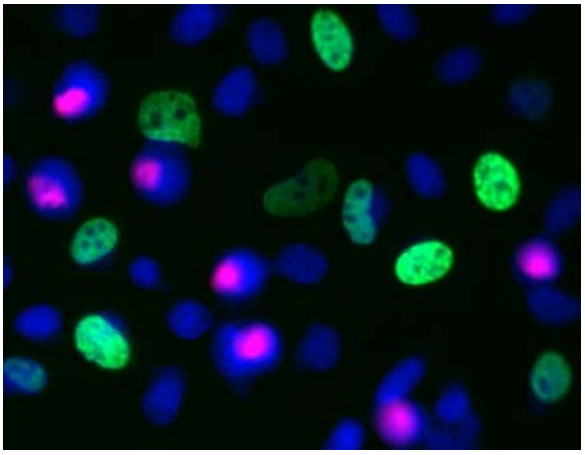
Technical Data Sheet

Cell Cycle Kit

Product Information

Catalog Number: **558662**
Size: 100 tests



Confocal image, using the BD Pathway™ 435 Bioimaging system and a 20x (0.75 NA) objective, of HeLa cells that were stained with the three kit components, Alexa Fluor® 488 Mouse anti-BrdU (pseudo-colored green), Alexa Fluor® 647 Rat anti-Histone H3 (pS28) (pseudo-colored red) and Hoechst 33342 (pseudo-colored blue). Co-staining of Hoechst 33342 and Histone H3 (pS28) appears pink.

Please Note:

For appropriate safety and disposal guidelines, refer to the MSDS.

The kit contains two parts, A and B, which are shipped separately.

Kit Contents and Storage

Upon arrival store Parts A and B as follows:

Part A, store at 4°C:

Description	# of vials	Component No.*
Bioimaging Certified Alexa Fluor® 488 Mouse anti-BrdU	1	51-9004981
Bioimaging Certified Alexa Fluor® 647 Rat anti-Histone H3 (pS28)	1	51-9004980
5x Fixation Buffer	1	51-9005210
Perm Buffer III	1	51-9004976
Stain Buffer (FBS)	1	51-9004979
PBS (10X) Concentrate	1	51-9004978
Hoechst 33342 Solution	1	51-9004975

Part B, store at -80°C:

Description	# of vials	Component No.*
DNase	5	51-2358KC
BrdU	1	51-2420KC

** Component numbers are provided for identification only. The components may not be ordered individually.*

BD Biosciences

bdbiosciences.com

United States	Canada	Europe	Japan	Asia Pacific	Latin America/Caribbean
877.232.8995	866.979.9408	32.2.400.98.95	0120.8555.90	65.6861.0633	55.11.5185.9995

For country contact information, visit **bdbiosciences.com/contact**

Introduction

The cell cycle consists of a series of processes involved in cell growth and replication. This coordinated division of cells can be separated into two major stages, interphase (the phase between mitotic events) and mitosis. There are three distinct, successive stages within interphase, namely G1, S, and G2 phases. During G1 (first gap), cells monitor their environment and grow (synthesize RNA and proteins). Once conditions are optimal, cells commit to DNA synthesis (S phase) and replicate their chromosomal DNA. The G2 phase (second gap) follows, when cells continue to grow and prepare for mitosis (division). The G2 gap allows time for the cells to complete DNA replication before initiating mitosis. During mitosis, the cell cycle is completed, giving rise to two daughter cells. Each daughter cell contains the same genetic material as the parent cell and approximately half of the parent's G2 level of cytoplasm. In addition to these specific stages, a G0 phase has been described for cells that exit from the cell cycle and enter a quiescent, non-dividing state. In response to external stimuli, cells may undergo reactivation and leave G0 to enter the G1 phase of the cell cycle. Another consequence of cellular activation may be the induction of programmed cell death (apoptosis).

Understanding the specific cell cycle phase is important when cells display heterogeneous responses to drugs, as is often the case in high-content screening applications. A combination of antibodies and dyes can be utilized to distinguish the S and M phases of the cell cycle.

The S phase can be identified using BrdU (an analog of the DNA precursor thymidine), which is incorporated into newly synthesized DNA in cells entering and progressing through S phase. The amount of BrdU incorporated is dependent upon the load time, rate of cell division, and stage of the cell within S phase (early, middle or late). Incorporated BrdU is detected by a fluorescently labelled mouse monoclonal antibody that specifically recognizes BrdU. BrdU analysis allows for the identification of cycling cells in asynchronous cell populations.

The M phase of the cell cycle can be identified with an antibody to phospho-Histone H3 (Histone H3 pS28). Histones are highly basic proteins that complex with DNA to form chromatin. Histone H3 is specifically phosphorylated at serine 28 during mammalian cell mitosis which coincides with chromosome condensation. Using these two antibodies, various time points and/or drug treatments can be examined to determine cell cycle kinetics.

This kit utilizes antibodies that are directly conjugated with fluorochomes that fluoresce in the green (Alexa Fluor® 488) and red (Alexa Fluor® 647) channels. This allows for an additional reagent in the orange (for example, Alexa Fluor® 555) channel to be multiplexed with the kit antibodies (see Sample Data). For a list of Bioimaging Certified Alexa Fluor® 555 conjugated primary antibodies please visit: http://www.bdbiosciences.com/bioimaging

Application Notes

Application

Bioimaging	Routinely Tested

Suggested Companion Products

Catalog Number	Name	Size	Clone
353219	Falcon® 96-well Imaging Plate	1 box	(none)
353962	Falcon® 384-well Imaging Plate	1 box	(none)

Falcon® is a registered trademark of Corning Incorporated.

Storage Conditions for Individual Kit Components

Once the kit has been opened, individual components can be stored at the following temperatures.

Component	Storage
Alexa Fluor® 488 Mouse anti-BrdU	4°C
Alexa Fluor® 647 Rat anti-Histone H3 (pS28)	4°C
5× Fixation Buffer	4°C
Perm Buffer III	-20°C
Stain Buffer (FBS)	4°C
BrdU	4°C
PBS (10X) Concentrate	4°C
DNase	-80°C
Hoechst 33342 Solution	4°C

All frozen components should be thawed at room temperature.

The components are sufficient to stain either one 96-well or one 384-well plate.

Read entire protocol prior to beginning experiments.

Alexa Fluor® 488 Mouse anti-BrdU is supplied as a bulk that requires 1:10 dilution in Stain Buffer (FBS). It is recommended to dilute the antibody immediately prior to use.

Alexa Fluor® 647 Rat anti-Histone H3 (pS28) is supplied as a bulk that requires 1:10 dilution in Stain Buffer (FBS). It is recommended to dilute antibody immediately prior to use.

5× Fixation Buffer is a formaldehyde solution used to fix the cells after loading with BrdU. The solution should be warmed to 37°C before use. For appropriate disposal of formaldehyde containing solutions, follow institutional guidelines.

Perm Buffer III is a methanol solution used for permeabilizing the cells post-fixation.

Stain Buffer (FBS) is used for blocking cells post-permeabilization and for diluting the antibody conjugates.

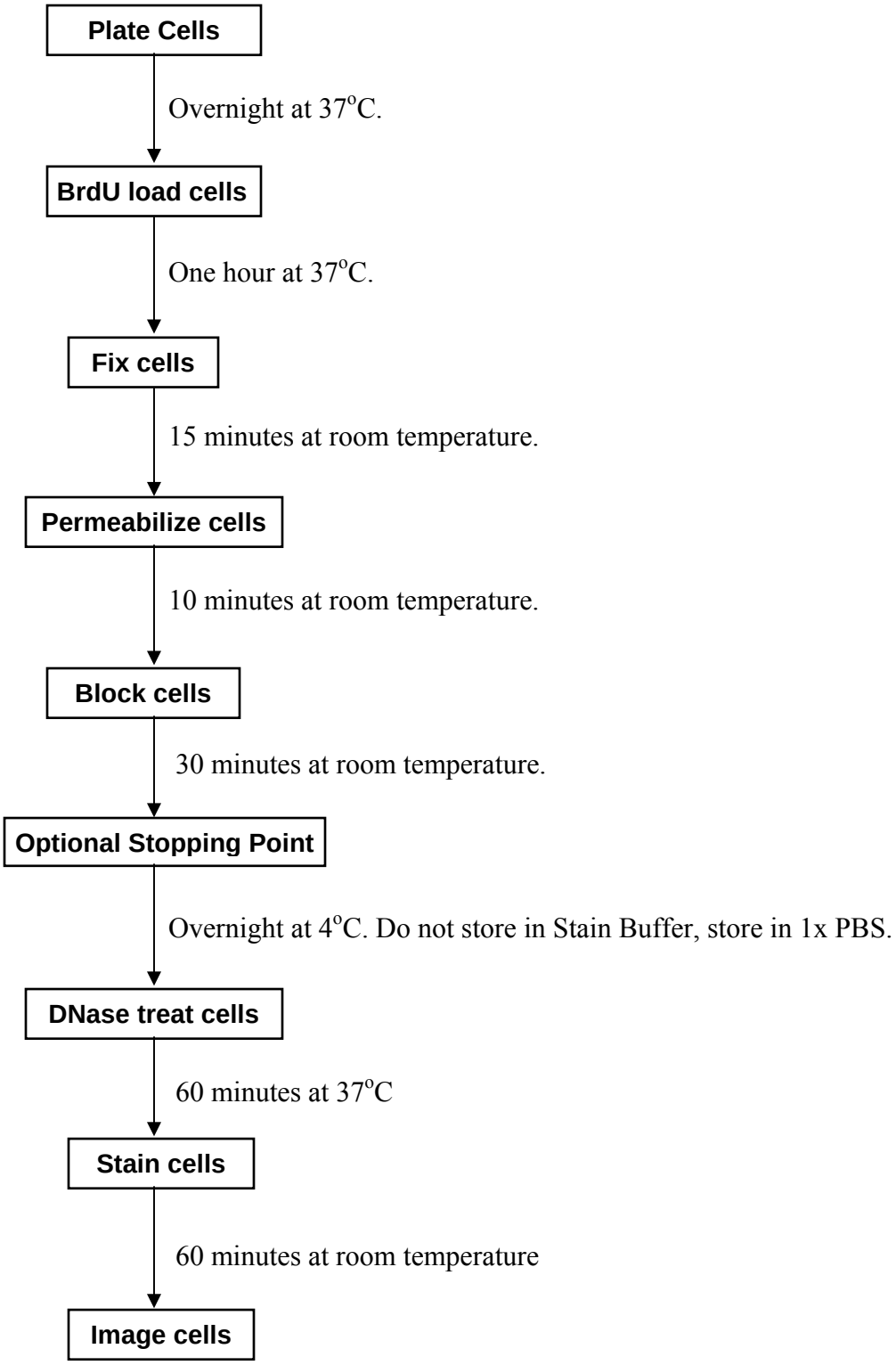
BrdU (10 mg/ml) is provided as a 0.22-micron filtered solution that contains no preservatives. **HANDLE UNDER ASEPTIC CONDITIONS.** Once thawed, the reagent is stable at 4°C for one year. Dilute the BrdU stock solution to 32 µg/ml in culture media immediately prior to use.

PBS (10X) Concentrate should be diluted 1:10 in sterile water. The resulting 1× PBS is used to dilute the DNase and to wash the plate between staining steps. 1× PBS should be stored at 4°C.

DNase (1 mg/ml) is provided in five 300-µl aliquots of a 1 mg/ml solution in 1× PBS. Undiluted DNase may be refrozen twice before losing activity. DNase should be diluted to 0.3 mg/ml in 1× PBS immediately prior to use.

Hoechst 33342 Solution (1 mg/ml) is provided to stain nuclei and thereby allow for the identification of cells in the field of view. The dye is specific for DNA. Because the solution may be located in the cap of the vial, it is recommended to do a quick spin of the vial (to ensure that the contents are spun to the bottom of the tube) prior to opening. Hoechst 33342 solution should be diluted to 2 µg/ml in 1× PBS immediately prior to use.

Flow chart of Cell Cycle Analysis procedure



Cell Cycle Analysis Protocol

NOTE: The recommended volumes of reagents are for cells plated in a 96-well plate. For a 384-well plate, use the volumes appearing in parentheses.

Calculations are given for preparation of reagents for an entire plate and also on a per well basis in case an entire plate is not being processed at one time. The volume of reagent prepared for an entire plate is for 100 (400) wells to allow some overage if reagent reservoirs are used.

Negative Control

Negative controls are recommended for establishing background staining intensities. Recommended negative controls include unstained cells and cells that are stained but without prior BrdU loading.

Plate Cells

Approximately 18 hours prior to BrdU loading, plate cells in 100 µl of medium per well in a 96-well bioimaging plate (Cat. No. 353219) or in 25 µl of medium per well in a 384-well (Cat. No. 353962) black-walled plate. Optimal cell concentrations will vary depending on the cell types used. We routinely plate HeLa, A549, and U-2 OS cells at 10×10^3 cells/well in a 96-well plate in 100 µl of culture medium or 2.5×10^3 cells/well in a 384-well plate in 25 µl of culture medium. The cells are cultured in a humidified incubator at 37°C in the presence of 5% CO_2.

Prepare the BrdU loading solution

For one plate a total volume of 5 ml is required. On a per well basis 50 µl (12.5 µl) is required. For an entire plate add 16 µl of the thawed BrdU stock solution to 4984 µl of cell culture medium and mix well; on a per well basis add 0.16 µl (0.04 µl) of the thawed BrdU stock solution to 49.84 µl (12.46 µl) of cell culture medium and mix well. The final BrdU concentration equals 104 µM.

Prepare 1x PBS Buffer

For one plate a total volume of 120 ml of 1x PBS is required for all washes and dilution of reagents. On a per well basis 1.2 ml (0.3 ml) is required. For an entire plate, add 12 ml of 10x PBS to 108 ml of sterile distilled water and mix well; on a per well basis add 120 µl (30 µl) of 10x PBS to 1.08 ml (270 µl) of sterile distilled water and mix well.

BrdU load cells

Remove the tissue culture plate from the incubator. Add 50 µl (12.5 µl) of the diluted BrdU loading solution per well, excluding negative control well(s). The solution is added directly to the medium from the overnight culture. Incubate for 1-2 hrs at 37°C. This load time is sufficient for cells with a doubling time of 24 to 48 hours (such as HeLa, A549, and U-2 OS cells). A longer load may be needed for cells with longer doubling times. It is up to each investigator to determine the optimal load time for the specific cells being used.

Fix cells

The cell fixation buffer is provided as a 5× concentrate. This allows users to add fixative directly to the wells to obtain a 1× concentration and avoid losing cells that may be less tightly adhered (M phase or apoptotic cells). Cells may lift off the plate if cold reagents are added directly to the wells, therefore the fixative must be pre-warmed. For an entire plate the whole bottle of 5x fixation buffer should be warmed to 37°C prior to use; on a per-well basis prepare 37.5 µl (9.375 µl).

After incubation with the BrdU solution, add 37.5 µl of pre-warmed 5× fixation buffer per well (150 µl volume in well prior to addition) of a 96-well plate (15.6 µl pre-warmed 5× fixation buffer per well (37.5 µl volume in well prior to addition) of a 384-well plate) and incubate for 15 minutes at room temperature.

Discard the fixation buffer and wash the wells twice with 100 µl (25 µl) of 1× PBS per well.

Permeabilize cells

Discard the 1× PBS from the wells after the second wash. Add 100 µl (25 µl) per well of ice-cold Perm Buffer III (stored at -20°C) and incubate for 10 minutes at room temperature. Discard the Perm Buffer III and wash the wells twice with 100 µl (25 µl) of 1× PBS per well.

Block cells

Discard the 1× PBS from the wells after the second wash. Add 100 µl (25 µl) of Stain Buffer (FBS) to each well and incubate for 30 minutes at room temperature. Discard the Stain Buffer (FBS) and wash the wells once with 100 µl (25 µl) of 1× PBS. Add 100 µl (25 µl) of 1× PBS to each well.

Note: If the procedure can not be completed in one day, this is the recommended stopping point. Store cells overnight at 4°C in 1× PBS and continue with the DNase treatment on the following day.

DNase treat cells

To detect the BrdU incorporated into the DNA chains, the DNA must be denatured to allow the anti-BrdU antibody access to the incorporated BrdU. DNA denaturization is accomplished using DNase. Thaw the appropriate number of DNase vials. Each vial contains sufficient DNase to denature 20 wells of a 96-well plate or 80 wells of a 384-well plate. For an entire plate pool the contents of the 5 vials and add 1.5 ml of DNase to 3.5 ml of 1x PBS; on a per well basis dilute 15 µl (3.75 µl) of the DNase stock solution in 35 µl (8.75 µl) of 1× PBS. Remaining DNase stock can be refrozen and used at a later time. Incubate the plate for one hour at 37°C. Discard the DNase solution and wash the wells once with 100 µl (25 µl) of 1× PBS.

Stain cells

Dilute the conjugated antibody stocks 1:10. For an entire plate add 500 µl of Alexa Fluor® 488 Mouse anti-BrdU and 500 µl of Alexa Fluor® 647 Rat anti-Histone H3 (pS28) to 4 ml of Stain Buffer (FBS); on a per well basis add 5 µl (1.25 µl) of Alexa Fluor® 488 Mouse anti-BrdU and 5 µl (1.25 µl) of Alexa Fluor® 647 Rat anti-Histone H3 (pS28) to 40 µl (10 µl) of Stain Buffer (FBS). Discard the 1× PBS from the wells and add 50 µl (12.5 µl) of the diluted antibody cocktail to each well, excluding the negative control well(s) to which 1× PBS should be added. Incubate in the dark for one hour at room temperature. Wash the cells twice with 100 µl (25 µl) of 1× PBS. Prepare Hoechst 33342 staining solution: For an entire plate add 20 µl of Hoechst 33342 Solution to 9.98 ml 1× PBS; on a per well basis add 0.2 µl (0.05 µl) Hoechst to 99.8 µl (24.95 µl) of 1x PBS. Add 100 µl (25 µl) per well and incubate in the dark for 15 minutes at room temperature. The stained cells are now ready to be imaged. Sealed plates can be stored in the dark at 4°C and should be imaged within 24 hours.

Note: If nuclear staining with DAPI is preferred, then substitute DAPI (1 µg/ml) for Hoechst at this stage.

Imaging cells and data analysis

Stained cells can be imaged on the BD Pathway™ 855 or 415/435 Bioimager Systems or on other imaging systems or fluorescent microscopes. The filter sets used for the fluorescent dyes on BD Pathway™ systems are shown in Tables 1 and 2. Cell populations are analyzed for nuclear fluorescence intensity using Pathway software tools. Users of other imaging platforms should consult the system's users manual for filter selection and analysis methods.

Table 1. BD Pathway 855 Bioimager Filter Sets

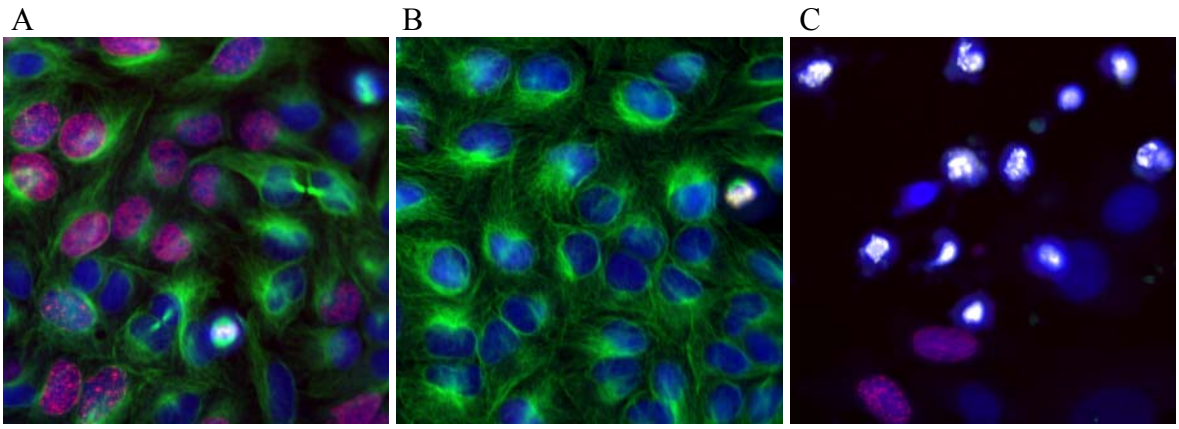
Dye	Excitation	Emission	Dichroic
Alexa Fluor® 488	488/10	515LP	Fura/Fitc
Alexa Fluor® 647	560/55	645/75	595LP
Hoechst Dye 33342	360/10	435LP	400DCLP

Table 2. BD Pathway 415/435 Bioimager Filter Sets

Dye	Excitation	Emission	Dichroic
Alexa Fluor® 488	482/35	536/40	FF506
Alexa Fluor® 647	628/40	692/40	FF660
Hoechst Dye 33342	377/50	FF409	435LP

Sample Data

U-2 OS cells were either left untreated, were treated with aphidicolin (250 ng/ml) for an hour, or were treated with colchicine (500 ng/ml) for 16 hours prior to BrdU loading. Aphidicolin, a DNA polymerase inhibitor, blocks cells in early S phase while colchicine, a microtubule polymerization inhibitor, blocks cells in M phase. Cells were stained as described in the protocol. However, in addition to the two antibodies provided in the kit, an Alexa Fluor® 555 mouse anti-β-tubulin (Cat. No. 558608) was used at the same time to monitor the depolymerization of microtubules. Representative 20x images are shown in the figure below. Hoechst staining is pseudo colored blue, BrdU staining is pseudo colored red (appears pink when co-localized with the blue), Histone H3 (pS28) staining is pseudo colored yellow (appears white when co-localized with the blue) and β-tubulin staining is pseudo colored green. Analysis of the images (data not shown) revealed that the untreated U-2 OS cells (Panel A) had approximately 30% cells in S phase (pink) and approximately 3% cells in M phase (yellow/white). Aphidicoline treatment (Panel B) blocked cells from entering S phase but there was no effect on β-tubulin or on cells entering M phase. Conversely, no β-tubulin staining (cytoplasmic intensity was measured using an 8 pixel ring dilated out from the nuclear mask identified by the Hoechst stain) was detected when cells were treated with Colchicine (Panel C) and the percent cells in M phase increased to 50%.



Danger: *5X Fixation Buffer (component 51-9005210) contains 19.1% formaldehyde (w/w) and 6.71% methanol (w/w).*
Hazard statements
Combustible liquid.
Harmful if swallowed or in contact with skin.
Toxic if inhaled.
Causes skin irritation.
Causes serious eye damage.
May cause an allergic skin reaction.
Suspected of causing genetic defects.
May cause cancer. Route of exposure: Inhalative.
May cause damage to organs. May cause respiratory irritation.
Precautionary statements
Wear protective gloves / eye protection.
Wear protective clothing.
Do not breathe mist/vapours/spray.
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell.

Danger: *Perm Buffer III (component 51-9004976) contains 87.68% methanol (w/w).*
Hazard statements
Highly flammable liquid and vapor.
Toxic if swallowed, in contact with skin or if inhaled.
Causes damage to the central nervous system. Route of exposure: Oral.
Precautionary statements
Keep away from heat/sparks/open flames/hot surfaces. - No smoking.
Wear protective clothing / eye protection.
Wear protective gloves.
Do not breathe mist/vapours/spray.
IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.
IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Troubleshooting Guide

Problem	Suggested Explanations and Solutions
No/weak signal for BrdU staining	1. Improper reagent storage – refer to manual. 2. Incorrect incubation time – refer to manual. 3. Incorrect dilution – refer to manual. 4. BrdU load omitted – refer to manual. 5. Load time too short or long – refer to manual. 6. No DNase treatment – refer to manual. 7. Improper DNase storage – refer to manual. 8. Incorrect filter set used – refer to manual. 9. Exposure too low – check exposure on positive control wells 10. Plate stored for too long prior to imaging – refer to manual
No/weak signal for Histone H3 (pS28) staining	1. Improper reagent storage – refer to manual. 2. Incorrect incubation time – refer to manual. 3. Incorrect dilution – refer to manual. 4. Too few cells imaged – percent cells in M phase in normal populations is usually low, image sufficient number of cells. 5. Incorrect filter set used – refer to manual. 6. Exposure too low – check exposure on positive control wells
No/weak signal for Hoechst 33342 DNA staining	1. Improper reagent storage – refer to manual. 2. Incorrect incubation time – refer to manual. 3. Improper dilution – refer to manual. 4. Hoechst 33342 dye not added – refer to manual. 5. Incorrect filter set used – refer to manual. 6. Exposure too low – check exposure on positive control wells
High Background	1. Inadequate washing – refer to manual. 2. Antibody not diluted – refer to manual. 3. Cells stored in stain buffer - refer to manual. 4. Exposure too high – check exposure on positive control wells
Loss of cells	1. Cytotoxic effect of compound treatment – microscopically inspect wells before fixation to assess cell density, modify compound concentration as needed. 2. Inadequate fixation – refer to manual. 3. Sheer stress from dispensing or aspiration of reagents – avoid directing the flow from the pipette vertically down into the well, flow all reagents down the side walls of the well. Use gentle flow rates for all reagent additions. Aspirate solutions gently using a manual pipette. 4. Improper setting on automated plate washer – make sure the plate washer has been set up and tested to process cells appropriately (especially after compound treatment).

Technical Support

For image and data analysis support please contact BD Biosciences Bioimaging Technical Support US 1-800 245-2614, International 301 340-7320.

For reagent support please contact BD Biosciences Technical Support US, International.

Product Notices

1. This product is sold under license from Shigei Medical Research Institute, Okayama, Japan.
2. The Alexa Fluor®, Pacific Blue™, and Cascade Blue® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc. for research use only, excluding use in combination with microarrays, or as analyte specific reagents. The Alexa Fluor® dyes (except for Alexa Fluor® 430), Pacific Blue™ dye, and Cascade Blue® dye are covered by pending and issued patents.
3. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
4. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.