36 Measurement of DNA Damage in Spermatozoa by TUNEL Assay

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Abstract

Infertile men with severe male-factor infertility have significantly more DNA damage than fertile men. Sperm DNA damage has been shown to affect ART outcomes. DNA damage is increasingly being evaluated as a test in establishing its utility in ART. Various assays have become more common than others. This chapter describes one of the more commonly used assays to measure sperm DNA damage by the terminal deoxynucleotidyltransferase dUTP nick end labeling or the TUNEL assay [1–5].

Keywords

Spermatozoa • Sperm chromatin • Terminal deoxynucleotidyltransferase dUTP nick end labeling • Flow cytometry

DNA fragmentation is a process that results from the activation of endonucleases during apoptosis. These nucleases degrade the higher order sperm chromatin structure into fragments of ~30 kb and subsequently into smaller DNA pieces about ~50 kb in length. This method is used to detect fragmented DNA and utilizes a reaction catalyzed by exogenous terminal deoxynucleotidyltransferase (tdt) and is termed as "end labeling" or "TUNEL" (terminal deoxynucleotidyltransferase dUTP nick end labeling) assay [1–5].

Assay Principle

This single-step staining method labels DNA breaks with FITC-dUTP followed by flow-cytometric analysis. Tdt catalyzes a template-independent addition of bromolated deoxyuridine triphosphatase to the 3'-hydroxyl (OH) termini of double- and single-stranded DNA. After incorporation, these sites are identified by flow-cytometric means by staining the sperm.

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Specimen Collection

1. Ideally, sample should be collected after a minimum of 48 h and not more than 72 h of sexual abstinence. The name of the patient, period of abstinence, date, and time and place of collection should be recorded on the form accompanying each semen analysis.
2. The sample should be collected in private in a room near the laboratory. If not, it should be delivered to the laboratory within one hour of collection.
3. The sample should be obtained by masturbation and ejaculated into a clean, wide-mouth plastic specimen cup. Lubricants should not be used to facilitate semen collection.
4. Coitus interruptus is not acceptable as a means of collection because it is possible that the first portion of the ejaculate, which usually contains the highest concentration of spermatozoa, will be lost. Moreover, cellular and bacteriological contamination of the sample and the acid pH of the vaginal fluid adversely affect sperm quality.
5. Incomplete samples should be analyzed, but a comment should be entered on the report form.
6. The sample should be protected from extremes of temperature (not less than 20°C and not more than 40°C) during transport to the laboratory.
7. Note down any unusual collection or condition of specimen on the report form.

Equipment and Reagents

- APO-DIRECT™ kit (BD Pharmingen, Catalog % 556381)
- Pipettes
- Pipette tips (200 μL and 1,000 μL)
- Microcell counting chamber
- 3.7% Paraformaldehyde in PBS
- Microfuge ependorf tubes
- Ethanol
- Flow cytometer

Sample Preparation

1. Following liquefaction, evaluate semen specimens for volume, sperm concentration, total cell count, motility, and morphology.
2. Aliquot and load a 5-μL aliquot of the sample on a Microcell slide chamber (Conception Technologies, San Diego, CA) for manual evaluation of concentration and motility. Check the concentration of sperm in the sample. Adjust it to $2–5 \times 10^6$/mL.
3. Using a cryomarker, label one 5-mL tube. Label specimen 1 with the patient name, identification number, and date, i.e., as follows:
   (I) TUNEL
   (II) Smith, John
   (III) No. X-XXX-XXX-X
   (IV) Date
4. Preparation of paraformaldehyde:
   (a) To 10.0 mL of formaldehyde (37%), add 90.0 mL of PBS (pH 7.4).
5. Check the concentration of sperm in the sample. Adjust the volume to give $3–5 \times 10^6$/mL. Spin the sample and remove seminal plasma. Add 1.0 mL of 3.7% paraformaldehyde.
6. Place the cell suspension on ice for 30–60 min/overnight.
7. Store cells in 1 mL of ice-cold 70% (v/v) ethanol at −20°C until use. Cells can be stored at −20°C several days before use.

   Note: The samples can be processed from A-G, batched and shipped.

Staining Protocol

1. Resuspend the positive (6552LZ) and negative (6553LZ) control cells by swirling the vials. Remove 2-mL aliquots of the control cell suspensions (approximately 1×10^6 cells/mL) and place in 12×75 mm centrifuge tubes. Centrifuge the control cell suspensions for 5 min at $300 \times g$ and remove the 70% (v/v) ethanol by aspiration, being careful to not disturb the cell pellet.

2. Resuspend each tube of control and sample tubes with 1.0 mL of Wash Buffer (6548AZ) (Blue cap) for each tube. Centrifuge as before and remove the supernatant by aspiration.
3. Repeat the Wash Buffer treatment.
4. Resuspend each tube of the control cell pellets in 50 μL of the *Staining Solution* (prepared as described below).
5. Staining solution (single assay)

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

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 1 assay. Mix only sufficient volumes of Staining Solution to complete the number of assays prepared per session. The Staining Solution is active for approximately 24 h at 4°C.

6. Incubate the sperm in the Staining Solution for 60 min at 37°C. The reaction can also be carried out at room temperature overnight for the control cells. For test samples, the 60-min incubation time at 37°C may need to be adjusted to longer periods of time.
7. At the end of the incubation time, add 1.0 mL of Rinse Buffer (6550AZ) (Red cap) to each tube and centrifuge each tube at 300×*g* for 5 min. Remove the supernatant by aspiration.
   Note: If the cell density is low, decrease the amount of PI/ RNase Staining Buffer to 0.3 mL.
8. Repeat the cell rinsing with 1.0 mL of the Rinse Buffer. Centrifuge and remove the supernatant by aspiration.
9. Resuspend the cell pellet in 0.5 ml of the PI/ RNase Staining Buffer (6551AZ).
10. Incubate the cells in the dark for 30 min at RT.
11. Analyze the cells in PI/ RNase solution by flow cytometry.
    Note: The cells must be analyzed within 3 h of staining, as they may begin to deteriorate if left overnight before the analysis.

Reference range: Percentage of cells showing DNA fragmentation is calculated.

Normal range: ≤19% DNA damage.

Panic values: >19% DNA damage.

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