

Measurement of DNA Fragmentation in Spermatozoa by TUNEL Assay Using Bench Top Flow Cytometer

24

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1 Introduction

DNA fragmentation endonucleases are activated during apoptosis. These nucleases degrade the higher-order sperm chromatin structure into fragments ~30 kb in length and then subsequently into smaller DNA pieces about ~50 kb in length. This fragmented DNA can be detected by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay [1, 2]. It is a single-step staining method that labels DNA breaks with FITC-dUTP; flow cytometry is then used to identify the sites of the strand breaks [1, 2].

2 Principle

TUNEL utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3' hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3'-OH break ends of DNA (Fig. 24.1) [1, 2]. The more DNA strand break sites that are present, the more labels that are incorporated within a cell.

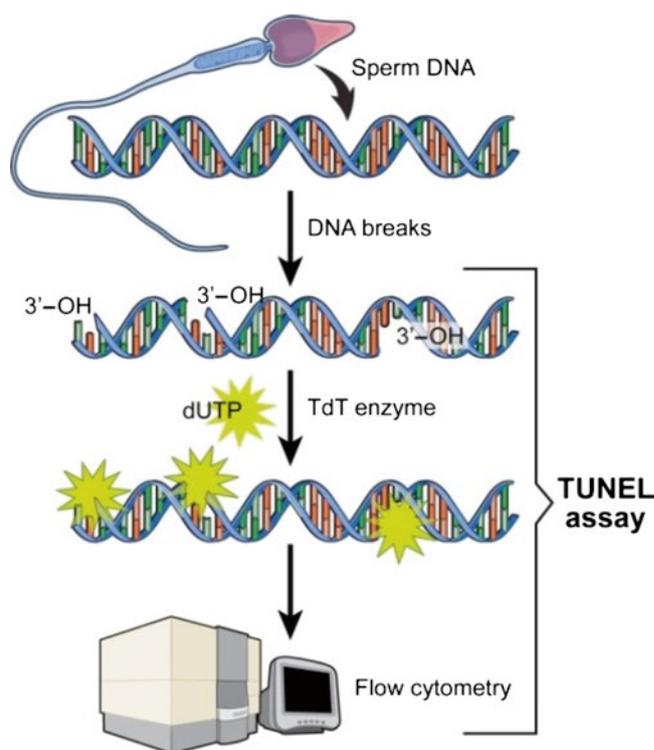


Fig. 24.1 Schematic of the DNA staining by the TUNEL assay

3 Specimen Collection

- Ideally, the sample is collected after a minimum of 48 h and not more than 72 h of sexual abstinence. Record the name of the patient, period of abstinence, date, time, and place of collection on the form accompanying each semen analysis.
- The sample should be collected in the privacy of a room near the laboratory. If not, it should be delivered to the laboratory within one hour of collection.

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Fig. 24.2 Decontamination concentrate, cleaning concentrate, extended flow cell clean, and bacteriostatic concentrate solutions [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

- C. The sample obtained by masturbation only and ejaculated into a clean, wide-mouth sterile plastic specimen cup. Lubricants should not be used to facilitate semen collection.
- D. The sample should be protected from extreme temperatures (not less than 20 °C and not more than 40 °C) during transport to the laboratory.
- E. Note any unusual collection or condition of specimen on the report form.
- D. Extended flow cell clean (PN 653159)—extended flow cell cleaning solution provided in working concentrate (Fig. 24.2, 3rd bottle).
- E. APO-DIRECT™ kit (BD Pharmingen, San Diego, CA Catalog #556381):
- PI/RNase staining buffer (Fig. 24.3).
 - Reaction buffer (Fig. 24.4).
 - FITC-dUTP (Fig. 24.4).
 - TdT enzyme (Fig. 24.4).
 - Rinsing buffer (Fig. 24.5).
 - Wash buffer (Fig. 24.6).

4 Reagents

Note: Use only approved fluids in the operation or cleaning of the instrument.

- A. Sheath fluid (**blue** bottle)—0.22 μm filtered, deionized water with, or without, bacteriostatic concentrate solution (PN 653156):
1. If bacteriostatic concentrate solution (Fig. 24.2, 4th bottle) is used (optional), add 1 bottle per 1 L of water.
- B. Cleaning solution (**green** bottle) (ON 653157)—cleaning concentrate solution is diluted:
1. Add 3 mL of cleaning concentrate (Fig. 24.2, 2nd bottle) to 197 mL of filtered deionized water.
 2. Use the solution within 2 weeks.
- C. Decontamination solution (**yellow** bottle) (PN653154)—decontamination concentrate solution, diluted:
1. Add entire bottle (Fig. 24.2, 1st bottle) to 180 mL of filtered, deionized water.



Fig. 24.3 Bottle of PI/RNase staining buffer [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.4 Reaction buffer, TdT enzyme, and FITC-dUTP vials [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.7 Positive and negative assay controls [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.5 Rinsing buffer [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.8 Bottle of 3.7% paraformaldehyde in PBS [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.6 Wash buffer [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

- Negative control cells (Fig. 24.7).
 - Positive control cells (Fig. 24.7).
- F. Serological pipettes (2, 5 mL).
- G. Eppendorf pipette and tips (20, 100 μ L and 1000 μ L).
- H. Sperm counting chamber.
- I. Paraformaldehyde (3.7% in PBS) (Fig. 24.8).
Preparation of paraformaldehyde:
Add 90.0 mL of PBS (pH 7.4) to 10.0 mL of formaldehyde (37%).
Note: Store at 4 °C.
- J. Microfuge tubes
- K. Ethanol (70%).
Preparation of ethanol (70%):
Add 30 mL of distilled water to 70.0 mL of ethanol (>99% vol./vol.).
Note: Store at 4 °C.



Fig. 24.9 Flow cytometer instrumentation [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]



Fig. 24.10 8-peak Validation Beads bottle [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

- L. Flow cytometer (fluorescence-activated cell sorting caliber, Becton and Dickinson, San Jose, CA) (Fig. 24.9).
- M. 8-peak validation beads—one bottle (Fig. 24.10).

5 Basic Instrument Instructions/Setup

Caution: A tube of 0.22 μm filtered deionized (DI) water must be placed on the sheath injection port (SIP) at all times to keep the SIP from drying out—before use, during use, and even after the machine is shut down.

5.1 First-Time Setup

- A. Make sure the sheath bottle (**blue** bottle) is filled with sheath fluid.

- B. Dilute cleaning concentrate as well as the decontamination concentrate per the above instructions (**B1** and **C1**) and then fill the **green** bottle with the cleaning solution and **yellow** bottle with the decontamination solution.
- C. Make sure all bottles are closed securely.
- D. Add 100 mL of 0.5% NaOCl to the waste bottle (**red** bottle).
- E. Place the bottles in the fluidic bottle tray.
- F. Slide the red tab on the black end of the fluidic harness to the unlock position and attach the black end of the fluidic harness to the back of the cytometer.
- G. Slide the red tab to the lock position.
- H. Attach the other end of the fluidic harness to the appropriate bottles:
 1. Red line—waste bottle.
 2. Blue line—sheath bottle.
 3. Yellow line—decontamination bottle.
 4. Green line—cleaner bottle.

5.2 General Setup

Note: Deselect all boxes in the Collect Tab.

1. Open the software by double-clicking the “BD Accuri C6 software” icon on desktop.
2. Bottles in steps 2–4 above should always be filled as indicated and seated in the fluidic bottle tray.
3. Check the fluid levels in all bottles. The waste bottle must be empty and the sheath, cleaner, and decontamination bottles full.
4. Pull the sample stage forward underneath the SIP.

Note: The sample stage accommodates any brand of 12×75 mm tube and most microcentrifuge tubes. Be careful not to bend or catch the SIP when inserting tubes.
5. If no tube is currently on stage, place a tube with 0.22 μm filtered DI water (Fig. 24.11).
6. Firmly press the power button on the front of the cytometer unit.
7. While starting up, the BD Accuri software “traffic light” should turn yellow, and the pumps should start to run.
8. Wait 5 min for the machine to flush the fluidic line with sheath fluid.

Warning: Do not open the lid of the cytometer during this time. This will disrupt the laser warm-up process.
9. The BD Accuri software “traffic light” will turn green and display “C6 is connected and ready” when complete.
10. To remove bubbles from the system, place a tube of 0.22 μm filtered DI water on the SIP.
11. Select “run with limits” and set to 15 min.
12. Select “fluidic” speed to “fast.”
13. Click the “RUN” button.



Fig. 24.11 Tube filled with 0.22 μm DI water placed on SIP [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

14. Save file as “flush.”

Note: Save location is not important. This file can be deleted at any time.

15. Leave tube on SIP.

Note: Validate the performance of the cytometer using the QC beads designed for the instrument, provided by BD before processing any samples.

5.3 Machine Shutdown

- Place a tube with 2 mL of bleach (diluted decontamination solution) on the SIP.
- Select an empty “data well” in the collect tab of the BD Accuri software.
- Set a time limit of “2 min” and set fluidic speed to “fast.”
- Click the “RUN” button.
- Once the run is finished, remove the tube from the SIP.
- Place a tube with 0.22 μm filtered DI water on the SIP and select another empty “data well” in the BD Accuri software.
- Repeat steps C and D, above.
- When the run is finished, leave the tube on the SIP.
- Press the power button to start the shutdown cycle.
- The cycle will take 15 minutes to complete and then the cytometer should automatically shut down.

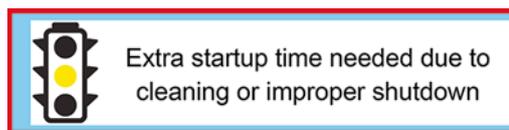


Fig. 24.12 Improper shutdown sign on flow cytometer [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

Note: The automatic shutdown cycle can be bypassed by pressing down the power button for 5 s. However, the cytometer will take additional time to recover and return to the steady state if it is shut down in this manner. The BD Accuri software will display the following message if the machine is shut down using this method: (see Fig. 24.12).

Note: It is unnecessary to shut down the software or computer during the shutdown process.

6 Quality Control

The performance validation process consists of measuring the coefficient of variation (CVs) on a daily basis and tracking CVs using a Levey-Jennings chart. Validation is done by using 8-peak beads provided by the company. The 8-peak validation beads are used for monitoring the FL1, FL2, and FL3 channels and are excited by a blue laser. The beads are 3.2 μm particles that emit light at eight different wavelengths.

6.1 Preparing the QC Beads

- Label one 12 \times 75 mm tube as “8-Peak QC Beads” with date of preparation.
- Add 1 mL of HPLC or deionized water.
- Vortex the vial.
- Add four drops of 8-peak beads to the tube.
- Vortex the tube.

Note: After preparing, store the beads in the dark until use.

6.2 Preparing the Run of the QC Beads

- Double-click the “8 Peak Template.c6t” as a QC file folder on the computer.

Note: A new folder and template should be created semiannually, i.e., 2016-S1 QC, 2016-S2 QC, etc.
- If the cytometer is off, press the power button on the front of the instrument to start.

- C. Under the collect tab, the cytometer software will display a “green traffic light” when the machine is ready to process samples.
- D. Click an empty data well (ex. A1).
- E. Place a tube with 2 mL of 0.22 µm filtered DI water on the SIP.
- F. Check “run with limits” and set the time field to “15 min.”
- G. Set “fluidics” speed to “fast.”
- H. “Click the “RUN” button.
- I. If asked to save, save the file in the directory listed above in step A.
- J. Once the run is finished, click the “delete events” tab at the bottom-left of the BD Accuri software window.

6.3 8-Peak Bead Run

Note: Use the file “8 Peak Template.c6” for this run located in a file under the BD Accuri QC folder. The 8-peak QC beads measures the following channels: FL1-H, FL2-H, and FL3-H; this corresponds to the following plot locations and x- vs. y-axes on the bead template:

FSC-H vs. SSC-H	FL1-H vs. Count	FL2-H vs. Count
FL3-H vs. Count		

- A. Select an empty field from A1-H12.
Note: Start by selecting wells from left to right at A1. The next QC performed will be A2, A3, A4, and so on until each of the wells is filled. This allows for 96 QC runs.
- B. In the empty field above the wells, name the sample “8-Peak Bead-DATE-TECH INITIALS;” for example, “8-Peak Bead-4-16-14-RKS.”
- C. Under the collect tab, deselect the “time” check box next to “min” and “sec.”
- D. Select the “events” check box and enter “50000” in the “events” field.
- E. Select “ungated sample” from the drop-down menu, if it is not already selected.
- F. Set “fluidics” speed to “slow.”
- G. Vortex the sample containing the already prepared “8-Peak QC Bead” mixture.
- H. Remove tube of DI water from the SIP.
I. Place the “8-Peak QC Bead” tube under the SIP.
J. Click the “RUN” button to start the acquisition.
- K. Save the file on the computer in the QC folder as indicated above. The file should be saved as “8-Peak QC-DATE-TECH INITIALS”.
- L. Acquisition will stop after 50,000 events.
- M. When the run is finished, remove “8-Peak QC Bead” tube from SIP and clean the SIP using a lint-free wipe.
- N. Place the tube containing 2 mL of DI water on the SIP.

6.4 Ending the Run

- A. With the 2 mL tube of DI water on the SIP, select an empty well in the BD Accuri software.
- B. Select the “time” check box and set the time to “2 min.”
- C. Set “fluidics” speed to “fast.”
- D. Click the “RUN” button.
- E. When the run is finished, keep the tube with 2 mL of DI water on the SIP.
- F. Before running any other samples, click “delete events” to erase the data collection from the water run.
- G. If shutting down instrument, proceed to “Machine Shutdown” section above and follow all the steps. Otherwise, skip this step.

6.5 Analyzing the Data

Note: This analysis should be performed under the “collect” tab.

6.5.1 8-Peak Beads

- A. Select the well position (e.g., A1) where the data were collected for the 8-peak run.
- B. Adjust the R1 gate so it contains 75%–85% of all events.
- C. In the first FSC-H VS SSC-H plot (top-left), click the border of the R1 gate. The border becomes bold and handles appear for adjustment.
- D. Adjust R1 to encompass the main bead population (singlets).
Note: Do not include bead doublets in the R1 gate. These appear as light-gray dots outside of the dark black (singlet) population.
- E. Verify that the FL1-H, FL2-H, and FL3-H histograms are gated on R1.
- F. Measure the CV of the brightest peak (right-most peak) of the FL1-H, FL2-H, and FL3-H histograms (Fig. 24.13).
- G. If necessary:
 1. Click on the zoom tool in the FL1-H histogram.
 2. Click and drag inside the histogram to zoom on the brightest peak.
 3. Adjust the M1 marker tightly around the brightest peak by clicking on the marker and dragging its edges.
- H. Repeat step D to measure the brightest peak CV in the FL2-H and FL3-H histograms.
Note: The above steps are necessary for the first time setup only. Minor adjustments may be necessary to ensure that the singlets are within the R1 gate. The CV’s for each peak must be less than 5% to meet validation specification.

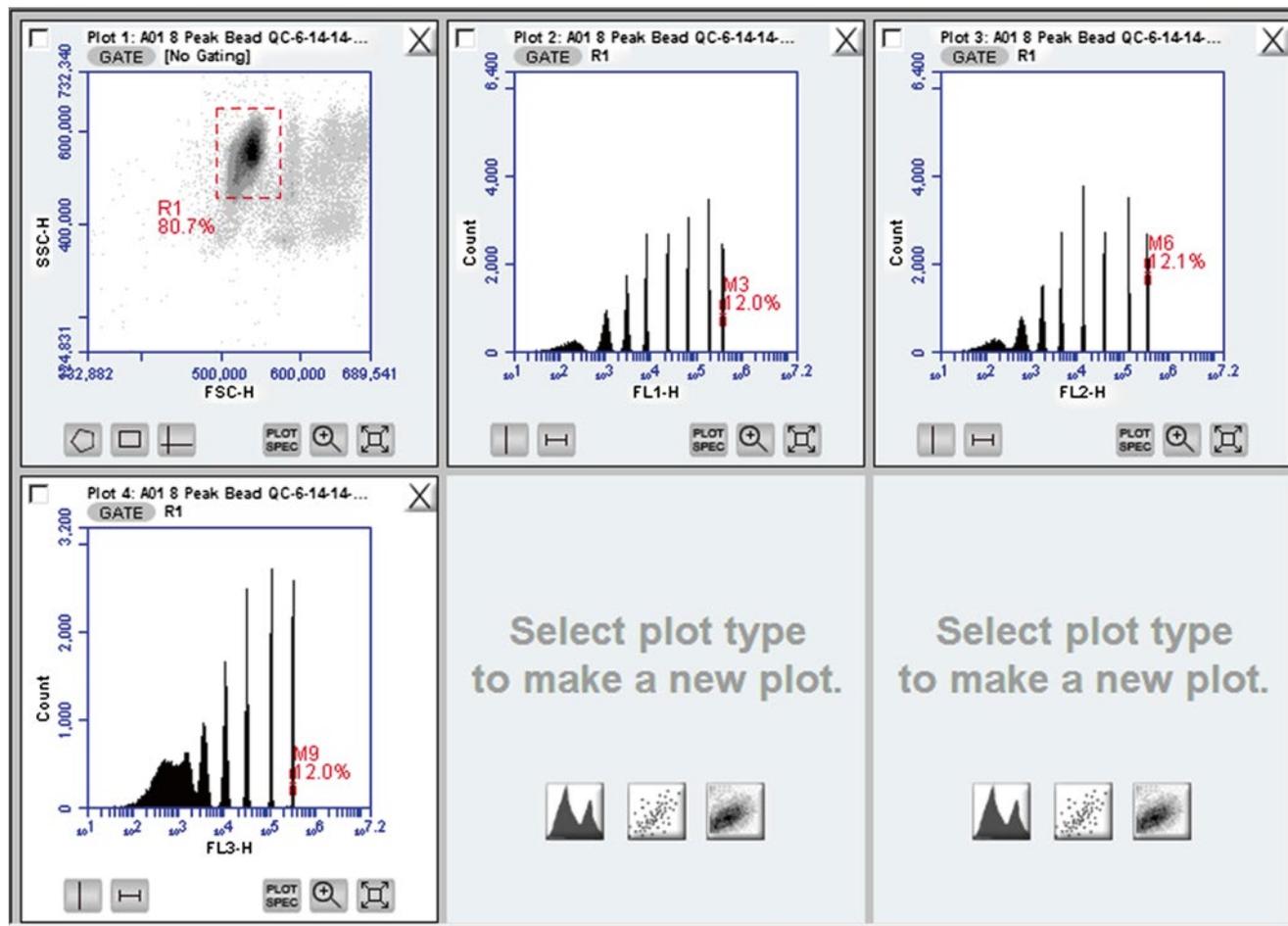


Fig. 24.13 8-peak quality control beads as seen after analysis in software; measure the CV of the brightest peak (M3, M6, M9) [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

6.5.2 Tracking 8-Peak Bead Cytometer Performance

Creating the Statistics

- Click the statistics tab.
- In the “sample selector,” check all the boxes next to the 8-peak bead runs (see Fig. 24.14).
- In the “statistics column selector,” check the boxes for the mean and CV of the brightest peak (M3, M6 and M9) for the following parameters: FL1-H, FL2-H, and FL3-H.

Note: The data will populate in the order in which the boxes are selected.

Displaying Data in Levey-Jennings Plot

- Open Excel.
- Select file>open and navigate to QC file folder
- Click “Bead Tracking.xlsx” and then “Open.”

- Verify that the 8-Peak tab is displayed.
- In the BD Accuri C6 software, highlight all the statistics in the Master Statistics Table.
- Select Edit>Copy.
- In Excel, paste the statistics for the 8-peak bead.
- The Levey-Jennings plots will be populated with your data.
- View the data that is populated.
- Save the spreadsheet.

Table 24.1 Fluorescence intensity in the FSC-H/SSC-H, FL1, FL2 and FL3 plots.

Plot	Fluorescence intensity	CV
R1 (FSC/SSC plot)	521,276	1.87
FL1 (M3)	384,290	1.78
FL2 (M6)	310,837	1.24
FL3 (M9)	243,852	2.81

Sample Selector		
Add rows to your master statistics table by selecting samples.		
Preview	Add to Table	Sample Name
<input type="radio"/>	<input checked="" type="checkbox"/>	HPB
<input checked="" type="radio"/>	<input checked="" type="checkbox"/>	HPB Auto, CD11b,C...
<input type="radio"/>	<input checked="" type="checkbox"/>	HPB CD3-F CD4,C...

Fig. 24.14 Sample selector [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved]

7 Semen Sample Preparation for TUNEL Assay

- A. Following liquefaction, evaluate semen specimens for volume, round cell concentration, sperm concentration, total cell count, motility, and morphology. Unless using a reference lab sample, skip to step D.

For Reference Lab Samples

- B. Samples from the reference labs are received frozen. These are not fixed in paraformaldehyde (unless otherwise indicated).
 C. Thaw the sample by incubating at 37 °C for 20 min.
 D. Aliquot and load 5 µL of the sample on a sperm counting chamber for manual evaluation of concentration and motility. Check the concentration of sperm in the sample. Adjust it to $2.5 \times 10^6/\text{mL}$.

This can be done using the following calculation:

$$\frac{2.5}{\text{Sperm Conc.} (10^6 / \text{mL})} \times 1000 = X \mu\text{L}$$

Example: Sperm concentration is $25 \times 10^6/\text{mL}$, and this has to be resuspended in 1.0 mL of paraformaldehyde:

$$\frac{2.5 \times 1000}{25} = 100 \mu\text{L}$$

- E. Using a cryomarker, label 4–6 (5 mL) tubes. Label each tube with the following:
 TUNEL.
 Patient name.
 MRN #.
 Date.

- F. Add the required amount of seminal ejaculate into the tube. Spin the sample at 1600 rpm for 7 min. and remove seminal plasma. Add 1 mL of PBS.
 G. Centrifuge for 7 min at 1600 rpm, remove supernatant and replace with 1 mL of PBS.

Preparation of the Spermatozoa Positive Control

- A. Prepare a hydrogen peroxide diluted solution (1:15 dilution) from the stock of the Andrology Laboratory (Hydrogen Peroxide 30%) by adding, for example, 100 µl of the stock to 1400 µl of PBS 1X.
 B. Resuspend the spermatozoa of the tube “Surname, Name, positive control” in 1 ml of the diluted H₂O₂ solution.
 C. Place the tube in the heater at 50 °C for one hour
 D. Centrifuge for 7 min at 1600 rpm.
 E. Remove the supernatant and replace with 1 ml of PBS.
 F. Centrifuge for 7 min at 1600 rpm.
 G. Remove the supernatant and replace with 1 ml of PBS.
 H. Together with the test and the negative samples, centrifuge for 7 min at 1600 rpm. Remove the supernatant and proceed to Fixation and Permeabilization step and PERMEABILIZATION.

Fixation and Permeabilization

- A. Prepare a paraformaldehyde 3.7% solution by diluting the 10 mL stock formaldehyde 37% solution in 90 mL PBS 1X.
 B. After removing the supernatant from the samples and spermatozoa controls, add 1 ml of the 3.7% paraformaldehyde solution. Incubate at room temperature for 15 min.
 C. Centrifuge for 4 min at 600 g.
 D. Remove the paraformaldehyde and add 1 ml of PBS 1X.
 E. Centrifuge for 4 min at 600 g. Remove the supernatant and replace with 1 mL of ice cold ethanol (70%).
 F. Perform a second wash with PBS (Repeat steps D and E)

8 Staining Protocol

8.1 Preparation of Kit Controls and Internal Test Samples

- A. Vortex the negative (Cat# 6553LZ; White cap) and positive (Cat# 6552LZ; Brown cap) samples provided in the kit.
Note: Verify the catalog numbers and the cap color to match each vial.
 B. Mix the contents of each vial by vortex. Remove 2 mL aliquots of the control cell suspensions (approximately 1×10^6 cells/mL) and place in 12×75 mm centrifuge tubes.

- C. Return the vials to -20°C .
- D. Include 3–4 samples with known DNA damage along with the kit controls.
- E. Centrifuge at 1600 rpm for 7 min and discard the supernatant.
- F. Centrifuge the control cell suspensions for 5 min at 1600 RPM and remove the 70% (v/v) ethanol by aspiration, being careful to not disturb the cell pellet.
- G. To the control and test samples, add 1.0 mL of “Wash Buffer” (6548AZ) (blue cap) and vortex. Centrifuge as before and discard the supernatant.
- H. Repeat the “Wash Buffer” treatment. Centrifuge and discard the supernatant.
- I. Number the tubes consecutively beginning with negative and positive kit controls, test samples, and internal controls.
- F. Resuspend the pellet in each tube in 50 μL of the staining solution.
Note: The same tip can be used to add the stain as long as the stain is added on the side of the tube and the tip does not come in contact with the solution.
- G. Incubate the sperm in the staining solution for 60 min at 37°C . Cover the tubes with aluminum foil.
Note: Record the incubation time on the aluminum foil.
- H. At the end of the incubation time, add 1.0 mL of “Rinse Buffer” (Cat# 6550AZ) (red cap) to each tube and centrifuge at 1600 rpm for 7 min. Discard the supernatant.
- I. Repeat the cell rinsing with 1.0 mL of the “Rinse Buffer”, repeat centrifugation, and discard the supernatant.
- J. Resuspend the cell pellet in 0.5 mL of the PI/RNase staining buffer (Cat# 6551AZ).
- K. Incubate the cells in the dark for 30 min at room temperature.

9 Staining for TUNEL Assay

- A. Check the number of tubes that will be required for the TUNEL assay. It is helpful to prepare the stain for additional 5–7 tubes.
- B. Remove the reaction buffer (green cap) from 4°C and the TdT (yellow cap) and FITC-dUTP (orange cap) from -20°C and place them at room temperature for 20 min to thaw.
Note: Give a quick spin to the TdT vial to bring the reagent to the bottom of the vial.
- C. Prepare the stain as shown in Table 24.2 for a single assay and calculate the required volumes. Always prepare additional 4–5 tubes to ensure that adequate stain is available for all the tubes.
- D. Add the stain in the same sequence as shown in Table 24.2.
Note: The preparation of the stain and all subsequent steps must be carried out in the dark. (See Table 24.2).
- E. Return the stains to appropriate storage temperature.
Note: The appropriate volume of Staining solution to prepare for a variable number of assays is based upon multiples of the component volumes needed for one assay. Mix only enough Staining solution to complete the number of assays prepared per session. The Staining solution is active for approximately 24 h at 4°C .

- L. Number the tubes according to the sample list. Cap the tubes and carefully cover the tubes with aluminum foil. The tubes are now ready to be taken to analyzed with flow cytometry.
Note: The cells must be analyzed within 3 h of staining. Cells may begin to deteriorate if left overnight before analysis.

10 Running Kit Controls

- Note:** Kit controls are run under the “Collect” tab.
All data will be saved in Kit Control Template folder with the following format:
“Kit Controls-DATE-TECH INITIALS”
- A. Double-click “Kit Control Template”.
- B. Select well “A1” (move to adjacent cell if occupied).
- C. In field “A01,” type “kit negative-date-tech initials.”
- D. Set the run parameters as follows:
1. “Run with limits”: 10,000 events in ungated samples.
 2. “Fluidics” speed: slow.
 3. Threshold: 80,000 on FSC-H.
- E. Remove tube of DI water from the SIP.
- F. Vortex and place negative control on the SIP.
- G. Click “RUN” button.
- H. Run will finish after collecting 10,000 events.
- I. Data will populate in plots 1 to 4 (Fig. 24.15).

Table 24.2 Preparation of the Staining solution

Staining solution	1 assay	6 assays	12 assays
Reaction buffer (green cap)	10.00 μL	60.00 μL	120.00 μL
TdT enzyme (yellow cap)	0.75 μL	4.50 μL	9.00 μL
FITC-dUTP (orange cap)	8.00 μL	48.00 μL	96.00 μL
Distilled H_2O	32.25 μL	193.5 μL	387.00 μL
Total volume	51.00 μL	306.00 μL	612.00 μL

Plot 1: FSC-A/SSC-A
 Plot 2: FSC-A/FL3-A
 Plot 3: FL2-A/ FL2-H
 Plot 4: FL1-A/FL2-A

- J. Select well “A2” and repeat steps C–I.
- K. Remove negative kit control from SIP/stage.
- L. Clean the SIP with a lint-free paper.
- M. Place a positive control tube on the SIP.
- N. Click well “A3.”
- O. Name “A03” as “kit positive-date-tech initials.”
- P. Repeat steps D–I.
- Q. Select well “A4” and repeat steps for positive control run (steps O and P) (Fig. 24.15).

11 Running Patient Samples

Note: Patient samples are run under the “Collect” tab, utilizing patient template created on the computer.

All data should be saved in this folder with the following format:

“TUNEL-DATE-TECH INITIALS.”

- A. Double-click on the “Assay Template.”
- B. Wait for the software to load.
- C. Check each well to ensure no data already exists inside.
- D. If necessary, click “delete events” for any wells which contain data.
- E. Select well “A5.”
- F. Begin with tube #5 (first test sample).
- G. Remove DI water from the SIP.
- H. Vortex the tube (test sample) and place on the SIP.
- I. In the name field, name the field as “TUNEL-SAMPLE#-DATE-TECH INITIALS.”
- J. Set the run parameters as follows:
 1. “Run with limits”: 10,000 events.
 2. “Fluidics” speed: slow.
 3. Threshold: 80,000 on FSC-H.
- K. Click the “RUN” button to start the collection.
- L. After 10,000 events, the run will finish.
- M. Remove tube from SIP and clean the SIP with a lint-free paper.
- N. Vortex and place the subsequent tube on the SIP.
- O. Select the next well (A6 and so on) for the new sample.
- P. Repeat steps I–O until all samples have been processed.
- Q. Remove final tube and place the “bleach tube” on the SIP.
- R. Set the parameters as follows:
 1. “Run with limits”: 2 min.
 2. “Fluidics” speed: fast.
 3. Threshold: 80,000 on FSC-H.
- S. Click the “RUN” tab.
- T. When the run is finished, wipe SIP.
- U. Remove tube and replace with DI water tube.

- V. Repeat steps Q–S with DI water.
- W. Proceed to shutdown step.

12 Data Acquisition

12.1 Kit Controls (Kit Control Template)

Note: Maintain a written record of all results.

- A. Under the collect tab, click on well A1 for the first negative kit control.
- B. Observe the graph for the negative control.
- C. The kit control template automatically separates the negative (left side of the graph) and positive (right side of the graph) regions of the graph with a vertical marker. Observe only the positive (FITC+) value.
- D. Record the result in the following template: Kit Control Result folder.
- E. Click on well A2 and follow steps B–D for the second negative kit control (Fig. 24.15).
- F. Click on well A3 for the first positive kit control.
- G. Observe the graph for the positive kit control.
- H. Follow steps C–D for the positive kit control.
 - I. Click on well A4 for the second positive kit control and repeat steps C–D (Figs. 24.16).

12.2 Data Analysis

The following strategies will be used for data analysis:

1. Alignment strategy and Data analysis in the Collect tab: Use a standard file and sample to align all the samples. This strategy is done in the Collect tab.
2. Data analysis in the analyze tab: Align each sample to its respective negative. This strategy is used in the analyze tab.

12.3 Alignment Strategy and Data Analysis in the Collect Tab

- A. Click on File, open Workspace or template (TUNEL template modified quadrant 2016)
- B. Click on the well where the standard is to be imported. It is important to have an internal standard with a known amount of DNA damage. In the Analyze tab, the Quadrant will be adjusted to coincide with this DNA damage.
- C. Go to the standard template and select it and click on the file import
- D. Click on the open workspace
- E. Go to the New Protocol 2016 Patient Results, click on TUNEL results (ex:050516SGResult)

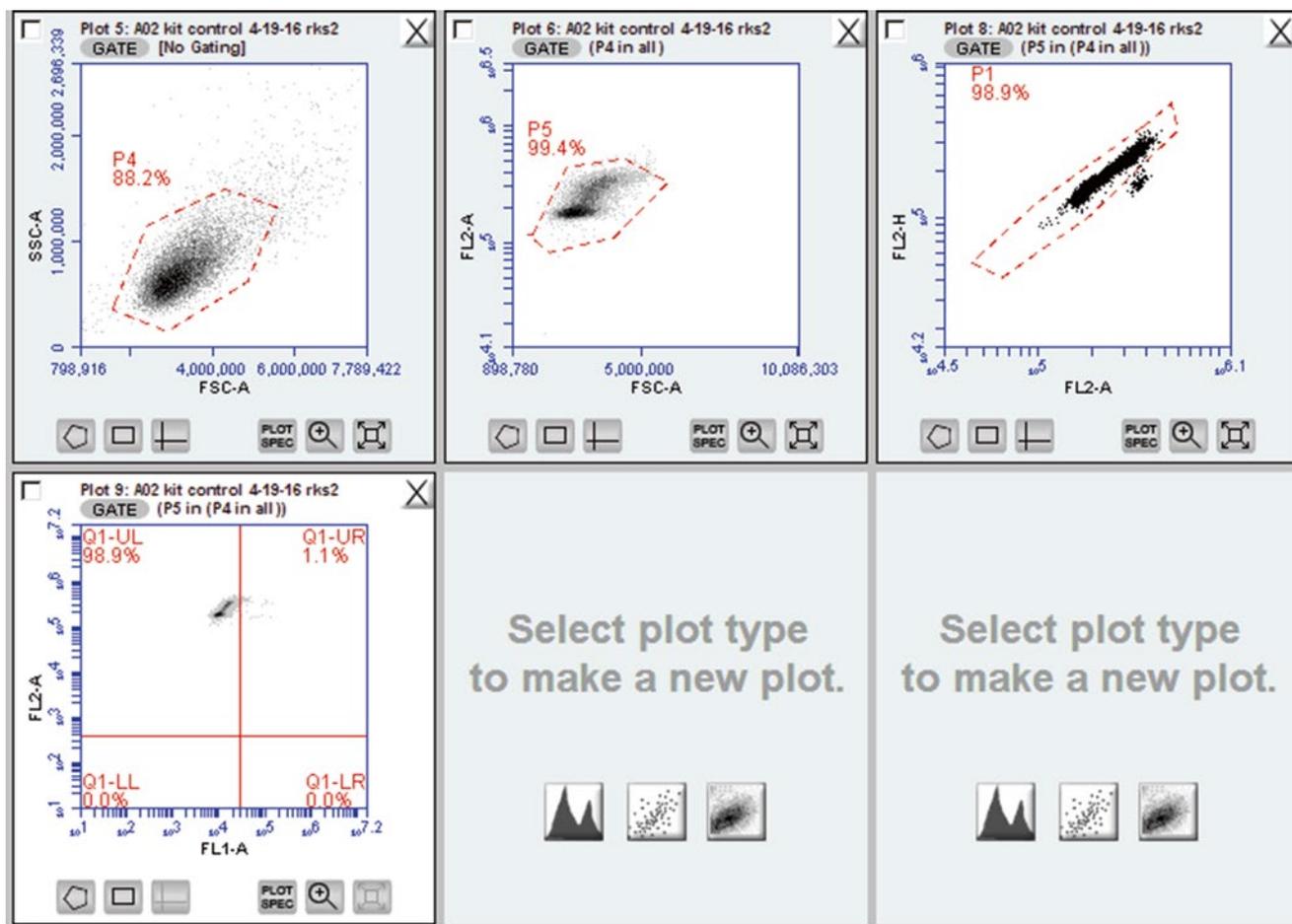


Fig.24.15 Representative plot showing the plots for the Assay kit - Negative sample

- F. Select the negative peak of the standard sample as the standard to be applied to all samples.
- G. Click on F1 well.
- H. Click on the histogram.
- I. Change the X-axis parameter from FSC-A to FL1-A.
- J. Change the gate to P3 in P1 for plot 5. This gate is the same as plot 4, which is a quadrant gate.
- K. Select the vertical line icon at the bottom left of the histogram plot.
Align the selected red line to the center of the histogram to obtain 50% cell population on either side (Fig. 24.18)
Note: Zoom on the histogram for easy alignment of the red bar in the middle of the peak.
- L. Right click on the X-axis and click on virtual gain.
- M. Align the blue line to the center of the peak of the histogram plot (Figs. 24.19, 24.20, and 24.21).
- N. Next, pick the sample to be aligned--for example, A5.
- O. Align the blue line to the center of the peak of the sample.
- P. Click on the “Preview”, “Apply”

- Q. Chose option “Apply” to this sample only and close.

NOTE: DO NOT CHANGE ANY OF THE SETTINGS IN THE FOUR PLOTS (Figs. 24.22, 24.23, 24.24, 24.25, and 24.26). If these are changed, all the values in these plots will also change affecting the result of DNA fragmentation in the final plot 4.

- R. Go to file and save the changes (SAVE WORKSPACE AS A RESULT AND ANALYSIS FILE)

12.4 Data Analysis in Analyze Tab

This strategy is utilized if there is a need to change something only for one patient and it is necessary to create a new set of 3 plots for that patient.

- A. The analysis of the data acquired is done using the Accuri C6 Software in the ANALYZE tab.
- B. When the Analyze tab is opened for the first time, the workspace is empty.

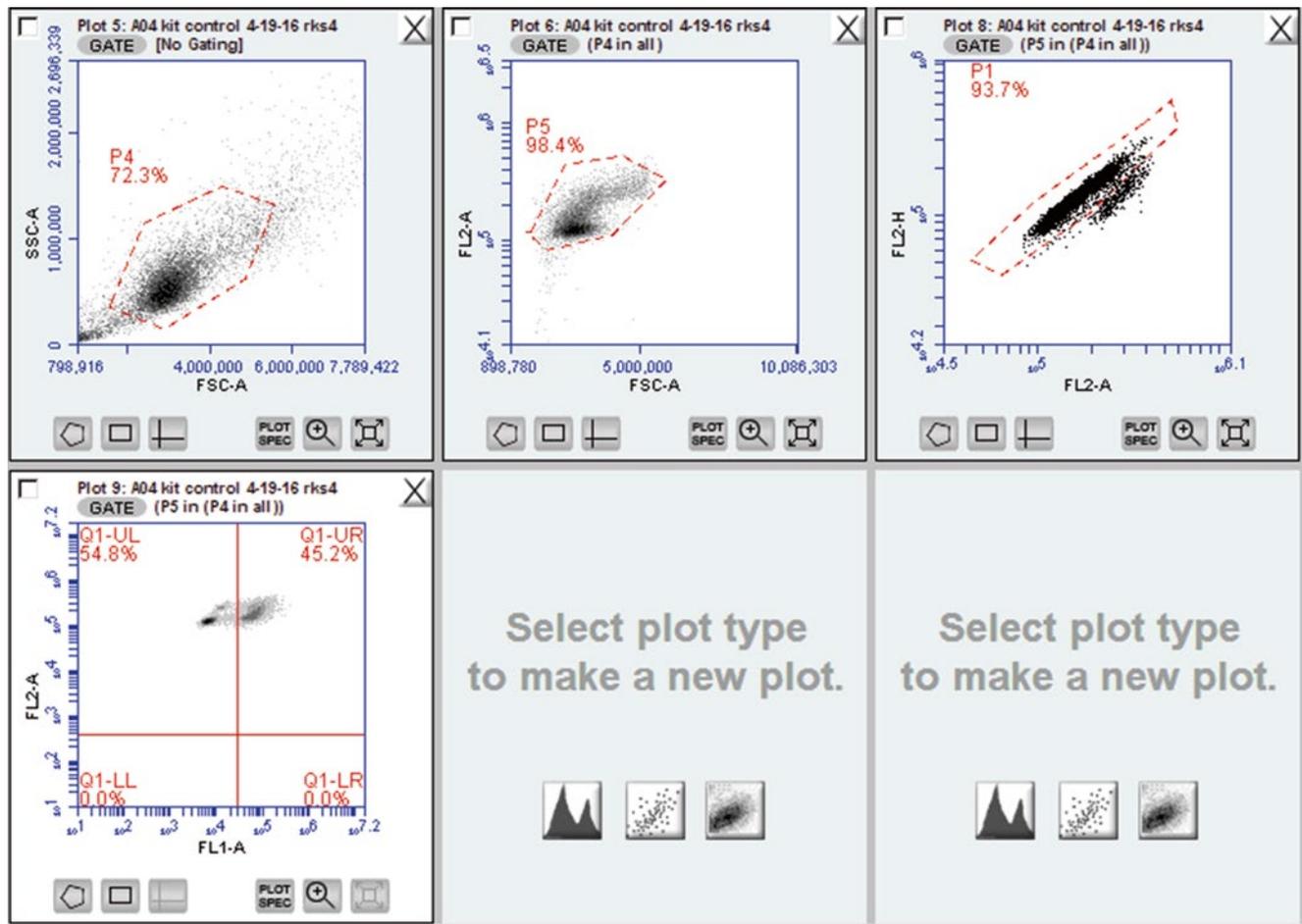


Fig.24.16 Representative figure showing the plots for the Assay kit - Positive sample

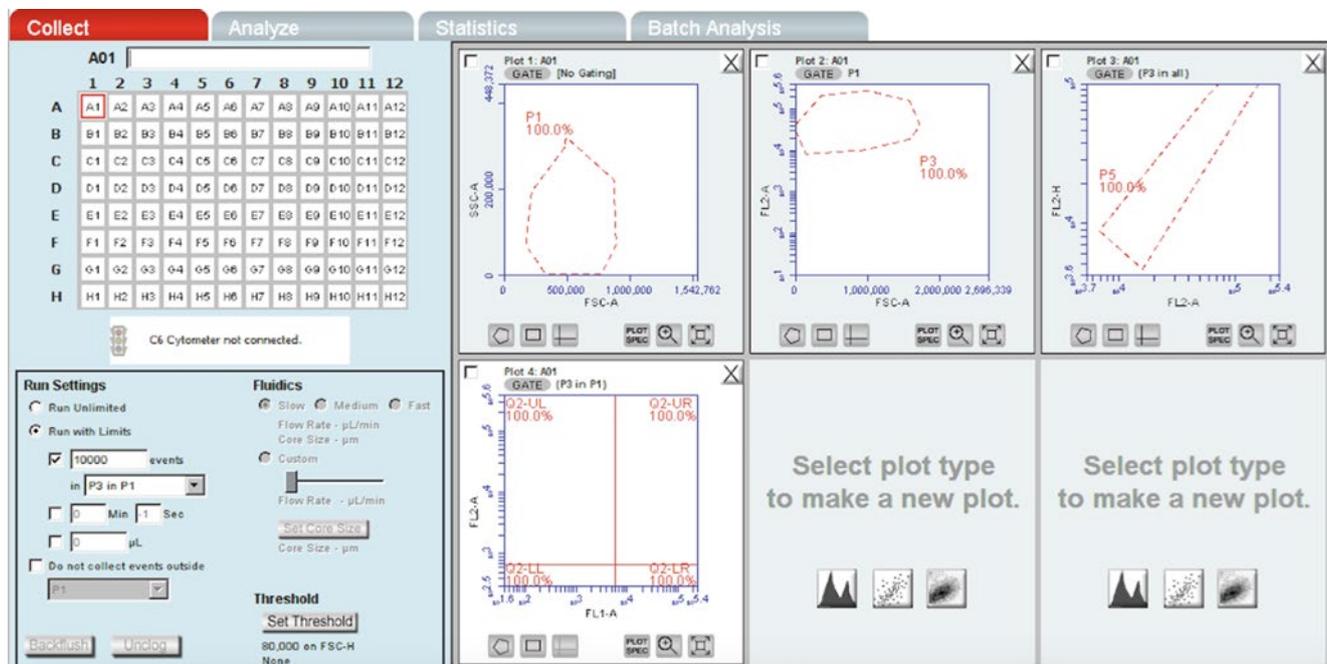


Fig.24.17 Patient sample analysis template showing the gating strategy

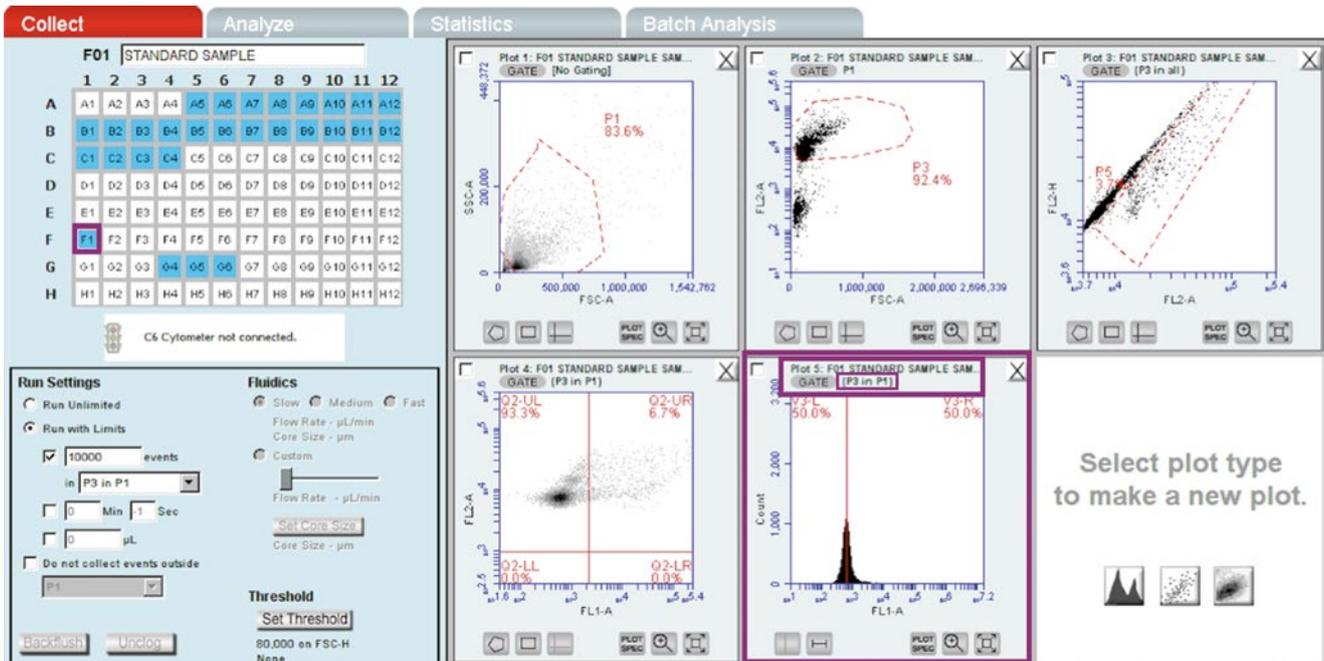


Fig.24.18 Depiction of Standard Sample alignment

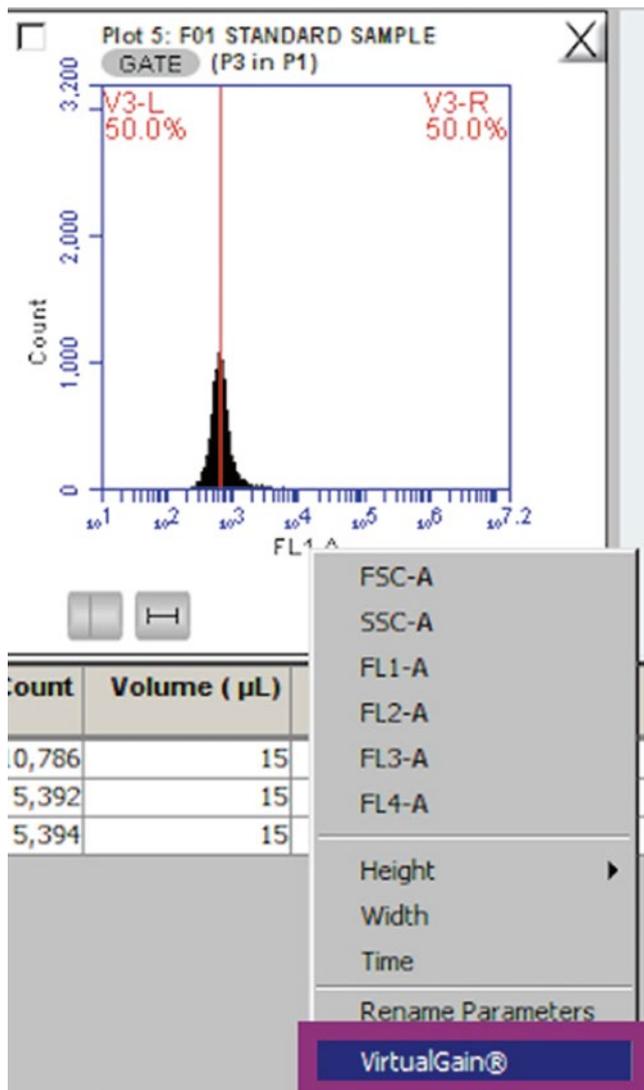


Fig.24.19 Steps in the virtual gain selection of the standard sample

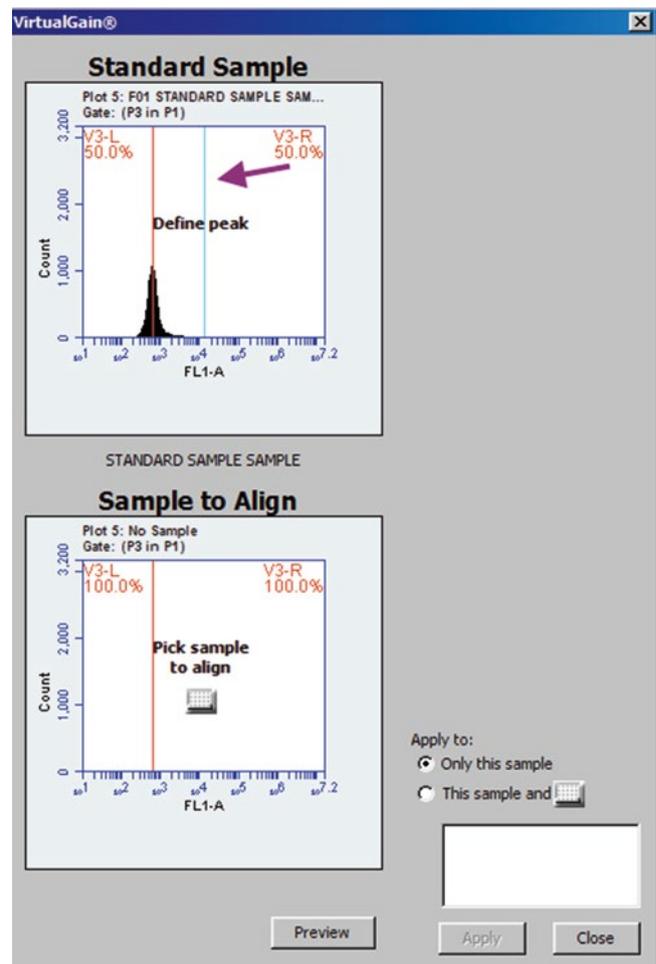


Fig. 24.20 Steps in the insertion of virtual gain in the standard sample

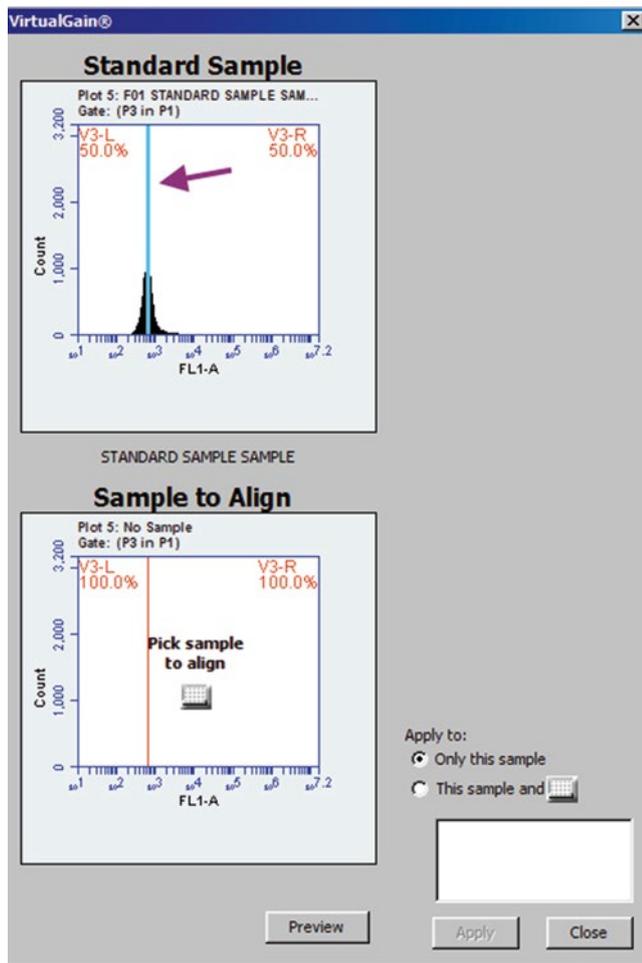


Fig. 24.21 Insertion of virtual gain in the standard sample

Note:

1. The plots are automatically selected from the original template.
 2. Make sure the original gates are used (Fig. 24.17).
 3. To close the plot, do not click on the x at the corner but click in the box with a horizontal line.
- C. Plots need to be copied from the Collect tab:
- D. Gating strategies that were set up in the Collect tab are applied in the Analyze tab as well.
- E. Select the samples acquired and create a three plot group for each sample:
1. FSC-A/SSC-A
 2. FSC-A/FL2-A
 3. FL1-A/FL2-A (Fig. 24.27)
- F. The first plot has no gating and the cell population is P9 (Fig. 24.28).
- G. The gate in the second plot will be P9 in all events. The population is P8 (Fig. 24.29).
- H. The gate in the 3rd plot will be P8 in P9 (in all events) (Fig. 24.30).

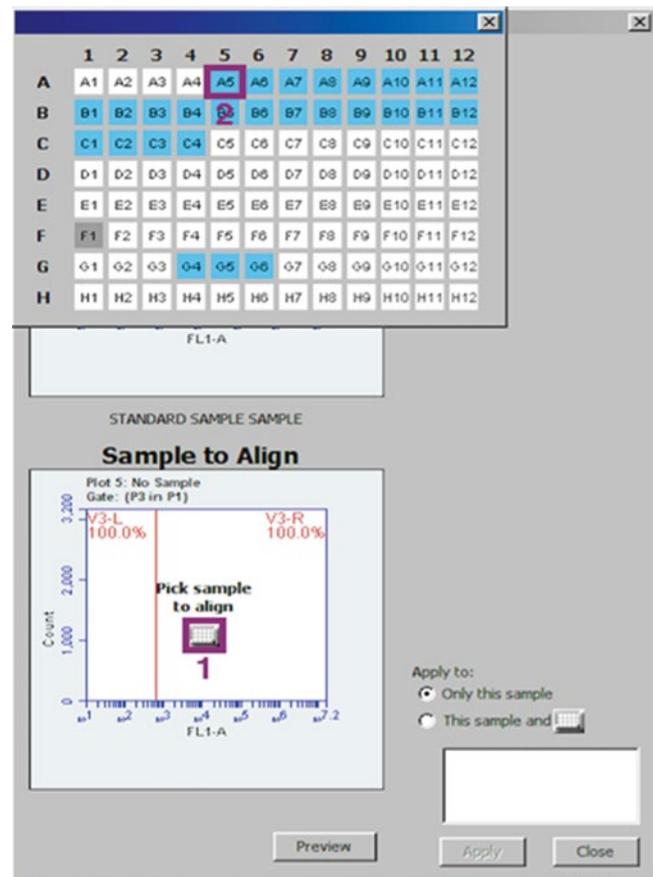


Fig. 24.22 Selecting a sample to align

- I. The adjustment is recorded only in the BD Accuri C6 Software file.
- J. The plots in which Virtual Gain is applied will appear with an asterisk in the FL1 axis (Fig. 24.25, Fig. 24.26 and Fig. 24.31).
- K. The percentage damage is recorded from the FL1-A/FL2-A PLOT
- L. For more information about how to apply Virtual Gain, consult the BD Accuri C6 Software User Guide pages 69-74 (located adjacent to the Accuri cytometer).
- M. Write the preliminary results of the analysis in the TUNEL Laboratory Report Form.
- N. Go to File and save changes (Save workspace as a Result and Analysis file)

12.5 Final Sperm DNA Fragmentation Result Calculation

- A. Calculate the average negative sample value for each patient.
- B. The average value of the negative samples of each patient must be subtracted from the average value obtained from the data analysis. This is done to subtract the autofluorescence in the sample.

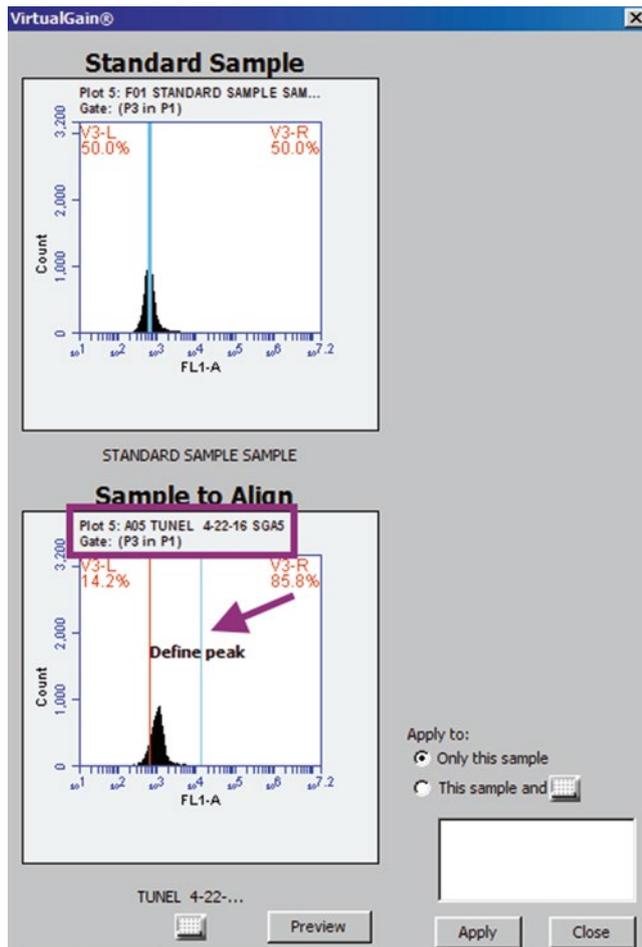


Fig. 24.23 Aligning a test sample to the standard sample.

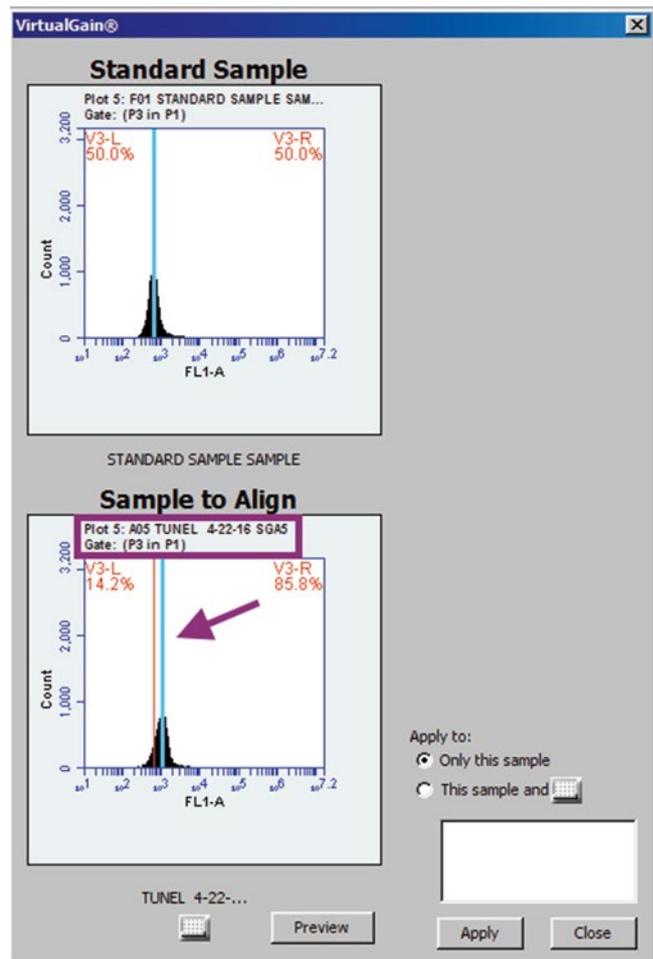


Fig. 24.24 Aligning the test sample to match the standard sample

13 Validation of the TUNEL Test Performed

To confirm that the TUNEL test was correctly performed and that the DNA fragmentation was accurately detected, two conditions must be fulfilled:

- A. The percentage of spermatozoa positive for TUNEL in the spermatozoa positive control sample must be higher than the percentage for the non-control spermatozoa samples.
- B. The percentage of cells positive for TUNEL in the kit positive control cells has to be higher than 40 %.

If both these conditions were verified the assay is considered correct.

14 Cleaning/Maintenance

- A. Cleaning SIP: **Daily**.
 1. Place a blotter or empty sample tube under the SIP to catch dripping fluid.

2. Do one of the following:
 - Click on the *Backflush* button in the *Collect* tab.
 - Select *Instrument > Run Backflush Cycle*.
- B. Decontaminating fluidic line: **Daily**.
 1. Place a tube with 2 mL of 0.22 µm filtered DI water on the SIP.
 2. Select *Instrument > Run decontamination fluid cycle*.
- C. Replace the fluidic bottle filters: **2 months**.
 1. Disconnect quick connect lines from each bottle.
 2. Carefully remove the lid from each bottle.
 3. Disconnect the filter Luer lock at the end of the fluidic tubing. Discard the filter according to standard lab protocols and regulations.
 4. Replace the filter with:
 - Sheath bottle: large disk filter.
 - Cleaner and decontamination bottles: small disk filter.
 5. Reassemble the bottles and reconnect the quick connect lines.

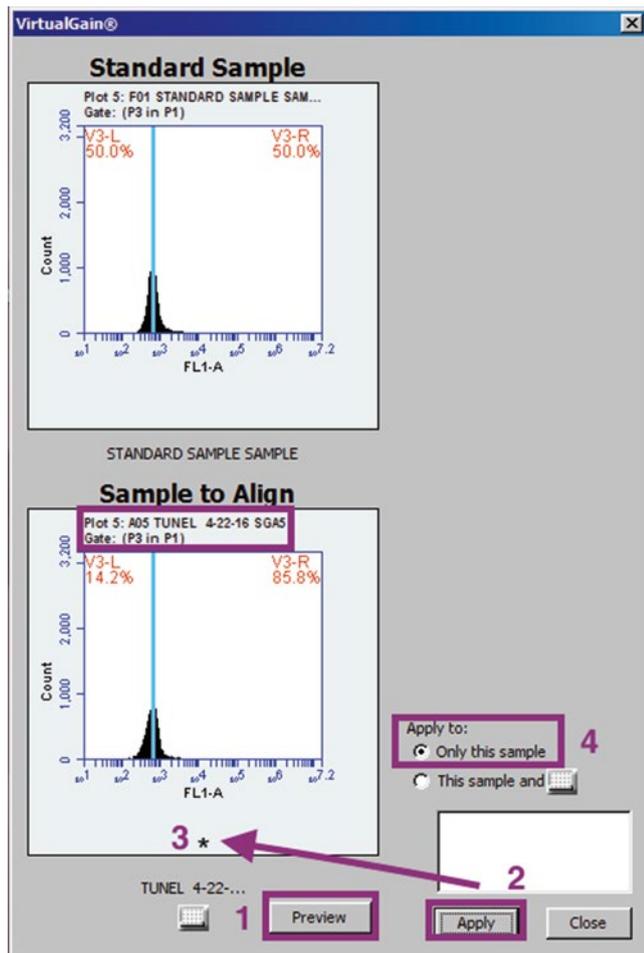


Fig.24.25 Applying the alignment to the test sample. This is indicated by an asterisk at the bottom of the histogram confirming alignment of the sample to the standard file

- D. Replace the in-line sheath filter: **2 months**.
 1. Gently lift the cytometer cover.
 2. Remove the plastic storage compartment.
 3. Twist the Luer locks on the in-line sheath filter to disconnect the locks.
 4. Discard the filter according to standard laboratory protocols and regulations.
 5. Install a new in-line sheath filter. This filter has a male and female end to ensure that it can only be installed in the correct orientation.
- E. Replace peristaltic tubing: **2 months**.

Note: There are two peristaltic pumps: a sheath pump and waste pump. Tubing should be considered hazardous; please take proper precautions when handling the tubing.

 1. Disconnect Luer locks from the peristaltic pumps by unscrewing them. Blue tubing is connected to the sheath pump and red tubing to the waste pump.
 2. Squeeze the grip marks on the pump element retainer clip to remove the clip.
 3. Lift and pull the Luer lock connectors outward and slide the Luer lock fittings off the pump head.
 4. Remove the peristaltic pump tubing and discard according to standard lab protocols and regulations.
 5. Replace with new peristaltic pump tubing by sliding the Luer lock fittings on the pump head.
 6. Snap the pump element retainer clip into place.

Note: Make sure that the blue and red tubes are not twisted, crimped, or over-tightened.
 7. Reconnect the Luer lock fitting on the outside of the pumps.
 8. Gently close the cytometer lid.

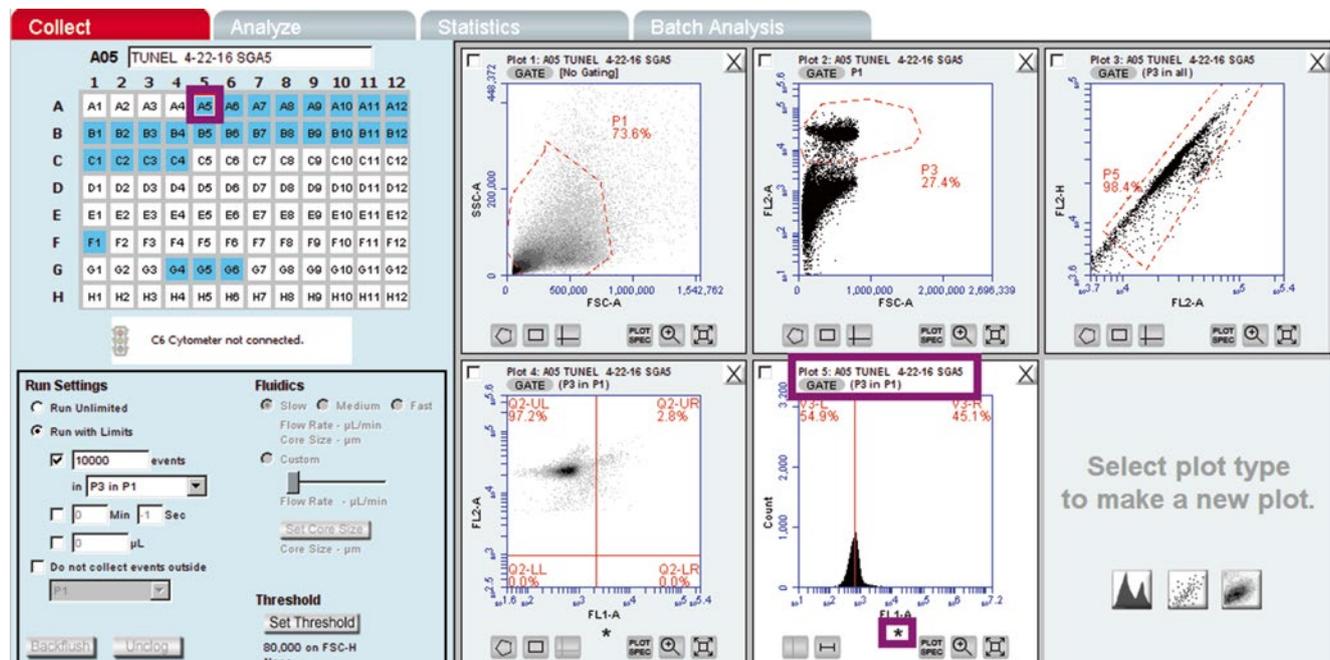


Fig.24.26 Step showing the alignment of the test sample with a star saved under the histogram plot

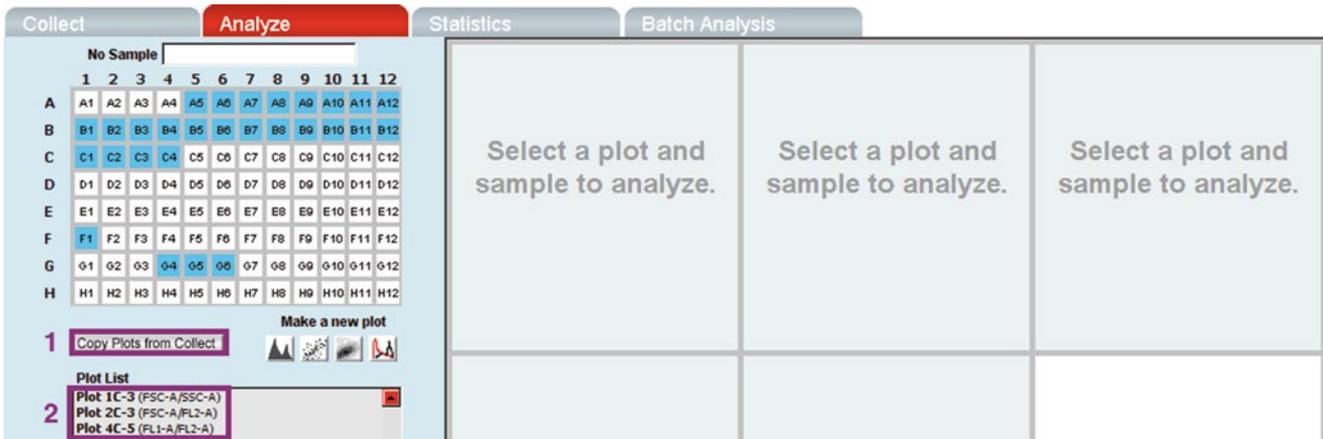


Fig.24.27 Selection of plots from the collect tab in the Analyze tab.

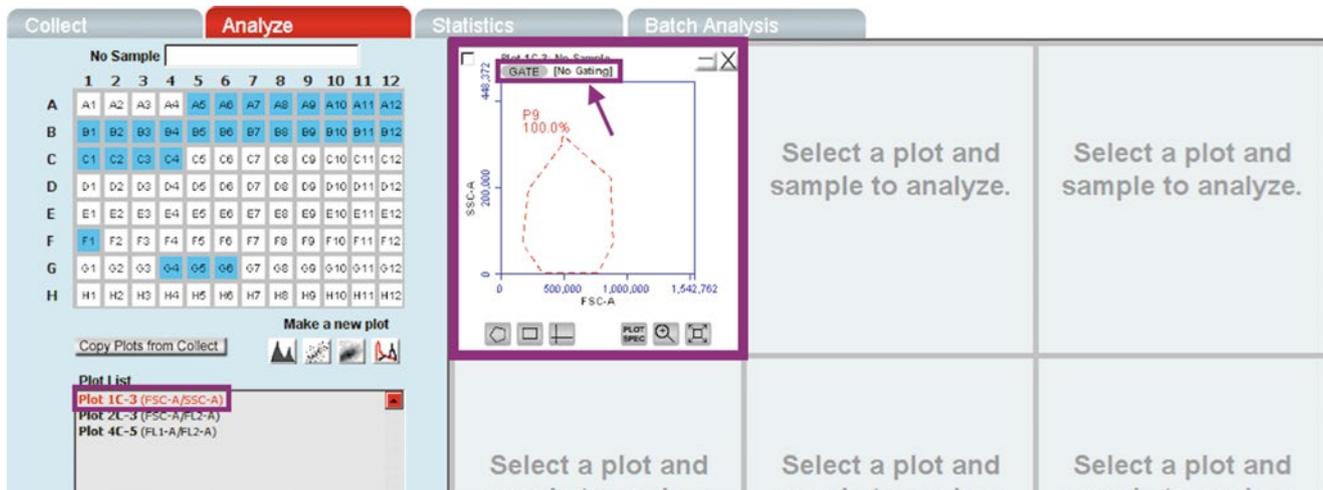


Fig.24.28 Showing first plot with no gating in the Analyze tab.

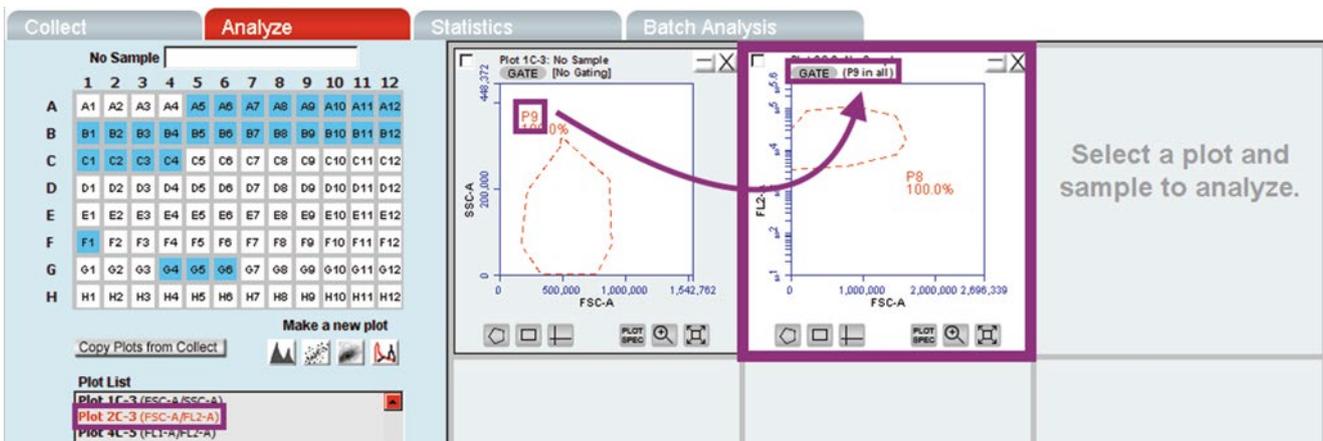


Fig.24.29 Inserting the Gate in the second plot with P9 in all events.

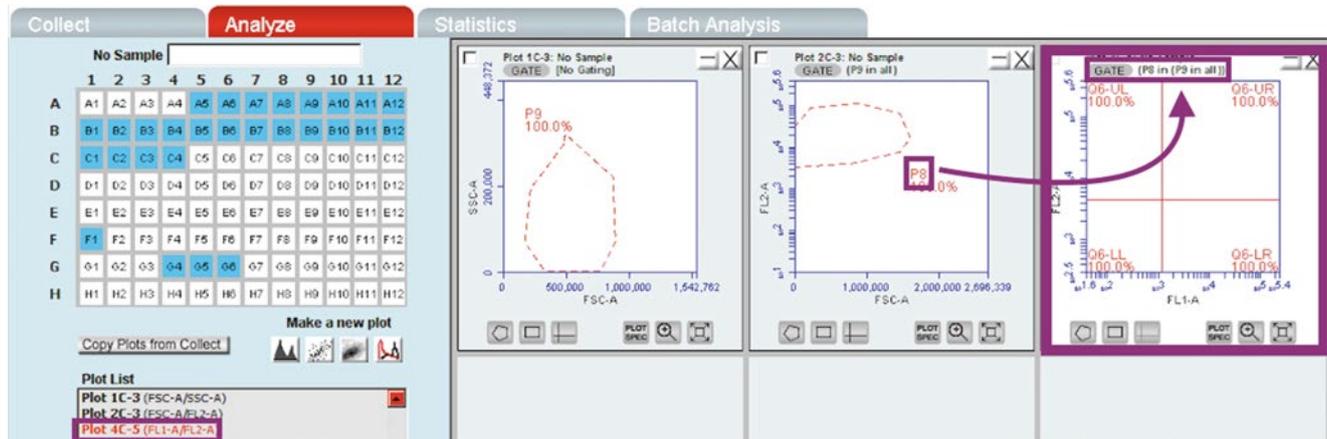


Fig. 24.30 Third plot showing gate P8 in P9 in all events.

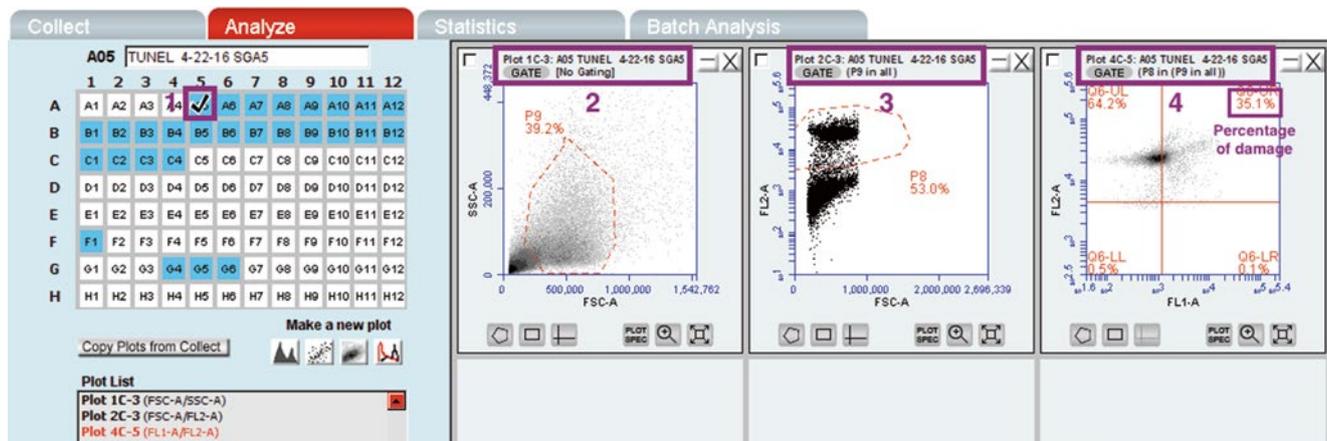


Fig. 24.31 Plot in the Analyze mode showing the percentage of DNA damage in the Upper Right Quadrant (Q6-UR)

15 Reference Values

A cutoff of 12% with >95% specificity can differentiate infertile men with DNA damage from healthy men. The high sensitivity and specificity make this an ideal test.

16 Factors Affecting the Assay Results

Several factors are important to consider when performing this test:

- Accessibility of the DNA.
- Sperm preparation
- Presence of dead cells
- Number of cells examined
- Interobserver and intraobserver as well as inter-assay and intra-assay variations

17 Troubleshooting

- Cleaning the fluid lines of the BD Accuri flow cytometer.
 - With the C6 on, remove the fluidic lines from the sheath, decontamination, and cleaning fluid bottles. Leave the fluid line connected to the waste bottle.
 - Place the fluidic lines in a 500 mL beaker containing approximately 300 mL of hot, but not boiling, DI water. Make sure that the ends of the fluid lines are submerged (Fig. 24.32).
- From the software, run the Decontamination Cycle (Instrument → Decontamination Cycle) at least two times. Replenish hot water, if necessary.
- Return the fluidic lines to their respective bottles and run the Decontamination Cycle again.
- Replace the in-line sheath filter.
- Run validation beads or samples to check the consistency of the fluidics.



Fig. 24.32 Fluidic lines placed in 500 mL beaker of hot water before running decontamination cycle to clean the lines

B. Using the SIP wire to clear the SIP

1. Locate the black knurled retaining ring at the top of the SIP and remove it by turning counterclockwise. Slide the ring off the SIP.
2. Grasp the upper black portion of the SIP and pull it straight down to remove (it may be difficult as it is a friction fit). Inspect the top of the SIP to ensure that the small O-ring is still in place.

3. Insert the wire into the hole in the bottom (sample end) of the SIP and slide it all the way through. If you feel resistance, repeat the procedure until it slides freely.
- #### C. Rinse and Reinstall the SIP
1. Locate the inlet port on the side of the black portion of the SIP. Press the nozzle of a squirt bottle containing DI water against the port and flush the SIP—a stream of water will shoot from the top of the SIP.
 2. Using the squirt bottle, squirt some water into the flow nozzle (hole where the SIP was removed).
 3. Replace the SIP. Note that there is a guide pin that allows the SIP to be installed in only one orientation.
 4. Replace the black knurled retaining ring and ensure that it is secured firmly (hand tight).
 5. Perform backflush and unclog a few times each.
 6. With a tube of water on the SIP, run water for 5 min on “fast.”
 7. Remove the tube from the SIP and perform backflush and unclog a few times each.
8. Rerun the 6-peak and 8-peak beads on “slow” (50,000 events).

Note: If the clog is aggressive and rinsing does not help, the SIP can be placed in a sonicator for a minute. Flush SIP with water with pressure using a syringe or transfer pipette with a fine end pipette tip. If necessary, repeat this step a couple of times until water flows freely through the SIP.

18 Unscheduled Maintenance

Note: This may be performed to troubleshoot common problems.

(See Table 24.3).

Table 24.3 Troubleshooting options for common problems

Procedure	When to do	What it does
Backflush	Clog in the SIP	Purges fluid from the below the flow cell out of the SIP
Unclog	Clog in the flow cell	Purges fluid from above the flow cell out of the SIP
Extended clean of flow cell	Large amounts of debris after a cleaning cycle	Allows the cell to soak in the extended flow cleaning solution
Purging air from the cytometer	Setting up the cytometer for the first time Cytometer not used for more than 2 months Air may have been introduced in the system	Removes air from the system after long periods of nonuse Runs sheath through the system
Hot water flush	If clog and backflush do not eliminate the clog	Runs hot water through the system
Fluidic cleaning routine	Large amount of background present	Runs decontaminating fluid, cleaning fluid, and sheath fluid through the sheath lines
SIP rinse	Unclog and backflush do not eliminate the clog	Flushes sheath through the SIP

References

1. Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A. TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology*. 2010;2010(76):1380–6.
2. Sharma RK, Agarwal A. Laboratory evaluation of sperm chromatin: TUNEL assay. In: Zini A, Agarwal A, editors. *Sperm chromatin: biological and clinical applications in male infertility and assisted reproduction*. New York, NY: Springer Science + Business Media; 2011. p. 201–15. Chapter 14.

DNA Fragmentation in Spermatozoa (TUNEL) BD Accuri™ C6 Flow Cytometer

Procedure

DNA fragmentation, both single and double stranded can be measured by the TUNEL assay using a bench top flow cytometer and the apoptosis detection assay kit. The reagents used for instrument and assay quality control are described below.

Note: Only approved fluids should be used in the operation or cleaning of the instrument, that are recommended and provided by the manufacturer (Figure 1). The various reagent bottles that are attached to the flow cytometer are color coded and described below:

Instrument Reagents

- Sheath fluid (Blue bottle) – 0.22 µm filtered, deionized water with, or without, bacteriostatic concentrate solution:
If bacteriostatic concentrate solution (Figure 1) is used (optional), add 1 bottle per 1 L of water concentrate solution, diluted:
- Cleaning solution (Green bottle) cleaning concentrate solution, diluted:
Add 3 mL of cleaning concentrate (Figure 1) to 197 mL of filtered deionized water
Solution should be used within 2 weeks
- Decontamination solution (Yellow bottle) decontamination concentrate solution, diluted:
Add entire bottle (Figure 1) to 180 mL of filtered, deionized water
- Extended flow cell clean extended flow cell cleaning solution provided in working concentrate (Figure 1)



Figure 1. Decontamination concentrate, cleaning concentrate, extended flow cell clean and bacteriostatic concentrate solutions.

- APO-DIRECT™ Kit (BD Pharmingen, Catalog #556381):
The following reagents are necessary to run the TUNEL assay:

- Paraformaldehyde (3.7%) (Figure 2)
- Positive (Brown Cap) and Negative kit (White Cap) controls (Figure 3)
- Wash Buffer (Blue Cap) (Figure 4)
- Rinse Buffer (Red Cap) (Figure 4)
- PI/RNase staining buffer (Figure 5)



Figure 2. Bottle of 3.7% paraformaldehyde in PBS



Figure 3. Positive and negative controls



Figure 4. Wash and Rinse Buffer



Figure 5. Bottle of PI/RNase staining Buffer

Preparation of Paraformaldehyde:

Add 90.0 mL of PBS (pH 7.4) to 10.0 mL of formaldehyde (37%).

Note: Store at 4°C.
F. Ethanol (70%)

Ethanol (70%):

Add 30 mL of distilled water to 70.0 mL of ethanol (>99% vol./vol.).

Note: Store at 4°C.

Staining reagents consist of 3 vials (Figure 6)

- Reaction buffer (green cap); TdT enzyme (Yellow cap) and FITC-dUTP (Orange cap)



Figure 6. Reaction buffer, TdT enzyme and FITC-dUTP vials

- Flow cytometer (Fluorescence activated cell sorting caliber, Becton and Dickinson, San Jose, CA) (Figure 7)



Figure 7. View of the bench top flow cytometer

I. Semen Sample Preparation for Tunel Assay

- A. Following liquefaction, evaluate semen specimens for volume, round cell concentration, sperm concentration, total cell count, motility, and morphology.
- B. Adjust the sperm concentration to 2-5 x10⁶ / mL.
A negative and a positive sample must be included with each batch run. The negative control is prepared by omitting the TdT from the staining solution.
 - i. Prepare a hydrogen peroxide diluted solution (1:15 dilution) from the stock of the Andrology Laboratory (Hydrogen Peroxide 30%) by adding, for example, 100 µl of the stock to 1400 µl of PBS 1X.
 - ii. Resuspend the spermatozoa of the tube "Surname, Name, positive control" in 1 ml of the diluted hydrogen peroxide solution.
 - iii. Place the tube in the heater at 50°C for one hour
 - iv. Centrifuge for 7 minutes at 1600 rpm.
 - v. Remove the supernatant and replace with 1ml of PBS.
 - vi. Together with the test and the negative samples, centrifuge for 7 minutes at 1600 rpm. Remove the supernatant and proceed to FIXATION and PERMEABILIZATION.

- C. Add 1.0 mL of 3.7% paraformaldehyde to the tube, vortex it and store it in the refrigerator at of 4°C.

II. Preparation for Tunel Staining

- A. Remove the paraformaldehyde by spinning the samples at 1600 rpm for 7 min. Discard the supernatant and resuspend with 1 mL of ice-cold ethanol (70% vol./vol.) for at least 30 minutes. Samples can also be batched and kept at -20°C until ready for TUNEL staining.

III. Staining Protocol

Preparation of Kit Controls and Internal Test Samples:

- A. Vortex the negative (White cap) and positive (Brown cap) samples provided in the kit.
Note: Verify the catalog numbers and the cap color to match each vial.

- B. Mix the contents of each vial by vortex. Remove 2 mL aliquots of the control cell suspensions (approximately 1 x 10⁶ cells/mL) and place in 12 x 75 mm centrifuge tubes.

- C. Return the vials to -20°C.

- D. Centrifuge at 1600 rpm for 7 min and discard the supernatant.

- E. Centrifuge the control cell suspensions for 5 min at 1600 rpm and remove the 70% (v/v) ethanol by aspiration, being careful to not disturb the cell pellet.

- F. To the control and test samples, add 1.0 mL of Rinse Buffer (6548AZ) (blue cap) and vortex. Centrifuge as before and discard the supernatant.

- G. Repeat the Wash Buffer treatment. Centrifuge and discard the supernatant.

- H. Number the tubes consecutively beginning with negative and positive kit controls, test samples and internal controls.

IV. Staining for Tunel Assay

- A. Check the number of tubes that will be required for the TUNEL assay. It is helpful to prepare the stain for an additional 5 to 7 tubes.

- B. Remove the reaction buffer (green cap) from 4°C and the TdT (yellow cap) and FITC-dUTP (orange cap) from -20°C and place them at 37°C for 20 min to thaw.

- C. Prepare the stain as shown in the Table 1 for 1 assay and calculate the required volumes. Always prepare an additional 4 to 5 tubes to ensure that adequate stain is available for all the tubes.

- D. Add the stain in the same sequence as shown in the table.

Note: The preparation of the stain and all subsequent steps must be carried out in the dark.

Table 1. Preparation of the Staining solution

Staining Solution	1 Assay	6 Assays	12 Assays
Reaction buffer (green cap)	10.00 µL	60.00 µL	120.00 µL
negative sample (yellow cap)	0.75 µL	4.50 µL	9.00 µL
FITC-dUTP (orange cap)	8.00 µL	48.00 µL	96.00 µL
Distilled H ₂ O	32.25 µL	193.5 µL	387.00 µL
Total volume	51.00 µL	306.00 µL	612.00 µL

- E. Return the stains to appropriate storage temperature.

Note: Mix only enough Staining solution to complete the number of assays prepared per session. The Staining solution is good for approximately 24 hr at 4°C.

- F. Resuspend the pellet in each tube in 50 µL of the Staining solution.

Note: The same tip can be used to add the stain as long as the stain is added on the side of the tube and the tip does not come in contact with the solution.

- G. Incubate the sperm in the Staining solution for 60 min at 37°C. Cover the tubes with aluminum foil.

Note: Record the incubation time on the aluminum foil.

- H. At the end of the incubation time, add 1.0 mL of Rinse Buffer (cat 6550AZ red cap) to each tube and centrifuge at 1600 rpm for 7 min. Discard the supernatant.

- I. Repeat the cell rinsing with 1.0 mL of the Rinse Buffer, repeat centrifugation and discard the supernatant.

- J. Resuspend the cell pellet in 0.5 mL of the PI/RNase Staining Buffer.

- K. Incubate the cells in the dark for 30 minutes at room temperature.

- L. Number the tubes according to the sample list. Cap the tubes and carefully cover the tubes with aluminum foil. The tubes are now ready to be analyzed.

Note: The cells must be analyzed within 3 hours of staining. Cells may begin to deteriorate if left overnight before analysis.

Basic Instrument Set Up

- A. 8-Peak Validation Beads – 2 Bottles (Figure 8)



Figure 8. Bottle of 8 Peak Validation Beads for Quality Control of the instrument

Basic Instrument Instructions / Setup

Caution: A tube of 0.22 μm -filtered deionized (DI) water should be placed on the SIP at all times to keep the SIP from drying out—before use, during use and even after the machine is shut down.

First-Time Setup:

- A. Make sure the sheath bottle (Blue bottle) is filled with sheath fluid.
- B. Dilute cleaning concentrate as well as the decontamination concentrate per the above instructions then fill the Green bottle with the cleaning solution and Yellow bottle with the decontamination solution.
- C. Make sure all bottles are closed securely.
- D. Add 100 mL of 0.5% NaOCl to the waste bottle (Red bottle).

General Setup:

Note: All boxes should be deselected or “unchecked” before any boxes are selected or “checked.”

1. Open the software by double-clicking the “BD Accuri C6 software” icon on desktop.
2. Check the fluid levels in all bottles. The waste should be empty and the sheath, cleaner, and decontamination bottles full.
3. Pull the sample stage forward underneath the SIP.

Note: The sample stage accommodates any brand of 12x75 mm tube and most microcentrifuge tubes.

Be careful not to bend the SIP when inserting tubes.

4. If no tube is currently on stage, place a tube with 0.22 μm -filtered deionized (DI) water (Figure 9).



Figure 9. Tube filled with 0.22 μm DI water placed on SIP

5. Firmly press the power button on the front of the cytometer unit.
 6. While starting up the BD Accuri software “traffic light” should turn yellow and the pumps should start to run.
 7. Wait 5 minutes for the machine to flush the fluidics line with sheath fluid.
- Warning:** Do not open the lid of the cytometer during this time. This will disrupt the laser warm-up process.

8. The BD Accuri software “traffic light” will turn green and displays “C6 is connected and ready” when complete.
9. To remove bubbles from the system, place a tube of 0.22 μm -filtered DI water on the SIP.

10. Select “run with limits” and set to 15 min.
11. Select “fluidics” speed to “fast.”
12. Click the “RUN” button.
13. Save file as “flush.”

Note: Save location is not important. This file can be deleted at any time.

14. Leave tube on SIP.

Note: Validate the performance of the cytometer using the QC beads designed for the instrument, provided by BD before processing any samples.

Running Kit Controls

Note: Kit controls are run under the “Collect tab”. All data should be saved in the folder with the following format:

“Kit Controls-DATE-TECH INITIALS”

- A. Double-click “Kit Control Template” on the share drive.
- B. Select well “A1” (move to adjacent cell if occupied).
- C. In field “A1” type “kit negative-date-tech initials.”
- D. Set the run parameters as follows:
 1. “Run with limits”: 10,000 events
 2. “Fluidics” speed: slow
 3. Threshold: 80,000 on FSC-H
- E. Remove tube of DI water from the SIP.
- F. Vortex and place negative control on the SIP.
- G. Click “RUN” button.
- H. Run will finish after collecting 10,000 events.
- I. Data will populate in plots 1 to 4.

The 4 plots are:

1. Plot 1: FSC-A/SSC-A
2. Plot 2: FSC-A/FL3-A
3. Plot 3: FL2-A/ FL2-H
4. Plot 4: FL1-A/FL2-A

- J. Select well “A2” and repeat steps C-I.
- K. Remove negative kit control from SIP/stage.
- L. Clean SIP with lint-free wipe.
- M. Put positive control tube on SIP.
- N. Click well “A3.”
- O. Name “A3” as “kit positive-date-tech initials.”
- P. Repeat steps D-I.
- Q. Select well “A4” and repeat steps for positive control run (steps O & P).

Basic Instrument Set Up

Data Acquisition

Kit Controls (Kit Control Template):

Note: Maintain a written record of all results.

- Under the collect tab, click on well A1 for the first negative kit control.
- Observe the graph for the negative control.
- The last plot is a Quadrant: Lower left (Q-L); Lower right (Q-LR); Upper left (Q-UL) and Upper Right (Q-UR). Observe only the percent positive (FITC+) value in the upper right quadrant (Q-UR).
- Record the result in the following template:

TUNEL Kit Results\KitControl\Results.xlsx

- Click on well A2 and follow steps B-D for the second negative kit control.
- Click on well A3 for the first positive kit control.
- Observe the graph for the positive kit control.
- Follow steps C-D for the positive kit control.
- Click on well A4 for the second positive kit control and repeat steps C-D.

Running Patient Samples

Note: A separate template is created for the patient samples. Patient samples are run under the "Collect tab":

All data should be saved in a folder with the following format:

"Tunel-Date-Tech Initials"

- Double-click on the "Assay Template."
- Wait for the software to load.
- Check each well to ensure no data already exists inside
- If necessary, click "delete events" for any wells which contain data.
- Select well "A5."
- Begin with tube #5 (first test sample).
- Remove DI water tube from the SIP.

Validation of the TUNEL test performed

- The percentage of spermatozoa positive for TUNEL in the spermatozoa positive control sample has to be higher than the percentage for the non-control spermatozoa samples.
- The percentage of cells positive for TUNEL in the kit positive control cells has to be higher than 40%.

If both these conditions were verified the assay is considered correct.

Note: The automatic shutdown cycle can be bypassed by pressing down the power button for 5 seconds. However, the cytometer will take additional time to recover and return to the steady state if it is shut down in this manner. The BD Accuri software will display the following message if the machine is shut down using this method:

Note: It is unnecessary to shut down the software or computer during the shutdown process.

Machine Shutdown

- Place a tube with 2 mL of bleach (diluted decontamination solution) on the SIP.
- Select an empty "data well" in the collect tab of the BD Accuri software.
- Set a time limit of "2 min" and set fluidics speed to "fast."
- Click the "RUN" button.
- Once the run is finished, remove the tube from the SIP.
- Place a tube with 0.22 µm-filtered DI water on the SIP and select another empty "data well" in the BD Accuri software.
- Repeat steps #3 and #4, above.
- When the run is finished, leave the tube on the SIP.
- Press the power button to start the shutdown cycle.
- The cycle will take 15 minutes to complete then the cytometer should automatically shut down.

H. Vortex the tube (test sample) and place on the SIP.

I. In the name field, name the field as "TUNEL-SAMPLE#-DATE-TECH INITIALS."

J. Set the run parameters as follows:

- "Run with limits": 10,000 events
- "Fluidics" speed: slow
- Threshold: 80,000 on FSC-H
- Gate P1 in P3.

K. Click the "RUN" button to start the collection.

L. After 10,000 events the run will finish.

M. Remove tube from SIP and clean the SIP with a lint-free paper.

N. Vortex and place the subsequent tube on the SIP.

O. Select the next well (A6, and so on) for the new sample.

P. Repeat steps I-O until all samples have been analyzed.

Q. Remove final tube and place the "bleach tube" on the SIP.

R. Set the parameters as follows:

- "Run with limits": 2 min
- "Fluidics" speed: fast
- Threshold: 80,000 on FSC-H
- Click the "RUN" tab.

S. When the run is finished, wipe SIP.

T. Remove tube and replace with DI water tube.

V. Repeat steps Q-S with DI water.

W. Proceed to shutdown step.

Test Samples (Assay Template)

A. Select the average value of the test sample to be used for applying the gate to all other samples. Select the negative peak of patient test sample as the standard to be applied to all samples.

B. Click on a new plot.

C. Click on the histogram.

D. Change the X-axis parameter FSC-A to FL1-A

E. Right click on the X-axis and click on virtual gain.

F. Align the blue line to the center of the peak of the histogram plot.

G. Next pick the sample to be aligned for example A5.

H. Click on the preview, apply and close.

I. Obtain reading for each sample, click on the well.

J. Upper right quadrant will display the percentage DNA fragmentation.