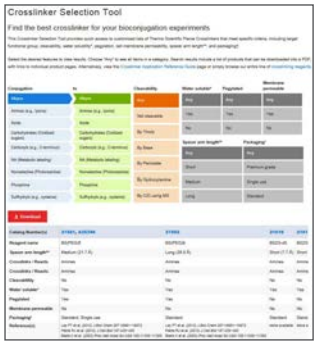
Watch the video and see for yourself at **thermofisher.com/endotoxin**.

From the video "How to use the Pierce Chromogenic Endotoxin Quantitation Kit".

Product	Quantity	Cat. No.
Pierce™ Chromogenic Endotoxin Quant Kit	30 reactions	A39552S
	60 reactions	A39552
	240 reactions	A39553

New product selection tool for crosslinking applications

Are you looking for ways to improve your protein crosslinking results? Our interactive Crosslinker Selection Tool enables you to quickly and easily find the optimal protein crosslinker based on target functional group, cleavability, water solubility, pegylation, cell membrane permeability, spacer arm length, and packaging. Download your selections to a PDF that includes a list of key references where applicable. Find the Crosslinker Selection Tool at **thermofisher.com/crosslinkers**.



New product selection tool for biotinylation applications

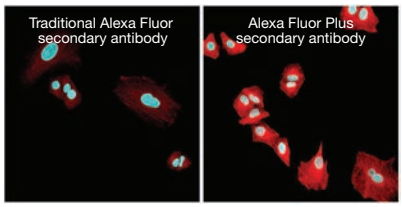
With so many biotinylation options to choose from, it can be time-consuming and cumbersome to find the right reagent. That's why we created the interactive Biotinylation Reagent Selection Tool to enable you to quickly and easily find the right biotin or desthiobiotin labeling reagent. This tool allows you to download your selections to a PDF that includes a list of key references where applicable. Find the Biotinylation Reagent Selection Tool at **thermofisher.com/biotinylation**.

Elevate your images with Alexa Fluor Plus secondary antibodies

Visualize your low-abundance targets, spend less time optimizing signals, and make every one of your samples count with Invitrogen™ Alexa Fluor™ Plus secondary antibodies. These fluorescent antibody conjugates provide up to 5.8 times higher signal-to-noise ratios than traditional Alexa Fluor secondary antibodies, due to both the proprietary formulation of the Alexa Fluor Plus dyes and preadsorption of the antibodies to minimize cross-reactivity.

With the set of 36 Alexa Fluor Plus secondary antibodies, you can now perform enhanced multiplex experiments, mixing and matching the six species and target combinations or combining them with traditional Alexa Fluor antibody conjugates for full spectral coverage. In addition, we have recently improved the original Alexa Fluor 594 dye and conjugated it to six of our best secondary antibodies, enabling more sensitive red-fluorescent detection in immunofluorescence (IF) applications. The Alexa Fluor Plus secondary antibodies are recommended for imaging and western blotting. Learn more at **thermofisher.com/alexafluorplus**.

Secondary antibody	Alexa Fluor Plus 488	Alexa Fluor Plus 555	Alexa Fluor Plus 594	Alexa Fluor Plus 647	Alexa Fluor Plus 680	Alexa Fluor Plus 800
Goat anti–rabbit IgG	A32731	A32732	A32740	A32733	A32734	A32735
Goat anti–mouse IgG	A32723	A32727	A32742	A32728	A32729	A32730
Goat anti–chicken IgY	A32931	A32932	A32759	A32933	A32934	A32935
Donkey anti–goat IgG	A32814	A32816	A32758	A32849	A32860	A32930
Donkey anti–rabbit IgG	A32790	A32794	A32754	A32795	A32802	A32808
Donkey anti–mouse IgG	A32766	A32773	A32744	A32787	A32788	A32789

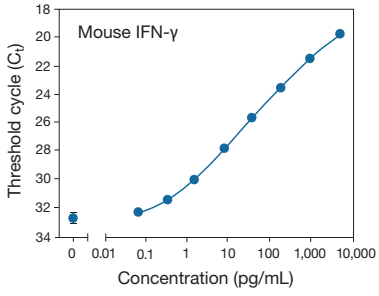


Tubulin detection with either Alexa Fluor 594 or Alexa Fluor Plus 594 conjugates. An Alexa Fluor 594 secondary antibody from another supplier (left) or Invitrogen™ Alexa Fluor™ Plus 594 donkey anti–mouse IgG secondary antibody (right, Cat. No. A32744) was used for immunofluorescent detection of tubulin (red) in U2OS cells. Nuclei were counterstained with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605), and images were obtained using the Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective.

The future is now with ProQuantum high-sensitivity immunoassays

The Invitrogen™ ProQuantum™ high-sensitivity immunoassay platform is an affordable, next-generation, no-wash method for cytokine detection and quantitation that requires only very small sample volumes. Collecting samples from mouse models can be particularly challenging. While conventional ELISAs typically require 50 µL of serum per well, the ProQuantum Immunoassay Kits only use 2 µL of serum to obtain results in duplicate or even triplicate. The small sample requirements combined with the high sensitivity and large dynamic range inherent in real-time PCR amplification make ProQuantum immunoassays the go-to solution when you need to find that needle-in-a-haystack analyte in your mouse serum samples. For more information, go to **thermofisher.com/proquantum**.

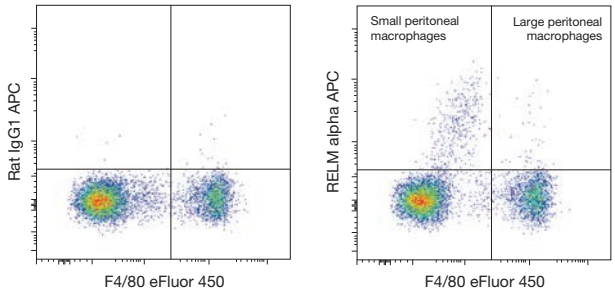
Mouse ProQuantum™ Immunoassay Kits	Quantity	Cat. No.
IFN gamma Mouse ProQuantum™ Immunoassay Kit	96 tests	A41150
IL-1 alpha Mouse ProQuantum™ Immunoassay Kit	96 tests	A42894
IL-2 Mouse ProQuantum™ Immunoassay Kit	96 tests	A42892
IL-4 Mouse ProQuantum™ Immunoassay Kit	96 tests	A42893
TNF alpha Mouse ProQuantum™ Immunoassay Kit	96 tests	A43658



Standard curve for the IFN gamma Mouse ProQuantum Immunoassay Kit. This standard curve shows an assay range of 0.064–5,000 pg/mL.

Flow cytometry antibodies: More options to fit your research

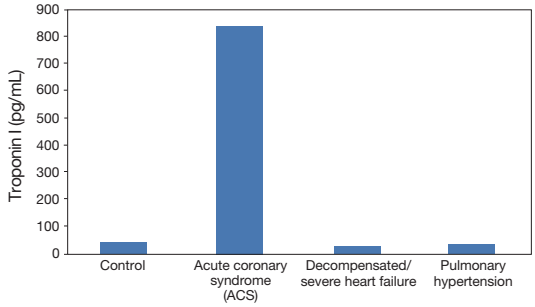
At Thermo Fisher Scientific, we understand the need for choice when selecting antibodies for multicolor flow cytometry panels, and we continue to release new conjugates every month. For example, we have recently expanded our macrophage marker portfolio. Highlights include antibodies that recognize arginase-1, RELM alpha, CD163, MARCO, AXL, and CXCL13, several of which are discussed in Invitrogen™ Behind the Bench blog posts at **thermofisher.com/flowblog**. In addition, the growing portfolio of Super Bright antibodies now includes nearly 700 antibody conjugates labeled with one of the five Super Bright dyes—Super Bright 436, Super Bright 600, Super Bright 645, Super Bright 702, and Super Bright 780—all of which are compatible with excitation from the violet laser. Choose from our wide selection of flow cytometry antibodies at **thermofisher.com/flowantibodies**.



Immunophenotyping with anti–mouse RELM alpha antibody. C57BL/6 mouse resident peritoneal exudate cells were surface-stained with Invitrogen™ F4/80 monoclonal antibody (clone BM8), eFluor™ 450 (Cat. No. 48-4801-82), fixed, permeabilized, and then intracellularly stained with either Invitrogen™ Rat IgG1 kappa Isotype Control (clone eBRG1), APC (left, Cat. No. 17-4301-82); or Invitrogen™ RELM alpha Monoclonal Antibody (clone DS8RELM), APC (right, Cat. No. 17-5441-82). All peritoneal cells were used for analysis; the majority of double-negative cells are B cells.

ProcartaPlex multiplex immunoassays for cardiovascular research

Cardiovascular diseases (CVDs), which are disorders of the heart and blood vessels, are considered to be the major cause of death globally. Cardiovascular research is mainly focused on understanding the complex molecular interactions and regulatory mechanisms involved in normal and pathological cardiovascular states. Newly released Invitrogen™ ProcartaPlex™ Panels for the Luminex® xMAP® instrument platform allow researchers to simultaneously study multiple protein biomarkers relevant to cardiac disease, cardiac remodeling, arteriosclerosis, atherosclerosis, and angiogenesis in human serum, plasma, and cell culture supernatant samples. For more information, go to **thermofisher.com/procartaplex**.



Dectetion of troponin I using the ProcartaPlex immunoassay. Troponin I levels in human plasma from patients with different cardiovascular diseases were measured using the Invitrogen™ Cardiac Disease 9-Plex Human ProcartaPlex™ Panel (Cat. No. EPX090-15809-901.

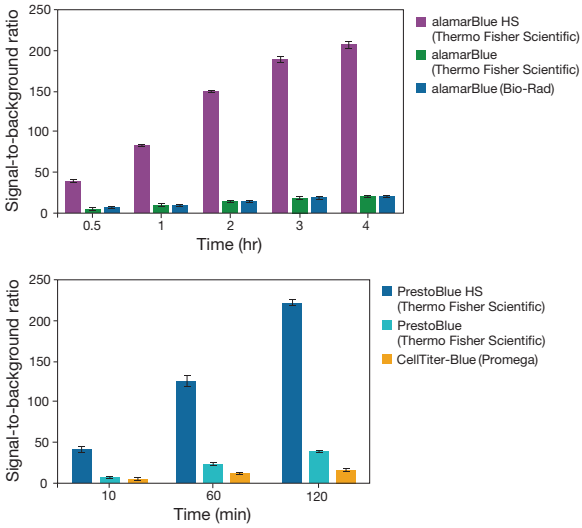
Product	Quantity	Cat. No.
Cardiac Disease 9-Plex Human ProcartaPlex™ Panel (NTproBNP, CTF-1, GDF-15, myoglobin, LDH-B, FABP-3, Big ET-1, TNI, CKMB)	96 tests	EPX090-15809-901
Cardiac Remodeling 8-plex Human ProcartaPlex™ Panel (PTX3, TREM-1, Gal-3, IL-33, IL-33R, LAP, IP-10, OPN)	96 tests	EPX080-15819-901
Arteriosclerosis 5-plex Human ProcartaPlex™ Panel (BMP-2, COMP, FGF-23, RANKL, OPG)	96 tests	EPX050-15821-901
Atherosclerosis 9-plex Human ProcartaPlex™ Panel 1 (tenascin C, HSP 60, LOX-1, MPO, FGF-2, PAI-1, TRAIL, IL-8, IL-6)	96 tests	EPX090-15822-901
Atherosclerosis 4-plex Human ProcartaPlex™ Panel 2 (Lp-PLA2, CHI3L1/YKL-40, ICAM-1, adiponectin)	96 tests	EPX040-15824-901
Angiogenesis 18-Plex Human ProcartaPlex™ Panel (angiopoietin-1, BMP-9, EGF, EMMPRIN, FGF2, follistatin, G-CSF, HB-EGF, HGF, IL-8, leptin, LYVE-1, PDGF-BB, CD31, syndecan-1, TIE-2, VEGF-A, VEGF-D)	96 tests	EPX180-15806-901
Angiogenesis 3-Plex Human ProcartaPlex™ Panel 2 (angiogenin, angiostatin, endostatin)	96 tests	EPX030-15807-901

More sensitive cell viability assays: Introducing alamarBlue HS and PrestoBlue HS reagents

All resazurin-based cell viability reagents contain a detectable amount of intensely fluorescent resorufin (the oxidized product of resazurin), which contaminates the assay and leads to increased background signal and reduced sensitivity. Using an innovative process to purify resazurin and remove contaminants, we have developed the Invitrogen™ alamarBlue™ HS and PrestoBlue™ HS Cell Viability Reagents. Formulated with highly purified resazurin, these high-sensitivity (HS) reagents enable:

- >50% reduction in background, due to contaminant removal
- >100% increase in signal-to-background ratios, producing a linear response and a large assay window
- Detection of as few as 20 cells per well
- Convenient add-and-read format, with no mixing, washing, or cell lysis required
- Analysis on either fluorescence- or absorption-based instrumentation

Both alamarBlue HS and PrestoBlue HS Cell Viability Reagents are nontoxic, aseptic reagents that do not require cell lysis. Therefore, once the assay is completed, the diluted alamarBlue HS and PrestoBlue HS reagents can be removed and replaced with complete growth medium to allow cells to be further cultured. PrestoBlue HS reagent is recommended for quick viability determination, whereas alamarBlue HS reagent is recommended in cases of extended viability studies or when using a high cell density. Learn more about our microplate assays for cell viability at **thermofisher.com/microplate-cell-viability**.



Sensitivity comparison between conventional cell viability reagents and the new alamarBlue HS and PrestoBlue HS reagents. A549 cells were assayed with the indicated cell viability reagents according to the manufacturers' protocols. Resorufin fluorescence was measured using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

Product	Quantity	Cat. No.
alamarBlue™ HS Cell Viability Reagent	25 mL	A50100
	100 mL	A50101
PrestoBlue™ HS Cell Viability Reagent	25 mL	P50200
	100 mL	P50201

Super new look. Same SuperSignal product.

Thermo Scientific™ SuperSignal™ chemiluminescent horseradish peroxidase (HRP) substrates are getting a new look, but the products inside are still the same high-quality substrates, developed and manufactured by the same dedicated experts you have trusted for years. The new flip-top box design allows reagent bottles to stand upright. Additionally, the clear bottle has a transparent label so you can easily see the volume remaining. Find out more at **thermofisher.com/supersignal**.

SuperSignal West Pico PLUS Chemiluminescent Substrate.

Product	Quantity	Cat. No.
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	200 mL	34577
	500 mL	34580
SuperSignal™ West Dura Extended Duration Substrate	200 mL	34076
SuperSignal™ West Femto Maximum Sensitivity Substrate	200 mL	34096

The powerful, new EVOS M7000 Imaging System

The Invitrogen™ EVOS™ M7000 Imaging System is a hardware refresh of the popular and fully automated Invitrogen™ EVOS™ FL Auto 2 Imaging System, with upgraded cameras that yield higher sensitivity and resolution and a high-performance PC for faster image acquisition and data processing. It was developed to meet today's expectations for image quality, user–interface interactions, speed of data generation, and flexibility. The automated image acquisition tools with enhanced autofocus algorithms are designed for demanding applications, including 3D imaging, time-lapse experiments, and large tile-stitch samples. With the optional Invitrogen™ Celleste™ 5.0 Image Analysis Software, the EVOS M7000 system becomes an exceptionally powerful tool for image acquisition, analysis, and rendering. Find out more at **thermofisher.com/evosm7000**.

EVOS M7000 Imaging System.

Product	Quantity	Cat. No.
EVOS™ M7000 Imaging System	1 system	AMF7000

The EVOS M5000 Imaging System: A fluorescence imaging workhorse

Released in late 2018, the Invitrogen™ EVOS™ M5000 Imaging System has already generated tremendous interest among researchers who need a robust, flexible, and easy-to-use microscope. It is a fully integrated digital inverted benchtop microscope that affords 4-color fluorescence, transmitted-light, and color imaging. With interchangeable optics and autofocus, single-click multichannel image acquisition, and an interface designed for usability, the EVOS M5000 system allows you to acquire publication-quality images in minutes. In addition, post-capture tools—from brightness/contrast to annotations and automated cell counting and confluence measurements—make it an exceptionally versatile microscope that is ideal for a broad range of imaging applications. Learn more at **thermofisher.com/evosm5000**.

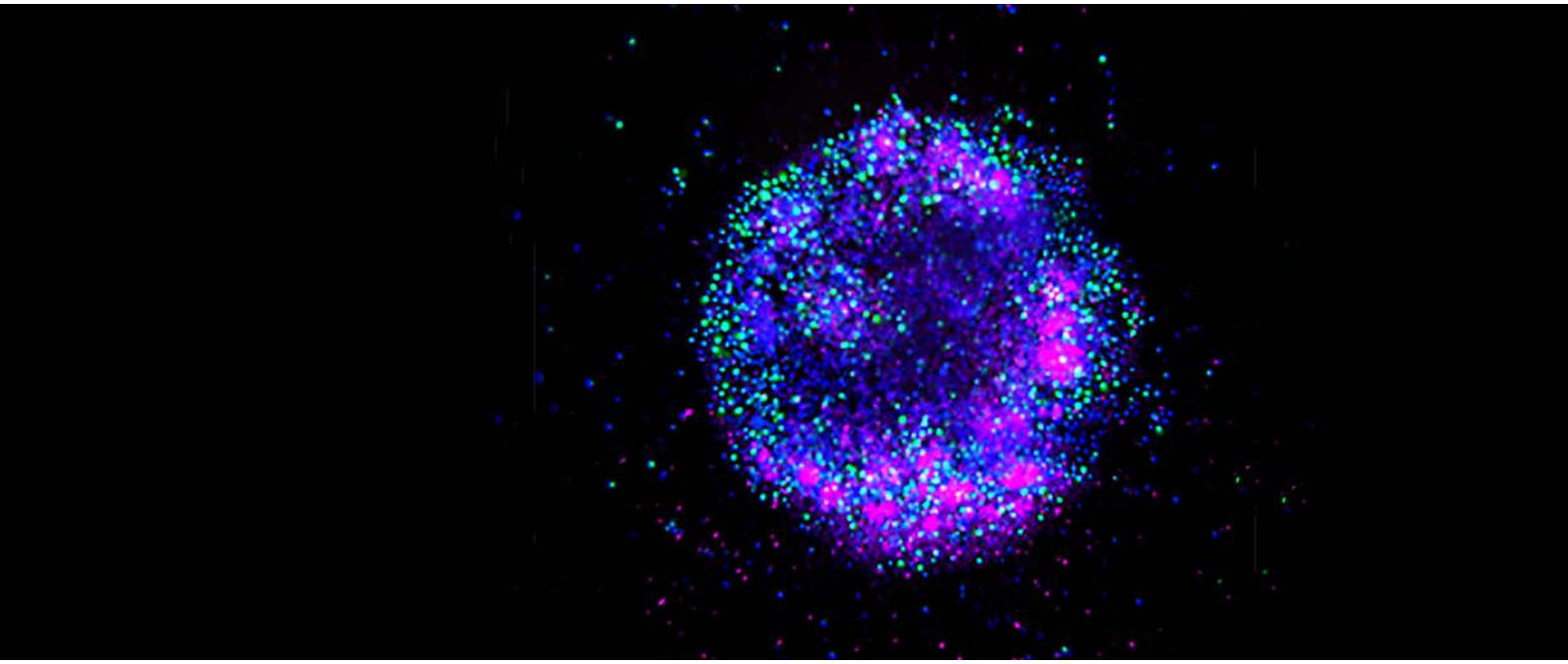
EVOS M5000 Imaging System.

Product	Quantity	Cat. No.
EVOS™ M5000 Imaging System	1 system	AMF5000

EVOS long working distance objectives

Together with the Invitrogen™ EVOS™ M7000 system, we introduced a line of long working distance (LWD) objectives designed for 1.0 mm–thick sample vessels. These objectives are available in achromat and fluorite glass quality, with phase options for both. They are ideal for imaging cells on most microscope slides and some plasticware. As with all EVOS objectives, they offer outstanding optical performance with light from visible to near-infrared. Together with the existing EVOS LWD objectives designed for 1.2 mm–thick sample vessels, these objectives offer more choices to provide the best image quality, particularly at higher magnifications of 40x and 60x. See our complete selection at **thermofisher.com/evos**.

EVOS long working distance (LWD) objectives.



Adoptive cell therapies in immuno-oncology research

Analyze immune cell cytotoxicity in 3D tumor models.

Recent advances in immuno-oncology (I-O) research are paving the way for the discovery of novel classes of cancer therapeutics that enhance or enable antitumor immune responses, overcome tumor evasion mechanisms, and promote conditions that favor immune protection. Two principal areas of research include the development of biotherapeutic antibodies that target immune-checkpoint pathways and the expansion of cellular approaches such as adoptive cell therapies (ACT), which rely on reintroduction of a patient's T cells after genetic modification. Such immunotherapies may offer distinct advantages over standard cancer treatment modalities. For example, tumor-specific immune cells have the ability to migrate to areas of the body that are inaccessible by surgery, as well as to seek out and target microscopic disease and disseminated metastases. In addition, unlike radiation and chemotherapy, the goal of immunotherapies is to act exclusively against the tumor, thereby lowering the risks of damage to surrounding healthy tissue and potentially minimizing other harmful side effects.

Figure 1 (above). Natural killer (NK) cells invading breast cancer spheroids. See details in Figure 3 caption. NK cell penetration and tumor cytotoxicity were evaluated using live-cell whole-spheroid imaging on the Thermo Scientific™ CellInsight™ CX7 LZR HCA Platform using confocal mode with 10 µm Z slicing.

In 1988, autologous T cell adoptive transfer of *ex vivo* expanded cells was used with relative success to treat patients with metastatic melanoma resistant to conventional therapies, and incremental improvements in efficacy have emerged over time [1]. More recently, investigators have realized gains with the development of chimeric antigen receptor (CAR) T cell therapy for treating certain forms of cancer, and two CAR T cell therapies were approved by the US Food and Drug Administration (FDA) in 2017. In CAR T cell therapy, the patient's (autologous) or a healthy donor's (allogeneic) T cells are genetically modified with CARs made up of antibody-based domains that recognize antigens expressed on the surface of the cancer cells [2].

Working with 3D tumor spheroid cultures

Increasingly, ACT research is focusing on the treatment of solid tumors such as those found in lung and breast cancers. Tumors and their associated microenvironments within intact organisms contain highly complex and dynamic sets of interactions between different cell types, as well as multiple chemical gradients and a variety of extracellular matrix components. Unlike their 2D tissue culture counterparts, 3D tumor spheroids can provide physiological and biochemical conditions that more closely resemble the tumor microenvironment in an intact organism. Thermo Fisher Scientific is developing 3D cell culture techniques and low–cell attachment microplates, along with assays and instruments for analyzing cell function, including fluorescent reagents for detecting viability, proliferation, and apoptosis. Here we demonstrate the use of several of these techniques and reagents to study immune cell infiltration in 3D tumor spheroid cultures and the subsequent death, by apoptosis, of spheroid cells (Figures 1–3).

Immune cell cytotoxicity in 3D cell models

Figures 2 and 3 show our investigation of the penetration and potency of cytotoxic T cells and natural killer cells in 3D cell models of lung and breast cancer tumors, respectively. We used the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis (HCA) Platform with Thermo Scientific™ HCS Studio™ Cell Analysis Software to segment and measure fluorescence intensities of individual cells within the 3D spheroid. The ability to conduct this type of detailed analysis of immune cell cytotoxicity may facilitate our understanding of the mechanisms of cytotoxic immune cells and their potency in immunotherapies.

Figure 2 shows the response of lung cancer spheroids (generated with A549 cells) in the presence of increasing numbers of T cells. The T cells were labeled with a far-red–fluorescent cell tracer (Invitrogen™ CellTracker™ Deep Red Dye), and the spheroids were labeled with an apoptosis indicator (Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent). As the T cell dose increased, we observed more A549 cells in a lung cancer spheroid undergoing apoptosis, →

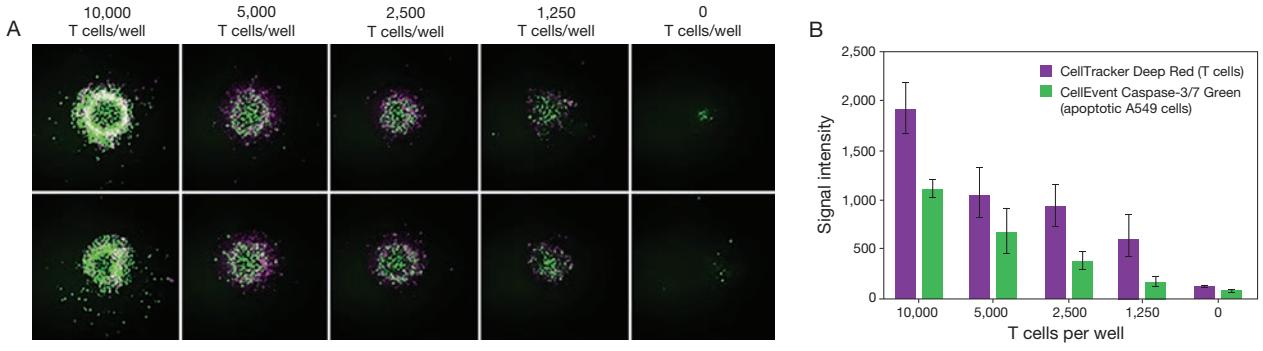


Figure 2. T cell–dependent killing of lung cancer spheroids. T cells were isolated from human peripheral blood and expanded for 5 days with Gibco™ Dynabeads™ Human T-Activator CD3/CD28 (Cat. No. 11131D) in Gibco™ CTS™ OpTmizer™ T Cell Expansion SFM (Cat. No. A1048501). Cells were analyzed with the Invitrogen™ Countess™ II FL Automated Cell Counter to determine cell viability and concentration, and cell concentration was adjusted to 1 x 10^6 cells/mL in Gibco™ PBS, pH 7.2. T cells were then labeled with 2 µM Invitrogen™ CellTracker™ Deep Red Dye (purple, Cat. No. C34565) for 15 min and washed with CTS OpTmizer medium. For spheroid formation, A549 (adenocarcinomic human alveolar basal epithelial) cells were plated in a Thermo Scientific™ Nunclon™ Sphera™ 96-well microplate at a density of 7,500 cells/well, incubated overnight in a cell culture incubator with 5% CO_2 at 37°C, and analyzed on the Invitrogen™ EVOS™ XL Core Imaging System to confirm spheroid formation. Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (2 µM final concentration) (green, Cat. No. C10723) and the indicated number of labeled T cells were added to each well, and the mixture was incubated for 4 hr at 37°C. **(A)** Two examples of the response of a lung cancer spheroid to 4 different T cell concentrations are shown, each imaged on the Thermo Scientific™ CellInsight™ CX7 LZR HCA Platform using confocal mode with 10 µm Z slicing. **(B)** Apoptosis in A549 spheroids and the T cell dose response were quantified using the CellInsight CX7 LZR platform and Thermo Scientific™ HCS Studio™ Cell Analysis Software. The spheroids were segmented as single objects based on the brightfield image using HCS Studio software, and cells were counted within the object. Mean signal intensities of cells within the spheroid were used to quantify immune cell cytotoxicity.

which was quantified using the mean signal intensities of cells within the spheroid.

Figure 3 shows the results of an investigation of antibody-dependent cell killing in breast cancer spheroids by natural killer (NK) cells. The NK cells were labeled with the far-red–fluorescent CellTracker Deep Red Dye, and the SKBR3 cells in the breast cancer spheroids were labeled with Hoechst 33342 nucleic acid stain and the CellEvent Caspase-3/7 Green reagent. While the addition of NK cells alone to the spheroids induced moderate cytotoxicity, the combination of NK cells and trastuzumab (a monoclonal antibody used to treat breast cancer) resulted in increased apoptosis and degradation of the spheroid structure.

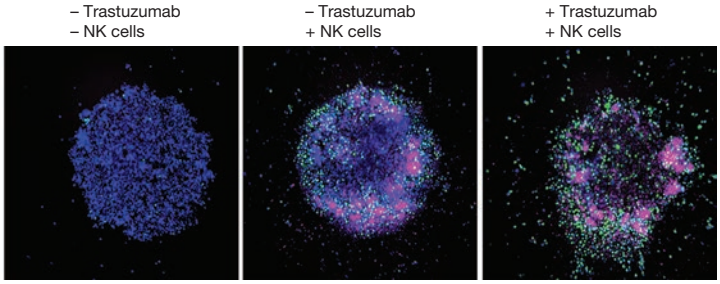


Figure 3. Antibody-dependent cell killing in breast cancer spheroids. Natural killer (NK) cells were isolated from human PBMCs using the Invitrogen™ Dynabeads™ Untouched™ Human NK Cells Kit (Cat. No. 11349D) and labeled with Invitrogen™ CellTracker™ Deep Red Dye (purple, Cat. No. C34565). These cells were added to SKBR3 breast cancer spheroids labeled with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605) and Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (green, Cat. No. C10723), with or without 30 nM trastuzumab, and the mixture was incubated for 2 hr at 37°C. NK cell penetration and tumor cytotoxicity were evaluated using live-cell whole-spheroid imaging on the Thermo Scientific™ CellInsight™ CX7 LZR HCA Platform using confocal mode with 10 µm Z slicing. The spheroids were segmented as single objects based on the brightfield image using Thermo Scientific™ HCS Studio™ Cell Analysis Software, and the cells were counted within the object. Mean signal intensities of cells within the spheroid were used to quantify immune cell cytotoxicity (data not shown). The addition of NK cells induced moderate cytotoxicity (middle), whereas the addition of NK cells and trastuzumab resulted in substantial apoptosis and degradation of the spheroid structure (right).

High-content imaging and analysis

The CellInsight CX7 LZR HCA Platform is an ideal platform for high-resolution imaging and analysis of live 3D cell cultures. This integrated benchtop system offers widefield, confocal (critical for axial resolution in 3D acquisition), and brightfield imaging, with extremely bright illumination to penetrate thick samples, and microscope objectives from 2x to 60x. It also provides fast image acquisition with shorter exposure times and laser autofocus capabilities.

With seven fluorescence detection channels on the CellInsight CX7 LZR system, and expanded excitation options provided by the near-infrared (785 nm) laser, it is possible to conduct multiplex analysis using a combination of structural and functional probes to investigate complex biological processes in both the immune cells and the individual cancer cells that make up the tumor spheroids. Live-cell imaging and analysis also benefit from advanced instrument features that allow you to control the amount of light reaching the sample, helping to minimize photobleaching and phototoxicity. The CellInsight CX7 LZR HCA Platform is compatible with a broad range of plate formats and types and offers optional onstage incubation and robotic plate handling, while the HCS Studio software allows access to all instrument configuration and control functions. This intuitive, icon-driven tool helps to manage the experimental design and workflow, starting with plate maps and protocol setup, all the way through image acquisition and data analysis.

Explore 3D culture and analysis

Thermo Fisher Scientific offers a suite of culture media, cultureware, cell analysis reagents, and fluorescence instrumentation for 3D cell culture. Learn more at **thermofisher.com/spheroid**. ■

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Product	Quantity	Cat. No.
CellEvent™ Caspase-3/7 Green Detection Reagent	25 µL 100 µL	C10723 C10423
CellInsight™ CX7 LZR High-Content Analysis Platform	1 each	CX7A1110LZR
CellInsight™ CX7 LZR HCA Platform with HCS Studio™ Cell Analysis Software Extended Warranty Package	1 each	A37014
HCS Studio™ 2.0 Cell Analysis Software	1 each	SX000041A
CellTracker™ Deep Red Dye	20 x 15 µg	C34565
CTS™ OpTmizer™ T Cell Expansion SFM, bottle format	1,000 mL	A1048501
Dynabeads™ Human T-Expander CD3/CD28	10 mL	11141D
Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation	0.4 mL 2 mL 5 x 2 mL	11161D 11131D 11132D
Dynabeads™ Untouched™ Human NK Cells Kit	1 kit	11349D
EVOS™ XL Core Imaging System	1 system	AMEX1000
NucBlue™ Live ReadyProbes™ Reagent	1 kit	R37605

Best practices for multiparameter flow cytometry

Experimental setup and panel design for accurate results.

Flow cytometry is an elegant quantitative technology, allowing the interrogation of single cells among tens of thousands or even millions of cells in minutes. Advantages of multiparameter flow cytometry include the ability to probe single cells with multiple functional markers, to correlate protein expression levels using multiple antibodies, and ultimately to more accurately define cell populations. Increasing the number of targets and fluorophores, however, also increases the complexity of the experiment and requires greater attention to detector optimization, panel design, controls, and other setup details. Here we describe a few best practices for designing a multiparameter flow cytometry experiment. While not comprehensive, they encompass some of the most important features of good experimental setup and panel design [1,2].

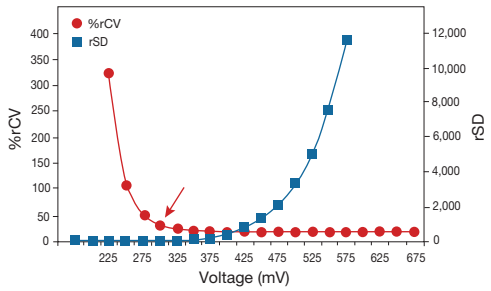

Figure 1. Determination of the optimal voltage setting for a flow cytometer detector using a voltage walk. The voltage walk method shown uses a single dimly fluorescent hard-dyed bead. Data are acquired at each voltage setting increment in a given detector, and the percent robust coefficient of variation (%rCV) and robust standard deviation (rSD) are exported and plotted vs. voltage to visualize the point of inflection. The lowest voltage on the %rCV curve before the increase in the rSD should be used for the detector. In this example, the MVR is determined to be 300 mV (arrow).

Voltage walk for highest-quality data

Cytometer manufacturers provide a performance test that certifies the instrument is performing optimally with respect to a precise set of specifications. Detector optimization takes this process a step further, enabling the highest-quality data to be obtained in each flow cytometer channel. For each detector, the voltage (or gain) chosen must provide the best separation between positive and negative signals and ensure all measurements are within the detector's linear range.

Typically, the voltage walk method (Figure 1) is used to determine the minimum voltage requirement (MVR) that allows clear resolution of dim fluorescent signals from the background noise of the instrument. In this method, dimly fluorescent beads are run using a series of increasing voltage settings, and the spread of the signal (or the coefficient of variation, CV) is plotted against the voltages. Decreasing the voltage for a detector below its MVR can result in the loss of resolution of dim populations, and increasing the voltage above its MVR gives no advantage for population resolution. Because this method does not ensure that the brightest signals do not exceed the upper limit of the detector's range, alternative methods have been developed in which both unstained and brightly stained beads or cells are used to determine MVR [3,4].

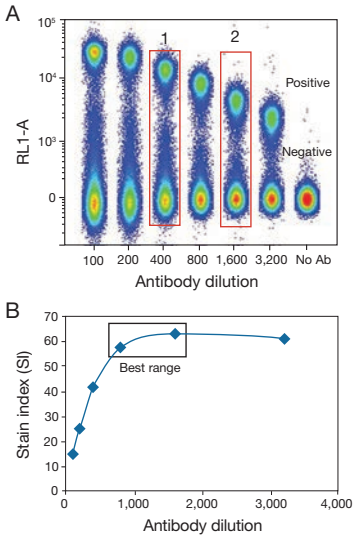

Figure 2. Antibody titration example. (A) Cells were incubated with serial dilutions of an APC-conjugated mouse anti-CD8 antibody, run on the Invitrogen™ Attune™ NxT Flow Cytometer, and analyzed using FlowJo™ software. Data are shown on a single graph for easier comparison. Note that the sample highlighted in box 1 is the saturation concentration of antibody, whereas the box 2 sample is the separation concentration. **(B)** The stain index (SI) was calculated for the data in (A) using the equation: (Mean (positive cells) – Mean (negative cells)) / (2 x SD (negative cells)), and plotted as a function of the antibody dilution.

Antibody titration in panel design

Antibody titration is also an important optimization technique for multiparameter flow cytometry and is the best way to minimize non-specific binding and increase signal detection. It can also be used to minimize spillover spreading, which occurs when the signal from dyes that emit fluorescence over a broad range of wavelengths is captured in multiple detectors, complicating data interpretation. To perform a simple antibody titration, start with the manufacturer's →

recommended concentration, perform serial 2-fold dilutions, and plot the stain index (SI), which is a measure of the relative brightness of a fluorophore-conjugated antibody [5]. The SI for a specific antibody–dye conjugate and its spillover spreading will help to determine if a separating concentration (at which negative and positive cells display the greatest difference in fluorescence), or a saturating concentration (at which the antibody has saturated the antigen available in the cells) of antibody should be used (Figure 2). A separating concentration provides good separation of labeled vs. unlabeled cells (e.g., when identifying percent-positive populations in immunophenotyping experiments), reduces spreading error, and conserves antibody. Saturating antibody concentrations—sometimes required for the detection of low-abundance antigens—can lead to increased spillover spreading and difficulty detecting dim signals in other detectors.

Fluorophore selection and allocation

One of the biggest challenges in multiparameter flow cytometry is selecting the combinations of fluorophores and antibody conjugates that minimize the need for compensation and spillover adjustments without compromising data quality. The more dyes included in a flow cytometry panel, the more likely that spillover spreading will reduce the ability to distinguish the specific signal of one fluorophore in the presence of others. When choosing fluorescent labels:

- Use bright fluorophores with antibodies for low-abundance targets and dim fluorophores with antibodies for highly expressed antigens
- Minimize the spectral overlap of fluorophores to reduce spillover
- Use fluorophores that are spectrally distinct for the detection of coexpressed markers
- Use fluorophores that are spectrally similar for different cell subpopulations that will be gated separately

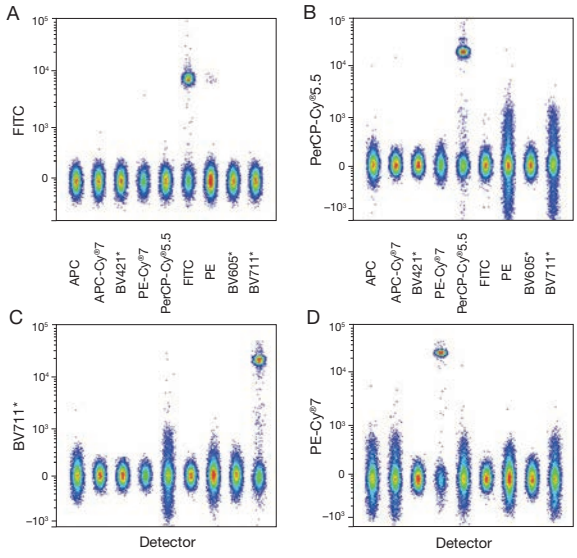

Figure 3. Spreading error visualization. Single-stained samples were run on the Invitrogen™ Attune™ NxT Flow Cytometer and analyzed using FlowJo™ software. Data from each detector were combined into a single plot. **(A)** Staining with the FITC antibody conjugate appears robust when analyzed in the FITC detector; minimal spreading error is observed in other channels. **(B)** Staining with PerCP-Cy®5.5 contributes high spreading error into the PE and BV711 channels. **(C)** Staining with BD Horizon Brilliant™ Violet 711 (BV711) contributes noticeable spread into the PerCP-Cy®5.5, APC, and PE channels. **(D)** Staining with PE-Cy®7 demonstrates extensive spreading error in multiple channels. *BD Horizon Brilliant™ Violet dyes (Becton, Dickinson and Company).

Figure 3 shows a method for visualizing spillover spreading error due to spectral overlap. Although commonly used, the tandem fluorophore PE-Cy®7 exhibits significant spreading due to low-energy

Flow Cytometry Panel Builder—A tool for all flow cytometrists

Whether you are a novice or an expert, designing a panel for flow cytometry is a highly complex process. If you are a beginner, let the Invitrogen™ Flow Cytometry Panel Builder lessen your anxiety over panel building by making the pairing of markers and fluorophores quick and simple using a highly visual format. Are you an expert? Then you will appreciate using the Flow Cytometry Panel Builder to easily review the spectral signals and filters per laser line and check fluorophore spillover values per channel. With access to information on over 13,000 antibodies for flow cytometry, this tool allows quick selection of antibodies for flow cytometry panels. Get started building your panel today at **thermofisher.com/flowpanel**.

(long-wavelength) photons, which in turn negatively impacts the resolution of fluorescent labels in other channels, especially those associated with poorly expressed antigens. Use of a spillover spread matrix is another way to visualize the spread into all other detectors for a given fluorophore [6].

Controls, controls, controls

Controls—e.g., fluorescence minus one (FMO) controls, compensation controls, and viability controls—are critical for evaluating multiparameter flow cytometry data. FMO controls are required for setting gates when multiple fluorophores are used together and when markers are expressed on a continuum. They help to account for the signal introduced from all other fluorescent labels in the channel being gated. FMO controls, which contain all markers except the one of interest, can provide clarity for low-density or smeared populations and can help to delineate two populations that are not easily resolved.

Also required in every multiparameter flow cytometry panel is a viability control, a fluorescent probe that specifically identifies dead cells so that they can be properly excluded from data analysis [7]. Dead cells are sticky and can nonspecifically bind antibodies and other probes, complicating the analysis (Figure 4).

Guidelines for flow cytometry

Follow these best practices for multiparameter flow cytometry:

- Optimize the voltage settings for each flow cytometer detector
- Titrate each antibody for optimal performance in the panel
- Carefully consider the pairing of dyes with targets and minimize spillover spreading
- Use FMO and viability controls to set gates correctly

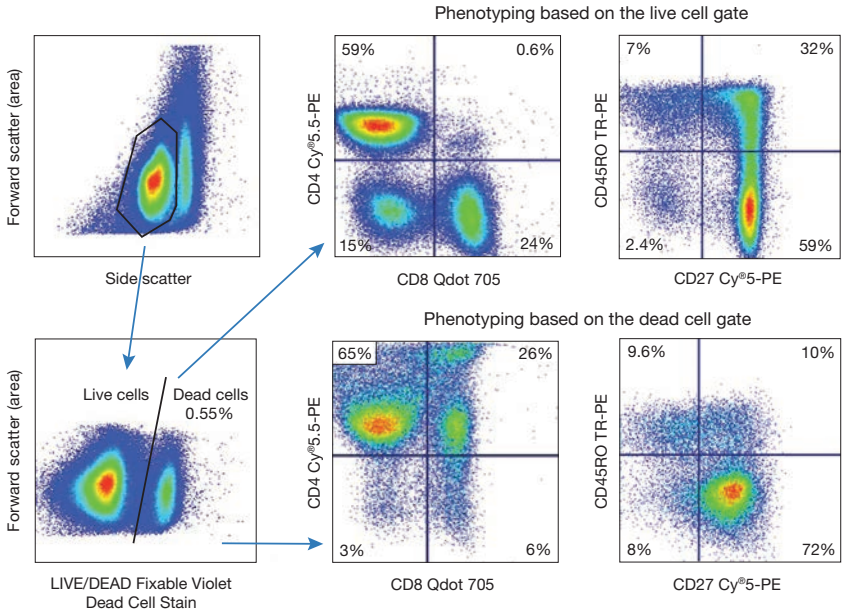

Figure 4. Effects of viability gating on population statistics. The inclusion or exclusion of a viability dye can drastically affect population statistics obtained from an experiment, and discriminating live and dead cells using only scatter parameters can be subjective and inaccurate [7]. In this example from Perfetto et al. [7], after application of a lymphocyte gate (forward scatter vs. side scatter), live and dead cells were discriminated using the Invitrogen™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Cat. No. L34955); note the significant number of dead cells despite a scatter gate. Subsequent analysis of live cells and dead cells shows the dramatic difference in apparent phenotypes between the two cell populations. Reprinted from Perfetto SP, Chattopadhyay PK, Lamoreaux L Nguyen R, Ambrozak D, Koup RA, Roederer M (2006) *J Immunol Methods* 313:199–208, with permission from Elsevier.

Panel design is an iterative process that requires testing all combinations and reviewing the spillover spread matrix at each iteration. Resources—such as webinars, eLearning courses, instrument information, and a library of application notes and protocols, as well as a link to the Invitrogen™ Flow Cytometry Panel Builder—are available at the Flow Cytometry Learning Center at **thermofisher.com/flowlearning**.

For more information

For a more in-depth discussion of best practices for multiparameter flow cytometry, watch the 1-hour webinar "Panel design for multiparameter flow cytometry", which expands on the topics included in this article. Find it at **thermofisher.com/flowwebinars**. ■

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Detect bacteria in both research and industrial samples

New applications for flow cytometric analysis.

Immunologists have long applied flow cytometry to their field of study because it is capable of delivering phenotypic statistics at the single-cell level for subsets within heterogeneous cell populations. Over the last 50 years, this technology, which began with simple 1-laser, 2-channel instruments, has advanced to include instruments with as many as 10 lasers and 50 channels. As the capabilities of flow cytometry technology have expanded, so have the applications that can now be analyzed by flow cytometry.

In part, this expansion of applications for flow cytometry is the result of the pairing of this technology with other detection platforms. The combination of fluorescence imaging and flow cytometry has led to the development of imaging cytometry, which, although much slower and less efficient than standard flow cytometry, is beneficial in applications where protein localization or cell-to-cell interaction is of interest. The combination of mass spectrometry and flow cytometry has led to the development of mass cytometry, which allows for the rapid interrogation of individual cell phenotypes using antibodies labeled with heavy metal ions instead of fluorescent dyes. While these advancements have provided additional analytical power, they also have limitations; generally they are not capable of quickly analyzing large cell populations (e.g., >1 million events) or effectively evaluating difficult sample types without extensive and potentially damaging manual processing.

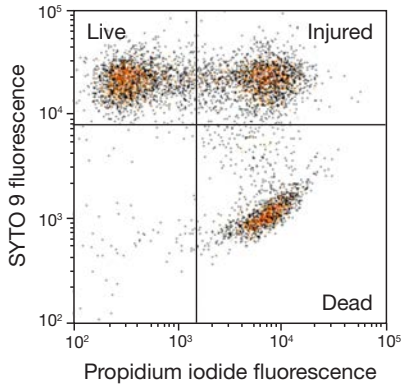


Figure 1. Staining of *Pasteurella multocida* using the LIVE/DEAD *Bac*Light Bacterial Viability Kit. *P. multocida* samples were analyzed using the Invitrogen™ LIVE/DEAD™ *Bac*Light™ Bacterial Viability and Counting Kit (for flow cytometry, Cat. No. L34856) on the Invitrogen™ Attune™ NxT Flow Cytometer at a flow rate of 25 µL/min with an event rate of approximately 5,000 events/sec. Samples were detected using the blue 488 nm laser and 530/30 nm emission filter for detection of Invitrogen™ SYTO ™ 9 Green Fluorescent Nucleic Acid Stain, and the yellow 561 nm laser and 620/15 nm emission filter for detection of propidium iodide (PI). The live, SYTO 9 dye–positive population is clearly distinguished from the dead, PI-positive population. Cells that are stained by both SYTO 9 and PI dyes are classified as injured cells, with some degree of damage to cell membranes.

Acoustics-assisted hydrodynamic focusing

While there have been many technologies that have been coupled with flow cytometry over the last 50 years, the introduction of acoustic focusing and positive displacement–based fluidics has opened the doors to new application areas in the environmental, food, and agriculture sciences, to name just a few. With its syringe-driven sampling, larger flow cell, and acoustic focusing–assisted cell alignment, the Invitrogen™ Attune™ NxT Flow Cytometer addresses many of the drawbacks of conventional hydrodynamic focusing–based flow cytometry. The Attune NxT Flow Cytometer provides faster run times (up to 10 times faster than traditional flow cytometers) and resistance to clogging, even with difficult samples that include larger or sticky cells. The anti-clogging design of the Attune NxT Flow Cytometer, attributable to its positive displacement–based fluidic system, has allowed previously incompatible samples types to be analyzed by flow cytometry. Here we describe advances in both instrumentation and reagents that facilitate flow cytometric analysis of bacteria in a wide variety of sample types.

Fluorescence-based detection of bacteria

Determining the size and viability of a bacterial population is a prerequisite for microbiology research, as well as for antibiotic, probiotic, and food and beverage industries. The traditional approach to microbial viability assessment is based on counting colony-forming units (CFUs), which requires several dilution and plating steps, appropriate growth media, and manual colony counting [1]. In addition, this method generally needs a minimum of 24 hours to complete, does not account for viable but unculturable microorganisms, and can be inaccurate due to the miscounting of cell clumps as single colonies. Thus, an alternative is needed to produce faster and more accurate counts of viable bacterial cells.

Using flow cytometry in conjunction with fluorescence-based viability assays allows thousands of bacteria to be analyzed in seconds,

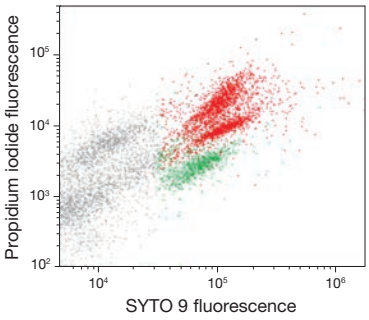
and population viability can be determined immediately. Moreover, high-throughput options allow large volumes and dilute samples to be analyzed. Early flow cytometry bacterial analyses were limited to studies of cellular aggregation because the instrumentation could not achieve enough resolution for single-bacterium identification [2]. Recent improvements in flow cytometers coupled with the availability of fluorescent bacterial viability dyes have led to increases in both resolution and the capabilities of multiparameter analysis.

The Invitrogen™ LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kits provide two different nucleic acid stains—the green-fluorescent Invitrogen™ SYTO™ 9 dye and the red-fluorescent propidium iodide—to rapidly distinguish live bacteria with intact plasma membranes from dead bacteria with compromised membranes. The SYTO 9 and propidium iodide stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population—those with intact membranes as well as those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 fluorescence when both dyes are present. With an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes fluoresce bright green, whereas bacteria with damaged membranes fluoresce red (Figure 1). The LIVE/DEAD *Bac*Light Bacterial Viability Kits yield consistent results in studies that use a variety of eubacterial genera.

Wastewater analysis

Through bacterial detection and removal, wastewater treatment is intended to help protect the public from disease and infection caused by pathogenic organisms. Recycling of potable water is increasingly important, especially in areas affected by water shortages due to drought, overpumping of groundwater, or arid climates. Accurate bacterial counts can be difficult and time-consuming with traditional flow cytometers because environmental samples are often very dilute, making the time required for acquisition of large volumes of wastewater unfeasible.

With its acoustics-assisted technology, the Attune NxT Flow Cytometer allows for fast (up to 1 mL/minute) and accurate analysis of very dilute samples. In addition, total bacteria and live and dead populations can be quantified without the use of reference counting beads (Figure 2). This rapid acquisition rate supports the use of flow cytometry as a standard method for water testing in an industrial setting.



Name	Gate	Count	%Total	Concentration
All Events	All Events	29,169	100.0	29.140
cells	cells	10,257	35.2	10.247
Live	Live	1,007	3.5	1.006
Dead	Dead	2,813	9.6	2.810

Figure 2. Flow cytometric analysis of bacteria in treated municipal wastewater. A 3 mL sample of municipal wastewater was labeled using the Invitrogen™ LIVE/DEAD™ *Bac*Light Bacterial Viability and Counting Kit (for flow cytometry, Cat. No. L34856) and analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer at a flow rate of 1 mL/min, allowing quick and accurate detection of a small number of bacteria. Concentrations of live and dead bacteria where determined without using reference counting beads. The two-parameter dot plot (propidium iodide vs. Invitrogen™ SYTO™ 9 fluorescence) shows well-separated live (green) and dead (red) bacterial populations; the statistics table displays the concentration measurements (cells/µL) for labeled bacteria in the acquired sample. Wastewater samples may also include small eukaryotes and types of bacteria that are potentially viable but nonculturable, each of which may be fluorescently stained with these dyes, as well as debris or other components that can contribute to background fluorescence.

Learn more about bacterial detection by flow cytometry

Find out about bacterial viability and vitality assays for flow cytometry at **thermofisher.com/flow-bacterial-viability** and about the Attune NxT Flow Cytometer at **thermofisher.com/attune**. ■

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Product	Quantity	Cat. No.
LIVE/DEAD™ *Bac*Light™ Bacterial Viability and Counting Kit, for flow cytometry	1 kit	L34856
LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit	1 kit	L13152
SYTO™ 9 Green Fluorescent Nucleic Acid Stain	100 µL	S34854
Attune™ NxT Acoustic Focusing Cytometer, blue/red/violet6/yellow	1 each	A29004
Attune™ NxT Acoustic Focusing Cytometer, blue/red/violet6	1 each	A29003
Attune™ NxT Acoustic Focusing Cytometer, blue/violet6	1 each	A29002
Attune™ NxT Violet Laser Upgrade Kit	1 kit	100022777

Fluorescent detection of senescence for imaging and flow cytometry applications

Multiplex with the CellEvent Senescence Green Probe for a more complete picture.

Aging is considered a significant risk factor for developing many chronic diseases, including cardiovascular diseases, cancers, and neurodegenerative diseases, and research indicates that many of these diseases are associated with cellular senescence. Senescent cells are cells that remain metabolically active but have stopped dividing due to telomere shortening, stress, or damage [1-4]. Although senescence appears to be an important pathway for controlling unlimited cell division, senescent cells that are not removed (e.g., as a result of cancer chemotherapy) contribute to a chronic, pro-inflammatory environment, increasing the risk of many age-related diseases. Recent work has shown that specific targeting of senescent cells results in increased life expectancy in a progeroid Ercc1$^{-/\Delta}$ mouse model, which has a premature aging phenotype [1,2]. As such there is great interest in identifying, characterizing, and modifying senescent cells.

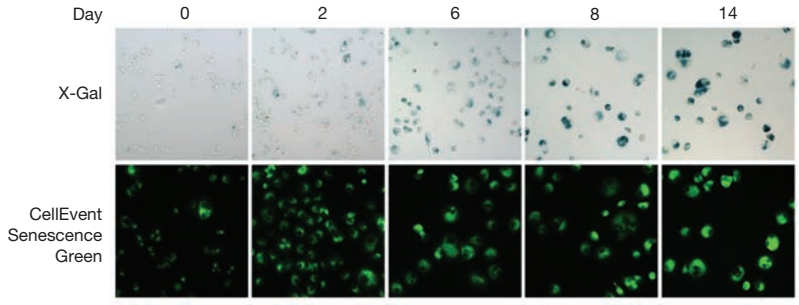

Figure 1. Comparison of senescent and nonsenescent cells labeled with CellEvent Senescence Green Probe or X-Gal. T47D human epithelial cells were untreated (on day 0) and then treated with 5 µM palbociclib every other day for 15 days to induce senescence. Cells were stained using the Invitrogen™ CellEvent™ Senescence Green Detection Kit (Cat. No. C10850) for 90 min or with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) overnight, and imaged using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform and the FITC filter for CellEvent Senescence Green fluorescence, or brightfield for colorimetric X-Gal detection.

Senescence biomarkers

As the mechanisms of senescence are examined, several biomarkers have been identified, including those related to the release of pro-inflammatory cytokines and chemokines, as well as to an increase in beta-galactosidase activity (called senescence-associated β-gal, or SA-β-gal) and senescence-associated heterochromatin foci (SAHF). Of the currently available methods for identifying senescent cells, the detection of SA-β-gal is considered the gold standard for both cells in culture and vertebrate tissue. SA-β-gal activity is the result of increased acidic lysosomal β-galactosidase, which can be detected at near-neutral pH because it is so highly overexpressed. It is typically detected by adding a buffered X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution to fixed and permeabilized cells, where the substrate is cleaved by lysosomal SA-β-gal, producing a blue-green precipitate. This colorimetric assay has several drawbacks, including inconsistent signal production, a lengthy protocol, and incompatibility with other cell function probes and flow cytometry.

In order to identify and quantify senescent cells by flow cytometry, researchers either detect other senescence biomarkers using the limited number of specific antibodies available, or detect SA-β-gal using the fluorescein-based β-gal substrate C_{12}FDG. Once inside the cell, the lipophilic, nonfluorescent C_{12}FDG is cleaved by intracellular β-gal, including SA-β-gal, producing a green-fluorescent product. However, even though it has been used since the mid-1990s, C_{12}FDG has limited utility because it tends to leak out of cells and is sensitive to fixation.

Introducing a fluorescent SA-β-gal probe

We have developed a sensitive substrate for β-gal that can be used for the fluorescent detection of senescent cells in both imaging and flow cytometry applications. The Invitrogen™ CellEvent™ Senescence Green Probe is a fluorescence-based β-gal substrate that contains two galactoside moieties, as well as an additional moiety that reacts with several functional groups found in proteins. This nonfluorescent substrate is cleaved by intracellular β-gal to produce a green-fluorescent product (excitation/emission maxima = 490/514 nm) that is well retained in cells due to its covalent binding to intracellular proteins. In addition, the CellEvent Senescence Green Probe is easy to use: simply fix the cells, add the reagent, incubate, and detect the fluorescence by imaging or flow cytometry.

To demonstrate the reliable and consistent detection of senescence, T47D human epithelial cells were induced with palbociclib (a specific CDK4/6 inhibitor that induces cell cycle arrest) for up to 15 days. Cell samples were removed throughout this time course and assayed using either CellEvent Senescence Green Probe or X-Gal, according to standard protocols. Figure 1 shows that the CellEvent Senescence reagent detected the same relative number of senescent cells as X-Gal, and that both substrates exhibited increased signal over time as senescence progresses. Furthermore, the CellEvent Senescence reagent only required a 90-minute incubation, whereas the X-Gal reagent required an overnight incubation to generate an equivalently sensitive signal.

Multiplexing with CellEvent Senescence Green Probe

Because the CellEvent Senescence reagent covalently binds to intracellular proteins upon enzymatic cleavage with β-gal, this reagent is better retained in cells compared with the traditional fluorescence-based β-gal substrate C_{12}FDG. In addition, the CellEvent Senescence reagent is compatible with cell fixation protocols, as well as with multiplex analysis with antibodies and other fixable cell health indicator reagents. Figure 2 demonstrates a multiparameter flow cytometry experiment in which cells were labeled with CellEvent Senescence reagent and antibodies specific for cyclins, which are differentially expressed throughout the cell cycle and downregulated in senescent cells.

Its compatibility with fixation means that the CellEvent Senescence Green reagent can be multiplexed with a variety of other cell health indicator reagents, including the Invitrogen™ Click-iT™ EdU cell proliferation assay (Figure 3) or the fixable Invitrogen™ FxCycle™ DNA stains for cell cycle analysis (data not shown).

Learn more about CellEvent Senescence Green Probe

In summary, we have developed a sensitive fluorescent substrate for β-gal that can be used for the detection of senescent cells using either fluorescence microscopy or flow cytometry. The CellEvent Senescence Green Probe is compatible with cell fixation and can therefore be multiplexed with antibodies or other fixable cell health indicator reagents for more complete immunophenotyping studies. Learn more at **thermofisher.com/cellevent-senescence**. ■

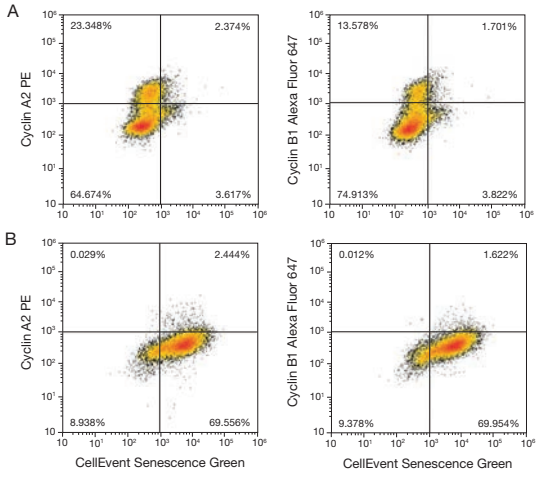

Figure 2. Decreased cyclin A2 and B1 with the onset of senescence. T47D human epithelial cells were **(A)** left untreated or **(B)** treated with palbociclib in media every other day for 15 days to induce senescence. Cells were stained using the Invitrogen™ CellEvent™ Senescence Green Flow Cytometry Assay Kit (Cat. No. C10840), in addition to anti–cyclin A2, PE conjugate, and anti–cyclin B1, Alexa Fluor™ 647 conjugate (Invitrogen™ Cyclin B1 Monoclonal Antibody (clone GNS1, Cat. No. MA5-14319) labeled using the Invitrogen™ Zenon™ Alexa Fluor™ 647 Mouse IgG1 Labeling Kit (Cat. No. Z25008)). Cells were analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer. Untreated T47D cells progressed through the various cell cycle stages, as indicated by the expression of cyclin A2 and B1. The lack of cyclin expression in the treated T47D cells is consistent with cell cycle arrest in senescent cells.

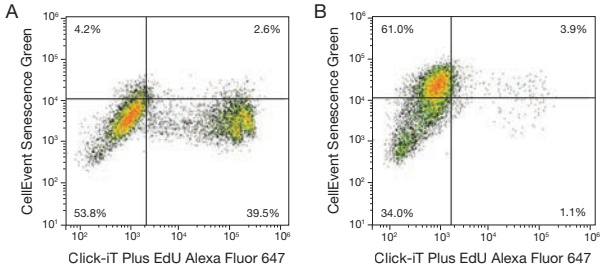

Figure 3. Decreased DNA synthesis in senescent cells. (A) Cycling, nonconfluent control WI-38 human lung fibroblast cells and **(B)** confluent cells that were passaged for 5 weeks to induce nonreplicative senescence were incubated with 10 µM EdU for 2 hr to allow incorporation of the thymidine analog into synthesizing DNA. Cells were subsequently trypsinized, resuspended in 1X PBS, fixed with 4% formaldehyde, and stained using the Invitrogen™ CellEvent™ Senescence Green Flow Cytometry Assay Kit (Cat. No. C10840). Following 1X PBS wash and permeabilization steps, EdU incorporation was detected using the Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Cat. No. C10634). Cells were analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer using 488 nm and 637 nm lasers and 530/30 nm and 670/14 nm bandpass filters. As confluency increases over time, cells stop dividing and DNA synthesis ceases, as indicated by the increase in CellEvent Senescence Green staining and the decrease in EdU-positive cells.

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Product	Quantity	Cat. No.
CellEvent™ Senescence Green Flow Cytometry Assay Kit	50 assays 200 assays	C10840 C10841
CellEvent™ Senescence Green Detection Kit	25 µL 100 µL	C10850 C10851

Monitor drug-induced calcium flux in iPSC-derived cardiomyocytes

Fluo-4 meets the EVOS FL Auto 2 Imaging System and Celleste Image Analysis Software.

Pluripotent stem cells (PSCs) have the ability to differentiate into any one of the different cell types in the human body and are an important research tool for disciplines such as developmental biology, regenerative medicine, and oncology, to name a few [1,2]. The discovery that a terminally differentiated cell such as a dermal fibroblast can be reverted back to a stem cell–like state has opened up the possibility of growing patient-specific tissue and organs that originate from a patient's own cells and therefore have less chance of rejection when transplanted [3,4]. Differentiation of induced pluripotent stem cells (iPSCs)—using specialized media with the appropriate growth and signaling factors—into complex cell culture models such as cardiomyocytes or dopaminergic neurons has opened up new avenues for basic research and translational medicine.

Role of calcium in cardiac action potentials

During an action potential, cardiomyocytes contract by membrane depolarization, which is caused by the rapid influx of calcium into the cell through voltage-gated calcium channels. Ion indicators that increase in fluorescence upon binding calcium, such as Invitrogen™ fluo-4 dye, are commonly used to measure cardiomyocyte action potentials and contraction rate. As calcium enters the cell, it binds to intracellular fluo-4, causing the fluorescence of the calcium indicator to increase. Likewise, the fluorescence of the indicator is reduced as intracellular calcium concentration decreases when calcium is pumped back out of the cell during the repolarization phase of the action potential [5-8].

Improper functioning of action potentials in cardiomyocytes can lead to a variety of human diseases such as hypertension. Drugs for treating high blood pressure, including the calcium channel blocker verapamil, act by helping to control cardiac action potentials. Furthermore, several compounds are produced by the body to increase the action potential rate in response to stimuli, such as the release of norepinephrine during the fight-or-flight response. Here we demonstrate a simple method for monitoring drug-induced changes in the rate of action potentials in human iPSC-derived cardiomyocytes.

Quantify calcium influx rate

To quantify the rate of calcium influx in iPSCs, we used the calcium indicator fluo-4 in a live-cell kinetic assay. Cell fluorescence was detected and analyzed on the Invitrogen™ EVOS™ FL Auto 2 Imaging System using Invitrogen™ Celleste™ Image Analysis Software. This live-cell imaging and analysis system enables quick and easy quantitative calcium imaging (Figure 1)—which is required when studying physiological responses to drugs in muscle cells, neurons, and other cell systems—without the need for specialized equipment or techniques.

The EVOS FL Auto 2 system was used to record videos of pulsing cardiomyocytes, allowing visualization of rapid calcium flux based on changes in the fluorescence intensity of the fluo-4 calcium indicator over time (Figure 1A). By simply selecting the "measure:intensity" tracking function after defining the region of

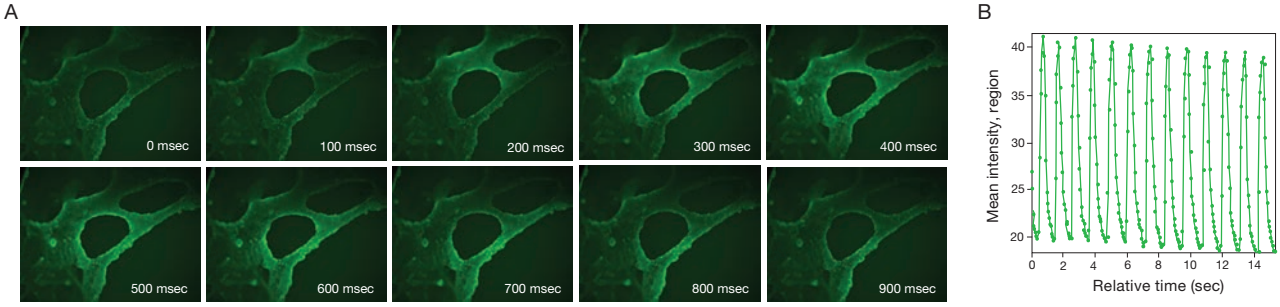

Figure 1. Time-lapse imaging and quantitation of cardiomyocyte pulse rate. (A) Time-lapse images (obtained from a video of contracting cardiomyocytes stained using the fluorescent fluo-4 calcium indicator provided in the Invitrogen™ Fluo-4 NW Calcium Assay Kit (Cat. No. F36206) and recorded using the Invitrogen™ EVOS™ FL Auto 2 Imaging System) show the flux of calcium moving across the cells over time. **(B)** Analysis of the video using the measure:intensity tracking function in Invitrogen™ Celleste™ Image Analysis Software shows that these cardiomyocytes have a pulse rate of approximately 1 beat per second.

interest in each video of contracting cardiomyocytes, Celleste software was used to generate a graph of the calcium flux and contraction rate based on fluorescence intensity changes observed (Figure 1B).

This function allowed quantitation of the baseline calcium flux and contraction rate in cardiomyocytes, along with changes in the pulse rate with different treatments. Application of 10 µM norepinephrine nearly doubled the rate of contraction compared with that seen in cardiomyocytes receiving no drug treatment (Figures 2A and 2B). Treatment with 50 nM verapamil reduced the rate of contraction to about half of the baseline cardiomyocyte contraction rate, whereas 1 µM verapamil prevented calcium flux, resulting in no contractions (Figures 2C and 2D).

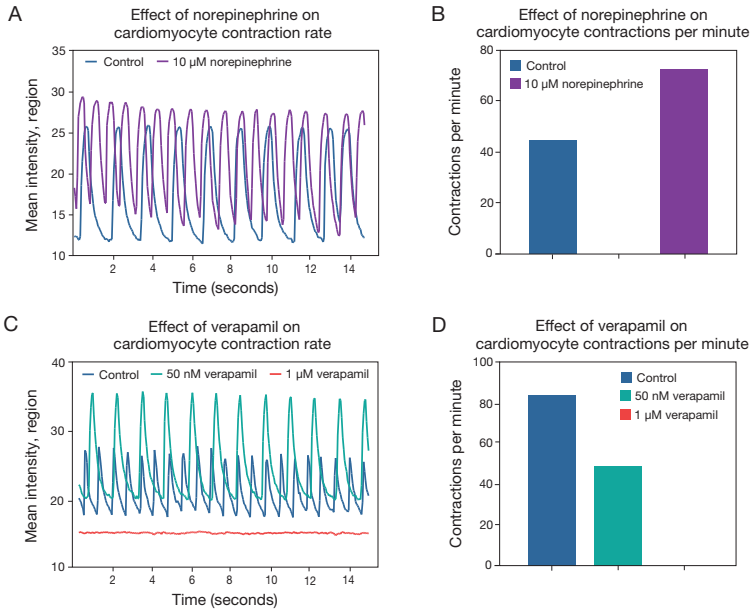

Figure 2. Effect of norepinephrine and verapamil on cardiomyocyte pulse rate. Overlays of intensity measurements of the defined region of interest were obtained using Celleste software for **(A)** control and 10 µM norepinephrine–treated cardiomyocytes and **(C)** control, 50 nM verapamil–treated, and 1 µM verapamil–treated cardiomyocytes. Quantitation of the calcium flux using fluo-4 shows that cardiomyocyte contraction rate is **(B)** nearly doubled with 10 µM norepinephrine and **(D)** reduced by about half with 50 nM verapamil or stopped completely with 1 µM verapamil, as compared with the baseline contraction rate with no drug treatment (control).

Fluorescence imaging in real time

Drug-induced changes in cardiomyocyte calcium flux, measured with the fluorescent calcium indicator fluo-4, can easily be imaged and quantified using the EVOS FL Auto 2 Imaging System and Celleste Image Analysis Software. Real-time fluorescence videos of calcium flux in pulsing cardiomyocytes are recorded by selecting the "record video" option on the EVOS FL Auto 2 system, and then changes in fluorescence intensity are quantified by defining a region of interest and using the "measure:intensity" tracking function with the Celleste software. This simple quantitation system can be applied to cell physiology and drug discovery assays using other fluorescent indicators. EVOS microscopes incorporate high-resolution cameras, bright and digitally controlled LED light cubes, and interfaces structured for usability that allow the acquisition of stunning, publication-quality images and data. Find out more about EVOS microscopes or request an in-lab demonstration at thermofisher.com/evos. ■

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Product	Quantity	Cat. No.
Invitrogen™ fluorescent ion indicator		
Fluo-4 NW Calcium Assay Kit	10 microplates 100 microplates	F36206 F36205
Gibco™ cells and media		
Essential 8™ Medium	500 mL	A1517001
Human Episomal iPSC Line	1 x 10^6 cells/vial	A18945
PSC Cardiomyocyte Differentiation Kit	1 kit	A2921201
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	1 mL 10 mL	A14700 A31804
Invitrogen™ fluorescence instrumentation		
EVOS™ FL Auto 2 Imaging System	1 system	AMAFD2000
EVOS™ 4x Objective, fluorite, LWD	1 each	AMEP4622
EVOS™ 10x Objective, fluorite, LWD	1 each	AMEP4623
EVOS™ Light Cube, GFP	1 each	AMEP4651
Celleste™ Image Analysis Software	1 each	AMEP4816

Visualize microtubule dynamics in live cells

With the highly photostable Tubulin Tracker Deep Red reagent.

Fluorescence imaging of live cells allows researchers to visualize dynamic cell processes such as those associated with the microtubule component of the cytoskeleton. Generally, microtubules are imaged in live cells after transfection or transduction with genes encoding fusion proteins of a fluorescent protein (e.g., GFP) and the tubulin monomer. There are a number of challenges inherent to this approach. Transfection or transduction can induce cellular stress and cytotoxicity, and expression of the transgene via plasmid or viral delivery requires significant time before enough protein is synthesized to generate a fluorescent signal suitable for live-cell imaging. Additionally, fluorescent proteins are largely limited to green and red detection channels, which can limit the ability to multiplex with commonly used markers. Most notably, transgene expression tends to produce vastly heterogeneous protein levels, yielding uneven labeling across the sample and making quantitative characterization of the microtubule cytoskeleton intractable by fluorescence imaging.

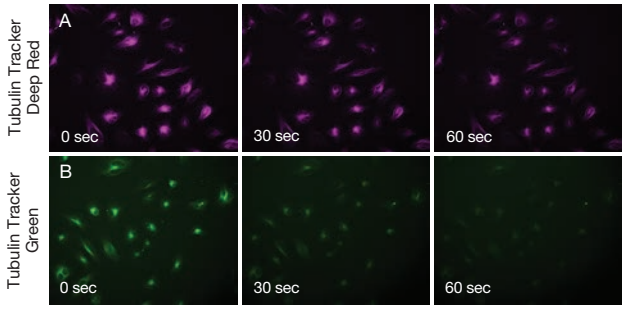

Figure 1. Superior photostability of Tubulin Tracker Deep Red in live cells. HeLa cells were labeled with Invitrogen™ Tubulin Tracker™ Deep Red (Cat. No. T34077) and Invitrogen™ Tubulin Tracker™ Green (Cat. No. T34078) according to the recommended protocol and then imaged continuously for 60 sec on the Invitrogen™ EVOS™ FL Auto 2 Imaging System using a 20x/0.75NA objective. Whereas Tubulin Tracker Green was largely photobleached after 30 sec, Tubulin Tracker Deep Red retained >85% of its initial signal, even after a full 60 sec of continuous illumination.

Tubulin Tracker Deep Red

To address these challenges, we have developed Invitrogen™ Tubulin Tracker™ Deep Red, a novel membrane-permeant fluorescent molecule that selectively stains microtubules in live cells and exhibits deep-red emission. Eliminating the need for genetic modification, Tubulin Tracker Deep Red provides uniform labeling of the microtubule cytoskeleton in live cells with a rapid and simple staining protocol.

The far-red spectral properties of Tubulin Tracker Deep Red—with excitation/emission maxima of 652/669 nm—result in minimal phototoxicity to the cells, allow the signal to be detected using common Cy®5/deep red filter sets, and make it amenable to multiplexing with blue, green, orange, red, and near-IR fluorophores. Moreover, Tubulin Tracker Deep Red shows superior photostability, as we show using HeLa cells labeled with this deep-red fluorescent stain and then imaged continuously for 60 seconds on the Invitrogen™ EVOS™ FL Auto 2 Imaging System (Figure 1A).

Our data demonstrate that Tubulin Tracker Deep Red can be used to label microtubules in live cells in as little as 30 minutes, with no measurable cytotoxicity or disruption of normal cellular function during a 72-hour incubation. Furthermore, Tubulin Tracker Deep Red is compatible with a no-wash protocol. With its advanced photostability and the inherent low phototoxicity of far-red–emitting labels, Tubulin Tracker Deep Red is ideal for long-term time-lapse imaging of live cells, as well as for rapid tubulin counterstaining to facilitate analysis in endpoint assays.

Invitrogen™ Tubulin Tracker™ Green has long been used to visualize microtubules in live cells. However, its short-wavelength excitation produces rapid photobleaching of the signal (Figure 1B) and increases phototoxicity to the cells during extended time-lapse imaging. Additionally, Tubulin Tracker Green cannot be multiplexed with commonly used GFP–tubulin monomers and related fluorescent protein fusions. Given its more photostable deep-red fluorescence, Tubulin Tracker Deep Red resolves many of these limitations without introducing any measurable cytotoxic side effects.

Tubulin labeling in live cells with minimal cytotoxicity

One of the primary concerns with using fluorescent taxanes to label microtubules is the possibility of cytotoxicity upon continuous incubation. The microtubule cytoskeleton is an essential regulator of the cell cycle, and taxane-mediated inhibition of microtubule depolymerization has been used as a common chemotherapeutic strategy in oncology for decades.

To assess the cytotoxicity of Tubulin Tracker Deep Red and Tubulin Tracker Green compared with that of their parent taxane molecules docetaxel and paclitaxel, respectively, HCASM (human cardiac arterial smooth muscle) cells and HeLa cells were incubated with 1 µM of

either the unconjugated taxanes or Tubulin Tracker reagents for up to 24 hours, and cell viability was assessed using Invitrogen™ PrestoBlue™ Cell Viability Reagent and the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. Our results indicate that after 2 hours of continuous incubation, cell viability was not significantly affected by any of the treatments (Figure 2B). After 24 hours, however, cells incubated with the unlabeled taxanes showed decreased viability whereas cells incubated with the Tubulin Tracker reagents did not show a significant change in viability (Figures 2A and 2B). These results were confirmed by a qualitative assessment of cell number and morphology. Additionally, Tubulin Tracker Deep Red remained in cells for the entire duration of treatment, thus demonstrating exceptional retention of the probe over extended periods.

Ideal for extended time-lapse imaging

Tubulin Tracker Deep Red can also be used to track microtubules over the course of multiple days for extended time-lapse imaging. To demonstrate this functionality, we labeled HeLa cells with 100 nM Tubulin Tracker Deep Red and placed the samples in a live-cell chamber on a laser-scanning confocal microscope for 72 hours. The dye was not removed from the medium, and images were captured at 20-minute intervals; low levels of extracellular background signal were subtracted using the offset function.

This experimental setup allowed us to track cellular movement, visualize microtubule dynamics, and observe cell proliferating throughout the entire 72-hour acquisition period; we also detected the successful completion of mitotic spindle assembly, disassembly, and cytokinesis (Figure 3).

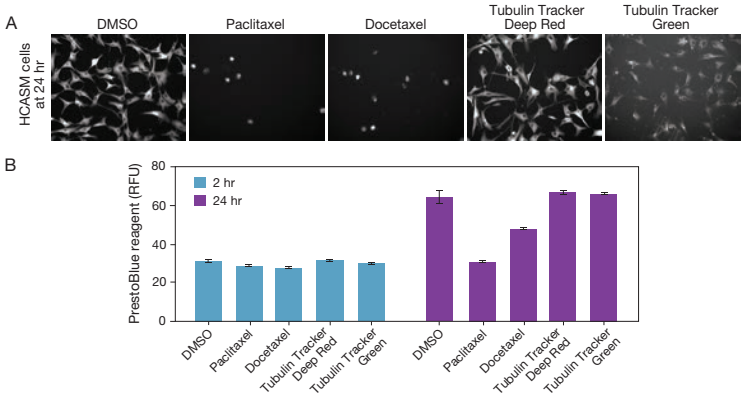

Figure 2. Minimal cytotoxicity and superior retention of Tubulin Tracker Deep Red in HCASM cells. HCASM (human cardiac arterial smooth muscle) cells were incubated with vehicle (DMSO), 1 µM unconjugated taxanes (paclitaxel or docetaxel), or 1 µM Invitrogen™ Tubulin Tracker™ reagent (Tubulin Tracker Deep Red, Cat. No. T34077; or Tubulin Tracker Green, Cat. No. T34078) for 24 hr. **(A)** After 24 hr, cells were imaged on the Invitrogen™ EVOS™ FL Auto 2 Imaging System using a 20x/0.75NA objective. **(B)** Cell viability was assessed at 2 hr and 24 hr using Invitrogen™ PrestoBlue™ Cell Viability Reagent (Cat. No. A13261) on the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

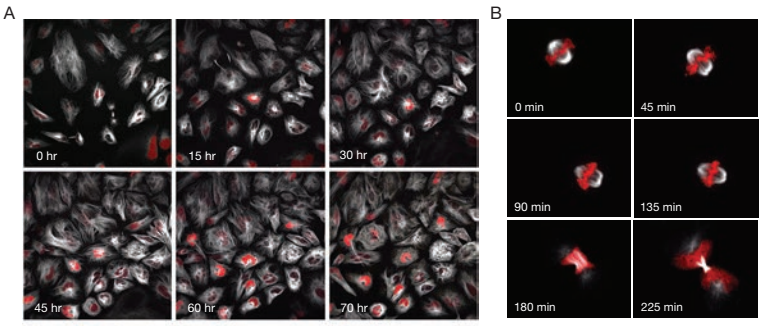

Figure 3. Extended time-lapse imaging of cells labeled with Tubulin Tracker Deep Red. Live HeLa cells were labeled with Invitrogen™ CellLight™ Histone 2B–RFP (red, Cat. No. C10595) for 24 hr and 100 nM Invitrogen™ Tubulin Tracker™ Deep Red (white, Cat. No. T34077) for 1 hr before imaging every 20 min over 72 hr on a Zeiss™ LSM 710 confocal microscope using a 40x/1.2NA water-immersion objective. **(A)** Zoomed-out view of a field of cells showing cell movement, microtubule dynamics, and cell proliferation. **(B)** Zoomed-in view of a cell undergoing mitosis.

Tubulin labeling made easy

Tubulin Tracker Deep Red can be used to visualize microtubules in a wide variety of live cell types, and its far-red spectral properties and superior photostability enable multiplexing with many common dyes and fluorescent proteins. Learn more at **thermofisher.com/tubulintracker**. ■

Product	Quantity	Cat. No.
Tubulin Tracker™ Deep Red	60 slides 300 slides	T34077 T34076
Tubulin Tracker™ Green (Oregon Green™ 488 Taxol™, Bis-Acetate), for live-cell imaging	60 slides 300 slides	T34078 T34075
Tubulin Tracker™ Variety Pack	2 x 60 slides	T34079
PrestoBlue™ Cell Viability Reagent	25 mL 100 mL	A13261 A13262
EVOS™ FL Auto 2 Imaging System	1 system	AMAFD2000
Varioskan™ LUX Multimode Microplate Reader	1 system	VLBLATD0

Assess the cell viability of *Staphylococcus aureus* biofilms

PrestoBlue HS and alamarBlue HS reagents for microplate viability assays.

Biofilms, a community life form of bacteria, are organized ensembles of sessile bacterial cells embedded in a self-produced matrix. The majority of chronic, antibiotic-resistant microbial infections are thought to be associated with biofilms. A signature trait of biofilms is their increased tolerance to antibiotics and to the host immune system, making successful eradication of biofilms a very challenging task. Different methods have been used for high-throughput screening (HTS) of biofilm inhibitors, primarily focused on measuring bacterial viability (i.e., metabolism) [1] or biofilm biomass [2]. Because biomass quantitation is typically a slow process and does not distinguish between live and dead cells, a more common first-tier approach to identifying biofilm inhibitors is to screen for the effects of investigational compounds on biofilm viability.

For HTS applications, a viability assay is ideally performed in an add-and-read format, requiring only three steps—addition of reagent, incubation, and measurement of output—with no mixing, washing, or cell lysis required. Add-and-read resazurin-based assays are commonly used to quantify metabolic activity of cells, including microorganisms. Upon entering live cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. In addition to ease of use, resazurin-based assays provide extended linear ranges, ample signal windows, and the flexibility to measure in either endpoint or kinetic mode and with either fluorescence- or absorption-based instrumentation. The endpoint mode is often used in primary screenings, in which the goal is to perform fast screening campaigns to identify hit compounds from large chemical collections. The kinetic mode is more applicable to secondary screenings, in which the time-dependent cytotoxic effects of selected hits are assessed for mechanistic purposes. Resazurin-based assays have been extensively used in anti-biofilm screening approaches [3,4].

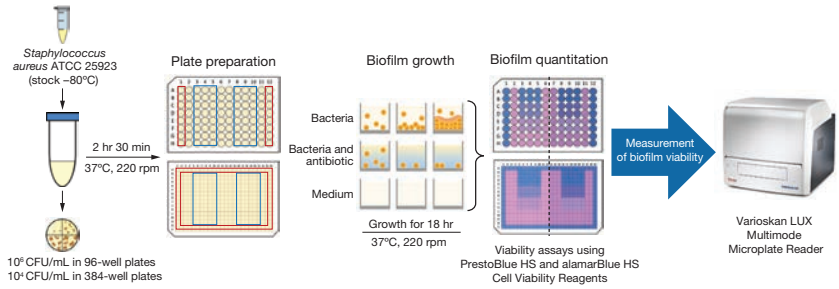

Figure 1. Workflow for quantitation of *S. aureus* biofilms with PrestoBlue HS and alamarBlue HS reagents. Exponentially grown cultures of *S. aureus* were prepared in tryptic soy broth medium as in [5] and added to microplates; medium-only controls were included. Biofilms were allowed to form without and with antibiotics at 37°C, 220 rpm; final assay volume was 200 µL in 96-well plates or 40 µL in 384-well plates. After 18 hr, planktonic cells were carefully removed, and biofilms were washed once with PBS prior to assaying with Invitrogen™ PrestoBlue™ HS or alamarBlue™ HS Cell Viability Reagent (Cat. No. P50201 or A50101). Resorufin fluorescence was measured using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader in either endpoint or kinetic mode.

PrestoBlue HS and alamarBlue HS reagents for anti-biofilm discovery

Two of the most popular resazurin-based dyes for cytotoxicity studies are the Invitrogen™ PrestoBlue™ and alamarBlue™ Cell Viability Reagents. Recently, Thermo Fisher Scientific introduced two new versions of these dyes—the Invitrogen™ PrestoBlue™ High Sensitivity (HS) and alamarBlue™ High Sensitivity (HS) Cell Viability Reagents—which contain highly purified resazurin (see "Just Released" on page 6). We have tested the suitability of PrestoBlue HS and alamarBlue HS reagents for measuring the viability of *Staphylococcus aureus* biofilms in an HTS workflow, such as that used when screening biofilm inhibitors. We have simulated the exposure to chemical libraries by performing a pilot study with model antibiotics, which were added to microplates just after the addition of *S. aureus* suspensions (Figure 1). The viability of attached biofilms was quantified in parallel using PrestoBlue HS and alamarBlue HS reagents. Fluorescence measurements of resorufin were performed using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader paired with Thermo Scientific™ SkanIt™ Software to allow fast readout of fluorescence signals and straightforward access to data processing steps such as calculations of cell viability, inhibition percentages of cell viability, and signal-to-background ratios.

Bacterial biofilm viability measurements: Endpoint mode

To model a primary screening workflow, PrestoBlue HS and alamarBlue HS reagents were used to assess biofilm viability with endpoint measurements. The reagents were added to *S. aureus* biofilms grown in 96- or 384-well plates and incubated at room temperature for

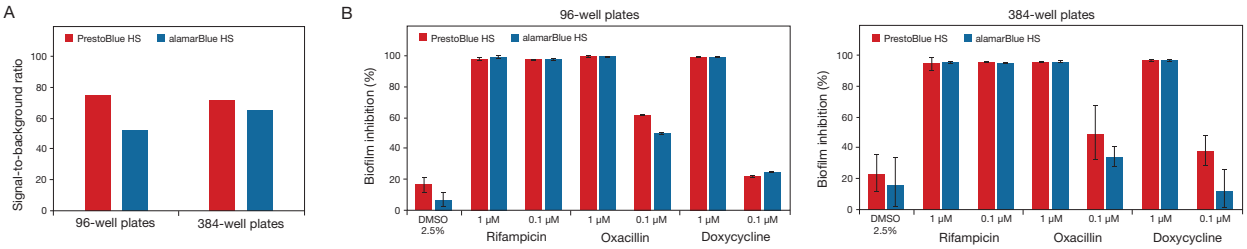

Figure 2. Performance of PrestoBlue HS and alamarBlue HS reagents in endpoint measurements of *S. aureus* biofilm viability. (A) Signal-to-background ratios were calculated from the fluorescent signals generated in untreated biofilm (signal) and media controls (background). **(B)** Rifampicin, oxacillin, and doxycycline were dissolved in anhydrous DMSO and added with the planktonic bacteria. After 18 hr, the quantity of formed biofilm per area was similar in the 96- and 384-well plates, as confirmed by viable plate counts (~1 x 10^8 CFU/cm^2). The viability assays with Invitrogen™ PrestoBlue™ HS and alamarBlue™ HS Cell Viability Reagents were performed as described in Figure 1. Fluorescence of resorufin was measured with top optics (12 nm excitation bandwidth) and excitation/emission = 560/590 nm using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. All data were measured in two independent experiments, with 16 replicates (in A) and 2 replicates (in B) in each experiment.

40 minutes prior to fluorescence measurements with the Varioskan LUX reader (Figure 2A). The signal-to-background ratio was 2 to 3 times higher than historical values obtained with other commercial resazurin reagents [6,7], which can be attributed to a significant decrease in background fluorescence. Inhibitory effects of three model antibiotics were easily quantified, with rifampicin being the most active, consistent with reported findings [5]. For each antibiotic treatment, cell viability measurements using PrestoBlue HS reagent were noticeably similar to those using alamarBlue HS reagent in both 96- and 384-well plates (Figure 2B). These results demonstrate the suitability of both reagents for fast and reliable measurements of biofilm viability.

Bacterial biofilm viability measurements: Kinetic mode

In kinetic mode on the Varioskan LUX reader, biofilms incubated with either PrestoBlue HS or alamarBlue HS reagent showed linear increases in the production of resorufin during the 40-minute incubation. Both reagents exhibited kinetic rates that were remarkably similar, regardless of the biofilm's age (Figure 3), indicating that both reagents likely diffuse at similar rates through the matrix and are similarly metabolized. The inhibitory effects of antibiotics were also readily measurable in kinetic mode with both reagents. Killing of biofilms by doxycycline was less efficient once biofilms matured (Figure 3A vs. 3B), demonstrating the well-known resilience of biofilms to antimicrobial therapy.

Learn more about microplate assays for viability

Find out more about PrestoBlue HS and alamarBlue HS Cell Viability Reagents at **thermofisher.com/microplate-viability**. ■

Acknowledgments: This study was conducted in collaboration with Shella Gilbert-Girard and Kirsi Savijoki, Faculty of Pharmacy, University of Helsinki, Finland.

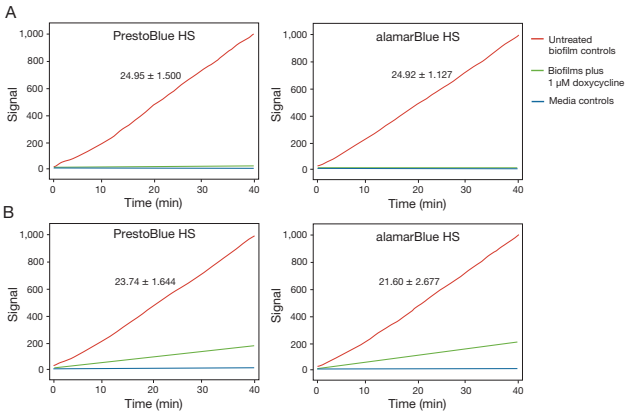

Figure 3. Kinetic curves of PrestoBlue HS and alamarBlue HS reagents showing linear resazurin reduction by *S. aureus* biofilms. Biofilms in 96-well plates were grown **(A)** for 18 hr in the presence of antibiotics or **(B)** for 18 hr and then an additional 24 hr in the presence of antibiotics; untreated biofilms and media controls were included. Invitrogen™ PrestoBlue™ HS or alamarBlue™ HS Cell Viability Reagent was added, and fluorescence was measured at excitation/emission = 560/590 nm every 2 min over 40 min using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. The average fluorescence/min (indicated above red lines) was calculated for untreated biofilm controls using Thermo Scientific™ SkanIt™ Software.

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Product	Quantity	Cat. No.
alamarBlue™ HS Cell Viability Reagent	25 mL 100 mL	A50100 A50101
PrestoBlue™ HS Cell Viability Reagent	25 mL 100 mL	P50200 P50201
SkanIt™ Software for Microplate Readers, Research Edition	1 each	5187139
Varioskan™ LUX Multimode Microplate Reader	1 each	VL0000D0

SNAP-ChIP: A robust method for determining histone antibody specificity in ChIP

Barcoded synthetic nucleosomes serve as ChIP internal controls.

Histones are the core protein components of nucleosomes, which package DNA into the fundamental repeating units of the eukaryotic chromosome. These highly alkaline proteins are decorated with posttranslational modifications (PTMs) that serve as epigenetic signatures for gene expression and chromatin structure regulation; individual PTMs also play a significant role in processes such as DNA compaction, transcription, translation, genome integrity, and cell cycle. Distinguishing between PTMs is essential for accurate data interpretation and is often achieved using antibodies. However, determining the specificity of antibodies for particular histone PTMs is challenging because these antibodies need to recognize the difference between highly similar modifications such as mono-, di-, or trimethylation of a single histone residue.

In recent years, the assay for determining the specificity of antibodies that recognize histone PTMs has been the peptide array [1-3], a reliable method for screening an antibody's ability to distinguish its target PTM from similar modifications and in the context of neighboring modifications. However, peptide arrays use denaturing conditions and are therefore likely to be most useful in reporting an antibody's ability to recognize and distinguish its intended histone PTM in applications such as western blotting, in which linear epitopes are displayed under denaturing conditions.

Introducing SNAP-ChIP controls

The application of chromatin immunoprecipitation (ChIP) is critical to our understanding of histone PTM regulation and distribution in its native chromatin configuration. Unlike peptide arrays and western blotting, ChIP and ChIP-sequencing (ChIP-Seq) require that an antibody recognize its intended histone PTM in the context of a functional nucleosome engaged in varying levels of chromatin compaction. The distinct antibody requirements for these two different sets of applications were not fully appreciated until recently. The methods for validating histone PTM antibodies have advanced over the years but generally consisted of using dot blots or peptide arrays to infer antibody specificity for a PTM. These histone PTM antibodies were then employed in ChIP assays to demonstrate enrichment or depletion of a specific PTM at expected regions in the chromatin, leading to reports of histone occupancy in the literature that are based on antibody specificity validated by peptide arrays. However, a bead-based flow cytometry assay that mimics the immunoprecipitation step of ChIP using peptides containing histone PTMs revealed that antibody specificity as determined by peptide arrays does not always correlate with specificity determined by peptide immunoprecipitation [4].

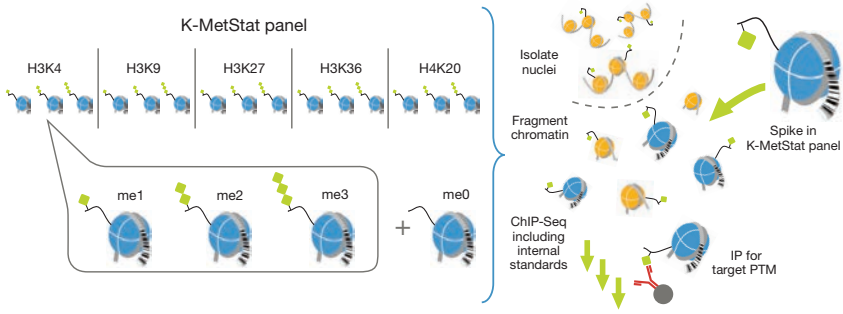

Figure 1. SNAP-ChIP technique for assessing antibody specificity. In a SNAP-ChIP™ assay, a panel of semi-synthetic nucleosomes containing specific histone PTMs is spiked in during the normal ChIP workflow. Because these nucleosomes are wrapped with unique DNA barcodes, they can later be quantified by qPCR to determine how much of each histone PTM is immunoprecipitated in the ChIP reaction. The full K-MetStat panel of histone PTMs includes H3K4, H3K9, H3K27, H3K36, and H4K20, in unmethylated and mono-, di-, and trimethylated forms. Figure used with permission from EpiCypher Inc.

Recently, histone PTM antibody specificity in ChIP applications has been further challenged using the Internal Standard Calibrated ChIP (ICeChIP) assay, a method in which a synthetic nucleosome containing a specific histone PTM is wrapped with a unique DNA barcode and then spiked into the normal ChIP workflow [5]. ICeChIP, which has been commercialized by EpiCypher Inc. as SNAP-ChIP™ (Sample Normalization and Antibody Profiling for Chromatin Immunoprecipitation) reagents and assays (Figure 1), can be used to determine if the antibody is pulling down the intended modification, as well as if the antibody is pulling down any other modifications among a panel of histone PTMs, distinguished on the basis of their DNA barcodes.

The K-MetStat panel (EpiCypher SNAP-ChIP™ Spike-In Controls) is currently available for testing antibody specificity in SNAP-ChIP assays and includes unmethylated and mono-, di-, and trimethylated H3K4, H3K9, H3K27, H3K36, and H4K20, each with a unique DNA barcode

that can be analyzed by either qPCR or DNA sequencing. Figure 2 shows the use of the K-MetStat panel for testing the specificity of an Invitrogen™ anti-H3K27me3 monoclonal antibody (Cat. No. MA5-11198). Antibody specificity was assessed by spiking HEK293 cell lysates with the K-MetStat panel and proceeding with a standard ChIP workflow. The amount of each of the spiked-in nucleosomes in the resulting immunoprecipitate was then quantified using qPCR. The data show that this Invitrogen antibody exhibited high specificity for H3K27me3 nucleosomes, with less than 15% cross-reactivity across the K-MetStat panel, and high efficiency of IP, with ~12% of the target nucleosome immunoprecipitated relative to the input.

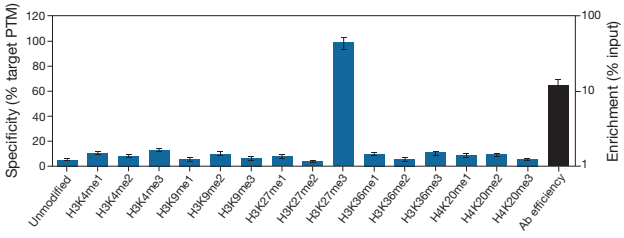

Figure 2. Histone PTM specificity analysis of an Invitrogen anti-H3K27me3 antibody. A SNAP-ChIP™ assay using an Invitrogen™ anti-H3K27me3 monoclonal antibody (Cat. No. MA5-11198) shows that it exhibits specificity to its target PTM. The antibody was tested in a SNAP-ChIP assay using chromatin isolated from HEK293 cell lysates, with the K-MetStat panel spiked in. Specificity (left y-axis, blue bars show mean ± SEM) was determined by qPCR for each modified nucleosome in the K-MetStat panel (x-axis) from six independent ChIP experiments. The black bar is antibody efficiency (right y-axis, log scale) and indicates % target nucleosome immunoprecipitated relative to input. Data used with permission from EpiCypher Inc.

Using SNAP-ChIP controls for antibody validation

The K-MetStat panel was used in a study of 54 commercially available antibodies, and no correlation was found between antibody peptide array specificity and antibody ICeChIP specificity [6]. The consequences of the lack of antibody specificity are significant. Using several H3K4 antibodies, Shah et al. performed ChIP-Seq, in which the ChIP protocol is followed by DNA sequencing to identify the histone occupancy. When the antibodies had similarly high specificity (>85% specific for the intended target), the histone occupancy (ChIP-Seq tracks) looked similar. In contrast, when a highly specific antibody was compared to one that was only 60% specific, the tracks looked different and the less specific antibody had additional peaks suggesting it recognized other histone PTMs and not just the intended target modification. This lack of antibody specificity can lead to incorrect assignment of histone occupancy and ultimately a misunderstanding of the biological role of a histone PTM.

Comparing ChIP-Seq data to previously published data is not always the best practice for validating a histone PTM antibody for ChIP because some less specific ChIP antibodies tested by Shah et al. had originally been used by the ENCODE project and others. Antibody specificity is of the utmost importance, particularly when performing ChIP for histone PTMs, and Thermo Fisher Scientific has begun employing SNAP-ChIP assays to validate Invitrogen histone PTM antibodies for ChIP. The SNAP-ChIP workflow can be used to determine both the efficiency and specificity of the antibody. Consistent with the literature, our own portfolio has revealed histone antibodies that are either concordant or discordant when tested in peptide array and SNAP-ChIP assays (Table 1).

These findings demonstrate the necessity of antibody specificity testing at an application level. As the histone PTM panels expand beyond the currently available K-MetStat panel, antibody testing for other histone methylation and acylation modifications will commence. Our goal is to develop a comprehensive antibody portfolio evaluated in the appropriate specificity test for a given application, providing antibodies that are highly specific for western blotting and for ChIP. To learn more about our expanded testing methodology, visit **thermofisher.com/antibodyvalidation**. ■

Table 1. Recommended antibodies for western blot (WB) based on specificity by peptide array and for ChIP based on specificity by SNAP-ChIP assay.

Target	Recommended for WB	Recommended for ChIP
H3K4me1	710795	710795
H3K4me2	710796	710796
H3K4me3	PA5-17420 and MA5-11199	PA5-27029
H3K9me1	710814	720091
H3K9me2	720092 and 710815	
H3K9me3	49-1008 and PA5-31910	
H3K27me1	49-1012	
H3K27me2		
H3K27me3	MA5-11198	MA5-11198
H3K36me1	701766	49-1016
H3K36me2	701767	
H3K36me3	MA5-24687	MA5-24687
H4K20me1	PA5-17027	MA5-18067
H4K20me2	720085	
H4K20me3	701777	MA5-18074

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Hunting for Hippo proteins

With highly specific ABfinity recombinant monoclonal antibodies.

The Hippo signaling pathway is an evolutionarily conserved pathway that has been shown to play a critical role in controlling organ size through the regulation of both cell proliferation and apoptosis. Dysregulation of the Hippo pathway results in aberrant cell growth and neoplasia. Given its involvement in these vital cell processes, it is not surprising that mutations in key Hippo pathway proteins are linked to a variety of cancers [1,2].

At the cellular level, the Hippo pathway integrates signals through several mechanisms, including G protein–coupled receptor (GPCR) signaling and the apicobasal polarity fundamental to epithelial cell function [3]. Kinase cascade and nuclear transcription modules form the backbone of the Hippo pathway. The kinase cascade includes serine/threonine kinases such as mammalian STE20–like protein kinases (MST1/2) and large tumor suppressors (LATS1/2), along with adaptor proteins including Salvador homolog 1 (SAV1), MOB kinase activator 1A (MOB1A), and MOB kinase activator 1B (MOB1B). The kinase cascade functions to restrict the activity of two transcriptional coactivators—Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ)—which are further modified by several Hippo regulators.

Antibodies are essential to the study of pathway proteins. However, for many components of the Hippo pathway, specific antibodies are either unavailable or poorly characterized. To address this need, Thermo Fisher Scientific has made a concerted effort to develop highly specific, application-tested antibodies directed against key proteins in this pathway.

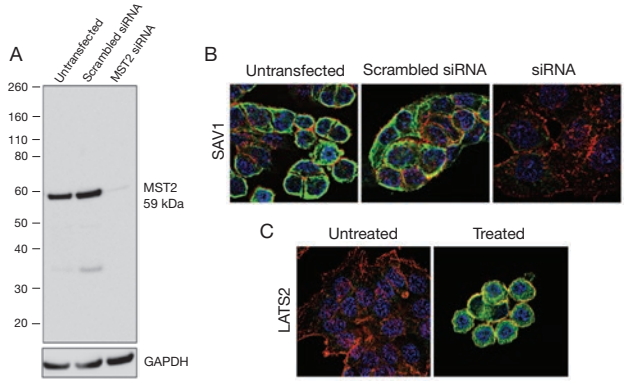

Figure 1. Antibodies directed against proteins in the Hippo pathway kinase cascade. (A) The specificity of Invitrogen™ MST2 Antibody (clone 19H19L39), ABfinity™ Rabbit Monoclonal (Cat. No. 703027) was determined by western blot analysis using siRNA-mediated MST2 knockdown in A549 cells. **(B)** The specificity of Invitrogen™ SAV1 Antibody (clone 6H5L16), ABfinity™ Rabbit Monoclonal (Cat. No. 703002) was demonstrated by immunofluorescence/immunocytochemistry (IF/ICC) using siRNA-mediated SAV1 knockdown in HCT116 cells. **(C)** The specificity of Invitrogen™ LATS2 Antibody (clone 17H14L2), ABfinity™ Rabbit Monoclonal (Cat. No. 703621) was demonstrated by IF/ICC; increased LATS2 expression was observed in U2OS cells treated with nocodazole. For IF/ICC, primary antibodies were detected with Invitrogen™ Goat Anti–Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor™ 488 (green, Cat. No. A27034), nuclei were stained using Invitrogen™ ProLong™ Diamond Antifade Mountant with DAPI (blue, Cat. No. P36962), and cytoskeletal F-actin was labeled with Invitrogen™ Rhodamine Phalloidin (Cat. No. R415). Chemiluminescence detection was performed using Invitrogen™ Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution; Cat. No. A27036) and Invitrogen™ Novex™ ECL Chemiluminescent Substrate Reagent Kit (Cat. No. WP20005) on the Invitrogen™ iBright™ FL1000 Imaging System (Cat. No. A32752).

Antibodies for kinase cascade proteins

In the kinase arm of the Hippo pathway, MST1/2-mediated phosphorylation of SAV1 and MOB1A/B leads to the recruitment, phosphorylation, and subsequent activation of LATS1/2. In conjunction with MOB1, the activated LATS1/2 kinases phosphorylate YAP/TAZ. These phosphorylation events result in the cytoplasmic sequestration and degradation of YAP/TAZ, mediated by 14-3-3 proteins. Figures 1A and 1B demonstrate the specificity of Invitrogen™ anti-MST1/2 and anti-SAV1 antibodies in western blot and immunofluorescence/immunocytochemistry (IF/ICC) applications using siRNA-mediated knockdown. In response to nocodazole treatment, LATS2 translocates from the cytoplasm into the nucleus, then binds to and activates p53, inducing LATS2 expression [4]. Figure 1C shows the specificity of the Invitrogen™ anti-LATS2 antibody in IF/ICC using this nocodazole-induced upregulation of LATS2 expression.

Antibodies for transcription factors and Hippo regulators

In the nuclear transcription module of the Hippo pathway, several DNA-binding proteins regulate the transcription of genes encoding Hippo pathway proteins and also undergo modification themselves. When not phosphorylated, YAP and TAZ translocate to the nucleus and serve as transcriptional coactivators for Hippo pathway genes. Their most significant interacting partners are the TEA-domain family member transcription factors (TEAD1–4) [5]. Figure 2A shows the specificity of the Invitrogen™ anti-TEAD4 antibody in western blot analysis using siRNA-mediated knockdown. Another transcription regulator, VGLL4, selectively binds to TEAD and interferes with the YAP–TEAD interaction. The specificity of the Invitrogen™ anti-VGLL4 antibody has been demonstrated with IF/ICC using siRNA-mediated knockdown (Figure 2B).

Angiomotin proteins (AMOT), which play a central role in tight junction maintenance, are also important negative regulators of YAP/TAZ,

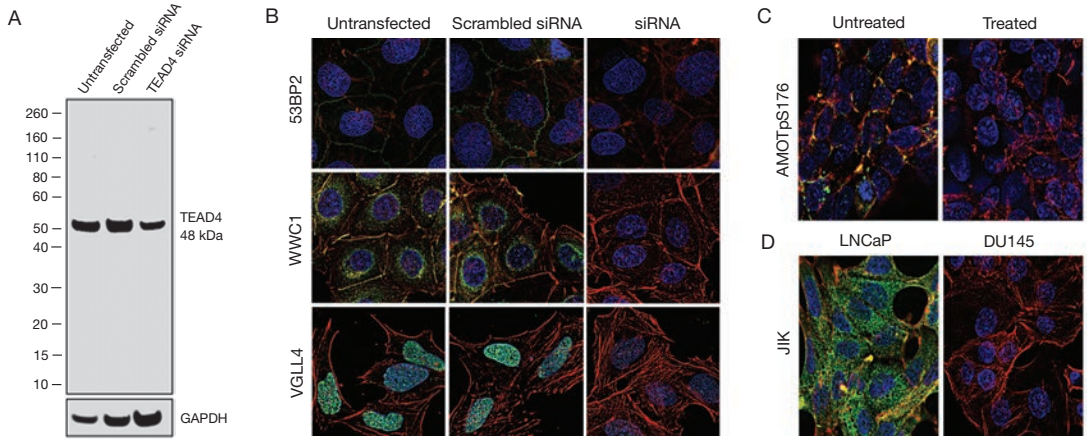

Figure 2. Antibodies that recognize Hippo pathway regulators. (A) The specificity of Invitrogen™ TEAD4 Polyclonal Antibody (Cat. No. 720430) was determined by western blot analysis using siRNA-mediated TEAD4 knockdown in HeLa cells. **(B)** The specificity of Invitrogen™ 53BP2 Antibody (clone 8H3L19), ABfinity™ Rabbit Monoclonal (Cat. No. 703010) and Invitrogen™ WWC1 Antibody (clone 1H4L22), ABfinity™ Rabbit Monoclonal (Cat. No. 703009) was demonstrated by immunofluorescence/immunocytochemistry (IF/ICC) using siRNA-mediated knockdown in Caco-2 cells. The specificity of Invitrogen™ VGLL4 Antibody (clone 16H12L24), ABfinity™ Rabbit Monoclonal (Cat. No. 703012) was demonstrated by IF/ICC using siRNA-mediated VGLL4 knockdown in HCT116 cells. **(C)** The specificity of Invitrogen™ Phospho-AMOT (Ser176) Antibody (clone 18H4L17), ABfinity™ Rabbit Monoclonal (Cat. No. 702980) was demonstrated by IF/ICC using untreated and serum-starved HEK293 cells, which exhibit reduced AMOTpS176 expression. **(D)** The specificity of Invitrogen™ JIK Antibody (clone 1HCLC), ABfinity™ Rabbit Oligoclonal (Cat. No. 712043) was demonstrated by IF/ICC to observe the differential basal expression of JIK in androgen-dependent (LNCaP) and -independent (DU145) cells. Fluorescent and chemiluminescent labeling reagents are detailed in Figure 1.

either by binding YAP/TAZ or by promoting their inhibitory phosphorylation. Phosphorylated AMOT (ser176) is downregulated during serum starvation, a characteristic that was used to show the specificity of the Invitrogen™ anti-AMOTpS176 antibody in IF/ICC (Figure 2C). In contrast to AMOT, 53BP2 (ASPP2) is a positive regulator of YAP, facilitating the dephosphorylation of YAP/TAZ. Specificity of the Invitrogen™ anti-53BP2 antibody has been demonstrated in IF/ICC using siRNA-mediated knockdown (Figure 2B).

WWC1 (KIBRA), a mechanical regulator that associates with tight junctions and other cell polarity complexes, can induce the phosphorylation of LATS1/2 [6]. The specificity of the Invitrogen™ anti-WWC1 antibody is shown in IF/ICC using siRNA-mediated knockdown (Figure 2B). The Hippo kinase cascade can also be initiated by TAO kinases, which phosphorylate MST1/2 [7]. TAO kinases, including TAO3 and JIK, are differentially expressed in androgen-dependent and -independent cell lines, a feature that was used to show the specificity of the Invitrogen™ anti-JIK antibody (Figure 2D).

Invitrogen antibodies for Hippo pathway proteins	Tested application*	Quantity	Cat. No.
Antibodies for Hippo pathway kinases			
SAV1 Antibody (clone 6H5L16), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	703002
MST2 Antibody (clone 19H19L39), ABfinity™ Rabbit Monoclonal	WB	100 µg	703027
LATS2 Antibody (clone 17H14L2), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	703621
Antibodies for Hippo pathway transcription regulators			
TEAD4 Polyclonal Antibody	WB	100 µg	720430
VGLL4 Antibody (clone 16H12L24), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	703012
Phospho-AMOT (Ser176) Antibody (clone 18H4L17), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	702980
53BP2 Antibody (clone 8H3L19), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	703010
JIK Antibody (clone 1HCLC), ABfinity™ Rabbit Oligoclonal	IF, ICC, WB	100 µg	712043
WWC1 Antibody (clone 1H4L22), ABfinity™ Rabbit Monoclonal	IF, ICC	100 µg	703009

*IF = immunofluorescence, ICC = immunocytochemistry, WB = western blot. The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. The product(s) was not validated for clinical or diagnostic use.

Find Hippo pathway antibodies

Visit **thermofisher.com/antibodies** to find specific, application-tested antibodies—including Invitrogen™ ABfinity™ recombinant antibodies—that recognize proteins in the Hippo signaling pathway. ■

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Role of metabolic pathways in cancer

Using siRNA knockdowns to verify antibody specificity.

Metabolic reprogramming has emerged as an important indicator of cancer cells [1]. Even in the presence of oxygen, cancer cells tend to metabolize glucose into pyruvate and then lactate by aerobic glycolysis (also known as the Warburg effect) rather than by the more efficient oxidative phosphorylation pathway [2]. This observation was instrumental in linking metabolic disorders to cancer. In an elegant review by Pavlova and Thompson [3], cancer-associated metabolic characteristics are classified as: (1) deregulated uptake of glucose and amino acids, (2) use of opportunistic modes of nutrient acquisition, (3) use of glycolysis or tricarboxylic acid (TCA) cycle intermediates for biosynthesis and NADPH production, (4) increased demand for nitrogen, (5) alterations in metabolite-driven gene regulation, or (6) metabolic interactions with the microenvironment. Of these metabolic characteristics, the deregulation of glucose and glutamine metabolism has been studied extensively, and the key proteins in these pathways are being explored for potential therapeutic interventions [4,5]. Here we detail several Invitrogen™ antibodies that are proving useful for the study of cancer metabolism and highlight the strategies we employ to ensure antibody specificity.

Antibodies for studying glucose metabolism

Hypoxia has been found to enhance both lactate production and tumor progression through the activation of hypoxia-inducible transcription factor 1-alpha (HIF1α), glucose transporter 1 (GLUT1), hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), pyruvate dehydrogenase kinase 1 (PDK1), enolase 1 (ENO1), and lactate dehydrogenase A (LDHA). PKM2 is upregulated in most cancer cells, leading to the redirection of metabolic pathways towards lactate production [6]. Thermo Fisher Scientific supplies antibodies against many of these metabolic markers (see product table on page 30), and the specificity of these antibodies has been validated using siRNA-mediated knockdown to reduce target expression. Figure 1 shows the specificity of the Invitrogen™ anti-LDHA and anti-PKM2 antibodies on western blots using various cell lines and in the presence of siRNA-mediated knockdown.

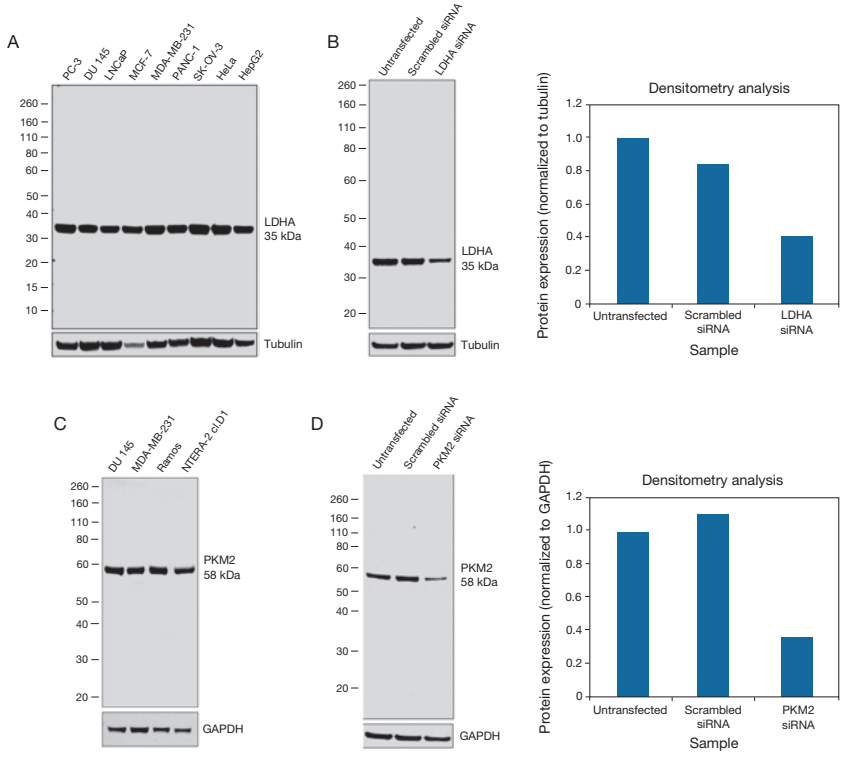

Figure 1. Confirmation of LDHA and PKM2 antibody specificity. (A) Western blot analysis of LDHA was performed using membrane-enriched extracts of various cell lines. The blot was probed with Invitrogen™ LDHA Antibody, ABfinity™ Rabbit Oligoclonal (2.5 µg/mL, Cat. No. 711782) and detected using Invitrogen™ Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution; Cat. No. A27036). A 35 kDa band corresponding to LDHA was observed. **(B)** The specificity of this LDHA antibody was determined by western blot analysis using siRNA-mediated LDHA knockdown; densitometry analysis shows decreased LDHA expression in the presence of the specific siRNA, but not the scrambled siRNA. **(C)** Western blot analysis of PKM2 was performed using membrane-enriched extracts of various cell lines. The blot was probed with Invitrogen™ PKM2 Polyclonal Antibody (1:1,000 dilution, Cat. No. PA5-23034) and detected using Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution). A 58 kDa band corresponding to PKM2 was observed. **(D)** The specificity of this PKM2 antibody was determined by western blot analysis using siRNA-mediated PKM2 knockdown; densitometry analysis shows decreased PKM2 expression in the presence of the specific siRNA, but not the scrambled siRNA. Chemiluminescence detection was performed using Invitrogen™ Novex™ ECL Chemiluminescent Substrate Reagent Kit (Cat. No. WP20005) on the Invitrogen™ iBright™ FL1000 Imaging System (Cat. No. A32752).

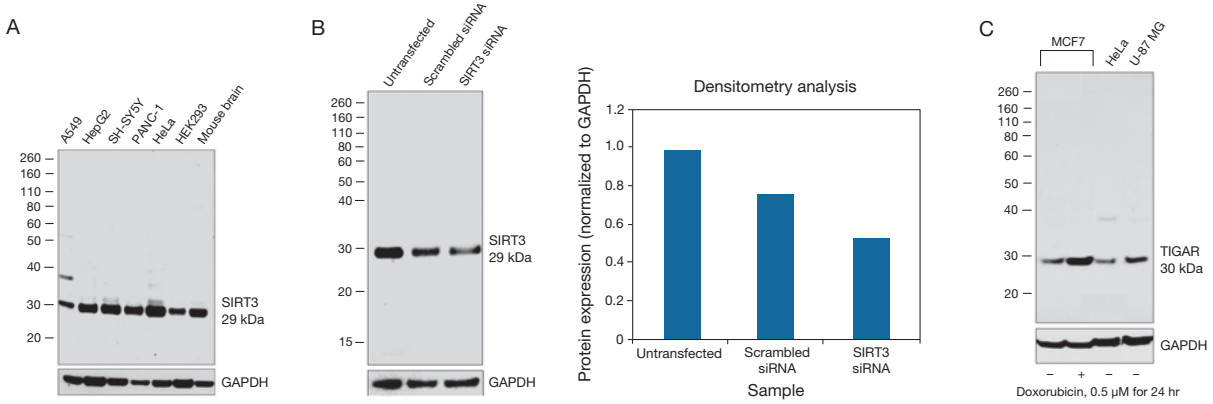

Figure 2. Confirmation of SIRT3 and TIGAR antibody specificity. (A) Western blot analysis of SIRT3 was performed using membrane-enriched extracts of various cell lines. The blot was probed with Invitrogen™ SIRT3 Polyclonal Antibody (1:1,000 dilution, Cat. No. PA5-28402) and detected using Invitrogen™ Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution; Cat. No. A27036). A 29 kDa band corresponding to SIRT3 was observed. **(B)** The specificity of this SIRT3 antibody was determined by western blot analysis using siRNA-mediated SIRT3 knockdown; densitometry analysis shows decreased SIRT3 expression in the presence of the specific siRNA, but not the scrambled siRNA. **(C)** Western blot analysis of TIGAR was performed using whole cell extracts of various cell lines. The blot was probed with Invitrogen™ TIGAR Polyclonal Antibody (1:5,000 dilution, Cat. No. PA5-29151) and detected using Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution). A 30 kDa band corresponding to TIGAR was observed, and TIGAR expression increased in doxorubicin-treated MCF7 cells. Chemiluminescence detection was performed as described in Figure 1.

Epigenetic control of glucose metabolism

Epigenetic control of glucose metabolism is primarily exerted though the sirtuin family of NAD^+-dependent deacetylases (SIRT1–7). In particular, SIRT6 is observed to upregulate glucose metabolism through induction of GLUT1, phosphofructokinase 1 (PFK1), aldolase C (ALDOC), PDK1, and LDHA. The mitochondrial deacetylase SIRT3 acts as a tumor suppressor; deactivating SIRT3 leads to disordered mitochondrial metabolism [7]. Figures 2A and 2B show the specificity of the Invitrogen™ anti-SIRT3 antibody on western blots using various cell lines and in the presence of siRNA-mediated SIRT3 knockdown.

Overlap of oncogenes and glucose metabolism

Likewise, oncogenes and tumor suppressors are linked to the regulation of glucose metabolism in cancer cells [8]. The expression of TP53-inducible glycolysis and apoptosis regulator (TIGAR) is upregulated by the wild-type tumor suppressor p53, resulting in the inhibition of fructose 2,6-bisphosphate production and redirection of glucose metabolism towards a pentose phosphate pathway [9]. Figure 2C shows the specificity of the Invitrogen™ anti-TIGAR antibody on western blots using extracts from MCF7 cells without and with doxorubicin treatment, which causes an increase in TIGAR expression.

Antibodies for studying glutamine metabolism

Glutamine is a nonessential amino acid that provides carbon for the TCA cycle and lipid biosynthesis, and elevated glutamine levels have been observed in many cancer cells. Glutaminolysis aided by glutaminases (GLS) leads to the production of various catabolites, including glutamate, which is subsequently converted to α-ketoglutarate by glutamate dehydrogenases (GLUD). Oncogenic molecules such as c-Myc and K-Ras can activate GLS1 and glutamic-oxaloacetic transaminases (GOT), whereas K-ras inhibits GLUD1 in proliferating cells, and therefore these glutamine metabolism enzymes have emerged as potential therapeutic targets for cancer [10]. Figure 3 shows the specificity testing of Invitrogen™ anti-GLS and anti-GLUD antibodies by immunofluorescence and western blot analysis, respectively, using siRNA-mediated knockdown of the target expression.

Explore our metabolic marker antibodies for cancer research

In addition to the antibodies for glucose metabolism described above, Thermo Fisher Scientific offers an extensive collection of specific, application-tested antibodies directed against various TCA cycle intermediates and lipid and fatty acid metabolism →

markers that play a role in tumorigenesis and disease progression (see product table below). Explore our complete portfolio of primary antibodies for metabolism research at **thermofisher.com/antibodies**. Use the antibody search tool to filter primary antibodies by target (or gene symbol or antigen), application, and target species. ■

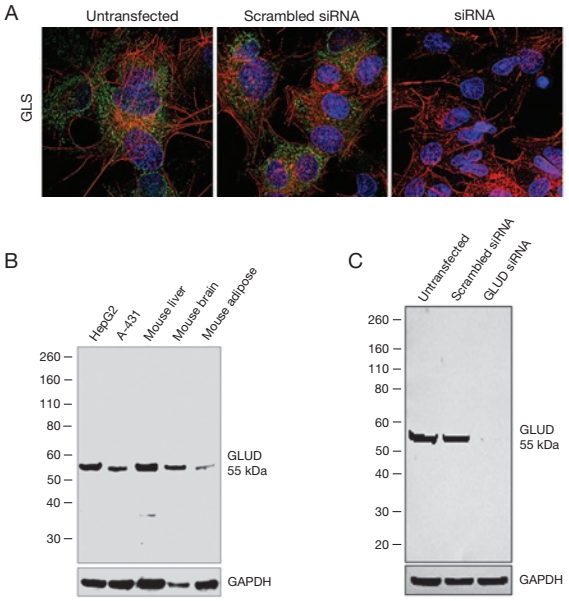

Figure 3. Confirmation of glutaminase (GLS) and glutamate dehydrogenase (GLUD) antibody specificity. **(A)** The specificity of Invitrogen™ GLS Antibody (clone 6H5L15), ABfinity™ Rabbit Monoclonal (5 µg/mL, Cat. No. 701965) was demonstrated by immunofluorescence/immunocytochemistry (IF/ICC) using siRNA-mediated knockdown in HepG2 cells; the siRNA-treated HepG2 cells showed reduced mitochondrial GLS expression. The anti-GLS antibody was detected with Invitrogen™ Goat Anti–Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor™ 488 (green, Cat. No. A27034), nuclei were stained using Invitrogen™ ProLong™ Diamond Antifade Mountant with DAPI (blue, Cat. No. P36962), and cytoskeletal F-actin was labeled with Invitrogen™ Rhodamine Phalloidin (Cat. No. R415). **(B)** Western blot analysis of GLUD was performed using membrane-enriched extracts of various cell lines. The blot was probed with Invitrogen™ GLUD Polyclonal Antibody (1:2,000 dilution, Cat. No. PA5-29492) and detected using Invitrogen™ Goat Anti–Rabbit IgG (H+L) Secondary Antibody, HRP (0.25 µg/mL, 1:4,000 dilution; Cat. No. A27036). A 55 kDa band corresponding to GLUD was observed. **(C)** The specificity of this GLUD antibody was determined by western blot analysis using siRNA-mediated GLUD knockdown. Chemiluminescence detection was performed as described in Figure 1.

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Selected Invitrogen antibodies for cancer metabolism research	Tested application*	Quantity	Cat. No.
Citrate Synthetase Polyclonal Antibody	IF, ICC, IHC (P), IP, WB	100 µL	PA5-22126
GLDC Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-22102
GLUT1 Monoclonal Antibody (clone SPM498)	IF, ICC, IHC (P)	1 mL	MA1-37783
Glutamate Dehydrogenase (GLUD) Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-29492
Glutaminase (GLS) Antibody (clone 6H5L15), ABfinity™ Rabbit Monoclonal	IF, ICC, WB	100 µg	701965
GOT1 Polyclonal Antibody	IF, ICC, IHC (P), pep-ELISA, WB	100 µg	PA5-18845
IDH1 Polyclonal Antibody	Flow, IF, ICC, IHC (P), WB	400 µL	PA5-14358
HIF1A Polyclonal Antibody	ELISA, IF, ICC, IP, WB	100 µg	PA1-184
LDHA Antibody, ABfinity™ Rabbit Monoclonal	IF, ICC, WB	100 µg	711782
MDH2 Polyclonal Antibody	IHC (P), WB	100 µL	PA5-21700
c-Myc Antibody (clone 27H46L35), ABfinity™ Rabbit Monoclonal	IF, ICC, WB	100 µg	700648
OGDH Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-28195
PKM2 Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-23034
SIRT3 Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-28402
SIRT6 Polyclonal Antibody	IF, ICC, IP, WB	100 µL	PA5-17215
TALDO1 Polyclonal Antibody	IHC (P), WB	100 µL	PA5-27614
TIGAR Polyclonal Antibody	IF, ICC, IHC (P), WB	100 µL	PA5-29151
TPI1 Polyclonal Antibody	IF, ICC, IHC (P), pep-ELISA, WB	100 µg	PA5-18342

*ELISA = enzyme-linked immunosorbent assay; Flow = flow cytometry; IF = immunofluorescence; ICC = immunocytochemistry; IHC (P) = immunohistochemistry (paraffin); IP = immunoprecipitation; pep-ELISA = peptide-ELISA; WB = western blot. The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. The product(s) was not validated for clinical or diagnostic use.

Dissect signaling pathways with multiplex western blots

Using fluorescent antibody conjugates in combination with the iBright Imaging System.

Signaling pathways control and coordinate all aspects of cell function. Dissecting these pathways leads to the identification of key receptors, enzymes, and signaling molecules that regulate both normal development and disease states. Great advances in examining signaling pathways have been made using CRISPR-Cas9 technology, which allows precise editing or excision of genes of interest, thus knocking out expression of targeted proteins. Using these gene knockouts, researchers can study the upstream and downstream interactions of the targeted protein.

Analyze gene knockouts with multiplex western blotting

The effect of gene knockouts on individual proteins in a signaling pathway can be determined using western blotting with pathway-specific antibodies. For example, antibody pairs that recognize a specific protein and corresponding phosphoprotein can be used to determine the phosphorylation state of a putative downstream protein in a signaling pathway. Probing the western blot with several fluorescently labeled antibodies that emit light at different wavelengths enables the simultaneous detection of multiple protein targets on a single blot. In contrast, traditional chemiluminescent western blotting requires multiple rounds of probing, stripping, and reprobing to collect the same pool of data.

By minimizing the need to run multiple protein gels or reprobe blots, multiplex western blotting saves a significant amount of time, effort, and cost. Furthermore, advances in western blot imaging equipment and software enable streamlined protein quantitation. To demonstrate its utility, we used multiplex western blotting to illustrate the efficient knockout of the gene encoding the epidermal growth factor receptor (EGFR), and the effects of this knockout on the downstream mitogen-activated protein kinase 1 (MEK1) (Figure 1). EGFR knockout cells created using CRISPR-Cas9 gene editing technology were treated with epidermal growth factor (EGF). Cell lysates were then prepared, separated by protein gel electrophoresis, transferred to a membrane, and probed using spectrally distinct fluorescent antibodies specific for EGFR and MEK1 and the corresponding phosphoproteins (Table 1). The blot was imaged using the Invitrogen™ iBright™ FL1000 Imaging System; the signals were normalized using an antibody specific for the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Figure 1 shows that EGF-induced phosphorylation of EGFR and MEK1 was observed in control cell lines (lanes 1–4) and not in the EGFR knockout cell lines (lanes 5 and 6).

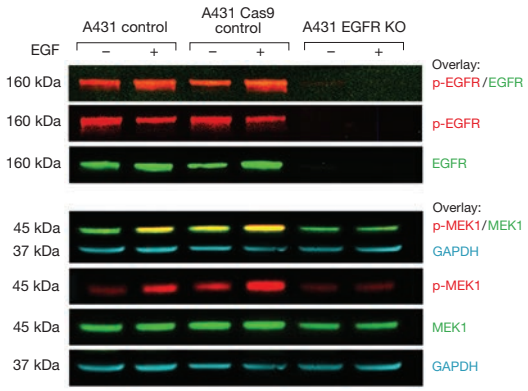

Figure 1. Western blot analysis of EGFR and its downstream target MEK1 using EGFR knockout cells. Electrophoresis was performed using an Invitrogen™ NuPAGE™ Bis-Tris 10% gel (Cat. No. NP030A) loaded with 30 µg of whole cell extracts from (1) A431 control cells, (2) EGF-treated A431 cells (200 ng/mL for 10 min), (3) A431 Cas9 control cells, (4) EGF-treated A431 Cas9 cells, (5) A431 EGFR knockout (KO) cells, (6) EGF-treated A431 EGFR KO cells, and Invitrogen™ Novex™ Sharp Pre-Stained Protein Standard (Cat. No. LC5800). Proteins were transferred to a nitrocellulose membrane using the Invitrogen™ iBlot™ 2 Gel Transfer Device (Cat. No. IB21001), blocked with 5% skim milk for 1 hr, and incubated with Invitrogen™ primary antibodies and Invitrogen™ Alexa Fluor™ Plus secondary antibodies (Table 1). Blots were imaged on the Invitrogen™ iBright™ FL1000 Imaging System (Cat. No. A32752).

Table 1. Primary and secondary antibodies, western blotting conditions, and imaging channels used to produce the data in Figure 1.

Target	Primary Ab* host (Cat. No.)	Secondary Ab* (Cat. No.)	Imaging channel
Phospho-EGFR (Tyr1068)	Rabbit (PA5-17848)	Alexa Fluor Plus 800 donkey anti–rabbit IgG (A32808)	800 nm
EGFR	Mouse (MA5-13343)†	Alexa Fluor Plus 680 donkey anti–mouse IgG (A32788)	680 nm
Phospho-MEK1 (Thr386)	Rabbit (702581)	Alexa Fluor Plus 800 donkey anti–rabbit IgG (A32808)	800 nm
MEK1	Mouse (MA5-15093)	Alexa Fluor Plus 680 donkey anti–mouse IgG (A32788)	680 nm
GAPDH	Goat (PA1-9046)	Alexa Fluor Plus 488 donkey anti–goat IgG (A32814)	488 nm

*Primary antibody (Ab) dilutions were determined empirically and ranged from 1:500 to 1:2,000; incubations were performed overnight at 4°C. Secondary antibody dilutions were 1:1,000, and incubations were performed for 1 hr at room temperature, protected from light. †MA5-13343 has been replaced with EGFR Antibody Cocktail (Cat. No. MA5-13697). Search our extensive antibody portfolio at **thermofisher.com/antibodies**.

Learn more about multiplex western blotting

Significant advances in instrumentation as well as in immunodetection have facilitated the migration from ECL to fluorescence-based western detection. Learn more about multiplex western blotting at **thermofisher.com/5steps-multiplexwesterns**. ■

ELISA workflows just got easier

With Instant ELISA kits, E1-ClipTip electronic pipette, and Wellwash Versa Microplate Washer.

Since the 1970s, the enzyme-linked immunosorbent assay (ELISA) has served as the mainstay for single-target protein quantitation, despite several cumbersome steps in its workflow. Specifically, assay steps associated with preparation, incubation, and washing can be vastly improved using innovative tools of the trade developed by Thermo Fisher Scientific. Here we describe three tools designed to simplify ELISA protocols.

Simplify preparation and incubation steps

Invitrogen™ Instant ELISA™ Kits are a family of immunoassays that have a condensed workflow format designed to alleviate the inconveniences of multiple preparation and incubation steps (Table 1). In conventional ELISA kits, the precoated plates provide only the capture antibody, to which the sample and detection reagents must be added sequentially. In contrast, Instant ELISA Kits come with ready-to-use plates that contain all necessary assay components—including capture antibody and lyophilized detection antibody, streptavidin-HRP, and sample diluent. These prepared plates greatly reduce pipetting time and enable a simple one-wash protocol (Table 1). Furthermore, serially diluted, lyophilized protein standards are provided in additional strip wells, eliminating the need for plate setup or serial dilutions to create a standard curve and leaving less room for error.

Table 1. Comparison of workflows for conventional ELISAs vs. Instant ELISA Kits.

17 steps for conventional ELISA kits	7 steps for Instant ELISA Kits
1. Washing of coated plates	
2. Reconstitution of standard proteins	
3. Addition of sample diluent to standard wells	
4. Titration of standard curve	1. Rehydration of plate
5. Addition of sample diluent	
6. Sample addition	2. Sample addition
7. Dilution of biotin conjugate	
8. Addition of biotin conjugate	
9. Incubation	3. Incubation
10. Preparation of streptavidin-HRP conjugate	
11. Washing	
12. Addition of streptavidin-HRP conjugate	
13. Incubation	
14. Washing	4. Washing
15. Addition of TMB substrate	5. Addition of TMB substrate
16. Addition of stop solution	6. Addition of stop solution
17. Calculation of results	7. Calculation of results

Execute a preprogrammed protocol

Rehydrating standards, introducing samples, and adding reagents can be simplified using a preprogrammed pipetting protocol that can be

A

B

C

1. Add 150 µL of distilled water to Standard and Blank wells.
2. Add 100 µL of distilled water to Sample well.
3. Add 50 µL of Sample in duplicate and mix.
4. Cover plate and incubate for 3 hours on microplate shaker if available at 400 rpm.
5. Remove plate cover, empty wells, and wash the microwell strips 6 times with 400 µL Wash Buffer per well with thorough aspiration of microwell contents.
6. Pipette 100 µL of TMB Substrate Solution to all wells, including the blank.
7. Incubate the microwell strips at room temperature (18° to 25°C) for 10–30 minutes (avoid direct exposure to intense light).
8. Add 100 µL of Stop Solution to each well.

Figure 1. Preprogrammed pipetting tools. (A) The programmable Thermo Scientific™ E1-ClipTip™ Multichannel Pipette. **(B)** The Thermo Scientific™ My Pipette™ Creator app (available at **apps.thermofisher.com**, powered by our Connect cloud-based platform) makes it easy to access and create assay protocols. **(C)** An example of a protocol for an Invitrogen™ Instant ELISA™ Kit.

easily transferred to the Thermo Scientific™ E1-ClipTip™ Electronic Multichannel Pipette (Figure 1A), saving valuable time and minimizing potential errors. A preprogrammed pipetting protocol can be downloaded from the Thermo Scientific™ My Pipette™ Creator app (Figure 1B) to an E1-ClipTip pipette. Using the My Pipette Creator app, custom pipetting programs can be quickly created, edited, and then transferred to a E1-ClipTip pipette through a USB or, for some models, a wireless connection. Once programmed, the pipette will automatically step you through the protocol (Figure 1C) with concise and clear instructions, even prompting when it is time for the plate to be incubated.

Automate wash steps

During an ELISA protocol, wash steps are extremely important for decreasing background levels, which can impact both the variability and sensitivity of the assay. Manual wash steps with either squirt bottles or multichannel pipettes can be awkward and unwieldy, introducing another potential source of variation. The Thermo Scientific™ Wellwash™ Versa Microplate Washer (Figure 2) makes wash steps easier, more efficient, and more consistent. With programmable volumes and soak times, the Wellwash Versa instrument is designed for high performance and versatility and can be configured with a 2 x 8 head to accommodate the 2 x 8-well standard curve strip format.

Figure 2. Automation of wash steps. Thermo Scientific™ Wellwash™ Versa Microplate Washer (Cat. No. 5165010).

Figure 3. Standard curve data with an average CV of 3.3% using the TNF alpha Human Instant ELISA Kit. The Invitrogen™ TNF alpha Instant ELISA™ Kit (Cat. No. BMS223INST) was used according to the manufacturer's protocol using a programmable Thermo Scientific™ E1-ClipTip™ Multichannel Pipette and the Thermo Scientific™ Wellwash™ Versa Microplate Washer (Cat. No. 5165050). Data were analyzed using the Thermo Scientific™ Multiskan™ Sky Microplate Spectrophotometer with Thermo Scientific™ SkanIt™ Software (Cat. No. 51119700).

Streamline ELISAs in your lab

The ELISA workflow can be significantly streamlined when combining these three time-saving tools, generating data with low coefficients of variation (CVs) (Figure 3). The Instant ELISA Kit format simplifies both the preparation and incubation steps and reduces hands-on time. Instead of tending to your plate every hour to perform washes, you have a 3-hour window to focus on something else before wrapping up the assay run. The My Pipette Creator app and E1-ClipTip electronic pipette make the steps in the ELISA workflow more efficient by reducing pipetting time and minimizing errors. And the Wellwash Versa Microplate Washer automates the single Instant ELISA wash step, providing consistency to help prevent incomplete washing and associated adverse outcomes, as well as convenience. To learn more about how these Thermo Fisher Scientific tools can provide an overall easier and more efficient ELISA workflow, go to **thermofisher.com/elisa**. ■

Product	Quantity	Cat. No.
TNF alpha Human Instant ELISA™ Kit	128 tests	BMS223INST
ClipTip™ 1250 Filtered Sterile Pipette Tips	Case of 768	94420813
E1-ClipTip™ Bluetooth™ Electronic Single-Channel Pipette, 15 to 1,250 µL	1 each	4670040BT
E1-ClipTip™ Bluetooth™ Electronic 8-Channel Pipette, 15 to 1,250 µL	1 each	4671100BT
Multiskan™ Sky Microplate Spectrophotometer, with touchscreen and cuvette	1 each	51119700
SkanIt™ Software for Microplate Readers, Research Edition	1 each	5187139
Wellwash™ Versa Microplate Washer, 2 x 8 model	1 each	5165010
Wellwash™ Versa Microplate Washer, 2 x 12 model	1 each	5165050

Measure secreted protein and mRNA levels with a single instrument platform

ProcartaPlex and ProQuantum immunoassays augment mRNA quantitation.

Given the investigative tools available, biomedical researchers are sometimes constrained to choose either the study of genes (genomics) or the study of proteins (proteomics) to further the understanding of disease mechanisms and pursue the analysis of potential treatments, even though it is widely held that interrogation at both the gene and protein levels is valuable and necessary. By leveraging existing instrument platforms and lab expertise as well as crossover detection technologies, genomic and proteomic workflows can be combined without compromising data interpretation or sensitivity.

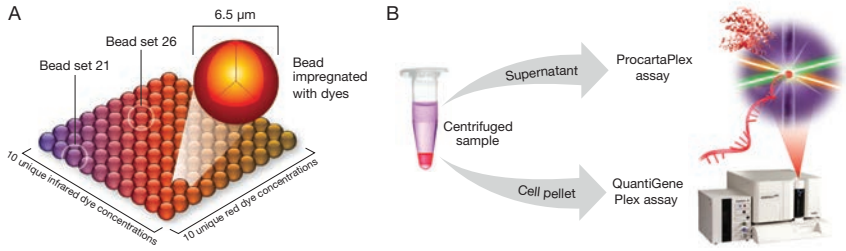

Figure 1. Levels of protein (ProcartaPlex assay) and gene expression (QuantiGene Plex assay) measured in a single sample using the Luminex® detection system.

Protein and mRNA detection with the Luminex detection system

One example of such a "dual-omic" platform is the Luminex® detection system and associated xMAP® magnetic bead technology, capable of multiplex analyte detection in a single microplate well (Figure 1). The Luminex® MagPlex® superparamagnetic microsphere beads are internally dyed with precise proportions of red and infrared fluorophores to create 100 spectrally unique signatures that can each be identified by the Luminex xMAP detection systems, including the Luminex® 200™, FLEXMAP 3D®, and MAGPIX® systems.

ProcartaPlex immunoassays. Predominantly adopted for protein quantitation—for example in the Invitrogen™ ProcartaPlex™ immunoassays—the xMAP bead technology allows the multiplex detection of up to 80 protein targets in a single microplate well, providing significant time and sample input savings over conventional ELISAs. ProcartaPlex immunoassays are antibody-based magnetic-bead reagent kits and panels for multiplex high-throughput quantitation of protein. Similar to a sandwich ELISA, the ProcartaPlex assay uses matched antibody pairs to identify the protein of interest; unlike conventional ELISAs, the capture antibody is conjugated to a free-floating magnetic bead and not adsorbed to the microplate well. In a ProcartaPlex multiplex assay, each spectrally unique bead is labeled with an antibody specific for a single target protein, and bound proteins are identified with biotinylated antibodies and streptavidin–R-phycoerythrin (RPE). The conjugation of protein-specific antibodies to distinct beads allows for analysis of multiple analytes in a single well. For detection, the Luminex 200 instrument, for example, contains two lasers, one to distinguish the spectral signature of each bead and the second to quantify the amount of RPE fluorescence, which is proportional to the amount of protein present in the sample. ProcartaPlex assays are available as preconfigured multiplex panels (2- to 65-plex) or singleplex kits that can be combined to create custom multiplex assays.

QuantiGene Plex mRNA assays. The xMAP technology has also been expanded and applied to the multiplex measurement of mRNA gene expression in the Invitrogen™ QuantiGene™ Plex Gene Expressions Assays, which are compatible with Luminex systems. QuantiGene Plex assays provide a method for multiplex high-throughput quantitation of mRNA, allowing the simultaneous measurement of up to 80 genes of interest in a single well of a 96- or 384-well plate using a Luminex xMAP detection system. With an ELISA-like workflow, the QuantiGene Plex assay begins with the direct hybridization of transcripts to magnetic beads, followed by signal amplification using branched DNA (bDNA) technology. In contrast with other gene expression assays, the QuantiGene Plex assay depends on signal amplification rather than target amplification for direct measurement of transcripts; i.e., no RNA purification or reverse transcription is required.

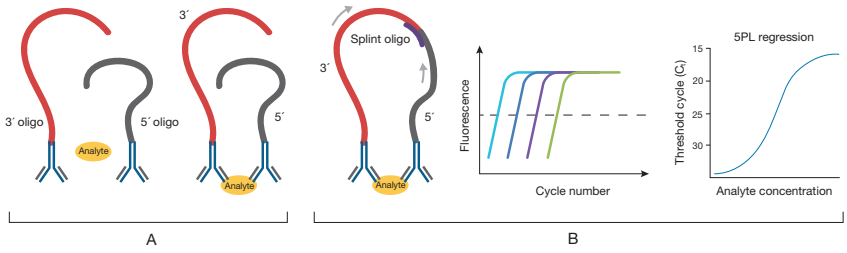

Figure 2. How ProQuantum immunoassays work. (A) Antibody conjugates bind to target during a 1 hr incubation. **(B)** With addition of a splint oligonucleotide and DNA ligase, the two antibody-conjugated oligonucleotides are ligated to create a 100-base template that is amplified through 40 qPCR cycles. The amount of DNA produced, measured via fluorescence, is directly proportional to the number of amplicons generated.

Protein quantitation using a qPCR platform: ProQuantum immunoassays

With qPCR reagents and instrumentation now so prevalent in research labs, Thermo Fisher Scientific has also recently launched a qPCR-based protein quantitation technology called the Invitrogen™ ProQuantum™ Immunoassay. ProQuantum high-sensitivity immunoassays provide an affordable and easily accessible protein quantitation method that can be used on the same instrument platform for mRNA quantitation.

Similar to ELISA kits, ProQuantum ready-to-use kits enable single-protein target detection and quantitation using matched antibody pairs. ProQuantum immunoassays, however, take advantage of Applied Biosystems™ TaqMan® fluorescence-based qPCR, providing both sensitivity and dynamic ranges that exceed those of conventional ELISAs (Figure 2). This immunoassay is based on an optimized pair of antibodies that bind to specific epitopes in close proximity on the analyte. These antibodies are preconjugated at the 3´ end of a 60-base oligonucleotide or the 5´ end of a 40-base oligonucleotide. When added to a sample containing the specific analyte, the two antibodies bind to their respective epitopes. In the presence of DNA ligase and a third splint oligonucleotide, the two antibody-conjugated oligonucleotides are ligated together to create a 100-base strand that can serve as a DNA amplification template. This template is then amplified through 40 qPCR cycles, and the amount of amplified product after each cycle, as measured by fluorescence, is directly proportional to the number of ligated templates created by the antibody–analyte binding. ProQuantum high-sensitivity immunoassays can quantify analytes over a concentration range of up to 5 orders of magnitude or more, minimizing the need for sample dilutions.

The ProQuantum immunoassay features a streamlined workflow with no wash steps. Moreover, only a total of 2 µL of serum is needed to obtain results in duplicate or triplicate, as compared with 50 µL volumes typically required for a single data point with an ELISA. The small sample-volume requirement means less work, not just during the assay run itself but also during sample collection procedures. Translational investigators working with finite human clinical samples must obtain as much data as they can from their often limited sample volumes. In addition, for small animal models like mice, longitudinal studies are often limited by the blood serum volumes required for each time point. With the small volume requirement of ProQuantum immunoassays, a simple tail prick is usually all that is required for each time point, and by using the same animal throughout the study, animal variability is also minimized.

Despite the small sample volumes, ProQuantum immunoassays can detect lower levels of proteins in samples than a traditional ELISA. For example, when comparing 40 endogenous natural sample measurements using either a mouse IL-1 alpha ELISA or a mouse IL-1 alpha ProQuantum immunoassay, only a single sample exhibited detectable levels of the IL-1 alpha protein using an ELISA, whereas 28 samples were quantifiable using the corresponding ProQuantum immunoassay (Figure 3). The assay range of the ProQuantum immunoassay spanned 0.32 to 5,000 pg/mL, whereas the ELISA provided an assay range of 8 to 500 pg/mL.

Explore these protein quantitation assays

To learn more about ProcartaPlex multiplex panels, ProQuantum high-sensitivity immunoassays, and the protein targets currently available for each assay, visit **thermofisher.com/immunoassays** (where you can also download a free biomarker quantitation assay guide). ■

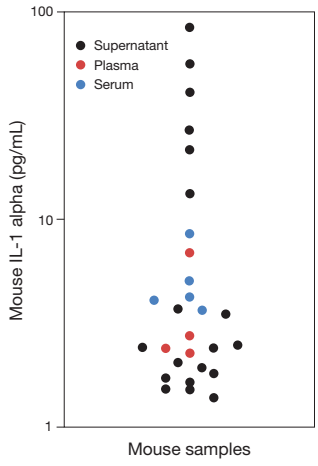

Figure 3. Use of a ProQuantum immunoassay with natural samples. Mouse IL-1 alpha levels were measured in natural samples (serum, plasma, and supernatant) using the Invitrogen™ IL-1 alpha Mouse ProQuantum™ Immunoassay Kit (Cat. No. A42894).

Therapeutic targeting of sunitinib-induced androgen receptor (AR) phosphorylation in renal cell carcinoma

Adelaiye-Ogala R, Damayanti NP, Orillion AR, Arisa S, Chintala S, Titus MA, Kao C, Pili R (2018) *Cancer Res* 78:2886–2896.

Androgen receptor (AR) expression is known to play a critical role in the development and progression of solid tumors, including prostate cancer [1] and renal cell carcinoma (RCC) [2-6]. However, the biological role of AR signaling and its potential as a target for treatment of RCC is not well understood and is of great interest to researchers.

Pili and his team at Indiana University utilized a range of technologies—including protein array, gene silencing, western blot, and immunofluorescence cell imaging—to identify the phosphorylation target within the AR protein that correlated with AR activation and to examine how alterations with key therapeutics could be used to effectively treat RCC [7]. Phosphorylation of AR by cyclin-dependent kinase 1 (CDK1) at serine 81 results in AR activation; inhibition of this phosphorylation decreases AR activity [8]. Sunitinib is a receptor tyrosine kinase inhibitor (RTKi) that is an effective treatment for RCC; however, in sunitinib-resistant cell models for RCC, AR expression and activity are elevated (Figure 1). To counter this resistance, Adelaiye-Ogala and coworkers treated the sunitinib-resistant cells with enzalutamide, an AR antagonist that acts by preventing androgen binding to AR as well as AR transcription. This antagonist also degrades any phosphorylated AR, restoring the sensitivity of RCC cells to sunitinib treatment.

This team also reported that resistance to sunitinib may result in an increase in CDK1, and that kinome alterations arising from constant inhibition by RTKi may lead to acquired resistance in RCC [9]. Because AR expression is observed to increase as cells become resistant to sunitinib treatment, they treated cells with a combination of enzalutamide and sunitinib. This drug cocktail proved to be more effective in inhibiting AR expression and reducing the observed tumor size than treatment with either drug alone. These results suggest that AR may be a key target for therapeutic drugs capable of mitigating a poor response to RTK inhibitors in RCC. ■

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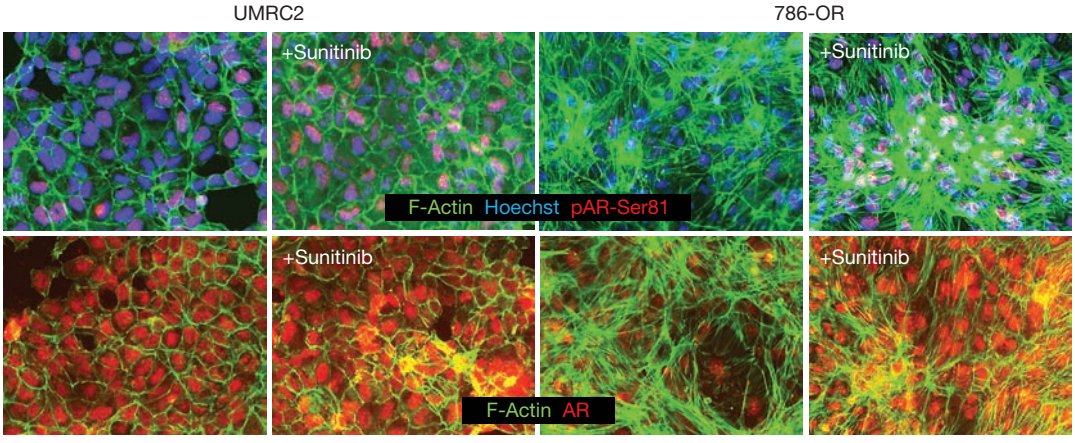
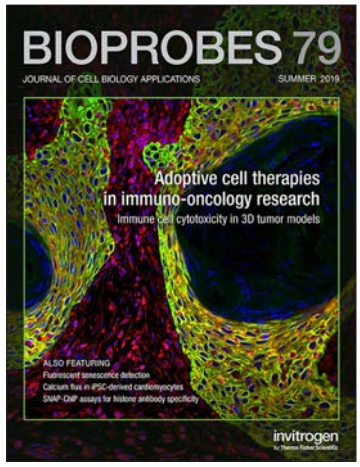


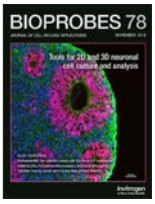
Figure 1. Increased sunitinib-induced AR expression is associated with activation of AR-targeted genes and increased AR phosphorylation. The renal cell carcinoma (RCC) cell line UMRC2 and the sunitinib-resistant cell line 786-OR were left untreated or exposed to sunitinib for 48 hr, and then immmunostained (after a 3–4 week sunitinib washout) for AR phosphorylated at serine 81 (pAR-Ser81) or for the AR C-terminal domain. Primary antibodies were visualized with fluorescent Invitrogen™ Alexa Fluor™ or FITC secondary antibody conjugates (red). F-actin and nuclei were stained with Invitrogen™ ActinGreen™ 488 ReadyProbes™ Reagent (green, Cat. No. 37110) and Hoechst™ 33258 (blue), respectively. Cells were analyzed using the Invitrogen™ EVOS™ FL Imaging System. Reprinted from Adelaiye-Ogala R, Damayanti NP, Orillion AR, Arisa S, Chintala S, Titus MA, Kao C, Pili R (2018) *Cancer Res* 78:2886–2896, with permission from AACR.



Cover image

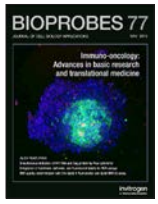
Hit the bull's eye. A human skin cancer section was probed with antibodies for keratin 14 and diaphanous 1, followed by detection with Invitrogen™ Anti–Mouse IgG Secondary Antibody, Alexa Fluor™ 488 (for keratin 14) and Invitrogen™ Anti–Rabbit IgG Secondary Antibody, Alexa Fluor™ 568 (for diaphanous 1). The section was counterstained with DAPI nucleic acid stain. The image was acquired using an Olympus™ FLUOVIEW™ FV1000 confocal laser-scanning microscope. Image contributed by Mohsin Bin Bashir, A*STAR Institute of Medical Biology (IMB), Singapore.

Previous issues



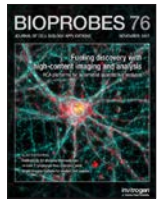
BIOPROBES 78

In this issue, we describe media, cultureware, and reagents for 2D and 3D neuronal cell culture and fluorescence imaging, as well as time-saving immunoassays such as Invitrogen™ Instant ELISA™ kits, ProQuantum™ kits, and ProcartaPlex™ multiplex panels. We also highlight the Thermo Scientific™ Multiskan™ Sky Microplate Spectrophotometer and the Invitrogen™ Attune™ NxT Autosampler for high-throughput assays.



BIOPROBES 77

This issue focuses on advances in immuno-oncology such as immune checkpoint inhibitors and T cell immunotherapy, and the use of stem cell differentiation models to verify antibody specificity. Also described are tools for flow cytometry and high-content imaging and analysis, as well as Invitrogen™ iWestern™ workflow instrumentation and the updated Invitrogen™ Qubit™ 4 Fluorometer and RNA IQ assay.



BIOPROBES 76

This issue highlights recent publications citing the use of Thermo Scientific™ high-content analysis (HCA) platforms. Also discussed are fluorescent probes for autophagy, antibody internalization, and low-density lipoproteins, as well as a 14-color T cell immunophenotyping flow cytometry panel and Invitrogen™ iBright™ Imaging Systems for western blot analysis.

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