



BD Cycletest™ Plus DNA Kit

For the analysis of nuclear DNA from solid tissue or cell suspensions

Catalog No.
340242

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

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RESEARCH APPLICATIONS

Research applications include:

- Characterization of clonal diversity of human neoplasms¹⁻¹²
- Research on the potential for progression of human neoplasms¹⁻¹²
- Characterization of cell-cycle phase distribution

DESCRIPTION

The BD Cycletest™ Plus DNA kit provides a set of reagents for isolating and staining cell nuclei from surplus fresh or frozen solid tissue specimens or cell suspensions. Flow cytometric analysis of differentially stained normal and tumor cells is used for research to identify abnormal DNA stemlines and to estimate the DNA index (DI) and cell-cycle phase distributions of these stemlines.

Uniform suspensions of single nuclei are prepared for DNA staining and flow cytometric analysis from surplus solid tissue specimens or cell suspensions. The *in vitro* fine needle aspiration technique ensures broad sampling of the tumor to maximize the extraction of malignant cells that might be present in the specimen, while minimizing cellular debris.

The method involves dissolving the cell membrane lipids with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with an enzyme, and stabilizing the nuclear chromatin with spermine.^{13,14} Propidium iodide (PI) is stoichiometrically bound to the clean, isolated nuclei, which are then run on a flow cytometer equipped with electronic doublet-discrimination capability.^{15,16} PI-stained nuclei emit fluorescent light primarily at wavelengths between 580 nm and 650 nm. The BD FACScan™ flow cytometer's fluorescence 2 (FL2) detector, equipped with a 585/42 bandpass filter, is used to analyze light emitted between 564 nm and 606 nm by the stained cells. The resulting fluorescence histograms are analyzed to detect the presence of an abnormal DNA stemline (DNA aneuploidy).

Normal cells obtained from the same tissue or peripheral blood mononuclear cells (PBMCs) are mixed with the sample in a second tube before staining and used as a control to determine the degree of DNA content aberration. The DI is obtained by dividing the mode (or mean) of the relative DNA content of the test sample G_0/G_1 population by the mode (or mean) of the normal control G_0/G_1 population. The coefficient of variation for each G_0/G_1 peak is also reported.¹⁷

MATERIALS

Reagents provided

The BD Cycletest Plus DNA kit contains the following:

- Buffer Solution (3 vials, 50 mL per vial)
Contains sodium citrate, sucrose, and dimethyl sulfoxide (DMSO) for the collection and/or freezing of cell suspensions.
- Solution A (10 mL)
Contains trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons. Use Solution A at room temperature (20°C–25°C).
- Solution B (8 mL)
Contains trypsin inhibitor and ribonuclease A in citrate-stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA. Use Solution B at room temperature (20°C–25°C).
- Solution C (8 mL)
Contains PI and spermine tetrahydrochloride in citrate stabilizing buffer. The PI stoichiometrically binds to the DNA at a final concentration of at least 125 µg/mL. Protect Solution C from light and keep ice cold (2°C–8°C).

Materials required but not provided

In addition to the reagents provided in this kit, the following reagents and materials are recommended.

Biological material

- Cell suspension from tissue culture, body fluids, or fine needle aspiration of a solid tissue sample
- PBMCs, fresh or thawed, to be used as a control

Chemicals, solutions, reagents, and beads

- BD FACSTFlow™ sheath solution (Catalog No. 342003)

NOTE Using phosphate-buffered saline (PBS) instead of sheath fluid in the flow cytometer can result in different performance characteristics.

- BD™ DNA QC particles kit (Catalog No. 349523)
See the BD DNA QC Particles reagent data sheet for instructions.
- Ficoll-Paque™ separation medium for Ficoll-Hypaque density gradient centrifugation

Consumables

- Powder-free gloves
- Falcon® disposable 17 x 100-mm capped polypropylene tubes, or equivalent
- Falcon disposable 12 x 75-mm capped polypropylene test tubes, or equivalent
- 50- μ m nylon mesh or a Falcon disposable 12 x 75-mm tube with cell strainer cap
- 25-gauge x 1.5-inch hypodermic needles for tissue preparation
- 20-cc syringes
- Transfer pipets or disposable pipets
- 200- μ L to 1000- μ L adjustable micropipet and disposable tips
- Aluminum foil
- Disposable tissues

Equipment

- Vortex mixer
- Low-speed centrifuge (300g) with swinging-bucket rotor and tube carriers for the 17 x 100-mm and 12 x 75-mm sample tubes
- Vacuum aspirator with trap
- BD FACS™ brand flow cytometer

For PI excitation, an argon-ion laser emitting at 488 nm and a filter detecting light at 580 nm–650 nm is optimal. See the appropriate cytometer user's guide for information.

- Hemacytometer and microscope
- Ice bath

HANDLING AND STORAGE

When stored at -18°C , the reagents are stable until the expiration date shown on the BD Cycletest Plus DNA kit label when stored as directed. Do not use after the expiration date. Do not expose reagents to direct light during storage or incubation with cells. Do not heat reagents to 37°C . However, brief exposure to a 37°C water bath while thawing is acceptable.

The thawed reagents are stable for 1 month when stored at 2°C – 8°C . Do not refreeze after thawing. Alteration in the appearance of the reagents indicates instability or deterioration. Do not use if discoloration occurs or a precipitate forms.

WARNING All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection^{18,19} and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.

PROCEDURE

Control and specimen preparation

PBMC Controls

Control cells with known DNA content (PBMCs) provide a reference point for determining the DI for a test sample. To use such a control, these cells must be prepared using the same reagents as the test sample and should ideally be added to an aliquot of the test sample before staining. The DI can be calculated from the ratio of the mode (or mean) of the G_0/G_1 peaks of the test and control (PBMC) populations for a particular specimen.¹⁷

1. Purify the PBMCs using density-gradient centrifugation following the manufacturer's instructions for use of the separation medium.
2. Place the white blood cell suspension into a labeled 17 x 100-mm tube.
3. Add 5 mL of Buffer Solution and resuspend the cells by gently vortexing at low speed.
4. Centrifuge for 5 minutes at 300g at room temperature (20°C–25°C).
5. Aspirate the supernatant, leaving approximately 50 μ L of residual fluid in the tube to avoid disturbing the pellet.
6. Resuspend the pellet in 1.5 mL of Buffer Solution by gently vortexing at low speed.
7. Centrifuge for 5 minutes at 300g at room temperature (20°C–25°C).
8. Aspirate the supernatant, leaving approximately 50 μ L of residual fluid in the tube to avoid disturbing the pellet.
9. Resuspend the pellet in 1 mL of Buffer Solution by gently vortexing at low speed.
10. Count the PBMCs using a hemacytometer according to standard laboratory procedures.
11. Adjust the concentration to 1.0×10^6 cells/mL with Buffer Solution.

PBMCs are now ready for immediate staining and flow cytometric analysis. Cells can also be frozen for

later testing. Freeze samples according to standard laboratory procedures.

Solid tissue

CAUTION Process surplus tissue specimens immediately upon receipt.

If the solid tissue specimen has been frozen, allow tissue to thaw at room temperature (20°C–25°C) before performing fine-needle aspiration.

CAUTION Areas of the tumor specimen appropriate for sampling should be indicated by a trained pathologist. Use a new syringe and needle for each tumor specimen to avoid cross-contamination of samples. If the specimen is not to be processed immediately, collect cells in freezer-safe vials and store frozen, according to standard laboratory procedures.

1. Label specimen collection tubes with the appropriate identification number and add 1 mL of Buffer Solution to each tube.
2. Assemble the syringe into a pistol-grip syringe holder and attach a 25-gauge x 1.5-inch needle.
3. Introduce the needle tip into the tumor and when positioned, pull back gently on the syringe plunger to produce a vacuum. In larger samples, pass the needle through as many aspects of the mass as possible, without breaking the vacuum. In some laboratories, quadrant areas are designated for multiple aspirations in the tumor specimen to ensure heterogeneous sampling. When processing small samples, attempt to aspirate as much tissue as possible.
4. When a visible amount of material appears in the needle hub (not in the barrel of the syringe), release the vacuum and then withdraw the needle.
5. Carefully expel the aspirated cells into 1 mL of Buffer Solution. Carefully rinse out cells remaining in the hub by aspirating Buffer Solution to fill the needle hub only, not the syringe. Expel the wash buffer into the sample tube.

Enough cell suspension (approximately 2 drops) will remain in the needle to make a cytology slide. A drop or two of the recovered sample suspension can also be used.

6. If sample size allows, prepare a histopathology slide according to standard laboratory procedures.

The suspension should be evaluated by a trained pathologist for adequate tumor representation using standard cytological methods.

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- Count the cells using standard laboratory procedures. The dissolution of cell clumps during the subsequent enzyme and detergent steps might improve the recovery of nuclei compared to the original cell count. If clumps are present, estimate the cell number recovered from tissue fragments at this stage.
 - Repeat steps 3 through 5 until the cell concentration in the Buffer Solution is at least 1.0×10^6 cells/mL. This should provide sufficient cells for both a test sample tube and a control tube.

Cells are now ready for immediate staining and flow cytometric analysis. Cells can also be frozen for later testing. Freeze samples according to standard laboratory procedures.

Cell suspensions

Samples received containing cells already in suspension from tissue culture or body fluids can also be used for DNA analysis. Process cell suspensions immediately upon receipt.

- Place cell suspension into a labeled 17 x 100-mm tube.
- Centrifuge for 5 minutes at 300g at room temperature (20°C – 25°C).
- Aspirate the supernatant, leaving approximately 50 μL of residual fluid in the tube to avoid disturbing the pellet.
- Add 1 mL of Buffer Solution and resuspend the cells by gently vortexing at low speed.
- Centrifuge for 5 minutes at 300g at room temperature (20°C – 25°C).
- Repeat step 3 through step 5.
- Aspirate the supernatant, leaving approximately 50 μL of residual fluid in the tube to avoid disturbing the pellet.
- Resuspend the pellet in 1 mL of Buffer Solution by gently vortexing at low speed.
- Count the cells using a hemacytometer according to standard laboratory procedures.
- Adjust the concentration to 1.0×10^6 cells/mL with Buffer Solution. This should provide sufficient cells for both a test sample tube and a control tube.

Cells are now ready for immediate staining and flow cytometric analysis. Cells can also be frozen for later testing. Freeze samples according to standard laboratory procedures.

Staining

The staining procedure for DNA ploidy analysis requires a test sample of 5.0×10^5 cells. Prepare an additional sample tube of the specimen mixed or “spiked” with PBMCs to use as a control. Use at least a 2:1 ratio of tumor cells to PBMCs.

NOTE Use Solution A and B at room temperature (20°–25°C). Keep Solution C ice cold (2°C–8°C) and protected from light.

1. Centrifuge the cell suspensions at 400g for 5 minutes at room temperature (20°C–25°C).
2. Carefully decant all the supernatant, and tap off the last drop onto a tissue.
3. Add 250 μ L of Solution A (trypsin buffer) to each tube and gently mix by tapping the tube by hand. **Do not vortex.**
4. Incubate for 10 minutes at room temperature (20°C–25°C). Do not aspirate Solution A.
5. Add 200 μ L of Solution B (trypsin inhibitor and RNase buffer) to each tube and gently mix by tapping the tube by hand. **Do not vortex.**
6. Incubate for 10 minutes at room temperature (20°C–25°C). Do not aspirate Solution A and B.
7. Add 200 μ L of cold (2°C–8°C) Solution C (PI stain solution) to each tube and gently mix by tapping the tube by hand. **Do not vortex.**
8. Incubate for 10 minutes in the dark on ice or in the refrigerator (2°C–8°C).
9. Filter the sample through 50- μ m nylon mesh into a labeled 12 x 75-mm tube, or use a 35- μ m cell strainer cap and filter into a 12 x 75-mm tube.

The samples are now ready to be analyzed on the flow cytometer.

10. Cap or cover the prepared tubes and store at 2°C–8°C in the dark until flow cytometric analysis.

For optimal results, acquire samples on the flow cytometer within 3 hours after the addition of Solution C. Prior to acquisition, mix the sample in the tubes by tapping the tube by hand to resuspend the cells.

ACQUISITION AND ANALYSIS

Instrument

The BD Cycletest Plus DNA kit kit is designed to prepare samples for DNA analysis on a flow cytometer equipped with appropriate computer hardware, software, and gating electronics. The flow cytometer must have linear

fluorescence amplification capabilities with forward-scatter (FSC) and side-scatter (SSC) detection. The flow cytometer should be equipped with a light source providing excitation in the blue-to-green range. For PI excitation, an argon-ion laser emitting at 488 nm is optimal. We recommend:

- BD FACScan or BD FACSort™ flow cytometer system equipped with a doublet discrimination module (DDM) or BD FACS Vantage™ flow cytometer equipped with pulse processing, and three-color fluorescence detection and two-parameter light-scatter detection.

Instrument setup

We recommend using the BD DNA QC particles kit to set the photomultiplier tube (PMT) voltages and check instrument resolution and linearity, and:

- On the Apple® Macintosh® platform, BD CellQuest™ software, version 1.0 (or later), with the DNA Experiment Document and appropriate analysis software. For detailed information on use, see the *BD CellQuest Software User's Guide*.
- On the Hewlett-Packard (HP) platform, BD CellFIT™ software, version 2.0 (or later) or 2.01.2 (or later). For detailed information on use, see the *BD CellFIT Software User's Guide*.

Acquisition

During acquisition of the stained samples, we recommend:

- Running a control with each test sample. See PBMC Controls on page 6.
- Running samples at an acquisition rate of at least 60 events per second. For samples prepared for DNA analysis according to this method, the BD FACScan flow rate should be set on LO.
- Acquiring at least 20,000 list-mode data events for each sample. More data events might be required for samples containing several smaller subpopulations.
- Leaving the flow cytometer on RUN between samples to allow the sample injection port to backflush.
- Ensuring that the fluidics are rinsed thoroughly before placing the flow cytometer on STANDBY. This prevents debris from adhering to the inside of the flow cell.
- Analyzing the histograms with appropriate DNA analysis software.

RESULTS

Results were obtained using BD Cycletest Plus DNA kit reagents and BD CellFIT software on a DDM-equipped BD FACScan flow cytometer. Figure 1 and Figure 2 show analysis of PBMCs. Figure 3 and Figure 4 show analysis of breast tumor tissue prepared by in vitro fine needle aspiration.

Figure 1 FL2-W vs FL2-A dot plot of PBMCs showing a singlet gate, which excludes aggregates

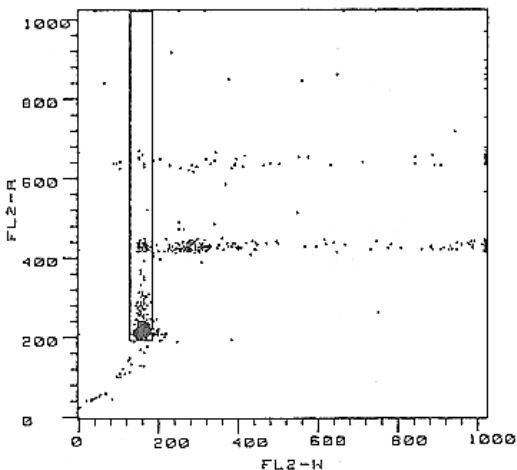


Figure 2 FL2-A DNA histogram of PBMCs, which has been gated to exclude aggregates

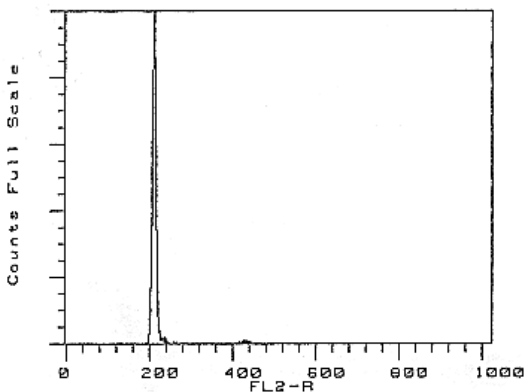


Figure 3 FL2-W vs FL2-A dot plot of breast tumor tissue showing a singlet gate, which excludes aggregates

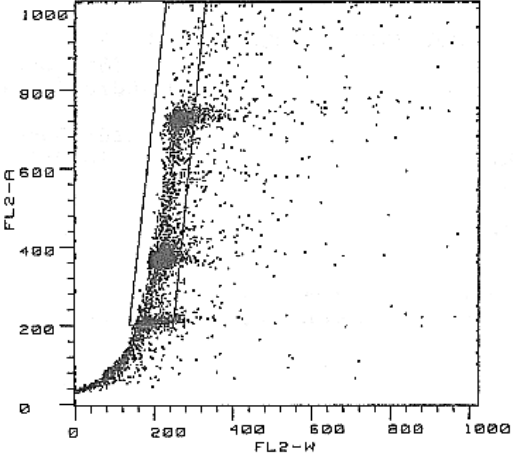
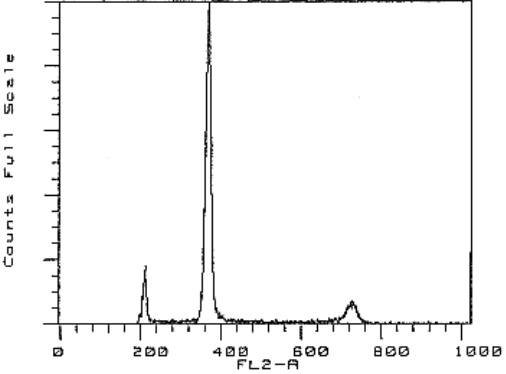


Figure 4 FL2-A DNA histogram of breast tumor tissue, which has been gated to exclude aggregates



Limitations

- This kit is for Research Use Only. Not for use in diagnostic or therapeutic procedures.
- The information obtained from this kit must be combined with other relevant information. Interpretation of this information by a medically qualified diagnostician is necessary.

- You must ensure the inclusion of tumor cells by carefully following the sample preparation procedure described in this document. Light microscopy confirmation must be performed to ensure that the suspension adequately represents the tumor under study. The presence of malignant cells must be confirmed by a trained pathologist using a standard cytological evaluation of the tumor.
- Performance characteristics have been determined with BD FACScan flow cytometers. Performance characteristics using other instruments have not been established.
- Stained samples should be analyzed on the flow cytometer within 3 hours of staining.
- Changing reagent volumes or incubation times from those specified might yield erroneous results. The reagents are formulated to appropriately process a specific number of cells. Using excessive numbers of cells might produce suboptimal results.
- The fluorescence of PI is dependent on the pH and ionic strength of the solution. The use of PBS instead of sheath fluid in the BD FACScan instrument might result in different performance characteristics.
- Incubation times, centrifugation times, or temperatures other than those specified might be a source of error.

TROUBLESHOOTING

Problem	Cause	Solution
Sudden change or slow drift of diploid G ₀ /G ₁ peak location.	Air bubbles or clogs in the fluidics system.	Flush the fluidics and clear the lines of air.
	Different tonicity of sheath and sample fluids.	Check the quality of the sheath fluid.
	Insufficient PI staining.	Check the concentration of the nuclei and adjust.
	Insufficient incubation of the nuclei in PI.	Increase the incubation time.
Presence of excess debris in fluorescence histogram.	Necrosis in the original specimen.	Visually check the tissue specimen for necrosis.
	Incomplete cell lysis.	Check the suspension for incomplete lysis.
	Particles appear in the sheath fluid.	Clean the system and replace the sheath fluid and/or sheath filter if necessary.

Problem	Cause	Solution
Broadened peaks and/or increased CVs.	Flow rate is too high.	Run the samples using a low flow rate.
	Air bubbles in the fluidics.	Check the fluidics for air.
	Necrotic cells are included in sample.	Check for the presence of necrotic cells and prepare another sample.
Shifting of the peaks due to a loss of fluorescence.	Too many cells.	Check the concentration of nuclei and adjust to optimum.
	DNA not saturated with propidium iodide.	Stain another sample with the correct concentration and volume of the PI stain.
	Residual bleach left in the sample injection probe (SIP).	Ensure that the fluidics are adequately rinsed after exposure to bleach.
Few or no events are being acquired.	Incorrect mounting of the sample tube.	Re-mount the sample tube or check for cracks in the sample tube.
	No nuclei in the sample.	Check for presence of nuclei in the sample using a microscope.
	Sample line is clogged.	Backflush and clean the sample line.
	Debris in flow cell.	Ensure that fluidics are rinsed thoroughly of sample fluid before placing the flow cytometer's fluidics control knob into STANDBY mode to prevent debris from adhering to the inside of the flow cell.

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WARRANTY

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