EZ-BrdU™ Kit

A Complete Kit for Measuring Apoptosis by Flow Cytometry

TNB-6600-KIT

Description

The EZ-BrdU™ Kit provides a two color staining method for measuring cell proliferation by multiparameter flow cytometric analysis of DNA replication and cellular DNA content/cell cycle position.

Contents

- BrdU solution for incorporation into cells actively synthesizing DNA
- Washing and rinsing buffers for processing individual steps in the assay
- Denaturation and neutralization buffers for making the incorporated BrdU accessible for labeling
- Fluorescein labeled anti-BrdU (PRB-1) for detection of incorporated BrdU
- Propidium iodide/RNase A solution for counter staining the total DNA
- Positive control cells to confirm the performance of the assay

The reagent bottles have colored caps to aid in their identification. Sufficient reagents are provided to process 50 cell suspensions and an additional 5 Positive Controls. The Positive Controls are provided at approximately 1 x 10^6 cells per mL in 70% (v/v) ethanol. The control cells are derived from a human lymphoma cell line that has been fed BrdU and have been fixed as described in this manual.

Component	Color Code	Part Number	Volume	Storage
Positive Control Cells	White Cap	TNB-6600-NC12	5.0 mL	-20° C
BrdU Solution	Pink Cap	TNB-6600-BP13	2.0 mL	-20° C
Wash Buffer	Blue Cap	TNB-6600-WB15	175.0 mL	2-8° C
Rinse Buffer	Red Cap	TNB-6600-RB17	130.0 mL	2-8° C
Denaturation Buffer	Clear Cap	TNB-6600-DB16	65.0 mL	2-8° C
Neutralization Buffer	Green Cap	TNB-6600-NB14	65.0 mL	2-8° C
FITC anti-BrdU (PRB-1)	Orange Cap	TNB-6600-FM20	325 µL	2-8° C
PI/Rnase Staining Buffer	Amber Bottle	TNB-6600-PR18	23.5 mL	2-8° C

Reagents and Materials Required, but not supplied:

- Flow cytometer capable of measuring red and green fluorescence.
- Distilled water
- 70% (v/v) ethanol
- Ice bucket
- 12 x 75 mm flow cytometry polystyrene test tubes
- Pipets and pipetting aids

Shipping

The EZ-BrdU™ Kit is shipped in one container which houses two packages. One package is provided at ambient temperature and should be stored at 2-8° C upon arrival. The other is packaged in a styrofoam container with frozen ice packs and the contents should be stored at -20° C upon receipt. We have determined the shipping method is adequate to maintain the integrity of the kit components. Upon arrival, store the reagents at the appropriate temperatures.

Precautions and Warnings

The components of this kit are for ***research use only*** and are not intended for diagnostic procedures.

The EZ-BrdU™ Kit (TNB-6600-KIT) contains the components TNB-6600-NC12, TNB-6600-WB15, TNB-6600- RB17 and TNB-6600-PR18 which contain 0.05% (w/v) sodium azide as a preservative. *These materials are harmful if swallowed; avoid skin contact, wash immediately with water.* See Material Safety Data Sheets.

The component TNB-6600-DB16 contains 2N HCL. *This material is harmful if swallowed; avoid skin contact; wash immediately with water.* See Material Safety Data Sheets.

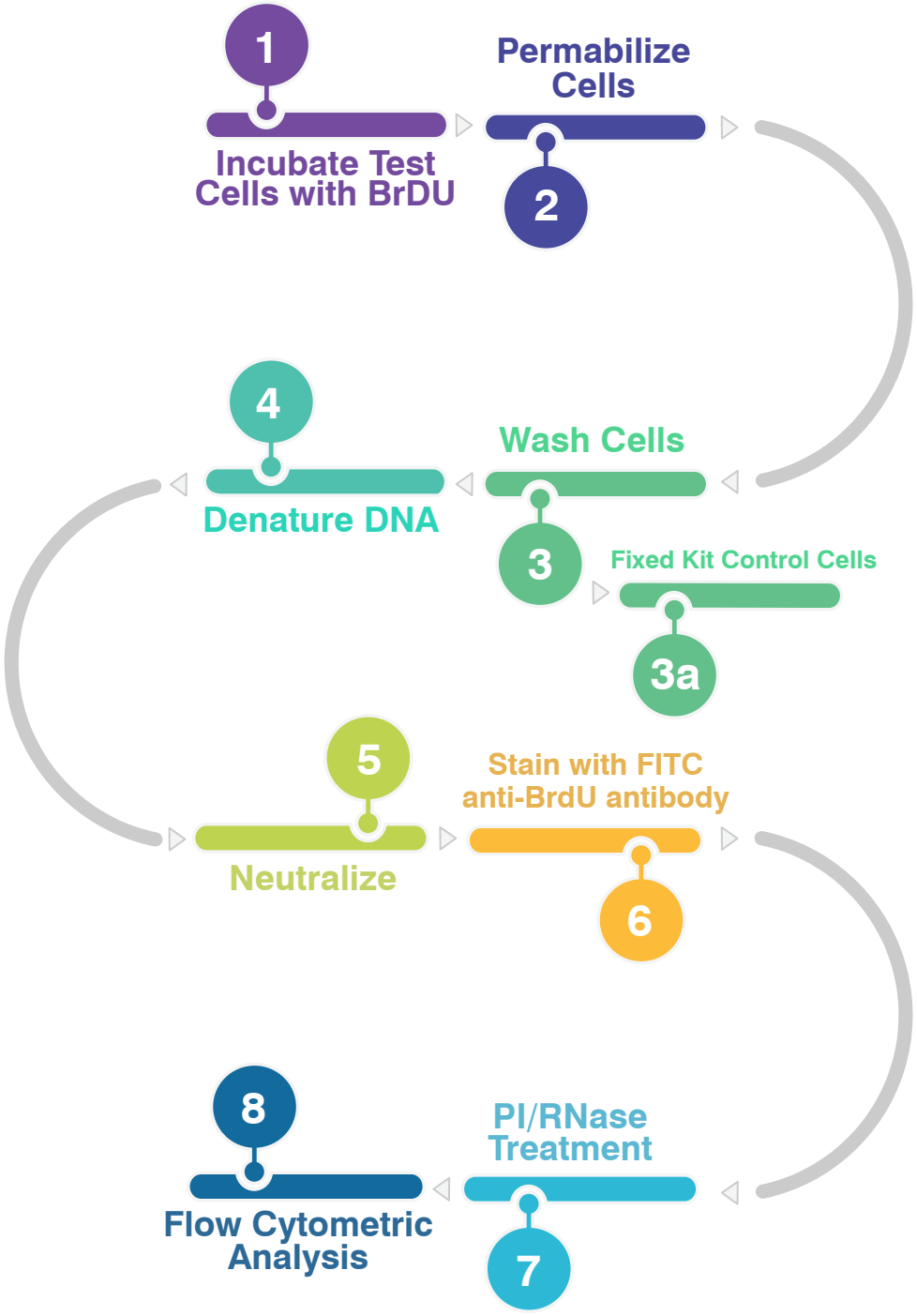
Assay Principle

The incorporation of BrdU into newly synthesized DNA by actively cycling cells is one method for measuring the changing amount of cellular DNA during cell proliferation through each of the cell cycle phases. As a thymidine analog, BrdU is preferentially incorporated into newly replicated DNA which can then be subsequently detected and analyzed to determine relative DNA content and cell cycle position.

Incorporation of BrdU is most commonly detected using anti-BrdU antibodies. This requires that cellular DNA be denatured in order for the BrdU epitope to be accessible to the antibody. This can be accomplished through heat treatment, acid treatment, enzymatic digestion or UV light exposure and each method has advantages and disadvantages.

The EZ-BrdU Kit employs an acid denaturation step and the low acid method used helps reduce damage to other cellular proteins. After the denaturation step, cells are stained with a FITC anti-BrdU antibody and total DNA is counterstained with a PI/RNase A solution. Two color flow cytometry can then be used to analyze cells that have incorporated BrdU (proliferating cells) in terms of their cell cycle position (G0/1, S, or G2/M phase).

EZ-BrdU™ Cell Proliferation Assay Workflow



Labeling of Cells with BrdU

Labeling of newly synthesized cellular DNA requires prolonged exposure of actively cycling cells to BrdU. There are many different protocols available and the methods described in this manual are intended to be used as guidelines. Determine the doubling time of the test cell population at least a day ahead of beginning the experiment in order to optimize pulse labeling times. As a negative control, cells from the same population that are not BrdU-pulsed can be used to determine background staining levels.

Labeling Cells in Culture

On the day of the experiment, add the BrdU solution at a point prior to the completion of the synthesis phase (S-phase) of the cycle. The cells should be in an exponential growth phase for a minimum of two passages before beginning the experiment. Take care when adding the BrdU solution so as not to disturb the cells and disrupt their cycling time.

Add 20 µL of the BrdU stock solution *(TNB-6600-BP13, pink cap)* per 10 mL of cell culture medium. Incubate the cells at 37° C in a CO_2 incubator for the desired length of time, as previously determined. An actively proliferating cell line may require a 20-40 minute incubation, while slow growing cells could require incubation times of up to 36 hours. For this reason, investigators should determine the optimal BrdU pulse duration for each cell system under investigation.

Labeling Cells *in vivo*

There are two common methods for BrdU labeling of cells in a live mouse or rat. Intraperitoneal injection of a BrdU solution, or ingestion of BrdU that has been added to drinking water.

- To label through injection, use 334 µL (1 mg) of BrdU solution and inject intraperitoneally. Incorporated BrdU can be detected in the bone marrow and elsewhere within as little as 1 hour post injection.

- To label through ingestion, add 300 µL of BrdU solution per mL of drinking water. Water should be prepared fresh daily and feeding should extend over the course of a full week for best incorporation with little associated toxicity.

Cell Fixation Procedure

After cells have been grown in the presence of the BrdU solution, they must be fixed to permeabilize the membranes. This allows the anti-BrdU antibody access to the nucleus of the cell. A convenient method to accomplish this is with 70% ice cold ethanol. Once cells are fixed in ethanol, they may be stored at -20° C until ready to use.

Permeabilization and Fixation Procedure

1. Centrifuge the cells for 5 minutes (300 x g) and remove the supernatant by aspiration.
2. Resuspend the cells at a concentration of 1 to 5 x 10^6 cells / mL in Wash Buffer *(blue cap)*.
3. Repeat #1 above.
4. Resuspend the cell pellet in the residual Wash Buffer (blue cap) left after aspiration by gently vortexing the tube.
5. Adjust the cell concentration to 1 to 2 x 10^6 cells/mL in 70% (v/v) ice cold ethanol.
6. Store cells in 70% ethanol at -20° C over night or until ready to use (after at least 18 hours)

Technical Tips

- To minimize cell loss during the assay, we recommend using a single 12 x 75 mm polystyrene flow cytometry tube per sample throughout the staining procedure and analysis.
- An electrostatic charge can build up on the sides of the tube causing cells to adhere to the tube wall.
- The sequential use of multiple tubes can result in significant cell loss during the assay.
- We also recommend that care is taken throughout the staining procedure to wash cells from the side of the tube.
- Cells can also be lost through the use of pipetting for mixing steps, as cells can adhere to the plastic pipette tips.

EZ-BrdU™ Protocol

The following protocol describes the method for measuring cell proliferation in the positive control cells that are provided in the EZ-BrdU™ Kit. The same procedure should be employed for measuring cell proliferation in the cells that have undergone fixation and permeabilization by the researcher.

1. Resuspend the positive (white cap) control cells by swirling the vial. Remove a 1 mL aliquot of the control cell suspension (approximately 1 x 10^6 cells per 1 mL) and place in 12 x 75 mm centrifuge tubes. Centrifuge (300 x g) the control cell suspension for 5 minutes and remove the 70% (v/v) ethanol by aspiration, being careful to not disturb the cell pellet.
2. Resuspend the tube of control cells with 1 mL of Wash Buffer (blue cap) per tube. Centrifuge as before and remove the supernatant by aspiration.
3. Repeat the Wash Buffer treatment (#2 above).
4. Resuspend the cell pellet in 1 mL of the Denaturation Buffer (**clear** cap).
5. Incubate 30 minutes at room temperature.
6. Centrifuge cells for 10 minutes at 400 x g.
7. Aspirate supernatant and immediately add 1 mL of Neutralization Buffer (green cap).
8. Centrifuge cells for 10 minutes at 400 x g.
9. Wash cells one time with 2 mL Rinse Buffer (red cap).
10. Resuspend the cell pellet in 0.1 mL of the Antibody Solution (prepared as described below).
11. Incubate the cells with Antibody Solution in the dark for 60 minutes at room temperature.

Antibody Solution	1 Assay	5 Assays	10 Assays
FITC anti-BrdU (orange cap)	5.00 µL	25.00 µL	50.00 µL
Rinse Buffer (red cap)	95.00 µL	475.00 µL	950.00 µL
Total Volume	100.00 µL	500.00 µL	1000.00 µL

12. Add 0.5 mL of the PI/RNase A solution (amber bottle) to the tube containing the antibody solution.
13. Incubate the cells in the dark for 30 minutes at room temperature.
14. Analyze the cells in the PI/RNase A Solution by flow cytometry.
15. Analyze the cells within 3 hours of staining.

Flow Cytometric Analysis - Cytek® Aurora

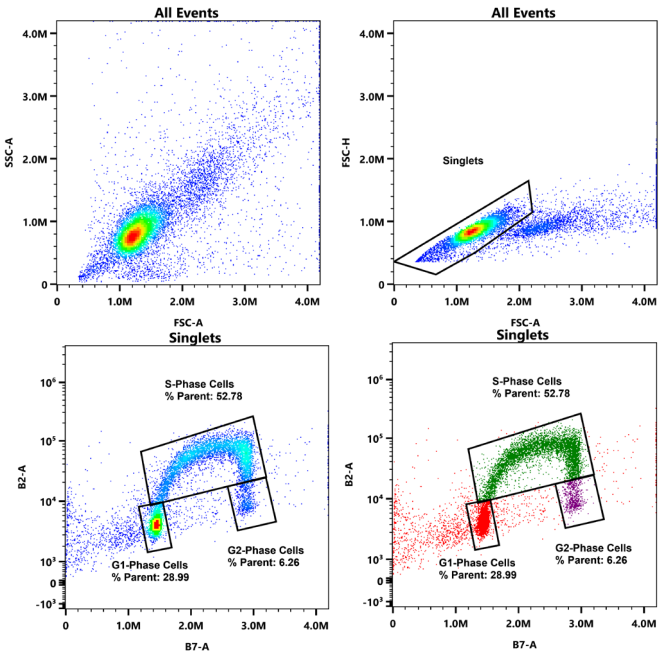
Data from this assay was acquired on a Cytek® Aurora platform with a 488 nm laser. Initial scatter plot (FCS-A vs SSC-A) allowed for on-scale positioning of the cells of interest. For doublet discrimination, single cells were gated using a FSC-A vs FSC-H plot. Alternatively, a DNA content approach can also be used. Singlets are identified in a B7-W vs B7-A plot.

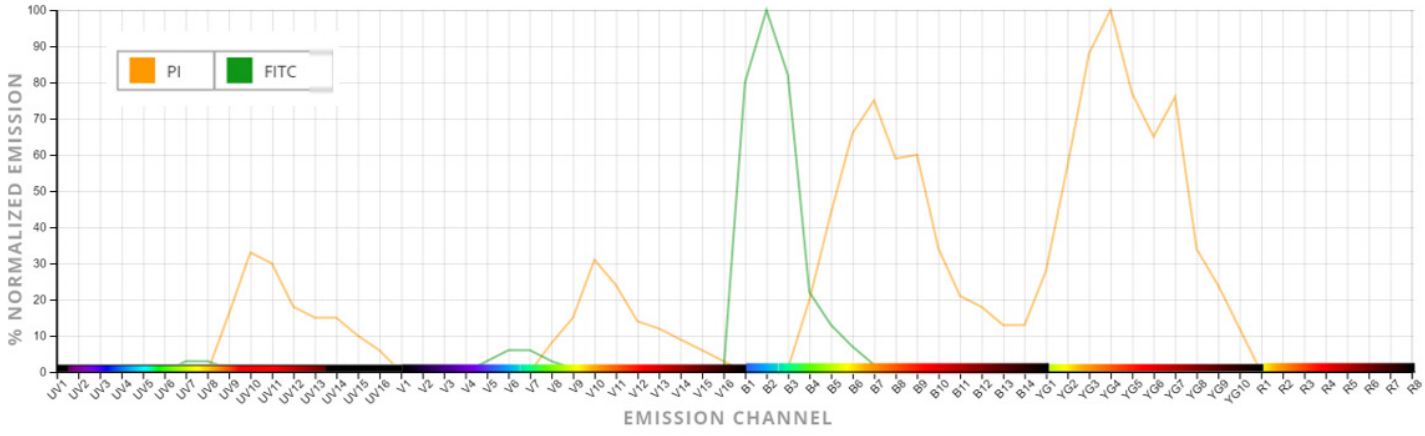
Total cellular DNA stained with propidium iodide peaks in the B7 channel. The fluorescence of fluorescein-conjugated anti-BrdU antibody has maximal emission that is detected by the B2 channel.

In the dual fluorescent parameter plots, B7-A (PI) vs B2-A (FITC), both cell cycle phases and cycling cells are distinguishable. These are shown as pseudocolor density plots density or as dot plots.

To acquire data on the Cytek Northern Lights or Aurora platforms, download the experiment template from cytekbio.com. For use with SpectroFlo 3.0 installed, select the file “EZ BrdU Template 3.0.zip”. For users with Spectroflo 2.2 installed, select” EZ BrdU Template 2.2.zip” and “EZ BrdU Settings. UST”.

Import this setting into Spectroflo and select it for acquisition. Acquire samples at Low flow rate, and collect a minimum of 10,000 singlet events. No spectral unmixing is required to accurately visualize the data. Gates may need to adjusted, however.





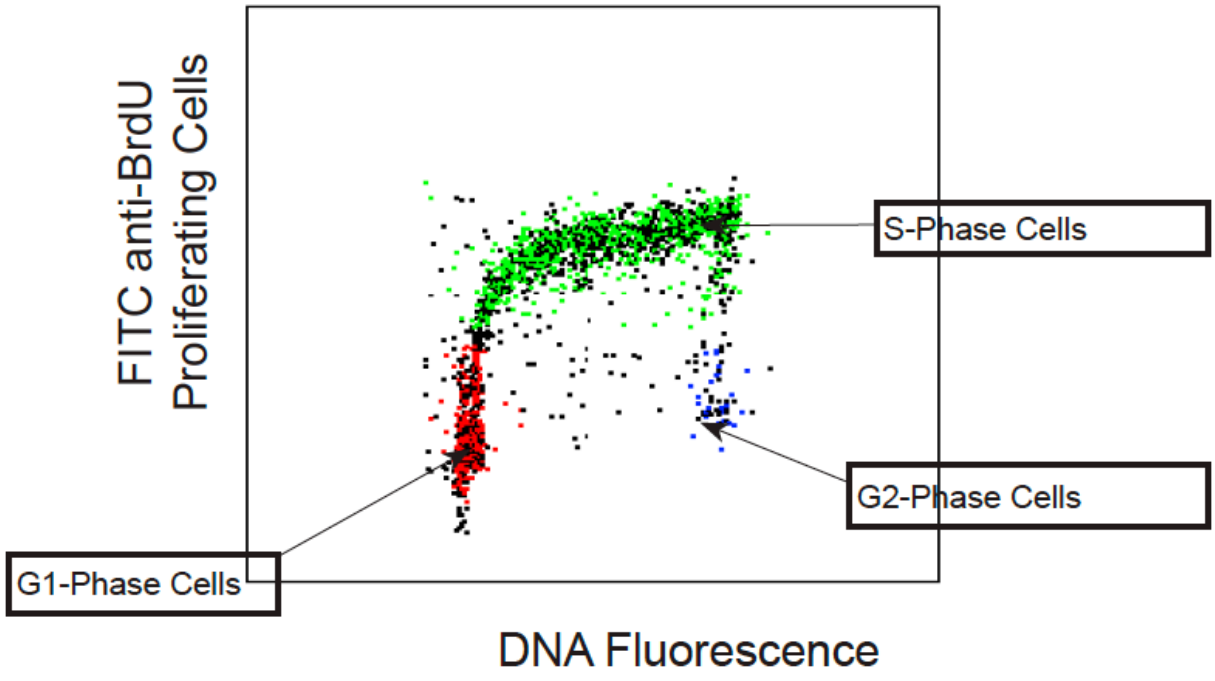
Flow Cytometric Analysis

This assay is run on a flow cytometer equipped with a 488 nm Argon laser. Propidium Iodide (total cellular DNA) and Fluorescein (proliferating cells) are the two dyes being used. Propidium Iodide (PI) fluoresces at about 623 nm and FITC at 520 nm when excited at 488 nm. No fluorescence compensation is required. Two dual parameter and two single parameter displays are created with the flow cytometer data acquisition software.

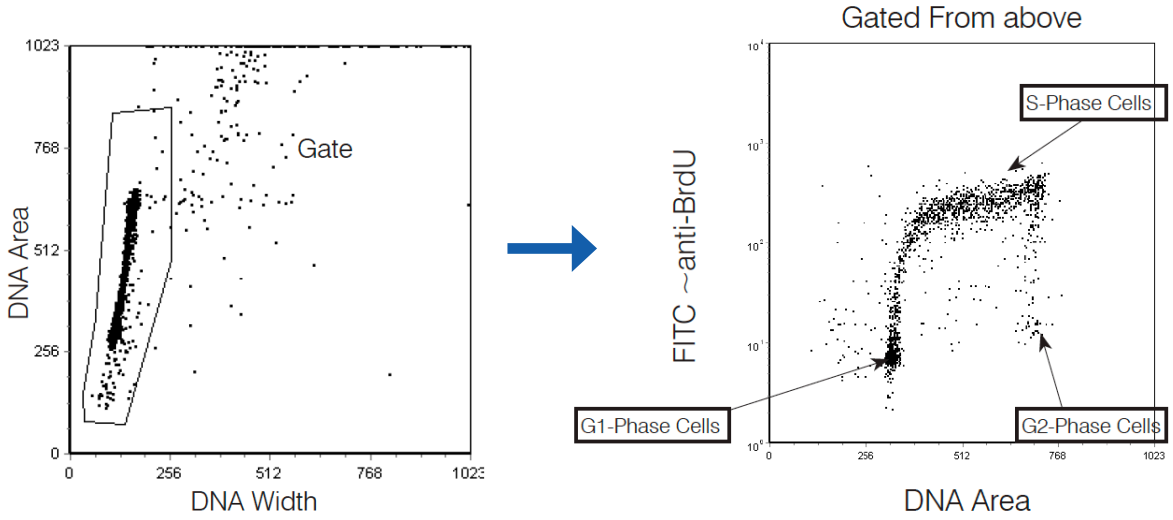
The gating display should be the standard dual parameter DNA doublet discrimination display with the DNA Area signal on the Y-axis and the DNA Width (Becton-Dickinson, Figure 4) or DNA Peak/Integral (Beckman Coulter, Figure 5) signal on the X-axis. From this display, a gate is drawn around the non-clumped cells and the second gated dual parameter display is generated.

The normal convention of this display is to put DNA (Linear Red Fluorescence) on the X-axis and the FITC anti-BrdU (Log Green Fluorescence) on the Y-axis (see bottom plot, Figure 4). Two single parameter gated histograms, DNA and FITC anti-BrdU, can also be added but are not necessary.

By using the dual parameter display method, not only are cycling cells resolved but the total cell cycle is displayed. The dual parameter histograms of the control cells should look like Figure 3 below.



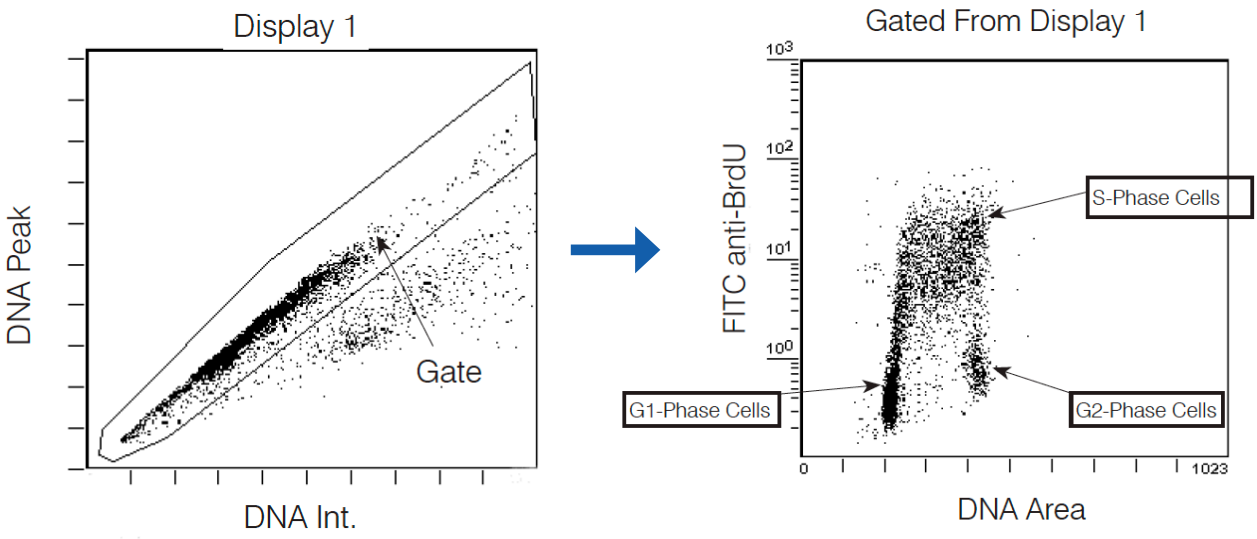
Flow Cytometer Setup for Becton Dickinson Hardware



Typical FACSCaliber™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	400 Volts
FL 3	1.46	430 Volts
FL 3 Width	.87	
FL 3 Area	3.25	
	Threshold- FL 3, 40	

Flow Cytometer Setup for Beckman Coulter Hardware



Typical XL™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	589 Volts
FL 3	2.00	698 Volts
AUX(FL3 Peak)	1.00	250 Volts

Discriminator-AUX (FL3 Peak)

Need techincal support?

Please reach out to Tonbo's Support Team. We can be reached via:

Email: support@tonbobio.com

Phone: 1-855-848-6626 toll-free

(858) 888-7300 local

Hours of Operation: 8:30am - 5:30pm PST

Additional support documents are available at: https://tonbobio.com/pages/technical-data-sheet

References

Gratzner HG. 1982. Science. 218: 474-475.

Dolbeare F, Gratzner HG, Pallavicini MG and Gray JW. 1983. Proc Natl Acad Sci USA. 80: 5573-5577.

Begg AC, McNally NJ, Shrieve DC and Karchner H. 1985. Cytometry. 6: 620-626.

Falini B, Canino S, Sacchi S, Ciani C, Martinelli MF, Gerdes J, Stein H, Pileri S, Gobbi M, Fagioli M, Minelli O and Flenghi L. 1988. Br J Hematol. 69: 311-320.

Williamson K, Halliday I, Hamilton P, Ruddell J, Varma M, Maxwell P, Crockard A and Rowland B. 1993. Cell Prolif. 26: 115-124.

Li X, Tragano F, Melamed MR and Darzynkiewicz Z. 1994. Int J Oncol. 4: 1157-1161.