Inter-and Intra-Laboratory Standardization of TUNEL Assay for Assessment of Sperm DNA Fragmentation

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The functional aspects of sperm activity such as sperm chromatin integrity and ability to fertilize cannot be characterized by routine semen parameters. Men with unexplained infertility and idiopathic infertility, as well as men with normozoospermic semen profiles, show high DNA fragmentation. Molecular anomalies in the sperm can be detected by a sperm DNA fragmentation (SDF) assay which can be used in adjunct to conventional semen analysis. While the sperm chromatin structure assay (SCSA) remains the “gold standard,” the TUNEL assay using flow cytometry is becoming popular among the different tests that are currently available to measure sperm DNA fragmentation. In this unit, we describe the inter-laboratory and intra-laboratory standardization of the TUNEL assay using a benchtop cytometer. The article also provides a step-by-step protocol for measuring sperm DNA fragmentation using the TUNEL assay and a bench-top flow cytometer, and also points out the inherent challenges with this test. © 2017 by John Wiley & Sons, Inc.

Keywords: Accuri C6 • flow cytometry • DNA fragmentation • sperm • TUNEL

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INTRODUCTION
Male reproductive dysfunction is a key issue and public health concern that leads to reproductive disorders such as male infertility, miscarriages, recurrent pregnancy loss, and anomalies of the newborn (Aitken, Smith, Jobling, Baker, & De Iuliis, 2014). The poor predictive value of conventional semen analysis is attributed to the high inter- and intra-observer variability in assessment of routine semen parameters such as sperm count, motility, and morphology (Guzick et al., 2001; Keel, 2006; Tielemans, Heederik, Burdorf, Loomis, & Habema, 1997), and also to inherent variability in the semen parameters within subjects (Keel, 2006). In this unit, we present step-by-step protocols established for the measurement of sperm DNA fragmentation (SDF) in human spermatozoa using a benchtop flow cytometer. We provide the details of sample collection, processing, and staining using the TUNEL assay. Also included are steps for data acquisition and analysis, and details of quality control for the cytometer. These steps should enable other andrology laboratories to follow the protocols and standardize them in their laboratory settings, and establish reference ranges for the TUNEL assay applicable to their patient population.
Importance of DNA Fragmentation in Male Infertility

Clinical relevance with ART outcomes

Molecular biology techniques have evolved over the past few decades. There are several state-of-the-art techniques that are utilized to assess sperm DNA fragmentation (Irvine et al., 2000). The assessment of SDF or DNA integrity is a reflection of the integrity of the genetic material that a spermatozoon is going to transmit to the offspring. Sperm DNA fragmentation is predictive of both natural and assisted fertility. Elevated levels of sperm DNA fragmentation assessed by SCSA were associated with a high odds ratio of failure to conceive naturally (Zini, 2011), as well as a longer time interval to conception or reduced fecundity for couples planning their first pregnancy (Spano et al., 2000). SDF assessment is proposed to be an integral part of the algorithm for management of infertile couples. SDF affects ART outcomes with impact on all types of assisted conception techniques such as, IUI, IVF, and ICSI, being associated with high pregnancy loss after IVF or ICSI (Duran, Morshedi, Taylor, & Oehninger, 2002; Jin et al., 2015; Zhao, Zhang, Wang, & Li, 2014).

STRATEGIC PLANNING

Validation of the Inter- and Intra-Laboratory Standardization of the TUNEL Protocol

Several literature reports have highlighted the need for standardization of the TUNEL assay across laboratories as well as within a laboratory (Muratori et al., 2008; Ribas-Maynou et al., 2013; Sharma et al., 2010; Sharma, Ahmad, Esteves, & Agarwal, 2016; Sharma, Masaki, & Agarwal, 2013). This is due to large variability reported in terms of diagnostic accuracy and precision of the sperm DNA fragmentation results assessed by the TUNEL assay in different studies. The differences in results obtained across different laboratories are due to different techniques of sample preparation, fixation, and protocols for staining, and differences in the instruments, as well as in the instrument settings for data acquisition and analysis.

Conducting the standardization experiment requires that two to three measurements for each subject be planned and recorded. Two independent observers are required to conduct the measurements both from the same subjects and from different subjects. Evaluation of inter- and intra-observer variability is done using all TUNEL measurements (Sharma et al., 2010). The difference in the results between the two observers (inter-observer variability) is analyzed by assessing the difference in the values between the two observers. The differences in the different readings by the same observer for the same samples need to be calculated to record intra-observer variability. Our group has established the reference ranges for the TUNEL assay to discriminate between infertile and fertile males (Sharma et al., 2010). We published a study on validation of the benchtop flow cytometer for TUNEL assay (Sharma et al., 2016; Sharma et al., 2010). We have also reported the standardization of the TUNEL assay across laboratories and validated TUNEL as a reproducible and robust assay within and across laboratories (Ribeiro et al., 2017). This study was performed to standardize the TUNEL assay in two established laboratories across two continents. The same samples were assessed by two independent, experienced observers. Furthermore, identical TUNEL protocols and lot numbers of kits, as well as similar acquisition settings with an identical template, were utilized for the standardization, with comparable results for both labs. We have established, for the first time, a standardized protocol for use in different andrology laboratories across the globe.

LABORATORY MEASUREMENT OF SPERM DNA FRAGMENTATION BY TUNEL ASSAY USING BENCHTOP FLOW CYTOMETER

This TUNEL assay protocol defines the procedure for sperm DNA fragmentation assessment (Figure 16.11.1) with the benchtop BD Accuri C6 flow cytometer (Figure 16.11.2;
Agarwal, Gupta, & Sharma, 2016). Apoptosis in the spermatozoa results in activation of endonucleases, and these enzymes induce sperm DNA fragmentation (Muratori, Marchiani, Maggi, Forti, & Baldi, 2006). High-order sperm chromatin is broken down into smaller DNA fragments of ~50 kb by the endonucleases. The DNA strand breaks are labeled by the fluorescein isothiocyanate deoxyuridine triphosphate (FITC-dUTP) stain. This is accomplished in the presence of the template-independent enzyme terminal deoxynucleotidyl transferase (TdT), which helps transfer the deoxyribonucleotides to the 3'-hydroxyl (3'-OH) end of the single- and double-strand breaks. The intensity of labeling is proportional to the number of DNA strand break sites.

**Collection of Semen Specimen**

**Materials**
- Donor of test specimen
- Viscosity treatment enzyme, e.g., chymotrypsin (optional)
Semen analysis form
Wide-mouth, sterile plastic specimen container

1. Collect the semen sample after a minimum of 48 hr and not more than 72 hr of sexual abstinence. A notation is made of the patient name, medical record number, and days of abstinence on the semen analysis form.

2. The sample should be collected by masturbation only. It should be collected into a clean, wide-mouth sterile plastic specimen container. All lubricants should be avoided.

3. Make a note on the semen analysis worksheet of any unusual collection conditions for the sample or any unusual findings such as sperm agglutination, high viscosity, or large amount of cellular debris. The sample should be incubated at 37°C for 20 min for complete liquefaction before being used in the TUNEL assay (see Support Protocol 2).

   For viscous samples, a viscosity treatment enzyme such as chymotrypsin should be added and the sample placed in the incubator for additional 10 min for complete liquefaction.

General Setup of the Benchtop Cytometer
The major components of the flow cytometer are (1) optical assembly, (2) blue laser, (3) red laser, (4) sheath pump, and (5) waste pump and in-line sheath filter.

Materials
Sheath fluid (blue bottle; BD Biosciences, PN 653156): 0.22-µm filtered deionized water with or without bacteriostatic concentrate solution; if bacteriostatic concentrate solution is used (optional), add 1 bottle per 1 liter of water
Cleaning solution (green bottle; BD Biosciences, PN 653157); dilute 3 ml of cleaning concentrate in 197 ml of filtered deionized water (use the solution within 2 weeks)
Decontamination solution (yellow bottle; PN653154); add entire bottle to 180 ml of filtered, deionized water
Benchtop flow cytometer (Accuri C6; BD Bioscience; Fig. 16.11.2)
BD Accuri C6 software

1. Open the software by double-clicking the “BD Accuri C6 software” icon on the desktop.
2. Inspect all the reagent bottles to ensure that the fluid levels are adequate.
3. The waste bottle should be empty.
4. The sheath fluid, cleaning solution, and decontamination bottles must be full.
5. Turn on the cytometer by pressing the power button firmly.
6. While starting the software the “traffic light” will turn yellow. This is an indication that the peristaltic pump has started to run.
7. Allow 5 min for the fluidics line to get flushed with the sheath fluid.
8. Wait for the cytometer software light to turn green, indicating that the C6 Accuri is connected and ready.
9. Flush the tubing to remove any bubbles from the cytometer system.
10. Place a 0.22 µm-filtered deionized (DI) water tube on the sheath injection port (SIP).
11. Run a cycle with criteria selected as “Run with limits.”
12. Select “Fluidics” speed as “Fast.”
13. After selection of the above criteria, click the “Run” button.
14. Leave the SIP tube on the tube holder. Save the file as “Flush.”

**Instrument Quality Control**

Quality control for the FL1, FL2, and FL3 channels is performed and validated with 8-peak beads. The 8-peak beads are 3.2-µm particles excited by the blue laser. The beads emit light at eight different wavelengths. The validation of the benchtop flow cytometer is done by running the 8-peak beads and determining the coefficient of variation (CV) and mean fluorescence intensity (MFI) each time the instrument is used. These can be plotted as CV and MFI in the Levy-Jennings chart.

**Materials**

- Spherotech 8-peak validation beads (BD Biosciences, cat. no. 653144)
- 12 × 75-mm tubes
- Benchtop flow cytometer (Accuri C6; BD Bioscience; Fig. 16.11.2), set up as in Support Protocol 1
- BD Accuri C6 software
- Computer running Microsoft Excel

**Preparation of 8-peak beads**

1. Use a 12 × 75–mm tubes and label it as “8-Peak QC Beads.” Also mark the date of preparation.
2. Add 1 ml of deionized water to each of the tubes.
3. Vortex each of the bead vials provided by manufacturer for 5 sec. Place four drops of 8-peak beads in the tube and vortex. Cover the tube with an aluminum foil.

**Preparation for the run of the 8-peak QC beads**

4. Double click and open the 8-peak bead template (Figure 16.11.3).
5. Turn on the cytometer by pressing the power button located in front of the cytometer.
6. A green light will be displayed under the ‘Collect’ tab, indicating that the machine is ready for sample acquisition.
7. Start the acquisition by clicking on the well “A1.”
8. Place a tube with 2 ml of 0.22-µm deionized water on the SIP.
9. Check “Run with limits” and set the time limit to “15 min.”
10. Set “Fluidics” speed to “Fast.”
11. “Click” the “Run” button.
12. The software will prompt to “Save” the file.
13. After completion of the “Run,” place the tube with deionized water on the SIP.

**Acquisition of the 8-peak bead data**

14. Select an empty field from left heading towards the right with selecting one well at a time from A1 to H12.
15. Enter in the empty space above the wells the acquisition date for the 8-peak beads as “8 peak-beads—‘date’—‘technician initials’.”
Figure 16.11.3  8-peak quality-control beads as seen after analysis in software; the CV of the brightest peak (M3, M6, M9) is measured.

16. The acquisition is performed under the “Collect tab.”
17. Unselect the “Time” check-box next to “Min.” and “Sec.”
18. Select the “Events” check-box and check the “50,000” option in the “Events” field.
19. From the drop-down menu, click on “Ungated sample.”
20. Set “Fluidics” speed to “Slow.”
21. Mix the 8-peak QC bead suspension by vortexing the tube.
22. Remove tube of deionized water from the SIP.
23. Place the “8-Peak QC Bead” tube under the SIP.
24. Click the “RUN” button to start the acquisition.
25. Save the file as “8 Peak QC—’date’—’technician initials’.”
26. After the cytometer has recorded 50,000 events, acquisition will stop.
27. When the run is finished, remove “8-Peak QC Bead” tube from SIP and clean the SIP using a lint-free wipe.
28. Place the tube containing 2 ml of deionized water on the SIP.

Ending the run
29. With the 2-ml tube of deionized water on the SIP, select an empty well in the BD Accuri software.
30. Check “Time” and set the time to “2 min.”
31. Set “Fluidics” speed to “Fast.”
32. Click the “Run” button.
33. When the run is finished, place the tube with 2 ml of deionized water on the SIP.
34. Before running any other samples, click “delete events” to erase the data collection from the water run.
35. If shutting down instrument, proceed to the “Machine Shutdown” section run with a 10% solution of bleach for 2 min, followed by the deionized water run.

**Analyzing the 8-peak bead acquisition data**

36. The analysis is done in the “Collect” tab only.
37. Select the well (example: well A1) where the data was acquired for the 8-peak beads run.
38. Adjust the R1 gate to include 75% to 85% of all events.
39. In the first plot—‘FSC-H’ on the x axis and ‘SSC-H’ on the y axis—click on the border of the ‘R1’ gate. The border will become bold and handles will appear to adjust the gate settings.
40. Include all the “Singlets” or the main bead population, making sure to exclude all the doublets which appear as light-gray dots.
41. FL1-H, FL2-H, and FL3-H must be gated on R1.
42. Measure the CV of the brightest peak (right most peak) of the FL1-H, FL2-H, and FL3-H histograms (Figure 16.11.3).

**Criteria for successful 8-peak bead QC**

The CV for all three peaks must be less than 5% for validation of the three channels of the instrument.

43. To select the brightest peak, use the zoom tool over the histogram and zoom in on the brightest peak in the FL1-H histogram.
44. The ‘M1’ marker is adjusted tightly around the brightest peak.
45. The above two steps need to be repeated around the FL2-H and FL3-H histograms as well.
46. Save this template for future runs of the 8-peak quality control.

**Performance tracking of 8–peak bead quality control**

47. Open the file for the acquisition data obtained from the 8-peak bead run. Highlight all the statistics that need to be copied and transferred to the Excel spread sheet. 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. The Levy-Jennings chart gets populated by the data and the data is saved.

**Sample Preparation for TUNEL Assay**

**Materials**

- Semen sample (Basic Protocol 1)
- Phosphate-buffered saline (PBS; **APPENDIX 2A**)
- Sperm counting chamber (Spectrum Technologies, cat. no. SC-20-01-02-B)
- Centrifuge
1. Semen sample is kept in the incubator for 20 min at 37°C to undergo liquefaction.

2. After liquefaction, sample is evaluated for volume, sperm concentration, total sperm count, sperm motility, and round cell concentration.

   Total sperm count is calculated as: concentration × sample volume.

   Sperm motility is calculated as: \( \frac{\text{(Average number of motile sperm)}}{\text{(Average number of total sperm)}} \times 100 \)

   Round cell concentration (using a 20× objective) is calculated as: \( \frac{\text{(Average number of round cells)}}{\text{(20)/(100 round cells)}} \times 106 \) cells

3. Aliquot 5 µl of the sample with the appropriate pipet. The 5 µl of sample is loaded into the fixed cell chamber well.

4. Assess the sperm concentration by counting sperm in five different fields.

5. The sample volume for TUNEL needs to be adjusted to 2.5 × 10⁶/ml. This can be achieved by the following formula:

   \( \frac{(2.5 \times 1000 \text{ µl})}{[\text{sperm concentration (10⁶/ml)]}} = x \text{ µl} \)

6. Save two tubes each for the test sample, negative control, and positive control samples. If the sample is inadequate, a single tube may be saved for the negative and the positive control sample.

7. Label tubes with the following information:

   TUNEL
   Patient name
   Medical record number
   Date.

8. Aliquot the required volume for an adjusted sperm concentration of 2.5 × 10⁶/ml cells to each of the four tubes.

9. Spin the aliquotted sample 7 min at 300 × g, 25°C.

10. Remove the supernatant after the spin.

11. Replace the supernatant with 1 ml PBS

12. Centrifuge 7 min at 300 × g, 25°C.

13. Remove the supernatant and replace with 1 ml PBS.

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**Preparation of the ‘Positive Control’ Sample**

**Materials**

- 37% hydrogen peroxide
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- Positive control (semen sample from healthy donor)
- 3.7% paraformaldehyde: add 90.0 ml of phosphate buffered saline (PBS) pH 7.4 ( *APPENDIX 2A*) to 10.0 ml of formaldehyde (37%); stored at 4°C
- 70% ethanol, ice cold
- 50°C heating block
- Centrifuge
Prepare cells for fixation/permeabilization

1. Add 100 μl of 37% solution hydrogen peroxide to 1400 μl of PBS prepare a 1:15 dilution of H₂O₂.
2. Add and suspend the sperm cells in 1 ml of the diluted H₂O₂ solution.
3. Place the sperm cell resuspended in H₂O₂ on a heating block at 50°C for 60 min.
4. After incubation, centrifuge the tube for 7 min at 300 × g, 25°C.
5. Aspirate the supernatant with a transfer pipet and resuspend in 1 ml PBS and centrifuge 7 min at 300 × g, 25°C.
6. Remove the supernatant and replace with 1 ml PBS. Along with the ‘Test’ and ‘Negative’ control sample tubes, repeat centrifugation step for 7 min at 300 × g, 25°C.
7. Remove the supernatant and replace with 1 ml PBS.

Fixation and permeabilization

Fixation of the sperm cells is done with paraformaldehyde.

8. The supernatant from the ‘Test’ sample, ‘Negative’, and Positive’ control samples is removed after centrifugation 7 min at 300 × g, 25°C, followed by addition of 1 ml of 3.7% paraformaldehyde solution.
9. Incubate the samples at room temperature for 15 min.
10. Centrifuge the samples 4 min at 300 × g, 25°C.
11. Carefully aspirate the paraformaldehyde and replace it with 1 ml of PBS.
12. Centrifuge 4 min at 300 × g, 25°C.
13. Aspirate the supernatant and replace with 1 ml of ice-cold 70% ethanol. Place the sample at 4°C for 15 to 30 min.
14. Perform a second wash with PBS at 25°C.

TUNEL Staining with the APO Direct Kit

Materials

Test samples
APO-DIRECT™ Kit (BD Pharmingen, cat. no. 556381):
- PI/RNase Staining Buffer
- Reaction Buffer
- FITC-dUTP
- TdT Enzyme
- Rinsing Buffer
- Wash Buffer
- Negative Control Cells
- Positive Control Cells

12 × 75–mm polystyrene tubes
Benchtop flow cytometer (Accuri C6; BD Bioscience; Fig. 16.11.2), set up as in Support Protocol 1
BD Accuri C6 software
Computer running Microsoft Excel
Table 16.11.1  Components of the Staining Solution

<table>
<thead>
<tr>
<th>Staining solution</th>
<th>1 Assay</th>
<th>6 Assays</th>
<th>12 Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer (green cap)</td>
<td>10.00 µl</td>
<td>60.00 µl</td>
<td>120.00 µl</td>
</tr>
<tr>
<td>TdT enzyme (yellow cap)</td>
<td>0.75 µl</td>
<td>4.50 µl</td>
<td>9.00 µl</td>
</tr>
<tr>
<td>FITC-dUTP (orange cap)</td>
<td>8.00 µl</td>
<td>48.00 µl</td>
<td>96.00 µl</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>32.25 µl</td>
<td>193.5 µl</td>
<td>387.00 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>51.00 µl</td>
<td>306.00 µl</td>
<td>612.00 µl</td>
</tr>
</tbody>
</table>

Prepare samples and tubes for TUNEL assay

The negative and positive controls are provided as kit components.

1. The negative controls, positive control, and test samples should be mixed well by vortexing them.

2. Aliquot 2 ml of the well-mixed kit control suspensions into 12 × 75–mm polystyrene tubes.

   *The 2 ml of suspension contains approximately 1 × 10⁶ cells/ml.*

3. Include internal controls—both positive and negative semen samples—with each run.

   *These are semen samples with known DNA fragmentation.*

4. The kit control samples, test samples, and internal control samples should be centrifuged for 7 min at 300 × g, 25°C.

5. Remove 70% ethanol with a transfer pipet by aspirating it, without disturbing the cellular pellet.

6. Add 1.0 ml of the ‘Wash Buffer’ from the kit (blue cap) and mix well.

7. Centrifuge 7 min at 300 × g, 25°C.

8. Aspirate and remove the supernatant from the tubes.

9. Repeat the washing step with the ‘Wash Buffer’ and discard the supernatant.


Staining for TUNEL assay

11. Count the total number of tubes or test samples including the kit controls and the internal controls.

12. Prepare stain for an additional five to seven tubes as described in the following steps.

13. Remove “Reaction Buffer” vial (green cap) from 4°C and the TdT (yellow cap) and FITC-dUTP (orange cap) vials from −20°C storage and place them at room temperature for 20 min. Give a quick vortex to bring the reagent to the bottom of the vial.

14. Prepare the stain as shown in Table 16.11.1.

15. Add the reagents in the same sequence as indicated in the table.

16. All the steps for the stain preparation must be carried out in the dark.

17. Omit the TdT from the negative controls.

18. Resuspend the pellet in each tube in 50 µl of the ‘Staining Solution’.

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19. Incubate the sperm suspension for 60 min at 37°C.

20. The tubes should be covered with aluminum foil.

21. After the 60-min incubation, add 1.0 ml of ‘Rinse Buffer’ (red cap) to each tube. Centrifuge the tubes for 7 min at 300 × g, 25°C. Aspirate and remove the supernatant.

22. Repeat the wash with addition of 1 ml of ‘Rinse Buffer’.

23. Repeat the centrifugation step for 7 min at 300 × g, 25°C.

24. Aspirate and discard the supernatant.

25. Resuspend the pellet in 0.5 ml of PI/RNase buffer.

26. Incubate the suspension mixture for 30 min at room temperature.

**Run kit controls and acquire data for kit controls**

27. Run the kit controls using the “kit control template” (Figures 16.11.4 and 16.11.5). The settings include “Run with limits” for a total of 10,000 events with a slow fluidics speed and threshold set at 80,000 on FSC-H. Data is recorded on four plots: FSC-A/SSC-A, FSC-A/FL2-A, FL2-A/FL2-H, and FL1A/FL2-A. Observe the right upper quadrant and record the FITC positive as the percent positive value for each kit control.

**Running patient samples**

Patient samples are run under the “Collect” tab. Use the standardized data acquisition template (Figure 16.11.6). The complete acquisition data should be saved in a designated folder for patient results.


29. Wait until the software loads.
30. Ensure that there is no data in any of the wells in the template file. If there is data in any of the wells, select the “delete all events icon” at the bottom left of the screen and remove the data.

31. Select well “F1” and import the standard sample file (.fcs; Figure 16.11.7).

32. Select the first well “A1.” In the space provided above the well, insert “TUNEL patient result,” technician initials, date, and well number. Hit the Save button.
33. Begin with tube #1 (first test sample).
34. Remove deionized water tube from the SIP.
35. Vortex the sample and place on the SIP.
36. The run parameters are set as follows for patient samples:
   a. “Run with limits”: check ‘10,000 events’.
   b. “Fluidics” speed: select ‘Slow’.
   c. Select gate ‘P3 in P1’.
   d. Threshold: set at 80,000 on ‘FSC-H’.
37. The acquisition of data is begun by clicking on the “Run” button.
38. After 10,000 events, the run will be completed.
39. Remove the tube from the SIP.
40. Use a lint-free wipe to clean the SIP.
41. Vortex and place the second sample on the SIP.
42. Select the next well (A2, and so on) for the next samples.
43. The above steps are repeated for each sample to allow processing of all samples.
44. The data acquisition workspace is saved in a subfolder—e.g., TUNEL Patient Results' 'date', 'technician initials'.C6. Save the workspace and close the file.
45. Remove the tube from the SIP and replace with “bleach tube” on the SIP.
46. A “bleach cycle” is run at the end with the following parameters:
   a. “Run with limits”: 2 min.
   c. Threshold: 80,000 on FSC-H.
47. Wipe the SIP at the end of the run.
48. Remove the tube and replace it with deionized water tube.
49. Repeat step 46 with deionized water.
50. Follow the shutdown steps at the end of the run.

**Cleaning and Maintenance of the Benchtop Cytometer**

The machine requires some elements of maintenance such as SIP cleaning and fluid cycle to be performed daily. The SIP needs to be cleaned daily by performing a back flush. A decontamination of the fluidics line can be done daily by running a decontamination fluid cycle using deionized water. The fluidic bottle filters and the in-line sheath filters should be changed every 2 months. The two peristaltic tubings for the sheath pump and the waste pump are also changed every 2 months.

**DATA ANALYSIS**

The dual strategy outlined below is used for data analysis

1. Alignment strategy is performed under the “Collect” tab. A standard sample file is used for alignment of all the samples.
2. Data analysis is performed under the “Analyze” tab. Each sample must be aligned to “Standard sample” under the “Analyze tab.”

**Alignment Strategy and Data Analysis in the Collect Tab**

**Materials**

BD Accuri C6 software

1. Go to File, open workspace, or template. Select the acquisition data saved in the TUNEL template (TUNEL Patient Template).
2. Select an empty well where the standard sample data acquisition file has to be imported.
3. Standard sample should be selected as a sample which has a known percentage of DNA fragmentation. Go to the “Standard template” and select it.
4. Click on “File” > “import.”
5. Select an open workspace.
6. Save the workspace as TUNEL patient acquisition—data analyzed—technician initials—date.
7. A single sample is selected as “Standard sample.”
8. Select the negative peak of the “Standard sample” and use it as a reference to be applied to all samples for alignment (Figure 16.11.8).
9. Click on the histogram for the “Standard sample.”
10. Change the x-axis parameter from FSC-A to FL1-A.
11. Right click below the x axis (FL1-A) and select the “Virtual gain” module for alignment.
12. Change the gate to P3 in P1 for plot 5. This gate is the same as plot 4, which is a quadrant gate.
13. Select the vertical line icon at the bottom left of the histogram plot.
14. Align the selected blue line to the center of the histogram to obtain 50% cell population on either side.
15. Select the sample to be aligned from the grid of wells.
16. Next, align the blue line to the center of the peak of the selected sample.
17. Click the tabs of “Preview” and “Apply.”
18. An asterisk will appear below the histogram. This confirms the alignment of the sample (Figures 16.11.9 and 16.11.10).
19. Hit the Close button. Go to File and hit “Save” after each sample is aligned.
Figure 16.11.9 Applying the alignment to the test sample. This is indicated by an asterisk at the bottom of the histogram confirming the alignment of the sample to the standard file.

Data Analysis in “Analyze” Tab

**Materials**

- BD Accuri C6 software

1. The data acquired in the “Collect” tab is utilized for analysis under the “Analyze” tab within the Accuri C6 software. Open the “Analyze” tab. Create a set of three plots for each sample: FSC-A/SSC-A, FSC-A/FL2-A, and FL1-A/FL2-A.

2. Apply the same gating strategy as used in the “Collect” tab (Support Protocol 4).

3. The first plot, “FSC-A/SSC-A” has no gating. The cell population is PX.

4. The second plot “FSC-A/FL2-A” will have the gate PX in all events. The cell population is PY.

5. The third plot “FL1-A/FL2-A” will have gate of PY in PX in all events.
6. Record the percent damage reflected in the upper right quadrant from the “FL1-A/FL2-A” plot (Figure 16.11.10).

7. Record the preliminary results in the TUNEL Laboratory record form.

**Final Result Calculation for Sperm DNA Fragmentation Percent Value and Verification of the Validity of the TUNEL Assay Performed**

**Materials**

BD Accuri C6 software

1. Calculate the average value of the “Negative samples” where no TdT was added.

2. Subtract the average negative value from the percent damage for each sample recorded from the “FL1-A/FL2-A” in the right upper quadrant of the plot (Figure 16.11.11).

3. The following two conditions need to be verified for the assay to be considered valid:
a. The spermatozoa “positive control” samples must have a higher percentage of sperm positive for TUNEL than the percentage of positive spermatozoa in the actual spermatozoa samples.

b. The positive kit control sample should have greater than 30% of spermatozoa positive for TUNEL.

**COMMENTARY**

**Background Information**

Unlike other somatic cells, sperm nuclear DNA is highly compacted because of its protamine content. DNA fragmentation can be attributed to a number of factors such as oxidative stress (Agarwal et al., 2014; Lewis & Aitken 2005; Sakkas, Seli, Bizzaro, Tarozzi, & Manicardi, 2003), abortive apoptosis (Sakkas et al., 2003), failure to repair DNA strand breaks (Lewis & Aitken 2005), environmental exposure of sperm DNA to toxins, defective chromatin packaging, and protamine deficiency (Aitken et al., 2014). This has led to the introduction of DNA integrity as an important parameter for clinical assessment of male infertility. Sperm DNA fragmentation is a biomarker for evaluation of male infertility.

Accurate assessment of sperm DNA integrity expressed as SDF is a good predictor of semen quality (Feijo & Esteves 2014; Ribas-Maynou et al., 2013; Sergerie, Laforest, Bujan, Bissonnette, & Bleau, 2005; Sharma et al., 2010). There are four main techniques for assaying SDF: (1) sperm chromatin structure assay (SCSA), which measures proportion of sperm that are susceptible to DNA damage (red fluorescence) compared with those that are not (green fluorescence), utilizing flow cytometry acid denaturation (Evenson et al., 1999); (2) TdT-mediated dUTP nick-end labeling (TUNEL), which measures the intensity of fluorescence proportional to the number of DNA strand breaks (Sharma et al., 2016), quantifying the incorporation of fluorescently labeled dUTP at the 3′OH strand breaks with TdT (Ribas-Maynou et al., 2013); (3) single-cell gel electrophoresis assay (SCGE, also known as Comet), which measures the displacement of the nuclear and tail material utilizing fluorescently labeled cells embedded in agarose by electrophoresis after they are lysed (Ribas-Maynou et al., 2014; Ribas-Maynou et al., 2013; Ribas-Maynou et al., 2012; Singh, McCoy, Tide, & Schneider, 1988); and (4) sperm chromatin dispersion (often known as HALO), utilizing microscopy to detect cells with intact DNA (large halos) versus those that have damaged DNA (small or no halo) (Fernandez et al., 2003; Fernandez, Cajigal, Lopez-Fernandez, & Gosalvez, 2011; Ribas-Maynou et al., 2013).

Each of these tests is related to properties of the DNA damage and provides semi-quantitative estimates only. However, they do not provide information regarding the specific DNA sequences that may be affected (Ni, Spiess, Schuppe, & Steger, 2016). A significant association has been reported, utilizing SDF tests, between sperm DNA damage and pregnancy outcomes. High DNA integrity is generally seen in fertile men with normal semen parameters, whereas infertile men with abnormal semen parameters have low DNA integrity. In addition, many men with normal semen parameters have abnormal DNA integrity (Spano et al., 2000; Zini et al., 2002). There is, however, some evidence to suggest that increased DNA fragmentation is associated with reduced fertility. Yet, this evidence is not conclusive for these tests to be truly predictive of fertility status.

The literature reports that 5% of women experience two consecutive miscarriages and approximately 1% suffer three or more consecutive miscarriages (ASRM Practice Guidelines, 2012; Rai & Regan 2006), and that these observations are linked with sperm DNA damage. A number of systematic reviews have examined the effects of SDF on ART outcome (IUI/IVF/ICSI), and the results have been inconclusive (Bareh et al., 2016; Carlini et al., 2017; Coughlan et al., 2015; Osman et al., 2015; Robinson et al., 2012; Simon, Zini, Dyachenko, Ciampi, & Carrell, 2017; Zini, 2011). At present, current data does not support a consistent association between abnormal DNA integrity and reproductive outcomes achieved through natural conception, IUI, IVF, or ICSI. More well-designed studies using standard techniques are needed for the predictive ability of SDF in recurrent pregnancy loss.

**Alternate kits**

**Evaluation by Apo-BrdU apoptosis detection kit**

There are other Apo-direct kits available from companies such as Biovision and Thermo Fisher. BioVision has a kit on the
market named “Apo-BrdUTP in situ DNA fragmentation kID.” The instructions from the manufacturer have to be adapted for spermatozoa. The kit also includes positive and negative controls. The DNA labeling is done with BrdUTP, which binds to the 3′-OH terminals of the DNA strand breaks and terminal deoxynucleotidyl transferase (TDT). The antibodies to the BrdUTP molecule are linked to fluorescein. These will be detected in the FL1 channel (Anzar, He, Buhr, Kroetsch, & Pauls, 2002; Ribeiro et al., 2013). Sample analysis is done with a flow cytometer. The FL3 channel records the propidium iodide fluorescence. The percentage of TUNEL-positive cells can be obtained from the right upper quadrant in the FL1-A/FL3-A, 4-quadrant plot.

**Historical background of comparable techniques and description of the alternate techniques**

Both SCSA and TUNEL tests using flow cytometry have been shown to correlate with each other. TUNEL-based assays show higher accuracy than the SCD and Comet assay (Cui et al., 2015).

**Critical Parameters**

Factors that can influence the protocol and need special attention are:

a. **Viscous semen samples:** These make it difficult to assess sperm concentration and subsequent sample preparation for TUNEL assay. Viscosity can be reduced by treating with chymotrypsin (5 mg) and incubating the sample for an additional 10 min. before examining for concentration.

b. **Oligozoospermic samples:** Samples that have extremely low sperm concentration (<10 × 10⁶ sperm/ml) will require larger sample volumes. In such cases, it is important to remove the seminal plasma to avoid clumping/fixation of the seminal plasma proteins with paraformaldehyde. This will make all the subsequent washing and staining steps difficult. Also it will likely clog the SIP during the analysis step.

c. **Aspiration of the supernatant must be very carefully done, as sperm will be lost in each washing and re-suspension step.**

d. **The reagent volumes that need to be aliquotted for preparing the stain must be carefully calculated and verified as described to avoid over staining or under staining of the samples.**

e. **It is helpful to give a quick spin to the TdT vial to bring the volume to the bottom of the vial.**

f. **The preparation of the stain and all subsequent steps must be conducted in indirect light or in the dark.**

g. **The incubation step with the stain should not exceed 60 min. at 37°C. The incubation time can be noted on the aluminum foil.**

h. **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 analysis.**

i. **The ‘staining solution’ is active for approximately 24 hr at 4°C.**

j. **The cells must be analyzed within 3 hr after staining. They will start to deteriorate if left overnight before analysis.**

k. **Always run the ‘Kit controls’ and ‘Patient samples’ in the ‘Collect Tab’.**

l. **The gate should be changed to ‘P3 in P1 for sample data acquisition.**

m. **Do not change the settings in the four plots.**

n. **The test must always be validated to confirm that the test was correctly performed as per the validation criteria provided above.**

o. **It is important to clean the SIP each time by clicking on the ‘Back flush’ button and following the steps in the manual.**

p. **The flow cytometer quality control must be performed as recommended for optimal performance of the instrument.**

It is important that proper quality control for reagents and instrument be in place for the test be precise and accurate. The instrument quality control using the ‘8 peak’ beads provided by the manufacturer must be performed regularly. In addition, negative and the positive kit controls that are provided with the TUNEL kit must be included with each run. Appropriate negative (no TdT) and positive controls (treated with hydrogen peroxide to induce DNA fragmentation) should also be included with each run. Internal controls with known sperm DNA fragmentation are also included in the run for validation of the assay.

**Troubleshooting**

**Common problems with the protocols, their causes, and potential solutions**

Common problems with contemporary protocols measuring sperm DNA fragmentation include the lack of standardized reference values for DNA fragmentation. This is largely
attributed to the variations in the methodology for measuring DNA fragmentation.

The four common methods described are the SCSA, TUNEL, Comet, and SCD assay. Each of them measures a different endpoint/aspects of DNA damage. TUNEL measures a definitive end point: DNA strand breaks at the 3’ terminal end. Some tests measure single-strand breaks; others measure double-strand breaks. While SCSA and TUNEL using flow cytometry, which is a robust technique, are objective, there are other reports using microscopy and manual assessment, which are subjective, with counting of 200 or 500 cells to derive the percentage of DNA fragmentation.

The manual methods account for the large variability in the reference value, which eventually affects the sensitivity and the specificity of each of these tests. These ultimately influence the overall diagnostic accuracy of these tests.

In order to overcome some of the potential difficulties, it is important that these tests be standardized in each laboratory and that instrument/assay variability as well as observer variability be minimized. These goals can be accomplished with adequate training, following completely vetted protocols with established reference ranges.

Statistical Analyses

Appropriate statistical methods must be used. The differences in the distribution of TUNEL-positive sperm DNA fragmentation levels between infertile patients and donors should be assessed using the Wilcoxon rank-sum test for paired samples. For a comparison between patients and donors, an independent sample test must be used. If samples are normally distributed, a parametric test should be used. Once adequate number of subjects have been tested for SDF, the receiver operating characteristic (ROC) curve can be generated to establish the cutoff, sensitivity, specificity, area under the curve, and positive and negative predictive values, as well as the accuracy of the test (Sharma et al., 2016; Sharma et al., 2010).

Alternative statistical approaches

To examine the inter-laboratory standardization of the TUNEL test, after a test for normal distribution is done, Spearman’s rank correlation (non-parametric) can be used to determine the strength of the relationship between the TUNEL values obtained in each laboratory and can also be used to categorize the degree of correlation—i.e., moderate, strong, or very strong (Evans, 1996). The average DNA fragmentation rates can compared using the Wilcoxon Signed Rank Test. The statistical significance level is set at 5%.

Anticipated Results

Sample concentration should be between 1-5 × 10⁶ sperm for appropriate run and to avoid clumping of the cells. SDF for the negative kit control should be 3% to 5%, and for the positive kit control, >30% to 40%, using the apoptosis detection kit. If the control results are outside this range, there are issues with the staining. The percent DNA fragmentation values of positive control spermatozoa must be greater than the percent DNA fragmentation of the spermatozoa samples. The kit positive control percent DNA fragmentation must be greater than 30%.

Time Considerations

Adequate time must be set aside to conduct this test. Conducting initial semen analysis to examine sperm concentration and preparation of the test samples along with appropriate negative and the positive controls takes about 2 to 3 hr. Processing and staining of samples takes about 2.5 hr. Finally, the running of the samples by flow cytometer takes about 1 hr. The analysis of the data and calculation of sperm DNA fragmentation takes another 60 min.

Acknowledgements

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Literature Cited


This chapter describes in detail the steps involved in the analysis of semen samples for TUNEL measurement, as well as a short protocol highlighting the main steps.

Sharma et al. (2016). See above.

This article describes the measurement of DNA fragmentation in semen samples from healthy controls and infertile men. It also provides the cutoff and sensitivity and specificity of the TUNEL test.

Sharma et al. (2013). See above.

This article is a step-by-step guide explaining how to set up the TUNEL assay as a clinical test using flow cytometry or fluorescence microscopy.

Sharma et al. (2010). See above.

This paper establishes the inter-and intra-observer and inter-and intra-assay variability, cutoff values, and the distribution of DNA fragmentation in infertile men referred to a clinical andrology laboratory.

Ribeiro et al. (2010). See above.

This paper describes the standardization of the method and comparison of data across two reference laboratories in different continents using identical semen samples, assay kit, protocol, acquisition settings, and flow cytometers. It validates the TUNEL assay and establishes it as a robust test for measuring DNA fragmentation.